Multivalent Chelators for In Vivo Protein Labeling

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With the advent of single-molecule methods, chemoselective and site-specific labeling of proteins evolved to become a central aspect in chemical biology as well as cell biology. Protein labeling demands high specificity, rapid as well as efficient conjugation, while maintaining low concentration and biocompatibility under physiological conditions. Generic methods that do not interfere with the function, dynamics, subcellular localization of proteins, and crosstalk with other factors are crucial to probe and image proteins in vitro and in living cells. Alternatives to enzyme-based tags or autofluorescent proteins are short peptide-based recognition tags. These tags provide high specificity, enhanced binding rates, bioorthogonality, and versatility. Here, we report on recent applications of multivalent chelator heads, recognizing oligohistidine-tagged proteins. The striking features of this system has facilitated the analysis of protein complexes by single-molecule approaches.

1. Introduction

Life sciences demand for strategies to label and track proteins with high-accuracy in native environments. In chemical biology, the visualization of complex cellular processes by fast, stoichiometric, and site-specific protein labeling is a fundamental focus. Beyond that, the ability to control protein function, dynamics, and interaction by precise protein modification is desirable and requires the attachment of distinguishable modifiers or reporter probes. For widespread use in diverse applications, methods for the incorporation and attachment of reporter probes must allow the site-specific and fast labeling of proteins. Parallel to advances in imaging technologies, protein-labeling methods, such as SNAP,[5a] CLIP,[5b] and Halo[6a] tags coding for enzymes (greater than 25 kDa), as well as FIAsh,[6a] ReAsh,[6a,5] and histidine tags of less than or equal to 1 kDa in molecular weight, contemporaneously evolved for localizing and staining proteins. To specifically bind reporter molecules, all current methods involve the genetic tagging of elected target proteins either by fusion with a small protein or extension with a minimalistic peptide sequence. Enzyme-based tags combine high specificity with fast labeling, however, the tag size, typically above 25 kDa, can perturb protein trafficking, localization or interaction networks. Labeling of the tetracycline motif with FIAsh and ReAsh can lead to unspecific interactions and often require additional experimental steps, as well as optimized flanking sequences for each target.[6a,5] Both of these techniques require high labeling concentrations (1–100 μM), provoking unspecific binding and thus background staining.[6b] Despite their emerging potential in site-specific incorporation of chemical reporters, such as small organic fluorophores, photo cross-linkers, or post-translational modifications, labeling techniques have been constantly refined and advanced to compensate for limitations of low affinity and reactivity or restrictions for their use in living cells.

Apart from the fusion to autofluorescent proteins or enzyme-mediated labeling methods, a major focus is currently set on small peptide-based recognition tags, owing to their small size (0.6–6 kDa), versatility, and generic introduction into the protein of interest (POI) by genetic fusion, and their minimal disturbance on protein function, dynamics, or localization. Within the target protein, a genetically encoded, unique peptide sequence serves as a tag to direct reporter probes to the POI. The small size and flexible incorporation provides great advantages to avoid potential inference with the behavior and performance of the POI. Ever since its application as an affinity tag for protein purification by F. Hoffmann-La Roche, the N- or C-terminal oligohistidine peptide fusion, termed His-tag, has found widespread use for the purification and detection of recombinant proteins.[3] Histidine (His) constitutes a special position as its imidazole moiety serves as an electron-rich ligand for transition metal ions, such as Zn²⁺, Cu²⁺, Ni²⁺, and Co²⁺.[4] Furthermore, it is a rarely occurring amino acid (2.5%). The histidine-rich sequence contributes multiple coordinating ligands that can form thermodynamically stable chelating sites with bivalent metal ions.[5] Generally, the His-tag consists of 6–12 consecutive histidines that can tightly interact with transition-metal complexes. By exploiting the high-affinity of iminodiacetic acid (IDA) or N-nitrotriacetic acid (NTA) for transition metal ions, complexes between histidines and the respective chelators can be formed. To this end, two consecutive histidines occupy 2–3 vacant coordination sites within the metal-chelating complex (Me²⁺-IDA, Me²⁺-NTA). To increase the affinity of monovalent chelator complexes, multiple NTAs were grafted to various scaffolds, yielding bis-, tris-, tetrakis-, and hexa-NTA with two to six NTA arms (Figure 1). The synthetic strategy for generating multivalent chelator heads (MCHs) employs linear, cyclic, or dendritic scaffolds. For NTA-based MCHs, glutamate or lysine derivatives have been mostly explored and two additional carboxylic moieties have been introduced at the α-amino group by alkylation with α-bromoacetic acid.[5] Similar, IDA entities have been generated by introducing two acetate groups on a nitrogen functionality. NTA and IDA moieties have been conjugated on cyclic scaffolds (for...
example, cyclam and cyclen) by amide coupling chemistry to engineer cyclic MCHs. For linear MCHs, the chemical synthesis involves solid-phase peptide synthesis and introduction of the chelator heads via Michael-addition or click-chemistry.

The architecture of the dendritic MCH is based on either glutamate or lysine as branching points. Integration of the MCH synthons can directly follow, or take place after further branching, resulting in bis-, tris- or tetrakis-NTA. Conjugation with reporter probes is permitted via coupling to primary amines, carboxylic acids, or thiols, additionally introduced on the MCHs. Generally, orthogonal protecting group chemistry (Bzl/tert-butyl) and standard amide coupling protocols have been used. The developed MCHs achieve a strong, yet reversible binding to the His-tag via multivalent interactions, enabling their efficient and rapid attachment to POIs even under physiological conditions.

In our review, a special focus is set on trivalent NTAs, due to their unique features, such as high-affinity and reversibility. Besides trisNTA, MCHs are currently undergoing a renaissance for a diverse set of applications, such as single-molecule localization microscopy or live-cell fluorescence imaging. We report on recent trends and selected applications of the trisNTA MCH in advanced microscopy and spectroscopy techniques, as well as briefly outline challenges and prospects for future implementations. For comprehensive reviews on peptide-based recognition tags or other protein-labeling methods, we kindly refer to other excellent review articles.

Figure 1. a) Chemical structures of trivalent chelator heads. Diverse scaffolds based on cyclic, linear, and dendritic trisNTAs were explored. Reproduced with permission from ref. [9b]. Copyright Wiley-VCH, 2018. b) High-affinity trisNTA–His-tag interaction. Cyclam-Glu-trisNTA (trisNTA) in complex with a His$_6$-tagged protein of interest (POI). c) Chemical structure of the newly developed super-chelator hexaNTA. Reproduced with permission from ref. [19]. Copyright Wiley-VCH, 2018.

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Robert Tampé is known for his seminal contributions in the mechanistic understanding of antigen processing and viral immune evasion. In addition, he discovered the ribosome recycling factor and provided structural and mechanistic insights into ribosome splitting and mRNA surveillance. His major passions are membrane biology, control of mRNA translation, as well as chemical and synthetic biology.
lization have previously been described.\textsuperscript{[9]} For the synthesis of the diverse MCHs, we kindly refer the reader to the original work.\textsuperscript{[6b,9]} Here, we primarily focus on the application of trisNTA and selected MCHs as a facile method for site-specific modification and labeling of POIs in vitro and in living systems.

\section*{2. Key Principles and Recent Developments}

An advantage of Ni\textsuperscript{II}–NTA chelation is its affinity to histidine with an approximate dissociation constant ($K_d$) of 10 $\mu$m.\textsuperscript{[10]} The nickel ion is in an octahedral coordination within the NTA–His\textsubscript{6}-tag complex, in which four coordination sites are occupied by NTA and two by imidazole moieties of the His-tag. To mimic the multipoint interactions between the His-tagged protein and immobilized metal ions, several molecular architectures for MCHs have been employed to generate structurally defined recognition moieties. By observing a multivalent effect of monoNTA chelator lipids at high surface density,\textsuperscript{[11]} the Piehler and Tamp/C216 labs were amongst the first to explore MCHs by assembling two to four NTAs on cyclic\textsuperscript{[12]} linear\textsuperscript{[13]} or dendritic scaffolds\textsuperscript{[4e,6a]} (Figure 1a). As the His\textsubscript{6}-tag is the most commonly used one, trisNTAs are dominant.

The trisNTA MCH and the genetically encoded His\textsubscript{6,12}-tag mutually result in a minimalistic lock-and-key pair (Figure 1b). As a result of multivalency, trisNTA shows exceptionally high affinities for His-tagged proteins (His:\ $K_d = 10$ nM; His\textsubscript{10}:: $K_d = 0.1$ nM), which are accompanied by a kinetically stable binding to the oligohistidine sequence ($k_{\text{off}} = 0.18$ h\textsuperscript{-1}).\textsuperscript{[10]} In general, the binding between MCHs and His-tagged proteins is a multivalent interaction. With increasing numbers of NTA heads (multivalency) an increase in complex stability is observed. The higher affinity of various MCHs can be explained by thermodynamic and kinetic means. Thermodynamically, an increasing number of chelator heads directly increases the binding enthalpy ($\Delta H$).\textsuperscript{[40]} A higher probability of simultaneous coordination and of re-binding events is observed with increasing multivalency. This probability further rises with increasing numbers of cumulated histidines. Since the free binding enthalpy also depends on the entropy term, the latter one can outbalance enthalpic contributions. For the MCH/His-tag complex formation, the substantial higher $\Delta H$ than $\Delta G$ values compensates for the high loss in entropy. Responsible for this are the drastic decrease of conformational freedom upon complex formation as well as the drop in flexibility of both interaction partners. Thus, the mobility of the linkers as well as the different MCH topologies (dendritic, linear, or cyclic) can severely affect the net free energy of MCH complex formation.\textsuperscript{[98]} Kinetically, the nanomolar $K_d$ values for MCH/His-tag complexes are marked by very fast $k_{\text{on}}$ and extremely slow $k_{\text{off}}$ rates ($K_d = k_{\text{on}}/k_{\text{off}}$; for example, trisNTA–His\textsubscript{6}-tag $k_{\text{on}} = 1.6 \pm 0.4 \times 10^7$ M\textsuperscript{-1}s\textsuperscript{-1} and $k_{\text{off}} = 0.34 \pm 0.01 \times 10^{-3}$ s\textsuperscript{-1}).\textsuperscript{[99]} With increasing length of the His-tag, the dissociation rates decrease significantly.\textsuperscript{[14]} The enhanced feasibility of collisions and fast re-binding of the chelator heads with the His-tag become more pronounced and are thus mainly responsible for this. Importantly, the determined binding affinities only represent apparent $K_d$ values, since a simple 1:1 binding model during the steady state phase is assumed. For the implementation of MCHs as generic tools for labeling, the very fast $k_{\text{on}}$ rates, as well as the subnanomolar $K_d$ values, are beneficial as the resulting stable interaction by multivalent interactions greatly promotes the selectivity and specificity at low probe concentration. Furthermore, the coordination of the imidazole side chain with the metal-loaded chelator heads also influences the affinity as well as the rebinding behavior. In this regard, the trisNTA–His\textsubscript{6}-tag interaction stands out as it apparently adopts an optimized binding geometry and exhibits a fine-balanced enthalpic and entropic contribution to the complex formation process.

The multiple metal-ion-mediated contacts maintain reversibility in the presence of imidazole, histidine, and ethylenediaminetetraacetic acid (EDTA). Because of patent issues, various architectures, including linear, dendritic, and cyclic scaffolds, have been employed for the alignment of two to six monomeric NTAs. Most common for MCHs is the assembly of 2–4 NTAs on cyclic or dendritic scaffolds. A systematic investigation of the impact of trisNTA architectures revealed that cyclic trisNTAs grafted to a cyclam or cyclen scaffold in combination with a glutamate-linked NTA are superior in terms of kinetic stability and affinity.\textsuperscript{[80]} In contrast, the linear as well as dendritic trisNTAs displayed a significantly lower kinetic stability compared to cyclic trisNTAs as examined by surface plasmon resonance (SPR) spectroscopy. It is most likely that the spatial proximity and conformational flexibility of the cyclic trisNTA best match the pattern of the consecutive histidines. Without substantial steric hindrance, efficient His-tag complexation is permitted by the conformational freedom of the trisNTA. Importantly, a strict 1:1 stoichiometry between Ni–trisNTA and His-tagged protein is maintained for both the His\textsubscript{6} and His\textsubscript{10}-tag.\textsuperscript{[12a]} For the decahistidine sequence, a 100-fold higher affinity in the subnanomolar range was determined ($K_d = 0.1$ nM), compared to the His\textsubscript{6}-tag.\textsuperscript{[40]} It is assumed that the multivalent ligands assist a “sliding/zipper-like” mechanism and thus allow the NTA arms to effectively rebind to the His-tag.\textsuperscript{[14]} A consequence of the multiple-site interactions is a significant increase in protein binding affinity as well as kinetic stability that are finely balanced by the scaffold, linker length, and chelator head.

Another parameter is the transition metal itself. Other divalent ions such as Co\textsuperscript{2+}, Cu\textsuperscript{2+}, and Zn\textsuperscript{2+} can also be used as the coordinating metal between the His-tag and NTA with similar affinity.\textsuperscript{[83]} Hamachi et al. reported on the design of a binuclear, diamagnetic zinc complex based on IDA with a binding constant of $K_{\text{app}} = 0.13$ $\mu$m for a His\textsubscript{10}-tagged peptide.\textsuperscript{[145]} Interestingly, the zinc complex of NTA (Zn\textsuperscript{II}–NTA) and a His\textsubscript{10} peptide was apparently weaker than that of Zn\textsuperscript{II}–IDA. However, the combination of the tetradeinate ligand NTA and Ni\textsuperscript{II} or Co\textsuperscript{II} has gained acceptance as it ensures strong interaction.

For a permanent interaction between NTAs and the His-tagged protein, Spatz and co-workers established the mild oxidation of the transition metal Co\textsuperscript{II} to Co\textsuperscript{III} within the chelator complex.\textsuperscript{[15]} In contrast to the more common nickel-
mediated interaction, Co$_{3+}$ complexes between the His-tagged protein and the NTA resulted in a high thermodynamic and kinetic stability of the complex with extended long-term stability ($t_{1/2}$ ≈ 7 days). Moreover, the complex was inert towards ligand exchange and reducing agents. A stable, quasi-reversible interaction with the His-tagged protein was obtained. The oxidation of Co$_{3+}$ to Co$_{2+}$ was also explored to decorate liposomes with His-tagged proteins as well as antigens. Here, cobalt chelated in porphyrin-phospholipidid was embedded in a lipid bilayer and allowed the stable capturing of His-tagged conjugates via non-covalent interactions, resulting in an essentially irreversible anchoring to liposomes.[13]

Exploiting a molecular avidity effect, trisNTA was recently advanced to distinguish between His$_{12}$-tagged and His$_{6}$-tagged proteins. By linking two cyclic trisNTAs via a short peptidic linker, a new class of hexavalent NTA (hexaNTA, Figure 1c) chelators was defined, exhibiting subnanomolar affinities and extremely stable protein labeling even in the presence of 30 mM imidazole ($k_{d}$ ≈ 10$^{-5}$ s$^{-1}$).[19] The 100-fold higher dissociation constant of hexaNTA compared to trisNTA allowed a kinetic discrimination between both MCHs in the presence of the competitor imidazole. Based on this, trisNTA bound to His$_{6}$-tagged proteins dissociated within minutes in the presence of imidazole, whereas the hexaNTA-Alexa Fluor 647 showed kinetically stable and persistent binding to His$_{12}$-tagged proteins. Conversely, the tunable and distinct kinetic profile of hexaNTA compared to trisNTA enabled the labeling of His$_{12}$-tagged proteins with hexaNTA prior to the decoration of His$_{6}$-tagged proteins with trisNTA.

For MCHs in general and especially trisNTA, an intricate interplay between the molecular architecture of the scaffold and the MCH multivalency is apparent. As a result of our comprehensive study, a cyclic scaffold as well as a glutamate-based linker is seen as optimal and superior for high-affinity and stable binding of trisNTA to His-tagged proteins.[16] With regard to high-accuracy protein labeling, key factors, including site-selectivity and specificity, efficiency and rate of labeling, bioorthogonality to other labeling methods, close-proximity as well as accessibility of the cognate partner, must all be considered. Equipped with such attributes, MCHs and in particular trisNTA emerged as very promising and powerful tools for protein targeting as they fulfill the demands by attaching to the unique peptide sequence of the His-tag. Furthermore, versatility is given by the straightforward conjugation to a wide range of functional entities, such as fluorescent dyes, lipids, biotin, polymers, or nanoparticles.

3. Conformational Dynamics and Structural Analysis

For the study of structural and conformational changes in proteins, Förster resonance energy transfer (FRET) is routinely applied. A methodological challenge in conducting FRET measurements is the ability to site-specifically label proteins with donor and acceptor fluorophores capable of undergoing energy transfer. Ebright and co-workers pioneered bisNTA conjugated to Cy3 and Cy5 dyes for FRET measurements.[20] By employing bivalent NTAs, the ryanodine receptor type 1 (RyR1) was investigated by FRET methods.[21] Furthermore, Vogel and co-workers reported on the His-tag-specific labeling with monoNTA probes for FRET measurements on the G-protein-coupled receptor (GPCR) 5HT$_3$.[10] The Gierasch lab utilized a bisNTA based on dibromobimane as a FRET donor to His-tagged cellular retinoic-acid-binding protein (CRABP) I, equipped with a FlAsH acceptor.[22] Following these FRET experiments with bisNTA and monoNTA, the Pichler and Tampé labs used the high-affinity and His-tag-specific targeting of trisNTA conjugated to acceptor fluorophores for in situ protein labeling.[12a] Thus, the kinetics of the ATP hydrolysis cycle of the mitochondrial ABC transporter Mdr1,[23] the conformational changes of interferon receptor subunit 1 (IFNAR1) upon ligand binding (Figure 2a),[24] ternary complex formation between IFNAR1/2 in presence of its ligand IFNβ,[25] and ring formation by virus-bound V33333-His, were characterized by changes in FRET efficiency.[26] In addition to fluorescent donor/acceptor conjugation, trisNTA and other MCHs were successfully linked to fluorescence quencher molecules for FRET studies. In this regard, an interesting MCH utilization is based on a fluorescent “turn-on” system in response to a His-tagged protein.[27] Here, a reversible complex between a quencher-conjugated His$_{6}$-peptide and a dye-conjugated dendritic trisNTA was preassembled. The quencher dissociates from the complex in the presence of a His-tagged protein and fluorescence emission is restored, displaying specific protein labeling.

Single-molecule FRET (smFRET) has become widely used for quantifying the conformational heterogeneity and structural dynamics of biomolecules. To probe the viral RNA conformations that occur during RNA polymerase (RNAP) binding and initial replication, specific labeling of RNAP by trisNTA was applied and revealed insights in the dynamic motion of RNA during influenza replication.[28] The study of conformational dynamics via smFRET was further realized by a simultaneous, four-color single-molecule fluorescence methodology.[29] Here, the co-localization of the mismatch repair protein MutSb on DNA and the assembly of membrane-tethered SNARE protein complexes were monitored. Another field of application in smFRET is the utilization of trisNTA for the site-specific, oriented, and high-affinity attachment of His-tagged proteins on surfaces. For instance, Listeria monocytogenes Ca$^{2+}$-ATPase (LMCA1), a Phosphorylation-type (P-type) ATPase, was anchored to a sparsely trisNTA-decorated interface to observe conformational changes associated with functional transitions.[30] Remarkably, the trisNTA–His-tag interaction enabled the highly specific, reversible, and oriented attachment of LMCA1. The controlled protein immobilization further allowed the investigation of the prokaryotic neurotransmitter:sodium symporter LeuT by smFRET.[31] The immobilization strategy helped to purify the protein within the microfluidic chamber assembly. Combining electron microscopy and FRET, subunits of RNAP were mapped with nanometer precision. The hybrid method exploits the attachment of a trisNTA–nanogold probe to the His-tagged C-terminus of Tfg2 for its nano-positioning.
Figure 2. Conformational and mechanistic studies facilitated by site-specific trisNTA protein labeling. a) Detection of ligand-induced receptor assembly by FRET measurements. Fluorescence quenching between trisNTA–OG488-labeled His10-tagged IFNAR2 and trisNTA–ATTO 565-labeled His10-tagged IFNAR1 before and after binding of IFNβ (left). Recovery of the donor fluorescence of His10-tagged IFNAR2 decorated with trisNTA–OG488 upon addition of non-labeled IFNAR1 (right). Adapted with permission from ref. [12a]. Copyright (2006) American Chemical Society.


c) FCCS quantification of IL-4R subunit interactions in the plasma membrane. Scheme of complex formation between trisNTA–Alexa Fluor 647-labeled His6-tagged IL-4Ra and an eGFP-tagged second receptor subunit (left). Correlation curves showing increased cross-correlation (blue, arrow) in the presence of IL-4 (right). Reproduced with permission from ref. [33]. Copyright Elsevier, 2014.

d) His6-tagged EB1 conjugated to gold nanoparticles decorated with a single trisNTA (left). Z projections of a few slices at the middle of the microtubule and cross-sections along the microtubule axis that reveal the organization of the trisNTA–gold-nanoparticles around the lattice (1–5, right). Adapted from ref. [39]. Copyright Nature Publishing Group, 2016.
Escherichia coli system of extraction of structural constraints within the maltose import D3-pyrroline-3-methyl) methanethiosulfonate) allowed the tris dimerization is a strictly ligand-induced process (Figure 2c). Structural and conformational information can also be obtained by EPR and NMR spectroscopy. Conjugation of trisNTA to the spin probe MTSL ((1-oxyl-2,2,5,5-tetramethyl-D3-pyrroline-3-methyl) methanethiosulfonate) allowed the extraction of structural constraints within the maltose import system of Escherichia coli and its multisubunit assembly by EPR spectroscopy. Distance information was retrieved both in vitro and in situ by the site-specific spin labeling in cell lysates and under in-cell conditions.

Along the same line, the direct attachment of two NTA-based ligands via disulfide bond formation to a protein α-helix resulted in a chelating motif for paramagnetic lanthanide ions. The ordered alignment of two proximal monoNTAs ligands, though not necessarily targeting a peptide recognition element or an MCH, enabled the complex formation with paramagnetic lanthanide ions. The ions were rigidly bound with high-affinity, leading to the generation of useful pseudo-contact shifts and residual dipolar couplings in the NMR spectrum. In this case, the principle of MCH found a different use, demonstrating the value and great versatility of the adaptor molecules.

By high-resolution atomic force microscopy (AFM), the specific binding of two different ligands to a GPCR was simultaneously detected. The human protease-activated receptor (PAR1) was reconstituted in proteoliposomes and the binding of two different ligands was monitored to simultaneously localize and quantify their interaction. For this purpose, a bifunctional AFM tip, equipped with the trisNTA group as well as the native receptor-activating peptide, was assembled. While imaging native PAR1, binding to an extracellular His₆-tag and to the intracellular surface via the native ligand enabled the distinction between two different receptor sites. In general, AFM-tip functionalization with trisNTA is a versatile technique and allowed the study of the interaction between the T4-bacteriophage gp37 adhesion and bacterial lipopolysaccharide or between the von Willebrand factor (VWF) A2 domain with single A1 domains and VWF A1A2 fragments.

Cryo-electron tomography (cryo-ET) can resolve cellular ultrastructures in their native environment and the molecular sociology of macromolecular assemblies in vivo. Thus, macromolecular complexes can be imaged yielding nanometer-scale information. In this context, insights into the conformational changes experienced by tubulin dimers during microtubule assembly and the architecture of the GTP-cap region were obtained via conjugation of end-binding protein 1 (EB1) to gold nanoparticles. For oriented and site-specific attachment, gold nanoparticles were decorated with trisNTA as an adaptor molecule for the anchoring of His₆-tagged EB1 (Figure 2d). Importantly, a single trisNTA on the gold nanoparticle was able to generate a stoichiometric EB1/nanoparticle construct (molar ratio: 0.8:1), demonstrating the specificity and 1:1 stoichiometric interaction of the trisNTA–His-tag system. Thus, structural information about dynamic microtubule assembly and the interaction of EB1 with growing microtubule ends was obtained. In a similar approach, the ordered nanoscale organization of E-cadherin molecules at the cell membrane was investigated. In this case, a His-tagged anti-GFP nanobody was used to decorate gold nanoparticles via interaction with trisNTA. The study revealed the existence of oligomeric cadherins at cell–cell contacts and indicated that the formation of oligomeric clusters controls the anchoring of cadherin to actin and cell–cell contact fluidity. Taken together, the versatile functionalization of trisNTA with fluorophores, quenchers, or gold nanoparticles, as well as its stoichiometric binding to His-tagged proteins, is orthogonal to other protein-labeling methods and can thus extend the scope of these techniques.


Single-molecule localization microscopy and super-resolution microscopy, in general, have enabled the investigation of the organization and interaction of biomolecules at the nanometer scale and thus substantially improved the study of cellular processes. For accurate and precise protein labeling, the POI needs to be specifically and efficiently decorated with ultra-bright (organic) fluorophores. Besides low background staining, the close proximity to and precise installation of fluorophores on the POI can further enhance the resolution of the target structure. Likewise, the photochemical properties of the fluorophores are also important for imaging cellular structures with high resolution. As trisNTA has high specificity and small size, the combination of the trisNTA–His-tag recognition with modern imaging techniques is advantageous because it offers great flexibility in the choice of fluorophores combined with the generic, site-specific labeling of the POIs.

Vogel and co-workers established one of the first His-tag-specific labeling approaches. They investigated the ionotropic 5HT₃ serotonin receptor by fluorescence analyses and characterized the monoNTA–His-tag interactions in vivo. The rapid reversibility of the binding of the monoNTA to oligohistidine sequences allowed not only FRET experiments to be performed, but also the visualization and localization of the 5HT₃ receptor in live HEK293 cells. However, the labeling with monoNTA fluorophores was hampered by the relatively low affinity of monoNTA for the His₆-tag (Kₐ ≈ 10 µM). One avenue to increase the half-life of the complex was to extend the oligohistidine sequence to 10 histidines, increasing the half-life by a factor of 5.

The enhanced stability of the multivalent trisNTA for His-tagged proteins was utilized to visualize and localize proteins in vitro and in vivo. The noncovalent trisNTA interaction was
exploited for protein labeling under immunostaining conditions, exploiting its high-selectivity and high-affinity targeting of POIs. To this aim, we established trisNTA–His-tag labeling for single-molecule localization microscopy called SLAP (small labeling pair).\[41\] Taking advantage of bright, photostable organic dyes conjugated to trisNTA, different intracellular POIs were efficiently decorated with trisNTA–fluorophore constructs for confocal laser scanning microscopy (CLSM) and direct stochastic optical reconstruction microscopy (dSTORM) in various cell types. The cytoskeletal protein β-actin (Figure 3a), the nuclear envelope protein lamin A, and the transporter associated with antigen processing (TAP) in the endoplasmic reticulum (ER) membrane were visualized at 40 nm resolution. In the case of TAP, nanometer-sized and clearly distinct TAP clusters were observed, a structural detail that had previously been hidden from conventional fluorescence microscopy (Figure 3b). Interestingly, by comparing conventional immunostaining (primary- and secondary-antibody (AB) labeling) with trisNTA targeting, a severely increased cluster dimension for immunostained TAP was determined (Figure 3c; ABs: 71 ± 8 nm vs. trisNTA: 49 ± 7). Notably, the radius difference of 22 nm is in perfect agreement with the increment calculated for two antibodies (2 × 10 nm). This showed that the trisNTA–
His-tag pair could be used as a robust protein-labeling system in vitro and highlights the value of an accurate, precise as well as close-proximity attachment of fluorophores to the POI. A second feature of the labeling method is its reversibility and the simple exchange of dye-labeled trisNTA, which is hardly achievable through immunofluorescence or covalent dye attachment (e.g., the SNAP-tag). This modularity and high rate of labeling offers rapid iterative targeting of proteins for fluorescence microscopy, free choice of the reporter group, and sequential analysis by different (super-resolution) microscopy techniques. Exploiting the reversibility of the SLAP technique, whole-cell 3D super-resolution microscopy was established by the repetitive application of trisNTA–fluorophore conjugates to His-tagged proteins by single-epitope repetitive imaging (SERI). Repetitive imaging, washing, and re-labeling of single-planes enabled the 3D super-resolution imaging of a complete cell (Figure 3d,e). This demonstrates that multiple rounds of labeling and imaging can compensate photobleaching effects in multi-planes. Beyond these studies, quantum-yield-optimized fluorophores based on trisNTA were designed for reversible, site-specific labeling of GPCRs. His-tagged AMPA receptors (GlutA1) in spatially restricted synaptic clefts were imaged by stimulated emission depletion (STED) microscopy with high spatiotemporal resolution (less than 50 nm). Even in neuronal synapses, the developed high-quantum yielding trisNTA fluorophores specifically visualized single cellular targets. It is important to note that the synaptic cleft can be difficult to access with conventional, bulky probes.

Recently, the hexaNTA was utilized in combination with trisNTA for the multiplexed labeling of His₆- and His₂₁₂-tagged proteins. Conventional fluorescence microscopy usually allows multiplexing owing to orthogonal excitation and emission spectra of different fluorophores. However, the kinetic discrimination of different His-tags by hexaNTA and trisNTA enabled the precise localization of two target POIs based on probes coupled to the same fluorophore. Their tunable kinetics and distinct dissociation profiles provided a simple and generic approach for the use of a single fluorophore to image different targets. Kinetic profiling was utilized in single-molecule-based super-resolution imaging and is, in principle, able to surpass the limitations of multicolor microscopy.

Nickel ions are efficient photostabilizers for single-molecule fluorophores, such as Cy5, Alexa Fluor 647, and ATTO 647N. Single-molecule fluorescence studies on various trisNTA–Alexa Fluor 647 constructs demonstrated that the photostability of Alexa Fluor 647 was improved by the presence of three nickel ions in complex with trisNTA. By rational optimization of the linker between the trisNTA and the fluorophore, an increased photon output of up to 25-fold was reported and led to improved photostability. Thus, the enhanced brightness of trisNTA–Alexa Fluor 647, combined with specific POI labeling, makes them ideal candidates for extended single-molecule imaging techniques.

5. Protein Tracking and Trafficking

To study subcellular protein dynamics, single-molecule tracking in live cells is the primary approach. However, both the tag and the probe size in combination with photostability issues of the fluorophores can severely limit its implementation. The Vogel lab first exploited reversible His-tag labeling by monodNTA to acquire single-molecule trajectories. By labeling engineered His-tags of the serotonin-gated 5HT₃ receptor, its mobility in the non-activated and activated state on the surface of live mammalian cells was studied. Also on living cells, local diffusion properties of specific receptors in distinct membrane compartments of adherent cells or neuronal synapses were obtained by the newly developed universal point accumulation for imaging in nanoscale topography (uPAINT) method. The transmembrane domain of the PDGF receptor, fused to a His₆-tag, was specifically labeled with trisNTA–ATTO 647N. In doing so, two-dimensional spatial maps of the molecular densities, as well as single-molecule trajectories, were recorded on a single cell (Figure 4a–c). Beneficial for the development of uPAINT was the nanomolar affinity of trisNTA for the His-tagged POI as well as the low trisNTA concentration (15 nM) used to prevent receptor labeling saturation. The tracking of single receptors in native cell membranes was also realized by quantum-yield-optimized trisNTA fluorophores. Furthermore, through the application of single-particle tracking on live cells, the dynamic nano-organization of integrins inside focal adhesions was revealed. Extracellular labeling of His₆-tagged β₃-integrin with trisNTA–ATTO 647N confirmed that β₃-integrins reversibly switch between immobilization and free-diffusion (Figure 4d–f). An increased immobilization inside focal adhesions or after integrin activation was detected, as well as a decreased immobilization of integrins unable to bind fibronectin or actin binding proteins (ABPs). Notably, longer trajectories could only be obtained by POI labeling with the more photostable fluorophore ATTO 647N via the trisNTA–His-tag interaction compared to the autofluorescent protein mEos2.

Besides bright organic dyes, fluorescent quantum dots (QDs) combine high brightness and photostability with wide absorbance and narrow emission spectra, making them ideal for long-term fluorescence observations. However, QD surface functionalization for specific protein targeting is non-trivial. Therefore, the groups of Pfeiler and Dahan designed and engineered trisNTA-functionalized QDs. By incorporating trisNTA into the amphiphilic, micellar coating of a QD, specific targeting of QDs to His-tagged POIs in solution, on functionalized surfaces, and on the membrane of living cells was facilitated. Apart from live-cell labeling of plasma-membrane-anchored His₁₀₉-tagged CFP (anchor: transmembrane domain of PDFR), QD tracking of the type-1 interferon receptor subunits at the plasma membrane was performed to examine receptor diffusion dynamics with extended duration times. In a subsequent report, the functionalization degree of QDs with trisNTA was controlled to be 1:1 by electrostatic repulsion. The monovalent trisNTA QDs were again successfully targeted to membrane-anchored CFP via its N-terminal His₁₀₉-tag and further enabled the measurement of...
the trajectories of His$_{10}$-tagged IFNAR2 on live cells by single-molecule tracking. Collectively, using trisNTA to label POIs with small and bright organic dyes allows for control over the labeling ratio as well as stable recognition, offering advantages for imaging at the single protein level.

6. Intracellular Delivery and Live-Cell Labeling

Early attempts to label POIs via the trisNTA–His-tag interaction were exclusively performed on the surface of living cells. Already in the seminal report by Vogel and co-workers, the His-tagged 5HT$_3$ receptor was visualized on live HEK293 cells by dye-conjugated monoNTA.\[10\] The emergence of multivalent trisNTA extended the selective fluorescence labeling of POIs on the surface of live, intact cells. Thus, full-length His$_{10}$-tagged IFNAR2 in the plasma membrane of live insect cells [12a] or His$_{10}$-tagged AMPA receptor complexes (GluA1) on a rat hippocampal neuron [43] were selectively stained with dye-conjugated trisNTA. QD-functionalized trisNTAs were likewise used to label extracellularly exposed His-tags of plasma-membrane-localized proteins on live cells.[48,49] An interesting no-wash, “turn-on” labeling approach was reported by Arai et al.[27b] Here, the displacement of a quencher peptide in complex with a dendritic trisNTA–tetramethylrhodamine (TMR) led to the specific binding of the fluorescent probe to the His-tagged protein on COS-7 cells and thus their fluorescence labeling. The Tsien and Hamachi labs established attractive alternatives for NTA-based MCHs to specifically label His-tagged proteins.[16,50] The fluorescent zinc complex HisZiFiT strongly binds to the His-tag and was utilized to label STIM1 (stromal interaction molecule 1) at the cell surface.[50] Remarkably, N-terminal fluorescent protein fusions interfered with STIM1 cell surface expression, whereas the short His$_{6}$-tag was accessible to the HisZiFiT dye conjugate.[50] Furthermore, zinc ions in complex with two IDA moieties were reported as fluorescent probes for His-tagged proteins and helped to visualize GPCRs on live-cell surfaces.[16]

Notwithstanding, the intracellular application of trisNTA or other MCHs in living mammalian cells has remained challenging owing to shortcomings in the efficient delivery of labeled MCHs. Dowdy and co-workers reported on the intracellular delivery of functional macromolecules, such as Cre recombinase, via complexation of His-tagged cargos with a trisNTA linked to a peptide transduction domain.[51] Orthogonally, we developed a carrier-complex-mediated delivery of fluorescent trisNTA conjugates for in-cell labeling.[52] The HIV-TAT$_{49-57}$ sequence was extended with a His$_{6}$-tag to deliver trisNTAs via complexation. After internalization, efficient and rapid intracellular targeting of His-tagged proteins in mammalian cells was observed. The in vivo labeling of the ER-resident TAP and the DNA-silencing complex methyl-CpG-binding protein 2 (MeCP2) was investigated. His-tag-specific labeling was further monitored in real-time and demonstrated high specificity and a high degree of co-localization, even in the crowded cellular environment. The specificity of trisNTA is highlighted by the fact that MeCP2 harbors an endogenous sequence of seven consecutive histidines in an unstructured C-terminal region.[52] Similarly, Hamachi and co-workers facilitated the in-cell labeling of His-tagged proteins with monoNTA and bisNTA.
via the cell-penetrating His$_x$-Arg$_y$ sequence.[53] Here, the MCH-induced proximity was used to covalently attach the probe via a cysteine-specific α-chloroacetamide group, an approach analogous to that already established for proteins at the plasma membrane.[54] A breakthrough for the delivery of MCHs into cells was achieved with the cell-squeezing-mediated delivery of tris-NTA.[55] Here, mammalian cells were mechanically pushed (squeezed) through defined micrometer constrictions in the presence of nanomolar concentrations of dye-conjugated trisNTA (Figure 5a). Cell squeezing allowed for high cell survival (more than 90%) and efficient uptake of trisNTA (up to 80%). The simplified and effective transfer of trisNTA probes into living mammalian cells enabled the precise control of the intracellular labeling concentration (0.2–100 nM). The high-affinity and target-specific tracing of His$_{10}$-tagged TAP, lamin A, and histone 2B by trisNTA was demonstrated with superior signal-to-noise and excellent colocalization of trisNTA and the target POI in various subcellular compartments (Figure 5b). Moreover, the delivery of trisNTA by cell squeezing allowed multiplexed labeling of POIs, combined with high specificity and low cytotoxicity. Finally, the robust in-cell targeting facilitated the visualization of target structures with nanometer precision at subnanometer resolutions.[19] This approach permitted instantaneous imaging and lead to live-cell super-resolution imaging of the nuclear lamina by dSTORM. The specificity, high-affinity, and bioorthogonality of the trisNTA–His-tag labeling can thus complement other prominent protein-labeling methods, including SNAP- or Halo-tag modification for extracellular and in-cell targeting, respectively.

### 7. Summary and Outlook

Site-specific modifications of proteins have significantly expanded the range of applications for exploring their function, localization, and dynamics, including the application of live-cell fluorescence microscopy and single-molecule imaging. The site-specific, close-proximity, and high-affinity protein modification with MCHs, and in particular trisNTA, via the small His$_{12}$-tag has high potential for selective protein targeting and the study of protein-driven processes by advanced imaging techniques. It offers an attractive alternative to complementary labeling methods, such as SNAP-tagging, not only for labeling, but also for protein manipulation in native environments. Despite all the advantages of the MCH–His-tag interaction, the method has also some limitations as does any other. For instance, the genetic modification of target cells, often the need for cell fixation before imaging, possible inaccessibility of the His-tag due to steric shielding or unexpected changes of protein properties (for example, decreased solubility, misfolding, or dimerization) need to be considered before implementation of this method. Aside from the studies presented here, the value of the trisNTA–His-tag interaction is further revealed by its application in 1) protein tethering to membranes,[12b,64] liposomes,[6b] glass surfaces,[65] gold interfaces,[9a,66] and nanoparticles,[67] 2) guided high-affinity protein trans-splicing and “traceless” labeling,[50] 3) stochastic sensing of single mole-

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**Figure 5.** Live-cell labeling of His-tagged protein assemblies in distinct subcellular compartments. a) Delivery of trisNTA–fluorophore conjugates into living cells by microfluidic cell squeezing. Cells are pressed through micrometer constrictions, which cause the formation of transient holes in the plasma membrane and enable trisNTA to transfer into the cytosol. b) Specific labeling of His-tagged proteins in living cells at diverse subcellular localizations. trisNTA–Alexa Fluor 647 was delivered by squeezing cells transfected with His$_{10}$-tagged TAP, lamin A, or histone 2B (H2B). High target specificity of trisNTA–Alexa Fluor 647 was observed. Scale bar: 5 μm. c) Reconstructed dSTORM image of His$_{10}$-tagged lamin A labeled with trisNTA–ATTO 655 in a living HeLa Kyoto cell. Increased spatial resolution (<40 nm) was obtained in live-cell super-resolution imaging of trisNTA–ATTO 655 by dSTORM (left and magnification right), compared with the wide-field image (left corner, bottom).[25] Scale bar: 2 μm. Open access, no permission required.
cules in nanopores,[37] 4) ultracentrifugation[38] and flow cytometry,[39, 5] protein manipulation[39] and knock-down.[40]
A high degree of versatility of the labeling system is given by the relatively simple construction of novel NTA-based probes or metal-chelation methods. An interesting enhancement of the small lock-and-key interaction pair is provided by photo-activatable trisNTA, as it allows spatial and temporal control of protein manipulation.[41] Nevertheless, several challenges lie ahead, including enhanced accuracy, multiplexing, and protein tagging in tissues or even animals. With the continuous advancements in spectroscopy and microscopy techniques, novel requirements and applications for MCHs will arise. In this regard, the ease of MCH design, their rapid synthetic adaptability, and functional versatility will synergistically help to enhance MCHs for chemical biology approaches as well as life science studies.

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Conflict of interest

The authors declare no conflict of interest.

These are not the final page numbers!
His-tags reloaded: Protein labeling demands high specificity, rapid as well as efficient conjugation, while maintaining low concentration and biocompatibility under physiological conditions. In this context, recent progress in the recognition of multivalent chelator heads with the small, genetically engineered His-tag fused to proteins is highly relevant.