APPLICATION NOTE

Optimizing Filter Sets for FRET Applications

Overview

FRET, or Forster Resonance Energy Transfer, is a phenomenon where closely matched pairs of fluorophores are used to determine spatial or temporal proximity and specificity in molecular, protein-protein interactions. More specifically, this energy transfer occurs when the emission energy of one fluorophore—the donor—is non-radiatively transferred to the second fluorophore—the acceptor—producing a secondary emission. When this occurs, donor fluorescence is quenched and acceptor fluorescence increases.

Biologically, in order for this transfer to occur, the cellular conditions need to be such that the distance between the molecules being measured is no more than 1-10nm. Spectrally, the fluorophores being used need to have a large overlap, which while creating the conditions for effective energy transfer, also results in spectral bleed through (SBT), defined as the overlap of the donor and acceptor emission spectra, and can be a problem in FRET measurements.

The development of SBT correction techniques have been critical to the evolution of FRET as a useful and more widely used application. These SBT correction techniques—which include software development, fluorescence lifetime imaging (FLIM) correlation, and photo bleaching techniques—have reached a degree of sophistication that improves the efficacy of FRET. Similarly, the development of microscopy techniques such as one-photon, two-photon (multi-photon), confocal, and TIRF are all contributing to the growing effectiveness and ease of FRET experiments.

While much has been written about the physical and biological aspects of FRET, as summarized above, this application note will review the best suited fluorophore pairs and summarize the considerations surrounding the hardware configuration and selection of optical filters required for successful capture, differentiation, and measurement of FRET.

FILTERS & MICROSCOPE CONFIGURATIONS

The filter components required for FRET experiments are not esoteric. As in any fluorescence microscopy application, an excitation filter is required for exciting the donor fluorophore, and a dichroic mirror is required for separating donor excitation energy from both donor and acceptor emission energy. Unlike other fluorescence applications, however, two emission filters are required, one for the acceptor fluorophore, or FRET emission, and one for the donor fluorophore in order to correct for single bleed thru. As for choosing specific filters, the same filter components and sets can be applicable for FRET as those which are matched to specific fluorophores and used in other single color, epifluorescence applications.

More important in the selection of filters is an understanding of the physical configuration of the microscope hardware to be used in the FRET experiment. At issue are critical experimental variables, such as time and image registration. While the ideal set-up may not be affordable or available to all researchers interested in FRET studies, it is nonetheless important to understand the pros and cons of the available hardware and filter set configurations.

1. Multi-View Configurations

Most ideal for the viewing and measurement of molecular, protein-protein interactions with critical spatial and temporal characteristic is a set-up which allows for simultaneous viewing of both donor and acceptor emission energy. This is only possible using a device which provides a simultaneous split-screen view of the sample. These multi-view accessories are mounted to the microscope in front of the detector and use filters integrated into the unit to split the donor and acceptor emission fluorescence into two images.

When FRET viewing is handled this way, the two critical variables—time and registration—are eliminated. The time of donor and acceptor imaging is simultaneous, and given a properly aligned unit, the image registration is identical, providing a duplicate view of the sample. The only difference between the two images is that one image is captured with an emission filter for donor emission while the other image uses an emission filter for acceptor emission.

2. Emission Filter Wheels

When multi-view accessories are not available, an automated emission filter wheel is the next best alternative. With this configuration, a filter cube/holder with a donor excitation filter and dichroic mirror are placed in the microscope. The emission filters for both the donor and acceptor fluorophores, in turn, are mounted in the emission filter wheel, which can be rapidly switched from one to the other.

Collecting donor and acceptor emission energy using this hardware configuration, while not simultaneous, can be accomplished with time delays of only 40-75msec (depending on make and model), given the state-of-the-art of automated filter wheels as well as camera and detector technology. Both temporal changes in the sample during live cell imaging and registration shift resulting from equipment movement, while almost negated, must still be considered when analyzing experimental results.

3. Separate Filter Cubes

Without a multi-view attachment or emission filter wheel, researchers must fall back on a third hardware configuration for FRET, which involves using a separate filter cube for both donor and acceptor fluorescence. In this configuration, while each cube has an exciter, dichroic and emitter, it is extremely important to remember that the exciter and dichroic in both sets are identical and are those filters that are typically used with the donor fluorophore.

While this third filter configuration allows for the discreet collection of donor and acceptor fluorescence, it is the configuration most susceptible

to the time and image registration variables mentioned previously. The time variable can be minimized as a result of the automated turrets, which are a standard feature on many new microscopes but may not be typical on older, installed models. In addition, alignment of filters within cube tolerances allows more room for registration error than in the two other configurations. The time and resolution variables that are inherent with this configuration must be thoughtfully weighed when using a spatially and temporally sensitive technique such as FRET.

FLUOROPHORE PAIRS

While certain fluorophore pairs such as CFP/YFP, have dominated the scientific literature and provided the foundation for successful FRET studies to date, there has been continued development of new monomeric fluorescent proteins such as Midoriishi Cyan and Kusabira Orange, for FRET experiments. These fluorophore developments have been stimulated by the refinement of procedures and ratio correction techniques, as well as microscopy applications that are FRET friendly.

On the most basic level, the success of any given pair of fluorophores centers on their spectral characteristics. First, there must be sufficient separation of excitation spectra for selective stimulation of the donor. Second, there must be sufficient overlap (>30%) between the emission spectrum of the donor and the excitation spectrum of the acceptor in order to obtain efficient energy transfer. And third, there must be sufficient separation of the donor and the acceptor emission spectra so that the fluorescence of each fluorophore can be collected independently.

Development of new fluorescent proteins has centered on meeting these criteria, while producing new colors and fluorophores that bind to varied proteins and biological molecules. The newest developments are cited in the links and references to recent literature listed below:

Fluorophore References

Wallrabe, H., and Periasamy, A. (2005) FRET-FLIM microscopy and spectroscopy in the biomedical sciences. Current Opinion in Biotechnology. 16: 19-27.

Karasawa, S., Araki, T., Nagai, T., Mizuno, H., Miyawaki, A. (2004) Cyan-emitting and orange-emitting fluorescent proteins as a donor/acceptor pair for fluorescence resonance energy transfer. Biochemical Journal, April 5.

Shaner, N., Campbell, R., Steinbach, P., Giepmans, B., Palmer, A., Tsien, R. (2004) Improved monomeric red, orange, and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nature Biotechnology, Vol. 22, Number 12, December. pp.1567-1572.

FRET FILTER SETS

The products listed in the catalog include the most commonly used FRET fluorophore pairs, as well as those recently developed pairs that are worthy of attention. For each FRET fluorophore pair the chart lists a filter set that is useful in the emission filter wheel configuration. These sets are comprised of the exciter and dichroic for the donor fluorophore and emitters for both the donor and acceptor fluorophores.

Individual filter set part numbers for both donor and acceptor fluorophores are also listed so components can be purchased individually, dependent on the specifics of the hardware set-up. If purchasing filters individually, it is important to remember that the exciter and dichroic from the acceptor fluorophore set are never used.

It is important that you provide hardware details and related mounting instructions when ordering filters for FRET applications