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Chemical proteomics for subcellular proteome analysis

Hao Zhu[1], Tomonori Tamura[1] and Itaru Hamachi[1,2]

Protein functions are tightly regulated by their subcellular localization and dynamic alteration. Chemical proteomics offers convenience and efficiency for profiling protein features in a native context. In this review, we summarize the recent progress of subcellular-compartment-focused chemical proteomics which do not rely on organelle fractionation. Organelle-specific activity-based protein profiling (ABPP) and engineered ascorbate peroxidase (APEX) have been developed for proteome analysis within organelles and even sub-organelles. In parallel, our lab designed organelle-localizable reactive molecules (ORMs) to selectively tag nuclear and mitochondrial proteins. ORMs-based proteomics is applicable to primary neurons and brain slices, as well as cultured cell lines. In addition, we invented a conditional proteomics approach to elucidate zinc homeostasis by labeling and identifying proteins localized in Zn^{2+}-rich space of live cells.

Addresses
[1] Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan
[2] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Sanbancho, Chiyodaku, Tokyo 102-0075, Japan

Corresponding author: Hamachi, Itaru (ihamachi@sbchem.kyoto-u.ac.jp)

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Introduction

Protein functions are closely associated with its subcellular distribution in live cells. Each subcellular compartment, or organelle, contains varied protein compositions that underlie the diversity of biochemical reactions in a single cell. Protein localization and expression level are dynamically varied in response to environmental changes. The spatiotemporal changes of proteomes reflect the biological states of resided organelles. Organelle-focused proteomics relying on subcellular fractionation have been used to identify the components of cellular organelles [1–3]. However, the conventional methods [1] often suffer from the limited specificity and the low coverage. Moreover, they can neither accurately report temporal dynamics of proteins, because of the time-consuming biochemical purification processes, nor readily access the sub-organelle proteomes.

Chemical proteomics is now a powerful strategy for the focused protein profiling [4–6]. The proteome of interest is covalently tagged with chemical reagents in live cells, followed by standard enrichment and mass spectrometry (MS) analysis, which allows for fixing protein information before cell lysis and, thus is able to obtain a snapshot of dynamically altered subcellular proteomes that cannot be addressed by the organelle fractionation. Organelle-focused chemical proteomics exploits spatially limited reactions by directing labeling reagents or enzymes to specific subcellular compartments. These may provide organelle and even sub-organelle proteome mapping with high spatiotemporal resolutions.

In addition to subcellular localization, cellular microenvironments, such as pH, concentrations of metals, hypoxia/hyperoxia and redox states, tightly regulate local protein structures and activities. Such environmental conditions are spatially heterogeneous and dynamically fluctuated in live cells and tissues. Useful methods to precisely address the local proteomes are enormously desirable for comprehensive elucidation of proteome dynamics. We firstly proposed a strategy termed 'conditional proteomics' [7••] as a powerful approach to selectively label and identify the conditional proteomes and profile their dynamics.

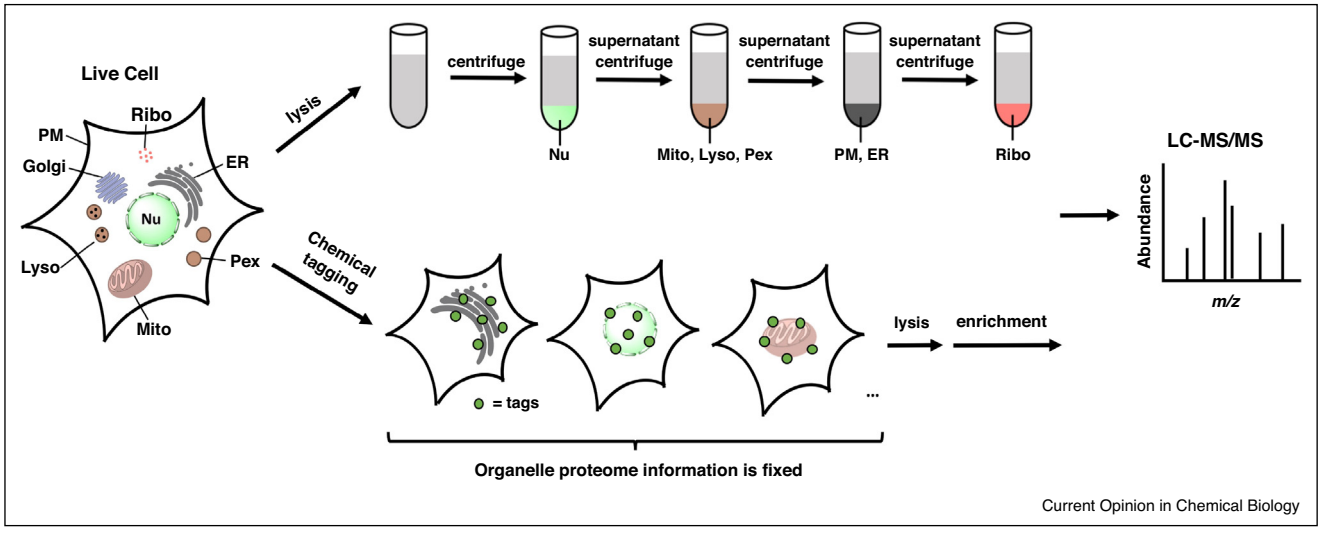
In this review, we introduce the recent progress of chemical proteomics that focus on subcellular compartments, including organelle-focused proteomics and conditional proteomics (Figure 1).

Organelle-focused proteomics

Organelle-specific ABPP

Activity-based protein profiling (ABPP) represents one of the most successful examples of chemical proteomics, which was invented by Cravatt's group [8,9]. Proteins exhibiting an enzymatic activity-of-interest are selectively labeled by activity-based probes (ABPs) through a bioorthogonal reaction. For organelle-specific ABPP, Wright and co-workers developed a lysosome-targeting ABP by conjugating a weakly basic amine (DAMP) to a cathepsin-reactive warhead [10•]. Liquid chromatography (LC)–MS/MS analysis of labeled macrophages showed an increased enzyme activity of cathepsins B and Z during starvation-induced autophagy.

Figure 1



Organelle-focused proteomics workflow. Top: conventional methods isolate cell organelles by subcellular fractionation after cell lysis. Bottom: chemical proteomics can fix organelle proteome information by chemical tagging before cell lysis. *Abbreviations*: Nu, nucleus; Mito, mitochondria; Lyso, lysosomes; Pex, peroxisomes; PM, plasma membrane; ER, endoplasmic reticulum; Ribo, ribosomes.

Proximity proteomics

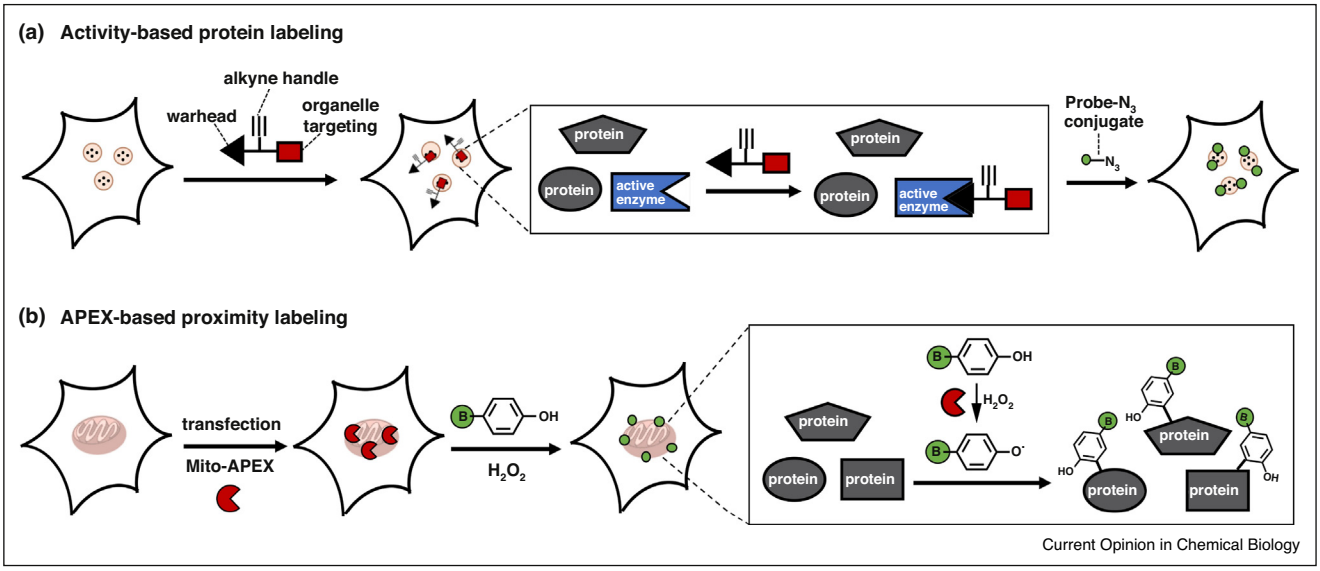
In the past few years, several groups have independently developed a class of methods termed 'proximity labeling' for protein network mapping [11–14]. The basic strategy exploits engineered peroxidase or biotin ligase to generate highly reactive and short-lived reagents that covalently tag proteins with biotin in the vicinity (within nanometers) of the enzyme. For organelle-focused proteomics, the enzyme is directed to specific cellular compartments, using genetically encodable organelle localization signals. Ting and co-workers explored the engineered ascorbate peroxidase (APEX or APEX2) in proteome profiling of various organelles and even organelle subcompartments, such as sub-mitochondrial spaces, ER lumen and membranes [15•,16–19]. It now turns out that APEX-based organelle proteomics is powerful to uncover novel components of organelles and determine the subcellular localization of unknown proteins. Recently, the same group used the engineered horseradish peroxidase in live neurons and characterized the proteomes of excitatory and inhibitory synaptic clefts [20], many of which are membrane-unbounded and biochemically unpurifiable. The resultant proteome lists are highly specific to synaptic clefts with high coverages, where new synaptic proteins were discovered and subtype localizations of some of synaptic proteins were revealed. In addition, APEX-based organelle proteomics can serve as spatially specific references, for example, endosome and plasma membrane, to reveal protein interaction networks that necessitate higher spatial resolution [21] (Figure 2).

Organelle-localizable reactive molecules

In parallel, we developed a new method for organelle-focused proteomics that does not require genetic manipulation and is not limited to active enzymes as targets. The organelle-localizable reactive molecules (ORMs) composed of an organelle-localizing moiety and a chemically reactive moiety were designed in this technology. ORMs can penetrate biomembranes and spontaneously accumulate in target organelles of live cells, where the protein labeling is facilitated by the condensation effect [22•,23••]. In the case of mitochondria-localizable reactive molecules (MRMs), for example, the concentrations of inside cells are 4 to 21-fold higher than those extracellularly added [23••] (Figure 3).

For nucleus-focused proteomics, nucleus-localizable reactive molecules (NRMs) were firstly prepared [22•], in which the Hoechst acts as a molecular vehicle to the nucleus [24,25] and chloroacetyl is employed as a reactive handle that shows potential reactivity to cysteine. Incubation of HeLa cells with NRMs allowed for their selective localization in the cellular nucleus as observed by CLSM imaging, and plenty of labeled proteins were detected by SDS–PAGE and Western blotting (WB) analysis. The labeled proteins were enriched by immunoprecipitation using anti-Hoechst antibody, and characterized by mass proteomics. 67 proteins were identified in total, among which 58 proteins (87%) were assigned as nucleus-localized proteins. This first report successfully demonstrated the feasibility of our strategy for organelle-focused proteomics by ORMs.

Figure 2



Organelle-focused chemical labeling for proteomics. **(a)** Activity-based protein labeling. ABPs are guided to target organelles and label a limited number of active enzymes. **(b)** APEX-based proximity labeling. APEX is engineered to be selectively expressed in target cellular compartments. Biotin-phenol and H_2O_2 are added to initiate the labeling. *Abbreviation*: B, biotin.

Since ORMs are modular, proteomes of other organelles can be analyzed by varying the organelle-localizing moiety and their chemical reactivity. In MRMs, for instance, tetraethyl-rhodamine (Et4-Rhod) was employed for targeting mitochondria [26] and various reactive functionalities exhibiting distinct amino acid preference [27] were tethered as the reactive moiety [23••]. Rapid and spontaneous localization of MRMs in mitochondria were observed by CLSM. The labeled proteins could be detected by *in-gel* fluorescence imaging, interestingly which showed distinct band patterns by different labeling reagents. Further, LC–MS/MS analysis determined the Et4-Rhod-modified peptides, clearly indicating the amino acid preference of these reactive moieties in live cells. Annotation analysis of the whole labeled proteins revealed that 69% are assigned to mitochondrial proteins that mainly localize in matrix and inner membrane of mitochondria.

Our organelle-focused chemical labeling is free of pretreating cells with gene transfection, thus, it is supposed to be applicable to sensitive and complicated biological samples that are not easily amenable to exogenous gene manipulation [28]. We explored the use of MRMs in primary cultured neurons and acutely prepared mouse brain slices. Among the whole identified proteins, ~61% and 64% were assigned to mitochondrial proteins for cultured neurons and brain slices, respectively.

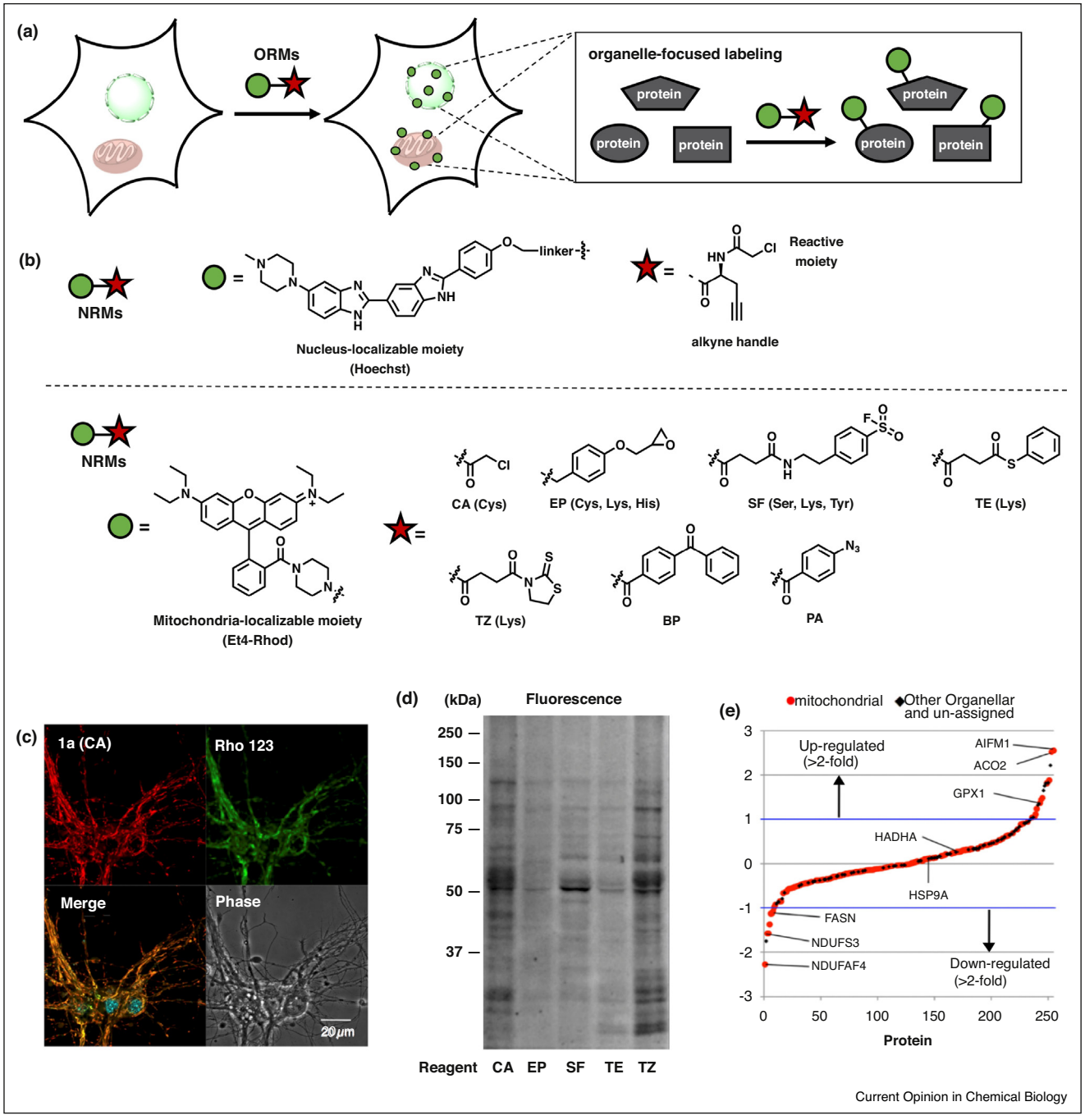
Finally, the MRM approach was combined with quantitative MS technique, SILAC (stable isotope labeling by amino acids in cell culture) [29], to explore the dynamics of mitochondrial proteome of HeLa cells during an apoptosis process induced by paraquat (PQ)-mediated oxidative stress [30,31]. In PQ-treated cells, 65% of identified proteins were classified into mitochondrial proteins. Of the quantified proteins, 21 proteins were up-regulated by more than 2-fold in PQ-treated cells and 7 proteins were down-regulated by more than 2-fold. Among such significantly changed proteins, some have been established to regulate oxidative stress and apoptosis, while some were discovered for the first time, for example, up-regulated AIFM1 and down-regulated NDUFAF4 which are supposed to be associated with mitochondrial diseases. This new finding clearly demonstrated that the MRM-based method is useful for identification and quantification of key proteins in dynamically dysfunctioned mitochondria.

Conditional proteomics

As described above, organelle-focused chemoproteomic approaches enabled to profile protein localizations, the dynamically altered expression levels of organelle proteins and these activities. Recently, we reported a new method that can explore an 'environment' in which proteins exist in live cells [7••].

It is now discussed that Zn (II) ion (Zn^{2+}) acts as a signaling molecule inside and outside cells, and the local concentration of Zn^{2+} dynamically changes. Zinc signaling and dynamics play significant roles in many physiological responses and diseases. To decipher the physiological roles

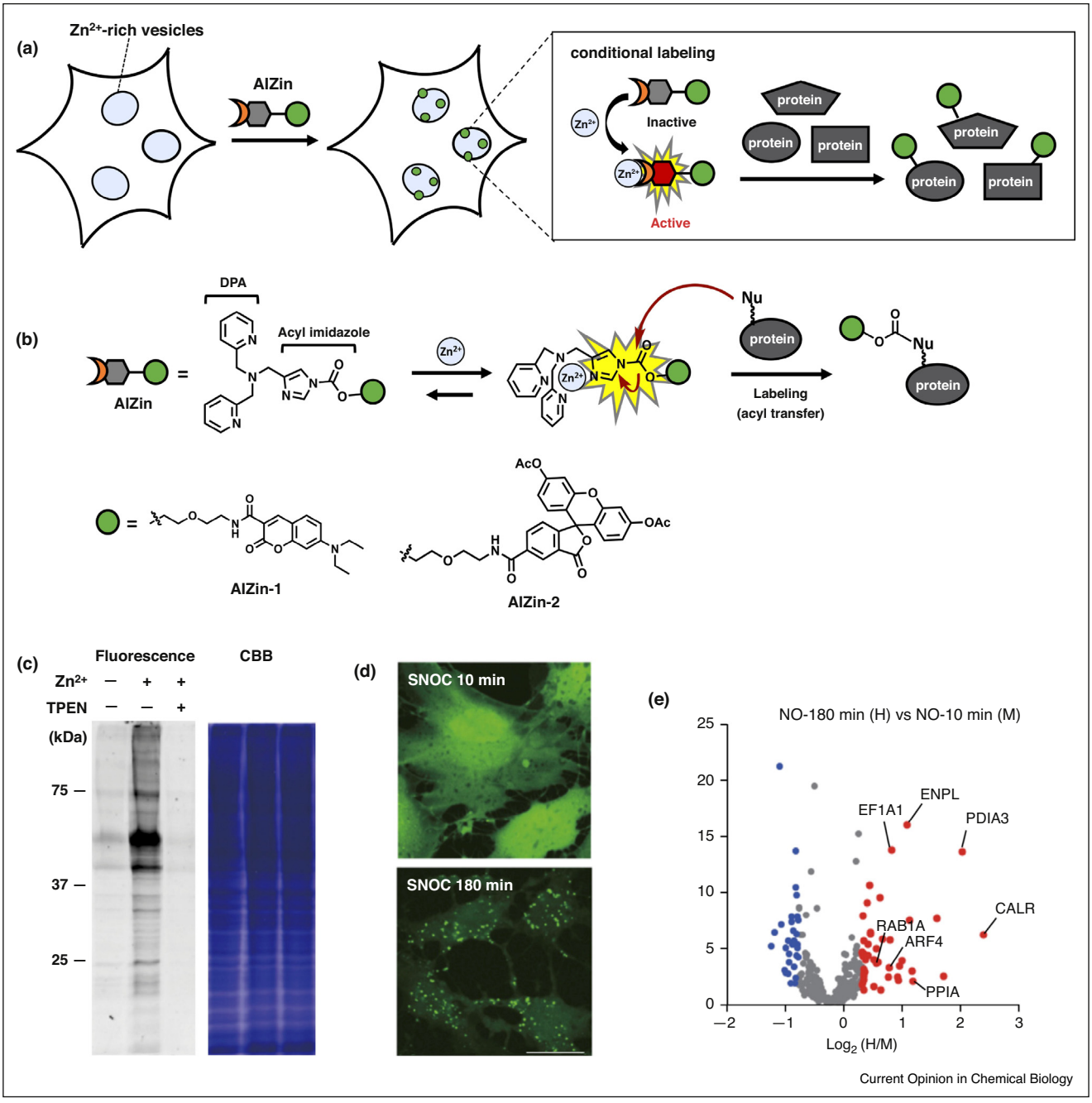
Figure 3



Organelle-focused proteomics by organelle-localizable reactive molecules (ORMs). **(a)** ORMs-based organelle labeling. **(b)** Structures of NRMs and MRMs. *Abbreviations*: NRMs, nucleus-localizable reactive molecules; MRMs, mitochondria-localizable reactive molecules; Et4-Rhod, tetraethylrhodamine; CA, chloroacetyl; EP, epoxide; SF, sulfonyl fluoride; TE, thiophenyl ester; TZ, thiazolidinethione; BP, benzophenone; PA, phenylazide. **(c)** Colocalization of CA with Rhodamine 123 in neurons. Blue staining: Hoechst. **(d)** Distinct labeling patterns by different MRMs in brain cerebrum slices. **(e)** Dynamic change of mitochondria proteome upon PQ stimulation in HeLa cells. The variation in expression levels of highlighted proteins was validated by Western blotting analysis.

of Zn^{2+} in detail, comprehensive characterization of proteins under high concentration of mobile Zn^{2+} is crucial. Toward this end, we developed a conditional proteomics approach to identify Zn^{2+}-associated proteins [7••]. This method relies on designer labeling reagents (AIZin) that are selectively activated in response to Zn^{2+} concentration. AIZin is comprised of a Zn^{2+}-binding site, dipicolylamine (DPA), conjugated to an electrophilic reactive group, acyl

Figure 4



Conditional proteomics by Zn^{2+}-responsive chemical labeling. **(a)** Zn^{2+} conditional protein labeling. **(b)** Schematic illustration of Zn^{2+}-promoted protein labeling. *Abbreviations*: DPA, dipicolylamine; FL, fluorophore; Nu, nucleophilic amino acid. **(c)** *In-gel* fluorescence imaging of Zn^{2+}-responsive protein labeling in HeLa cell lysates. **(d)** Imaging of NO-induced zinc segregation process by FluoZin-3 AM in C6 cells. **(e)** Time-dependent change of zinc-associated proteome in SNOC-treated C6 cells. The highlighted proteins were validated, showing complete or partial colocalization with zinc distribution.

imidazole (AI), which was originally developed by our lab for ligand-directed chemical labeling of proteins [32,33]. Upon Zn^{2+} coordination with DPA and closely connected AI, the electron density of the imidazole ring is reduced, which enhances the reactivity of the carbamate unit of AI for covalent conjugation with nucleophiles on surrounding proteins.

The reactivity of AI moiety in AIZin-1 is enhanced by 23 folds upon zinc complexation, and the activation was

selectively induced by Zn^{2+} among several metal cations. In the analysis of Zn^{2+}-responsive protein labeling in HeLa lysates, *in-gel* fluorescence imaging showed intense fluorescent bands over the whole molecular weight range in the presence of Zn^{2+}. This labeling disappeared when depleting Zn^{2+} by TPEN, clearly demonstrating the Zn^{2+}-dependent protein labeling. The labeling sites cover a wide range of nucleophilic residues, including Lys, Ser, Thr, Tyr, and His. For live-cell labeling, we applied AIZin-2, which has a diacetyl fluorescein as the tag moiety and can spontaneously penetrate live cell membranes. This probe uniformly distributed inside cells and can be activated in response to an increase in Zn^{2+} levels.

We applied our AIZin-based conditional proteomics to clarify zinc homeostasis under NO-triggered oxidative stress in glioma cells. Without stress, most of Zn^{2+} is bound to metalloproteins and mobile Zn^{2+} is negligible [34]. Upon exposure to NO, NO-mediated S-nitrosylation of metalloproteins causes the rapid release of mobile Zn^{2+} in live cells [35]. It is reported that excess Zn^{2+} are sequestered in small cytoplasmic vesicles [36,37], however, these Zn^{2+}-rich vesicles have remained largely uncharacterized. In our experiment, C6 glioma cells were treated with AIZin-2 after stimulation with S-nitrosocysteine (SNOC), a NO donor, for 0, 10, or 180 min. Fluorescent bands indicating labeled proteins in whole cell lysates dramatically increased after NO stimulation. The labeled proteins were captured by immunoprecipitation and analyzed by TMT (tandem mass tags)-based quantitative MS technique [38], by which 331 proteins were identified and quantified in total. Interestingly, the labeled proteome changed remarkably in a NO stimulation time-dependent manner, revealing reduced enrichment of nuclear proteins and increased enrichment of vesicle and endoplasmic reticulum (ER) proteins from 10 min to 180 min incubation after NO stimuli. The most enriched proteins, such as calreticulin, peptidyl–prolyl isomerase, and endoplasmin, colocalized with the Zn^{2+}-rich vesicles imaged by FluoZin-3, which validated our MS proteomics results. The detailed analysis of our proteomics data and colocalization imaging finally allowed for characterizing the unknown zinc-rich vesicles generated by oxidative stress as ER-related and Golgi-related vesicles (Figure 4).

Conclusions and perspectives

Chemical proteomics has been proved powerful for subcellular proteome analysis. It does not rely on conventional organelle purification and can provide detailed insights into the microenvironments of diverse subcellular compartments. Proximity labeling, especially by APEX, have been successfully applied to high-resolution proteome mapping restricted in specific organelles and even organelle sub-compartments. The two types of chemical proteomics developed by our group, using ORMs or AIZin, do not require the expression of engineered fusion proteins and thus these complement genetically encoded methods such as proximity labeling.

Given the modular structure of ORMs, we envision the extension of our organelle-focused-labeling strategy to other organelles such as ER and lysosomes by varying the localizable moiety. After proteome analysis within one individual organelle, multi-organelle proteomics may be carried out by a couple of ORMs to investigate protein trafficking between organelles. As for AIZin, there is still a big scope of applying it to identify the composition of Zn^{2+}-rich environments, such as Zn^{2+} spark in fertilization, synaptic vesicles and insulin granules, that play many crucial roles in biological zinc signaling. Like ORMs, the conditional proteomics could be further developed to target other conditions, such as other metals and reactive oxygen species, by metal-coordination-enhanced reactivity (like AIZin) or stimuli-activated unmasking of latent electrophiles. Furthermore, our conditional labeling for imaging (in both live and fixed cells) and proteomics hold great potentials on applications to tissues and whole animal bodies, by which protein localization and dynamics associated with cell types would be elucidated in molecular details.

Conflict of interest statement

Nothing declared.

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