Spectral imaging and its use in the measurement of Förster resonance energy transfer in living cells

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Fürstere resonance energy transfer (FRET) imaging is a form of microscopy that allows the visualization of interaction between two fluorophores on a 1–10 nm scale. FRET imaging is based on measuring subtle changes in fluorescence that arises from nonradiative energy transfer from a “donor” fluorophore to an “acceptor.” Interest in applying FRET to image molecular interactions inside living cells has been growing, but has been hampered by technical problems encountered when accurate and precise measurements of fluorescence is necessary. Ironically, a requirement for FRET to occur, spectral overlap of donor emission with acceptor absorption makes it technically difficult to accurately and efficiently measure the fluorescent signals required to quantify FRET. Spectral imaging is a relatively new form of fluorescence light microscopy where
a fluorescent emission spectrum is recorded at each location in an image. Many of the technical problems encountered when acquiring FRET images can be eliminated by analyzing the data encoded in spectral images with an image analysis algorithm called linear unmixing. In this chapter, we will cover the theory of linear unmixing of spectral images, and describe how it can be used to acquire accurate FRET measurements.

8.1. Introduction

Förster resonance energy transfer (FRET) is a physical phenomena in which photon energy absorbed by a fluorophore is transferred by nonradiative dipole–dipole coupling to a nearby chromophore [1]. While this arcane phenomenon was first observed in the 1920s, there has been a reemergence of interest in FRET driven in part by the need for a microscope based assay for monitoring protein–protein interactions inside living cells. Commercial interest in FRET has also grown, driven by the conviction that FRET can be used effectively as the basis for developing new biosensors. Spectral imaging microscopy [2–4] is a relatively new form of multidimensional fluorescence microscopy that can potentially eliminate several of the obstacles one encounters in FRET imaging [5–7]. In spectral imaging, each picture element or pixel maps to a specific Cartesian coordinate within a sample and encodes the complex spectrum emitted from the population of fluorophores present at each specific location. Compared with more conventional forms of fluorescence microscopy in which the emission intensity is detected through a filter, spectral imaging holds the promise of potentially revealing information about the abundance and identity of the fluorescent species present. Vis-à-vis FRET, the emission spectrum of a donor should be attenuated and the emission spectrum of acceptors should be potentiated as energy transfer increases. Moreover, spectral imaging has the potential to detect
these FRET related spectral changes in a photon efficient manner, which is critical for effective live-cell imaging. One goal of this chapter is to describe how spectral imaging can be used to detect and measure FRET and to convey the strengths and weaknesses of the approach. FRET is not a rare event in biology as it is fundamental to the process of photosynthesis [8]. FRET can also occur, often unintentionally, upon the introduction of fluorophores into a biological milieu, particularly into crowded environments such as in membranes [9]. Fluorophore tagged membrane proteins, that are not commonly thought to specifically interact, often undergo non-specific FRET by virtue of their close proximity [10, 11]. Thus, it should be clear that the apparent abundance of fluorophores discerned by both conventional light microscopy through emission filters, as well as spectral imaging will be erroneous if energy transfer is occurring [12]. We call this the FRET problem. Accordingly, a second goal of this chapter will be to convey an appreciation of this fundamental problem in quantitative fluorescence microscopy, and to outline how spectral imaging can be used to measure the true abundance of fluorophores, even when FRET is occurring. Finally, we wish to state that this chapter is not intended to be a review of the literature addressing spectral imaging or how it has been used to measure FRET, rather, our goal is to convey a more intuitive appreciation of the spectral FRET method, the strengths and weaknesses of the approach, as well as to identify some of the current technical limitations that we are hopeful will soon be overcome, perhaps by some of our readers.

8.2. Understanding spectral imaging

As mentioned above, spectral imaging microscopy is a form of multidimensional fluorescent microscopy where a fluorescent emission spectrum is acquired at each coordinate location in the sample. This mode of imaging has been implemented for wide field, confocal, and two-photon laser scanning microscopy, and several excellent
reviews on spectral imaging microscopy have been published \[2, 4, 13–18\]. To illustrate and contrast spectral imaging with conventional filter-based imaging, we imaged three glass capillaries (Fig. 8.1). The first capillary (from top to bottom) contained Cerulean (10 \( \mu \)M) [19], a cyan spectral variant of green-fluorescent protein (GFP); the second contained Venus (10 \( \mu \)M) [20], a yellow spectral variant of GFP; and the third capillary contained a mixture of Cerulean and Venus at unknown concentrations. The monomeric variant of Cerulean and Venus were used to avoid nonspecific interactions between these proteins [21]. Two-photon microscopy with 900 nm infrared excitation light was used to acquire a spectral image of the three capillaries (Fig. 8.1). In panel A, we see 32 individual images of the capillaries. In the implementation of spectral imaging used to acquire these images (a Zeiss 510 NLO/META), the fluorescence emitted from a sample is spectrally dispersed by a diffraction grating and projected onto a 32-node photomultiplier tube array. Each of the images depicted in panel A was acquired from a single node of this photomultiplier array and represents a \(~10–11\) nm spectral slice of the emission spectrum. The center wavelength is indicated in yellow in the upper left corner of each image. You can see that none of the three capillaries contained samples that emit below 446 or above 596 nm. Between 457 and 510 nm, the top and bottom capillaries are emitting but not the center. Between 521 and 596 nm, all three capillaries emit. The emission wavelength and intensity information encoded in the stack of images depicted in Fig. 8.1A can be distilled down into a color-coded ‘spectral image’ shown in panel B. This image depicts how the sample would appear if observed by the human eye. The color at each pixel is based on the weighted mean of the emitted photon wavelengths, and the intensity is based on the number of photons detected at that pixel location. In panel C, we show the emission spectra for regions of interest (ROI) centered over the Cerulean capillary (blue solid line), Venus capillary (yellow solid line), the mixture capillary (green dashed line), and from an ROI centered over a region that did not have a capillary (background, red dotted line).
Fig. 8.1. Spectral Imaging. Panel A shows 32 individual images that comprise a spectral image of three capillaries containing from top to bottom 10 μM Cerulean, 10 μM Venus, and a mixture of Cerulean and Venus of unknown stoichiometry acquired on a Zeiss 510 META/NLO laser scanning confocal microscope with 900 nm two-photon excitation. Each image was acquired from a single node of a 32 node photomultiplier array, and represents sequential ∼10 nm portion of the emission spectrum that are detected on each node after being dispersed by a diffraction grating. The emission wavelength measured by each node is indicated in yellow. Panel B shows a color-coded representation of the images shown in panel A. The color (wavelength) is the intensity weighted average of the 32 individual emission wavelengths, and the brightness is proportional to the total number of photons detected at each pixel. Each capillary is labeled as is a background region. Size bar is 100 μm. Panel C shows emission spectra calculated measured from ROI centered over the Cerulean capillary, Venus capillary, mixture capillary or from a background region.
It is useful to monitor the background spectrum because it can indicate if there is any back-scattered excitation light or higher-order scattering reaching the detector [12], as well as an indicator of the presence of any autofluorescence in a sample. These spectra were calculated from the mean pixel intensity of the images in panel 1A for the specific ROI regions indicated, and plotted as a function of wavelength.

An important control for any quantitative spectral imaging experiment is to compare emission spectra of pure fluorophore samples obtained on the microscope with spectra obtained using a fluorimeter [22]. Differences between spectra measured with a spectral microscope and those obtained using a fluorimeter can occur due to (1) the presence of filters or dichroic beam splitters in the emission light path, (2) the spectral throughput of the objective and other optics in the light-path, (3) wavelength aliasing due to the limited bandwidth of the spectral detector, (4) misalignment of the dispersed emission beam and the spectral detector, and as mentioned previously, (5) contamination by backscattered excitation light including higher-order scattering. The impact of these potential artifacts must be considered when quantitative spectral imaging is desired. Because the emission spectra of the capillaries containing only Cerulean or Venus shown in panel C closely matched the emission spectra obtained for these samples as measured on conventional nonimaging fluorimeters, these artifacts could be eliminated from further consideration.

One of the major advantages of spectral imaging can be appreciated by noting that the emission spectrum of Cerulean in Fig. 8.1C completely overlaps the emission spectrum of Venus. At the excitation wavelengths used here, conventional filter-based imaging can never isolate Venus emission from Cerulean emission. To quantify the abundance of Venus in the presence of Cerulean using filter-based technology requires the existence and use of excitation wavelengths that excite Venus without exciting Cerulean. As we shall see shortly, this is not a requirement for quantifying Venus and Cerulean using spectral imaging.
Next, we will explore how spectral images change as a function of excitation wavelength. In Fig. 8.2A, we see spectral images of the same three capillaries depicted in Fig. 8.1 but now obtained with 820, 900, 920, and 940 nm two-photon excitation. With 820 nm excitation Cerulean is readily excited while Venus is poorly excited. In contrast, with 940 nm excitation, Cerulean is poorly excited while Venus is excited well. With 900 and 920 nm excitation, intermediate excitation behavior is observed. The Cerulean capillary emitted blue fluorescence at all excitation wavelengths. Similarly, the middle Venus capillary appeared green at all excitation wavelengths.

Fig. 8.2. Spectral Images can change with excitation wavelength. Spectral images of the same three capillaries depicted in Fig. 8.1 were imaged at four different excitation wavelengths: 820, 900, 920, and 940 nm (A). Normalized emission spectra (at all four excitation wavelengths) from ROI’s centered over the Cerulean capillary (B), the Venus capillary (C), and the mixture capillary (D) are co-plotted. Note that the color and normalized emission spectra do not change for a sample containing a single fluorophore. In contrast the color and normalized spectrum changed as a function of excitation wavelength in the capillary containing both fluorophores.
In contrast, the color of the Cerulean–Venus mixture capillary (bottom) appeared blue at 820 nm, blue-green at 900 and 920 nm, and green at 940 nm. The normalized emission spectra of the Cerulean (Fig. 8.2 B) and Venus (Fig. 8.2 C) capillaries did not change as a function of excitation wavelength, yielding the expected characteristic spectrum of these fluorophores. In stark contrast, the normalized emission spectra of the Cerulean–Venus mixture capillary was different at each excitation wavelength used (Fig. 8.2 D) with subtle differences between the emission spectra observed with 820, 900, and 920 nm excitation, and a dramatic difference with 940 nm excitation. These differences can be understood if one considers that while the shape of the emission spectrum of a fluorophore is typically not a function of excitation wavelength, the emission intensity of a fluorophore is a function of excitation wavelength [23]. The emission intensity is also a function of the fluorophore’s abundance and the intensity of the excitation light source [23]. With regard to the Cerulean and Venus mixture capillary shown in Fig. 8.2, the concentrations of the two fluorophores and their relative abundance were obviously the same at each excitation wavelength (they are after all the same sample). In this demonstration, however, excitation energy was not the same at each excitation wavelength (though the excitation energy for the mixture capillary was the same for the Cerulean and Venus capillaries), and it is known that Cerulean and Venus have significantly different absorption spectra [19, 20] and two-photon absorption cross sections [12]. At a Venus concentration far less than 1–3 mM, there should not be any appreciable FRET between Cerulean “donors” and Venus “acceptors” as a result of molecular crowding [23]. Thus, for the mixture capillary, the complex spectrum observed should be a linear sum of the Cerulean and Venus emission spectrum (a function of excitation light intensity and their absorption coefficients at each excitation wavelength) weighted by their respective abundance. A mathematical formalism, called linear unmixing, based on this idea of the abundance-weighted summation of individual fluorophore spectra, can be used to
measure the concentration of individual fluorophores in a mixed population, but only if energy transfer between different fluorophores is not occurring [12, 24].

8.2.1. Linear unmixing and its limitations

As mentioned previously, the complex emission spectrum $F_i(\lambda)$ of samples containing multiple fluorophores is assumed to be the linear sum of individual component spectra $F_1(\lambda)$, $F_2(\lambda)$, $F_3(\lambda)$, weighted by their abundance $x_1, x_2, x_3$. Let $F_1^i(\lambda)$ and $F_2^i(\lambda)$ be the reference emission spectra of pure samples of fluorophore (e.g., Cerulean and Venus). The term reference emission spectra is used because these spectra describe the emission at excitation wavelength $\lambda_{\text{ex}}$ of a defined concentration of fluorophore (e.g., 10 $\mu$M) acquired using the same excitation light intensity as was used to acquire an emission spectra of an unknown sample mixture. Under these conditions, the shape and magnitude of the fluorophore mixture spectra will be:

$$F^i(\lambda) = x_1 F_1^i(\lambda) + x_2 F_2^i(\lambda) + x_3 F_3^i(\lambda) + \ldots \tag{8.1}$$

If reference emission spectra of a set of pure fluorophores are available, and if an emission spectrum of an unknown mixture of any combination of these fluorophores is acquired under the same conditions, this equation can be used to determine the abundance of the different fluorophores in the mixture. The use of this equation to determine the abundance of the fluorophores present is called linear unmixing. To illustrate the basis of linear unmixing, we will first use this equation to analyze the emission spectra of the mix capillary containing an unknown mixture of Cerulean and Venus depicted in Fig. 8.1. The unmixing approach we describe will utilize reasonable guesses for the values of $x_1$ (representing the abundance of Cerulean) and $x_2$ (representing the abundance of
Venus) in the linear unmixing equation, and then use these guesses to generate spectra to compare with actual data. In Fig. 8.3A, we see a 10 μM reference emission spectrum of Cerulean and Venus, as well as a spectrum of our unknown mixture. All three spectra were acquired at the same excitation wavelength (900 nm) at the same laser intensity. Thus, all of the requirements for linear unmixing outlined above have been met. In Fig. 8.3 B, the linear unmixing equation is used to model complex emission spectra of mixtures containing 10 μM Venus and either 14 μM Cerulean (blue dashed trace), 10 μM Cerulean (white dotted trace), or 6 μM Cerulean (red dashed trace). These models can be compared with the actual emission spectra from the mixture capillary (green circles). The model represented by the white trace is the closest match to the data set, particularly below 500 nm. Next, we explore models where we hold the Cerulean concentration at 10 μM and vary the Venus concentration (Fig. 8.3 B). In the blue trace, Venus is held at 10 μM; in the white trace, it has been reduced to 6 μM; and in the red trace, it has been reduced further to 2 μM. We can now see that while all three models are reasonable matches to the data, the blue model overestimates emissions between 500 and 600 nm and the red model underestimates emissions from this same spectral region. In contrast, the white model (10 μM Cerulean, 6 μM Venus) matched the experimental data well.

In this example of linear unmixing, the value $x_2$ was first held constant while the value of $x_1$ was varied. Next, the value of $x_1$ was held constant and the value of $x_2$ was varied. This illustration was used to provide an intuitive example of how linear unmixing finds values for the abundance of each fluorophore that are consistent with a given complex emission spectrum. In practice, linear unmixing software utilizes curve fitting algorithms [25] to rapidly find values of $x_1$, $x_2$, ... which can generate spectra that best match the experimental data. These calculations are repeated for every pixel in an image. Ultimately, separate images are created representing the abundance of each fluorophore present. An example set of images generated by linear unmixing of the data set presented in
Fig. 8.1 is shown in Fig. 8.4. In panel A, we show the Cerulean channel image (left) generated by linear unmixing, the Venus channel image (middle), and an overlay image of these two channels (right). In the Cerulean channel image, it is clear that the top and bottom capillaries each contain Cerulean at the same concentration. The Venus channel image revealed that the middle and lower capillaries each contain Venus, but the concentration of Venus in the middle capillary was higher than in the lower capillary. A more quantitative view of this data can be seen by plotting the intensities (calibrated to concentration) of a line scan across these images (Fig. 8.4 B). The dashed red line in panel A depicts the position of the line scan across all three capillaries. Because known concentrations of Cerulean and Venus were used in the top and middle capillaries (10 \mu M), the intensities measured for these capillaries (blue for the Cerulean channel, yellow for the Venus channel) could be calibrated to that concentration of fluorophore (dotted black line in Fig. 8.4 B). In essence, the Cerulean and Venus capillaries can be considered as calibration controls for interpreting the signals obtained by linear unmixing of the mixture capillary. In the line

Fig. 8.3. The basis of linear unmixing. Unnormalized emission spectra of the three capillaries are shown in panel A. The linear unmixing algorithm is based on the hypothesis that a complex emission spectrum (an emission spectrum of a sample containing 2 or more fluorophores) can be modeled as a weighted sum of the emission spectra of the individual fluorophores present. Thus, the Mix spectrum should be the sum of the Cerulean and Venus spectra after each is multiplied by an abundance factor. In panel B the abundance factor for Venus is held at a value of 1, while the value of the Cerulean abundance factor is varied from 0.6 to 1.4. Because the Cerulean and Venus capillaries each contained 10 \mu M of fluorophore, an abundance range of 0.6–1.4 corresponds to a concentration range of 6–14 \mu M. In panel C the Cerulean abundance factor is held at a value of 1 (10 \mu M) while the abundance factor for Venus was altered from 0.2 to 1 (2–10 \mu M). Note that when the Cerulean spectrum was multiplied by 1 (corresponding to 10 \mu M) and added to the Venus spectrum multiplied by 0.6 (corresponding to 6 \mu M), the linear unmixing model matched the complex spectrum measured for the mix capillary.
scan trace corresponding to the position of the mixture capillary (the far right), we see a Cerulean signal (blue trace) of 10 μM (dotted line), and a Venus signal (yellow trace) of 6 μM (dashed line). These values are consistent with the values generated by spectral analysis in Fig. 8.3.

Fig. 8.4. Linear unmixing with curve fitting algorithms. Linear unmixing image processing software utilize least-square curve fitting routines to fit a spectra from each pixel of a spectral image to the linear unmixing equation and predict values for the abundance factors for each fluorophore. These abundance factors for Cerulean and Venus are then multiplied by the concentration of the individual reference spectrum samples (10 μM) to produce a Cerulean Channel image (blue), a Venus Channel image (yellow), and an overlay image (panel A). Now it can be seen that the top and bottom capillaries have the same concentration of Cerulean. The middle and bottom capillaries both have Venus, but at different concentrations. The dashed red line indicates the location of a line scan across the two image channels. Line scan plots for each channel are useful for measuring the actual concentration of fluorophores observed in a sample, and are plotted in panel B.
Before describing how spectral imaging in conjunction with linear unmixing can be used to measure FRET, we should first consider what limitations exist for successful quantitative linear unmixing of spectral images. First and foremost, linear mixing will fail if the emission spectra of the fluorophores present in a sample are nearly indistinguishable. As a rule of thumb, if two fluorophores have emission maxima separated by at least 10 nm, conditions can be found where the emissions of those fluorophores can be separated by linear unmixing [2]. However, even if the fluorophores emission spectra are clearly different, quantitative linear unmixing can still fail if the emission from one fluorophore is significantly brighter than that from the others [24]. An obvious question is “At what point will quantitative linear unmixing fail due to differences in fluorophore intensity?” This question was addressed in a study using mixtures of purified CFP and YFP at different defined molar ratios (1:9, 1:1, and 9:1) and at different emission intensity ratios [24]. Spectral images of these mixtures were acquired and used to determine how well the CFP:YFP ratio predicted by linear unmixing matched the specific samples. It was found that linear unmixing could accurately predict the correct CFP:YFP ratio for all three mixtures. Linear unmixing yielded the correct molar ratios even under conditions where one fluorophore was 90 times brighter than the other. It must be pointed out, however, that the variance in the fluorophore ratio measurement observed under these extreme differences in intensity was quite large. Because of signal-to-noise limitations in spectral microscopes in conjunction with limitations in the dynamic range of data acquisition instrumentation (typically 12 bits), it is difficult to use linear unmixing to accurately separate signals whose intensities are greater than an order of magnitude apart. Essentially, if the dynamic range of a spectral detector is set to capture the peak emission of the brightest fluorophore (without clipping the signal), that dynamic range will be poorly matched to accurately measure the emission of a much dimmer fluorophore. Under these conditions, linear unmixing will yield accurate measurements for the
abundance of the bright fluorophore, but the accuracy of the dim fluorophores abundance may be compromised. This problem is compounded by the fact that it is often difficult to control the abundance of fluorophores in a biological experiment, and even more difficult to control their relative abundance in living cells. The relative brightness of the light emitted from the fluorophores present in a mixed sample is a function of their absorption spectra (and thus the excitation wavelength), their quantum yields, as well as their relative abundance. Thus, one solution to this problem is to empirically select an excitation wavelength for a given sample that yields a complex emission spectrum whose shape is significantly different from the emission spectrum of the individual fluorophores alone. To illustrate this approach, linear unmixing was applied to the four spectral images depicted in Fig. 8.2. The same set of capillaries was imaged at four different excitation wavelengths. At 820 nm, Cerulean was excited well but Venus was barely excited. At 900 and 920 nm, both were excited well, and at 940 nm Venus was excited more efficiently than Cerulean. In Fig. 8.5A, we replot the emission spectra of the Cerulean capillary (blue) and the Mix capillary (red) at each excitation wavelength, but here each spectrum is normalized to the intensity of its Cerulean emission peak at 478 nm. Because the Cerulean spectrum is overlaying the Mix spectrum in these graphs, the visible part of the Mix spectrum (red) represents the portion of the Mix spectrum resulting from Venus emission. With 820 nm excitation, the Mix emission spectrum is almost identical to the Cerulean spectrum (note the token red signal between 500 and 600 nm). In contrast, the Mix spectra resulting from 900, 920, and 940 nm excitation were all significantly different than the Cerulean alone spectrum (blue), as well as from a Venus alone spectrum (data not shown). Furthermore, at these three excitation wavelengths the fractional contribution of Cerulean and Venus to the Mix spectra emissions was all within an order of magnitude of each other. Thus, we would predict that the 900, 920, and 940 spectral images (but not the 820 nm spectral image) will yield accurate estimates of the abundance of Cerulean and
Fig. 8.5. The effects of excitation wavelength on linear unmixing. In panel A we see the Cerulean normalized emission spectra of the mixture capillary (red) overlaid with the normalized emission spectrum of Cerulean alone (blue) at the four different two-photon excitation wavelengths used in Fig. 8.2. Note that with 820 nm excitation the mix spectrum was not significantly different than the Cerulean spectrum, while with 900, 920, and 940 nm excitation they were different. When the four spectral images depicted in Fig. 8.2 were processed by linear unmixing they produced the four unmixing channel-sets depicted in panel B. The unmixed images with 900, 920, and 940 nm excitation were all identical, while with 820 nm excitation erroneous results were obtained. In panel C the results of linear unmixing of a spectral image of the same samples, obtained with one-photon excitation at 458 nm is shown. These images looked identical to the images obtained with 900, 920, and 940 nm two-photon excitation, and a line scan across the one-photon image confirmed this conclusion (compare panel D with Fig. 8.4B).
Venus with linear unmixing analysis. To test this prediction, the linear unmixing algorithm was applied to these data sets (Fig. 8.5 B). As expected, the Cerulean and Venus unmixing channels generated by linear unmixing of the 900, 920, and 940 nm excitation spectral images (as well as the overlay channel) were virtually identical. In contrast, the unmixing of the 820 nm spectral image dramatically underestimated the abundance of Venus in the Mix capillary. Clearly, judicious selection of excitation wavelength is important for quantitative linear unmixing of spectral images, and the guidelines mentioned earlier can help in the selection of those wavelengths.

8.2.2. Single- versus multiphoton spectral imaging

Can conventional one-photon excitation be used to acquire spectral images for quantitative linear unmixing, and if so, are there any advantages or disadvantages of two-photon excitation over one-photon? Fig. 8.5C depicts the Cerulean, Venus, and overlay channels generated by linear unmixing of a spectral image of our three capillaries acquired with one-photon excitation using the 458 nm laser line of an argon laser. These images were nearly identical to the linear unmixing images generated for the same sample excited with 900, 920, and 940 nm two-photon excitation (Fig. 8.5B). Similarly, a line scan across this unmixed one-photon image (red dotted line in Fig. 8.5C) was equivalent to a line scan across a linear unmixed two-photon image (compare Fig. 8.5D with Fig. 8.4B). Thus, we conclude that quantitative linear unmixing can work well with spectral images acquired with either one-photon or two-photon excitation. There are differences between two-photon and one-photon microscopy that may impact on their suitability for quantitative spectral imaging microscopy. First, the spectral band involved in two-photon excitation (typically from 700 to 1000 nm) is well separated from the spectral region where most commonly used fluorophores emit (400–600 nm). Thus, with two-photon
excitation, the whole emission spectrum of a sample can be easily collected. In contrast, because the wavelength for one-photon excitation often overlaps with a sample's emission spectrum a portion of the emission spectrum typically is lost. Even when one-photon excitation is left shifted from emission, the emission filters and dichroic beam splitters involved in preventing one-photon excitation from reaching the spectral detector can attenuate the emission signal over a portion of the emission spectrum. The ability to collect a full emission spectrum potentially translates into a more photon-efficient microscope, and this is key for successful live cell imaging. Second, because the lasers used in most two-photon microscopes are tunable, it is relatively easy and straightforward to select an excitation wavelength that is optimized for the specific fluorophores present in a sample as well as to their stoichiometry in the sample. In contrast, one-photon laser scanning confocal microscopes typically use lasers with a limited number of fixed laser lines and are thus poorly suited for optimizing the excitation wavelength for spectral imaging. Recently, however, new white light laser sources have become available which can support one-photon excitation over a broad range of wavelengths. Wide-field conventional fluorescence microscopes typically use broad wavelength light sources in conjunction with excitation filters. Although the excitation wavelength can be optimized for a particular sample, this would require having a large set of different excitation filters on hand that can be rapidly changed to optimize excitation. This is typically not available. One alternative is to replace excitation filters with a tunable programmable excitation filter. This could potentially allow easy and convenient matching of one-photon excitation to a particular sample. Third, the depletion of fluorophores by bleaching with two-photon excitation is typically much slower than with one-photon excitation because with two-photon excitation there is no fluorophore excitation occurring above and below the image plane [26]. If different fluorophores are bleached at different rates in a sample, emission spectra will change as a function of acquisition time. This will greatly complicate the
quantitative analysis of spectral images. Alternatively, for some fluorophores, multiphoton excitation can have different selection rules than one-photon excitation. Thus, it may be possible with two-photon excitation to directly populate a fluorophores triplet state. If this happens, the bleach rate, at least in the image plane, can be much faster [27]. Finally, two-photon absorption cross-sections are in general broader than that of one-photon absorption spectra. Thus, with two-photon excitation it is relatively easy to find wavelengths that excite multiple fluorophores simultaneously.

8.2.3. Baseline correction and emission wavelength selection

We have demonstrated how the selection of the excitation wavelength for acquiring spectral images can influence how accurate linear unmixing can be in predicting the abundance of fluorophores present in a mixed sample. For this reason, there is a great advantage in having a spectral microscope with a large, preferably continuous selection of excitation wavelengths so that the relative intensities of the fluorophores present can be modulated to optimize the signal to noise ratio obtainable for all fluorophores present. Two-photon microscopy is well suited for this task. We will now address two other factors that can influence how quantitative the results of linear unmixing can be: (1) baseline corrections of reference spectra, and (2) the wavelength range and resolution of the spectral detector used. The Cerulean, Venus, and Mix capillary spectra shown in Fig. 8.3A all have baseline intensities of zero (seen below 425 and above 625 nm). In practice, the baseline of spectra obtained using a spectral microscope will have a positive offset from zero as a result of dark noise and instrumentation noise that is added to the true signal. If these offsets are not corrected before applying the linear unmixing algorithm, significant errors can be introduced [24]. Baseline correction can be implemented in hardware by properly configuring the gain and offset of the spectral detector. Alternatively, Baseline corrections can also be implemented with
postprocessing by measuring the offset for the spectra and subtracting it from the signals. A third approach for Baseline correction is only partially effective. In this approach, in addition to using reference spectra for each fluorophore present in a sample, the spectrum of the background signal (e.g., see red trace in Fig. 8.1 C) can also be used by the linear unmixing algorithm. This will produce, in addition to image channels for each fluorophore, an additional image channel of the background which will not be used in any subsequent overlay images. While this approach can effectively remove the offset from the sample image, it does not remove offset from the individual fluorophore unit reference spectra. These offsets must be corrected before the linear unmixing algorithm is applied. For the best photon efficiency, the wavelength range of a spectral detector used for linear unmixing should cover the full range of a samples complex emission spectrum. For the sample depicted in Fig. 8.1, this range would be from 450 to 600 nm. To effectively implement the Baseline corrections described earlier, however, this range should be extended a bit (i.e., from 400 to 650 nm), so that the true baseline can be defined. In the example depicted in Fig. 8.1, the spectral detector used captured a spectral image comprised of 32 separate images (Fig. 8.1 A). Theoretically, linear unmixing of a sample containing \( n \) fluorophores will require a spectral image composed of at least \( n \) spectral points (or subimages) [28], and those images must cover the portions of the spectrum where the individual fluorophores emission spectra differ. Thus, theoretically, Cerulean and Venus emissions from the mixture capillary could be accurately separated using the linear unmixing algorithm if applied to a spectral image composed of only two spectral points; one recording from 450 to 500 nm, and the other covering 500–600 nm. Although the theory describing the influence of detector wavelength range, and resolution on linear unmixing has been described [28], experimental confirmation would be a welcome addition to the literature.
8.3. What is the spectral signature of FRET?

Finally, an underlying assumption of quantitative linear unmixing is the absence of mechanisms that can selectively alter the emission spectra of the individual fluorophores present in a sample. Because FRET shifts energy from donor to acceptor fluorophores, FRET activity will decrease the magnitude of a donors emission spectrum and increase the magnitude of the acceptors emission spectrum. In practice, this means that quantitative linear unmixing of a sample with a FRET efficiency greater than zero will fail to yield accurate estimates of the abundance of donors and acceptors present. This has been confirmed experimentally [12]. This is both good and bad news. The bad news is that studies that have used quantitative linear unmixing of spectral images without regard to the possibilities that the fluorophores present in their sample might be transferring energy by FRET may be based on erroneous unmixing data. The good news is that because linear unmixing will yield different results in the presence or absence of FRET, this behavior can be used to measure FRET from spectral data sets.

It has been shown that the linear unmixing equation used to estimate the abundance of fluorophores in a sample will fail if FRET is occurring between the fluorophores in a sample. Devising a strategy for using spectral imaging to measure FRET requires a quantitative understanding of how the shape and magnitude of a complex emission spectrum changes with FRET. A FRET adjusted spectral equation which accurately describes the shape and magnitude of the complex emission spectrum \(F^i(\lambda)\) from a population containing two fluorophores that might be transferring energy by FRET is:

\[
F^i(\lambda) = d(1 - E_D)F^i_{D,\text{ref}}(\lambda) + aF^i_{A,\text{ref}}(\lambda) + dE_D \frac{Q_A}{Q_D} k_i F^i_{A,\text{ref}}(\lambda)
\]

(8.2)
where $d$ and $a$ represents the donor and acceptor fluorophores concentrations, $F_{D,\text{ref}}^i(\lambda)$ and $F_{A,\text{ref}}^i(\lambda)$ are the reference emission spectra of the donor and acceptor at the same concentration as measured on the spectral microscope being used. It is important to note that these spectra are a function of the excitation wavelength, $\lambda_{\text{ex}}^i$. $E_D$ is the apparent FRET efficiency of the sample, that is the fraction of donor excitation events that results in energy transfer to an acceptor for all of the donors present at a specific pixel in an image. $Q_D$ and $Q_A$ are the quantum efficiency of the donor or acceptor, and $k^i$ is a transfer factor (see the Appendix) [12]. This equation can be understood intuitively by realizing that the complex emission spectrum of a population of donor and acceptor fluorophores (that may be transferring energy by FRET) is comprised of the sum of three parts. The first part describes the fluorescent emission from directly excited donor fluorophores in a sample:

$$d(1 - E_D)F_{D,\text{ref}}^i(\lambda)$$  \hspace{1cm} (8.3)

It is the reference emission spectrum of the donor, multiplied by its abundance, but attenuated by the fraction of donors that are not transferring energy by FRET. The second part of the equation describes emission from directly excited acceptors:

$$aF_{A,\text{ref}}^i(\lambda)$$  \hspace{1cm} (8.4)

It is simply the reference emission spectrum of the acceptor, multiplied by the abundance of acceptor. Finally, the last part of the equation represents the energy transferred from directly excited donors to acceptors by FRET, and then emitted as acceptor fluorescence:

$$dE_D \frac{Q_A}{Q_D} k^i F_{A,\text{ref}}^i(\lambda)$$  \hspace{1cm} (8.5)

It is the emission spectrum of the acceptor multiplied by the abundance of the donor (attenuated by the fraction of donor emissions that actually result in FRET), then multiplied by a factor
\[ Q_A/Q_D \cdot k^i \] that equates how the emission intensity increase of the acceptor corresponds to the attenuation of the donors emission. This factor is equal to the extinction coefficient ratio of the donor and acceptor at the used excitation wavelength, \( \lambda_{ex}^i \), see appendix. If FRET is not occurring in a sample (i.e., \( E_D = 0 \)), the whole equation reduces to:

\[ F^i(\lambda) = dF_{D,\text{ref}}^i(\lambda) + aF_{A,\text{ref}}^i(\lambda) \quad (8.6) \]

This is the same equation as the standard linear unmixing equation for two fluorophores.

Standard linear unmixing of a spectral image of a sample composed of two fluorophores yields a measure of the concentration of each fluorophore present for each pixel. If FRET is occurring, linear unmixing will produce an apparent donor concentration \( d_{\text{apparent}} \) that underestimates the true donor concentration \( d \) by a factor of \( 1 - E_D \):

\[ d_{\text{apparent}} = d(1 - E_D) \quad (8.7) \]

Linear unmixing will also produce an apparent acceptor concentration \( a_{\text{apparent}} \) that will over-estimate the true abundance of the acceptor \( a \):

\[ a_{\text{apparent}} = a + dE_D \frac{Q_A}{Q_D} k^i F_{A,\text{ref}}^i(\lambda) \quad (8.8) \]

A prediction based on these two equations is that the value of \( d_{\text{apparent}} \) will remain constant for spectral images taken at different excitation wavelengths, while values for \( a_{\text{apparent}} \) can change with different excitation wavelength (because \( a_{\text{apparent}} \) is a function of excitation wavelength) but only when FRET is occurring. This prediction has been experimentally verified [12], and is in essence the spectral signature of FRET. This behavior can also be used as a simple test to determine if FRET is or is not occurring in a sample. Spectral images are taken at two excitation wavelengths that yield good signal to noise ratios for both donors and acceptors.
8.3.1. Strategies for measuring FRET using spectral imaging

Strategies for using spectral imaging to measure FRET begin with the realization that the FRET adjusted spectral equation described above has three independent variables, $d$ the abundance of donor, $a$ the abundance of acceptor, and $E_D$ the FRET efficiency. In contrast, at a single excitation wavelength, quantitative linear unmixing produces only two observables, $d_{\text{apparent}}$, the apparent donor concentration in a sample and $a_{\text{apparent}}$, the apparent acceptor concentration. Thus, with three unknown variables and two observables there can and will be multiple combinations of values for $d$, $a$, and $E_D$ that can produce the same complex emission spectrum observed in a sample [7, 12]. Strategies for using spectral imaging to measure $d$, $a$, and $E_D$ therefore must either reduce the number of independent variables in the FRET adjusted spectral equation, or produce at least one additional observable to supplement the two values measured by spectral unmixing.

One way to reduce the number of independent variables in the FRET-adjusted spectral equation is to use samples with a fixed donor-to-acceptor ratio. Under these conditions, the values of $d$ and $a$ are no longer independent, but rather the concentration of $d$ is now a function of $a$ and vice-versa. This approach is typical for the situation of FRET-based biosensor constructs. These sensors normally are designed to have a donor fluorophore attached to an acceptor by a domain whose structure is altered either as a result of a biological activity (such as proteolysis or phosphorylation), or by its interaction with a specific ligand with which it has high affinity. In general, FRET based biosensors have a stoichiometry of one...
donor to one acceptor. FRET activity for biosensors is typically reported as a “FRET-ratio” index value rather than as a FRET efficiency. This is unfortunate because FRET indices are often nonlinear [11, 29]. For this example, where a single donor is linked to a single acceptor, the FRET-adjusted spectral equation will have only two variables, \(x\), the concentration of the donor and acceptor \((x = d = a)\), and \(E_D\), the FRET efficiency. Linear unmixing will yield:

\[
d_{\text{apparent}} = x(1 - E_D)
\]

\[
a_{\text{apparent}} = x\left(1 + E_D \frac{Q_A}{Q_D} k_i\right)
\]

In this situation because these equations have only two variables \(E_D\) and \(x\), and two observables \(d_{\text{apparent}}\) and \(a_{\text{apparent}}\) this problem is determined, and these two simultaneous equations can be solved for \(E_D\) and \(x\):

\[
E_D = \frac{a_{\text{apparent}} - d_{\text{apparent}}}{a_{\text{apparent}} + d_{\text{apparent}} \frac{Q_A}{Q_D} k_i}
\]

\[
x = \frac{a_{\text{apparent}} + d_{\text{apparent}} \frac{Q_A}{Q_D} k_i}{1 + \frac{Q_A}{Q_D} k_i}
\]

Using these equations, the values of \(E_D\) and \(x\) can be calculated from the measured values of \(d_{\text{apparent}}\) and \(a_{\text{apparent}}\). The advantage of this approach is that it is simple and only requires a microscope capable of producing spectral images, as well as software that can perform quantitative linear unmixing to produce \(d_{\text{apparent}}\) and \(a_{\text{apparent}}\) images, and then process those images according to the equations listed earlier to yield \(x\) and \(E_D\) images. The main limitation of this approach is that it will only work for samples with fixed donor-to-acceptor stoichiometry such as FRET-based biosensors.
In many biological applications of FRET, the donor to acceptor ratio of a specimen is not fixed, and unknown. Even with samples that do have a fixed donor to acceptor ratio such as in a biosensor, it is possible that cellular activities such as proteolysis might alter this ratio in unknown ways. Under these circumstances, an occult change in donor to acceptor ratio might be misinterpreted as a change in the biological activity that the biosensor was designed to monitor. Obviously, knowing the real donor to acceptor ratio in a sample as well as the actual concentrations of these fluorophores can be very useful for avoiding these types of errors, as well as for interpreting the meaning of FRET. For example, if the donor concentration in a FRET experiment is known to be much greater than the acceptor concentration, a low FRET efficiency would be expected (because most donors will not have even a single acceptor to interact with) even if the molecule that the donor fluorophore is attached to does have a high affinity for the acceptor tagged component. In contrast, a low FRET efficiency when the donor concentration is known to be much lower than the acceptor concentration might indicate that the molecule with an attached donor does not interact with the acceptor tagged molecule (though other reasons for having a low FRET efficiency must also be considered). Donor and acceptor stoichiometry and concentrations can be obtained by acquiring a spectral image in conjunction with FRET efficiency measurements obtained by another imaging method. Typically, FRET efficiency can be measured by monitoring the sensitized emission before and after acceptor bleaching [30–34], or by monitoring the fluorescence lifetime of the donor in the presence or absence of acceptors [34–38]. Regardless of the auxiliary methods used to measure the FRET efficiency hybrid approaches reduce the number of independent variables in the FRET adjusted spectral equation from three \((d, a, \text{ and } E_D)\) to two \((d \text{ and } a)\), by independently finding the value of the FRET efficiency \((E_D)\) at each location in an image. The benefit of this approach is that under these circumstances, the true donor \((d)\) and acceptor \((a)\) concentrations can be calculated at each pixel in a spectral image using the
independently measured FRET efficiency \((E_D)\), in conjunction with the \(d_{\text{apparent}}\) and \(a_{\text{apparent}}\) values produced by linear unmixing using the following equations:

\[
d = \frac{d_{\text{apparent}}}{1 - E_D} \tag{8.11a}
\]

\[
a = a_{\text{apparent}} - \frac{d_{\text{apparent}}}{1 - E_D} E_D Q_A \frac{k^i}{Q_D} \tag{8.11b}
\]

One criticism of this approach, however, is that in addition to requiring the specialized hardware for obtaining spectral images, additional instrumentation is often required to measure the FRET efficiency. Furthermore, the limitations specific to the FRET method used in conjunction with spectral imaging will also apply to this hybrid approach.

As mentioned previously, strategies for using spectral imaging to measure FRET (as well as the concentrations of donors and acceptors) must either reduce the number of variables in the FRET adjusted spectral equation, or increase the observables measured using linear unmixing. All of the methods mentioned so far work by reducing the number of variables in the spectral equation. Next, a method will be described whose approach is to measure an additional observable by acquiring two spectral images that will each be analyzed by linear unmixing. At each excitation wavelength: \((\lambda_{\text{ex}}^1\) and \(\lambda_{\text{ex}}^2\)), a spectral image of the sample is acquired. Reference spectra of samples containing known concentrations of donor and acceptor are also acquired at the same excitation wavelengths and excitation intensities. Quantitative linear unmixing of the first spectral image obtained at \(\lambda_{\text{ex}}^1\) using the references spectra also acquired at \(\lambda_{\text{ex}}^1\) will yield two observables:

\[
d^l_{\text{apparent}} = d(1 - E_D) \tag{8.12a}
\]

\[
a^l_{\text{apparent}} = a + dE_D Q_A \frac{k^i}{Q_D} \tag{8.12b}
\]
Linear unmixing of the spectral image acquired at $\lambda_{\text{ex}}^2$ will also yield two observables:

$$d_{\text{apparent}}^2 = d(1 - E_D)$$  \hspace{1cm} (8.13a)

$$a_{\text{apparent}}^2 = a + dE_D \frac{Q_A}{Q_D} k^2$$  \hspace{1cm} (8.13b)

Note that because the value of $d_{\text{apparent}}$ is not a function of excitation wavelength, the same values for $d_{\text{apparent}}$ should be observed at both excitation wavelengths ($\lambda_{\text{ex}}^1$ and $\lambda_{\text{ex}}^2$). In contrast, the values measured for $a_{\text{apparent}}^1$ and $a_{\text{apparent}}^2$ will be different if $k^1 \neq k^2$. Thus, acquiring spectral images of a sample at two different excitation wavelengths can allow three observables to be observed with linear unmixing: $d_{\text{apparent}}, a_{\text{apparent}}^1,$ and $a_{\text{apparent}}^2$. Because the FRET adjusted spectral equation has three unknowns ($d$, $a$, and $E_D$) and spectral imaging at two excitation wavelengths produces three observables, this problem is determined, and the three simultaneous equations describing these observables can be solved for $E_D, d,$ and $a$:

$$E_D = \frac{\Delta a}{\Delta a + d_{\text{apparent}} \Omega \Delta k}$$  \hspace{1cm} (8.14a)

$$d = \frac{\Delta a + d_{\text{apparent}} \Omega \Delta k}{\Omega \Delta k}$$  \hspace{1cm} (8.14b)

$$a = \frac{k^2 a_{\text{apparent}}^1 - k^1 a_{\text{apparent}}^2}{\Delta k}$$  \hspace{1cm} (8.14c)

Where the following substitutions have been made:

$$\Delta a = a_{\text{apparent}}^2 - a_{\text{apparent}}^1$$  \hspace{1cm} (8.14d)

$$\Omega = \frac{Q_A}{Q_D}$$  \hspace{1cm} (8.14e)

$$\Delta k = k^2 - k^1$$  \hspace{1cm} (8.14f)
Using these equations the values of $E_D, d, and a$ can be calculated from the measured values of $d_{\text{apparent}}, a^{1}_{\text{apparent}}, and a^{2}_{\text{apparent}}$. The advantage of this approach is that it only requires a microscope capable of producing spectral images as well as software for performing quantitative linear unmixing. Furthermore, not only does it not require knowledge of the donor–acceptor stoichiometry of a sample, it will actually yield this information. Another advantage of this approach is that the FRET measurements are based on changes in both the donors’ fluorescence signal as well as the acceptors’. Most other FRET methods are based on monitoring changes in either the donors’ fluorescence or the acceptors’ and are thus susceptible to artifacts caused by nonspecific quenching or de-quenching. The major limitation of this approach is that it requires acquiring two spectral images at two different excitation wavelengths. Thus, a light source with a choice of several excitation wavelengths is required. Furthermore, if cell components move between the acquisition of the first and second spectral images, motion artifacts will introduce errors in the calculations. This is true for any analysis whose calculations involve more than one image. Finally, any variance in the tuning, bandwidth, or power of the light source used in this approach will also necessitate obtaining reference spectra at the exact same settings. This typically means measuring reference spectra before tuning to the second wavelength.

8.3.2. Measuring FRET from spectral images: sRET

Many of the strategies for measuring FRET from spectral images that were mentioned above have been implemented to study FRET. We will now cover sRET [12], a specific implementation that uses the last approach where FRET is measured from a pair of spectral images collected at different excitation wavelengths. Recently, the sRET approach has been extended to explicitly consider paired and unpaired fluorophores, the impact of incomplete labeling (or for fluorescent proteins fractional maturation), and the
implementation of a calibration procedure that does not require purified fluorophores [39]. While it is important to realize that all of the approaches mentioned in the previous section are valid and may have specific advantages for particular biological problems, we have chosen to highlight the sRET approach because: (1) it can measure FRET efficiencies from samples with unknown donor to acceptor stoichiometry, (2) in addition to measuring the FRET efficiency it also measures the abundance of donors and acceptors, (3) it does not require the destruction of the sample (i.e., with sensitized emission by acceptor bleaching), (4) It measures FRET solely based on spectral images of the donor and acceptor, and (5) sRET has been shown to yield the same FRET efficiencies as obtained by fluorescence lifetime imaging (FLIM–FRET) and by a variant of the three-cube method (E-FRET) [40].

Spectral images of cells transfected with DNA encoding either a fluorescent protein construct that has a low FRET efficiency (CTV) or a high FRET efficiency (C5V) [12] were acquired with two photon excitation at 890 and 940 nm. Spectral images of capillaries containing either 7.8 μM Cerulean or Venus were also acquired at these excitation wavelengths to serve as reference spectra for linear unmixing of these spectral images, as well as to measure \( k^1 \) at \( \lambda^1_{\text{ex}} = 890\text{nm} \) and \( k^2 \) at \( \lambda^2_{\text{ex}} = 940\text{nm} \) for the Cerulean–Venus pair. Values measured for \( k^i \) will be specific for a particular fluorophore pair, and for the microscope used to measure spectra. It can also change as a function of the intensity, wavelength, and bandwidth of the light source used, and is therefore best measured along with the sample. These values, as well as the quantum efficiencies of Cerulean \( (Q_D = 0.62) \) and Venus \( Q_A = 0.57 \) will be needed to convert the linear unmixed images of our transfected cells into a FRET-efficiency image, a Cerulean-concentration image, and a Venus-concentration image.

Linear unmixing of each pair of spectral images for a given sample will produce an apparent Cerulean-image at \( \lambda^1_{\text{ex}} = 890\text{nm} \) \((d^1_{\text{apparent}})\), an apparent Venus Image at \( \lambda^1_{\text{ex}} = 890\text{nm} \)(\(d^1_{\text{apparent}}\)), an apparent Cerulean-image at \( \lambda^2_{\text{ex}} = 940\text{nm} \) \((d^2_{\text{apparent}})\), and an
apparent Venus-image at $\lambda_{\text{ex}}^2 = 940\,\text{nm}$ ($a_{\text{apparent}}^2$). As mentioned above, $d_{\text{apparent}}^1$ and $d_{\text{apparent}}^2$ should be indistinguishable, and therefore an average of these two images are used for further processing ($d_{\text{apparent}}$). A significant difference between these two images is indicative of quenching and/or bleaching in the sample. Additionally, sample motion during the period between acquiring the two spectral images can also be responsible for this type of artifact. Regardless, if a ratio of the pixel intensities of these two donor images are significantly different than 1 they should not be used for further processing. In contrast, $a_{\text{apparent}}^1$ and $a_{\text{apparent}}^2$ should be different if the FRET efficiency of the sample is greater than zero. Next, these three apparent images that were produced by linear unmixing are processed (for each pixel) using Eq. (8.14) explained above.

Image processing with these equations produces a donor-image ($d$), an acceptor-image ($a$), and a FRET efficiency image ($E_D$). Examples of images produced by this process can be observed for cells transfected with either CTV, a construct that has a low FRET efficiency (Fig. 8.6), or for cells transfected with C5V, a construct that has a high FRET efficiency (Figure 8.7). In both the figures, panel A shows the donor abundance (Cerulean concentration). Panel B shows the acceptor abundance (Venus concentration), and panel D shows the color-coded FRET-efficiency image where pink to red indicates increasing amounts of FRET and white indicates low FRET efficiencies. Panel C shows a color-coded ratio image formed by dividing the image in panel B by the image in panel A. This ratio image is useful for confirming that the proteins expressed and imaged have the same donor:acceptor stoichiometry as encoded in the construct and transfected into the cell. It is also important to realize that in live cell experiments, particularly when a FRET pair is composed of spectral variants of GFP, that differential expression, maturation, folding, as well as post-translation modifications (such as proteolysis) can all modify the observed acceptor/donor ratio of an expressed construct. Both the CTV and C5V constructs encode a single Cerulean molecule
Fig. 8.6. sRET analysis of CTV, a Cerulean-Venus construct with a low FRET efficiency. sRET analysis is based on linear unmixing of two spectral images obtained at two different excitation wavelengths. Spectral images of cells expressing the CTV construct were acquired with 890 and 940 nm excitation. These spectral images, and their matching reference spectra were processed using the sRET algorithm to produce (A) a Cerulean concentration image, (B) a Venus concentration Image, (C) a Venus/Cerulean ratio image, and (D) a FRET-efficiency image. The graphs in panels C and D show frequency histograms of the pixel values for the corresponding images. The red trace in panel C is a log-normal fit to the Venus/Cerulean histogram, while the black trace in panel D is a Gaussian fit to the FRET-efficiency histogram.
concatenated to a single Venus molecule. Thus, they should have a Venus/Cerulean ratio of 1 in these images (red). To the right of panels C and D in Figs. 8.6 and 8.7 are frequency histograms of the pixel values for the corresponding images.

Fig. 8.7. sRET analysis of C5V, a Cerulean-Venus construct with a high FRET efficiency. Spectral images of cells expressing C5V, and their matching reference spectra were processed using the sRET algorithm to produce (A) a Cerulean concentration image, (B) a Venus concentration Image, (C) a Venus/Cerulean ratio image, and (D) a FRET-efficiency image. The graphs in panels C and D show frequency histograms of the pixel values for the corresponding images.

concatenated to a single Venus molecule. Thus, they should have a Venus/Cerulean ratio of 1 in these images (red). To the right of panels C and D in Figs. 8.6 and 8.7 are frequency histograms of the
pixel values measured for the Venus/Cerulean ratio image (panel C) or for the FRET-efficiency images (panel D). The Venus/Cerulean ratio-image histograms (in panels C of Figs. 8.6 and 8.7) are plotted on a log-scale (grey bars) and are fit to a log-normal distribution (red trace). We can see that for both CTV and C5V, these distributions peak near a value of 1 confirming that on average each Cerulean expressed in these cells is attached to a single Venus molecule and vice-versa. The FRET-efficiency histograms (in panels D of Figs. 8.6 and 8.7) are plotted on a linear-scale (red bars) and are fit to a Gaussian distribution (black trace). The peak of the FRET-efficiency distribution for the CTV image (Fig. 8.6 D) was near 0 indicating little if any FRET. In contrast, the peak of the distribution for the C5V construct was between 0.4 and 0.5 indicating an average FRET efficiency of approximately 45%.

8.3.3. Testing and validating methods for measuring FRET

What are the relative merits of different methods of measuring FRET, and is the spectral approach to FRET measurement appropriate for a specific project? First and foremost, a method for measuring FRET must produce accurate results. Only then should other factors, such as the precision of the measurements, equipment costs, photon efficiency, and speed of data acquisition be considered when selecting a FRET method. Surprisingly, FRET reference standards (i.e., compounds with known FRET efficiencies) have only become available over the past year [40]. Without reference standards it was difficult to compare one type of FRET measurement with another or even to determine if a specific implementation of FRET was in fact accurate [11]. To correct this problem, our laboratory produced three genetically encoded FRET standards with the following ‘known’ FRET efficiencies: C5V ($E = 43 \pm 2\%$), C17V ($E = 38 \pm 3\%$), and C32V ($31 \pm 2\%$). These genetic constructs are based on two spectral variants of green fluorescent protein, Cerulean and Venus. The Förster radius ($R_0$) for energy
transfer from Cerulean to Venus is 5.4 nm [41]. In these constructs, the donor was separated from the acceptor by amino acid linkers of different lengths so that they would have different FRET efficiencies. To date, we have provided these DNA encoding FRET standards to over 75 different labs. All of the constructs mentioned above have one donor and one acceptor. We have also produced other related constructs that have two donors and one acceptor (CVC) and one donor and two acceptors (VCV) [12]. These are particularly useful for evaluating how well spectral FRET methods can measure the relative abundance of donors and acceptors. Finally, we have also produced a construct (CTV) that has a very low FRET efficiency. The CTV construct encodes a single Cerulean donor attached to a single Venus acceptor. When this construct is expressed it assembles into trimers composed of three donors (in close proximity) and three acceptors (also in close proximity). The donors, however, are thought to be separated from the acceptors by at least 8 nm, and therefore this construct has a very low FRET efficiency. The CTV construct is useful as a negative FRET control, and can also be used to evaluate if different FRET methods are susceptible to errors caused by homo-FRET occurring between donors and/or acceptors.

In Table 8.1 the FRET-efficiency values measured for these constructs by a spectral FRET method (sRET), as well as by fluorescence lifetime imaging (FLIM–FRET) [43, 44], and by a variant of the three-cube method (E-FRET) [42, 45] are shown. As can be seen, the spectral method produced FRET efficiencies that were similar to the other two methods. Furthermore, the sRET method also successfully determined the C5V, CVC, and VCV acceptor donor stoichiometries, demonstrating that the spectral FRET method can successfully and accurately measure FRET efficiency over a range of 3–69% with varying donor to acceptor ratios.

Another important lesson from Table 8.1 is that all three of the methods tested yielded virtually the same FRET efficiencies for the same samples. The sRET method as implemented used two-photon excitation on a Zeiss 510 META/NLO microscope, as did the FLIM–FRET method, but FLIM–FRET used auxiliary time
correlated single photon counting hardware, detectors, and software [46]. In contrast, the E-FRET method used one photon excitation on a standard automated fluorescence microscope, using a CCD detector and custom written software. For reasons that we do not fully understand, the standard deviations observed with the sRET measurements were at least two times greater than those observed with FLIM–FRET or with E-FRET. We suspect that this is not intrinsic to the spectral approach, but arises from some aspect of the sRET hardware/software implementation. Even though the standard deviations observed for FLIM–FRET and E-FRET were lower than that of sRET, all three methods were capable of differentiating a 5% change in FRET efficiency [40].

### TABLE 8.1

FRET efficiencies and acceptor to donor ratios of FRET standards. The FRET efficiencies of six genetic constructs expressed in cell culture were evaluated by three different methods: sRET, FLIM–FRET, and E-FRET. The acceptor to donor ratio (V/C) was also measured for each construct by the sRET method. Key: C5V, Cerulean-5 amino acid linker-Venus; C17V, Cerulean-17 amino acid linker-Venus; C32V, Cerulean-32 amino acid linker-Venus; CVC, Venus flanked on each side by a Cerulean; VCV, Cerulean flanked on each side by a Venus; CTV, Cerulean-Traf2 protein domain-Venus

<table>
<thead>
<tr>
<th>Construct</th>
<th>FRET efficiency by Method</th>
<th>V/C of constructs measured by sRET</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5V</td>
<td>41 ± 9, n = 62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.3, n = 12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C17V</td>
<td>35 ± 9, n = 91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>C32V</td>
<td>30 ± 8, n = 81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>CVC</td>
<td>41 ± 5, n = 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.3, n = 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VCV</td>
<td>70 ± 6, n = 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1 ± 1.0, n = 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CTV</td>
<td>2 ± 7, n = 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.4, n = 12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>From Ref. [40].
<sup>b</sup>From Ref. [12].
<sup>c</sup>From Ref. [42].
Another difference in these FRET methods is the cost of the microscopes. The two-photon microscope and its mode-locked laser used for sRET and FLIM–FRET cost approximately an order of magnitude more than the E-FRET system. Clearly, if cost is a limiting factor then the E-FRET approach is superior.

Theoretically, spectral imaging should be among the most photon efficient methods for measuring FRET because every photon emitted by either the donor or acceptor (if detected) can be used in the FRET calculation. With FLIM–FRET only emissions from the donor are typically used, and any part of the donors emission spectrum that overlaps with the acceptors emission spectrum is also not typically used. Clearly, FLIM–FRET, as implemented, is not very photon efficient. E-FRET is typically more photon-efficient than FLIM–FRET because emissions from both donors and acceptors are used. However, the use of emission filters to isolate donor emissions from acceptor emissions prevents many photons from being detected. Complicating this analysis of photon-efficiency is the fact that three separate excitation periods must occur to acquire the images required for E-FRET analysis. FLIM–FRET requires only one excitation period but it can last tens of minutes, and sRET requires two excitation periods, one for each excitation wavelength. Finally, not all photon detectors have the same quantum efficiency. Photomultipliers used for FLIM–FRET (and in confocal microscopes) typically have quantum efficiencies for detecting photons in the range of 10–40% [46]. A recent study compared the efficiencies of the spectral detector used in a Zeiss META confocal with nonspectral detectors and concluded that the spectral detectors were fivefold less efficient in detecting photons [47]. State of the art CCD cameras that can be used for E-FRET imaging, can have quantum efficiencies as high as 90%. Because of all of these different factors, it is often difficult to predict the photon efficiency of different FRET methods. Nonetheless, an empirical estimate of their relative photon efficiencies can be derived from the time each method requires to acquire a FRET-efficiency image. A single FRET image acquired on a time-domain
FLIM–FRET system (Becker and Hickl SPC 830 with a Hamamatsu R3809 detector mounted on a Zeiss 510 META/NLO) can take between 5 to 20 min to acquire. On a sRET system (Zeiss 510 META/NLO) a single FRET image (i.e., two spectral images) takes approximately 100 s to acquire, but tuning the laser (Coherent Chameleon) to the second excitation wavelength adds an additional minute or two. Acquiring a FRET-efficiency image on an E-FRET system typically requires only a few seconds. Thus, even though E-FRET does not produce thin optical sections as does sRET and FLIM–FRET (with a two-photon pulsed laser), E-FRET’s low cost and rapid acquisition time affords it a great advantage, particularly for time-lapse studies. It is also important to realize that empirical comparisons like these only contrast specific implementations of these FRET methods. For example, a comparison of FRET methods similar to our own reached noticeably different conclusions [48]. With future improvements in the quantum efficiencies of spectral detectors, improvements in their signal to noise ratio, as well as technology to rapidly and reproducibly tune lasers [49], we predict that spectral imaging will ultimately become the most photon efficient, and rapid method for accurately measuring FRET while simultaneously measuring the abundance of donors and acceptors.

Acknowledgments

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Appendix

The detected fluorescence emission spectrum \( F^i(\lambda) \) at excitation wavelength \( \lambda^i_{\text{ex}} \) is composed of three components. These three components are (analogous to the notation in Chapter 7) the
spectrum originating from (partly) quenched donors $I_{D-S}^i(\lambda)$ (corresponding to $I_{DA}$ in the general notation, referring to residual donor fluorescence in the presence of the acceptor), the spectrum originating from sensitized emission ($I_S^i(\lambda)$) and the spectrum due to direct acceptor excitation $I_{A}^i(\lambda)$.

$$F^i(\lambda) = I_{D-S}^i(\lambda) + I_S^i(\lambda) + I_A^i(\lambda) \quad (8.1)$$

The spectra are a product of the number of molecules ($N$), the laser intensity $I(\lambda_{ex})$, the (excitation wavelength dependent) extinction coefficient $\varepsilon^i$, the quantum yield $Q$, the corrected emission spectra $F(\lambda)$, and the instrument response or gain $g(\lambda)$ that is typically wavelength dependent. If we have $N_s$ molecules that show FRET with an efficiency $E$, and $N_D$ (total) donor molecules and $N_A$ (total) acceptor molecules, Eq. (8.1) can be rewritten:

$$F^i(\lambda) = I^i(\lambda) \left\{ (N_D - N_S)\varepsilon_D^i Q_DF_D(\lambda) + N_S[\varepsilon_D^i Q_D(1 - E)F_D(\lambda) + \varepsilon_D E Q_A F_A(\lambda)] + N_A \varepsilon_A^i Q_A F_A(\lambda) \right\} \quad (8.2)$$

If we define $E_D \equiv \frac{N_S}{N_D} E = f_D E$ where $f_D$ is the fraction of Donor molecules involved in FRET (8.2) can be rewritten as:

$$F^i(\lambda) = I^i(\lambda) \left\{ N_D(1 - E_D)\varepsilon_D^i Q_D F_D(\lambda) + \left( N_A + N_D \frac{\varepsilon_D^i}{\varepsilon_A^i} \right) \varepsilon_A^i Q_A F_A(\lambda) \right\} \quad (8.3)$$

If we consider two reference samples of pure donor and acceptor with a known concentration, two reference spectra can be recorded according to Eq. (8.4):

$$F^i_{D,ref}(\lambda) = I^i(\lambda) \varepsilon_D^i Q_D F_D(\lambda) N_D,ref$$

$$F^i_{A,ref}(\lambda) = I^i(\lambda) \varepsilon_A^i Q_A F_A(\lambda) N_A,ref \quad (8.4)$$
Then we can perform the following integration:

\[
\int_{\lambda} \frac{F_{D,ref}^i(\lambda)}{F_D(\lambda)} = \ell^i e_D^i Q_D N_{D,ref} \int_{\lambda} g(\lambda)
\]

\[
\int_{\lambda} \frac{F_{A,ref}^i(\lambda)}{F_A(\lambda)} = \ell^i e_A^i Q_A N_{A,ref} \int_{\lambda} g(\lambda)
\]

From which it is apparent that:

\[
\frac{e_D^i}{e_A^i} = k^i \frac{N_{A,ref}}{N_{D,ref}} \frac{Q_A}{Q_D}
\]

with

\[
k^i = \int_{\lambda} \frac{F_{D,ref}^i(\lambda)}{F_D(\lambda)} / \int_{\lambda} \frac{F_{A,ref}^i(\lambda)}{F_A(\lambda)}
\]

Note that for the calculation of \(k^i\), two reference samples of known concentrations are required in addition to calibrated unit area spectra of donor and acceptor fluorophores. In case the instrument response curve is not wavelength dependent \((g(\lambda) = g)\) then unit reference spectra are not required because in this case:

\[
k^i = \int_{\lambda} F_{D,ref}^i(\lambda) / \int_{\lambda} F_{A,ref}^i(\lambda)
\]

With Eqs. (8.A4–8.A6), (8.A4) can be reformulated according to:

\[
F^i(\lambda) = \frac{N_D}{N_{D,ref}} (1 - E_D) F_{D,ref}^i(\lambda)
\]

\[
+ \frac{N_A}{N_{A,ref}} F_{A,ref}^i(\lambda) + \frac{N_D}{N_{D,ref}} E_D \frac{Q_A}{Q_D} k^i F_{A,ref}^i(\lambda)
\]

If we define the relative donor and acceptor concentration as:

\[
d = \frac{N_D}{N_{D,ref}} \text{ and } a = \frac{N_A}{N_{A,ref}}
\]

(8.A8)
Then Eq. (8.48) rewrites as:

\[ F^i(\lambda) = d(1 - E_D)F_{D,\text{ref}}^i(\lambda) + aF_{A,\text{ref}}^i(\lambda) + dE_D \frac{O_A}{Q_D} k^i F_{A,\text{ref}}^i(\lambda) \]  

which is identical to Eq. (8.2).

Note that if from a separate experiment the molar extinction coefficients of the donor and acceptor at both excitation wavelengths are known, the determination of \( k^i \) is not required since Eq. (8.49) rewrites in:

\[ F^i(\lambda) = d(1 - E_D)F_{D,\text{ref}}^i(\lambda) + aF_{A,\text{ref}}^i(\lambda) + dE_D \frac{e_D^i}{e_A^i} \frac{N_{D,\text{ref}}}{N_{A,\text{ref}}} F_{A,\text{ref}}^i(\lambda) \]  

\[ = d(1 - E_D)F_{D,\text{ref}}^i(\lambda) + a \left( 1 + \frac{e_D^i}{e_A^i} E_A \right) F_{A,\text{ref}}^i(\lambda) \]  

with

\[ E_A \equiv \frac{N_S}{N_A} E = f_A E \]  

However, in case of multiphoton excitation, the determination of \( \frac{e_D^i}{e_A^i} \) will be difficult.

References


