SMAP user guide

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1 Introduction

SMAP is a Superresolution Microscopy Analysis Platform for all steps of data analysis in single-molecule localization microscopy (SMLM). It is easily extendible with own functionality, contains already many analysis plugins and has a configurable graphical user interface (GUI) to allow advanced data analysis without any programming experience.

In this user guide for SMAP we present detailed step-by-step protocols on how to analyze SMLM data. It complements other user guides in `SMAP/Documentation/`:

`Getting_Started.xml`: *A step-by-step guide that introduces SMAP on the example of a simple 2D single-color data set. We recommend starting with this guide if you are new to SMAP.*

`Example_SMAP_Step_by_step.xml`: *A step-by-step guide that explains how to fit dual-color 3D data.*

`ProgrammingGuide.xml`: *Explains the architecture of SMAP and is useful if you want ot extend the functionality of SMAP.*

`SMAP_manual_NPC.xml`: *explains specific plugins used to quantitatively analyze data of the nuclear pore complex.*

1.1 Some definitions

SMLM (Single-molecule localization microscopy) comprises any superresolution microscopy technique that relies on the measurement of the position of individual fluorophores. Prominent examples are PALM, STORM, *d*STORM, PAINT, DNA-PAINT, MINFLUX and similar. SMAP can be used with any of these; it is, at least currently, not suitable for STED, SIM and others that yield different data types.

Raw data refers to camera images, saved e.g. as tiff files or in a proprietary format. The pixel gray values are *Analog Digital Units (ADU)* and include an offset. These images are internally converted into *photon units* using the camera metadata. **Raw data** can also refer to these converted images.

Metadata is data contained in the raw data that describes the acquisition settings. Additional camera metadata might be required for the conversion and is supplied by the ***camera manager***.

A Localization corresponds to a single fitted molecule position.

Localization attributes are parameters that describe the single localization. They contain coordinates such as *x*, *y* and *z*, *number of photons per localization*, *background values*, *localization precisions, frame in which localization was found* etc. Any other descriptor can be added to the localization attributes in SMAP. All attributes can be used for filtering or coloring the rendered image. In SMAP attributes are also called localization **fields**.

Grouping or **Merging** of localizations describes combining localizations that persist over several adjacent frames and thus belong to one activation event of the same fluorophore into one localization.

Filtering of localizations. For rendering and analysis, localizations with attributes outside of user-defined boundaries can be removed. This filtering can be used to further evaluate only localizations with a certain fitting quality (filter on log-likelihood ratio or on localization precision), those close to the focus (filter on the size of the PSF), those belonging to a certain color channel etc.

Region of interest (ROI). In SMAP, a ROI can describe two different user-defined regions:

1. Users can draw a ROI in the rendered image. Only coordinates in this ROI are then used for further analysis.
2. Users can define specific positions in many data sets and evaluate them using the ROI manager. A single position is called a ***ROI*** or a ***site***.

2 Installation

2.1 Requirements

1. MATLAB newer 2019a and newer. Toolboxes: *Optimization, Image processing, Curve fitting, Statistics and Machine Learning.*
2. A compiled stand-alone version will run without a MATLAB license, but will be limited in extensibility.
3. Mac or Windows (Linux users will have to compile some C code into mex functions).
4. 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.

2.2 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. Install Micromanager 1.4.22 or later from https://micro-manager.org.
3. If you used software other than Micromanager to acquire your data, install Bio-Formats (MATLAB toolbox):
    www.openmicroscopy.org/bio-formats/downloads.
4. In MATLAB: run `SMAP.m` from the SMAP installation folder, if prompted, change folder.
5. In the Menu select **SMAP/Preferences...** Switch to the **Directories** tab and select the directories 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) and of the `bioformats_package.jar`. Press Save and exit.

2.3 Installation of stand-alone versions

You can download compiled versions of SMAP for Windows, Mac, and Linux here: https://www.embl.de/download/ries/SMAPCompiled/. More detailed installation notes can be found under the same link (Installation_notes_SMAP_compiled.rtf).

3 Overview of the GUI

Start SMAP by executing `SMAP.m`. Figure 1 shows the main parts of the GUI. Note that most elements in the GUI have a tool tip: hover with the mouse over the control to display it.

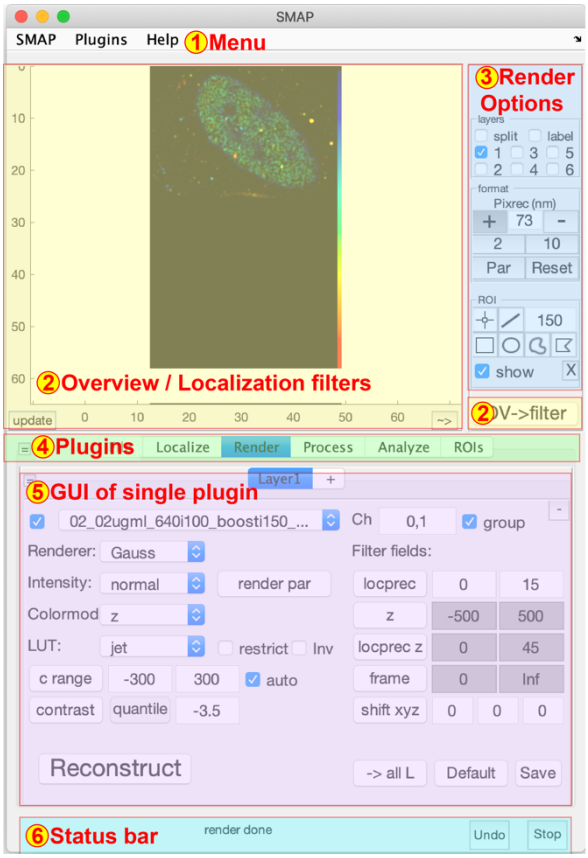

Figure 1: Main parts of the SMAP GUI.

1. *Menu*. There are three menu items:
    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: Here you can search in the help files of all plugins to find a plugin for a specific task (*Search Plugin*) and you can open this user guide and additional help files.
    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, but not all, 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:

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

4 Import and Export

Most import and export functions can be accessed via the **File** tab (Figure 2).

The GUI has the follwowing components:

1. File list. SMAP saves all data in a proprietary format with the ending `*_sml.mat`. All loaded SMAP files appear in this list.
2. Loaders. Select a specific loader plugin from the list. Currently these include loaders for several coordinate-based file formats and for tiff files.
3. Savers for coordinate-based file formats and for image files are found here. You can activate auto-save.
4. Grouping is applied directly after loading files using the parameters of this GUI (i.e. localizations in adjacent frames stemming from one and the same fluorophore are combined into one localization). dX (in nm) is the maximum distance two localizations can be apart, linking occurs if they are separated by not more than dT frames without a localization in this vicinity. Note that the ungrouped localizations are always available as well. If you change the grouping parameters, press Group to regroup.

4.1 Loading

4.1.1 Load localizations

1. In the **File** tab press Load and select a file containing localizations (`'*_sml.mat'`, or also `'.csv'`).
2. When load Gui Parameters is checked and the `*_sml.mat` file contains information about the GUI settings, these are restored upon loading.
3. The localizations are automatically grouped.
4. After loading, the **Render** tab is opened and an overview image is displayed. By clicking in the overview image or pressing Render, the rendered superresolution image is calculated and displayed in a separate window.
5. Load clears current data before loading. Add adds a file to the current localizations without clearing already loaded localizations.
6. After loading localizations, you can add single tiff images (diffraction limited markers) and associate them to a localization file as described below (section 4.1.2).

4.1.2 Load images

Image data (e.g. raw camera frames, additional tiff images) cannot be loaded by themselves but need to be assigned to a localization file. Load tiff images with Add, select a tiff file or other image file, then in the dialog select a localization data set that you want this image to be associated to. You can display the image in the **Render** tab by selecting Renderer: Tiff, and the loaded tiff image under Image.

4.2 Saving

4.2.1 Save SMAP SML files

1. Select SMLMSaver as a saver
2. You can select which attributes to save with Fields to save (only available with advanced controls). Not saving unimportant attributes reduces the file size.
3. If only save visible is checked, only the localizations that are displayed in the figure window (after filtering) are exported.
4. As default, all loaded data sets are saved in a single file. With only you can choose to save only one data set.

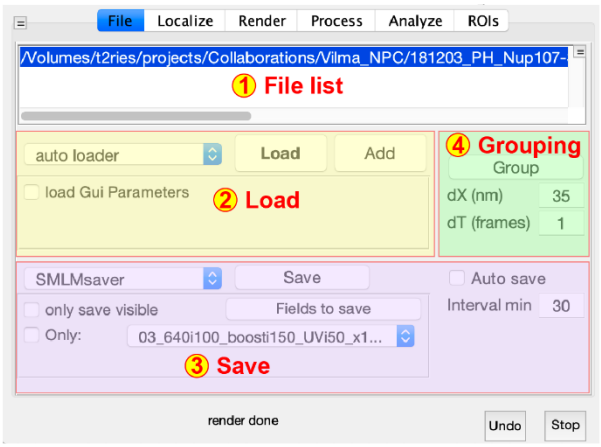

Figure 2: The File GUI

4.2.2 Export coordinates

1. Select export_coordinates as a saver.
2. If all is selected, all localizations are exported, then select if to export ungrouped or grouped localizations. If visible ROI is selected, only filtered localizations within a defined ROI (or

currently displayed in the figure window if no ROI is selected) are exported.
3. Choose the export file format (csv, txt, dat. xls).
4. With select you can select the attributes (fields) you want to export.

4.2.3 Saving images

1. Choose TifSaver to save the rendered superresolution image exactly as it is currently displayed in the figure window.
2. When saving Tiff images, the reconstruction parameters (such as pixel size, applied filters, scale bar size,…) are saved as comments. When opened in Fiji, the size shown in the title is in nanometers (although Fiji calls them pixels).

5 Fitting of single molecules

Fitting (or localizing) single molecules is the most important step in SMLM analysis. It itself consists of many steps including loading of the raw camera images, converting those to photons, filtering of images, finding of candidate positions, fitting of single molecule positions and saving of the results. In SMAP these steps are implemented as *workflows*, a collection of plugins that are executed one after the other. Users can define their own workflows in a GUI, or exchange individual plugins by others.

Micromanager users: Save your images as single or multi-image Tiff stacks, turn on "save metadata" in preferences.

5.1 Camera Manager

Many fitting algorithms depend on the correct conversions of raw images (ADU units) to photon units. Especially when different cameras or different camera settings are regularly used in a lab, inserting wrong camera parameters is a likely source of errors. Thus, SMAP contains a camera manager in which such parameters are stored. During loading of images and fitting, SMAP automatically recognizes the camera and the camera settings based on the metadata and thus always uses the correct camera settings.

Add your camera and camera settings to SMAP:

1. If you want to use your own camera collection choose a new name for the configuration file in **Menu: SMAP/Preferences/File.** Here you can also toggle between different camera configuration files.
2. Acquire a data set with the same camera settings as used for SMLM.
3. Open **Menu: SMAP/Camera Manager** (Figure 3).
4. Use Load images to load the data set. If the camera has not been recognized you will be prompted to add a new camera. Otherwise you can add and remove cameras with a right click on the camera list.
5. Click on the cell ‘ID field’ belonging to your camera and select a metadata field that uniquely describes the

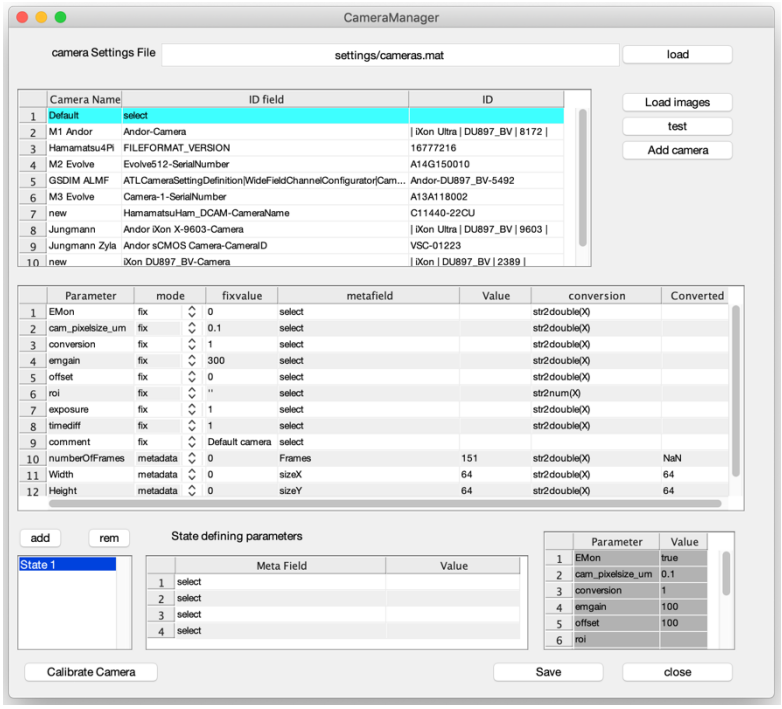

Figure 3: The Camera Manager

camera. This field is used to distinguish between different cameras based on their metadata.

6. The “Default” camera will be used if no camera is recognized.
7. The camera manager allows extracting acquisition parameters from the metadata. SMAP uses the following parameters:
    a. ***EMon*** (logical, if EM gain is on).
    b. ***cam_pixelsize_um*** (pixel size in the object plane in micrometers, a vector [*px py*] for non-square pixels).
    c. ***conversion*** (in ADU/e-, used to calculate photons from the camera units, by default it does not include the EM gain. Usually, this value is provided in the data sheet of the camera).
    d. ***emgain*** (EM gain value).
    e. ***offset*** (camera image offset in ADU, this does not include background).
    f. ***roi*** (acquisition ROI position and dimension on the camera chip in pixels, given as vector [*minx miny width height*]).
    g. ***exposure*** (camera exposure time)***, timediff*** (time difference between frames)***, comment*** (any user defined string). These values are stored for information only, they are not used in SMAP. Per convention, times are usually in milliseconds.
    h. ***numberOfFrames*** (number of frames).
    i. ***Width, Height*** of the image, in pixel units.
    j. For sCMOS cameras you can specify the path of the correctionfile, a file that contains the flatfield, offset and variance maps. You can also select the file by clicking ‘select’ in the correction file row. You can generate this file with the plugin ***Process/Images/calibrateSCMOS*** or with the ImageJ/Micromanager plugin Accent (https://github.com/ries-lab/Accent).
8. For each parameter that SMAP uses define the mode:
    a. *fix*: uses the value in the column *'fixvalue'*.
    b. *metadata*: uses the field defined in *'metafield'* together with the parser defined in the 'conversion' column. Here *X* is substituted by the metadata corresponding to the *metafield*. Choose the *metafield* by clicking on it.
    c. *State dependent*: This allows you to define camera parameters in dependence on camera settings. This is useful e.g. for the conversion factor which might change depending on the camera-internal amplifier setting. The state is defined by the parameters in the list *state defining parameters*. Select the metadatafields whose values define the camera state. Define the respective values in the list to the right.
9. Save and Close.

5.2 Fitting GUI

For fitting of SMLM data please select the **Localize** tab in the plugins tab group (Figure 4). In the bottom of the GUI you can select a new workflow with Change or get Info on the current workflow. Further workflow functionality is accessible with a right-click on the workflow name. Toggle between a simple and advanced fitting GUI with the small - button next to Info.

The user interfaces of all workflow plugins are mapped to different tabs and are grouped by functionality. You can switch between them and adjust fitting parameters. To test the current settings press Preview (select the frame of choice with the slider next to it beforehand). By pressing Batch you can save the current workflow settings as a batch file that you can later apply/use for fitting with the batch Processor (see 5.5).

The tabs can vary depending on the workflow, but usually they contain the following items:

1. Input Image: here you load the image and set the parameters to convert the raw data into photons.
2. Peak Finder: here you set parameters for the candidate finding, such as thresholds and ROIs in the image that you want to include/exclude from fitting.
3. Fitter: here you set the parameters for a specific fitter.

4. Localizations: here you set how fitted localizations are rendered online, filtered and saved.

5.2.1 Selection of a localization Workflow

1. A workflow for single-molecule localization can be selected in the **Localize** tab with the Change button close to the bottom right of the window. For most cases `'fit_fastsimple'` is a good choice.
2. You can find a description of the current fitting workflow and a graphical representation of the modules by pressing Info next to Change button.

You can create your own workflows from existing plugins as described in the "Assembling workflows" section in the *SMAP programming guide*.

5.3 Basic fitting

Here we show how to do simple 2D fitting using the `fit_fastsimple` workflow. To change a workflow, click on Change in the lower right and choose the txt file of the desired workflow. In section *5.4 Fitting with an experimental PSF model* we describe how to perform 3D fitting.

1. In the **Localize** tab, load images: Select one image inside a directory containing all the tiffs, or a (single file of a) tiff-stack. Alternatively, you can select any OME-readable file, but import of metadata then is limited and needs to be adjusted in the Camera Manager (section Camera Manager). If the right loading plugin is not recognized change auto to the specific loader in the popup menu.
2. If sufficient metadata could be extracted and if the camera has been added in the Camera Manager, the acquisition parameters are automatically set. Otherwise you get a message in the MATLAB command window ('Camera not recognized'). Then you can either load metadata from a previous experiment or manually set Cam Parameters. It is a good idea to check with set Cam Parameters if the metadata has been parsed correctly.
3. Many EM-CCD cameras mirror the images when the EM gain is used, compared to the case when the conventional gain is used. This can create problems if data is acquired with different settings at different occasions (e.g. bead calibration with conventional gain for optimal SNR, EM gain for single-molecule detection). Because of this, SMAP mirrors all images acquired with EM gain back. You can avoid this by unchecking ☐mirror if EM mode (part of advanced GUI parameters, show by clicking the v button on the bottom right side of the GUI). This is useful if the EM state is not recognized properly, or if you never switch between EM gain and conventional gain.
4. If you use an sCMOS camera and selected the file containing flatfield/offset maps in the ***CameraManager***, you can select to ☐correct flatfield/offset here.
5. You can specify a frame range for fitting. Check ☐Online analysis if you want to fit during the acquisition (then the maximum frame is ignored, and SMAP waits for new images). For this, use load images to open a file that is currently written to.
6. In the **Peak Finder** tab you can set the parameters for the initial estimation of single-molecule positions. Usually this is done on a background-corrected image or on an image convoluted with a *difference of Gaussians (DoG)* kernel. Use tool tips (hover mouse over control) to get information about specific parameters.
7. By pressing Preview after selecting a frame with the slider next to it, an image will open which shows the positions of the found localizations. Use this preview to optimize the peak finding parameters. The preview mode determines which images (raw images, or the normalized ones on which peak finding is performed) are shown here.

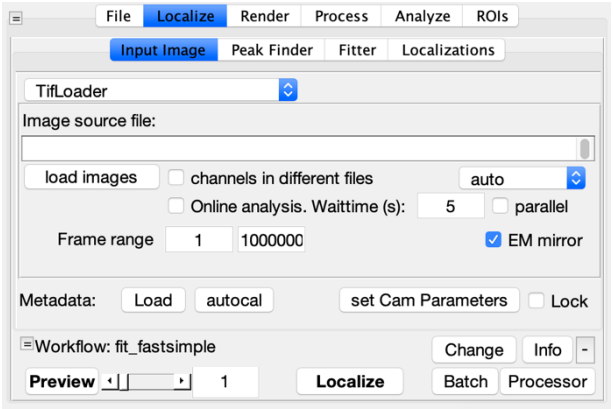

Figure 4: The GUI to fit (localize) raw camera images.

8. You can restrict the fitting to pre-defined regions in the image. Make sure to Preview at least once. Select either an elliptical or rectangular ROI in the popup menu. With ROI to include you can select a region in which to fit, with ROI to exclude you can select a region which to exclude from the fit. You can define multiple ROIs. Clear ROI to fit everything. Draw the ROI in the preview window and double-click on it. With Preview you see the ROI overlaid on the image.
9. In the **Fitter** Tab you set
    a. The size of the ROI in which the fitting of single molecules is performed. For 2D fitting 7-11 pixels (corresponding to ~1 µm) are a good choice.
    b. The fitter module and its parameters. For Gaussian 2D fitting choose PSF free.
    c. Here you can also select to fit with an sCMOS noise model (see Huang, et al. "Video-Rate Nanoscopy Using SCMOS Camera-Specific Single-Molecule Localization Algorithms." Nature Methods doi.org/10.1038/nmeth.2488). To this end, check □ sCMOS and make sure the file containing the variance map is selected in the ***CameraManager***.
10. In the **Localizations** tab you can switch on the rendering during the fitting and set the update time. You can also press Now to render the localizations fitted so far (the update occurs only after a block of many frames is fitted, so you might have to wait for a few seconds). In addition, you can do simple pre-filtering of the data. To set these parameters you can again use Preview. Found candidate positions are marked with a box, found localizations which pass the filters are marked in addition by a circle.
11. Now you can fit the whole data by pressing Localize.
12. You can abort fitting by pressing Stop at the bottom of the GUI. Press it again before you fit again.
13. The fitted localizations are automatically saved in the base directory of the images with an extension `'_sml.mat'`.

5.4 Fitting with an experimental PSF model

Here we show how to use an experimentally calibrated PSF model for 3D fitting using the `fit_fastsimple` workflow. For more information see *Li et al., Real-time 3D single-molecule localization using experimental point spread functions. Nature Methods (2018)*.

5.4.1 Calibrate Astigmatic or any other 3D PSF

Plugin: *Analyze/sr3D/calibrate3DsplinePSF*

This plugin generates a cspline model of your experimental PSF. For this you need to acquire several *z*-stacks of beads immobilized on a coverslip. A range from -1 µm to +1 µm (with respect to the focal plane on the coverslip) and a distance between the planes of 10-50 nm works well. Please consult the `User_guide_Ries` in the `fit3Dcspline` directory for further information.

1. Press Run in the plugin *Analyze/sr3D/calibrate3DsplinePSF* to open the GUI to calibrate the 3D PSF.
2. Select camera files to open a file dialog box to select several files at the same time
    a. With add you can add a single file or several files in the same directory
    b. With add dir you can add several directories. SMAP will automatically try to find image files in those directories and add them.

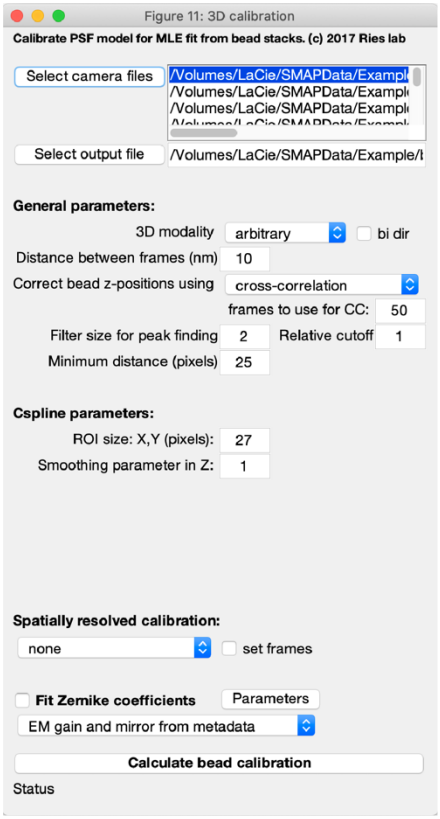

Figure 5: GUI to calibrate experimental PSF models.

c. Press Done.
3. The output file is set automatically, but you can change it manually with Select output file.
4. Select the 3D modality: arbitrary is the most common choice.
5. If your PSF does not show strong variations along *z* or a symmetry with respect to the focus (e.g. because it is an unmodified 2D PSF) check bi dir.
6. Enter the distance between the frames you used for acquiring the *z*-stacks.
7. Leave other parameters as they are, but if you have dense beads decrease Minimum distance and ROI size.
8. Calculate bead calibration calculates and saves the PSF model.

5.4.2 Fitting with an experimental PSF model

You can use this model for fitting as described above using e.g. the `fit_fastsimple` workflow using the following modifications:

1. In the **Fitter** tab select Spline as the fitting model.
2. Load the PSF model you previously generated with Load 3D cal.
3. If you have a symmetric PSF you can use different start parameters that you can insert in z start (nm). Default is 0.
4. With additional Gauss fit you can perform a standard Gauss fit in addition to the fit with an experimental PSF. This can have the advantage that a Gauss fit might be more robust for *x* and *y* coordinates under certain conditions.
    a. Choose which Gauss model you want to use (fix: fixed Gauss, free: symmetric Gauss, elliptical: asymmetric Gauss, this is used for an astigmatic 3D PSF.
    b. Select if you want to get the final *x* and *y* coordinates from the spline (=experimental model) or Gauss fit.
5. You can overwrite the pixel size stored in the camera manager with New pixelsize X,Y (um). This might be necessary if the insertion of the cylindrical lens distorts the image in one direction and thus leads to a change in the aspect ratio (unequal pixel sizes in *x* and *y*). Insert the size of the pixels in *x* and *y* direction in micrometers.
6. RI mismatch allows you to rescale the fitted *z*-positions with a constant factor (typically 0.8). This might be necessary for oil objectives, because here a movement of the objective (as performed during the calibration process when acquiring bead stacks) results in a smaller movement of the focal plane (see Huang et al, “Three-Dimensional Super-Resolution Imaging by Stochastic Optical Reconstruction Microscopy.” Science (2008), doi.org/10.1126/science.1153529.).

5.5 Batch fitting

1. You can save your acquisition fit settings (previous paragraph) in a file with the Batch button.
2. Open the batch Processor (Figure 6).
3. The batch file you just saved is already set as the main batch file. But you can replace it by another with load main batchfile. If use for all is checked, this will be used for all the fits, otherwise only for the datasets which are not imported to the batch processor with a batch file.
4. With add you can add a) further batch files, b) one single image from a stack or c) a tiff stack. These appear in the list on the left.
5. You can add multiple directories with add directories. These directories contain a) tiff images, b) further directories with Tiff images inside (here use the filter string below to specify which directories to load and fit, and the >#images to set a lower limit for the number of images required to start fitting).
6. You can remove items from the list, but don't empty it. With Batch process the fitting starts.
7. If you add online directory and start the batch processor, it checks for new fittable directories in this directory and automatically fits them (used e.g. for automated microscopy). Make sure you have checked the Online analysis in the batch file that is used for fitting.

Figure 6: Plugin for batch fitting of SMLM data.

6 Post-processing

Before analyzing SMLM data, localizations are typically processed to e.g. group them over adjacent frames, filter out spurious/bad localizations, correct for drift, align channels or assign colors. These steps are called post-processing. SMAP allows for an interactive exploration of post-processing steps and parameters by applying a post-processing step and directly monitoring the results in the reconstructed image.

6.1 Grouping

Grouping (merging) of localizations persistent over several frames is automatically performed after fitting and upon loading of SMLM data (see section 4.1.1). At any time you can change the grouping parameters dX (maximum distance two localizations can be apart) and dT (maximum number of dark frames between localizations) and press Group in the **File** tab to recalculate the grouping.

SMAP implements two modes of grouping:

1. Fix dX. Here the maximum distance (in nm) is directly provided.
2. Locprec. Here the maximum distance is calculated from the localization precison of the two candidate localizations. That means that localizations are only grouped if it is likely they stem from the same event.
   Localizations are grouped if $(\mathbf{x}_1 - \mathbf{x}_2)^2 < d_{12}^2$
   $d_{12}^2 = \max(\min(2.5(lp_1^2 + lp_2^2), dX_{max}^2), dX_{min}^2)$

6.2 Filtering of localizations

Filtering of localizations is a very important step during post-processing. It allows you to reject badly fitted or dim localizations, or to display only localizations in focus (for 2D data this means filtering the PSF size). SMAP allows you to filter on any attribute of the localization data. Most SMAP analysis plugins use directly the filtered localizations for further analysis. Filtering is performed for every layer independently.

1. You can toggle between the overview image and the filter GUI by pressing OV -> filter.
2. The upper table lists all properties (fields) of the single molecule localizations together with their minimum, mean and maximum value. You can set minimum and maximum values. To filter according to a specific attribute/field, activate it with the ☐ filter checkbox.
3. With ☐invert you can invert the filter to only display the filtered-out localizations. This can be useful when adjusting the filter range to remove bad localizations but not good ones belonging to the structure of interest.
4. Below, you see a histogram representation of a specific field. You can select the field by either pressing on a row in the table or on a button in a Layer corresponding to the specific field (locp, frame, PSF, locprec z, z). The green graph shows a histogram of the value of the field for all localizations, the red curve a histogram for the filtered localizations.

5. You can switch the filter on and off and change the range with the sliders. If □Auto update is checked, the image is directly rendered on the fly. If you check □range fix and move the sliders, the difference between minimum and maximum slider is fixed to the value below □range fix.

6.3 Drift correction

Plugin: Process/drift/driftcorrection

This implementation uses redundant cross-correlations between images reconstructed for short time windows for drift correction. It is based on the localizations of your structures of interest, but works also very well in case fiducial markers are present (in that case render the image ungrouped). It is designed to correct for relatively slow and smooth drift, caused e.g. by temperature fluctuations, but won't be able to correct for vibrations or abrupt jumps.

1. Select parameters to render a large part of the image. The part of the superresolution image in the ROI (or if no ROI is defined the part displayed in the rendered image) is used for drift correction.
2. Choose the number of time points to perform the drift correction on (typically 7-25, this algorithm rather corrects for drifts than for fast jumps or oscillations). The other parameters usually do not need to be optimized (use tool tips to understand what they mean).
3. Use □Reference is last frame to drift correct the first of two consecutive measurements.
4. Press Run. With □show results you can display the results of the procedure.
5. The drift-corrected localizations are by default automatically saved as a `'_driftc_sml.mat'` file. To suppress the saving, untick □ Save driftcorrected SML

7 Rendering

SMAP provides a 2D renderer that allows exploration (moving, zooming) in real time. It can overlay different layers containing different channels, files, and even image data (e.g. diffraction limited images) with independent localization filters and render options.

In addition, SMAP provides a 3D renderer as a plugin (section 7.6).

7.1 The render GUI

The 2D renderer is an integral part of the SMAP GUI, and controls are found in several panels (see Figure 7):

1. The tab group to select a layer. You can add new layers by pressing +, or rename or remove the active layer (right click in an empty spot next to the tabs to access context menu).
2. The GUI of a single layer in which you can set all parameters that control the appearance of the respective data.
3. An overview image for navigation
4. A panel to switch layers on and off quickly and to display layers not overlaid, but next to each other.
5. A panel to set layer-independent parameters of the rendered image (e.g. pixel size).
6. A panel to define a ROI in the rendered image for further analysis.
7. The rendered image itself in a separate window.

7.2 Modify the size and location of the image

1. Set the pixel size in the Format GUI (or use the mouse wheel to zoom in and out). You can use pre-defined pixel sizes. By default, one pixel in the superresolution image corresponds to one pixel on the monitor.
2. When you change the size of the image window and press Render, the size of the superresolution image is changed while keeping the pixel size constant.
3. There are several ways to navigate the superresolution image: (i) left-click on a point in the Overview image; (ii) left-click-hold within the Rendered image and move/drag it; (iii) right-

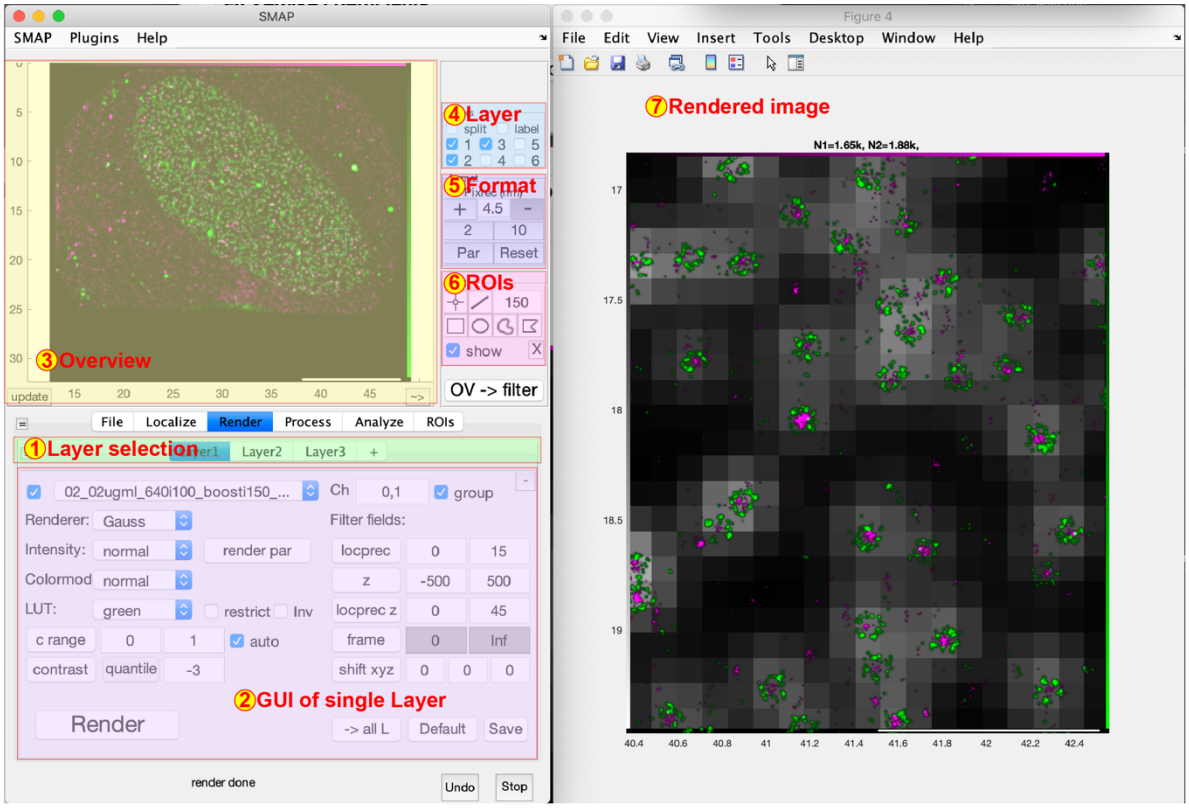

Figure 7: GUI panels related to rendering of superresolution reconstructions.

click on a point within the Rendered image to re-center it; (iv) double-click resets the view to display all localizations (same as Reset in the Format GUI).

4. In Par you can specify a size of the image different from the screen resolution and binning of pixels.
5. In this part of the GUI you can also quickly turn individual layers or the split view on and off.

7.3 Set the appearance of the image

1. The parameters in the **Layer** tab determine how the image is rendered and which localizations are rendered.
2. You can define multiple layers (click **+** tab), which are overlaid in the rendered superresolution image.
3. The checkbox in the upper left corner (or the one in the Layer part of the main GUI at the top right corner, these checkboxes are linked, i.e. if you change one, the other one also changes) determines if the layer is displayed.
4. Select the file to display and the channel indices. You can display several channels in the same layer by separating them with a comma or a space, or use the MATLAB `a:b` notation.
5. Select Colormode: Normal uses the value of the reconstructed image for coloring, but you can also color the image according to the z-coordinates or any other field (attribute) of the localizations.
6. Select a look-up table (LUT).
    a. The values of c range determine the range of the parameters used for coloring that are mapped onto the entire LUT.
    b. Use restrict to remove localizations outside the LUT, otherwise they will be displayed as saturated, i.e. with the minimum or maximum color of the LUT.
7. You can select with the group checkbox if to display grouped or ungrouped localizations.
8. The contrast button brings up a histogram to select which image intensity is mapped to the maximum intensity (higher intensities are saturated). You can toggle to use absolute intensities, or the fraction of pixels to be not saturated (quantile). The quantile parameter can be between 0 and 1 (typically: 0.995) or a negative number Q (typically -3.5). Then the fraction 1-10^Q is not saturated.

9. With the remaining fields you can determine minimum and maximum values for filtering.
10. By pressing save you can save the current settings of the Layer. Pressing default loads those settings. -> all L copies the settings of the current layer to all other layers.

7.4 Advanced Render settings

1. You can rename a **Layer** tab by right clicking next to the tab group. You can display a label (upper right corner of the GUI) with the name of the layer in the rendered image.
2. Select the renderer:
    a. Gauss, every localization is rendered as a Gaussian with a sigma proportional to the localization precision (set the proportionality factor in render par, see point 4 below).
    b. histogram, which gives the number of localizations binned per pixel
    c. constant Gauss, set the size of the Gaussian in nm. If empty, the median of the localization precision is used.
    d. DL, diffraction limited reconstruction.
    e. Other, external renderer, e.g. Voronoi.
    f. tiff, in case you have attached a Tiff image to the data, you can choose here to display it.
    g. raw, a few raw camera frames are attached to the data set, you can display them here.
3. You can choose what determines the displayed integrated intensity of single localizations. It can be set equal to 1 (normal: every localization has the same integrated intensity), equal to the number of photons or equal to the number of blinks (only makes sense if grouped data is displayed).
4. The render par button opens a dialog to set additional parameters for Gaussian rendering: minimum size of the Gaussian kernel (to avoid too small points) in pixels and nanometers and factor gauss, the proportionality constant k between localization precision and width of Gaussian kernel (we use a value of 0.4, as a value of 1 might further decrease the resolution of the rendered image, see Baddeley et al, “Visualization of Localization Microscopy Data.” Microscopy and Microanalysis 2010, doi: 10.1017/S143192760999122X).
5. Shift xyz shifts the image in the associated layer, this can be used to correct for lateral offsets (and offsets in z for 3D data) between images.
6. Color bars corresponding to the LUTs of the first four layers are displayed around the rendered image. You can change their thickness with the Par dialog in the Format GUI (upper right corner). Set the thickness to zero to suppress the color bars.
7. A scale bar is automatically plotted on the lower right corner of the reconstructed image. Its length is a power of ten (nanometers). You can switch it off in the Par dialog.

7.5 Image ROIs

1. You can define a region of interest with the buttons in ROIs (below Format GUI). Define point, line, rectangular, elliptical, free or polynomial ROIs. These ROIs are used by various plugins. For line and point ROIs you can specify the thickness of the ROI (in nm).
2. You can toggle redrawing of ROIs with show or delete a ROI with X.
3. The size of the ROI is displayed as well. Use the line ROI to measure lengths in the rendered image.

7.6 3D viewer

Plugin: Analyze/sr3D/Viewer3D

This plugin opens a 3D viewer. The localizations to be rendered in 3D are defined by a line ROI (see section 7.5). The 3D viewer uses all render settings from the **Render/Layer** tabs of the main GUI, including all filtering.

1. Define a line ROI ⟋ (not a rectangular ROI) in the superresolution image.
2. Press Run. A window with the side-view reconstruction is opened.

3. If pixelsize x,z is checked, you can set the pixel size manually (pass on a vector px,pz for a different pixel size in *x* and *z*, in nanometers); otherwise the pixel size of the current reconstruction is used. If fill is checked the pixel sizes are chosen so that the rendered image fills the entire window.
4. With Show Controls you can use a control panel to translate, rotate or zoom. 0 resets the view (centers the origin).
5. When the sideview window is selected and on top, you can use key shortcuts to translate, rotate (Mac: command / PC: strg) or zoom (alt, this changes the size of the ROI). The direction is defined by the arrow keys. '->', '<-' move along the line roi, up and down in the z-direction. The direction perpendicular to the screen can be accessed with the '.' and ',' keys.
6. Pressing additionally 'shift' results in a smaller movement.
7. You can also manually move the ROI in the superresolution image, the 3D reconstruction is updated on-the-fly.
8. When using transparency instead of projection, localizations closer to you partially block localizations in the background for a better 3D impression.

For performance, the 3D renderer stores a local copy of a subset of localizations. If you change filtering or render settings in the **Layer** tabs, you need to press Run again to update this local copy.

8 ROI manager

The ROI manager allows for simple automated, semi-automated or manual selection of ROIs that can be then annotated, sorted and run through an evaluation pipeline. The results of this evaluation can then be statistically analyzed. ROIs (in the context of the ROI manger often called ***sites***) are assigned to ***cells*** (larger ROIs), cells are part of files. This three-layer hierarchy allows visualization and selection of small sites in large data sets across different files.

The ROI manager should not be confused with the ROI selection tools found in the format panel that allow selecting regions in a reconstructed superresolution image.

8.1 ROI manager GUIs

The ROI manager consists of two GUIs. On one hand, it has its own tab **ROIs** with subtabs in the SMAP main GUI. Here you define all parameters, annotate individual ROIs and define the evaluation plugins. On the other hand, it has an own window called ROI manager that opens by clicking show ROI manager in sub-tab **Settings** in the **ROIs** tab.

The GUI in SMAP has the following tabs:

Settings: Here you define display parameters for the ROI manager.

Annotation: Here you can annotate individual ROIs.

Evaluate: Here you can select evaluation plugins and define their parameters. Evaluation plugins typically work on single ROIs at a time.

Helper: Here you find a list of helper plugins, e.g. to sort the ROIs according to any parameter.

Analyze: Here you find plugins that analyze the result of evaluation plugins. Typically, they work on many ROIs simultaneously.

Segment: Here you find plugins to automatically segment ROIs.

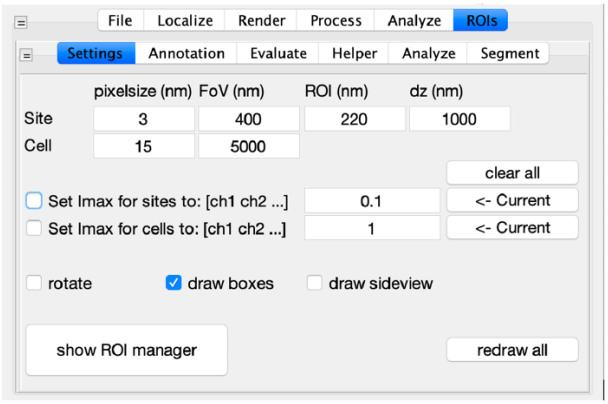

Figure 8: GUI to set parameters for the ROI manager.

8.2 Manually generating a list of ROIs

1. In tab **ROIs/Settings** click show ROI manager. Then click on one file name to render the file overview image.
2. The ROI manager window (Figure 9) contains panels that show the *File image* (2), the *Cell image*, i.e. a part of the *File image* (4) and the *Site image*, i.e. a ROI (6), as well as lists to select stored ROIs (5) and cells (3). The file list (1) is linked to the file list of the **File** tab and cannot be edited here.
3. On the subtab Settings in the ROI tab (main GUI window), define the pixel size for reconstruction and the FoV for the cells and sites (regions around the ROIs), as well as the ROI size itself. The ROI is used for the evaluation, the FoV is used for rendering and navigation of the site context.
4. Check ☐rotate to rotate sites if a rotation angle has been defined and ☐draw boxes to show the positions of the selected cells or sites. For 3D data you can ☐draw sideview in a separate window. To see the effect of the rotation, click redraw.

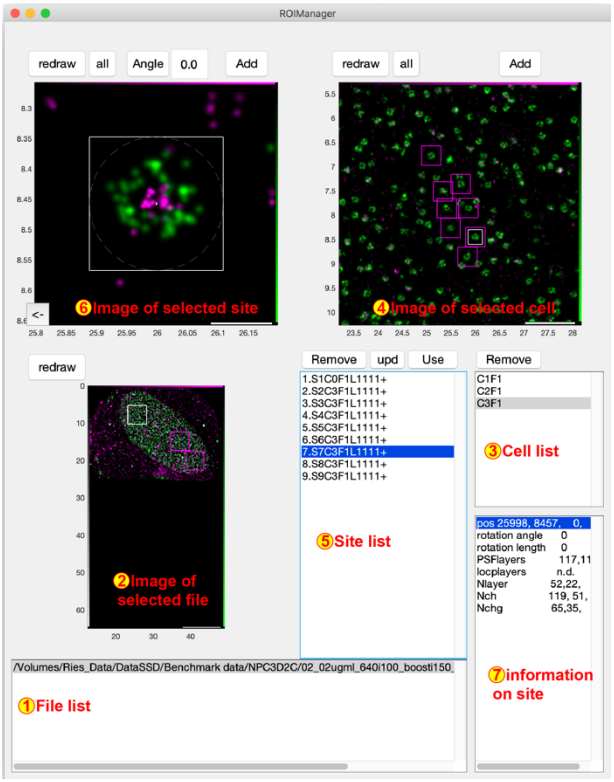

Figure 9: The ROI manager main GUI.

5. By clicking on an item in a list in the ROI manager you can select and render (draw) it.
6. For fast scrolling through sites, the reconstructions are saved. Therefore, if you change any parameters (e.g. size of the FoV, or render parameters in the **Layers**) you need to redraw. You can redraw all in the **ROI/Settings** tab or the ROI manager.
7. By left-clicking in the *File image* you can define the location of a cell. Add it with the Add button above the cell image to the list. You can move a cell by right-clicking in the *Cell image* which will re-center this cell on that spot.
8. Analogously, you can define a site by left-clicking in the *Cell image* and then adding it with the Add button, and move it by right-clicking it in the *Site image*.
9. You can rotate a ROI by pressing Angle and drawing a line. Click redraw, the ROI is rotated so that the line is horizontal.

8.3 Annotate ROIs manually

1. In the tab **ROIs/Annotation** ROIs can be manually annotated. There are four lists to choose from. The items are defined in text files (look at `settings/parlistdefault.txt` and modify accordingly). You can load your settings file with <-load.
2. Use left-arrow and right-arrow keys to go to the previous and next site, respectively.
3. Annotate site by clicking on the lists. Keyboard shortcuts are: up-arrow and down-arrow to choose list entries and shift + left-arrow/right-arrow to go to the previous/next list.
4. You can draw additional lines (two buttons on the right). Line positions, lengths and angles serve as additional annotations.
5. You can define a separate area-ROI for each site.
6. Add an additional comment if desired.
7. The checkbox ☐use site lets you decide if a specific site is used for analysis. By selecting many sites in the ROI manager, you can toggle their ‘use’ annotation at the same time.

8.4 Sorting of ROIs

1. In the tab **ROIs/Helper** select the SortROIs plugin. You can sort the ROIs according to up to four criteria.
2. Define if you want to sort in ascending or descending order.
3. Choose the category and then select the parameter that should be used for sorting:
    a. Use: sort according to the 'use' attribute of each ROI.
    b. Annotation + select any of the annotation lists or the lines (length).
    c. Evaluation + select to select the results of any evaluator. Use the list to navigate through all results.
    d. Statistics + select: Number of photons, PSF size etc...
    e. Cell/File + select: File ID, Cell ID, Site ID.
4. Sort with the Run button. The sequence of sites in the ROI manager will update according to the sorting criteria.

8.5 Evaluation

1. In the tab **ROIs/Evaluate** you can select several evaluation processors, which evaluate each site and return results that are then saved with the ROIs.
2. Use add module and remove to select additional processors or remove them.
3. If an individual module is checked, it is used for evaluation. Evaluations are carried out with each draw of a site if the evaluate on is checked.
4. Clicking on a module in the list opens its GUI on the right. You can adjust parameters here and run the evaluation with preview or by redrawing a site.
5. Re-evaluate all ROIs with the same settings with redraw all.
6. To speed up the process of redraw all you can untick display to not draw every individual site in the ROI manager. If you only want to redraw a subset of ROIs, select them in the ROI list (using shift) and then click on redraw all.

Some useful evaluation plugins:

generalStatistics: Calculates mean brightness, number of localizations etc. for each ROI.

CenterSites: Calculates the centroid or median coordinates of the localizations in the ROI and shifts the ROI to this new position.

circleFitter: Fits circular model to localizations in the ROI to determine the radius and center coordinates of circular structures.

clusterAnalysis: performs clustering on data using dbscan, k-means, k-medoid, Gaussian mixture models or hierarchical clustering and returns positions of clusters and statistics.

8.6 Analyze evaluation results

In **ROIs/Analyze** you can find plugins to analyze results.

Some useful analysis scripts and plugins:

SaveSiteParameters: Exports evaluation results and additional ROI parameters.

FilterSites: Lets you define mathematical equations based on annotation or evaluation results. The result of a logical combination of those then sets the 'use' flag of the ROIs.

RemoveEmptySites: Removes ROIs without any localizations in them.

RemoveDoubleSites: Removes ROIs that are too close to other ROIs.

Export_tiffs: Exports ROIs as tiff images.

ROI_montage: Exports a gallery of ROIs.

AverageSites: Shifts all localizations in ROIs to the same reference position location and creates a new data set for those.

Sitenumbers2loc: Adds two fields (attributes) to the localization data containing the site number and the cell number, respectively.

8.7 Automatic segmentation

In **ROIs/Segment** you can find plugins to automatically segment files and store the result as cells or sites.

Some useful segmentation plugins:

MakeCellGrid: Creates cells across the whole field of view with a size determined in the **Settings** tab.

SegmentNPC: Segments circular or ring-like structures (e.g. the nuclear pore complex, NPC).

SimulateSites: a localization-based simulation engine for SMAP. It uses as an input a list of localizations, a MATLAB function that returns coordinates or an image which defines a 2D structure. It returns simulated localizations to SMAP using a realistic model for the photophysics of the dye.

9 Dual-color and dual-channel SMLM

There are many ways of performing dual-color SMLM:

1. **Sequential, same fluorophore** (e.g. DNA exchange PAINT). In this case, just load data for each color as separate files. Drift-correct each file separately, for the first file choose reference is last frame.
2. **Sequential, different fluorophores** (e.g. Alexa-Fluor 647 and Atto 488). As above. But in addition, you will have to correct for chromatic aberrations. To this end, acquire many fields of view of Tetraspeck beads on a coverslip. Use this to calculate the transformation T (see 9.1.1) and apply it to the target data set (see 9.1.3).
3. **Quasi simultaneous on the same FoV, same color** (e.g. different activator/reporter pairs). Treat this as a single-color data set and assign the color based on the frame, e.g. using the ***MathParser*** (see section 10.3).
4. **Simultaneous, two cameras or two areas on the same camera** (e.g. Alexa Fluor 647 and mEos3.2). Generate a transformation file as described at item 2 and use it to transform the target channel onto the reference channel.
5. **Ratiometric** (e.g. Alexa Fluor 647 and CF680 on two parts of the same camera or on two cameras). See section 9.3.

9.1 Registration

9.1.1 Find a transformation to register two data sets

Plugin: Process/Register/Register Localizations

1. Select the target (usually bottom or right), and if to mirror the target half-image. For two synchronized cameras, use center for target.
2. Select a transformation type (try projective, if that is not sufficient use polynomial with the parameter 3).
3. Under Parameters you can choose additional parameters
    a. Pixel size for correlation. Around size of the localization precision. If the correlation image is dotty and the wrong maximum is found, increase this size.
    b. Max shift for correlation: reduce, if wrong maximum is found. Increase, if true maximum is outside.
    c. Max locs for matching: eg. 10^7. Number of localizations used to determine transformation. Precision increases with this, as does computation time. Rather use large values here.
    d. Initial magnification: Estimate for the magnification of the second channel to facilitate initial registration.
    e. Initial shift X, Initial shift Y: starting translocation parameters, in nm. Use a rough estimate e.g. from drawing a rectangular ROI with two opposing corners positioned on the same feature in the two channels and using its dimensions.

f. Max shift matching: distance that corresponding localizations can be apart (after shift is applied). 250-500 nm typically. If this value is too large, random localizations are matched, this can introduce systematic error.
g. For all of these parameters you can pass on a vector to go through iterations of registrations (e.g. start with a pixel size of 250 nm, reduce this to 50 in the second step).

4. Press Run and judge results (the plugin might perform several iterations, look at the last iteration):
   a. If the transformation does not work and a message is displayed in the MATLAB command window that no maximum was found, increase maximum shift for correlation and/or enter a realistic initial shift.
   b. The **corr** image should show a clear maximum, the square should be on that maximum. If you see many dots around this maximum, increase the pixel size.
   c. The **dxy** should show a clear maximum in the center. *dx* and *dy* should be on the order of the localization precision and for beads < 0.2 pixels. The number of anchor points should be at least a few %. **hist** is just a profile through the scatter image.
   d. The **pos** image should show paired (blue) localizations across the entire field of view, not only locally.
   e. If the transformation is good, save it with save T. Note that by performing a registration, the found transformation automatically becomes the default transformation also in other plugins, making it optional to load a transformation file.
5. For difficult data you can also first find an approximate transformation (e.g. projective) as described before (or load one, with load T). Then check ☐use initial T. This transformation is then applied before finding anchor points.
6. If ☐use layers is checked, the plugin does not use all localizations, but only those displayed in T: and R: layers (e.g. for two synchronized cameras).

9.1.2 Find the shift between two images

Plugin: Process/Register/Register Images

Finds the shift between two layers based on the rendered images.

1. Render the two channels that are not registered in two different layers and select the corresponding layers.
2. If you ☐set pixelsize (nm) the images are re-rendered with the corresponding pixel size.
3. The *x*- and *y*- shifts between the images are determined using image cross-correlation.
4. By default, the shifts are written to the corresponding fields in the **Render/Layer** tabs.
5. If you set ☐correct coordinates the `xnm` and `ynm` fields are corrected by the shift. For this you need to select the channel and data set to which to apply the transform to.

9.1.3 Apply Transformation

Plugin: Process/Register/Apply Transformation

This plugin applies a saved transformation to localizations or images. You can use this to move all localizations from the second channel into the first channel.

1. Load a transformation with load T.
2. Select a dataset. If ☐transform all files is selected, all loaded files are transformed with the same transformation.
3. Select what to transform: reference (to target), target (to reference) or all (meaning all localizations are transformed. For this you can choose either reference to target or target to reference transformation).
4. Select if to transform only localizations or tiffs or both.
5. If ☐set channel is selected, the channel field is overwritten depending on the localizations being reference or target localizations. Otherwise, use a number with ☐add to target

channel to create a new channel for the transformed localizations. The latter is useful to inspect the quality of the registration, i.e. with ratiometrically acquired dual-color data.
6. Click Run.

9.1.4 Combine Channels

Plugin: Process/Register/Combine Channels.

Similar to ***Apply Transformation***. In addition, fluorophores in both channels which can be associated to a single fluorophore (same frame, close proximity after transformation) are averaged and presented as a single localization. This is useful for e.g. ratiometric dual-color imaging or bi-plane 3D imaging to avoid duplication of localizations. However, any error in the transformation then leads to an error in the position.

9.2 Color assignment

9.2.1 Get intensities from camera images

Plugin: Process/Assign2C/2C intensities from images 2 cam

This plugin uses a transformation (determined e.g. with ***Register Localizations***) to find for every localization the position in the other channel and then determines the intensity in both channels.

1. Fit your data set and make sure it is loaded in SMAP.
2. Load tif files corresponding to the raw data.
3. If you use two cameras, load also the tif files for the reference channel, otherwise leave it empty, then the same file as for the target is used.
4. Load the transformation with Load T.
5. If you want to subtract a background based on a median filter, check calculate BG. Otherwise the fitted background is used.
6. Check reference if you want to analyze the reference channel as well.
7. Check EM for the data sets that were acquired with EM gain and need to be mirrored.
8. Select one or several plugins which determine the intensity. By clicking on the list, the GUI of the plugin is opened and you can adjust parameters.
    a. ***Roi2int_sumG***: uses a ROI (set size) to determine intensity, and a larger ROI for the background.
    b. ***Roi2int_fitG***: Uses a Gaussian fit to determine intensity and background. The position is fixed to the fitted position. You can use the fitted PSF size or fix it. If subtract BG before fitting is checked, the background is subtracted prior to fitting and the fit is performed with background set to zero. Otherwise the background is a fitting parameter.
    c. ***roi2int_expPSF:*** Uses an experimental PSF model.
9. Press Run. The results are automatically saved with a `'_dc'` (for dual-color) in the file name.

9.2.2 Determine channel from intensities

Plugin: Process/Assign2C/Intensity2ManyChannels

This plugin assigns channel values to the localizations based on two fields of the localization data (usually intensity in camera channel 1 vs camera channel 2).

1. Select the two fields that encode the intensity in both channels, eg. fit_n1 and fit_n2. From this selection an image is generated in which the x-axis corresponds to the first intensity value and the y-axis corresponds to the second intensity value (logarithmic scaling). With log scale you can also use a logarithmic scaling for the contrast.
2. Press ROI 1 to draw a polygon ROI around the area the image that encloses all localizations corresponding to channel 1.
3. Repeat the same for channel 2 and additional channels (for channel 4 and onwards put the channel number in the respective field).

4. With Show ROIs you can display all ROIs. You can always adjust all ROIs.
5. With Delete ROIs you can delete all ROIs and start over.
6. With load and save you can load and save all defined ROIs for later use.
7. Press Run to assign a channel number to all localizations based on the defined ROIs.
    a. If use grouped is selected, this assignment is based on grouped localizations.
    b. Localizations outside of any ROI are assigned the channel value 0. This is also the channel value for all data before channel assignment.

9.3 Ratiometric Dual-Color Imaging

1. Fit your data
    a. 2D or 3D fit.
    b. Do not yet apply drift correction. This you can do later after channel assignment.
2. Find transformation for both channels with ***Process/Register/Register Localizations***.
3. Determine intensities of localizations in both channels with ***Process/Assign2C/2C intensities from images 2 cam.***
4. Assign channel from relative intensities with **Assign2C/*Intensity2ManyChannels***.
5. You can now render both channels individually with in two layers.
6. Optional: transform target localizations onto reference with ***Process/Register/Apply Transformation*** or ***Process/Register/Combine Channels***.
7. Now apply the drift correction.

10 Selected plugins

1. Plugins are found in the **Plugins** menu. A selection of regularly used plugins can be found in the **Analyze** and the **Process** tabs in subtabs (configurable).
2. Select a plugin, edit the parameters and press Run.
3. With Show results you can toggle the window with the output of the module on and off.
4. Info displays a description text of the module in the results window.

10.1 Batch analysis

Plugin: Analyze/other/BatchAnalysis

Runs various process and analysis plugins on many data sets and saves the output.

1. Load an example file and set localization filters.
2. In the Analyze Tab create a new tab called Batchanalysis:
    a. Right-click on the tabs.
    b. Select ‘add’ in the context menu.
    c. Set the name ‘Batchanlaysis’.
3. Add analysis plugins:
    a. In the new tab right-click on ‘empty’ and select ‘add plugin’.
    b. Click through the hierarchy until you find the analysis plugin of choice.
    c. Click on the name of the plugin and configure all parameters in the GUI.
    d. Repeat for all plugins.
4. Go back to **Analyze/other/BatchAnalysis** and select a directory with all *_sml.mat files you want to run the analysis on or a *.txt file .with the full paths for all *_sml.mat files.
5. Set the path to a directory where to write the output files.
6. Select if to save output as *.png or *.fig or both.
7. Press ‘Run’ in the BatchAnalysis plugin.

10.2 Statistics and Resolution

Localization statistics, *Plugin: Analyze/measure/Statistics*

Get single-molecule statistics

1. If use Roi is checked, only the localizations in the current ROI /FoV are evaluated.

2. If □use layers/filters is checked, each layer is evaluated individually; otherwise statistics for grouped and ungrouped data are shown.
3. With □plot overview you can have all results in one figure (e.g. for saving) rather than in individual tabs.

FRC resolution, *Plugin: Analyze/measure/FRC resolution*

Calculates the FRC resolution according to: R. P. J. Nieuwenhuizen, K. A. Lidke, M. Bates, D. L. Puig, D. Grunwald, S. Stallinga, and B. Rieger, "Measuring image resolution in optical nanoscopy," Nat Methods,vol. 10, no. 6, pp. 557--562, Apr. 2013.

10.3 Mathematics

Mathematics parser, *Plugin: Process/Modify/MathParser*

This plugin allows you to evaluate a mathematical expression using localization attributes and assign the result to a new attribute. Use MATLAB notation and access localization attributes directly via their name. Don't forget to use the `'.'` in front of `'*'`, `'/'` and `'^'`. For example, to calculate a 3D localization precision type in the equation field:

```
sqrt(2*locprecnm.^2+locprecznm.^2)
```

The last 10 expressions are saved and can be recovered using the dropdown menu.

Spatial mean median max min filter, *Plugin: Process/modify/SpatialFilterLocs*

Calculates mean / median / max / min / quantile of a specific field (attribute) of localizations in an area around each localization. Useful for displaying color-coded images.

10.4 Other useful plugins

Remove localizations, *Plugin: Process/Modify/RemoveLocs*

Allows you to remove all localizations inside or outside a user-defined image ROI.

Keep only filtered localizations, *Plugin: Process/Modify/keepfiltered*

Keeps only filtered localizations. Can be used to reduce file size.

Define x and y coordinate fields for rendering,

Plugin: Process/Modify/DefineMainCoordinates

Define any fields as main x, y and z coordinates

Evaluate line profiles: *Plugin: Analyze/measure/Lime profiles*

Calculates profiles along a linear ROI and fits it with a model of choice. Flat: step function convolved with Gaussian (=Erf). Disk: Projection of a homogeneously filled disk, convolved with Gaussian. Ring: Projection of a ring, convolved with Gaussian. Distance: Two Gaussians at a distance *d*.

Density calculator, *Plugin: Analyze/cluster/density_calculator*

Looks at the neighbourhood of each localizations and counts number of neighbours in a defined region.

Rendering using any two attributes as x and y*, Plugin: Analyze/other/VersatileRenderer*

Render any field vs any other using any 3rd field for coloring (the LUT is taken from the **Render** tab).

Show history*, Plugin: Analyze/other/Show History.*

Shows the parameters of all plugins used previously that change the data.

11 Configuring the GUI

11.1 Simple vs advanced mode

Often, analysis plugins have essential input parameters that need to be set and changed often, and additional parameters that work well with default values and are used for fine tuning of the algorithms. SMAP offers a way of hiding controls for optional parameters, thus making a plugin GUI less cluttered and easier to use. You can select in the Menu/SMAP/Hide advanced controls. For individual plugins you can toggle between advanced and simple mode by using the [-] and [v] buttons in the top right corner.

11.2 Saving and loading a GUI configuration

You can save a current GUI configuration with **Menu/SMAP/Save GUI appearance** under any name. You can then choose **SMAP/Preferences/GUI** to select the GUI settings file as a default. When you save the file, you are asked if you want to save all GUI parameters. If you do so, then all user provided parameters are saved as well. This might slightly slow down loading of the SMAP GUI, but gives you the possibility to save default parameters with the GUI.

Load a GUI configuration file with **Menu/SMAP/Load GUI appearance**. This writes the GUI settings file in the preferences. Restart SMAP to have an effect.

11.3 Adding plugins to the GUI

For quick access, plugins can be displayed in the main GUI in the **Process**, **Analyze** and **ROIs** tabs. Right-clicking next to the tabs in one of these panels opens a menu. Here you can add another tab and name it.

Now you can add plugins to this tab by right-clicking on the plugin list. A dialog opens in which you can select categories, then sub-categories, then the plugin.

Set the default parameters in the GUI of the plugin and don't forget to save the GUI as described in section 11.2.

11.4 Resizing and detaching components

Most GUIs can be resized, controls then adapt to the new size.

Some components can be detached from the main GUI and appear then in their own window. This can be useful for re-arranging components on a large screen.

Tabs can be detached in the context menu (right-click).
The overview window can be detached with the [~>] button.
The Format panel can be detached in the context menu (right-click).
If you start a plugin from the menu it will be opened in its own window.

Except for the overview window it is not possible to attach components again. For this, just restart SMAP.

11.5 User-defined menu

You can add your own plugins to the menu by providing a text file and selecting it in **SMAP/Preferences/GUI/custom menu**. Restart SMAP to show the custom menu.

Each line in the menu definition file contains the hierarchy in the menu, each level separated by a `'.'`. End with `.module=` and then provide the position of the plugin with `category,subcategory,pluginname`. You can rename categories or plugins by providing another line that ends with `.name = New Plugin Name`. In `settings/custommenu.txt` you find an example for a menu definition file.