TrackIt manual

Release 1.6

written by
Timo Kuhn and Johannes Hettich

publication
Single molecule tracking and analysis framework including theory-predicted parameter settings. Sci Rep 11, 9465 (2021).

Gebhardt lab, University of Ulm
March 2024

Contents

1 General Information

1.1 Licence

1.2 Requirements

- Operating system: Windows 10 64-bit
- Matlab version 2017b and above
- For full functionality, the following Matlab toolboxes are required: Optimization, Image Processing, Statistics and Machine Learning, Parallel Computing
- You may need to manually install the "Visual C++ Redistributable for Visual Studio 2012 Update 4" which you can download from www.microsoft.com

1.3 Installation

1. TrackIt is available from `https://gitlab.com/GebhardtLab/TrackIt`
2. Extract
3. Open the TrackIt_v1_6.m file with Matlab
4. Run TrackIt by clicking the run button (F5) or from the command line

2 Workflow

2.1 Overview of the main GUI

TrackIt is a tracking and analysis pipeline for single-molecule fluorescence microscopy movies. The Software uses a unique batch data structure to handle and save all data created by the user. This can be stored in a .mat file and loaded into the software at any point of the workflow. Only one .mat file is needed per data-set which makes it easy to analyze and compare many sets of experiments.

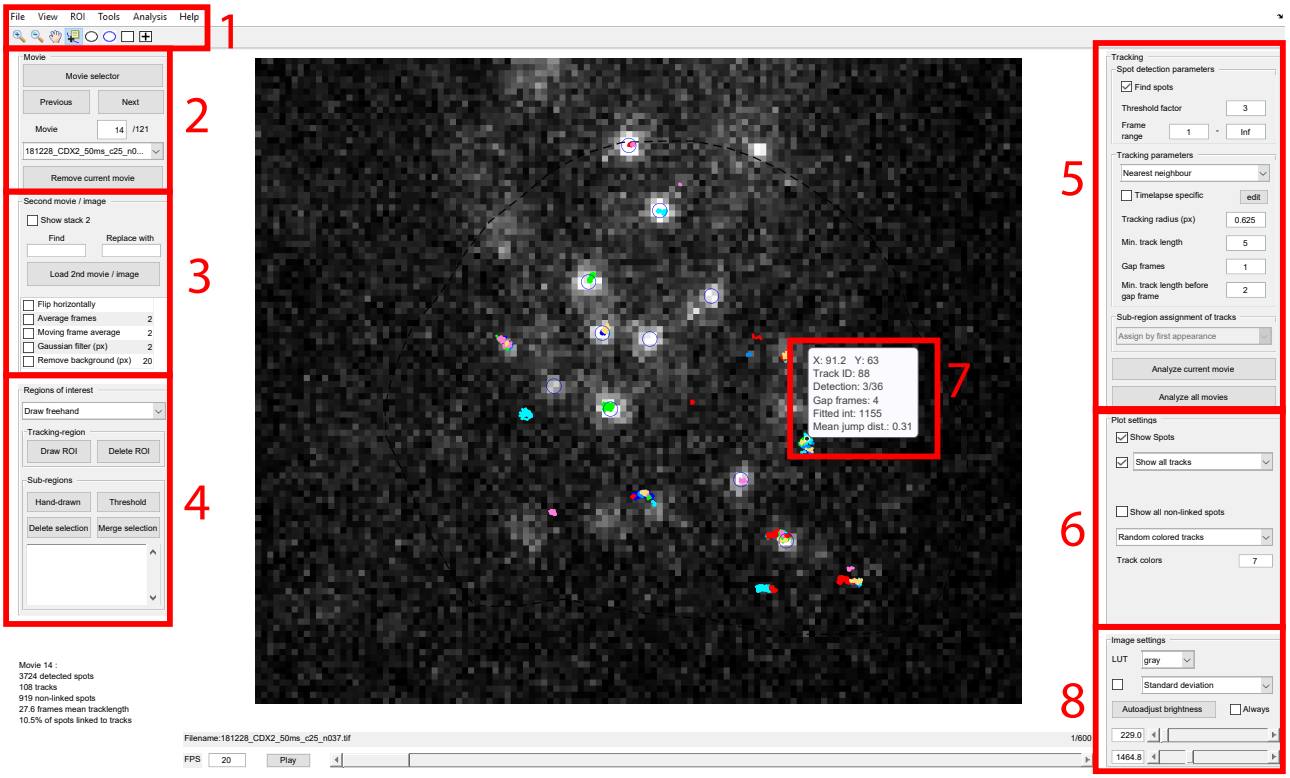


Figure 2.1: Screenshot of the main user interface. 1) menu- and toolbar 2) movie panel 3) second movie / image panel 4) region of interest (ROI) panel 5) tracking panel 6) plot properties panel 7) information on selected track 8) brightness adjustments and z-projections.

1. **Menu bar and toolbar** Saving and loading batch files, exporting data as well as several analysis tools can be accessed via the menu bar. The toolbar contains tools to zoom or move the plot area and the data-tip to select specific tracks. "Scale to ROI" and "Scale to subROI" tools on the right of the toolbar restrict the visible image section to the tracking region or the sub-regions, respectively.

2. **Movie panel** Add movies via the "Movie selector" button. Navigate through movies of the current batch with the "next" and "previous" button, by entering a movie number or by selecting a movie from the drop-down list. Single movies can be removed from the current batch with the "Remove current movie" button (the movie is not deleted from your folder). See section 2.2.

3. **Second movie/ image panel** Load reference channels carrying information about regions of interest (ROIs) such as the cell nucleus via the "Load 2nd movie / image" button. "Find" and "Replace with" help selecting the filename directly without the need of searching through file lists (can also be applied to all movies in a batch via the "Find stack 2 for all movies" function in the "Tools" menu bar). The reference movie or image can be manipulated with common filtering and averaging operations. See section 2.3.

4. **Region of interest panel** To restrict tracking to a specific region, e.g. cell outline, a tracking-region can be drawn in the "tracking-region" panel. Further region specific classification of tracks can be done by creating sub-regions. These can either be drawn by hand or through an intensity threshold in the sub-regions panel. Existing regions can be loaded from a .roi file which is created automatically every time a tracking-region or sub-region is drawn. See section 2.4.

5. **Tracking panel** All relevant parameters concerning spot detection and tracking can be set. By clicking "Analyze current movie" or "Analyze all movies" fluorescent molecules are tracked in the current movie or all movies, respectively. Tracking parameters can also be applied with respect to the corresponding frame-cycle-time of each movie by checking the "Timelase specific" box. See section 2.5.

6. **Plot properties panel** All plot related properties such as size, style and coloring of spots and tracks, the range in which tracks are shown and scalebar options are accessible here. Clicking the button "Plot selected track" opens a single track analysis window of the track selected with the "Data tip" in the toolbar. The "track explorer" includes kymographs, intensity, angle and position plot of the track and a mean squared displacement (MSD) analysis (see section 2.6).

7. **Information on selected track** Selecting a track with the "Data tip" in the toolbar shows basic track information such as track ID, current position, fitted intensity and mean jump distance.

8. **Brightness adjustments and z-projections** Settings concerning displayed pixels like the color lookup table (LUT) or brightness and contrast can be adjusted in the lower right corner. Additionally z-projections of the displayed movie or detection and jump distance mappings can be displayed.

2.2 Import movies using movie selector

Clicking the "Movie Selector" Button in the main GUI opens the "Movie selector" window.

Movies can be added in two ways:

1. **Add movies button** .tiff files can be selected directly.

2. **Search folder button** All .tiff files in a selected folder will be added. Optionally, a "Search string for folder scan" can be entered before clicking "Search folder for movies", so that only filenames containing the user specified string are added.

Automatic recognition of frame cycle times
The software is able to recognize the frame cycle times (the time between two consecutive frames) if the filename contains a string consisting of an underline followed by a number and either "ms" (milliseconds), "s" (seconds) or "Hz" (Hertz). For example "_50ms", "_1s" or "_50Hz" (see fig. 2.2). The files will then be grouped according to their frame cycle time. The number of files of each frame cycle time will be displayed together with the time in milliseconds.

Manually set frame cycle times
If no frame cycle time is recognized, the corresponding field will display -1. The time can be manually entered by clicking the frame cycle time field. Movies of different timelapse conditions have to be added separately while entering the timelapse condition for each case.

Set movie order
The user can choose on how the movies should be sorted:

1. Filename: Movies are sorted by their filename

2. Frame cycle time: Movies are sorted according to their frame cycle time (or timelapse condition)

3. String pattern: Movies are sorted by the part of the filename that follows a given string pattern (eg. "_n"), ignoring the part of the filename before the string pattern.

By clicking "OK" the movie selector closes and the first movie will be loaded and displayed in the main GUI. Now is a good time to save the workflow for the first time by clicking "File" » "Save batch file as".

In order to change the filenames of the movies stored in a batch afterwards without creating a new batch file, click "Tools" » "Change filenames in current batch". A user dialog will appear where the part of the filename that should be replaced or deleted can be entered in the "Search for" field. This part of the filename can be replaced with a different string which can be entered in the field "Replace with". This will not rename the original .tiff files but only the stored filenames in the batch file!

2.3 Load reference channel

The tracking channel can be overlaid with a second .tiff movie or image (e.g. brightfield image, DAPI or membrane marker). This can be useful for drawing a region of interest (ROI) or localization of single molecules with respect to cellular compartments.
A reference channel can be loaded by pressing "Load 2nd movie / image".

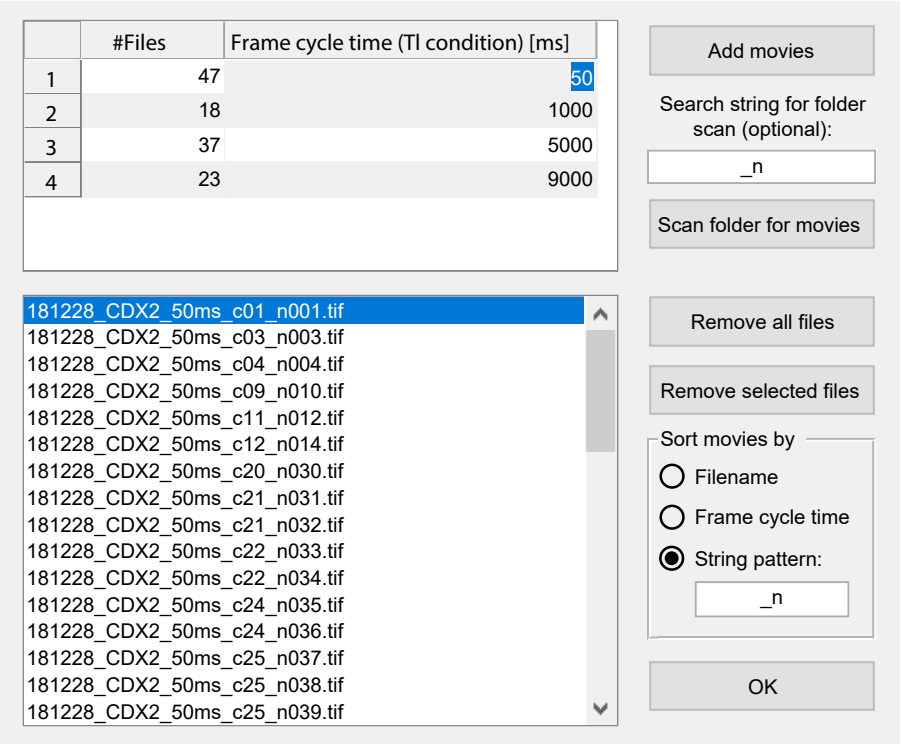


Figure 2.2: Screenshot of transcription factor CDX2 data-set displayed in the movie selector. The search string "_n" was used in combination with the search folder function to add all files in a folder containing that string. The software automatically recognized the frame cycle times (timelapse conditions) as given by the filename.

In order to ease a laborious search through lists of filenames, a replacement function is implemented. This can be used if the tracking movie and the reference channel share a similar filename for example "cell1_488nm_n001.tif" and "cell1_561nm_n001.tif" and are located in the same folder. The string to replace must be written in the field depicted with "Find", likewise the string which should be inserted must be written in the field depicted with "Replace with" (see fig. 2.4). When "Show stack 2" is checked the image/movie will directly be loaded or if "Load 2nd movie / image" is pressed the desired filename is directly inserted in the file selection dialog and can easily be opened by hitting "Open".
If you want to perform this for all movies in the current batch, press "Tools" » "Find stack 2 for all movies".

For the reference channel, following post-processing steps can be applied:

- Flip horizontally: Useful for two color-experiments where a second detection path is split-of by a beam-splitter.
- Average frames: The second movie is grouped and averaged in frame packages given by a user specified number. The amount of frames is reduced by a factor equal to this number
- Moving frame average: The second movie is averaged by calculating a moving average of the frames. Each frame is built by averaging over a user specified amount of frames before

and after each frame. The amount of frames therefore stays the same. The window size is automatically truncated at the beginning and the end of the movie.

- Gaussian filter: Uses Matlabs "imgaussfilt" function to apply a 2-D Gaussian image smoothing filter with a standard deviation (width of the filter kernel) specified by the user (in pixels).
- Remove Background: Subtracts a morphologically opened image from the original image. The radius of the disk element used for the opening operation can be specified by the user (in pixels).

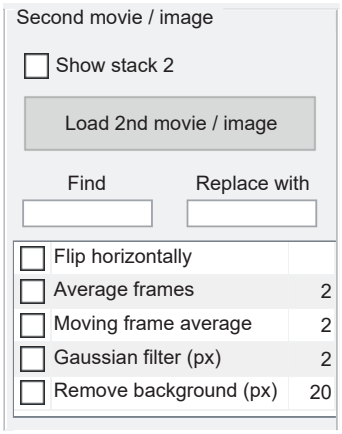


Figure 2.3: Screenshot of the reference channel panel.

2.4 Regions of interest

In TrackIt regions can be drawn either to restrict tracking to a specific region (tracking-region) or to assign tracks to multiple compartments (sub-regions). Four drawing functions are implemented to draw the tracking-region and sub-regions by hand: "Draw freehand", "Assisted freehand", "Draw polygon", "Draw ellipse". Sub-regions can additionally be drawn using an intensity threshold.

TrackIt automatically saves a .roi file in a subfolder called "ROI" located in the same folder as the original movie every time a region is added or deleted. Whenever movies are selected via the "Movie selector", the software will check whether a .roi file exists for this file and will load it automatically. The regions saved in a .roi file can also be loaded manually for a single movie by clicking "Load ROI from .roi file" or for all movies by clicking "Tools" » "Reload all ROIs from ROI folder".

To remove a specific region (tracking-region or sub-region) from all movies of a batch select "Tools" » "Remove ROI from all movies".

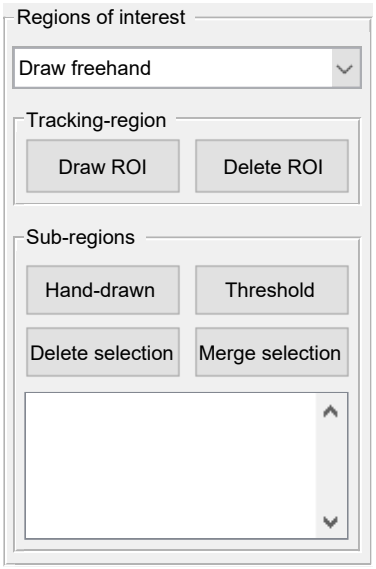


Figure 2.4: Screenshot of the ROI panel.

2.4.1 Tracking-region

If desired, a tracking-region can be drawn by pressing the button "Draw ROI" or deleted with the "Delete ROI" button located in the tracking-region panel. Spot detection will then be restricted to this specific area. If no ROI is drawn, the whole image will be used for detection and tracking.

2.4.2 Sub-regions

Sub-regions can either be drawn by hand or using an intensity threshold on the reference channel by clicking "Hand drawn" or "Threshold" in the sub-regions panel, respectively. Existing sub-regions are shown in a list-box in the lower part of the sub-regions panel. Selected regions in

the list box can be merged or deleted by clicking the "Delete selection" or "Merge selection" buttons, respectively. The assignment of tracks to sub-regions is performed subsequent to tracking which is why changes to sub-regions or the assignment option are only visible after tracking is performed.

Drawing sub-regions using an intensity threshold
When clicking "Threshold" a small window appears where the intensity threshold and the minimum area size of a region can be selected via a slider or entered directly. The "Fill holes" checkbox can be enabled to also consider the pixels below the intensity threshold that are surrounded by pixels above the threshold as a part of the same region.
The intensity threshold is applied on the images in the reference channel (stack 2) where all pixels above the threshold are considered to be part of the sub-region (see figure 2.5). The sub-region is drawn for each of the frames of the reference channel separately. It is recommended to enable the "Gaussian filter" with a kernel size of at least 1 pixel to smoothen the region borders.

Assignment of tracks to regions
There are four options to choose from on how the assignment of spots and tracks to sub-regions should be conducted:

- Assign by first appearance: The position of the first spot in a track determines the region to which it is assigned, regardless where the rest of the track is located.
- Split tracks at borders: Tracks crossing the border from one region to another (ie. appearing in several different regions) are split into multiple tracks so that each of the track fragments is located completely inside of a specific region.
- Delete tracks crossing borders: Tracks that cross the border from one region to another are discarded. The spots making up discarded tracks are considered as non-linked spots.
- Only use tracks crossing borders: Tracks that lie completely inside a region are discarded. The spots making up discarded tracks are considered as non-linked spots. Only tracks that cross region borders are kept.

2.5 Tracking

Before tracking is started, several parameters can be adjusted in the "Tracking" panel (see fig. 2.6).

2.5.1 Spot detection and tracking parameters

Threshold factor A user defined threshold factor is used to calculate an automatic threshold for spot detection. The threshold is calculated for each movie separately providing comparable detection thresholds between all movies. Reliable detection of single fluorophores is commonly achieved with values between 1 and 5. Movies with a good single-molecule signal (i.e. high signal-to-noise ratio) can usually be analyzed with higher threshold factors.

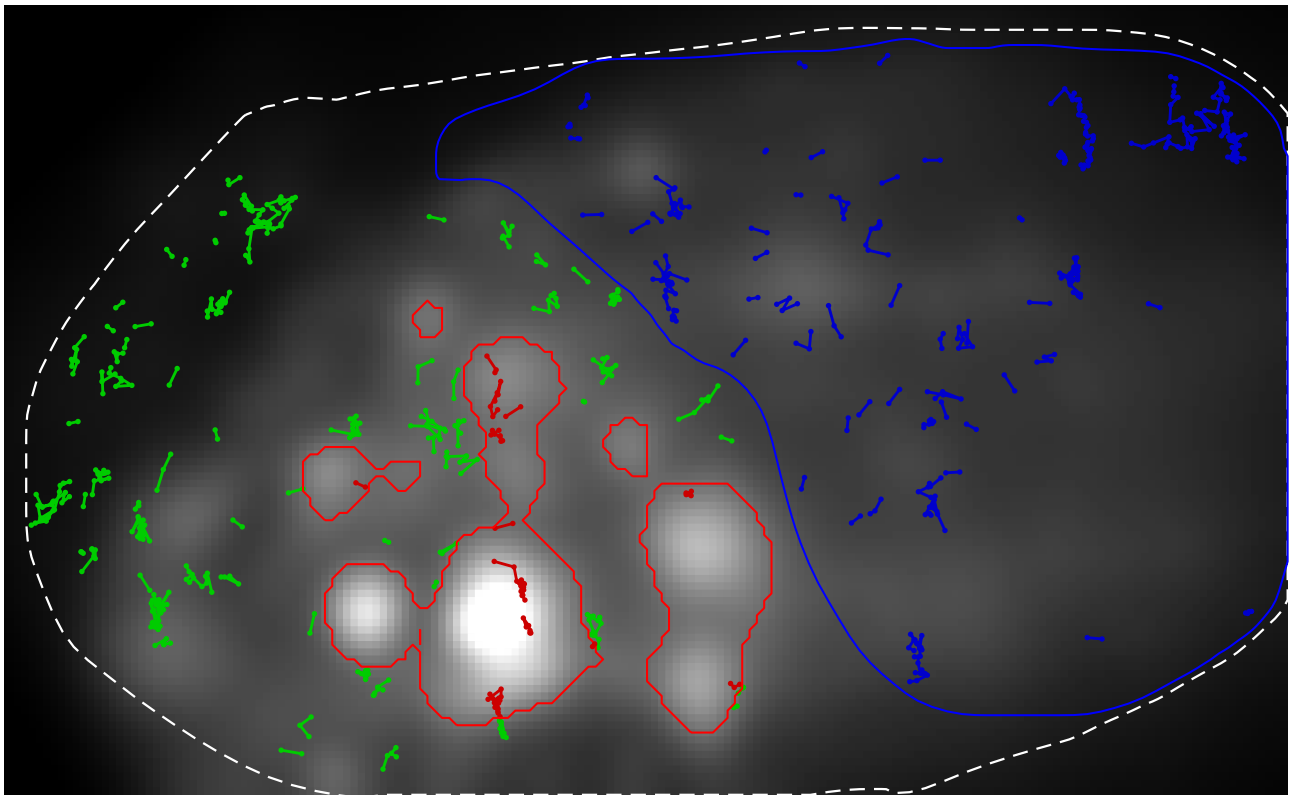


Figure 2.5: Example of a tracked single molecule movie with different regions. The tracking-region (dashed white line) restricts the spot detection and tracking to this region. The blue sub-region is hand drawn and the tracks assigned to this region are plotted in the same color. The sub-region depicted in red is created via an image threshold that was conducted on the reference channel (stack 2) showing the Gaussian filtered signal of a Hoechst-stained nucleus. The tracks assigned to this region are plotted in the same color. Tracks crossing sub-region borders are splitted because the option "Split tracks at borders" was selected.

Frame range Specifies the frames between which the spots should be detected and tracked

Tracking algorithm Ideally the concentration of fluorescent molecules is low enough so that each spot is spatially well separated from others at all times. In this case the nearest-neighbor delivers fast and reliable tracking results. For higher concentrations the u-Track algorithm might provide better results. For spot density considerations see section 2.7.6, Avg. #spots per frame.

Tracking radius Maximum distance at which spots are linked between two consecutive time points. The tracking radius can also be entered in microns by right clicking onto the tracking radius field and selecting "convert from µm". In case of several different frame cycle times as used eg. in time-lapse experiments, consistent tracking radii can be predicted automatically, see 2.5.3.

Min. track length Minimum number of frames a fluorescent molecule has to persist to be accepted as a track.

Gap frames Number of frames a fluorescent molecule can disappear or stay undetected so that tracking is still continued and the fluorescent molecule is combined into a single track.

Min. track length before gap frame Number of frames a track has to exist before closing of gaps (gap frames) is allowed. This can help to prevent connecting random detections in overly crowded movies or movies with bad signal-to-noise ratio (SNR).

Timelapse specific tracking parameters Increasing movement of cellular compartments (eg. chromatin diffusion) with longer frame cycle times, makes it necessary to chose less restrictive tracking parameters. Therefore, above described tracking parameters can be set dependent on their frame cycle time (timelapse condition) (see fig. 2.7).

Sub-region assignment of tracks Choose how the assignmenet of spots and tracks to sub-regions should be conducted (only applicable if sub-regions are drawn). See chapter 2.4.2.

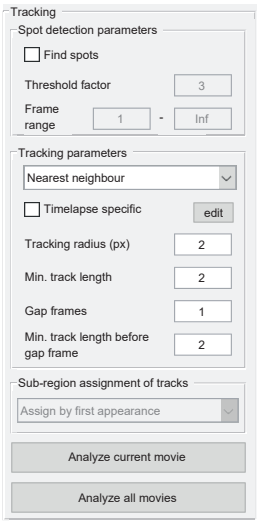
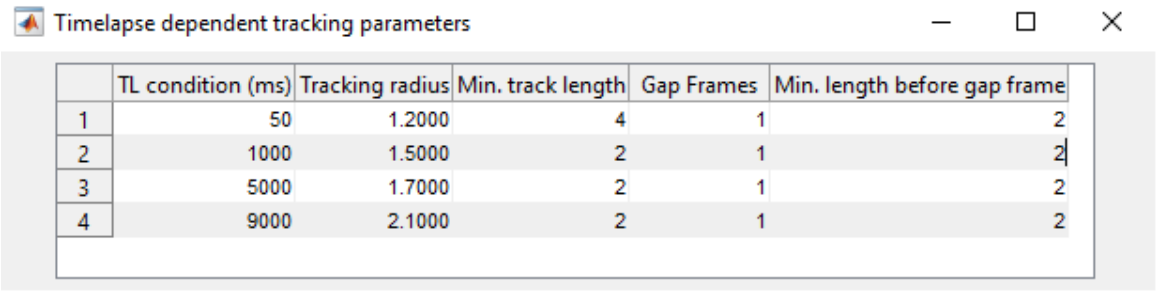


Figure 2.6: Screenshot spot detection and tracking parameter panel.



	TL condition (ms)	Tracking radius	Min. track length	Gap Frames	Min. length before gap frame
1	50	1.2000	4	1	2
2	1000	1.5000	2	1	2
3	5000	1.7000	2	1	2
4	9000	2.1000	2	1	2

Figure 2.7: Screenshot of the timelapse specific tracking parameters window where a different set of tracking parameters is used for each of the four timelapse conditions.

2.5.2 Tracking routine

By clicking either "Analyze current movie" or "Analyze all movies" tracking is performed with the predefined parameters. The tracking routine consists of four steps:

1. **Filtering raw data** Movies are filtered using a wavelet filter as described in [7].
2. **Detecting candidates** First a threshold is applied to the wavelet filtered image to separate signal from background noise. The threshold is calculated by multiplying a user defined threshold factor with the standard deviation of the background noise. Candidate detection is based on local maxima finding at the pixel level. In brief, all pixels that have the same value before and after image dilation are selected.
3. **Position refinement** Candidate positions are refined to a sub-pixel precision by fitting a Gaussian function using the freely available psfFit_Image.m from the TrackNTrace software as described in [15]. We included a maximum distance allowed for spot refinement through gaussian fitting. If the distance between candidate and refined position is larger than 2 pixels, the fit is discarded and the position is refined using the center of mass inside a window of 5 pixels centered at the candidate position. This usually happens if two detections are too close together for correctly fitting a single peak. For spots laying closer together than 2 pixels, the smaller peak is discarded after refinement in order to avoid multiple detections per spot.
4. **Tracking** Linking spots in time and space can be carried out using either a fast nearest-neighbor algorithm or the more sophisticated u-Track algorithm of [9].

2.5.3 Automatic determination of tracking parameters

Click "Tools" » "Predict tracking radii" to open a separate window for tracking radii prediction. The table gives an overview of all parameters. Before running the program, choose wheter gap-frames shall be allowed (=1) or not (=0). Furthermore the shortest track length can be adapted. Continue by clicking "execute". Fill in the inverse of the trageted track length, i.e. for a targeted mean tracklength of 100, fill in 1e-2. Once the program is done, the table will be updated with the new tracking radii.

2.6 Visualization of tracks

2.6.1 Plotting options

Show spots Shows all detected spots in the current frame

Plotting tracks

- Show tracks in Range: Tracks are visible up to the displayed frame number. The parameter "#frames track is visible" defines how long tracks are plotted after the end of the track. A value of 0 will only show tracks visible in the current frame, for "inf" all tracks are plotted until the current frame.

- Show all Tracks: All tracks of the current movie are plotted
- Show initial positions: Only the position of first appearance of a track will be plotted
- Show all tracked positions: show only the positions of tracked molecules without connecting them with lines.

Show non-linked spots All positions of non-linked detections are marked with yellow dots.

Coloring of tracks

- Random colored: The track color for each track is chosen randomly and the amount of colors can be entered in the field "#track-colors".
- Colored by track length: Matlabs "parula" color map is used to plot tracks with a color corresponding to their length. Shortest tracks are colored blue while longest tracks are colored in yellow.
- Colored by track length regime: The track colors are chosen according to user defined track length classes. Tracks with a minimum duration of the value entered in the field *min. length to count as long track* are plotted green whereas shorter tracks are shown in red.
- Colored by mean jump distance: Matlabs "parula" color map is used to plot tracks with a color corresponding to their mean jump distance. Tracks with the lowest mean jump distance are colored blue while tracks with the highest mean jump distance are colored in yellow.
- Colored by sub-region: Tracks are plotted in the same color as the sub-region to which they are assigned. tracks that are not assigned to any sub-region are plotted in green.

Advanced plot properties Clicking the "Advanced" button opens a window where further adjustments can be made regarding the appearance of the plotted tracks and spots like the line width and size of the tracks or the style, size and color of the spot marker.

Scalebar Clicking the "Scale bar" button opens a window where the position, size, text and font size of the scale bar can be entered.

2.6.2 Detection mapping and jump distance mapping

Detected spots fit to sub-pixel precision with a 2D Gaussian fit are used to create a super-resolved heat map of localizations. Here, fitted positions of spot are accumulated in a 2D histogram. The pixel values therefore correspond to the amount of detections in each pixel (see figure 2.8 c). Detection maps can be created either using all spots (including non-linked spots) by selecting "Detection map incl. non-linked" or using only the spots that have been linked into tracks by selecting "Detection map w/o non-linked.

Similar to localization mapping, a heat map of jump distances can be created. For all jumps within a track, a virtual line is drawn between the start and end position of a jump. Each pixel touching this line is assigned with the corresponding jump distance (see fig. 2.8 b). The resulting 2D histogram is then normalized by the amount of events in each pixel to obtain the average jump distance in each pixel.

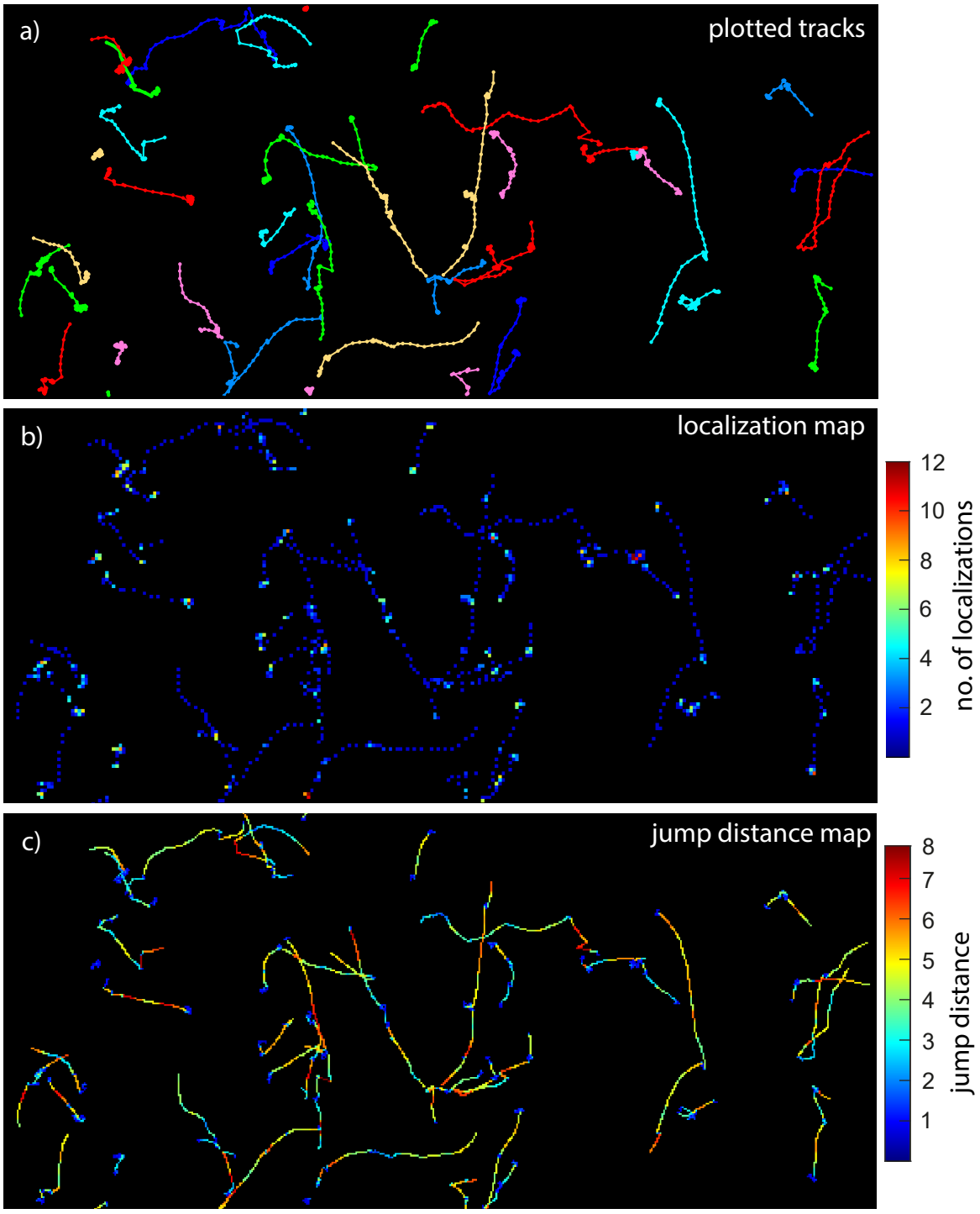


Figure 2.8: a) Plotted tracks of particles from a simulated single molecule movie [1] exhibiting a mixture of confined and linear motion. b) Localization map of detections showing blue pixels where a low number of molecules are localized whereas higher number of localizations per pixels are shown in green, yellow and red. c) Jump distance map of the track particles where blue pixels indicate regions of confined motion whereas pixels which are passed with high jump distances are colored in green, yellow and red.

For both kinds of heat maps the bin size can be chosen as a multiple of the original pixel size resulting in an up –or down-scaled image and can be entered in the field "Scaling factor".

Both detection maps and jump distance map can be accessed in the lower right of the main TrackIt GUI via the pop-up menu where also z-projections like standard deviation, average or maximum intensity can be displayed.

2.6.3 Track explorer

Single tracks can be selected via the *Data Tips* function located in the toolbar of the main GUI. A click on "Plot selected track" in the main GUI opens the track explorer showing a visualization and analysis of the selected track. Alternatively the track explorer can be opened via "Tools" » "Track explorer". The results can either be displayed in units of pixels and frames (see fig. 2.9) or microns and seconds (see fig. 2.10) by selecting the corresponding entry in the "Units" panel. Switching between different tracks can be directly done by clicking the "Previous" or "Next" button or by entering a number in the "TrackID" field.

Position plot The selected track is plotted in the upper left corner of the figure. The background image of the track can be selected in the "Track background" panel and can be switched between showing a cutout of the original movie frame or to be dark. The track segments can be colored in four different styles which can be set in the "Track color" panel:

- Uniform: track is uniformly colored in green (see fig. 2.10).
- Time: Matlabs "parula" color map is used to plot track segments with a color corresponding to their time of persistence. The first segments of the track are colored in blue and with increasing time the segments are plotted from light blue over green to yellow.
- Intensity: Matlabs "parula" color map is used to plot track segments with a color corresponding to the intensity of the detected spot. The detection with lowest intensity is colored blue whereas the detection with highest intensity is colored yellow.
- Jump distance: Matlabs "parula" color map is used to plot track segments with a color corresponding to the jump distances between the spots of the track in subsequent frames (see fig. 2.9). The minimum jump distance between two subsequent frames is colored blue and with increasing distances the segments are colored from light blue over green to yellow.

Kymographs Horizontal (XT, y-axis projected) and vertical kymographs (YT, X-axis projected) of the track are shown in the bottom left corner of the figure. Here only one dimension is plotted at each timepoint while the other dimension is maximum projected. There are two types of projection windows which can be chosen in the panel "Kymograph projection window":

- Propagating with spot center: The maximum projection is performed within a window of 13 pixels around the spot center. The projection window therefore "travels" with the spot center leading to a kymograph where the spot position is always lying on a horizontal line in the center of the kymograph image.

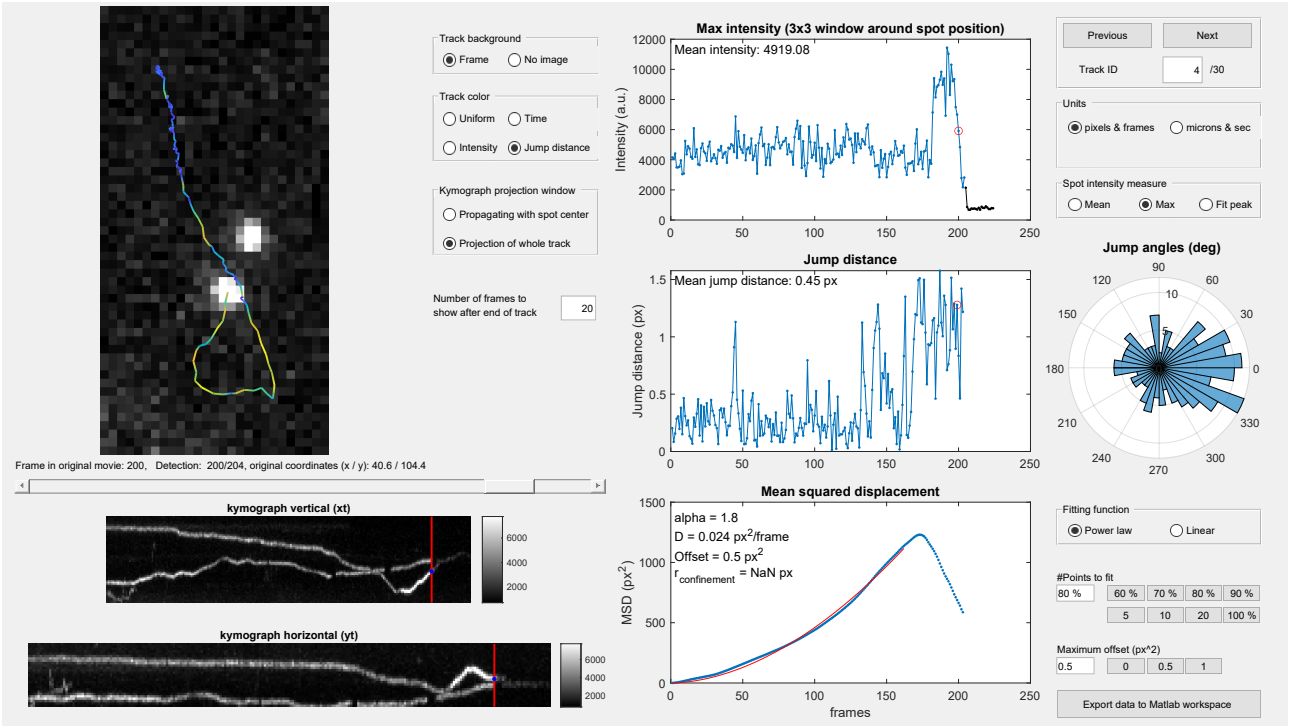


Figure 2.9: Track explorer showing a track of a moving nuclear Myosin VI molecule [3]. The coloring of the track indicates the jump distance from frame to frame which can also be seen in the jump distance plot. Vertical and horizontal kymographs show the movement of the molecule as a projection of one of the spatial dimensions over time. The currently viewed frame in the track plot can be selected with a slider and is indicated in the kymograph as a red bar. A blue dot inside the red bar further indicates the position of the track in the kymographs. Additional plots show the spot intensity and jump distance over time, the mean squared displacement including power law fit and a jump angle histogram. The abundant population of angles between $330° - 30°$ in the angle histogram and an alpha value of 1.8 are characteristic for a linear motion.

- Projection of whole track: The maximum projection is performed within the window seen in the position plot on the top left corner. Here the window is kept static so that a movement of the spot results in a movement in the kymograph image.

Kymographs can be continued for additional frames after the track ended by entering a positive number in the field "Number of frames to show after end of track". If "Propagation with spot center" is selected the kymograph projection window is kept at the position of the last spot in the track.

Intensity plot Shows the intensity of the detected spot for each frame of the track. The intensity measure can be chosen in the panel "Spot intensity measure" with the following options:

- Mean: Shows the mean intensity inside a 3x3 pixel window around the spot center for each frame of the track.
- Max: Shows the maximum intensity inside a 3x3 pixel window around the spot center for each frame of the track.

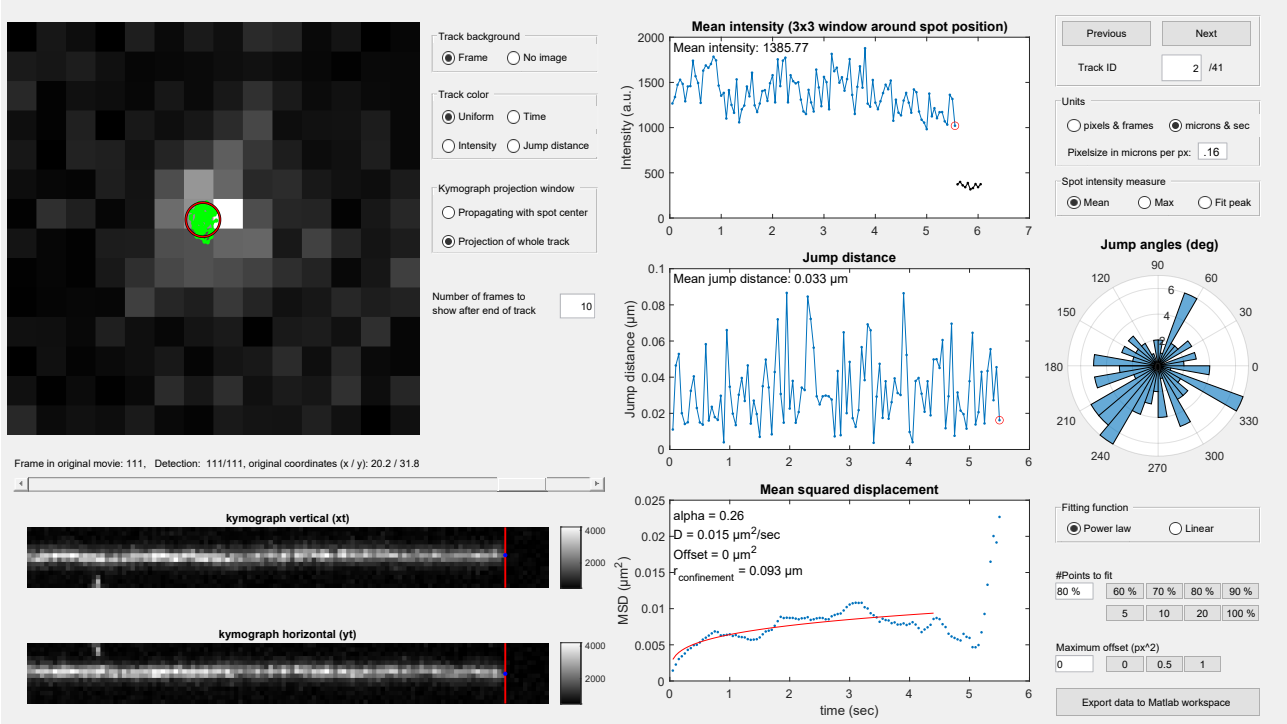


Figure 2.10: Track explorer showing a track of a binding CDX2 transcription factor molecule. In addition to the track, a red circle is plotted with its center at the mean track position and a radius equal to the confinement radius. The confinement radius is calculated by fitting the mean MSD with a confined diffusion model. An angular histogram mostly populated with values between $120° - 240°$ and an alpha value of 0.26 are characteristic for the confined nature of the molecule.

- Fit peak: Shows the peak amplitude of the fitted 2D gaussian function in each frame of the track.

If a number > 0 is entered in the field "Number of frames to show after end of track" the plot is continued after the track ended (drawn in black). In this case the window in which the intensity is determined is held constant at the position of the last spot of the track and is applicable only for the mean and maximum intensity measure. This can be used to check whether the fluorescent molecule has bleached or if the track was lost during tracking process.

Jump distance plot Shows the distances between spots of subsequent frames which were connected to a track.

Angular histogram Displays a polar histogram of jump angles where the jump angle is defined as the change in direction between each step of a track. See also section 2.7.7.

Mean squared displacement plot Shows the mean squared displacement (MSD) of the track. The MSD can either be fitted with a power law or a linear function. The fitted function is plotted on top of the MSD and the fitted parameters are shown in the top left corner of the plot. If a power law is chosen as fit function, an additional fit is used to calculate the confinement radius $r_{\text{confinement}}$ with a confined diffusion model. If the fit was successful, the

resulting confinement radius is written below the other fit results and a circle with radius $r = r_{\text{confinement}}$ centered at the mean track position is drawn into the position plot (see fig. 2.10). If a confinement radius could not be determined the result shows "NaN". For more information regarding the MSD analysis see section 2.7.7.

Export to Matlab workspace By clicking this button all results visible in the track explorer are exported to the Matlab workspace.

2.7 Data analysis tool

The data analysis tool can be accessed via "Analysis" » "Tracking data analysis.

2.7.1 Overview

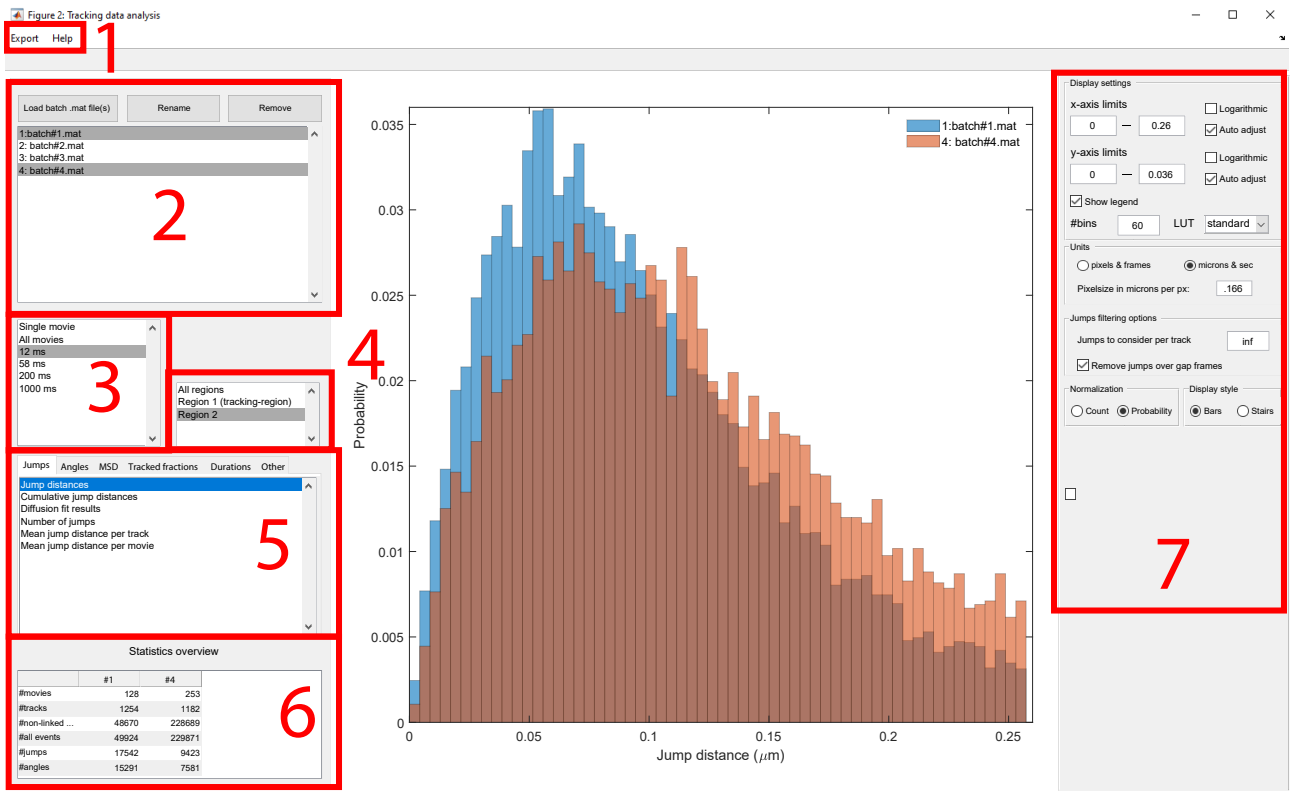


Figure 2.11: Screenshot of the track analysis tool. 1) Export data to Matlab workspace and open manual. 2) Add, select, rename and delete batch files 3) Frame-cycle-time selection 4) Sub-region selection 5) Analysis parameter selection window 6) Statistics overview of loaded batches 7) Plotting and analysis options.

1. Exports two variables to the Matlab workspace: "currentPlotValues" contains the data which is shown in the current plot, "allHistogramResults" contains all the raw data that is used to display the different parameters implemented in the histogram tool.

2. Batches analyzed and saved with the TrackIt software can be loaded into the data analysis tool via the button "Load batch .mat file(s)". The window below shows a list of loaded batch files where batches to be included in the analysis can be selected. Multiple batches can be selected using the "Ctrl" key.

3. The frame-cycle time selection window in the central left lists all frame-cycle times that are involved in the selected batches and can be used to select subsets of movies. Either a single movie, or a certain frame-cycle time or all movies can be selected to be included into the analysis.

4. The sub-region selection window contains a list of all sub-regions that exist in the selected batches. The list is only visible if sub-regions exist.

5. The parameter selection window lists all the analysis parameters which are grouped into three tabs: mobility, tracked fractions and statistics. The selected parameter is then displayed in the central plot.

6. The statistics overview windows gives information on movie numbers and molecule counts for each loaded batch.

7. Shows a number of plotting options and settings regarding calculations of the different parameters:
    - #bins: Amount of bins in a histogram.
    - LUT: The lookup table can be switched between the Matlab colormaps lines(standard), winter, parula, jet, copper, gray.
    - Jumps to consider: The maximum number of jumps that are considered in each track. This can be useful to prevent an over representation of slow diffusing or bound molecules in the histogram.
    - Normalization: Histogram normalization can be switched between "Count" and "Probability". Bin heights are either given by the number of events in each bin or normalized so that the height of all bins sums up to 1, respectively.
    - Display style: Display histogram either as filled bars or as stairs.
    - Remove jumps over gap frames: Angles and jump distances extracted from jumps involving gap frame(s) are not considered if the checkbox is checked.
    - Units: Can be switched between "pixels & frames" and "microns & sec". If "pixels & frames" is selected, all results concerning length scales are given in the unit of pixels and all results concerning temporal units are in the unit of frames. If "microns & sec" is selected, a conversion factor for the size of one pixel in μm must be entered. All results are then displayed in micrometers using this conversion factor and seconds using the frame-cycle time stored for each movie (displayed in the frame-cycle time window).
    - Normalize by ROI-size: Most of the statistical evaluations can be normalized by the size of the ROI in each movie in order to give information on densities (eg. spot densities).

- Illumination pattern: Can be switched between "periodic" and "ITM" (interlaced time-lapse microscopy). ITM is an illumination scheme where two subsequent image acquisitions are followed by a dark time. This scheme is specifically designed to gather quantitative information on chromatin-bound fractions and proportions of stable bound molecules [14]. Due to a non-periodic image acquisition, the illumination scheme has to be taken into account when calculating the number of dark periods that an immobile molecule survived (see Section 2.7.5).
- x-axis: Whenever a parameters is selected that represents movie-wise results (eg. number of tracks, average track length, ROI size etc.), the results of each movie can either be grouped by their batch file number by selecting "batch file" or plotted versus a variety of other movie-wise values on the x-axis (eg. movie number, average track length etc.).

2.7.2 Jumps analyis tab

Jump distances Displays the distribution of jump distances in a histogram. The jump distance is defined as the Euclidean distance between the positions of two linked spots of a track in consecutive frames.

Show diffusion fit curves To visualize diffusion analysis results, the probability distribution p(r), as obtained from fitting the cumulative jump distance distribution, can be plotted together with the histogram of jump distances. The bin wdith for the cumulative jump distance distribution has to be set separately in the "fit settings" panel on the lower right.

In the case of the three-rate model, the plotted distribution is:

$$p(r) = \frac{1}{2\tau} r \cdot \Delta r \left(\frac{A_1}{D_1} \exp\left(-\frac{r^2}{4\tau D_1} \right) + \frac{A_2}{D_2} \exp\left(-\frac{r^2}{4\tau D_2} \right) \right. \tag{2.1}$$

$$\left. + \frac{A_3}{D_3} \exp\left(-\frac{r^2}{4\tau D_3} \right) / 1 - \exp\left(-\frac{r_{tr}^2}{4\tau D_3} \right) \right) \tag{2.2}$$

where r is the jump distance, Δr is the bin width of the jump-distance histogram (tracking radius r_{tr} divided by the number of bins), τ is the frame cycle time and D_i and A_i are the diffusion coefficients and fractions resulting from the cumulative jump distance distribution fit. The last term is normalized to account for the cut-off due to the lower and upper limit of jump distances.

Cumulative jump distances Displays the cumulative distribution of jump distances. This distribution can be used to fit either a 1-rate, 2-rate or 3-rate diffusion model (see below). The fitted diffusion functions can be visualized by checking the "Show diffusion fit curves" box.

Diffusion parameters Cumulative distributions of squared jump distances are fitted with one, two or three exponential components corresponding to one, two or three effective diffusion coefficients D_{1-2} or D_{1-3} with amplitudes A_{1-2} or A_{1-3}, respectively [2]. It is important to set a reasonable bin width in the "Bin width" field (we recommend to use around 1 nm per bin).

If the **one-rate model** is selected in the pop-up menu, the following fit function is applied

$$f_1(X) = \left(1 - e^{-\frac{X}{D_1}}\right) / \left(e^{-\frac{C_1}{D_1}} - e^{-\frac{C_2}{D_1}}\right) \tag{2.3}$$

If the **two-rate model** is selected in the pop-up menu, the following fit function is applied

$$f_2(X) = A_1 \left(1 - e^{-\frac{X}{D_1}}\right) + (1 - A_1) \left(e^{-\frac{C_1}{D_2}} - e^{-\frac{X}{D_2}}\right) / \left(e^{-\frac{C_1}{D_2}} - e^{-\frac{C_2}{D_2}}\right) \tag{2.4}$$

If the **three-rate model** is selected in the pop-up menu, the applied fit function is

$$\begin{aligned} f_3(X) =& A_1 \left(1 - e^{-\frac{X}{D_1}}\right) + A_2 \left(1 - e^{-\frac{X}{D_2}}\right) \\ &+ (1 - A_1 - A_2) \left(e^{-\frac{C_1}{D_3}} - e^{-\frac{X}{D_3}}\right) / \left(e^{-\frac{C_1}{D_3}} - e^{-\frac{C_2}{D_3}}\right) \end{aligned} \tag{2.5}$$

where $X = (x^2 + y^2)/(4\tau)$ with the camera frame cycle time τ. Functions are normalized to account for the lower and upper limit of jump distances $C_1 = 0$ and $C_2 = r_{max}$ where r_{max} is the tracking radius. To ensure that the fit converges to a global minimum, diffusion constant start values can be entered on the right side of the figure. For a visualization and control of the fitted function see *cumulative jump distances* above.

Effective D The effective are average diffusion coefficient, here defined as $D_{eff} = \sum_i D_i \cdot A_i$, can be used as a measure of the overall mobility of the tracked molecules.

Displayed value and error:

Pooled data, 95% confidence interval Uses the cumulative jump distance distribution of all movies of a batch and displays the 95% confidence interval of the fit as an error.

Mean & stand. dev. of movie-wise values The jump distance distribution of each movie is fitted individually. The average value over all movies are shown and the error bar indicates the standard deviation of the movie-wise values.

Mean & stand. dev. of resampling values In order to get an idea of the robustness of your data and for further error estimation a resampling can be performed. A specified percentage of jumps from the pooled distribution is then drawn n-times and each randomly drawn distribution is fitted individually. The average value over all resamplings are shown and the error bar indicates the standard deviation of the resampling values.

Important: fitting distributions of jump distances from movies with mixed frame-cycle times or tracking radii will lead to wrong results!

Number of jumps

No. of jumps Shows the total number of jumps (track segments) in each movie. It is also the number of jumps that goes into diffusion analysis and changes if the number of jumps to consider is changed. The total number of jumps in each batch is shown in the statistics overview in the row #jumps.

Mean jump distance per track Shows a histogram of the mean jump distances of all tracks. The mean jump distance $\bar{d}$ of a track is defined as:

$$\bar{d} = \frac{1}{n} \sum_{i=1}^{n} d_i \tag{2.6}$$

where n is the number of jumps in a track and d_i are the jump distances of the track.

Mean jump distance per movie Shows the movie-wise average jump distance.

2.7.3 Angles analyis tab

Jump angles Displays a polar histogram of jump angles which are defined as the change in direction between each step of a track. The jump angle histogram can give additional information about diffusion properties or directional behavior. A circular polar histogram may indicate a random, brownian motion. A polar histogram where bins are concentrated around 180° indicates either a confined diffusion or a bound state (where the apparent jump distance due to a localization imprecision is higher than the fluorescent molecule movement). Tracks with its polar histogram centered near 0° tend to have only small directional changes and may indicate a directed motion. See also [8] and [**?**].

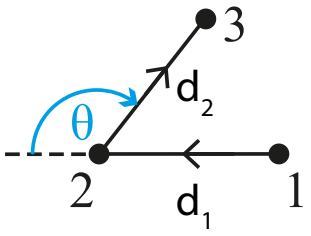


Figure 2.12: Definition of the jump angle between two consecutive jumps, i.e. three consecutive localizations 1-3. An angle of 0° indicates no change, i.e. a direct forward movement of the molecule, an angle of 180° indicates that a molecule moved backward in the opposite direction. Angles <180° or >180° indicate a movement to the right or left, respectively. The average of the jump distances d_1 and d_2 of the jumps making up the angle is used in the Jump angle anisotropy vs. mean jump distance analysis tool.

Jumps making up the angle In certain cases it might be beneficial to just consider angles where both jump distances between the three localizations that make up the angle should have a maximum or minimum length. This can be specified in the "Jumps making up the angle" panel. One possible application could be to make sure that the displacements making up the angle are be larger than the localization error.

Jump angle anisotropy Shows the degree of reverse or forward motion as calculating by the 'fold anisotropy' metric, $f_{180/0}$, which measures how many-fold more likely a backward jump is, compared to a jump forward, using:

$$f(180/0) = \frac{BWD}{FWD} = P\left(\frac{180° \pm 30°}{0° \pm 30°}\right) \tag{2.7}$$

where BWD is the probability for a backward jump with an angle Θ of 150°-210° and FWD is the probability for a forward jump with an angle Θ of 330°-30°. Noteworthy references for this kind of analysis are [4] and [11] which both use the jump angle anisotropy and jump angle anisotropy vs. mean jump distance analysis. It might be useful to first separate bound and mobile track segments and only analyse the anisotropy on mobile track segments. This can be done in the main TrackIt UI: Menu -> Tools -> Classify bound and free segments (vbSPT), see chapter 2.10.1.

Jump angle anisotropy vs. mean jump distance Shows the anisotropy of jump angles in dependence of the average jump distances of the jumps making up the angle $\bar{d} = (d_1 + d_2)/2$ (see figure 2.12). For the analysis, the mean displacements and their corresponding angles are separated in to histogram bins and the anisotropy is calculated for each bin. The bin number can be set #bins field. It might be useful to first separate bound and mobile track segments and only analyse the anisotropy on mobile track segments. This can be done in the main TrackIt UI: Menu -> Tools -> Classify bound and free segments (vbSPT), see chapter 2.10.1.

Number of jump angles Shows the number of jump angles in each movie. The total number of angles in each batch is shown in the statistics overview in the row #angles.

2.7.4 MSD analyis tab

Diffusion constants from MSD fit Shows the distribution of diffusion constants which are extracted by either fitting the the mean squared displacement (MSD) with a linear function

$$\text{MSD} = 4 \cdot D \cdot t + \text{offset} \tag{2.8}$$

or with a power law

$$\text{MSD} = 4 \cdot D \cdot t^{\alpha} + \text{offset} \tag{2.9}$$

where D is the diffusion coefficient, α a coefficient that indicates the motion type and t is the time interval given by the frame cycle time. For the offset a maximum allowed value can be set in the field "Max. offset".

The MSD is calculated as

$$\text{MSD} = \frac{1}{N-n}\sum_{i=1}^{N-n}(x_i - x_{i+n})^2 + (y_i - y_{i+n})^2, \quad n = 1, ..., N-1 \tag{2.10}$$

where x_i and y_i are the coordinates of the i-th spot within a track. *N* is the number of spots in a track (track length in frames) and *n* is the time lag between two spots of a track. The maximum time lag that should be considered can be set in the field "#Points to fit". Here, either a numeric value can be entered or a percentage value (eg. 90%). Entering a percentage value between 60% and 90% can be useful as the statics that goes into each point in the MSD gets worse with n reaching n_{max} = N-1. A minimum track length can be entered in the field "shortest track".

Alpha values from MSD fit Shows the distribution of alpha-values which are extracted by fitting the MSD (see above).

Confinement radius Shows the distribution of confinement radii calculated by fitting the MSD with a confined diffusion model [10]

$$MSD_{circle} = R^2 \cdot \left(1 - e^{-4 \cdot D \frac{t}{R^2}}\right) + \text{offset} \tag{2.11}$$

where R is the radius of confinement and D is the local diffusion coefficient. The offset is introduced to account for the finite localization precision. To use exclusively tracks which show a confined motion, tracks with alpha-values above a threshold specified in the field "upper alpha limit" are discarded.

Confinement radius vs. mean jump distance Displays a 2-dimensional plot where the mean jump distance of a track is plotted versus its confinement radius (see above) [10]. This representation can give insights into different mobility classes of single molecules.

2.7.5 Tracked fraction analysis tab

Bound fractions (Bf) Bound fractions can either be analyzed from movies with continuous or interlaced illumination. The illumination pattern is set in the "Illumination pattern" panel and can be switched between two modes:
Interlaced (ITM): The fractions of molecules belonging to two different binding time classes can be approximated using the interlaced time-lapse microscopy (ITM) scheme [6, 14]. In ITM, two frame acquisitions separated by a short dark time are followed by a longer dark time. This illumination scheme allows to classify molecules by their binding time. Molecules count as long bound (N_{long}) if they survive at least one long dark time, short bound (N_{short}) if the molecule survives only one short dark time and diffusive if the molecule is detected in only one frame ($N_{\text{non-linked}}$). The threshold for counting tracks as long bound can be set in the field "#survived dark periods to count as long track"
Continuous: Bound fractions can also be calculated from continuous movies, but come with high errors (eg. due to bleaching or because diffusing molecules are falsely counted as bound) and have to be handled with care. The threshold for counting tracks as long

bound can be set in the field "Count as long track if track is longer than"

Generally, accurate bound fractions can only be obtained if bleaching is corrected [14]. Three different types of bound fractions can be extracted:

Tracks vs. all events Shows the fraction of all bound molecules with respect to all counted events

$$\mathrm{Bf}_{\text{all bound}} = \frac{N_{\text{bound}}}{N_{\text{all events}}} = \frac{N_{\text{long}} + N_{\text{short}}}{N_{\text{long}} + N_{\text{short}} + N_{\text{non-linked}}} \tag{2.12}$$

where N_{bound} is the number of all molecules classified as bound and $N_{\text{all events}}$ is the number of all events.

Long tracks vs. all events Shows the fraction of long bound molecules with respect to all counted events

$$\mathrm{Bf}_{\text{long vs. all}} = \frac{N_{\text{long}}}{N_{\text{all events}}} = \frac{N_{\text{long}}}{N_{\text{long}} + N_{\text{short}} + N_{\text{non-linked}}} \tag{2.13}$$

where $N_{\text{all events}}$ is the number of all events.

Short tracks vs. all events Shows the fraction of short bound molecules with respect to all counted events

$$\mathrm{Bf}_{\text{short vs. all}} = \frac{N_{\text{short}}}{N_{\text{all events}}} = \frac{N_{\text{short}}}{N_{\text{long}} + N_{\text{short}} + N_{\text{non-linked}}} \tag{2.14}$$

where $N_{\text{all events}}$ is the number of all events.

Long tracks vs. long + short tracks Shows the fraction of long bound molecules with respect to all bound molecules

$$\mathrm{Bf}_{\text{long bound}} = \frac{N_{\text{long}}}{N_{\text{bound}}} = \frac{N_{\text{long}}}{N_{\text{long}} + N_{\text{short}}} \tag{2.15}$$

Pooled and movie-wise values Bound fractions are determined for each movie (displayed as blue triangles) and can show a significant variance between cells or movies. The average of these movie-wise values is calculated and displayed as a red dot. To avoid an over-representation of outliers or movies with low molecule counts, events for each binding time class can be summed up over all movies resulting in a single “pooled” bound fraction of all movies which is displayed as a black dot.

Error estimation The error for the movie-wise bound fraction (red error bar) is given by the standard error of the mean

$$\Delta_{\mathrm{BF_{movie\text{-}wise}}} = \frac{\mathrm{stdev}(\mathrm{BF_{movie\text{-}wise}})}{\sqrt{N_{\mathrm{movies}}}} \tag{2.16}$$

where $\mathrm{stdev}(\mathrm{BF_{movie\text{-}wise}})$ is the standard deviation of the movie-wise bound fraction values and N_{movies} is the amount of movies for which bound fractions are calculated.

The error for the pooled bound fraction (black error bar) is calculated through linear error propagation

$$\Delta_{\mathrm{BF_{pooled}}} = \frac{1}{N_{\mathrm{denominator}}} \cdot \delta_{N_{\mathrm{numerator}}} + \frac{N_{\mathrm{numerator}}}{N^2_{\mathrm{denominator}}} \cdot \delta_{N_{\mathrm{denominator}}} \tag{2.17}$$

where $N_{\mathrm{numerator}}$ is either N_{long} or N_{bound} and $N_{\mathrm{denominator}}$ is either N_{bound} or $N_{\mathrm{all\ events}}$. The errors δ are estimated by the counting errors with $\delta_{N_{\mathrm{numerator}}} = \sqrt{N_{\mathrm{numerator}}}$ and $\delta_{N_{\mathrm{denominator}}} = \sqrt{N_{\mathrm{denominator}}}$.

No. of tracks Shows the total number of tracked molecules in each movie. It is equal to the above mentioned number of all bound molecules N_{bound}.

No. of non-linked detections Shows the number of non-linked detections. This can either be single detections that have not been linked to tracks or detections of a track with a track length shorter than the minimum track length set by the user (see fig. 2.1 panel 4).

No. of all events (tracks + non-linked) Shows the number of total events in each movie calculated by the total number of tracks plus the number of non-linked detections.

No. of long tracks In the case of continuous illumination the amount of tracks which are longer than the threshold entered in the field "count as long track if track is longer than" is shown. If the interlaced illumination pattern (ITM) is selected, the displayed values are given by the amount of tracks which survive a specific number of long dark periods entered in the field "#survived dark periods to count as long track".

No. of short tracks Shows the amount of short tracks in each movie which is given by the total number of tracks minus the number of long tracks (see above).

2.7.6 Durations analysis tab

If the unit is set to "pixels & frames", the track length is defined as the amount of frames a track survives. If the units is set to "microns & sec", the track length is calculated by: $t = (\text{amount of frames} - 1) \times$ frame cycle time i.e. the number of jumps times the frame cycle time. A track that is composed of localizations in three consecutive frames connected via two "jumps", therefore has a track length of three frames and a duration of 20 ms at a frame cycle time is 10 ms.

Track lengths Histogram showing the lengths of tracks.

Survival time distribution Shows the survival probability of tracks calculated by the cumulative track duration histogram. The bin size of the histogram equals the time between two consecutive frames. The probability distribution is calculated separately for each frame cycle time in the batch. This distribution is equal to the one that is used in GRID.

Avg. track length Shows the average track length in each movie.

2.7.7 Other analyis tab

Avg. no. tracks per frame The average number of tracks per frame in a movie is calculated by counting the number of tracks visible in each frame divided by the number of frames in the corresponding movie.

Avg. no. spots per frame Shows the average number of spots per frame in each movie calculated by dividing the total amount of spots by the number of frames in the movie. If "Normalize by ROI-size" is selected, the values are divided by the size of the ROI resulting in the average spot density per frame. Based on experience, the average spot density should be below $2.5 \cdot 10^{-3} \frac{\text{spots}}{\text{px}\cdot\text{frame}}$ for the nearest neighbor algorithm to link trajectories correctly.

Total number of spots Shows the total number of detected spots in each movie, regardless of whether they were linked to tracks or not.

ROI size Shows the size of the region of interest (ROI) of each movie either in μm^2 or in number of pixels depending on the selected units.

Dist. to ROI border Histogram showing the closest distance of the average position of a track to its region border. If a track is part of a sub-region, the distance is the minimum distance to the sub-region border it has been assigned to. If the track is not part of a sub-region (ie. only party of the tracking region) it resembles the minimum distance to the tracking-region. The distance calculation can be executed by pressing the "Calculate distances" button.

Dist. to ROI border vs. mean jump dist. Shows a 2D scatter plot where the distance to region border (see above) is plotted versus the mean jump distance of the same track.

2.8 Analysis of dissociation rates with GRID

Via "Analysis" » "Analyse dissociation rates (GRID)" tracking results can be directly analyzed using the GRID software. Therefor track lengths (in seconds) are passed over to the GRID tool. For a detailed description of GRID please read the GRID user manual or refer to [13]. GRID is fully compatible with batch files created with the TrackIt software and can be loaded directly from the GRID GUI via "Files" » "Load File". GRID is available under `https://gitlab.com/GebhardtLab/GRID`.

2.9 Data export and movie creation

Export all data to Matlab workspace
By selecting "File" » "Export all data to Matlab workspace" in the menu bar of the main GUI, the data structure is saved in a variable of type "struct" with the name "currentBatch" in the Matlab workspace.

Export tracks
An export dialog window (see fig. 2.13) can be opened by clicking "File" » "Export tracks to .mat or .csv". In the "Compatibility" popup-menu .mat files can be chosen to be compatible either with Spot-On [5] or with vbSPT [12]. Only .csv files are compatible with Spot-On. Spot-On is available under `https://spoton.berkeley.edu/` and vbSPT under `https://github.com/bmelinden/vbSPT`.

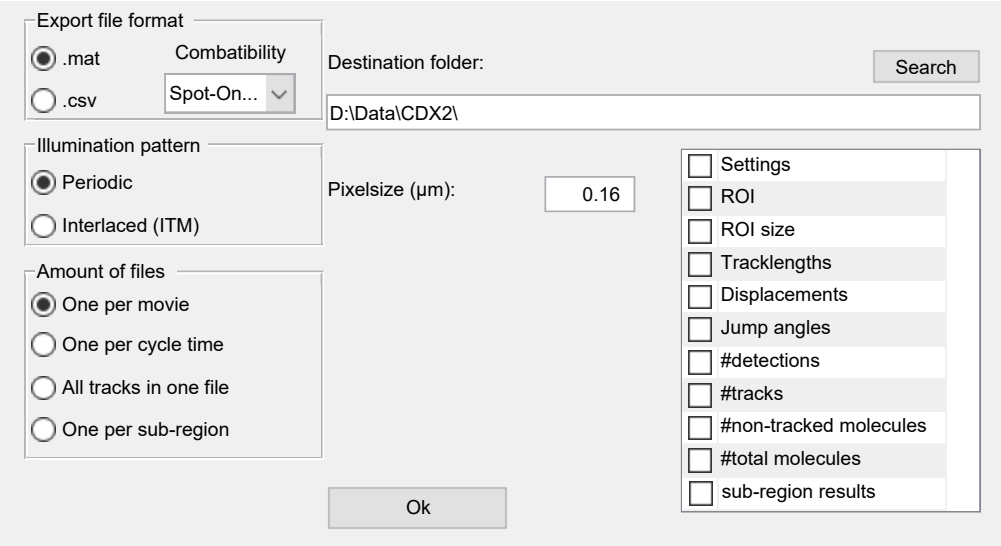


Figure 2.13: Track export window.

Movie creation
A movie inlcuding all selected plot elements can be created by clicking "File" » "Create .avi movie". A .avi movie file will be created using the exact same plot properties as currently shown in the main GUI. The playback speed (frames-per-second, FPS) can be set in the "FPS" field at the lower part of the main GUI.

2.10 Additional tools

2.10.1 Classify bound and free track segments using vbSPT

Tracks can be classified into segments of bound (immobile) and free (mobile) track segments using a Hidden Markov Model approach implemented within the vbSPT software [12]. The tool can be opened via Menu » "Tools" » "Classify bound and free segments (vbSSPT)". Tracks are

separated into bound and free segments and are then saved in two separate batch files with the filename add-on "_free.mat" and "_bound.mat", into the same folder as the original batch file. Before classification, tracks with gap frames are split at gap positions into separate tracks, as vbSPT cannot handle gap frames. The minimum track length field defines the minimum amount of frames of a track after splitting. Advanced settings can be set in the "runinput.m" file int the "vbSPT" folder. Noteworthy references where this kind of separation is used for single-molecule data analysis are [4] and [11].

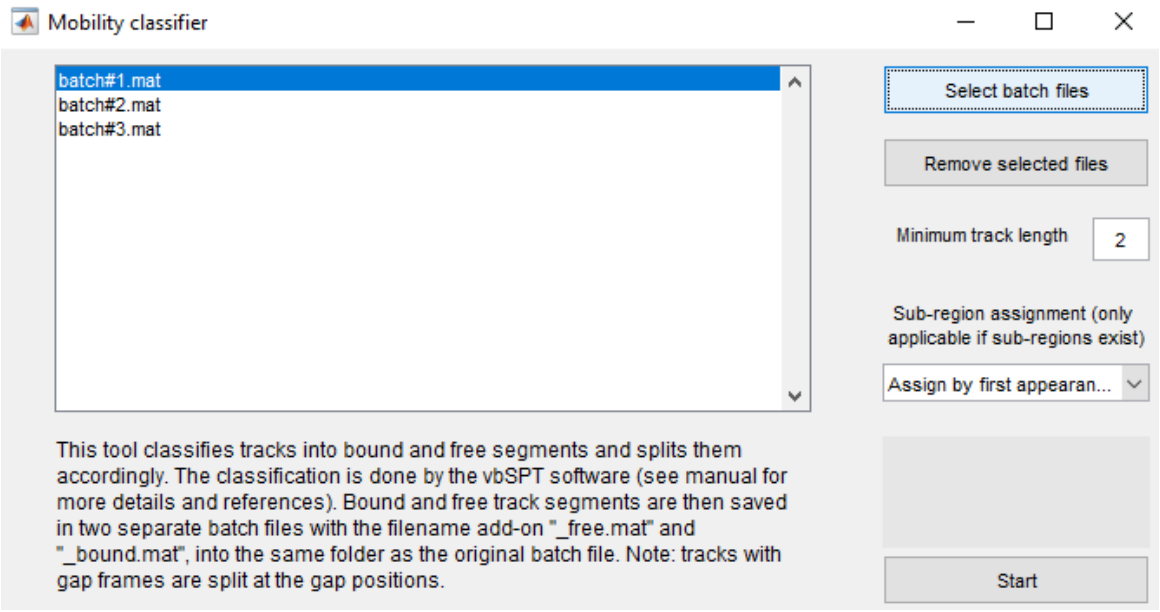


Figure 2.14: Tool to separate tracks into bound and free segments.

2.10.2 Spot statistics

A tool showing information about the detected spots can be opened via "Analysis" » "Spot statistics" where the following data can be shown and retrieved:

Peak spot intensity Shows a histogram of the peak intensity of all detected spots. The peak intensity is defined as the highest pixel value of all pixels within a radius of 2 px around the spot center.

Fitted spot intensity Shows a histogram of the fitted intensities of all detected spots as given by the maximum of the 2D Gaussian fit.

Spot SNR Histogram of the signal-to-noise ratio (SNR) of all detected spots. The SNR is calculated in a square window with side length of 17 pixels around the spot peak position at the center pixel (see fig. 2.16) as follows:

$$\mathrm{SNR} = \frac{\overline{I}_{\mathrm{blue}} - \overline{I}_{\mathrm{white}}}{\sigma_{\mathrm{white}}} \tag{2.18}$$

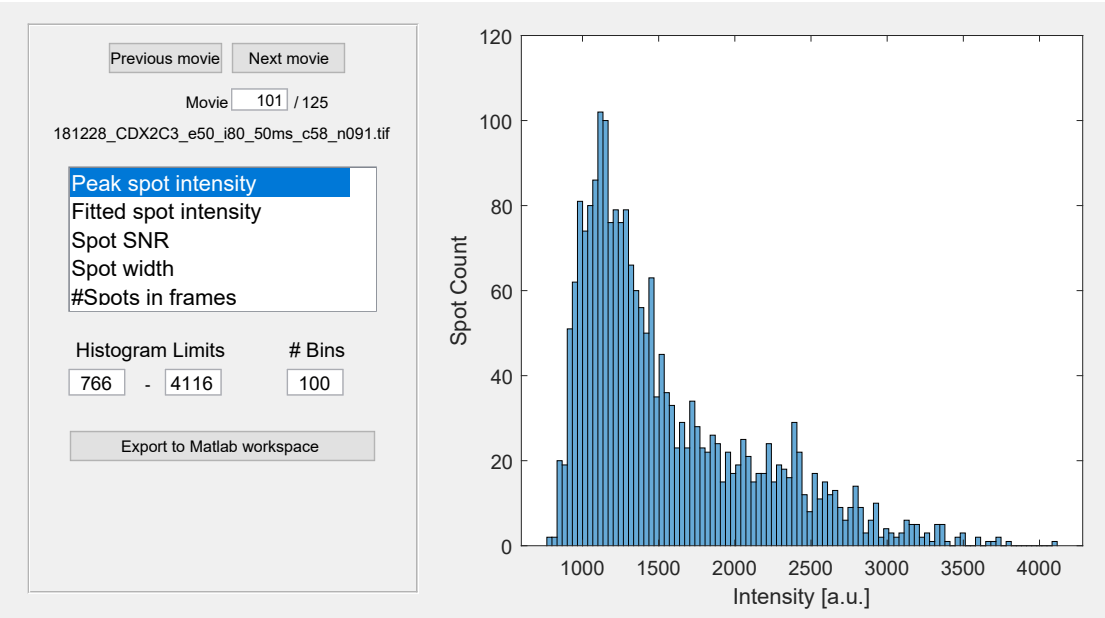


Figure 2.15: Spot statistics tool.

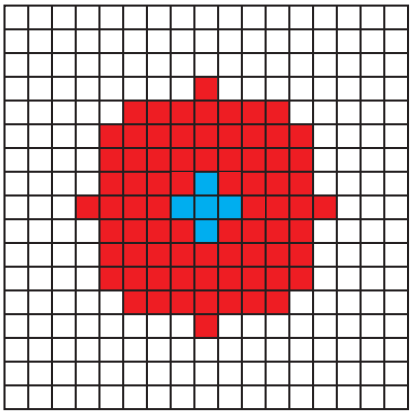


Figure 2.16: Pixel mask used for calculating the spot signal-to-noise ratio (SNR). **White**: Area where the mean background signal $\overline{I}_{\text{white}}$ and standard deviation of background noise σ_{white} are calculated. **Blue**: Area where the mean intensity $\overline{I}_{\text{blue}}$ of the spot is calculated. **Red**: Not used for calculation

where $\overline{I}_{\text{blue}}$ and $\overline{I}_{\text{white}}$ is the mean intensity of the pixel values of the respective area and σ_{white} denotes the standard deviation of the the pixel values of the white area.

Spot width Histogram of the spot width of all detected spots as given by the standard deviation σ of the Gaussian fit.

#spots in frames Shows a plot of the number of detected spots in each frame.

2.10.3 Kymograph

The kymograph tool can be started by clicking "Tools" » "Kymograph". As soon as a rectangular region is drawn the kymograph opens (see fig. 2.17). The cutout region of the original movie is shown in the upper left part and the current frame can be chosen using the slider. Horizontal (XT, Y-axis projected) and vertical kymographs (YT, X-axes projected) of the region are shown in the lower part of the figure. Here only one dimension is plotted at each time point while the other dimension is maximum projected. A red bar indicates the currently shown movie frame. In the top right part of the figure a plot shows the maximum pixel value in each frame of the kymograph. The amount of frames shown in the kymograph and the intensity plot can be entered in the field "No. of frames shown in kymograph and intensity plot". A click on "Export to Matlab workspace" the original movie cutout, the intensity and kymographs are exported to the Matlab workspace.

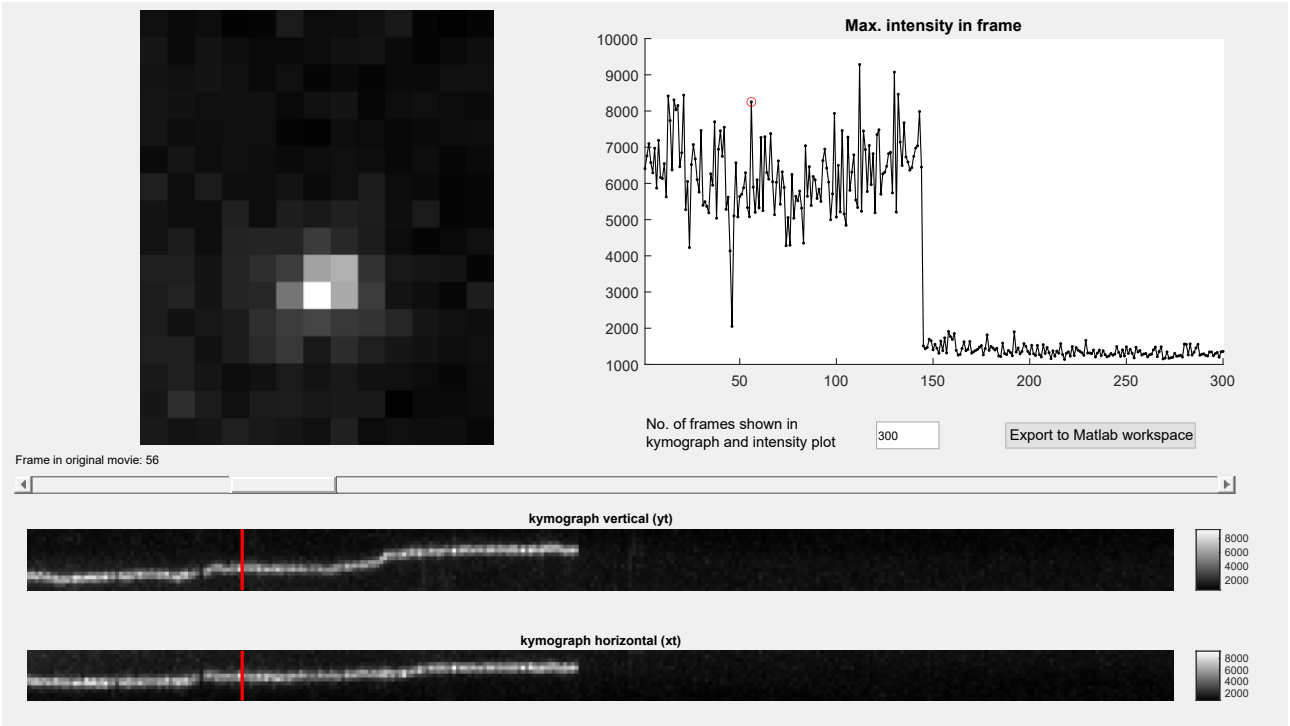


Figure 2.17: Kymograph tool. Top left: cutout of the original movie frame. Bottom: horizontal and vertical kymographs with red bars indicating the currently viewed frame. Top right: maximum pixel value of each frame in the kymograph plotted versus the frame number.

2.10.4 Movie splitter

Movies containing dark frames or movies containing more than one channel, as commonly originated from multiple-color experiments, can be split using the "Movie splitter" tool located in the "Tools" menu bar (see fig. 2.18). The amount of splits as well as the number of frames in each sequence can be defined by the user. A name entered in the field "Add-on to original filename" will be added to the original filename for each splitted movie part. If desired a .txt file containing the .tiff metadata can be created for each original movie. TrackIt uses the Bio-Formats library (`https://www.openmicroscopy.org/bio-formats/`) to extract metadata. Settings can be saved in different presets and are stored into the "MovieSplitterPresets.txt" when pressing the "Save all presets" button. Upon opening the movie splitter tool, presets are automatically loaded from the "MovieSplitterPresets.txt" file.

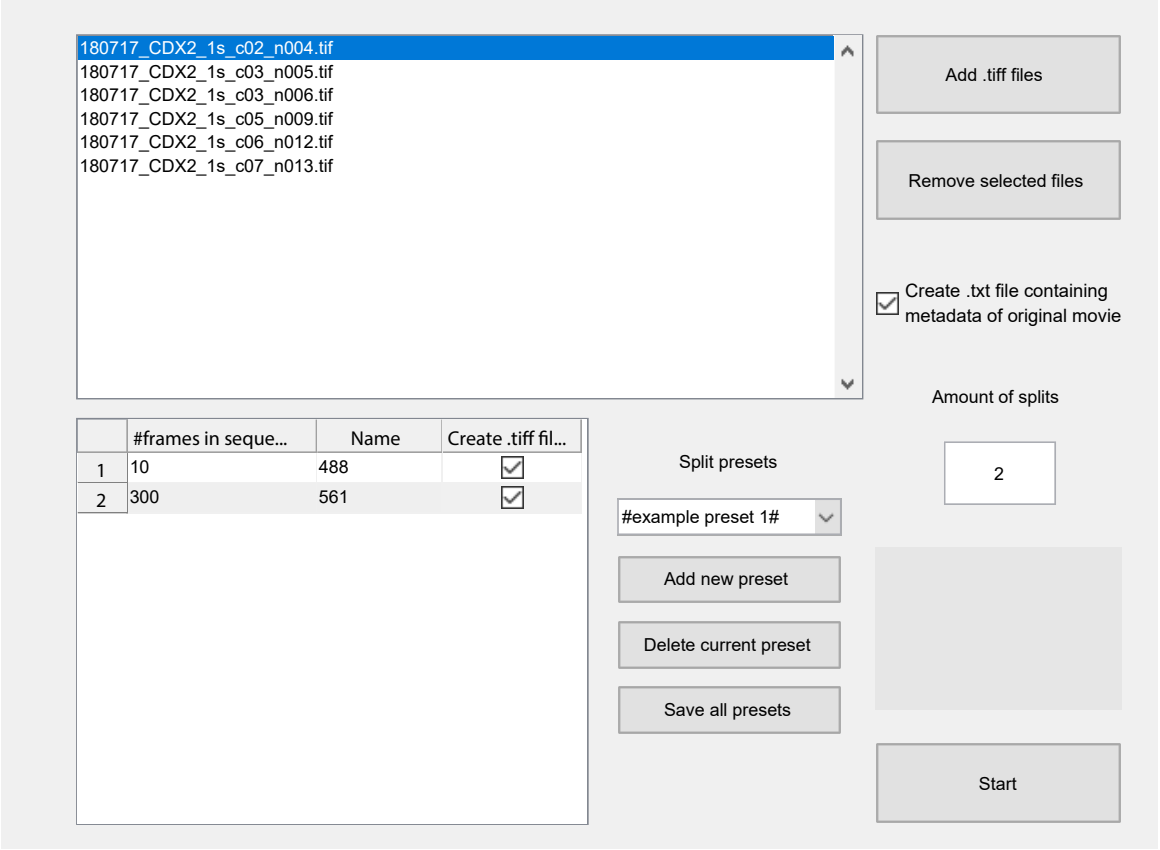


Figure 2.18: Movie splitter tool.

2.10.5 Merge multiple batch files

A tool to merge multiple batch files containing different movies can be accessed via "File" » "Merge multiple batch files" (see figure 2.19). Clicking "Start merge" will merge the batch files that have been added to the list via the "Add files" button and will be saved as a single batch in a .mat file to the destination specified in the field in the top row.

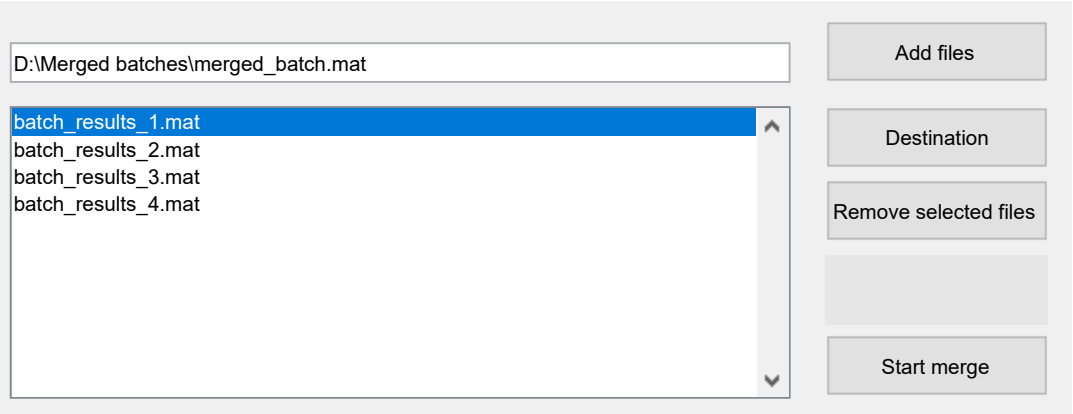


Figure 2.19: Tool for merging multiple batch files.

2.10.6 Subdivide batch file

The batch currently opened in TrackIt can be split into smaller batches based on the filenames of the movies that the batch contains. When clicking "File" » "Subdivide batch file" a window appears where a regular expression can be entered that will be used to divide the movies of the current batch into several filename classes. Example: "\d6" represents a sequence of 6 numeric digits and you can use it eg. if you want one batch file per measurement day. Each batch file will then contain all the movies that have the same sequence of 6 digits (ie. the same measurement day).
Or "_c\d+" represents an underline followed by a "c" and a series of digits. If you have filenames containing "_c01, c_02" etc. there will be one batch file containing all movies with "_c01", one containing all movies with "_c02" and so on. See `https://de.mathworks.com/help/matlab/matlab_prog/regular-expressions.html` for more information.
The new batch files will be saved in a folder specified by the user.

2.10.7 Re-analyze multiple batch files

A tool to analyze multiple batch files at once with multiple sets of tracking parameters can be opened by clicking "File" » "Re-analyze multiple batch files" (see figure 2.20). Batch files can be added by clicking the "Select batch files" button. The new batch files will be saved in the same folder as the original batch files where the filenames are extended with the tracking parameters that have been used to analyze the batch.

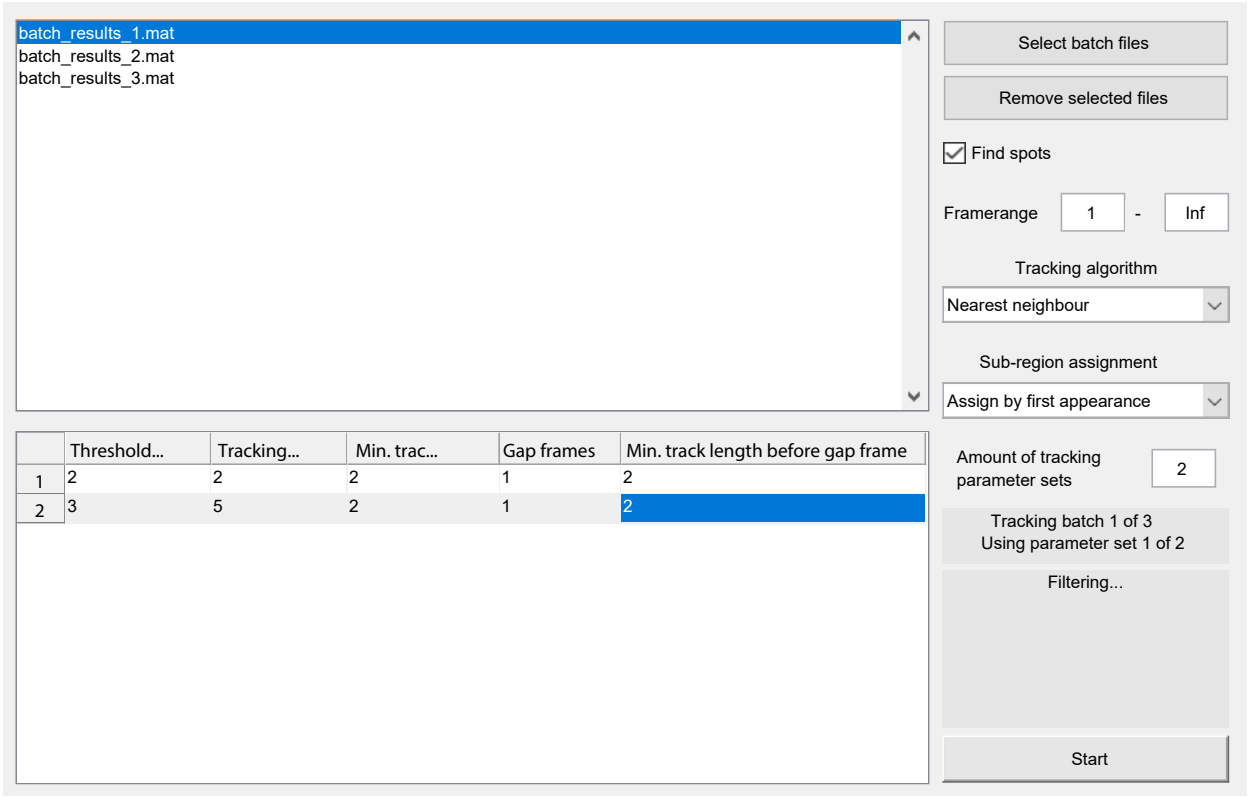


Figure 2.20: Tool to re-analyze multiple batch files with several sets of tracking parameters.

3 Benchmark

3.1 Tracking performance

In this section the reliability and performance of the TrackIt tracking routine is evaluated using simulated single molecule movies from a particle tracking challenge [1]. The image data plus a stand-alone software for performance evaluation is publicly available under `http://www.bioimageanalysis.org/track/index.php#datac`. Tracking and timing performances are evaluated using the datasets "Vesicles" and "Receptors" and a comparison is made between TrackIt and 14 other software tools which participated in the particle tracking challenge.
The "Vesicles" dataset shows particles diffusing in a two-dimensional plane simulated with a Brownian motion model. The "Receptor" dataset also shows particles simulated in a two-dimensional plane where the movement can switch between confined and linear motion. Both datasets were simulated with three different densities (100, 500 and 1000 molecules per frame, or 0.0004, 0.0019 and 0.0038 molecules per pixel and frame respectively) at four different signal-to-noise ratios (1, 2, 4 and 7), summing up to a total of 12 simulation movies per dataset.
Two different performance measures were considered for assessing the tracking accuracy and comparing the results with other software tools:

alpha value indicates to which extent estimated tracks and ground truth tracks overlap: $\alpha(X,Y) = 1 - d(X,Y)/d(X,\emptyset)$ where $d(X,Y)$ denotes the total distance between a set of estimated tracks X and a set of paired ground truth tracks Y. $\emptyset$ denotes a set of dummy tracks so that $d(X,\emptyset)$ is the maximum possible total distance from the ground truth. The value of α therefore ranges from 0 to 1 (perfect match) and does not penalize nonpaired tracks.

beta value additionally penalizes estimated tracks where no corresponding ground truth tracks where found (nonpaired tracks): $\beta(X,Y) = (d(X,\emptyset) - d(X,Y))/d(X,\emptyset) + d(\bar{Y},\emptyset))$ where $\bar{Y}$ is the set of nonpaired tracks, and $d(\bar{Y},\emptyset)$ is the penalty score. It takes the value α if no nonpaired tracks exist and converges to zero with higher number of nonpaired tracks.

For more information on the tracking performance measures, please refer to [1].

Figure 3.1 shows the performance results of the participants of the particle tracking challenge plotted together with the results of our TrackIt software using the nearest neighbor algorithm.

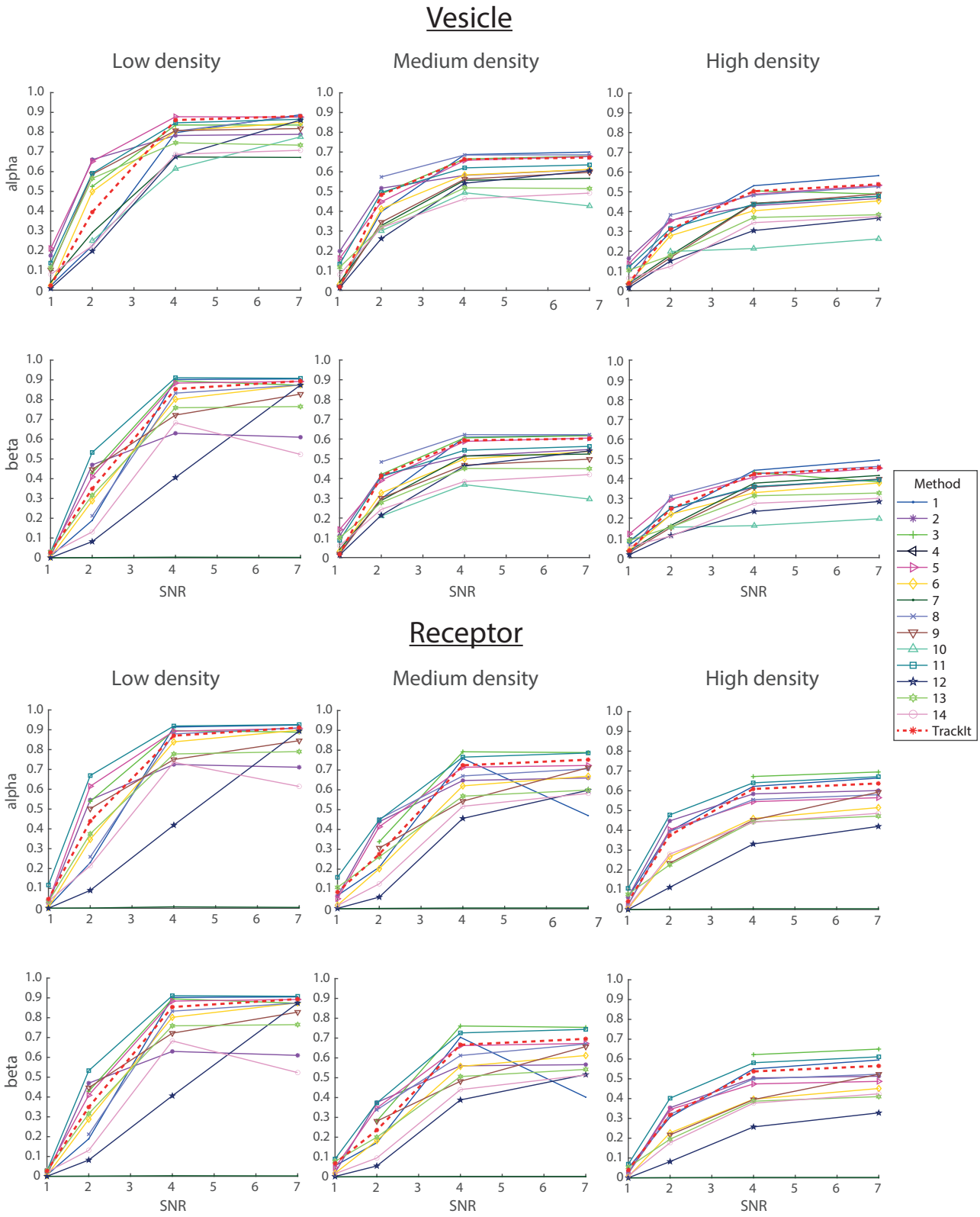


Figure 3.1: Tracking performance of participants of the particle tracking challenge [1] and TrackIt in the two scenarios "Vesicle" and "Receptor". Values of the performance measures α and β are plotted as a function of SNR for three different densities (low, medium and high). Tracking with the TrackIt software was performed using the nearest neighbour algorithm.

For low densities and medium to high signal-to-noise ratios (4 and 7), TrackIt delivers reliable, above average tracking results. With lower SNR and higher density the performance drops as expected. TrackIt is not designed to work for very low SNR or high single molecule densities. Still it is evident that all the methods that participated in the particle tracking challenge show a drastically reduced tracking performance for low SNR (2 and 1) and for medium to high particle densities. In order to obtain reliable tracking results we recommend to use particle densities below 0.0025 molecules per pixel and frame and a SNR above 3.

3.2 Computation times

An overview of the computation times of each method for the two scenarios are shown in table 3.1. Computation times for Method 1-14 are taken from [1] where tracking was performed on a workstation with a 64-bit Intel Xeon X5550 processor (2.67 GHz), 24 GB RAM and Windows 7 operating system. Tracking with the TrackIt software using the nearest neighbor algorithm was performed on a workstation with a 64-bit Intel Core i5-6500 processor (3.2 GHz), 16 GB RAM and Windows 10 operating system. Although tracking was performed on different workstations, it is evident that TrackIt is in a class with the fastest participating softwares in the particle tracking challenge.

Scenario	Receptor											
Density	Low				Mid				High			
SNR	1	2	4	7	1	2	4	7	1	2	4	7
Method 1	13	13	13	13	15	15	14	60	19	36	30	26
Method 2	21	10	8	9	402	149	141	119	1433	995	499	439
Method 3	-	33	25	29	-	58	113	109	-	-	356	344
Method 4	-	-	-	-	-	-	-	-	-	-	-	-
Method 5	206	205	62	67	468	320	274	256	948	636	819	705
Method 6	688	1097	541	529	945	1681	1340	1219	1020	3043	2414	2274
Method 7	4948	3556	2371	2313	6405	5609	4719	4186	5817	11438	9260	9796
Method 8	-	319	364	383	-	6449	2925	2719	43375	9505	9873	9545
Method 9	-	14	11	14	-	19	26	39	-	29	39	74
Method 10	-	-	-	-	-	-	-	-	-	-	-	-
Method 11	94	10	8	8	270	76	117	104	2081	3117	2006	1838
Method 12	164	162	153	158	161	167	168	147	163	207	167	156
Method 13	25	19	9	9	65	45	17	18	97	44	31	29
Method 14	839	592	45	45	819	2706	103	106	4150	10931	503	399
TrackIt	5	3	3	2	7	6	9	8	6	35	52	48
Scenario	**Vesicles**											
Density	**Low**				**Mid**				**High**			
SNR	**1**	**2**	**4**	**7**	**1**	**2**	**4**	**7**	**1**	**2**	**4**	**7**
Method 1	13	13	10	13	13	21	16	16	39	49	43	39
Method 2	21	14	12	12	410	406	231	201	2229	1998	991	733
Method 3	-	34	32	38	-	126	157	165	-	-	418	425
Method 4	-	-	-	-	-	-	-	-	-	-	-	-
Method 5	69	20	17	19	90	111	133	136	177	326	402	387
Method 6	842	703	743	702	1569	4119	2554	2626	2334	6578	5244	5490
Method 7	7012	3028	2388	2396	8282	6015	7716	19879	9335	11203	19644	21980
Method 8	-	-	622	632	-	5240	4714	4310	10279	13214	16802	15479
Method 9	12	14	13	17	12	25	42	45	11	33	104	122
Method 10	-	46	46	40	-	103	116	112	-	119	193	179
Method 11	107	11	10	9	282	604	408	430	3348	7113	10854	9135
Method 12	57	56	289	134	51	58	156	149	59	77	252	156
Method 13	25	17	11	10	51	20	20	20	80	33	38	38
Method 14	869	220	37	26	4287	495	134	126	2773	1118	475	311
TrackIt	5	4	3	3	4	18	16	16	5	59	104	96

Table 3.1: Timing in seconds for all particle tracking methods for the scenarios "Receptor" and "Vesicle".

3.3 Diffusion coefficient analysis

In order to validate our diffusion coefficient analysis routine we simulated 50 videos each containing one molecule diffusing in a two-dimensional plane switching between two diffusion states. The corresponding diffusion coefficients were $D_1 = 2\ \mu m^2/s$ and $D_2 = 20\ \mu m^2/s$. The dwell time before switching was set to 1 second for both states implying equally distributed fractions of states $F_1 = 0.5$ and $F_2 = 0.5$. The movies had a window size of 150 x 150 pixels with a pixel size of 160 nm/px and a length of 500 frames with a frame cycle time of 10 ms. After tracking with TrackIt we fit the cumulative density distribution of squared displacements with a Brownian diffusion model with two diffusion components (see section (2.7.7)). We additionally estimated the precision of the diffusion constants and fractions by performing a resampling where we fit 500 subsets of the jump distance distribution each containing 80% of randomly drawn jump distances out of the full dataset and calculated the standard deviation of the results. We further analyzed the tracking results with Spot-On [5] and vbSPT [12], to which the tracks can be directly exported from TrackIt (see section (2.9)). The results in Table 3.2 show that all three methods produce similar results close to the ground truth values. The overall fitting error Spot-On delivered from its Matlab version was 9.7e-05. Please note that the choice of the right diffusion analysis software is always dependent on the type of data but also on individual requirements and shall not be discussed here.

	D_1(µm^2/s)	**D_2**(µm^2/s)	**F_1**	**F_2**	**Loc. err.** (µm)
Ground truth	2.00	20.00	0.500	0.500	-
TrackIt	2.32 $\pm$ 0.03	19.98 $\pm$ 0.13	0.434 $\pm$ 0.004	0.566 $\pm$ 0.004	-
vbSPT	2.37 $\pm$ 0.03	20.09 $\pm$ 0.93	0.439	0.561	-
Spot-On	2.11	19.60	0.440	0.560	0.045

Table 3.2: Diffusion analysis results including diffusion constants $D_{1,2}$ and fractions of molecules in each state $F_{1,2}$. Spot-On additionally fits the localization error.

Bibliography

[1] N. Chenouard, I. Smal, F. De Chaumont, M. Maška, I. F. Sbalzarini, Y. Gong, J. Cardinale, C. Carthel, S. Coraluppi, M. Winter, A. R. Cohen, W. J. Godinez, K. Rohr, Y. Kalaidzidis, L. Liang, J. Duncan, H. Shen, Y. Xu, K. E. Magnusson, J. Jaldén, H. M. Blau, P. Paul-Gilloteaux, P. Roudot, C. Kervrann, F. Waharte, J. Y. Tinevez, S. L. Shorte, J. Willemse, K. Celler, G. P. Van Wezel, H. W. Dan, Y. S. Tsai, C. O. De Solórzano, J. C. Olivo-Marin, and E. Meijering. Objective comparison of particle tracking methods. *Nature Methods*, 11(3):281–289, 2014.

[2] J. C. M. Gebhardt, D. M. Suter, R. Roy, Z. W. Zhao, A. R. Chapman, S. Basu, T. Maniatis, and X. S. Xie. Single-molecule imaging of transcription factor binding to DNA in live mammalian cells. *Nature Methods*, 10(5):421–426, 2013.

[3] A. Große-Berkenbusch, J. Hettich, T. Kuhn, N. Fili, A. W. Cook, Y. Hari-Gupta, A. Palmer, L. Streit, P. J. Ellis, C. P. Toseland, and J. C. M. Gebhardt. Myosin vi moves on nuclear actin filaments and supports long-range chromatin rearrangements. *bioRxiv*, 2020.

[4] A. S. Hansen, A. Amitai, C. Cattoglio, R. Tjian, and X. Darzacq. Guided nuclear exploration increases CTCF target search efficiency. *Nature Chemical Biology*, 16(3):257–266, 2020.

[5] A. S. Hansen, M. Woringer, J. B. Grimm, L. D. Lavis, R. Tjian, and X. Darzacq. Robust model-based analysis of single-particle tracking experiments with spot-on. *eLife*, 7:1–33, 2018.

[6] L. Hipp, J. Beer, O. Kuchler, M. Reisser, D. Sinske, J. Michaelis, J. C. M. Gebhardt, and B. Knöll. Single-molecule imaging of the transcription factor SRF reveals prolonged chromatin-binding kinetics upon cell stimulation. *Proceedings of the National Academy of Sciences of the United States of America*, 116(3):880–889, 2019.

[7] I. Izeddin, J. Boulanger, V. Racine, C. Specht, A. Kechkar, D. Nair, A. Triller, D. Choquet, M. Dahan, and J. Sibarita. Wavelet analysis for single molecule localization microscopy. *Optics Express*, 20(3):2081, 2012.

[8] I. Izeddin, V. Récamier, L. Bosanac, I. I. Cissé, L. Boudarene, C. Dugast-Darzacq, F. Proux, O. Bénichou, R. Voituriez, O. Bensaude, M. Dahan, and X. Darzacq. Single-molecule tracking in live cells reveals distinct target-search strategies of transcription factors in the nucleus. *eLife*, 2014(3):1–27, 2014.

[9] K. Jaqaman, D. Loerke, M. Mettlen, H. Kuwata, S. Grinstein, S. L. Schmid, and G. Danuser. Robust single-particle tracking in live-cell time-lapse sequences. *Nature Methods*, 5(8):695–702, 2008.

[10] J. Lerner, P. A. Gomez-Garcia, R. L. McCarthy, Z. Liu, M. Lakadamyali, and K. S. Zaret. Two-Parameter Mobility Assessments Discriminate Diverse Regulatory Factor Behaviors in Chromatin. *Molecular Cell*, 79:1–12, 2020.

[11] M. Mazzocca, A. Loffreda, E. Colombo, T. Fillot, D. Gnani, P. Falletta, E. Monteleone, S. Capozi, E. Bertrand, G. Legube, Z. Lavagnino, C. Tacchetti, and D. Mazza. Chromatin organization drives the search mechanism of nuclear factors. *Nature Communications*, 14(1):6433, oct 2023.

[12] F. Persson, M. Lindén, C. Unoson, and J. Elf. Extracting intracellular diffusive states and transition rates from single-molecule tracking data. *Nature Methods*, 10(3):265–269, 2013.

[13] M. Reisser, J. Hettich, T. Kuhn, A. A. P. Popp, A. Große-Berkenbusch, and J. C. M. Gebhardt. Inferring quantity and qualities of superimposed reaction rates from single molecule survival time distributions. *Scientific Reports*, 10(1):1–13, 2020.

[14] M. Reisser, A. Palmer, A. P. Popp, C. Jahn, G. Weidinger, and J. C. M. Gebhardt. Single-molecule imaging correlates decreasing nuclear volume with increasing TF-chromatin associations during zebrafish development. *Nature Communications*, 9:5218, 2018.

[15] S. C. Stein and J. Thiart. TrackNTrace: A simple and extendable open-source framework for developing single-molecule localization and tracking algorithms. *Scientific Reports*, 6(November):1–7, 2016.