

Quantitative Measurements Of FRET Using Standard Fluorescence Microscopy

by Dr. Wolf Malkusch

Introduction

Fluorescence resonance energy transfer (FRET) is a technique used for quantifying the distance between two molecules conjugated to different fluorophores. Since Förster first described the direct energy transfer from a donor fluorophore to a neighboring acceptor molecule without the involvement of a photon,⁽¹⁾ many approaches have been tried to quantify these effects.

By combining optical microscopy with FRET, it is possible to obtain quantitative temporal and spatial information about the binding and interaction of proteins, lipids, enzymes, DNA, and RNA in vivo. This requires resolution below what is normal for a light microscope. In conjunction with the recent development of a variety of mutant green fluorescent proteins (mtGFPs), FRET microscopy provides the potential to measure the interaction of intracellular molecular species in intact living cells where the Donor and Acceptor fluorophores are actually part of the molecules themselves.

However, steady-state FRET microscopy measurements can suffer from several sources of distortion, which need to be corrected. These include direct excitation of the Acceptor at the Donor excitation wavelengths and the dependence of FRET on the concentration of the Acceptor.

Implementation of various methods into an easy-to-use software package

The Carl Zeiss AxioVision FRET system (Figure 1) offers various methods described in the literature to determine the amount of FRET.

Any method includes the acquisition of single or time-lapse series

of images with the options to measure directly the amount of FRET inside selected regions of interest (ROI). These regions may be defined by threshold settings, circles with variable sizes, or freehand. The results of time-lapse series may be displayed as time dependent curves for each selected ROI. In addition, color encoded images may be produced for each time point.

Depending on the method selected, these color-encoded images are calculated for each pixel individually and either show an intensity value for FRET (Fc - Youvan), a FRET ratio image in relation to Donor times

Acceptor (Fn - Gordon), a FRET ratio image in relation to the square root of Donor times Acceptor (NF - Xia), or in relation to the Acceptor alone (Ac).

As an alternative method B measures FRET after bleaching of the Acceptor by calculating the increase of the Donor emission (BL - Siegel).

Either a motorized Axioplan 2 imaging, or an Axiovert 200 mot (Figure 2) may be used as the microscope platform. The method for direct FRET measurement uses a combination of three filter sets, the Donor, FRET, and Acceptor filter set. These filter sets are designed to isolate and maximize three signals: the Donor fluorescence, the Acceptor fluorescence due to FRET, and the directly excited Acceptor fluorescence, respectively.

Obtain quantitative temporal and spatial information about the binding and interaction of proteins, lipids, enzymes, DNA and RNA in vivo.

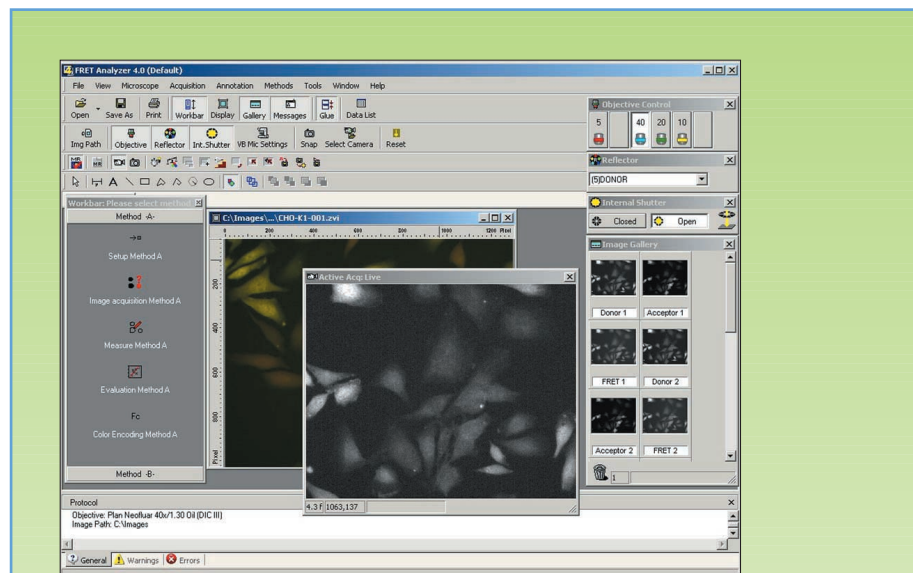


Figure 1. The easy-to-understand user interface of the AxioVision FRET software extremely facilitates all kinds of FRET experiments.



Figure 2. Axiovert 200 mot — the optimum platform for FRET investigations in living cells.

FRET determination using correction methods

As Donors such as CFP generally show a long-wavelength tail in their emission spectrum, there is significant fluorescence at YFP emission wavelengths from CFP alone. It is therefore difficult to estimate FRET accurately from conventional measurements of sensitized emission without elaborate mathematical corrections. Four various methods may be used for the direct FRET measurement. Youvan's method (Fc) simply measures the FRET image and performs a correction by the amount in the Donor and Acceptor channel. For the determination of these correction factors 2 additional specimens are necessary. The first specimen has to be labeled with the Donor fluorochrome only, and the second one only with the Acceptor fluorochrome. From these specimens the correction factors for the cross talk can accurately be determined.

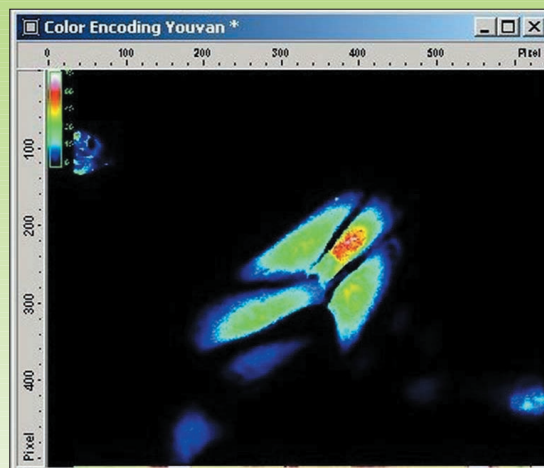


Figure 3. AxioVision FRET — color coded FRET image due to Youvan's method. CHO-K1 cells labeled with a CFP-YFP fusion as a positive control.

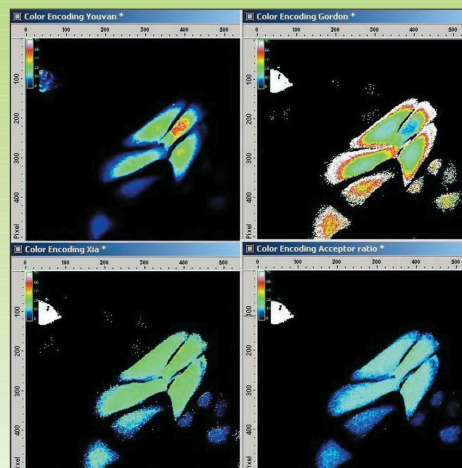


Figure 4. AxioVision FRET — color coded FRET image using correction methods. CHO-K1 cells labeled with a CFP-YFP fusion: top left, 4a, Youvan's method; top right, 4b, Gordon's method; bottom left, 4c, Xia's method; bottom right, 4d, Acceptor ratio method.

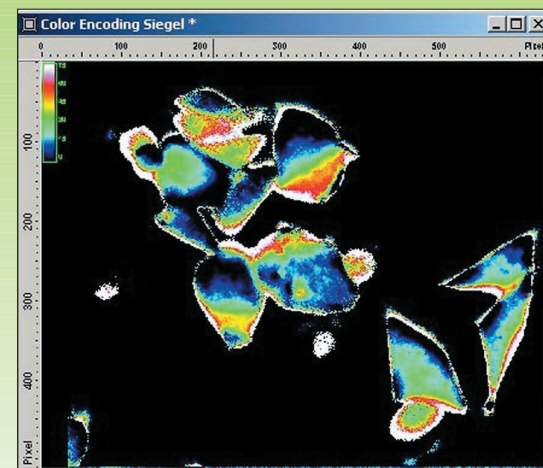


Figure 5. AxioVision FRET — color coded FRET image due to Siegel's method. CHO-K1 cells labeled with a CFP-YFP fusion.

Youvan's method is the base of all other correction measurement types of AxioVision FRET.⁽²⁾ As a result an intensity value is determined (Figure 3). The directly measured FRET intensity from the FRET filter set is corrected for the cross talk from the Donor alone and from the Acceptor alone. The intensity from each filter set will be first corrected by the background intensity prior to the calculation.

The calculation is done by:

$$F_c = \frac{(f_{ret} - bg_{fret}) - cf_{don} * (don_{gv} - bg_{don})}{cf_{acc} * (acc_{gv} - bg_{acc})}$$

F_c = FRET concentration according to Youvan et al.

gv = intensity as gray value

bg = background intensity

cf = correction factor

$fret$ = FRET image

don = Donor image

acc = Acceptor image

With Gordon's method (F_n) this result is additionally normalized to the direct Donor and Acceptor signal.⁽³⁾ As a result a ratio value is determined (Figure 4 b). FRET is measured according to Youvan's method (as described before). The result is divided by the product of the Donor and Acceptor intensity.

$$F_n = F_c / [(don_{gv} - bg_{don}) * (acc_{gv} - bg_{acc})]$$

F_n = FRET concentration according to Gordon et al.

F_c = FRET concentration according to Youvan et al.

gv = intensity as gray value

bg = background intensity

don = Donor image

acc = Acceptor image

Due to the multiplication of Donor and Acceptor this method is calculating the ratio twice, which has the effect, that in many regions relatively low intensities are expressed with very high FRET values. This method might be of special interest when cell structures with low signal intensities are investigated, such as vacuoles or membranes.

The third method from Xia (NF) is similar to Gordon, but using a different formula.⁽⁴⁾ FRET according to Youvan is divided by the square root of the product of Donor and Acceptor. In this way the ratio is calculated only once, and FRET is determined independently from the direct Donor and Acceptor concentrations (Figure 4 c). With this method relative FRET differences can easily be determined.

$$NF = F_c / \sqrt{[(don_{gv} - bg_{don}) * (acc_{gv} - bg_{acc})]}$$

NF = FRET concentration according to Xia et al.

F_c = FRET concentration according to Youvan et al.

$\sqrt{}$ = square root

gv = intensity as gray value

bg = background intensity

don = Donor image

acc = Acceptor image

The fourth method is calculating the ratio of FRET in relation to the Acceptor alone (Ac). FRET according to Youvan is divided by the Acceptor intensity.

$$Ac = F_c / (acc_{gv} - bg_{acc})$$

Ac = FRET concentration as Acceptor ratio

F_c = FRET concentration according to Youvan et al.

gv = intensity as gray value

bg = background intensity

acc = Acceptor image

As with Xia's method the ratio is also only measured once, but the amount of FRET is depending on the Acceptor concentration, resulting in relatively lower FRET values with high Acceptor values, and higher FRET values with low Acceptor concentrations due to the division (Figure 4 d).

FRET determination using Acceptor Bleaching method

An alternative easy way to quantify the absolute efficiency of FRET between the

Donor (CFP) and the Acceptor (YFP) is to measure the Donor emission before and after selective photo bleaching of the Acceptor. The increase, or de-quenching, of Donor emission is a direct measure of the FRET efficiency.

$$BL = [1 - (don_{gv}^{bef} - bg_{don}^{bef}) / (don_{gv}^{aft} - bg_{don}^{aft})] * 100$$

BL = FRET concentration by Acceptor bleaching according to Siegel et al.

don = Donor image

gv = intensity as gray value

bg = background intensity

bef = before bleaching

aft = after bleaching

This procedure uses each cell or sub region as its own internal standard after photo bleaching and is, according to Siegel et al.,⁽⁵⁾ far more accurate than comparing cells with unknown FRET against separate control cells with strong or zero FRET. Values as low as a few percent in this way can be quantified with reasonable confidence. This method was first described for FRET investigations in combination with a Laser Scanning Microscope.⁽⁶⁾

The result is a percentage for the Donor concentration, as only the Donor is directly determined (Figure 5). The authors claim this method the only quantitative method. But this method has decisive disadvantages with living cells, due to even small movements of the cells, or organelles inside the cells.

About the author

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More information about FRET microscopy is available from **Carl Zeiss, Inc.**

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