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FRET microscopy imaging

FRET microscopy relies on the ability to capture weak and transient fluorescent signals efficiently and rapidly from the interactions of labeled molecules in single living or fixed cells. The occurrence of FRET signal (sensitized signal) can be verified by acquiring the two-emission signal bands of the double labeled cells excited with donor wavelength. If FRET occurs the donor channel signal will be quenched and the acceptor channel signal will be sensitized or increased (Herman et al., 2001). In principle, the measurement of FRET in a microscope can provide the same information that is available from the more common macroscopic solution measurements of FRET; however, FRET microscopy has the additional advantage that the spatial distribution of FRET efficiency can be visualized throughout the image, rather than registering only an average over the entire cell or population. Because energy transfer occurs over distances of 1–10 nm, a FRET signal corresponding to a particular location within a microscope image provides an additional magnification surpassing the optical resolution (~0.25 μm) of the light microscope (Kauzmann, 1957). Thus, within a voxel of microscopic resolution, FRET resolves average donor-acceptor distances beyond the microscopic limit down to the molecular scale. This is one of the principal and unique benefits of FRET for microscopic imaging: not only colocalization of the donor- and acceptor-labeled probes within ~0.09 μm^2 can be seen, but intimate interactions of molecules labeled with donor and acceptor can be demonstrated. Different intensity-based imaging techniques that apply the method of FRET include Wide-field, Confocal, and Multi-photon microscopy (Periasamy et al., 2001). All FRET microscopy systems require neutral density filters to control the excitation light intensity, a stable excitation light source (Hg or Xe or combination arc lamp; UV, Visible or Infrared lasers), a heated stage or a chamber to maintain the cell viability and appropriate filter sets (excitation, emission, and dichroic) for the selected fluorophore pair. It is important to carefully select filter combinations that reduce the spectral bleed through (SBT) to improve the signal-to-noise (S/N) ratio for the FRET signals. Some possible filter combinations for FRET imaging using several of the different fluorophore combinations are shown in Table SI (Chroma Technology Corp.; Omega Opticals, Inc.).

Wide-field FRET microscopy. In addition to the above-mentioned features, the wide-field microscopy requires a tripartite system: a conventional fluorescence microscope equipped with a movable z-axis stage that permits imaging of the specimen at different focus positions, a higher quantum efficiency CCD camera for quantification of the light emitted by the specimen and an appropriate software package that is capable of synchronizing hardware, acquiring images and correcting them for distortions and information loss inherent in the imaging process (Periasamy and Day, 1999). To allow simultaneous monitoring of spectral emissions at two wavelengths for a donor/acceptor FRET, a dichroic filter is used that reflects the donor excitation wavelength to excite the double-labeled cells and transmit the two emission (donor and acceptor) bands.

With wide-field microscopy, the cells expressing the GFP-fusion protein are initially identified using the appropriate GFP filter set and a reference image is obtained. A second image is then acquired for Donor (D) fluorescence from the same field of cells using the donor filter set, followed by acquisition of a third image of the same field using the acceptor filter set. The camera gain, neutral density (ND) filter and image acquisition time are kept constant for the donor and acceptor images. Camera dark-current noise and background contributions due to medium, cells and optics are digitally subtracted from donor (I_D) and acceptor (I_A) images.

Some of the FRET signal results from background SBT for the combination of the donor and acceptor fluorophores. To obtain the optimal FRET signal, it is important to correct for the bleed through background (or cross-talk) signal in

Table SI. **Filter configuration for wide-field FRET imaging for several of the different fluorophore combinations**

Pair		Excitation (nm)	Emission (nm)		Dichroic (nm)
Donor	Acceptor	(Donor)	Donor	Acceptor	
BFP	GFP	365/15	460/50	535/50	390
BFP	YFP	365/15	460/50	535/26	390
BFP	dsRED1	365/15	460/50	610/60	390
CFP	dsRED1	440/21	480/30	610/60	455
CFP	YFP	440/21	480/30	535/26	455
GFP	Rhod-2	488/20	535/50	595/60	505
FITC	Rhod-2	488/20	535/45	595/60	505
FITC	Cy3	488/20	535/45	595/60	505
Cy3	Cy5	525/45	595/60	695/55	560
Alexa®488	Cy3	488/20	535/45	595/60	505

the acceptor channel. As an approach for cross-talk correction, a very narrow-band filter can be used and the exposure time can be increased to improve the S/N ratio in the acquired images (Periasamy et al., 2001).

Computer software designed to correct for the SBT signal is then used to process the FRET image. This correction requires that a series of images from cells labeled with either the donor or acceptor proteins alone be obtained using the same filter configuration that is to be used for FRET data acquisition. The computer algorithm then determines the percentage of SBT in each channel from the donor and acceptor control images, and this dataset is then used to correct the SBT in the FRET images (Elangovan et al., 2003).

Table SII. **Filter configurations for confocal image acquisition for selected fluorophore pairs**

Fluorophore	Excitation wavelength (nm)	Emission filter (nm)
Alexa®488 or GFP	Argon 488	515/30 or 535/50
Cy3 or Rhod-2	Green HeNe 543	590/70
CFP	Argon 457	485/30
dsRED1	HeNe 543	590/70
CFP	Argon 457	485/30
YFP	Argon 514	528/50
Cy3	Green HeNe 543	590/70
Cy5	HeNe 633 or HeNe 594	660LP

Laser scanning confocal FRET microscopy. A major limitation to the Wide-field microscopy technique is that emission signals originating from above and below the focal plane contribute to out-of-focus signal that reduces the contrast and seriously degrades the image. Laser scanning confocal FRET (C-FRET) microscopy can overcome this limitation owing to its capability to reject signals from outside the focal plane. This capability provides a significant improvement in lateral resolution and allows for the use of serial optical sectioning of the living specimen.

A disadvantage of this type of microscopy is that the wavelengths available for excitation of different fluorophore pairs are limited to standard laser lines. The use of fluorophores that are excited by near-UV light in these live cell-imaging studies is limited by problems associated with photobleaching and photodamage. The FRET images acquired using confocal microscope also require processing software (Elangovan et al., 2003). The various filter configurations used for a typical Nikon PCM 2000 and Radiance 2100 Confocal Microscopes are given in Table SII.

Multiphoton FRET microscopy. A significant improvement over C-FRET can be achieved by eliminating the out-of-focus signal altogether by limiting excitation to only the fluorophore at the focal plane. This is precisely what multiphoton FRET (MP-FRET) microscopy does. Because multiphoton excitation occurs only in the focal volume, the detected emission signal is exclusively in-focus light. Furthermore, because multiphoton excitation uses longer wavelength light, it is less damaging to living cells, thus limiting problems associated with fluorophore photobleaching and photodamage as well as intrinsic fluorescence of cellular components.

In multiphoton FRET (MP-FRET) imaging, we select the appropriate filters and high sensitivity photomultiplier tubes (PMTs) to acquire donor and acceptor images. For MP-FRET, the ti:sapphire laser is tuned to detect the maximum and minimum signal for the donor and acceptor proteins expressed individually. The wavelength corresponding to maximum donor signal and minimum acceptor signal will be used to collect the FRET signal from the doubly expressed cells. For example, in the case of cells expressing C/EBPD244 protein tagged with CFP (donor) and YFP (acceptor), the laser wavelength is tuned from 700–1000 nm. The maximum CFP signal and minimum YGFP signal are seen at 820 nm. Maximum YFP signal and minimum CFP signal are seen at 920 nm. Hence, an excitation wavelength of 820 nm is used to acquire the FRET images from doubly expressed (CFP-YFP-C/EBPD244) cells. This method of selecting donor and acceptor excitation wavelengths can be used for any possible MP-FRET fluorophore pairs. Since the selected donor excitation wavelength can also excite (about $<$10%) the acceptor fluorophore molecule present, the technique requires correction to remove unwanted fluorescence signal in the FRET image (Elangovan et al., 2003). It is important to note that appropriate average power should be used to reduce photobleaching. The filter configurations for selected fluorophore pairs used in Multiphoton FRET imaging microscopy are given in Table SIII.

Other FRET imaging techniques

Fluorescence lifetime imaging microscopy (FLIM). Each of the fluorescence microscopy techniques described uses intensity measurements to reveal fluorophore concentration and distribution in the cell. Recent advances in camera sensitivities and resolutions have improved the capability of these techniques to detect dynamic cellular events. Unfortunately, even with the improvements in technology, these fluorescence microscopic techniques do not have high-speed ($<$s) time resolution to fully characterize the organization and dynamics of complex cellular structures.

In contrast, the time-resolved fluorescence microscopic technique allows the measurement of dynamic events at very high temporal resolution (nanoseconds). FLIM merges the information of the spatial distribution of the probe with probe lifetime information to enhance the reliability of the concentration measurements. The technique monitors the localized changes in probe fluorescence lifetime (Gerritsen and de Grauw, 2001) and provides an enormous advantage for imaging dynamic events within the living cells. The one- and two- photon confocal FLIM techniques measure environmental changes within the living cells (Bastiaens and Squire, 1999) and can be used in multilabeling experiments. An important advantage of FLIM measurements is that they are independent of change in probe concentration, photobleaching and other factors that limit intensity-based steady-state measurements. Additionally FLIM enables the discrimination of fluorescence coming from

Table SIII. **Filter configurations for multiphoton image acquisition for selected fluorophore pairs.**

Fluorophore	Excitation (nm)	Emission (nm)
Alexa®488	790	515/30
Cy3	735	590/70
BFP	740	450/80
eGFP	880	515/30
CFP	820	485/30
YFP	920	528/30
CFP	820	485/30
dsRED1	780	590/70
BFP	740	450/80
S65T (GFP)	770	515/30

different dyes, including autofluorescent materials that exhibit similar absorption and emission properties but show a difference in fluorescence lifetime.

Instrumental methods for measuring fluorescence lifetimes are divided into two major categories, frequency-domain (Lakowicz, 1999; Verveer et al., 2001) and time-domain (Periasamy et al., 1996). With the time-domain method (or pulse method), the specimen is excited with a short pulse and the emitted fluorescence is integrated in two or more time windows (Elangovan et al., 2002). The relative intensity capture in the time windows is used to calculate the decay characteristics. The determination of prompt fluorescence with lifetime in the range of 0.1 to 100 ns requires elaborate fast excitation pulses and fast-gated detection circuits. As an alternative to the time-domain method, the frequency-domain method uses a homodyne detection scheme and requires a modulated light source and a modulated detector. The excitation light is modulated in a sinusoidal fashion. The fluorescence intensity shows a delay or phase-shift with respect to the excitation and a smaller modulation-depth. The phase-shift and modulation-depth depend on the decay constants of the fluorescent material and the modulation frequency (unpublished data). The lifetime is determined from the phase-shift by the relation:

$$\tau_{\upsilon} = 1/\omega(\tan\upsilon),$$

where w is the angular frequency of the modulation and u is the phase shift. Lifetime can also be determined from the modulation-depth m, which is the relative modulation depth of the emission signal as compared with the excitation, by the relation:

$$\tau_{m} = 1/\omega(1/m^2 - 1)^{1/2}.$$

To measure fluorescence lifetimes in the range of 0.5–20 ns, modulation frequencies of 5–100 MHz can be used.

The FLIM system can be coupled to any wide-field microscope (Elangovan et al., 2002; Lakowicz, 1999). The lifetime method can also be applied in a laser-scanning confocal microscope and in multiphoton microscopy (Becker & Hickl GmbH).

When combined with FRET, this approach can provide direct evidence for the physical interactions between proteins with very high spatial and temporal resolution. Because only one protein partner, the donor, is monitored, it is unnecessary to use spectral bleed-through correction in FRET-FLIM images. The one- and two- component analysis of the donor molecule lifetime in the presence of acceptor demonstrates the distance distribution between interacting proteins (Fig. S1).

Fluorescence correlation spectroscopy (FCS). Fluorescence correlation spectroscopy (FCS) is a technique in which spontaneous fluorescence intensity fluctuations are measured in a microscopic detection volume of $\sim 10^{-15}$ Liter as defined by a tightly focused laser beam (Molecular Probes, Inc.). FCS is a special case of fluctuation correlation techniques, where the laser-induced fluorescence out of a very small probe volume is autocorrelated in time. The solution under investigation contains fluorescent molecules in 10^{-9} - 10^{-12} M concentration. The raw data for FCS analysis is the fluorescence signal. Fluorescence intensity fluctuations measured by FCS represent changes in either the number or the fluorescence quantum yield of molecules resident in the detection volume. Small, rapidly diffusing molecules produce rapidly fluctuating intensity patterns, whereas larger molecules produce more sustained bursts of fluorescence. If no further effects on fluorescence characteristics are present, fluctuations in the emission light simply arise from occupation number changes in the illuminated region by random particle motion. Average occupation numbers are very low, down to single molecules or even less. By fluctuation analysis, a noninvasive determination of molecular dynamics in the single molecule range can be performed yielding chemical reaction constants or diffusion coefficients.

Image Correlation Spectroscopy (ICS) was developed as the imaging analogue of FCS for measuring protein aggregation in biological membranes. The ICS method entails collecting fluorescence intensity fluctuations as a function of position by using a laser scanning microscope imaging system and analyzing the imaged intensity fluctuations by spatial autocorrelation analysis (Peterson et al., 1998). The amplitude of the normalized spatial autocorrelation function is directly related to the absolute concentration of fluorophore in the focal volume and the state of aggregation of the fluorescent entities. Extension of ICS to temporal autocorrelation analysis of image time series also permits measurement of molecular transport occurring on slower time scales characteristic of macromolecules within the plasma membrane. The other related technique, Image Cross-Correlation Spectroscopy (ICCS) allows direct measurement of the interactions of two colocalized proteins labeled with fluorophores having different emission wavelengths. ICS and ICCS involve the use of laser scanning confocal microscopy to obtain fluorescence images of fluorescently labeled cell culture membranes. Using the technique of ICS, individual high magnification images are used to calculate spatial autocorrelation functions, which can quantify an average cluster density of the fluorescent particle of interest, giving information about the surface distribution. Using the technique of ICCS, images are taken using two spectrally separated fluorophores on the same area of the cell and calculating the spatial cross-correlation function enables quantification of the fraction of associations for the two differently labeled particles of interest.

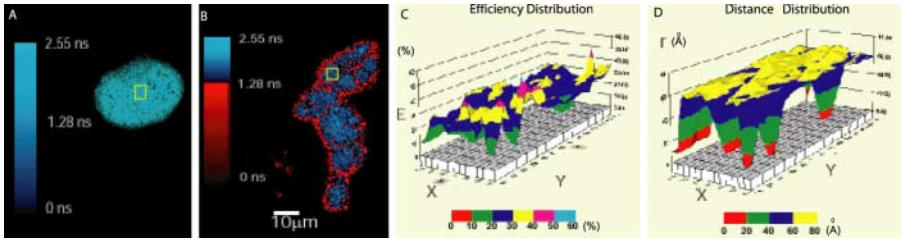

Figure S1. **One-component lifetime distribution of CFP-YFP-C/EBPD244 protein dimerization in mouse pituitary GHFT1-5 live cells.** The mean lifetime of the donor FRET-FLIM image (A) in the absence of the acceptor was 2.2 ns. In a protein complex such as CFP-C/EBPD244, proteins have different lifetime distributions. In the presence of acceptor, most of the proteins in the protein complex participate in the energy transfer processes as shown in one-component (B) FRET-FLIM image. The image obtained was spatially and temporally resolved as compared to a patch of protein complex in intensity based FRET image (Elangovan et al., 2002). Three-dimensional plots of the efficiency (E) and the distance (r) distribution of a region of interest are shown in figures (C) and (D).

Spectral FRET imaging microscopy. Recently, Carl Zeiss MicroImaging, Inc. introduced the Laser Scanning Microscope (LSM) 510 META system, with the revolutionary emission fingerprinting technique permitting the clean separation of several (even spectrally overlapping) fluorescence signals of a specimen. The number of dyes, which can be used and detected in the experiment, is almost unlimited. The new system overcomes the limits of existing detection methods and permits both qualitative and quantitative analyses, quickly and precisely, in vitro and in vivo. Furthermore, it is beneficial in many cases for the elimination of unwanted signals, such as background noise or autofluorescence.

Photobleaching FRET (pbFRET) methods. Photobleaching methods, in which either the donor or acceptor molecules are selectively photodestructed, provide a sensitive means for establishing and quantitating energy transfer efficiencies in cells (Bastiaens and Jovin, 1998). The photobleaching time of a fluorophore is inversely proportional to the excited state lifetime; any process such as energy transfer that shortens the lifetime will decrease the photobleaching rate. Thus, the photobleaching time constant of the donor fluorophore is expected to be longer in the presence of acceptor molecules.

In the acceptor photobleaching scenario, destruction of the acceptor leads to a corresponding increase in the donor emission quantum yield, i.e., intensity. In the acceptor bleaching method, the difference between quenched donor and unquenched donor is used to calculate the energy transfer efficiency (Kenworthy et al., 2000; Wouters et al., 1998). While this approach has the advantage of using a double-labeled specimen, it cannot be used for living cells, since the exposure to extended laser energy is believed to cause ill effects. The donor photobleaching approach is based on the idea that the donor signal will decay more slowly in the presence of an acceptor molecule, due to the introduction by FRET of an additional deactivation pathway shortening the lifetime of the excited state. FRET using photobleaching techniques establishes the possibility of studying on a very localized spatial scale the interactions between a receptor-ligand pair.

FRAP. FRAP is based on the principal of observing the rate of recovery of fluorescence due to the movement of a fluorescent marker into an area of the membrane which contains this same marker but which has been rendered nonfluorescent via an intense photobleaching pulse of laser light. The diffusion of the fluorophore is related to both its rate and extent of recovery. The fluorescence recovery of a two-phase system, originally at equilibrium, is monitored by a microscope-mounted CCD camera as it relaxes to a new equilibrium. During this relaxation, the concentrated profiles of the probe solute are measured on both sides of the interface as a function of time, yielding information about the transport characteristics of the system. To minimize the size of the meniscus between the two phases, a photolithography technique is used to selectively treat the glass walls of the cell in which the phases are contained. This allows concentration measurements to be made very close to the interface and increases the sensitivity of the FRAP technique. FRAP is being used to measure the lateral diffusion of various membrane or cytoplasmic constituents. FRAP has been used to characterize the mobility of plasma membrane receptors and lipids under various condition; e.g., during cell locomotion and hypoxic injury.

Fluorescence loss in photobleaching (FLIP). In the technique of Fluorescence Loss In Photobleaching (FLIP), a region of fluorescence within the cell is subjected to repeated photobleaching (by increased illumination). Over time, this will lead to a loss of the fluorescent signal from throughout the cell if all the fluorescent molecules can diffuse past the point of increased illumination. The rate at which fluorescence is lost from the entire cell is monitored and in the case where the fluorescent signal is provided by a suitably tagged membrane protein, provides an indication of the diffusional mobility of the protein in the plane of the lipid bilayer. FRAP and FLIP are valuable for studies of Golgi/ER trafficking in animal cells (Cole et al., 1996).

Single molecule FRET microscopy. The sensitivity of FRET is extended to the single molecule level by measuring energy transfer between a single donor fluorophore and a single acceptor fluorophore. The classical equations used to measure energy transfer on ensembles of fluorophores are modified for single-molecule measurements. Multi-parameter fluorescence detection (intensity, lifetime, anisotropy, spectral range) of single molecules in a confocal setup and generation of multi-dimensional frequency histograms can offer high precision and accuracy in the determination of structures and conformational dynamics of individual molecules in solution. The techniques of single-pair FRET (SP-FRET) and single molecule fluorescence polarization anisotropy (SMFPA) can be used to observe the conformational fluctuations and interactions of molecules at single molecule resolution (Ha et al., 1999).

Bioluminescence resonance energy transfer (BRET). Bioluminescence resonance energy transfer (BRET), based on energy transfer between fusion proteins containing *Renilla* luciferase (Rluc) and a mutant of the GFP, is an advanced, nondestructive, cell-based assay technology that is perfectly suited for proteomics applications, including receptor research and the mapping of signal transduction pathways (Xu et al., 1999). BRET has shown promise as a tool to chart the physical contacts between specific cellular proteins, and their time-resolved interactions, in living tissues and in real time. BRET offers the advantages of FRET but avoids the consequences of fluorescence excitation. By choosing the proper luciferase/GFP mutant combination, BRET can be used to measure protein interactions in vitro and in vivo.

New FRET modalities. With the development of new FRET modalities like Homotransfer or energy migration FRET (emFRET) and photochromic FRET (pcFRET), the future of FRET is bright, making it possible to study a broad range of interesting applications involving cellular events coupled to specific molecular signaling processes and simultaneous observation of a series of reversible molecular processes in living cells. In pcFRET, the fluorescent emission of the donor is modulated by cyclical transformations of a photochromic acceptor (Giordano et al., 2002). Light induces a reversible change in the structure and absorption properties of the acceptor. Both emFRET and pcFRET are efficient techniques for studying the protein-protein associations and dynamics in cellular signal transduction.

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