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# A new pair for inter- and intra-molecular FRET measurement

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**Abstract**

Fluorescence resonance energy transfer between mutant green fluorescent proteins provides powerful means to monitor in vivo protein–protein proximity and intracellular signaling. However, the current widely applied FRET pair of this class (CFP/YFP) requires excitation by expensive UV lasers, thereby hindering FRET imaging on many confocal microscopes. Further challenges arise from the large spectral overlap of CFP/YFP emission. Another FRET pair GFP/DsRed could obviate such limitations. However, the use of DsRed as a FRET acceptor is hampered by several critical problems, including a slow and incomplete maturation and obligate tetramerization. A tandem dimer mutant of DsRed (TDimer2) has similar spectral properties as those of DsRed. The rapid maturation and non-oligomerization make TDimer2 a promising substitute for DsRed in FRET experiments. Here, we have explored the possibility of using TDimer2 as a FRET acceptor for the donor EGFP. FRET was demonstrated between the EGFP–TDimer2 chimeric fusion protein. By substituting CFP/YFP in the $Ca^{2+}$-sensor cameleon with EGFP/TDimer2, dynamic changes in cytosolic free $Ca^{2+}$ concentrations were observed with 488 nm excitation under conventional wide-field microscopy. The EGFP/TDimer2 pair was further successfully employed to monitor inter-molecular interaction between Syntaxin and SNAP25. These results reveal EGFP/TDimer2 as a promising FRET pair in monitoring intra-molecular conformation change as well as inter-molecular interaction.


*Keywords:* FRET; EGFP; TDimer2; Cameleon

At early state, fluorescence resonance energy transfer [1] served mainly as an optical ruler to measure the distance of two interacting biomolecules [2]. FRET initially fostered much progress in defining biomolecular dimensions and interactions, primarily in the in vitro setting [3,4]. As the development of molecular biology, especially the discovery of green fluorescent protein [5] color mutants and the application in labeling functional proteins in living cells, FRET between genetically encoded proteins is revolutionizing widespread detection of protein–protein interactions in vivo, as they occur in single, living cells [1,6].

Among the genetically encoded fluorophores, two green fluorescent protein [5] color mutants, CFP and YFP, have emerged as the leading donor/acceptor pair for FRET experiments. These fluorophores afford reasonable spectral separation and brightness. However, the spectral properties of this pair are suboptimal for FRET in two aspects, thus limiting its full promise in experimental employment. First, the high degree of overlap between emission spectra for CFP and YFP entails substantial "cross-talk" of CFP emission in the YFP detection channel [7], thereby complicating quantification of FRET signal. Second, FRET experiments with these fluorophores require excitation of CFP,

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which requires expensive UV laser and is unavailable on most confocal microscopes [5].

A potential solution to these challenges has come up with the discovery of a growing class of sea coral fluorescent proteins that have dramatically shifted emission spectra toward longer wavelengths, some with genuinely red emission [8,9]. The advantages of this red shift for FRET experimentation are illustrated by a GFP analog from Discosoma coral, DsRed [8]. FRET pairs comprised of CFP/DsRed or GFP/DsRed manifest superb wavelength separation of donor and acceptor emission spectra, implicating minimal donor emission cross-talk in the acceptor emission channel. For example, a 600-nm longpass emission filter would sensitively report DsRed emission with little contribution from either GFP or CFP. Such selective detection of DsRed emission would greatly simplify quantification of FRET. Furthermore, the spectral characteristics of GFP/DsRed mimic those of fluorescein/rhodamine, thus permitting efficient excitation of the donor [5] by a standard Argon laser (488-nm line). However, slow chromophore maturation and oligomerization have hindered the use of DsRed in FRET applications [10,11]. Although many strategies were developed to overcome the complications of slow maturation presents in employing DsRed as a FRET partner for GFP or CFP [5], the obligate tetramerization of DsRed, with the potential for larger-scale aggregation [12,13], adds further potential complexity to the assessment of FRET.

One important approach to overcoming these problems is targeted mutagenesis to produce variant fluorophores with accelerated maturation and attenuated oligomerization. Targeted mutagenesis based on the DsRed crystal structure [14] has provided a tandem dimer DsRed variant, TDimer2, which, compared to DsRed, exhibits favorable spectral characteristics of fast maturation and non-oligomerization [14]. TDimer2 and EGFP can easily be excited at 488 nm simultaneously, which may avoid the complexity of switching wavelength and enhance the time resolving power. Another important advantage of TDimer2 is its pH-insensitivity, making TDimer2 a better fluorescence marker, especially for acidic organelles. These characteristics imply the possibility of TDimer2 as a FRET partner with GFP/CFP.

Here, we tested the possibility of using TDimer2 as a FRET acceptor for EGFP. FRET between EGFP and TDimer2 was demonstrated with EGFP–TDimer2 chimeric fusion protein. By substituting CFP/YFP in the $Ca^{2+}$-sensor cameleon with EGFP/TDimer2, dynamic changes in cytosolic free $Ca^{2+}$ concentrations were observed with 488 nm excitation under conventional wide-field microscopy. We further demonstrated intermolecular FRET signal between EGFP-Syntaxin and SNAP25-TDimer2.

## Materials and methods

### *Molecular biology*

*Construction of EGFP–TDimer2.* Mammalian expression plasmid pcDNA3.1 was purchased from Invitrogen (San Diego, CA, USA). EGFP was PCR amplified from pEGFP-N1 (Clontech, Palo Alto, CA) using the following primers: 5′-cgtaagcttccaccatggtgagcaag-3′ and 5′-tcaggatccctcttgtacagctcgtccatgc-3′ and cloned into *Hin*dIII/*Bam*HI-digested pcDNA3.1Zeo vector (Invitrogen) to get plasmid pcDNA3.1-EGFP. The plasmid pRSETB-TDimer2 was kindly provided by Dr. R.Y. Tsien (University of California at San Diego). TDimer2 was digested and ligated into pcDNA3.1-EGFP using *Bam*HI and *Eco*RI to get pEGFP–TDimer2.

*Construction of EGFP–cameleon–TDimer2.* The cDNA of the EGFP was amplified by PCR with a sense primer containing a *Hin*dIII site and a reverse primer containing a *Sph*I site eliminating the EGFP stop codon. Similarly, TDimer2 was amplified with a *Sac*I site and an *Eco*RI site introduced to the 5′ and 3′ ends of the gene, respectively. Two restriction sites (*Sph*I and *Sac*I) were introduced by PCR into the 5′ and 3′ ends of the calmodulin–M13 gene, respectively.

*Construction of EGFP-Syntaxin and SNAP25-TDimer2.* Plasmid encoding CFP-Syntaxin 1A (Stx1A) was kindly provided by Dr. Yuechueng Liu (University of Oklahoma Health Sciences Center, Oklahoma, USA). A 900-bp fragment containing the Stx1A open reading frame (ORF) was amplified with Pfu DNA polymerase (Stratagen, La Jolla, CA). The primers induced an in-frame *Bgl*II site before the start codon and a *Kpn*I site after the stop codon. The sequences of Stx1A primers were: forward 5′-gcgcagatctatgaagga ccgaacccagg-3′ and reverse 5′-ccggtaccttatccaaagatgcccccgatg-3′. The PCR product was digested with *Bgl*II and *Kpn*I, and then ligated into *Bgl*II- and *Kpn*I-digested Mammalian expression vector pEGFP-C1 (Clontech) to get pEGFP-Stx1A.

For construction of SNAP25-TDimer2 fusion construct, the TDimer2 fragment was digested with *Bam*HI and *Eco*RI from pRSETB-TDimer2, and ligated into *Bam*HI- and *Eco*RI-digested pcDNA3.1Zeo vector (Invitrogen) to produce subclone pcDNA3.1-TDimer2. The primer sequences for SNAP25 fragment were: forward 5′-ggcggtacc accaccatggcccccattgg-3′ and reverse 5′-ggcggagatctgcactacttattt cttcgtctgttttattcagc-3′. The *Kpn*I- and *Bgl*II-digested PCR fragment was ligated into *Kpn*I- and *Bam*HI-digested pcDNA3.1-TDimer2.

All constructs were verified by sequencing and the subcellular localization of expressed proteins was checked using fluorescence spectroscopy.

### *Cell culture*

INS-1 cells (passages 70–90) were cultured as previously described [15]. Cell cultures were maintained in RPMI 1640 culture medium containing 10 mM Hepes, 11.1 mM glucose, 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. INS-1 cells were passaged by trypsinization and subcultured once a week.

PC12 cells were cultured at 37 °C in a humidified $CO_2$ (5%) incubator in DMEM (Life Technology, Grand Island, NY, USA) supplemented with 5% fetal bovine serum and 10% horse serum.

### *Fluorescence imaging*

PC12 cells or INS-1 cells were grown on high refractive-index glass coverslips ($n = 1.78$) and transfected by Effectene Transfection Reagent (Qiagen GmbH, Hilden, Germany) with cDNA encoding fluorescent proteins. Cells were viewed with a fluorescence microscope system (IX70; Olympus) as described previously [16].

We took advantage of the high numeric aperture objective (APO × 100 OHR, NA = 1.65, Olympus) to take high-resolution

fluorescence images of transfected cells. Excitation light from a fiber optical coupled monochromator (Polychrome IV; TILL Photonics GmbH, Germany) was passed through a shutter that opened only during camera exposure. The wavelength selection and switch were controlled by the image acquiring software (TILL vision 4.0; Till Photonics GmbH). Images were acquired with a cooled CCD (PCO SensiCam; Germany) with pixel size of 0.067 μm at the specimen plane. Simultaneous GFP and TDimer2 imaging was conducted by placing a Dual-View Micro-Imager (Optical-Insights) between the microscope and camera. Appropriate dichroic mirror (505 DCLP from Chroma) and emission filters were used for imaging. Images were viewed, processed, and analyzed in TILL Vision (T.I.L.L.Photonics, Germany) and Adobe Photoshop (Adobe Systems).

*FRET measurements*

For FRET study, EGFP and TDimer2 chimeric proteins were imaged under fluorescence microscope. Emission within the band of 505–535 and 605–655 nm was collected for EGFP and TDimer2 fluorescence, respectively. To quantify the FRET signal, we calculated the effective FRET efficiency as [17]

$$E_A = \gamma\left[\frac{I_F - \beta I_D}{\alpha I_A} - 1\right], \quad (1)$$

where $I_A$ was obtained with 568 nm excitation and $630 \pm 30$ nm emission, $I_D$ and $I_F$ were excited with 488 nm and collected through a dual-View Micro-Imager which enabled simultaneous fluorescence collection at $520 \pm 15$ and $630 \pm 25$ nm emission. Raw spectra were corrected for background emission by subtracting spectra obtained from untransfected cell regions. $\alpha$ is the proportionality constant relating acceptor fluorescence at the acceptor excitation to the donor excitation, $\beta$ is the proportionality constant relating donor fluorescence detected at the acceptor emission relative to that detected at the donor emission, and $\gamma$ is the ratio of the extinction coefficients of the acceptor to the donor over the donor's excitation wavelength. The constants $\alpha$, $\beta$, and $\gamma$ were calibrated in our imaging system to be 0.77, 0.04, and 0.5, respectively, using cells expressing EGFP, TDimer2, or tandem EGFP–TDimer2 (each from ~25 cells).

## Results

*Spectral properties favoring TDimer2 as a FRET partner with GFP*

Whether fluorophore pairs are suitable for FRET or not is determined largely by the overlap between donor emission and acceptor excitation spectra. In this regard, the overlap region appears substantial for EGFP/TDimer2 pair. The spectral characteristics of TDimer2 are similar to those of DsRed [18]. EGFP/TDimer2 pair manifests superb wavelength separation of donor and acceptor emission spectra, implicating almost no donor emission cross-talk in the acceptor emission channel. Furthermore, the spectral characteristics of EGFP/TDimer2 permit efficient excitation of both the donor and the acceptor [5] by a standard Argon laser (488-nm line). We thus check the possibility of using EGFP/TDimer2 pair for inter- and intra-molecular FRET measurement by constructing fluorescence protein-tagged chimeras as depicted in Fig. 1.

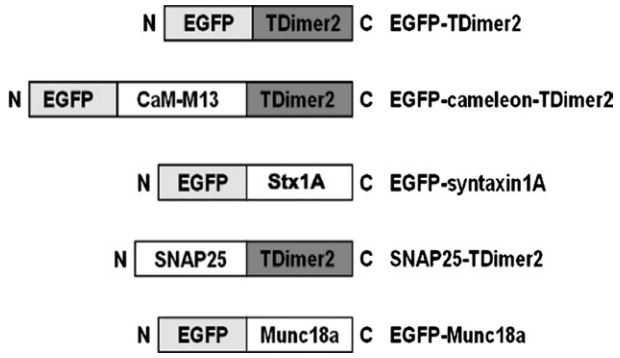


Fig. 1. A schematic representation of the constructs used in this study. Left and right sides are the N and C termini, respectively.

*The maturation time course of TDimer2 in PC12 cells*

A major challenge for using DsRed in FRET experiments is the slow maturation and tetramerization of the DsRed chromophore [10,11,19]. The non-oligomerizing TDimer2 is supposed to overcome these two critical problems associated with the wild-type DsRed. To verify the rate of maturation of TDimer2, we explicitly expressed engineered EGFP–TDimer2 concatemers in PC12 cells. As shown in Fig. 2, the EGFP fluorophore matures much rapidly than TDimer2. Shortly after transfection, the fluorescence of EGFP emission from such a concatemer was stronger than that of TDimer2, hence cells appeared green (Fig. 2A, top). However, when the associated TDimer2 fluorophore matures, the concatemer gradually changes from yellow to orange, and is finally dominated by the red fluorescence of TDimer2 (Fig. 2A, bottom). To quantify this process, we plotted the ratio between the average fluorescence of TDimer2 emission and that of EGFP as a function of time after transfection. As shown in Fig. 2B, the ratio reaches a steady level after 36 h from transfection. Thus, for the subsequent FRET measurement, the experiments were carried out 36 h after transfection.

*Demonstration of FRET between EGFP and TDimer2 with photobleaching methods*

The occurrence of FRET could be reliably determined by acceptor photobleaching. Since FRET is a non-radiative energy transfer process, the absence (or destruction) of the acceptor should result in a slightly increased emission from the donor. We selectively photobleached the acceptor TDimer2 using monochrome light at 568 nm. As expected, this maneuver significantly decreased the fluorescence of TDimer2 accompanied by concurrent increase in the fluorescence of EGFP (Fig. 3). The EGFP fluorescence increased by $40.6 \pm 2.5\%$ after photobleaching, demonstrating significant FRET in the concatemer EGFP–TDimer2. No FRET was

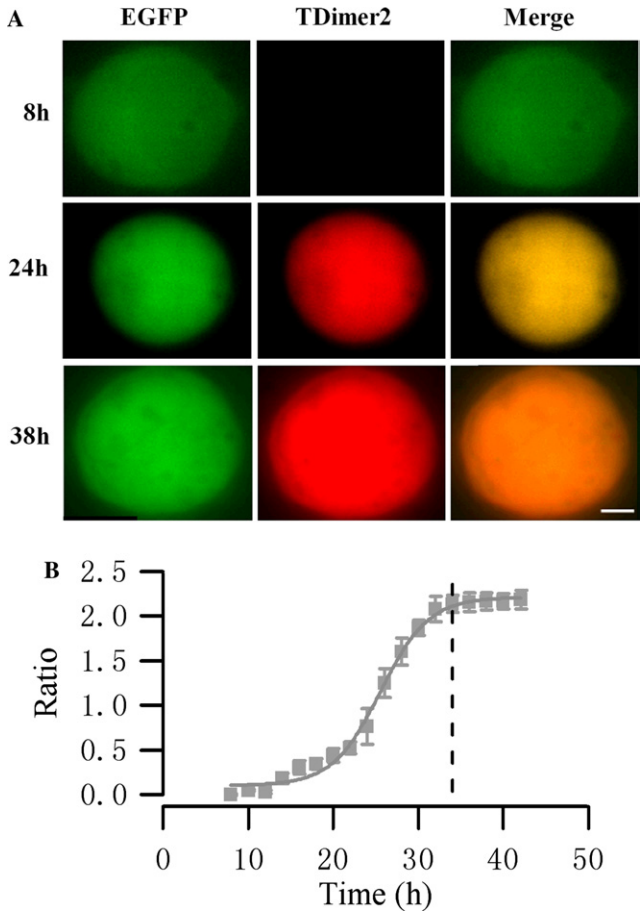


Fig. 2. Time course of the expression of EGFP–TDimer2 chimera. (A) Images of PC12 cells expressing EGFP–TDimer2 at different times after transfection. The cells exhibit from green, yellow to orange-red color, depending on the relative amounts of EGFP and mature TDimer2. Scale bar, 5 μm. (B) The averaged ratio ($n = 14$) of fluorescence intensity of EGFP to that of TDimer2 in PC12 cells. The fluorescence intensities of EGFP and TDimer2 were measured every 2 h after transfection from 8 to 42 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

observed in cells expressing EGFP and TDimer2 as separate proteins (data not shown).

### *Reporting intra-molecular FRET in a $Ca^{2+}$-dependent cameleon protein with EGFP/TDimer2*

In neuron and many endocrine cells, exocytosis could be stimulated by elevating $[Ca^{2+}]_i$. Cameleon is a FRET based fluorescent indicator to measure $Ca^{2+}$ signals in the cytosol. Binding of $Ca^{2+}$ makes calmodulin wrap around the M13 domain, hence increasing the FRET signal between the flanking GFPs [20]. We constructed a tandem fusion protein consisting of EGFP, calmodulin, the calmodulin-binding peptide M13, and TDimer2. After transfection of the chimeric protein EGFP–cameleon–TDimer2 in PC12 cells, we employed the $Ca^{2+}$ ionophore, ionomycin [21], to bring

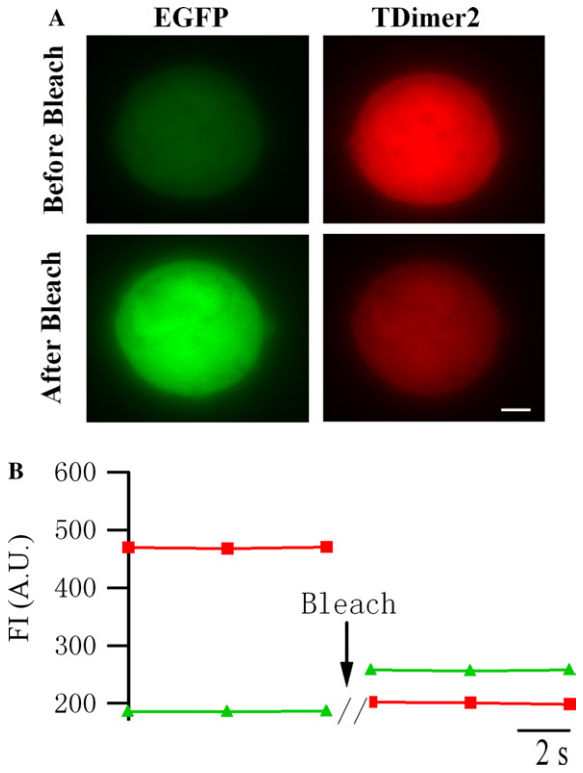


Fig. 3. Demonstration of FRET between EGFP and TDimer2. (A) The images of EGFP and TDimer2 emission from a PC12 cell expressing EGFP–TDimer2 chimera before and after photobleaching by monochrome light of 568 nm. Scale bar, 5 μm. (B) The fluorescence intensities (FI) of EGFP and TDimer2 in arbitary unit (AU) were collected before and after photobleaching. The photobleaching experiments were carried out 48 h after transfection.

$Ca^{2+}$ into the cytoplasm. The time course of the spatially averaged emission fluorescence intensities was measured before and after adding ionomycin. Prior to stimulus, the emission ratio was constant, signifying a steady state of $[Ca^{2+}]_i$. However, upon addition of 5 μM ionomycin, there was a sharp increase of fluorescence intensity of TDimer2 accompanied by a concurrent decrease in EGFP fluorescence (Fig. 4A), manifesting the increased FRET from EGFP to TDimer2 due to the conformational change of cameleon upon binding of $Ca^{2+}$. The rise in emission ratio from $1.21 \pm 0.08$ to $1.32 \pm 0.09$ ($n = 6$) thus reflects an increase in $[Ca^{2+}]_i$ (Fig. 4A). Another stimulus by addition of 60 mM KCl also induced an increase in TDimer2 fluorescence and a simultaneous decrease in EGFP fluorescence (Fig. 4B). The emission ratio between TDimer2 and EGFP changed from $1.20 \pm 0.08$ to $1.32 \pm 0.09$ ($n = 6$) upon KCl stimulus.

In a parallel experiment, we also tested the FRET between mRFP and EGFP. mRFP is another DsRed variant which will not form tetramer and exhibit much faster maturation as compared to DsRed [18]. When 60 mM KCl was added in single PC12 cells transfected with EGFP–cameleon–mRFP, we could observe a

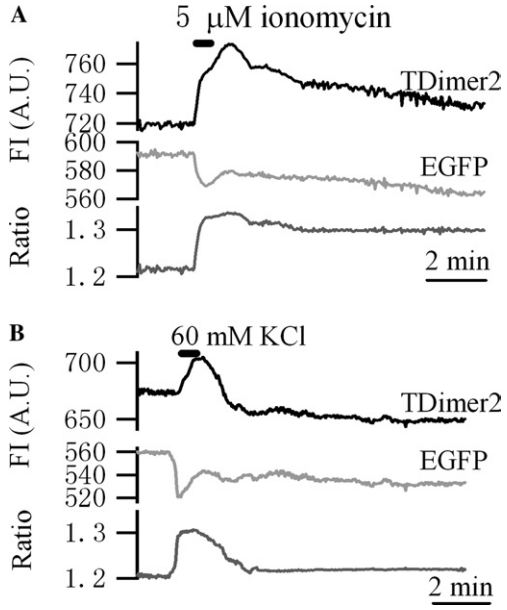


Fig. 4. $[Ca^{2+}]_i$ dynamics induced by either 5 μM ionomycin (A) or 60 mM KCl (B) in PC12 cells transfected with EGFP–cameleon–TDimer2. Displayed traces are TDimer2, EGFP fluorescence (in arbitrary unit) and the ratio between them. The fluorescence was excited at 488 nm and measured at $520 \pm 15$ and $630 \pm 25$ nm emission, respectively. The emission intensities of EGFP and TDimer2 were sampled every 4 s.

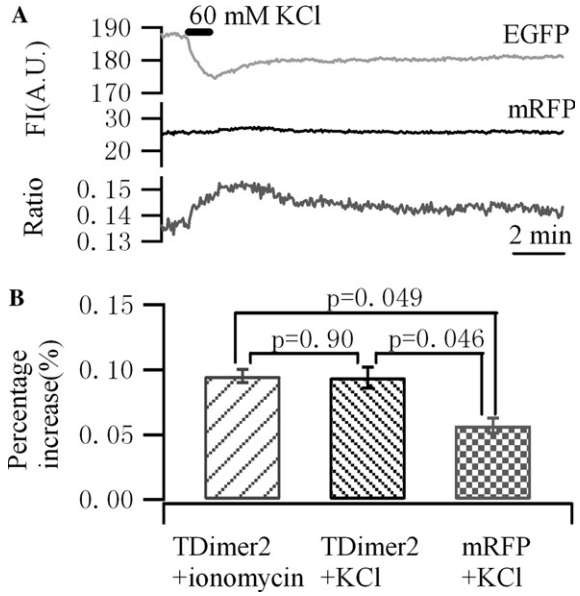


Fig. 5. mRFP displays less FRET with EGFP than that of TDimer2. (A) $[Ca^{2+}]_i$ dynamics induced by 60 mM KCl in PC12 cells expressing EGFP–cameleon–mRFP. The fluorescence intensities of EGFP and mRFP were excited and sampled as in Fig. 4. As exemplified in the graph, mRFP fluorescence displays less change which results in a less increment in the ratio between mRFP and EGFP fluorescence. (B) Summary of the percentage increase in ratio upon different stimuli. To calculate the percentage increase, the peak of the ratio during stimulation was subtracted by the pre-stimulation value and then normalized to the pre-stimulation value. Results are expressed as means and SEM from 6 to 12 cells, respectively.

significant decrease in EGFP fluorescence intensity. However, the increase in the mRFP fluorescence is less obvious as compared with TDimer2 (Fig. 5A). The emission ratio slightly increases from $0.139 \pm 0.001$ to $0.147 \pm 0.002$ ($n = 6$), which is significantly less than that using EGFP/TDimer2 pair. The result suggests that the red spectral shift of mRFP makes the energy transfer from EGFP to mRFP less efficient.

### *Quantitative FRET measurement between Stx1A and SNAP25 using EGFP/TDimer2 pair*

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) have been shown to play essential roles in vesicular fusion [22,23]. In nervous and endocrine systems, SNARE complex is a tight ternary complex composed of vesicle (v-) SNARE Synaptobrevin, and target (t-) SNAREs Syntaxin and SNAP25 [24]. In vitro binding studies have shown that SNAP-25 binds syntaxin at a 1:2 molar ratio [25]. The interaction of Stx1A and SNAP25 has been reported in vivo by FRET between CFP-Syntaxin and YFP-SNAP25 [24]. Here we use EGFP and TDimer2 to label Stx1A and SNAP25, respectively. When EGFP-Stx1A and SNAP25-TDimer2 were coexpressed in INS-1 cells, we observed colocalization of the two proteins at the plasma membrane as expected. To quantify the FRET efficiency, we collected the EGFP and TDimer2 emission with excitation at both 488 and 568 nm. Then we calculated the apparent FRET efficiency [17] between EGFP and TDimer2 according to Eq. (1). In agreement with previous study [24], we have observed a significant FRET signal between Stx1A and SNAP25, whereas Munc18a and SNAP25, as a negative control, showed no FRET signal (Fig. 6).

## Discussion

GFP and its blue, cyan, and yellow variants have found widespread use as donor/acceptor pairs for fluorescence resonance energy transfer [1] to study the interaction of proteins in vitro and in vivo. However, the leading FRET pair of CFP/YFP entails suboptimal donor excitation by Argon lasers, thereby hindering FRET imaging on many confocal microscopes. The large spectral overlap of CFP/YFP emission further complicates the quantification of FRET signal. Extending the spectrum of available colors to red wavelengths would provide a FRET acceptor that has superb wavelength separation in emission spectra from the EGFP (or a suitable variant) donor, resulting in minimal donor emission cross-talk in the acceptor emission channel.

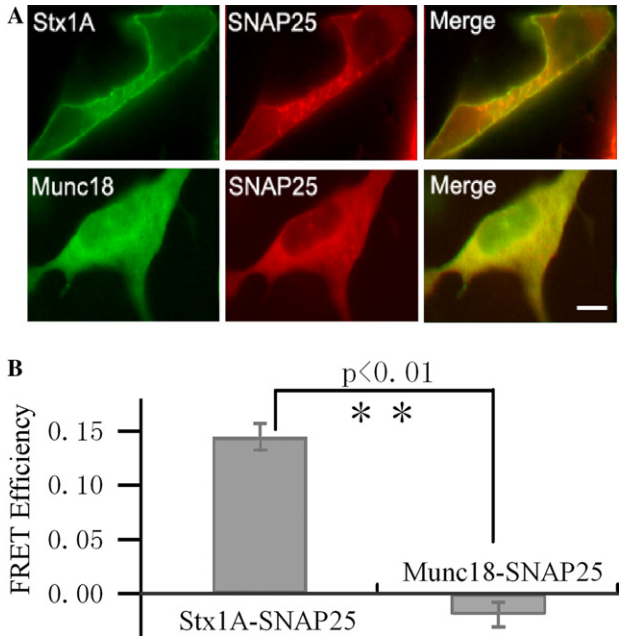


Fig. 6. In vivo inter-protein FRET measurement using EGFP/TDimer2 pair. (A) The EGFP/TDimer2 FRET pair was employed to assess the interaction between Stx1A or Munc18a with SNAP25. EGFP-labeled Stx1A or Munc18a and TDimer2-tagged SNAP25 were coexpressed in INS-1 cells, and the emission images of EGFP (green images) and TDimer2 (red images) were collected with excitation at both 488 and 568 nm, respectively, after 36 h transfection. Scale bar, 5 μm. (B) The apparent FRET efficiency calculated according to Eq. (1) was used to quantify the FRET signal. Strong FRET signal between Stx1A and SNAP25 ($n = 20$) is demonstrated, whereas no FRET signal is detected between Munc18a and SNAP25 ($n = 10$). **Significant difference ($p < 0.01$, $t$ test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

The spectral characteristics of DsRed have favored it as a good FRET partner with GFP/CFP [5]. However, the use of DsRed has been hampered by several critical problems, including a slow and incomplete maturation, and obligate tetramerization [18]. Thus, fluorophores with accelerated maturation, attenuated oligomerization, and favorable spectral characteristics are desirable for FRET experiments. Several attempts have been made to mend the rate and/or speed of maturation of DsRed [17], e.g., an engineered variant of DsRed, known as T1, has recently become available and effectively solved the problem of slow maturation. Although these strategies represent significant advances, there still remains the serious issue of oligomerization, which could complicate quantification of FRET via intricate second-order mechanisms such as homotransfer among adjacent DsRed molecules [11]. Moreover, oligomerization could disrupt native targeting or association of molecules tagged with DsRed. Thus, the tendency of DsRed tetramerization and slow maturation [11] restricts the wide application of DsRed fluorophores as an acceptor in FRET measurement.

Remarkably, targeted mutagenesis based on the DsRed crystal structure [14] has provided a tandem dimer of DsRed variant, TDimer2, which exhibits favorable characteristics of fast maturation, intense brightness, and non-oligomerization [18]. As a FRET pair EGFP/TDimer2 has better wavelength separation in donor and acceptor emission spectra than that of CFP/YFP, implicating less emission cross-talk when quantifying the FRET efficiency. Furthermore, the spectral characteristics of EGFP/TDimer2 permit efficient excitation of both the donor and the acceptor [5] by a standard Argon laser (488-nm line), which would be advantageous for time-resolved FRET measurement by sparing the alternative excitation with different laser lines.

The non-oligomerizing TDimer2 simultaneously overcomes the two critical problems associated with the wild-type DsRed. Besides its non-oligomerizing property, the rate of maturation of TDimer2 is greatly accelerated over that of DsRed. Within 24 h the transfected cells displayed strong red fluorescence. The high extinction coefficient and fluorescence quantum yield of TDimer2 [18] make it very bright and display significant FRET with EGFP. However, as TDimer2 is twice as big as EGFP or DsRed, it may interfere with the distribution, function, and fate of the labeled proteins. Regarding this point, another monomer DsRed variant, mRFP [18], which also exhibits fast maturation compared to DsRed, may serve as an alternative substitute for TDimer2. Unfortunately, mRFP showed severely attenuated brightness [18], which makes it difficult to quantify the change in fluorescence intensities. Furthermore, as compared with TDimer2, mRFP showed less FRET signal with EGFP (Fig. 5), which is likely due to the longer red shift in excitation spectrum (~30 nm) than that of TDimer2 [18]. In conclusion, we propose TDimer2 as a promising acceptor for EGFP in FRET measurement for in vivo characterizing protein conformation change and protein–protein interaction.

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