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FRET with broadband LED Light Source - Localization of protein-protein interactions in living cells

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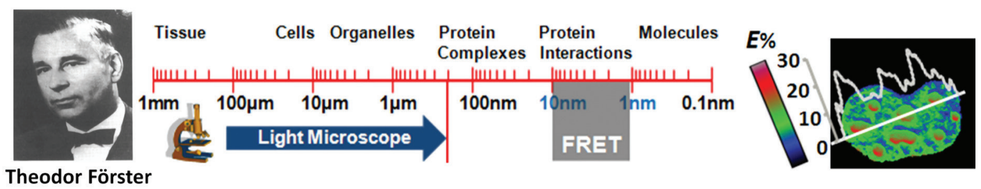
Abstract

FRET is a powerful tool used to localize protein-protein interactions in living cells. Based on the detection of the sensitized emission of acceptors, we demonstrate FRET imaging using the X-Cite® mini+ light source with a widefield, conventional FRET microscopy system. This light source is an excellent replacement for traditional HBO lamps or high-end laser systems, with zero consumables and a low carbon footprint. We demonstrate FRET efficiency using FRET standards with genetic constructs where Cerulean and Venus fluorescent proteins are tethered by five amino acid linkers (cerulean-5aa-Venus). The results are comparable with other FRET imaging methods such as FLIM.



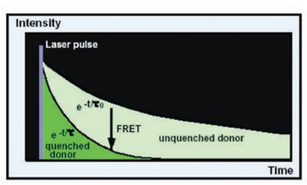
Introduction

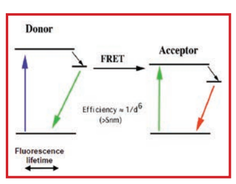
Förster resonance energy transfer (FRET) methodology is a powerful tool used to localize protein-protein interactions in living specimens. Various FRET microscopy imaging techniques and light sources utilized with these techniques have been evaluated and established over decades. Based on the detection of the sensitized emission of acceptors, we have demonstrated FRET imaging methodologies using the X-Cite mini+™ light source that can be used with wide-field, conventional FRET microscopy system. This light source is an excellent replacement for traditional HBO lamps or high-end laser systems, with zero consumables and a low carbon footprint. FRET microscopy methods have the capability to interpret the change in proximity between donor and acceptor through measuring the apparent FRET efficiency (E%) [1-3]. The measured FRET efficiency (E%) from donor to the acceptor can be used to interpret biological events. Here, we demonstrate FRET efficiency using FRET standards with genetic constructs where Cerulean and Venus fluorescent proteins are tethered by five amino acid linkers (cerulean-5aa-Venus).

The efficiency and the distance between the molecules is comparable with other FRET imaging methods such as fluorescence lifetime imaging microscopy (FLIM).

FRET Basics

FRET is a process by which radiationless transfer of energy occurs from a fluorophore in the excited state to an acceptor molecule in the ground state in close proximity.





Importance of studying protein-protein interactions

Protein-protein interactions mediate the majority of cellular processes

Protein Localization studies can indicate:

- What proteins are expressed
- Where proteins are expressed
- Where they go over time

By tracking these parameters (in healthy versus diseased tissue and in control versus treated tissue), researchers can gain a greater understanding of these proteins' functions and determine which are likely to be the best drug targets.

FRET in Protein Studies

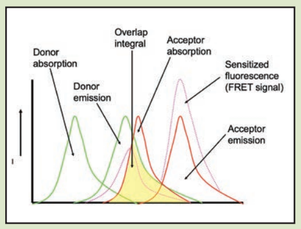
Traditional Biophysical or Biochemical methods used for protein studies are:

- Affinity chromatography
- Nondenaturing electrophoresis
- Co-immunoprecipitation
- Spectroscopic methods
- X-ray diffraction
- Covalent cross-linking
- Electron microscopy

Conditions for FRET to Occur

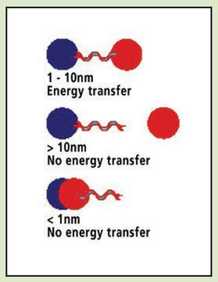
- FRET- process involving the radiationless transfer of energy from a donor fluorophore to an appropriately positioned acceptor fluorophore.

- FRET can occur when the emission spectrum of a donor fluorophore significantly overlaps (>30%) the absorption spectrum of an acceptor (Figure 1). If the spectrum is not overlapped there cannot be any FRET.



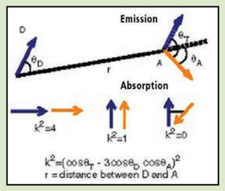
- Because the efficiency of energy transfer varies inversely with the sixth power of the distance separating the donor and acceptor fluorophores, the distance over which FRET can occur is limited to between 1-10 nm.

  r = Ro{ (1/E) - 1}1/6
  r – distance between Donor & Acceptor
  Ro – Forster distance
  E – Energy transfer efficiency



- The emission dipole of the donor and the acceptor absorption dipole must be oriented to each other and it must not be oriented perpendicular to each other.

- Dipole is an electromagnetic field that exists in a molecule that has one region with oppositely charged areas, a negative charge and another region with a positive charge. If the spectra are overlapped, the donor's oscillating emission dipole will look for a matching absorption dipole of an acceptor to oscillate in synchrony. overlapped there cannot be any FRET.



- The magnitude of the relative orientation of the dipole-dipole coupling value is 1-4.

FRET - Pair

It is important to optimize the system with FRET standard. Here we used FRET standard cerulean and Venus purchased from addgene.org and expressed in GHFT1 live pituitary cells. We used for the light source testing cerulean alone, Venus alone and C5V, cerulean linked to Venus with 5 amino acid [3]. The biological example for FRET application used here concerns the basic region-leucine zipper (bZip) domain of the CCAAT/enhancer binding protein alpha (C/EBPα) transcription factor. The bZip family proteins form obligate dimers through their leucine-zipper domains, which positions the basic region residues for binding to specific DNA elements. Immunocytochemical staining of differentiated mouse adipocyte cells showed that endogenous C/EBPα is preferentially bound to satellite DNA-repeat sequences located in regions of centromeric heterochromatin [7-9]. When the C/EBPα bZip domain is expressed as a fusion fluorescent protein in cells of mouse origin, it is localized to the well-defined regions of centromeric heterochromatin in the cell nucleus [5].

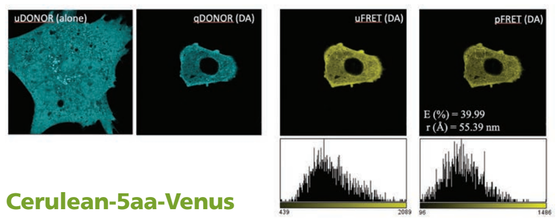
FRET Microscope

We used an Olympus IX71 Wide-field FRET microscopy system at the Keck Center for cellular imaging, University of Virginia to test the effectiveness of the light source for FRET. This is an inverted microscope coupled with an Ex and Em filter wheels and a Hamamatsu Flash 4 CCD camera. Imaged under Olympus IX71 wide-field inverted fluorescence microscope at 60 x NA1.4 with Hamamatsu ORCA Flash4.0 CCD camera. FRET images were processed using custom developed pFRET software (Keck Center, UVA). The donor and acceptor excitation wavelengths were selected from the X-Cite mini fluorescence illumination system using 436/20 nm and 500/20 nm band-pass filters, respectively. 470/30 nm and 535/30 nm band-pass filters were used for the donor and acceptor emission channels, respectively.

FRET Data Analysis - pFRET software

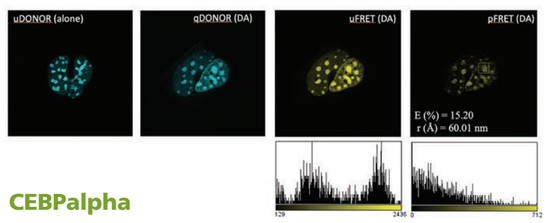
This custom developed software by the Keck Center provides great opportunity to remove the spectral bleed-through in FRET imaging. This image J or Fiji plugin-based software requires 7 images to process the images collected from the widefield microscopy system [3]. The E% calculated using the pFRET software results were comparable with the FLIM technique [4-6]

Results and Discussion



Cerulean-5aa-Venus

Figure 1. C5V FRET standard. The quenched donor intensity (qdonor) in the presence of acceptor (C5V) intensity less than the unquenched donor (udonor) without an acceptor. Before processing using pFRET software, the uFRET image is brighter than the corrected FRET (pFRET), after removing the bleed-through and other contamination in the image, represented in the histogram below the images. The distribution of histogram for pFRET and the uFRET is shown. The histogram was plotted number pixels at each gray level intensity. The result clearly demonstrates the selected light source for this experiment is useful to implement the localization of protein association in live cells.



CEBPalpha

Figure 2. A FRET system for investigating this biological model in living cells was built by fusing the C/EBPα bZip domain to Cerulean (bZip-Cerulean, FRET donor) and Venus (bZip-Venus, FRET acceptor) fluorescence proteins separately. To use the pFRET software for processing require three coverslips, donor alone (D - Cerulean), acceptor alone (A - Venus) and D+A. The processed FRET (pFRET) is shown in the figure. The interaction between Cerulean-and Venus-tagged C/EBPα is demonstrated by the E% value indicates the homodimerization of C/EBPα in regions of centromeric heterochromatin of the cell nucleus. The histogram for corrected FRET very low level of histogram compared to the uncorrected FRET.

Conclusion

The FRET results indicates that the protein dimerization and its distribution was accountable. The X-Cite mini+ fluorescence light source was useful to investigate protein associations in biological samples.

- Alignment Free: As there are no bulbs involved, there is no need for any alignment
- Zero Consumables: X-Cite mini+ is direct coupled so there is no need for light guides or replacements over time
- Field Uniformity: The LED based system provided good sample field uniformity
- Stability: LED technology is inherently more stable than lamp technology
- Fine Illumination Intensity Tuning: 1% intensity tuning eliminates the need for neutral density filters
- Long Lifetime: The LEDs are guaranteed for 15,000 hours of ON time

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