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Determination of tumor necrosis factor receptor-associated factor trimerization in living cells by CFP→YFP→mRFP FRET detected by flow cytometry

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ABSTRACT

The availability of protein fluorophores with appropriate spectral properties has made it possible to employ fluorescence resonance energy transfer (FRET) to assess interactions between three proteins microscopically. Flow cytometry offers excellent sensitivity, effective signal separation and the capacity to assess a large number of events, and, therefore, should be an ideal means to explore protein interactions in living cells. Here, we report a flow-cytometric FRET technique that employed both direct energy transfer from CFP→YFP→mRFP and donor quenching to assess TRAF2 trimerization in living cells. Initially, a series of fusion proteins incorporating CFP, YFP and mRFP with spacers that did or did not permit FRET were employed to document the magnitude of CFP→YFP and YFP→mRFP FRET and to calculate the efficiency of CFP→YFP→mRFP two-step FRET. Based upon this, TRAF2 homotrimerization could be detected. This method should have great utility in studying the dynamics of interactions between three specific proteins *in vivo*.

INTRODUCTION

FRET (fluorescence resonance energy transfer) is a physical process in which energy will transfer from one fluorophore (donor) to another (acceptor) when the donor emission spectrum significantly overlaps the acceptor absorption spectrum and these two fluorophores are closely approximated (within 10 nm). The efficiency of energy transfer is inversely proportional to the sixth-power of the distance between donor and acceptor. Thus, FRET can be used as a molecular ruler to determine relative molecular distance, typically between two interacting proteins (1–5).

Because of the spectral properties of the GFP mutants, CFP and YFP, interactions between two proteins in living cells have been successfully revealed using FRET, which can be detected by spectrofluorimetry, confocal microscopy and/or flow cytometry (6–10). However, there are few reports of visualization of interactions between three proteins by FRET in the literature (11). One of the reasons is the difficulty in identifying a third fluorophore, which can be used as a final acceptor in a FRET chain of energy transfer (A→B→C), and will be maximally excited by the emissions from the second fluorophore, but minimally excited by those emitted from the first fluorophore. For example, the GFP-unrelated anthozoa-derived red fluorescent protein (dsRed) can be used as an acceptor for YFP-derived energy (12). However, there is significant spectral overlap between the emission of CFP and the excitation of dsRed and, therefore, this fluorophore is not useful to detect CFP→YFP→dsRed specifically as a linked two-step FRET process. Additionally, dsRed as well as a second anthozoa-derived protein, far-red HcRed are slow to mature in cells and have limited utility for FRET because they form oligomers in living cells (13,14).

Recently, a monomeric mutant of dsRed, monomeric RFP (mRFP) has been generated and appears to be useful for FRET studies (14). Of importance, excitation and emission maxima of mRFP occur at 584 nm (from 520 to 630 nm) and 607 nm (from 580 to 700 nm), respectively. As a result, it is likely to be a FRET acceptor for YFP-derived energy, but only minimally for energy from CFP. Therefore, using mRFP as a final FRET acceptor, we hypothesized that it would be possible that CFP→YFP→mRFP linked FRET could be employed to detect physical interactions between proteins that form trimers in living cells. Recently, the method

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of using CFP, YFP and mRFP, and confocal microscopy to detect linked CFP→YFP→mRFP FRET as an indicator of heterotrimer formation in living cells has been described (11).

In the present study, we used flow cytometry to detect two-step linked FRET employing CFP, YFP and mRFP. Unlike confocal microscopy, flow cytometry has the advantages of being a population-based analysis, having independent laser pathways that permit precise signal discrimination, and also permitting the analysis of large numbers of events rapidly in unfixed living cells. Because FRET could be detected both by direct energy transfer as well as by donor quenching, the efficiency of energy transfer could be accurately assessed. We developed a six-color three-laser flow cytometric system in which CFP→YFP FRET (FRET1), YFP→mRFP FRET (FRET2) and CFP→YFP→mRFP FRET (two-step-FRET) could be simultaneously detected when each fluorophore was incorporated into a fusion protein (CFP-YFP-mRFP) or fused to TRAF2. Using this approach and a series of fusion proteins, we report that FRET between YFP and mRFP is readily detectable, and occurs with significantly greater efficiency than between CFP and mRFP. With this system, two-step FRET was detected in the cells transfected with CFP-YFP-mRFP, but not those transfected with a construct in which a spacer (10 nm) was positioned between either CFP and YFP or YFP and mRFP. Employing this system, TRAF2 was demonstrated to form spontaneous homotrimers *in vivo*. The ability to employ flow cytometric FRET to detect interactions between three proteins in living cells should be of great value in assessing the nature of protein interactions that control cellular function *in vivo*.

MATERIALS AND METHODS

Plasmid construction

CFP-TRAF2 and YFP-TRAF2 plasmids were described previously (7). mRFP-TRAF2 was prepared by inserting the TRAF2 fragment directly into the BglII and HindIII sites of mRFP-C1 vector, which was prepared by a standard PCR technique using mRFP as a template and primers 5′-GCG CTA CCG GTC GCC ACC ATG GCC TCC TCC GAG GAC GTC-3′ and 5′-CGC TCC GGA GGC GCC GGT GGA GTG GCG-3′ into the AgeI and BspEI sites of pEYFP-C1 (Clontech, Palo Alto, CA). The CFP-YFP and CFP-TRAF2TD-YFP fusion plasmids were described previously (2,3). In order to make CFP-YFP-mRFP and CFP-TRAF2TD-YFP-mRFP constructs, the mRFP fragment that was amplified by PCR using mRFP-C1 as a template and a pair of primers (the forward primer mRFP-HindIII 5′-GCT CAA GCT TCG ATG GCC TCC TCC GAG GAC GTC-3′and the reverse primer mRFP-KpnI 5′-CGC GGT ACC TTA GGC GCC GGT GGA GTG GCG-3′) was digested with HindIII and KpnI first and then directly cloned into the same sites of the CFP-YFP and CFP-TRAF2TD-YFP fusion constructs, respectively. The CFP-YFP-TRAF2TD-mRFP was cloned by inserting the HindIII-digested TRAF2 TRAF domain PCR fragment, which was obtained using the forward primer 5′-GCT CAA GCT TCG GAG AGC CTG GAG AAG AAG ACG GCC-3′ and the reverse primer 5′-ATT CAA AGC TTG GAA CCC TGT CAG GTC CAC AAT GGC-3′, directly into the same site of the CFP-YFP-mRFP construct.

Cell culture and transfection

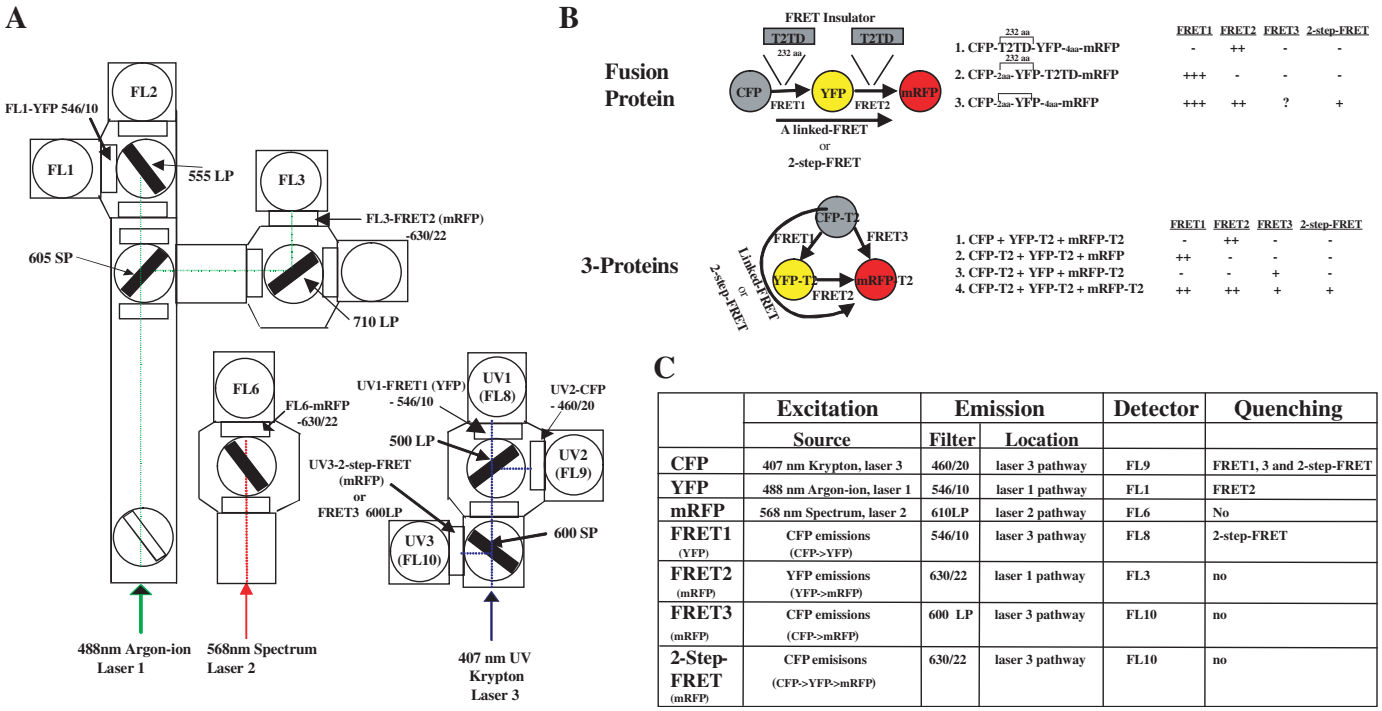
Hela and 293 cells were purchased from ATCC (Manassas, VA). The methods of cell culture and transfection were described previously (2,3). All the data presented in this study were obtained with Hela cells, but similar results were obtained with 293 cells.

FRET detection by flow cytometry

All flow cytometric data were collected with the optical configuration shown in Figure 1A using a MoFlo cytometer (DakoCytomation, Fort Collins, CO). The optical configuration for measurement of FRET (FRET1) between CFP and YFP was described previously (2,3). Briefly, the argon-ion 488 nm laser lines at 150 mW and the krypton-ion UV 407 nm laser lines at 30 mW were employed to excite YFP and CFP, respectively. YFP signals were collected using a 546/10 nm bandpass filter in the primary laser pathway (laser 1, FL1). CFP signals were collected using a 460/20 nm bandpass filter in the third laser pathway (laser 3, FL9). FRET1 signals directly emitted from YFP during CFP→YFP FRET were collected using a 546/10 nm bandpass filter in the third laser pathway (UV1-FL8). To study FRET (FRET2) signals between YFP and mRFP, a 630/22 nm bandpass filter was used to detect emission signals from mRFP in the primary laser pathway (laser 1, FL3) whereas mRFP was excited by 568 nm lines emitted from the spectrum laser at 30 mW power and its emission was monitored by the signals detected in the second pathway (laser 2) using a 630/22 bandpass filter (FL6). The detector in the UV1-FL10 position of the UV-laser pathway was also used to collect either two-step-FRET signals emitted from mRFP during CFP→YFP→mRFP FRET or possible FRET3 signals emitted from mRFP during CFP→mRFP FRET using a 600 nm long-pass filter. All data were analyzed using Summit software (DakoCytomation, Fort Collins, CO).

FRET detection by confocal microscopy in a meta-mode

The method was described previously (20,21). All imaging experiments were performed on a Carl Zeiss confocal microscope equipped with an acousto-optical beamspitter, a 100 mW argon laser (457, 488 and 514 nm), 100 mW He–Ne laser with 543 nm lines and a 20 mW blue diode laser for the 405 nm excitation. When documenting meta-FRET in cells transfected with CFP-TRAF2TD-YFP-4aa-mRFP, CFP-2aa-YFP-TRAF2TD-mRFP or CFP-2aa-YFP-4aa-mRFP, the 405 nm line was used to excite the CFP component only and sensitized YFP and mRFP emissions were scattered first and collected continuously in a 12 nm interval from 400 to 660 nm. Zeiss 510 software was used to analyze FRET spectra of each cell. Only cells expressing equivalent amounts of mRFP among all transfectants were analyzed. Because YFP alone was directly, albeit minimally, excited by the 405 nm line of the blue diode laser, subtraction of the directly excited YFP emissions from the FRET signals was required. To achieve statistical significance, up to 38 individual cells were analyzed.



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	Excitation	Emission		Detector	Quenching
	Source	Filter	Location		
CFP	407 nm Krypton, laser 3	460/20	laser 3 pathway	FL9	FRET1, 3 and 2-step-FRET
YFP	488 nm Argon-ion, laser 1	546/10	laser 1 pathway	FL1	FRET2
mRFP	568 nm Spectrum, laser 2	610LP	laser 2 pathway	FL6	No
FRET1 (YFP)	CFP emissions (CFP->YFP)	546/10	laser 3 pathway	FL8	2-step-FRET
FRET2 (mRFP)	YFP emissions (YFP->mRFP)	630/22	laser 1 pathway	FL3	no
FRET3 (mRFP)	CFP emissions (CFP->mRFP)	600 LP	laser 3 pathway	FL10	no
2-Step-FRET (mRFP)	CFP emisisons (CFP->YFP->mRFP)	630/22	laser 3 pathway	FL10	no

Figure 1. Optical configuration of the DakoCytomation MoFlo cytometer and its use in simultaneously measuring three distinct FRET signals, CFP→YFP, CFP→YFP→mRFP and YFP→mRFP. (**A**) Optical configuration of the MoFlo used to detect CFP, YFP, mRFP, FRET1 (CFP→YFP), FRET2 (YFP→mRFP), potential FRET3 (CFP→mRFP) and two-step FRET (CFP→YFP→mRFP). Briefly, mRFP, an acceptor fluorophore for YFP (YFP→mRFP), was excited by the 568 nm line emitted by the Spectrum laser (laser 2) and its emission signal was collected with a 630/22 bandpass filter (FL6) in the second laser pathway. YFP is either a donor (YFP→mRFP) or an acceptor (CFP→YFP) or both (CFP→YFP→mRFP). It was excited by the 488 nm line emitted from the argon-ion laser (laser 1) and its signal was detected with a 546/10 bass-pass filter (FL1). The FRET2 signal emitted from sensitized mRFP molecules that were excited by closely approximated excited YFP moieties during YFP→mRFP FRET was collected with a 630/22 bandpass filter (FL3) in the first laser pathway. CFP, as a donor fluorophore (CFP→YFP FRET), was excited by the 407 nm line emitted from the krypton-ion laser (laser 3) and its signal was reflected with a 500 nm long-pass dichroic filter first and then detected with a 460/20 bandpass filter (FL9) in the third laser pathway. The FL8 detector in the third laser pathway was used to collect CFP→YFP FRET1 signals with a 546/10 bandpass filter. The FL10 detector in the third laser pathway was employed to collect the two-step-FRET signals emitted from mRFP in a two-step linked FRET reaction (CFP→YFP→mRFP) or possible mRFP FRET3 signals between CFP and mRFP with a 600 longpass filter, along with 600 shortpass splitter. (**B**) Fusion proteins composed of CFP, YFP and mRFP as well as TRAF2 (Ta) labeled with CFP, YFP or mRFP were used to validate the nature of the FRET signals. In the fusion protein system, the TRAF2 TRAF domain (T2TD), which forms a mushroom-shaped structure with a distance of 95 Å, as a FRET insulator, was inserted between fluorophores to prevent energy transfer. (**C**) Summary of excitation sources and emission signals for the different fluorophores (CFP, YFP, mRFP, FRET1, FRET2, FRET3 and two-step-FRET). The potential for donor quenching during the FRET process is also indicated.

RESULTS

Optical configuration by flow cytometry to detect three distinct FRET signals

CFP and YFP have been successfully used in FRET assays to document an interaction between two proteins in living cells (2). However, few reports have described a FRET assay that can reveal an interaction between three proteins *in vivo* (11) and none has employed flow cytometry. Required for detection of two-step FRET would be a third fluorophore that is excited maximally by YFP, but minimally by CFP emission. A newly cloned fluorophore, mRFP, has been employed for this purpose (11) and appears to be a good candidate because its absorption spectrum ranges from 520 to 630 nm, maximally overlapping the YFP emission spectrum (500–570 nm), but minimally overlapping the CFP emission spectrum (460–520 nm). Therefore, a specific two-step-FRET reaction using CFP, YFP and mRFP should be able to reveal interactions between three proteins in living cells. Recently, this approach has been reported to be effective to detect heterotrimerization in living cells using confocal microscopy (11). However, the linked FRET (CFP→YFP→mRFP) cannot be easily separated from the individual steps of the linked reaction (CFP→YFP FRET1 and YFP→mRFP FRET2) by confocal microscopy, without extensive mathematical modeling. Additionally, flow cytometry has the ability to compensate electronically in order to delete any bleed-over signals and thus can be used effectively to discriminate the FRET signal accurately. We, therefore, developed a flow cytometric six-color three-laser system (Figure 1A) to measure distinct signals from CFP, YFP, mRFP and four possible FRET signals, including FRET1 (FL8) emitted from YFP during CFP→YFP FRET (third laser), FRET2 (FL3) emitted from mRFP during YFP→mRFP FRET (first laser), two-step-FRET (FL10) emitted from mRFP during CFP→YFP→mRFP FRET (third laser) and possibly FRET3 (FL10) emitted from mRFP during CFP→mRFP FRET (third laser). The optical configuration to detect these signals specifically is summarized in Figure 1C. Of importance, quenching of CFP→YFP FRET1 can also be employed to assess linked CFP→YFP→mRFP two-step FRET and to calculate FRET efficiency, as has been described for CFP→YFP FRET (2).

In order to validate the system, we first generated positive and negative FRET control plasmids that were direct fusions of CFP, YFP and mRFP fluorophores with or without a FRET insulator, TRAF2 TRAF domain (T2TD), between any two of them (Figure 1B). That T2TD (tumor necrosis receptor-associated factor 2 TRAF domain) acts as a FRET blocker or insulator was previously documented (2). As shown in Figure 1B, CFP-T2TD-YFP-10aa-mRFP serves as a FRET2 (YFP→mRFP)-positive control and a FRET1 (CFP→YFP) as well as a two-step-FRET negative control, whereas CFP-2aa-YFP-T2TD-mRFP acts as a FRET1-positive control and a FRET2- as well as a two-step-FRET-negative control. In contrast, CFP-2aa-YFP-10aa-mRFP serves as a FRET1-, FRET2- and two-step-FRET-positive control.

It is important to note that the quenching of donor emission has been successfully employed with flow cytometric CFP→YFP FRET to calculate FRET efficiency as well as relative distance between two interacting molecules (2). For example, CFP emission quenches during CFP→YFP (FRET1), CFP→mRFP or CFP→YFP→mRFP FRET, whereas YFP emission quenches only during YFP→mRFP FRET (Figure 1C). Thus, CFP→YFP FRET1 signals, along with CFP and YFP signals, will be attenuated when two-step FRET (CFP→YFP→mRFP) occurs. Importantly, mRFP as a final acceptor will not be quenched during any of these FRET reactions.

Validation of more efficient FRET between YFP and mRFP than between CFP and mRFP

To assess whether FRET occurs from YFP or CFP to mRFP, we prepared direct fusion constructs between mRFP and YFP or CFP (YFP-10-aa-mRFP, YFP-2aa-mRFP and CFP-10aa-mRFP), and then transfected them into Hela cells. As shown in Figure 2A, FRET2 (YFP→mRFP) only occurred in the cells transfected with YFP-10-aa-mRFP (**4**) or YFP-2aa-mRFP (**6**), but not in those transfected with YFP-T2TD-mRFP (**3**) or those transfected with either mRFP or YFP alone (**1** and **2**). To calculate FRET2 efficiency, donor quenching was calculated by mixing cells transfected with YFP-T2TD-mRFP with cells transfected with YFP-10aa-mRFP or those transfected with YFP-2aa-mRFP and then directly examining YFP emission intensity in cells in which FRET could or could not occur (**5** and **7**). Donor YFP quenching was calculated in a plot of mRFP versus YFP, using a previously described method (2), when equal mRFP intensity was achieved by adjusting the position of R2 and R3 to encompass the FRET-positive and the FRET-negative population, respectively. The FRET2 efficiencies for the YFP-10aa-mRFP and YFP-2aa-mRFP constructs are 0.20 and 0.30, respectively.

In order to determine whether energy transfer occurs from CFP to mRFP, Hela cells were transfected with the CFP-10aa-mRFP fusion construct and then assessed for FRET by flow cytometry. As can be seen in Figure 2B, weak FRET3 (CFP→mRFP) was detected (**4**). Similarly, FRET efficiency was calculated to be 0.14 for the probe with the 10-aa linker between CFP and mRFP, which is less than that noted with YFP-10aa-mRFP (0.14 versus 0.20). As a control, we also calculated FRET1 efficiency from CFP to YFP with the same 2-aa linker between the fluorophores as the one used between YFP and mRFP (Figure 2C, **3**–**5**). The FRET1 efficiency (0.54) was much higher for the CFP-2aa-YFP probe. Thus, energy transfer is most efficient from CFP to YFP, less from YFP to mRFP and least from CFP to mRFP.

Three distinct FRET signals (CFP→YFP FRET1, YFP→mRFP FRET2 and CFP-YFP→mRFP linked FRET) can be measured simultaneously by flow cytometry

As experimental controls, several fusion constructs with or without the T2TD spacer between CFP and YFP or YFP and mRFP were prepared. As a result, only FRET2 (YFP→mRFP), but not FRET1 (CFP→YFP) or two-step FRET was detected in the cells transfected with CFP-T2TD-YFP-4aa-mRFP (Figure 3, **1**). In addition, FRET1 (CFP→YFP), but not FRET2 (YFP→mRFP) or two-step FRET, was detected in those transfected with CFP-2aa-YFP-T2TD-mRFP (Figure 3, **2**). In contrast, FRET1 (CFP→YFP), FRET2 (YFP→mRFP) and two-step-FRET were detected in the cells transfected with the intact CFP-2aa-YFP-4aa-mRFP construct with no spacer (Figure 3, **3**). Additionally, there were no FRET signals observed in cells transfected with CFP-T2TD-mRFP that has 232 amino acids between CFP and mRFP, eliminating the possibility of direct energy transfer from CFP to mRFP in the CFP-2aa-YFP-4aa-mRFP that has 271 amino acids between CFP and mRFP (data not shown).

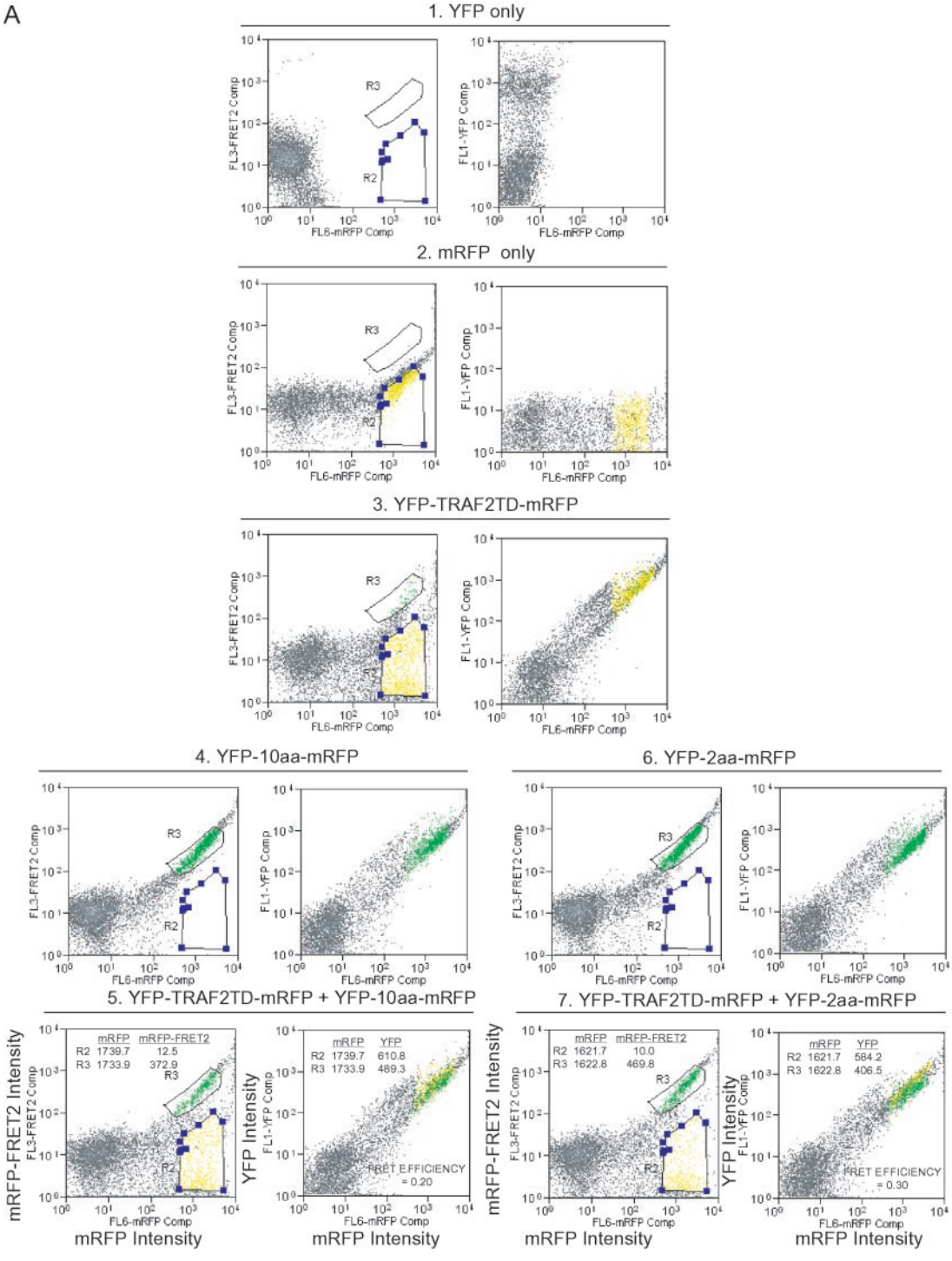
As mentioned previously, mRFP intensity remains unchanged during any of these FRET reactions. Thus, to analyze the data quantitatively, **R3** (green) with equal mRFP intensities for all transfected samples and **R4** (yellow) representing untransfected cells were gated in a plot of YFP versus mRFP, and then their FRET1, FRET2 and two-step-FRET signals were directly visualized quantitatively in histograms or in a plot of CFP versus FRET1, YFP versus FRET2 and mRFP versus two-step-FRET, respectively (Figure 3). Cells (**R3** in green) transfected with CFP-2aa-YFP-4aa-mRFP (**3**) displayed more intense two-step-FRET signals than those transfected with CFP-T2TD-YFP-4aa-mRFP (**1**) or CFP-2aa-YFP-T2DN-mRFP (**2**) (MFI: 75.0:15.3:13.3). As expected, only FRET2 (YFP→mRFP) was detected in cells transfected with CFP-T2TD-YFP-4aa-mRFP (**1**) whereas only FRET1 (CFP→YFP) was detected in those transfected with CFP-2aa-YFP-T2DN-mRFP (**2**).

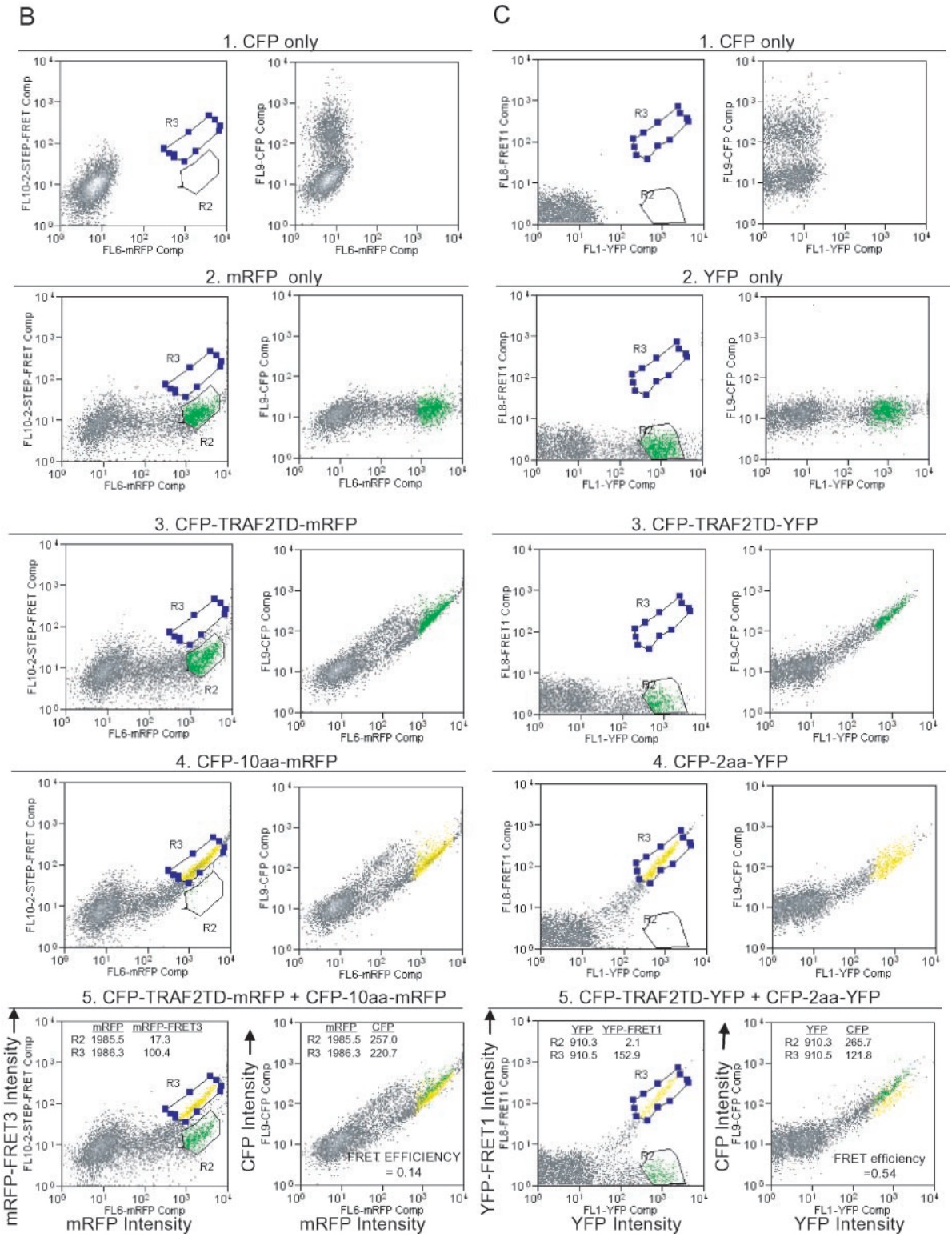
FRET efficiency was calculated using donor quenching for FRET1, FRET2 and two-step-FRET (Figure 4). By comparing CFP intensity from cells transfected with CFP-T2TD-YFP-4aa-mRFP (**1**) to that obtained from cells transfected with CFP-2aa-YFP-T2DN-mRFP (**2**), FRET1 (CFP→YFP) efficiency was calculated to be 0.50 (Figure 4A), which was very close to the value (0.54) previously determined by direct energy transfer using the same linker in Figure 2C. FRET2 (YFP→mRFP) efficiency was calculated by donor quenching by comparing YFP intensity from cells transfected with CFP-2aa-YFP-T2DN-mRFP (**2**) with those transfected with CFP-T2TD-YFP-4aa-mRFP (**1**) and was determined to be 0.24. FRET2 efficiency was also calculated by CFP→YFP FRET1 quenching by comparing FRET1 intensity in cells transfected with CFP-2aa-YFP-T2DN-mRFP (**2**) with those transfected with CFP-2aa-YFP-4aa-mRFP (**3**) and was found to be 0.20 (Figure 4B). Importantly, FRET1 quenching itself indicates the occurrence of two-step-FRET in this system. Therefore, as shown in Figure 4C, two-step-FRET

efficiency was theoretically calculated to be 0.11 (FRET1 × FRET2). Similar results were obtained when donor quenching was calculated by comparing CFP intensities in cells transfected with CFP-2aa-YFP-T2DN-mRFP (**2**) with those transfected with CFP-2aa-YFP-4aa-mRFP (**3**).

Calculation of FRET R_0

The fusion proteins can be used to calculate R_0 (the distance to achieve 50% energy transfer) between YFP and mRFP or between CFP and mRFP using the equation: $E = R_0^6/(R_0^6 + R^6)$, where E = FRET efficiency and R = the biophysical distance between fluorophores. The R_0 for a CFP-YFP FRET pair is estimated to be nearly 45 Å (1). FRET efficiency (E) for CFP-2aa-YFP was determined to be 0.54 by the donor quenching method (Figure 2C). Thus, the relative biophysical distance (R) between the CFP and YFP fluorescent 'core' is estimated to be 43.8 Å. This is close to that expected from the GFP crystal structure, in which the distance between the fluorescent 'core' and the surface was estimated to be 20–22 Å





(15–19). Therefore, the distance between CFP and YFP with the 2-aa linker can be used as a relative marker of distance between YFP and mRFP in a YFP-2aa-mRFP construct (with the same 2-aa as employed in CFP-2aa-YFP) because mRFP shares structural features with GFP (14). With the known FRET efficiency (0.30) determined by donor quenching, R_0 from YFP to mRFP is estimated to be 38.1 Å. Similarly, the distance between YFP and mRFP with a 10-aa linker was determined to be 48.0 Å. Using this set of assumptions, the R_0 from CFP to mRFP is estimated to be 35.4 Å based on the following information: $R = 48.0$ Å and $E = 0.14$ for the CFP-10aa-mRFP probe. Taken together, a CFP-YFP pair (R_0 = 45 Å) is the most efficient at energy transfer, whereas the YFP-mRFP FRET pair is less efficient

(R_0 = 38.1 Å) and the CFP-mRFP pair is the least efficient (R_0 = 35.4 Å). These data are consistent with a previously reported microscopic study (11). Similarly, with the relative distance and transfer efficiency from CFP to mRFP, the calculation could be made to estimate potential direct energy transfer efficiency from CFP to mRFP in CFP-2aa-YFP-4aa-mRFP. The efficiency is estimated to be 0.007, indicating little contribution to the signals detected from CFP→mRFP transfer.

Two-step-FRET confirmed by confocal microscopy

The method employing 405 nm excitation to detect CFP→YFP FRET by confocal microscopy was previously described (20,21), and was applied to detect CFP→YFP→mRFP two-step FRET. As can be seen in Figure 5B, CFP→YFP FRET1 was detected at 524 nm as YFP emissions in cells transfected with CFP-2aa-YFP-T2TD-mRFP (**2**) and CFP-2aa-YFP-4aa-mRFP (**3**), but not those transfected with CFP-T2TD-YFP-4aa-mRFP (**1**). FRET1 and two-step linked FRET signals can be quantitatively expressed as the ratio of the emission intensity at 524 nm (FRET1) to that at 470 nm (CFP) and the emission intensity at 600 nm (two-step FRET) to that at 524 nm (FRET1), respectively. Notably, significantly less YFP emission was detected in cells transfected with CFP-2aa-YFP-4aa-mRFP (**3**) than CFP-2aa-YFP-T2TD-mRFP (**2**) (FRET1 ratio 524/470, 1.90 versus 2.26) because of donor quenching resulting from two-step FRET. Notably, statistically significant two-step-FRET at 600 nm emission (two-step-FRET ratio 600/524, 0.19) was documented only in cells transfected with CFP-2aa-YFP-4aa-mRFP (**3**). Fifteen to thirty-eight cells were analyzed to achieve statistical significance for each transfection with representative images shown in Figure 5A. Using the donor CFP quenching method, two-step-FRET efficiency was calculated to be 14.1% (Figure 5B). Similarly, when FRET1 was used as the donor quenching parameter, two-step-FRET was calculated to be 15.9%. Both of these calculations are similar to those obtained by flow cytometry.

Two-step-FRET demonstrates TRAF2 trimerization in living cells

The next experiments examined whether this system could be used to assess interactions between three proteins in living cells. TRAF2 were chosen since it is known to form homotrimers in solution (22). As can be seen in Figure 6, FRET2 (YFP→mRFP), but not FRET1 (CFP→YFP), was detected in cells co-transfected with CFP, YFP-TRAF2 and mRFP-TRAF2 (**1**), and FRET1 (CFP→YFP) but not FRET2 (YFP→mRFP) was detected in those co-transfected with CFP-TRAF2, YFP-TRAF2 and mRFP (**2**). As a control, cells co-transfected with CFP-TRAF2, YFP and mRFP-TRAF2 (**3**) manifested a modest FRET3 (CFP→mRFP) signal (MFI of 32.7 in **3** versus 17.0 in **2** and 14.9 in **1**). However, two-step FRET was significantly greater (MFI: 58.6) in cells co-transfected with CFP-TRAF2, YFP-TRAF2 and mRFP-TRAF2 (**4**) whereas both FRET1 and FRET2 were positive (Figure 6B). FRET1 quenching was again observed in these cells (3.9 in **4** and 10.9 in **2**, Figure 6B). These data are consistent with the formation of TRAF2 homotrimers.

DISCUSSION

In the present study, we describe a six-color three-laser flow cytometric system in which three distinct FRET signals from CFP→YFP (FRET1), YFP→mRFP (FRET2) and CFP→YFP→mRFP (two-step-FRET) can be simultaneously distinguished and measured. Using this approach, direct evidence was provided for spontaneous development of TRAF 2 homotrimerization in living cells.

The use of specific fluorophores to detect two-step FRET requires that the final acceptor, mRFP, receives energy more efficiently from the intermediate, YFP, than from the initiator, CFP. With the same 10-aa spacer, energy transfer to mRFP was significantly more efficient from YFP, than from CFP (efficiency of 0.20 in YFP-10aa-mRFP versus 0.14 in CFP-10aa-mRFP). These data demonstrate that mRFP is a favored FRET partner for YFP. However, the capacity of CFP to transfer energy to mRFP indicates that energy transfer from CFP to YFP must also be monitored to obtain an accurate assessment of the degree of CFP energy that is transferred to mRFP by linked two-step FRET and, therefore, represents the interaction of three proteins. Flow cytometry is an ideal way to analyze these interactions simultaneously because the laser pathways are separated electronically.

For the first time, we describe a six-color three-laser flow cytometric optical configuration in which three distinct FRET signals (CFP→YFP FRET1, YFP→mRFP FRET2 and CFP→YFP→mRFP linked FRET) were simultaneously distinguished. With a 546/10 nm bandpass filter positioned in the FL8 UV laser pathway, the FRET1 signal (CFP→YFP) could be measured, the FRET2 signal (YFP→mRFP) was detected in FL3 (630/22) positioned in the primary laser pathway,

Figure 2. Flow cytometric profiles showing the presence of strong FRET (FRET2) between YFP and mRFP and weak FRET (FRET3) between CFP and mRFP. (**A**) FRET occurs from YFP to mRFP fluorophores when YFP is fused directly to mRFP with a 2 or 10-aa linker. Transfected cells were excited with the 488 nm line emitted from the argon-ion laser (laser1) and the 568 nm line emitted from the spectrum laser (laser2) and assessed for FRET2 (YFP→mRFP) shown on the *y*-axis or YFP emission also shown on the *y*-axis and mRFP emission shown on the *x*-axis. FRET2 (shown in **R3**) was positive in cells transfected with YFP-10aa-mRFP (**4**) and those transfected with YFP-2aa-mRFP (**6**), but not in those transfected with YFP-T2TD-mRFP (**3**) or transfected with either YFP or mRFP alone (**1** and **2**). FRET-negative control cells (**3**) were mixed with FRET-positive controls with 10-aa spacer (**4**) or with 2-aa linker (**6**) in order to determine FRET2 efficiency (**5** and **7**). FRET2 efficiency was calculated based on the donor YFP quenching from R3 to R2 where these two regions had equal amounts of mRFP acceptor intensity and significant differences in FRET2 signals. FRET efficiency was 0.3 and 0.2 for the YFP-mRFP constructs with 2-aa and 10-aa linkers between YFP and mRFP, respectively. FRET2 mean fluorescence intensity (MFI) for each region is shown in each panel. (**B**) Weak FRET3 was detected between CFP and mRFP. Transfected cells were excited with the 407 nm line from krypton-ion laser (laser3) and the 568 nm line from the spectrum laser (laser2) and assessed for potential FRET3 (CFP→mRFP) shown on the *y*-axis or CFP also on the *y*-axis and mRFP shown on the *x*-axis. The same 10-aa linker used in the YFP-10-aa-mRFP construct was inserted between CFP and mRFP and this fusion protein (CFP-10aa-mRFP) was examined for CFP→mRFP FRET3 detected as FRET3 (**R3**) in FL10 (**4**). Positive FRET3 was detected between CFP and mRFP, but with less FRET efficiency (0.14, **B5**, determined by mixing cells transfected with CFP-10aa-mRFP and those transfected with the FRET3-negative control CFP-T2TD-mRFP) than was noted between YFP and mRFP (0.20, **A5**). (**C**) As a control, strong FRET1 was noted between CFP and YFP when these two fluorophores are fused with a 2-aa linker. FRET1 was detected in cells transfected with CFP-2aa-YFP with a FRET1 efficiency of 0.54 (**5**), but not in those transfected with CFP-TRAF2TD-YFP (**3**) or with either CFP or YFP alone (**1** and **2**).

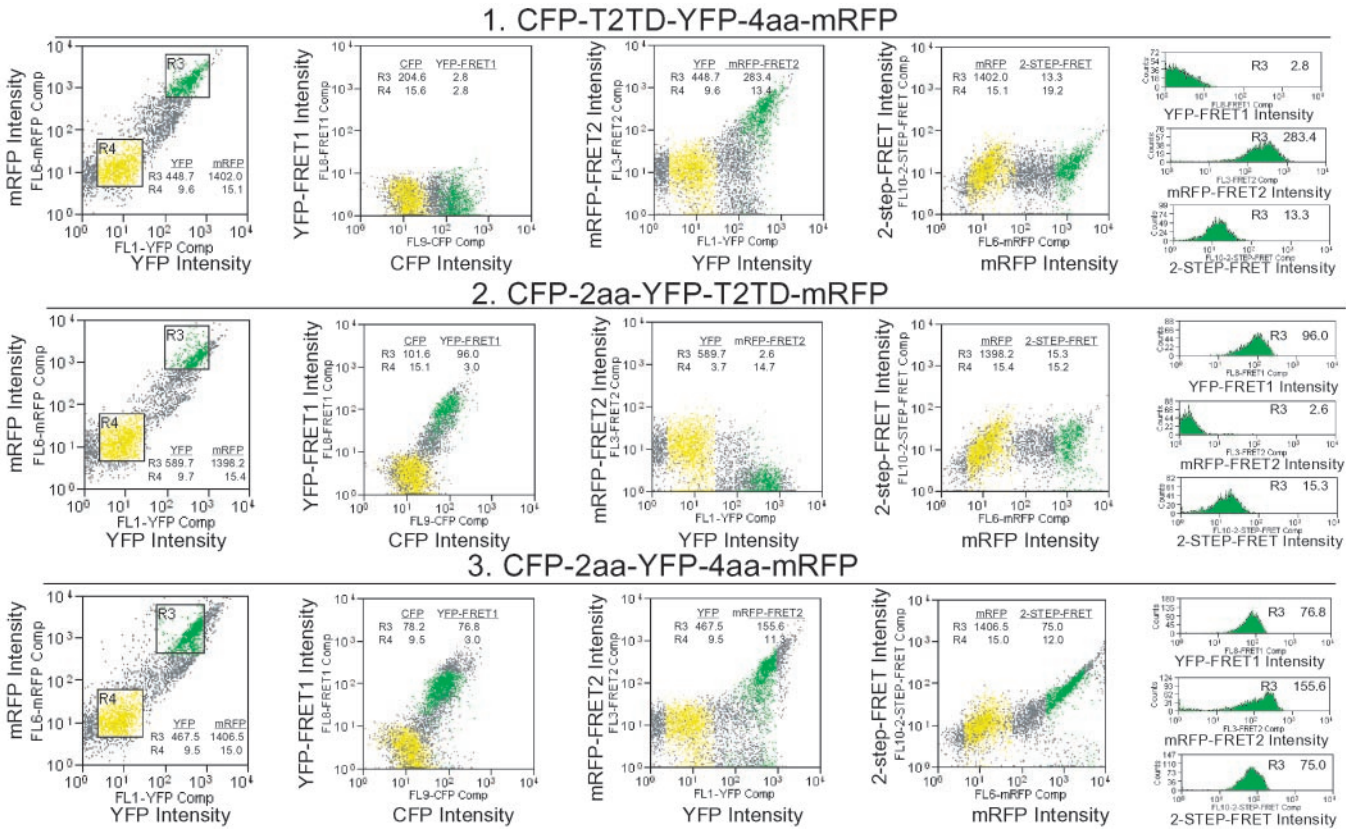


Figure 3. Validation of the two-step FRET measurement in living cells by flow cytometry using the CFP-YFP-mRFP fusion proteins. Signals of FRET1 (CFP→YFP), FRET2 (YFP→mRFP) and two-step-FRET or potential FRET3 (CFP→mRFP) were measured simultaneously using flow cytometry as described in Figure 1. All FRET1, FRET2 and two-step-FRET measurement were carried out in cells transfected with CFP-2aa-YFP-4aa-mRFP (**3**). Only FRET2 was detected in the cells expressing CFP-T2TD-YFP-4aa-mRFP (**1**) whereas only FRET1 was detected in the cells expressing CFP-2aa-YFP-T2TD-mRFP (**2**). Region 3 (R3, in green) and region 4 (R4, in yellow) were gated with equal mRFP intensity in all transfectants as positively transfected cells and negatively transfected cells, respectively. The MFIs for FRET1, FRET2 and two-step-FRET are shown for each FRET panel. The histograms are shown in the right-hand side panels.

whereas two-step FRET was measured in FL10 (600LP) in the UV laser pathway. As expected, FRET1, FRET2 and two-step linked FRET were positive in the cells expressing CFP-2aa-YFP-4aa-mRFP, whereas only FRET1 or FRET2 was detected in those expressing CFP-2aa-YFP-T2TD-mRFP and CFP-T2TD-YFP-4aa-mRFP, respectively. Similarly, two-step linked FRET signals were detected to a significant degree in cells co-expressing CFP-TRAF2, YFP-TRAF2 and mRFP-TRAF2 compared with those co-expressing CFP-TRAF2, YFP and mRFP-TRAF2 although weak energy transfer from CFP-TRAF2 to mRFP-TRAF2 was detected. The ability to measure these various modes of energy transfer made it possible to confirm that homotrimerization of TRAF2 occurred in these cells in an unambiguous manner. Since flow cytometry summates fluorescence in a specific cell, it was not possible to identify the stoichiometry of trimer formation quantitatively. The high proportion of cells in which two-step-linked FRET occurred, the relative uniformity of the linked FRET signal, along with the consideration that only 22% of trimers containing TRAF2-CFP would also contain a TRAF2-YFP as well as a TRAF2-mRFP suggested the possibility that higher order TRAF2 interactions might occur. Consistent with the conclusion that TRAF2 formed multimeric structures, microscopic imaging studies showed that TRAF2 forms visible punctate dots in the cytoplasm of transfected cells (7). These data suggest that TRAF2 might form larger oligomeric structures in the transfected cells.

Donor quenching is an important means to quantify FRET between two flurophores when they are approximated (11). In CFP→YFP→mRFP two-step linked FRET, the CFP→YFP FRET1 is intermediate and would be quenched as a result of subsequent FRET1→mRFP FRET. Calculating the efficiency of the two-step linked FRET can be accomplished by calculating CFP quenching or CFP→YFP FRET1 quenching. Combined with the measurement of direct energy transfer, donor quenching analysis confirmed unequivocally that two-step FRET was being measured and also showed that the accuracy of the analysis was independent of the optical pathway employed. Moreover, the use of donor quenching permitted an accurate assessment of the efficiency of energy transfer between the three fluorophores and an assessment of the relative biophysical distance between the fluorophores. This coincided with the distances documented by structural studies and provided an important internal control for the validity of the approach. It needs to be emphasized that the observance of two-step linked FRET and the FRET efficiency calculation based on donor quenching are more accurate using the fusion proteins than the system in which CFP, YFP or mRFP are fused to the protein of interest, such as TRAF2, as examined here.

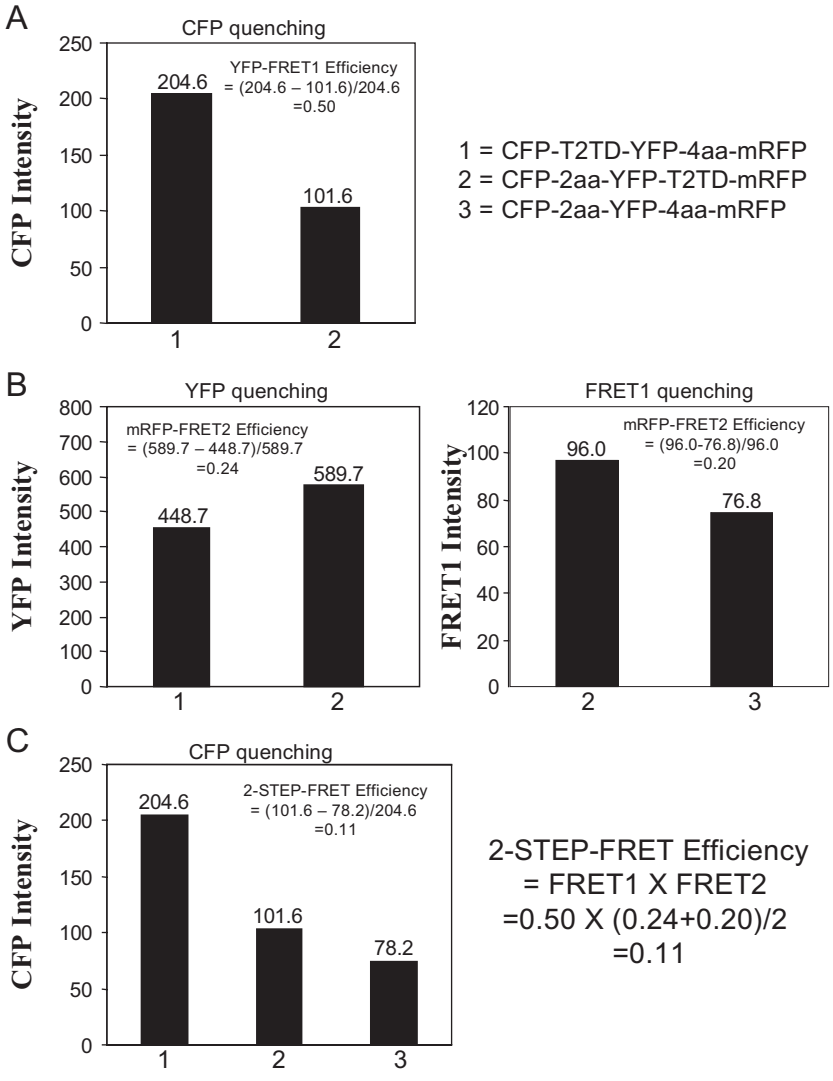


Figure 4. Calculations of FRET1, FRET2 and two-step-FRET efficiency by directly visualizing donor quenching. (**A**) CFP donor quenching was used to calculate the FRET1 efficiency from CFP to YFP with a 2-aa linker. CFP donor quenching was obtained by subtracting the intensity of CFP emission in cells transfected with CFP-2aa-YFP-T2TD-mRFP (**2**) from that in cells transfected with CFP-T2TD-YFP-4aa-mRFP (**1**) and the difference was used to calculate FRET1 efficiency. FRET1 efficiency is 0.50 for the 2-aa liner between CFP and YFP, and similar to that shown in Figure 2C. (**B**) The calculation of FRET2 efficiency from YFP to mRFP with 10-aa linker by visualizing YFP donor or FRET1 donor quenching. YFP donor quenching during YFP→mRFP was obtained by comparing YFP emission intensity between cells transfected with CFP-T2TD-4aa-mRFP (**1**) and those transfected with CFP-2aa-YFP-T2TD-4aa-mRFP (**2**). FRET1 quenching was observed by comparing FRET1 intensity between cells transfected with CFP2aa-YFP-T2TD-4aa-mRFP (**2**) and those transfected with CFP-2aa-YFP-4aa–mRFP (**3**). Both YFP and FRET1 quenching were used to calculate the efficiency of FRET2 from YFP to mRFP with a 4-aa linker, and both calculations produced a similar value. (**C**) The calculation of two-step-FRET efficiency by CFP quenching or simply by FRET1 efficiency times FRET2 efficiency that were obtained previously was comparable. CFP quenching because of two-step-FRET contribution was obtained by subtracting CFP intensity in cells transfected with CFP-2aa-YFP-4aa-mRFP (**3**) from those transfected with CFP-2aa-YFP-T2TD-mRFP (**2**). Based on CFP loss, two-step-FRET efficiency is 0.11, which is the same as determined by FRET1 times FRET2 efficiency.

It should be noted that distinguishing three distinct FRET processes can be easily and rapidly achieved using flow cytometry because flow cytometry detects the signals in physically separated laser pathways. In addition, flow cytometry uses electronic compensation to delete bleed-over signals, making it possible to assess FRET immediately during sample acquisition. These features afford considerable advantage over confocal microscopy, which mostly depends only on filters to distinguish signals and also cannot be used to isolate cells based upon FRET. Although acceptor photobleaching can be employed to enhance the ability to detect FRET accurately by confocal microscopy, donor quenching can be employed by flow cytometry to quantitate FRET accurately. Because of these features and the ability to analyze a large number of cells without bias and the capacity to sort cells based on their FRET properties, flow cytometry becomes an excellent means to assess two and three protein FRET accurately and efficiently.

In summary, we have for the first time provided direct evidence to demonstrate TRAF2 trimerization in living cells using a CFP→YFP→mRFP FRET assay and flow cytometry. With this flow cytometric optical configuration, three distinct FRET signals (CFP→YFP, CFP→YFP→mRFP and YFP→mRFP) can be measured simultaneously in a high throughput

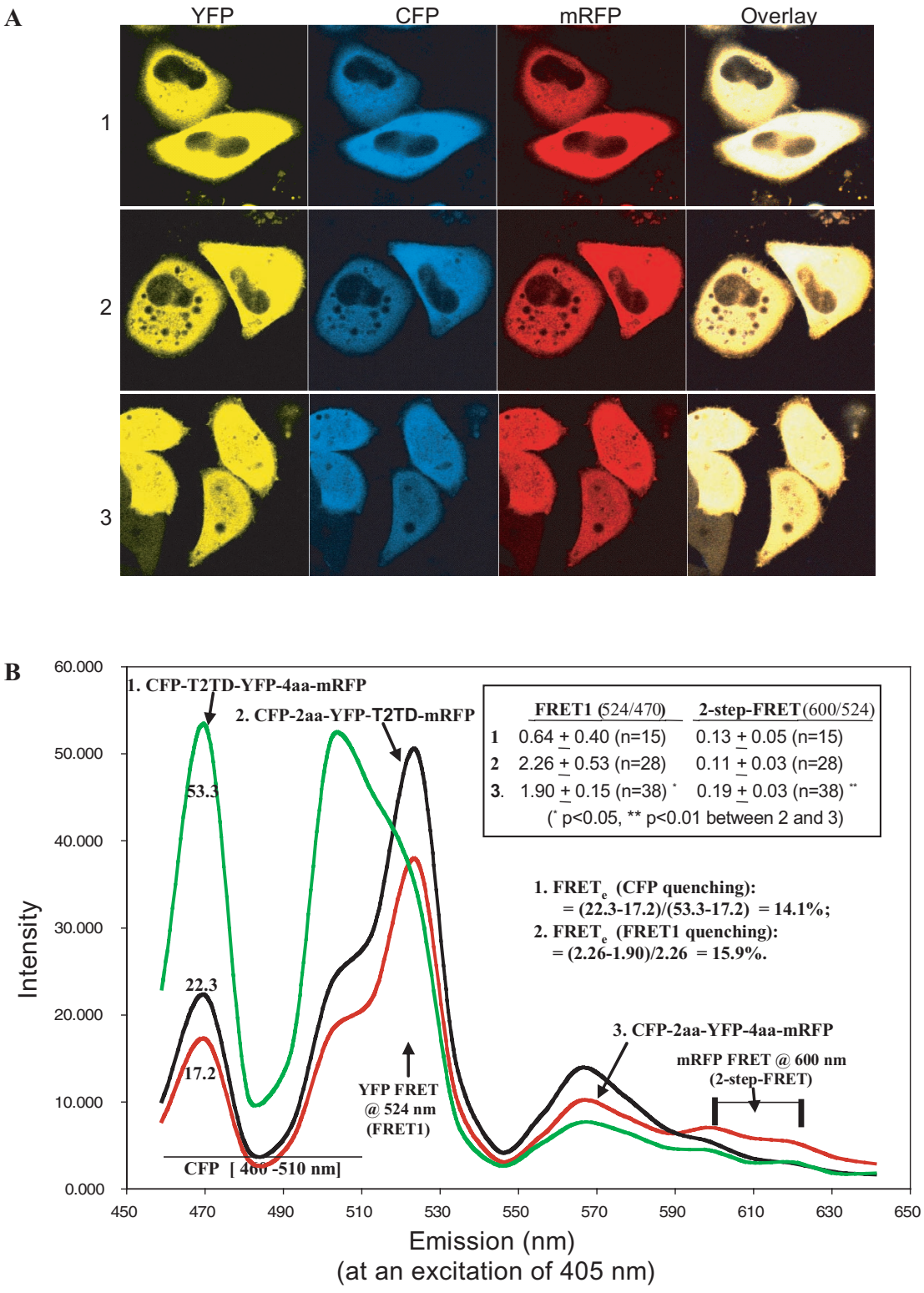
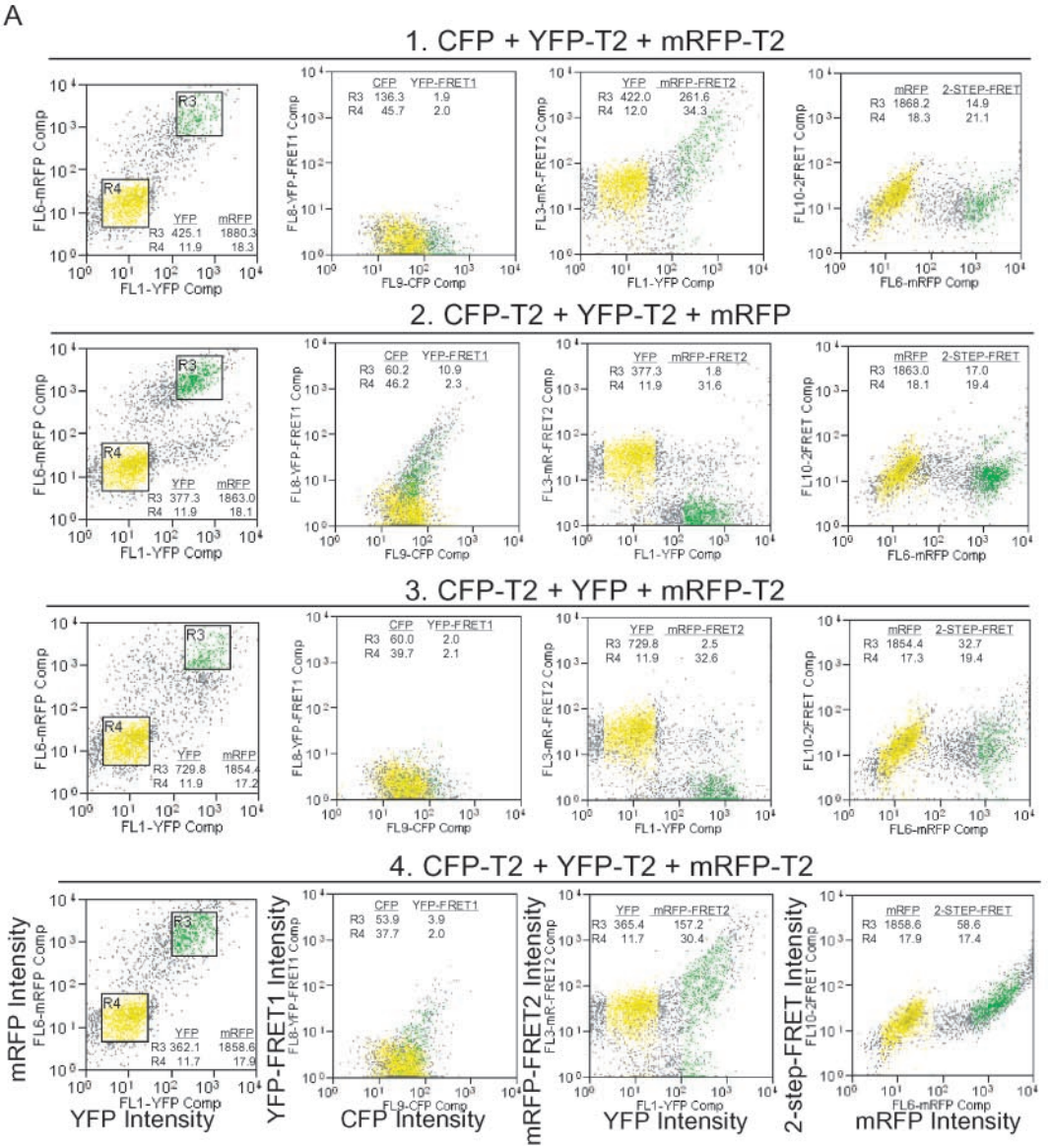


Figure 5. Validation of linked FRET in the CFP-YFP-mRFP fusion protein using confocal microscopy. (**A**) Images of YFP, CFP, mRFP and their overlays in cells transfected with CFP-T2TD-YFP-4aa-mRFP (**1**), CFP-2aa-YFP-T2TD-mRFP (**2**) or CFP-2aa-YFP-4aa-mRFP (**3**). CFP was excited with the 405 nm line and its emissions were collected with a 480/20 filter. YFP was excited with the 488 nm line and its emissions were collected with a 520/20 filter, whereas mRFP was excited with 543 nm and its emissions were collected with a 600 LP filter. (**B**) The spectra of the mean emissions ranging from 460 to 640 nm for cells transfected with CFP-T2TD-4aa-mRFP (**1**), CFP-2aa-YFP-T2TD-mRFP (**2**) or CFP-2aa-YFP-4aa-mRFP (**3**). Two valleys at 488 and 543 nm resulted from the use of 488 and 543 filters in order to delete laser scatter noise in the pathway. The sensitized YFP or FRET1 emissions and two-step-FRET emissions are indicated by an arrow at 524 and 600 nm, respectively. A large number of transfected cells were analyzed for the FRET1 ratio (524/470 nm) and the two-step-FRET ratio (600/524 nm). The occurrence of FRET1 quenching and two-step-FRET signals was noted in cells transfected with CFP-2aa-YFP-4aa-mRFP (**3**) compared to those tranfected with CFP2aa-YFP-T2TD-mRFP (**2**). CFP intensity for each of the transfectants is indicated at 470 nm in the panel.



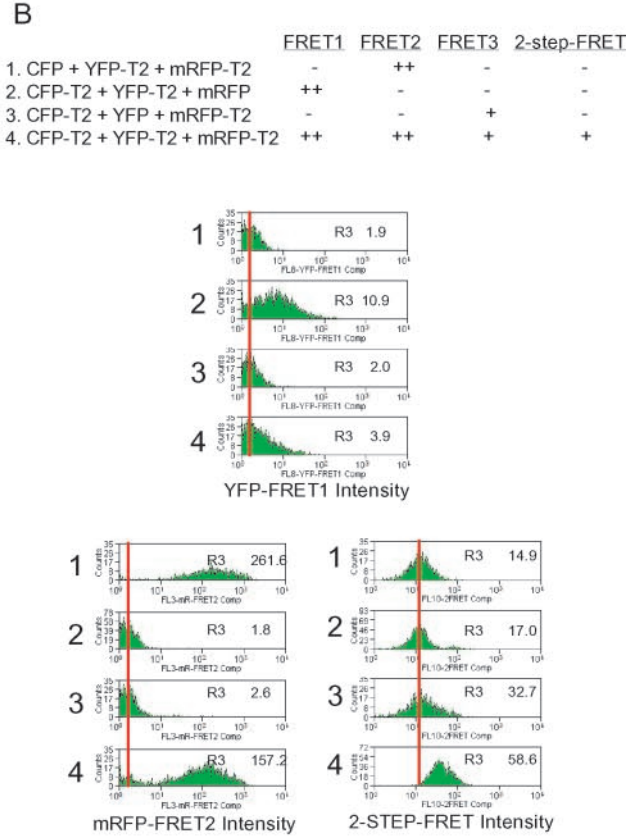


Figure 6. Validation of TRAF2 homotrimer formation in living cells by positive two-step FRET. (**A**) Flow cytometric profiles showing simultaneous measurements of FRET1 (CFP→YFP), FRET2 (YFP→mRFP) and two-step-FRET (CFP→YFP→mRFP) or potential FRET3 (CFP→mRFP). Region 3 (**R3**) with equal mRFP intensity for all transfected cells and region 4 (**R4**) were gated as positively and negatively transfected cells, respectively. The positive FRET1, FRET2 and two-step-FRET signals were detected in cells co-expressing CFP-TRAF2, YFP-TRAF2 and mRFP-TRAF2 (**4**). In contrast, only FRET2 was detected in the cells co-expressing CFP/YFP-TRAF2/mRFP-TRAF2 (**1**), whereas only FRET1 was detected in the cells co-expressing CFP-TRAF2/YFP-TRAF2/mRFP (**2**). Weak positive FRET3 was detected in cells co-expressing CFP-TRAF2, YFP and mRFP-TRAF2 (**3**), indicating potential energy transfer from CFP-TRAF2 to mRFP-TRAF2. However, this transfer was significantly less than that detected in sample **4** (MFI: 32.7 versus 58.6). The MFIs of FRET1, FRET2 and two-step-FRET or potential FRET3 are shown for each FRET panel. (**B**) Flow cytometric histogram profiles showing the CFP→YFP FRET1 quenching and strong two-step-FRET signals, indicating trimerization of TRAF2 in cells co-expressing CFP-TRAF2, YFP-TRAF2 and mRFP-TRAF2 (**4**).

manner, which is not easily achieved with other approaches, such as confocal microscopy and spectrofluorimetry. This method thus provides a tool to study the dynamic process of protein–protein interactions between three proteins in living cells.

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Conflict of interest statement. None declared.

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