

Measuring FRET in Flow Cytometry and Microscopy

This unit presents protocols describing the measurement of protein associations using FRET by flow and image cytometry. The theoretical background of FRET is described in detail in *UNIT 1.12*, and will not be discussed here. FRET is ideal for the investigation of protein associations, but can also be used for the sorting of cells in which interaction of one protein with another is detected by FRET (Szöllősi et al., 1998; Mátyus et al., 2001; Nagy et al., 2005; van Wageningen et al., 2006). The proteins under investigation can be labeled by fluorescent antibodies or fluorescent protein (FP) variants. The protocols described are applicable to both situations, except where indicated. Four protocols will be presented. Basic Protocol 1 describes flow cytometric FRET based on the measurement of donor quenching. This method provides a FRET value on a population basis. Basic Protocol 2 covers flow cytometric FRET based on the measurement of fluorescence intensities in the donor, FRET, and acceptor channels, providing cell-by-cell FRET values. Alternate Protocol 1 is based on cell-by-cell correction for autofluorescence and requires the measurement of four fluorescence intensities. The algorithm described can be applied for image cytometric FRET as well. Alternate Protocol 2 is a procedure for application of the FRET protocol to microscopy. Basic Protocol 3 describes image cytometric FRET resolved by donor photobleaching. Consult Table 12.8.1 for applicable combinations of donor and acceptor dye pairs.

A protocol for the measurement of FRET by acceptor photobleaching in microscopy is described in *UNIT 12.7*.

Table 12.8.1 Applicable Fluorophore Combinations for FRET Measurements^a

| Donor | Acceptor |
|------------------------------------|--------------------------|
| Fluorescein, Alexa488, Cy2 | Rhodamine, Alexa546, Cy3 |
| Rhodamine, Cy3, Alexa546, Alexa555 | Cy5, Alexa633, Alexa647 |
| Phycoerythrin | Cy5, Allophycocyanin |
| CFP | YFP, Citrine, Venus |
| BFP | GFP |
| GFP | DsRed |

^aOwing to the very large number of available fluorophores, it is impossible to present a thorough listing of even the most widely used combinations. *UNIT 1.12* contains a detailed list of dye combinations for FRET measurements. Excitation and emission spectra of molecules can be checked on one of the following web sites for donor-acceptor pair selection: Bio-Rad fluorochrome database and charting application (<http://fluorescence.nexus-solutions.net>), Becton-Dickinson Fluorescence Spectrum Viewer (<http://www.bdbiosciences.com/spectra>), Invitrogen-Molecular Probes Spectrum Collection (<http://probes.invitrogen.com/servlets/spectra>). The number of available GFP variants has exploded in recent years. A paper to aid the selection of the optimal GFP variant has been published by the Tsien Laboratory (Shaner et al., 2005). A detailed characterization of classical GFP variants for FRET experiments is also available (Patterson et al., 2000).

FLOW CYTOMETRIC FRET RESOLVED BY DONOR QUENCHING

This protocol is the simplest realization of FRET measurements on a flow cytometer, requiring only a single laser line for excitation of donor fluorescence. However, this approach is probably the most error-prone; it does not provide the FRET efficiency on a cell-by-cell basis. Consequently, experimental results should be interpreted carefully.

Materials

- Cells of interest
- Donor-conjugated antibody
- Acceptor-conjugated antibody
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- 1% to 3.7% (v/v) paraformaldehyde in PBS (optional)
- Flow cytometer capable of detecting donor fluorescence intensity

1. Label individual samples containing $\sim 10^6$ cells with the appropriate donor- and/or acceptor-conjugated antibodies in 50 μ l PBS by incubating 30 min on ice.

At least three different samples are required: (a) unlabeled cells for autofluorescence subtraction; (b) cells labeled with donor-conjugated antibodies; (c) cells labeled simultaneously with both donor-conjugated antibody and acceptor-conjugated antibody (double-labeled sample).

2. Wash twice in PBS. If cells are measured immediately after labeling, keep them on ice all the time to prevent internalization of membrane proteins labeled by the antibodies.

Washing technique and conditions vary with cell type. If cells are not going to be measured promptly, they can be fixed, following washing, in 1% to 3.7% formaldehyde (prepared using factory-made formaldehyde solution or paraformaldehyde crystals). Fixed samples can be stored at 4°C for a few weeks.

3. Adjust detector gain on the flow cytometer so that the brightest (donor-only) and darkest (unlabeled) samples are on the top and bottom part of the scale, respectively. Run the three samples on the flow cytometer. Gate on the forward- versus side-scatter dot plot and determine the mean fluorescence intensity in the donor channel.
4. Calculate the average FRET efficiency (E) of the measured population according to the following equation:

$$E = 1 - \frac{I_{DA} - I_0}{I_D - I_0}$$

Equation 12.8.1

where I_0 is the fluorescence intensity of unlabeled sample, I_D that of the donor-labeled sample, and I_{DA} that of the double-labeled sample.

FLOW CYTOMETRIC FRET MEASUREMENTS BASED ON DETECTION OF THREE FLUORESCENCE INTENSITIES

This method has been published previously (Szöllősi et al., 1984; Trón et al., 1984). The exact form of the equations depends on the fluorophores and flow cytometer used. The protocol has been made as general as possible. In most cases, the equations can be significantly reduced in complexity owing to negligible spectral spillover factors (S_1 to S_6). This method is generally referred to as flow cytometric energy transfer (FCET).

Materials

Cells of interest
Donor-conjugated antibody
Acceptor-conjugated antibody
Phosphate buffered saline (PBS; APPENDIX 2A)
1% to 3.7% paraformaldehyde in PBS
Flow cytometer capable of detecting donor fluorescence and acceptor fluorescence intensities in the donor, FRET, and acceptor channels

1. Label individual samples containing $\sim 10^6$ cells with the appropriate donor- and/or acceptor-conjugated antibodies in 50 μl PBS by incubating 30 min on ice.

This approach is applicable to cells labeled with fluorescent antibodies or transfected with suitable FP variants. The four necessary samples are the following: (a) cells without donor and acceptor labeling for autofluorescence correction; (b) cells labeled with donor-conjugated antibody or cells transfected with donor FP variant for the determination of spectral spillover factors for the donor; (c) cells labeled with acceptor-conjugated antibody or cells transfected with acceptor FP variant for the determination of spectral spillover factors for the acceptor; (4) double-labeled or double-transfected cells for measuring FRET.

For the determination of factor α , an antibody has to be labeled separately with donor and acceptor. One of the samples is labeled with the donor-conjugated antibody, and the other is labeled with the acceptor-conjugated antibody, i.e., the two samples are identically labeled except for the color of the fluorophore. Both antibodies have to saturate the binding sites.

2. Wash twice in PBS. If cells are measured immediately after labeling, keep them on ice all the time to prevent internalization of membrane proteins labeled by the antibodies.

Washing technique and conditions vary with cell type. If cells are not going to be measured promptly, they can be fixed, following washing, in 1% to 3.7% formaldehyde (prepared using factory-made formaldehyde solution or paraformaldehyde crystals). Fixed samples can be stored at 4°C for a few weeks.

3. Use a flow cytometer capable of detecting the following three fluorescence intensities:
 - a. Donor fluorescence (excited at the donor absorption wavelength, detected at the donor emission wavelength). Designate this value as I_1 .
 - b. FRET (excited at the donor absorption wavelength, detected at the acceptor emission wavelength). Designate this value as I_2 .
 - c. Direct acceptor fluorescence (excited at the acceptor absorption wavelength, detected at the acceptor emission wavelength). Designate this value as I_3 .

4. Adjust detector gains so that the brightest and darkest samples can be measured using the same voltage.

The gain of photomultiplier tubes changes in a nonlinear fashion with the voltage; therefore, it cannot be corrected for. Linear gain factors (using linear amplifiers) can be taken into consideration during analysis.

5. Run the samples on the flow cytometer.
6. Use a software program capable of performing arithmetic operations between parameters (Szentesi et al., 2004).

A program written specifically for flow cytometric FRET calculations is ReFlex, which can be downloaded from <http://www.freewebs.com/cytoflex>.

- Determine intensities I_1 , I_2 , and I_3 for the unlabeled sample, and subtract these mean intensities from the corresponding fluorescence channels of the donor, acceptor, and FRET samples.

IMPORTANT NOTE: All intensities in the following discussion are assumed to be background corrected.

- For the donor-only sample, determine the spectral correction factors S_1 and S_3 , characterizing the spillover of donor fluorescence from the donor channel into the FRET and acceptor channels, respectively, as follows:

$$S_1 = \frac{I_2}{I_1}, S_3 = \frac{I_3}{I_1}$$

Equation 12.8.2

- For the acceptor-only sample determine the spectral correction factors S_2 and S_4 , characterizing the spillover of acceptor fluorescence from the acceptor channel into the FRET and donor channels, respectively, as follows:

$$S_2 = \frac{I_2}{I_3}, S_4 = \frac{I_1}{I_3}$$

Equation 12.8.3

- Determine factor α using a donor- and an acceptor-labeled sample labeled by antibodies with the same idiotype but conjugated to different fluorophores. Calculate the mean background-corrected I_1 fluorescence intensity of the donor-only sample, and the mean background-corrected I_2 fluorescence intensity of the acceptor-labeled sample. Determine α according to the following equation:

$$\alpha = \frac{I_{2,a} \epsilon_d L_d}{I_{1,d} \epsilon_a L_a}$$

Equation 12.8.4

where ϵ_d and ϵ_a are the molar absorption coefficients of the donor and the acceptor, respectively, at the donor excitation wavelength (i.e., the excitation wavelength used for I_1 and I_2), and L_d and L_a are the labeling ratios (i.e., number of fluorophores/antibody) of the donor- and acceptor-labeled antibodies, respectively.

The use of robust estimators of central tendency (trimmed mean, median) instead of the mean is preferable if the distribution is wide or if there are outlier events significantly distorting the mean. If cells transfected with FP variants are used, a different approach has to be used for the determination of α , which is described in detail elsewhere (Nagy et al., 2005).

- Run the double-labeled sample on the flow cytometer. In this sample, the I_1 , I_2 , and I_3 intensities can be expressed according to the following equations:

$$I_1 = I_D(1 - E) + I_A \cdot S_4 + I_D \cdot E \cdot \alpha \cdot \frac{S_4}{S_2}$$

Equation 12.8.5

$$I_2 = I_D(1 - E) \cdot S_1 + I_A \cdot S_2 + I_D \cdot E \cdot \alpha$$

Equation 12.8.6

$$I_3 = I_D(1 - E) \cdot S_3 + I_A + I_D \cdot E \cdot \alpha \cdot \frac{1}{S_2} \cdot \frac{\epsilon_{\lambda_A}^D \cdot \epsilon_{\lambda_D}^A}{\epsilon_{\lambda_D}^D \cdot \epsilon_{\lambda_A}^A}$$

Equation 12.8.7

where E is the FRET efficiency, I_D and I_A are the unquenched donor and direct acceptor fluorescence intensities, respectively, and ϵ denotes the molar absorption coefficient of the donor (superscript D) or acceptor (superscript A) labeled in the upper index, at the donor (subscript λ_D) or acceptor (subscript λ_A) excitation wavelengths.

From the above system of equations, E can be calculated as follows:

$$E = \frac{S_2(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4)}{\alpha \left(\frac{\epsilon_{635}^D \cdot \epsilon_{488}^A}{\epsilon_{488}^D \cdot \epsilon_{635}^A} - 1 \right) (I_2S_4 - I_1S_2) + S_2(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4)}$$

Equation 12.8.8

In most cases, the above equation can be simplified by neglecting some of the constants. For example S_3 , S_4 , and the absorption ratio in parentheses in the numerator on the right-hand side of Equation 12.8.8 are negligible for the Cy3-Cy5 donor-acceptor pair measured on a FACSCalibur. In this case the equation can be rewritten in the following form:

$$E = \frac{S_2(I_2 - I_1S_1 - I_3S_2)}{\alpha I_1S_2 + S_2(I_2 - I_1S_1 - I_3S_2)}$$

Equation 12.8.9

CELL-BY-CELL CORRECTION FOR AUTOFLUORESCENCE

If the fluorescence intensity of the samples is comparable to autofluorescence, subtraction of a constant autofluorescence value can result in serious errors in the calculation. In a slightly modified version of the approach described above, a fourth fluorescence intensity is measured, corresponding to autofluorescence. The excitation and emission wavelengths for this channel, designated by I_0 , are chosen such that donor and acceptor fluorophores will not have considerable contribution to the fluorescence intensity measured in the autofluorescence channel. The method has been published in Sebestyén et al. (2002). In addition to the procedures described in Basic Protocol 1 perform the following steps:

1. When running the unlabeled sample, determine factors B_1 , B_2 , and B_3 , characterizing the spillover of autofluorescence from the autofluorescence channel to the donor, FRET, and acceptor channels, respectively:

$$B_1 = \frac{I_1}{I_0}, B_2 = \frac{I_2}{I_0}, B_3 = \frac{I_3}{I_0}$$

Equation 12.8.10

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12.8.5

2. When running the donor-labeled sample, determine factor S_5 characterizing the spectral spillover of donor fluorescence from the donor channel to the autofluorescence channel:

$$S_5 = \frac{I_0}{I_1}$$

Equation 12.8.11

3. When running the acceptor-labeled sample, determine factor S_6 characterizing the spectral spillover of acceptor fluorescence from the acceptor channel to the autofluorescence channel:

$$S_6 = \frac{I_0}{I_3}$$

Equation 12.8.12

4. Express the fluorescence intensities of the double-labeled sample by the following set of equations:

$$I_0 = AF + I_D(1-E) \cdot S_5 + I_A \cdot S_6 + I_D \cdot E \cdot \alpha \cdot \frac{S_6}{S_2}$$

Equation 12.8.13

$$I_1 = AF \cdot B_2 + I_D(1-E) + I_A \cdot S_4 + I_D \cdot E \cdot \alpha \cdot \frac{S_4}{S_2}$$

Equation 12.8.14

$$I_2 = AF \cdot B_3 + I_D(1-E) \cdot S_1 + I_A \cdot S_2 + I_D \cdot E \cdot \alpha$$

Equation 12.8.15

$$I_3 = AF \cdot B_4 + I_D(1-E) \cdot S_3 + I_A + I_D \cdot E \cdot \alpha \cdot \frac{1}{S_2} \cdot \frac{\epsilon_{635}^D \cdot \epsilon_{488}^A}{\epsilon_{488}^D \cdot \epsilon_{635}^A}$$

Equation 12.8.16

where AF denotes the autofluorescence intensity of single cells.

The above set can be converted to a system of linear equations by designating the term $I_D \cdot E$ as X :

$$I_0 = AF + I_D S_5 + I_A \cdot S_6 + X \cdot \left(\alpha \cdot \frac{S_6}{S_2} - S_5 \right)$$

Equation 12.8.17

$$I_1 = AF \cdot B_1 + I_D + I_A \cdot S_4 + X \cdot \left(\alpha \cdot \frac{S_4}{S_2} - 1 \right)$$

Equation 12.8.18

$$I_2 = AF \cdot B_2 + I_D \cdot S_1 + I_A \cdot S_2 + X \cdot (\alpha - S_1)$$

Equation 12.8.19

$$I_3 = AF \cdot B_3 + I_D \cdot S_3 + I_A + X \cdot \left(\alpha \cdot \frac{1}{S_2} \cdot \frac{\epsilon_{635}^D \cdot \epsilon_{488}^A}{\epsilon_{488}^D \cdot \epsilon_{635}^A} - S_3 \right)$$

Equation 12.8.20

5. Calculate the FRET efficiency according to the following equation:

$$X = \begin{vmatrix} 1 & S_5 & S_6 & I_0 \\ B_1 & 1 & S_4 & I_1 \\ B_2 & S_1 & S_2 & I_2 \\ B_3 & S_3 & 1 & I_3 \end{vmatrix}, I_D = \begin{vmatrix} 1 & I_0 & S_6 & \alpha \frac{S_6}{S_2} - S_5 \\ B_1 & I_1 & S_4 & \alpha \frac{S_4}{S_2} - 1 \\ B_2 & I_2 & S_2 & \alpha - S_1 \\ B_3 & I_3 & 1 & \alpha \cdot \frac{1}{S_2} \cdot \frac{\epsilon_{635}^D \cdot \epsilon_{488}^A}{\epsilon_{488}^D \cdot \epsilon_{635}^A} - S_3 \end{vmatrix}$$

$$X = \begin{vmatrix} 1 & S_5 & S_6 & \alpha \frac{S_6}{S_2} - S_5 \\ B_1 & 1 & S_4 & \alpha \frac{S_4}{S_2} - 1 \\ B_2 & S_1 & S_2 & \alpha - S_1 \\ B_3 & S_3 & 1 & \alpha \cdot \frac{1}{S_2} \cdot \frac{\epsilon_{635}^D \cdot \epsilon_{488}^A}{\epsilon_{488}^D \cdot \epsilon_{635}^A} - S_3 \end{vmatrix}, I_D = \begin{vmatrix} 1 & S_5 & S_6 & \alpha \frac{S_6}{S_2} - S_5 \\ B_1 & 1 & S_4 & \alpha \frac{S_4}{S_2} - 1 \\ B_2 & S_1 & S_2 & \alpha - S_1 \\ B_3 & S_3 & 1 & \alpha \cdot \frac{1}{S_2} \cdot \frac{\epsilon_{635}^D \cdot \epsilon_{488}^A}{\epsilon_{488}^D \cdot \epsilon_{635}^A} - S_3 \end{vmatrix}$$

Equation 12.8.21

And finally:

$$E = \frac{X}{I_D}$$

Equation 12.8.22

If S_3 , S_4 , S_6 , and the absorption ratio (i.e., the ratio term containing the epsilons) are negligible (which is the case when using the Cy3-Cy5 donor-acceptor pair and detecting autofluorescence in the FL1 channel of a FACSCalibur (Sebestyén et al., 2002)), the equation takes on a much simpler form:

$$E = 1 + \frac{\alpha(I_0 B_1 - I_1)}{I_2 - B_2 I_0 + \alpha(I_1 - B_1 I_0) - I_1 S_1 + B_1 I_0 S_1 - I_3 S_2 + B_3 I_0 S_2 + S_5(B_2 I_1 - B_1 I_2 - B_3 I_1 S_2 + B_1 I_3 S_2)}$$

Equation 12.8.23

APPLICATION OF THE FRET PROTOCOL TO MICROSCOPY

This protocol can be applied to the measurement of FRET in fluorescence microscopy (Nagy et al., 1998). The principal difference is in the determination of α . According to one approach, a sample is labeled with a donor-conjugated and an acceptor-conjugated antibody against the same protein. It is required that the antibodies not compete with each other, and that there be no FRET between them. If these conditions are met, the same number of donor-conjugated and acceptor-conjugated antibodies are bound to the sample, and they fluoresce independently of each other. In this case, Equation 12.8.4 can be used

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12.8.7

to calculate α if both I_1 and I_2 are pure donor and acceptor intensities, respectively (Nagy et al., 1998). An alternate approach can be used when a photosensitive (i.e., bleachable) acceptor is used. If the acceptor is bleached, the donor fluorescence intensity increases owing to the elimination of FRET (dequenching). The lost acceptor intensity divided by the gain in donor intensity yields α . In this case, it must also be ensured that pure donor and acceptor intensities are considered in the calculation (Zal and Gascoigne, 2004).

DONOR PHOTBLEACHING FRET MEASUREMENTS IN MICROSCOPY

FRET measurements based on the measurement of donor photobleaching kinetics present an extremely easy method for the pixel-by-pixel determination of FRET efficiencies. The required instrumentation is available in most laboratories. However, as in the case of FRET measurements based on the measurement of donor quenching, simplicity comes at a price: FRET is only one of the parameters influencing the rate of donor photobleaching. Therefore, carefully designed control experiments are required to establish that the observed effects are due to FRET.

Materials

- Cells labeled with donor-conjugated antibody on coverslip
- Cells double-labeled with donor-conjugated antibody and acceptor-conjugated antibody on coverslip
- Fluorescence microscope with filters appropriate for the fluorophores used

1. Mount the donor-labeled sample on the microscope, and select an area of interest. Bleach the donor and record a time series until the donor fluorescence disappears or becomes constant.

The implementation of bleaching is microscope dependent. Basically, the area of interest has to be illuminated with a strong beam capable of exciting the donor. According to one approach, the bleaching beam is used for imaging as well, i.e., the sample is illuminated with the beam for a given duration, and an image is taken during this time. In an alternative method, the bleaching beam, and a usually less strong imaging beam, are alternately shined on the sample. The acquisition time of the images should be adjusted such that the number of collected images should be ~ 30 , for accurate fitting. In general, the more parameters to be fitted, the more images are needed.

The following is a brief outline of the procedure with the Zeiss LSM 510 microscope, which provides a streamlined interface for photobleaching FRET measurements. Under Time Series, choose "Manual" for "Start series," and adjust the number of exposures under "Stop series." The number depends on the fluorophore and the strength of the bleaching beam. These should be adjusted such that ~ 30 images are acquired. Adjust the delay between cycles to zero. Press StartT to begin the acquisition of the specified number of images. In this case, the same exposure is used for bleaching and image acquisition. Under EditBleach, one can define the region to be bleached with square-, polygon-, and ellipse-shaped ROIs, and select the laser line for bleaching. Adjust the number of iterations, i.e., how many times the selected ROI is scanned with the bleaching beam. This option actually adjusts the amount of bleaching taking place between two successive images. Adjust the "Bleach after number of scans" box to 1, so that one image is taken by the imaging beam followed by one bleaching shot. Start the cycle with the StartB button.

2. Repeat step 1 with the double-labeled sample.
3. In an image-analysis program, open the first image in the bleaching sequence of the donor-only sample, place regions of interest (ROIs) on the cells, and analyze the decline of donor fluorescence intensity as a function of time. Export the intensity data so that they can be imported later to a program (e.g., Microcal Origin) capable of fitting exponential functions to the data.
4. Repeat step 3 with the recorded images of the double-labeled sample.

Photobleaching kinetics of fluorochromes can usually be reasonably well approximated by an exponential function with a constant background term (Young et al., 1994; Song et al., 1995, 1996, 1997). One must decide how many exponential terms have to be included. In many cases, a single exponential term is sufficient, and the photobleaching kinetics of the donor can be described by the following function:

$$I(t) = I_0 e^{-\frac{t}{\tau_D}}$$

Equation 12.8.24

where $I(t)$ is the time-dependent intensity of the donor, I_0 is the initial, maximal intensity of the donor, t is time, and τ_D is the photobleaching time constant of the donor in the absence of the acceptor. If a single exponential term is not sufficient to fit the bleaching kinetics without systematic deviation, increase the number of exponential terms. If the number of exponential terms is n , the equation takes the following form:

$$I(t) = \sum_{k=1}^n I_k e^{-\frac{t}{\tau_{Dk}}}$$

Equation 12.8.25

5. From the τ_{Dk} time constants, calculate an effective bleaching time constant:

$$\tau_D = \frac{\sum_{k=1}^n I_k \tau_{Dk}}{\sum_{k=1}^n I_k}$$

Equation 12.8.26

6. Calculate the mean of the pixel-by-pixel or ROI-by-ROI τ_D values ($\langle \tau_D \rangle$).
7. Repeat the calculations of step 5 for the donor-acceptor double-labeled sample to yield the bleaching time constant of the donor in the presence of the acceptor (τ_{DA}). Calculate the mean value of the τ_{DA} values ($\langle \tau_{DA} \rangle$).

The mean FRET efficiency can be found according to the following equation:

$$E = 1 - \frac{\langle \tau_D \rangle}{\langle \tau_{DA} \rangle}$$

Equation 12.8.27

Alternatively, the FRET efficiency can be determined on a pixel-by-pixel basis in the donor-acceptor double-labeled sample according to the following equation:

$$E_i = 1 - \frac{\langle \tau_D \rangle}{\tau_{DAi}}$$

Equation 12.8.28

where E_i is the pixel-by-pixel (or ROI-by-ROI) FRET, and τ_{DAi} is the photobleaching time constant of the donor in the presence of the acceptor in a single pixel (or ROI). An analysis program, "pbFRET," has been written for the evaluation of a donor-photobleaching FRET image series and is available at <http://www.freewebs.com/cytoflex> (Szentesi et al., 2005).

COMMENTARY

Background Information

Measurement of donor quenching is probably the easiest way to perform a FRET experiment, but simplicity comes at a price. In these kinds of measurements, the average fluorescence intensities of two different samples (donor-labeled and double-labeled) are compared. Therefore, FRET cannot be calculated on a cell-by-cell basis; instead, a population average is measured, i.e., heterogeneities cannot be resolved. Consequently, a large enough number of cells has to be measured (at least 10,000 cells) so that the population mean can be determined accurately. Microscope measurement of FRET based on donor quenching is therefore not feasible. It is assumed that the only difference between the donor-only and the double-labeled samples is the presence of the acceptor. Since it is practically impossible to meet this requirement for cells transfected with FP variants, donor quenching–resolved FRET measurements can be done only on antibody-labeled cells. There is always one additional control to make, to check for competition of the antibody carrying the acceptor with donor labeling. This should be done with the unlabeled antibody against the “acceptor” epitope, and any decrease of donor fluorescence caused by adding the unlabeled antibody should be attributed to competition rather than donor quenching that results from FRET. Needless to say, competition between labeling antibodies is likely also a sign of molecular proximity, albeit not as readily quantitated as FRET. In some rare cases, an antibody increases the binding of another antibody. This enhancement can also lead to misinterpretation of FRET data. For example, if the acceptor-labeled antibody increases the binding of the donor-labeled antibody, the unquenched donor intensity of the donor-acceptor double-labeled sample is larger than that of the donor-only sample, so the FRET calculated by comparing the donor intensity of the donor-acceptor double-labeled sample and the donor-only sample will be underestimated.

In some cases, the acceptor fluorescence may spill over to the donor channel, and the assumption that the background (i.e., non-donor) fluorescence intensity of the double-labeled sample is equal to the fluorescence intensity of the unlabeled sample does not hold. In such a case, a sample labeled by the acceptor-conjugated antibody and the unlabeled antibody against the donor epitope (to correct for the competition between the two antibodies)

is to be used for background subtraction. An equation taking acceptor spillover and competition effects into account can be written in the following form:

$$\text{FRET} = 1 - \frac{I_{\text{DA}} - I_{0\text{A}}}{I_{\text{D0}} - I_0}$$

Equation 12.8.29

where $I_{0\text{A}}$ and I_{D0} denote, respectively, the fluorescence intensities (measured in the donor channel) of the sample labeled by the unlabeled antibody against the donor epitope and the acceptor-conjugated antibody, and that of the sample labeled by the donor-conjugated antibody and the unlabeled antibody against the acceptor epitope.

Critical Parameters and Troubleshooting

Flow cytometry

Flow cytometric FRET measurements provide the opportunity to measure energy transfer on a cell-by-cell basis and resolve heterogeneities in cell populations. Although the spectroscopic spillover factors S_1 to S_6 are not expected to show any cell-by-cell heterogeneity, their cell-by-cell determination also has certain advantages. Performing mathematical calculations with cells having low fluorescence intensity introduces a large error into the calculations. Omitting these cells from the determination of the S factors greatly increases the reliability of these calculations.

As already pointed out, large autofluorescence (compared to the donor and acceptor intensities) makes the calculations error-prone. Therefore, it is advisable to decrease the autofluorescence level as much as possible. A straightforward way to achieve this is to use yellow or red fluorescent dyes, since cellular autofluorescence becomes progressively weaker in the red region of the visible spectrum.

In a similar vein, the higher the amount of spectral spillover compared to the pure FRET signal, the lower the reliability of the experiment. Therefore, every effort has to be made to minimize spectral spillover between different fluorescence channels. This can be achieved by carefully selecting the fluorophores used as well as the excitation and emission filters (Horváth et al., 2005).

In addition to the distance dependence, FRET efficiency is also a function of the

relative orientation of the fluorophores. This makes the interpretation of FRET values complicated. A decrease in FRET may be the result of an increased average separation between the donor and the acceptor. Alternatively, the distance between the fluorophores may not have changed, and the lower FRET efficiency can be the consequence of a less favorable relative orientation between the fluorophores. It is advisable to use alternative approaches in order to convincingly prove the reason for the observed changes in FRET efficiency. For example, if fluorescent antibodies are used, antibodies against different epitopes can also be tested, if available. On the other hand, in most practical cases the relative orientation of the fluorophores does not significantly influence FRET experiments, owing to the fact that the fluorophores rotate very rapidly on the time scale of the FRET interaction, resulting in dynamic averaging of the orientations (Dale et al., 1979). This is the case for fluorescently labeled antibodies and proteins labeled with FP variants, but dyes rigidly interacting with a large molecule (e.g., DNA) may be considered stationary during the FRET interaction. In the latter case, the orientation substantially influences the FRET phenomenon.

Although the presence of FRET unquestionably implies a short separation distance between the donor and the acceptor, it begs the question whether the detected association is the result of a biologically relevant interaction or is a chance association of fluorophores present at large densities. One can use model calculations to predict the average distance of donors and acceptors, assuming random distribution, and compare the predictions of the calculations to the observed FRET efficiencies (Wolber and Hudson, 1979; Trón et al., 1984; Szöllősi et al., 1989). Alternatively, the examination of the dependence of FRET on the acceptor density and on the donor-acceptor ratio can make it possible to decide whether the examined proteins form clusters or are randomly distributed (Kenworthy and Edidin, 1998). It has to be noted that even random associations resulting from high expression levels of certain oncoproteins must not be regarded as biologically irrelevant.

FRET microscopy

This method carries with it inherent advantages and disadvantages. Donor photobleaching kinetics are assumed to be independent of the expression density of the protein under investigation; therefore, the method is not sensitive for the cell-by-cell or sample-by-sample

variation of protein expression level. While subcellular distribution of E is derived from the measurement, this distribution should be judged carefully, since pixel-by-pixel variations of bleaching times can also result from variations of the local molecular environment or oxygenation, as well as from previous bleaching of neighboring cells or even pixels. For this reason, the method offers more reliable results in full-field microscopy, while confocal laser scanning microscopes tend to fare worse in implementing this approach. Nonetheless, donor photobleaching FRET is relatively simple to implement and is rather sensitive—FRET efficiencies of 2% to 4% can be measured reasonably well if labeling is good and images are free of noise. Adherent cells are the best targets for investigations with this approach, as they are for other image cytometric FRET measurements. However, suspension cells and cell lines can also be measured after making the cells adhere to a substrate, either by sedimentation onto poly-L-lysine or collagen-coated coverslips, or by using a cytocentrifuge.

Some disadvantages and pitfalls should also be considered when choosing and implementing this method. Primarily, the measurements are not self-controlled in the conventional sense (i.e., the assumption that the donor-labeled and the donor-acceptor double-labeled samples differ only in the presence of the acceptor is not necessarily true). Therefore, care should be taken to execute bleaching sequences alternately between the donor only- and the donor plus acceptor-labeled samples, even more so as fluctuations of temperature, illumination intensity, and oxygenation are factors that greatly influence the photobleaching rate. Mixing donor-labeled and donor-acceptor double-labeled sample on the same slide and bleaching them simultaneously is an easy and effective way to minimize the effects of different environmental factors on the FRET measurement. A dye that is easily photobleached should be chosen as the donor—e.g., fluorescein. This should minimize movement artifacts that hinder the proper exponential fitting. Should such artifacts persist, the microscope needs to be checked for mechanical stability, and the cells for proper adherence. Sequential images can be corrected for registration (*UNIT 12.2*), but in the case of a long sequence (~30 images are necessary for a good fitting), this may be cumbersome even if using a simple FFT (fast Fourier transform)-based algorithm.

Special consideration ought to be given to the actual kinetics of photobleaching. This mostly depends on the number and nature of various excited-state reactions the donor can undergo. Fluorescein, for example, shows a rather complex behavior in this respect (Song et al., 1995); therefore, although it offers the advantage of fast bleaching, a multiexponential fitting may be necessary to obtain the proper bleaching time constants. In practice, quite often, a double exponential works very well. Here, for easy comparison of pixels or cells, an amplitude-weighted average can be calculated from the two bleaching time constants (Young et al., 1994). Other considerations are the initial bleaching that occurs during the adjustment of the microscope, localization of the spot to be measured, and focusing. If the bleaching is not monoexponential, this will lead to an overestimation of the time constant, since the initial fast-bleaching components will not be analyzed. Furthermore, it is most necessary to choose a highly photostable acceptor, since photobleaching of the acceptor after excitation via FRET will primarily destroy the nearest acceptors and eliminate FRET between the tightest donor-acceptor pairs. This will certainly lead to an underestimation of E . A sign that hints at such a possibility is the relative stability or unexpected increase of donor fluorescence in the initial phases of the bleaching curve.

Comparison between flow cytometric FRET measurements and the donor photobleaching image cytometric approach has revealed that consistently higher transfer values are obtained with the donor photobleaching FRET method. This overestimation is independent of the pixel size: pixel sizes as large as a cell give similar results to those obtained with smaller ones, reinforcing the view that energy transfer values are independent of fluorescence intensity in the samples. Some of this discrepancy can be attributed to the different weighting of energy transfer values in the donor photobleaching FRET and the ratiometric energy transfer methods. Using Monte Carlo simulation, it was demonstrated that this overestimation in donor photobleaching FRET is proportional to the heterogeneity in the pixel-by-pixel FRET efficiency values. Therefore, discrepancies between FRET efficiency values obtained with donor photobleaching FRET and ratiometric approaches should be interpreted with caution (Nagy et al., 1998).

Anticipated Results

Flow and image cytometric FRET measurements, if carefully carried out and interpreted, can strongly support the association of the investigated proteins if the observed FRET efficiencies are larger than a threshold value. The threshold value is experiment dependent; therefore, a negative control (nonassociating proteins) should be checked as well. The lack of FRET between two investigated proteins does not prove that they do not associate with each other. Suboptimal orientation of fluorophores may prevent FRET from happening even if the molecules are within FRET distance from each other. In addition, the labeled epitopes on the protein may be on opposing sides of the proteins, leading to a relatively large separation distance even though the proteins are in close apposition. Flow cytometric FRET measurements based on donor quenching provide a single FRET efficiency for the whole population. FCET measurements yield single-cell FRET efficiencies and, owing to the high number of cells measured in a reasonably short time, the statistical accuracy is high. Microscopic FRET measurements give sub-cell resolution, but their statistical reliability is inferior compared to those obtained via flow cytometric approaches.

Time Considerations

Labeling of cells with antibodies takes 1 to 2 hr; both the measurement and evaluation of the basic set of samples require ~ 1 hr. Microscopic FRET experiments are usually more laborious than flow cytometric ones. Processing time for one image (bleaching and data analysis) can range from 1 to 5 min, and the total time depends upon how many images are analyzed from a sample. For reliable statistics, at least 20 to 30 cells are analyzed from the donor-only sample and 20 to 30 cells from the double-labeled sample. One experimental set can be analyzed in 1 to 5 hr.

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Contributed by Péter Nagy, György Vereb,
Sándor Damjanovich, László Mátyus,
and János Szöllősi
University of Debrecen
Debrecen, Hungary

The authors wish to acknowledge support from the Hungarian Academy of Sciences (grant numbers: OTKA F049025, T043509, T043061, T037831), the Péter Pázmány Program for Regional Knowledge Centers (contract number: 06/2004), and the European Commission (contract numbers: LSHC-CT-2005-018914, LSHB-CT-2004-503467).