Fluorescent Probes

DOI: 10.1002/ange.201200867

Development of Protein-Labeling Probes with a Redesigned Fluorogenic Switch Based on Intramolecular Association for No-Wash Live-Cell Imaging**

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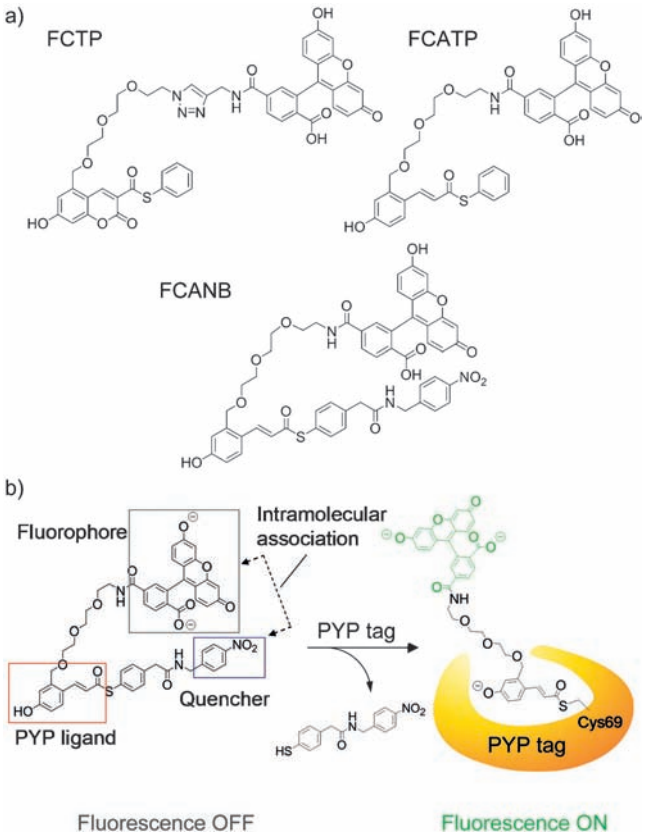
Fluorescence labeling of proteins is a powerful technique for studying precise protein localization and movement in living cells. Currently, fluorescent proteins (FPs) are the primary tools in this field owing to their technical feasibility and convenience.[1,2] Although various FPs have been reported,[2] their properties such as protein size and brightness are not completely satisfactory for some biological applications.[1–3] As an alternative to FPs, chemical methods utilizing synthetic fluorescence probes and fusion proteins have recently emerged.[4–7] In this imaging technique, a ligand-binding domain is fused to the protein of interest (POI) as a protein (peptide) tag and is reacted with a fluorophore-conjugated ligand. Thus, the POI is labeled by the fluorophore through the linkage of the tag and the ligand. Representative examples of previously commercialized protein (peptide) tags are the HaloTag,[5] the SNAP tag,[6] and the tetracysteine tag.[7] The key characteristics of these techniques are that POIs are conditionally labeled by the temporal addition of probes, and various fluorophores can easily be incorporated into probes by replacing just the fluorophore moiety. However, since the fluorescence of free probes or probes bound to nontarget biomolecules interfere with the identification of the labeled target protein, thorough washing of cells is necessary to remove free probes. This is a time-consuming process, and incomplete washing causes a decrease in the signal-to-noise ratio. To solve this problem, we have developed protein-labeling probes that do not require any washing procedure for live-cell imaging.

As possible approaches to the problem, we and other groups have reported turn-on fluorescence labeling systems, in which the fluorescence of a probe is quenched in a free state and is recovered in a protein-tag-bound state.[7–11] These fluorogenic probes minimize background fluorescence and overcome the limitation of conventional protein-labeling systems. Although many protein-labeling methods are known, fluorogenic methods that do not require any washing are still restricted to a few protein-tagging systems.[11] Therefore, the further development of novel fluorogenic systems is required. Based on the quenching mechanism of intramolecular association, we have recently created a fluorogenic probe, FCTP, for labeling the photoactive yellow protein (PYP) tag (Scheme 1 a).[8] The PYP tag is derived from purple bacteria[12] and binds to the thioester derivatives of 4-hydroxycinnamic acid, a natural cofactor, or 7-hydroxycoumarin through transthioesterification with residue Cys69.[8,13] The small size of the PYP tag (14 kDa; half the size of the green fluorescent protein, GFP) makes this protein particularly interesting, as



Scheme 1. a) Chemical structures of FCTP, FCATP, and FCANB. b) Principle of fluorogenic labeling of PYP tag with FCANB.

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[**] This work was supported by MEXT of Japan (Grants 20675004, 22108519 to K.K. and 22685016, 23114710 to Y.H.), by the Funding Program for World-Leading Innovative R&D on Science and Technology from JSPS, by the Grant-in-Aid from the Ministry of Health, Labour and Welfare (MHLW) of Japan, by CREST from JST, and by Asahi Glass Foundation.

Supporting information (including experimental details) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201200867.

well as promising for protein-tagging technology. Other than the tetracysteine tag, the PYP tag is the smallest protein tag covalently labeled by a fluorogenic substrate. The fluorogenic probe FCTP is nonfluorescent because of the intramolecular association between the fluorescein and coumarin moieties, but the fluorescence is restored by the reversal of this association upon protein labeling. The probe FCTP, however, requires more than 24 h to complete this labeling reaction. For this reason, a washing step is essential for expeditious live-cell imaging with a high signal-to-noise ratio. Hence, fluorogenic probes with much more rapid kinetics are required for no-wash imaging.

To achieve this, we have employed a design strategy based on a new probe scaffold consisting of cinnamic acid thioester as a PYP-tag ligand and nitrobenzene as a fluorescence quencher (Scheme 1). By introducing fluorescein as a fluorophore into this scaffold, we have designed and synthesized a fluorogenic probe, FCANB. Cinnamic acid thioester was chosen in place of the coumarin ligand, which had been utilized in FCTP, to reduce the intramolecular stacking interaction between the ligand and fluorophore moieties. This interaction is undesirable, because it could cause steric hindrance around the ligand toward the PYP tag, and this hindrance was observed for FCTP in our previous study.[8] Nitrobenzene has desirable properties as a fluorogenic switch. The first is a synthetic advantage, in that it is easily incorporated into fluorescence probes owing to its simple structure. A more important feature is that nitrobenzene associates with fluorophores, including fluorescein, and thereby quenches the fluorescence.[9,14] Furthermore, this quenching mechanism can be utilized as a general strategy for the design of turn-on fluorescence probes. It is envisioned that the fluorescein of the probe loses its fluorescence by the association with nitrobenzene, and this association inhibits the unwanted interaction between the ligand and fluorophore moieties, thereby leading to rapid protein labeling (Scheme 1b). Moreover, the probe will become fluorescent if the thiophenyl leaving group that is connected to nitrobenzene is dissociated from the probe upon protein labeling. To confirm the effects of nitrobenzene, we have also synthesized a probe, FCATP, which does not contain nitrobenzene (Scheme 1a).

First, the binding characteristics of the probes with the PYP tag were investigated. Purified PYP tag was incubated with the probes FCATP or FCANB and analyzed by SDS-PAGE after heating at 95 °C. In both cases, a fluorescent band appeared in the gel and the molecular weight was in accordance with that of the PYP tag, thus indicating that the probes bind to PYP tag (Figure 1a, Figure S1 in the Supporting Information). Since the samples were denatured by SDS and heat, the binding mode is considered to be covalent. The probes were also reacted with PYP tag in cell lysate to verify binding specificity (Figure 1b, Figure S1 in the Supporting Information). No fluorescence was detected in the gel image when the probes were added to cell lysate in the absence of PYP tag. In contrast, in the case of PYP-tag-containing cell lysate, a single fluorescent band appeared in the gel at the band position of the PYP tag. These results confirm that the probes specifically bind to the PYP tag.

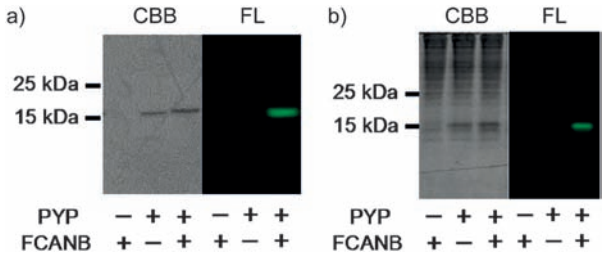

Figure 1. SDS-PAGE experiments of labeling reactions of PYP tag with the probe FCANB. Images of Coomassie Brilliant Blue (CBB)-stained and fluorescence gel are displayed on the left and right, respectively. PYP tag (a: 10 μM, b: 5 μM) was reacted with FCANB (a: 16 μM, b: 8 μM). Images (a) and (b) represent the reactions in the absence and presence of cell lysate, respectively.

Next, fluorescence spectra were measured to examine whether the fluorescence intensity of the probes increased upon binding to the PYP tag (Figure 2a, Table 1). Unexpectedly, FCATP exhibited only a slight fluorescence in the absence of PYP tag (fluorescence quantum yield $\Phi_f = 0.05$), even though no quenching group was introduced into this molecule. The binding of FCATP to the PYP tag led to a 9.3-fold enhancement of the fluores-

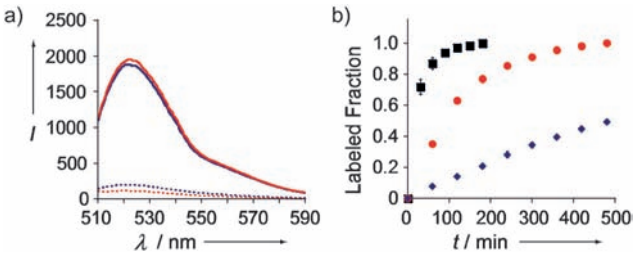

Figure 2. Fluorescence spectroscopy analyses of labeling reactions. a) Fluorescence emission spectra of FCATP (blue lines) and FCANB (red lines) in the absence (dotted lines) or presence (solid lines) of PYP tag. b) Time course of labeling reactions of FCTP (blue diamonds), FCATP (red circles), and FCANB (black squares) with PYP tag. All measurements were conducted using samples in tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer (20 mM Tris·HCl, 150 mM NaCl (pH 7.4)) at 37 °C. The probe and protein concentrations were 8 μM.

Table 1: Spectral and kinetic properties of PYP-tag probes.

Probe	λ_{abs} [nm]	λ_{em} [nm]	ε [$M^{-1}cm^{-1}$]	Φ_f	k_2[a] [$M^{-1}s^{-1}$]
FCTP	505	522	37300	0.02	1.11
FCATP	501	522	51100	0.05	11.1
FCANB	501	521	44400	0.04	125
Probe–PYP tag[b]	498	521	57300	0.47	–

[a] All measurements were made in triplicate. k_2 = second-order rate constant. [b] Spectroscopic data of PYP-tag-bound probe were obtained after the labeling reaction of PYP tag with FCANB was completed.

cence intensity of FCANB was weak ($\Phi_f = 0.04$) and was augmented by the binding of FCANB to the PYP tag ($\Phi_f = 0.47$); its fluorescence change was 15-fold and thus larger than that of FCATP. Neither of the probes displayed a time-dependent alteration of the fluorescence intensity in the absence of PYP tag (Figure S2 in the Supporting Information). These results demonstrate that both FCATP and FCANB are fluorogenic probes for labeling PYP tag. Moreover, they show that the nitrobenzene moiety in FCANB contributes to the quenching efficiency by lowering the fluorescence quantum yield and extinction coefficient of the fluorophore under these experimental conditions (Table 1). The absorption spectra of free FCATP and FCANB show that the maximum wavelength was shifted by 7 nm in comparison to that of the fluorescein derivative without a ligand or nitrobenzene moiety (Figure S3 in the Supporting Information). This spectral change strongly suggests that intramolecular association occurs between the fluorophore and the ligand or nitrobenzene, and this association could cause fluorescence quenching for both probes. This type of quenching mechanism has also been reported, and it has been shown that fluorescence quenching is triggered by the close contact of intramolecular fluorophores.[8,15]

The kinetic analyses of protein labeling were carried out by monitoring the increase in the fluorescence intensity of the probes. The time required for 50% labeling, $t_{1/2}$, was estimated using the labeling reactions, in which the concentrations of the probes and proteins were 8 μM (Figure 2b, Figure S2 in the Supporting Information). FCANB has the shortest $t_{1/2}$ value (ca. 15 min), followed by FCATP ($t_{1/2}$ ca. 78 min) and FCTP ($t_{1/2} > 470$ min), thus demonstrating that FCANB binds to the PYP tag most rapidly. In further kinetic analyses, second-order rate constants were also calculated (Table 1, Figure S4 in the Supporting Information). Consistent with the $t_{1/2}$ values, the k_2 values of FCATP and FCANB are 10-fold and 110-fold higher than that of FCTP, respectively. These kinetic data suggest that the introduction of the 4-hydroxycinnamic acid ligand in probes FCATP and FCANB leads to the fast protein labeling. There is a possibility that the PYP tag could intrinsically bind to the 4-hydroxycinnamic acid ligand more rapidly than to the coumarin ligand. However, this possibility was excluded, because it was found that the PYP tag binds to both ligands, neither of which contains the fluorophore, with almost the same kinetics (Figure S5 in the Supporting Information). Therefore, it is more likely that the strength of intramolecular interaction or the structure of the intramolecular complex between the fluorophore and ligand moieties affects the protein-labeling kinetics of both probes FCANB and FCATP. As well as the 4-hydroxycinnamic acid ligand, nitrobenzene also gave a promotional effect on the protein labeling kinetics of FCANB, as expected. This result suggests that the fluorophore interacts more favorably with the nitrobenzene rather than with the ligand moiety, and thereby the steric hindrance around the ligand is diminished.

Finally, live-cell imaging was conducted using the probes. Epidermal growth factor receptor (EGFR) fused with the PYP tag at the N-terminal extracellular domain (PYP–EGFR) was expressed on the surface of human embryonic kidney (HEK293T) cells (Figure S6 in the Supporting Information), and each of the probes was incubated with the cells for 30 min. After washing the cells to remove free probes, fluorescence images were taken using confocal laser-scanning microscopy (Figure 3a, Figure S7a in the Supporting Information). Clear fluorescence was observed along the plasma membrane in the cells treated with either FCATP or FCANB. No fluorescence was detected in cells expressing EGFR without PYP tag. These results indicate that both probes specifically label the PYP–EGFR fusion protein on the cell surface. We also tried to label PYP-tagged proteins inside cells with the probes. This attempt, however, failed, because the probes were not cell-permeable. Taking advantage of the fluorogenic properties of the probes, direct imaging of cell-surface proteins without washing was performed immediately after the labeling reaction (Figure 3b, Figure S7b). As in images obtained when the washing procedure was used, distinct fluorescence was detected only on the surface of the cells expressing the PYP–EGFR fusion protein, while the fluorescence of the free probe was not seen in the media or in other parts of cells. Nonspecific labeling was also confirmed to be absent in cells that do not express the PYP–EGFR fusion protein. Importantly, specific imaging of proteins was accomplished by utilizing these fluorogenic probes without washing.

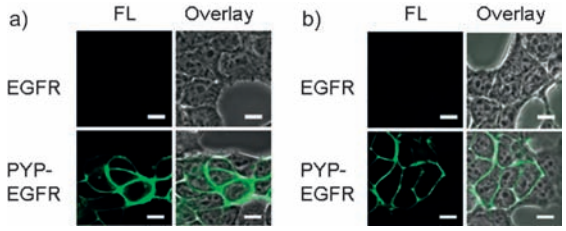

Figure 3. Live-cell imaging of PYP-tagged EGFR on cell surfaces with the probe FCANB (5 μM) a) with or b) without washing procedures. Fluorescence images and their overlays with phase contrast images are shown in the left and right of each panel, respectively. Scale bars: 10 μm.

In summary, we have developed fluorogenic probes, FCATP and FCANB, for labeling PYP tag. Kinetic properties of the probes were significantly improved compared to the previous probe, FCTP. In particular, FCANB binds to the PYP tag 110 times more rapidly than FCTP. This acceleration effect is induced by the introduction of both the cinnamic acid ligand and the nitrobenzene quencher into the probe structure. A possible reason for this effect is that the fluorophore preferably interacts with the nitrobenzene instead of the ligand and that thereby the steric hindrance around the ligand is reduced. The kinetic enhancement and fluorogenicity of the probes enabled the specific labeling of PYP-tagged proteins on the cell surface with a procedure that does not require washing steps. The most notable point is that the PYP tag is the smallest protein among existing protein tags that can be covalently labeled by small fluorogenic compounds without the requirement of washing cells. This no-wash labeling system combined with the small PYP tag offers an attractive tool for the imaging of rapid movement and

trafficking of cell-membrane proteins with a high signal-to-noise ratio.

Received: February 1, 2012
Revised: March 19, 2012
Published online: April 25, 2012

Keywords: fluorescence imaging · fluorescent probes · intramolecular association · protein modifications · protein tags

[1] A. Miyawaki, *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 656 – 668.
[2] a) J. Wiedenmann, F. Oswald, G. U. Nienhaus, *IUBMB Life* **2009**, *61*, 1029 – 1042; b) D. M. Chudakov, M. V. Matz, S. Lukyanov, K. A. Lukyanov, *Physiol. Rev.* **2010**, *90*, 1103 – 1163.
[3] a) C. L. Thomas, A. J. Maule, *J. Gen. Virol.* **2000**, *81*, 1851 – 1855; b) C. S. Lisenbee, S. K. Karnik, R. N. Trelease, *Traffic* **2003**, *4*, 491 – 501; c) P. R. Dahlgren, M. A. Karymov, J. Bankston, T. Holden, P. Thumfort, V. M. Ingram, Y. L. Lyubchenko, *Nanomedicine* **2005**, *1*, 52 – 57; d) A. Raney, A. Y. Shaw, J. L. Foster, J. V. Garcia, *Virology* **2007**, *368*, 7 – 16.
[4] a) Y. Yano, K. Matsuzaki, *Biochim. Biophys. Acta Biomembr.* **2009**, *1788*, 2124 – 2131; b) C. Jing, V. W. Cornish, *Acc. Chem. Res.* **2011**, *44*, 784 – 792; c) M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 707 – 708; d) H. Nonaka, S. Tsukiji, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* **2007**, *129*, 15777 – 15779; e) S. S. Gallagher, J. E. Sable, M. P. Sheetz, V. W. Cornish, *ACS Chem. Biol.* **2009**, *4*, 547 – 556; f) Y. Hori, Y. Egashira, R. Kamiura, K. Kikuchi, *ChemBioChem* **2010**, *11*, 646 – 648.
[5] a) G. V. Los, K. Wood, *Methods Mol. Biol.* **2007**, *356*, 195 – 208; b) H. L. Lee, S. J. Lord, S. Iwanaga, K. Zhan, H. Xie, J. C. Williams, H. Wang, G. R. Bowman, E. D. Goley, L. Shapiro, R. J. Twieg, J. Rao, W. E. Moerner, *J. Am. Chem. Soc.* **2010**, *132*, 15099 – 15101.
[6] a) A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol.* **2003**, *21*, 86 – 89; b) A. Gautier, A. Juillerat, C. Heinis, I. R. Correa, Jr., M. Kindermann, F. Beaufils, K. Johnsson, *Chem. Biol.* **2008**, *15*, 128 – 136.
[7] a) B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269 – 272; b) S. R. Adams, R. E. Campbell, L. A. Gross, B. R. Martin, G. K. Walkup, Y. Yao, J. Llopis, R. Y. Tsien, *J. Am. Chem. Soc.* **2002**, *124*, 6063 – 6076; c) M. J. Roberti, C. W. Bertoncini, R. Klement, E. A. Jares-Erijman, T. M. Jovin, *Nat. Methods* **2007**, *4*, 345 – 351.
[8] Y. Hori, H. Ueno, S. Mizukami, K. Kikuchi, *J. Am. Chem. Soc.* **2009**, *131*, 16610 – 16611.
[9] K. K. Sadhu, S. Mizukami, S. Watanabe, K. Kikuchi, *Chem. Commun.* **2010**, *46*, 7403 – 7405.
[10] S. Mizukami, S. Watanabe, Y. Hori, K. Kikuchi, *J. Am. Chem. Soc.* **2009**, *131*, 5016 – 5017.
[11] a) T. Komatsu, K. Johnsson, H. Okuno, H. Bito, T. Inoue, T. Nagano, Y. Urano, *J. Am. Chem. Soc.* **2011**, *133*, 6745 – 6751; b) X. Sun, A. Zhang, B. Baker, L. Sun, A. Howard, J. Buswell, D. Maurel, A. Masharina, K. Johnsson, C. J. Noren, M. Q. Xu, I. R. Corrêa, Jr., *ChemBioChem* **2011**, *12*, 2217 – 2226; c) C. J. Zhang, L. Li, G. Y. Chen, Q. H. Xu, S. Q. Yao, *Org. Lett.* **2011**, *13*, 4160 – 4163.
[12] a) T. E. Meyer, *Biopchim. Biophys. Acta* **1985**, *806*, 175 – 183; b) M. Kumauchi, M. T. Hara, P. Stalcup, A. Xie, W. D. Hoff, *Photochem. Photobiol.* **2008**, *84*, 956 – 969.
[13] a) Y. Imamoto, T. Ito, M. Kataoka, F. Tokunaga, *FEBS Lett.* **1995**, *374*, 157 – 160; b) W. D. Hoff, B. Devreese, R. Fokkens, I. M. Nugteren-Roodzant, J. Van Beeumen, N. Nibbering, K. J. Hellingwerf, *Biochemistry* **1996**, *35*, 1274 – 1281; c) R. Cordfunke, R. Kort, A. Pierik, B. Gobets, G.-J. Koomen, J. W. Verhoeven, K. J. Hellingwerf, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7396 – 7401.
[14] G. S. Beddard, S. Carlin, L. Harris, G. Porter, C. J. Tredwell, *Photochem. Photobiol.* **1978**, *27*, 433 – 438.
[15] a) A. P. Wei, D. K. Blumenthal, J. N. Herron, *Anal. Chem.* **1994**, *66*, 1500 – 1506; b) B. Z. Packard, D. D. Toptygin, A. Komoriya, L. Brand, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 11640 – 11645; c) H. Takakusa, K. Kikuchi, Y. Urano, T. Higuchi, T. Nagano, *Anal. Chem.* **2001**, *73*, 939 – 942.

