The characterization of optimized fluorescent proteins for Förster resonance energy transfer microscopy

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ABSTRACT

The genetically encoded fluorescent proteins (FP), used in combination with Förster resonance energy transfer (FRET) microscopy, provide the tools necessary for the direct visualization of protein interactions inside living cells. Currently, the FPs most commonly used for live-cell FRET studies are the Cerulean and Venus variants of the cyan and yellow FPs. However, there are problems associated with this donor-acceptor pair, and these might be overcome by exploiting the characteristics of some of the newer FPs. For example, earlier we showed that the monomeric teal FP (mTFP) has advantages over Cerulean as a FRET donor for Venus. Here, using mTFP as the common donor fluorophore, we characterize a variety of different yellow, orange and red FPs as potential acceptors of FRET. We employed a "FRET standard" genetic construct to minimize variability in the separation distance and positioning of the fused donor and acceptor FPs. Using spectral FRET imaging and fluorescence lifetime measurements from living cells expressing the fused proteins, we characterized both sensitized acceptor emission and the shortening of the donor lifetime resulting from quenching for each of the fused FP pairs. Surprisingly, we found disagreements between the spectral FRET and lifetime measurements for some of the different FP pairs. Our results appear to indicate that some of the orange and red FPs can quench the mTFP donor while yielding little sensitized emission. We are characterizing the basis for this observation.

Key words: fluorescence lifetime imaging microscopy (FLIM), fluorescence resonance energy transfer (FRET) microscopy, Teal fluorescent protein, Venus, protein-protein interactions

1. INTRODUCTION

Proteins inside the living cell interact with one-another to form transient complexes that function in the regulation of homeostasis. Biochemical methods and screening approaches are typically used to characterize the assembly of these protein complexes, but these techniques cannot detect weak or transient interactions that occur only within the context of the intact cell. Therefore, the development of non-invasive imaging techniques that can detect dynamic protein behavior in living specimens have become increasingly important for biomedical research. The genetically encoded fluorescent proteins (FPs), used in combination with fluorescence microscopy provide the tools necessary for the direct visualization of protein interactions inside living cells.[1,2] Recently, many different color FPs with optimized characteristics have become available through the cloning and engineering of proteins from marine organisms.[3-7] The genetic sequence that encodes a FP can be attached to the sequence coding for any defined cellular protein. When produced in living specimens, fluorescence microscopy can be used to monitor behavior of these fusion proteins. However, because the spatial resolution of the conventional light microscope is insufficient to directly detect specific protein-protein interactions, other techniques are required to obtain this information. One technique that can be used for this purpose is Förster resonance energy transfer (FRET) microscopy.

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1.1. FRET Microscopy

When a fluorophore is illuminated by excitation light, it behaves as an oscillating dipole that creates its own characteristic electric field. If a suitable acceptor enters the electric field, direct electric field interactions will drive the acceptor to transition to the excited state. The efficiency of the energy transfer (E_{FRET}) changes as the inverse of the sixth power of the distance (r) that separates the donor and acceptor. The sixth power dependence is described by the equation:

$$E_{FRET} = \frac{R_0^6}{R_0^6 + r^6} \quad (1)$$

where R_0 is the Förster distance at which the efficiency of energy transfer is 50%. Because E_{FRET} varies as the inverse of the sixth power of the separation distance between the probes, the efficiency falls off sharply over the range of 0.5 R_0 to 1.5 R_0.

The efficient transfer of energy requires that the donor emission spectrum strongly overlap with the absorption spectrum of the acceptor. This is described by the spectral overlap integral ($J\lambda$), which can be determined by

$$J(\lambda) = \left(\frac{SO}{Dem}\right) (\varepsilon_A \lambda) (\lambda_A)^4 \quad (2)$$

where the SO is the area of the spectral overlap and Dem is the area of the donor emission spectrum. However, the strong spectral overlap also generates significant spectral bleedthrough (SBT) background signals detected in the FRET channel. The accurate measurement of FRET signals requires the removal of both the donor and the acceptor SBT background from the signal acquired in the FRET channel.[8-10] In this regard, spectral imaging approaches, when combined with linear unmixing, are among the most accurate methods for removing the background SBT from the FRET signal.[9-10] In this study, we used spectral FRET (s-FRET) measurements to characterize different yellow, orange and red FPs as acceptor fluorophores for the monomeric teal FP (mTFP).[11,12]

1.2. Fluorescence Lifetime Imaging Microscopy

These intensity-based imaging methods measure a time averaged fluorescent signal. In contrast, fluorescence lifetime imaging microscopy (FLIM) measures the fluorescence lifetimes of fluorophores within a cell to reveal heterogeneity in the probe environments. The excited-state lifetime is an intrinsic property of a fluorophore, and is not influenced by probe concentration, excitation light intensity, or light scattering, making FLIM particularly useful for biological applications. Since FRET is a dynamic process that non-radiatively depopulates the excited state of the donor fluorophore, it can be detected by FLIM as a reduction in the donor fluorescence lifetime. There is a direct and inverse relationship between the donor fluorescent lifetime and the FRET efficiency. When donor lifetimes are determined in the absence (τ_D) and in the presence of the acceptor (τ_{DA}), and ratio of the lifetimes provides a straightforward method to estimate E_{FRET} by

$$E_{FRET} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (3)$$

The FRET-FLIM approach provides a method that is independent of intensity-based measurements to quantify energy transfer.[10, 13-15]

In this study, we used the combination of s-FRET and FRET-FLIM microscopy to test systematically several different orange and red FPs as FRET acceptor fluorophores for mTFP. Our findings appear to indicate that factors besides quantum yield and spectral overlap play a critical role in determining whether a particular pair of FPs will be useful for FRET measurements. We have observed that, despite having strong spectral overlap with mTFP, not all FPs behave as expected for FRET applications. Thus, it is important not only to consider the photophysical properties, but also to test the new FP pairings for their efficacy as FRET partners using different FRET-based assays.

2. MATERIALS AND METHODS

2.1. Expression plasmids and cell transfection

The plasmid encoding the mTFP[11] (Gene bank accession: DQ676819) is available from Allele Biotechnology & Pharmaceuticals (San Diego, California). The cDNA for Venus was obtained from Dr. Atsushi Miyawaki (RIKEN, Japan).[16] The plasmid encoding the fusion protein consisting of Cerulean tethered to Venus by a 5 amino acid (aa) linker (SGLRS) was a gift from Dr. Steven Vogel (NIH).[17] This plasmid was used to generate the mTFP fusion protein

(mTFP-5aa-Venus) by substituting the coding sequence for Cerulean with the cDNA for mTFP that was generated by PCR with primers incorporating suitable restriction enzyme sites. Site directed mutagenesis of mTFP-5aa-Venus plasmid was used to generate the mTFP-5aa-Amber. In Amber, the chromophore tyrosine was changed to cysteine, producing a protein that folds correctly, but does not act as a FRET acceptor.[17] This same parent plasmid was also used to generate the sequences that encode mTFP and the 5 aa linker coupled in-reading frame to the sequence encoding the different orange or red FPs. The cDNA sequences encoding the following FPs were used to generate the mTFP-5aa fusion protein constructs. The sequence encoding mOrange2[18] (DQ336159), mTagRFP-T[18] (EU582019), and tandem dimer (td) Tomato[5] (AY678269) are from Dr. Roger Tsien (UCSD, San Diego, California). The monomeric Kusabira orange 2[19] (mKO2) construct is available from MBL International (Woburn, Massachusetts). The monomeric Katushka[20] (mKate or TagFP635) is available from Evrogen (Moscow, Russia). All plasmid constructs were verified by automated nucleotide sequencing.

2.2 Tissue culture and transfection

Mouse pituitary GHFT1 cells[21] were maintained as monolayer cultures in Dulbecco's Modified Eagles Medium containing 10% newborn calf serum. The cells were transfected with the indicated plasmid DNA by electroporation as described earlier.[12] The amount of DNA was kept constant for each transfection using empty vector DNA. Suspensions of the transfected cells were added drop-wise onto a sterile 25 mm cover glass in culture dishes, and the cells were allowed to attach to the glass prior to gently flooding the culture dish with media. The cultures were maintained in an incubator for 24 h before imaging. The cover glass with attached cells was then inserted into a chamber, which was filled with CO_2-independent medium and placed on the microscope stage.

2.3 Spectral imaging

A Zeiss Axiovert 200M epifluorescent motorized microscope equipped with a plan-apochromat 63x oil NA 1.4 objective lens, and coupled to the Zeiss 510 confocal-multiphoton-spectral imaging system, was used for the studies described here. The system was controlled using the LSM software (version 3.5). For spectral FRET imaging, the HFT 458/514 or 488/561 beamsplitter was used in combination with the 32-channel spectral META detector and optical grating system to collect lambda stacks consisting of a series of x-y images at emission wavelengths separated by 10.7 nm steps. An algorithm that defines the spectral signature for each pixel of the scanned confocal image was used for digital separation of the component signals (ps-FRET). The corrected FRET signal (pF) was determined after linear unmixing of the donor SBT and correction for acceptor SBT based on measurements from control cells, and the FRET efficiency (E%) was determined after correction for PMT sensitivity and quantum yield using the equation:

$$E\%_{S\text{-}FRET} = \frac{c\,(pF)}{D_q + c\,(pF)} \tag{4}$$

where c is the correction coefficient for PMT sensitivity and quantum yield, and Dq is the quenched donor signal. PMT sensitivities were obtained from the manufacturer.

2.4 Fluorescence lifetime measurements

The fluorescence lifetime measurements were made using a Nikon TE300 epi-fluorescent microscope equipped with a Plan Fluor 60x NA 1.2 water IR objective lens. The microscope was coupled to the Radiance 2100 confocal multiphoton system and a 10W Verdi pumped, tunable (700 to 1,000 nm) mode-locked ultrafast (78 MHz) pulsed (150 femtosec) laser (Mira 900, Coherent, Inc.). The LaserSharp2000 multiphoton configuration was used to scan the specimen, and the photon-counting module board (SPC-730, Becker & Hickl GmbH, Berlin, Germany) in the Radiance2100 computer matched the X and Y scan synchronizing pulses with the pixel clock signal from control unit, allowing pixel-by-pixel registration of the accumulated photons with the laser scanning. The detector was a fast photomultiplier tube (PMT), with a response time of approximately 150 picoseconds (PMH-100, Becker & Hickl GmbH, Berlin, Germany). The minimum temporal resolution of the imaging system was approximately 50 picoseconds.

The excitation beam was tuned to 870 nm for mTFP, and the lifetime images were acquired using a 480/30 nm emission filter. The laser excitation power was measured at the specimen plane using a power meter (SSIM-VIS-IR, Coherent, Inc.), and was typically 0.7 to 1.5 mW. At this power, a data acquisition time of 120 sec resulted in peak photon counts sufficient for high confidence double exponential decay fitting. Fluorescence lifetimes were measured by the time

correlated single photon counting (TCSPC) method. A fluorescence decay histogram of photon emission times relative to the laser excitation pulse was generated from the distribution of inter-pulse intervals at each pixel of the image. The data analysis software (SPCImage version 2.9, Becker & Hickl GmbH) allows multi-exponential curve fitting of the acquired data on a pixel-by-pixel basis using a weighted least-squares numerical approach.

3. RESULTS AND DISCUSSION

Currently, the optimized cyan and yellow FPs Cerulean and Venus are the most commonly used FPs for FRET studies.[17] However, the attributes of some of the newer FPs suggest that they might also be useful for FRET measurements from living cells. For example, the ideal donor FP should be photostable and have a high quantum yield. Further, the donor FP should have comparatively narrow spectra and possess simple lifetime decay kinetics. The mTFP has these characteristics, and earlier studies showed that mTFP is an improvement over Cerulean as a FRET donor.[12] For intensity-based FRET measurements, the ideal acceptor FP will also be bright and photostable, and have an absorption spectrum that strongly overlaps the emission spectrum for mTFP. Venus shares a significant spectral overlap with mTFP (Figure 1), and was shown earlier to be an effective acceptor for mTFP.[12] However, Venus is not particularly photostable (Table 1) and the spectral characteristics of some of the new orange and red FPs (Table 1) suggest that they could serve as improved acceptor fluorophores for the monomeric teal FP (mTFP).

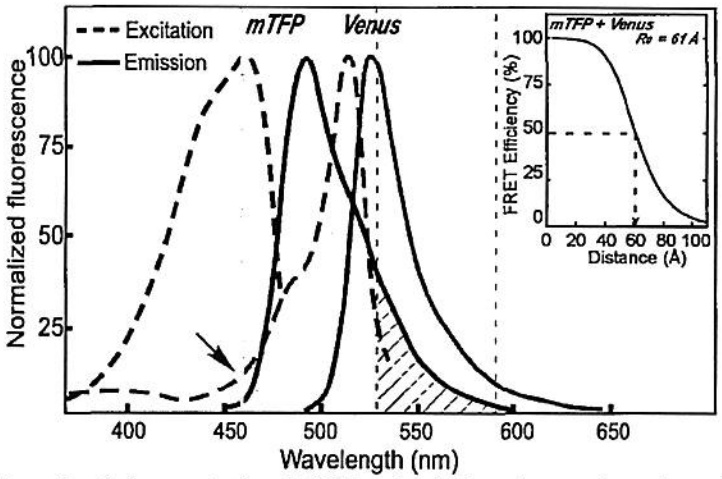

Fig. 1. The excitation and emission spectra for mTFP (donor) and Venus (acceptor) are shown, illustrating their spectral overlap (grey shaded area). The 458 nm laser line and the FRET emission channel (dashed lines at 535 nm and 590 nm) are shown, with the donor SBT into the FRET channel indicated by hatching. The arrow indicates the excitation of the acceptor by the 458 nm laser line. (Inset) The efficiency of energy transfer is plotted as a function of the separation distance, and was determined according to the equation (1). The R_0 value was determined from the overlap integral, J_λ (equation (2); *see* Table 1).

3.1. Spectral FRET measurements of the mTFP-5aa-acceptor fusion proteins

Each of the orange or red FPs chosen for this study is bright, photostable, monomeric (except the linked dimer tdTomato), and has significant spectral overlap with mTFP (Table 1). These properties suggest that each could potentially serve as an effective FRET acceptor for mTFP. To directly compare the different FPs as acceptor fluorophores, a series of plasmids was constructed that encoded each different FP linked to mTFP by the same 5 aa linker (see *Materials and Methods*). Spectral imaging was used to collect the emission spectrum for each of the individual FPs at a bandwidth of 10.7 nm using the donor excitation laser line (458 nm; Figure 2). In addition, spectral data were also obtained for each of the acceptor FPs using the available laser lines (514 nm or 561 nm; Figure 2A-C). These reference spectra were used for linear unmixing and SBT correction to determine FRET efficiency.

Each different mTFP-5aa-acceptor plasmid was then produced in living cells, and the efficiency of energy transfer was determined by s-FRET under identical conditions. The 458 nm laser line was used to optimally excite mTFP (see Figure

1), and lambda stacks were collected to generate the emission spectrum for each fusion protein. The spectral characteristics for three of the different fusion proteins are shown in Figure 2D. Each spectrum includes the quenched donor emission, sensitized acceptor (FRET) emission, as well as the donor and acceptor SBT signals. For the Teal-5aa-

Table 1. The photophysical properties of the donor and acceptor FPs used in this study.

FP	Peak Ex (nm)	Peak Em (nm)	Extinction coefficient, Quantum yield [a,b]	Photo-stability [b]	Spectral overlap (J_λ), [c] Förster Distance (R_0) [d]	Ref.
Donor						
mTFP	462	492	64 0.85	+++	NA	11
Acceptor						
Venus	515	528	92 0.57	+	4.02×10^{15} 61 Å	16
mOrange2	549	565	58 0.6	++++	3.29×10^{15} 59 Å	18
mKO2 (Kusabira)	551	565	63.8 0.62	+++	3.49×10^{15} 60 Å	19
tdTomato	554	581	138 0.69	+++	9.98×10^{15} 71 Å	5
mTagRFP-T	555	584	81 0.41	++++	3.22×10^{15} 59 Å	18
mKate	588	635	45 0.33	++++	2.53×10^{15} 57 Å	20

[a] $(\times 10^{-3})(M^{-1} cm^{-1})$.

[b] Adapted from references 5, 7, 15, 18, 20.

[c] Determined using Equation (2).

[d] Determined by: $R_0 = 0.211 [(k^2) (n^{-4}) (QY_D) (J\lambda)]^{1/6}$

The orientation factor (k^2) and index of refraction (n) used were 0.667 and 1.4, respectively.

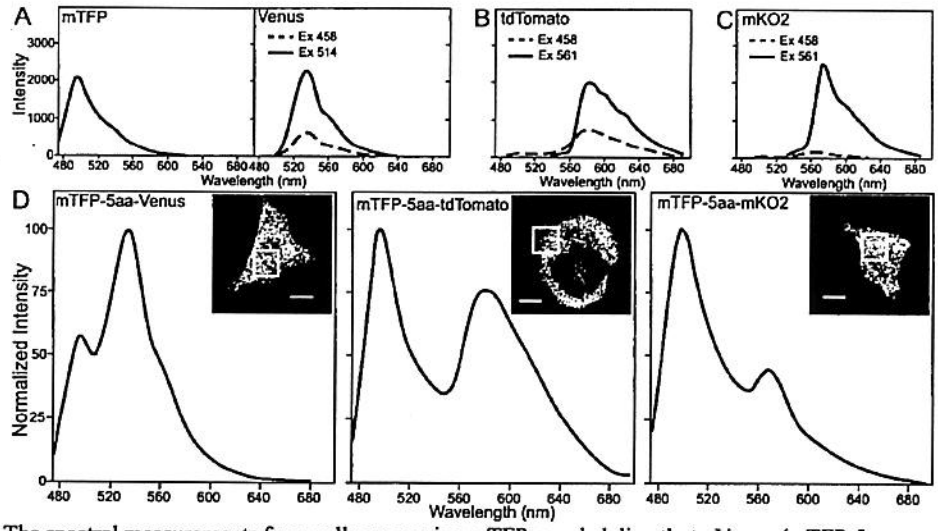

Fig. 2. The spectral measurements from cells expressing mTFP coupled directly to Venus (mTFP-5aa-Venus), tdTomato (mTFP-5aa-tdTomato), or mKO2 (mTFP-5aa-mKO2). (A) Spectral images were acquired from cells that expressed mTFP or Venus only using the 458 nm laser line. Additionally, the emission spectrum for Venus was obtained using the 514 nm laser line. (B, C) The emission spectrum was also acquired from cells that expressed only tdTomato or mKO2 using the indicated laser lines for excitation. (D) Spectral images were then acquired from cells expressing the indicated fusion proteins using the 458 nm laser line for excitation. The spectrum was measured in the boxed region of each cell; the calibration bar is 10 μm.

Venus fusion protein, there was a strong signal at the acceptor emission wavelengths (peak 528 nm) relative to the donor emission (peak 492 nm). This is consistent with donor quenching and sensitized emission from the acceptor (FRET), and this was quantified using the ps-FRET approach (Table 2). By comparison, there was lower signal at the acceptor emission wavelengths relative to the donor signal for the Teal-5aa-tdTomato fusion protein, despite the presence of the tandem acceptor fluorophores (Figure 2D). Further, an even more modest signal was detected at the acceptor emission wavelengths relative to the donor emission for the Teal-5aa-mKO2 fusion protein (Figure 2D). Since the 458 nm laser line was very inefficient for direct excitation of mKO2 (Figure 2C), this result indicated that little sensitized emission was detected from the Teal-5aa-mKO2 fusion protein.

To quantify the FRET signals from these and the other fusion proteins, the reference spectra obtained from the cells expressing mTFP alone (Figure 2A) were used for the linear unmixing to remove the donor SBT from the signals obtained from the cells that expressed the different fusion proteins. However, because the spectra for both the FRET signal and the acceptor SBT are identical (Figure 2A), other methods are required to correct for the acceptor SBT component. We developed an approach to remove the ASBT component from the spectral FRET (ps-FRET) data that is similar to other established methods for SBT correction in filter-based FRET microscopy systems.[9] The ps-FRET approach was used to quantify FRET signals from each of the different fusion proteins expressed in living cells (Table 2).

Consistent with earlier results[12] the ps-FRET measurements from cells expressing mTFP-5aa-Venus indicated strong FRET signals could be detected from this fusion protein. In comparison, the FRET efficiency for the mTFP-5aa-tdTomato fusion protein was significantly reduced (Table 2). This was surprising because the strong spectral overlap of tdTomato with mTFP, and the exceptional quantum yield of the tandem chromophores (Table 1), predicts that tdTomato would be an improved acceptor. What is more, each of the other monomeric orange and red FPs also proved to be poor acceptors for mTFP when analyzed by the ps-FRET method (Table 2). Together, these results suggest that, unlike the *Aequorea*-based Venus FP, the coral-derived orange and red FPs are either poor FRET acceptors, or alternatively, that there were problems in detecting the sensitized acceptor emission in the orange and red spectrum. To address these possibilities, we sought to verify and extend the measurements using FRET-FLIM.

Table 2. ps-FRET and FRET-FLIM measurements.

FP Pair	ps-FRET (%) [a]	τ_{DA} (ns) [a]	χ^2 [b]	E_{FRET} (%) [a,c]
Donor				
mTFP-5aa-Amber [d]	NA	2.653 - 2.752 [e]		
Donor-Acceptor				
mTFP-5aa-Venus	50.2 ± 2.6	1.358	1.05	48.8 ± 2.7
mTFP-5aa-tdTomato	32.5 ± 2.6	1.818	1.06	35.4 ± 2.6
mTFP-5aa-mKO2	18.5 ± 3.7	1.454	1.05	47.24 ± 2.6
mTFP-5aa-mOrange2	14.6 ± 3.8	2.403	1.05	12.5 ± 1.6
mTFP-5aa-mTagRFP-T	12.2 ± 2.8	1.973	1.04	28.3 ± 1.6
mTFP-5aa-mKate	15.9 ± 1.7	1.968	1.04	28.5 ± 1.9

[a] ± SD, n = 5-9 cells
[b] Fitting for double-exponential decays.
[c] Determined by equation (3); see text.
[d] Amber is Venus Y67T, which does not act as a FRET acceptor; see text.
[e] Range of lifetimes for mTFP-5aa-Amber determined for all experiments.

3.2. FRET-FLIM measurements of the mTFP-5aa-acceptor fusion proteins

Because FRET-FLIM relies on measurement of the donor fluorescence lifetime in the absence and presence of an acceptor, it provides a method for acquiring FRET measurements that is independent of the intensity-based approaches described above. We used 2p excitation and the TCSPC method to acquire the donor fluorescence lifetime measurements (see Sec. 2.4, *Material and Methods*). The peak 2p excitation for mTFP occurred near 870 nm[12], and 2p excitation conditions that avoided photobleaching of the fluorophores were used. Here, the mTFP-5aa-Amber fusion protein was used to obtain the donor lifetime of mTFP. Amber is a mutant variant of Venus in which the chromophore tyrosine was changed to cysteine. This generates a protein that folds correctly, but cannot function as a FRET acceptor.[17]

This fusion protein more accurately replicates the molecular environment of the donor fluorophore in living cells than does expression of mTFP alone.

The measurements of the mTFP fluorescence lifetime when linked to Amber were consistent within each experiment, but varied slightly for experiments conducted on different days (Table 2). The average lifetime determined for the mTFP-5aa-Amber was 2.738 ns, but the donor lifetime measured for each individual experiment was used as τ_D to determine the FRET efficiency (equation 3, Section 1.2). Consistent with the earlier results[12] the FRET-FLIM measurements from cells expressing the mTFP-5aa-Venus fusion verified the results obtained by ps-FRET, indicating about 50% FRET efficiency detected from the cells expressing this fusion protein (Table 2). Additionally, the lifetime measurements also verified the results obtained for the mTFP-5aa-tdTomato fusion protein, indicating about 35% FRET efficiency to the tandem fluorophores. Interestingly, there was a stark difference between the ps-FRET and FRET-FLIM results obtained for the mTFP-5aa-mKO2 fusion protein. Whereas the ps-FRET measured less than 20% efficiency for this donor-acceptor pair, the lifetime measurements showed more efficient energy transfer had occurred, indicating that mKO2 was equal to Venus as an acceptor for mTFP (Table 2). There were also significant discrepancies between the ps-FRET and lifetime measurements for both the mTagRFP-T and mKate fusion proteins. Only the mTFP-5aa-mOrange2 fusion protein showed a consistently low FRET efficiency when measured using the two different methods (Table 2). Taken together, the results showed unexpected and significant differences between the results obtained using the two different methods to determine FRET efficiency from cells expressing the fusion proteins.

3.3. Differences between ps-FRET and FRET-FLIM measurements

Here, we used a FRET-standard fusion protein approach to compare several of the newer orange and red FPs as potential acceptors for mTFP. However, in using two independent methods to determine FRET efficiency, we observed discrepancies between the measurements. The ps-FRET approach detected efficient FRET between mTFP and Venus or tdTomato, but none of the other mTFP-5aa-acceptor fusion proteins showed particularly robust FRET signals. The FRET-FLIM approach confirmed the results obtained for mTFP linked to Venus or tdTomato, but surprisingly also indicated that mKO2 was also an effective acceptor for mTFP. In addition, there were also differences between the two FRET methods for the measurements of the red fluorophores mTagRFP-T and mKate. There are several possible explanations for the differences in the FRET efficiencies detected by the two methods for the same fusion proteins.

One possibility was that there were problems associated with detecting the sensitized acceptor emission in the orange and red spectrum. When determining the ps-FRET efficiencies, we accounted for different sensitivities of the PMTs at the longer wavelengths, as well as the different quantum yields of the acceptor proteins (Equation (4), section 2.3). However, for the red FP mKate, its lower quantum yield, coupled with poor PMT sensitivity at its peak emission resulted in a large correction factor. This reduces confidence in the ps-FRET measurements, and could account for some of the disagreement with the lifetime measurements (Table 2). More striking, however, was the difference between the ps-FRET and lifetime measurements for the mTFP-5aa-mKO2 fusion protein. Here, the hardware setup of the confocal microscope used for the measurements appears to contribute to poor detection of acceptor sensitized emission. Because the microscope was equipped with the 561 nm laser line, there is a pin in the light path that blocks the laser line from reaching the detector. Since this is near the peak emission wavelength for mKO2, we suspect the poor FRET efficiency detected by ps-FRET for the mTFP-5aa-mKO2 relates to this hardware feature. To confirm this, we made ps-FRET measurements using another confocal microscope system with a different light path configuration. The ps-FRET results obtained on this system (not shown) were in very close agreement with the lifetime measurements (Table 2), suggesting that the light path configuration did lead to an underestimation of the ps-FRET efficiency for the mTFP-5aa-mKO2 in our study. Therefore, it appears that, of the new orange and red FPs tested here, at least mKO2 functions as an effective acceptor for mTFP.

This raises the question of why the other FPs are not especially suited as acceptors for mTFP. Although the identical linker peptide (-SGLRS-) was used for all the constructs, the different acceptor proteins are not of identical size, and this could contribute to differences in the relative positioning of the donor and acceptor chromophores. Therefore, we cannot rule out variations in the orientation of the dipoles as a contributing factor for different FRET efficiencies. However, at least for tdTomato, the tandem acceptor fluorophores would be expected to be minimized this type of effect. The potential contribution of the linker could be tested by using a longer linker, or by changing its composition by inserting a proline residue into the existing linker.

Another possible explanation is that, unlike the *Aequorea*-based Venus FP, the coral-derived orange and red FPs are simply poor FRET acceptors. In this regard, the *Aequorea* GFP evolved as a FRET partner for the aequorin chemiluminescent protein.[23] In contrast, the coral FPs evolved to function in photoprotective mechanisms,[24] or to support symbiotic relationships between the corals and algae.[25] Therefore, it is possible that subtle differences in protein structure could contribute to the ability of the *Aequorea*-based Venus to function as an effective acceptor, while the *Discosoma* Red-derived mOrange2 protein does not. Here, it is interesting that the mOrange2 and the mKO2 FPs share very similar spectral properties (Table 1), but the lifetime measurements showed that mKO2, but not mOrange2, functioned as an efficient FRET acceptor (Table 2). We are investigating whether differences in protein structure might account for this observation.

4. CONCLUSIONS

Intensity-based FRET imaging methods that detect protein interactions in their natural environment within the living cell are important tools for the biomedical sciences, so it is important to identify FPs with optimal characteristics for these assays. Moreover, as with any method used for cell-based measurements, it is critical to verify the results using an independent assay. The FLIM approach is increasingly being used for this purpose, since measurements made in the time-domain are independent of variations in the probe concentration, excitation intensity, and other factors that can limit steady-state intensity-based measurements. Here, we used ps-FRET and FRET-FLIM to determine whether the newer orange and red FPs could replace Venus as an acceptor for mTFP. Our results indicated that mKO2 is an effective acceptor fluorophore for mTFP, but also raised the caveat that some confocal imaging systems may not be optimally configured to detect sensitized emission from this fluorophore. Further, the results indicated that another fluorophore, mOrange2, which is nearly identical to mKO2 in its spectral characteristics, was a relatively poor FRET acceptor for mTFP. This suggests that there may be subtle protein structural differences that permit some FPs to be optimal acceptor fluorophores.

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