TAMT - Intensity versus time trace of fluorescent spot extraction and analysis software

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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,
  - fluorescent spot from 2 channels co-localization,
  - intensity versus time traces extraction for selected type of fluorescent spots,
  - filtering of traces
  - noramlization 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 single-molecule data formats.
- Software has internal memory for entered parameters, size of windows and their positions.
- Each part of the program gives output as tables of igor binary format. There is a additional function in the main panel to convert them into delimited text format.
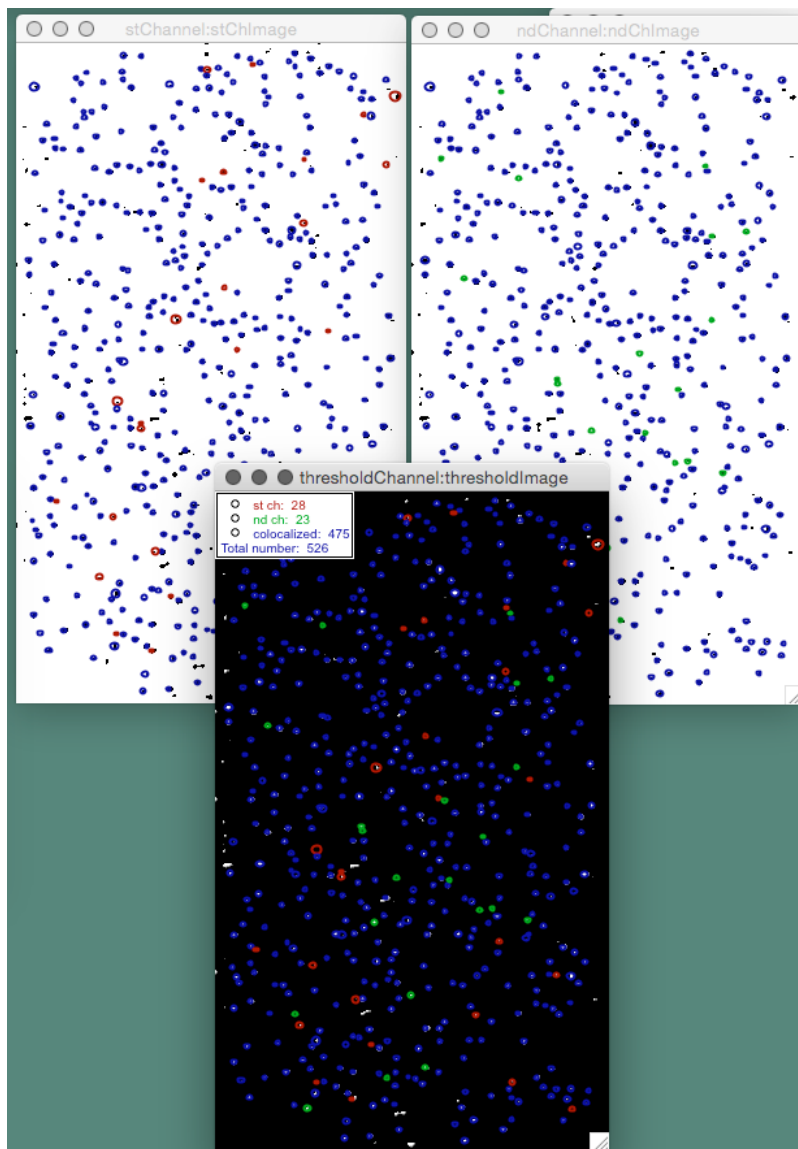- Software development was under MacOS, therefore it may happen that Windows users finds some small problems.
- I am making updates of software constantly therefore feed-back is highly appreciated: marijonas@ar.fi.lt or marijonas.tutkus@gmail.com

Installation

- To install this software just copy paste the entire TAMT 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. Then you will be able to 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. This allows you to use "Analyse many" button (see page #15). 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 su-sub-folder and the movie in first of the sub-sub-folders.
- 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 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 #8) 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").
- Once this done, you can click "Analyse single" button (main panel) and program will ask to define folders containing images. Once folders are defined, you can check out the output (page # 13 and # 6-7) of each of the analysis part with these images and adjust parameters to achieve best performance.

Recomendation for image distribution

**main folders**   **sub-folders**   **sub-sub—folders**
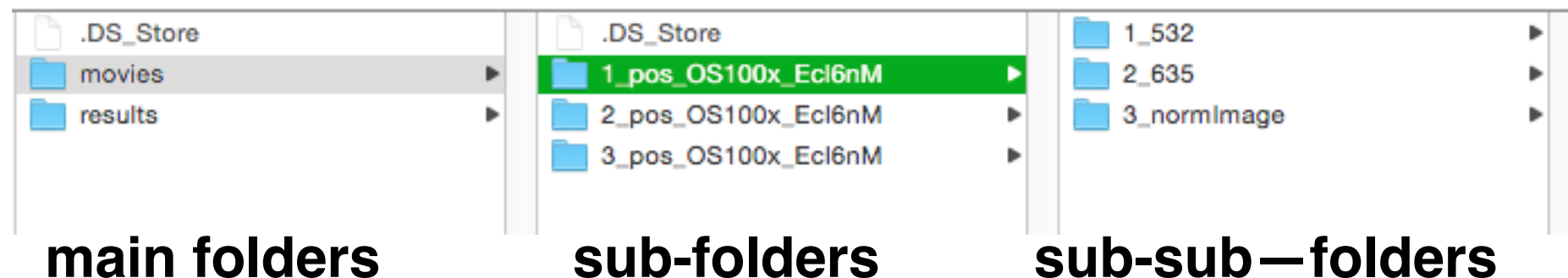
Alignment of dual-view or dual-camera

- To enable alignment of the 2nd channel image in the TAMT you have to select this option in the additional panel pop-up menu entitled "Aligning 2nd ch image". 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 "align channels" button (main panel). It performs image registration (see Igor Pro manual) on the binarized (threshold applied) reference images.
- You will be asked to navigate into the main directory containing sub-folders for the different positions and in these sub-folders you should have sub-sub-folders 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.
- 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 rigth section of the additional settings panel.

Arrangement of files
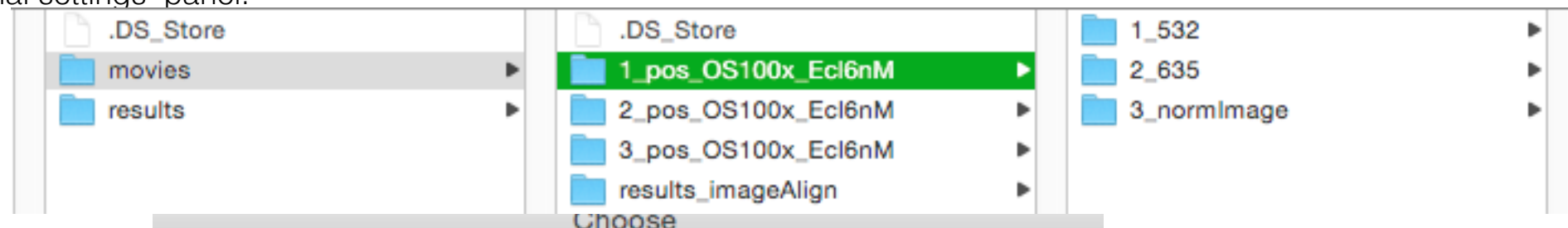
Options for alignment
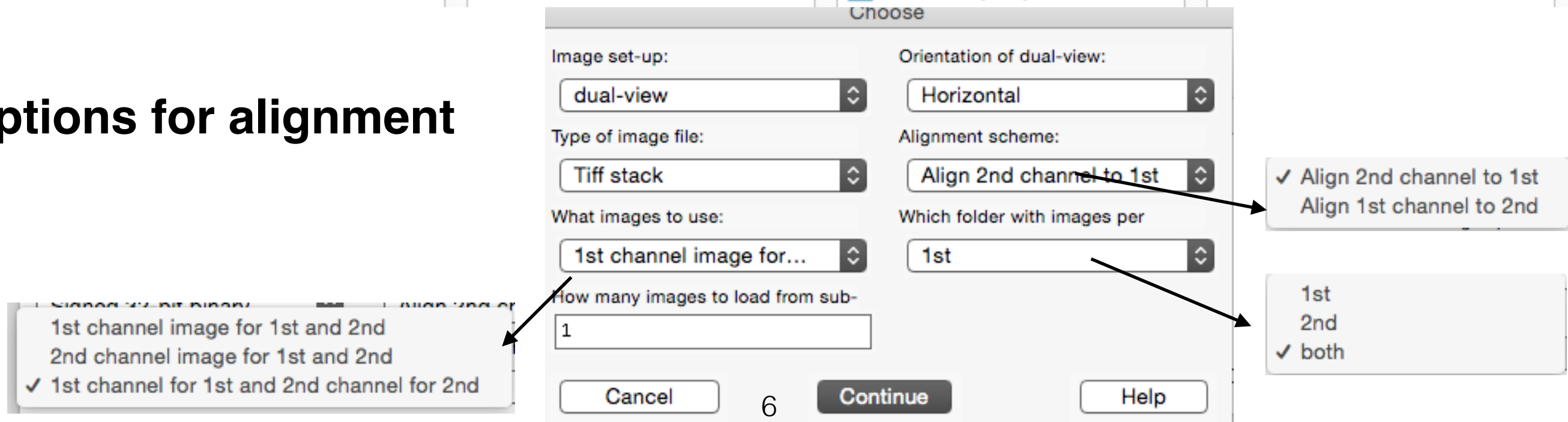
Detection of fluorescent spots in 2 channels and their co-localization

- 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 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 (additional settings panel > image filtering parameters > # images avg. ch1 or ch2)
  - If enabled, subtracts background from this average image by the rolling ball algorithm (additional settings panel > checkbox "Remove backg from ch1 image or ch2 image". See ImageJ reference) with the given ball radius (additional settings panel > image filtering parameters > "ball radius ch1 or ch2"). Typical value of ball radius for 512 x 512 image is 13 pix.
  - if enabled, further performs smoothing of the image using Gaussian convolution (additional settings panel > checkbox "Filter ch1 or ch2 image by Gaussian"), using given mask width (additional settings panel > image filtering 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 (additional settings panel > checkbox "Filter ch1 or ch2 image by low-pass filter"), using given freq. cut-off (additional settings panel > image filtering parameters > "Freq. ch1 or ch2"). Frequency can take values from 0 till 0.5. I am typically using 0.35.
- Next step is 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 > min area ch1 and ch2) and then binarize it using given threshold (main panel > 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 > 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.

Benefit of this procedure - spots of different intensity and high density are easier detectable.

- if selected, program will perform 2D Gaussian fitting (additional settings panel > 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 > Dev. of fit ch1 and ch2). You should also specify crop radius for Gaussian fitting (main panel > Crop size for fitting). It has to be as big as a half of largest spot of interest.
- Next, program will co-localize the detected spots in 1st and 2nd channels, with given co-localization distance (main panel > Co-loc. distance). Overlaping spot removal: if there is 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_allIdentifiedPartInfo.ibw. 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 > pop-up menu "make traces from") by the 2D Gaussian fitting with given crop radius for fitting (main panel > Crop size for fitting). During the fitting procedure center and width of Gaussian can be either constant or varying (additional settings panel > pop-up menu "Vary Gauss width" and "center"). In the end intensity can be saved as Gaussian amplitude or Gaussian integral: amplitude * width (additional settings panel > pop-up menu "trace intensity").
- You should also define number of images to extract intensity from (additional settings panel >"# 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 > pop-up menu "extract traces from").
- Gaussian fitting is performed on multiple processors so that each particle is fitted on separate core. This speeds up the processes in comparison to multi-core 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 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_partContTrExtract. It is the same type of table as in the spot detection.

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_ (Efret) , 031_1_ (ch1), 031_2_ (ch2).
- Before saving Efret traces are recalculated from filtered ch1 and ch2 traces.

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.
- Program can normalize the extracted filtered traces 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.
- Switch normalization on or off by the pop-up menu in additional settings panel > pop-up menu "normalize traces"
- Normalized traces will be saved in the results folder: 032_0_ (Efret) , 032_1_ (ch1), 032_2_ (ch2). Efret traces will not be normalized.

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

- For FRET efficiency traces, program automatically detects time point, where acceptor and donor bleached. It analyses only non bleached part of this trace.
- There are two methods for ICP detection in this software: Bayesian Factor (expression rewritten in the form of natural logarithm) or Hidden Markov Modeling (as in the original article, just parameter optimisation based on Metropolis or Simulated annealing algorithm). Choose which of them to use in the additional settings panel > pop-up menu "ICP detection". In the case of Bayesian factor ICP detection method you have to provide threshold value of Bayesian factor (from 0 till infinity, in main panel > "ICP detection parameter") and minimal amplitude of step (if "refine detected ICP"checkbox in the additional settings panel is selected). In the case of Hidden Markov Modeling, you have to define groups of intensity by clicking the "Define intensity groups" button on the bottom right of the main panel. It will ask you to save the table (000_groupsInfoTable.ibw) with intensity group info (max and min intensity of group) in the result folder.
- Output of the detection is table containing idealised traces. It will be saved in the results folder: 033_0_signal_IdealTr.ibw.

My recommendation is to use Bayesian ICP detection whenever possible.

More info on Bayesian Factor ICP detection: Bayesian Detection of Intensity Changes in Single Molecule and Molecular Dynamics Trajectories. Ensign et al., J. Phys. Chem. B 2010.
More info on Hidden Markov Modeling: Analysis of Single-Molecule FRET Trajectories Using Hidden Markov Modeling. McKinney et al., Biophysical Journal, 2006.

Auto selection of states and traces

- This part of program performs several tasks:
  - If "exclude multi-molecules" check box is checked (additional settings panel), program tests whether ch1 or ch2 trace exceeds maximal single-molecule intensity (additional settings panel > multi-molecule intensity ch1 and ch2) and for how many points it is exceeded (additional settings panel > # pnts above MM). 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 "test anti-correlation" check box is checked (additional settings panel), program checks whether detected ICPs in the Efret trace has anti-correlated intensity change of ch1 and ch2 trace at the same spot. Amplitude of the intensity change in ch1 and ch2 has to be above defined threshold value (additional settings panel > anti-corr amp. ch1 and ch2). ICP satisfying this criteria has a value of 1 in the stateInfoTable column labeled "antiCorr".
  - 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 these tables in the results folder:
  - 050_stateInfoTable.ibw: rows corresponds ICP index, column different information - index of trace, start point of state and its end point, duration, average intensity, average intensity of following state, standard deviation and anti-correlation indicator.
  - 051_accRejAutoIndic.ibw: single column table, containing accepted (1) /rejected (0) indicators. Rows stands for trace index.
  - 034_0_antiCorrTr_Acc.ibw - table containing markers for anti-correlated ICP location.
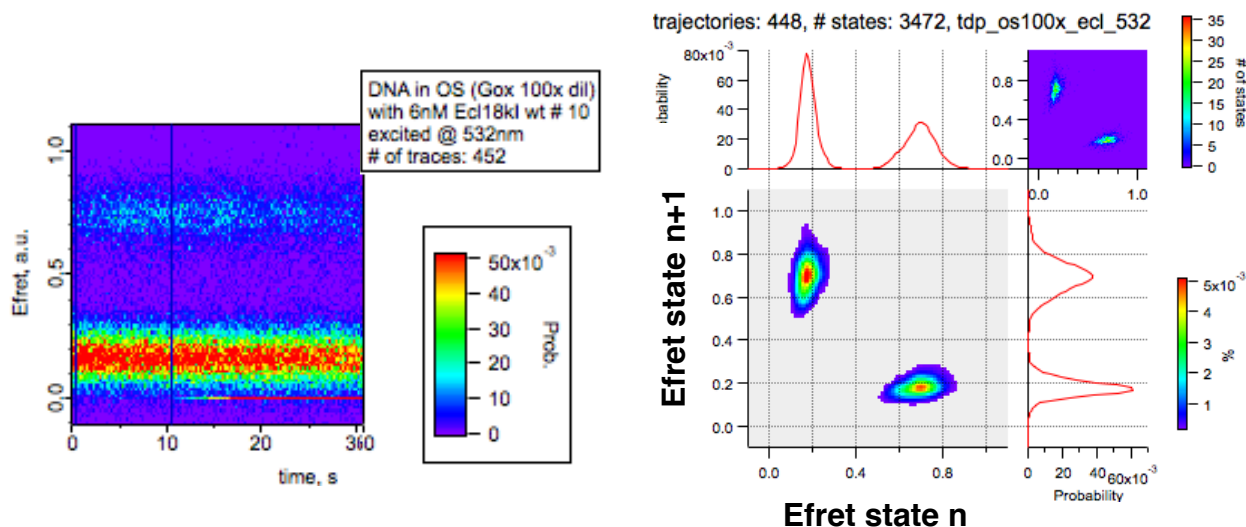
Manual examination/ selection of traces

- Explanation how to check out the results and modify accepted/rejected indicator table:
  - Click "examine results manually" button in the main panel.
  - Then 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
  - New control panel will open:
    - Select what type of traces you would like to load:check-boxes on the bottom left corner of the panel.
    - Next, click "load data" button and the first trace from table with corresponding fluorescent spot cropped image will appear.
    - You can also select which signals you would like to use for histograms and enter the histogram parameters, which are used to make histogram and adjust the axis limits.
    - Use "previous" and "following" buttons to navigate through the traces.
    - 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.
    - Once you are done with examination of traces, click "Save data" button. It saves the accepted/rejected trace indicator table in the results folder with a name "051_accRejAutoIndic.ibw".
    - Then click button "Close" to close the opened windows.

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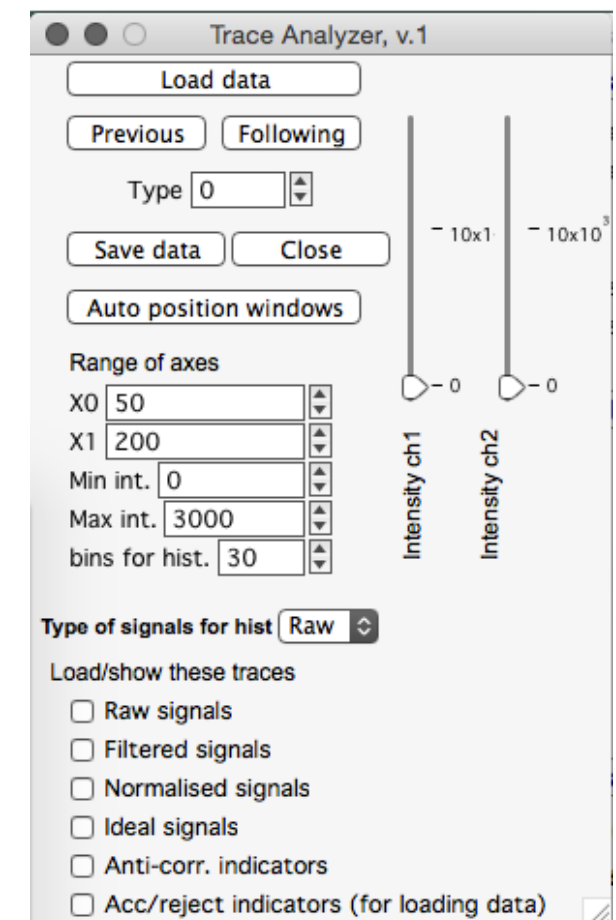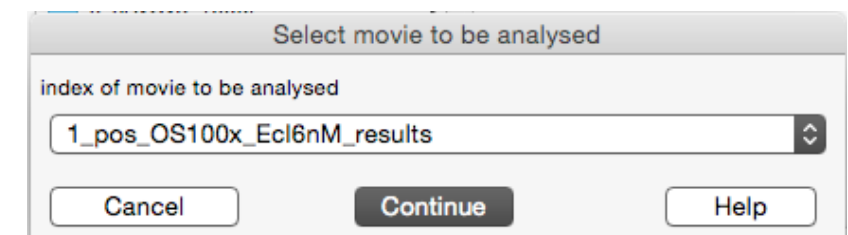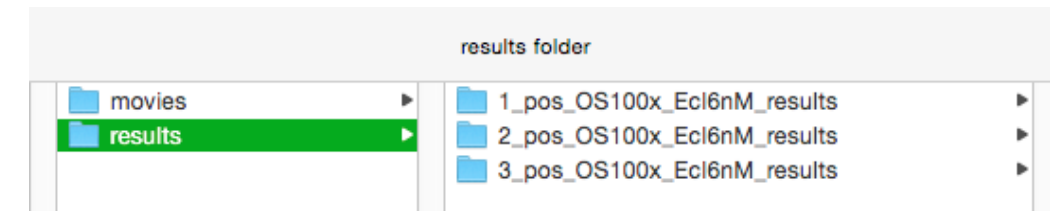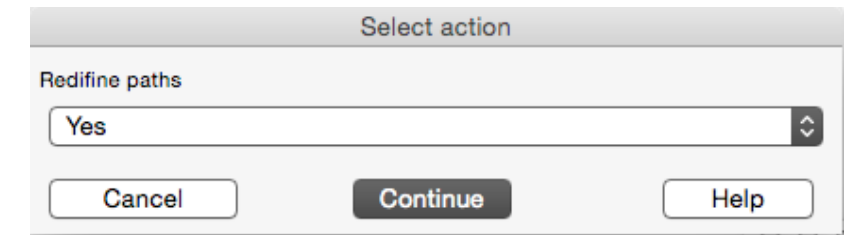
Making plots

- 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.
  - Then select type of 2D histogram plot - overlaid traces or state info, enter exposition time of CCD camera and 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 indicator table, and if decided to use it type in value of the accepted traces indicator.
  - For state info plot
    - In the new pop-up menu select information of state which will be plot on x and y axes, type in the title of plot and select whether you would like to define a point in the plot as a regular pixel or to use Gaussian approximation. Finally enter number of bins for this plot and select whether you would like to draw only anti-correlated states (passed anti-correlation test in the auto selection part, page #12).
    - In the following part you will be asked to 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.
    - Finally you will be asked to specify number of 1D histograms to make which will be added on the sides of the prepared 2D histogram plot, and to specify specify their range. 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 and so on.
  - For overlaid traces plot
    - In the new pop-up menu 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.
- All of the plots are normalised to their integral.

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Automatical analysis of many movies

- 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 left bottom corner of the additional settings panel.
- Click "Analyze many" button and 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".

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.