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Fluorescent "Turn-on" system utilizing a quencher-conjugated peptide for specific protein labeling of living cells

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ABSTRACT

A specific protein fluorescent labeling method has been used as a tool for bio-imaging in living cells. We developed a novel system of switching "fluorescent turn on" by the recognition of a fluorescent probe to a hexahistidine-tagged (His-tag) protein. The tetramethyl rhodamine bearing three nitrilotriacetic acids, which was used as a fluorescent probe to target a His-tagged protein, formed a reversible complex with the quencher, (Dabcyl)-conjugated oligohistidines, in the homogeneous solution, causing fluorescence of the fluorophore to be quenched. The complex when applied to living cells (COS-7) expressing His-tagged proteins on the cell surface caused the quencher-conjugated oligohistidines to be dissociated from the complex by specific binding of the fluorescent probe to the tagged protein, resulting in the fluorescent emission. The complex that did not participate in the binding event remained in the quenched state to maintain a low level of background fluorescence.

1. Introduction

The fluorescent probe has become the leading candidate as a tool for tracking a target protein in living cells because of its inherent sensitivity and temporal–spatial resolution [1–3]. The method using gene construction of a fused fluorescent protein such as green fluorescent protein (GFP) variants for fluorescent labeling of a target protein has become a powerful tool for biologists [4–6]. However, use of this method is restricted when the large molecular weight of a fused protein interferes with the folding and trafficking of the target protein [7]. A small fluorescent compound could therefore be expected to minimize the interference of protein dynamics and resolve this restriction [8,9]. Some studies have recently been reported to satisfy this requirement [10–12]. A general strategy has described that a short peptide tag was introduced to a protein, and a fluorophore was then designed to specifically recognize the peptide tag. In particular, a fluorescent probe for labeling a hexahistidine-tagged (His-tag) protein has attracted researchers in various fields, because the His-tag protein has been widely applied as the tag for affinity purification of a recombinant protein and for localization studies on molecules [13–16]. However, the challenge of providing a general small chemical probe remained, so that fluorescent molecules not participating in specific binding to a target protein do not need to be removed by washing with a buffer [17]. A functional fluorescent probe exhibiting enhanced fluorescence in response to His-tag recognition, *i.e.* a "fluorescent turn-on probe", is therefore required. An earlier report has described a field-sensitive system by which the Dansyl-conjugated nitrilotriacetic acid nickel complex (Dansyl-NTA-Ni^{2+}) bound to the protein tethering the hexahistidine coupled to a hydrophobic motif, resulting in a wavelength shift of the fluorescence emitted by Dansyl adjacent to the hydrophobic motif [18]. Higuchi et al. have reported a different functional probe utilizing a weakly fluorescent complex involving intramolecular coordination of the fluorophore by metals [19,20]. Although both approaches are elegant, they have never been applied for imaging living cells to our knowledge. It is assumed that the strategies just described would require sophisticated structural design utilizing a probe or a functional peptide sequence, implying that it would not be widely applicable as a general chemical probe. In addition, the type of fluorophore that can be used is restricted, although selection of the wavelength is significant in an application to living cell imaging.

We demonstrate in this paper a simple "fluorescent turn-on" system that applies a fluorescence-quenching pair with a fluorescent chemical probe to target the tagged protein and reversible quencher-conjugated peptide. The turn-on system was applied to

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living cells and evaluated by using a fusion protein with a His-tag and trans-membrane domain. The new system showed a low artificial background and a distinct signal after binding to the His-tagged protein without needing any washing procedure. Such requirements as the recognition ability, quenching effect, and reversibility are also discussed to establish the turn-on system.

2. Materials and methods

2.1. General methods (reagents, characterization and antibodies)

FITC-NTA was synthesized according to the previous report [21]. All solvents were purchased from Kanto Chemical Industry Co. (Tokyo, Japan) and were used as received. All organic reagents were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and used without further purification. The synthesized compounds were characterized by ^{1}H NMR and electrospray ionization mass spectrometry (ESI-MS). NMR spectra were recorded in a deuterated solvent by an Oxford NMR AS400 spectrometer (400 MHz). Electrospray ionization mass spectra (ESI-MS) were recorded by an LCQ Fleet mass spectrometer (Thermo Fisher Scientific, USA). Plastic sheets coated with 0.2-mm silica gel 60 without a fluorescent indicator (Merck, Germany) were used for thin-layer chromatography (TLC). The anti-His6 monoclonal mouse antibody (His6 Ab) was purchased from Roche (11922416001, Germany) for the cell study. Anti-mouse IgG conjugated with horseradish peroxidase (HRP) was from Vector Laboratories (PI-2000, USA), and Alexa Fluor-633 goat anti-mouse IgG was from Molecular Probes (A21050, USA). The reagents for enhanced chemiluminescence labeling (ECL) and western blotting detection were from Amersham (GE Healthcare, UK). The Mini Complete™ protease inhibitor cocktail (PI) was from Roche (Mannheim, Germany), and the PVDF membranes were from Bio-Rad Laboratories (62-0177, USA). Phenyl-methyl-sulfonylurea-fluoride (PMSF) and *p*-formaldehyde were from Sigma (P7626 and P6148, USA).

2.2. Synthesis of TMR-triNTA

TMR-triNTA was synthesized according to the literature procedures [21,24]. *N,N*-Diisopropylethylamine (DIPEA, 10 μl) was added to a CH_2Cl_2 solution (1 ml) of NH_2-tri-NTA(t-Bu)$_3$ (21.0 mg, 15 μmol) and 5(6)-carboxy-tetramethylrhodamine *N*-succinimidyl ester (4.6 mg, 7.0 μmol), and the resulting mixture was stirred overnight at room temperature. The reacted solution was evaporated *in vacuo* and purified by column chromatography on silica gel ($CHCl_3$/CH_3OH = 40/1 v/v) to give TMR-triNTA(t-Bu)$_3$. This was then dissolved in TFA (2 ml) and stirred overnight. The solvent was evaporated *in vacuo* to give TMR-triNTA as a solid (5.1 mg, 42%). ^{1}H NMR (400 MHz, D_2O/CD_3CN) δ: 8.61, 8.30, 8.15, 8.10, 7.66, 7.42 (m, 3H), 7.04 (d, 2H), 6.92–6.83 (m, 6H), 3.66–3.13(m, 28H), 1.87–0.76 (m, 36H). ESI-MS *m/z*: 467.92 $[M-3H^+]^{3-}$.

2.3. Synthesis of 2′,7′-bis(2-pyridylsulfonamido)-4′,5′-dimethylfluorescein (HisZiFiT)

2′,7′-Bis(2-pyridylsulfonamido)-4′,5′-dimethylfluorescein (HisZiFiT) was synthesized according to the previous report [23]. To a solution of 2′,7′-diamino-4′,5′-dimethylfluorescein (60.8 mg, 0.156 mmol) in anhydrous pyridine (4 ml) was added 2-pyridine sulfonylchloride (200.4 mg, 0.936 mmol). The mixture was stirred at room temperature for 12 h, poured into an aqueous solution of 1 M HCl (80 ml), and the precipitate was collected. The crude compound was purified by flash column chromatography on silica gel ($CHCl_3$/CH_3OH/acetic acid = 9/1/0.05 (v/v/v)) to give HisZiFiT (10.5 mg, 10.0%) as a solid. ^{1}H NMR (CD_3OD) δ: 2.23 (6H, s), 6.34 (2H, br-s), 6.94–7.00 (1H, m), 7.42–7.48 (2H, m), 7.57 (2H, d, J = 8.0 Hz), 7.69–7.73 (2H, m), 7.79–7.85 (2H, m), 8.12–8.22 (1H, m), 8.40 (1H, d, J = 4.4 Hz) ESI-MS *m/z*: only the fragment peak was detected.

2.4. Synthesis of the quencher-conjugated peptide (Dabcyl-His6)

Quencher-conjugated His6 (Dabcyl-His6) was synthesized by Fmoc solid-phase peptide synthesis (SPPS), using Fmoc-His (Trt)-OH and HBTU/HOBt/NMM as coupling reagents (Hayashi Kasei Co.). The compound was deprotected with DMF/piperidine (20%) and TFA/EDT/Thioanisole/TIS/H_2O. The final compound was characterized by HPLC (Shimadzu Prominence) and MS (HP1100 series LC/MSD). HPLC was performed at a flow rate of 1.0 ml/min. in a C18 column (Shiseido Capcell Pak C18). MS *m/z*: 1093 $[M+H]^+$.

2.5. Fluorescence measurements

All fluorescence spectra were recorded by an RF5300PC spectrofluorophotometer (Shimadzu). The bandwidth was 5.0 nm for excitation and emission, using a 10-mm quartz cell.

2.6. Plasmid construction

His-pDisplay was constructed by replacing a DNA fragment encoding for hexahistidine residues with the sequence between the SmaI and SacII sites in pDisplay (Invitrogen, USA). In the first round of PCR, primers P1, the T7 primer (5′-TAA TAC GAC TCA CTA TAG GGA GAC-3′) and P2 (5′-ATG ATG ATG ATG ATG ATG GGG AGA TCT GGC CGG CTG-3′) were used to introduce the hexahistidine coding sequence at the 3′ end of the PCR fragment. Similarly, primers P3 (5′-CAT CAT CAT CAT CAT CAT CGG CTG CAG GTC GAC GAAC-3′) and P4, the BGH reverse primer (5′-CTA GAA GGC ACA GTC GAG GC-3′) were used to generate an overlapping region of the hexahistidine coding sequence at the 5′end. pDisplay was used as a template for the two reactions. The two PCR-amplified DNA fragments were then used as templates for the second round of PCR with primers P1 and P4. Plasmid pFLAG CMV–neuregulin containing cDNA [22] for the EGF domain of mouse neuregulin is preserved in our laboratory. His-EGF-TD was constructed by amplifying the cDNA fragments of EGF by PCR from pFLAG CMV–neuregulin, using primers P1 (5′-GCGTCGAC TCA AAC GCC ACA TCT ACA TCC-3′) and P2 (5′-GCGTCGAC CTC CTC CGC TTC CAT AAA TTC-3′) which introduced SalI restriction enzyme sites. The amplified PCR product was ligated into the TA vector, and the confirmed sequence was subcloned into the SalI site of His-pDisplay.

2.7. Cell application

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. All chemicals used were purchased from Gibco (USA). The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. COS-7 cells were seeded at 7×10^4 cells onto 35-mm glass-bottom dishes containing 2 ml of the fresh culture medium and transfected with the plasmid (0.5 μg) of His-EGF-TD by using the Effectene reagent (Qiagen, USA) according to the manufacturer's protocol. After 1 or 2 days, the cells were washed twice with Hanks's balanced salt solution (HBSS; Gibco, USA) and stained at room temperature by treating with either of the dyes described in the legend to Fig. 2 or His6 Ab (see Section 2) at room temperature. A His6 Ab stock solution (0.1 mg/ml) was diluted 1:20 in DMEM with 10% FBS, and the mixture added to the cells for 15 min. The cells were next washed twice with HBSS and stained for 15 min with the Alexa Fluor-633 secondary antibody (see Section 2) at a 1:300 dilution

of the stock solution (2 mg/ml). The cells were then washed twice with HBSS and viewed by a laser-scanning confocal microscope (Olympus FV1000) under a 60× oil lens.

2.8. Immunoblotting

The cells were washed with phosphate-buffered saline (PBS, 10 mM phosphate and 150 mM sodium chloride, at pH 7.2) and lysed in a sodium dodecyl sulfate (SDS) buffer (100 mM Tris, at pH 6.8, 2% SDS, 15% glycerol and 0.1 mM dithiothreitol (DTT)). After scraping the cells from the dishes, the sample was homogenized and boiled at 95 °C for 5 min. A 10-μg amount of the sample and a 4× sample buffer (0.26 M Tris–HCl, at pH 6.8, 40% glycerol, 16% SDS, 0.08 mg/ml of bromophenol blue, and 100 mM DTT) were diluted to 1× and loaded into each well. The sample was separated by SDS gel electrophoresis, transferred to a polyvinylidenefluoride (PVDF) membrane, blocked with 5% skim milk in Tween 20 Tris-buffered saline (TBST; 150 mM NaCl, 0.05% Tween 20, and 20 mM Tris–HCl, at pH 7.4), and probed with His6 Ab. The membrane was incubated with an anti-mouse secondary antibody conjugated with HRP at a 1:20,000 dilution for 1.5 h. The blot was detected with an ECL reagent.

3. Results and discussion

3.1. Operating principle of the fluorescent turn-on probes and materials

We have proposed a simple "fluorescent turn-on" system that was useful for detecting a specific protein in living cells. The schematic strategy is presented in Fig. 1. Fluorescent labeling of a fusion protein with a peptide tag was achieved by designing a fluorescent probe which comprised the fluorophore and adaptor molecule as the tag recognition site. We then attached a non-fluorescent quenching molecule to the N or C terminus of a short peptide possessing a similar structure to that of the target peptide tag so that it could be recognized by the probe. When the quencher-conjugated peptide was mixed with the fluorescent probe, a complex was formed by non-covalent bonding, causing the fluorescence of the fluorophore to be quenched due to the closely situated quenching moiety (Fig. 1A). If the complex at the quenched state was applied to cells expressing the protein tethering the peptide tag, the quencher-conjugated peptide was dissociated from the complex through binding of the fluorescent probe to the tagged protein, resulting in fluorescence of the fluorophore, since the quencher was separated from the fluorophore. This phenomenon gave a fluorescence "turn-on" system in response to the recognition of a target protein (Fig. 1B). We planned to demonstrate this concept for a His-tag targeting probe which involved two important requirements: (i) a tag targeting probe needs to be able to stably bind a tagged protein in living cells. (ii) After complexing the fluorescent probe with the quencher-conjugated peptide, the fluorescence needs to be efficiently quenched and the complexation must be reversible.

Three kinds of representative fluorescent probes showing different affinity to His-tag were initially synthesized as shown in Fig. 1C. *N*-Triacetic acid (NTA) is a well-known molecule showing high affinity to His-tag and has been used as the immobilized resin for purifying His-tagged proteins [13]. The recognition of NTA for His-tag was based on the reversible interaction between NTA,

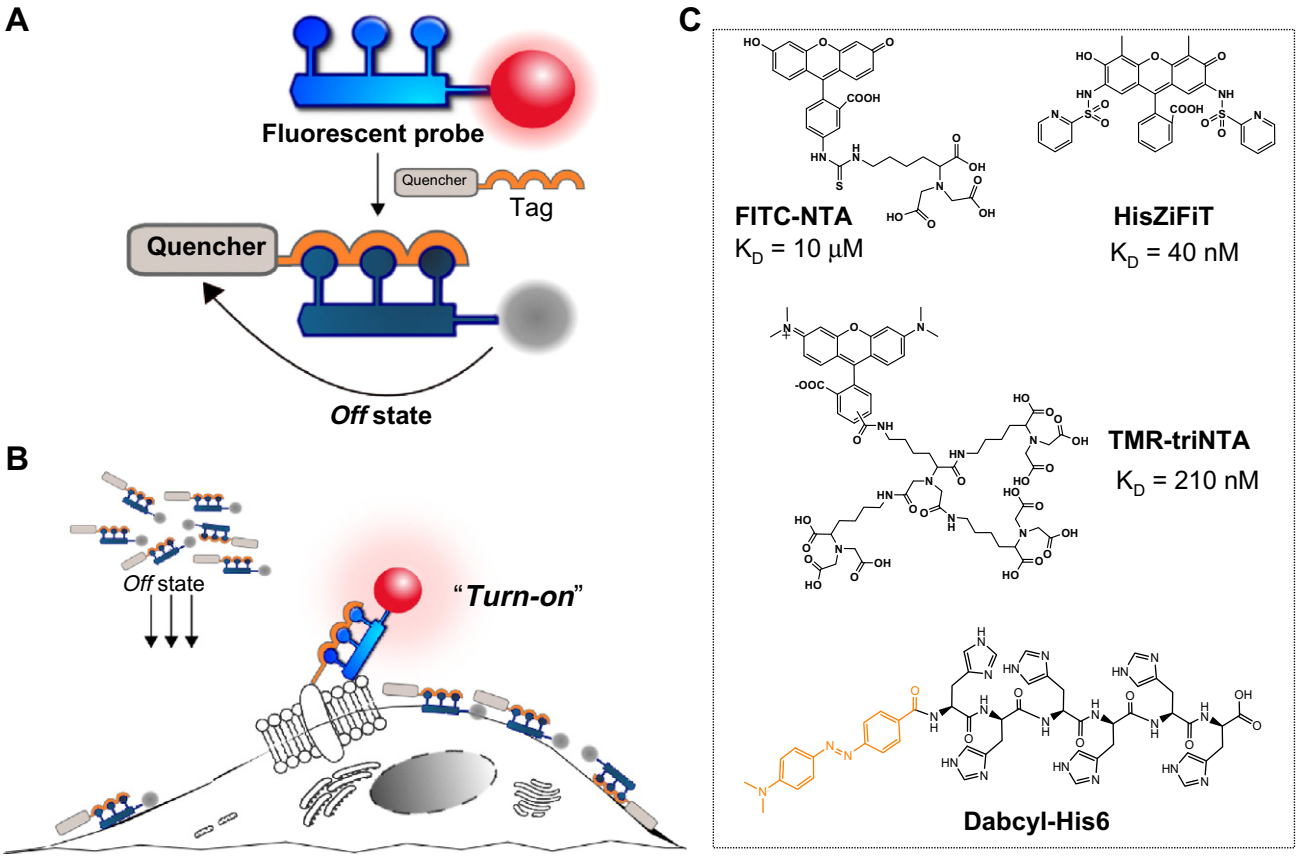

Fig. 1. Schematic images. (A) Complexation with the small fluorescent probe and quencher-conjugated peptide. (B) The turn-on system was applied to a tagged protein expressed on the cell membrane. (C) Three representative fluorescent probes and the quencher-conjugated peptide.

nickel ions and the imidazole rings of histidines. A simple fluorescent probe bearing one NTA moiety, FITC-NTA, has been prepared by Vogel and coworkers, and the affinity of mono-NTA to the His-tag was found to be *ca.* 10 μM [15,16,21]. Multi-branched NTA moieties have generated higher affinity to the His-tag than that of FITC-NTA [14,15]. A fluorescent probe bearing multi-branched NTA, such as TMR-triNTA, has previously been reported to work under a diluted condition (K_d = 210 nM) [24]. Furthermore, Tsien et al. have reported a unique fluorescent probe targeting His-tag that used coordinated bonding of 2-pyridyl sulfonamide *via* the zinc ion (HisZiFiT) [23]. The 2-pyridylsulfonamide functionalities and phenolate anions would satisfy the three valances of the zinc ion, while the other vacancies were bound to two lone pairs provided by two imidazoles. In spite of only two binding sites, the affinity of HisZiFiT was relatively high (K_d = 40 nM). Tsien et al. have explained that the virtue of its structural rigidity would surpass any unfavorable entropic loss involved in the recognition of a His-tagged protein.

3.2. Staining ability of FITC-NTA, HisZiFiT and TMR-triNTA to the His-tagged protein expressed on the cell membrane

The His_6-EGF-TD plasmid was transfected into COS7 cells to examine the staining ability of the probes for living cells. The plasmid was constructed by subcloning the EGF-like and transmembrane domains into pDisplay in which the fusion gene product has its own signal peptide [25,26]. The expression of the His-tagged protein on the cell surface was confirmed by staining with His6 Ab, suggesting that our construct was good for testing the surface-staining ability of the three probes as shown in Fig. 2. The cells were respectively stained with 1 μM of FITC-NTA (1 μM $NiCl_2$, 1 eq.), TMR-triNTA (3 μM $NiCl_2$), and HisZiFiT (10 μM $ZnCl_2$) in a Tris–HCl buffer (200 mM, at pH 7.4). The cell surface was specifically stained in the presence of TMR-triNTA and HisZiFiT (see Fig. 2B).

Staining of the cell surface with each of these dyes was completed in less than 1 min. The staining intensity induced by TMR-triNTA was much higher than that induced by HisZiFiT at the same apparent concentration of the sample. The high background signal shown in Fig. 2B was due to the panels being subjected to an identical confocal setup which was based on the detection of a very low signal from HisZiFiT (the reason will be discussed in the next section). In the case of FITC-NTA, no specific staining was apparent, even when the concentration was increased 10-fold (data not shown). These results suggest that the dissociation equilibrium constant of 10 μM provided by monomeric NTA was not enough to stably label the tagged protein on the cell surface. Consequently, TMR-triNTA and HisZiFiT, which showed higher affinity than 210 nM, should be more suited for application to imaging living cells.

3.3. Evaluation of the quenching effect of the quencher-conjugated peptide

4-(4′-Dimethylamino-phenylazo) benzoic acid (Dabcyl) was selected as a quenching molecule, because the dye had no native fluorescent property and has been widely used as a dark quencher of various fluorescent molecules. We attached a quenching molecule to the N terminus of hexahistidine (His6) as a short peptide tag, named Dabcyl-His6. HPLC and MS analyses of Dabcyl-His6 showed high purity (99.5%). The purity of Dabcyl-His6 was significant, because contamination related to quencher-free hexahistidine should influence the precise evaluation of the quenching effect. To test this quenching effect, an equimolar amount of Dabcyl-His6 was mixed with 5 μM of TMR-triNTA (15 μM $NiCl_2$, 3 eq.) or HisZiFiT (50 μM $ZnCl_2$, 10 eq.) in a 20-mM Tris–HCl buffer at pH 7.4 as shown in Fig. 3.

Upon complexation with Dabcyl-His6, the fluorescence emitted from HisZiFiT was dramatically quenched to 0.33% of the intensity of corresponding quencher-free HisZiFiT. However, when an equimolar amount of quencher-free hexahistidine (His6) was added to the solution of the complex, and the mixture allowed to stand for more than 24 h, no fluorescence enhancement could be detected. When we added excess His6 to the solution of the complex, slight fluorescence was observed (data not shown). The excess addition of EDTA as a chelating reagent restored the fluorescence of the

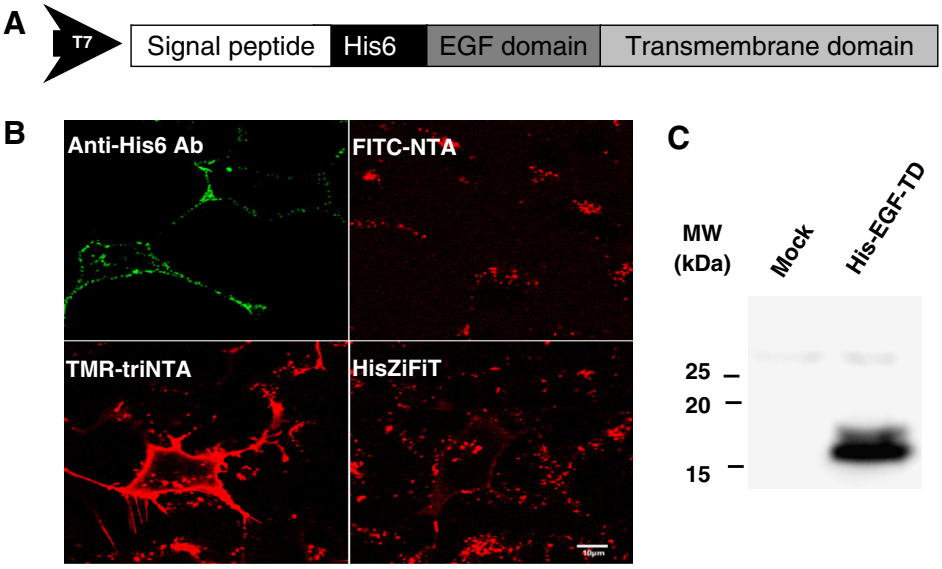

Fig. 2. Specific membrane staining by TMR-triNTA and HisZiFiT in living cells was observed. (A) Gene construction of the His-tag plasmid. The hexahistidine and EGF domain were subcloned into the pDisplay vector (Invitrogen) which allowed display of the fused protein on the cell surface. (B) Cos-7 cells transfected with His-EGF-TD were respectively stained with the anti-His6 antibody, FITC-NTA, TMR-triNTA and HisZiFiT. Images were captured by using an Olympus confocal microscope. The panels stained with FITC-NTA, TMR-triNTA and HisZiFiT involved an identical confocal setup. Immunofluorescence was induced as described in Section 2. The bar represents 10 μm. (C) Immunoblots for His-EGF-TD were developed with anti-His6 (1:1000). A 10-μg amount of the total lysate prepared from a 65-mm dish of His-EGF-TD transfected or mock-transfected cells was loaded in each lane.

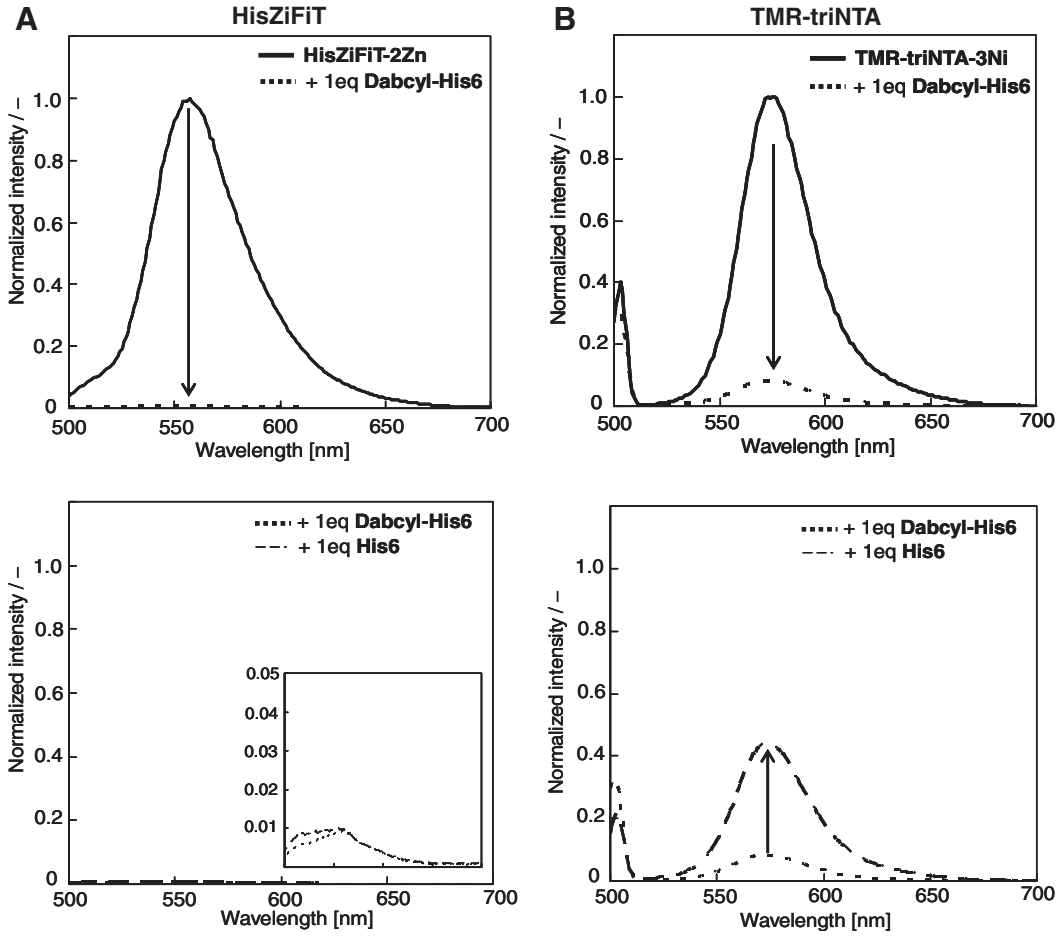

Fig. 3. The quenching effect of HisZiFiT and TMR-triNTA was evaluated by using Dabcyl-His6. (A) Fluorescence spectra of 5 μM of HisZiFiT with 10 eq. $ZnCl_2$ in a 20 mM Tris–HCl buffer at pH 7.4 (λ_{ex} = 500 nm). The upper graph shows the effects before and after adding equimolar Dabcyl-His6, and the lower graph, the effects before and after adding equimolar His6. (B) Fluorescence spectra of 5 μM of TMR-triNTA with 3 eq. $NiCl_2$ in a 20 mM Tris–HCl buffer at pH 7.4 (λ_{ex} = 500 nm). As shown for HisZiFiT, the upper graph shows the effect of equimolar Dabcyl-His6, and the lower graph shows the recovery effect by adding equimolar His6.

fluorophore due to dissociation of the complex. This means that the complex formed of HisZiFiT with Dabcyl-His6 did not cause irreversible decomposition and not involve non-specific aggregation by hydrophobic interaction between such aromatic moieties as TMR and Dabcyl. We also found that the simple addition of His6 as a quencher-free peptide caused quenching of HisZiFiT in a solution state. This result agrees with the observation by confocal microscopy showing a low signal from HisZiFiT (Fig. 2B), although the reason for this remains obscure. It would therefore be difficult to evaluate the difference between the quenching effect with Dabcyl-His6 and the enhancement in response to the addition of His6.

The quenching of TMR-triNTA by Dabcyl-His6 was also effective due to the formation of the fluorescence-quenching complex (8%). When an equimolar amount of His6 was added to the solution of the complex, the fluorescence emitted by TMR-triNTA increased to 44% (5.4-fold), suggesting that the dissociation equilibrium constant of His6 and Dabcyl-His6 was almost the same, this being followed by reversible displacement between Dabcyl-His6 of the complex and His6. In respect of the conceptual requirements for the quenching effect and its reversibility, the complex of TMR-triNTA and Dabcyl-His6 is considered to have been appropriate proof of the concept.

3.4. Application of the turn-on system to a cultured cell

The complex of Dabcyl-His6 and TMR-triNTA was used to examine if the system would function in living cells. When COS-7 cells transfected with His-EGF-TD were incubated with only TMR-triNTA or the complex with Dabcyl-His6, the fluorescence intensity was respectively measured before and after washing with dye-free HBSS (Fig. 4). As expected, compared to only TMR-triNTA before washing, the complex with Dabcyl-His6 appeared to reduce the non-specific fluorescence emitted by the dye absorbed in the background, this being similar to the case from washing with HBSS (see Fig. 4A).

The fluorescence intensity of TMR-triNTA dissociated from the complex was similar to that incubated with TMR-triNTA alone on single-cell level as shown in Fig. 4B, S (surface) indicating that an exchange between Dabcyl-His6 and a His-tagged protein should effectively occur on the cell membrane. In addition, the signals shown inside the cells were significantly lower than in those treated with TMR-triNTA alone ($p < 0.01$) (Fig. 4B, I (inside)). It was assumed that the signal observed inside the cell would have resulted only from TMR-triNTA partially penetrating the cell. On the other hand, the complex with Dabcyl-His6 might have prevented penetration of the dye inside the cell, resulting in a lower signal due to the internal signal.

In conclusion, we have demonstrated the fluorescent turn-on system in response to a His-tagged protein being expressed on the cell membrane. The fluorescent probe targeting the His-tagged protein formed a reversible complex with the quencher-conjugated hexahistidines *via* coordinated bonding, causing the fluorescence of the fluorophores in close proximity to each other to be quenched. When the complex was applied to a living cell

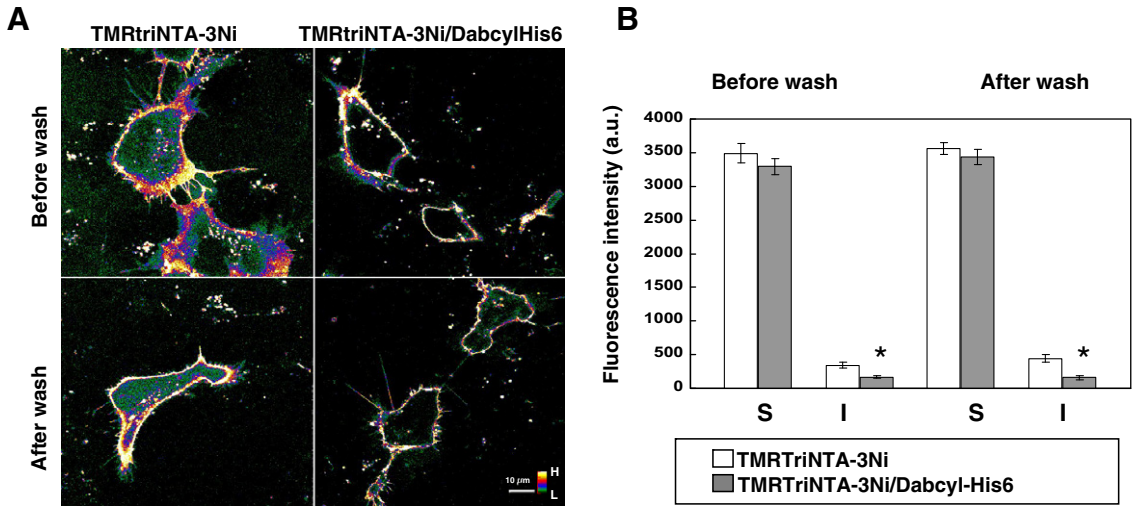

Fig. 4. The turn-on system reduced non-specific absorption. (A) Cos-7 cells transfected with His-EGF-TD were incubated with TMR-triNTA or a complex of TMR-triNTA and Dabsyl-His6. Images were captured with an Olympus confocal microscope before washing (upper half) and after washing with HBSS (lower half). All panels involved an identical confocal setup. The bar represents 10 μm. (B) The fluorescence intensity was measured on both S (surface) and I (inside) the cells. Each value is expressed as the mean ± S.E.M of 6–9 cells chosen from three different experiments. $^{*}p < 0.01$ compared to the dye only by an unpaired two-tailed *t*-test.

expressing the His-tagged protein, the quencher-conjugated hexahistidine in the complex was replaced by the tagged protein, resulting in enhanced fluorescence, whereas the complex that did not participate in labeling the tagged protein remained in the quenched state to reduce the background signal. This system would be attractive for microarray application, because the washing process to remove free dye is unnecessary. In addition, the system should offer high potential for application to cytoplasmic and *in vivo* labeling, in which washing can cause difficulties.

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References

[1] M.F. Suarez, A.Y. Ting, Fluorescent probes for super-resolution imaging in living cells, Nat. Rev. Mol. Cell Biol. 9 (2008) 929–943.
[2] B.N.G. Giepmans, S.R. Adams, M.H. Ellisman, et al., The fluorescent toolbox for assessing protein location and function, Science 312 (2006) 217–224.
[3] N. Johnsson, K. Johnsson, Chemical tools for biomolecular imaging, ACS Chem. Biol. 2 (2007) 31–38.
[4] R.Y. Tsien, Constructing and exploiting the fluorescent protein paintbox (Nobel Lecture), Angew. Chem. Int. Ed. 48 (2009) 5612–5626.
[5] R. Ando, H. Hama, M. Yamamoto-Hino, et al., An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein, Proc. Natl. Acad. Sci. USA 99 (2002) 12651–12656.
[6] J.L. Schwartz, G.H. Patterson, Development and use of fluorescent protein markers in living cells, Science 300 (2003) 87–91.
[7] Y. Hori, H. Ueno, S. Mizukami, et al., Photoactive yellow protein-based protein labeling system with turn-on fluorescence intensity, J. Am. Chem. Soc. 131 (2009) 16610–16611.
[8] I. Chen, A.Y. Ting, Site-specific labeling of proteins with small molecules in live cells, Curr. Opin. Biotechnol. 16 (2005) 35–40.
[9] N. Soh, Selective chemical labeling of proteins with small fluorescent molecules based on metal-chelation methodology, Sensors 8 (2008) 1004–1024.
[10] H. Nonaka, S. Tsukiji, A. Ojida, et al., Non-enzymatic covalent protein labeling using a reactive tag, J. Am. Chem. Soc. 129 (2007) 15777–15779.
[11] B.A. Griffin, S.R. Adams, R.Y. Tsien, Specific covalent labeling of recombinant protein molecules inside live cells, Science 281 (1998) 269–272.
[12] T.L. Halo, J. Appelbaum, E.M. Hobert, D.M. Balkin, et al., Selective recognition of protein tetraserine motifs with a cell-permeable, pro-fluorescent bis-boronic acid, J. Am. Chem. Soc. 131 (2009) 438–439.
[13] E. Hochuli, H. Dobeli, A. Schacher, New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues, J. Chromatogr. 411 (1987) 177–184.
[14] A.N. Kapanidis, Y.W. Ebright, R.H. Ebright, Site-specific incorporation of fluorescent probes into protein: hexahistidine-tag-mediated fluorescent labeling with (Ni^{2+}:nitrilotriacetic acid)$_n$-fluorochrome conjugates, J. Am. Chem. Soc. 123 (2001) 12123–12125.
[15] S. Lata, A. Reichel, R. Brock, et al., High-affinity adaptors for switchable recognition of histidine-tagged proteins, J. Am. Chem. Soc. 127 (2005) 10205–10215.
[16] E.G. Guignet, R. Hovius, H. Vogel, Reversible site-selective labeling of membrane proteins in live cells, Nat. Biotechnol. 22 (2004) 440–444.
[17] Y. Urano, D. Asanuma, Y. Hama, et al., Selective molecular imaging of viable cancer cells with pH-activatable fluorescence probes, Nat. Med. 15 (2009) 104–109.
[18] N. Soh, D. Seto, K. Nakano, et al., Methodology of reversible protein labeling for ratiometric fluorescent measurement, Mol. BioSyst. 2 (2006) 128–131.
[19] M. Kamoto, N. Umezawa, N. Kato, et al., Novel probes showing specific fluorescence enhancement on binding to a hexahistidine tag, Chem. Eur. J. 14 (2008) 8004–8012.
[20] M. Kamoto, N. Umezawa, N. Kato, et al., Turn-on fluorescent probe with visible light excitation for labeling of hexahistidine tagged protein, Bioorg. Med. Chem. Lett. 19 (2009) 2285–2288.
[21] Z. Huang, J.I. Park, D.S. Watson, et al., Facile synthesis of multivalent nitrilotriacetic acid (NTA) and NTA conjugates for analytical and drug delivery applications, Bioconjugate Chem. 17 (2006) 1592–1600.
[22] M. Ozaki, K. Tohyama, H. Kishida, et al., Roles of neuregulin in synaptogenesis between mossy fibers and cerebellar granule cells, J. Neurosci. Res. 59 (2000) 612–623.
[23] C.T. Hauser, R.Y. Tsien, A hexahistidine-Zn^{2+}-dye label reveals STIM1 surface exposure, Proc. Natl. Acad. Sci. USA 104 (2007) 3693–3697.
[24] A. Murata, S. Arai, S.I. Yoon, M. Takabayashi, et al., Construction of a 'turn-on' fluorescent probe system for his-tagged proteins, Bioorg. Med. Chem. Lett. (2010). <http://www.ncbi.nlm.nih.gov:80/pubmed/21035333>.
[25] Z.M. Yang, E.M. Li, B.C. Lai, Y.L. Wang, et al., Anti-CD3 scFv-B7.1 fusion protein expressed on the surface of HeLa cells provokes potent T-lymphocyte activation and cytotoxicity, Biochem. Cell Biol. 85 (2007) 196–202.
[26] M. Ozaki, K. Itoh, Y. Miyakawa, et al., Protein processing and releases of neuregulin-1 are regulated in an activity-dependent manner, J. Neurochem. 91 (2004) 176–188.