

Getting started with SMAP using a simple example

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1 Installation

SMAP can be run either as a fully functional stand-alone version or within MATLAB. This information can also be found in the *SMAP user guide*.

1.1 Installing the stand-alone version

On www.rieslab.de scroll to *Software* and click on download compiled version for Windows and Mac. In the web page that opens you find a link to the installation guide: https://www.embl.de/download/ries/SMAPCompiled/Installation_notes_SMAP_compiled.rtf

SMAP needs write access to its settings directory:

- **Mac:** You need to grant write access to all files in **/Applications/SMAP/application/settings** and **/Applications/SMAP/application/Documentation/pdf**. For this, right-click the **/Applications/SMAP** directory, select Get info, select 'Read & Write' privilege for the current user (or everyone, if this doesn't work, for current user and administrator), click on the lock symbol and enter the computer password to allow for changes, and below in the settings icon click 'Apply to enclosed items'.
- **PC:** Right-click on **Program Files/SMAP/applications/** to show Properties. In the **Security** tab, allow *full control* to **Users** and **ALL RESTRICTED APPLICATION PACKAGES**.

Finally, start SMAP from the task bar or application folder with a double click. In case of problems concerning access rights, please have a look at the trouble shooting guide in the installation notes.

1.2 Installing the MATLAB version

Requirements

1. MATLAB 2019a and newer. Toolboxes: *Optimization, Image processing, Curve fitting, Statistics and Machine Learning*.

Box 1: The example sample.

Nup96-SNAP labeled with BG-AlexaFluor647. Homozygous CRISPR cell lines, imaged in BME-based oxygen depleted imaging buffer as described in Thevathasan et al, Nature Methods 2019. This sample was acquired under very slow imaging conditions with 500 ms exposure time and an excitation intensity of 0.14 mW, leading to a high localization precision and a large number of re-activations. Images were acquired on a custom microscope using an sCMOS camera.

2. Mac or Windows (Linux users will have to compile some C code into mex functions).
3. For GPU fitting: Windows, NVIDIA graphics card. CUDA driver (recommended: version 7.5). All fitters also come with a CPU version that is used when these specifications are not met.

Installation

1. Clone git repository:
 - a. Install git (<https://git-scm.com/>) if needed. Use Terminal (MacOS) or Cmd (Win). Use cd to navigate to the target directory. (e.g. cd git).
 - b. Type: git clone <https://github.com/jries/SMAP.git> and type in your username and password for your git account.
2. To start SMAP in MATLAB: run `SMAP.m` from the SMAP installation folder, if prompted, change folder.

1.3 Installing third-party software

1. As the simple example was acquired using Micromanager, install Micromanager 1.4.22 or later from <https://micro-manager.org>.
2. In the Menu select **SMAP/Preferences...** Switch to the **Directories** tab and select the directory of Micro-Manager (main Micro-Manager directory, where you find the ij.jar, which however is not displayed in the file dialog on a PC). Press **Save and exit**.

2 Preparation

1. Download and unzip the example file `Gettingstarted2D_Nup96-AF647.zip` from www.rieslab.de. For details on the sample see **Box 1**.
2. You can select **Menu/SMAP/hide advanced controls** to have a simple GUI and hide advanced controls with default parameters. This guide is based on this simple GUI.
3. If during the following protocol things don't work as expected, try restarting SMAP. You can load the last *.sml file that was produced by clicking on **Load** in the **File** tab and selecting this file.

3 Overview of the SMAP GUI

This part is copied from the SMAP User Guide. After starting SMAP the SMAP GUI opens. Note that most elements in the GUI have a tool tip: hover with the mouse over the control to display it. Figure 1 shows the main parts of the GUI:

1. *Menu*. There are three menu items:

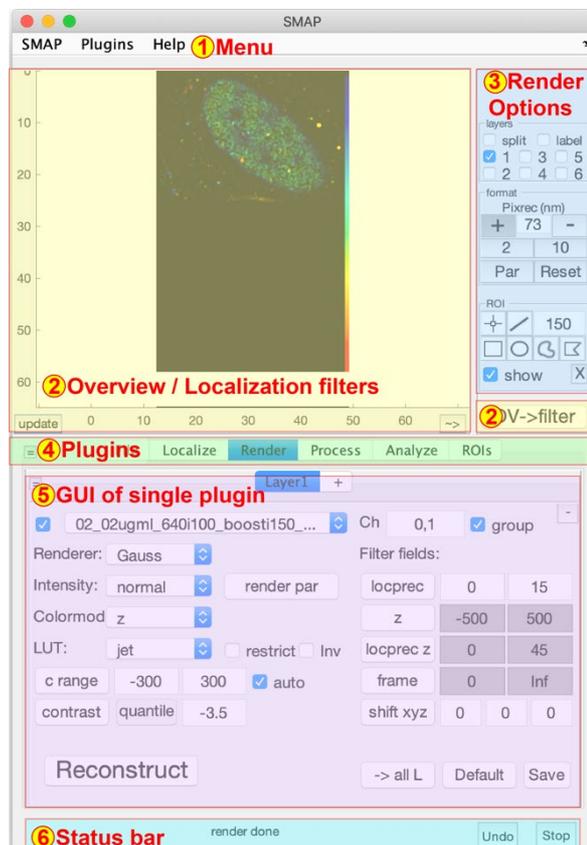


Figure 1: Main parts of the SMAP GUI.

- a. SMAP: gives access to Preferences and the Camera Manager, allows saving and loading the GUI appearance, and allows toggling between a simple version of the GUI, in which optional parameters are hidden, and the advanced version of the GUI with all GUI elements shown.
 - b. Plugins: here any plugin (except those that work only in the context of other plugins) can be called.
 - c. Help: shows this documentation.
 - d. Optional user-defined menus appear here as well.
2. *Overview/Filters*. In this part of the GUI an overview image of the data is shown. Clicking on the image centers this part in the separate image window. Alternatively, this part of the GUI shows the filters that are applied to the localization data. The button **OV -> filter** toggles between these two modes.
 3. *Render options*. Controls in this panel relate to the rendered image (channels, magnification, user-defined ROIs).
 4. *Plugin tabs*. Each tab corresponds to a main task (loading/saving, fitting of single-molecules, rendering, ROI manager) or a collection of user-defined plugins (post-processing and analysis).
 5. *Plugin GUI*. The GUI of a specific plugin is shown here.
 6. *Status bar*. The status bar reports the progress. The execution of some plugins can be stopped using the **Stop** button. Some plugins change attributes of the single localizations. The **Undo** button can undo the last execution of such plugins. Pressing **Undo** a second time reapplies the changes.

Many GUI components have tool tips defined, which are displayed when you hover the mouse over the component. Also, some lists or tab groups have a context menu attached (accessible by right-click). In most cases, the existence of a menu is denoted by a  symbol.

Additional windows belong to the GUI:

7. A figure window in which the rendered image is displayed
8. Plugins called from the menu open their own GUI window
9. The ROI manager has its own window.

4 Fit SMLM data with a Gaussian PSF model

1. After starting SMAP select the **Localize** tab.
2. Check at the bottom of the **Localize** tab that the fitting workflow `fit_fastsimple` is selected. Otherwise load that workflow by pressing **Change** and selecting `fit_fastsimple.txt`.
3. In the main SMAP GUI select the **Localize/Input Image** tab (Figure 2).
4. **Load images** and select the tiff file `Gettingstarted2D_Nup96-AF647.tif` that you just unpacked. Ignore an error from the Micro-manger loader that says you cannot load the image. If the file name appears in the field, it worked. The loader might display an **error message** that it cannot load the images. Ignore this for the moment and try the next steps. This error message comes from the micro-manager code; thus it cannot easily be fixed in SMAP.
5. If you like, you can check the camera parameters with **set Cam Parameters**.
6. In the **Peak Finder** you can set parameters for the candidate finding.

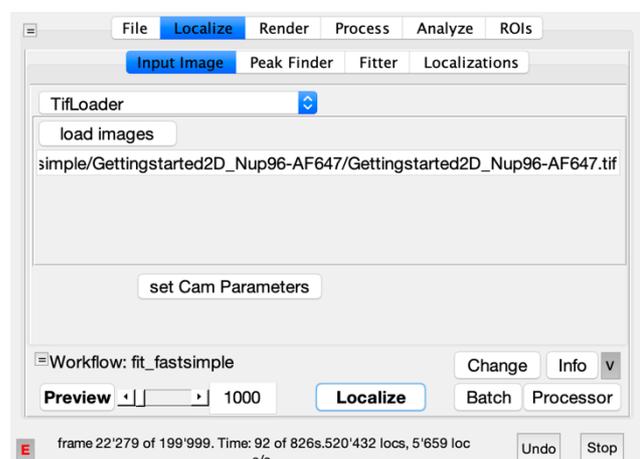


Figure 2: The localization GUI

The cutoff parameter is an empirical relative parameter to distinguish background noise from real localizations. Select a value between 1.3 (very sensitive) to 3 (only fits clear localizations), for example 2.

7. In the **Fitter** tab you can set fit options. For the fit with a symmetric Gaussian PSF of variable size (due to different z positions of the fluorophore with respect to the focus) select **PSF free fit** and a **ROI size** of 7 pixels.
8. Select a large frame in the field next to the **Preview** button at the bottom of the GUI (e.g. frame 1000, as the first frames might be still too dense to optimize fitting parameters) and press **Preview**. A figure opens that shows the camera image and denotes with squares which regions are found as candidates for fitting. The results should be comparable to those in Figure 3. You can change the cutoff parameter to find more or less candidates.
9. By changing the **preview mode** in the **Peak Finder** tab, you can select what image is shown. Either the filtered image, on which candidate finding is performed, or the original camera image.
10. Press **Localize** and wait for the fitting to be done (this might take up to 20 minutes depending on your computer). The number of fitted localizations and frame, and the estimated time are shown continuously in the status text of the GUI. Note that this example file was reduced in size after acquisition for faster downloading, thus the number of frames (100 000) does not correspond to the number of frames SMAP extracts from the metadata (200 000).
11. The fitting results are automatically saved in the directory of the camera data as `Gettingstarted2D_Nup96-AF647_sml.mat`.

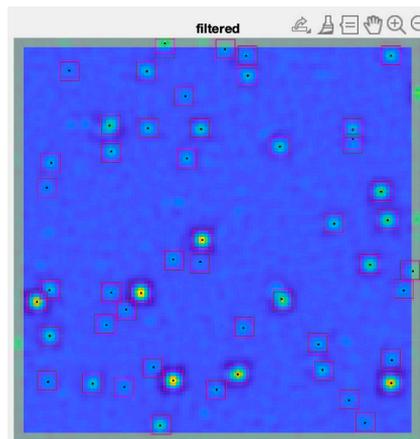


Figure 3: Preview allows you fine-tuning the peak-finding parameters.

5 Render a superresolution image

Here we discuss how to do a first superresolution rendering. In section 7 we will discuss additional render options to render the images according to the user's preferences.

1. Select the **Render** tab and press the **Render** button. A figure opens that shows the rendered superresolution image.
2. Navigate in this image:
 - a. Drag the image with your mouse.
 - b. Right-click on a part of the image to center it.
 - c. In the main GUI in the format panel press **filter->OV** to show the overview image. Click in this image to center the superresolution image to that part.
 - d. Change the pixel size (zoom factor) by using the scroll functionality of your mouse.
 - e. Alternatively set the pixel size in the main GUI in the format panel, either directly or by pressing the **-** and **+** buttons, or the pre-defined pixel sizes.
 - f. You can always reset the view to show all localizations by double clicking on the reconstructed image or by pressing **Reset** in the format panel.
3. Zoom into the image to show only a few nuclear pores. You can change the number next to the contrast button to increase (e.g. to -3) or decrease (e.g. to -4) the brightness. Press the **Render** button to confirm the change.
4. Under **colormode** select **field** and then in the new list select **frame**. Select the jet LUT below. Render. Now every localization is color coded by the frame they were detected in. You can clearly see a 'rainbow effect' with localizations at the upper left more blue and at the lower right more red. This indicates sample drift, which we need to correct for. See Figure 4.

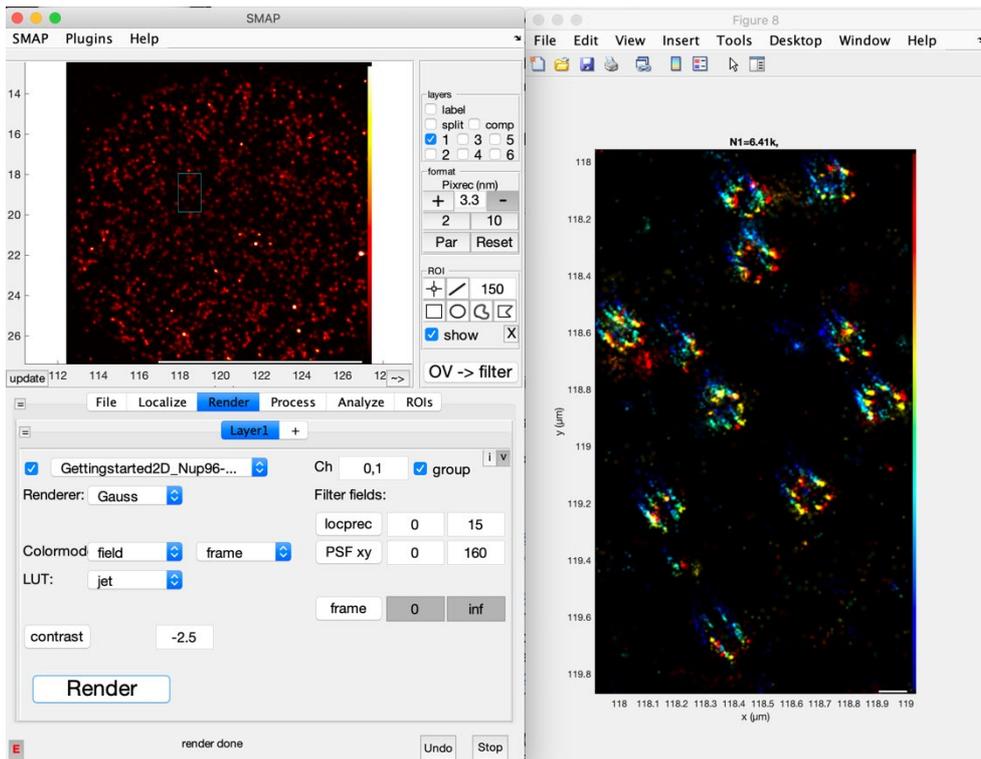


Figure 4: The render GUI and zoomed-in nuclear pores, color-coded by the frame, indicating drift.

6 Perform drift correction

1. Reset view as described above, e.g. by double clicking on the image. Drift correction is performed only on the localizations that are rendered in the superresolution figure. Sometimes, it helps to filter the localizations before drift correction, as described in section 7. However, the render parameters (Gauss vs histogram, pixel size, LUT color coding) do not have any effect on drift correction.
2. Select the **Process** tab in the main GUI and then the **Drift** tab. Select the plugin **drift correction X,Y,Z** by clicking on it.
3. Make sure Correct xy drift is selected.

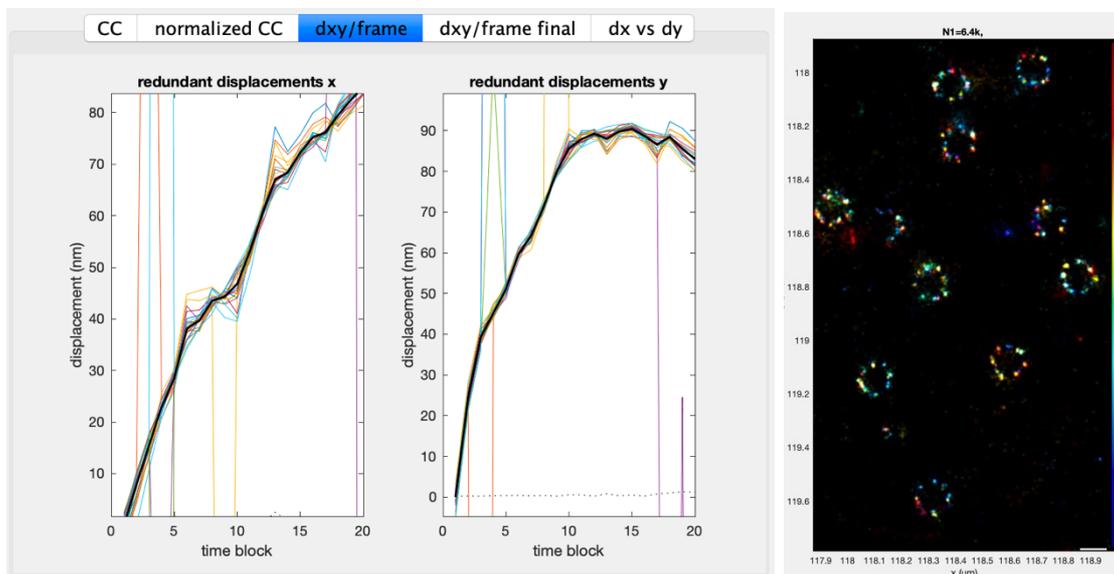


Figure 5: Drift correction: Left: redundant cross-correlations mostly overlap, indicating reasonable drift correction. Right: drift-corrected rendered image.

4. Select the number of time points (blocks) you want the data divided into for drift correction. The stronger and discontinuous the drift, the larger this number. But if the number of blocks gets too large, the pairwise distance estimation gets noisy and the accuracy suffers, especially for small fields of view with few defined structures. 10-50 is a good value. For this example with strong drift you can try 20 time points.
5. Click **Run**. The drift-corrected localizations are saved automatically with the ending *_driftc_sml.mat.
6. Check if the majority of curves in the dxy/frame tab scatter without too much error around the black curve, this indicates that all redundant displacements are compatible with each other and that drift correction worked. You can **Undo** the drift correction, change the parameters and try again (Figure 5).
7. Render the image and zoom in to appreciate that the nuclear pore complexes seem much better defined.

7 Improve rendering and filtering of localizations

Select the **normal** colormode and the **red hot** LUT. Now we will explore how different render and filter options change the appearance of the rendered image. After changing a parameter press **Render** to update the image.

7.1 Basic render options

Grouping: You can always toggle between showing all localizations in each frame (ungrouped) or showing the merged localizations that were obtained by grouping localizations that persist over several frames and belong to the same fluorophore and blinking event. Select and deselect **group** to see the effect. Also note how that changes the number of displayed localizations (the number on top of the superresolution image).

Contrast: Change the value to see how the brightness of the image changes.

Renderer: Investigate the different render options, how **Gauss** vs **constGauss** with varying sizes for the Gaussian kernel and **hist** compare. We usually use **Gauss**.

Colormode: As described before in section 5, you can explore different fields for color coding and different LUTs.

7.2 Filtering of localizations

It is a common practice to not display all localizations, but only a selected subset to improve the image quality. In SMAP, there are three (internally equivalent) ways of selecting filters for specific attributes of the localizations. Note that filtering is performed for every Layer separately.

- a. In the **Layer** tab you can directly access filters for localization precision in x,y and in z, PSF size, z range and frame. Insert the minimum and maximum value. Press the corresponding button, e.g. **locprec**. This will activate the filter. If the minimum/maximum fields are grayed out, the attribute is not filtered, if it is white, the attribute is filtered.
- b. With the **OV->filter** button in the format panel you can toggle between showing the overview image or advanced filter options. On the left, on top you see a list of all attributes. Here you can set a check mark for filtering, and edit the minimum and maximum values (min set, max set). The checkbox **inv** for invert lets you invert the specific filter. This is a useful option to directly see what localization are rejected by the filter.
- c. By pressing a button belonging to a localization attribute in the **Layer** tab or by selecting an attribute in the table, you select this attribute in the histogram below. The histogram in green is a histogram of the value for all localizations. The red curve is a histogram only for the filtered and displayed localizations. The sliders below allow you to select the minimum and maximum range. If **auto update** is selected, any change directly causes the image to be rendered. You can check **fix**

range to a value specified below, then if you change the minimum or maximum, the maximum or minimum are changed automatically, respectively.

Important attributes for filtering:

locprec: Localization precision in x,y in nanometers. This filters out dim localizations with a poor precision (e.g. worse than 30 nm), increasing the effective resolution of the superresolution image at the expense of localization density. Note that even if this filter is not used, there is a filtering on the number of photons and thus localization precision that comes from the peak detection step. As very dim fluorophores are not detected, they are already filtered out then.

locprecz: as above, but for z-coordinates.

zrange: z-range to display only a slice in z.

PSF xy: Size of the PSF when using a Gaussian fit. This allows rejecting fluorophores that are out-of-focus and thus blurred, leading to effective sectioning of the image.

frame: The frame in which the localization was first detected. Remove early frames if the localization density in the beginning was very high. Remove late frames if most fluorophores have been bleached already and these frames contain mostly background localizations.

Ch (channel or color): This is for multi-color data sets where each localization has an attribute *channel* that denotes the color.

LLrel: relative log-likelihood. This is a measure for the goodness of fit and can be used to reject poorly fitted localizations that mostly stem from overlapping fluorophores. A suitable value cuts off the long tail at negative values but leaves most of the peak in.

Several analysis plugins calculate additional localization fields. For example, the **count neighbors** plugin counts the number of neighbors of each localization within a specific radius and writes this number as a field. You can then use these filter options to only display localizations within a structure, i.e. with a sufficient number of neighbors.

7.3 Advanced render options

Show the advanced GUI options by clicking in the top right corner of the **Layer** tab and explore the following render parameters:

render par: This opens a dialog box in which you can set parameters that are specific for the selected renderer. For the Gauss renderer you can select the minimum size of the Gaussian in pixels and nanometers and the proportionality factor between the localization precision

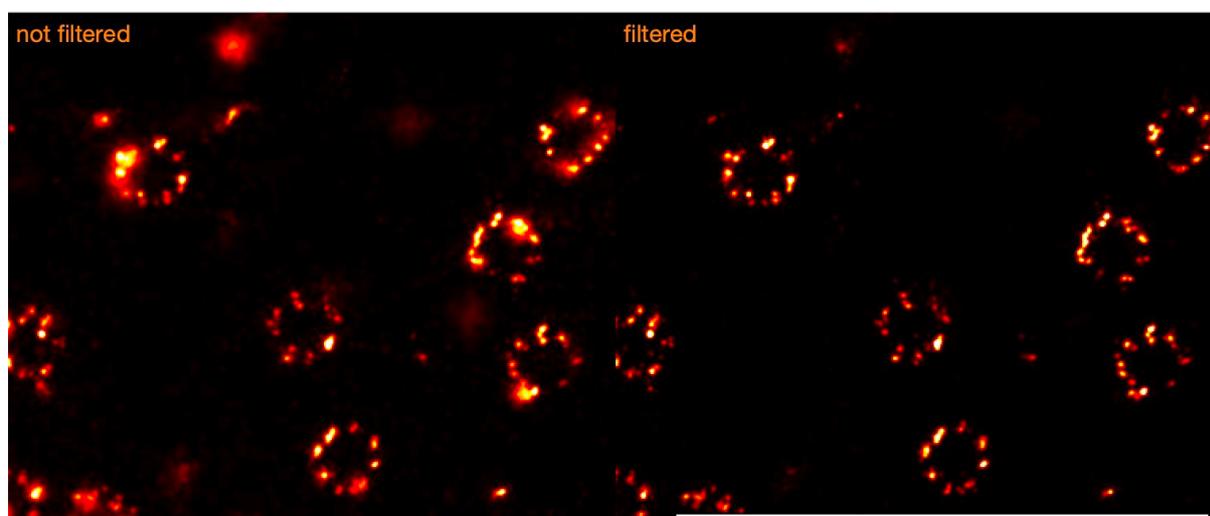


Figure 6: Effect of filtering and rendering parameters. Left: ungrouped, unfiltered data. Right: grouped and filtered data, rendered with the intensity normalization $\sqrt{\text{photons}}$. Scale bar 1 μm .

and the size of the Gauss. The gamma factor of the image can be set here as well, but we recommend leaving it at 1.

Intensity coding: Here you can select how the Gaussians for rendering are normalized. The integral over the Gaussian is: *normal*: 1; *photons*: the number of photons in the localization; *blinks*: the number of merged localizations in that constitute a specific localization, use only for displaying grouped data; $\sqrt{\text{photons}}$: square root of photons and $\sqrt{\text{blinks}}$: square root of merged localizations. These normalizes the noise, as due to Poisson statistics the standard deviation of photons per localization is given by $\sqrt{\text{photons}}$. And it can be a good compromise between normalizing according to photons and blinks and standard normal normalization.

Renderer: Try out the **DL** (diffraction-limited) renderer to render an image as it would look like if taken with a diffraction limited microscope and **raw** to display some raw camera frames. The raw image with the number 0 corresponds to the average of all camera frames.

7.4 Reconstruction in multiple layers

You can reconstruct data with different rendering parameters in different layers and overlay the reconstructions. Most importantly, this is used for multi-color data. But it can also be useful to compare different render modalities.

As an example, we describe here how to render a superresolution image and to overlay it with a diffraction-limited reconstruction (Figure 7).

1. Render your superresolution image as described above.
2. Click on the **+** tab next to the **Layer1** tab. This opens a new tab called **Layer2**.
3. In this tab select the **DL** renderer and the **gray** LUT and press **Render**. The composite image is rendered.
4. You can hide or show specific layers by a) the check boxes in the format GUI (top right in the main GUI) or b) by clicking the left top checkmark in the **Layer** tab.
5. You can rename the Layers by right-clicking next to the tab and select **rename layer**.
6. In the format GUI you can check **label** to display the label names in the reconstructed image. With **split** you can show images corresponding to each layer next to each other. With check **comp** you can additionally render the composite image.
7. Explore different reconstruction options by comparing them directly using this feature (see Figure 6).
8. If you like, you can save the reconstructed image as a tiff file by selecting the **File** tab. Then next to the **Save** button select **TifSaver** and press **Save**.

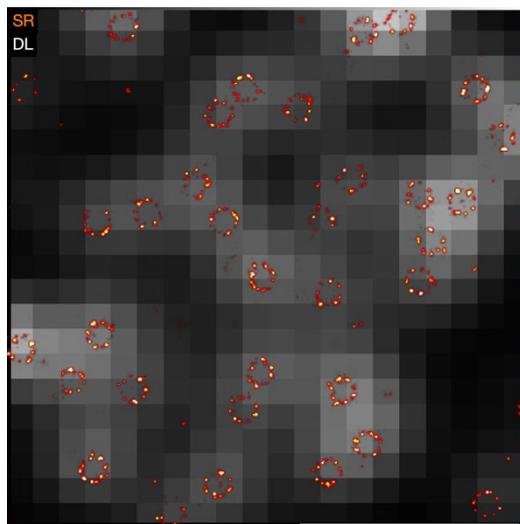


Figure 7: Overlay of diffraction-limited and superresolution rendering using two layers.

8 Perform simple analyses

Here we discuss a few simple analysis routines. For more information, consult the User Guide. You can also search for specific plugins for a given task in the **Help/Search plugin** menu. Consult the help files with detailed description by pressing the **info** button. Get information about the GUI parameters from the tool tips by hovering with the mouse above them.

8.1 ROIs

SMAP allows the user to draw ROIs in the reconstructed image. Many plugins then only evaluate localizations inside the ROI.

Measuring distances: The Line ROI is also useful to quickly measure distances in the image. Click the line icon in the format GUI and draw a line in the reconstructed image. The length of the line is displayed next to it. You can then drag the end points of the line to change the ROI (Figure 8).

Selecting Localizations: You can select localizations with the line ROI, then specify the width of the line ROI next to the icon.

8.2 Fitting intensity profiles with Gaussian functions

Select the **Analyze** tab. In this, select the **measure** tab. Select **Line profiles**. As a model, select **Distance**, then a double Gaussian will be fitted to the profile. Use the line profile to draw a line through two clusters in one nuclear pore complex. The ROI should extend beyond the clusters. Select a small line width on the order of the cluster size (e.g. 20 nm). Press **Run**. In the **yprofile** tab you see the line profile (as a histogram of localizations along the line ROI) and a fit with a double Gaussian. The FWHM is the distance from the first to the last data point that is above half the maximum value of the profile. More useful are the distance (in nm) and the width of the Gaussians, given by their standard deviation (Figure 8).

8.3 Statistics on localizations

Delete the line ROI. You can either zoom into the region you want to analyze or draw a larger ROI, e.g. a rectangular ROI. In the **measure** tab select **Statistics** and the default parameters, press **Run**. The individual tabs of the resulting figure show histograms and statistics on various parameters, such as the photons per localization, the localization precision, the on-time of the fluorophores in frames and the background.

8.4 FRC resolution

In the **measure** tab you can also select **FRC resolution**. Execute with default parameters with **Run**. The FRC resolution curve with the resolution estimate is shown.

8.5 Color code according to density

In the tab **Analyze**, **cluster** select the plugin **density calculator**. **Run** it with default parameters. This might take a while. This plugin creates a new attribute for the localizations called **clusterdensity** (in reality it is the number of neighbors). In the **Render** tab you can select it for color coding by selecting **field** as **colormode** and **clusterdensity** as the field to use for color coding. Use e.g. the **jet** LUT.

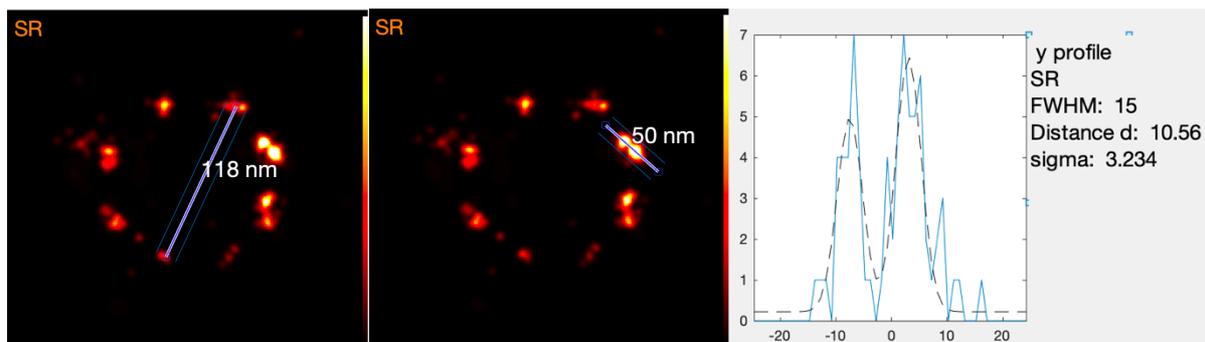


Figure 8: Left: line ROI tool to manually measure distances. Center, right: line-ROI tool to define line profiles, that can be fitted with the plugin: Line profiles. Scale bar 100 nm.

Select the advanced GUI options with the button on the top right of the **Layer** GUI. Click on range. Use the sliders under the histogram to adjust the color range that is mapped to the number of neighbors.

8.6 Versatile renderer

This plugin allows plotting any localization attribute vs any other and can be found in the **Analyze, other** tab. We will illustrate this by plotting the coordinate of the localizations for a single nuclear pore vs the frame to see when individual fluorophores were activated. Color coding is according to the same field as chosen in the **Render, Layer** tab.

1. Select a single nuclear pore with a circular ROI or by zooming into the image until only one nuclear pore is shown.
2. For the horizontal axis select frame (even if it is already selected).
3. For the vertical axis select xnm.
4. Run.
5. You can adjust the parameters, e.g. the pixel size and the blurring of the image (given in sigma of the pixels).