"FRET and Colocalization Analyzer"

a method to validate the measurements of sensitized-emission FRET acquired by confocal microscopy and available as an ImageJ Plug-in

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I) FRET by sensitized emission of the acceptor fluorophore with confocal microscopy

Fluorescence Resonance Energy Transfer (FRET) is a physical process, distance-dependent, in which energy is transferred in a non-radiative manner, from a Donor fluorophore to an Acceptor fluorophore. This technique is widely used by biologists who study the interaction between two proteins inside cells by expressing one of the proteins of interest fused to a Donor fluorophore and the other protein fused to the adequate Acceptor fluorophore.

The plug-in analyses images obtained by confocal microscopy using the « sensitized emission FRET » technique in which the Donor fluorophore is excited and the emission of the Acceptor fluorophore due to FRET is measured in the so-called FRET Channel. Donor/Acceptor pairs of fluorophores, that give rise to efficient FRET signals, require that the emission spectrum of the Donor partially overlaps the excitation spectrum of the Acceptor. This often results in a leakage of the Donor emission into the FRET Channel and of an emission of the Acceptor through direct excitation at the excitation wavelength of the Donor. These two signals, called Spectral Bleed-Through, have to be evaluated for each confocal microscope acquisition session and subsequently subtracted from the FRET images acquired during the same acquisition session.

The Plug-in calculates the Bleed-Through of the pair of fluorophores as constant values and subtracts them from the raw FRET Channel Image using an equation similar to the one described in Youvan et al. 1997 *Calibration of Fluorescence Resonance Energy Transfer in Microscopy using genetically engeniered GFP derivatives on nickel chelating beads.* ***Biotechnology*** 3:1-18. The resulting FRET image displays intensities of Acceptor emission due to FRET in each pixel. These intensity values are relative values of FRET that can be compared with other values of images acquired during the same session. For more details of the methods and image analysis process, see Hachet-Haas et al. *in press*, for Bleed-Through coefficients evaluation with ImageJ other than a constant, see the "PixFRET" plug-in, and for quantitative rather than qualitative FRET values with ImageJ, see the "FRET Stoichiometry" plug-in).

II) Originality of the Plug-in "FRET and Colocalization Analyzer"

The interest of this plug-in is that it provides control images that allow the user to evaluate whether the Bleed-Through coefficients that are calculated are valid. It also allows the user to eliminate false FRET signals in the images by correlating FRET with the co-localization of the two fluorophores. The method described in the original "Microscopy Research and Technique" article also proposes a new way of normalizing the FRET data that is not yet implemented in the plug-in. For more details see Hachet-Haas et al. *in press*.

We have specially adapted this plug-in to transient expression of the fluorescent fusion proteins and to biological samples in which the two proteins of interest are co-localized in small structures a few pixels in size, such as vesicles. We also recommend starting the analysis with fixed samples such that they can be re-used over several acquisition sessions in order to get familiar with all the parameters important for a good analysis and to acquire the maximum of fields during one day of acquisition in order to improve the statistical analysis.

III) Plug-in installation

The plug-in "FRET and colocalization Analyzer" runs under ImageJ and is freely downloadable at ***http://rsb.info.nih.gov/ij/download.html***. To install the plug-in, download "Fret_Analyzer.jar" from ImageJ web site at ***http://rsb.info.nih.gov/ij/plugins/fret-analyzer.html*** and copy it in the ImageJ "plugins" folder.

IV) Biological material

FRET index measurements require the image acquisition of 3 types of samples at each confocal microscope acquisition session :

1- one sample with cells expressing only the Donor fluorophore to evaluate the Donor Bleed-Through coefficient,

2- one sample with cells expressing only the Acceptor fluorophore to evaluate the Acceptor Bleed-Through coefficient, and
3- one sample with cells co-expressing the two fluorophores in which FRET signals will be measured. We recommend using a mixture of the three types of cells (cells expressing each ones of the fluorophores individually, together with cells co-expressing them) as described in Hachet-Haas et al. 2006.

V) Confocal acquisition settings

This step is essential for a good evaluation of FRET indices. Do not try to use this plug-in with old images that were not acquired properly. At each acquisition session, all the controls have to be included. The settings given as an example below are for the CFP/YFP pair of fluorophores.

Definition of the Channels

Donor Channel Image : emission of the Donor upon excitation of the Donor
For CFP : excitation with the 458nm of the argon laser-line and emission window collected between 470-500nm with PMT1.

Acceptor Channel Image : emission of the Acceptor upon excitation of the Acceptor
For YFP : excitation with the 514nm of the argon laser-line and emission window collected between 530-600nm with PMT2.

FRET Channel Image : any emission corresponding to the Acceptor emission window upon excitation with the laser-line of the Donor.
For CFP/YFP pairs : excitation with the 458nm of the argon laser-line and emission window collected between 530-600nm with PMT2.

Control Channel Image : any emission corresponding to the Donor emission window upon excitation with the laser-line of the Acceptor. This Channel should contain no signal apart from the instrumental noise of the acquisition system.

Settings of the Photomultiplier Tubes, PMTs

Amplification of the signal should be in the linear zone and should give a good signal to noise ratio. Do not put any offset.

Settings of the laser-lines

Adapt the laser-lines power in order to get the maximal dynamics (i.e. maximal number of grey-scale values) in the images of the Donor in the Donor Channel and of the Acceptor in the Acceptor Channel. Some pixels that are not essential for the analysis can even be saturated.

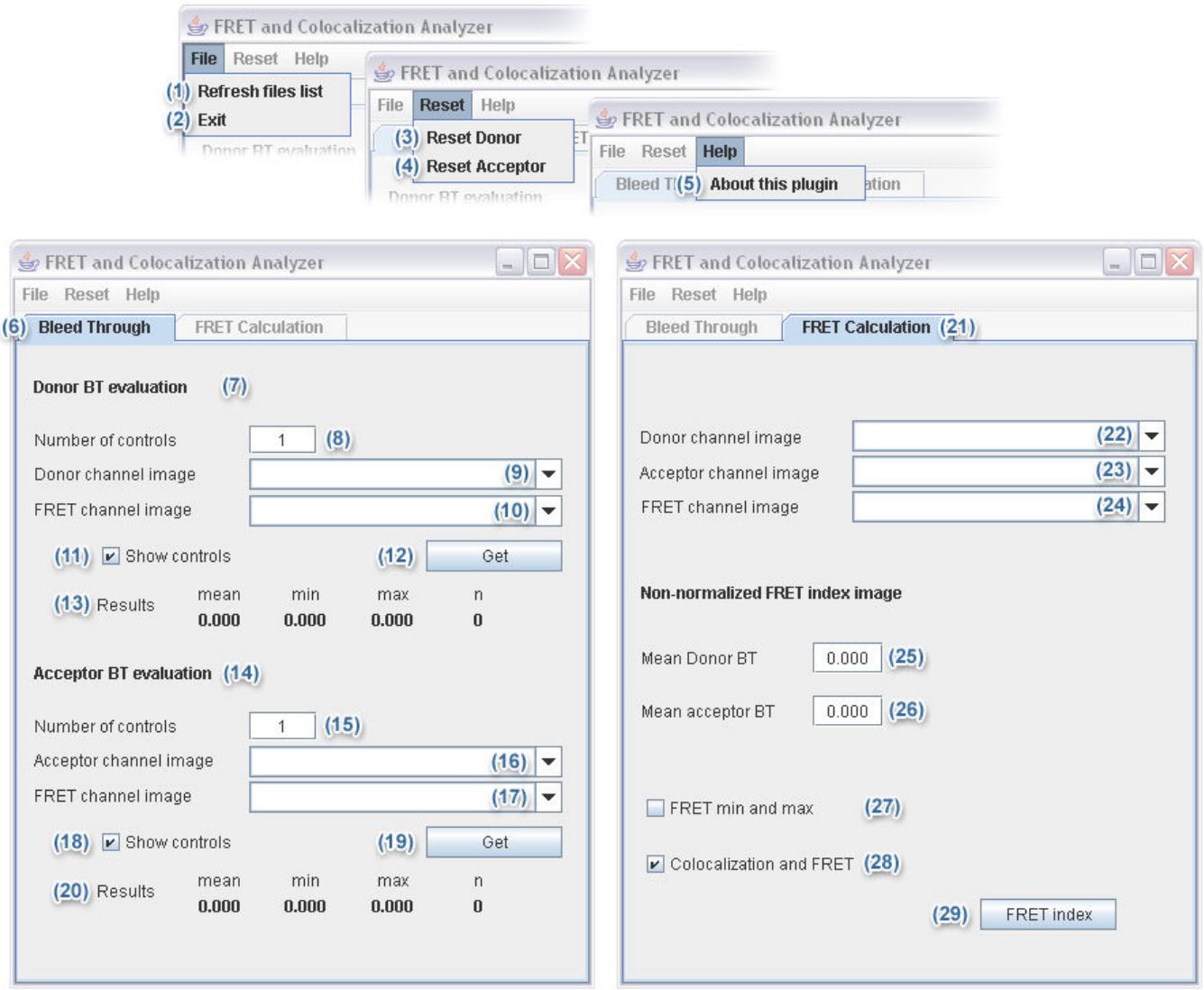
Use the same Channels and PMTs, once they are correct, during all the different acquisition sessions and at each individual session, re-adjust only the power of the laser-lines in order to always get the maximal dynamics. Do not change the settings over the acquisition period and start the collection of the images that will be used for FRET analysis only once the laser-lines are stable, usually around one hour after having adjusted the laser-lines power.

FRET analysis :

A) Launch the plug-in

To launch the plug-in, open some images with the ImageJ software. Since the first step in the image analysis consists of evaluating the Donor Bleed through, it is better to open both the Donor Channel Image of the field number 1 and the corresponding FRET Channel Image acquired on the Donor alone expressing sample. Then the "**Fret analyzer**" can be launched by selecting it in the "plugins" tools bar. Note that the plug-in works with 8-bits images.

B) Plug-in overview



- File menu -

(1) **Refresh files list:** refresh list of open images in **(9), (10), (16), (17), (22), (23), (24)** boxes.
(2) **Exit:** end the program.

- Reset menu -

(3) **Reset Donor:** allows user to reset the donor parameters: "Number of controls" **(8)** and associated "Results" table **(13)**.
(4) **Reset Acceptor:** allows user to reset the acceptor parameters: "Number of controls" **(8)** and associated "Results" table **(13)**.

- Help menu -

(5) **About this plug-in:** gives information about the conception of the plug-in.

- Bleed Through -

(6) **Bleed Through:** Select in order to determine bleed through calculations and controls.
(7) **Donor BT evaluation:** Part of the plug-in that calculates the Donor Bleed-Through parameters
(8) **Number of controls:** The user indicates here the number of control images that will be used to calculate the mean Donor Bleed-Through coefficient.
(9) **Donor channel image:** Select here the image acquired with the donor settings.
(10) **FRET channel image:** Select here the corresponding image acquired with the FRET settings.
(11) **Show controls :** If selected, the plug-in will display two additional images for data analysis.
(12) **Get:** Press to start the Bleed-Through analysis process.
(13) **Results:** Table summarizing the results obtained
(14) **Acceptor BT evaluation:** Part of the plug-in that calculates the Acceptor Bleed-Through parameters
(15 to 20) like **(8 to 13)**.

- FRET Calculation -

(21) **FRET Calculation:** Select in order to determine the FRET between the two fluorophores in cells co-expressing them.
(22) **Donor channel image:** Select here the image acquired with the Donor settings.
(23) **Acceptor channel image:** Select here the corresponding image acquired with the Acceptor settings.
(24) **FRET channel image:** Select here the corresponding image acquired with the FRET settings.
(25) **Mean Donor BT:** Displays the "mean" value of the Donor Bleed-Through calculation table.
(26) **Mean Acceptor BT:** Displays the "mean" value of the Acceptor Bleed-Through calculation table.
(27) **FRET min and max:** If selected, the plug-in will display two additional control images that give an interval of confidence of the calculated FRET index.
(28) **Colocalization and FRET:** If selected, the plug-in displays two additional data: first, an interactive diagram that correlates FRET and colocalization between the two fluorophores ; second, the Colocalized Image resulting from the selection in the interactive diagram, that shows only the level of FRET in pixels in which the two fluorophores are colocalized .
(29) **FRET index:** Press to start the FRET analysis process.

C) Donor Bleed-Through evaluation

Select **(6)** «**Bleed Through**» inlet of the plug-in. First for the Donor Bleed-Through, and then for the Acceptor Bleed-Through evaluations, here are the steps to follow.

- Indicate the number n of fields of control images that will be used to evaluate the mean of the Donor Bleed-Through (maximum 10) «**Number of controls**» **(8)** for the Donor alone expressing samples.

- Open the images of the first field required for the analysis: i.e. Donor Channel Image 1 and FRET Channel Image 1 of the same field number 1 of cells expressing the Donor fluorophore alone. Press **(1)** «**Refresh files list**» such that the names of the images appear in the rolling menus in **(9)** and **(10)** for the Donor BT evaluation and select in the rolling menu the name of the images that you want to analyse pair-wise.

- Recommended Option : «**Show controls**» in **(11)**. If selected, the plug-in will display a «**Regression Control**» image 1 and a «**Regression Graph**» 1, that give indications of the pixels in the images of field 1 that follow the linear regression function used to calculate the Donor Bleed-Through parameters and of the pixels that are divergent from the equation.

- Select «**Get**» in **(12)** in order to get the calculation of the Donor Bleed-Through parameters of the field number 1.

- Interpretation of the Regression Graph, of the «**Results**» text file and of the Regression Control image of each individual result

  - the «**Regression Graph**» 1 together with the «**Regression Control**» image 1 allow the user to decide whether the Bleed-Through coefficient is indeed correctly defined by the linear equation that is given in the «**Results**» text file.
  - The «**Regression Graph**». Each pixel has an intensity in the Donor Channel Image and in the corresponding FRET Channel Image. The graph plots a point for each pixel with the Donor Channel Image 1 intensity as abscissa and the FRET Channel Image 1 intensity as ordinate. A linear regression and standard error of regression are calculated predicting the FRET Channel Image 1 from the Donor Channel Image 1 pixel intensities. The hue of each pixel in the color graph expresses the residual, the difference between the predicted and actual FRET Channel Image 1 intensity divided by the standard error of the regression.

  One can thus rapidly detect the intensities of the pair of pixels that are significantly dispersed from the linear regression. In blue appear the points representing the pair of pixels that are far below the line and in red those that are far above the line.

  The red points will be under-corrected by the coefficient of Bleed-Through and thus will be those that give false FRET signals. (Parts of this code were taken from plug-ins by Bill O'Connell and by Christophe Laummonerie and Jerome Mutterer, "Color Comparison" and "Colocalization Finder", respectively).

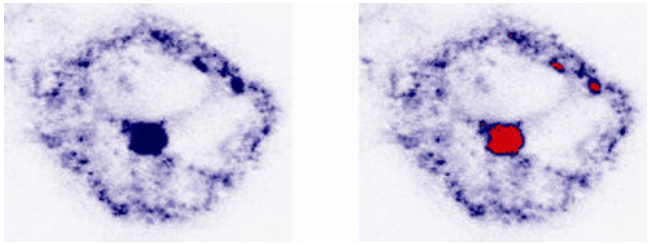
  - The linear regression function consists of :
    Regression : y = ____ * x + constant,

    in which the coefficient of the line will define the Bleed-Through coefficient.

    Statistical parameters of the pair of pixels distribution around the line:
    Standard Error = ____ Pearson = ____ ,
    Obviously, the smaller the Standard Error (Std Err) and the closer to 1 the Pearson, are indications of a good evaluation of the Bleed-Through parameters.

- The hue of each pixel in the «**Regression Control**» image expresses the residual, the difference between the predicted and actual FRET Channel Image 1 intensity divided by the standard error of the regression. Thus, the points of pair of pixels that are too dispersed from the linear regression, whether in blue or in red, are presented in a spatial way. With a good evaluation of the linear regression, these pair of pixels should be homogenously distributed in the image or be in structures that are not of biological interest for the user.

Note : With the method, saturated pixels are given the value 0 and are thus eliminated from the analysis.



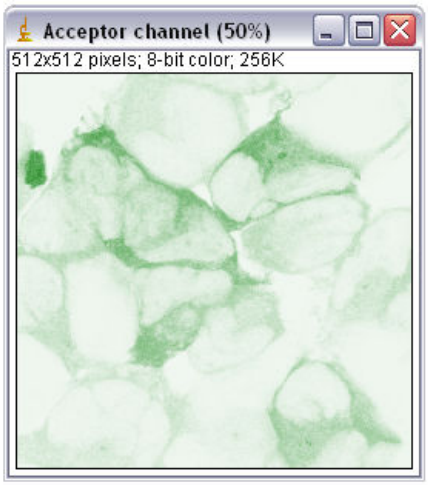
- After the analysis of the first control images pair, open the images of the second field required for the analysis: i.e. Donor Channel Image 2 and FRET Channel Image 2 of the same field number 2 of cells expressing the Donor fluorophore alone. Press **(1)** «**Refresh files list**» such that the names of the images are appearing in the "rolling menus in **(9)** and **(10)** for the Donor BT evaluation and select in the rolling menu the name of the images that you want to analyse pair-wise.

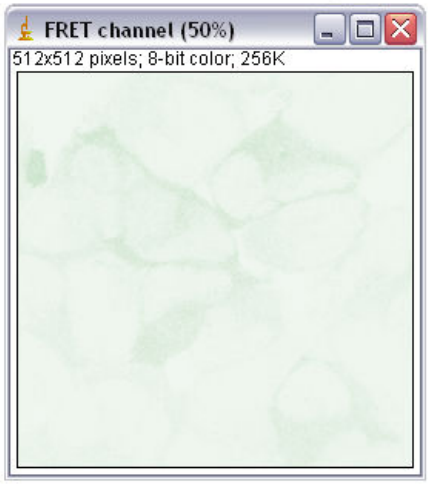
- Select «**Get**» in **(12)** in order to get the calculation of the mean Donor Bleed-Through parameters of the two fields number 1 and 2.

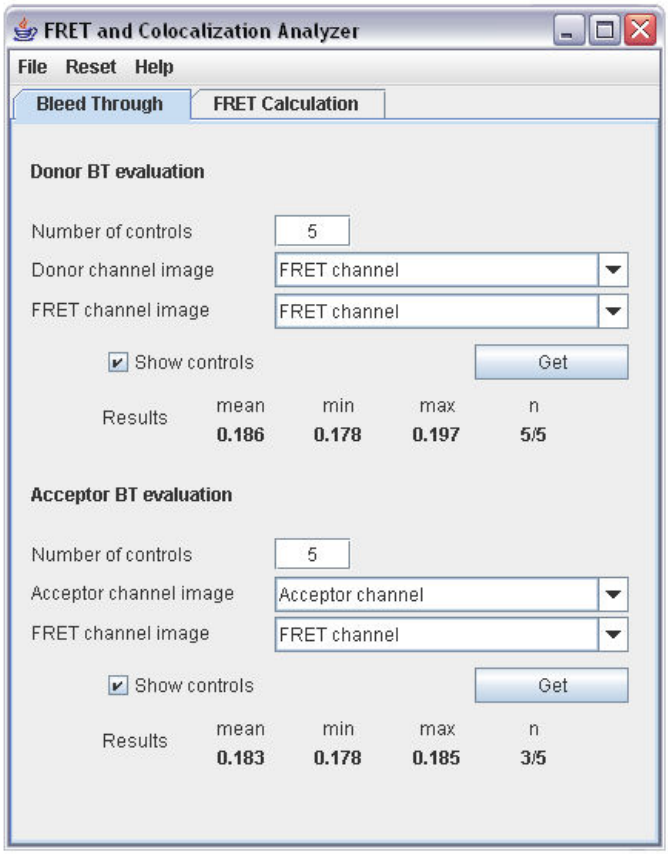
- If selected, «**Show controls**» in **(11)**, the plug-in will display a «**Regression Control**» image 2 and a «**Regression Graph**» 2, that give indications of the pixels in the images of field number 2 that follow the linear regression function used to calculate the Bleed-Through parameter and of the pixels that are divergent from the equation.

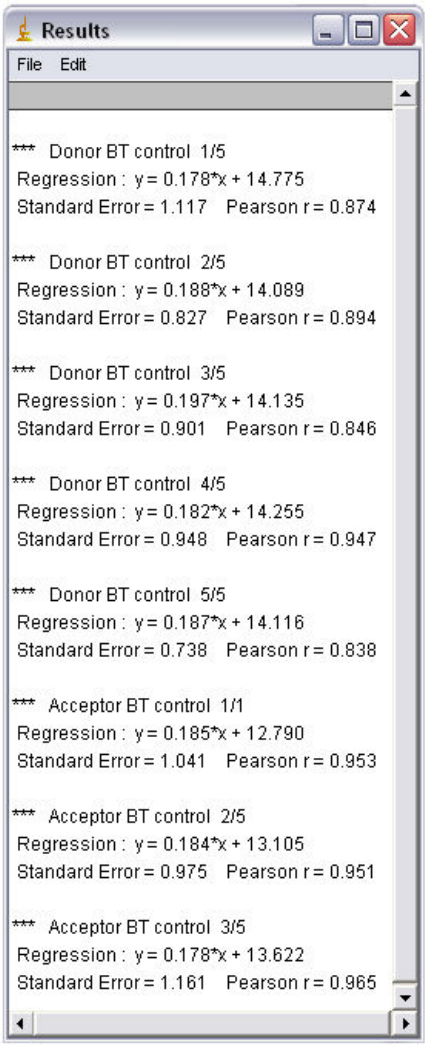
- After each additional field used to evaluate the Donor Bleed-Through coefficient, the **«Results»** **(13)** table is automatically incremented. This table displays the mean value of the coefficient «**mean**» and the minimal and maximal values «**min**» and «**max**» of the Bleed-Through. «**n**» stands for the number of control calculated as compared with the total number of controls that will be performed. The text file, «**Results**», increments all the individual measurements.

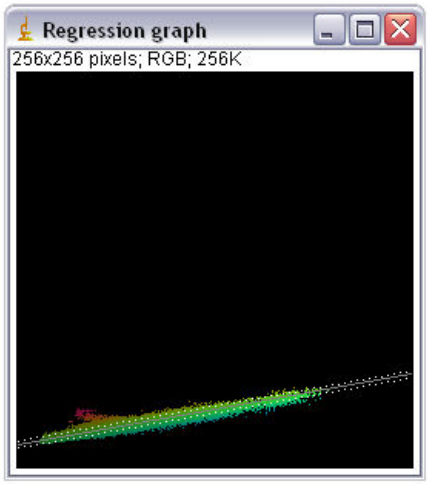
- In case of a pair of images that are not presenting a linear regression and that one want to exclude from the Bleed-Through calculation, the **«Results»** table can be re-adjusted to 0 with the function «**Reset Donor**» **(3)** while the text file will still display all the erased data.

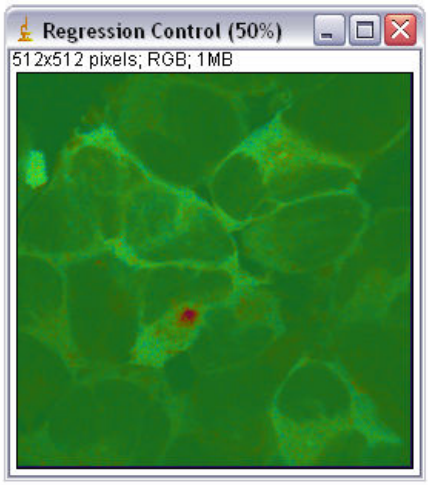

Acceptor channel (50%)
512x512 pixels; 8-bit color; 256K


FRET channel (50%)
512x512 pixels; 8-bit color; 256K


FRET and Colocalization Analyzer
File Reset Help
Bleed Through
FRET Calculation
Donor BT evaluation
Number of controls 5
Donor channel image FRET channel
FRET channel image FRET channel
Show controls
Get
Results mean min max n
0.186 0.178 0.197 5/5
Acceptor BT evaluation
Number of controls 5
Acceptor channel image Acceptor channel
FRET channel image FRET channel
Show controls
Get
Results mean min max n
0.183 0.178 0.185 3/5


Results
File Edit
*** Donor BT control 1/5
Regression : y = 0.178*x + 14.775
Standard Error = 1.117 Pearson r = 0.874
*** Donor BT control 2/5
Regression : y = 0.188*x + 14.089
Standard Error = 0.827 Pearson r = 0.894
*** Donor BT control 3/5
Regression : y = 0.197*x + 14.135
Standard Error = 0.901 Pearson r = 0.846
*** Donor BT control 4/5
Regression : y = 0.182*x + 14.255
Standard Error = 0.948 Pearson r = 0.947
*** Donor BT control 5/5
Regression : y = 0.187*x + 14.116
Standard Error = 0.738 Pearson r = 0.838
*** Acceptor BT control 1/1
Regression : y = 0.185*x + 12.790
Standard Error = 1.041 Pearson r = 0.953
*** Acceptor BT control 2/5
Regression : y = 0.184*x + 13.105
Standard Error = 0.975 Pearson r = 0.951
*** Acceptor BT control 3/5
Regression : y = 0.178*x + 13.622
Standard Error = 1.161 Pearson r = 0.965


Regression graph
256x256 pixels; RGB; 256K


Regression Control (50%)
512x512 pixels; RGB; 1MB

D)

D) Acceptor Bleed-Through evaluation

- Indicate the number of fields of control images that will be used to evaluate the mean of the Bleed-Through (maximum 10) «**Number of controls**» **(15)** for the Acceptor alone expressing samples.

- Open the images of the first field required for the analysis: i.e. Acceptor Channel Image 1 and FRET Channel Image 1 of the same field number 1 of cells expressing the Acceptor fluorophore alone. Press **(1)** «**Refresh files list**» such that the names of the images appear in the rolling menus in **(16)** and **(17)** for the Acceptor BT evaluation and select in the rolling menu the name of the images that you want to analyse pair-wise.

- Recommended Option : «**Show controls**» in **(18)**. If selected, the plug-in will display a «**Regression Control**» image and a «**Regression Graph**» that give indications of the pixels in the images that follow the linear regression function used to calculate the Acceptor Bleed-Through parameters and of the pixels that are divergent from the equation.

- Select «**Get**» in **(19)** in order to get the calculation of the Acceptor Bleed-Through parameters.

- After each additional field used to evaluate the Acceptor Bleed-Through coefficient, the **«Results»** **(20)** table is automatically incremented. This table displays the mean value of the coefficient «**mean**» and the minimal and maximal values «**min**» and «**max**» of the Bleed-Through. «**n**» stands for the number of controls calculated as compared with the total number of controls that will be performed. A text file, «**Results**», increments all the individual measurements.

- In case of an error, the **«Results»** table can be re-adjusted to 0 with the function «**Reset Acceptor**» **(4)** while the text file will still display all the erased data.

E) FRET calculation

Select **(21)** «**FRET Calculation**» inlet of the plug-in. Here are the steps to follow.

- Open the images of the first field to be analysed: i.e. Donor Channel Image 1, Acceptor Channel Image 1 and FRET Channel Image 1 of the same field number 1 of cells co-expressing the two fluorophores. Press **(1)** «**Refresh files list**» such that the names of the images are appearing in the rolling menus in **(22)**, **(23)** and **(24)**.

- The non-normalized FRET index image will be generated using the «**mean**» values of the Donor and Acceptor Bleed-Through coefficients determined in the Bleed-Through inlet and presented in the «**Results**» tables in **(13)** and **(20)**. These mean coefficients, appearing in **(25)** and **(26)**, can be modified by the user if a method of coefficients refinement is applied (see Hachet-Haas et al. *in press*).

- Recommended Options : «**FRET min and max**» **(27)** and «**Colocalization and FRET**» **(28)**.

  If selected, the «**FRET min and max**» option will display in addition to the non-normalized FRET index image, both «**Minimum FRET index**» and «**Maximum FRET index**» images.

  If selected, the «**Colocalization and FRET**» option will display both a «**Colocalization diagram and FRET**» and a «**Colocalized FRET index**» image.

- To launch the processing of the non-normalized FRET index image, press «**FRET index**» **(29)**. Once finish, the «**FRET index**» image will appear.

- Interpretation of the resulting FRET images and of the control Images

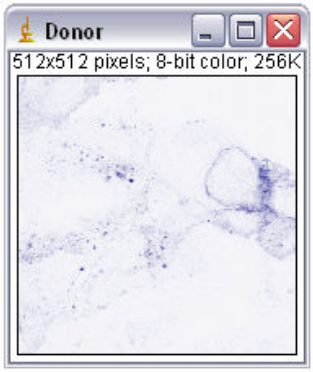
  - The «**FRET index**» image is generated using an equation similar to the one described in Youvan et al. 1997 and using the mean Bleed-Through coefficients determined in the Bleed Through inlet for both the Donor alone and the Acceptor alone (see Hachet-Haas et al. i*n press* for more details). The image displays intensities of Acceptor emission due to FRET in each pixel. The color of the pixel is the "fire" Look-Out-Table from ImageJ software such that blue no FRET and red is high FRET. These intensity values are relative values of FRET that can be compared with other FRET images acquired during the same acquisition session. The method described in the original "Microscopy Research and Technique" article also proposes a new way of normalizing the FRET data that is not yet implemented in the plug-in.

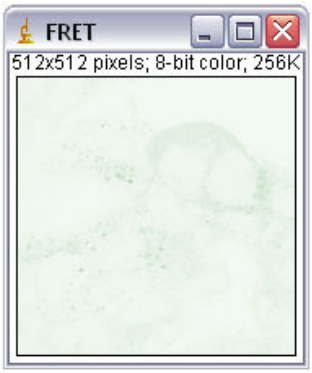
  - The «**Minimum FRET index**» image is generated using the maximal Bleed-Through coefficients determined in the Bleed Through inlet for both the Donor alone and the Acceptor alone while the «**Maximum FRET index**» image is processed using the minimal values of the Bleed-Through coefficients. These images are helping the user to evaluate the significance of the variation of the FRET indices.

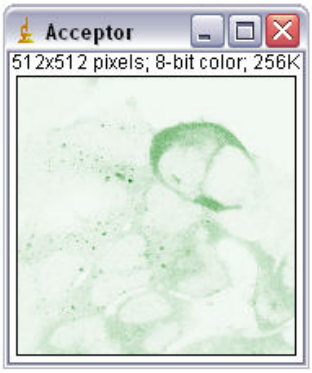
  - The «**Colocalization diagram and FRET**» plots a point for each pixel with the Donor Channel Image intensity as abscissa and the Acceptor Channel Image as ordinate. Such a configuration of diagram is often used to evaluate co-localization between two fluorophores. The color of the points represents the mean of the indices of FRET calculated for the corresponding pixels. In other words, this diagram, first, combines the information of co-localization and of FRET indices. Second, this diagram is interactive and the user can adjust the yellow square in which the co-localized pixels are indeed present. This allows the elimination of occasional false FRET signal, in as much as this artefact signal comes from pixels that do not contain both Donor and Acceptor fluorophore signal together.

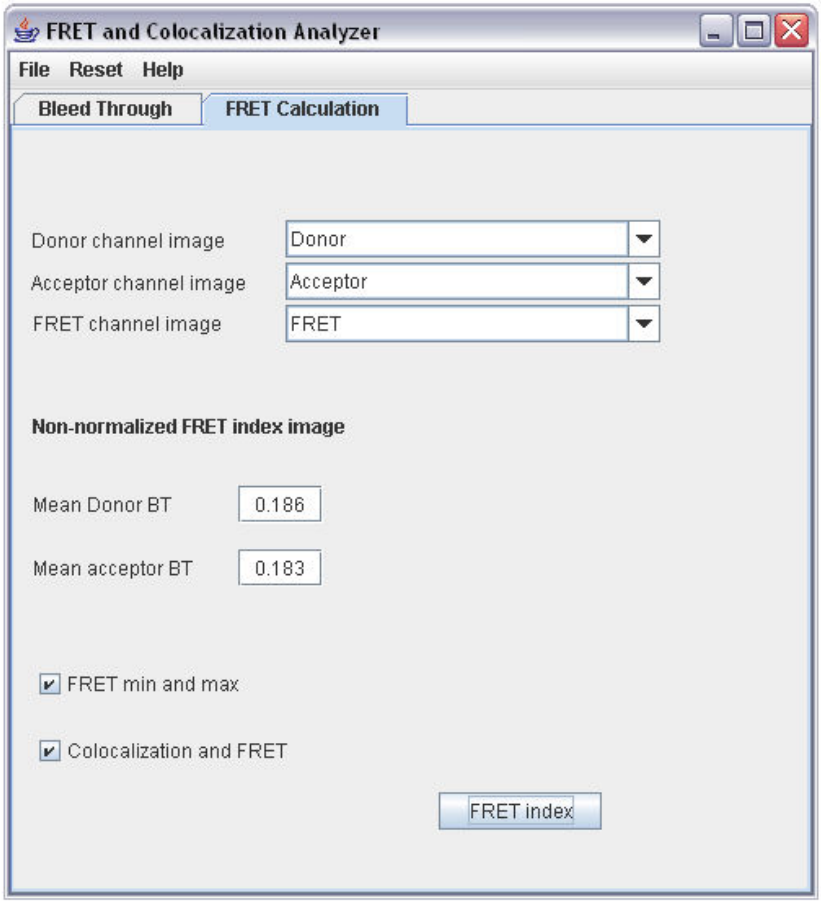
  - The «**Colocalized FRET index**» image corresponds to the «**FRET index**» image in which are masked the pixels that are excluded from the interactive square that defines co-localized pixels in «**Colocalization diagram and FRET**». As such, this image is re-adjusted by the user until only the pixels that correspond to co-localization are shown. Note that the mask appears in blue.

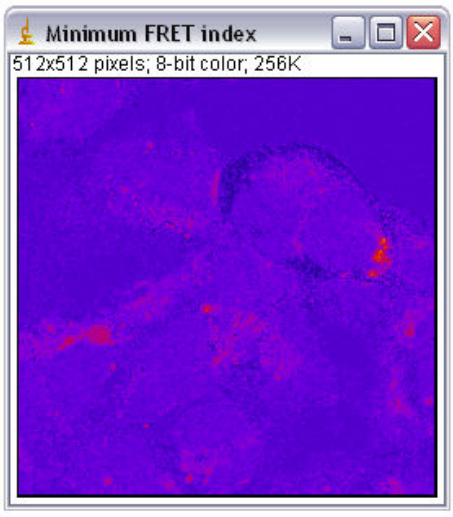
  - In the example below, the yellow square of the «**Colocalization diagram and FRET**» has been placed such that it excludes a series of low to middle intensity pixels of the Donor Channel Image that present high FRET indices (in red) but that corresponds to background intensities in the Acceptor Channel Image.

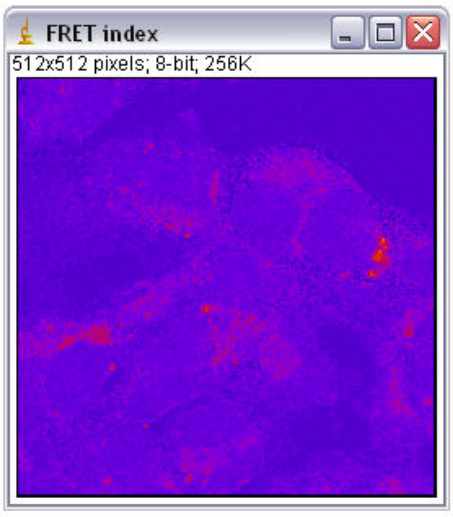

Donor
512x512 pixels; 8-bit color; 256K

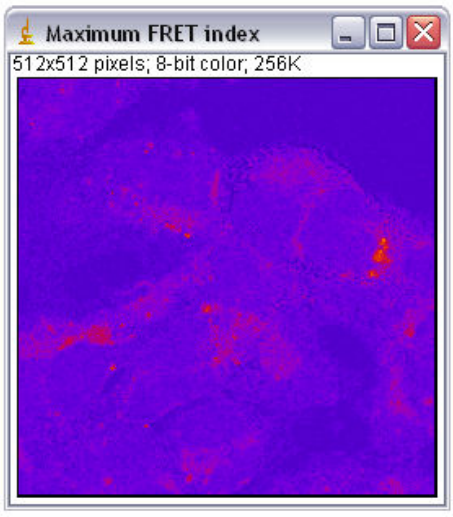

FRET
512x512 pixels; 8-bit color; 256K

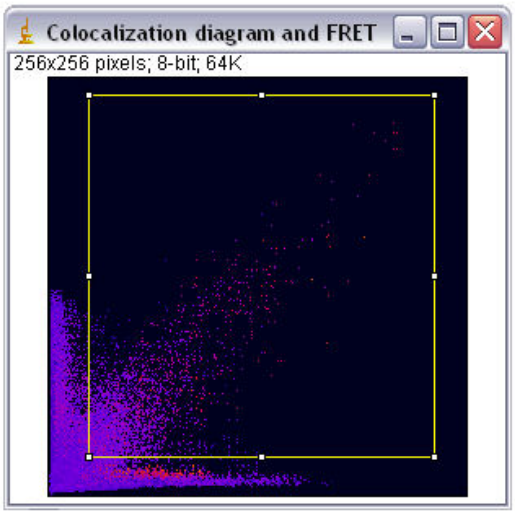

Acceptor
512x512 pixels; 8-bit color; 256K

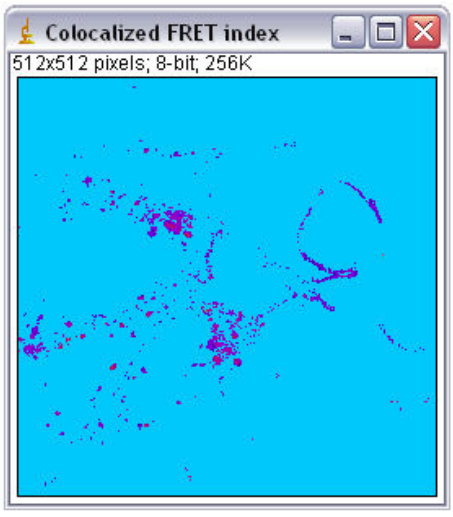

FRET and Colocalization Analyzer
File
Reset
Help
Bleed Through
FRET Calculation
Donor channel image
Donor
Acceptor channel image
Acceptor
FRET channel image
FRET
Non-normalized FRET index image
Mean Donor BT
0.186
Mean acceptor BT
0.183
FRET min and max
Colocalization and FRET
FRET index


Minimum FRET index
512x512 pixels; 8-bit color; 256K


FRET index
512x512 pixels; 8-bit; 256K


Maximum FRET index
512x512 pixels; 8-bit color; 256K


Colocalization diagram and FRET
256x256 pixels; 8-bit; 64K


Colocalized FRET index
512x512 pixels; 8-bit; 256K