J. Biophotonics **4**, No. 6, 391–402 (2011) / **DOI** 10.1002/jbio.201100018

Journal of BIOPHOTONICS

EDITOR'S CHOICE

Chemical tags: applications in live cell fluorescence imaging

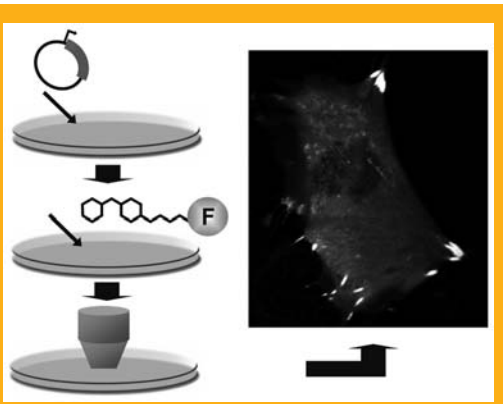
Richard Wombacher[*,1] *and Virginia W. Cornish*[2]

[1] Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany
[2] Department of Chemistry, Columbia University, 3000 Broadway, New York, New York 10027, USA

Received 8 March 2011, revised 8 April 2011, accepted 9 April 2011
Published online 12 May 2011

Key words: protein labeling, cells/chemistry, fluorescent dyes, protein binding, ligands

Technologies to visualize cellular structures and dynamics enable cell biologists to gain insight into complex biological processes. Currently, fluorescent proteins are used routinely to investigate the behavior of proteins in live cells. Chemical biology techniques for selective labeling of proteins with fluorescent labels have become an attractive alternative to fluorescent protein labeling. In the last ten years the progress in the development of chemical tagging methods have been substantial offering a broad palette of applications for live cell fluorescent microscopy. Several methods for protein labeling have been established, using protein tags, peptide tags and enzyme mediated tagging. This review focuses on the different strategies to achieve the attachment of fluorophores to proteins in live cells and cast light on the advantages and disadvantages of each individual method. Selected experiments in which chemical tags have been successfully applied to live cell imaging will be discussed and evaluated.



Principle of chemical tagging strategies: the interaction of genetically encoded tags with small molecules allows for fluorescent labeling of proteins in live cells.

1. Introduction

One of the greatest challenges in life science is the understanding of the sophisticated interplay between structure and function of molecules within complex systems. Selective labeling of biomolecules with biophysical probes in principle allows for investigation and manipulation of proteins, enzymes or biochemical processes *in vitro* as well as *in vivo*. The invention of fluorescent proteins (FPs) as genetically encoded reporters in the early nineties revolutionized the cell biologist's ability to gain insight into cellular processes in living cells. Since the first application of the original *Aequorea Victoria* wild type green fluorescent protein (GFP) in nematodes [1] researchers started to generate improved FP versions which have better photophysical properties and optimal behavior in cells and organisms [2–3]. Nowadays, the scientific community has access to a vast number of FPs which cover a broad spectral range and have become an indispensable tool to study cell biology by fluorescence microscopy. However, there are yet a

* Corresponding author: e-mail: wombacher@uni-heidelberg.de; Phone: +49 (0) 6221 544879

few drawbacks associated with the application of FPs. The structural architecture and size of FPs, a β-barrel of $\sim$27 kDa, cannot be altered without the loss of fluorescent properties. The size of a reporter tag however, plays an important role, as the natural behavior of a protein of interest (POI) should be minimally perturbed by the reporter tag. Compared to fluorescent proteins, chemical tags are smaller and ideally can be combined with arbitrary biophysical probes. In recent years highly selective chemical tags have been invented as tools to address biological questions. Whereas *in vitro* labeling of purified proteins is routinely used in biology and pharmaceutical research, chemical methods are now being developed for *in vivo* labeling. This requires strategies which ensure the labeling of a specific target molecule in a cell that contains a daunting number of reactive and functional molecules. The demand for specificity is the most challenging task in the development of chemical labeling technologies. We describe the different strategies to achieve specific labeling with chemical tags and their application in live cell experiments.

2. What makes a good chemical tag?

Chemical tags are based on the interaction of a small molecule to a genetically encoded amino acid sequence (a small peptide or protein). This interaction has to be highly orthogonal, enabling the specific labeling of the tag sequence within highly functionalized environments. The ideal chemical tag for *in vivo* protein labeling should fulfill the following criteria: (i) the tag should be as small as possible so as not to perturb the properties of the molecule of interest; (ii) the labeling must be as specific as possible in order to achieve an optimal signal to noise ratio; and (iii) the labeling reaction should be fast and quantitative to ensure high labeling density; (iv) the method should enable the introduction of a multitude of biophysical probes; (v) the fluorophore conjugates must be cell permeable and well-behaved within cells for intracellular labeling, (vi) the tag as well as the fluorophore conjugates should not exhibit cytotoxic properties; (vii) it should work *in vitro* as well as *in vivo,* on cell surfaces, within cells and on or in cell compartments. Certainly, to meet all these criteria is nearly impossible. Each chemical tag has its own advantages and disadvantages, and, currently, none of the chemical tags fulfill all the requirements. For the design of a successful experiment, it is helpful to know the strengths of each approach to find the best labeling strategy for each individual experiment. In the following sections we will present different classes of chemical tags and discuss the individual pros and cons of each method.

3. The different types of chemical tags

Three general strategies have been developed for protein labeling in living cells, all based on the specific interaction between a genetically encoded tag and a small molecule (Table 1). In the case of fluorescent labeling, the small molecule is either fluorescent itself or covalently linked to a fluorophore. In this review we will use the term fluorescent label as we will mainly focus on the application of chemical tags for fluorescent labeling. The binding of a fluorescent label to the tag sequence occurs either by formation of a covalent bond (by self-modification), a non-covalent high affinity binding, or the formation of a covalent bond mediated by an enzyme (enzyme mediated tagging). The polypeptide tag can be an intact protein or a short peptide, expressed as fusion with the protein of interest (POI). Protein tags exploit the highly specific interaction between protein domains and the fluorescent label which thereby gets directed to the POI. The "covalent" protein tag forms a covalent bond with the label (self-modification), whereas the "non-covalent" exploits the high affinity interaction between a protein domain and the fluorescent label. Compared to protein tags, peptide tags have the advantage of being small in size, potentially minimizing the perturbation in folding of the protein and its natural behavior. Similar to protein tags, peptide tags have been developed for covalent and non covalent attachment of a fluorescent label to the peptide sequence. To achieve highly specific and strong binding of a fluorescent label to the interface of a short peptide sequence is a significant challenge with peptide tags. This problem can be solved when labeling is mediated by an enzyme that specifically recognizes the peptide as substrate and catalyzes the formation of a covalent bond to the fluorescent label.

4. Protein tags

4.1 Covalent protein tags

The group of Kai Johnsson was the first who exploited the reaction of a small molecule to a protein's active site for protein labeling purpose. They employ the ubiquitous human DNA repair enzyme O^6-alkylguanin-DNA alkyltransferase (hAGT) to label the POI with a substrate-probe conjugate. The hAGT is 20 kDa in size and responsible for the repair of O^6-alkylated guanine residues in DNA strands. The hAGT protein transfers alkyl groups from the O^6-position of the guanine in a one turnover self-modification reaction to a cysteine residue in its active site [4]. Johnsson and colleagues showed

Table 1 Chemical tagging methods.

protein tags

tag	substrate / ligand	size	live cell application
covalent			
hAGT (SNAP-tag)	benzyl guanine derivatives	20 kDa	extra- / intracellular
hAGT version (CLIP-tag)	cytosin derivatives	20 kDa	extra- / intracellular
dehalogenase (Halo-tag)	alkylhalide derivatives	33 kDa	extra- / intracellular
cutinase	pNpp	22 kDa	extracellular
noncovalent			
eDHFR (LigandLink)	trimethoprim derivatives	18 kDa	extra- / intracellular
FKBP(F36V)	SLF'-derivatives	12 kDa	extra- / intracellular
proximity induced covalent			
eDHFR(L28C)	trimethoprim derivatives	18 kDa	intracellular / in vitro
native proximity induced covalent			
native (e.g. CA)	ligand (e.g sulfonamide tosyl deriv.)	-	intracellular / in vivo

peptide tags

tag	substrate / ligand	size	live cell application
covalent			
tetracystein helix motif	biarsenical derivatives e.g. (FlAsH)	6 (12) aa	extra- / intracellular
noncovalent			
Texas red aptamer	Texas red derivatives	38 aa	intracellular
His_6	NTA-derivatives, HisZiFit	6 aa	extracellular
lanthanide binding tag	lanthanides	12-33 aa	extracellular
enzyme mediated covalent			
biotin acceptor peptide	biotin derivatives, BirA	15 aa	extracellular, in vitro
lipoic acid acceptor peptide	lipoic acid derivatives, LplA	22 aa	extra- / intracellular
lipoic acid acceptor peptide	coumarin derivatives, LplA(W37V)	22 aa	extra- / intracellular
sortase acceptor peptide	polyglycin conjugates, sortase A	5 aa	extracellular

that modified O^6-benzyl guanine substrates carrying fluorophores in the para position of the benzyl group result in labeling of hAGT at the active cysteine residue [5]. By mutational analysis they identified a hAGT mutant that catalyzed the self-labeling reaction 50 times faster than the wild type hAGT, thus eliminating the need for hAGT deficient cell lines [6–7]. In additional studies, they introduced a variety of probes that tag hAGT and its fusion proteins using this chemical labeling approach that they named SNAP-tag approach [8]. To date, the SNAP-tag is the most widely used chemical labeling technology, applied in live cell experiments for intra- as well as extracellular labeling. In 2008, the Johnsson

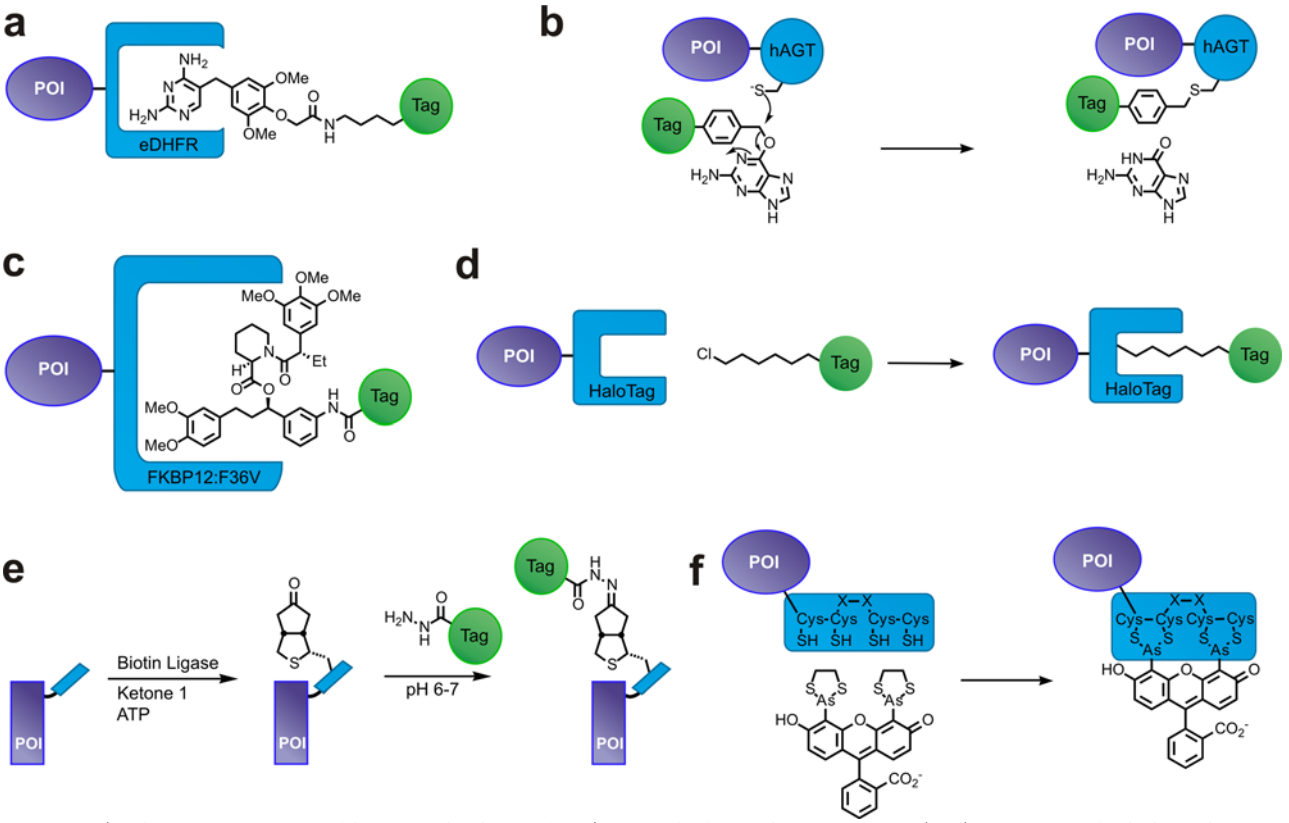


Figure 1 (online color at: www.biophotonics-journal.org) Chemical Tagging Methods. (**a**–**f**) Cartoons depicting different chemical tagging methods. (**a**) TMP-eDHFR labeling; (**b**) BG-hAGT labeling (SNAP-Tag); (**c**) SLF-FKBP12(F36V) labeling; (**d**) Halo Tag labeling strategy from Promega; (**e**) Biotin Ligase peptide tag labeling; e) FlAsH tag labeling.

lab reported the direct evolution of a hAGT mutant version which exhibits a 100-fold greater preference of cytosine over guanine substrate [9]. This mutant protein tag, named CLIP-tag, was shown to be selective enough to be used orthogonally to the SNAP-tag. For this reason, it is possible to simultaneously and specifically label two fusion proteins with different fluorophores allowing for multicolor labeling in one cell. As the SNAP- and the CLIP-tag have the same hAGT origin, this also demonstrates the potential of directed evolution for the development of new protein tags. Both the SNAP-tag and the CLIP-tag and their respective substrates are now commercially available from New England Biolabs (NEB).

Another self-modifying protein tag is based on a mutant version of a dehalogenase enzyme (marketed as "Halo Tag" by Promega) [10]. The 33 kDa wild type haloalkane dehalogenase hydrolyses alkylhalides by transferring the alkyl group covalently to an asparagine residue in the active site with the halide ion as the leaving group. In a second step, the previously formed covalent bond between the substrate and the protein is hydrolyzed to recycle the dehalogenase. The mutation of the His289 residue in the active site prevents the hydrolysis of the covalent bond [11]. The transferred alkyl group stays covalently bound and the mutant version of the dehalogenase can be used as a self modifying protein tag. Because eukaryotes lack endogenous dehalogenases, the "Halo Tag" can function as a specific labeling strategy in eukaryotic cells with low unspecific background staining. The small size of the substrate and the little synthetic effort to convert fluorophores to substrates makes the Halo-tag an attractive tag for applications in live cell experiments.

Bonasio et al. report the use of the fungal serin esterase cutinase (22 kDa) and its suicide substrate p-nitrophenyl phosphonate (pNPP) to covalently link fluorophores to an integrin on the surface of living cells [12]. The substrate p(NPP) is not able to permeate cell membranes and therefore the cutinase protein tag is restricted to extracellular protein labeling.

A very small protein tag (only 9 kDa in size) has been engineered based on the acyl-carrier protein (ACP) [13]. The ACP-tag developed by Yin et al. is an enzyme mediated protein tag. The covalent linkage of a 4′-phosphopantetheine-probe to a serin residue in ACP is formed by the enzyme phosphopantetheine transferase (PPTase). However, the charged

substrate has difficulty crossing cell membranes, which is why the ACP-tag is only suited for labeling cell surface proteins [13–15].

4.2 Noncovalent protein tags

All the above mentioned protein tags have the covalent bond formation between protein tag and fluorescent label in common, resulting in the irreversible attachment of the fluorophore. However, noncovalent receptor-ligand pairs can also be exploited for labeling, provided that the interaction is of high selectivity and affinity. Beneficial for the development of noncovalent protein tags is the longstanding experience in drug research providing an arsenal of potential receptor-ligand pair candidates. The Cornish group invented the first noncovalent protein tag which exploits the high affinity binding of folate analogs to the protein dihydrofolate reductase (DHFR) from *Escherichia coli* (*E. coli*). Methotrexate-fluorophore conjugates have been successfully used to label *E. coli* DHFR (eDHFR) expressed in DHFR deficient mammalian cells [16]. This was necessary to circumvent background staining from binding of methotrexate conjugates to endogenous DHFR. To overcome this limitation for further applications, methotrexate was substituted with the antibiotic trimethoprim (TMP). TMP binds to eDHFR with high affinity (1 nM K_D) but exhibits minimal binding to mammalian DHFR ($K_D > 1$ μM) [17]. It has been demonstrated that trimethoprim conjugates can be used to selectively label eDHFR in wild type mammalian cells. The synthetic access to the TMP conjugates is straightforward. Modifications introduced to the para-methoxy position of the benzene ring minimally affect the binding to eDHFR, representing an ideal position for the linkage to fluorophores [18–21]. TMP itself has excellent cell permeability, as expected from its use as therapeutic agent. The 18 kDa monomeric eDHFR behaves well when expressed in mammalian cells, and TMP-fluorophore conjugates with standard linker and protection group chemistry show good cell permeability. Labeling of nuclear proteins, plasma membrane proteins, and cytoplasmic proteins have been demonstrated in various cell types, showing that the eDHFR-TMP receptor-ligand pair can be used as a robust noncovalent protein labeling system in living cells [17–18]. The TMP-tag is marketed as LigandLink by Active Motif (Carlsbad, CA).

Marks et al. made a noncovalent protein tag based on the small molecule immunosuppressant FK506 and its target FKBP12 [22], a system pioneered by the laboratory of Stuart Schreiber [23, 24]. The 12 kDa human protein FKBP12 binds the natural ligand rapamycin with a K_D of 0.2 nM and a synthetic ligand (SLF) with subnanomolar affinity [25]. Furthermore, Clackson et al. developed a non-native receptor ligand pair, using the "bump-hole" approach. A FKBP12(F36V) mutant selectively binds a "bumped" SLF analog, also referred to as SLF', with very high affinity ($K_D = 0.094$ nM) [26]. In 2009 Robers et al. reported optimized SLF' fluorophore conjugates, validated by morphology analysis using a number of FKBP(F36V) fusion proteins in live cell imaging [27]. Currently, SLF' itself or SLF'-fluorophore conjugates are not commercially available, limiting the access to the FKBP based labeling strategy for cell biologists. In comparison to covalent attachment strategies, the non covalent labeling has experimental limitations for long term studies and also cannot be used for pulse-chase experiments.

4.3 Proximity induced covalent protein tags

Recent studies have shown that noncovalent tagging methods can be successfully converted to covalent tagging methods using proximity-induced reactivity. This method, applied in pharmaceutical research for the design of irreversible inhibitors, can also be adapted for protein labeling. Gallagher et al. engineered a covalent TMP-tag based on proximity-induced reactivity using an eDHFR(L28C) variant of the wild type eDHFR [28]. The high affinity binding of TMP is used to direct a reactive acrylamide electrophile in close proximity to the nucleophilic cysteine residue on the protein surface of the eDHFR(L28C) variant. It could be shown that eDHFR(L28C) fusions can be used for covalent protein labeling in cell lysates as well as in living cells. This report demonstrates the potential of proximity induced reactivity for the development of new covalent chemical tags from existing receptor-ligand pairs.

Whereas all previously discussed protein tags rely on genetic manipulations, namely the expression of the POI as fusion to the particular protein tag, Tsukiji et al. reported a native protein labeling strategy [29]. In this method, the ligand binds with high affinity to the native target protein directing a reactive group to the surface of the protein that forms a covalent bond through proximity-induced reactivity. To achieve labeling of the native protein carboanhydrase (CA), Tsukiji et al. used the CA inhibitor benzenesulfonamide conjugated to a fluorophore via an electrophilic phenylsulfonate ester (tosyl). Upon binding of the inhibitor conjugate to CA, the tosyl forms a covalent bond to a nucleophilic residue on the protein surface in a S_N2-type reaction with benzenesulfonamide as the leaving group. Thus, the inhibitor itself is not covalently bound to the target, but the fluorophore is covalently attached to the protein. The traceless labeling of CA using ligand directed to-

syl-chemistry (LDT) has been shown in living cells, tissues, and mice. Despite its elegance, such native labeling is limited to a small number of proteins with known high affinity ligands. The general reactivity of tosyls to nucleophiles might limit the application to highly abundant proteins. However, the method demonstrates the enormous potential of classic organic chemistry reactions to be exploited by chemical biology. Recently, Tsukiji et al. presented the application of LDT-chemistry for the construction of a fluorescent biosensor which is only fluorescent when covalently bound to the POI [30]. Such fluorogenic probes are of high interest for chemical labeling strategies as they can help to overcome the problem of background fluorescence from unbound probes.

5. Peptide tags

5.1 Non-enzymatic peptide tags

The ideal tag for chemical labeling should be as small as possible to minimally perturb the protein's function and behavior. Therefore the development of peptide tags, which consist of only a few amino acids, is of high interest for protein labeling. The inherent challenge for peptide labeling is to yield highly selective recognition of a small molecule from a short amino acid sequence. The first specific protein labeling using a peptide tag has been reported by Griffin et al. in 1998. A 15-amino acid tetracysteine tag with the consensus sequence CCPGCC shows high affinity to a bis-arsenical fluorescein probe named FlAsH (Fluorescein Arsenical Helix binder) [31, 32]. The design of the small tag enables the formation of arsenic-sulfur bonds between peptide and probe which are highly cooperative and entropically favored, resulting in a strong binding with a dissoziation constant of ~10 pM. Additionally, binding of the FlAsH-dye to the cysteine motif enhances the fluorescence 1000-fold, making it a fluorogenic tag. In further studies, the method was expanded to a red fluorescent version called ReAsH [33] and a biarsenical cyanine dye probe with the binding sequence CCKEAACC, orthogonal to the standard tetracysteine tag [34]. Although there have been further improvements to the tetracysteine-biarsenical system [31], it can show problems with unspecific background labeling and toxicity. The tag motif CCXXCC can not be found in the genome, but similar cysteine rich sequences are present in other proteins and can lead to non-specific labeling [35]. Although nonspecific interactions can be minimized by the addition of dithiols, the background labeling and the unspecific binding to hydrophobic sites in cellular components limits the potential of tetracysteine-biarsenical probes. Nevertheless, the FlAsH-tag has been used in a number of cell biology studies [36–38] and is the most established peptide tag to date.

A very similar strategy of labeling exploits the poly-His peptide tag, well established in protein purification and immobilization methods. Both reported probes, a NTA-chromophore conjugate [39] and a zinc-chelating fluorophore called HisZiFit [40], show moderate binding affinity to the poly-His peptide (NTA I with $K_d \sim 2\,\mu M$; HisZiFit-Zn^{2+} with $K_d \sim 40\,nM$). Weak binding and poor dye characteristics limit the poly-His tag approach to labeling on the cell surface. In contrast to these rational design strategies, peptide-ligand pairs have also been developed from peptide libraries using *in vitro* selection methods. Marks et al. selected a peptide aptamer against the fluorophore Texas red using phage display [41]. They obtained a 38-amino acid peptide sequence with picomolar affinity to a Texas red calcium sensor that has been applied in live cells for calcium imaging. Another peptide tag has been selected for lanthanide binding from a peptide library. Lanthanide ions have ionic radii similar to calcium and can be coordinated by short peptide sequences. Lanthanide-binding tags (LBTs) are only 15–20 amino acids in size and can bind Tb(III) with high affinity [42]. LBTs are genetically encodable luminescent tags and have the advantage of long-lived luminescence [43]. Franz et al. reported LBTs that bind lanthanides in the low micromolar range and that can be applied in extracellular labeling [44].

5.2 Enzyme mediated peptide tags

Since there are inherent difficulties to achieve high-specificity binding with short peptide tags, enzyme-mediated peptide labeling has been developed as an alternative strategy to label small peptides with fluorophores. The development of enzyme-modified peptide tags greatly benefit from cell posttranslational modifications (PTMs) carried out by enzymes that form covalent bonds between specific peptide sequences and their corresponding substrate [45]. Selectivity for the modification of a peptide is mediated by an enzyme that recognizes both the specific peptide sequence and the substrate for modification (e.g. farnesylation, prenylation, myristoylation, biotinylation). This concept has been adapted to develop enzyme-mediated peptide tags. For example, Biotin ligase is an enzyme that attaches the cofactor biotin to a specific peptide sequence [46]. The *E. coli* biotin ligase BirA covalently attaches biotin to a lysine residue in a 15 amino acid biotin acceptor peptide sequence (BAP) [47] and is orthogonal to eukaryotic biotinylation. Ting and colleagues synthe-

sized a biotin analog with a ketone functionality that is accepted as substrate by BirA [48]. Recently, they also utilized other biotin ligases from *yeast* (yBL) and *Pyrococcus horikoshii* (PhBL) to label corresponding acceptor peptides with azide- and alkyne functionalized biotin analogs [49]. The labeling with biotin analogs containing alkynes, ketones or azides allow for downstream modifications with fluorophores using click chemistry. However, the biotin ligase approach is restricted to protein labeling on the cell surface as endogenous biotin still serves as a much better substrate. Further mutational analysis of the individual biotin ligases are needed to reduce the cross reactivity and make the biotin ligase mediated modification of BAP a useful peptide tagging strategy for intracellular protein labeling. Another example of an enzyme mediated peptide tag is the *E. coli* lipoic acid ligase (LplA) that couples the prosthetic group lipoic acid to proteins involved in oxidative metabolism [50, 51]. LplA is structurally related to biotin ligase and shows mechanistic parallels to BirA. Similar to BirA, the Ting lab optimized LplA for substrates carrying an azide functionality [52]. By mutational analysis, the residue W37 was identified to be the most important for expanding the substrate specificity. Based on this finding Uttamapinant et al. recently evolved LplA mutants that accept a fluorescent coumarin derivative as substrate [53]. The two most promising candidates, LplA(W37V) and LplA(W37I), have succeeded in intracellular labeling of peptide tagged proteins and did not exhibit cross-reactivity to endogenous sequences or substrates. Ting and colleagues demonstrated for the first time that it is feasible to convert a natural ligase to a fluorophore ligase. This new method, which they call PRIME (probe incorporation mediated by enzymes) can serve as a superior alternative to the FlAsH-tag, having the advantage of low background labeling. However, PRIME does not allow for free choice of probes because the mutant LplA only accepts coumarin derivatives. The attractiveness of having a very small tag which minimizes protein perturbation comes with a trade off in the photophysical properties of the fluorophore. Although coumarins can be used for fluorescence microscopy, they are only moderately suited for high resolution fluorescence imaging. Nevertheless, PRIME is a breakthrough for the application of enzyme mediated peptide tags by making it a one-step labeling approach. Future studies will show if probe selectivity can be further expanded to more attractive fluorescent probes, allowing for widespread use of PRIME as tagging approach for fluorescence imaging. The shortest known enzyme-mediated peptide tag is the sortase tag [54–56]. The bacterial enzyme sortase A from *Staphylococcus aureus* recognizes the 5 amino acid peptide tag sequence LPXTG, cleaves between the T and G residues and subsequently forms a new peptide bond to a polyG-probe conjugate. The sortase tag is only applicable for cell surface labeling because the polyG substrates cannot cross the cell membrane.

6. Applications of chemical tags in live cell imaging

In recent years, some of the chemical tagging methods have surpassed the proof-of-principle stage and have been increasingly applied to address various biological questions. Chemical tags have been used to study the localization and dynamics of proteins in living cells, especially in experiments that can not be easily performed with fluorescent proteins. In the last few years, chemical tags have been used to label proteins with fluorophores suited for advanced imaging technologies such as super-resolution (SR) microscopy [57–59], Ca^{2+}-imaging [60–62], pH sensing [63], hydrogen peroxide detection [64], chromophore assisted light inactivation [36, 65, 66], and multi-photon microscopy [19]. Recently, Kosaka et al. demonstrated the use of the Halo-tag to perform *in vivo* imaging studies in live animals for the first time [67]. In the following, we focus on three applications of chemical tags that have shown advantages over fluorescent proteins, namely chromophore assisted light inactivation, Ca^{2+}-imaging, and super-resolution microscopy.

6.1 Fluorescent chemical tags for chromophore assisted light inactivation of proteins

Fluorophores can be used as photosensitizers that generate reactive oxygen species (ROS) upon light irradiation at the appropriate wavelength. This photochemical property can be utilized for acute, spatially and temporally controlled inactivation of proteins in chromophore-assisted light inactivation (CALI) [68]. Photosensitizers can damage proteins by the reaction of the ROS with methionine or by crosslinking [69]. This damage only occurs to the proteins in close proximity to the fluorophore due to the inactivation distance of approximately 3–4 nm [70]. However, the difficulty in CALI applications is to direct the damage and inactivation by ROS solely to the POI. Fluorescent proteins do not serve as good sources for ROS, presumably because the barrel structure screens the chromophore. Nevertheless, the FP called “killer red” was found to be a good photosensitizer but has the drawback of dimerization [71]. Chemical tags offer the ideal technology to localize photosensitizers in close proximity to the target protein. When directly attached to the POI, the damage from the generated ROS to other proteins

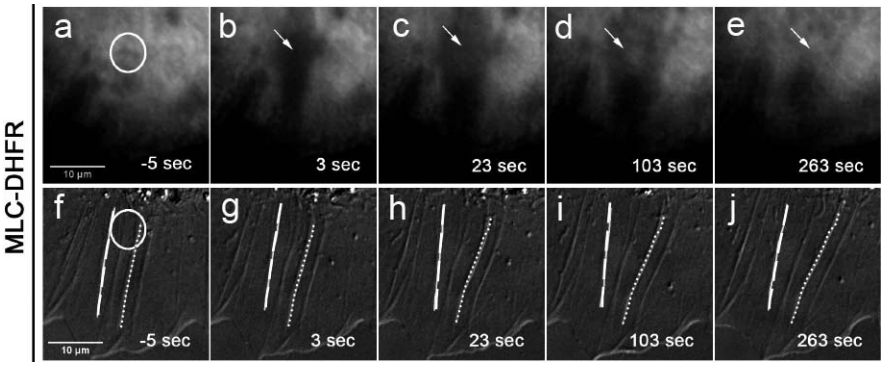


Figure 2 Chromophore assisted light inactivation of NMII using the TMP-fluoresceine tag. Laser inactivation of TMP-fluorescein labeled MLC-eDHFR fusion proteins by irradiation of a ~7.0 µm diameter for 1 sec. (**a**–**j**) CALI of cytoplasmic myosin II locally destroyed the contractile actomyosin II network, revealed by the curvature of the actin cables close to the irradiated region marked with the dashed and dotted lines [66].

will most likely be minimized. The FlAsH-tag was the first chemical tag to be used in inactivation experiments. Kurt et al. utilized the FlAsH-tag for CALI in neuronal cells from *Drosophila,* to study the role of synaptotagmin I (syt IaC) in vesicle formation [36] and demonstrated that FlAsH-tag CALI can be successfully combined with electrophysiological methods. Fluorescein with a high singlet oxygen quantum yield is a good photosensitizer and, therefore, a suitable reagent for CALI experiments. Cai et al. successfully used eDHFR-tag with TMP-fluorescein in CALI experiments to reveal that nonmuscle myosin II (NMII) is required to maintain cytoplasmic coherence [66]. The myosin light chain (MLC) is part of the NMII hexamer and can be labeled with TMP-fluorescein when expressed as a MLC-eDHFR fusion. Inactivation of the cytoplasmic NMII using CALI caused a loss of local coherence in the actin cytoskeleton network that was demonstrated by the curving of actin cables around the irradiated region (those denoted by dashed and dotted lines (Figure 2 f–j) upon inactivation of MLC (Figure 2 g–i).

The SNAP-tag has also been applied in CALI using the photosensitizer fluorescein. Keppler et al. studied the effect of inactivation of α- and γ-tubulin on cell division and spindle function [65]. Inactivation of fluorescein tagged SNAP-α-tubulin causes spindle shortening and arrest in metaphase, whereas inactivation of centrosomal γ-tubulin affected microtubule nucleation and growth rate. Recently, an eosin probe for Halo-tag technology was successfully used in CALI. It was demonstrated that the eosin photosensitizing probe shows superior effectiveness in ROS generation over fluorescein [72].

6.2 Localization of fluorescent Ca^{2+}-sensors by chemical tags

Intra- and extracellular Ca^{2+} is central to a multitude of physiological and biochemical processes such as signal transduction or muscle contraction. One of the earliest breakthroughs for live cell imaging was the invention of chemical Ca^{2+}-indicators by Roger Tsien [73]. The functional principle is based on Ca^{2+} chelating fluorophores that only fluoresce when complexed with Ca^{2+}. Nowadays, there is a large number of commercially available Ca^{2+}-indicators with different Ca^{2+}-affinities, applicable systems with Ca^{2+}-concentrations ranging from 50 nM to 50 µM [74]. The particular disadvantage of chemical Ca^{2+}-sensors is the difficulty of controlling cellular localization, for example, the targeting to specific organelles. As an alternative to the chemical sensors, the Tsien lab and others developed genetically encoded Ca^{2+}-indicators (GECIs) based on fusions of FPs with calcium responsive elements like calmodulin or troponin [75–78]. These indicators allow for the dissection of the spatial and temporal control of calcium signaling processes in cells because they are genetically encoded and therefore specifically localized. However, these protein based sensors named "cameleons" or "camgaroos" do not cover the full detection range as well as chemical Ca^{2+}-indicators do. Therefore, chemical methods have been developed to combine the advantages of genetically encoded tags and chemical Ca^{2+}-indicators. Chemical tags are ideally suited to localize chemical sensors to a specific organelle or protein.

There are currently three reports from the Johnsson laboratory using the SNAP-tag approach in combination with chemical Ca^{2+}-indicators. Bannwarth et al. demonstrated calcium sensing with Indo-1 and Fura-2FF SNAP-tag conjugates and verified the Ca^{2+}-sensitivity of the probes in live cell experiments. It was shown that the derivatization and covalent attachment to proteins did not affect the Ca^{2+}-sensing capabilities of the two well known chemical Ca^{2+}-indicators [60, 61]. A new sensitive calcium indicator based on BODIPY was developed by Kamiya et al. [62]. A benzyl guanine derivative of the BODIPY sensor was successfully coupled to the SNAP-tag (localized to the nucleus) and used for sensitive Ca^{2+}-imaging in live cells. The SNAP-tag Ca^{2+}-sensors are still in the proof-of-principle stage. Further experiments will show if they can serve as

robust tools in calcium imaging to address further biological questions.

A FlAsH-tag based calcium sensor has been shown to provide new insights into the mechanisms by which voltage-gated calcium channels regulate signaling pathways [79]. Tour et al. used the FlAsH-tag method to localize the calcium sensor calcium green-FlAsH (CsGF) to the mouth of the calcium channel $Ca_v1.2$. By measuring the calcium signals generated at the pore of calcium channels, they showed that spatially elevated calcium concentrations are not caused by single channels but by channel clusters. This study demonstrates the strength of chemical tags for measuring calcium concentrations close to ion channels in cells. Further applications using genetically encodeable tags in combination with chemical sensors will greatly expand the ability to study fast calcium signals in domains next to proteins.

6.3 Chemical tags for super-resolution imaging in live cells

The diffraction of light classically limits the resolution of fluorescence microscopy to ~200 nm. In the last few years, this constraint has been removed by the invention of new microscopy technologies that break the diffraction limit [80–82]. These new technologies, termed super-resolution (SR) microscopy, can improve the spatial resolution of fluorescence microscopy over 10-fold to length scales approaching those necessary to determine the structures of macromolecular complexes in cells. For more details about each individual technology, we refer to reviews about SR microscopy [83–85]. Important to all super-resolution microscopy technologies is the ability to switch emitters between fluorescent "on" and fluorescent "off" states.

Among the super-resolution microscopy methods, photoactivated localization microscopy (PALM) [80], and stochastic optical reconstruction microscopy (STORM) [82], has the unique advantage of simple setup and the versatility for multicolor colocalization. As illustrated in Figure 3, in PALM/STORM individual fluorophores are localized at resolutions well beyond the ~200 nm diffraction limit by (i) stochastically photoactivating small percentages of the total population of fluorophores over time and (ii) determining the centroid position of these individual fluorophores from the Gaussian fits of their point spread functions (Figure 3). Given, that single-fluorophore localization precisions as low as ~10 Å have already been achieved *in vitro* [86, 87], technical advances are expected to further improve the resolutions in live cells. SR-microscopy

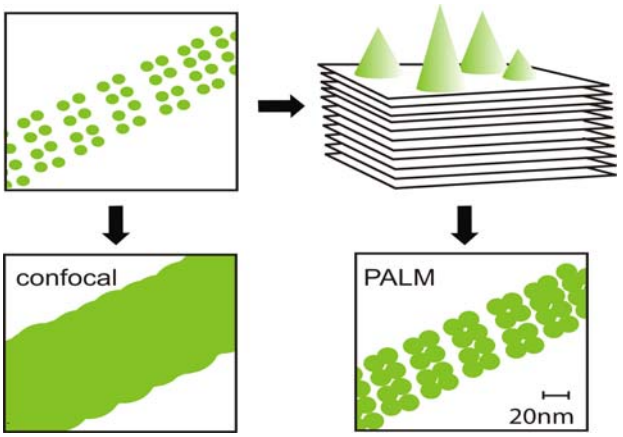


Figure 3 (online color at: www.biophotonics-journal.org) PALM/STORM. Schematic cartoons of conventional confocal fluorescence microscopy (left) and super-resolution imaging using PALM/STORM (right). In conventional confocal microscopy, the resolution set by the diffraction of light is ~200 nm, and thus individual protein molecules, typically 1–5 nm themselves and densely packed, cannot be resolved. In PALM/STORM, small percentages of the total population of fluorophores are randomly photoactivated over time, allowing all individual emitter to be localized to resolutions of ~20 nm in the x, y-plane from the Gaussian fits of their point spread functions.

has so far done mostly in fixed cells. Recently, it could be shown that chemical tagging strategies are ideal platforms to introduce fluorophores that have the photophysical characteristics to allow for SR-microscopy with live cells [88].

Dellagiacoma et al. used SNAP-tag probes that contain Cy3-Cy5 pairs to label SNAP-α-tubulin and SNAP-β-tubulin fusions, demonstrating the capability of the probe for STORM-based SR imaging [58]. Cy3-Cy5 pairs allow for controlled switching between fluorescent "on" and "off" states [82, 89], but they only can be used in fixed cells, as cyanine-pairs require a deoxygenated environment and do not perform switching in the reductive environment of living cells. In another report, Schroder et al. applied Halo-tag labeling to integrins on the cell surface for SR imaging by stimulated emission depletion (STED) microscopy [90]. Hein et al. used the SNAP-tag to label cytoskeletal and membrane proteins with fluorophores suitable for STED and demonstrated that chemical tags have the great potential to introduce fluorophores that enable SR imaging in living cells.

Recently it has been reported that the noncovalent eDHFR TMP-tag is suitable for SR-imaging based on dSTORM (directSTORM) [59]. Wombacher et al. successfully labeled the histone protein H2B-eDHFR fusion with a TMP-ATTO655 conjugate. The fluorophore ATTO655 shows inherent

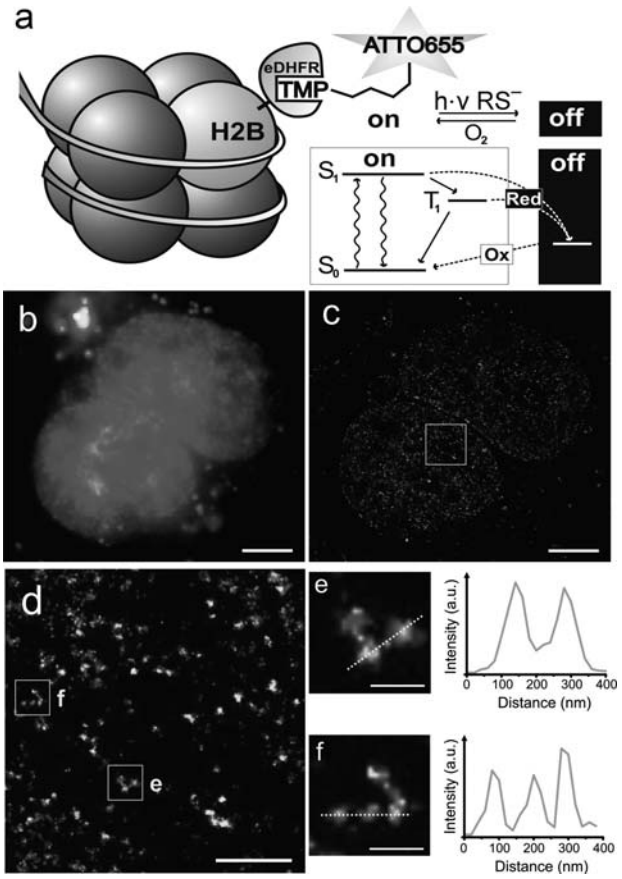


Figure 4 Super-resolution imaging of H2B labeled with TMP-ATTO655 conjugates. (**a**) Schematic representation of super-resolution imaging according to the *d*STORM method with the TMP-eDHFR labeling system. TMP is covalently attached to ATTO655 and binds to eDHFR fused to H2B. Upon irradiation at 647 nm ATTO655 is efficiently transferred to a reduced non-fluorescent species in the presence of millimolar concentrations of glutathione (RS^-) in living cells. The fluorescent state is recovered upon reaction with molecular oxygen. (**b**) wide field image (**c**) dSTORM image reconstructed from 10000 images recorded upon excitation at 647 nm with 5 kW/cm^2 at a frame rate of 50 Hz (~3 min acquisition time). The expanded views and cross-sectional profiles (**d**, **e**, **f**) demonstrate superior resolution well below the diffraction barrier. Adjacent histone proteins separated by 50–100 nm are clearly resolved [59].

photoswitching catalyzed by the reducing environment of the cell as a function of excitation laser power [91, 92]. Pronounced photoswitching of ATTO655 under physiological conditions and a high stability of the "off" state has been observed. Therefore, the fluorophore fulfills very well the prerequisites for dSTORM based SR-imaging and is compatible with super-resolution imaging in live cells. Applying dSTORM imaging increased the resolution to ~20 nm, and allowed the identification of histones within the nucleoprotein complex (Figure 4). Furthermore, Wombacher et al. could exploit the label to follow nucleosome movements in living cells by reconstructing SR-images of 500 frames with a temporal resolution of 10 s (50 Hz). In a recent publication, Klein et al. demonstrated the use of SNAP-tag based H2B labeling with a rhodamine type fluorophore showing switching behavior in live cells similar to ATTO655. They successfully validated the capability of rhodamines for SR-imaging in live cell dSTORM experiments [93].

Acknowledgments The Authors would like to thank Prof. Michael P. Sheetz and Dr. Sarah Gallagher for contributing to the figures and Sarah Schlatterer, Dr. Joerg Schlatterer and Chaoran Jing for critical reading and helpful comments on the manuscript.

Richard Wombacher is a junior group leader in the Institute of Pharmacy and Molecular Biotechnology (IPMB) at the Heidelberg University. He graduated in chemistry at the Free University of Berlin and received his Ph.D. at the Heidelberg University working in the field of RNA catalysis. After a post-doc in the laboratory of Prof. V. W. Cornish at the Columbia University he started his own research group at the Heidelberg University, working in the field of chemical biology. His research is focused on the development and application of chemical tools for protein modification and control of protein function.

Virginia W. Cornish is a Professor in the Department of Chemistry, 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 post-doctoral research in the Department of Biology at the Massachusetts Institute of Technology. Her research has been recognized by numerous awards, including 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 methods for carrying out chemistry in living cells, with application to tools for live cell imaging.

References

[1] M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher, Science **263**, 802 (1994).
[2] B. N. Giepmans, S. R. Adams, M. H. Ellisman, and R. Y. Tsien, Science **312**, 217 (2006).
[3] N. C. Shaner, G. H. Patterson, and M. W. Davidson, J. Cell Sci. **120**, 4247 (2007).
[4] A. E. Pegg, Mutat. Res. **462**, 83 (2000).
[5] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, and K. Johnsson, Nat. Biotechnol. **21**, 86 (2003).
[6] A. Keppler, M. Kindermann, S. Gendreizig, H. Pick, H. Vogel, and K. Johnsson, Methods **32**, 437 (2004).
[7] A. Keppler, H. Pick, C. Arrivoli, H. Vogel, and K. Johnsson, Proc. Natl. Acad. Sci. USA **101**, 9955 (2004).
[8] A. Keppler, C. Arrivoli, L. Sironi, and J. Ellenberg, Biotechniques **41**, 167 (2006).
[9] A. Gautier, A. Juillerat, C. Heinis, I. R. Correa, Jr., M. Kindermann, F. Beaufils, and K. Johnsson, Chem. Biol. **15**, 128 (2008).
[10] G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit, and K. V. Wood, ACS Chem. Biol. **3**, 373 (2008).
[11] F. Pries, J. Kingma, G. H. Krooshof, C. M. Jeronimus-Stratingh, A. P. Bruins, and D. B. Janssen, J. Biol. Chem. **270**, 10405 (1995).
[12] R. Bonasio, C. V. Carman, E. Kim, P. T. Sage, K. R. Love, T. R. Mempel, T. A. Springer, and U. H. von Andrian, Proc. Natl. Acad. Sci. USA **104**, 14753 (2007).
[13] J. Yin, F. Liu, X. Li, and C. T. Walsh, J. Am. Chem. Soc. **126**, 7754 (2004).
[14] J. Yin, P. D. Straight, S. M. McLoughlin, Z. Zhou, A. J. Lin, D. E. Golan, N. L. Kelleher, R. Kolter, and C. T. Walsh, Proc. Natl. Acad. Sci. USA **102**, 15815 (2005).
[15] Z. Zhou, P. Cironi, A. J. Lin, Y. Xu, S. Hrvatin, D. E. Golan, P. A. Silver, C. T. Walsh, and J. Yin, ACS Chem. Biol. **2**, 337 (2007).
[16] L. W. Miller, J. Sable, P. Goelet, M. P. Sheetz, and V. W. Cornish, Angew. Chem. Int. Ed. Engl. **43**, 1672 (2004).
[17] L. W. Miller, Y. Cai, M. P. Sheetz, and V. W. Cornish, Nat. Methods **2**, 255 (2005).
[18] N. T. Calloway, M. Choob, A. Sanz, M. P. Sheetz, L. W. Miller, and V. W. Cornish, Chembiochem **8**, 767 (2007).
[19] S. S. Gallagher, C. Jing, D. S. Peterka, M. Konate, R. Wombacher, L. J. Kaufman, R. Yuste, and V. W. Cornish, Chembiochem **11**, 782 (2010).
[20] N. Gahlaut and L. W. Miller, Cytometry A **77**, 1113 (2010).
[21] H. E. Rajapakse, D. R. Reddy, S. Mohandessi, N. G. Butlin, and L. W. Miller, Angew. Chem. Int. Ed. Engl. **48**, 4990 (2009).
[22] K. M. Marks, P. D. Braun, and G. P. Nolan, Proc. Natl. Acad. Sci. USA **101**, 9982 (2004).
[23] B. E. Bierer, P. K. Somers, T. J. Wandless, S. J. Burakoff, and S. L. Schreiber, Science **250**, 556 (1990).
[24] D. M. Spencer, T. J. Wandless, S. L. Schreiber, and G. R. Crabtree, Science **262**, 1019 (1993).
[25] T. Clackson, W. Yang, L. W. Rozamus, M. Hatada, J. F. Amara, C. T. Rollins, L. F. Stevenson, S. R. Magari, S. A. Wood, N. L. Courage, X. Lu, F. Cerasoli, Jr., M. Gilman, and D. A. Holt, Proc. Natl. Acad. Sci. USA **95**, 10437 (1998).
[26] C. T. Rollins, V. M. Rivera, D. N. Woolfson, T. Keenan, M. Hatada, S. E. Adams, L. J. Andrade, D. Yaeger, M. R. van Schravendijk, D. A. Holt, M. Gilman, and T. Clackson, Proc. Natl. Acad. Sci. USA **97**, 7096 (2000).
[27] M. Robers, P. Pinson, L. Leong, R. H. Batchelor, K. R. Gee, and T. Machleidt, Cytometry A **75**, 207 (2009).
[28] S. S. Gallagher, J. E. Sable, M. P. Sheetz, and V. W. Cornish, ACS Chem. Biol. **4**, 547 (2009).
[29] S. Tsukiji, M. Miyagawa, Y. Takaoka, T. Tamura, and I. Hamachi, Nat. Chem. Biol. **5**, 341 (2009).
[30] S. Tsukiji, H. Wang, M. Miyagawa, T. Tamura, Y. Takaoka, and I. Hamachi, J. Am. Chem. Soc. **131**, 9046 (2009).
[31] B. A. Griffin, S. R. Adams, J. Jones, and R. Y. Tsien, Methods Enzymol. **327**, 565 (2000).
[32] B. A. Griffin, S. R. Adams, and R. Y. Tsien, Science **281**, 269 (1998).
[33] S. R. Adams, R. E. Campbell, L. A. Gross, B. R. Martin, G. K. Walkup, Y. Yao, J. Llopis, and R. Y. Tsien, J. Am. Chem. Soc. **124**, 6063 (2002).
[34] H. Cao, Y. Xiong, T. Wang, B. Chen, T. C. Squier, and M. U. Mayer, J. Am. Chem. Soc. **129**, 8672 (2007).
[35] K. Stroffekova, C. Proenza, and K. G. Beam, Pflugers Arch. **442**, 859 (2001).
[36] K. W. Marek and G. W. Davis, Neuron **36**, 805 (2002).
[37] L. Rudner, S. Nydegger, L. V. Coren, K. Nagashima, M. Thali, and D. E. Ott, J. Virol. **79**, 4055 (2005).
[38] O. Tour, R. M. Meijer, D. A. Zacharias, S. R. Adams, and R. Y. Tsien, Nat. Biotechnol. **21**, 1505 (2003).
[39] E. G. Guignet, R. Hovius, and H. Vogel, Nat. Biotechnol. **22**, 440 (2004).
[40] C. T. Hauser and R. Y. Tsien, Proc. Natl. Acad. Sci. USA **104**, 3693 (2007).
[41] K. M. Marks, M. Rosinov, and G. P. Nolan, Chem. Biol. **11**, 347 (2004).
[42] M. Nitz, K. J. Franz, R. L. Maglathlin, and B. Imperiali, Chembiochem **4**, 272 (2003).
[43] J. C. Bunzli, Acc. Chem. Res. **39**, 53 (2006).
[44] K. J. Franz, M. Nitz, and B. Imperiali, Chembiochem **4**, 265 (2003).
[45] C. T. Walsh, S. Garneau-Tsodikova, and G. J. Gatto, Jr., Angew. Chem. Int. Ed. Engl. **44**, 7342 (2005).
[46] M. G. Cull and P. J. Schatz, Methods Enzymol. **326**, 430 (2000).
[47] D. Beckett, E. Kovaleva, and P. J. Schatz, Protein Sci. **8**, 921 (1999).

[48] I. Chen, M. Howarth, W. Lin, and A. Y. Ting, Nat. Methods **2**, 99 (2005).
[49] S. A. Slavoff, I. Chen, Y. A. Choi, and A. Y. Ting, J. Am. Chem. Soc. **130**, 1160 (2008).
[50] J. E. Cronan, X. Zhao, and Y. Jiang, Adv. Microb. Physiol. **50**, 103 (2005).
[51] X. Zhao, J. R. Miller, Y. Jiang, M. A. Marletta, and J. E. Cronan, Chem. Biol. **10**, 1293 (2003).
[52] M. Fernandez-Suarez, H. Baruah, L. Martinez-Hernandez, K. T. Xie, J. M. Baskin, C. R. Bertozzi, and A. Y. Ting, Nat. Biotechnol. **25**, 1483 (2007).
[53] C. Uttamapinant, K. A. White, H. Baruah, S. Thompson, M. Fernandez-Suarez, S. Puthenveetil, and A. Y. Ting, Proc. Natl. Acad. Sci. USA **107**, 10914 (2010).
[54] M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, and H. L. Ploegh, Nat. Chem. Biol. **3**, 707 (2007).
[55] M. W. Popp, J. M. Antos, and H. L. Ploegh, Curr. Protoc. Protein Sci. **15**, Unit 15.3 (2009).
[56] T. Tanaka, T. Yamamoto, S. Tsukiji, and T. Nagamune, Chembiochem **9**, 802 (2008).
[57] H. L. Lee, S. J. Lord, S. Iwanaga, K. Zhan, H. Xie, J. C. Williams, H. Wang, G. R. Bowman, E. D. Goley, L. Shapiro, R. J. Twieg, J. Rao, and W. E. Moerner, J. Am. Chem. Soc. **132**, 15099 (2010).
[58] C. Dellagiacoma, G. Lukinavicius, N. Bocchio, S. Banala, S. Geissbuhler, I. Marki, K. Johnsson, and T. Lasser, Chembiochem **11**, 1361 (2010).
[59] R. Wombacher, M. Heidbreder, S. van de Linde, M. P. Sheetz, M. Heilemann, V. W. Cornish, and M. Sauer, Nat. Methods **7**, 717 (2010).
[60] M. Bannwarth, I. R. Correa, M. Sztretye, S. Pouvreau, C. Fellay, A. Aebischer, L. Royer, E. Rois, and K. Johnsson, ACS Chem. Biol. **4**, 179 (2009).
[61] A. A. Ruggiu, M. Bannwarth, and K. Johnsson, Org. Biomol. Chem. **8**, 3398 (2010).
[62] M. Kamiya and K. Johnsson, Anal. Chem. **82**, 6472 (2010).
[63] H. Benink, M. McDougall, D. Klaubert, and G. Los, Biotechniques **47**, 769 (2009).
[64] D. Srikun, A. E. Albers, C. I. Nam, A. T. Iavarone, and C. J. Chang, J. Am. Chem. Soc. **132**, 4455 (2010).
[65] A. Keppler and J. Ellenberg, ACS Chem. Biol. **4**, 127 (2009).
[66] Y. Cai, O. Rossier, N. C. Gauthier, N. Biais, M. A. Fardin, X. Zhang, L. W. Miller, B. Ladoux, V. W. Cornish, and M. P. Sheetz, J. Cell Sci. **123**, 413 (2010).
[67] N. Kosaka, M. Ogawa, P. L. Choyke, N. Karassina, C. Corona, M. McDougall, D. T. Lynch, C. C. Hoyt, R. M. Levenson, G. V. Los, and H. Kobayashi, Bioconjug. Chem. **20**, 1367 (2009).
[68] D. G. Jay, Proc. Natl. Acad. Sci. USA **85**, 5454 (1988).
[69] P. Yan, Y. Xiong, B. Chen, S. Negash, T. C. Squier, and M. U. Mayer, Biochemistry-US **45**, 4736 (2006).
[70] K. G. Linden, J. C. Liao, and D. G. Jay, Biophys. J. **61**, 956 (1992).
[71] M. E. Bulina, D. M. Chudakov, O. V. Britanova, Y. G. Yanushevich, D. B. Staroverov, T. V. Chepurnykh, E. M. Merzlyak, M. A. Shkrob, S. Lukyanov, and K. A. Lukyanov, Nat. Biotechnol. **24**, 95 (2006).
[72] K. Takemoto, T. Matsuda, M. McDougall, D. H. Klaubert, A. Hasegawa, G. V. Los, K. V. Wood, A. Miyawaki, and T. Nagai, ACS Chem. Biol. DOI: 10.1021/cb100431e [Epub ahead of print] (2011).
[73] G. Grynkiewicz, M. Poenie, and R. Y. Tsien, J. Biol. Chem. **260**, 3440 (1985).
[74] R. M. Paredes, J. C. Etzler, L. T. Watts, W. Zheng, and J. D. Lechleiter, Methods **46**, 143 (2008).
[75] A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura, and R. Y. Tsien, Nature **388**, 882 (1997).
[76] N. Heim and O. Griesbeck, J. Biol. Chem. **279**, 14280 (2004).
[77] J. E. McCombs and A. E. Palmer, Methods **46**, 152 (2008).
[78] A. E. Palmer, Y. Qin, J. G. Park, and J. E. McCombs, Trends Biotechnol. **29**(3): 144–152 (2011).
[79] O. Tour, S. R. Adams, R. A. Kerr, R. M. Meijer, T. J. Sejnowski, R. W. Tsien, and R. Y. Tsien, Nat. Chem. Biol. **3**, 423 (2007).
[80] E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, and H. F. Hess, Science **313**, 1642 (2006).
[81] T. A. Klar, S. Jakobs, M. Dyba, A. Egner, and S. W. Hell, Proc. Natl. Acad. Sci. USA **97**, 8206 (2000).
[82] M. J. Rust, M. Bates, and X. Zhuang, Nat. Methods **3**, 793 (2006).
[83] S. W. Hell, Science **316**, 1153 (2007).
[84] G. Patterson, M. Davidson, S. Manley, and J. Lippincott-Schwartz, Annu. Rev. Phys. Chem. **61**, 345 (2010).
[85] L. Schermelleh, R. Heintzmann, and H. Leonhardt, J. Cell Biol. **190**, 165 (2010).
[86] A. Yildiz, J. N. Forkey, S. A. McKinney, T. Ha, Y. E. Goldman, and P. R. Selvin, Science **300**, 2061 (2003).
[87] A. Yildiz, M. Tomishige, R. D. Vale, and P. R. Selvin, Science **303**, 676 (2004).
[88] M. Fernandez-Suarez and A. Y. Ting, Nat. Rev. Mol. Cell Biol. **9**, 929 (2008).
[89] M. Bates, B. Huang, G. T. Dempsey, and X. Zhuang, Science **317**, 1749 (2007).
[90] J. Schroder, H. Benink, M. Dyba, and G. V. Los, Biophys. J. **96**, L01 (2009).
[91] M. Heilemann, S. van de Linde, A. Mukherjee, and M. Sauer, Angew. Chem. Int. Ed. Engl. **48**, 6903 (2009).
[92] J. Vogelsang, T. Cordes, C. Forthmann, C. Steinhauer, and P. Tinnefeld, Proc. Natl. Acad. Sci. USA **106**, 8107 (2009).
[93] T. Klein, A. Loschberger, S. Proppert, S. Wolter, S. van de Linde, and M. Sauer, Nat. Methods **8**, 7 (2011).

