Installation

Compiled standalone SharpViSu with integrated SplitViSu & ClusterViSu for Windows. Download and run "SharpViSu_web_installer.exe" https://github.com/andronovl/SharpViSu/tree/master/Installer and follow instructions. If not already present, the required version of the MATLAB Compiler Runtime will be downloaded from the web and installed automatically.

MATLAB application. Add folder "*SharpViSu*" to the MATLAB search path. SharpViSu2 was developed in MATLAB R2021b but should work also in older versions. Toolboxes required: Image Processing Toolbox, Signal Processing Toolbox, Statistics and Machine Learning Toolbox

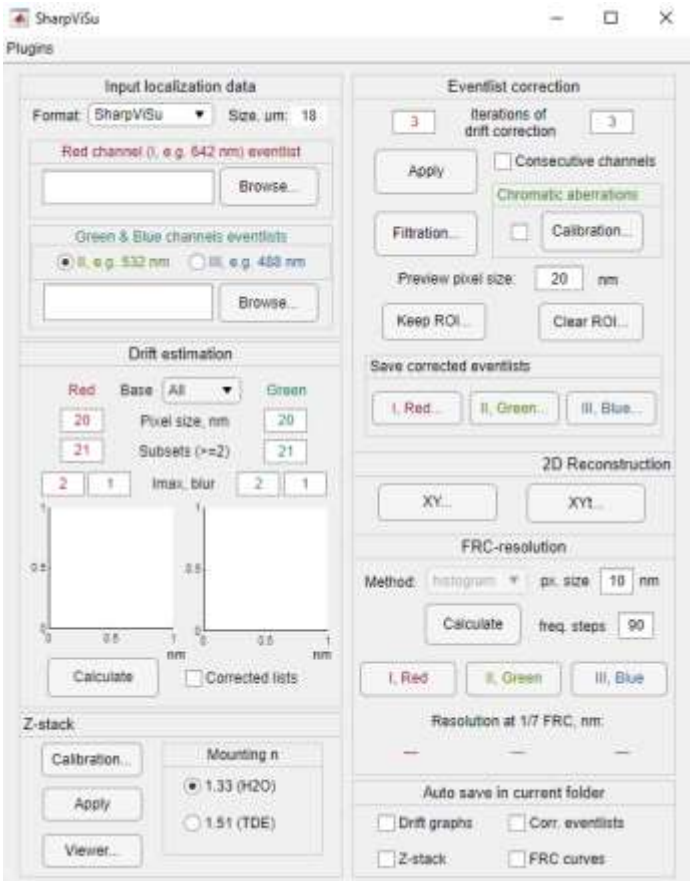
Running SharpViSu

Standalone application. Run "SharpViSu.exe" (default path: "%ProgramFiles%\SharpViSu\application\SharpViSu.exe", could has been changed during installation)

MATLAB scripts. Type in the MATLAB Command Window:

>> SharpViSu_app

This will launch the function SharpViSu_App.mlapp - the main window of the SharpViSu GUI will open:



Loading localization data

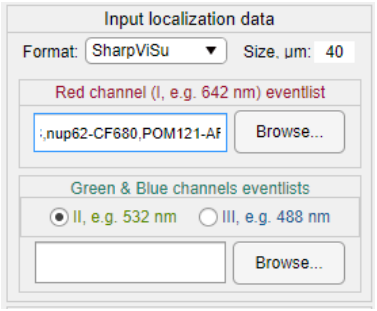
Before loading localization data, select the input data format in the popup menu “Format”. The following formats are supported:

- Leica LAS (.ascii or .bin, both 2D and 3D versions supported);
- QuickPALM (text files);
- RapidSTORM (text files);
- Localization Microscopy plugin for Micro-Manager (.txt, decimal separator = comma);
- ThunderStorm (text files);
- ViSP;
- Zeiss ZEN (text files);
- SharpViSu (.ascii or .mat, internal format of SharpViSu).

Press “Browse…” and choose the localization table first for the “Red” channel. After loading, the geometrical size of the datasets will be displayed in the field “Size, µm”. This parameter is the size of the biggest dimension of the biggest loaded dataset, rounded to the higher integer, and is at least 18 µm. If the displayed value is not reasonable, re-check the format of the data.

One, two or three datasets (color channels) can be processed at the same time.

The second and the third datasets can be loaded under the “Green & Blue channels eventlists”



Estimation of drift

First, select the Base for the estimation:

“All” – the drift will be estimated on each channel separately (useful for sequential or independent acquisitions);

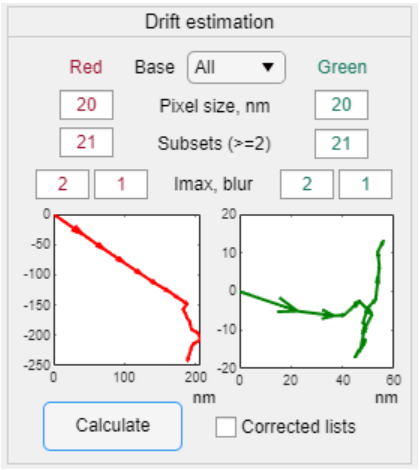
“Sum” – the drift will be estimated on the sum of all channels (useful for simultaneous acquisitions);

“Red”, “Green” or “Blue” – the drift will be estimated using only the corresponding dataset

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. For the Green/Blue eventlist, only drift estimation for the active dataset will be displayed.



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.

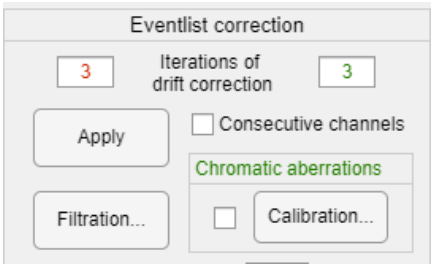
To use previously corrected data for the drift estimation, check “Corrected lists” prior to “Calculate” (see below). Do not use this option on data that has not been corrected yet.

If satisfied with the initial estimation of drift, go the following step.

Correction of drift and chromatic aberrations

Choose desired number of iterations for the correction of the drift (default 3). Check "*Sequential channels*" if your double-color acquisition was performed in a sequential way (first red, then green channel). In this case, the "red" channel data will be moved towards the end of the acquisition, the "green" channel data – towards its beginning, compensating for a shift between the channels. If all channels were acquired simultaneously, uncheck this option. In this case, all channels 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.

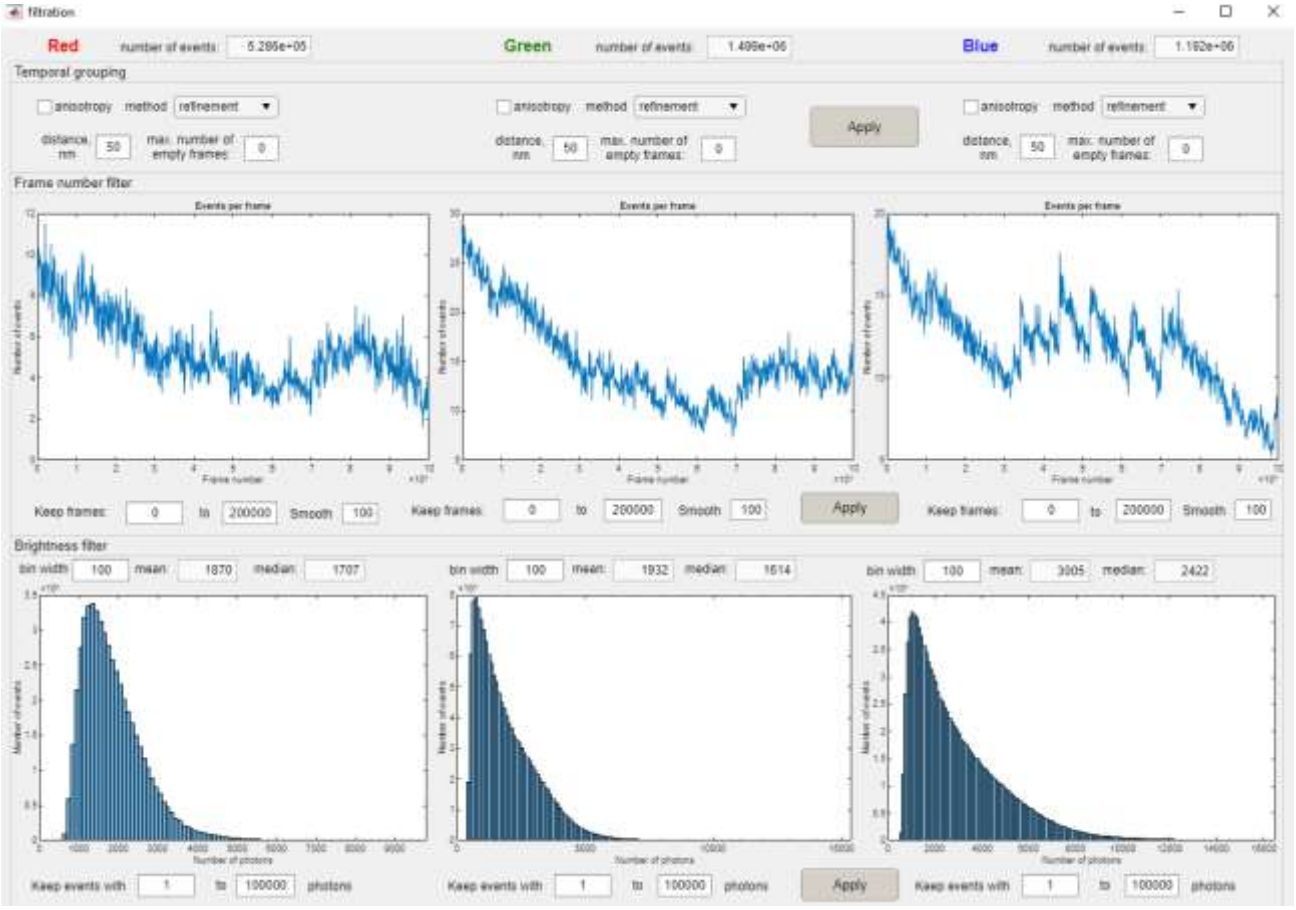
After the correction, the residual drift will be calculated and displayed in the "Drift" panel.

Chromatic aberrations are corrected by shifting the localizations of the "green" channels relatively to the "red" one. To perform corrections, the checkbox should be active before pressing "Apply". For chromatic aberration calibration, see below.

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.

Filtration of data

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.

If you would like to use elliptic (instead of circular) search regions for consecutive events, check “anisotropy” before “Apply”. The axes of the ellipse for the i-th event will be equal to $a_i = r*\sigma_{xi}/100$, $b_i = r*\sigma_{yi}/100$, where

σ_{xi} and σ_{yi} are the x- and y- widths of the diffraction spot for the i-th localization. This option can be useful for 3D experiments with astigmatism, where the uncertainty of localization is strongly anisotropic.

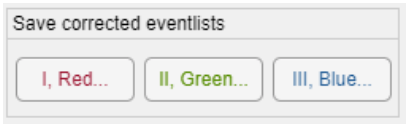
In the “Frame number filter” section, you can see graphs with the number of localizations per frame, smoothed within blocks of specified size. You can keep localizations originating only from a range of frames using the corresponding options under the plots. The plots will change every time you enter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

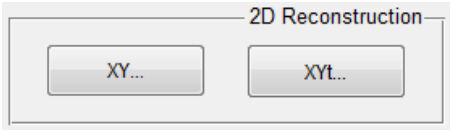
Saving the corrected datasets

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).

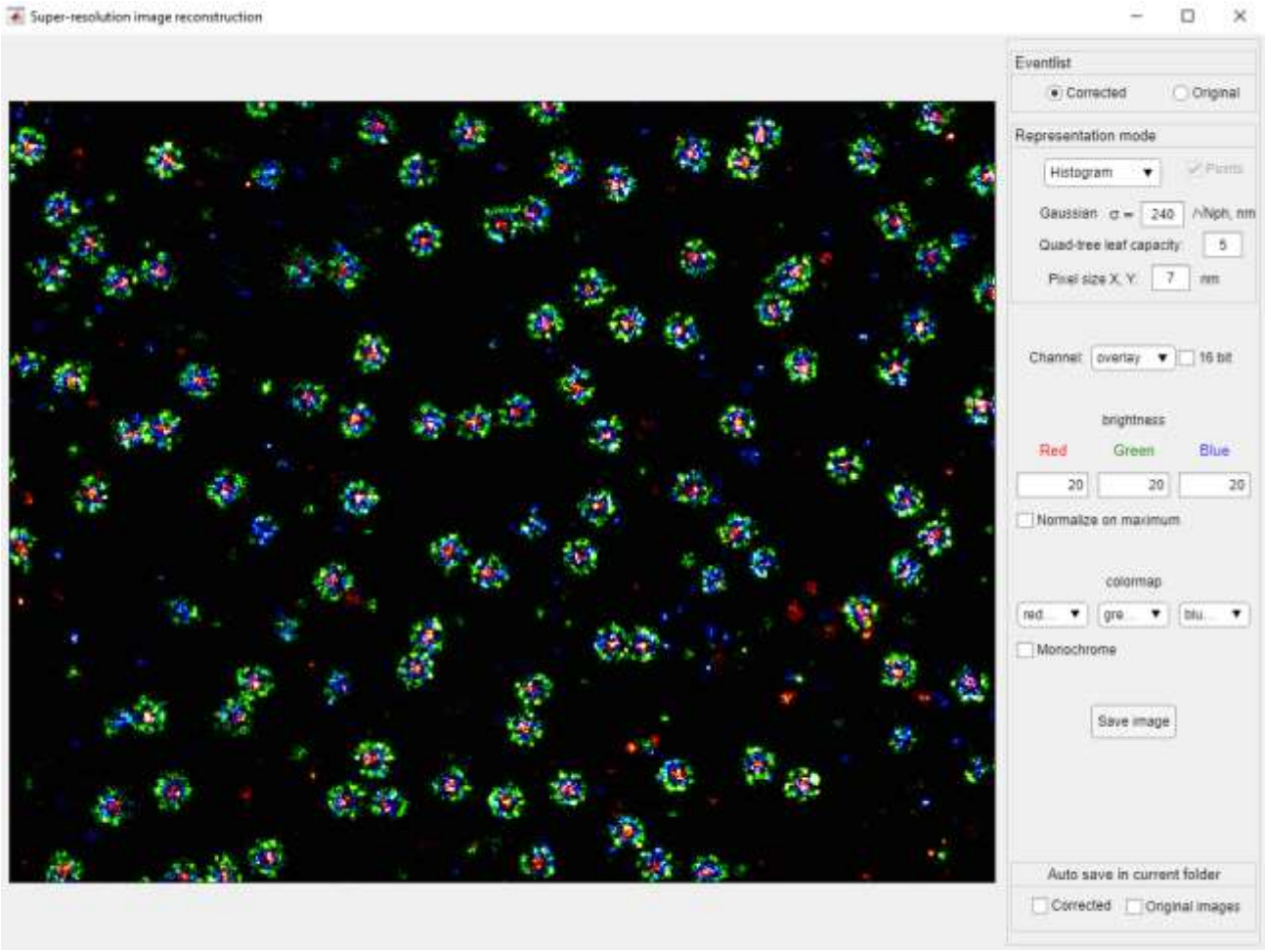


Super-resolution image reconstruction

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”, “Gaussian” or “Voronoi smooth”, “Quad-Tree histogram” or “Voronoi tessellation”. Histogram provides the fastest result, the other modes can be very slow, depending on dataset.

Put the desired pixel size (10 nm by default).

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

“brightness” is a constant with 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.

In the histogram mode the grey value of a pixel will be equal to the number of molecules detected within the pixel’s area. In the Gaussian mode each event is rendered as a 2D Gaussian function, centered on the pixel where the event is located. The sum of all pixel values originating from one event equals to 1. The standard deviation of the Gaussian equals $\sigma = A/(Nph)^{1/2}$, the constant A should be put in the corresponding field (A = 240 nm by default).

For the Quad-tree histogram representation, you can choose the leaf capacity (the maximal number of events in histogram bins), it will affect the final size of the image. Beware that a very small leaf capacity can lead to a huge image which can provoke a “lack of memory” error.

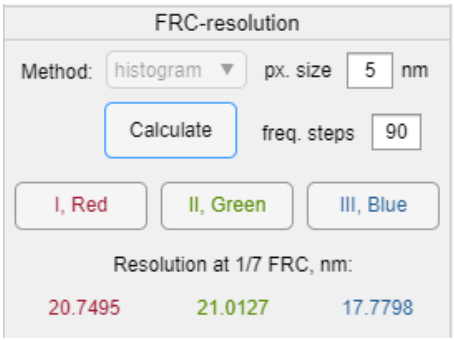
In the Voronoi diagram mode the polygons will be colored proportionally to the inverse value of their area using the selected colormap; the data points can be displayed as white pixels after checking the corresponding box. This mode is not compartible with the “overlay” channel mode, please display the two channels separately. Visualization including zooming is slow for big datasets in this mode.

You can choose a colormap for each channel. The available colormaps are: red, green, gray, blue, hot, hsv, jet, cool, spring, summer, autumn, winter, bone, copper, pink and others. If checked “Monochrome”, the both channels will be shown in the grey colormap, and the picture will be subsequently saved in an 8-bit monochrome tiff file.

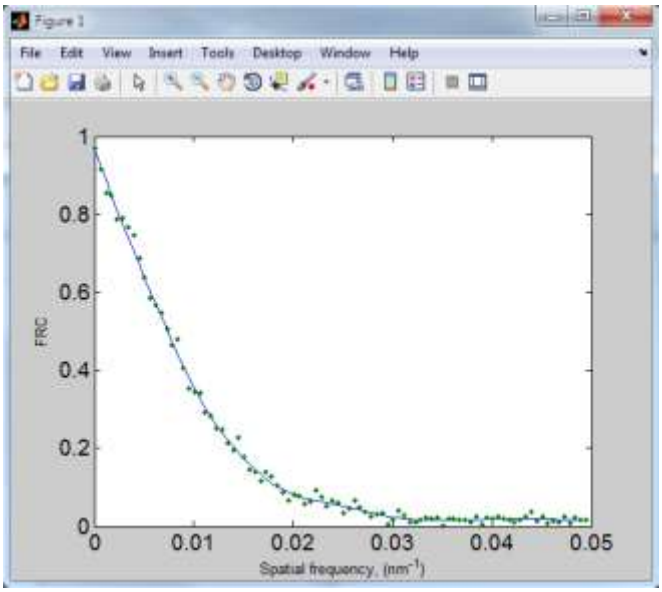
By pressing “Save image” you can save the image in full resolution with the current settings (a dialog window for file saving will appear).

If the checkboxes “Corrected” and/or “Original images” are checked in the “Auto save in current folder” panel, the corresponding super-resolution images will be saved during closing of the window. The current folder is the directory where the last opened eventlist is situated. The image from the non-corrected dataset will be saved only if it has been rendered by checking the box “Original” in the “Eventlist” panel. The filename of automatically saved images is ‘Xm_multr_multg.tif’, where X = 0 for non-corrected data, X = 1 for the corrected one; m = h for histogram mode, m = g for Gaussian mode, m = v for Voronoi mode; multr and multg are the multipliers for the red and the green channel, accordingly. The format of saved files is 24-bit RGB LZW-compressed tiff.

Estimation of resolution with Fourier ring correlation

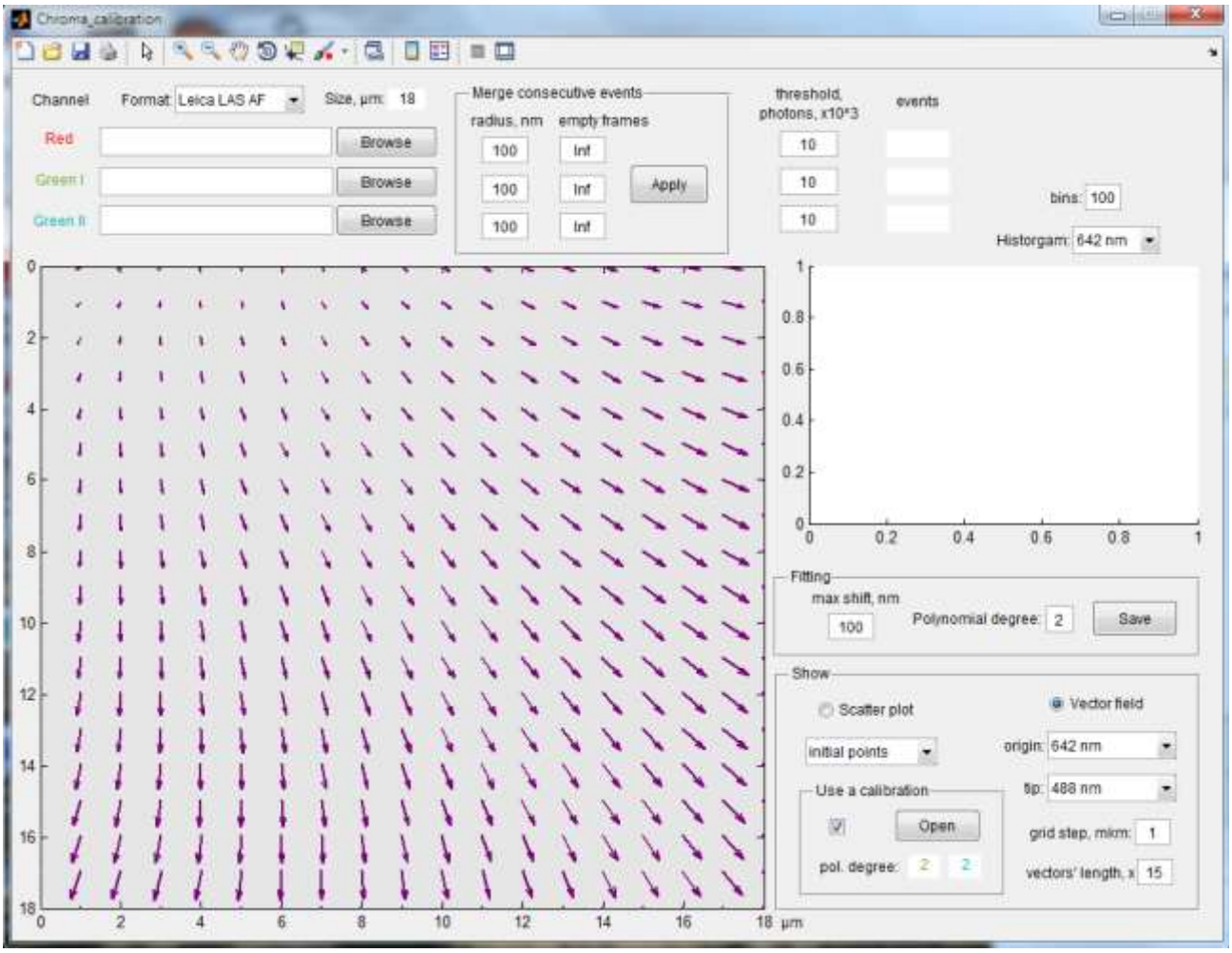


Select the pixel size and the number of frequency steps (default 90) and press “Calculate”. The number of frequency steps equals to the number of points in the final FRC graph. The resolution at 1/7th FRC will be displayed for each channel after the calculation ends. You can view the curves by clicking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



Calibration of chromatic aberrations

Press “Calibration…” in the “Chromatic aberrations” subpanel. The calibration window will open. If the installation folder already contains calibration files, the current chromatic aberrations will be shown with arrows.



The calibration relies only on localization coordinates. The recommended procedure is to acquire for each color a short dataset using a sample containing multi-color beads. The density of the beads should not be too high allowing separation of the signals during single-molecule localization. In addition, the beads should be distributed evenly throughout the field of view providing a sufficient number of points for proper fitting. The localization table for each color will contain positions of the beads, possibly with some noise.

Select the format of input data (Leica LAS by default). Press “Browse” and load an eventlist for the corresponding color. Repeat the procedure for each color. On the right-hand side, you will see the number of events in each table and a histogram of the number of photons per event.

Go to the panel “Merge consecutive events”, put the search radius (100 nm by default) and number of empty frames (0 by default), press “Apply”. You will see a strongly reduced number of events and increased photon counts. The number of events should be equal or more than the number of beads in the sample. The incorrect

localizations originating from noise have much less brightness because they were not summed with the localizations from beads. Delete them by imposing a threshold on the photon number ($10 \cdot 10^3$ by default). Ideally, you should see the same number of events for each channel that is equal to the number of beads.

Go to the panel “Fitting”. Put “max shift” (default 100 nm) and “Polynomial degree” (default 2). “Max shift” means maximal distance between images of the same bead in different colors. To automatically find a pair of beads, the software will search for a neighbor in the other color within this radius.

Go to the panel “Show” and uncheck “Use a calibration”. Here you can choose different representations of the calibration data. In the part “Scatter plot” you can choose one of the following options:

- Initial points – will show all the points present in the input data
- Filtered – points that remain after merging and filtration
- Used for fitting – points that were chosen as belonging to the pairs originating from the same bead
- Corrected – the filtered points corrected with the fit

“Vector field”, the fit will be shown with arrows. Here you can choose the color for the origins and the tips of the arrows, the grid period (default 1 μm) and the scale for lengths of the arrows (default 15). Here you can assess the goodness of the fit and change some of its parameters (namely, the degree of the polynomial) if needed.

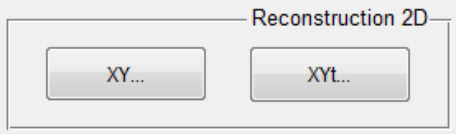
If satisfied with the fit, press “Save”. The calibration files named ‘532.mat’ and ‘488.mat’ will be saved in the installation folder. The files contain a Matlab’s geometric transformation object that describes the transformation from the positions of the Green (‘532.mat’) or the Blue (‘488.mat’) channels to the position of the Red channel.

You can also display any previously saved calibration using panel “Use a calibration”. Check the checkbox, press “Open” and choose the calibration file for the Green I channel; then a second window will open, choose there the calibration file for the Green II channel. The polynomial degree of the loaded fit will be displayed below. If the box “Use a calibration” is checked, the data from calibration files in the installation folder will be shown by default.

There is another (optional) possibility for correction of chromatic aberrations. For this purpose, the installation folder should contain files named ‘488.dat’, ‘532.dat’ and ‘642.dat’ with x and y coordinates of beads, each row stands for the same bead in every file. If the installation folder does not contain .mat calibration files, the coordinates from the .dat files will be fitted with a 2-degree polynomial and the fit will be applied to experimental data upon correction.

Image reconstruction in the time-in-color mode

Press “XYt…” in the panel “Reconstruction 2D”



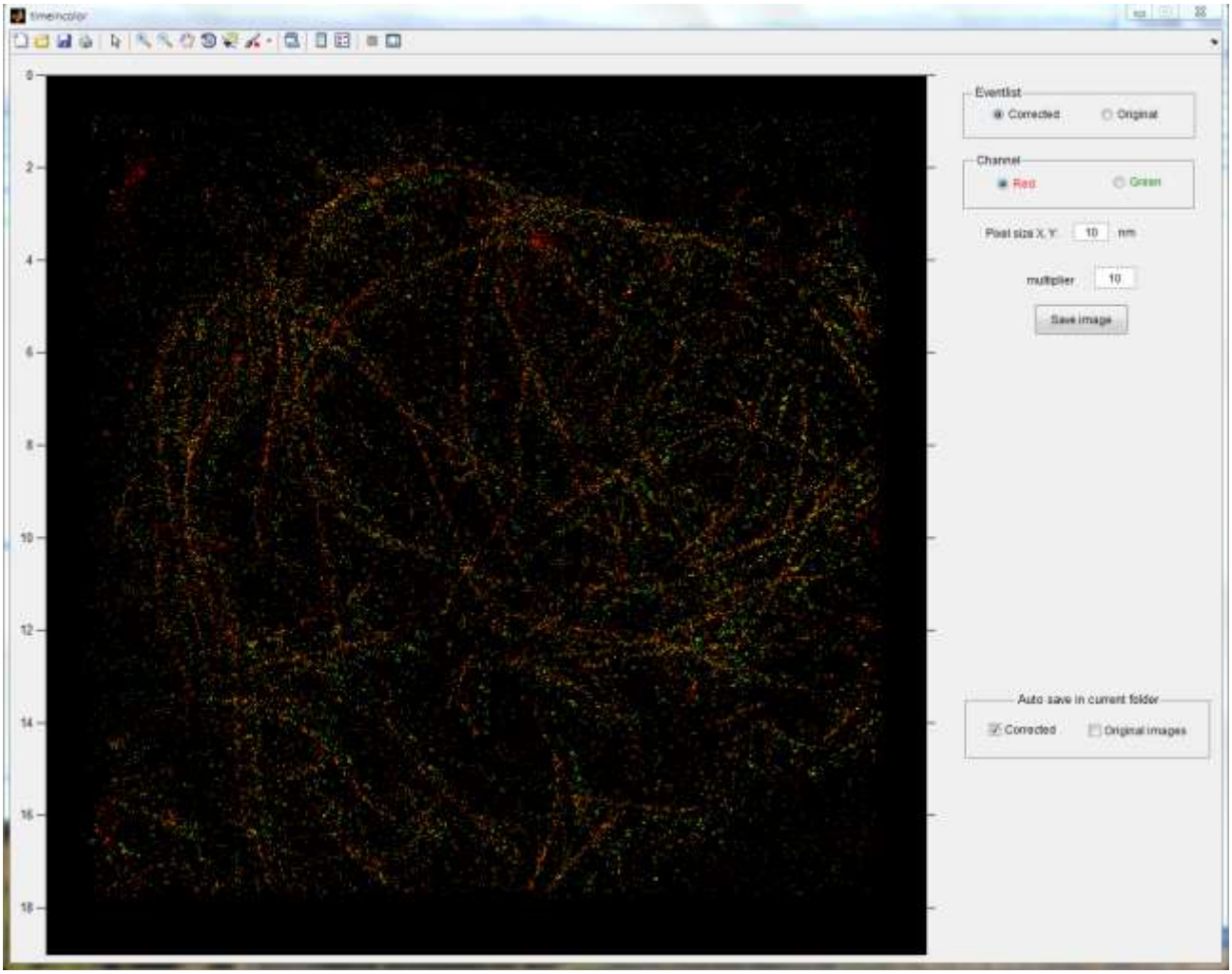
A new window will open (after some delay in case of large datasets).

The image here is built in the HSV colorspace with H values representing the time where H = 0° (red) corresponds to the first frame and H = 240° (blue) corresponds to the last one. The V values represent the relative number of events in the pixel comparing to the brightest pixel (the pixel with the highest number of events has the value V = 1, the other pixels have values $V = N/N_{max}$ ($0 \le V \le 1$), where N is the number of events detected in the area of the given pixel and N_{max} in the maximal number of events in the pixel through the image. The S value is always set to 1.

The V values are multiplied by a specified multiplier (default 10) to increase contrast.

As in the case of histogram/Gaussian representation it is possible to choose either the corrected or the initial dataset and one of the two color channels. You can also save the current image by pressing “Save image”. It is also possible to save the images automatically in the current folder using the corresponding option. Only the current color channel will be saved in this case. In order to automatically save the initial image you should build it before.

The name of automatically saved files is the following: ‘TN_xxx.tif’, where N = 0 or 1 (raw or corrected data), xxx = red or green (the name of the channel). The format is also 24-bit RGB LZW-compressed tiff.


timeincolor
0
2
4
6
8
10
12
14
16
18
Eventlist
Corrected
Original
Channel
Red
Green
Pixel size X, Y:
10
nm
multiplier
10
Save image
Auto save in current folder
Corrected
Original images

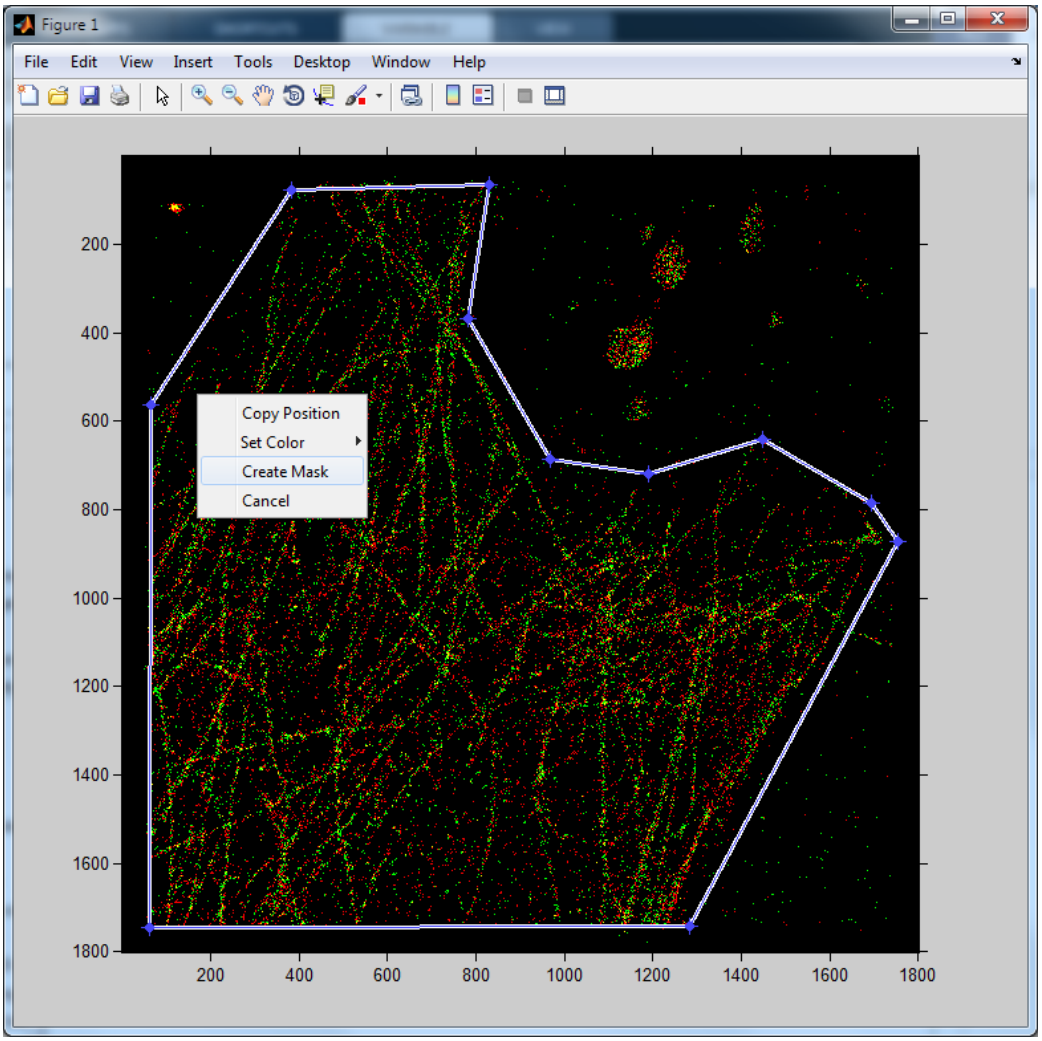
Selection of a region of interest (ROI)

Choose the desired pixel size for preview (default 10 nm). Press “Keep ROI…” or “Clear ROI…”.

A window with preview (high contrast, histogram mode, screen colors correspond to channels) will appear. Pressing left button of mouse will build the vertices of the polygon representing the ROI. Close the polygon with selecting the first vertex. Double click or choice “Create Mask” from the context menu (on the figure) will create a mask corresponding to the drawn polygon. The localizations inside (for “Clear ROI”) or outside (“Keep ROI”) of the mask will be removed from the dataset.

You can close the preview window without drawing a polygon. This allows for fast evaluation of the data.

Another possibility is drift correction with fiducial markers (fluorescent beads). Press “Keep ROI…” and select an image of a bead. Then go the “Drift” panel. Choose “Corrected lists” there and press “Calculate”. You will see the trace of the movement of the bead. You can apply this movement to your data.

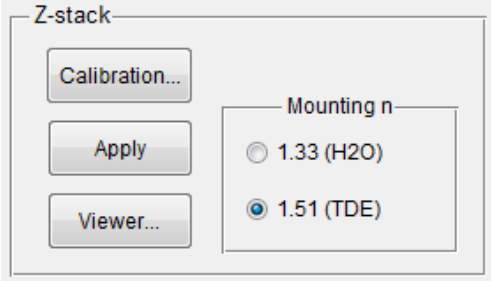


3D super-resolution with astigmatism

SharpViSu provides the following functionality for 3D STORM (PALM, GSDIM) experiments:

Calibration of the system for the determination of the axial position of fluorophores by astigmatic deformation of the PSF; determination of the positions using the calibration; slice-by-slice view of reconstructed 3D data.

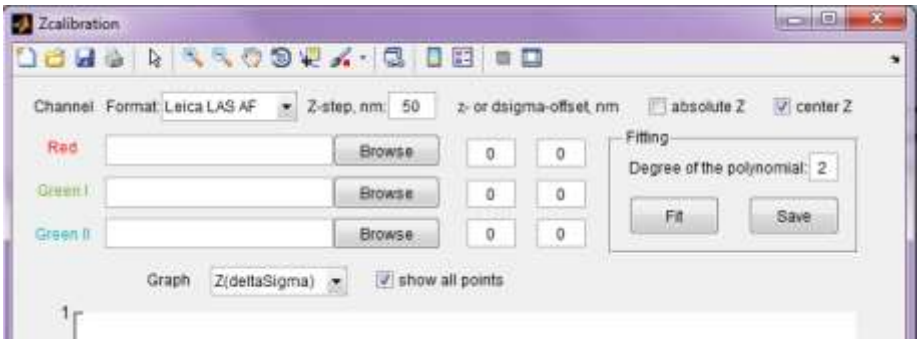
As the software works exclusively with localization tables, the fitted widths of the PSFs in X and Y direction should be present in the input data both for calibration and determination of positions. You should use the same fitting software to produce both calibration and experimental data.



Calibration of astigmatism

For the calibration you should have, for each color, a set of localization tables where each table corresponds to one Z-position of objective and contains widths of PSFs in X and Y (e.g. σ_x and σ_y) for this position. For this purpose you can use multi-color beads spread in a single plane (on a coverslip, for example).

Press “Calibration…” A new window will show up.



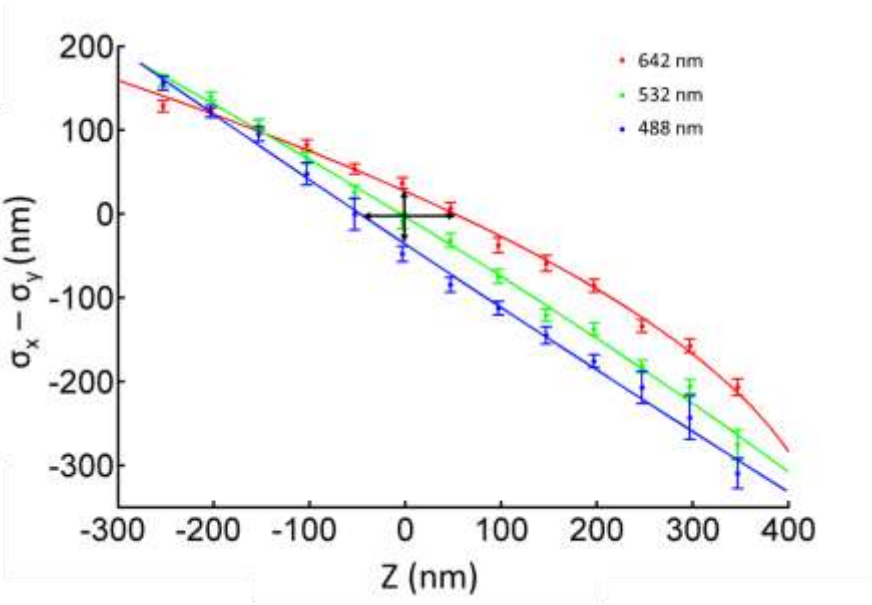
Choose the format of the input data (Leica LAS AF, 3D version, default; QuickPALM; RapidSTORM or (x, y, dx, xy), a table with columns with the corresponding content). Put the experimental step size between different axial positions of the objective (default 50 nm).

Press “Browse” and select a set of localization tables each corresponding to one position in Z. Repeat it for the other channels.

If the eventlists correspond to exactly the same Z-positions for all channels, check “absolute Z”. If you wish to center the calibration around Z = 0, check “center Z”. In this case the axial positions will have values [$-Z_{max}/2$ … $Z_{max}/2$] instead of [0 … Z_{max}].

Put the degree of polynomial for fitting (default 2) and press “Fit”. The experimental points and the fitted curve will appear on the graph. You can invert the graph by selecting “Graph Z(deltaSigma) or deltaSigma(Z)”. All the experimental points will be shown if checked “show all points”. For the deltaSigma(Z) vertical error bars indicate standard deviation of the data.

If the Z-positions are different for different channels (for example, each channel was calibrated with a different sample), you should know the axial chromatic aberration of your system in order to retrieve the correct Z-coordinates of the dyes. You can find the offset between channels in Z at the positions where $\sigma_x = \sigma_y$, or the offset in $\sigma_x - \sigma_y$ for the same Z around the position $\sigma_x - \sigma_y = 0$ (see the figure below, the horizontal arrow indicates the offset in Z and the vertical one is the offset in $\sigma_x - \sigma_y$). To consider the axial chromatic aberration, uncheck “absolute Z”, check “center Z” and put the offset for each channel in the corresponding fields.



If satisfied with the fit, press “Save”. The calibration data (the coefficients of the polynomial) will be saved in the files “Znnn.dat”, where nnn = 488, 532 and 642, in the installation folder of the application.

Calculation of Z-coordinates of fluorophores

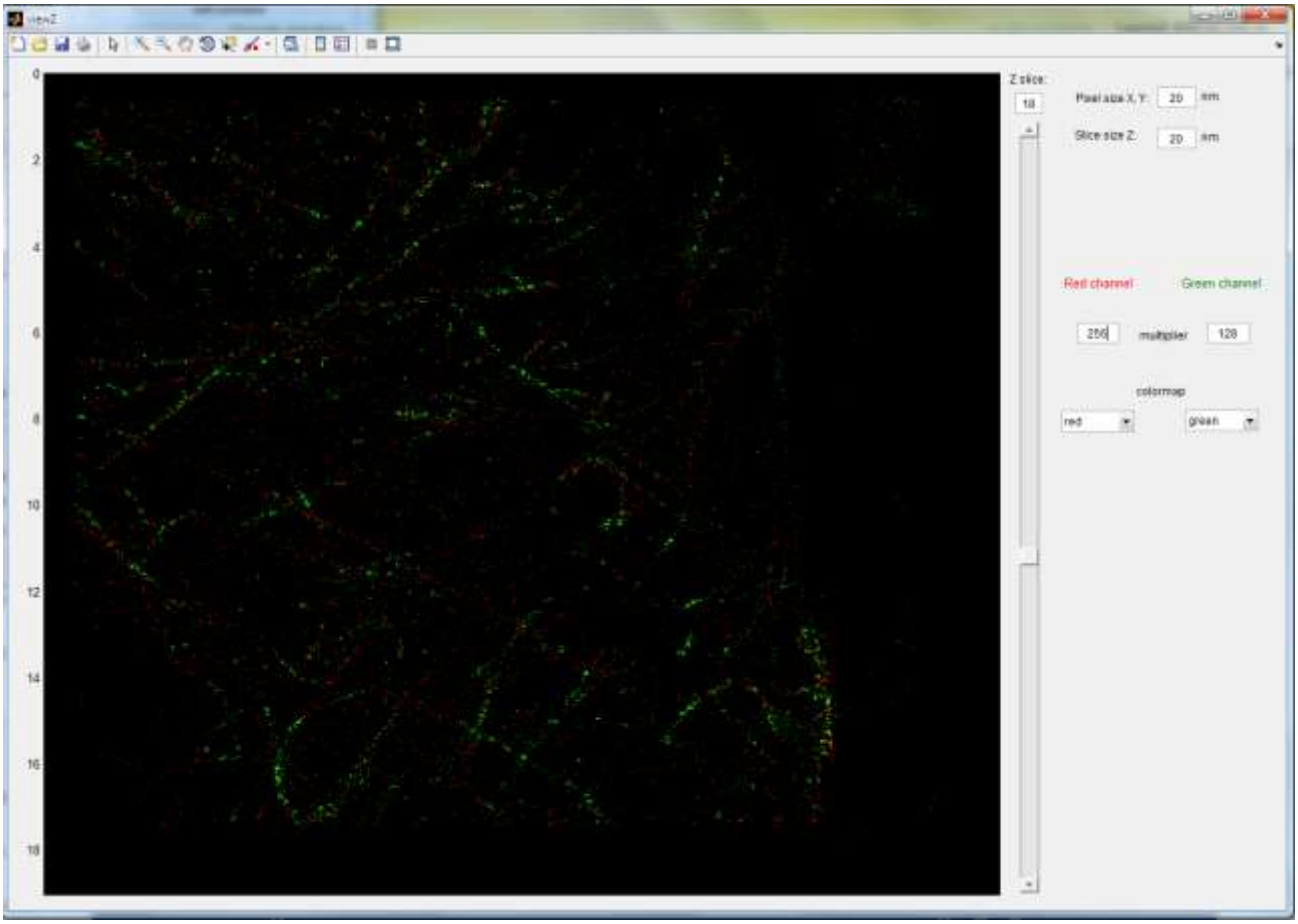
Select the proper refractive index of the mounting medium (1.33 for aqueous solution: PBS, MEA, Glucose Oxidase etc.; 1.51 for the medium TDE + Vectashield).

Press “Apply”. The Z-coordinates of dyes will be calculated using the calibration (see the previous step) and widths of the PSFs. The coordinates will be saved in the corrected eventlist.

Z-stack viewer

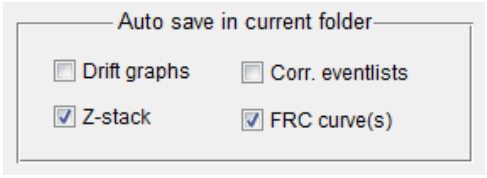
After applying corrections to your data, press “Viewer…” in the “Z-stack” panel. The viewer window will open (after a delay in the case of big datasets).

You can choose the pixel size in X,Y; slice size in Z; multiplier and colormap for each channel. Go through slices using slider. Upon closing of the viewer, the calculated stack will be sent to the main program. To save the Z-stack, check “Z-stack” in the panel “Auto save in current folder” (see next section) before pressing “Viewer…”



Save data automatically

It is possible to save some output data automatically in the current folder. Choose the desired output in the panel “Auto save in current folder”.



Drift graphs: each graph of calculated drift, displayed in the panel “Drift”, will be saved in a file ‘drift_xxx_nnn.png’, where xxx = ‘red’ or ‘green’; nnn = total drift length (sum of magnitudes of the vectors in the graph).

Corr. eventlists: localization tables after drift correction will be saved in a file ‘el_xxx_corrected.ascii’, where xxx = ‘red’, ‘green’ or ‘blue’.

Z-stack: the last Z-stack generated by the stack viewer will be saved upon closing the viewer. The slices in the .tif format will be saved in the subdirectory “stack” automatically created in the work folder.

FRC curve(s): FRC curve for each channel will be saved after calculation of the FRCs in a file ‘FRCxxx.png’, where xxx = ‘red’, ‘green’ or ‘blue’.

Plugins

Spectral demixing in SplitViSu

To run the plugin for demixing of splitSMLM data, go to the menu bar – Plugins – SplitViSu demixing. The current data (if present) will be transferred to the plugin. Further instructions, can be found in the SplitViSu user manual under https://github.com/andronovl/SharpViSu

ClusterViSu

To run the cluster analysis plugin, go to the menu bar – Plugins – ClusterViSu. The current corrected data (if present) will be transferred to the plugin. For further instruction, refer to the ClusterViSu user manual under https://github.com/andronovl/SharpViSu

Add your own plugins

It is possible to connect to SharpViSu other Matlab GUIs. To do so, open 'SharpViSu.fig' in Matlab GUIDE, go to Tools – Menu Editor…, select the folder 'Plugins' and press the button 'New Menu Item' in the toolbar of the menu editor. The new menu item will appear, you can call it at your convenience. Press Callback … View. The callback function will open in the Editor. This is the function that will be executed upon selecting your plugin from the plugin menu. Here you can call the main function of your plugin's GUI and pass to it variables from the SharpViSu workspace (you can look at a function '`clusters_Callback`' from 'SharpViSu.m' as an example, this is the callback for calling ClusterViSu plugin). All useful variables inside SharpViSu are stored in the 'handles' structure. Some examples: '`handles.ABcorr{1}`' and '`handles.ABcorr{2}`' are the 'red' and the 'green' eventlists, accordingly, after the latest correction; '`handles.AB{1}`' and '`handles.AB{2}`' are the 'red' and the 'green' non-corrected eventlists; '`handles.folder`' is the address of the directory from which the latest eventlist was loaded.