



## Fluorescence Resonance Energy Transfer (FRET)

Fluorescence Resonance Energy Transfer (FRET) is a powerful technique for characterizing distance-dependent interactions on a molecular scale. It is one of the few tools available that is able to measure intermolecular and intramolecular distance interactions both in-vivo and in-vitro.

FRET involves the excitation of a donor fluorophore by incident light within its absorption spectrum. This radiative absorption elevates the donor fluorophore to a higher-energy excited state that would normally decay (return to the ground state) radiatively with a characteristic emission spectrum. If, however, another fluorophore molecule (the acceptor) exists in proximity to the donor with its energy state characterized by an absorption spectrum that overlaps the emission spectrum of the donor, then the possibility of non-radiative energy transfer between donor and acceptor exists. As an example, Figure 1 shows the overlap of the cyan fluorescent protein (CFP) emission spectrum and the yellow fluorescent protein (YFP) absorption spectrum; this pair supports a strong FRET interaction.

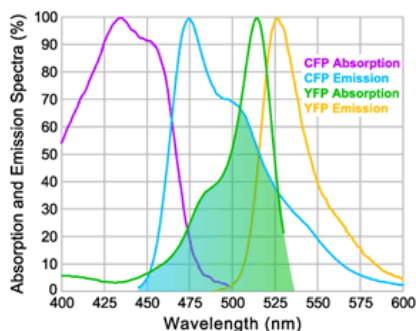


Figure 1: CFP (donor) and YFP (acceptor) absorption and emission spectra. Overlap between CFP emission and YFP absorption (shaded region) leads to efficient FRET interaction.

The radiationless energy transfer described above is mediated by dipole-dipole interactions (Van der Waals forces) between the donor and acceptor fluorophore molecules that vary as the inverse 6<sup>th</sup> power of distance between the two molecules. The rate of energy transfer from donor to acceptor,  $k_F$ , is approximately

$$k_F \sim k_D(r_0/r)^6$$

where  $k_D$  is the radiative decay rate of the donor fluorophore, or inverse of the fluorescence emission lifetime in the absence of the acceptor fluorophore (typically 1 – 50 ns),  $r$  is the distance between the two molecules, and  $r_0$  is the "Förster distance" that characterizes the 50% efficiency point of the energy transfer. FRET is suited to measuring changes in distance on the order of the Förster distance, which is typically 20 to 90 Å. This length scale is far below the Rayleigh-criterion resolution limit of an optical microscope (typically 2500 Å for visible light at high numerical aperture), thus illustrating the power of FRET for measuring extremely small distance interactions.

After energy transfer occurs from donor to acceptor, the acceptor fluorophore is excited to its fluorescence emission state. Because the observed rate of fluorescence emission from the acceptor is rate-limited by energy transfer from donor to acceptor, the quantitative measurement of FRET emission can therefore provide an inferred measurement of distance using the equation above. Accurate FRET determination generally involves comparison of the donor and donor-acceptor fluorescence emission intensities in samples with and without the acceptor present. A ratio measurement is necessary because, as Figure 1

The two measurements illustrated in Figures 2 and 3 may be made sequentially with two different filter cubes mounted in a fluorescence microscope turret, each of which has identical exciter and dichroic filters, but different emitter filters. However, due to the extreme sensitivity of FRET image analysis to relative motion of the two images – exacerbated by the vibrations and longer time required to exchange filter cubes in a turret – this approach is not recommended for FRET analysis using wide-field microscopy. Instead, a more rapid, low-vibration filter change or true simultaneous detection of the donor and acceptor emission are recommended, especially when performing FRET on live-cell samples which are moving.

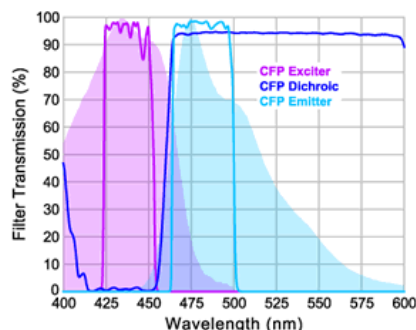


Figure 2: CFP exciter, dichroic, and emitter filters (from the BrightLine [FRET-CFP/YFP-A](#) set) for quantitative measurement of donor emission.

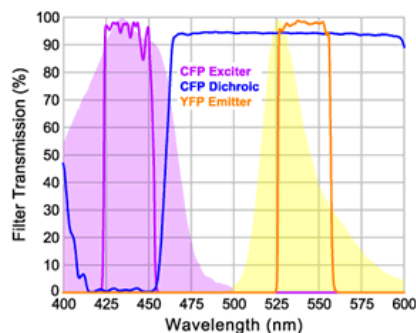


Figure 3: CFP exciter and dichroic filters with YFP emitter filter (from the BrightLine [FRET-CFP/YFP-A](#) set) for quantitative measurement of acceptor emission.

Rapid, low-vibration filter changes can be achieved simply by using an emission filter wheel. This is the most common microscope configuration for wide-field FRET measurement. Some researchers prefer the flexibility of using a dual filter wheel system (one for excitors and one for emitters), though such systems are more expensive and more complex in terms of software control, due to the synchronization required between the two filter wheels. For dual-filter wheel systems, the best filter set choice is a "Sedat" multiband set – for example, the BrightLine [CFP/YFP-2X2M-A](#) is ideal for CFP/YFP FRET measurements. Finally, it is also possible to detect both donor and acceptor emission channels simultaneously using two cameras. Several manufacturers make microscope attachments for this application. In addition to the standard FRET filter set (such as the [FRET-CFP/YFP-A](#)), and additional dichroic beamsplitter is required to separate the two emission paths in the attachment.

The imaging performance of optical filters used for FRET is also critical, since for accurate measurements a ratio of the intensities measured on two separate images must be computed. If there is

demonstrates, there is typically overlap between the donor and acceptor emission spectra, thus making it difficult to determine with a single measurement exactly what fraction of the fluorescence measured with an acceptor emission filter derives from only the acceptor. Fluorescence lifetime measurements provide more direct results for the energy transfer rate, are not susceptible to concentration variations, and can be made using time domain or phase modulation lifetime measurement techniques. These types of measurement can also provide information regarding conformational changes due to molecular interactions based on further detailed knowledge of the bound and/or conjugated FRET fluorophore pair systems.

Application conditions for observing FRET are often characterized by extremely low molecular fluorophore concentrations thus requiring detection of very faint fluorescence emission levels. [Optical fluorescence filters](#) are a critical part of an optical system for FRET detection. Semrock [BrightLine® fluorescence filters](#) offer the highest possible transmission for maximizing the FRET emission signal, as well as carefully optimized deep blocking out of the transmission passbands, for maximum possible signal-to-background ratios (highest contrast).

Figure 2 shows the transmission spectra for a filter combination optimized for measuring CFP, and Figure 3 shows the same exciter and dichroic filter spectra with the spectrum of a YFP emitter filter (all of these filters are found in the complete BrightLine filter set [FRET-CFP/YFP-A](#)). The filter combination in Figure 2 is used to quantify the fluorescence from the donor alone, while the combination in Figure 3 is used to quantify the FRET fluorescence from the acceptor.

any relative movement of the images (even for a perfectly stationary sample), the ratio calculation will be distorted. The simplest and most accurate way to avoid this problem is to use filter sets which cause no "pixel shift," or relative variation of the beam deviation caused by non-zero wedge angles of the dichroic and emission filters. Semrock's [BrightLine ZERO™ filter sets](#) offer certified zero-pixel-shift performance to eliminate the pixel-shift problem. This unique performance is made possible by the patented BrightLine filter technology (U.S. Patent No. 6,809,859 and pending) that is based on a single glass substrate coated by durable, hard coatings.

Selected references for further reading on FRET:

1. J. R. Lakowicz, "Principles of Fluorescence Spectroscopy" (Kluwer Academic / Plenum Publishers, New York, 1999).
2. Paul R. Selvin, "The Renaissance of Fluorescence Resonance Energy Transfer," *Nature Structural Biology*, Vol. 7, No. 9, pp. 730-734 (2000).
3. Handbook of Fluorescent Probes and Research Products, Molecular Probes, [www.probes.com/handbook](http://www.probes.com/handbook).

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