Chapter 2

An Introduction to Fluorescence Imaging Techniques Geared Towards Biosensor Applications

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Abstract

After providing a brief overview of the basics of fluorescence and FRET, this chapter discusses the most commonly used methods to record FRET. Emphasis is on microscopy methods that are widely used for biosensor imaging. We cover choice of instruments, describe various ways to detect FRET based on intensity as well as on donor lifetime, and provide some guidelines to match particular recording methods with specific scientific experiments. We end with an extensive discussion on further practical considerations that may greatly affect the success of the experiments.

Key words Wide-field fluorescence microscopy, Laser scanning microscopy, Ratio-imaging, Fluorescence lifetime imaging

1 Introduction

After providing a brief overview of the basics of fluorescence and FRET, this chapter discusses the most commonly used methods to record FRET. Emphasis is on microscopy methods that are widely used for biosensor imaging. We provide some guidelines to match particular recording methods with specific scientific experiments, and we end by discussing further practical considerations that may greatly affect the success of the experiments.

2 Fluorescence Basics

The term fluorescence was coined by Sir George Gabriel Stokes in the nineteenth century. It describes the process in which a material (the so-called fluorophore) is excited by absorbing light which is subsequently emitted at a longer wavelength. After absorbing light, the molecule is in an excited state. The excited state is short-lived, typically in the nanosecond (10^{-9} s) time range.

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The light emitted when the molecule returns to the ground state is known as fluorescence emission light or fluorescence for short.

Typically, the excitation light is several orders of magnitude stronger than the emission. To obtain pure fluorescence emission signals, the fluorescence signal needs to be isolated somehow. While this is easy to achieve in a fluorimeter by detecting fluorescence emission from a cuvette at an angle perpendicular to the excitation source, it is more complicated in a microscope. The most popular configuration to observe fluorescence with a microscope is the epifluorescence mode, in which both the excitation light and emission light pass through the objective (Fig. 1). Excitation light is typically obtained by filtering suitable wavelength from a white-light source using a interference filter. A dichroic mirror reflects the excitation light onto the sample while allowing the fluorescence emission light to freely pass. An emission filter is required to completely block any excitation light that is reflected (scattered) by the sample and might leak through the dichroic mirror. Emission filters typically have a transmittance of 10^{-4}–10^{-5} % at the excitation wavelengths whereas they allow light to pass with an efficiency close to 100 % at the emission wavelengths.

Although the epifluorescence mode provides high signal to noise, it is important to note that only a fraction of the emission light is detected (about 10–25 %). The detection efficiency scales with the square of the numerical aperture (NA) of the objective. Therefore, the use of high NA objectives (NA 1.2–1.4) is recommended when high sensitivity is desired.

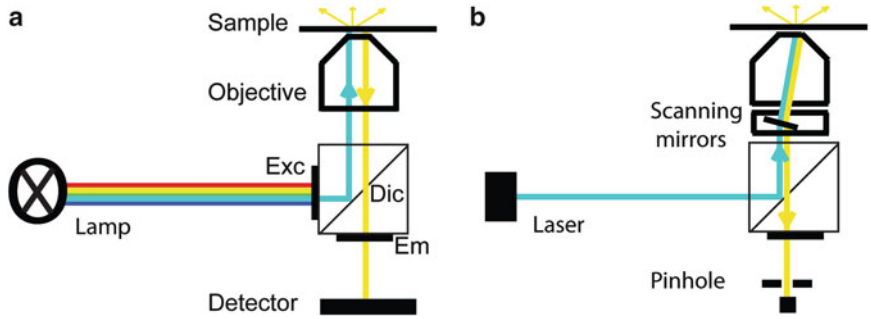

Fig. 1 (**a**) Basic setup of filters in fluorescence microscopy. The excitation source, here a mercury lamp, produces a broad range of light of various colors. A restricted range of wavelengths (here *blue*) is selected by the excitation filter (Exc) and reflected towards the sample by the dichroic mirror (Dic), placed under an angle of 45°. The light is focused by an objective lens into the sample resulting in a full illumination of the sample, resulting in fluorescence (*yellow*). Since the fluorescence has a different wavelength, light passes the dichroic mirror. An emission filter (Em) allows detection of the fluorescent signal while providing an additional block for the intense excitation light. In the case of a laser scanning confocal microscope (**b**), a monochromatic laser beam is focused into a single point in the sample via the scanning mirrors. By moving the scan-mirrors and therefore moving the excitation spot, the whole sample is imaged point-by-point. Detection of out-of-focus light is reduced by placing an aperture, the pinhole, in the detection path resulting in optical sectioning

3 FRET

Förster resonance energy transfer (FRET) is the radiationless transfer of energy from the excited state of a donor fluorophore to an acceptor. FRET occurs when a donor and acceptor are in close proximity, usually within 1–10 nm of each other. Importantly, this range matches the scale at which protein–protein interactions take place. Since the FRET efficiency depends on the inverse sixth power of distance ($E \equiv 1/r^6$), it is very sensitive to distance. Other than distance, the efficiency of FRET also depends strongly on the orientation between the donor and acceptor dipole moments, which allows FRET-based detection of conformational changes.

The applications of FRET include detection of protein–protein interactions, detecting protein conformational changes, and, most relevant to this volume, as readout of so-called "sensors", i.e., genetically encoded constructs that are designed to spy on cellular biochemistry, such as the activity of proteases or the concentration of second messengers. Literally hundreds of such sensors have been described and the development and optimization of FRET sensors is a very active field. The application of FRET sensors allows us to detect any process with high spatial and temporal resolution in single living cells, giving us an unprecedented view into cellular functioning.

The basic spectroscopic changes that occur upon FRET are quenching of donor fluorescence, a decrease in excited state lifetime of the donor and, if the acceptor is a fluorophore, emission of fluorescence by the acceptor. The latter is known as sensitized emission. All these parameters can be used to detect and quantify FRET by fluorescence microscopy. Other spectroscopic changes, including fluorescence anisotropy and a change in photobleaching kinetics, can also be used to measure FRET but will not be discussed here. Further details on FRET recording and the required instrumentation will be described in next section.

FRET requires matched fluorophores, which means that the spectra of donor emission and acceptor absorbance should have substantial overlap. In addition the quantum yield of the donor and extinction coefficient of the acceptor should be sufficiently high. The cyan and yellow fluorescent protein pair is currently the most widely employed pair for FRET based genetically encoded biosensors, both for historical reasons as well as favorable spectroscopic characteristics. For more information about suitable fluorophores for FRET we refer to Chapter 1. It has been pointed out that the method of FRET detection also affects the choice of donor and acceptor fluorophores, see for instance [1].

4 Fluorescence Microscopes

Two kinds of fluorescence microscopy setups are commonly available in most cell biology laboratories: the wide-field fluorescence microscope and the confocal (laser scanning) microscope. Both instruments can be readily used to perform FRET imaging. We will discuss some of the basic principles and differences.

4.1 The Wide-Field Microscope

In wide-field microscopy, the sample is illuminated uniformly over the field of view. The emitted fluorescence is captured by a camera. In contrast to confocal microscopy, there is no optical sectioning, resulting in the image being somewhat deteriorated by blur from out-of-focus parts of the cells. The typical resolution for a wide-field microscope is about half the wavelength of the used light, or ~250 nm, in the XY-plane and whereas the resolution along the Z-axis is about 700 nm.

High power Mercury and Xenon lamps are often used as excitation sources. These lamps emit broad-spectrum (white) light, which needs to be filtered to obtain the correct wavelength for excitation. High quality excitation filters are used that only transmit a small bandwidth of light (Fig. 1a). More recently, colored Light Emitting Diodes (LEDs) are replacing the arc lamps. For quantitative microscopy, sensitive cameras that linearly detect fluorescence are crucial. Cooled CCD (Charge-Coupled Device) cameras provide good sensitivity and high resolution. Current state-of-the-art imaging is performed with Electron-Multiplied CCD cameras which combine optimal sensitivity (95 % quantum efficiency for back-illuminated CCD cameras), fast acquisition (>1,000 frames/s) and high resolution (>1 megapixels).

4.2 The Confocal Laser Scanning Microscope

In laser scanning microscopy, for excitation a focused light spot is used to scan the sample point-by-point (Fig. 1b). The emission light is passed through a pinhole and fluorescence intensity of each point, the pixel, is recorded with a detector. The pinhole rejects out-of-focus fluorescence, which results in "optical sectioning" Thus, the resolution is increased, especially along the z-axis. The confocal resolution is only marginally better than that of the wide-field microscope, but since it lacks out-of-focus blur it yields crisp optical sections.

A laser is usually employed as the excitation source, since it produces intense light that can be focused to a small so-called diffraction-limited spot. Since lasers are monochromatic, one is limited to the available lasers for excitation but excitation filters are not necessary. Detection of fluorescence is usually by a photomultiplier tube (PMT). The avalanche photodiode (APD) is a more sensitive alternative, but it requires more careful handling. Important parameters when purchasing a PMT are the sensitivity and speed needed in the experiment. The detection efficiency of

the classical PMTs that are included in most confocal microscopes is 10–15 % and it decreases sharply in the red part of the spectrum. Recent, more sensitive variants may have efficiencies up to 50 %. PMTs and APDs may be operated either in linear mode or in photon-counting (Geiger) mode. It is important to note that, at very low and at high photon fluxes, detectors may not be linear which would of course hamper quantitative measurements. In photon counting mode, detector dead-time (the time period required to process the signal of the photon, during which it is insensitive for further photons) is an important consideration because it limits detection speed as well as efficiency.

4.3 Alternative Excitation Modes

Several alternative approaches to image fluorescence are available. For wide-field microscopes, TIRF excitation [2] or Nipkow spinning disk scanning [3] can be implemented to increase *z-resolution*. Laser scanning confocal microscopes can be equipped with a source for two-photon excitation, providing better tissue penetration for intravital imaging. Although useful for FRET, a detailed explanation of these techniques is outside of the scope of this introductory chapter.

5 FRET Detection Methods

For a detailed description of FRET imaging we refer to the authoritative volume edited by Gadella Jr. [4] and to Jares-Erijman [5]. Useful suggestions on selecting the right imaging approach for an application are in ref. 6. Here, we will describe the most common techniques to measure FRET in single living cells, emphasizing those most useful for fluorescent biosensors. Besides intensity-based methods (ratio-imaging and acceptor photobleaching) which are readily performed on ordinary fluorescence microscopes we will also discuss fluorescence lifetime imaging as a robust method to detect FRET. FLIM detection equipment is now commercially available, either as integrated "push-button" system or as add-on for wide-field or laser scanning microscopes.

5.1 Intensity-Imaging

In *ratio-imaging* (Fig. 2a), the intensity of both donor emission and sensitized emission (i.e., the emission detected from the acceptor upon excitation of the donor) are detected. This is by far the most popular method for (dynamic) biosensor imaging due to its simplicity, sensitivity and speed. Ratio-imaging is often not carried out in a quantitative manner, although addition of extra corrections or use of an end-point calibration can easily remedy that. This method is very photon-efficient and most useful to record FRET changes. As said, ratiometry is not, in general, quantitative because the recorded sensitized emission image is contaminated by bleedthrough of the donor molecules, and because it also contains

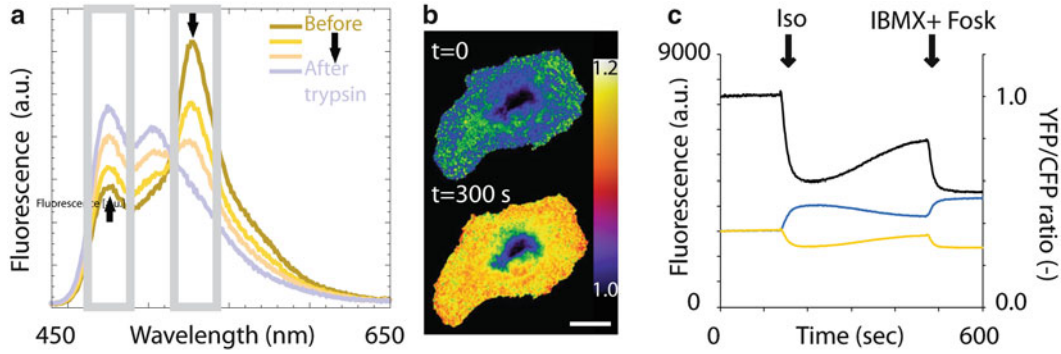

Fig. 2 (**a**) Fluorescence spectra of a purified CFP-YFP fusion protein After addition of trypsin, the fusion protein is progressively cleaved, resulting in a loss of FRET that can be monitored over time as a loss in sensitized emission (~530 nm) and an increase in CFP donor fluorescence (~480 nm). A fast readout of this sensor may be achieved by monitoring the 530/480 intensity ratio using emission filters specific for these spectral regions, as indicated by the *gray* blocks. (**b**) YFP/CFP ratio image of HUVEC cells transfected with a FRET sensor. A significant ratio change was observed 300 s after stimulation of the cells. (**c**) Dual-PMT ratio experiment of ^{T}EPAC^{W} expressed in HeLa cells. Upon stimulation with isoproterenol or IBMX and forskolin, the FRET efficiency is lowered thereby increasing the CFP (*blue*) and lowering the YFP (*yellow*). *Black* denotes the ratio (YFP/CFP)

emission from acceptors that are directly excited by the excitation at donor wavelength, so-called cross-excitation.

Experiments usually start with recording of a baseline from a cell or population of cells. Cells are then stimulated with an agonist that alters FRET, and the changes are expressed as deviation (in percentage) of that change from the baseline, or as a percentage of the change induced by a control stimulus at the end of the experiment (Fig. 2b).

An extension of this technique, *filterFRET*, allows quantitative FRET recording from emission intensities. To this goal, ratiometry is upgraded by simply recording an additional image that reflects the emission of acceptors when excited at their own characteristic wavelength. It can be shown that from such image triplets, FRET can be quantified in a straight-forward manner [4, 7]. Detailed description is beyond the scope of this chapter but provided in the literature.

For ratio-imaging, the speed of acquisition requires some consideration. Fast-moving structures such as cellular vesicles or the leading edge of the plasma membrane of a moving cell may lead to an artifact when the ratio of the two images is taken. Thus, the acquisition of donor and acceptor channels should be faster than those changes to avoid movement artifacts. In confocal microscopes, simultaneous detection of two or more channels is a basic feature, allowing donor and acceptor channels to be imaged without delay. In wide-field microscopes, the emission filters are often located in a filter cube or emission filter wheel, and the images have to be taken consecutively. The integration time and time added by changing the

filter will limit the application for the fastest biological processes. Parallel imaging can, however, be achieved in several ways. It is relatively straightforward to incorporate a secondary dichroic mirror and two CCD cameras, but this is rather expensive. An excitation-economic alternative is implementation of an image splitter (marketed under the name of optosplit or dualview), which projects two emission channels side-by-side on a single CCD camera. Another option is to combine an emission beamsplitter with two point detectors (PMTs), a method known as "dual-photometer detection". At the expense of spatial information, this allows the fastest and highest sensitivity detection by far. Dual photometer detection is often used when very dim excitation intensities are necessary to avoid photodamage to sensitive cells (Fig. 2c).

5.2 Acceptor Photobleaching

The basic idea behind acceptor photobleaching is that the donor is quenched due to FRET. By destroying the acceptor, FRET is no longer taking place and the donor becomes dequenched (Fig. 3). The FRET efficiency can be directly quantified from the intensity increase of the donor.

Laser scanning systems are popular for this technique as these have high-intensity monochromatic excitation lasers which can be

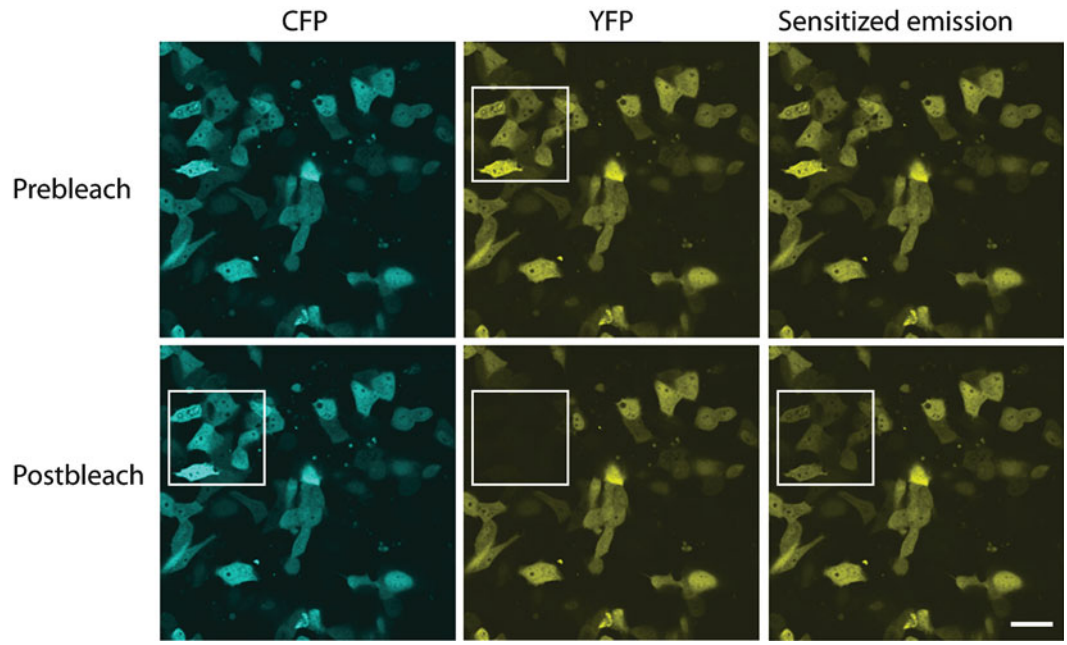

Fig. 3 Acceptor photobleaching experiment of a FRETing CFP-YFP fusion protein expressed in HeLa cells. By selectively exciting the YFP acceptor protein in a region of interest (*box*) using the full intensity of a 514 nm laser line, the YFP protein was destroyed locally. In this ROI the FRET was abolished resulting in an increase of the CFP donor fluorescence and a reduction of the sensitized emission. The remaining fluorescence in the sensitized emission panel is CFP cross-talk (leak-through) in the YFP detector

used to excite/bleach the acceptor with high specificity without exciting and possibly destroying the donor. For instance, the 514 nm laser line selectively bleaches YFP without exciting CFP, and 561/568 nm laser lines can be used to specifically bleach red fluorescent proteins that act as acceptors to green-shifted variants. Note that this technique is destructive so it cannot provide temporal information; it is, however, very useful as an extra control (endpoint calibration) for other methods.

5.3 Lifetime Imaging

Imagine a population of fluorophores that is excited by a very brief (ps) pulse of light. These molecules will not emit fluorescence all at the same time; rather, they emit randomly and the population will thus send out light in an exponentially decaying manner (Fig. 4a). The decay time or characteristic fluorescence lifetime, τ, for common fluorophores in biology is between ~0.1 and 5 ns. FRET can be quantified with high precision by detecting lifetimes because it causes a reduction of the lifetime. Since the lifetime is a kinetic parameter, it is independent of excitation or emission intensity, and not affected by expression level, cell thickness and local concentration. Fluorescence lifetime imaging microscopy (FLIM) requires dedicated and expensive equipment but delivers trouble-free "push-button" FRET pictures.

Two acquisition modes are popular, the so-called *time-domain* and *frequency-domain* methods [4]. Time-domain FLIM uses the above-described brief excitation pulse and subsequently measures (many) photon arrival times to construct a decay curve in a technique known as time-correlated singles photon counting (TCSPC). The kinetics of the decay curve can be analyzed to obtain the excited state lifetime. This technique presents the ultimate in precision, but since it depends on individual photons being collected

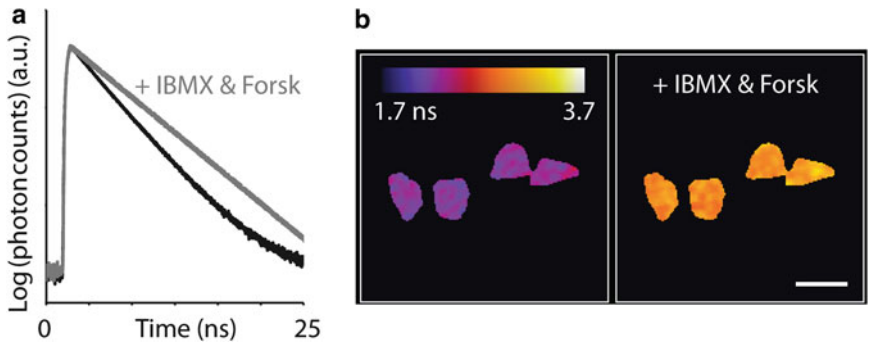

Fig. 4 (**a**) Fluorescence lifetime decay histogram of purified cAMP sensor ^{T}EPAC^{W} (*black*). Upon stimulation with IBMX and forskolin, FRET is lowered, resulting in increased fluorescence lifetime and thus a slower decay of the curve (*gray*). The amplitudes of the curves were normalized for clarity. (**b**) Frequency-domain FLIM analysis of ^{T}EPAC^{W} expressed in HeLa cells before and after stimulation. The images were intensity thresholded to reduce background contribution and pseudo-colored to display the phase lifetimes

sequentially for each pixel, image acquisition times of several minutes are common.

In frequency domain FLIM, the sample is irradiated with light that is intensity-modulated, typically at 40–80 MHz [4]. The fluorescence emitted by the cells is therefore also modulated with the same frequency, but it will be shifted in phase and its amplitude will be demodulated, depending on the lifetime. Calculation of the lifetime based on phase-shift and demodulation yields not one, but two parameters with usable information. For mono-exponentially decaying fluorophores, both lifetimes should be identical, but when the decay is multi-exponential, the modulation lifetime is higher than the phase lifetime.

Acquisition of lifetime images (Fig. 4b) with frequency-FLIM is much faster than with TCSPC but still slower than with intensity-based methods (ratio-imaging) and therefore this approach is less suitable for fast FRET imaging (<1 s). Care should be taken that the acquisition time is short relative to possible movement of structures in the sample in order to obtain reliable data. Quantification of FRET by FLIM may be complicated if the donor has a multi-exponential decay, like most CFP variants. EGFP [8] and the new CFP variants mTurquoise and mTurquoise2 [9] are mono-exponential and therefore popular for FLIM.

Both frequency-domain and time-domain FLIM can be implemented on fluorescence microscopes. For technical reasons, frequency-domain FLIM is usually implemented on wide-field systems, while the time-domain method is more easily compatible with confocal laser scanning microscopes.

6 Spying on Intracellular Messengers by FRET: Practical Considerations

Many practical considerations guide readout of FRET sensors by microscopy. These can be loosely grouped in issues relating to biology, to the fluorophores and, most important in the context of this chapter, to the choice of acquisition method.

6.1 Biology

Biosensors should interfere minimally with normal cell physiology. It is important to carefully ensure in the cells to be studied that the biosensor has minimal effects of the readout, for example by comparing responses in cells with high expression levels to those with (very) low levels of the sensor. Whereas the noise levels may be high in the latter case, both experiments should reveal at least similar kinetics of the process under study. This ensures that the sensor neither buffers the analyte nor affects the reaction steps in another manner. Any unwanted activity of the sensor, such as catalytic activity [10] or binding to endogenous components [11], must be removed. Note that the choice of fluorophores may be important too. For example, we noticed that FRET sensors employing monomeric

GFP variants may behave remarkably different from similar sensors that incorporate "dimerizing" fluorophores in, for example, their tendency to dimerize and localize to organelles (unpublished observations and ref. 1).

The various techniques described in the previous paragraphs require widely differing amounts of excitation light to achieve a good signal-to-noise ratio of the readout. In order not to disturb sensitive cellular processes such as mitosis, it may be necessary to be less demanding on the required spatiotemporal resolution and precision. Simple ratiometric imaging is very photon-efficient, and both sensitized emission and FLIM may be sped up by summing, so-called binning, camera pixels.

6.2 Fluorophores

Stable fluorescence signals are essential for quantitative imaging, regardless of the approach. The sensors should exhibit stable (i.e., no photobleaching and no photochromism) and bright signals. Large differences exist in the performance of individual FPs, in this respect. Therefore, photostable FPs should be used at the lowest practical intensity of illumination.

Theoretically, it is preferable to employ fluorescent proteins that emit in the red part of the visible spectrum, since cells show less autofluorescence in this region. In addition, the Förster radius, a parameter that quantifies the FRET efficiency of a certain donor–acceptor pair, is higher while the excitation light is of lower energy, causing it to be less harmful for the cells. However, several issues impede the wide application of red acceptors. First of all, the red acceptors reported thus far have rather low quantum yield.

Secondly, some red fluorescent proteins mature via green intermediates. Therefore, maturation should be fast, a requirement which is met by only a few variants [1, 12]. For example, we find that the majority of inter-cell variability when reading out resonance levels with, e.g., the GFP-tdTomato FRET pair, is caused by poor maturation of tdTomato. Unmatured acceptors will cause erroneous readout of FRET in ratio measurements, as well as in FLIM. We anticipate that red-shifted FRET biosensors will become more popular once maturation- and brightness issues have been solved. Fluorophore choice is discussed in much more detail in Chapter 4 of this textbook.

6.3 Acquisition

Not all biosensors are equally suited for the different acquisition methods described in this chapter. Ratio-imaging and *filterFRET* rely on detection of sensitized emission and therefore profit from bright (high quantum yield) acceptors. An elegant way to improve acceptor brightness is by increasing the number of acceptors [1]. FLIM, on the other hand, is based on donor fluorescence only and thus the light yield of the acceptor is not important. Rather, FLIM may benefit from so-called "dark" acceptors in biosensors [13]. These acceptors are chromophores, rather than fluorophores, and absorb but do not emit light. Therefore, the acceptor channel is

not occupied by the biosensor and can be used for another probe or sensor for multiplexing applications. A disadvantage may be that no information on the actual distribution of the acceptor can be obtained. Furthermore, since the donor is quenched when FRET occurs, the signal obtained by FLIM decreases when FRET increases. This means that the FRET efficiency should not be too high for reliable analysis by FLIM; in practice, this is hardly ever a problem since, in fluorescent protein-based FRET, efficiencies are usually well below 50 %.

Other considerations concern the quantitation of the response. Simple ratiometry effectively cancels out excitation intensity fluctuations, image shading (lateral intensity variations present in the image) and local abundance of the sensor. However, in triple-image *filterFRET* images, the acquired acceptor image may contain a differing amount of shading, and this should be corrected in order to get the best possible quantitation, particularly on older confocal microscopes. For an excellent review on details of quantitative microscopy and necessary corrections, see Waters [14].

Pixel-shift between donor and acceptor channel may be introduced by image-splitting devices on wide-field microscopes and needs to be meticulously corrected for, e.g., by imaging submicron beads. Post-acquisition, the pixel-shift can be corrected using image processing (for a detailed procedure see Kardash et al. [15]). A detailed procedure for sensitized emission–based FRET imaging using confocal microscopy, including all corrections has also been reported [4, 7]. Note that FLIM measurements are essentially free of shading and pixel-shift issues.

Finally, it must be emphasized again that the various acquisition methods differ widely in sensitivity, speed and photon efficiency. TCSPC represents the ultimate in precision, requiring no calibration at all, but the acquisition is so slow that for imaging live-cell dynamics it is usually not an option. However, when coupled to a confocal microscope, high-resolution FRET images can be obtained. On the other hand, depending on sensor expression levels, frequency-domain FLIM yields quantitative results in ~1 to a few seconds. With current instrumentation, this technique is not very photon-efficient, requiring an estimated 5–20 times more excitation to achieve a good quality image. In addition, in our experience, very small FRET changes usually escape detection by these FLIM methods. Intensity-based FRET readings, on the other hand, are not quantitative unless additional calibrations and corrections are carried out, but they are very sensitive, even to small changes in FRET. The photon efficiency is high and readout rate is, in principle, only limited by the emission intensity; for example, using a resonant scanning confocal, full-frame emission ratios can be easily detected at video rate. The ultimate in speed, finally, is represented by dual-photometer readout: sub-ms recording is readily achieved, even at very dim excitation, but spatial resolution is sacrificed completely with this method. We detect FRET changes

as little as 0.25 % with our setup, but quantification is usually out of the question. Thus, depending on the questions to be answered, it pays to invest in more than just one acquisition setup.

7 Conclusion

In many cell biology labs fluorescence microscopy setups are available that can be employed for measuring FRET-based biosensors. The first priority is to make sure that the right excitation wavelength and correct filters (dichroic and emission filters) are in place to do the FRET measurements. Additional modifications may be necessary for specific requirements (fast imaging or high spatial resolution). To set up the system, it is worthwhile to use FRET biosensors which exhibit high contrast and are easy to manipulate. Good examples are YCam3.6 and the new generation of EPAC-based sensors (^{T}EPAC^{VV}). These biosensors are readily expressed in eukaryotic cells, have a high dynamic range and a FRET change that can be conveniently induced by adding small molecule reagents (for more information on measuring cAMP in cells with the EPAC-based sensor see Chapters 4 and 5 in this textbook).

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