



# Designs and applications of fluorescent protein-based biosensors

Andreas Ibraheem and Robert E Campbell

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.

## Address

University of Alberta, Department of Chemistry, Edmonton, Alberta, Canada T6G 2G2

Corresponding author: Campbell, Robert E  
([robert.e.campbell@ualberta.ca](mailto:robert.e.campbell@ualberta.ca))

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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 strat-

egies 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<sup>pro</sup> 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<sup>2+</sup>, researchers have used an MRE composed of calmodulin and a Ca<sup>2+</sup>/calmodulin-binding peptide. This MRE has served as the basis of the ever-expanding family of Ca<sup>2+</sup> biosensors known as Cameleons [14,15]. Using similar designs, FRET-based biosensors have been developed for Zn<sup>2+</sup> [16] and cyclic nucleotides [17].

### Group II: intermolecular FRET-based biosensors

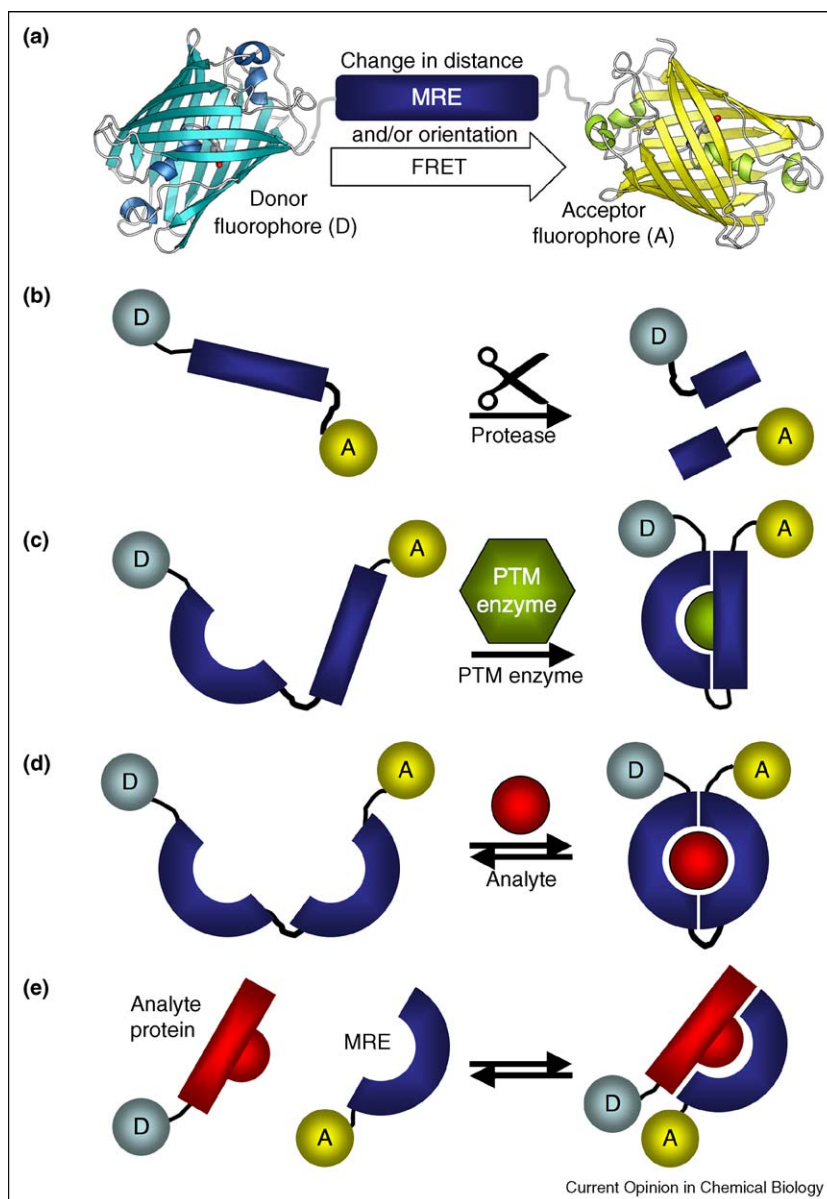
Biosensors belonging to this group are necessarily split constructs, in which the MRE is fused to one of the FPs and the analyte protein is fused to the other (Figure 1e). This design of biosensors is particularly useful for the study of protein–protein interactions. Intermolecular FRET has been applied 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  $\beta$ -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

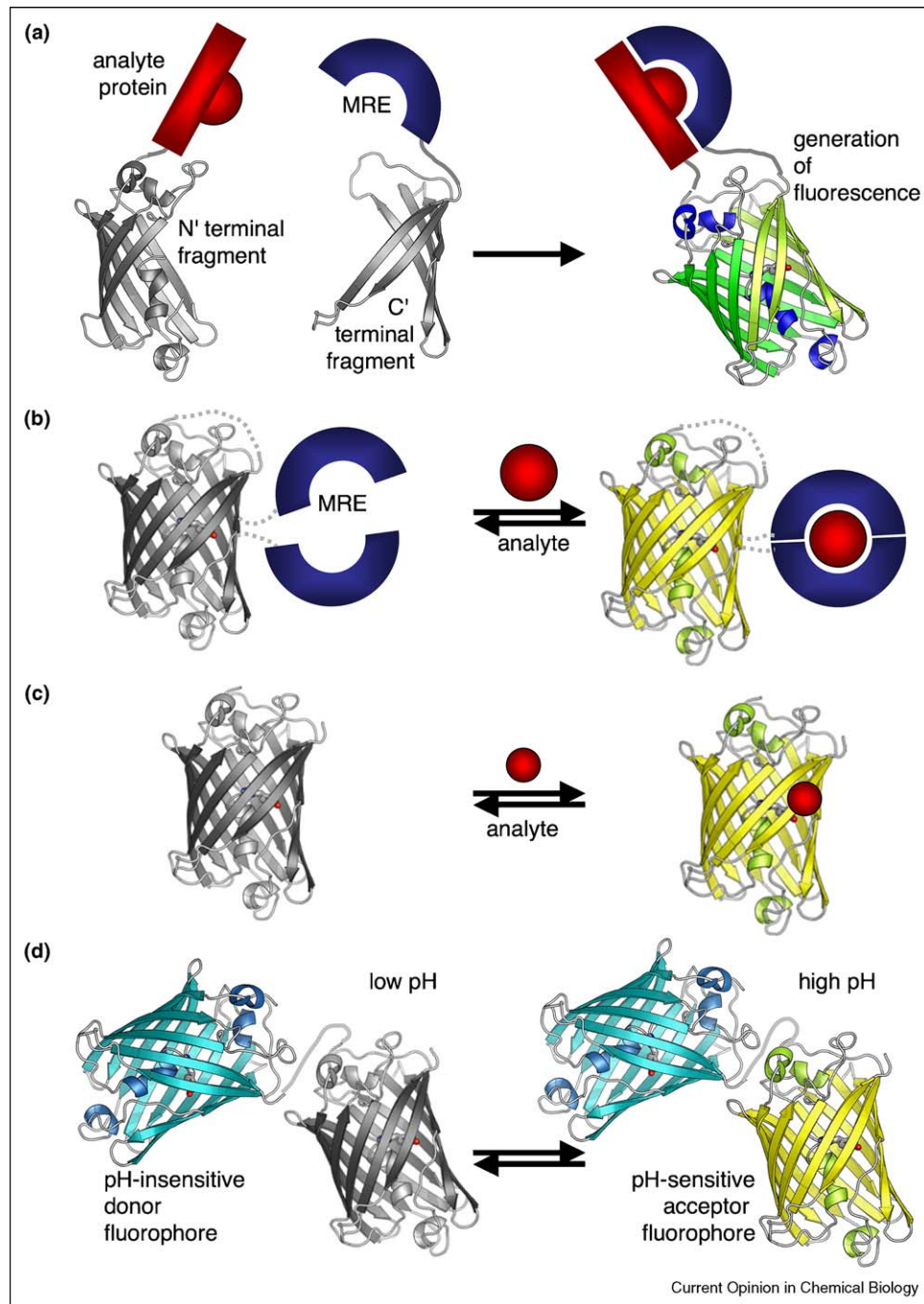


FRET-based biosensor designs. **(a)** Schematic model of a generic intramolecular FRET-based biosensor. A FP FRET pair flanks an MRE that undergoes a conformational change that alters the distance and/or orientation of the FPs relative to each other. **(b)** An MRE suitable for the detection of protease 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  $\text{Ca}^{2+}$  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 *Drosophila* 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 intensimetric 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 measure-

ment 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 intensimetric 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.

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