Installation

Stand-alone application. Download and run "SharpGSDIM_web_installer.exe" and follow instructions. If not already present, the required version of the MATLAB Compiler Runtime will be downloaded from the web and installed automatically.

You may also want to use a portable stand-alone version. Unpack archive "SharpGSDIM.zip" to a preferred location. To run it, you will need to have MATLAB R2014a Compiler Runtime (v. 8.3) installed on your computed. It can be downloaded for free form the website of Matlab: http://fr.mathworks.com/products/compiler/mcr/

Matlab code. Add folder "SharpGSDIM" to the search directory of Matlab. Note that the code was tested and compiled in Matlab R2014a.

Running SharpGSDIM

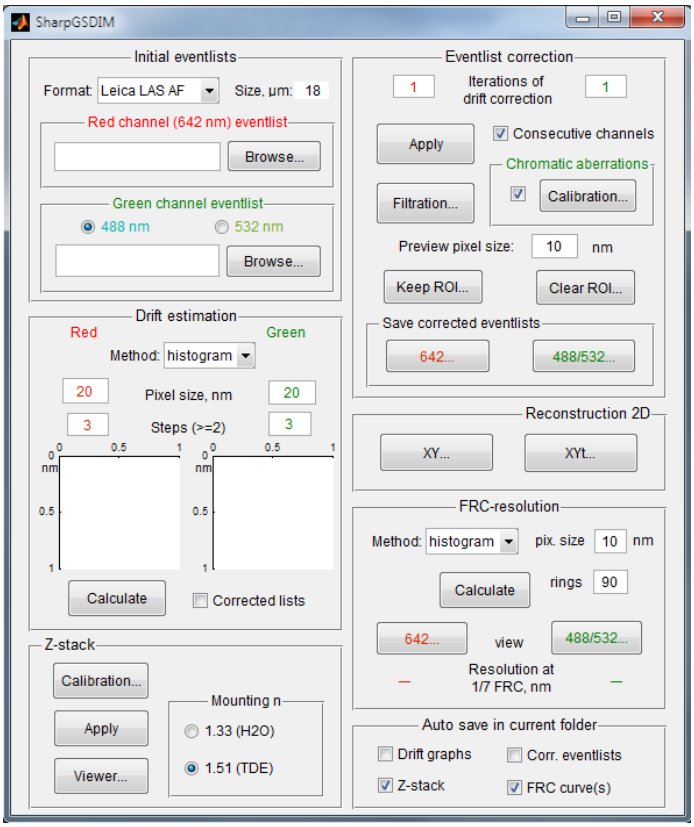
Stand-alone application. Run "SharpGSDIM.exe" (default path: "%ProgramFiles%\IGBMC\SharpGSDIM\application\SharpGSDIM.exe", could have been changed during installation)

Matlab code. Type in the command line:

>> SharpGSDIM

It will run the function SharpGSDIM.m

The main window of the program will appear



Loading localization tables

Before loading the eventlists, select the type of the data in the popup menu “Format”. The following formats are supported:

Leica LAS AF (ascii, both 2D and 3D versions supported), default;

QuickPALM;

RapidSTORM;

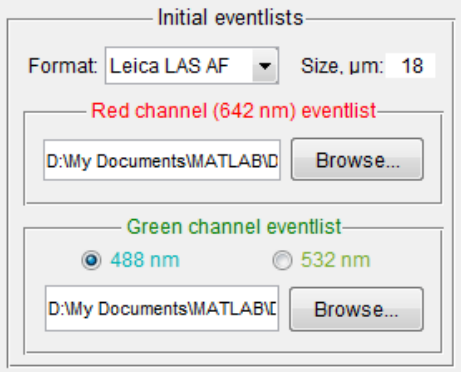
Micro-Manager Localization Microscopy plugin (text files);

SharpGSDIM (internal format used in the software).

Press “Browse…” and choose the localization table for the corresponding color channel. After the loading, the geometrical size of the datasets will be displayed in the field “Size, µm”. This size in the size of the biggest dimension of the biggest loaded dataset, rounded to the higher integer, and is not less than 18 µm. If the displayed value is not reasonable, re-check the format of the data. Please note that for Micro-Manager LM data all the commas will be replaced by dots in the original file.

Either one or two channels can be processed in the same time.

Choose the proper wavelength (488 nm or 532 nm) for the “green” channel, if present.



The channels are named for convenience and do not oblige to use the exact excitation wavelengths, but the system should be precalibrated for chromatic aberrations and astigmatism (see below) for the color channels used for imaging.

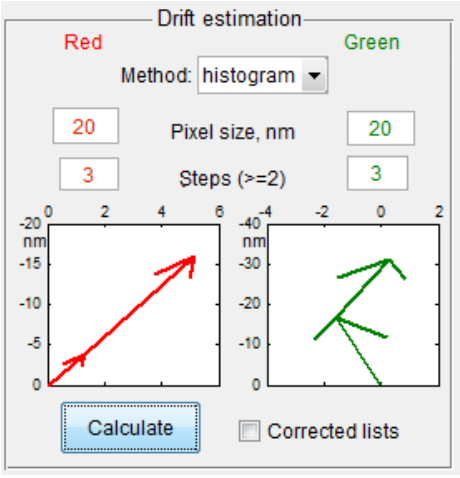
Estimation of drift

Type desired pixel size (p, default 20 nm), number of steps (n, default 3) and method (default histogram) for each channel in the corresponding fields.

Press “Calculate”. During the calculation, the entire eventlists will be divided into n consecutive sublists and from each of them an image with the pixel size of p nm in the selected mode (histogram, Gaussian or Voronoi)

will be built. The shift between each of the images will be calculated with a subpixel precision yielding (n-1) values.

The calculated values of drift will be displayed by (n-1) arrows in the corresponding windows.



You can change the parameters of pixel size, steps, method and recalculate drift. The corrections do not apply to the data in this step. If the estimated values are inconsistent or too big, it means that there is not enough redundancy in the data for the calculation. Try to use bigger pixel sizes, less number of steps or another method. From experience, the histogram representation is fastest and usually yields robust results.

Checking “Corrected lists” prior to “Calculate” will use for the calculation already corrected data (see below), do not use this option before correction of the data.

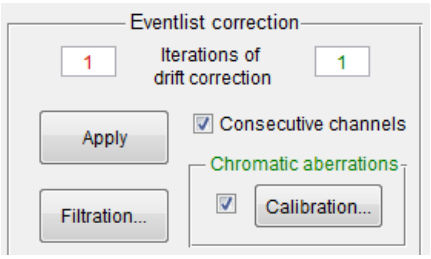
If satisfied with the initial estimation of drift, go the following step.

The current step is not mandatory if you do not plan to perform the correction for drift.

Correction of drift and chromatic aberrations

Choose desired number of iterations for the correction of the drift (default 1). Check “Consecutive channels” if your double-color acquisition was performed in a successive way (first red, then green channel). Otherwise (both channels were acquired in the same time), uncheck this option. If selected, the red channel data will be corrected toward its end, the green channel one – towards its beginning. Otherwise, both channels will be moved towards their beginnings. Press “Apply”.

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



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.

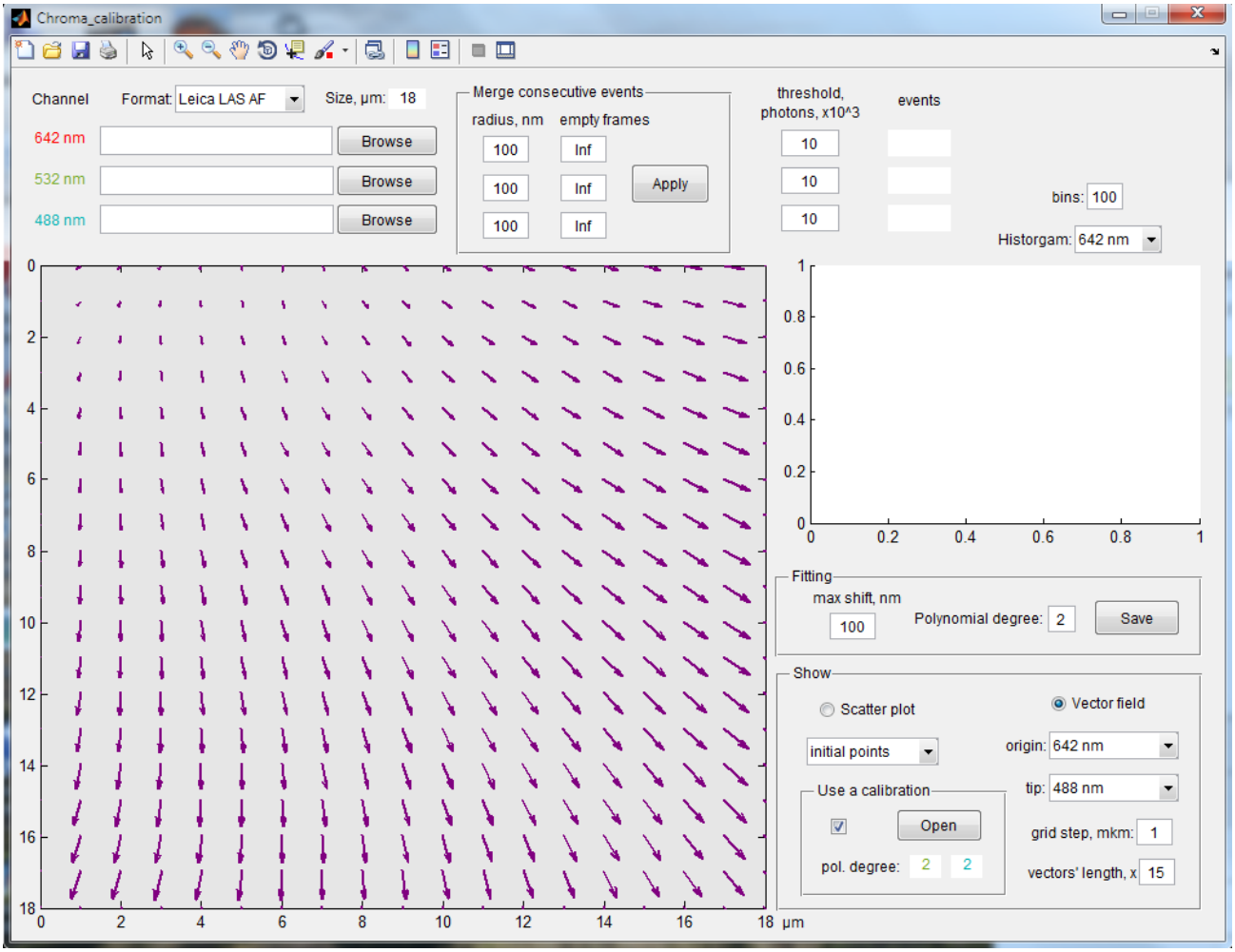
Chromatic aberrations are corrected by shifting the localizations of the “green” channel relatively to the “red” one. To perform corrections, the checkbox should be active (by default) before pressing “Apply”. For chromatic aberration calibration, see below.

If you want to remake the correction with different number of iterations, repeat the process starting from the preceding section, “Estimation of drift”: calculate the drift once and go to the correction. Usually the correction converges after 1-3 iterations.

If you do not want to perform any corrections for you data at this point, put zeros as number of iterations, uncheck “Chromatic aberrations” and press Apply. Otherwise, further processing may not work.

Calibration for chromatic aberrations

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



The calibration totally relies on localization tables. A recommend procedure is to acquire a short dataset with a sample containing multi-color beads, for each color. The density of the beads should not be too high allowing nice separation of the signals during localization. In addition, the beads should be distributed more

or less evenly through the field of view enabling proper fit of aberration in X, Y. The localization table for each color will contain positions of the beads, possibly with some noise.

Select the format of input data (Leica LAS AF 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 number of photons in events.

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 strongly reduced number of events and increased photon statistic. Number of events should be equal or more than the number of beads in the sample. The incorrect localizations originating from noise were not averaged with the localizations from beads, and so the formers have much less brightness. Delete them by imposing a threshold on photon number ($10*10^3$ 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 the pairs of bead, the software will search for the other color’s neighbor 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 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 as a “quiver” plot of 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 (notably the degree of the polynomial) if needed.

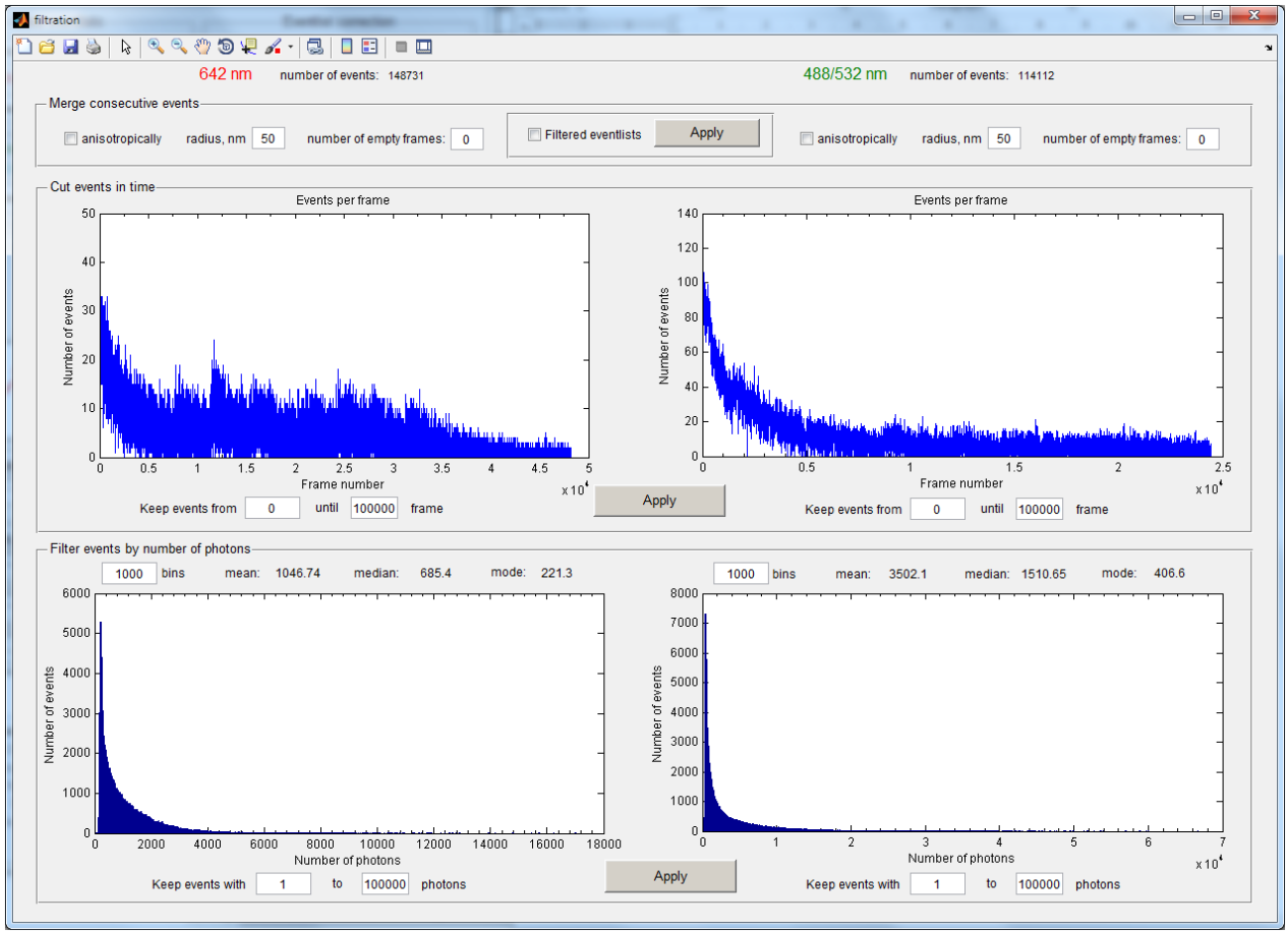
If satisfied with the fit, press “Save”. The calibration files named ‘488.mat’ and ‘532.mat’ will be saved in the installation folder. The files contain a geometric transformation object that describes the transformation from the positions of the 488- or 532-nm channel to the position of the 642-nm channel.

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 532 nm channel; then a second window will open, choose there the calibration file for the 488 nm channel. The polynomial degree of the loaded fit will be displayed below. If the “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.

Filtration of data

After applying the corrections, press “Filtration”. A new window will appear.



At the top, you will see the number of localization events for each of the used channels.

For averaging consecutive events put the desired search radius (r, default 50 nm) and number of empty events (default 0) and press “Apply”. “Number of empty frames” means maximal number of the consecutive empty circles in the batch being averaged. The number of empty blocks in the batch is not limited. After a few instants of computing, the number of events will normally drop and the distributions at the bottom will change.

If you would like to use ellipse-like search regions for consecutive events, check “anisotropically” before “Apply”. The axes of the ellipse for the i-th event will be equal $a_i = r*\sigma_{xi}/100$, $b_i = r*\sigma_{yi}/100$, where σ_{xi} and σ_{yi} are taken from the i-th row of the eventlists. This option can be very useful for 3D experiments with astigmatism, where the uncertainty of localization is strongly anisotropic.

In the middle you see a plot: quantity of events as a function of the frame number. If needed, you can keep a region in time by putting the first and the last frame to keep. The statistics will update immediately after putting new values, but the eventlists will be actually filtered only after pressing “Apply”. The option is useful, for example, to delete frames with too high density at the beginning of acquisition.

At the bottom, there are histograms of photon counts in the datasets. You can change the bin size of them. The mean, median and mode values are directly shown permitting to get an idea of corresponding localization

precision or resolution of you data. You can also choose a range in photon counts to keep (statistics updates immediately). The events with photon counts out of the selected range will be removed from the datasets after pressing “Apply”.

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

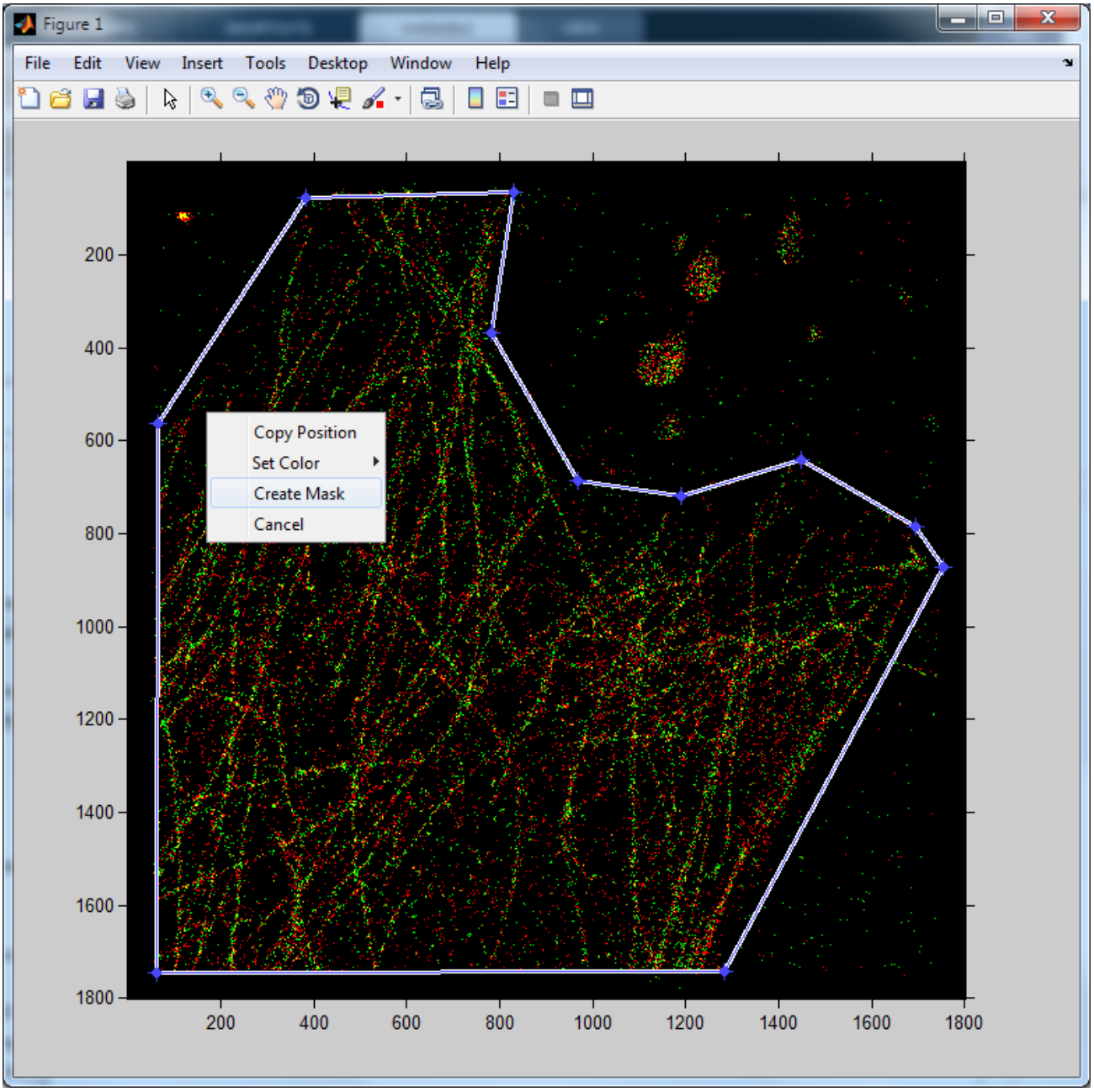
Selection of a region of interest (ROI)

Choose 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 vertices of polygon. Close the polygon by 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 lying inside (if “Clear ROI”) or outside (“Keep ROI”) of the mask will be removed from the dataset.

You can just close the preview window without drawing a polygon. It allows 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 you data: put 1 into the number of iterations and press “Apply”. The procedure will subtract the calculated drift of the bead from the dataset. The first iteration of the drift correction always works with raw data.



Saving the corrected datasets

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

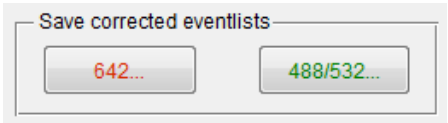
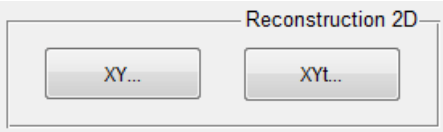
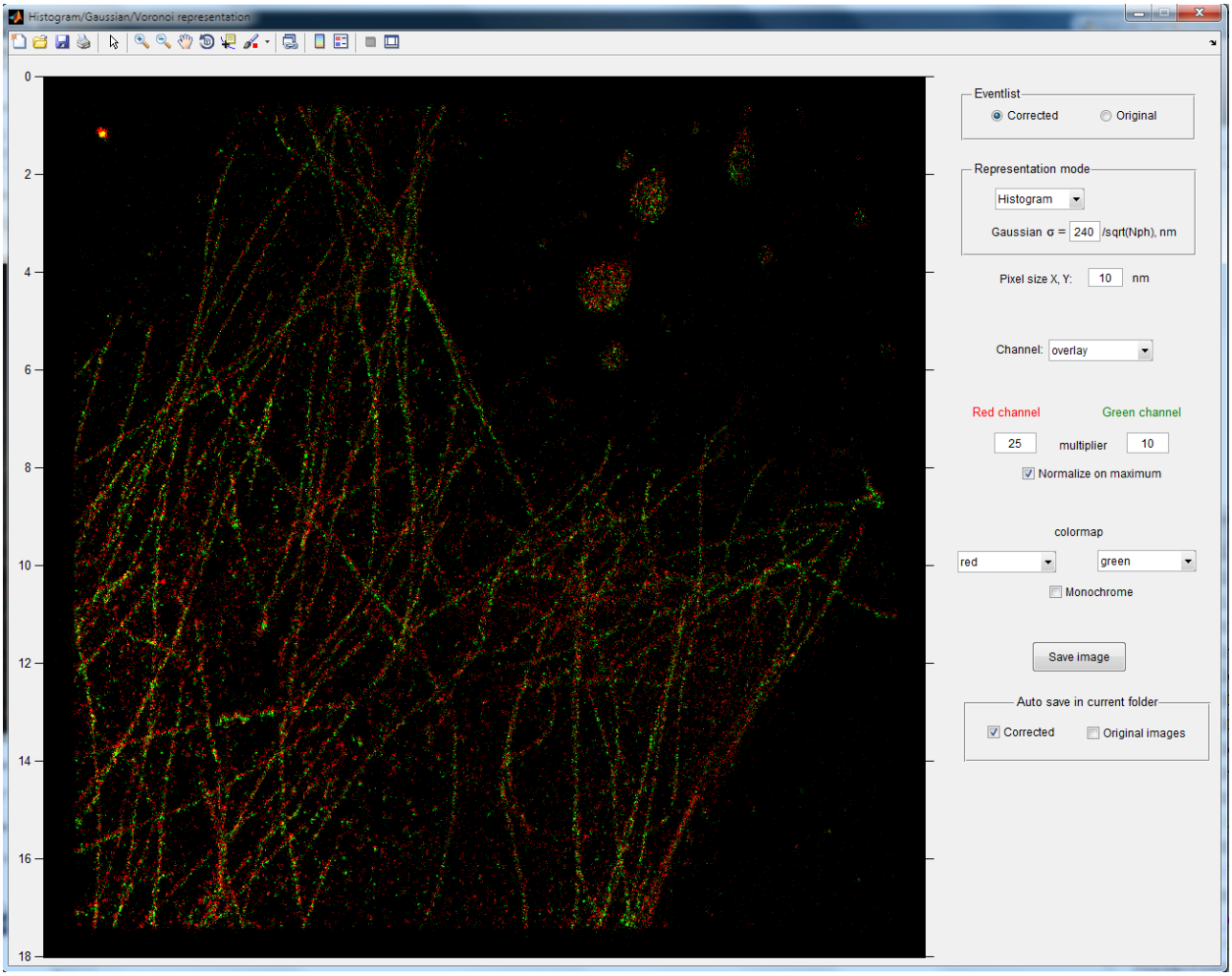


Image reconstruction in histogram, Gaussian or Voronoi mode

After applying corrections to the data, press “XY…” in the panel “Reconstruction 2D”



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”.

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

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

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

In the histogram mode the grey value of pixel will equal to number of events collected inside the area of the pixel. In the Gaussian mode each event is rendered as a 2D Gaussian function, centered on the pixel where the event is situated. 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).

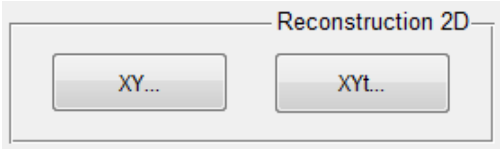
You can choose a colormap separately for each channel. The available colormaps are: red, green, gray, blue, hot, hsv, jet, cool, spring, summer, autumn, winter, bone, copper, pink (for detailed description of the colormaps see the manual of Matlab). If checked "Monochrome", the both channels will be shown in the grey colormap, and the picture will be subsequently saved in an 8-bit monochrome tiff.

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

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 "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.

Image reconstruction in time-in-color mode

Press "XYt…" in the panel "Reconstruction 2D"



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

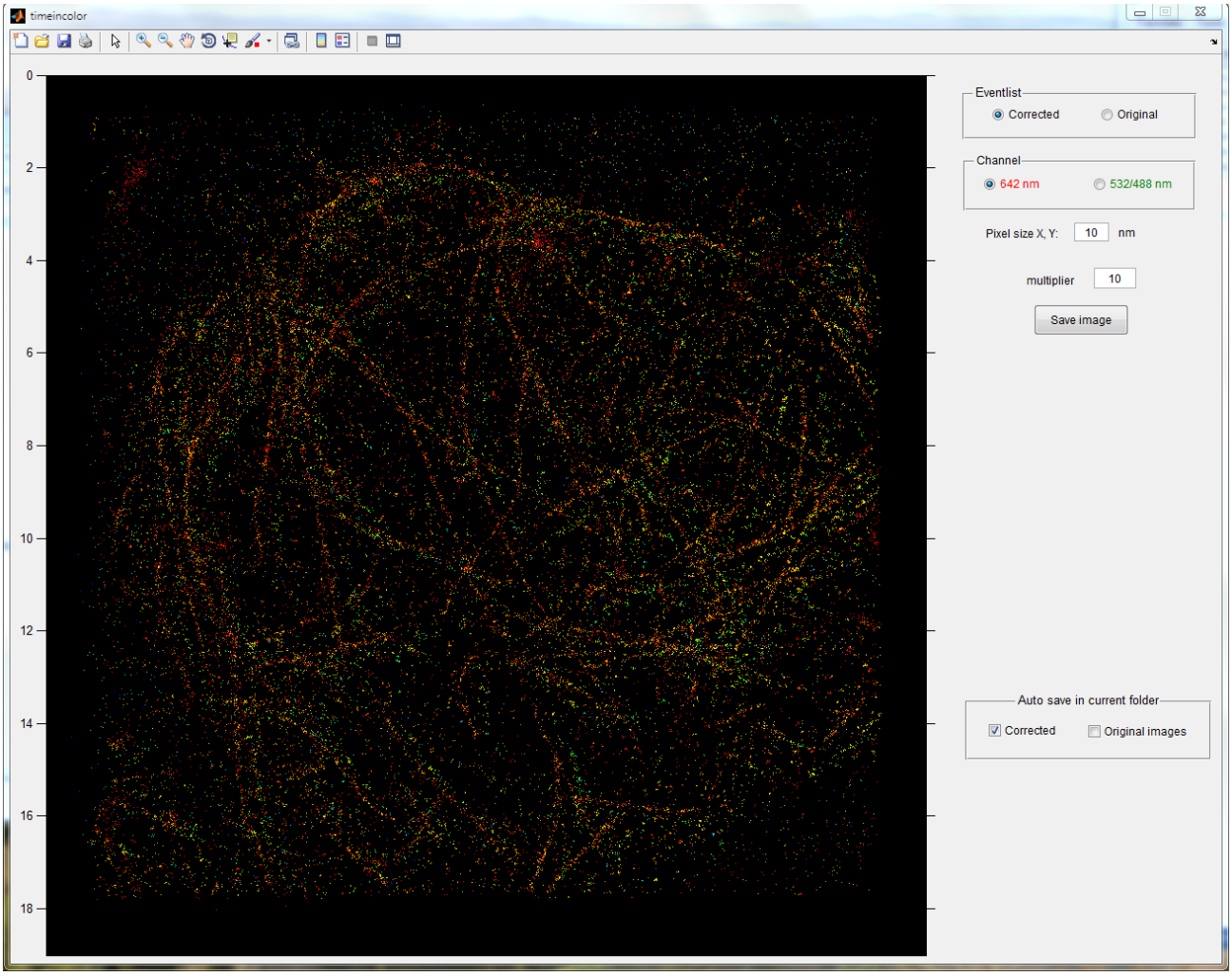
The image here is built in the HSV colorspace with H values representing the time where H = 0° (red) corresponds to the first frame and H = 240° (blue) corresponds to the last one. The V values represent the relative number of events in the pixel comparing to the brightest pixel (the pixel with the highest number of events has the value V = 1, the others 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.

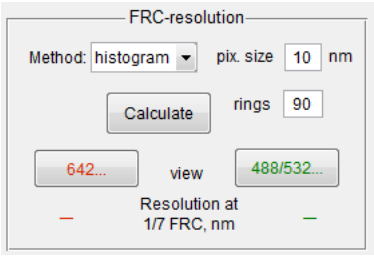
As in the case of histogram/Gaussian representation it is possible to choose either corrected or initial dataset and one of two channels. You can also save the current image by pressing "Save image". It is also possible to

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.

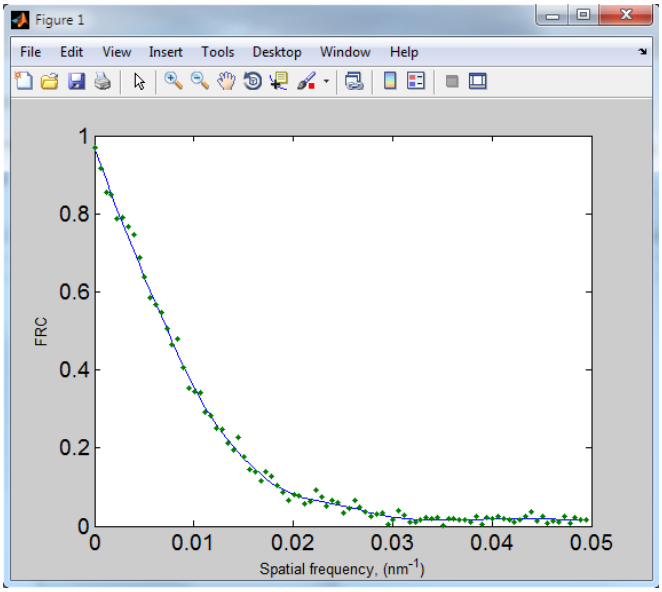
The name of automatically saved files is the following: 'TN_xxx.tif', where N = 0 or 1 (raw or corrected data), xxx = red or green (the name of the channel). The format is also 24-bit RGB LZW-compressed tiff.



Fourier ring correlation and estimation of resolution



Select a method (histogram, Gaussian or Voronoi), number of rings (default 90) and press “Calculate”. The number of rings equals to the number of points in the final FRC graph. The resolution at 1/7th of the FRC will be shown for each channel after the calculation ends. You can view the curves by pressing the button for the corresponding channel.

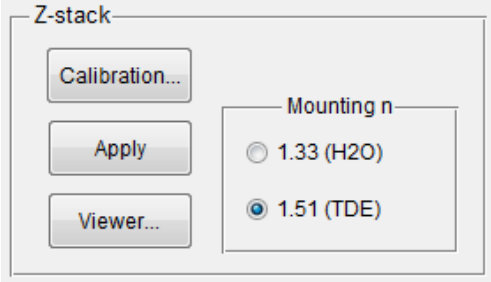


3D super-resolution with astigmatism

SharpGSDIM provides following functionality for 3D STORM experiments:

Calibration of the system for determination of 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.

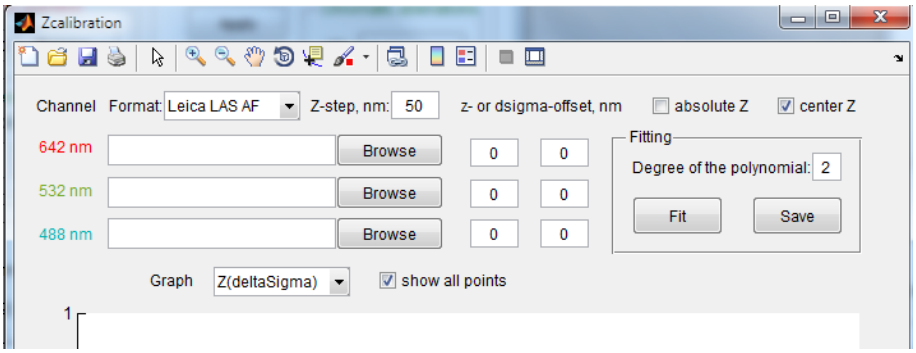
As the software works exclusively with localization tables, the fitted widths of the PSFs in X and Y direction should be present in the input data both for calibration and determination of positions. You should use the same fitting software for calibration and for experiments.



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 be opened.



Choose the format of 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 objective (default 50 nm).

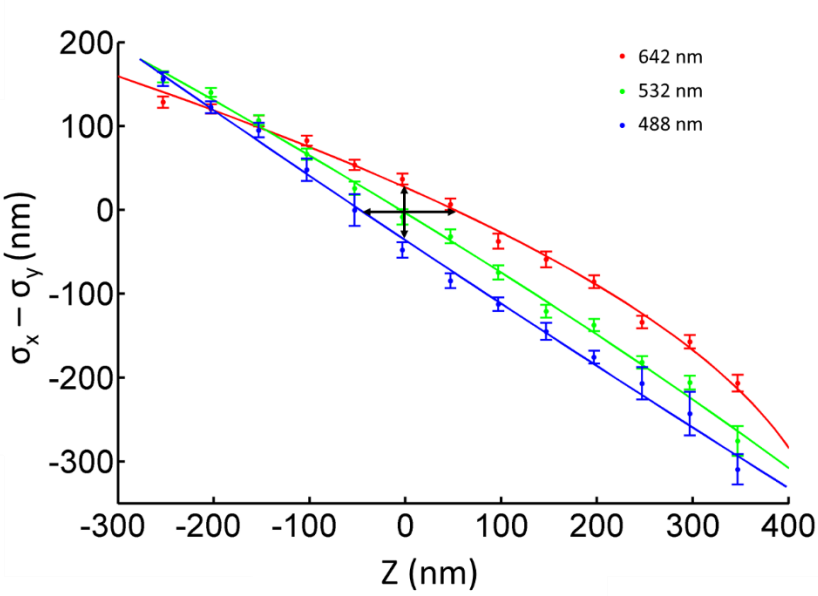
Press “Browse” and select a set of localization tables each corresponding to one position in Z. Repeat it for the other channels.

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

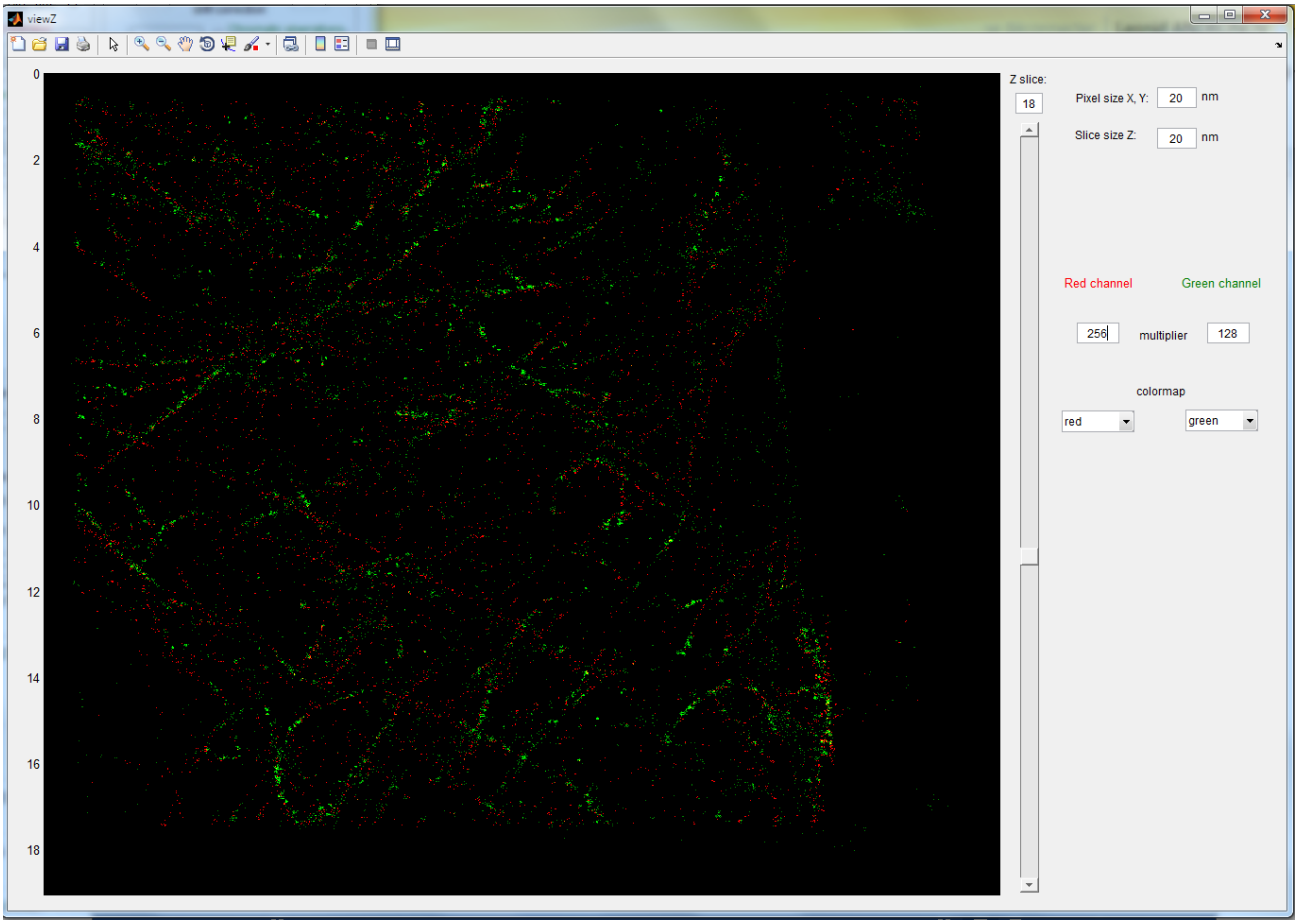
Select the proper refraction index of the mounting medium (1.33 for aqueous solution: PBS, MEA, Glucose Oxidase etc.; 1.51 for the medium TDE + Vectashield).

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

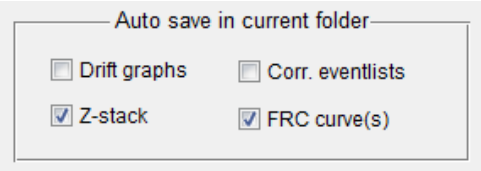
After applying corrections to your data, press “Viewer…” in the “Z-stack” panel. The viewer window will open (after a delay in case of big datasets).

You can choose 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. In order to save the Z-stack, check “Z-stack” in the panel “Auto save in current folder” (see next section) before pressing “Viewer…”



Save the 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_nnn_corrected.ascii’, where nnn = 488, 532 or 642.

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 in a file ‘FRCnnn.png’, where nnn = 488, 532 or 642 after calculation of the FRCs.