FRETcalc v4.0 plugin for analysis of FRET by acceptor photobleaching

David Stepensky
Department of Pharmacology
Ben-Gurion University of the Negev, Beer-Sheva, Israel
davidst@bgu.ac.il

1. Introduction

FRETcalc plugin for ImageJ program allows pixel-by-pixel analysis of FRET by acceptor photobleaching. FRETcalc uses thresholding $FRET_{TH}$ algorithm to exclude from data analysis pixels that do not match user-defined thresholds of donor and/or acceptor fluorescence intensity, bleaching efficiency, and %FRET. Subsequently, FRET signal-containing pixels on the analyzed images could be separated from the fluorophore-free background pixels, making possible analysis of FRET signal arising from non-continuous intracellular organelles/compartments (e.g., endoplasmic reticulum, endosomal system, etc.). Effect of the threshold-based pixel selection on the estimated FRET values can be determined by comparing $FRET_{TH}$ and $FRET_{ALL}$ (that is calculated using all the pixels in the selected region of interest (ROI)). The plugin allows easy visualization of the analysis results (as plots and/or histograms), thus helping to estimate the statistical robustness of FRET analysis. FRETcalc plugin, its validation, and application to study protein-protein interactions in the MHC class I loading complex in the endoplasmic reticulum has been described in: Stepensky D. FRETcalc plugin for calculation of FRET in non-continuous intracellular compartments. Biochem Biophys Res Commun 2007; 359(3):752-8.

2. Installation

Install ImageJ program (http://rsb.info.nih.gov/ij/). Copy FRETcalc_.java to the plugins folder, or subfolder, compile and run it using Plugins/Compile and Run. Restarting ImageJ will add a "FRETcalc" command to the Plugins menu or a submenu of the Plugins menu. FRETcalc was tested for ImageJ version 1.37c.

3. The format of the analyzed images

Prior to running the FRETcalc plugin, stack image with experimental data should be opened in ImageJ. The stack image should be 8-bit Tiff file and should include slices with Pre- and Post-bleach images for donor and acceptor. FRETcalc_sample.tif file attached to this manual contains the images in the required format and could be used for testing and learning the FRETcalc plugin.

4. FRETcalc plugin menus: settings & options

4.1 Image manipulations

Bg subtraction – subtracts the average intensity of the pre-defined ROI from each pixel on the selected slice/s.

Smooth – applies 3x3 filter to smooth all the slices of the image, is equivalent to Process->Smooth command.

LUT->Rainbow2 – changes the LUT of each slice to Rainbow2; is recommended for easy visualization of the images with low fluorescence intensities. Requires rainbow2.lut file in the ImageJ LUT directory.

Px by Px subtract – performs pixel-by-pixel subtraction of one slice from another, could be used to visualize the difference between Pre- and Post-bleached images for donor or acceptor. The subtraction result is added as a slice at the end of the original stack file.

4.2 Settings

Pre- & Post-bleach images – specifies the location of the images in the stack. The default settings could be used directly with the FRETcalc_sample.tif file.

Settings for data analysis

Donor and acceptor thresholds – only pixels above the specified thresholds would be used for analysis. The threshold values should be in the 0-255 range for the 8-bit images.

% bleached thresholds – allows exclusion of pixels with %bleaching of the acceptor that are not in the range of the specified thresholds. 'Use %bleached thresholds' option should be selected to use these thresholds in image analysis.

%FRET thresholds – allows exclusion of pixels with values of %FRET that are not in the range of the specified thresholds. 'Use %FRET thresholds' option should be selected to use these thresholds in image analysis.

4.3 Output options

Plots – visualization of the donor or acceptor intensities, %bleached for acceptor, and %FRET for the pixels that matched the applied thresholds

and gave rise to $FRET_{TH}$ value. %bleached and %FRET plots are added as slices at the end of the original stack file. All the other plots appear in separate windows.

Histograms – display the donor and acceptor intensity histograms (Post- and Pre-bleach, respectively), and $\%FRET_{TH}$ histogram for the pixels that matched the applied thresholds and gave rise to $FRET_{TH}$ value.

Tables – outputs the thresholds and calculation results for the selected ROI (Summary Results Table) and/or for individual pixels (Raw Results Table).

Thresholds: Donor thresholds (min and max), Acceptor thresholds (min and max), %bleaching (min and max), %FRET thresholds (min and max).

Summary results: DPre & DPost - donor Pre- & Post-bleach (median values). APre & APost - acceptor Pre- & Post-bleach (median values). %bleach_th and %FRET_th - median acceptor photobleaching efficiency and $FRET_{TH}$ values for the pixels in the selected ROI that matched the applied thresholds. %bleach_all and %FRET_all - median acceptor photobleaching efficiency and $FRET_{ALL}$ values for all the pixels in the selected ROI.

Raw results: Xstart, Ystart, DPre, DPost, APre, APost, %bleach, and %FRET: location, pixel fluorescence intensities, acceptor photobleaching efficiency, and %FRET for the individual pixels in the selected ROI that matched the applied thresholds.

Important! %bleached and %FRET plots show only subset of pixels that could be plotted on 8-bit images (i.e., pixels with values within 0-100% range. Pixels with negative values and higher than 100% are plotted as 0). **These plots are useful for visualization purposes only and should not be used for calculations**! The plotted values are presented on the 8-bit (0-255) scale; i.e., 0 corresponds to 0% FRET, 128 to 50% FRET, and 255 to 100% FRET. Histograms and tables include all the pixels that were used in the $FRET_{TH}$ calculation in their original scale, including pixels with values below 0% and higher than 100%.

4.4 Calculation

Calculate – performs the calculation with the applied settings & output options. FRET is calculated from the sum of the individual pixel's fluorescence intensities (I) as:

$$\%FRET = \frac{I_{DonorPostBleaching} - I_{Donor\,Pre\,Bleaching}}{I_{DonorPostBleaching}} \cdot 100\%$$

For $FRET_{ALL}$ the calculation is based on sum of the fluorescence intensities of all the pixels in the selected ROI, and for $FRET_{TH}$ on the sum of the fluorescence intensities of the pixels that passed the thresholds specified in *Settings*.

Important! In earlier versions of FRETcalc (v. 1.0 and 2.0) FRET values were calculated as the median of individual pixels' FRET values, and not from the sum of the fluorescence intensities. Although both approaches could be used for FRET calculation, the later approach is less sensitive to the background noise and is expected to be more robust and reliable for estimation of FRET values in the analyzed images.

4.5 Other buttons

Help – opens the help window

Save Image as – allows saving of the active image file only, Histograms, Results Table & ROIs should be saved separately

Close – quits the FRETcalc plugin

5. Performing the analysis

1. Open the stack file with analyzed data (donor and acceptor images Pre- & Post-bleaching)
2. Open the FRETcalc plugin (Plugins->FRETcalc)
3. Smooth all the images (optional)
4. Settings:
   Check the locations of the images in the stack (slice numbers)
   Input the donor and acceptor thresholds (use 15 and 15 for the FRETcalc_sample.tif file)
   Change the %bleached & %FRET thresholds (optional)
5. Output options:
   Change the output options (optional)
6. Define region of interest (usually polygonal ROI) on the active image that will serve for analysis
7. Press the calculate button to perform the analysis
8. $\%FRET_{TH}$ and $\%FRET_{ALL}$ values appear in the Summary Results Table (if this output is selected in Options). Robustness of $FRET_{TH}$ analysis could be assessed from the $\%FRET_{TH}$ histogram and $\%FRET_{TH}$ plot. Reanalyze the same images with different settings/options/ROI selection, if required.