

BACKGROUND CORRECTION AND SPECTRAL OVERLAP COMPENSATION IN FRET ASSAYS

When proximity between two fluorescent molecules leads to FRET, the total fluorescence emission spectrum of the mixture is different from the spectrum of the same molecules mixed randomly in solution. The spectral differences reflect changes in the magnitudes of the donor and acceptor emission spectra, added together and superimposed on background fluorescence from various sources. These components of the complex emission spectra are identified and discussed to illustrate the principals behind the various methods of calculating FRET results (TNPJ100.04 FRET Calculations).

In all types of FRET assays, whether TR-FRET, PB-FRET or other configurations (TNPJ100 *Considerations for Development of FRET Assays*), several factors combine to yield the raw fluorescence intensity seen in the acceptor detection window. These include:

- Fluorescence due to FRET (signal)
- Fluorescence due to off-peak excitation of the acceptor molecule (acceptor background)
- Fluorescence due to off-peak emission of the donor molecule (donor background)
- Background fluorescence due to instrument or buffer characteristics (instrument/buffer background)
- Sample autofluorescence
- Sample quenching of acceptor fluorescence
- Sample quenching of excitation illumination, and possibly,
- Sample quenching of the donor through resonance energy transfer

The first four factors must be routinely incorporated into the calculation of FRET results to maximize the use of information

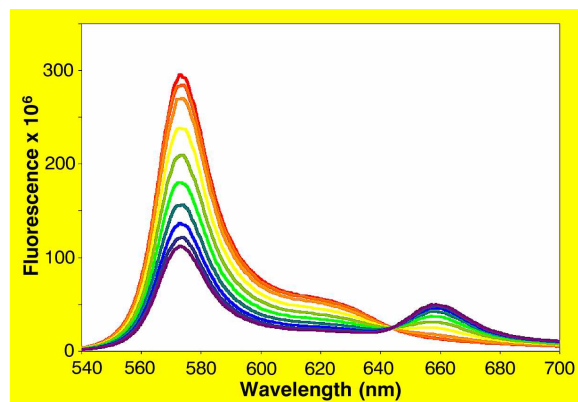


Figure 1 - Changes in emission spectra with increasing FRET between RPE (Donor, 575 nm) and APC (Acceptor, 660 nm)

available from the assay. The remaining factors relate to the characteristics of the candidates to be screened rather than the assay itself, and are discussed in TechNote TNPJ100.19 *Evaluation of Sample Interference in FRET Results*.

In addition to measurements in the acceptor window (Channel A), analysis of FRET data has traditionally incorporated measurements in a second window (Channel B) that primarily encompasses direct donor fluorescence. When FRET occurs, the fluorescence of the donor is reduced; thus, changes in Channel B are also indicative of molecular proximity.

When TR-FRET was first introduced, users were advised to view their results as the ratio of A:B, the raw counts in each channel. This treatment ignored most of the factors listed above, based on the assertion that time-resolution made most of them negligible. Recently, TR-FRET proponents have begun to recognize the benefits of including the appropriate corrections in their calculations.

Use of the A:B ratio is a viable option, in both PB-FRET and TR-FRET, for users who wish to minimize computational complexity and can accept the loss of sensitivity and information that results from less than complete utilization of available data. Please note that almost all of the considerations discussed here apply to both of these FRET methodologies (and others besides). This discussion is directed towards those with an interest in fully utilizing the data output from FRET assays.

A full interpretation of the results of FRET assays requires an understanding of the spectral characteristics of the underlying signals. Figure 1 shows an example of changes in emission spectra with increasing FRET for R-Phycoerythrin (RPE) and Allophycocyanin (APC), *i.e.* a decline in the RPE donor fluorescence offset by an increase in the APC acceptor signal.

These changes in the fluorescence emission spectrum are the consequence of changes in several contributing fluorescence components. The changes in these component spectra are diagrammed in Figure 2:

FRET - No signal due to FRET is seen in Figure 2 (top), while this signal appears in Figure 2 (bottom).

Acceptor background - This signal is unchanged, at least to a first approximation, barring saturation of acceptor fluorophores. (The magnitude of this signal is exaggerated in the figures for improved visibility.)

Donor background - While donor background contributes to overall Channel A fluorescence in both panels, the total amount of donor background is significantly lower in Figure 2 (bottom) due to donor quenching as a result of FRET.

Instrument/buffer background - This signal is unchanged. The magnitude is exaggerated for improved visibility. Also, the spectral characteristics are simplified for illustration purposes: background levels may in fact vary across the spectrum.

All three sources of background must be subtracted from the total fluorescence in Channel A to arrive at FRET fluorescence. These factors can be measured or calculated:

- Acceptor background is measured in wells containing the acceptor reagent and all other reagents in the proper assay quantities, with the exception of the donor reagent which is omitted.
- Donor background is calculated as a fraction of donor fluorescence in Channel B, as described below in “Compensation.”
- Instrument/buffer background is measured in wells containing no fluorescent reagents. For some instruments and assays, this background may be negligible and can be ignored, but only after this has been explicitly confirmed.

These values should be determined in advance of actual assay measurements, in highly replicated trials leading to confident estimates of their values. If desired, a few wells on each assay plate can then be allocated to wells for background measurement, with data analysis routines set to “flag” any plates for which measured background levels fall statistically outside of the range determined in detailed measurements.

COMPENSATION

The term “compensation” carries over from flow cytometry, where it is common to measure fluorescence in several different wavelength windows to assess multiple analytical parameters simultaneously. The fluorescence emission spectrum of any given label in a multilabel system may extend into detection windows set to measure other labels, and this overlapping signal must be removed. Figure 2 shows how this applies to

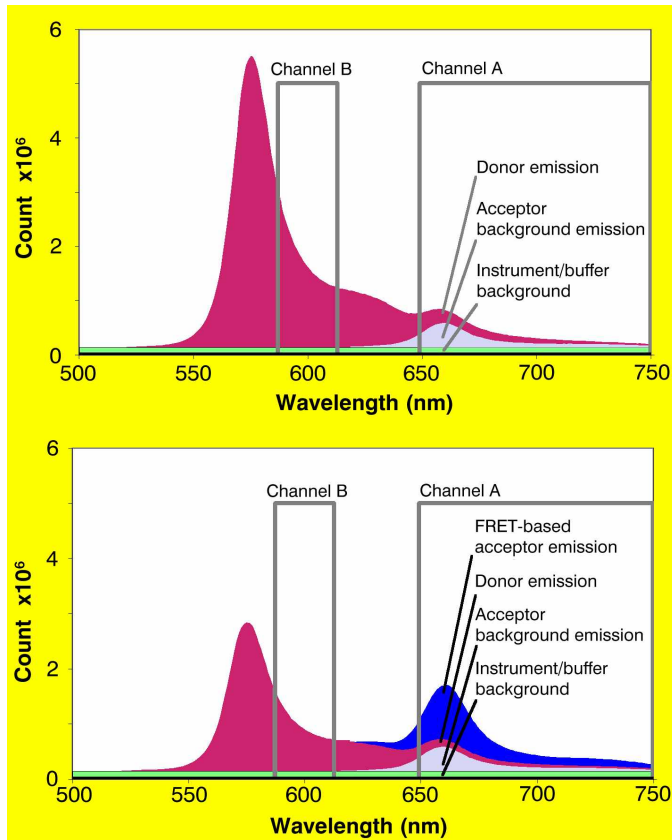


Figure 2 - Predicted composition of Raw Signal in Channel A in the absence (top) and presence (bottom) of FRET

[Note that the detection window for channel B is offset from the donor emission peak to provide for more similar counting levels in Channel A and Channel B, and to take advantage of reduced sample autofluorescence at higher wavelengths.]

FRET assays. The tail of the RPE emission spectrum clearly extends into Channel A, the window selected for measurement of APC (FRET) fluorescence. Compensation is the deduction of spectral overlap fluorescence from total fluorescence.

The calculation of compensation relies on the fact that the shape of the fluorescence emission curve of any given substance does not change where fluorescence is reduced (or increased). For example, when donor fluorescence is reduced by FRET quenching, it is reduced proportionally across the entire emission spectrum. As a result, the total donor fluorescence in Channel A will be a fixed fraction of the donor fluorescence measured in Channel B.

To calculate this compensation factor, measurements are taken in wells containing all of the assay reagents except the acceptor. The ratio of Channel A counts to Channel B counts, each corrected for instrument/buffer background if necessary, then becomes the

compensation factor used to calculate donor fluorescence in Channel A measurements of test samples.

In most FRET assays, it is often possible to select a donor emission window (Channel B) such that the only contributors to fluorescence therein are donor fluorescence and instrument/buffer background (as in Figure 2). If other sources of fluorescence, such as acceptor fluorescence, are present, more complicated calculations of cross-compensation are required.

To calculate Channel A compensation for donor fluorescence, first subtract the instrument/buffer background, if significant, from Channel B fluorescence. The resulting value is multiplied by the compensation factor determined above. This compensation value is subtracted from Channel A counts, along with other background values, to complete the calculation of net FRET in Channel A.

The manner in which corrections and compensation are applied in calculations of FRET results are discussed in detail in TNPJ100.04 *FRET Calculations*.

TECHNICAL SERVICE

This and other TechNotes are available on PROZYME's webpage located at:

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