Review

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In situ Construction of Protein-based Semisynthetic Biosensors

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ABSTRACT: Chemically constructed biosensors consisting of a protein scaffold and an artificial small molecule have recently been recognized as attractive analytical tools for the specific detection and real-time monitoring of various biological substances or events in cells. Conventionally, such semisynthetic biosensors have been prepared in test tubes and then introduced into cells using invasive methods. With the impressive advances seen in bioorthogonal protein conjugation methodologies, however, it is now becoming feasible to directly construct semisynthetic biosensors in living cells, providing unprecedented tools for life-science research. We discuss here recent efforts regarding the *in situ* construction of protein-based semisynthetic biosensors and highlight their uses in the visualization and quantification of biomolecules and events in multi-molecular and crowded cellular systems.

KEYWORDS: *semisynthetic biosensor, live cell imaging, protein modification, peptide-tag, protein-tag, ligand-directed chemistry, chemosensor, ^{19}F-probe, FRET.*

Elucidating the inner workings of complicated cellular processes requires a powerful set of bioanalytical tools that allow for the visualization of various biological parameters, such as pH, ionic concentrations, metabolites, and protein activities. Over the past two decades, a large number of biosensors based on fluorescent proteins (FP) have been developed for the real-time tracking of a range of biological events and substances *in vitro*, inside cells, or even *in vivo*.[1–7] These biosensors typically consist of a recognition domain that can specifically bind or respond to a target analyte, and a FP-based fluorescent transducer to convert the recognition of the analyte into a change in the fluorescent signal, either in its intensity or wavelength.[6] Since these sensors can be genetically encoded, they are relatively easy to construct in live cells using conventional transfection protocols, in which cellular translational machinery spontaneously produces the biosensors. These biosensors can be confined to a particular subcellular compartment by tethering a specific signal sequence tag,[8–14] which provides more precise spatial localization of the analyte. These advantages make FP-based biosensors indispensable tools for quantitative analysis in live-cell imaging using fluorescent microscopy. However, there are still many biological substances that are difficult or impossible to detect with conventional FP-based biosensors,[2, 3, 15] along with other situations in which a detection mode other than fluorescence is desirable, as with deep tissue imaging in animals.[16–19]

Chemistry-based protein labeling techniques offer a complimentary approach for the construction of protein-based biosensors.[2, 6, 7, 20–26] Remarkable progress in chemical modification methods have recently accelerated the development of protein-based semisynthetic biosensors as an alternative to FP-based biosensors. Semisynthetic biosensors are generally constructed in a site-specific process by introducing a small synthetic probe to a protein domain, which acts as a recognition or a subcellular localization motif. Although they are more difficult to prepare, compared with FP-based biosensors, semisynthetic biosensors possess several unique features, including a small probe size (< 1 kDa), which may minimize perturbations to the structure and function of the protein scaffold. Also, they allow for a wide variety of fluorescent probes and sensors with diverse spectroscopic properties, such as wavelength, brightness, stability against photobleaching, and microenvironment sensitivity, and can be made into sensing modules. Finally, imaging modalities other than fluorescence, such as NMR or MRI probes, can be incorporated into the protein scaffold as a unique reporter. In early studies, semisynthetic biosensors were constructed *in vitro* and subsequently transferred into living cells with microinjection, or with the help of cell-penetrating peptides,[27–32] because of the limited number of bioconjugation strategies then available (Figure 1a). In recent years, however, several biocompatible and/or bioorthogonal methods for the selective chemical modification of target proteins in live cells have emerged,[21, 22, 33–41] which allow for the direct conversion of intracellular proteins into semisynthetic biosensors (Figure 1b).

In this review, we focus on chemical strategies for the *in situ* construction of protein-based

semisynthetic biosensors and successful biological applications. A wide variety of semisynthetic biosensors described herein were summarized in Table 1. Due to limitations of space, we omit descriptions of FP-based biosensors and studies for the *in vitro* construction of semisynthetic biosensors, the details of which are available in previous reviews.[1, 3, 4, 7, 42]

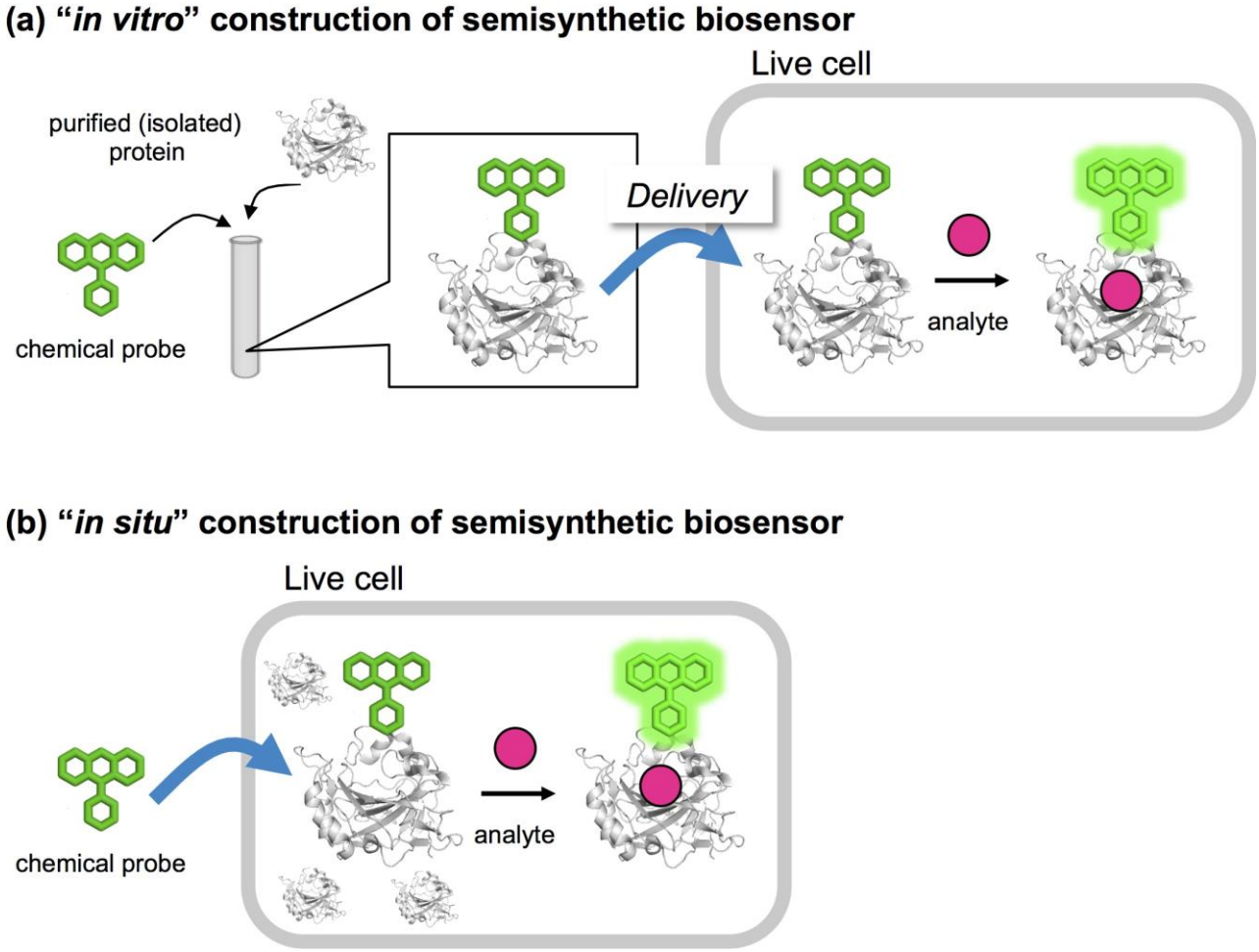


Figure 1. General schemes of (a) *in vitro* and (b) *in situ* construction of semisynthetic biosensors for live-cell applications.

Table 1. Summary of semisynthetic biosensors for *in situ* analysis.

Method	Analyte	Binding/sensing module	Modality	Context	Reference
tetracysteine-tag	Ca^{2+}	CaGF (Ca^{2+} sensor)	fluorescence	inside cell	82
SNAP-tag	Zn^{2+}	Zn^{2+} sensor	fluorescence	inside cell	83
	Ca^{2+}	Ca^{2+} sensor			84
	H_2O_2	H_2O_2 sensor			86, 87
	NO	NO sensor			88

	H_2S	H_2S sensor			89
Halo-tag	K^+	K^+ sensor	fluorescence	cell surface	85
Snifit	HCA inhibitor and Zn^{2+}	HCA	FRET	cell surface	93, 94
	glutamate	$iGluR_5$-S_1S_2			95
	GABA and $GABA_BR$ inhibitor	GB_{1a}			96
	ACh and AChE inhibitor	AChE			97
LUCID	methotrexate	cpDHFR	BRET	in vitro	91
		antibody fragment		(paper device)	92
	tacrolimus and sirolimus	FKBP12			91
	cyclosporine A	cpCyA			
	topiramate	HCA			
	digoxin	DIG10.3			
	theophylline	antibody fragment			92
	quinine	antibody fragment			
LDT	HCA inhibitor	HCA	^{19}F chemical shift	inside cell	101, 105, 106
	interaction of FKBP12 and FRB		FRET	inside cell	107
Q-LDT	HCA inhibitor	HCA	BFQR	cell lysate	104
	phosphotyrosine peptide	SH2 domain			
LDAI	FR inhibitor	FR	fluorescence	cell surface	108
	$GABA_AR$ inhibitor	$GABA_AR$	BFQR		110
AGD	B_2R inhibitor	B_2R	BFQR	cell surface	114
ligand-probe	FR inhibitor	FR	fluorescence	cell	128, 129

conjugate			surface
	HCA inhibitor	HCA	inside cell
	Hsp90 inhibitor	Hsp90	

■ Semisynthetic biosensors constructed by peptide/protein tagging systems

In-situ conjugation of chemosensors with proteins for targeting subcellular compartments

One straightforward approach to the design of a semisynthetic biosensor is the hybridization of a protein with an artificial fluorescent chemosensor.[8, 43–48] Many fluorescent chemosensors have been used for the detection of biologically active species, including metal cations,[49–52] anions,[53, 54] carbohydrates,[55, 56] pH,[57, 58] and reactive oxygen and nitrogen species.[59–62] These chemosensors have greatly contributed to deciphering complicated biological processes. Currently, it is thought that the local concentration distributions of these biological species inside cells may be critical to their biological roles. Thus, chemosensors that can localize in an organelle are strongly desirable. Unfortunately, however, spatial control of conventional chemosensors in certain subcellular compartments remains quite challenging, although there have been some successful examples.[63–71] In contrast, the spatial distribution of proteins can be genetically controlled using localization signal sequences.[8–14] To localize chemosensors in a specific subcellular region, a chemosensor-protein conjugate strategy has been proposed, in which the protein scaffold is employed as a controlled localization unit.

Genetically encodable peptide/protein tag systems[72–81] are widely used to conjugate a chemosensor with a localized protein scaffold (Figure 2). As the first example, Tsien *et al.* installed a calcium-responsive dye, called Calcium Green FlAsH (CaGF), in connexin 43 expressed at the gap junctions of HeLa cells, using the tetracysteine–biarsenical system which relied on the strong interaction between the thiol group and arsenicum.[82] This CaGF-connexin 43 conjugate displayed ten-fold brighter fluorescence upon Ca^{2+} binding, with a K_d of 100 μM, so Ca^{2+} waves could be sensed near the gap junctions in live cells. Also, the same group constructed CaGF-modified α_{1C} L-type calcium channels, and successfully visualized μm-sized hot spots of calcium influx at the cellular membrane (Figure 2a).

Despite the importance of this labeling technique, the inherent toxicity of the biarsenical compounds prevents it from being widely applied in living cells. As another strategy for *in situ* incorporation of chemosensors into intracellular proteins, self-labeling protein-tag systems are valuable because of their high biocompatibility. Several protein-tag systems have been developed so far,[36, 38, 40, 81] among which the SNAP-tag and Halo-tag systems are the most robust and widely used

for intra- and extracellular protein labeling.[78–80] A SNAP-tag, an engineered mutant of 19-kDa human O^6-alkylguanine-DNA alkyltransferase (AGT), catalyzes the covalent bond formation of O^6-benzylguanine (BG) derivatives with an active cysteine residue of the enzyme. A Halo-tag is created from haloalkane dehalogenase, 33-kDa bacterial enzyme, to attach a haloalkane derivative to a reactive aspartate residue at the enzyme's active site. The nontoxic and highly selective labeling of these protein-tag systems greatly facilitates *in situ* construction of semisynthetic biosensors in a specific intracellular compartment (Figure 2b). Lippard *et al.* detected Zn^{2+} ions in Golgi and mitochondria by tethering Zn^{2+} fluorescent chemosensors to SNAP-tag fusion proteins with a localization unit.[83] Johnsson *et al.* measured the Ca^{2+} concentration inside nuclei, using the dye Iodo-1 conjugated with the fused SNAP-tag nuclear localization signal protein.[84] Urano *et al.* analyzed physiological K^+ dynamics at cellular membranes using TLSHalo, which consists of a K^+ ion-sensitive probe conjugated with a GPCR/Halo-tag fusion protein.[85] In addition, several research groups proved that such protein-tag strategies are useful with a variety of chemosensors in a specific organelle to sense biofunctional molecules, such as H_2O_2,[86, 87] NO,[88] and H_2S.[89]

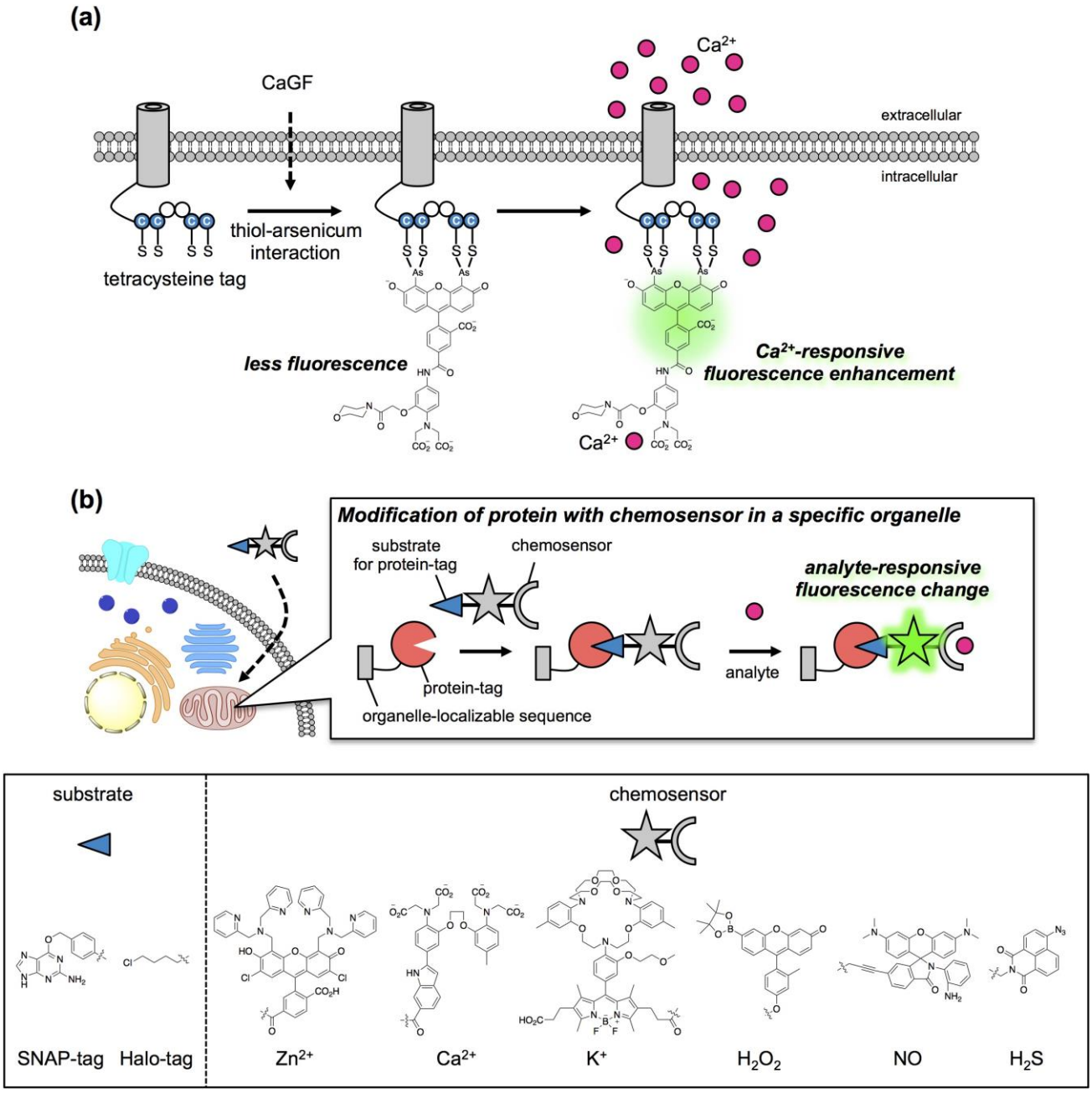


Figure 2. (a) Schematic illustration of a Calcium Green FlAsH (CaGF)-based biosensor that can report highly localized, rapid Ca^{2+} dynamics in live cells. (b) General strategy for organelle-targeting chemosensors. Chemosensors can be targeted to various subcellular compartments through the organelle-localized protein-tags, such as the SNAP-tag and Halo-tag systems.

Snifits

Although there are a vast array of chemosensors equipped with a synthetic chelator and/or reactive group for the detection of inorganic ions or redox species, it is still difficult for the chemosensors to selectively recognize large and structurally complex analytes, such as metabolites and drugs. On the other hand, natural proteins exhibit excellent recognition ability with regard to

these analytes, showing high selectivity and affinity. Thus, if a protein-based recognition module is equipped with the appropriate transducer functionality, such constructs are able to act as semisynthetic biosensors for complex analytes. Among a few of the signal transduction mechanisms, Förster resonance energy transfer (FRET)-based techniques are valuable for biosensor construction.[42] FRET is a quantum-mechanical phenomenon of nonradiative energy transfer from a donor fluorophore in its excited state to an acceptor fluorophore. This occurs only when the two fluorophores are in very close proximity to each other (1-10 nm), and the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. The analyte-induced conformational changes in distance and/or relative orientation of the two fluorophores affect the FRET efficiency, which induces a ratiometric fluorescence readout from the FRET-based biosensors.

Johnsson and coworkers exquisitely constructed FRET-based semisynthetic biosensors called Snifits, for SNAP-tag based indicator with a fluorescent intramolecular tether.[7, 90] This strategy uses an orthogonal labeling technology, employing both SNAP-tag and CLIP-tag.[77] A CLIP-tag is a variant of the SNAP-tag that selectively reacts with benzylcytosine (BC) derivatives as the substrates. Thus, the SNAP-tag and CLIP-tag systems can be simultaneously attached to two different fluorophores, the FRET donor and acceptor, in an analyte-binding protein domain. The general design of a Snifit is shown in Figure 3a. The SNAP-tag is modified with a bifunctional molecule containing both the FRET acceptor and an affinity ligand for the analyte-binding protein, while the CLIP-tag is labeled with a FRET donor. In the absence of the analyte, the tethered ligand binds to the protein, resulting in a closed conformation for the Snifit. In the presence of the analyte, the equilibrium is shifted toward an open conformation, with a competition between the tethered ligand and the analyte. Such conformational changes induce alterations of the FRET between the two attached fluorophores. In contrast with FP-based FRET sensors, Snifit sensors have many advantages. These include removing the need for ligand-induced conformational change, a wide variety of options for the synthetic fluorophores of the FRET pair (eg. Cy5/DY-547, Cy3/Cy5, Alexa Fluor 488/DY-547, and Alexa Fluor 488/Alexa Fluor 594), and a detection range of that can be tuned in light of the desired analyte concentration with an appropriately chosen tethered ligand. Furthermore, replacement of one fluorophore with a luciferase can generate bioluminescent resonance energy transfer (BRET)-based sensors, termed luciferase-based indicators of drugs (LUCIDs), which permit therapeutic drug monitoring for point-of-care diagnosis with portable paper devices.[91, 92]

Using various binding proteins, such as human carbonic anhydrase,[93, 94] ionotropic glutamate receptor 5,[95] $GABA_B$ receptor,[96] and acetylchlolinesterase,[97] Johnsson's group successfully demonstrated that the Snifits strategy can be used for the ratiometric detection of several drugs and metabolites in live cells. As an impressive example, the GABA-Snifit visualized GABA concentrations in the μM to mM range on the surface of a live cell. In addition, the GABA-Snifit

module was able to sense and quantify the relative binding affinity of agonists, antagonists, and allosteric modulators for the $GABA_B$ receptor (Figure 3b).

Snifits often suffered from modest dynamic ranges, with a maximum of value 5,[94–98] because the large size of the fluorophores carrying the SNAP-tags and CLIP-tags prevents them from getting very close in their closed conformation. Recently, Johnsson *et. al.* solved this problem by conjugating a fluorophore at the vicinity of a binding site of the analyte-binding protein using unnatural amino acids, a process they termed uSnifit.[98] The group created semisynthetic methotrexate (MTX)-uSnifit using a DHFR scaffold as the binding protein, which sensitively detected MTX with a large dynamic range both *in vitro* (up to 32) and on the surface of a living cell (up to 13).

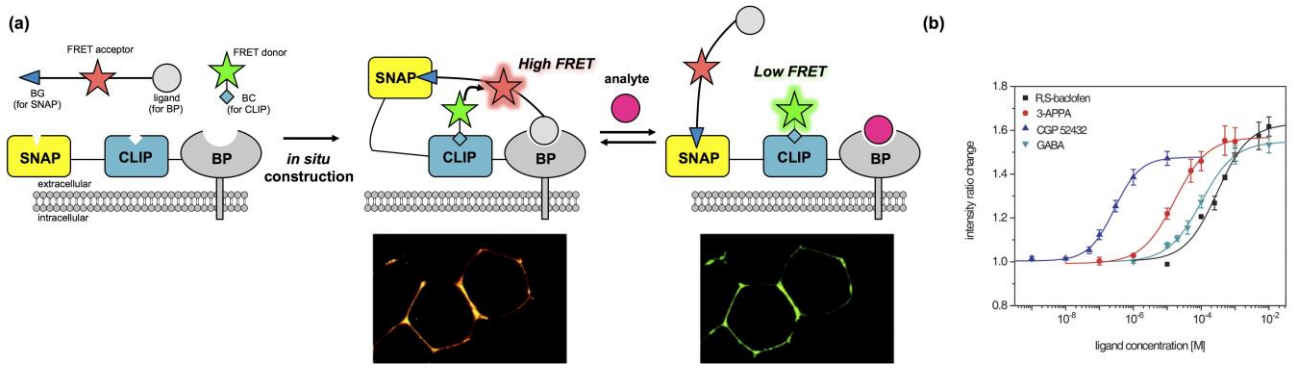


Figure 3. (a) Schematic illustration of the principle of Snifit. Adapted with permission from ref 94. Copyright 2011 American Chemical Society. (b) Titration curves of a GABA-Snifit system constructed on the surface of HEK 293 cells with different $GABA_B$ receptor ligands [R,S-baclofen (black squares), GABA (cyan triangles), 3-APPA (red circles), and CGP 52432 (blue triangles)]. Adapted with permission from ref 96. Copyright 2012 American Chemical Society.

■ Semisynthetic biosensors constructed using traceless affinity-based modification

In addition to the engineered proteins exogenously expressed in cells, native endogenous proteins are attractive as the recognition module for biosensor construction. This is because some of the endogenous proteins are also drug targets and/or biomarkers for specific diseases, such as cancer and neuronal disorders. Also, in the case of structurally complicated proteins composed of heterooligomers in nature, the direct conversion of native proteins into semisynthetic biosensors in their native habitats is preferable to avoid undesired perturbations. However, such conversions have remained challenging, because suitable methods for native protein modification without the help of genetic manipulation have yet to be adequately developed. To date, traceless affinity-based protein labeling developed by Hamachi and coworkers offers the only means for the *in-situ* construction of "native protein-based" semisynthetic biosensors.[21, 22, 99, 100] This method uses a designed compound connecting a protein ligand and a probe through a cleavable reactive group, termed ligand-directed

(LD) chemistry. In LD chemistry, the ligand part 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. Importantly, the ligand part can be removed after labeling using the cleavable linker of the LD reagents, so that the labeled protein retains its original function. This labeling mechanism permits biosensor construction, unlike in conventional affinity-based protein labeling. A few cleavable reactive groups in LD chemistry, such as phenylsulfonate ester (LDT),[101–107] acyl imidazole (LDAI),[108–111] and dibromophenylbenzoate (LDBB),[112] have been developed to date. As another class of traceless affinity-based protein labeling, called affinity-guided DMAP (4-dimethylaminopyridine) (AGD) catalysts, have been used for target-selective acylation with a thiophenyl ester acyl donor carrying a fluorophore.[113–117] Both LD and AGD chemistry are used to transform endogenous proteins into semisynthetic biosensors even under the crowded multi-molecular conditions of a living cell.

LD chemistry is able to introduce various probes close to the ligand-binding site of a target protein. This feature allows the researchers to directly convert an endogenous protein into a semisynthetic biosensor inside live cells when the environmentally sensitive probe is tethered to the ligand-binding pocket. For example, a semisynthetic ^{19}F NMR-based biosensor was constructed in red blood cells (RBCs) by tethering a ^{19}F NMR probe to native carbonic anhydrase I (CAI) proteins (Figure 4a).[101, 105, 106] Since the chemical shift of ^{19}F nuclei is very sensitive to the surrounding microenvironment, the ligand-free and bound states of CAI can be distinguished using in-cell ^{19}F NMR spectroscopy, thereby detecting the presence or absence of a CAI ligand (Figure 4b). The crystal structure of the ^{19}F-modified CAI revealed the ligand sensing mechanism (Figure 4c).[106] The ^{19}F-probe attached to an active pocket, proximal His (His67), was inserted deeply into the hydrophobic active pocket of CAI in the ligand-free state, while the ^{19}F-probe extruded from the pocket upon ligand binding (Figure 4). Such a slight change in the microenvironment surrounding the ^{19}F-probe was transduced to a chemical shift change in the ^{19}F NMR spectrum. The ^{19}F NMR-based titration curves yielded the association constants of various CAI inhibitors, both *in vitro* and in live RBCs. The relative order of the affinity of these inhibitors obtained using ^{19}F-modified CAI was very similar to those reported for native CAI. Interestingly, however, the affinity value of acetazolamide (AAZ) in RBCs in the natural environment was significantly lower than those found in the *in vitro* experiment. This difference can be attributed to the lower effective concentration of AAZ inside RBCs, which is due to its limited cell permeability, as well as nonspecific interactions with other biomolecules, such as cell membranes or off-target proteins.

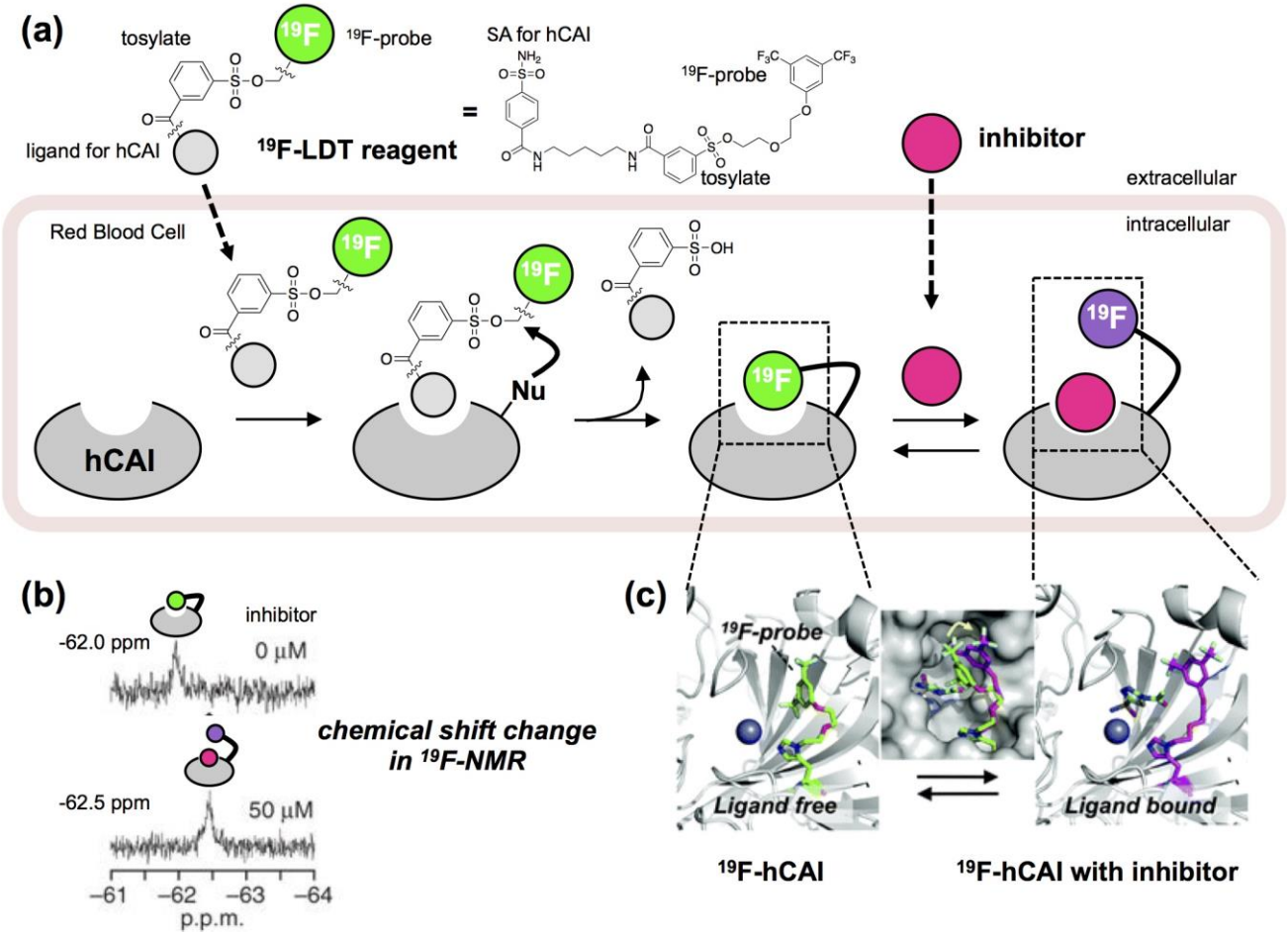
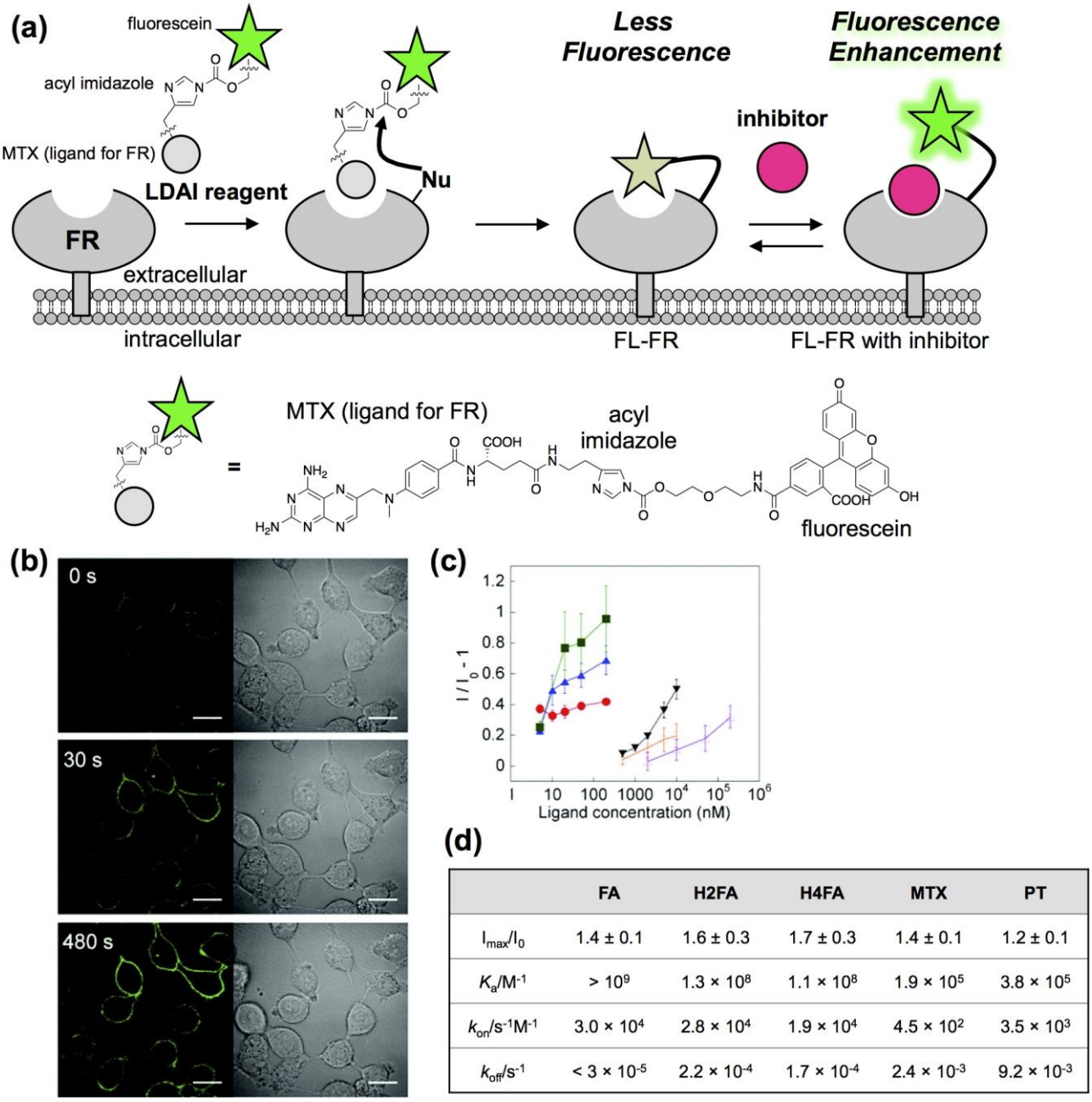


Figure 4. (a) *In situ* conversion of endogenous human carbonic anhydrase isoform I (hCAI) to a semisynthetic ^{19}F NMR biosensor in red blood cells using LDT chemistry. (b) In-cell detection of CA inhibitors using ^{19}F NMR spectroscopy. (c) Crystal structures of the bound and free states of ^{19}F-labeled hCAI. The ^{19}F-probe in the free and bound states are shown as green and purple sticks, respectively. A ligand of hCAI (acetazolamide) is also shown as a stick model, and the Zn^{2+} ion at the active site of hCAI is depicted by a purple sphere.

In many cancer cells, folate receptors (FR) are overexpressed, which is an important biomarker. LD chemistry has successfully labeled endogenous FR with several fluorophores. It turned out that fluorescein (FL)-modified FR worked as a fluorescent biosensor for FR ligands on a surface of a live cell (Figure 5a).[108] The fluorescence intensity of FL attached to FR increased with the addition of folic acid (FA), which allowed for the evaluation of the binding kinetics and affinities of various FR ligands in live cells. The time-profile of the fluorescence intensity changes yielded an apparent association rate constant (k_{on}) for FA of $3.0 \times 10^4\ M^{-1}s^{-1}$ (Figure 5b). Similarly, the k_{on} values for other ligands, such as dihydrofolic acid (H2FA), tetrahydrofolic acid (H4FA), MTX, and pterine (PT) can also be determined. In addition, by varying the ligand concentrations, the fluorescence titration experiments gave the association constants (K_a/M^{-1}) for the FR ligands. The dissociation

rate constants (k_{off}) for these ligands were determined by these k_{on} and K_a values. Such analysis on live cells clarified that the affinity of FR ligands was controlled by the dissociation rate, not the association rate. This was the first report of the binding kinetics of FR ligands in live cells, highlighting the utility of the native protein-based FL-biosensor (Figure 5c, d).



	FA	H2FA	H4FA	MTX	PT
I_{max}/I_0	1.4 ± 0.1	1.6 ± 0.3	1.7 ± 0.3	1.4 ± 0.1	1.2 ± 0.1
K_a/M^{-1}	> 10^9	1.3 × 10^8	1.1 × 10^8	1.9 × 10^5	3.8 × 10^5
$k_{on}/s^{-1}M^{-1}$	3.0 × 10^4	2.8 × 10^4	1.9 × 10^4	4.5 × 10^2	3.5 × 10^3
k_{off}/s^{-1}	< 3 × 10^{-5}	2.2 × 10^{-4}	1.7 × 10^{-4}	2.4 × 10^{-3}	9.2 × 10^{-3}

Figure 5. (a) *In situ* construction of a folate receptor (FR)-based biosensor with LDAI reagent in live cells. (b) Time-dependent fluorescence enhancement of fluorescein-labeled FR after the addition of folic acid (FA). Adapted with permission from ref 108. Copyright 2012 American Chemical Society. (c) Fluorescence titration profiles of fluorescein-labeled FR upon the addition of various ligands: FA (red solid circles), H2FA (blue solid triangles), H4FA (green solid squares), MTX (black solid down-triangles), or PT (orange open circles). Adapted with permission from ref 108. Copyright 2012

American Chemical Society. (d) Binding parameters of ligands to fluorescein-labeled endogenous FR in cells determined by a live-cell imaging study.

Despite the few successful examples mentioned, the chemical labeling of endogenous proteins by LD chemistry does not always directly produce useful biosensors, and it is still difficult to predict the appropriate probe and labeling site to achieve a detectable response. The coupling of traceless affinity labeling with a bimolecular fluorescence quenching and recovery (BFQR) system has proven to be a powerful tool for rationally designing native protein-based fluorescent biosensors. By taking advantage of the unique reaction mechanism of LDT chemistry, a quenched LDT (Q-LDT) method for the one-step construction of turn-on fluorescent semisynthetic biosensors has been reported.[104] The Q-LDT reagent contains a fluorophore and its quencher (Figure 6a), so that the fluorescence of the reagent itself is initially suppressed. Like the LDT chemistry, the Q-LDT reagent is also able to attach a fluorophore to a native protein, but the cleaved ligand-quencher fragment remains noncovalently bound to the ligand binding pocket, so it remains quenched. Subsequently, upon the binding of specific analytes to the protein, the fluorescence can be recovered by expelling the quencher fragment from the ligand-binding site. This Q-LDT strategy was successfully used to directly transform the CAII and SH2 (Src homology 2) domains into semisynthetic turn-on fluorescent biosensors in test tubes and cell lysates.

Recently, the BFQR method was extended to the construction of turn-on fluorescent biosensors on the surface of a live cell. Both bradykinin B_2 receptors (B_2R), which are G-protein coupled receptors, and $GABA_A$ receptors, which are inhibitory neurotransmitter receptors, are important drug target proteins, and can be converted to BFQR-based biosensors in live cells. In a B_2R study, a FL-labeled B_2R was prepared on the surface of a live cell using AGD chemistry, followed by the addition a B_2R antagonist-quencher (DABCYL) conjugate, to generate a quenched state of FL-B_2R.[114] The fluorescence is enhanced (tuned on) in response to the antagonist ligand by the competitive exchange with the ligand-quencher conjugate (Figure 6b). Similarly, the $GABA_A$ receptor-based turn-on fluorescent biosensor was fabricated on live cells by using LDAI chemistry to introduce Oregon-green (OG) dye to $GABA_AR$.[110] To exclusively sense the orthosteric (GABA) site ligands and allosteric benzodiazepine site ligands, a pair of the corresponding labeling reagents and ligand-quencher conjugates was used (Figure 7). The fluorescence of the OG-labeled $GABA_AR$ was effectively quenched by the corresponding ligand-quencher conjugate, which was clearly visualized using confocal laser scanning microscopy. The quenched fluorescence was recovered by the addition of the appropriate ligand, such as GABA for the orthosteric site, or flumazenil for the allosteric benzodiazepine site, demonstrating that this system can specifically discriminate between orthosteric and allosteric ligands. The observed fluorescence recovery allowed for the determination of the

ligand affinity in live cells.

Interestingly, the BFQR-based $GABA_AR$ biosensor in live cells can be used for the high-throughput screening of a chemical library of pharmacologically active compounds (LOPAC1280) to discover novel ligands for the benzodiazepine site. The fluorescent imaging-based screening resulted in the identification of four candidate compounds, including flumazenil, isoliquiritigenin (ILTG), 4,4',4"-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT), and 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB). Although flumazenil and ILTG were already known to be ligands of $GABA_AR$, there have been no previous reports that PPT and TBB can interact with $GABA_AR$. A competitive radiolabeled ligand binding assay using [^{3}H]flumazenil indicated that flumazenil and ILTG bind to the benzodiazepine sites, whereas PPT and TBB did not bind competitively to the benzodiazepine sites. An electrophysiological assay clearly showed that these compounds indeed bind to $GABA_AR$ allosterically at the non-benzodiazepine site, which structurally perturbed the benzodiazepine sites. These results highlight the great potential of biosensors constructed *in situ* as invaluable tools for drug discovery.

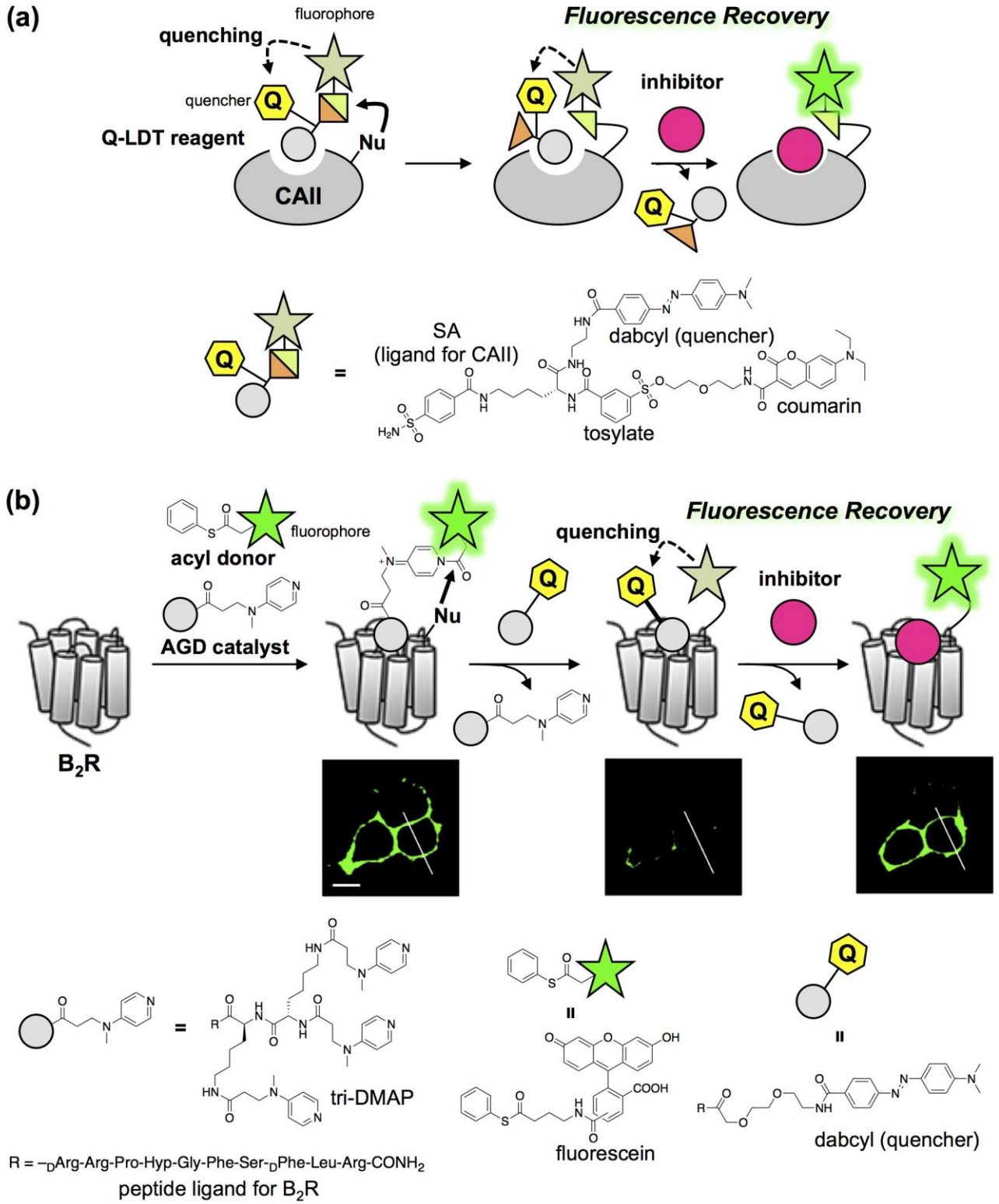


Figure 6. (a) One-step construction of turn-on fluorescent semisynthetic biosensors using Q-LDT reagents in test tubes and bacteria lysates. (b) AGD chemistry-mediated construction of B_2R-based fluorescent biosensors on live cells using the BFQR mechanism. Adapted with permission from ref 114. Copyright 2011 American Chemical Society.

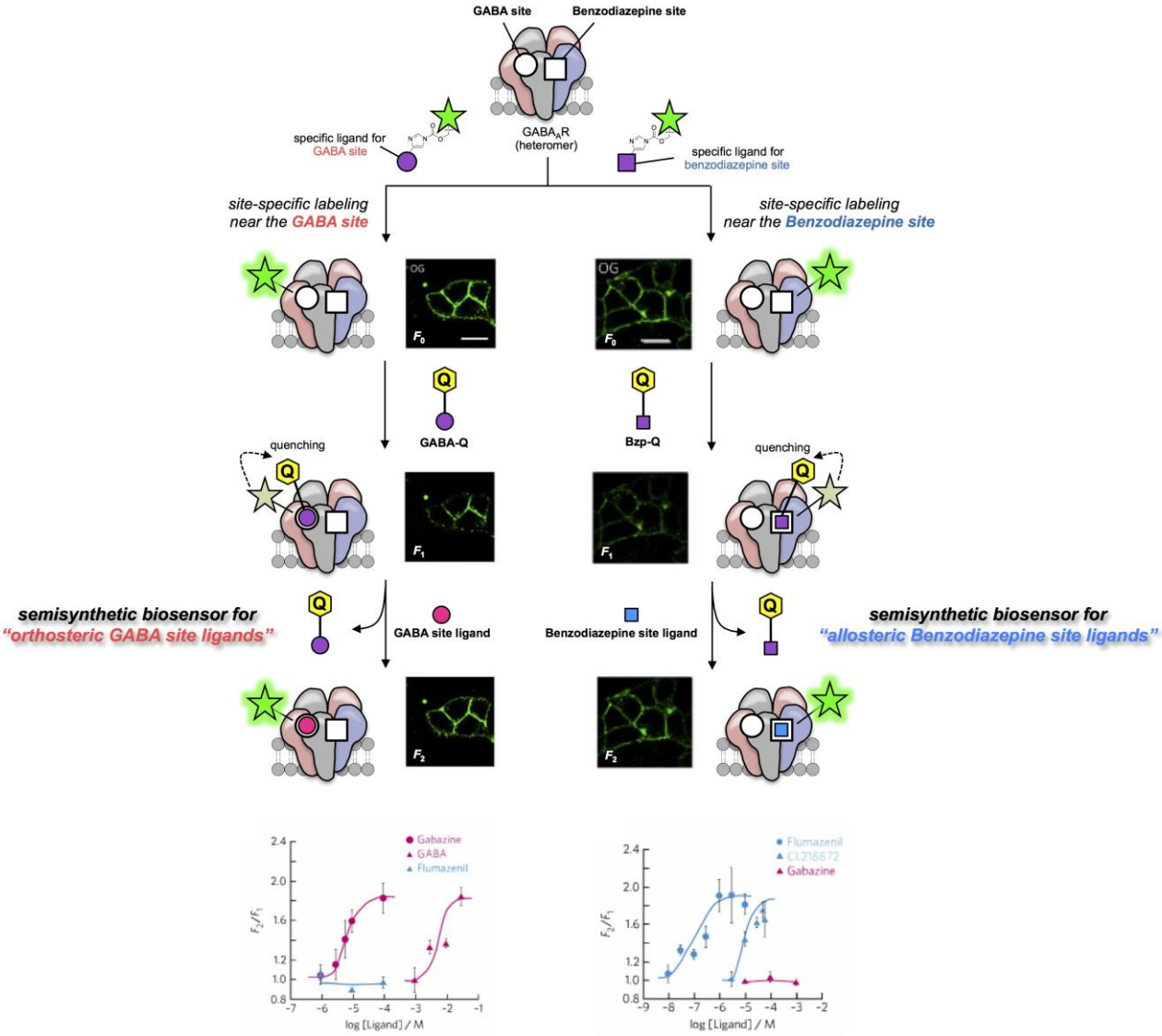


Figure 7. *In situ* construction of $GABA_A$ receptor-based fluorescent biosensors for testing orthosteric GABA site ligands and allosteric benzodiazepine site ligands with LDAI chemistry.

The semisynthetic biosensors constructed using LD chemistry can be applied to the detection of not only protein-ligand binding, but also protein-protein interactions.[107] It is well known that FK506-binding protein 12 (FKBP12) has an interaction with the FKBP-rapamycin-binding domain (FRB) mediated by rapamycin. To image the formation of this ternary complex, intracellular endogenous FKBP12 was selectively labeled with OG dye using LDT chemistry. The rapamycin-induced interaction of the OG-modified FKBP12 with the FP (DsRed)-tagged FRB in a single live cell could be monitored using an intermolecular FRET signal. This biosensor clarified the interaction between FKBP12 and FRB and could be completed within five minutes under cellular conditions.

■ Inhibitor sensing using noncovalently bound protein/ligand-probe complexes

As an alternative to covalently modified proteins, *in situ* noncovalent protein labeling is available for the construction of protein-based biosensors. Ligand-probe conjugates are widely used to visualize specific subcellular localization and the concentrations of the corresponding proteins.[118–123] These conjugates enable the noncovalent attachment of a probe to a ligand-binding pocket in a target protein scaffold. If a signal coming from the probe can be reversibly altered by the presence of the target protein in a live cell, such ligand-probe conjugates can allow for not only protein detection (imaging), but also for the evaluation of the ligand-protein interaction process. In the case of targeting intracellular proteins without washing, a turn-on mechanism in response to the target protein is highly desirable to reduce background noise.

Although conventional ligand-probe conjugates generally cannot change their fluorescence after binding with a target protein, it was reported that some of the ligand-probe conjugates with self-assembling properties may generate a turn-on signal in response to a target protein. For instance, the monomeric state of the conjugate may exhibit strong fluorescence, while the fluorescence is significantly quenched upon the self-assembly. Or, the reverse may occur, as with aggregation-induced emission (AIE) probes.[124–127] Therefore, it is expected that supramolecular assemblies that can be selectively collapsed by protein/ligand recognition even in live cells may become a promising system for the turn-on detection of target proteins with low background noise. In a pioneering work, Hamachi's group developed a disassembly-driven turn-on protein detection system using ligand-probe conjugates. Using elaborated designed ligand-probe conjugates, whose assembly-disassembly properties can be reversibly controlled upon protein recognition, they carried out the construction of noncovalent biosensors (Figure 8a). These were composed of a ligand-probe conjugate and folate receptor (FR), carbonic anhydrases (CAs), or heat shock protein 90 (hsp90), and used *in vitro*, or even in living cells.[128, 129] Moreover, they demonstrated that such noncovalent biosensors were useful for assaying inhibitors of these proteins, due to the reversibility of the self-assembled and disassembled state. As a representative example, the ligand-probe conjugate for Hsp90 consists of PU-H71 as the Hsp90 ligand and tetramethylrhodamine (TMR) as the detection module (Figure 8b), the fluorescent properties of which are significantly different between the monomeric and assembled states, with the latter being quenched ten-fold (Figure 8c, d). When the PU-H71-TMR conjugate was used in SKBR3 cells endogenously expressing Hsp90, a strong TMR fluorescent signal was detected in the cytosol region due to conjugate-Hsp90 complexation. The fluorescent intensity inside the cells substantially decreased upon the addition of PU-H71 or other Hsp90 inhibitors (Figure 8e), suggesting that the PU-H71-TMR conjugate was expelled from the ligand-binding site of the Hsp90, and then reformed into less fluorescent supramolecular assemblies. Such a fluorescence change induced by the recognition-driven assembly or disassembly yielded IC_{50}

values for the Hsp90 ligands for the first time.

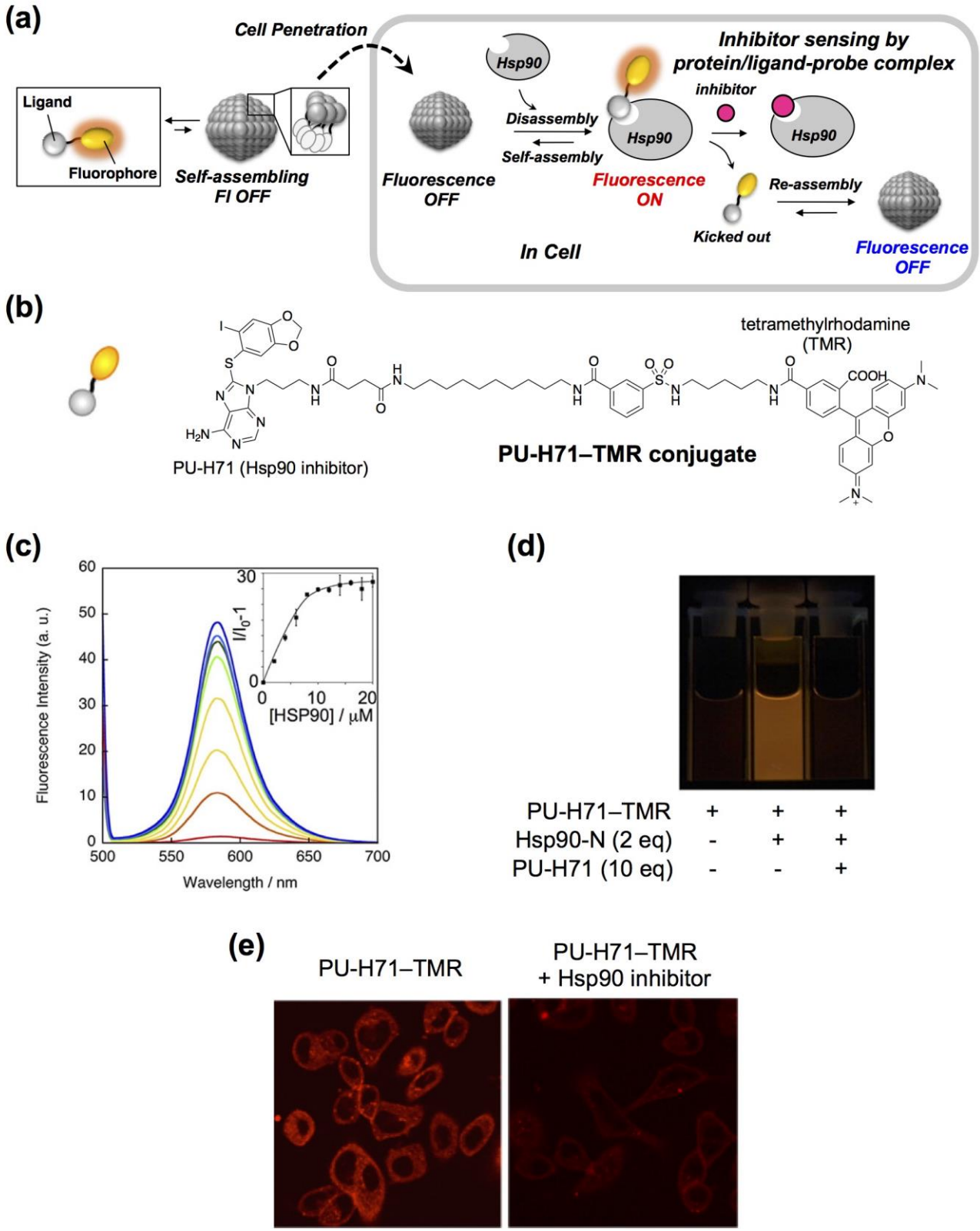


Figure 8. (a) Schematic illustration of inhibitor sensing by noncovalently bound protein/ligand-probe complexes in live cells. (b) Molecular structure of PU-H71–tetramethylrhodamine (TMR) conjugate for noncovalently labeling intracellular Hsp90. (c) Fluorescence spectra change for PU-H71–TMR

(10 μM) upon the addition of the ligand binding domain of Hsp90 (Hsp90-N) (0−20 μM) (λ_{ex} = 480 nm) in 50 mM HEPES buffer. (d) Photographs of PU-H71–TMR (10 μM) in the absence or presence of HSP90-N (20 μM, left and middle, respectively), and after the addition of PU-H71 (100 μM) to the solution of PU-H71–TMR and HSP90-N (right). Adapted with permission from ref 129. Copyright 2014 American Chemical Society. (e) Confocal images of cells treated with PU-H71–TMR in the absence or presence of Hsp90 inhibitor (PU-H71, left and right, respectively). Adapted with permission from ref 129. Copyright 2014 American Chemical Society.

Conclusion and perspective

Here, we briefly described semisynthetic biosensors constructed *in situ*, which allow for the visualization of multiple classes of biomolecules and drugs in living cells and the development of new therapeutics and diagnostics. The unique sensing mechanisms and the availability of diverse semisynthetic probes provide an invaluable platform to study cellular phenomena that cannot be investigated by conventional biological tools. One of the important challenges for chemistry-based biosensors is their transition for use in non-cultured cells and *in vivo*, beyond the cultured cells. Although some pioneering studies have achieved chemical protein modification in tissues and living animals,[101, 111, 130–134] there are still many problems involving stabilities, toxicities, unspecific binding to non-target proteins and tissue distributions of chemical probes, as well as labeling specificities and efficiencies. Thus, future work in this field will likely focus on the further development of new protein chemical labeling technologies applicable to more complicated biological environments and the improvement of ADME (absorption, distribution, metabolism and excretion) properties of synthetic molecules to realize the *in vivo* construction of semisynthetic biosensors. We envision that such research will facilitate an understanding of complex biological systems, and contribute to new insights regarding the *in situ* behavior of biomolecules in detail.

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The manuscript was written through contributions of all authors.

Notes

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■ Vocabulary

Semisynthetic, Composed of both natural and synthetic materials; Protein-based biosensor, The sensor which contains a protein scaffold for biological substances or events; Chemistry-based protein labeling, The protein modification method with synthetic molecules in order to explore biological processes; BFQR system, Turn-on fluorescent detection system based on a bimolecular fluorescence quenching and recovery; ADME properties, Key factors of the drug molecules in pharmacokinetics and pharmacology. The abbreviation for absorption, distribution, metabolism and excretion.

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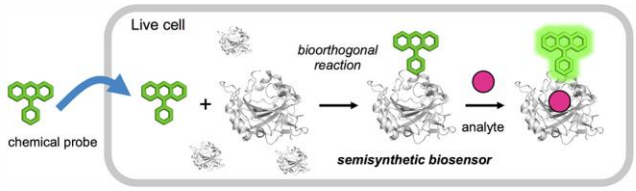
Live cell
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