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Live-Cell Targeting of His-Tagged Proteins by Multivalent *N*-Nitrilotriacetic Acid Carrier Complexes

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Supporting Information

ABSTRACT: Selective and fast labeling of proteins in living cells is a major challenge. Live-cell labeling techniques require high specificity, high labeling density, and cell permeability of the tagging molecule to target the protein of interest. Here we report on the site-specific, rapid, and efficient labeling of endogenous and recombinant histidine-tagged proteins in distinct subcellular compartments using cell-penetrating multivalent chelator carrier complexes. In vivo labeling was followed in real time in living cells, demonstrating a high specificity and high degree of colocalization in the crowded cellular environment.

Live-cell labeling and tracking of proteins with therapeutic relevance pose a significant challenge.[1−3] To understand the interplay of proteins and cellular pathways in cells, a range of fluorescent reporters have been explored. Live-cell labeling demands high specificity, high labeling density, and cell permeability of the tagging molecule to track the protein of interest (POI) in the crowded cellular environment at a protein concentration of 300 mg/mL.[4] Large efforts have been made to site-specifically incorporate chemical reporters such as small organic dyes, genetically encoded amino acids, or autofluorescent proteins to label, track, and manipulate specific targets.[5] Selective tools such as probe incorporation mediated by enzymes (PRIME)[6] and the fusion proteins SNAP,[7,8] CLIP,[9] and Halo[10] emphasize these efforts. Organic probes are preferred over autofluorescent proteins because of their significantly smaller size, superior quantum yield, photostability, and broader spectral range. Small tags, such as the tetracysteine tag,[11] the retro-Diels−Alder reaction of tetrazines with alkenes,[12,13] *N*-nitrilotriacetic acid (NTA),[14,15] or its multivalent derivative *tris*NTA have further contributed to the analysis of structure, function, and dynamics of proteins.[16] The (sub)nanomolar affinity of the *tris*NTA moiety toward His-tagged proteins enables the site-specific and stoichiometric labeling of POIs. In contrast to *mono*NTA, *tris*NTA offers kinetically stable binding while the small size of the *tris*NTA/His-tag interaction pair keeps the influence on the physiological function of the POI as low as possible.[17] Both properties are of special interest for super-resolution microscopy, single-molecule tracking, and other emerging techniques.[18,19] The *tris*NTA/His-tag interaction pair has unique potential for highly specific labeling of POIs in living cells and intracellular delivery of functional His-tagged proteins in vivo.[20] However, its poor cell permeability has limited its in vivo applications to date.

Several technologies have been established for the intracellular delivery of proteins, nucleic acids, therapeutic agents, or other chemical reporters to study and perturb biological networks.[21,22] Cell-penetrating peptides (CPPs) have often been used to target cytosolic proteins without harming the cell.[23,24] One of the first identified CPPs was the transactivator of transcription (TAT) of HIV-1, which is responsible for cellular uptake and translocation into the nucleus.[25,26] The basic domain of TAT ($_{49}$RKKRRQRRR$_{57}$) has been identified to be responsible for cell penetration.[27] Since then, the TAT sequence has been conjugated to other proteins to facilitate fast and efficient internalization. In addition to TAT, other CPPs have been identified, including polyarginines,[28] Penetratin,[29] Transportan,[30] and cyclic arginine-rich peptides.[31] The low cytotoxicity of CPPs and the high diversity of the delivered cargoes make them an attractive choice for intracellular delivery.[32] Here, we describe a cell-penetrating *tris*NTA/His$_6$-TAT$_{49-57}$ carrier complex to internalize fluorescent *tris*NTA moieties for the efficient and rapid intracellular targeting of His-tagged proteins in mammalian cells.

We non-covalently linked HIV TAT$_{49-57}$ to Ni(II)-loaded *tris*NTA by forming a stoichiometrically defined carrier complex. HIV TAT$_{49-57}$ (RKKRRQRRR) equipped with six N-terminal histidines separated by a short flexible GGGS linker (Scheme 1) was synthesized using solid-phase Fmoc chemistry [see the Supporting Information (SI)]. For sensitive detection, the *tris*NTA was modified with different fluorophores, e.g., ATTO565 or AlexaFluor 647 (AF647). After Ni(II) loading, the *tris*NTA/His$_6$-TAT$_{49-57}$ carrier complex was formed to deliver the fluorescent multivalent chelator into the cytosol and nucleus. Once there, the *tris*NTA preferentially bound to the His$_{10}$-POI and released the TAT$_{49-57}$ carrier peptide because of its higher affinity. As previously shown, the affinity of *tris*NTA toward a His$_{10}$-tagged protein is 10-fold higher (K_D = 0.1 nM) compared with a His$_6$ tag, but the complex stoichiometry remains 1:1.[16] This allows for efficient and rapid exchange of the *tris*NTA carrier complex in vivo.

Here we focused on two medically relevant proteins located in distinct cellular compartments. First, we selected the antigen

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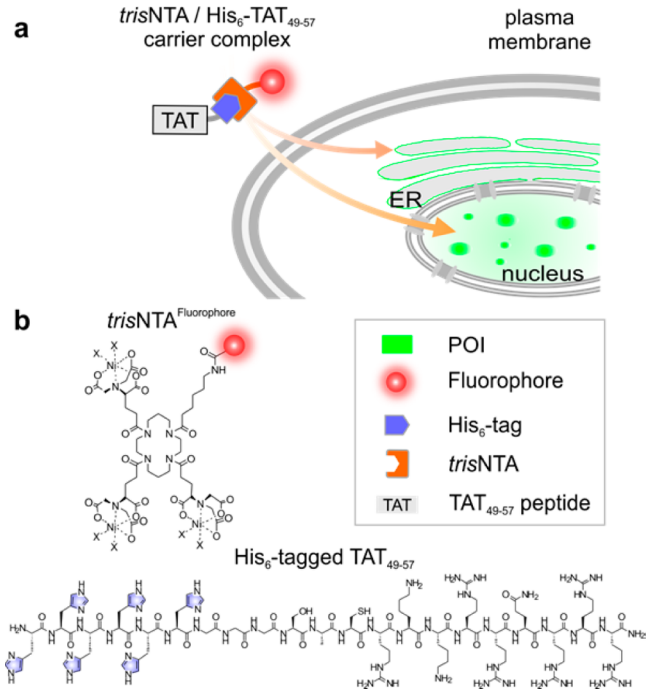
Scheme 1. (a) Live-Cell Labeling of His-Tagged Proteins in Distinct Cellular Compartments Using a Cell-Penetrating Non-covalent *tris*NTA Carrier Complex Formed by (b) Fluorescent *tris*NTA and His_6-Tagged TAT_{49-57}



translocation complex TAP, which resides in the endoplasmic reticulum (ER) membrane. Within the adaptive immune response, TAP shuttles proteasomal degradation products into the ER for loading of major histocompatibility complex (MHC) class I molecules.[33] Second, the DNA-silencing complex methyl CpG binding protein 2 (MeCP2) localized in the nucleus was investigated. MeCP2 is known to bind to methylated DNA, and mutations in MeCP2 are linked to neurodevelopmental disorders (Rett syndrome).[34] For colocalization studies, both POIs were fused to an autofluorescent protein, TAP^{mVenus} and $MeCP2^{GFP}$.[35,36] Notably, MeCP2 harbors an endogenous sequence of seven consecutive histidines in an unstructured C-terminal region, whereas the TAP construct displays a recombinant C-terminal His_{10} tag.

First, human cervical cancer (HeLa) cells expressing membrane-bound $TAP1^{mVenus}$-His_{10} were fixed, permeabilized, and incubated with only 100 nM $trisNTA^{ATTO565}$ to target the His_{10}-tagged POI in the ER membrane. Confocal laser scanning microscopy revealed a perfect colocalization (Pearson coefficient 0.90) of the His_{10}-tagged TAP and $trisNTA^{ATTO565}$ (Figure S1 in the SI). Notably, cells that did not express $TAP1^{mVenus}$-His_{10} did not show any fluorescence signal in the $trisNTA^{ATTO565}$ channel, indicating the specificity of the intracellular labeling. Similar results were obtained for the MeCP2 construct (Figure S2). Here, C2C12 mouse myoblasts expressing $MeCP2^{GFP}$ in the nucleus were labeled with $trisNTA^{AF647}$ as noted above. Colocalization of the fluorescence signals emanating from both reporter dyes (GFP and AF647) was detected (Pearson 0.78), demonstrating the applicability of the *tris*NTA staining and full access of this internal naturally occurring His tag for subsequent live-cell labeling studies.

After demonstrating the site-specific labeling of His-tagged POIs in fixed cells, we carried out cellular uptake and colocalization studies in living cells. We anticipated that the *tris*NTA carrier complex would be taken up by cells and delivered *tris*NTA to the POI for labeling, as has been seen for other non-covalent TAT adducts. To address this, we performed in vivo labeling experiments of $TAP1^{mVenus}$-His_{10} and $MeCP2^{GFP}$ by tracing the uptake of the *tris*NTA/His_6-TAT_{49-57} carrier complex in living cells. HeLa cells expressing TAP^{mVenus}-His_{10} were incubated with 500 nM $trisNTA^{ATTO565}$/His_6-TAT_{49-57} carrier complex. After transduction for 1 h, site-specific labeling was imaged using confocal laser scanning microscopy. To facilitate the uptake of *tris*NTA/His_6-TAT_{49-57} in the nanomolar range, cells were preincubated with 0.25 mM chloroquine (1 h at 37 °C) prior to the transduction step. Chloroquine is an inhibitor of endosomal sequestration and is known to increase the transduction efficiency.[37] Representative images of the live-cell labeling of His_{10}-tagged TAP by complex formation with $trisNTA^{ATTO565}$ demonstrate specific ER staining (Figure 1; see Figure S3 for further examples).

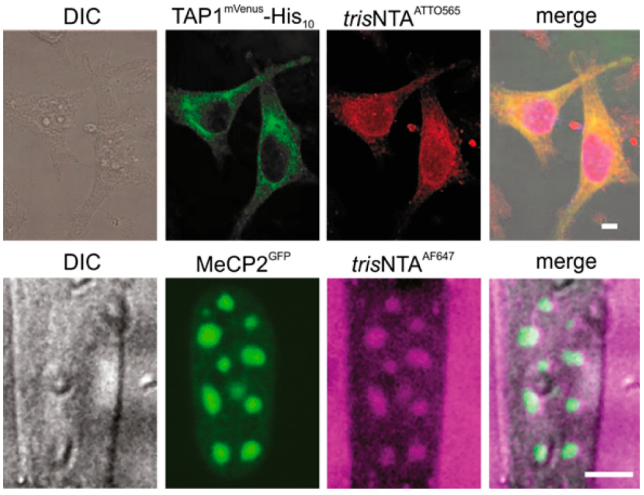


Figure 1. Live-cell labeling of His-tagged proteins by cell-penetrating *tris*NTA/His_6-TAT_{49-57} carrier complexes. $TAP1^{mVenus}$-His_{10}-transfected HeLa cells (upper row) and $MeCP2^{GFP}$-transfected C2C12 cells (lower row) were treated with the *tris*NTA carrier complex. Colocalization of $trisNTA^{fluorophore}$ (red/magenta) and the His-tagged POIs (green) was detected after cell incubation with the carrier complex for 60 min (TAP1) or 30 min (MeCP2). Site-specific recruitment of *tris*NTAs to the POI *via* complex formation was observed in the overlaid images (merged channel). See the SI for more examples. Scale bar: 5 μm.

Colocalization of the fluorescent signals for the His_{10}-tagged TAP1 and the red-emitting $trisNTA^{ATTO565}$ provided evidence for the site-specific and high-affinity labeling in living cells by the minimal lock-and-key element.

Similar results were obtained for the in vivo labeling of the transcription regulator $MeCP2^{GFP}$ (Figure 1). Transiently transfected C2C12 mouse myoblasts were incubated with the *tris*NTA/His_6-TAT_{49-57} carrier complex (9 μM) and the uptake was imaged 30 min after addition. In this case, the *tris*NTA was conjugated with an AF647 dye for readout. Merging the red channel of $trisNTA^{AF647}$ with the green channel of $MeCP2^{GFP}$ revealed colocalization of the small lock-and-key element and thus the site-specific targeting of the endogenous His-tag at heterochromatic regions of chromosomes (chromocenters). In all our live-cell approaches, strong ATTO565 or AF647 fluorescence of the *tris*NTA was observed that perfectly coincided with the fluorescence of the POI in the green

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channel (see the SI for further images). No colocalization of the *tris*NTA/His_6-TAT_{49-57} carrier complex was detected in cells expressing a TAP construct without the His tag or a truncated MeCP2 lacking the intrinsic heptahistidine sequence (Figure S4). It is important to note that no uptake of fluorescently labeled *tris*NTA was observed in the absence of the TAT_{49-57} carrier peptide (Figure S5) or in complex with a cumulative histidine sequence lacking the TAT_{49-57} peptide (His_5 peptide; Figure S6). In addition, the formation of the carrier complex is essential for cellular uptake, since no transduction of the *tris*NTAs was observed upon removal of the coordinated Ni(II) by addition of EDTA (Figure S7).

Finally, we followed the cellular uptake and labeling of POIs in real time by live-cell confocal microscopy. Imaging of $MeCP2^{GFP}$ in transiently transfected C2C12 cells and subsequent treatment with the preformed *tris*NTA/His_6-TAT_{49-57} carrier complex demonstrated rapid uptake within 10−30 min (Figure 2). Labeling of the cumulative histidines of

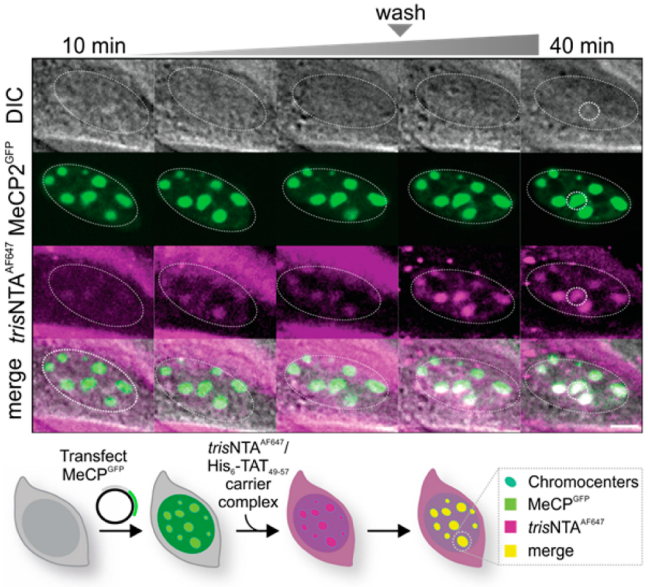

Figure 2. Site-specific targeting of $MeCP2^{GFP}$ by cell-penetrating *tris*NTA carrier complexes followed in real time. $MeCP2^{GFP}$ (green)-transfected C2C12 cells were treated with the *tris*NTA^{AF647}/His_6-TAT_{49-57} carrier complex and analyzed by live-cell microscopy. Colocalization of *tris*NTA^{AF647} (magenta) and $MeCP2^{GFP}$ was already detected after 10−30 min, demonstrating rapid and site-specific targeting of the POI by *tris*NTAs (merged channel). Scale bar: 5 μm.

MeCP2 proved to be fast and colocalization of the two fluorophores was already detected in the nucleus after 10 min. The two dyes accurately overlapped, indicating that our labeling approach enables efficient, rapid, and site-specific targeting of POIs in living cells. Notably, Ni(II) ions complexed to *tris*NTA at the given concentrations and incubation periods were nontoxic for the cells, as observed in long-term cell cultures and cell viability assays (Figure S8).

In summary, we have developed a TAT-mediated carrier system of fluorescent *tris*NTA for in vivo and real-time labeling of POIs harboring an endogenous or recombinant His-tag. This cell-penetrating *tris*NTA carrier system underwent rapid cellular uptake and showed site-specific labeling of His-tagged targets in distinct cellular compartments of living cells. Moreover, these studies prove the eminent potential for live-cell targeting of cumulative histidines by *tris*NTA groups, since the His tag is the smallest and one of the most frequently used tags for protein tagging and purification. This highly sensitive and rapid labeling method will have a great impact on live-cell imaging techniques, and we are currently investigating the use of this labeling method in super-resolution microscopy and in real-time tracking analysis of His-tagged targets within the interior of living cells.

■ ASSOCIATED CONTENT

Supporting Information

Methods, Figures S1−S8, and Schemes S1 and S2. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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