**Abstract**  Interacting proteins assemble into molecular machines that control cellular homeostasis in living cells. While the *in vitro* screening methods have the advantage of providing direct access to the genetic information encoding unknown protein partners, they do not allow direct access to interactions of these protein partners in their natural environment inside the living cell. Using wide-field, confocal, or two-photon (2p) fluorescence resonance energy transfer (FRET) microscopy, this information can be obtained from living cells and tissues with nanometer resolution. One of the important conditions for FRET to occur is the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor. As a result of spectral overlap, the FRET signal is always contaminated by donor emission into the acceptor channel and by the excitation of acceptor molecules by the donor excitation wavelength. Mathematical algorithms are required to correct the spectral bleed-through signal in wide-field, confocal, and two-photon FRET microscopy. In contrast, spectral bleed-through is not an issue in FRET/FLIM imaging because only the donor fluorophore lifetime is measured; also, fluorescence lifetime imaging microscopy (FLIM) measurements are independent of excitation intensity or fluorophore concentration. The combination of FRET and FLIM provides high spatial (nanometer) and temporal (nano-second) resolution when compared to intensity-based FRET imaging. In this paper, we describe various FRET microscopy techniques and its application to protein-protein interactions.

**Key words**  fluorescence resonance energy transfer (FRET) · confocal FRET (C-FRET) · two-photon FRET (2p-FRET) · fluorescence lifetime imaging microscopy (FLIM) · FLIM-FRET · CCAAT enhancer binding protein x (C/EBPα) · dimerization · traumatic brain injury · tissue FRET · acceptor-photobleaching FLIM-FRET

**Introduction**

In living cells, proteins interact to achieve cellular homeostasis. Protein assemblies are traditionally studied using biophysical or biochemical methods such as affinity chromatography or co-immunoprecipitation. Recently, two-hybrid and phage-display methods have been used for detecting protein-protein interactions. These *in vitro* screening methods have the advantage of providing direct access to the genetic information encoding unknown protein partners (Cunningham, 2001). These techniques, however, do not allow direct access to interactions of these protein partners in their natural environment inside the living cell. Using FRET microscopy, this information can be obtained with nanometer resolution (Clegg, 1996; Gordon et al., 1998; Cubitt et al., 1999; Miyawaki et al., 1999; Ng et al., 1999; Kenworthy et al., 2000; Kraynov et al., 2000; Periasamy, 2001; Day et al., 2003; Jares-Erijman and Jovin, 2003).

New imaging technologies, coupled with the development of new genetically encoded fluorescent labels and sensors and the increasing capability of computer...
software for image acquisition and analysis, have enabled more sophisticated studies of protein functions and processes ranging from gene expression to second-messenger cascades and intercellular signaling (Roessel and Brand, 2002). FRET microscopy relies on the ability to capture fluorescent signals from the interactions of labeled molecules in single living or fixed cells and tissues. If FRET occurs, the donor channel signal will be quenched and the acceptor channel signal will be sensitized or increased (Herman, 1998; Herman et al., 2001). By measuring these effects, FRET microscopic imaging can verify close molecular associations between colocalized donor- and acceptor-labeled fusion proteins that are far beyond the resolution of traditional fluorescent microscopy.

FRET is a process involving the radiationless transfer of energy from a donor fluorophore to an appropriately positioned acceptor fluorophore (Förster, 1965; Stryer, 1978; Van Der Meer et al., 1994; Wu and Brand, 1994; Lakowicz, 1999). FRET can occur when the emission spectrum of a donor fluorophore significantly overlaps (>30%) the absorption spectrum of an acceptor (Fig. 1), provided dipoles of the donor and acceptor fluorophores are in favorable mutual orientation. 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 and 10 nm. When the spectral, dipole orientation, and distance criteria are satisfied, excitation of the donor fluorophore results in sensitized fluorescence emission from the acceptor, indicating that the tagged proteins are separated by <10 nm.

Several FRET techniques exist based on wide-field, confocal, and 2p microscopy as well as FRET/FLIM, each with its own advantages and disadvantages. They are used for various biological applications such as studies of organelle structure, conjugated antibodies, cytochemical identification, and oxidative metabolism (Sekar and Periasamy, 2003). This paper describes and compares the above microscopy techniques, using dimerization of a transcription factor expressed as fusion proteins with CFP and YFP to demonstrate the utility of the various approaches. Also, we discuss the implementation of confocal and two-photon FRET in tissue in an in vivo model of neurological disease using aldehyde-fixed rat brain tissue sections.

**Different FRET techniques**

Different intensity-based imaging techniques that can utilize the FRET method include wide-field, confocal, and two-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. Very important are good sensitivity detectors, filters, and objective lenses. High-sensitivity detectors help to reduce data acquisition time, and narrow band pass filters for excitation and emission reduce the spectral bleed-through noise. These carefully selected filter combinations also improve the signal-to-noise (S/N) ratio for the FRET signals. Basically, the FRET signal is created by exciting the double-labeled (donor and acceptor) protein(s) of interest within the cell with the donor excitation wavelength and collecting both donor emission and acceptor emission. When all the conditions for FRET are satisfied, the donor signal will be quenched due to the transfer of energy and the acceptor signal will be sensitized (increased). The acceptor emission is the FRET signal, which is usually contaminated by spectral bleed-through, which should be corrected as explained in the FRET data analysis section.

**Wide-field FRET (W-FRET) microscopy**

Any fluorescence microscope (inverted or upright) can be converted to W-FRET microscopy. There are a number of papers in the literature for various protein studies using the W-FRET system (Jovin and Arndt-Jovin, 1989; Kam et al., 1995; Day, 1998; Gordon et al., 1998; Varma and Mayor, 1998; Periasamy and Day, 1999; Krainov et al., 2000; Day et al., 2003). For W-FRET, it is advisable to use a single dichroic to acquire the donor (D) and acceptor (A) images for the donor excitation wavelength in the double-labeled specimen. This can be achieved by using excitation and emission

**Fig. 1** Absorption and emission spectra of CFP-YFP fluorophores. DSBT, donor spectral bleed-through into the acceptor (or FRET channel); ASBT, acceptor molecule spectral bleed-through signal due to the acceptor being excited by the donor wavelength. Wide-field donor excitation (ExD) 440/21 nm, emission (EmD) 480/30 nm; acceptor (ExA) 520/15 nm, EmA 535/26 nm. Confocal ExD 457, EmD 485/30 nm; ExA 514, EmA 528/50 nm.
filter wheels in the microscope system. This option helps to reduce any spatial shift of donor and acceptor channel images, because the processed FRET image is obtained through pixel-by-pixel calculation as described in the FRET data analysis section.

Even though W-FRET microscopy is the simplest and most widely used technique, there is a major limitation to W-FRET in that the emission signals originating from above and below the focal plane contribute to out-of-focus signals that reduce the contrast and seriously degrade the image. Digital deconvolution microscopy in the W-FRET system helps to localize the proteins at different optical sections, but this requires an intensive computational process to remove the out-of-focus information from the optical sectioned FRET images (Periasamy and Day, 1998, 1999). For protein interactions taking place homogeneously over a wider area of a cell (e.g., nucleus), as described in this paper, W-FRET is an entirely suitable technique.

Confocal FRET (C-FRET) microscopy

Laser scanning confocal FRET (C-FRET) microscopy overcomes the limitation of out-of-focus information owing to its capability of rejecting signals from outside the focal plane and acquiring the signal in real time (Kenworthy et al., 2000; Pozo et al., 2002; Wallrabe et al., 2003). This capability provides a significant improvement in lateral resolution and allows the use of serial optical sectioning of the living specimen (Pawley, 1995; Lermers et al., 2001). By selecting appropriate filter combinations, one can configure any commercially available confocal microscopy system for FRET imaging. A disadvantage of this technique is that the wavelengths available for excitation of different fluorophore pairs are limited to standard laser lines. Standard laser lines do allow C-FRET to be used for a number of fluorophore combinations including CFP-YFP or ds-RED, GFP-rhodamine or Cy3, FITC or Alexa488-Cy3, Alexa488-Alexa555, and Cy3-Cy5 (Kenworthy et al., 2000; Periasamy, 2001; Day et al., 2003; Elangovan et al., 2003; Mills et al., 2003; Wallrabe et al., 2003).

Also, in one-photon wide-field or confocal microscopy, illumination occurs throughout the excitation beam path, in an hourglass-shaped pattern. This results in absorption along the excitation beam path, giving rise to substantial fluorescence emission both below and above the focal plane. Excitation of other focal planes contributes to photobleaching and photodamage in the specimen planes that are not being involved in imaging. This can be ameliorated by multi-photon/2-photon microscopy.

Two-photon FRET (2p-FRET) microscopy

As mentioned above, the advantage of C-FRET over W-FRET lies in the ability to reject the out-of-focus signal that originates from outside the focal plane. A significant improvement over W-FRET and C-FRET is achieved by eliminating the above/below-focal-plane signal altogether, by limiting excitation to only the fluorophores at the focal plane. This is precisely what two-photon excitation microscopy does (Denk et al., 1990; Periasamy et al., 1999). The fluorophores exhibit two-photon absorption at approximately twice (740 nm) their one-photon absorption wavelengths (370 nm), while the emission for below-focal-plane (BFP) labeled specimen is the same as that of one-photon (420 nm), allowing the specimen to be imaged in the visible spectrum. When an infrared laser beam is focused on a specimen, illumination takes place at a single point and the fluorescence emission is localized to the vicinity of the focal point. The fluorescence intensity then falls off rapidly in the lateral and axial direction. The infrared illumination in two-photon excitation penetrates deeper into the specimen than visible light excitation due to its higher energy, making it ideal for many applications involving depth penetration through thick sections of tissue.

Two-photon absorption was theoretically predicted by Goppert-Mayer (1931) and was experimentally observed for the first time in 1961 using a ruby laser as the light source (Kaiser and Garrett, 1961). Denk and others have experimentally demonstrated two-photon imaging in laser scanning confocal microscopy (Denk et al., 1990). Two-photon excitation microscopy has been widely used in the area of biomedical sciences including tissue engineering, protein-protein interactions, cell, neuron, molecular, and developmental biology (Denk et al., 1995; Periasamy et al., 1999; Svoboda et al., 1999; Periasamy, 2001; Diaspro, 2002; König and Riemann, 2003; Samkoe and Cramb, 2003; Soeller et al., 2003). Because pulsed lasers are used as an excitation source, this configuration is an ideal system for fluorescence lifetime imaging (FLIM).

In 2p-FRET microscopy, the excitation process is different compared to 1p-FRET microscopy (W-FRET and C-FRET), while the emission process is the same. The 2p-system is suitable for any FRET fluorophore pair because wavelengths are tunable from 700 to 1000 nm. The emission spectrum is well separated from the excitation spectrum when compared to 1p-FRET microscopy. In the case of CFP-tagged molecules, the emission spectrum separation for 1p is about 40 nm (Ex-440 nm and Em-480 nm), compared to 340 nm (Ex-820 nm and Em-480 nm) in the 2p-FRET systems. The disadvantage is that a single wavelength may excite the donor and acceptor molecule at different rates. For example, at 820 nm, the CFP molecule is excited maximally but the YFP molecule is excited minimally. It is important to remove the YFP molecule fluorescence signal excited by the CFP excitation wavelength (820 nm) from the FRET channel, as described in the FRET data analysis section. Two-photon-FRET micro-
scopy is also advantageous for time-lapse FRET imaging because the cells can be maintained for a longer duration of time compared to the 1p-FRET microscopy systems, as there is less photodamage above and below the focal plane.

**FRET data analysis**

In principle, the spectral bleed-through (SBT) signal is the same for 1p- or 2p-FRET microscopy. In addition to SBT, the FRET signals in the acceptor channel also require correction for spectral sensitivity variations in donor and acceptor channels, autofluorescence, and detector and optical noise, which contaminate the FRET signal. The details of the corrections and the relevant biological applications have been reported in the literature (Elangovan et al., 2003; Mills et al., 2003; Wallrabe et al., 2003; www.circusoft.com, accessed November, 2003).

In brief, to remove the SBT or cross-talk for 1p- or 2p-FRET, seven images are acquired: double-labeled (three images: donor excitation/donor and acceptor channel; acceptor excitation/acceptor channel), single-labeled donor (two images: donor excitation/donor and acceptor channel), and single-labeled acceptor (two images: donor excitation/acceptor channel; acceptor excitation/acceptor channel) with appropriate filters for PFRET data analysis as described in the literature (Elangovan et al., 2003). Our approach works on the assumption that the double-labeled cells and single-labeled donor and acceptor cells, imaged under the same conditions, exhibit the same SBT dynamics. The hurdle we had to overcome was the fact that we had three different cells (D, A, and D+A), where individual pixel locations cannot be compared. What could be compared, however, were pixels with matching fluorescence levels. Our algorithm follows fluorescence levels pixel-by-pixel to establish the level of SBT in the single-labeled cells, and then applies these values as a correction factor to the appropriate matching pixels of the double-labeled cell.

Then, the PFRET, the contamination-removed FRET signal, is (Elangovan et al., 2003)

\[
PFR = uFRET - DSBT - ASBT
\]  

where \( uFRET \) is uncorrected FRET (signal in the FRET/acceptor channel), DSBT is donor spectral bleed-through in the FRET/acceptor channel, and ASBT is the acceptor bleed-through signal due to excitation of the acceptor molecule by the donor wavelength (see Fig. 1).

Conventionally, energy transfer efficiency (\( E \)) is calculated by ratioing the donor image in the presence (\( I_{DA} \)) and absence (\( I_{D} \)) of acceptor. To execute this calculation, either the acceptor in the double-labeled specimen has to be bleached or the donor fluorescence averages of two different cells (single and double label) with most likely different dynamics are used in the efficiency calculation. When using the algorithm as described, we indirectly obtained the \( I_D \) image by using the PFRET image (Elangovan et al., 2003). The sensitized emission in the acceptor channel is due to the quenching of the donor or energy transferred signal from the donor molecule in the presence of acceptor. Therefore, if we add the PFRET to the intensity of the donor in the presence of acceptor, we obtain the \( I_D \). This \( I_D \) is from the same cell used to obtain the \( I_{DA} \). Hence, the efficiency equation will be modified to obtain the new transfer efficiency (\( E_n \)) from the same cell, as shown in Equation 3.

\[
E = 1 - \left( \frac{I_{DA}}{I_D} \right)
\]  

\[
E_n = 1 - \left[ \frac{I_{DA}}{(I_{DA} + PFRET)} \right]
\]  

where

\[
I_D = I_{DA} + PFRET
\]  

It is important to note that there are a number of other processes involved in the excited state during energy transfer. The new efficiency (\( E_n \)) is calculated by generating a new \( I_D \) image by including the detector spectral sensitivity of donor and acceptor channels and the donor quantum yield with PFRET signal as shown in Equation 5 (Elangovan et al., 2003).

\[
E_n = 1 - \left[ \frac{I_{DA} + PFRET^*(\Psi_{dd}/\Psi_{aa})^*Q_d}{I_{DA} + PFRET^*(\Psi_{dd}/\Psi_{aa})^*Q_d + PFRET} \right]
\]  

where

\[
(\Psi_{dd}/\Psi_{aa}) = [(PMT \text{ gain of donor channel}/PMT \text{ gain of acceptor channel}) \times (\text{spectral sensitivity of donor channel}/\text{spectral sensitivity of acceptor channel})]
\]  

where \( Q_d \) - donor quantum yield

In Equation 7, for estimating the distance between donor and acceptor, \( r \) has changed to \( r_n \). Förster’s distance \( R_0 \) value was calculated for various fluorophore pairs

\[
r = R_0 \{(1/E) - 1\}^{1/6}
\]  

\[
r_n = R_0 \{(1/E_n) - 1\}^{1/6}
\]

The energy transfer efficiency was calculated and compared for the conventional (two different cells, \( I_{DA}/I_D \)) and new method (same cell, \( I_{DA}/I_{DA} + PFRET^*(\Psi_{dd}/\Psi_{aa})^*Q_d \)). The error in efficiency between the same cells versus different cells was about 43%. In the same way, we compared the distance between the donor and acceptor molecule and the error was about 8% (Elangovan et al., 2003). It is important to note that we did not consider the quantum yield of the acceptor.
molecule in our calculation because the energy transfer is from donor to acceptor. Also, the self-interaction dominates over the FRET signal in the case of overexpressed cells.

**Fluorescence lifetime imaging FRET (FLIM-FRET) microscopy**

Each of the fluorescence microscopy techniques described above 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 these improvements in technology, currently available fluorescence microscopic techniques do not have high-speed (<s) time resolution to fully characterize the organization and dynamics of complex cellular structures. In contrast, time-resolved fluorescence microscopy (or lifetime) allows the measurement of dynamic events at very high temporal resolution. FLIM was developed to image and study the environmental behavior of the living specimen and to study the dynamic behavior from single cell to single molecule (Gadella et al., 1993; Dowling et al., 1998; Straub and Hell, 1998; Hanley et al., 2002). Lifetime measurement is independent of spectral bleed-through, fluorophore concentration, or unintended photobleaching.

The fluorescence lifetime ($\tau$) is defined as the average time that a molecule remains in an excited state prior to returning to the ground state. In practice, the fluorescence lifetime is defined as the time in which the fluorescence intensity decays to 1/e of the intensity immediately following excitation (Lakowicz, 1999). Excited-state lifetime measurements are independent of change in excitation light intensity, probe concentrations, and light scattering, but highly dependent on the local environment of the fluorophore, such as the occurrence of FRET, changes in pH and temperature, and the presence/absence of calcium ions. Instrumental methods for measuring fluorescence lifetimes are divided into two major categories, frequency-domain (Gratton et al., 1984, 2003; Lakowicz, 1999) and time-domain (Demas, 1983; O’Connor and Phillips, 1984). In this paper we describe the development of the time-domain method of data acquisition and processing.

The combination of lifetime and FRET (FLIM-FRET) provides high spatial (nanometer) and temporal (nanosecond) resolution (Baekskai et al., 2003; Elango-van et al., 2002; Krishnan et al., 2003). The presence of acceptor molecules within the local environment of the donor that permit energy transfer will influence the fluorescence lifetime of the donor. By measuring the donor lifetime in the presence and absence of the acceptor, one can accurately calculate the distance between the donor- and acceptor-labeled proteins. While 1p-FRET produces “apparent” E%, that is, efficiency calculated on the basis of all donors (FRET and non-FRET), the double-label lifetime data in 2p-FLIM-FRET usually exhibit two peaks of donor lifetimes (FRET and non-FRET), allowing a more precise estimate of distance based on FRET donors only (see Figs. 6, 7). The former may be sufficiently accurate for many situations, and the latter may be vital for establishing comparative distances of several proteins from a protein of interest.

The energy transfer efficiency (E), the rate of energy transfer ($k_T$), and the distance between donor and acceptor molecule ($r$) are calculated using the following equations (Lakowicz, 1999):

$$E = 1 - \left(\frac{\tau_{DA}}{\tau_D}\right)$$ (9)

$$k_T = \frac{1}{\tau_D}(R_0/r)^6$$ (10)

$$r = R_0\left(\frac{1}{E} - 1\right)^{1/6}$$ (11)

$$R_0 = 0.211\left(\frac{x^2 - 4Q_DJ(\lambda)}{6}\right)^{1/6}$$ (12)

where $\tau_D$ and $\tau_{DA}$ are the donor excited state lifetime in the absence and presence of the acceptor; $R_0$ is the Förster distance—that is, the distance between the donor and the acceptor at which half the excitation energy of the donor is transferred to the acceptor while the other half is dissipated by all other processes, including light emission; $n$ is the refractive index; $Q_D$ is the quantum yield of the donor; and $x^2$ is a factor describing the relative dipole orientation (normally assumed to be 2/3; Lakowicz, 1999).

**FRET cellular assay**

*Transcription factor C/EBPα: FRET microscopy* was used to characterize intra-nuclear dimer formation for the transcription factor C/EBPα in living pituitary GHFT1-5 cells. Members of the C/EBP family of transcription factors are critical determinants of cell differentiation. C/EBPα controls the transcription of genes involved in energy, including those encoding anterior pituitary growth hormone (GH) and prolactin (PRL) (Jacob and Stanley, 1999). C/EBPα is a basic region-leucine zipper (α-zip) transcription factor that forms dimers through contacts in the leucine zipper and binds to specific DNA elements via the basic region. Day et al. recently showed that GFP-tagged C/EBPα expressed in mouse pituitary GHFT1-5 cells was localized to sub-nuclear sites associated with pericentromeric heterochromatin (Day et al., 2001), and this pattern was identical to that for the endogenous protein in differentiated mouse adipocytes (Tang and Lane, 1999). Studies indicate that the b-zip region of C/EBPα (amino acids 244–358) fused to GFP was sufficient for
sub-nuclear targeting of the fusion protein in pituitary GHFT1-5 cells (Day et al., 2001). Because this region contains the dimerization domain, we sought to determine whether the expressed fusion proteins were associated as dimers in these sub-nuclear sites.

**Tissue FRET pair preparation**

**Animal surgery:** Adult male Sprague-Dawley rats were subjected to an impact acceleration injury. Specifically, rats weighing between 350 and 400 g received induction anesthesia, were endotracheally intubated, and maintained on a modified medical anesthesia machine. A 3 cm midline incision in the scalp was then made, and periostial membranes were separated, exposing bregma and lambda. A metal disc 10 mm in diameter and 3 mm thick was attached to the skull with cyanoacrylate and centered between bregma and lambda. The animal was then placed prone on a foam bed with the metal disk directly under a plexiglass tube. A 450 g brass weight was dropped through the tube from a height of 2 meters striking the disk. Sham surgery animals underwent an identical procedure with the exception of the weight striking the metal disk. Six hours following injury, or 1 hr following sham injury, animals were perfused transcardially, and the brain was removed. The brainstem, including the corticospinal tracts and the medial lemnisci, was then sagittally cut on a vibratome into 40, 100, or 200 micron thick sections.

All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Virginia, and were performed according to the principles of the Guide for the Care and Use of Laboratory Animals, published by the Institute of Laboratory Resources, National Research Council (NIH publication 85-23-2985).

**Immunohistochemistry:** The tissue sections were incubated in polyclonal antibody raised in rabbit against Bcl-xL amino acids 126–188 (sc-7195; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 in 1% normal goat serum (NGS) in PBS overnight at 4°C. Following incubation in primary antibody, the tissue was washed three times in 1% NGS in PBS, and then incubated in a secondary anti-rabbit IgG antibody conjugated with Alexa555 fluorochrome (Molecular Probes, Eugene, OR) for 2 hr. The tissue was washed three times in 1% NGS in PBS, and preincubated in 10% NGS for 40 min. The tissue was then incubated in the second primary antibody, monoclonal anti-BAD amino acids 1–168 raised in mouse (sc-8044; Santa Cruz Biotechnology), at a dilution of 1:50 in 1% NGS in PBS overnight at 4°C. The tissue was again washed three times in the 1% NGS solution, and incubated in a secondary anti-mouse IgG antibody conjugated with Alexa488 (Molecular Probes) for 2 hr. The tissue underwent a final wash in 0.1 M phosphate buffer and was then mounted using an antifade agent (Molecular Probes) and coverslipped.

**Biological processes used for different FRET microscopy approaches**

There is great interest in studying the dynamics of protein molecules under physiological conditions because protein-protein interactions mediate many cellular processes. Identification of the interacting protein partners is critical in understanding its function and place in the biochemical pathway, thereby establishing its role in important disease processes. The microscopy techniques described above allow the study of proteins in multiple ways, including what proteins are expressed, where they are expressed, and where they move over time.

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. FRET microscopy has the 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 (~ 0.2 μm) of the light microscope. Thus, within a voxel of microscopic resolution, FRET resolves average donor-acceptor distances beyond the microscopic limit down to the molecular scale (0.001–0.01 μm). This is one of the principal and unique benefits of FRET microscopic imaging: not only colocalization of the donor- and acceptor-labeled probes can be seen, but intimate interactions of molecules labeled with donor and acceptor can be demonstrated.

**Positive and negative control for FRET:** Two important aspects have to be observed in order to confirm the occurrence of FRET in protein-protein interactions in any biological process: (1) removal of the optical, electrical noises and the spectral bleed-through, and (2) demonstration of positive and negative controls. In the data presented here, for a positive FRET control, images of cells expressing CFP coupled directly to YFP through a 15 amino acid linker (CFP-15aa-YFP) were used. Using three coverslips (donor [D] and acceptor [A] alone expressed cells; and D + A, expressed cells), seven images were acquired using the W-FRET microscopy system and processed as described in the literature (Day, 1998; Periasamy and Day, 1999; Elangovan et al., 2003). The filter configuration for various FRET pairs has been listed in the literature (Periasamy, 2001; Sekar and Periasamy, 2003; www.kcci.virginia.edu; www.chroma.com; www.omegafilters.com; each accessed November, 2003). As shown in Fig. 2, the contamination was removed and the PFRET signal was used to calculate the efficiency and the distance between the donor and acceptor molecule. The estimated average distance separating the linked CFP and YFP deter-
The shortest distance from the fluorophore to the outside of the fluorescent protein (FP) β-barrel is 11 Å (Rekas et al., 2002), which limits the minimum distance that can separate the donor and acceptor fluorophores to ~22 Å. The 15 amino acid linker separating the FPs would have a maximum extended chain length of ~50 Å, but flexibility in its conformation should allow a range of distances separating the fluorophores at any given time. When expressed in the living cell, it is difficult to predict the conformation that this fusion protein would adopt, but the FRET results provide an estimate of the spatial relationship between the linked FPs. For negative control, seven images were collected as described above. In this case, in cells co-expressing the unlinked CFP- and YFP-tagged 4-hep C/EBPα.
which were co-localized but did not interact, no FRET signal (or negligible) was observed, as demonstrated in the histogram (see Fig. 3). As seen elsewhere in dynamic biological processes, one observes a heterogeneous distribution of efficiency and distances, also demonstrated in data presented in this paper.

The W-FRET system is simple and widely used, but images acquired using this system contain out-of-focus information, which can be removed by digital deconvolution (Periasamy and Day, 1999). In contrast, C-FRET and 2p-FRET provide very sharp images of protein localization because these configurations reject out-of-focus information. In addition, two-photon microscopy offers advantages over one-photon by causing less autofluorescence and photobleaching, and being able to excite any fluorophore using infrared excitation wavelengths from 700 to 1000 nm. Moreover, 2p-FRET microscopy is a very effective system for tissue FRET imaging (Mills et al., 2003).

**Tissue FRET in traumatic axonal injury:** Based on experiments in other model systems, we hypothesized that following traumatic axonal injury, increased levels of intra-cellular Ca\(^{2+}\) should activate calcineurin, resulting in the dephosphorylation and translocation of BAD to the mitochondria, where it binds to and suppresses the anti-apoptotic protein Bcl-xL, leading to cytochrome c-dependent caspase activation and apoptosis (Wang et al., 1999). According to this hypothesis, prior to injury, BAD (donor) and Bcl-xL (acceptor) should remain separate, the distance between them should be greater than 100 Å, and no FRET signal should be detected. Conversely, following injury, if there exists heterodimerization between BAD and Bcl-xL, the distance between them should be less than 100 Å, resulting in FRET signal detection.

For tissue FRET, we performed a series of preliminary immunohistochemical dilution and control experiments to determine the appropriate working antibody concentrations and to verify the absence of nonspecific or cross-reactive interactions (Mills et al., 2003). Using serial sections of tissue, we simultaneously labeled injured tissue with donor only (BAD/Alexa488), acceptor only (Bcl-xL/Alexa555), and with both donor and acceptor. The specimens were then examined for axons demonstrating vacuolization or formation of retraction bulbs, morphological characteristics of axonal injury. Implementing the C-FRET and 2p-FRET methodologies as described above in the FRET data analysis section, seven images were acquired and processed (Elangovan et al., 2003; Mills et al., 2003). In tissue processed from animals receiving a sham injury, we observed axons with normal morphologies and ubiquitous labeling with both BAD and Bcl-xL, with no FRET (not shown). Six hours post-injury,
swollen injured axons continued to be ubiquitously labeled for both BAD and Bcl-xL; however, these axons demonstrated energy transfer efficiencies approaching 30%. Comparing the PFRET images based on C-FRET and 2p-FRET shows that the energy transfer efficiency was considerably higher in the latter, demonstrating the advantage of the more sensitive two-photon technique for this particular application of localizing protein heterodimerization in tissue up to 200 microns thick (see Fig. 4). Additionally, the 2p-FRET image has less background compared to the C-FRET image (see Fig. 4).

**Lifetime measurements of green fluorescent proteins (GFPs):** FLIM measurements are sensitive to competing environmental and competing physical processes, such as resonance energy transfer and quenching, which can alter the fluorescence lifetime; thus, measurements of fluorescence lifetimes provide a very accurate reflection of the probe's local environment (Ng et al., 1999; Elangovan et al., 2002; Chen and Periasamy, 2004). To measure the lifetime of a fluorophore molecule, we integrated the Becker & Hickl (Berlin, Germany; www.becker-hickl.de, accessed November, 2003) FLIM system with the Bio-Rad Radiance2100 confocal/multiphoton microscopy (www.cellscience.bio-rad.com, accessed November, 2003) system (Chen and Periasamy, 2004). A Coherent pulsed Ti:sapphire laser was used as an excitation source (Santa Clara, CA; www.coherent.com, accessed November, 2003). The lifetime values were determined at each pixel of the intensity image by the nonlinear least square curve-fitting method (Eliceiri et al., 2003; Chen and Periasamy, 2004). The lifetime values are calculated based on a single exponential decay analysis (for single labeled donor images) and double exponential decay analysis (for both donor- and acceptor-labeled cells). We used the system to measure the lifetime for various mutant forms of green fluorescent proteins (blue, cyan, yellow, and green) fused to C/EBPα protein and expressed in the GHFT1-5 cell nucleus. As shown in the Fig. 5, the lifetime was obtained using single exponential decay and is found to be different for different color variants as well as the lifetime distribution. The lifetime distribution is a heterogeneous phenomenon in a biological system due to various environmental variations. The lifetime is insensitive to photobleaching but is sensitive to environmental changes such as temperature, pH, calcium signaling, protein-protein interactions, and so on (Sanders et al., 1995; Periasamy et al., 1996; Periasamy, 2001; Chen and Periasamy, 2004), which is very useful to observe the effect of different experimental conditions.

**Comparison of C-FRET, 2p-FRET, and FLIM-FRET:** We describe here the basic methodology of extracting and interpreting data using different FRET microscopy techniques. As explained in the FRET data analysis section, seven images were acquired for C-FRET and 2p-FRET and processed to remove SBT. The resultant data are shown in Fig. 6A–6D. In the case of FLIM-FRET, the donor lifetimes were measured in the absence (Fig. 6E) and presence of acceptor molecules; if FRET occurs, the lifetime of the donor is expected to decrease in the presence of acceptors (Fig. 6F). Lifetime changes occur in the double-labeled CFP/CFP

![Fig. 4 Tissue FRET in traumatic axonal injury. Six hours post-injury; tissue labeled with BAD/Alexa488 (donor) and Bcl-xL/Alexa555 (acceptor) demonstrates energy transfer consistent with BAD-Bcl-xL heterodimerization. The same tissue was used for C-FRET and 2p-FRET imaging using Bio-Rad Radiance2100 confocal/multiphoton microscopy. A confocal PFRET image was obtained at a 40 micron depth and we were unable to achieve decent signal beyond that depth. On the other hand, we obtained two-photon (or multiphoton) PFRET images up to 200 microns in depth. As shown in the figure, the signal appears to be less when we moved from 40 to 200 microns. This can be attributed to the concentration of the fluorophore deep inside the tissue and also to the visible FRET signal lost in the tissue before reaching the detector. The scale bar represents 10 μm; 20× MIMM NA 0.75; confocal-ExD 488, EmD 528/30, ExA 543, EmA 590/70; two-photon-ExD 790, ExA 730.](image-url)
YFP-tagged C/EBPα as the labeled molecule dimerizes and FRET takes place. The lifetime data of the donor in the presence and absence of acceptor are crucial for the efficiency and distance calculation, based on Equations 9 and 11, respectively.

As shown in the table in Fig. 6, the efficiency (E) and distance (r) between the donor and acceptor molecules are calculated using the respective protein complex in C-FRET and 2p-FRET (Fig. 6C), and mean values were estimated for about eight protein complexes. We would expect the same r values in both cases, but the 2p-FRET values were higher than those of C-FRET, which we attribute to the difference in signal acquired from the 2p system. The lifetime distribution is different for different cellular environments and in this case most of the molecules are quenched (Fig. 6F), as shown in a single peak (compared to Fig. 7), but less than the donor lifetime in the absence of acceptor. The FLIM-FRET methodology provides an accurate measurement of distance between interacting protein molecules. The quenched lifetime in Fig. 6F is in the range of 0.7–2.2 ns. The efficiency (E) distribution is in the range of 15%–73%, and the distance between donor and acceptor molecules is in the range of 44.58–70.55 Å. A single donor and acceptor molecule can exist at different proximities, which can be detected or calculated using FLIM-FRET techniques. Moreover, it helps to identify multiple molecular interactions because we are following the fingerprint of each molecular native fluorescent lifetime change. Multiple protein interactions or distance distribution cannot be measured with C-FRET and 2p-FRET microscopy.

**Acceptor-photobleaching FLIM-FRET microscopy:** As described earlier, FLIM-FRET microscopy was used to characterize intra-nuclear dimer formation for the transcription factor C/EBPα in living pituitary GHFT1-5 cells. As shown in Fig. 7, the dimerization reduces donor lifetime at the occurrence of FRET when energy transfer quenches the donor, resulting in different lifetime distributions compared with non-FRET/unquenched donors. Non-FRET/unquenched donors in the nucleus are those that did not dimerize as shown by the distribution in Fig. 7D. The first peak is the quenched donor molecule ($\tau_1 = 1.7$ ns), and the second peak represents the donor molecule ($\tau_2 = 2.4$ ns), which did not participate in the energy transfer process. As mentioned, in the intensity-based method, we speak of apparent energy transfer efficiency as that calculation is based on all donor molecules, including those that do not participate in FRET. We have also demonstrated that when we bleach the acceptor molecule (using 514 nm), the quenched molecular peak disappears and all that is left is the lifetime distribution of the unquenched molecule alone (Fig. 7E; $\tau = 2.5$ ns). This clearly demonstrates the occurrence of FRET. In FLIM, we can separate the FRET and non-FRET donors on the basis of lifetime distributions, and we believe that this is a more realistic measurement. We have experienced experimental conditions when only one peak appears in the double-labeled specimen, suggesting that all or the majority of donors have actually participated in energy transfer (Fig. 6F).

**Estimation of number of molecules involved in the energy transfer process:** As shown in Figs 6E, 6F, and 7, at the occurrence of FRET, energy transfer takes place and results in extreme quenching of donor
fluorescence and decrease of fluorescence lifetime. Thus, with the measured values of donor lifetime in the presence and absence of acceptor, the concentration of the acceptor species can be determined. Moreover, the fluorescence decay function contains the fluorescence of quenched and unquenched donor molecules at a pixel, and is therefore double exponential (Fig. 8; graph is from the Becker-Hickl processing software). For example, if the lifetime of the donor molecule at a single pixel in the absence and presence of acceptor is $t_2 = \tau_2 = 2.359$ ns and $t_1 = \tau_1 = 0.715$ ns, respectively, the estimated decay component values are 32.1% ($a_1$) and 67.9% ($a_2$), as shown in Fig. 8. It is clear that 32.1% of the total donor molecules are quenched at that particular pixel. The ratio of quenched to unquenched molecules is $a_1/a_2 = 32.1/67.9 = 0.472$. This confirms that quenching has occurred. In the unquenched condition, the ratio would be equal to 1.0. By this method, the distribution of quenched and unquenched molecules under any given experimental conditions becomes available. Thus, with the lifetime measurements, complete quantitative characterization of the molecular interactions can be made.

**Conclusion**

We have demonstrated the feasibility of various FRET microscopy techniques to characterize the dimerization of C/EBP$\alpha$ proteins in the GHFT1-5 cell nucleus and detecting FRET signals deep inside tissue. These methodologies can be used in any biological system with various FRET-pair combinations. A particular technique should be chosen depending on the experi-

<table>
<thead>
<tr>
<th>Protein Complex</th>
<th>Confocal</th>
<th>2Photon</th>
<th>2P-Lifetime</th>
</tr>
</thead>
<tbody>
<tr>
<td>E (%)</td>
<td>r (Å)</td>
<td>E (%)</td>
<td>r (Å)</td>
</tr>
<tr>
<td>Mean</td>
<td>24.7</td>
<td>63.59</td>
<td>15.08</td>
</tr>
<tr>
<td>± 1.95</td>
<td>± 1.15</td>
<td>± 0.53</td>
<td>± 0.49</td>
</tr>
</tbody>
</table>

Fig. 6 Comparison of C-FRET, 2p-FRET, and FLIM-FRET. C-FRET and 2p-FRET images of the quenched donor (A, C) and PFRET images (B, D) are shown. The respective efficiency (E) and distance (r) are shown in the table below the figure. The distance between donor and acceptor molecules appears to be higher for 2p-FRET compared to C-FRET. This may be due to the difference in methodology of acquisition of photons. Both C-FRET and 2p-FRET signals were collected in the same cell and optics using the Bio-Rad Radiance2100 confocal/multiphoton microscopy system. For the same cell, the donor lifetime images were acquired in the absence (E) and presence (F) of acceptor. As stated in the text, the natural lifetime of the donor (2.62 ns) was reduced to 1.9 ns (mean value) due to FRET. Lifetime measurements are the accurate values of the distance distribution of the dimerization of C/EBP$\alpha$444 protein molecules in the mouse pituitary GHFT1-5 cell nucleus. The distribution values are provided in the table below the figure.
mental conditions. It is important to note that irrespective of the selection of techniques, the FRET signal is always contaminated, which should be corrected with appropriate software. The alternative is FLIM-FRET microscopy. Even though this technique is somewhat more complex, it provides an unprecedented level of information about the protein molecular associations under physiological conditions at a very high temporal and spatial resolution. Moreover, the complimentary techniques of fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS; time and image, not described here) provide information about the mobility of the protein molecules and confirm the occurrence of FRET (Wiseman and Petersen, 1999; Lippincott-Schwartz et al., 2001).

Fig. 7 Acceptor-photobleaching FLIM-FRET microscopy. Acceptor-photobleaching method to demonstrate the occurrence of FRET (dimerization of CFP-YFP-C/EBP244 protein molecules in the mouse pituitary GHFT1-5 cell nucleus). Donor lifetime image in the presence (A, D) and absence (B, E) of acceptor, and (D) and (E) are the respective lifetime distributions, (C) Lifetime decays at the same pixel before ($\tau_1 = 1.7$ ns) and after ($\tau = 2.5$ ns) bleaching at 514 nm (100%; 7 min). Nikon objective lens 60 x 1.4 NA1.4 was used, laser average power at Ex 820 nm was 5 mW, and the acquisition time was 35 s. $\chi^2 = 1.4$.

Fig. 8 Estimation of energy transferred between molecules. Graphical demonstration of the existence of two components of decay ($t_1 = \tau_1$ and $t_2 = \tau_2$) for the donor molecule in the presence of acceptor at a pixel. The $a_1$ and $a_2$ represent the quenching ($a_1$) during FRET and non-quenching ($a_2$) molecules. These lifetime measurements allow determination of the interacting and non-interacting protein molecules in a single living cell.
Acknowledgments We wish to thank Dr. Richard Day for providing cells and for his valuable discussion. We would like to thank Mr. Horst Wallrabe for the help and support provided in preparation of this manuscript. We appreciate Drs. Ty Voss, James Stone, David Okonkwo, and Gregory Helm for discussion. We acknowledge the funds supported by the University of Virginia and Virginia Commonwealth Neurotrauma Initiative.

References


