Pixel by Pixel analysis of sensitized-emission FRET with the ImageJ PixFRET plug-in

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I - Introduction.

Fluorescence Resonance Energy Transfer (FRET) is a technique used to investigate interactions between fluorescent partners that has gained great interest for cell biologists with the recent introduction of auto-fluorescent proteins which can be coupled to a protein of interest to produce a fluorescent chimera.

A plethora of methods exist to evaluate FRET, depending on the protocol used (sensitized emission, acceptor photobleaching, fluorescence lifetime) and the precision that is pursued (see (1,2) for reviews).

The ImageJ plug-in PixFRET allows to generate images of sensitized-emission FRET, and hence to visualize where FRET occurs within a cell or a population of cells, by computing pixel by pixel the images of a sample acquired in a three channel setting, according to the formulas and the methodologies described by Gordon et al. (3) and Xia and Liu (4). The plug-in allows either to enter manually the spectral bleed-through (SBT) parameters for the donor and the acceptor, or to use stacks of FRET and donor or acceptor images to determine these values. Moreover, the type of normalization of the FRET values can be chosen by the user among 4 possibilities: by dividing by the donor intensity, by the acceptor intensity, by the product of donor and acceptor intensities or by the square root of the product of donor and acceptor intensities.

II – Plug-in installation.

The PixFRET plug-in runs under ImageJ, a freeware downloadable at http://rsb.info.nih.gov/ij/ and has been tested for version 1.33. To install PixFRET, download the PixFRET_.jar file from the website http://www.unil.ch/cig/page16989.html and copy it to the "plugins" folder in the ImageJ program.

III - Images used by the PixFRET plug-in.

Before starting the PixFRET plug-in, different stacks of images must be created:

i) For FRET analysis, a stack of three images of the cell or group of cells to analyze: the first one in the FRET setting (excitation of the Donor, detection of the Acceptor), the second one in the Donor setting (excitation of the Donor, detection of the Donor) and the third one in the Acceptor setting (excitation of the Acceptor, detection of the Acceptor).

ii) For Donor Bleed-Through (BT) determination (see also chapter V for more information about SBTs), one stack of two images of cells expressing the Donor only: the first one in the FRET setting, the second one in the Donor setting.

iii) For Acceptor Bleed-Through (BT) determination, one stack of two images of cells expressing the Acceptor only: the first one in the FRET setting, the second one in the Acceptor setting.

1. Create a stack of 3 images for each cell or group of cells on which FRET will be analyzed (cells expressing both the Donor and the Acceptor).

   a. Open the images in the following order: FRET image, Donor image, Acceptor image.

   b. Convert images to stack:
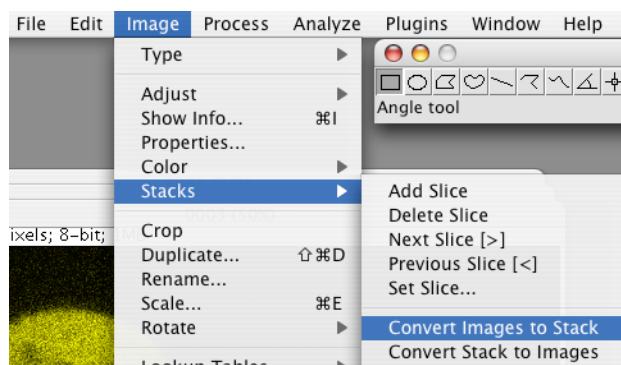
   c. Save the stack as a TIFF file.

   d. If the SBT parameters have been determined offline, go to point III.

2. Create a stack for Donor SBT determination (cells expressing the Donor only), opening images in the following order: FRET image, Donor image.
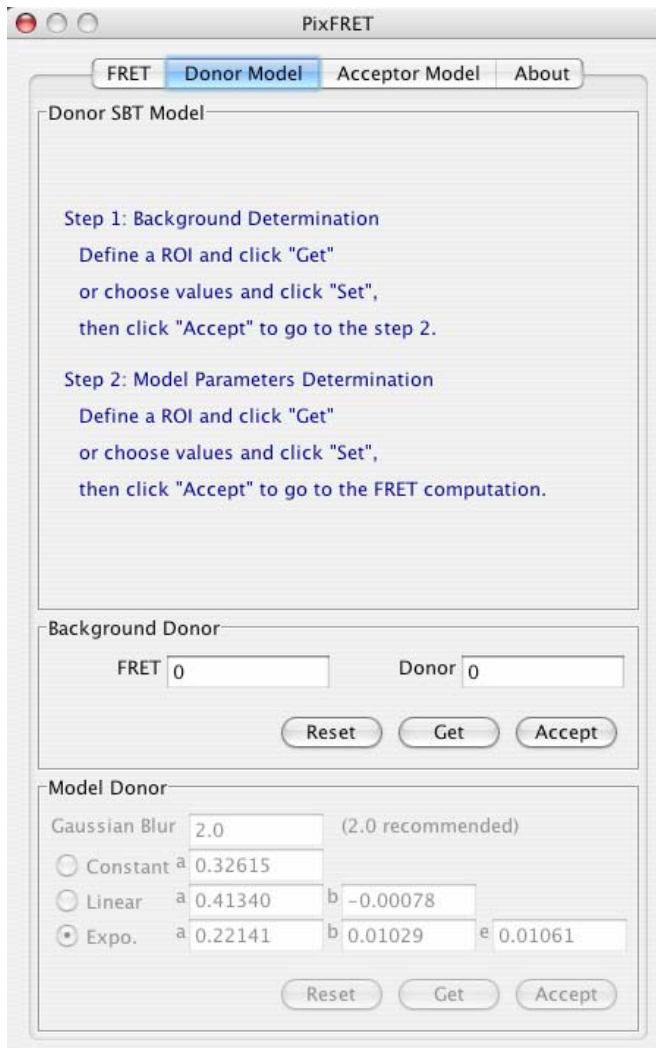
3. Create a stack for Acceptor SBT determination (cells expressing the Acceptor only), opening the images in the following order: FRET image, Acceptor image.

IV – FRET calculation and display.

1. Launch PixFRET from the plugins menu
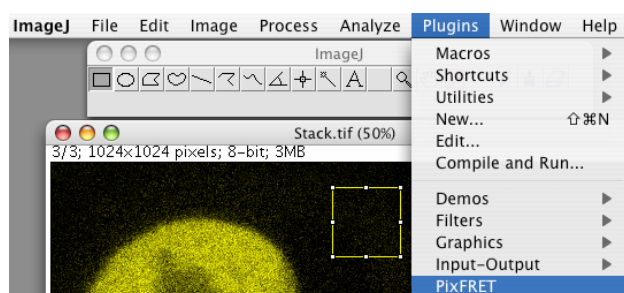
2. Click on the "Donor Model" bookmark, to enter or acquire donor SBT parameters.


3. If SBT parameters have been determined offline, clicking "Accept" in the background dialog box allows to directly select the appropriate model for donor SBT estimation in the dialog boy beneath (see chapter V for more details) and to enter the values manually. Click on the "Accept" button and then skip to point 8.

4. Open the stack for Donor SBT determination.

5. Select a region-of-interest (ROI) to determine the background (BG) outside cells and click on the "Get" button. The BG will be determined in all images of the stack at the same place. The average BG intensity values in the ROI are displayed in the two boxes below. You can reset values to zero by clicking the "Reset" button. You can also enter manually these values. Once these values have been entered (by the plug-in or manually), click on the "Accept" button to move on to the SBT determination.

6. Select a ROI in which the SBT will be determined and click the "Get" button (see V. for more explanations about SBT ratios). We generally use a ROI covering the whole image for SBT determination. However, in images where saturated pixels are present, more restricted areas must be used. Note that SBT ratios should be constant. However, we noticed that under some experimental circumstances, SBT ratios vary with fluorophore intensity in a manner that could

be fitted with a linear, or an exponential model (see figure below, constant value in red, linear fit in blue, exponential fit in green). However, the model used may vary, depending on the instrument used.
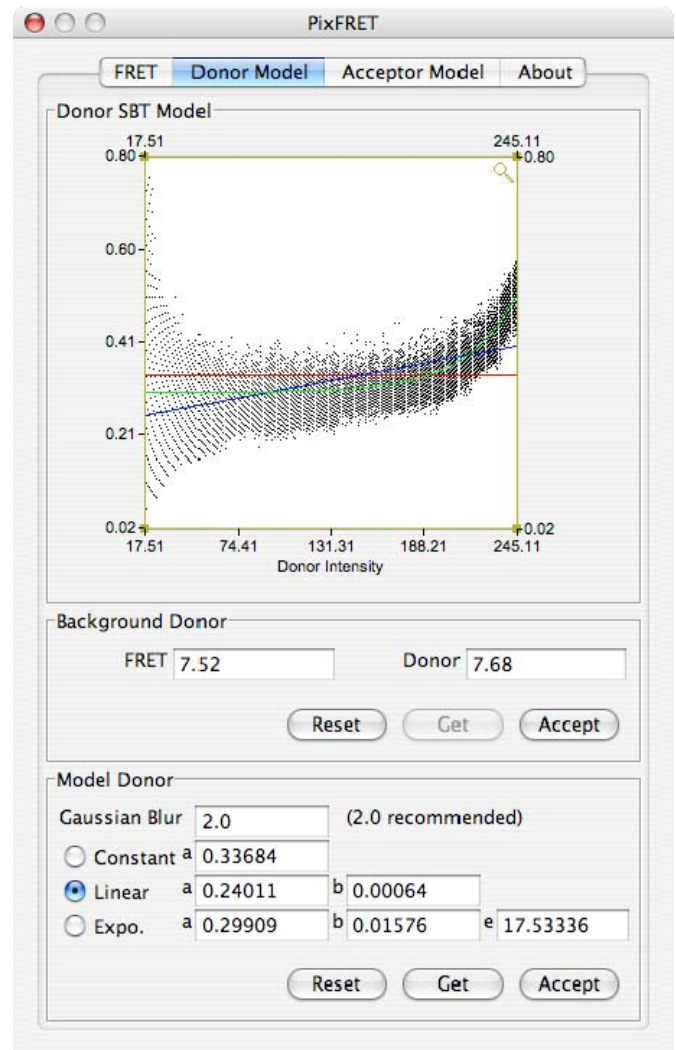

We also highly recommend at this point to apply a Gaussian blur to the stack to improve SBT determination by the plug-in. A value of 2.0 is generally satisfactory but this parameter should be tested for each set of images. If the value 0.0 is entered, the images are not changed. Note that the blurred images are not displayed.

Aberrant values are often generated at low intensities where BG noise has a big impact. On the graph, the regions where the SBT is calculated can be selected by moving the edges of the yellow window with the cursor:
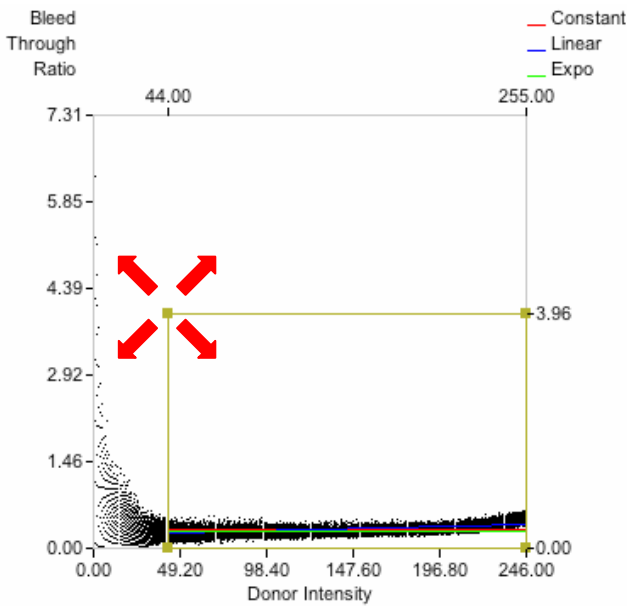
Once the frame has been repositioned, the SBT values are re-calculated automatically by the plug-in, do not click the "Get" button again. Clicking on the "zoom" icon located at the upper right corner of the graph allows to rescale the graph to the selected area.

Zoom icon
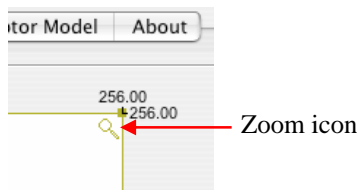
The values displayed at the bottom of the dialog window are the parameters corresponding to the different fits which are drawn on the graph and calculated on the points included in the zoom window: a red line shows the average SBT value calculated from the points selected, the blue line shows the linear fit and the green line the exponential fit of the values.

Importantly, if the ROI corresponds to only a subset of the image, drawing a new ROI and clicking a second time on the "Get" button does not replace but adds the new values to the former ones, making the SBT calculation more precise. This operation can be repeated several times to refine SBT calculation on ROI selected in different representative areas of the image.

Clicking on the "Reset" button erases all values from the memory. New ROIs can then be defined.

7. Click on the "Accept" button to use the SBT values obtained.

8. Repeat operations 2. to 7. for the Acceptor. Note that the models used for the donor and acceptor SBT determination are independent; thus, distinct models can be selected for the donor and the acceptor.

9. Go to the FRET bookmark and open the stack with the images where FRET should be calculated.
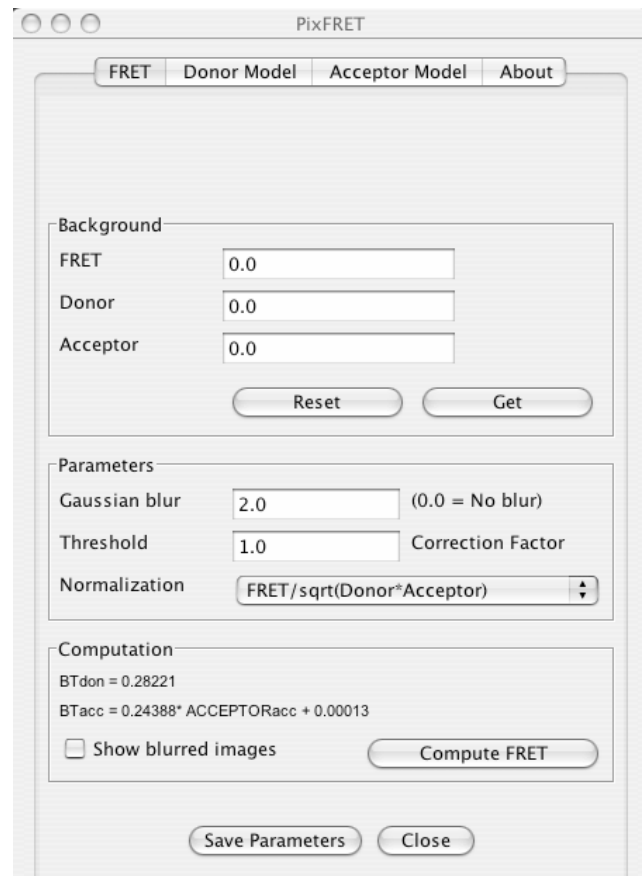
10. Select a ROI in the background and click "Get" in the background box to display background values. Note that the values can also be entered manually.

11. Enter the value for the Gaussian Blur. The Gaussian blur is an option offered here to smoothen the images of the stack. If the value 0.0 is entered, the images are not changed. We recommend to apply a factor from 1.0 to 2.0 to improve the rendering of the computed image. This notably smoothens the images and improves the resulting image by preventing aberrant FRET values. It is possible to visualize the blurred images by clicking the "show blurred images" box.

The images will be displayed with that of FRET, once the "Compute FRET" button will have been clicked (see below)

12. Enter the "threshold correction factor". FRET and NFRET are calculated only if pixel values in each image are above a given threshold, set by default to average background values. The threshold correction factor is a multiplication factor applied to background values in order to modify this threshold. The pixels below the threshold are displayed in blue in the computed FRET and NFRET images.

Note that when computing FRET, all the parameters are saved in a preference file in the plugins folder: pixFret_settings.txt, and reloaded automatically at the next use of the plug-in. Alternately, if the user wants to save parameters without computing FRET, the "Save Parameters" button should be clicked.

13. Select then either a normalization method or to display FRET efficiency. With normalization, the FRET values for each pixel will be divided by the value in the same pixel of the donor intensity, the acceptor intensity, the product of donor and acceptor intensities or the square root of

the product of donor and acceptor intensities (see below equation (2)).

14. Generation of FRET images:

After clicking the "Compute FRET" button, the software calculates for each pixel the FRET (1), or the normalized FRET (NFRET) value (2) and displays the two images. Note that in the following formulas, the SBTs may be considered as constants or functions of fluorophore intensities. The factor N can be either the donor intensity, the acceptor intensity, the product of donor and acceptor intensities or the square root of the product of donor and acceptor intensities (see point 13).

$$\text{FRET} = I_{FRET} - BT_{Donor} * I_{Donor} - BT_{Acceptor} * I_{Acceptor} \quad (1)$$

$$\text{NFRET} = \frac{I_{FRET} - BT_{Donor} * I_{Donor} - BT_{Acceptor} * I_{Acceptor}}{N} * 100 \quad (2)$$

15. Interpretation of the computed images:

FRET and NFRET are calculated only if pixel values in each image are above a given threshold. Otherwise the value is set to zero in the computed image and the pixel is displayed in blue. This allows to visualize all the FRET values of the image, including those that happen to be negative.

The computed images are encoded on 32 bits but visualized on 256 color levels; the minimum value displayed in black and the maximum value in white. Hence, in a cell where no FRET occurs (negative control), an artificially amplified signal may be observed. Users should therefore always:

i) check the amplitude of the FRET signal by performing a profile plot through the cell of interest and compare this amplitude to that of a negative control.
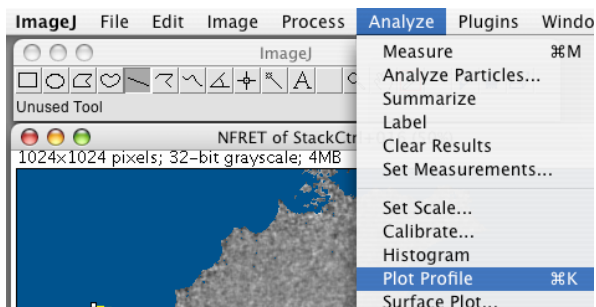
ii) stack the image of interest with an image of a negative or a positive control in order to visualize both images with the same color scale.

16. Save the image and the LUT:

Save the computed FRET image as a TIFF file, as well as the corresponding LUT file to be able to visualize it with the same blue background later. When this is needed, just open the TIFF file and import the LUT from the File menu. For exporting a snapshot of the image with the blue look-up table, the image can also be converted to 8-bit and saved as TIFF but this will lose pixel information and should not be considered as a way of storing processed images.

17. The same operations from 9. to 16. can be repeated for another image. The plug-in not only keeps the SBT values in memory for subsequent image analyses but also saves

them to a log file. Hence, if numerous images have to be analyzed, even over several days, the software can be closed and opened again, the last entered values being automatically reloaded.

V - SBT determination methods.

One crucial parameter in sensitized emission FRET calculation is the evaluation of spectral bleed-throughs, i.e. the amount of light detected in the FRET channel which is not due to energy transfer. The main causes of bleed-through are the emission of the donor in the acceptor channel and the excitation of the acceptor when exciting the donor. To estimate the donor (and acceptor) SBTs, average fluorescence intensities in cells expressing only the donor (or the acceptor) must be quantified, both in the FRET and in the donor (or acceptor) channels. The SBT ratios are the ratios between the fluorescence intensities in each channel after background subtraction:

$$BT_{donor} = \frac{I_{FRET}}{I_{Donor}}, \text{ when the donor alone is expressed,}$$

$$BT_{acceptor} = \frac{I_{FRET}}{I_{Acceptor}}, \text{ when the acceptor alone is expressed.}$$

PixFRET plots the SBT ratio of each individual pixel within a ROI as a function of fluorophore intensity. Determining SBT ratios with PixFRET on a whole image increases the probability to get values for the whole dynamic range. Otherwise, the number/size of ROIs analyzed should be adapted to have a good representation of fluorophore intensities over the dynamic range.

SBTs are often considered constant. However, plotting the SBTs as a function of fluorophore intensity may reveal some correlation between the two parameters (5). We hence propose here the possibility to fit the SBT values with a linear or an exponential model in order to improve the precision of the calculation.

V - References.

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5.   Feige, J. N., Sage, D., Wahli, W., Desvergne, B., and Gelman, L. (2005) *Microscopy Research and Technique* **68**, 51-58

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