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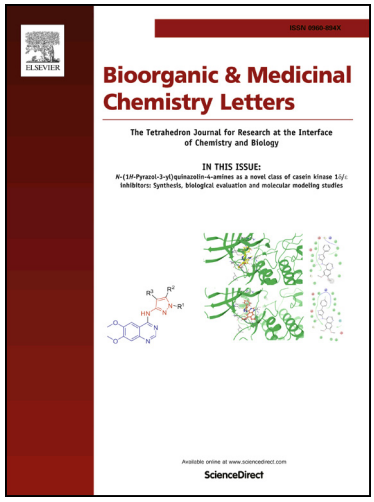

Development of an ON/OFF switchable fluorescent probe targeting His tag fused proteins in living cells

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Development of an ON/OFF switchable fluorescent probe targeting His tag fused proteins in living cells

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Keywords: cell-penetrating peptide; maleimide; ON/OFF switch; His tag fused protein

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Abstract

The fluorescent labeling of target proteins is useful for analyzing their functions and localization in cells, and several fluorescent probes have been developed. However, the fusion of tags such as green fluorescent protein (GFP) to target proteins occasionally affects their functions and/or localization in living cells. Therefore, an imaging method that uses short peptide tags such as hexa-histidine (the His tag) has been attracting

increasing attention. Few studies have investigated ON/OFF switchable fluorescent probes for intracellular His-tagged proteins. We herein developed a novel ON/OFF switchable probe for imaging targeted intracellular proteins fused with a CH6 tag, which is composed of one cysteine residue and six histidine residues.

The fluorescent labeling of target proteins is useful for analyzing their functions and localization in cells, and a number of fluorescent probes have been developed to date. The green fluorescent protein (GFP) is a representative fluorescent probe and the genetic fusion of GFP to target proteins is broadly used to visualize the behaviors of these proteins in cells.[1,2] However, the fusion of GFP to a target protein of interest (POI) sometimes affects its function and/or localization due to the large molecular size of GFP.[3] In order to overcome these issues, several fluorescent labeling methods using short peptide tags such as the FlAsH[4], ReAsH[5] tags, and tetraserine motifs[6] have recently been reported. The hexa-histidine tag (His tag) is a well-known short peptide tag that interacts with metal ions such as copper (Cu), nickel (Ni), and cobalt (Co) through non-covalent bonds. Therefore, the His tag represents a promising approach to POI

labeling because its molecular size has minimal effects[7], and it is often utilized in the affinity purification of genetically modified proteins. Several Ni (II)-NTA (nickel-nitrilotriacetic acid)-based probes that target His-tagged proteins have been reported to date.[8-10] However, Ni (II)-NTA-based probes for His-tagged proteins are limited to the labeling of membrane proteins because Ni (II)-NTA itself is impermeable to the cell membrane. Recent studies reported that a Ni (II)-NTA-based fluorescent probe bearing a chloroacetamide moiety targeted the CH6 tag, which comprises one cysteine residue and the His tag, through a covalent bond between a cysteine residue on the CH6 tag and the chloroacetamide group on the fluorescent probe, thereby increasing labeling efficiency.[11,12]

The ON/OFF switching of fluorescence is also important for improving its sensitivity. An ON/Off switchable probe, which only emits fluorescence when it interacts with POI, efficiently enables observations of the behavior of POI in living cells without the washing of excess amounts of the fluorescent probe.[13-15] Therefore, ON/OFF switchable probes have been attracting increasing attention as an effective imaging tool.

We herein developed an ON/OFF switchable imaging method that targets intracellular

CH6-tagged POI in living cells. We specifically designed and synthesized fluorescent probe **1** and the carrier peptide **R9-H4A2**, as shown in Figure 1. 7-(Diethylamino) coumarin as a fluorophore was conjugated with three Ni (II)-NTA moieties to strengthen binding affinity to the His tag[16], and with a maleimide moiety not only to form a covalent bond with the cysteine residue of the CH6 tag, but also to quench fluorescence by photoinduced electron transfer (PeT).[17] We also synthesized the carrier peptide **R9-H4A2**, consisting of the representative cell-penetrating peptide nona-arginine (R9) and **H4A2** (Sequence: HAHAHH, H: Histidine, A: Alanine) fragment. The **H4A2** moiety interacts with probe **1** through the Ni-NTA moiety and delivers hydrophilic fluorescent probe **1** into cells by the R9 fragment. Due to the substitution of two histidines with two alanines, the binding affinity of the **H4A2** moiety is weaker than that of the His tag to POI. Therefore, we expected probe **1** to dissociate from the carrier peptide and target the CH6 tag in cells.

In the fairly recent past, we have developed the small molecules that mediated degradation of His-tag fused protein using the same strategy.[18] In this report, MV-1, which induces the ubiquitination of target proteins, was conjugated with three Ni

(II)-NTA moieties. The hybrid compound was internalized by the supports of carrier peptide and formed the covalent bond with cysteine residue of CH6 tagged target proteins, subsequently the selective degradation of target proteins occurred. These results indicated that the probe **1** is capable of selectively reacting with CH6 tagged proteins and visualize the target proteins in living cells.

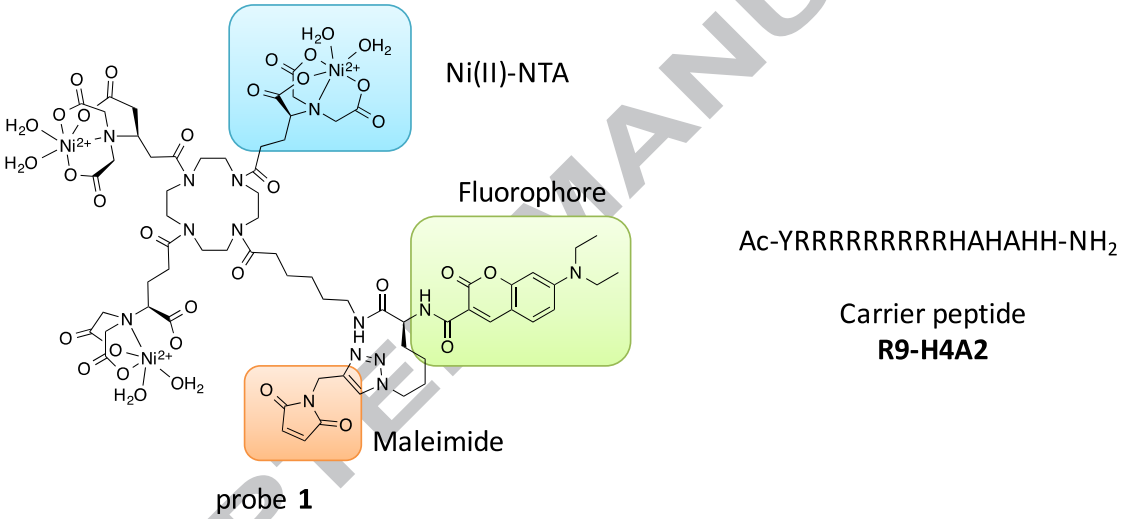


Figure 1. Chemical structure of fluorescent probe **1** and the sequence of the carrier peptide **R9-H4A2**.

The mechanism proposed for the ON/OFF switchable imaging method of CH6-tagged POI is shown in Figure 2. Fluorescent probe **1** and the carrier peptide **R9-H4A2** initially form a complex through interactions between the Ni (II)-NTA and **H4A2**

moieties. The complex then penetrates the cell membrane through the effects of R9. Fluorescent probe **1** dissociates from the complex and interacts with CH6-tagged POI. The cysteine residue of the CH6 tag is physically close to the maleimide moiety of **1** and forms a covalent bond. The fluorescence of probe **1** is recovered by the loss of PeT effects and enables the visualization of CH6-tagged POI (Figure 2).

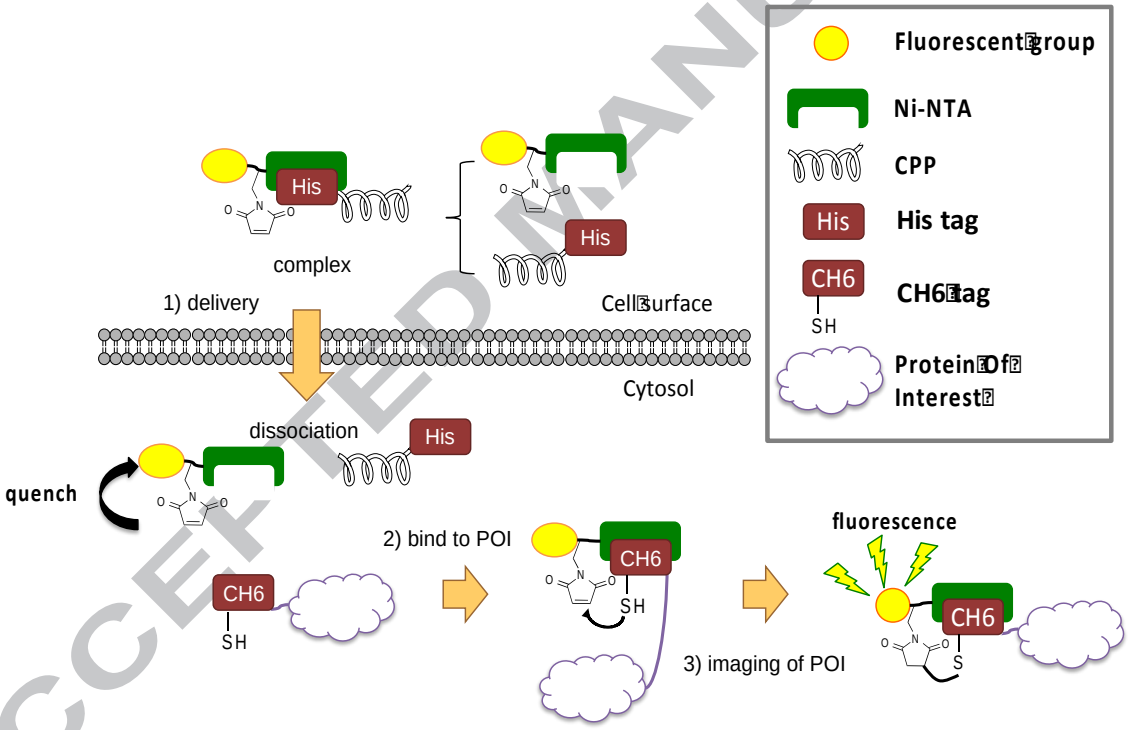
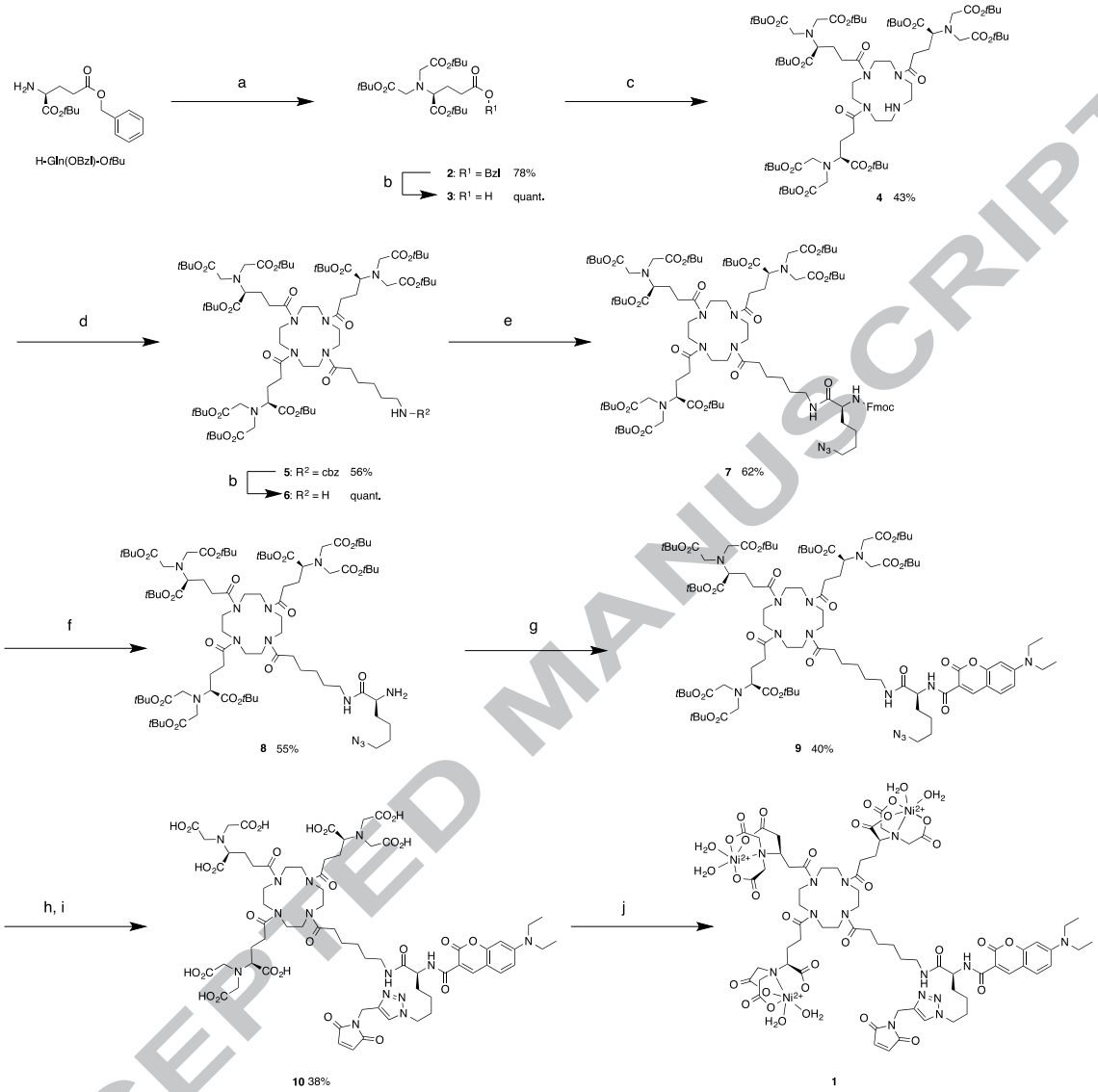


Figure 2. Proposed mechanism for the ON/OFF switchable imaging method.

The synthetic route of fluorescent probe **1** was shown in Scheme 1. H-Glu(OBzl)-O*t*Bu was treated with *tert*-butyl 2-bromoacetate to form a NTA moiety and the benzyl group

was removed using Pd/C under a H_2 atmosphere to give compound **3**. Compound **4** was synthesized by the condensation of 3 eq. compound **3** and 1,4,7,10-tetraazacyclododecane. The resulting amino group of **4** was condensed with 6-(carbobenzoxyamino)hexanoic acid [Cbz-Acp(6)-OH]] and the subsequent deprotection of the Cbz group to give compound **6**. Fmoc-azidolysine was coupled with **6** and the Fmoc group was subsequently removed by diethylamine to afford compound **8**. Compound **8** was coupled with 7-(diethylamino)coumarin, the maleimide moiety was attached by 1,3-dipolar cycloaddition, and all *t*ert-butyl ester groups were then removed under acidic conditions to afford target compound **10**.[19] The solution of compound **10** was added to 3 eq. $NiCl_2$ in PBS solution to form the Ni ion chelate **1**, and the complex was then used in assays. The carrier peptide **R9-H4A2** was synthesized using the microwave-assisted solid phase method. [18]



Scheme 1. (a) *tert*-Butyl 2-bromoacetate, DIPEA, DMF, 55°C, 16 h; (b) Pd/C, H_2, MeOH, rt. 6 h; (c) 1,4,7,10-Tetraazacyclododecane, EDC, HOBT, DIPEA, DCM, rt., 12 h; (d) Cbz-Acp(6)-OH, HATU, HOAT, DIPEA, DCM, rt., 1 d; (e) Fmoc-azidolysine, EDC, HOBT, DIPEA, DCM, rt., 20 h; (f) Diethylamine, THF, rt., 6 h; (g) 7-(Diethylamino)coumarin-3-carboxylic acid, EDC, HOBT, DIPEA, DCM, rt., 20 h; (h)

N-Propargylmaleimide, $CuSO_4$, sodium ascorbate, rt., 20 h; (i) TFA, DCM, rt., 4 h; (j) $NiCl_2$, PBS, rt., 2 h.

We initially investigated whether the fluorescence intensity of probe **1** is switchable *in vitro* using fluorescence spectra. Probe **1** showed an absorption maxima at 435 nm and emission at 475 nm. Furthermore, the addition of 6 μM glutathione (GSH) increased fluorescence intensity, indicating that the maleimide moiety quenches the fluorescence intensity of probe **1**, and the cysteine residue of GSH reacts with the maleimide moiety, resulting in the regulation of the ON/OFF switch (Figure 3).

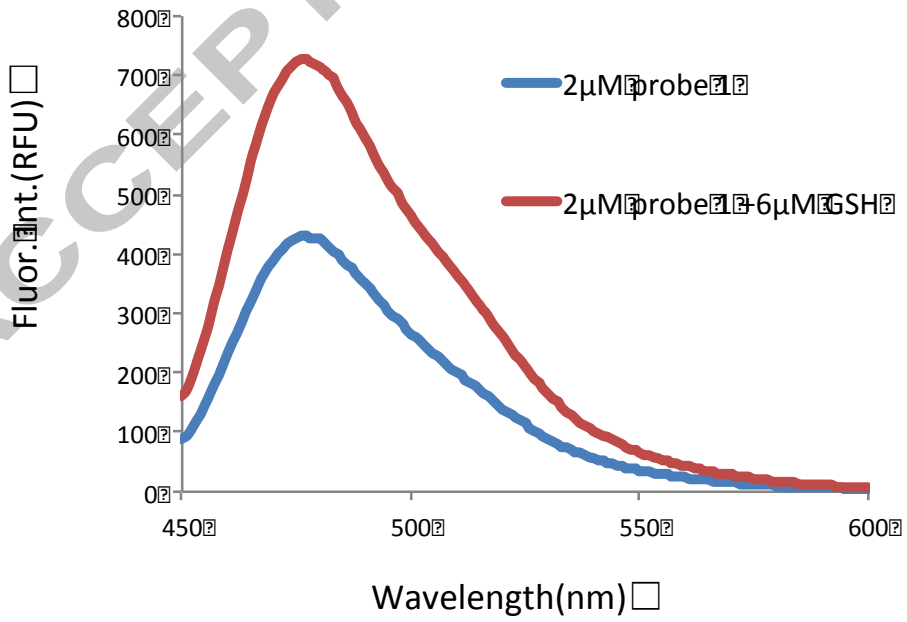
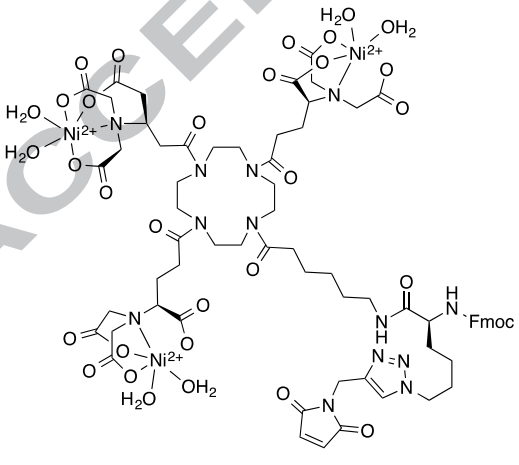


Figure 3. Fluorescence intensity of 2 μM of probe **1** with or without 6 μM of GSH.

Fluorescent probe **1** is possible to react with thiol groups of other proteins in the cells. Therefore, we evaluated that the selectivity of probe derivatives **11** to CH6 tagged POI over 5 mM GSH. We monitored the reaction between compound **11** and CH6 peptide in the coexistence of 5 mM GSH.[20] As shown in Figure 4, compound **11** and CH6 peptide were detected in 6 min, 20.5 min respectively. Compound **11** reacted with CH6 peptide with or without 5 mM GSH and the resulting product was detected (lanes 2, 3; retention time = 18.3 min). On the other hand, the mixture of compound **11** and 5 mM GSH gave another peak in 17.8 min (Lane 4). These results suggest that the fluorescent probe **1** could preferentially react with CH6 tagged POI over a thiol group of other proteins.



11

Ac-YCHHHHHH-NH_2

CH6 peptide

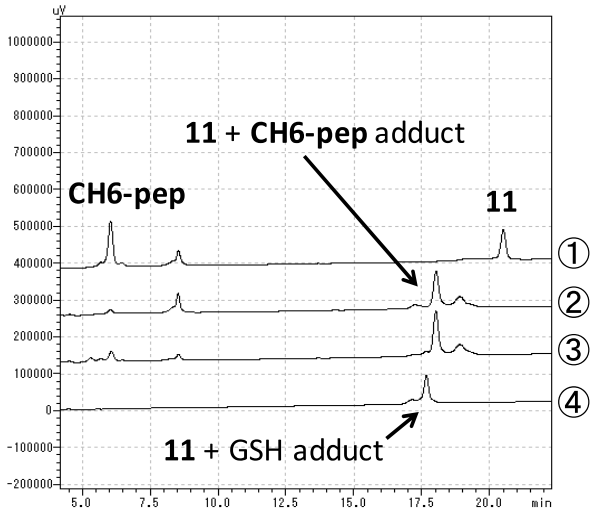


Figure 4. The reaction monitoring between compound **11** and CH6 peptide in coexistence of 5 mM GSH by HPLC. Condition 1: CH6 pep and compound **11** (reaction time = 0, Lane 1), Condition 2: CH6 pep and compound **11** (Lane 2), Condition 3: CH6 peptide, compound **11**, 5mM GSH (Lane 3), Condition 4: Compound **11** and 5 mM GSH (Lane 4).

We then examined the effects of probe **1** on fluorescence intensity against CH6-tagged POI-expressing HT1080 cells. HT1080 cells were transfected with and stably expressed CH6-tagged CRABP2 2×(CH6)-CRABP2), which was prepared as previously reported[18]. HT1080 cells constitutively expressing 2×(CH6) -CRABP2 were treated with the solution of 20 μM of probe **1** and 20 μM of **R9-H4A2** at 37 °C for 2.5 h.[21] Fluorescence intensity was measured and quantified using flow cytometry. As shown in Figure 5, fluorescence intensity against HT1080 cells stably expressing 2×(CH6)-CRABP2 was 2-fold higher than that against HT1080 cells. Moreover, fluorescence intensity without **R9-H4A2** was markedly decreased, indicating that **R9-H4A2** played a pivotal role in the delivery of probe **1** into cells.

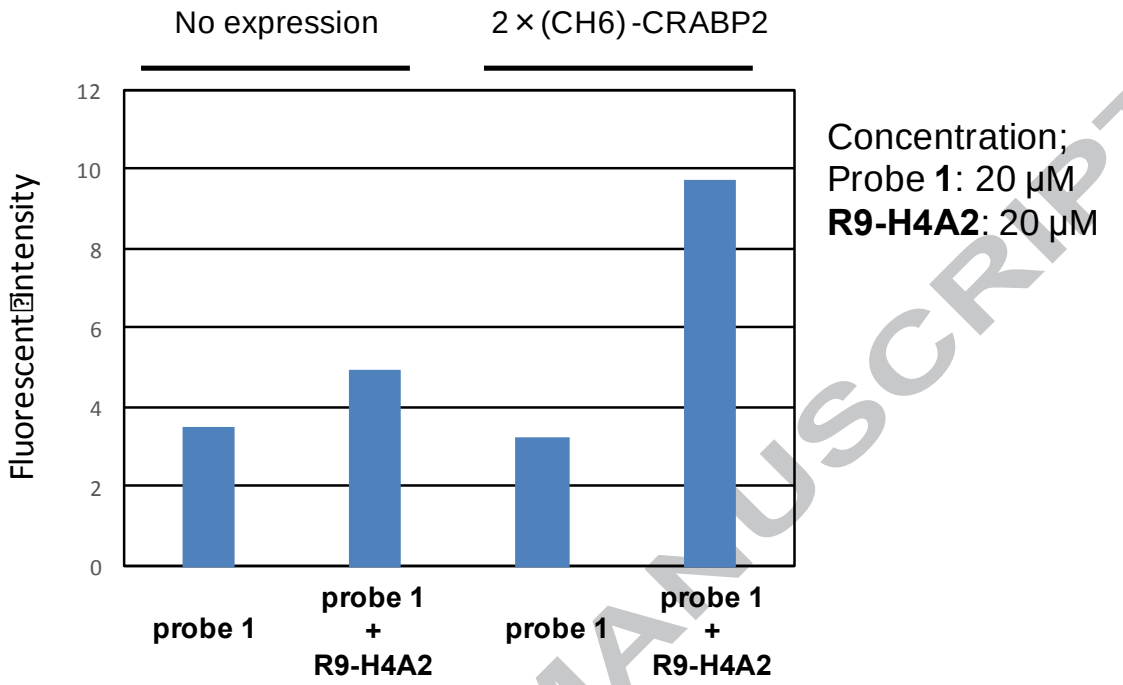


Figure 5. Effects of probe **1** and **R9-H4A2** against HT1080 cells and HT1080 cells stably expressing 2×(CH6)-CRABP2.

We then investigated whether CH6-tagged POI is observed using our method without washing. HT1080 cells stably expressing 2×(CH6)-CRABP2 were treated with the solution of 20 μM of probe **1** and 20 μM of **R9-H4A2** at 37 °C for 2.5 h and fluorescence was observed using confocal laser scanning microscopy.[22] An increase in fluorescence was detected in living cells without washing. The increase observed in fluorescence against HT1080 cells stably expressing 2×(CH6)-CRABP2 was greater

than that against HT1080 cells (Figure 6).

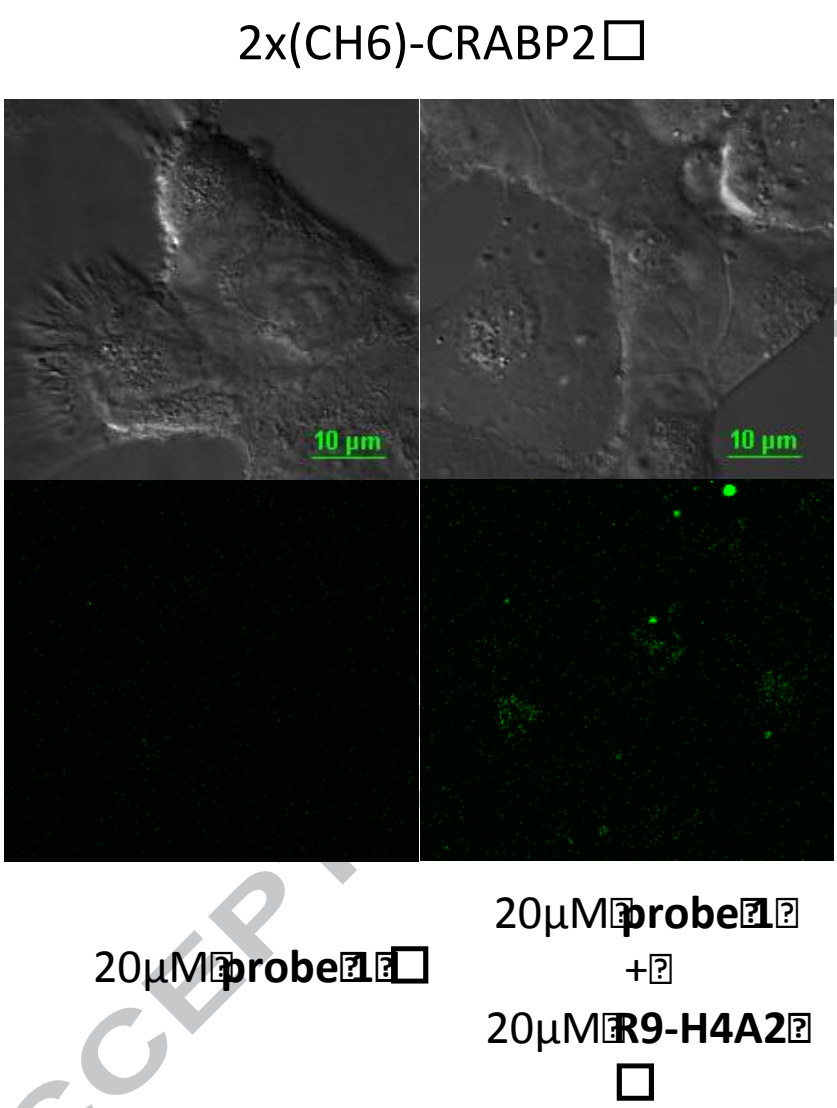


Figure 6. Confocal laser scanning microscopy analyses. Fluorescent (upper panels) and phase contrast (lower panels) images are shown. Scale bars represent 10 μm.

We performed a cytotoxicity assay using cell counting kit-8 according to the

manufacturer's instructions.[23] The results of the cytotoxicity assay revealed that fluorescent probe **1** and **R9-H4A2** did not exhibit marked cytotoxicity against HT1080 cells at 30 μM. These results suggest that our imaging method is broadly applicable to CH6-tagged POI (Figure 7). Ni ions are generally cytotoxic; however, the Ni ion of probe **1** was chelated by the NTA moiety and, thus, was not cytotoxic.

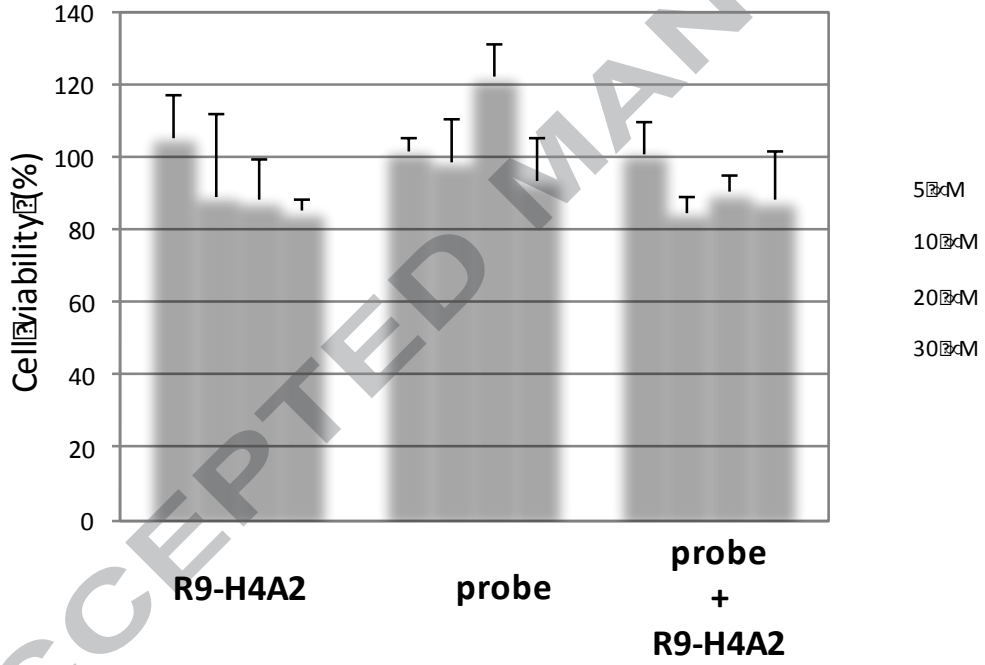


Figure 7. Cytotoxicities of probe **1** and **R9-H4A2**.

In summary, we herein developed an ON/OFF switchable fluorescent probe for CH6-tagged POI. The fluorescence intensity of probe **1** was increased by the presence

of cysteine residues *in vitro* and in living cells, and was detected using confocal laser scanning microscopy without washing. Furthermore, synthesized compounds did not exhibit significant cytotoxicity. These results demonstrate that our method is applicable to the imaging of several CH6-tagged POI. Further biological analyses are currently underway.

Acknowledgments

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[19] Synthesis of precursor **10**: Compound **8** (50 mg, 26 μmol) was dissolved in *t*-BuOH (4 mL) and H_2O (2 mL) followed by the addition of propargylmaleimide (35 mg, 0.26 mmol), copper (II) sulfate (6.4 mg, 40 μmol), and sodium ascorbate (7.9 mg, 40 μmol). After continuous stirring at room temperature for 20 h, the mixture was concentrated *in vacuo* to give a crude compound, which was used in the next reaction without further purification.
The above compound was dissolved in DCM (2 mL), followed by the addition of trifluoroacetic acid (5 mL). The mixture was stirred at room temperature for 4 h. The solvent was then removed, and the residue was purified by reversed-phase HPLC using a Discovery® Bio Wide Pore C18 column (25 cm x 21.2 mm). After being purified, the solution was lyophilized to give 15 mg (38%) of compound **10** as a yellow solid. Compound purity was assessed using analytical HPLC with a Discovery® Bio Wide Pore C18 column (25 cm x 4.6 mm; Retention time = 19.5 min, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate 1.0 mL/min, gradient: 10-60% gradient of solvent B over 30 min). MS [HR-ESI(+)]: m/z calcd. $[M+2H]^{2+}$: 777.3176; found: 777.3199.

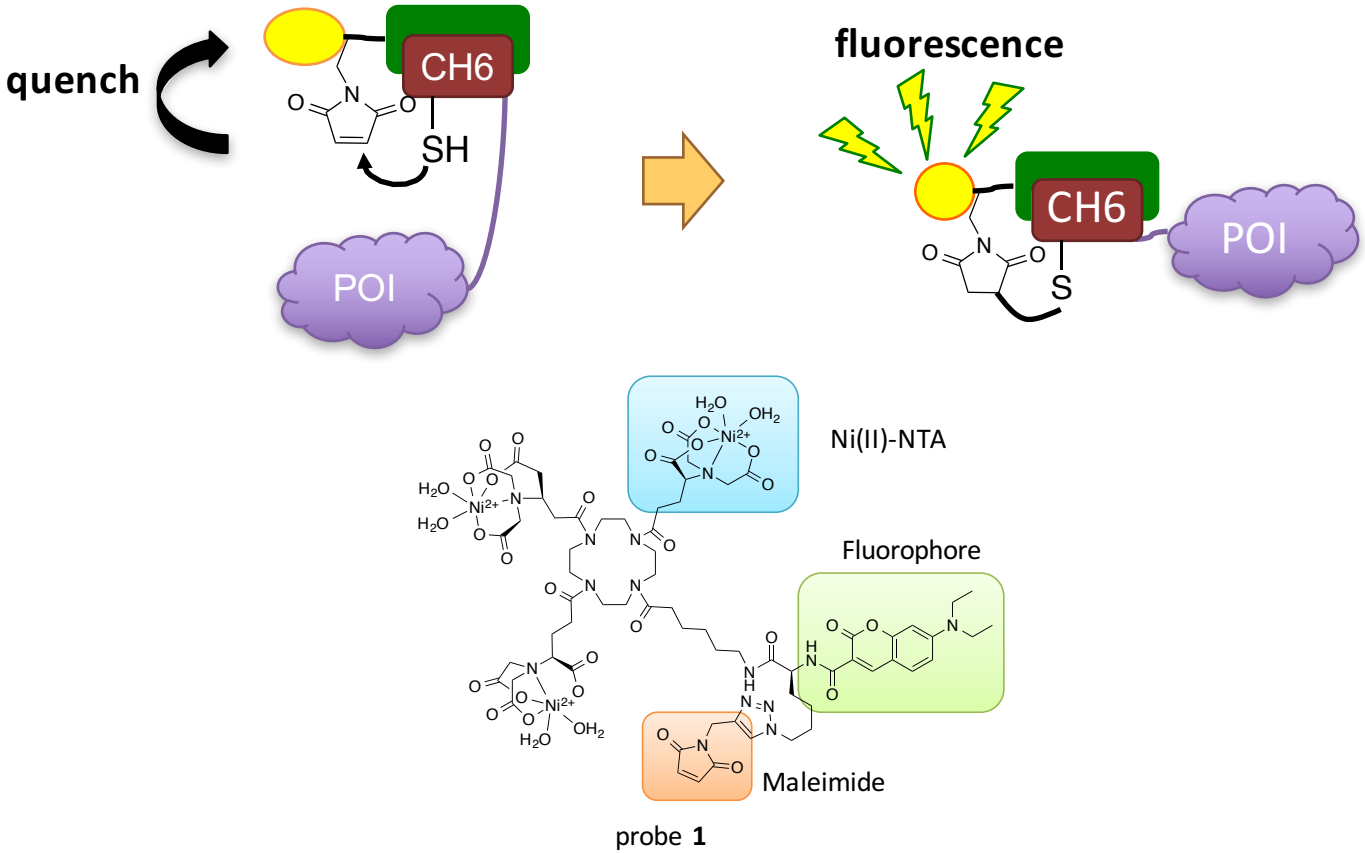
[20] Reaction monitoring between compound **11** and CH6 peptide with or without 5 mM GSH was performed using analytical HPLC with a Discovery® Bio Wide Pore C18 column (25 cm x 4.6 mm; Retention time = 19.5 min, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate 1.0 mL/min, gradient: 10-60% gradient of solvent B over 30 min).

[21] Flow cytometry: HT1080 cells were seeded on 6-well dishes at a density of 2.0 x 10^5 cells/well and cultured in DMEM for 24 h. Cells were treated with compound solution and incubated for 2.5 h. Cells were then washed with phosphate buffer (PBS) and detached by a treatment with trypsin-EDTA. The cells collected were pelleted by centrifugation at 3000 rpm for 5 min and the supernatant was removed. The cells collected were suspended in 500 μL of PBS and the mean fluorescence intensity of cells was measured using flow cytometry.

[22] Confocal laser microscopy: HT1080 cells were seeded onto glass-bottomed dishes (Greiner

Bio-one, Tokyo, Japan) (10,000 cells/well) and incubated overnight in 2 mL of DMEM containing 10% FBS. Medium was replaced with fresh medium containing 10% FBS, and compound solution was applied to the wells. After cells had been incubated for 3 h, they were observed using LSM 710 (Carl Zeiss, Oberkochen, Germany) equipped with a 63X objective lens (Plan-Apochromat, Carl Zeiss) at an excitation wavelength of 488 nm (Ar laser) for the compound.

[23] Cytotoxicity; HT1080 cells were seeded on 96-well culture plates (5,000 cells/well) and incubated for 24 h in DMEM containing 10% FBS. Compound solution in fresh DMEM was added at each concentration. After 24 h, cell viability was evaluated using cell counting kit-8 (DOJINDO) following the manufacturer's protocol. Results are presented as the mean and standard error of 3 samples. The significance of differences was analyzed by the Student's *t*-test.


quench
CH6
SH
POI
fluorescence
CH6
POI
S
Ni(II)-NTA
Fluorophore
Maleimide

probe **1**