Correction of Emission Crosstalk for FRET sensors

By Teng-Leong Chew and Rex L. Chisholm

Excerpt from "Monitoring Protein Dynamics Using FRET-based Biosensors"
Live Cell Imaging Laboratory Manual, Cold Spring Harbor Press

Emission Crosstalk

Although CFP and YFP are great fluorophore pair for energy transfer, the long trailing tail of CFP emission spectrum overlaps that of the YFP to a significant extent (Figure 4), potentially leading to emission crosstalk. This is a problem that cannot be simply corrected by filter design due to the extent of overlapping.

The degree of CFP "bleed-through" can be established by acquiring a CFP-expressing cell using the YFP set-up. The easiest way to accurately separate the overlapping CFP and YFP spectra would be to perform spectral unmixing based the emission fingerprints of these two fluorophores. However, the accurate extent of emission crosstalk can also be determined with conventional epifluorescent microscopes using a simple algorithm developed by Youvan *et al.* (Youvan et al., 1997) To perform the correction, the extent of crosstalk must first be determined either with cells singly expressing each of the FRET fluorophores or with fluorescent beads with the same fluorescent properties. Overall, the Youvan method can be established using the following notation:

$$X_u^y$$

where X can be "D", "A", or "F", indicating an monochrome image acquired through the Donor , Acceptor or FRET channels. The subscript 'u', can be substituted by 'd' or 'a' to represent a pixel from a donor or acceptor fluorophore. The substantial overlap of the donor and the acceptor spectra will ensure that crosstalk fluorescent be seen in unprocessed channels. The superscript "y" can be replaced by "b" to indicate that the monochrome image has been background subtracted. For example:

F_d^b represents a background-subtracted image of the donor fluorophore taken using the FRET channel.

Using this notation, the following two ratios can be established:

F_d^b / D_d^b = ratio of FRET channel intensity to Donor channel intensity for a 'pure donor' pixel after background subtraction on each monochrome image.

F_a^b / A_a^b = ratio of FRET channel intensity to Acceptor channel intensity for a 'pure acceptor' pixel after background subtraction on each monochrome image.

These ratios are essentially the fractional 'bleed' of donor and acceptor, respectively, into the FRET channel. They are then used to correct the FRET channel pixel intensities. The pixel in the corrected FRET image (F^c) is then given by:

$$F^c = F^b - \left(F_d^b / D_d^b\right) \times D^b - \left(F_a^b / A_a^b\right) \times A^b$$

Fluorescent crosstalk can also be performed using method devised by Gordon *et al.* (Gordon et al., 1998). Using the same notation as the Youvan method, the Gordon FRET correction can be expressed as:

$$F^c = [F_d - (D_d \times R_D) - (A_a \times R_E)] / A_a$$

where R_D is the ratio of the detection efficiencies of the donor emissions intensities through the acceptor and donor filter sets. It can be obtained from a sample that has only donor molecules by exciting at a single wavelength and dividing the total intensities detected through the acceptor and donor channels. R_E is the ratio of the extinction coefficients of the acceptor when excited at the acceptor and donor wavelengths. It

can be determined by exciting a sample with only the acceptor molecules at the donor and acceptor wavelengths, and dividing the total intensities detected through the acceptor channel. These two ratios are constant factors and are assumed to be spatially invariant in the image.

Some commercially available software packages, such as Zeiss FRET Analyzer Package, now include these correction methods, thus allowing users to easily correct for the resultant FRET ratio images.

FRET crosstalk can also be empirically eliminated by adjusting the illumination intensity until crosstalk is absent, as determined by spectral analysis. However, depending on relative protein concentration between the two fluorophores, and other intracellular conditions, this empirical method may not always be feasible.

Acceptor Photobleaching

The hallmark of a true FRET spectrum is that the energy-transfer from the donor to the acceptor. This phenomenon dictates that if FRET occurs, the donor emission peak will drop, concomitant with an increase in the acceptor emission. These spectra will all intersect at one point referred to as the isosbestic point. To test whether the biosensor actually undergoes true FRET *in vivo*, a technique called acceptor photobleaching can be employed. Photobleaching will cause the acceptor to lose its capability to absorb the energy from the donor, thus causing the donor to surge to the maximum as if there is no FRET. This will confirm that the emission detected by the FRET channel comes from true FRET, and not due to channel crosstalk, or cross-excitation of the acceptor by the donor excitation light. In fact, an apparent energy transfer efficiency $E_D(i)$ can be can be determined in each position i of an image as follows:

$$E_D(i) = 1 - F^{D}(i) / F^{D}_{pb}(i)$$

where $F^{D}(i)$ and $F^{D}_{pb}(i)$ are the donor emission images before and after photobleaching. It is important to note this correction is best performed in fixed biospecimen. Photobleaching requires prolonged illumination during which translocation of the donor-tagged molecules can occur, generating a motion artifact.

References

Youvan, D. C., Silva, C. M., Bylina, E. J., Coleman, W. J., Dilworth, M. R. and Yang, M. M. (1997). Calibration of fluorescence resonance energy transfer in microscopy using genetically engineered GFP derivatives on nickel chelating beads. *Biotechnology et alia* 3, 1-18.

Gordon, G. W., Berry, G., Liang, X. H., Levine, B. and Herman, B. (1998). Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J* 74, 2702-13.