INTRODUCTION

Green fluorescent protein (GFP) is a true workhorse of modern cellular biology. GFP and its spectral mutants have been used primarily as passive fluorescent markers to monitor the location of tagged polypeptides within living cells and organisms. A relatively recent but important trend has been the development of GFP-based active indicators of cellular processes and microenvironments (Tsien 1998). It is now possible to measure fluctuations in the concentration of cellular ions (Miyawaki et al. 1997; Romoser et al. 1997; Miesenböck et al. 1998), to monitor the activity of proteases (Pollok and Heim 1999), to assess the translational and rotational motion of polypeptides (Yokoe and Meyer 1996; Swaminathan et al. 1997), to observe misfolding and aggregation of polypeptides (Waldo et al. 1999), to show differences in potential across electrically active membranes (Siegel and Isacoff 1997), and, of interest to readers of this volume, to detect protein–protein interactions.

Fluorescence resonance energy transfer (FRET) between appropriately chosen spectral variants of GFP has been used successfully to detect protein–protein interactions in vivo as well as in vitro (see Chapter 10). FRET is well suited for monitoring heterotypic protein–protein interactions but suffers from some drawbacks when monitoring homotypic protein interactions. This
chapter introduces GFP-PRIM (Proximity Imaging), a technique specializing in the detection of homotypic protein interactions.

Detecting Homotypic Protein Interactions with FRET

FRET requires that two proteins of interest be fused, respectively, to a fluorescence donor (typically cyan fluorescent protein, CFP) and a fluorescence acceptor (typically yellow fluorescent protein, YFP). If the two proteins interact, the close proximity of CFP and YFP facilitates energy transfer from excited CFP to YFP. The efficiency of energy transfer is highly dependent on the distance separating the chromophores and on the relative orientation of their transition dipoles. Energy transfer can be monitored by a decrease in the ratio of cyan to yellow fluorescence emission by the complex following excitation of CFP (see Chapter 10). Heterotypic protein–protein interactions are more readily detected than homotypic interactions when using FRET. If two different proteins respectively labeled with CFP and YFP interact stably and with high affinity, every complex formed will contain the fluorescence donor and acceptor in close proximity (Fig. 1A). Thus, all heterotypic protein interactions can result in a FRET signal if the separation between CFP and YFP is suitable. In contrast, if two versions of a protein capable of stable and high-affinity homodimerization (labeled with CFP and YFP, respectively) are incubated together, proximity of the donor and acceptor fluorophores will occur in 50% at most of complexes formed (Fig. 1B). The remaining 50% will consist of interactions that do not produce FRET (CFP/CFP or YFP/YFP).

Detecting Homotypic Protein Interactions with GFP-PRIM

Recently, we reported that tagging a protein with a GFP variant can provide clues about the protein’s oligomeric status (De Angelis et al. 1998). The particular mutant used for these experiments

![Figure 1. Detection of heterotypic protein–protein interactions versus homotypic interactions with FRET. Energy transfer from CFP to YFP is indicated by a YFP with jagged contour. (A) Heterotypic protein–protein interactions between protein X and protein Y bring CFP and YFP in close proximity 100% of the time. (B) Detection of homotypic protein interactions with FRET between CFP and YFP. Only 50% of homotypic interactions involving protein X will bring CFP and YFP together; the rest will either bring CFP with CFP or YFP with YFP.](image-url)
is thermotolerant GFP (ttGFP) (Siemering et al. 1996). ttGFP is essentially wild-type (wt) GFP with two amino acid substitutions (V163A and S175G) that promote efficient folding and maturation of the protein at 37°C, likely by minimizing the aggregation tendency of wt GFP (Yokoe and Meyer 1996; Fukuda et al. 2000). ttGFP has two excitation peaks with respective maxima at 395 nm and 475 nm (see Fig. 2A). Illumination of the protein at wavelengths within either peak results in the emission of green light (508 nm) characteristic of GFP. The ratio of the two excitation maxima (the excitation ratio, also $R_{395/475}$) is the same for any monomeric ttGFP fusion protein when measured in the same buffer and at constant temperature. When ttGFP is fused to a protein that is capable of self-association, the excitation ratio in the homo-oligomeric state can differ from that of the monomeric state in a characteristic way, depending on the attached protein (Fig. 2B). PRIM exploits these spectral changes to detect the extent of self-association of ttGFP-tagged proteins in vitro and in vivo.

**FIGURE 2.** Detection of homotypic protein interactions with PRIM. (A) Excitation spectrum of ttGFP fused to FK506 binding protein (FKBP, labeled F in the diagram) obtained by reading the intensity of green fluorescence emitted at 508 nm as a function of excitation wavelength from 350 to 500 nm in a Perkin Elmer LB-50 spectrofluorometer. The spectrum displays two excitation peaks, with maxima at 395 nm and 475 nm. The excitation ratio ($R_{395/475}$) of this fusion protein and every ttGFP fusion protein in the monomeric state is 5.0 at 25°C in phosphate-buffered saline. (B) Excitation spectrum of ttGFP-FKBP following addition of the dimerizer drug FK1012 (dumbbell-shaped molecule). Dimers isolated from gel permeation chromatography display a $R_{395/475}$ of 0.5, which is a tenfold change from the monomeric ttGFP-FKBP excitation ratio. The change in shape of excitation spectrum is represented by a change in the shape of the ttGFP molecule.
Differences between PRIM and FRET

PRIM and FRET are fundamentally very different (outlined in Table 1). The emission ratio changes measured with FRET derive from quantum mechanical interactions between any spectrally matched fluorescence donor and acceptor. Efficient interactions between CFP and YFP always result in a decrease in the CFP/YFP emission ratio; fluorescence changes are therefore unidirectional with respect to proximity. In contrast, PRIM is specific to some GFP mutants displaying both 395-nm and 475-nm excitation peaks. Current evidence suggests that the excitation ratio changes measured by the method result from direct structural interactions between two ttGFP moieties (De Angelis et al. 1998). The changes in PRIM are bidirectional: Homotypic interactions between ttGFP-labeled proteins can result in either increases or decreases from the baseline monomeric $R_{395/475}$. Although further studies are required to elucidate the precise mechanism giving rise to GFP-PRIM, it is easy to see the advantages of this method over FRET for the detection of homotypic protein interactions. FRET requires CFP-labeled and YFP-labeled versions of the protein, and these must be expressed at similar levels. Moreover, only half of the homotypic protein interactions will contribute to a CFP-YFP FRET signal, as mentioned above (Fig. 1). In contrast, PRIM only requires expression of the candidate protein labeled with ttGFP, and every homotypic interaction contributes to the signal.

GFP-PRIM can monitor changes in the degree of self-association of a given protein in vitro and in vivo. Ideal candidates for a GFP-PRIM experiment are proteins that can exist in one of two states (monomeric or self-associated, whether dimeric or trimeric or tetrameric, etc.) and that undergo self-association or dissociation in response to a given stimulus. There are two prerequisites for a successful GFP-PRIM experiment: (1) Experimental conditions must exist under which monomers can be resolved from homo-oligomers and (2) the excitation ratios of ttGFP obtained in each of these states must differ sufficiently. In these cases, PRIM can be a very powerful method to monitor the dynamics of homotypic interactions. The following sections outline the major steps of carrying out GFP-PRIM experiments in vitro by spectrofluorometry and in vivo by excitation ratio imaging of live cells.

**TABLE 1. Summary of the Principal Differences between FRET and PRIM**

<table>
<thead>
<tr>
<th>FRET</th>
<th>PRIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Requires two GFP mutants of different colors. The emission of the FRET donor has to overlap with the excitation of the FRET acceptor.</td>
<td>Requires ttGFP, which has two excitation maxima (395 nm and 475 nm).</td>
</tr>
<tr>
<td>Readout is a change in the emission ratio of the donor/acceptor pair upon proximity.</td>
<td>Readout is a change in the excitation ratio of ttGFP upon proximity.</td>
</tr>
<tr>
<td>Proximity always results in a decrease of the donor/acceptor emission ratio (unidirectional).</td>
<td>Proximity can result in either an increase or a decrease in the excitation ratio of ttGFP (bidirectional).</td>
</tr>
<tr>
<td>Relative orientation of the donor and acceptor within a complex affects the efficiency of the FRET signal.</td>
<td>Relative orientation of the two copies of ttGFP within a complex determines both the efficiency and the direction of the PRIM signal.</td>
</tr>
<tr>
<td>Quantum mechanical interaction between matched donor/acceptor fluorophore pair.</td>
<td>Structural interaction between adjacent ttGFP molecules.</td>
</tr>
<tr>
<td>Ideal to measure heterotypic protein–protein interactions.</td>
<td>Ideal to measure homotypic protein interactions.</td>
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GFP-PRIM In Vitro: Measuring the Excitation Ratio by Spectrofluorometry

Determination of the Dynamic Range of PRIM

Spectral changes seen with GFP-PRIM are not predictable a priori: On dimerization, the $R_{395/475}$ of a ttGFP-labeled polypeptide can either increase, decrease, or stay the same. The outcome ultimately depends on the relative orientations of the ttGFP moieties within a homotypic complex. To maximize the probability of success, it is advisable to construct both amino- and carboxy-terminal ttGFP fusions of the protein of interest and to test them independently. In principle, insertion of ttGFP within the polypeptide is also feasible when one or both termini must remain free to preserve function of the candidate protein. The excitation ratio of the resulting chimeric proteins is measured in a spectrofluorometer by recording the intensity of green light emitted at 508 nm following illumination of the protein solution at 395 nm and 475 nm. The quotient of these intensities is the excitation ratio or $R_{395/475}$. An excitation spectrum, as shown in Figure 2, is obtained by recording the intensity of 508-nm light emitted as a function of increasing excitation wavelength from 350 nm to 500 nm.

If the excitation ratio of the ttGFP fusion protein differs from ttGFP alone, chances are that the protein is capable of a certain degree of self-association. The extent of self-association can be determined biochemically by gel filtration chromatography, a method that separates proteins in the native state according to molecular weight (Fig. 3). Theoretical and practical aspects of this technique, also known as size exclusion chromatography or gel permeation chromatography, have been reviewed previously (Stellwagen 1990). Gel filtration achieves two important goals: (1) to resolve monomers from homo-oligomers, thus allowing their excitation ratios to be independently measured, and (2) to determine the exact proportion of ttGFP–protein X in the monomeric and oligomeric state. When relatively crude extracts are applied on sizing columns, both the size and the excitation ratio of ttGFP–protein X can be determined by spectrofluorometry of the collected fractions. When in the monomeric state, every ttGFP-labeled protein tested so far has the same excitation ratio as ttGFP alone. In most cases, the $R_{395/475}$ of homo-oligomers differs significantly from the monomeric $R_{395/475}$ (De Angelis et al. 1998). The dynamic range of PRIM for a given ttGFP fusion protein—a quantitative measure of the difference in excitation ratio between each state—is obtained by dividing the $R_{395/475}$ of the monomer by that of the homo-oligomer (or the reciprocal if the result is below 1).

Knowing the excitation ratios of 100% homo-oligomeric and 100% monomeric ttGFP fusion proteins allows one to calculate the relative fraction of monomeric protein from a mixed population displaying an intermediate $R_{395/475}$ value, using the equation:

$$R_{395/475} \text{ [mixed population]} = x \left(R_{395/475} \text{ [monomer]}\right) + \left(1 - x\right) \left(R_{395/475} \text{ [homo-oligomer]}\right)$$

where $x$ is the relative fraction of monomers and $\left(1 - x\right)$ is the relative fraction of homo-oligomers. This can only be calculated if there is a single homo-oligomeric state of the protein (dimeric or trimeric, etc.). A greater dynamic range will enable a more accurate determination of the proportion of the ttGFP–fusion protein in each state. With such information, the kinetics of self-association or dissociation of the protein following stimulation by a test substance can be assayed in a spectrofluorometer.
Sensitivity of the Excitation Ratio to Variables Other Than Proximity

The excitation ratio of wt GFP can vary with changes in temperature, pH, and ionic strength (Ward et al. 1982); this is also true of ttGFP. Although these parameters may not change significantly over time during the assay of a given sample, care should be exercised in keeping them as uniform as possible from sample to sample.

A more important concern is that under certain illumination conditions, ttGFP undergoes photoisomerization. This is manifested by a decrease in the intensity of the first excitation peak.
and a reciprocal increase in that of the second peak, resulting in a gradual decrease in excitation ratio over time. Light in the UV range is particularly potent at causing photoisomerization, as is illumination at the 395-nm peak, albeit to a lower extent (Cubitt et al. 1995). This phenomenon can be minimized by (1) reducing the intensity of light applied to the sample and (2) illuminating it for shorter periods of time (by shuttering off excitation between data acquisition). In cases where this is impractical, the excitation ratio change of a test sample must be normalized to that of a control sample that does not undergo changes in self-association under the same illumination conditions. This will distinguish between excitation ratio changes due to photoisomerization and those due to changes in self-association.

**Experimental Controls, False Negatives, and False Positives**

Whether the assays are conducted with pure protein or crude extracts, several controls should be included in GFP-PRIM experiments. Subjecting ttGFP to the same challenge as the ttGFP–fusion protein should not result in a change in excitation ratio. If possible, a fusion between ttGFP and a non-oligomerizing mutant of the protein should also be included; such a protein should not undergo a change in $R_{395/475}$. Another negative control consists of challenging the ttGFP-labeled protein with an inactive version of the oligomerizing agent. Such negative controls are necessary to correct for the effects of photoisomerization on the excitation ratio (discussed above). Competition between ttGFP-labeled and nonlabeled versions of the polypeptide should also be performed. Inducing self-association in the presence of increasing amounts of the nonlabeled protein should result in a progressive decrease in the PRIM signal.

Similarity between the excitation ratio of ttGFP and the ttGFP-labeled protein does not rule out the possibility of protein homo-oligomerization. False negatives could occur, for instance, if the ttGFP subunits within a homotypic complex are too far apart to interact, or if their relative orientation is such that structural interactions between them fail to generate a PRIM signal. Independently testing two versions of the polypeptide with ttGFP fused at either terminus will help maximize the chances of success.

False positives have not yet been encountered; however, they are conceivable because of the structural (as opposed to spectral) requirement for generating a PRIM signal. One can imagine a ttGFP–fusion protein in which part of the attached protein folds back onto the surface of ttGFP, such that a change in excitation ratio is now caused intramolecularly (between the protein and ttGFP) instead of intermolecularly (between adjacent ttGFP modules).

At high concentrations, purified wt GFP has a tendency to form a specific dimer (Ward et al. 1982; Palm et al. 1997). The dimerization tendency is diminished in so-called “folding” variants of GFP that harbor the V163A point mutation, such as ttGFP (Yokoe and Meyer 1996). In their pioneering work, Ward and colleagues reported increases in the excitation ratio of highly concentrated wt GFP. Interestingly, proximity of ttGFP induced via fusion to certain proteins (such as glutathione-S-transferase, GST) also results in an increase in excitation ratio relative to the monomer. The relative geometry of ttGFP moieties in a ttGFP-GST dimer, therefore, might resemble that of the specific wt GFP dimer obtained at high concentrations or seen in some of the crystal structures (see, e.g., Yang et al. 1996). However, fusion of ttGFP to other dimerizing proteins causes decreases in $R_{395/475}$ (e.g., ttGFP-FKBP; Fig. 2). This completely opposite spectral behavior suggests that proximity of two ttGFP molecules does not necessarily lead to the formation of a specific dimer analogous to the one formed at high concentrations of wt GFP. Although the tendency for ttGFP to form specific dimers is lower than that of wt GFP, the protein concentration should be kept constant from assay to assay to avoid possible misinterpretations of changes in $R_{395/475}$.
GFP-PRIM in Live Cells: Imaging Homotypic Protein Interactions

The point of a GFP-PRIM experiment is to show dynamic changes in homotypic interactions brought about by a challenge—usually an externally administered substance. A series of 410-nm and 470-nm image pairs is acquired prior to stimulus application, to determine the baseline excitation ratio (Fig. 4B). During recording, a solution of the test substance can be applied by careful pipetting onto the cells. More complex perfusion setups are also available (see, e.g., Rieder and Cole 1998). Additional image pairs can then be recorded for the estimated duration of the response (the appropriate amount of time will vary for each ttGFP–fusion protein, but can range from a few minutes to several hours). Excitation ratios can be calculated and displayed during image acquisition (on-line), or after all primary images have been collected (off-line). The series of ratio images produced displays the spatiotemporal dynamics of homotypic interactions of ttGFP–protein X in pseudocolor over the recorded interval. (For an example, see De Angelis et al. 1998, Fig. 3.)

MATERIALS

A list of the principal components and a brief description of their function follows (the specific models used in our laboratory are indicated in parentheses).

Instrumentation

- Computer equipped with software to control the image acquisition and perform the image analysis (Metamorph/Metafluor 3.0 software; Universal Imaging)
- Dichroic mirror and bandpass filter combination to capture the green light emitted from ttGFP while rejecting the 410-nm and 470-nm excitation wavelengths (respectively, 500DCXR and HQ535/550; Chroma Technology)
- Experimental chamber with temperature controller (Medical Systems Corp.)
- Light source capable of emitting light of the wavelengths to be ratioed, with an output that can be coupled to a microscope (Polychrome II grating monochromator fitted with a 50-W Xenon lamp; Till photonics)
- Low-light-level camera (Pentamax 512EFT frame transfer camera with fiber-coupled Gen IV image intensifier; Princeton Instruments cooled 12-bit EEV 37 charge-coupled device chip)
- Microscope (Zeiss Axiovert 135TV)
Cuvette assays with intact cells are complicated by the fact that cells have a high level of intrinsic autofluorescence that can interfere with, or even mask, the PRIM signal. This hinders a precise assessment of the extent of self-association in live cells by spectrofluorometry. The spatiotemporal dynamics of homotypic interactions between ttGFP-labeled proteins can be analyzed in single live cells using excitation ratio imaging. In vivo proximity imaging experiments are conceptually similar to in vitro assays using a spectrofluorometer: In both cases, the excitation ratio of a ttGFP-labeled protein is followed over time after application of a stimulus. Using microscopy, additional information can be gathered concerning the subcellular localization of homotypic interactions within live cells.

The light path is more complex in microscopy than in spectrofluorometry: Light needs to travel through several optical components before and after the sample, prior to forming an image that can be digitally acquired, stored, and analyzed. The objectives must be corrected for spherical and chromatic aberrations. The light source must provide flat and even illumination for the whole field of view. A thorough discussion of excitation ratio imaging systems is beyond the scope of this chapter, and the reader is referred to more exhaustive reviews of the subject (Bright et al. 1989; Dunn and Maxfield 1998; Silver 1998).

METHOD

Recording the Excitation Ratio from Live Cells

In a typical experiment:

1. Grow cultured cells expressing a specific protein fused to ttGFP as a monolayer on glass coverslips.
   
   The fusion protein can be introduced into cells by a variety of means (usually transfection, infection, or microinjection).

2. For imaging, place a coverslip into the experimental chamber in defined medium and identify a field containing one or more positive cells.

3. For every ratio, acquire two digital images by illumination of the sample at 410 nm and 470 nm (Fig. 4A).

   These wavelengths, slightly shifted from the 395 nm and 475 nm used in the spectrofluorometer, were empirically determined to be optimal for imaging ttGFP in our system (Miesenböck et al. 1998). Because each ratio represents one time point in the experiment, the image pair has to be acquired in rapid succession (within a few milliseconds at most).

4. The two images are produced as pixel arrays stored on the computer hard drive; each pixel has a location and an intensity value. Background correction of images is necessary. After subtracting the average intensity of a region devoid of fluorescence from the entire image, the excitation ratio is subsequently obtained by dividing the intensity of the 410-nm image with the 470-nm image on a pixel-by-pixel basis. The resulting ratios are displayed on a third image, typically color-coded from red (high) to blue (low) ratios.

Determination of the Dynamic Range In Vivo

In intact cells, monomeric and multimeric ttGFP–fusion proteins cannot be readily resolved as is the case in vitro (described above), where proteins can be fractionated according to size. For this reason, the respective excitation ratios of the ttGFP–fusion protein in the monomeric and multimeric state should be determined in vitro prior to embarking on an imaging experiment whenever possible. In cases where this is impractical, control conditions that induce maximal and minimal self-association of the candidate protein, respectively, should be known a priori for the cell type under study. The two excitation ratios thus obtained will represent the upper and lower lim-
its of the PRIM signal for this system. Again, a large dynamic range will facilitate the detection of small shifts in the proportion of monomeric versus multimeric \textit{ttGFP}–fusion protein. The dynamics of self-association initiated by the application of different test substances will be detectable in real time and with spatial resolution.

### Experimental Controls and Caveats for In Vivo Imaging

Experimental controls for GFP-PRIM in vivo are the same as in vitro (see above): No change in $R_{410/470}$ should result from treatment of cells with an inactive version of the oligomerizing agent. Cells expressing \textit{ttGFP} alone, or \textit{ttGFP} fused to a version of the protein incapable of self-association, should not undergo changes in $R_{410/470}$.

If a \textit{ttGFP}–fusion protein capable of homodimerization is introduced into cells that endogenously express the protein, interactions between the labeled and nonlabeled versions of the protein will occur. These interactions will not result in a PRIM signal, because two \textit{ttGFP} moieties are required to produce an excitation ratio shift. To maximize the strength of the PRIM signal, the \textit{ttGFP}–fusion protein should be expressed at a level higher than that of the endogenous protein. Cells with different expression levels of the \textit{ttGFP}–fusion protein might display different excitation ratios, even when maximal self-association is induced. In these cases, cells with similarly high expression levels should be selected and analyzed.
The precautions taken with the sample in vitro with spectrofluorometry also apply with in vivo cell imaging. The same medium should be used for imaging, and the temperature should be kept constant. Photoisomerization must be minimized by both (1) shuttering off illumination between image acquisitions and (2) using as little light intensity as possible to illuminate the sample (the use of very sensitive intensified cameras helps reduce the amount of illumination required to produce an image). To correct for the effects of photoisomerization, images from untreated samples must be acquired under the same exact conditions as test samples. The ratio changes due to photoisomerization will then be distinguishable from those due to changes in homo-oligomerization status.

CONCLUSION

PRIM and FRET are two techniques that complement one another in the study of protein–protein interactions. FRET is best suited for the detection of heterotypic protein interactions, whereas PRIM is specialized for the study of homotypic protein interactions.

Conditions for a successful GFP-PRIM experiment are twofold. First, the monomeric state must be experimentally resolvable from the multimeric state. For in vitro experiments by spectrofluorometry, this can be achieved by sizing cellular extracts by gel filtration. For cellular imaging, conditions must be known a priori under which the protein will be maximally monomeric or maximally self-associated in the cells of interest. Second, the excitation ratios of monomers must differ sufficiently from multimers. When these two conditions are met, PRIM is a very powerful method to detect homotypic protein interactions in real time. Structural studies are clearly required to understand fully the basis for excitation ratio changes seen with PRIM. Despite the fact that the magnitude and direction of ratio change cannot be predicted a priori for any protein that can self-associate, the method can nevertheless be useful for monitoring the dynamics of homotypic protein–protein interactions in vivo and in vitro.

REFERENCES


