Contents

1. Introduction

The MATLAB app allows to evaluate single molecule localization microscopy data in the form of tiff image stacks (time series) taken in a total internal reflection fluorescence (TIRF) microscope. It estimates 3D position, signal and background level for each individual molecule.

To avoid localization biases, the app allows the user to measure optical aberrations present in the microscope by taking a z-stack of a small (e.g. 100 nm) fluorescent bead. This feature makes it also possible to evaluate data taken with engineered PSFs (e.g. using a cylindrical lens).

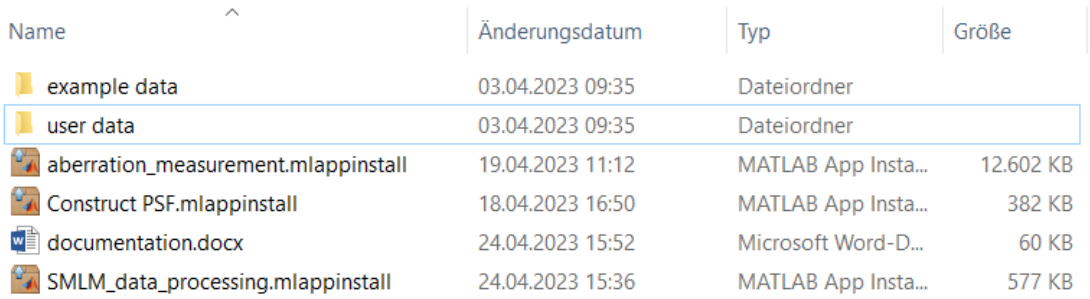
The app requires the ImageJ plugin "Thunderstorm" for doing a coarse, 2D pre-localization.

The app consists of three parts that can be also run individually:

„SMLM_data_processing.mlapp“	The main app
“construct_PSF.mlapp”	A sub-app to construct point spread functions, which can be loaded into and used in the main app
“aberration_measurement.mlapp”	A sub-app to estimate pupil phase aberrations from a previously recorded z-stack of a single fluorescent bead

2. Installation

Unpack the zip archive and double click on the apps to install them in Matlab. They will appear in the MATLAB “apps” bar. **When executing the apps, be sure that the Matlab path is set to the path where the folders “example data” and “user data” are located**. The latter contains files that characterize the PSF, objective lenses, cameras and aberrations. Otherwise, the app fails to load the default PSF at startup.



3. APP: "SLM_DATA_PROCESSING"

a. General workflow

The app consists of three tabs: "**define PSF**", "**prelocalization**" and "**precise localization**". The general workflow comprises:

- the definition of the PSF that persisted when the raw image data was recorded (in tab: "define PSF")
- processing the raw image data using Thunderstorm (2D localization) and saving the results as csv-table. Note that the correct pixel size must be set in Thunderstorm under "camera settings". The other parameters there are not relevant.
- loading the raw image data + Thunderstorm csv-table into the app (in tab: "prelocalization")
- performing precise 3D localization (in tab: "precise localization")

b. Detailed explanations of the app

Fig. 3.1 shows the "define PSF" tab as it should appear at startup.

The text box contains info about the PSF such as name, defocus value, oversampling value, the used camera and objective lens (explanations to these parameters can be found in chapter xxx). The figure on the right allows one to inspect the PSF for all z-values it supports. The z-value is defined as the position of a molecule form the coverslip and ranges from 0 to $\lambda_{em}/3$ (a third of the peak emission wavelength).

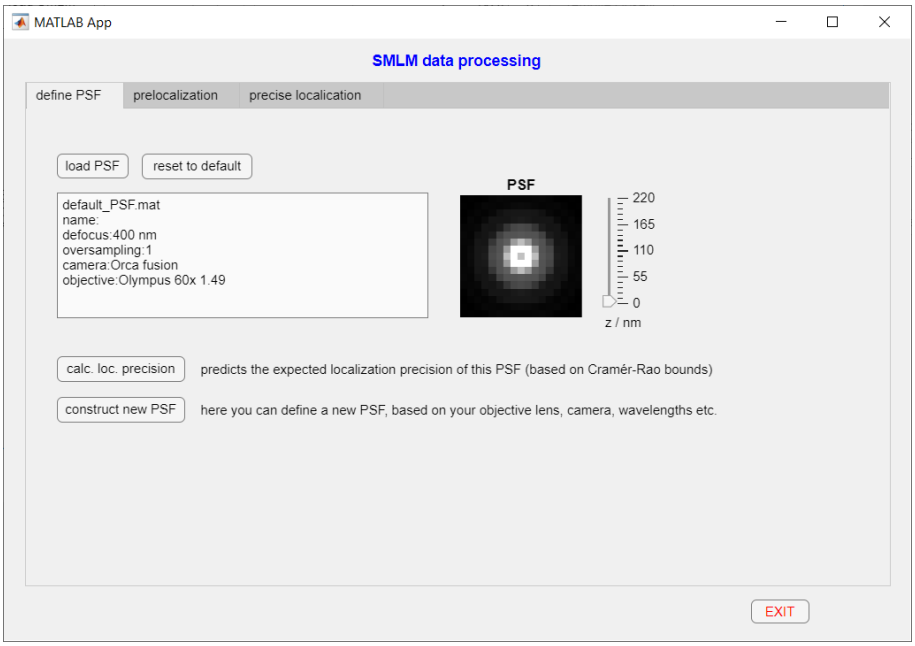


Fig. 3.1 The "define PSF" tab of the main app. It allows the user to load and inspect point spread functions (PSF) created by the app "construct_PSF.mlapp".

reset to default	loads the default PSF. To replace the default PSF by a user-defined one, you have to construct it in “construct_PSF.mlapp” and save it as “default_PSF.mat” in the folder “.\user data\PSFs”.
calc. loc. precision	displays the expected localization precision of the PSF using Cramér Rao lower bounds.
construct new PSF	launches the app “construct_PSF.mlapp”

Fig. 3.2 shows the “prelocalization” tab as it should appear at startup.

Fig. 3.2 The „prelocalization“ tab of the main app. Here you can load the raw image stack and 2D localization results from Thunderstorm.

load SMLM image stack	For importing the raw image stack in tiff format
load TS data (csv) & crop molecules	For importing the results table created in Thunderstorm. The table should contain the columns “id”, “frame”, “x / nm”, “y/nm”. After data import, single molecule images are cropped from the raw image stack, using the position information in the Thunderstorm table. Therefore, the correct pixel size must be also set in Thunderstorm under “camera settings”.
show random molecule	Shows a random molecule to verify if they have been cropped correctly: The molecule should appear in the center of the small image.
remove closely spaced molecules	Removes molecules detected in Thunderstorm that are too closely spaced. A threshold (minimum) distance can be specified in “d_min (AU)”, which is given in Airy units (AU = 1.22 * λ_{em}/NA)
save preloc data	Saves the stack of cropped molecules and the table as mat-file. The mat-file contains two variables, a 3D array (“images”) and a table (“positions”), e.g. like this: Name ▲ Value images 17x17x21090 double positions 21090x4 table
load preloc data	Previously generated preloc data can be loaded here. The data can then be processed in the “precise localization” tab. It can also be filtered again using “remove closely spaced molecules”.

Fig. 3.3 shows the “precise localization” tab as it appears at startup.

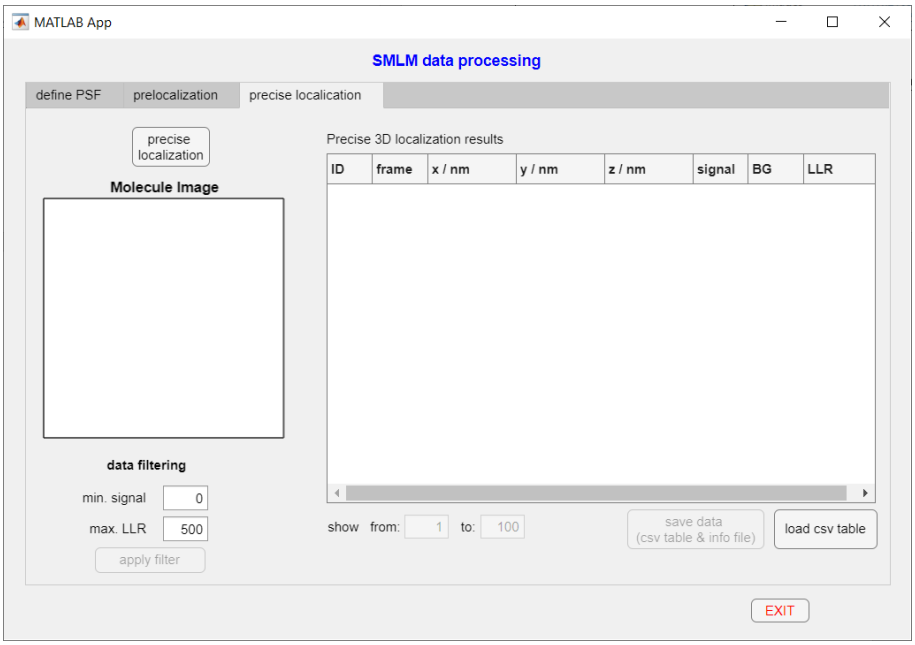


Fig. 3.3 The "precise localization" tab

Precise localization	Performs 3D fine localization using a Levenberg Marquardt algorithm and maximum likelihood estsimation for Poissonian noise.
Precise 3D localization results	The table is automatically filled afer the precise localization has finished. Description of columns: “**ID**” and “**frame**” denote the molecule identification number and the raw image stack frame number which contains the molecule. The data is copied from the Thunderstorm table. **x/y/z** contain estimated molecule positions in nm. **signal/BG** contain estimated signal and background level in photons. Note that the photon number corresponds to the estimated number of photons that are collected and transmitted by the objective lens (NOT the number of photons in the actual single molecule image, which is smaller) **LLR** Log-Likelihood ratio; this provides a measure for the fit quality. The smaller this number the better the fit.
Data filtering	Allows basic data filtering. A minimum value for the signal (in photons) and a maximum number for the LLR value can be defined. Note that “apply filter” must be clicked in order to perform the filtering process.
save data (csv table & info file)	Saves the shown table “precise 3D localization results” in csv format and a mat file containing the used PSF. Note that also info about the used camera and objective lens are part of the PSF definition, so this info is stores as well.
load csv table	For importing and visualizing previously processed data, or data that has been drift-corrected in Thunderstorm.

c. Drift correction

The app cannot correct the data for x-y drifts that occurred during the measurement. For drift correction, follow these steps:

- Export the final data table by clicking on “save data (csv table & info file)”
- Import the csv table in Thunderstorm and perform the drift correction there
- Export from Thunderstorm again and import data in the app for visualization

4. APP: "CONSTRUCT_PSF"

You can construct new PSFs here for use in "SLM_data_processing.mlapp". It is also possible to modify existing PSFs. The PSF of your microscope depends on your microscope objective, camera, emission wavelength of your fluorophore and phase aberrations that may be present in your system.

The objective properties are defined in the "**Objective lens**" tab shown in Fig. 3.1.

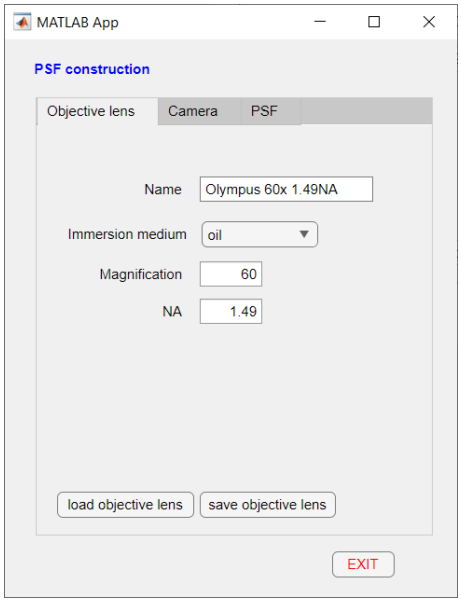


Fig. 4.1 The „objective lens" tab

Name	Choose a name of your liking.
Immersion medium	Immersion medium of your objective lens.
Magnification	Total system magnification. Note that if you have a home-built system the magnification may deviate from the one specified on the objective (E.g. if you are using an Olympus lens with magnification M together with a 200 mm focal length tube lens, your magnification is $\frac{M}{180} \cdot 200$.
NA	Numerical aperture specified on your objective lens.

The camera parameters are defined in the “Camera” tab show in Fig. 4.2. The parameters should be specified on the data sheet of your camera.

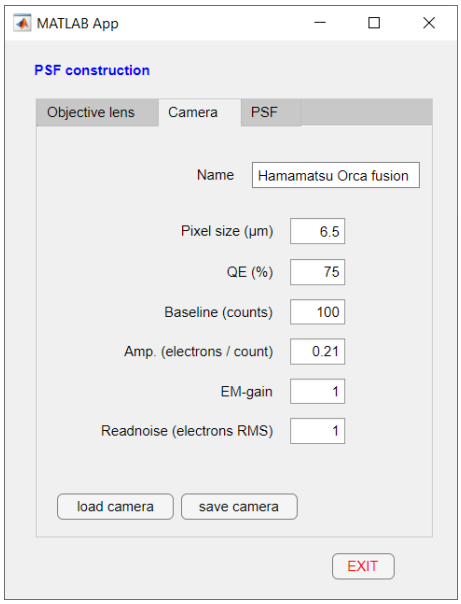


Fig. 4.2 The „Camera“ tab.

Name	Choose a name of your liking.
Pixel size (µm)	Physical size of a camera pixel in micrometer.
QE (%)	Quantum efficiency of your camera at the wavelength you are using
Baseline (counts)	This value is an “offset” (measured in counts) that the camera automatically adds to all of your images in order to prevent negative values.
Amp. (electrons/count)	The amplification parameter defines how many digital counts result from one created electron in a pixel.
EM-gain	The electron-multiplying gain is only required for EMCCD cameras. Set this value to 1 if you work with a CMOS camera.
Readnoise (electrons RMS)	The number of noise electrons produced in each pixel when they are read out.

The 3D PSF can be finally generated in the “PSF” tab shown in Fig. 4.3. The axial range of the PSF covers z-values from 0 (= the coverslip surface) to $\frac{\lambda_{em}}{3}$.

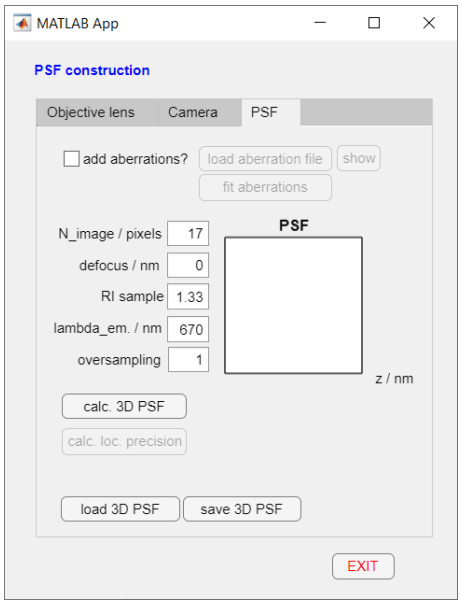


Fig. 4.3 The „PSF“ tab.

add aberrations	Activate this checkbox if you want to add phase aberrations to the PSF. Then you can load an aberration file by clicking on “load aberrations”. Aberrations can be measured from a z-stack of a small fluorescent bead. To start the corresponding app, click on “fit aberrations” (find more in chapter 5).
N_image / pixels	The sidelength (x-y size) of the PSF in pixels. It should be large enough to safely contain the PSF (no cropping), but not larger. This value determines the size at which single molecule images are cropped from the raw image stack (see **APP: “SLM_DATA_PROCESSING**chapter 3): The sidelength of the cropped molecule images is N_image – 2.
defocus / nm	Axial shift of your objective lens with respect to the coverslip surface during data recording (e.g. if you are using “off-focus” imaging as described in Refs. [1, 2])**.** **Positive values denote shifts towards the sample.**
RI sample	Refractive index of your sample (i.e. the buffer solution).
lambda_em / nm	Peak emission wavelength of your fluorophore.
oversampling	The oversampling-factor determines the spatial resolution at which the PSF should be calculated. If the camera pixel size divided by the magnification is much larger than ~100 nm, if can make sense to set this value to 2. This will avoid localization biases, but the fitting time will increase.
calc. 3D PSF	Calculates the PSF and displays the result in the box on the right.
calc. loc. precision	displays the expected localization precision of the PSF using Cramér Rao lower bounds.
load 3D PSF	You can load previously defined PSFs for editing.
save 3D PSF	You can save the PSF here to be used later in “SMLM_data_processing.mlapp”.

5. APP: "ABERRATION_MEASUREMENT"

This app can be either run independently or called from the "construct_PSF.mlapp" by clicking on "fit aberrations" in the "PSF" tab. It allows the user to estimate the phase aberrations present in the imaging system from a z-stack of a single small fluorescent bead (e.g. "PS Speck" microbeads from Thermo Fisher Scientific).

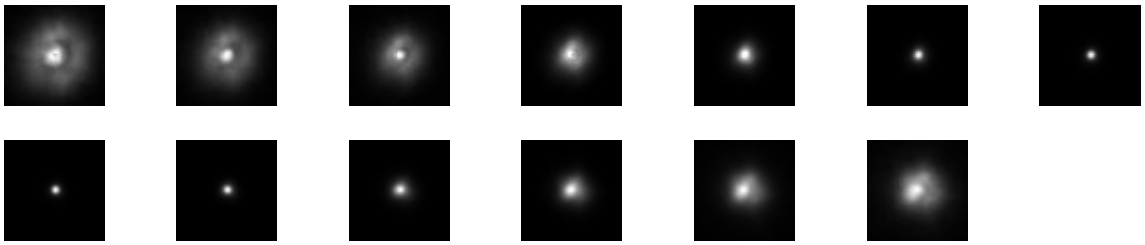
a. Taking the z-stack of a single fluorescent bead

The beads should be dried onto a coverslip of the same thickness as used later in the biological experiments, then covered by water or a mounting medium and finally glued onto a glass slide (e.g. using nail polish). Make sure that the beads are sparsely distributed, since it is required to record only a single one of them. According to our experience, good x-y-z dimensions of the stack are roughly 5 µm x 5 µm x 3 µm, with an axial spacing of around 200 nm.

It is important that the first image in the stack corresponds to the smallest distance between objective and bead and that this distance gradually increases for the following frames. This is important because the PSF it not mirror-symmetric with respect to the x-y plane, in particular if you are using astigmatic PSFs.

When taking the z-stack, it is further important to obtain a really good signal-to-noise ratio, as too much noise creates erroneous fits. Take especially care in frames where the bead appears out of focus. Since the fit algorithm normalizes every single frame of the z-stack, you are free to choose different camera exposure times for the individual frames. For instance you can choose short exposure times for images where the bead appears in focus and longer ones when it appears out of focus.

The table below contains a full exemplary stack (13 images ranging from z = -1.3 µm to z = +1.3 µm around the bead.



b. Detailed explanations of the app

Fig. 5.1 shows the panel of the app as it appears at startup.

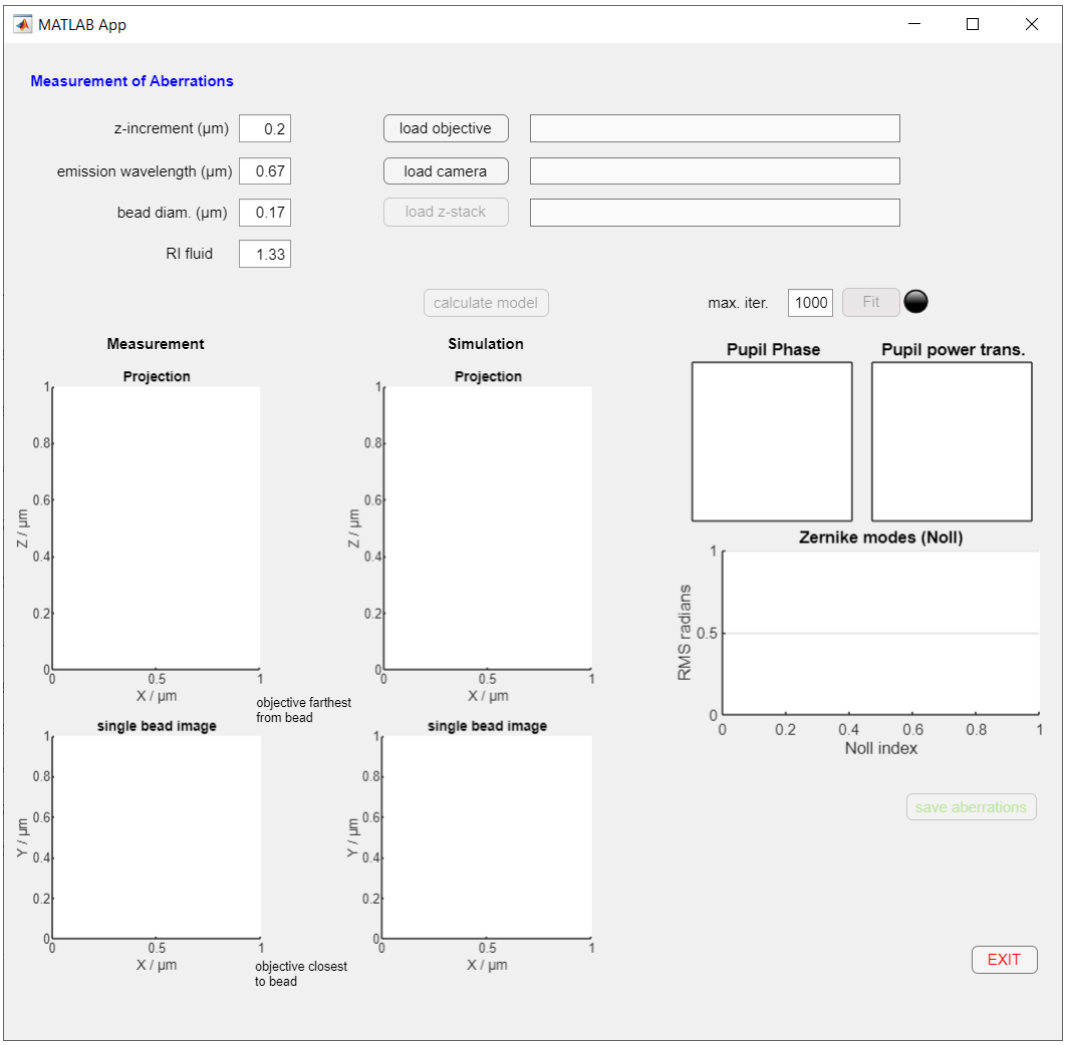


Fig. 5.1 The „Measurement of Aberrations" app.

z-increment (µm)	Axial interspacing between two successively taken frames of the z-stack
emission wavelength (µm)	Peak emission wavelength of the microbead. Note that this value can be different from the wavelength parameter set in the app "PSF_construction.mlapp" (i.e. the wavelength you are using in your actual experiments).
bead diam. (µm)	Diameter of the microbeads you are using. The diameter of your microbeads should be as small as possible. A diameter of $\lambda_{em}/4$ should be fine.
RI fluid	Refractive index of the solution your beads are embedded in (e.g. mounting medium or water). Note that this value can be different from the "RI sample" parameter in the app "PSF_construction.mlapp".
load objective	Load objective file. This field gets auto-filled if you call the app from "PSF_construction.mlapp" by clicking there on "fit aberrations".
load camera	Load camera file. This field gets auto-filled if you call the app from "PSF_construction.mlapp" by clicking there on "fit aberrations".
load z-stack	For importing the image z-stack of a single fluorescent bead. A frontal projection of your stack and x-y-sice will be shown in the two "Measurement" image panels on the left.
calculate model	Calculates the theoretical, aberration free PSF according to the parameters specified. Frontal projection and x-y slice of the theoretical PSF will be shown in the two "Simulation" images panels in the center.
max. iter	maximal allowed iteration number of the fit algorithm before it aborts.
Fit	Starts the fitting procedure. It typically takes around 1 minute. The fit uses a Levenberg-Marquardt algorithm that tries to minimize the sum of squared intensity differences between the experimental and simulated z-stacks by varying the phase

	and amplitude functions in the objective pupil. The results will be shown in the panels below the “Fit” button.
save aberrations	You can save the aberration file to be later loaded into the app “PSF_construction.mlapp” by clicking on the button “load aberration file” in the “PSF” tab.

6. EXAMPLES

The folder “`.\example data\synthetic testdata`” contains computer-generated image stacks of molecules, which you can use to practice the entire SMLM procedure. The folder further contains the PSF and objective mat-files that are compatible with these stacks.

You should be able to get a result similar as shown in Fig. 6.1 if you process the image stack “spiral_8fold_dz=10nm_R=100nm_s2000 _bg20.tif” The ground truth object consist of eight molecules aligned along a spiral with 100 nm radius in x-y. Each molecule is “on” in 10% of the frames (randomly chosen) and “off” in the others. The z-positions of the molecules range from z = 10 nm to z = 80 nm in steps of 10 nm. In this example, I applied an LLR filter of 80, which removed many fits on images where more than one molecule was in an “on” state.

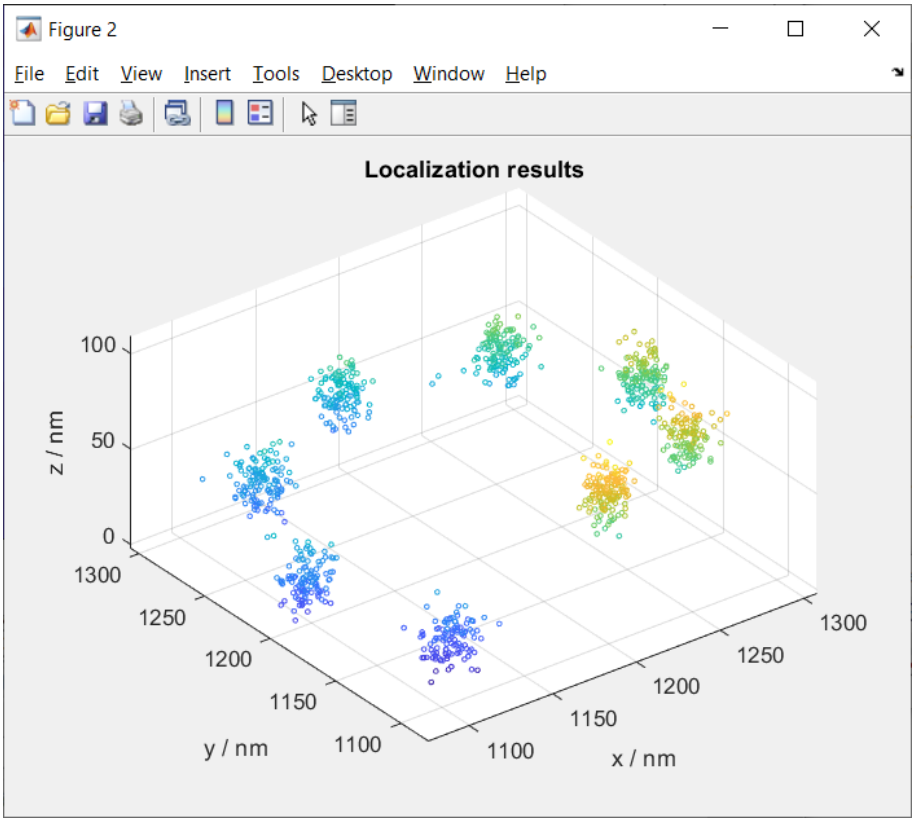


Fig. 6.1 Result of processing the synthetic testdata file „ TS_8fold_spiral_R=100nm_dz=10nm_s2000_bg20.tif”.

7. Acknowledgements

The following third-party software packages are used in our apps:

- ZernikeCalc.m
  Robert Gray,
  (2023). ZernikeCalc (https://www.mathworks.com/matlabcentral/fileexchange/33330-zernikecalc), MATLAB Central File Exchange. Retrieved April 26, 2023.

We would like to thank the authors for sharing their work.

8. References

1. Zelger, P., L. Bodner, L. Velas, G.J. Schütz, and A. Jesacher, *Defocused imaging exploits supercritical-angle fluorescence emission for precise axial single molecule localization microscopy.* Biomedical Optics Express, 2020. **11**(2): p. 775-790.
2. Zelger, P., L. Bodner, M. Offterdinger, L. Velas, G.J. Schütz, and A. Jesacher, *Three-dimensional single molecule localization close to the coverslip: a comparison of methods exploiting supercritical angle fluorescence.* Biomedical Optics Express, 2021. **12**(2): p. 802-822.