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# No-Wash Protein Labeling with Designed Fluorogenic Probes and Application to Real-Time Pulse-Chase Analysis

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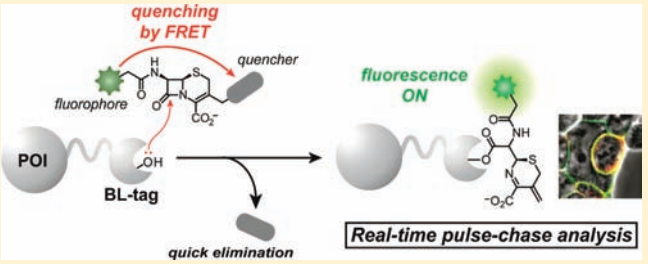
*W* *Web-Enhanced Feature* *S* *Supporting Information*

**ABSTRACT:** Small molecule labeling techniques for cellular proteins under physiological conditions are very promising for revealing new biological functions. We developed a no-wash fluorogenic labeling system by exploiting fluorescence resonance energy transfer (FRET)-based fluorescein-cephalosporin-azopyridinium probes and a mutant $\beta$-lactamase tag. Fast quencher elimination, hydrophilicity, and high resistance against autodegradation were achieved by rational refinement of the structure. By applying the probe to real-time pulse-chase analysis, the trafficking of epidermal growth factor receptors between cell surface and intracellular region was imaged. In addition, membrane-permeable derivatization of the probe enabled no-wash fluorogenic labeling of intracellular proteins.




## INTRODUCTION

By imaging protein dynamics, valuable information about target proteins can be obtained, such as their intracellular localization, expression and degradation timings, and interactions with other proteins. Fluorescent proteins (FPs) have been utilized for this purpose because advances in fluorescence microscopy have enabled the real-time visualization of target proteins genetically fused with FPs in living cells.[1] Combining the use of FPs with probe design strategies based on fluorescence resonance energy transfer (FRET) has also led to the production of innovative probes for biological studies.[2] However, such genetic engineering approaches have critical limitations. For instance, we cannot label target proteins expressing at specific timings by using FPs. Although imaging the dynamics of cell cycle progression was achieved by exploiting the cell-cycle dependent proteolysis of two FP-labeled ubiquitination oscillators,[3] the strategy cannot be generally applied to other dynamic biological processes. In addition, organic or inorganic chemistry-based synthetic molecules provide a wider range of functions. Therefore, techniques for labeling cellular proteins with synthetic molecules under physiological conditions are obviously useful and promising in revealing new biological functions.

Many research groups have reported tag-based protein labeling techniques, in which the target proteins are genetically fused with a peptide tag or a small-protein tag.[4] These tags are specifically modified with the corresponding small-molecule probes by a chemical or enzyme reaction, coordination, protein−ligand interaction, etc. By using such labeling technologies, proteins expressed at specific timings can be visualized. However, most labeling techniques, except the tetracysteine-tag technology,[4a] do not exhibit a fluorogenic property. In other words, the washout of the unlabeled probes is required prior to microscopic imaging. Tetracysteine-tag technology also requires the washing procedure for imaging with a high signal/noise ratio to avoid nonspecific labeling.[5] Because the washout procedure takes at least 15 min, precise pulse-chase experiments with shorter time intervals are practically impossible with conventional labeling technologies. In addition, the complete washout of the unreacted probes inside cells is difficult, because synthetic probes often accumulate in various organelles.

We developed a fluorogenic protein labeling system that exploits a mutant of 29-kDa TEM-1 $\beta$-lactamase (BL-tag) and the synthetic $\beta$-lactam probes.[6] This technology is quite versatile, and various applications such as multicolor fluorescence imaging,[7] a pulse-chase experiment with luminescent quantum dots via specific biotinylation,[8] and time-resolved protein imaging with a lanthanide-based probe[9] have been demonstrated. In addition, the use of FRET-based labeling probes[6] in this system conferred both specificity and a turn-on fluorescence property. The labeling mechanism involves two steps: an initial noncatalytic enzyme reaction and subsequent quencher elimination via a self-immolative reaction (Scheme 1).

Effective FRET quenching of the probes and fluorescence recovery after *in vitro* protein labeling via a self-immolative reaction have been observed. However, the no-wash labeling of live-cell proteins has not been performed because of a slow turn-on fluorescence response. In addition, the high hydro-

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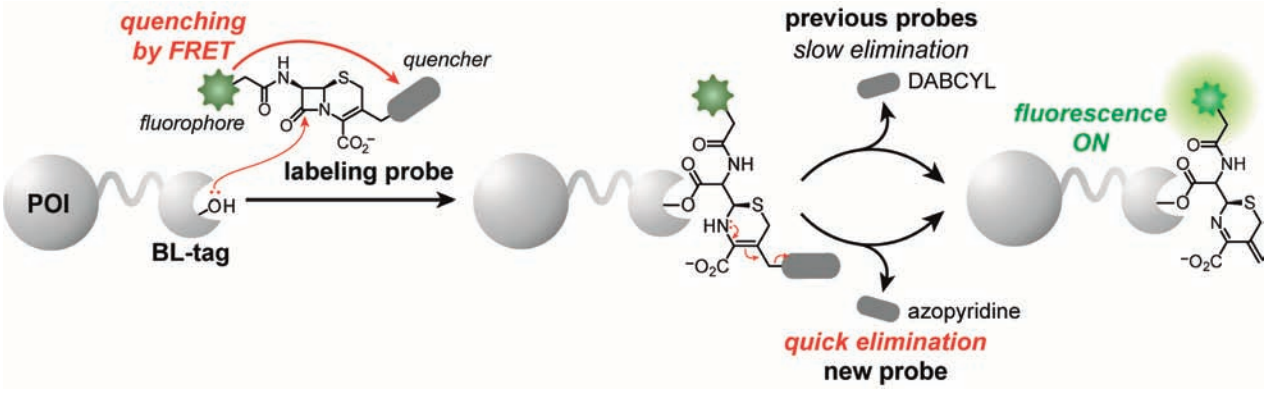
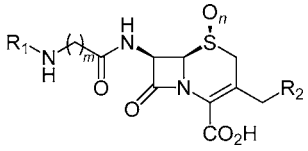
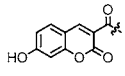
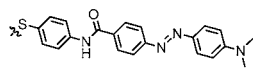
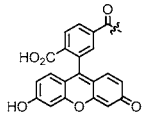
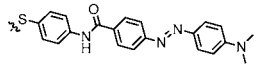
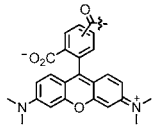
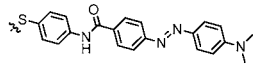
**Scheme 1. Schematic Illustration of Fluorogenic Protein Labeling Mechanism Using Previous and New Probes Based on Fluorescence Resonance Energy Transfer (FRET)[a]**

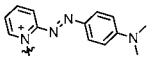
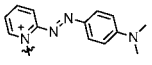
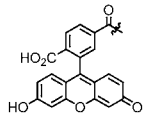
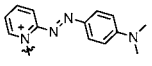
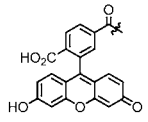
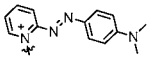
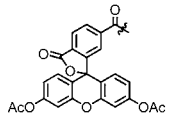
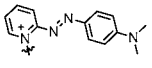



[a]POI: protein of interest.

**Chart 1. Structures of Synthesized Compounds**



| | $R_1$ | $R_2$ | $m$ | $n$ |
|---|---|---|---|---|
| CCD |  |  | 1 | 0 |
| FCD |  |  | 1 | 0 |
| RCD |  |  | 1 | 0 |

| | $R_1$ | $R_2$ | $m$ | $n$ |
|---|---|---|---|---|
| CAP1 | H |  | 1 | 0 |
| CAP2 | H |  | 2 | 0 |
| FCAP2 |  |  | 2 | 0 |
| FCAPO2 |  |  | 2 | 1 |
| FCAPO2-DA |  |  | 2 | 1 |

phobicity of the probes, mainly of the DABCYL group, caused a tendency to accumulate in the hydrophobic regions of cells such as plasma membranes, although nonspecific accumulation disappeared after washing the cells. When analyzing the quick dynamics of target proteins using pulse-chase experiments, the labeling rate should be as high as possible. Thus, it is very important to develop superior labeling probes that exhibit a quick turn-on fluorescence response and thus enable real-time pulse-chase experiments without the washing procedure. However, no such probes have been reported thus far.

Herein, we report the rational development of a novel probe that can exhibit a fast fluorogenic labeling response for BL-tag technology. The probe did not require the washing procedure in live cell imaging and thus provided an innovative protein analytical method using real-time pulse-chase analysis. In addition, the membrane-permeable derivatization of the probe by the esterification enabled the no-wash fluorogenic labeling of intracellular proteins.

## RESULTS

**Design, Synthesis, And Photophysical and Biochemical Properties of Novel Labeling Probes.** We investigated the optimization of the leaving quencher for no-wash fluorogenic labeling. As described above, the fluorogenic labeling process is divided into two steps: namely, enzymatic labeling and subsequent elimination reaction. Preliminary absorption analysis of the synthesized labeling probes showed that the nucleophilic enzyme reaction proceeds quickly and the following elimination is the rate-determining step. Elimination rates generally depend on the elimination ability of the leaving groups, which is correlated with the p$K_a$ value of the conjugate acid. The p$K_a$ of the thiophenol group of FCD (Chart 1) was estimated to be about 6.5,[10] and it was thus expected that a decrease in p$K_a$ would accelerate the elimination rate. It has been reported that azopyridinium-conjugated cephalosporin induces fast elimination from the 3′-position after hydrolysis of $\beta$-lactam, because the corresponding conjugate acid has a lower p$K_a$ (4.3).[11] Because of its fast elimination, hydrophilicity, and absorption spectra, we chose 2-(4-dimethylaminophenylazo)-pyridinium as the quencher. Although this azopyridinium compound is not widely known as a FRET quencher, as compared with DABCYL, the absorption spectral property from 450 to 600 nm fulfills the requirement for quenching green or orange fluorescence of fluorescein or tetramethylrhodamine, respectively. In addition, the hydrophilicity of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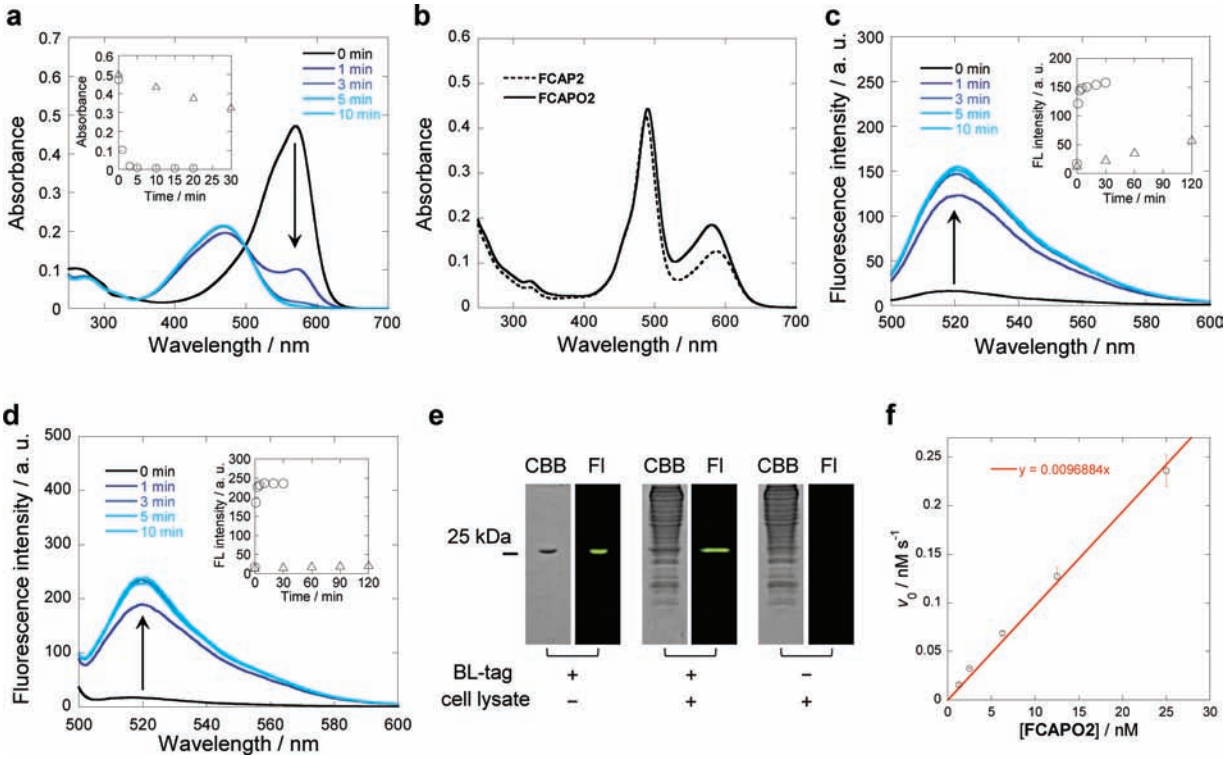


**Figure 1.** Spectral and protein labeling properties of synthesized probes. All reactions were performed in 100 mM HEPES buffer (pH 7.4) at 25 °C. (a) Time-dependent absorption spectra of 10 $\mu$M CAP1, obtained by incubation with 40 nM WT TEM. (inset) Time-dependent absorbance ($\lambda$ = 570 nm) of CAP1 with (circle) or without (triangle) WT TEM. (b) Absorption spectra of 5 $\mu$M FCAP2 (dotted line) and 5 $\mu$M FCAPO2 (solid line). (c, d) Time-dependent emission spectra of 500 nM FCAP2 (c, $\lambda_{ex}$ = 487 nm) or 500 nM FCAPO2 (d, $\lambda_{ex}$ = 490 nm), obtained by incubation with 1 $\mu$M BL-tag. (inset) Time-dependent fluorescence intensity of 500 nM FCAP2 (c, $\lambda_{ex}$ = 487 nm, $\lambda_{em}$ = 518 nm) or 500 nM FCAPO2 (d, $\lambda_{ex}$ = 490 nm, $\lambda_{em}$ = 518 nm) with (circle) or without (triangle) BL-tag. (e) Electrophoresis gel images of BL-tag incubated with FCAPO2 in the presence or absence of HEK293T cell lysate. CBB: Coomassie Brilliant Blue staining. Fl: fluorescence. (f) Plot of initial labeling reaction rate $v_0$ versus probe concentration [FCAPO2]. [BL-tag] = 125 nM.

azopyridinium (calculated logP = −1.9) was expected to enhance the practical utility in live cell imaging.

We designed and synthesized CAP1 (Chart 1 and Scheme S1, Supporting Information) as the key compound, which can be conjugated with various fluorophores, to develop novel fluorogenic probes. The elimination reaction rate of CAP1 was investigated using wild-type TEM-1 $\beta$-lactamase (WT TEM). The absorption maximum of CAP1 in HEPES buffer (pH 7.4) was observed at 580 nm, and the peak quickly decreased by the addition of WT TEM (Figure 1a). This spectral change indicates that azopyridinium was eliminated, getting converted into the neutral azopyridine form.[11a] However, the absorption spectrum of CAP1 gradually changed in an enzyme-free buffer solution (Figure 3a inset), probably because CAP1 degraded automatically in neutral aqueous solution. This degradation was also observed in dimethyl sulfoxide (DMSO)-$d_6$. Furthermore, fluorophore conjugation to the amino group was unsuccessful because of the instability of CAP1. We suspected that the instability might be caused by intramolecular aminolysis via the six-membered ring formation (Scheme S2, Supporting Information).

To avoid the intramolecular cyclization, we designed an improved synthetic scheme (Scheme S3, Supporting Information) via CAP2 (Chart 1), which has an aminoethyl group instead of the aminomethyl group of CAP1. As a result, CAP2 showed good stability in solution. CAP2 showed equally quick absorption spectral change by the addition of WT TEM as CAP1 (Figure S1a, Supporting Information). CAP2 also showed enough stability in neutral buffer solution (Figure S1b, Supporting Information). Thus, the intramolecular cyclization observed in the case of CAP1 could be completely suppressed by linker elongation in CAP2.

A new fluorescent probe, FCAP2 (Chart 1), was successfully synthesized from CAP2 (Scheme S3, Supporting Information). FCAP2 showed the absorption maxima at 487 nm ($\lambda$ = 85 000 M$^{-1}$ cm$^{-1}$) and 589 nm ($\varepsilon$ = 25 200 M$^{-1}$ cm$^{-1}$) for fluorescein and azopyridinium, respectively (Figure 1b, dotted line). As expected, the fluorescence of FCAP2 was largely quenched ($\Phi$ = 0.03) by FRET and recovered by incubation with BL-tag protein (Figure 1c). The fluorescence increase, as well as the absorption spectral change, was very quick and completed within a few minutes (Figure 1c inset, circle). This indicates the great potential of FCAP2 in real-time fluorescence imaging. However, the fluorescence of FCAP2 was unexpectedly increased in the absence of BL-tag, probably as a result of autohydrolysis (Figure 1c inset, triangle).

A previous report has suggested that stability of cephalosporin derivatives is improved by oxidizing the sulfides of cephalosporins to sulfoxides.[12] Therefore, FCAPO2 (Chart 1), the oxidized compound of FCAP2, was designed and synthesized (Scheme S4). FCAPO2 showed an absorption spectrum very similar to that of FCAP2, with two peaks for fluorescein ($\lambda$ = 490 nm, $\varepsilon$ = 89 000 M$^{-1}$ cm$^{-1}$) and azopyridinium ($\lambda$ = 581 nm, $\varepsilon$ = 37 000 M$^{-1}$ cm$^{-1}$) (Figure 1b, solid line). The fluorescence of FCAPO2 was largely quenched ($\Phi$ = 0.02) by FRET and quickly recovered by


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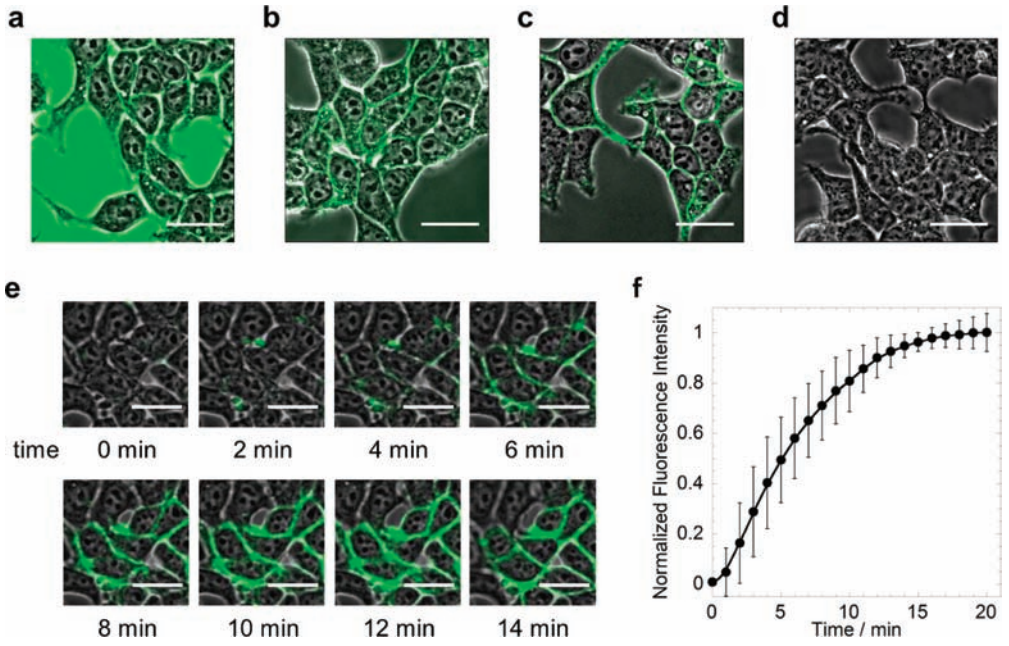


**Figure 2.** No-wash fluorogenic labeling of cell surface proteins. (a–d) HEK293T cells expressing BL-EGFR (a–c) or EGFR (d) were treated with 500 nM FA (a,b) or FCAPO2 (c,d) at 37 °C. Fluorescence images ($\lambda_{ex}$ = 473 nm) were captured using a confocal fluorescence microscope without (a, c, d) or with (b) the washing procedure. (e, f) Time-lapse fluorogenic labeling of cell surface BL-EGFR with 10 nM FCAPO2 at 37 °C. Time-course graph of the fluorescence images (e) and the averaged fluorescence intensity for the complete field of view ($n$ = 4) (f). Scale bar: 20 $\mu$m. See also Movie 1 in the HTML version of this paper.

incubation with BL-tag protein (Figure 1d). The fluorogenic labeling and elimination reactions were very quick and completed within a few minutes (Figure 1d inset, circle). The stability of FCAPO2 in neutral aqueous solution was considerably increased, as compared with that of FCAP2 (Figure 1d inset, triangle) and was considered enough for practical use in physiological studies. With regard to specificity, FCAPO2 labeled only BL-tag, even in the presence of HEK293T cell lysate (Figure 1e). All these results indicate that we successfully developed a highly stable labeling probe that showed quick fluorogenic response after specific protein labeling.

Then, the precise labeling kinetics of FCAPO2 toward BL-tag was studied. To a solution of the excess tag protein (125 nM), 1.25–25 nM FCAPO2 was added, and the initial reaction rate calculated from the fluorescence increase (Figure S4, Supporting Information) was plotted against the probe concentration (Figure 1f). As a result, the bimolecular labeling rate constant was determined to be $7.8 \times 10^4$ $M^{-1}$ $s^{-1}$. This value indicates that the combination of FCAPO2 and BL-tag is the fastest fluorogenic labeling system among the highly selective labeling technologies, as shown in Table S1 in the Supporting Information.

**Fluorogenic Labeling of Cell Surface Proteins with FCAPO2.** Fluorogenic labeling of cell surface proteins with FCAPO2 was examined using living cells. BL-tag-fused epidermal growth factor receptor (BL-EGFR) proteins were expressed in HEK293T cells, following treatment with FCAPO2 or nonfluorogenic FA (Figure S2a, Supporting Information) at a concentration of 500 nM. After 10 min of incubation, the cells were observed with a confocal fluorescence microscope without washout of the probe. In the case of FA, high background fluorescence signals were observed in the medium (Figure 2a), although the fluorescence labeling of the membrane proteins was visible after the washing procedure (Figure 2b), as previously reported.[7] On the other hand, when the cells were incubated with FCAPO2, green fluorescence increased along the plasma membranes within a few minutes without the washing procedure (Figure 2c). This fluorescence increase was not observed in the cells expressing EGFR without BL-tag (Figure 2d).

Next, we performed time-lapse imaging of protein labeling with FCAPO2. Under confocal fluorescence microscopy, 10 nM FCAPO2 was added to HEK293T cells expressing BL-EGFR. Consecutive capture of cell images without the washing procedure showed time-dependent fluorescence enhancement along the plasma membranes (Figure 2e and Movie 1). The fluorogenic labeling rate of the living cell surface proteins was investigated in time-lapse fluorescence images. Even with highly diluted probe ([FCAPO2] = 10 nM), the labeling rate was fast and labeling was almost completed within 15 min (Figure 2f). Based on the results of these *in vitro* and live cell experiments, we conclude that this new labeling system is faster than previously reported fluorogenic labeling systems, including our previous reports and the SNAP-tag-based fluorogenic labeling system reported.[13]

**Real-Time Imaging of Cell Surface Expression and Internalization of Target Proteins by Using Dual Probes.** Although imaging of protein trafficking is one of the promising applications of protein labeling techniques, the washing procedures in the current technologies restrict wide application of the techniques. Therefore, we performed real-time pulse-chase analysis of protein expression on cell surface by using FCAPO2. As pulse-chase analysis visualizes target protein expression at a specific time, quick protein labeling that does not require the washing procedure would be very effective for pulse-chase analysis of protein trafficking with high temporal resolution.

We constructed a protein analytical method, in which internalization of cell surface proteins and cell surface


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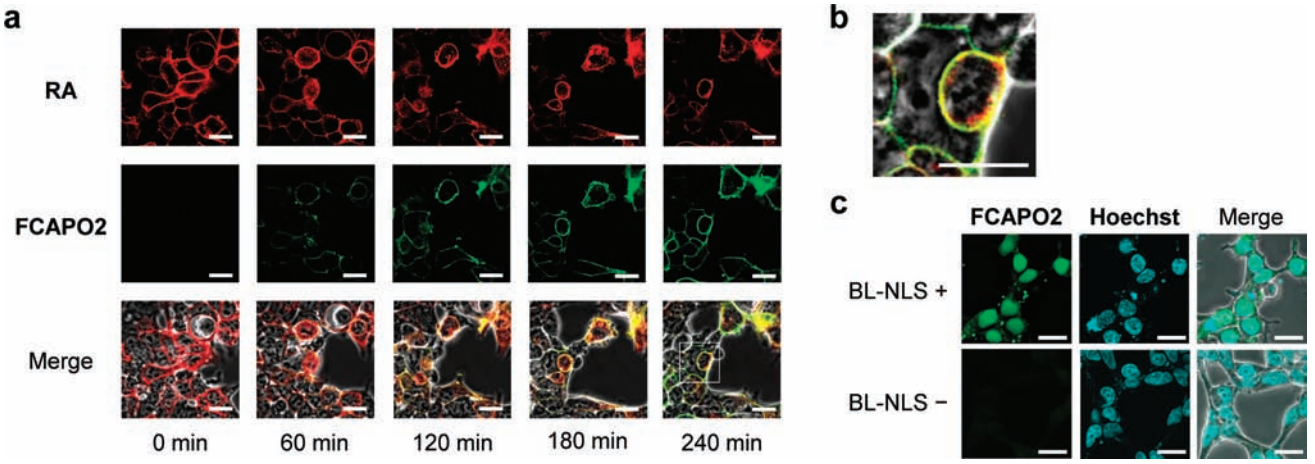


**Figure 3.** Real-time pulse-chase analysis and no-wash fluorogenic labeling of intracellular proteins. (a, b) Real-time fluorescence imaging of BL-EGFR trafficking by using FCAPO2 and RA. HEK293T cells were labeled with 100 nM RA prior to incubation with 20 nM FCAPO2 at 37 °C. (a) (top row) Fluorescence images ($\lambda_{ex}$ = 559 nm) for RA, (middle row) fluorescence images ($\lambda_{ex}$ = 473 nm) for FCAPO2, (bottom row) merged images of fluorescence and phase contrast microscopy. (b) Magnified image of the square marked in the bottom row in part a. (c) Intracellular protein labeling with FCAPO2-DA ($\lambda_{ex}$ = 473 nm). HEK293T cells expressing BL-NLS (top) and nontransfected cells (bottom) were incubated with 5 $\mu$M FCAPO2-DA and 100 ng mL$^{-1}$ Hoechst33342 at 37 °C for 2 h. Scale bar: 20 $\mu$m.

expression of genetically identical proteins can be discriminatively visualized. In the protocol, BL-EGFR proteins displayed on the cell surface were labeled in advance with a membrane-impermeable rhodamine probe, RA (Figure S2b, Supporting Information). After the culture medium was replaced with a fresh medium, FCAPO2 was added, and cell images were captured using a confocal fluorescence microscope (Figure 3a and b). Initially, only the red fluorescence of RA was detected around the plasma membranes. Thereafter, the red fluorescence signals gradually internalized and formed bright spots in the cells in a time-dependent manner. This trafficking indicates the endocytosis of EGFR caused by the stimulation of epidermal growth factor (EGF) included in the serum. On the other hand, the green fluorescence of FCAPO2 was scarcely observed in the beginning. However, the green fluorescence signals gradually increased along the plasma membranes, probably due to the fluorogenic labeling of newly expressed proteins on the cell surface. These results suggest that this imaging methodology can discriminately visualize EGFR trafficking from the cell surface to the intracellular region, as well as in the opposite direction.

**Fluorogenic Labeling of Intracellular Proteins with the Cell-Permeable Probe FCAPO2-DA.** We developed cell-permeable labeling probes for intracellular proteins by utilizing bacampicillin, which is a clinical penicillin-type $\beta$-lactam prodrug developed for improving intestinal absorption.[14] However, these probes do not have fluorogenic property because it is very difficult to incorporate leaving groups in the bacampicillin structure. Therefore, we extended the probe design of FCAPO2 to achieve fluorogenic labeling of intracellular proteins. FCAPO2 has three anionic groups and one cationic group in its structure. Of the three anionic groups, two are in the fluorescein structure, and the remaining anionic carboxylate and the cationic group are in cephalosporin and azopyridinium, respectively. Because the latter two groups are very close, they are likely to form an intramolecular ion pair. Hence, we hypothesized that the protection of only two anionic groups in fluorescein in FCAPO2 might enable probe introduction into living cells, although previous reports suggest that the carboxylate of cephalosporin-based probes needs to be protected to permeate living cell membranes.[15]

We acetylated FCAPO2 to obtain FCAPO2-DA (Chart 1 and Scheme S5 in the Supporting Information). The absorption spectra of FCAPO2-DA indicated that it was quickly hydrolyzed by WT TEM (Figure S3a, Supporting Information), although it was considerably stable for several hours in aqueous solution (Figure S3b, Supporting Information). FCAPO2-DA showed no fluorescence (Figure S3c, Supporting Information), similar to the other diacetylated fluorescein derivatives.[16]

To assess the intracellular labeling property of FCAPO2-DA, we used a BL-tag protein that localizes in cell nuclei, BL-tag-fused nuclear localization signal (BL-NLS).[14] BL-NLS has three consecutive simian virus 40 (SV40) large T antigen NLS at the C terminus of BL-tag. HEK293T cells expressing BL-NLS were incubated with FCAPO2-DA, and the cell permeability and the fluorogenic labeling property of FCAPO2-DA were confirmed by confocal fluorescence microscopy. As expected, FCAPO2-DA exhibited cell permeability, and the fluorescence gradually accumulated at the cell nuclei (Figure 3c top). No noteworthy fluorescence accumulation was detected in the nuclei of the control cells transfected with empty vector plasmids (Figure 3c bottom), even though a slight fluorescence increase was observed in whole cells, which is now under investigation. As a result, we finally developed a cell-permeable fluorogenic probe to label intracellular proteins. This probe, FCAPO2-DA, enabled no-wash intracellular protein labeling, as in the case of extracellular protein labeling with FCAPO2.

## ■ DISCUSSION

The key problem in developing a practical fluorogenic protein labeling technology by extending our previous strategy[6] was the slow quencher elimination after labeling. In principle, elimination reaction rate is correlated with the p$K_a$ of the conjugate acid of the leaving group. The p$K_a$ of 2-(4-dimethylaminophenylazo)pyridinium, which is the conjugate acid of the leaving group in FCAPO2, is 4.3.[10] This value is much smaller than that of thiophenol, which is the leaving group in our previous probes such as CCD and FCD. The fast turn-on fluorescence property of FCAP2 indicates that the p$K_a$


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of the leaving quencher was indeed an important factor for the probe design strategy. However, the fast elimination step induced undesirable autodegradation of FCAP2. This instability was overcome by oxidizing the sulfur atom in the design of FCAPO2. In addition, the linker length between the fluorophore and the $\beta$-lactam was optimized to achieve high synthetic yields.

Another essence of the probe design is the physical properties of the leaving quencher. The quencher in FCAPO2 is the cationic azopyridinium form, and it converts into the neutral azopyridine form after elimination. The absorption spectrum of the azopyridinium shows the maximum at 581 nm and broadly ranges from 500 to 600 nm. Thus, this compound works as an efficient quencher for fluorescein. This wavelength region is also suitable for the quenching of orange to light-red fluorescent dyes such as rhodamines. Although DABCYL is often used as the quencher in many quenching FRET-based probes,[17] the shorter-wavelength absorption ($\lambda_{max}$ = 462 nm) restricts its application to red fluorescent dyes. The higher molar extinction coefficient of the azopyridinium ($\varepsilon$ = 37 000 $M^{-1}$ $cm^{-1}$) than that of DABCYL ($\varepsilon$ = 25 500 $M^{-1}$ $cm^{-1}$)[7] also confirms its utility in quenching FRET applications. Hydrophilicity of azopyridinium provides another practical advantage in biological experiments. In live cell imaging, hydrophobic probes often cause localization at membranes or subcellular organelles. With regard to this point, FCAPO2 including azopyridinium showed less accumulative properties, as compared with DABCYL-based probes. In addition, the cationic aromatic heterocycle may compensate the anionic charge of the adjacent carboxy group in cephalosporin by forming an inner salt, because FCAPO2-DA permeates living cells without the protection of the carboxylate in cephalosporin.

In widely used protocols for other protein labeling techniques, at least 15-min incubation with the probes and subsequent washing procedure are required.[18] Such procedures restrict temporal resolution and diverse experimental designs. In this study, we achieved real-time pulse-chase analysis, and discriminatively visualized the internalization of cell surface-displayed proteins and the translocation of newly expressed proteins to plasma membranes. This innovative real-time imaging provided the information about how fast protein expression occurs on the plasma membranes and how fast the proteins enter the cells. Conventional methods using FPs cannot afford to discriminate proteins that have the same amino acid sequence but different functions or history. Urano et al. and Correa, Jr. et al. separately reported similar real-time fluorogenic labeling probes based on SNAP-tag technology.[13,19] However, our BL-tag-based system with FCAPO2 exhibited much faster labeling kinetics (Table S1, Supporting Information) and requires a lower probe concentration.

## CONCLUSION

In conclusion, we developed second-generation fluorogenic labeling probes by exploiting an azopyridinium compound as the leaving quencher. By rational optimization of the probe structures, we reached the design of FCAPO2, which specifically labeled BL-tag proteins and very quickly recovered its fluorescence. In a detailed kinetic study of fluorogenic labeling, the bimolecular reaction rate was the highest among the known selective and fluorogenic protein labeling methods. The outstanding properties of FCAPO2 provided a versatile cell surface protein labeling system that does not require any washing procedure. In addition, membrane-permeable derivatization of FCAPO2 to FCAPO2-DA enabled no-wash fluorogenic labeling of intracellular proteins. No-wash fluorogenic labeling for intracellular proteins is very rare, although we have developed an alternative no-wash labeling method by using highly diluted prodrug-based $\beta$-lactam probes.[14] Our no-wash fluorogenic labeling system described herein would evidently be an innovative technique that provides various applications in cell biology, one of which is described in this paper. Moreover, the fluorophore can be extended to near-infrared (NIR) fluorescent dyes, which are more suitable for *in vivo* studies. These results and discussion confirm the practical utility and promise of the mutant $\beta$-lactamase-tag-based protein labeling technology in biological studies, especially those on protein trafficking, gene expression, protein degradation, etc.

## ASSOCIATED CONTENT

### Supporting Information

Materials, instruments, synthesis and characterization of compounds, experimental procedures, and supplementary figures and schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

### Web-Enhanced Features

Movie 1, showing fluorogenic labeling of cell surface BL-EGFR with 10 nM FCAPO2 at 37 °C over 20 min, is available in the HTML version of this paper.

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## REFERENCES

(1) Chudakov, D. M.; Lukyanov, S.; Lukyanov, K. A. *Trends Biotechnol.* **2005**, *23*, 605−613.

(2) Truong, K.; Ikura, M. *Curr. Opin. Struct. Biol.* **2001**, *11*, 573−578.

(3) Sakaue-Sawano, A.; Kurokawa, H.; Morimura, T.; Hanyu, A.; Hama, H.; Osawa, H.; Kashiwagi, S.; Fukami, K.; Miyata, T.; Miyoshi, H.; Imamura, T.; Ogawa, M.; Masai, H.; Miyawaki, A. *Cell* **2008**, *132*, 487−498.

(4) (a) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269−272. (b) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86−89. (c) Chen, I.; Howarth, M.; Lin, W.; Ting, A. Y. *Nat. Methods* **2005**, *2*, 99−104. (d) Yin, J.; Liu, F.; Li, X.; Walsh, C. T. *J. Am. Chem. Soc.* **2004**, *126*, 7754−7755. (e) O'Hare, H. M.; Johnsson, K.; Gautier, A. *Curr. Opin. Struct. Biol.* **2007**, *17*, 488−494. (f) Hinner, M. K.; Johnsson, K. *Curr. Opin. Biotechnol.* **2010**, *21*, 766−776. (g) Rabuka., D. *Curr. Opin.*

*Chem. Biol.* **2010**, *14*, 790−796. (h) Sunbul, M.; Yin, J. *Org. Biomol. Chem.* **2009**, *7*, 3361−3371.
(5) (a) Stroffekova, K.; Proenza, C.; Beam, K. G. *Pfluegers Arch.* **2001**, *442*, 859−886. (b) Machleidt, T.; Robers, M.; Hanson, G. T. *Methods in Molecular Biology*; Humana Press: Totowa, 2007; Vol. *356*, pp 209−220.
(6) Mizukami, S.; Watanabe, S.; Hori, Y.; Kikuchi, K. *J. Am. Chem. Soc.* **2009**, *131*, 5016−5017.
(7) Watanabe, S.; Mizukami, S.; Hori, Y.; Kikuchi, K. *Bioconjug. Chem.* **2010**, *21*, 2320−2326.
(8) Yoshimura, A.; Mizukami, S.; Hori, Y.; Watanabe, S.; Kikuchi, K. *ChemBioChem* **2011**, *12*, 1031−1034.
(9) Mizukami, S.; Yamamoto, T.; Yoshimura, A.; Watanabe, S.; Kikuchi, K. *Angew. Chem., Int. Ed.* **2011**, *50*, 8750−8752.
(10) Dean, J. A. *Lange's Handbook of Chemistry*, 14th ed.; McGraw-Hill: New York, 1972.
(11) (a) Jones, R. N.; Wilson, H. W.; Novick, W. J. *J. Clin. Microbiol.* **1982**, *15*, 677−683. (b) Faraci, W. S.; Pratt, R. F. *J. Am. Chem. Soc.* **1984**, *75*, 1489−1490.
(12) Gao, W.; Xing, B.; Tsien, R. Y.; Rao, J. *J. Am. Chem. Soc.* **2003**, *125*, 11146−11147.
(13) Komatsu, T.; Johnsson, K.; Okuno, H.; Bito, H.; Inoue, T.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* **2011**, *133*, 6745−6751.
(14) Watanabe, S.; Mizukami, S.; Akimoto, Y.; Hori, Y.; Kikuchi, K. *Chem.—Eur. J.* **2011**, *17*, 8342−8349.
(15) Zlokarnik, G.; Negulescu, P. A.; Knapp, T. E.; Mere, L.; Burres, N.; Feng, L.; Whitney, M.; Roemer, K.; Tsien, R. Y. *Science* **1998**, *279*, 84−88.
(16) Takakusa, H.; Kikuchi, K.; Urano, Y.; Kojima, H.; Nagano, T. *Chem.—Eur. J.* **2003**, *9*, 1479−1485.
(17) Marras, S. A. E. *Mol. Biotechnol.* **2008**, *38*, 247−255.
(18) *HaloTag Technology: Focus on Imaging*, Technical Manual; Promega: U.S.A., 2009; Available online: http://www.promega.com/.
(19) Sun, X.; Zhang, A.; Baker, B.; Sun, L.; Howard, A.; Buswell, J.; Maurel, D.; Masharina, A.; Johnsson, K.; Noren, C. J.; Xu, M. Q.; Correa, I. R. *ChemBioChem.* **2011**, *12*, 2217−2226.