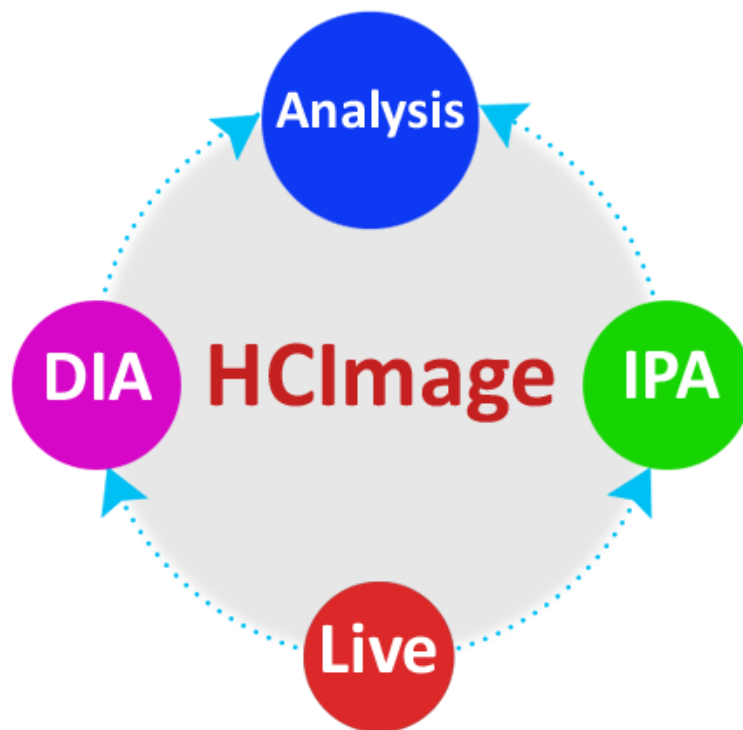


HCIimage FRET

Getting Started Guide



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HCImage FRET Analysis

HCImage Analysis has FRET specific analysis for research into protein-protein interactions. It incorporates selection and customization of algorithms based on published methods for accurate crosstalk correction, FRET measurement and visualization. FRET is an acronym for Fluorescence Resonance Energy Transfer. This protein-protein interaction is characterized by non-radiative transfer of energy from an excited donor fluorophore to another acceptor fluorophore in very close proximity (approximately 30-60 angstroms). FRET is an important technique for imaging biological phenomena where distances, conformational changes and interactions between molecules are of interest. FRET Analysis is Available in HCImage DIA and HCImage Analysis.

HCImage offers both the ability to acquire new images for FRET analysis or to perform analysis on previously collected images. To begin your FRET analysis, you will need to open a target FRET image, select FRET Analysis from the Choose Type of Analysis drop-menu and proceed to Step 1: Method Selection. See FRET Image Acquisition below if you have yet to acquire your target image.

Batch FRET works the same way as FRET Analysis except that instead of using a single image, Batch FRET performs calculations on an image sequence. All of the features and functions outlined in this section apply to both FRET Analysis and Batch FRET except for Acceptor Photobleaching. Acceptor Photobleaching may only be calculated using single images.

FRET Image Acquisition

To begin your FRET analysis, you will need to open a target FRET image. Typically images will be acquired with 2 or 3 channels of data representing the fluorescence intensity in both control images and target images. It is required that the acquisition of these images be under carefully monitored identical conditions including illumination, magnification and registration.

FRET images can contain three channels each using specific filter sets as follows:

- **FRET filter set** (acceptor excitation, donor emission)
- **Donor filter set** (donor excitation, donor emission)
- **Acceptor filter set** (acceptor excitation, acceptor emission)

The Side Panel allows the specification of capture parameters for each of the three channels required, including camera Exposure, Gain, Offset, Sensitivity (multiplication) Gain and the associated emission, dichroic and excitation filter and shutter combination to provide the correct illumination for each channel. These Capture settings can be saved in the HCImage Workfile and reloaded for routine use, allowing the user to maintain the consistent acquisition parameters necessary between image acquisitions. Automated shutter control provides protection for sensitive specimens by minimizing the exposure to excitation illumination. Camera properties including binning, sub-region, etc. should also be kept constant for experimental comparisons.

The image acquisition will generate a three channel image. For consistency the same filter sets should be used with the same channels. HCImage will automatically cycle through the acquisition of each channel and merge these three channels together for display in a color image.

The typical order for the capture and display of the image channels is as follows:

- **Red display channel** - FRET filter set (acceptor excitation, donor emission)
- **Green display channel** - Donor filter set (donor excitation, donor emission)
- **Blue display channel** - Acceptor filter set (acceptor excitation, acceptor emission)

Acquisition for FRET analysis requires excitation/emission filter sets for correct spectral separation of the fluorophores involved. Unavoidable crosstalk or bleed through between channels requires mathematical processing corrections. These algorithms have been published and are discussed in the literature. The more complete correction methods require more control images to determine the correction coefficients to use.

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	f = donor and acceptor fluorophore
D = Donor filter set	d = donor fluorophore
A = Acceptor filter set	a = acceptor fluorophore

The combination of Upper case and Lower case are combined to indicate the filter set and fluorophore (e.g., Ff, Df, Af).

Step 1: Method Selection

To start your FRET analysis, you must enable FRET by going to View in the Menu bar and selecting FRET Analysis. Now you may select **FRET Analysis** from the **Choose Type of Analysis** drop menu. The next step is to choose the FRET correction method, this step determines the algorithm to apply, the filter sets to use and the image combinations required for the calculations. To choose a FRET method, click on one of the methods in the list. The available methods are described below.

Complete FRET

This method corrects for all possible crosstalk situations, including visible donor signal using acceptor filter set and visible acceptor signal using donor filter set. G is the factor relating the FRET induced loss of donor signal with the subsequent increase in acceptor signal. G may be arbitrarily set to 1. Complete FRET requires 3 filter sets and 3 labeled specimens for a total of 9 images.

FRET Correction

$$F_1 = \frac{F_f - D_f \left(\frac{F_d}{D_d} \right) - A_{fa} \left[\left(\frac{F_a}{A_a} \right) - \left(\frac{F_d}{D_d} \right) \left(\frac{D_a}{A_a} \right) \right]}{G \left[1 - \left(\frac{D_a}{F_a} \right) \left(\frac{F_d}{D_d} \right) \right]}$$

Normalization Values

$$\overline{D_{fd}} = D_f + F_a \left[1 - \left(\frac{D_a}{A_a} \right) \right] - A_{fa} \left(\frac{D_a}{A_a} \right)$$

$$\overline{A_{fa}} = \frac{A_f - F_f \left(\frac{A_d}{F_d} \right)}{1 - \left(\frac{F_a}{A_a} \right) \left(\frac{A_d}{F_d} \right)}$$

FRET Efficiency

$$E = \frac{F_1}{\overline{D_{fd}}}$$

MicroFRET

This method assumes simplified crosstalk. No signal visible with acceptor labeled fluorophore and donor filter set. ($D_a = 0$). No signal visible with donor labeled fluorophore and acceptor filter set. ($A_d = 0$) MicroFRET requires 3 filter sets and 3 labeled specimens for a total of 7 images.

FRET Correction

$$F_c = F_f - D_f \left(\frac{F_d}{D_d} \right) - A_f \left(\frac{F_a}{A_a} \right)$$

Normalization Values

$$\overline{D_f} = \frac{D_f}{A_f}$$

FRET Efficiency

$$E = \frac{F_c}{\overline{F_c + D_f}}$$

2-Filter FRET

This method assumes the concentration of donor and acceptor are correlated: Acceptor concentration is whole number multiple of the Donor concentration. Our calculations assume a 1:1 ratio. 2-Filter FRET requires 2 filter sets and 3 labeled specimens for a total of 6 images.

FRET Correction

$$F_2 = \frac{F_f - D_f \left(\frac{F_d' + F_a'}{D_d' + D_a'} \right)}{\left[G - \left(\frac{F_d}{D_d} \right) \right] + \left[1 - G \left(\frac{D_a'}{F_a'} \right) \right] \left(\frac{F_d' + F_a'}{D_d' + D_a'} \right)}$$

Normalization Values

$$\overline{D_{fd}} = \frac{D_f + F_2 \left[1 - G \left(\frac{D_a'}{F_a'} \right) \right]}{1 + \left(\frac{D_a'}{D_d'} \right)}$$

FRET Efficiency

$$E = \frac{F_2}{D_{fd}}$$

Acceptor Photobleaching

This method measures the donor emission before and after acceptor photobleaching. It provides an alternative method of quantifying the FRET efficiency and can be used to provide a double check on previous FRET methods. Acceptor Photobleaching requires 1 filter set and 1 labeled specimen at two time points for a total of 2 images.

FRET Correction

$$F_c = I_D - I_{DA}$$

Normalization Values

$$I_{DA}$$

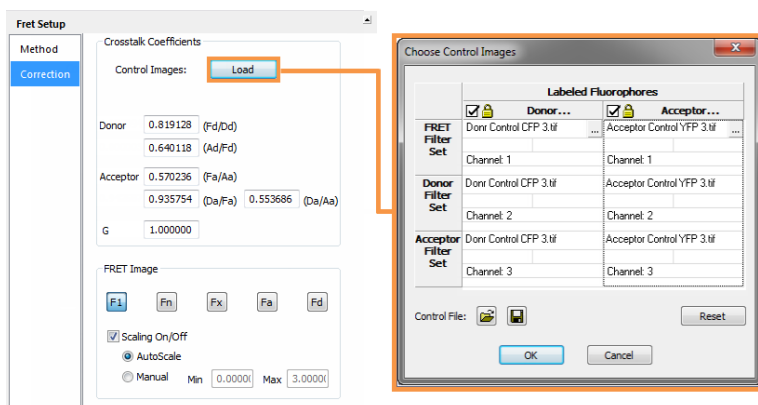
FRET Efficiency

$$E = \frac{I_D - I_{DA}}{I_D}$$

Once you have chosen the FRET method, click Correction to go to the next step.

Step 2: Correction

The second step determines the source of the image channels necessary for performing the calculations and calculates the Crosstalk Coefficients. Select Correction from the FRET Setup panel (shown below) and click **Load**.

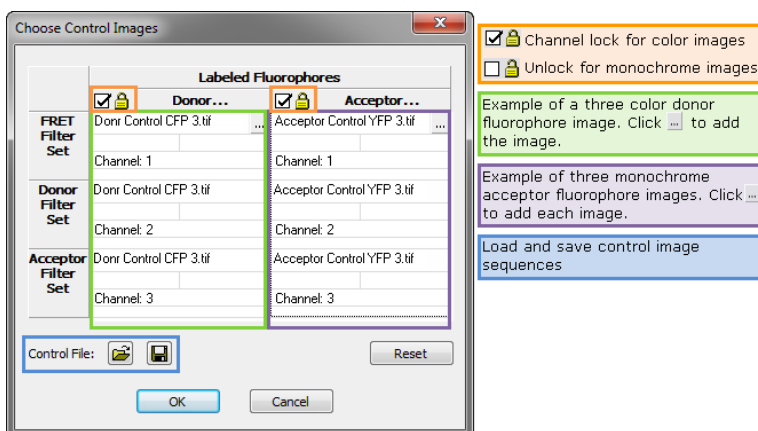


Typically, the target FRET image is normally the open document and automatically entered into the image selection list, so only the control images need to be selected. If you are manually entering known crosstalk coefficients to calculate your FRET correction, no control images are necessary.

Load Images from Disk

FRET images can be selected individually or grouped by fluorophore. You can load three individual monochrome images for the FRET labeled specimen or you can load one three-color image where each component is representative of a filter set (i.e., Red - FRET filter set, Green - Donor filter set, Blue - Acceptor filter set).

To select the control images click the **Load** button in the **FRET Setup** panel.



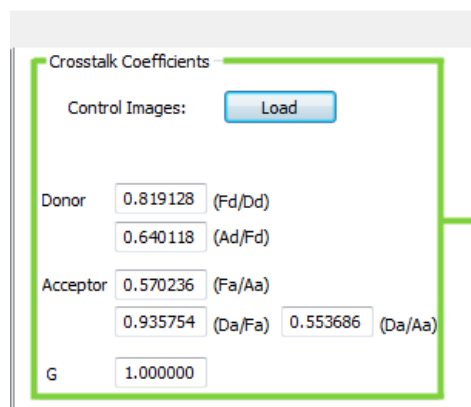
To load a three-color image, make sure the **Locked** option is checked for that fluorophore. Click the [...] button to select the image. To load individual monochrome images separately, uncheck the **Locked** option and there is a [...] button for each component.

Control Files

For an experiment, the same control images may be used if the capture settings are kept constant. When the control images are fully loaded, use the **Save** button to save both the donor and acceptor control image sets to a data document. Click the **Open** button in the **Choose Control Images** dialog to load the control file.

Crosstalk Coefficients

The Crosstalk Coefficients are automatically calculated from the selected control images. Known Crosstalk Coefficients can be entered manually if correction images are not available, or if the values can reliably re-used rather than re-calculated.



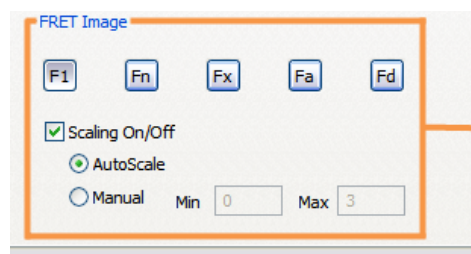
Donor Coefficients
Fd/Dd: The amount of crosstalk of the Donor signal into the FRET channel
Ad/Fd: The amount of crosstalk of the FRET signal into the Acceptor channel

Acceptor Coefficients
Fa/Aa: The amount of crosstalk of the Acceptor signal into the FRET channel
Da/Aa: The amount of crosstalk of the Acceptor signal into the Donor channel
Da/Fa: The amount of crosstalk of the FRET signal into the Donor channel

G is the factor relating the FRET induced loss of Donor signal with the subsequent increase in Acceptor signal. G may be arbitrarily set to 1.

FRET Image

The raw and normalized FRET data will be calculated and reported in the data document. Select one of the FRET image buttons to display the raw or normalized image in the data document.



F1 - Not normalized, raw FRET value
Fn - Normalized using the Acceptor/Donor product
$$Fn = FRET1 / (Acceptor * Donor)$$
Fx - Normalized using the square root of the Acceptor/Donor product
$$Fx = FRET1 / \sqrt{Acceptor * Donor}$$
Fa - Normalized using only the Acceptor
$$Fa = FRET1 / Acceptor$$
Fd - Normalized using only the Donor
$$Fd = FRET1 / Donor$$

AutoScale: The FRET image will be scaled according to the results of the analysis
Min/Max: Set the lower and upper limits for the FRET percentages

The **Min** and **Max** scaling values can be used to control the range of intensities displayed, allowing flexibility in highlighting a specific range of FRET values in the image. You can enter these values manually or select **AutoScale** and these values will be calculated for you.

Step 3. FRET Analysis

After selecting a correction method and the control images, the next step is to define the areas of interest and then calculate FRET. The resulting data is stored in a data document. The diagram below provides an example of the controls and function of the Analysis panel for FRET and Batch FRET Analysis.

The diagram illustrates the FRET Analysis software interface. At the top, the 'Choose Type of Analysis' dropdown is set to 'FRET Analysis' (highlighted in orange), and 'Choose Image(s) to Analyze' is set to 'Fret CFP-YFP 3.tif' (highlighted in red). Below these are five icons for 'Detect Objects': Enhance, Identify, Modify, Qualify, and Draw/Erase. A 'Measure' panel on the left shows a fluorescence image with a table of data:

Obj#	Ave_FRET 1.1
1	27097.706826
2	39502.453409
3	42288.911868
4	39004.625058

Below the table, it says 'Measure multiple objects in a single image'. A 'Select Measure' button is also present. At the bottom, there are two buttons: 'Measure to DataDoc' and 'Measure to Spreadsheet'. Callouts provide further details:

- FRET Analysis** - calculate FRET values, including raw and normalized, for multiple objects in a single image
- Batch FRET** - calculate FRET values for an entire data document
- Select from open image and data documents or choose **Live Scan** to use the image in the Image Display

Below the main interface is a detailed view of the 'Detect Objects' tools:

- Enhance**: Optimize using image processing filters
- Identify**: Identify objects of interest with a binary image layer
- Modify**: Improve object size, shape and connectivity
- Draw/Edit**: Manually create and modify the binary layer
- Qualify**: Remove unwanted objects

Define Areas of Interest

This provides a method for restricting the FRET image display to avoid distracting artifacts or other image details. Use the Enhance, Identify, Modify, Draw/Erase, and Qualify features in the Measurement Tools to define the areas of interest. Anything outside of the defined area will evaluate to zero during FRET calculations.

Calculation

The next step is to decide whether to measure to a data document or to a spreadsheet. By default, all of the available FRET measurements are selected when **FRET Analysis** or **Batch FRET** is selected. So once the objects or areas of interest have been defined, simply click **Measure to DataDoc** or **Measure to Spreadsheet** to collect the data.