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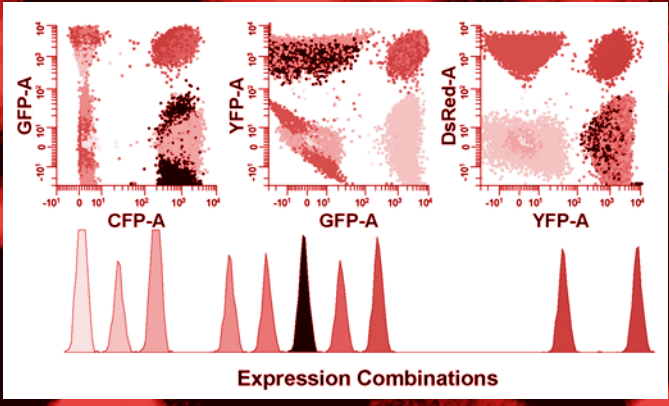

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Flow Cytometric Analysis of Fluorescence Resonance Energy Transfer

A Tool for High-Throughput Screening of Molecular Interactions in Living Cells

Francis Ka-Ming Chan and Kevin L. Holmes

Summary

The study of cellular processes has been facilitated by the use of methods to detect molecular associations both in vivo and in vitro. An invaluable tool to study molecular associations associated with dynamic processes in living cells utilizes the phenomenon of fluorescence resonance energy transfer (FRET), together with selected fluorophores that are attached to molecules of interest. Many reports have utilized fluorophores conjugated to antibodies for FRET pairs. However, these methods are restricted to extracellular molecules and dependent upon the availability of appropriate antibodies. The recent development of green fluorescent protein (GFP) variants suitable for FRET has expanded the utility of this methodology by permitting the study of intracellular as well as extracellular processes. Combining FRET with flow cytometric analysis results in a powerful high-throughput assay for molecular associations. This article details the use of green fluorescent protein (GFP) mutants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to measure the association of the signaling component TRAF2 with the TNFR-2 receptor to illustrate the versatility of this methodology.

Key Words

Energy transfer, flow cytometry, fluorescence resonance energy transfer, green fluorescent protein, molecular interactions, tumor necrosis factor receptor.

1. Introduction

The various biological functions of the cell are coordinated by the interaction of distinct molecular machineries. Numerous methods have been developed over the years to detect molecular associations both in vivo as well as in vitro.

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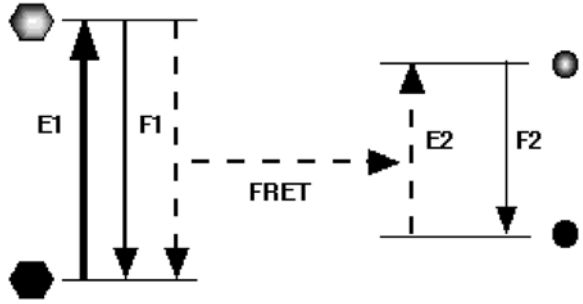

Fig. 1. Principles of FRET. The excitation of the first fluorophore (E1) results in the emission of energy (F1) at a longer wavelength as it returns to the ground state. Fluorescence emission from the first fluorophore (F1) can be "absorbed" by a nearby second fluorophore with an excitation wavelength (E2) that overlaps with the emission from the first fluorophore (F1) through dipole–dipole interactions. The net result is the transfer of energy in the form of fluorescence emission from the second fluorophore (F2 or FRET) and the "quenching" of fluorescence emission from the first fluorophore.

Many of these methods, however, do not allow the examination of dynamic processes in the living cells. The phenomenon of fluorescence resonance energy transfer (FRET) describes the transfer of energy from a fluorophore (donor) in an excited state to a neighboring fluorophore (acceptor) through dipole–dipole interaction ***(1,2)*** **(Fig. 1)**. FRET can be a sensitive means to measure molecular distances within 100 Å **(Fig. 1)** ***(3,4)***. Many methods utilizing the concept of FRET have been used successfully for the measurement of biological interactions ***(5–8)***. For example, subunit associations of the interleukin-2 (IL-2) receptor and epidermal growth factor (EGF) receptor have been detected with FRET-compatible, fluorochrome-labeled antibodies using the flow cytometer ***(9,10)***. Furthermore, molecular interactions between the dimeric subunits of the transcription factor Pit-1 and the interaction between the apoptotic regulatory proteins Bcl-2 and Bax were determined using microscopic FRET measurement ***(11,12)***. These methods are superior to conventional biochemical methods in that they allow the investigators to monitor biological processes in living cells. However, their use was sometimes limited by the availability of the appropriate antibodies that allow FRET without disrupting the interaction under examination or the relatively small sample size of microscopic measurements.

Recently, several spectral variants of green fluorescent protein (GFP) ***(13)*** were developed that have compatible fluorescence excitation and emission properties for FRET analysis **(Table 1)**. In particular, the excitation and emission spectra of the cyan (CFP) and yellow (YFP) versions of the GFP proteins allow them to be used in FRET analysis as donor and acceptor fluorophores,

Table 1
Spectral Properties of GFP Variants

Clone name	Mutations	Excitation peak (nm)	Emission peak (nm)
BFP (blue)	F64L, Y66H, Y145F	383	447
GFP (green)	F64L, S65T, H231L	488	507
CFP (cyan)	K26R, F64L, S65T, T66W, N146I, M153T, V163A, N164H, H231L	434 (452)	476 (505)
YFP (yellow)	S65G, S72A, T203Y, H231L	514	527

respectively. By creating fusion proteins to these GFP variants and introducing them into the appropriate cellular system, one can monitor the molecular associations of different biological machineries in living cells using flow cytometric methods. Combining FRET analysis with flow cytometry is advantageous as the investigator can screen a large number of cells within a short time. Unlike fluorescently labeled antibodies, the use of GFP fusion proteins permits the examination of extra- as well as intracellular associations. The versatility of flow cytometric FRET analysis is illustrated here using the interaction between p80 TNFR-2 and TRAF2 as an example. TRAF-2 is an important signal transduction component to many of the members of the tumor necrosis factor (TNF) receptor superfamily. On activation with TNF-α, TRAF2 is directly recruited to the preassembled TNFR-2 receptor (reviewed in **refs. *14*** and ***15***). This interaction will be monitored by the expression of fusion proteins of CFP and YFP to p80 TNFR-2 and TRAF2 in 293T cells.

2. Materials

1. Plasmids: pEF6-myc-HisB (Invitrogen, Carlsbad, CA), pECFP-N1, pEYFP-N1 (BD Biosciences Clontech, Palo Alto, CA).
2. cDNAs for p80 TNFR-2, TRAF2 and SODD (silencer of death domain).
3. Oligonucleotide primers for polymerase chain reaction (PCR).
4. Restriction enzymes, T4 DNA ligase.
5. QIAEX gel purification kit (Qiagen, Valencia, CA).
6. Competent *E. coli* cells for transformation (e.g., XL-1 blue from Stratagene, La Jolla, CA).
7. HEK 293T cells.
8. Complete Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD): DMEM without phenol red, 10% fetal calf serum (FCS), 100 units of penicillin and streptomycin, and 2 m*M* L-glutamine.

9. FuGENE 6 (Roche Applied Science, Indianapolis, IN).
10. Phosphate-buffered saline (PBS).
11. Nonidet *P*-40 (NP-40) lysis buffer: 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, and 1% NP-40.
12. Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).
13. 10% Bis-Tris NuPAGE protein gels (Invitrogen).
14. Rabbit polyclonal antiserum against TRAF2 (Santa Cruz Biotechnology, Santa Cruz, CA).
15. 6-mL fluorescent-activated cell sorter (FACS) tubes (Falcon brand).
16. 500 µg/mL of propidium iodide (PI) solution.
17. FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA) (*see* **Subheading 3.3.3.**).
18. FlowJo analytical software (Tree Star, Inc., San Carlos, CA) or other flow cytometry analytical softwares.

3. Methods

*3.1. Design of the Constructs (*see *Note 1)*

The crystal structure of TRAF2 homotrimer bound to the TNFR-2 peptide reveals a "mushroomlike" conformation of TRAF2 with the C- and N-termini resembling the cap and the stalk of a mushroom, respectively ***(16)***. The p80 TNFR-2 polypeptide makes contact with TRAF2 at the edge of the mushroom cap. To maximize the potential for interaction and therefore FRET, CFP was cloned at the C-terminus of full-length p80 TNFR-2 and YFP was cloned at the N-terminus of the full length TRAF2 protein. The silencer of death domain (SODD) protein ***(17)***, which binds to only TNFR-1 but not TNFR-2, was used as a non-interacting negative control.

*3.1.1. The Vectors and the Generation of the CFP and YFP Vectors (*see ***Note 2****)*

1. Design PCR primers for CFP and YFP cDNA inserts.
2. Perform PCR using pECFP-N1 or pEYFP-N1 as templates.
3. Resolve PCR products on 1% agarose gel. Excise and purify the correct fragments using QIAEX.
4. Digest PCR fragments with:
   a. *BamH*I/*EcoR*V (YFP fragment)
   b. *EcoR*V/*Xba*I (CFP and YFP fragments)
5. Digest vector pEF6-myc-HisB with:
   a. *BamH*I/*EcoR*V
   b. *EcoR*V/*Xba*I
6. Ligate fragment from **step 4a** with vector from **step 5a**. Resulting plasmid is pEF6B-YFP-C. Ligate CFP or YFP fragment from **step 4b** with vector from **step 5b**. Resulting plasmids are pEF6B-CFP-N and pEF6B-YFP-N.

7. Transform into competent XL-1 blue cells.
8. Screen resulting clones for PCR inserts in minipreps.
9. Sequence miniprep DNAs to confirm sequence integrity.

3.1.2. Cloning of the cDNAs

1. PCR amplify cDNAs for p80 TNFR-2, TRAF2 and SODD.
2. Digest p80 PCR product with *BamH*I/*EcoR*V. Digest TRAF2 and SODD PCR products with *EcoR*V/*Xba*I.
3. Digest pEF6B-YFP-C with *EcoR*V/*Xba*I. Ligate TRAF2 and SODD cDNA inserts from **step 2** to create pEF6B-YFP-TRAF2 and pEF6B-YFP-SODD.
4. Digest pEF6B-CFP-N and pEF6B-YFP-N with *BamH*I/*EcoR*V. Ligate p80 fragment from **step 2** to create pEF6B-p80-CFP and pEF6B-p80-YFP.
5. Repeat **steps 7–9** as described in **Subheading 3.1.1.**

3.2. Examination of the Expression of the Constructs

The next step in this process involves the confirmation of protein expression from the FRET donor and acceptor plasmids. To determine the expression of the plasmids, they were introduced into the human kidney epithelial cell line HEK 293T cells.

3.2.1. Transfection in 293T Cells (see ***Note 3***)

1. Seed 2.5×10^5 cells in each well of a 12-well plate in 1 mL of complete DMEM medium. Incubate at 37°C for 16–20 h.
2. Transfect 2 µg of each plasmid into HEK 293T cells using 6 µL of FuGENE 6 to give a DNA/FuGENE 6 ratio of 1:3.
3. Grow and incubate cells at 37°C for 24 to 48 h.
4. Harvest cells for subsequent analyses by Western blotting or flow cytometry.

3.2.2. Western Blotting Analysis

1. Aspirate medium from wells. Add 1 mL of PBS to each well. Gently resuspend cells in PBS and transfer cell suspension to microfuge tubes.
2. Centrifuge the cells for 5 min at 480*g*. Aspirate PBS.
3. Resuspend the cell pellet in 100 µL of NP-40 lysis buffer and incubate for 15 min on ice.
4. Centrifuge at 17,900*g* at 4°C in a microfuge for 10 min.
5. Transfer the supernatant to a fresh microfuge tube. Measure lysate concentration using the Bio-Rad protein assay reagent.
6. Load 50 µg of cell lysates on a 10% Bis-Tris NuPAGE gel and transfer onto nitrocellulose membrane.
7. Probe the membrane with antibody against TRAF2 and secondary HRP-conjugated antibody against rabbit IgG. **Figure 2** shows the expression of YFP-TRAF2 (lane 2) and untagged TRAF2 (lane 3) in HEK 293T cells (*see* **Note 4**).

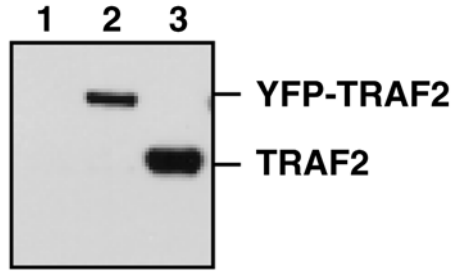

Fig. 2. Expression of YFP-TRAF2 in HEK 293T cells. HEK 293T cells were transfected with (1) pEF6-myc-HisB, (2) pEF6B-YFP-TRAF2, and (3) pcDNA3-TRAF2. Twenty-four hours later, cells were harvested for whole cell lysates. Equal amounts (50 μg) of lysates were loaded on a 10% Bis-Tris NuPAGE gel and resolved by electrophoresis. The expression of TRAF2 was examined in Western blotting using antibody against TRAF2. YFP-TRAF2 and TRAF2 were indicated.

3.2.3. Monitoring Fluorescence of the Expressed Proteins

As an alternative to Western blot analysis, expression of the transfected plasmids can be monitored by flow cytometry.

1. Wash cells twice in 1 mL of PBS supplemented with 2% FCS by centrifuging for 5 min at 4°C at 480*g*.
2. Resuspend cells in 1 mL of PBS supplemented with 2% FCS.
3. Add 2 μL of PI solution (500 μg/mL) to the cell suspension.
4. Acquire events on the flow cytometer.

3.3. Performing Flow Cytometric FRET Analysis

Once the expression of the FRET plasmids is confirmed, they can be tested in flow cytometric FRET analysis. Unlike the transfection procedure described in **Subheading 3.2.1.**, transfections with a combination of FRET donor and acceptor plasmids were performed here.

3.3.1. Transfections

The transfection procedure was similar to what was described in **Subheading 3.2.1.** using FuGENE 6 (*see* **Note 5**).

Sample number	Sample description
1	2 μg of pEF6-myc-HisB
2	0.5 μg of pEF6B-p80-CFP + 1.5 μg of pEF6-myc-HisB
3	1.5 μg of pEF6B-p80-YFP + 0.5 μg of pEF6-myc-HisB

(continued)

4	1.5 μg of pEF6B-YFP-TRAF2 + 0.5 μg of pEF6-myc-HisB
5	1.5 μg of pEF6B-YFP-SODD + 0.5 μg of pEF6-myc-HisB
6	0.5 μg of pEF6B-p80-CFP + 1.5 μg of pEF6B-p80-YFP
7	0.5 μg of pEF6B-p80-CFP + 1.5 μg of pEF6B-YFP-TRAF2
8	0.5 μg of pEF6B-p80-CFP + 1.5 μg of pEF6B-YFP-SODD

3.3.2. Cell Harvesting

1. After 24–48 h, harvest cells by washing and centrifuging twice in PBS supplemented with 2% FCS in FACS tubes.
2. Resuspend cells in PBS supplemented with 2% FCS.
3. Pass cells through a nylon filter prior to analysis.
4. Keep cells at 4°C until they are ready for analysis. Alternatively, if further manipulations of the cell samples are required (such as ligand stimulation), they can be kept at room temperature.

*3.3.3. Acquisition of Events on the Flow Cytometer (see **Notes 6** and **7**)*

Cells were analyzed on a FACSVantage SE flow cytometer. Two lasers were used on the FACSVantage SE; an ILT air-cooled argon laser and a krypton laser (Spectra-Physics model 2060, Spectra-Physics, Mountain View, CA) that is equipped with violet optics. The argon laser was tuned to 514 nm for direct excitation of YFP (**Fig. 3**, laser 1). The krypton laser was tuned to 413 nm for excitation of CFP (**Fig. 3**, laser 2). Forward (FSC) and side scatter (SSC) filters were replaced with 513/10-nm bandpass (BP) filters. CFP fluorescence was detected in FL5 (P6) using a 470/20-nm BP filter. A 505LP dichroic mirror (DM) was used for separating CFP fluorescence and FRET emission from laser 2. FRET signal was detected in FL4 (P5) using a 546/10-nm BP filter. Direct YFP fluorescence was detected using a 546/10-nm BP filter in the FL1 (P3) channel but was directed to the P7 channel to allow P5-P7 interlaser compensation using the Omnicomp option (*see* **Fig. 3** and **Note 8**). Samples were collected with fluidics pressure at 30 pound per square inch (psi). This was done to shorten the pulse timing between the argon laser and the krypton laser in order to allow interlaser compensation using the standard delay module with a maximum delay of 17.5 μs. One microgram per milliliter of PI was added prior to the acquisition of events. Fifty thousand live cells were collected.

3.3.4. Analyzing the Data Using FlowJo

1. Draw live cells gate by plotting SSC against FSC **(Fig. 4A)** or PI (detected in P8) against FSC.
2. Construct a two-dimensional dot-plot of CFP fluorescence (FL5/P6) vs FRET (FL4/P5) using cells in the live cells gate **(Fig. 4B)**.

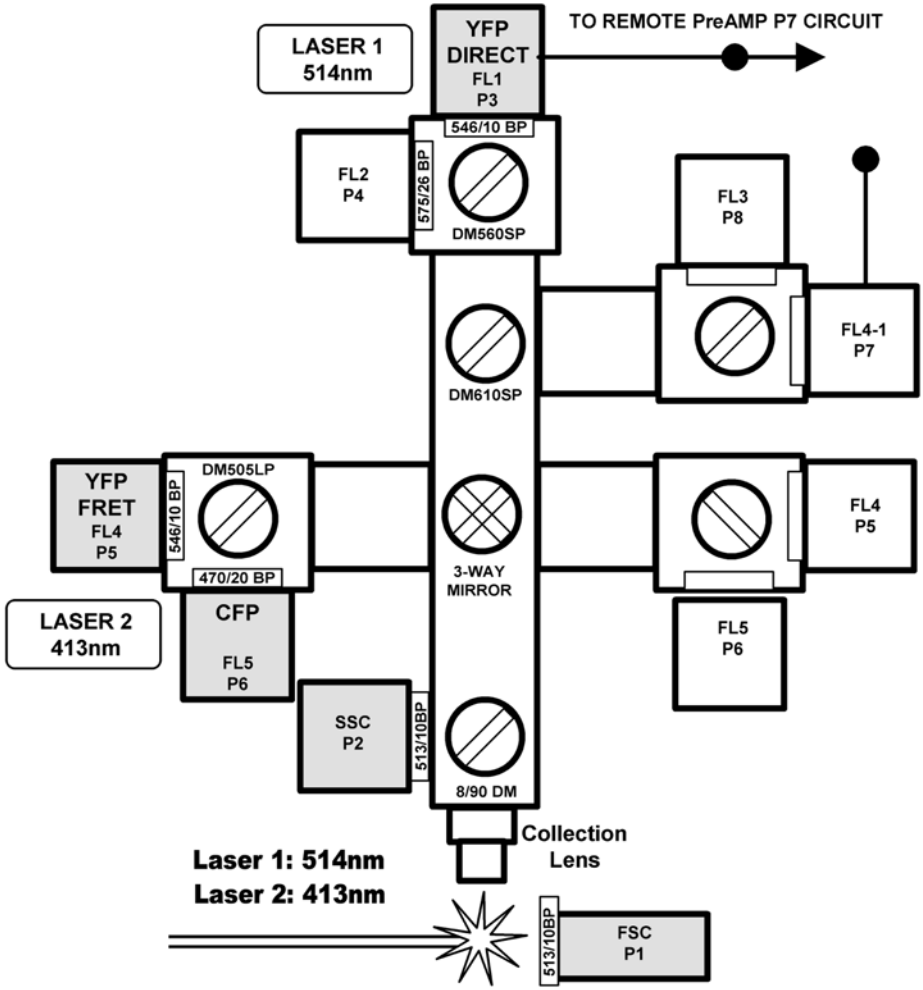

Fig. 3. Schematic setup of the FACSVantage SE flow cytometer. Only the relevant filters and mirrors are labeled. The channels that were used for FRET analysis were colored gray. Other unused channels were shown for reference only.

3. Use vector-transfected sample (sample 1; *see* **Subheading 3.3.1.**) as the negative control to set the cutoff between CFP-positive and CFP-negative populations. Draw the CFP-positive gate accordingly.
4. Using cells in the CFP-positive gate, perform histogram analysis for FRET intensity (*see* **Note 9**) (**Fig. 4C**).
5. Perform FRET histograms overlay using p80-CFP (sample 2) as the negative control (**Fig. 4D**, dashed lines). Overlay histograms from samples 6, 7, or 8 on

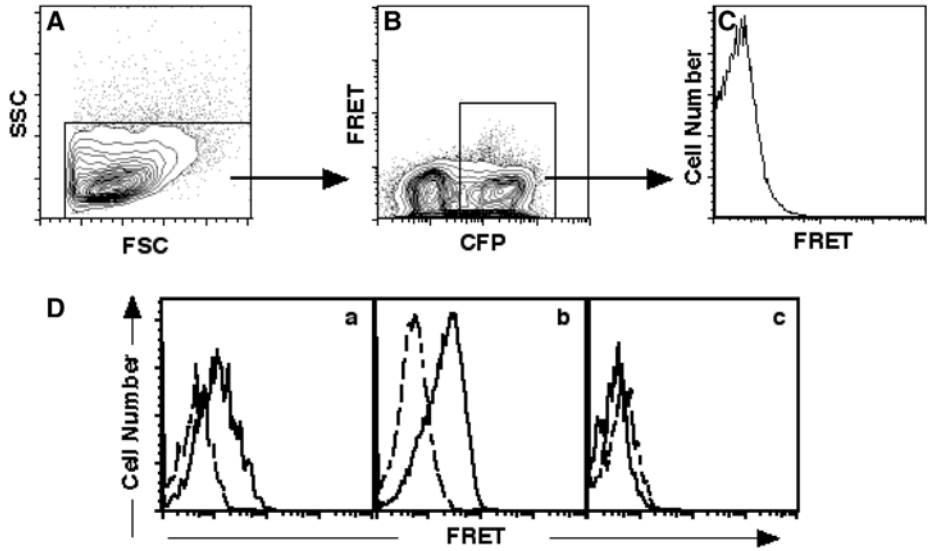

Fig. 4. Analysis of FRET data using FlowJo software. **(A)** Live cells *(box)* were determined by their forward and side scatter profile. **(B)** The cells that were in the live gate in **(A)** were analyzed for their CFP fluorescence and FRET signal on two dimensional dot-plot analysis. The transfected cells (CFP-positive cells in the *box*) were gated for histogram analysis of FRET. **(C)** Histogram analysis of FRET on CFP-positive cells from **(B)**. **(D)** Histograms overlay showed that only cells coexpressing **(a)** p80-CFP and p80-YFP or **(b)** p80-CFP and YFP-TRAF2 exhibited significant level of FRET *(solid lines)*. **(c)** Cells expressing p80-CFP and YFP-SODD did not exhibit any FRET *(solid line)*. *Dashed lines:* baseline level of FRET in cells expressing only p80-CFP.

histogram from sample 2. Expression of p80-CFP and p80-YFP (sample 6) resulted in a strong FRET signal (**Fig. 4D**, panel a). Coexpression of p80-CFP and YFP-TRAF2 (sample 7) also resulted in strong FRET signal (**Fig. 4D**, panel b). However, the noninteracting control YFP-SODD failed to generate any significant FRET signal with p80-CFP (sample 8) when cotransfected with p80-CFP (**Fig. 4D**, panel c).

4. Notes

1. The success of FRET is most dependent on two criteria: (a) the physical distance between the fluorophores and (b) the relative orientation of the fluorophores ***(18)***. In using CFP and YFP as FRET donor and acceptor molecules, the investigator should also pay particular attention to the spacer length between the CFP (or YFP) moiety and the protein polypeptide itself. Owing to the size of CFP and YFP (~30 kDa), it is not uncommon that their fusion to another protein may affect

the protein function itself. In fact, fusion of CFP and YFP to the extracellular preassembly domain of TNFRs can severely hamper the ability of the receptor to bind ligand ***(19)***. However, this effect is ameliorated by increasing the spacer length between YFP and the receptor.

2. The vector pEF6-myc-HisB, which has an EF promotor, was chosen because of its ability to drive strong expression of the protein of interest in mammalian cells. The readers should note that the pECFP and pEYFP series of plasmids from BD Biosciences Clontech can be used directly for the introduction of the cDNA insert of interest. This will save time in the cloning steps.
3. HEK 293T cells are ideal for high protein expression because of the presence of the SV40 large T-antigen. However, other cell lines including Jurkat T cells have also been used successfully in FRET analysis. For 293T cells, FuGENE 6 is the transfection reagent of choice because of its consistency in yielding high protein expression. Cell culture conditions should be carefully monitored as they can significantly affect the transfection efficiency. Typically, seeding 2.5×10^5 cells on 12-well plates will yield a 70–80% confluent culture after 16–20 h of incubation, which generally results in >50% transfection efficiency.
4. To detect protein expression for the fusion proteins, monoclonal antibody against GFP can also be used. GFP-specific monoclonal antibody from Roche Applied Science can cross-recognize both CFP and YFP in Western blotting analysis (data not shown).
5. To optimize the FRET signal, it is necessary to have the acceptor molecule in slight excess relative to the donor molecule. To achieve that, a 1:3 ratio of CFP plasmids and YFP plasmids were used in the experiments described in this chapter. However, the investigators should empirically determine the optimal DNA ratio for each pair of FRET plasmids. Because the phenol red present in most tissue culture media can sometimes affect the autofluorescence level of the cells, phenol red-free medium is recommended for culturing cells for FRET analysis.
6. The more standard 488-nm laser found in most flow cytometers can sufficiently excite the YFP protein and therefore can replace the argon laser tuned to 514 nm. However, the 488-nm laser is not optimal for CFP excitation. Therefore, a separate laser tuned to ≤440 nm is needed (*see* **Subheading 3.3.3.**) for CFP excitation.
7. The LSR II model of flow cytometer from BD Biosciences has an optional fourth laser (405-nm wavelength) that can be used for CFP excitation and therefore will be compatible for FRET analysis as well. Other models of flow cytometer may also work provided that they have the proper lasers installed. The investigators should consult the individual vendor for more information.
8. The use of flow cytometers equipped with digital electronics (such as the FACSVantage SE with FACSDiVa option) will simplify the acquisition, particularly for interlaser compensation, since the output of P3 can be compensated against P7 without redirection of the signal output.
9. In the example given in this chapter, we used two-dimensional dot-plot of CFP vs FRET to define the CFP positive gate for subsequent FRET signal analysis.

However, other ways of defining the transfected populations such as plotting CFP fluorescence versus YFP fluorescence and gating on the double positive population are plausible alternatives for determining the transfected populations from which FRET is analyzed.

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