Site-specific labeling of proteins with small molecules in live cells

Irwin Chen and Alice Y Ting

The principal bottleneck for the utilization of small-molecule probes in live cells is the shortage of methodologies for targeting them with very high specificity to biological molecules or compartments of interest. Recently developed methods for labeling proteins with small-molecule probes in cells employ special protein or peptide handles that recruit small-molecule ligands, harness enzymes to catalyze small-molecule conjugation or hijack the cell's protein translation machinery.

Addresses
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Corresponding author: Ting, Alice Y (ating@mit.edu)

Current Opinion in Biotechnology 2005, **16**:35–40

This review comes from a themed issue on
Analytical biotechnology
Edited by Keith Wood and Dieter Klaubert

Available online 23rd December 2004

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2004.12.003

Abbreviations

ACP	acyl carrier protein
DHFR	dihydrofolate reductase
GFP	green fluorescent protein
hAGT	human O^6-alkylguanine transferase
Ni-NTA	nickel-nitrilotriacetic acid
PPtase	phosphopantetheine transferase

Introduction

Parallel advances in reporter and imaging technologies have led to the widespread use of probes such as green fluorescent protein (GFP), luciferase, β-galactosidase, and β-lactamase to visualize recombinant protein expression and/or localization in single living cells. Although powerful, these probes are limited by their large size (and thus potential to interfere with the structure and/or function of the proteins to which they are fused; see [1,2] for examples) and reliance on optical forms of readout. As our understanding of cell signaling improves, researchers are increasingly interested in probing the more sophisticated functions of proteins in their natural habitats; for example, photoaffinity probes could reveal information about protein–protein interactions, photocaging groups could be used to regulate protein activity, positron emission tomography or magnetic resonance imaging probes could allow imaging of tissue in live animals, and electron microscopy probes could provide much higher resolution spatial information after cell fixation. The principal barrier to the utilization of such probes in the cellular context is the shortage of site-specific protein labeling methodologies. Whereas the strength of reporters like GFP lies in the perfect specificity of genetic fusion, the chemoselective reaction of a small-molecule probe with a single functional group on a single protein to the exclusion of thousands of other competing cytoplasmic proteins, as well as DNA, RNA, carbohydrates and small molecules, poses an enormous chemical problem. Here we review the state-of-the-art in methods for labeling recombinant proteins in cells with small-molecule probes.

Considerations for site-specific protein labeling inside cells

For labeling proteins with small-molecule probes *in vitro*, biochemists have exploited the unique reactivities of cysteine (which reacts with maleimide- or haloacetamide-functionalized molecules at slightly basic pH) and, less commonly, the protein N terminus (which has reduced pK_a compared to lysine sidechains). These strategies cannot be used for cellular labeling, because nearly all endogenous proteins bear cysteine sidechains and a free N terminus. Thus, strategies for achieving specificity have relied upon unique combinations of amino acids (i.e. peptide or protein sequences fused to the protein under investigation) that recruit the small-molecule probe of interest. For some methods the information contained in the targeting sequence alone is sufficient to confer specificity; in other cases, an enzyme mediates the conjugation of the probe to the target sequence. Another very different approach to labeling involves the incorporation of unnatural amino acids into the protein of interest. Other considerations in making the transition from labeling *in vitro* to labeling in cells are the membrane permeance of the probe (or, for cell-surface labeling applications, impermeance), the potential for the probe or targeting sequence to affect the cellular system (via toxicity or more complicated interactions), the labeling timescale (the faster the labeling, the more biological processes that can be studied), and the stability of the probe–protein conjugate (does the probe dissociate or the conjugate degrade over time?). Table 1 summarizes existing site-specific labeling methods and highlights the advantages and disadvantages of each.

Small-molecule labeling using protein targeting sequences

The most straightforward method for targeting a small molecule to a protein sequence is to use a binding

Table 1

Different methods for labeling proteins with small-molecule probes and their relative attributes.

Method	Tag size[a]	Specificity	Restrictions	Other comments
Small-molecule labeling using protein targeting sequences				
DHFR	157	Labels endogenous DHFR	None	Protein–ligand dissociation
FKBP12 (F36V)	108	Excellent	None	Protein–ligand dissociation
hAGT	207	Labels endogenous AGT	None	
ACP(PCP)/PPtase	77	Excellent	Cell-surface proteins	
Intein/native chemical ligation	~150→1[b]	Competition with endogenous cysteine	Reducing environment	Labeling process is slow
Small-molecule labeling using peptide targeting sequences				
His_6/Ni-NTA	6	Unclear	None	Fast off-rate; Ni quenches fluoresence
Texas-red-binding peptide	23–38	Excellent	Texas-red only	Ligand dissociation
Tetracysteine biarsenicals	6–10	Background due to affinity for monothiols	Reducing environment	Fluorogenic; possible residual arsenic toxicity; long labeling times
Biotin ligase/hydrazide	15	Excellent One natural substrate in *E. coli*	Cell-surface proteins	Two-step labeling
Small-molecule labeling using other methods				
Incorporation of unnatural amino acids	0	Excellent	Not yet demonstrated in all cell types	Dominant negative effect due to truncations

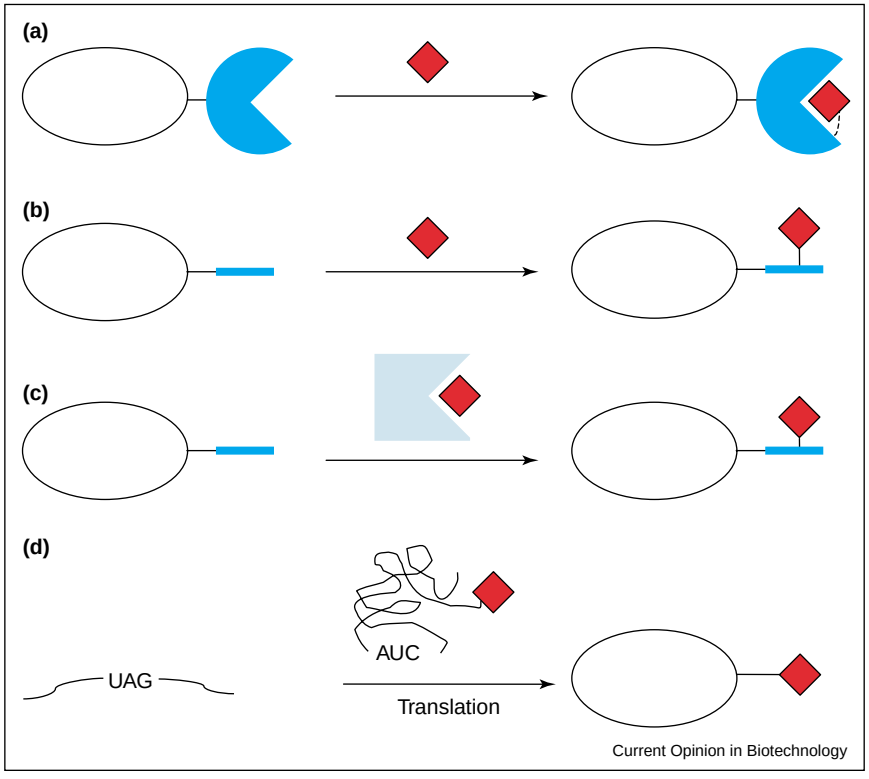
[a]The tag size is given as the number of amino acids. [b]The tag is temporary and is cleaved off inside the cell.

interaction (Figure 1a). In this approach, the protein of interest is expressed fused to a protein tag that is capable of binding to a small-molecule ligand. For example, Farinas and Verkman [3] used antibody tags fused to localization signal sequences to target various hapten–fluorophore conjugates to specific subcellular compartments in live cells. More recent approaches have employed dihydrofolate reductase (DHFR) and the Phe36Val mutant of FK506-binding protein 12 (FKBP12(F36V)), which bind to the small-molecule ligands methotrexate and SLF′ (synthetic ligand for FKBP12), respectively, with dissociation constants of 25 pM and 94 pM [4,5]. Appending various biophysical probes to these ligands is synthetically straightforward and does not reduce binding affinity for the protein targets. Nuclear- and plasma-membrane-localized DHFR fusion proteins were labeled in DHFR-deficient Chinese hamster ovary (CHO) cells using a membrane-permeant methotrexate–Texas-red conjugate [6]. Similarly, a fluorescein–SLF′ conjugate was used to label several FKBP12(F36V) fusion proteins in NIH3T3, COS-7, Jurkat and HeLa mammalian cell lines [7•]. Labeling with these tags is highly specific, and the method is versatile because it can accommodate probes of many different structures. However, like GFP, the protein tags are large (DHFR is 157 amino acids and FKBP12(F36V) is 108 amino acids) and ligand dissociation can cause the signal to deteriorate over time. The methotrexate–Texas-red conjugate, for instance, remains localized to DHFR fusion proteins for only about one hour after its introduction. DHFR-based labeling has the additional disadvantage of high background in mammalian cells resulting from the labeling of endogenous DHFR, unless DHFR-knockout cell lines are used. By contrast, the SLF′ ligand has a >1000-fold preference for the F36V mutant over wild-type FKBP12.

Stabilizing the protein–small-molecule binding event with a subsequent covalent reaction reduces the problem of signal loss through ligand dissociation. Johnsson and coworkers [8,9,10•] exploited the alkylation of Cys145 of human O^6-alkylguanine transferase (hAGT) with various O^6-benzylguanine suicide substrates to covalently label nuclear, cytosolic, cytoskeletal, and cell-surface recombinant proteins with fluorophores in *Escherichia coli*, yeast, and hAGT-deficient mammalian cells. In a separate approach, Yin *et al.* [11] and George *et al.* [12•] employed an ~80 amino acid protein tagging sequence derived from either peptide carrier protein (PCP) [11] or acyl carrier protein (ACP) [12•], which was labeled using the enzyme phosphopantetheine transferase (PPtase). PPtase transfers 4′-phosphopantetheine-linked probes from coenzyme A (CoA) to a serine sidechain of ACP or PCP to form a specific covalent attachment. This enzyme-mediated ligation has been used for fluorophore tagging of α-agglutinin receptor Aga2p and human G-protein-coupled receptor NK_1 in yeast and mammalian cells [12•].

Both the hAGT and PPtase labeling methods have excellent specificity, because the initial binding interactions orient the reactive groups and ensure site-specific ligation. Both methods are also quite versatile, because the derivatization sites on the benzylguanine and CoA cores are permissive and nearly any biophysical probe can be appended without affecting the specificity or rate of the ligation chemistry. Another benefit of these approaches is

Figure 1



Strategies for the site-specific labeling of proteins with small molecules in live cells. **(a)** Small-molecule labeling using protein tags. The protein of interest (white oval) is expressed fused to a protein tag (e.g. DHFR or hAGT; blue), which is able to bind noncovalently or covalently to a small molecule (e.g. methotrexate or O^6-benzylguanine conjugate; red diamond)). **(b)** Small-molecule labeling using peptide tags. The protein of interest is expressed fused to a peptide tag (e.g. the tetracysteine motif; blue), which is able to bind to a small molecule (e.g. fluorogenic biarsenical; red diamond). **(c)** Small-molecule labeling using enzyme-mediated covalent attachment to a peptide tag. The protein is expressed fused to a peptide tag (e.g. acceptor peptide; blue). An enzyme (e.g. biotin ligase; cyan) labels the peptide tag with the small molecule directly or with a functional handle to which the small molecule ligates through a bio-orthogonal reaction. **(d)** Small-molecule labeling using unnatural amino acid mutagenesis. An amber stop codon (UAG) is introduced within the gene coding the protein of interest by mutagenesis. During translation, a tRNA bearing the anticodon (AUC), which has been aminoacylated with an unnatural amino acid, suppresses the stop codon and enables production of the full-length protein containing the unnatural amino acid (red).

the short labeling time: fluorescent benzylguanine or CoA probes are typically administered to cells for only 5–10 min before washout of unreacted probe (probably an additional 5–10 min). The development of fluorogenic probes, which become fluorescent only after ligation to the target sequence, would further reduce the labeling time because the 'invisibility' of the unreacted probe eliminates the need for a washout. The principal drawback of both methods is the large tag size: 207 amino acids for hAGT and ~80 amino acids for ACP or PCP. In addition, background labeling is observed for the hAGT method, as AGT is naturally found in mammalian cells. Mutant hAGTs that react up to 20 times faster with the benzylguanine probes have been engineered [13], but the milder labeling protocol used with these probes can still label endogenous hAGT to a significant extent. Cys145-alkylated hAGT is also targeted for degradation at a rate of ~10%/h [9] so, ironically, signal attenuation is still a problem, despite covalent attachment of the probe to the target protein. The PPtase method does not suffer from these problems, but is restricted to the labeling of cell-surface proteins, because the probes used are not membrane-permeable.

Lastly, we consider the use of inteins and ubiquitin as protein-tagging sequences. Inteins and ubiquitin can be cleaved 'tracelessly' from the protein under study using protein splicing or deubiquitination to produce a protein with an N-terminal cysteine. The N-terminal cysteine is then chemoselectively ligated via native chemical ligation to a thioester-functionalized probe [14]. This method is versatile and should work in any cell type, but the precursor intein and ubiquitin fusions are still large, even if temporary. In addition, the rates of splicing and thioester ligation are slow, restricting the methodology to the study of biological processes that take place over several hours. Moreover, intracellular cysteine and cysteamine compete with the target protein for reaction with the

thioester, reducing the labeling efficiency and necessitating extensive washouts to reduce background.

Small-molecule labeling using peptide targeting sequences

The fusion of a peptide sequence to a protein of interest presents a much less invasive option than the fusion of a protein sequence. As with proteins, small molecules can be targeted to peptides via high-affinity binding interactions. For instance, Nolan and colleagues [15] have evolved a 38 amino acid peptide ('fluorette') that binds with a K_d of 25 pM to Texas red fluorophore; labeling of this sequence in cells was demonstrated using a test GFP construct fused to a membrane localization signal sequence from Lyn kinase. Even though the measured *in vitro* binding affinity is quite high, in practice the use of non-covalent targeting strategies leads to signal deterioration, as observed for the protein–ligand interactions described above (for FKBP12 and DHFR). Another drawback of the fluorette method is that a new peptide partner must be evolved for every new probe of interest, so the versatility is limited. In a separate approach, Vogel and coworkers [16] have shown that cell-surface receptors bearing a hexahistidine or decahistidine tag can be labeled with nickel-nitrilotriacetic acid (Ni-NTA)-functionalized chromophores. Labeling with Ni-NTA had to be demonstrated indirectly via quenching of a fluorescently tagged receptor, probably because direct imaging of the Ni-NTA conjugated fluorophore is made difficult (or impossible) owing to fluorescence quenching by Ni^{2+} and the modest affinity of Ni-NTA for the oligohistidine sequences (0.2–10 μM). Signal deterioration is far more severe in this case than in the previous examples.

The use of fluorogenic biarsenical (FlAsH) compounds to label a six amino acid tetracysteine peptide (Cys-Cys-X-X-Cys-Cys, where X-X were initially undefined amino acids but are now preferably Pro-Gly) was pioneered by Tsien and coworkers [17,18] (Figure 1b). The probes are membrane-permeant and become intensely fluorescent upon high-affinity binding (2–4 pM K_d) to tetrathiol sequences. The interaction is strong enough to survive SDS–PAGE so the signal deterioration problem observed with other non-covalent targeting strategies is not pertinent in this case. Recent improvements to the original methodology have greatly enhanced the utility of this approach: new biarsenicals that span the visible spectrum have been developed [19], dyes that can also moonlight as photosensitizers to polymerize diaminobenzidine for electron microscopy [19] or inactivate protein function via singlet oxygen generation [20] have been reported, and new tetracysteine sequences that bind with higher affinity to the biarsenical probes have improved the specificity of the method [19]. These probes have been used to study connexin [21], AMPA receptor [22], Ebola virus matrix protein VP40 [23], and synaptotagmin I [24•] trafficking in live mammalian cells and *Drosophila*, often by employing a clever two-color pulse-chase labeling strategy that distinguishes younger protein from older protein. This methodology has been employed in *E. coli* [25] and yeast [26], in addition to the mammalian and *Drosophila* cell lines mentioned above. Unlike most other methods described in this review, the FlAsH methodology has already begun to make significant contributions to cell biology; however, several drawbacks still limit its utility. The biarsenical compounds retain significant affinity for isolated thiols. Thus, lengthy (>1 h) and complex washouts are required to compete out arsenic-monothiol interactions, even though the probes are fluorogenic, and in many cases even the extensive washing does not fully eliminate background staining [27]. Although acute toxicity of arsenic is alleviated by dithiol antidotes, longer-term toxicity remains a concern. Tetracysteines in oxidizing environments such as the secretory pathway or cell surface can be labeled only if acutely reduced, which adds technical complexity and the risk of side effects.

Lastly, our laboratory has recently developed methodology for labeling of a 15 amino acid peptide sequence ('acceptor peptide' or AP) with small-molecule probes using the *E. coli* enzyme biotin ligase (BirA; Figure 1c) (I Chen *et al.*, unpublished). BirA catalyzes the ligation of biotin to the acceptor peptide, but we have found that a ketone analog of biotin can efficiently substitute for biotin (the k_{cat} is reduced by less than fourfold). As ketones are absent from cell surfaces, the introduced ketone analog can be selectively derivatized with hydrazide- or hydroxylamine-functionalized probes [28]. The combination of a small tag, covalent probe attachment, and extremely high labeling specificity distinguish this technique from others, but currently it is limited to cell-surface labeling. Further re-engineering of BirA to allow it to accept azide-functionalized biotin analogs that can react with triarylphosphine- [29] or alkyne-containing [30,31] small-molecule probes, or to accept fluorophores directly, should enable extension of the methodology to intracellular labeling. Also, the rate of labeling, currently 10–20 min at best, could be improved. Modifications to the hydrazide/hydroxylamine probe structure might improve the reaction rate of the second conjugation step.

Small-molecule labeling using unnatural amino acids

The use of amber codon suppression mutagenesis to introduce unnatural amino acids in a site-specific manner has also been used to label proteins in living cells (Figure 1d) [32]. Biophysical probes such as benzophenone and aryl azide cross-linkers have been incorporated at these sites [33•,34]. Like the biotin ligase methodology, the versatility of this method is enhanced through cell-compatible chemoselective ligation reactions. For instance, unnatural amino acids bearing ketones [35], azides [34] or alkynes [36] have been incorporated, allowing further derivatization using hydrazone formation,

Staudinger ligation [29] or azide/alkyne cycloaddition reactions [30,31]. The major advantages of unnatural amino acid labeling are the excellent specificity, versatility with respect to small-molecule label, and minimal structural perturbation to the protein of interest. However, unnatural amino acid mutagenesis is not yet broadly applicable in all cell types; most demonstrations, including the ones with the photoaffinity labels and functional handles mentioned above, have been in bacteria and yeast. Nevertheless, the recent report of 5-hydroxytryptophan incorporation into the bacteriophage T4 fibritin foldon domain in 293T mammalian cells suggests that more examples are forthcoming [37]. Another serious concern is that ribosomes do not readily tolerate highly polar amino acids or amino acids considerably larger than trytophan. This technique therefore places a limit on the types of probe structures that can be directly introduced, unless two-step labeling protocols employing chemoselective ligation reactions are used. Finally, a large amount of truncated protein product is inevitably generated as a result of competition between the amber suppressor transfer RNA and release factors that normally terminate protein synthesis at the amber stop codon. This can lead to a dominant negative effect, especially if the protein of interest is multimeric in its functional state.

Conclusions

Site-specific labeling methods represent the major bottleneck for the utilization of small-molecule probes in the cellular context. The methods described here illustrate various approaches to address this difficult chemical problem. Among the existing techniques, there is generally a trade-off between tag size and labeling specificity, although this can be surmounted through the use of additional mediating factors, such as enzymes (e.g. the biotin ligase method) or the cell's translation machinery (unnatural amino acid mutagenesis). The unnatural amino acid labeling method is notable for its minimal tag size (no additional amino acid), but future approaches that enable the introduction of probes into endogenous, unmodified proteins will be highly desirable. For future work, it is useful to look to creative methods that have been developed for *in vitro* protein labeling (e.g. transglutaminase-mediated labeling [38,39], selenocysteine labeling [40], sortase-mediated ligation [41] or epoxide-based labeling [42]) to explore the feasibility of extending these to cell labeling applications.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Marguet D, Spiliotis ET, Pentcheva T, Lebowitz M, Schneck J, Edidin M: **Lateral diffusion of GFP-tagged H2Ld molecules and of GFP-TAP1 reports on the assembly and retention of these molecules in the endoplasmic reticulum**. *Immunity* 1999, **11**:231-240.

2. Lisenbee CS, Karnik SK, Trelease RN: **Overexpression and mislocalization of a tail-anchored GFP redefines the identity of peroxisomal ER**. *Traffic* 2003, **4**:491-501.

3. Farinas J, Verkman AS: **Receptor-mediated targeting of fluorescent probes in living cells**. *J Biol Chem* 1999, **274**:7603-7606.

4. Sasso SP, Gilli RM, Sari JC, Rimet OS, Briand CM: **Thermodynamic study of dihydrofolate-reductase inhibitor selectivity**. *Biochim Biophys Acta - Prot Struct Mol Enzymol* 1994, **1207**:74-79.

5. Clackson T, Yang W, Rozamus LW, Hatada M, Amara JF, Rollins CT, Stevenson LF, Magari SR, Wood SA, Courage NL *et al.*: **Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity**. *Proc Natl Acad Sci USA* 1998, **95**:10437-10442.

6. Miller LW, Sable J, Goelet P, Sheetz MP, Cornish VW: **Methotrexate conjugates: a molecular *in vivo* protein tag**. *Angew Chem Int Ed Engl* 2004, **43**:1672-1675.

7. • Marks KM, Braun PD, Nolan GP: **A general approach for chemical labeling and rapid, spatially controlled protein inactivation**. *Proc Natl Acad Sci USA* 2004, **101**:9982-9987.
Various FKBP12(F36V) fusion proteins in live mammalian cells were labeled via a noncovalent binding interaction with a fluorescein-conjugated SLF′ ligand. Moreover, the function of fluorophore-labeled fusion proteins could also be abolished with spatiotemporal control using fluorophore-assisted light inactivation.

8. 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.

9. Keppler A, Kindermann M, Gendreizig S, Pick H, Vogel H, Johnsson K: **Labeling of fusion proteins of O-6-alkylguanine-DNA alkyltransferase with small molecules *in vivo* and *in vitro***. *Methods* 2004, **32**:437-444.

10. • Keppler A, Pick H, Arrivoli C, Vogel H, Johnsson K: **Labeling of fusion proteins with synthetic fluorophores in live cells**. *Proc Natl Acad Sci USA* 2004, **101**:9955-9959.
AGT fusion proteins localized to the nucleus, cytoplasm, cytoskeleton and the cell surface of mammalian cells were covalently labeled with a variety of fluorophore-conjugated benzylguanine suicide substrates. Fluorophore-labeled fusion proteins were used for multicolor analysis of protein subpopulations and fluorescence resonance energy transfer measurements.

11. Yin J, Liu F, Li XH, Walsh CT: **Labeling proteins with small molecules by site-specific posttranslational modification**. *J Am Chem Soc* 2004, **126**:7754-7755.

12. • George N, Pick H, Vogel H, Johnsson N, Johnsson K: **Specific labeling of cell surface proteins with chemically diverse compounds**. *J Am Chem Soc* 2004, **126**:8896-8897.
Using the enzyme PPtase, ACP fusions to α-agglutinin receptor Aga2p in yeast and human G-protein-coupled receptor NK_1 in HEK293 cells were covalently labeled with coenzyme A derivatives bearing cyanine fluorophores or a biotin affinity handle. The dependence of the labeling reaction on enzyme activity can also be exploited for exceptional spatial control of labeling by immobilizing the enzyme via genetic fusion.

13. Juillerat A, Gronemeyer T, Keppler A, Gendreizig S, Pick H, Vogel H, Johnsson K: **Directed evolution of O_6-alkylguanine-DNA alkyltransferase for efficient labeling of fusion proteins with small molecules *in vivo***. *Chem Biol* 2003, **10**:313-317.

14. Yeo DSY, Srinivasan R, Uttamchandani M, Chen GYJ, Zhu Q, Yao SQ: **Cell-permeable small molecule probes for site-specific labeling of proteins**. *Chem Comm* 2003:2870-2871.

15. Marks KM, Rosinov M, Nolan GP: ***In vivo* targeting of organic calcium sensors via genetically selected peptides**. *Chem Biol* 2004, **11**:347-356.

16. Guignet EG, Hovius R, Vogel H: **Reversible site-selective labeling of membrane proteins in live cells**. *Nat Biotechnol* 2004, **22**:440-444.

17. Griffin BA, Adams SR, Tsien RY: **Specific covalent labeling of recombinant protein molecules inside live cells**. *Science* 1998, **281**:269-272.

18. Griffin BA, Adams SR, Jones J, Tsien RY: **Fluorescent labeling of recombinant proteins in living cells with FlAsH**. *Methods Enzymol* 2000, **327**:565-578.

19. • Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY: **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.

Significant improvements to the original tetracysteine-biarsenical methodology are described, including the development of red- and blue-fluorescing dyes, development of biarsenical ligands for electron microscopy, and optimization of the tetracysteine motif. Kinetic characterization of the labeling process and determination of the sensitivity of the method are also described.

20. Tour O, Meijer RM, Zacharias DA, Adams SR, Tsien RY: **Genetically targeted chromophore-assisted light inactivation**. *Nat Biotechnol* 2003, **21**:1505-1508.

21. Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH: **Multicolor and electron microscopic imaging of connexin trafficking**. *Science* 2002, **296**:503-507.

22. Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC: **Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors**. *Nat Neurosci* 2004, **7**:244-253.

23. Panchal RG, Ruthel G, Kenny TA, Kallstrom GH, Lane D, Badie SS, Li L, Bavari S, Aman MJ: ***In vivo* oligomerization and raft localization of Ebola virus protein VP40 during vesicular budding**. *Proc Natl Acad Sci USA* 2003, **100**:15936-15941.

24. • Poskanzer KE, Marek KW, Sweeney ST, Davis GW: **Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis *in vivo***. *Nature* 2003, **426**:559-563.

Fluorescein biarsenicals were used to label and photoinactivate tetracysteine-tagged Synaptotagmin I (Syt I) in living *Drosophila*. Thus, it was demonstrated that Syt I plays a role in synaptic vesicle endocytosis, in addition to its well-documented role in vesicle exocytosis. Direct testing of the *in vivo* role of Syt I specifically during synaptic vesicle endocytosis was made possible by the combination of site-specific protein labeling and fluorophore-assisted light inactivation.

25. Ignatova Z, Gierasch LM: **Monitoring protein stability and aggregation *in vivo* by real-time fluorescent labelling**. *Proc Natl Acad Sci USA* 2004, **101**:523-528.

26. Rice MC, Czymmek K, Kmiec EB: **The potential of nucleic acid repair in functional genomics**. *Nat Biotechnol* 2001, **19**:321-326.

27. Stroffekova K, Proenza C, Beam KG: **The protein-labeling reagent FLASH-EDT2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins**. *Pflugers Arch* 2001, **442**:859-866.

28. Mahal LK, Yarema KJ, Bertozzi CR: **Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis**. *Science* 1997, **276**:1125-1128.

29. Saxon E, Bertozzi CR: **Cell surface engineering by a modified Staudinger reaction**. *Science* 2000, **287**:2007-2010.

30. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG: **Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition**. *J Am Chem Soc* 2003, **125**:3192-3193.

31. Agard NJ, Prescher JA, Bertozzi CR: **A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems**. *J Am Chem Soc* 2004, **126**:15046-15047.

32. Wang L, Schultz PG: **Expanding the genetic code**. *Chem Commun (Camb)* 2002:1-11.

33. • Chin JW, Martin AB, King DS, Wang L, Schultz PG: **Addition of a photocrosslinking amino acid to the genetic code of *Escherichia coli***. *Proc Natl Acad Sci USA* 2002, **99**:11020-11024.

The photocrosslinking amino acid para-benzoyl-L-phenylalanine was incorporated via site-specific amber stop codon suppression into *Schistosoma japonica* glutathione-*S*-transferase (GST) expressed in living *E. coli* cells. Model crosslinking experiments with GST suggest that site-specific substitution with this unnatural amino acid could be used to map protein–protein interactions *in vivo* and to define the specific amino acids involved.

34. Chin JW, Santoro SW, Martin AB, King DS, Wang L, Schultz PG: **Addition of *p*-azido-L-phenylalanine to the genetic code of *Escherichia coli***. *J Am Chem Soc* 2002, **124**:9026-9027.

35. Zhang Z, Smith BA, Wang L, Brock A, Cho C, Schultz PG: **A new strategy for the site-specific modification of proteins *in vivo***. *Biochemistry* 2003, **42**:6735-6746.

36. Deiters A, Cropp TA, Mukherji M, Chin JW, Anderson JC, Schultz PG: **Adding amino acids with novel reactivity to the genetic code of *Saccharomyces cerevisiae***. *J Am Chem Soc* 2003, **125**:11782-11783.

37. Zhang Z, Alfonta L, Tian F, Bursulaya B, Uryu S, King DS, Schultz PG: **Selective incorporation of 5-hydroxytryptophan into proteins in mammalian cells**. *Proc Natl Acad Sci USA* 2004, **101**:8882-8887.

38. Huff T, Ballweber E, Humeny A, Bonk T, Becker C, Muller CS, Mannherz HG, Hannappel E: **Thymosin β_4 serves as a glutaminyl substrate of transglutaminase. Labeling with fluorescent dansylcadaverine does not abolish interaction with G-actin**. *FEBS Lett* 1999, **464**:14-20.

39. Dutton A, Singer SJ: **Crosslinking and labeling of membrane proteins by transglutaminase-catalyzed reactions**. *Proc Natl Acad Sci USA* 1975, **72**:2568-2571.

40. Johansson L, Chen C, Thorell J, Fredriksson A, Stone-Elander S, Gafvelin G, Arnér ESJ: **Exploiting the 21st amino acid — purifying and labeling proteins by selenoate targeting**. *Nat Methods* 2004, **1**:61-66.

41. Mao H, Hart SA, Schink A, Pollok BA: **Sortase-mediated protein ligation: a new method for protein engineering**. *J Am Chem Soc* 2004, **126**:2670-2671.

42. Chen G, Heim A, Riether D, Yee D, Milgrom Y, Gawinowicz MA, Sames D: **Reactivity of functional groups on the protein surface: development of epoxide probes for protein labeling**. *J Am Chem Soc* 2003, **125**:8130-8133.