

Protein functional analysis and proteomics
by novel chemical labeling

Takayuki Miki

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Preface and Acknowledgements

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General Introduction

Introduction

Numerous proteins play significant roles in many physiological responses and are building complex systems in our bodies. To provide insights into mysterious biological systems, two different (bidirectional) approaches are presented (**Fig. 1**). In the first approach (protein analysis), a protein of interest is studied in its biological roles, such as enzymatic function, ligand-binding, protein-protein interaction, localization, and movement. In the other approach (focused proteomics), a particular biological event is focused and proteins involved in the event are analyzed and identified.

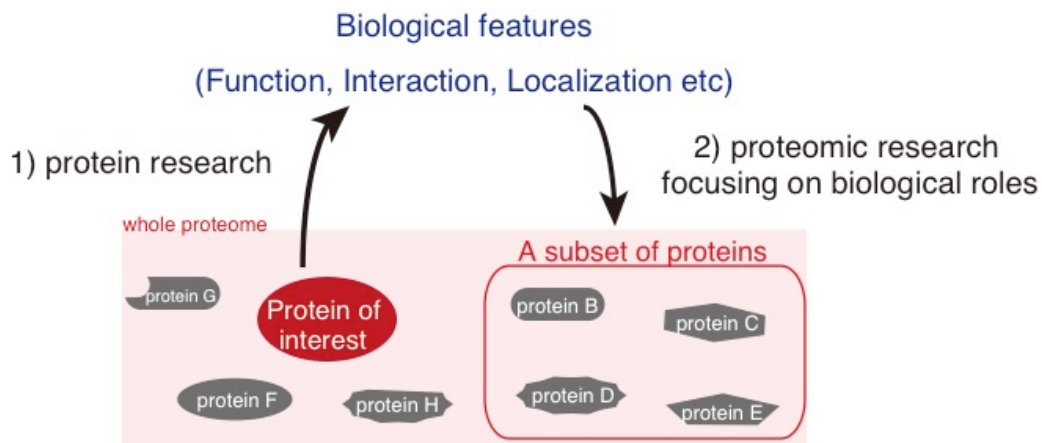


Figure 1. Two different approaches to evaluate the relationships between protein and its biological features.

Biological systems often fluctuate spatio-temporally in response to intra- or extracellular environmental changes. Accompanied with the dynamics, the biological role of each protein is precisely regulated. For instance, kinase families are activated by phosphorylation¹, and some enzymes associated with metabolic processes are modulated by metabolites^{2,3}, as allosteric modulators. Membrane proteins, such as GPCR, are internalized in response to the agonist binding, resulting in the desensitization⁴. Therefore, these roles are quite susceptible to experimental artifacts and must be evaluated in native contexts. To sidestep this problem, various techniques have been developed to date in both approaches, a single protein analysis and

proteomics. In such background, chemical strategies for protein labeling have been recognized as powerful tools for both approaches. Chemical labeling offers a great advantage that the intact proteins are labeled and analyzed in native contexts with minimum perturbation. Here, I will focus on the chemical strategies.

Genetical and/or chemical approaches for specific protein labeling and imaging

Fluorescent protein techniques

Evaluation of biological roles of proteins substantially contributed to unveil physiological events. One of the world-widely applied probes for molecular imaging is genetically encoded fluorescent proteins (FPs)⁵ (**Fig. 2**). In this method, a protein of interest is genetically fused to FP, and the dynamics is monitored by the fluorescent reporters. The diversity of FPs in fluorescent wavelengths enables multi-color imaging^{6,7}, expanding the utility to the more complex experiments. For instance, FRET (Förster resonance energy transfer)-based approaches enable dynamic observation of protein-protein interactions, conformational change of protein, enzymatic activity, and so on in living systems^{5,8}. Moreover, genetic engineering efforts have developed a various FPs with attractive properties. Miyawaki group has developed a photo-activatable FP named “Dronpa”⁹, revealing the fast dynamics of ERK (extracellular signal-regulated kinase). For studies of protein interactions, Kerppola group constructed a bimolecular fluorescence complementation (BiFC) assay^{10,11} based on complementation of two fragments of YFP (yellow fluorescent protein). Rothman group has developed pH-sensitive GFP (“pHlurins”)¹², visualizing protein localization in acidic vesicles in neuron. The various FPs are exploited to evaluate biological roles of protein in various aspects.

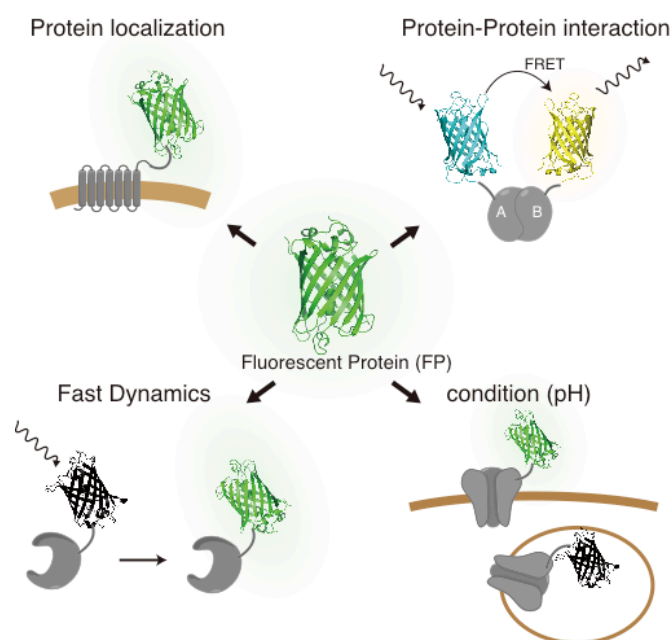


Figure 2. Engineered GFP derivatives for evaluation of protein functions.

Genetically encoded tag techniques in combination with synthetic probes

In recent years, tag-based technologies in combination with synthetic probes have also been actively developed¹³. These are expected to improve two crucial limitations of the FP methods. First, synthetic probes can be used for diverse objectives such as fluorescent imaging, cross-linking, biotin-tagging for immune precipitation, while FPs offer only fluorescent modality probe. Second, the behavior of FP-fusions is sometimes distinct from that of intact proteins in case due to its large size of FPs (27 kDa).

Tag-proteins make covalent bounds to their ligands in living system, providing bio-orthogonal reactions to achieve specific labeling of a tag-fused protein of interest. As a representative, K. Johnsson group has developed a SNAP tag¹⁴, a modified variant of *O*⁶-guanine nucleotide alkyltransferase that irreversibly reacts with *O*⁶-benzyl guanine (**Fig. 3a**). The SNAP tag can be applicable in living cell and *in vivo* to observe protein localization, and pulse-chase analyses are performed to study its protein half-life (**Fig. 3b-c**)^{15,16}. Due to the attractive property of its small size (2/3 size

of GFP), SNAP-tag is compatible with time-resolved FRET approach, and Pin group succeeded to obtain evidence for the GPCR oligomerization states on living cell surface (**Fig. 3d**)¹⁷. Recently, this technique is expanded to the application for drug screening assay and quantification of concentration of drug¹⁸. Other tag-protein, CLIP tag, Halo tag¹⁹, BL tag²⁰ and TMP tag²¹ also have been developed. Among them, SNAP tag and Halo tag are commercially available and applied world-widely. And, these combinations are also utilized for multi-labeling experiments.

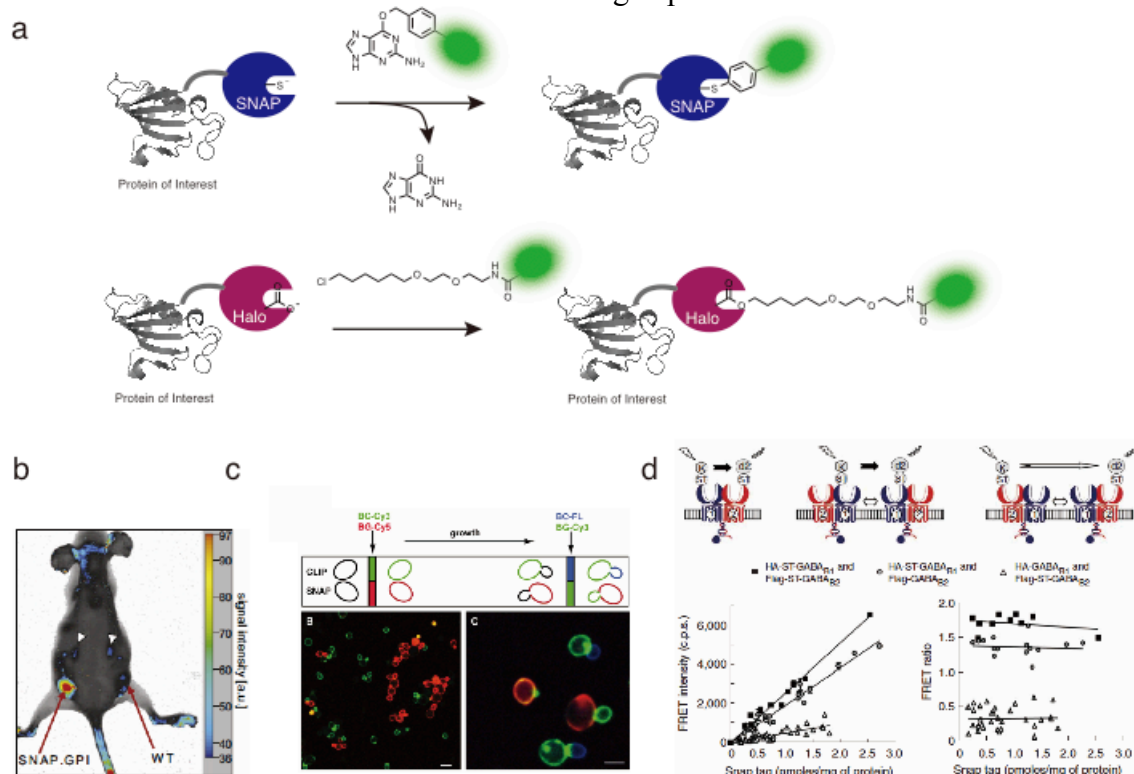


Figure 3. Genetically encoded tag-protein techniques for visualizing a target protein. (a) Schematic illustrations of chemical reactions of SNAP-tag (up) and Halo-tag (bottom). (b) Labeling SNAP-fused proteins *in vivo*¹⁵. (c) Multi-color pulse-chase imaging of two different proteins¹⁶. (d) Detection of SNAP-fused GABA_B dimers by FRET signals on live cells. The SNAP-GABA_{B1} and SNAP-GABA_{B2} are labeled by two different fluorescent probes¹⁷.

In addition to tag-protein techniques, alternative labeling methods using short peptide tags have been developed. FLaSH tag^{22,23}, the firstly reported peptide tag designed by Tsien group, tightly bind to short peptides containing a core motif

(CCXXCC) in living cell (**Fig. 4a**). Hamachi group also developed reactive D4-tag²⁴ and His-tag²⁵ for selective covalent labeling (**Fig. 4b**). While these tag-peptide techniques are based on metal-peptide complex formation, Alice Ting group has developed enzyme-mediated labeling methods for short-peptide tethered proteins of interest²⁶.

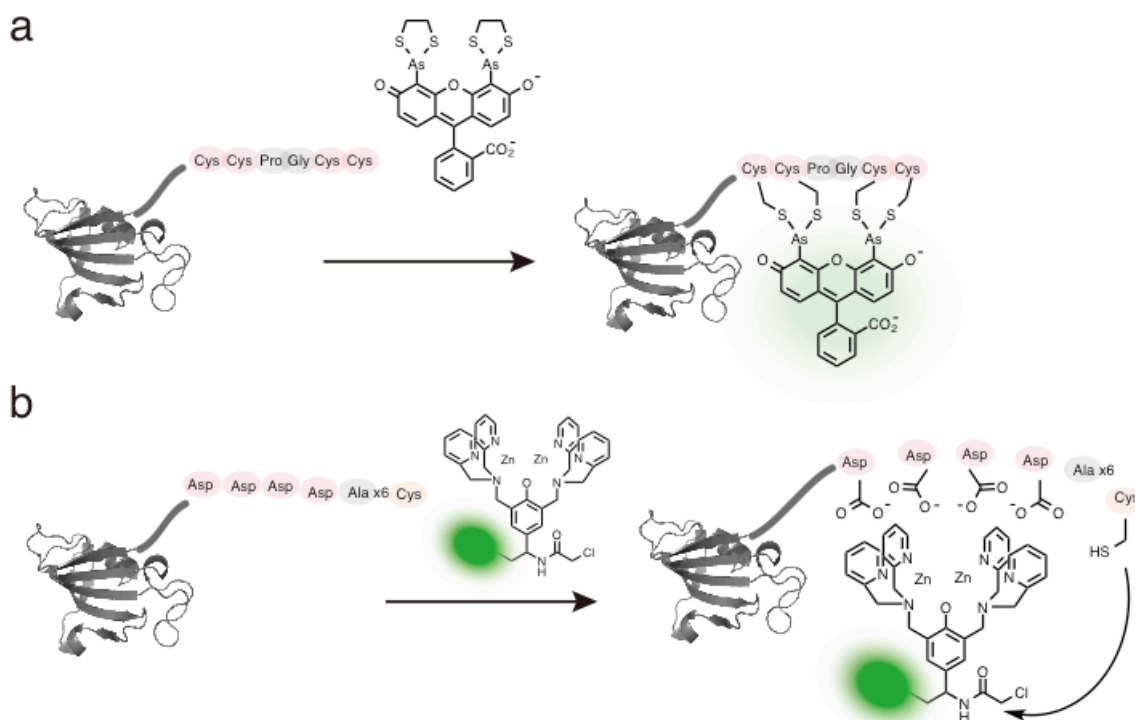


Figure 4. Schematic representations of labeling of genetically tag-fused proteins. (a) FLAsH-peptide complex formation. (b) Covalent labeling of D4 tag-fused proteins via a complex formation.

Bio-orthogonal reaction with unnatural amino acid incorporated into target protein

One of the biggest disadvantages of above methods is that an additional protein- or peptide- tag has to be fused or inserted into a protein of interest. This limits the precise site-specific modification, and these structural changes may sometimes disturb their functions. To address these problems, several groups incorporated unnatural amino acids into target proteins for site-specific protein labeling by using the orthogonal tRNA synthetase-tRNA pairs^{27,28}. Jason Chin group shows that the

genetically encoded unnatural amino acids, containing a norbornene, allow the site specific labeling with synthetic probes via an inverse electron-demand Diels-Alder cycloaddition²⁹. In same manner, Ben Davis group reported Suzuki-Miyaura Pd(0) mediated coupling reaction for labeling a residue on protein surface³⁰. Qing Lin group succeeded in Sonogashira Cross-Coupling for site specific labeling of proteins bearing an alkyne tag^{31,32}. However, these techniques require not only the coexpression of orthogonal tRNA synthetase-tRNA pairs and protein of interest, but also the addition of unnatural amino acids, and the treatment with synthetic probes, which may perturb natural conditions of live cells³³.

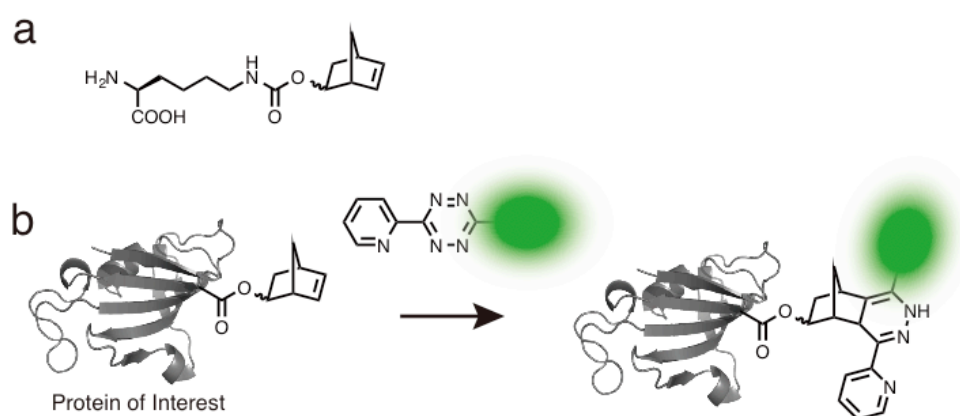


Figure 5. Site-specific labeling via a rapid inverse electron-demand Diels-Alder cycloaddition. (a) Chemical structure of an unnatural amino acid bearing norbornene. (b) Schematic illustration of the labeling reaction.

Chemical approaches for specific protein labeling

All methods mentioned above require genetic manipulations and the observed proteins are not intact and endogenous ones. The artificial over-expression of engineered proteins perturbs the native context and cause artificial localization or protein-interactions especially in case of proteins consisting a hetero-dimer or oligomer. The recent explosion in diverse of powerful genome editing technologies, such as CRISPR/Cas system³⁴, enables to express the edited protein in endogenous level. However, besides these techniques are reducing these problems, there are a numerous

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samples, which genome not able to be edited, such as excised organs from patients. Therefore, chemical-based approaches without any genetic manipulations are necessary for specific labeling.

Hamachi and his colleges have developed Ligand-directed chemical labeling methods³⁵. As a representative, LDT (Ligand directed Tosyl) chemistry is widely used for visualizing endogenous proteins, the kinetic studies of ligand-binding, and identification of interaction sites between a target protein and interaction sites³⁶⁻³⁹. The manipulation is quite simple, that is the addition of a labeling reagent, in which a ligand is conjugated to a functional probe via phenyl sulfonyl ester as an electrophilic group. The reagent makes a physiological complex with the target protein, and then the S_N2 reaction with nucleophilic amino acids (His, Tyr, Ser, Glu, Cys etc) on protein surface is accelerated by the proximity effects. In this method, the ligand moiety can be removed by washing, resulting in trackless modification. The utility of the method were also demonstrated in labeling of an endogenous CA (carbonic anhydrase) in a living mouse body³⁷.

In the series of ligand-directed labeling, LDAI (ligand-directed acyl imidazole) chemistry⁴⁰ is lately developed. LDAI reagents consist of an acyl imidazole electrophile instead of Tosyl group. Dr. Fujishima demonstrated that the LDAI is also applicable to an endogenous membrane protein, folate receptor, and succeeded in kinetic studies of ligand-binding in living cells. The other various methods based on chemical labeling have been developed from his group and other groups^{41,42}. One of the most different points between chemical approaches and genetic methods is that the intact proteins that has already expressed, can be labeled in the former cases.

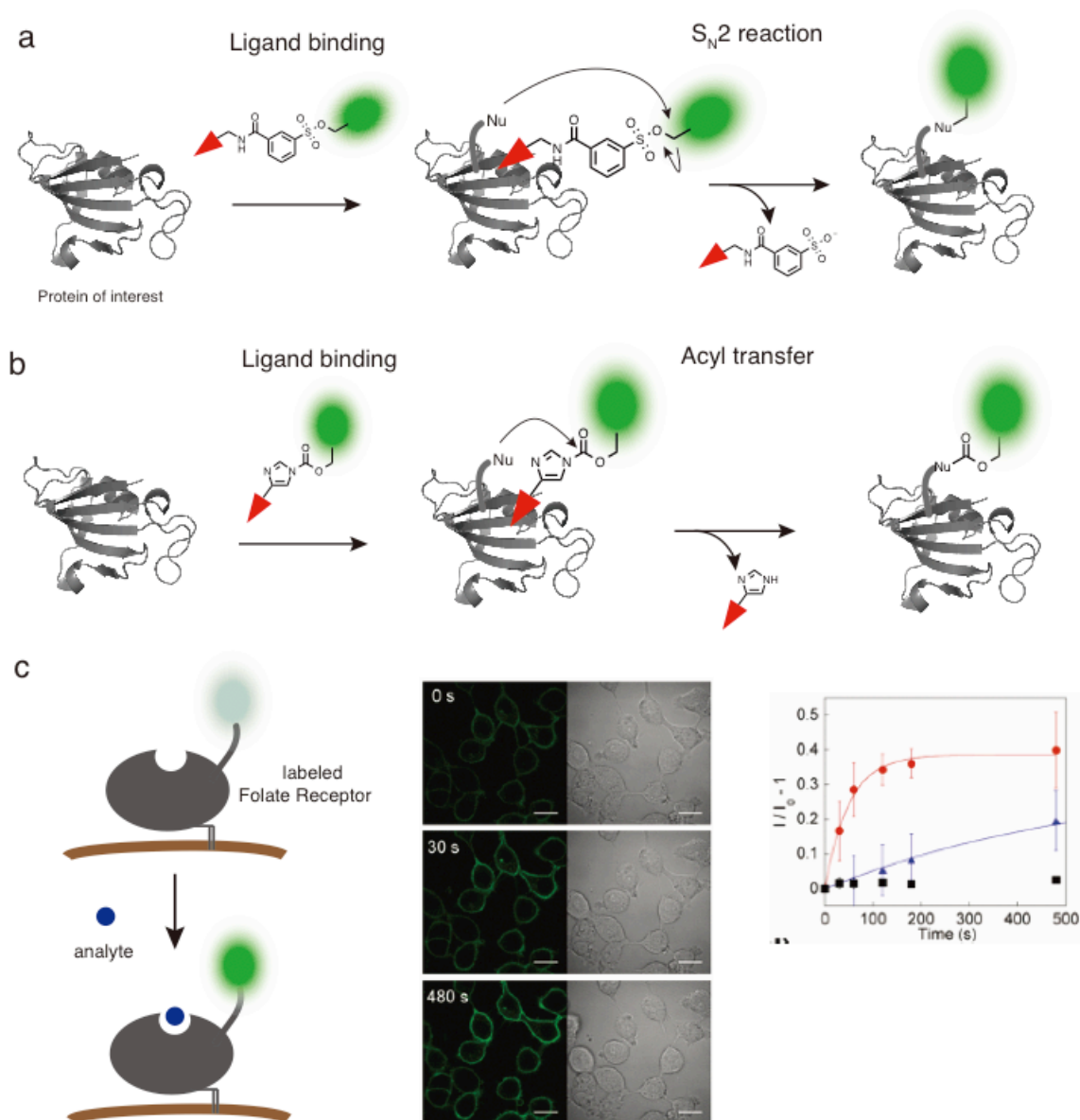


Figure 6. Ligand-directed protein labeling. (a) Scheme for LDT chemistry. (b) Scheme for LDAI chemistry. (c) Kinetic studies of ligand-protein binding on living cells. The fluorescent signal of the labeled fluorescein on the folate receptor is responsive to the analyte-binding⁴⁰.

Chemical labeling for proteomic research

For understanding proteins (key players) involved in a specific biological function, the “focused proteomics” approach is powerful. In this approach, a subset of proteins related to a biological question that you are interested in is extracted or enriched from whole proteome, and identified by using mass spectrometry. In terms of focused proteomics, antibody-based methods have conventionally been used to target proteins, these complexes, or vesicles from homogenized cells. In particular, chemical proteomics is now recognized as a powerful method to offer large amounts of information about various protein features in a native context, where the proteome of interest is labeled with chemical tags in living cells, which can facilitate a precisely focused proteomics (**Fig. 7**). Currently, various chemical tagging techniques have been developed to categorize and annotate proteins in various aspects. Here I will describe several examples of chemical techniques.

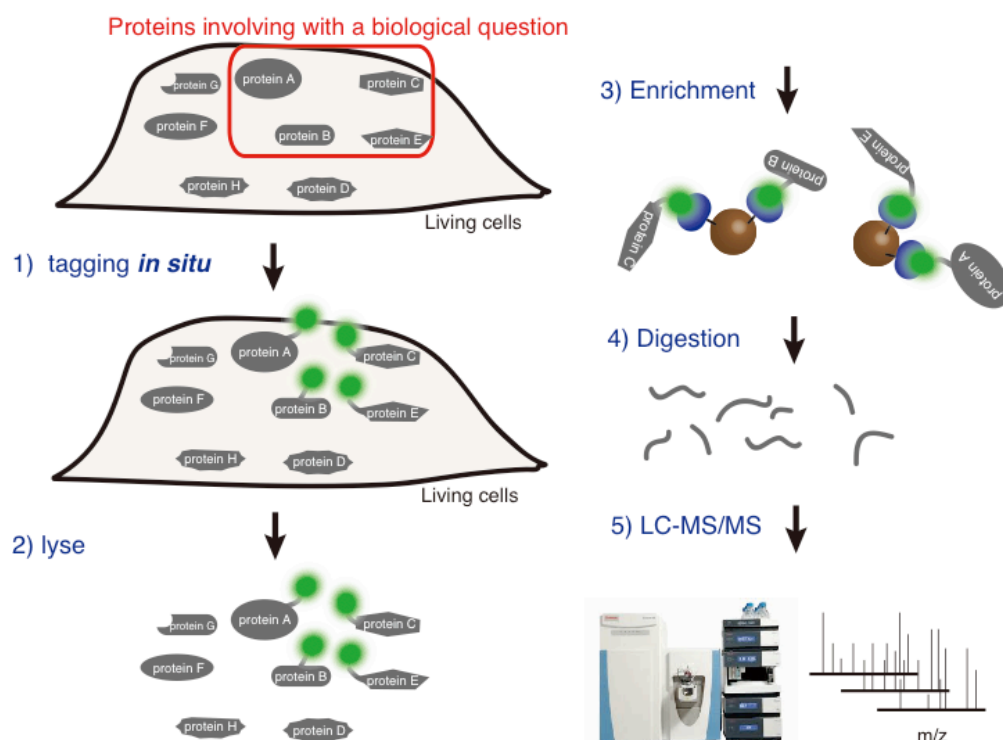


Figure 7. Workflow of focused proteomics. A subset of proteins related to a biological question, are tagged and analyzed by a conventional peptide mass fingerprinting method.

Focusing on protein function and activity

Activity-based protein profiling pioneered by Cravatt and coworkers, facilitates function-based assignment such as specific enzymatic activity. In these methods, chemical probes consist of an irreversible inhibitor and a chemical tag, such as a fluorescent dye or alkyne tag. The probes label proteins based on their catalytic properties. For example, they focused on serine hydrolase families, and labeled them by the ABPP probe⁴³ in which, a fluorophosphonate irreversible inhibitor is linked to a fluorescein, reporters group. The families are labeled by their own hydrolase activity. The ABPP combined with quantitative MS^{44,45} enables to perform a proteome-wide analysis. For identifying the targets of covalent kinase inhibitors that form a bond with a nucleophilic residue in ATP binding pocket, the labeled proteins are identified by quantitative MS analysis⁴⁶ (**Fig. 8a**). In this report, they discovered the off-targets of the inhibitors (**Fig. 8b-c**). Up to now, various ABPP probes have been developed for enzyme families^{47,48}.

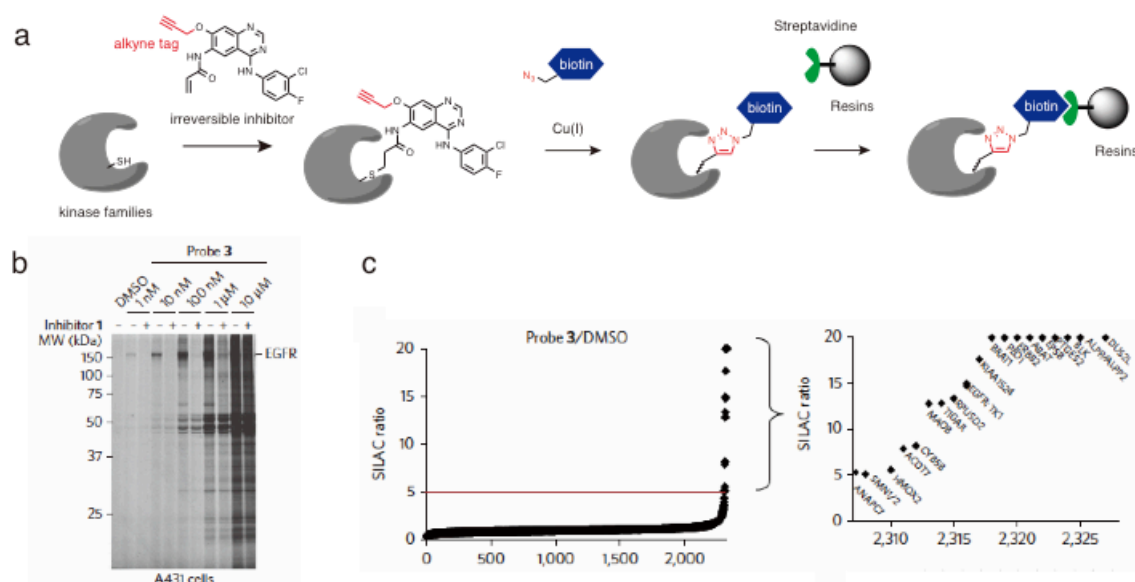


Figure 8, The ABPP methods with quantitative MS spectrometry. (a) The kinase families are labeled with alkyne tags by their own catalytic activity, conjugated with biotins or fluorescent tags by click reaction, and enriched by pull-down manipulation. (b) The labeled proteins are monitored by SDS-PAGE and in-gel fluorescent imaging. (c) Quantitative MS spectrometry of the labeled proteins. Comparing with the negative controls, the catalytic active proteins are identified⁴⁶.

Focusing on proteins-ligand physiological interaction

To analyze the proteins interacting with a target molecule, photo-affinity labeling has been conventionally applied. Photo-activatable group such as benzophenone and diazirine in probes generates an active intermediate to form a covalent bond with the physiologically interacting proteins⁴⁹. For instance, Cravatt group demonstrated that lipid type of probes determined protein targets in live cells (**Fig. 9**)^{50,51}. Other groups have reported a variety of probes⁵².

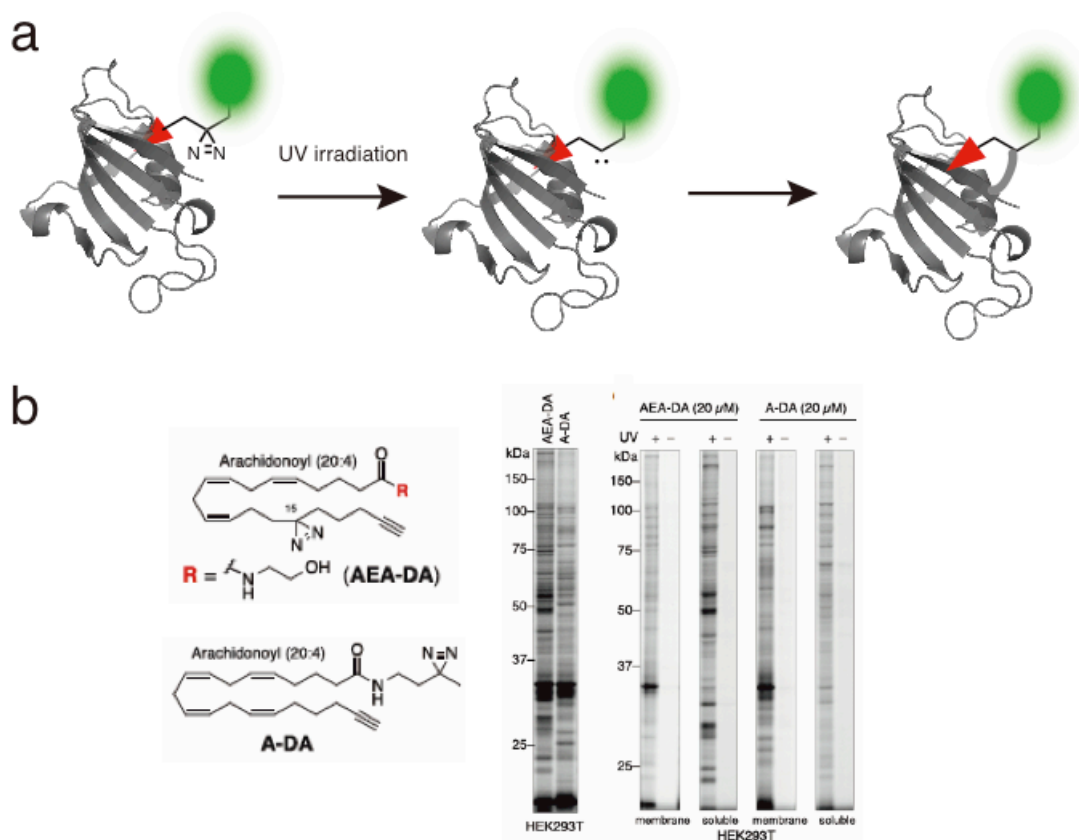


Figure 9, Photo-affinity labeling for identifying the interacting proteins. (a) Schematic illustration of chemical labeling using a diazirine group. **(b)** Mapping the lipid-interacting proteome

Focusing on local spaces

Recently, Alice Ting and her coworkers reported a spatially restricted enzymatic tagging for identifying what proteins localized in the space. The engineered ascorbate peroxidase (APEX)^{53,54} as a genetic reporter is expressed in mitochondria, and the tagging were performed by addition of phenol-biotin and H₂O₂ (**Fig. 10a**). The phenol-biotins are converted into reactive phenyl radical species catalyzed by H₂O₂/APEX and labeled proteins in the local space. They identified 495 proteins within the mitochondrial matrix, including 31 not previously annotated as mitochondrial localization (**Fig. 10b**). This technique is applicable in live *Drosophila* tissues⁵⁵.

Tirrell group reported a cell-specific proteomic analysis in *C. elegans*⁵⁶. The subset of proteins in specific cells is metabolic labeled with unnatural amino acid by construction of a engineered *C. elegans*. The proteins bearing azide group were conjugated with a biotin by bioorthogonal reaction, enriched and identified. These spatial information are readily vanished by the following lyse process of conventional experimental protocol. Therefore, these methods tagging proteins in living states are powerful.

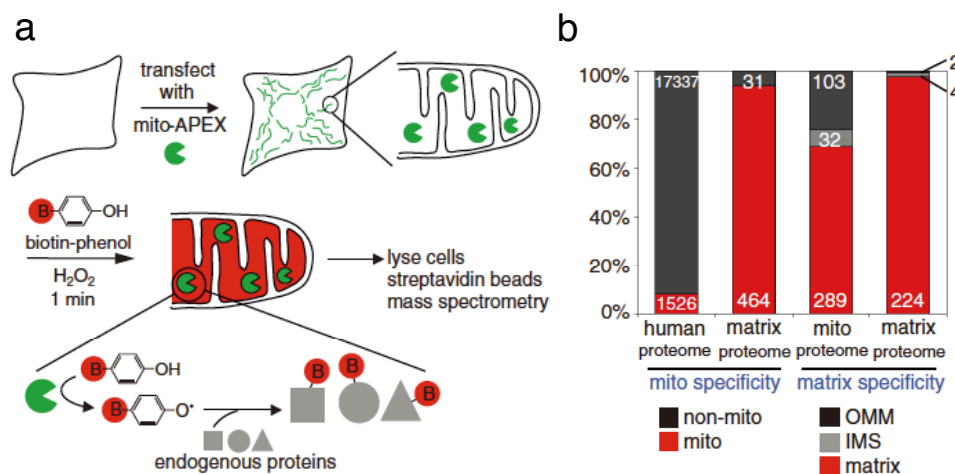


Figure 10. Spatially restricted proteomics via engineered APEX⁵³. (a) The work flow chart of the spatial proteomic. (b) The proteins localizing in matrix are highly enriched in the identified proteome.

Focusing on post-translation modifications

Protein glycosylation also plays an essential role in biological phenomena. However, the heterogenous modification complicates the proteome-wide analysis. Bertozzi and her coworkers have developed IsoTaG (Isotope-targeted glycoproteomics)⁵⁷ as a mass independent platform for intact glycopeptide discovery (**Fig. 11a**). This comprises the metabolic labeling with azide-containing glycan, isotope-tagging, enrichment, and mass spectrometry step. The MS² and MS³ charts offer the information about the peptide sequence and the modification fragments. In this report, they identified 32 intact N-glycopeptide and over 500 intact glycopeptides from 250 glycoproteins. Hamachi group also achieved to label and profile glycoproteins on living cell surfaces by using DMAP-tethered lectin catalyst⁵⁸ (**Fig. 11b**). The semi-synthetic probe can bind to the glycoproteins, and catalyze the acyl-transfer reaction on the protein surfaces.

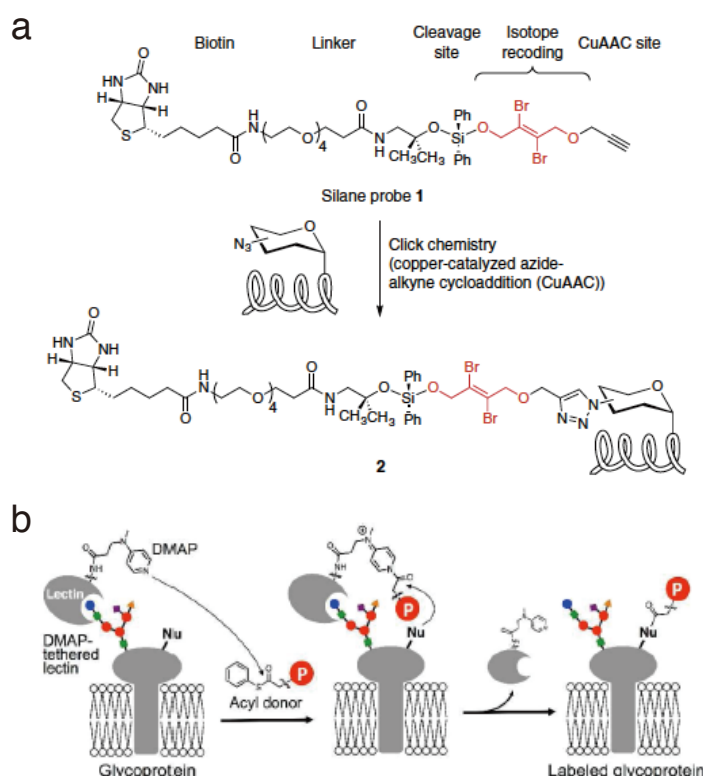


Figure 11. Glycoprotein focused proteomics. (a) Chemical labeling of glycoproteins with IsoTaG. The metabolic labeled glycoproteins are tagged, digested and analyzed by MS, MS² and MS³ analysis⁵⁷. (b) Schematic representation of glycoprotein labeling by a

DMAP-tethered lectin⁵⁸.

These reports highlight that chemical strategies have been developed for proteome-wide analysis, offering the various insights into biological function and roles of proteins.

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Summary of thesis

When I focus on chemical based approach, including ligand-directed chemical labeling, the selectivity is mainly based on the ligand moiety (**Figure 12**). And, the engaged reaction mechanism is not bioorthogonal such as S_N2 reaction, acyl transfer reaction and so on. However, I doubted that the selectivity of the protein labeling depends only on the selectivity of ligand binding. In chapter 1, I investigated the isozyme-selectivity in LDAI chemical labeling, and revealing that the chemical reaction also contributes to enhance the selectivity. In other words, the LDAI reagent can bind to different proteins, yet the reactions are facilitated in only one protein. I reasoned this selectivity as the different number of nucleophilic amino acid near the active site. In chapter 2, I demonstrated the utility of LDAI labeling for tracking intact membrane proteins and succeeded in pulse-chase analysis to determine the half-lives and degradation pathway.

Focusing on the selectivity of chemical labeling mechanism once again, the ligand moiety of reagents is crucial for selective labeling. If the ligand-protein interaction is highly selective comparable to bioorthogonal, the labeling reaction must be specific. Conversely, when I used biological abundant compounds such as a sugar, lipid and nucleotide, the protein labeling may not be specific but selective, and it becomes a proteome-wide labeling. In aspect of focused-proteomes, this ligand moiety is also considered as a moiety focusing the proteins interacting with a ligand. In chapter 3, I expanded the LDT chemical labeling to a proteome-wide search for sterol-interacting proteomics. To validate this LDT-based proteomic research, I applied this methods to nematode as a model of *in vivo* experiment.

Considering the diversity of focused proteomics, I thought the focused proteomics should not be restricted in functional assignment, and it should be expanded. In chapter 4, I converted the ligand moiety into a metal chelator to develop a

zinc-responsive labeling reagent. By using the reagent, I successfully demonstrated a novel “conditional proteomics” that chemical proteomics focusing on Zn^{2+} distribution. I envision the selective moiety will be more expanded to selective labeling to other native conditions, and features.

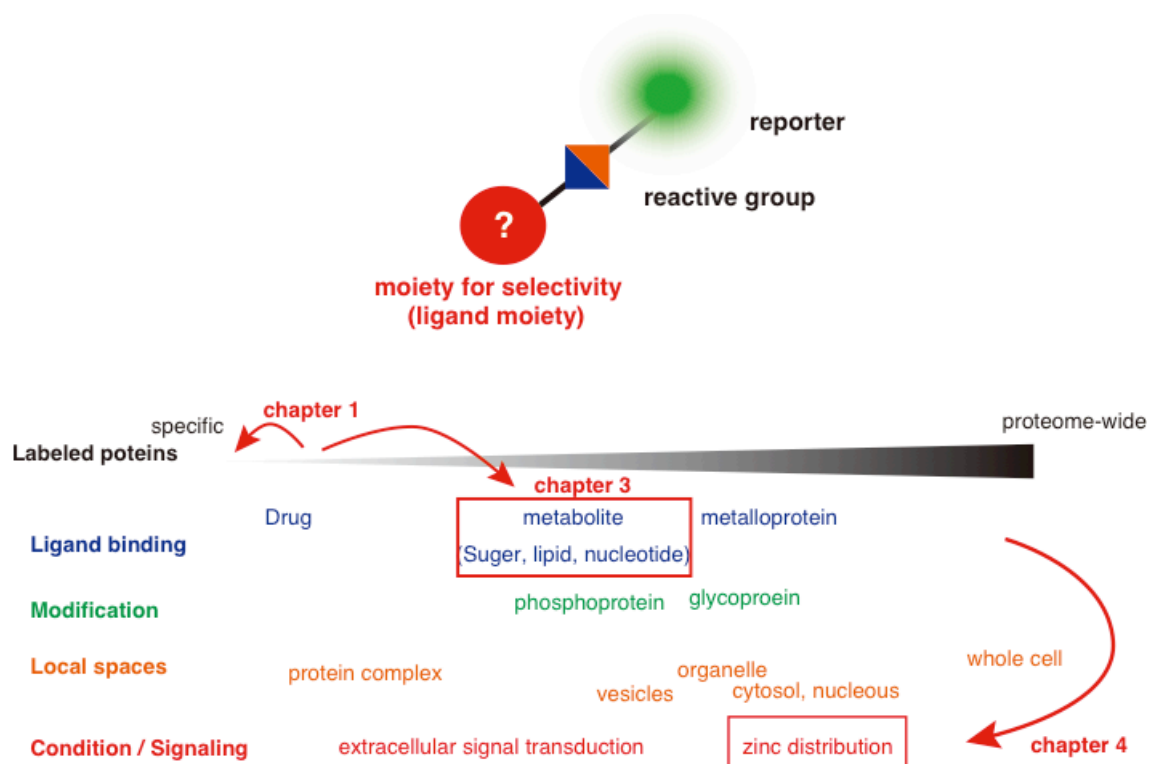


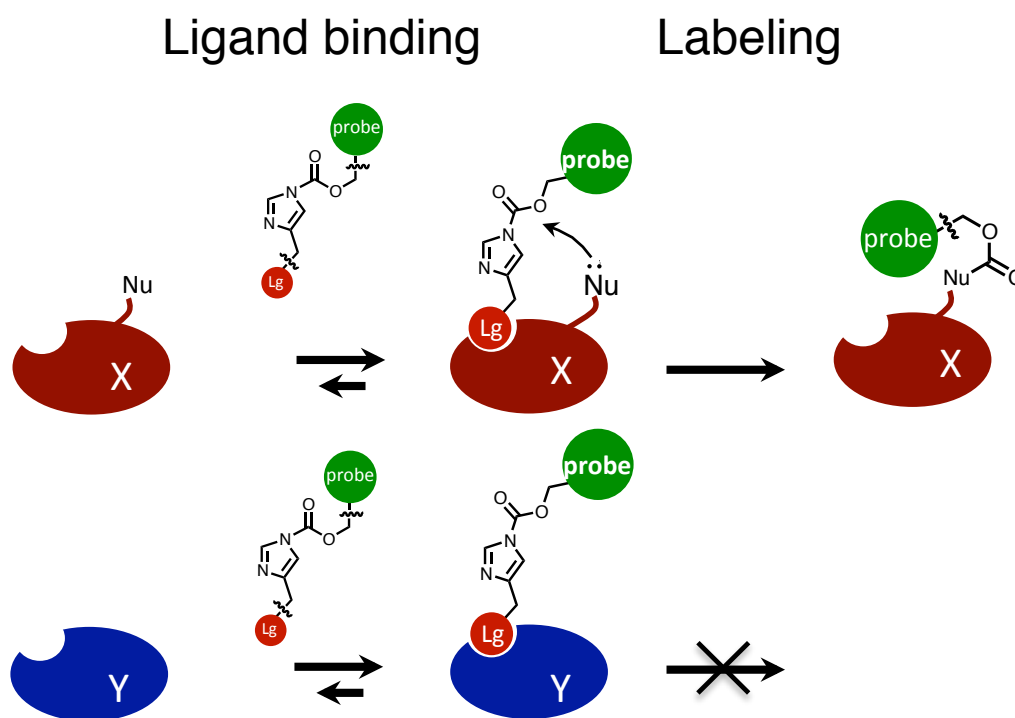
Figure 12. Selectivity of chemical labeling and expanding the methods in this thesis.

Chapter 1

Isozyme-Selective Labeling in CA Family Using Ligand-Directed Acyl Imidazole Chemistry

Abstract

Enzyme has an active site conserved in its families. While each isozyme has its own biological functions, the development of isozyme-selective drug and analyses based on chemical strategies are quite difficult. On the other hand, the outside of active sites is less preserved and attractive to generate the selectivity. Given that Ligand-directed Acyl Imidazole (LDAI) labeling which is based on acyl transfer reaction with protein surface near the active sites, I hypothesized that LDAI labeling can generate the selectivity by it reaction. Here, I demonstrated the isozyme-selective labeling in carbonic anhydrase families *in vitro* and *in cellulo* by kinetic studies and mapping the labeling sites.



1-1, Introduction

Fluorescent imaging of a target protein offers invaluable information about its localization change and interactions in living cells. As fluorescent labeling techniques, a various of genetic engineering techniques, such as green fluorescent protein, have been developed in a few decades. However, in almost all case, there are two critical disadvantages. First, the observed proteins are chimera products with fluorescent protein (GFP, 27 kDa) or tag proteins (Halo tag, 33 kDa)¹, and it has already not intact form. Second, these are artificially expressed, not endogenously. These sometimes cause artifacts to its localization and its dynamic changes. On the other hand, Ligand-Directed protein labeling techniques², which our group has developed, enable us to selectively monitor a target protein expressed endogenously. These techniques are based on interaction between a target protein and its ligand, so that specific labeling can achieve. However, this also means that the labeling selectivity is highly dependent on the ligand selectivity, and the specific labeling is impossible if you use a ligand containing less-selectivity.

In many cases, the active sites of protein structure are conserved in its protein family. Due to this feature, a lot of drugs and ligands exhibit insufficient selectivity among the family. In order to enhance its isozyme-selectivity, several approaches are focusing on the outside of active site, which residues are less conserved than the active site. For instance, Getzoff and his colleagues revealed that iNOS selective drug reached to 2nd and 3rd shell of the active site to improve its selectivity rather than other NOS, i.e. eNOS and nNOS³. Several new kinase inhibitors, named Type II and Type III, were designed that the interaction extends to the allosteric binding site, relatively less conserved, from ATP binding site, well-conserved in kinase family^{4,5}.

Considering our Ligand-directed labeling mechanism, I noticed that this reaction also occur at the surface near the active site, less conserved than the active site, and set a

hypothesis that the Ligand-directed labeling may have a potential for the isozyme-selectivity.

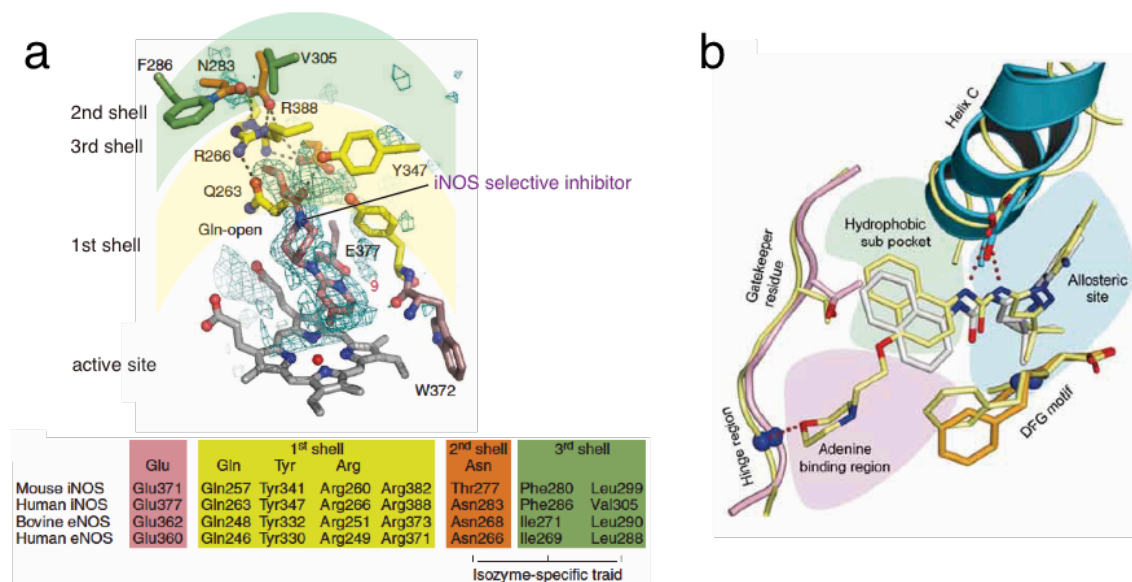


Figure 1. Isozyme-/subtype-selective drug development. (a) An aminopyridine inhibitor binding to iNOS. The isozyme-selectivity was generated because the binding causes the conformation change of the 2nd and 3rd shell in iNOS³. (b) A type III kinase inhibitor binding to cSrc. The inhibitor reach to the allosteric site, generating the cSrc-selectivity⁴.

1-2, Results and discussion

1-2-1, Isozyme selectivity of LDAI chemistry between CA1 or CA2

When I started this project, my colleagues, Dr. Fujishima and Mr. Yasui, succeed to label carbonic anhydrase 1 (CA1) using LDAI method⁶. Thus, CA family was employed as a target protein to evaluate the isozyme selective labeling by LDAI in this study. It has been known that there are endogenous CA1 and CA2 in human red blood cell⁷. In addition, it was reported that some of the transmembrane-type human carbonic anhydrase (CA9 and CA12) are substantially over-expressed in tumors under hypoxia conditions, which make them valuable biomarkers for cancer diagnostic and treatment⁷. I initially compared the labeling rate and efficiency for purified CA1 or CA2 labeling using LDAI reagent **1** in test tubes. The reagent **1** contains SA (sulfonamide) ligand, which affinity (K_i value) has already reported as 1,300 and 45 nM toward CA1 and CA2, respectively⁸.

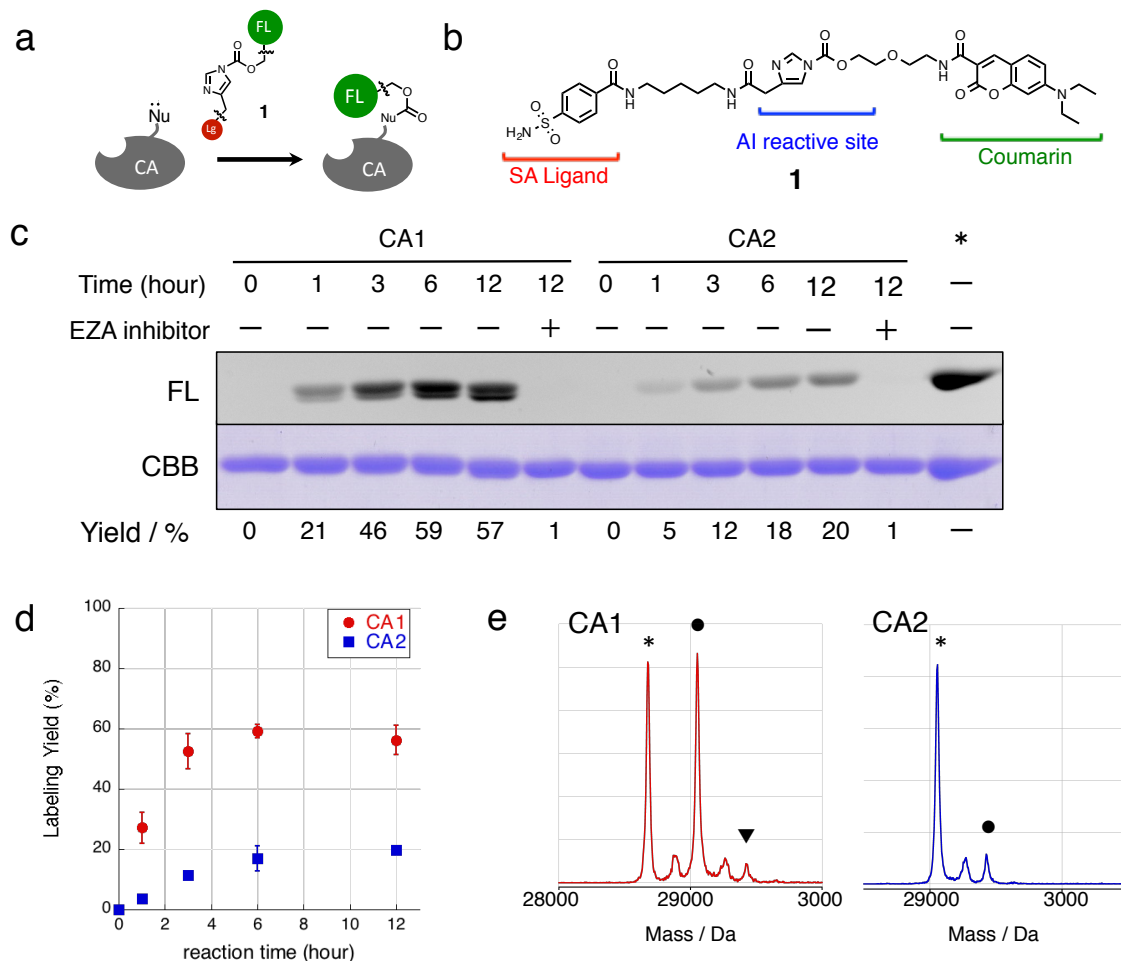


Figure 2. CA1/CA2 protein labeling by LDAI chemistry *in vitro*. (a) Schematic illustration of LDAI labeling. (b) Chemical structure of reagent **1**. (c) In-gel fluorescence analysis of the labeling reaction. CA protein (5 μ M) was mixed with **1** (5 μ M) in 50 mM HEPES (pH 7.2). As a fluorescent standard, a purified Coumarin-modified CA2 was loaded (*). (d) Time course of the labeling reaction of CA1 (●) or CA2 (■) with **1**. (e) MALDI-TOF MS analysis of the labeled CA1 or CA2 after 6 h-incubation. The peaks of unlabeled CA, singly or doubly labeled CAs are marked with *, ●, and ▼, respectively.

The labeling reaction was monitored by SDS-PAGE. **Figure 2a** showed the time course of the labeling reaction for CA1 and CA2 using **1**. While the fluorescent labeled proteins were intensified gradually in both CAs, the labeled CA1 was produced apparently more higher than labeled CA2. The results showed that the labeling rate for CA1 was about 5-fold faster than that of CA2, and the labeling yield was 3 times better than for CA1 (59%) relative to for CA2 (20%). The reaction did not occur in both CAs

in the presence of ethoxzolamide (EZA), a strong inhibitor for CA, indicating that both labeling are driven by ligand-protein recognition. The isozyme selectivity was also observed by MALDI-TOF mass (**Fig. 2e**). The labeled CA1 was strongly detected, while the labeled CA2 was observed as a small peak. The labeling yield estimated by mass is consistent with that by gel-based assay.

1-2-2, Kinetic studies of LDAI labeling to CA1 and CA2

In order to understand factors controlling the present isozyme selectivity in detail, I conducted kinetic studies. The mechanism of the labeling reaction consists on two steps, protein-ligand interaction and acyl transfer reaction on protein surface. Based on previous researches, the 1st step, protein-reagent ligand interaction, is extremely fast (CA2, $k_{on} = 5.9 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 0.049 \text{ s}^{-1}$)⁹ than the following reaction. Then, the 1st step and reagent exchanging are negligible, and I focus on 2nd step when the reagent and protein was mixed in a 1:1 ratio. The LDAI reagent intrinsically undergo nonproductive hydrolysis at the acyl-imidazole group in CA-reagent complex, along with the Acyl transfer reaction for protein labeling as shown in **Figure 3a**. To determine the rates of the labeling reaction (k_L) and the hydrolysis reaction (k_H) in CA-reagent complex, I monitored the reaction by HPLC. Using nonlinear least-squares curve fitting of the first-ordered decay of **1**, the sum of the rates of the labeling rate and hydrolysis reaction ($k_L + k_H$) were determined. From the ratio of the formation of labeled CA to the decay of **1**, the labeling rates were determined to be 0.27 and 0.049 h^{-1} , and the hydrolysis rates were determined to be 0.079 and 0.086 h^{-1} for CA1 and CA2, respectively. There is little difference in the hydrolysis rate. On the other hand, the labeling rate of CA1 is 5.5-fold higher than that of CA2. These results mean that the conversion is affected mainly by collision probability and reactivity of nucleophilic amino acids on CA surface, not by the local environment, such as pH and base.

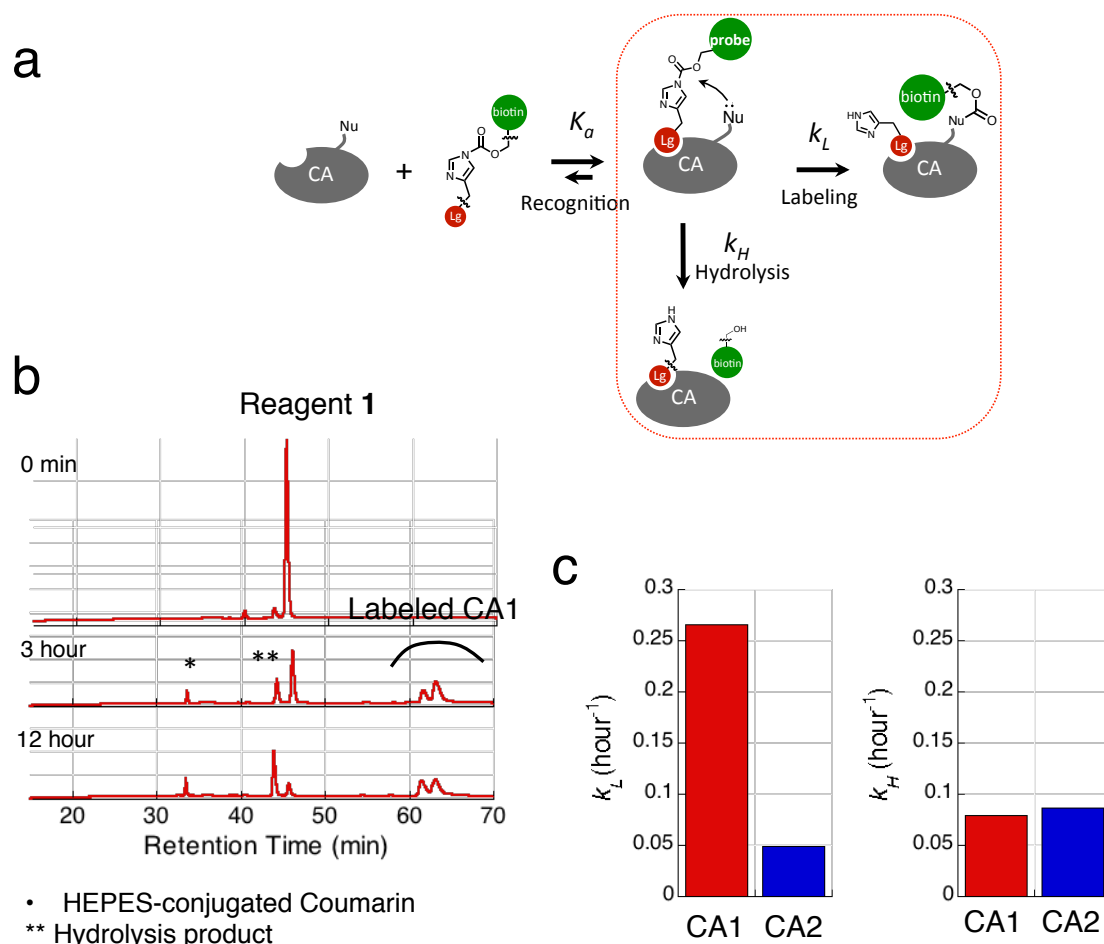


Figure 3. Kinetics studies of CA labeling in *vitro*. (a) Overall scheme of the hydrolysis and labeling reaction processes. In this study, I focused on the later steps, which is labeling and hydrolysis. (b) HPLC charts of CA1 labeling reaction. Abs. (430 nm) was detected. (c) The rates of labeling (k_L) and hydrolysis (k_H)

1-2-3, Comparison of the labeling sites between CA1 and CA2

The labeling sites of CA1 have been determined using the peptide mapping analysis by Mr. Yasui, which revealed that several sites were labeled in CA1 that is Ser2 was identified as a major labeling site (38%), and Tyr20 (12%), Ser136 (6%), Lys170 (6%), and Lys57 (3%) were determined as minor sites (**Fig. 4a-b**)⁶. On the other hand, I identified only Ser2 (20%) as the labeling sites and the rest was unidentified (8%) by **1** in the case of CA2 (**Fig. 4c-d**). The binding affinity of sulfonamide (SA) ligand to CA1 is not substantially different from that to CA2, which is reasonable because the active

sites are not distinguished by the SA ligands that coordinate to Zn^{2+} ion. In contrast, by carefully comparing their 3D structure, it was found that the variety and number of nucleophilic amino acid exposed on the CA surface in 22 Å length form S atom of sulfonamide (SA) ligand, corresponding to its linker length, are considerably different each other. There are Ser2, Tyr20, Lys57, Ser136, and Lys170 in CA1 and Ser2, His3, His4, and Lys170 in CA2 (**Fig. 4a**). Among them, the stable carbonate or carbamate bond formation was reasonably expected for Ser, Lys, Tyr, whereas His cannot form such a stable bond. It is crucial that the reactive residues including Tyr20, Lys57 and Ser136 in CA1 are not preserved in CA2. This indicates the probability of labeling reactions in CA2 is potentially lower than that in CA1. However, the selectivity can not be explained only by the number of reactive residues. The preserved residues, Ser2 and Lys170 in CA2 must be less reactive than CA1. This is probably caused by the slightly different steric configuration. It was consistently considered that both effects suppressed the labeling efficiency for CA2, compared to CA1. Taken together, the isozyme selectivity of LDAI is ascribed to the difference in the CA surfaces proximal to the active pocket. This instructively implies that the isozyme selectivity is controlled not only by the ligand binding affinity, but also by the subsequent reaction of AI toward an amino acid located on a protein surface in LDAI-based protein labeling.

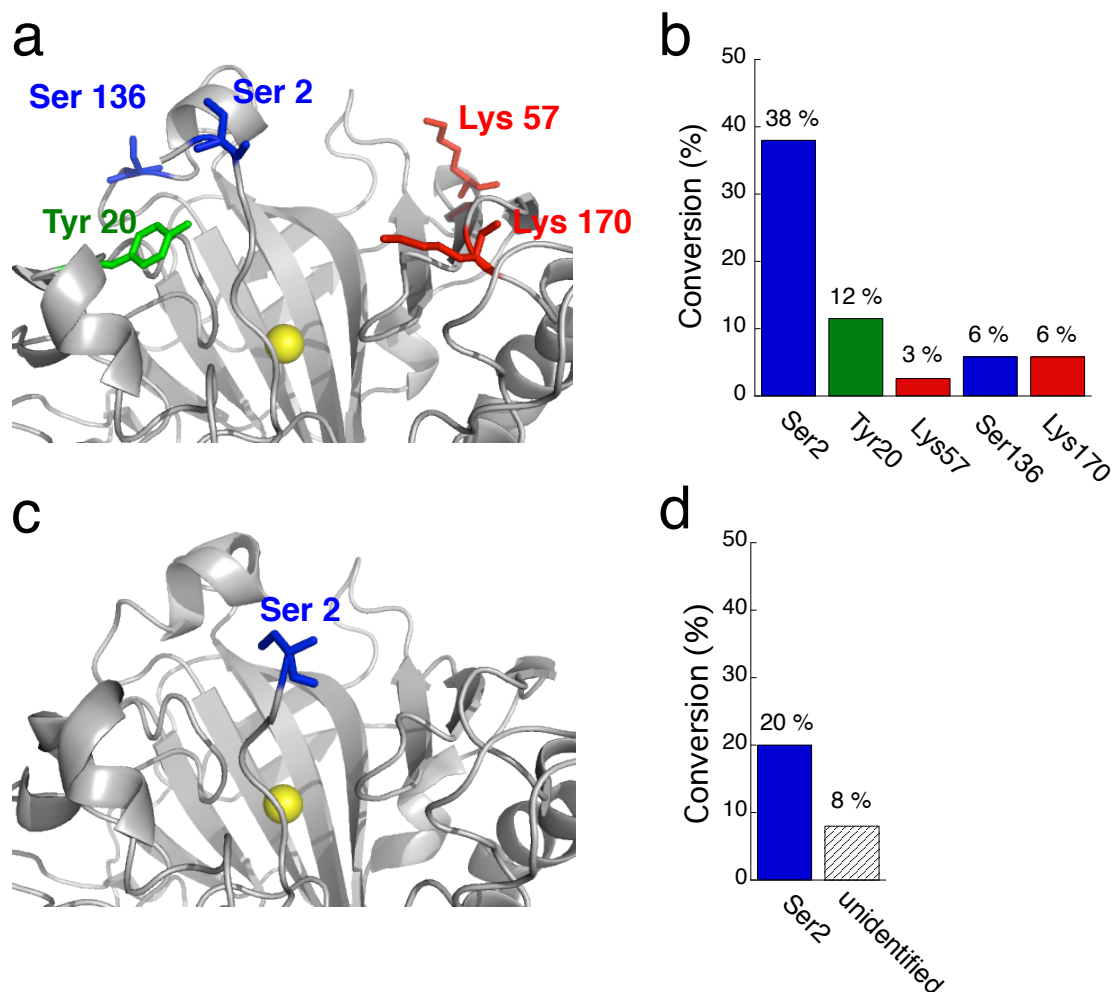


Figure 4. Labeling sites and conversion by LDAI reaction. (a, c) Structural models of CA1 protein (PDB ID: 1BZM) (a) or CA2 protein (3M2Y) (b). The amino acids (Ser2, Tyr20, Lys57, Ser136, and Lys170 for CA1; Ser2 for CA2) are highlighted by a colored stick model. The reaction conversion of each amino acid was shown in (b) for CA1 and (d) for CA2.

1-2-4, Isozyme selective CA12 labeling in living systems

As mentioned before, some in the CA family were reported to be useful biomarkers for solid tumors, because of their over-expression. For instance, both CA9 and CA12 are strongly induced in A549 cell membrane under hypoxia conditions¹⁰. I therefore next sought to label these endogenous CAs by LDAI chemistry and examined whether the isozyme selectivity is accomplished even under living cell conditions. After

culturing A549 cells under normoxia or hypoxia conditions, LDAI reagent **2** was mixed with A549 cells and incubated for 3 hr. After the labeling, I rinsed with medium 3 times, the cells were lysed and analyzed by Western Blotting. **Figure 5** showed the blotting data stained by anti-CA9 antibody, anti-CA12 antibody, or horseradish peroxidase-conjugated streptavidin (SAv-HRP). Comparison of the blotting by anti-CA9 with that by anti-CA12 indicated both CA9 and CA12 over-expressed in A549 cells under hypoxia conditions. Nevertheless, only CA12 was stained by SAv-HRP, implying that biotin-labeling by LDAI **2** selectively took place for CA12 not CA9. It is conceivable that the affinity of the SA ligand for CA9 is not largely different from that for CA12⁸. In addition, I checked the expression level of both proteins by western blotting. This showed the expression level of CA12 is 5.4-fold higher than CA9, while the labeled amount of labeled CA12 is 34-fold higher than that of labeled CA9. This result indicates LDAI reagent **2** reacted with CA12 6.2-fold selectively toward CA9 (**Fig. 6**). This explicitly demonstrated the isozyme selectivity can be accomplished by LDAI chemistry even under living cell contexts.

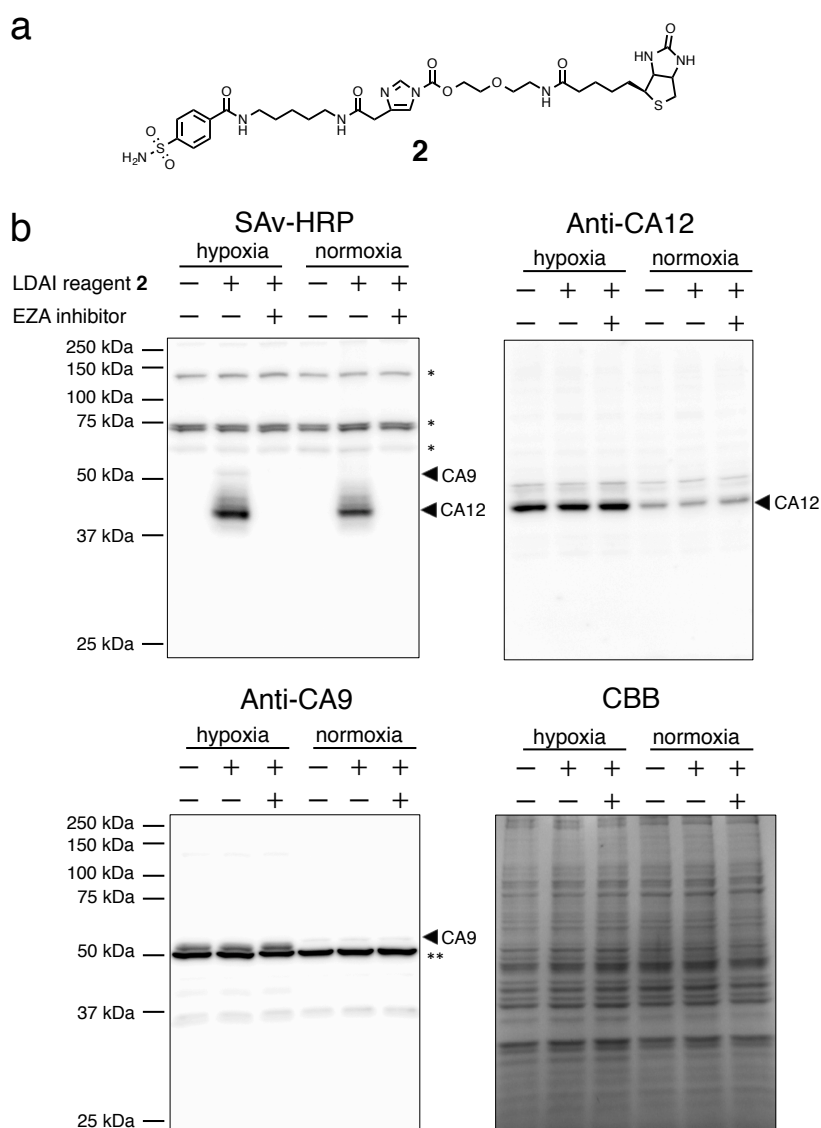


Figure 5. Endogenous CAs labeling on living cells. A549 cells cultivated in hypoxia (<0.1% O₂) or normoxia (20% O₂) were treated with **2** (1 μM) in D-MEM (10% FBS) at 37 °C for 3 hr with or without EZA (50 μM). The images show western blotting analysis using HRP-conjugated streptavidin, mouse anti-CA12 antibody, rabbit anti-CA9 antibody, and CBB stain. * endogenous biotinylated protein; ** non-specific band of anti-CA9 antibody.

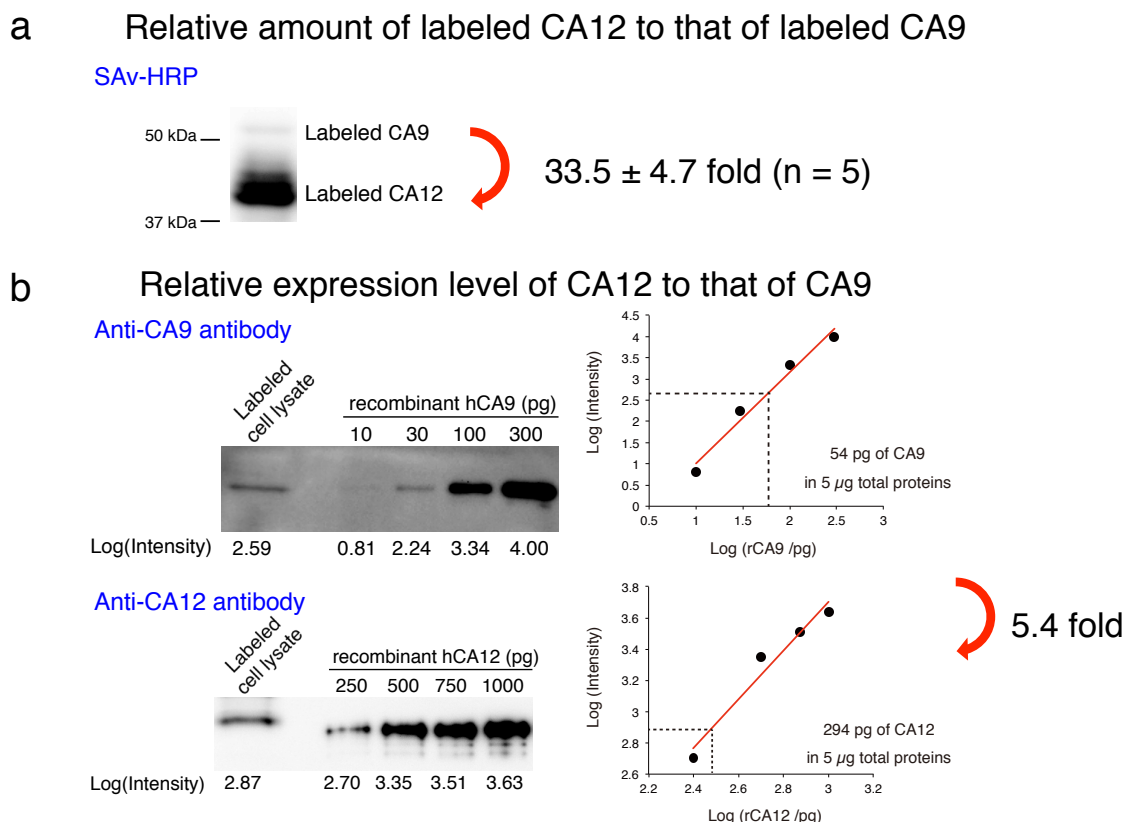


Figure 6. CA12 selectivity of LDAO labeling on living cell. (a) Biotin blotting analysis using SAv-HRP of labeled cell lysate. The amount of labeled CA12 is 33.5-fold higher than that of labeled CA9. (b) Relative expression level of CA12 to that of CA9. By using recombinant proteins as standard, the expression level of CA is determined. CA12 is expressed 5.4-fold higher than CA9.

1-2-5, Identification of labeled site of CA12

I thought that this selectivity may be explained by the difference in the reactive amino acids localized on the enzyme surface. Thus, I decided to identify the labeling sites by peptide mapping analysis using nanoLC-MS/MS systems. To achieve the labeling site identification of the membrane protein labeled on living cell, there are three following critical problems. (1) the amount of CA12 protein in cell lysate are largely less than purified proteins. (2) I needed to purify the labeled CA12 to simplify the LC-MS/MS chart. (3) Appropriate digestive enzyme should be used to adjust the length of labeled peptide fragments to mass detectable range. Trypsin, the most widely

used enzyme, cleave peptides at the carbonyl side of Lys and Arg residues, but not modified Lys residues. This modification causes the labeled peptide fragments much larger. In a bunch of trial and error, I finally succeeded in detecting the labeled peptides by the following protocol. (a) reagent **2** was mixed with A549 cells (6 dishes of 10 cm), which were under hypoxia condition and labeled for 3 hr. (b) the cells were homogenized under hypotonic condition and removed the soluble fraction (cytosol fraction). (c) the insoluble fraction was resuspended in DDM (*n*-Dodecyl- β -D-maltopyranoside) buffer and purified by anion-exchange chromatography, (d) the target fraction was electrophoreses on 1D SDS-PAGE gel and silver-stained, (e) the CA12 band was digested in-gel by treatment with chymotrypsin, (6) using LC-MS/MS analysis of the peptides, labeled peptide were detected (**Fig. 7a-c**).

The identified labeling sites were Lys97 and Lys196, close to the active pocket¹¹ of CA12 (**Fig.7d**). Interestingly, these residues are not preserved in CA9¹². This implies the chance of labeling reaction in CA9 is potentially lower than that in CA12. Similar to the selective labeling of CA1 over CA2 in test tube, the present isozyme selectivity can be explained by the difference in the reactive amino acids localized on the enzyme surface near the active site.

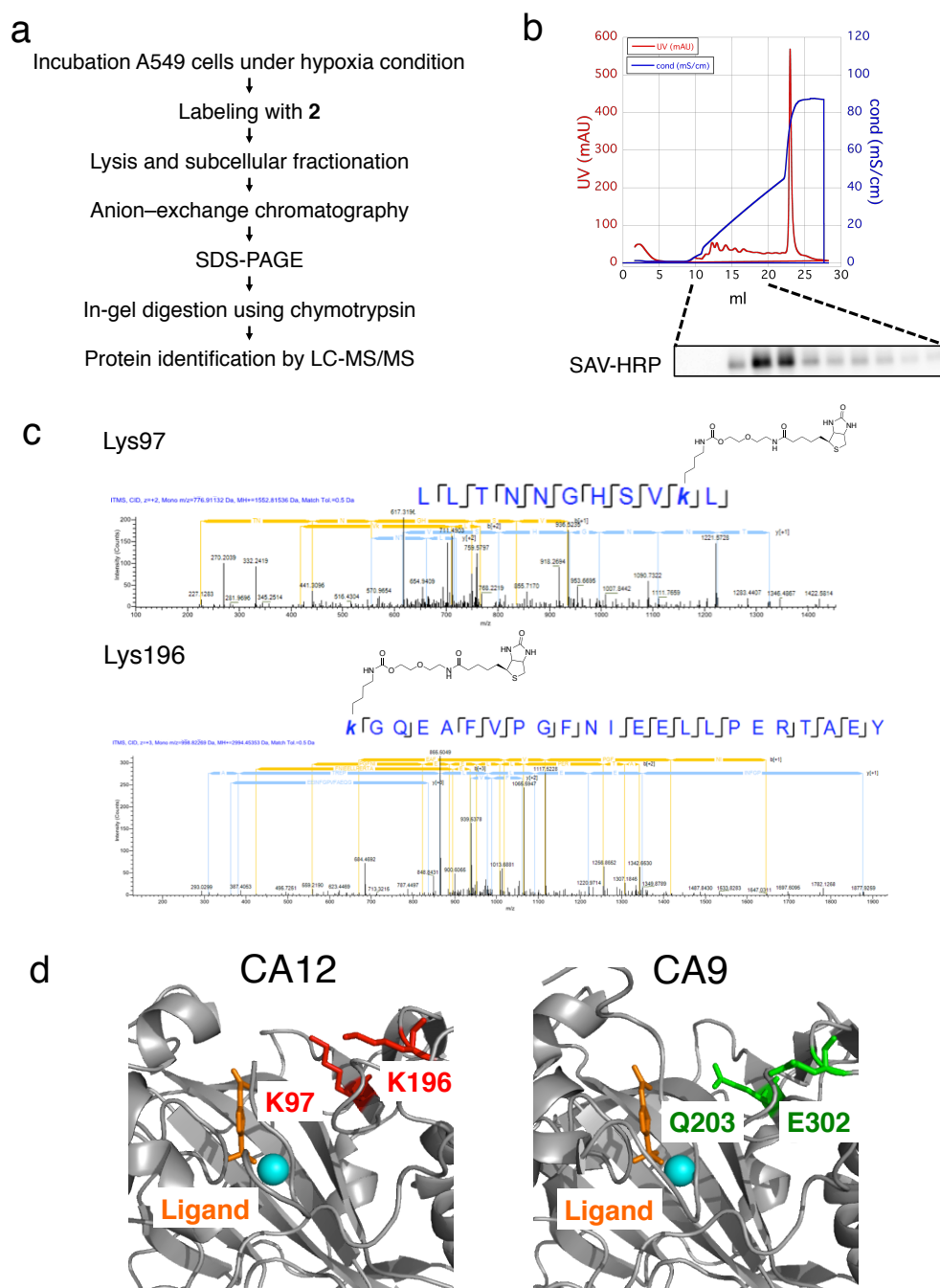


Figure 7. Identification of labeled site of CA12. (a) Schematic illustration of identification procedure. (b) Anion-exchange chromatogram of labeled A549 cell lysate. (c) MS/MS charts of labeled peptide. (d) Structural models of CA12 protein (left, IJD0) and CA9 protein (right, 3IAI). K97 and K196 residues, which are identified as labeled sites were highlighted by a colored stick model (left). These residues are not preserved in CA9 (right).

1-3, Conclusion

The isozyme-selectivity of Ligand-directed chemical labeling has not been thoroughly investigated. In this study using carbonic anhydrase family as a model, I explicitly revealed that Ligand-directed chemical labeling exhibits isozyme-selectivity and the selectivity is driven from (1) the number of nucleophilic amino acids, which locate near the active site and (2) nucleophilicity of these residues. Furthermore, its selectivity was also carried out on the live cell. By combination of optimized enrichment procedure of labeled-CA12 with nanoLC-MS/MS techniques, I determined the labeled residues to reason that the non-conserved Lys residues causes its selectivity.

Given the sequences among the CA family, the active site was highly conserved. On the other hand, the residues outside of the active site ($> 10 \text{ \AA}$) were variable (**Fig. 8a**). The length between SA ligand (S atom) and AI reactive site has $22 \text{ \AA}$, this enables the AI group to reach the outer side of the less-conserved surface, resulting the residues around $12\text{--}20 \text{ \AA}$ from S atom of sulfonamide ligand were labeled (**Fig. 8b-c**). It is considered that this is the main factor of isozyme selectivity.

Previous researches in Ligand-Directed Labeling showed the linker structure of labeling reagents strongly affect to the labeling yield and labeled residues¹³. This suggests that the linker structure can also affect to the isozyme selectivity. The precise design may be attributed to improvement of its selectivity.

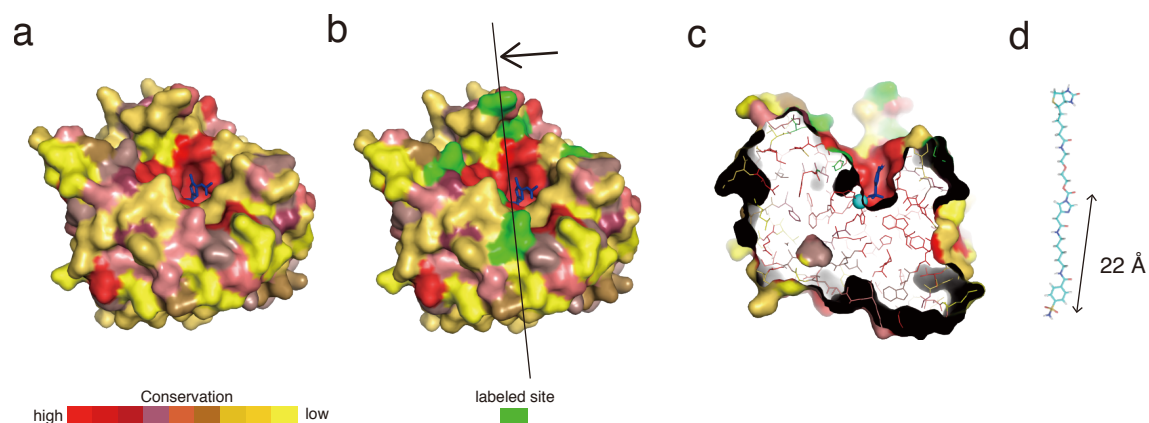


Figure 8. Conserved residues of CA family and labeled sites. (a) Conserved residues are colored in red in the structural models of CA1 protein (left, 1BZM). (b), (c) Labeled residues are shown in green. Sequence alignment was conducted using ClustalW (<http://clustalw.ddbj.nig.ac.jp/>) and ConSurf Server (<http://consurf.tau.ac.il/>) among CA catalytic familis (CA1, CA2, CA3 CA4, CA5A, CA5B, CA6, CA7, CA9, CA12, CA13, CA14). Structure model of LDAI reagent **1** was shown in (d) in same scale with (c).

1-4, Experimental Section

Chemical synthesis of AI reagents

Reagent 1 and 2 were synthesized in the same manner as reference 6.

Protein labeling of CA1 and CA2

A solution of 5 μ M of human CA1 (Sigma) or CA2 (Sigma) was incubated with 5 μ M of reagent 1 (1 equivalent to CAs) in HEPES buffer (50 mM, pH 7.2) at 37 °C for 1, 3, 6 and 12 h. As a negative control, I pre-treated with 50 μ M of EZA. The labeled reaction was monitored by MALDI-TOF mass and in-gel fluorescent assay. For the in-gel fluorescent assay, the samples were mixed with same volume of 2 $\times$ Leammli buffer (pH 6.8, 125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 100 mM dithiothreitol) and incubated for 1 h at r.t.. These were subjected to SDS-PAGE, followed by fluorescent imaging (LAS4000, Fuji Film). For fluorescent standard, I also subjected Coumarin-labeled CA2 proteins which was modified by LDT chemistry as shown in reference.

Kinetic studies of CAs labeling¹⁴

The CAs labeling was performed in the same condition as described above. The labeling was quenched by addition of EZA (10 μ M) and subjected to RP-HPLC (column; COSMOSIL, 5C18-AR-I, 250 $\times$ 4.6 mm, mobile phase; CH₃CN (containing 0.1 % TFA) / H₂O (containing 0.1 % TFA) = 0/100 (5 min) $\rightarrow$ 30/70 (linear gradient over 20 min), $\rightarrow$ 35/60 (linear gradient over 25 min), flow rate; 1.0 mL/min, detection; UV-Vis (220-600 nm)). All fractions containing absorbance at 430 nm were collected and analyzed by MALDI-TOF mass. The concentration of reagent was estimated by determining the relative peak area. The first order rate constant of reagent decay ($k_L + k_H$, protein labeling and hydrolysis reaction) were obtained by fitting a plot of reagent concentration ([1]) as a function of incubation time (t) to eqn (1) using Excel Solver.

Chapter 1

$$[1] = [1]_0 \exp\{-(k_L + k_H)\} \quad (1)$$

where $[1]_0$ is the initial concentration of the LDT reagent (5 μ M),

To determine the reaction rates of protein labeling (k_L), fluorescent value assigned as labeled CA was plotted toward concentration of degraded reagent ($[1]_0 - [1]$).

Based on eqn (2), the value of k_L and k_H was estimated.

$$[\text{labeled CA}] = \frac{k_L}{k_L + k_H} ([1]_0 - [1]) \quad (2)$$

Peptide mapping of labeled CA2

A solution of human CA2 (25 μ M) and reagent 1 (50 μ M) in HEPES buffer (50 mM, pH 7.2) was incubated for 24 h at 37 °C. The labeled CA2 was purified by size-exclusion chromatography (TOYO-PEARL HW-40F). The fraction was concentrated by ultrafiltration (Amicon Ultra 10K cut off). The labeling yield (30%) was determined based on the relative absorbance at 280 and 430 nm. For protein digestion, the solution was incubated with lysyl endopeptidase (LEP) (20wt% of total CA2) at 37 °C for 21 h in the presence of urea (2 M). The digested sample was subjected to RP-HPLC (column; YMC-pack ODS-A, 250 $\times$ 4.6 mm, mobile phase; CH₃CN (containing 0.1 % TFA) / H₂O (containing 0.1 % TFA) = (5/95 to 55/45) (linear gradient over 100 min), flow rate; 1.0 mL/min, detection; UV-Vis (220-600 nm) Fluorescence (Ex; 430 nm, Em; 480 nm). All fluorescent fractions were collected and analyzed by MALD-TOF MS and MS/MS. The modification yield of each residue was calculated by multiplying the protein labeling yield and ratio of fluorescent signal together.

Labeling of Endogenous CA12 on Living Cells

A549 cells were maintained in 5% CO₂ atmosphere at 37 °C in DMEM (Sigma) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (199

μg/ml), and amphotericin B (250 ng/ml). A549 cells (1×10^5 cells) were incubated under the hypoxic condition ($<0.1\% \text{ O}_2$) generated with AnaeroPack (Mitsubishi Gas Chemical Company) for 1 day and treated with 1 (1 μM) at 37 °C in DMEM for 3 hr. As a control experiment, the labeling was conducted in the presence of EZA (50 μM). The labeled cells were lysed using NP40 buffer (pH 7.4, 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40) containing 1% protease inhibitor cocktail set III (Calbiochem). The insoluble material was removed by centrifugation at 12,000 rpm for 10 min. The supernatant was mixed with the same volume of 2×Laemmli buffer and incubated for 1 h at r.t.. The samples were subjected to SDS-PAGE (10% Acryl Amide) and then transferred onto Immun-Blot PVDF membrane (Bio-Rad). The biotin-labeled CA12 was detected by chemiluminescence analysis using SA_v-HRP (Invitrogen, $\times 10,000$). The immune-detection of CA9 and CA12 was performed with a rabbit anti-CA9 antibody (Abcam, ab107257, $\times 1,000$), a mouse anti-CA12 antibody (Abcam, ab54917, $\times 500$), an anti-rabbit IgG-HRP conjugate (GR Healthcare, $\times 2,000$) and an anti-mouse IgG-HRP conjugate (GR Healthcare, $\times 2,000$).

Determination of relative expression level of CA12 to CA9

The incubation and labeling protocols are mentioned above. Protein concentration in labeled cell lysate was determined by BCA assay (Thermo). I subjected the 5 μg proteins to SDS-PAGE and western blotting analysis. For protein standard, I used recombinant human CA12 (R&D) for CA12 and recombinant human CA9 (R&D) for CA9. The immune-detection of CA9 and CA12 was performed with a rabbit anti-CA9 (Abcam, ab10471, $\times 1,000$), mouse anti-CA12 (Abcam, ab54917, $\times 500$), an anti-rabbit IgG-HRP conjugate (GR Healthcare, $\times 2,000$) and an anti-mouse IgG-HRP conjugate (GR Healthcare, $\times 2,000$). For the biotin-blotting, I used SA_v-HRP (Invitrogen, $\times 10,000$).

Modification site determination of biotin-labeled CA12

The labeling site identification was processed as follows. (1) 80-90 % confluence of A549 cells (6 dishes of 10 cm) was incubated in hypoxia condition for 1 day. (2) To the dishes, the compound **2** was added to final concentration 1 μ M. Then, the cells were incubated for 3 h in hypoxia. (3) The cells were homogenized under hypotonic condition (10 mM HEPES buffer, pH 7.2), centrifuged (105,000G, 30 min, 4 °C), and the supernatant was removed. The pellet was dissolved with DDM buffer (50 mM DDM, 20 mM HEPES, 150 mM NaCl). (4) The fractionated crude was diluted with 10 mM HEPES (pH 7.5), and purified by anion-exchange chromatography using ÄKTA purifier system and HiTrap Sepharose XL (Q) column (column volume; 1 mL, GE healthcare). The detection of labeled-protein was carried out by UV (280 nm) and biotin blotting analysis. (5) The target fraction was mixed with 2xLaemmli buffer, and electrophoresed on 1-D SDS-PAGE gel. (6) After silver-staining, the CA12 band (45 kDa) was cut, alkylated by iodoacetamide (IAA), and digested in-gel by treatment with chymotrypsin using 15 μ L of the chymotrypsin (Promega) solution (30 ng/ μ L) in 25 mM ammonium bicarbonate buffer for overnight at 30 °C¹⁵. (7) The sample was analyzed by nano-flow reverse phase liquid chromatography followed by tandem MS, using a LTQ-orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) as follows.

A capillary reverse phase HPLC-MS/MS system composed of an Agilent 1100 series gradient pump equipped with Valco C2 valves with 150- μ m ports, and LTQ-Orbitrap XL hybrid mass spectrometer equipped with an XYZ nano-electrospray ionization (NSI) source (AMR, Tokyo, Japan). Samples were automatically injected using PAL system (CTC analytics, Zwingen, Switzerland) into a peptide L-trap column (Chemical Evaluation Research Institute, Tokyo, Japan) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water

containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent C), the peptides were loaded into a separation capillary reverse phase column (MAGIC C18 packed with gel particles of 3 μm in diameter and 200-Å pore size, 150×0.2 mm, Michrom BioResources) by switching the valve. The eluents used were: A, 100% water containing 0.1% formic acid, and B, 100% acetonitrile containing 0.1% formic acid. The column was developed at a flow rate of 1 $\mu\text{L}/\text{min}$ with the concentration gradient of acetonitrile: from 5% B to 20% B in 10 min, 20% B to 40% B in 45 min, then from 40% B to 95% B in 30 min, sustaining 90% B for 15 min, from 95% B to 5% B in 1 min, and finally re-equilibrating with 5% B for 20 min. Xcalibur 2.1 system (Thermo Fisher Scientific) was used to record peptide spectra over the mass range of m/z 350–1500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 150–2000. Repeatedly, MS spectra were recorded followed by three data-dependent collision induced dissociation (CID) MS/MS spectra generated from three highest intensity precursor ions. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by Proteome Discoverer 1.3.0.339 (Thermo Fisher Scientific). Searches were performed by using the SEQUEST (Thermo Fisher Scientific) against latest Swissprot database for protein identification. Searching parameters were set as follows (i) SEQUEST: enzyme selected as used with five maximum missing cleavage sites, species limited to *Human*, a mass tolerance of 5 ppm for peptide tolerance, 0.5 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of oxidation (M) and chemical modification (K, S, T, Y). Positive protein identifications were based on significant Xcorr (5% false discovery rate). Protein identification and modification information returned from SEQUEST were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS. Human Carbonic anhydrase 12 was identified with high sequence coverage (68.36 %, 44 peptides hit).

Chapter 1

Two chemically-labeled sites in CA12 were identified at Lys196 and Lys97. The MS score of Lys97-labeled peptide was not sufficiently high, however the peptide was constantly detected in several same experiments.

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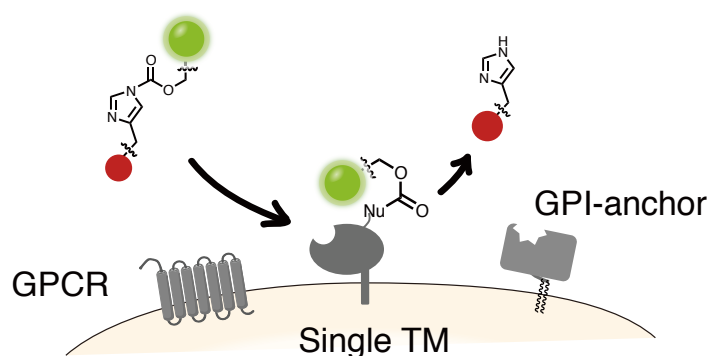
Chapter 2

Pulse-Chase Analysis of Endogenous Membrane Proteins Using LDAI-Based Chemical Labeling

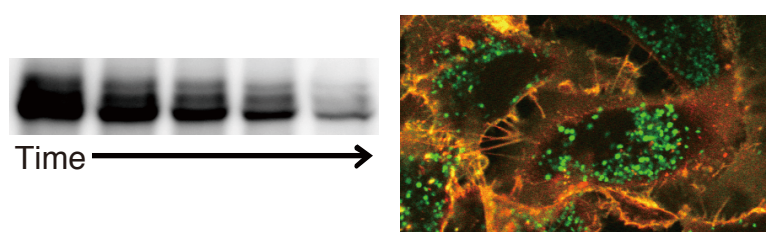
Abstract

The functions of membrane proteins are precisely regulated by the dynamics such as protein trafficking and degradation. The widely used techniques for protein imaging are based on genetic engineering. However, they are not applicable to intact proteins in principle. In this chapter, I demonstrated that ligand-directed acyl imidazole (LDAI) chemistry is broadly applicable to selective chemical labeling of various types of intact membrane-bound proteins under native contexts without a need for any tag fragments. Moreover, the LDAI chemistry enables pulse-chase analysis of these proteins to determine the half-life, as well as their degradation pathways by the imaging, under almost natural cellular conditions.

LDAI labeling



Pulse-chase analysis



2-1, Introduction

The functions of membrane proteins are tightly controlled by the dynamics such as protein trafficking and degradation¹. In most cases, membrane proteins are constantly internalized and degraded. However, the half-lives of these proteins vary from minutes to days, and the degradation pathways depend on the type of proteins and the existing conditions. For instant, some GPCRs (G-protein conjugated receptor) are desensitized by agonist-induced endocytosis and/or down regulation². Therefore, valuable methods that offer both half-life value and live cell imaging are greatly desirable for investigating membrane-bound proteins in intact cellular contexts.

Conventionally, protein half-life has been evaluated by isotope pulse-chase labeling using [³⁵S]-methionine³ or by protein synthesis inhibition using cycloheximide (CHX). However, the isotope labeling needs laborious manipulation and CHX perturbs cell context strongly. Recently, some groups developed a novel method based on protein labeling using protein-tag, such as SNAP-tag⁴ or Halo-tag⁵. These techniques offer advantages of measurement of protein half-lives in cell and *in vivo* with minimal perturbation and also protein trafficking in living cell imaging. However, in these techniques, genetic manipulations are necessary and protein-tag must be fused to the target proteins. On the other hand, as shown in chapter 1, LDAI chemical labeling is applicable to intact proteins. Therefore, I hypothesized that LDAI chemistry enables to measure the half-life and monitor the protein in living cell without any (bulky protein) tag, which is applicable to endogenous proteins.

Here, I demonstrated the pulse-chase analysis and tracking of endogenous CA12 in living cell by using LDAI chemistry to determine its half-life and as well as degradation pathway. Moreover, I succeeded to demonstrate that LDAI chemistry is broadly applicable to selective chemical labeling and pulse-chase analysis of various types of membrane-bound proteins, i.e., integral proteins containing a single TM or GPCR, and a glycosylphosphatidylinositol (GPI)-anchored protein under living cell.

2-2, Results and discussion

2-2-1, General strategy of membrane protein labeling and pulse-chase analysis

LDAI chemistry is based on a nucleophilic acyl substitution reaction on protein surface, which driven by the proximity effect via a ligand-protein interaction⁶. And it is capable of releasing the ligand moiety upon covalent labeling of a target protein, resulting in traceless labeling. In order to evaluate the validity of the LDAI method, a variety of membrane proteins including human carbonic anhydrase 12 (CA12)⁷, a membrane-bound enzyme containing a single TM helix; bradykinin B2 receptor (B2R)⁸, which is classed into the family of GPCRs with seven TM domains; and Folate receptor (FR)⁹, a typical GPI-anchored protein, were employed here without introducing any tags for labeling. All of these proteins are different from each other in their membrane topology and size, and are attractive as tumor biomarkers or drug targets (**Fig. 1a**).

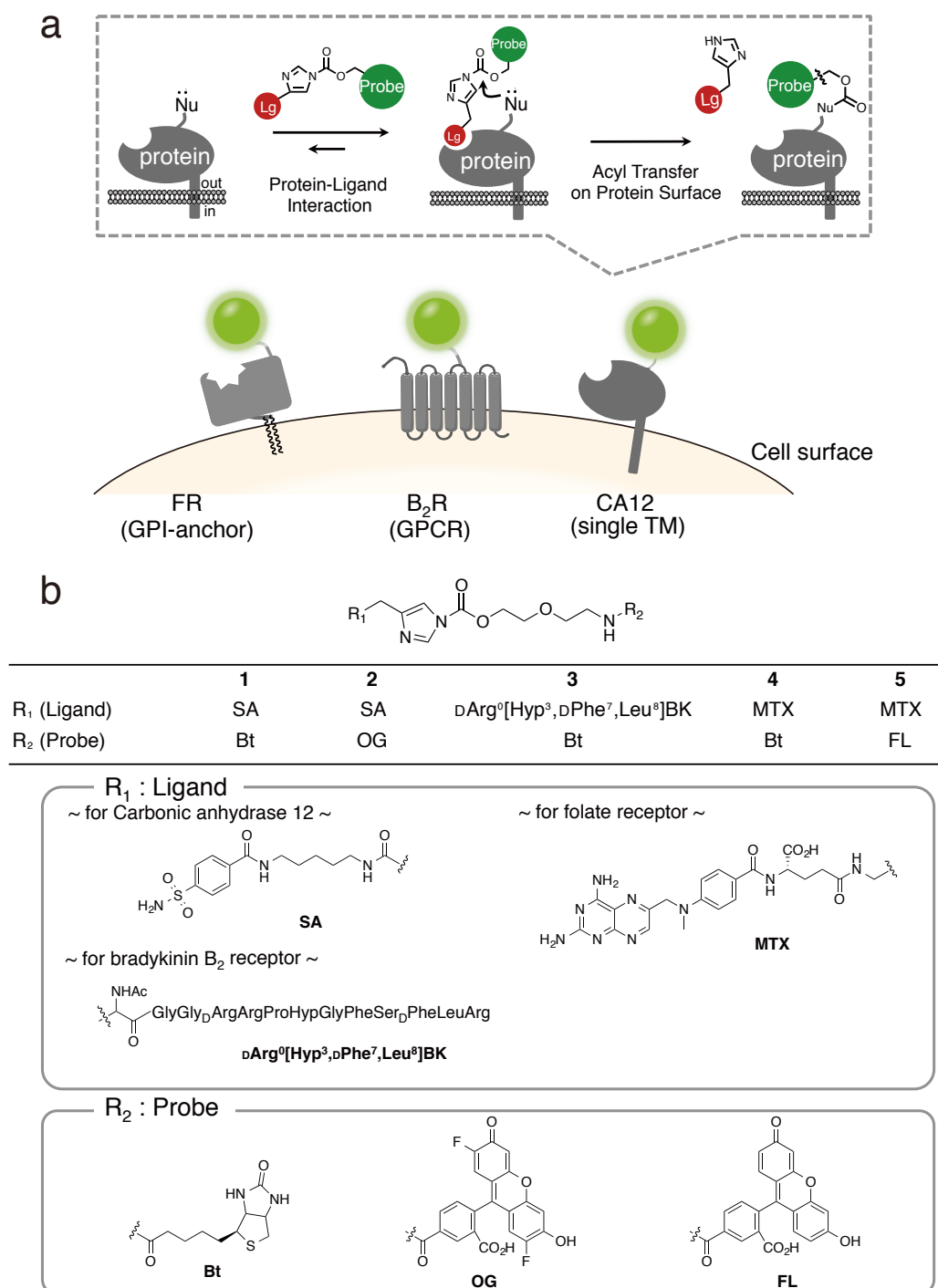
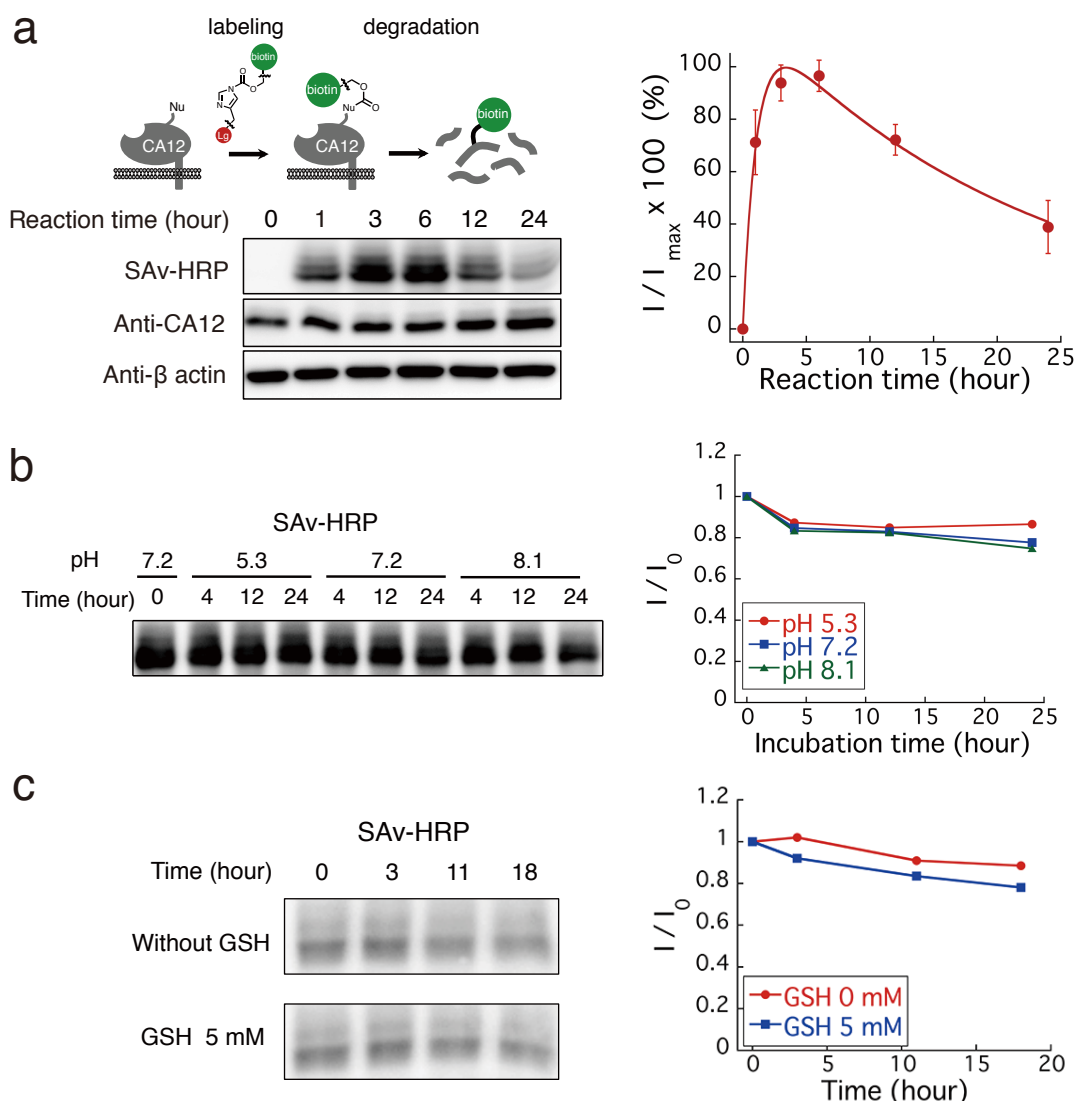


Figure 1, LDAI Chemistry for Selective Protein Labeling. (a) Schematic illustration of affinity-based chemical labeling of membrane proteins. Lg; Ligand, Nu; nucleophilic amino acid. (b) LDAI reagents used in this chapter. **1** and **2** for carbonic anhydrase 12 (CA12), **3** for bradykinin B₂ receptor (B₂R), **4** and **5** for folate receptor (FR).

2-2-2, Time course of CA12 labeling and half-life determination in living cell

In chapter 1, I demonstrated selective labeling of endogenous CA12 by using reagent **1** (1 μ M). During the study of the CA12 labeling on A549 living cells, I noticed that the amount of labeled CA12 is strongly dependent on the incubation time. Up to 3 hr incubation, the biotin-labeled CA12 increased, but thereafter it gradually decreased until 24 hr (**Fig. 2a**). This biphasic change suggests that the first increment step can be attributed to the CA12 labeling and the second decrease step may be due to the proteolysis. The other possibility in the later step is cleavage of the carbamate bond of the labeled CA. Therefore, I separately confirmed the carbamate bond stability *in vitro* comparable to the physiological conditions, i.e., pH and high concentration of GSH (**Fig. 2b-c**). Only a minor decrease of the band intensity was detected, which indicates that the decrease of the labeled protein in the later step is attributed to the CA proteolysis.



To determine the half-life of CA12, I washed out with the LDAI reagent after 3 hr of labeling and monitored the change in the band intensity of labeled CA12. The monotonic decrease in the labeled CA12 band was detected with a half-life of 9 hr (**Fig. 3a**). In contrast, the band intensity did not considerably change by incubation at 4 °C (**Fig. 3b**). It should also be pointed out that the total amount of CA12 never decreased during this time range in all cases, as confirmed by the anti-CA12 antibody-stained bands. Next, the resultant decay profiles of the labeled CA12 band was compared with the anti-CA12 antibody-stained band change in presence of cycloheximide (CHX), an inhibitor of protein biosynthesis in eukaryotic cells, which is traditionally used to determine the half-life of a protein (**Fig. 3c**). The decay curve was similar to that of the labeled CA12 band at 37 °C, while the almost steady state of the band intensity after the initial decrease (until 6 hr) by CHX was somewhat different from the exponential decay of the labeled CA12 (**Fig. 3d**). This discrepancy may be ascribed the fact that all protein synthesis was halted by addition of CHX, which confer substantial disturbance to normal living cell habitats. In contrast, it was clear that our method of using selective CA12 labeling by LDAI in living cells enabled us to study half-life of the target CA under almost natural conditions.

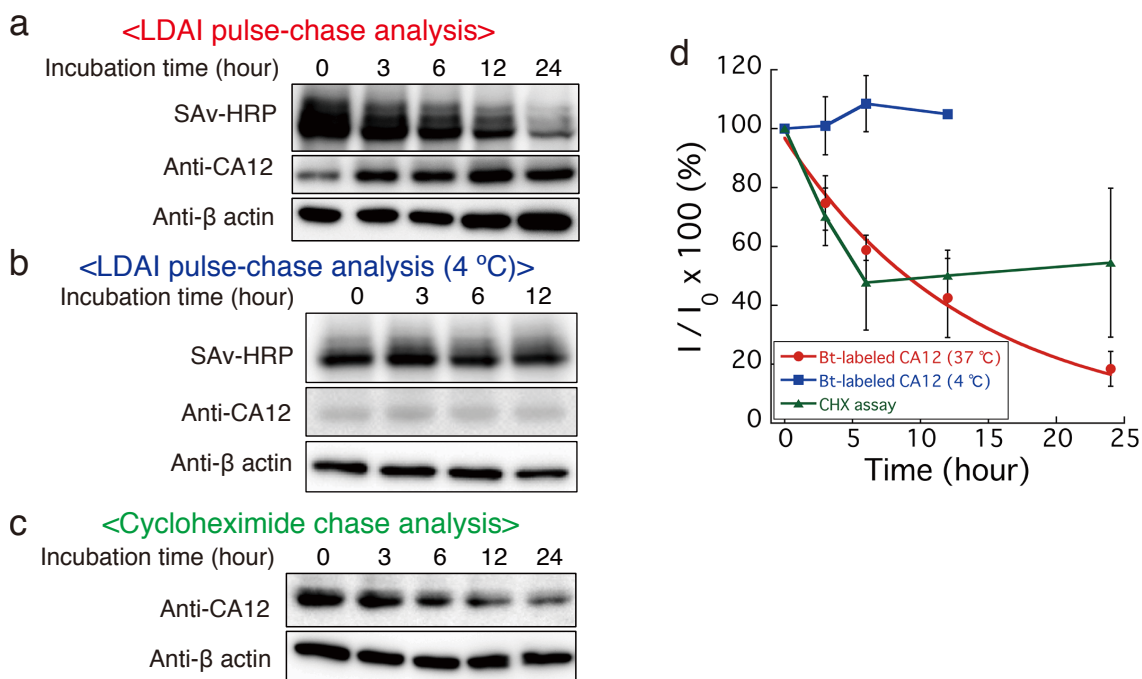


Figure 3. LDAI Pulse-chase analysis and conventional CHX half-life study of CA12. (a-b) Half-life study of CA12 using LDAI pulse-chase analysis. A549 cells were labeled with **1** (1 μM) and incubated for 0, 3, 6, 12, and 24 hr at 37 °C (a) or 4 °C (b), after washing three times with medium. Western blotting analysis using SAv-HRP, mouse anti-CA12 antibody, and mouse anti-β-actin antibody are shown. (c) Half-life study of CA12 using CHX chase analysis. A549 cells were incubated with 50 μM CHX in DMEM for 0, 3, 6, 12, and 24 hr at 37 °C. (d) The time course plot of CA12 degradation. Red, Bt-labeled CA12 degradation at 37 °C (n=4); blue, Bt-labeled CA12 degradation at 4 °C (n=3); green, CHX degradation at 37 °C (n=3) (error bars means SD).

2-2-3, Pulse-chase imaging of labeled CA12 in living cells

Given the modular feature of the LDAI method, a suitable fluorophore such as OG was attached to endogenous CA12 by LDAI reagent **2** and the fluorescent bio-imaging of CA12 was carried out using confocal laser scanning microscopy (CLSM). This provided important information on the endogenous CA12 degradation pathway in living A549 cells. As shown in the CLSM images, strong OG fluorescence was initially detected at the cell membrane region just after labeling, whereas such images were not obtained in the presence of the inhibitor EZA (**Fig. 4a**). After incubation for 3 hr, a few fluorescent granules appeared inside the cell, and 12 hr later

many granules were observed predominantly inside of the cell, while the fluorescence became very smeared at the cell membrane region (**Fig. 4b**). The intracellular fluorescent image showing the OG-labeled CA12 merged well with that of the fluorescent lysosome marker, LysoTracker, in many places after 10 hr incubation, revealing that most of the membrane-bound CA12 was translocated to lysosomes within 10 hr (**Fig. 4c**). Sequential multi-color pulse-chase imaging was also accomplished by two LDAI reagents bearing distinct fluorophores, OG (as the first pulse) and Bt/streptavidin-HiLyte647 (SAv-HiLyte647) (as the second pulse), which helped precise elucidation of the dynamic trafficking process in living A549 cells (**Fig. 4d**). Just after the second pulse, only OG fluorescent granules were observed inside the cell. After 3-12 hr, not only green OG spots but also red (HiLyte647, internalized at a late stage) spots appeared, together with yellow spots. The yellow spots generated by overlapping the green and red spots suggest that the endosomes that were internalized at a late stage were fused with the previously produced endosome. After 24 hr, the fluorescence on the membrane was scarcely detected and instead red fluorescent spots were predominantly observed, suggesting that almost all of the labeled CA12 proteins were internalized and the first labeled CA12 proteins were degraded.

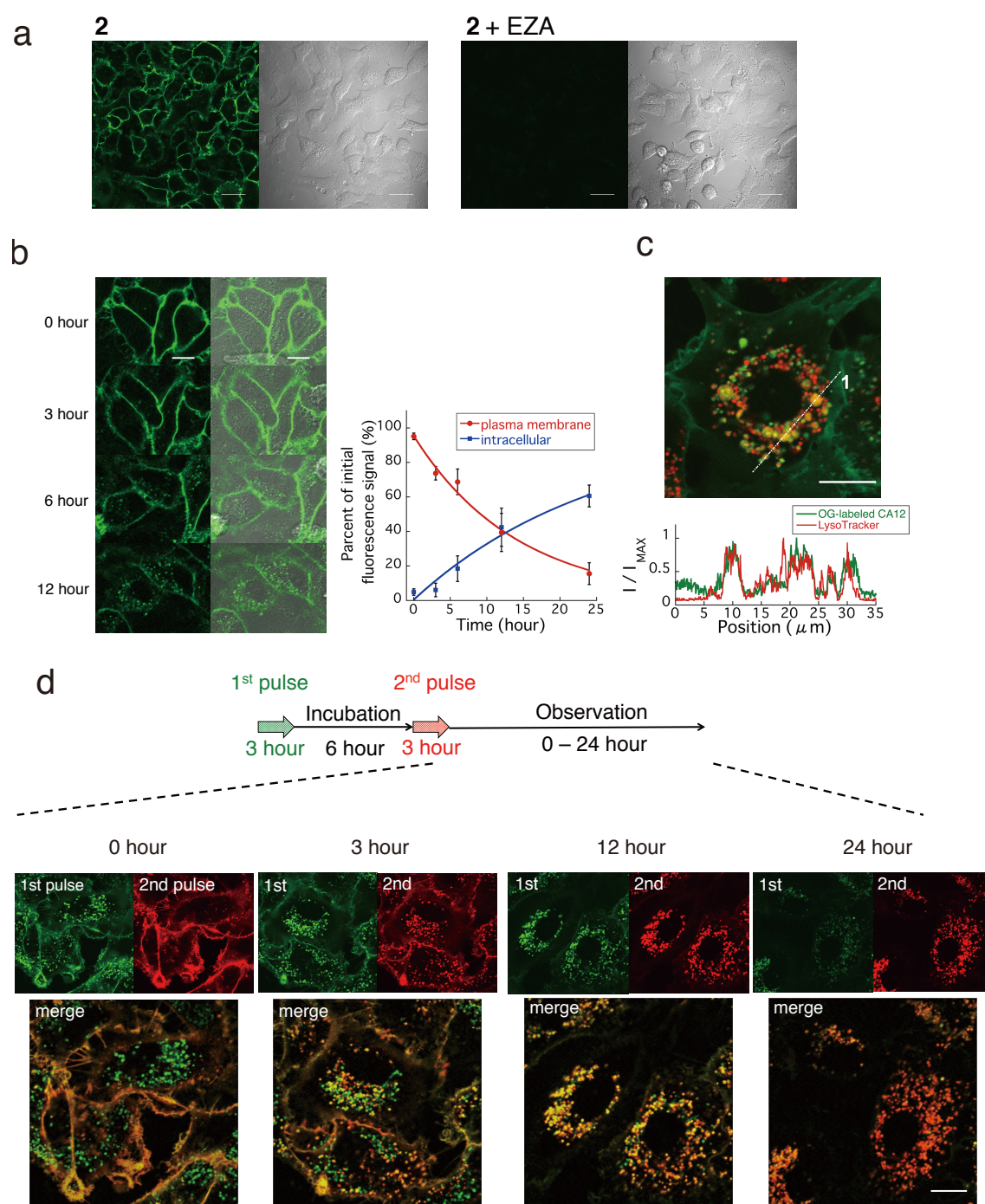


Figure 4. Pulse-Chase Imaging of Endogenous CA12 on Living Cells. (a) CLSM images of CA12 labeling by using **2** (1 μ M). As a control experiment, the labeling was conducted in presence of EZA competitive inhibitor (50 μ M). (b) Time-lapse fluorescent imaging of CA12 internalization. The labeling reaction was conducted with 1 μ M of **2** in DMEM (10% FBS) at 25 $^{\circ}$ C for 3 hr. The labeled cells were incubated in normoxia at 37 $^{\circ}$ C after washing three times with the medium. Scale bar = 15 μ m. Right panel shows the time profile of the fluorescent intensity (red, Plasma membrane fraction; blue, intracellular fraction) (error bar means SD). (c) Colocalization of LysoTracker staining (red) and OG-labeled CA12 (green) at 10 hr incubation. Scale bar = 15 μ m. In lower panel, fluorescent intensity of the dashed line is plotted. (d)

Multicolor pulse-chase imaging of CA12 degradation. The top panel shows a workflow of sequential pulse-chase imaging protocol. The bottom panel shows time-lapse fluorescence images of A549 cells upon labeling with LDAI reagent **2** for first pulse and **1** and SAv-HiLyte647 for second pulse. The labeling reaction was conducted with 1 μ M of **2** in DMEM (10% FBS) at 25 °C for 3 hr. After 6 hr, the second labeling reaction was conducted with 1 μ M of **1** at 37 °C for 3 hr. The labeled cells were stained with SAv-HiLyte647 after washing three times with the medium, and incubated in normoxia at 37 °C. Scale bar = 10 μ m.

Based on these imaging data showing that membrane-bound (OG-labeled) CA12 was internalized via endocytosis followed by transport to lysosome, it is reasonable to consider that the above-mentioned CA12 degradation takes place mainly inside the lysosome. Indeed, the half-time of the endocytosis process was estimated to be 9 hr by this time-lapse imaging, which is identical to that of the degradation decay. In addition, I confirmed that the degradation of Bt-labeled CA12 was suppressed (half-life of 27 hr) by addition of NH_4Cl (20 mM), a lysosomal protease inhibitor (**Fig. 5**). Given these experimental results, I demonstrated that the LDAI-based pulse-chase labeling of membrane-bound CA12 is useful chemical tool for evaluating the half-life of a protein in living cells, as well as examining its degradation process.

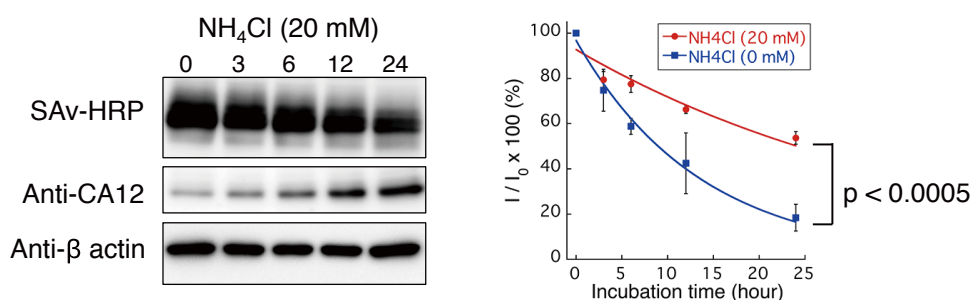


Figure 5. Inhibition of the lysosomal degradation of CA12 by 20 mM NH_4Cl . The labeled A549 cells using **1** for 3 h were incubated in DMEM containing 20 mM NH_4Cl . The time profile of band intensity shows that the proteolysis was inhibited by NH_4Cl , known to neutralize acidic environments in lysosome (Error bar = $\pm$ S.D., $n = 3$ for 20 mM NH_4Cl , $n = 4$ for without NH_4Cl , $p < 0.0005$ (at 24 hour)).

2-2-4, Selective labeling of B2R in living cells by LDAI chemistry

Once I had a potentially useful tool for analyzing membrane protein dynamics. I next sought to extend this method to other membrane protein-bound proteins, such as B2R and FR. Like other GPCRs, B2R, an important drug target, plays crucial roles in biological events such as vasodilation and edema¹⁰. Analytical tools for GPCRs in molecular detail are warranted, although handling GPCRs is generally difficult because of their complicated physicochemical properties. For B2R labeling, an antagonist peptide $\text{DArg}^0[\text{Hyp}^2, \text{DPhe}^7, \text{Leu}^8]\text{BK}^{11}$ was used as the ligand part of LDAI reagent **3** and a tripeptide (HisGlyGly) was newly added at its N-terminal where the His site was modified as the AI moiety containing a biotin. The labeling experiment was conducted in the same manner as in the case of CA12: that is, the labeling reagent **3** was mixed with HEK293 cells expressing B2R, followed by incubation for 3 hr.

The B2R labeling was analyzed by WB analysis using SAV-HRP and anti-B2R (**Fig. 6a**). The immature B2R bands without *N*-glycosylation (I in lane 1–3, 35 and 40 kDa) and a few of the mature B2R bands due to the heterogeneous saccharide modification (from 50 to 70 kDa) were detected in WB using anti-B2R (M in lane 1–3)^{12,13}. On the other hand, the biotin blotting stained only the mature bands, revealing selective labeling of mature B2Rs (50–75 kDa), which were transported to the cell surface after post translational modification. These broad bands of the labeled B2R were not detected in the presence of HOE140 (lane 3), a strong inhibitor of B2R, or in the absence of B2R transfection, suggesting that the labeling is extremely selective to the mature B2Rs and nonspecific labeling was negligible. The CBB-stained gel pattern with the B2R transfection was not apparently different from that without the B2R transfection, which means the expression level of B2R was low. It is clear that the LDAI labeling is applicable to such a low abundant target protein.

The labeled B2R on living cells was also detected by living cell imaging using complexation of the biotinylated B2R with SAV-HiLyte647. In order to ensure that the

labeling does not perturb its intact properties, I observed the agonist-induced B2R internalization. After B2R labeling followed by bradykinin (agonist) addition, the fluorescence on plasma membrane moved into the cell interior within 20 min, whereas such a rapid internalization was not observed in the case of HOE140 (antagonist) addition (**Fig. 6b**). The rate of bradykinin-induced internalization is almost same as that of previous reports¹⁴ determined by the radioisotopic ligand, strongly indicating that LDAI-labeling does not perturb the trafficking properties of B2R.

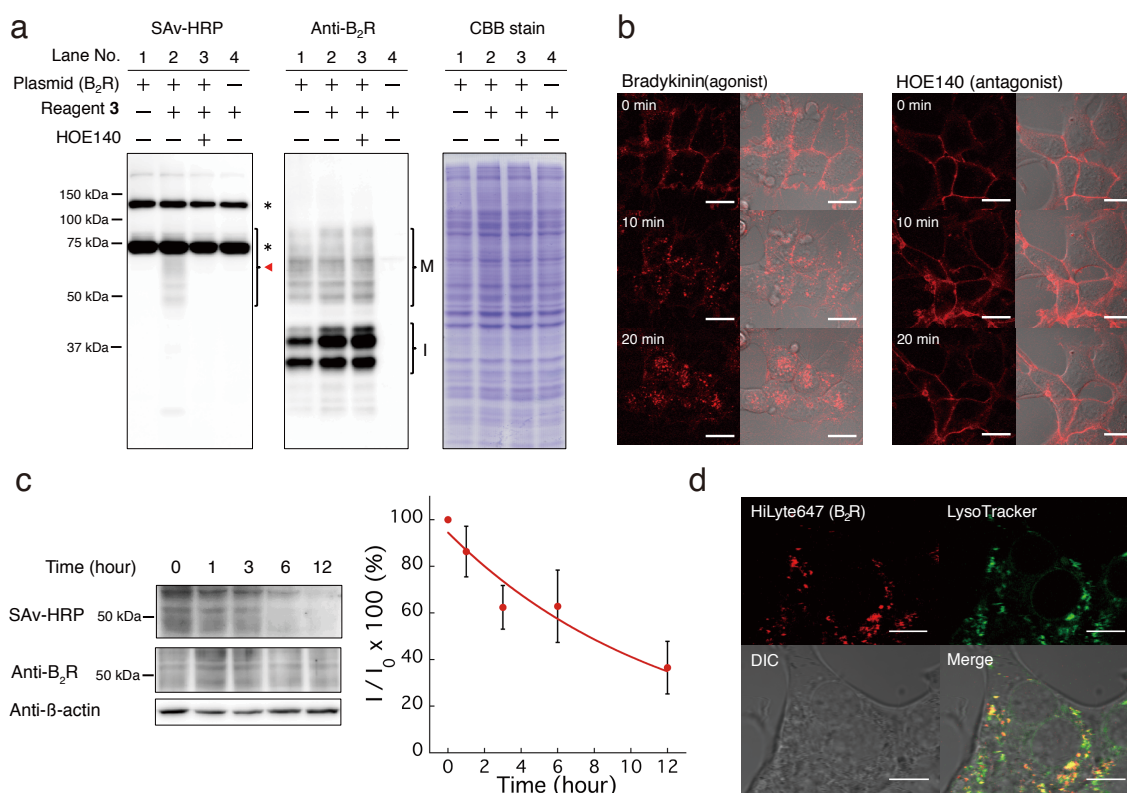


Figure 6. B2R labeling and pulse-chase study using LDAI chemistry. (a) B2R labeling on living cells. B2R-translated or vector-translated HEK239 cells were treated with **3** (5 μ M) in DMEM at 37 °C for 3 hr with or without HOE140 (2.5 μ M). The images show western blotting analysis using SAv-HRP, rabbit anti-B2R antibody, and CBB-staining of the gel. Red arrow head, labeled B2R; *, endogenous biotinylated proteins; M, mature B2R band; I, immature B2R without *N*-glycosylation. (b) Fluorescent imaging of agonist-induced internalization of labeled B2R are shown in left panel. In contrast, the localization of labeled B2R did not mainly change by addition of HOE140 (1 μ M) in right panel. (c) Pulse-chase analysis of B2R. The cells were treated with **3** and rinsed with DMEM and incubated for 0, 1, 3, 6, and 12 hr in DMEM containing HOE inhibitor. The plot represents the time course of band intensity ratio ($n=3$, error bar means SD). (d) Colocalization of LysoTracker staining (green) and

labeled-B2R (red) at 6 hr incubation. B2R expressed on HEK293 cells was biotin-labeled with **3** and stained with SAV-HiLyte647. Scale bar = 10 μ m.

2-2-5, Pulse-Chase Study of B2R

Next, I conducted the half-life study of B2R almost in the same manner as in the case of CA12. By tracing these labeled bands, the half-life of B2R was determined to be 8 hr, which is comparable to that of CA12 (**Fig. 6c**). From the time-lapse imaging data in **Figure 6d**, it is clear that B2R was internalized within a few hours and transported into lysosomes through endosomes until 6 hr, similarly to the CA12 endocytosis process mentioned above. It was thus concluded that the lysosomal degradation pathway plays a predominant role in the B2R degradation under living cell condition.

2-2-6, Pulse-chase study of folate receptor (FR) on KB cells.

Using the same method, the degradation of endogenous FR in living human KB carcinoma cells was successfully monitored, determining the half-life to be 42 hr (**Fig. 7a-b**). This was in good agreement with the previously reported value, which was evaluated by the isotope pulse-chase labeling method and the CHX method¹⁵, indicating that there are no substantial effects on the FR half-life by LDAI labeling. Its half-life is 5-fold longer than that of CA12 and B2R. Thus, I carefully monitored the dynamics of endogenous FR by attaching an imaging probe (FL) by LDAI reagent **5**, showing that the fluorescent intensity of the whole cells gradually decreased in the time range coincident with the WB analysis data (**Fig. 7c**). However, the subcellular distribution of the fluorescence between the plasma membrane and the cell interior did not substantially change after for 24 hr. On the basis of previous reports that FR on KB cell membranes was converted to a soluble folate binding domain (FBD) by a membrane-associated metalloprotease^{16,17}, I decided to evaluate the content of the labeled FBD during the pulse-chase experiments. By collecting the medium and

immune precipitation of the soluble FBD by an anti-FR antibody, the labeled FBD band was detected from the medium by biotin blotting and its intensity increased during the incubation (**Fig. 7a**). This implies that the biotin-labeled FR on living KB membranes was secreted to the medium by protease cleavage, which may be assigned as the main pathway of FR degradation.

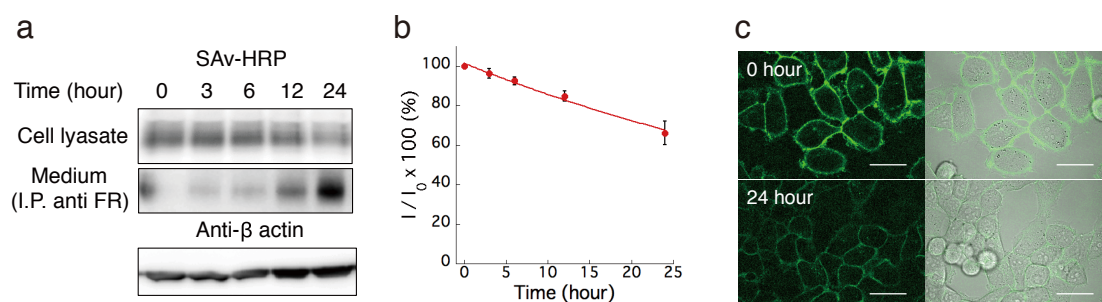


Figure 7. Pulse-chase study of FR using LDAI chemistry. (a) Biotin blotting analysis of labeled FR on cell and in medium. KB cells were treated with **4** (1 μ M) for 3 hr, rinsed and incubated. The labeled FR in cell lysate was analyzed by biotin blotting using SAy-HRP. Secreted FBD in the medium was immune precipitated by anti-folate receptor antibody and detected by biotin blotting. (b) Time profile of degradation of FR ($n = 3$, error bar means SD). (c) Time-lapse images of Fluorescent labeled FR on KB cells (scale bar means 30 μ m).

2-3, Conclusion

I demonstrated here that LDAI chemistry-based protein labeling can be broadly applied to diverse membrane proteins from rather simple proteins such as GPI- or single TM-anchored proteins to the more complicated GPCR. Moreover, the pulse-chase LDAI labeling allowed us to determine the protein half-life, as well as to examine its degradation pathway, by combining biochemical western blotting analysis and live cell imaging. I envision that a more precise analysis of a variety of membrane proteins that are substantially complicated in their structure and functions would be facilitated by LDAI chemistry.

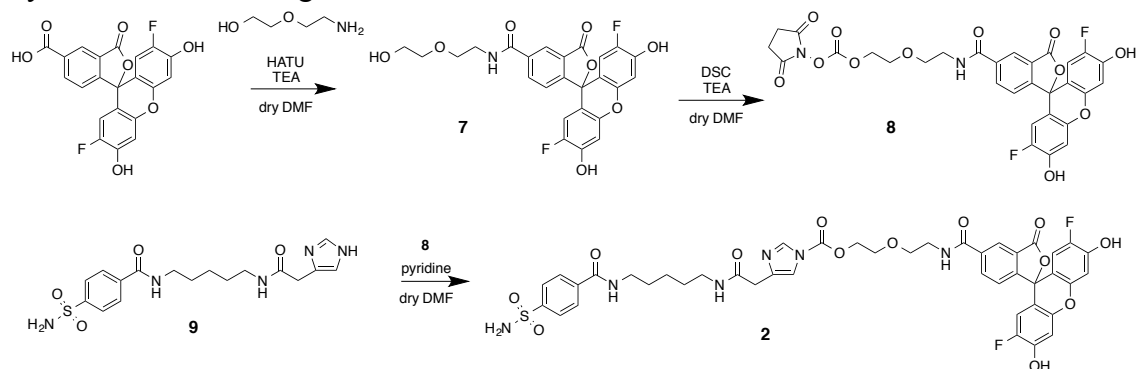
2-4, Experimental Section

General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Acros Organics, Sasaki Chemical, or Watanabe Chemical Industries) and used without further purification.

Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminium sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was conducted by flash column chromatography on silica gel 60 N (neutral, 40–50 μ m, Kanto Chemical). ¹H NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer or JEOL JNM-ECA600 (600 MHz) spectrometer calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, br s = broad singlet. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was measured by an Autoflex II instrument (Bruker Daltonics) using a-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid as the matrix. High-resolution electrospray ionization quadrupole fourier transform mass spectroscopy (HR-ESI Qq-LTMS) were measured by a JEOL JMS-HX110A mass spectrometer or Bruker apex-ultra (7T) mass spectrometer.

Synthesis of compound **1**, **5**, **6**, **9**, **10** was previously reported^{6,18}.

Synthesis of LDAI reagents **2**Scheme S1. Synthetic scheme of compound **2**Compound **7**

A solution of Oregon Green® 488 carboxylic acid (300 mg, 0.73 mmol), HATU (360 mg, 0.95 mmol), DIPEA (250 μ L, 1.5 mmol), and 2-(2-aminoethoxy) ethanol (87 μ L, 0.87 mmol) in DMF (5 mL) was stirred for 3 h at room temperature. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO₂ (CHCl₃ : MeOH : AcOH = 8 : 1 : 0.02) to yield compound **7** (148 mg, 0.30 mmol, 41 %) as a red solid.

¹H NMR (400 MHz; CD₃OD): δ 8.50 (s, 1H), 8.20 (d, J = 8.0 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 6.82 (d, J = 7.6 Hz, 2H), 6.49 (d, J = 11.2 Hz, 2H), 3.68 – 3.73 (m, 4H), 3.65 (t, J = 4.8 Hz, 2H), 3.60 (t, J = 4.6 Hz, 2H).

MALDI-TOF MS (CHCA) m/z calc. for $[M+H]^+$ = 500.11 obsd. 500.04

Compound **8**

To a solution of compound **7** (20 mg, 0.11 mmol) and triethylamine (14 μ L, 0.1 mmol) in DMF (1.0 mL) was added *N,N'*-disuccinimidyl carbonate (26 mg, 0.1 mmol) at room temperature. The reaction solution was allowed to stir for 5 h. The residue was concentrated under reduced pressure and precipitated by diisopropyl ether to give compound **8** as a red solid (19 mg) containing some impurities, which was used for the

next step without further purification.

MALDI-TOF MS (CHCA) m/z calc. for $[M+H]^+ = 641.12$, found 641.22

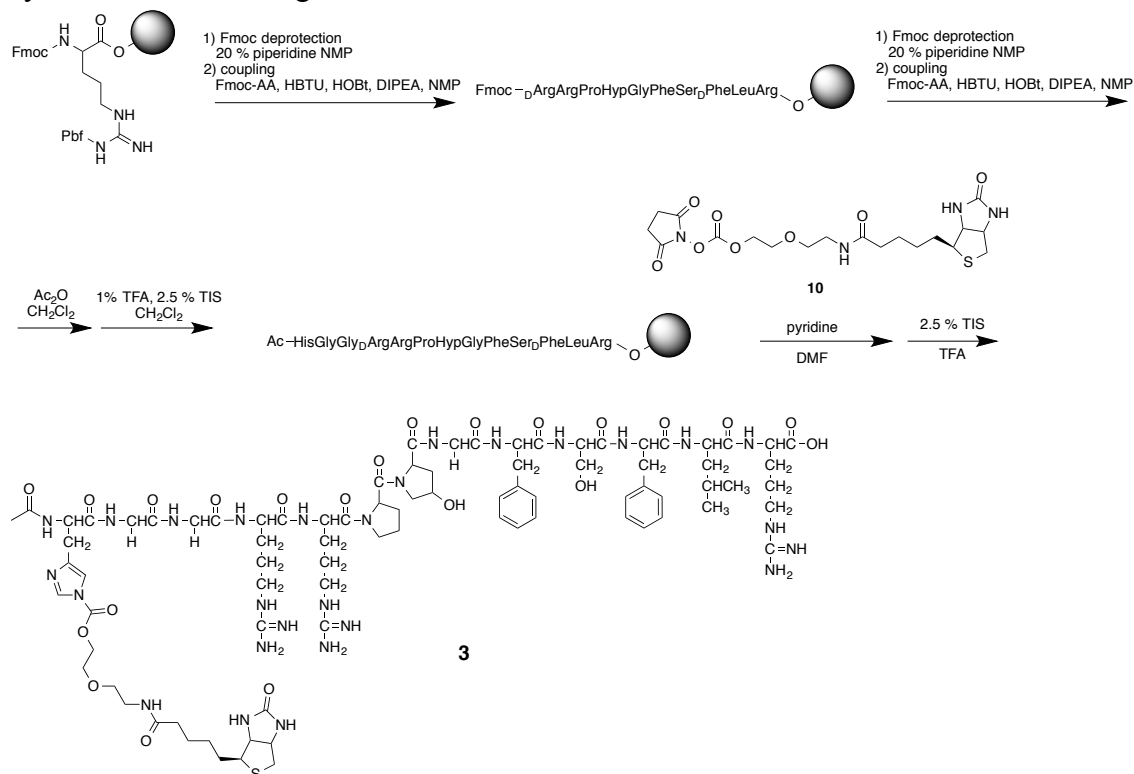
Compound 2

To a solution of compound **9** (7.9 mg, 0.02 mmol) and pyridine (3.2 μ L, 0.04 mmol) in DMF (500 μ L) was added compound **8** (19 mg). The solution was allowed to stir for 12 h at room temperature. The crude solution was purified by RP-HPLC (column ; YMC-packODS-A, 250 x 25 mm, mobile phase; CH₃CN ; 10 mM NH₄OAc = 5 : 95 $\rightarrow$ 35 : 65 (linear gradient over 60 min), flow rate ; 10 mL/min, detection; UV (220 nm)) to give compound **2** (11 mg, 63 %) as a red powder.

¹H NMR (600 MHz; (CD₃)₂SO): δ 8.83 (t, $J = 5.7$ Hz, 1H), 8.70 (t, $J = 6.6$ Hz, 1H), 8.46 (s, 1H), 8.14 (s, 1H), 8.05 (br, 1H), 7.97 (d, $J = 8.4$ Hz, 2H), 7.94 (t, $J = 6.6$ Hz, 1H), 7.85 (d, $J = 8.4$, 2H), 7.48 (br s, 2H), 7.36 (s, 1H), 7.32 (br , 1H), 6.36 (br, 4 H), 4.50 (t, $J = 4.5$ Hz, 2H), 3.79 (t, $J = 4.5$ Hz, 2H), 3.64 (t, $J = 5.7$ Hz, 2H), 3.48 (q, $J = 5.7$ Hz, 2H), 3.50 (s, 2H), 3.21 (q, $J = 6.6$ Hz, 2H), 3.00 (q, $J = 6.6$ Hz, 2H), 1.49 (m, 2H), 1.39 (m, 2H), 1.27 (m, 2H).

HR-ESI MS m/z calcd for $[M+H]^+ 919.2415$, found 919.2404

Synthesis of LDAI reagents 3



Scheme S2. Synthetic scheme of compound **3**

Compounds were synthesized by standard Fmoc solid-phase methods using a peptide synthesis system attached to a rotary shaker (Kokusan chemical company, Japan). Fmoc-Arg(Pbt)-Alko-PEG resin (0.22 mmol/g, 100-200 mesh) was used for the synthesis. Fmoc deprotection was performed with 20% piperidine in *N*-methylpyrrolidine (NMP), and coupling reaction was carried out in NMP containing Fmoc-amino acid (3 equiv), HBTU (3 equiv), HOBT (3 equiv), and DIPEA (6 equiv). Kaiser test was performed to confirm the completion of Fmoc deprotection and coupling reaction. After acetyl capping of N-terminal, the Mtt group of His residue was deprotected by 1% TFA solution (CH₂Cl₂ : TFA : TIS = 94 : 1 : 5). The condensation reaction with compound **10** (3 equiv) was carried out in DMF containing pyridine (4 equiv). Peptides were cleaved from the resin with TFA containing 2.5 % TIS and precipitated with cold diisopropyl ether. Peptides were purified by RP-HPLC (column ;

YMC-packODS-A, 250 x 25 mm, mobile phase; CH₃CN ; 10 mM NH₄OAc (pH = 5.5) = 10 : 90 → 40 : 60 (linear gradient over 50 min), flow rate ; 10 mL/min, detection; UV (220 nm)) to give compound **3** (7 mg, total-yield 15%) as a white powder.

MALDI-TOF MS (CHCA) m/z calcd for $[M+H]^+ = 1898.94$, found 1899.38

HR-ESI MS m/z calcd for $[M+2H]^{2+} 949.9758$, found 949.9738

General materials and methods for biochemical/biological experiments

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were carried out using a Bio-Rad Mini-Protean III electrophoresis apparatus. Chemiluminescent signals generated with Chemi-Lumi One (nacalai tesque) or ECL Prime (GE Healthcare) were detected with an LAS4000 imaging system (Fuji Film). Cell imaging was performed with a confocal laser scanning microscope (CLSM, Olympus, FV1000, IX81) equipped with a 60× objective lens. Fluorescence images were acquired using 488 nm line of an argon laser for excitation of Oregon Green or Fluorescein (emission, 500-600 nm) and 633 nm line of a HeNe Red laser for excitation of streptavidin modified with HiLyte647 (SAv-HiLyte647) (emission, 645-745 nm).

Labeling of endogenous CA12 or FR on live cells and their half-life studies

A549 cells or KB cells were maintained in 5% CO₂ at 37 °C in D-MEM (Sigma, for A549 cells) or folate free RPMI1640 (Gibco, for KB cells) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (250 ng/mL). For the endogenous CA12 labeling, A549 cells (1×10^5 cells) were incubated under the hypoxic condition (< 0.1% O₂) generated with AnaeroPack (Mitsubishi Gas Chemical Company, Inc.) for 1 day, and treated with **1** (1 µM) at 37 °C in D-MEM (10% FBS) for 3 h in hypoxic condition. As a control experiment, the labeling was conducted in the presence of EZA (50 µM). For half-life study, the labeled cells were washed 3 times with the same medium, and incubated

Chapter 2

for 0, 3, 6, 12, 24 h in normoxia condition, and lysed using NP40 buffer (pH 7.4, 50 mM Tris·HCl, 150 mM NaCl, 1% Nonidet P-40) containing 1% protease inhibitor cocktail set III (Calbiochem®). The insoluble material was removed by centrifugation at 12 000 rpm for 10 min. The supernatant was mixed with the same volume of 2× Laemmli buffer (pH 6.8, 125 mM Tris·HCl, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 100 mM dithiothreitol) and incubated for 1 h at room temperature. The samples were subjected to SDS-PAGE (10%) and then electrotransferred onto an Immun-Blot® PVDF membrane (Bio-Rad). The biotin-labeled CA12 was detected by chemiluminescence analysis using SAv-HRP (Invitrogen, x10,000). The immunodetection of CA12 was performed with a mouse anti-CA12 antibody (Abcam, x500) and anti-mouse IgG-HRP conjugate (GE Healthcare, x2000).

For the FR labeling, KB cells (1×10^5 cells) were treated with **4** or **5** ($1 \mu\text{M}$) at 37°C in RPMI1640 (10% FBS) for 3 h. The cells were washed 3 times with the medium, incubated for 0, 3, 6, 12, 24 h, and subjected to western blotting for protein half-life study. The culture medium (1 mL) was collected, pre-cleared with Protein G Agarose resin (Invitrogen, $50 \mu\text{L}$), and the cleaved FBD was immunoprecipitated by using mouse anti-FR antibody (abcam, x2000) and Protein G Agarose resin. The cells were lysed with Ripa buffer (pH 7.6, 25 mM Tris·HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40) containing 1% protease inhibitor cocktail set III, mixed with the same volume of 2x Laemmli buffer, and subjected to SDS-PAGE and western blotting. The biotin-labeled FR or FBD was detected by chemiluminescence analysis using SAv-HRP (Invitrogen, x10,000). β -actin, detected by an antibody (abcam, x2,000), was used as a loading control.

Cycloheximide degradation assay

A549 cells (1×10^5 cells) were treated with Cycloheximide ($50 \mu\text{M}$) at 37°C in D-MEM (10% FBS) for 0, 3, 6, 12, 24 h, and washed with PBS (-). For western blotting, the cells were lysed using NP40 buffer containing 1% protease inhibitor cocktail set III. The lysed sample was

centrifuged, and the total amount of protein in supernatant were analysed by BCA assay. The samples were diluted to 0.25 μ g (total protein) / μ L, and subjected to WB analysis.

Cell culture and transfection of B₂R cDNA

The expression plasmid for human B₂R, pCI(B₂R) was previously constructed (Nonaka et al., 2010). Human embryonic kidney (HEK293) cells were maintained in 5% CO₂ at 37 °C in D-MEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μ g/mL), and amphotericin B (250 ng/mL). Transfection of cDNA plasmid was carried out in a 35 mm dish using Lipofectamine 2000 (Invitrogen) according to a general protocol. HEK293 cells were used for B₂R transfection and subjected to labeling experiments after 48 h of the transfection.

Labeling of B₂R and the half-life studies

The B₂R expressing on HEK293 cells were labeled by the treatment with 1 mL D-MEM containing 5 μ M of reagent **3** for 3 h in 5% CO₂ at 37 °C. As a control experiment, the labeling was conducted in the presence of HOE140 (5 μ M). For pulse chase analysis, the cells were washed with D-MEM for 3 times after removing the probe solution, and incubated in D-MEM with HOE140 (1 μ M) for 0, 1, 3, 6, 12 hours. The dishes were washed with 1 mL HBS for 3 times, and lysed with Ripa buffer containing 1% protease inhibitor cocktail set III. After mixing with the same volume of 2x Laemmli buffer, the following electrophoresis processes are similarly performed as described above. The immunodetection of B₂R was performed with a rabbit anti-B₂R antibody (Santa Cruz, $\times$ 500) and anti-rabbit IgG-HRP conjugate (Santa Cruz, $\times$ 5000). For the pulse chase imaging, HEK293 cells stably expressing human B₂R (Wang et al., 2011) were used. The cells were labeled with reagent **3** for 3 h in D-MEM. After the incubation, the cells were washed with D-MEM for 3 times, and stained using SAV-HiLyte647.

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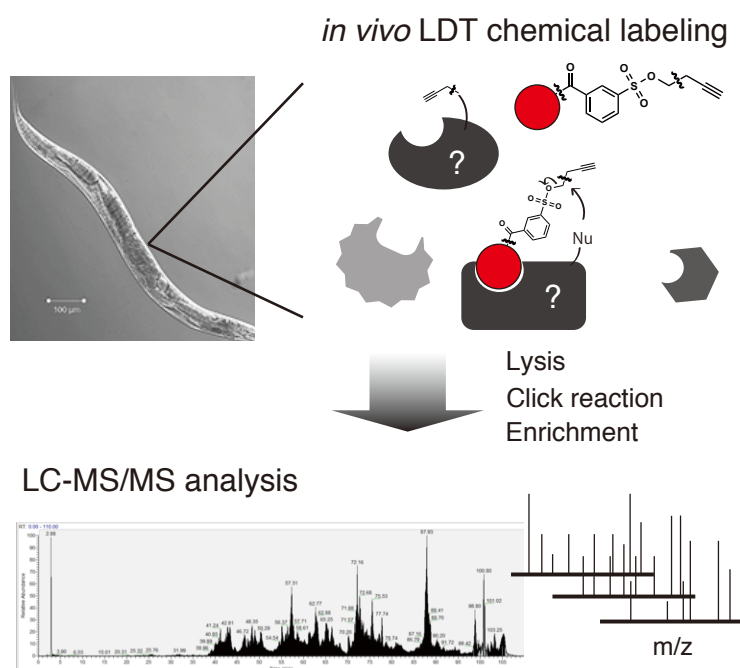
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Chapter 3

Proteomic Analysis of Cholesterol-/Sterol-Binding Proteins Using Ligand-Directed Tosyl Chemical Labeling in Living Nematode

Abstract

Proteome-wide analysis of protein-ligand interactions offers insights to answer various biological questions. Photo-affinity labeling is known as a conventional method for tagging proteins interacting with the ligand. However, this is impossible to apply *in vivo*, due to critical problems such as low labeling efficiency, requirement of UV-irradiation. To overcome these problems, here I extended the Ligand-Directed Tosyl Chemistry which has been developed for specific protein labeling, to a proteome-wide labeling tool. By using LDT chemistry, I succeeded in identifying 60 proteins interacting with cholesterol in living nematode.



3-1, Introduction

Affinity labeling is a powerful tool to identify the proteins interacting with a ligand of interest. Once the interacting proteins are modified with a chemical tag such as biotin, the proteome can be enriched by using streptavidine or antibodies, and identified in combination with mass fingerprinting methods. For instance, Cravatt and his colleagues have developed activity-based protein profiling (ABPP), in which labeling reagents consist of a covalent inhibitor and a chemical tag in order to label and identify the active enzyme families¹⁻³. On the other hand, for studying the proteins physiologically but not covalently interacting with a molecule of interest, photo-affinity labeling is conventionally used⁴⁻⁶.

The widely used photoactive groups are benzophenone and diazirine, which generate diradical and carbene species as reactive intermediates, respectively. However, these groups have three drawbacks^{7,8}; 1) low labeling efficiency, 2) low selectivity in same case, and 3) UV-irradiation is inevitably needed, which prevent us from applying this method *in vivo* where UV can not well reach. Therefore, this method is limited *in vitro* or in cell experiments. Given Ligand-directed Tosyl (LDT) chemistry^{9,10} that is based on S_N2 reaction with nucleophilic amino acids on protein surface, I thought that LDT chemistry can overcome these problems. Here, I sought to apply the LDT chemistry that has been used for labeling of a specific target protein to a proteome-wide analysis, and demonstrated the utility of LDT chemistry in *in vivo* chemoproteomics.

3-2, Results and Discussions

3-2-1, Molecular designs of reagents and a nematode as a model *in vivo*

Cholesterol is one of the most essential molecules in biological systems as a component of cellular membranes and a precursor for steroid hormones, such as estradiol¹¹. It is assumed that cholesterol interacts with many proteins and is converted to derivatives by various enzymes. Therefore, to evaluate the utility of LDT chemistry in proteome-wide analysis, I employed cholesterol and estradiol as ligands of interest. And, I sought to label and identify a set of proteins interacting with steroids. I initially synthesized a LDT-type reagent **1**, where cholesterol is conjugated to an alkyne tag through a phenyl sulfonyl ester, and the similar reagents **2** and **3** for estradiol ligand. In addition, I synthesized reagent **4** containing a Cbz group instead of these ligands as a negative control. A diazirine-tethered reagent **5** was previously reported for photoaffinity labeling¹². These molecules are characterized by ¹H-NMR and mass spectrometry.

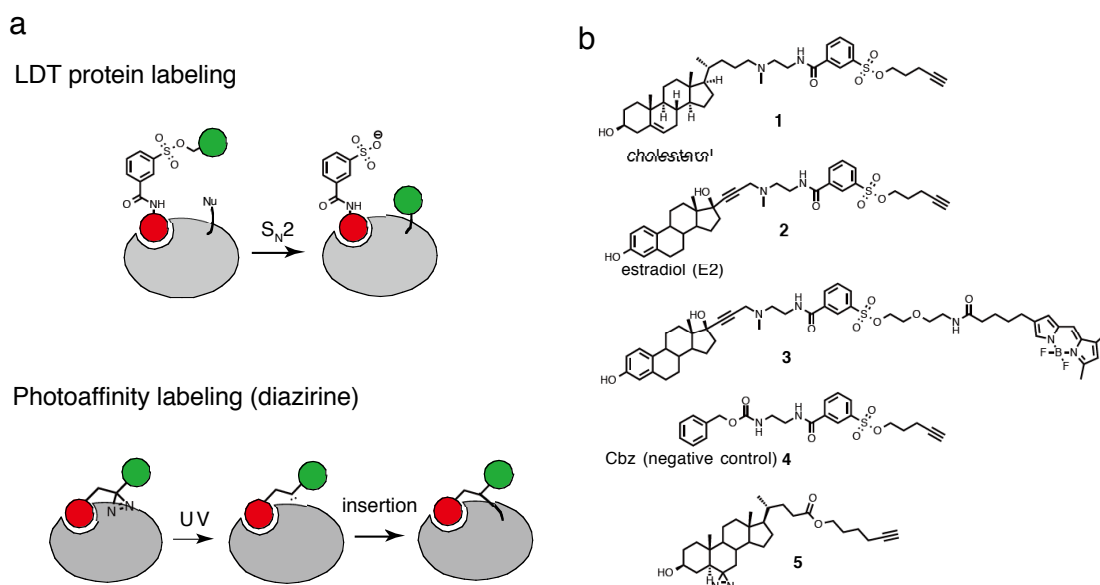


Figure 1. Comparison between LDT reaction and photo affinity labeling methods. (a) Schematic illustration of reaction mechanisms, (b) Chemical compounds of labeling reagents (LDT-type reagents; **1-4**, diazirine-type reagent; **5**)

I used a nematode worm *Caenorhabditis elegans* (*C. elegans*) as a live model of *in vivo* experiences, due to following reasons. (1) the genome sequence had been completely unveiled and available, so that this animal is suitable to proteomic research. (2) Steroid hormones synthesized from cholesterol in the body are key signaling molecules for a various physiological events such as growth, metabolism, and life of *C. elegans*¹³⁻¹⁶. (3) This animal can't synthesis cholesterols, yet it is essential. Then, I can supply the cholesterol-type reagents instead of intact cholesterols to the medium.

3-2-2, Proteomic labeling using LDT-type or diazirine-type reagents

The utility of LDT chemistry in proteomic labeling was initially examined using a *C. elegans* lysate. I added reagent **1** (5 μ M) to the lysate and incubated it for 16 h at 20 °C. In these experiments, alkyne tags should be modified to the protein surfaces. For detection of the alkyne-labeled proteins, Oregon Green (OG) fluorophore was conjugated with the alkyne tag by click reaction. The labeling was then evaluated by SDS-PAGE, followed by fluorescent imaging. **Figure 2** shows many fluorescent bands due to labeled proteins were detected. To ensure the reaction was based on cholesterol-protein interactions, I added cholesterol (C, 20 eq for reagent **1**) or 3 β -Hydroxyl- Δ 5-cholenic acid (CA, 10 eq.) to the lysate before the treatment of reagent as competitive inhibition experiments. In **Figure 2**, the fluorescent bands remained in presence of cholesterol, probably because the cholesterol was not dissolved enough due to its strong hydrophobicity. On the other hand, in case of 3 β -Hydroxyl- Δ 5-cholenic acids, the major fluorescent bands disappeared, indicating that labeling of these proteins are driven by the cholesterol-protein interactions. These data strongly suggested that LDT chemistry applicable to proteomic-wide labeling.

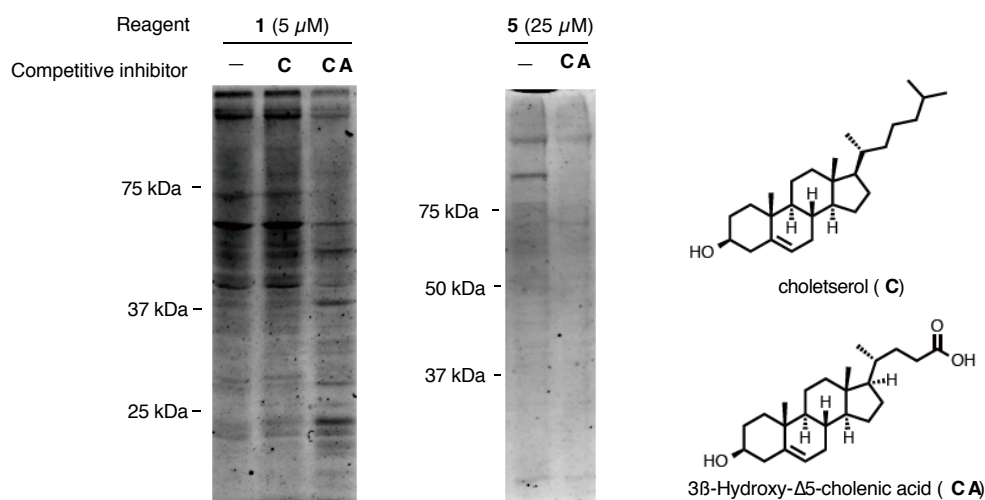


Figure 2. Comparison of proteome-wide chemical labeling of cholesterol-interacting proteins. The labeled proteins by LDT-type reagent **1** and diazirine-type reagent **5** were shown in left and middle panels, respectively. For LDT labeling, 5 μM of **1** was added to the lysate and incubated for 16 h at 20 $^{\circ}\text{C}$. To the lysate, OG- N_3 (20 μM), click ligand (10 μM), CuSO_4 (1 mM) and TCEP (1 mM) was added to conjugate OG to alkyne tagged proteins. For photo-affinity labeling, 25 μM of **5** was added to lysate and irradiated UV light 365 nm on ice. The chemical structures of competitive inhibitors, cholesterol (C) and 3 β -Hydroxyl- Δ^5 -cholenic acid (CA) were shown in right panel.

I evaluated the proteome-wide labeling by using the diazirine-type reagent **5**. I added 25 μM of **5** to the lysate and irradiated UV light (365 nm) for 15 min on ice. Although the many fluorescent bands were detected, the fluorescent intensities were lower than that in case of LDT reagent **1**. I also noticed that the band pattern was largely distinct from that by LDT labeling, indicating that the labeled proteome was different each other. I supposed such difference was caused by (1) the reaction mechanism and (2) the sites of a reactive group in the reagent. Diazirine group generates a reactive carbene intermediate, which can less-selectively react with amino acids by C-H or O-H insertion. In general, due to its high reactivity, the half-life of carbene species is extremely short. Therefore, in most case, diazirine group must be directly attached or incorporated into the ligand to enhance the proximity effect for the high labeling yield. As a representative, in many photoactive probes for cholesterol, a diazirine is inserted into the C4 carbon of cholesterol^{12,17} as shown in **5**. On the other hand, the half-life of

LDT reagents are about 1 day in aqueous system, and it selectively reacts with nucleophilic side chains such as Tyr, Ser, His, Glu, strongly dependent on the proximity effect. This feature allows for flexible designs and need not compromise the intact structure of the ligand.

3-2-3, Proteomic labeling *in vivo*

It was expected that LDT chemical labeling is potentially applicable *in vivo*, because UV-irradiation is not necessary. Although *C. elegans* is a skeleton organism and UV may reach the whole bodies, I apply LDT chemistry to this worm as a model animal *in vivo*. I incubated the nematodes in S-medium containing 5 μ M reagent **1** for 16 h at 20 °C. Then, I homogenized the worms and modified the alkyne-labeled protein with OG by click reaction. Similar to the protocol in lysate experiment, **Figure 3** shows a variety of labeled proteins, whereas these intensities were dramatically decreased in presence of extra cholesterol 20 mg/L ($\sim 53 \mu$ M)¹¹, the concentration same as that of normal medium for nematode. Moreover, reagent **4** as a negative control does not label these proteins. Taken together, it is indicated that the LDT reagent can apply to the proteome-wide labeling *in vivo*.

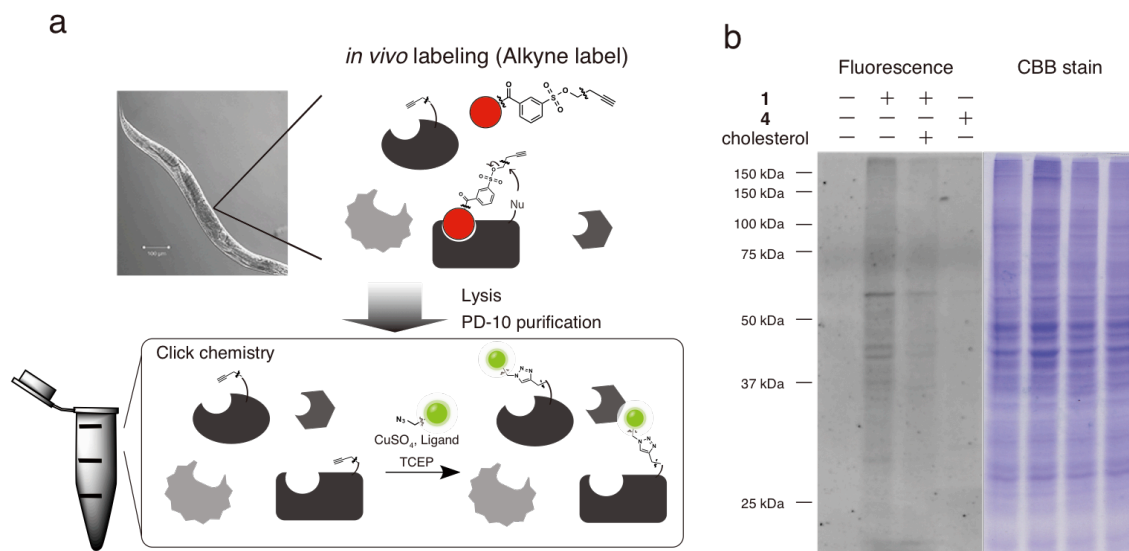


Figure 3. *in vivo* proteome-wide labeling using LDT chemistry. (a) Workflow of chemical labeling and identification of cholesterol-interacting proteins. (b) SDS-PAGE analysis followed by in-gel fluorescent image of labeled proteins. Worms were suspended in S-medium containing 5 μ M of reagent **1** or **4** for 16 hr at 20 °C. The labeled worm were lysed and modified with OG by click reaction.

3-2-4, Mapping of cholesterol-interacting proteins

I finally tried to identify the labeled proteins in combination with the peptide mass finger printing method. The cholesterol-interacting proteins were labeled by alkyne tags *in vivo*, conjugated to OG by click reaction, enriched by immune precipitation using anti-fluorescein (FL) antibody, followed by in-gel digestion using trypsin. To decrease the false-positive detection caused by contamination from non-specific interaction with the FL-antibody, and absorption with tubes or beads, I conducted the quantitative mass analysis using stable isotope dimethyl labeling¹⁸, and compared between positive and negative control (competitive inhibition by cholesterol). The digested peptides were mixed with *d*²-formaldehyde (for positive control) or formaldehyde (for negative control) in the presence of cyanoborohydride (NaBH₃CN). The reaction was quenched with 5% hydroxylamine and both samples were mixed in a 1:1 ratio, and subjected to nanoLC-MS/MS measurement (**Fig. 4a**). Only proteins

showing at least two unique peptides (FDR < 0.001) were judged as “identified proteins” (150 proteins). **Figure 4b** shows the dimethyl isotopic ratio values (Heavy; positive, light; competitive control). Among this proteome, I set proteins showing the ratio value more than 2.0 as “sterol-interacting proteins” (60 proteins). I found that some proteins in this group have been reported as sterol-binding proteins or the relevant complexes in other organisms, such as IDOL (Inducible Degradator of the LDL receptor; top 1/152), ERp57 (Protein disulfide isomerase-3; 28/152)¹⁹ and PDI-2 (Protein disulfide isomerase-2; top 35/152)²⁰ or in sterol-rich membrane domains such as MyoI (Myosin-1; top 36/152)²¹. For the broader survey, I also analyzed the group with gene ontology term enrichment by using PANTHER Classification System. The result showed proteins implicated in developmental processes (larval development ; 31 proteins ; $p = 9.2 \times 10^{-18}$, reproduction ; 31 proteins ; $p = 7.9 \times 10^{-15}$, multicellular organismal development ; 37 proteins ; $p = 2.0 \times 10^{-14}$ and so on), metabolic process (38 proteins, $p = 1.5 \times 10^{-13}$) were enriched (**Table 1**). This strongly implied that cholesterol-interacting proteins play critical roles for worm growths, being consistent with biological role of cholesterol. Taken together, it is concluded that the chemical proteomics studies *in vivo* can perform by using LDT chemistry.

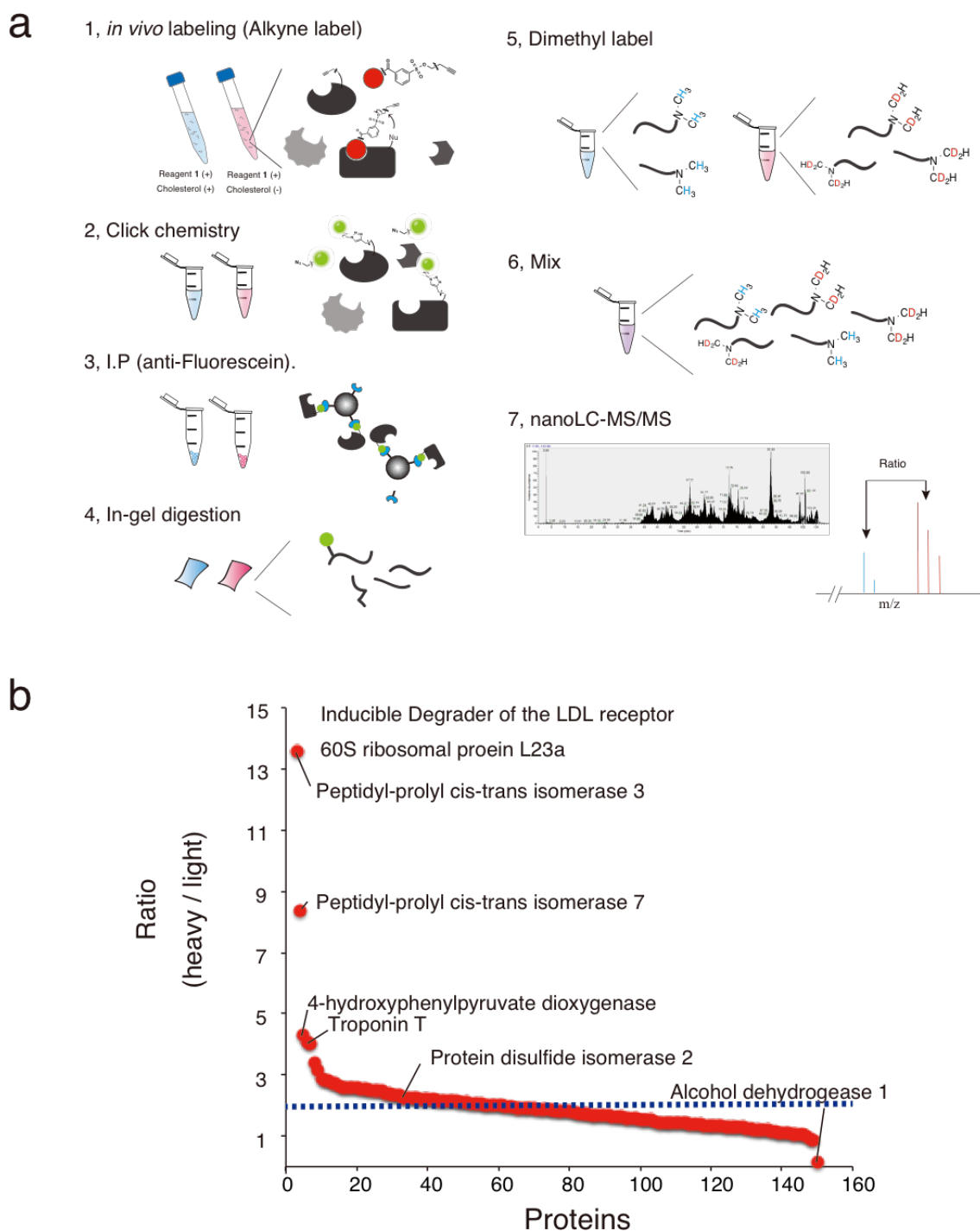


Figure 4. Mapping of cholesterol-interacting proteins by using LDT labeling in combination with quantitative proteomics. (a) Schematic illustration of the procedure from protein labeling to nanoLC-MS/MS measurement. (b) Dimethyl isotope ratio values of identified proteins.

Table.1 Gene Ontology enrichment analysis (Top 9)

Gene ontology term (GO ID)	protein	-log P value
larval development (GO:0002164)	31	17.0
post-embryonic development (GO:0009791)	31	16.9
nematode larval development (GO:0002119)	30	15.8
reproduction (GO:0000003)	31	14.1
multicellular organismal development (GO:0007275)	37	13.7
anatomical structure development (GO:0048856)	36	13.2
single-organism developmental process (GO:0044767)	37	12.9
developmental process (GO:0032502)	37	12.8
metabolic process (GO:0008152)	38	12.8

3-2-5, Labeling and identification of estradiol-interacting proteins

Estradiol (E2), one of the steroid hormones produced by cholesterol plays important roles in growth and memories in nematode¹³. Therefore, it was interesting to compare the labeling behavior by using LDT-type reagent **2** and **3**, with that by cholesterol-tethered reagent **1**. First of all, I labeled estradiol-interacting proteins *in vitro* by using reagent **3** containing a BODIPY dye instead of alkyne. Surprisingly, only a single fluorescent band of 56 kDa, was observed (**Fig. 5a**). I separated this band by 2D-PAGE, digested in-gel and analyzed it by nanoLC-MS/MS, indicating that this is protein disulfide isomerase-2 (PDI-2) (**Fig. 5b**). Next, I investigated the *in vivo* labeling by using reagent **2**, showing that PDI-2 was again selectively labeled (**Fig 5c**). Although the interaction between E2 and human PDI was previously analyzed in test tubes²², to the best of my knowledge this is the first report to show the direct interaction of E2 with PDI *in vivo*. PDI exhibits hydrophobic domains to assist its function, protein folding. Besides, PDI is supposed to store and buffer the hydrophobic compounds, such as

thyroid or steroid hormones, to maintain these the concentration at low level²³.

To identify other compounds interacting with PDI-2, I next conducted the competition assay on the basis of LDT labeling in the *C. elegans* lysate. I added a various steroid hormones or biologically active compounds, i.e. rapamycin, FK506, or its derivatives to the lysate together with reagent **3** and examined the inhibition level in the PDI-2 labeling. **Figure 4d** shows that Estrone (E1) and Estradiol (E2) substantially inhibited the labeling reactions, although Testosterone (T) didn't. Both E1 and E2 contains hydroxyl group in C3 position of steroid, suggesting this hydroxyl group is crucial to interact with PDI-2. Previously, Zhu group evaluated the interaction model between E2 and hPDI using the computer simulation²⁴, finding a significant hydrogen bond between this hydroxyl group and His256 are preserved in nematode PDI-2. It is reasonably assumed that the PDI-2 may bind E2 in the manner same to hPDI. To my surprise, rapamycin, FK506 and its derivatives also showed inhibition of the reaction. This is the first experimental data showing their interaction with PDI-2. The biological function of these compound are inhibition of immune responses, suggesting PDI-2 is either directly or indirectly related with immune responses.

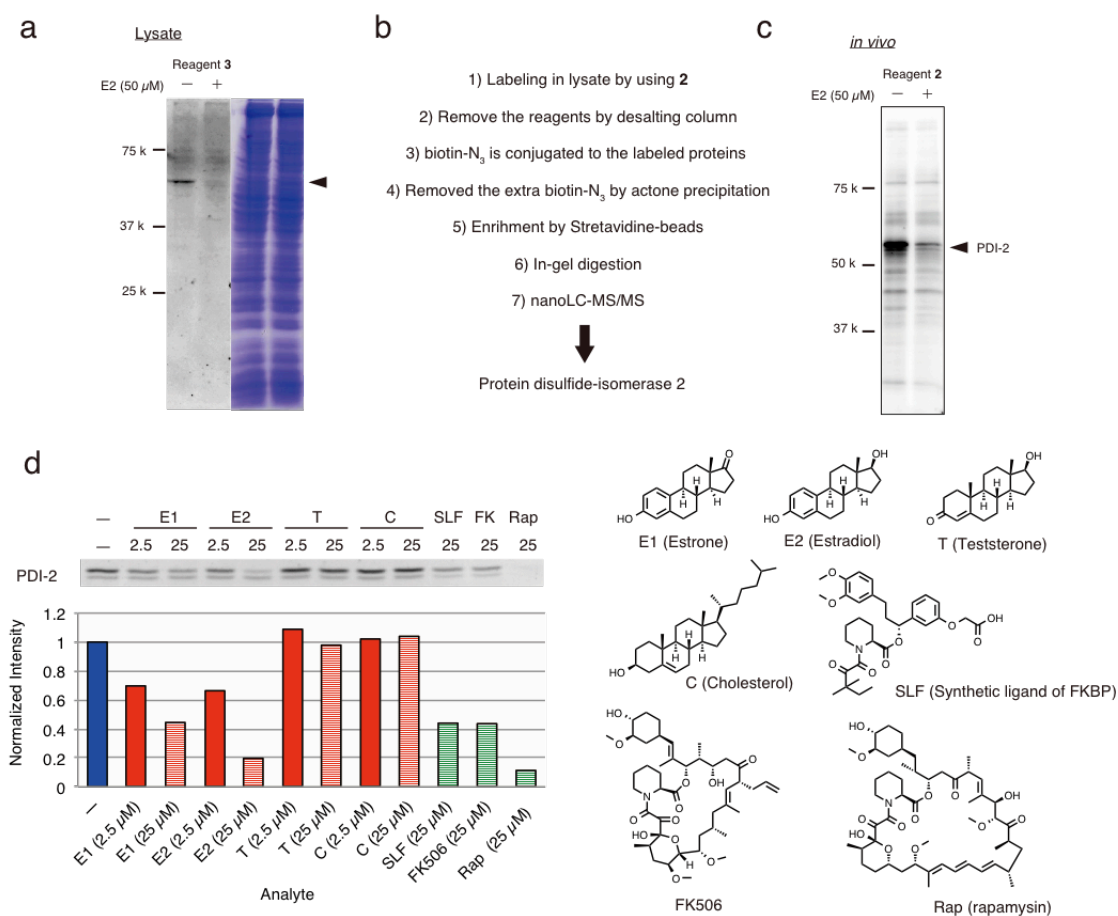


Figure 5. *In vitro* and *in vivo* labeling of E2-interacting proteins and competitive inhibition of labeling reaction by various hormone and hydrophilic compounds. (a) SDS-PAGE and in-gel fluorescent image of labeled proteins by using reagent **3** (1 μ M) *in vitro*. (b) Workflow of identification of the 56 kD protein. (c) Western blotting analysis of labeled protein by using **2** (1 μ M) *in vivo*. (d) Competitive assay of PDI-2 labeling. Fluorescent gel image is shown in left panel. The chemical structures of tested compounds are shown in right panel. To the lysate, I added reagent **3** (1 μ M) with competitive inhibitors (2.5 or 25 μ M) and incubated for 16 h at 20 $^{\circ}$ C.

3-3, Conclusion

For proteomic research to uncover proteins interacting with a molecule of interest, photoactive reagents are conventionally and widely used. I herein demonstrated LDT chemistry is also a powerful tool for chemical proteomics. I demonstrated here that LDT chemistry has two great advantages over this method; (1) the labeling efficient is high, (2) UV-irradiation is not required and it is applicable *in vivo*. These utilities were highlighted by labeling of cholesterol-interacting proteins *in vitro* and *in vivo*, and identification of the proteome.

Recently, Cravatt and his colleges demonstrated proteomic wide competitive inhibition assays to find the new interaction pairs between drugs and proteins, in which they used covalent inhibitor or photoactive reagents. Here I showed that LDT chemistry is also applicable to this competitive assay.

3-4, Experimental Section

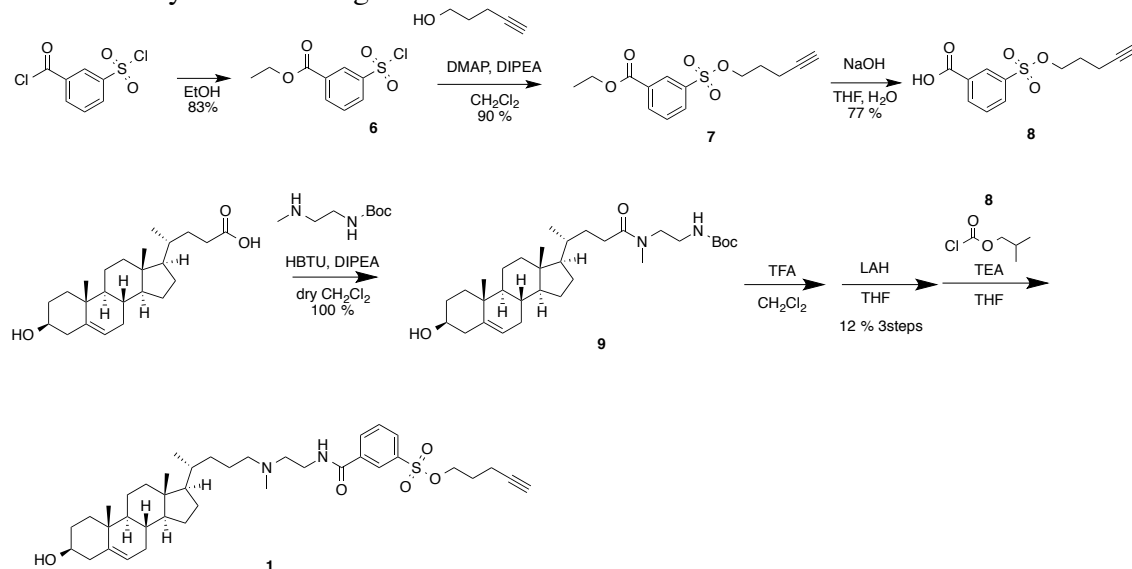
General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Acros Organics, Sasaki Chemical, or Watanabe Chemical Industries) and used without further purification.

Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminium sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was conducted by flash column chromatography on silica gel 60 N (neutral, 40–50 μ m, Kanto Chemical). ¹H NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer and calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, br s = broad singlet. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was measured by an Autoflex II instrument (Bruker Daltonics) using α -cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid as the matrix.

Synthesis of the LDT reagents

Scheme 1. Synthetic of reagent **1**



Synthesis of **6**

To an ice-cold 3-(chlorosulfonyl)benzoyl chloride (2.0 g, 0.083 mol, 1.0 eq) was added anhydrous ethanol (10 mL). The solution was stirred for 30 min at r.t.. The solution was monitored by TLC (AcOEt/hexane = 1/1) to confirm the end of the reaction. After evaporation, the crude was purified by silica column chromatography (AcOEt/hexane = 1/5). The solvent was evaporated and dried *in vacuo* to yield **6** 1.74 g (colorless oil, 7.0 mmol, 83 %). ¹H-NMR (400 MHz) δ 8.69 (t, *J* = 1.6 Hz, 1H), 8.40 (dt, *J* = 7.8, 1.6 Hz, 1H), 8.21 (dt, *J* = 7.8, 1.6 Hz, 1H), 7.72 (t, *J* = 7.8 Hz, 1H), 4.45 (q, *J* = 7.2 Hz, 2H), 1.44 (t, *J* = 7.2 Hz, 3H).

Synthesis of **7**

Compound **6** (500 mg, 2.01 mmol, 2.0 eq), DIPEA (520 μL, 3.03 mmol, 3.0 eq) and DMAP (36.7 mg, 0.3 mmol, 0.3 eq) was dissolved in dry CH₂Cl₂ (4 mL). To the 4-Pentyn-1-ol (93 μL, 1.0 mmol, 1.0 eq) solution in dry CH₂Cl₂ (4 mL) was added the solution. The mixture was stirred for 2 hr at r.t. and monitored by TLC (AcOEt/hexane

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= 1/2) to confirm the end of the reaction. After evaporation, the crude was purified by silica gel column chromatography (AcOEt/hexane = 1/4). The solvent was evaporated and dried *in vacuo* to yield **7** 269 mg (colorless oil, 0.91 mmol, 90 %). ¹H-NMR (400 MHz) δ 8.58 (t, J = 1.6 Hz, 1H), 8.33 (dt, J = 7.8, 1.6 Hz, 1H), 8.10 (dt, J = 7.8, 1.6 Hz, 1H), 7.70 (t, J = 7.8 Hz, 1H), 4.43 (q, J = 7.2 Hz, 2H), 4.22 (t, J = 6.0 Hz, 2H), 2.28 (dt, J = 2.4, 6.0 Hz, 2H), 1.92–1.85 (m, 3H), 1.43 (t, J = 7.2 Hz, 3H).

Synthesis of **8**

To an ice-cold solution of **7** (250 mg, 0.844 mmol, 1.0 eq) in THF (5 mL) and H₂O (2.5 mL) mixture was added 2 M NaOH (422 μ L, 0.844 mmol, 1.0 eq). The solution was stirred for 3 hr at r.t., and monitored by TLC (AcOEt/hexane = 1/1 with a few drops of AcOH) to confirm the end of the reaction. Then the reaction was neutralized with 4 M HCl dioxane solution and extracted with CHCl₃ (10 mL x 3 times). The combined organic layers were washed with brine, dried over MgSO₄ and evaporated. The crude was purified by silica gel column chromatography (AcOEt/hexane = 1/1 with a few drops of AcOH). The solvent was co-evaporated with toluene and dried *in vacuo* to yield **8** 175 mg (white solid, 0.65 mmol, 77 %). ¹H-NMR (400 MHz) δ 8.65 (t, J = 1.6 Hz, 1H), 8.37 (dt, J = 7.8, 1.6 Hz, 1H), 8.17 (dt, J = 7.8, 1.6 Hz, 1H), 7.70 (t, J = 7.8 Hz, 1H), 4.24 (t, J = 6.0 Hz, 2H), 2.28 (dt, J = 6.0, 2.4 Hz, 2H), 1.93–1.86 (m, 3H).

Synthesis of **9**

To a stirred solution of 3 β -hydroxy- Δ^5 cholenic acid (50 mg, 0.13 mmol, 1 eq), HBTU 54 mg (0.14 mmol, 1.1 eq) and DIPEA (45 μ L, 0.26 mmol, 2 eq) in dry DCM 1 mL was added the *N*-(*tert*-butoxycarbonyl)-*N'*-methylethylenediamine (25 mg, 0.13 mmol, 1 eq). The solution was allowed to be stirred for 10 hours. The reaction mixture was monitored by TLC (AcOEt/hexane = 1/1) to confirm the end of the reaction. The crude was washed with sat. NaHCO₃ aq. (10 mL, 2 times) and 1N HCl (6 mL, 2 times) and

dried over MgSO_4 . The product was filtered, evaporated and purified by silica gel column chromatography ($\text{AcOEt/hexane} = 3/1$). The solvent was evaporated and dried *in vacuo* to yield **9** (71 mg, white solid, 0.13 mmol, 100 %). $^1\text{H-NMR}$ (400 MHz) δ 5.34 (d, $J = 5.2$ Hz, 1H), 4.96 (br s, 1H), 3.56–3.40 (m, 3H), 3.30–3.23 (m, 2H), 3.03 (s, 2H), 2.93 (s, 1H), 2.38–2.15 (m, 4H), 1.98 (m, 2H), 1.88–1.75 (m, 4H), 1.62–1.40 (m, 30H), 1.37–1.21 (m, 4H), 1.19–1.03 (m, 5H), 1.01 (s, 3H), 0.98–0.91 (m, 4H), 0.68 (s, 3H).

Synthesis of reagent **1**

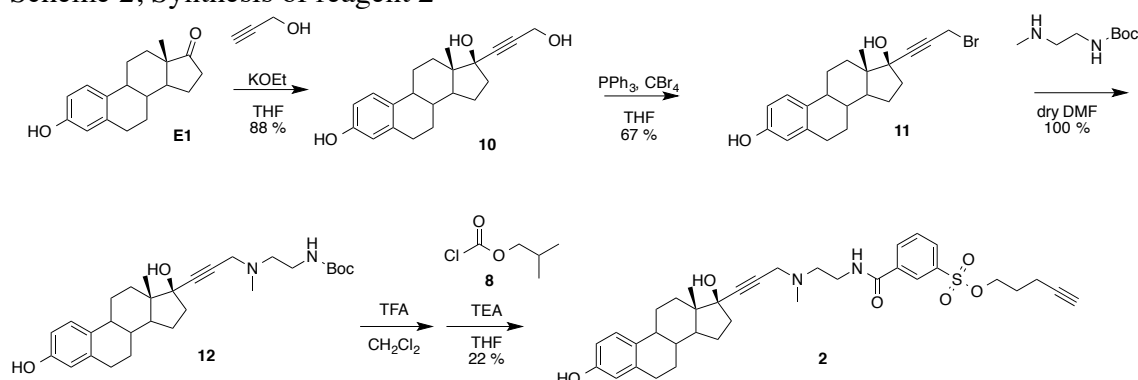
To the solution of **9** (35 mg, 0.066 mmol) in DCM (1 mL) was added TFA (500 μL). The solution was stirred for 45 min. The mixture was co-evaporated with toluene (3 times) and diluted by sat. NaHCO_3 aq. (10 mL). The deprotected compound was extracted with CHCl_3 (10 mL, 3 times), evaporated and dried *in vacuo*.

To the solution of deprotected compound in dry THF (600 μL) was added LAH (9 mg) at 0 °C under N_2 atmosphere. The mixture was stirred for 2 hours. To the mixture was added LAH (2 mg) and stirred for another 2 hours. To confirm the end of the reaction, the mixture was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 5/1$ with a few drop of NH_3 aq.). To the solution water (40 μL) was slowly added and 2N NaOH aq. was added. The solution was stirred for overnight and dried over MgSO_4 . The mixture was filtered, evaporated, and dried *in vacuo*.

To the solution of reduced product in THF/DMC mixture (1 mL, 1/1) was added the solution of **8** (18 mg, 0.066 mmol, 1 eq), isobutyl chloroformate (8.6 μL , 0.066 mmol, 1 eq), and TEA (40 μL , 0.03 mmol, 4 eq) in THF (300 μL). The mixture was stirred for 2 hours at r.t.. The mixture was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$) to confirm the end of the reaction. After evaporation, the crude was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH} = 20/1$). The solvent was evaporated and dried *in vacuo* to yield **1** (5.4 mg, colorless oil, 8.1 μmol , 12% in three steps). $^1\text{H-NMR}$ (400 MHz) δ 8.43 (s, 1H), 8.14 (d, $J = 8.4$ Hz, 1H), 8.04 (d, $J = 8.4$ Hz, 1H), 7.64 (t, $J = 8.4$ Hz, 1H),

5.31 (m, 1H), 4.21 (t, $J = 6.0$ Hz, 2H), 3.79–3.65 (m, 2H), 3.52 (m, 1H), 3.20–2.50 (m, 7H), 2.31–2.20 (m, 4H), 2.00–1.80 (m, 8H), 1.80–1.32 (m, 9H), 1.30–0.68 (m, 18H), 0.65 (s, 3H). MALDI TOF-MS (CHCA) m/z calcd for $C_{39}H_{59}N_2O_5S$ $[M+H]^+$ 667.414, found 667.249.

Scheme 2, Synthesis of reagent **2**



Synthesis of **10**

To an ice cold **E1** solution (2.0 g, 7.4 mmol, 1.0 eq) in anhydrous THF (40 mL) was added KOEt 9.3 g (0.11 mol, 15 eq) and propargyl alcohol (3.3 mL, 55.5 mmol, 7.5 eq). The solution was stirred for 15 min at 0 °C. The solution was allowed to reach the room temperature and stirred for 2 hours. The reaction mixture was monitored by TLC (AcOEt/haxane = 1/1) to confirm the end of the reaction. Then the reaction mixture was poured into ice water, neutralized with acetic acid (about 8 mL) and extracted with EtOAc (20 mL, 3 times). The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated. The crude was purified by silica gel column chromatography (AcOEt/hexane = 2/1 $\rightarrow$ 1/0). The solvent was evaporated and dried *in vacuo* to yield **10** (2.14 g, 6.55 mmol, 88 %) as white solid. 1H -NMR (400 MHz, $CDCl_3$) δ 7.13 (d, $J = 8.4$ Hz, 1H), 6.62 (dd, $J = 8.4, 2.4$ Hz, 1H), 6.56 (d, $J = 2.4$ Hz, 1H), 4.29 (s, 2H), 2.81–2.74 (m, 2H), 2.34–2.22 (m, 2H), 2.22–2.16 (m, 1H), 2.00 (dt, J

= 13.2, 4.0 Hz, 1H), 1.87–1.80 (m, 2H), 1.80–1.62 (m, 3H), 1.51–1.31 (m, 4H), 0.87 (s, 3H).

Synthesis of **11**

To an ice-cold stirred solution of **10** (500 mg, 1.53 mmol, 1.0 eq) and triphenylphosphine (803 mg, 3.06 mol, 2.0 eq) in anhydrous THF (5 mL) was added carbon tetrabromide (763 mg, 2.3 mmol, 1.5 eq). The solution was allowed to reach the room temperature and stirred for 2 hours. The reaction mixture was monitored by TLC (AcOEt/hexane = 1/1) to confirm the end of the reaction. Then the reaction mixture was evaporated. The crude was purified by silica gel column chromatography (AcOEt/hexane = 1/2). The solvent was evaporated and dried *in vacuo* to yield **11** (401 mg, 1.03 mmol, 67 %) as colorless solid. ¹H-NMR (400 MHz, CDCl₃) δ 7.16 (d, *J* = 8.4 Hz, 1H), 6.63 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 4.53 (s, 1H), 4.00 (s, 1H), 2.85–2.70 (m, 2H), 2.40–2.17 (m, 3H), 2.00 (dt, *J* = 13.2, 4.0 Hz, 1H), 1.90–1.61 (m, 6H), 1.53–1.31 (m, 4H), 0.88 (s, 3H).

Synthesis of **12**

To a stirred solution of *N*-(*tert*-butoxycarbonyl)-*N'*-methylethylenediamine (224 mg, 1.29 mmol, 5 eq) in anhydrous DMF (1.5 mL) was added the **11** (100 mg, 0.258 mmol, 1.0 eq) solution in dry DMF (1.5 mL). The solution was allowed stirred for 11 hours. The reaction mixture was monitored by TLC (CHCl₃/MeOH = 20/1) to confirm the end of the reaction. Then the reaction mixture was evaporated. The crude was purified by silica gel column chromatography (CHCl₃/MeOH = 20/1). The solvent was evaporated and dried *in vacuo* to yield **12** (127 mg, 0.263 mmol, 100 %) as colorless oil. ¹H-NMR (400 MHz, CDCl₃) δ 7.14 (d, *J* = 8.4 Hz, 1H), 6.63 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 5.00 (s, 1H), 3.41 (s, 2H), 3.28–3.19 (m, 2H), 2.86–2.76 (m, 2H), 2.58 (t, *J* = 6.2 Hz, 2H), 2.40–2.24 (m, 5H), 2.18 (m, 1H), 2.02 (dt, *J* = 4.0, 13.2 Hz, 1H), 1.90–

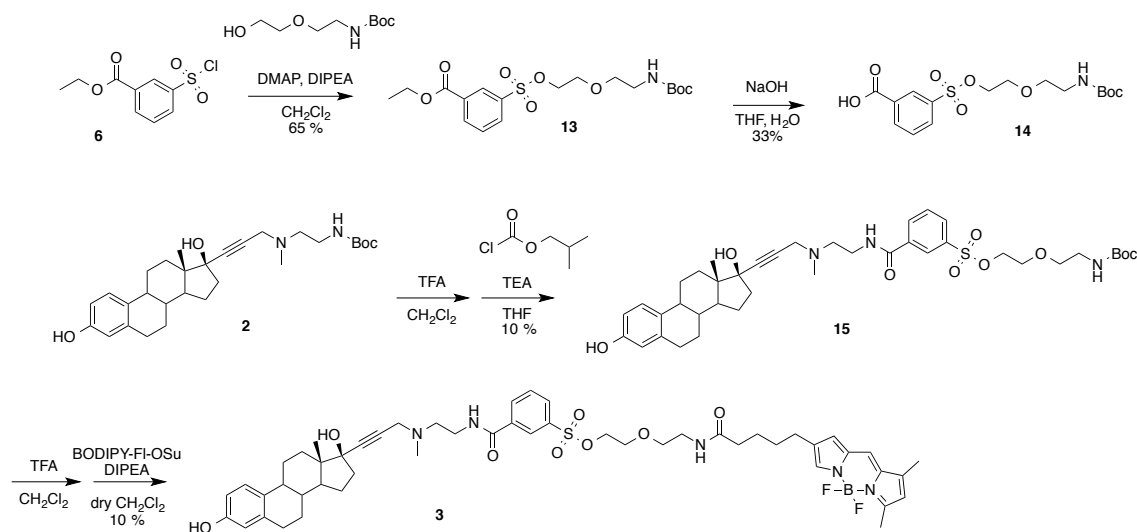
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1.31 (m, 18H), 0.88 (s, 1H).

Synthesis of **2**

The compound **12** (25 mg, 0.052 mmol, 1.0 eq) was added to TFA (500 μ L) and dry CH_2Cl_2 (1 mL) mixture. The solution was stirred for 30 min at r.t. and co-evaporated with toluene (2 mL x 3 times). To a solution of **8** (14 mg, 0.052 mmol, 1.0 eq) in anhydrous THF (250 μ L), TEA (36 μ L, 0.26 mmol, 5.0 eq) and isobutyl chloroformate (6.8. μ L, 0.052 mmol, 1.0 eq) were added. The solution was stirred for 10 min. To the solution was added the deprotected **12** (0.052 mmol, 1.0 eq). The mixture was stirred for 2 hour at room temperature. The solution was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$) to confirm the end of the reaction. After evaporation, the crude was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH} = 30/1 \rightarrow 20/1$). The solvent was evaporated and dried *in vacuo* to yield **2** (7.2 mg, 11 μ mol, 22 %) as colorless oil. ^1H -NMR (400 MHz, CDCl_3) δ 8.36 (t, $J = 1.6$ Hz, 1H), 8.14 (dt, $J = 7.8, 1.6$ Hz, 1H), 8.02 (dt, $J = 7.8, 1.6$ Hz, 1H), 7.63 (t, $J = 8.0$ Hz, 1H), 7.10 (d, $J = 8.4$ Hz, 1H), 6.63 (dd, $J = 8.4, 2.4$ Hz, 1H), 6.54 (d, $J = 2.4$ Hz, 1H), 4.20 (t, $J = 6.0$ Hz, 2H), 3.59 (t, $J = 5.8$ Hz, 2H), 3.46 (s, 2H), 2.80–2.74 (m, 4H), 2.41 (s, 3H), 2.27–2.02 (m, 4H), 2.14–1.63 (m, 13H), 1.46–1.25 (m, 4H), 0.86 (s, 1H).

Scheme 3, Synthesis of reagent **3**



Synthesis of **13**

Compound **6** (500 mg, 2.01 mmol, 2.0 eq), DIPEA (520 μL , 3.03 mmol, 3.0 eq) and DMAP (36.7 mg, 0.3 mmol, 0.3 eq) was dissolved in dry CH_2Cl_2 (4 mL). To the solution of 2-(2-boc-aminoethoxy)ethanol (205 μL , 1.0 mmol, 1.0 eq) in dry CH_2Cl_2 (4 mL) was added the solution of **2**. The mixture was stirred for 2 hr at r.t. and monitored by TLC (AcOEt/hexane = 1/1) to confirm the end of the reaction. After evaporation, the crude was purified by silica gel column chromatography (AcOEt/hexane = 1/2 $\rightarrow$ 1/1 $\rightarrow$ 2/1). The solvent was evaporated and dried *in vacuo* to yield **13** (269 mg, 0.65 mmol, 65 %) as colorless oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.58 (t, J = 1.6 Hz, 1H), 8.33 (dt, J = 7.8, 1.6 Hz, 1H), 8.10 (dt, J = 7.8, 1.6 Hz, 1H), 7.67 (t, J = 8.0 Hz, 1H), 4.83 (s, 1H), 4.44 (q, J = 7.2 Hz, 2H), 4.24 (t, J = 4.6 Hz, 2H), 3.65 (t, J = 4.6 Hz, 2H), 3.47 (t, J = 4.6 Hz, 2H), 3.26 (t, J = 4.8 Hz, 2H), 1.44 (s, 9H), 1.43 (t, J = 7.2 Hz, 3H).

Synthesis of **14**

To an ice-cold solution of **13** (100 mg, 0.24 mmol, 1.0 eq) in THF (2 mL) and H_2O (1 mL) mixture was added 2N NaOH (120 μL , 0.24 mmol, 1.0 eq). The solution was stirred for 4 hr at r.t. and monitored by TLC (AcOEt) to confirm the end of the reaction. Then the reaction was neutralized with 4N HCl dioxane solution (60 μL) and extracted with CHCl_3 (10 mL x 3 times). The combined organic layers were washed

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with brine, dried over MgSO_4 and evaporated. The crude was purified by silica gel column chromatography (AcOEt). The solvent was evaporated and dried *in vacuo* to yield **14** (31.3 mg, 0.08 mmol, 33 %) as colorless oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.60 (s, 1H), 8.37 (d, $J = 7.6$ Hz, 1H), 8.15 (d, $J = 7.6$ Hz, 1H), 7.70 (t, $J = 7.6$ Hz, 1H), 4.88 (s, 1H), 4.27 (t, $J = 4.6$ Hz, 2H), 3.68 (t, $J = 4.8$ Hz, 2H), 3.48 (t, $J = 4.6$ Hz, 2H), 3.26 (t, $J = 4.8$ Hz, 2H), 1.45 (s, 9H).

Synthesis of **15**

The compound **12** (20 mg, 0.042 mmol, 1.0 eq) was added to TFA (500 μL) and dry CH_2Cl_2 (1 mL) mixture. The solution was stirred for 30 min at r.t. and co-evaporated with toluene (2 mL x 3 times). To a solution of **14** (17.7 mg, 0.042 mmol, 1.0 eq) in anhydrous THF (500 μL), TEA (24 μL , 0.168 mmol, 4.0 eq) and isobutyl chloroformate (5.5 μL , 0.042 mmol, 1 eq) were added. The solution was stirred for 10 min. To the solution was added the deprotected **12** (0.042 mmol, 1.0 eq). The mixture was stirred for 2 hour at room temperature. The solution was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$) to confirm the end of the reaction. After evaporation, the crude was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH} = 20/1 \rightarrow 10/1$). However, the purified product contains impurity. The solvent was evaporated and dried *in vacuo* to yield impure **15** (10.5 mg, about 7.0 μmol , 10 %) as colorless oil. I conducted next step without further purification. MALDI TOF-MS (CHCA) m/z calcd for $\text{C}_{40}\text{H}_{56}\text{N}_3\text{O}_9\text{S}$ $[\text{M}+\text{H}]^+$ 776.355, found 776.356.

Synthesis of **3**

To the compound **15** (5.0 mg, 6.6 μmol , 1.0 eq) was added TFA (500 μL) and dry CH_2Cl_2 (1 mL) mixture. The solution was stirred for 30 min at r.t. and co-evaporated with toluene (2 mL x 3 times), BODIPY-Fl succinimidyl ester (2.0 mg, 4.8 μmol , 0.7 eq), dry DIPEA (10 μL , 56 μmol , 8.4 eq) and anhydrous DMF (1 mL)

was added. The mixture was stirred for 2 hour at room temperature. The solution was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$) to confirm the end of the reaction. After evaporation, the crude was purified by HPLC (ODS-A, A/B = 20/80 $\rightarrow$ 80/20 for 60 min, A: 0.1% TFA CH_3CN , B: 0.1% TFA H_2O). The solvent was lyophilized to yield pure **3** (0.6 mg, 0.6 μmol , 10 %) as orange oil. MALDI TOF-MS (CHCA) m/z calcd for $\text{C}_{51}\text{H}_{64}\text{BF}_2\text{N}_5\text{O}_8\text{SNa}$ $[\text{M}+\text{Na}]^+$, $\text{C}_{51}\text{H}_{64}\text{BF}_2\text{N}_5\text{O}_8\text{SK}$ $[\text{M}+\text{K}]^+$, found 978.66 and 994.69.

Materials and General Methods for biological experiments

SDS-polyacrylamide gel electrophoresis and western blotting were carried out using a Bio-Rad Mini-Protean electrophoresis apparatus. Fluorescent and chemi-luminescent signals generated with ECL Prime (GE Healthcare) were detected with a LAS4000 imaging system (Fuji Film). Cell imaging was performed with a confocal laser scanning microscope (CLSM, FV10i, Olympus) equipped with a 60x objective lens, and LD laser (473 nm and 559 nm).

Lyse and labeling *in vitro*

Wild-type (strain N2) was cultured on NGM agar plates with *E. Coli* (OP50) at 20 °C. For the preparation of lysed solution. *C. elegans* were collected from agar plates by PBS addition (10 mL). The worms were centrifuged (3,000 G , 2 min) and the supernatant was removed. The worms were rinsed with PBS buffer (10 mL) for 3 times. I added 400 μ L of a lysis buffer (PBS buffer containing 5% glycerol, 1/100 protease inhibitor cocktail) and homogenized by sonication on ice. The lysed solution was centrifuged (15,000 G, 10 min) and the supernatant was collected to a new tube. I determined the protein concentration by BCA assay (Thermo). For LDT chemical labeling *in vitro*, I added 5 μ M of labeling reagents **1** with or without competitive inhibitors (100 μ M C; cholesterol or 50 μ M of CA; 3 β -Hydroxyl- Δ 5-cholenic acids), and incubated at 20 °C for 16 h. For photo-affinity labeling, I added 25 μ M of reagent **5** to lysate with or without 500 μ M CA, and irradiated (365 nm) them for 15 min on ice. To these solution, 20 μ M of Oregon Green 488 Azide (Thermo), 100 μ M of click ligand (1H-1,2,3-Triazole-1-acetic acid, 4,4',4''-[nitrilotris(methylene)]tris-,1,1',1''-triethyl ester), 1 mM CuSO₄, and 1 mM TCEP were added, and incubated for 1 hr at r.t.. The samples were separated by SDS-PAGE, followed by fluorescent imaging.

***In vivo* labeling by LDT reagents**

Worms were rinsed with PBS (3 times) and suspended in S-medium with or without cholesterol (20 $\mu\text{g/mL}$). To the S-medium, 5 μM of reagent **1** or **2** was added and incubated for 16 h at 20 °C. The worms were centrifuged (2000 G, 2 min), the supernatant was removed and rinsed with PBS (10 mL). These processes were repeated for 3 times. The worms were suspended to the lysis buffer (300 μL) and homogenized by sonication. To remove labeling reagents from lysate, the lysate was desalted by ZebaTM Desalting Column (Thermo). Then, The labeled proteins were conjugated to Oregon Green by Click reaction in same manner as described. The samples were mixed with 5xLeammli buffer and subjected to SDS-PAGE, followed by fluorescent imaging.

Enrichment and dimethyl labeling for quantitative MS measurement

In vivo labeling and following click reaction was performed in same manner as described. The 400 μL of OG-labeled lysates (1 mg/mL) were precipitated by addition of -30°C cold acetone (1.6 mL), and incubated for overnight at -30 °C. The samples were centrifuged (10,000 G, 15 min), and the supernatant were removed. The pellets were washed with ice-cold methanol (2 mL), and reconstituted in 100 μL of Ripa buffer, and diluted in 900 μL of NP-40 buffer (pH 7.4, 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40). To reduce contamination caused by non-specific binding to the sepharose resins, I treated the lysate with 10 μL of 50% sepharose resins for 15 min at 4 °C, and the supernatant was collected to a new tube. To the pre-cleared lysate, I added 1.5 μL of anti-fluorescein antibody (abcam) and incubated at 4 °C for 10 hr with rotation. Then, I added 30 μL of proteinA sepharose resin (GE healthcare) to the solution, incubated for overnight, and washed with Ripa buffer (1 mL) for three times. The labeled proteins were eluted in 2xLeammli (20 μL) at 95 °C. The supernatant was collected, and loaded on a SDS-PAGE gel and run for 10 min at 15 mA (the migration distance of dye front is about 10 mm). The gel was fixed and silver stained, then each

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lane was cut (Height, 10 mm) and destained, according to manufacturer's protocol. I reduced and alkylated the proteins in gel fragments by using the In-Gel Tryptic Digestion Kit (Thermo). To the alkylated samples, I added 10 ng/mL trypsin and incubated for overnight at 37 °C. The peptides are extracted from gels by repetitive addition of acetonitrile (0.1% TFA). The peptide solution was lyophilized and reconstituted by addition of 100 μ L of 100mM TEAB (triethylamine bicarbonate). To the solution, I added 4 μ L of 4% formaldehyde (for negative control, in presence of competitive inhibitor) or d^2 -formaldehyde (for positive control) and 4 μ L of 0.6M cyanoborohydride. The reactions were quenched by an addition of 1% NH_3 aq. (16 μ L) and 5% formic acid (8 μ L). Two different samples were mixed in a 1:1 ratio, and subjected to a C18 Spin Column (Thermo). The peptides were eluted in 15 μ L of extraction solution (acetonitrile/ H_2O = 7/3 with 0.1% TFA). Then, the eluent was lyophilized and reconstituted in 15 μ L of solution (2% acetonitrile, 0.1% TFA).

NanoLC-MS/MS analyses

A capillary reverse phase HPLC-MS/MS system composed of an Agilent 1100 series gradient pump equipped with Valco C2 valves with 150- μ m ports, and LTQ-Orbitrap XL hybrid mass spectrometer equipped with an XYZ nano-electrospray ionization (NSI) source (AMR, Tokyo, Japan). Samples were automatically injected using PAL system (CTC analytics, Zwingen, Switzerland) into a peptide L-trap column (Chemical Evaluation Research Institute, Tokyo, Japan) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent C), the peptides were loaded into a separation capillary reverse phase column (MAGIC C18 packed with gel particles of 3 μ m in diameter and 200-Å pore size, 150×0.2 mm, Michrom BioResources) by switching the valve. The eluents used were: A, 100% water containing 0.1% formic acid, and B, 100% acetonitrile containing 0.1% formic acid.

The column was developed at a flow rate of 1 μ L/min with the concentration gradient of acetonitrile: from 5% B to 20% B in 10 min, 20% B to 40% B in 45 min, then from 40% B to 95% B in 30 min, sustaining 90% B for 15 min, from 95% B to 5% B in 1 min, and finally re-equilibrating with 5% B for 20 min. Xcalibur 2.1 system (Thermo Fisher Scientific) was used to record peptide spectra over the mass range of m/z 350–1500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 150–2000. Repeatedly, MS spectra were recorded followed by three data-dependent collision induced dissociation (CID) MS/MS spectra generated from three highest intensity precursor ions. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by Proteome Discoverer 1.3.0.339 (Thermo Fisher Scientific). Searches were performed by using the SEQUEST (Thermo Fisher Scientific) against latest Swissprot database for protein identification. Searching parameters were set as follows (i) SEQUEST: enzyme selected as used with five maximum missing cleavage sites, species limited to *C. elegans* a mass tolerance of 5 ppm for peptide tolerance, 0.5 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of oxidation (M). Positive protein identifications were based on significant Xcorr (5% false discovery rate). Protein identification and modification information returned from SEQUEST were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS.

Data processing and GO term enrichment analysis.

Only proteins that showed at least two unique identified peptides (FDR < 0.001) were considered as “identified proteins” (150 proteins). In the “identified proteins” list, I set proteins showing the ratio value more than 2.0 as “sterol-interacting proteins” (60 proteins). For gene ontology enrichment analyses, I uploaded these group

lists to the Gene Ontology Consortium website (<http://geneontology.org/>). I used the PANTHER Overrepresentation Test (release 20150430) as an analysis type and the GO Ontology database Released 2015-08-06 as an annotation version. In the GO enrichment analysis in biological processes, I used the GO biological PANTHER GO-Slim Biological Process as an annotation data set.

***In vitro* labeling and identification of PDI-2 as an E2-binding protein**

In vitro labeling by using reagent **2** and biotin-conjugation via click reaction was performed in the same manner as cholesterol-type reagents. The biotin-labeled proteins are precipitated by addition of -30°C acetone, and incubated for overnight. After the centrifugation (10,000 G, 15 min), The pellets were dissolved in 100 μ L of buffer (6M Urea, 2% SDS, 20% glycerol, 373 mM Tris, pH8.8) and diluted in 900 μ L of Ripa buffer. To the solution, I added 100 μ L of Nutraavidin-sepharose resins (Thermo) and incubate for 4 h at 4 °C with rotation. The resins were rinsed with Ripa buffer (1 mL for 5 times). The labeled proteins were eluted by boiling in 2xLeammuli buffer containing 100 mM DTT (dithiothreitol) for 10 min at 95 °C. The sample was loaded on SDS-PAGE to separate proteins. After silver-stain, I cut the gel fragment in 50-60 kDa range. The gel fragment was destained, reduced, alkylated and digested in same manner as described. The peptide was extracted, lyophilized, reconstituted and subjected into nanoLC-MS/MS measurement.

3-5, References

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Chapter 3

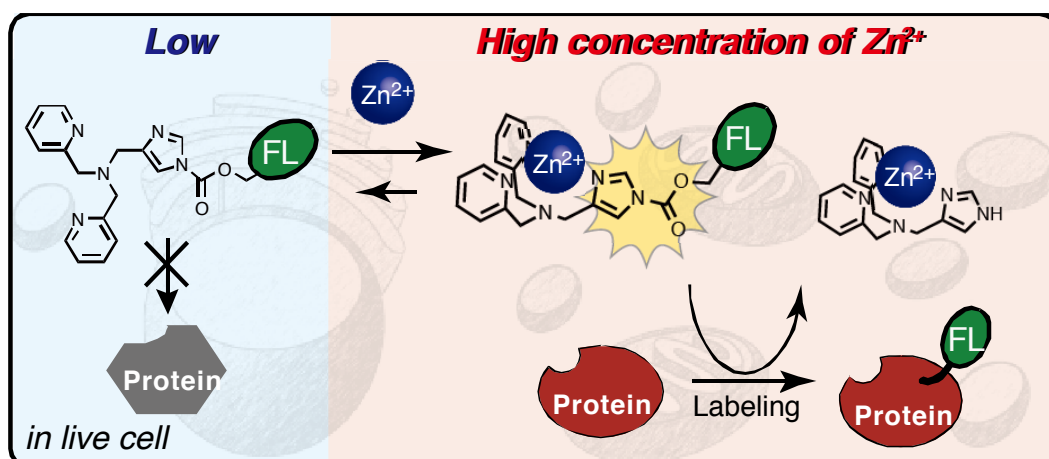
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Chapter 4

Zinc Conditional Labeling in Living Cells for Novel Spatial Proteomics

Abstract

Zinc signaling and dynamics play significant roles in many physiological responses and diseases. To unveil the physiological roles of zinc ion in detail, comprehensive identification of proteins localizing in zinc-rich spaces is crucial. In this chapter, I developed a novel “conditional proteomics” based on a chemical proteomic strategy with designer zinc-responsive labeling reagents (named “AIZin”). In this conditional proteomics, a subset of protein in zinc-rich spaces is tagged and identified in combination with quantitative mass spectrometry. This method was successfully applied to elucidate zinc dys-homeostasis induced by nitric oxide-triggered oxidative stress in glioma cells and evaluated the dynamic changes of the zinc-related proteomes. Moreover, a subset of identified proteins allowed the characterization of unknown zinc-rich vesicles generated by oxidative stress as endoplasmic reticulum-Golgi intermediate compartments.



4-1, introduction

Zinc distribution in living cell spatio-temporally fluctuates, associated with many biological functions¹⁻³. In neuron, zinc ion is released together with glutamate from presynaptic vesicles, affecting that activities of several membrane proteins on post-synapse. This is considered to be crucial for physiological process in memorizing and learning. Additionally, zinc ion takes part in the oxidative stress signaling. In a various excitotoxic neuronal and glial cell death, such as cerebral ischemia, the oxidative stress increases the cytosolic zinc concentration, inducing an apoptosis signal. To analyze the zinc distribution, various fluorescent probes have developed for sensing zinc ion for real-time imaging of released Zn^{2+} and Zn^{2+} -stored vesicles⁴⁻⁶. For studying proteins whose involvement in zinc homeostasis and signaling are assumed, such as Zn^{2+} -binding or transporting ones, genetic and pharmacological methods are widely used³. However, these techniques are insufficient for comprehensive elucidation of the physiological roles of zinc ions and cannot characterize new proteins that should be indirectly, yet significantly associated with zinc distribution, for example, constituent proteins of zinc-rich vesicles.

As I described in chapter 3, chemical proteomics is utilized to annotate a subset of proteins with the ligand binding or enzymatic profiles. Recently, the chemical proteomics have been also recognized as a powerful method for assignment of spatial information. Alice Ting and her colleagues developed organelle specific proteome analysis, in which spatially restricted APEX (engineered ascorbate peroxidase) produce phenoxyl radicals that can label proteins in mitochondria^{7,8}. David A. Tirrell and his colleagues achieved cell-selective proteomic analysis in *C. elegans* by incorporating reactive noncanonical amino acid using engineered mutant phenylalanyl-tRNA synthetase⁹. Both methods can identify proteins in physiologically restricted spaces. However, any method that can selectively label proteins in specific spaces defined as dynamic condition has not been developed.

Inspired by these chemical proteomics, I sought to develop a novel proteomics focusing on zinc distribution, “conditional proteomics”, that can reveal what proteins localize in zinc-rich spaces for unveiling the zinc homeostasis in detail (**Fig. 1a**). In this novel method, I rationally designed zinc-enhanced protein labeling reagent named AIZin. AIZin can be activated in presence of zinc ion, which allows the selective labeling of a proteome of Zn^{2+} concentrated local space. Using this method, I demonstrated that NO-triggered Zn^{2+} release caused a dramatic change of the zinc-related proteome in a time dependent manner. I also succeeded in identifying a subset of proteins in Zn^{2+} -rich vesicles that were generated by NO-promoted oxidative stresses.

4-2, Results

4-2-1, Molecular design and synthesis of zinc responsive labeling reagent

For design of zinc responsive reagent, I focused on Acyl Imidazole (AI)^{10,11} reactive group already described in chapter 1 and 2. In previous works, Dr. Fujishima, a pioneer of LDAI protein labeling, showed the AI group is easily degraded in pH low condition ($\text{pH} < 4$) by hydrolysis reaction due to the protonation of N-atom on imidazole rings (unpublished data). Inspired by this feature, I set a hypothesis that AI reactivity can be activated accompanied with zinc coordination to the imidazole. In other words, a zinc ion, strong Lewis acid, reduces the electron density of the imidazole ring, resulting in activation of AI group. Therefore, I designed an AI-based reactive group, in which the AI was conjugated with one dipicolylamine (Dpa), a tridentate ligand for Zn^{2+} , through one methylene unit. I named this AI reagent responsive to zinc ion as “AIZin”. I prepared two AIZin reagents, that tethered coumarin or diacetyl fluorescein to the terminal of AI moiety. Synthesis of AIZin-1 (**1**), AIZin-2 (**2**) and its derivatives were conducted and the characterization of these molecules was done by ^1H -NMR, ^{13}C -NMR and HR-MS.

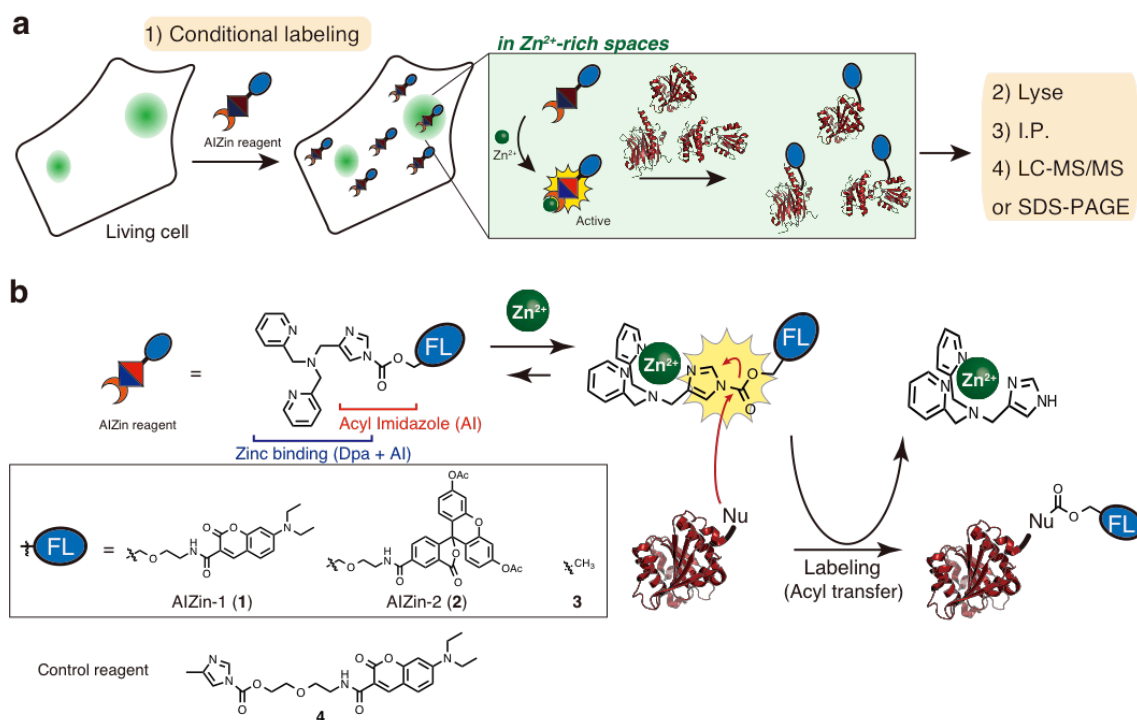


Figure 1. Conditional proteomics by Zn^{2+} -enhanced chemical labeling. (a) Conditional proteomics work flow. (b) Schematic illustration of Zn^{2+} -promoted protein labeling. FL = fluorescent dye; Nu = nucleophilic amino acid.

4-2-2, Zinc-enhanced reactivity of AlZin reagent

For proof-of principle experiment, the coordination geometry of Zn^{2+} with AlZin derivative (**3**) was confirmed by the crystal structure of the 1:1 complex ($\text{Zn}:\mathbf{3}$). The data clearly indicated that a zinc ion directly coordinates to the imidazole ring of AI unit with 2.01 Å in the distance between nitrogen of imidazole and Zn atom, together with Dpa unit in the trigonal bipyramidal fashion (**Fig. 2a**). The impact of zinc coordination on the AI moiety was examined by ^1H -NMR (**Fig. 2b**). Zn^{2+} addition induced the large down-field shift of both two protons of the imidazole ring, indicating that zinc ion, strong Lewis acid, reduced the electron density of imidazole of AI unit.

The zinc enhanced reactivity change of AlZin-1 (**1**) was evaluated by comparing the hydrolysis rate of the AI moiety in the presence and absence of Zn^{2+} . Without Zn^{2+} , **1**

was less susceptible to hydrolysis for a few hour (0.0011 min^{-1} as the first order rate constant). On the other hand, addition of Zn^{2+} dramatically promoted the reaction (0.024 min^{-1}) by 23-fold, demonstrating the activation of AI group by Zn^{2+} complex formation (**Fig. 2c**). By varying the Zn^{2+} concentrations in this hydrolysis experiment, I determined the zinc ion binding affinity of **1** to be 0.9 nM, while reagent **4** lacking DPA moiety was not activated even in high concentration of zinc ion (**Fig. 2d**). I also checked the zinc ion selectivity among other metal ions in the hydrolysis of **1** (**Fig. 2e**). While the reaction was enhanced strongly by Zn^{2+} , alkali earth metals such as Ca^{2+} or Mg^{2+} are not effective even though in mM order of the high concentration. Although some of the transition metal cations, i.e. Co^{2+} , Cu^{2+} , Cd^{2+} and Pd^{2+} , also activate the hydrolysis, it is conceivable that AIZin mainly respond to Zn^{2+} in living cells, given the abundance of the mobile of zinc ion¹².

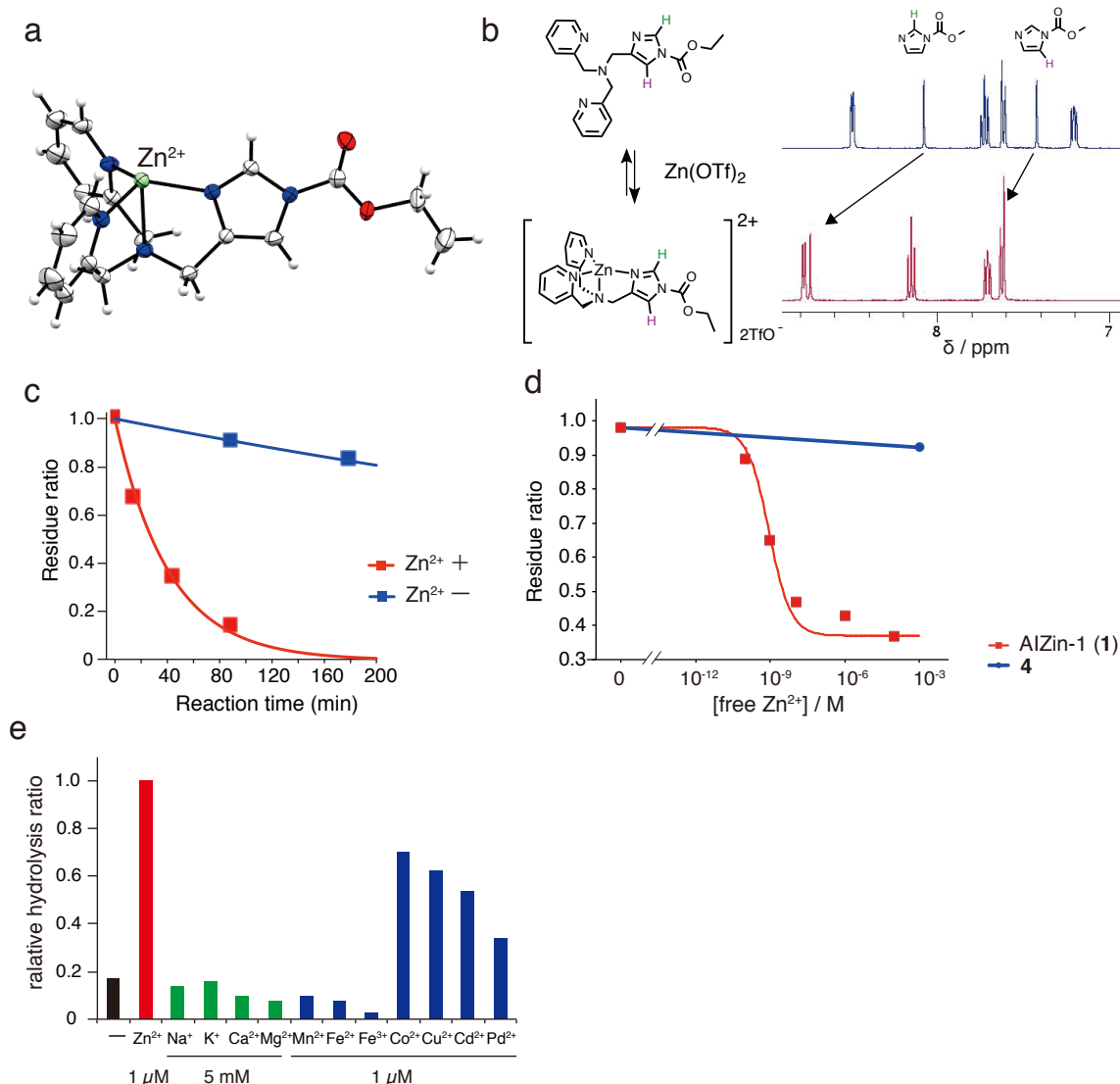


Figure 2. Activation of AlZin reagents by Zn²⁺ coordination. (a) X-ray structure of the 3-Zn(OTf)₂ complex. Two TfO⁻ anions were omitted. (b) Comparison of ¹H-NMR spectrum between reagent 3 and 3-Zn(OTf)₂ complex. (c) Time course of hydrolysis in presence (red) or without Zn²⁺ (blue). The 1 μM of 1 was incubated with 0 or 1 μM of ZnSO₄ in 100 mM HEPES buffer (pH 7.4, *I* = 0.1 (KOH/KCl)) for 37 °C. (d) concentration dependency of hydrolysis reaction of 1 and control 4. To measure the apparent dissociation constant (*K_d*), I mixed 1 μM of 1 or 4 with various concentration of Zn²⁺ buffer, prepared by using 0–9 mM ZnSO₄/10 mM NTA system. The solution was incubated for 90 min at 25 °C and subjected to RP-HPLC immediate after addition of TPEN. (e) Relative hydrolysis ratio in various metal ions. The 1 μM of 1 was incubated with 1 μM or 5 mM of metal ions in 100 mM HEPES buffer (pH 7.4, *I* = 0.1 (KOH/KCl)) at 25 °C for 90 min.

4-2-3, The impact of Zn^{2+} coordination on protein labeling

Zn^{2+} -promoted protein labeling was initially evaluated using HeLa cell lysate as a proof-of-principle. In these experiments, a coumarin fluorophore is covalently labeled by Zn^{2+} -facilitated acyl transfer reaction with nucleophilic amino acids on proteins surface. The cell lysate with or without zinc ion was treated with 50 μM of **1** for 30 min, followed by SDS-PAGE and fluorescent gel image analyses (**Fig. 3a**). Many intense labeled proteins were detected in whole range of molecular weight in presence of Zn^{2+} , whereas these bands almost disappeared by addition of TPEN, a Zn^{2+} chelator. Additionally, when I treated the HeLa lysate with reagent **4** lacking the DPA moiety, fluorescent bands were negligibly smear even in the presence of excess Zn^{2+} . From the time course data, the fluorescent intensity reached a plateau within 20 min and it takes 5 min to reach half-maximum, suggesting that AIZin is applicable to biological events longer than 5 min (**Fig. 3b**). The metal ion selectivity in the protein labeling was found to be almost identical to that of its hydrolysis, indicating that hard metals such as Ca^{2+} or Mg^{2+} does not affect to the reactivity of **1** (**Fig. 3c**). These results clearly showed that AIZin is able to comprehensively label many proteins in response to the increase of Zn^{2+} concentration.

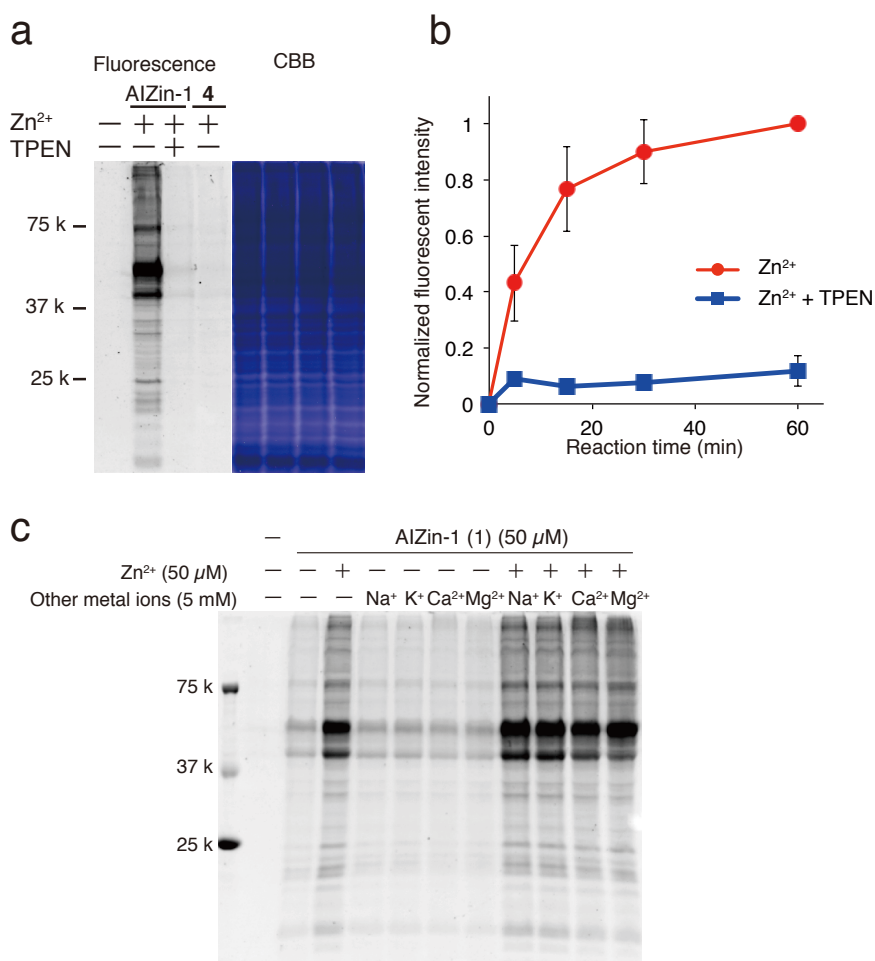


Figure 3. Zinc-responsive protein labeling in test tube. (a) SDS-PAGE and in-gel fluorescence imaging of Zn²⁺-responsive labeling in HeLa lysates. To a HeLa lysate containing 4.8 mg/mL protein, ZnSO₄ (50 μM) was added, followed by addition of **1** or control **4** (50 μM). The mixture was incubated at 37 °C with or without TPEN (1 mM) for 30 min. (b) Time course of protein labeling in HeLa cell lysate containing ZnSO₄ (50 μM) in the presence (blue) or absence (red) of TPEN (1 mM) (n = 3, error bar means S.D.). (c) Independency of alkali or alkali earth metals in the Zn²⁺ response. To HeLa cell lysate, I added 5 mM NaCl, KCl, CaCl₂, or MgSO₄ with or without ZnSO₄ (50 μM). The reactions were started with addition of **1** (50 μM) and incubated for 30 min.

4-2-4, Uniform distribution and zinc promoted-labeling of AIZin-2 in living cells

For living cell application, the labeling reagent should be cell permeable and uniformly distributed inside the cell. Moreover, in order to capture and enrich the labeled proteins for LC-MS/MS analysis, the chemical tag must be an epitope for

immune precipitation. I subsequently designed AIZin-2 (**2**), which has a diactyl fluorescein as a fluorescent dye, because the diactyl fluorescein improves hydrophilicity and cell permeability, and the anti-fluorescein antibody is commercially available and works well. I added 1 μM of **2** to living HeLa cells and monitored its distribution by using confocal fluorescence microscopy. The intracellular fluorescence from fluorescein appeared with in 5 min and it was uniformly distributed across the whole area of live cells including nuclei (**Fig. 4a**). These properties seemed favorable for unbiased protein labeling in live cells. To check the Zn^{2+} responsive labeling, I added the exogenous Zn^{2+} as a form of Zinc pyrithione to living HeLa cell that was pre-treated with 1 μM of **2**. Many labeled proteins were detected as shown in the SDS-PAGE analysis of **Figure 4b**, whereas these bands almost disappeared in the case contained TPEN. Take together, I concluded that AIZin-2 can spontaneously penetrate through living cell membranes, uniformly distribute inside cell and be activated in response to the increased Zn^{2+} .

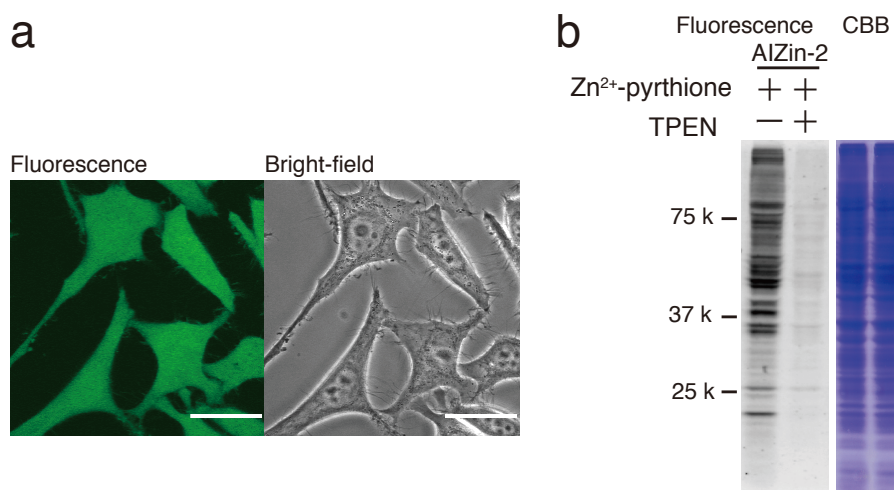


Figure 4. Distribution of AIZin-2 and zinc responsive labeling in living cell. (a) CLSM image of AIZin-2 distribution in living cells. To living HeLa cells 1 μM of AIZin-2 was added to the medium HBSS, and incubated for 10 min. Scale bar means 30 μm . (b) SDS-PAGE and in-gel fluorescence imaging of zinc-responsive protein labeling in living HeLa cells. Pre-incubated HeLa cells with TPEN (0 or 400 μM) were treated with 1 μM of **2** for 20 min, followed by incubation with 200 μM zinc-pyrithione for 20 min.

4-2-5, Conditional protein labeling in living cell in response to zinc ions

Accumulating reports show that nitric oxide (NO) generated by the NO synthase family induces the elevation of intracellular Zn^{2+} , resulting in neuronal apoptosis and death *in vitro* and *in vivo*^{13–15}. The major source of free Zn^{2+} is considered to be Zn^{2+} -binding proteins such as metallothioneins (MTs), which are abundantly expressed and bind heavy metals in living cells. NO-mediated S-nitrosylation of MTs causes the rapid release of mobile zinc ion¹⁶. The increased cytosolic Zn^{2+} not only interfere with glycolytic metabolism, but also is uptaken in mitochondria to disrupt the function, initiating the apoptosis signaling^{17,18}. On the other hand, it is also recently reported that excess Zn^{2+} may be segregated in cytosolic vesicles as a transient pool^{19,20}, in order to buffer the cytosolic Zn^{2+} for minimizing the detrimental effects of Zn^{2+} . However, characteristics of such Zn^{2+} -rich vesicles have been poorly understood in their constituents, the origin, and the relationship to other organelles. Therefore, I decided to apply the AIZin-based “conditional proteomics” to this event to characterize the Zn^{2+} -rich vesicles by identifying relevant proteins.

Initially, I observed a NO-triggered Zn^{2+} distribution change in living C6 glioma cells, a glial cell strain, using FluoZin-3 AM (a fluorescent Zn^{2+} imaging probe) (**Fig. 5a,b**). Immediately after addition of NO-donor, S-nitrosocysteine (SNOC), the strong fluorescence appeared throughout the cell. Subsequently, many punctate fluorescence assigned to Zn^{2+} -rich vesicles were clearly observed three hours later along with the weakened cytosolic fluorescence. This time profile revealed that mobile Zn^{2+} is generated by NO stimuli and the Zn^{2+} localization dramatically changed from the broad distribution to sequestration into vesicles.

I subsequently conducted Zn^{2+} -responsive protein labeling of C6 glial cells. After stimulation with SNOC, followed by incubation for 0, 10, or 180 min, I treated it with **2** (1 μM) for 20 min. As shown in **Figure 5c**, fluorescent bands due to labeled proteins in cell lysate dramatically increased after NO stimulation and the subset of labeled

proteins mostly changed from 10 min to 3 hours. The labeling was inhibited in the presence of TPEN, indicating this labeling is promoted by mobile Zn^{2+} . To investigate the localization of labeled proteins, I performed a fractionation of whole lysate (W) by differential centrifugation into two fractions, organelle (Or) and cytosol (Cy) (**Fig. 5d**). The labeled proteins were detected in both fractions at first 10 min, whereas the labeled proteins after 3 hours were predominantly detected in the Or fraction. Additionally, the band pattern was largely distinct from that observed after NO-10min incubation.

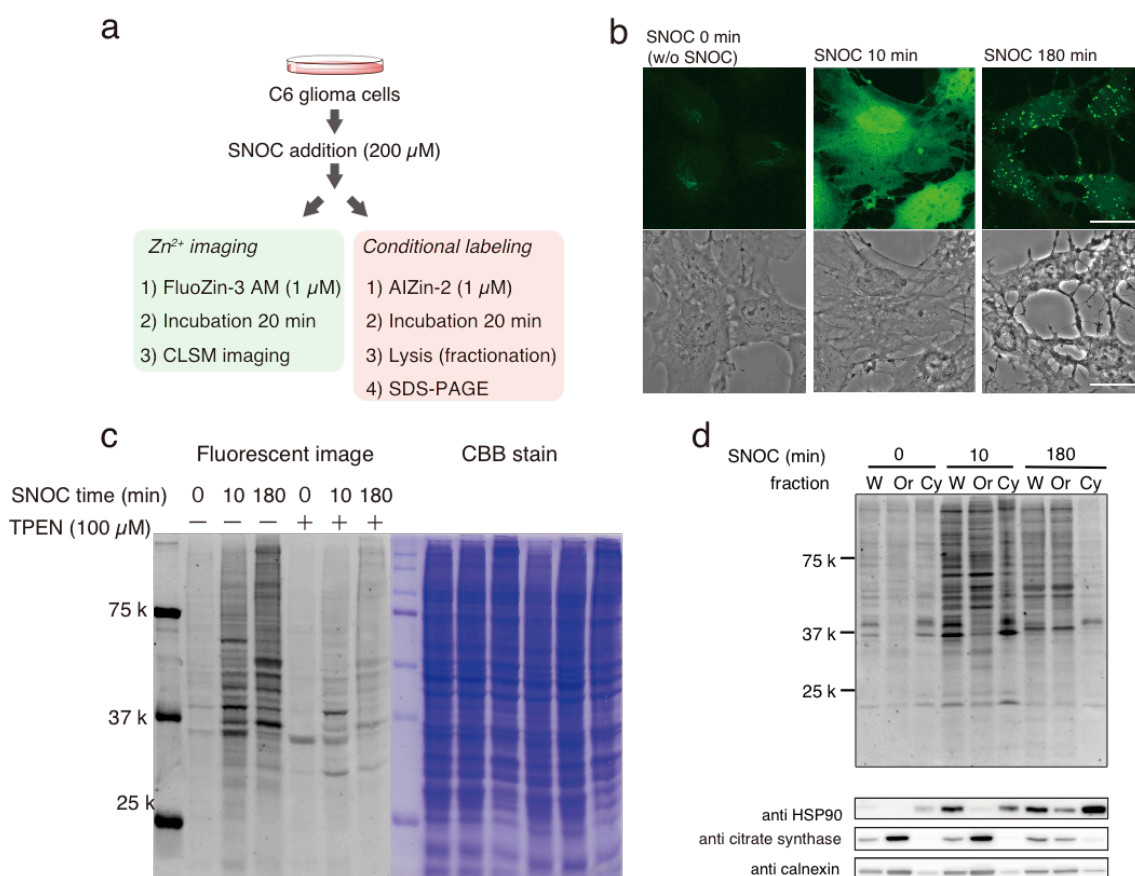


Figure 5. Conditional protein labeling in living C6 cells treated with SNOC. (a) work flow of Zn^{2+} imaging by FluoZin-3 and conditional labeling followed by analysis of the NO-induced Zn^{2+} dyshomeostasis. (b) CLSM imaging of the NO-induced zinc release and segregation processes. C6 cells were treated with 200 μM SNOC. Before (0 min), 10 min after, or 180 min after incubation, the cells were treated with 1 μM FluoZin-3 AM and CLSM observation was conducted. Scale bar = 20 μm . (c) SDS-PAGE and in-gel fluorescent image of labeled proteins by 2. Before (0 min), 10 min after, or 180 min after NO-stimulation, I treated with or without 100 μM TPEN for 5 min, followed by incubation with 1 μM AIZin-2 (2) for 20 min. (d) In-gel fluorescent

analysis of fractionation of labeled C6 cells. The labeled C6 cells were homogenized and separated into cytosol and organelle fractions by different centrifugation processes. W; whole cell, Or; Organelle fraction, Cy; Cytosol fraction. HSP90, citrate synthetase, and calnexin are cytoplasmic, mitochondria, and ER markers, respectively.

4-2-6, Profiling of spatio-temporal changes of zinc-related proteomes

To profile the zinc-related proteome, I identified labeled proteins by use of a coupling mass-fingerprinting technique with AIZin-based chemical labeling. The labeled proteins were enriched by immune precipitation using anti-fluorescent antibody, tryptic digested in-gel, and subjected to LC-MS/MS measurements. To clear the dynamic changes of identified proteins at each time point (0 min, 10 min, and 3 h after NO stimuli), I conducted quantitative mass analysis using TMTs (tandem mass tags)²¹. The digested peptides were modified with light-(L-), medium-(M-), or heavy-(H-) TMTs for w/o NO (0 min), NO-10 min, or NO-3 h samples, respectively, and I mixed three modified peptides in a 1:1:1 ratio. Then, the mixed sample was subjected to LC-MS/MS analysis (**Fig. 6a-b**). During the MS/MS measurements, peptides were quantified as signal ratio values of three isotopic fragments. I performed three independent biological replicates, in which proteins detected twice (at least) were selected as identified proteins, resulting in 331 proteins identified and quantified in total.

Comparing the labeled proteins under NO-stimulated cells with non-stimulated cells (that is 0 min vs. 10 min) by the M/L ratio, 328 (99%) proteins showed more than zero in $\text{Log}_2(\text{M/L})$ values, indicating that protein labeling was greatly facilitated with Zn^{2+} release triggered by SNOC addition within 10 min (**Fig. 6c**). I focused on the top 20% of proteins, which are highly enriched by NO stimuli and analyzed GO (gene ontology) cellular component enrichment where the proteins can be annotated with GO terms (protein features), based on GO database maintained by GO consortium (<http://geneontology.org/>). The results of this analysis showed that proteins located in the mitochondrion, vesicles, cytosol, and nucleus are enriched (**Fig. 6d**). This subcellular distribution is coincident with both imaging data by FluoZin-3 (10 min after

NO stimuli) and previous reports describing zinc level increases in these spaces caused by oxidative stress¹⁷.

Interestingly, the proteome remarkably changed in a time-dependent manner. That is, for comparison between 10 min- and 3 h- samples, the number of proteins showing positive $\text{Log}_2(\text{H/M})$ values were almost equivalent to the number of proteins with negative $\text{Log}_2(\text{H/M})$ values as shown in **Figure 6e**. When I categorized the bottom and top 20% of $\text{Log}_2(\text{H/M})$ values into group A and B, respectively, it was clear that proteins in the nucleus (70%, $p = 2.7 \times 10^{-13}$), vesicle (48%, $p = 5.5 \times 10^{-9}$), cytosol (33%, $p = 1.2 \times 10^{-6}$), and ribosome (13%, $p = 5.5 \times 10^{-6}$) are enriched in group A, whereas vesicle (77%, $p = 1.9 \times 10^{-27}$), endoplasmic reticulum (ER) (44%, $p = 2.6 \times 10^{-16}$), and mitochondrion (30%, $p = 3.3 \times 10^{-5}$) are enriched in group B (**Fig. 6f**). These results strongly suggested that the free zinc level decreased in the nucleus, some vesicles, and cytosol from 10 min to 180 min incubation after NO stimuli, while the zinc level increased in different vesicles, ER, and mitochondria over this time period. These changes in the labeled proteome agree well with the imaging and fractionation data.

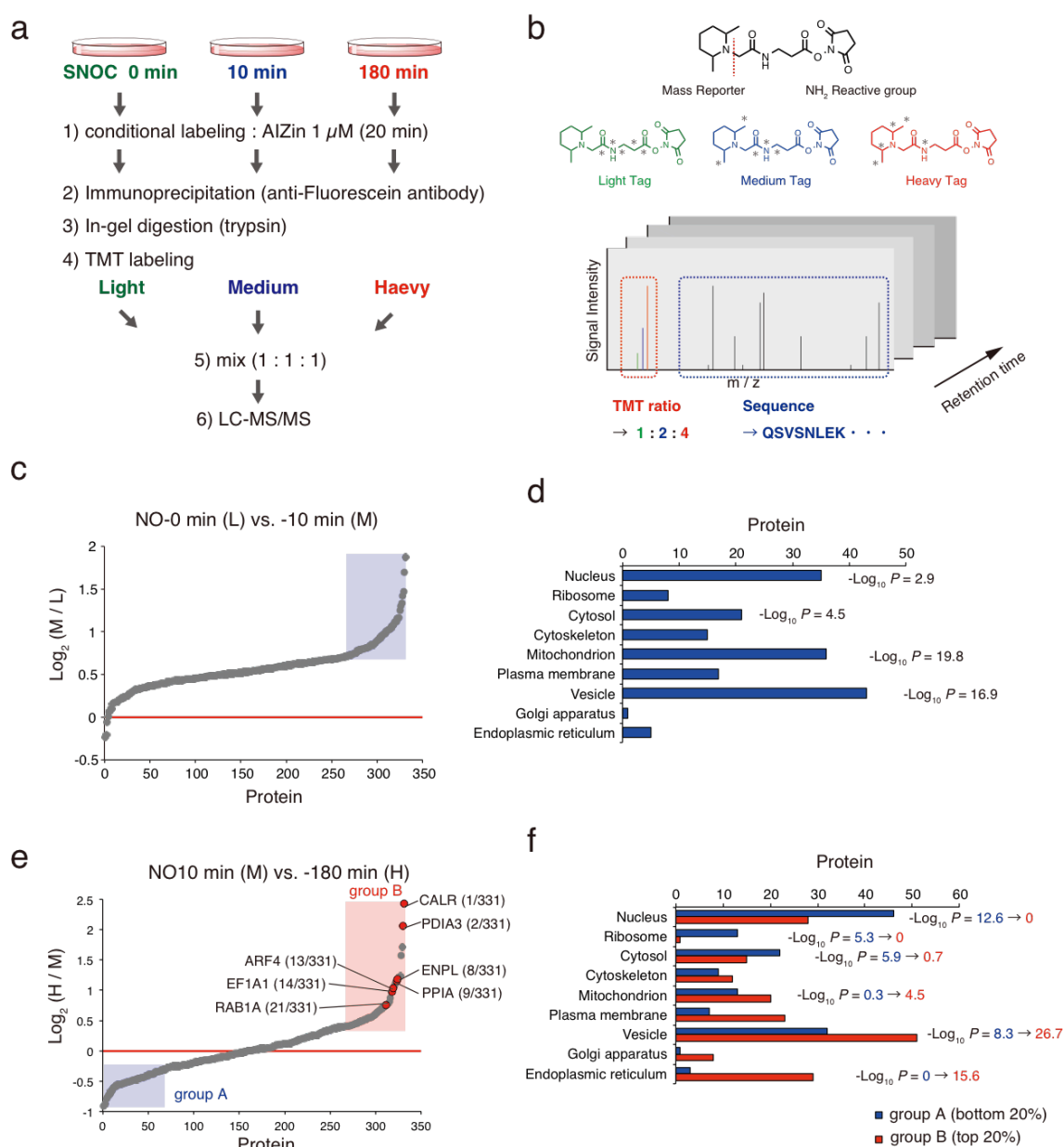


Figure 6. Proteomic analysis of labeled proteins in NO-stimulated C6 cells. (a) Workflow of TMT quantitative analysis of labeled proteins using LC-MS/MS measurements. (b) TMT chemical structures and MS/MS quantification. After in-gel digestion the peptides of 0, 10 and 180 min-stimulated proteomes were labeled by light- (L-), medium- (M-) and heavy- (H-) TMTs, respectively. (c) TMT $\log_2$ ratio plot (NO-0 min (L) vs. NO-10 min (M)). (d) Cellular component analysis of top 20% proteins in the ratio of NO-10 min/0 min. (e) TMT $\log_2$ ratio plot (NO-10 min (M) vs. -180 min (H)). (f) Cellular component analysis of bottom 20% (group A, colored in blue) and top 20% (group B, colored in red).

4-2-7, Validation of zinc responsive conditional proteomics

To confirm that identified proteins depend on free Zn^{2+} , the labeled samples were quantitatively compared with that treated with TPEN as a negative control. All of the identified protein shows over zero value in TMT log ratio, indicating that the proteomes directly reflect the zinc distribution (**Fig. 7a**). In addition, I checked, by western blotting analysis for several proteins, that the ratio values were not mainly reflected by expression level changes (**Fig. 7b**).

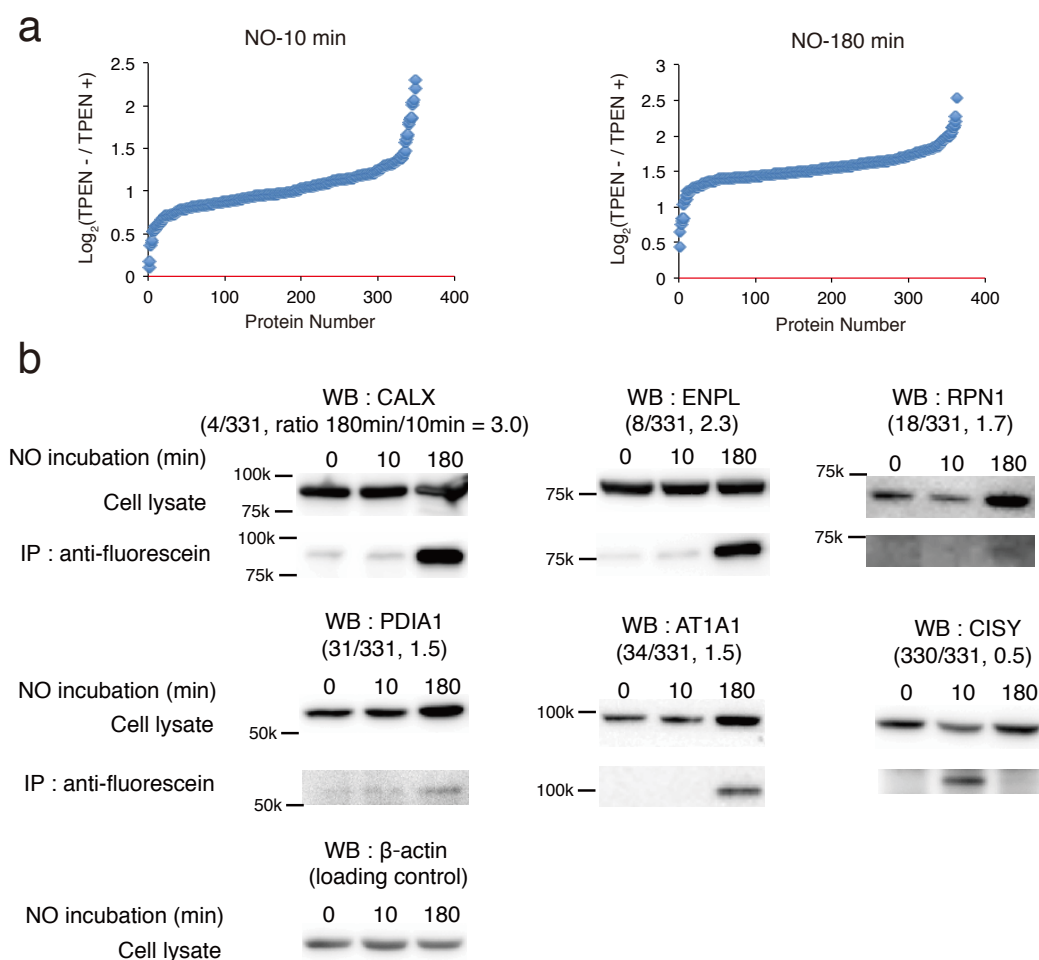


Figure 7. Negative control experiments of proteomic analysis. (a) Quantitative decrease of the identified proteins by an addition of TPEN. After incubation with 200 μM of SNOC for 10 or 180 min, C6 cells were treated with or without TPEN (100 μM) and labeled using AIZin-2 (1 μM). The labeled proteins were enriched by immune precipitation and digested in gel. The peptides were labeled by TMT reagents (Heavy;

without TPEN, Light; with TPEN). The H/L log-ratio plots of NO-10 min and -180 min treated cells are shown in left and right panel, respectively. (b) Western blotting analysis of expression level in cells and enrichment of labeled proteins. The labeled cell lysate (pre-enrichment) and IP product (post-enrichment) were subjected to SDS-PAGE and western blotting analysis. Beta-actin is a loading control. I tested the expression level and IP-enrichments for 6 protein in the top 11% of the TMT ratio and for 1 protein in the bottom 1% of the ratio. For each protein, Uniprot ID, its order in TMT ratio, and its TMT ratio value (H/M ; NO-180 min/-10min) are shown in top. Western blotting imaging data of cell lysates and IP products are shown in middle and bottom, respectively.

To validate the results obtained by this new conditional proteomics, I subsequently investigated whether the identified proteins would localize in the FluoZin-3-stained vesicles at 3 h incubation after SNOC stimuli by live cell imaging. As representatives, seven proteins were selected in group B according to the below-mentioned criteria and mCherry-fused ones were constructed. The criteria were: i) in the top 10% of the $\text{Log}_2(\text{H/M})$ values; ii) whose fluorescent-protein fusion recombinants have already been reported; and iii) mitochondrial localization is not reported because it is not possible to image the mitochondrial Zn^{2+} by FluoZin-3. These seven proteins are marked in **Figure 6e**, that is, Calreticulin (CALR, top 1/331), Protein disulfide-isomerase A3 (PDIA3, 2/331), Endoplasmic (ENPL, 8/331), Peptidyl-prolyl cis-trans isomerase A (PPIA, 9/331) ADP-ribosylation factor 4 (ARF4, 13/331), Elongation factor 1 alpha 1 (EF1A1, 14/331), and Ras-related protein RAB1A (RAB1A, 21/331). All these proteins have not been reported to localize in Zn^{2+} -rich vesicles generated by oxidative stress, although three proteins (PDIA3, EF1A1, and Rab1A) are known to be in other types of Zn^{2+} -pooled vesicles such as synaptic vesicles²². **Figure 8** show that the localization is completely (for RAB1A) or partially (for the other 6 proteins) overlapped with the Zn^{2+} distribution visualized by FluoZin-3. Given all the data, I conclude that AIZin-2 is able to label proteins localized in zinc-rich subcellular spaces and the following mass fingerprinting analysis indeed identified the zinc-related proteome.

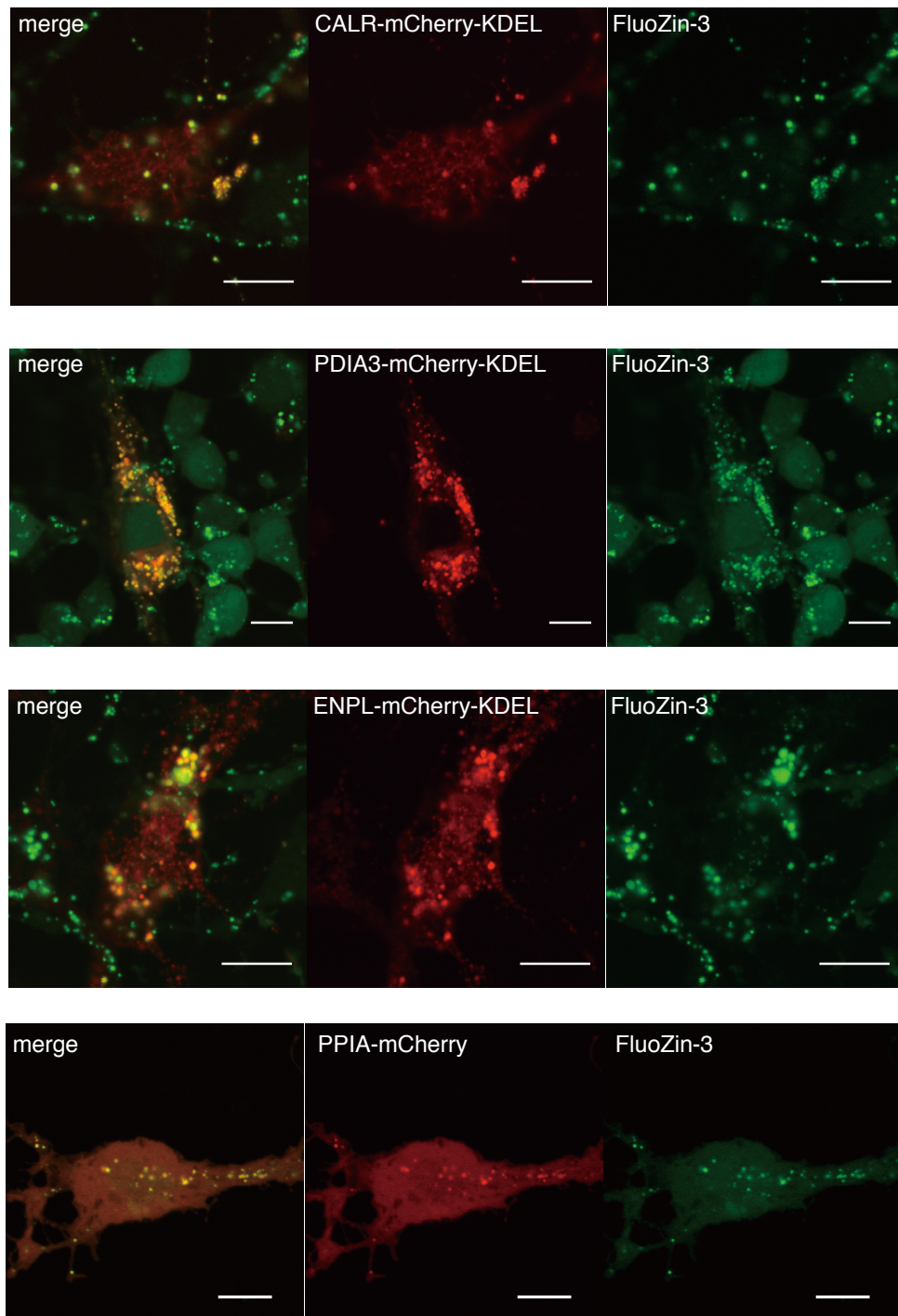


Figure 8. Fluorescence co-localization of zinc distributions (FluoZin-3, green) and mCherry-fused proteins (red). The C6 cells transfected with plasmids were grown for 2 days. I treated the transfected cells with 200 μ M of SNOC and incubated for 3 hours. I added 1 μ M of FluoZin-3 AM and observed using confocal microscopy. Scale bar = 10 μ m. The punctate mCherry fluorescence partially or completely overlapped with FluoZin-3 fluorescence.

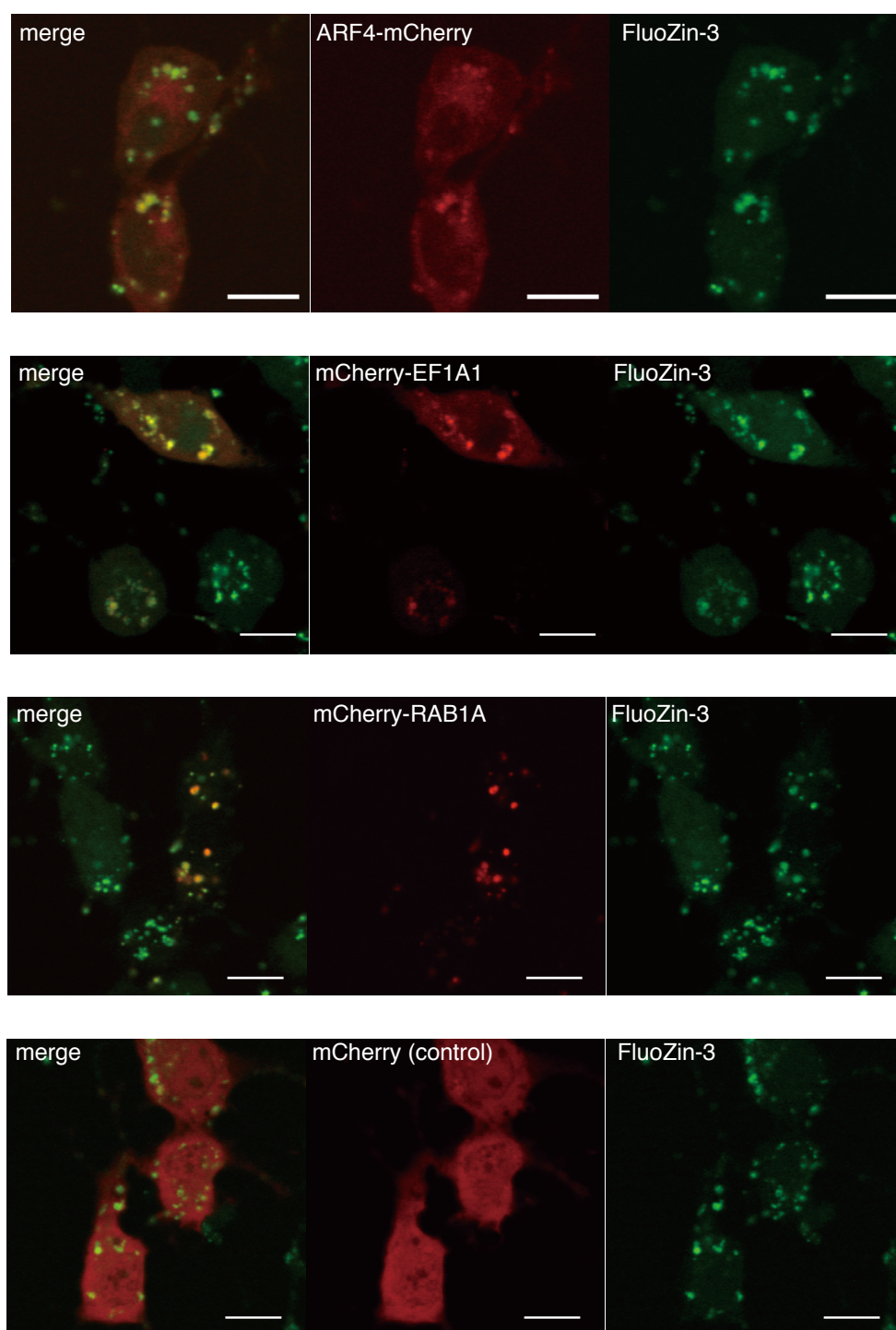


Figure 8 (continued).

4-2-8, Characterization of the zinc-rich vesicles

I finally sought to characterize these zinc-rich vesicles on the basis of our conditional proteomics data. It was reported previously that zinc-rich vesicles generated by oxidative stresses are assigned as a lysosome, autophagosome, or another vesicle named zincosome^{23–25}. In our experimental conditions, FluoZin-3-stained vesicles are overlapped in only limited portions with those imaged with LysoTracker red (a lysosome marker) and with mCherry-LC3 (an autophagosome marker), indicating that the zinc-rich vesicles generated by NO stimuli in C6 cells are not homogenous and the majority are distinct from lysosome and autophagosome (**Fig. 9a-b**). From careful analysis of our proteomics data, I found that 20 of the 51 proteins in group B are annotated as being both vesicle and ER localized, and 8 among 51 proteins are annotated as Golgi localized. Besides, proteins implicated in protein folding ($p = 2.1 \times 10^{-7}$) and protein metabolic process ($p = 5.6 \times 10^{-7}$) are highly enriched in group B (**Fig. 9c**). These results enable us to propose that vesicles trafficking between the ER and Golgi, including COPII (coat protein complex II, anterograde cargo from ER), COPI (retrograde cargo), and ERGIC (ER-Golgi intermediate compartment)²⁶, store zinc ions 3 h after NO stimuli. This hypothesis is supported by the following four points: i) unique cargo proteins, four COP proteins (β -COP, β' -COP, γ 2-COP, and δ -COP) for ERGIC-COPI and SEC13 for COPII are identified in the top 28% of our analysis. ii) RAB1 and ARF4, proteins validated by our method, were reported to localize on COPII and ERGIC, respectively²⁷. In particular, ARF4 forms a complex with γ 2-COP²⁸. iii) Our imaging experiments showed that FluoZin-3-stained vesicles localized spatially close to objects stained by an ER marker (**Fig. 9d**). iv) I confirmed that DsRed-ERGIC53, known as the ERGIC marker²⁹, localized in FluoZin-3-stained vesicles (**Fig. 9e**).

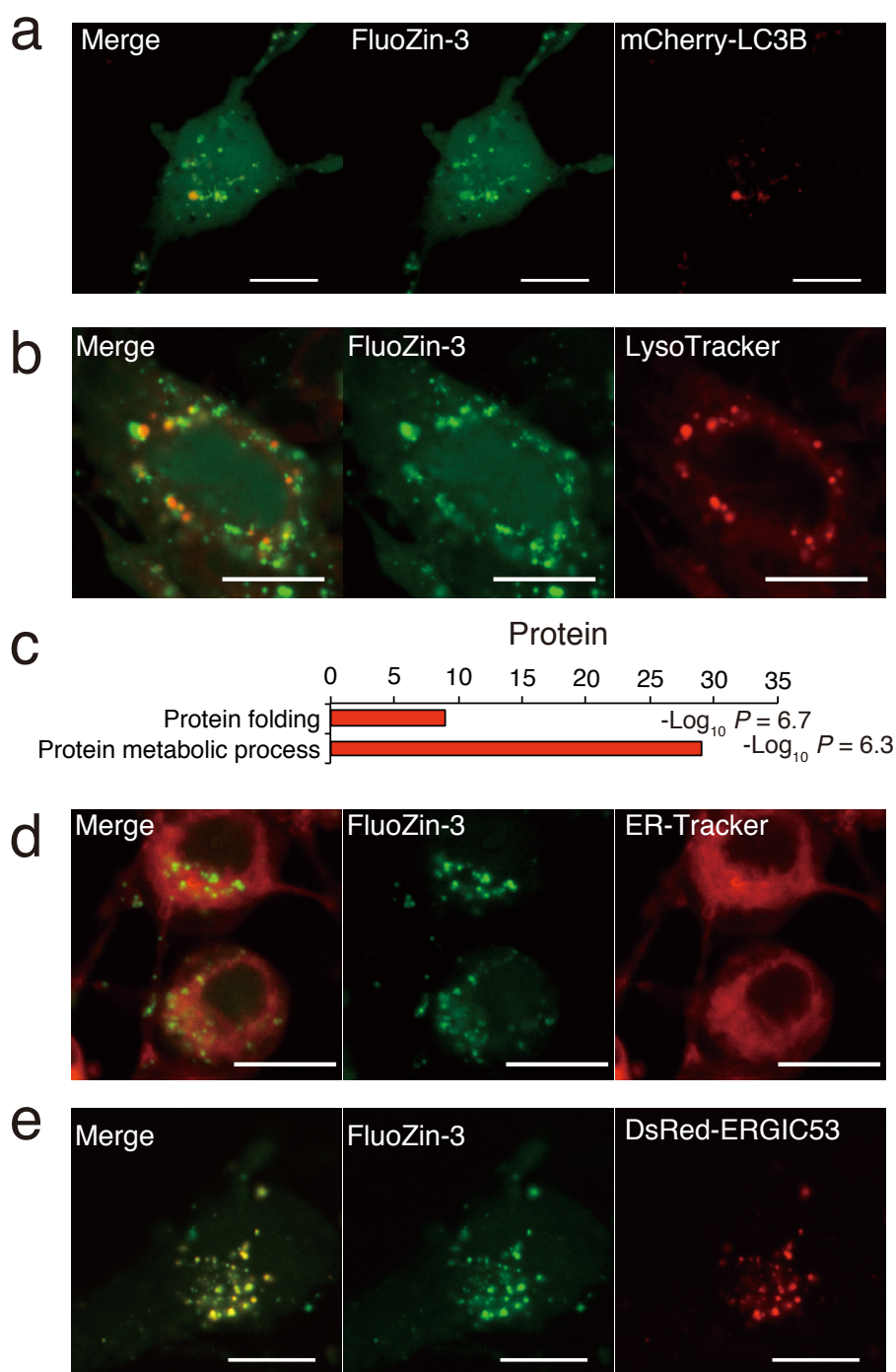


Figure 9. Characterization of zinc-rich vesicles. (a-b, d-e) CLSM imaging of zinc-rich vesicles (green) and organelle markers (red). The C6 cells were treated with SNOc (200 μ M) for 3 hour. The co-localization of zinc distribution was observed using FluoZin-3 AM (green) with (a) mCherry-LC3B as an autophagosome marker, (b) LysoTracker Red DND-99 for lysosomes, (d) ER-Racker Red for endoplasmic reticulum, and (e) DsRed-ERGIC53 as an ERGIC marker. Scale bar = 10 μ m. (c) Gene Ontology enrichment analysis of biological processes term for group B. The top two terms are shown here.

4-3, Conclusion

I have developed zinc-responsive labeling reagents “AIZin”. In combination with quantitative mass spectrometry, a novel method was successfully established for conditional proteomics that can identify various proteins localized in zinc-rich environments. This is the first report on a condition-focused proteome analysis, to the best of our knowledge. In addition to synaptic vesicles or insulin granules whose proteomic studies are being conducted, various uncharacterized zinc vesicles have been discovered, such as the zinc secretory systems in the mammalian egg³⁰, vesicles implicated in Ehlers–Danlos syndrome³¹, and lysosome-related vesicles in nematodes³². Some vesicles are reported to dynamically form, move, and sometimes fuse with the plasma membrane. By Zn^{2+} -responsive chemical tagging, our method can take a snapshot of a dynamically altered condition that is readily destroyed by a lyse process for conventional mass proteomics. The simple experimental protocol that involves neither genetic manipulation nor laborious (and time-consuming) organelle fractionation is beneficial in zinc-responsive proteomics. Although I focused on the zinc-associated signaling, I envision that this strategy can be extended to other conditional proteomics by the development of appropriate stimuli-responsive chemical labeling reagents, which provides useful platforms to elucidate many biological processes associated with a variety of conditional changes.

4-4, Experimental Section

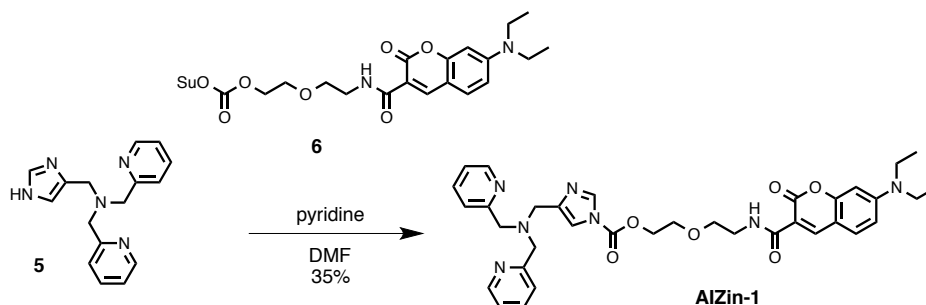
General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, or Sasaki Chemical) and used without further purification.

Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ precoated aluminium sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was conducted by flash column chromatography on silica gel 60 N (neutral, 40–50 μ m, Kanto Chemical). ¹H-NMR or ¹³C-NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer or JEOL JNM-ECA600 (600 MHz) spectrometer calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, br s = broad singlet. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was measured by an Autoflex II instrument (Bruker Daltonics) using a-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid as the matrix. High-resolution electrospray ionization quadrupole fourier transform mass spectroscopy (HR-ESI Qq-LTMS) were measured by a JEOL JMS-HX110A mass spectrometer and Bruker apex-ultra (7T) mass spectrometer, respectively.

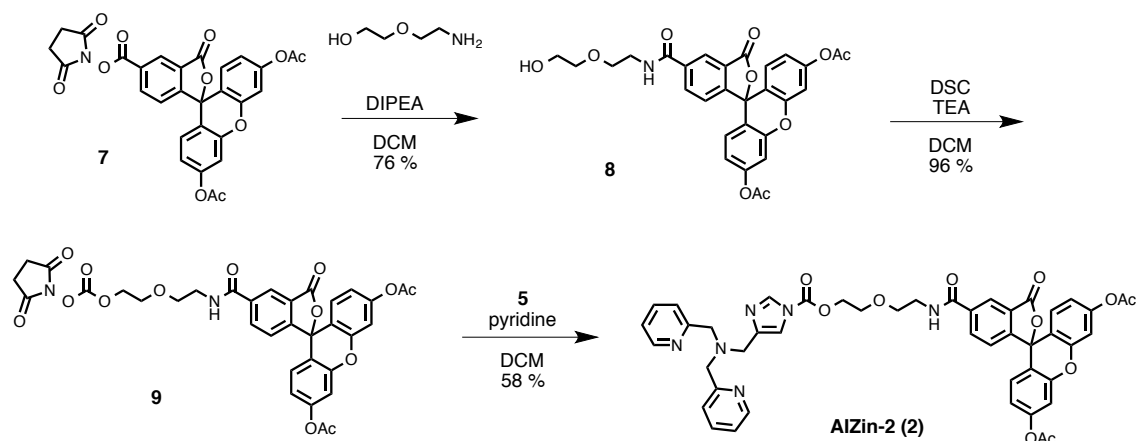
(1*H*-Imidazol-4-yl)-*N,N*-bis((pyridin-2-yl)methyl)methanamine (5),
 2-[2-[[[7-(7-(diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl)carbonyl]ethoxyl]ethyl
 2,5-dioxo-1-pyrrolidinyl ester (6), 5-Carboxyfluorescein diacetate *N*-succinimidyl ester
 (7) were synthesized as described in ref 33, 34, and 35, respectively.

Synthesis of 1-4

Scheme 1. Synthetic scheme of compound **AlZin-1 (1)**Compound **AlZin-1 (1)**

To a mixture of **5** (17 mg, 0.061 mmol, 1.0 eq) and pyridine (6 μ L, 0.074 mmol, 1.2 eq) in DMF (500 μ L), compound **6** (35 mg, 0.072 mmol, 1.2 eq) was added. The solution was stirred at r.t. for 13 hour. The reaction was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$) to confirm the end of the reaction. The mixture was evaporated and purified by silica gel column chromatography (SiO_2 , $\text{CHCl}_3/\text{MeOH} = 20/1$). The fraction was evaporated and dried *in vacuo* to yield **AlZin-1 (1)** (14 mg, 0.021 mmol, 35%) as a yellow oil. ^1H -NMR (400 MHz, CDCl_3) δ 9.07 (t, $J = 5.4$ Hz, 1H), 8.67 (s, 1H), 8.51–8.49 (m, 2H), 8.15 (d, $J = 1.2$ Hz, 1H), 7.66–7.60 (m, 4H), 7.46 (d, $J = 1.2$ Hz, 1H), 7.40 (d, $J = 9.2$ Hz, 1H), 7.12 (ddd, $J = 6.6, 4.8, 1.8$ Hz, 2H), 6.63 (dd, $J = 9.2, 2.4$ Hz, 1H), 6.47 (d, $J = 2.4$ Hz, 1H), 4.57–4.55 (m, 2H), 3.88 (s, 4H), 3.85–3.83 (m, 2H), 3.72–3.65 (m, 6H), 3.44 (q, $J = 7.2$ Hz, 4H), 1.23 (t, $J = 7.2$ Hz, 6H). ^{13}C -NMR (150 MHz, CDCl_3) δ 163.35, 162.61, 159.64, 157.68, 152.56, 148.97, 148.74, 148.06, 141.18, 137.09, 136.47, 131.12, 122.92, 121.89, 115.51, 110.16, 109.91, 108.34, 96.62, 70.00, 68.40, 67.04, 59.87, 51.30, 45.07, 39.28, 12.44.

HR-ESI MS m/z calcd. for $\text{C}_{35}\text{H}_{40}\text{N}_7\text{O}_6$ $[\text{M}+\text{H}]^+$ 654.3035, found 654.3029.



Scheme 2. Synthetic scheme of compound **AlZin-2 (2)**

Compound **8**

To a solution of **7** (358 mg, 0.64 mmol, 1.0 eq) and 2-(2-aminoethoxy)ethanol (64 μL , 0.64 mmol, 1.0 eq) in DCM (7 mL) was added DIPEA (111 μL , 0.64 mmol, 1.0 eq). The mixture was stirred for 90 min at r.t.. To the mixture CHCl_3 (20 mL) was added. The crude was washed with 1N HCl (20 mL, twice) and brine (20 mL), dried over MgSO_4 , and filtered. The solution was evaporated and dried *in vacuo* to yield **8** (267 mg, 0.49 mmol, 76%) as a colorless oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.44 (d, $J = 1.6$ Hz, 1H), 8.22 (dd, $J = 8.4, 1.6$ Hz, 1H), 7.25 (d, $J = 8.4$ Hz, 1H), 7.21 (br s, 1H), 7.11 (d, $J = 1.6$ Hz, 2H), 6.84–6.80 (m, 4H), 3.79 (t, $J = 4.6$ Hz, 2H), 3.72–3.70 (m, 4H), 3.65 (t, $J = 4.6$ Hz, 2H), 2.32 (s, 6H).

Compound **9**

To a solution of **8** (50 mg, 0.091 mmol, 1.0 eq) and DSC (47 mg, 0.18 mmol, 2.0 eq) in DCM (1 mL) was added TEA (25 μL , 0.18 mmol, 2.0 eq). The mixture was stirred for 120 min at r.t.. To the solution CHCl_3 (10 mL) was added. The crude was washed with 1N HCl (10 mL, twice), sat. NaHCO_3 aq. (10 mL) and brine (20 mL), dried over MgSO_4 , and filtered. The solution was evaporated and dried *in vacuo* to yield **9** (60 mg,

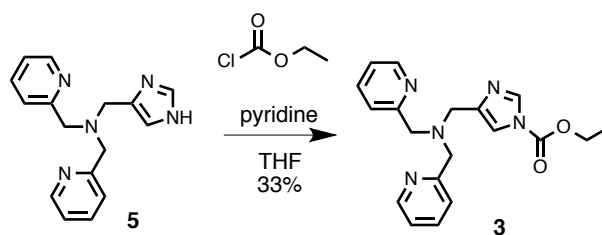
Chapter 4

0.087 mmol, 96%) as a colorless solid. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.41 (d, $J = 1.6$ Hz, 1H), 8.29 (dd, $J = 8.4, 1.6$ Hz, 1H), 7.35 (s, 1H), 7.25 (d, $J = 8.4$ Hz, 1H), 7.10 (d, $J = 1.6$ Hz, 2H), 6.85–6.78 (m, 4H), 4.55 (t, $J = 4.4$ Hz, 2H), 3.78–3.72 (m, 4H), 2.90–2.80 (m, 6H), 2.31 (s, 6H).

Compound **AlZin-2 (2)**

To a mixture of **5** (10 mg, 0.036 mmol, 1.0 eq) and compound **9** (33 mg, 0.048 mmol, 1.3 eq) in DCM (500 μL), pyridine (4 μL , 0.050 mmol, 1.4 eq) was added. The solution was stirred at r.t. for 12 hour. The reaction was monitored by TLC ($\text{CHCl}_3/\text{MeOH} = 10/1$) to confirm the end of the reaction. The mixture was evaporated, dissolved in DMF, and purified by HPLC (ODS-A, preparative, A/B = 40/60 to 70/30 for 30 min, A = CH_3CN , B = 5 mM NH_4OAc pH 5.5). The fraction was lyophilized to yield **AlZin-2 (2)** (18 mg, 20 μmol , 58%) as a white powder.

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 8.51 (d, $J = 4.8$ Hz, 2H), 8.40 (s, 1H), 8.19 (d, $J = 8.4$ Hz, 1H), 8.10 (s, 1H), 7.64 (dt, $J = 7.8, 1.8$ Hz, 2H), 7.59 (d, $J = 7.8$ Hz, 2H), 7.45 (s, 1H), 7.25 (d, $J = 8.4$ Hz, 1H), 7.14–7.10 (m, 4H), 6.84 (br s, 1H), 6.82 (dd, $J = 8.4, 2.4$ Hz, 2H), 6.79 (d, $J = 8.4$ Hz, 2H), 4.59 (t, $J = 4.6$ Hz, 2H), 3.90–3.85 (m, 6H), 3.75–3.70 (m, 6H), 2.31 (s, 6H); $^{13}\text{C-NMR}$ (600 MHz, CDCl_3) δ 168.77, 168.21, 165.63, 159.46, 155.28, 152.26, 151.54, 149.01, 148.77, 141.13, 136.99, 136.79, 136.54, 134.72, 128.86, 126.61, 124.64, 123.42, 123.02, 121.99, 117.93, 115.74, 115.51, 110.57, 81.94, 69.84, 68.71, 66.71, 59.72, 51.08, 40.07, 21.13; HR-MS (ESI) m/z calcd. for $\text{C}_{46}\text{H}_{41}\text{N}_6\text{O}_{11}$ $[\text{M}+\text{H}]^+$ 853.2828, found. 853.2823.



Scheme 3. Synthetic scheme of compound **3**

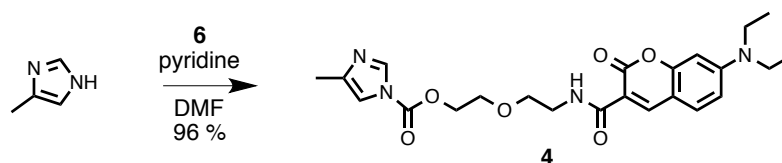
Compound **3**

To a mixture of **5** (3.0 g, 0.011 mol, 1.0 eq) and pyridine (1.7 mL, 0.022 mmol, 2 eq) in dry THF (50 mL), ethyl chloroformate (1.62 mL, 0.016 mmol, 1.5 eq) was added. The solution was stirred at r.t. for 1 hour. The reaction was monitored by TLC (CHCl_3 / MeOH = 10/1) to confirm the end of the reaction. The mixture was evaporated and purified by silica gel column chromatography sequentially (SiO_2 , $\text{CHCl}_3/\text{MeOH}$ = 30/1 $\rightarrow$ 40/1). The fraction was evaporated and dried *in vacuo* to yield **3** (1.26 g, 3.58 mmol, 33%) as a colorless oil.

^1H -NMR (400 MHz, CDCl_3) δ 8.53 (dd, J = 4.8, 2.0 Hz, 2H), 8.09 (d, J = 1.2 Hz, 1H), 7.68-7.60 (m, 4H), 7.39 (d, J = 1.2 Hz, 1H), 7.13 (ddd, J = 6.6, 4.8, 1.2 Hz, 2H), 4.46 (q, J = 7.2 Hz, 2H), 3.88 (s, 4H), 3.72 (s, 2H), 1.44 (t, J = 7.2 Hz, 3H); ^{13}C -NMR (150 MHz, CD_3CN) δ 160.68, 149.71, 149.60, 141.69, 137.68, 137.21, 123.59, 122.80, 116.31, 65.14, 60.33, 51.59, 14.23.

[**3**-Zn](OTf)₂ complex

Solid $\text{Zn}(\text{OTf})_2$ (5.0 μmol , 1 eq) was dissolved in DCM (100 μL) containing 50 mM of **3**. The mixture was sonicated, filtered and transferred into a glass tube. I slowly poured diethyl ether (400 μL) into the tube. Colorless needle crystals formed liquid-liquid diffusion after incubation at r.t. for 2 days.



Scheme 4. Synthetic scheme of compound **4**

Compound **4**

To a mixture of 4-methyl imidazole (12.5 mg, 0.15 mmol, 1.0 eq) and pyridine (37 μ L, 0.45 mmol, 3.0 eq) in DMF (1 mL), compound **6** (88 mg, 0.18 mmol, 1.2 eq) was added. The solution was stirred at r.t. for overnight. The reaction was monitored by TLC (AcOEt) to confirm the end of the reaction. The mixture was evaporated and purified by silica gel column chromatography (SiO_2 , AcOEt to $\text{CHCl}_3/\text{MeOH} = 10/1$). The fraction was evaporated and dried *in vacuo* to yield **4** (66 mg, 0.14 mmol, 96 %) as a yellow oil.

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 9.07 (br s, 1H), 8.67 (s, 1H), 8.10 (s, 1H), 7.43 (d, $J = 9.2$ Hz, 1H), 7.17 (s, 1H), 6.65 (dd, $J = 9.2, 2.4$ Hz, 1H), 6.49 (d, $J = 2.4$ Hz, 1H), 4.54 (t, $J = 4.6$ Hz, 2H), 3.83 (t, $J = 4.6$ Hz, 2H), 3.65-3.71 (m, 4H), 3.46 (q, $J = 7.2$ Hz, 4H), 2.20 (s, 3H), 1.24 (t, $J = 7.2$ Hz, 6H); HR-MS (ESI) m/z calcd for $\text{C}_{23}\text{H}_{29}\text{N}_4\text{O}_6$ $[\text{M}+\text{H}]^+$ 457.2082, found 457.2078.

X-ray crystallography of Zn:3 complex

Data were processed with direct methods of SHELX-97 and SIR97. All non-hydrogen atoms were refined anisotropically. The positions of hydrogen atoms were calculated, and their contributions in structural factor calculations were included. A summary of crystallographic data for Zn:3 complex data is as follows. $C_{21}H_{21}F_6N_5O_8S_2Zn$ (Mw 714.94); Crystal dimensions (mm): 0.40 x 0.20 x 0.05 ; Crystal System: monoclinic; Space group: P2₁; Z = 2; Cell parameters: $a = 8.832(3)$ Å, $b = 12.317(4)$ Å, $c = 13.554(4)$ Å; $T = 173(2)$ K; $V = 1437.3(8)$ Å³; Density(calculated): 1.652 g/cm³; $3.01 \leq \theta \leq 27.50$; refl. Measured = 6158; ref. unique = 4271; GOF = 0.667; $R(I > 2\sigma(I)) = 0.0391$; R_w (all data) = 0.1126.

Hydrolysis assay

To valuate the zinc-modulated reactivity change of the AIZin reagent, I measured the hydrolysis rate by HPLC. I added 1 µM of reagent **1** (1 mM DMSO stock) to a Zn²⁺ containing buffer (1 µM of ZnSO₄, 100 mM HEPES, $I = 0.1$ (KCl/KOH), pH 7.4) or a Zn²⁺ deleted buffer³⁶ (100 mM HEPES, $I = 0.1$ (KCl/KOH), 10 mM NTA, pH 7.4) to start the hydrolysis. After incubation at 37 °C, the solutions were mixed with TPEN to quench the reactions and subjected to RP-HPLC analyses (column; YMC-pack ODS-A, 250×4.6 mm, mobile phase; CH₃CN (containing 0.1 % TFA) / H₂O (containing 0.1 % TFA) = (30/70 to 60/40) (linear gradient over 30 min), flow rate; 1.0 mL/min, detection; UV-Vis (220-600 nm)). The hydrolysis ratios were determined by the Abs420 area. To investigate the selectivity in various metal ions, I added each of various metal ions (5 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 5 mM MgSO₄, 1 µM MnSO₄, 1 µM FeSO₄, 1 µM FeCl₃, 1 µM CoSO₄, 1 µM CuSO₄, 1 µM CdSO₄, 1 µM Pb(NO₃)₂ or 1 µM ZnSO₄) and incubated for 90 min at 25 °C.

General materials and methods for biochemical/biological experiments

SDS-polyacrylamide gel electrophoresis and western blotting were carried out using a Bio-Rad Mini-Protean electrophoresis apparatus. Fluorescent and chemi-luminescent signals generated with ECL Prime (GE Healthcare) were detected with a LAS4000 imaging system (Fuji Film). Cell imaging was performed with a confocal laser scanning microscope (CLSM, FV10i, Olympus) equipped with a 60x objective lens, and LD laser (473 nm and 559 nm).

Protein labeling *in vitro*

To prepare a cell lysate, cultured HeLa cells were rinsed with PBS, suspended in lysis buffer (100 mM HEPES, 58.5 mM NaCl, pH 7.4), homogenized by ultrasonic treatment, and centrifuged (2,740 G, 7 min). The supernatant was collected and measured the protein concentrations by bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Fisher Scientific). To evaluate the Zn^{2+} -enhanced protein labeling, I added ZnSO_4 (50 μM , 1 eq. for reagent) with or without TPEN (1 mM, 20 eq.) to the lysate (protein; 4.8 mg/mL). The reactions were initiated with addition of reagent **1** or **4** (50 μM), followed by incubation for 30 min at 37 °C. The lysates were mixed with 2xLeammli buffer (pH 6.8; 125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 100 mM dithiothreitol, 1 mM EDTA). In order to monitor the time course, the reactions were quenched by addition of 1 mM TPEN (20 eq) and mixed with 2xLeammli buffer. To investigate the selectivity on metal ions, I added each of various metal ions (5 mM NaCl, 5 mM KCl, 5 mM CaCl_2 , 5 mM MgSO_4 , 50 μM MnSO_4 , 50 μM FeSO_4 , 50 μM FeCl_3 , 50 μM CoSO_4 , 50 μM CuSO_4 , 50 μM CdSO_4 , 50 μM $\text{Pb}(\text{NO}_3)_2$, 50 μM NiSO_4 or 50 μM ZnSO_4) to the lysate. I added reagent **1** (50 μM) to these lysates and incubated for 30 min at 37 °C.

Cell culture and zinc-responsive labeling

HeLa cells were maintained in DMEM (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (250 ng/ml). For proof of principle experiments, I rinsed HeLa cells (2×10^5) with HBSS, added AIZin-1 (1 μ M) and incubated for 20 min, followed by treatment with 200 μ M of zinc pyrithione complex for 20 min. The cells were lysed using 1xLaemmuli buffer (pH 6.8; 62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.005% bromophenol blue, 50 mM dithiothreitol, 1 mM EDTA) and loaded on a SDS-PAGE (12.5 % poly-acrylamide) gel. For TEPN control experiment, I added 100 μ M of TPEN before the addition of AIZin-1.

Protein labeling in living C6 glioma cell

C6 cells were grown in a F-12K medium (Sigma) supplemented with 15% (v/v) House serum (HS), 2.5% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 mg/ml) under 5% CO₂ at 37 °C. For zinc-responsive labeling experiments, the culture mediums were replaced with HBSS, and treated with 200 μ M SNOC. The NO-stimulated C6 cells were incubated for 0, 10 or 180 min under 5% CO₂ at 37 °C. After the incubation, I treated 1 μ M AIZin-2 for 20 min and washed with PBS containing 50 μ M TPEN twice. For fractionation experiments, I used two dishes (10 cm) of semi-confluent C6 cells for each condition. The labeled organelle fraction and cytosolic fraction were obtained by using Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific) according to the manufacturer's protocol. I conducted the procedure according to Option B in the protocol (isolation of mitochondria using Dounce Homogenation) to separate cytosolic fraction and the organelle mixture including mitochondrial, lysosomal, peroxisomal and ER proteins. Both fractions were dissolved in Ripa buffer (pH 7.4, 25 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25 % DOC) containing 1% protease inhibitor cocktail set I. I

determined the protein concentrations by BSA assay, diluted in a Ripa buffer to adjust the concentration, mixed with 2xLaemmuli buffer. The samples were loaded on a 12.5% SDS-PAGE gel, separated and imaged.

Conditional labeling and enrichment of labeled proteins

I used two dishes (10 cm) of semi-confluent C6 cells for each condition. I treated C6 cells with 200 μ M SNOC and added 1 μ M AIZin-2 in the same manner as described above. I rinsed the labeled C6 cells twice with PBS containing 50 μ M of TPEN and harvested by scraping and centrifugation (600 G, 3 min). I removed the supernatant and dissolved the pellet in 450 μ L of a NP-40 buffer (pH 7.4, 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% protease inhibitor cocktail set I) for 30 min on ice. After centrifugation (12,000 G, 10 min), I applied the supernatants to a ZebaTM Spin Desalting Coulmn 7k MWCO 2 mL (Thermo Fisher Scientific) to remove extra AIZin-1 reagents and its hydrolyzed products. I determined the protein concentrations using the BCA protein assay and diluted in NP-40 buffer to concentrations of 1 mg/mL. To these solutions (300 μ L), I added rabbit anti-fluorescein antibody (abcam) conjugated DynaBeads (Thermo Fisher Scientific) slurry (Antibody/Beads = 15 μ g/ 4.5 mg) and incubated at 4 °C for overnight with rotation. The Beads were rinsed with PBS buffer for 4 times and the labeled proteins were then eluted by addition of a 50 mM Glycine-HCl buffer (40 μ L).

In-gel tryptic digestion and extraction

Labeled proteins eluted from magnetic beads were mixed with the same volume of a 2xLaemmuli buffer, loaded on a 10% SDS-PAGE gel (Mini-PROTEAN[®] TGX Gels), and run for 10 min at 15 mA (the migration distance of dye front is about 10 mm). The gel was fixed in a 1:1 mixture of acetonitrile : pure water (5% acetic acid) for 20 min, in a 1:1 mixture of acetonitrile : pure water for 10 min, and in pure water for 10 min. Each

lane was cut (Hight, 10 mm), rinsed with pure water, and dehydrated with acetonitrile. The dehydrated gels were swelled with 200 μ L of 10 mM DTT in a 50 mM TEAB (triethylamine bicarbonate) buffer (Sigma Aldrich) and heated at 56 °C for 30 min. The DTT solutions were replaced with a 55 mM iodoacetamide solution in 50 mM TEAB buffer and allowed to react for 45 min in the dark. The gel fractions were washed with a 50 mM TEAB buffer, dehydrated in acetonitrile, rehydrated in 50 mM TEAB buffer, and dehydrated in acetonitrile again. The gels were swelled in a 50 mM TEAB buffer containing 10 ng/ μ L Sequencing Grade Trypsin (Promega) and incubated for overnight at 37 °C. After the digestion, I transferred the supernatant to a new tube and added 50 μ L of an extraction solution (50% acetonitrile, 0.1% TFA) to the gel piece. The supernatants were collected after 10 min incubation and I repeated this process two additional times. Extracted peptides were lyophilized.

Preparation of TMT-labeled peptides mixture

I reconstituted the lyophilized peptides in 25 μ L of a 100 mM TEAB buffer. To the solutions, I added 10.25 μ L of TMT reagents (0.8 mg/ 41 μ L) in acetonitrile. I used 6-plex TMT reagents (Thermo Fisher Scientific) for NO-0, -10, -180min comparison (light tag for NO-0min, medium tag for NO-10min, and heavy tag for NO-180min). The peptides were allowed to react for 1 h at room temperature, and the reactions were quenched by addition of 5% hydroylamine (2 μ L). Three different solutions were mixed in a 1:1:1 ratio and lyophilized. The peptides were reconstituted in 200 μ L of a 5% acetonitrile/0.1% TFA mixture and loaded onto a GL-Tip SDB (GL Sciences). The tip was washed with 5% acetonitrile/0.1% TFA mixture, and peptides were eluted by 80% acetonitrile/0.1% TFA. I conducted the biological replication for three times.

NanoLC-MS/MS analyses

NanoLC-MS/MS analyses were performed on a Q-Exactive mass spectrometer (Thermo Fisher Scientific), an Ultimate 3000 nanoLC pump (Thermo Fisher Scientific) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). A self-pulled needle (150-mm length $\times$ 100 μ m ID, 6- μ m opening) packed with Reprosil-C18 3 μ m reserved phase material (Dr. Maisch, Ammerbuch, Germany) was used as the analytical column³⁷. The injection volume was 5 μ L and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A three-step linear gradient of 5–10% B in 5 min, 10–40% B in 60 min, 40–100% B in 5 min, and 100% B for 10 min was employed. Spray voltages of 2400 V were applied. The mass scan ranges were m/z 350–1500, and top ten precursor ions were selected in each MS scan for subsequent MS/MS scans. The normalized collision energy was set to be 33.0. A lock mass function was used for the system to obtain constant mass accuracy during gradient³⁸.

MS data analysis

The raw MS data files were analyzed by ProteoWizard to create peak lists based on the recorded fragmentation spectra. Peptides and proteins were identified by means of automated database searching using Mascot v2.4 (Matrix Science, London) against UniprotKB/Swiss-Prot release 2015_11 of 11-Nov-15 with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.02 Da and strict trypsin specificity³⁹ allowing for up to 1 missed cleavage. TMT tags on lysine residues and peptide N-termini and cysteine carbamidomethylation were set as fixed modifications. Methionine oxidation was allowed as a variable modification. Peptides were considered identified if the Mascot score was over the 95% confidence limit ($p < 0.05$) for each peptide. A reversed decoy database search was conducted to set false discovery rates (FDRs) of less than 1% both at peptide and protein levels. Proteins were quantified by

averaging relative peak intensities of the TMT reporter ions across all identified peptides.

Data processing and GO term enrichment analysis

I performed three independent biological replicates in TMT quantitative analysis. In each experiment, only proteins that showed at least two identified and quantified peptides were considered as hit proteins. In addition, only proteins that were hit more than twice in triplicate were considered as “identified proteins” (331 proteins) in which several keratins (contaminants) are removed. In the “identified proteins” list, I categorized the bottom and top 20% (66 proteins) of $\text{Log}_2(\text{H/M})$ ratio values as “group A” and “group B”, respectively. For gene ontology enrichment analyses, I uploaded these group lists to the Gene Ontology Consortium website (<http://geneontology.org/>). I used the PANTHER Overrepresentation Test (release 20150430) as an analysis type and the GO Ontology database Released 2015-08-06 as an annotation version. In the protein localization studies, I used following GO IDs (Nucleus; GO:0005634, Ribosome; GO:0005840, Cytosol; GO:0005829, Cytoskeleton; GO:0005856, Mitochondrion; GO:0005739, Plasma membrane; GO:0005886, Vesicle; GO:0031982, Golgi apparatus; GO:0005794, Endoplasmic reticulum; GO:0005783). In the OG enrichment analysis in biological processes, I used the GO biological PANTHER GO-Slim Biological Process as an annotation data set.

cDNA cloning and construction of the expression plasmids

To obtain cDNA of the identified proteins and LC3B, I carried out the Marathon cDNA Amplification according to the manufacturer's protocol with Marathon-Ready cDNA from rat brain (Clontech) as templates. Following primers for PCR and nested PCR were used. The PCR products were inserted into EcoRV sites of pBluescript II SK (-). All plasmids were amplified using DH5 α competent cells and purified by QIAprep Spin MiniPrep Kit (Qiagen). The cDNA sequence was confirmed by DNA sequence analyses.

CALR-forward primer: gctgctgaagatcggttttaaggccagtg
 CALR-reverse primer: ctctgtggcggggtgttcaggcctc
 CALR-forward primer for nested PCR (XhoI): caactcgaggccaccatgctccttcgggtgccgctc
 CALR-reverse primer for nested PCR (SacII): gttccgcggcagctcatccttgcttgccagtgg
 PDIA3-forward primer: ggcggtctctccatctcggttctctgg
 PDIA3-reverse primer: ccattctgtctctctgatggtttgctttctc
 PDIA3-forward primer for nested PCR (XhoI): gaactcgaggccaccatgctccttcagctgccttgccg
 PDIA3-reverse primer for nested PCR (SacII): gttccgcgggagggtcctctgtgccttcttcttctcttag
 ENPL was cloned in divided two species (ENPL-1, ENPL-2).
 ENPL-1-forward primer: cgaccgaaaaggacttgcgactctcc
 ENPL-1-reverse primer: gcagatgtaggcacaacaaaattgactgaaggtg
 ENPL-1-forward primer for nested PCR (XhoI): gaactcgaggccaccatgagggtcctgtgggtgctg
 ENPL-1-reverse primer for nested PCR (NcoI): cagtaaagtggatataagccatgggggtcatcactttcc
 ENPL-2-forward primer: ccaaagaagtagaagaagacagtagtacaagctttctac
 ENPL-2-reverse primer: gtcacattccctctccacacaggattcatag
 ENPL-2-forward primer for nested PCR (NcoI): ggaaagtgtatgaccccatggcttataccactttactg
 ENPL-2-reverse primer for nested PCR (SacII): ctccgcggcaattcatccttctctgtagggtcctttctgtttg
 PPIA-forward primer: gccgtgtctcttttcgccgttg
 PPIA-reverse primer: ggaatggttgatgggtaaaatgcccgcaag
 PPIA-forward primer for nested PCR (XhoI): caactcgaggccaccatgggtcaacccaccgtgttcttcgac
 PPIA-reverse primer for nested PCR (BamHI): ctggatccacgagttgtccacagtcggagatggtgatc
 ARF4-forward primer: ctcaattgtgagccggaaccgtgaaggc
 ARF4-reverse primer: gtaacaagcctagaccaattctatcaaacatgtccttg
 ARF4-forward primer for nested PCR (XhoI): caactcgagccaccatgggctcaccatctcctctctctctc
 ARF4-reverse primer for nested PCR (BamHI): ctggatccccgcgttttgaaagttcatttgacagccaatccagc
 EF1A1-forward primer: gggtttccgtcagaacgcaggtgtg
 EF1A1-reverse primer: ctgagaccgttctccaccactgattaagac
 EF1A1-forward primer for nested PCR (XhoI): caactcgagctatgggaaaggaaaagactcacatcaacatcgctc
 EF1A1-reverse primer for nested PCR (BamHI): ctggatcctcatttagccttctgagctttctgggcagac
 RAB1A-forward primer: cttagctcattctcccccttcattacc
 RAB1A -reverse primer: caatctctgacctgttggaacggtgaagaatc
 RAB1A -forward primer for nested PCR (XhoI):
 ccactcgagctatgaatccgaatatgattatttcaagtactcctgattggc
 RAB1A -reverse primer for nested PCR (BamHI): gttggtatccttagcagcagcctccacctgactg

LC3B -forward primer: gttgaggagacacacaagggaagtgatcg
 LC3B -reverse primer: cggaaccagatcgtctgaccaggac
 LC3B -forward primer for nested PCR (EcoRI):caagaattccatgccgtccgagaagaccttaaacagc
 LC3B -reverse primer for nested PCR (BamHI): ctgtgggtggtgagctgaccagc

In order to construct pmCherry-KDEL-N1 vector containing KDEL, ER retention signal peptide, at C-terminus of mCherry sequence (before TAG), I carried out QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Tech.) to insert KDEL (tagcggccg) into pmCherry-N1 vector by using forward primer (5'-caagtctccacccattgac-3') and reverse primer (5'-gacaaaccacaactagaatgcag-3').

The cDNA of the identified protein sub-cloned into pBS was digested with restriction enzymes and ligated into vector (pmCherry-N1, pmCherry-C1 or pmCherry-N1-KDEL) to obtain their expression vectors. I summarized the combinations in the bellow table.

Name	Protein	Vector	Ref.
CALR-mCherry-KDEL	Calreticulin	KDEL-N1	40
PDIA3-mCherry-KDEL	Protein disulfide-isomerase A3	KDEL-N1	41
ENPL-mCherry-KDEL	Endoplasmin	KDEL-N1	42
PPIA-mCherry	Peptidyl-prolyl cis-trans isomerase A	N1	43
ARF4-mCherry	ADP-ribosylation factor 4	N1	44
mCherry-EF1A1	Elongation factor 1-alpha 1	C1	45
mCherry-RAB1A	Ras-related protein Rab-1A	C1	46
mCherry-LC3B	Microtubule-associated proteins 1A/1B light chain 3B	C1	47

Transfection and fluorescent imaging studies

Transfection of cDNA plasmid was carried out in a 35 mm dish using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's instruction. After 48

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hour of the transfection, the medium were replaced with HBSS buffer and the C6 cells were treated with 200 μ M SNOC. After 10 min or 3 h stimuli, I added FluoZin-3 AM (1 μ M), incubated for 10–20 min, and observed using a confocal laser scanning microscope (CLSM, FV10i).

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List of Publication

Chapter 1-2

LDAI-Based Chemical Labeling of Intact Membrane Proteins and Its Pulse-Chase Analysis under Live Cell Conditions

Takayuki Miki, Sho-hei Fujishima, Kazuhiro Komatsu, Keiko Kuwata, Shigeki Kiyonaka, and Itaru Hamachi

Chem. Biol. **21**, 1013–1022 (2014)

Chapter 3

Proteomic Analysis of Cholesterol-/Sterol-Binding Proteins Using Ligand-Directed Tosyl Chemical Labeling in Living Nematode

Takayuki Miki, Takuma Sugi and Itaru Hamachi

Manuscript in preparation.

Chapter 4

Zinc-responsive labeling in live cells for novel conditional proteomics

Takayuki Miki, Masashi Awa, Sigaki Kiyonaka, Masaki Wakabayashi, Yasushi Ishihama and Itaru Hamachi

Nat. Methods under revision

List of Publications and Presentations

Other Publications

1) Ligand-Directed Acyl Imidazole Chemistry for Labeling of Membrane-Bound Proteins on Live Cells

Sho-hei Fujishima, Ryosuke Yasui, Takayuki Miki, Akio Ojida, Itaru Hamachi

J. Am. Chem. Soc. **134**, 3916-3964 (2012)

2) Fluorophore Labeling of Native FKBP12 by Ligand-Directed Tosyl Chemistry Allows Detection of Its Molecular Interactions in Vitro and in Living Cells

Tomonori Tamura, Yoshiyuki Kioi, Takayuki Miki, Shinya Tsukiji, Itaru Hamachi

J. Am. Chem. Soc. **135**, 6782-6785 (2013)

3) One-step construction of caged carbonic anhydrase I using a ligand-directed acyl imidazole-based protein labeling method

Kazuya Matsuo, Yoshiyuki Kioi, Ryosuke Yasui, Yousuke Takaoka, Takayuki Miki,

Sho-hei Fujishima, Itaru Hamachi

Chem. Sci. **4**, 2573-2580 (2013)

4) Extended Affinity-guided DMAP Chemistry with Finely-tuned Acyl Donor for Intracellular FKBP12 Labeling

Zhining Song, Yousuke Takaoka, Yoshiyuki Kioi, Kazuhiro Komatsu, Tomonori

Tamura, Takayuki Miki, Itaru Hamachi

Chem. Lett. **44**, 333-335 (2015)

Reviews and Books

1) 第 23 章 高選択的 in vivo タンパク質ラベル化による新イメージング技術
浜地 格, 三木 卓幸, 高岡 洋輔

『がんの分子イメージング』(浦野泰照 編), **2015**,化学同人, 京都, p.216–225

List of Presentations

1) Construction of Fluorescent Biosensor by Ligand-Directed Tosyl Chemistry and Schiff Base Formation Reaction.

Takayuki Miki, Tomonori Tamura, Yoshiyuki Kioi, Shinya, Tsukiji, and Itaru Hamachi
5th Joint Symposium on Biofunctional Chemistry, Tsukuba, September **2011**.

2) Protein labeling with acyl transfer chemistry (1) Development of fluorescent sensor on living cell by acyl imidazole labeling reagent.

Takayuki Miki, Sho-hei Fujishima, Ryosuke Yasui, Akio Ojida, and Itaru Hamachi
92nd Annual Meeting of Chemical Society of Japan, Kanagawa, March **2012**.

3) Receptor labeling by using LDAI chemistry : on cell kinetics of ligand binding

Takayuki Miki, Sho-hei Fujishima, Ryosuke Yasui, Akio Ojida, and Itaru Hamachi
Summer School in 24th Symposium on Biofunctional Chemistry, Fukuoka, July **2012**.

4) Isozyme selective labeling by LDAI chemistry

Takayuki Miki, Sho-hei Fujishima, Ryosuke Yasui, and Itaru Hamachi
The 27th Forum on Biomolecular Chemistry Sapporo, September **2012**.

5) Isozyme selective labeling by using LDAI chemistry

Takayuki Miki, Sho-hei Fujishima, Ryosuke Yasui, and Itaru Hamachi
6th Joint Symposium on Biofunctional Chemistry, Sapporo, September **2012**.

6) Progress of LDAI chemistry (2) Pulse-chase analysis of endogenous membrane proteins in live cells.

Takayuki Miki, Sho-hei Fujishima, Ryosuke Yasui, and Itaru Hamachi
93rd Annual Meeting of Chemical Society of Japan, Shiga, March **2013**.

7) LDAI chemical labeling and pulse-chase analysis of endogenous membrane proteins.

Takayuki Miki, Sho-hei Fujishima, Ryosuke Yasui, and Itaru Hamachi
The 8th Annual Meeting of Japanese Society for Chemical Biology, Tokyo, June **2013**

List of Publications and Presentations

8) Analysis of membrane protein degradation by using LDAI chemistry.

Takayuki Miki, Sho-hei Fujishima, and Itaru Hamachi

Summer School in 24th Symposium on Biofunctional Chemistry, Tokyo, July **2013**.

9) Multi-color pulse chase imaging of endogenous CA12 by using LDAI chemistry.

Takayuki Miki, Sho-hei Fujishima, and Itaru Hamachi

7th Joint Symposium on Biofunctional Chemistry, Nagoya, September **2013**.

10) Multi-color pulse imaging of endogenous membrane proteins by using LDAI chemistry.

Takayuki Miki, Sho-hei Fujishima, and Itaru Hamachi

4th Vivid Workshop –Multi-Dimensional Fluorescence Live Imaging of Cellular Functions and Molecular Activities-, Ishikawa, February 2013.

11) LDAI chemical protein labeling (1) Pulse chase analysis of membrane protein

Takayuki Miki, Sho-hei Fujishima, and Itaru Hamachi

94th Annual Meeting of Chemical Society of Japan, Nagoya, March **2014**.

12) A novel conditional protein labeling method (1): Zinc ion responsive focused proteome analysis in live cell

Takayuki Miki, Masashi Awa, Shigeki Kiyonaka, and Hamachi Itaru

95th Annual Meeting of Chemical Society of Japan, Ibaraki, March **2015**.

13) Development of zinc-responsive reagent and its proteomics.

Takayuki Miki, Masashi Awa, and Hamachi Itaru

Summer School in 27th Symposium on Biofunctional Chemistry, Tokyo, July **2015**.

14) Conditional proteomics using zinc ion responsive labeling reagent.

Takayuki Miki, Masashi Awa, Shigeki Kiyonaka, and Hamachi Itaru

9th Joint Symposium on Biofunctional Chemistry, Kumamoto, September **2015**.

List of Honors

- 1) Japan Society for the Promotion of Science (JSPS) research fellowships for young scientist (DC1, 2013–2016)
- 2) Poster award at the 27th Forum on Biomolecular Chemistry Sapporo, September **2012**.
- 3) Poster award at Summer School in 24th Symposium on Biofunctional Chemistry, Tokyo, July **2013**.
- 4) Poster award at Summer School in 27th Symposium on Biofunctional Chemistry, Tokyo, July **2015**.
- 5) Poster award of Organic & Biomolecular Chemistry at 9th Joint Symposium on Biofunctional Chemistry, Kumamoto, September **2015**.

List of Publications and Presentations