

A Guide to Fluorescence Lifetime Microscopy and Förster's Resonance Energy Transfer in Neuroscience

Daniel J. Liput,^{1,2} Tuan A. Nguyen,³ Shana M. Augustin,¹ Jeong Oen Lee,¹ and Steven S. Vogel^{3,4}

¹Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland

²Laboratory of Molecular Physiology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland

³Laboratory of Biophotonics and Quantum Biology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland

⁴Corresponding author: stevevog@mail.nih.gov

Fluorescence lifetime microscopy (FLIM) and Förster's resonance energy transfer (FRET) are advanced optical tools that neuroscientists can employ to interrogate the structure and function of complex biological systems in vitro and in vivo using light. In neurobiology they are primarily used to study protein-protein interactions, to study conformational changes in protein complexes, and to monitor genetically encoded FRET-based biosensors. These methods are ideally suited to optically monitor changes in neurons that are triggered optogenetically. Utilization of this technique by neuroscientists has been limited, since a broad understanding of FLIM and FRET requires familiarity with the interactions of light and matter on a quantum mechanical level, and because the ultra-fast instrumentation used to measure fluorescent lifetimes and resonance energy transfer are more at home in a physics lab than in a biology lab. In this overview, we aim to help neuroscientists overcome these obstacles and thus feel more comfortable with the FLIM-FRET method. Our goal is to aid researchers in the neuroscience community to achieve a better understanding of the fundamentals of FLIM-FRET and encourage them to fully leverage its powerful ability as a research tool. Published 2020. U.S. Government.

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INTRODUCTION

Neuroscience is a rich and diverse field that combines disciplines in the effort to understand the brain and its behavioral and cognitive functions. During the evolution of this interdisciplinary field, neuroscientists have worked closely with other disciplines to develop and implement many techniques, such as light microscopy paired with Golgi staining

(Golgi, 1873; Ramón & Cajal, 1904), patch-clamp electrophysiology (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981), whole-brain imaging (Ogawa, Lee, Nayak, & Glynn, 1990; Reivich et al., 1979), and now a diverse toolkit of optical techniques ranging from 2-photon microscopy (Denk, Strickler, & Webb, 1990) to in vivo fiber photometry (Cui et al., 2013, 2014). The combinatorial approach of

microscopy and fluorescent molecules, which uses photon emission scaling from seconds to picoseconds to monitor molecular dynamics and protein-protein interactions, has allowed researchers to explore signal transduction in cells within the brain with high spatial and temporal resolution. FLIM-FRET microscopy is an emerging powerful tool to study molecular dynamics in live cells in vitro and in vivo. Here, we describe the general principle of FLIM-FRET microscopy, quantification of FRET efficiency using fluorescence lifetime, applications of FLIM-FRET in neuroscience research ranging from molecular neurobiology to its use in vivo in behaving animals, and discuss the experimental challenges and considerations of FLIM-FRET techniques.

See the APPENDIX at the end of this article for a list of abbreviations used in the discussion of FLIM-FRET and background topics.

FLIM-FRET

FLIM-FRET is a fluorescence-microscopy based tool (Algar, Hildebrandt, Vogel, & Medintz, 2019; Gadela, 2009; Periasamy, Mazumder, Sun, Christopher, & Day, 2015) that can be and has been used to measure dynamic changes in the proximity of molecules on a 1-10 nm scale under physiological conditions. To understand how to design and interpret FLIM-FRET experiments, as well as to be aware of the limitations and potential artifacts of this approach, it is helpful to understand the photophysical basis of FLIM and FRET. One major obstacle when trying to understand FLIM-FRET is confronting the realization that a comprehensive understanding of FLIM and FRET typically requires being comfortable with the physics that describes the interactions of light and matter on a molecular level. Unfortunately, extensive training in this subject is not typically part of the repertoire or comfort zone of many neuroscientists. The objective of this section is to provide an intuitive and mostly math-free understanding of FLIM and FRET. To do this, the reader will be asked to accept certain quantum mechanical phenomena without a rigorous explanation of the basis for those beliefs.

First, let us set the stage. In a FLIM-FRET experiment, molecules of interest are tagged with chromophores; at least one of these must be a fluorophore (see NOTE 1), and usually they all are. Next, our fluorophore-tagged sample is irradiated with light to excite one

of the fluorophores, and we monitor changes in certain aspects (see NOTE 2) of the light that has interacted with our sample. Changes observed then allow one to infer changes in the proximity of the tagged molecules. These inferences typically are based in part on some experimental assumptions; thus, any inference of molecular proximity from a FLIM-FRET experiment will only be valid if these underlying assumptions are valid. In this scheme, FRET is a photophysical phenomenon that can only occur when fluorophores come into close proximity (typically <10 nm) (Algar et al., 2019; Gadela, 2009; Medintz & Hildebrandt, 2013; Periasamy et al., 2015; Vogel, Thaler, & Koushik, 2006), and FLIM is a microscopic technique that facilitates the observation and quantification of changes in the amount of FRET (Bastiaens & Squire, 1999; Becker, 2005; Gadela, 2009; Gadella, Jovin, & Clegg, 1993; Periasamy et al., 2015). In the next three sections, we will first explain the basis of FLIM, then we will explain the basis of FRET, and finally we will discuss how FLIM can be used to measure FRET.

FLIM is designed to measure the fluorescence lifetime of a fluorophore. FLIM can both be used to generate lifetime images as well as in a photometry mode to generate lifetime decay traces. Here are the key questions we need to answer:

1. What is a fluorescence lifetime?
2. How does a FLIM microscope measure a fluorescence lifetime?
3. How is a fluorescence lifetime parametrized?
4. What experimental factors can influence and/or limit FLIM measurements?

NOTE 1: Chromophores are molecules that can absorb photons, which are particles of light. Fluorophores are chromophores that can subsequently reemit photons with a longer wavelength (lower energy).

NOTE 2: In quantum mechanics, light behaves as both a wave and as particles (photons). Some aspects of light are easier to understand as a wave, while other aspects are easier to understand as particles. For example, the amount of energy in a photon is related to the wavelength (color) of a beam of light. The number of photons in a beam of light is related to the intensity of the light. Light also has an orientation; this orientation is the basis of polarization in waves of light and is related to the spin of individual photons.

What is a Fluorescence Lifetime?

A fluorophore is a chromophore; thus, it can transiently absorb a photon (a quanta of light). The absorption of a photon elevates the chromophore, actually one of its electrons, into a higher energy excited-state orbital. Eventually, that electron will return to its ground state, and the energy that was initially absorbed (from the photon) will be dissipated. Chromophores can have multiple pathways by which the excitation energy can be dissipated; these include the dampening of molecular vibrations, the loss of energy through collisions with molecules surrounding the chromophore, undergoing chemical reactions in the excited state, etc. Invariably some energy is lost from the excited state as heat to the surrounding environment. What distinguishes a fluorophore from a chromophore is that the former have a unique additional mechanism for how excitation energy from the absorbed photon can be dissipated: a fluorophore in its excited state can emit a photon to dissipate at least some of its excited-state energy. An important concept to keep in mind here is conservation of energy; the energy that a chromophore absorbs (when it is elevated to an excited state) must be greater than or equal to the energy liberated (when it returns to its ground state). Since, in addition to the emission of a photon, energy is lost from the excited state by other mechanisms, the energy of the photon emitted always (see NOTE 3) has less energy (is red shifted) when compared to the energy of the photon that excited the fluorophore. **The average time that a fluorophore spends in the excited-state (between when a photon is absorbed, and when it emits a red-shifted photon to return to the ground-state) is called its fluorescence lifetime tau (τ).** Typical lifetime values can range from picoseconds to nanoseconds. The experimentally measured value of a fluorophore's lifetime (τ_{apparent}) is a time-constant value sensitive to environmental factors. Thus, τ_{apparent} represents an average lifetime value of a population of fluorophores in a sample and is inversely proportional to the rate at which the population of excited fluorophores emits red-shifted photons, k_E .

$$k_E = \frac{1}{\tau_{\text{apparent}}}$$

Equation 1

This emission rate is a product of the radiative photon emission rate intrinsic to each fluorophore, K_R , as determined by the Strickler-

Berg equation (Strickler & Berg, 1962), and the quantum yield, Q , of the fluorophore:

$$k_E = K_R \cdot Q$$

Equation 2

Q is the probability that a fluorophore will emit a photon when it is excited, and is calculated by dividing the intrinsic radiative rate of a fluorophore by the intrinsic radiative rate plus the sum of all non-radiative (see NOTE 4) energy transfer rates specific for each of the multiple pathways available to a fluorophore to dissipate energy from its excited state (k_{NRa} , k_{NRb} , $k_{NR...}$).

$$Q = \frac{k_R}{k_R + k_{NRa} + k_{NRb} + k_{NR...}}$$

Equation 3

These non-radiative energy transfer rates may be influenced by environmental factors, decreasing or increasing the quantum efficiency of a fluorophore and thus the observed value of k_E (and inversely τ_{apparent}). While an in-depth description of fluorescence is beyond the scope of this overview, extensive information can be found in the literature (Anthony, Guo, & Berland, 2010; Lakowicz, 2006; Valeur & Berberan-Santos, 2012).

NOTE 3: We are only considering one-photon excitation here.

NOTE 4: Non-radiative here refers to mechanisms that result in the loss of excited state energy without emitting a photon.

How Does a FLIM Microscope Measure a Fluorescence Lifetime?

The two main methods used are the Time Domain approach (Becker, 2005; Becker et al., 2006; Gerritsen, Agronskaia, Bader, & Esposito, 2009; Suhling, French, & Phillips, 2005) and the Frequency Domain approach (Kremers, van Munster, Goedhart, & Gadella, 2008; Lakowicz, Szmazinski, Nowaczyk, Berndt, & Johnson, 1992; van Munster, Goedhart, Kremers, Manders, & Gadella, 2007; Verveer & Hanley, 2009; Verveer, Squire, & Bastaens, 2001). In the Time Domain approach, the fluorophore of interest is excited by a very short light pulse, and the interval between the excitation light pulse and when a red-shifted emitted photon is detected is recorded. This type of measurement is repeated tens of thousands of times to calculate the average time that these fluorophores remain in the excited state. In the Frequency

Domain approach, the fluorophore of interest is excited by a frequency-modulated light source, and the phase shift observed in the resulting frequency-modulated red-shifted emitted photons (relative to the excitation light source) and their modulation amplitude is used to calculate the fluorescence lifetime. Both approaches require highly specialized excitation light sources, light detectors, and processing instrumentation to accurately measure fluorescence lifetimes. In this overview, we will only describe the Time Domain approach because: (1) We believe it is a more intuitive approach for those new to FLIM-FRET; (2) one of the key, and most expensive pieces of instrumentation required for the time-domain approach, an ultra-fast pulsed laser (such as a Ti:Sapphire laser used in two-photon excitation), is readily available in many neuroscience laboratories and/or departments; and (3) the authors' expertise in FLIM-FRET was primarily gained using this approach on modified two-photon microscopes. Nonetheless, it is important to realize that both approaches are commonly used for measuring fluorescent lifetimes, and we encourage those interested in the Frequency Domain approach to read the many excellent reviews on this technique (Lakowicz et al., 1992; Verveer & Hanley, 2009; Verveer et al., 2001). Similarly, other approaches have been used to measure lifetimes, such as streak cameras (Krishnan, Masuda, Centonze, & Herman, 2003; Ramanujan, Jo, Ranjan, & Herman, 2010) or gated photo-detectors (Sun, Demas, & Periasamy, 2010), but these approaches will not be discussed here.

In addition to a microscope platform, the three main pieces of equipment required for implementing the time-domain approach for FLIM are: (1) an ultra-fast pulsed light source, typically a laser, needed for photo-excitation of the fluorophore; (2) photo-detectors capable of detecting single photons of emitted light from the fluorophore; and (3) time-correlated single photon counting (TCSPC) electronics that can accurately and precisely measure the time delay between an excitation light pulse and when a photon is detected, record these values, and count these detection events as a function of the delay time between excitation and emission to generate a histogram of these events. It is important to realize that the apparent lifetime measured with this approach will be a convolution of the actual lifetime of the sample convolved with the dynamics of the excitation pulse, the instrument response function (IRF) of the detector, and the electronics

used to process detected photons. Thus, the instrumentation component with the slowest response time, typically the photodetector, will set a limit on the fastest lifetime decay components that can be observed. In Figure 1A, we can see a cartoon depicting the principle of how TCSPC is used to measure a fluorescence lifetime in the time domain. Regularly spaced laser excitation pulses are depicted in green, and emitted photons are occasionally detected and are depicted in yellow. The time interval between an excitation pulse and when an emitted photon is detected is called the micro-time. A histogram (Fig. 1C) of measured micro-times (Fig. 1B) constitutes a fluorescence lifetime decay curve, the raw data for measuring a lifetime in the time domain.

The most important trait when selecting a pulsed laser for FLIM measurements is that its wavelength and power must be capable of efficiently exciting the fluorophore whose lifetime is being measured. Other characteristics of the laser that should be considered are its pulse width and repetition rate. In general, to accurately measure a fluorescence lifetime, the pulse width of the laser used for excitation should be much smaller than the lifetime of the fluorophore. For example, two-photon microscopes use lasers that typically have a pulse width of a few hundred femtoseconds, thousands of times smaller than the lifetimes of most fluorescent proteins (FPs, between 2 and 4 ns). For one-photon excitation, a pulsed laser with a pulse width of under 50 ps is typically used, still over an order of magnitude smaller than the lifetimes of most fluorophores of neurobiological interest. It is important to note that if the fluorophores lifetime is much shorter than the excitation pulse width, lifetime measurements will not accurately reflect the true lifetime of the fluorophore. If the lifetime is on the same order of magnitude as the laser pulse width, measured lifetime will effectively be a convolution of the laser pulse shape and duration with the true underlying lifetime of the fluorophore. Deconvolution methods might be useful for extracting an accurate fluorophore lifetime under low-noise conditions.

Another attribute of the pulsed excitation light source that must be considered is its repetition rate, the number of laser pulses per second. Most Ti:Sapphire lasers used for FLIM measurements operate at 80 MHz, or 1 laser pulse every 12.5 ns. Lasers used for one-photon FLIM typically have a choice of repetition rates, ranging from 20-80 MHz (1 laser pulse every 50-12.5 ns, respectively).

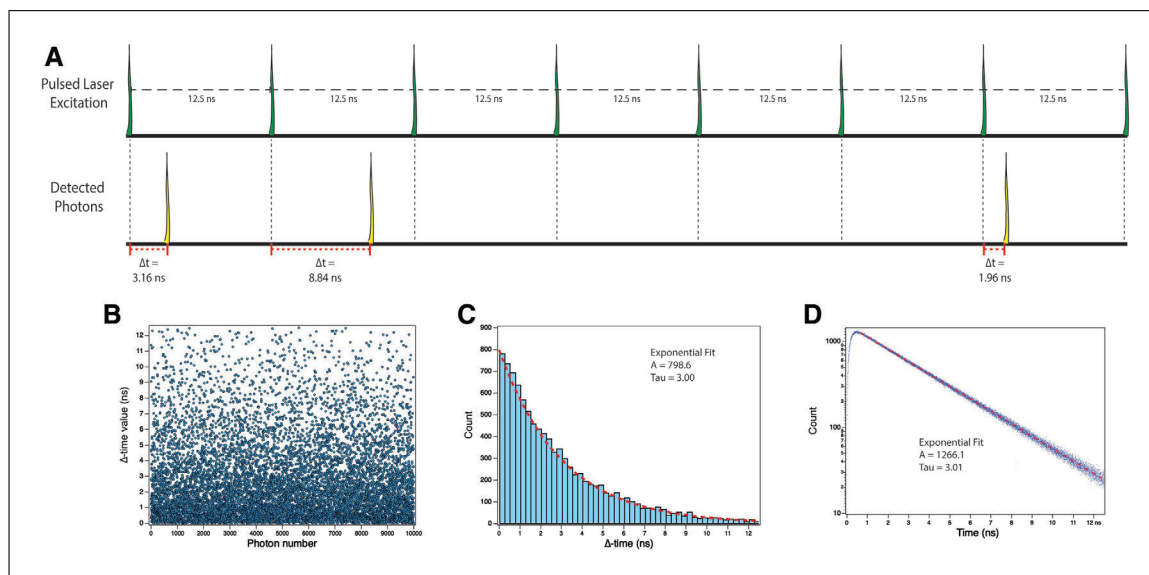


Figure 1 Lifetime measurements using TCSPC. (A) Schematic depicting laser pulses from an 80-MHz pulsed laser (green spikes) used to excite a sample containing a fluorophore, and detected photons (yellow spikes) emitted from the sample. The time interval (Δt) between an excitation pulse and a detected photon is measured (see dashed red lines). (B) A Monte Carlo—simulated population of 10,000 Δt measurements from a fluorophore having a 3-ns fluorescence lifetime. (C) A histogram of the Δt values (blue bars). Dashed red line is a fit of the histogram values to a single exponential decay model which accurately recovers the simulated 3-ns lifetime. (D) A more realistic rendition of a simulated lifetime decay plotted on a semi-log plot. Note the rising phase and peak count (observed here between 0 and 1 ns) resulting from the convolution of the true underlying lifetime decay (3 ns) and the instrument response function (150 ps). The red dashed line indicates a fit to a single exponential decay model using only the data between the peak count and the count at 12.5 ns. This fit successfully recovered the 3 ns tau value. On a semi-log plot, a single exponential decay looks like a straight line with a negative slope. Also note how the fractional noise of the photon count gets larger (see the spread of blue dots around the dashed red fit) as the count gets smaller with time.

Ideally, for FLIM, the time interval between laser pulses should be long enough to allow for the vast majority of fluorophores in a population to decay to their ground state after an excitation pulse, approximately $5 \times$ the fluorophore's fluorescence lifetime. In practice, this is not always possible, and corrections can be applied to compensate for incomplete decay. Conversely, if the time interval between laser pulses is much greater than $5 \times$ the fluorescence lifetime being measured, the time needed to measure a fluorescence lifetime will be adversely affected.

The most important trait for selecting photo-detectors for FLIM measurements is that they should be able to efficiently detect the wavelength of the photons being emitted by the fluorophore of interest. Photo-detectors have a range of quantum efficiencies, the detector's probability of detecting a photon of a specific wavelength if it strikes the detector surface. Quantum efficiencies of detectors used for FLIM typically range from 10% to 90%, where a higher value will be able to detect a higher fraction of the photons that interact with its detector surface. Quantum ef-

ficiency, however, is only half the issue, as the cross-section of a detector—the surface area of the detector that can sense photons—can vary greatly. A photon-sensor's ability to detect a photon is a function of both its quantum efficiency as well as the probability that an emitted photon will be captured by its cross-section. A detector with a very high quantum efficiency, such as an avalanche photo-diode, but with a very small cross-section, may not always be a superior choice for FLIM experiments compared to a detector with an intermediate quantum efficiency, but with a very large cross-section, such as a hybrid detector. Ideally, for FLIM measurements a detector with both a high quantum efficiency and a large cross section is desirable.

Other important photo-detector traits to consider when selecting a photo-detector for FLIM are its dark noise and its IRF. A detector's dark noise is the number of counts per second a detector records when placed in a dark environment. The dark count of a detector sets limits on the best signal-to-noise ratio possible in a FLIM experiment. For example, if a detector with a dark noise of 500

counts per second detects 1000 counts per second when the sample is irradiated with the excitation laser, only half of these ‘detected’ counts are actually photons emitted from the sample, and the signal-to-noise will be 1:1. In contrast, if a detector with the same quantum efficiency and cross section is used, but with a dark noise of 10 counts per second, ~510 counts per second will be detected and the signal-to-noise will be 50:1.

The function of a photo-detector and the subsequent TCSPC electronics used in FLIM is to detect photons as a function of time, with high accuracy and precision, and to convert that data into a format that a computer can use for further analysis. Ideally, when a photon is detected, the time of its incidence on the detector surface should be instantaneous. In reality, the process of photon detection typically involves signal-amplification steps that take time, and this delay will degrade the timing precision of a FLIM measurement and set limits on the fastest lifetime decay components that can be measured. The IRF of a detector and the subsequent electronics used to detect and process photons is a measure of how long the photon-detection instrumentation takes to measure a near-instantaneous pulse of light. Typical photodetectors used for FLIM have IRFs ranging from ~70 to 300 ps, but recently some detectors have had IRFs as fast as 20 ps. In general, it is difficult, if not impossible, to measure lifetimes much shorter than the IRF of the photodetector used.

How is a Fluorescence Lifetime Parametrized?

An ideal fluorophore has a single excited state, and once it is excited it will have a fixed probability per unit time of decaying back to its ground state. Under these circumstances, a homogenous population of this fluorophore, when excited by a short laser pulse, should emit a fluorescence signal whose intensity, I , decays exponentially with time, t (Fig. 1D). This signal can be fit to a single exponential decay model:

$$I(t) = A \cdot e^{-t/\tau}$$

Equation 4

This model has two fitting parameters, A and τ . A is the amplitude of the signal at time zero (proportional to the number of photons emitted immediately after photoexcitation, referred to as the peak count), and τ is a decay constant. Since in a TCSPC FLIM measurement emitted photons are integer values result-

ing from counting, the Poisson error in the intensity value of the signal (the count) at any value of t should be the square root of the count (Bevington & Robinson, 1992; J. R. Taylor, 1997). Thus, the fractional error in $I(t)$ values:

$$f_{error} = \frac{\sqrt{I(t)}}{I(t)}$$

Equation 5

increases at longer times because they have lower $I(t)$ values. This can be directly observed in decay curves as the spread of $I(t)$ values increases with time (Fig. 1D). Conversely, the higher the peak count in a FLIM experiment (typically resulting from longer data acquisition time; see NOTE 5), the smaller the fractional error in the lifetime measurements. For a single exponential decay of photons emitted by a population of fluorophores, mathematically it turns out that τ is the average time that the fluorophore spends in its excited state, a.k.a. its lifetime. Thus, the value of τ , as measured by curve fitting, can be used to parameterize the lifetime of a fluorophore. Clearly, the less fractional error present in a fluorescence lifetime decay curve, the more accurate and precise a τ measurement will be. This is typically not a problem for fluorophores that decay as a single exponential but is an important experimental consideration if a sample has a more complex decay. We will cover this momentarily. It is also worth noting that the total number of emitted photons detected in a FLIM experiment is simply the area under the $I(t)$ decay curve. It turns out that τ is also proportional to this value, and this will be useful when we discuss FRET measurements.

Many fluorophores do not decay as single exponentials. This might indicate that they have multiple excited states, that the fluorophore’s environment in the sample population is not homogeneous, or even that the sample itself is not homogeneous. Often, under these conditions, the fluorescence decay curve from these samples can still be well fit by a double exponential decay model:

$$I(t) = A_1 \cdot e^{-t/\tau_1} + A_2 \cdot e^{-t/\tau_2}$$

Equation 6

or it might require a multi-exponential decay model for a better fit:

$$I(t) = \sum_{i=1}^n A_i \cdot e^{-t/\tau_i}$$

Equation 7

It is important to realize that these models have 4 or more fitting parameters, respectively. Accordingly, they have many more degrees of freedom for fitting FLIM data as compared to a single exponential decay model with only 2 parameters. For this reason, it is often possible to fit a sample with a broad distribution of decay components with a simple double exponential decay model. In other words, just because a double exponential model fits a data set well does not necessarily mean that that a sample only has two tau values. What it does mean is that a multi-exponential decay model can be used to parameterize a more complex FLIM decay curve and measure an accurate average lifetime value, $\langle\tau\rangle$. The amplitude-weighted average lifetime value for a multi-exponential decay model is:

$$\langle\tau\rangle = \frac{\sum_{i=1}^n A_i \cdot \tau_i}{\sum_{i=1}^n A_i}$$

Equation 8

where i is an integer value, and n equals 2 for a double exponential model, 3 for a triple exponential model, etc. Again, it is worth noting that $\langle\tau\rangle$ is proportional to the area under the lifetime decay curve of a sample with a multi-exponential decay.

NOTE 5: Peak count can also be increased by increasing the intensity of the excitation laser, although care must be exercised to avoid photo-damage to the sample or bleaching of the fluorophore.

What Experimental Factors can Influence and/or Limit FLIM Measurements?

From a practical viewpoint, a neuroscientist using or evaluating FLIM experiments needs to understand what experimental factors can influence and/or limit FLIM measurements. These fall into three broad categories: factors based on the instrumentation used for the measurements, environmental factors that might alter radiative and non-radiative energy transfer rates from the fluorophore's excited state, and the presence of other fluorophores with their own distinct lifetimes that might corrupt the apparent lifetime of the fluorophore of interest. While a comprehensive description of these factors is beyond the scope of this manuscript, we will give examples of each category that we feel is specifically relevant to FLIM-FRET experiments in neurons.

As mentioned above, the apparent fluorescence decay curve measured by a FLIM experiment is a convolution of the “real” underlying fluorescence lifetime decay with both the shape of the excitation light pulse and the IRF of the photo-detector and subsequent electronics. In practice, the slowest component in a FLIM-FRET microscope is typically the photo-detector IRF, and accordingly this is usually the component that limits the fastest decay components that can be observed in a FLIM experiment. This concept is illustrated in Figure 2. Panel A shows a hypothetical double exponential decay curve. Each of the two decay components in this curve had the same amplitude, and therefore account for 50% of the total decay. The first component was assigned a decay constant of 100 ps, and the second component had a decay constant of 1000 ps. These values were successfully recovered by curve fitting with a double exponential decay model (see red dashed line). Thus, using Equation 8, we can calculate an average lifetime for this sample of 550 ps. The inset in the upper right corner of panel A in Figure 2 shows three exponential IRFs set at 20 ps (black trace), 70 ps (blue trace), and 150 ps (green trace). In Figure 2, panels B-D, we show the normalized convolution of the black trace in panel A with the three different IRFs. Note that unlike the black trace in panel A which decays from its highest value at $t = 0$, the traces in panels B, C, and D have a rapid rising phase as a result of the convolution, and their peak values occur slightly after $t = 0$. The amplitude of each of these traces was normalized to the peak value. For each convolution curve, we attempt to recover the amplitudes and tau values of the decay components (red dashed traces). The 20 ps IRF convolution decay (panel B) appeared very similar to the decay in panel A, and curve fitting recovered two decay components with tau values of 115 and 1009 ps; their relative amplitudes were approximately 4:6. With these values, we can calculate an average apparent lifetime of 651.4 ps (a value that is ~18% larger than the real $\langle\tau\rangle$). When the 70 ps IRF was used (panel C), curve fitting still recovered two decay components, but now with tau values of 192 and 1018 ps, and their amplitudes were approximately 2:8 yielding an average lifetime of ~852.8 ps (~55% > than the real $\langle\tau\rangle$). When the 150 ps IRF was used (panel D), only a single exponential decay was observed, with a tau value of 985 ps, reflecting a $\langle\tau\rangle$ that is 79% larger than the real value. In all three examples the

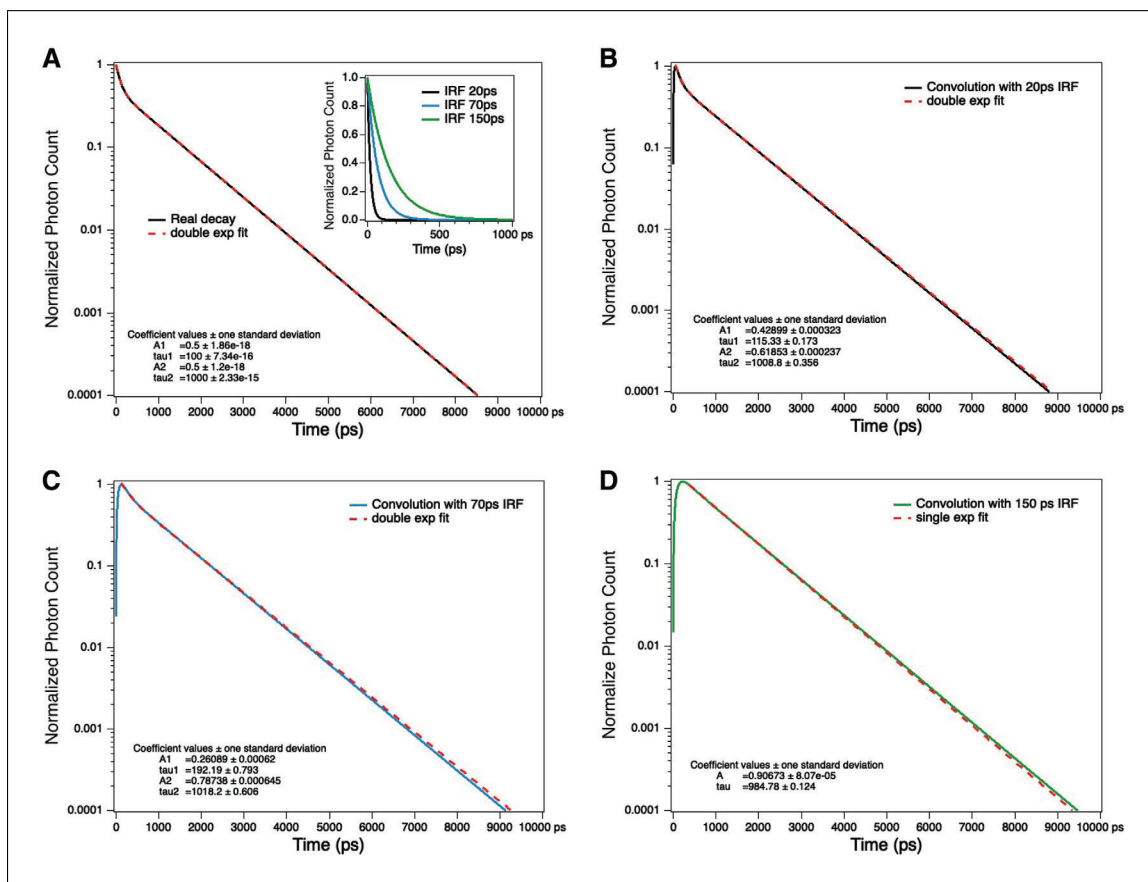


Figure 2 How the instrument response function (IRF) influences our ability to recover lifetime decay information. **(A)** Simulated lifetime decay (black line) with two decay components (100 ps and 1000 ps) of equal amplitude. The red dashed line is a fit of the decay data to a double exponential decay model which accurately recovers both Tau1 and Tau2 as well as their relative amplitudes (A1 and A2). The inset in panel A shows three IRFs based on commercially available photo-detectors with 20 (black trace), 70 (blue trace), and 150 ps (green trace) decay constants. The un-convolved lifetime decay depicted in panel A was convolved with either the 20 ps IRF **(B)**, the 70 ps IRF **(C)**, or the 150 ps IRF **(D)**. A rising phase and peak count is seen in all three cases. In panels B and C, data between the peak count and 9 ns was fit to a double exponential decay model, while the data in panel D was fit to a single exponential decay model (red dashed lines).

tau of the slow decay component (1000 ps) was reasonably recovered (1009, 1018, and 985 ps, respectively), but the tau of the fast decay component (100 ps) varied, ranging from 115 to 192 ps to being undetectable as the IRF changed from 20 to 70 ps, to 150 ps. You should also note that in this example there was no Poisson or Dark noise in our decay curve or IRFs. In reality the presence of noise will complicate curve fitting, particularly if attempts are made to deconvolve the IRF with the apparent decay curve. The main take-away point is that the choice of instrumentation used for FLIM measurements will limit the fastest decay components that can be observed. Since it is exceedingly difficult to detect decay components that are faster than the IRF, this may dramatically alter the apparent lifetime observed in a FLIM experiment.

Environmental factors in a biological milieu that might alter radiative and non-radiative energy transfer rates include pH, refractive index, endogenous quenchers, viscosity and even the fluorophores concentration itself. Further complicating this issue in living cells is that it cannot always be assumed that the distribution of these environmental factors within neurons is homogenous. If possible, efforts must be taken in FLIM experiments to keep environmental factors constant.

Finally, we must consider the impact of having other fluorophores present in our FLIM sample. Under these conditions, the apparent lifetime measured in a FLIM experiment will be a combination of the lifetime of the different fluorophores present, weighted by the relative number of photons they emit under the experimental conditions. Neurons are known to typically have high levels of

endogenous fluorescence, so this is a major issue for FLIM measurements in neurons. One strategy to deal with this problem is to manipulate either the excitation wavelength and/or the emission wavelength collected by the photodetectors to selectively excite and/or collect photons from the fluorophore of interest. This, however, cannot always be achieved. Another strategy is to manipulate the system, typically by over-expressing the fluorophore of interest, so that it is contributing the vast majority of the emitted photons being detected as compared with endogenous fluorophores. This approach, however, is also often problematic, as expressing high levels of exogenous fluorophores in living cells might be toxic, and/or perturb the behavior of the cell. Finally, we would like to point out a common dynamic artifact we have observed in live-cell FLIM experiments due to having autofluorescence present. Autofluorescence typically has much shorter lifetimes than most exogenous fluorophores used in FLIM experiments. In general, the longer the lifetime of a fluorophore, the easier it is to bleach over time. So, if at the start of a time-course FLIM experiment 90% of collected photons comes from the exogenous fluorophore and 10% might come from autofluorescence, over the duration of the experiment the exogenous fluorophore will bleach at a much faster rate than the short-lifetime autofluorescence, such that by the end of the time course, 50% of the collected photons might now be coming from the exogenous fluorophore and 50% might come from autofluorescence. Under these conditions, it will appear that the apparent lifetime of the fluorophore is getting shorter with time when in reality what is happening is that the relative contributions of the long-lived fluorophore of interest is decreasing.

What is FRET?

FRET is a physical phenomenon where excited-state energy from a fluorophore is transferred, non-radiatively, to another nearby chromophore. Here are the key questions we need to answer about FRET:

1. What is FRET?
2. How is FRET parameterized?
3. How can FLIM be used to measure FRET?
4. What is needed for FRET to occur?

As mentioned above, after being excited by the absorption of a photon, most fluorophores, after a short delay (related to its lifetime), will emit a red-shifted photon and return to

its ground state. When FRET occurs, a fluorophore absorbs a photon to reach its excited state, but instead of emitting a red-shifted photon, it transfers its excitation energy to a nearby ground-state chromophore. The fluorophore that initially absorbs a photon to reach its excited state is called the FRET donor, and the chromophore that receives excited-state energy from the donor is called the FRET acceptor. When FRET occurs, the excited-state energy of the donor is transferred to a ground-state acceptor using a non-radiative mechanism—photons are not involved in the transfer. The transfer of energy from donor to acceptor is discrete (quantal); the energy is thought to hop from the donor to the acceptor. The net result of FRET is that the donor will return to its ground-state without emitting a photon and the ground-state acceptor is elevated to its excited-state without absorbing a photon. If the acceptor, now in its excited-state, is also a fluorophore, it will be free to emit a photon, just as if it had absorbed a photon to reach its excited state. FRET is based on coulombic coupling between the transition dipoles (see NOTE 6) of the donor and acceptor.

NOTE 6: The transition dipole moment is the multidimensional vector describing the electric charge separation created when a fluorophore's ground-state electron is elevated to its excited state.

How are FRET Experiments Parameterized?

All too often in the literature, FRET has been parametrized based on the specific experimental approach used to measure it. This has resulted in FRET indices or ratios that are instrument specific and inherently not reproducible from lab to lab. This practice has led to confusion, particularly for FRET dilettantes, as it is hard for them to compare FRET experiments conducted in one lab, using one FRET technique, with FRET experiments conducted in another lab. Ideally, when the amount of FRET in a sample is measured, its magnitude should be identical regardless of the FRET technique used to measure it (assuming that the technique is valid, and the environmental conditions were the same). Furthermore, these FRET values should be reproducible by any lab (i.e., be sample specific but not instrument specific). Additionally, it is important to realize that FRET measurements are donor-centric; that is to say, they are measurements related to the photonic behavior of

the donor fluorophore. Thus, even though the FRET acceptor used in a FRET experiment, as well as its stoichiometry, can dramatically influence the amount of FRET observed experimentally, the amount of FRET is reported from the donor's perspective. Furthermore, FRET is reported assuming a single donor, or as an average of a population of single donors. Two ways of parameterizing FRET that meet the criteria mentioned above (and that can be measured using FLIM) are by reporting the donor FRET rate, K_{FRET} , (along with the donor emission rates in the absence of an acceptor, $1/\tau$), or more typically by combining these values into a donor FRET efficiency. An advantage of using K_{FRET} to parameterize FRET as compared to using the FRET efficiency is that K_{FRET} reveals dynamic information on how rapidly FRET is occurring, while FRET efficiency does not. In fact, one of the prime advantages of using FLIM to measure FRET is to use information embedded in the dynamics of energy transfer to better understand heterogeneities in the experimental system. Nonetheless, in most FRET studies, FRET efficiency is used to parameterize FRET.

The FRET efficiency, denoted as E , is the probability that a donor transfers its excited-state energy to a FRET acceptor, via a FRET mechanism (as compared to the donor dissipating that energy by any and all other pathways). On a single-molecule level, FRET is quantal—a donor will either transfer its excited-state energy or it will not. Experimentally, a FRET efficiency is measured by monitoring the energy-transfer behaviors of a large population of donors, either one by one using single-molecule techniques (Deniz et al., 1999), or as an ensemble. In biological FLIM-FRET experiments FRET is typically measured for a population of donors and acceptors whose interactions and environments may be heterogeneous (Vogel, Nguyen, van der Meer, & Blank, 2012). Under these conditions, FRET values should be reported as the average FRET efficiency of the population of donors, $\langle E \rangle$.

How Can FLIM Be Used to Measure FRET?

The average FRET efficiency, $\langle E \rangle$, can be calculated by comparing the average lifetime of a donor in the presence, τ_{DA} , or absence, τ_D , of a FRET acceptor:

$$\langle E \rangle = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle}$$

Equation 9

Since τ_{DA} is proportional to the number of photons emitted from a donor when an acceptor is present (i.e., the area under the DA decay curve), and τ_D is proportional to the number of photons emitted from a donor in the absence of an acceptor (i.e., the area under the D decay curve), then τ_{DA}/τ_D is the fraction of donor excitation events, in the presence of an acceptor, that result in the donor emitting a photon, and $1 - \tau_{DA}/\tau_D$ is the fraction of donor excitation events where the donor transfers its excitation energy, presumably to the acceptor. The underlying assumption of Equation 9 is that the introduction of a FRET acceptor creates a new FRET-based non-radiative energy dissipation pathway that is now available to the donor. Thus, if FRET occurs (when an acceptor is introduced), a decrease in τ_{apparent} , as outlined in Equations 2 and 3, should occur, and that decrease in lifetime (from $\langle \tau_D \rangle$ to $\langle \tau_{DA} \rangle$) is used to calculate FRET efficiency.

The FRET efficiency can perhaps be more intuitively understood by considering the relative rates of emission in the absence of a FRET acceptor (K_E) and when a new additional FRET energy dissipation rate, (K_{FRET}) is initiated by introducing an acceptor into the system (Fig. 3):

$$\langle E \rangle = \frac{\langle k_{FRET} \rangle}{\langle k_{FRET} \rangle + k_E}$$

Equation 10

Analogous to quantum yield (see Equation 3), FRET efficiency is also a probability that can be calculated from energy transfer rates, but instead of defining the probability that the donor will emit a photon, the FRET efficiency is the probability that excited-state energy will be transferred from the donor to an acceptor using a FRET mechanism. The problem with using Equation 10 to parameterize FRET is that in a FLIM experiment, k_{FRET} is not directly measured and must be inferred (see NOTE 7). Nonetheless, Equations 9 and 10 can be used to derive the relationship between k_{FRET} and lifetime values that are readily obtained from FLIM experiments:

$$k_{FRET} = \frac{1}{\tau_{DA}} \cdot \left(1 - \frac{\tau_{DA}}{\tau_D} \right)$$

Equation 11

Equation 11 states that the FRET transfer rate is equal to the product of two factors, the FRET efficiency ($1 - \tau_{DA}/\tau_D$) multiplied by the reciprocal of the τ_{DA} lifetime.

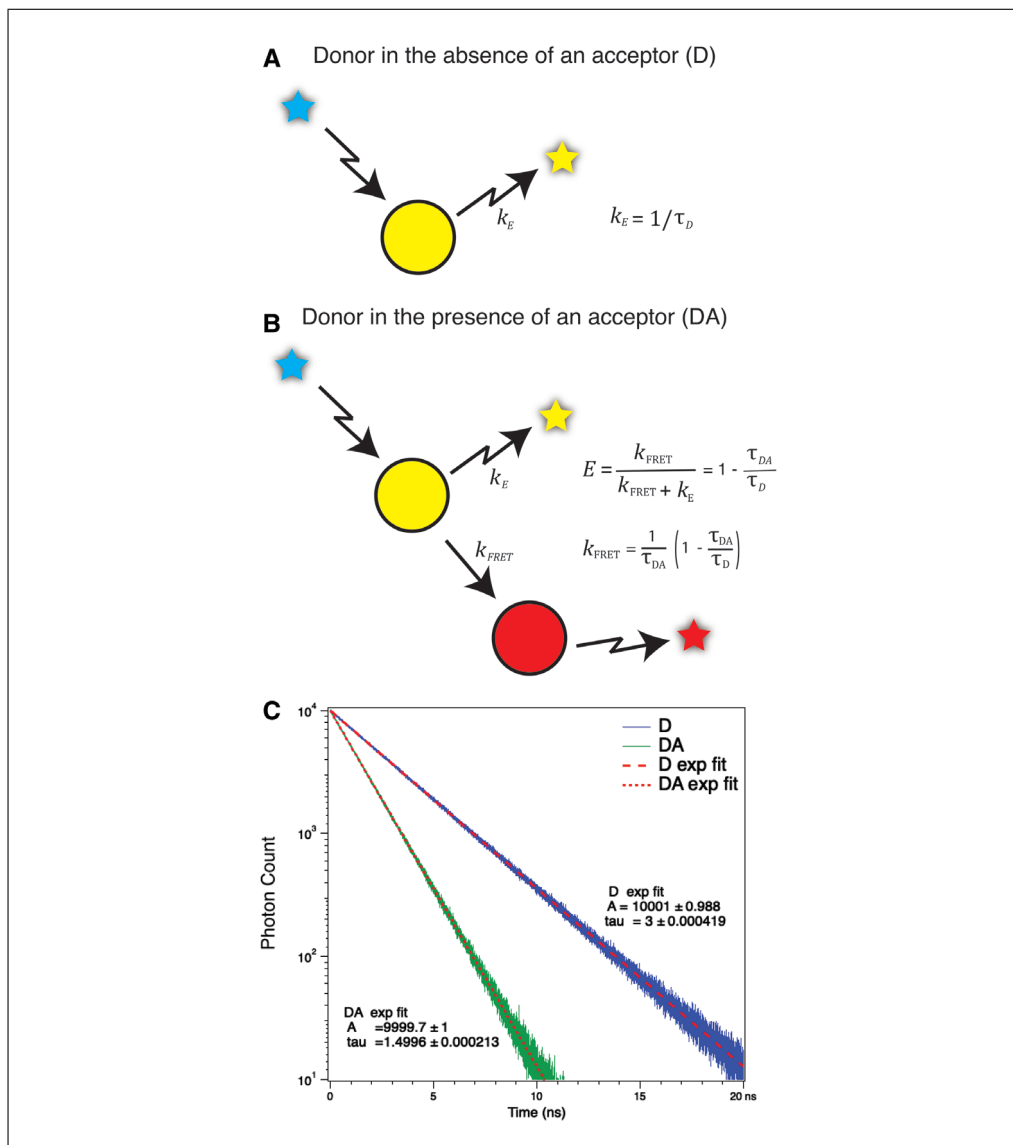


Figure 3 FRET and how it influences donor lifetime decays. **(A)** Cartoon depicting a donor fluorophore in the absence of a FRET acceptor (yellow circle) being excited by a photon (blue star) and emitting a red-shifted photon (yellow star) at rate k_E . **(B)** Cartoon depicting a donor fluorophore (yellow circle) in the presence of a FRET acceptor (red circle). The donor fluorophore is excited by a photon (blue star), but now has a choice of either emitting a red-shifted photon (yellow star) or transferring its excitation energy via FRET to a nearby acceptor (red circle). **(C)** Simulated lifetime decay data for a donor alone (blue trace) or a donor in the presence of an acceptor (green trace). In this simulation, the donor alone had a lifetime of 3 ns, the acceptor was Föster's distance (R_0) away from the donor, and it was assumed that the donor and acceptor were in the isotropic dynamic regime with κ^2 equal to 2/3. Dashed and dotted red lines are fits to a single exponential decay model.

NOTE 7: Using time-resolved anisotropy measurements, k_{FRET} can be directly measured under some conditions.

FRET measurement. Essentially, being able to evaluate and/or interpret a FRET experiment requires some level of understanding the FRET mechanism.

What is Needed for FRET to Occur?

What is needed for FRET to occur and why is this important to know? From a practical perspective, knowing what is needed for FRET can give insight into what experimental variables might be influencing a

Requirements for FRET

- (1) **Proximity:** The donor and the acceptor need to be in close proximity, typically under 10 nm,
- (2) **Orientation:** The acceptor's transition dipole must be oriented in such a way that

it can sense the electric field generated by the donors transition dipole,

- (3) **Energy Overlap:** The quantal energy that is liberated when an excited donor transitions to its ground state must be equal to the energy that its acceptor needs to transition from its ground state to an excited state
- (4) **Independence:** The donor and acceptor must act independently of each other. We will now discuss each of these requirements in greater detail.

Proximity

In most biological FRET experiments, an investigator wishes to use the magnitude of a FRET measurement to infer the proximity between the molecules that a donor and the acceptor are attached to (Stryer, 1978; Stryer & Haugland, 1967). For each specific donor-acceptor FRET pair, a Förster distance, R_0 , can be calculated based on the fluorophore's photophysical characterization (see NOTE 8). A useful database of commonly used FRET donors and acceptors (<https://www.fpbse.org/fret/>) includes a web tool for calculating R_0 . Once R_0 is known, the theoretical relationship between the distance between donor and acceptor, R_{DA} , and the measured FRET efficiency, E , can be determined with a few assumptions. If we assume that all of the donor and acceptor transition dipoles in a population are randomly oriented in space, isotropically, then the average FRET efficiency can be plotted as a function of separation (Fig. 4). The difference between the two curves in Figure 4A relates to a further assumption on how rapidly the donors and acceptors can rotate (see NOTE 9). Specifically, we are interested in how rapidly their transition dipoles rotate relative to the fluorescence lifetime of the donor. In the first curve (dashed blue), it is assumed that the donor and acceptor dipoles rotate much faster than the fluorescent lifetime of the FRET donor. This is called the dynamic energy transfer regime (van der Meer, van der Meer, & Vogel, 2013). Assuming an isotropic dynamic regime, R_{DA} can be calculated from an experimentally measured FRET efficiency and the R_0 value:

$$R_{DA} = R_0 \cdot \sqrt[6]{\frac{1}{E} - 1}$$

Equation 12

It is worth noting that in the isotropic dynamic regime, when $\langle E \rangle = 0.5$, $R_{DA} = R_0$.

In other words, the Förster distance is the separation where 50% energy transfer occurs.

In the second curve (solid red), it is assumed that the donor and acceptor dipoles rotate much slower than the fluorescent lifetime of the FRET donor, as is the case for a fluorescent protein (FP). This is called the isotropic static regime (van der Meer et al., 2013; Vogel, van der Meer, & Blank, 2014). The approximate value of R_{DA} as a function of $\langle E \rangle$ is:

$$R_{DA} \approx R_0 \cdot \sqrt[6]{\frac{(1 - \langle E \rangle)^2}{\langle E \rangle}}$$

Equation 13

Note that in the isotropic static regime $\langle E \rangle < 0.5$ when $R_{DA} = R_0$. It is also worth noting that Equations 12 and 13 are only valid if their underlying assumptions are valid. We can also see in Figure 4A that when R_{DA} is greater than $2.5 \times$ the Förster distance, virtually no FRET occurs. Thus, for a FRET donor-acceptor pair with a R_0 value of 5 nm, virtually no FRET will be observed at R_{DA} values greater than 12.5 nm (125 Å).

For evaluating FRET experiments, it is often worth considering how distance between molecules changes as a function of concentration in aqueous environments. A conservative estimate of the average distance between molecules in solution can be calculated (Chandrasekhar, 1943):

$$\langle D \rangle = \frac{0.55}{\sqrt[3]{C}}$$

Equation 14

where $\langle D \rangle$ is the average distance between molecules in nanometers, and C is the molar concentration of the molecule in solution. Using Equation 14, we can readily calculate that a 200 μ M solution of acceptors will have on average 9.4 nm separation between molecules. Under these circumstances, if we introduce a FRET donor into this solution, it will always have a R_{DA} distance ≤ 9.4 nm. If we again assume a typical R_0 value of 5 nm, some FRET should be observed under these conditions, not because of a specific interaction between a donor-tagged molecule and an acceptor-tagged molecule, but because the acceptor-tagged molecule was present at such a high concentration that wherever a donor-tagged molecule is placed it will be within 10 nm. This non-specific form of FRET is called bystander FRET.

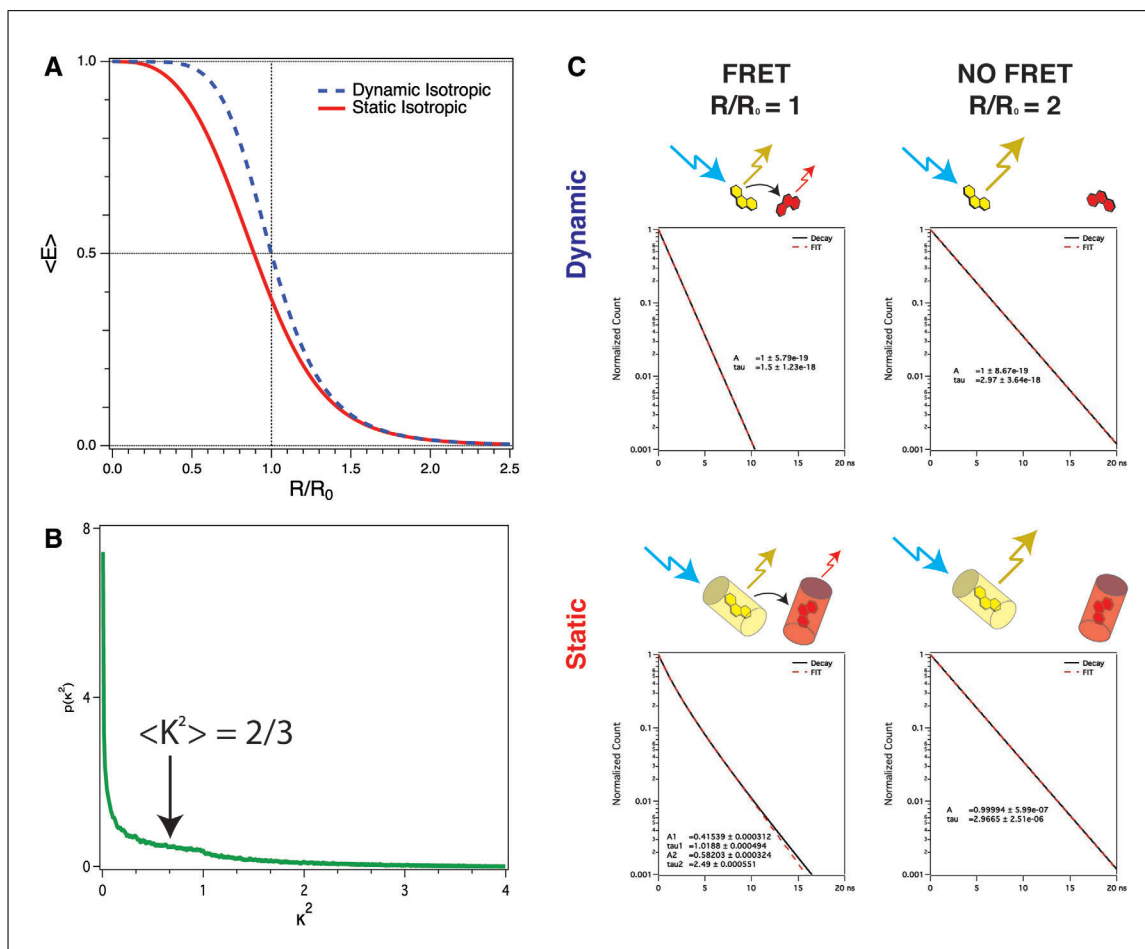


Figure 4 The speed of molecular rotation relative to the donor lifetime can influence FRET donor-lifetime decays. **(A)** The average FRET efficiency ($\langle E \rangle$) as a function of the donor-acceptor separation (R) divided by the Förster distance (R_0) in the isotropic dynamic regime (dashed blue line) or in the isotropic static regime (red line). **(B)** The κ^2 probability distribution (green trace). Note that when an isotropic population of fluorophores rotate faster than their excited state lifetime, it can be assumed that κ^2 has a value of $2/3$, the average value of the κ^2 probability distribution. If isotropic fluorophores rotate slower than their excited state lifetime, the heterogeneous nature of the isotropic κ^2 probability distribution may impart heterogeneous lifetime decay characteristics to a lifetime decay curve. **(C)** The influence of dynamic (top panels) versus static (bottom panels) regimes on FRET donor lifetime decays. Dynamic regime is typically observed with small organic fluorophores that rotate rapidly, while the static regime is typically observed with large fluorophores, such as FPs, which rotate slowly. Four Monte Carlo–simulated decay curves are depicted: when $R/R_0 = 1$ (FRET) or 2 (no FRET), for the dynamic or static regimes. Note that molecular rotation does not alter the donor lifetime decay in the absence of FRET. In the presence of FRET, the donor lifetime decays as a single exponential in the dynamic regime, but decays multiexponentially in the static regime.

NOTE 8: R_0 is a function of the refractive index of the local environment, the donor's quantum yield, the acceptor's absorption coefficient, the emission spectra of the donor, and the absorption spectrum of the acceptor; in the literature it assumes an isotropic distribution of donors and acceptors in the dynamic energy transfer regime.

NOTE 9: Analogous to tau value to parameterize lifetimes, molecular rotation is parameterized by a rotational correlation time, a time-constant which can be measured using time-resolved anisotropy.

Orientation

How the relative orientation of donor and acceptor transition dipoles influences FRET is perhaps the most confusing aspect of FRET for both newcomers and old-timers alike (van der Meer, 2002). The key aspect to understand is that when a donor fluorophore transitions into its excited state by absorbing a photon, an electron in the donor's ground-state electron probability cloud moves outward to occupy the donor's excited-state probability cloud. The relative shapes and different symmetries of these clouds define a vector of the average direction in which the negative charge of the

electron moves when the fluorophore is excited. This vector is called a transition dipole. The electron cloud surrounding a fluorophore might typically extend ~ 0.5 nm away from the center of the atoms that comprise the fluorophore. When a fluorophore is excited, and an electron becomes free to move back and forth within the confines of the fluorophore's extended excited-state electron cloud, an electric field is generated that is symmetrical around the transition dipole. This electric-field symmetry is analogous to the magnetic-field lines extending from the positive and negative tips of a bar magnet's poles. The strength of the electric-field lines connecting the two ends of a fluorophore's transition dipole is a function of the fluorophore's absorption coefficient (see NOTE 10). In any specific direction (relative to the axis of the dipole), the strength of the electric field will diminish the further away they extend from the dipole. The field strength weakens more quickly when moving in a direction perpendicular to the dipole axis as compared to when moving away from the dipole along the dipole axis. The key point is that the electric-field lines emanating from a FRET donor can extend tens of nanometers away from the fluorophore, in an asymmetrical way. This forms the basis of FRET-based energy transfer. When a FRET acceptor is in close proximity to a FRET donor, the acceptor's transition dipole can potentially 'sense' the electric field generated by the donor. If it can 'sense' the donor's electric field, excitation energy can be transferred from the donor to the acceptor via a resonance mechanism. The acceptor's ability to 'sense' the donor's electric field will depend on three factors: the acceptor's distance from the donor (R_{DA} , as outlined in the preceding section), an angle defining the acceptor's position in space relative to the axis of the donor's dipole orientation (θ), and the angle between the acceptor's dipole axis and the orientation of the donor's electric field at the acceptor's location (ω). Together, the angles θ and ω define an orientation factor called kappa-squared (κ^2)(van der Meer et al., 2013):

$$\kappa^2 = \cos^2\omega \cdot (1 + 3 \cdot \cos^2\theta)$$

Equation 15

From Equation 15, it is clear that κ^2 can have any value ranging from 0 to 4. It turns out that the magnitude of the FRET transfer rate, k_{FRET} , discussed above, is a linear function of κ^2 . Thus, when $\kappa^2 = 4$, the k_{FRET} energy

transfer rate will be four times larger than if $\kappa^2 = 1$. More importantly, if $\kappa^2 = 0$, the k_{FRET} transfer rate will also be zero and FRET cannot occur, regardless of the distance separating donors and acceptors. Unfortunately, the actual value of κ^2 is almost never known in a biological FRET experiment. Nonetheless, with some reasonable assumptions, the value or values of κ^2 in a population can be estimated. The probability distribution of κ^2 values for an isotropic population of donors and acceptors, depicted in Figure 4B, was first calculated by Richard E. Jones when he was a graduate student in Lubert Stryer's group at Stanford University (Jones, 1970). We can see that when donors and acceptors are randomly oriented, most FRET pairs will have a κ^2 value near zero—no FRET. In contrast, it is very rare to have a FRET pair with a κ^2 value of 4. The average value of the κ^2 probability distribution is $2/3$. In the dynamic energy transfer regime, donors and acceptors rotate much faster than their fluorescence lifetime. This means that during the time that a donor is in its excited state, the relative orientation of the donor and acceptor dipoles will assume many different κ^2 values. This forms the basis for the assumption that the average κ^2 value in a FRET experiment is $2/3$. It must be noted that, assuming this value for κ^2 is only valid in the dynamic regime, and when the donors and acceptors have an isotropic distribution of orientations. In the static energy transfer regime, such as the case for FPs, donors and acceptors rotate much slower than their fluorescence lifetime. This means that during the time that a donor is in its excited state, the relative orientation of donor and acceptor dipoles for each FRET pair in the population will barely change. Thus, an average κ^2 value of $2/3$ is not a valid assumption, but the underlying distribution of κ^2 values is still valid if the donor and acceptor orientations are isotropic. The difference in how randomly oriented dipoles changes their orientation during the excited-state lifetime of a FRET pair is the basis for the two curves depicted in Figure 4A.

NOTE 10: The fluorophore's absorption coefficient describes how light, of a specific frequency, is attenuated (absorbed) as it passes through a substance.

Energy overlap

The energy overlap requirement for FRET is rooted in the concept of conservation of energy and the quantal nature of light. The

energy liberated in a FRET reaction when an excited-state donor transitions to its ground state must be equal to the energy absorbed by an acceptor's ground state when it is elevated to its excited state. Fluorophores are complex molecules with multiple vibration modes in both their ground and excited states, and at physiological temperatures they exist in a complex environment that influences these vibrational modes. Effectively this means that under biological conditions there are typically a large array of discrete energy values that are capable of elevating an acceptor in the population to its excited state. In the same fashion, there are also characteristically a large array of discrete energy values that are liberated when an excited-state donor returns to its ground state. Remembering that the color of light is related to the energy of photons absorbed or emitted during fluorescence, the acceptor's absorption spectrum and the donor's emission spectrum essentially map out the arrays of different energies involved in these transitions. Any spectral overlap between a donor emission spectrum and an acceptor absorption spectrum indicates that this FRET pair shares a common energy transition that could support the energy overlap requirement for FRET. A large overlap between these spectra indicates that there are multiple discrete energy values that fulfill this requirement. The greater the spectral overlap between a donor emission and an acceptor absorption, the greater the probability that FRET can occur. In FRET calculations, a factor based on the overlap of the donor emission spectrum and the acceptor absorption spectrum is called the overlap integral, $J(\lambda)$. The Förster distance, R_0 , used in Equations 12 and 13, is itself a function of $J(\lambda)$ and also assumes that κ^2 is 2/3:

$$R_0 = 0.211 \cdot \sqrt{\kappa^2 n^{-4} Q_D J(\lambda)}$$

Equation 15

where R_0 is in Angstroms (Å), n is the refractive index of the medium surrounding the FRET pair, and Q_D is the quantum efficiency of the donor. The important point here is that dipole orientation and energy overlap influence FRET measurements by altering the values of κ^2 and $J(\lambda)$, and thus the value of R_0 . It is also worth noting that refractive index can also influence FRET measurements.

Finally, for completeness, we need to mention the last requirement for FRET, **Independence**. When FRET occurs, excitation

energy is thought to hop discretely from the donor to the acceptor (Clegg, 2006). The rate of these energy hops is k_{FRET} . Under specialized conditions, a donor and acceptor can be so strongly coupled to each other that they no longer behave independently (Förster, 1965; Kasha, 1963; Kenkre & Knox, 1974; kim et al., 2019). While still being composed of two fluorophores, quantum mechanically they behave as a single quantum entity. Under these conditions, excitation energy is thought to almost instantaneously be distributed between donors and acceptors. This mechanism of energy transfer, while related to FRET, is not FRET per se. This form of energy transfer is called coherent energy transfer or excitonic coupling, which typically transfers energy much faster than FRET and theoretically can transfer excitation energy over much greater distances than FRET.

Fluorescence Anisotropy for Characterizing Homo-FRET

While FLIM is an excellent way to monitor FRET between spectrally distinct donors and acceptors (hetero-FRET), FLIM cannot detect FRET between the same fluorophore acting as both donor and acceptor (homo-FRET), since it is not associated with a net-change in fluorescence lifetime. However, FRET between like fluorophores results in a net change in the polarization, or anisotropy, of emitted light, and this can be measured to detect homo-FRET. In this section, we will briefly discuss fluorescence anisotropy, how it can be measured using a variant of FLIM called time-resolved fluorescence anisotropy, and why it is useful for FRET experiments. A comprehensive discussion on anisotropy theory is beyond the scope of this review, so we point interested readers to a previously published book chapter written on this subject (Vogel, Thaler, Blank, & Koushik, 2009). Fluorescence anisotropy is a parameter that describes the polarization, or electric field orientation, of light emitted from a population of excited fluorophores. Fluorescence polarization is determined by properties of the excitation light source, the fluorophore's transition dipole orientations, and how this orientation changes over the fluorophore's excited state lifetime.

When an isotropic population of fluorophores are irradiated by a linear polarized light source, such as typical pulsed lasers used in a FLIM instrument, a subset of the fluorophores will be preferentially excited based on their orientation relative to the electric field

of the laser. Fluorophores whose transition dipole is parallel to the laser electric field will be excited with high probability, and those with an orthogonal orientation will not be excited at all. This process of ‘photoselection’ generates an anisotropic subpopulation of excited fluorophores that will have similar dipole orientations. Thus, the polarization of the light emitted from this subpopulation will be highly correlated to the electric field orientation of the laser, immediately after excitation. Between the moment of photoselection and emission of a photon, fluorophores might rotate, or transfer excited-state energy to nearby fluorophores by homo-FRET, and these processes will change the polarization of the emitted light as a function of time after excitation (Fig. 5). This dynamic change in polarization can be measured using a FLIM instrument that is configured with polarization optics. Photons emitted from excited fluorophores are split into two channels using a polarizing beam splitter and directed to single photon counting detectors. Photons detected in the parallel channel ($I_{\parallel}$) have an electrical field orientation parallel to the electric field of the excitation light source, while photons detected in the perpendicular channel ($I_{\perp}$) are oriented orthogonally (Fig. 5B). The time-resolved emission anisotropy (r) can be calculated using the photon decay histograms of the $I_{\parallel}$ and $I_{\perp}$ channels according to the following equation:

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}$$

Equation 16

It should be noted that the denominator of Equation 16, $I_{\parallel}(t) + 2I_{\perp}(t)$, is equivalent to the fluorescence lifetime decay of the fluorophore. Thus, the $r(t)$ decay curve shows the time-dependent change in the orientation of a population of excited fluorophores ($I_{\parallel} - I_{\perp}$) normalized to the lifetime decay. As $I_{\parallel} - I_{\perp}$ approaches 0, the excited fluorophores in the population become randomly oriented. Demonstrated by the anisotropy decay curves shown in Figure 5C, the fluorescence depolarizes exponentially over the course of a fluorophore’s lifetime. As mentioned above, and illustrated in Figure 5C, two factors account for depolarization on this timescale: molecular rotation and homo-FRET. The depolarization due to molecular rotation is parameterized by the anisotropy decay time constant associated with rotation, and is called the rotational cor-

relation time of the fluorophore. So, for small organic fluorophores, such as fluorescein, that rotate much faster than their fluorescence lifetime, depolarization is rapid, with a time constant of ~ 140 ps in water. In contrast, for fluorophores that rotate much slower than their lifetime, as is the case for FPs, the rate of depolarization is slow. For example, the anisotropy decay constant of mVenus in a dilute homogenate is 11.4 ns (Fig. 5C). The second factor that influences fluorescence depolarization is homo-FRET. When energy is transferred from a donor fluorophore to an acceptor, their transition dipoles are often not parallel. As a result, light emitted from an acceptor fluorophore will typically have an electric field orientation that is less correlated to the emission dipole of the donor (and the electric field of the excitation light) and is thus depolarized (Fig. 5A). Depolarization due to homo-FRET occurs with time constants ranging from picoseconds to a few nanoseconds, which is significantly faster than the depolarization caused by the molecular rotation of a FP. Therefore, both the fast anisotropy decay component caused by homo-FRET and the slow rotational decay component can be measured by multi-exponential fitting of time-resolved anisotropy decay curves for slowly rotating fluorophores such as FPs. This is demonstrated in Figure 5C for a population of mVenus-17-mVenus (i.e., two mVenus proteins tethered by a 17-amino-acid linker) molecules. The anisotropy decay curve of this sample was fit with a double exponential model, yielding a FRET-dependent anisotropy decay constant of 910 ps. Importantly, in this example, the fitting takes into account the change in rotationally dependent depolarization that can occur when an FP is tethered to another molecule. For example, when mVenus is tethered to mUranus with a 17-amino-acid linker, the rotational correlation time changes from 11.4 to 13.0 ns. Since the primary difference between mVenus-17-mVenus and the mUranus-17-mVenus constructs in Figure 5 is a point mutation in mUranus that prevents chromophore formation, the rotational correlation time of mUranus-17-mVenus can be used to constrain the slow decay constant of the double exponential fitting of the mVenus-17-mVenus anisotropy decay curve. This fitting strategy provides a more accurate measurement of the FRET-dependent depolarization for mVenus-17-mVenus. The fast anisotropy decay constant is inversely proportional to K_{FRET} and, under certain circumstances, K_{FRET} can be used to parameterize homo-FRET.

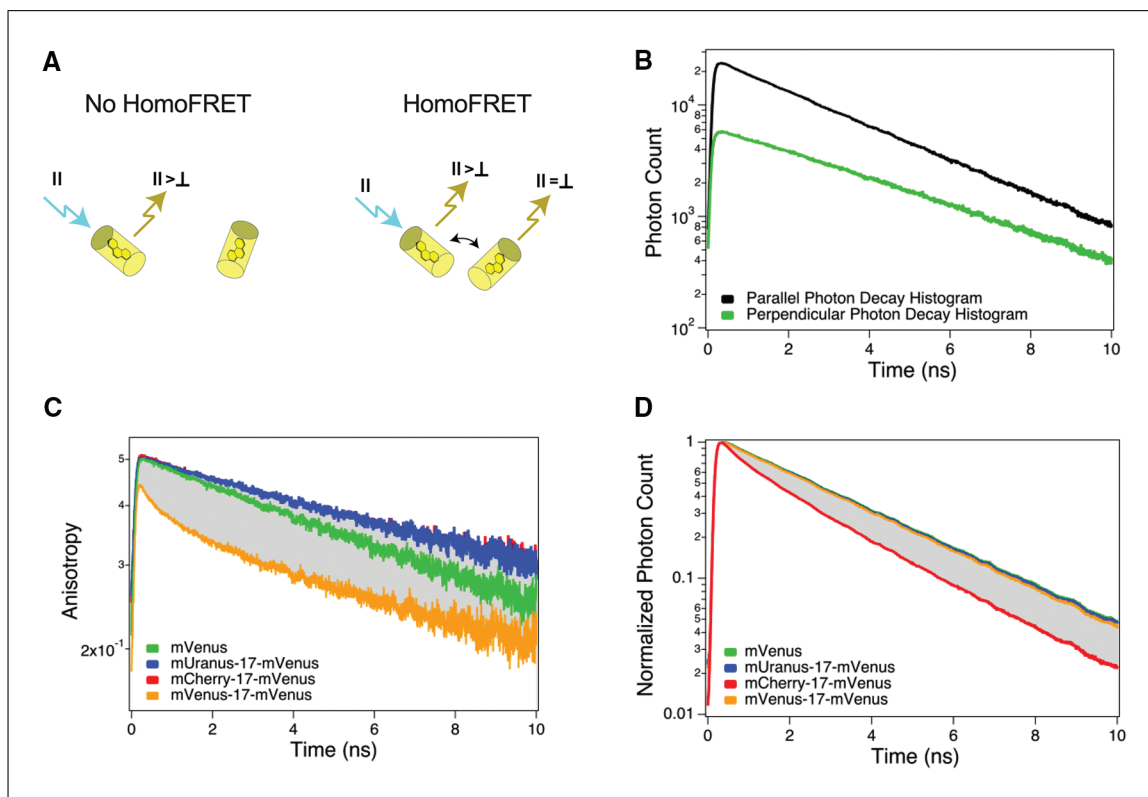


Figure 5 Homo-FRET is measured using a variant of FLIM called time-resolved anisotropy, a technique that measures the time-dependent polarization of light emitted from a fluorophore. **(A)** Cartoon depicting the polarization of light emitted from a fluorophore in the absence or presence of homo-FRET. On the left is shown an excited fluorophore emitting light with an orientation correlated with the orientation of the linear polarized excitation light (i.e., polarized emission). In contrast, the right depicts how the orientation of emitted light changes due to homo-FRET. In homo-FRET, excited energy can migrate back and forth between like fluorophores before de-excitation. Light emitted from the initially excited 'donor' fluorophore is correlated to the excitation light, while light emitted from the 'acceptor' fluorophore is uncorrelated or depolarized. **(B)** Photon decay histograms collected from a sample of mVenus fluorophores. Lifetime decays measured using either a detector configured to collect light that had an orientation parallel to the linear polarized excitation light (black trace) or from a detector configured to collect light that had an orientation perpendicular to the excitation light (green trace). **(C)** Anisotropy decay curves, calculated from orthogonal polarization lifetime decay histograms similar to those shown in **(B)** using Equation 16, for mVenus (green trace), mUranus-17-mVenus (blue trace), mVenus-17-mVenus (orange trace), or mCherry-17-mVenus samples (red trace, 17 in these construct names denotes the length of the amino acid linker used, and mUranus is a point mutant of an FP that cannot form its chromophore). Starting the moment after excitation, mVenus anisotropy decays over time and is well described by a single exponential decay model. The decay time constant of this curve is the rotational correlation time of mVenus, and represents depolarization due to molecular rotation. Similarly, the mVenus-17-mUranus anisotropy curve decays according to a single exponential model. This curve (and the mCherry-17-mVenus curve) demonstrates how molecular rotation can slow down when a fluorophore is tethered to another protein. The mVenus-17-mVenus anisotropy decay curve is well described by a double exponential model. The fast decay time constant is associated with homoFRET-dependent depolarization, while the slow decay constant is due to molecular rotation. The gray-shaded area in between the mUranus-17-mVenus and mVenus-17-mVenus decay curves represents depolarization due primarily to homo-FRET. **(D)** Photon decay histograms for the samples described in **(C)**. A decrease in donor fluorescence lifetime due to hetero-FRET is clearly shown as the gray-shaded area in between the mUranus-17-mVenus and mCherry-17-mVenus decay curves. Notice that the change in anisotropy for homo-FRET and lifetime for hetero-FRET in **(C and D)**, respectively, are qualitatively similar.

Hetero-FRET and Homo-FRET: How Are They Different and How Are They Useful?

Non-radiative energy transfer can occur between distinct fluorophores (i.e., mVenus to a mCherry), which is referred to as hetero-FRET (Fig. 5D), or it can occur between like

fluorophores (i.e., mVenus to mVenus), which is called homo-FRET (Fig. 5C). Although hetero-FRET is by far the most familiar and utilized variant of FRET across neuroscience laboratories, experimental designs using homo-FRET can be advantageous in certain situations. In this section, we will compare the

photophysical properties of these two FRET variants and discuss the advantages of each one in the context of experimental questions commonly tackled in modern neuroscience where FRET techniques may be implemented.

Both homo-FRET and hetero-FRET are dependent on the same photophysical requirements, including close proximity between fluorophores, permissible orientation of transition dipoles, and spectral overlap. For a hetero-FRET pair, the donor fluorophore is excited, and energy from the excited donor can transfer to the acceptor. In this situation, there is typically negligible energy overlap between the absorption spectra of the donor fluorophore and emission spectra of the acceptor fluorophore, so energy transfer is considered unidirectional. In contrast, for a homo-FRET pair, either fluorophore can be excited, and the absorbed energy can migrate back and forth between fluorophores in close proximity prior to de-excitation, as long as there is overlap between their absorption and emission spectra (i.e., a short Stoke's shift) and they have a permissive dipole orientation. This critical difference between hetero-FRET and homo-FRET accounts for the different ways they are parameterized in a FLIM experiment and the different types of information about a protein complex you can extract from them.

For a hetero-FRET pair, the donor and acceptor have different emission spectra which can be easily separated using optical filters. Therefore, hetero-FRET can be quantified in a FLIM experiment by measuring the fluorescence lifetime of the donor fluorophore. In contrast, in homo-FRET, the donor and acceptor fluorophores have the same emission spectra, so the donor's lifetime cannot be experimentally separated from the acceptor's. Accordingly, homo-FRET cannot be quantified in the same way as most hetero-FRET experiments. However, there is a high probability that the emission dipoles of the acceptor and donor are not oriented in parallel; therefore, if the sample is excited by linear polarized light, the fluorescence will depolarize at a faster rate if FRET occurs (Fig. 5).

Hetero-FRET and homo-FRET have many applications in neuroscience fields ranging from detecting intermolecular interactions and intramolecular conformation changes to the use of biosensors to monitor intracellular and extracellular signaling dynamics. Hetero-FRET approaches are by far the most common strategy for these applications; however, hetero-FRET is not always the most appro-

priate approach. Hetero-FRET experiments are most useful for studying protein-protein interactions (PPIs) between different protein species or for studying intramolecular conformational changes where controlling the donor-to-acceptor ratio is feasible.

In some cases, homo-FRET may be advantageous. A major application of homo-FRET, largely overlooked by neuroscientists, is for studying homodimers and molecular assemblies consisting of identical subunits. Although researchers have used hetero-FRET experimental designs in these situations, with varying levels of success (Bosch et al., 2014; Dore, Aow, & Malinow, 2015), we argue that homo-FRET designs can generate superior data and can be easier to implement from the molecular biology perspective. To illustrate the utility of homo-FRET to study PPIs of like molecules, it is first useful to present the shortcomings of a hetero-FRET design. As an example, in a typical hetero-FRET experiment designed to study homodimers, two expression constructs are designed so that a protein of interest is labeled with either a donor or an acceptor. Then, the two constructs are co-expressed in a cell type of interest and a FLIM experiment is carried out. When these two constructs are co-expressed, three combinations of labelled dimers can form, which can be approximated by a binomial distribution. In this example, if both constructs are expressed at equal levels, 25% of dimers will contain only acceptor fluorophores, which cannot contribute to a FLIM measurement and will drop out, 25% of dimers will consist of only donor fluorophores, which is counterproductive to FLIM-FRET measurements as this will result in an underestimation of FRET efficiency, and only 50% of dimers will have the desired 1:1 donor-acceptor pair configuration that is ideal for FRET measurements. On the other hand, in a homo-FRET design, only one fluorophore is used to label the protein of interest, so all potential homodimers will have the desired 1:1 donor-to-acceptor stoichiometry and all homo-FRET pairs will contribute to a time-resolved anisotropy measurement. In addition to bypassing this major limitation of hetero-FRET in studying homodimerization, the average number of fluorophore-tagged subunits in a molecular complex is related to the amplitude of the FRET component of a time-resolved anisotropy curve (Runnels & Scarlata, 1995), the molecular biology of a homo-FRET experiment is simpler since only one construct needs to be made and expressed,

and the use of only one fluorophore frees up spectral bandwidth to measure another physiological parameter simultaneously. It is worth noting some of the obstacles surrounding homo-FRET approaches. First, it can be difficult to implement time-resolved anisotropy for homo-FRET imaging because relatively long photon-collection times are required for calculating $r(t)$ using Equation 16. To speed up image-acquisition time, most investigators use steady-state anisotropy with the assumption that changes in anisotropy are primarily driven by homo-FRET as opposed to molecular rotation. Second, several factors that can influence anisotropy measurements have to be accurately accounted for if one wants to convert anisotropy decay constants to K_{FRET} , such as depolarization due to molecular rotation and coherent energy transfer.

FRET-based genetically encoded fluorescent sensors are rapidly gaining popularity for monitoring neural dynamics in *ex vivo* models and in freely behaving animals. To date, the vast majority of these sensors are designed using a hetero-FRET approach, and donor lifetime can be measured to determine the biosensor's activation state. There is an emerging trend in the field to multiplex FRET sensors to measure the dynamics of multiple endogenous molecules simultaneously or pair optical measurements with optogenetic stimulation. Although progress in this area has been made, this is a difficult task because of the limited spectral bandwidth available for measuring multiple hetero-FRET pairs while avoiding optical crosstalk, and the spectra of most optical actuators overlap with spectra of common FPs used for hetero-FRET sensors. An interesting approach to circumvent this limitation is the design of homo-FRET-based biosensors (Cameron et al., 2016; Ross et al., 2018; Snell et al., 2018; Thaler, Koushik, Puhl, Blank, & Vogel, 2009; Warren, Margineanu, Katan, Dunsby, & French, 2015). In this case, the spectral bandwidth used for a single sensor is restricted to the spectra of a single fluorophore. Although the use of homo-FRET-based biosensors has not been widely adopted in neuroscience research, the feasibility of this approach has been demonstrated, and the conversion of hetero-FRET-based biosensors into homo-FRET biosensors, with the goal of multiplexing, is a reasonable approach (Ross et al., 2018).

One of the great strengths of both FLIM and time-resolved anisotropy is that they can easily be adapted for measuring FRET in *vitro*, where the researcher has great control

over the experimental milieu, but can also be tailored to monitoring FRET in more challenging conditions such as live cells and even intact organisms (where behavioral tasks can be simultaneously monitored). Thus, these FRET approaches can potentially bridge molecular events in neurons and the behaviors they mediate. Now that we have presented how hetero- and homo-FRET can be measured using FLIM and time-resolved anisotropy, respectively, we will discuss in greater detail the three main neuroscience applications for these approaches, specifically, for studying protein-protein interactions, for studying protein conformational changes, and for monitoring FRET-based biosensors.

FLIM-FRET for Studying Protein-Protein Interactions (PPIs)

Proteins rarely function in isolation, but rather dynamically interact with each other to perform complex computations that support a biological system. For example, AMPA receptors can interact with dozens of other proteins that determine the receptors' trafficking, post-synaptic localization, and gating properties, all of which contribute to dynamic changes in synaptic strength in response to a stimulus. PPIs are so fundamental to biology that tremendous efforts have been dedicated to monitoring these interactions in the context of nearly every biological function. In neuroscience, techniques have been developed and implemented to study how PPIs support basic functions of the nervous system, such as synaptic transmission, neural plasticity, and gene transcription and translation, and, importantly, to study how these basic molecular and cellular functions endow an organism with the ability to sense and interpret its environment and translate that information into a behavioral output to support the organism's survival.

Various optical microscopy techniques have been used to study PPIs in the nervous system. Perhaps the most important parameter of an optical system used to detect a PPI is the spatial resolution of the system. Confocal and 2-photon microscopes have been widely used to colocalize proteins of interest in biological preparations; however, the spatial resolutions of these systems range from ~ 200 nm to $1\ \mu\text{m}$, about $100\text{--}1000\times$ less than the resolution required to detect a PPI. Subdiffraction-limited resolution can be obtained with more sophisticated super-resolution microscopes, but these systems can only resolve distances of ~ 20 nm, which still creates uncertainty in detecting bona fide PPIs. FRET

occurs over a fluorophore separation (R_{DA}) of ~ 1 -10 nm and the transfer rate (k_{FRET}) is proportional to the inverse 6th power of the distance; thus, FRET microscopy, particularly FLIM-FRET, is positioned to be the prime optical technique available to study PPIs. In addition to the inherent high resolution of FRET microscopy, these approaches are advantageous compared to other high-resolution microscopy techniques, such as cryo-EM, because PPI dynamics can be monitored over time and under physiological conditions. Indeed, FLIM-FRET has been used to study snare complexes involved in neurotransmitter release (Degtyar, Hafez, Bray, & Zucker, 2013; Takahashi et al., 2015; Zhao et al., 2013), ion channel assembly and dynamics (Dore et al., 2015), and intracellular signaling mechanisms underlying neuroplasticity (Aow, Dore, & Malinow, 2015; Bosch et al., 2014; Dore, Labrecque, Tardif, & De Koninck, 2014) and gene transcription (Laviv et al., 2020), all of which involve dynamic PPIs.

When designing a FLIM-FRET experiment to study a PPI, one should carefully consider several experimental variables, the most critical of which we will discuss here. First, fluorophores constituting a FRET pair can be selected so that the calculated Förster distance (R_0) is within the expected distance of the PPI, taking into consideration the location of the fluorophores on the labeled proteins. This selection process can be aided by structural models of the proteins of interest. Second, the best labeling sites should be determined to maximize the probability of a FRET reaction. Typically, a good starting point is to label the amino and carboxy termini of each protein of interest with donor or acceptor. Third, expectations of measured lifetimes should be developed to help interpret if changes in donor lifetime are due to FRET, as opposed to other possible variables that can influence lifetime as discussed in previous sections. These expectations can be developed based on the calculated Förster distance of the FRET pair and structural models.

Additionally, several potential pitfalls should be considered. For a PPI FRET study, many neuroscientists choose to use FPs to label their proteins of interest. This approach is advantageous over other labeling techniques because FPs are genetically encoded, which allows precise protein labeling using standard molecular biology procedures, easy heterologous expression in molecularly defined neurons (whether in culture, brain slice, or in vivo), and mutagenesis of FPs to generate

powerful controls to confirm FRET mechanisms (including acceptor FPs with broken chromophores). However, because FPs are large fluorophores (~ 27 kDa), one must consider the impact of FP labeling on the function of the protein of interest, and efforts should be made to confirm that the protein's function and the biology it supports are not perturbed.

It is most common for a FLIM-FRET experiment to be carried out using FP-labeled proteins of interest that are heterologously expressed in neurons using expression vectors such as DNA plasmids or adeno-associated viruses (AAVs). Using this approach, it is possible to achieve robust expression that leads to acceptable signal-to-noise ratios. However, it is also possible that such robust expression can lead to bystander FRET due to molecular crowding. This effect can be compounded depending on the subcellular compartment of interest. For example, many neuroscientists are interested in PPIs occurring at the postsynaptic density (PSD) or in the plasma membrane, which are among the most densely packed compartments within a neuron.

To determine if bystander FRET is occurring, several control constructs can be implemented. It is common for investigators to control for bystander FRET by expressing free acceptor. This is a good control in many instances, because there should be no mechanism that brings the acceptor in close enough proximity to the donor to support a FRET reaction. This control can be useful for studying interactions between two soluble proteins that can freely move throughout the cytosol or within large organelles; however, this control is less appropriate for studying PPIs that are restricted to a subcellular compartment such as the PSD or plasma membrane. In these cases, expressing a free acceptor does not recapitulate the environment of the PPI of interest and could lead to erroneously concluding that bystander FRET is not occurring. Control constructs can be created that traffic the acceptor to the subcellular compartment of interest. For example, one could tether a transmembrane domain to anchor the acceptor to the plasma membrane if the acceptor tagged protein is localized to the plasma membrane. As an alternative to using free acceptor to control for bystander FRET, one can tether the acceptor to a protein that is localized to the same subcellular compartment as the PPI of interest but does not participate in the interaction. For example, this type of control was implemented in a FLIM-FRET study examining activity-dependent regulation of NMDAR and PSD95

interactions (Dore et al., 2014). In this study, the authors tethered a donor GFP to the C-terminus of the GluN1 subunit of the NMDA receptor and an mCherry to the C-terminus of PSD95, a strategy that they showed supports a FRET reaction. To control for bystander FRET, the investigators expressed a control construct containing mCherry tethered to Homer, a protein that localizes to the PSD but is not thought to directly interact with NMDA receptors. Indeed, FRET was not observed when this construct was expressed with GluN1-GFP, suggesting that the FRET observed between GluN1-GFP and PSD95-mCherry was dependent on a bona fide interaction between these two proteins. Yet another approach to control for bystander FRET is to mutate one or both proteins of interest to disrupt the interaction between them, which should preclude a FRET reaction from occurring. In contrast to the other controls discussed, knowledge of the mechanism of interaction is required in order to perform mutagenesis. This type of control was implemented in a recent study that used in vivo 2-photon FLIM-FRET to monitor activation of the transcription factor cAMP response element-binding protein (CREB) by monitoring a PPI between CREB and the KIX domain of CREB binding protein (Laviv et al., 2020). It is well established that CREB binding to KIX is dependent on phosphorylation of S133 in CREB. With this knowledge, the authors of the aforementioned study generated a S133A CREB mutant, which prevents S133 phosphorylation and blocks CREB interaction with KIX, to demonstrate the specificity of their FLIM-FRET measurements.

Additionally, one must consider how well FPs satisfy the assumptions made when calculating R_{DA} to determine the distance between interacting proteins. FRET efficiency (E) can be calculated in a FLIM experiment using Equation 9, and from $\langle E \rangle$ a population average R_{DA} can be estimated given certain assumptions. It is commonly assumed that the donor-to-acceptor dipole orientation factor, κ^2 , is equal to $2/3$, the average value in an isotropic population of fluorophores. Although this value is a good approximation for small organic fluorophores with rotational coefficients much less than their fluorescence lifetimes in an aqueous environment, this assumption is not valid for FPs in a viscous intracellular environment where they rotate much slower than their fluorescence lifetimes (see Fig. 4C). Given that most FRET experiments in neuroscience are designed using FPs

and evaluated in a viscous cellular environment, Equation 12 cannot be used to calculate R_{DA} , but rather Equation 13 should be used to estimate R_{DA} under these circumstances.

FLIM-FRET for Studying Protein Conformational Changes

Proteins comprise the primary machinery of cells and are often flexible and dynamic molecules. Their shape, or conformation, may alter in response to changes in their environment, such as upon ligand binding, or due to other local factors. Transition between possible structures is called a conformational change and is frequently the basis for how proteins perform their function. Therefore, characterizing and following protein conformational changes has become a major method for understanding cellular mechanisms in neurons.

Changes in protein structure occur on a length scale comparable to the dimensions of proteins or their domains, ranging from a few Å to hundreds of nanometers. Structural transitions greater than 200 nm can be followed by light microscopy. At higher resolution, alterations greater than 20–30 nm can be observed using super-resolution microscopy. For more advanced requirements, detection of structural changes below 10 nm, even though well beyond the resolution of light microscopy, can be monitored by FRET. Thus, FRET microscopy has become a standard spectroscopic tool for investigations of protein conformational changes on a nanometer scale under physiological conditions. In a FLIM-FRET experiment to investigate conformational changes, donor and acceptor fluorophores are fused to distinct domains or subunits of the protein of interest and FRET between them is monitored before and after an environmental perturbation. A high FRET efficiency indicates a “closed” conformation where the fluorophore-tagged domains/subunits are in close proximity, and a low FRET efficiency indicates an “open” conformation where the donor and acceptor are separated. Depending on whether the donor and acceptor used are different (hetero-FRET) or identical (homo-FRET) fluorophores, changes in FRET can be detected through measurements of donor’s fluorescence lifetime, or time-resolved/steady-state anisotropy, respectively.

Using hetero-FRET to study protein conformational changes

In neuroscience, FLIM-based hetero-FRET has been employed to investigate

structural dynamics of receptors (Degtyar et al., 2013; Dore et al., 2015; Ferreira et al., 2017; Zachariassen et al., 2016), ion channels (Yang et al., 2020), motors (Ems-McClung et al., 2013) and proteins related to neurodegenerative disorders such as Huntington disease (Caron, Desmond, Xia, & Truant, 2013; Caron, Munsie, Keillor, & Truant, 2012), Alzheimer disease (Bacsikai, Skoch, Hickey, Allen, & Hyman, 2003; Berezovska et al., 2005; Caron et al., 2012; Jones et al., 2011; Lleo et al., 2004; Wang et al., 2015), Parkinson's disease (Caron et al., 2012; Kasai et al., 2017) and spinocerebellar ataxia type 14 (Verbeek, Goedhart, Bruinsma, Sinke, & Reits, 2008). In these studies, the investigated proteins were tagged with a pair of non-identical fluorophores, and fluorescence lifetime of the donor was monitored under physiological conditions. For a monomeric protein like, for example, transglutaminase type 2 (TG2), a multifunctional enzyme related to several neurodegenerative disorders including Huntington, Parkinson, and Alzheimer, Caron and colleagues fused a donor mCerulean FP and an acceptor yellow FP (eYFP) to the amino and carboxyl termini of TG2, respectively (Caron et al., 2012). The fluorescence lifetime of the donor was monitored under a series of conditions that affect the GDP/GTP ratio and calcium binding, as well as interactions with irreversible (NC9) and reversible (CP4d) inhibitors. The measured lifetime was converted to FRET efficiency, which should alter if the distance between mCerulean and eYFP changes as a result of transition between closed and open protein conformations. In contrast, for a multimeric protein like tetrameric NMDA receptor composed of two GluN1 and two GluN2 subunits, Dore and colleagues chose to tag either donor GFP or acceptor mCherry to the C-terminus of GluN1 to form GluN1-GFP or GluN1-mCherry, correspondingly (Dore et al., 2015). Then, the GluN1-GFP and GluN1-mCherry were co-expressed at a ratio of 1:3 to minimize GluN1-GFP/GluN1-GFP pairings. FRET between GluN1-GFP and GluN1-mCherry within NMDA receptor was monitored through measurements of fluorescence lifetime of GluN1-GFP in different environments such as spines and dendrites, or with agonist applications containing the non-competitive NMDA receptor antagonist 7CK or the NMDA receptor ion-channel blocker MK-801. Changes in the FRET efficiency reflected fluctuations in distance between

GluN1-GFP and GluN1-mCherry subunits during NMDAR conformational transitions.

Labeling raises a few very important points and FRET practices that one may want to be aware of when designing a FRET-based conformational study. For physiological relevance, it is essential to demonstrate that the act of fluorophore tagging does not interfere with the protein of interest's functional activity, its structural transition, and its ability to interact with substrates, cofactors, or other protein binding partners. On a practical level, this leads to considerations of finding proper locations for the attachments as well as optimizing linkers between the fluorophores and protein. The former, for instance, was showed by Zachariassen et al. (2016). In their conformational study of GluA2, the authors fused mVenus, a yellow variant of GFP, to various insertion points on GluA2m, and measured glutamate responses of each fusion construct. It was revealed that among eleven mVenus-tagged GluA2 (GluA2-mVenus) constructs, five did not respond to glutamate, one exhibited a significantly weaker response, and only five displayed robust responses similar to those obtained from the parent untagged GluA2m and wild-type GluA2. The authors found four locations suitable for FP tagging that preserved the glutamate response with robust fluorescence. Next, the GluA2 was double tagged with mCerulean3 and mVenus at these permissive positions, and the glutamate/fluorescence verifications were repeated for all possible combinations. This time, it was shown that five dual-tagged GluA2-mCerulean3/mVenus constructs had responses similar to that of the parent untagged GluA2m construct. It is noteworthy that four of five constructs did not display any FRET signal due to large separation distances ($\sim 120\text{--}170$ Å) between the mCerulean3 and mVenus. As a result, only the construct (referred to as GluA2-6mVenus-10mCerulean3) that had a FRET efficiency of 13.4% was used for further conformational investigations (Zachariassen et al., 2016). Therefore, while looking for tagging positions that have minor effects on the protein of interest, one should also make sure that the FRET pair is in close enough proximity to support energy transfer. To maximize the magnitude of a FRET change, the donor-acceptor distance in the initial conformation should ideally be close to the Förster distance R_0 .

In addition to tagging location, linkers between the tagged fluorophores and protein

should also be carefully examined. In the TG2 study (Caron et al., 2012), the authors demonstrated that adding four glycine residues as a linker between the FPs and TG2 resulted in a higher FRET efficiency in the closed conformation, suggesting that the FPs were more flexible and introduced less steric hindrance of protein dynamics. Thus, optimizing linker lengths can optimize the dynamic range of a FRET change as well as minimize the tagging impact. Furthermore, for studies that need to accurately estimate the distance between donors and acceptors, ensuring flexibility of FPs, such that the donor and acceptor can rotate freely while still being attached to the protein, is a prerequisite for using Equation 13, which is only valid if dipole-dipole orientation is random in the population.

Careful consideration is needed when selecting FRET-pair fluorophores in order to enhance the detection and interpretation of FRET. In general, the donor should have a mono-exponential lifetime decay and broad spectral overlap with the acceptor. The former simplifies lifetime decay calculations in the absence of FRET, and hence the FRET efficiency, while the latter allows for a larger Förster distance. Moreover, the donor should have a lifetime that is not altered due to homo-FRET between donor molecules, for reasons that will be explained below. Furthermore, when using FPs for the donor and acceptor, to avoid the risk of erroneous FRET measurements on samples with functional donors but where the acceptor has not yet fully formed, it is important to use an acceptor FP that matures faster than the donor FP. Because mVenus is one of the fastest-maturing FPs (Nagai et al., 2002), it often makes an ideal FRET acceptor.

Another very important consideration in a FRET measurement is to avoid unwanted FRET signals. In a conformational investigation, one wants to ensure that only changes in intramolecular FRET, i.e., FRET between a donor and acceptor on the same molecule, are associated with the protein's structural transitions that cause changes in the fluorescence lifetime of the donor. If, however, intermolecular FRET between a donor on one molecule and an acceptor on another occurs, the donor's fluorescence lifetime will be altered by that interaction as well. In addition, for ECFP and mCerulean, homo-FRET is known to shorten their lifetimes (Koushik & Vogel, 2008; Rizzo, Springer, Granada, & Piston, 2004). Accordingly, if these FPs are used as donor, any inter- or intra-molecular homo-FRET will complicate the interpretation of FLIM-FRET

experiments. Generally, intermolecular FRET appears when two or more molecules are in very close proximity (<10 nm) due to high concentration (>200 μ M), usually as a consequence of overexpression or FP multimerization. The former could be avoided by controlling expression level and selecting cells with relatively weak fluorescence intensity for FLIM measurements. Regarding the latter, to prevent dimerization of GFP and its derivatives, an A206K mutation has been introduced into Cerulean (Rizzo & Piston, 2005), creating its monomeric version, mCerulean, which was used in the studies mentioned above (Caron et al., 2012; Zachariassen et al., 2016). It is an important control in FRET studies to verify the absence of intermolecular FRET. In the TG2 study (Caron et al., 2012), the authors prepared negative controls including mCerulean-TG2 expressed alone, mCerulean-TG2 co-expressed with eYFP, and mCerulean-TG2 co-expressed with TG2-eYFP. A lifetime of about 2.8 ns was obtained from FLIM measurements for all these controls, indicating that under their experimental conditions no intermolecular FRET was detected between mCerulean-TG2 and free eYFP, or between mCerulean-TG2 and TG2-eYFP. Thus, the authors concluded that donor-lifetime changes observed with the mCerulean-TG2-eYFP tagged molecule were due to conformational changes in TG2 that altered the mCerulean-eYFP separation and thus intramolecular FRET.

Last but not least, it is worth noting that tagging an FP to a protein can alter its lifetime due to a change in local refractive index (Tregidgo, Levitt, & Suhling, 2008). Therefore, using the fluorescence lifetime of free donor as τ_D , the fluorescence lifetime of donor in the absence of acceptor, in Equation 9, may decrease the accuracy of FRET efficiency calculations. To avoid this problem, the AMPA GluA2 subunit study (Zachariassen et al., 2016) did not use the lifetime of free mCerulean3 as the τ_D ; instead, the YFP in GluA2-6Y-10C was replaced with Amber, the Y67C mutation of YFP that folds correctly but does not form a chromophore and thus cannot act as a FRET acceptor (Koushik, Chen, Thaler, Puhl, & Vogel, 2006), to create GluA2-6A-10C construct, and used its fluorescence lifetime as τ_D in FRET calculations (Zachariassen et al., 2016).

Using homo-FRET to study protein conformational changes

Homo-FRET can also be used to detect protein conformational changes. One

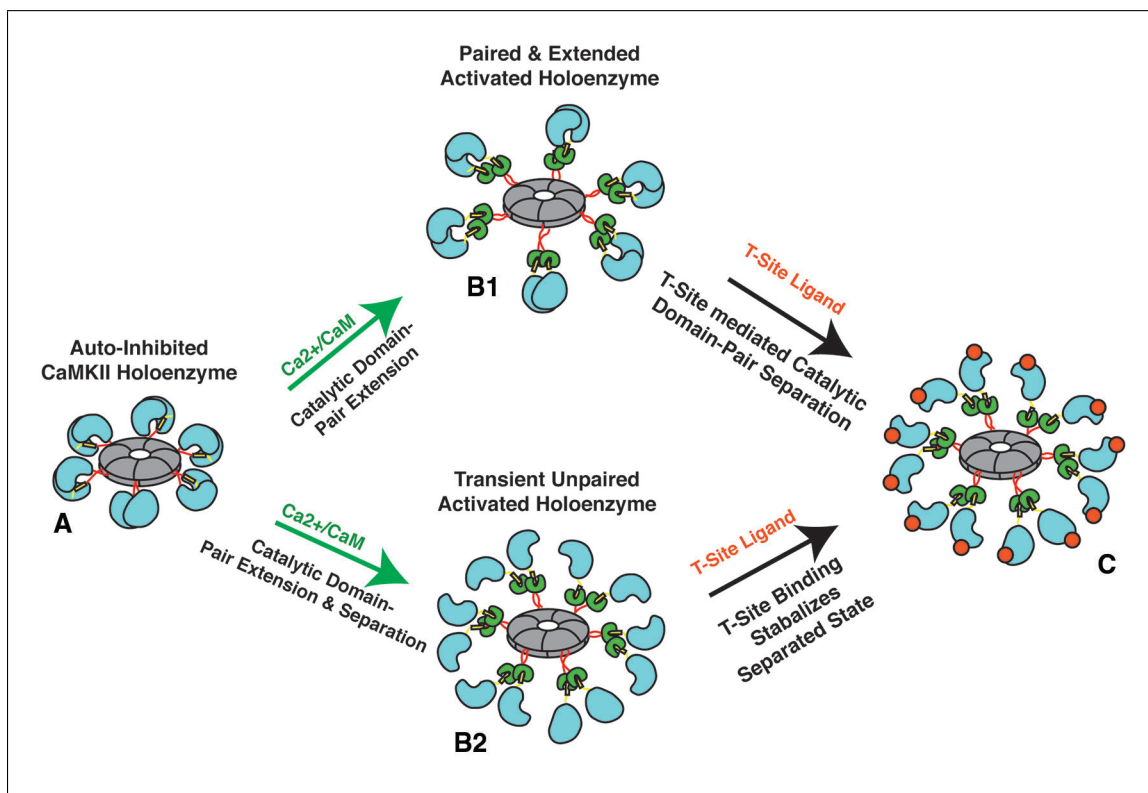


Figure 6 Cartoon depicting conformational changes in the CaMKII holoenzyme structure observed, in part, using FRET microscopy. The autoinhibited enzyme, as depicted in (A), is composed of an assembly of 12 subunits each having a catalytic domain (blue) on its N-terminal end and an oligomerization domain (gray) on its C-terminal end with a regulatory (yellow)/linker (red) domain connecting the two. The pairing of catalytic domains was clearly observed by both homo-FRET and hetero-FRET microscopy. The holoenzyme is activated when calcium-calmodulin (green U-shaped blobs) bind to the regulatory domain. If the kinase is activated in the absence of T-site ligands (orange circles), the holoenzyme assumes a paired but extended structure as depicted in (B1). This was observed by a combination of homo-FRET microscopy and FCS diffusion analysis. If the kinase is activated in the presence of T-site ligands (orange circles), the holoenzyme assumes an unpaired extended structure as depicted in (C). This was also observed by a combination of homo-FRET microscopy and FCS diffusion analysis. It is not known if the binding of T-site ligands upon activation, such as to the NMDA receptor, proceeds via an (A to B1 to C) pathway or alternatively via an (A) to (B2 to C) pathway. Some homo-FRET experiments suggest that a transient unpaired activated holoenzyme structure as depicted in (B2) might exist.

of the early attempts was implemented by Thaler and colleagues to investigate structural rearrangement of catalytic domains of Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) upon activation by calcium calmodulin (Ca^{2+} /CaM) in hippocampal neurons (Thaler et al., 2009). Activation of CaMKII was known to alter this holoenzyme structure, initiating a biochemical cascade resulting in long-term potentiation, a molecular model for memory (Silva, Paylor, Wehner, & Tonegawa, 1992). Electron microscopy images of CaMKII showed that the kinase has a sophisticated holoenzyme structure with between 8 and 14 subunits (Kanaseki, Ikeuchi, Sugiura, & Yamauchi, 1991; Kolodziej, Hudmon, Waxham, & Stoops, 2000). While four subtypes of CaMKII exist (α , β , γ , and δ), and in the brain the holoenzyme is primarily

composed of the α and β subtypes, most biochemical studies of the holoenzyme are performed on homogeneous holoenzyme comprised of only the α subtype. Because of this, and the complex structure of the holoenzyme, homo-FRET microscopy, where only one type of fluorophore is needed for labeling, was used for studying conformational changes in the CaMKII α holoenzyme. Here, the authors used mVenus, a monomeric yellow GFP spectral variant, to label each subunit of the holoenzyme on its N-terminus, and monitored homo-FRET between Venus molecules. One of the key findings in this paper, revealed by homo-FRET measurements, is that the kinase catalytic domains are organized around the central core domain as pairs. In Figure 6A, we see a model of the auto-inhibited holoenzyme based in part on homo-FRET measurements.

Time-resolved anisotropy of autoinhibited CaMKII α tagged with mVenus on its N-terminus was measured and compared with anisotropy decays of mVenus monomers, dimers, and trimers. Despite having between 8 and 14 subunits, the mVenus-tagged holoenzyme had an anisotropy decay that looked like a mVenus dimer. Next, steady-state anisotropy of mVenus-CaMKII α transfected in neuronal cultures was monitored before, during and after perfusion of glutamate/glycine. A decrease of mVenus-CaMKII α anisotropy was observed after perfusion, indicating a reduced homo-FRET and hence larger distance between mVenus molecules tagged on catalytic domains. This led to the conclusion that activation of CaMKII α in hippocampus neurons initiates separation of the catalytic domain dimers (Fig. 6B2).

Surprisingly, in a follow-up study in 2015, Nguyen and colleagues failed to observe catalytic domain separation when mVenus-CaMKII α was expressed in HEK cells upon activation with a calcium ionophore (see Fig. 6B1; Nguyen et al., 2015). Catalytic domain separation was, however, observed if CaMKIIN protein, a known CaMKII T-site ligand (see NOTE 11), was transfected into the HEK cells along with mVenus-CaMKII α (Fig. 6C). In vitro homo-FRET experiments confirmed that adding T-site ligands promoted catalytic domain separation, but only after activation with Ca/CaM. Thus, it was concluded that hippocampal neurons must have abundant T-site ligands compared to HEK cells. From these studies, it remains unclear if T-site ligands, in conjunction with Ca/CaM, are directly responsible for catalytic domain separation (A $\rightarrow$ B1 $\rightarrow$ C), or alternatively if activation with Ca/CaM causes transient catalytic domain pair separation and T-site ligands simply stabilize a conformation with catalytic domains separated (A $\rightarrow$ B2 $\rightarrow$ C).

Similar to hetero-FRET experiments, it is important to carefully select the fluorophore used for a homo-FRET study. Ideally, the molecule should support strong homo-FRET (large overlap of the fluorophores emission and absorption spectra) and have a lifetime that does not change with homo-FRET. In the two studies mentioned above, mVenus was chosen because a mVenus-mVenus dimer had a larger change in its anisotropy decay than a mCerulean-mCerulean dimer despite both constructs having the same donor-acceptor distance. This, presumably, is due to the greater spectral overlap integral of the mVenus emission and absorption spectra as compared

to those of mCerulean. In addition, while the mCerulean lifetime decreases due to homo-FRET, the mVenus lifetime is not altered by homo-FRET (Koushik & Vogel, 2008). This suggests that mVenus has a single excited state, and therefore simplifies the estimation of mVenus-mVenus distance within a catalytic domain dimer of the mVenus-CaMKII holoenzyme (Thaler et al., 2009). Moreover, to prevent dimerization of a construct caused by dimerization of the attached FP, it is important to use monomeric FPs; in the case of Venus, an A206K mutation was introduced creating its monomeric form, mVenus. Last but not least, in the studies mentioned above, a 15-amino acid linker was inserted between mVenus and the CaMKII α subunit to allow the attached fluorophore to rotate freely.

NOTE 11: T-site ligands include several proteins of interest to neuroscience that can bind to CaMKII following kinase activation. T-site ligands include the NR2B subunit of the NMDA receptor and L-type voltage-gated calcium channels.

FLIM-FRET BIOSENSOR CONSIDERATIONS

Structural Design of FLIM-FRET Biosensors

Parallel to the development of genetically encoded fluorescence intensity-based sensors, there has been development of genetically encoded fluorescent biosensors that rely on FRET to report conformational changes in proteins in response to signaling events inside living cells (Greenwald, Mehta, & Zhang, 2018; Zhang, Campbell, Ting, & Tsien, 2002; Zhou, Herbst-Robinson, & Zhang, 2012). Although a variety of design strategies have been developed, all FRET-based sensors have a common modular design consisting of two functional units, sensing and reporting domains (Fig. 7). The sensing domain typically is derived from proteins that recognize and bind to the specific biological signaling molecules of interest. Ideally, a sensor should only respond to the ligand of interest, have a binding constant that is physiologically relevant, and do this without perturbing cell signaling. For example, to minimize the risk of perturbing cell signaling, the optimization of FRET-based calcium sensors has included the replacement of the original calcium-sensing domain from calmodulin, a ubiquitous protein that interacts with a multitude of other proteins, with the calcium-sensing domain of

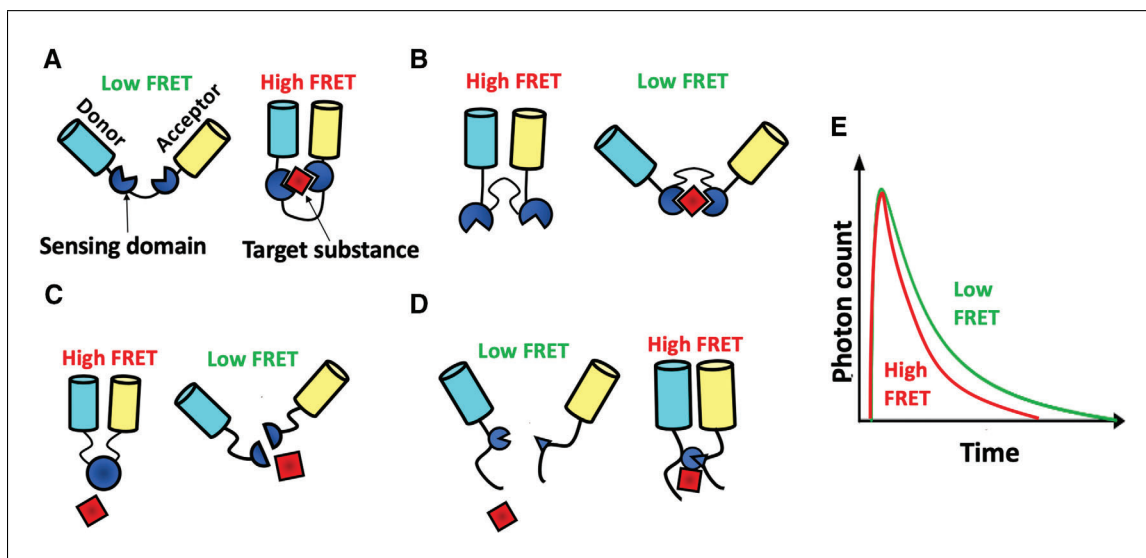


Figure 7 Schematic of FRET biosensors. **(A)** Enhanced FRET can be observed when the biological factor of interest (red square) interacts with the sensing domain (blue circles) of the biosensor. **(B)** Similarly, in some biosensors, diminishing FRET can be observed when the biological factor of interest interacts with the sensing domain. **(C)** Diminishing FRET can also be observed when a protease (red square) cleaves the protease's specific peptide substrate (blue circle) linking the donor and acceptor. **(D)** For some biosensors, FRET can only be detected when a biological factor promotes the association of two separate sensor components. **(E)** Cartoon depicting the expected fluorescence lifetime decay curve changes in response to FLIM-FRET biosensor activity.

troponin C, a protein primarily found in muscle (Heim & Griesbeck, 2004; Mank et al., 2006). An ideal sensing domain should undergo a large conformational change when its ligand binds. The reporting domain, consisting of two FPs, a donor and an acceptor, can then transform this conformational change into a fluorescence emission readout, based on a change in FRET. FRET efficiency is dependent primarily on the distance and relative orientation of the donor-acceptor pair (Berezin & Achilefu, 2010), which can be influenced by ligand binding. It is worth mentioning that FPs that are genetically encoded are usually preferred in live-cell FRET imaging due to their high cellular specificity and intracellular stability (Bajar, Wang, Zhang, Lin, & Chu, 2016). Ideally, a large change in the FRET efficiency will be observed when a ligand binds to the biosensor. In the example shown in Figure 7A, we expect the FRET efficiency to increase when the ligand binds, but biosensors can also be designed where a decrease in FRET efficiency can occur (Fig. 7B and 7C). Since the protein-sensing domain has an affinity for a specific ligand, the cellular concentration of that ligand will modulate the fraction of biosensors with bound ligands. Thus, the experimentally measured fluorescence lifetime will be a function of the lifetimes of the biosensor with or without the ligand bound, weighted by the relative

abundance of these two species. In this example, as FRET increases when the ligand is bound, its donor lifetime (i.e., the decay constant of the curve) will get shorter (Fig. 7E). In contrast, when FRET decreases, as depicted in Figure 7B, the donor lifetime will get longer. As depicted in Figure 7, FRET efficiency can be affected by conformational change and enzymatic cleavage, as well as enzymatic activity (not depicted). The overall design and the fundamental principal of the FRET biosensors allow for the spatiotemporal interrogation of molecules in living cells in a minimally invasive manner (DiPilato & Zhang, 2010; Zhang et al., 2002).

Several factors should be considered when choosing FPs for biosensors (Shaner, Steinbach, & Tsien, 2005). Clearly, the donor emission and the acceptor absorption should have good spectral overlap (Bajar et al., 2016; Lam et al., 2012). Since many FPs dimerize or even tetramerize during maturation, use of these might inadvertently cause biosensor multimerization. For this reason, monomeric FPs are preferred when designing FRET-based biosensors (Lam et al., 2012). Molecular brightness of the FPs is also an important consideration. Molecular brightness is the measure of relative fluorescence emission rate per molecule. Since the accuracy of lifetime measurements increases with photon count, high molecular brightness is important. If the

photon count is limited due to experimental limitations, alternative, more photon-efficient methods for measuring FRET efficiency should be considered. There are several other parameters that can influence emission intensity, such as temperature, excitation intensity, pH, and FP photostability. For longitudinal studies, photostability is critical. If an FP bleaches easily, this will attenuate photon count. Furthermore, if an acceptor bleaches faster than its donor, the FRET efficiency may decrease with time due to bleaching. One remedy for bleaching is to reduce excitation intensity, sampling rate, and excitation duty cycle. Over the last decade, substantial progress has been made in improving FP brightness and photostability (Bajar et al., 2016; Lam et al., 2012), and these FP variants should be considered when photo-bleaching is an issue.

In most FLIM-FRET applications, only the donor's lifetime is monitored. Thus, technically, FRET acceptors do not have to be fluorescent: they can also be dark absorbers, chromophores that can absorb a photon but do not emit a red-shifted photon. If their absorption spectrum has significant overlap with the donor's emission spectrum, FRET can occur. An advantage of using a dark absorber as a FRET acceptor is that their use can potentially free up spectral bandwidth to allow multiplexing with other fluorophores (Ganesan, Ameer-Beg, Ng, Vojnovic, & Wouters, 2006). Nonetheless, care must be exercised to make sure that a "dark absorber" really does not emit any photons. If a "dark absorber" is simply a very dim fluorophore with a very short lifetime, it is essential to make sure that its emitted photons are not detected in the donor's channel.

In addition to development of FRET-based sensors that allow for spectral multiplexing, considerable efforts have been made to target FRET-based probes to different subcellular locations using targeting sequences or translocation proteins, (Pendin, Greotti, Lefkimiatis, & Pozzan, 2017). Targeting FRET sensors to subcellular components where the targeted molecule is enriched can increase signaling specificity and signal-to-noise. This strategy has been extremely successful for studying cell signaling in vitro (for example, DiPilato, Cheng, & Zhang, 2004, and Galpersin and Sorkin, 2003), but limited for in vivo application (Ma et al., 2018; Sprenger et al., 2015). When targeting FRET sensors to subcellular compartments, care must be taken to avoid local overexpression and thus the potential for bystander FRET.

Measurement of FRET-Based Biosensors in Neuroscience

A biosensor's conformational changes can be measured by monitoring the ratio of the fluorescence intensity emitted from the donor and acceptor (ratiometric) or by measuring the fluorescence lifetime of the donor (FLIM-FRET) (Bajar et al., 2016; Pendin et al., 2017). Although both FRET detection methods can offer sub-second temporal resolution, only FLIM-FRET can detect kinetic changes on a picosecond timescale. Ratiometric measurements are not standardized, and vary with different microscope systems and setups. This is due, in part, to the many factors that can influence fluorescence intensities. For example, filters used in the light path, excitation intensity, spectral bleed-through, direct activation of acceptors, and camera sensitivity can all influence the fluorescence intensity measured (Bajar et al., 2016). FRET sensors consisting of different FPs are intrinsically ratiometric (i.e., emit fluorescence), but only a few have been optimized for fluorescence lifetime measurements (Berezin & Achilefu, 2010). One of the advantages of using FLIM to interrogate FRET biosensors is that only the donor lifetime needs to be measured, and its lifetime should be independent of the biosensor's donor fluorescent intensity or its abundance (see NOTE 12) (Becker, 2012). Another appealing advantage of using FLIM for neuroscience applications is that lifetime measurements are independent of imaging depth, thus, allowing deep brain structures to be imaged despite wavelength-dependent light scattering. In mixed populations where both activated and ground-state biosensors are present, the fitting of the population decay of the donor with a double exponential fluorescence decay model can potentially determine the proportion of fluorophores that have undergone FRET activity (Day & Davidson, 2012; Pelet, Previte, & So, 2006). However, since FRET between FPs is inherently in the static regime (Fig. 4C), the fitting of a double exponential decay curve may not properly estimate the fraction of FRET biosensors in the activated state. Another disadvantage of FRET-FLIM measurements for monitoring biosensors is that they require a good signal-to-noise ratio, particularly for multiexponential decay analysis. One way to achieve higher signal-to-noise is to increase acquisition sampling time and/or increase excitation light intensity. These methods, however, can also increase the likelihood of phototoxicity or bleaching.

NOTE 12: As long as the biosensor concentration is kept below the threshold concentration where bystander FRET can occur, $\sim 200 \mu\text{M}$ (see Equation 14).

FLIM-FRET techniques for monitoring biosensors in neuroscience

While FLIM measurement techniques have been associated with FRET-based biosensors in neurobiology research, there are only a few successful demonstrations where FLIM-FRET was used to measure the modulation of physiological parameters in vitro or in vivo (Chen, Saulnier, Yellen, & Sabatini, 2014; Dore et al., 2014; Rinnenthal et al., 2013; Zheng et al., 2015; Zheng, Jensen, & Rusakov, 2018). Based on these studies, we believe there is enormous potential for using FLIM-FRET as a physiological tool in neuroscience. Below we will discuss the two most basic optical configurations for FLIM-FRET in neuroscience: the microscopy imaging approach (Grewe & Helmchen, 2009; Trautmann et al., 2013) and the fiber-based photometry approach (Adelsberger, Garaschuk, & Konnerth, 2005).

Scanning confocal microscopy is a fluorescence imaging technique that generates thin optical sections by attenuating out-of-focus fluorescence. The higher depth resolution is achieved by focusing a one-photon laser onto a defined spot at a specific depth in the sample, while a pinhole inside the optical path is used to cut off fluorescence generated from out-of-focus fluorophores. Confocal microscopy can be combined with FLIM (Levitt et al., 2020) for neuroscience applications. For example, activation of AMPA receptor was measured by confocal FLIM using CFP/YFP-tagged GluA2 subunit constructs, and electrophysiological properties of the cultured cells were simultaneously measured with a patch clamp technique (Zachariassen et al., 2016).

Two-photon scanning microscopy (Denk et al., 1990) is another imaging approach that can be adapted for FLIM imaging. It is particularly conducive to fluorescence imaging of thicker samples, such as living tissue and intact brains up to approximately one millimeter in depth (Kobat, Horton, & Xu, 2011). Using two-photon excitation, most cortical structures (Layer 2/3 and Layer 5) in mouse brains (Helmchen & Denk, 2005; Mittmann et al., 2011; Yaeger, Ringach, & Trachtenberg, 2019) can be monitored through a cranial window (Helmchen & Denk, 2005; Kondo, Kobayashi, Ohkura, Nakai, & Matsuzaki, 2017) with minimal perturbation of the tissue. Even deeper imaging in a mouse brain

is possible by using an implantable optical component, such as a GRIN lens (Murray & Levene, 2012), in conjunction with two-photon microscopy. Using this approach, layers under the superficial cortex or even deeper subcortical structures of the brain, such as mPFC (Otis et al., 2017), SNc, and VTA (Engelhard et al., 2019) can be imaged. Since two-photon microscopes use pulsed lasers for excitation, these strategies can be adapted for two-photon FLIM-FRET imaging (Rosenegger, Tran, Ledue, Zhou, & Gordon, 2014) by incorporating a time-resolved photon-counting module and compatible photon detectors. Two-photon FLIM microscopy has been demonstrated in both in vitro pharmacological studies with acute brain slices (Chen et al., 2014; Dore et al., 2014; Rinnenthal et al., 2013; Zheng et al., 2015; Zheng et al., 2018) and in vivo imaging of cortical projection neurons on head-fixed animals (Ma et al., 2018). Another advantage of two-photon microscopy is that it can be used for second harmonic generation (Pavone & Campagnola, 2013). Unlike fluorescence, second harmonic generation occurs instantaneously; thus, it is an ideal method for measuring the IRF of a two-photon FLIM microscope.

Fiber photometry (Adelsberger et al., 2005; Cui et al., 2014; Falkner, Grosenick, Davidson, Deisseroth, & Lin, 2016) is an alternative technique that enables the spectroscopic interrogation of the brain with optic fibers while animals are freely moving and learning behaviors (Calipari et al., 2016; Cui et al., 2014; Gunaydin et al., 2014; Kupferschmidt, Juczewski, Cui, Johnson, & Lovinger, 2017; Reed et al., 2018). A fiber-based photometry system can use either continuous wave light sources (e.g., LEDs and lasers) for photoexcitation of genetically encoded biosensors (Jing et al., 2018; Patriarchi et al., 2018; Piatkevich et al., 2018; Tian et al., 2009) or a pulsed laser using the TCSPC approach for fiber-based FLIM (Cui et al., 2014). In these schemes, the fluorescence signal detected represents the population of photons generated by biosensors within a defined region of the brain where the optical fiber is implanted (Adelsberger et al., 2005; Cui et al., 2014). Since fiber photometry does not image the brain, it cannot be used to reconstruct spatial information of the collected fluorescence (Resendez & Stuber, 2015). Despite this limitation, fiber photometry typically has a much better signal-to-noise than deep brain imaging techniques, and cell-type-specific expression of genetically encoded biosensors can often compensate

for their inability to image which neurons are expressing the biosensor. In addition, the implementation of the fiber-based FLIM photometry system (Lee, Chen, Lodder, & Sabatini, 2019) can be relatively cost-efficient compared to confocal or two-photon FLIM imaging systems. Within these constraints, the fiber-photometry approach can be a powerful tool for studying behaving animals expressing genetically encoded FRET biosensors in specific subsets of neurons.

Challenges Associated with FLIM-FRET Biosensor Analysis

FP biosensors may be sensitive to environmental factors, such as ionic strength, pH, and oxidation state (Shinoda, Shannon, & Nagai, 2018; Stepanenko, Stepanenko, Kuznetsova, Verkhusha, & Turoverov, 2013). These factors, independent of sensing domain activation, can potentially alter the fluorescence lifetime observed from a FLIM-FRET biosensor, thus complicating the interpretation of FLIM-FRET data. This issue becomes even more complicated if these environmental factors have a non-homogeneous and/or dynamic distribution in the cellular milieu. How then can we compensate for environmental impacts when interpreting FLIM-FRET experiments? One common approach to evaluate the impact of environmental factors is to run control experiments using a point-mutant variant of the biosensor that does not respond to the ligand of interest (Ma et al., 2018). A point-mutated biosensor is identical to the functional biosensor in every way, but its functional site is mutated and therefore it cannot respond to its biological trigger. Any environmental factors that directly influence the biosensor's FPs most likely will still occur. Thus, lifetime decay differences between the actual biosensor and the mutated biosensor may reveal the influence of environmental factors. An alternative approach to deal with uncontrolled environmental factors is to develop the methodology to monitor arrays of multiplexed biosensors that can simultaneously monitor the specific molecule of interest as well as the most likely environmental factors that might adversely influence its read-out.

Another potential experimental issue to consider when evaluating *in vivo* biosensor experiments is that a biosensor's dynamic range for detecting changes in the biomolecule of interest may not match the actual physiological levels of that factor within the brain. Under these conditions, the biosensor may not respond due to either low affinity or saturated

responses even though the biological factor of interest is in fact changing. For this reason, it is important to test FLIM-FRET biosensor readout in (1) neuronal cell lysates where the biological factor as well as environmental factors can be easily controlled and manipulated; (2) neurons in culture and/or brain slices where biosensor responses can be evoked by electrical stimulation, modulated pharmacologically, and compared to electrophysiological read-out; and, finally, (3) animal brains where biosensor responses can be evoked optogenetically or by behaviors, and activities can be modulated pharmacologically. A bioengineering approach to deal with this issue is the development of highly selective FLIM-FRET biosensors with large dynamic ranges (Bajar et al., 2016; Rizzo et al., 2004).

FUTURE TRENDS OF FLIM-FRET IN NEUROSCIENCE

From its inception, neuroscience has been multidisciplinary, embracing a vast array of new technologies and innovations to help understand how the brain and neural circuits work. Experimental neuroscience typically involves two fundamental components: 'interrogation' and 'manipulation'. Traditionally, electrical recording (Jun et al., 2017; Neher & Sakmann, 1992; Sakmann & Neher, 1984; Verkhratsky, Krishtal, & Petersen, 2006) and stimulation (Fibiger, LePiane, Jakubovic, & Phillips, 1987; Kandel, Spencer, & Brinley, 1961; Kringelbach, Jenkinson, Owen, & Aziz, 2007) have been the prime method used to assay neuronal activity in living cells and tissues, and to stimulate or inhibit neuronal activity. Optical techniques, such as used by Ramón y Cajal at the end of the 19th century (Llinas, 2003; Sotelo, 2003), have been used to interrogate stained neurons in nerve systems, and more recently video light microscopy, confocal microscopy, and multiphoton microscopy have extended this approach to living neurons. Similarly, photo-activated reagents, such as caged calcium (Ellis-Davies, 2008), have been introduced to stimulate neuronal systems. While these optical techniques have opened up new strategies for studying neuronal systems, electrophysiology has remained the predominant methodological tool for studying neuronal activity. Recently, remarkable breakthroughs have been made in genetically encoded fluorescence biosensors (Yang & St-Pierre, 2016; Jing et al., 2018; Nakai, Ohkura, & Imoto, 2001; Patriarchi et al., 2018), deep

brain fiber photometry (Adelsberger et al., 2005; Calipari et al., 2016; Cui et al., 2014; Gunaydin et al., 2014; Reed et al., 2018) and optogenetic neuronal stimulation (Bernstein & Boyden, 2011; Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005). We believe these developments are driving a paradigm shift in neuroscience towards the acceptance of optical ‘interrogation’ and ‘manipulation’ strategies as members of a core neuroscience toolbox. Some of the great advantages of optical approaches are that they enable simultaneous real-time monitoring of neural activity of thousands of cells (Aharoni, Khakh, Silva, & Golshani, 2019; Ziv et al., 2013), they allow access to regions of the intact brain typically inaccessible by electrophysiology, and they allow on-demand excitatory or inhibitory control of neural activities with cell-type and anatomical specificity (Yizhar, Fenno, Davidson, Mogri, & Deisseroth, 2011). These optical tools are not a stand-alone technology, but a complementary toolbox to the existing electrical methodologies. Owing to the seamless integration of the novel optical techniques and classical electrical techniques, neuroscientists can now leverage another degree of assessment ability in answering the challenging scientific question of how the brain works (Kim, Adhikari, & Deisseroth, 2017).

The FLIM-FRET technology discussed in this overview is a relatively new and advanced form of light microscopy that brings in new capabilities that neuroscientists can use for ‘optical interrogation’ of neuronal systems. Two aspects of FLIM-FRET’s capabilities make it a unique tool for interrogating living neuronal systems: first, it can detect changes in distance on a 1-10 nm scale, well below the resolution of classical light microscopy and even below that of super-resolution microscopy. Second, while not typically exploited, FLIM-FRET can potentially detect dynamic changes in biological systems with a temporal resolution around 100 ps if those changes can be synchronized to the laser excitation pulses. To put this into perspective, synaptic transmission takes a few hundred microseconds, a million times slower than the temporal resolution of FLIM-FRET. The most promising opportunity afforded by FLIM-FRET technology is its ability to monitor dynamic protein-protein interactions and conformational changes in living cells and behaving animals in real time with these spatial and temporal resolutions. FLIM-FRET measurement techniques have already been adopted as useful tools to investigate intracellular signaling and protein

properties in cell biology (Long et al., 2017; Marx, 2017). As the technology advances and becomes more accessible, there is interest in interrogating intracellular signaling processes in neurons of behaving animals with FLIM-FRET. Although a few pioneering studies have demonstrated the feasibility of using the FLIM-FRET technology in neuroscience applications (Chen et al., 2014; Ma et al., 2018; Murakoshi, Lee, & Yasuda, 2008), there are still many challenges in implementing FLIM-FRET experiments *in vivo*.

In neuroscience, there is great interest in understanding how intracellular signaling cascades, protein-protein interactions, and conformational change of protein structures influence neuronal function under physiological conditions. FRET-based biosensors have been developed that can report activity of kinases (Castro, Guiot, Polito, Paupardin-Tritsch, & Vincent, 2014; Demeautis et al., 2017; Violin, Zhang, Tsien, & Newton, 2003), proteases (Allen & Zhang, 2006; Goryashchenko, Khrenova, & Savitsky, 2018), or GTPases (Kiyokawa, Aoki, Nakamura, & Matsuda, 2011; McGhee et al., 2011) in living cells. FRET has also been used to monitor signaling pathways associated with receptors (Muller, Joseph, Slesinger, & Kleinfeld, 2014; Ziegler, Batz, Zabel, Lohse, & Hoffmann, 2011), GPCR activity (Albizu et al., 2010; Hoffmann et al., 2005), protein localization (Chen, Mills, & Periasamy, 2003), and downstream signaling events (Castro et al., 2014; Taylor, Buechler, & Yonemoto, 1990) in cell culture and in freshly prepared cell lysates. In the near future, we anticipate more widespread use of FRET biosensors in intact animal models using viral or transgenic strategies to introduce genetically encoded FRET biosensors.

In this overview, we have discussed fundamental concepts of FLIM-FRET systems to help neuroscientists better implement and interpret FLIM-FRET experiments. We have also discussed several important concepts of time-resolved fluorescence anisotropy measurement and homo-FRET systems that are closely related to FLIM-FRET but not significantly covered in the neuroscience literature. Like FLIM-FRET, time-resolved fluorescence anisotropy could be used as a powerful research tool in neuroscience. Particularly, we have highlighted the applications of anisotropy measurement and lifetime measurement techniques in the understanding protein-protein interactions (Long et al., 2017; Sun, Day, & Periasamy, 2011), protein conformational changes (Caron et al., 2012), and

interrogating intracellular signaling (Taylor et al., 1990) using FRET-based biosensors.

Another exciting opportunity for using FLIM-FRET technology in neuroscience is in exploiting its potential to enable multiplexed detection of several FRET-based biosensors, and/or combining biosensor readout with optogenetic neuronal stimulation of specific circuits. Successful optical multiplexing requires having sufficient spectral bandwidth for each biosensor or optogenetic component to prevent signal crosstalk. Since FLIM-FRET measurements require a narrower spectral bandwidth than ratiometric FRET approaches, they are well suited for this application. Furthermore, multiplexed optical signals can be isolated based on the fluorophore's spectral emission profile, its spectral absorption profile, its lifetime profile, or a combination of these traits. This reduction in spectral bandwidth requirements is even more striking for applications that monitor multiple homo-FRET-based biosensors (Bunt & Wouters, 2017; Ross et al., 2018; Warren et al., 2015), and potentially in applications simultaneously monitoring homo-FRET and hetero-FRET based sensors using simultaneous FLIM and time-resolved anisotropy measurements (Nguyen, Puhl, Pham, & Vogel, 2018).

Other exciting FLIM-FRET applications involve combining FLIM-FRET microscopy with other advanced optical imaging techniques. For example, in a recent report, a wide-field TCSPC-based fluorescence lifetime imaging was combined with a light-sheet illumination configuration for rapid 3D lifetime imaging (Hirvonen et al., 2020). In another recent study, a STED super-resolution microscope was augmented with TCSPC instrumentation to perform high-resolution FLIM-FRET measurements in cultured hippocampal neurons (Tardif et al., 2019). Finally, in our lab, FLIM-FRET microscopy has been combined with time-resolved anisotropy and fluorescence correlation spectroscopy (FCS) to enhance the ability to detect protein conformational changes (Nguyen et al., 2015; Sarkar et al., 2017; Thaler et al., 2009).

In summary, this review describes FLIM-FRET, a form of advanced optical photometry that can be used as a potential tool for neuroscientists to interrogate molecular-level dynamics of protein conformational changes and resulting interactions within the brain. We have explored the basis for how it works, the environmental factors that might influence our ability to accurately monitor this phenomenon, and the technology required for

its implementation. We also discussed FLIM-FRET's strengths and limitations to help the novice interpret FLIM-FRET experiments, and provided examples of how it can be used to specifically monitor protein-protein interactions and protein conformational changes, as well as for developing and monitoring biosensors. In the great interdisciplinary tradition of neuroscience, the development and adaptation of FLIM-FRET microscopy as a tool to study neurobiological problems has involved the concerted efforts and expertise of physicists, biophysicists, biochemists, engineers, and neuroscientists. While such interdisciplinary efforts positively exploit the vast knowledge base of these different fields, the specific jargon of each specialty can sometimes create barriers that impede the adoption of the interdisciplinary approaches developed. This review has pointed out and defined key expressions used in the FLIM-FRET community that might not be familiar to neuroscientists, and therefore unintentionally act as impediments. The prime objective of this review has been to help neuroscientists overcome these obstacles and thus feel more comfortable with the FLIM-FRET approach. We hope this review aids researchers in the neuroscience community to achieve a better understanding of the fundamentals of FLIM-FRET systems and encourages them to fully leverage its powerful ability as a research tool. Despite the strengths and capabilities of FLIM-FRET, there remain substantial opportunities to improve and adapt FLIM-FRET for neuroscience applications. Consequently, we look forward to seeing close interdisciplinary interplay among a broad spectrum of scientific researchers to achieve this goal.

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AUTHOR CONTRIBUTIONS

Daniel J. Liput: Conceptualization; project administration; writing-original draft; writing-review & editing. **Tuan A. Nguyen:** Conceptualization; investigation; writing-original draft; writing-review & editing. **Shana M. Augustin:** Conceptualization; writing-original draft; writing-review &

editing. **Jeong Oen Lee:** Conceptualization; writing-original draft; writing-review & editing. **Steven S. Vogel:** Conceptualization; funding acquisition; supervision; writing-original draft; writing-review & editing.

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APPENDIX: ABBREVIATIONS USED IN THIS ARTICLE

AAV	Adeno-associated virus
Absorption coefficient	A coefficient that describes how light is attenuated (absorbed) as it passes through a substance.
Acceptor	The fluorophore in a FRET pair that transitions from its ground to its excited state.
Bystander FRET	A non-specific form of FRET caused by molecular crowding.
Coherent energy transfer	A form of energy transfer where energy is transferred coherently between donors and acceptors, also called excitonic coupling
Conformational change	A transition between possible structures.
Cross-section	The surface area of the detector (or chromophore) that can sense a photons.
Dark noise	The number of counts per second a detector records in a dark environment.
Donor	The fluorophore in a FRET pair that transitions from its excited to its ground state.
FLIM	Fluorescence Lifetime Microscopy.
Fluorescence anisotropy	A trait of light, related to the spin of a photon, that parameterizes the orientation of emitted light relative to the electric field orientation of the excitation light.
Fluorescence lifetime, τ	The average time that a fluorophore spends in the excited-state.
Förster Distance, R_0	The distance between donors and acceptors where the FRET efficiency is 50% assuming an isotropic distribution in the dynamic regime.
FP	Fluorescent protein (e.g., Green Fluorescent Protein, GFP).
FRET	Förster's Resonance Energy Transfer.

<i>FRET efficiency, E</i>	The probability that a donor transfers its excited-state energy to a FRET acceptor.
<i>Hetero-FRET</i>	FRET between spectrally distinct donors and acceptors.
<i>Homo-FRET</i>	FRET between spectrally identical donors and acceptors.
<i>Intermolecular FRET</i>	FRET between a donor and acceptor on different molecules.
<i>Intramolecular FRET</i>	FRET between a donor and acceptor on the same molecule.
<i>IRF</i>	Instrument Response Function.
<i>Isotropic dynamic regime</i>	The energy transfer regime that assumes random donor and acceptor orientations and when their rotation is much faster than their fluorescent lifetime.
<i>Isotropic static regime</i>	The energy transfer regime that assumes random donor and acceptor orientations and when their rotation is much slower than their fluorescent lifetime.
<i>Non-radiative</i>	A mechanisms that result in the loss of excited state energy without emitting a photon.
<i>Orientation factor, κ^2</i>	A coefficient that describes the relative orientation and position of a FRET pair's transition dipoles.
<i>Overlap integral, $J(\lambda)$</i>	A coefficient that describes the spectral overlap of a FRET pair's donor emission spectrum and its acceptor's absorption spectrum.
<i>Peak count</i>	The number of photons emitted immediately after photoexcitation.
<i>PPI</i>	Protein-protein interactions.
<i>PSD</i>	Postsynaptic density.
<i>Quantum efficiency</i>	The detector's probability of detecting a photon at a specific wavelength.
<i>Quantum yield, Q</i>	The probability that a fluorophore will emit a photon when it is excited.
<i>Rotational correlation time</i>	An anisotropy decay time constant related to molecular rotation.
<i>TCSPC</i>	Time-correlated single photon counting.
<i>Time-resolved fluorescence anisotropy</i>	A method that monitors the change of fluorescence anisotropy as a function of time after photo-excitation.
<i>Transition dipole moment</i>	The multidimensional vector describing the electric charge separation created when a fluorophore's ground state electron is elevated to its excited state.