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# Recent Progress in the Construction Methodology of Fluorescent Biosensors Based on Biomolecules

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## 1. Introduction

The creation of novel molecular tools for detection and monitoring of the transitional concentration and localization changes of biologically important molecules, such as biomacromolecules, signaling small molecules and biologically important ions, is a great challenge in the field of chemical biology. Therefore, much attention has been devoted by chemists and biologists to develop sensing tools that allow real-time tracking of the molecules of interests *in vivo* or *in vitro*. (Thevenot, D. R. et al., 2001; Jelinek, R. et al., 2004; Borisov, S. M. et al., 2008) Among them, the fluorescent biosensor, which is defined as the sensor that converts a molecular recognition event to a measurable fluorescent signal change, has recently emerged as a powerful tool for the following reasons. (Hellings, H.W. et al., 1998; Johnsson, N. et al., 2007; Johnsson, K., 2009; Wang, H. et al., 2009; Liu, J. et al., 2009) Biomacromolecular receptors, such as nucleic acids (DNA or RNA) or proteins, have superior characteristics as the recognition platform because they play crucial roles in numerous biological processes to mediate and regulate a range of strict recognition and chemical reactions within cells. As for the tools for the transducer, the fluorescence detection has the superior physical properties, such as high sensitivity, excellent spatial resolution, good tissue penetration and low cost for the detection system, in contrast to the other detection method including optical, electrical, electrochemical, thermal, magnetic detections. Thus, transducing the molecular recognition events with the fluorescence signals is very appealing and has been one of the most widely adapted methods. (Giepmans, B.N. et al., 2006; Rao, J. et al., 2007) The rational design strategies of fluorescent biosensors have not been matured as generally considered by the researchers in the biological field. A simple strategy to construct a biosensor with tailored characteristics would be to conjugate a recognition module with a signal transducer unit, although there is no simple methodology to conjugate the recognition module and the transducer unit to afford a usable fluorescent biosensor. Here we focus to overview the progress in the design strategy of fluorescent biosensors, such as the auto-fluorescent protein-based biosensor, protein-based biosensor covalently modified with synthetic fluorophores and signaling aptamers.

## 2. Auto-fluorescent proteins (AFPs) based biosensors

Auto-fluorescent proteins (AFPs) such as green fluorescent protein (GFP) from jellyfish (Shimomura, O. et al., 1962) are widely used as noninvasive fluorescent markers for gene expression, protein localization, and intracellular protein targeting (Chalfie, M. et al., 1994; Lippincott-Schwartz, J. et al., 2001). The application of AFPs is not limited to the fluorescent markers. Various kinds of AFP-based biosensors have recently been developed by fusion of reporter proteins or mutation of AFPs for imaging and sensing important molecules and key events in living cell. (Zhang, J. et al. 2002; Zhang, J. et al. 2007; Mank, M. et al., 2008; VanEngelenburg, S. B. et al. 2008; Lawrence, D. S. et al. 2007; Ozawa, T. 2006; Prinz, A. et al. 2008) The advantage of AFP-based biosensor is that it can be endogenously expressed in cells or tissues simply by transfection of the plasmid DNA encoding it. This approach is a noninvasive method and therefore avoids damage to the cell. Because AFPs based biosensor can be produced automatically, the influence of dilution due to vital activity, such as cell growth and division, is minimal. Moreover, it is possible to control the localization of biosensors to the sites of interest within cell by introducing a certain organelle-specific targeting signal. These biosensors have been powerful tool for in vivo applications.

### 2.1 Single AFP based biosensor

In the case of biosensors based on a single AFP, analyte binding events affect directly or indirectly to fluorescent properties or formation, respectively, of the chromophore moiety of AFP. The former is classified as analyte-sensitive sensors and the latter as conformation-sensitive sensors.

The design of analyte-sensitive sensors utilizes AFP variants, whose fluorescent properties are directly affected by the interaction between a target molecule and a chromophore moiety in AFP. In general, the fluorescence of most of AFP variants is affected reversibly by moderate acidification of the chromophore. To exploit such intrinsic properties of AFPs, pH sensitive AFP variants have been developed. (Kneen, M. et al. 1998; Llopis, J. et al. 1998; Miesenbock, G. et al. 1998; Matsuyama, S. et al. 2000) Mutants of YFPs showing pH sensitivity bind to halide ion selectively and the binding of anion leads to fluorescence quenching due to the induced pKa shift. (Wachter, R. M. et al. 1999; Jayaraman, S. et al. 2000; Wachter, R. M. et al. 2000) The fluorescence of AFP becomes sensitive to other signals by the introduction of specific mutation in close proximity to the chromophore or within the barrel structure. In this manner, biosensors specific for Mercury (II) ion (Chapleau, R.R. et al. 2008) and Zinc (II) ion (Barondeau, D. P. et al. 2002) have been created. The receptor function of the sensor was directly integrated into the chromophore by alteration of the chemical nature around the chromophore.

Another design strategy of a single AFP based biosensor relies on circularly permuted AFP (cpAFP), which is classified as a conformation-sensitive sensor, that is, a conformational change of the receptor associated with the ligand-binding event results a formation of the AFP chromophore. The cpAFP is a regenerated AFP variant, in which the original N- and C termini are connected with a flexible peptide linker to regenerate novel N and C termini at specific positions. (Baird, G. S. et al. 1999) A number of cpAFPs with novel termini retained their fluorescence even when a foreign receptor was inserted at the termini. Indeed, cpAFP variants that detect  $\text{Ca}^{2+}$  (Nakai, J. et al. 2001; Souslova, E. A. et al. 2007; Baird, G. S. et al. 1999, Nagai, T. et al. 2001), cGMP (Nausch, L. W. et al. 2008),  $\text{H}_2\text{O}_2$  (Belousov, V. V. et al. 2006; Dooley, C. T. 2004),  $\text{Zn}^{2+}$  (Mizuno, T. et al. 2007) and an inositol

phosphate derivative (Sakaguchi, R. et al. 2009), have been developed by inserting appropriate receptor modules.

Morii and coworkers developed a cpAFP-based sensor for D-*myo*-inositol-1,3,4,5-tetrakisphosphate, Ins(1,3,4,5)P<sub>4</sub>, by utilizing a newly designed split PH domain of Bruton's tyrosine kinase (Btk) and cpGFP (Sakaguchi, R. et al. 2009) (Figure 1). Interestingly, the conjugate Btk-cpGFP realized a ratiometric fluorescence detection of Ins(1,3,4,5)P<sub>4</sub> by the excitation of each distinct absorption band, and retained ligand affinity and selectivity of the original PH domain.

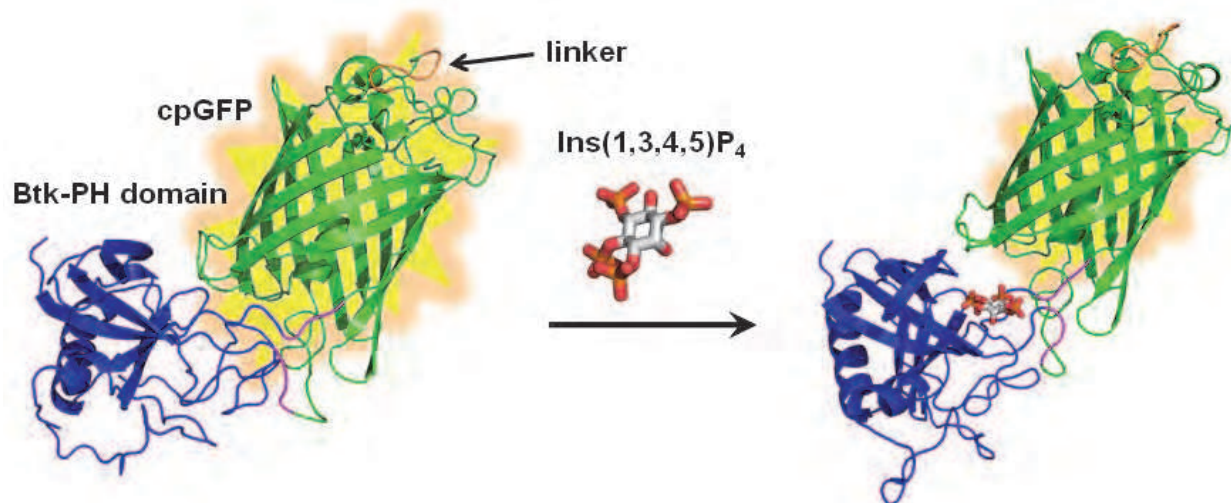


Fig. 1. Schematic illustration shows a fluorescent biosensor for Ins(1,3,4,5)P<sub>4</sub> based on the split Btk PH domain-cpGFP conjugate (Sakaguchi, R. et al. 2009). The original N and C termini of GFP were linked with a short peptide linker (orange), and the novel terminal of cpGFP (purple) was fused to the split Btk PH domain (blue). The conformational change of the PH domain induced by the ligand-binding event was transduced to the structural change of the chromophore of cpGFP, and then resulted in the ratiometric fluorescence change of cpGFP.

## 2.2 Split AFP based biosensor

It is considered that the formation of a AFPs chromophore requires a properly folded and an intact structure. However, many experimental data indicate that slight structural modifications of AFPs, like circular permutation and insertion of recognition domains as described in the previous section, still give fluorescent AFPs constructs. Therefore, AFP sensors in the absence of targets often reveal unavoidable background fluorescence. An excellent strategy to accomplish full suppression of the initial fluorescence utilizes an AFP variant that was split into two non-fluorescent fragments. (Shyu, Y.J. et al. 2008; Kerppola T. K. 2006) Regan and co-workers first demonstrated that a split GFP displayed a quite low background fluorescence in the separated state and a fluorescence emission was significantly recovered by the reassembly of the two fragments when they were placed in close proximity by strongly interacting antiparallel leucine zippers. (Ghosh, I. et al. 2000) Based on this strategy, a receptor composed of two subunits that are associated by binding to the analyte can be converted into a fluorescent biosensor by connecting each of the two subunits with each split AFP fragment (Figure 2). Actually, several types of biosensors have



been developed for fluorescent detection of specific DNA sequences (Stains, C. I. et al. 2005; Demidov, V. V. et al. 2006), DNA methylation (Stains, C. I. et al. 2006), mRNA (Ozawa, T. et al. 2007; Valencia-Burton, M. et al. 2007) and protein interactions (Nyfeler, B. et al. 2005; Hu, C. -D. et al. 2003; Wilson, C. G. et al. 2004).

Unlike the above-mentioned split AFP reconstitution, in which split AFP halves reform into a fluorescent structure via noncovalent association, another reconstitution strategy, intein-mediated reconstitution, has been developed by Ozawa and co-workers (Ozawa, T. et al. 2000). In this strategy, split inteins were fused to split EGFPs. Each split intein-EGFP fusion is attached to a protein of interest. The split inteins are brought into close proximity to trigger protein splicing when an analyte induces the association between proteins of interest. As a result, the two EGFP fragments are linked with a covalent bond and emit fluorescence. More comprehensive information on this reconstitution strategy is available in other excellent reviews (Ozawa, T. 2006; Awais, M. et al. 2011).

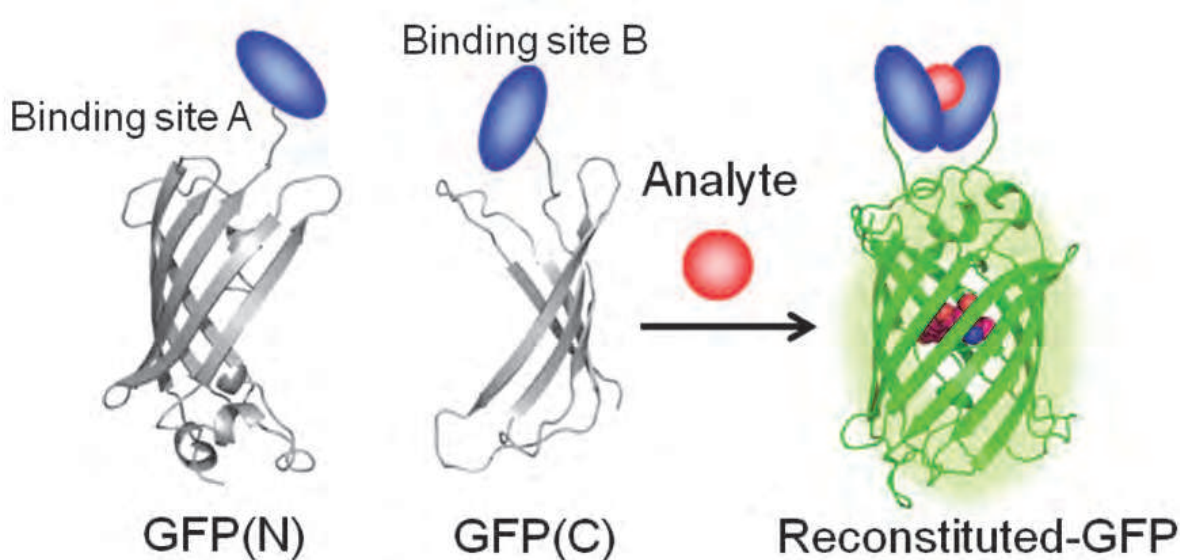


Fig. 2. Schematic illustration shows split GFP based fluorescent biosensor. A fluorescent protein such as GFP is split into two halves [GFP(N) and GFP(C)], which connect each of the two binding subunits, are associated by binding to the analyte.

### 2.3 FRET based biosensor

Non-radiative transfer of energy from an excited donor fluorophore to an acceptor chromophore is known as fluorescence resonance energy transfer (FRET). In order to induce FRET, the excitation spectrum of the acceptor must overlap with the emission spectrum of the donor, and the two fluorophores must be close in proximity ( $< 10$  nm) and in a favorable orientation (Sapsford, K. E. et al. 2006). The efficiency of FRET is sensitive to the distance and the orientation between the donor and acceptor groups. To obtain the expected energy transfer efficiency for biological applications, the following two issues in the sensor design should be considered. First, suitable FRET pairs in which the donor emission spectrum overlaps the acceptor absorption spectrum should be chosen. In the AFP-based FRET strategy, CFP and YFP mutants have been favorably utilized as a FRET donor and an acceptor, respectively (Piston, D.W. et al. 2007). Second, the donor and the acceptor fluorophores should be placed at a rational distance which can drastically change

the efficiency of FRET before and after the sensing event. (Ohashi, T. et al. 2007) Therefore, a FRET based biosensor can sense the analyte in a ratiometric manner by comparing the donor and acceptor emission intensities that are result from the analyte induced distance and/or conformational changes. Based on the mechanism by which FRET efficiency changes, AFP-based FRET biosensors can be divided into two classes, that is, an intramolecular and an intermolecular FRET systems (Figure 3). In the case of intramolecular FRET biosensors, the two fluorophores are attached at two ends of a peptide sequence in the receptor protein or the concatenation of interacting domains. The feasibility of this strategy strongly depends on the magnitude of the structural change of the receptor. In the case of a receptor that displays a large structural change upon binding to the substrate, this strategy would be the most straightforward way to integrate the signal transduction function into the receptor of interest. Based on this strategy, various FRET biosensor for imaging intracellular events such as enzyme activities [e.g. protease (Mahajan, N. P. et al. 1999; Luo, K. Q. et al. 2001; Rehm, M. et al. 2002; Ai, H. W. et al. 2008), kinase (Sato, M. et al. 2002; Nagai, Y., et al. 2000), phosphatase (Newman, R. H. et al. 2008)] and dynamics of intracellular second messengers [e.g.  $\text{Ca}^{2+}$  (Miyawaki, A. et al. 1997; Romoser, V. A. et al. 1997), cAMP (Nikolaev, V. et al. 2004), cGMP (Sato, M. et al. 2000),  $\text{IP}_3$  (Sato, M. et al. 2005)] have been developed. It should be noted that careful optimization, such as tuning the position of AFPs relative to the sensing domain by changing the linker between each of protein units, is frequently necessary to realize the satisfactory response of the FRET change. Most importantly, the

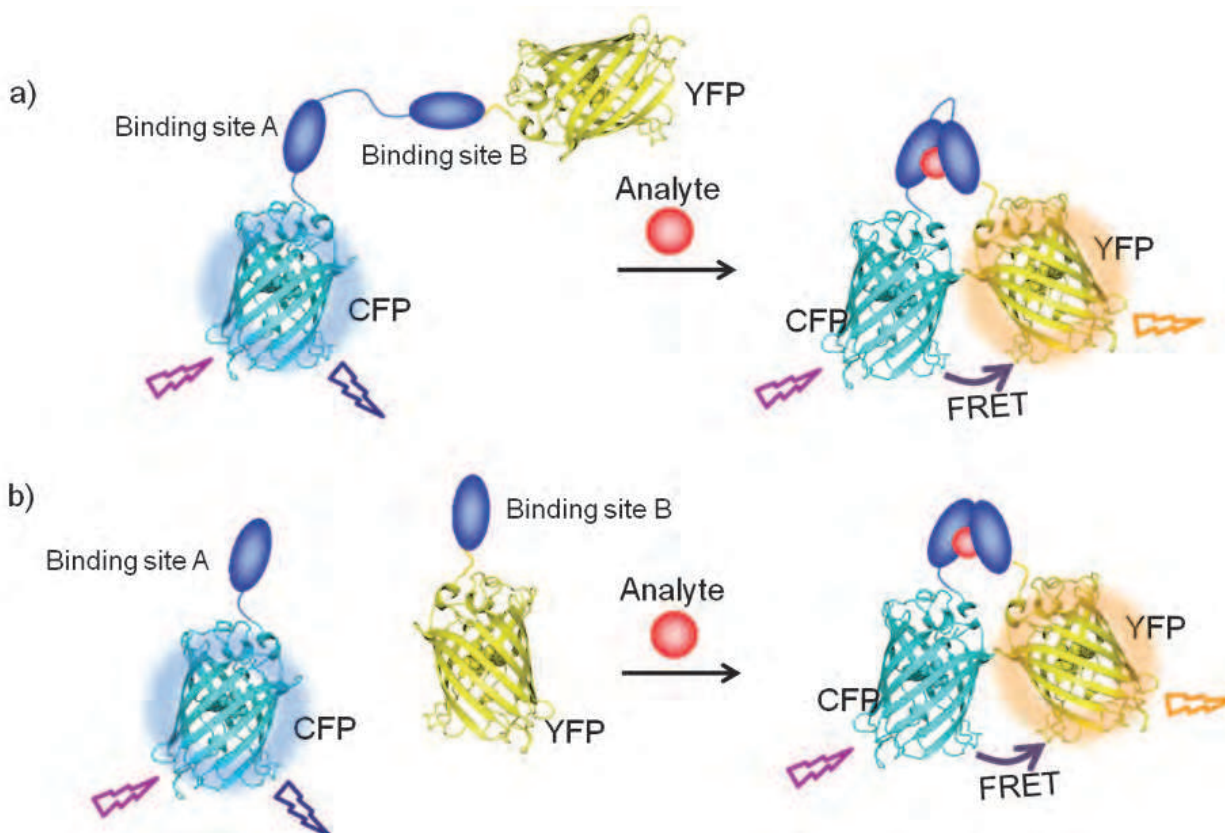


Fig. 3. AFP-fused FRET based biosensors. (a) Intramolecular FRET-based biosensor: The protein domains with a large structural change upon the analyte binding event. (b) Intermolecular FRET-based biosensor: The change of FRET efficiency is induced by the dissociation or association of the subunit upon the analyte-binding event.

obligatory conformational change in the receptor protein severely limits the choice of proteins available for the construction of FRET biosensors by this strategy. Recently, Johnsson and co-workers have demonstrated a new type of FRET biosensor based on their SNAP-tag technique, for which conformational changes upon analyte binding were not required (Brun, M. A. et al. 2009). Intermolecular FRET biosensors have been developed by employing two protein domains separated from each other, to which AFPs of FRET donor and acceptor are attached, respectively. Zaccaro and co-workers constructed FRET biosensor for cAMP by applying this strategy to the regulatory and catalytic subunit of protein kinase A (PKA) (Zaccolo, M. et al. 2000; Zaccolo, M et al. 2002). This biosensor can detect the rise of intracellular cAMP concentration by the decrease in the FRET efficiency induced by dissociation of the catalytic subunit from the regulatory subunit. Although this strategy shows a potential to effect a dynamic FRET change by the analyte-induced association and/or dissociation of protein subunits, the stoichiometry of the FRET donor and acceptor may vary between either cells or intracellular compartments. In these cases, they cause difficulty in analysis of the FRET efficiency changes. More comprehensive information on dual FRET-based biosensors is available in other excellent reviews (Souslova, E. A. et al. 2007; VanDngelenburg, S. B. et al. 2008; Carlson, H. J. et al. 2009).

### **3. Protein-based biosensor covalently modified with fluorescent artificial molecules**

Another useful strategy to construct fluorescent biosensors is a structure-based design of a protein covalently modified with a fluorescent dye. Advantages for the use of fluorescent dyes are as follows. First, the relatively smaller size of the synthetic fluorophore is likely to less perturb the property of the original receptor protein. Second, a superior characteristic of dye, that is, the fluorescence changes in intensity and wavelength and the microenvironmental sensitivity such as pH, polarity or molecular recognition, could be introduced to the receptor protein. Not only simple dyes but also functional molecules, such as artificial receptors, can be incorporated. Third, the attachment of dye to the protein framework is more flexible than the use of AFPs. While the attaching positions of AFP are generally limited to the N- and C termini of receptor proteins, the incorporation of small dye to proteins is also possible in the middle of loop regions or at close proximity to the binding pocket. On the other hands, unlike AFPs based biosensor, this type of protein-based biosensor generally require the invasive technique for translocating across the plasma membrane, such as electroporation (Marrero, M.B. et al. 1995; Fenton, M. et al. 1998; Sakaguchi, R. et al. 2010), lipofection (Zelphati, O., et al. 2001; Zheng, X. et al. 2003), microinjection (Abarzua, P. et al. 1995), and tagging cell-permeable peptide sequences (Wadia, J.S. et al 2005; Sugimoto, K. et al 2004). In addition, the central issue for the construction of these types of biosensors is the way to introduce a dye into the receptor protein site-selectively. Here, a variety of fluorescent biosensors that use fluorescent molecules is described according to a classification of the incorporation methodologies of fluorescent dye.

#### **3.1 Introduction of a thiol reactive fluorophore on a unique cysteine residue of engineered receptor protein**

The most important process to success this methodology is that all of the original cysteine residue of the receptor protein must be initially substituted with other amino acids to avoid

the nonspecific labeling of cysteine reactive fluorophores. Following the process, a unique cysteine residue was introduced at specific position. The position to introduce a fluorophore is most conveniently determined by the three-dimensional structure of the receptor protein. As a pioneering work, bPBPs (bacteria periplasmic binding protein), a representative protein scaffold, were converted to fluorophore-modified biosensors by Hellinga *et al.* (Dwyer, M. A. *et al.* 2004) or others (Gilardi, G. *et al.* 1994; Brune, M. *et al.* 1998; Hirshberg, M. *et al.* 1998). Most of bPBPs consist of two domains connected by a hinge region, with a ligand binding site located at the interface between the two domains, which can permit dynamic conformational changes induced upon ligand binding. Therefore, two distinct approaches are used to establish an efficient signal transduction mechanism that would sense the ligand-binding event. In the first approach, an environmentally sensitive fluorophore is positioned in the binding pocket so that the ligand-induced changes in the fluorescence are produced by the direct fluorophore-ligand interactions. This approach often has a disadvantage that unfavorable steric interactions between the introduced fluorophore and the ligand lower the binding affinity. The second approach introduces environmentally sensitive fluorophore at the region that is distant from the ligand-binding site but exhibits dynamic domain movement in response to the ligand binding. This allosteric sensing mechanism shows an advantage that the ligand binding is essentially unaffected by introducing a fluorophore.

On the other hand, there are number of proteins that do not undergo such a dynamic conformational change upon ligand binding, but they are capable of recognizing the various substances of biological importance. The useful methodology to convert such non-allosteric proteins to fluorescent biosensors is to introduce an environmentally sensitive fluorophore within the proximity of the ligand-binding site, though this strategy might have some drawbacks as mentioned above. But several successful examples demonstrated that such a methodology is applicable for obtaining biosensors (Chan, P. H. *et al.* 2004; Nalbant, P. *et al.* 2004; Chan, P. H. 2008). Morii and coworkers constructed novel biosensors for inositol 1,4,5-trisphosphate [Ins(1,4,5)P<sub>3</sub>] and 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P<sub>4</sub>] by utilizing the pleckstrin homology (PH) domain of phospholipase C (PLC)  $\delta_1$  (Morii, T. *et al.* 2002) and the general receptor for phosphoinositides 1 (GRP1) (Sakaguchi, R. *et al.* 2010) (Figure 4), respectively. In these biosensors a synthetic fluorophore was attached at the proximity of the ligand-binding site based on the three dimensional structures of proteins so that the changes in orientation of the fluorophore induced by the substrate binding lead to a sufficient fluorescence response. This structure-based design of synthetic fluorophore-modified biosensors is a powerful method to produce biosensors with high selectivity and appropriate affinity to target inositol derivatives in living cells (Sakaguchi, R. *et al.* 2010; Sugimoto, K. *et al.* 2004; Nishida, M. *et al.* 2003).

### 3.2 Site-specific unnatural amino acid mutagenesis with an expanded genetic code

As mentioned above, the post-labeling of unique cysteine residues required preliminary preparation that all of the original cysteine residue of the receptor protein must be substituted with other amino acids. The process might cause the instability of the receptor protein mutant. A mutagenesis technique for direct incorporation of synthetic fluorophores as unnatural amino acids into desired positions in proteins can avoid such a problem. A site-specific mutagenesis with an expanded genetic code that employed an amber suppression method (Wang, L. 2005; *et al.* Xie, J. *et al.* 2006) or a four-base codon method (Hohsaka, T. *et al.*, 2002) in cell-free translation systems has provided a variety of fluorescently modified



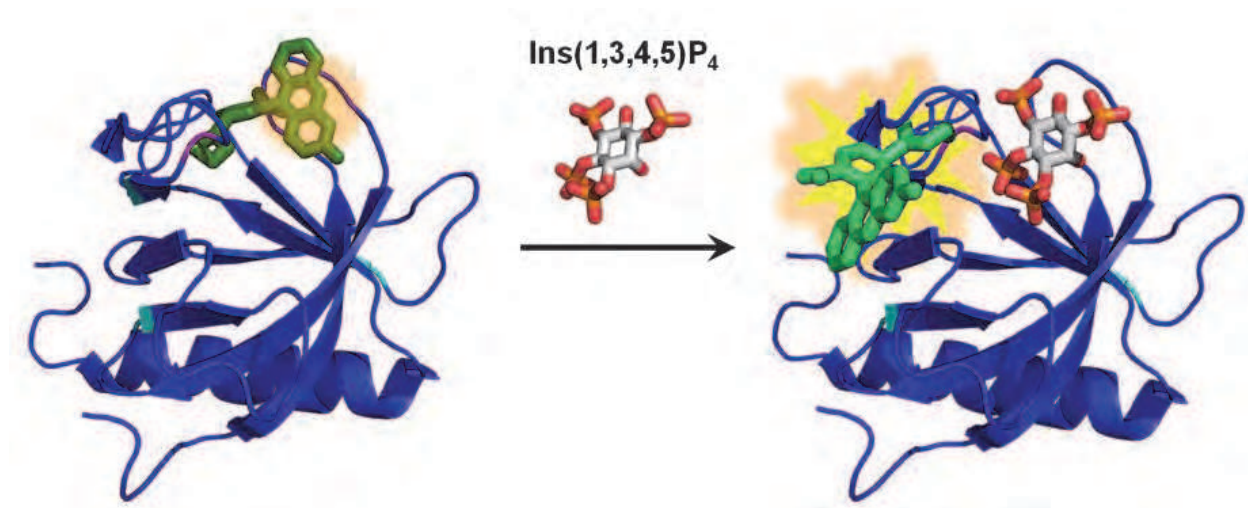


Fig. 4. A schematic illustration shows a fluorescent biosensor for Ins(1,3,4,5)P<sub>4</sub> based on the GRP1 PH domain (Sakaguchi, R. et al. 2010). Firstly, the original cysteine residues (cyan) of GRP PH domain were replaced with other amino acids. Second, a unique cysteine residue (magenta) was introduced to the resultant mutant followed by labeling with thiol reactive fluorescein (green) as an environment sensitive fluorophore to give Ins(1,3,4,5)P<sub>4</sub> sensor. The local environmental change of the fluorophore induced by the ligand-binding event was transduced to the fluorescence enhancement.

proteins (Anderson, R. D. et al. 2002; Taki, M. et al. 2002; Kajihara D. et al. 2006). As an excellent example, Hohsaka and co-workers prepared a series of semisynthetic calmodulins, two different position of which were replaced with unnatural amino acids bearing a FRET pair of BODIPY derivatives by using two sets of four-base codons. Some of the doubly modified calmodulin sensed calmodulin-binding peptide by substantial FRET signal changes. This is a powerful tool for site-specific introduction of unnatural amino acids into protein, though the examples of the construction of fluorescent biosensor based on these methods are still limited.

### 3.3 Covalent introduction of fluorescent molecules by chemical modification

Modification of a protein by using genetic method often causes the lower activity or instability of the mutated protein as mentioned in the previous section. In addition, the method is not appropriate when the three dimensional structure of a receptor protein is not known. In that case, an approach to site-specifically incorporate a signal transducer proximal to the binding pocket of intact receptor protein by using selective chemical modifications is valid.

As the primary example, Schultz and co-workers constructed an antibody-based fluorescent biosensor by using an affinity-labeling method (Pollack, S. J. et al. 1988). The chemically engineered antibody, of which the proximal antigen-recognition site was modified by fluorescent molecule, can detect antigen binding by fluorescence decrease. Hamachi and co-workers constructed a lectin-based fluorescent biosensor using an improved photo affinity labeling method, termed as P-PALM (post-photoaffinity labeling modification) (Hamachi, I. et al. 2000; Nagase, T. et al. 2001, Nagase, T. et al. 2003). This methodology can introduce artificial molecules (e.g. fluorophore, artificial receptor) proximal to the active site of a target protein without genetically modifying the protein framework. In a proof-of-principle experiment, P-PALM was demonstrated by using concanavalin A (Con A), an extensively

studied lectin (saccharide-binding protein). Introduction of a thiol group as a chemoselective modification site in proximity of the ligand-binding pocket of Con A is conducted by a designed photoaffinity labeling molecule, which is composed of a ligand module, a photo reactive module and a cleavable disulfide module. Depending on the nature of the subsequent modification by a thiol reactive artificial molecule, not only environmental sensitive fluorophore (Koshi, Y. et al. 2005; Nakata, E. et al. 2005; Nakata, E. et al. 2008) but also fluorescent artificial receptor (Nakata, E. et al. 2004) can be introduced to Con A. Intact Con A can be converted to a various type of fluorescent biosensors that successfully sense the saccharide derivatives in different manners. Because the initial P-PALM strategy based on thiol chemistry shows limited bioorthogonality, this method is not applicable to many proteins. To overcome this drawback, Hamachi group adopted the ketone/aldehyde-based hydrazone/oxime exchange reaction (Takaoka, Y. et al. 2006) and the organometallic Suzuki reaction (Wakabayashi, H. 2008) as bioorthogonal chemoselective modifications. Recently, Hamachi and co-workers also developed ligand-directed tosyl (LDT) chemistry-based approach as a more general and simple strategy of target selective chemical modification (Tsukiji, S. 2009). A detailed description of their strategies is described in other review articles (Nakata, E. et al. 2007; Wang, H. et al. 2009).

#### 4. Signaling aptamers

Protein based biosensors are generally constructed by using native or slightly modified proteins as the scaffold. Therefore, the function of the constructed biosensor, such as the specificity and the affinity toward the substrate, depends on that of the native receptor. Unlike receptor proteins, DNA or RNA based receptors (aptamers) which have appropriate affinity and specificity for various targets ranging from small molecules to proteins can be generated by using *in vitro* selection, also known as SELEX (systematic evolution of ligands by exponential enrichment) (Ellington, A. D. 1994; Ellington, A. D. et al. 1990; Gold, L. et al. 1995; Osborne, S. E. et al. 1997; Wilson, D. S. et al. 1999). That is, aptamers that bind to the substrate of interest with tailor made functions, such as the specificity and the affinity, can potentially be generated through *in vitro* selection. Previous work indicated that most of the structurally characterized aptamers underwent induced-fit type of conformational change upon ligand binding [Westhof, E. et al. 1997]. Introduction of the signal transduction module such as a fluorophore at an appropriate site of the aptamer enables a read out of the ligand-binding event as a local environmental change of the fluorophore. Thus, the design of aptamer-based fluorescent sensors represents an attractive and promising alternative to the protein-based sensors. Some excellent reviews of aptamer sensors have already covered the selection and evolution techniques and sophisticated applications of the aptamer sensors [Liu, J. et al 2009; Mok, W. et al. 2008]. Here we focus on unique modular strategies to construct aptamer sensors, which would avoid the cumbersome trial-and-error process to construct a sensor with an optimized function.

##### 4.1 Modular strategies for tailoring aptamer sensors

Sophisticated design strategies have successfully provided fluorescent biosensors based on biomolecules such as DNA, RNA or proteins, but these strategies usually require the redundant optimization of sensor functions. For example, introduction of the fluorophore often impairs the original receptor function and does not always ensure the fluorophore-labelled receptor to act as an expected sensor. It is quite difficult to empirically apply the

obtained findings from the previously constructed biosensor to the other one, because the communication between the substrate binding and the signal-transduction is not so simple and is unique to the individual biosensor. On the other hand, a modular strategy that permits facile preparation of biosensors with tailored characteristics by a simple combination of a receptor and a signal transducer has recently emerged as a new paradigm for a versatile design of fluorescent biosensors. Stojanovic and co-workers have proposed a modular design of signaling aptamers based on the allosteric regulation of binding events (Stojanovic, M. N. et al. 2004). The target binding aptamers were fused with the reporter dye binding aptamers, which can drastically increase the fluorescent intensity of reporter dye, and the reporter dye binding was significantly enhanced upon target binding. Fluorescent sensors for adenosine triphosphate (ATP), flavin mononucleotide (FMN) and theophylline have been demonstrated based on this design, showing the generality of the approach. Later, several groups reported various allosteric aptamer sensors based on the methodology (Kolpashchikov, D. M. 2005; Xu, W. et al. 2010; Furutani, C. et al. 2010).

The application of the selection and evolution technique is not limited to obtain functional macromolecules solely composed of RNA or DNA. Morii and co-workers have recently developed a conceptually new strategy for preparation of fluorescent biosensors with diverse functions based on a framework of ribonucleopeptide (RNP), such as the structurally well characterized complex of the Rev Responsive Element (RRE)-HIV Rev peptide (Rev peptide) and RRE RNA (Figure 5) (Tainaka, K. et al. 2010). In the first step to construct the fluorescent RNP sensor, a randomized nucleotide sequence was introduced into the RNA subunit of RNP to construct RNP library. In vitro selection method was applied to the RNP library to afford a series of RNP receptors for a given target (Morii, T. et al. 2002). In the second step, the Rev peptide was modified with a fluorophore as the transducer of binding event without greatly disturbing the affinity and specificity of the RNP receptor. The constructed fluorescent RNP sensor showed the fluorescent intensity changing upon binding to the target molecule as the result of the conformational change of RNA subunit by inducing target binding. In similar to RNA aptamers, the RNP receptors, which obtained by in vitro selection, are considered as a RNP receptor library, because a variety of RNA structures and reveal different affinity to the target molecule were included. The combined peptide subunit is also easily converted to functionalized Rev peptide libraries, such as various fluorophore modified Rev peptide libraries with a variety of excitation and emission wavelengths. By taking the advantage of such the noncovalent nature of the RNP complex, RNP sensors with desired affinity, selectivity and optical sensing properties could be selected in a high-throughput manner by combining a series of RNA subunits derived from each of the library. Actually, a variety of fluorescent biosensors for targeting ATP (Hagihara, M. et al. 2006), GTP (Hagihara, M. et al. 2006), histamine (Fukuda, M. et al. 2009), phosphotyrosine (Hasegawa, T. et al. 2005), and phosphotyrosine-containing peptide fragment (Hasegawa, T. et al. 2008) have been produced by the group, showing the generality of the approach. Recently, the group showed that ATP-binding RNP sensor was rationally converted to GTP-binding RNP sensor to have realized the detail of the recognition mechanism (Nakano, S. et al. 2011). Though the noncovalent configuration conveniently provides fluorescent RNP sensors in the selection stage, it have a possibility to becomes a disadvantage for the practical measurements after optimization of the sensor function, for instance, the RNP complex would dissociate to each component under reducing condition such as the nanomolar range. A covalently linking of RNA and peptide subunits without sacrificing the sensing function would overcome such disadvantages.

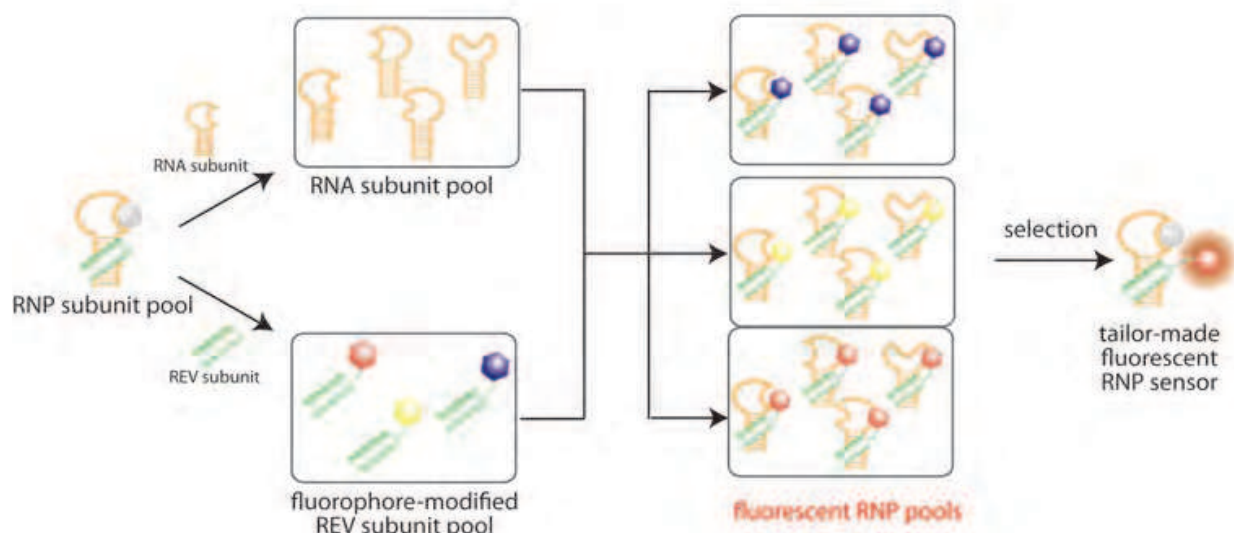


Fig. 5. Screening methodology of a tailor-made RNP fluorescent sensor [Hagihara, M. et al. 2006]. Combination between the RNA subunit library and several dye-labeled Rev peptide subunits generates combinatorial fluorescent RNP receptor libraries, from which RNP sensors with desired function, such as optical property, affinity and selectivity, are selected.

## 5. Perspective

Here we overviewed construction methodologies of fluorescent biosensor based on biomolecules, that is, protein-based biosensor and aptamer-based biosensor. The systematic developments of these technologies have expanded the applicability of fluorescent biosensors. In the case of the protein based biosensor, there is no doubt that these sensors represent the most practical and reliable tools for the real-time measurements of various biologically important molecules in living cells. Actually, the function of second messengers, for example, in the cell has been progressively clarified owing to significant contribution of these new biosensors. However, the wide varieties of the construction strategies, which have both the advantages and drawbacks as mentioned above, strongly indicated the lack of general approach to conjugate a recognition module with a signal transducer unit. Further effort in the fields for establishing a general and simple strategy to construct usable biosensors will realize tailor-made fluorescent biosensors.

Aptamer-based biosensors have potential to realize the tailor-made biosensor with finely tuneable affinity and selectivity based on in vitro selection technique, and to visualize intracellular molecules. However, this type of sensor is practically passive with challenges in cell application owing to the inherent liability of RNA molecules in the intracellular condition. Such the drawbacks will be overcome by the improved selection and evolution technique to construct the aptamers that resist to the cellular degradation activity.

## 6. Reference

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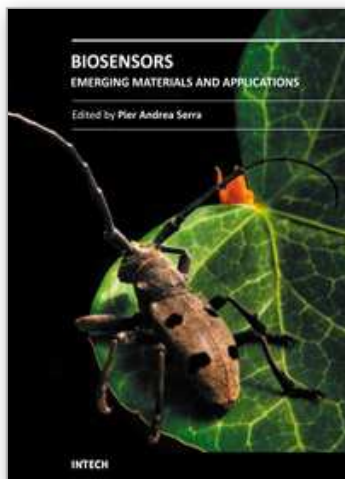
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## **Biosensors - Emerging Materials and Applications**

Edited by Prof. Pier Andrea Serra

ISBN 978-953-307-328-6

Hard cover, 630 pages

**Publisher** InTech

**Published online** 18, July, 2011

**Published in print edition** July, 2011

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Eiji Nakata, FongFong Liew, Shun Nakano and Takashi Morii (2011). Recent progress in the construction methodology of fluorescent biosensors based on biomolecules, Biosensors - Emerging Materials and Applications, Prof. Pier Andrea Serra (Ed.), ISBN: 978-953-307-328-6, InTech, Available from: <http://www.intechopen.com/books/biosensors-emerging-materials-and-applications/recent-progress-in-the-construction-methodology-of-fluorescent-biosensors-based-on-biomolecules>

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