Introduction

SimplePCI **Q**uantitative **F**luorescent **A**pplications - **F**luorescent **R**esonant **E**nergy **T**ransfer (**QFA-FRET**) adds FRET specific analysis for research into protein-protein interactions. **QFA-FRET** incorporates selection and customizing of algorithms based on published methods for accurate crosstalk correction, FRET measurement and visualization.

The modules support the following features:

- Corrections: complete, micro, 2 filter, and acceptor photobleaching
- Normalizations: acceptor/donor, donor, acceptor, square root of acceptor/donor
- Integrates with **AIC** module for efficient capture of FRET, Donor, and Acceptor images

Expand the functionality of **QFA-FRET** by adding the following optional modules:

- **AIC,** automated control and image acquisition
- **DIA**, dynamically measures intensity changes on- or off-line
- **IPA**, develops icon-driven work files for automatic image analysis and processing
- **IPA-MTA**, track and analyze moving objects
- **VIS-MD**, provides rapid 3D visualizations of multi-dimensional data sets
- **DNN**, remove or restore blur in images using fast algorithms
- **DNN-2D**, a Point Spread Function is derived and used in restoration

Getting Started

Quick Start contains examples of how to utilize **QFA-FRET**. For further assistance, refer to the online help, manual, or visit support at http://www.cimaging.net, for access to the latest **How to's** and frequently asked questions. Additional support is available via e-mail: support@cimaging.net or Tel: 412-741-7920.

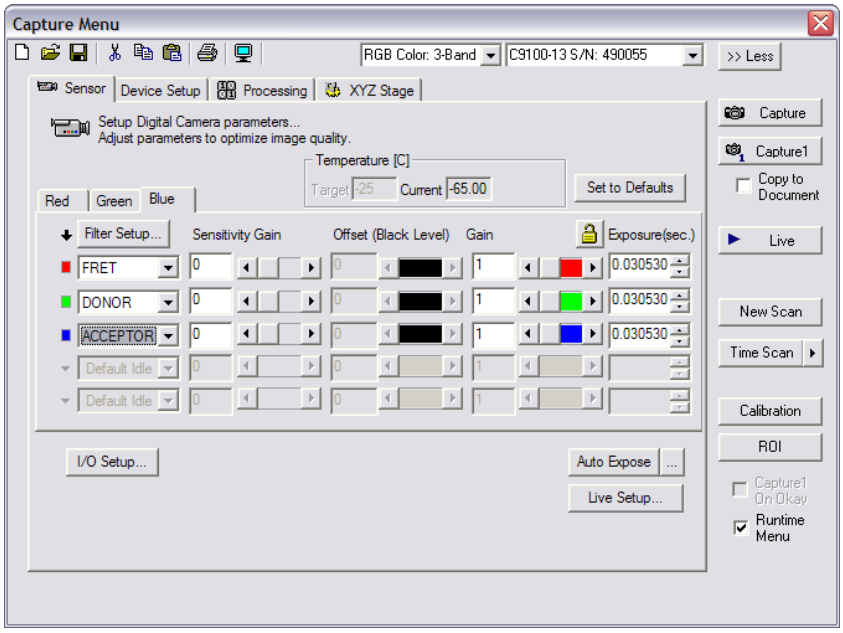
Examples guides:

Definition of Conventions

For consistency, the same filter sets should be used with the same channels. The typical order for the capture and display of the image channels is as follows:

1. Red display channel - FRET filter set (donor excitation, acceptor emission)
2. Green display channel - Donor filter set (donor excitation, donor emission)
3. Blue display channel - Acceptor filter set (acceptor excitation, acceptor emission)

A mnemonic for the recommended sequence is “FDA” - Fret, Donor, Acceptor, in order, RGB, as below:



Up to three image sets are required, one for each fluorophore, each acquired with up to three filter sets. The convention for referencing each channel is as follows:

Capital letter = Filter Set
Lowercase letter = Fluorophore

F = FRET filter set (donor excitation and acceptor emission)
f = donor and acceptor fluorophore

D = Donor filter set
d = donor fluorophore

A = Acceptor filter set
a = acceptor fluorophore

Combinations of Upper case and Lower case are combined to indicate the filter set and fluorophore.

Examples:
Ff, for Fret filter set + Donor and Acceptor fluorophores,
Da for Donor filter set + Acceptor fluorophores, Etc.

Run a complete FRET analysis

1. Open Three Channel Image, which has the Ff image in the Red Channel, Df in Green, and Af in the blue channel. (fig. 1)

2. Select the FRET properties icon. (fig. 2)

fig.2

3. Select “Complete FRET” method for analysis. This determines several factors including the algorithm to apply, the filter sets to use and the image combinations required for the calculations. (fig. 3)

4. Select “Image Selection” - this second screen determines the source of the image channels necessary for performing the calculations. This display shows the 9 images required for a Complete FRET analysis. (fig. 4)

5. Select image by clicking on the [...] button for file selection dialog (fig. 5). Images can be selected individually, for instance as single monochrome image files or as a single channel from a color image file, or components may be selected from an existing data document. If a color image is selected, the channels will be detected and selected automatically according to our “FDA” convention, however it can be overridden if desired.

Note: Selected Item information box indicates which of the filter set/fluorophore combinations are currently selected. This is also indicated by the highlighted rectangle around the selected images.

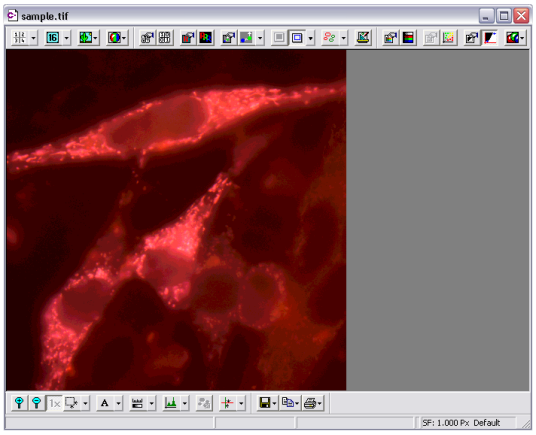


fig.1

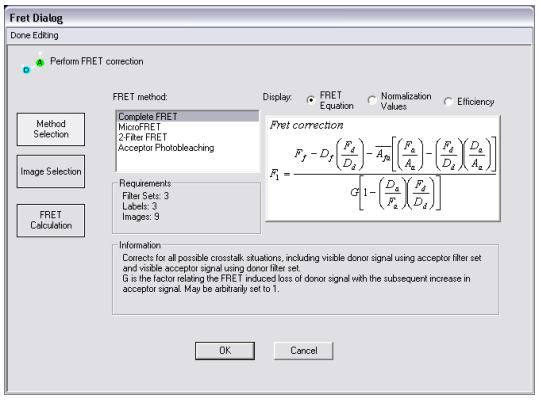


fig.3

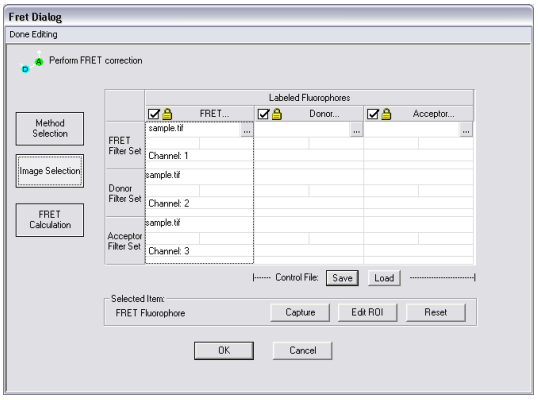


fig.4

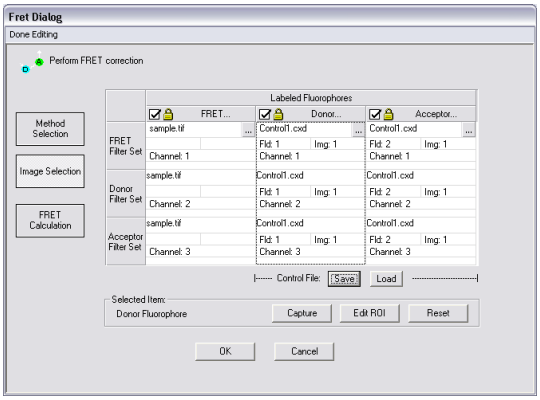


fig.5

Run complete FRET analysis

6. The intensities used for FRET correction may be localized to specific areas within the control images. The ROIs for each of the fluorophore control images are independent, and can be defined by selecting the respective column. Select a column, then select the “Edit ROI” button to use standard ROI tools to identify these areas where the control intensity is, and to avoid any artifacts in the control images. This will bring up a new window (fig. 6). The image may be magnified and contrast enhanced to accurately locate the areas for measurement. Areas not included in the measurement shapes will not be used for the correction.

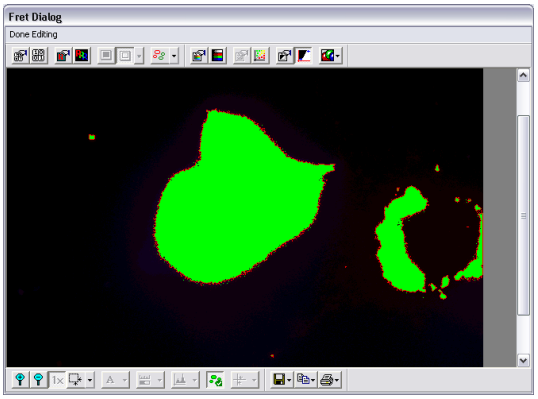


fig.6

7. After selecting the Images, and editing the ROI’s for each column, you may save your control as a special data file for later reuse. Use the “Save” and “Load” buttons in the highlighted “Control File” box. (fig.7)

8. Select “FRET Calculation” to move to the third screen. (fig.8)

9. In the “FRET Calculation” dialog, select and set the appropriate values for your sample. (fig.8)

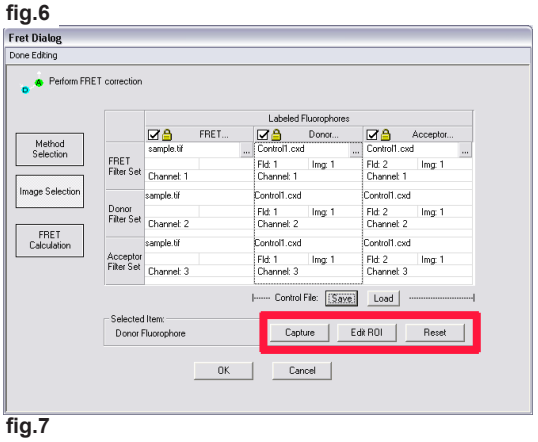


fig.7

A. Crosstalk coefficients and concentration values may be entered if the correction images are not available, or if the values can be reliably re-used.

B. Min and Max scaling values can be used to control the range of intensities displayed in the visualization image, allowing flexibility in highlighting a specific range of FRET values in the image. The Min and Max settings do not change the values, only what is displayed.

C. The “G” value is a correction which is usually set to “1”, but it may be necessary to change it depending on experimental conditions.

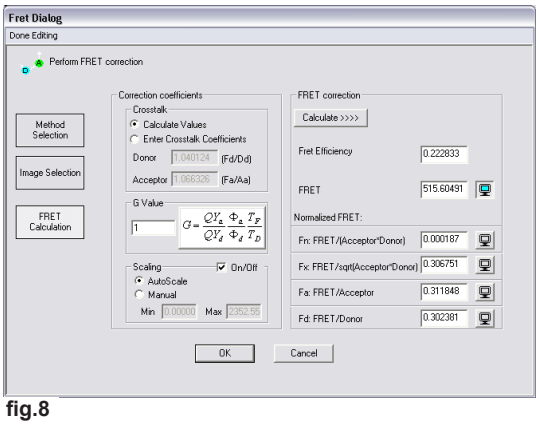


fig.8

10. Select “Calculate”, then select either raw FRET value, or desired normalization for visualization and data reporting. (fig.8)

11. Press “OK” to return to the source document and to view the Results of the FRET calculations. (fig.9)

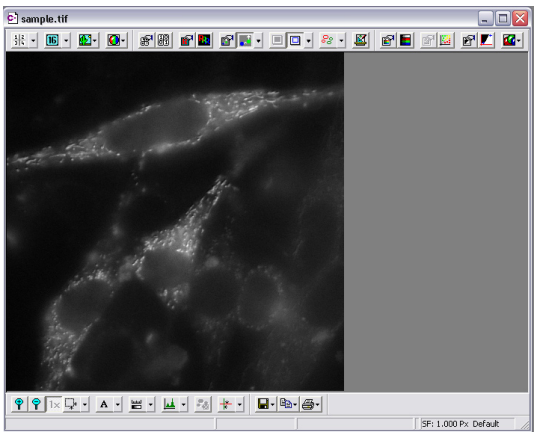


fig.9

How to access raw FRET values

1. Perform FRET calculations on your image, (fig. 10) then select the “Current View” from the Image Data Views tool bar (fig. 11). If you have selected any normalizations, corrections, or other adjustments to the FRET calculation, those will be reflected in the current view.

2. Select “Spreadsheet View” (fig. 11) from the list. Selecting the “Spreadsheet Histogram View” will give you the distribution of the calculated FRET values. Use the “Bin Increment” and “Bin Decrement” icons to change the bin size in the Histogram.

3. Spreadsheet View shows the calculated FRET value at each pixel in the Image, the columns show the X coordinate, the rows show the Y coordinate.

4. Select “Edit” from the main menu bar, then “Copy to Spreadsheet” (fig. 12) to copy the pixel by pixel FRET values to a new XLS spreadsheet.

5. Save the new spreadsheet (fig. 13) by selecting “File” from the main menu bar, then “Save”. Use the keyboard shortcut “Ctrl-S” to save the file immediately to the default spreadsheet location.

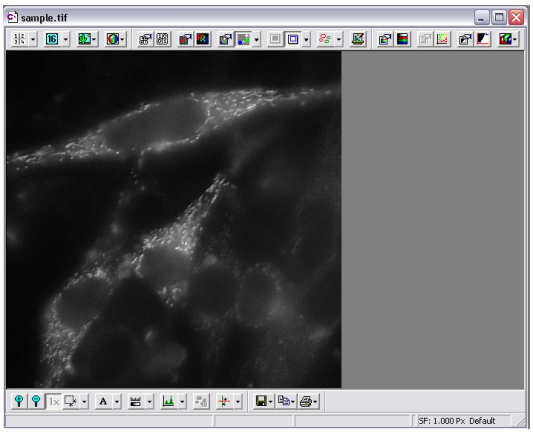


fig.10

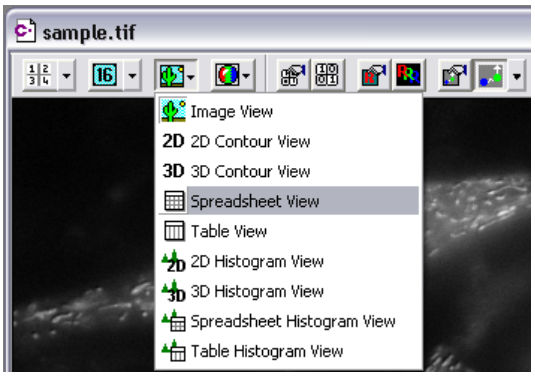
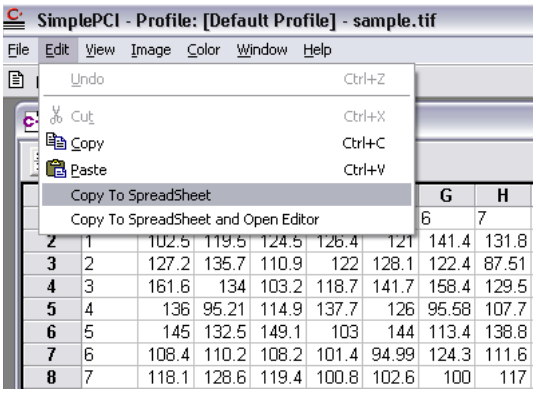
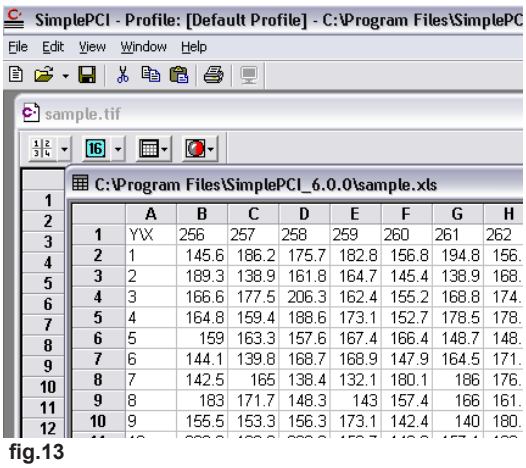


fig.11



							G	H
							6	7
2	1	102.5	119.5	124.5	126.4	121	141.4	131.8
3	2	127.2	135.7	110.9	122	128.1	122.4	87.51
4	3	161.6	134	103.2	118.7	141.7	158.4	129.5
5	4	136	95.21	114.9	137.7	126	95.58	107.7
6	5	145	132.5	149.1	103	144	113.4	138.8
7	6	108.4	110.2	108.2	101.4	94.99	124.3	111.6
8	7	118.1	128.6	119.4	100.8	102.6	100	117

fig.12



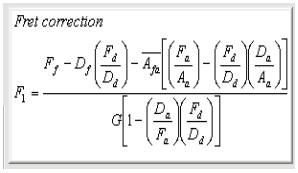
	A	B	C	D	E	F	G	H
1	Y\X	256	257	258	259	260	261	262
2	1	145.6	186.2	175.7	182.8	156.8	194.8	156.
3	2	189.3	138.9	161.8	164.7	145.4	138.9	168.
4	3	166.6	177.5	206.3	162.4	155.2	168.8	174.
5	4	164.8	159.4	188.6	173.1	152.7	178.5	178.
6	5	159	163.3	157.6	167.4	166.4	148.7	148.
7	6	144.1	139.8	168.7	168.9	147.9	164.5	171.
8	7	142.5	165	138.4	132.1	180.1	186	176.
9	8	183	171.7	148.3	143	157.4	166	161.
10	9	155.5	153.3	156.3	173.1	142.4	140	180.

fig.13

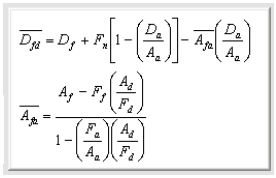
Glossary

Complete FRET
It requires 3 Filter sets and 3 labeled specimens for a total of 9 images.

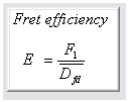
FRET Equation:



Normalization

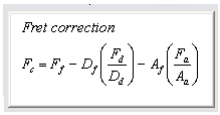


Efficiency:



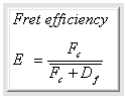
Micro FRET
It requires 3 Filter sets and 3 labeled specimens for a total of 7 images.

FRET Equation:



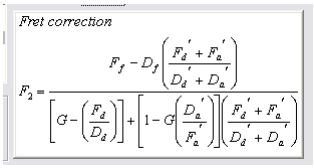
Normalization

Efficiency:

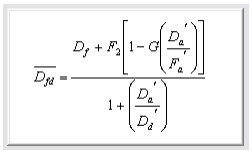


2-Filter FRET
It requires 2 Filter sets and 3 labeled specimens for a total of 6 images.

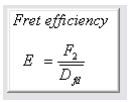
FRET Equation:



Normalization

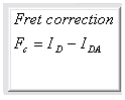


Efficiency:



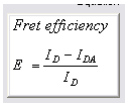
Acceptor Photobleaching
It requires 2 Filter sets and 1 labeled specimen and requires a total of 2 images.

FRET Equation:



Normalization

Efficiency:



References

1. T. Zimmermann, J. Rietdorf, A. Girod, V. Georget, R. Pepperkok;_Spectral imaging and linear un-mixing enables improved FRET efficiency with a novel GFP-YFP FRET pair; FEBS Letters; 531 (2002) 245-249

2. Z. Xia, Y. Liu; Reliable and Global Measurement of Fluorescence Resonance Energy Transfer Using Fluorescence Microscopes; Biophysical Journal; Volume 81 October 2001 2395–2402

3. M. ELANGOVAN, R. N. DAY, A. PERIASAMY; Nanosecond fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy to localize the protein interactions in a single living cell; Journal of Microscopy, Vol. 205, Pt 1 January 2002, pp. 3–14

For more information on QFA-FRET, please see the file QFA-FRET-addendum.pdf on the Examples Disc.