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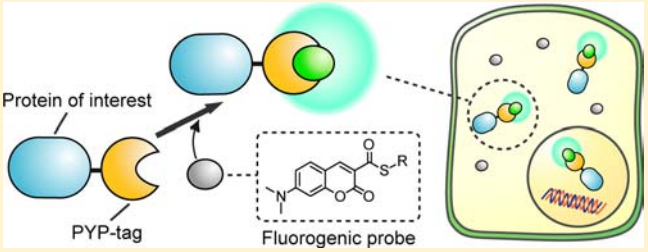
Development of Fluorogenic Probes for Quick No-Wash Live-Cell Imaging of Intracellular Proteins

Yuichiro Hori,[†,‡,§] Tomoya Norinobu,[†] Motoki Sato,[†] Kyohei Arita,[∥] Masahiro Shirakawa,[⊥] and Kazuya Kikuchi*[,†,‡]

[†]Graduate School of Engineering, and [‡]Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan
[§]JST, PRESTO, Suita, Osaka 565-0871, Japan
[∥]Graduate School of Medical Life Science, Yokohama City University, Yokohama, Kanagawa 230-0045, Japan
[⊥]Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan

Supporting Information

ABSTRACT: We developed novel fluorogenic probes for no-wash live-cell imaging of proteins fused to PYP-tag, which is a small protein tag recently reported by our group. Through the design of a new PYP-tag ligand, specific intracellular protein labeling with rapid kinetics and fluorogenic response was accomplished. The probes crossed the cell membrane, and cytosolic and nuclear localizations of PYP-tagged proteins without cell washing were visualized within a 6-min reaction time. The fluorogenic response was due to the environmental effect of fluorophore upon binding to PYP-tag. Furthermore, the PYP-tag-based method was applied to the imaging of methyl-CpG-binding domain localization. This rapid protein-labeling system combined with the small protein tag and designed fluorogenic probes offers a powerful method to study the localization, movement, and function of cellular proteins.



INTRODUCTION

Protein labeling by synthetic fluorescence probes is a powerful technique used to investigate protein function and localization in living cells.[1] Advances have been made in this field through the development of specific pairs of fluorescence probes and protein (peptide) tags, which allow live-cell imaging of proteins. In this technique, objective proteins are fused with a protein (peptide) tag and are labeled by a fluorescent probe through the binding of the probe and tag. An early report of this technique described the pairing of tetracystein tag and its probes (FlAsH/ReAsH).[2] Since then, protein-labeling systems using various tags such as SNAP-tag,[3] HaloTag,[4] TMP-tag,[5] LAP,[6] Oligo-Asp tag,[7] coiled-coil tag,[8] and BL-tag[9,10] have been developed. This method has attracted attention as an alternative to fluorescent proteins because it has the advantage of various bright fluorophores that can be incorporated into probes. In addition, the timing of the labeling can be easily controlled, allowing for precise spatiotemporal analyses of protein movement. On the other hand, unlabeled probes remaining inside cells have the potential to cause an increase in the background fluorescence and hinder the identification of labeled proteins. Therefore, rigorous washing of cells is required to remove free probes.

Recently, fluorogenic probes have been created as a solution to this problem.[2,10−16] These probes exhibit only low background fluorescence in a free state and, as a result, enable live-cell imaging of proteins with a high signal-to-noise ratio. Furthermore, because the removal of free fluorescence-quenched probes is not necessary, prompt imaging is possible. It is, however, still challenging to visualize intracellular proteins by fluorogenic probes without a wash-out process. Successful examples have been restricted to SNAP-tag,[11−13] BL-tag,[14] and fluorogen-activating proteins (FAPs).[15] However, most of these reports showed some limitations. One of them is that special treatment for probe introduction into cells was required.[12] Another limitation is that long incubation caused the nonspecific illumination of cellular components.[15] Moreover, in some of the reports, quick imaging of intracellular proteins was not shown and labeling reactions were conducted with long incubation time (2 h or more).[11,14] Only silicon−rhodamine (SiR) probes for SNAP-, CLIP-, and Halo-tags allowed protein imaging with moderate incubation time (30 min).[13] In this study, by adopting a fluorogenic mechanism based on an environmental-sensitive fluorophore, we created novel fluorogenic probes for specific rapid labeling of proteins inside living cells with no-wash procedures to overcome these limitations.

We have previously reported a protein-labeling system using Photoactive yellow protein (PYP) tag and its fluorogenic probes, FCTP and FCANB.[16,17] PYP-tag is a soluble protein derived from purple bacteria.[18] PYP-tag covalently binds to the thioester derivative of cinnamic acid or coumarin through

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transthioesterification with Cys69.[16,17,19] The attractive feature of this protein tag is its small size (125 amino acids). FCTP is nonfluorescent because of the intramolecular association between ligand and fluorophore moieties, and it becomes fluorescent through the dissociation of the dyes upon protein labeling. This probe requires more than 24 h for full PYP-tag labeling. Owing to the slow labeling kinetics, a wash-out process is necessary for protein imaging. FCANB was designed to improve the labeling kinetics and enable the imaging of cell surface proteins without a cell-washing procedure. Unfortunately, wash-free imaging of intracellular proteins has not been possible because the probe is not cell-permeable.

To solve this problem, we developed cell-permeable fluorogenic probes that label PYP-tagged proteins expressed inside living cells with a short incubation time. The probes contain a novel PYP-tag ligand with a fluorogenic mechanism, which is based on the environment sensitivity of a fluorophore. The fluorescence was enhanced by the capture of the probes into the low-polar pocket of PYP-tag. This mechanism allowed us to design quencher-free fluorogenic probes with a simple structure that can cross the cell membrane and rapidly label intracellular proteins. In addition, this system was applied to the imaging of localization of methyl-CpG-binding domain, MBD, which is known to bind DNA containing 5-methylcytosine.[20] Using this labeling method, DNA methylation in living cells was successfully visualized.

RESULTS AND DISCUSSION

Probe Design. For no-wash imaging of intracellular proteins, we explored a new PYP-tag ligand, which crosses the cell membrane and shows a fluorogenic response upon protein labeling. To this end, we focused on ligand structures, which were known to be accommodated by PYP-tag. According to previous reports, the protein binds to 4-dimethylaminocinnamic acid thioester as well as 4-hydroxycinnamic acid and 7-hydroxycoumarin thioesters.[21] Considering the structural similarity between cinnamic acid and coumarin structures, we hypothesized that 7-dimethylaminocoumarin thioester derivatives could bind to PYP-tag (Figure 1a). Importantly, 7-dialkylaminiocoumarin derivatives are environment-sensitive fluorophores, which are scarcely fluorescent in polar solvents but become fluorescent in low-polar solvents.[22] Therefore, it was thought that a PYP-tag probe with this structure would weakly fluoresce in aqueous buffer and emit strong fluorescence when the probe binds to PYP-tag and, thereby, is brought into protein interior, which is generally in a low-polar environment. Based on this idea, we designed novel probes, TMBDMA and CMBDMA, by incorporating trimethylamine or carboxylic acid into the coumarin thioester derivative to increase water solubility (Figure 1b). The ligand moiety, 7-dimethylaminocoumarin-3-carboxylic acid, was synthesized from *p*-dimethylaminosalicylaldehyde in three steps and was then coupled with 2-(4-mercaptobenzamido)-*N*,*N*,*N*-trimethylethanaminium or 4-(carboxymethyl)thiophenol to yield TMBDMA or CMBDMA, respectively (Schemes S1, S2).

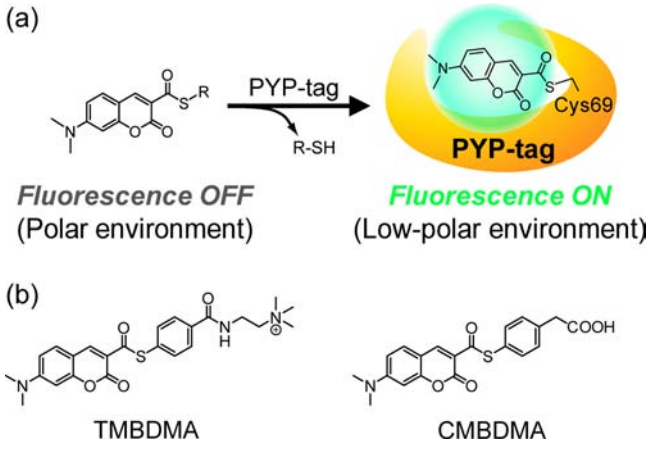

Figure 1. (a) Principle of labeling system based on PYP-tag and environment-sensitive fluorogenic probe. (b) Structures of new probes for labeling PYP-tag.

Labeling Reactions with Fluorogenic Response. First, in vitro experiments were conducted to examine whether the probes bind to PYP-tag. The labeling reactions of PYP-tag with TMBDMA or CMBDMA were analyzed by SDS-PAGE. In both cases, fluorescence was observed from the band of PYP-tag (Figure 2a, b). This result demonstrates that these probes

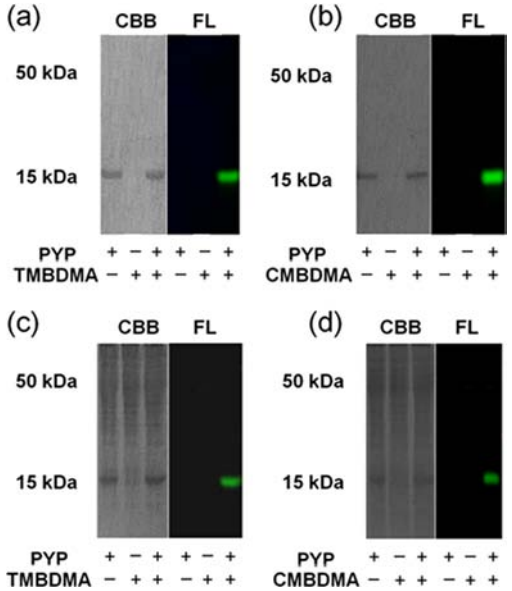

Figure 2. Labeling reactions of PYP-tag with TMBDMA and CMBDMA. PYP-tag (15 μM) was reacted with TMBDMA (25 μM) (a, c) or with CMBDMA (25 μM) (b, d). The reactions were carried out in the absence (a, b) or presence (c, d) of cell lysate. Images of CBB-stained and fluorescence gel are shown on the left and right, respectively.

bind to PYP-tag, and 7-dimethylaminocoumarin is accommodated as a ligand by PYP-tag. The binding mode was considered to be covalent because the reaction mixture was denatured by SDS and heat shock prior to analyses. To investigate the binding specificity, labeling reactions were carried out in cell lysate prepared from HEK293T. Although no fluorescence was detected in the gel when the reaction mixtures were incubated in cell lysate without PYP-tag, a clear single fluorescent band appeared in a gel lane when the cell lysate containing PYP-tag and each of the probes were loaded (Figure 2c, d). The band position was consistent with the molecular weight of PYP-tag. Because glutathione is the most abundant in living cells among thiol compounds, which may compete with the labeling reactions, the effect of glutathione was also examined. Even in the presence of physiological concentrations

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of glutathione (up to 10 mM),[23] the probes were found to label PYP-tag, although the fluorescence intensity of the labeled band was slightly reduced (Figure S1). These results indicate that the probes specifically bind to PYP-tag even in the presence of various biomolecules.

Next, spectral studies were conducted (Figure 3, Table 1, Figure S2). Fluorescence spectra were recorded to clarify the

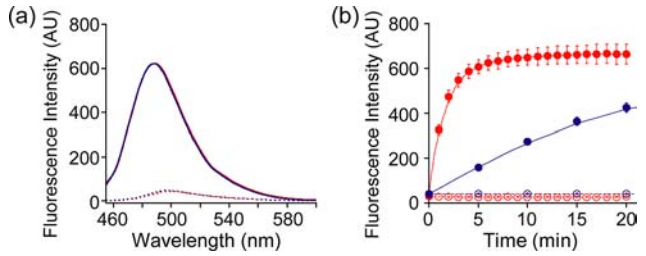

Figure 3. Fluorescence analyses for labeling reactions of PYP-tag with its probes. (a) Fluorescence spectra of TMBDMA (red lines) and CMBDMA (blue lines) in the absence (dotted lines) or presence (solid lines) of PYP-tag. (b) Time course of fluorescence intensity of TMBDMA (red circles) and CMBDMA (blue circles) at 487 nm in the absence (disks) or presence (circles) of PYP-tag. The measurements were conducted using samples in HEPES buffer (20 mM HEPES, 150 mM NaCl (pH 7.4)) at 37 °C. The protein and probe concentrations were 5 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})
TMBDMA	451	501	34600	0.02	3950
CMBDMA	449	499	36400	0.02	126
PYP-probe[b]	450	487	27800	0.38	—

[a]All data were obtained in triplicate experiments. [b]Spectroscopic data of PYP-tag-bound probe were obtained after the labeling reaction of PYP-tag with CMBDMA was completed.

fluorescence properties of the probes. Both probes exhibited only weak fluorescence in the absence of PYP-tag (TMBDMA, Φ_f = 0.02; CMBDMA, Φ_f = 0.02) (Table 1). The binding of the probes with PYP-tag induced remarkable fluorescence enhancement (Figure 3a). Upon protein labeling, the fluorescence intensity of both probes at 487 nm increased 22-fold and 16-fold, respectively. There was no substantial change of the fluorescence intensity of the probes in the absence of PYP-tag during the 3-h incubation period (Figure S3). These results demonstrate that both TMBDMA and CMBDMA are fluorogenic probes for labeling PYP-tag. In addition, this fluorescence response suggests that the dimethylaminocoumarin was captured into the low-polar binding pocket of PYP-tag.

Kinetic Analyses of Labeling Reactions. The kinetic experiments of the protein labeling were conducted by monitoring the fluorescence intensity of the probes. TMBDMA labeled 50% of PYP-tag for 1.1 min ($t_{1/2}$) under an experimental condition, in which the protein and probe concentrations were 5 μM (Figure 3b). On the other hand, CMBDMA required a longer reaction time ($t_{1/2}$ = 13 min) under the same experimental conditions (Figure 3b, Figure S4). For further detailed kinetic analyses, the second-order rate constant for reactions between PYP-tag and TMBDMA/CMBDMA was determined. TMBDMA showed a remarkable value (k_2 = 3950 M^{-1} s^{-1}) that was 32-fold higher than FCANB (k_2 = 125 M^{-1} s^{-1}).[17] By contrast, the value of CMBDMA (k_2 = 126 M^{-1} s^{-1}) was comparable to that of FCANB (Table 1, Figure S5).

Despite the similar probe structures of TMBDMA and CMBDMA, TMBDMA showed better kinetic feature than CMBDMA. There are two possible reasons for this difference. One is that electrostatic interaction occurs between PYP-tag and the probes. PYP-tag is anionic under the physiological conditions because the pI of the protein is 4.3.[24] This finding implies that the interaction of PYP-tag with cationic TMBDMA is preferable to anionic CMBDMA. The other probable reason is that TMBDMA contains a better leaving group than CMBDMA. When Cys69 in PYP-tag attacks the thioester of the probe, a thiophenyl compound is released from the probe. Its leaving ability correlates with the labeling kinetics and is dependent on the pK_a of the thiol. The leaving group of TMBDMA could have the thiol with a lower pK_a than CMBDMA because the leaving group of TMBDMA is composed of thiophenol directly connected to an electron-withdrawing carbonyl group, whereas the corresponding thiophenol in CMBDMA is linked with a methylene group. Accordingly, it is suggested that lowering of the pK_a results in increased kinetic constant of TMBDMA. The effect of charge and pK_a on labeling kinetics can also explain why TMBDMA shows faster kinetics than FCANB, because FCANB is anionic and contains a thiophenol leaving group linked with a methylene group.

Live-Cell Imaging of PYP-Tagged Proteins on Cell Surface. No-wash live-cell imaging of cell surface proteins on cell membrane was carried out using TMBDMA and CMBDMA. For the labeling of cell surface proteins, a PYP-tag-encoding construct was prepared by fusing PYP-tag to the epidermal growth factor receptor at the N-terminal extracellular domain (PYP-EGFR). The protein expression on plasma membrane of HEK293T cells was ascertained by immunofluorescence staining as described in our previous report.[17] The probes were incubated with the cells for 30 min, and then fluorescence images were taken with a confocal laser scanning microscope without washing the cells. Fluorescence was observed along the plasma membrane of the cells expressing PYP-EGFR (Figure 4a). In contrast, nontransfected cells in the image were not stained, and fluorescence was hardly detected in the culture media. Additionally, nonspecific labeling was not detected from cells expressing EGFR without PYP-tag. Similar results were obtained with CMBDMA (Figure S6). When cells were washed after the labeling reactions, the images displayed

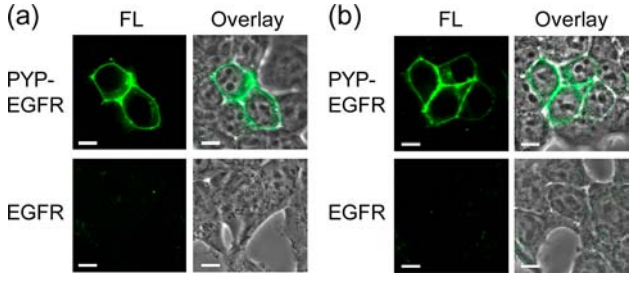

Figure 4. Live-cell imaging of PYP-EGFR by utilizing TMBDMA (1 μM) without (a) or with (b) a wash-out step. Images of PYP-EGFR- and EGFR-expressing HEK293T cells are shown in upper and lower panels, respectively. Fluorescence images and their overlays on phase contrast images are displayed in the left and right of each panel, respectively. The images were obtained with the excitation at 473 nm by using a 490−590 emission filter. Scale bar: 10 μm.

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fluorescence localization, which were essentially similar to the images acquired by the no-wash protocol (Figure 4b). These results indicate that the probes specifically imaged PYP-tagged proteins on the cell surface without washing.

Quick Imaging of Intracellular Proteins without Wash-Out Process. We investigated whether the probes pass through the cell membrane and label intracellular proteins without washing cells. Genes encoding PYP-tag fused to maltose-binding protein (MBP-PYP), and nuclear localization signals (PYP-NLS) were created and expressed in HEK293T cells. From the immunofluorescence images, MBP-PYP was expressed mainly in the cytosol (Figure S7a), and PYP-NLS was localized to the nuclei, as expected (Figure S7b). Live-cell images were obtained with a no-wash protocol immediately after TMBDMA was incubated with cells expressing MBP-PYP for 30 min. As a result, fluorescence was detected mostly in the cytosol of the cells (Figure 5a). This localization pattern

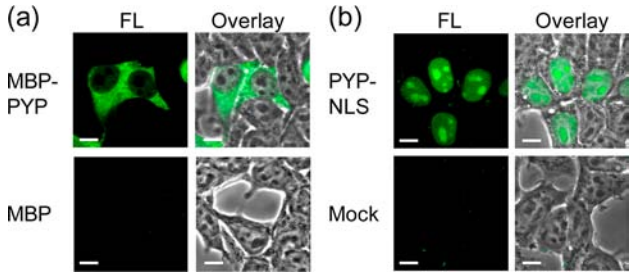

Figure 5. No-wash live-cell imaging of intracellular proteins with TMBDMA. PYP-tagged MBP or NLS-fused PYP-tag was localized to cytosol (a) or nuclei (b) of HEK293T cells and was labeled with TMBDMA (1 μM). Fluorescence images and their overlays on phase contrast images are displayed in the left and right of each panel, respectively. The images were obtained with the excitation at 473 nm by using a 490−590 emission filter. Scale bar: 10 μm.

coincided with the results of immunofluorescence staining. No cell expressing MBP without PYP-tag exhibited fluorescence. Specific labeling of PYP-NLS by TMBDMA was also observed (Figure 5b). Co-staining of the cells with Hoechst 33342 and TMBDMA confirmed that the probe visualized the nuclear localization of PYP-NLS (Figure S8). CMBDMA also imaged the specific localization of MBP-PYP and PYP-NLS, as with the images obtained with TMBDMA (Figure S9). This is surprising for us because an anionic probe does not efficiently permeate the cell membrane in general. All of the no-wash imaging data were indistinguishable from those obtained in labeling experiments with a washing step (Figure S10). These results clearly show that both of the probes are cell-permeable and specifically label intracellular PYP-tag fusion protein in targeted subcellular regions without the requirement of washing.

We performed time-lapse imaging experiments to determine the incubation time necessary for fluorescence detection of PYP-tag inside cells (Figure 6, Figure S11). After addition of TMBDMA to cells expressing PYP-NLS, fluorescence images were periodically recorded. Fluorescence started to appear in nuclei just 2 min after addition of the probe. The labeling reaction was almost completed within 6 min. In the case of CMBDMA, the fluorescence intensity was saturated 30 min after the addition of the probe. Consistent with in vitro kinetic analyses, it took longer to finish the labeling reaction in comparison with TMBDMA. Nevertheless, the incubation time of CMBDMA for labeling intracellular proteins was comparable

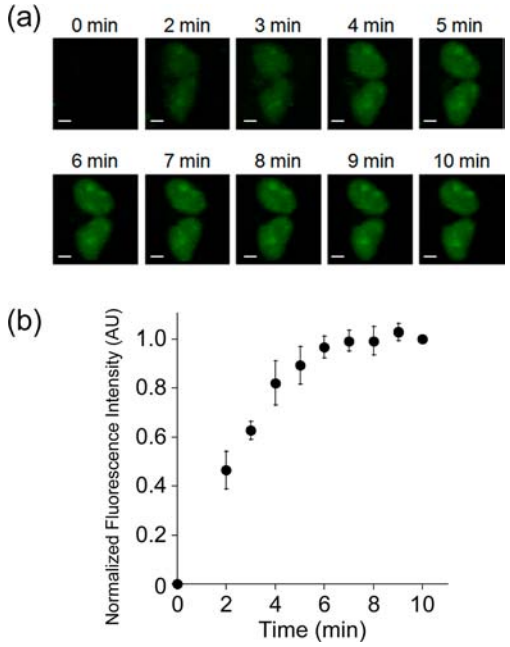

Figure 6. Time-lapse live-cell imaging of PYP-NLS with TMBDMA. (a) Measurement was made every 1 min after the addition of the probe (1 μM). PYP-NLS was expressed in HEK293T cells. The images were obtained with the excitation at 473 nm by using a 490−590 emission filter. (b) Plots of average fluorescence intensity of TMBDMA against incubation time (N = 3). Scale bar: 5 μm.

to that of previously reported fluorogenic protein-labeling probes, SiR derivatives.[13]

Imaging Analyses of DNA Methylation in Nuclei. DNA methylation is an important epigenetic reaction, which controls gene expression.[25] Methylation occurs in the 5-position of cytosine and is typically observed in CpG sequences. DNA sequences rich in 5-methylcytosine (5-mC) generally form heterochromatin, in which gene expression is suppressed. Methyl-CpG-binding domain 1 (MBD1) specifically binds to 5-mC[20] and is involved with heterochromatin formation. In mammals, this epigenetic modification plays a significant role in the homeostasis of somatic cells, the differentiation of embryonic and induced-pluripotent stem cells (ES and iPS cells), and carcinogenesis.[25] Hence, the live-cell data of the DNA methylation state offers valuable epigenetic information. We applied the probes to the imaging of 5-mC by labeling the fusion protein of PYP-tag and MBD1 (1-112) (PYP-MBD). After NIH3T3 cells were transfected with the gene of PYP-MBD, incubation with TMBDMA or CMBDMA was carried out for 10 or 15 min, respectively. Fluorescence signals were clearly detected as characteristic puncta in the nuclei (Figure 7a). The localization of the fluorescence overlapped with Hoechst33342. Since Hoechst33342 mainly stains heterochromatin,[26] which is rich in 5-mC, it is suggested that PYP-MBD was predominantly localized to heterochromatin in these cells. This result is consistent with a previous report.[27]

DNA methylation inhibitor, 5-aza-2-deoxycytidine (5-azadC), was used to examine whether the localization pattern of the fusion protein was altered. This inhibitor is mis-incorporated into DNA instead of dCTP, and it inactivates DNA methyltransferases by trapping the enzyme.[28] Thus, the incubation of the inhibitor during cell division leads to the suppression of DNA methylation. In the presence of 5-azadC, fluorescence derived from the complex between the probes and

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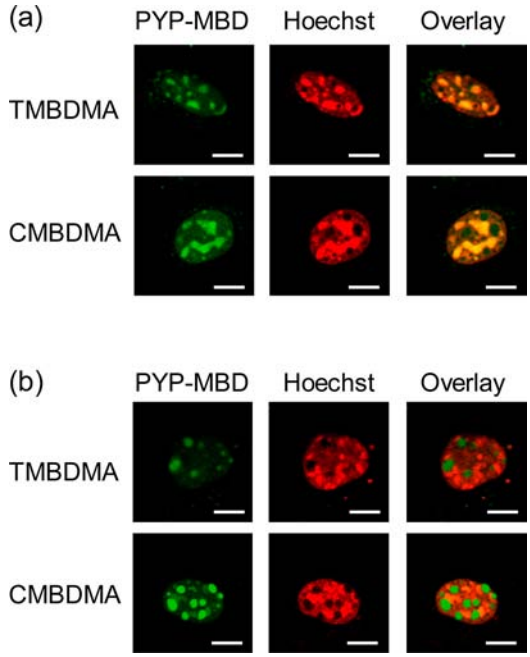

Figure 7. Live-cell imaging of localization of PYP-MBD in the absence (a) or presence (b) of 5-azadeoxycytidine. PYP-MBD was expressed in NIH3T3 cells. Images of cells treated with TMBDMA and CMBDMA are shown in upper and lower panels, respectively. Fluorescence derived from PYP-MBD and Hoechst33342 is displayed as green and red colors, respectively. In their overlay images, orange colors indicate the overlap of the localizations of PYP-MBD and Hoechst33342. The images were obtained with the excitation at 473 nm by using a 490–590 emission filter. Scale bar: 10 μm.

PYP-MBD was detected in some spots, which were not stained with Hoechst33342 (Figure 7b). These results clearly indicate that PYP-MBD was bound to 5-mC-containing DNA in the absence of 5-azadC and was released from the DNA by the addition of the inhibitor. In other words, this observation indicates that DNA methylation was imaged by monitoring the localization of PYP-MBD. Importantly, the probe precisely visualized PYP-MBD in living cells. The precision of the imaging analyses was accomplished by the simple probe design that solved the problem of cell-permeability, labeling specificity, labeling kinetics, and fluorogenic property for protein-labeling probes.

CONCLUSION

We created novel PYP-tag-probes with a fluorogenic ligand, 7-dimethylaminocoumarin. The labeling rate of TMBDMA dramatically accelerated in comparison with a previous probe, FCANB. Furthermore, no-wash labeling of intracellular proteins as well as cell surface proteins was successfully achieved, and the localization of the proteins was specifically visualized. Importantly, clear live-cell images were acquired in a short incubation time (<6 min). Moreover, the probes were utilized for the imaging of MBD localization, and DNA methylation was visualized by combining the labeling method with inhibitor analyses. As far as we know, TMBDMA is the most rapid imaging probe for intracellular proteins among reported fluorogenic probes that covalently label protein tags inside living cells.

The pair of PYP-tag and the fluorogenic probes contains the advantage of a small tag size, rapid in-cell-labeling kinetics, and high signal-to-noise ratio. This protein-labeling system offers a practical method for the analyses of protein expression and localization, and it will be an attractive tool for verifying versatile biological events, including epigenetic phenomena.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and supplemental results. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

kkikuchi@mls.eng.osaka-u.ac.jp

Notes

The authors declare no competing financial interest.

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