User manual of
TEA_MT - Intensity versus time trace of fluorescent spots extraction and analysis software

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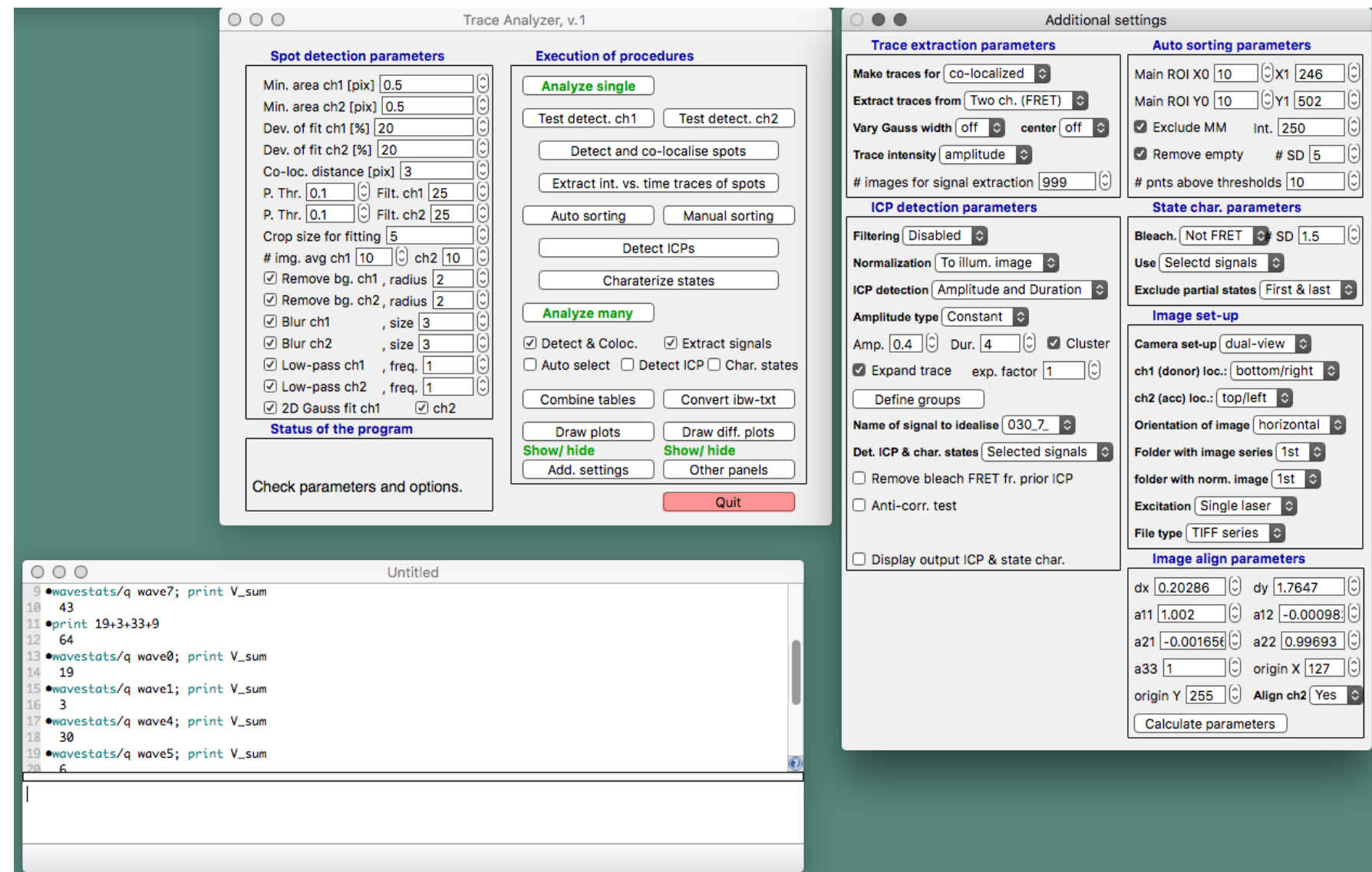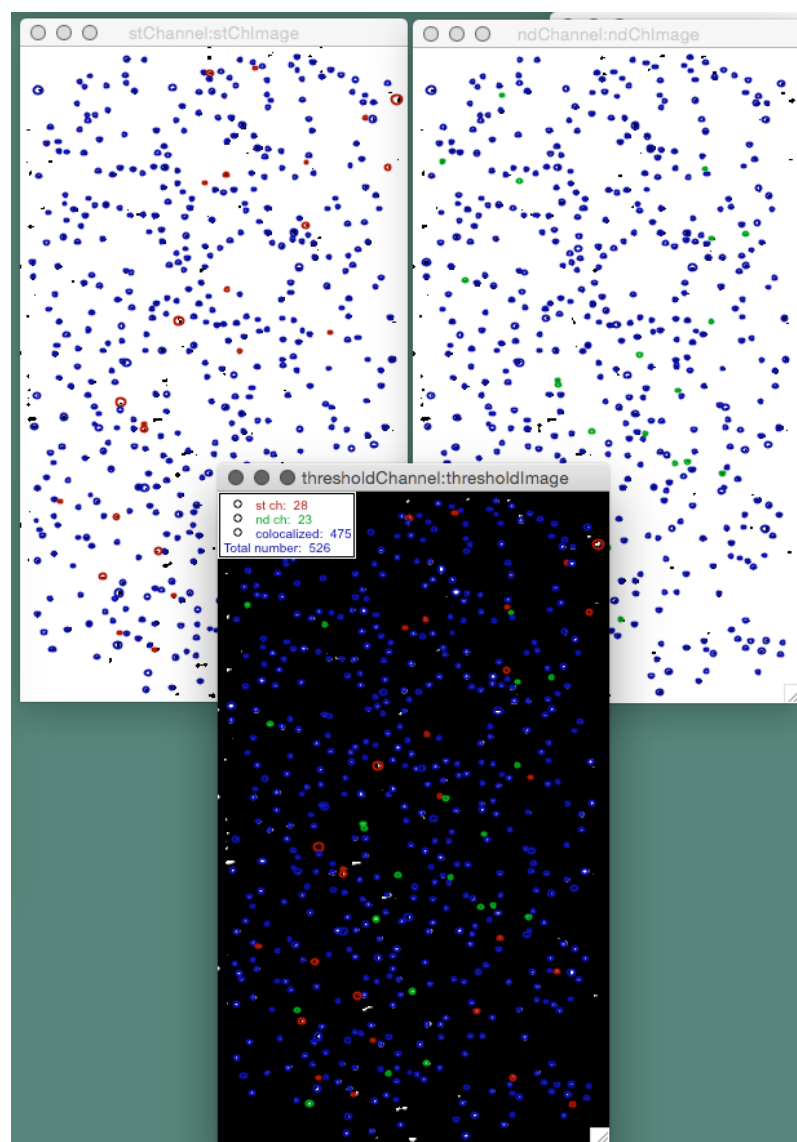
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General info

- <u>This software can only be distributed upon direct request to the author!</u>
- The software was written so that all of the daily single-molecule data analysis tasks are accessible at one place:
    - fluorescent spot detection,
    - co-localization of the fluorescent spots from 2 channels,
    - intensity versus time traces extraction for selected type of fluorescent spots,
    - filtering of traces
    - normalization of traces to an excitation field
    - intensity change point detection in the traces,
    - characterization of detected states,
    - manual selection of extracted traces,
    - 2D histogram plotting of the analysis results.
- It can handle most of the single-molecule data formats.
- The software has internal memory for entered parameters, the size of windows and their positions.
- Each part of the program gives output as tables of igor binary format. There is an additional function in the main panel to convert them into
- delimited text format.
- Software development was under MacOS, therefore, it may happen that Windows users find some small problems.
- I am making updates of software constantly, therefore, feedback is highly appreciated: marijonas@ar.fi.lt or marijonas.tutkus@gmail.com

Installation

• To install this software just copy paste the entire TEA_MT folder into the Igor Pro folder > Igor procedures
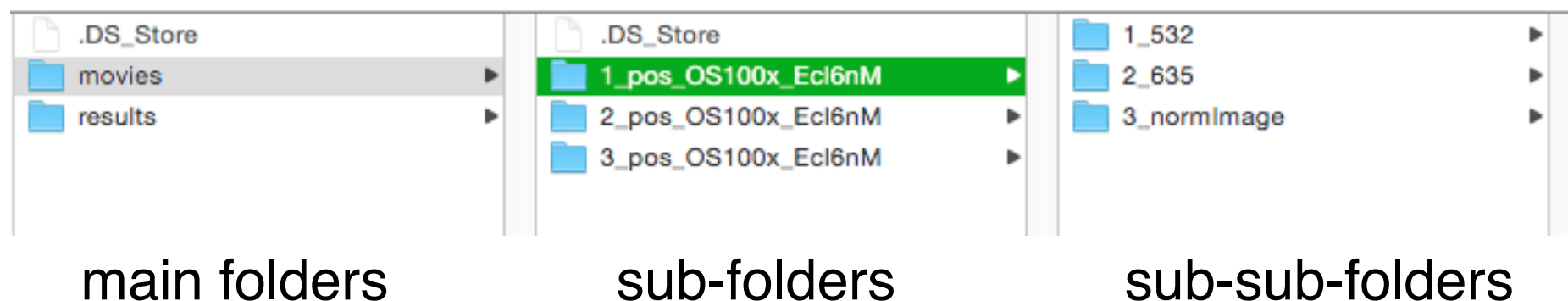
General user interface

• You can execute the software under Igor Pro by clicking cmd+2 (Mac) or ctrl+2 (Windows)
• There are two ways to run analysis: single movie at a time (main panel > "Analyze single" button) or many movies at once (main panel > "Analyze many" button).
But first, I will explain what each button and parameter does.

Loading data

• Recommend distribution of images showed below. Next, you should select in the "image set-up" parameter box (additional settings panel), that you have both donor and acceptor signal containing images (image series or stack) in the first sub-sub-folder (this case 1_532) or in the second sub-sub-folder (this case 2_635) or donor in the first and acceptor in the second sub-sub-folder. Now you can use the "Analyse many" button (see page #16). The reason to give such an option: sometimes I capture few images of acceptor signal with acceptor laser excitation before acquiring movie with donor laser, just to be able to detect all of the acceptor and donor spots. Then I would place acceptor images in the second sub-sub-folder and the movie in first of the sub-subfolders.

• The software can handle these data formats: TIFF stack, TIFF image series and signed 32-bit binary image stack (additional settings panel, pop-up menu "type of image file"). More formats are available, I just never needed other.

• Choose parameters for the set-up of your image (additional settings panel, "image set-up" parameter box): dual-view or dual-camera image. If it is dual-view you have to define splitting of image: vertical or horizontal and then were is your donor and acceptor: top/left or bottom/right. If it is dual-camera, select in which of the sub-sub-folder is acceptor and in which donor located.

• If you decide to employ normalization of signals to the excitation profile (slide #9) you should select this option in the pop-up menu "normalize traces" (additional settings panel) and select in the image set-up in which of the sub-sub-folder normalisation image is located (this case the third sub-folder "3_normimage").

• Finally, you should specify the type of excitation - single laser or alternating laser (ALEX).

• Once this done, you can click "Analyse single" button (main panel) and the program will ask to define folders containing images. Once folders are defined, you can check out the output (page # 14 and # 6-8) of each of the analysis part with these images and adjust parameters to achieve the best performance.

Recomendation for image distribution

main folders   sub-folders   sub-sub-folders

Alignment of dual-view or dual-camera

· To enable alignment of the 2nd channel image in the TEA_MT you have to select this option in popup menu "Align ch2" located in the additional panel section entitled "Image align parameters". This will enable translation and skewing of the 2nd channel image. If your images are perfectly aligned ignore this part.

· To get image alignment parameters, which you will need to provide for program later on (additional settings panel), kick "Calculate parameters" button (main panel). It performs image registration (see Igor Pro manual) on the binarized (threshold applied) reference images.

· You will be asked to navigate to the main directory containing sub-folders for the different positions and in these sub-folders you should have sub-subfolders for the 1st and 2nd channel images. If you have separate images for first and second channel - place them separately to these folders. In this case select in the bottom left pop-up menu the third option and the third option from bottom right pup-menu. You can as well have a single image for both channels. In that case place it in the 1st or 2nd sub-sub-folder and select in the bottom left pop-up menu first or second option and first or second option in the bottom left pop-up menu.

· Define other parameters of the image set-up: dual-view or dual-camera, number of images from sub-sub-folder to average before applying intensity threshold and type of image: TIFF stack, signed 32-bit binary, TIFF series, type of excitation, number of images to accumulate per position and starting threshold values (later on you will be able to change these threshol values).

· Select the alignment scheme: align 2nd to first channel image or first to second channel image.

· Select continue and then program will produce an average image per position and further average these with different positions. Resulting images will be displayed and you will be asked to enter intensity threshold value.

· Once you find the right threshold, confirm that it is good, and generated alignment parameters will be printed in the history window and saved in the results_imageAlign folder. There you will find a split, processed (threshold applied) and aligned images. Which you can load into the imageJ into a stack and check out whether the result is satisfactory. Also there you will find a regParams.txt file, containing parameters of the alignment. You should enter them into the bottom right section of the additional settings panel.

Arrangement of files

Options for alignment

Detection of fluorescent spots in 2 channels and their co-localization, part 1

· It is shown in the slide #4 that program has 3 image windows: stChannel, ndChannel and thresholdChannel. Images after filtering with particle specific

filter will be displayed in the first two and particle probability image in the last.

· Here the task is to find the right parameters for the detection giving a maximum number of co-localized spots.

· Since single-molecules are typically changing intensity, program before detecting fluorescent spots improves quality of image by these steps:

· Average given number of images (main panel > spot detection parameters > # images avg. ch1 or ch2)

· If enabled, subtracts background from this average image by the rolling ball algorithm (main panel > spot detection parameters > checkbox "Remove backg

from

ch1 image or ch2 image". See ImageJ reference) with the given ball radius (main panel > spot detection parameters > "ball radius ch1 or ch2"). A typical value

of ball radius for 512 x 512 image is 13 pix.

· if enabled, further performs smoothing of the image using Gaussian convolution (main panel > spot detection parameters > checkbox "Filter ch1 or ch2 image

by Gaussian"), using given mask width (main panel > spot detection parameters > "Gauss size ch1 or ch2"). Size should be similar to the smallest spot width

in the image. It is ~ 3pix.

· if enabled, further filters the image using low-pass filter (main panel > spot detection parameters > checkbox "Filter ch1 or ch2 image by low-pass filter"),

using given freq. cut-off (main panel > spot detection parameters > "Freq. ch1 or ch2"). Frequency can take values from 0 till 0.5. I am typically using 0.35.

Detection of fluorescent spots in 2 channels and their co-localization, part2

· Next step is the detection of fluorescent spots in the improved quality image. Instead of regular intensity threshold application for spot detection program filters this image with spot selective convolution filter of given radius (main panel > spot detection parameters > min area ch1 and ch2) and then binarize it using given threshold (main panel > spot detection parameters > filt. thresh ch1 and ch2). Next, it converts this filtered image into the particle probability image and detects spots having value above the defined probability value (main panel > spot detection parameters > P. Thresh ch1 and ch2). Typical minimal area value is 1 pixel, filtering threshold depends on the intensity of spots and background (in the range of 5 to infinity), probability threshold will depend on the filtering threshold (can have values from 0 till 1). You can check out what probability to use by employing Igor built-in image threshold panel (which is in the top menu bar > image > threshold…) on the thresholdChannel. More info on the spot detection algorithm here: An adaptive non-local means filter for denoising live-cell images and improving particle detection. Lei Yang et al, Journal of Structural Biology, 2010. The benefit of this procedure - spots of different intensity and high density are easier detectable.

· if selected, the program will perform 2D Gaussian fitting (main panel > spot detection parameters > Fit spots from 1st and 2nd channel to 2D Gauss) to the original image with given coordinates from the previous step of spot detection in the improved quality image. Only those spots, which fit error will be less than the defined value will be detected (main panel > spot detection parameters > Dev. of fit ch1 and ch2). You should also specify crop radius for Gaussian fitting (main panel > spot detection parameters > Crop size for fitting). It has to be as big as a half of largest spot of interest.

· Next, the program will co-localize the detected spots in 1st and 2nd channels, with given co-localization distance (main panel > spot detection parameters > Co-loc. distance). Overlapping spot removal: if there are more than 2 spots: a spots per channel, within the co-localization distance program will remove them all.

· Output is saved in the results folder in file - 01_*. It contains parameters of gaussian fit and type of spot: 1 - only detected in the 1st channel, 2 - only detected in the 2nd channel, and 3 - co-localized.

· To test spot detection parameters use "Test thresh. ch1 and ch2" buttons located in the main panel.

Extraction of fluorescence intensity over time traces from detected spots

· Program will extract intensity of the detected spots of the given type: co-localized or other (additional settings panel > trace extraction parameters > pop-up menu "make traces from") by the 2D Gaussian fitting with given crop radius for fitting (main panel > spot detection parameters > Crop size for fitting). During the fitting procedure center and width of Gaussian can be either constant or varying (additional settings panel > trace extraction parameters > pop-up menu "Vary Gauss width" and "center"). In the end, intensity can be saved as the Gaussian amplitude or Gaussian integral: amplitude * width (additional settings panel > trace extraction parameters > pop-up menu "trace intensity").

· You should also define a number of images to extract intensity from (additional settings panel > trace extraction parameters > "# of images for signal extraction").

· Also, you can select whether to have traces extracted from the 1st, 2nd or both (for FRET) channels (additional settings panel > trace extraction parameters > pop-up menu "extract traces from").

· Gaussian fitting is performed on multiple processors so that each particle is fitted on the separate core. This speeds up the processes in comparison to multicore fitting for one spot at least 10 times.

· Output is saved in the results folder:
   · Tables intensity traces: 030_0_ (Efret),030_1_ (ch1) and 030_2_ (ch2). Each column is the separate spot, row indicates time point.
   · If width was variable, it saves width of Gaussian fit trace in tables: 030_3_ (ch1), 030_4_ (ch2). Useful for tethered fluorophore motion experiments.
   More info on this method: Capturing reaction paths and intermediates in Cre-loxP recombination using single-molecule fluorescence. Pinkney et.al., PNAS, 2012.
   · ParticleInfo table for selected type of spots is saved: 040_*. It is the same type of table as in the spot detection.

Intensity change point (ICP) detection in intensity over time traces

· Select wich of the trace you would like to run ICP detection on by entering unique fraction of table name (e.g. "030_0_") in the "name of signal to idealise" (additional settings panel > ICP detection parameters > pop up menu "name of signal to idealise").

· You can analyse only manually selected signals (page # 13?) or all extracted signals, by selecting either option in popup menu "Det. ICP & char. states" (additional settings panel > ICP detection parameters > check box "Det. ICP & char. states").

· Prior to ICP detection you can normalise and filter trace which you are going to perform ICP detection on (see page # 12).

· For FRET efficiency traces, program automatically can detect time point, where acceptor and donor bleached. It analyses only non bleached part of this trace. To enable this select "Remove bleach FRET fr. prior ICP" (additional settings panel > ICP detection parameters > check box "Remove bleach FRET fr. prior ICP").

· If "Anti-corr. test" check box is checked (additional settings panel > ICP detection parameters > check box "Anti-corr. test"), program checks whether detected ICPs in the Efret trace has anti-correlated intensity change of ch1 and ch2 trace at the same point. Amplitude of the intensity change in ch1 and ch2 has to be above defined threshold value (additional settings panel > ICP detection parameters > set variable control "amp. ch1" and "ch2"). ICP satisfying this criteria has a value of 1 in the stateInfoTable column labeled "antiCorr".

· There are two methods for ICP detection in this software: "amplitude and duration with clustering" developed by M. Tutkus (page # 16) or Hidden Markov Modeling (as in the original article, just parameter optimisation based on Metropolis or Simulated annealing algorithm) (additional settings panel > ICP detection parameters > pop-up menu "ICP detection"). More info on Hidden Markov Modeling: Analysis of Single-Molecule FRET Trajectories Using Hidden Markov Modeling. McKinney et al., Biophysical Journal, 2006.

Intensity change point (ICP) detection in intensity over time traces

- In the case of "amplitude and duration with clustering" ICP detection method you have to select type of amplitude (additional settings panel > ICP detection parameters > pop-up menu "Amplitude type") provide amplitude threshold, duration threshold and select whether to cluster neighbouring states or not (they are located below the "Amplitude type" popup menu).

- "Amplitude and duration with clustering" ICP detection algorithm scans trajectory point by point with a 8 frame window, takes an average of the first 4 points and last 4 points within that window and calculates step amplitude (difference between averages). In case amplitude is higher than defined value, the center position of this window is recorded as a putative intensity change point (ICP). There are two criteria for accepting putative ICP: duration and slopes of two neighbouring states that it separates (e.g., state n and state n+1 are separated by ICP_n). If either state n or n+1 has the duration shorter than half of the scanning window length ICP_n is rejected. Next criteria for accepting ICP_n is the slope of the line fits to state n and n+1 if the sum of absolute slope values of state n and n+ 1 is higher than a set value the ICP_n is rejected. Detected states are further clustered — if average intensity (or E_{FRET}) of state n and n+1 falls into the same group, they are combined by removing ICP_n.

- Using any of method you can select to expand trace (additional settings panel > ICP detection parameters > "Expand trace"): extra points defined by exp. factor (additional settings panel > ICP detection parameters > "Exp. factor") are inserted into the trace and then the trace is smoothed using box-car smoothing with box size equal to expansion factor.

- You can check out result of ICP detection for each trace online by selecting check box "Display output ICP & state char." (additional settings panel > ICP detection parameters).

- Output of the detection is table containing idealised traces. It will be saved in the results folder: 033_0_*.

Filtering of the intensity over time traces

· Program can filter the extracted traces using three different methods: box-car smoothing, low-pass filtering and discrete wavelength transformation. You can define method of filtering (additional settings panel > pop-up menu "trace filtering") and enter the parameter for filtering (main panel > "trace filtering parameter"). Filtering parameter for smoothing is from 0 till length of trace, DWT and low-pass filtering can take values from 0 till 0.5.
More info on discrete wavelength transformation: Structural landscape of isolated agonist-binding domains from single AMPA receptors. Landes et al., Nature chemical biology 2011.

· Filtered traces will be saved in the results folder: 031_0_*.

Normalization of the intensity over time traces

· TIRF images are heterogeneous in excitation power. Here method for normalization to unevenness of excitation power is provided.

· There are two methods for signal normalisation (goes after signal filtering step, if it was enabled) implemented:

1. Using provided excitation profile image (e.g. high concentration streptavidin-cy3 cojugate incubated on the silanised and methoxy/biotin-PEG modified surface. Excess of this protein has to be washed out before taking images). Program fits this image the 3rd order 2D polynomial, normalize the polynomial to unity and divides each extracted trace by intensity of this polynomial in the corresponding location.

2. By fitting double Gaussian fit to the histogram of signal intensity. Then it normalises the signal to the center position of the first peak.

· Select type of normalization or switch it off by the pop-up menu in additional settings panel > pop-up menu "Normalization"

· Normalized traces will be saved in the results folder: 032_0_*.
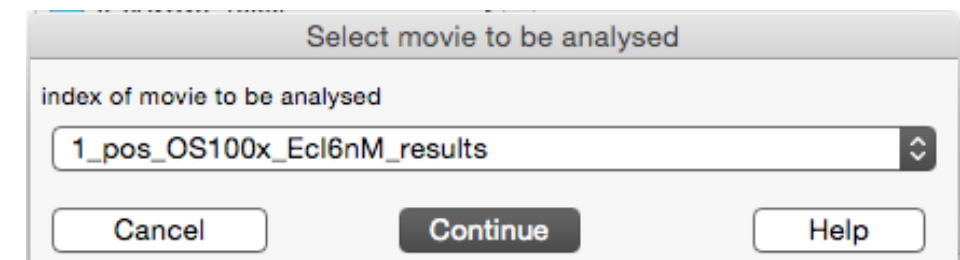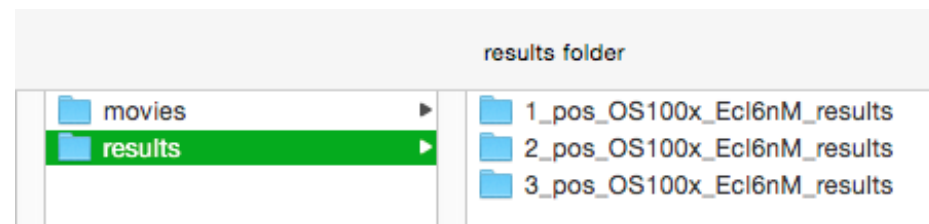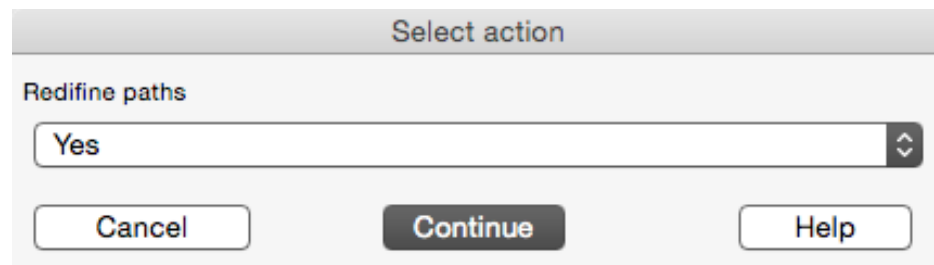
Auto sorting of the extracted traces

- If "exclude multi-molecules" check box is checked (additional settings panel > auto sorting parameters > check box "Exclude MM"), program tests whether the selected trace exceeds maximal single-molecule intensity (additional settings panel > auto sorting parameters > set variable control "Int.") and for how many points it is exceeded (additional settings panel > auto sorting parameters > set variable control "# pnts above thresholds"). If this trace has more points above maximum intensity than the defined threshold value, program sets indicator in the accRejTable for that trace to 0. Otherwise indicator is set to 1.

- If enabled (additional settings panel > auto sorting parameters > check box "Remove empty") program identifies empty traces and sets indicator to 1 in the accRejTable for each trace, which has number of interconnected points above defined intensity value (additional settings panel > auto sorting parameters > set variable control "# SD") higher than defined value (additional settings panel > auto sorting parameters > set variable control "# pnts above thresholds"). Otherwise, indicator is set to 0. Intensity level threshold is set by standard deviations calculated from 10 % counting from the end of the trace.

- Program sets indicator to 1 in the accRejTable for each trace which is inside the main ROI defined by x0, x1 and y0, y1 (additional settings panel). Otherwise, indicator is set to 0.

- It saves/updates table - 051_*: single column table, containing accepted (1) /rejected (0) indicators. Rows stands for trace index.

Characterization of the detected states

- If "Bleach." pop up menu (additional settings panel > state char. parameters > pop up menu "Bleach.") is selcted on "FRET", program detects when Efret trace bleached, and will characterize states only in the non bleached fraction of the trace. If the "non FRET" option is selcted, then you will have to provide "# SD" for bleaching step detection in the traces. Program will examine the trace from its end and will identify last state from begiing of the trace which has higher avg. int. value than the number of defined SD (calculated for 10% from the end of trace). If disabled program will characterise all of the detected states.

- Program can characterize states either for all extracted or only for selcted traces (additional settings panel > state char. parameters > pop up menu "Use").

- Program can skip characterization of partially defined states (additional settings panel > state char. parameters > pop up menu "Exclude partial states"). Four options are available: 1) none, 2) first, 3) last, 4) first and last.

- Based on the selection of above mentioned parameters program will calculate characteristics for detected states and will save them to the results folder

- It saves table - 050_*: several column table, containing inforamtion about detected states. Rows stands for state index. Column info: 0) "index" - current trace index , 1) "startX" - begining of state indicator [points], 2) "endX" - end of state indicator [points], 3) "dur" - duration of state [points], 4) "currInt" - avg. intensity/ Efret of the current state, 5) "fwInt" - avg. intensity/ Efret of the following state, 6) "amp" - amplitude of step (fwInt-currInt), 7) "sdev" - standard dev. intensity/ Efret of the current state, 8) "currEfret" - avg. Efret of state current state.

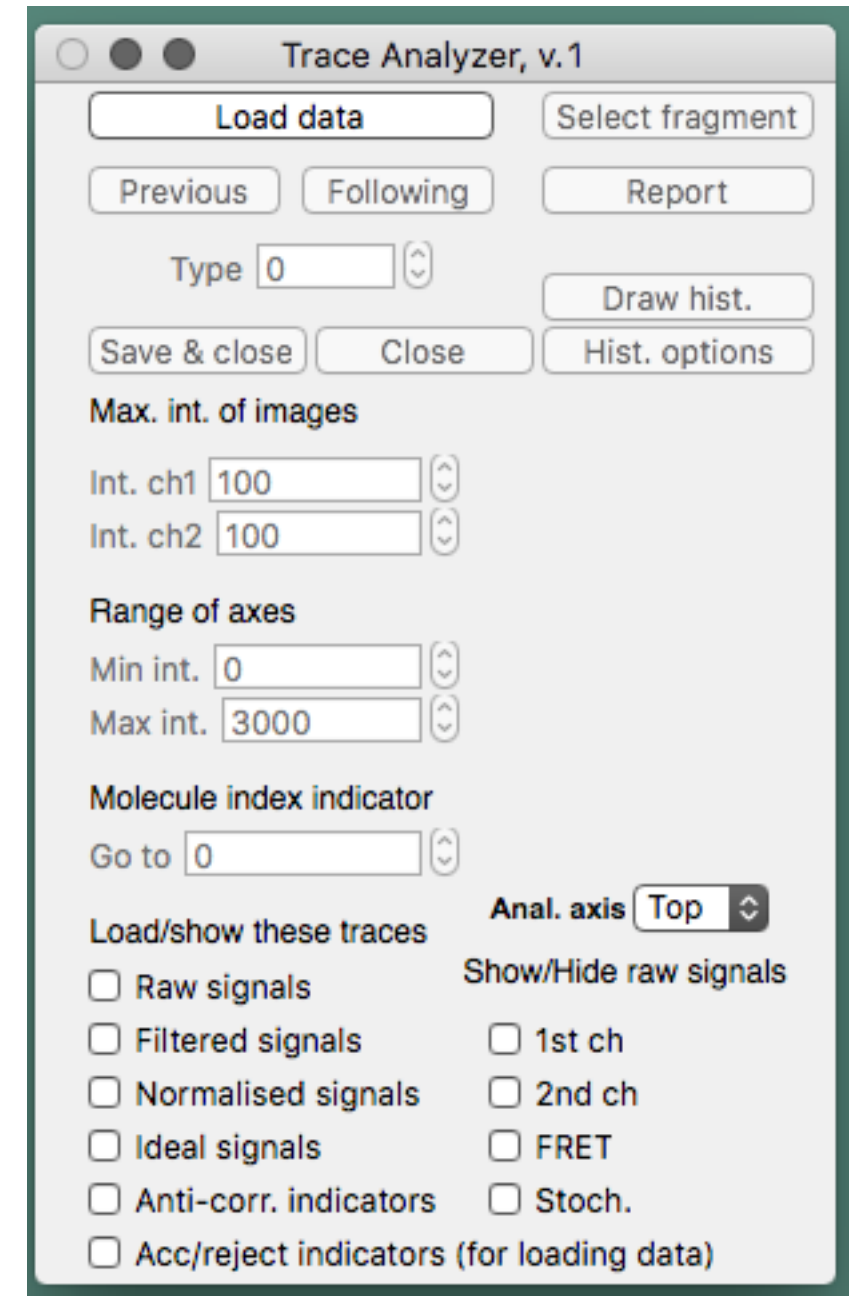# Manual examination/ selection of traces

- Explanation how to check out the results and modify accepted/rejected indicator table:

- Click "examine results manually" button in the main panel.

- If you are clicking this button for the first time in the pop-up menu select to redefine paths and click continue. Otherwise, click not to redefine paths and select one of the folder from previously defined main results directory.

- Navigate to main results folder containing results sub-folders for different positions or experiments (as shown in the second from top illustration) and click "choose" button.

- Next, in the pop-up menu select which of the sub-folder with results you would like to examine and click continue
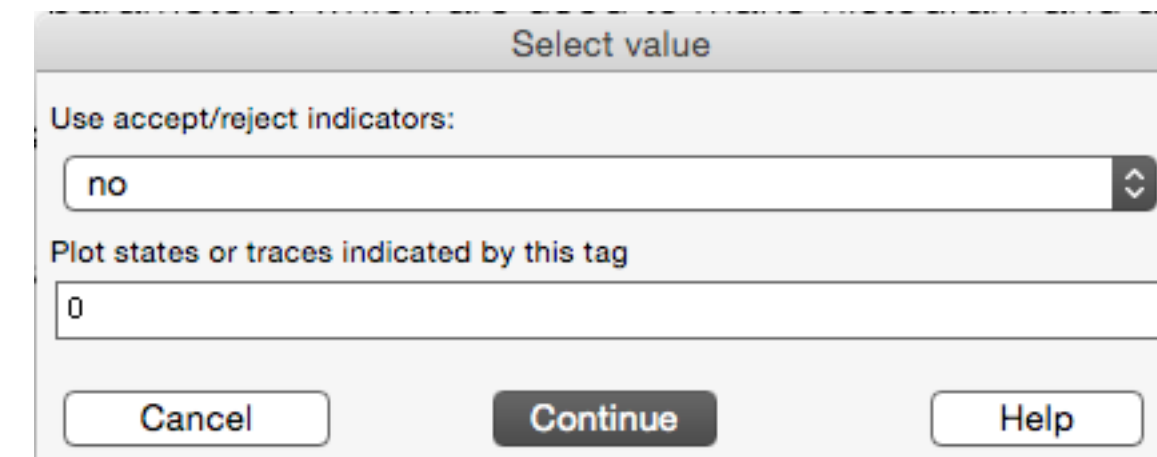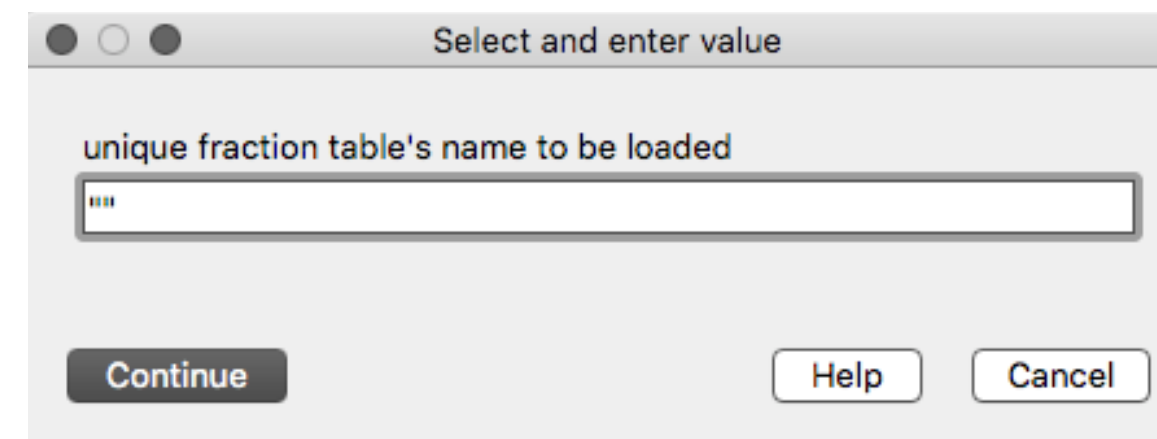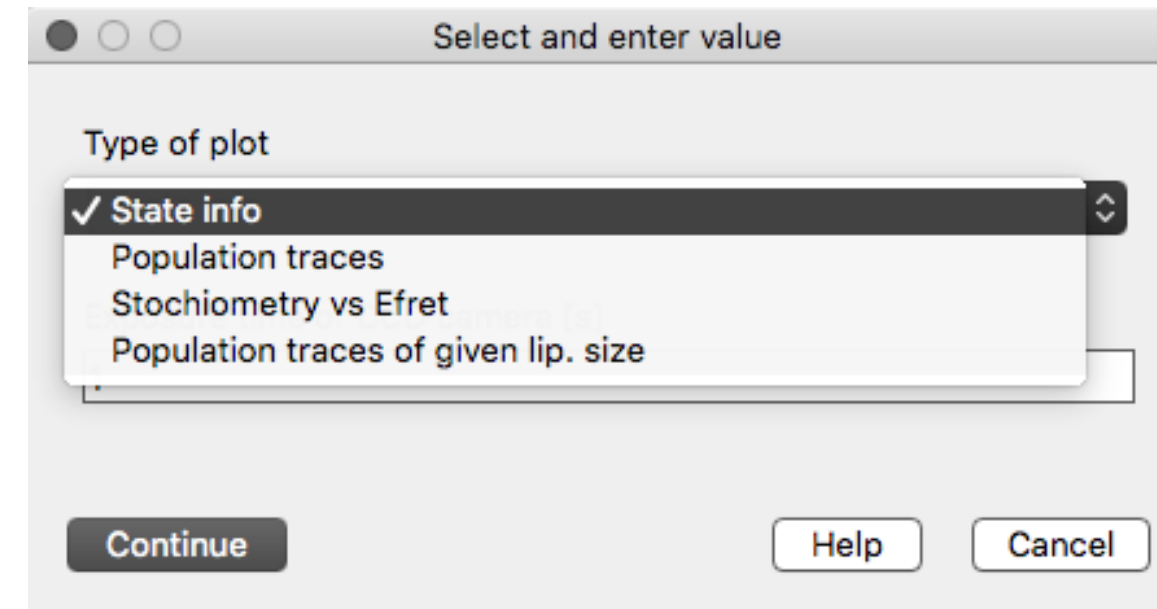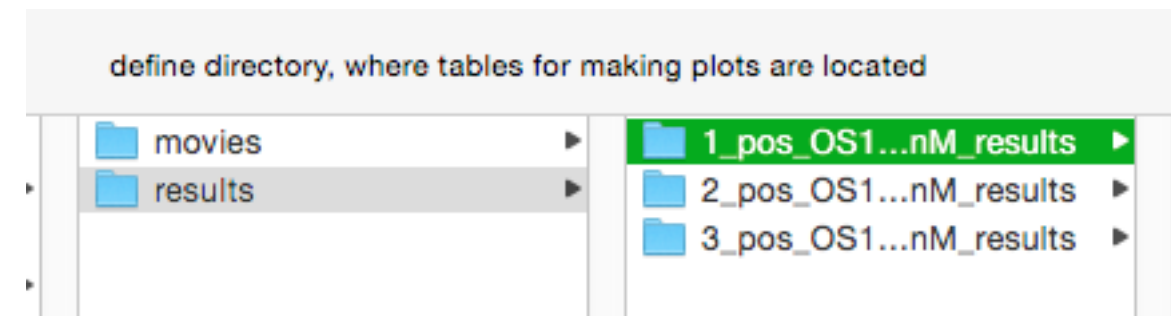
Manual examination/ selection of traces

- New control panel will appear:
- Select type of traces you would like to load: check-boxes on the bottom left corner of the panel. Also, if you would like to see which of traces has been auto selected or previously manually selected, you should check the "Acc/reject indicators…" option,
- Select from which of the channels signals you would like to see (check boxes in the bottom rigth corner),
- Select axis in which idealized signals are represented (you can also change this later on)
- click "load data" button and the first trace with corresponding fluorescent spot cropped image will appear.
- You can also draw histograms for shown signals and modify the histogram parameters (buttons on the top right corner).
- Use "previous" and "following" buttons to navigate through the traces. Or keybord arrows: 1) left - previous trace, 2) rigth - following trace, 3) up - type of trace indicator +1, 4) down - type of trace indicator -1
- Use "type" value entering field for changing the indicator value in the accepted/ rejected indicator table. This way you can add a tag for later sorting or plotting traces.
- Also, you can select fragment from the trace - click "Select fragment" button on the to right cornerr and use indicator in the bottom of the graph for saving begining and end of the fragments.
- Once you are done with examination of traces, click "Save data" button. It saves the accepted/ rejected trace indicator table with a name "051_*", slected fragments - "052_*" in the results folder and closes the examination windows.
- If you would like to quit the examination without saving what was done - click button "Close".

Trace Analyzer, v.1

Making plots

define directory, where tables for making plots are located

- Explanation how to produce 2D or 1D histogram plots (e.g. bottom left corner of this page):
- Clik "draw plots" button in the main panel.
- If you are clicking this button for the first time in the pop-up menu select to redefine paths and click continue. Otherwise, click not to redefine paths and select one of the folder from previously defined main results directory.
- Navigate to main results folder containing results sub-folders for different positions or experiments and click "choose" button.
- Select type of 2D histogram plot: state info, Population traces, Stochiometry vs. Efret, Population traces of given lip. size, and enter exposition time of CCD camera
- Enter unique fraction of table name (e.g. "050_" for state info table) and click "continue" button.
- In the following pop-up menu select whether you would like to use: accepted/ rejected traces, selected fragments of traces or all extracted traces. If decided to use acc/rej indiacotrs, type in value of the selected traces indicator.

Making plots

For state info or Stoch/ Efret plot:

- Select char. of state which will be plot on x and y axes, type in the title of plot and select whether to define a point in the plot as a regular pixel or to use Gaussian approximation. Finally enter number of bins for this plot.
- Specify minimum and maximum value of each axis, and if selected to enter the Gaussian width.
- You can choose to normalise each added point to the duration of state.
- Specify number of 1D histograms to make (on the sides of the 2D histogram plot). Original data which was used to make each 1D histogram is saved with this name: dataHoriz0_mapName or dataVertical0_mapName. If more than one histogram per axis was made, then you will have also dataHoriz1_mapName.
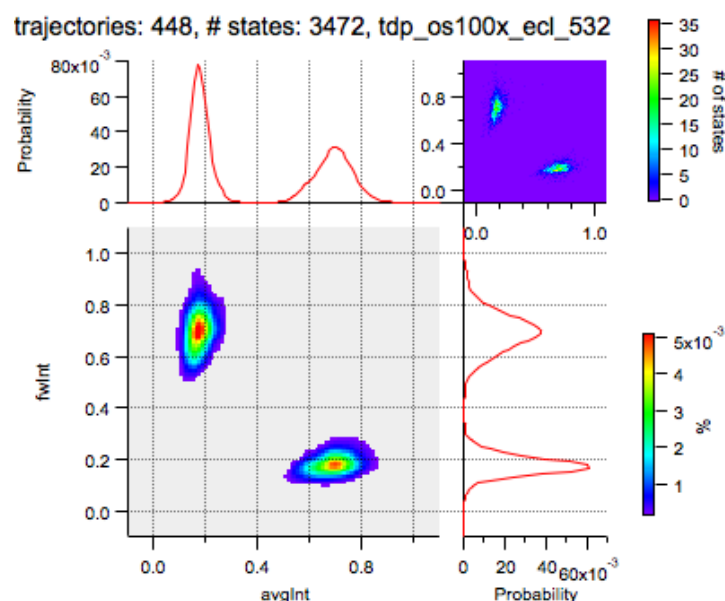
For population traces plot:

- Enter number of bins in x and y direction for this 2D plot, minimal and maximal value of Y axis, type in the title of plot and select whether to normalise each trace before putting in into the plot to unity or not (To avg. minimum;Double Gauss fit - using first peak center position;No). The plot will be normalised to PDF.

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Analysis of many movies at once

- Once you have identified satisfying parameter values, you can execute analysis of multiple movies in one go.
- You can define what parts of the analysis you would like to perform at this time, select/unselect check-boxes on the rigth bottom corner of the main settings panel.
- Click "Analyze many" button and navigate to the main folder with image series, click "choose" button, then navigate to the main folder for storing results and click "choose" button.
- Program will generate results sub-folders having the same name as the movie sub-folder and will add "_results".

Combining results tables of many movies

- Once analysis is done, you may want to make a single table containing all extracted traces from different positions or conditions of experiment.
- Click button "Combine tables" (main panel), and navigate to the main results folder containing results sub-folders with tables, which you are willing to combine.
- Click "Choose" and select in the new pop-up menu whether it is a state info table (it applies for the acc/rej indicator table as well) or traces table, and enter unique fraction of table name (e.g. 030_0_ for raw Efret traces) and click continue.
- Program will combine defined tables and generated combined table will be stored in the main results folder.