Application Forum

Quantifying the efficiency of various FRET constructs using OptiMiS™

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Introduction

The method of Förster Resonance Energy Transfer (FRET) (1, 2) is a powerful tool for quantifying intermolecular interactions as well as probing supramolecular assembly of, e.g., proteins into dimeric/oligomeric complexes. However, numerous nuances exist in FRET-based measurements which need to be addressed by the measuring system before the full capabilities of the method can be realized. Firstly, in order to maximize the amount of FRET signal occurring, the emission spectrum of the donor molecule must overlap appreciably with the excitation spectrum of the acceptor for efficient energy transfer. While good coupling between donor and acceptor is desirable, this situation can also lead to overlapping emission profiles, making it difficult to separate the contributions of each molecule to the detected emission. Therefore, the spectral resolution of the measurement system needs to be high enough to separate FRET pairs of closely spaced emission spectra; this typically means a spectral resolution of five nanometers or better. Secondly, the measuring system needs to acquire all spectrally resolved emission in a single scan of the sample. When multiple excitation scans are used to detect FRET, the resulting pixel-level information is compromised due to mixing caused by diffusion of molecular complexes moving during the image acquisition period. Nevertheless, detecting the presence of permutations of oligomer constructs inside the cell is vital to understanding the complexity of an oligomeric construct of interest.

An exquisitely suitable tool to performing measurements of this nature is the Optical Micro-Spectroscopy System (OptiMiS™, Aurora Spectral Technologies), which is used in conjunction with standard multiphoton microscope (MPM) setups. OptiMiS either may be added to an existing MPM setup or a whole new system may be set up around it, incorporating the customer's choice of laser, microscope platform (from any major microscope manufacturer) and EMCCD (or CMOS) camera. In this article, we will provide several

examples of OptiMiS uses in FRET-based imaging of living cells.

Spectrally resolved fluorescence emission collection

Single cells measured using OptiMiS were typically scanned with a pulsed (femtosecond) infrared laser. The fluorescence emitted from excited portions of the sample is separated into its spectral components before striking the detection hardware. The software controlling the laser scanning mirrors of OptiMiS also collects the data from the detector and reconstructs images of the samples' fluorescence intensity at various wavelengths. The wavelength channels are separated by as little as 1.25 nm and range in wavelength from 400 nm to 650 nm. Displayed in Figure 1 is a small selection of fluorescence spatial maps resulting from a single scan of yeast cells expressing GFP₂ (3) in their cytoplasm.

The fluorescence spectrum of each pixel of the spectrally resolved images was unmixed (4) to reveal the pixel-level

Figure 1. A selection of 6 fluorescence spatial maps at various emission wavelengths resulting from a scan of yeast cells expressing the fluorophore GFP₂ in their cytoplasm. The scale displayed in the upper left-hand corner indicates fluorescence intensity; the maximum obtainable signal level is 16,383 (i.e. the data obtained are 14 bit-depth images). A total of 200 wavelength channels of this kind were obtained in a single scan of the sample (ranging from 400 nm to 650 nm) and were separated by 1.25 nm. Images, collected with a system consisting of a KM Labs ultrafast Ti:sapphire laser, an Evolve 512 EMCCD camera (Photometrics), and OptiMiS, all attached to an Axio Observer Z1 inverted microscope (Zeiss).

Figure 2. Two-dimensional fluorescence distribution maps for the donor (Cerulean, 'C') in the presence of the acceptor (FDA) and acceptor (Venus, 'V') in presence of the donor (FAD) along with the map of FRET efficiencies calculated from FDA and FAD for (A) a CHO cell expressing a VCVV construct, and (B) a CHO cell expressing a VCV construct. Images were collected using an imaging system consisting of a KM Labs ultrafast Ti:Sapphire laser, an iXon X3 EMCCD camera (Andor Technologies), and OptiMiS, all attached to a custom-made upright microscope. (C) FRET efficiency displayed in histogram form obtained from the FRET efficiency measurements of the CHO cells shown in A and B. Solid blue circles, FRET efficiency distribution of the cell expressing VCVV; red open circles, that of the cell expressing VCV.

contributions to the detected emission from each fluorophore species inherent to the scanned sample. For fluorophore combinations participating in FRET, the contributions to the detected emission arise from the fluorescence of the donor in the presence of the acceptor (FDA) and the fluorescence of the acceptor in the presence of the donor (FAD). By a careful selection of the FRET pairs (5), it is possible to directly excite only the donor fluorophore with the laser; in this way, any emission emanating from the acceptor molecule is occurring solely due to FRET. Thus, the fluorescence intensity maps of the donor and acceptor emission, obtained from a single scan of the sample, are sufficient for calculating pixel-level FRET efficiencies, with no photobleaching steps required (5). The pixel-level FRET efficiencies were further analyzed by binning them according to their values to build a histogram.

Efficiency determination for some FRET standards

Chinese hamster ovary (CHO) cells were transiently transfected with two FRET oligomer standards comprised of

Figure 3. Imaging of a single CHO cell co-expressing the rat M1 muscarinic receptor and the Premo Cameleon FRET biosensor. The CHO cell was imaged (A) prior to and (B) 190 s after the addition of 500 μM carbachol. Symbols used are the same as in Figure 2. Images were collected using an imaging system consisting of a KM Labs ultrafast Ti:Sapphire laser, an iXon X3 EMCCD camera (Andor Technologies), and OptiMiS, all attached to an Axio Observer Z1 inverted microscope (Zeiss).

different combinations of a donor (Cerulean, 'C') and acceptors (Venus, 'V'); the plasmids for the FRET standards were a gift from Dr. Steven S. Vogel (NIAAA, NIH) (6). One of the oligomers was a tetramer consisting of a single donor and three acceptors, denoted by the symbol VCVV, while the other construct was a trimer, denoted by the symbol VCV.

The CHO cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Two days prior to imaging, cells were seeded in 6-well plates and cultured overnight. The following day, plasmid DNA (2 μg per transfection reaction) and Lipofectamine 2000 were diluted in Opti-MEM medium (both from Life Technologies) and added to the cells. Analysis was performed 24 hr following transfection.

Using OptiMiS, spectrally resolved fluorescence information was obtained from CHO cells expressing either VCV or VCVV constructs. Examples of results obtained with both types of samples are displayed in Figure 2. The Cerulean and Venus emission intensity maps (FDA and FAD, respectively) are shown along with the corresponding FRET efficiency distributions. The histograms constructed using the FRET efficiency distributions of the two CHO cells are shown in Figure 2C. The addition of the third Venus fluorophore to the tetramer construct causes an increase in the FRET efficiency of the construct due to the presence of an additional acceptor (Venus molecule) in the vicinity of the Cerulean fluorophore. As can be seen from the graphs in Figure 2, this slight difference in FRET efficiency between the two FRET constructs is resolvable from a single scan of a cell using OptiMiS.

Probing calcium concentration changes in living cells

The Premo Cameleon FRET biosensor (Life Technologies) consists of two fluorophore subunits, eCFP and cpVenus, connected by a calmodulin-M13 moiety. When the calmodulin

Figure 4. Measurements of the FRET distributions in CHO cells expressing the rat M1 muscarinic receptor and the Premo Cameleon FRET Biosensor. (A) FRET efficiency histograms collected for a single cell before and 190 s after addition of agonist (carbachol). (B) Average value of FRET efficiency histograms obtained at several time points before and after addition of carbachol (solid black squares). The open squares are the average of the FRET efficiency histogram for a cell scanned in a similar manner as the carbachol-treated cell, but never exposed to carbachol.

Figure 5. Two-dimensional fluorescence distribution maps of donors in the presence of acceptors (FDA) and acceptors in presence of the donors (FAD) along with the map of FRET efficiencies, calculated using the corresponding FDA and FAD, for a yeast cell co-expressing two populations of sterile two alpha-factor (ste2p) receptor: one tagged with GFP₂ as a donor and the other with YFP as an acceptor. Images were collected using an imaging system consisting of a KM Labs ultrafast Ti:Sapphire laser, an iXon EMCCD camera (Andor Technologies), and OptiMiS, all attached to a custom-made upright microscope.

binds four Ca²⁺ molecules, the biosensor undergoes a conformational change which brings the fluorescent protein domains closer to one another. This configuration change causes an increase in FRET occurring between the eCFP (donor) and cpVenus (acceptor) molecules. Hence, by monitoring the FRET efficiency distribution over single cells or even subcellular regions, it is possible to detect changes in the calcium concentrations of said regions.

CHO cells expressing the rat M1 muscarinic acetylcholine receptor (CHO M1WT3, ATCC Inc) were plated on a glass bottom culture dish (MatTek Corporation) at a density of 250,000 cells per plate. The CHO cells were cultured in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.5 mg/ml G418 and 10% fetal bovine serum. The M1-expressing CHO cells were then transduced with the Premo Cameleon Calcium Sensor using the BacMAM transduction protocol provided by the manufacturer. Following the transduction procedure, the cells were incubated in their normal growth medium for 40 hours at 37°C and 5% CO₂. Individual cells expressing the Ca²⁺ biosensor were scanned with OptiMiS a number of times in order to establish a

baseline FRET efficiency value for the sensor expressed in that particular cell. Then, 500 μM carbachol, a muscarinic receptor agonist, was added to the culture dish, and the cell was imaged repeatedly over an extended period of time at intervals of 15 seconds.

The FRET efficiency distribution of the CHO cell displayed in Figure 3 qualitatively illustrates a change in the configuration of a large number of the Cameleon sensors expressed in that cell. FRET efficiency distributions were constructed in this manner for each time point in the series of OptiMiS scans following carbachol stimulation of the cell. For each time point, a histogram of the FRET efficiencies was constructed; two such histograms are plotted in Figure 4A. An average FRET value over the entire cell was calculated from each histogram and also plotted as a function of time (Figure 4B). Plotted on the same graph in Figure 4B is the average FRET efficiency for a cell which was repeatedly scanned as a function of time, but at no point subjected to carbachol stimulation.

Apparent from the plots of Figure 4 is a time-dependent change in the FRET efficiency measured for the cell displayed in Figure 3. This was likely due to the binding of calcium to the calmodulin-M13 moiety of the Cameleon sensor and subsequent change in configuration of the Cameleon sensor. Stimulation of the M1-expressing cells with carbachol is known to lead to an increase in inositol phosphates, which in turn releases intracellular Ca²⁺ stores (7). The Ca²⁺-induced configuration change in the Cameleon biosensor leads to the donor and acceptor being brought closer to each other, and hence produces the increase in FRET efficiency.

Careful inspection of the shape of the plot of Figure 4B allows elucidation of the cellular events leading to the observed changes in Ca²⁺ concentration. The initial steep increase in FRET occurring over a time period of thirty seconds signifies the point when the carbachol concentration in the exterior near to this particular cell was high enough to initiate a cascade of events which eventually leads to the opening of the internal calcium stores within the cell. Following this rapid increase in average FRET efficiency is a slight decrease to a prolonged plateau of FRET efficiency values. This plateau state is indicative of the opening of calcium release activated channels following the depletion of the internal calcium stores (7).

Stoichiometry and structure of a G protein-coupled receptor oligomer

Yeast cells (*Saccharomyces cerevisiae*) were genetically engineered to express the pheromone receptor ste2 fused to either GFP₂ (donor) or YFP (acceptor), as described previously (5, 8). Yeast cell colonies selected for the two plasmids of interest were placed on the surface of a microscope slide

Figure 6. The histogram of measured FRET efficiencies (blue circles) obtained from the cell in Figure 5. Individual Gaussian functions (dashed lines) are summed to simulate (solid red line) the measured values as predicted by a rhombus-shaped tetramer. The peak centers of all five Gaussians are determined by a single FRET efficiency value, called pair-wise FRET efficiency, E_p (9).

and imaged using OptiMiS. Typical images obtained with this system are shown in Figure 5, while the corresponding histogram of FRET efficiencies is pictured in Figure 6. Notably, this histogram differs in shape and width when compared to similarly constructed histograms of the CHO cells expressing oligomeric constructs characterized by a single FRET efficiency (see Figure 2). Specifically, multiple peaks are observed in the histogram of Figure 6, suggesting the population of ste2 oligomers consists of sub-species characterized by different FRET efficiency values. These subpopulations occur in oligomer complexes of size greater than two due to the naturally occurring permutations of the ratio and placement of the donor- and acceptor-tagged protomers in the oligomeric complex. The measured data (blue circles) displayed in Figure 6 are simulated with a model of a rhombus-shaped tetramer, which predicts five Gaussian functions (red solid line). Each Gaussian corresponds to a certain combination of number of donors to acceptors in the cell (9). This result is consistent with previously published reports (5).

Conclusion

OptiMiS gives researchers the ability to finely discern, through a single scan, the FRET efficiency of dimeric or oligomeric molecular complexes within single cells. This level of FRET efficiency resolution is highly valuable in protein complex stoichiometry and configuration studies, as well as when using sensors based on FRET. In addition, OptiMiS may be used for techniques which have not been illustrated in this short note, e.g. Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Correlation Spectroscopy (FCS). Because OptiMiS acquires all the necessary spectral infor-

mation in a single excitation scan of the sample, the acquisition time can be reduced such that changes in FRET efficiency, which may be occurring over time due to changes to the structure of the molecular complex, can be accurately monitored.

References

1. Selvin, P.R., *The renaissance of fluorescence resonance energy transfer*. Nat Struct Biol, 2000. **7**(9): 730-4.
2. Clegg, R.M., *Fluorescence Resonance Energy Transfer*, in *Fluorescence Imaging Spectroscopy and Microscopy*, X.F. Wang and B. Herman, Editors. 1996, Wiley-Interscience: New York.
3. Zimmermann, T., et al., *Spectral imaging and linear un-mixing enables improved FRET efficiency with a novel GFP2-YFP FRET pair*. FEBS Lett, 2002. **531**(2): 245-249.
4. Raicu, V., et al., *Protein interaction quantified in vivo by spectrally resolved fluorescence resonance energy transfer*. Biochem J, 2005. **385**(Pt 1): 265-77.
5. Raicu, V., et al., *Determination of supramolecular structure and spatial distribution of protein complexes in living cells*. Nat Photonics, 2009. **3**: 107-113.
6. Koushik, S.V., P.S. Blank, and S.S. Vogel, *Anomalous surplus energy transfer observed with multiple FRET acceptors*. PLoS One, 2009. **4**(11): e8031.
7. Jones, S.V., O.H. Choi, and M.A. Beaven, *Carbachol induces secretion in a mast cell line (RBL-2H3) transfected with the m1 muscarinic receptor gene*. FEBS Lett, 1991. **289**(1): 47-50.
8. Stoneman, M., D. Singh, and V. Raicu, *In vivo quantification of G protein coupled receptor interactions using spectrally resolved two-photon microscopy*. J Vis Exp, 2011. **47**: e2247.
9. Raicu, V., *Efficiency of resonance energy transfer in homo-oligomeric Complexes of Proteins*. J Biol Phys, 2007. **33**: 109-127.

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Sponsored Paper. *BioTechniques* 52:192-195 (March 2012)
doi 10.2144/000113828

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