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Small-Molecule-Based Protein-Labeling Technology in Live Cell Studies: Probe-Design Concepts and Applications

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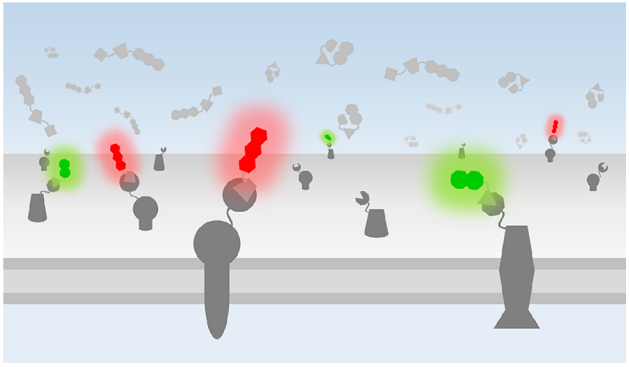
CONSPECTUS

The use of genetic engineering techniques allows researchers to combine functional proteins with fluorescent proteins (FPs) to produce fusion proteins that can be visualized in living cells, tissues, and animals. However, several limitations of FPs, such as slow maturation kinetics or issues with photostability under laser illumination, have led researchers to examine new technologies beyond FP-based imaging. Recently, new protein-labeling technologies using protein/peptide tags and tag-specific probes have attracted increasing attention.



Although several protein-labeling systems are commercially available, researchers continue to work on addressing some of the limitations of this technology. To reduce the level of background fluorescence from unlabeled probes, researchers have pursued fluorogenic labeling, in which the labeling probes do not fluoresce until the target proteins are labeled. In this Account, we review two different fluorogenic protein-labeling systems that we have recently developed.

First we give a brief history of protein labeling technologies and describe the challenges involved in protein labeling. In the second section, we discuss a fluorogenic labeling system based on a noncatalytic mutant of β-lactamase, which forms specific covalent bonds with β-lactam antibiotics such as ampicillin or cephalosporin. Based on fluorescence (or Förster) resonance energy transfer and other physicochemical principles, we have developed several types of fluorogenic labeling probes. To extend the utility of this labeling system, we took advantage of a hydrophobic β-lactam prodrug structure to achieve intracellular protein labeling. We also describe a small protein tag, photoactive yellow protein (PYP)-tag, and its probes. By utilizing a quenching mechanism based on close intramolecular contact, we incorporated a turn-on switch into the probes for fluorogenic protein labeling. One of these probes allowed us to rapidly image a protein while avoiding washout. In the future, we expect that protein-labeling systems with finely designed probes will lead to novel methodologies that allow researchers to image biomolecules and to perturb protein functions.

1. Introduction

Proteins are one of the most important components in living systems and function through interactions with various biomolecules. To discuss the physiological roles of proteins, it is important to investigate protein localization, transportation, functions, and so on. For this purpose, bioimaging with fluorescent proteins (FPs) has been widely utilized to visualize proteins of interest (POIs) under a microscope by constructing fusion proteins.[1] In addition, FP-based functional probes can be genetically localized at specific subcellular organelles, such as the nucleus, mitochondria, and endoplasmic reticulum. Despite these advantages, there are some limitations. Precise regulation of protein expression timing and application to various modalities other than fluorescence imaging are difficult. Moreover, fluorescence intensity and photostability need to be improved for strong laser-based imaging technologies such as super-resolution imaging and single molecule imaging.

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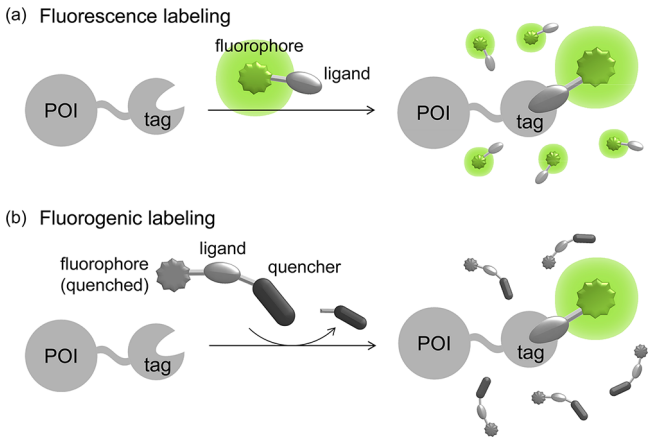


FIGURE 1. Two different protein-labeling modes: (a) fluorescence labeling and (b) fluorogenic labeling.

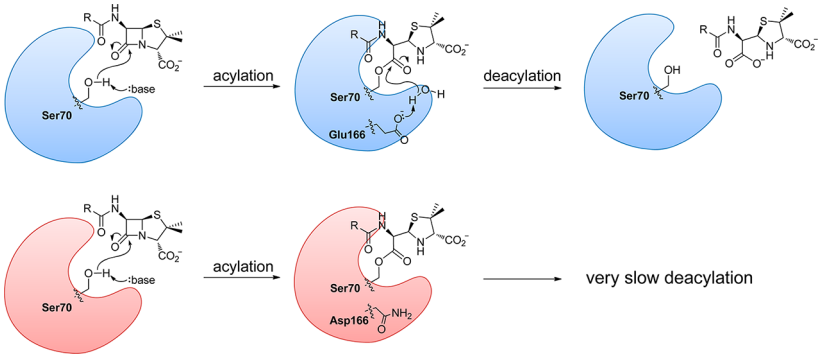
Furthermore, near-infrared FPs for visualizing deep tissue regions in living animals are still in short supply.

In contrast, some organic dyes are much more photoresistant and would be applicable for use in various modalities, including near-infrared fluorescence imaging.[2] In fact, small-molecule fluorescent probes have played indispensable roles in physiological studies.[3] In addition, rational design strategies of various fluorescent sensors have been established.[4] Therefore, the combination of protein and small molecules can produce novel research strategies when used with the latest instrumental analytical techniques. To label POIs with functional molecules, unnatural amino acids can be directly incorporated into the POIs.[5] This technique uses an orthogonal aminoacyl-tRNA synthetase–tRNA pair that incorporates the unnatural amino acid in response to a nonsense or a four-base codon. In addition, by conjugating POIs using bioorthogonal reactions such as azide–phosphine ligation[6] or azide–alkyne cycloaddition "click" chemistry,[7] more extensive functions can be incorporated into proteins.[8]

Alternatively, POIs can be fused with a genetically encodable peptide or protein tag and can be specifically modified with various functional groups at the location of the tag under physiological conditions such as in cultured cells (Figure 1a). As the pioneering study, Tsien and co-workers developed a tetracysteine motif (CCXXCC) and an arsenical fluorescein-based labeling probe, FlAsH-EDT$_2$.[9] POIs fused with this small peptide tag were fluorescently labeled with FlAsH-EDT$_2$ in living cells. This technology contributed to many protein studies[10,11] despite slight drawbacks with nonspecific background. To achieve specific labeling on the short peptide tag, Ting and co-workers exploited enzymatic reactions by biotin ligase,[12] transglutaminase,[13] and lipoic acid ligase.[14] Another type of protein labeling technology utilizes protein tags, which are larger but have more specificity than peptide tags, in labeling probes. Cornish and co-workers exploited the specific binding of trimethoprim and *Escherichia coli* dihydrofolate reductase (eDHFR).[15] Waggoner and co-workers developed an interesting method for noncovalent protein labeling by screening a library of single-chain antibodies.[16] Although the noncovalent tag–ligand binding induces dissociation under highly diluted conditions, this property is advantageous in some cases due to its reversibility. Meanwhile, covalent protein labeling technologies such as SNAP-tag[17] or HaloTag[18] are more broadly utilized in biological studies, especially in single molecule imaging that requires highly diluted conditions. SNAP-tag was developed by altering human O^6-alkylguanine DNA alkyl transferase (hAGT) to accelerate the alkyl transfer reaction that gives rise to stable enzyme–substrate adducts. HaloTag is a similar labeling tag based on a noncatalytic mutant of bacterial haloalkane dehalogenase. Both labeling technologies are widely used, because many ligands with various functional groups, such as blue to far-red fluorescent dyes and biotin, are commercially available. By using these labeling technologies, biologists can pigment POIs with various fluorescent colors, as well as having a choice of FPs.

So, what is the current challenge for protein labeling technologies? For intracellular protein labeling with commercial fluorescent probes, we have very few choices: coumarin, fluorescein, rhodamine green, or tetramethylrhodamine derivatives. We postulate that this is because most of the red to far-red fluorescent dyes tend to accumulate in hydrophobic organelles in living cells, and it is difficult to wash them out of cells completely. To minimize the influence from nonspecific labeling or residual labeling probes in cells, fluorogenic labeling probes, in which the fluorescence is quenched before labeling POIs, would be useful. So far, only a few fluorogenic protein-labeling methods have been reported. Tetracysteine-tag[9] by Tsien et al. is the first example. However, a washout step is necessary in this method. Fluorogen-activating single-chain antibody[16] by Waggoner et al. is a very promising fluorogenic method; although it includes a noncovalent system, the method's ligand-replaceability is promising. Recently, Urano et al. and Correa et al. separately reported fluorogenic probes based on the SNAP-tag system.[19,20] On the other hand, we also have developed two novel fluorogenic protein-labeling technologies. Thus, in this Account, we focus on these two technologies using a mutant β-lactamase and a photoactive yellow protein (PYP) as the protein tags, respectively.

SCHEME 1. Enzymatic Reaction Schemes of TEM-1 β-Lactamase (top) and Its E166N Mutant (bottom)



2. Protein Labeling System Based on Mutant β-Lactamase

The ideal tag for protein labeling in live cell imaging requires the following traits: (1) the tag is not endogenous, (2) the tag does not associate or react with endogenous molecules, and (3) fusion with the tag does not inhibit the functions of POIs. On the basis of the first two criteria concerning bioorthogonality, plant or bacterial peptides or proteins are preferable for mammalian cell studies. To satisfy the third criterion, a peptide or protein with hydrophilicity, impartial charge, and small size would be suitable. Considering these criteria, we focused on a class A β-lactamase, TEM-1, a small bacterial enzyme that hydrolyzes β-lactam antibiotics, as a suitable protein tag.

The reaction of TEM-1 with β-lactam substrates involves acylation and deacylation steps (Scheme 1). In the deacylation step, a water molecule activated by Glu166 hydrolyzes the ester bond of the enzyme–substrate intermediate to complete the catalytic reaction. It was previously reported that the E166N mutant of TEM-1 causes the acyl–enzyme intermediate to accumulate due to the marked reduction in the rate of deacylation.[21] Because this mutant β-lactamase fulfilled the above-mentioned requirements for protein tags, we named this mutant protein "BL-tag" and designed specific fluorescent labeling probes that had a β-lactam structure. Synthetic methods for β-lactam compounds have been extensively pursued, and many intermediates for β-lactam antibiotics are commercially available due to the enormous clinical demands. Therefore, it is possible to functionally tune labeling probes by choosing the appropriate β-lactam structures.

2.1. Ampicillin-Based Labeling Probes. We designed and synthesized the first probe, CA, a conjugate of coumarin and ampicillin, which is one of the penicillin-type β-lactam antibiotics (Chart 1).[22] To confirm its labeling ability, CA was incubated with the purified BL-tag protein in neutral buffer solution, and the mixture was analyzed by SDS-PAGE (Figure 2a). UV excitation showed that the 29 kDa BL-tag was specifically labeled with CA even in the presence of cell lysate. In order to show the generality of the BL-tag system, we also synthesized other fluorophore-conjugated probes such as FA and RA (Chart 1), and these probes were also specifically labeled with the BL-tag (Figure 2a).[23]

To investigate the fluorescence labeling of living cellular proteins, BL-tag was fused to the extracellular N-terminus of epidermal growth factor receptor (EGFR), which is a transmembrane receptor protein, and the fusion protein was expressed in HEK293T cells. The specific labeling of BL-tag-fused EGFR (BL-EGFR) with the synthesized probes was examined with a confocal laser scanning microscope (CLSM) after washout of the excess probes. Fluorescent signals from each fluorophore were observed on the cell membrane (Figure 2b). Nonspecific labeling was not observed in the control cells, in which untagged EGFR was expressed.

Next, the influence of the BL-tag toward the inherent functions of the labeled proteins was examined. In response to the physiological ligand EGF, BL-EGFR was internalized into the cells via phosphorylation. This ligand-induced internalization and phosphorylation indicates that the fusion of the BL-tag with the N-terminus of EGFR did not inhibit the intrinsic functions of EGFR in live cells. Results of the ampicillin-based labeling probes confirmed that BL-tag-based protein-labeling technology is useful for fluorescence labeling of cell surface proteins in live mammalian cells.

As another ampicillin-based probe, we developed a photoluminescent lanthanide complex labeling probe.[24] This probe enabled not only autofluorescence-free imaging of POIs by time-resolved fluorescence microscopy but also lifetime-based dual color imaging with a single optical filter

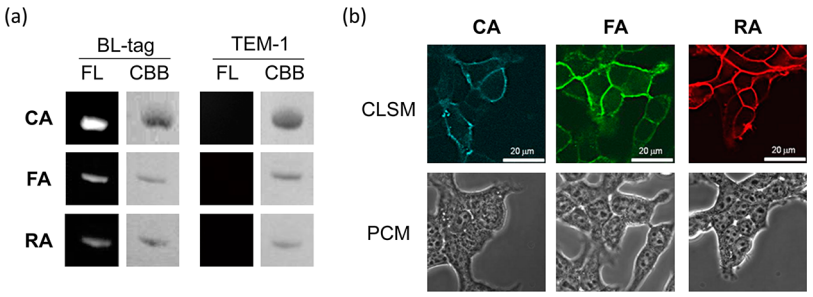
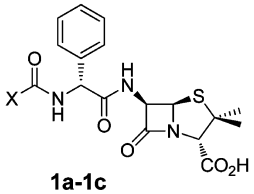


FIGURE 2. (a) SDS-PAGE to confirm protein labeling by the synthesized probes. FL, fluorescence image; CBB, Coomassie Brilliant Blue-stained image. (b) Fluorescence labeling of membrane proteins with penicillin-based probes CA, FA, and RA. CLSM, CLSM image; PCM, phase-contrast microscopic image.

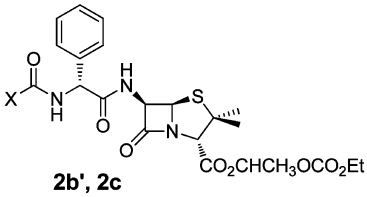
CHART 1. Structures of β-Lactam-Based Labeling Probes for BL-Tag

ampicillin-based probes



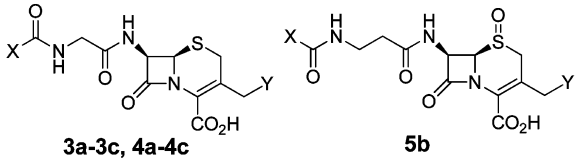
1a-1c

bacampicillin-based probes



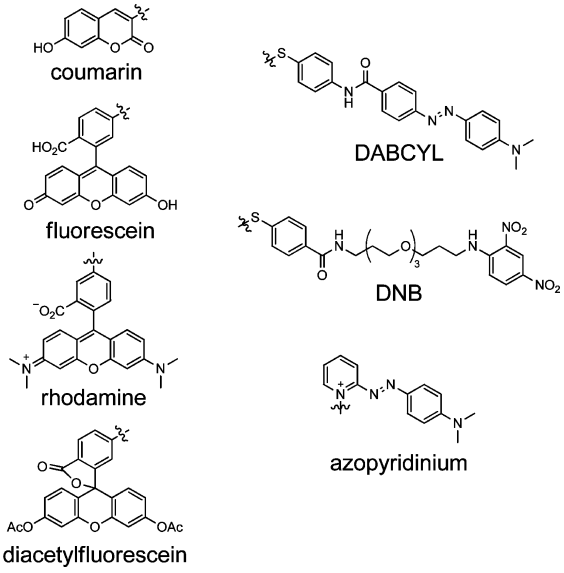
2b', 2c

cephalosporin-based probes



3a-3c, 4a-4c **5b**

Compound	X	Y	Name
1a	coumarin	-	**CA**
1b	fluorescein	-	**FA**
1c	rhodamine	-	**RA**
2b'	diacetylfluorescein	-	**FB-DA**
2c	rhodamine	-	**RB**
3a	coumarin	DABCYL	**CCD**
3b	fluorescein	DABCYL	**FCD**
3c	rhodamine	DABCYL	**RCD**
4a	coumarin	DNB	**CCDNB**
4b	fluorescein	DNB	**FCDNB**
4c	rhodamine	DNB	**5RCDNB**
5b	fluorescein	azopyridinium	**FCAPO2**



set. We also developed a biotinylation probe and achieved pulse-chase imaging of EGFR by using the probe together with commercial streptavidin-conjugated quantum dots.[25]

2.2. Bacampicillin-Based Probes for Intracellular Proteins. Fluorescence labeling of intracellular proteins is more challenging than labeling cell surface proteins, because labeling probes need to fulfill both sufficient hydrophilicity and cell membrane permeability criteria. Ampicillin has an anionic carboxy group; thus ampicillin-based labeling probes showed no membrane permeability, and protection of the carboxy group seemed essential for intracellular protein labeling. We focused on hydrophobic β-lactam antibiotics such as bacampicillin or pivampicillin, in which the carboxylate is protected as a noncharged ester.[26] In particular, bacampicillin is industrially available and shows good absorptivity in living body as well as high stability at a neutral pH.[27] Hence, we designed and synthesized bacampicillin-based probes FB-DA and RB (Chart 1),[28] which are cell-permeable derivatives of FA and RA, respectively. To confirm the intracellular protein labeling ability of the

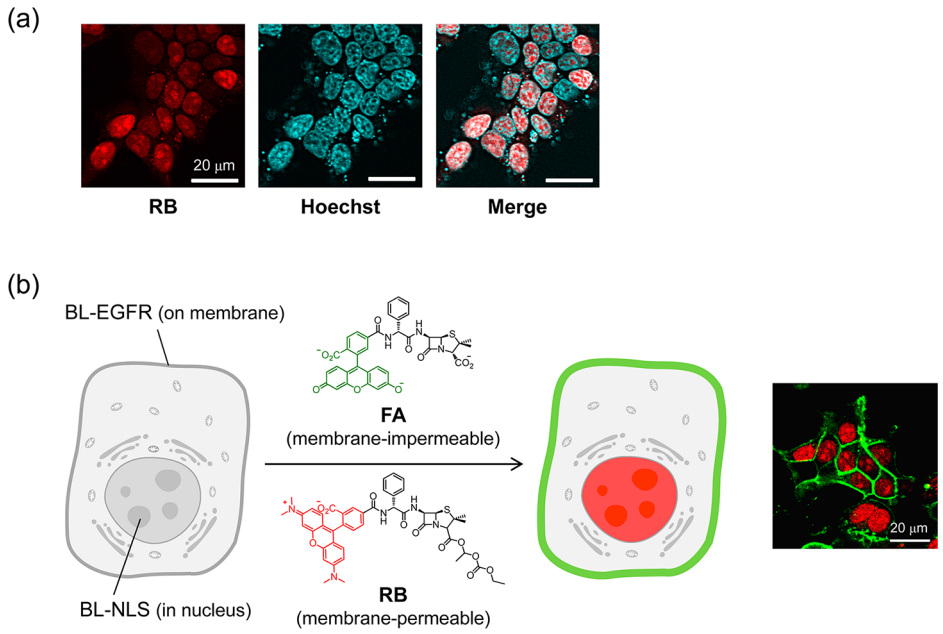


FIGURE 3. (a) CLSM images of HEK293T cells expressing BL-NLS costained with RB and Hoechst33342. (b) Scheme (left) and CLSM image (right) of simultaneous discriminative labeling of intracellular and cell surface BL-tag fusion proteins by FA and RB. Copyright 2011 Wiley. Used with permission from ref 28.

bacampicillin-based probes, we constructed BL-NLS, a nucleus-localized BL-tag that had three consecutive nuclear localization sequences (NLS)[29] from SV40 large T antigen at the C-terminus. When HEK293T cells expressing BL-NLS were treated with FB-DA or RB, strong fluorescence from the labeling probes were observed in the cell nuclei (Figure 3a).

Generally, esterification of the carboxy group of ampicillin largely decreases the reaction rate of enzymatic cleavage by β-lactamases.[30] Therefore, the binding rates of bacampicillin-based probes and BL-tag were much lower than those of ampicillin-based probes. When ampicillin-based and bacampicillin-based labeling probes FA and RB were simultaneously used to treat cells, cell surface tag-fused proteins were selectively labeled with FA, and intracellular tag-fused proteins were labeled only with the membrane-permeable probe RB (Figure 3b, left). This result verified the theory that the same tag could be labeled with different fluorophores in different locations at the same time. Transmembrane BL-EGFR and nuclear BL-NLS were coexpressed in HEK293T cells, and the cells were treated with 100 nM RB and 100 nM FA. When the cells were examined under CLSM after incubation and washing procedures, green fluorescence from FA and red fluorescence from RB was observed to be in separate locations (at the plasma membrane and in the nucleus, respectively) (Figure 3b, right).

2.3. Cephalosporin-Based Probes for Fluorogenic Labeling. Although penicillin-type probes have many advantages, such as their quick labeling rates and straightforward synthesis, these probes do not alter their fluorescence properties when they label target proteins. Thus, unlabeled probes essentially need to be washed out prior to imaging experiments, which is also the case with several commercial labeling systems. However, it is difficult to completely wash out probes from live cells in many cases. Therefore, we addressed the development of fluorogenic labeling probes, which do not fluoresce until after they label the target protein. In principle, washout of the excess labeling probes is not required in complete fluorogenic labeling systems.

Cephalosporins are another class of clinically used β-lactams. Some cephalosporins show substituent elimination after β-lactam hydrolysis. This property has been exploited to develop several probes to detect β-lactamase activity.[31−33] We also focused on this property to develop fluorogenic labeling probes for the BL-tag (Figure 1b). Our first fluorogenic labeling probe, CCD, which was named after coumarin−cephalosporin−DABCYL, contains the DABCYL (4-(4-dimethylaminophenylazo)benzoyl) group as the FRET (fluorescence (or Förster) resonance energy transfer) quencher.[22] Because the absorption spectrum of DABCYL substantially overlaps with the emission spectrum of 7-hydroxycoumarin, coumarin fluorescence was efficiently suppressed ($\Phi = 0.005$) by FRET to DABCYL. As a result, CCD covalently labeled a BL-tag to the same degree as ampicillin-based probes did, and fluorescence was increased by the elimination of DABCYL after the labeling (Figure 4a). We also developed the fluorescein-based labeling probe FCD and the tetramethylrhodamine

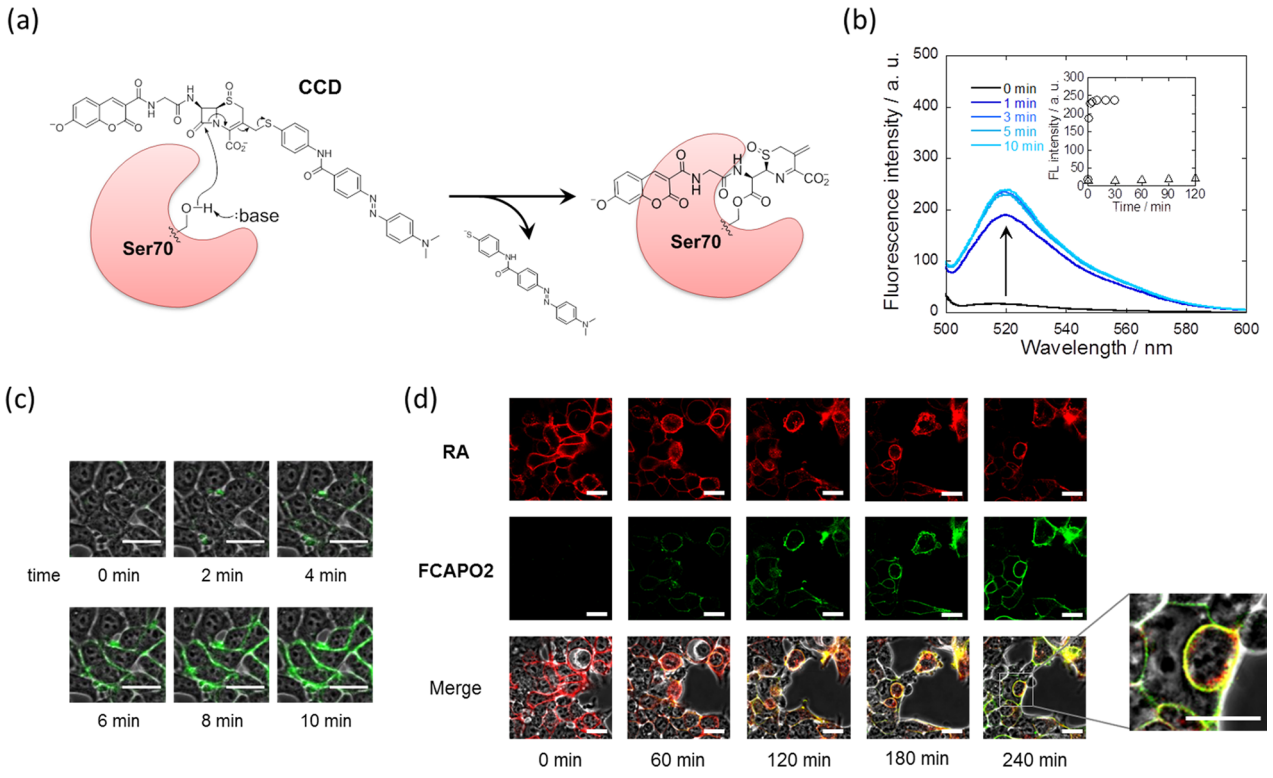


FIGURE 4. (a) Fluorogenic protein labeling scheme with cephalosporin-based probe CCD. (b) Time-dependent emission spectra of 500 nM FCAPO2 (λ_{ex} = 490 nm), obtained by incubation with 1 μM BL-tag. Inset, time-dependent fluorescence intensity of 500 nM FCAPO2 (λ_{ex} = 490 nm, λ_{em} = 518 nm) with (circle) or without (triangle) BL-tag. (c) Real-time fluorogenic labeling of cell surface BL-EGFR with 10 nM FCAPO2 at 37 °C. (d) Real-time fluorescence imaging of BL-EGFR trafficking by using FCAPO2 and RA: (top row) CLSM images (λ_{ex} = 559 nm) for RA; (middle row) CLSM images (λ_{ex} = 473 nm) for FCAPO2; (bottom row) merged images of CLSM and PCM. Scale bar: 20 μm. Reprinted with permission from ref 35. Copyright 2012 American Chemical Society.

(TMR)-based probe RCD.[23] Although FCD fluorescence was largely quenched (Φ = 0.005), the quenching of RCD fluorescence was less efficient (Φ = 0.07). This is probably because DABCYL is not an efficient FRET quencher for TMR due to the small spectral overlap between TMR emission and DABCYL absorption.

Fluorogenic labeling was conceptually achieved by using DABCYL-conjugated cephalosporin probes. However, there remained a practical problem to be solved, the slow elimination of the quencher: the first-order kinetic parameter of the autoelimination reaction was estimated to be 7.2×10^{-5} s^{-1}. To analyze quick dynamics of target proteins by pulse–chase experiments, a prompt turn-on fluorescence response after the labeling is important. Spontaneous elimination rates generally depend on the conjugate acid pK_a of the leaving group. Thus, it was predicted that the elimination rate for an azopyridine group (p$K_a \approx 4.3$) would be faster than that previously reported for the thiophenolate group (p$K_a \approx 6.5$) in FCD.[34] Considering its low pK_a, its hydrophilicity, and its absorption spectra (which ranges from 450 to 600 nm), we chose 2-(4-dimethylaminophenylazo)pyridine as the leaving FRET quencher. The new probe FCAPO2 (Chart 1) showed absorption peaks for both fluorescein and azopyridinium, and the fluorescence of FCAPO2 was mostly quenched (Φ = 0.02) by FRET.[35] The fluorescence was quickly recovered after incubation with BL-tag protein, and the fluorescence recovery was completed within a few minutes (Figure 4b). In live cell imaging, when HEK293T cells expressing BL-EGFR were treated with FCAPO2, green fluorescence increased along the plasma membranes within a few minutes without the washing procedure (Figure 4c). The bimolecular labeling rate constant of FCAPO2 and BL-tag was 7.8×10^4 M^{-1} s^{-1}, and this value indicates that this is the fastest fluorogenic labeling system among selective covalent labeling technologies.

By using the superior fluorogenic probe FCAPO2, we constructed an analytical method for protein trafficking, in which internalization of cell surface proteins and cell surface expression of genetically identical proteins could be discriminatively visualized. Cell surface BL-EGFR proteins were labeled in advance with the membrane-impermeable labeling probe RA, and FCAPO2 was added after medium replacement. As a result, the internalization of cell surface-displayed EGFR and the translocation of newly expressed

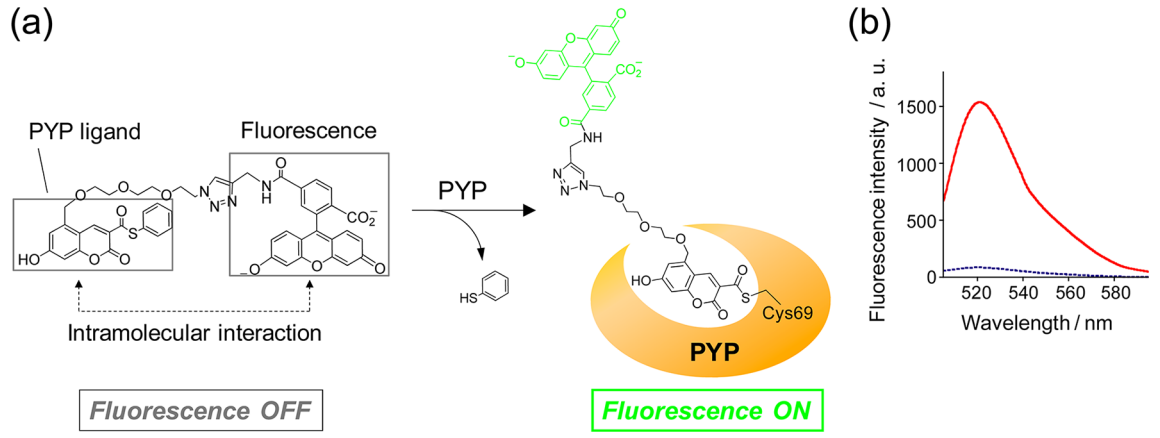


FIGURE 5. (a) PYP-tag fluorogenic labeling system utilizing FCTP. Fluorogenic mechanism is based on the intramolecular interaction between fluorophore and ligand. (b) Fluorescence spectra of FCTP in the absence (blue dashed line) or presence (red solid line) of PYP-tag.

EGFR to plasma membranes were discriminatively imaged (Figure 4d). This real-time imaging method can provide information about how quickly protein expression occurs on plasma membranes and how quickly proteins enter cells.

We also exploited different quenching strategies to design fluorogenic labeling probes. Dinitrobenzene-modified labeling probes CCDNB, FCDNB, and 5RCDNB were quenched through the static aggregation quenching mechanism.[36,37] A different probe, CC3DNB, was designed by reducing the linker length to inhibit intramolecular aggregation between the fluorophore and the quencher. This probe was also quenched, and the quenching mechanism was shown to be via photoinduced electron transfer.[38] One of the advantages of non-FRET quenching probes is that the quencher can be effective for a wide color range of fluorophores. Although there are still a few challenges that need to be overcome prior to practical use, non-FRET type fluorogenic labeling probes will be increasingly important.

3. Protein Labeling System Based on PYP

We developed another protein labeling system based on the PYP-tag, which is a small protein consisting of only 125 amino acids. In general, a small protein is preferred as a fusion tag because there is a concern that a large protein tag may have undesirable steric effects on the function or intracellular trafficking of POIs. In fact, some studies on FPs suggested that their relatively large size compromises the natural properties of proteins fused to FPs.[39−41] From this standpoint, the PYP-tag is an attractive choice and has an advantage over FPs because the size of the PYP-tag is almost half that of FPs (238 amino acids).[42] PYP is derived from *Halorhodospira halophila* and is a putative photoreceptor for negative phototaxis.[43] PYP-tag covalently binds to a 4-hydroxycinnamic acid derivative, which forms a thioester bond with Cys69.[44] In addition to the natural ligand, it is known that a 7-hydroxycoumarin-3-carboxylic acid derivative also acts as a ligand for the protein.[45] This artificial ligand was the key to the initial design of a fluorogenic probe. According to a previous report, when fluorescein is linked to coumarin through a flexible linker, intramolecular association between the compounds occurs, and the fluorescence of the fluorescein quenches owing to the formation of a nonfluorescent ground-state complex.[46] We made use of this principle to devise a fluorogenic labeling system for PYP-tag. A probe, FCTP, was designed by connecting the phenyl thioester of 7-hydroxycoumarin-3-carboxylic acid, which is a PYP-tag ligand, to fluorescein as a fluorophore moiety through a poly(ethylene glycol) linker (Figure 5a).[47] It was envisioned that the free state of the probe would be weakly fluorescent or nonfluorescent because of the static quenching by the association between coumarin and fluorescein. Since the ligand-binding pocket of PYP-tag has no room for the fluorescein, the intramolecular interaction was assumed to diminish upon protein labeling, triggering the fluorescence enhancement of the probe.

SDS-PAGE analysis demonstrated that FCTP specifically bound to PYP-tag in cell lysate and that the binding mode was covalent. As expected, the fluorescence intensity of FCTP was weak in the absence of PYP-tag and significantly increased when the labeling reaction occurred (Figure 5b). In live cell imaging experiments, FCTP visualized the expression of PYP-tag displayed on the cell surface. However, in vitro kinetic analyses showed that the labeling reaction was slow and did not completely finish for 24 h ($k_2 = 1.1\ M^{-1}\ s^{-1}$). For this reason, a washout process was necessary to obtain live cell images with high contrast after short incubation

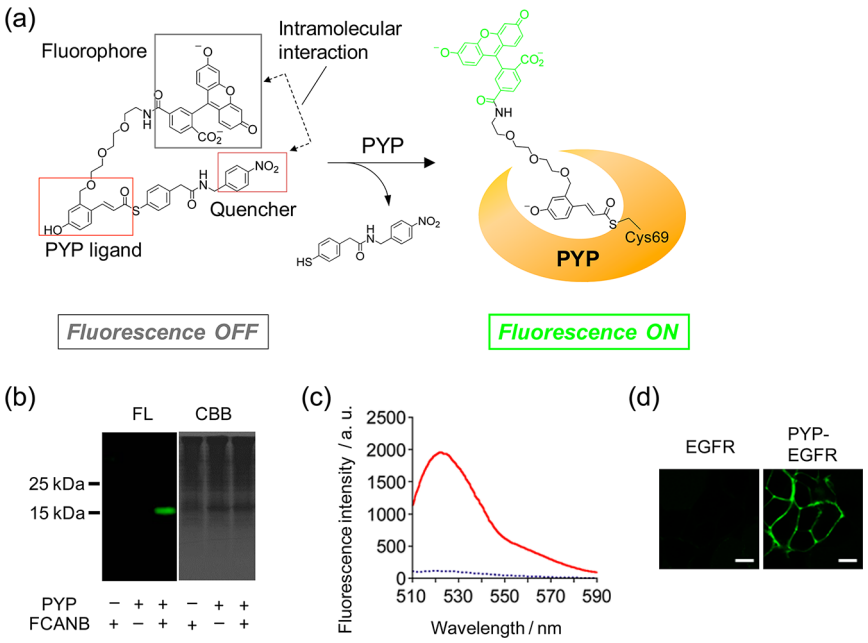


FIGURE 6. (a) Principle of fluorogenic labeling of PYP-tag with FCANB. (b) SDS-PAGE analysis of labeling reactions of PYP-tag (5 μM) with FCANB (8 μM). Images of CBB-stained and fluorescence gels are displayed on the right and left, respectively. FCANB was reacted with PYP-tag in cell lysate. (c) Fluorescence spectra of FCANB in the absence (blue dashed line) or presence (red solid line) of PYP-tag. (d) Live-cell imaging of PYP-tagged EGFR on cell surfaces with FCANB (5 μM). Scale bar: 10 μm.

periods. A possible cause of the slow kinetics was that the bulky intramolecular complex between the ligand and the fluorophore generated steric hindrance when the ligand moiety bound to the PYP-tag. Thus, a new labeling mechanism was required to reduce the steric hindrance around the ligand and improve the kinetic property.

To overcome this problem, we focused on the leaving group of the PYP-tag probe. Upon the labeling reaction, thiophenol is released from the ligand moiety of the probe. When a fluorescence quencher is linked to the thiophenol, it was predicted that the fluorescence of the probe would be restrained in the absence of PYP-tag and would be switched on by the dissociation of the quencher upon the labeling reaction. In addition, by selecting a quencher that interacted with the fluorophore, the fluorophore would be attracted to the quencher instead of the ligand, and consequently, the steric hindrance around the ligand would be minimized. Based on this strategy, a new probe, FCANB, was designed by incorporating fluorescein and its quencher into a natural PYP-tag ligand, 7-hydroxycinnamic acid (Figure 6a).[48] Nitrobenzene was chosen as the quencher because it interacts with fluorescein and reduces its fluorescence as described above. Another important feature of the probe design is that 4-hydroxycinnamic acid was employed as a ligand moiety instead of coumarin, in the hope of further repression of undesirable $\pi-\pi$ stacking interaction between the ligand and the fluorophore.

As with FCTP, FCANB was shown to form a covalent bond with PYP-tag, and the specific binding of the probe to the protein occurred when the labeling reaction was conducted in cell lysate (Figure 6b). Fluorescence measurements demonstrated that FCANB in a free state was scarcely fluorescent and increased fluorescence intensity by binding to PYP-tag (Figure 6c). As expected, the kinetic property of FCANB was remarkably improved over that for FCTP. The second-order rate constant of FCANB was calculated to be 125 M^{-1} s^{-1}, which is 110-fold higher than that of FCTP. Since the absorption peak of FCANB shifted to a wavelength that was 7 nm longer than that for fluorescein, it was suggested that the probe's fluorescein interacts intramolecularly with the nitrobenzene in a ground state. The structural difference of the ligand moieties between FCTP and FCANB did not affect the labeling kinetics. Therefore, these results support the hypothesis that the labeling rate of FCANB was promoted because the fluorophore preferentially interacted with the nitrobenzene instead of the ligand in the probe, leading to the reduction of the steric hindrance around the ligand.

Live cell imaging was conducted using FCANB and the PYP-EGFR fusion gene. After the probe was added to cells expressing the fusion protein on cell surface and the cells were washed with buffer, fluorescence microscopic measurement was performed. Fluorescence was observed along

the plasma membrane of the cells. Taking advantage of the fluorogenic property of FCANB, direct microscopic imaging without a washing process was carried out after the addition of the probe. As a result, fluorescence was detected only from the cell membrane. No significant fluorescence was seen in the medium, other cell parts, or cells not expressing PYP-fusion proteins. Moreover, the image was similar to that obtained with a washout step. This no-wash labeling system in combination with the small PYP-tag provides a promising tool for the imaging of rapid movement and trafficking of cell membrane proteins with a high signal-to-noise ratio.

4. Conclusions and Perspectives

In summary, we briefly surveyed our recent studies on the development of two different protein-labeling technologies. One technology, BL-tag, exploits a noncatalytic mutant of class A β-lactamase, and the other, PYP-tag, utilizes a photoactive yellow protein. BL-tag is attractive for pulse–chase analysis because BL-tag-fused POIs can be rapidly labeled by using probes with different emission wavelengths. When the steric effect of a protein tag is critical, a PYP-tag is preferable owing to its small size. Both technologies achieved no-wash fluorogenic protein labeling through the rational design of their own labeling probes. Additionally, appropriate derivatization of the probe structures enabled intracellular protein labeling. Therefore, these technologies will contribute to the clarification of various physiological phenomena by fluorescence microscopic imaging methodologies.

For future perspectives, the applications of protein labeling technology are increasingly expanded. One possible application is the localization of fluorescent sensing probes, such as fura-2[49] or fluo-3,[50] at specific organelles. Although FP-based sensors achieved such applications,[51] the combination of protein labeling technology and small molecule sensors will lead to a wider variety of sensing methodologies that can be used to study intracellular local signaling.[52−54] Another promising future direction of protein labeling technology is regulation of protein functions. Recently, a photo-triggered neuronal cell activation system called "optogenetics" has come under the spotlight. Since various protein functions are regulated by protein translocation and protein–protein interaction, many physiological phenomena could be regulated using protein labeling technology and functional labeling probes. Although there have been few reports about regulation of intracellular signaling[55,56] or protein degradation,[57] such artificial regulation of cellular functions will likely become a new trend in next-generation chemical biology.

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FOOTNOTES

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