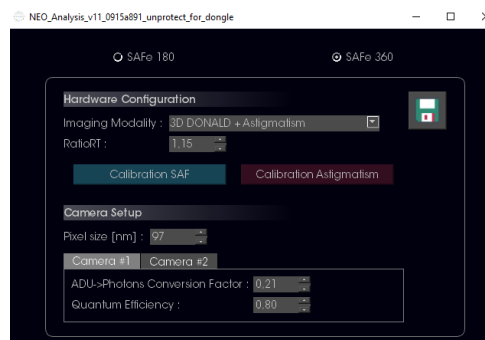


abbelight user guide

Data processing with NEO software

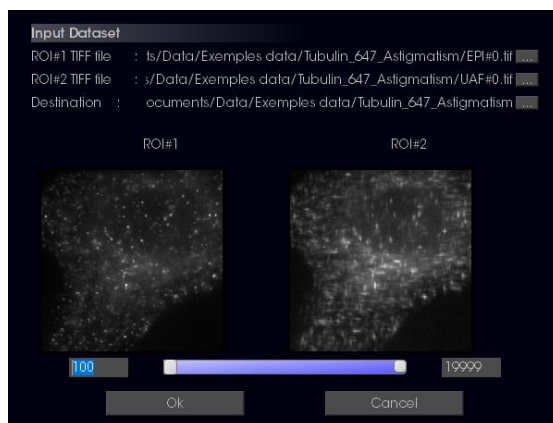
1. Open **NEO_Analysis** software. You will be able to (i) process your datasets individually and get reporting on super-localization in the "Process" tab, (ii) process your datasets in batch in the "Batch" tab.
2. **Process**: in this tab, you can load raw data, adjust the processing parameters, see software localizations, and process to obtain coordinate table and reconstructed image.
 - a. Click on "Select files to import"
 - b. Choose the setup you used for acquisition (SAFe 180 for single camera or SAFe 360 for dual camera).



- c. Choose the appropriate imaging mode.
- d. (If needed) Indicate the RatioRT, pixel size and camera info (photon conversion factor and quantum efficiency). All these parameters were provided to your facility manager on the day of the installation.
- e. Click on the save icon to save these parameters. *Note: the software will remember the saved parameters, so you do not have to indicate them every time.*



- f. If processing data in 3D using astigmatism, click on "Calibration Astigmatism". Calibration curves for Gaussian Fitting and Phasor algorithms appear. Load the correct calibrations made on your setup, by clicking on "Import file..." and selecting the appropriate calibration curve. Note: click on "Use demo curve" if you want to use a standard calibration curve; however, keep in mind that demo curves were not made on your setup.
- g. Select the raw data to analyze ("ROI*.tif"). For SAFe180, only one file is needed. For SAFe360, you need to load the files from both camera (ROI1.tif and ROI2*.tif).

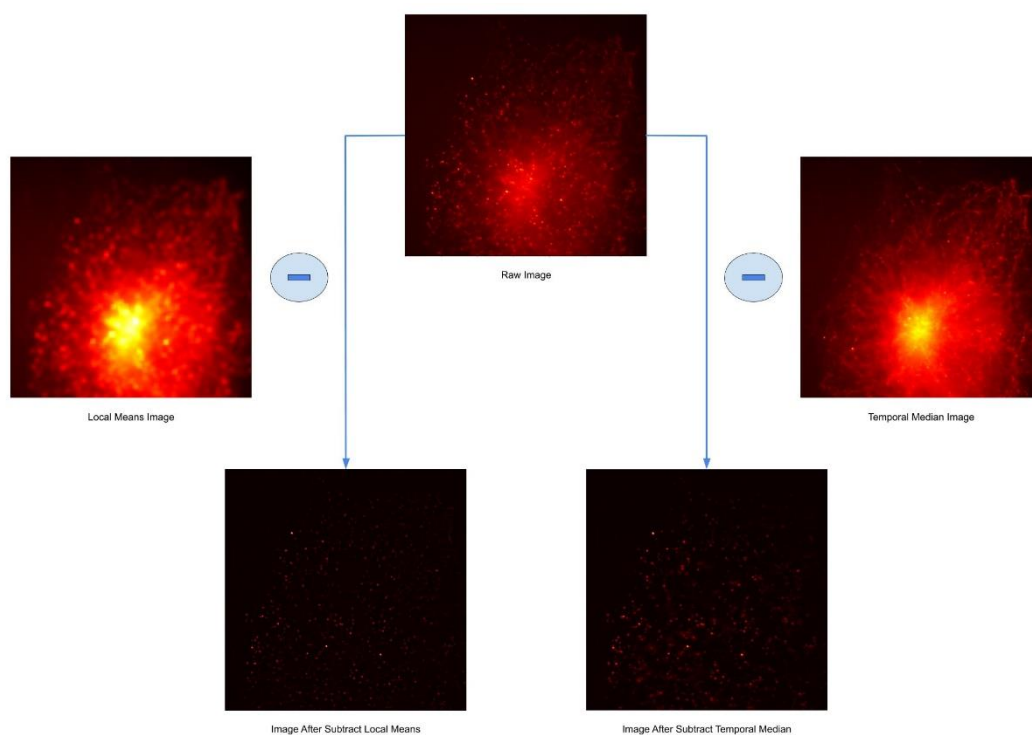


- h. Choose the directory where you want the processed results to be saved. We recommend creating a specific "Analysis" folder for clarity.
- i. Use the slider to remove frames at the beginning or at the end of the acquisition. Make sure you start the processing when fluorophores are properly blinking. Click on "Ok".
- j. You can adjust the localization parameters in the corresponding tab. For beginner users, the default localization parameters are sufficient to reconstruct nanoscopy data. Depending on the quality of the signal, you can adjust the detection threshold (see table, "Detection threshold"). *Note: for single-particle tracking (SPT), make sure you inactivate drift correction.*
For more experienced users, the table below describes how to adjust all localization parameters:

Category	Parameter	Detail
General parameters	Microscopy image pixel size (nm)	Camera pixel size in nm. This parameter is fixed and dependent on your system.
	Nanoscopy image pixel size (nm)	Nanoscopy data are coordinate tables with x, y and z positions of all molecules. It is possible to generate TIFF images where each pixel intensity represents the density of molecules in the pixel. As it is a reconstructed image, the pixel size can be chosen by the user.
	Nanoscopy image rendering mode	The nanoscopy image can be rendered using two visualization options: <ul style="list-style-type: none"> • Normalized Gaussian: each localization is represented by a gaussian distributed signal corresponding to the lateral uncertainty. • Uniform Gaussian: each localization is represented by a uniform gaussian distributed signal
	ADU → Photons Conversion Factor	Conversion Factor to Convert ADU (Arbitrary Digital Units) pixels intensities given by the camera to a number of photon-electrons. This spec is sometimes referred to as the "conversion coefficient" or "conversion factor". All digital cameras have a «conversion factor» and generally come with documentation giving this value for each individual camera.
	Quantum efficiency	Conversion of photon-electrons to photons requires dividing the number of photon-electrons in each pixel by the quantum efficiency (QE) of the sensor at the wavelength you are imaging (for example, if the QE is 80%, enter 0.80). This parameter is dependent on each individual camera for each wavelength (see camera documentation).
Detection and super-localization parameters	Subtract temporal median	The sample may have inhomogeneous densities leading to significant differences in background signal in the acquired image. In order to accurately detect PSFs, background removal is essential. NEO offers two ways to subtract background: <ul style="list-style-type: none"> - Local mean subtraction (box unchecked). A local mean filter is a spatial filter that considers the neighborhood pixels around a given pixel. For every pixel in the image, the algorithm looks at its neighbors and replaces its intensity with the average of its neighbors. The local mean filter creates a local background estimation image (comparable to a blurred version

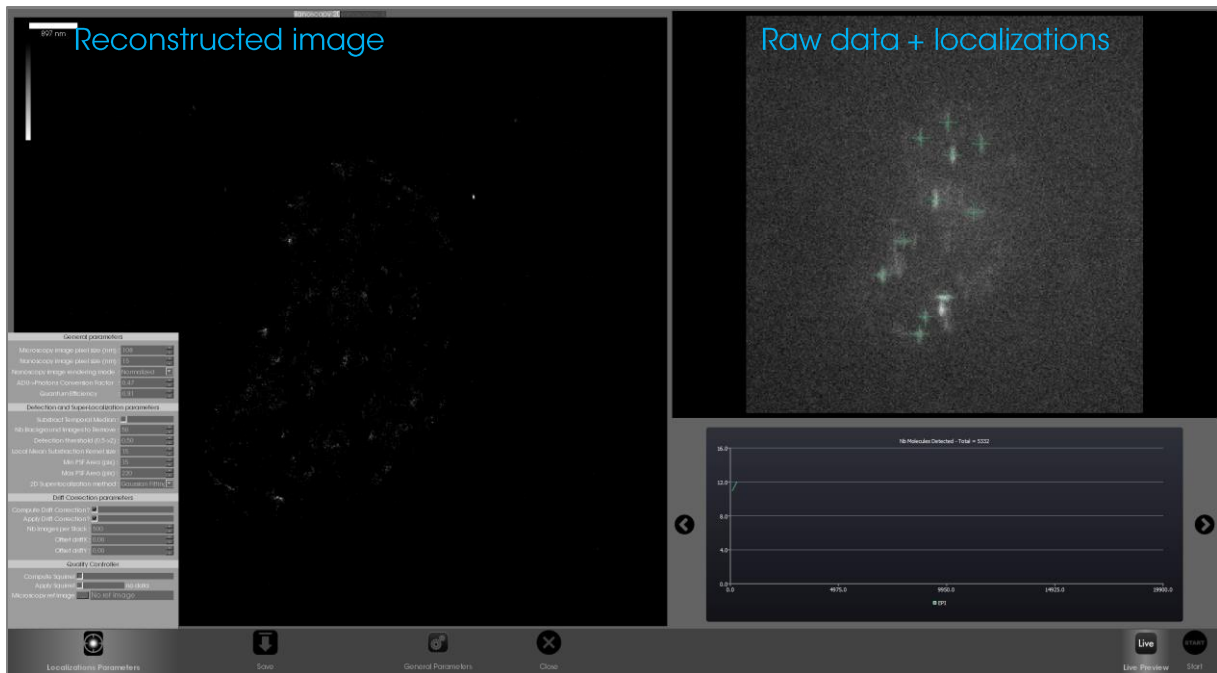
		<p>of the image), which can be subtracted from the raw data to reduce background.</p> <p>- Temporal median subtraction (box checked). This method considers the temporal component: since acquisitions are movies, what happens at a given pixel over time provides useful information for background removal. In single-molecule experiments, molecules emit fluorescence for a limited duration. Measuring signal intensity before and after this ON state provides an estimation of the background at that precise location. The temporal median filter creates a background estimation image, which can be subtracted from the raw data to reduce background.</p>
	Nb Background images to remove	<p>N= Nb Background images to remove</p> <p>For a given pixel in a given frame, the median intensity over N preceding frames can be calculated and subtracted from the raw pixel intensity. This is used twice:</p> <ul style="list-style-type: none"> - in the detection process, if the subtract temporal median box is checked. - in the calculation of the PSF intensity. If N=0, subtraction of global mean is used instead, in order to remove global background. When imaging molecules that do not blink (such as fluorescent beads), this parameter should be set to 0.
	Detection threshold	The detection threshold can be adjusted to allow detection of blinking spots with more or less stringency, depending on the quality of the signal: between 0.5 (least stringent) and 2 (most stringent). The user can start by using 0.5 for astigmatism-deformed signal, and 1 otherwise, then adjust.
	Local mean subtraction Kernel size	This parameter sets N= Kernel window size. If generating the local mean filter, the neighborhood is a kernel window of NxN pixels centered around the pixel. The kernel size must be at least 10 times the PSF (Point Spread Function) pixel area in order to smooth the PSF signal and not take it into account in the background estimation. This parameter determines the size N of the kernel window.
	Min PSF area (pix)	Detected spots that are smaller (in number of pixels) than this min parameter are rejected.
	Max PSF area (pix)	Detected spots that are bigger (in number of pixels) than this max parameter are rejected.
	Super-localization method	<p>Algorithm used to calculate the super-localized lateral positions (x, y) of each detected spot. Three algorithms are possible:</p> <ul style="list-style-type: none"> - Gaussian Fitting - Center of mass - Phasor (Martens et al. 2018) - Deep Learning
Drift correction parameters	Compute drift correction	<p>To correct for lateral drift (x, y), an algorithm based on cross-correlation is used (add ref Wang et al.). The algorithm collects partial nanoscopic images at different steps of the acquisition to realign them. A partial image I_{n+1} at t_{n+1} is compared to a partial image I_n at an earlier time point t_n by generating a cross-correlation map between the two images. The algorithm finds the displacement between the two, and uses it to correct for drift and realign I_{n+1} to I_n. This process is repeated, so that eventually the entire acquisition is drift-corrected. When the "Compute correction" box is checked, drift is measured.</p> <p><i>Note: for single-particle tracking (SPT), the drift correction should be disabled.</i></p>
	Apply drift correction	When the box is checked, the algorithm corrects for measured drift in the output data. The final image and coordinate tables are drift-corrected.
	Nb images per stack	This parameter N is the number of frames in each stack used to reconstruct partial images in the cross-correlation algorithm. It needs to be high enough to ensure that partially reconstructed images feature structures that can be compared between stacks. However, it needs to be low enough in order to detect as many drift events as possible. For example, for an acquisition at 20 FPS (frames per second) with dense recognizable structures, try with N=500. For more pointillistic samples, increasing N is required.
	Offset drift X and Y	When acquiring several colors sequentially, it is important to ensure that different colors are properly aligned. When drift is corrected for the first color, the end of the acquisition is shifted back to the position of the sample at the beginning of the acquisition. Similarly, the acquisition for the second color is shifted to the beginning of the second color. In order to shift it to the beginning of the first color - to ensure proper alignment -, X and Y offsets can be applied when processing the second color, corresponding to the drift measured at the end of the acquisition of the first color. These offsets can be entered manually or detected

		automatically by loading the drift table of the first color when processing the second color.
Quality controller	Compute Squirrel	In single-molecule imaging, enough localizations must be accumulated in order to have a complete reconstructed image. In order to know when to stop an acquisition, decision-guiding tools can be used. SQUIRREL is an algorithm developed by Culley et al. 2017. It simulates a diffraction-limited image based on the reconstructed nanoscopy image and compares it to an image of reference (typically, the fluorescence image before blinking). As long as the simulated image is not able to recapitulate the entire image of reference, the reconstruction is not complete, and the acquisition should continue. <i>Note: no automatic method can determine whether a reconstructed image is satisfying for the user or not. Therefore, it is safer for the user to monitor the live reconstruction directly during the acquisition in addition to decision-guiding methods, to guarantee image quality.</i>
	Apply Squirrel	When the box is checked, the SQUIRREL algorithm runs to give guidance on image quality during the acquisition.
	Microscopy ref image	The user needs to take a snapshot of the epifluorescence image (before blinking) as a reference for the SQUIRREL algorithm.



Effect of background subtraction using temporal median subtraction (right) or local mean subtraction (left).

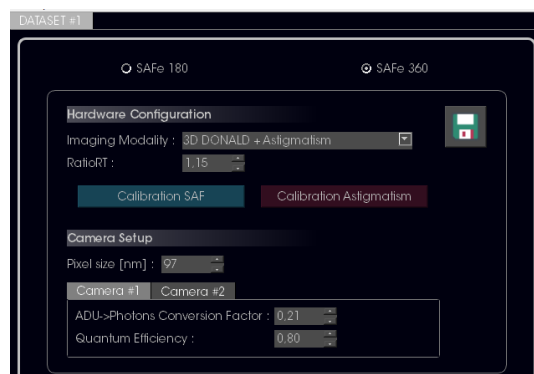
- k. Click on "Live Preview" to get a preview of the processing. On the right, you can see the raw data with the localizations as determined by the software; on the left, you can see the reconstructed image. You can still adjust the localization parameters at this point. Once the parameters are set, click on "Start" to start the processing.



- l. When the processing is over, click on "Save" and save the 2D and 3D nanoscopy image.
- m. Go the saving directory you selected.
 - i. "CoordTable2D.csv" and "CoordTable3D.csv" are the coordinate tables.
 - ii. "Nano*D_*.tif" are the reconstructed images, which you can open in ImageJ.
 - iii. "Paramlogger" contains the processing information.
 - iv. "DriftTable" contains the drift as measured by the drift correction algorithm.

3. **Batch:** in this tab, you can process several datasets in parallel.

- a. Choose the setup you used for acquisition (SAFe 180 for single camera or SAFe 360 for dual camera).

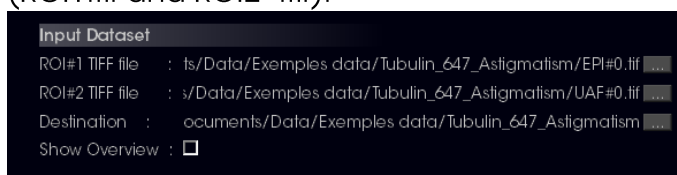


- a. Choose the appropriate imaging mode.
- b. (If needed) Indicate the RatioRT, pixel size and camera info (photon conversion factor and quantum efficiency). All these parameters were provided to your facility manager on the day of the installation.

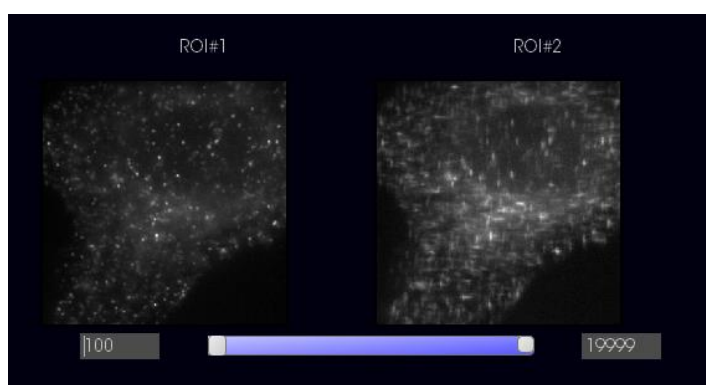
- c. Click on the save icon to save these parameters. *Note: the software will remember the saved parameters, so you do not have to indicate them every time.*



- d. If processing data in 3D using astigmatism, click on "Calibration Astigmatism". Calibration curves for Gaussian Fitting and Phasor algorithms appear. Load the correct calibrations made on your setup, by clicking on "Import file..." and selecting the appropriate calibration curve. *Note: click on "Use demo curve" if you want to use a standard calibration curve; however, keep in mind that demo curves were not made on your setup.*
- e. Select the raw data to analyze ("ROI*.tif"). For SAFe180, only one file is needed. For SAFe360, you need to load the files from both camera (ROI1.tif and ROI2*.tif).



- f. Choose the directory where you want the processed results to be saved. We recommend creating a specific "Analysis" folder for clarity.
- g. Check the "Show Overview" box to visualize the raw data (this may take a few seconds). Use the slider to remove frames at the beginning or at the end of the acquisition. Make sure you start the processing when fluorophores are properly blinking. Click on "Ok".



- h. You can adjust the localization parameters on the right. For beginner users, the default localization parameters are sufficient to reconstruct nanoscopy data. Depending on the quality of the signal, you can adjust the detection threshold (see table in section 2.h, "Detection threshold"). *Note: for single-particle tracking (SPT), make sure you inactivate drift correction.* For more experienced users, the table in section 2.j describes how to adjust all localization parameters.

- i. The default settings are appropriate for most STORM acquisitions, but if needed (depending on the quality of the acquisition and how bright the PSFs are) you can adjust the detection threshold: between 0.5 (least stringent) and 2 (most stringent). *Note: for single-particle tracking (SPT), make sure you inactivate drift correction.*

The screenshot displays a software interface with a dark background and white text. It is divided into three main sections: 'Background Removal', 'Detection and Super-Localization parameters', and 'Drift Correction'. In the 'Background Removal' section, there is a checkbox for 'Subtract Temporal Median' which is unchecked, and a numeric input field for 'Nb Background Images' set to 50. The 'Detection and Super-Localization parameters' section features a table with two columns, 'ROI#1' and 'ROI#2'. Below the table, several parameters are listed with their corresponding values in input fields: 'Detection threshold (0.5->2)' is 1.00, 'Local Mean Subtraction Kernel Size [pix]' is 15, 'Min PSF Area [pix]' is 9, 'Max PSF Area [pix]' is 50, '2D Super-localization method' is 'Gaussian Fitting', and 'Integration Mask Size [pix]' is 21. The 'Drift Correction' section includes input fields for 'Offset Drift X' and 'Offset Drift Y', both set to 0.00000, a 'Load Drift Table of Previous Color' button, a checked checkbox for 'Compute Drift Correction?', and a numeric input field for 'Nb Images per Stack' set to 500.

ROI#1	ROI#2

Background Removal

Subtract Temporal Median :

Nb Background Images : 50

Detection and Super-Localization parameters

ROI#1	ROI#2

Detection threshold (0.5->2) : 1.00

Local Mean Subtraction Kernel Size [pix] : 15

Min PSF Area [pix] : 9

Max PSF Area [pix] : 50

2D Super-localization method : Gaussian Fitting

Integration Mask Size [pix] : 21

Drift Correction

Offset Drift X : 0.00000

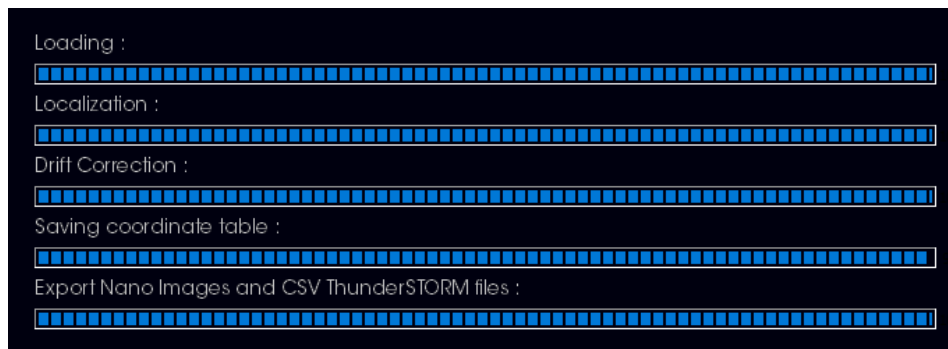
Offset Drift Y : 0.00000

Load Drift Table of Previous Color

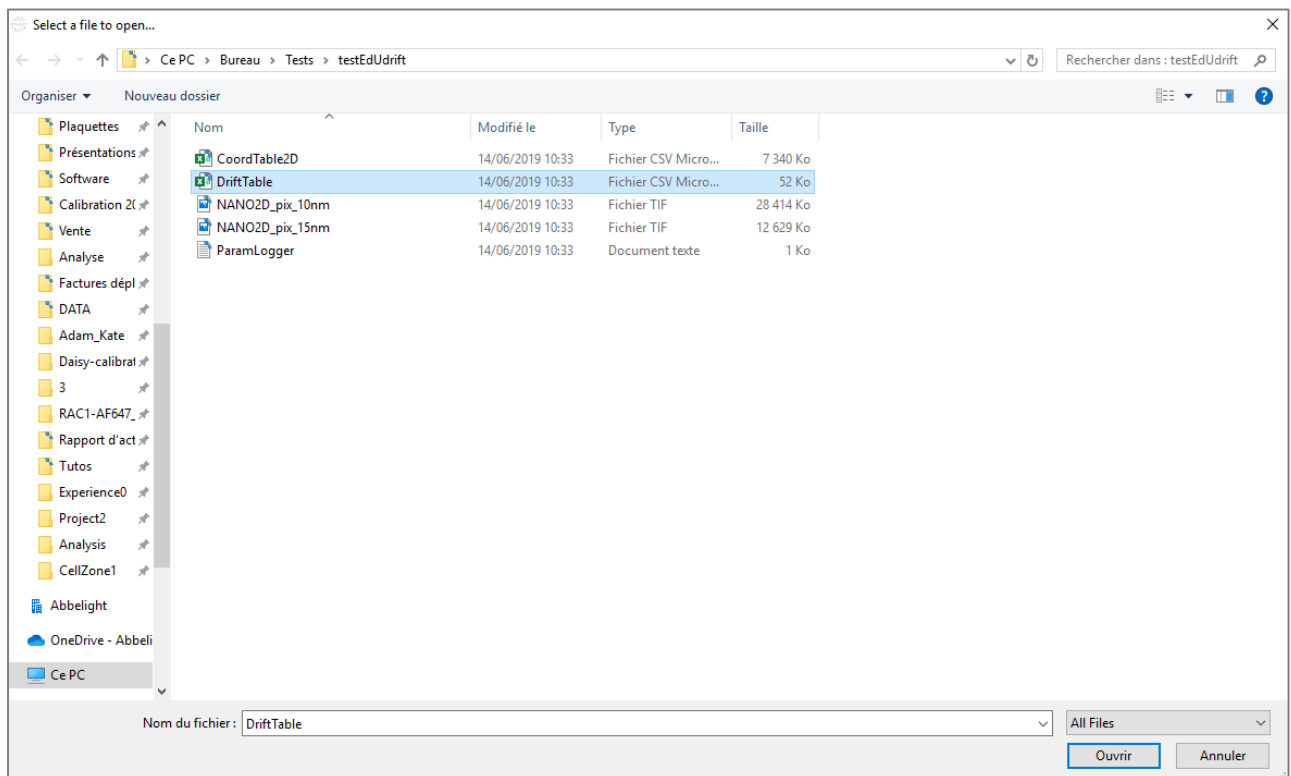
Compute Drift Correction?

Nb Images per Stack : 500

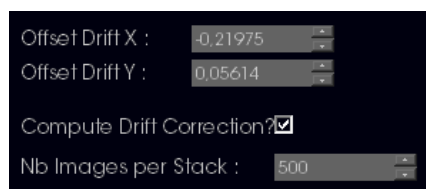
- j. Click on "Add Dataset" and repeat this process for all the datasets to be processed. *Note: be careful, the "Reset" button will remove all the datasets.*
- k. When ready, click on "START". You can see the progression of the processing on the right for each dataset by clicking on the corresponding tab.



- I. In the case of sequential multicolor imaging, it is important to ensure that different colors are properly aligned. When processing the second color:
 - i. Click on “load Drift Table of Previous color”.
 - ii. Select the “DriftTable.csv” file from the previous color (located in the output directory you chose for the previous color):



- iii. The Offset Drift X and Offset Drift Y should be updated:



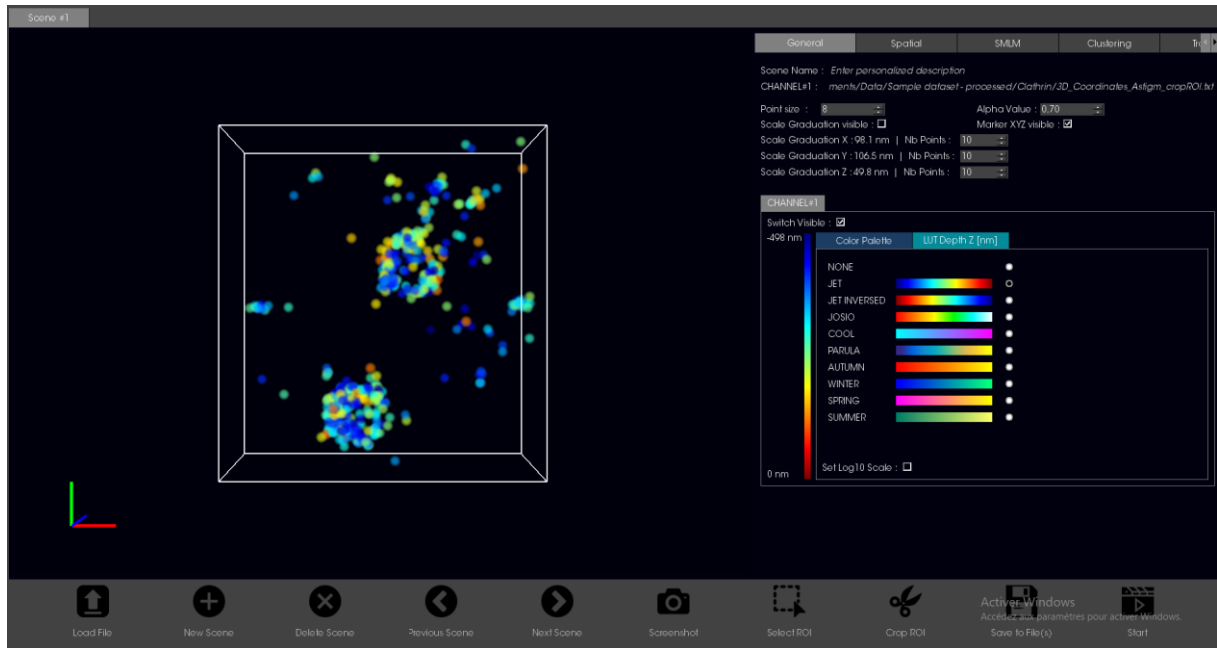
- iv. Start the processing.
- m. When the processing is over, go the saving directory you selected.
- v. “CoordTable2D.csv” and “CoordTable3D.csv” are the coordinate tables.

- vi. "Nano*D_*.tif" are the reconstructed images, which you can open in ImageJ.
- vii. "Paramlogger" contains the processing information.
- viii. "DriftTable" contains the drift as measured by the drift correction algorithm.

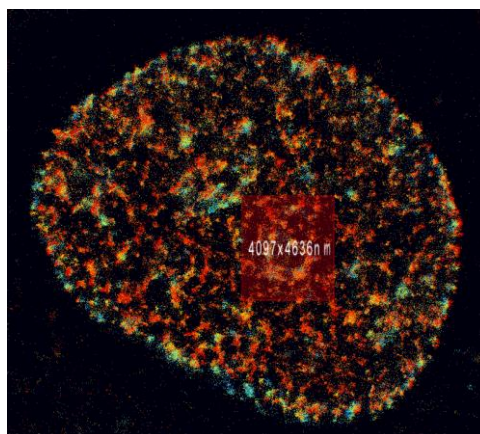
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Data analysis with NEO software

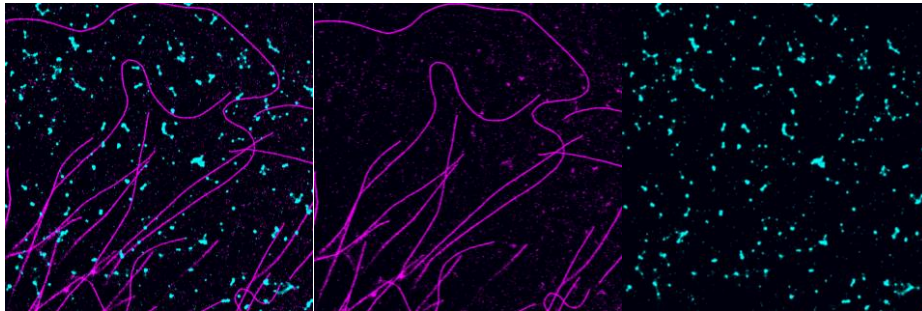
1. Open **NEO_Analysis** software.
2. Go to the Viewer tab (make sur the Process and Batch tabs are closed). Here, you will be able to visualize and quantify your data.
3. Click on "Load File" and select the "CoordTable*D.csv" file corresponding to the dataset you want to analyze. Your image should appear in the viewer.



4. You can visualize several datasets in parallel:
 - a. To open another dataset, click on "New scene" then "Load File".
 - b. You can navigate between scenes by using the "Previous scene" and "Next scene" arrows.
 - c. You can choose your region of interest by clicking on "Select ROI", drawing a rectangle around your ROI, and clicking on "Crop ROI". The new ROI appears in a new scene.

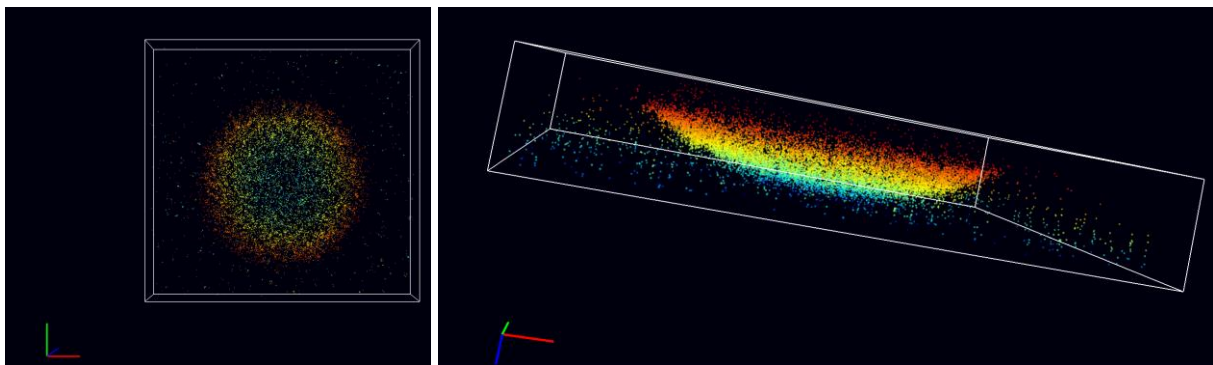


5. You can use "Save to file" to export modified coordinate tables, images and quantifications, and "Screenshot" to save a screenshot of the viewer.
6. On the right, you have access to several tabs: General, Spatial, SMLM, Clustering, Tracking, Spectral Demixing.
7. **General:** in this tab you can adjust visualization features:
 - a. The size of each localization "Point Size".
 - b. Its brightness "Alpha Value".
 - c. Whether the frame and XYZ marker are visible and the scale.
 - d. The colormap for 3D datasets, where color represents the z coordinate.
 - e. Use the "Switch visible" button to visualize, or not, a dataset (for example in the case of multicolor imaging).

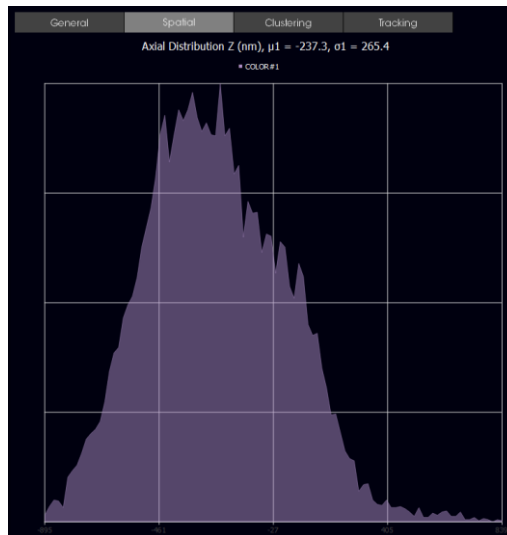


8. **Spatial:**

- a. In the **Spatial Distribution** tab:
 - i. Use the left click of your mouse to rotate your image and see the 3D visualization. The XYZ marker indicates the orientation.



- ii. The graph shows the z distribution. You can use the clipping tools to crop in X, Y, and Z.



Clipping Tools (nm)

x min : 1136.3 y min : 928.2 z min : -600.0

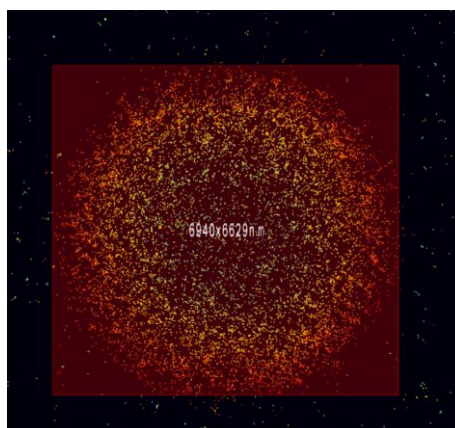
x max : 47545.4 y max : 47466.5 z max : 600.0

[Crop ROI](#)

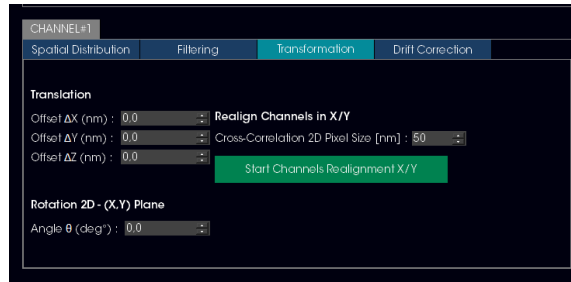
iii. The table gives information on volume or surface area, number of localizations, and average density on the total dataset or on a selected ROI.

	Total Dataset :	ROI Selected :
Volume (μm^3) :	3754.24	5.50671
Nb of Locs :	21791	1247
Average Density ($\#/\mu\text{m}^3$) :	5.804	226.451

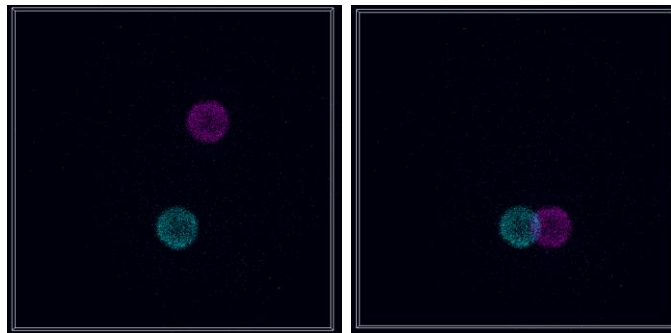
iv. When selecting a ROI, its size appears on the image in nm. You can use it as a measuring tool.



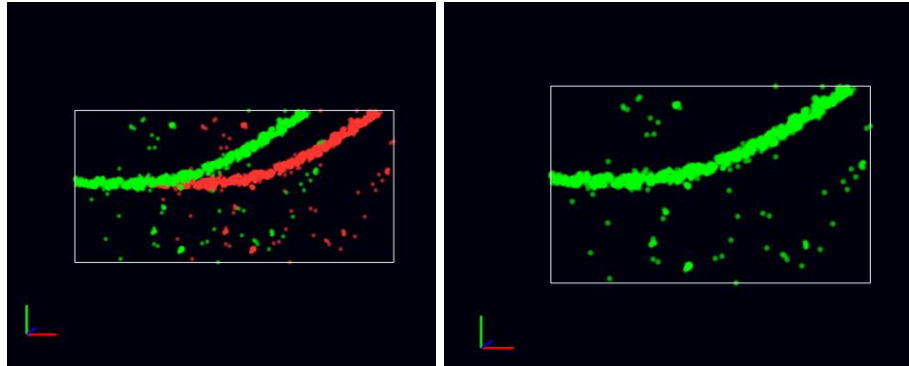
b. In the **Translation** tab:



- i. The **Translation** tool allows you to manually translate the dataset using "Offset ΔX (nm)", "Offset ΔY (nm)", and "Offset ΔZ (nm)". This can be done specifically for one color by choosing the proper "Channel#*" tab.



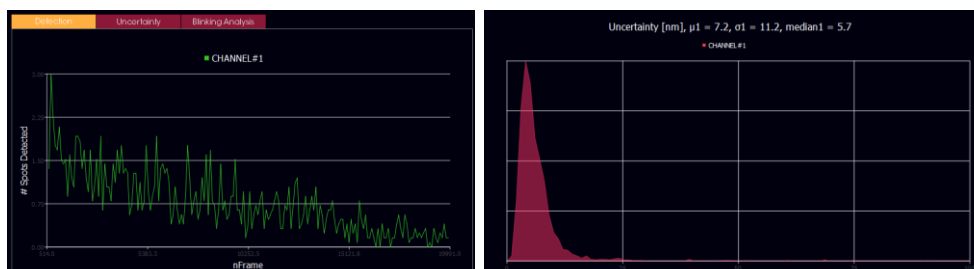
- ii. Use the "Realign Channels in X/Y" tool to align two channels automatically using cross-correlation:



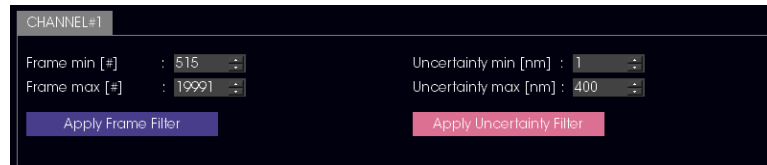
- iii. Use the **Rotation** tool to add an angle to your dataset.

9. **SMLM**: in this tab, you can have access to quantitative information on the quality of your blinking:

- a. Visualize graphs showing the number of localizations found throughout your acquisition as well as the uncertainty:



b. You can filter based on the frame number and the uncertainty:



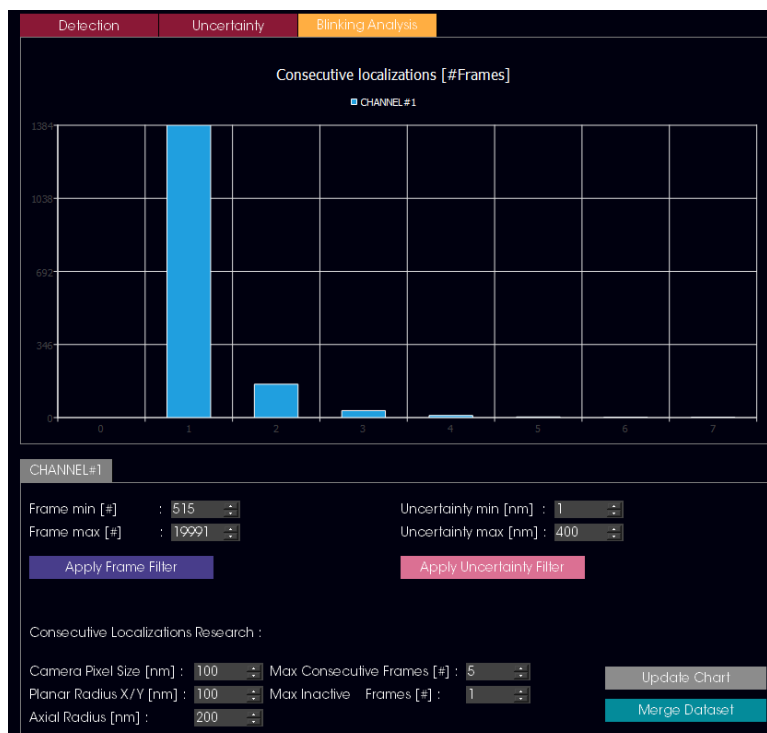
CHANNEL#1

Frame min [#] : 515 :
Frame max [#] : 19991 :
Uncertainty min [nm] : 1 :
Uncertainty max [nm] : 400 :

Apply Frame Filter Apply Uncertainty Filter

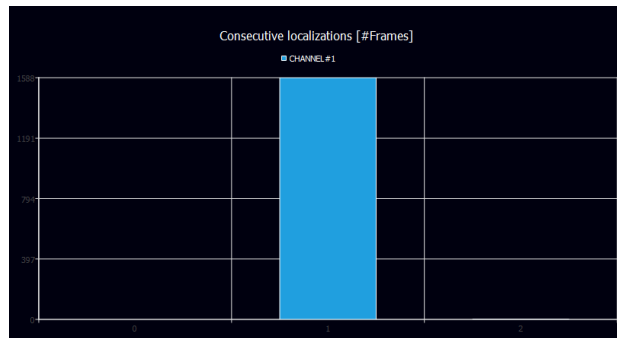
c. **Blinking analysis:** this tool indicates how many localizations appear in several consecutive frames and allows you to merge them. Indeed, a localization found at the same place in several consecutive frames can be considered the same molecule. To only count it once in your data, this section allows you to merge consecutive-frame localizations.

i. Click on "Update Chart" to visualize the bar chart of consecutive localizations.



In the example above, the chart shows that most (1384) localizations only appear in one frame, about 175 localizations appear in 2 consecutive frames, etc.

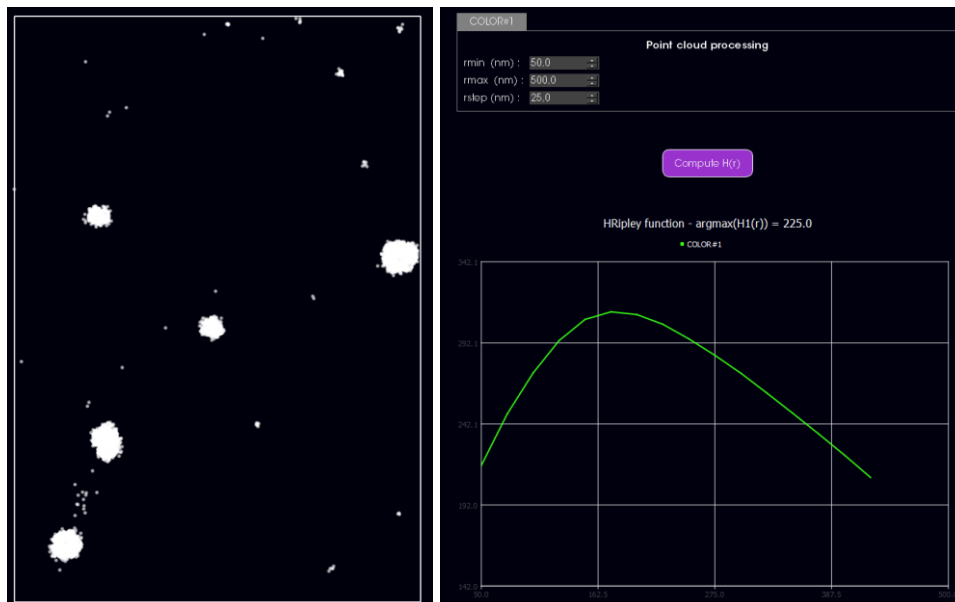
- ii. If you click on "Merge Dataset", consecutive localizations are merged, creating a new coordinate table and therefore a new scene.
- iii. Click on "Update Chart" again. This time, you will see that all localizations are found on only one frame. The dataset was successfully merged.



iv. Use the "Save to File" button to export this merged dataset.

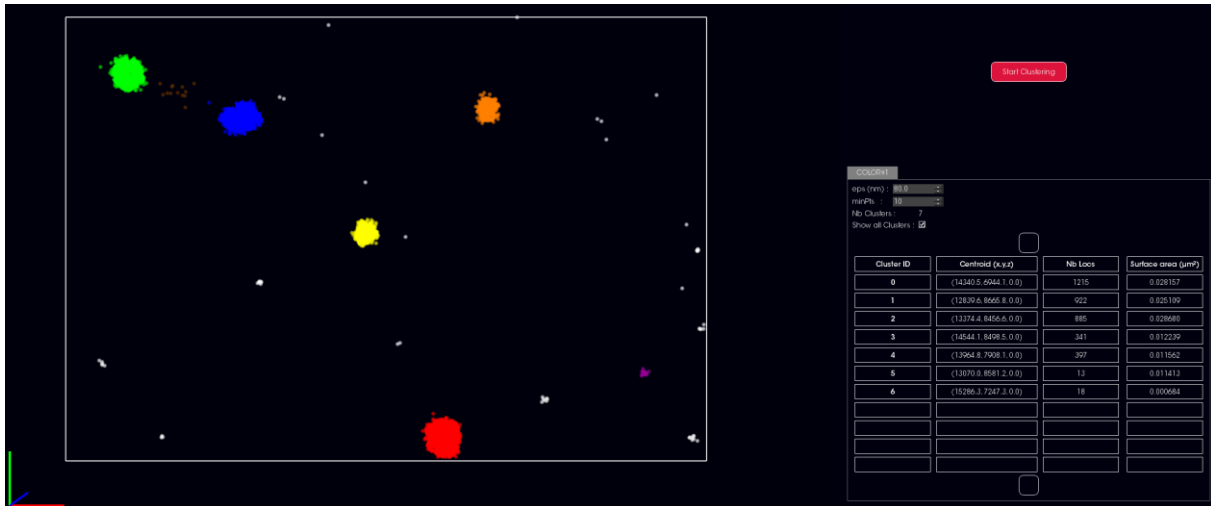
10. **Clustering:** in this tab, you have access to three different methods to study clustering.

a. K Ripley (see Ripley et al. 1976) is a function to determine if a population of localizations is clustered or randomly distributed. It is based on number of neighbors within each search distance.

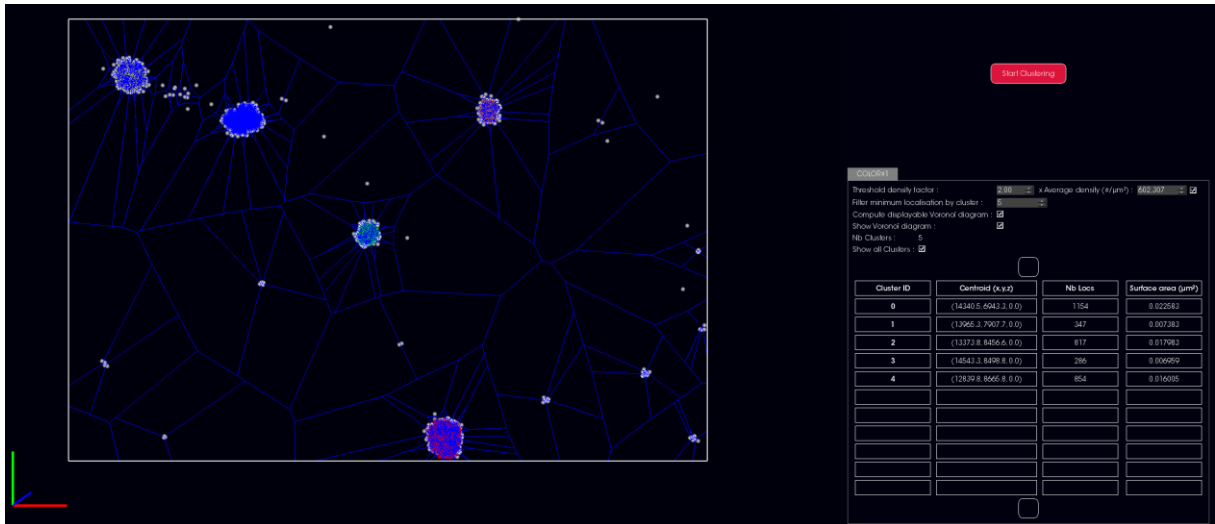


b. DBSCAN (Density-Based Spatial Clustering of Applications with Noise, see Ester et al. 1996) is a method to detect individual clusters in an image.

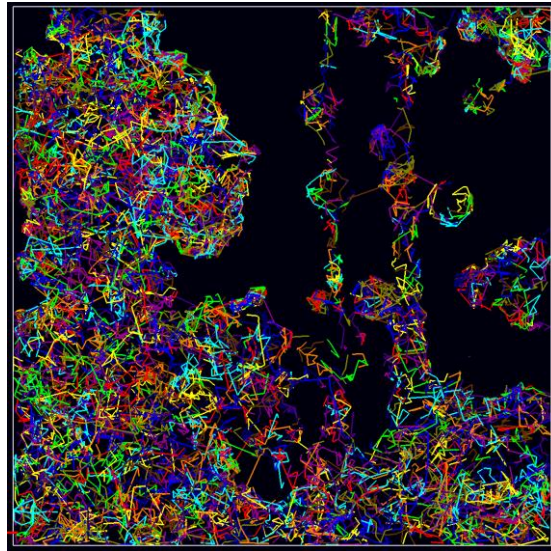
- i. You can adjust two parameters: eps (nm) and MinPts. The software counts a localization as part of a cluster if at least MinPts localizations are located within a distance of eps. Increasing eps will lead to detection of bigger clusters and increasing MinPts will lead to detection of clusters with more localizations.
- ii. The detected clusters appear in different colors.
- iii. On the right, the list of individual clusters appears with: cluster ID, coordinates of centroid, number of localizations in cluster, surface area (um²) or volume (um³).
- iv. Clicking on a cluster ID singles it out on the image on the left.



- c. Voronoi (see Levet et al. 2015) is a method to detect individual clusters in an image.
 - i. A Voronoi diagram partitions the image into polygons called Voronoi cells. Each cell contains one and only one localization. Therefore, cells in high-density regions of the image are small, while cells in low-density regions are big. Localizations are counted as part of a cluster if they are located in a region whose density is above a certain threshold. In the Voronoi tab, you can adjust the "threshold density factor" and "average density". By default, the threshold density factor is set at 2, and the average density factor is the average density of the whole image, which means that a region has to be at least twice as dense as the average density of the image to be considered a cluster. You can increase or decrease the threshold density factor for more or less stringency, and manually type in an average density.
 - ii. The detected clusters appear in different colors and you can see the Voronoi diagram by selecting the corresponding box.
 - iii. On the right, the list of individual clusters appears with: cluster ID, coordinates of centroid, number of localizations in cluster, surface area (um²) or volume (um³).
 - iv. Clicking on a cluster ID singles it out on the image on the left.



11. **Tracking:** this tab is dedicated to the study of single-particle tracking (SPT).



a. Track reconstruction:

- i. The algorithm for track reconstruction is based on Jaquaman et al. 2008. The principle of the algorithm is to list trajectories T_i at a frame t , and list localizations P_j present at frame $t+1$, calculate costs associated with assigning each T_i to a P_j , and to choose the outcome that minimizes the costs. Costs are calculated based on (1) the distance between T_i and P_j and (2) the speed of T_i .
- ii. When the "Constant speed" box is unchecked, only the distance (1) is taken into account: tracks will be reconstructed based on closest localization in the next frame. When the "Constant speed" box is checked, the speed (2) is also taken into account: tracks that maintain a constant speed will be favored. Increasing the Linking Cost Weight will give more importance to this method (set at 1 by default: cost of distance and cost of speed weigh equally).

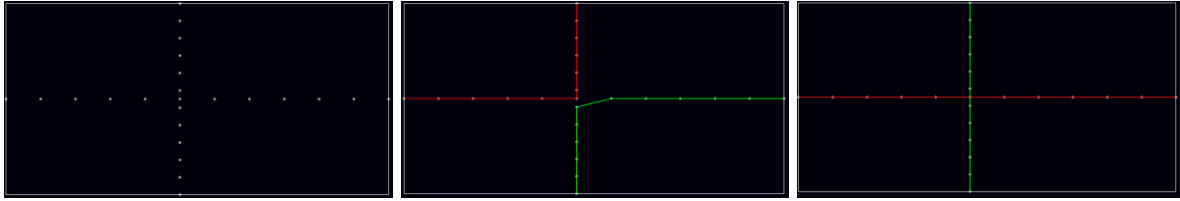


Figure - left : localizations for track reconstruction; middle: track reconstruction based on distance ("Constant speed" box unchecked); right: track reconstruction based on distance and speed ("Constant speed" box checked).

- iii. Frame to Frame Linking: adjusting the max linking distance d_{lim} limits the search area. Track reconstruction in the next frame will only search in a maximum distance of d_{lim} .

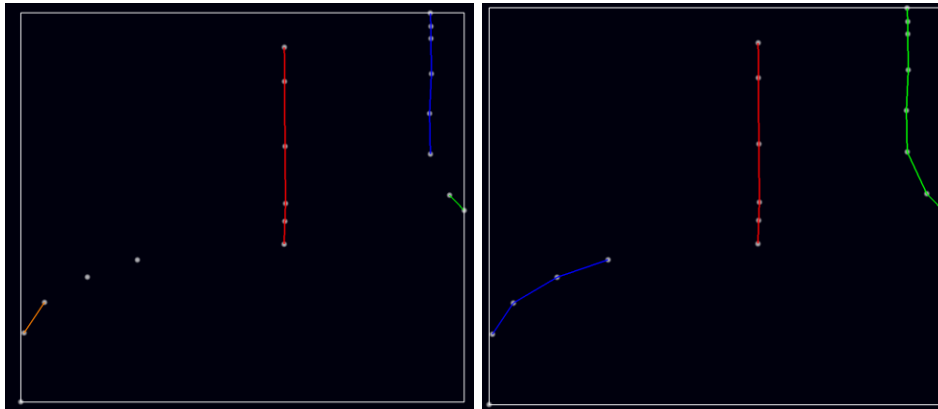


Figure - Left : maximum linking distance $d_{lim}=250$ nm; right: $d_{lim}=500$ nm.

- iv. Gap closing: the algorithm allows for temporary disappearance of molecules within a track. You can choose the maximum number of frames in which you allow a molecule to disappear. If the "Synchro with frame linking settings" box is checked, when there is a gap, the algorithm searches in a larger search area ($1 \text{ frame/gap} \rightarrow 2 * \text{max linking distance}$, etc). You can uncheck this box and manually input the max linking distance for the gap closing.

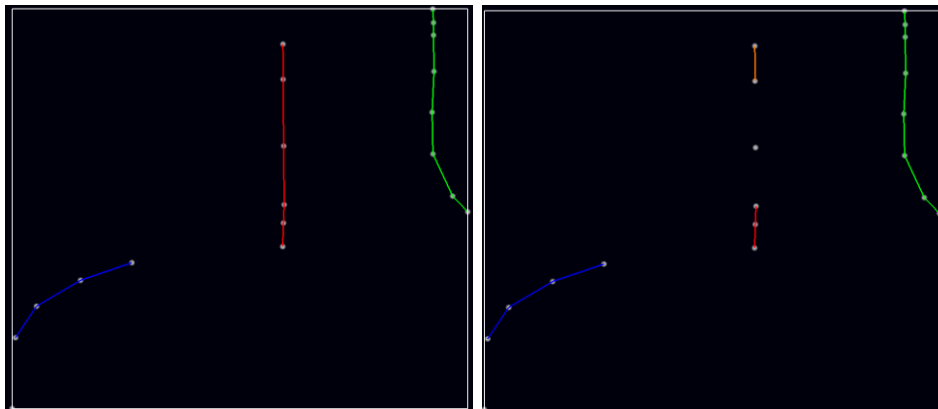
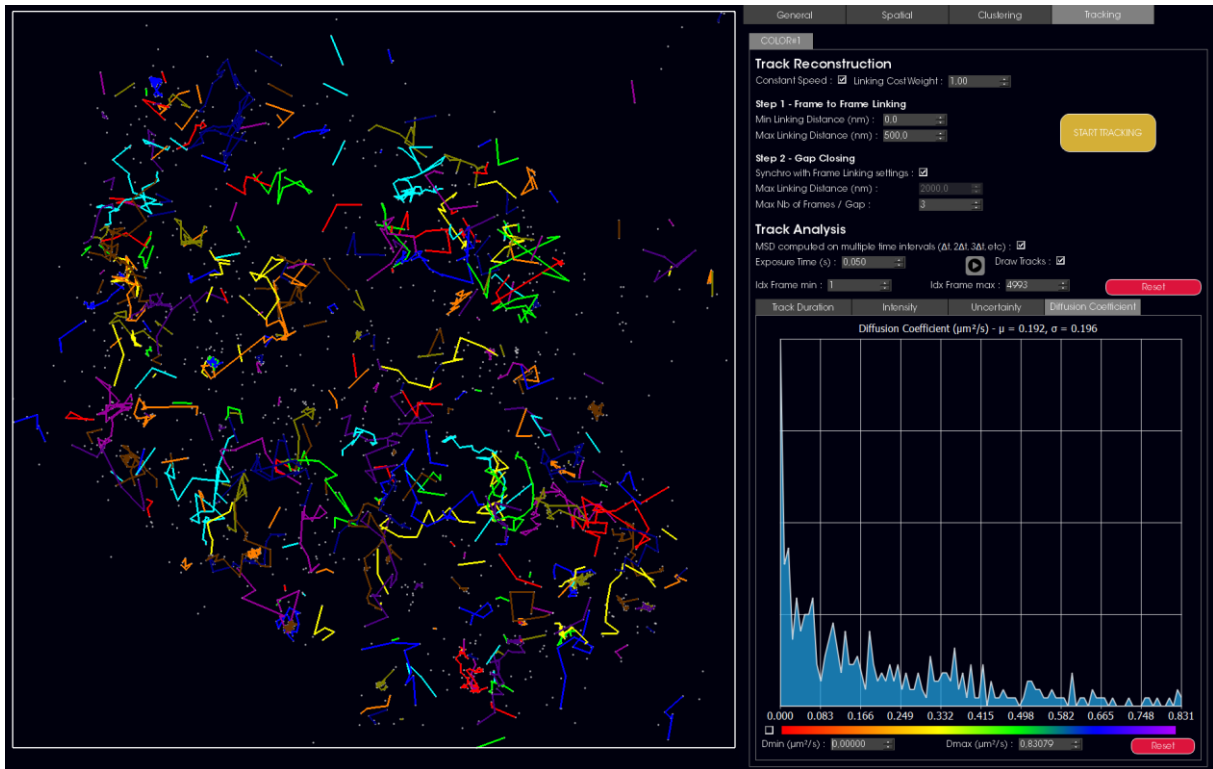
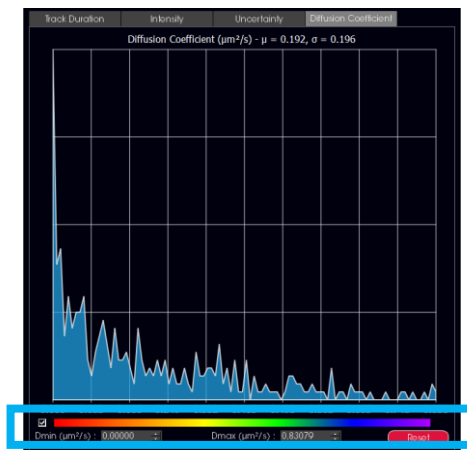


Figure - Gap closing: left: max number of frames/gap = 3; right: max number of frames/gap = 0.

- v. Track analysis: indicate the exposure time of your acquisition. The diffusion coefficient is based on MSD analysis (see Weimann et al. 2013). If you anticipate your tracks to be long enough, check the box "MSD computed on multiple time intervals". However, if you anticipate your tracks to be short, unchecking that box will limit MSD calculation to intervals of $1 * \Delta t$, but keep in mind that MSD is not the most appropriate method for short tracks.
- vi. Click on "Start Tracking". The tracks appear on the left.



- vii. The graphs on the right indicate distribution of track duration, intensity, uncertainty, and diffusion coefficient. Filters can be applied on these parameters to visualize corresponding tracks. A color code can be applied to the tracks based on the diffusion coefficient.



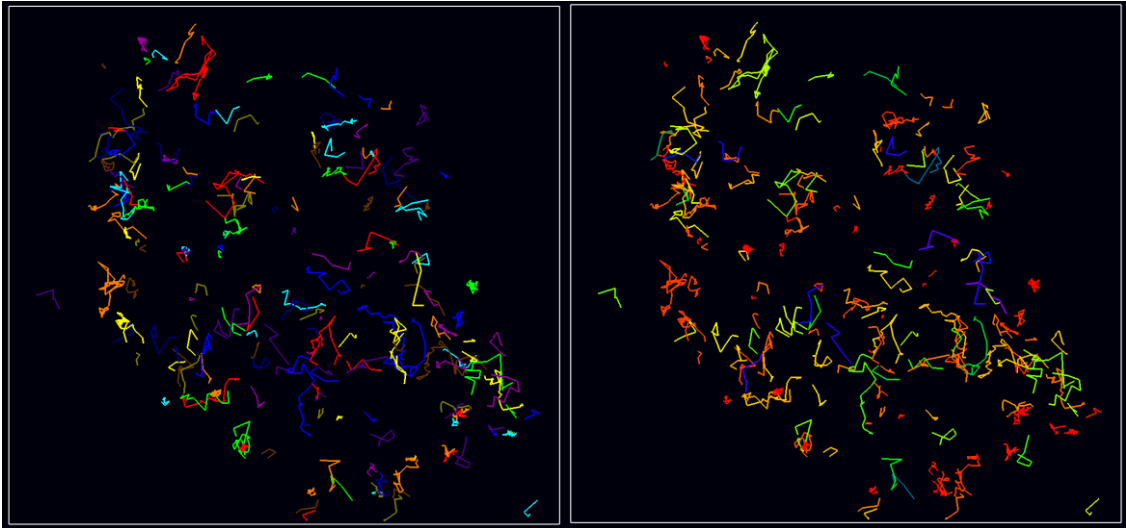
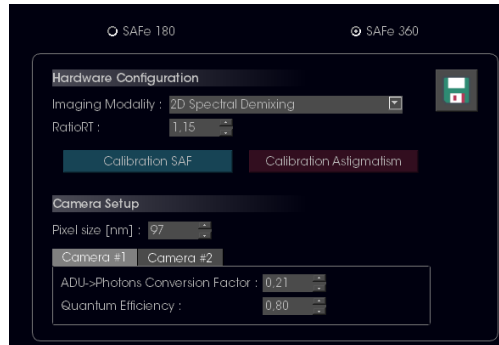


Figure – top: filter and color code based on diffusion coefficient; bottom left: color code random; bottom right: color code based on diffusion coefficient.

abbelight user guide

Spectral Demixing with NEO software

1. Open **NEO_Analysis** software.
2. Go to the **Batch** to process your data (*.TIFF) using the SAFe 360 - 2D Spectral Demixing mode.



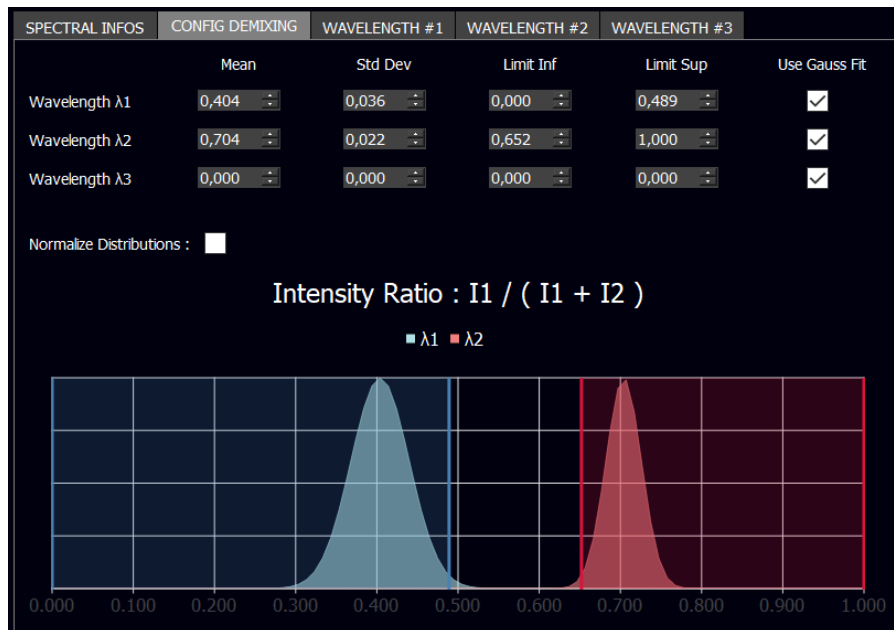
Three output files are generated :

- [CoordTable_SAFE360_2D_MULTIPLEXING_ROI1.csv](#)
- [CoordTable_SAFE360_2D_MULTIPLEXING_ROI2.csv](#)
> reconstruction of each channel independantly
- [CoordTable_SAFE360_2D_MULTIPLEXING.csv](#)
> reconstruction recombining channels information

3. Go to the **3D Viewer**.
Here, you can perform demixing on your reconstructed data.
4. Click on "Load File" in the bottom menu to open the CSV file(s) of your multicolor dataset to demix by following one of these two procedures:
 - Open the [CoordTable_SAFE360_2D_MULTIPLEXING.csv](#) file, or;
 - Open sequentially [CoordTable_SAFE360_2D_MULTIPLEXING_ROI1.csv](#) and [CoordTable_SAFE360_2D_MULTIPLEXING_ROI2.csv](#) files in the same scene (the 'recombining channels' step by PSF matching will be performed before demixing)
5. Go to the Spectral Demixing tab:
 - In the SPECTRAL INFOS menu, you can click on "Compute Distribution" to visualize the spectral intensity ratio of your dataset loaded in the central scene
 - In the CONFIG DEMIXING menu, you can set a configuration to perform demixing by following one of these two procedures:
 - Click on the "Import Calib" button to load an existing calibration. It will fill directly the config params with the calibration imported values.

(Please find below the supplementary user guide to generate your own calibration for Spectral Demixing)

- Enter values manually for each wavelength (mean, std dev, limit inf, limit sup)



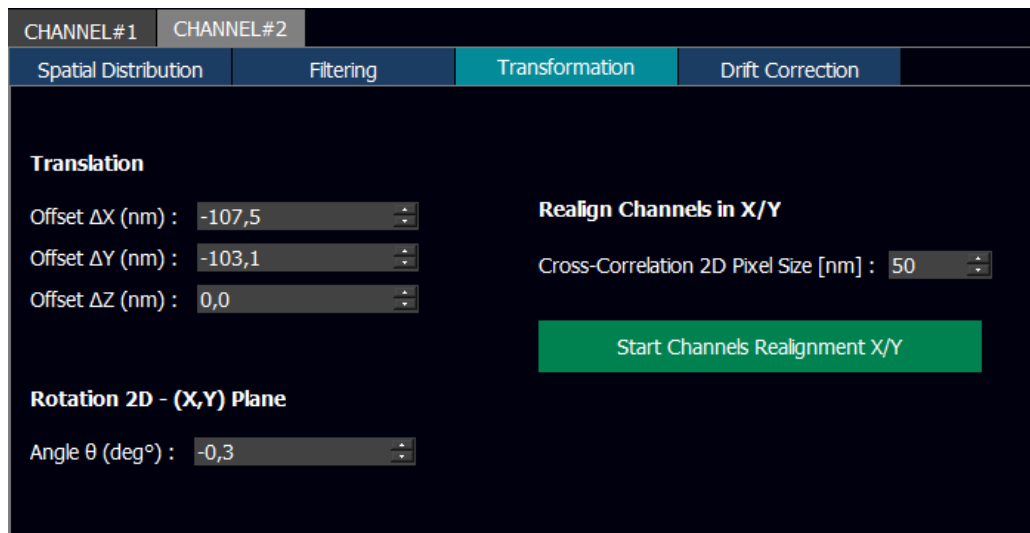
6. If you loaded the two **CoordTable_SAFE360_2D_MULTIPLEXING_ROI1.csv** and **CoordTable_SAFE360_2D_MULTIPLEXING_ROI2.csv** files: we offer additional tools to recombine channels before demixing:

- **Realign channels in X/Y**

Channels reconstructed independently may not be properly aligned due to hardware cameras misalignment.

Go to the **Spatial > Transformation** tab and follow one these procedures:

- Fill manually the 'Offset' and 'Angle' values to apply transformations on channels.
- Click on **Start Channels Realignment X/Y**. It will compute the best affine transformation (translation + rotation) that realigns channels using a cross-correlation algorithm.



- **PSF association parameters** (Spectral Demixing tab)
 - **Radius PSF Matching [nm]** : maximum distance between PSFs on the two channels to be considered as the same single-emitter detection (set to 500 nm for low density datasets and try smaller values in case of higher density)
 - **Enable mono-channel PSF detection** : check this option to maintain demixing on PSFs detected only on one channel, even if no PSF matching is found on the other channels.
 Due to the spectral demixing hardware setup, single-emitters with a high intensity ratio may have PSFs with low SNR (Signal-to-Noise Ratio) on one of the channels, so the PSF may not be detected. If this option is checked, these PSFs will be assigned to the wavelength with the lowest/highest ratio.
Example: if a PSF appears only on channel 1, we can consider that $I_1 \gg I_2$, with a high ratio_intensity close to 1 ($ratio_intensity = I_1 / (I_1 + I_2)$), so we can assign this PSF to the wavelength of highest ratio.
 If unchecked, PSFs detected only on one channel are discarded.

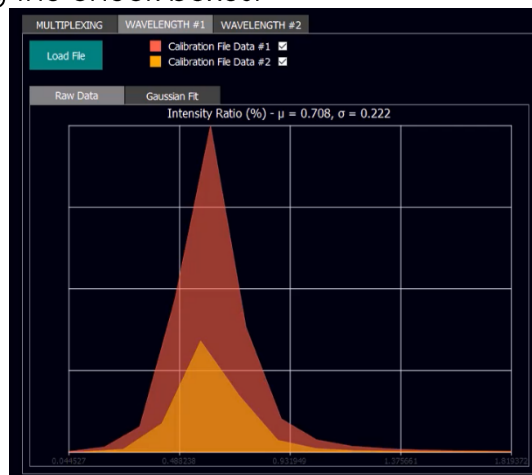
7. Click on the **Start Demixing** button to demix the dataset loaded in the scene. The demixed data appears in a new scene.
8. Use the **Save to file(s)** button, in the general bottom menu, to export:
 - a. The coordinate tables of the separated colors in *.CSV format.
 - b. The TIFF images of the different colors. *Note: in order to overlay TIFF images of different colors in ImageJ, it is essential to generate them from the same scene. If different colors are open in different scenes, the resulting TIFF images will have slightly different dimensions (determined by the min and max x and y coordinates in each table), and, as a result, ImageJ won't be able to overlay. **As a general rule, in the case of multicolor datasets, always load the coordinate tables of different***

colors in the same scene in order to generate TIFF images with common dimensions.

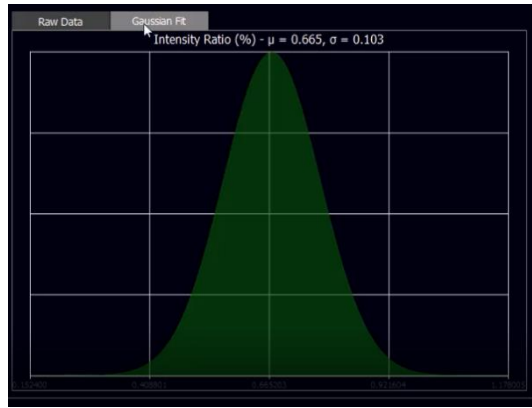
- c. General quantitative information

Supplementary tutorial – Calibration

1. First, you need to generate calibrations for each wavelength you want to demix:
 - a. Go to the WAVELENGTH#1 tab.
 - b. Click on [Load File](#).
Load a coordinate table *.CSV file corresponding to a monocolour staining of wavelength 1. *Reminder: in order to properly perform spectral demixing, you need to acquire monocolour images for each staining in the same hardware conditions to use as calibration. For example, if you want to demix Tubulin-CF680 and Clathrin-AF647, you need to prepare three samples:*
 - i. Tubulin-CF680 alone (for calibration)
 - ii. Clathrin-AF647 alone (for calibration)
 - iii. Tubulin-CF680 and Clathrin-AF647 together
 - c. The distribution of the intensity ratio for this wavelength will appear below.
 - d. You can load more of such coordinate tables. The distributions will all appear in the graph. You can choose which ones you want to use for calibration using the check boxes.



- e. In the Gaussian Fit tab, you can see the fit of all the distributions:



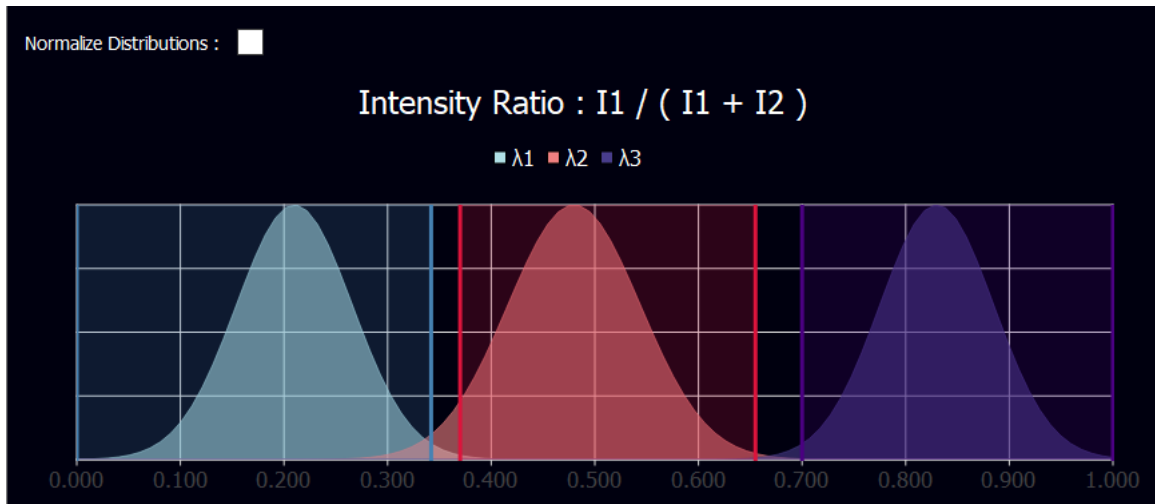
f. Repeat this process for Wavelength 2, 3, etc.

2. Go to the CONFIG DEMIXING tab to visualize and adjust your calibration.

a. For each wavelength, the mean and standard deviation computed from the calibration data have appeared. You can choose to use the Gaussian fit calibrations (box checked) or raw calibrations (box unchecked).

SPECTRAL INFOS	CONFIG DEMIXING	WAVELENGTH #1	WAVELENGTH #2	WAVELENGTH #3	
	Mean	Std Dev	Limit Inf	Limit Sup	Use Gauss Fit
Wavelength λ1	0,210	0,056	0,000	0,342	<input checked="" type="checkbox"/>
Wavelength λ2	0,480	0,064	0,370	0,655	<input checked="" type="checkbox"/>
Wavelength λ3	0,830	0,055	0,700	1,000	<input checked="" type="checkbox"/>

b. You can visualize the calibration distributions in the graph below. If using raw calibration ("Use Gauss Fit" unchecked), you can also visualize the normalized distributions by checking the "Normalize distribution" box. If using the Gaussian Fit calibration, the distributions are by default normalized.



- c. On the graph, the vertical lines indicate the limits used for the spectral demixing. You can adjust these limits manually using the "Limit Inf" and "Limit Sup" input boxes. Any localization that has an intensity ratio between the inferior and the superior limit of wavelength X will be assigned to wavelength X. *Note: in case of overlap between the ranges of different wavelengths, the localization is assigned to both wavelengths; therefore it's best to avoid overlap between wavelengths.*
3. Once you have chosen the appropriate spectral demixing limits, you can export the calibration for future use by clicking on the "Export calib" button. The calibration file saved contains only the final infos from the CONFIG DEMIXING tab: mean, standard deviation, inferior and superior limits, and the distributions.