Characterization of Two-photon Excitation Fluorescence Lifetime Imaging Microscopy for Protein Localization

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ABSTRACT Two-photon excitation fluorescence resonance energy transfer (2P-FRET) imaging microscopy can provide details of specific protein molecule interactions inside living cells. Fluorophore molecules used for 2P-FRET imaging have characteristic absorption and emission spectra that introduce spectral cross-talk (bleed-through) in the FRET signal that should be removed in the 2P-FRET images, to establish that FRET has actually occurred and to have a basis for distance estimations. These contaminations in the FRET signal can be corrected using a mathematical algorithm to extract the true FRET signal. Another approach is 2P-FRET fluorescence lifetime imaging (FLIM). This methodology allows studying the dynamic behavior of protein-protein interactions in living cells and tissues. 2P-FRET-FLIM was used to study the dimerization of the CAATT/enhancer binding protein α (C/EBPα). Results show that the reduction in donor lifetime in the presence of acceptor reveals the dimerization of the protein molecules and also determines more precisely the distance between the donor and acceptor. We describe the development and characterization of the 2P-FRET-FLIM imaging system with the Bio-Rad Radiance2100 confocal/multiphoton microscopy system. *Microsc. Res. Tech. 63:72–80, 2004.*

INTRODUCTION

Developments in fluorescent dyes that display the chemical and molecular dynamics of intact cells have significantly broadened the usage and applications of optical microscopy. These developments allow real-time observation and measurement of the activities of dye-marked constituents in cells and tissues. All light microscopy techniques can be used to view these dye-marked constituents and processes. However, all digitized fluorescence microscopy imaging techniques including the wide-field, confocal, and two-photon microscopy provides clear details of the molecules in 3 and 4 dimensions (Diaspro, 2002; Pawley, 1995; Periasamy, 2001).

Advancements in high-speed lasers and the development of high-speed, high sensitivity detection devices and image-processing techniques have aided the development of fluorescence lifetime imaging (FLIM) microscopy (Dowling et al., 1998; Gadella et al., 1993; Lakowicz and Berndt, 1991; Ng et al., 1999; Straub and Hell, 1998). The FLIM technique measures the nanosecond duration of the excited state of fluorophores within the living cell (Periasamy et al., 1996). The fluorescence lifetime of a fluorophore is critically dependent upon the local environment that surrounds the probe. Because biological interactions occur over a similar time-scale, monitoring the localized changes in probe fluorescence lifetime provides an enormous advantage for imaging dynamic cellular events (Herman et al., 1997). An important advantage of these lifetime measurements is that they are independent of change in probe concentration, photobleaching and other factors that limit intensity-based steady-state measurements (Herman et al., 1997; Lakowicz, 1999). When combined with FRET, this approach can provide direct evidence for the physical interactions between proteins, representing one type of change in the environment, with very high temporal resolution, and details of dynamic protein interactions in 2- to 4-dimensions (Elangovan et al., 2003; Periasamy, 2001; Verveer et al., 2001). Importantly, because only one protein partner, the donor, is monitored, it is unnecessary to use spectral bleed-through correction in FRET-FLIM images.

In this report, we describe the development and characterization of a two-photon excitation FRET-FLIM (2P-FRET-FLIM) microscopy system that uses a time-correlated single photon counting imaging mode hardware from Becker-Hickl, Bio-Rad Radiance 2100 confocal/multiphoton microscopy, and the high-speed Coherent ti:sapphire laser systems. We implemented the 2P-FRET-FLIM technologies to visualize the distribution of localization of interactions of the transcription factor CAATT/enhancer binding protein alpha (C/EBPα) in living pituitary cells.

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MATERIALS AND METHODS

Green Fluorescent Proteins (GFPs)

The increasing interest in fluorescence microscopy has been driven by the availability of the different mutant form of green fluorescent proteins (Cubitt et al., 1999; Ellenberg et al., 1998; Heim and Tsien, 1996; Sullivan and Kay, 1999). Mutagenesis of the *Aequoria vicotoria* green fluorescent protein (GFP) has yielded proteins that fluoresce from blue to red; some of these expressed protein tags have proven to be suitable as donor and acceptor pairs for FRET microscopy (Periasamy et al., 2001). A cyan (blue-green) color variant is resistant to photobleaching and shares an extensive spectral overlap with the yellowish fluorescent protein (YFP), allowing this combination to be used in FRET-FLIM studies (Bacskai et al., 2003; Elangovan et al., 2002).

For the studies described here, the sequence encoding the DNA binding and dimerization domain of the transcription factor C/EBPα was fused (or unfused) in-frame to the commercially available CFP or YFP or GFP color variants (www.clontech.com) to generate CFP-C/EBP Δ154 and YFP- C/EBP Δ154 (Day et al., 2003). For transfections, mouse pituitary GHFT1-5 cells were harvested and transfected with the indicated plasmid DNA(s) by electroporation (Day, 1998; Schaufele et al., 2001). The total input DNA was kept constant using empty vector DNA. Cell extracts from transfected cells were analyzed by Western blot to verify that the tagged proteins were of the appropriate size as described previously (Day, 1998). For imaging, the cells were inoculated drop-wise onto a sterile cover glass in 35-mm culture dishes, allowed to attach prior to gently flooding the culture dish with media, and maintained for 18 to 36 hours prior to imaging. The coverglass with cells attached was inserted into a chamber containing the appropriate medium and the chamber was then placed on the microscope stage.

Two-photon Excitation FRET-FLIM (2P-FRET-FLIM) Imaging Microscopy

FRET Microscopy

In the late 1940s, Förster proposed the theory of FRET, which described how energy could be transferred directly from a fluorophore in the excited state (the donor, D) to a non-identical acceptor (A) fluorophore (Förster, 1948, 1965; Lakowicz, 1999; Sekar and Periasamy, 2003; Stryer, 1978). The transfer of excited state energy occurs without the production of heat and does not require that a collision occur between D and A. The energy from the D molecule, without releasing a photon, can be transferred directly to A under the following four conditions: (1) the emission spectrum of D has to overlap the absorption spectrum of A by a considerable percentage (>30%); (2) the two fluorophores are within ~1 to ~10 nm of each other; (3) when the D emission dipole moment, the A absorption dipole moment and their separation vectors are in favorable mutual orientation; and (iv) when the emission of D has a reasonably high quantum yield.

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):

$$E = 1 - (\tau_{DA}/\tau_D) \quad (1)$$

$$k_T = (1/\tau_D)(R_0/r)^6 \quad (2)$$

$$r = R_0\{(1/E)-1\}^{1/6} \quad (3)$$

$$R_0 = 0.211\{\kappa^2 n^{-4} Q_D J(\lambda)\}^{1/6} \quad (4)$$

where τ_D and τ_{DA} is 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,κ^2 is a factor describing the relative dipole orientation (normally assumed to be 2/3; Lacowicz, 1999).

Two-photon Excitation Microscopy

In one-photon (wide-field or confocal) fluorescence microscopy, the absorption of laser energy excites the fluorescent molecules to a higher energy level and results in the emission of one photon of fluorescence. The fluorescence intensity increases at a linear rate with the excitation intensity. Typically, some of the absorbed light energy is dissipated as heat, so the emission wavelength is longer than the absorption wavelength. For example, a fluorophore might absorb one-photon at 480 nm and fluoresce at a green wavelength around 530 nm. Two-photon (2P) excitation occurs when two photons of $h\omega$ and $h\omega'$ are absorbed simultaneously and a molecule is excited to the state of energy $E = h\omega + h\omega'$. The probability that two-photon absorption will occur depends on the co-localization of two photons within the absorption cross section of the fluorophore. The rate of excitation is proportional to the square of the instantaneous intensity. This extremely high local instantaneous intensity is produced by the combination of diffraction-limited focusing of a single laser beam in the specimen plane and the temporal concentration of a femtosecond (fsec) mode-locked laser (typically of the order of 10^{-50} to 10^{-49} cm^4 s/photon/molecule) (Denk et al., 1995). Two-photon absorption was theoretically predicted by Göppert-Mayer in 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 a laser scanning confocal microscopy (Denk et al., 1990).

The fluorophores exhibit two-photon absorption at approximately twice (960 nm) their one-photon absorption wavelengths, while two-photon emission is the same as that of one-photon (535 nm), allowing the specimen to be imaged in the visible spectrum. When an infrared laser beam is focused on a specimen, it illuminates 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. In one-photon microscopy, illumination occurs throughout the excitation beam path, in an hourglass-shaped path. This results in absorption along the excitation beam path, giving rise to substantial fluorescence emission both below and

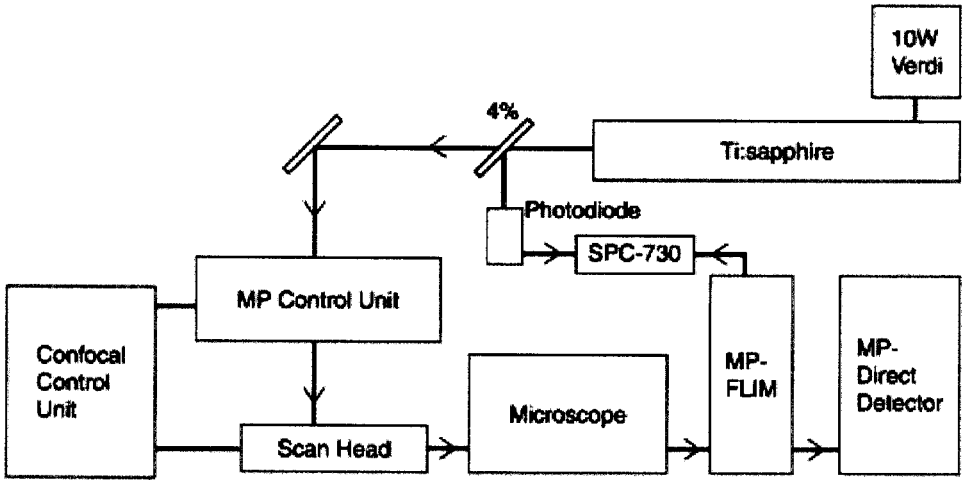

Fig. 1. Schematic illustration of two-photon excitation FRET-FLIM microscopy (also see Fig. 2).

above the focal plane. Excitation from other focal planes contributes to photobleaching and photodamage in the specimen planes that are not being involved in imaging. 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 excitation microscopy has been widely used in the area of biomedical sciences including tissue engineering, protein-protein interactions, cell, neuron, molecular, and developmental biology (Diaspro, 2001, 2002; Periasamy et al., 1999; Periasamy, 2001; Samkoe and Cramb, 2003; Soeller et al., 2003). Since pulsed lasers are used as an excitation source, this configuration is an ideal system for fluorescence lifetime imaging (FLIM). The FLIM system is not only used for protein-protein interactions but also for various biological applications from single cells to single molecules as well as deep tissue cellular imaging (Bastiaens and Squire, 1999; Clegg et al., 1994; Elangovan et al., 2002; Gerritsen and de Grauw, 2001; Gratton et al., 2003; Krishnan et al., 2003; Periasamy, 2001).

Fluorescence Lifetime Imaging (FLIM) Microscopy

The fluorescence lifetime is the average time that a molecule remains in an excited state prior to returning to the ground state. For single exponential decay of fluorescence, the fluorescence intensity as a function of time after a brief pulse of excitation light is described as (Lakowicz, 1999)

$$I(t) = I_0 \exp(-t/\tau) \quad (5)$$

where I_0 is the initial intensity immediately after the excitation pulse; thus, the time in which the fluorescence intensity decays to 1/e of the intensity immediately following excitation is the lifetime (τ). In practice, fluorescence decay is often multiexponential, leading to complex decay curves. The average time that a fluorophore spends in an excited state is typically less than 100 nanoseconds, and the duration of the excited state, i.e., its fluorescence lifetime, is critically dependent upon the local environment surrounding the probe.

Conventional fluorescence microscopy gives measurements of intensity that provide images to reveal primarily the distribution and amount of stain in the cell. In contrast, the fluorescence lifetime microscopic technique allows the measurement of dynamic events at very high temporal resolution and can monitor interactions between cellular components at very high spatial resolution as well. Because biological interactions occur over a similar time-scale, monitoring the localized changes in probe fluorescence lifetime provides an enormous advantage (Clegg et al., 1994; Elangovan et al., 2002; Gadella et al., 1993; Krishanan et al., 2003; Periasamy et al., 1996). For example, proteins labeled with fluorophores may exist in environmentally distinct regions of a cell that all have similar fluorescence intensity distribution, but may exhibit regional differences in the fluorescence lifetimes for the probe. The measurement of fluorescence intensity alone would not indicate these differences, but fluorescence lifetime imaging will reveal these different subcellular environments (Elangovan et al., 2002).

Instrumentation

Microscope and Excitation Light Source. The system consists of a Nikon TE300 epifluorescent microscope with a 100W Hg Arc Lamp. A Plan Fluor 60× NA 1.4 oil IR objective lens was used for 2p-FRET and 2p-FRET-FLIM image acquisition. TE300 was coupled to Bio-Rad Radiance2100 confocal/multiphoton system (www.cellscience.bio-rad.com). A 10W Verdi pumped, tunable (model 900 Mira, www.coherent.com) mode-locked ultrafast (78 MHz) pulsed (<150 femtosecond) laser was coupled to the laser port of a Radiance2100 (see Fig. 1). This laser is equipped with x-wave optics for easy tunable range of the entire wavelength (700 to 1,000 nm). The system was equipped with laser spectrum analyzer (Model E201; www.istcorp.com) to monitor the excitation wavelength and power meter to measure the laser power at the specimen plane (Model SSIM-VIS & IR; www.coherent.com). The Radiance system was equipped with an external detector and

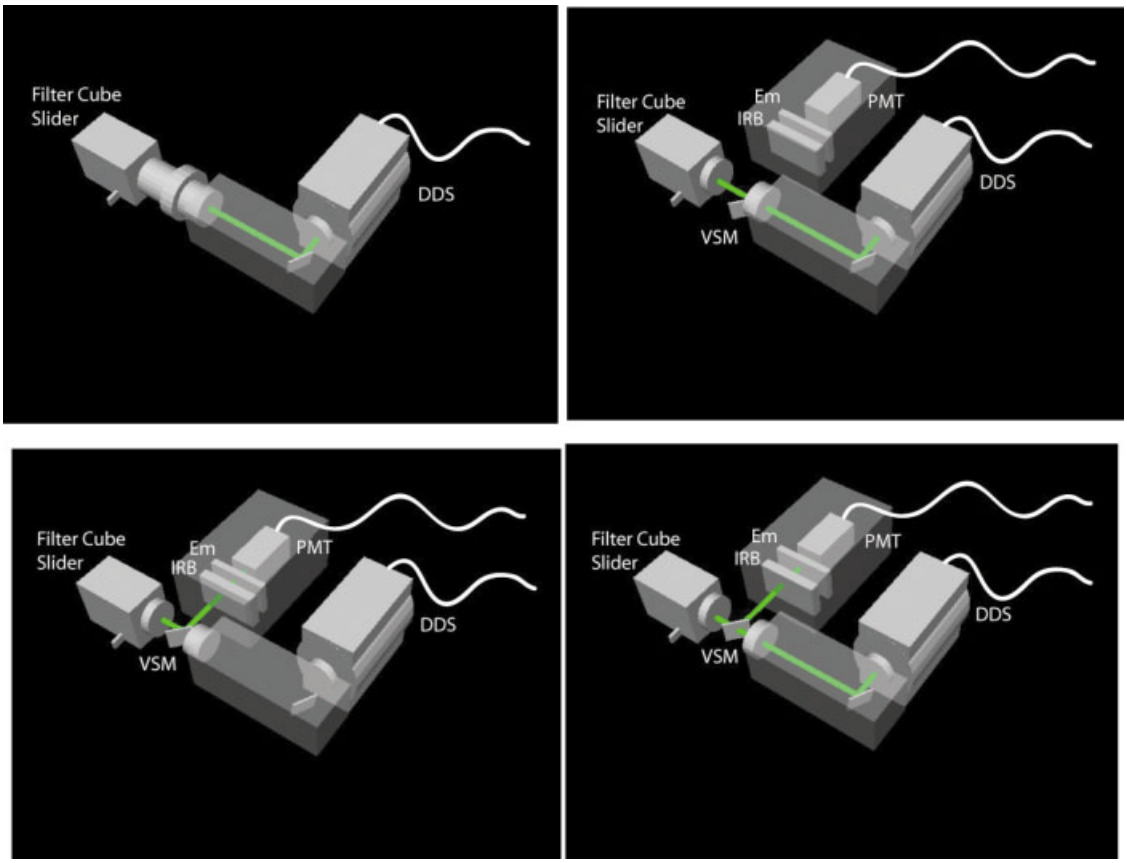

Fig. 2. Optical path configuration of 2P-FRET-FLIM microscopy. Integration of the FLIM hardware in the Bio-Rad Radiance2100 confocal/multiphoton microscopy. Emission signal from the specimen is directed either to the direct detector (DDS) or to the FLIM detector (photomultiplier tube, PMT) or both using a flip mirror (VSM) or a beam splitter. Two six-position filter wheels are used between the flip mirror and the PMT for appropriate selection of the emission filter (Em) and for the excitation infrared light-blocking filter (IRB). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

four internal detectors for fluorescence imaging. The transmission detector was used for transmission imaging and also for Second Harmonic Generation (SHG) Imaging. LaserSharp2000 software was used to acquire both 2p-FRET and FLIM images using the internal or direct (external) detectors.

The Becker & Hickl GmbH, (http://www.becker-hickl.de) company photon-counting module (TCSPSC, SPC-730), is widely used to acquire FLIM images for various biological applications (Bacskai et al., 2003; Eliceiri et al., 2003; König and Riemann, 2003). This board was installed in the Radiance2100 computer and the X and Y scan synchronizing pulses, together with a pixel clock signal from Radiance2100 control unit, are used to synchronize data collection in the SPC-730 board. This allows pixel-by-pixel registration of the accumulated photons with the laser scanning. Laser pulses are detected by a high-speed PIN photodiode and used by the SPC-730 board to determine the detection time of a photon (anode pulse from the PMT) relative to the laser pulses. This measurement system requires that the timer (a time-to-amplitude converter (TAC)) be activated only on receipt of a detected photon rather than at every laser pulse. The SPC-730 system starts timing at the receipt of a detected photon and measures the time interval until the next laser pulse. A fluorescence decay histogram of photon emission times relative to the laser excitation pulse is generated from the distribution of inter-pulse intervals at each pixel of the image. The detector is a fast photomultiplier tube, with a FWHM response time of about 150 ps (PMH-100, Becker & Hickl). This detector is fast enough to resolve lifetimes due to environmental changes in a biological system or protein-protein interactions. The data analysis software (SPCImage, Becker & Hickl) allows multi-exponential curve fitting of the acquired data on a pixel-by-pixel basis using a weighted least-squares numerical approach. The sum of all time bins is equivalent to the intensity image and this is displayed to an image, pseudo-colored according to the curve fit results. Therefore, each image can be easily displayed in a meaningful way to compare lifetimes within or between other images.

The FLIM PMT (model no. PMH-100) was coupled in the middle of the arm connected to the direct detector as shown in Figure 2. The coupler was removed in the arm and a flip mirror was inserted to direct the emission fluorescence signals from the specimen to the FLIM detector or to the direct detector. It is also possible to detect simultaneously both 2p-FRET and FLIM signal using this configuration. The six-position dual filter wheel is installed between the FLIM detector and

the flip mirror in order to select appropriate emission filter depending on the fluorophore used for protein molecular imaging. In another filter wheel, we used BG-36 glass filter, which blocks the excitation IR laser light and also transmits the visible spectrum, about 70% at 500 nm (www.chromatech.com). The whole system including the microscope was covered with a black box in order to reduce the background counts to as low a level as possible.

Acquisition of FLIM and FRET Images. Any fluorescence imaging requires careful selection of excitation power at the specimen plane in order to reduce photobleaching on the one hand, but on the other hand to obtain reasonable photon counts in the acquired data to obtain a good statistical fit. We found that 30 seconds is the ideal data collection time for our CFP expressed cells to obtain a lifetime of 2.4–2.6 ns. The same data acquisition time was used for all FLIM and FRET images. We used unfused cells to obtain multiphoton excitation intensity spectra for CFP, YFP, GFP, BFP, and dsRED within the ti:sapphire laser tuning range from 700–1,000 nm. We selected the peak excitation wavelength from these spectra to excite unfused cells of the above-mentioned GFPs for FLIM images and the lifetime numbers were measured using the lifetime data processing software (SPCImage; Becker & Hickl) at every pixel.

This FLIM imaging methodology acquired lifetime images of CFP-C/EBPΔ154 (donor) in the absence and presence of the acceptor YFP-C/EBPΔ154. Mouse pituitary GHFT1-5 cells expressing CFP-C/EBPΔ154 alone (i.e., donor alone) were identified using an arc lamp light source. As described above, the 820-nm laser line was used as an excitation wavelength to illuminate the cell and the SPC-730 board was used to acquire lifetime images using 480/30 nm emission filters. The FLIM PMT was synchronized (see explanation in the Instrumentation Section) to the excitation laser pulse. The accumulation time was 30 sec to obtain reasonable photon counts. The acquired image was processed using the SPCImage software to obtain a single exponential decay FRET-FLIM image (τ_D).

We next imaged cells expressing the double-label combination of CFP-C/EBPΔ154 and YFP-C/EBPΔ154 (i.e., donor in the presence of the acceptor). The lifetime images were acquired using the same excitation (820 nm laser line) and emission filter (480/30 nm) used for donor alone lifetime image acquisition. The double exponential fluorescence lifetimes were processed and calculated. Both sets of images (τ_D and τ_{DA1}, τ_{DA2} were processed for lifetime FRET images. All images were acquired at room temperature (74°F). We did not observe any detectable autofluorescence signals using the unlabeled cells in the same media used for FRET-FLIM imaging.

RESULTS AND DISCUSSION

We described the integration of the FLIM instrumentation with Bio-Rad Radiance2100 confocal/multiphoton microscopy to study the intranuclear dimer formation for the transcription factor C/EBPα in living pituitary cells. As mentioned in the literature, the intensity based or steady-state protein-protein interaction imaging introduces errors in estimating the distance between the donor and acceptor molecules. Moreover, the spectral bleed-through, or cross talk, is a problem to recognize whether one is observing true sensitized emission, the bleed-through signals, or a combination of both (Elangovan et al., 2003; Gordon et al., 1998; Mills et al., 2003; Wallrabe et al., 2003). The strength of the signal also depends on the excitation intensity and the fluorophore concentration. In contrast, 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 (Bastiaens and Squire, 1999; Elangovan et al., 2002).

Two-photon microscopy offers considerable advantages over one-photon because it causes less autofluorescence, photobleaching, and is able to excite any fluorophore using infrared excitation wavelengths from 700 to 1,000 nm. To establish the optimal wavelengths for different mutant forms of green fluorescent proteins (BFP, GFP, CFP, YFP, and dsRED), we used these in an unfused form, i.e., not inserted into a protein. For each color variants, the wavelength was tuned in 10-nm steps from 700–1,000 nm and the intensity was measured at the specimen plane and at the nosepiece of the microscope using a detector (Coherent, model no. LM-2). The measured intensity at different wavelength was normalized and plotted as shown in Figure 3. The peak wavelength for various color variants shown in Figure 3 was used to excite the C/EBPΔ154 proteins in the nucleus for lifetime measurements. Also, we measured the lifetime of these green fluorescent proteins as shown in Table 1.

We also characterized the 2P-FRET-FLIM systems with known lifetime specimens such as fluorescein, rhodamine, and cyan fluorescent protein. The measured lifetime values appear to be very close to the lifetime values shown in the literature (Elangovan et al., 2003; Lakowicz, 1999; Periasamy et al., 1996). As mentioned, one of the advantages of lifetime measurements is the independence of photobleaching, clearly demonstrated in Figure 4, where no change in the lifetime occurs. We used single-label cells expressing either CFP-C/EBPΔ154 or YFP-C/EBPΔ154 proteins and measured lifetimes by repeatedly collecting data at the appropriate excitation power at the specimen plane. We then photobleached the same cells at various periods of time and measured their lifetimes and observed no change in their lifetime values (see Fig. 4a), confirming their independence from photobleaching. The situation is quite different in the case of cells expressing both CFP-C/EBPΔ154 and YFP-C/EBPΔ154 protein molecules. In the presence of an acceptor, the lifetime of the CFP donor is shorter than that of a single-label donor on account of FRET, which represents an additional pathway for de-excitation. When in the same double-label cell the acceptor YFP-C/EBPΔ154 is selectively bleached—we use our one-photon confocal at 514 nm to achieve this—the CFP donor lifetime approaches that of the single-label donor as with the removal of the acceptor, no FRET takes place. This is clearly demonstrated in Figure 4b where the two peaks observed, one for the protein dimerization and another one for the protein molecules, were not involved in the dimerization process. A single peak was observed after acceptor photobleaching since the donor molecule does not have a partner to dimerize or

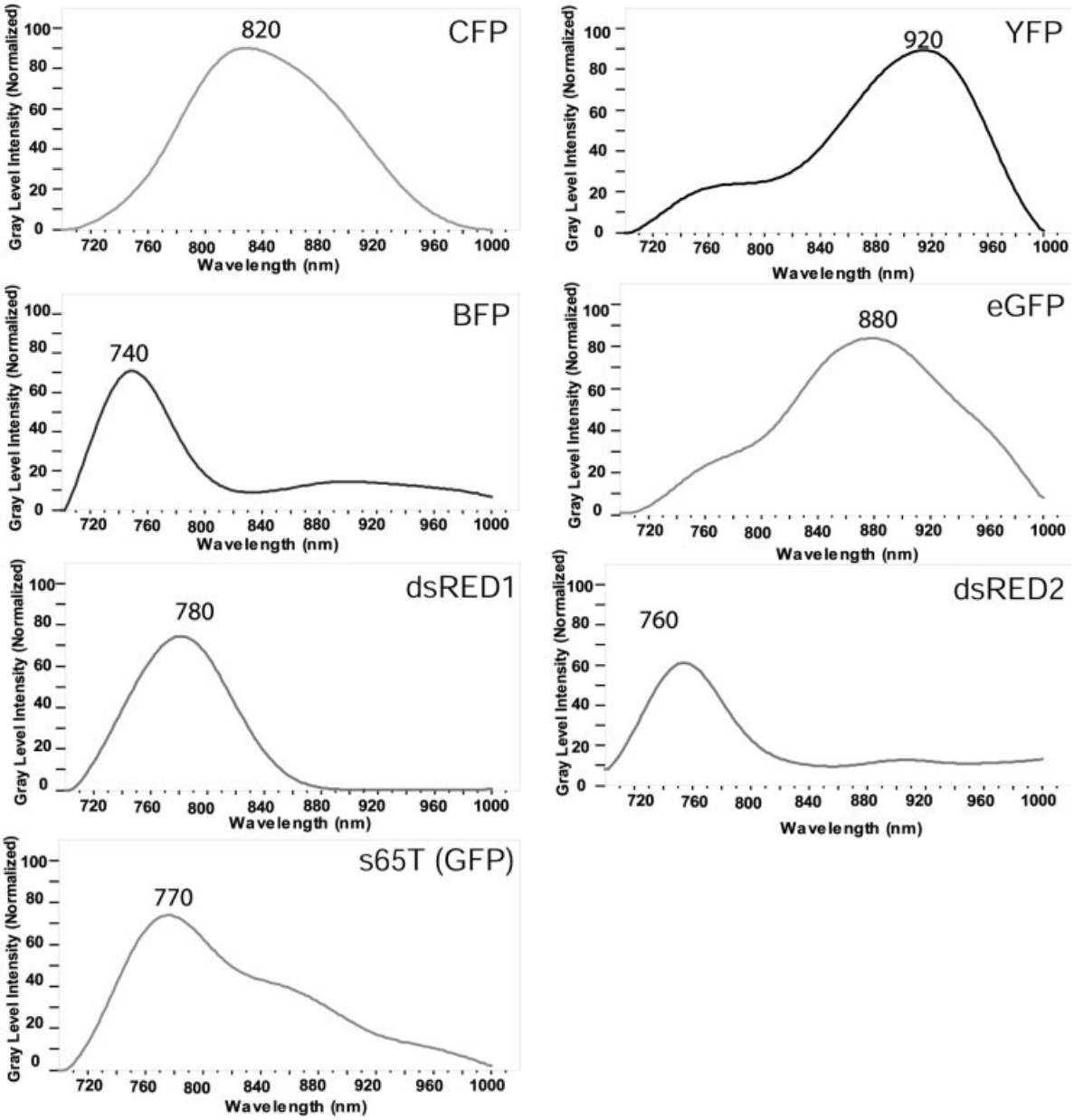

Fig. 3. Two-photon excitation spectra for cyan, yellow, blue, S65T, dsRED1, dsRED2, and eGreen fluorescent proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1. Measured Lifetime for blue, cyan, yellow, and green fluorescent proteins

Fluorophore	Lifetime (τ) ns
eBFP	1.69
eGFP	2.51
eCFP	2.62
eYFP	2.82

to satisfy the condition for FRET to occur. While under these experimental conditions photobleaching was used to demonstrate the influence of the FRET event on donor lifetimes and FRET's utility to measure molecule proximity, it is important to note that photobleaching should be minimized because of unknown impact on the cellular environment. This can be achieved by choosing appropriate excitation power levels. Nevertheless, some degree of photobleaching may be unavoidable depending on experimental conditions. Two-photon FLIM at least reassures that lifetimes remain unaffected.

Other factors can potentially affect microscopy-based data generation such as different immersion media or different objective lenses. In the case of lifetime measurements, none of these elements has any impact. We used a Plan Fluor Nikon 20× lens to image the CFP-

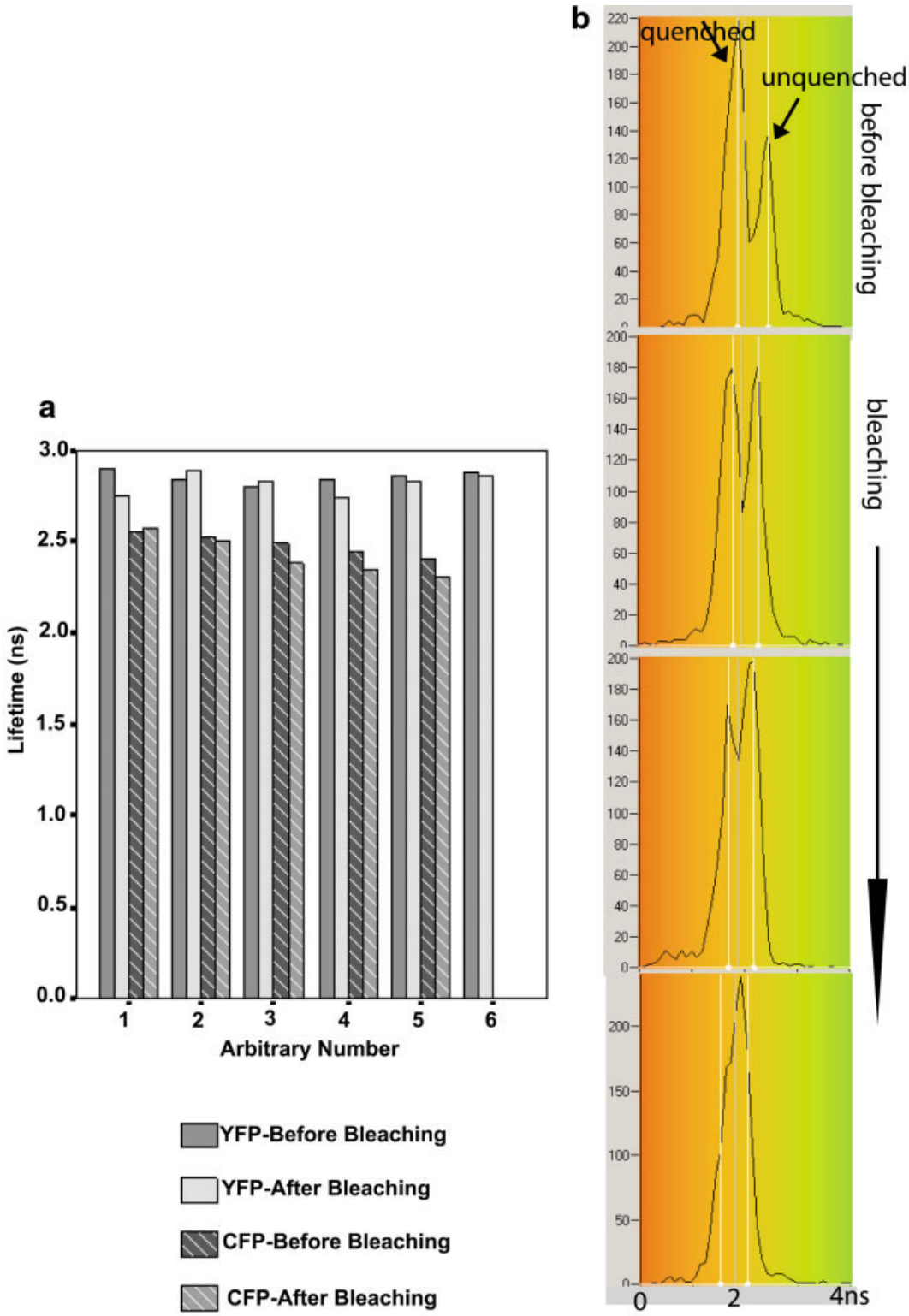

Fig. 4. Lifetime vs. photobleaching. **a:** CFP-C/EBPΔ154 and YFP-C/EBPΔ154 expressed in GHFT1-5 cells was bleached at different levels and then the lifetime data was acquired and processed. The lifetime changes are negligible with photobleaching of the fluorophore molecules. But the lifetime tends to goes down if its bleached more than 60% of the gray level intensity of the original image. This clearly demonstrates that the lifetime is not sensitive to photobleaching. **b:** CFP-YFP-C/EBPΔ154 expressed in GHFT1-5 cells was imaged using the lifetime instruments at various acceptor photobleaching levels. The lifetime distribution shows two peaks before bleaching the acceptor. The quenched donor peak started disappearing while bleaching the acceptor molecule. This clearly demonstrates that the donor lifetime changes due to the environmental changes (change in the acceptor level). Objective lens-60× oil IR; Average power was measured at the specimen plane. 820 nm = 1.9 mW, 920 nm = 2.9 mW. The laser power used for photobleaching CFP at 457 nm = 41 μW and for YFP at 514 nm = 100 μW. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

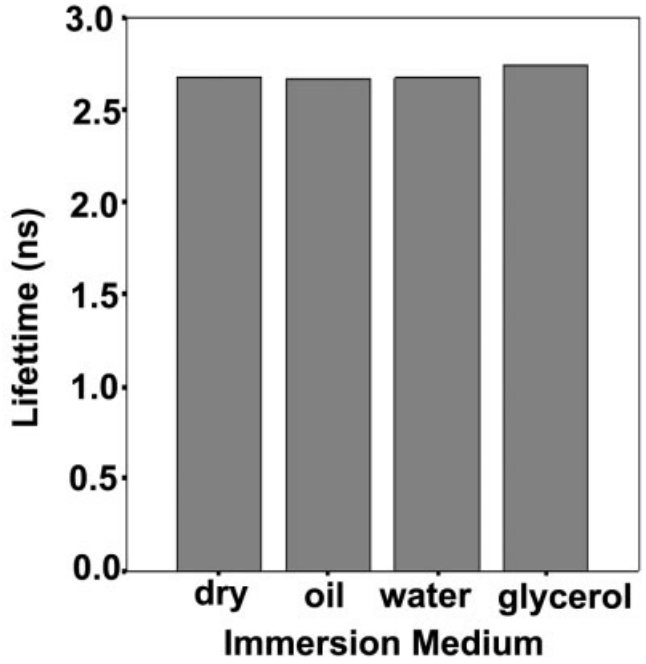

Fig. 5. Lifetime vs. immersion medium. Lifetime does not change due to the change in immersion medium.

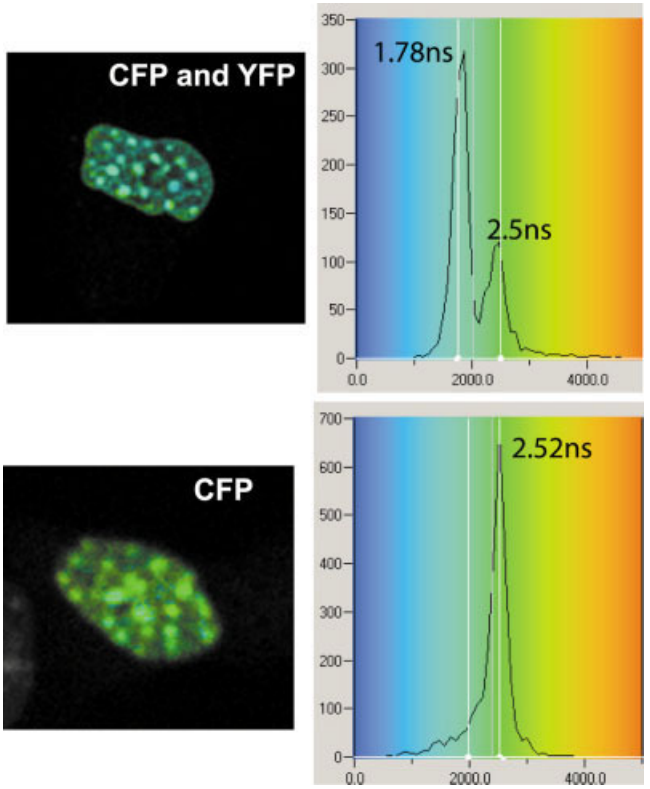

Fig. 6. Demonstration of lifetime distribution for the CFP-YFP-C/EBPΔ154 protein dimerization in GHFT1-5 cell nucleus. The mean lifetime of the donor in the absence of the acceptor was 2.52 ns and in the presence of acceptor were 1.78 ns. In a protein complex such as CFP-C/EBPΔ154, proteins have a different lifetime distribution as shown. In the presence of an acceptor, not all the proteins participate in the energy transfer process, shown as two peaks. One peak was quenched at 1.78 ns (τ_{DA1}) ns due to resonance energy transfer and the other one represents the unquenched (τ_{DA2} = 2.5 ns) protein molecules lifetime distribution. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

C/EBPΔ154 expressed in GHFT1-5 cells. We acquired the images in air, water, oil, and glycerol immersion medium. As shown in the Figure 5, the lifetime is comparable, clearly demonstrating that the optical environment does not influence the lifetime changes.

The above observations are important considerations when deciding to use the FLIM methodology for studying the dynamic behavior of the interacting protein molecules. Whether fused or unfused, single-label CFP, YFP, and GFP in GHFT1-5 cells show comparable mean lifetimes. Lifetime changes only occur when in the double-label CFP/YFP cell the labeled molecule dimerizes and FRET takes place. In this study, we used 2P-FRET-FLIM microscopy to characterize intranuclear 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) (Jacobs and Stanley, 1999). Day et al. (2003) indicated that the b-zip region of C/EBPα fused to GFP was sufficient for subnuclear targeting of the fusion protein in pituitary GHFT1-5 cells. Since this region contains the dimerization domain, we sought to determine whether the expressed fusion proteins were associated as dimers in this subnuclear sites 2P-FRET. As shown in Figure 6, 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 two peaks in Figure 6. The first peak is the quenched donor molecule (τ_{DA1} = 1.78 ns), the second peak represents the donor molecule (τ_{DA2} = 2.5 ns), which did not participate in the energy transfer process. The single labeled donor molecule CFP-C/EBPΔ154 lifetime was also measured (τ_D = 2.52 ns). We also calculated the energy transfer efficiency (E%) using two methods to estimate the distance between donor and acceptor for the same fluorophore pair (CFP/YFP): Intensity-based two-photon microscopy (r = 7.0 nm) and two-photon FLIM (r = 5.8 nm). It should be noted that for 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. In FLIM, we can separate the FRET and non-FRET donors on the basis of lifetime distributions and, therefore, believe that this is a more realistic measurement.

CONCLUSION

We have demonstrated the integration of the FLIM hardware with the existing laser scanning two-photon microscopy to measure the fluorescence lifetime for protein molecules in living cells. We have shown the advantages of FLIM in that lifetime measurements are independent of types of immersion media, different objective lenses, or different detectors and are not im-

pacted by sometimes unavoidable photobleaching. The 2P-FRET-FLIM imaging system has the added advantage of tracking dynamic protein-protein interactions and providing more precise determinations of the distance between donor and acceptor molecules.

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