Visible Fluorescent Proteins for FRET-FLIM

Richard N. Day

3.1 INTRODUCTION
The use of noninvasive approaches such as fluorescence microscopy to detect events as they occur inside living cells is providing remarkable insight into molecular processes. Just in the last decade, the development of new genetically encoded probes, coupled with advances in digital image acquisition and analysis, has dramatically improved our ability to obtain quantitative measurements from living cells. Specifically, the cloning of the jellyfish *Aequorea victoria* green fluorescent protein (GFP; Prasher et al. 1992) sparked a revolution in studies of cell biology and physiology. For the first time, it became possible to produce genetically encoded fluorescent markers inside living cells and organisms (Chalfie et al. 1994; Inouye and Tsuji 1994). The utility of the fluorescent proteins (FPs) as noninvasive probes has been repeatedly proven by their integration into a variety of different living systems (reviewed by Hadjantonakis et al. 2003; Stewart 2006).

In the years since its cloning, the sequence encoding the *Aequorea* GFP has been engineered to yield new FPs emitting light from the blue to yellowish green range of the visible spectrum (Tsien 1998; Cubitt, Woollenweber, and Heim 1999; Nagai et al. 2002; Rizzo et al. 2004; Ai et al. 2007). Furthermore, it is now well appreciated that many marine organisms produce FPs that are homologous to the *Aequorea* GFP (Matz, Lukyanov, and Lukyanov 2002; Labas et al. 2002; Shagin et al. 2004). Recently, some of the GFP-like proteins that are responsible for the bright colors we see in reef corals have become available for live-cell imaging applications (Matz et al. 1999; Karasawa et al. 2004; Shcherbo et al. 2007).

The FP palette now spans the visible spectrum from deep blue to deep red, giving investigators a wide choice of genetically encoded markers for studies in cell biology (Shaner, Patterson, and Davidson 2007; Day and Schaufele 2008). The new colors expanded the repertoire of uses of the FPs to include multicolor imaging of protein co-localization and behavior inside living cells or to detect changes in intracellular activities, such as pH or ion concentration. However, their use for Förster resonance energy transfer (FRET) microscopy in living
cells has generated the most interest in these probes (Tsien 1998; Lippincott-Schwartz, Snapp, and Kenworthy 2001; Zhang et al. 2002; Giepmans et al. 2006; Shaner et al. 2007).

Chapters 1 and 2 introduced FRET and microscopy and how to detect the transfer of excited-state energy nonradiatively from a donor fluorophore and nearby acceptor probes. This chapter will discuss the spectral properties of the different FPs that allow them to be used as donor and acceptor probes in FRET microscopy. Because energy transfer is limited to the scale of less than 100 Å, FRET provides unique information about the spatial relationships of proteins inside the living cell that is beyond the optical resolution limit of the conventional light microscope.

Although there are the many different methods for detecting FRET (reviewed by Jares-Erijman and Jovin 2003; Periasamy and Day 2005), the measurement of the donor fluorescence lifetime is considered to be the most rigorous method (see Chapters 1 and 9; Day and Piston 1999; Yasuda 2006; Piston and Kremers 2007). Intensity-based imaging measures a time-averaged fluorescent signal, and it is sometimes difficult to distinguish spectrally overlapping fluorophores and determine concentrations. Fluorescence lifetime imaging microscopy (FLIM) can map the spatial distribution of lifetimes in a sample to reveal heterogeneity in the probe environments. FLIM is particularly useful for biological applications because 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 (see Chapters 1 and 2). Importantly, FRET is a dynamic process that nonradiatively depopulates the excited state of the donor fluorophore. This lowers the fluorescence lifetime of the donor, which is detected by FLIM, and provides a direct measurement of FRET.

This chapter presents selected practical guidelines for using the FPs as labels for FRET-FLIM studies. The objective is to relate the important features and photophysical properties of different FPs to their use as probes for FRET-FLIM measurements in biological systems. The intent is not to provide a comprehensive listing of all the FPs currently available, but rather to highlight a few exceptional probes and discuss their application for FRET-FLIM studies. The goal is to introduce the properties of the newer FPs in sufficient depth to be useful to those interested in pursuing these techniques in more detail.

The limitations, pitfalls, and critical considerations for FRET studies using these probes will be demonstrated using simplified theoretical frameworks, as well as experimental results. These considerations are especially relevant when making FRET-FLIM measurements in living cells because awareness of the potential pitfalls will help avoid complications and increase the reliability of these fluorescence measurements. To gain an appreciation of the applications of FRET and FLIM, the reader can then explore these topics in more depth in the references.

3.2 BACKGROUND

3.2.1 Overview of the Fluorescent Proteins

In 1962, Shimomura, Johnson, and Saiga purified the blue-light-emitting photoprotein, aequorin, from the jellyfish Aequorea victoria. They also reported the presence of an
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autofluorescent protein in extracts from the jellyfish. The protein was GFP, and it was later isolated and shown to be a companion protein for aequorin, absorbing its blue light emission and then reemitting it as green light (Morise et al. 1974). Prasher et al. (1992) cloned the gene encoding the Aequorea GFP, and the utility of this new probe for in vivo fluorescence labeling was proven by its expression in bacteria, mammalian and plant cells, and transgenic organisms (Chalfie et al. 1994; Inouye and Tsuji 1994; Plautz et al. 1996; van Roessel and Brand 2001; reviewed by Hadjantonakis et al. 2003; Stewart 2006).

To be fluorescent, GFP must fold into a tightly woven 11n-strand beta-barrel structure (see Figure 3.1). Nearly the entire 238 amino acid sequence is required for its proper folding. The tripeptide sequence serine65–tyrosine66–glycine67 is positioned at the core of the

![Figure 3.1](See color insert following page XXX.) The β-barrel structure of the FPs. (A) The structure of GFP is shown illustrating the 11-strand β-barrel that surrounds the chromophore. The excitation (Ex) and emission (Em) spectra for wtGFP and EGFP are plotted, showing how the S65T mutation in EGFP shifts the spectrum to a single peak excitation at 489 nm. (B) The tetrameric structure of the DsRed FP. (C) The structure of its monomeric derivative, mCherry. The illustration of DsRed looks down the barrel of two subunits (1,2) and views the other two subunits from the side. The approximate dimension of the β-barrel for the monomer mCherry is shown. (Data from the National Center for Biotechnology Information Molecular Modeling DataBase. The structures were rendered using the Cn3D software.)
beta-barrel as the protein matures, and this drives the cyclization and dehydration reaction that forms the chromophore (reviewed by Tsien 1998; Ward 1998). Once formed, the wild type (wt) *Aequorea* GFP displays a complex absorption spectrum, with maximal excitation occurring at 397 nm and a minor secondary peak of excitation at 476 nm (Figure 3.1A). Chattoraj et al. (1996) examined the excited-state dynamics of the wtGFP protein and showed that green fluorescence resulted from deprotonation within the chromophore, populating the state favoring excitation at 397 nm. In addition, a charged intermediate state is thought to relax slowly to a second stable state that is only rarely formed, accounting for the minor secondary excitation peak at 476 nm (Chattoraj et al. 1996).

The measurement of the fluorescence lifetime of the wtGFP revealed a multiexponential decay with components that corresponded to the different excited-state species (Striker et al. 1999). A mutated wtGFP, called Sapphire, was generated in which a chromophore contacting amino acid in the beta-barrel, threonine$^{203}$, was changed to isoleucine (Tsien 1998). As we will see in Section 3.2.2, this amino acid in the beta-barrel is a critical determinant of the photophysical properties of the chromophore. Changing the threonine$^{203}$ to isoleucine abolished the secondary excitation peak at 475 nm, generating a GFP with an exceptionally large Stokes shift. The Sapphire FP was further optimized for expression in mammalian cells and was shown to be a useful FRET donor for the red FPs described later (Zapata-Hommer and Griesbeck 2003).

### 3.2.2 Spectral Variants from the *Aequorea* GFP

Over the last decade, both targeted and random mutagenesis strategies have been used to modify the spectral and physical characteristics of the wtGFP, yielding new, enhanced (E) FPs ranging in color from blue to yellow-green (reviewed in Patterson, Day, and Piston 2001; Zhang et al. 2002; see Table 3.1). The sequences encoding the E FPs incorporate preferred human codon usage and silent mutations that improved the efficiency of production and maturation of the proteins in mammalian cells. In addition, many laboratories have contributed mutant variants that have altered spectral properties, providing different color fluorescent probes for live-cell imaging.

For example, one of the earliest color variants of the wtGFP is a blue FP (BFP) that results from substitution of tyrosine66 with histidine (Heim and Tsien 1996; Cubitt et al. 1999). The original BFP, however, has a low quantum yield and is very susceptible to photobleaching. Recently, several groups used mutagenesis strategies to develop new blue FP variants with much higher quantum yields and photostabilities, greatly improving the utility of these deep blue probes (Ai et al. 2007; Kremers et al. 2007; Mena et al. 2006). The EBFP2, generated by Ai et al (2007), is the brightest and most stable of the blue FPs available and has been shown to be an excellent donor for FRET studies (see Table 3.1; Shaner et al. 2007). This new EBFP should be useful for long-term imaging in living cells where a blue probe is required, especially when two-photon excitation is used, which avoids cellular damage by near UV excitation (Wallrabe et al. 2003).

The development of cyan color variants (CFP) provided an early alternative to the blue FP. ECFP resulted from substitution of tyrosine66 with tryptophan in combination with mutations in several other residues within the surrounding beta-barrel structure (Heim,
Prasher, and Tsien 1994; Cubitt et al. 1999). Although ECFP is optimally excited at 433 nm, its absorption spectrum is broad, with a second excitation peak near 445 nm. As had been observed for the wtGFP, the complex excitation spectrum of ECFP indicates more than one excited-state species. This was confirmed by fluorescence lifetime measurements (Tramier et al. 2002).

Efforts to improve the characteristics of ECFP yielded a mutant variant, called Cerulean, which results from substitutions on the solvent-exposed surface of ECFP (tyrosine145 and histidine148). Cerulean has an increased quantum yield compared to ECFP, and Cerulean has been reported to have a single excited state (Table 3.1; Rizzo et al. 2004). However, when expressed in living cells, the fluorescence decay for Cerulean, similarly to ECFP, still indicates the presence of more than one excited-state species (Yasuda et al. 2006; Millington et al. 2007).

Improved green color variants result from changing the central chromophore, serine65, to threonine (S65T). This stabilizes the chromophore in a permanently ionized form with a single peak absorbance at 489 nm and a peak emission at 507 nm (see Figure 3.1A; Heim et al. 1994; Brejc et al. 1997; Cubitt et al. 1999). Importantly, this simplifies the lifetime decay kinetics to a monoexponential (Traimer et al. 2006; Yasuda et al. 2006). Currently, the EGFP with the best imaging characteristics is called Emerald, which incorporates several additional point mutations that improve folding and increase its brightness (Table 3.1; Cubitt et al. 1999). The longest wavelength emission variants of Aequorea GFP result from

<table>
<thead>
<tr>
<th>Table 3.1</th>
<th>Selected FP Color Palette Including Proteins Based on the Aequorea GFP, DsRed, and FPs Cloned from Other Marine Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent protein</td>
<td>Color</td>
</tr>
<tr>
<td>EBFP2</td>
<td>Blue</td>
</tr>
<tr>
<td>Cerulean</td>
<td>Cyan</td>
</tr>
<tr>
<td>mTFP</td>
<td>Teal</td>
</tr>
<tr>
<td>EmGFP</td>
<td>Green</td>
</tr>
<tr>
<td>Venus</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>mKO (Kusabira)</td>
<td>Orange</td>
</tr>
<tr>
<td>mOrange2</td>
<td>Orange</td>
</tr>
<tr>
<td>TagRFP-T</td>
<td>Orange</td>
</tr>
<tr>
<td>tdtTomato</td>
<td>Orange</td>
</tr>
<tr>
<td>mCherry</td>
<td>Red</td>
</tr>
<tr>
<td>mKate (Katushka)</td>
<td>Deep red</td>
</tr>
<tr>
<td>REACH</td>
<td>Weak yellow</td>
</tr>
</tbody>
</table>

a Intrinsic brightness is the product of quantum yield and extinction coefficient.


c Proven useful for applications that involve photobleaching.

d Cellular autofluorescence is low at longer wavelengths, improving the signal-to-noise ratio.

e Dark probe useful for FRET-FLIM; see Section 3.3.6.
again targeting the chromophore contacting amino acid in the beta-barrel, threonine203—in this case, changing it to tyrosine (Ormö et al. 1996). This generates a bright yellow green FP (YFP) that is optimally excited at 514 nm and has a peak emission at 527 nm.

The EYFP variant, however, is sensitive to both pH and halides, limiting its usefulness for studies in living cells. Starting with EYFP, Nagai et al. (2002) discovered that the substitution of phenylalanine46 with leucine improves the maturation efficiency and reduces the halide sensitivity of YFP. Combined with several additional mutations, this results in a variant called Venus, which is among the brightest and most red shifted of the mutant variants based on the Aequorea GFP currently available (Table 3.1).

### 3.2.3 Aequorea Fluorescent Proteins and Dimer Formation

Most of the FPs from marine organisms that have been characterized were isolated as dimers, tetramers, or part of higher order complexes (see Figure 3.1B, for example; Shagin et al. 2004). Although the Aequorea GFP was isolated as a monomer, it can form dimers when the protein is highly concentrated (Ormö et al. 1996; Brejc et al. 1997). This self-association is not typically observed when the FPs diffuse freely within the cell, but dimers have a tendency to form when diffusion is restricted, such as in the two-dimensional space of biological membranes. Here, the formation of Aequorea FP dimers can cause the proteins that they label to form atypical complexes (Kenworthy 2002). To overcome this problem, Zacharias et al. (2002) developed monomeric forms of the Aequorea-based FPs by substitution of the alanine206 with lysine, which blocked the dimer formation without altering the fluorescence characteristics of the Aequorea-based FPs.

### 3.2.4 New Fluorescent Proteins from Corals

Much of the color diversity in reef corals results from GFP-like proteins (Matz et al. 2002; Labas et al. 2002; Shagin et al. 2004). It is thought that these proteins evolved to fulfill a photoprotective function (Leutenegger et al. 2007) or, alternatively, to support symbiotic relationships between the corals and algae (Field et al. 2006). Over the past decade, some of these GFP-like proteins have been characterized, cloned, and optimized for imaging applications (Matz et al. 1999; Karasawa et al. 2004; Shcherbo et al. 2007). Among the first of the new FPs from coral is a protein called DsRed that was isolated from the mushroom anemone Discosoma striata (Matz et al. 1999). DsRed is the first red FP (RFP) to become available, with a peak absorbance at 558 nm and a maximum emission at 583 nm.

Unfortunately, the characteristics of DsRed do not particularly lend themselves to live cell imaging. For instance, DsRed is a very slowly maturing protein that generates a green intermediate as it matures. Even more problematic is that it is an obligate tetramer (Figure 3.1B) with a strong tendency to form oligomers when produced inside cells (Baird, Zacharias, and Tsien 2000). The fluorescence lifetime of the native complex has been determined to be about 3.6 ns (Heikal et al. 2000), but continued illumination causes photoconversion events, leading to more heterogeneous lifetimes ranging from 1.5 to 3.6 ns (Cotlet et al. 2001).

To overcome these problems, both random and directed mutagenesis strategies have been used to improve this novel red FP. Bevis and Glick (2002) used this strategy to address the
problem of slow maturation, and they generated a rapid maturing variant called DsRedT.1. Starting with the DsRedT.1 variant, Campbell et al. (2002) applied directed mutagenesis to break the tetramer, but this generated a nonfluorescent species. They then used many rounds of random mutagenesis and selected for proteins with improved red fluorescence (an approach called directed evolution). This yielded a rapid maturing monomeric RFP (mRFP) that overcame the critical problems associated with DsRed4, and also shifted the fluorescence emission deeper into the red spectrum (Campbell et al. 2002).

However, this new mRFP still has problems that limit its use as a probe for quantitative imaging studies. As expected, the fluorescence quantum yield is only about 25% of that of the DsRed tetramer. Further, there is an absorbance peak at 503 nm; however, this species is nonfluorescent, which might indicate a fraction of the protein that never fully matures. To generate additional FPs with improved characteristics, mRFP was subjected to many rounds of directed evolution using both the error-prone polymerase chain reaction and somatic hypermutation in B-lymphocytes (Wang et al. 2004; Shaner et al. 2004). When combined with cell-based screening methods, these approaches yielded a variety of new FPs. This new crop of FPs included mCherry (Figure 3.1C), which is a rapidly maturing, brighter, more stable version of mRFP (Table 3.1; Shaner, Steinbach, and Tsien 2005). In addition, a dimeric FP called tdTomato has been generated that is currently the brightest of the available FPs (Table 3.1). This probe is useful for applications that require minimal exposure to excitation illumination to maintain cell viability, but it is limited by its larger size (54 kDa).

Still other novel FPs have recently been cloned from corals and engineered to improve their utility for live-cell imaging. For example, the sequence encoding a cyan-colored protein from the coral Clavularia was modified by directed mutagenesis to generate a monomeric teal FP (mTFP) with remarkable brightness and photostability (Ai et al. 2006; see Table 3.1). Unlike CFP, which has a tryptophan residue at the central chromophore position, mTFP has a tyrosine residue in this position, which is typical of the GFPs. Indeed, both the excitation and emission spectra of the mTFP are shifted to the green wavelengths when compared to CFP. The mTFP protein has a high quantum yield and displays a relatively narrow emission spectrum that strongly overlaps the excitation spectrum of the yellow and orange FPs. The mTFP is also noteworthy because it fills the spectral gap between the cyan and green FPs and is optimally excited by the 457 nm laser line that is available on most confocal microscopes. This new blue green protein is also an excellent donor fluorophore for FRET studies using the Venus FP (Day, Booker, and Periasamy 2008; discussed later).

Several orange and red FPs are currently available that share significant spectral overlap with other commonly used FPs, providing alternative acceptor fluorophores. For example, a protein called Kusabira orange (KO) was isolated from the mushroom coral Fungia concinna. This FP was cloned and engineered to a bright, photostable monomeric protein called mKO (Karasawa et al. 2004; see Table 3.1). Another monomeric orange (mOrange) protein was generated during the directed evolution of mRFP (Shaner et al. 2004, 2005), but its use was limited by problems with photostability. Recently, an improved variant, mOrange2, was evolved and selected for increased photostability (Shaner et al. 2008; Table 3.1).

This same approach was applied to another orange FP called TagRFP, which was originally cloned from the sea anemone Entacmaea quadricolor (Merzlyak et al. 2007). The
directed evolution yielded the TagRFP-T protein, with much improved photostability (Shaner et al. 2008; Table 3.1). Shcherbo et al. (2007) also applied this approach to the Entacmaea FP, but instead selected for deep red FPs. This yielded a dimeric RFP called Katushka, which was then engineered to the monomeric protein called mKate. The mKate protein is currently the brightest and most photostable of the deep RFPs (Shcherbo et al. 2007; Table 3.1). This makes mKate potentially useful for studies that combine FRET imaging in the deep red spectral window with blue or cyan probes for other protein activities.

3.3 METHODS

3.3.1 Visible Fluorescent Proteins for FRET Measurements

As we have seen in the earlier chapters in this book, the Förster distance ($R_0$) for a fluorophore pair depends on the quantum yield of the donor and the spectral overlap integral ($J_\lambda$) of the donor emission with the absorption of the acceptor. The importance of the spectral overlap is illustrated by comparing the absorption and emission spectra for Cerulean CFP and Venus YFP (Figure 3.2), which share significant spectral overlap (shaded area), making them efficient FRET partners. When energy is transferred from Cerulean to Venus, the Cerulean emission detected in the donor channel is quenched and emission from the acceptor is increased (sensitized); this can be detected in the FRET channel (Figure 3.2).

**FIGURE 3.2** FRET efficiency as a function of distance separating Cerulean and Venus. (A) Cerulean CFP and Venus YFP are commonly used in FRET studies, and the excitation (- - -) and emission (——) spectra are illustrated here. The spectral overlap is illustrated by shading. Excitation with the 457 nm laser line is shown, and typical band-pass filters for detection of the donor and the acceptor are illustrated (vertical dotted lines). The spectral bleed-through caused by the direct excitation of the acceptor (arrow) and donor bleed-through fluorescence into the FRET channel (hatching) are also illustrated. (B) The Förster distance, $R_0$, for this fluorophore pair is 54 Å, and the efficiency of energy transfer, $E_{\text{FRET}}$, is plotted as a function of the separation distance ($r$). The distance spanning the range of 0.5–1.5 $R_0$ is shaded.
However, the strong spectral overlap also produces spectral bleed-through signals that hinder the accurate measurement of FRET using filter-based methods.

For intensity-based measurements, the spectral bleed-through signals result from the direct excitation of the acceptor (arrow, Figure 3.2) and the donor emission that bleeds into the FRET channel (hatching, Figure 3.2). Therefore, the accurate measurement of FRET signals requires correction methods that detect and remove the different spectral bleed-through components (see Periasamy and Day 2005). Significantly, the accuracy of these spectral bleed-through correction methods is degraded as the spectral overlap between the FPs is increased to the point where spectral bleed-through components overwhelm the FRET signal (discussed later; see Berney and Danuser 2003).

The alternative is to measure the effect of FRET on the donor fluorophore, which requires only measurements in donor channel and therefore is less prone to bleed-through artifacts (see Figure 3.2). The FRET-FLIM approach uses optical filtering to isolate the donor fluorescence emission signal and then measure the fluorescence lifetime (for example, see Figure 3.2; this is discussed further later in the chapter). When FRET occurs, FLIM will detect at least two donor populations: the unquenched donors (free donor) and the donors that are quenched by the acceptors (bound donor). These different populations will be reflected in the donor fluorescence decay kinetics, which will be described by at least two exponential components.

Here, the accurate assignment of the donor populations will be improved if the donor fluorophore has simple decay kinetics. In addition, it is important to choose an optical filter that efficiently collects the donor emission signal while eliminating the acceptor emission bleed-through (discussed in Section 3.3.5); however, this may be at the expense of photon counts (Bastiaens and Squire 1999; Peter et al. 2005). If these requirements are met, the measurements of the donor lifetime provide a robust method to quantify FRET (Yasuda 2006; Piston and Kremers 2007).

3.3.2 Standards for Live-Cell FRET Imaging

As mentioned in Chapter 1 and other chapters in this book, numerous methods can be used to measure FRET (for examples, see Jares-Erijman and Jovin 2003 and Periasamy and Day 2005). However, it has been difficult to compare the accuracies of the different methods. An elegant solution to this problem is the development of genetically encoded FRET “standard” proteins. The direct coupling of donor and acceptor FPs to one another by a protein linker has been used to develop a variety of different FRET-based biosensor probes (reviewed by Zhang et al. 2002; Giepmans et al. 2006).

The Vogel laboratory adopted this strategy and developed a set of genetic constructs encoding fusion proteins containing donor and acceptor FPs separated by protein linkers of defined length (Thaler et al. 2005; Koushik et al. 2006). These fusion proteins were then produced in living cells, and two different intensity-based methods (sensitized acceptor emission and emission spectra measurements) and FRET-FLIM were used to measure the FRET efficiency. For each of the fusion proteins tested there was consensus in the results obtained by the different FRET methods, demonstrating that the genetic constructs could serve as FRET standards.
More importantly, other laboratories can verify and calibrate their FRET measurements using these same genetic constructs. For example, Thaler et al. (2005) showed that the Cerulean-5aa-Venus fusion protein yielded an average FRET efficiency of approximately 45%, and we confirmed these results in our laboratory using spectral FRET measurements (Chen et al. 2007). Furthermore, we used the FRET standard approach to compare another FP—the new Teal colored variant mTFP (see Section 3.2.4)—directly to Cerulean to determine its utility as a FRET donor for Venus (Day et al. 2008).

A genetic construct substituting the sequence coding for Cerulean with the cDNA for mTFP was generated to produce the mTFP-5aa-Venus fusion protein. The FRET-FLIM method was then used to acquire fluorescence lifetime measurements from cells that expressed Cerulean, mTFP, or the two different FRET standard constructs. The fluorescence lifetime measurements for Cerulean and mTFP expressed living cells shows that they have similar mean lifetimes (Figure 3.3 and Table 3.2). The FRET-FLIM measurements

![Figure 3.3](image.png)

**Figure 3.3** (See color insert following page XXX.) Donor lifetime measurements for the fusion proteins consisting of Cerulean or mTFP linked to Venus. (A) Cells expressing Cerulean or mTFP alone, or the FRET standards Cer-5aa-Venus or mTFP-5aa-Venus were used to acquire fluorescence lifetime measurements as described in the text. The fluorescence lifetime decay kinetics for donor fluorophores alone or in the presence of Venus were determined by fitting the data to a double exponential decay. The lifetime distributions are shown for representative cells expressing (B) Cerulean (the calibration bar indicates 10 µm), (C) mTFP, (D) Cer-5aa-Venus, or (E) mTFP-5aa-Venus. The results of the fluorescence lifetime analysis are summarized in Table 3.2. (Adapted from Day, R. N. et al. 2008. Journal of Biomedical Optics 13:031203.)
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from cells expressing the FRET standard proteins consisting of either Cerulean or mTFP tethered to Venus are shown in Figure 3.3(B–E).

For both Cer-5aa-Venus and mTFP-5aa-Venus, strong quenching of the donor results in a shortening of the mean donor lifetime (Table 3.2 and Figure 3.3A). These measurements can be used to determine the FRET efficiency (see Table 3.2), and the results are in good agreement with measurements obtained using other methods (Day et al. 2008). Thus, mTFP, with its increased brightness and photostability and optimal excitation using the standard laser line, is an excellent donor fluorophore for FRET studies.

3.3.3 Using FRET-FLIM to Detect Protein Interactions in Living Cells

Once the instrumentation and FRET measurement methods have been verified using the standard protein, the goal is then to use the system to address biological questions. For example, we have used FRET-FLIM to characterize the protein interactions involving the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα). The C/EBP family proteins bind to specific DNA elements as obligate dimers and function to regulate genes involved in energy metabolism and programs of cell differentiation (Wedel and Ziegler-Heitbrock 1995). Immunocytochemical staining in differentiated mouse adipocyte cells shows that the endogenous C/EBPα protein preferentially bound to repeated DNA sequences located in regions of centromeric heterochromatin (Tang and Lane 1999).

Similarly, when the FP-labeled C/EBPα is produced in cells originating from the mouse, it is also preferentially localized to the regions of centromeric heterochromatin (Schaufele et al. 2001; Enwright et al. 2003). In earlier studies, we showed that only the basic-region leucine zipper (BZIP) domain, which is sufficient for dimerization and specific DNA-binding, is necessary for this subnuclear positioning (Day et al. 2003). This positioning of C/EBPα BZIP domain dimers in discrete heterochromatin islands within the cell nucleus is clearly seen in the images shown in Figure 3.4.

The fluorescence lifetime distribution for the CFP-labeled BZIP proteins has been measured by the time-correlated single-photon counting (TCSPC) method (see Chapter 7 for method details), and the fluorescence decay histograms of photon emission times relative to the laser excitation pulse were generated from the distribution of interpulse intervals at each pixel in the image. This method was used to determine a mean lifetime ($\tau_m$) for CFP-BZIP of 2.57 ns (Figure 3.4A). Time-resolved images were then acquired from cells that

### Table 3.2  FRET Measurements of Cerulean or mTFP Fusions to Venus

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>$\tau_D$ (ns)</th>
<th>$E_{\text{FRET}}^b$</th>
</tr>
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<tr>
<td>Cerulean</td>
<td>2.7 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Cerulean-5aa-Venus</td>
<td>1.21 ± 0.12</td>
<td>51</td>
</tr>
<tr>
<td>mTFP</td>
<td>2.65 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>mTFP-5aa-Venus</td>
<td>1.11 ± 0.06</td>
<td>55</td>
</tr>
</tbody>
</table>

- ±SD; $n$ = five to six cells.
- $E_{\text{FRET}} = 1 - \frac{\tau_D}{\tau_D}$. 

AU: substituted "Karsten" for "Kusten"
The BZIP proteins must dimerize to bind to the DNA elements in regions of centromeric heterochromatin. Therefore, if the donor- and acceptor-labeled proteins are produced at similar levels (discussed later) and the fluorophores are favorably positioned, it should be possible to detect dimerization events as a decrease in the mean donor lifetime ($\tau_m$).

For the cells that coexpress the CFP- and YFP-labeled BZIP, the FLIM results show that lifetime decay kinetics for the CFP-labeled proteins are best fitted by a double-exponential decay, and the $\tau_m$ is 2.05 ns in the presence of the acceptor (Figure 3.4B). A slow lifetime component ($\tau_{DA2}$) was measured at 2.65 ns, which is similar to that measured for coexpressed the CFP- and YFP-labeled BZIP proteins. The BZIP proteins must dimerize to bind to the DNA elements in regions of centromeric heterochromatin. Therefore, if the donor- and acceptor-labeled proteins are produced at similar levels (discussed later) and the fluorophores are favorably positioned, it should be possible to detect dimerization events as a decrease in the mean donor lifetime ($\tau_m$).

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the donor alone (Figure 3.4A) and represents the population of donor labeled proteins not quenched by the acceptor. A fast lifetime component ($\tau_{DA1}$), representing the donor population quenched by FRET, is 1.05 ns (Figure 3.4B). The relative amplitudes for the $\tau_{DA2}$ and $\tau_{DA1}$ decay components are 71 and 29%, respectively, yielding a distribution ratio of quenched to unquenched donor molecules of 0.4. These results illustrate how FRET-FLIM can be used to detect the association of donor- and acceptor-labeled proteins and, importantly, provide an estimate of the molar ratio of quenched donor to donor not associated with the acceptor.

3.3.4 Verifying Protein Interactions Using Acceptor Photobleaching FRET

If a shortening of the donor lifetime results from energy transfer, then destroying the acceptor fluorophore will eliminate FRET, and the donor signal should increase (dequench). The technique of acceptor photobleaching FRET exploits this effect and measures the dequenching of the donor signal in the regions of the cell where FRET has occurred (Bastiaens and Jovin 1996; Kenworthy and Edidin et al. 1998). The photobleaching approach requires that bleaching of the acceptor is selective because any bleaching of the donor fluorophore will lead to an underestimation of the dequenching. Further, the bleaching of the acceptor must be nearly complete because any remaining acceptor will still be available for FRET—again resulting in an underestimation of the donor dequenching.

The acceptor photobleaching method is used here to demonstrate that the fast donor lifetime component observed by FLIM was the result of FRET. The 514 nm laser line was used selectively to photobleach the YFP labeling the BZIP proteins. The donor lifetime was then reacquired, and the measurements revealed that the fast decay component disappeared after acceptor photobleaching, leaving only the unquenched donor population (Figure 3.5). The donor $\tau_m$ was found to be 2.49 ns, with over 90% of the population falling into the slow lifetime distribution.

These results provide independent verification of the FLIM measurements and clearly demonstrate that the average distance separating the fluorophores attached to the BZIP proteins bound to the heterochromatin is less than 80 Å. Although acceptor photobleaching is an end-point assay that cannot be repeated on the same cells, it does provide a straightforward method to verify FRET measurements made using other techniques, including FLIM.

3.3.5 Alternative Fluorophore Pairs for FRET-FLIM

Earlier chapters in this book describe how the spectral overlap, $J(\lambda)$, between the donor and acceptor fluorophores is a critical determinant of FRET efficiency. By using fluorophores that share more spectral overlap, it is possible to increase the distance over which FRET can be detected (Patterson, Piston, and Barisas 2000). For example, Emerald is a bright, stable GFP that shares substantial spectral overlap with Venus (Table 3.1), making it an effective donor (see Figure 3.6). However, the strong spectral overlap that improves the FRET efficiency will also cause a profound increase in the background spectral bleed-through signals (Section 3.3.1). As the spectral bleed-through components become larger, they overwhelm the FRET signal, severely limiting the accuracy of sensitized acceptor
emission measurements (compare Figures 3.2 and 3.6). This problem is not limited to intensity-based bleed-through correction methods, however, because the increased overlap also leads to acceptor signal bleed-through into the donor channel (back-bleed-through; see Figure 3.6).

This would appear to eliminate the GFP/YFP pair for FRET-FLIM measurements as well, but a strategy was developed that exploits the overlapping signals from the donor and acceptor fluorophores. Because energy transfer causes an increase in the acceptor lifetime (see Chapters 1 and 9), the lifetime of the combined donor and acceptor will also be increased, and this can be measured using FLIM (Harpur, Wouters, and Bastiaens 2001; Calleja et al. 2003). A broad band-pass filter is used to collect the emissions from both GFP and YFP simultaneously, allowing the combined lifetimes of the donor and acceptor to be determined. This method obviates the need for exclusive filtering to isolate the donor signal. However, the measurements of FRET using the combined spectral regions results in an increased number of lifetimes and will be less accurate than methods that are able to isolate the donor lifetime.

An alternative approach is to use acceptor probes with improved photophysical characteristics that do not fluoresce at donor emission wavelengths. For example, the red fluorescent cyanine dye Cy3 and the similar Alexa Fluor 555 are excellent acceptors for GFP (Bastiaens and Squire 1999; Ng et al. 1999). The Cy3 probe attached to antibodies or Fab fragments can be used in combination with expressed GFP-labeled proteins for FRET-FLIM experiments. Fixed cells expressing the GFP-labeled donor protein can be labeled with antibodies raised against a specific epitope or the entire interacting partner protein, thus increasing the chance of a favorable orientation of the tagged antibody to the donor fluorophore.
This approach was used to study the epidermal growth factor receptor (EGFR), which becomes autophosphorylated when the ligand binds. Wouters and Bastiaens (1999) used FRET-FLIM to map the fluorescent lifetimes of GFP-tagged EGFR that was bound by Cy3-labeled antiphosphotyrosine antibodies in cells fixed after EGF stimulation. Their FRET results, acquired at different time points after EGF addition, provided evidence for the recruitment of signaling proteins to phosphorylated EGFRs that were internalized in endosomes. Their lifetime maps also showed differences between the antibody-labeled phosphotyrosine and the FRET distributions. It is important to point out that antibody staining used by these investigators does not provide a quantitative measure of the extent of phosphorylation. However, because FRET-FLIM only detected the donor fluorophore (here, GFP), the antibody labeled with acceptor could be used in excess because the non-specific interactions of the antibody would not be detected.

### 3.3.6 Fluorescent Proteins Designed Specifically for FLIM Applications

For live-cell imaging, using probes that are excited at longer wavelengths offers important advantages. The longer wavelength spectral windows decrease phototoxicity in the living specimens and also reduce the sample autofluorescence background. Here, we would expect the new orange and red FPs (Table 3.1) to have advantages for FRET studies; however, few published studies have used these probes for intensity-based FRET measurements. The reason for this is likely that sensitized acceptor emission measurements favor fluorophores with a high quantum yield, and most of the red FPs have low intrinsic brightness (Table 3.1). In stark contrast, the acceptor quantum yield is irrelevant if FLIM is used to detect the lifetime of the donor. Therefore, probes that are optimal for FLIM measurements have requirements different from those of probes that are best for intensity-based methods.
Ideally, the donor fluorophore used for FRET-FLIM should have simple lifetime decay kinetics to allow unambiguous assignment of quenched and unquenched fractions. In this regard, the complex decay kinetics of ECFP and Cerulean can be problematic for lifetime analysis (Traimer et al. 2004; Yasuda et al. 2006; Millington et al. 2007). Here, because of variations in the protonation of the chromophore (see Section 3.2.1, for example), multiple decay pathways are available to the donor fluorophore in the excited state. Because the fluorescence decay of the donor alone is already complex, this must be accounted for when extracting quantitative information from FRET-FLIM measurements.

Further, acceptor probes with high absorption coefficients are efficient in quenching the donor fluorophore, but the acceptor does not have to be fluorescent. An acceptor probe with optimal spectral overlap, but low quantum yield, will have decreased acceptor back-bleed-through detected in the donor channel (see Figure 3.6). Here, some FPs that have not been particularly useful for intensity-based FRET measurements have turned out to be most useful for FRET-FLIM studies.

For FRET-FLIM, there are several advantages to using GFP as a donor fluorophore. First, the donor quantum yield determines the $R_0$ for the FRET pair, and GFPs have a higher intrinsic brightness than the Cerulean CFP (Table 3.1). Second, GFP is excited in a spectral window that generates less autofluorescence than that for CFP. Third, as mentioned in Section 3.3.1, simple decay kinetics are an important characteristic of donor fluorophores for FLIM studies. The emission decay of GFP is monoexponential (Traimer et al. 2006; Yasuda et al. 2006), which allows the unambiguous assignment of quenched and unquenched fractions in FRET studies. These characteristics have prompted the search for optimal FRET acceptors for GFP.

Because of their spectral overlap with GFP, both mRFP and its variant, mCherry (Table 3.1), have been used as acceptors for EGFP in FLIM studies (Peter et al. 2005; Tramier et al. 2006; Yasuda et al. 2006). The low quantum yield of mRFP improves the signal-to-noise ratio because there is less bleed-through from the acceptor detected in the donor fluorescence channel (Yasuda et al. 2006). Still, acceptors that have increased spectral overlap with EGFP emission would improve the FRET efficiency (see Section 3.3.4). As we have seen, a substantial spectral overlap exists between GFP and YFP (Figure 3.6), but the strong back-bleed-through from the bright YFP variants prevented the use of this pair for donor-based measurements.

Recently, novel YFPs have been developed that have a high absorbance coefficient, but extremely low quantum efficiency. This class of chromophore, called resonance energy-accepting chromoproteins (REACh; see Table 3.1), permits the optimal use of GFP as a donor for FRET-FLIM (Ganesan et al. 2006; Murakoshi, Lee, and Yasuda 2008). Their very low quantum yield overcomes the problem of acceptor back-bleed-through emission into the donor channel. This allows the use of filters with a wider donor spectral window to collect optimally the donor signal. The measurement of a double-exponential fluorescence lifetime decay curve for EGFP in the presence of the dark chromoproteins will now accurately reflect the populations of free donor and donor quenched by the REACh probe (Ganesan et al. 2006).

What is more, the absence of fluorescence from REACh probes means that the spectral window normally occupied by the acceptor is now available for the detection of another
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probe. This opens the possibility of correlating the protein–protein interactions detected by FRET with the behavior of another labeled protein expressed inside the same living cells—the cellular biochemical network (Ganesan et al. 2006; Murakoshi et al. 2008).

3.4 CRITICAL DISCUSSION

3.4.1 General Considerations and Limitations

As we have seen in Section 3.3.2, the genetically encoded FRET standard proteins are useful tools for checking cell culture conditions for protein expression and for calibrating imaging systems for the detection of FRET. The standards developed by Thaler et al. (2005) also included FRET standards with low FRET efficiency. These low FRET efficiency standards are especially useful for assessing the background noise in the system. In addition, FRET standards were developed that included a mutant form of Venus, called Amber, in which the chromophore tyrosine was changed to cysteine. The Amber protein folds correctly, but does not act as a FRET acceptor (Koushik et al. 2006). For some methods, such as FLIM and anisotropy approaches (see Chapter 10), the Amber-mutated FRET standard constructs may be more suitable for obtaining the donor-alone measurements because the donor-Amber fusion protein will have the same size, geometry, and mobility as the intact donor–acceptor fusion protein.

The linked Cerulean–Venus or mTFP-Venus constructs described here (see Section 3.3.2) also serve as a starting point for the design of biosensor probes. The biosensor proteins use a bioactive linker peptide to separate the donor and acceptor fluorophores. They use FRET to report changes in the conformation of the linker resulting from its modification or the binding of a substrate (reviewed by Zhang et al. 2002). These intramolecular FRET-based indicator proteins have been used to measure diverse intracellular events, including changes in intracellular calcium or protein kinase activity (Nagai et al. 2000; Miyawaki and Tsien 2000; Ting et al. 2001; Zhang et al. 2001). The direct tethering of the donor to the acceptor fixes the ratio of the expressed fluorophore pair at 1:1 and allows simple ratio imaging of acceptor-to-donor fluorescence.

The major limitation to the intramolecular FRET approach has been the poor dynamic range of the FRET sensor probes. To improve the range of response, a directed mutagenesis strategy was applied to a linked CFP-YFP construct, and mutant sensor proteins with increased FRET efficiency were selected (Nguyen and Daugherty 2002). This approach yielded sensor probes with substantially enhanced FRET signals, even though the coevolved FPs had similar spectral characteristics and slightly decreased quantum yields when compared to the original FPs. This paradox was recently resolved when it was shown that the enhanced FRET signals resulted from mutations that promoted the stabilization of an intramolecular complex formed between the linked FPs (Ohashi et al. 2007; Vinkenborg et al. 2007).

Although the self-association of the FPs is typically an unwanted characteristic for most experiments, the increased dynamic range of these probes can be an advantage for high-throughput screening methods. For example, the FRET sensor probes with markedly improved dynamic range were recently used in large-scale screening approaches (You et
al. 2006). There are concerns, however, that the stabilized intramolecular association of the FPs will also contribute to increased false positives (Section 3.4.4). Therefore, the biosensor probes based on Cerulean or Teal coupled to Venus might still be preferable, and FRET-FLIM can be particularly useful for the biosensor measurements. Here, variations in the biosensor probe lifetimes can be accurately mapped throughout the cell to reveal localized changes in probe activity. Further, the dark chromoproteins such as REACh have the added advantage of allowing the biosensor probes to be used in combination with a separate probe for protein activity.

In contrast to the intramolecular FRET measurements, intermolecular FRET experiments are designed to detect the association of independently produced proteins that are labeled with either the donor or acceptor FPs. In this case, the ratio of donor to acceptor is not fixed, and the ratio can be highly variable between individual cells within the transfected population. Adjustments in the amount of the input plasmid DNAs in the cell transfections will only influence the average relative expression levels of donor- and acceptor-labeled proteins within the population. Furthermore, because the donor–acceptor ratio varies from cell to cell, the spectral bleed-through background signal will also be different for each transfected cell. Therefore, intensity-based measurements of FRET are highly dependent upon the donor-to-acceptor ratio and will work best over a limited range of ratios (Berney and Danuser 2003). FRET-FLIM is less restricted in this respect because the determination of donor lifetimes will also provide an estimate of the molar ratio of quenched donor to donor not involved in FRET (Section 3.3.3).

3.4.2 Overexpression Artifacts

The intermolecular FRET experiments in living cells typically involve transfection methods to introduce DNA constructs encoding the proteins labeled with the donor or acceptor FPs. This type of approach offers great flexibility for the analysis of protein interactions using FRET. However, it is important to recognize that any amount of exogenous protein that is produced in a cell is, by definition, overexpressed relative to its endogenous counterpart. The transfection approach can yield very high levels of the fusion proteins in the target cells, especially when strong promoters are used. This can result in improper protein distribution and protein dysfunction that could lead to erroneous interpretations of protein activities.

It is critical that immunostaining approaches be used to demonstrate that the subcellular distribution of the expressed proteins is the same as that for the endogenous protein. Moreover, several different approaches should be used to verify that FP-fusion proteins produced in living cells retain proper functions. Only a small percentage of the expressed protein detected by fluorescence microscopy might actually be involved in the cellular function that is being monitored. Therefore, it is important to work with cells expressing near-physiological ranges of the fusion proteins and to quantify the effect of protein concentration on the FRET results carefully.

3.4.3 Factors Limiting FRET-FLIM

The major limitation to FRET-FLIM studies has been the complexity of the imaging systems. Recently, however, commercial user-friendly systems have become available, making
the FLIM approach much more accessible. Another limitation of FLIM is that the acquisition of the data is typically slow. For example, acquiring sufficient photon counts to assign lifetimes using the TCSPC method described earlier in Section 3.3.3 required 2 minutes for each image, which limits its utility for monitoring dynamic events. Some commercial systems use the frequency domain method, which can be faster and more photon efficient (see Chapter 5). Finally, fluorescence lifetime provides detailed information about any local environmental event that influences the excited state. Environmental factors, such as changing pH or collisional quenching, will also shorten the measured fluorescence lifetime. Therefore, care must be taken in interpreting FRET-FLIM data from living cells.

3.4.4 False Positives and False Negatives

Just as with any other imaging method, it is critical to identify the sources of noise in FRET-FLIM measurements in order to determine the reliability of the data analysis and to avoid overinterpretation of data. Instrumental errors are always a possible source of erroneous results. Here, the FRET&' standard proteins (Section 3.3.2) are valuable tools because they should report the same range of FRET signals each time they are used, and they will effectively reveal problems in the imaging system. A potential source of false-positive results, mentioned in Sections 3.2.3 and 3.4.1, is interactions driven by the association of the FPs themselves. This can be avoided by using the monomeric versions of the FPs (see Section 3.2.3). Further, false-positive signals can arise because of the overexpression artifacts mentioned previously. This can be avoided by choosing cells during imaging that express the labeled proteins at low levels.

In contrast, false-negative results are common because it is often difficult to achieve the required spatial relationships for FRET between the FPs that tag proteins—even when the proteins are interacting. For this reason, a negative FRET result provides information only in cases where the donor and acceptor are physically linked, such as the biosensor probes. Here, it is also important to recognize that the detection of FRET provides information about the spatial relationship of the fluorophores themselves and does not necessarily indicate the direct interactions between the proteins that they label. Rather, the FPs serve as surrogates for the relative spatial relationships between the specific protein domains to which they are attached.

Thus, FRET measurements alone are not sufficient to prove direct protein–protein interactions. Additional biochemical approaches are required to demonstrate that the protein partners that are being studied are actually in physical contact with each other. However, intermolecular FRET measurements with the FPs do provide direct evidence of protein associations within the natural environment of the living cell at less than 100 Å, revealing information about the structure of protein complexes that form at specific subcellular sites.

3.4.5 Analysis in the Cell Population

FRET results from single cells are, by themselves, not sufficient to characterize the associations between proteins in living cells. Although the FRET measurements, when collected and quantified properly, are remarkably robust, there is still heterogeneity in the measurements. Furthermore, substantial cell-to-cell heterogeneity may also be present for some types
of interactions, and it is possible that only a subpopulation of cells responds to a particular stimulus. Therefore, data must be collected and statistically analyzed from multiple cells to prevent the user from reaching false conclusions from a nonrepresentative measurement.

### 3.5 SUMMARY

This chapter has presented some of the characteristics of the FPs that make them useful labels for FRET-FLIM studies in biological systems. The results presented here illustrated how the FRET standard proteins can be used to calibrate measurements and characterize new fluorophore pairs. The results also showed how FRET-FLIM can be used to detect the intermolecular interactions between proteins produced in living cells and how these measurements can be verified by acceptor photobleaching. In addition, the chapter outlined how some of the FPs that have not been useful for intensity-based FRET measurements are finding great utility in FRET-FLIM studies. Furthermore, FPs designed specifically for FLIM applications, such as the REACh probes, are overcoming some of the limitations to the FRET-FLIM approach in living cells. The reader is encouraged to answer the following critical questions and to explore these topics in greater depth using the references provided.

### 3.6 FUTURE PERSPECTIVE

The combination of the FPs and lifetime imaging provides a powerful tool for studies in cell biology. This approach will certainly come into the mainstream as user-friendly and less expensive commercial systems become available. The measurements of probe lifetimes can enhance the contrast in biological and medical imaging and have the potential to be used for large-scale screening applications in living cells. In addition, as more FPs with unique properties are discovered, the palette of useful probes for FLIM will expand. In this regard, new FPs have been identified that can be switched on by light or that change their emission characteristics when illuminated at specific wavelengths (reviewed by Day and Schaufele 2008). The photoswitching properties of these novel FPs are being used to measure the dynamic behaviors of proteins inside living cells (for example, see Demarco et al. 2006), and it may be possible to exploit these characteristics for FLIM studies as well.

### REFERENCES


