Step-by-step guide to fit 3D dual color example data

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Preparation

1. Install SMAP according to the *User guide* (on www.rieslab.de) section 2. To install the compiled version, download the zip files on www.rieslab.de and *follow the installation notes*: https://www.embl.de/download/ries/SMAPCompiled/Installation_notes_SMAP_compiled.rtf.
2. Download and unzip the example *beads.zip* and *Nup107_AF647_WGA_CF680.zip*, or for a smaller example *beads_subset_small.zip* and *Nup107_AF647_WGA_CF680_subset_small.zip*, from www.rieslab.de. (see Box 1 for details)
3. You can select **Menu/SMAP/hide advanced controls** to have a simple GUI.
4. If during the following protocol things don't work as expected, try restarting SMAP and load the last file that was produced.
5. For an introduction to SMAP, we recommend following the *getting started* example first.

Box 1: The example sample.

Nup107-SNAP labeled with BG-AlexaFluor647 and the central channel labeled with WGA-CF680. Homozygous CRISPR cell lines, imaged in an MES-based oxygen depleted imaging buffer as described in Thevathasan et al, Nature Methods 2019. The data was taken on a custom microscope with two spectral channels, split by a LP665 beam splitter and imaged on two parts of the EMCCD camera. Astigmatism was introduced with a cylindrical lens.
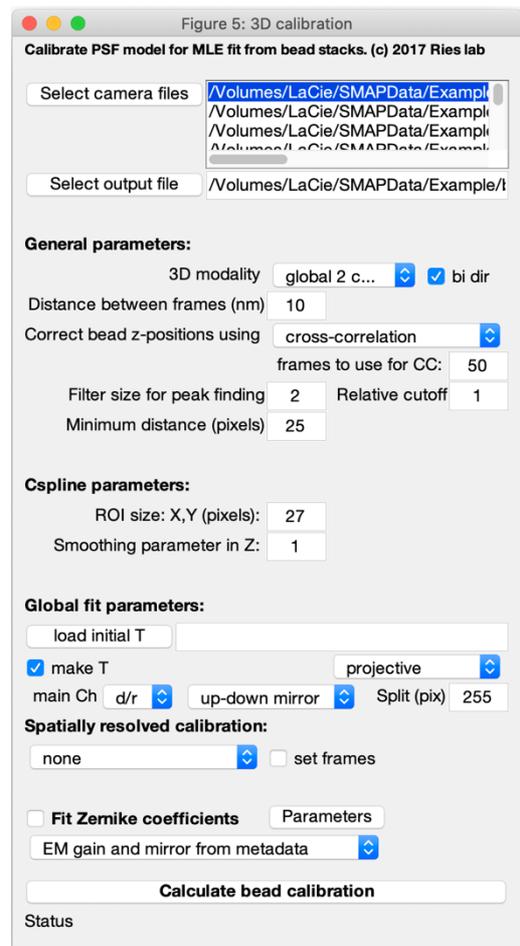
Figure 1: GUI to calibrate the experimental PSF model.

1 Calibrate experimental PSF model from bead stacks

1. Start SMAP.
2. Find the plugin *calibrate3DsplinePSF* either from the **menu/Plugins/Analyze/sr3D** or in the **Analyze** tab, **sr3D** subtab and press Run . Now the GUI for the spline calibration opens.
3. Select camera files to load all the bead stacks. A new window opens. Here press add dir and select all directories in the `bead` directory. Press Done .
4. Set the parameters according to Figure 1.
    a. As we have dual-color data in two spectral channels in two parts of the camera we choose the 3D modality: global 2 channel.
    b. For nicer visualization of the validation of the model you can select ☐ bi dir to activate bi-directional fitting.
    c. Set the distance between slices in the stack (in nanometers).
    d. You can adjust the other parameters according to the *User guide* section 5.4.1.
    e. An initial transformation is calculated from the bead stacks. You can choose the main channel (in this case the bottom channel) and specify that the upper and lower channels are mirrored horizontally with respect to each other.
    f. We let SMAP determine the EM gain and mirror from the metadata. Otherwise, set here if beads were acquired with the conventional or EM mode.

5. Calculate bead calibration . This step might take 5-10 minutes, you can see the progress in the status bar of the plugin. You can compare your saved files with example_3dcal.fig. Specifically, compare the tabs of the output figure with those in Figure 2.
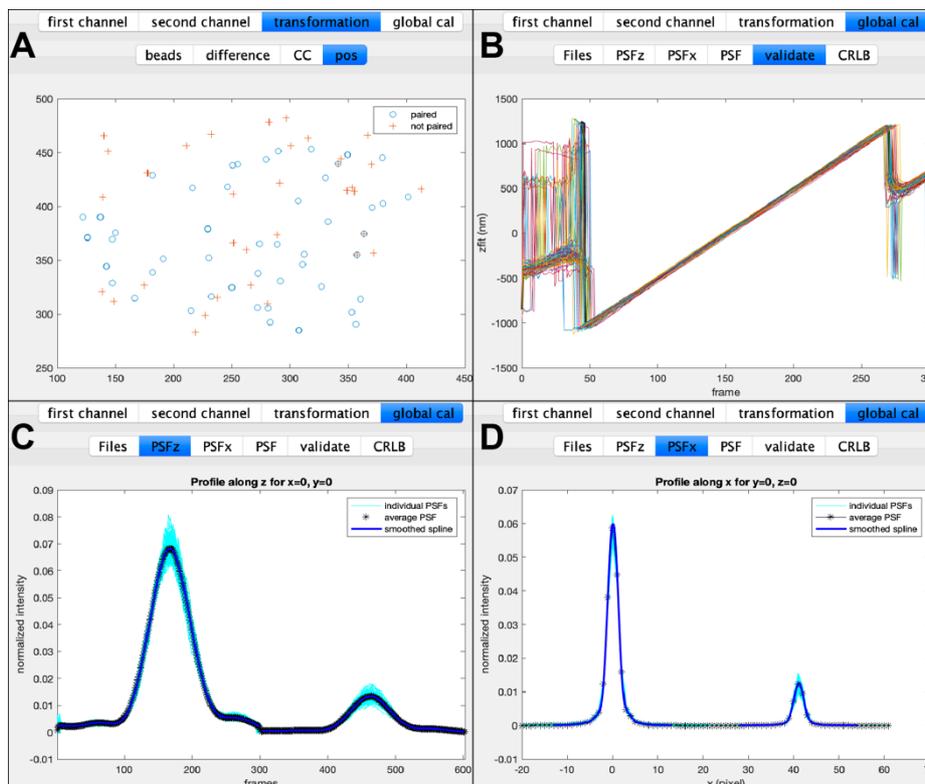
Figure 2: Output of the GUI calibration. A) to check if beads across the whole field of view were paired. B) to check if the model could properly fit the individual beads. C) Cross-section along z-axis through model to check if the model represents the individual beads. D) as C. but along x-axis.

2 Fit 3D dual-color SMLM data with experimental PSF model

1. Check in the SMAP GUI at the bottom of the **Localize** tab that the fitting workflow `fit_fastsimple` is selected. Otherwise load that workflow by pressing Change.
2. In the main SMAP GUI select the **Localize/Input Image** tab.
3. Load images and select one of the files in the directory `Nup96_AF647_WGA_CF680`.
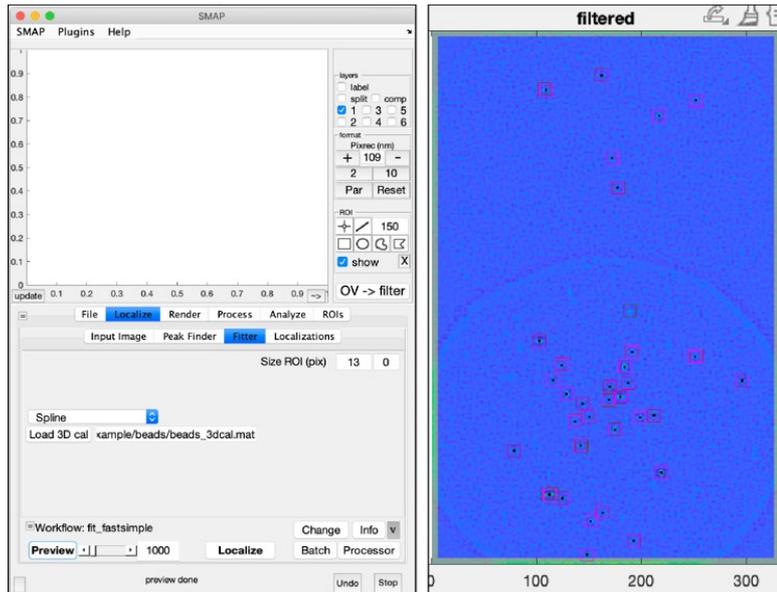
Figure 3: Fitting GUI and result of preview.

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.
4. You can adjust the parameters in the **Peak Finder** tab according to the *User guide* section 5.3.
5. In the **Fitter** tab select Spline and with Load 3D cal load the bead calibration file that you just generated.
6. Select a large frame (e.g. 1000 as the first frames are still too dense to optimize fitting parameters) and press preview. The results should be comparable to those in Figure 3.
7. Press Localize and wait for the fitting to be done (this might take up to one hour depending on your computer).
8. You a have a first look at the results by pressing Render in the **Render** tab.

3 Calculate transformation between channels

See *User Guide* section 9.1.1

1. In the Format part of the GUI (Figure 4) reset the field of view with Reset and delete any ROI you might have drawn with X.
2. Filter out the bad localizations by selecting a maximum localization precision (e.g. 10 or 15 nm) and a minimum LogLikelihood (see *User Guide* section 6.2).
3. Choose the plugin **Menu/Plugins/Process/Register Localizations** or in the tab **Process/Register/Register Localizations**.
4. Select use layers and layer1 both for reference and target.
5. Select for target bottom and for mirror up-down. Press Run.
6. Compare the output to Figure 5.
7. Save the transformation with save T.
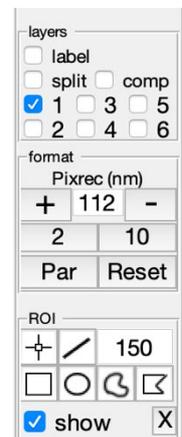
Figure 4: Format GUI
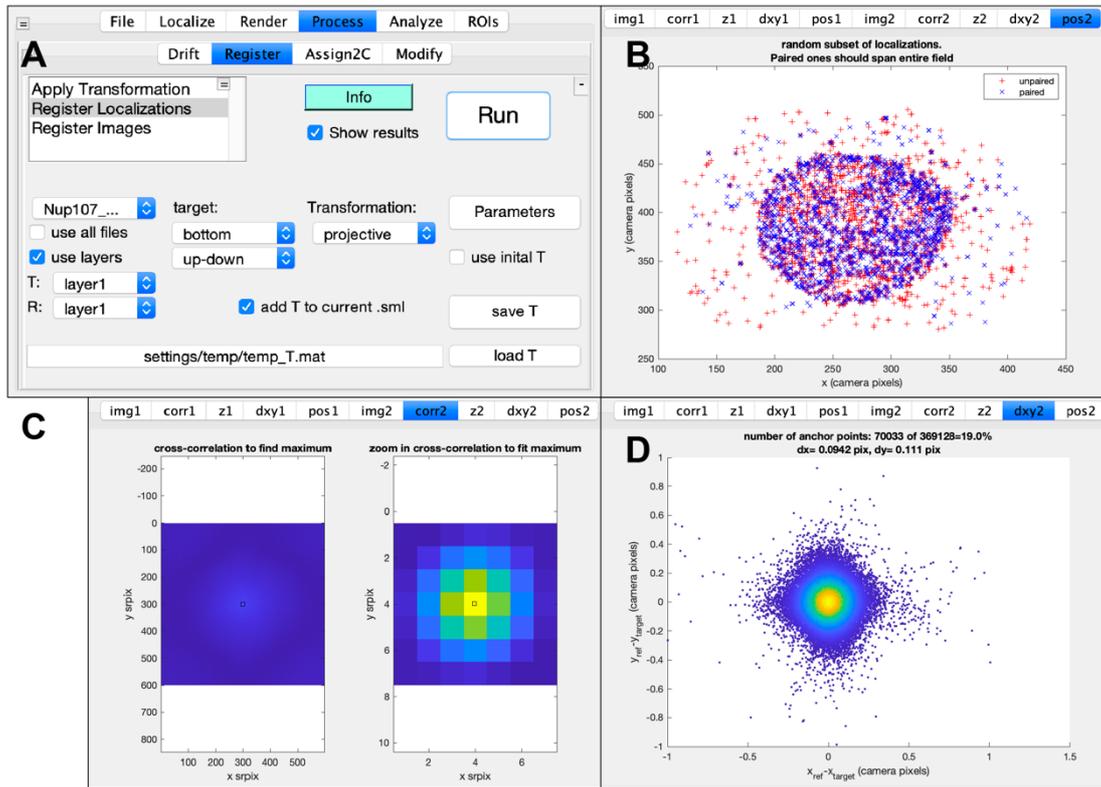
Figure 5: A) GUI to calculate transformation. B) the paired localizations should span the whole field of view. C) the correlation should have a clear peak. D) the errors should form a clear maximum in the center, the standard deviations dx and dy should be << 1 pixel.

4   Assign intensities in both channels

Here we discuss how to assign to each localization the intensity in both channels, which is later used to assign the color.

1. Start the plugin: **menu/Plugins/Process /Assign2C/2C intensities from images 2 cam** or in the **Process/Assign2C/2C intensities from images 2 cam** tab.
2. Load the raw camera images in the target field with load tif, leave the reference field empty (as both channels are in one file). As the images were acquired with EM gain, leave the ☐EM ticked, as well as ☐target and ☐reference.
3. Load the transformation file you generated above with load T.
4. Have only ☐roi2int_expPSF selected.
5. Click on roi2int_expPSF and load the bead calibration file that defines the PSF (*_3Dcal.mat).
6. Press Run. This process can take up to one hour again. The results are automatically saved with the ending _dc_sml.mat.
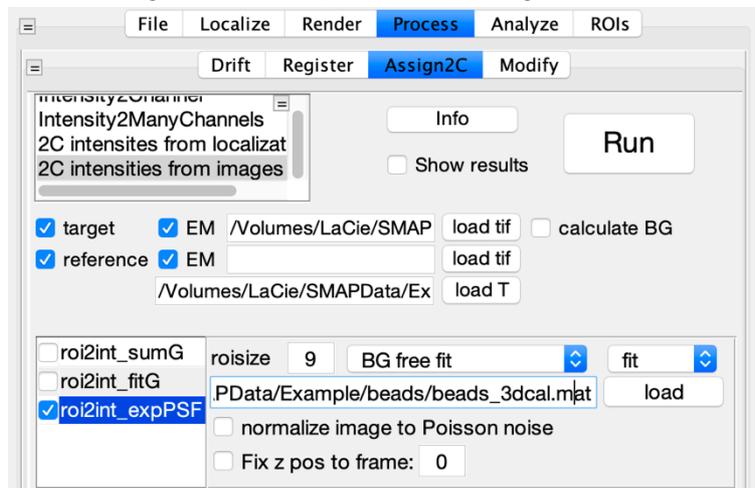
Figure 6: The GUI to extract intensities from raw camera images.

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5 Assign color from intensities

1. Select the plugin **Menu/Plugins/Process/Assign2C/Intensity2ManyChannels** or the same in the tabs.
2. Show ROIs.
3. Click ROI 1 and select the region of intensities for the first fluorophore.
4. Click ROI 2 and select the region of intensities for the second fluorophore.
5. Compare to Figure 7 and Run.
6. In the **Render** tab next to **Layer1** click the **+** tab to create Layer2. Type in 2 in the *Ch* field of the tab **Layer2** to only display localizations assigned to channel 2. Type in 1 in the *Ch* field of the tab **Layer1** to only display localizations assigned to channel 1.
7. Select a different look up table in the LUT menu.
8. Press Render. You should already be able to see the dual-color image.
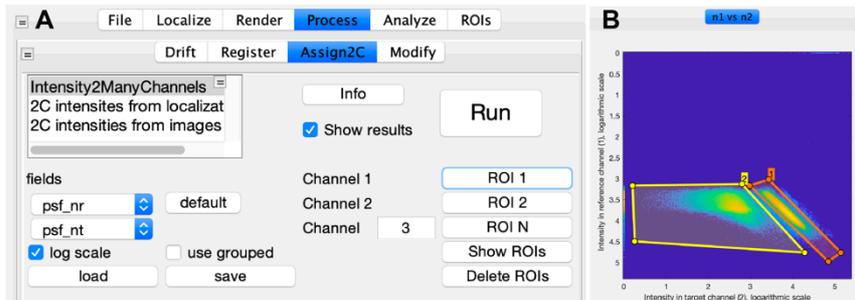
Figure 7: A) GUI. B) Regions corresponding to the two fluorophore species AF647 and CF680.

6 Transform second channel onto first.

To avoid any errors due to channel misalignments, you can analyze just one of the two channels exclusively. This is especially recommended if you split the two colors not in the middle of the spectra, but at the low end of the spectra as is done in this example. Then the long wavelength channel is exclusively used for rendering and carries all positional information, whereas the short wavelength channel is only used for color assignment.

You, however, have the option to transform both channels onto each other using the transformation file you generated in section 3. It is important that this is done prior to drift correction.

1. Select the plugin **Menu/Process/ Register/Apply Transformation** or the corresponding plugin in the tab.
2. Load the transformation file with load T.
3. Select ☐add to target channel 10 and Run.
4. Create two more layers as Layer 3 and type in at Ch 11, and Layer 4 and type in Ch 12. Alternatively display channels 1 and 11 in Layer1 by typing Ch 1,11 and 2 and 12 in Layer2 by typing Ch 2,12.
5. You should check if the transformation onto each other was successful, otherwise this points to problems with the transformation.
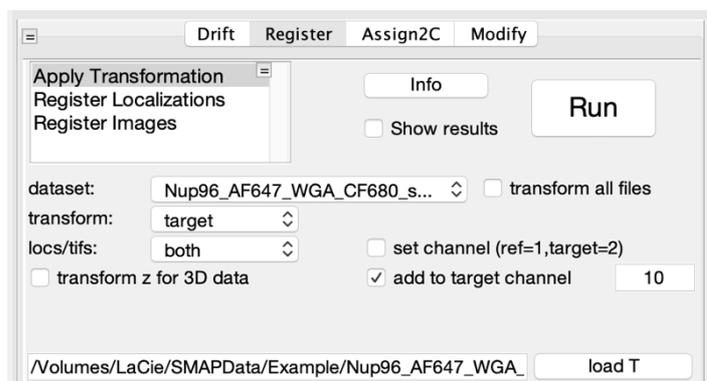
Figure 8: GUI to transform channels onto each other.

7 Perform drift correction

1. Reset view and remove any ROI you selected (see 3.1).

2. Select the plugin **menu/Plugins/Process/Drift/drift correction X,Y,Z** or the corresponding tab.
3. Click the v symbol in the upper right corner to show the advanced controls.
4. Check both ☐Correct x-y drift and ☐Correct z-drift.
5. If you have transformed the channels onto each other select no mirror, otherwise select 2 channels, mirrored, vertical split to drift correct both channels independently. Click Run.
6. Check if all curves 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.
7. Play around with the rendered image by zooming and panning and explore the effect of filters as described in the *User guide* sections 6 and 7.
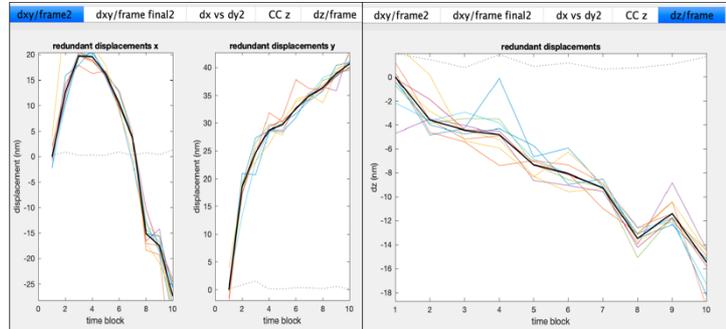
Figure 9: Relative displacement between blocks in redundant cross-correlation.

8 Generate a movie of a rotating nuclear pore complex

1. Zoom into a region of interest with nice looking NPCs.
2. Draw a line ROI through the pores, change the width of this ROI e.g. to 250 nm. Only localizations in the rectangular ROI will be rendered in the 3D viewer (Figure 9).
3. Select the plugin **menu/Analyze/sr3D/Viewer 3D** or the corresponding tab.
4. Press Run. The side view image should open. The render parameters (color, filtering,…) are the same as for the main rendered image and set in the **Render/Layer** tab.
5. You can drag the line ROI; the side view reconstruction will automatically follow.
6. You can start a dynamic rotation by pressing Animate. To reset to the side view press Polar angle.
7. Adjust zmin/max so that the NPCs are in the center of the z range. As the rotation axis is centered with the FOV, it is necessary to center the structure with the FOV to have proper rotation animation.
8. Save movie renders a rotating animation.
9. The animation is saved as a tiff stack. You can use Fiji to export it to avi or mp4.
10.   For more information on the 3D viewer see the *User guide* section 7.6.
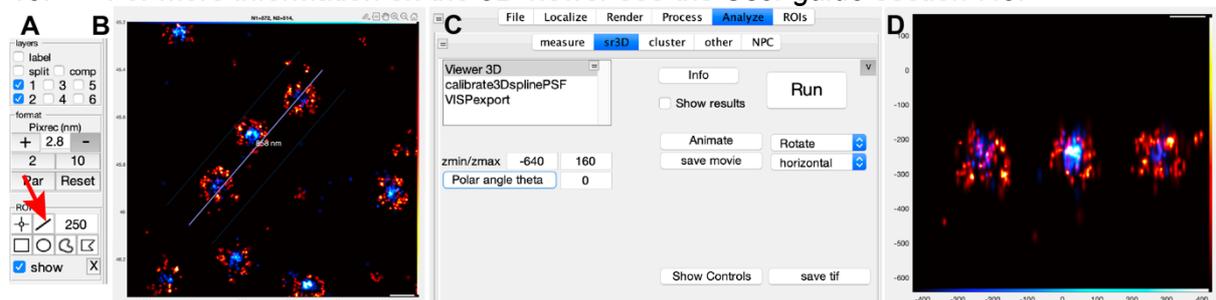
Figure 10: A) Format GUI to draw a line ROI. B) Main 2D reconstruction window with a line ROI. C) GUI for the 3D renderer. D) Side-view rendering of the localizations in the line ROI.