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Construction of ligand assay systems by protein-based semisynthetic biosensors

Seiji Sakamoto, Shigeki Kiyonaka and Itaru Hamachi

Proteins as causative agents of diseases such as cancers, diabetes and neurological disorders are attractive drug targets. For developing chemicals selectively acting on key disease-causing proteins, one useful concept is the direct conversion of such target proteins into biosensors. This approach provides ligand-binding assay systems based on protein-based biosensors, which can quantitatively evaluate interactions between the protein and a specific ligand in many environments. Site-specific chemical modifications are used widely for the creation of protein-based semisynthetic biosensors *in vitro*. Notably, a few bio-orthogonal approaches capable of selectively modifying drug-targets have been developed, allowing conversion of specific target proteins into semisynthetic biosensors in live cells. These biosensors can be used for quantitative drug binding analyses in native environments. In this review, we discuss recent efforts for the construction of ligand assay systems using semisynthetic protein-based biosensors and their application to quantitative analysis and high-throughput screening of small molecules for drug discovery.

Address
Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

Corresponding author: Hamachi, Itaru (ihamachi@sbchem.kyoto-u.ac.jp)

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Introduction

Over the last two decades, a large number of protein-based biosensors have been created using fluorescent proteins (FPs) [1–7]. Since FPs are genetically encodable, they can be easily expressed in live cells to allow quantitative evaluation of various analytes, such as pH, second messengers and metabolites, in a spatiotemporal manner. The concentration and distribution of such analytes in live cells are considered to be critical parameters associated with many biological phenomena. Most FP-based biosensors are designed to target a particular analyte of interest, where an appropriate analyte-binding domain of a protein scaffold is carefully selected, and the subsequent fusion of FPs is conducted without loss of function. Ligand binding is often read-out by Förster resonance energy transfer (FRET). In these situations, a large conformational shift upon analyte binding is required. Analyte-induced conformational changes in the distance and/or relative orientation of two FPs affect FRET efficiency, which induces ratiometric fluorescence readout from FRET-based biosensors.

Proteins/enzymes that are causative agents of disease states such as cancer, diabetes and neurological disorders are attractive drug targets. Conversion of these drug-target proteins to biosensors should enable the construction of ligand-binding assay systems using protein-based recognition. These systems should be powerful for detailed quantitative evaluation of interactions between the protein and a specific ligand not only *in vitro* but also in live cells and more complex biological samples. Such a set up would provide a unique platform for drug screening, which is crucial for the discovery of new therapeutics and diagnostics. However, conversion of disease-related proteins to biosensors is more difficult when compared with the preparation of conventional FP-based biosensors. Also, it is ideal to employ the whole target protein, rather than simply the ligand-binding domain. This is because the whole protein enables assaying allosteric as well as orthosteric ligands.

Chemical modification of proteins may allow the creation of protein-based semisynthetic biosensors. Semisynthetic biosensors composed of a protein scaffold and an artificial small molecule offer a complementary approach for the construction of FP-based biosensors [2,6,7,8–13]. The relatively small sizes (<1 kDa) of synthetic probes such as fluorescence, NMR, MRI and Raman may confer minimal effects on the structure and function of modified proteins and their incorporation site can be flexibly tuned in the protein of interest (POI). Traditionally, semisynthetic biosensors were prepared by a site-specific chemical modification strategy targeting a Cys mutation in the protein scaffold [14–16,17•,18–21]. The optimal mutation site for introducing the Cys would be chosen to cause a sensitive and selective signal change upon ligand binding. In addition to such a conventional method conducted in test tubes, a few bio-orthogonal approaches capable of site-selectively and protein-selectively modifying POIs

have been developed recently, which allow the direct conversion of a target protein into a semisynthetic biosensor in live cells. Herein, we discuss recent efforts for the construction of ligand assay systems using semisynthetic protein-based biosensors and their application to the quantitative analysis and high-throughput screening of small chemicals for drug discovery.

Cutting-edge strategies for constructing semi-synthetic biosensors under live cell conditions

Snifit and LUCID technology

Johnsson and co-workers have explored a rational method that allows the conversion of POIs to FRET-based biosensors called Snifits (SNAP-tag-based indicator proteins with a fluorescent intramolecular tether) [8]. This strategy relies on an orthogonal labeling technology employing a SNAP-tag and a CLIP-tag, which are specifically and covalently labeled with O^6-benzylguanine (BG) and O^2-benzylcytosine (BC) derivatives, respectively (Figure 1a) [22]. The Snifit strategy involves simultaneous tethering of two different fluorophores, a FRET donor and acceptor pair, to a POI (Figure 1b). In the general design of Snifit biosensors, the SNAP-tag is modified with a bifunctional synthetic molecule containing a FRET-acceptor and a POI-affinity ligand, while the CLIP-tag is labeled with a FRET-donor. In the absence of the analyte, the tethered ligand binds to the fused POI in an intramolecular fashion, resulting in a closed conformation of the Snifit. In the presence of analytes, in contrast, the analytes compete for binding to the POI, resulting in a shift of the equilibrium to the open conformation. This conformational change alters the FRET efficiencies between the two attached fluorophores. Snifit enables the construction of a specific ratiometric semisynthetic biosensor in live cells without the need for a large conformational change within the ligand-binding domains. In addition, a wide variety of synthetic fluorophores are available as FRET pairs (Cy3/DY-547, Cy3/Cy5, Alexa Fluor 488/DY-547), and the detection ranges can be tuned easily in light of the desired analyte concentrations by choosing the appropriate tethered ligand. In early studies, Brun *et al.* constructed protein-based biosensors using human carbonic anhydrase 2 (CA2) as a POI. The CA2 inhibitors are used in the field of antiglaucoma and diuretic agents for the treatment of altitude sickness and some anticonvulsants, and possibly against some cancer cells. The biosensors allowed the ratiometric sensing of CA inhibitors in a quantitative manner in live cells [23,24]. The same group also created a ratiometric fluorescent biosensor using acetylcholine esterase (AchE) [25]. Furthermore, replacement of the CLIP-tag with a luciferase (NanoLuc) generates bioluminescent resonance energy transfer (BRET)-based sensors, termed luciferase-based indicators of drugs (LUCIDs), which permit monitoring of therapeutic drugs and biologically important metabolites for point-of-care diagnosis with portable paper devices [26•,27•,28•]. These achievements reveal the potential generality and flexibility of Snifit technology as a rational design for semisynthetic biosensors available for drug screening assays. Although powerful, the molecular weights of fused enzyme tags are still comparable to FPs and may be too large in some cases to ensure the fused POIs retain their native structure, function, dynamics and localization.

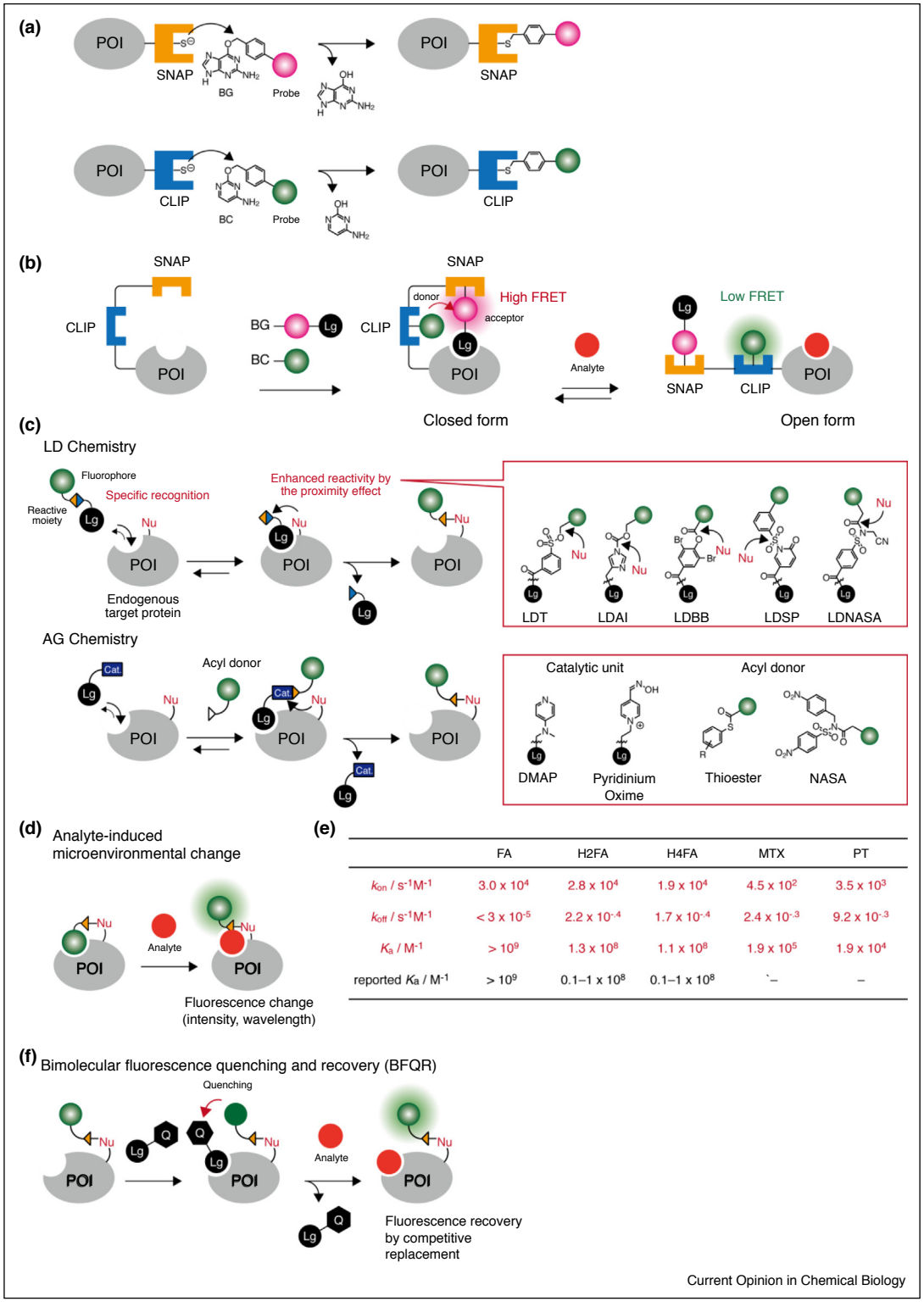
Ligand-directed and affinity guided chemical labeling

Conventional FP-based biosensors basically require the artificial expression of exogenous protein constructs in cells. In sharp contrast, traceless affinity-based protein labeling methods may be an alternative that allows the construction of native (endogenous) protein-based semisynthetic biosensors in live cells with minimal perturbation of cellular conditions. Tsukiji *et al.* developed a new recognition-driven method for endogenous protein labeling, termed ligand-directed (LD) chemistry, in which a cleavable electrophile is employed as a linker of a labeling reagent for connecting the affinity ligand and the reporter group (Figure 1c) [29]. In LD chemistry, the ligand moiety selectively recognizes and binds to the target protein, facilitating the chemical reaction of the reactive group with an amino acid located near the ligand-binding site through a proximity effect [29,30]. Hamachi and co-workers have also developed another type of traceless affinity-based labeling strategy, affinity-guided 4-dimethylaminopyridine (DMAP) chemistry (AGD chemistry) [30,31]. Here, an affinity ligand tethered to the DMAP catalyst promotes the acyl transfer reaction from an appropriate acyl donor to a nucleophilic amino acid residue near the ligand-binding pocket of POIs (Figure 1c). Both LD and affinity-guided (AG) chemistries can modify an amino acid proximal to the ligand binding pocket of POIs, which may directly convert a target endogenous protein to a corresponding biosensor even under multimolecular crowding found under live cell conditions (Figure 1d). For example, Hamachi's group labeled endogenous folate receptors (FRs) with several fluorophores on the surface of live cells by LD chemistry [32]. The FR is overexpressed in some cancer cell lines, and is thus, considered to be a drug target and biomarker for cancer diagnosis. Hamachi's group finally demonstrated that the fluorescein-labeled FR could behave as a fluorescent biosensor. Using this molecule, quantitative evaluation of the binding kinetics and dissociation constants of folic acid (FA) and its analogues [dihydrofolic acid (H2FA), tetrahydrofolic acid (H4FA), pterine (PT) and methotrexate (MTX)] to the FR under live cell conditions was undertaken (Figure 1e).

The BFQR strategy

The chemical labeling of proteins by LD and AG chemistries does not always produce suitable biosensors because changes in the microenvironment around the tethered fluorophore are usually required for the ligand-binding induced fluorescence changes. In order

Figure 1



	FA	H2FA	H4FA	MTX	PT
k_{on} / $s^{-1}M^{-1}$	3.0×10^4	2.8×10^4	1.9×10^4	4.5×10^2	3.5×10^3
k_{off} / $s^{-1}M^{-1}$	$< 3 \times 10^{-5}$	2.2×10^{-4}	1.7×10^{-4}	2.4×10^{-3}	9.2×10^{-3}
K_a / M^{-1}	$> 10^9$	1.3×10^8	1.1×10^8	1.9×10^5	1.9×10^4
reported K_a / M^{-1}	$> 10^9$	$0.1–1 \times 10^8$	$0.1–1 \times 10^8$	–	–

Conversion of proteins into semisynthetic biosensors. **(a)** Labeling mechanism of SNAP-tag and CLIP-tag. **(b)** Schematic illustration of the Snifit sensor. **(c)** Schematic illustration of the LD and AG chemistry. **(d)** Analyte sensing mechanism of LD-chemistry based biosensors. **(e)** Binding parameters of ligands to fluorescein-labeled FR. **(f)** Analyte sensing mechanism of LD-chemistry based biosensors. Lg, ligand; Nu, nucleophilic amino acid; Cat, catalytic unit; Q, quencher.

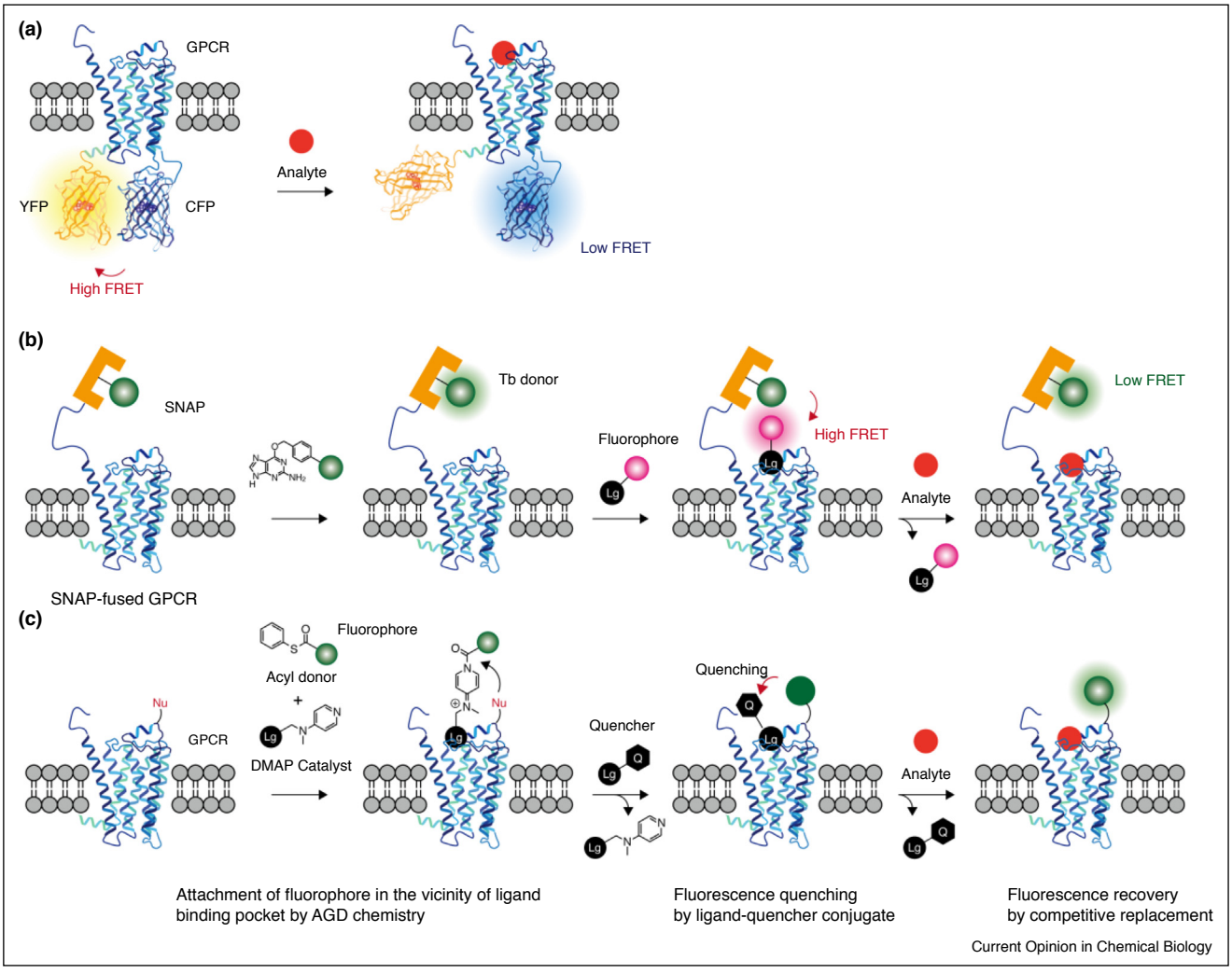
to sidestep this limitation, Hamachi and co-workers exploited a bimolecular fluorescence quenching and recovery (BFQR) strategy. In this manifold, the fluorescence of a dye attached to the protein in the vicinity of the ligand binding pocket is quenched by the addition of a ligand–quencher conjugate. Fluorescence is recovered upon binding of competitive ligands that can replace the ligand–quencher conjugate in the binding site (Figure 1f).

Semisynthetic biosensors of endogenous or artificial ligands for cell-surface receptors

G-protein coupled receptors (GPCRs)

The superfamily of G-protein-coupled receptors (GPCRs) represents the largest class of cell surface receptors and is, therefore, a prominent family of drug targets. FRET-based FP-biosensors have been constructed by inserting a YFP-CFP FRET pair into appropriate positions of GPCR scaffolds, which include the α2A-adrenergic and parathyroid hormone receptors [33], the β-adrenergic receptor [34] and the muscarinic acetylcholine receptor (Figure 2a) [35]. As semisynthetic biosensors, Zwier *et al.* developed a FRET-based ligand assay system by using SNAP-tag fused GPCRs (Tag-lite) (Figure 2b) [36]. In the Tag-lite strategy, SNAP-tag fused GPCRs were selectively labeled with a Terbium cryptate–BG (Tb–BG) conjugate as a FRET donor, and a ligand–fluorophore conjugate was applied as a FRET acceptor. Ligand binding is detected via a decrease of the FRET signals in a competitive manner, which would be suitable for high-throughput screening applications. Using AGD chemistry, Wang *et al.* demonstrated the selective labeling of a GPCR, the bradykinin B2 receptor (B2R), on the surface of live cells (Figure 2c)

Figure 2



Protein-based biosensors for GPCR ligands. **(a)** FP-based FRET biosensor. **(b)** Semisynthetic biosensor using SNAP-tag (Tag-lite). **(c)** Semisynthetic biosensor by a combination of AGD chemistry and BFQR method.

[31]. Because the labeled B2R did not show a fluorescence response upon addition of any tested ligands, a BFQR strategy was applied for the fluorescent biosensor construction. The rational combination of AG chemistry with the BFQR method provided a turn-on fluorescent sensor, which allowed the quantitative evaluation of the binding properties of B2R ligands under live cell conditions.

Excitatory neurotransmitter receptors

Glutamate (Glu) is a major excitatory neurotransmitter in the central nervous system (CNS), and fast excitatory neurotransmission is mediated mainly via AMPA-type Glu receptors (AMPARs). In early studies, Glu concentrations were monitored by FP-based biosensors that fused bacterial periplasmic Glu-binding proteins (iGluSnFR and FLIPE) [37–39]. They allowed the spatiotemporal monitoring of Glu concentrations in live cells and live neurons; however, the ligand-binding properties of these biosensors are largely different from those of mammalian Glu-receptors [38,39]. Hirose and co-workers have reported fluorescent sensors consisting of the ligand-binding domain (S1S2) of an AMPAR subunit (GluA2) (Figure 3a) [15,16]. Matthias *et al.* also developed Snifit-based biosensors consisting of a SNAP-tag, CLIP-tag and S1S2 of a kainate receptor subunit (GluK1) (Figure 3b) [40]. While these biosensors enabled imaging of the extracellular Glu concentration under live cell conditions, S1S2 domains, an artificially prepared ligand-binding region of Glu (AMPA or kainate) receptors, are noticeably different when compared with the size and structure of tetrameric complexes of full-length receptors. Therefore, the pharmacological properties of S1S2 are different from those of full-length AMPARs. Semisynthetic biosensors using full-length receptor proteins that retain their original function are the most suitable receptors for drug screening. Kiyonaka *et al.* recently demonstrated the selective labeling of AMPARs by ligand-directed acyl imidazole (LDAI) chemistry on live cells (Figure 3c) [41•]. The fluorophore-labeled AMPARs act as turn-on-type biosensors that allow the selective detection and quantitative analysis of AMPAR ligands under live cell conditions. They successfully determined the affinities of agonists or antagonists for AMPARs under live cell conditions. As show in Figure 3d, the agonist-binding properties of AMPARs are clearly different between live-cell environments and non-cellular conditions. The dissociation constants of AMPAR agonists determined by LDAI-based live cell biosensor were almost identical to those observed in functional assays using live cells. However, these dissociation constants are largely different from those obtained by radioisotope-based binding assays using membrane fractions or by LDAI-based biosensor using S1S2. A plausible explanation for the difference between LDAI/functional assays and the radioisotope assays could be the cellular versus non-cellular conditions used to take measurements. Thus, Kiyonaka *et al.* claimed that biosensors constructed from full-length receptors in natural environments would be valuable for quantitative drug binding analysis.

Inhibitory neurotransmitter receptors

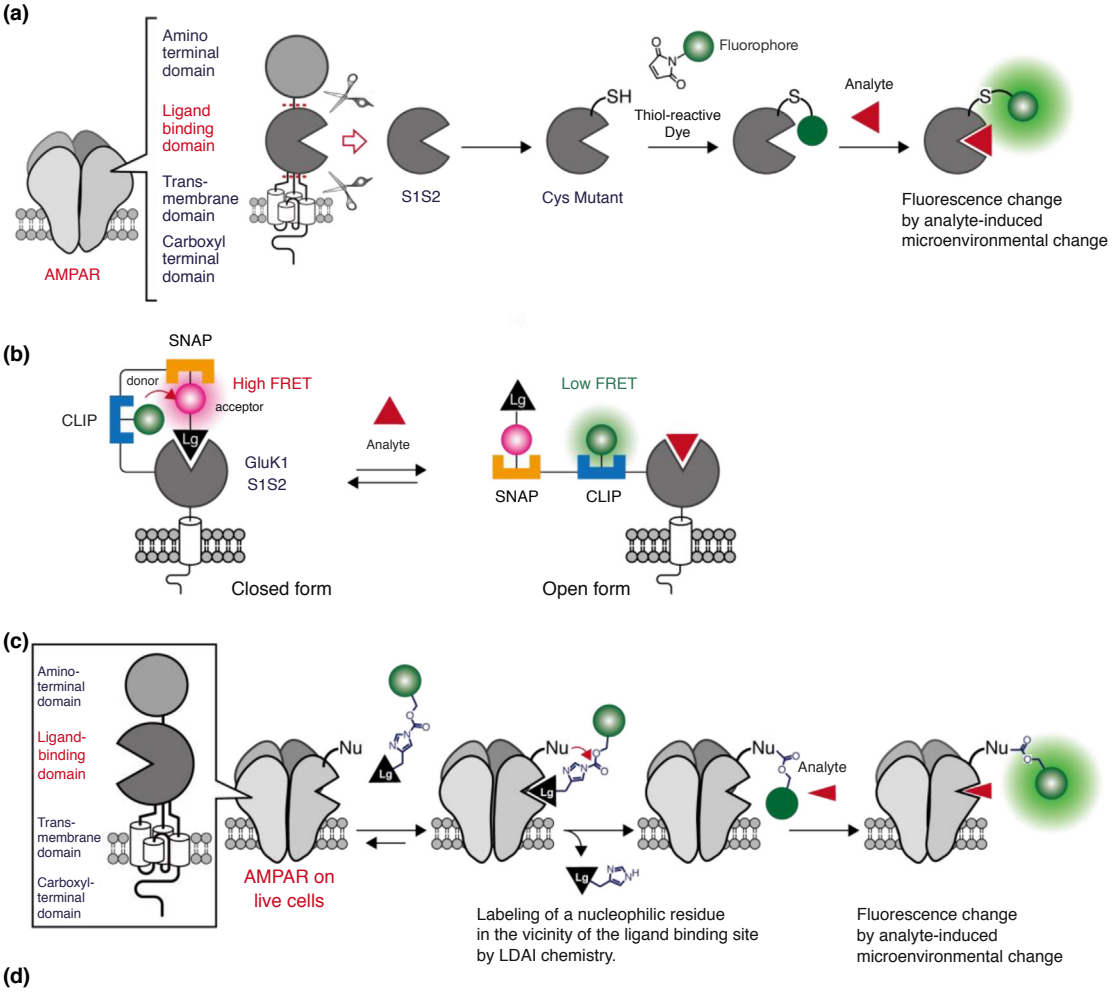
In contrast to Glu, γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the CNS, and inhibitory neurotransmission in the brain is mediated via ion-channel-type $GABA_A$ and GPCR-type $GABA_B$ receptors ($GABA_A$Rs and $GABA_B$Rs). The $GABA_B$-based ratiometric biosensor using the Snifit strategy enables detailed analysis of the binding properties of $GABA_B$R ligands on live cells (Figure 4a) [42]. $GABA_A$Rs are regarded as important targets for a number of pharmaceuticals, such as anticonvulsants, anesthetics, sedatives, anxiolytics and antidepressants. A total of 19 different subunits of $GABA_A$Rs have been identified and they assemble in limited combinations to form functional heteropentamers [43,44]. Given the complexity of the active heteropentamer, application of the Snifit strategy to $GABA_A$Rs is challenging. By a combination of LDAI chemistry and the BFQR strategy, Yamaura *et al.* successfully constructed $GABA_A$R-based turn-on fluorescent biosensors on live cells (Figure 4b) [45••]. The $GABA_A$R-based fluorescent biosensors enable high-throughput screening for $GABA_A$R ligands under live cell conditions. Using these biosensors, four hit compounds were discovered from a library of pharmacologically active compounds (LOPAC1280). Among them, two compounds were newly identified negative allosteric modulators of $GABA_A$Rs, suggesting BFQR-based biosensors are able to sensitively detect subtle conformational changes induced by ligand-binding. This example demonstrates the versatility of LD and AG chemistries for construction of ligand assay systems for a particular protein.

Semi-synthetic biosensors for intracellular protein ligands

Drug screening for intracellular enzymes and receptors *in vitro*

Intracellular proteins are also attractive drug targets and/or biomarkers for specific diseases, such as cancer and neurological disorders. Rauh and co-workers have established a new *in vitro* screening system capable of identifying kinase inhibitors using kinase–fluorophore conjugates [17•,18,19]. According to the structural information of active and inactive forms of kinases, they introduced an acrylodan fluorophore, the emission intensity of which is known to be sensitive to its local environment, into a Cys side chain located in a regulatory loop region of the cSrc kinase. The ligand-binding event is transduced into a fluorescence change via a conformational change of the regulatory loop. Thus, the development of a direct ligand-binding assay for characterizing small molecule inhibitors that stabilize the inactive form of the kinase was possible. Rauh and co-workers subsequently improved this system by using red-shift fluorophores to circumvent the intrinsic fluorescence of

Figure 3



	radioligand binding assay (K_d/ μM)		functional-based assay (EC_{50} or K_d / μM)	LDAI-based biosensor (K_d/ μM)	
	AMPAR	S1S2	AMPAR (GluA2)	AMPAR (GluA2)	S1S2
	non-cellular	test tube	live cell	live cell	test tube
Glu	0.17–0.48	0.45	294–634	254 ± 14	1.67 ± 0.01
AMPA	0.012–0.025	0.025	11–66	18.3 ± 2.3	0.023 ± 0.003

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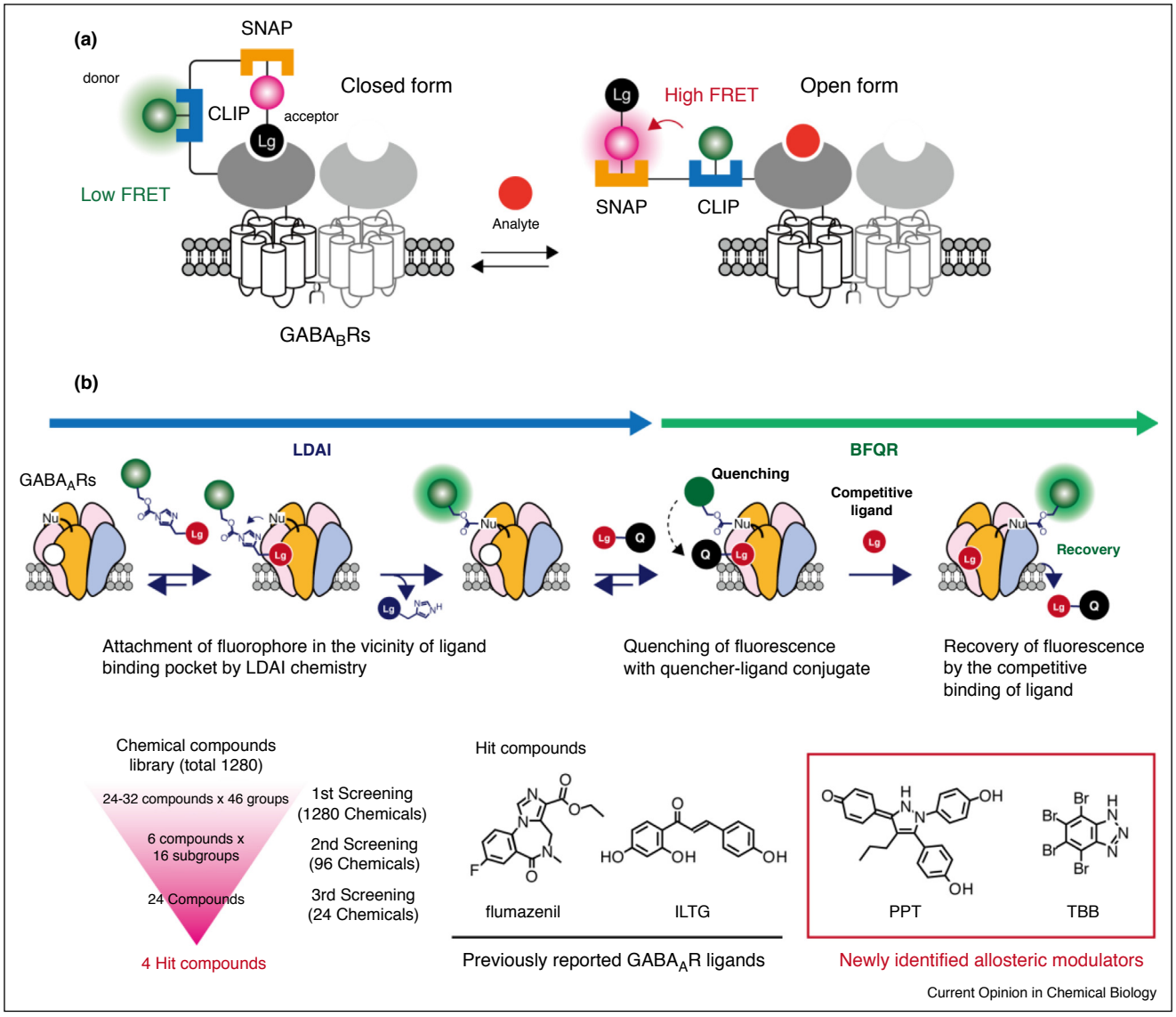
Protein-based biosensors for excitatory neurotransmitter receptor ligands. **(a)** S1S2-based semisynthetic biosensor. **(b)** Snifit-based biosensor using S1S2 domain of GluK1 on live cells. **(c)** Semisynthetic biosensor based on full-length AMPARs using LDAI chemistry on live cells. **(d)** Previously reported K_d and EC_{50} values of AMPAR agonists (Glu or AMPA) and K_d values determined by LDAI-based biosensor.

inhibitors, and, therefore, reduce the number of false-positive and false-negative hits. The same group extended this strategy to phosphatases and estrogen-receptors for identifying their specific inhibitors via high-throughput screening assays [20,21]. Although pioneering, these are examples of *in vitro* experiments and live-cell screening remains highly desirable for more precise and straightforward assays.

Figure 4



Protein-based biosensors for inhibitory neurotransmitter receptor ligands. **(a)** Snifit biosensor constructed from GABA$_B$R. **(b)** Biosensor constructed from GABA$_A$R by a combination of LDAI-chemistry and BFQR method.

Biosensors using intracellular proteins in live cells

A few efforts have been initiated for intracellular construction of semisynthetic biosensors for drug assays. By combining LD chemistry with the BFQR strategy, Matsuo *et al.* recently converted the endogenous intracellular CA2 (as well as membrane-bound CA12) into fluorescent biosensors, which allowed the quantitative characterization of a variety of CA inhibitors inside live cells [46•]. The semisynthetic biosensors constructed using LD chemistry can detect both protein–ligand binding and protein–protein interactions [47]. The interaction between FK506-binding protein 12 (FKBP12) and the FKBP-rapamycin binding domain (FRB) is mediated by rapamycin. For visualizing the formation of this ternary complex, intracellular endogenous FKBP12 was selectively labeled with a fluorophore using LD chemistry. The rapamycin-induced interaction of the fluorophore modified FKBP12 with the FP-tagged FRB could be monitored in a single live cell using an intermolecular FRET signal. This biosensor clarified the interaction between FKBP12 and FRB and afforded detailed kinetics under cellular conditions. Scarabelli *et al.* have also reported in-cell biosensors using a SNAP-tag technology, which allows the evaluation of cell permeability and kinetics of drugs and drug candidates [48]. Although a few successful examples have been reported, the direct

conversion of intracellular proteins into biosensors is more difficult compared with that for cell surface receptors. To accelerate the progress of this research field, efforts for the development of chemical protein labeling, which are selective enough to be employed in intracellular environments, are needed. Another important point is efficient delivery of chemical reagents into cells in this case. Moreover, rapid clearance of excess amount of the chemical reagents from intracellular space is also essential after the labeling, because unbound reagents cause background fluorescence. In this context, turn-on-type probe, which shows high fluorescence only when the probe binds to target protein, may be powerful. Although more efforts are still required, the direct conversion of intracellular proteins into biosensors for screening ligands would represent a powerful approach to develop novel platforms for drug screening assays in next generation therapeutics.

Conclusions

In this review, we briefly described recent advances in the construction of ligand assay systems using semisynthetic protein-based biosensors and their application to the quantitative analysis and high-throughput screening of small molecules for drug discovery. An important challenge in chemistry-based protein biosensor development is the application of biosensors in tissues and whole organisms. While some pioneering studies have achieved chemical modifications in tissues and living animals, there remain a number of drawbacks, such as insufficient labeling specificities and efficiencies [49•,50–56]. We believe that further expansion of the reaction modules and efficient catalysts available under multimolecular crowding conditions should facilitate practical applications for medical therapeutics and diagnosis.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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