

Energy migration alters the fluorescence lifetime of Cerulean: implications for fluorescence lifetime imaging Forster resonance energy transfer measurements

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Abstract. Förster resonance energy transfer (FRET) is a physical phenomenon used to study molecular interactions in living cells. Changes in the fluorescence lifetime of proteins genetically tagged with a donor fluorophore, such as cyan fluorescent protein or Cerulean, are used to measure energy transfer to a protein tagged with an acceptor fluorophore (yellow fluorescent protein or Venus). Increased transfer efficiency is usually interpreted as closer proximity. Resonance energy transfer is also possible between identical fluorophores. This form of FRET is called energy migration resonance energy transfer (EM-RET). Theoretically, EM-RET should not alter the lifetime or emission spectrum measured from a population of fluorophores. We find a change in the fluorescent lifetime of Cerulean that correlates with energy migration and can result in significant errors when using Cerulean as a donor to measure fluorescence lifetime imaging (FLIM)-FRET efficiencies. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2940367]

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1 Introduction

Förster resonance energy transfer (FRET) is a nonradiative mechanism for transferring energy between fluorophores in close proximity.¹⁻⁴ FRET can occur if three criteria are fulfilled: (1) the emission spectrum of the donor fluorophore overlaps with the absorption spectrum of an acceptor; (2) the donor and acceptor are within 10 nm of each other; and (3) the orientation of the transition dipole moments are not perpendicular. In living cells, FRET can be used to study molecular interactions between proteins that are genetically tagged with two different fluorophores (hetero-FRET).⁵ Typically, cyan fluorescent protein (CFP) is used as a donor and yellow fluorescent protein (YFP) as an acceptor. The efficiency of energy transfer is interpreted as an indicator of proximity, and is measured by following the changes in the fluorescence lifetime (FLIM-FRET⁶), intensity (E-FRET^{7,8} P-FRET),⁹ or spectrum (sRET).¹⁰ Recently, FLIM-FRET has emerged as the “gold standard” of FRET methods because there is a direct and inverse relationship between the fluorescent lifetime of the donor fluorophore and the FRET efficiency. As FRET efficiency increases, the fluorescence lifetime of the donor decreases. Typically, the lifetime of a donor in the presence of an acceptor is compared with the lifetime of the donor in the absence of an acceptor to calculate the FRET efficiency.

Since the spectral overlap of the donor emission and acceptor absorption in hetero-FRET is usually much greater

than the overlap of the acceptor emission and donor absorption, the forward transfer rate is almost always faster than the reverse rate. However, some fluorescent proteins (FPs), such as YFP, have a significant overlap between their emission and absorption spectra (small Stokes shift). For these fluorophores, excited-state energy can be exchanged between two identical fluorophores in close proximity. In this case the forward and backward transfer rate will be identical, because both fluorophores have the same excitation and emission spectra. Thus, once energy transfer is initiated, the excited-state energy can migrate back and forth between the fluorophore pair until the energy is eventually dissipated by a radiative or nonradiative event. This form of resonance energy transfer (RET) is called energy migration-RET (EM-RET)^{11,12} or homo-FRET.⁵

Energy migration cannot be monitored by changes in fluorescence lifetime, intensity, or spectrum because any change in the donor's fluorescence is thought to be reciprocated by an equal but opposite change in the acceptor. EM-RET can, however, be measured by monitoring changes in the polarization of emitted photons.⁵ Typically, EM-RET is monitored by a method called time-dependent fluorescence anisotropy decay analysis.¹³ This method can detect both molecular rotation as well as EM-RET. Molecular rotation for large fluorophores such as green fluorescent proteins (GFPs) is manifested as a slow anisotropy decay component with a lifetime greater or equal to 15 to 20 ns.¹⁴⁻¹⁷ In contrast, EM-RET is detected as a fast temporal component in the fluorescence anisotropy de-

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cay curve.¹⁸ RET-based measurements in cells presumably can occur in the presence of multiple donors and acceptors. The presence of multiple donors in close proximity could potentially lead to EM-RET. Although FP properties such as lifetime, spectra, and intensity are not thought to be affected by energy migration, the impact of EM-RET on hetero-FRET has been poorly studied.

Recently EM-RET has been used to reveal energy migration between two CFP molecules tethered together by a short poly-peptide linker. Surprisingly, the fluorescent lifetime of CFP in this construct was shorter than the lifetime of CFP alone.¹⁹ A change in fluorescent lifetime is problematic for any FLIM-FRET study using CFP as a donor because even in the absence of an acceptor, such as YFP, the lifetime will become shorter if several donor CFP molecules are in close proximity.¹⁹ To circumvent this problem, an improved variant of CFP, Cerulean, was developed for use as an optimized FRET donor. Unlike CFP, Cerulean was reported to have a single exponential decay that significantly simplified FLIM-FRET analysis.¹⁹ Furthermore, a purified Cerulean-Cerulean construct did not exhibit the aberrant fluorescence-lifetime behavior observed with tethered CFPs.¹⁹ Unfortunately, energy migration was not demonstrated for the Cerulean-Cerulean construct. Thus, several plausible trivial reasons can be envisioned to explain why a change in Cerulean lifetime was not observed: (1) a large separation distance between tethered Ceruleans, (2) a dipole-dipole orientation close to perpendicular, (3) linker proteolysis during isolation of the Cerulean-Cerulean protein, or (4) improper folding of one of the two Ceruleans during bacterial expression, resulting in a product similar to free Cerulean. Any of these factors could prevent energy migration between Ceruleans, leading to the normal Cerulean fluorescence lifetime decay detected. In this report we demonstrate that, like CFP, Cerulean also exhibits shorter lifetimes that correlate with EM-RET. This study also demonstrates that attenuation of fluorescence lifetimes due to EM-RET, if unaccounted for, could produce erroneous FRET efficiencies.

2 Materials and Methods

All media and transfection reagents were purchased from Invitrogen (Carlsbad, California). HEK 293 cells (ATCC) were grown as a monolayer on T75 cell culture flasks (Corning) at 37°C and 5% CO₂ in DMEM with high glucose and 10% FBS, 1X sodium pyruvate, 1X glutamax, and 1X nonessential amino acids. Two days prior to imaging, the cell monolayer was resuspended using TrypLE Express, replated onto 35-mm glass bottom tissue culture dishes (FluoroDish, World Precision Instruments), and incubated overnight. On the second day, the cells were transfected overnight with 1 µg of DNA encoding specific FP constructs using Lipofectamine 2000. Prior to imaging on day three, the medium in the dishes was replaced with phosphate-buffered saline with calcium and magnesium.

2.1 Clone Construction

The construction of Venus C1 (containing the A206K monomeric mutation), Cerulean C1 (containing the A206K monomeric mutation), 6xHis tagged Cerulean, 6xHis tagged Venus, Cerulean-5-amber (C5A), Venus-5-amber (V5A), Venus-5-

Venus (V5V), Cerulean-Traf-Venus (CTV), Cerulean-K-Ras (C-K-Ras) are described elsewhere.^{10,20} The open reading frame (ORF) of Cerulean without the translation initiation codon (ATG) was amplified by PCR using *pfu* ultra polymerase (Stratgene). A BglII site (underlined) and an EcoRI site were engineered into the sense 5'-GCAGATCTGTGAGCAAGGGCGAGGAGCTGTTACC-3' and antisense 5'-GCCGAATTCCTTGACAGCTCGTCCATGCCGAGAGTG-3' primers, respectively, for directional cloning into the Cerulean C1 vector. The PCR fragment was cloned into the Zero Blunt-II Topo vector (Invitrogen) and sequenced. The sequence-verified cDNA for Cerulean was subsequently cloned into the BglII/EcoRI digested Cerulean C1 to generate C5C. The ORF of Cerulean without the first two codons (ATG GTG) was amplified using oligos containing an Asp718 (Roche) site on the sense primer (underlined) 5'-AGTCGGTACCAGCAAGGGCGAGGAGCTGTT-3' and a BamHI (New England Biolabs) site on the antisense primer (underlined) 5'-AGTCTCGGATCCCTTGACAGCTCGTCCATGCCGAGAGTGATC-3. The ORF was cloned into Asp718/BamHI (New England Biolabs) digested Cerulean C1 to generate C17C. The C32C clone was generated by excising the Cerulean ORF from C32V (Ref. 20) using NheI/AgeI (New England Biolabs) and cloning it into Cerulean N1. The cDNA coding for Traf was excised from CTV using BspE1 and cloned into C5A or V5A to generate CTA and VTA, respectively. The oligonucleotide coding for the K-Ras²¹ membrane targeting sequence was excised from Cerulean-K-Ras²⁰ using BsrGI and NotI (New England Biolabs). The insert was cloned into linearized Venus N1 and EGFP N1 to generate V-K-Ras and EGFP-K-Ras, respectively.

2.2 Protein Purification

BL21(DE3)pLysS bacteria (Invitrogen) transformed with either His tagged Cerulean or 22Venus were plated onto LB Agar plates with chloramphenicol and ampicillin and grown overnight at 37°C. Five ml of liquid LB cultures with chloramphenicol and ampicillin were inoculated with a single colony of either Cerulean or Venus expressing bacteria and shaken overnight at 37°C. The next day, a 1-ml bacterial culture was added to 100 ml fresh LB with antibiotics and the culture flasks were shaken until the OD₆₀₀ of the culture reached 0.4 to 0.6. Protein expression was induced by adding 1 ml of 1M IPTG (Sigma), and cell cultures were incubated at room temperature overnight, then centrifuged and frozen (-80°C). Before purification, the cells were thawed, resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM Imidazole), and sonicated. Protein was purified by Ni-NTA magnetic agarose beads (Qiagen) using manufacturer suggestions.

2.3 Refractive Index (η) Studies

FP dilutions containing 0, 10, 30, 50, and 70% glycerol (w/w) (MG Scientific Inc.) with identical concentrations of either Cerulean or Venus (5 µM each) were prepared. The refractive index of each dilution was measured at room temperature using a refractometer (Milton Roy Company). The dilutions were loaded into 0.1-mm-square capillaries (Vetrocon), and the FP lifetimes were measured by time-correlated single-photon counting (TCSPC).²²

2.4 Microscopy and Imaging

All imaging and spectroscopy was performed using a Zeiss 510 META/NLO scan head attached to an upright Zeiss Axioptan 2 microscope. A mode-locked Ti:sapphire laser (Coherent Chameleon), tunable from 710 to 950 nm, was attached to the NLO laser “in” port of the scan head through an acoustic optic modulator.

2.5 Spectral Imaging

Spectral images of Cerulean-K-Ras, EGFP-K-Ras, and Venus-K-Ras were acquired using a 40×0.8 numerical aperture water immersion objective with the Ti:sapphire laser mode-locked at 880 nm, as previously described in Ref. 23.

2.6 FLIM-FRET Measurements

Time-domain FLIM and spectroscopy were performed using TCSPC.²² Cerulean constructs were excited with a mode-locked laser tuned to 820 nm. GFP constructs were excited with 850-nm excitation. Venus constructs were excited with 950-nm excitation. For lifetime determinations, emitted photons were filtered through a BG39 filter, a polarizer set to 54.7 deg, a 700-nm short-pass filter (Chroma Optical, e700sp-2p), and either a 460- to 490-nm bandpass filter for Cerulean or a 520- to 550-nm filter for Venus. They were detected on a bialkali microchannel plate photomultiplier (Hamamatsu R3809U-52) attached to a Zeiss 510 non-descanned detector port placed in the transmitted light pathway. Photons emitted from all the pixels of a FLIM image were pooled, correlated with excitation laser pulses using a Becker and Hickl SPC830 module to generate a lifetime spectrum. The fluorescence lifetimes (τ) of the constructs were determined using SPCImage software using a measured system response.²⁰ The FRET efficiency (E) of the constructs was calculated from lifetimes using Eq. (1):

$$E = 1 - \left(\frac{\tau_{DA}}{\tau_D} \right), \quad (1)$$

where τ_{DA} and τ_D are the lifetime of the donor in the presence or absence of the acceptor, respectively.

2.7 Time-Dependent Anisotropy Measurements

For time-dependent fluorescence anisotropy measurements, a mode-locked Ti:sapphire laser was scanned over a region of interest and detected photons pooled, counted, and correlated with excitation laser pulses to generate a fluorescence lifetime decay curve. The polarizer was set first at 0 deg and then repeated with the polarizer set at 90 deg relative to the laser polarization. The decay curves obtained from parallel and perpendicular polarization settings were exported into Igor Pro 5.04 (Wavemetrics) and the fluorescence anisotropy (r) was calculated using the following equation:

$$r = \left(\frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} - 2GI_{\perp}} \right), \quad (2)$$

where I_{\parallel} is the decay curve comprised of photons detected through a parallel oriented polarizer, and I_{\perp} is the decay curve comprised of photons that were detected through a perpendicular oriented polarizer. The parallel and perpendicular data

were collected with the same detector using the same optical pathway. Therefore, the G factor for our instrument was found to be 1 (as expected).

2.8 Simultaneous Anisotropy and Lifetime Measurements

For simultaneous acquisition of anisotropy and lifetime curves, a Ti:sapphire laser mode-locked at 820 nm was scanned over a region of interest. The emitted photons were filtered through a BG39 filter and a polarizing beamsplitter that separated the photons parallel and perpendicular to the excitation light source. The parallel and perpendicular photons were counted using two bialkali microchannel plate photomultipliers (Hamamatsu R3809U-52) attached to a Zeiss 510 non-descanned detector port placed in the transmitted light pathway. Anisotropy curves for each clone (at least six cells each) were generated using Eq. (2) as described earlier. Since the parallel and perpendicular data were collected through different optical paths and different detectors, the G factor was found to be 1.26.

Fluorescence lifetime curves were generated using the equation

$$I = (I_{\parallel} + 2 \times G \times I_{\perp}). \quad (3)$$

Individual lifetime curves were normalized to peak photon counts, averaged, and plotted.

3 Results and Discussion

3.1 EM-RET affects the lifetime of Cerulean

Since the fluorescence lifetime of a fluorophore is typically unaffected by the intensity of incident light or the concentration of fluorophores, FLIM is frequently used to calculate hetero-FRET efficiencies in living cells. The fluorescence lifetime (τ) of a molecule is a measure of the average time a fluorophore spends in the excited state.⁶ FRET provides a competing pathway by which excited state energy can decay, so FRET results in a faster decay time. Unfortunately, any additional mechanism that decreases the donor fluorescence lifetime such as photobleaching, dynamic quenching, or changes in refractive index (n) might be misinterpreted as FRET. To test if the lifetime of the Cerulean was altered in the presence of EM-RET, a DNA construct called C5C encoding two Ceruleans separated by a 5 amino acid linker was generated. Three additional related constructs, composed of Cerulean, Venus,²⁴ or Amber (a point mutation in Venus that destroys the fluorophore²⁰) called C5A, V5A, and V5V, were also generated as controls for C5C. In all live-cell experiments, the Cerulean intensity was monitored throughout the data acquisition period to ensure that the fluorophore did not bleach, because even small amounts of photobleaching can alter the lifetime.²⁵ Fluorescence anisotropy curves of Cerulean, C5A, and C5C are shown in Fig. 1(a). The anisotropy decay of cells transfected with the Cerulean (green circles) or C5A (red inverted triangles) were both well fit by a single exponential with time constants (ϕ) of 16.7 ± 1.1 ns (mean \pm SD $n=5$) and 25.0 ± 1.8 ns ($n=5$), respectively. These time constants correspond to the molecular rotation of the Cerulean monomer and the Cerulean in C5A construct. Note that the Cerulean in C5A rotated slightly slower than

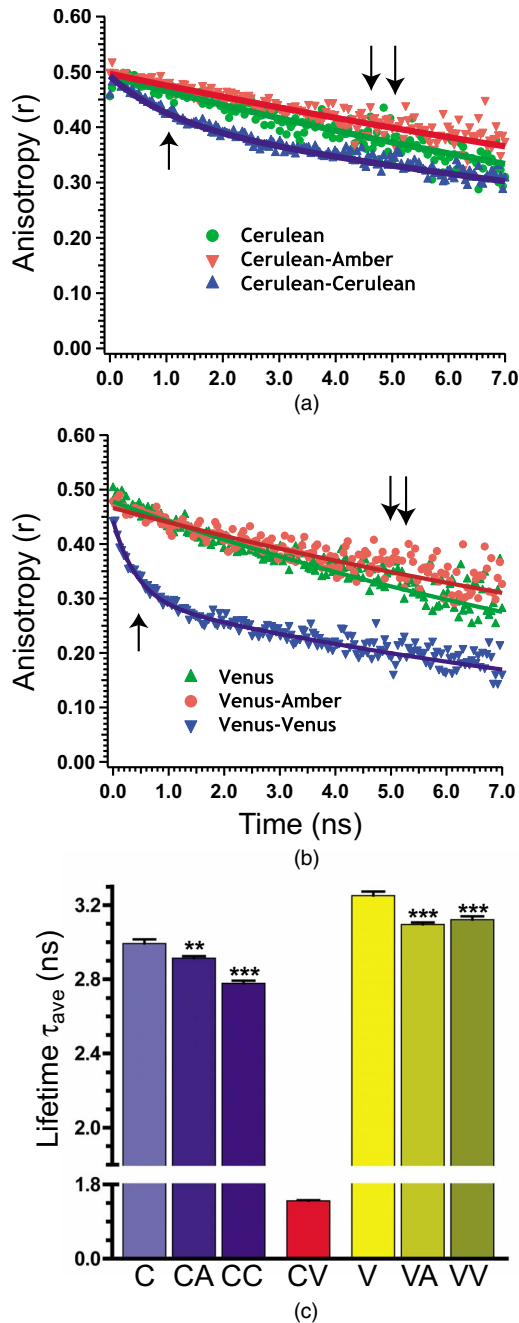


Fig. 1 Attenuation of Cerulean's fluorescence lifetime correlates with EM-RET. (a) C5C exhibits EM-RET (blue triangles) as seen in the rapid depolarization detected in the early part of the anisotropy decay (single arrow) when compared to C5A (red inverted triangles) and Cerulean (green filled circles). Also note that the rotational component of the anisotropy curves (double arrows) of C5A and C5C are slower than Cerulean due to increased mass. Furthermore, because all experiments were conducted using two-photon excitation, our $r_0 > 0.40$. (b) Anisotropy curve of V5V, like that of C5C, also exhibits EM-RET. (c) Fluorescence lifetimes of Cerulean, C5A, and C5C (blue bars), C5V (red bar) data originally used to determine FRET efficiency of C5V in (Ref. 20) and Venus, V5A and V5V (yellow-green bars). C5A and C5C have significantly different fluorescence lifetimes compared to Cerulean alone. Similarly, V5A and V5V have significantly different lifetimes than Venus alone, suggesting that both Cerulean and Venus lifetimes are affected by attaching a protein to them. The fluorescence lifetime of C5C is significantly different from C5A, while fluorescence lifetimes of V5V and V5A are not different. (Color online only.)

Cerulean alone, as expected for a larger construct. In contrast, the C5C has two functional fluorophores in close proximity, so energy migration (EM-RET) can occur between the two Cerulean subunits. As shown in Fig. 1(a), the anisotropy decay curve obtained from cells transfected with C5C had two decay components (blue triangles). C5C had a slow rotational decay component similar to C5A and a fast component (observed between 0 to 3 ns) corresponding to EM-FRET.

The fluorescent lifetimes (τ) of the Cerulean, C5A, and C5C were measured using TCSPC of cells transfected with these constructs [Fig. 1(c)]. The fluorescence lifetimes were calculated for individual curves using SPCImage and could be fit to a single exponential based on the χ^2 rule.²⁰ Other laboratories have shown that Cerulean decays as a double exponential.²⁶ Cerulean had a τ of 2.99 ± 0.11 ns (mean \pm SD, $n=21$), C5A decayed slightly faster with a τ of 2.91 ± 0.05 ns ($n=21$), and C5C decayed the fastest with a τ of 2.78 ± 0.62 ns ($n=21$). These lifetimes were all significantly different from each other as determined by an analysis of variance (ANOVA). Apparently, the simple act of attaching a protein to Cerulean can slightly alter its fluorescence lifetime decay, as seen in the difference in τ between C5A and Cerulean. Furthermore, because the τ of C5C was significantly faster than that of C5A (Tukey's multiple comparison test $p < 0.001$), energy migration might be responsible for the further decrease in the Cerulean's fluorescence lifetime. The τ_{ave} of C5A fit to a double exponential (based on the χ^2 rule) from previous experiments was included [Fig. 1(c) red bar] to assist in visualizing the magnitude of lifetime attenuation seen in Cerulean.²⁰

To test if an energy migration correlated reduction in fluorescent lifetime is a unique attribute shared by CFP and Cerulean as opposed to a general characteristic of spectral variants of GFP, we measured the fluorescence anisotropy decay [Fig. 1(b)] and fluorescence lifetime decay [Fig. 1(c)] of Venus, and the related yellow fluorescent constructs V5A and V5V. The anisotropy decay of Venus was virtually indistinguishable from the decay of Cerulean with a ϕ of 17.0 ± 1.4 ns (mean \pm SD, $n=6$). Similarly, the anisotropy decay of V5A was virtually indistinguishable from the decay of C5A with a ϕ of 22.7 ± 1.6 ns ($n=4$). Like C5C, V5V was well fit by a double exponential, indicating the presence of energy migration in this construct. The amplitude of V5V's fast decay component was larger than that of C5C [compare bottom trace in Figs. 1(a) and 1(b)]. This is indicative of a faster energy transfer rate in V5V than in C5C and is consistent with the measured Förster distance for Cerulean-Cerulean RET (3.52 nm) and Venus-Venus RET (4.95 nm).²⁷ An ANOVA revealed that the fluorescence lifetime for the Venus monomer (3.25 ± 0.10 ns, $n=20$) [Fig. 1(c)] was significantly different from the lifetimes for V5A (3.10 ± 0.05 ns, $n=20$) and V5V (3.12 ± 0.08 ns, $n=21$). (Tukey's multiple comparison test $p < 0.001$). These results demonstrate that the addition of protein adducts to either Cerulean or Venus can result in a slightly faster fluorescence lifetime decay. Additionally, since the fluorescence lifetimes of V5A and V5V were indistinguishable from each other (Tukey's multiple comparison test $p > 0.05$), the Venus constructs did not demonstrate a further reduction in lifetimes that were correlated with energy migration.

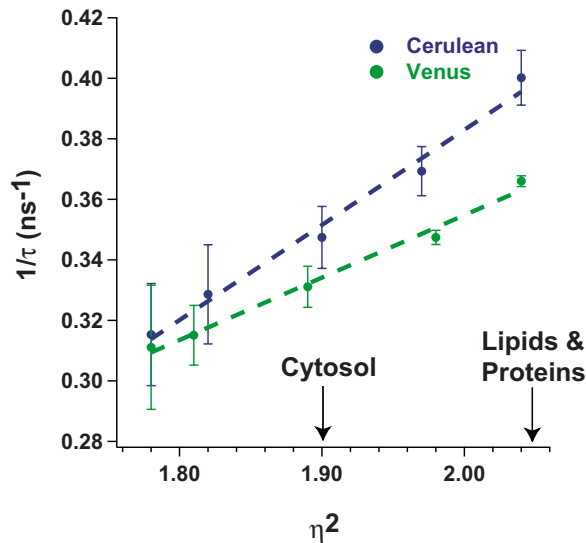


Fig. 2 Refractive index (η) dependence of FP lifetimes. Cerulean and Venus fluorescence lifetimes decrease with increasing refractive index. Changes in the lifetime of Venus were less than that observed in Cerulean. Note the lifetime of a FP attached to another protein or membrane is predicted to be faster than that of an FP in cytosol as a result of being in an environment with a higher η .

3.2 Refractive index affects fluorescence lifetimes

We were interested in understanding why attaching a protein to either Cerulean or Venus resulted in a slight yet significant reduction in fluorescence lifetime. It is known that a change in refractive index alters the fluorescence lifetimes of FPs. Refractive indices for proteins ($\eta=1.58$ to 1.613) to (Ref. 28) and lipids ($\eta=1.4$ to 1.6) (Ref. 29) are larger than that of cytosol ($\eta=1.38$) (Ref. 30). Therefore, adding a protein to a fluorophore or targeting a fluorophore to a lipid environment might decrease its lifetime.^{29,31,32} The relationship between refractive indices and lifetimes can be seen for purified Cerulean and Venus (Fig. 2). Cerulean or Venus was diluted into different concentrations of glycerol, and the refractive index (η) and τ for each sample were measured. As predicted by the Strickler-Berg equation,³³ τ got shorter as the refractive index increased. This supports the idea that attaching a protein adduct to a FP may alter the refractive index that its fluorophore perceives, thus altering its lifetime.

We have shown that a construct containing two covalently linked Cerulean fluorophores has a shorter τ than a Cerulean covalently attached to Amber. This was not observed for a nearly identical construct comprised of two covalently linked Venus molecules (Fig. 1). This anomalous behavior appears to be correlated with energy migration between Cerulean molecules. We next wanted to test if a covalent attachment between two Ceruleans was required for this attenuation of lifetime. DNA constructs encoding either Cerulean, Venus, or EGFP ligated to the membrane-targeting domain of the monomeric GTP binding protein K-Ras were produced and transfected into HEK 293 cells. A typical plasma membrane localization was observed for these constructs [Figs. 3(a)–3(c)]. Unlike cytoplasmic proteins, overexpressed membrane proteins are trapped in the same small compartment and thus readily reach local concentrations sufficient to support energy

migration.³⁴ All K-Ras constructs, when examined by anisotropy decay analysis, exhibited EM-RET (data not shown). In contrast, only Cerulean-K-Ras had a fluorescence lifetime that was significantly shorter than the lifetime of the FP alone [Fig. 3(d)], suggesting that the covalent attachment between Ceruleans is not required for this effect. The lifetimes of Venus and Venus-K-Ras were indistinguishable, as were the lifetimes of EGFP and EGFP-K-Ras (unpaired t-test, $p > 0.05$). The lack of decrease in the lifetimes of Venus or EGFP, even though they were targeted to the plasma membrane (which has a high η), suggests that refractive index changes may not completely account for the lifetime changes seen in the V5A and C5A constructs. This might be explained by the fact that FPs respond to refractive index changes over a distance range of micrometers.^{28,31} An alternative explanation for the shortening of the C5A and V5A lifetimes could be due to the addition of a bulky protein that may alter the shape of their β -barrels. The absence of fluorescence lifetime attenuation with the K-Ras membrane targeting domain,²¹ a small 22 amino acid polypeptide added to the C-terminus of either Venus or GFP, supports this explanation.

3.3 Attenuation of the lifetime of Cerulean correlates to the amount of EM-FRET

Having demonstrated that increasing the local concentration of a Cerulean can alter its lifetime, we wanted to further explore the relationship between EM-RET and lifetime attenuation observed with Cerulean. Specifically, if EM-RET could alter the fluorescence lifetime, then decreasing the separation distance between two Cerulean molecules in a protein dimer should result in a corresponding decrease in the lifetime. To test this hypothesis, we generated two additional constructs in which the two Cerulean ORFs were separated by 17 and 32 amino acids and called C17C and C32C, respectively. Photons that were emitted parallel and perpendicular to the excitation light source were simultaneously counted and correlated with the laser pulses to generate lifetime spectra. These parallel and perpendicular lifetime spectra were processed using Eqs. (2) and (3) to generate fluorescence anisotropy and lifetime decay curves, respectively. Individual curves obtained from HEK 293 cells transfected with C ($n=7$), C5C ($n=6$), C17C ($n=6$), and C32C ($n=6$) were averaged and plotted [Figs. 4(a)–4(c)]. All three constructs containing two Ceruleans showed EM-RET as demonstrated by the change in slope of the early part of the anisotropy decay curve when compared to C [arrow in Fig. 4(a)]. Note that as the separation distance between the Ceruleans increased the EM-RET decreased [Fig. 4(b)]. A plot of the normalized and average fluorescence lifetime decays [Fig. 4(c)] for C, C5C, C17C, and C32C shows that Cerulean lifetimes decreased as EM-RET increased. Therefore, increasing the separation between the two Ceruleans resulted in longer lifetime decays. These data support our conclusion that the lifetime of a Cerulean is attenuated by EM-RET.

3.4 The presence of EM-FRET affects Cerulean FRET efficiencies

The decrease in Cerulean lifetime that correlates with energy migration is rather small. For example, the change in lifetime measured between C5A and C5C was approximately 100 ps,

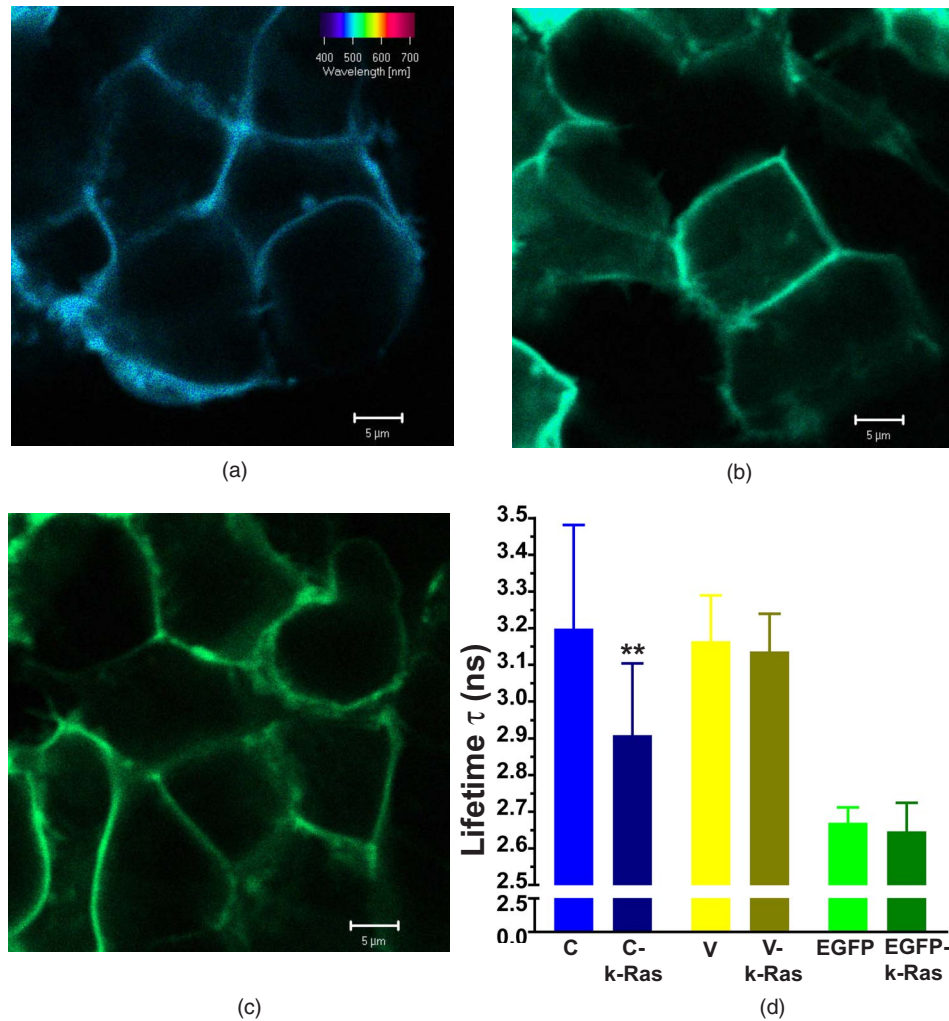


Fig. 3 Targeting Cerulean to membrane alters its fluorescence lifetime. (a) Spectral image of C-K-Ras. (b) Spectral image of EGFP-K-Ras. (c) Spectral image of Venus-K-Ras. (d) Average fluorescence lifetimes (mean \pm SD) for Cerulean ($n=11$), C-K-Ras ($n=12$), Venus ($n=18$), V-K-Ras ($n=20$), EGFP ($n=10$), and EGFP-K-Ras ($n=11$) are graphed. The fluorescence lifetime of Cerulean-K-Ras is significantly different from Cerulean alone ($p<0.05$ unpaired t -test). However, the lifetimes of Venus and V-K-Ras were not different ($p>0.05$ unpaired t -test). Similar results were obtained from the EGFP constructs.

and the difference between Cerulean and Cerulean-K-Ras was approximately 300 ps. To illustrate the impact that such small changes in donor lifetime can have on a FLIM-FRET measurement, we measured the FRET efficiency of a protein domain whose crystal structure had been solved and was known to form a cytoplasmic mushroom-shaped trimeric structure as a result of subunit association through a coiled-coil motif [see Fig. 5(a)]. We produced a DNA construct encoding the Traf 2 domain of the Traf protein where a Cerulean was attached to its N-terminus, and Venus was attached to its C-terminus (CTV).¹⁰ Related control constructs comprised of the Traf 2 domain attached to Cerulean and Amber (CTA) or Venus and amber (VTA) were also generated. The Traf 2 crystal structure predicts that the C- and N-termini should be separated by at least 8 nm, not including the distance added by linkers or the size of the FP β -barrel structure (2.4-nm diameter and 4-nm high).³⁵ A conservative estimate of the total separation distance between Cerulean and Venus in CTV is ~ 10 nm. With a Cerulean to Venus Förster distance of 5.4 nm, a Cerulean lifetime of ~ 3 ns, and assuming a random dipole-dipole ori-

entation ($\kappa^2=2/3$), the predicted FRET efficiency for this fluorophore pair separated by 10 nm is 0.024 (with a FRET transfer rate of 0.008 ns^{-1}). Because the Traf 2 domain forms a trimeric assembly, the three Cerulean donors are predicted to be in close proximity [see Fig. 5(a)]. This was verified in living cells by fluorescence anisotropy decay analysis [Fig. 5(b)]. A fast anisotropy decay component associated with energy migration was observed in cells transfected with CTA. A similar but more prominent drop in anisotropy was observed for VTA (data not shown). Likewise, it is expected that three acceptors should also be present at the top of the mushroom structure. Thus, for Traf 2 labeled with Cerulean and Venus, we expect that an excited Cerulean could transfer energy to not one but three potential acceptors, each approximately 10 nm away. Accounting for multiple acceptors, we revised our FRET efficiency prediction for the Traf 2 domain trimeric assembly to 0.067 based on an ensemble transfer rate of 0.024 ns^{-1} ($3 \times 0.008 \text{ ns}^{-1}$). TCSPC was used to measure the fluorescent lifetimes of Cerulean alone, as well as Cerulean in CTV construct and in the CTA construct [Fig. 5(c)]. Cerulean

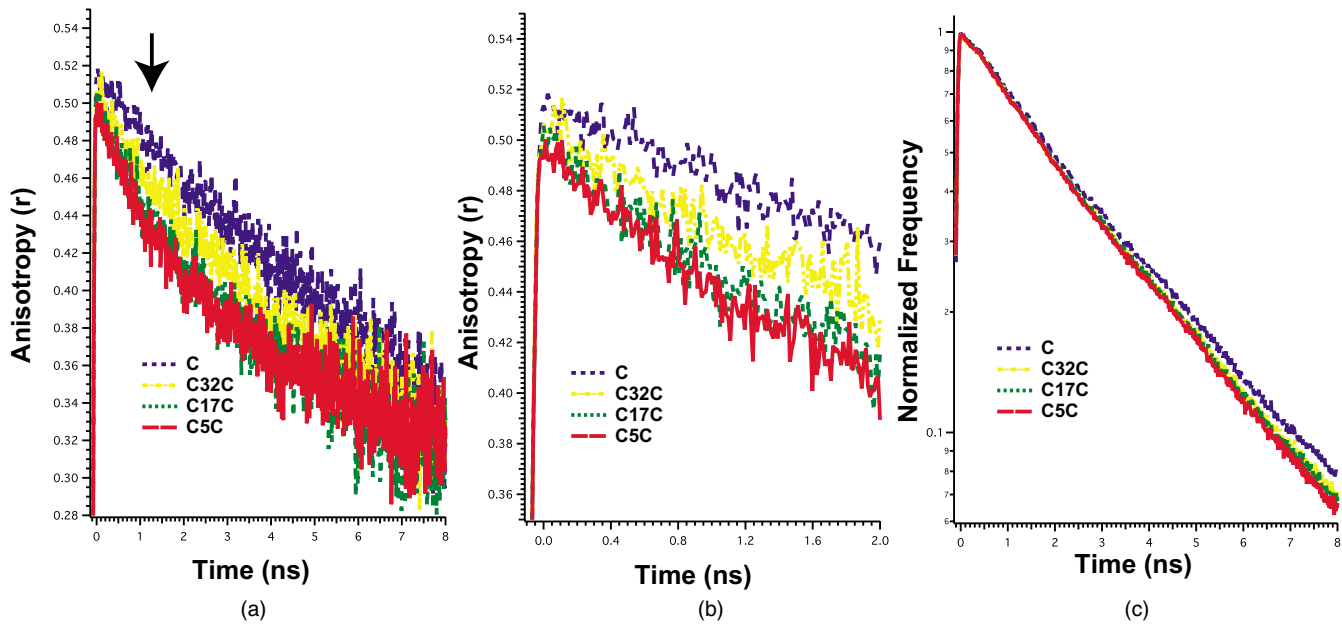


Fig. 4 Effect of energy migration on lifetime. (a) Anisotropy of C, C5C, C17C, and C32C. Note the change in slope in the early part of the curves (arrow). (b) Anisotropy increases with increased linker size. (c) Lifetimes of C, C5C, C17C, and C32C. Note the Cerulean lifetimes decrease as a function of linker length.

had a lifetime of 2.94 ± 0.11 ns (mean \pm SD, $n=10$), CTV had a lifetime of 2.38 ± 0.13 ns ($n=10$), and CTA had a lifetime of 2.59 ± 0.11 ns ($n=10$). In the FLIM-FRET method, the FRET efficiency is calculated by subtracting from 1 the ratio of the donor lifetime in the presence of acceptors divided by its lifetime in the absence of acceptors [see Eq. (1)]. If we use the lifetime of Cerulean alone as the denominator in this equation, we calculate a FRET efficiency for CTV of 0.19 ± 0.05 ($n=10$). Using the lifetime of Cerulean alone, however, fails to account for an energy migration correlated decrease in Cerulean lifetime that is expected in this construct. In contrast, if we use the lifetime of CT or CTA as the denominator, we calculate FRET efficiencies for CTV of 0.06 ± 0.02 ($n=30$)¹⁰ and 0.08 ± 0.02 ($n=10$), respectively. These FRET efficiency calculations do account for Cerulean energy migration. Furthermore, the fact that the FRET efficiencies of CT and CTA were not significantly different ($p > 0.05$, Tukey's multiple comparison test) suggests that adding amber does not alter the lifetime of CT. The estimated FLIM-FRET efficiency of CTV using the Cerulean lifetime differed from the values estimated using CTA or CT by 0.13. The FRET efficiency of CTV calculated using the Cerulean lifetime was significantly different ($p < 0.001$) from the values obtained using CT or CTA lifetimes. The FRET efficiency estimate based on using the lifetime of CTA was consistent with our predictions based on the crystal structure of Traf 2 (0.07; see discussion above). We have previously shown that a spectral method of measuring FRET efficiencies, sRET, has an accuracy of ± 0.05 .²⁰ We have also previously used sRET to measure a FRET efficiency for CTV of 0.02 ± 0.07 .¹⁰ We repeated those sRET measurements and obtained a FRET efficiency for CTV of 0.08 ± 0.06 (mean \pm SD, $n=24$). Both of these values were consistent with the FLIM-FRET value calculated using CTA's lifetime in the denominator.

We have observed a decrease in Cerulean fluorescence lifetime in five different constructs: C5C, C17C, C32C, C-K-Ras, and CTV. The constructs C5C, C17C, C32C, and CTV have a cytoplasmic localization while C-K-Ras was membrane targeted. Thus, this change in lifetime did not correlate with a specific cellular localization. Common to cells transfected with these five constructs is the existence of a fast fluorescence anisotropy decay component. This suggests that the decrease in Cerulean lifetime is correlated with EM-RET. This phenomenon was not observed for similar constructs (V5V, V-K-Ras, and VTA) built using a yellow spectral variant of GFP, e.g. Venus. Similarly, EGFP-K-Ras also failed to express this anomalous behavior. Thus, it appears that the energy migration correlated attenuation of fluorescent lifetime is specific to the cyan chromophore found in Cerulean and formed from Thr₆₆-Trp₆₇-Gly₆₈ but not observed in the chromophores of Venus (Gly₆₆-Tyr₆₇-Gly₆₈) or EGFP (Thr₆₆-Tyr₆₇-Gly₆₈). Presumably the contribution of Trp₆₇ to the chromophore is responsible for this behavior. Consistent with this hypothesis is the finding that a similar construct composed of two covalently linked CFP molecules also expressed a drop in its fluorescent lifetime. Like Cerulean, the chromophore in CFP is also formed from Thr₆₆-Trp₆₇-Gly₆₈. While we do not know the mechanism of this phenomenon, we speculate that this cyan chromophore might have multiple excited states, one of which has a faster decay time, and is either not populated, or poorly populated by direct excitation, but efficiently populated by energy migration.

3.5 Conclusions

Our result differs from a previous report, which argued that the lifetime of Cerulean was not altered by the presence of EM-RET.¹⁹ One major difference between the two studies was that our experiments were performed in living cells while the

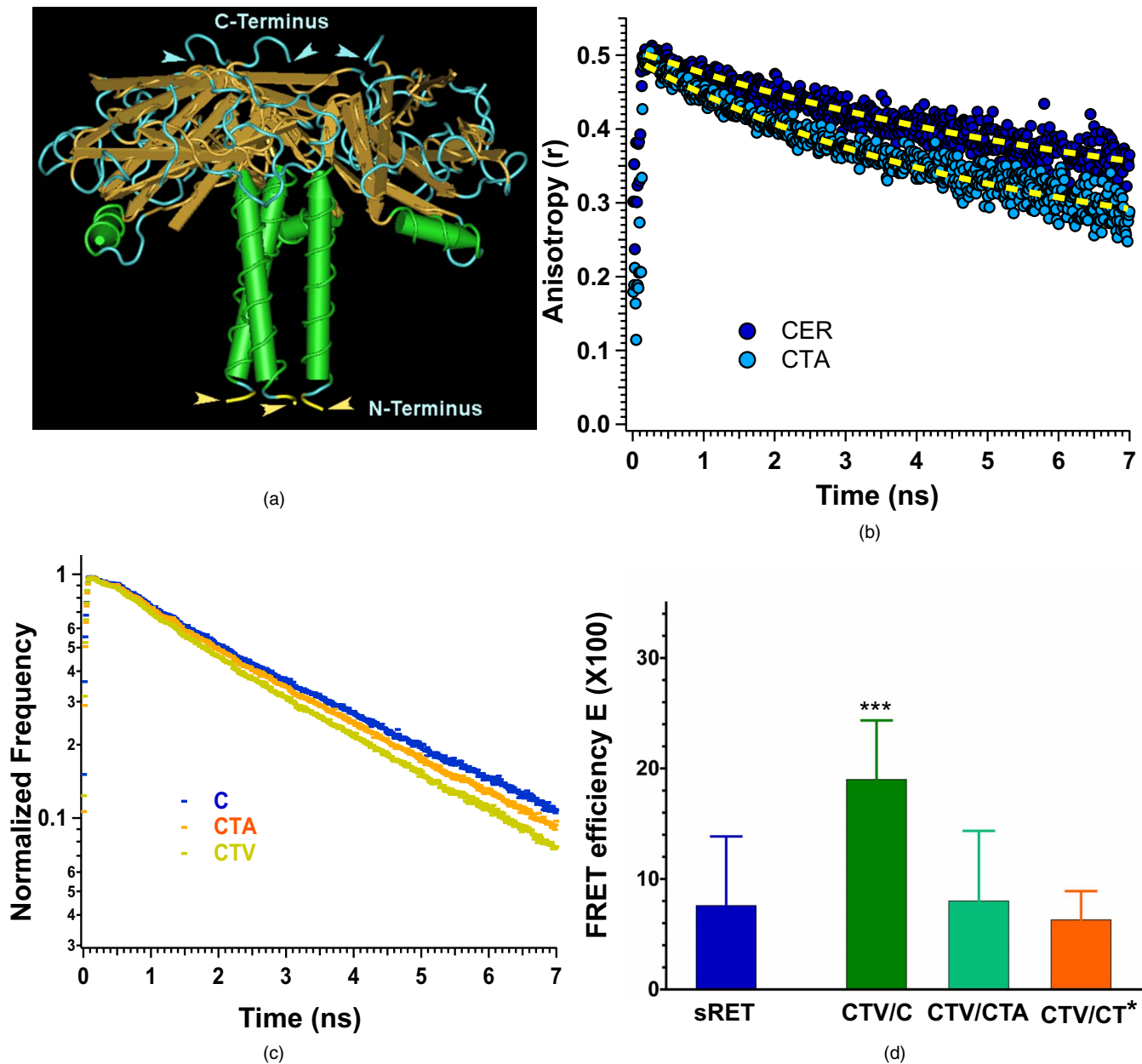


Fig. 5 Effects of EM-RET correlated lifetime attenuation on FRET. (a) The structure of Traf 2 domain of the Traf protein, used in CTV and CTA, shows that Traf exists as a mushroom-shaped homotrimer. Alpha helices near the N-terminus (yellow arrowheads), which are attached to Cerulean in CTV and CTA, associate to form a coiled-coiled structure. The C-terminus (blue arrowheads) of the protein forms the head of the mushroom and is tagged to amber or Venus in CTA and CTV, respectively. (b) Anisotropy analysis of CTA (light blue circles) is compared to Cerulean (CER) shows that CTA exhibits EM-RET as seen from the rapid depolarization in the early part of the anisotropy decay curve. (c) Lifetime curves of cells transfected with Cerulean (C, $n=10$), CTA ($n=20$), and CTV ($n=20$) were normalized to peak counts, averaged, and plotted. The slopes of C, CTA, and CTV are different. (d) Depicts the FRET efficiency of CTV. The FLIM-FRET efficiency of CTV calculated using the lifetime of Cerulean (dark green) is different from the value measured by sRET (blue). The use of the CTA lifetime in the FLIM-FRET calculations produced FRET efficiencies similar to those measured by sRET (light green). The * denotes FRET efficiencies for CTV calculated using the lifetime of CT as the denominator that were previously published.¹⁰

other study was based primarily on spectroscopy of purified proteins. Additionally, the presence of EM-RET was not demonstrated in the other study's Cerulean-Cerulean construct. Therefore, several plausible explanations could explain the discrepancies between the two reports, including linker proteolysis during isolation, incomplete Cerulean maturation or folding in the bacterial host, suboptimal, separation distance (27 as compared to a 5 amino-acid linker between the Cer-

uleans), or a dipole orientation factor with a value close to zero in the expressed protein.

While the changes in Cerulean fluorescent lifetime that were correlated with energy migration were modest, we have shown that these changes can result in significant errors when measuring FRET efficiencies using fluorescent lifetime imaging. This is particularly true for samples with low FRET efficiencies. Our findings suggest that the practice of using un-

tagged CFP or Cerulean as a donor-alone control in FLIM-FRET analysis should be avoided if there is any possibility that multiple donors might be localized in close proximity. Under these circumstances, a conservative approach for a donor-alone sample would be to measure lifetimes from cells transfected with the same Cerulean donor construct but substituting Amber for Venus in the acceptor construct. Since Amber differs from Venus by a single point mutation, it is unlikely to alter any molecular assemblies formed between a particular Cerulean construct and a Venus construct encoding a putative interacting protein. Obviously, it is also prudent to check FLIM-FRET results with other FRET methods that are not based on lifetime measurements.

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