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A Mixed Cell Protocol for Sensitized Emission FRET Experiments

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INTRODUCTION: Fluorescence resonance energy transfer (FRET) involves the nonradiative transfer of electronic excitation from a donor to an acceptor molecule. This process has a $(R_o/R)^6$ dependence on the separation of the donor and acceptor (R). R_o is typically on the order of 50Å, so FRET measurements enable detection of interactions on a spatial scale which is inaccessible in optical image formation.. We consider a FRET experiment based on three measurements, in which the observables are the fluorescence intensities with the excitation and emission combinations shown in Table 1. The FRET emission nF is given by $nF = I_{da} - a*I_{dd} - b*I_{aa}$ where $a = I_{da}/I_{dd}$ is the fraction of fluorescence emitted by the donor which will be measured in the acceptor channel, and $b = I_{da}/I_{aa}$ is the fraction of acceptor which are directly excited by illumination in the donor excitation band. [1,2] Excitation of the donor by illumination in the excitation band, and emission of the acceptor into the donor detection channel, are assumed to be negligible. a and b must be measured on cells containing only the donor or the acceptor, respectively. Thus, a FRET experiment consists of three fluorescence measurements, as well as two control experiments (to measure a and b).Here we present a mixed cell protocol which allows automatic calculation of the crosstalk coefficients, a and b, and FRET (nF) from a single mixed plate.

EXPERIMENTAL:
Cells expressing fluorescent proteins: Most experiments were performed with NIH3t3 cells stably expressing CFP, YFP, HcRed and mRFP alone, in combinations or as fusion proteins. HEK293 cells transiently transfected with the same plasmids were also used. Cells expressing donor only, acceptor only, and fusion constructs of the FRET pair were mixed in about equal proportions into a single culture dish.

Microscope: FRET measurements were carried out on a widefield Olympus IX-70 microscope with motorized excitation and emission filter wheels, and a 100W Hg excitation source. Images were acquired with an Optronics Magnafire 3P 10-bit digital CCD camera. A fixed three-band dichroic mirror was used since the filter turret was not motorized. The dichroic, as well as the CFP, YFP and RFP excitation and emission filters were from a multiband filter set (#86006, Chroma Technology Corp, Rockingham, VT), and the appropriate excitation and emission pair was selected using the motorized filter wheels.

ANALYSIS: The results of the sensitized emission experiments were processed using Image Pro Plus (Media Cybernetics, Silver Spring, MD). A macro was used to calculate FRET emission (nF) and to normalize nF to either the donor intensity I_{dd} or the acceptor intensity I_{aa}. This macro also classified the cells as donor-only, acceptor only, or donor-acceptor. Donor-only and acceptor-only cells were used to calculate a and b, while FRET emission was derived from the donor-acceptor group. The images were first processed with a 3x3 median filter to remove point noise. Background was then estimated based on large spectral filters, and subtracted from the images. After preprocessing, the FRET and crosstalk were determined automatically. Finally, normalized

sensitized emission was measured in those cells which express both donor and acceptor. Figure 1 shows the three input images as well as the outputs of this process.
In addition to reducing the number of experiments that must be done, this protocol measures the controls in the same plate as the experiment, thus reducing many sources of error in estimation of the crosstalk parameters. Measurement of sensitized emission usually requires subtraction of relatively large numbers in order to derive a small value (*nF*). The reason for this is that FRET efficiencies of common fluorescent protein pairs are in the 20-40% range, even before considering stochiometric effects. Thus, accurate determination of the crosstalk parameters is vital to acquiring scientifically significant sensitized emission data.

References

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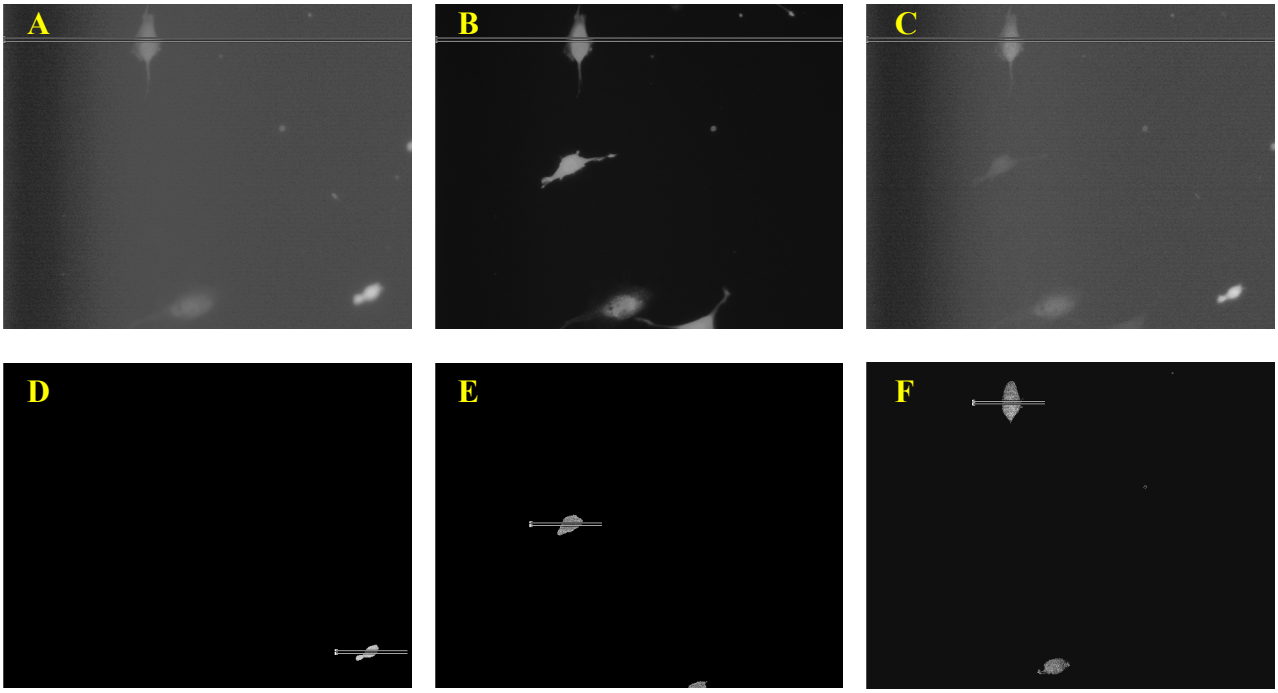

Figure 1: A: Donor image I_{dd} . Is this case crosstalk coefficient a=0.1; B: Acceptor image I_{aa} . b=0.012; C: FRET image I_{da} ; D: Ratio of I_{da}/I_{dd} for donor only cell; E: Ratio of I_{da}/I_{aa} for acceptor only cell; F: cells with donor-acceptor fusion; The FRET pair was YFP-RFP.

Table 1: Filter Combinations for Three-Measurement FRET Experiment

symbol	ex channel	em channel
I_{dd}	donor	donor
I_{aa}	acceptor	acceptor
I_{da}	donor	acceptor

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