Measurement of Molecular Interactions in Living Cells by Fluorescence Resonance Energy Transfer Between Variants of the Green Fluorescent Protein

Richard M. Siegel, Francis Ka-Ming Chan, David A. Zacharias, Ruth Swofford, Kevin L. Holmes, Roger Y. Tsien, and Michael J. Lenardo

(Published 27 June 2000)

INTRODUCTION

Many signal transduction pathways operate through oligomerization of proteins into multisubunit complexes. Although biochemical assays can identify potential protein-protein interactions, studying these interactions in living cells is more challenging. Fluorescence resonance energy transfer (FRET) has been used as a "spectroscopic ruler" to measure molecular proximity (*1*). Molecular biology and the use of the versatile green fluorescent protein (GFP) have led to new and more powerful applications of FRET (*2, 3*). We will briefly review the theory behind FRET, and present two methods of quantifying FRET between proteins linked to spectral variants of GFP. These techniques can be used to measure dynamic interactions between many pairs of molecules in living cells.

The principle of resonance energy transfer is based on the ability of a higher energy donor fluorophore to transfer energy directly to a lower energy acceptor molecule, causing sensitized fluorescence of the acceptor molecule and simultaneous quenching of the donor fluorescence (Fig. 1). Donor and acceptor molecules must be matched so that the emission wavelengths of the donor overlap with the excitation wavelengths of the acceptor. However, excessive overlap of emission spectra between the donor and acceptor should be avoided so that FRET signals can be easily detected. To test for FRET, the samples are irradiated with light at an appropriate wavelength within the donor excitation range, and then the emission of light at the acceptor emission wavelength is measured.

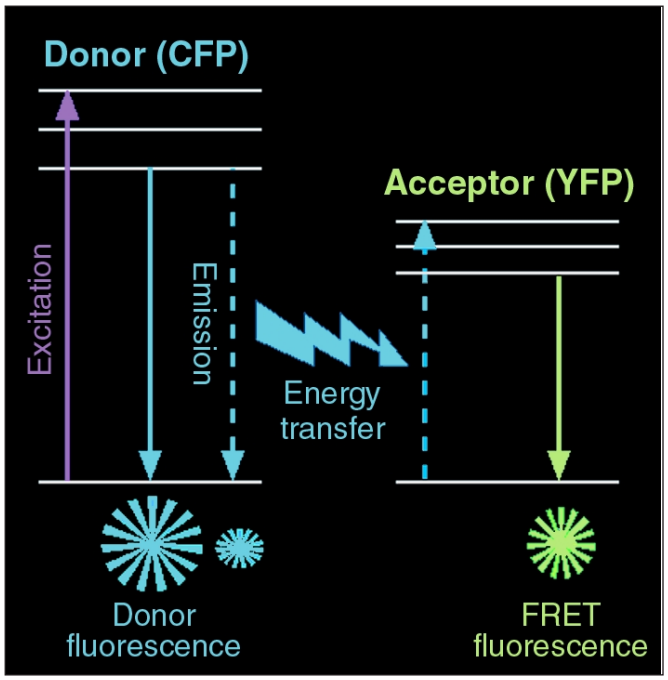

Fig. 1. Schematic of fluorescence resonance energy transfer. The donor (CFP) fluorescence, released during transition to a lower quantum state, is shown by the blue arrows. In the presence of an acceptor fluorescent molecule, donor fluorescence is quenched (dashed blue arrow) and energy is transferred to the acceptor (YFP), which then releases photons at the acceptor emission wavelength (yellow-green arrow)

The efficiency of energy transfer (E) is highly dependent on the distance (r) between the donor and acceptor chromophores, as described by the Förster equation (4): $E = R_o^6/(R_o^6 + r^6)$, where the Förster radius (R_o) is the distance at which the efficiency of energy transfer is 50% of maximum and can be calculated from the spectral properties and relative orientation of each fluorescent molecule. For example, increasing the distance between acceptor and donor from $R = R_o$ to $R = 2R_o$ will decrease the efficiency (E) of energy transfer from 50 to 1.5%.

Richard Siegel, Francis Ka-Ming Chan, and Michael J. Lenardo are at the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA. E-mail: lenardo@nih.gov

Ruth Swofford and Kevin L. Holmes are at the Flow Cytometry Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

David A. Zacharias and Roger Y. Tsien are at the Howard Hughes Medical Institute and Department of Pharmacology, UCSD School of Medicine, La Jolla, CA 92093–0647, USA.

Thus, FRET provides a very sensitive measure of small changes in intermolecular distances. In most cases, no FRET can be observed at distances greater than 100 Å, so the presence of FRET is a good indicator of close proximity, implying biologically meaningful protein-protein interactions.

The first uses of FRET in biological systems relied on intrinsic fluorescence of tryptophan donors or direct labeling of macromolecules with pairs of donor and acceptor dyes such as fluorescein and tetramethylrhodamine. With the advent of monoclonal antibodies, it became possible to tag cell-surface molecules with donor- and acceptor-labeled antibodies to analyze receptor interactions. These techniques were successfully used to measure (i) self-interactions between major histocompatibility complex (MHC) molecules and between epidermal growth factor (EGF) family receptors, and (ii) heterologous interactions between the T cell receptor (TCR) and the CD4 coreceptor and between the intercellular cell adhesion molecule ICAM-1 and the interleukin 2 (IL-2) receptor, among other studies (*5*–*7*). However, these techniques were limited to probing interactions of extracellular portions of receptors, which may differ from interactions of the intracellular signaling domains of these receptors. In addition, the antibodies used in these experiments may perturb the conformation and associations of the bound receptors.

To overcome these limitations, we and others have used FRET between spectral variants of GFP fused to proteins of interest. All the variants described here are "enhanced" versions of the original GFP selected for brighter fluorescence. Molecular techniques allow placement of the autofluorescent protein at different positions in the molecule to be studied. The large size of GFP (28 kD) can potentially alter the function of the target protein. However, hundreds of GFP fusion proteins produced in the past 5 years have been reported without exhibiting any obvious deleterious effects from adding GFP.

Two pairs of GFP mutants have excitation and emission properties favorable for FRET: blue fluorescent protein (BFP) with GFP and cyan fluorescent protein (CFP) with yellow fluorescent protein (YFP) (Table 1). R_o for BFP as the donor and GFP as the acceptor is 40 to 43 Å, whereas R_o for CFP as the donor and YFP as the acceptor is 49 to 52 Å, if we assume that the mutual orientation of the two fluorescent proteins is random. Of these, the CFP/YFP pair is generally superior because of the greater extinction coefficient, quantum yield, and photostability of CFP compared with BFP, the easier photobleaching of YFP, and the greater Förster radius R_o. In addition, the short wavelengths needed for BFP excitation could be cytotoxic, and autofluorescence is more of a problem for the range of BFP emission. The interfluorophore angle of orientation κ^2 also affects the efficiency of FRET. A random orientation (i.e., free rotation, $\kappa^2 = 2/3$) of the GFP is generally assumed even when GFP is fused to macromolecules. This assumption may not be correct for real macromolecular assemblies but is customarily made because the actual orientation is quite difficult to determine. This uncertainty, plus the uncertainty in chromophore location within the entire chimeric protein, means that FRET with GFP variants is more suited to detecting changes in conformation or percentages of association than to quantifying absolute distances.

PROCEDURES

Materials

ECFP and EYFP expression vectors from Clontech (http://www.clontech.com/gfp/) for creation of the fusion protein constructs

6- or 12-well tissue culture plates

Sterile coverslips

293T: a SV40 T antigen-expressing variant of the 293 adenovirus-transformed human embryonic kidney cell line (ATCC CRL-1573)

Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum

FuGENE (Roche) (http://biochem.roche.com/fugene/), SuperFect (Qiagen) (http://www.qiagen.com/tools/), or conventional calcium phosphate precipitation reagents for transfection

Equipment

Flow cytometry

Dual-laser, fluorescence-activated cell sorter (FACS) with interlaser compensation module (e.g., FACS Vantage SE flow cytometer with Omnicomp module) [BD Immunocytometry Systems (BDIS) at http://www.bdfacs.com/literature/catalog/ins_02.asp]

Krypton laser (Spectra-Physics 2060, http://www.spli.com)

Air-cooled argon laser [Fraunhofer Institute for Lasertechnology (ILT) model 413]

470 nm/20 nm bandpass filter for CFP channels

546 nm/10 nm bandpass filter for FRET and YFP channels

Microscopy

Epifluorescence microscope with appropriate filter sets, and image acquisition and analysis software (Table 2).

Recipe

Recipe 1: Phosphate-buffered saline (PBS)

KH_2PO_4	0.26 g
Na_2HPO_4-$7H_2O$	2.17 g
NaCl	8.71 g

Dissolve in 800 ml distilled H_2O, adjust the pH to 7.4, then adjust volume to 1 l.

Instructions

Generation of cell expressing CFP and YFP fusion proteins

Fusion proteins expressing ECFP and EYFP linked to proteins of interest can be generated by standard molecular biology techniques. Vectors designed to express all of the GFP variants as fusion proteins described here are available from Clontech (http://www.clontech.com/gfp/). The fluorescent proteins can be

fused either to the NH_2- or COOH-termini of the host proteins, but it is often difficult to predict in advance which order will perturb the host protein the least and give the largest FRET. Control constructs expressing ECFP and EYFP with proteins targeted to the same intracellular compartment should be used to rule out nonspecific interactions between fusion proteins or the fluorescent proteins themselves. The method described here is for the generation of transfected 293T human embryonic kidney cells. 293T cells allow high-level expression and replication of plasmids containing the SV40 origin of replication, but any highly transfectable cell line should give similar results. The 293 cells are particularly useful for flow cytometry because they are semiadherent and can be resuspended in media without the use of trypsin.

1. Plate 293T cells in 6- or 12-well plates and grow to 50% confluence.

2. Transfect cells in each well with 1 to 2 mg of each of the plasmids encoding the ECFP and EYFP fusion proteins. Transfect some wells with only one of the fusion proteins to be used as controls.

Note: Commercial transfection reagents, such as SuperFect (Qiagen) or FuGENE (Roche), or conventional calcium phosphate precipitation may be used to perform transfection. The ratio of YFP to CFP plasmid may need to be optimized for each system. In general, a slight excess of acceptor molecules (YFP) will allow the most efficient energy transfer. Generation of stable clones allows selection of cells expressing desired ratios of donor to acceptor molecules.

3. Analyze cells 36 to 48 hours after transfection.

Flow cytometric measurement of FRET between CFP and YFP

Flow cytometry has been used to measure FRET between surface molecules identified by differentially labeled monoclonal antibodies (8). We have adapted this technique for the analysis of FRET between CFP and YFP fusion proteins (9, 10). The major advantage of flow cytometry is that a large number of cells can be rapidly analyzed. The effects of expression levels of the interacting proteins can also be studied in heterogeneous mixtures of transfected cells. Compensation can be used to mitigate the effects of spectral overlap between CFP and YFP emissions. However, the heterogeneity of expression of each protein and use of compensation make it difficult to estimate accurately the absolute FRET efficiency, which requires knowledge of the absolute donor and acceptor fluorescence (8). Nonstandard lasers are also necessary for excitation of CFP and YFP. We use a FACS Vantage flow cytometer with a krypton laser tuned to 413 nm for CFP excitation and an air-cooled argon laser tuned to 514 nm for YFP excitation. However, a standard 488-nm laser can also excite YFP, and a helium-cadmium laser line at 442 nm may also be appropriate for CFP excitation.

When two interacting proteins are coexpressed, a significant signal is observed in the YFP channel with CFP excitation, indicating energy transfer (Fig. 2). In our experiments with tumor necrosis family (TNF) receptor family members, we observed some dependence of FRET on the concentration of acceptor molecules. With a control protein in which CFP and YFP were covalently linked through a short peptide (11), a more linear relationship between expression levels and FRET was seen (9). The fact that FRET requires a threshold level of YFP expression may reflect either the statistics of mixing unlabeled, CFP-labeled, and YFP-labeled Fas receptors or a true density-dependence of the extent or stoichiometry of receptor preassociation.

1. Thirty-six to 48 hours after transfection, resuspend cells from each well with vigorous pipetting in 0.5 to 1 ml of PBS at 4°C. Analyze the transfected cells that have been resuspended in PBS in the flow cytometer. Cells transfected with a single construct, as well as cells transfected with both the ECFP and EYFP constructs, should be tested.

2. Configure the cytometer to sequentially illuminate the cells with the 514- and 413-nm lasers so that all three signals (CFP, YFP, and FRET) can be detected from each cell. High flow rates may be necessary to capture both events from the same cell.

3. Detect fluorescence using the following filter sets: CFP is detected with a 470 nm/20 nm bandpass filter, YFP emission is detected with a 546 nm/10 nm bandpass filter and 514 nm exci-

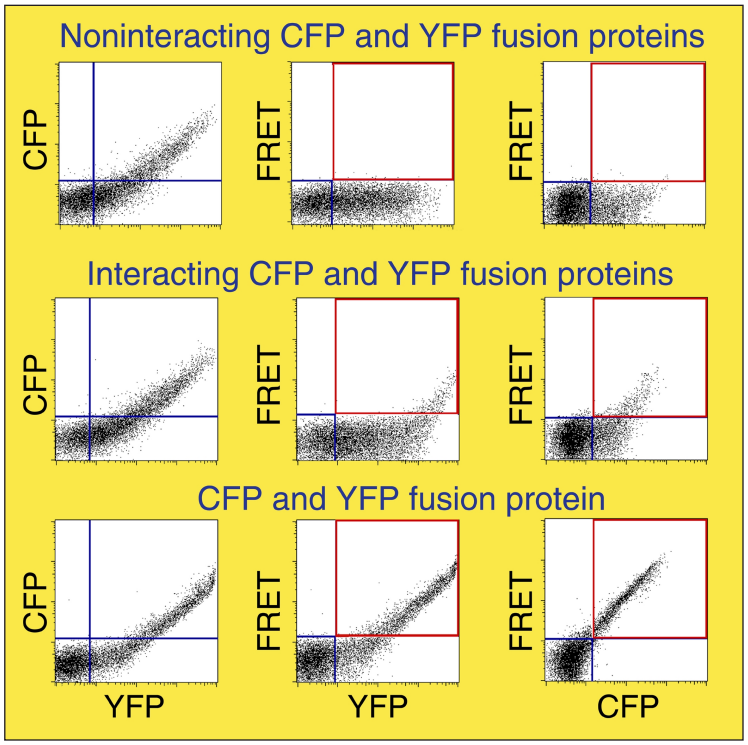

Fig. 2. Sample FACS profiles of FRET between ECFP and EYFP fusion proteins. 293T cells were transfected with ECFP and EYFP fusion proteins as described in the protocol. Two-color dot-plots of live cells with the fluorescence channels shown on the *x* and *y* axes. Fluorescence is on a four-log arbitrary scale. The red boxes outline the quadrants in which FRET signals are seen.

tation, and FRET is detected by measuring emission with a 546 nm/10 nm bandpass filter using 413 nm excitation.

4. Apply fluorescence compensation with the FACS acquisition software so that there is no FRET signal visible from cells transfected with ECFP or EYFP alone. Compensation electronically removes the fluorescence derived from the spectral overlap in emission between two fluorophores and must be performed with appropriate controls for every experiment.

Note: We use the Omnicomp module on the FACS Vantage flow cytometer for interlaser compensation between the YFP and FRET signals. Without this module, CFP and FRET channels can still be recorded with single-laser compensation.

5. Record the fluorescence intensities and plot the CFP signal versus the YFP signal and the FRET signal versus the CFP and YFP signal (see Fig. 2).

Microscopic measurement of FRET between CFP and YFP using donor dequenching

Because donors such as CFP generally show a long-wavelength tail in their emission spectrum, there is significant fluorescence at YFP emission wavelengths from CFP alone. It is therefore difficult to estimate FRET accurately from conventional measurements of sensitized emission without elaborate mathematical corrections (*12*). However, FRET also manifests itself as decreases in the donor's excited-state lifetime and fluorescence intensity. Measurements of the donor lifetime are an elegant way to measure FRET, but equipment to do lifetime measurements through a microscope is currently expensive, custom-built, and therefore, not generally available. In our view, the easiest way to quantify the absolute efficiency of FRET between the donor (CFP) and the acceptor (YFP) is to measure the CFP emission before and after selective photobleaching of the YFP. The increase or dequenching of CFP emission is a direct measure of the FRET efficiency *E*:

$$E\% = [1 - (\text{CFP emission before YFP photobleach} / \text{CFP emission after YFP photobleach})] \times 100$$

This procedure uses each cell or subregion as its own internal standard after photoboleaching and is, therefore, far more accurate than comparing cells with unknown FRET against separate control cells with strong or zero FRET. Values of *E%* as low as a few percent can be quantified with reasonable confidence.

1. Grow cells on sterile coverslips to 50% confluence.

2. Transfect cells with 1 to 2 mg of each of the plasmids encoding the ECFP and EYFP fusion proteins. Transfect some cells with only one of the fusion proteins to be used as controls.

Note: Commercial transfection reagents, such as SuperFect (Qiagen) or FuGENE (Roche), or conventional calcium phosphate precipitation may be used to perform transfection. The ratio of EYFP to ECFP plasmid may need to be optimized for each system. In general a slight excess of acceptor molecules (YFP) will allow the most efficient energy transfer. Generation of stable clones allows selection of cells expressing desired ratios of donor to acceptor molecules.

3. Thirty-six to 48 hours after transfection, transfer coverslips to slides without any antibleaching agent.

4. Examine cells with an epifluorescence microscope with appropriate filter sets (Table 2), and record the YFP and CFP

Clone (Color)	Mutations	Excitation peak (nm)	Emission peak (nm)
EBFP (Blue)	F64L Y66H Y145F	383	447
ECFP (Cyan)	K26R F64L S65T Y66W N146I M153T V163A N164H H231L	434 (452)	476 (505)
EGFP (Green)	F64L S65T H231L	488	507
EYFP (Yellow)	S65G S72A T203Y H231L	514	527
DsRED (Red)	Different protein	558	583

Table 1. Fluorescence characteristics of "enhanced" GFP variants. Numbers in parentheses denote secondary excitation and emission peaks.

fluorescence of a region of interest using standard imaging capture and analysis software, such as Metafluor (http://uicweb.image1.com/products/metafluor/).

5. Irradiate the area of the region of interest for 2 to 3 min through a 525 nm/40 nm bandpass filter with no other attenuation of the excitation beam to photobleach the YFP.

Note: On our microscope with a 40 × 1.3 NA objective, these conditions destroy YFP with very little or no direct bleaching of the CFP donor. The optimal exposure conditions that bleach YFP maximally with minimal direct effect on CFP should be determined for each microscope and filter set, using droplets of ECFP and EYFP under oil, cells transfected with ECFP and EYFP fusion proteins alone, or cells cotransfected with noninteracting ECFP and EYFP fusion proteins.

6. Remeasure the fluorescence intensities of the cell region of interest after photobleaching.

7. Average data from many regions of interest to determine the final *E%* value as described by the formula above. When energy transfer is present, there should be a gain in intensity of CFP emission after photobleaching of YFP. Care should be taken to collect data from relevant parts of the cell (e.g., cell surface areas for membrane proteins), so that the most biologically relevant interactions are measured.

Note: If background FRET signals are present, test controls with ECFP or EYFP fusion proteins alone and correct for any "bystander" quenching of CFP occurring during the photobleaching of YFP.

TROUBLESHOOTING

No FRET signal is observed: If fluorescence from both donor and acceptor proteins is visible, a positive control, usually a construct in which the two GFP proteins are covalently fused, or two proteins known to interact by FRET should be used to confirm that the experimental setup is correctly configured to measure FRET. If protein expression is not adequate, FRET signals may be weaker. Try repeating the transfection with more plasmid DNA until levels of protein equivalent to the positive control are seen. If FRET is still not seen, the molecules may not be interacting at distances close enough to produce FRET, or the fluorophores may be forced into a conformation that does not favor energy transfer.

NOTES AND REMARKS

Interpretation of FRET signals and other applications

We have applied FRET imaging to examine the interactions between members of the TNF receptor family on the cell surface. Although these receptors were previously thought to be randomly distributed as monomers on the cell surface, we found significant FRET between receptor subunits of fusion proteins of the same TNF receptor family member but not between different receptors. FRET between spectral variants of GFP has also been used to look at the interaction of Bcl-2 family members in the cytosol and Pit-1 transcription factors in the nucleus (*13, 14*). The disappearance of FRET between chimeric molecules containing donor and acceptor GFP variants on either side of a proteolytic cleavage site has been used to measure caspase proteolytic activity in apoptotic cells (*11*). Changes in FRET have also been used to measure calcium concentrations in living cells by detecting calcium-sensitive conformational changes in calcium-binding proteins fused to GFP variants (*15*). FRET may be used in the future to examine receptor-ligand interactions, DNA-protein complexes, and conceivably to screen for novel protein interactions and inhibitors of target molecules in many different signal transduction pathways.

Filter set	Bandpass excitation filter (nm)	Longpass dichroic beam splitter (nm)	Bandpass emission filter (nm)
CFP	440/20	455	480/40
YFP	495/10	525	535/25
FRET	440/20	455	535/25
Photobleaching	525/40	525	

Table 2. Filter sets for FRET measurement by fluorescence microscopy. The bandpass gives the total wavelength allowed through the filter surrounding the peak, e.g., a 440/20 filter passes light from 410 to 430 nm. Appropriate filter sets can be obtained from Chroma Technologies (http://www.chroma.com) or Omega Optical (http://www.omegafilters.com). Note that because CFP has significant emissions in wavelengths up to 600 nm, it may be difficult to detect a specific FRET signal unless acquisition hardware with electronic compensation circuitry is used.

Useful links

From the Molecular Probes handbook, a detailed discussion of FRET theory and tabulation of R_o values for commonly used fluorophores is available at

http://www.probes.com/handbook/sections/0002.html

A discussion of the spectral properties of GFP and spectral shift variants is available at http://www.clontech.com/archive/OCT99UPD/RFP.html

A description of the mathematics and techniques of FRET measurement using flow cytometry by one of the groups that pioneered this approach is available at http://www.visi.com/~softflow/fcap/FRET.htm

REFERENCES

1. Stryer, L. (1978) Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* **47:** 819-846.
2. Tsien, R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* **67:** 509-544.
3. Miyawaki, A., and Tsien, R. (in press) Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.*
4. Förster, T. (1948) Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann. Physik (Leipzig)* **2**: 55-75.
5. Burton, J., Goldman, C.K., Rao, P., Moos, M., and Waldmann, T.A. (1990) Association of intercellular adhesion molecule 1 with the multichain high-affinity interleukin 2 receptor. *Proc. Natl. Acad. Sci. U.S.A.* **87:** 7329-7333.
6. Gadella, T.W., Jr., and Jovin, T.M. (1995) Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy: A stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol.* **129**: 1543-1558.
7. Mittler, R.S., Goldman, S.J., Spitalny, G.L., and Burakoff, S.J. (1989) T-cell receptor-CD4 physical association in a murine T-cell hybridoma: induction by antigen receptor ligation. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 8531-8535.
8. Tron, L., Szollosi, J., Damjanovich, S., Helliwell, S.H., Arndt-Jovin, D.J., and Jovin T.M. (1984) Flow cytometric measurement of fluorescence resonance energy transfer on cell surfaces. Quantitative evaluation of the transfer efficiency on a cell-by-cell basis. *Biophys. J.* **45**: 939-946.
9. Siegel, R.M., Frederiksen, J.K., Zacharias, D.A., Chan, F.C., Johnson, M., Lynch, D., Tsien, R., and Lenardo, M.J. (2000) Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* **288**: 2354-2356.
10. Chan, F., Chun, H.J., Zheng, L., Siegel, R., Bui, K., and Lenardo, M.J. (2000) A domain in TNF receptors that mediates ligand-dependent receptor assembly and signaling. *Science* **288**: 2351-2353.
11. Mahajan, N.P., Harrison-Shostak, D.C., Michaux, J., and Herman, B. (1999) Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis. *Chem. Biol.* **6**: 401-409.
12. Gordon, G.W., Berry, G., Liang, X.H., Levine, B., and Herman, B. (1998) Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J.* **74**: 2702-2713.
13. Day, R.N. (1998) Visualization of Pit-1 transcription factor interactions in the living cell nucleus by fluorescence resonance energy transfer microscopy. *Mol. Endocrinol.* **12**: 1410-1419.
14. Mahajan, N.P., Linder, K., Berry, G., Gordon, G.W., Heim, R., and Herman, B. (1998) Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer. *Nature Biotechnol.* **16**: 547-552.
15. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**: 882-887.