SplitViSu

Software for spectral demixing of splitSMLM data (SMLM with a dichroic image splitter).

SplitViSu can be used either as a standalone Windows application or as an application for MATLAB

Installation

***Standalone application for Windows*:**

Full functionality: download and run 'SharpViSu2.x.x._web_installer' under https://github.com/andronovl/SharpViSu/tree/master/Installer and follow instructions

Only spectral demixing: download and run 'SplitViSu1.x.x._web_installer' under https://github.com/andronovl/SharpViSu/tree/master/Installer and follow instructions

***MATLAB application (installed MATLAB is required, tested under version 2021b)*:**

Download the folder https://github.com/andronovl/SharpViSu/tree/master/SharpViSu and add it to the MATLAB search path. SharpViSu2 and SplitViSu were developed in MATLAB R2021b but should work also in older versions. Toolboxes required: Image Processing Toolbox, Signal Processing Toolbox, Statistics and Machine Learning Toolbox

Running

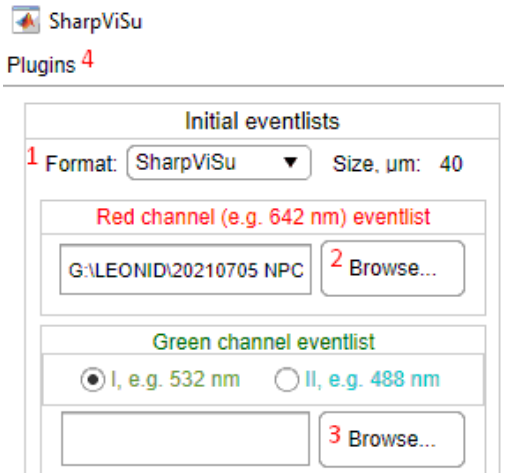
Standalone application: run file 'SharpViSu.exe' (full functionality) or 'SplitViSu.exe' (spectral demixing only)

MATLAB application: run 'SharpViSu_App.mlapp' (full functionality) or 'SplitViSu.mlapp' (spectral demixing only)

Data input

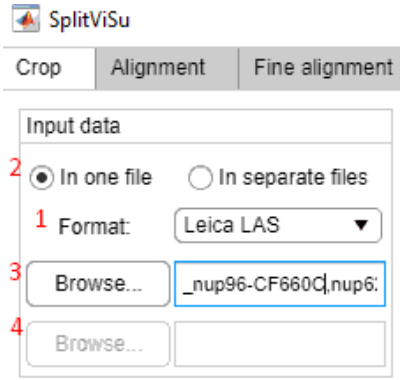
EITHER from SharpViSu (full functionality):

1. Select input Format
2. If both channels of the image splitter are localized within a single localization file, load the single dataset as the ‘Red channel (e.g. 642 nm) eventlist’
3. If each channel of the image splitter is localized within an individual localization table, load the first channel as the ‘Red channel (e.g. 642 nm) eventlist’ and the second channel as the ‘532 nm Green channel eventlist’
4. Press 'Plugins - SplitViSu demixing' - the main window of SplitViSu will open



OR Inside SplitViSu:

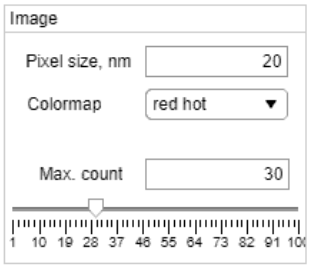
1. Choose the format of your input data
2. Select the number of input files with localizations: 'In one file' (both channels are in one file) or 'In separate files' (one file per channel)
3. Press 'Browse...' and select the (first) file to upload
4. (only if 'In separate files') Press 'Browse...' and select the second file to upload



Visualization of input data

- If 'In one file' - the data will be automatically visualized after the localization list has been loaded
- If 'In separate files' - select from the 'Display data from input list' the channel to display
- The total number of input localizations will be displayed as well 

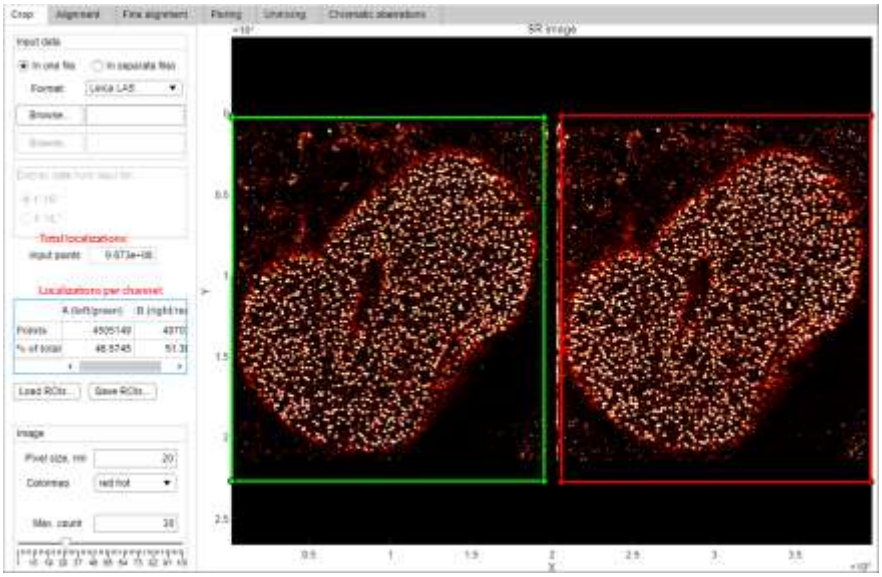
- The super-resolution image is reconstructed as a 2D histogram of the input localizations
- The 'Image' section allows to change the pixel size of the image, the colormap and the pixel capacity ('Max. count')



Cropping of input data

<u>Only for an 'In one file' input</u>

- Two ROIs for two channels (green and red) will appear after the localization list has been loaded
- Adjust the position of the ROIs with a mouse so that each ROI would correspond to each channel of the image splitter
- (optional) Press 'Save ROIs...' to save the ROIs
- (optional) Press 'Load ROIs...' to use previously saved ROIs
- The displayed number of localizations in each channel will be updated every time you change a ROI



Translational alignment of channels

Go to the tab '**Alignment**'

The number of localizations and frames in each channel will be displayed.

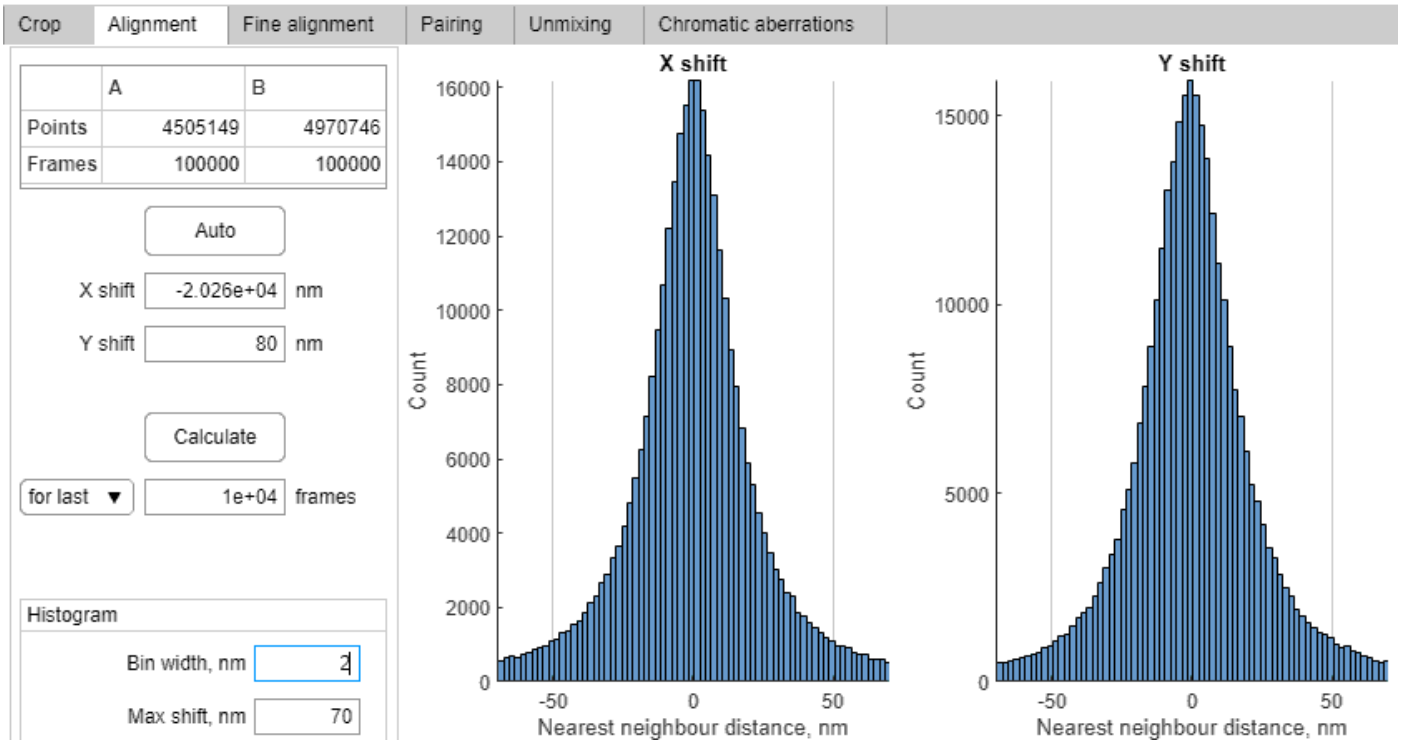
If known, the translational shift in X and Y can be entered in the corresponding fields.

If the shift is not know precisely, press 'Auto' - the software will attempt to calculate the shift automatically.

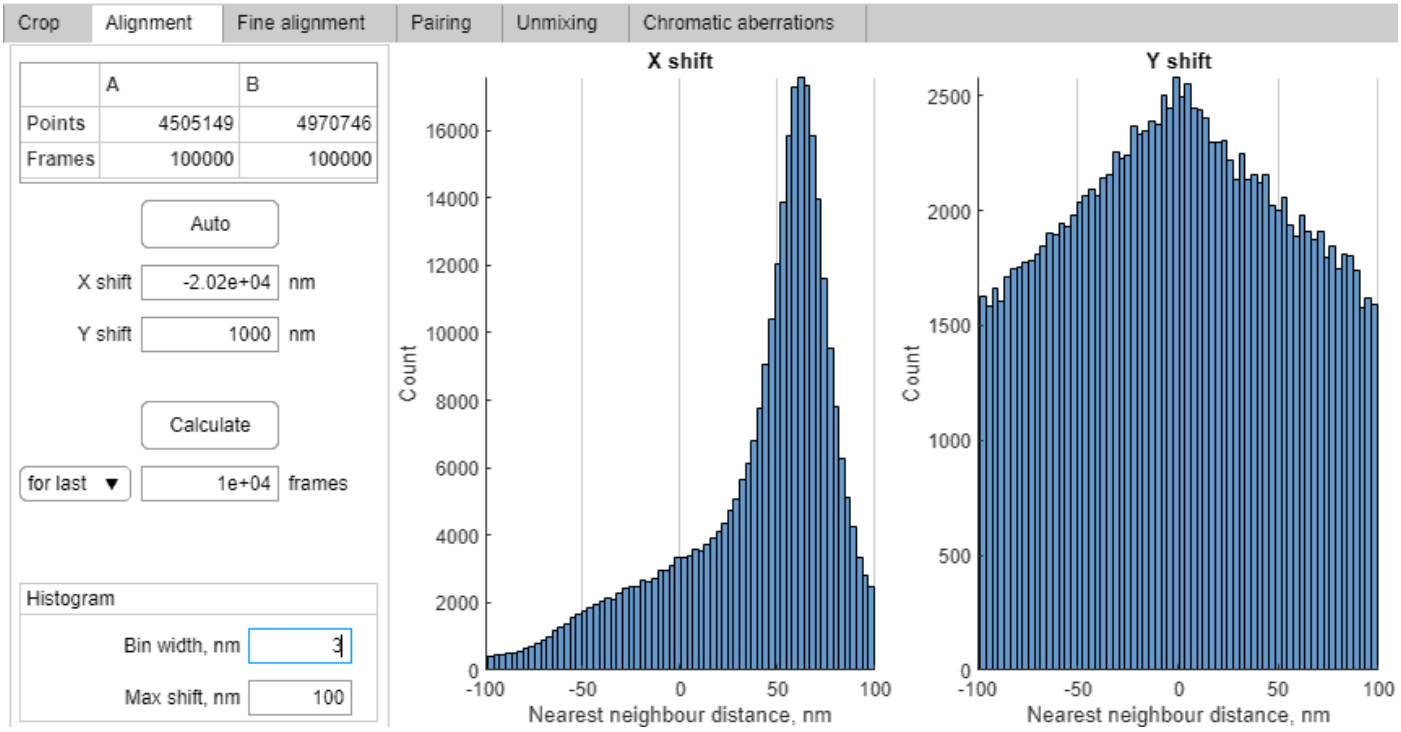
To check the quality of the alignment, SplitViSu can calculate the distance between the pairs of fluorophores from two channels:

1. Choose the range of frames to calculate the shift on: for first or for last user-defined number of frames
2. Press 'Calculate'
3. The shift between the channels will be represented as two histograms, one for X and one for Y direction

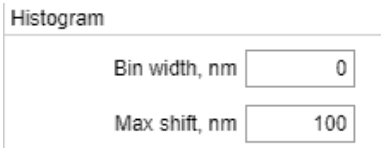
If the alignment was correct, the histograms should have a narrow peak close to the zero distance on both histograms:



Signs of an incorrect alignment: the peak is not at the zero or the histograms are very broad even if the peak is at the zero distance:



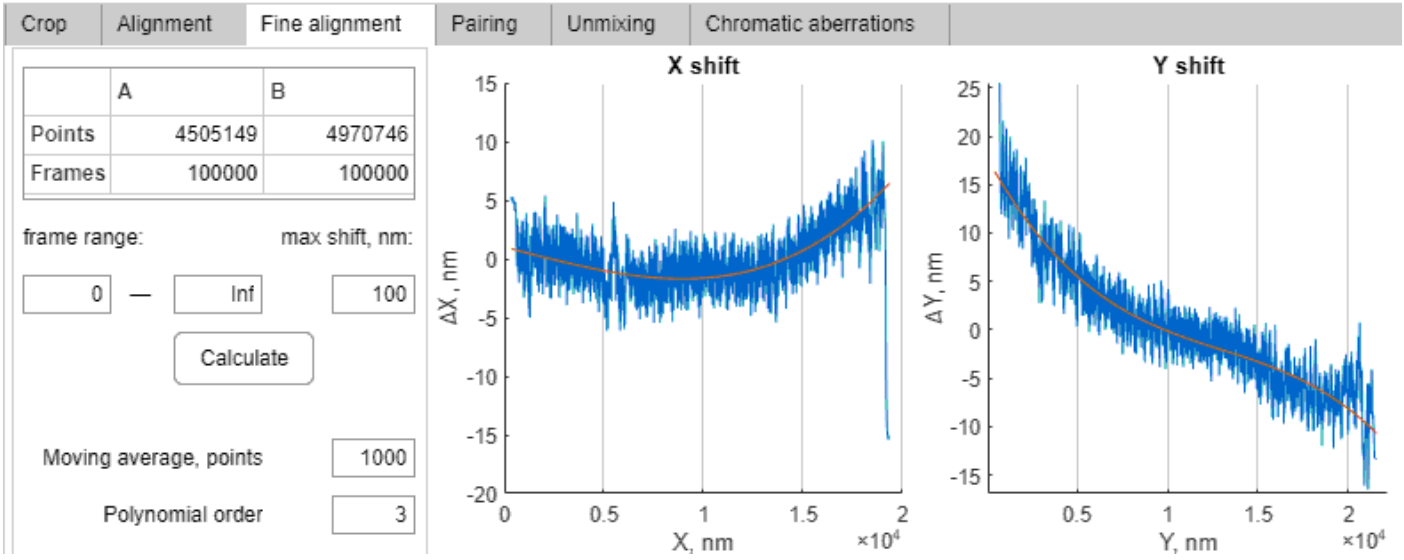
The range and the bin width of the displayed histograms can be changed (a zero bin width will produce bins with a width of 1 nm)



Fine scale alignment (optional)

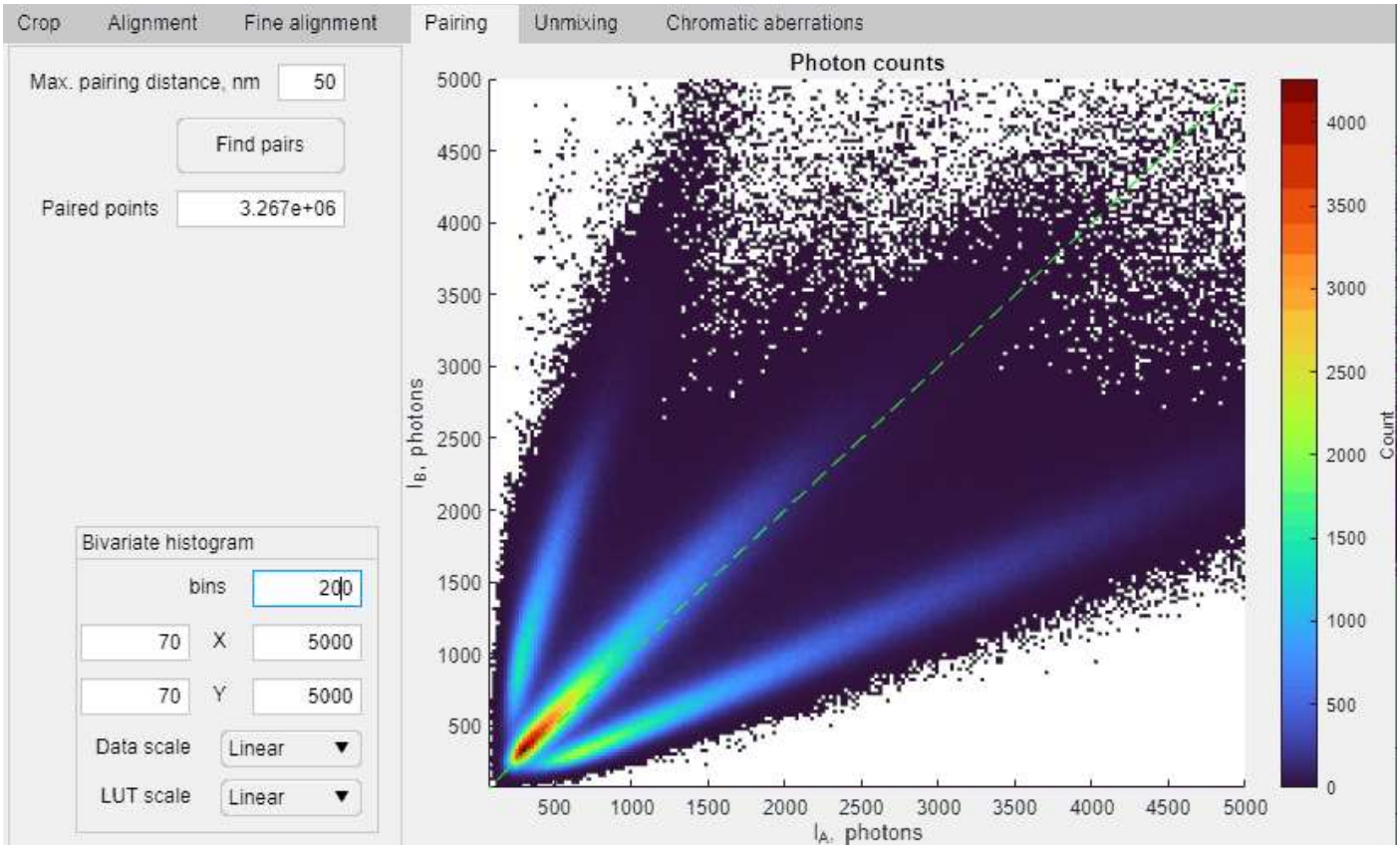
SplitViSu can align the scale of channels, caused by e.g. chromatic aberrations or unequal magnifications of the channels. The software will look and correct for a systematic shift between pre-aligned localizations, as a function of the coordinates.

1. After pre-alignment (see above), go to the tab '**Fine alignment**'
2. Choose the frame range and maximal shift.
3. Press 'Calculate'
4. The shift within each localization pair will be displayed (in blue), separately for X and Y direction.
5. For smoother appearance, the shift will be averaged using a moving mean of a user-defined number of points
6. This shift will be fitted and corrected using a polynomial of a user-defined order (the fit is shown in red)
7. The procedure can be repeated iteratively by pressing 'Calculate' again - the shifts should become smaller after each iterations.
8. After the fine alignment, one can return to the '**Alignment**' tab and re-calculate the histograms of the distances - if the fine alignment was successful, the histograms should become more narrow.
9. To undo the fine alignment, manually change the translational alignment parameters or press 'Auto' in the '**Alignment**' tab.



Pairing of localizations

- After alignment, go to the 'Pairing' tab
- Here one can define the maximum pairing distance, i.e. the maximal distance between the localizations from two channels to be considered as a pair. Allow ~40...100 nm for localization imprecision and misalignments. One can use the histograms in the Alignment tab to visualize the shifts between the localizations from two channels and to estimate the pairing distance to use.
- Press 'Find pairs'
- After the computation, the number of paired points as well as the bivariate histogram of photon counts within two channels will be displayed:

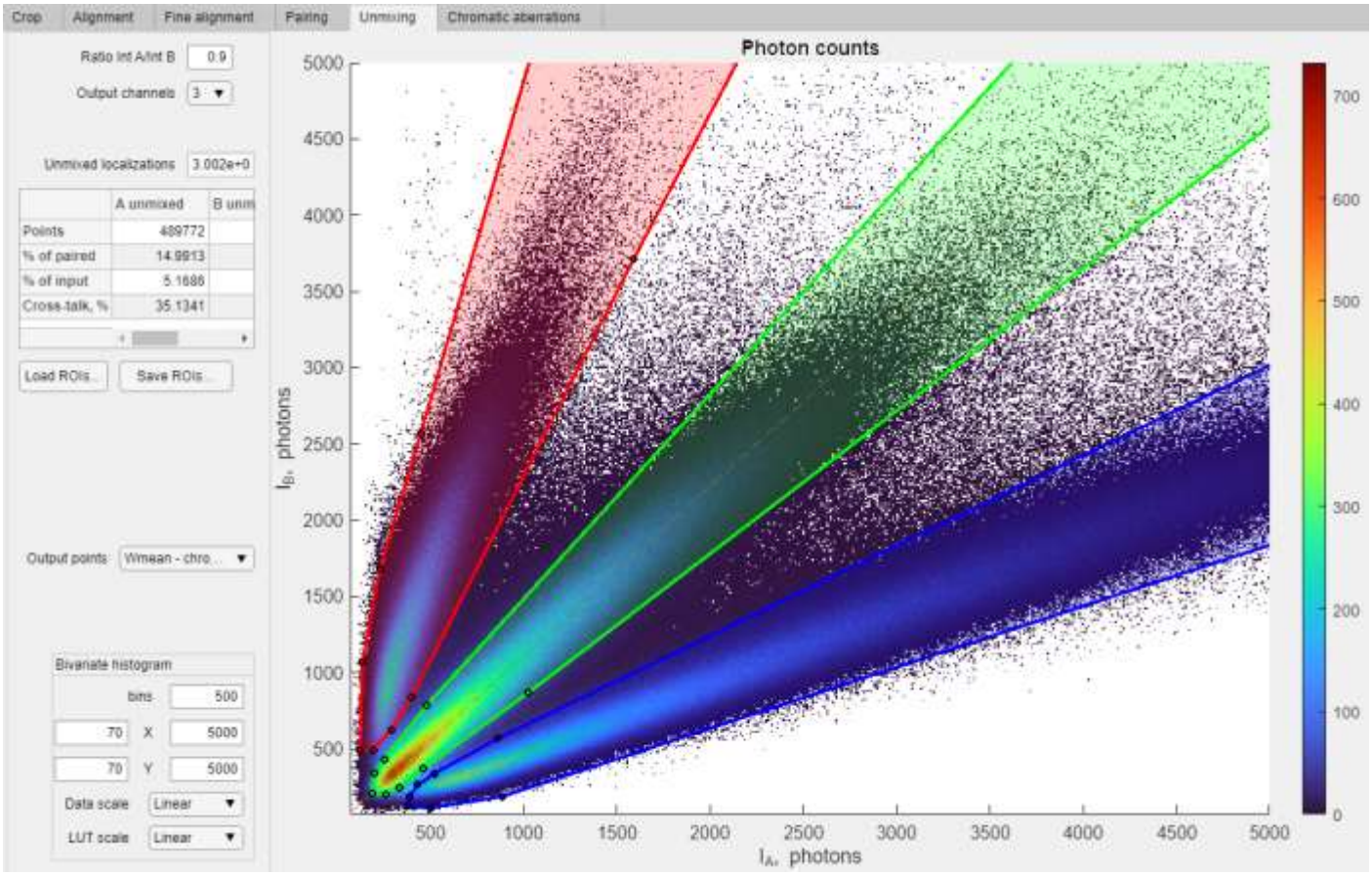


The appearance of the histogram (number of bins, axes limits, data & LUT scales) can be adjusted using the corresponding parameters.

Demixing of localizations

1. Go to the ‘Unmixing’ tab
2. Select the number of output channels (1, 2 or 3)
3. Manually adjust the ROIs for each channel so that each ROI would encircle the region of the histogram corresponding to a fluorophore or use saved ROIs under ‘Load ROIs…’
4. (optional) Save the ROIs under ‘Save ROIs…’
5. Select a method for calculation of the output points:

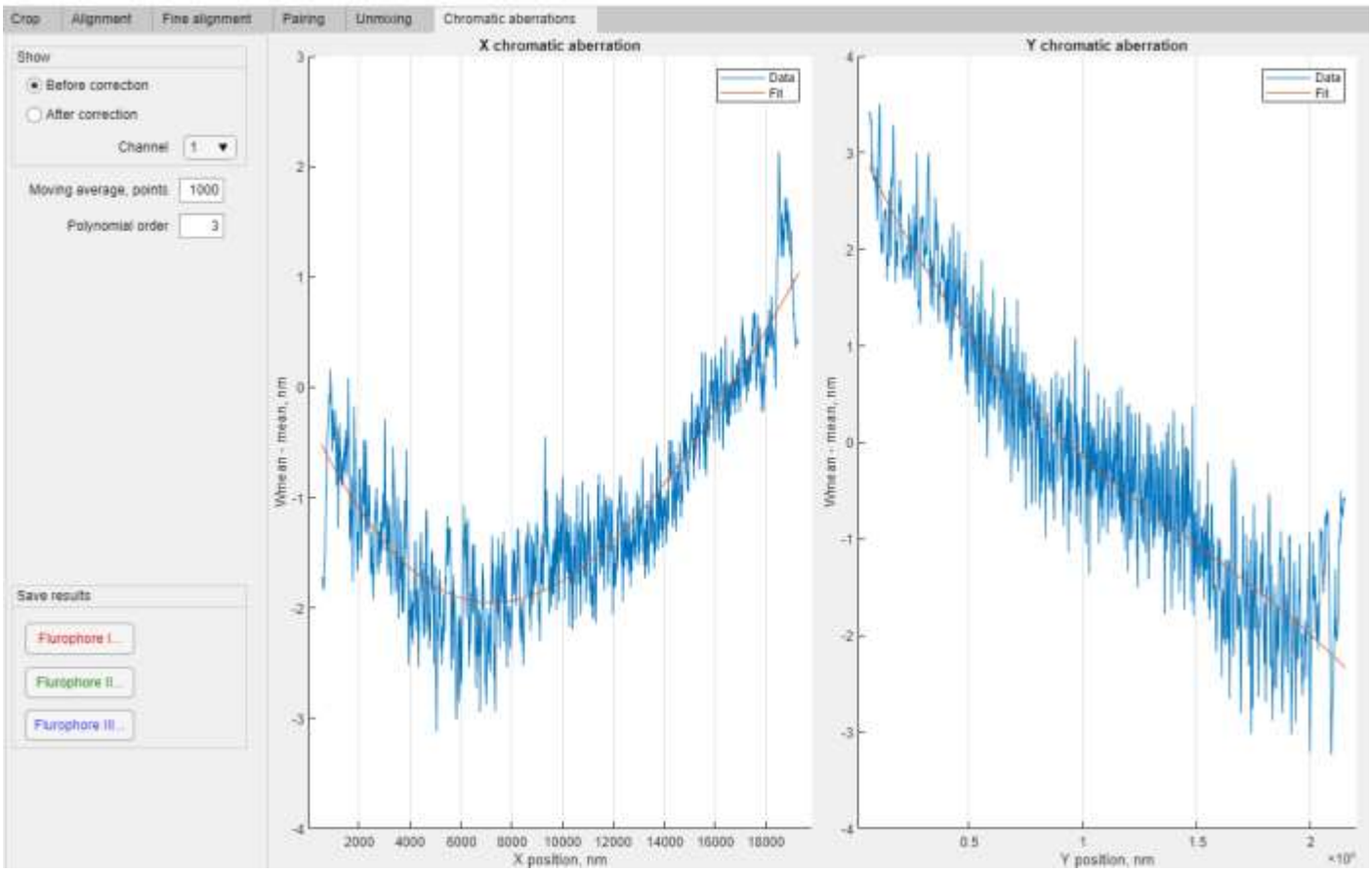
- All from input A – the output localizations will adopt coordinates from the input channel A
- From brightest input – the output localizations for a given output channel will adopt coordinates from input channel that has highest brightness for the given fluorophore. This method can produce relatively strong chromatic shifts!
- All from input B – the output localizations will adopt coordinates from the input channel B
- Simple mean – the output localizations will adopt coordinates, calculated as a simple mean of the coordinates of the input channels A & B. This method provides best correction of chromatic aberrations.
- Weighted mean – the output localizations will adopt coordinates calculated as a weighted mean of the coordinates of the input channels A & B. The weight for a given input channel is proportional to the brightness of the molecule within this channel. This method provides best localization precision as it optimally uses photons from both input channels, but the chromatic aberrations might not be corrected perfectly.
- Wmean – chroma (default) – the output localizations will adopt coordinates calculated as a weighted mean of the coordinates of the input channels A & B minus the chromatic shift. This method provides best precision and chromatic aberration correction (see below).


Crop
Alignment
Fine alignment
Pairing
Unmixing
Chromatic aberrations
Ratio Int A/Int B
0.9
Output channels
3
Unmixed localizations
3.002e+0
A unmixed
B unm
Points
489772
% of paired
14.9913
% of input
5.1686
Cross-talk, %
35.1341
Load ROIs...
Save ROIs...
Output points
Wmean - chro...
Bivariate histogram
bins
500
70
X
5000
70
Y
5000
Data scale
Linear
LUT scale
Linear
Photon counts
I_B, photons
I_A, photons
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
700
600
500
400
300
200
100
0

Correction of residual chromatic aberrations

<u>Only for the “Wmean – chroma” method</u>

- Go to the ‘Chromatic aberrations’ tab – two graphs (for X and Y directions) with a shift ‘Wmean – mean’ will appear in blue and their polynomial fit in red
- Use ‘Before correction’ to display the actual shift and its fit
- Use ‘After correction’ to display the corrected result
- ‘Moving average’ allows for averaging out fluctuations due to localization imprecision
- Changing the ‘Polynomial order’ can help to improve the fit



Saving the results

Go to the 'Chromatic aberrations' tab

In the "Save results" box, click on the name of the fluorophore to save a localization table for the corresponding output channel. The output SharpViSu .mat format provides smallest file sizes and can be opened in SharpViSu or in MATLAB. It contains: the frame index in the column 2; the localization index within the frame in the column 3; X, Y, Z coordinates in the columns 4,5,6, accordingly; photon count in the column 7; fitted (X, Y) PSF widths in the columns 8 and 9, accordingly.

If used under SharpViSu, the demixed data are automatically exported to main SharpViSu and can be further processed and saved from the main SharpViSu application. SharpViSu user manual

References

SplitViSu:

SharpViSu: Andronov, L., Lutz, Y., Vonesch, J.-L. & Klaholz, B. P. SharpViSu: integrated analysis and segmentation of super-resolution microscopy data. *Bioinformatics* **32** (2016) https://doi.org/10.1093/bioinformatics/btw123

ClusterViSu: Andronov, L., Orlov, I., Lutz, Y., Vonesch, J.-L. & Klaholz, B. P. ClusterViSu, a method for clustering of protein complexes by Voronoi tessellation in super-resolution microscopy. *Scientific Reports* **6**, 24084 (2016) http://www.nature.com/articles/srep24084

3DClusterViSu: Andronov L., Michalon J., Ouararhni K., Orlov I., Hamiche A., Vonesch J-L, Klaholz B.P. 3DClusterViSu: 3D clustering analysis of super-resolution microscopy data by 3D Voronoi tessellations. *Bioinformatics* **34** (2018) https://doi.org/10.1093/bioinformatics/bty200