ACCOUNTS
of chemical research

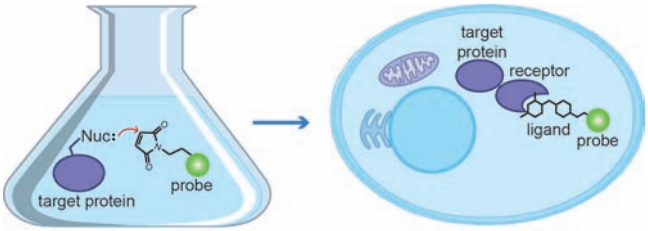
Chemical Tags for Labeling Proteins Inside Living Cells

CHAORAN JING AND VIRGINIA W. CORNISH*

Department of Chemistry, Columbia University, 550 West 120th Street, MC 4854, NWC Building, New York, New York 10027, United States

RECEIVED ON MARCH 31, 2011

CONSPECTUS



20th century - labeling purified proteins in test tubes

21st century - labeling proteins in the complex environment of live cells

To build on the last century's tremendous strides in understanding the workings of individual proteins in the test tube, we now face the challenge of understanding how macromolecular machines, signaling pathways, and other biological networks operate in the complex environment of the living cell. The fluorescent proteins (FPs) revolutionized our ability to study protein function directly in the cell by enabling individual proteins to be selectively labeled through genetic encoding of a fluorescent tag. Although FPs continue to be invaluable tools for cell biology, they show limitations in the face of the increasingly sophisticated dynamic measurements of protein interactions now called for to unravel cellular mechanisms. Therefore, just as chemical methods for selectively labeling proteins in the test tube significantly impacted in vitro biophysics in the last century, chemical tagging technologies are now poised to provide a breakthrough to meet this century's challenge of understanding protein function in the living cell.

With chemical tags, the protein of interest is attached to a polypeptide rather than an FP. The polypeptide is subsequently modified with an organic fluorophore or another probe. The FlAsH peptide tag was first reported in 1998. Since then, more refined protein tags, exemplified by the TMP- and SNAP-tag, have improved selectivity and enabled imaging of intracellular proteins with high signal-to-noise ratios. Further improvement is still required to achieve direct incorporation of powerful fluorophores, but enzyme-mediated chemical tags show promise for overcoming the difficulty of selectively labeling a short peptide tag.

In this Account, we focus on the development and application of chemical tags for studying protein function within living cells. Thus, in our overview of different chemical tagging strategies and technologies, we emphasize the challenge of rendering the labeling reaction sufficiently selective and the fluorophore probe sufficiently well behaved to image intracellular proteins with high signal-to-noise ratios. We highlight recent applications in which the chemical tags have enabled sophisticated biophysical measurements that would be difficult or even impossible with FPs. Finally, we conclude by looking forward to (i) the development of high-photon-output chemical tags compatible with living cells to enable high-resolution imaging, (ii) the realization of the potential of the chemical tags to significantly reduce tag size, and (iii) the exploitation of the modular chemical tag label to go beyond fluorescent imaging.

Introduction

Chemical methods for site-specifically labeling proteins with organic fluorophores and other biophysical probes significantly impacted fundamental studies of proteins in vitro in the last century. Evidence of the utility of these chemical probes, chemical modification of purified proteins for microinjection into cells, is still utilized today for live cell imaging, despite the fact that microinjection is technically demanding and damaging to the cell.[1] The chemical probes designed to react selectively with Cys and Lys residues that are so effective at labeling purified proteins in vitro, however, simply do not provide the selectivity required to label an individual protein of interest in the sea of proteins and other reactive species present in the cell.

Published on the Web 08/31/2011 www.pubs.acs.org/accounts
10.1021/ar200099f

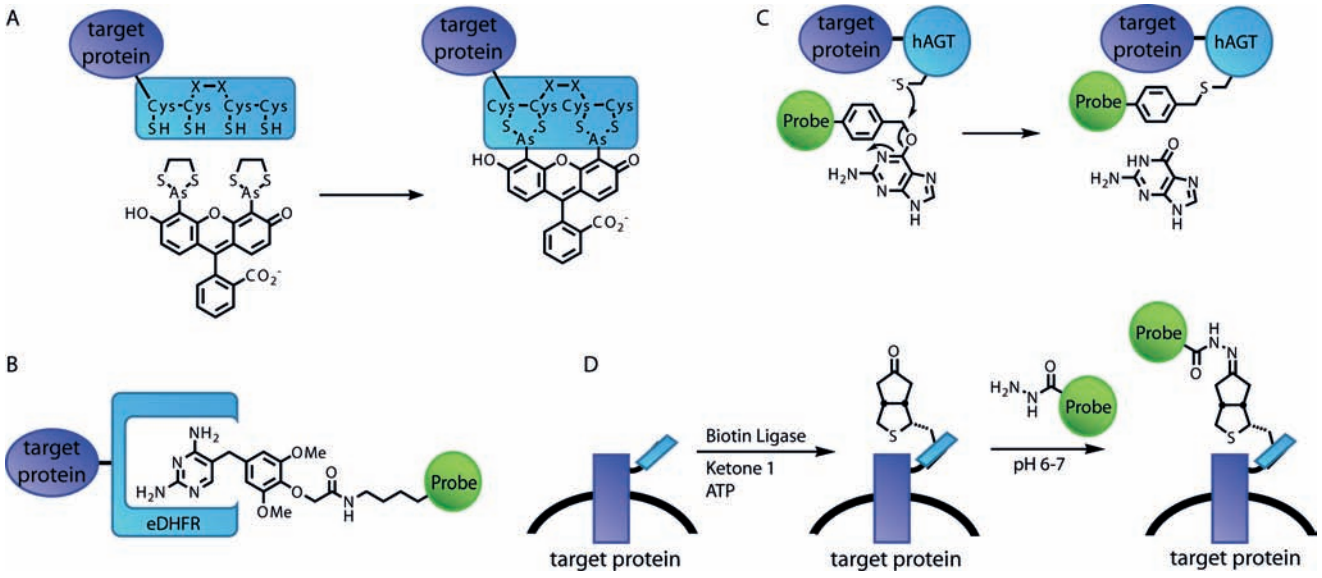


FIGURE 1. Schematic illustration of representative example technologies of different strategies for selectively labeling proteins in living cells. (A) The FlAsH tag features a short peptide with a tetracysteine core that directly binds bisarsenical fluorogens. (B) The eDHFR/TMP labeling strategy is based on noncovalent, high-affinity binding of TMP-probe heterodimers by the protein eDHFR. (C) In the SNAP-tag, the enzyme hAGT utilizes a guanine-probe heterodimer as a suicide substrate. (D) Biotin ligase enzymatically modifies a short peptide tag with a biotin analogue, which then reacts with the probe in a second step.

A breakthrough for live cell imaging came with the introduction of fluorescent proteins (FPs) in 1994 as selective, genetic protein tags.[2,3] The original green fluorescent protein (GFP) from *A. victoria* is a 238 amino acid protein, which upon folding spontaneously forms a fluorescent chromophore by rearrangement and oxidation of Ser, Tyr, and Gly residues in the core of the 11-stranded β-barrel.[4,5] Since these original reports, naturally occurring and engineered FPs have been optimized for spectral variation, increased brightness and other properties to provide a wealth of reagents for cell biologists.[6,7] FP tags are used routinely to observe the timing and location of protein expression in living cells, often providing significant mechanistic insight.[4,6,8] However, FPs have limitations for more sophisticated biophysical and mechanistic studies. As ~30 kD proteins, FPs can disrupt the assembly, interaction, or function of the labeled protein; FPs typically have broad absorption and emission spectra, making it technically demanding to monitor even just three different proteins simultaneously using multicolor imaging;[7] FPs can suffer from oligomerization and/or slow folding and chromophore maturation; it is difficult to manipulate the fluorophore for specialized properties since it is inherent to the sequence of the FP; and significantly, none of the FPs can be compared to the best organic fluorophores, much less nanoparticles or other emerging chemical probes, in terms of photon output (often measured as brightness and photostability), critical for single-molecule resolution. Thus, chemical tags have been developed to provide an alternative for labeling proteins with chemical probes directly in living cells.

Protein Labeling via Chemical Tags

With chemical tags, rather than tagging the target protein with an FP, the protein is tagged with a polypeptide, which is subsequently modified with an organic fluorophore. Technically, labeling a target protein with a chemical tag is very similar to labeling with FPs: a plasmid encoding a fusion between the target protein and polypeptide tag is constructed using standard molecular cloning techniques and then introduced into the desired cell. The transfected cells are briefly incubated with the organic fluorophore, which diffuses into cells and is then specifically bound by or conjugated to the polypeptide tag. Thus, chemical tags retain the specificity of protein labeling achieved with FPs through genetic encoding but provide smaller, more robust tags and modular use of organic fluorophores with high photon-output and tailored functionalities.

The first report of a chemical surrogate to FPs for labeling proteins with organic fluorophores in living cells was FlAsH from Tsien and co-workers in 1998.[9] In design, FlAsH is the ideal chemical tag, a short 15 amino acid polypeptide tag with a tetracysteine core (CCXXCC) that is covalently labeled with a fluorogenic bisarsenical fluorescein ligand whose fluorescence increases upon binding to the polypeptide

tag (Figure 1A). To date, a number of bisarsenical fluorophores and corresponding tetracysteine (TC) tags have been reported,[10−12] among which the original green-fluorescent FlAsH and the red-fluorescent ReAsH are most frequently utilized. Despite its elegant design, the FlAsH technology suffers practically from nonspecific labeling of thiol-rich biomolecules in the cell and toxicity of the bisarsenical ligands and labeling conditions.[13] Nonetheless, benefiting from its small size, FlAsH often is the only viable tag for labeling proteins or complexes impaired by the ~250 amino acid FPs and is widely reported and has enabled many experiments not otherwise possible.[14]

Protein-Based Chemical Tags. To overcome the selectivity limitations of a short peptide tag, protein-based chemical tags were introduced that allowed the target protein to be tagged with a protein receptor or enzyme, which can be subsequently labeled with a cell-permeable organic ligand- or substrate-probe heterodimer. There are several critical design issues for these protein receptor−ligand or enzyme−substrate tags. One, and perhaps most importantly, the organic fluorophore ligand or substrate must be readily cell-permeable and not binding nonspecifically to endogenous proteins or other biomolecules or, equally important but perhaps less appreciated, otherwise partitioning to particular organelles within the cell. Two, the synthesis of the ligand or substrate derivatives should be straightforward and minimally disruptive to the receptor binding or enzyme function. Three, the protein receptor or enzyme should be small, monomeric, and well behaved for minimal perturbation of the biological pathway being studied. Fourth, the labeling reaction between the protein tag and the ligand/substrate-probe should be rapid and near quantitative. To date, the advantage of protein tags over other chemical tags is that they are sufficiently selective and efficient to enable intracellular proteins to be imaged with high signal-to-noise.

In collaboration with the Sheetz group, our laboratory has exploited the high-affinity interaction between dihydrofolate reductase (DHFR) and folate analogues to label proteins in living cells. Briefly, we tag the target protein with *E. coli* DHFR (eDHFR). Because eDHFR binds trimpethoprim (TMP) with high affinity (1 nM K_D) and selectivity (affinities for mammalian DHFRs are $K_D > 1\ \mu$M), the eDHFR tag can then be labeled with near stoichiometric concentrations of TMP-probe heterodimers that bind to eDHFR noncovalently with a dissociation half-life of tens of minutes (Figure 1B).[19,30,31] Consistent with TMP's use therapeutically as an antibiotic, the TMP-probe heterodimers have excellent cell permeability and solubility properties. As anticipated based on high-resolution structural and structure−activity relationship data, commercially available TMP can be modified at the para-methoxy position with only minor perturbation of high-affinity binding to eDHFR, making the synthesis of TMP-probe labels very straightforward. As a 159 amino acid, monomeric, well-behaved protein, eDHFR is about two-thirds the size of FPs, does not suffer from oligomerization and expression problems, folds rapidly, and circumvents the issue of chromophore maturation half-life.[32,33] Among chemical tags, the TMP-tag stands out for enabling the imaging of *intracellular* proteins with high resolution in living cells.

Our efforts to image individual proteins in the focal adhesion complex in mammalian cells using the TMP-tag taught us that the key performance issue for the tag is not so much the cell-permeability of the TMP-probe label, but really the solubility of the TMP-probe label once it is inside the cell. Significantly, through optimization of protecting groups and linkers, we obtained TMP-fluorophore labels that exhibited minimal nonspecific partitioning to lipid-rich cellular compartments and could thus be utilized to image more dilute, rapidly diffusing cytoplasmic proteins with high signal-to-noise.[19] The palette of TMP-fluorophores able to image intracellular proteins has been expanded from fluorescein-based green and red dyes to include a far-red photoswitching Atto-655, a two-photon fluorophore BC575, and lanthanide probes.[20,34,35] Important for adoption by cell biologists and biophysicists (i.e., laboratories not specializing in organic synthesis), the TMP-tag is commercially available from Active Motif as LigandLink.

Remarkably, the TMP-tag now has also been rendered covalent by installing a unique Cys residue on eDHFR in position to react with a latent acrylamide electrophile added to the TMP-probe molecule via a classic proximity-induced Michael addition.[36] While under optimization, already this first-generation covalent TMP-tag undergoes rapid, quatitative covalent labeling (in vitro $t_{1/2} \sim 50$ min) and enables imaging of nuclear-localized eDHFR in live NIH3T3 cells. This work demonstrates that an advantage to a chemical tag based on high-affinity binding is that it does not require the high concentration of ligand−probe conjugate necessary with enzyme-based chemical tags, where K_M's typically range from micromolar to millimolar, leading to high background noise from unbound fluorophore and necessitating extensive washing steps.[29,37]

An alternative strategy to protein-based chemical tags, the "SNAP-tag" utilizes O^6-modified guanine-fluorophore heterodimers to covalently label proteins fused to human O^6-alkylguanine-DNA-alkyltransferase (hAGT), a 20 kD,

TABLE 1. Chemical Tags and Corresponding Fluorophores Used to Image Functional Proteins Inside Living Mammalian Cells

chemical tag	fluorophore	target protein	ref
FlAsH; ReAsH	Fluorescein; Oxazine	Cx43; α-tubulin; Cytochrome *c*; β-actin	14−17
TMP-tag	Fluorescein	MLC	18, 19
	Hexachlorofluorescein	MLCK; α-tubulin	19
	Atto655	H2B	20
SNAP-tag	Tetramethylrhodamine (TMR)	CENA; MAP2; Vimentin; Cx43	21−23
	Atto633	STAT5b	24
	Fluorescein	α-tubulin; γ-tubulin	25
	Peroxy-Green	NK1R; H2B	26
	Quenched Fluorescein (Q-Fl)	MEK1; FRB protein	27
HaloTag	TMR	p65, IκBα	28
Coumarin ligase	Coumarin	β-actin, Vimentin; MAP2	29

monomeric DNA repair protein that naturally dealkylates O^6-alkylated guanine residues in damaged DNA by a single turnover alkylation of an active-site Cys residue (Figure 1C).[37] A fast-reacting SNAP-tag variant has been engineered to minimize background labeling of endogenous mammalian AGT.[38] Impressively, an orthogonal AGT variant that selectively uses cytosine fluorophores as substrates, called CLIP-tag, has been evolved, although it will require further optimization to be as robust as the SNAP-tag.[37,39] A wide range of SNAP- and CILP-fluorophores are commercially available from New England Biolabs, and a subset of these have been confirmed to work inside living cells (Table 1). Likewise, Promega has developed a covalent chemical tag based on the reaction of an engineered dehalogenase enzyme with a suicide substrate, "HaloTag",[26,28] which has been shown to be a useful tag in vitro, but reports indicate it may suffer efficiency and selectivity issues inside cells.[40]

New protein-based chemical tags continue to be introduced, but most of these are yet to be sufficiently vetted, particularly for the demanding task of labeling proteins intracellularly, to judge their practical performance at this time. These tags include a cutinase variant that reacts with a suicide substrate;[41] a fluorogenic tag in which a β-lactamase variant displaces a quencher from a cephem suicide substrate;[42,43] and a noncovalent tag where synthetic ligand of FK506 (SLF) binds to FKBP12:F36 V.[44]

Peptide-Based Chemical Tags. With chemical tagging, it should be possible to replace protein tags, which can interfere with protein interactions and pathway function, with very short peptide tags. The challenge for the field now is to devise conceptually new strategies for chemically modifying short peptides with fluorophores that overcome current limitations in the selectivity with which a short peptide tag can be recognized over the many other reactive molecules present in a living cell.

The Ting laboratory has pioneered the re-engineering of natural enzymes that modify short peptides with organic molecules to fluorophore labeling.[45] The most advanced of these chemical tags, the *E. coli* biotin ligase (BirA) enzyme, whose natural function is biotinylation of proteins containing a peptide recognition motif, is used to label a 15 amino acid peptide tag with a biotin analogue, which is subsequently modified with a fluorophore (Figure 1D).[45,46] To date BirA has resisted more dramatic modification to enable direct incorporation of a biotin−fluorophore conjugate. Intracellular protein labeling, therefore, is still difficult because the second reaction between the biotin analogue and the fluorophore is slow and incompletely selective at the micromolar concentrations typical in the cell. An exciting recent advance for the potential it illustrates, a lipoic acid ligase variant was evolved to use a coumarin derivative directly, although coumarin itself is not an ideal fluorophore for live cell imaging.

A variety of enzyme-mediated peptide tags have now been reported, including the acyl-carrier protein (ACP)-based tag modified by phosphopantetheine transferase (PPTase);[47] the 6-amino acid peptide modified by sortase;[48,49] and the formylglycine-generating enzyme-based tag reported by Bertozzi et al.[50] To date, all of these aforementioned peptide tags have only been demonstrated to work on the cell surface.

Beyond enzyme-mediated peptide tags, other clever approaches to short peptide tags are being explored. Similar to the FlAsH tag, a tetraserine peptide tag was demonstrated to bind a fluorogenic bis-boronic acid derivative.[51] In a very recent report, Chang and co-workers developed a peptide tag that undergoes a Michael addition with a BODIPY fluorophore.[52] Inspired by the popular polyhistidine tag for protein purification, various metal-chelating peptides have been adapted for protein labeling.[53−57] In an interesting

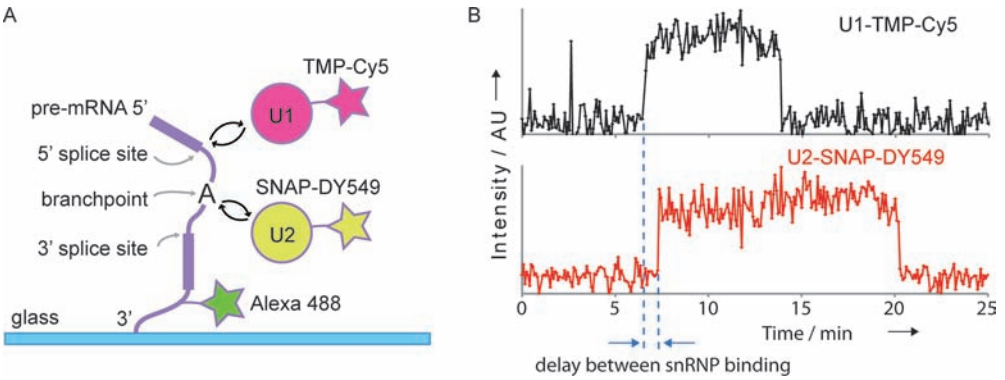


FIGURE 2. Experimental setup and representative single-molecule trace of pre-mRNA splicing in yeast cell extracts imaged with chemical tags, providing a unique way to access the dynamic mechanism of pre-mRNA splicing. (A) Pre-mRNA labeled with Alexa488 is immobilized on a glass slide. Pairs of the snRNP complexes that make up the spliceosome, shown here U1 and U2, are genetically encoded in fusion with eDHFR and hAGT, respectively, allowing for orthogonal labeling with TMP-Cy5 and SNAP-DY549 in yeast cell extract. (B) The arrival and departure of each snRNP complex is visualized as the appearance and disappearance of fluorescence signal above the baseline. The delay between U1 binding and U2 binding provided direct evidence that assembly of the spliceosomal components is a highly ordered process and, surprisingly, the single-molecule traces also showed the association and disassociation of each component is highly dynamic.

approach, Nolan and co-workers evolved a 38-amino acid peptide that binds the Texas red fluorophore with high affinity.[58] A N-terminal Cys residue generated in vivo with a sequence-specific protease has been labeled with thioesters, analogous to native chemical ligation.[59] Although these different strategies are at an early stage of development, they illustrate the creativity with which chemistry can be exploited in a living cell, making a significant impact not only for live cell imaging, but more broadly for synthetic biology.

Application of Chemical Tags

The chemical tags have come of age, and the measure of their value is now their ability to enable experiments in living cells that are difficult or not otherwise possible with FPs. Because the best organic fluorophores emit about 10 times as many photons as their FP counterparts,[60,61] there is intense interest in exploiting chemical tags for single-molecule imaging in living cells. Moreover, with their modular design, chemical tags are not limited to fluorescent labeling and can be creatively co-opted as useful tools for a variety of applications in living cells, and also in vitro. Here we highlight just a few of the numerous applications of chemical tags to outstanding questions of biological mechanism that illustrate some of the different unique capabilities of chemical tags.

High-Resolution Imaging Enabled by Chemical Tags. In very recent results, our TMP-tag has been exploited in combination with the SNAP-tag to enable single-molecule imaging of the spliceosome in yeast cell extracts (Figure 2), an experiment not possible with the lower photon-output FPs.[62] Despite intense interest in understanding the mechanism by which pre-mRNAs are correctly spliced to mature mRNAs, the splicoesome is difficult to study because it is a complex 2-3 MD machinery of protein and RNA and cannot be reconstituted in vitro from purified components. The TMP- and SNAP-tags allowed pairs of the small nuclear ribonucleoprotein (snRNP) components of the spliceosome to be labeled directly in cell extracts with high photon-output dyes and imaged as they assembled on individual pre-mRNAs. The next advance will be to have 2-3 orthogonal, high photon-output chemical tags enabling single-molecule imaging *inside* cells (the cyanine dyes employed in this work require a deoxygenated environment).

Likewise, the high photon-output chemical tags are beginning to impact "super-resolution" (SR) imaging technologies that break the diffraction barrier to allow fluorescence imaging at the nanometer length scale of natural proteins (Figure 3).[63−65] Stochastic SR imaging technologies including PALM (photoactivatable localization microscopy) and STORM (stochastic optical reconstruction microscopy) hinge on *photoswitchable* fluorophores with high photon-output that enable the locations of subsets of the total fluorophore population to be determined precisely over time. Until recently, SR imaging has typically relied on either photoactivatable FPs, which have limited photon-output and palletes, or antibodies conjugated with organic fluorophores, which offer higher resolution and many more colors, but are incompatible with live cells. Chemical tags have the potential to combine the advantages of these two contrasting labeling methodologies; they are genetically encoded and thus compatible with live cells, and they are modular and

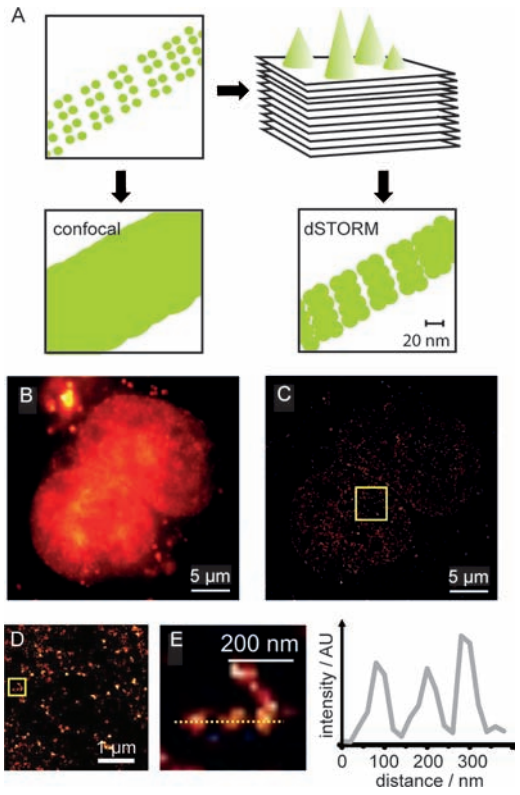


FIGURE 3. Super-resolution dSTORM imaging of histone protein 2B (H2B) using TMP-tag. (A) In conventional confocal microscopy, the resolution set by the diffraction limit of light is ~200 nm, and thus individual protein molecules cannot be resolved. In PALM/STORM, small percentages of the total population of fluorophores are randomly photoactivated over time, allowing all individual fluorophores to be localized to resolutions of ~20 nm from the Gaussian fits of their point spread functions. (B) Total internal reflection fluorescence image of TMP-Atto655 tagged H2B in the nucleus of living HeLa cells and (C) corresponding dSTORM image with improved resolution. The expanded views and cross-sectional profiles (D, E) demonstrate superior resolution well below the diffraction barrier. Adjacent histone proteins separated by ~100 nm are clearly resolved.

thus allow use of photoswitchable high photon-output organic fluorophores.[20,66,67] Bringing this potential to reality, this past year, building on the discovery by Sauer et al. that the reducing environment of the cell catalyzes reversible photoswitching of high photon-output Atto organic fluorophores at time scales well suited for direct STORM (dSTORM) maging,[68] we demonstrated dynamic dSTORM imaging of H2B labeled with TMP-Atto-655.[20] Significantly, we achieved exceptional spatial (~20 nm) and temporal (~10 s) resolutions, which are not possible with FPs.

Themes and Variations on Cell Imaging with Chemical Tags. Unlike FPs, chemical tags are not limited to traditional fluorescent imaging. For example, in a creative adaptation of classic pulse-chase labeling, the FlAsH tag was exploited for correlative fluorescence and electron microscopy (EM) of the

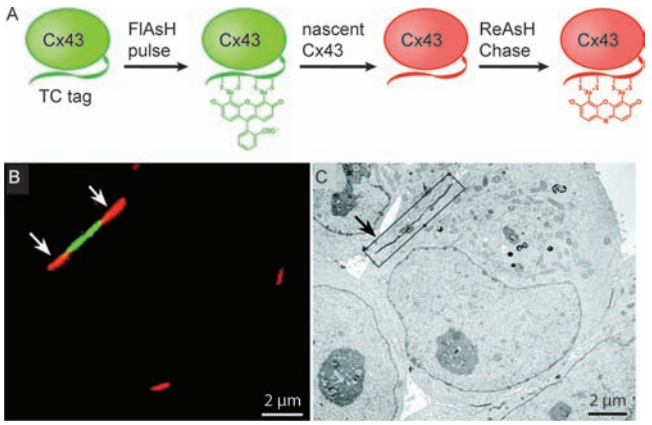


FIGURE 4. Pulse-chase labeling of Cx43 with FlAsH and ReAsH and correlative fluorescence and EM images. Oligomers of Cx43 form gap junctions on plasma membranes through which metabolites and signaling molecules are exchanged between cells. Tetracysteine tags have enabled pulse-chase experiments to observe the dynamic assembly and turnover of junctional plaque with minimal disturbance on Cx43 structure and function. (A) Cellular Cx43 is first "pulsed" with green-fluorescent FlAsH tag, and then the nascent Cx43 is "chased" with red-fluorescent ReAsH tag. (B) Nascent Cx43s are observed to be added to the periphery of the junctional plaque, indicated by the arrows. (C) Correlative EM shows high-resolution image of Cx3 juncitional plaque (indicated by arrow) in the context of other subcellular structures. (B, C courtesy of R.Y. Tsien.)

assembly of connexin43 (Cx43) to form gap junctions between cells (Figure 4). Cx43 is a small protein forming tightly packed oligomers at gap junctions on the plasma membrane. Tagged Cx43 was labeled with FlAsH (pulse) and, after a period of time, ReAsH (chase). Fluorescence and EM unequivocally showed, for the first time, that nascent Cx43s are added to the periphery of the gap junction plaque.[14]

Enzyme-mediated peptide tags have been reinvented as unique reporters of endocytosis. To monitor the internalization of cell surface receptors, it is necessary to differentially label receptors on the cell surface and those internalized into the cell, which is difficult with FPs. Using chemical tags, however, the receptors on the cell surface can be selectively labeled with fluorophores that are not cell permeable and subsequently quenched if they are on the cell surface, in order to detect internalized receptors. The biotin ligase (BirA)/streptavidin labeling technology has been applied to image the endocytosis of low density lipoprotein (LDL) receptor, showing that LDL receptors are internalized as oligomers in the presence and absence of LDL ligand (Figure 5).[69]

There seems to be no limit to the creative extensions of chemical tags. The TMP-tag, SNAP-tag, and FlAsH-tag all have been exploited for chromophore assisted laser inactivation (CALI), in which the target protein is selectively

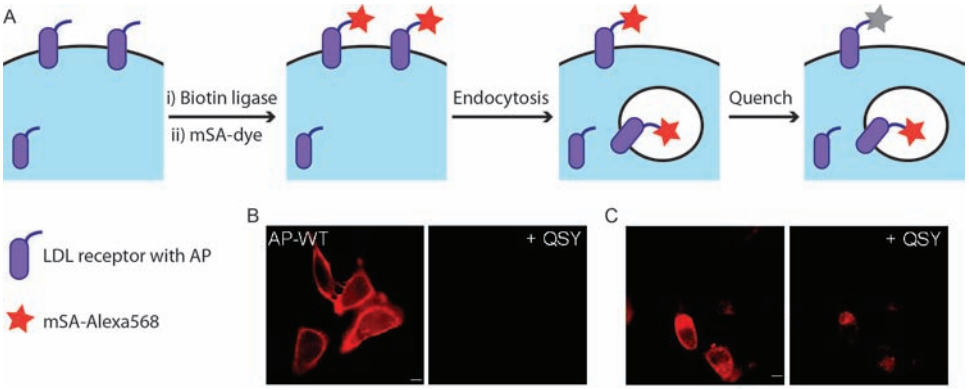


FIGURE 5. Cartoon and live cell imaging of the BirA-based peptide tag used to visualize internalization of low density lipoprotein (LDL) receptors. (A) LDL receptors are fused to biotin acceptor peptide (AP) and are expressed both inside and on the surface of the cell. Only cell surface receptors can be labeled with Alexa568 via monomerized streptavidin (mSA), which is not cell-permeable. After endocytosis, cell surface receptors are quenched with QSY quencher, such that internalized receptors can be selectively visualized. (B) Fluorescence images of cells immediately before and after surface fluorescence quenching (+QSY). With no incubation to allow for endocytosis, very few internalized receptors are detected. (C) With 5 min incubation at 37 °C, some LDL receptors are internalized and thus protected from QSY quenching. (B, C courtesy of A.Y. Ting.)

inactivated by locally damaging reactive oxygen species released by the fluorophore upon irradiation;[18,25,70] The SNAP- and CLIP-tags have been used in conjunction to detect protein−protein interactions;[71] and a variant of the tetracysteine-FlAsH-tag has been designed to read out protein folding and association.[72] Arguably, chemical tags are all "chemical inducers of dimerization" (CIDs) just applied specifically to fluorescent imaging, and thus, the corollary of this argument is that chemical tags can be utilized as CIDs for a myriad of applications.[73]

Chemical Tags Are Biotin/Streptavidin Surrogates for in Vitro Applications. While this Account focuses on live cell imaging, it should be noted that the chemical tags also can be viewed as a modern equivalent to biotin/streptavidin for in vitro engineering, with the benefits of being monomeric, possibly covalent, and more readily reversible.[28] Already chemical tags (notably HaloTag) have been utilized as orthogonal alternatives to biotin/streptavidin for protein purification and immobilization; labeling proteins in sensitive macromolecular complexes; and, in materials chemistry, for surface patterning, although a comprehensive survey on these in vitro applications is beyond the scope of this Account.[74]

Future Development of the Chemical Tagging Technology

As the chemical tagging technology transitions from a proof-of-principle stage to widespread adoption by cell biologists and biophysicists interested in studying protein function in the complex environment of the cell, there is an immediate need for multiple orthogonal chemical tags for multicolor imaging in living cells. Perhaps surprisingly, given that new chemical tags are now regularly reported in the literature, the field still needs additional chemical tags that are sufficiently selective for intracellular protein labeling. Moreover, while virtually any known fluorophore can be conjugated to the now large repertoire of chemical tags; there are still very few reports of high photon-output fluorophore tags that work inside the cell.

Another important advance will be the realization of the potential of chemical tags to dramatically decrease tag size. There are tantalizing recent results showing that it will be possible to render the existing enzyme-mediated peptide tags functional inside living cells. In our opinion, unnatural amino acid mutagenesis has the potential to provide a breakthrough with direct incorporation of organic fluorophores as amino acid side chains, although this advance will require (1) expanding the substrate specificity of the translational machinery for incorporation of the high photon-output, red-shifted organic fluorophores and (2) overcoming ubiquitous incorporation of the fluorescent amino acid in response to the numerous stop codons present in the transcriptome.

Finally, while chemical tags offer advantages for fluorescence imaging over FPs with modular incorporation of fluorophores with higher photon-output and specialized properties such as photoswitching, the potential of these modular tags for introducing probes beyond fluorophores is surprisingly largely unexplored. It will be exciting to see if chemists are able to exploit these modular tags in the coming decade to invent entirely new spectroscopies to meet the challenge of understanding biological mechanism in the context of the living cell.

We would like to thank Dr. Aaron Hoskins and Tracy Y. Wang for helpful discussions and advice on the manuscript. The authors are supported by grants to V.W.C. from the NIH (5RC1GM091804-02, U54GM087519-01A1).

BIOGRAPHICAL INFORMATION

Chaoran Jing is a graduate student at Columbia University in the Department of Chemistry. She completed her undergraduate degree in Chemistry from Tsinghua University in Beijing in 2008. She is developing next generation TMP-tags for single-molecule imaging and exploiting the TMP-tag to understand the mechanism of force-dependent signaling in cell motility.

Virginia W. Cornish is a Professor in the Department of Chemistry at Columbia University. She received a B.A. in Biochemistry *summa cum laude* from Columbia University in 1991 and a Ph.D. in Chemistry from the University of California, Berkeley, in 1996, followed by postdoctoral research in the Department of Biology at the Massachusetts Institute of Technology. Her research has been recognized by numerous awards, including most recently the ACS Pfizer Award in Enzyme Chemistry and the Protein Society Irving Sigal Young Investigator Award. Her laboratory focuses on the development of conceptually new synthetic methods for carrying out chemistry in living cells with application to tools for live cell imaging.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: vc114@columbia.edu.

REFERENCES

1 Danuser, G.; Waterman-Storer, C. M. Quantitative Fluorescent Speckle Microscopy of Cytoskeleton Dynamics. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 361–387.

2 Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C. Green Fluorescent Protein as a Marker for Gene Expression. *Science* **1994**, *263*, 802–805.

3 Heim, R.; Prasher, D. C.; Tsien, R. Y. Wavelength Mutations and Posttranslational Autoxidation of Green Fluorescent Protein. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12501–12504.

4 Tsien, R. Y. The Green Fluorescent Protein. *Annu. Rev. Biochem.* **1998**, *67*, 509–544.

5 Ormo, M.; Cubitt, A. B.; Kallio, K.; Gross, L. A.; Tsien, R. Y.; Remington, S. J. Crystal Structure of the Aequorea Victoria Green Fluorescent Protein. *Science* **1996**, *273*, 1392–1395.

6 Giepmans, B. N.; Adams, S. R.; Ellisman, M. H.; Tsien, R. Y. The Fluorescent Toolbox for Assessing Protein Location and Function. *Science* **2006**, *312*, 217–224.

7 Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y. A Guide to Choosing Fluorescent Proteins. *Nat. Methods* **2005**, *2*, 905–909.

8 Shaner, N. C.; Patterson, G. H.; Davidson, M. W. Advances in Fluorescent Protein Technology. *J. Cell Sci.* **2007**, *120*, 4247–4260.

9 Griffin, B. A.; Adams, S. R.; Tsien, R. Y. Specific Covalent Labeling of Recombinant Protein Molecules inside Live Cells. *Science* **1998**, *281*, 269–272.

10 Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. New Biarsenical Ligands and Tetracysteine Motifs for Protein Labeling in Vitro and in Vivo: Synthesis and Biological Applications. *J. Am. Chem. Soc.* **2002**, *124*, 6063–6076.

11 Bhunia, A. K.; Miller, S. C. Labeling Tetracysteine-Tagged Proteins with a Splash of Color: A Modular Approach to Bis-Arsenical Fluorophores. *ChemBioChem* **2007**, *8*, 1642–1645.

12 Cao, H.; Xiong, Y.; Wang, T.; Chen, B.; Squier, T. C.; Mayer, M. U. A Red Cy3-Based Biarsenical Fluorescent Probe Targeted to a Complementary Binding Peptide. *J. Am. Chem. Soc.* **2007**, *129*, 8672–8673.

13 Hoffmann, C.; Gaietta, G.; Zurn, A.; Adams, S. R.; Terrillon, S.; Ellisman, M. H.; Tsien, R. Y.; Lohse, M. J. Fluorescent Labeling of Tetracysteine-Tagged Proteins in Intact Cells. *Nat. Protoc.* **2010**, *5*, 1666–1677.

14 Gaietta, G.; Deerinck, T. J.; Adams, S. R.; Bouwer, J.; Tour, O.; Laird, D. W.; Sosinsky, G. E.; Tsien, R. Y.; Ellisman, M. H. Multicolor and Electron Microscopic Imaging of Connexin Trafficking. *Science* **2002**, *296*, 503–507.

15 Goldstein, J. C.; Munoz-Pinedo, C.; Ricci, J. E.; Adams, S. R.; Kelekar, A.; Schuler, M.; Tsien, R. Y.; Green, D. R. Cytochrome C Is Released in a Single Step During Apoptosis. *Cell Death Differ.* **2005**, *12*, 453–462.

16 Martin, B. R.; Giepmans, B. N.; Adams, S. R.; Tsien, R. Y. Mammalian Cell-Based Optimization of the Biarsenical-Binding Tetracysteine Motif for Improved Fluorescence and Affinity. *Nat. Biotechnol.* **2005**, *23*, 1308–1314.

17 Andresen, M.; Schmitz-Salue, R.; Jakobs, S. Short Tetracysteine Tags to Beta-Tubulin Demonstrate the Significance of Small Labels for Live Cell Imaging. *Mol. Biol. Cell* **2004**, *15*, 5616–5622.

18 Cai, Y.; Rossier, O.; Gauthier, N. C.; Biais, N.; Fardin, M. A.; Zhang, X.; Miller, L. W.; Ladoux, B.; Cornish, V. W.; Sheetz, M. P. Cytoskeletal Coherence Requires Myosin-Iia Contractility. *J. Cell Sci.* **2010**, *123*, 413–423.

19 Calloway, N. T.; Choob, M.; Sanz, A.; Sheetz, M. P.; Miller, L. W.; Cornish, V. W. Optimized Fluorescent Trimethoprim Derivatives for in Vivo Protein Labeling. *ChemBioChem* **2007**, *8*, 767–774.

20 Wombacher, R.; Heidbreder, M.; van de Linde, S.; Sheetz, M. P.; Heilemann, M.; Cornish, V. W.; Sauer, M. Live-Cell Super-Resolution Imaging with Trimethoprim Conjugates. *Nat. Methods* **2010**, *7*, 717–719.

21 Jansen, L. E.; Black, B. E.; Foltz, D. R.; Cleveland, D. W. Propagation of Centromeric Chromatin Requires Exit from Mitosis. *J. Cell Biol.* **2007**, *176*, 795–805.

22 Hein, B.; Willig, K. I.; Wurm, C. A.; Westphal, V.; Jakobs, S.; Hell, S. W. Stimulated Emission Depletion Nanoscopy of Living Cells Using Snap-Tag Fusion Proteins. *Biophys. J.* **2010**, *98*, 158–163.

23 Testa, I.; Wurm, C. A.; Medda, R.; Rothermel, E.; von Middendorf, C.; Folling, J.; Jakobs, S.; Schonle, A.; Hell, S. W.; Eggeling, C. Multicolor Fluorescence Nanoscopy in Fixed and Living Cells by Exciting Conventional Fluorophores with a Single Wavelength. *Biophys. J.* **2010**, *99*, 2686–2694.

24 Stohr, K.; Siegberg, D.; Ehrhard, T.; Lymperopoulos, K.; Oz, S.; Schulmeister, S.; Pfeifer, A. C.; Bachmann, J.; Klingmuller, U.; Sourjik, V.; Herten, D. P. Quenched Substrates for Live-Cell Labeling of Snap-Tagged Fusion Proteins with Improved Fluorescent Background. *Anal. Chem.* **2010**, *82*, 8186–8193.

25 Keppler, A.; Ellenberg, J. Chromophore-Assisted Laser Inactivation of Alpha- and Gamma-Tubulin Snap-Tag Fusion Proteins inside Living Cells. *ACS Chem. Biol.* **2009**, *4*, 127–138.

26 Srikun, D.; Albers, A. E.; Nam, C. I.; Iavarone, A. T.; Chang, C. J. Organelle-Targetable Fluorescent Probes for Imaging Hydrogen Peroxide in Living Cells Via Snap-Tag Protein Labeling. *J. Am. Chem. Soc.* **2010**, *132*, 4455–4465.

27 Maurel, D.; Banala, S.; Laroche, T.; Johnsson, K. Photoactivatable and Photoconvertible Fluorescent Probes for Protein Labeling. *ACS Chem. Biol.* **2010**, *5*, 507–516.

28 Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. Halotag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis. *ACS Chem. Biol.* **2008**, *3*, 373–382.

29 Uttamapinant, C.; White, K. A.; Baruah, H.; Thompson, S.; Fernandez-Suarez, M.; Puthenveetil, S.; Ting, A. Y. A Fluorophore Ligase for Site-Specific Protein Labeling inside Living Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10914–10919.

30 Miller, L. W.; Cai, Y.; Sheetz, M. P.; Cornish, V. W. Vivo Protein Labeling with Trimethoprim Conjugates: A Flexible Chemical Tag. *Nat. Methods* **2005**, *2*, 255–257.

31 Aaron Hoskins, J. G.; Moore, M. J.; Cornish, V. W. Personal communication, 2010

32 Baccanari, D.; Phillips, A.; Smith, S.; Sinski, D.; Burchall, J. Purification and Properties of Escherichia Coli Dihydrofolate Reductase. *Biochemistry* **1975**, *14*, 5267–5273.

33 Wallace, L. A.; Robert Matthews, C. Highly Divergent Dihydrofolate Reductases Conserve Complex Folding Mechanisms. *J. Mol. Biol.* **2002**, *315*, 193–211.

34 Gallagher, S. S.; Jing, C.; Peterka, D. S.; Konate, M.; Wombacher, R.; Kaufman, L. J.; Yuste, R.; Cornish, V. W. A Trimethoprim-Based Chemical Tag for Live Cell Two-Photon Imaging. *ChemBioChem* **2010**, *11*, 782–784.

35 Rajapakse, H. E.; Reddy, D. R.; Mohandessi, S.; Butlin, N. G.; Miller, L. W. Luminescent Terbium Protein Labels for Time-Resolved Microscopy and Screening. *Angew. Chem., Int. Ed.* **2009**, *48*, 4990–4992.

36 Gallagher, S. S.; Sable, J. E.; Sheetz, M. P.; Cornish, V. W. An in Vivo Covalent Tmp-Tag Based on Proximity-Induced Reactivity. *ACS Chem. Biol.* **2009**, *4*, 547–556.

37 Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. A General Method for the Covalent Labeling of Fusion Proteins with Small Molecules in Vivo. *Nat. Biotechnol.* **2003**, *21*, 86–89.

38 Gronemeyer, T.; Chidley, C.; Juillerat, A.; Heinis, C.; Johnsson, K. Directed Evolution of O6-Alkylguanine-DNA Alkyltransferase for Applications in Protein Labeling. *Protein Eng., Des. Sel.* **2006**, *19*, 309–316.

39 Gautier, A.; Juillerat, A.; Heinis, C.; Correa, I. R., Jr.; Kindermann, M.; Beaufils, F.; Johnsson, K. An Engineered Protein Tag for Multiprotein Labeling in Living Cells. *Chem. Biol.* **2008**, *15*, 128–136.

40 Lee, J.; Yu, P.; Xiao, X.; Kodadek, T. A General System for Evaluating the Efficiency of Chromophore-Assisted Light Inactivation (Cali) of Proteins Reveals Ru(Ii) Tris-Bipyridyl as an Unusually Efficient "Warhead". *Mol. Biosyst.* **2008**, *4*, 59–65.

41 Bonasio, R.; Carman, C. V.; Kim, E.; Sage, P. T.; Love, K. R.; Mempel, T. R.; Springer, T. A.; von Andrian, U. H. Specific and Covalent Labeling of a Membrane Protein with Organic Fluorochromes and Quantum Dots. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 14753–14758.

42 Mizukami, S.; Watanabe, S.; Hori, Y.; Kikuchi, K. Covalent Protein Labeling Based on Noncatalytic Beta-Lactamase and a Designed Fret Substrate. *J. Am. Chem. Soc.* **2009**, *131*, 5016–5017.

43 Watanabe, S.; Mizukami, S.; Hori, Y.; Kikuchi, K. Multicolor Protein Labeling in Living Cells Using Mutant Beta-Lactamase-Tag Technology. *Bioconjugate Chem.* **2010**, *21*, 2320–2326.

44 Marks, K. M.; Braun, P. D.; Nolan, G. P. A General Approach for Chemical Labeling and Rapid, Spatially Controlled Protein Inactivation. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9982–9987.

45 Chen, I.; Howarth, M.; Lin, W.; Ting, A. Y. Site-Specific Labeling of Cell Surface Proteins with Biophysical Probes Using Biotin Ligase. *Nat. Methods* **2005**, *2*, 99–104.

46 Howarth, M.; Takao, K.; Hayashi, Y.; Ting, A. Y. Targeting Quantum Dots to Surface Proteins in Living Cells with Biotin Ligase. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7583–7588.

47 Yin, J.; Liu, F.; Li, X.; Walsh, C. T. Labeling Proteins with Small Molecules by Site-Specific Posttranslational Modification. *J. Am. Chem. Soc.* **2004**, *126*, 7754–7755.

48 Popp, M. W.; Antos, J. M.; Grotenbreg, G. M.; Spooner, E.; Ploegh, H. L. Sortagging: A Versatile Method for Protein Labeling. *Nat. Chem. Biol.* **2007**, *3*, 707–708.

49 Yamamoto, T.; Nagamune, T. Expansion of the Sortase-Mediated Labeling Method for Site-Specific N-Terminal Labeling of Cell Surface Proteins on Living Cells. *Chem. Commun. (Cambridge, U.K.)* **2009**, 1022–1024.

50 Wu, P.; Shui, W.; Carlson, B. L.; Hu, N.; Rabuka, D.; Lee, J.; Bertozzi, C. R. Site-Specific Chemical Modification of Recombinant Proteins Produced in Mammalian Cells by Using the Genetically Encoded Aldehyde Tag. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 3000–3005.

51 Halo, T. L.; Appelbaum, J.; Hobert, E. M.; Balkin, D. M.; Schepartz, A. Selective Recognition of Protein Tetraserine Motifs with a Cell-Permeable, Pro-Fluorescent Bis-Boronic Acid. *J. Am. Chem. Soc.* **2009**, *131*, 438–439.

52 Lee, J. J.; Lee, S. C.; Zhai, D.; Ahn, Y. H.; Yeo, H. Y.; Tan, Y. L.; Chang, Y. T. Bodipy-Diacrylate Imaging Probes for Targeted Proteins inside Live Cells. *Chem. Commun. (Cambridge, U.K.)* **2011**, *47*, 4508–4510.

53 Goldsmith, C. R.; Jaworski, J.; Sheng, M.; Lippard, S. J. Selective Labeling of Extracellular Proteins Containing Polyhistidine Sequences by a Fluorescein-Nitrilotriacetic Acid Conjugate. *J. Am. Chem. Soc.* **2006**, *128*, 418–419.

54 Lata, S.; Gavutis, M.; Tampe, R.; Piehler, J. Specific and Stable Fluorescence Labeling of Histidine-Tagged Proteins for Dissecting Multi-Protein Complex Formation. *J. Am. Chem. Soc.* **2006**, *128*, 2365–2372.

55 Ojida, A.; Honda, K.; Shinmi, D.; Kiyonaka, S.; Mori, Y.; Hamachi, I. Oligo-Asp Tag/Zn(Ii) Complex Probe as a New Pair for Labeling and Fluorescence Imaging of Proteins. *J. Am. Chem. Soc.* **2006**, *128*, 10452–10459.

56 Hauser, C. T.; Tsien, R. Y. A Hexahistidine-Zn2+-Dye Label Reveals Stim1 Surface Exposure. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3693–3697.

57 Franz, K. J.; Nitz, M.; Imperiali, B. Lanthanide-Binding Tags as Versatile Protein Coexpression Probes. *ChemBioChem* **2003**, *4*, 265–271.

58 Marks, K. M.; Rosinov, M.; Nolan, G. P. Vivo Targeting of Organic Calcium Sensors Via Genetically Selected Peptides. *Chem. Biol.* **2004**, *11*, 347–356.

59 Yeo, D. S.; Srinivasan, R.; Uttamchandani, M.; Chen, G. Y.; Zhu, Q.; Yao, S. Q. Cell-Permeable Small Molecule Probes for Site-Specific Labeling of Proteins. *Chem. Commun. (Cambridge, U.K.)* **2003**, 2870–2871.

60 Yildiz, A.; Selvin, P. R. Fluorescence Imaging with One Nanometer Accuracy: Application to Molecular Motors. *Acc. Chem. Res.* **2005**, *38*, 574–582.

61 Yildiz, A.; Forkey, J. N.; McKinney, S. A.; Ha, T.; Goldman, Y. E.; Selvin, P. R. Myosin V Walks Hand-over-Hand: Single Fluorophore Imaging with 1.5-Nm Localization. *Science* **2003**, *300*, 2061–2065.

62 Hoskins, A. A.; Friedman, L. J.; Gallagher, S. S.; Crawford, D. J.; Anderson, E. G.; Wombacher, R.; Ramirez, N.; Cornish, V. W.; Gelles, J.; Moore, M. J. Ordered and Dynamic Assembly of Single Spliceosomes. *Science* **2011**, *331*, 1289–1295.

63 Klar, T. A.; Jakobs, S.; Dyba, M.; Egner, A.; Hell, S. W. Fluorescence Microscopy with Diffraction Resolution Barrier Broken by Stimulated Emission. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8206–8210.

64 Betzig, E.; Patterson, G. H.; Sougrat, R.; Lindwasser, O. W.; Olenych, S.; Bonifacino, J. S.; Davidson, M. W.; Lippincott-Schwartz, J.; Hess, H. F. Imaging Intracellular Fluorescent Proteins at Nanometer Resolution. *Science* **2006**, *313*, 1642–1645.

65 Rust, M. J.; Bates, M.; Zhuang, X. Sub-Diffraction-Limit Imaging by Stochastic Optical Reconstruction Microscopy (Storm). *Nat. Methods* **2006**, *3*, 793–795.

66 Jones, S. A.; Shim, S. H.; He, J.; Zhuang, X. Fast, Three-Dimensional Super-Resolution Imaging of Live Cells. *Nat. Methods* **2011**, *8*, 499–505.

67 Klein, T.; Loschberger, A.; Proppert, S.; Wolter, S.; van de Linde, S.; Sauer, M. Live-Cell Dstorm with Snap-Tag Fusion Proteins. *Nat. Methods* **2011**, *8*, 7–9.

68 Heilemann, M.; van de Linde, S.; Mukherjee, A.; Sauer, M. Super-Resolution Imaging with Small Organic Fluorophores. *Angew. Chem., Int. Ed.* **2009**, *48*, 6903–6908.

69 Zou, P.; Ting, A. Y. Imaging Ldl Receptor Oligomerization During Endocytosis Using a Co-Internalization Assay. *ACS Chem. Biol.* **2011**, *6*, 308–313.

70 Marek, K. W.; Davis, G. W. Transgenically Encoded Protein Photoinactivation (Flash-Fali): Acute Inactivation of Synaptotagmin I. *Neuron* **2002**, *36*, 805–813.

71 Gautier, A.; Nakata, E.; Lukinavicius, G.; Tan, K. T.; Johnsson, K. Selective Cross-Linking of Interacting Proteins Using Self-Labeling Tags. *J. Am. Chem. Soc.* **2009**, *131*, 17954–17962.

72 Krishnan, B.; Gierasch, L. M. Cross-Strand Split Tetra-Cys Motifs as Structure Sensors in a Beta-Sheet Protein. *Chem. Biol.* **2008**, *15*, 1104–1115.

73 Czlapinski, J. L.; Schelle, M. W.; Miller, L. W.; Laughlin, S. T.; Kohler, J. J.; Cornish, V. W.; Bertozzi, C. R. Conditional Glycosylation in Eukaryotic Cells Using a Biocompatible Chemical Inducer of Dimerization. *J. Am. Chem. Soc.* **2008**, *130*, 13186–13187.

74 Ohana, R. F.; Encell, L. P.; Zhao, K.; Simpson, D.; Slater, M. R.; Urh, M.; Wood, K. V. Halotag7: A Genetically Engineered Tag That Enhances Bacterial Expression of Soluble Proteins and Improves Protein Purification. *Protein Expression Purif.* **2009**, *68*, 110–120.