A quantitative protocol for intensity-based live cell FRET imaging.

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Summary
Förster resonance energy transfer (FRET) has become one of the most ubiquitous and powerful methods to quantify protein interactions in molecular biology. FRET refers to the sensitization of an acceptor molecule through transfer of energy from a nearby donor and it can occur if the emission band of the donor exhibits spectral overlap with the absorption band of the acceptor molecule. Numerous methods exist to quantify FRET levels from interacting protein labels including fluorescence lifetime, acceptor photobleaching, and polarization resolved imaging **(1--3)**. For live cell imaging, however, sensitized emission FRET (seFRET) is the most powerful and robust method of FRET signal quantification **(4)**. It is fast, can be applied using straight forward microscopy equipment and offers information not only on strength of interaction, but, uniquely, also on the relative changes between interacting and non-interacting moieties in the reaction, referred to as FRET stoichiometry. A rigorous and quantitative application of seFRET is far from trivial, however, and requires appropriate calibration experiments and constructs, control over hardware settings and appropriate image processing steps.
This protocol presents a rigorous method to perform quantitative seFRET measurements in live cells, providing the maximum possible information content from the measurement. The theoretical development and validation of the method is described in detail in **(5)** where it is also demonstrated in the kinetic ('time lapse') analysis of protein interactions governing mitosis. The present protocol gives a detailed recipe for application of seFRET. It is written specifically for use with CFP (cyan fluorescence protein) as donor fluorophore and YFP (yellow fluorescent protein) as acceptor fluorophore, a popular choice for many experiments. The protocol is however valid for any other FRET fluorophore pair, and we indicate how to adapt the protocol in such situations. We also provide a software programme that automates the calibration tasks outlined in this protocol and which is available for free to download **(6)**.

1. Introduction to seFRET methodology

The principal features of seFRET measurements are shown in figure 1. Two fluorescence filter sets are required: one which is optimized to detect fluorescence from the donor ('donor emission channel', here CFP) and similarly one optimized for emission from the acceptor ('acceptor emission channel', here YFP).

[Figure 1 near here]

Excitation of the donor molecule, *D*, (solid blue arrow), leads to fluorescence emission into the donor emission channel (light blue). In the absence of acceptors and FRET, this is the only signal emitted. If FRET occurs (red arrow, labeled FRET) the acceptor molecule, *A*, becomes sensitized, and the seFRET signal emitted from *A* is detected in the acceptor channel. This is indicated as pathway 3 (solid red arrow). The efficiency of energy transfer from *D* to *A* is denoted by *E* and the so called Förster radius is defined to be the distance at which *E*=50%.
However, in practice the seFRET signal is contaminated by **signal cross talk**. There are two contributions to cross talk in seFRET based on the CFP/YFP - and most other

fluorophore - pairings. *Cross excitation* refers to the direct excitation of the acceptor at the donor excitation wavelength (path 1, dashed blue arrow). For example, YFP has a finite probability of being excited at the CFP excitation wavelength. Contamination also arises from *donor bleed through* (path 2, red dashed red arrow), e.g. CFP fluorescence will leak into the YFP fluorescence band. Signal cross talk is indistinguishable from the true seFRET signals, and, if unaccounted for, can generate false positives.
To correct for these effects we calculate a corrected seFRET efficiency, which we refer to as *cFRET* **(5)**, to yield:

$$cFRET = I^{DA} - AER \times I^{AA} - DER \times I^{DD}$$

Here the first term on the right hand side denotes the uncorrected signal, the second term accounts for the acceptor cross excitation and the last term for the donor bleed through. Table 1 denotes the nomenclature in detail.

[Table 1 near here]

The *AER* and *DER* are dye specific parameters that are ideally constant for a given sample and can be determined from control samples containing, respectively, donor (CFP) only and acceptor only (YFP).
A measureable value for *cFRET* signifies that FRET takes place between donor and acceptor, however, its value also depends on the **stoichiometries** of free donors and acceptors and interacting FRET pairs. To obtain information on the relative number of free and interacting fluorophores we further normalize *cFRET* either by the acceptor or the donor concentration. This leads to the finally sought quantities:

$$dFRET = \frac{cFRET\frac{\alpha}{DER}}{I^{DD} + cFRET\frac{\alpha}{DER}} = \chi_D E$$

and

$$aFRET = \frac{cFRET}{AER\ I^{AA}\beta} = \chi_A E$$

The nomenclatures for the terms in these equations are given in table 2. *dFRET* is the true FRET efficiency *E* normalized by χ_D which is the fraction of the donor molecules that undergo FRET. Similarly *aFRET* is the acceptor normalized FRET efficiency. The two may be significantly different as Figure 2a and 2b illustrate. For the situation depicted in Fig 2a) we have *aFRET>>dFRET* signifying that the number of donor molecules participating in FRET interactions is much smaller than the number of acceptor molecules. Similarly in b) the opposite is true. The evolution of *aFRET* and *dFRET* and their interdependence thus offer important information in the stoichiometry of biological interactions, information that is usually hidden in other modalities of performing FRET (e.g. lifetime measurements). Examples of how this can be used to follow kinase activity in living cells are given in **(5)**.
In what follows we give a recipe on how to quantify *aFRET* and *dFRET* in biological samples. Example MATLAB scripts are provided together with example data as a guide to performing the image processing required for seFRET.

[Figure 2 near here]

[Table 2 near here]

2. Materials

Biological sample preparation

In addition to the biological FRET sample of interest 3 control samples are required to determine, α , β , *DER*, and *AER* (See Note 1).

1. Donor only control sample (used as negative control and for determination of *DER*): Transfect cells (the protocol is verified for mammalian cells) using the CFP-tagged protein of interest (see Note 2).
2. Acceptor only control sample (used as negative control and for determination of AER): Transfect cells with YFP-tagged protein of interest in analogy to step 1.
3. Positive control (required for determination of α , β): Transfect cells with CFP-YFP linker construct exhibiting a known amount of FRET. The protocol as follows is valid for a CFP-YFP tandem construct obtained by excising the EYFP gene (Enhanced Yellow Fluorescent Protein) from the pEYFP-N1 vector, and ligating it into the Multiple Cloning Site (MCS) of the pECFP-C1 vector (Enhanced Cyan Fluorescent Protein; both originally obtained from Clontech, Palo Alto, CA, USA) such that the two fluorophores in the construct are separated by a sequence of 18 amino acids (GLRSRAQASNSAVEGSAM). The plasmid vector maps are available from http://www.addgene.org/vector-database/2689/, and http://www.addgene.org/vector-database/2445/, respectively (see Note 3). Propagate the plasmid in *E.coli* (recommended host strains are DH5-alpha or HB101) and use kanamycin (30 μg/ml) to select positive strains. Isolate DNA and transfect cells according to standard protocols. If required, stable transformants can be selected by G418 (neomycin). See also Note 4.
4. Samples for FRET analysis: Transfect cells with pECFP-fusion- and pEYFP-fusion proteins. Aim to obtain similar expression levels for both proteins.

Microscope set-up
The protocol described here permits system independent determination of FRET efficiency and system specific attributes (filter transmission, detector gains etc.) are taken into account through control experiments. However, for optimal sensitivity such filter sets and excitation wavelengths should be used as to maximize FRET signals whilst minimizing donor bleed through and acceptor direct excitation. For CFP / YFP based FRET the following settings are representative examples for either widefield or confocal imaging set-ups.

Wide field microscope settings:
Sensitive CCD cameras with good dynamic range (12 bit or greater) should be chosen as detectors, and a linear response ensured. Note: camera / detector settings must remain constant for control and sample measurements. If acceptor and donor channels are measured sequentially, ensure that no sample movement occurs between images (see Note 5).

[Table 3 near here]

Confocal microscope settings.
Choose PMT voltage settings to ensure a linear signal response. Set laser excitation powers to obtain similar signal levels for cfp and yfp. Check for photobleaching and adjust settings to minimize this effect. Minimise sample movement artefacts (e.g. use line averaging rather than frame averaging if available and minimize time delays between consecutive measurements of donor and acceptor channels).

[Table 4 near here]

3. Methods

3.1 Measurement of DER

Use the donor only sample. Keep magnification and gain settings constant throughout and subtract any d.c. background signal from all obtained images before further processing (see Note 6). Set up the microscope in sequential acquisition mode (if available) and obtain the following set of images, from which the *DER* can be calculated:

1. Set excitation light source to donor excitation setting and the emission channel to the donor emission bandpass to obtain signal I^{DD} (e.g. 431-441 nm excitation / 455-485 nm emission filter for widefield system).
2. Set excitation light source to donor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{DA} (e.g. 431-441 nm excitation / 520-550 nm emission filter for widefield system).
3. Calculate the *DER* for every image pixel according to:

$$DER = \frac{I^{DA}}{I^{DD}}$$

4. Repeat this calibration measurement on at least five different areas of the donor-only sample.

3.2 Measurement of AER

Use the acceptor only sample. Keep magnification and gain settings same as above and constant throughout and subtract d.c. background signal from all obtained images before further processing (see Note 6). Set up the microscope in sequential acquisition mode (if available) and obtain the following set of images, from which the *AER* can be calculated. :

1. Set excitation light source to donor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{DA} (e.g. 431-441 nm excitation / 520-550 nm emission filter for widefield system).
2. Set excitation light source to acceptor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{AA} (e.g. 490-510 nm excitation / 520-550 nm emission filter for widefield system).
3. Calculate the *AER* for every image pixel according to:

$$AER = \frac{I^{DA}}{I^{AA}}$$

4. Repeat this calibration measurement on at least five different areas of the acceptor-only sample.

3.3 System calibration with tandem FRET construct

Use the sample containing the cfp-yfp linker construct. Keep magnifaction and gain settings same as above and constant throughout and subtract any d.c. background signal from all obtained images before further processing (see Note 6). Set up the microscope in sequential acquisition mode (if available) and obtain the following set of images, from which the cross talk corrected FRET signal, *cFRET*, can be calculated. :

1. Set excitation light source to donor excitation setting and the emission channel to the donor emission bandpass to obtain signal I^{DD}. (e.g. 431-441 nm excitation / 455-485 nm emission filter for widefield system).

2. Set excitation light source to donor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{DA} (e.g. 431-441 nm excitation / 520-550 nm emission filter for widefield system).
3. Set excitation light source to acceptor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{AA} (e.g. 490-510 nm excitation / 520-550 nm emission filter for widefield system).
4. Calculate the *cFRET* for every image pixel according to:

$$cFRET = I^{DA} - AER \times I^{AA} - DER \times I^{DD}$$

5. Select regions (pixels) in your image where FRET occurs and determine α through calculation of (See Note 7, 8):

$$\alpha = \frac{E}{1-E}\,\frac{DER \times I^{DD}}{cFRET}$$

6. Select regions (pixels) in your image where FRET occurs and determine β through calculation of (See Note 7):

$$\beta = \frac{cFRET}{AER \times I^{AA} \times E}$$

7. Repeat measurements on five areas of linker-only sample.

3.4 Measurement of *dFRET* and *aFRET*

Use the biological sample with cfp and yfp fusion proteins of interest. Keep magnifaction and gain settings same as above and constant throughout and subtract any d.c. background signal from all obtained images before further processing (see Note 6). Set up the microscope in sequential acquisition mode (if available) and obtain the following set of images, from which *dFRET* and *aFRET* can be determined:

1. Set excitation light source to donor excitation setting and the emission channel to the donor emission bandpass to obtain signal I^{DD}. (e.g. 431-441 nm excitation / 455-485 nm emission filter for widefield system).
2. Set excitation light source to donor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{DA} (e.g. 431-441 nm excitation / 520-550 nm emission filter for widefield system).
3. Set excitation light source to acceptor excitation setting and the emission channel to acceptor emission bandpass to obtain signal I^{AA} (e.g. 490-510 nm excitation / 520-550 nm emission filter for widefield system).
4. Calculate the *cFRET* for every image pixel according to:

$$cFRET = I^{DA} - AER \times I^{AA} - DER \times I^{DD}$$

5. Calculate *dFRET* for every image pixel according to:

$$dFRET = \frac{cFRET\frac{\alpha}{DER}}{I^{DD} + cFRET\frac{\alpha}{DER}} = \chi_D E$$

See Notes 9 and 10.

6. Calculate *aFRET* for every image pixel according to:

$$aFRET = \frac{cFRET}{AER\, I^{AA}\beta} = \chi_A E$$

See Notes 9 and 10.

4. Notes:

1) The protocol here is specifically written for use with cfp and yfp fusion proteins and for the linker construct presented in paragraph 3 of the Materials section. It has furthermore been validated for use in live HeLa cells. The protocol is however valid for other FRET fluorophore pairs, cell types, and linker constructs. In this case appropriate filter sets and excitation lines have to be chosen, and, crucially, the FRET efficiency of the positive FRET tandem control construct needs to be determined independently, as described in Note 7.

2) To ensure strong FRET signal levels it is advisable to devise constructs labeled near the binding sites of the proteins. E.g. if the interaction is likely to occur in the C-terminal region of the protein of interest, it is advisable to construct a C-terminal fusion protein, e.g. via use of the pECFP-N1 vector (see http://www.addgene.org/vector-database/2445/). Vice versa if the interaction is more likely to happen in the N-terminal region, use the pECFP-C1 plasmid, instead. Follow similar procedure for the yfp-fusion proteins.

3) The plasmid for the positive control construct is available from the authors. In live HeLa cells this construct was determined to yield a FRET efficiency of E=38 percent. Other linker constructs may also be used but require an independent determination of FRET efficiency (see **(5)** for details).

4) Optionally, a further negative control sample containing empty pECFP and pEYFP plasmids can be used. These are not strictly necessary but provide a useful means to validate the calibration procedure described in the Methods section 3.1, 3.2, and 3.3 and in Note 9.

5) Use other filter sets / laser settings as appropriate if FRET pairs other than cfp/yfp are used in the experiments.

6) For background subtraction identify dark regions in measured images. The average brightness of these regions allows for a d.c. background subtraction from the images. Incorrect background subtraction would yield erroneous FRET results. Perform background subtraction for all measurements in this protocol. The MATLAB script provided in **(6)** as an example of seFRET image processing already includes a background correction which is suitable for the sample data.

7) For the 18 aa cfp-yfp linker construct expressed in live HeLa cells (see Materials section) substitute E=0.38 in the equations for α and β . If fixed cells are used or a different calibration construct is used (e.g. different FRET fluorophores or different linker system) then E needs to be determined independently for this construct and the appropriate value for E substituted. E can be determined from a measurement of the donor fluorescence lifetime as:

$$E = 1 - \frac{\tau}{\tau_0}$$

where τ is the donor fluorescence lifetime in the presence of FRET and τ_0 is the donor lifetime in the absence of FRET (i.e. the natural lifetime of the donor). For details refer to **(5)**.

8) Measure in at least five different image areas to determine α and β , to check for consistency of and enable averaging.

9) A control experiment to check that the calibration has been successful and the correct data for AER and DER have been obtained is to measure a negative control sample, which contains non interacting pECFP and pEYFP plasmids (or corresponding plasmids if other fluorophores are used in the experiment). For this sample bot *aFRET* and *dFRET* should be 0 if correct values for *DER*, *AER* and α and β were used.

10) The consistency of obtained datasets can be verified through a repeat determination of the *AER* from a corresponding measurement of the acceptor only sample at the end of the experiment. If this value differs from that determined at the start of the experiment this is indicative of changes in the experimental conditions (e.g. laser power fluctuations, microscope settings, etc.) which may invalidate results.

5. References:

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Figure Captions

Figure 1. Principle of seFRET

Figure 2. Illustration to exemplify the difference between donor-normalised and acceptor-normalised FRET values. In a) there is an abundance of non interacting donors, whereas all acceptors are interacting. Here *dFRET* is less than *aFRET*. In b) there is an overabundance of acceptor and the opposite holds true. A simultaneous determination of *aFRET* and *dFRET* thus establishes both whether energy transfer is taking place and also whether there is an overabundance of non interacting donors / acceptors.

Table Captions

Table 1. Quantities required to correct for signal cross talk in seFRET

Table 2. Nomenclature of seFRET calibration constants

Table 3. Widefield microscope filter setup

Table 4. Confocal microscope filter setup

Tables

I^{DA}	Uncorrected 'FRET' signal: Signal in Acceptor channel upon donor excitation
AER	Acceptor excitation ratio: corrects for direct excitation of acceptor (path 1 in figure 1)
DER	Donor emission ratio: Determines bleed through of donor fluorescence into the acceptor channel (path 2 in figure 1)
I^{AA}	Signal obtained in acceptor channel, upon excitation at acceptor excitation wavelength
I^{DD}	Signal obtained in donor channel, upon excitation at the donor excitation wavelength

Table 1

$\chi_D E$	Donor normalized FRET efficiency (i.e. fraction of interacting donor χ_D times FRET efficiency *E*)
$\chi_A E$	Acceptor normalized FRET efficiency (i.e. fraction of interacting acceptor χ_A times FRET efficiency *E*).
α	Relates to relative quantum yields and signal detection efficiencies between donor and acceptors.
β	Relates to lineshapes and excitation efficiencies of donor and acceptor molecules

Table 2

Excitation wavelength	
Donor excitation filter	431-441 nm,
Acceptor excitation filter	490-510 nm
Donor emission filter	455-485 nm
Acceptor emission filter	520-550 nm

Table 3

Donor excitation wavelength	458 nm
Acceptor excitation wavelength	514 nm
Donor emission filter	470-494 nm
Acceptor emission filter	530-545 nm

Table 4

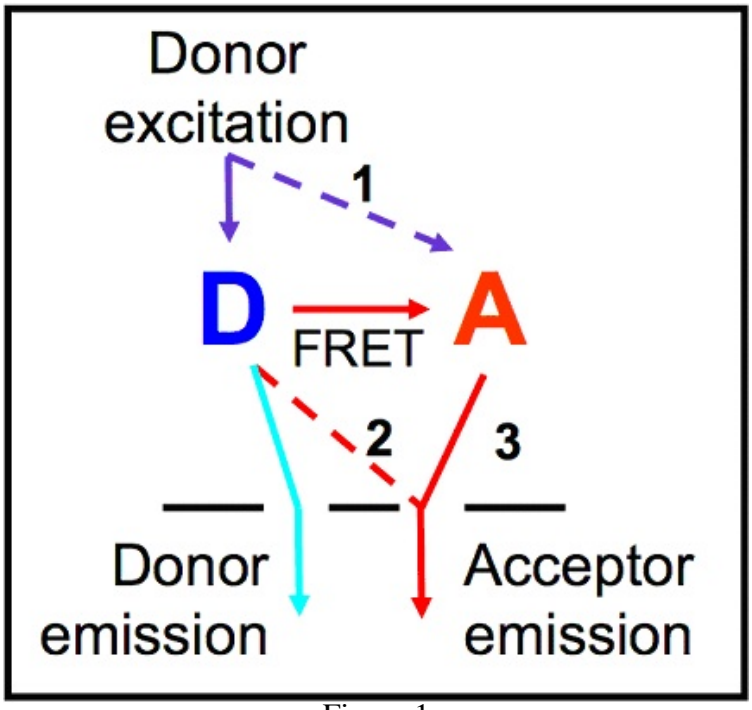

Figure 1

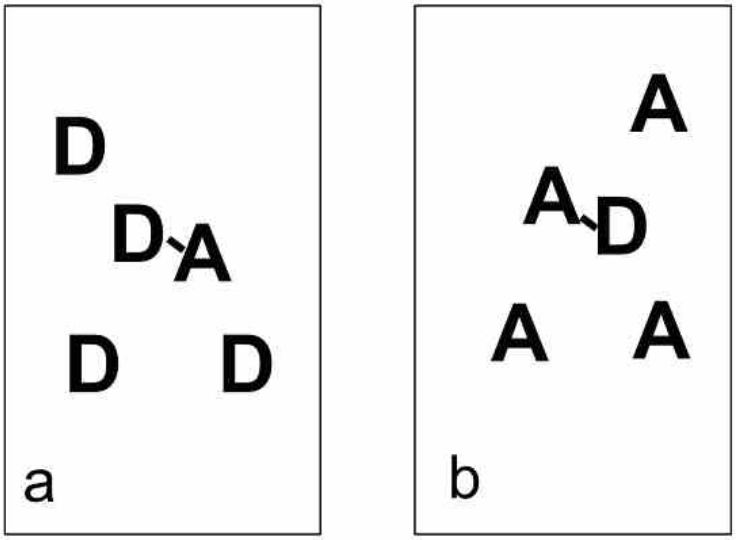

Figure 2