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Designs and applications of fluorescent protein-based biosensors

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Genetically encoded biosensors allow the noninvasive imaging of specific biochemical or biorecognition processes with the preservation of subcellular spatial and temporal information. *Aequorea* green fluorescent protein (FP) and its engineered variants are a critical component of genetically encoded biosensors, as they serve to provide a 'read-out' of the biorecognition event under investigation. The family of FP-based biosensors includes a diverse array of designs that utilize various photophysical characteristics of the FPs. In this review, we will discuss these designs and their read-outs through reviewing some of the recent works in this area.

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Introduction

Biosensing encompasses a diverse array of techniques for the generation of an experimentally accessible 'read-out' of a molecular interaction between a biomolecule-derived molecular recognition element (MRE) (e.g. a protein domain) and an analyte of interest (e.g. a small molecule, another protein, or an enzymatic activity). Molecular entities or devices that enable biosensing are generally referred to as biosensors. The primary challenge of creating biosensors is transducing the nanometer-scale event of a biorecognition process into an observable change in a macroscopic property such as color or fluorescence hue. One of the nanometer-scale changes that typically accompany biorecognition events is the change in molecular 'geometry' of the MRE. This change could be a distance change between the MRE and its analyte as in the case of protein–protein interaction, or a conformational change of the MRE as in the case of allosteric proteins. As will be discussed in this review, researchers have now devised a variety of strategies by which changes in the molecular geometry of an MRE can modulate the fluorescence hue or intensity of an intrinsically fluorescent protein (FP) belonging to the superfamily of *Aequorea* green FP-like proteins.

As described elsewhere in this issue [1], engineered FPs have revolutionized the ability of researchers to study protein localization and dynamics in live cells. FPs have also enabled the construction of genetically encoded FP-based biosensors that have numerous advantages relative to alternative technologies such as dye-based probes. Specifically, FP-based biosensors are relatively easy to construct using standard molecular biology techniques; able to be noninvasively introduced into living cells where they are produced using the cellular transcriptional and translational machinery; able to yield information about a biorecognition process in the natural habitat of the protein thus preserving spatial and temporal information of this interaction; able to be targeted to most cellular compartments using specific signal sequence tags.

Practically all genetically encoded FP-based biosensors can be classified into five groups depending on their structure. We define Group I as those biosensors based on intramolecular Förster Resonance Energy Transfer (FRET). Such biosensors have all of their components in a single polypeptide chain, and the analyte brings about a change in the structure or conformation of the MRE unit. This change is detected by ratiometric intensity measurements of the two FPs. Group II includes biosensors based on intermolecular FRET. In contrast to Group I, the two FPs are in two different polypeptide chains and are brought into proximity by a protein–protein interaction. Group III includes those biosensors based on bimolecular fluorescence complementation (BiFC). In this biosensing strategy, a biorecognition event is used to bring two fragments of a split FP suitable proximity for the reconstitution of an intact (and fluorescent) FP.

Groups IV and V are both based on single FPs encoded by a single polypeptide chain. The difference between these two groups is whether or not the MRE element of the biosensor is exogenous (Group IV) or endogenous (Group V) with respect to the FP. In the case of an exogenous MRE, the binding of the analyte causes conformational changes that are relayed to the chromophore environment and alter its spectral properties. In the case of an endogenous MRE, the FP plays a dual role: it is responsible for both the molecular recognition and the fluorescence read-out.

In this review, we will provide examples of the different designs of genetically encoded FP-based biosensors belonging to the aforementioned groups and describe recent progress in their development and application.

Group I: intramolecular FRET-based biosensors

FRET is the phenomenon of nonradiative energy transfer observed between an excited blue-shifted fluorescent chromophore (donor) and a chromophore with a red-shifted absorption spectrum (acceptor) through dipole–dipole coupling. FRET has proven to be extremely useful in the design of genetically encoded biosensors. The canonical structure of biosensors belonging to this group consists of two FPs flanking an MRE (Figure 1a). Changes in the MRE conformation alter the distance between the two FPs and thus affect the FRET efficiency. The FRET phenomenon manifests itself as a ratiometric change in ratio of acceptor (I_A) to donor (I_D) fluorescence intensity. That is, a change from a lower to a higher FRET efficiency results in an increase in I_A at the expense of I_D. This basic design of FP-based biosensors has been applied successfully to detect proteolytic activities, post-translational modification (PTM) enzymes activities, and small molecules. Each of these variations on this design is discussed in the following paragraphs. Practical aspects of performing FRET measurements in live cells have been discussed in recent reviews [2,3,4•].

An MRE to detect proteolytic activity consists of a polypeptide that is a substrate for the protease under investigation (Figure 1b). This enzymatic activity detection is manifested by a decrease in the intensity of the acceptor fluorophore with a concomitant increase in that of the donor's. Recently, biosensors with this design have been developed for the detection of $3C^{pro}$ and NS3-4A proteases that belong to human enterovirus (HEV) [5•] and hepatitis C virus (HCV) [6], respectively.

PTM enzymes catalyze the covalent modification (e.g. phosphorylation by a kinase) of a peptide substrate. An MRE capable of detecting PTM activity is composed of two parts: a specific substrate to the PTM of interest and a binding domain that preferentially binds to the modified substrate. These two units could be collectively regarded as a MRE that changes its geometry in response to the PTM activity (Figure 1c). This design of biosensor has been employed to detect a variety of kinase enzymes, with a recent example being an ERK activity biosensor [7]. Aye-Han *et al.* have recently reviewed different FRET-based biosensors that were designed to detect various PTM enzymatic activities [8].

Some proteins undergo a change in their conformation upon binding to their cognate small molecule analytes (Figure 1d). A celebrated family of proteins that exhibit this behavior is the bacterial periplasmic binding proteins [9,10]. These allosteric proteins have been used to construct various intramolecular FRET-based biosensors including the ones for glucose [11], maltose [12], and glutamate [13]. For the detection of Ca^{2+}, researchers have used an MRE composed of calmodulin and a Ca^{2+}/calmodulin-binding peptide. This MRE has served as the basis of the ever-expanding family of Ca^{2+} biosensors known as Cameleons [14,15]. Using similar designs, FRET-based biosensors have been developed for Zn^{2+} [16] and cyclic nucleotides [17].

Group II: intermolecular FRET-based biosensors

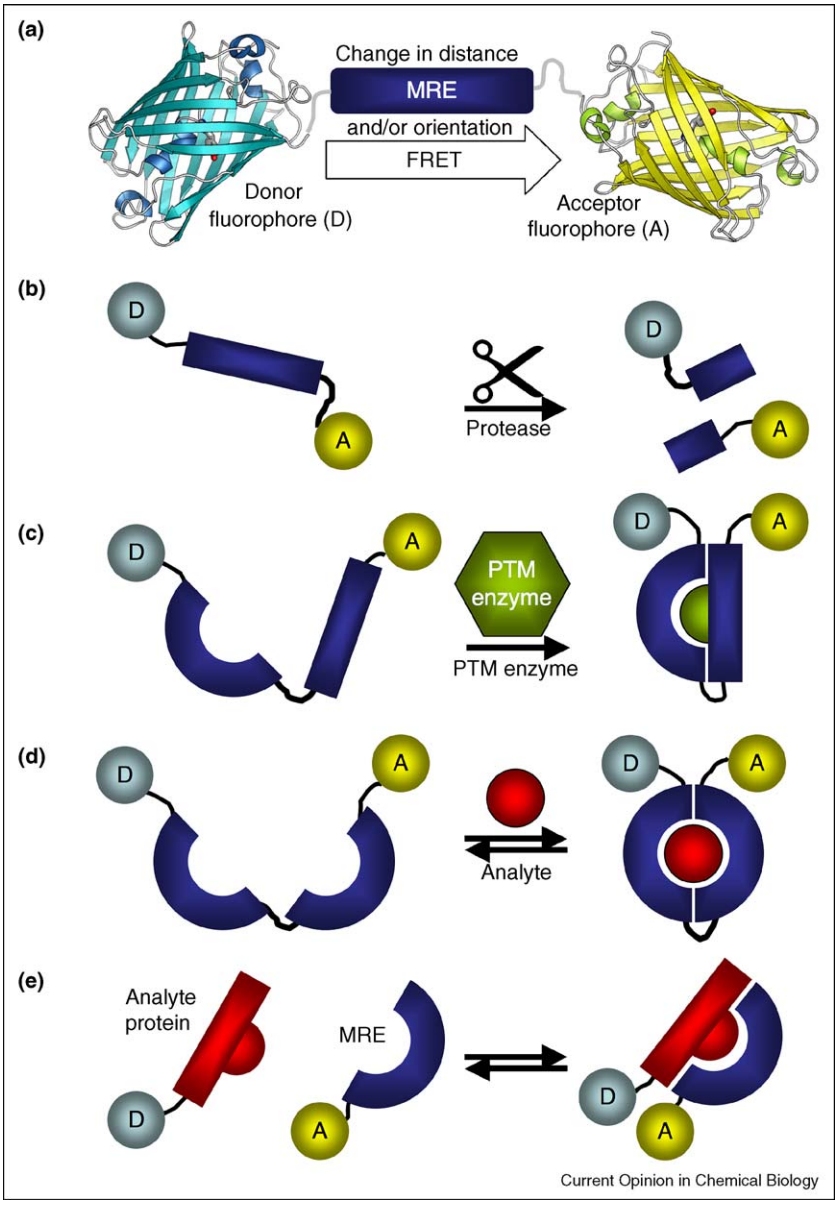
Biosensors belonging to this group are necessarily split constructs, in which the MRE is fused to one of the FPs and the analyte protein is fused to the other (Figure 1e). This design of biosensors is particularly useful for the study of protein–protein interactions. Intermolecular FRET has been applied to study the oligomerization state of different member of the G-protein-coupled-receptor (GPCR) superfamily [18,19]. However, the versatility of this design of biosensors does not end at merely deducing the oligomerization state of receptors. The conformational changes of the activated receptors, read-out by changes in FRET, can be used to determine the kinetic parameters of the receptor activation as was demonstrated in a recent study for mGluR1, a member of the GPCR superfamily [20••].

One of the problems that can hinder accurate and reproducible intermolecular FRET measurements is the variation in the expression level of the two biosensor halves. This could be a major concern when ratiometric measurements are employed. To circumvent the variations in concentration, fluorescence lifetime imaging (FLIM) can be employed [21]. Another caveat to the use of intermolecular FRET measurements is that caution must be exercised in the interpretation of results, since FRET also can sometimes occur between two proteins that do not directly interact. For example, in a recent study, Orthaus *et al.* observed FRET between FP fusions of CENP-A and CENP-B. However, *in vitro* studies suggest no interaction between these proteins [22].

Group III: BiFC-based biosensors

BiFC is dependent on the intrinsic ability of some FP variants, when expressed in a split form tagged to a pair of interacting proteins, to refold properly into the β-barrel structure and thus reconstitute the fluorescent form of the protein. BiFC-based biosensors are necessarily split constructs in which the MRE is genetically fused to one fragment of the FP and the analyte protein is fused to the other (Figure 2a). Several recent reviews provide a thorough treatment of the practical aspects of BiFC and guide how to correctly implement this technique [23•,24].

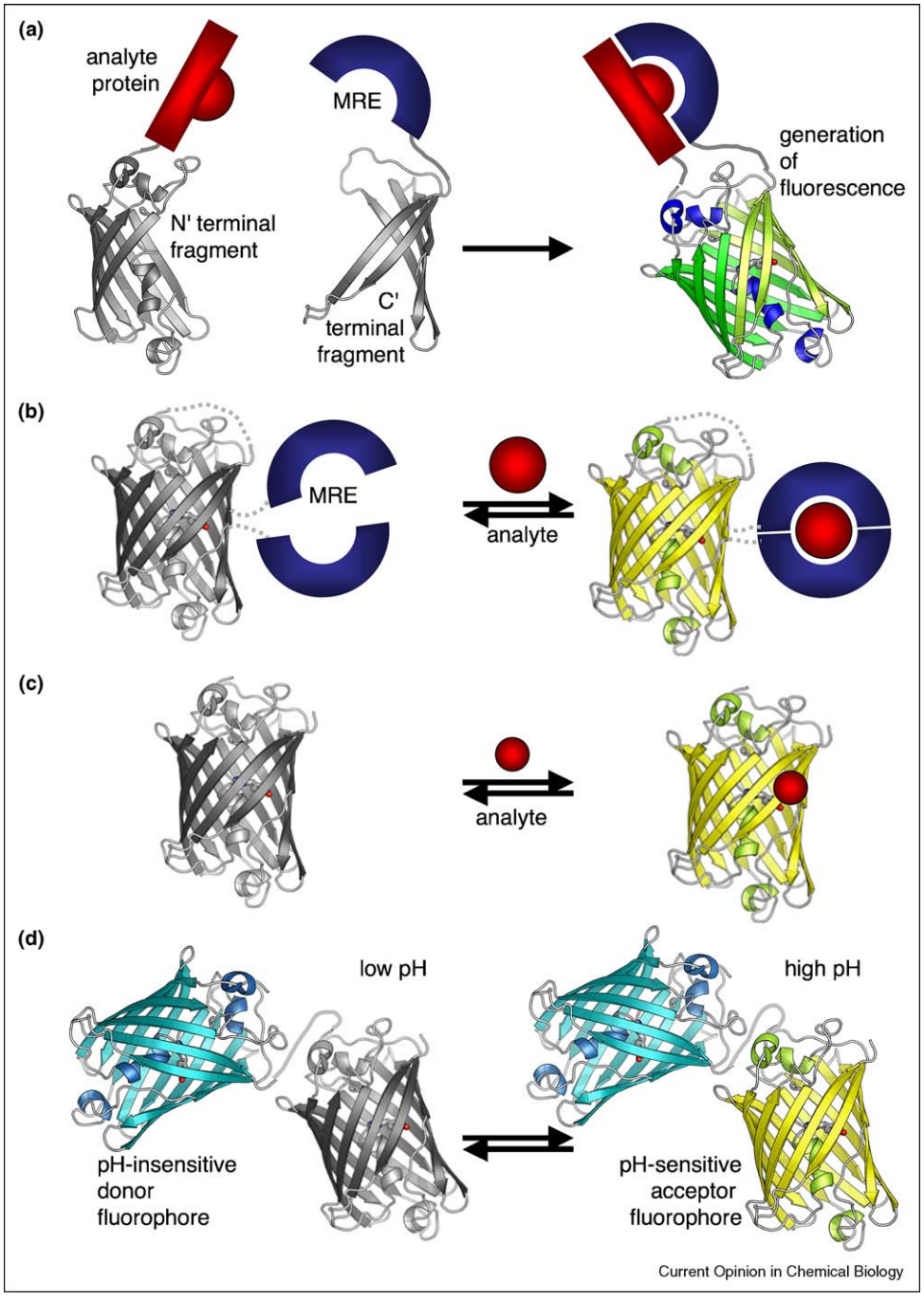
Figure 1



FRET-based biosensor designs. **(a)** Schematic model of a generic intramolecular FRET-based biosensor. A FP FRET pair flanks an MRE that undergoes a conformational change that alters the distance and/or orientation of the FPs relative to each other. **(b)** An MRE suitable for the detection of protease activity. **(c)** An MRE for the detection of PTM enzymatic activities where the modification of the peptide substrate creates a binding dock for the binding domain resulting in a FRET change. **(d)** An MRE in which the conformational change is triggered by the presence of its analyte. **(e)** Protein–protein interactions can be visualized in live cells by tagging each one of the proteins to one member of a FP FRET pair and observing the changes in donor/acceptor intensities.

BiFC-based biosensors have been employed to visualize a variety of protein–protein interactions in live cells. For example, BiFC was used to reveal the recruitment of members of the CBX family to different parts of the chromatin through their interaction with histone 3 [25]. The BiFC design of biosensors has also been used to elucidate the interaction between the three protein subunits that constitute the influenza A polymerase complex [26]. Interestingly, fluorescent reconstitution is sometimes possible between fragments belonging to different FPs, creating chimeras with a variety of fluorescent hues [27]. This allows for simultaneous imaging of more than one event in live cells. Utilizing the multicolor BiFC, it has been shown that the oligomerization between adenosine A_{2A} and dopamine D_2 receptors to form homodimers and heterodimers was ligand-dependant [28•].

Figure 2



Additional designs of FP-based biosensors. **(a)** Detection of a protein–protein interaction by BiFC. **(b)** Single FP biosensors with an exogenous MRE. **(c)** Single FP biosensors with an endogenous MRE. **(d)** A hybrid design that used a pH-sensitive acceptor fluorophore in a FRET pair.

Group IV: single FP-based biosensors with an exogenous MRE

This class of genetically encoded single FP-based biosensors depends on the ability of some of the variants of FPs to tolerate protein insertion and circular permutations at certain locations. This property has allowed researchers to construct ligand sensitive single FP-based biosensors. The biorecognition event is carried out by an exogenous MRE and information about this event is relayed to the chromophore changing its spectral properties (Figure 2b). Some examples of a biosensor with this design are Camgaroo [29] and Case 12 [30] for Ca^{2+} detection. Following

the same design, single FP-based biosensors have been reported for Zn^{2+} [31] and cGMP [32].

In a recent study, Yellen and coworkers utilized the GlnK1 protein as an ATP-specific MRE to construct a biosensor to determine the ATP concentration. Binding of ATP caused conformational changes in GlnK1 protein that ratiometrically altered the excitation profile of cpmVenus. However, the competition between ADP and ATP for the same binding location in GlnK1 made the sensor more appropriate to evaluate the ATP:ADP ratio in live cells [33••].

Group V: single FP-based biosensors with an endogenous MRE

Most FP variants show pH-dependent change in their spectral properties [34]. For example, the engineered avGFP variants known as EGFP, ECFP, and EYFP have pK_as for fluorescence quenching of 6.15, 6.4, and 7.1, respectively [35]. Recently an engineered variant of *Discosoma* RFP, known as mNectarine, was shown to exhibit a useful pH-dependency [36]. To demonstrate its potential, the authors fused mNectarine to the cytoplasmic amino acid terminus of human concentrative nucleoside transporter (hCNT3). The read-out of the mNectarine and the other pH-sensitive FPs mentioned above is a change in their fluorescence intensity. These intensity-based measurements have the disadvantages of not being easily calibrated and large cell-to-cell variation.

To overcome the concentration dependence, and other limitations that are inherent in intensiometric measurements, researchers have imaged the pH-dependent changes in EGFP fluorescence lifetime rather than intensity [37•]. Fluorescence lifetime is a characteristic parameter of a given fluorophore that does not depend on the fluorophore concentration and is not affected by the fluorophore photobleaching. Another way to overcome the intensity measurement shortcomings is to utilize a FP variant that shows ratiometric changes of its spectral properties such as ratiometric pHluorin [38]. In a recent application, the cytoplasmic and mitochondrial pHs were measured utilizing ratiometric pHluorin for a study of the relationship between internal pH and growth rate of *S. cerevisiae* [39]. A novel pH-sensitive GFP variant showing a pH-dependent shift of its emission spectrum was recently reported [40]. The employment of GFP variants as biosensors was the topic of a recent review [41].

Another example of a biosensor with an endogenous MRE is redox-sensitive GFP (roGFP). The substitution of two surface amino acid residues of a GFP variant with a cysteine pair at an appropriate distance from each other — to facilitate a disulfide bond formation — rendered this GFP variant sensitive to the redox state of its environment. This roGFP allows for ratiometric measurement of the cell redox status [42]. Recently, an improved redox biosensor was created by fusing roGFP to human glutaredoxin-1 (Glx1) which catalyzes rapid equilibration between roGFP and glutathione, thus improving the response rate of roGFP [43].

Hybrid strategies

Owing to continuous innovation in the development of biosensor designs and experimental techniques to detect protein–protein interaction, some designs do not fit in any of the aforementioned categories. For example, some FRET-based biosensors do not depend on conformational changes in the FRET construct but rather on spectral changes in the acceptor FP. Esposito *et al.* designed a FRET construct that consists of the pH-insensitive donor cyan FP and a pH-sensitive variant yellow FP variant (Figure 2d). As pH is lowered, the extinction coefficient of the pH-sensitive YFP decreases. This decrease lowers the overlap integral between the donor and the acceptor FPs and produces an increase in donor emission due to 'FRET frustration' [44••]. An advantage of this design is that it enables ratiometric measurement of pH changes which overcomes the drawbacks of the intensiometric measurement techniques. A similar biosensor design was used to measure the pH changes in extracellular microdomains [45].

Outlook

Ongoing protein engineering efforts will eventually provide researchers with a complete repertoire of genetically encoded biosensors, each with specific properties that are 'tuned' to the conditions of the event under investigation. It is apparent that a substantial amount of progress has already been made toward this goal. For example, genetically encoded Ca^{2+} biosensors with a range of different affinities to Ca^{2+} have been developed. It is likely that other classes of biosensor will see increased specialization and tuning of properties in order to better address specific types of questions. In addition to increased specialization, there are a number of other trends that are expected to direct future progress in the fields of genetically encoded biosensors. For example, we anticipate numerous efforts to improve the quality of these biosensors by increasing the specificity for detecting the target analyte and increasing response kinetics. Furthermore, we expect that simultaneous monitoring of more than one cellular event, by combining two or more biosensors of the types described in this review, will be a fruitful area of application for genetically encoded biosensors [46•,47].

In conclusion, we have seen remarkable progress in the development of genetically encoded biosensors in recent years. With these developments has come an increasing awareness of these powerful new tools among biologists. Accordingly, we expect that the number of papers describing applications of biosensors will soon come to

dwarf the number of papers describing the design of biosensors.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Piatkevich KD, Verkhusha VV: **Advances in engineering of monomeric fluorescent proteins and photoactivatable proteins with red emission**. *Curr Opin Chem Biol* 2010, **14**:23-29.

2. Campbell RE: **Fluorescent-protein-based biosensors: modulation of energy transfer as a design principle**. *Anal Chem* 2009, **81**:5972-5979.

3. Jares-Erijman EA, Jovin TM: **FRET imaging**. *Nat Biotechnol* 2003, **21**:1387-1395.

4. • Wang YX, Shyy JYJ, Chien S: **Fluorescence proteins, live-cell imaging, and mechanobiology: seeing is believing**. *Annu Rev Biomed Eng* 2008, **10**:1-38.
A thorough review that covers the available FP variants and microscopy techniques for live-cell imaging.

5. • Tsai MT, Cheng YH, Liu YN, Liao NC, Lu WW, Kung SH: **Real-time monitoring of human enterovirus (HEV)-infected cells and anti-HEV 3C protease potency by fluorescence resonance energy transfer**. *Antimicrob Agents Chemother* 2009, **53**:748-755.
An elegant study showing HEV 3C protease FRET-based biosensor being used to establish a stable cell line which in turn is used to assay different viral serotypes and test them for their inhibition susceptibility in live cells.

6. Sabariegos R, Picazo F, Domingo B, Franco S, Martinez MA, Llopis J: **Fluorescence resonance energy transfer-based assay for characterization of hepatitis C virus NS3-4A protease activity in live cells**. *Antimicrob Agents Chemother* 2009, **53**:728-734.

7. Harvey CD, Ehrhardt AG, Cellurale C, Zhong HN, Yasuda R, Davis RJ, Svoboda K: **A genetically encoded fluorescent sensor of ERK activity**. *Proc Natl Acad Sci U S A* 2008, **105**:19264-19269.

8. Aye-Han N-N, Qiang N, Zhang J: **Fluorescent biosensors for real-time tracking of post-translational modification dynamics**. *Curr Opin Chem Biol* 2009, **13**:392-397.

9. Dwyer MA, Hellinga HW: **Periplasmic binding proteins: a versatile superfamily for protein engineering**. *Curr Opin Struct Biol* 2004, **14**:495-504.

10. Deuschle K, Fehr M, Hilpert M, Lager I, Lalonde S, Looger LL, Okumoto S, Persson J, Schmidt A, Frommer WB: **Genetically encoded sensors for metabolites**. *Cytometry A* 2005, **64A**:3-9.

11. Takanaga H, Chaudhuri B, Frommer WB: **GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor**. *Biochim Biophys Acta Biomembr* 2008, **1778**:1091-1099.

12. Ha JS, Song JJ, Lee YM, Kim SJ, Sohn JH, Shin CS, Lee SG: **Design and application of highly responsive fluorescence resonance energy transfer biosensors for detection of sugar in living *Saccharomyces cerevisiae* cells**. *Appl Environ Microbiol* 2007, **73**:7408-7414.

13. Hires SA, Zhu YL, Tsien RY: **Optical measurement of synaptic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters**. *Proc Natl Acad Sci U S A* 2008, **105**:4411-4416.

14. McCombs JE, Palmer AE: **Measuring calcium dynamics in living cells with genetically encodable calcium indicators**. *Methods* 2008, **46**:152-159.

15. Mank M, Griesbeck O: **Genetically encoded calcium indicators**. *Chem Rev* 2008, **108**:1550-1564.

16. Dittmer PJ, Miranda JG, Gorski JA, Palmer AE: **Genetically encoded sensors to elucidate spatial distribution of cellular zinc**. *J Biol Chem* 2009, **284**:16289-16297.

17. Vincent P, Gervasi N, Zhang J: **Real-time monitoring of cyclic nucleotide signaling in neurons using genetically encoded FRET probes**. *Brain Cell Biol* 2008, **36**:3-17.

18. Lukasiewicz S, Faron-Gorecka A, Dobrucki J, Polit A, Dziedzicka-Wasylewska M: **Studies on the role of the receptor protein motifs possibly involved in electrostatic interactions on the dopamine D-1 and D-2 receptor oligomerization**. *FEBS J* 2009, **276**:760-775.

19. Rives ML, Vol C, Fukazawa Y, Tinel N, Trinquet E, Ayoub MA, Shigemoto R, Pin JP, Prezeau L: **Crosstalk between GABA(B) and mGlu1a receptors reveals new insight into GPCR signal integration**. *EMBO J* 2009, **28**:2195-2208.

20. •• Marcaggi P, Mutoh H, Dimitrov D, Beato M, Knopfel T: **Optical measurement of mGluR1 conformational changes reveals fast activation, slow deactivation, and sensitization**. *Proc Natl Acad Sci U S A* 2009, **106**:11388-11393.
An interesting employment of the FRET phenomenon to study the activation kinetics of mGluR1. The authors demonstrate that the changes in FRET correlate with activation of the receptor.

21. Levitt JA, Matthews DR, Ameer-Beg SM, Suhling K: **Fluorescence lifetime and polarization-resolved imaging in cell biology**. *Curr Opin Biotechnol* 2009, **20**:28-36.

22. Orthaus S, Biskup C, Hoffmann B, Hoischen C, Ohndorf S, Benndorf K, Diekmann S: **Assembly of the inner kinetochore proteins CENP-A and CENP-B in living human cells**. *Chembiochem* 2008, **9**:77-92.

23. • Kerppola TK: **Biomolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells**. *Annu Rev Biophys* 2008, **37**:465-487.
A comprehensive review that covers the BiFC technique, the practical aspects of BiFC experiments, and the kind of applications it is suitable for.

24. Kerppola TK: **Bimolecular fluorescence complementatlon: visualization of molecular interactions in living cells**. *Methods Cell Biol* 2008, **85**:431-470.

25. Vincenz C, Kerppola TK: **Different polycomb group CBX family proteins associate with distinct regions of chromatin using nonhomologous protein sequences**. *Proc Natl Acad Sci U S A* 2008, **105**:16572-16577.

26. Hemerka JN, Wang D, Weng YJ, Lu WX, Kaushik RS, Jin J, Harmon AF, Li F: **Detection and characterization of influenza A virus PA–PB2 interaction through a bimolecular fluorescence complementation assay**. *J Virol* 2009, **83**:3944-3955.

27. Kodama Y, Wada M: **Simultaneous visualization of two protein complexes in a single plant cell using multicolor fluorescence complementation analysis**. *Plant Mol Biol* 2009, **70**:211-217.

28. • Vidi PA, Chemel BR, Hu CD, Watts VJ: **Ligand-dependent oligomerization of dopamine D2 and adenosine A(2A) receptors in living neuronal cells**. *Mol Pharmacol* 2008, **74**:544-551.
A nice example of using the BiFC strategy to investigate the oligomerization state of A_{2A} and D_2 GPCRs, and how they are affected by the presence of certain drugs.

29. Baird GS, Zacharias DA, Tsien RY: **Circular permutation and receptor insertion within green fluorescent proteins**. *Proc Natl Acad Sci U S A* 1999, **96**:11241-11246.

30. Souslova EA, Belousov VV, Lock JG, Stromblad S, Kasparov S, Bolshakov AP, Pinelis VG, Labas YA, Lukyanov S, Mayr LM *et al.*: **Single fluorescent protein-based Ca^{2+} sensors with increased dynamic range**. *BMC Biotechnol* 2007, **7**:37.

31. Mizuno T, Murao K, Tanabe Y, Oda M, Tanaka T: **Metal-ion-dependent GFP emission in vivo by combining a circularly permutated green fluorescent protein with an engineered metal-ion-binding coiled-coil**. *J Am Chem Soc* 2007, **129**:11378-11383.

32. Nausch LWM, Lecloux J, Bonev AD, Nelson MT, Dostmann WR: **Differential patterning of cGMP in vascular smooth muscle cells revealed by single GFP-linked biosensors**. *Proc Natl Acad Sci U S A* 2008, **105**:365-370.

33. Berg J, Hung YP, Yellen G: **A genetically encoded fluorescent reporter of ATP:ADP ratio**. *Nat Methods* 2009, **6**:161-166.
•• An interesting example of a single FP-based biosensor where cpmVenus was inserted at a certain position of GlnK1 protein. Depending on the ATP:ADP ratio, this biosensor showed different excitabilities at wavelengths 405 nm and 490 nm, therefore allowing for ratiometric measurement of ATP:ADP ratio by excitation. This study is also a good example of tuning and optimizing biosensors.

34. Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW: **Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy**. *Biophys J* 1997, **73**:2782-2790.

35. Llopis J, McCaffery JM, Miyawaki A, Farquhar MG, Tsien RY: **Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins**. *Proc Natl Acad Sci U S A* 1998, **95**:6803-6808.

36. Johnson DE, Ai HW, Wong P, Young JD, Campbell RE, Casey JR: **Red fluorescent protein pH biosensor to detect concentrative nucleoside transport**. *J Biol Chem* 2009, **284**:20499-20511.

37. Nakabayashi T, Wang HP, Kinjo M, Ohta N: **Application of fluorescence lifetime imaging of enhanced green fluorescent protein to intracellular pH measurements**. *Photochem Photobiol Sci* 2008, **7**:668-670.
• An elegant example of utilizing FLIM to circumvent the disadvantages of intensiometric measurement. It also demonstrates how EGFP in lifetime measurements can be used for quantitative estimation of pH changes.

38. Miesenbock G, De Angelis DA, Rothman JE: **Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins**. *Nature* 1998, **394**:192-195.

39. Orij R, Postmus J, Ter Beek A, Brul S, Smits GJ: **In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth**. *Microbiology* 2009, **155**:268-278.

40. Serresi M, Bizzarri R, Cardarelli F, Beltram F: **Real-time measurement of endosomal acidification by a novel genetically encoded biosensor**. *Anal Bioanal Chem* 2009, **393**:1123-1133.

41. Bizzarri R, Serresi M, Luin S, Beltram F: **Green fluorescent protein based pH indicators for in vivo use: a review**. *Anal Bioanal Chem* 2009, **393**:1107-1122.

42. Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, Remington SJ: **Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators**. *J Biol Chem* 2004, **279**:13044-13053.

43. Gutscher M, Pauleau AL, Marty L, Brach T, Wabnitz GH, Samstag Y, Meyer AJ, Dick TP: **Real-time imaging of the intracellular glutathione redox potential**. *Nat Methods* 2008, **5**:553-559.

44. Esposito A, Gralle M, Dani MAC, Lange D, Wouters FS: **pHlameleons: a family of FRET-based protein sensors for quantitative pH imaging**. *Biochemistry* 2008, **47**:13115-13126.
•• An interesting example of FRET-based pH biosensor that is composed of a pH-insensitive donor fluorophore and a pH-sensitive acceptor fluorophore. Unlike the conventional FRET-based biosensors that depend on the changes in relative distance and/or orientation of the fluorophores, this biosensor depends on the spectral changes of the acceptor fluorophore that accompany pH changes which in turn change the overlap integral affecting FRET.

45. Urra J, Sandoval M, Cornejo I, Barros LF, Sepulveda FV, Cid LP: **A genetically encoded ratiometric sensor to measure extracellular pH in microdomains bounded by basolateral membranes of epithelial cells**. *Pflugers Arch* 2008, **457**:233-242.

46. Ai HW, Hazelwood KL, Davidson MW, Campbell RE: **Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors**. *Nat Methods* 2008, **5**:401-403.
• An interesting example of detecting caspase-3 activity in the cytoplasm and nucleus using two orthogonal FRET pairs simultaneously. This study shows how the utilization of these FRET pairs preserved the temporal resolution of the caspase-3 activity in the cytoplasm and in the nucleus.

47. Carlson HJ, Campbell RE: **Genetically encoded FRET-based biosensors for multiparameter fluorescence imaging**. *Curr Opin Biotechnol* 2009, **20**:19-27.