

FRET IMAGING OF PIT-1 PROTEIN INTERACTIONS IN LIVING CELLS

Ammasi Periasamy[†] and Richard N. Day[‡]

[†]University of Virginia, Department of Biology, Center for Cellular Imaging, Gilmer Hall, Charlottesville, Virginia 22903; [‡]University of Virginia Health Sciences Center, Departments of Medicine and Cell Biology, National Science Foundation Center for Biological Timing, Charlottesville, Virginia 22908

(Paper JBO-158 received June 12, 1997; revised manuscript received Dec. 6, 1997; accepted for publication Dec. 9, 1997.)

ABSTRACT

The combined use of fluorescence resonance energy transfer (FRET) microscopy and expression of genetic vectors encoding protein fusions with green fluorescent protein (GFP) and blue fluorescent protein (BFP) provides an exceptionally sensitive method for detecting the interaction of protein partners in living cells. The acquisition of FRET signals from GFP- and BFP-fusion proteins expressed in living cells was demonstrated using an optimized imaging system and high sensitivity charge coupled device camera. This imaging system was used to detect energy transfer signals from a fusion protein containing GFP physically linked to BFP expressed in living HeLa cells. In contrast, the co-localization of noninteracting GFP- and BFP-fusion proteins was not sufficient for energy transfer. The FRET imaging system was then used to demonstrate dimerization of the pituitary-specific transcription factor Pit-1 within the living cell nucleus. © 1998 Society of Photo-Optical Instrumentation Engineers. [S1083-3668(98)00802-8]

Keywords fluorescence resonance energy transfer (FRET); microscopy, green fluorescent proteins (GFPs); Pit-1.

1 INTRODUCTION

Microscope imaging of fluorescent probes in living cells provides an unparalleled perspective on the complex events that regulate cellular function. The jellyfish green fluorescent protein (GFP) has proven to be a versatile probe for microscopic imaging of living cells. GFP retains its characteristic fluorescence when fused to other protein sequences, allowing it to be used as a marker for protein localization within the living cell. Fluorescence microscopy has been used to localize the subcellular distribution of GFP-fusion proteins¹ and to visualize dynamic changes in their localization in intact living cells.^{2–6} Mutant forms of GFP with emission in both the green and the blue spectrum (blue fluorescent protein, BFP) have been isolated.^{5,6} These different color fluorophores provide a general method for simultaneously labeling two different proteins within the same living cells. Moreover, the excitation and emission spectra for the mutant GFP and BFP proteins are compatible with the technique of fluorescence resonance energy transfer (FRET), making this unique noninvasive imaging approach more generally applicable.^{7,8}

We have used the expression of genetic vectors encoding GFP- and BFP-fusion proteins in living cells to optimize an imaging system for the acquisition of GFP-, BFP-, and FRET-fluorescence signals from single living cells.⁹

We describe here the characterization of FRET signals from single living cells using this imaging system. We then describe the application of this approach to monitor the dimerization of a pituitary-specific transcription factor known as Pit-1 within the living cell nucleus. The Pit-1 protein is a member of the homeodomain transcription factor family that includes the octamer binding proteins,¹⁰ Oct-1 and Oct-2. Pit-1 is expressed in several different anterior pituitary cell types, where it functions as an important determinant of pituitary-specific gene expression.¹¹ The protein was cloned based upon its binding to cell-specific DNA elements in the promoter regions of the rat prolactin (PRL) and growth hormone (GH) genes.¹² We have chosen to use this important transcription factor in the development of the FRET imaging approach because the domain structure of the protein has been mapped, and the protein-protein interactions have been well characterized.^{13–15} Using GFP- and BFP-Pit-1 fusion proteins and the FRET imaging approach, we demonstrate Pit-1 protein dimerization in the living cell nucleus. Our results illustrate the power of the combination of GFP-fusion proteins, a high sensitivity detector and the FRET technique for visualizing where and when proteins interact in the living cell.

Address all correspondence to Ammasi Periasamy.
E-mail: ap3t@virginia.edu

2 FRET MICROSCOPY

FRET is a quantum mechanical effect that occurs when the emission energy of a donor fluorophore (D) coincides with the energy required to excite an acceptor fluorophore (A). The energy from D can be transferred directly to A if the proximity of the two fluorophores is within ~ 10 to ~ 100 Å. Energy is transferred nonradiatively by means of intermolecular long-range dipole-dipole coupling.^{16,17} The rate of energy transfer depends upon the extent of overlap of the emission spectrum for D with the absorption spectrum for A, the distance between D and A, and the relative orientation of D and A transition dipoles. For GFP-fusion proteins, it is not possible to know the precise positioning of the fluorophores relative to one another in space due to the flexibility of the protein structure. This flexibility does, however, provide dynamic averaging of the relative orientations of D and A, thus minimizing the contribution of the orientation factor.¹⁸ The efficiency of energy transfer varies inversely with the sixth power of the distance separating D and A; the range over which resonance energy transfer can occur is limited to 0.001–0.01 μm (10–100 Å). Further, the efficiency of energy transfer is extraordinarily sensitive to the distance separating D and A fluorophores.¹⁹ Based upon the significant overlap in the emission spectrum of BFP^{Y66H,Y154F} with the excitation spectrum of GFP^{S65T}, it was predicted that these fluorophores would be suitable as donor (D) and acceptor (A) for FRET.²⁰ The Förster distance (R_0), the distance separating D and A at which the average efficiency of energy transfer is 50%, was estimated by the energy transfer rate equation to be 40 Å for BFP^{Y66H,Y154F} and GFP^{S65T}, assuming a random orientation for the fluorophores.⁵ Since the efficiency of energy transfer decreases dramatically over the range of 0.5 R_0 –1.5 R_0 , the energy transfer signal at a particular location within the microscope image provides spatial information about the relative proximity of the two fluorophores that surpasses the optical resolution of the light microscope. When used in combination with GFP- and BFP-fusion proteins, FRET imaging provides a method to resolve colocalization of D- and A- proteins within $\sim 0.04 \mu\text{m}^2$ in the living cell, and allows real-time visualization of intimate interactions between protein partners.

2.1 FRET IMAGING SYSTEM DESCRIPTION

The FRET imaging system used in these studies is based on a conventional inverted microscope equipped for epi-fluorescence and transmitted illumination (IX-70, Universal infinity system; Olympus America Inc., Melville, NY).^{9,21} Fluorescence images were acquired using a Planapo 100 \times (oil, NA 1.4) or 60 \times (water, NA 1.2) objective lens. The excitation light source was a 100 W mercury-xenon arc lamp (Hamamatsu Corp., Middlesex, NY)

coupled to an excitation (ex) and neutral density (ND) filter wheels (Ludl Electronic Products Ltd., Hawthorne, NY). The emission (em) filter wheel is coupled between the output port of the microscope and the camera. The following filter sets from Omega (Omega Optical Inc., Brattleboro, VT) were used (also see Fig. 1): for BFP, excitation (ex)=365/15 nm, dichroic mirror (dm)=390 nm, emission (em)=460/50 nm; for GFP, ex=485/22 nm, dm=510 nm, em=535/50 nm; for FRET, ex=365/15 nm, dm=390 nm, em=520/40 nm. Fluorescence image acquisition was achieved using a slow scan, liquid nitrogen-cooled (-93°C) charge coupled device (CCD) camera with a back-thinned, back-illuminated imaging chip (CH260, Photometrics, Ltd., Tucson, AZ). This camera has extremely low dark current and very high sensitivity, and is significant for the FRET imaging approach described here, because of a quantum efficiency of approximately 32% for blue light, compared to $\sim 12\%$ for other conventional CCD cameras. The digital image output of the camera is 512 \times 512 pixels with 16 bits resolution. The Silicon Graphics, Inc. (SGI) based Isee software (Inovision Corp., RTP, Raleigh, NC) was used to integrate the operation of the camera, filter wheels, image acquisition, and image processing.

3 CHARACTERIZATION OF FRET MICROSCOPY

3.1 IMAGING GFP- AND BFP-FUSION PROTEINS IN LIVING CELLS

Construction of some of the GFP- and BFP-fusion protein expression vector DNAs, as well as cell culture conditions and transfection of the HeLa cell line were described previously.⁹ Briefly, the cDNA sequences encoding the GFP mutant proteins S65T and Y66H, Y154F with optimal human codon usage⁵ were inserted into the pCMV vector²² to generate cytomegalovirus (CMV) GFP^{S65T} and pCMV BFP^{Y66H,Y154F}, respectively. The CMV GFP-3aa-BFP vector encoding GFP fused to BFP by a 3 aa linker was prepared by ligation of the BFP^{Y66H,Y154F} cDNA into the polylinker region of the CMV GFP^{S65T}. This placed the BFP coding sequence in the reading frame of GFP, separating the two cDNA sequences by a unique EcoRI site (CCG-GAATTC) encoding the protein sequence PEF. The CMV GFP- and BFP-Pit-1 vectors were prepared by insertion of the Pit-1 cDNA into a unique restriction endonuclease site at the extreme 3' terminus of the fluorescent protein coding sequences. The resulting vectors encode GFP or BFP followed by the 5 aa sequence YPLVG and the complete Pit-1 protein sequence. The vector encoding GFP with a nuclear localization signal (NLS) was prepared by insertion of a duplex oligonucleotide sequence encoding the SV40 NLS protein sequence LYPKKRKGVEDQYK at the 3' terminus of the GFP coding sequence.

HeLa cells were transiently transfected with the indicated expression vectors by electroporation, diluted in phenol red-free medium containing serum and used to inoculate 35 mm culture dishes containing 25 mm glass cover slips. The cells were maintained at 33°C in a humidified 5% CO₂ incubator for 24–48 h prior to FRET imaging. This lower temperature was selected to optimize the folding of the GFP and BFP chromophores. For fluorescence microscopy, a cover slip with a monolayer of transfected cells was placed in a chamber designed specifically for the microscope stage.²¹ Transmitted illumination was used to acquire a focused image of the cells, and the field of cells was then scanned for fluorescence using the appropriate filter combinations for either GFP or BFP (Sec. 2.1). Neutral density filter and integration time on the cooled CCD chip were adjusted to minimize photobleaching and to obtain an optimal signal-to-noise (S/N) ratio. After acquiring an image, a background image was obtained under the same conditions and digitally subtracted from the original image. The background-subtracted images were stored on the computer for further FRET image analysis.

3.2 CHARACTERIZATION OF SPECTRAL OVERLAP IN FRET IMAGING

We previously described the characterization of excitation and emission filters used to discriminate the fluorescence from GFP- and BFP-fusion proteins expressed in the same cell, and to detect the transfer of energy from donor (BFP) to acceptor (GFP).⁹ In the present study, we acquired images from cells expressing different levels of the GFP- and BFP-fusion proteins using the different filter combinations to determine the amount of channel overlap for the two fluorophores. HeLa cells were transfected expression vectors encoding either the GFP- or the BFP-Pit-1 fusion proteins independently. Individual cells expressing the fusion proteins were identified by nuclear fluorescence at the wavelength appropriate for the fluorophore. Between eight and 12 individual cells expressing different levels of the fusion protein were imaged with each of the three filter combinations (see Sec. 2.1), and the gray level intensity of fluorescence signal in each channel was determined under constant conditions of neutral density and integration time. The results shown in Figure 1, represent graphically the level of signal detected with each of the three filter combinations for cells expressing different levels of either GFP-Pit-1 (top left) or BFP-Pit-1 (top right). With increasing levels of expression of GFP-Pit-1 we observed little change in the fluorescence signal monitored with either the BFP (Ex 365/15, Em 460/50) or FRET (Ex 365/15, Em 520/40) channels until the GFP signal approached the saturation level for the detector (cell 10, 65 K maximum). Moreover, no overlap of the BFP signal was detected with the GFP filter (Ex 485/22, Em 535/50), and the BFP sig-

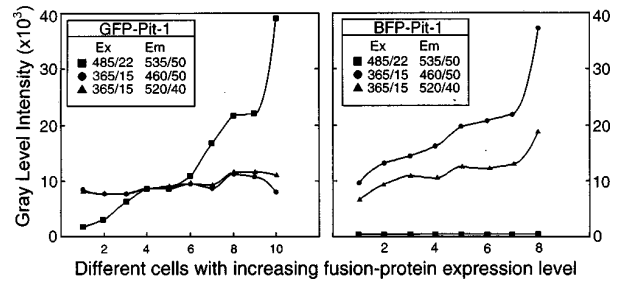


Fig. 1 Characterization of the filter combinations and channel overlap for cells expressing independently GFP and BFP. Little change in FRET signals occurred in the green-only transfected cells at higher expression levels of GFP (left). The graph on the right, blue-only transfected cells, shows the FRET signals are approximately constant and increase at higher expression levels of BFP.

nal detected in the FRET channel was constant at approximately 50% of the signal detected with the BFP channel. These results demonstrated that there was minimal overlap of the GFP signal into the FRET channel.

3.3 FRET IMAGING OF LIVING CELLS EXPRESSING GFP-BFP FUSION PROTEINS

We next used fusion proteins in which GFP was physically coupled to BFP through a protein linker to characterize the acquisition of FRET signals from single living cells. Protein fusions in which GFP was directly coupled to BFP were originally used in *in vitro* experiments to validate resonance energy transfer between these probes.^{5,6} We demonstrated previously the acquisition of a FRET signal from single living HeLa cells expressing GFP coupled directly to BFP through protein linker of 29 randomly assigned amino acids (*aa*).⁹ Here we extend this important control experiment by comparing the FRET signal generated by GFP separated from BFP by 3 *aa* linker (GFP-3*aa*-BFP). Cells expressing the fusion proteins were first identified by green fluorescence [Figure 2(B)]. This fusion protein does not contain a subcellular localization signal, and fluorescence is uniform throughout the cell. A second image is acquired of donor fluorescence (BFP) from the same cell, and a background-subtracted image is obtained [I_D , Figure 2(A)]. We then acquire a third image of the cell using the acceptor (FRET) filter set and the background-subtracted acceptor fluorescence is obtained (I_A , not shown). The camera gain, ND filter, and image acquisition time are kept constant for these two images (I_A and I_D). Acquisition times for the relatively dim donor fluorescence typically range from 1 to 5 s depending upon expression level and ND used. We then use the SGI based Isee software to obtain a pixel-by-pixel ratio of acceptor to donor fluorescence to yield the processed FRET image [I_A / I_D , Figure 2(C)]. A look-up table was applied to the resulting image and the red color indicates the highest gray level intensity [Figure 2(C)]. This image indicated that the accep-

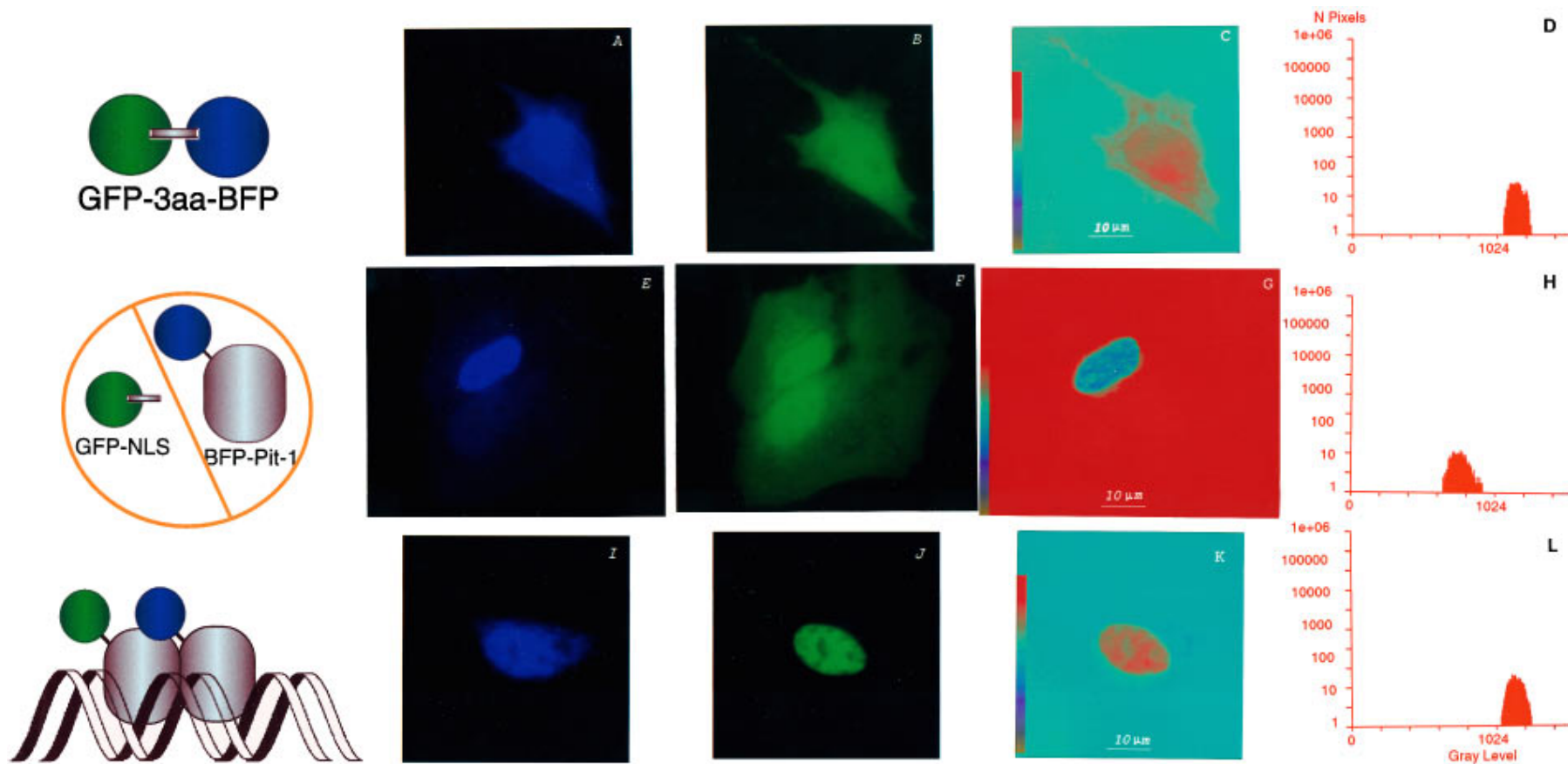


Fig. 2 FRET imaging of cells expressing GFP linked to BFP [Figures 2(A)–2(C)]. FRET imaging of a HeLa cell expressing GFP-3aa-BFP. A GFP image (B) and the background-subtracted BFP donor image (I_D ; A) are shown. A background-subtracted acceptor image (I_A) (not shown) was acquired and the ratio of acceptor and donor images was obtained (I_A/I_D ; C). Red color indicates maximum gray-scale intensity corresponding to an increased I_A/I_D , consistent with energy transfer. FRET imaging of a HeLa cell expressing nuclear localized GFP-NLS and BFP-Pit-1 [Figures 2(F)–2(G)]. Background-subtracted BFP donor image (I_D ; E), GFP image (F), and the ratioed image (I_A/I_D ; G) are shown. Blue color in the nucleus indicates a decrease in the I_A/I_D ratio in this region. FRET image of the nucleus of a cell expressing GFP-Pit-1 and BFP-Pit-1 [Figures 2(I)–2(K)]. A FRET image (I_A/I_D , K) was obtained from a HeLa cell co-expressing GFP-Pit-1 and BFP-Pit-1 and processed to resolve the spatial distribution of the energy transfer signal in the living cell nucleus. The red color represents the energy transfer signals indicative of Pit-1 dimerization. Figures 2(D), 2(H), and 2(L) illustrate the histogram of the FRET image of nuclei Figures 2(C), 2(G), and 2(K), respectively. This histogram clearly demonstrates that the nucleus has considerably less FRET signal [< 1024 gray level intensity, Figure 2(H)] for noninteracting but co-localized proteins compared to the 3aa linker [Figure 2(D)] and Pit-1 protein dimerization [Figure 2(D)] in the nucleus where FRET signal is > 1024 .

tor was significantly greater than the donor signal, consistent with energy transfer from BFP to GFP.

3.4 IMAGING CO-LOCALIZED, BUT NONINTERACTING, GFP- AND BFP-FUSION PROTEINS

To demonstrate that simple co-localization of the blue and green fluorophores was *not* sufficient to yield energy transfer we co-expressed GFP with a nuclear localization signal (GFP-NLS) and the BFP-Pit-1 fusion protein in the same HeLa cells (see Sec. 3.1. for details). The Pit-1 protein is exclusively localized to the nucleus and co-expression with the GFP-NLS resulted in both green and blue fluorophores in the nuclear compartment [Figures 2(F) and 2(E)]. We then applied the same imaging protocol described above for the GFP-3aa-BFP fusion to these cells. The same look-up table and contrast levels were applied to processed FRET image and the results revealed that I_A was less than the D signal in the nuclear compartment (indicated by the blue color in the cell nucleus compared to red color surrounding the cells) consistent with the expected result of no energy transfer between the noninteracting, but co-localized, proteins [Figure 2(G)]. The difference in gray level intensity in the histogram [Figures 2(D) and 2(H)] also confirms that the energy transfer occurs in 3aa linker but not in the co-localized proteins. Together, these results demonstrated that FRET signals can be monitored in single living cells using the digital imaging system described here, and that simple co-localization of the fluorophores was not sufficient for resonance energy transfer.

4 FRET IMAGING OF PIT-1 PROTEIN DIMERIZATION IN THE LIVING CELL NUCLEUS

The FRET imaging protocol was then applied to cells co-expressing GFP- and BFP-Pit-1 to determine if dimerized Pit-1 proteins could be detected in the living cell nucleus. This is significant because *in vitro* biochemical studies demonstrated that the Pit-1 proteins exist as monomers in solution, and only forms dimers when interacting with specific pituitary gene DNA elements.¹¹⁻¹⁵ Since the genes that are transcriptionally activated by Pit-1 are not expressed in HeLa cells, these biochemical studies imply that Pit-1 would not dimerize in these cells, and FRET imaging should fail to detect energy transfer. Alternatively, if the Pit-1 protein associates as a dimer with other structures within the nuclear compartment, it is possible that this would be detected by FRET imaging. HeLa cells were co-transfected with the individual expression vectors encoding the GFP-Pit-1 and BFP-Pit-1 fusion proteins as described above (Sec. 3.1.). The GFP and BFP fluorophores were positioned at the amino terminus of the Pit-1 protein, in proximity to the trans-

activation domain. Expression of the full length GFP-Pit-1 fusion protein was confirmed by Western blotting, the fusion protein was shown to bind with high affinity Pit-1 DNA elements using the gel mobility shift assay (data not shown). Moreover, transient transfection of the GFP-Pit-1 protein resulted in transactivation of the PRL gene promoter linked to luciferase in HeLa cells (data not shown).

Energy transfer signals from dimerized BFP-Pit-1 (D) and GFP-Pit-1 (A) proteins in the HeLa cell nucleus would be expected if the fluorophores are within 10–100 Å and the D and A transition dipoles are aligned. This would be evidenced by an increase in the I_A/I_D ratio, whereas no change or a decrease in the ratio would indicate that the fluorophores are not within the range of 10–100 Å, or not aligned. Cells co-expressing the BFP-Pit-1 and GFP-Pit-1 proteins were first identified by green fluorescence using the GFP filter set [Figure 2(I)]. Images of these same cells were then obtained with the donor (BFP-Pit-1) filter set and background-subtracted [I_D , Figure 2(I)]. As described above, camera gain, ND, and acquisition time were kept constant to acquire the background-subtracted acceptor fluorescence (I_A) image from this cell (not shown). The pixel-by-pixel ratio of the I_A to I_D fluorescence signal was obtained and the same look-up table and contrast levels used in the control experiments were applied to generate the processed FRET image [Figure 2(K)]. The results indicated that the I_A signal was significantly greater than the I_D signal, consistent with energy transfer from BFP-Pit-1 to GFP-Pit-1 (red color). This would require that the fluorophores be near the Ro of 40 Å, demonstrating that the Pit-1 fusion proteins were in physical contact within these HeLa cell nuclei.

To confirm and extend this observation, we examined the relationship between fluorescence signals detected in the GFP, BFP, and FRET channels for 12 individual HeLa cells co-expressing BFP-Pit-1 and different levels of GFP-Pit-1. The gray level intensity of the fluorescence signal from the nuclear compartment was determined for each cell using the three filter combinations and the results are shown in Figure 3. In contrast to the results shown in Figure 1, this experiment revealed that the FRET signal exceeded the signal detected in the BFP channel at all levels of GFP-Pit-1 expression, demonstrating that energy transfer from D to A occurred over a range of different A protein concentrations. Further, the FRET signal intensity paralleled the D signal, despite increasing levels of A protein. This demonstrated that the FRET signal is not a result of GFP signal overlap into the FRET channel. Taken together, these results demonstrate the utility of the FRET imaging approach to detect dimerization of the Pit-1 transcription factor in the nucleus of a living cell. Our results suggest that the Pit-1 protein is capable of homodimerization within the nucleus of nonpituitary cells, demonstrating that interaction

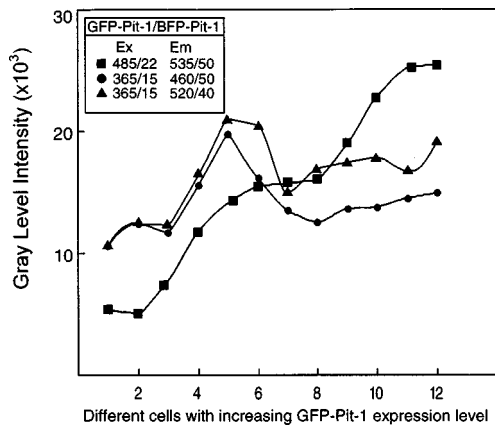


Fig. 3 Characterization of the filter combinations and channel overlap for cells expressing GFP- and BFP-Pit-1. The FRET signal increases with an increase in expression level of GFP (increase in concentration of acceptor signal) and then stabilizes with further increase in expression level. But the FRET signal in the FRET channel was not influenced by the GFP signal as compared with Figure 1.

with pituitary gene-specific DNA elements is not an absolute requirement for dimerization. Our results imply that dimerized Pit-1 is associated with macromolecular complexes within the nucleus, and we are currently using the imaging system to obtain three-dimensional FRET images to precisely localize Pit-1 within the nuclear compartment.²³ We are also investigating the utility of quantifying Pit-1 protein-protein interactions using the approach of photobleaching FRET (Day et al., in preparation).

Further improvements in the detection of FRET signals from single living cells will result from continued optimization of imaging system hardware. Both increased sensitivity of the detector and improved filter combinations for discrimination of the fluorescence signals will yield improvements in the FRET signal-to-noise ratio. The CCD camera chosen for these studies has exceptional sensitivity to both blue and green light, a characteristic that is critical for FRET imaging. The selection of the GFP and BFP as fluorescent probes provided the overlap of the D emission spectrum with the A excitation spectrum necessary for energy transfer. However, the broad excitation and emission spectrums for these probes makes the choice of filter sets critical to minimize channel overlap. For the experiments presented here, a narrow 365 nm excitation filter was chosen to provide a maximal excitation of BFP while minimizing the excitation of GFP. In addition a 520/40 nm em filter for FRET signal was chosen to further reduce channel overlap.

5 CONCLUSION

In this paper we discussed the use of a conventional inverted epi-fluorescence microscope and the technique of FRET microscopy to visualize protein-protein interactions in living cells. Our results show that FRET imaging of GFP-fusion proteins can fur-

nish a wealth of information regarding the physical interactions between protein partners within the cell. The broader application of FRET imaging to the detection and quantitation of protein associations will depend upon further optimization of hardware, software, and biological probes used in these experiments.

Acknowledgments

We wish to thank Dr. Roger Tsien and Dr. Roger Heim (University of California, San Diego, CA) for providing the mutant GFP S65T and Y66H/Y145F cDNAs. We thank Dr. Jen Sheen (Massachusetts General Hospital, Boston, MA) for providing the mutant GFP with optimized codon usage. We acknowledge the expert technical assistance of Diana Berry and Margaret Kawecki. This work was supported by awards from the Academic Enhancement Program of the University of Virginia (AP), the NSF DIR-8920162 Center for Biological Timing Technology Development sub-project (RND), NSF SGER IBN9528526 (RND) and NIH Grant No. RO1-DK-43701 (RND).

REFERENCES

1. J. D. Plautz, R. N. Day, G. M. Dailey, S. B. Welsh, J. C. Hall, S. Halpain, and S. A. Kay, "Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells," *Gene* **173**, 83-87 (1996).
2. H. H. Gerdes and C. Kaether, "Green fluorescent protein: applications in cell biology," *FEBS Lett.* **389**, 44-47 (1996).
3. B. Ludin, T. Doll, R. Meili, S. Kaech, and A. Matus, "Application of novel vectors for GFP tagging of proteins to study microtubule-associated proteins," *Gene* **173**, 107-111 (1996).
4. K. L. Carey, S. A. Richards, K. M. Lounsbury, and I. G. Macara, "Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import," *J. Cell Biol.* **133**, 985-996, (1996).
5. R. Heim and R. Y. Tsien, "Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer," *Curr. Biol.* **6**, 178-182 (1996).
6. R. D. Mitra, C. M. Silva, and D. C. Youvan, "Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein," *Gene* **173**, 13-17 (1996).
7. B. Herman, "Resonance energy transfer microscopy," *Methods Cell Biol.* **30**, 216-243 (1989).
8. T. M. Jovin and D. J. Arndt-Jovin, "FRET microscopy: Digital imaging of fluorescence resonance energy transfer: Application in cell biology," in *Cell Structure and Function by Microspectrofluorometry*, E. Kohen, and J. G. Hirschberg, Eds., pp. 99-117, Academic, San Diego, CA (1989).
9. A. Periasamy, S. A. Kay, and R. N. Day, "Fluorescence resonance energy transfer (FRET) imaging of a single living cell using green fluorescent protein," *Proc. SPIE* **2983**, 58-66, (1997).
10. W. Herr, R. A. Sturm, R. G. Clerc, L. M. Corcoran, D. Baltimore, P. A. Sharp, H. A. Ingraham, M. G. Rosenfeld, M. Finney, G. Ruvkin, and H. R. Horvitz, "The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and *Caenorhabditis elegans* unc-86 gene products," *Genes Dev.* **2**, 1513-1516 (1988).
11. B. Andersen, M. G. Rosenfeld, "Pit-1 determines cell types during development of the anterior pituitary gland. A model for transcriptional regulation of cell phenotypes in mammalian organogenesis," *J. Biol. Chem.* **269**, 29335-29338 (1994).

12. M. Bodner, J. L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin, "The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein," *Cell* **55**, 267–275 (1988).
13. H. A. Ingraham, S. E. Flynn, J. W. Voss, V. R. Albert, M. S. Kapiloff, L. Wilson, and M. G. Rosenfeld, "The POU-specific domain of Pit-1 is essential for sequence specific, high affinity DNA binding and DNA dependent Pit-1-Pit-1 interactions," *Cell* **55**, 519–529 (1990).
14. W. Chang, W. Zhou, L. E. Theill, J. D. Baxter, and F. Schaufele, "An activation function in Pit-1 required selectively for synergistic transcription," *J. Biol. Chem.* **271**, 17733–17738 (1996).
15. J. M. Holloway, D. P. Szeto, K. M. Scully, C. K. Glass, and M. G. Rosenfeld, "Pit-1 binding to specific DNA sites as a monomer or dimer determines gene-specific use of a tyrosine-dependent synergy domain," *Genes Dev.* **9**, 1992–2006 (1995).
16. T. Förster, "Intermolecular energy migration and fluorescence," *Ann. Phys. (Leipzig)* **2**, 55–75 (1948).
17. L. Stryer, "Fluorescence energy transfer as a spectroscopic ruler," *Annu. Rev. Biochem.* **47**, 819–846 (1978).
18. R. E. Dale, J. Eisinger, and W. E. Blumberg, "The orientational freedom of molecular probes. The orientation factor in intramolecular energy transfer," *Biophys. J.* **26**, 161–194 (1979).
19. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum Press, New York (1986).
20. A. B. Cubitt, R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross, and R. Y. Tsien, "Understanding, improving and using green fluorescent proteins," *Trends Biochem. Sci.* **20**, 448–455 (1995).
21. A. Periasamy and B. Herman, "Computerized fluorescence microscopic vision in the biomedical sciences," *J. Comput. Assist. Microsc.* **6**, 1–26 (1994).
22. G. R. MacGregor and C. T. Caskey, "Construction of plasmids that express E. coli beta-galactosidase in mammalian cells," *Nucleic Acids Res.* **17**, 2365 (1989).
23. A. Periasamy, "Digital deconvolution FRET microscopy: 3D visualization of protein-protein interactions in a single living cell (Invited Paper)," *Proc. SPIE* (in press).