MATLAB Code for Scanning fluorescence cross-correlation spectroscopy (scanning FCCS) analysis

System requirements: MATLAB 2020a or newer, at least 16 GB RAM system

Analysis pipeline:

Export

- Export acquired .czi line scan files as RGB .tif in *Zen Black* Software (e.g. version *2.3 SP1*). Save one .tif file per 2-channel measurement and make sure that the channels are saved according to RGB channels (i.e. the GFP channel ("*channel T1*") is saved in the green channel and the mCherry channel ("*channel T2*") is saved in the red channel).

Analysis

- Add subfolders (*Functions* and *Bioformats*) to path.
- Run main analysis script *analysis_2color_sFCS_VD.m.*
- Select exported .tif file of the measurement that you want to analyze.
- Select corresponding .czi file to load all metadata (e.g. line acquisition time) into workspace.
- Optional: Click through the kymograph using the GUI (*Intensitycarpets2ch*) and remove transient bright signal, e.g. appearing due to vesicles, if close to the membrane. Then click *Done*. Otherwise click *Done* directly.
- In *Figure1: Line Fluorescence – define ROI*: Do polygonal selection of membrane section in kymograph for green channel. Exclude bright intracellular structures (e.g. vesicles). Make sure to start and close the rectangle outside the plot window. Afterwards, if background correction is needed (*backgroundcorrectionCh1/…Ch2=1*), set whether the left or right side contains stronger background signal. Then do polygonal selection that encloses the background signal on the side of the membrane line, again selecting a window containing all time points. As a guide to the eye, use the plot of the time-averaged fluorescence for the first and second half of the measurement. Next, repeat the same steps for the red channel. (Note: The background region that is selected is only used to set the initial parameters for the block-wise background correction fit. Don't worry if you have selected *left* or *right*, the fit usually converges well in any case).
- Select directory where output files shall be saved
- If background correction is applied: The background fit and Gaussian component after background subtraction are shown for the first block in the first channel. Just press *space*. Afterwards, the histogram of fitted waist values (in units of pixels) is shown, again for the first channel. The average waist is fitted and calculated in physical units. It should be slightly larger than the waist of the PSF (typically 0.2-0.3 µm). Press space, then repeat the steps for the second channel.
- The lateral alignment of the kymographs proceeds and the selected regions are displayed.
- The bleaching correction is performed. As part of it, the GUI *Intensity2colors_new* pops up and allows you to remove intensity segments (e.g. bright peaks) that may distort the double-exponential fit in channel 1 or 2. Remove those segments if occurring and press *Done* afterwards. Press *exponential* unless you want to use a higher order polynomial correction. The fit will be determined for each channel and the bleaching correction applied.
- Raw correlation functions are plotted.
- The correlation functions are now calculated in segments and plotted as overlay.
- A GUI *Corrselection2Channelsindividualpreview_new* pops up allowing you to remove segments from the analysis. For both channels, evaluate the average CFs (solid lines) and scatter of points (CFs of the segment) as well as intensity traces (fluctuating around average

or long-term oscillations/ deviations?) to keep or remove segments with associated buttons. As a guide to the eye, the fits to ACFs and CCF are shown on the right side for the average CFs from all kept segments. Iteratively go through the segments and confirm with *Done* button at the end of the inspection. For stable measurements, very few or no segments need to be removed. For an illustration of the procedure, watch the video associated to Dunsing&Chiantia, JoVE 2018, doi: 10.3791/58582

- The final fitting is now performed and the segment-averaged correlation functions plotted together with particle numbers, diffusion times and the relative cross-correlation.
- Final graphs and output files (e.g. fit parameters) are saved in specified directory.

Pooling of SFSCS results from multiple analyzed measurements

- Run scripts *two_species_FCS_parametertxtfiles_pooled.m* to pool fit parameters from multiple analyzed files. Load the files from the same directory
- The script generates a table with header saved as a .txt file, containing Ns, rel.cc.'s, tau's, brightness values, and bleaching fractions