

Quantitative Ratiometric Imaging of FRET- Biosensors in Living Cells

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CHAPTER OUTLINE

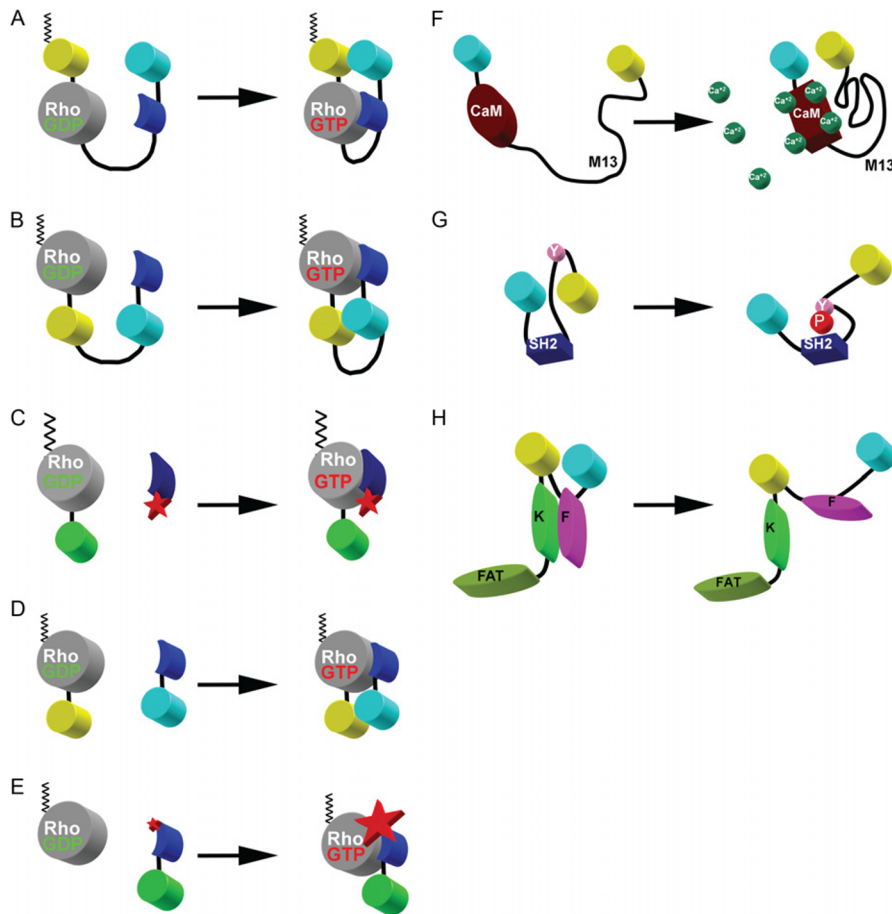
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Abstract

Biosensors based on FRET have been useful in deciphering the dynamics of protein activation events in living cells at subcellular resolutions and in time scales of seconds. These new systems allow observations of dynamic processes which were not possible previously using more traditional biochemical and cell biological approaches. The image data sets obtained from these sensors require careful processing in order to represent the actual protein activation events. Here, we will cover the basic approaches useful for processing the raw image data sets into relativistic ratiometric measurements, capable of depicting relative differences in the protein activation states within a single cell. We will discuss in detail the approaches for genetically encoded, single-chain biosensor systems based on FRET, as well as those that are based on intermolecular, dual-chain design. Additionally, the same analysis can be utilized for biosensor systems using solvatochromic dyes (Nalbant, Hodgson, Kraynov, Touthkine, & Hahn, 2004), useful for detection of endogenous protein activation states.

INTRODUCTION

Fluorescent biosensors based on the Forster resonance energy transfer (FRET) have now been established as powerful tools, allowing direct observations of protein activation states (posttranslational modification, conformational change, or protein–protein interactions) in living cells in their native environment (Hahn & Touthkine, 2002; Honda et al., 2001; Itoh et al., 2002; Machacek et al., 2009; Miyawaki & Tsien, 2000; Nakamura, Kurokawa, Kiyokawa, & Matsuda, 2006; Nalbant, Hodgson, Kraynov, Touthkine, & Hahn, 2004; Pertz, Hodgson, Klemke, & Hahn, 2006; Ting, Kain, Klemke, & Tsien, 2001; Zaccolo et al., 2000; Zhang, Ma, Taylor, & Tsien, 2001). The term “biosensor” implies that it is capable of reporting information other than the bulk localization changes of the protein, usually through molecular engineering, which are typically not possible to observe using traditional biochemical and cell biological methods. A few examples of these sensors are shown in Fig. 25.1. The primary detection modality is via the FRET, where the changes in the distance between the FRET donor and acceptor molecules as well as the angle of dipole coupling can significantly impact the amount of FRET transfer that occurs in a given state of the biosensor (Hodgson, Pertz, & Hahn, 2008; Hodgson, Shen, & Hahn, 2010; Lakowicz, 1983; Lankawoicz, 1986; Piston & Kremers, 2007; Stryer, 1978; Stryer & Haugland, 1967). Therefore, the “optimization” of the total FRET change as a function of all-on versus the all-off conditions of the biosensor in a cell constitutes the major developmental effort of these molecular constructs (Hodgson et al., 2008). Once properly optimized, however, the biosensors can reveal in real time and at subcellular spatial resolutions, the posttranslational modification states (or, activation states, binding events, etc.) of the protein of interest, which is not possible to do using traditional approaches. In order to harness their power, appropriate microscopy techniques and image processing approaches become critical components to obtaining meaningful readouts. Here, we outline the

**FIGURE 25.1**

Schematic drawings of some of the available types of biosensors which the image processing techniques presented here can be used. (A) Rho family GTPase biosensors in which the FRET donor and acceptor are placed on the N-/C-terminals of the molecule (Itoh et al., 2002; Kurokawa & Matsuda, 2005). This design does not allow for the correct interaction with the upstream guanine nucleotide dissociation inhibitor (GDI). (B) Rho family GTPase biosensor in which the full-length Rho GTPase is placed on the C-terminus, maintaining the correct C-terminal posttranslational modification of the GTPase thereby allowing for the correct GTPase–GDI interaction (Pertz et al., 2006). (C) The original Rac1 FLAIR biosensor, in which the binding of the small binding domain labeled with Alexa-546 to the activated, EGFP-labeled GTPase (bound to GTP), resulted in FRET between the EGFP (donor) and Alexa-546 (acceptor) (Kraynov et al., 2000). (D) Similar to (C) but fully genetically encoded version utilizing the FRET between ECFP and EYFP (Machacek et al., 2009). (E) MeroCBD biosensor for detecting the activity of endogenous, unlabeled GTPase (Nalbant et al., 2004). Solvent-polarity sensing dye is attached to a small binding domain (also labeled with EGFP).

(Continued)

basic image processing methods, useful for single-chain as well as dual-chain versions of the ratiometric FRET-biosensors. The approaches we outline here will give relativistic readouts of FRET ratios in cells expressing the biosensor. The ratiometric measurements of a system in which the FRET donor and acceptor halves are placed within single-chain system (Fig. 25.1A, B, and F–H) allows for the use of FRET/CFP ratio as a simple estimate of the relative FRET, as the concentrations of the FRET donors and acceptors are stoichiometrically equimolar everywhere within a cell (Miyawaki et al., 1997). However, it is important to note that the resulting ratiometric data is nonlinear in relation to the FRET efficiency changes; therefore, such data will be useful for observing the relativistic differences in FRET ratio within a single cell, or in an experiment performed under the same conditions using the same optical microscopy system. There are more rigorous methods available (Bastiaens & Squire, 1999; Hinde, Digman, Welch, Hahn, & Gratton, 2012; Zal & Gascoigne, 2004) in which the microscopy modality as well as the processing are performed in terms of the efficiency of FRET, that is, involving fluorescence lifetime imaging, etc. However, for routine purposes and for relativistic comparison purposes, the traditional ratiometric approach is still highly efficient and useful, especially if correct assumptions are made and users are aware of its limitations as well as its capabilities.

25.1 IMAGE PROCESSING METHODS

25.1.1 “Single-chain” systems

Ratiometric biosensor systems of the “single-chain” design broadly includes: (1) single-chain fluorescent protein–FRET-based systems, (2) sensors that require ratiometric calculations in which the intensity modulating fluorophore signal is ratioed against a constant intensity fluorophore signal, wherein both fluorophores are attached on the same molecule, or (3) single fluorophore that modulates the fluorescence emission intensities at two different emission wavelengths which are then

Figure 25.1—Cont’d Upon binding to active GTPase, the local solvent polarity changes due to the binding event, thus the fluorescence emission intensity from the dye changes. By monitoring the ratio between the dye intensity modulation and the EGFP emission, the activation states of the endogenous GTPase can be observed. (F) Cameleon biosensor for calcium ion concentration measurements (Miyawaki et al., 1997). (G) Substrate-based biosensor for focal adhesion kinase (FAK) (Seong et al., 2011). This biosensor uses a substrate which the activated FAK will phosphorylate, and this changes the conformation within the molecule to affect FRET. The substrate-based biosensor detects the local “balance” of the upstream kinase and phosphatase activities; thus, they are not strictly measuring the activities of kinases. (H) FAK biosensor that detects the activation of the molecule through sensing the conformational change (Cai et al., 2008). In the strict sense, this biosensor detects the “activation” of FAK through sensing the exposure of the kinase domain (“K”).

ratioed. For the purpose of this section, the procedure for the CFP–YFP FRET biosensor based on single-chain design will be described. The extension of the approach to these other systems should be straightforward.

25.1.1.1 Flatfield correction (shading correction)

We routinely use an arc-lamp-based illumination system for the widefield imaging of FRET-biosensors (Hodgson, Nalbant, Shen, & Hahn, 2006; Spiering & Hodgson, 2012). In this case, the arc-lamp condenser and fluorescence illumination train introduce shading effects in the field of view (FOV) where the center of the FOV is brighter than the periphery of the FOV (Fig. 25.2). This effect can be minimized by a careful adjustment of the condenser focus setting, or the use of other illumination modes including solid-state and laser illumination systems coupled to correctly configured beam expanders. Therefore, it is advisable to assess the extent of the field illumination unevenness prior to initiating any FRET biosensor imaging by simply mounting a chamber containing some imaging media and take a set of images of the blank FOV. In order to assess the extent of the field unevenness, make a linescan measurement diagonally across the FOV and observe the resulting intensity values across the FOV (Fig. 25.2). Any deviations from uniformity require the correction outlined here. The basic equation for the flatfield corrections is:

$$[\text{IMAGE}]_{\text{Corrected}} = \frac{\{[\text{IMAGE}]_{\text{Raw}} - [\text{DC}] + \overline{[\text{DC}]} \} \times [\text{SF}]}{\{[\text{SHADE}] - [\text{DC}] + \overline{[\text{DC}]} \}}. \quad (25.1)$$

The three components necessary for this correction are: (1) Data image stack for the CFP and FRET ([IMAGE]), (2) corresponding shading images in CFP and FRET ([SHADE]), (3) dark-current image set for the exposure times used in CFP

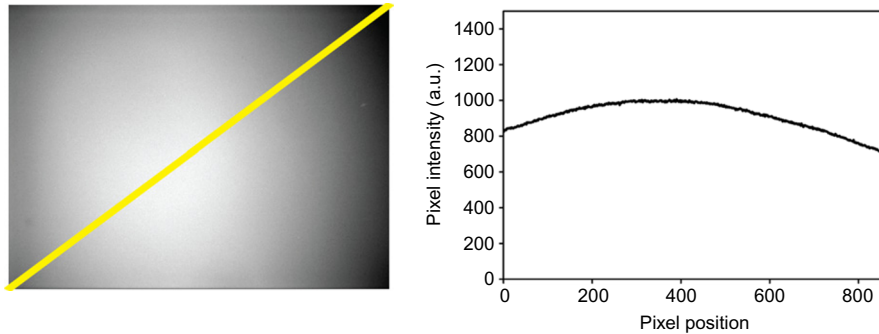


FIGURE 25.2

Shading present in the field of view. (Left) An empty field of view is imaged, and the linescan measurement (yellow diagonal line) is shown. (Right) The corresponding linescan measurement of the empty field of view.

and FRET image acquisitions ([DC]; $\overline{[DC]}$ denotes average pixel intensity value of the [DC] image used), and (4) a scaling factor ([SF]) to account for the floating point considerations. Following the calculation of the flatfield correction, a 3×3 median filter is applied in order to eliminate any hot pixels.

25.1.1.1.1 Shading images

Shading images are images of the blank FOV taken at *identical* conditions and settings (neutral density filters used, camera exposure times, and the CCD sensor mode used) as the actual data sets. Things that can directly affect the shading within an FOV are the focus location, relative amount of the lens immersion oil used and the volume and the type of media that exists above the specimen plane. All of these factors contribute to creating and affecting the specific shading of the data images in widefield microscopy and all must be accounted for. Here, a good practice is to keep a log notebook of the excitation neutral density filters and exposure times used for each time-lapse data set taken for CFP and FRET as one proceeds with the imaging experiments throughout the day. Another key point is the use of frame averaging when obtaining the shading images. We routinely take 20 FOVs and average in order to produce one shading correction image. This greatly reduces the stochastic noise (Hodgson et al., 2006).

25.1.1.1.2 Dark-current correction images

Dark-current correction images are taken at the exact exposure times used for each of CFP and FRET, but without any illumination. When obtaining these images, it is best to completely shut out the illumination paths; we routinely manually close off the fluorescence illumination train by using a sliding manual shutter and flipping the bottom camera port mirror to prevent any stray light entry into the CCD. These images correct for the thermal noise and readout noise associated with the CCD camera operation. The frame averaging is useful, and we routinely take 20 FOVs and average to produce the dark-current correction images for CFP and FRET. When Eq. (25.1) is used, the average pixel value of the dark-current correction image is added back as a single constant to both the raw data image and the shading image after the dark-current subtraction. This is done in order to maintain the pedestal intensity value within the FOV the same as prior to the operation.

25.1.1.1.3 Scaling factor

Scaling factor is used to account for the floating point calculation operation. Two things that determine the choice of the scaling factor are: (1) the typical intensity differences between the average intensity value within the shading image compared to the brightest intensity pixels in the data image and (2) the 16-bit depth in which the computations and the resulting tiff images must reside. The first point depends on the bit depth of the digitizer used in the CCD, whether this is 12, 14, or 16 bits, on the amount of autofluorescence in the medium, and the expression levels of the biosensor. Typically, the maximum possible value within the appropriate bit range is divided by the average intensity value of the shading image. This value in our system is usually in the order of 10–20. Therefore, in order to stay within the 16-bit space,

the scaling factor of 1000 is chosen. Once this value is set, one should be consistent for both the CFP and FRET data sets.

25.1.1.2 Background subtraction

Flatfield corrected, median-filtered images are then processed for background subtraction. Because of the flatfield correction, the cell-free areas within the FOV should have more or less uniform intensity level (Fig. 25.3). Select a small area away from the cell where there is no background debris and measure the average intensity value within such an area. Here, it is important to scroll through the time-lapse stack of images to make sure that no debris or cell protrusions come into the selected region as a function of time. The measured background intensity values will be different from frame to frame in a time-lapse series (due possibly to photobleaching of the autofluorescence in the imaging media, addition of stimulation media, etc.); therefore, it is important to measure the average value at each time point and subtract the

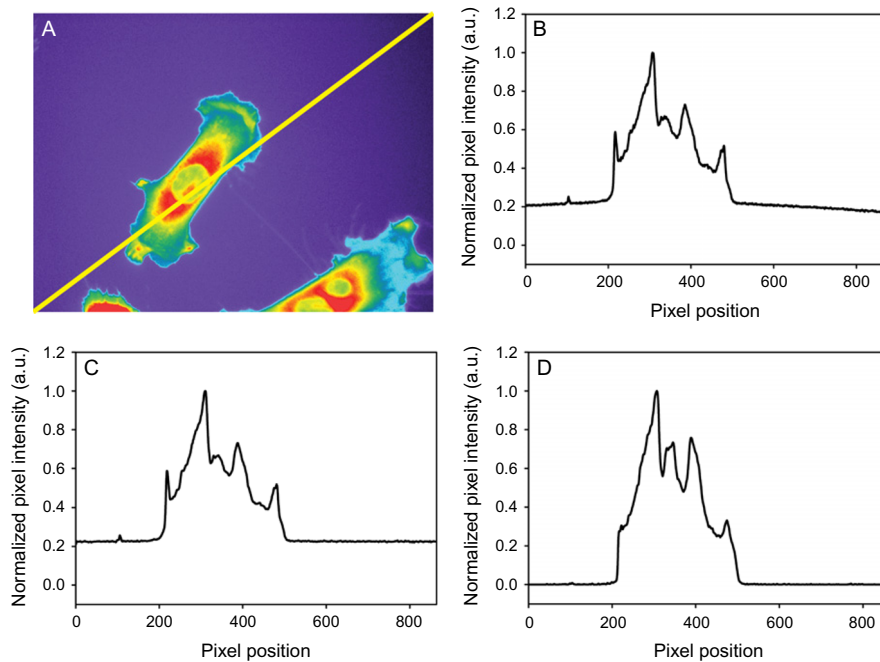


FIGURE 25.3

Effect of the shading correction on the background of images. (A) Raw image of a cell expressing a biosensor is shown. The yellow line corresponds to the linescan measurement location. (B) Linescan measurement of (A). (C) Linescan measurement of (A) after shading correction. (D) Linescan measurement of (A) after shading correction and background subtraction.

appropriate intensity value from each image plane in a time series. This means that one cannot simply measure the background value from the first image plane and subtract such a value from all image planes in a time-lapse series.

25.1.1.3 Morphing

In order to achieve a pixel-by-pixel matching of the CFP and FRET channels of the data set, a careful alignment of the data set is essential. When using multiple cameras to capture simultaneously the CFP, FRET, and any other fluorescence channels (Spiering & Hodgson, 2012), mounting of the cameras assigned to each channel will never be perfect; even the relative magnifications at each camera port could be different and these effects are compounded since different wavelengths of emission channels will impart differential lateral chromatic effects as a function of the location within a FOV. Even if a single camera is used to acquire sequentially CFP and FRET channels, the lateral chromatic dispersion will be dependent on the emission wavelengths so the pixel-by-pixel matching using computational methods is desired. In order to address this, a field of multispectral beads (Tetraspeck beads, Invitrogen) is used to produce a set of calibration images in CFP and FRET channels. Such calibration images are used to construct a set of morphing filters using programs such as Matlab, to result in pixel-by-pixel matching accuracy of sub-1/10th of a pixel (Hodgson et al., 2010). Particle tracking (Crocker & Grier, 1996) based morphing algorithm for Matlab which is useful for achieving this can be provided through contacting the authors of this chapter.

25.1.1.4 Image masking

In preparation for ratio calculations, the CFP and FRET image sets should be intensity thresholded and masked. The basic idea of masking is to set the regions that are outside of the cell uniformly zero, as the background subtraction of a single averaged value from every pixel in the FOV does not necessarily produce uniformly zero values outside the cell due to stochastic nature of the intensity distribution within the background regions. If the background regions are not masked to set equal to zero, noisy appearance in the ratio will be a problem (Fig. 25.4). Because of the changing total fluorescent intensity levels inside the cell as a function of time due primarily to photobleaching, images at the beginning of the time-lapse series are usually much brighter than those at the end of a time series. If a single intensity threshold value is used to produce the mask, it is likely to result in either overselection or underselection of cell area depending on how the thresholding is performed. To overcome this, we typically equalize the average intensity levels in a whole time series using image processing software, and use the equalized time series to threshold in order to produce the binary mask with the least amount of impact from the changing intensity levels due to photobleaching. Once such threshold value is determined, then a binary mask (pixel values inside the cell area is one and outside is zero) is produced for every time point in the time-lapse series. This series of binary masks is then multiplied into the CFP and FRET image sets to produce the masked CFP and FRET image sets. Here, it is important to produce the binary masks independently for CFP and FRET. Alternatively, an automated thresholding and masking (segmentation) approach is also available (Shen et al., 2006).

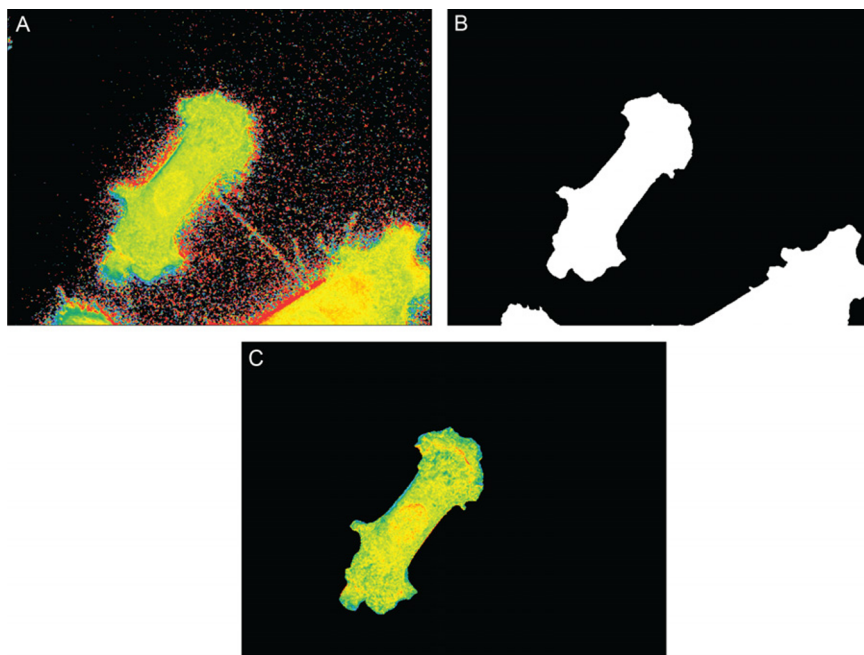


FIGURE 25.4

Effect of masking on the final ratio image. (A) Ratio image without masking. (B) Binary mask created using the intensity-based thresholding. (C) Ratio image after applying the binary mask to the data.

25.1.1.5 X–Y translational registration

We apply the X–Y subpixel translational registration to morphed and masked CFP and FRET image sets to correct for any image centering differences. This stems from the mounting of the two cameras used for simultaneous acquisition being not perfectly centered in the FOV with respect to each other. In situations, where the morphing-based registration may not be critical (single camera used, dual-excitation single-emission dye used, emission filters placed within the infinity space of the microscope), X–Y subpixel registration may also be sufficient in some cases. We use a previously published approach to achieve subpixel registration using an automated algorithm, available through contacting the authors of this chapter ([Shen et al., 2006](#)). Since this method is based on the optimization of the global cross-correlation coefficient between the two masked images, it works the best if there is only one cell within the FOV to attain the matching between two image sets.

25.1.1.6 Ratio calculation

Once the registration is complete, FRET image is divided by the CFP image. Here again, the floating point operation consideration is necessary. We previously showed the effect of the scaling factor on the resulting ratio histogram distribution ([Hodgson](#)

et al., 2006). Here, the dynamic range of the biosensor response between all-on versus all-off is important to consider when choosing a scaling factor. Our FRET-biosensors typically result in the dynamic range of 1:2–1:25, depending on the design and the construction. Therefore, we routinely use 1000 as the scaling factor to stay within the 16-bit depth.

25.1.1.7 Photobleach correction

The photobleaching effect of the resulting ratio image set depends on the rates of FRET donor and acceptor photobleaching during a time-lapse imaging session. Thus, this effect can be optimized by choosing photostable variants of fluorescent proteins. We have previously reported a method to correct for photobleaching of the Rho family GTPase biosensors during live cell time-lapse imaging (Hodgson et al., 2006). Here, the key point is to recall and to consider carefully the assumptions associated with this approach involving the biexponential fit method that we applied to our Rho GTPase sensors previously (Hodgson et al., 2006). The Rho family GTPases is such that the great majority of the GTPases are kept in an inactive state in complex with GDI, and only less than 5% of the total GTPase is activated at spatially distinct locations at any given time (Del Pozo et al., 2002). It is only possible to assume the biexponential intensity decay model because of this biological feature of the Rho family GTPases; where a great majority of the biosensor will remain in an inactive state and thus bleaching at a constant rate. This allows for a direct measurement of the average intensity change per time in the ratio data to approximate the rate of photobleaching by a biexponential function. When using a fluorescent biosensor other than for the Rho family GTPases, the biological regulation of such molecule must be carefully considered in order to determine whether or not this assumption holds.

25.1.2 “Dual-chain” systems

Ratiometric biosensor systems of the “dual-chain” design (i.e., Rac1 and RhoA sensors; Machacek et al., 2009) require additional calibration steps in order to account for the nonequimolar distribution of the FRET donor and acceptor halves of the biosensor components. This involves constructing calibration plots using cells expressing only the CFP or the YFP, and measuring the relative bleedthroughs of each signal into the FRET emission channel upon respective CFP or YFP excitation. The calibration curve is used to determine the amounts of bleedthrough and these values are then subtracted from the raw FRET intensity data to produce the corrected FRET images. Because of the need for such calibrations due to the nonequimolar distribution of the sensor halves, we image in sequence a set of CFP, FRET, and YFP images at every time point of a time-lapse series. The same calibrations outlined here can be applied to single-chain biosensor data sets to reduce the equimolar-associated assumptions or in cases if other analysis techniques including the efficiency-FRET (Zal & Gascoigne, 2004) are required so as to translate the measured relative ratios into absolute FRET transfer efficiencies.

25.1.2.1 Quantitative bleedthrough calibration

Because of the nonequimolar distribution of the FRET donor and acceptor halves of the biosensor in a given location within a cell, one must account for the amounts of bleedthroughs.

- a. CFP bleedthrough into the FRET channel: After the completion of imaging, calibration is performed using cell expressing only CFP. Here, images are taken at identical conditions as the actual imaging experiments, and the CFP and the FRET channel emissions are measured.
- b. YFP excitation by CFP excitation light: Here, calibration is performed using cells expressing only YFP. Images are taken at identical conditions as the actual imaging experiments, and the CFP-excited FRET channel emission is measured followed by YFP channel direct excitation and emission. The YFP emission into the CFP emission channel is usually negligible so we do not carry this quantity over in our calculations.
- c. Once the above set of images are obtained, they are processed (dark-current correction, flatfield correction, and background subtraction), and the pixel-by-pixel measurements are made to correlate between: (1) CFP calibration: FRET channel intensity per CFP channel intensity and (2) YFP calibration: FRET channel intensity per YFP channel intensity. These correlations are then linearly regressed through the origin (0,0) and the slope of the corresponding regression function becomes the correction factor with which to determine the bleedthroughs for each channel, according to the following equation:

$$[\text{FRET}]_{\text{Corr}} = [\text{FRET}]_{\text{Raw}} - \alpha[\text{CFP}] - \beta[\text{YFP}] \quad (25.2)$$

where α is the slope of the CFP calibration correlation and β is the slope of the YFP calibration correlation. Methods to automate this process are available.

25.1.2.2 Ratio versus $[\text{FRET}]_{\text{Corr}}$

When bleedthrough corrections are made as above in [Section 1.2.1](#), the resulting $[\text{FRET}]_{\text{Corr}}$ can be used to study the total FRET occurring in a cell. When this is ratioed against CFP and the CFP is attached to the GTPase, then the ratio is indicative of the relative FRET per localization of the GTPase. Both of these measures are useful in interpreting biological readouts such as whether the accumulation of $[\text{FRET}]_{\text{Corr}}$ is due to per GTPase activity levels being elevated (higher Ratio) or the spatial accumulation of modestly activated GTPase (lower Ratio) may be contributing to the increased $[\text{FRET}]_{\text{Corr}}$ (in the absence of elevated Ratio). These parameters should both be used to interpret the observed activation patterns.

25.1.2.3 Photobleach correction

A similar assumption as the single-chain sensor case applies here for the GTPase biosensor. Other types of sensors must be carefully considered in terms of the biological function so as to determine whether or not the observed photobleach rate can be attributed to majority of inactive material passively bleaching. The biexponential

correction should be directly applicable to $[\text{FRET}]_{\text{Corr}}$; however, correction of the Ratio ($[\text{FRET}]_{\text{Corr}}/[\text{CFP}]$) requires additional considerations. The Ratio is based on the following (Vanderklish et al., 2000):

$$R = \frac{[\text{FRET}]_{\text{Corr}}}{[\text{CFP}]} = \frac{[\text{FRET}]_{\text{Raw}} - \alpha[\text{CFP}] - \beta[\text{YFP}]}{[\text{CFP}]} \quad (25.3)$$

Rearranging Eq. (25.3) yields:

$$R = \frac{[\text{FRET}]_{\text{Raw}}}{[\text{CFP}]} - \beta \frac{[\text{YFP}]}{[\text{CFP}]} - \alpha \quad (25.4)$$

In Eq. (25.4), α is a constant therefore simply measuring the biexponential decay pattern of the $[\text{FRET}]_{\text{Raw}}/[\text{CFP}]$ and $[\text{YFP}]/[\text{CFP}]$ parameters, correcting them based on the fits, and linearly combining the corrected fractions with the appropriate α and β parameters, one can obtain the photobleach corrected Ratio. Algorithm to automate this is available.

25.2 IMAGING CONSIDERATIONS AND CAVEATS, PITFALLS

25.2.1 How to keep cells happy

The key to keeping cells alive and happy during imaging is rigorous light management. In order to avoid photodamaging cells, it is best to select the minimal intensity of excitation light to achieve the desired signal saturation in the CCD dynamic range (80% saturation of the dynamic range of the CCD) within reasonable exposure times. We routinely place neutral density filters in the excitation light path to significantly attenuate the light intensity. The usual target for the exposure times for our biosensor is approximately 600–1000 ms to achieve 80% saturation of the CCD dynamic range. We find that to achieve the same level of signal saturation levels, longer exposures at lower light intensity tend to minimize photodamage compared to rapid exposures using high intensity excitation light. This is due to the difference in the rate of reactive oxygen species production between the two conditions and in the slower situation, cells appear to process these oxygen radicals more effectively (Hodgson et al., 2010).

25.2.2 Minimizing photobleaching

Photobleaching of fluorescent material stems from the production of reactive oxygen species and how such oxygen radicals attack the fluorophore rendering it in nonfluorescent state. Therefore, it is critical to minimize spurious irradiation. We find it useful to set up a trigger device controlling the shutter system to rapidly open/close the fluorescence shutter and then place this device near the microscope controls. When trying to locate cells using the eyepiece, immediately close the shutter upon finding a potential cell target and switch to DIC for fine tuning the stage placement, focus, etc. In the DIC transillumination pathway, we use LED-based system with defined light

wavelength spectra that do not overlap with any of the excitation spectra of the fluorophores used to prevent spurious fluorescence excitation. Additional methods to control photobleaching includes bubbling the media with Argon gas to displace the dissolved oxygen and then to use Oxyfluor reagent (www.oxyrase.com) to remove oxygen from the media to minimize the formation of the reactive oxygen species. Such aggressive oxygen management may not be ideal or applicable in situations where long imaging duration is required. However, for imaging conditions where rapid kinetics is measured in the time scales of seconds to minutes, these approaches are useful.

25.2.3 Motion artifacts

When taking ratiometric image sets with a single camera where filter wheels are used to switch between different excitation/emission conditions, the time it takes to complete a single cycle of data acquisition can result in artifactual ratio signals simply from the motion of the cells. The use of simultaneous acquisition using multiple cameras, or the use of CCD image splitting device (dual/quad-view: photometrics) will eliminate these issues. However, if only a single camera system is available, one can estimate the extent of such motion effects by simply obtaining: (1) CFP, (2) FRET, and (3) CFP; in one data acquisition cycle. Comparing the ratio patterns of FRET (2) to the first CFP (1) acquired versus the last CFP (3) acquired, should be indicative of how much motion artifacts may be present in the ratiometric data. Once the extent of the motion artifact is determined, it is important to also keep the order of image acquisitions the same between each experiment so as to maintain this effect consistent between data sets.

25.2.4 Thickness artifacts

Depending on the distribution patterns of the biosensor within a cell and how the active versus the inactive compartments partition in different parts of a cell (i.e., plasma membrane vs. cytoplasmic, etc.), there will be a dilution effect in measured ratio values as a function of the cell thickness. In a traditional two-dimensional microscopic imaging, a three-dimensional cell is projected onto a 2D image plane; therefore, information in the z -dimension is lost and integrated into a single point in the plane. When examining cellular protein activities using biosensors, it is desirable to correct for the effects of cell thickness, especially for membrane bound proteins that specifically partition between membrane and bulk compartments as a function of protein activation. For the GTPase biosensor, because it is a membrane-active protein, this correction is not a simple division by the cell thickness to normalize for the thickness and is therefore more complex. The model we generated is:

$$\text{FRET} = \text{FRET}_m + \text{FRET}_c \quad (25.5)$$

$$\text{CFP} = \text{CFP}_m + \text{CFP}_c, \quad (25.6)$$

where FRET and CFP are the actual fluorescence of FRET and CFP measured by each CCD pixel in a camera, FRET_m is the plasma membrane FRET fluorescence, and FRET_c is cytoplasmic FRET fluorescence, CFP_m is the plasma membrane CFP fluorescence, and CFP_c is cytoplasmic CFP fluorescence. From Eqs. (25.5) and (25.6), for the volume above the pixel area:

$$\text{FRET} = \text{FRET}_m + \rho_{\text{FRET}}Ah \quad (25.7)$$

$$\text{CFP} = \text{CFP}_m + \rho_{\text{CFP}}Ah, \quad (25.8)$$

where ρ_{FRET} and ρ_{CFP} are the fluorescence densities of FRET and CFP (cytoplasmic, inactive state of the biosensor), respectively, A is the pixel area, and h is the thickness of the cell at the pixel coordinate. From empirical image data, FRET/CFP has a maximum value of α in a cell. The maximum value occurs at the thinnest part of the cell, and assuming this part of the cell has negligible cytoplasm:

$$\text{FRET}_m = \alpha \text{CFP}_m \quad (25.9)$$

Therefore, from Eqs. (25.7) and (25.8):

$$\frac{\text{FRET}}{\text{CFP}} = \frac{\alpha \text{CFP}_m + \rho_{\text{FRET}}Ah}{\text{CFP}_m + \rho_{\text{CFP}}Ah} \quad (25.10)$$

The cell thickness effect can be elucidated by plotting FRET/CFP versus h as shown in Eq. (25.10). Figure 25.5 shows the result of fitting the experimental data to

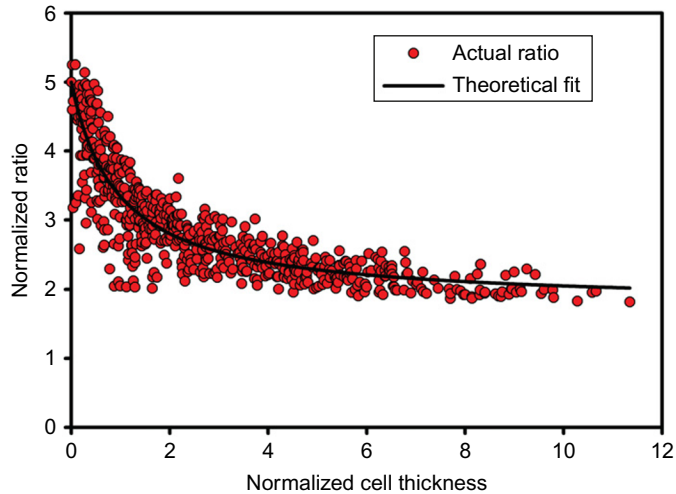


FIGURE 25.5

Effect of cell thickness of biosensor ratio measurements. The normalized ratio values at different regions of interests within a single-cell data were plotted against the normalized cell thickness indicator fluorescence intensity (red dots). The theoretical model derived in Eq. (25.10) was used to fit the data (solid line).

Eq. (25.10). As can be seen from the figure, the cell thickness has a dilution effect on sensing GTPase activity. With this relation known, a thickness indicator, such as a fluorescent dextran, can be introduced into the cell to measure h , and protein activity can be corrected accordingly.

SUMMARY

Here, we presented an overview of the procedures to follow when analyzing the image data from single-chain and bimolecular FRET-biosensors for the Rho family GTPases. The automation journals for the Metamorph software, as well as the computer codes written for Matlab program are available to automate almost all parts of the quantitative processing. These codes can be obtained by contacting the authors.

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