Example of spectral demixing in SplitViSu

Test data

Triple-labelled Nuclear Pore Complex in U2OS nup96-GFP cells: nup96-GFP-CF660C, nup62-CF680, POM121-Alexa Fluor 647 acquired with splitSMLM: download in a .mat format under
https://github.com/andronovl/SharpViSu/blob/master/Data/SplitViSu/NPC-3colors-cropped.mat

The dataset was cropped vertically in order to reduce its size.

Start

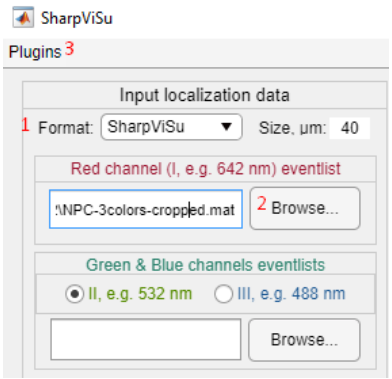
Standalone application: run file 'SharpViSu.exe' (full functionality) or 'SplitViSu.exe' (spectral demixing only)

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

Data input

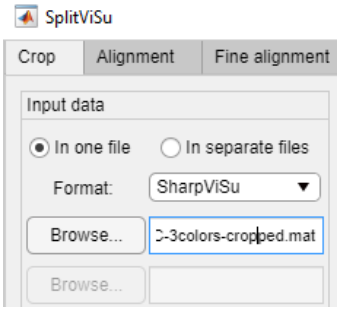
EITHER from SharpViSu (full functionality, recommended):

1. Select input Format: 'SharpViSu'
2. Load the single dataset as the 'Red channel (e.g. 642 nm) eventlist'
3. Click 'Plugins - SplitViSu demixing' - the main window of SplitViSu will open



OR Inside SplitViSu:

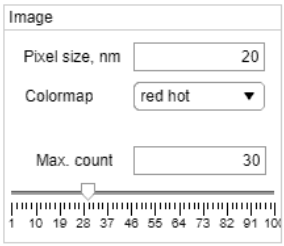
1. Choose the format of the input data: ‘SharpViSu’
2. Select the number of input files with localizations: 'In one file' (both channels are in one file)
3. Press 'Browse...' and select the file to upload



Visualization of input data

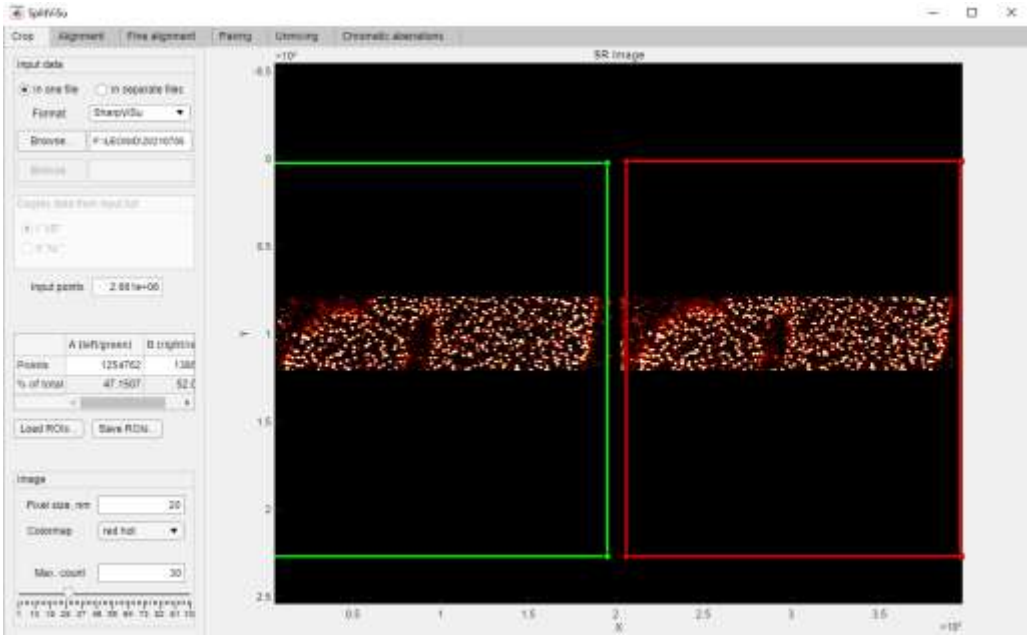
- The data will be automatically visualized after the localization list has been loaded
- The total number of input localizations will be displayed as well

- 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

- Two ROIs for two channels (green and red) will appear after the localization list has been loaded
- Press 'Load ROIs...' to use a previously saved ROIs, that can be downloaded under https://github.com/andronovl/SharpViSu/blob/master/Data/SplitViSu/roiCrop.mat
- (optional) Adjust the position of the ROIs with a mouse so that each ROI would correspond to each channel of the image splitter
- 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**'

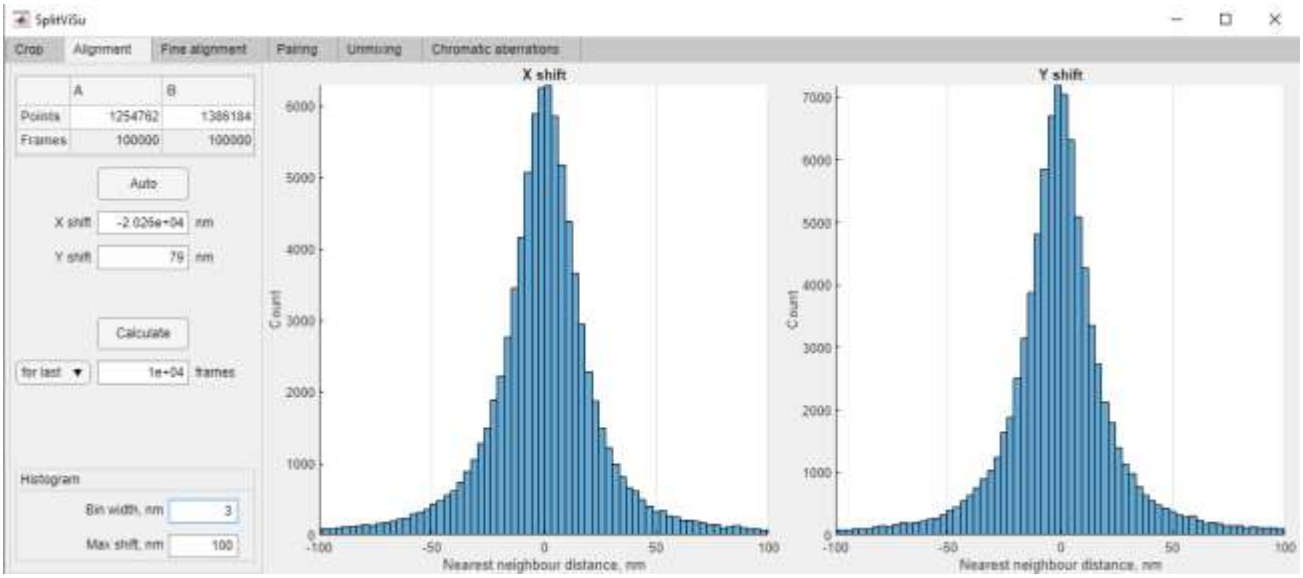
The number of localizations and frames in each channel will be displayed.

Press 'Auto' - the software will calculate the shift automatically.

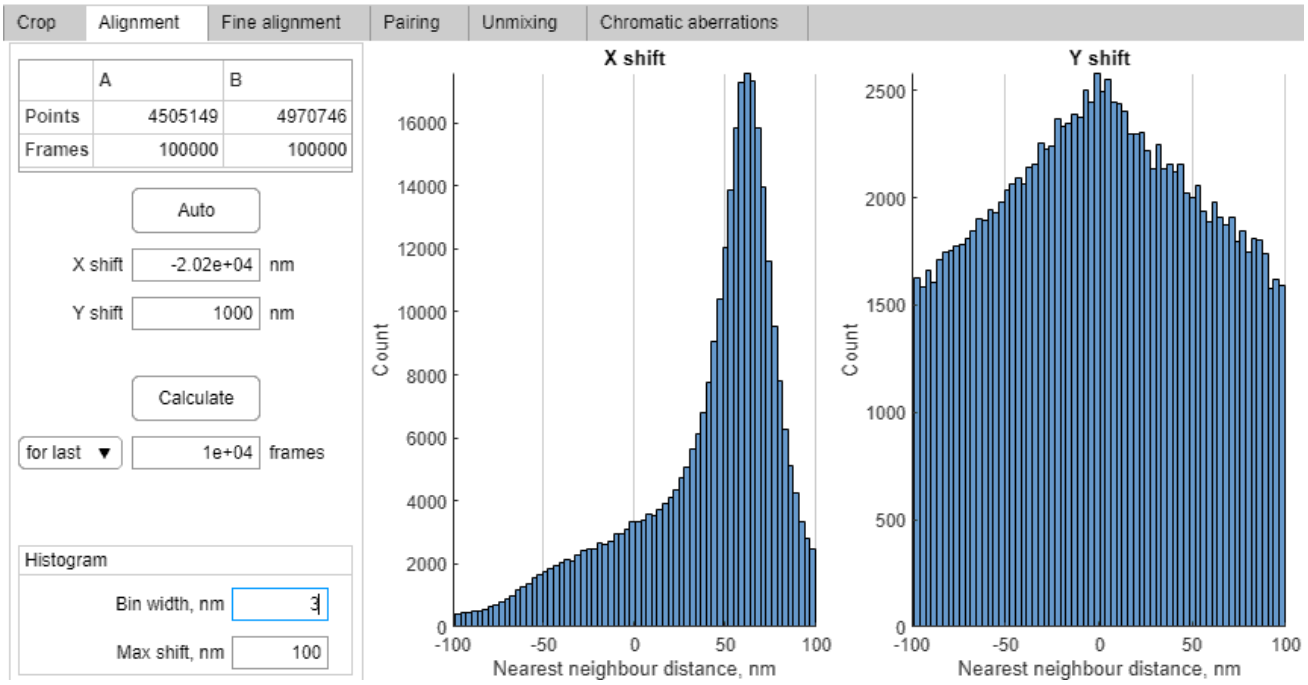
To check the quality of the alignment, SplitViSu can calculate the distance between the pairs of fluorophores from two channels:

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 the X and one for the Y direction

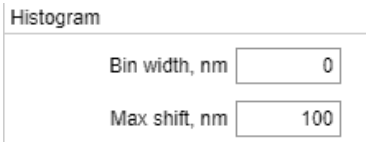
If the alignment was correct, the histograms should have a narrow peak close to the zero distance on both histograms:



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:

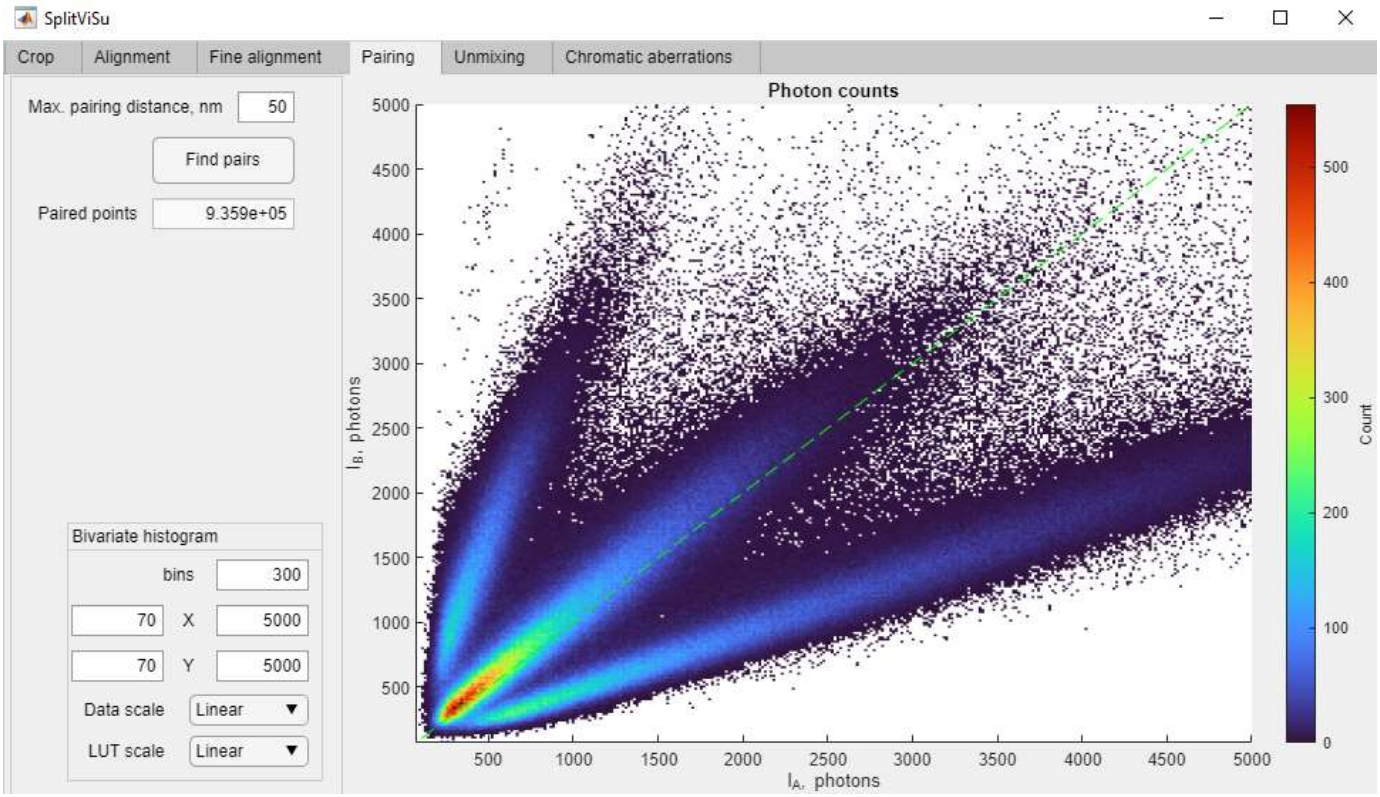


The range and the bin width of the displayed histograms can be changed (a zero bin width will produce bins with a width of 1 nm)



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:

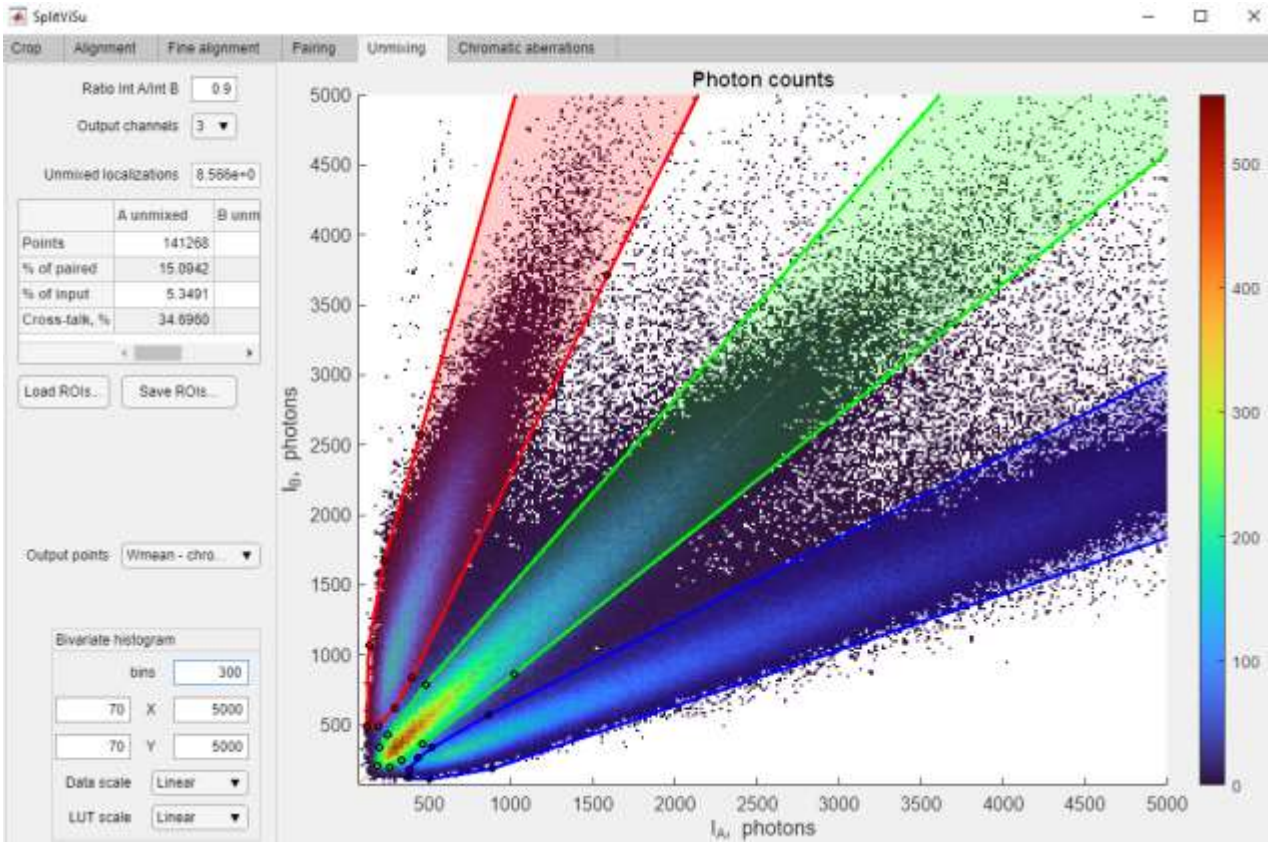


The appearance of the histogram (number of bins, axes limits, data & LUT scales) can be adjusted using the corresponding parameters.

Demixing of localizations

1. Go to the ‘Unmixing’ tab
2. Select the number of output channels (3)
3. Use saved ROIs under ‘Load ROIs…’: https://github.com/andronovl/SharpViSu/blob/master/Data/SplitViSu/roi.mat
4. The ROIs can be further adjusted manually
5. Select a method for calculation of the output points:

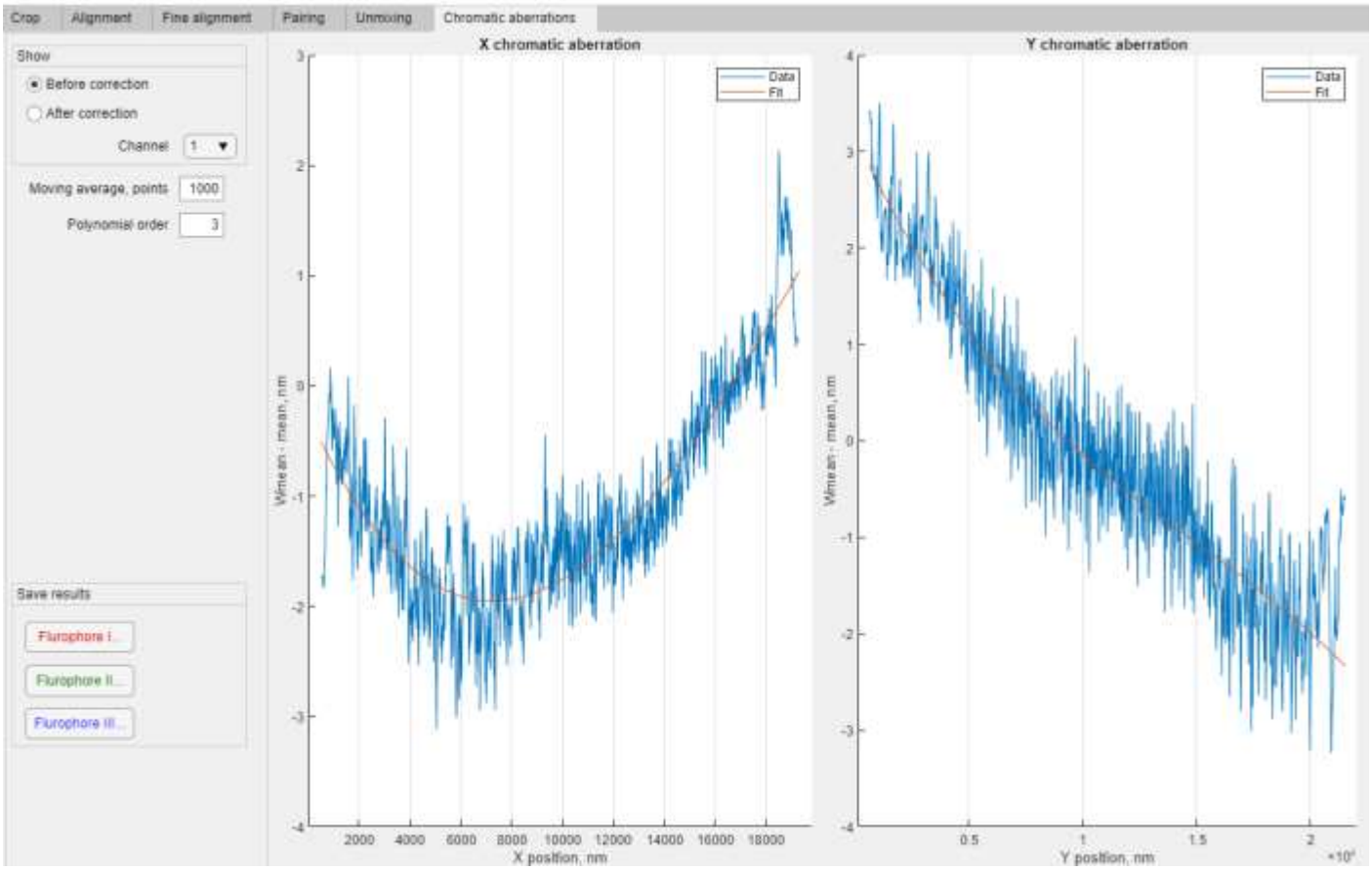
- All from input A – the output localizations will adopt coordinates from the input channel A
- From brightest input – the output localizations for a given output channel will adopt coordinates from input channel that has highest brightness for the given fluorophore. This method can produce relatively 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).


SpiteViSu
Crop
Alignment
Fine alignment
Pairing
Unmixing
Chromatic aberrations
Ratio Int A/Int B
0.9
Output channels
3
Unmixed localizations
8.566e+0
A unmixed
B unm
Points
141268
% of paired
15.0942
% of input
5.3491
Cross-talk, %
34.6960
Load ROIs...
Save ROIs...
Output points
Wmean - chro...
Bivariate histogram
bins
300
70
X
5000
70
Y
5000
Data scale
Linear
LUT scale
Linear
Photon counts
I_B, photons
I_A, photons
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
0
100
200
300
400

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

Further processing is performed in SharpViSu

Drift estimation

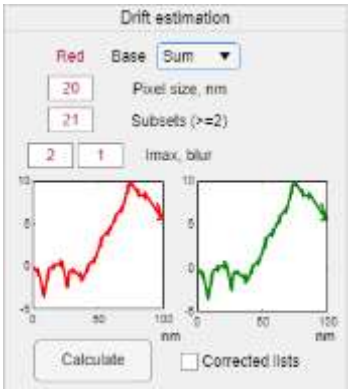
First, select the Base for the estimation:

“Sum” – the drift will be estimated on the sum of all channels (useful for simultaneous acquisitions)

Type desired pixel size (p, default 20 nm), number of subsets (n, default 21), maximum gray value of the subimages (*Imax*, default 2) and the standard deviation for the Gaussian blur (*blur*, default 1) for each channel in the corresponding field.

Press “Calculate”. During the calculation, each input eventlist will be divided into n consecutive sublists and from each of them an image with a pixel size of p will be reconstructed (in the histogram mode). To avoid correlation between individual bright spots on the images, the pixels with gray values higher than *Imax* will be assigned a gray value of *Imax*. The images will be blurred with a Gaussian blur with a standard deviation of *blur* pixels. The shift between each consecutive image will be calculated with a subpixel precision yielding (n-1) values.

The calculated values of drift will be represented by (n-1) arrows in the corresponding windows. Because we use the sum of three channels for drift estimation, the displayed drift estimation will be the same for all channels



It is possible to change any parameter and recalculate drift. The corrections are not applied to the data at this point. If the estimated values are inconsistent or too big, there is probably not enough redundancy in the data for the calculation. Try to use a larger pixel size or a smaller number of steps.

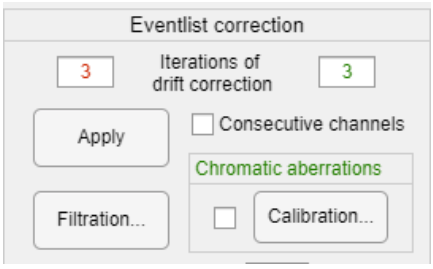
To use previously corrected data for the drift estimation, check “Corrected lists” prior to “Calculate” (see below). Do not use this option on data that has not been corrected yet.

If satisfied with the initial estimation of drift, proceed to the next step.

Drift correction

Choose a desired number of iterations for the correction of the drift (default 3). Uncheck “*Sequential channels*”. During correction, the localizations within all three datasets will be moved towards the beginning of the acquisition. Press “Apply”.

To skip drift correction for a given channel, put “0” as the number of iterations for this channel.



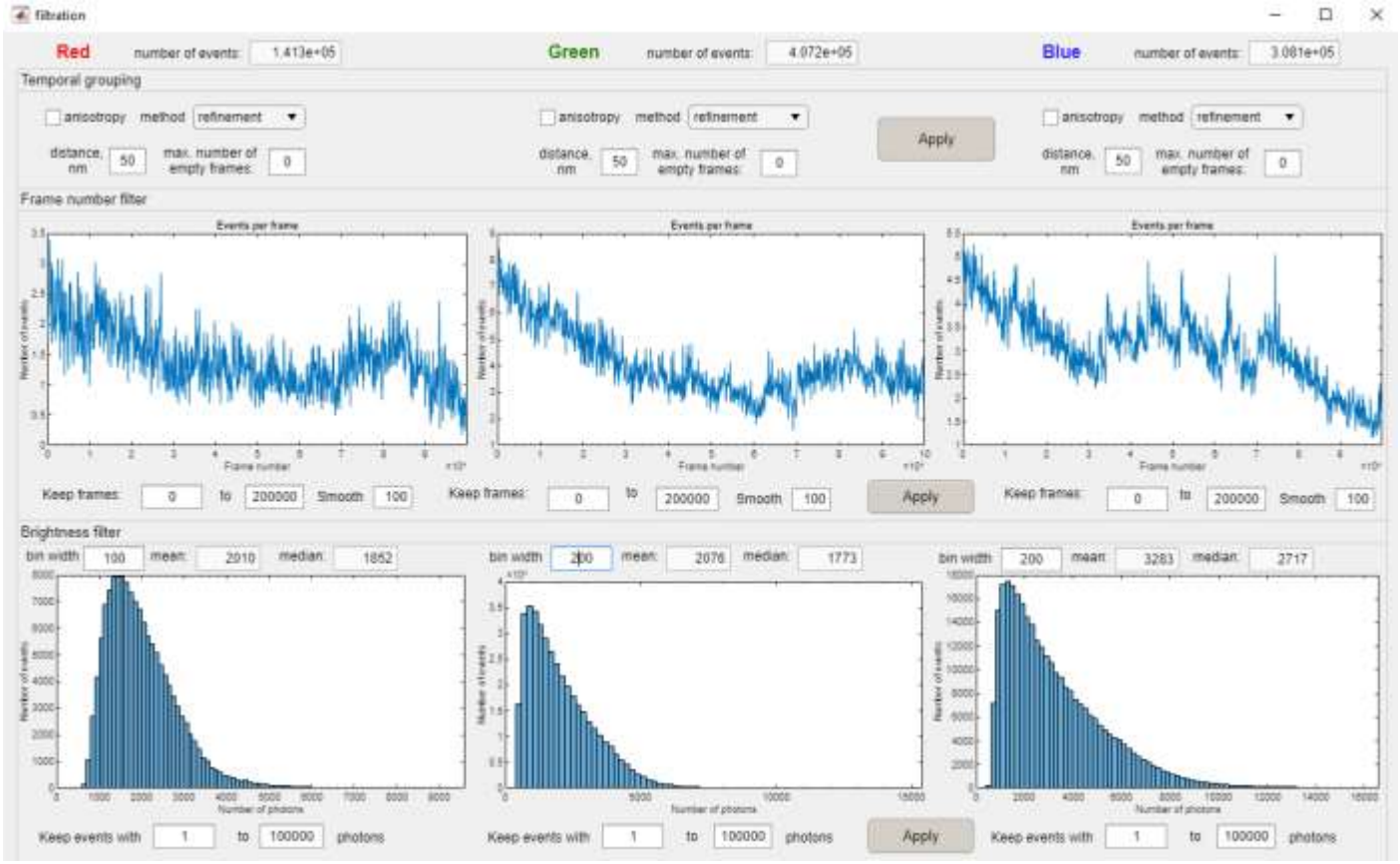
During the correction, the drift will be recalculated and shown after each iteration.

After the correction, the residual drift will be calculated and displayed in the “Drift” panel.

If you want to add more iterations of drift correction, check “Corrected lists” in the “Drift estimation” section. In this case, the new iterations of drift correction will be applied to the pre-corrected data.

Processing of multiple localizations and filtration

Press “Filtration” in the “Eventlist correction” box. A dedicated window will open.



On top, you will see the number of localizations for each channel.

For temporal grouping of consecutive localizations, select a method, put the desired search radius (r, default 50 nm), number of empty frames (default 0) and press “Apply”.

There are two methods:

- Refinement – the localizations within each series will be kept but will adopt new, more precise coordinates (usually provides better image resolution);
- Reduction – the localizations from each series will be reduced to a single localization at the average position of the input localizations (can be useful for single-molecule counting).

“Number of empty frames” is the maximum number of consecutive empty frames within the series of localizations being grouped.

In the “Frame number filter” section, you can see graphs with the number of localizations per frame, smoothed within blocks of specified size. You can keep localizations originating only from a range of frames using the corresponding options under the plots. The plots will change every time you enter new values in the “Keep frames” filter, but the

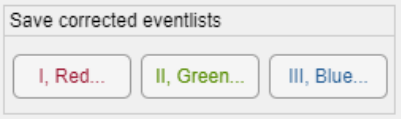
actual removal of frames happens only after you press “Apply”. This option is useful, for example, to delete very dense frames at the beginning of an acquisition.

In the “Brightness filter” section, you can see histograms of the photon counts of the eventlists. The mean and median values of the photon count distributions are displayed as well. You can keep localizations with photon counts within a specified range. This can be useful to remove dim molecules with low localization density. The histograms will change every time you enter new values in the “Keep events” filter, but the actual removal of localizations happens only after you press “Apply”.

After closing the window, the new filtered eventlists are transferred to SharpViSu replacing the old ones.

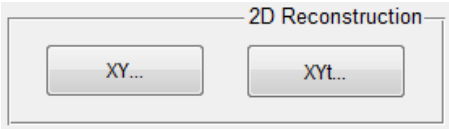
Saving the corrected datasets

You can save the current corrected eventlists with a click on the name of the channel in the corresponding panel. The output format is MATLAB .mat, tab-delimited .ascii or one of the formats for ViSP (.2d, .2dlp, .3d, .3dlp).

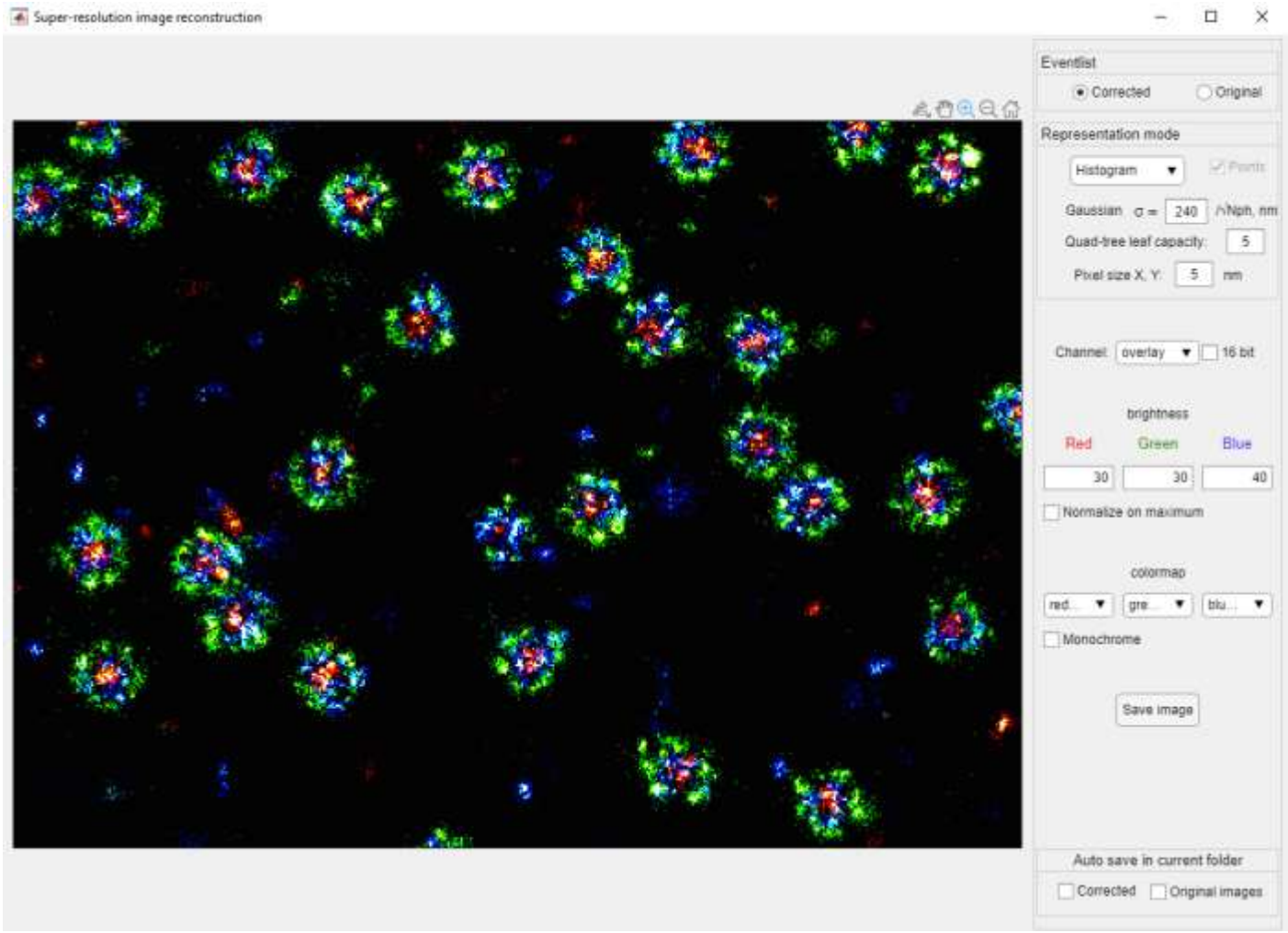


Super-resolution image reconstruction

Click “XY…” in the panel “2D Reconstruction”



A new window will open.



Select the corrected or the original dataset in the panel “Eventlist”.

In the panel “Representation mode” choose “Histogram”. Histogram provides the fastest result, the other modes can be very slow, depending on dataset.

Put the desired pixel size (10 nm by default, down to 5 nm is recommended for this dataset).

Choose the color channel to show: “red”, “green”, “blue” or “overlay” (three channels will be shown in the same picture).

“brightness” is a constant by which all the pixel values will be multiplied in order to be seen on the screen, it can be chosen saparately for each channel. If you have checked “Normalize on maximum”, the pixel values will be modified linearly in a way that the maximal pixel value = 255·brightness, the image will use the full dynamic range of an 8-bit image.

You can choose a colormap for each channel. Various colormaps are available.

By pressing “Save image” you can save the image in full resolution with the current settings (a dialog window for file saving will appear).

Estimation of resolution with Fourier ring correlation

Select the pixel size and the number of frequency steps (default 90) and press “Calculate”. The number of frequency steps equals to the number of points in the final FRC graph. The resolution at 1/7th FRC will be displayed for each channel after the calculation ends. You can view the curves by clicking at the button for the corresponding channel. Please remember that the resolution can not be higher than the double pixel size. E.g. for a pixel size of 10 nm, the smallest resolution value is 20 nm. If the pixel size is too big, the FRC curve might not go down to the 1/7 value.