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Perspective

Lighting Up and Identifying Metal-Binding Proteins in Cells

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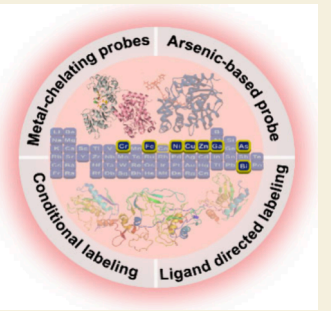
ABSTRACT: Metal ions, either essential or therapeutic, play critical roles in life processes or in the treatment of diseases. Proteins and enzymes are involved in metal homeostasis and the action of metallodrugs. Imaging and identifying these metal-binding proteins will facilitate the elucidation of metal-mediated life processes. The emerging research field of metallomics and metalloproteomics has significantly advanced our understanding of metal homeostasis and the roles that metals play in biology and medicine. Fluorescence-based metalloproteomics offers the possibility of not only visualization but also identification of metal-binding proteins in living cells and tissues. Herein, we summarize different strategies of labeling and tracking of metal-binding proteins with the aid of fluorescent probes. We highlight several examples as showcases of how this fluorescence-based metalloproteomics approach could be utilized in metallobiology and chemical biology. In conclusion, we also discuss the advantages and limitations of fluorescence-based metalloproteomics approaches and point out future directions of metalloproteomics including development of more sensitive and selective fluorescence probes, integration with other omics approaches, as well as application of emerging advanced super-resolution imaging techniques that utilize fluorescent molecules or proteins. We aim to attract more scientists to engage in this exciting field.



KEYWORDS: *metallomics, metalloproteomics, fluorescence imaging, cell biology, chemical biology*

1. INTRODUCTION

Metal ions are vital for microbes, animals, and humans, participating in numerous biological processes e.g. catalytic and electron transfer reactions in cells, primarily as cofactors. Their dysregulations are closely associated with various diseases.[1] For example, elevated iron contents in the tissue and accumulation of copper and zinc in the amyloid are characteristics of neurodegenerative diseases.[2] Increasing evidence show that metal ions also play important roles in innate and adapted immunity.[3] For example, it has been demonstrated recently that serological metals or metalloproteins are closely correlated with immunity in COVID-19 patients, providing an insight into the roles of metals/metalloproteins in immunity and disease pathogenesis as well as intervention.[4,5] To ensure the bioavailability of metal ions and to avoid toxicity of excess metal ions, metal ions must be tightly regulated through binding to a battery of proteins, namely, metalloproteins, involved in processes of uptake, storage, and secretion. About a quarter to one-third of the human proteome are metalloproteins with intrinsic metals (Fe, Zn, Co and Cu etc.) serving as catalytic, regulatory and structural roles, which are critical to protein functions.[6,7] Therefore, identification and analysis of a complete set of metalloproteins are needed to achieve a better understanding of the regulatory roles and functions of metals in biology. Moreover, metal ions are also incorporated into pharmaceuticals as diagnostics and therapeutics.[8−10] Given that metal ions are tightly regulated by proteins, mining the proteins involved in metal homeostasis or metallodrug response is of significance. In particular, proteome-wide tracking of metal- binding proteins, which could be metalloproteins or nonmetalloproteins, will facilitate unveiling their molecular mechanisms of action,[11,12] allowing new druggable targets to be identified for rational design of more potent drugs.[13]

How exactly metal ions take part in the life processes through binding to a variety of proteins at the system level is an extremely complicated issue to be addressed. This is due to complex metal-protein interactions in living systems as well as varied oxidation states and coordination numbers of a metal ion. Recently, metallomics has emerged as a new research field, addressing metals and metalloids within a cell or tissue/organ, in particular metalloproteomics, which focuses on the large-scale study of metals and their binding proteins, and offers an invaluable tool to investigate the role of metals in cell biology and disease processes[14,15] as well as in the elucidation of the molecular mechanism of metallodrugs.[16,17] However, there are considerable challenges to track proteins that bind to metallodrugs or metals, particularly in live cells, as the interaction of metals/metallodrugs with proteins *in vivo* can

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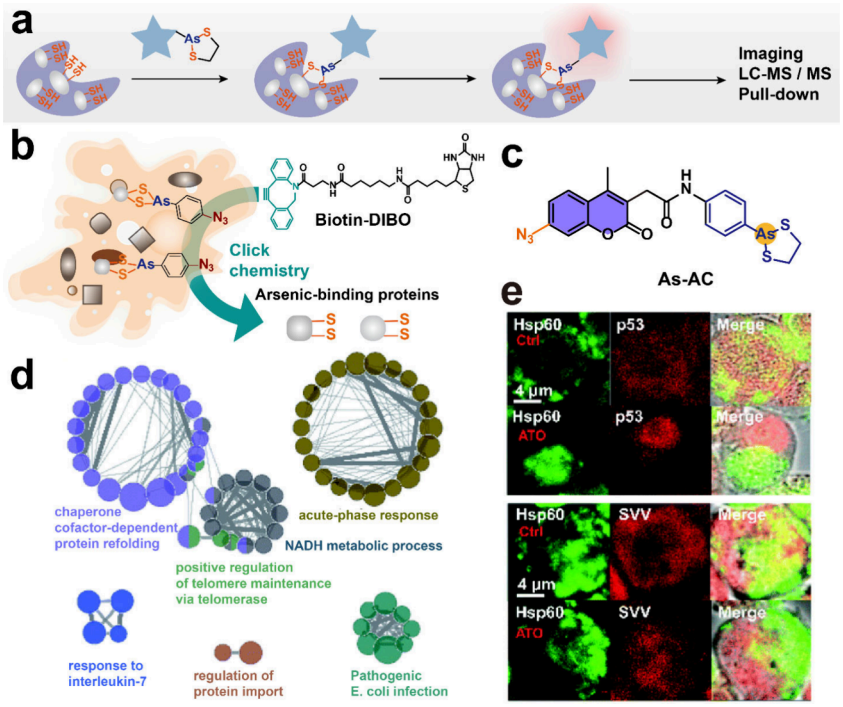

Figure 1. (a) Diagram illustrates the binding of As(III) to thiols in a protein. (b) Identification of the captured arsenic-binding proteins by PAzPAO. Adapted with permission from ref 39. Copyright 2016 Wiley-VCH. (c) Chemical structure of organoarsenic probe **As-AC**. (d) GO enrichment analysis of 37 As-binding proteins detected by **As-AC** probe. (e) Immunolocalization of endogenous Hsp60 (green) with p53 (red) and survivin (red), showing the disruption of the protein−protein interactions upon ATO treatment in cell. (d, e) were reproduced with permission from ref 40. Available under Creative Commons Attribution 4.0 International License.

be weak or even transient. Over the last two decades, different metalloproteomic approaches have been established and applied in the research related to metallobiology.[18] For example, the separation and detection-based GE-ICP-MS[19] and LC-GE-ICP-MS[20,21] have facilitated the uncovering of the molecular mechanism of action of different metallodrugs. However, such methods are not applicable for tracking proteins that bind to metal ions weakly. Alternative strategies are needed for mining these metal-binding proteins such as fluorescence-based metalloproteomic approaches.[12,18] It shall be pointed out that metalloproteins are metal-binding proteins with metal ions playing catalytic or structural roles, while metal-binding proteins are not necessary to be metalloproteins. Recently, a novel approach has been reported to identify metalloproteins (or meta-binding proteins) via thermal proteome profiling,[22] for which metal-binding proteins were first extracted by chelators such as EDTA, followed by monitoring the changes of protein stability via thermal proteome profiling. Such a method may provide a rich source of information for functional studies of metals in biology and medicine.

Fluorescent imaging has long been recognized as a powerful method for visualizing proteins or other biomolecules in cell and chemical biology. Labeling a protein with small molecular probes is a noninvasive imaging method in living cells without perturbing its function. Over the last decades, tremendous efforts have been made in the development of small molecule based fluorescent probes for labeling proteins, enabling the discovery of metalloproteins and analysis of their expression levels, and regulatory and functional roles.[23−26]

In this review, we mainly focus on visualization and identification of metal-binding proteins using fluorescence-based approaches via unique florescence probes. We highlight the arsenic-based and ligand-based probes as well as conditional labeling as showcases to demonstrate the applicability and utility of such a methodology in the research of metallobiology and cell biology. We discuss the advantages and limitations of these techniques and also point out the future direction of the metalloproteomics research field.

2. ARSENIC-BASED FLUORESCENT PROBES

Arsenic trioxide (ATO) is an ancient Chinese medicine that is highly effective in treating patients with acute promyelocytic leukemia (APL), with very low toxicity. According to registry data from the European Leukemia Net, >90% of patients achieved remission after treatment with ATO. Additionally, the survival rate of APL patients has also improved with the use of ATO in combination therapy.[27] In the year 2000, the U.S. Food and Drug Administration (FDA) approved the use of ATO for treating APL patients.[28] Therefore, ATO has attracted the attention of chemists worldwide. Since then, many arsenic-based anticancer agents and drugs have been developed. For example, S-dimethylarsino-glutathione (Darvias), an organic arsenic drug composed of a dimethylated arsenic linked to glutathione, exhibits significantly improved anticancer properties against a variety of tumors *in vitro* and *in vivo* including APL[29] and has been developed by Solasis Pharma K.K for the treatment of relapsed or refractory peripheral T-cell lymphoma (PTCL), and its intravenous formulation has been approved in Japan.[30] Its mechanism of action is incompletely understood.[31,32] The identification of arsenic-binding proteins is a prerequisite for understanding of the role of arsenic in biology and for the development of arsenic-based therapeutics. Arsenic(III) is known to have a

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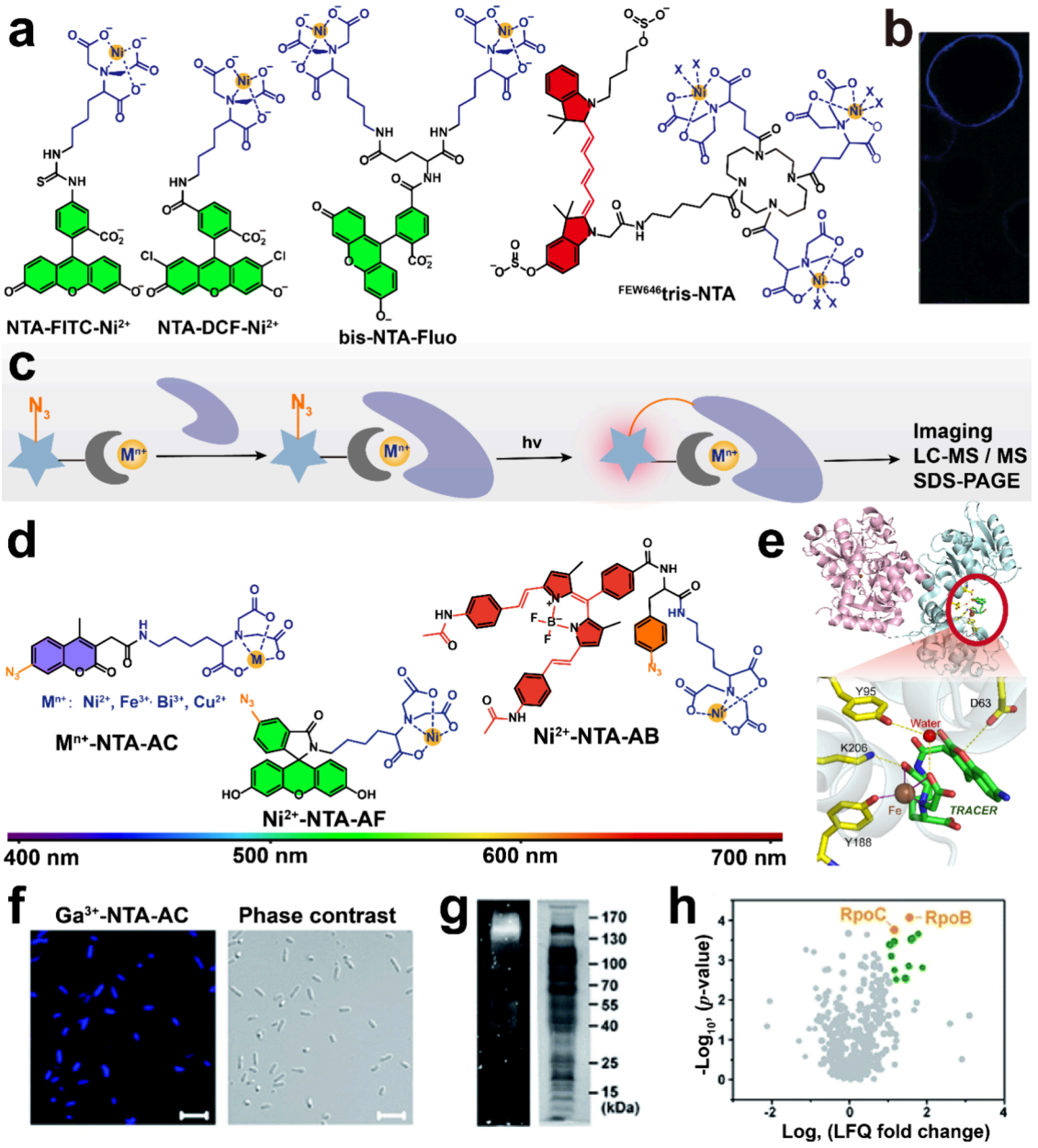

Figure 2. (a) Chemical structures of **NTA-FITC-Ni^{2+}**, **Bis-NTA-Fluo**, and ^{FEW646} tris-NTA. (b) Confocal image of ^{FEW646} tris-NTA labeling a plasma membrane protein. Adapted with permission from ref 55. Copyright 2016 American Chemical Society. (c) Schematic diagram of photoaffinity labeling of protein by *metal*-NTA based fluorescence probes. (d) Chemical structures of Ni-NTA-based photoaffinity labeling probes with NTA conjugated with different fluorophores. (e) Cartoon representation of transferrin with the **Fe^{3+}-TRACER** binding site at the N-lobe (*Light blue*). The binding site is enlarged with **Fe^{3+}-TRACER** interacting residues shown in sticks. Reproduced with permission from ref 58. Copyright 2018 the Royal Society of Chemistry. (f) Confocal imaging of *P. aeruginosa* cells treated with **Ga^{3+}-NTA-AC** (also known as **Ga(III)−TRACER**). (g) SDS-PAGE separation of *P. aeruginosa* cell lysate showing **Ga^{3+}-NTA-AC** labeled proteins. (h) Volcano plot showing the fold change and significance of protein intensities detected in the competitive Ga-IMAC experiment. Nodes in green color represent proteins with fold change >2 and *p*-value (of fold change) < 0.01. (f,g,h) were reproduced with permission from ref 60. Available under Creative Commons Attribution 4.0 International License.

high affinity for thiol groups, which provide an ideal protein binding site for arsenic-related drugs (Figure 1a). However, identifying arsenic-related proteins at the proteome-wide scale is of tremendous challenge because of the low abundance of cysteine in proteins (approximately 2.3% of the proteome)[33] and oxidative modifications of thiols in cysteine, such as sulfenylation, nitrosylation, and glutathionylation, which significantly increase the difficulty in the identifying arsenic-binding proteome.

Genetically Tagged Proteins

Vicinal dithiol-containing proteins (VDPs) are a class of proteins that contain at least one pair of closely positioned thiol groups in their reduced state.[34] The cyclic complex formed by As(III) and two closely arranged thiols is more stable than the linear structure formed by As(III) and a single thiol, which has led to a shift in the mainstream research trend.[35] Ethanedithiol (EDT) is known to induce quenching of fluorescent probes through facilitating rotation of the aromatic

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arsenic bond and vibrational deactivation or photoinduced electron transfer. However, after undergoing a thiol exchange reaction with the thiols in VDPs, this more rigid conformation would hinder the conjugation of the arsenic lone pair electrons with the orbitals of the probe, thereby terminating the fluorescence quenching (i.e., enhancing the fluorescence). A library of membrane-permeant fluorescence probes based on biarsenical(III) has been designed and modified, offering great versatility in protein attachment sites and the in situ visualization of proteins of interest. Since 1998, Roger Tsien and colleagues reported a group of bisarsenical *on-off* fluorescent probes, FLAsH-EDT2 and ReAsH-EDT2 (EDT = ethanedithiol), which can site-specifically recognize proteins fused with tetra-cysteines, which are present uncommonly in natural proteins.[36,37] To achieve higher absorbance, quantum yield, and greater photostability, halogen-substituted bisarsenical probes have been developed, such as F4FlAsH-EDT_2 and Cl4FlAsH-EDT_2.[38] These probes become ideal tools for tracking proteins of interest by constructing FRET complexes. However, introducing the tetracysteines (TC) sequence into native protein of interest might result in a change of redox property of the protein. VDPs can be considered as a part of the TC sequence and play a crucial role in biological activities. Therefore, monoarsenical probes developed for detecting VDPs have gained the attention of researchers.

Natural/Untagged Proteins

In 2016, p-Azidophenylarsenoxide (PAzPAO) was designed as a novel and compact arsenic "bait", which captured arsenic-associated proteins *in situ* and subsequently labeled these proteins using a copper-free click chemistry reaction within live cells.[39] Through enrichment and identification using shotgun proteomics, 48 arsenic-binding proteins were tracked in A549 cells. This approach utilizes the compact para-azidophenyl arsine oxide (PAzPAO), circumventing the interference of arsenic-biotin conjugates with endogenous biotin metabolism pathways within cells and providing valuable insights into the potential therapeutic targets of arsenic and key proteins responsible for arsenic health effects (Figure 1b).

In 2021, a new organoarsenic fluorescent probe, **As-AC** (Figure 1c), was designed and synthesized via conjugation of an arsenic moiety with a fluorophore and an arylazide. An arylazide was incorporated into the probe, allowing the protein of interest to be anchored to the probe upon photoactivation, and subsequently identified via high-throughput proteomics.[40] By using the fluorescence based metalloproteomics via **As-AC** together with quantitative proteomics, 37 arsenic-binding proteins and 250 arsenic-regulated proteins were identified in the human acute promyelocytic leukemia (APL) cell line NB4 (Figure 1d). Bioinformatics analysis unveiled multiple physiological processes being disrupted by ATO, in particular, chaperone-related protein folding and cellular response to stress. Among the identified arsenic-binding proteins, a heat shock protein 60 (Hsp60) was demonstrated to be a vital target of ATO. The binding of arsenic trioxide to Hsp60 disrupts the formation of Hsp60-p53 and Hsp60-survivin complexes, leading to the degradation of p53 and survivin (Figure 1e). This study provides significant insights into the mechanism of action of ATO at a system level and offers a guidance for rational design of arsenic and other metal-based anticancer drugs.

3. METAL CHELATING LIGAND-BASED LABELING

Affinity labeling based on metal chelation is a promising and attractive method due to the stable binding-site and high selectivity to metalloproteins.[41] These approaches have shown broad application perspectives in biological labeling, disease diagnosis, and molecular imaging.

Hexahistidine/Nickel-Complex System

Genetically Tagged Proteins. Hexahistidine tags (His_6-Tag) are one of the most commonly used tags in biotechnology for purification of recombinant proteins. Its minimal size has less effect on the protein itself or downstream applications. Moreover, a His_6-Tag fused to the N-terminus of a protein is compatible with bacterial transcriptional translation mechanisms, making it advantageous for protein expression. The His-tagged proteins can be purified by single-step Immobilized Metal Affinity Chromatography (IMAC), with Ni-NTA being the mostly widely used matrices.[42]

Given the large library of His_6-tagged proteins and the wide utility of His-Ni^{2+}-NTA (Ni^{2+}- nitrilotriacetate NTA) system in molecular biology and biotechnology, it of significant interest to image His-tagged proteins using Ni-NTA based fluorescence probes in order to further understand their cellular events, such as localization and expression levels inside cells. Enormous efforts have been made to site-selectively label His-tagged proteins via conjugation Ni-NTA with fluorophores.[43−49] However, most of this type of probes could be only used to label membrane His-tagged proteins.[50−52] One of the serious limitations for labeling protein using Ni^{2+}-NTA-His-tag system is the relatively low affinity of His-tag to Ni^{2+}-NTA complex (K_d values generally lie in the range of 1−20 μM), which may disturb stable labeling and imaging. To overcome this drawback and also to mimic previously reported arsenic-based fluorescence probe, FlAsH,[36,37] different NTA-based fluorescence probes with a fluorophore conjugated to multiple NTA molecules have been made (Figure 2a).[44,53−55] However, the highly negative charges of these moieties prevent these probes from entering cells, limiting their applications in labeling intracellular proteins (Figure 2b).[48,55]

To address this issue, we have designed a battery of Ni-NTA-based photoaffinity labeling (PAL) fluorescent probes (Figure 2c). We conjugated Ni-NTA with different fluorophores as well as an arylazide, which serves as the photoaffinity group to strengthen the binding between the probe and a protein of interest, resulting in a series of PAL fluorescent probes (Figure 2d).[25,56,57] These probes oriented by Ni-NTA bind specifically to a protein with a genetically fused His_6-tag and subsequently could be anchored to the labeled protein upon UV activation of arylazide, leading to significant fluorescence turn-on. Importantly, these probes could rapidly enter live cells and even plant tissues to light up His-tagged proteins, offering a new opportunity for labeling a large library of His_6-tagged proteins in both prokaryotic and eukaryotic cells.

Natural/Untagged Proteins. Significantly, the ability of NTA to bind to multiple metals expands the application of such probes. We developed a membrane-permeable, metal tunable fluorescent probe, namely **M^{n+}-NTA-AC** or **M^{n+}-TRACER**, by substitution of Ni^{2+} with other metal ions e.g. Bi^{3+}, Ga^{3+}, Cu^{2+}, Fe^{3+}, Cr^{3+}, that bind NTA with moderate to high affinity. The remaining open coordination sites of **M^{n+}-NTA** are available to bind to a target protein, providing a noncovalent capture approach to protein labeling. The

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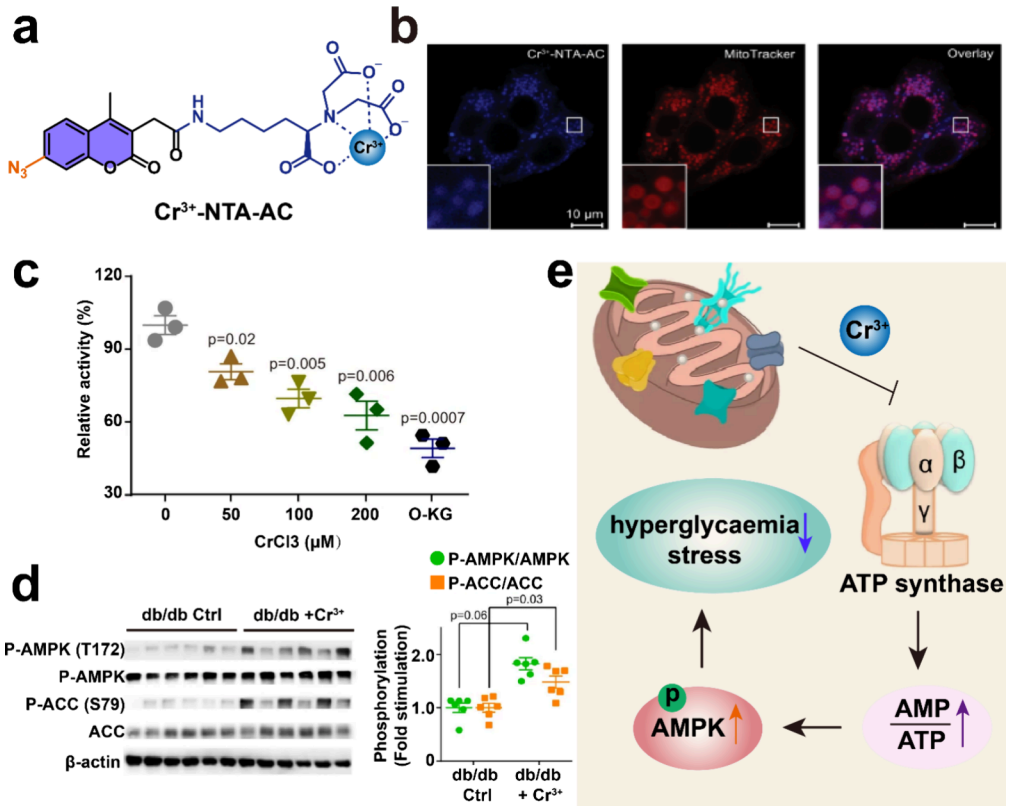

Figure 3. (a) Chemical structure of chromium probe **Cr^{3+}-NTA-AC**. (b) Colocalization confocal images of **Cr^{3+}-NTA-AC** in mitochondria. (c) Dose-dependent inhibition of ATP synthase activity by Cr^{3+} treatment in HepG2 cells under hyperglycaemia condition. ATP5B inhibitor Octyl-α-ketoglutarate (O-KG) is used as a control. $n = 3$; mean ± SEM (d) Cr^{3+} activates AMPK and ACC in db/db mice. $n = 6$; mean ± SEM (e) The proposed scheme shows that Cr^{3+} ameliorates hyperglycemia stress by inhibiting ATP synthase and subsequent activation of AMPK in diabetic mice. All images were reproduced with permission from ref 26. Available under Creative Commons Attribution 4.0 International License.

introduction of arylazide resulted in not only significant florescence enhancement (for **M^{n+}-TRACER**) but also downstream protein identification through formation of covalent bonds with labeled proteins upon UV activation. By integration of fluorescence imaging with a proteomics approach, different endogenous metal-binding proteins or metalloproteomes have been tracked in cells.[11,58,59]

Gallium(III)-based drugs have gained widespread clinical application as antimicrobials, and several gallium-based drugs have been developed to combat the crisis of antimicrobial resistance. However, the precise mechanisms of action of gallium in bacterial cells remain largely unknown.[60] By using **Ga^{3+}-TRACER** based metalloproteomics, together with metabolomics, and transcriptomics, two gallium-binding proteins, RpoB and RpoC in *Pseudomonas aeruginosa* were identified (Figure 2f, 2h, 2g). Ga(III) inhibited RNA synthesis was found by specifically targeting RNA polymerase, thereby disrupting metabolic activity and energy utilization. Moreover, exogenous supplementation of acetate salts enhances the antibacterial activity of Ga(III) compounds through the augmentation of Ga(III) absorption, reduction of the TCA cycle flux, and suppression of bacterial respiration. Novel mechanistic insights into the antibacterial activity of Ga(III) were found, leading to a safe and practical combination therapy strategy to be proposed, i.e., use of metabolites to enhance the efficacy of Ga(III)-based antibacterial agents against antimicrobial resistance (AMR).

The specific recognition of metalloproteins by the probe was also demonstrated by **Fe^{3+}-TRACER** and human serum transferrin.[35,58] **Fe^{3+}-TRACER** binds to specific iron binding site in the N-lobe of transferrin, with Fe^{3+} coordinating to Y188, while D63, Y95 and K206 form H-bonding to stabilize the structure, as revealed from the X-ray structure of the ternary complex. Interestingly, the probe folds into a sandwich structure, and Fe^{3+} coordinates to only two carboxylates and one nitrogen of the probe, leaving sufficient vacant sites for interacting with proteins (Figure 2e). The strategy opens a new horizon to image as well as to identify endogenous metalloproteins at a proteome-wide scale, providing a systematic platform for our understanding the role of metals in biology and medicine.

One of the significant advantages of this probe lies in its capability of tracking proteins that bind metal ions weakly or transiently, owing to the formation of covalent bonds between the probe and target protein upon photoactivation of the arylazide. This was evidenced by a showcase study for mining Cr(III)-binding proteins in cell (Figure 3a).[26] Cr(III) was shown to be able to increase blood-glucose removal in mice over 50 years ago;[61] subsequently, it has been extensively used either as a supplement for the treatment of diabetes mellitus or as a nutritional supplement for weight loss and muscle development. Despite decades of effort, the biomolecules responsible for its pharmacological effects are unclear owing to the failure in identification of its molecular target. Conventional separation and detection methods led to no Cr(III)-binding proteins being identified, possibly attributable to the dissociation of Cr(III) from its binding proteins during separation. By combining fluorescence imaging via **Cr^{3+}-**

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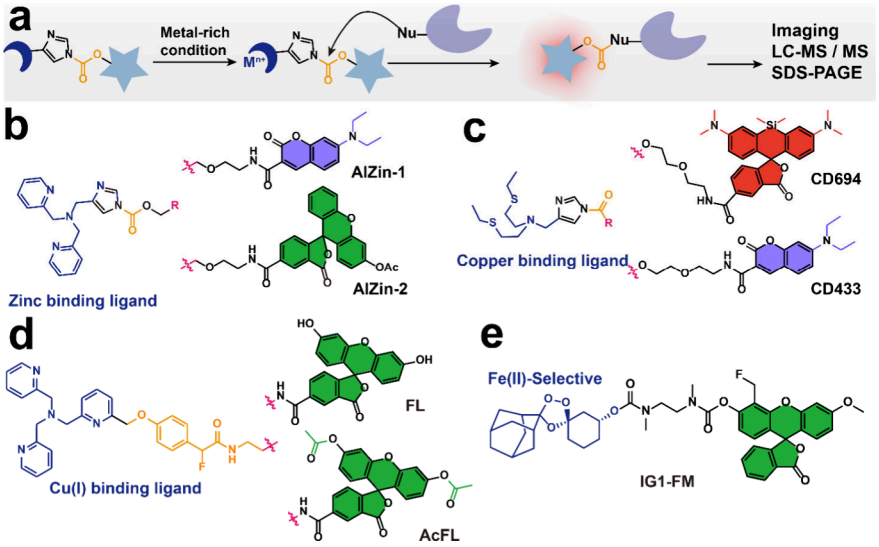

Figure 4. (a) Schematic diagram illustrating conditional protein labeling. (b) Chemical structures of Alzin-1 and Alzin-2. (c) Chemical structures of CD694 and CD433. (d) Chemical structure of Cu(I) ligand (*Blue*) with cross-linking moiety quinone methide (*Orange*) and fluorophores FL and AcFL. (e) Chemical structure of **IG1- FM**.

NTA-AC with a high-throughput proteomic approach, we could visualize Cr(III)-binding proteins, which are mainly localized in mitochondria (Figure 3b). Eight Cr(III)-binding proteins were subsequently identified, i.e., two subunits of ATP synthase (ATP5B and ATP5L), two redox-related proteins thioredoxin (TXN) and peroxiredoxin 1 (PRDX1), a mitochondrial heat shock protein 60 (Hsp60), a chloride intracellular channel protein 1 (CLIC1) and an enzyme catechol-*O*-methyltransferase (COMT). Bioinformatic analysis revealed that five proteins were associated with mitochondria. This strongly implicates that Cr(III) functions mainly in mitochondria. Subsequent studies showed that Cr(III) could rescue mitochondria from hyperglycaemia-induced fragmentation. The binding of ATP5B in the active site of ATP synthase, which resulted in the irreversible release of Mg^{2+}, inhibits the ATP synthase activity, leading to increased AMP/ATP ratio and activation of AMPK (Figure 3c-e). This mode of action also holds true in diabetic mice. This study resolved the long-standing issue on the molecular mechanisms of physiological/pharmacological effects of trivalent chromium, paving the way for further exploration of the pharmacological effects of chromium(III).[62]

4. CONDITIONAL LABELING

Unlike conventional protein labeling, conditional labeling is hypothesized to decipher proteins involved in certain homeostasis and physiological conditions. Specifically, metal homeostasis and redox homeostasis play important regulatory roles, and their dysregulation is related to many diseases. Unveiling proteins in specific malfunction locations can provide comprehensive and sufficient knowledge on their misregulation. For this type of labeling method, labeling reagents should have sufficient cell permeability that can be evenly distributed inside living cell. Then it should predominantly accumulate in certain malfunctioned areas where the reagent can be activated and facilitate the local protein labeling process. After which, the gathered proteins can be analyzed by conventional techniques such as LC-MS/MS, SDS-PAGE or confocal cell imaging (Figure 4a).

Hamachi's group first reported a conditional proteomics approach to identify proteins involved in zinc homeostasis using **AIZin-1** and **AIZin-2** probes[63] (Figure 4b). **AIZin** is composed of a tridentate zinc binding ligand conjugated with acyl imidazole as a reactive electrophilic unit for labeling protein though the attack of nucleophilic amino acid in the proteins. Coumarin and fluorescein were used as fluorophore units in **AIZin-1** and **AIZin-2**, respectively. AIZin probes can accumulate in Zn(II)-enriched cellular environments, where the probes activate and simultaneously label proteins in close vicinity. Subsequent analysis of labeled proteins revealed that Lys, Ser, Thr, Tyr, Cys, and His of a protein can react with probe AIzin-1. The hard metal ions such as Ca^{2+} do not affect the labeling, while mobile and borderline Zn^{2+} promotes protein labeling by the AIZn probes. Similarly, AIZin-2 spontaneously penetrates the cell membrane, being activated for labeling of proteins in response to elevated zinc levels. Such a conditional proteomic has been used to elucidate zinc dyshomeostasis induced by oxidative stress derived from nitric oxide (NO) in glioma cells as well as dynamic change of the zinc-related proteomes.

Chang and colleagues designed **C**opper-**D**irected acyl imidazole (**CD**) probe for labeling of proximal proteins at sites of elevated labile copper through copper-mediated activation of acyl imidazole electrophiles[64] (Figure 4c). By using coumarin and silicon-rhodamine as fluorophores, two probes i.e., **CD433** and **CD649** were synthesized, respectively. One of the advantages of such probes is that the probe is enriched in the copper-elevated area and coordinated with copper ions, leading to the formation of covalent bond between florescent reporters and proximal proteins in cell, which overcomes the major challenge of small-molecule based probe. Specifically, **CD649** can detect copper elevation and depletion caused by knockout of the copper exporter (ATP7A) in cell models. Importantly, **CD649** revealed distinct cell-specific response in labile copper dynamics in three different neuro cell lines, e.g., neurons, astrocytes, and microglia with inflammatory stimuli. Such probes respond in a copper-dependent manner by increased covalent labeling of the probe with proximal proteins in cells at copper enriched sites to

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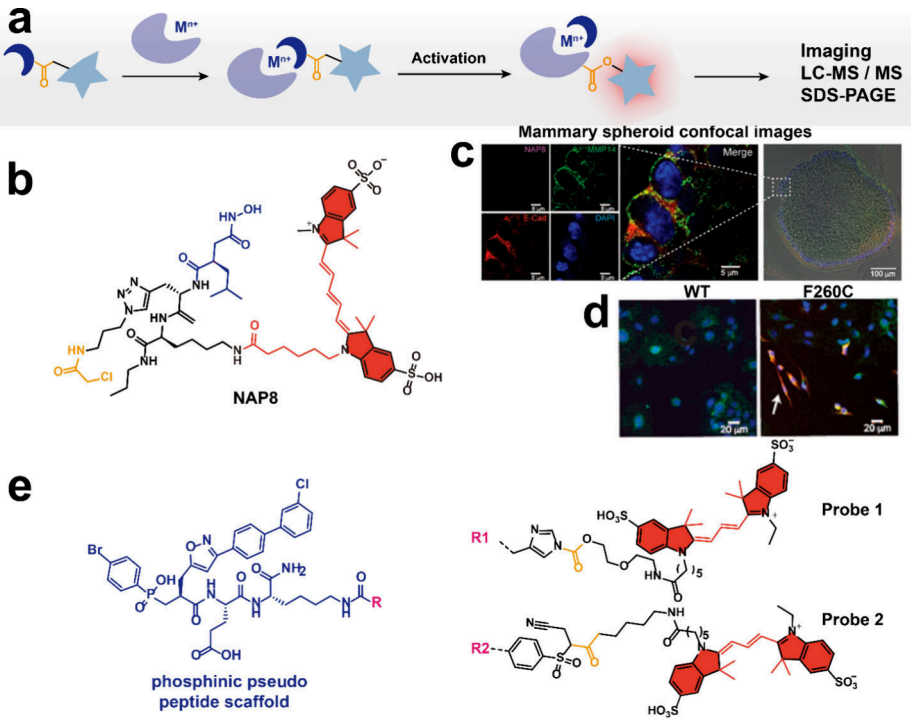

Figure 5. (a) Schematic diagram illustrating ligand-directed protein labeling. (b) Chemical structure of NAP8 that comprised fluorescent tag (*red*), war head (*yellow*) and affinity moiety (*blue*). (c) Immunofluorescence analysis of the presence of MMP14 on mammary spheroids. (d) Confocal images of active MMP-14 which are shown with white arrow. Noted that the 260C is a mutant form of MMP-14 without interfering with its activity. (e) Chemical structures of probe **1** and **2**. (c,d) were adapted with permission from ref 67. Copyright 2018 American Chemical Society.

minimize dye diffusion away from copper hotspots and preserve spatial information. The above-mentioned **CD** probes could only detect protein expression and label proteins in both Cu(I)/Cu(II) dysregulation environment. Recently, Hamachi and colleagues developed a new probe **CuRs** (**FL** and **AcFL**), which is only Cu(I)-responsible[65] (Figure 4d). **CuRs** includes a quinone methide (QM) that links the fluorophore and copper(I)-ligands together. When the probes are exposed to Cu(I)-rich environments, instantly highly reactive quinone methide is formed by releasing the ligand to immediately label nearby proteins of nucleophilic amino acids. The crucial step in the design of Cu(I)-selective protein labeling probes is employing a chelator with high specificity toward cuprous. The authors integrated TPA (tris(2-pyridylmethyl)amine) (K_d = 126 fM) due to its high selectivity for copper(I). They showed that probe FL selects Cu(I) over other tested metals including Cu(II), indicating that CuRs was activated only in response to Cu(I) for labeling proteins in Cu(I)-rich environments. Furthermore, **AcFL**, which exhibits better cell permeability, enabled a total of 60 and 152 proteins to be identified in WT and copper dysregulated ATP7A knockout cells. A similar approach has been reported by Chang and colleagues to label nucleophilic proteins under iron rich environments with probe **IG1- FM**[66] (Figure 4e).

5. LIGAND-DIRECTED APPROACH

Other than mining and identifying unknown metalloproteins, targeting specific metalloproteins for visualization has also been explored. The key to employing these probing methods is incorporating metal-specific ligands into the fluorophore as a warhead (Figure 5a). The most utilized warhead is hydroxamic acid as a zinc-binding group (ZBG) for visualizing matrix metalloproteases (MMPs). MMPs are a family of at least 23 zinc-containing metalloproteases that are involved in a diverse set of physiological and pathological processes and have important medical implications on diseases such as Alzheimer's disease, arthritis, and cancer. Different MMP members play different regulatory roles in the progression of certain diseases. Distinguishing individual MMPs can deepen our understanding of the specific roles, physical locations, and dynamics of proteolytic activation. In the case of design of MMPs targeting small molecular probes: 1) hydroxamic acid moiety is known to chelate to the active zinc site of the MMPs; 2) an electrophilic "warhead" to react with the catalytic nucleophile present within the protease active site and form a covalent bond with the target MMPs; and 3) a fluorophore for visualization of target enzymes.

With these strategies, Bogyo's groups developed the **NAP8** probe[67] (Figure 5b) for specifically targeting MMP-14 as it is the main protease that is involved in tumor progression. Namely, an elevated expression of MMP-14 is associated with increased tumor invasiveness. With the **NAP8**, hydroxamic acid first enriches around MMP-14 by chelating the zinc. Then the electrophilic warhead chloromethyl amide anchors the MMP-14. The fluorescent signal was emitted from the Cy5 fluorophore Cy5 tag. In Figure 5c, anti-MMP-14 (green) confirms the presence of MMP-14 on the apical membrane, colocalizing with E-cadherin, and forming a conjunction with neighboring protein. However, it should be noted that the MMP-14 on the spheroid is inactive, evidenced by the lack of probe fluorescence in the NAP8 channel (red). Through selectively labeling active MMP-14 with NAP8, it was shown that MMP-14 in its active state is present in stromal cell populations (Figure 5d). Since the activation is triggered by tumor-induced signaling pathways, NAP8 could be applicable

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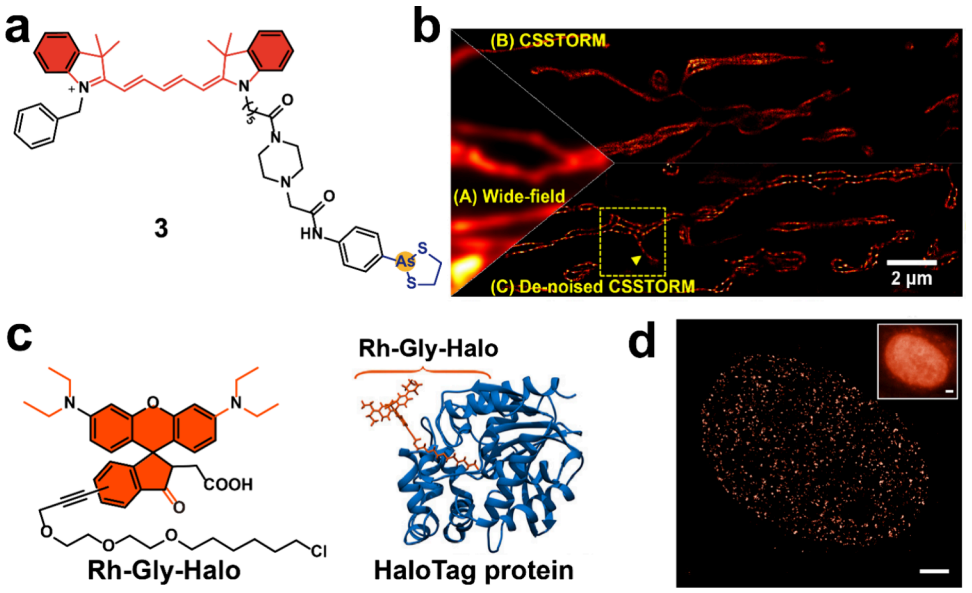

Figure 6. (a) Chemical structure of probe **3**. (b) Wild-field and STORM imaging comparisons of mitochondrial membranes labeled with probe **3**. Adapted with permission from ref 72. Copyright 2020 Elsevier B.V. (c) Structure of Halo tagged probe Rh-Gly-Halo and its bidding with HaloTag-protein. (d) PALM image of H_2B-Halo fusion proteins labeled with Rh-Gly-Halo. Panels (c,d) were adapted with permission from ref 74. Copyright 2019 American Chemical Society.

for studying the regulatory mechanism of active MMP-14 in the tumor microenvironment.

Such a strategy indeed enables monitoring the activity of a single MMP cell in vivo; however, it requires engineering the MMP to contain a reactive cysteine site, and this genetic manipulation is rather incompatible with physiological MMP forms. Therefore, Devel and colleagues proposed using RXP470.1 phosphinic pseudo peptide that includes a Zn-targeting group and further introduced a cleavable linker to the side for covalently modified active metalloproteases with Cy3 fluorophore for imaging purposes[68] (Figure 5e). Probes **1** and **2** were examined with 8 sets of MMPs and revealed that probes **1** and **2** have a higher affinity toward recombinant human MMP-12. Further, in-gel fluorescence study with model MMP-12 proteins ranging from pure human serum albumin to biological samples such as cells lysate (Hek), 4T1-mouse tumor, and mouse-liver extracts confirmed that probe **1** almost exclusively reacted with rhMMP-12 in contrast to probe 2, which has off-target activity, leading to a nonspecific labeling.

7. CONCLUSION AND PERSPECTIVE

Considering the crucial roles metal ions played in regulating biological processes, knowledge of how a metal executes its important role in biological systems may have a positive impact on human health. Given the complexity of metal-related events in life processes, it is of significance to *in situ* track the biomolecules, in particular proteins, that interact with metals. Small molecule-based fluorescence imaging technique is deemed as a promising strategy for real-time visualization of metalloproteins or metal-binding proteins.

In this review, we describe several protein labeling strategies, in particular, a metal-chelation based strategy to image and identify metal-binding proteins in live cells. Such a strategy has enabled imaging and identifying metal-protein interaction that could not be detected by other methods, which ultimately helps uncovering previously unknown protein targets of metal ions (or metallodrugs), e.g. Ga(III)[60] and Cr(III).[26] Moreover, strategies such as ligand-directed approaches enable mapping of metalloproteins that already coordinate with specific intrinsic metals. Conditional labeling, on the other hand, allows differentiation of protein expression under oxide/redox imbalance cellular environment or other specific cellular environments.

Aside from the advantages of these labeling techniques mentioned above, they also have limitations. The major drawback is that these probes could only identify more abundance proteins that bind to metal ions in cell owing to the relative low sensitivity of the probes, e.g. M^{n+}-NTA based probes. The design of fluorescent probes that could not only label but also enrich metal-binding proteins may resolve this issue. However, ligand-directed approaches may not be able to identify those metalloproteins with metal ions buried deep inside of the proteins. Moreover, all these labeling techniques are unable to distinguish the exact localization of metal-binding proteins. Therefore, it is a significant challenge to uncover all protein targets of a metallodrug or to track all metalloproteins in cells. It is necessary to design more sensitive fluorescent probes as well as to synthesize near-infrared fluorescent probes, which could be used to monitor metal-protein interactions in tissue and even in living organisms given their ability to penetrate tissues deeply, reduced photodamage to living organisms, and a high signal-to-noise ratio for NIR fluorescent probes.[69]

Single molecular imaging techniques may serve as a solution to detect the cellular localization of metal-binding proteins. For example, **S**tochastic **O**ptical **R**econstruction **M**icroscopy (STORM), one of the mostly used super resolution microscopy, could achieve in principle molecular-scale resolution through high-accuracy localization of photoswitchable fluorophores to reconstruct high resolution image.[70] Such stochastic activation of molecules is typically achieved using photo switchable dyes or fluorescent proteins.[70,71] The visualization of ultrafine mitochondrial structures by STORM was achieved by using a vicinal-dithiol-proteins targeting

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probe[72] (Figure 6a, 6b). In addition, **P**hoto**a**ctivated **l**ocalization **m**icroscopy (PALM) is another widely used method for optical imaging intracellular proteins at nanometer spatial resolution.[73] Similar to STORM, the success of PALM depends on the design of proper fluorophores. By using a new spirolactam based rhodamine probe, **Rh-Gly**,[74] a super-resolution image of live cell mitochondria was obtained with a good temporal resolution of 10 s and a satisfactory localization precision up to 25 nm. Moreover, a chloroalkane ligand conjugated derivative of **Rh-Gly**, i.e., Rh-Gly-Halo (Figure 6c) could be utilized to visualize histone H_2B protein with HaloTag genetically fused in live HeLa cells with super-resolution (Figure 6d). Super resolution imaging enables ultrafine cell structure and cell dynamics to be probed with previously unattainable spatial and temporal precision. Such imaging methods are definitely applicable to visualize metal-binding proteins, provided a suitable fluorescent probe is designed.

Overall, fluorescence imaging techniques may allow metal-involved life processes to be monitored in real-time. Given the complexity of metal−protein interactions under physiological conditions, it is necessary to integrate different metal-loproteomics approaches to uncover as much as possible the full scenarios of metal−protein interactions at different dynamic and thermodynamic scales. This provides a solid basis for understanding the role of metals in biology and medicine as well as unveiling the mode of action of a metallodrug, which potentially facilitates the development of metal-based drugs.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the paper. A.T. and H.Y.Z.: writing the original draft; J.X.C.: generating the image of the crystal structure of proteins. H.Y.L. and H.Z.S.: Conceptualization, writing-review and editing. CRediT: **Aliya Tiemuer** writing - original draft; **Hongyu Zhao** writing - original draft; **Jingxin Chen** methodology; **Hongyan Li** conceptualization, writing - review & editing; **Hongzhe Sun** conceptualization, supervision, writing - review & editing.

Notes

The authors declare no competing financial interest.

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