Inorganic Chemistry

Forum Article

pubs.acs.org/IC

Peptide Tag/Probe Pairs Based on the Coordination Chemistry for Protein Labeling

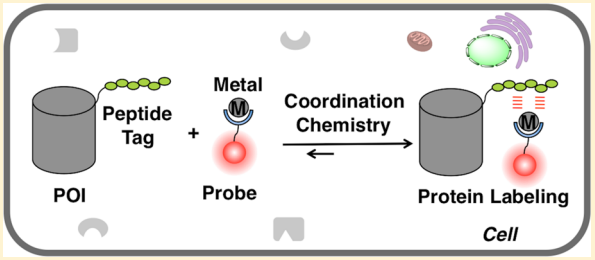
Shohei Uchinomiya,[†] Akio Ojida,[‡] and Itaru Hamachi*[,†,§]

[†]Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan

[‡]Graduate School of Pharmaceutical Sciences, Kyushu University, Maedashi, Higashi-ku, Fukuoka 819-0395, Japan

[§]Japan Science and Technology Agency (JST), CREST, 5 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan

ABSTRACT: Protein-labeling methods serve as essential tools for analyzing functions of proteins of interest under complicated biological conditions such as in live cells. These labeling methods are useful not only to fluorescently visualize proteins of interest in biological systems but also to conduct protein and cell analyses by harnessing the unique functions of molecular probes. Among the various labeling methods available, an appropriate binding pair consisting of a short peptide and a de novo designed small molecular probe has attracted attention because of its wide utility and versatility. Interestingly, most peptide tag/probe pairs exploit metal−ligand coordination interactions as the main binding force responsible for their association. Herein, we provide an overview of the recent progress of these coordination-chemistry-based protein-labeling methods and their applications for fluorescence imaging and functional analysis of cellular proteins, while highlighting our originally developed labeling methods. These successful examples clearly exemplify the utility and versatility of metal coordination chemistry in protein functional analysis.



1. INTRODUCTION

Proteins play vital roles in many biological events such as molecular recognition, biocatalysis, metabolism, and cell signaling among others. While proteins have been traditionally analyzed in purified form in vitro, recent researches has been focusing on studying them under more biologically relevant conditions such as in cell lysates or living cells. This is because protein analysis under such conditions is expected to give us real protein functions and structures with high cooperativity and spatiotemporal regulation rich in a variety of diverse natural substances.

Labeling and imaging of proteins of interest (POIs) under live cell conditions is necessary for understanding the fate of proteins in vivo (Figure 1a). The most widely used technology involves the fusion of fluorescent proteins (FPs) such as green fluorescent protein (GFP) to the POIs, which enables the fluorescent visualization of a target protein in live cells or animals.[1] Although FP-fusion technology is powerful and widely used in many biological research areas, the large size of FPs as a fluorescent tag (GFP: 27 kDa) may deleteriously affect the natural functions of the POIs, particularly the formation of delicate protein complexes through protein−protein interactions (PPIs). In addition, their usage is limited as fluorescent markers for bioimaging studies. To overcome these limitations and provide a more practical chemical tool for protein analyses, many researchers have actively developed new methods for selective labeling of POIs with a functional molecular probe.[2] Introduction of a tag specific for the POI is a valuable strategy to achieve labeling under crude biological conditions. Among the various protein tagging technologies available, the SNAP-tag (a protein tag based on human O^6-alkylguanine−DNA alkyltransferase)[3] and Halo-tag (a protein tag based on haloalkane dehalogenase)[4] systems are the most successful. These methods are based on the enzyme-catalyzed reactions of the protein tag with its suicide substrate molecule, allowing rapid and selective labeling of the protein-tag-fused POI with a variety of functional molecules. However, the large sizes of the protein tags (SNAP tag, 20 kDa; Halo tag, 33 kDa) may still impact the protein structure and function and the PPIs similar to the FP-fusion technologies. Instead, a short peptide that can serve as a substrate for a specific enzyme-catalyzed reaction was developed for the selective labeling of the POI. This short peptide tag, fused to the N or C terminal of the POI in most cases, is less likely to perturb the protein function because of its small size. This labeling technique includes the acyl carrier protein (ACP) tag,[5] sortase tag,[6] and lipoic acid ligase acceptor peptide (LAP) tag,[7] which were successfully applied to protein functional analyses in living cells. However, these labeling methods often suffer from limitations in the enzyme-dependent labeling conditions and the probe structure available as the corresponding substrate.

Special Issue: Imaging and Sensing

Received: June 25, 2013
Published: October 16, 2013

   dx.doi.org/10.1021/ic401612z | Inorg. Chem. 2014, 53, 1816−1823

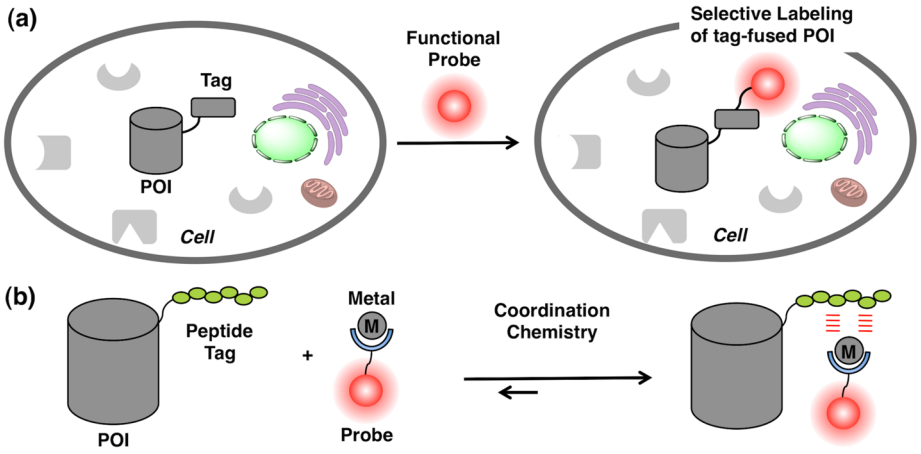


Figure 1. (a) Selective labeling of tag-fused POI with a functional molecular probe in cell systems. (b) Coordination-chemistry-based protein-labeling method using the pair of peptide tag and metal complex.

To overcome these problems, a de novo designed binding pair consisting of a short peptide and a small molecular probe has been developed (Figure 1b). Compared with other enzyme-dependent tag technologies, a peptide tag/probe pair has the following advantages: (1) small molecular sizes of both the tag and probe, which is less likely to perturb the protein functions and PPIs and (2) large flexibility in the labeling conditions and probe structure, which allows the introduction of various functional molecules to the target proteins. It is worth noting that most of the peptide tag/probe pairs use coordination interactions as the main binding force. In this article, we briefly describe the recent progress of coordination-chemistry-based protein-labeling methods and their applications for bioimaging and functional analysis of cellular proteins, mainly focusing on the labeling methods that we have originally developed. These successful examples clearly reveal the utility and versatility of metal coordination chemistry in protein functional analysis.

2. DEVELOPMENT OF NEW PEPTIDE TAG/PROBE PAIRS BASED ON COORDINATION CHEMISTRY

The metal−ligand interaction (coordination chemistry) is a versatile binding force for protein recognition because it can work effectively under physiological aqueous conditions.[8] This can be achievable by the careful design of metal complexes; that is, there must be vacant coordination sites on the metal centers for peptide-tag recognition as well as sufficient stability in the aqueous solution. A representative example is found in an affinity-based protein purification system that employs an oligohistidine tag (His-tag) and nickel(II) nitrilotriacetic acid complex (Ni^{II}-NTA) binding pair.[9] Besides this well-known binding system, the combination of a designed amino acid sequence and a metal complex might serve as a peptide tag/artificial probe pair with a strong binding affinity suitable for selective labeling of POIs. Tsien and co-workers' pioneering work was based on this concept. They found that a biarsenical fluorophore such as fluorescein arsenical helix binder (FlAsH) can interact with the tetracysteine motif (CCXXCC) with very strong binding affinity (K_d = 4−70 pM) and serve as a peptide tag/probe pair for fluorescence imaging of proteins in living cells (Figure 2a).[10] This pair was further applied to a chromophore-assisted light inactivation (CALI) method,[11] sensing of the local calcium concentration,[12] and detection of PPIs in living cells.[13] The conventional His tag was also applicable to the fluorescent bioimaging of proteins in living

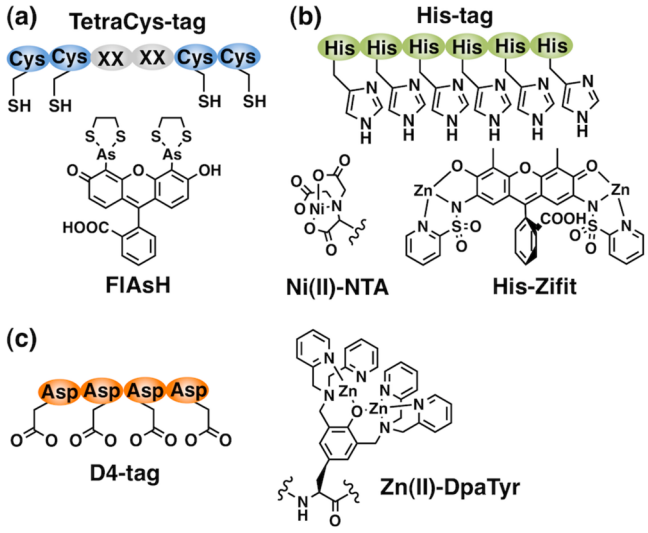


Figure 2. Representative coordination-chemistry-based tag/probe pairs: (a) TetraCys tag/FlAsH pair; (b) His tag/Ni^{II}-NTA or His-Zifit pair; (c) D4 tag/Zn^{II}-DpaTyr pair.

cells combined with fluorophore-appended Ni^{II}-NTA probes, a methodology that was demonstrated by the Vogel and Lippard groups (Figure 2b).[14] Although the binding affinity between the His6 tag (HHHHHH) and Ni^{II}-NTA was relatively weak (K_d = 13 μM), the Pieler group reported that this could be greatly improved by a multivalent strategy using a trimeric Ni^{II}-NTA probe that can strongly bind to a His6-tag-fused protein (K_d = 2.1 nM).[15] The Tsien group designed a binuclear zinc(II) complex termed His-Zifit (histidine−zinc fluorescent in vivo tag) as a binding probe for a His tag (Figure 2b).[16] Because of its strong binding affinity to the His6 tag (K_d = 40 nM) and bright fluorescent property, His-Zifit was successfully applied to the visualization of a His-tag-fused protein on live cell surfaces. The Imperiali group developed unique lanthanide binding tags (LBTs) that can bind to a terbium ion with high affinities (K_d = 19−220 nM).[17] This method is applicable not only to visualize tag-fused proteins in a cell lysate but also to detect PPIs by combination with luminescence resonance energy transfer.[18]

2-1. Oligoaspartate Tag and Metal-DpaTyr as a New Entity for a Tag/Probe Pair. As described above, the complementary tag/probe pairs useful for selective protein labeling are still limited. This is mainly because of the general

dx.doi.org/10.1021/ic401612z | Inorg. Chem. 2014, 53, 1816−1823

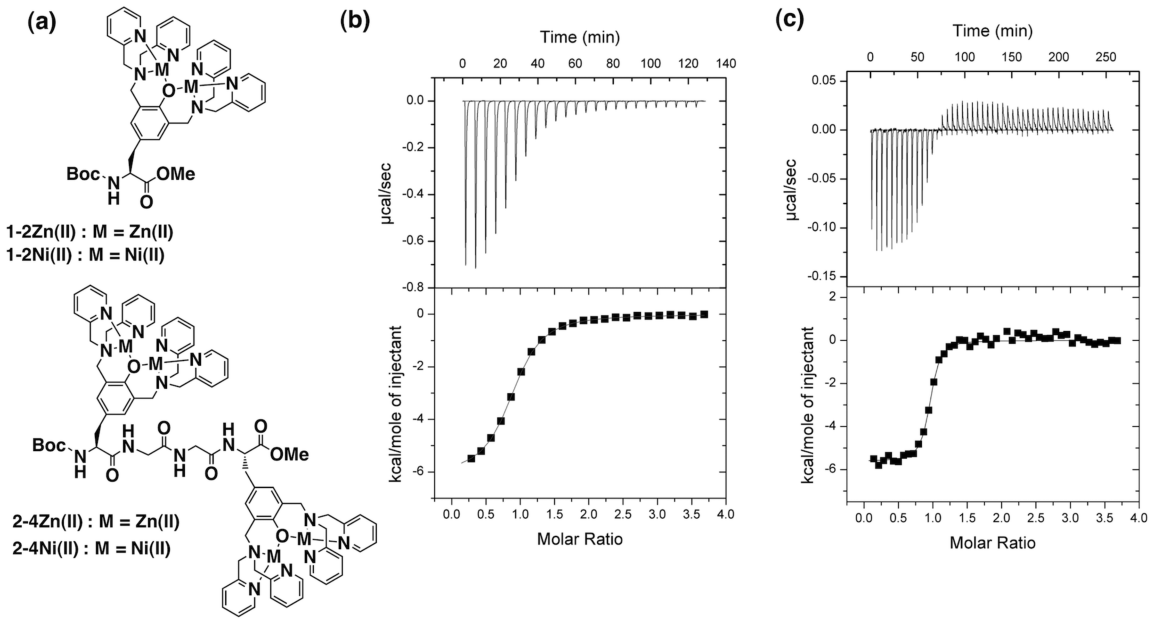


Figure 3. (a) Structures of metal-DpaTyr probes. (b) ITC titration curve (upper) and processed data (lower) in the titration of **1**-2Zn^{II} with D4 peptide. (c) ITC titration curve (upper) and processed data (lower) in the titration of **2**-4Zn^{II} with (D4)$_2$-RNase.

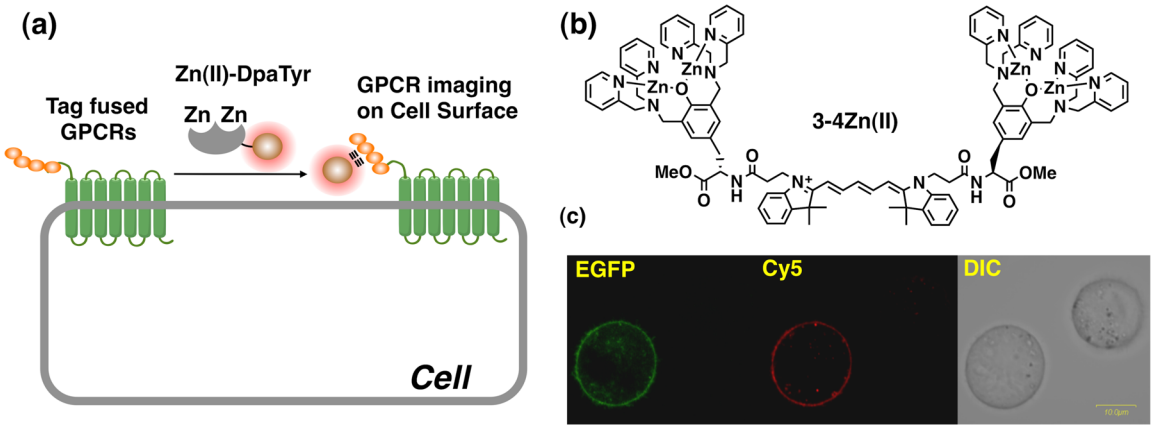


Figure 4. (a) Labeling of a D4-tag-fused GPCR protein with a Zn^{II}-DpaTyr probe on the cell surface. (b) Structure of Cy5-appended probe **3**-4Zn^{II}. (c) Fluorescence imaging of (D4×3)-tag-fused mAChR on the cell surface. EGFP was also fused to mAChR as a fluorescent expression marker.

difficulty in the de novo design of a molecular recognition system composed of a small molecular probe and a short-peptide tag. As a new entry for the peptide tag/probe pair, we originally developed the D4 tag (DDDD) and Zn^{II}-DpaTyr pair (Figure 2c).[19] The D4 tag was designed de novo by aligning the Asp residues in a short peptide in anticipation that the accumulated carboxylate groups may favorably interact with a cationic metal complex. The relatively rare sequence of oligoaspartate among naturally occurring proteins was also considered to be suitable for selective protein labeling. Among a number of metal complexes, we found that the binuclear Zn^{II}-Dpa (2,2′-dipicolylamine) complex based on the L-tyrosine scaffold (Zn^{II}-DpaTyr) was able to strongly bind to the D4 tag. In an initial attempt, the binding affinity of **1**-2Zn^{II} to a series of Asp-repeated peptides, D2−D5 (Boc-D*n*-NH$_2$, *n* = 2−5), was quantitatively evaluated by isothermal titration calorimetry (ITC; Figure 3a). The affinity increased incrementally as the number of Asp units increased and saturated with the D4 or D5 peptide (K_d values for the peptide in aqueous solution: D2, 140 μM; D3, 16 μM; D4, 1.4 μM; D5, 1.1 μM). Interestingly, the binding of **1**-2Zn^{II} with the tetraglutamate (E4) peptides (Boc-E4-NH$_2$) was 6-fold weaker than that with the D4 peptide. These results indicate that D4 is the optimal length of the tag for binding with Zn^{II}-DpaTyr. When the D4 tag was fused to ribonuclease (D4-RNase), **1**-2Zn^{II} bound to D4-RNase with a moderate dissociation constant of 83 μM. Interestingly, the binding affinity of a dimeric Zn^{II}-DpaTyr **2**-4Zn^{II} with a repeated D4 sequence [DDDDGDDDD; (D4)$_2$ tag] was dramatically enhanced by 1500-fold [56 nM for (D4)$_2$-RNase] compared with that of the monomeric pair (83 μM; Figure 3b,c). Overall, these results reveal that the use of coordination multivalency is a powerful strategy to enhance the binding affinity between the metal-complex probe and the tag peptide. We also observed that the binding of Zn^{II}-DpaTyr toward the His6 peptide (HHHHHH) is significantly weak (10^{-3} M < K_d) and the binding of Ni^{II}-NTA toward the D4 tag is also negligible (10^{-3} M < K_d). This indicated that the D4 tag/Zn^{II}-DpaTyr pair is orthogonal to the His tag/Ni^{II}-NTA pair.

To gain more insight into the molecular recognition of the D4 tag with the metal complex, the binding affinity of the

dx.doi.org/10.1021/ic401612z | *Inorg. Chem.* 2014, 53, 1816−1823

oligoaspartate peptides with various binuclear metal complexes of DpaTyr (metal ion = Zn^{II}, Ni^{II}, Mn^{II}, Cu^{II}, Cd^{II}, Co^{III}, and Fe^{III}) was examined by ITC.[20] The Zn^{II}- and Ni^{II}-DpaTyr complexes had reasonable binding affinities toward the oligoaspartate peptides, whereas the binding of the other metal complexes to the D4 tag was very weak ($K_d > 10^{-3}$ M). Ni^{II}-DpaTyr **1**-2Ni^{II} bound to the D4 tag with a 2:1 binding stoichiometry but displayed strong binding (K_d = 6.7 μM) for the triaspartate (D3) peptide with a 1:1 stoichiometry. This binding affinity is stronger than that of **1**-2Zn^{II} for the D4 tag (K_d = 83 μM).[19] Thus, the multivalent strategy was successful for ensuring the binding of the dimeric Ni^{II}-DpaTyr **2**−4Ni^{II} with the repeated D3 peptide (D3×2, DDDNGDDD), as evidenced by the dissociation constant that reached 0.50 nM, the value of which is 110-fold stronger than that of **2**-4Zn^{II} and D4×2 tag (K_d = 55 nM).

2-2. Fluorescent Imaging of Cell Surface Proteins Using the New Peptide/Probe Pairs. The utility of the D4 tag/Zn^{II}-DpaTyr pair was highlighted by the fluorescence imaging of G-protein-coupled receptors (GPCRs) on live mammalian cell surfaces (Figure 4a).[19] As an initial attempt, muscarinic acetylcholine receptor (mAChR) were selected as a target GPCR protein to which a triply repeated D4 tag (D4×3; DDDDGDDDDGDDDD) was tethered at the exoplasmic N-terminus. When CHO cells transiently expressing the tag-fused mAChR were treated with the Cy5-appended Zn^{II}-DpaTyr (**3**-4Zn^{II}; Figure 4b), the fluorescence attributed to Cy5 was observed on the cell surfaces. This fluorescence overlapped well with that of enhanced green fluorescent protein (EGFP), which was also fused to mAChR as an expression marker. In contrast, neither Cy5 nor EGFP fluorescence was detected in the other cell shown in Figure 4c, indicating that cells that did not express D4×3-EGFP-mAChR were not stained with **3**-4Zn^{II}. It is also worth noting that clear fluorescence images were never obtained using the monomeric Zn^{II}-DpaTyr (K_d = 10^{-4} M) as a fluorescent probe. This result implies that a sufficiently strong interaction, probably at least a K_d of 10^{-7} M as observed for the dimeric Zn^{II}-DpaTyr, might be crucial for fluorescence detection of membrane proteins on a living cell surface.

As mentioned above, the Zn^{II}-DpaTyr complex served as a binder for the D4 tag, whereas it was not bound to the His6 tag,[19] which suggests that stable zinc(II) complexes may be formed by a combination of the appropriate nitrogen-based ligands (such as pyridine and imidazole) and oxygen-based ligands (such as carboxylate and phenolate) under biological conditions. We therefore hypothesized that the replacement of the nitrogen-containing pyridine rings of Dpa in Zn^{II}-DpaTyr with carboxylates may give rise to a zinc complex exhibiting a high binding affinity to a His tag. To test this idea, a new binuclear zinc(II) complex (Zn^{II}-Ida) possessing two sets of iminodiacetic acid (Ida; Figure 5a) was designed.[21] As expected, Zn^{II}-Ida was able to bind to a His6 peptide with a moderate binding affinity (K_d = 310 μM, whereas the binding of Zn-Ida toward the D4 peptide is negligible). The multivalent strategy again enhanced the binding ability of the Zn^{II}-Ida probe, that is, the tetrameric Zn^{II}-Ida probe **4**-8Zn^{II} (Figure 5b) strongly interacted with the His10-tag-fused EGFP with a dissociation constant of 1.2 nM. This strong interaction was exploited to achieve the clear and lasting fluorescent detection of the bradykinin receptor (B2R), another GPCR, fused to EGFP, and a His10 tag (His10-EGFP-B2R) on live cell surfaces (Figure 5c).

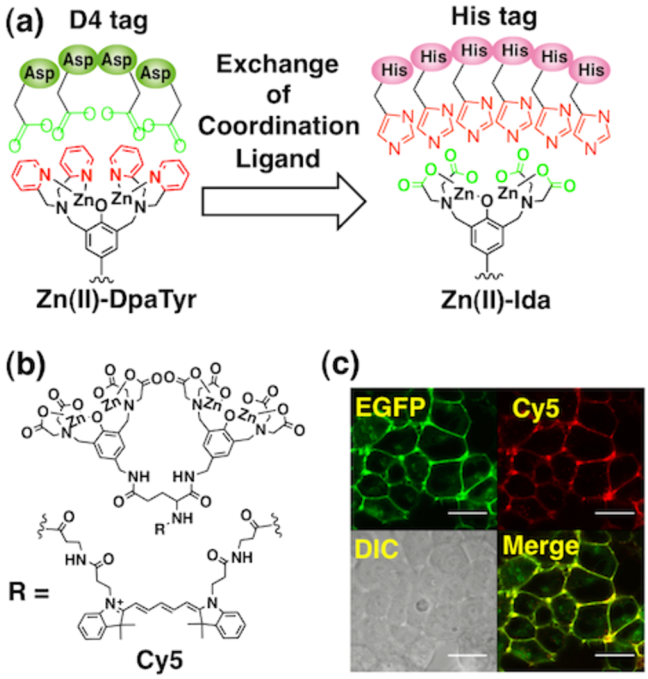


Figure 5. (a) Design strategy of the molecular probe for a His tag based on exchange of the coordination ligand. (b) Structures of Cy5-appended Zn^{II}-Ida probes **4**-8Zn^{II}. (c) Fluorescence imaging of His10-fused B2R on the cell surface using **4**-8Zn^{II}. EGFP was also fused to B2R as a expression fluorescence marker.

3. COVALENT PROTEIN LABELING ASSISTED BY COORDINATION CHEMISTRY

In the previous section, we described the successful applications of coordination-chemistry-based peptide tag/probe pairs to the fluorescence bioimaging of proteins. However, these imaging systems use noncovalent reversible coordination interactions, which cause unfavorable dissociation of the probe from the labeled protein in some cases. Such dissociation apparently makes it difficult to conduct long-term time-lapse imaging and precludes postlabeling analyses such assodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Therefore, we next sought to develop covalent-protein-labeling methods assisted by coordination chemistry.

3-1. Development of a Reactive-Tag System Based on the D4 tag/Zn^{II}-DpaTyr pair. Our covalent-protein-labeling method, termed a "reactive-tag system", was designed using the functional coupling of a chemical reaction with molecular recognition assisted by coordination chemistry.[22] In this labeling system, the designed probe has an electrophilic group with moderate reactivity that can form a covalent bond with a nucleophilic cysteine incorporated in the tag. It was expected that the reaction could be greatly facilitated by forming the binding complex in which the reaction site would be suitably oriented for the nucleophilic substitution reaction (Figure 6a). To develop the reactive-tag system based on the D4-tag/Zn^{II}-DpaTyr pair, an α-chloroacetyl group was introduced to Zn^{II}-DpaTyr as a reaction site with the cysteine-containing D4 tag. The screening of the Cys-containing peptides [Cys-$(Ala)_n$-DDDD, n = 0−8] revealed that their reactivity with the α-chloroacetyl-appended Zn^{II}-DpaTyr (**5**-2Zn^{II}; Figure 6b) largely depended on the number of alanines (Figure 6c). The CA6D4 peptide with six Ala residues showed a remarkably high reactivity, as evidenced by

dx.doi.org/10.1021/ic401612z | Inorg. Chem. 2014, 53, 1816−1823

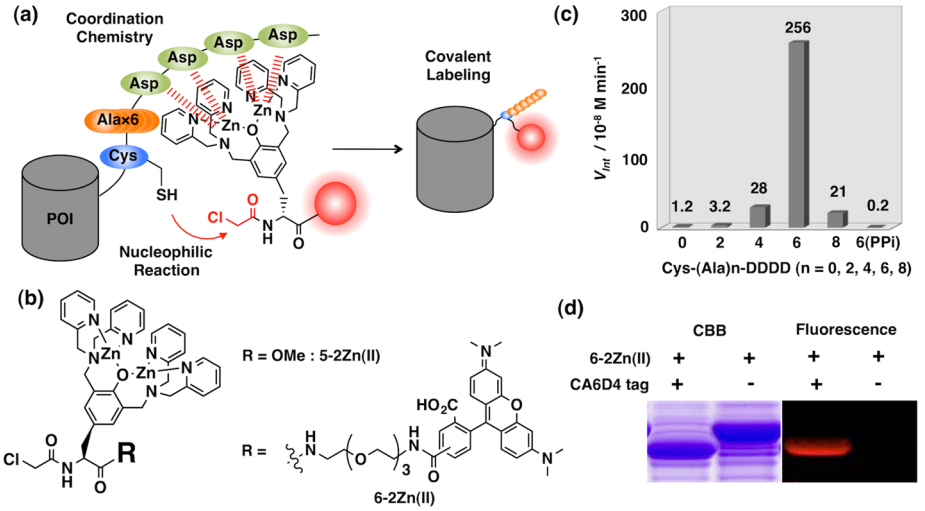


Figure 6. (a) Covalent labeling of POIs by the nucleophilic reaction of a reactive tag/probe pair. (b) Structures of the reactive Zn^{II}-DpaTyr probes. (c) Summary of the initial rate (V_{int}, M min^{-1}) of the labeling reaction of **5**−2Zn^{II} with the CAnD4 peptide (n = 0, 2, 4, 6, 8). 6(PPi) means reaction with the CA6D4 peptide in the presence of 3 mM PPi. (d) Coomassie staining (left) and in-gel fluorescence detection (right) of the labeling of CA6D4-tag-fused MBP with **6**-2Zn^{II} inside *E. coli* cells.

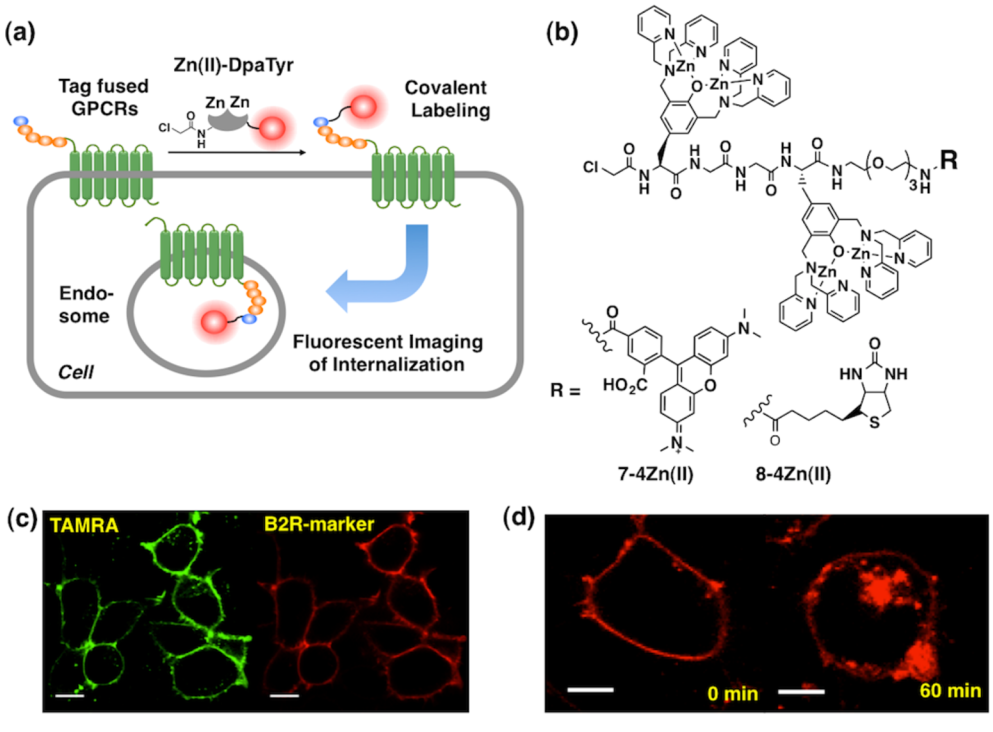


Figure 7. (a) Covalent labeling of the reactive D4-tag-fused proteins on the cell surface. (b) Structures of the Zn^{II}-DpaTyr probes used for on-cell protein labeling. (c) Fluorescence imaging of the tag-fused B2R expressing on a living cell surface. (d) Fluorescence visualization of B2R internalization.

the reaction that proceeded in an over 90% yield within 30 min. The covalent adduct was not observed in the presence of 3 mM inorganic pyrophosphate (PPi), a strong binder for Zn^{II}-DpaTyr even after 2 h. These results indicate that nucleophilic attack of the Cys residue on the chloroacetyl group is facilitated by the tight binding between **5**-2Zn^{II} and the peptide owing to their coordination interaction. The reactive-tag system was also applicable to the labeling of the CA6D4 tag-appended EGFP, which was rapidly modified with the reactive Zn^{II}-DpaTyr probes tethered with various functionalities such as fluorophores, biotin, and poly(ethylene glycol). We also confirmed that the reactive tag worked in *Escherichia coli* cells, where the tag-fused maltose binding protein (MBP) was selectively labeled with the tetramethylrhodamine (TAMRA)-appended probe **6**-2Zn^{II} (Figure 6d).

3-2. Covalent Labeling of GPCR Proteins on a Live Cell Surface. The reactive-tag system was successfully applied to fluorescence imaging and functional analysis of GPCR proteins on live cell surfaces (Figure 7a).[23] For this purpose, the dimer-type Zn^{II}-DpaTyr and the Cys-containing D4×2 tag (CAAAAAADDDDGDDDD) were designed to enhance the binding affinity. When HEK293 cells transiently expressing the

dx.doi.org/10.1021/ic401612z | *Inorg. Chem.* 2014, 53, 1816−1823

tag-fused B2R receptor were treated with the dimer probe **7**-4Zn^{II}, a bright fluorescence due to TAMRA was observed on the cell surfaces (Figure 7b,c). This fluorescence remained after extensive washing with a high concentration of PPi (1 mM), indicating that B2R was covalently labeled. Kinetic analysis of the increased rate of fluorescence on the cell surfaces indicated that the labeling reaction quantitatively proceeded for 30 min. The durable and irreversible covalent labeling by this reactive tag allowed us to conduct time-lapse imaging of GPCR proteins. Thus, the agonist-induced internalization process of the labeled B2R was traced, whereby the fluorescent endosomal particles were gradually accumulated inside the cells in a time-dependent manner (Figure 7d). When using the biotin-appended probe **8**-4Zn^{II} (Figure 7b), the labeled B2R protein was detectable by blotting analysis after the labeling. It should be pointed out that such postlabeling analysis was never accomplished by the noncovalent labeling techniques discussed above. This reactive-tag strategy was extended to other tags containing oligoaspartate sequences such as the FLAG tag (DYKDDDDK).[24] In this reactive-tag system, the Ni^{II}-DpaTyr probe having a thioester unit as an electrophilic counterpart can undergo an acyl-transfer reaction with the Lys residues of the FLAG tag to form the covalent bond of the tag-fused protein. An interesting feature was provided by the fact that the Ni^{II}-DpaTyr moiety is concomitantly released upon probe labeling of the FLAG-tag-fused protein. The utility of this method was demonstrated in the fluorescence imaging of FLAG-tag-fused proteins expressed on a cell surface.

3-3. Development of a His Tag/Ni^{II}-NTA-Based Reactive-Tag System. Given that the His tag/Ni^{II}-NTA pair is orthogonal to the D4 tag/Zn^{II}-DpaTyr pair in terms of its recognition, a reactive-tag orthogonal to the reactive D4 tag/Zn^{II}-DpaTyr pair can be constructed on the basis of the His tag/Ni^{II}-NTA pair.[25] In the protein-labeling and imaging fields, orthogonality between distinct methods is important because it allows the concurrent use of multiple methods such as multicolor fluorescence imaging and pulse-chase labeling to study dynamic cellular processes involving multiple proteins. We thus attempted to develop new reactive-tag systems using the modified Ni^{II}-NTA probe. As an initial attempt, a Ni^{II}-NTA probe **9**-Ni^{II}, which has an electrophilic benzenesulfonyl (so-called tosyl) ester group,[26] was designed (Figure 8a). This probe reacted with the His residues of the His tag through an S_N2-type reaction to form the covalent adduct. An advantage of this labeling system is that the His10-tag-fused protein is labeled with a small-sized functional unit because the Ni^{II}-NTA moiety is concomitantly released upon the substitution reaction. The reaction kinetic analysis reveals that ca. 80% of His10-tag-fused EGFP was labeled within 12 h in a test tube. The orthogonality of this reactive tag against the D4 tag/Zn^{II}-DpaTyr-based reactive tag was tested in a labeling experiment with an *E. coli* cell lysate sample containing His10-fused EGFP and CA6D4-fused MBP. The result showed that the His10-fused EGFP was exclusively labeled with **9**-Ni^{II}, while the CA6D4-fused MBP was exclusively labeled with the Zn^{II}-DpaTyr probe **6**-2Zn^{II} (Figure 8b,c), clearly demonstrating the orthogonality of the two labeling systems.

3-4. In-Cell Covalent Labeling Proteins Using the His Tag/Ni^{II}-NTA-Based Reactive-Tag System. Although we succeeded in the covalent labeling of the tag-fused GPCR protein on living cell surfaces using the reactive D4 tag/Zn^{II}-DpaTyr pair, chemical labeling of intracellular proteins was not achieved by this method. This was mainly because of inhibition

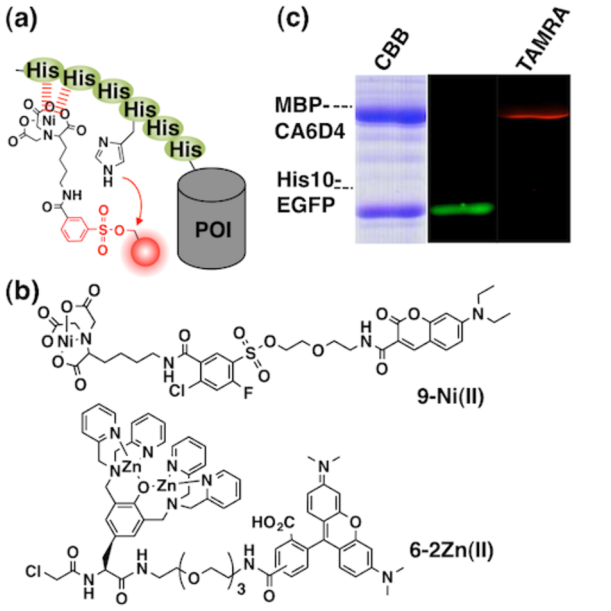


Figure 8. (a) Reactive-tag system using a His tag/Ni^{II}-NTA pair. (b) Structures of the fluorophore-appended Ni^{II}-NTA and Zn^{II}-DpaTyr probe. (c) Coomassie staining (left) and in-gel fluorescence detection (right) of orthogonal covalent labeling of His10-EGFP and CA6D4-MBP with the reactive probe **9**-Ni^{II} and **6**-2Zn^{II} in *E. coli* cell lysates. Coumarine and TAMRA mean that the detection channels correspond to coumarin and TAMRA emission, respectively.

of the interaction between the D4 tag and Zn^{II}-DpaTyr by abundant phosphate species such as adenosine triphosphate (ATP) inside living cells. By contrast, the Ni^{II}-NTA probe can tightly bind to a His-tag-fused protein even in the presence of these phosphate species. The biocompatibility of this pair might allow us to conduct in-cell covalent labeling of proteins (Figure 9a).[27] As a second-generation His reactive-tag system, we designed the cysteine-containing His6 tag (CHHHHHH) and the reactive Ni^{II}-NTA probe having an α-chloroacetyl group. This pair exhibited a rapid covalent bond formation reaction that resulted in approximately 80% of the His-tag (CHHHHHH)-fused EGFP being labeled with **10**-2Ni^{II} within 60 min (Figure 9). One issue in cell protein labeling using this pair was the cell membrane impermeability of the Ni^{II}-NTA probe because of its negatively charged hydrophilic character. We found that the Ni^{II}-NTA probe was effectively delivered into cells by employing a carrier peptide (HHHHRRRRRRRR), which consists of octaarginine (R8), a representative cell penetrating peptide (CPP),[28] and a short tetrahistidine (H4) as a coordination site for the Ni^{II}-NTA. With the help of the carrier peptide, this reactive-tag system was successfully applied to labeling of several intracellular His-tag-fused proteins such as 12-kDa FK506 binding protein (FKBP12), FK506-Rapamycin binding protein (FRB), farnesyl EGFP, and tublin with the biotin-appended probe **11**-2Ni^{II}, as confirmed by Western blotting analysis. Moreover, this labeling method was further applied to the detection of PPI between FKBP12 and FRB inside living cells by using a photo-cross-linker-appended probe **12**-2Ni^{II}. In general, detection of PPIs is difficult by conventional protein-tag techniques because of the large protein tag that might tend to quench the cross-linking reaction and suppress the PPI. The present results explicitly highlighted the benefit of small-sized tags and probes for analyzing in-cell PPIs. The limitation of this method is the

dx.doi.org/10.1021/ic401612z | *Inorg. Chem.* 2014, 53, 1816−1823

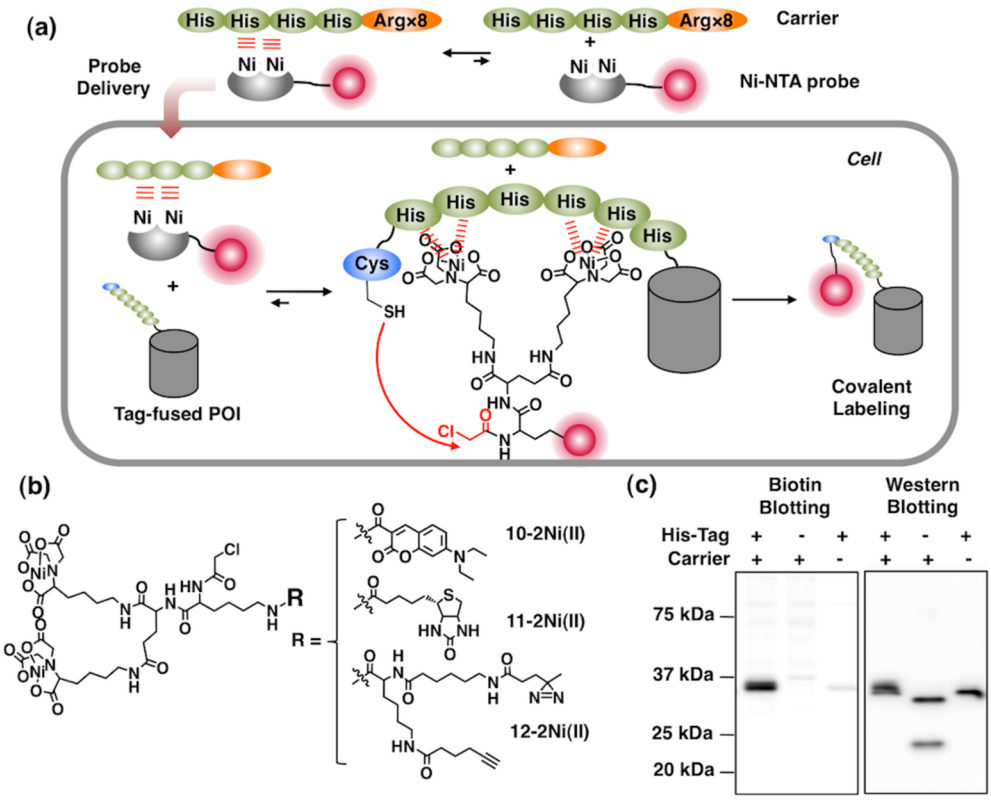


Figure 9. (a) Covalent labeling of the His-tag-fused proteins inside living cells. (b) Structures of the reactive Ni^{II}-NTA probes. (c) Blotting analysis of the in-cell labeling of the reactive His-tag-fused EGFP with **11**-2Ni^{II}. Labeled EGFP was detected by biotin blotting (left) and Western blotting (right) analyses using streptavidin–HRP conjugate and anti-GFP antibody, respectively.

difficulty in washing out the unlabeled Ni-NTA probes from live cells. Therefore, careful optimization of the labeling conditions is needed for protein imaging inside live cells to reduce background fluorescence from the unlabeled probes by controlling the amount of probes introduced into the cells.

4. CONCLUSION

We have described the development and biological applications of coordination-chemistry-based protein-labeling methods using a short peptide tag/probe pair. The characteristics of these methods are summarized in Table 1. It is apparent that the number of useful methods is still very small. From the viewpoint of coordination chemistry, we should point out that only limited metal–ligand interactions have been exploited for their binding affinities. This implies that there are still other ways to develop a more sophisticated molecular recognition system based on the elaborated design of the tag sequence and the probe with an appropriate choice of metal ion. Such efforts should give rise to a highly selective tag/probe pair compatible with the crude biological conditions present not only on the membrane surface but also in the intracellular spaces. In addition to the labeling specificity, the labeling stability is another important factor that is necessary to expand the utility of the protein-labeling methods. It is envisioned that development of the new pairs orthogonal to the known labeling methods enables us to conduct simultaneous analysis of plural distinct proteins, which still remains a challenge in the present research. Progress in both coordination-chemistry-based molecular recognition and protein organic chemistry should help the development of rich chemical tools suitable for obtaining a deeper understanding of the many protein functions present in complicated biological conditions.

Table 1. Coordination-Chemistry-Based Complementary Peptide Tag/Probe Pairs for Selective Protein Labeling

peptide tag	metal ion	probe	affinity (K_d)	labeling conditions
TetraCys tag	As^{III}	FlAsH	4–70 pM	test tube/membrane/ intracellular
His tag	Ni^{II}	NTA	13 μM^a	test tube/membrane
His reactive tag	Ni^{II}	NTA		test tube/membrane/ intracellular
His tag	Zn^{II}	His-Zifit	40 nM	test tube/membrane
His tag	Zn^{II}	Ida	3200 μM^a	test tube/membrane
D4 Tag	Zn^{II}	DpaTyr	14 μM^a	test tube/membrane
D4 reactive tag	Zn^{II}	DpaTyr		test tube/membrane
LBT	Tb^{III}		19–220 nM	test tube/cell lysates

[a]The peptide tag and probe were designed with multiple metal-binding units to improve the association constant.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ihamachi@sbchem.kyoto-u.ac.jp.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) (a) Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y. *Nat. Methods* **2005**, *2*, 905–909. (b) Schwartz, J. L.; Snapp, E.; Kenworthy, A. *Nat.*

Rev. Mol. Cell Biol. **2001**, *2*, 444−456. (c) Shaner, N. C.; Patterson, G. H.; Davidson, M. W. *J. Cell Sci.* **2007**, *120*, 4247−4260.
(2) (a) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974−6998. (b) Hinner, M. J.; Johnsson, K. *Curr. Opin. Biotechnol.* **2010**, *21*, 766−776. (c) Takaoka, Y.; Ojida, A.; Hamachi, I. *Angew. Chem., Int. Ed.* **2013**, *52*, 2−21. (d) Soh, H. *Sensors* **2008**, *8*, 1004−1024.
(3) (a) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86−89. (b) Lukinavičius, G.; Umezawa, K.; Olivier, N.; Honigmann, A.; Yang, G.; Plass, T.; Mueller, V.; Reymond, L.; Corrêa, I. R., Jr.; Luo, Z. G.; Schultz, C.; Lemke, E. A.; Heppenstall, P.; Eggeling, C.; Manley, S.; Johnsson, K. *Nat. Chem.* **2013**, *5*, 132−139.
(4) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, G. M.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ACS Chem.* **2008**, *6*, 373−382.
(5) George, N.; Pick, H.; Vogel, H.; Johnsson, N.; Johnsson, K. *J. Am. Chem. Soc.* **2004**, *126*, 8896−8897.
(6) Popp, M. W.; Antos, J. M.; Grotenbreg, G. M.; Spooner, E.; Ploegh, H. L. *Nat. Chem. Biol.* **2007**, *3*, 707−708.
(7) Suarez, M. F.; Baruah, H.; Hernandez, L. M.; Xie, K. T.; Baskin, J. M.; Bertozzi, C. R.; Ting, A. Y. *Nat. Biotechnol.* **2007**, *25*, 1483−1487.
(8) (a) Ojida, A.; Takashima, I.; Kohira, T.; Nonaka, H.; Hamachi, I. *J. Am. Chem. Soc.* **2008**, *130*, 12095−12101. (b) Sakamoto, T.; Ojida, A.; Hamachi, I. *Chem. Commun.* **2009**, 141−152. (c) Ojida, A.; Sakamoto, T.; Inoue, M.; Fujishima, S.; Lipens, G.; Hamachi, I. *J. Am. Chem. Soc.* **2009**, *131*, 6543−6548.
(9) Hochuli, E.; Dobeli, H.; Schacher, A. *J. Chromatogr.* **1987**, *411*, 177−184.
(10) (a) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269−272. (b) Adams, A. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063−6076. (c) Gaietta, G.; Deerinck, T. J.; Adams, S. A.; Bouwer, J.; Tour, O.; Laird, D. W.; Sosinsky, G. E.; Tsien, R. Y.; Ellisman, M. H. *Science* **2002**, *296*, 503−507.
(11) Tour, O.; Meijer, R. M.; Zacharias, D. A.; Adams, S. R.; Tsien, R. Y. *Nat. Biotechnol.* **2003**, *21*, 1505−1508.
(12) Tour, O.; Adams, S. R.; Kerr, R. A.; Meijer, R. M.; Sejnowski, T. J.; Tsien, R. W.; Tsien, R. Y. *Nat. Chem. Biol.* **2007**, *3*, 423−431.
(13) (a) Liu, B.; Archer, C. T.; Burdine, L.; Gillette, T. G.; Kodadek, T. *J. Am. Chem. Soc.* **2007**, *129*, 12348−12349. (b) Luedtke, N. W.; Dexter, R. J.; Fried, D. B.; Schepartz, A. *Nat. Chem. Biol.* **2007**, *3*, 779−784. (c) Rutkowska, A.; Haering, C. H; Schultz, C. *Angew. Chem., Int. Ed.* **2011**, *50*, 12655−12658.
(14) (a) Guignet, E. G.; Hovius, R.; Vogel, H. *Nat. Biotechnol.* **2004**, *22*, 440−444. (b) Goldsmith, C. R.; Jaworski, J.; Sheng, M.; Lippard, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 418−419.
(15) (a) Lata, S.; Reichel, A.; Brock, R.; Tampe, R.; Piehler, J. *J. Am. Chem. Soc.* **2005**, *127*, 10205−10215. (b) Lata, S.; Gavuits, M.; Tampe, R.; Piehler, J. *J. Am. Chem. Soc.* **2006**, *128*, 2365−2372.
(16) Hauser, C. T.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3693−3697.
(17) Franz, K. J.; Nitz, M.; Imperiali, B. *ChemBioChem* **2003**, *4*, 265−271.
(18) Sculimbrene, B. R.; Imperiali, B. *J. Am. Chem. Soc.* **2006**, *128*, 7346−7352.
(19) Ojida, A.; Honda, K.; Shinmi, D.; Kiyonaka, S.; Mori, Y.; Hamachi, I. *J. Am. Chem. Soc.* **2006**, *128*, 10452−10459.
(20) Ojida, A.; Fujishima, S.; Honda, K.; Nonaka, H.; Uchinomiya, S.; Hamachi, I. *Chem.—Asian J.* **2010**, *5*, 877−886.
(21) Fujishima, S.; Nonaka, H.; Uchinomiya, S.; Kawase, Y. A.; Ojida, A.; Hamachi, I. *Chem. Commun.* **2012**, 594−596.
(22) Nonaka, H.; Tsukiji, S.; Ojida, A.; Hamachi, I. *J. Am. Chem. Soc.* **2007**, *129*, 15777−15779.
(23) Nonaka, H.; Fujishima, S.; Uchinomiya, S.; Ojida, A.; Hamachi, I. *J. Am. Chem. Soc.* **2010**, *132*, 9301−9309.
(24) Nonaka, H.; Fujishima, S.; Uchinomiya, S.; Ojida, A.; Hamachi, I. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6696−6699.
(25) Uchinomiya, S.; Nonaka, H.; Fujishima, S.; Tsukiji, S.; Ojida, A.; Hamachi, I. *Chem. Commun.* **2009**, 5880−5882.
(26) Tsukiji, S.; Miyagawa, M.; Takaoka, Y.; Tamura, T.; Hamachi, I. *Nat. Chem. Biol.* **2009**, *5*, 341−343.
(27) Uchinomiya, S.; Nonaka, H.; Wakayama, S.; Ojida, A.; Hamachi, I. *Chem. Commun.* **2013**, *49*, 5022−5024.
(28) Takeuchi, T.; Kosuge, M.; Tadokoro, A.; Sugiura, Y.; Nishi, M.; Kawata, M.; Sakai, N.; Matile, S.; Futaki, S. *ACS Chem. Biol.* **2006**, *1*, 299−303.