 MCB, vol.1, no.2, pp.133-145, 2004

Illuminating the Dynamics of Intracellular Activity with 'Active' Molecular Reporters

A. Tsourkas and R. Weissleder[1]

Abstract: Traditionally, fluorescent and luminescent reporter proteins have been used as indicators of gene expression and protein localization. However, insightful mutagenesis and protein engineering strategies have transformed these simple passive reporters into active biological sensors. Molecular reporters are now being designed to alter their intrinsic optical properties in response to specific biomolecular interactions. Applications for these novel biological sensors range from monitoring intracellular pH and ion fluxes to detecting protein-protein interactions and enzymatic activity. The ability to monitor the dynamics of intracellular activity in response to external stimuli can help elucidate the cascade of events involved in complex processes such as mechanotransduction. Here we review some of the approaches used to create these novel biological sensors, including resonance energy transfer (RET) between reporter proteins and protein fragmentation strategies.

keyword: Imaging, GFP, luciferase, reporter, sensor, probe

1 Introduction

Over the past few decades, it has become well established that many types of mammalian cells not only respond to chemical stimuli but also mechanical stimuli. Mechanotransduction can lead to changes in cell morphology, growth rate, metabolism, and molecular expression and activity (Davies, 2002, Lehoux and Tedgui, 2003, Shyy and Chien, 2002). Insightful and systematic studies on cultured cells under mechanical loading have provided important information regarding many of the signaling pathways responsible for cell remodeling; however, this controlled setting may not always correlate well to a complex in vivo environment. Further, many approaches used for studying cellular responses to mechanical loading involve fixed time points, but since cells are very dynamic in nature some intracellular mechanisms could easily be overlooked. Recent advancements in molecular imaging techniques provide novel approaches to help study the dynamics of intracellular activity in vivo and could be used to help elucidate the intricacies of the mechanotransduction pathway (Stamatas and McIntire, 1999, Weissleder and Ntziachristos, 2003).

Molecular imaging is based on the development of novel molecular probes that can be used to identify specific biomolecules, molecular interactions, or enzymatic activity (Jaffer and Weissleder, 2004, van Roessel and Brand, 2002, Weissleder and Mahmood, 2001, Zhang, et al., 2002). In general, molecular probes can be synthesized either chemically (i.e. on the bench top) or genetically (within the cell). Both approaches have proven to be valuable and in many cases the information attained is complementary. Chemically based probes, which often consist of a contrast reagent linked to a targeting molecule or substrate, are introduced exogenously and are typically used to identify anatomical and pathological signs of disease. Alternatively, genetic or molecular reporters (i.e. green fluorescent protein, luciferase, and thymidine kinase), which are synthesized by intracellular transcriptional and translational machinery, are typically used to study the complex spatiotemporal dynamics of intracellular activity.

The introduction of molecular reporters into cells provides a simple means to study transcriptional control mechanisms by reporting the activity of promoter and activator elements. Moreover, molecular reporters allow for the visualization of protein transport and localization by serving as optical tags for proteins of interest (Lippincott-Schwartz and Patterson, 2003, Weijer, 2003). Mutagenesis and the discovery of a diverse array of molecular reporters (and substrates in the case of luciferase) with unique spectral properties have further allowed for the visualization of multiple processes simultaneously via multi-channel imaging (Contag and Ross, 2002, Hadjantonakis, et al., 2003, Hu and Kerppola,

[1] CMIR, Harvard-MGH, Boston, MA, U.S.A.

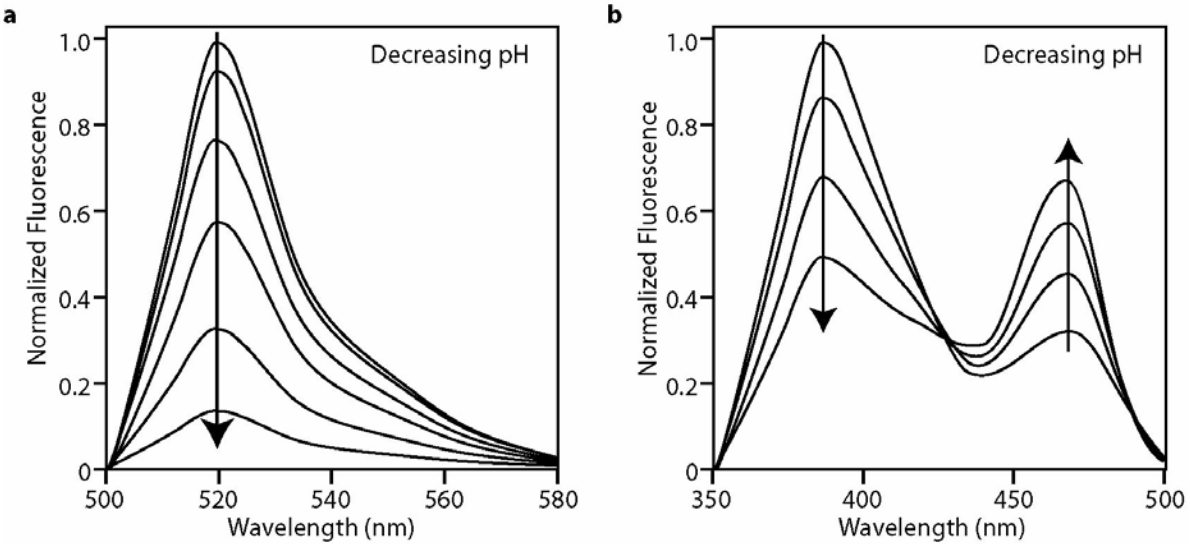

Figure 1 : **pH Sensitive Molecular Reporters.** Random mutagenesis of GFP-variants has led to the development of biological reporters capable of monitoring pH. (a) In general changes in pH are determined by a measurable reduction in fluorescent intensity. (b) However, a special class of pH sensitive molecular reporters known as ratiometric pHluorins exhibit a spectral shift in response to changes in pH.

2003, Kato, et al., 2002, Shah, et al., 2003). Molecular reporters are now available with peak emission wavelengths ranging from UV-visible to far red (Fradkov, et al., 2000, Fradkov, et al., 2002, Gurskaya, et al., 2001, Heim and Tsien, 1996, Hyun Bae, et al., 2003, Matz, et al., 1999, Murphy and Lagarias, 1997, Rizzo, et al., 2004, Wiedenmann, et al., 2002).

Recently, the utility of molecular reporters has been dramatically expanded by the development of novel approaches to transform these somewhat passive labels into active indicators of enzyme activity, protein-protein interactions, and intracellular dynamics of small molecules (van Roessel and Brand, 2002, Zhang, et al., 2002). Reporter molecules are now being designed to alter their optical properties in response to specific external stimuli or biological processes. The ability of these novel biological sensors to provide real-time information on complex biochemical networks will undoubtedly have a profound impact on our understanding of cell behavior under diverse conditions. This review focuses on the strategies currently used to develop these biological sensors and highlights their utility over a wide range of applications including mechanotransduction.

2 Random mutagenesis

Perhaps, the most indiscriminate approach used to develop biological sensors is random mutagenesis of relatively 'inactive' molecular reporters. The introduction of several mutations into the coding sequence of fluorescent proteins derived from the *Aequorea victoria* can transform this simple molecular reporter into a noninvasive intracellular pH indicator (Elsliger, et al., 1999, Kneen, et al., 1998). Although the spectral properties of GFP are intrinsically sensitive to pH, several mutations can greatly enhance their biosensing capability over a broader range of pH values. Variants of GFP that were identified by random mutagenesis have been used to monitor pH anywhere from 5 to 9 in a reversible manner. Decreases in pH typically correspond to a decrease in fluorescent intensity (Fig. 1a), but pH-sensitive variants of fluorescent proteins also exist that undergo shifts in wavelength (i.e. ratiometric pHluorin) (Miesenbock, et al., 1998). Ratiometric pHluorins exhibit a decrease in fluorescence intensity at 410 nm and an increase in fluorescence at 470 nm as pH decreases (Fig. 1b). The pH can therefore be quantified directly in cells by determining the ratio of intensities at these two wavelengths and then comparing it to a calibration curve. pH-senstive fluorescent proteins have been used successfully to monitor

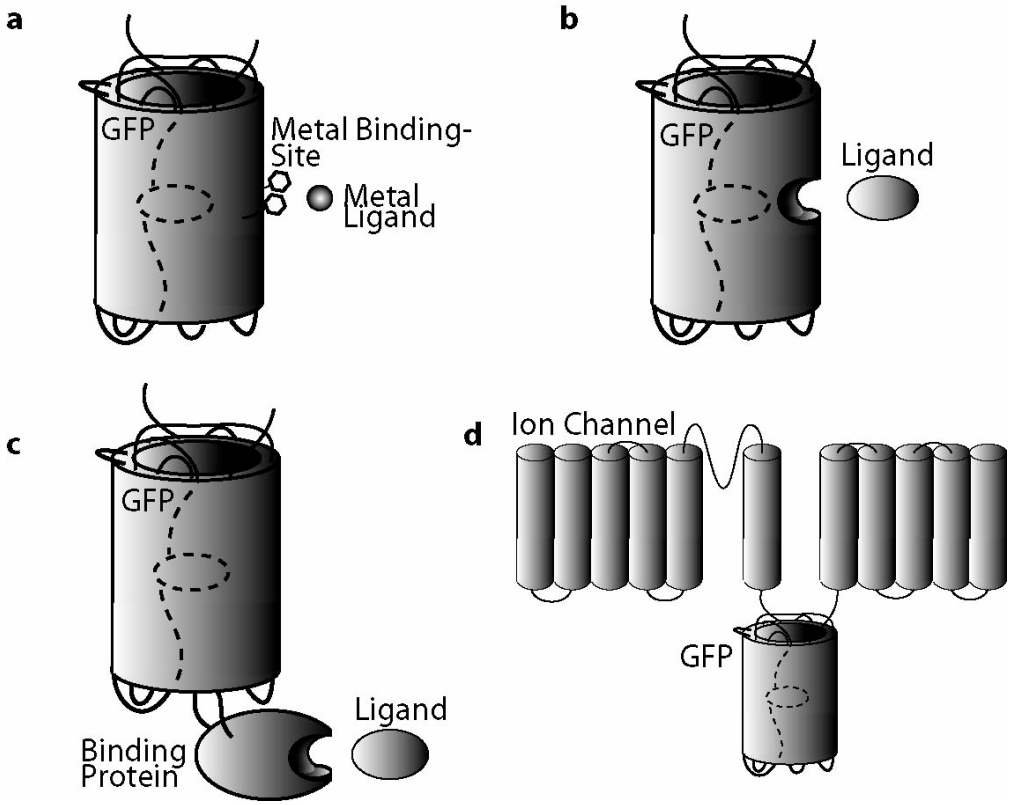

Figure 2 : **Protein Engineered Biological Sensors.** Several protein engineering strategies have been developed to transform GFP-variants into active biological sensors including (a) the introduction of metal binding sites into the b-barrel by site-selected mutagenesis (b) the insertion of small ligand binding sites into the b-barrel in proximity to the chromophore (c) the introduction of ligand binding sites into the loops connecting the b-strands and (d) N- and C- terminal fusions capable of mechanically perturbing the GFP-variant structure under diverse conditions.

cytoplasmic and organellar pH in individual cells under both steady-state conditions and in response to various external stimuli (Llopis, et al., 1998, Matsuyama, et al., 2000). They have also been used to monitor exocytosis and recycling of secretory and synaptic vessels at individual synaptic boutons (Miesenbock, et al., 1998).

In addition to serving as a pH indicator, it has been observed that mutants of GFP could be used to monitor ion concentrations. For example, increasing concentrations of halides or nitrates alter the intrinsic pKa of GFP-variants resulting in a reduction of fluorescence intensity (Wachter and Remington, 1999). Expression of the halide sensitive mutant has been utilized in cells for the sensitive monitoring of Cl^- transport across the plasma membrane in real-time (Jayaraman, et al., 2000). An alternate mutation can be used to transform GFP into a Zn^{2+} and Cu^{2+} biosensor (Barondeau, et al., 2002). Using this variant, the presence of Zn^{2+} results in up to a 2-fold fluorescence enhancement, while Cu^{2+} quenches the fluorescence.

3 Engineered scaffold (β-barrel) modifications

Although random mutagenesis has led to the development of several GFP-derived biosensors, the uncovering of the structure of GFP has allowed for a more systematic approach to designing activatable reporters. One tactic involves creating a binding-site in the β-barrel that encircles the chromophore. Typically, the introduction of a compound into close proximity of the chromophore quenches the fluorescence. For example, when two histidines were precisely incorporated into the β-barrel, GFP became a very sensitive indicator of Cu^{2+} concentration (Fig. 2a) (Richmond, et al., 2000). The presence of Cu2+ resulted in up to a 90% decrease in fluorescence

intensity.

Careful genetic engineering of the GFP scaffold has also allowed for the introduction of larger ligand binding sites into the β-barrel (Fig. 2b). The incorporation of a lipopolysaccharides and Lipid A binding site, consisting of a string of 5 amino acids, into one of the β-strands allowed for the successful detection of 100 pg/μL of lipopolysaccharide and 4 ng of Lipid A (Goh, et al., 2002). This biological sensor was further used to tag gram-negative bacteria in contaminated water sampled from the environment (Goh, et al., 2002).

Amazingly, the β-barrel of GFP has even been engineered to serve as the basis for a redox switch by the simple insertion of a pair of cysteine residues into the appropriate locations (Hanson, et al., 2004, Ostergaard, et al., 2001). Formation of a disulfide bond under oxidative conditions caused a strain in the polypeptide backbone that resulted in more than a 2-fold decrease in fluorescence. Subsequent reduction of the disulfide bond resulted in the restoration of fluorescence.

4 Engineered loop modifications

As an alternative to modifying the β-barrel, it has been found that modifications to the loop regions connecting the β-strands is also a viable approach for transforming GFP into an active biosensor. For example, a protease sensitive GFP variant was constructed by inserting a protease specific cleavage site into one of the loops (Chiang, et al., 2001). Proteolysis, subsequently, resulted in a 40% reduction in fluorescence. Loop regions of GFP have also been modified to include entire ligand-binding sites (Fig. 2c). When TEM1 β-lactamase was inserted into a loop region of GFP and subsequently exposed to directed evolution, one variant underwent an increase in fluorescence upon binding to β-lactamase-inhibitory protein (Doi and Yanagawa, 1999).

5 N- and C-terminal fusions

Traditionally, N- and C-terminal protein fusions of GFP are used to study protein trafficking and localization, however, under special circumstances fusion proteins can also serve as biological sensors. For example, When GFP was inserted into a loop domain of either a sodium or potassium channel, depolarization of the ion channel induced recordable changes in fluorescence, most likely due to mechanical perturbations (Fig. 2d) (Ataka and Pieribone, 2002, Siegel and Isacoff, 1997). Another example is the cytochrome b_{562}-GFP fusion protein (Takeda, et al., 2001, Takeda, et al., 2003). The reconstitution of cytochrome b_{562} with hemin resulted in nearly a 70% reduction in fluorescence due to fluorescence resonance energy transfer (FRET).

Luciferase activity can also be modulated by N- and C-terminal fusions. When estrogen receptor (ER) regulatory domains were fused to either side of the *Firefly* luciferase protein, the bioluminescent signal was quenched up to 90% (Laxman, et al., 2002). Cleavage of protease substrates inserted between the ER regulatory domains and the luciferase enzyme resulted in the restoration of bioluminescence. The high sensitivity of bioluminescent assays, due to the absence of an excitation light and subsequent autofluorescence, allowed for the detection of caspase-3 activity in animal models.

6 Intramolecular FRET

Although the fusion of any protein to the N- and/or C-terminus of GFP can potentially yield a biological sensor, a more versatile approach is to flank a catalytic substrate or a binding domain by two fluorescent proteins that form a FRET pair. Thus, any conformational change incurred by ligand binding or catalytic activity alters the FRET efficiency between the two fluorescent proteins and can be detected by the corresponding shift in the emission spectrum. This approach can be applied to a wide range of applications and often allows for quantitative measurements. Perhaps, the simplest example of a fluorescent protein-based biosensor utilizing an intramolecular FRET mechanism consists of a protease substrate flanked by two fluorescent proteins (Fig. 3a). Cleavage of the protease substrate leads to the dispersion of the two fluorescent proteins and a loss of FRET. These protease probes have given insight into a number of cellular activities including the spatial and temporal activation of specific members of the caspase family during apoptosis (Harpur, et al., 2001, Luo, et al., 2001, Luo, et al., 2003, Mahajan, et al., 1999, Rehm, et al., 2002, Xu, et al., 1998). Other applications include the detection of calpain activity in dendritic spines following a synapse and the presence Factor X_a or tobacco etch virus poteases in biological samples (Kohl, et al., 2002, Mitra, et al., 1996, Vanderklish, et al., 2000).

As mentioned previously, the introduction of a ligand-binding domain between two GFP-variants can also

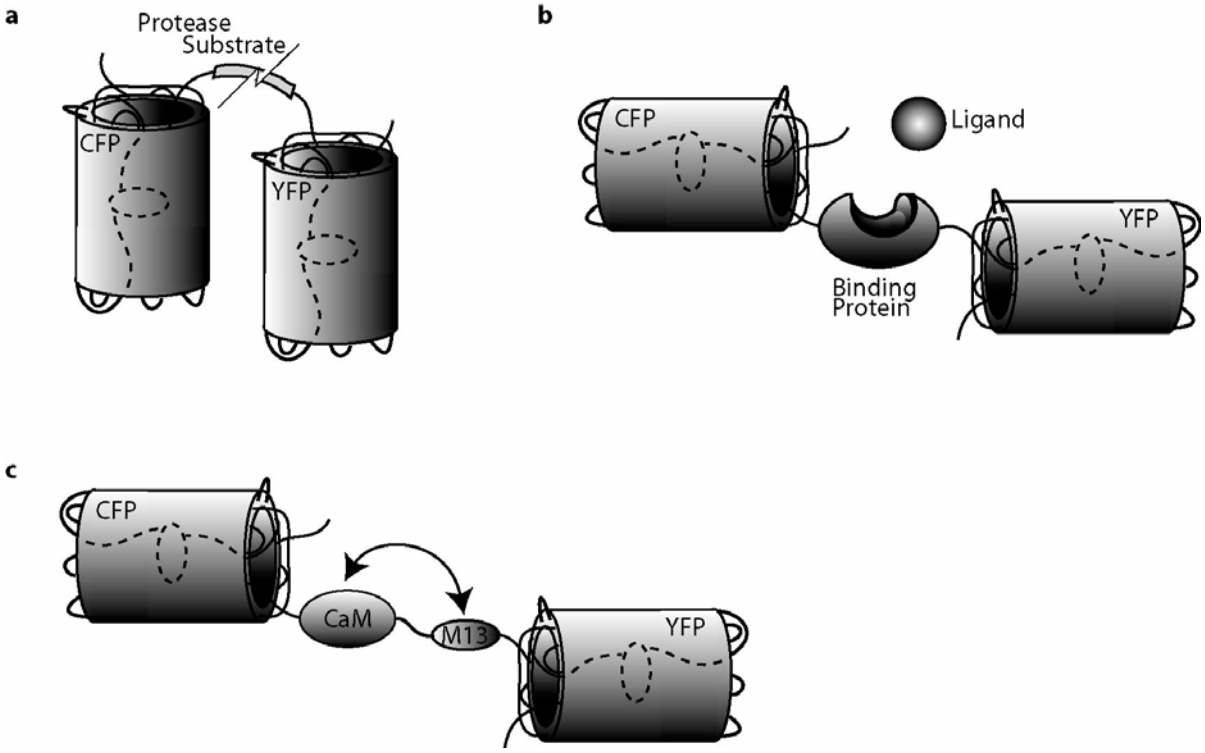

Figure 3 : **Intramolecular FRET-Based Biosensors.** Intramolecular FRET-based biosensors consist of a catalytic domain flanked by two molecular reporters with overlapping excitation-emission spectrums (e.g. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP)). Catalytic activity affects the relative orientation of the two reporters and can be monitored by the modulation in FRET efficiency. Possibilities for the catalytic site include (a) a substrate for proteases (b) a ligand binding protein (c) or a molecular substrate (i.e. calmodulin, CaM) in combination with a binding domain (i.e. M13), where the binding domain only binds to a modified form of the substrate. In this case, M13 is specific for Ca-calmodulin.

serve as a powerful approach to non-invasively observe the dynamics and localization of intracellular signaling molecules (Fig. 3b). For example, the insertion of a calmodulin-binding protein between two GFP-variants results in a spectral change upon the binding of $(Ca^{2+})_4$-calmodulin (Persechini and Cronk, 1999, Romoser, et al., 1997). Ligand-modulated FRET biosensors have also been designed to indicate the presence of glucose using a glucose binding protein (Ye and Schultz, 2003), cyclic GMP using cyclic GMP-dependent protein kinase (Honda, et al., 2001, Sato, et al., 2000), the small guanisine triphosphate RAN using a RAN binding domain (Kalab, et al., 2002), and numerous other ligand-binding protein combinations (Mochizuki, et al., 2001, Seth, et al., 2003).

A slightly modified strategy used to detect intracellular molecular activity involves the introduction of a substrate and a binding domain between two GFP-variants. The binding domain is selected such that it only binds the modified substrate. For example, a Ca^{2+} sensor was designed to consist of calmodulin and calmodulin binding peptide flanked by two GFP-variants (Fig. 3c) (Miyawaki, et al., 1999, Miyawaki, et al., 1997, Truong, et al., 2001). In the presence of Ca^{2+}, the calmodulin-Ca^{2+} complex wraps around the calmodulin binding peptide improving the efficiency of FRET. Intramolecular FRET-based biosensors have even proven capable of monitoring kinase activity in living cells. The simplest construct consists of a kinase inducible domain flanked by two GFP variants (Nagai, et al., 2000). Phosphorylation of the domain influences the FRET efficiency between the two GFP-variants. An alterative strategy involves the placement of both a phosphorylation substrate and a phosphorylation recognition domain between two

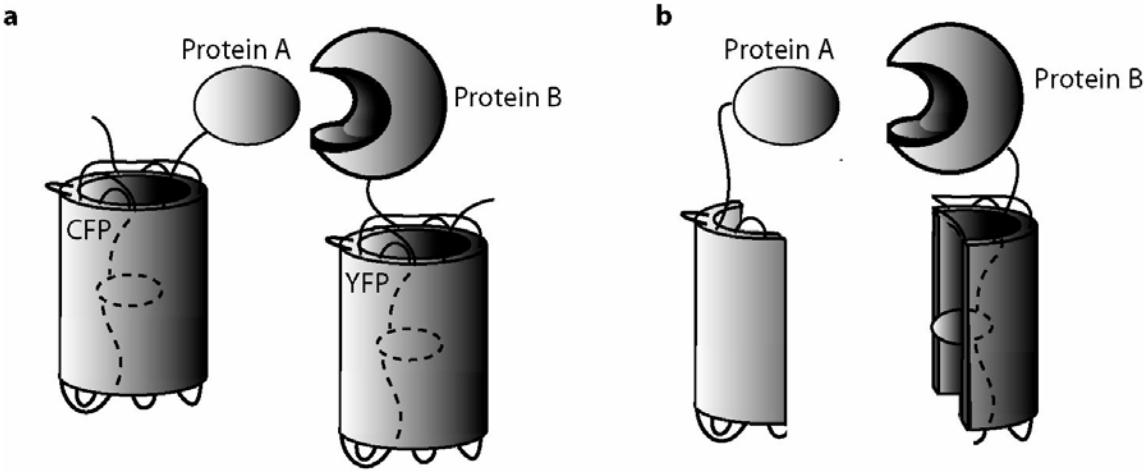

Figure 4 : **Visualizing protein-protein interactions.** Protein-protein interactions can be monitored by fusing two proteins of interest (i.e. protein A and protein B) to (a) molecular reporters that form a FRET pair or (b) to fragments of a single molecular reporter that restore functionality when brought into close proximity.

GFP-variants (Kurokawa, et al., 2001, Sato, et al., 2002, Ting, et al., 2001, Violin, et al., 2003, Zhang, et al., 2001). Upon phosphorylation the recognition domain binds the substrate and alters the FRET efficiency.

7 Intermolecular FRET/BRET

FRET based biosensors do not require the construction of a single fusion protein, but could also consist of two independent fluorescent moieties that undergo FRET when proteins to which they are tethered interact or co-localize (Fig. 4a). This approach has been used in a wide range of applications (Chan, et al., 2001, Erickson, et al., 2001, Jensen, et al., 2001, Kato, et al., 2002, Li, et al., 2001, Llopis, et al., 2000, Majoul, et al., 2001, Mas, et al., 2000, Schmid, et al., 2000, Siegel, et al., 2000, Sorkin, et al., 2000, Tertoolen, et al., 2001, Xia, et al., 2001) including monitoring the receptor-mediated dissociation of heterotrimeric G-proteins (Janetopoulos, et al., 2001), mapping interactions between nuclear transport factors (Damelin and Silver, 2000, Day, et al., 2001), and localizing the dissociation of protein kinase A subunits in response to cyclic AMP (Ruehr, et al., 1999, Zaccolo, et al., 2000, Zaccolo and Pozzan, 2002). It should be noted, however, that intermolecular FRET experiments are complicated by the possibility of unbalanced expression of donor and acceptor molecules and interactions of fusion proteins with endogenous proteins. To overcome these obstacles more sophisticated approaches must be undertaken to quantify FRET such as the mathematical processing of three images (Gordon, et al., 1998, Hoppe, et al., 2002), photo-bleaching the acceptor and measuring donor dequenching (Llopis, et al., 2000, Siegel, et al., 2000, Zacharias, et al., 2002), or fluorescence lifetime imaging (Harpur, et al., 2001, Ng, et al., 1999).

An advantage of intermolecular FRET is it allows for more flexibility in choosing FRET pairs. For example, proteins labeled exogenously with organic dyes could be used as either donors or acceptors. In one such case, a GFP-ErbB1 fusion protein was used as a donor and a Cy3 labeled antibody to phosphotyrosine was used as an acceptor in an experiment to image signal propagation in the plasma membrane (Verveer, et al., 2000). Another possibility is to use luciferase fusion proteins as donors resulting in bioluminescent resonance energy transfer (BRET) (Angers, et al., 2000, Baubet, et al., 2000, Boute, et al., 2002, Xu, et al., 1999). Bioluminescent proteins have the advantage that they do not require an excitation source allowing for less autofluorescence, no photobleaching, and easier quantification of resonance energy transfer (donor and acceptor concentrations can be determined independently by luminescence and fluorescence measurements, respectively). The lack of autofluorescence is particularly important in assays with populations of cells where autofluorescence can significantly impair the sensitivity of the assay. Fluorescent proteins, however, have the advantage of higher special resolution due

to the low emission intensity of bioluminescent proteins.

8 Protein Fragment Complementation

Protein fragment complementation provides another very novel approach for monitoring the dynamics of protein-protein interactions (Michnick, 2003). The strategy requires the fragmentation of a protein, such as a molecular reporter, into two or more components (Fig. 4b). The dissection must be conducted so as to avoid the undirected association of the fragments, while still retaining the ability to restore function when the fragments are held in close proximity. It is often necessary to tune the affinity by inserting several point mutations into the fragments. The fragments are brought into close proximity by the interaction between the proteins to which they are fused. The reconstitution of the fragmented protein is typically reversible but if desired the introduction of intein fragments into the fusion protein can result in the permanent splicing of the reporter (Ozawa, et al., 2001, Ozawa, et al., 2000, Ozawa, et al., 2001).

The fragmentation of GFP-variants has allowed for the study of known interactions such as that between the transcription factors bZIP and Rel and also for the uncovering of unknown interactions (Ghosh, et al., 2000, Hu, et al., 2002, Hu and Kerppola, 2003). For example, high-throughput screening methods have been implemented where one complementation fragment is fused to a known protein ('bait') and the second is fused to a library of unknown proteins ('prey') (Remy and Michnick, 2004). Colonies of cells where the 'bait' and 'prey' interact are identified by fluorescence. The simultaneously visualization of multiple protein-protein interactions can also be performed by conducting complementation assays with multiple optically distinct GFP-variants (Hu and Kerppola, 2003). Another option is to use reporters other than GFP-variants, such as β-galactosidase (Rossi, et al., 1997), β-lactamase (Galarneau, et al., 2002, Wehrman, et al., 2002), and *Firefly* and *Renilla* luciferase (Kaihara, et al., 2003, Paulmurugan and Gambhir, 2003, Paulmurugan, et al., 2004, Paulmurugan, et al., 2002). Luciferase constructs are particularly useful since they provide the benefit of being able to monitor protein-protein interaction in living subjects.

9 Circular Permutation

Circular permutation refers to the fragmentation of a gene into two components, a N-terminal fragment and the C-terminal fragment, and the subsequent fusion of the original N- and C-termini via a short peptide linker (Akemann, et al., 2001, Iwai, et al., 2001, Spotts, et al., 2002, Topell, et al., 1999). This new variant in many cases can exhibit unique optical properties or can retain the same properties, at least to some extent, as the wild-type protein. The addition of fusion proteins on the new N- and C-termini can then sometimes transform a circular permutated GFP into a biological sensor. For example, when calmodulin and its target peptide M13 were fused to the N- and C-termini of a circular permeated GFP construct, the presence of Ca^{2+} resulted in the reversible change of its spectral properties (Baird, et al., 1999, Nagai, et al., 2001, Nakai, et al., 2001).

10 Conclusion

The transformation of passive molecular reporters into active biosensors has provided unrivaled insight into the molecular dynamics of living cells under a diverse array of conditions. It is expected that the discovery of new molecular reporters with unique optical properties combined with novel approaches to transform them into biological sensors will continue to drastically improve our understanding of cell function. Of particular interest are probes that can be used for imaging molecular activity in living subjects. There, however, is still a clear need for the discovery and generation of new GFP-variants that are sensitive in the near infrared to allow for the observation of molecular activity in deeper tissues to complement luciferase biosensors. The ability to monitor molecular pathways and understand disease mechanisms in their natural environment will undoubtedly lead to significant insight into the cascade of events involved in the mechanotransduction pathway of endothelial cells.

References

Akemann, W.; Raj, C. D.; Knopfel, T. (2001): Functional characterization of permuted enhanced green fluorescent proteins comprising varying linker peptides *Photochem Photobiol*, vol. 74, pp. 356-63.

Angers, S.; Salahpour, A.; Joly, E.; Hilairet, S.; Chelsky, D.; Dennis, M.; Bouvier, M. (2000): Detection of

beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET) *Proc Natl Acad Sci U S A*, vol. 97, pp. 3684-9.

Ataka, K.; Pieribone, V. A. (2002): A genetically targetable fluorescent probe of channel gating with rapid kinetics *Biophys J*, vol. 82, pp. 509-16.

Baird, G. S.; Zacharias, D. A.; Tsien, R. Y. (1999): Circular permutation and receptor insertion within green fluorescent proteins *Proc Natl Acad Sci U S A*, vol. 96, pp. 11241-6.

Barondeau, D. P.; Kassmann, C. J.; Tainer, J. A.; Getzoff, E. D. (2002): Structural chemistry of a green fluorescent protein Zn biosensor *J Am Chem Soc*, vol. 124, pp. 3522-4.

Baubet, V.; Le Mouellic, H.; Campbell, A. K.; Lucas-Meunier, E.; Fossier, P.; Brulet, P. (2000): Chimeric green fluorescent protein-aequorin as bioluminescent Ca2+ reporters at the single-cell level *Proc Natl Acad Sci U S A*, vol. 97, pp. 7260-5.

Boute, N.; Jockers, R.; Issad, T. (2002): The use of resonance energy transfer in high-throughput screening: BRET versus FRET *Trends Pharmacol Sci*, vol. 23, pp. 351-4.

Chan, F. K.; Siegel, R. M.; Zacharias, D.; Swofford, R.; Holmes, K. L.; Tsien, R. Y.; Lenardo, M. J. (2001): Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein *Cytometry*, vol. 44, pp. 361-8.

Chiang, C. F.; Okou, D. T.; Griffin, T. B.; Verret, C. R.; Williams, M. N. (2001): Green fluorescent protein rendered susceptible to proteolysis: positions for protease-sensitive insertions *Arch Biochem Biophys*, vol. 394, pp. 229-35.

Contag, C. H.; Ross, B. D. (2002): It's not just about anatomy: in vivo bioluminescence imaging as an eyepiece into biology *J Magn Reson Imaging*, vol. 16, pp. 378-87.

Damelin, M.; Silver, P. A. (2000): Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex *Mol Cell*, vol. 5, pp. 133-40.

Davies, P. F. (2002): Multiple signaling pathways in flow-mediated endothelial mechanotransduction: PYKing the right location *Arterioscler Thromb Vasc Biol*, vol. 22, pp. 1755-7.

Day, R. N.; Periasamy, A.; Schaufele, F. (2001): Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus *Methods*, vol. 25, pp. 4-18.

Doi, N.; Yanagawa, H. (1999): Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution *FEBS Lett*, vol. 453, pp. 305-7.

Elsliger, M. A.; Wachter, R. M.; Hanson, G. T.; Kallio, K.; Remington, S. J. (1999): Structural and spectral response of green fluorescent protein variants to changes in pH *Biochemistry*, vol. 38, pp. 5296-301.

Erickson, M. G.; Alseikhan, B. A.; Peterson, B. Z.; Yue, D. T. (2001): Preassociation of calmodulin with voltage-gated Ca(2+) channels revealed by FRET in single living cells *Neuron*, vol. 31, pp. 973-85.

Fradkov, A. F.; Chen, Y.; Ding, L.; Barsova, E. V.; Matz, M. V.; Lukyanov, S. A. (2000): Novel fluorescent protein from Discosoma coral and its mutants possesses a unique far-red fluorescence *FEBS Lett*, vol. 479, pp. 127-30.

Fradkov, A. F.; Verkhusha, V. V.; Staroverov, D. B.; Bulina, M. E.; Yanushevich, Y. G.; Martynov, V. I.; Lukyanov, S.; Lukyanov, K. A. (2002): Far-red fluorescent tag for protein labelling *Biochem J*, vol. 368, pp. 17-21.

Galarneau, A.; Primeau, M.; Trudeau, L. E.; Michnick, S. W. (2002): Beta-lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions *Nat Biotechnol*, vol. 20, pp. 619-22.

Ghosh, I.; Hamilton, A. D.; Regan, L. (2000): Antiparallel leucine zipper-directed protein reassembly: application to the green fluorescent protein *J Am Chem Soc*, vol. 122, pp. 5658-9.

Goh, Y. Y.; Frecer, V.; Ho, B.; Ding, J. L. (2002): Rational design of green fluorescent protein mutants as biosensor for bacterial endotoxin *Protein Eng*, vol. 15, pp. 493-502.

Goh, Y. Y.; Ho, B.; Ding, J. L. (2002): A novel fluorescent protein-based biosensor for gram-negative bacteria *Appl Environ Microbiol*, vol. 68, pp. 6343-52.

Gordon, G. W.; Berry, G.; Liang, X. H.; Levine, B.; Herman, B. (1998): Quantitative fluorescence res-

onance energy transfer measurements using fluorescence microscopy *Biophys J*, vol. 74, pp. 2702-13.

Gurskaya, N. G.; Fradkov, A. F.; Terskikh, A.; Matz, M. V.; Labas, Y. A.; Martynov, V. I.; Yanushevich, Y. G.; Lukyanov, K. A.; Lukyanov, S. A. (2001): GFP-like chromoproteins as a source of far-red fluorescent proteins *FEBS Lett*, vol. 507, pp. 16-20.

Hadjantonakis, A. K.; Dickinson, M. E.; Fraser, S. E.; Papaioannou, V. E. (2003): Technicolour transgenics: imaging tools for functional genomics in the mouse *Nat Rev Genet*, vol. 4, pp. 613-25.

Hanson, G. T.; Aggeler, R.; Oglesbee, D.; Cannon, M.; Capaldi, R. A.; Tsien, R. Y.; Remington, S. J. (2004): Investigating Mitochondrial Redox Potential with Redox-sensitive Green Fluorescent Protein Indicators *J Biol Chem*, vol. 279, pp. 13044-53.

Harpur, A. G.; Wouters, F. S.; Bastiaens, P. I. (2001): Imaging FRET between spectrally similar GFP molecules in single cells *Nat Biotechnol*, vol. 19, pp. 167-9.

Heim, R.; Tsien, R. Y. (1996): Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer *Curr Biol*, vol. 6, pp. 178-82.

Honda, A.; Adams, S. R.; Sawyer, C. L.; Lev-Ram, V.; Tsien, R. Y.; Dostmann, W. R. (2001): Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator *Proc Natl Acad Sci U S A*, vol. 98, pp. 2437-42.

Hoppe, A.; Christensen, K.; Swanson, J. A. (2002): Fluorescence resonance energy transfer-based stoichiometry in living cells *Biophys J*, vol. 83, pp. 3652-64.

Hu, C. D.; Chinenov, Y.; Kerppola, T. K. (2002): Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation *Mol Cell*, vol. 9, pp. 789-98.

Hu, C. D.; Kerppola, T. K. (2003): Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis *Nat Biotechnol*, vol. 21, pp. 539-45.

Hyun Bae, J.; Rubini, M.; Jung, G.; Wiegand, G.; Seifert, M. H.; Azim, M. K.; Kim, J. S.; Zumbusch, A.; Holak, T. A.; Moroder, L.; Huber, R.; Budisa, N. (2003): Expansion of the genetic code enables design of a novel "gold" class of green fluorescent proteins *J Mol Biol*, vol. 328, pp. 1071-81.

Iwai, H.; Lingel, A.; Pluckthun, A. (2001): Cyclic green fluorescent protein produced in vivo using an artificially split PI-PfuI intein from Pyrococcus furiosus *J Biol Chem*, vol. 276, pp. 16548-54.

Jaffer, F. A.; Weissleder, R. (2004): Seeing within: molecular imaging of the cardiovascular system *Circ Res*, vol. 94, pp. 433-45.

Janetopoulos, C.; Jin, T.; Devreotes, P. (2001): Receptor-mediated activation of heterotrimeric G-proteins in living cells *Science*, vol. 291, pp. 2408-11.

Jayaraman, S.; Haggie, P.; Wachter, R. M.; Remington, S. J.; Verkman, A. S. (2000): Mechanism and cellular applications of a green fluorescent protein-based halide sensor *J Biol Chem*, vol. 275, pp. 6047-50.

Jensen, K. K.; Martini, L.; Schwartz, T. W. (2001): Enhanced fluorescence resonance energy transfer between spectral variants of green fluorescent protein through zinc-site engineering *Biochemistry*, vol. 40, pp. 938-45.

Kaihara, A.; Kawai, Y.; Sato, M.; Ozawa, T.; Umezawa, Y. (2003): Locating a protein-protein interaction in living cells via split Renilla luciferase complementation *Anal Chem*, vol. 75, pp. 4176-81.

Kalab, P.; Weis, K.; Heald, R. (2002): Visualization of a Ran-GTP gradient in interphase and mitotic Xenopus egg extracts *Science*, vol. 295, pp. 2452-6.

Kato, N.; Pontier, D.; Lam, E. (2002): Spectral profiling for the simultaneous observation of four distinct fluorescent proteins and detection of protein-protein interaction via fluorescence resonance energy transfer in tobacco leaf nuclei *Plant Physiol*, vol. 129, pp. 931-42.

Kneen, M.; Farinas, J.; Li, Y.; Verkman, A. S. (1998): Green fluorescent protein as a noninvasive intracellular pH indicator *Biophys J*, vol. 74, pp. 1591-9.

Kohl, T.; Heinze, K. G.; Kuhlemann, R.; Koltermann, A.; Schwille, P. (2002): A protease assay for two-photon crosscorrelation and FRET analysis based solely on fluorescent proteins *Proc Natl Acad Sci U S A*, vol. 99, pp. 12161-6.

Kurokawa, K.; Mochizuki, N.; Ohba, Y.; Mizuno, H.; Miyawaki, A.; Matsuda, M. (2001): A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo *J*

Biol Chem, vol. 276, pp. 31305-10.

Laxman, B.; Hall, D. E.; Bhojani, M. S.; Hamstra, D. A.; Chenevert, T. L.; Ross, B. D.; Rehemtulla, A. (2002): Noninvasive real-time imaging of apoptosis *Proc Natl Acad Sci U S A*, vol. 99, pp. 16551-5.

Lehoux, S.; Tedgui, A. (2003): Cellular mechanics and gene expression in blood vessels *J Biomech*, vol. 36, pp. 631-43.

Li, H. Y.; Ng, E. K.; Lee, S. M.; Kotaka, M.; Tsui, S. K.; Lee, C. Y.; Fung, K. P.; Waye, M. M. (2001): Protein-protein interaction of FHL3 with FHL2 and visualization of their interaction by green fluorescent proteins (GFP) two-fusion fluorescence resonance energy transfer (FRET) *J Cell Biochem*, vol. 80, pp. 293-303.

Lippincott-Schwartz, J.; Patterson, G. H. (2003): Development and use of fluorescent protein markers in living cells *Science*, vol. 300, pp. 87-91.

Llopis, J.; McCaffery, J. M.; Miyawaki, A.; Farquhar, M. G.; Tsien, R. Y. (1998): Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins *Proc Natl Acad Sci U S A*, vol. 95, pp. 6803-8.

Llopis, J.; Westin, S.; Ricote, M.; Wang, Z.; Cho, C. Y.; Kurokawa, R.; Mullen, T. M.; Rose, D. W.; Rosenfeld, M. G.; Tsien, R. Y.; Glass, C. K.; Wang, J. (2000): Ligand-dependent interactions of coactivators steroid receptor coactivator-1 and peroxisome proliferator-activated receptor binding protein with nuclear hormone receptors can be imaged in live cells and are required for transcription *Proc Natl Acad Sci U S A*, vol. 97, pp. 4363-8.

Luo, K. Q.; Yu, V. C.; Pu, Y.; Chang, D. C. (2001): Application of the fluorescence resonance energy transfer method for studying the dynamics of caspase-3 activation during UV-induced apoptosis in living HeLa cells *Biochem Biophys Res Commun*, vol. 283, pp. 1054-60.

Luo, K. Q.; Yu, V. C.; Pu, Y.; Chang, D. C. (2003): Measuring dynamics of caspase-8 activation in a single living HeLa cell during TNFalpha-induced apoptosis *Biochem Biophys Res Commun*, vol. 304, pp. 217-22.

Mahajan, N. P.; Harrison-Shostak, D. C.; Michaux, J.; Herman, B. (1999): Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis *Chem Biol*, vol. 6, pp. 401-9.

Majoul, I.; Straub, M.; Hell, S. W.; Duden, R.; Soling, H. D. (2001): KDEL-cargo regulates interactions between proteins involved in COPI vesicle traffic: measurements in living cells using FRET *Dev Cell*, vol. 1, pp. 139-53.

Mas, P.; Devlin, P. F.; Panda, S.; Kay, S. A. (2000): Functional interaction of phytochrome B and cryptochrome 2 *Nature*, vol. 408, pp. 207-11.

Matsuyama, S.; Llopis, J.; Deveraux, Q. L.; Tsien, R. Y.; Reed, J. C. (2000): Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis *Nat Cell Biol*, vol. 2, pp. 318-25.

Matz, M. V.; Fradkov, A. F.; Labas, Y. A.; Savitsky, A. P.; Zaraisky, A. G.; Markelov, M. L.; Lukyanov, S. A. (1999): Fluorescent proteins from nonbioluminescent Anthozoa species *Nat Biotechnol*, vol. 17, pp. 969-73.

Michnick, S. W. (2003): Protein fragment complementation strategies for biochemical network mapping *Curr Opin Biotechnol*, vol. 14, pp. 610-7.

Miesenbock, G.; De Angelis, D. A.; Rothman, J. E. (1998): Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins *Nature*, vol. 394, pp. 192-5.

Mitra, R. D.; Silva, C. M.; Youvan, D. C. (1996): Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein *Gene*, vol. 173, pp. 13-7.

Miyawaki, A.; Griesbeck, O.; Heim, R.; Tsien, R. Y. (1999): Dynamic and quantitative Ca2+ measurements using improved cameleons *Proc Natl Acad Sci U S A*, vol. 96, pp. 2135-40.

Miyawaki, A.; Llopis, J.; Heim, R.; McCaffery, J. M.; Adams, J. A.; Ikura, M.; Tsien, R. Y. (1997): Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin *Nature*, vol. 388, pp. 882-7.

Mochizuki, N.; Yamashita, S.; Kurokawa, K.; Ohba, Y.; Nagai, T.; Miyawaki, A.; Matsuda, M. (2001): Spatio-temporal images of growth-factor-induced activation of Ras and Rap1 *Nature*, vol. 411, pp. 1065-8.

Murphy, J. T.; Lagarias, J. C. (1997): The phytofluors: a new class of fluorescent protein probes *Curr Biol*, vol. 7, pp. 870-6.

Nagai, T.; Sawano, A.; Park, E. S.; Miyawaki, A. (2001): Circularly permuted green fluorescent proteins

engineered to sense Ca2+ *Proc Natl Acad Sci U S A*, vol. 98, pp. 3197-202.

Nagai, Y.; Miyazaki, M.; Aoki, R.; Zama, T.; Inouye, S.; Hirose, K.; Iino, M.; Hagiwara, M. (2000): A fluorescent indicator for visualizing cAMP-induced phosphorylation in vivo *Nat Biotechnol*, vol. 18, pp. 313-6.

Nakai, J.; Ohkura, M.; Imoto, K. (2001): A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein *Nat Biotechnol*, vol. 19, pp. 137-41.

Ng, T.; Squire, A.; Hansra, G.; Bornancin, F.; Prevostel, C.; Hanby, A.; Harris, W.; Barnes, D.; Schmidt, S.; Mellor, H.; Bastiaens, P. I.; Parker, P. J. (1999): Imaging protein kinase Calpha activation in cells *Science*, vol. 283, pp. 2085-9.

Ostergaard, H.; Henriksen, A.; Hansen, F. G.Winther, J. R. (2001): Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein *Embo J*, vol. 20, pp. 5853-62.

Ozawa, T.; Kaihara, A.; Sato, M.; Tachihara, K.; Umezawa, Y. (2001): Split luciferase as an optical probe for detecting protein-protein interactions in mammalian cells based on protein splicing *Anal Chem*, vol. 73, pp. 2516-21.

Ozawa, T.; Nogami, S.; Sato, M.; Ohya, Y.; Umezawa, Y. (2000): A fluorescent indicator for detecting protein-protein interactions in vivo based on protein splicing *Anal Chem*, vol. 72, pp. 5151-7.

Ozawa, T.; Takeuchi, T. M.; Kaihara, A.; Sato, M.; Umezawa, Y. (2001): Protein splicing-based reconstitution of split green fluorescent protein for monitoring protein-protein interactions in bacteria: improved sensitivity and reduced screening time *Anal Chem*, vol. 73, pp. 5866-74.

Paulmurugan, R.; Gambhir, S. S. (2003): Monitoring protein-protein interactions using split synthetic renilla luciferase protein-fragment-assisted complementation *Anal Chem*, vol. 75, pp. 1584-9.

Paulmurugan, R.; Massoud, T. F.; Huang, J.; Gambhir, S. S. (2004): Molecular imaging of drug-modulated protein-protein interactions in living subjects *Cancer Res*, vol. 64, pp. 2113-9.

Paulmurugan, R.; Umezawa, Y.; Gambhir, S. S. (2002): Noninvasive imaging of protein-protein interactions in living subjects by using reporter protein complementation and reconstitution strategies *Proc Natl Acad Sci U S A*, vol. 99, pp. 15608-13.

Persechini, A.; Cronk, B. (1999): The relationship between the free concentrations of Ca2+ and Ca2+-calmodulin in intact cells *J Biol Chem*, vol. 274, pp. 6827-30.

Rehm, M.; Dussmann, H.; Janicke, R. U.; Tavare, J. M.; Kogel, D.; Prehn, J. H. (2002): Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3 *J Biol Chem*, vol. 277, pp. 24506-14.

Remy, I.; Michnick, S. W. (2004): A cDNA library functional screening strategy based on fluorescent protein complementation assays to identify novel components of signaling pathways *Methods*, vol. 32, pp. 381-8.

Richmond, T. A.; Takahashi, T. T.; Shimkhada, R.; Bernsdorf, J. (2000): Engineered metal binding sites on green fluorescence protein *Biochem Biophys Res Commun*, vol. 268, pp. 462-5.

Rizzo, M. A.; Springer, G. H.; Granada, B.; Piston, D. W. (2004): An improved cyan fluorescent protein variant useful for FRET *Nat Biotechnol*, vol.

Romoser, V. A.; Hinkle, P. M.; Persechini, A. (1997): Detection in living cells of Ca2+-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators *J Biol Chem*, vol. 272, pp. 13270-4.

Rossi, F.; Charlton, C. A.; Blau, H. M. (1997): Monitoring protein-protein interactions in intact eukaryotic cells by beta-galactosidase complementation *Proc Natl Acad Sci U S A*, vol. 94, pp. 8405-10.

Ruehr, M. L.; Zakhary, D. R.; Damron, D. S.; Bond, M. (1999): Cyclic AMP-dependent protein kinase binding to A-kinase anchoring proteins in living cells by fluorescence resonance energy transfer of green fluorescent protein fusion proteins *J Biol Chem*, vol. 274, pp. 33092-6.

Sato, M.; Hida, N.; Ozawa, T.; Umezawa, Y. (2000): Fluorescent indicators for cyclic GMP based on cyclic GMP-dependent protein kinase Ialpha and green fluorescent proteins *Anal Chem*, vol. 72, pp. 5918-24.

Sato, M.; Ozawa, T.; Inukai, K.; Asano, T.; Umezawa, Y. (2002): Fluorescent indicators for imaging protein phosphorylation in single living cells *Nat Biotechnol*,

vol. 20, pp. 287-94.

Schmid, J. A.; Birbach, A.; Hofer-Warbinek, R.; Pengg, M.; Burner, U.; Furtmuller, P. G.; Binder, B. R.; de Martin, R. (2000): Dynamics of NF kappa B and Ikappa Balpha studied with green fluorescent protein (GFP) fusion proteins. Investigation of GFP-p65 binding to DNa by fluorescence resonance energy transfer *J Biol Chem*, vol. 275, pp. 17035-42.

Seth, A.; Otomo, T.; Yin, H. L.; Rosen, M. K. (2003): Rational design of genetically encoded fluorescence resonance energy transfer-based sensors of cellular Cdc42 signaling *Biochemistry*, vol. 42, pp. 3997-4008.

Shah, K.; Tang, Y.; Breakefield, X.; Weissleder, R. (2003): Real-time imaging of TRAIL-induced apoptosis of glioma tumors in vivo *Oncogene*, vol. 22, pp. 6865-72.

Shyy, J. Y.; Chien, S. (2002): Role of integrins in endothelial mechanosensing of shear stress *Circ Res*, vol. 91, pp. 769-75.

Siegel, M. S.; Isacoff, E. Y. (1997): A genetically encoded optical probe of membrane voltage *Neuron*, vol. 19, pp. 735-41.

Siegel, R. M.; Frederiksen, J. K.; Zacharias, D. A.; Chan, F. K.; Johnson, M.; Lynch, D.; Tsien, R. Y.; Lenardo, M. J. (2000): Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations *Science*, vol. 288, pp. 2354-7.

Sorkin, A.; McClure, M.; Huang, F.; Carter, R. (2000): Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy *Curr Biol*, vol. 10, pp. 1395-8.

Spotts, J. M.; Dolmetsch, R. E.; Greenberg, M. E. (2002): Time-lapse imaging of a dynamic phosphorylation-dependent protein-protein interaction in mammalian cells *Proc Natl Acad Sci U S A*, vol. 99, pp. 15142-7.

Stamatas, G. N.; McIntire, L. V. (1999): Novel optical methodologies in studying mechanical signal transduction in mammalian cells *Ind Eng Chem Res*, vol. 38, pp. 601-9.

Takeda, S.; Kamiya, N.; Arai, R.; Nagamune, T. (2001): Design of an artificial light-harvesting unit by protein engineering: cytochrome b(562)-green fluorescent Protein chimera *Biochem Biophys Res Commun*, vol. 289, pp. 299-304.

Takeda, S.; Kamiya, N.; Nagamune, T. (2003): A novel protein-based heme sensor consisting of green fluorescent protein and apocytochrome b(562) *Anal Biochem*, vol. 317, pp. 116-9.

Tertoolen, L. G.; Blanchetot, C.; Jiang, G.; Overvoorde, J.; Gadella, T. W. Jr.; Hunter, T.; den Hertog, J. (2001): Dimerization of receptor protein-tyrosine phosphatase alpha in living cells *BMC Cell Biol*, vol. 2, pp. 8.

Ting, A. Y.; Kain, K. H.; Klemke, R. L.; Tsien, R. Y. (2001): Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells *Proc Natl Acad Sci U S A*, vol. 98, pp. 15003-8.

Topell, S.; Hennecke, J.; Glockshuber, R. (1999): Circularly permuted variants of the green fluorescent protein *FEBS Lett*, vol. 457, pp. 283-9.

Truong, K.; Sawano, A.; Mizuno, H.; Hama, H.; Tong, K. I.; Mal, T. K.; Miyawaki, A.; Ikura, M. (2001): FRET-based in vivo Ca2+ imaging by a new calmodulin-GFP fusion molecule *Nat Struct Biol*, vol. 8, pp. 1069-73.

van Roessel, P.; Brand, A. H. (2002): Imaging into the future: visualizing gene expression and protein interactions with fluorescent proteins *Nat Cell Biol*, vol. 4, pp. E15-20.

Vanderklish, P. W.; Krushel, L. A.; Holst, B. H.; Gally, J. A.; Crossin, K. L.; Edelman, G. M. (2000): Marking synaptic activity in dendritic spines with a calpain substrate exhibiting fluorescence resonance energy transfer *Proc Natl Acad Sci U S A*, vol. 97, pp. 2253-8.

Verveer, P. J.; Wouters, F. S.; Reynolds, A. R.; Bastiaens, P. I. (2000): Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane *Science*, vol. 290, pp. 1567-70.

Violin, J. D.; Zhang, J.; Tsien, R. Y.; Newton, A. C. (2003): A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C *J Cell Biol*, vol. 161, pp. 899-909.

Wachter, R. M.; Remington, S. J. (1999): Sensitivity of the yellow variant of green fluorescent protein to halides and nitrate *Curr Biol*, vol. 9, pp. R628-9.

Wehrman, T.; Kleaveland, B.; Her, J. H.; Balint, R. F.; Blau, H. M. (2002): Protein-protein interactions monitored in mammalian cells via complementation of beta -lactamase enzyme fragments *Proc Natl Acad Sci U S A*,

vol. 99, pp. 3469-74.

Weijer, C. J. (2003): Visualizing signals moving in cells *Science*, vol. 300, pp. 96-100.

Weissleder, R.; Mahmood, U. (2001): Molecular imaging *Radiology*, vol. 219, pp. 316-33.

Weissleder, R.; Ntziachristos, V. (2003): Shedding light onto live molecular targets *Nat Med*, vol. 9, pp. 123-8.

Wiedenmann, J.; Schenk, A.; Rocker, C.; Girod, A.; Spindler, K. D.; Nienhaus, G. U. (2002): A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from Entacmaea quadricolor (Anthozoa, Actinaria) *Proc Natl Acad Sci U S A*, vol. 99, pp. 11646-51.

Xia, Z.; Zhou, Q.; Lin, J.; Liu, Y. (2001): Stable SNARE complex prior to evoked synaptic vesicle fusion revealed by fluorescence resonance energy transfer *J Biol Chem*, vol. 276, pp. 1766-71.

Xu, X.; Gerard, A. L.; Huang, B. C.; Anderson, D. C.; Payan, D. G.; Luo, Y. (1998): Detection of programmed cell death using fluorescence energy transfer *Nucleic Acids Res*, vol. 26, pp. 2034-5.

Xu, Y.; Piston, D. W.; Johnson, C. H. (1999): A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins *Proc Natl Acad Sci U S A*, vol. 96, pp. 151-6.

Ye, K.; Schultz, J. S. (2003): Genetic engineering of an allosterically based glucose indicator protein for continuous glucose monitoring by fluorescence resonance energy transfer *Anal Chem*, vol. 75, pp. 3119-27.

Zaccolo, M.; De Giorgi, F.; Cho, C. Y.; Feng, L.; Knapp, T.; Negulescu, P. A.; Taylor, S. S.; Tsien, R. Y.; Pozzan, T. (2000): A genetically encoded, fluorescent indicator for cyclic AMP in living cells *Nat Cell Biol*, vol. 2, pp. 25-9.

Zaccolo, M.; Pozzan, T. (2002): Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes *Science*, vol. 295, pp. 1711-5.

Zacharias, D. A.; Violin, J. D.; Newton, A. C.; Tsien, R. Y. (2002): Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells *Science*, vol. 296, pp. 913-6.

Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. (2002): Creating new fluorescent probes for cell biology *Nat Rev Mol Cell Biol*, vol. 3, pp. 906-18.

Zhang, J.; Ma, Y.; Taylor, S. S.; Tsien, R. Y. (2001): Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering *Proc Natl Acad Sci U S A*, vol. 98, pp. 14997-5002.