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# Tag–probe labeling methods for live-cell imaging of membrane proteins

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Abstract

Instead of using reconstituted proteoliposomes, in situ investigations of membrane proteins in living cell membranes are important because the heterogeneous and dynamic nature of biomembranes significantly affects their behavior. Protein-specific labeling is a key technique for the detection of a target protein by fluorescence measurements, particularly fluorescence microscopy. However, conventional genetic fusion with fluorescent proteins has several shortcomings. Post-translational labeling methods using a genetically encodable tag and synthetic probes targeting to the tag can overcome these limitations. This review summarizes emerging tag–probe techniques for labeling specific membrane proteins and their applications, including endocytotic internalization, partitioning to specific membrane domains, interprotein interactions, and conformational changes.

Contents

# 1. Introduction

Integral membrane proteins are essential for vital functions across cell membranes such as signal transduction, material transport, energy conversion, and intercellular communication. These dynamic functionalities of the proteins are based on a ligand-induced shift in conformational equilibrium, which, in some cases, sequentially induces new intermolecular interactions and translocation of the protein via membrane trafficking machinery. Lipids also highly influence the folding and conformation of integral membrane proteins. For example, the lipid compositions of reconstituted proteoliposomes could dramatically alter the activity of incorporated membrane proteins [1,2]. Experimental systems using model transmembrane helices have also revealed that the thermodynamics of helix–helix interaction, the major driving force for membrane protein folding, strongly depends on lipid composition [3–5]. A biomembrane contains several hundred or more different lipid species varying in physicochemical properties such as electric charge and fluidity. The distribution of the lipids is heterogeneous among organelles [6,7] and between the extracellular and cytosolic leaflets of a bilayer [7], and even laterally within a leaflet on a nanoscale [8]. These facts suggest the structure and function of membrane proteins to be sophisticatedly regulated by lipids in cell membranes. Therefore, experimental approaches for the in situ investigation of membrane proteins in living cell membranes are essential to

observe the dynamic behavior of the proteins, in addition to reconstituted systems using

isolated proteins.

Fluorescence microscopy, such as epifluorescence or confocal microscopy, has been

widely used to detect proteins in living cells in combination with protein-specific labeling

techniques. The visualization of membrane proteins in living cells can reveal dynamic

behavior such as endocytotic internalization (Fig. 1A) and partitioning to specific membrane

domains (Fig. 1B). In addition to simple observations of the intracellular dynamics of

proteins, Förster resonance energy transfer (FRET) between different fluorophores having a

spectral overlap detects changes in distance and/or orientation of the fluorophores in the range

<100 Å and thus is useful for monitoring interprotein interactions (Fig. 1C) and

conformational changes (Fig. 1D). Other advanced applications of fluorescence techniques

are reviewed in Ref. [9], including pulse-chase labeling and chromophore-assisted laser

inactivation of the target protein.

Genetic fusion of fluorescent proteins to a target protein has been widely used for

protein-specific labeling in living cells [9,10]. However, the large size of fluorescent

proteins (e.g. ~ 27 kDa for GFP) might disrupt the normal trafficking and function of target

proteins (see [11,12], for example). The large size also greatly restricts use for site-specific

labeling within a protein, for example, for detection of conformational changes (Fig. 1D).

Furthermore, modern imaging techniques such as single molecule microscopy require fluorophores with better photophysical properties, such as long-term photostability and greater brightness. Precise control of the labeling ratio in multicolor labeling for FRET measurements is not easy using fluorescent proteins either. To overcome these shortcomings, post-translational labeling methods using a genetically encodable tag and synthetic probes targeting the tag have recently emerged to specifically label proteins in living cells. Diverse fluorophores with improved brightness, photostability, and spectral properties, including quantum dots, can be specifically attached to target proteins using the tag–probe techniques, although the size of quantum dots could be a major problem. Integral membrane proteins expressed on the cell surface are generally available for most tag–probe techniques because probes are accessible to the tags attached to the extracellular domains of the proteins. This review summarizes tag–probe techniques for labeling membrane proteins and their applications. Several related reviews have also been published on selective labeling techniques generally used in living cells [13–16], fluorescent probes for super-resolution imaging [17], metal-chelation labeling [18] and bioorthogonal chemistry in living cells [19].

2. Labeling principles and applications

Various labeling methods developed so far rely on protein–ligand interaction,

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peptide–peptide interaction, peptide–fluorophore interaction, metal chelation, and enzymatic

reactions (Fig. 2 and Table 1).   A protein tag genetically fused to a target protein can be

labeled with a ligand conjugated to a fluorophore (Fig. 2A).   If the ligand is a peptide, it can

be used as a tag that is labeled by a protein probe (Fig. 2A').   Two peptides that form a tight

heterodimer are also used as a tag–probe combination (Fig. 2B).   A minimalist approach is

the use of a peptide tag that directly binds a fluorophore (Fig. 2C).   A combination of a

peptide and a chemical that cooperatively binds a metal ion is another principle for tag–probe

labeling (Fig. 2D).   Finally, an enzymatic reaction that covalently conjugates a substrate to a

specific site of a peptide is useful for covalent labeling (Fig. 2E).


2.1 Protein–ligand interaction

Noncovalent binding of a ligand to a protein has been used for specific labeling of

membrane proteins.   The ligand trimethoprim has been used to label *E. coli* dihydrofolate

reductase (eDHFR) at the plasma membrane by fusing the N-terminus of eDHFR to the

myristoylation/palmitoylation sequence (MGCIKSKGKD) (Table 1) [20].   A labeling kit

(LigandLink[TM]) is available from Active Motif (Carlsbad, CA).   Also, a mutant of the human

FKBP12 protein (FKBP12 (F36V)) and a synthetic ligand specific for the protein (SFL') can

label a variety of proteins (Table 1), including caveolin, rac, and rho in HeLa cells [21,22],

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although only two types of fluorophores [tetramethylrhodamine (TMR) and fluorescein] are

suitable for effective labeling in spite of the extensive examination of a number of

fluorophores with different linkers [22].    If the ligand is a peptide, it is useful as a tag that

binds to the protein probe (Fig. 2A').    A 13-amino acid peptide that binds to α-bungarotoxin

has been successfully used as a tag to label extracellular domains of target proteins (Table 1)

[23,24].    Membrane trafficking of AMPA receptors and a vesicle-associated protein VAMP2

has been observed.

A covalent bond can form if the ligand is metabolized by the protein.    For example,

a dysfunctional mutant of a bacterial haloalkane dehalogenase (HaloTag[TM], Promega,

Madison, WI), which forms a stable bond with the substrate chloroalkane, is useful for both

cell-surface and intracellular specific labeling (Table 1) [25].    In addition to organic

fluorophores such as TMR and fluorescein, quantum dots ($QD_{655}$) have been attached to the

extracellular domain of platelet-derived growth factor receptors for long-term imaging [26].

This labeling method was recently used to visualize endocytotic-like structures in filopodia

(diameters: 90–130 nm) of HeLa cells in combination with a super-resolution imaging,

stimulated emission depletion microscopy [27].    Johnsson and colleagues reported a method

involving the irreversible transfer of an alkyl group from $O^6$-alkylguanine-DNA to human

$O^6$-alkylguanine-DNA alkyltransferase (hAGT) (Table 1) [28], which is currently

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commercially available (SNAP-tag[TM], New England Biolabs, Ipswich, MA). In a recent publication, this technology was used to specifically label cell-surface G protein-coupled receptors (GPCRs) with synthetic fluorophores or luminescent europium cryptate to circumvent the insufficient fluorescence intensity and unnecessary fluorescence from receptors accumulated within intracellular compartments, both of which are often problematic in the labeling of a GPCR by fluorescent proteins [29]. Time-resolved FRET measurements revealed the presence of oligomers for various GPCRs in COS-7 cell membranes [29]. An alternative approach to the covalent labeling of a target protein is the use of a suicide inhibitor, for example, *p*-nitrophenyl phosphonate which binds to the fungal protein cutinase (Table 1) [30]. The N and C termini of cutinase are close to each other (28.2 Å) and opposed to the active site, offering the possibility of insertion into the target protein. The integrin LFA-1 expressed on the surface of BAF cells was labeled with Alexa Fluor 488 or quantum dots (QD$_{655}$) [30]. The inhomogeneous distribution of the integrin during cell locomotion was observed.

2.2 Peptide–peptide interaction

A promising way to reduce the label's size is the utilization of peptide–peptide interactions. Simple labeling without cofactors such as metals or enzymes is an advantage

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for universal use.   The de novo designed peptide pair (EIAALKE)$_n$ and (KIEELEK)$_n$ (n = 3,

4) is known to form a tight heterodimer in a coiled-coil fashion (Fig. 3A) [31].   We found

that the E3 tag peptide (EIAALKE)$_3$ and the probe peptide K3 (KIAALKE)$_3$ or K4

(KIAALKE)$_4$ are suitable pairs for specific labeling of cell-surface receptors, such as

prostaglandin EP3β, β2-adrenergic, and EGF receptors by virtue of the

membrane-impermeability of the probes (Table 1) [32].   The probes are nontoxic and the

labeled receptors maintain their functionality.   The K3 and K4 probes have apparent

dissociation constants of 64 and 6 nM against the E3 tag, respectively.   The labeling is

completed within 1 min.   The reversibility of the E3-K3 labeling enables a pulse–chase

labeling of internalized and cell-surface receptors with TMR and fluorescein, respectively

(Fig. 3B).


2.3 Peptide–fluorophore interaction

The minimal design of a tag–probe system consists of a fluorophore and a

polypeptide that directly binds it.   Sequence screening by phage display has been used to

optimize such a dye-binding peptide (Table 1) [33].   The resulting 38-mer peptide TR512

composed of two dimerization domains and a dye-binding domain was assumed to recognize

the xanthene core of Texas Red.   The tag peptide targeting plasma membranes of NIH3T3

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cells was stained with a calcium sensor derivative of Texas Red (X-rhod-5F) to detect local calcium responses.   The affinity between the Texas Red probe and a phage that has five tag sequences is 25 pM.   The stoichiometry of the tag–probe complex and the binding constant are yet to be determined.

2.4 Metal chelation

The first tag–probe labeling system in living cells, reported in 1998, was based on a reversible covalent bond formation between organoarsenicals and pairs of thiols [34].   The biarsenical derivative of FL [fluorescein arsenical helix binder (FlAsh)] was found to tightly bind to the tetracysteine tag motif (Table 1).   The tetracysteines in the tag were initially positioned in a helical secondary structure, and subsequently positioned into optimized sequences for specific labeling in mammalian cells (HRWCCPGCCKTF and FLNCCPGCCMEP) [35], which form a hairpin structure [36].   To minimize nonspecific labeling and the toxicity of arsenical compounds, 1,2-ethanedithiol (EDT) or other dithiols and/or suppression dyes are indispensable in the labeling and washing procedures [37].   An excellent property of FlAsh is that it is nonfluorescent in the EDT-form but becomes fluorescent after binding to the tag.   FlAsh and longer-wavelength biarsenical fluorophore ReAsh are membrane-permeable and therefore suitable for the labeling of intracellular

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domains of membrane proteins. On the other hand, the labeling of cysteines in extracellular

domains requires reducing agents because of the oxidization of the cysteines to disulfides.

Various biarsenical fluorophores including $Ca^{2+}$ indicators have been synthesized [18,38].

A noticeable application of biarsenical–tetracysteine labeling for membrane proteins is the

detection of conformational changes in GPCR following agonistic stimulation.    Hoffman and

colleagues constructed a FRET sensor mutant of adenosine $A_{2A}$ receptor in which a

tetracysteine tag and cyan fluorescent protein were fused to the intracellular third loop and the

C-terminus of the receptor, respectively [11].    After FlAsh labeling, the structural

rearrangement induced by an agonist (e.g. 100 μM adenosine) occurred in the order of tens of

milliseconds and could be read out as changes in fluorescence intensity of FlAsh and CFP,

reflecting alterations in FRET efficiency from CFP to FlAsh.    The intracellular third loop is

important for coupling to downstream G-proteins.    Nevertheless, the insertion of the

biarsenical–tetracysteine label (< 2 kDa) into the third loop did not affect the receptor activity,

in contrast to the insertion of yellow fluorescent protein which reduced the receptor activity.

In a recent report, FlAsh labels were positioned at different sites of the third intracellular loop

of $\alpha_{2a}$-adrenergic receptor: N-terminally close to the transmembrane helix V (I3-N), in the

middle of the loop (I3-M), and C-terminally close to the transmembrane helix VI (I3-C) [39]

(Fig. 4).    A full agonist evoked similar FRET changes in all three constructs whereas weak

partial agonists induced a change only in the construct I3-C.  These results demonstrate

distinct agonist-specific conformational changes of GPCR in living cells.  A kit for

biarsenical–tetracysteine labeling is available commercially (TC-FlAsh$^{TM}$ and TC-ReAsh$^{TM}$,

Invitrogen, Carlsbad, CA).

Most other methods using metal chelation are based on coordination among a

divalent metal cation, a tag peptide, and a probe molecule.  An example is the

membrane-impermeable HisZiFit probe that binds to a hexahistidine tag via $Zn^{2+}$ coordination

(Table 1) [40].  Surface exposure of a membrane protein, stromal interaction molecule

(STIM) 1, from the endoplasmic reticulum in HEK293 cells was successfully detected using

this method.  Another promising approach is the use of DpaTyr probes that bind to an

oligo-aspartate tag via $Zn^{2+}$ coordination (Table 1) [41].  This technique has been used to

visualize muscarinic acetylcholine receptors in CHO cells.  Based on the tag–metal–probe

assembly, the formation of a covalent bond between a cysteine residue optimally positioned in

the tag and the $N$-$\alpha$-chloroacetyl group attached to the probe is possible [42].  The

combination of the NTA probe and hexahistidine tags in the presence of $Ni^{2+}$ has also been

used to label 5HT$_3$ serotonin receptors in HEK-293 cells (Table 1) [43].  A nonfluorescent

chromophore was labeled at an intracellular or extracellular site of the receptor by the method

and used as a quencher for a fluorophore conjugated to the receptor antagonist to obtain

structural information about the quencher-binding site and the ligand-binding site. This

labeling system has several problems. The affinity of hexahistidine–NTA-Ni$^{2+}$ is weak.

Multimerization of the probe could improve the affinity [44,45]. The introduction of

dichlorofluorescein [46], for example, circumvents partial quenching of the fluorescence by

Ni$^{2+}$. Another possible problem is the cytotoxicity of Ni$^{2+}$ [47].


2.5 Enzymatic reactions

Enzymes that attach a substrate to a specific site of a polypeptide have been applied

to tag–probe labeling in living cells. An example is the use of phosphopantetheinyl

transferase which transfers part of a phosphopantetheinyl probe to an acyl carrier protein

(ACP) tag consisting of ~80 amino acids (Table 1) [48,49]. The lateral organization of

neurokinin-1 (NK$_1$) receptors in HEK293 cell membranes has been investigated by FRET

between the receptors [50]. ACP-NK$_1$ receptors expressed on plasma membranes were

simultaneously labeled with the Cy3 and Cy5 fluorophores at defined labeling ratios to

correctly estimate FRET efficiency. Because of the strong dependence of FRET efficiency

on the receptor concentration on the cell surface, the authors concluded that the receptors

tended to be concentrated in microdomains rather than self-associated. Interestingly, the

FRET signal was slightly sensitive to the depletion of cholesterol, which is an important

component of lipid raft microdomains.    This ACP labeling was also used to visualize odorant

receptors labeled with Cy5 down to the single molecule level [51].    After stimulation with

an agonist, the receptors were confined to small domains of ~190 nm, which are likely

precursors of clathrin-coated pits.    Currently two orthogonal tag–enzyme pairs using the

principle are available for multicolor labeling (ACP-tag[TM] and MCP-tag[TM], New England

Biolabs).    Shorter peptide tag sequences substituted for ACP have also been reported to

further reduce the label's size [52].    Another example of tag–probe labeling with an

enzymatic reaction is a system using *E. coli* biotin ligase (BirA) and a 15-amino-acid acceptor

peptide (Table 1) [53].    The labeling of fluorophores using the method is carried out in two

steps.    First, a ketone analog of biotin (ketone 1) is attached to the tag, and second, the

ketone group absent on the native cell surface is specifically reacted with a hydrazide group

conjugated to a fluorophore.    EGF receptors expressed on the surface of HeLa cells were

labeled by this procedure [53].    Similar two-step labeling was performed using lipoic acid

ligase and alkyl azide probes with an improved labeling time (total ~20 min) (Table 1) [54].

Recently, bacterial sortases have been applied to tag–probe labeling on living cell membranes

(Table 1) [55,56].    This enzyme recognizes the LPXTG motif and cleaves the peptide bond

between threonine and glycine, subsequently yielding a new peptide bond between the

C-terminus of the threonine and the N-terminus of the pentaglycine probe.    The tag should

be placed in a flexible region close to the C-terminus of the target protein [55].   Human CD

154 protein and osteoclast differentiation factor were successfully labeled and visualized in

HEK 293 cells using the method.   The unique features of the labeling system are 1)

protein–protein conjugation was possible on the cell surface, as exemplified by the

conjugation of externally added GGGGG-EGFP to ODF-LPETGG [56], and 2) tag and probe

sequences could be exchanged.   Another promising enzyme is transglutaminase, which

introduces a cadaverine-conjugated fluorophore to a glutamine side chain in Q-rich tag

sequences expressed on HeLa cells (Table 1) [57].


2.6 Pros and cons of various techniques

An ideal tag–probe labeling method should have several features: high specificity, a

small tag, no toxicity, no perturbation of the target protein, versatility in the choice of

available fluorophores, a short labeling time, and a simple labeling procedure, although these

features are often incompatible with each other.   In general, there is a trade-off between

specificity and size.   For example, tag–probe pairs based on protein–ligand interactions (Fig.

2A) enable highly specific labeling, when the tag is relatively large (typically comparable to

GFP).   On the other hand, labeling via metal chelation (Fig. 2D) could greatly reduce the

label's size (down to 1 kDa), however nonspecific staining, insufficient affinity, or metal

toxicity might restrict applications. A satisfactory balance between size and specificity could be achieved by utilizing an intermediate-sized peptide–peptide assembly (Fig. 2B) or peptide–fluorophore complex (Fig. 2C). Alternative approaches are based on enzymatic reactions that catalyze the formation of a covalent bond between the tag and the probe (Fig. 2E). A smaller size and tight labeling are achieved with this approach, although a longer labeling time in the presence of excess probes (= substrates) is usually required for efficient labeling.


## 3. Outlook

Most of the principles for labeling membrane proteins in living cells described above have been reported in the last 5 years. New principles are also emerging, although they are yet to be tested in living cells. For example, the tetraserine-bisboronic acid labeling system, in which a tetraserine motif specifically binds fluorophores having bisboronic groups, was reported [58], however labeling in living cells is currently unavailable presumably because of abundant natural Ser-rich sequences that bind to the probe. An approach without using organic fluorophores or quantum dots is the use of luminescence from lanthanides. Imperiali and colleagues have reported a lanthanide-binding tag (15 amino acids) that strongly binds $Tb^{3+}$ and becomes luminescent on excitation of Trp in the tag [59]. Although

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the excitation wavelength is too short for conventional fluorescence imaging, this labeling system has potential for measuring distances between domains in membrane proteins using lanthanide-based resonance energy transfer coupled with emission lifetime measurements, as demonstrated for potassium channels expressed in *Xenopus* oocytes [60]. An important but currently challenging technology is protein- and site-specific labeling of fluorophores in transmembrane regions of membrane proteins in living cells. A promising strategy for this is the genetic incorporation of nonnatural fluorescent amino acids [61]. An alternative might be the use of native chemical ligation and related biochemical techniques ('expressed protein ligation' and 'protein *trans*-splicing') to perform protein semisynthesis [62], allowing the site-specific incorporation of fluorophores.

4. Conclusions

A variety of tag–probe techniques based on physicochemical interactions or biochemical reactions have been successfully applied to the labeling of membrane proteins in living cells. These techniques have advantages over the conventional genetic fusion of fluorescent proteins in diverse choice of fluorophores, smaller size, and rapid and surface-specific labeling at a defined time. These features are useful for the visualization of intracellular translocation, interprotein interactions, and conformational changes of membrane

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proteins.   An appropriate choice of technique is possible depending on the intended use, although the use of a smaller label generally accompanies a rather complicated procedure or a lower specificity.   Multicolor labeling using multiple techniques orthogonal with each other particularly demands simple labeling procedures.   Although the further improvement of existing principles and the development of new principles will be actively studied, current tag–probe techniques for specific labeling in living cells have greatly contributed to elucidation of the behavior of membrane proteins in situ, the activities of which are regulated by interactions with diverse molecules in biological membranes.

Acknowledgement

References

[1] A.G. Lee, How lipids affect the activities of integral membrane proteins, Biochim. Biophys. Acta 1666 (2004) 62–87.

[2] A.M. Powl, J.M. East, A.G. Lee, Importance of direct interactions with lipids for the function of the mechanosensitive channel MscL, Biochemistry 47 (2008) 12175–12184.

[3] L. Cristian, J.D. Lear, W.F. DeGrado, Use of thiol-disulfide equilibria to measure the energetics of assembly of transmembrane helices in phospholipid bilayers, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 14772–14777.

[4] S. Mall, R. Broadbridge, R.P. Sharma, J.M. East, A.G. Lee, Self-association of model transmembrane α-helices is modulated by lipid structure, Biochemistry 40 (2001) 12379–12386.

[5] Y. Yano, M. Ogura, K. Matsuzaki, Measurement of thermodynamic parameters for hydrophobic mismatch 2: intermembrane transfer of a transmembrane helix, Biochemistry 45 (2006) 3379–3385.

[6] K. Mitra, I. Ubarretxena-Belandia, T. Taguchi, G. Warren, D.M. Engelman, Modulation of the bilayer thickness of exocytic pathway membranes by membrane proteins rather

than cholesterol, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 4083–4088.

[7] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell. Biol. 9 (2008) 112–124.

[8] C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V.N. Belov, B. Hein, C. von Middendorff, A. Schonle, S.W. Hell, Direct observation of the nanoscale dynamics of membrane lipids in a living cell, Nature 457 (2009) 1159–1162.

[9] B.N. Giepmans, S.R. Adams, M.H. Ellisman, R.Y. Tsien, The fluorescent toolbox for assessing protein location and function, Science 312 (2006) 217–224.

[10] D.M. Chudakov, S. Lukyanov, K.A. Lukyanov, Fluorescent proteins as a toolkit for in vivo imaging, Trends Biotechnol. 23 (2005) 605–613.

[11] C. Hoffmann, G. Gaietta, M. Bunemann, S.R. Adams, S. Oberdorff-Maass, B. Behr, J.P. Vilardaga, R.Y. Tsien, M.H. Ellisman, M.J. Lohse, A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells, Nat. Methods 2 (2005) 171–176.

[12] C.S. Lisenbee, S.K. Karnik, R.N. Trelease, Overexpression and mislocalization of a tail-anchored GFP redefines the identity of peroxisomal ER, Traffic 4 (2003) 491–501.

[13] I. Chen, A.Y. Ting, Site-specific labeling of proteins with small molecules in live cells,

Curr. Opin. Biotechnol 16 (2005) 35–40.

[14] M.Z. Lin, L. Wang, Selective labeling of proteins with chemical probes in living cells, Physiology (Bethesda) 23 (2008) 131–141.

[15] K.M. Marks, G.P. Nolan, Chemical labeling strategies for cell biology, Nat. Methods 3 (2006) 591–596.

[16] L.W. Miller, V.W. Cornish, Selective chemical labeling of proteins in living cells, Curr. Opin. Chem. Biol. 9 (2005) 56–61.

[17] M. Fernandez-Suarez, A.Y. Ting, Fluorescent probes for super-resolution imaging in living cells, Nat. Rev. Mol. Cell. Biol. 9 (2008) 929–943.

[18] N. Soh, Selective chemical labeling of proteins with small fluorescent molecules based on metal-chelation methodology, Sensors 8 (2008) 1004–1024.

[19] J.A. Prescher, C.R. Bertozzi, Chemistry in living systems, Nat. Chem. Biol. 1 (2005) 13–21.

[20] L.W. Miller, Y. Cai, M.P. Sheetz, V.W. Cornish, In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag, Nat. Methods 2 (2005) 255–257.

[21] K.M. Marks, P.D. Braun, G.P. Nolan, A general approach for chemical labeling and rapid, spatially controlled protein inactivation, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9982–9987.

[22] M. Robers, P. Pinson, L. Leong, R.H. Batchelor, K.R. Gee, T. Machleidt, Fluorescent labeling of proteins in living cells using the FKBP12 (F36V) tag, Cytometry A 75 (2009) 207–224.

[23] C.M. McCann, F.M. Bareyre, J.W. Lichtman, J.R. Sanes, Peptide tags for labeling membrane proteins in live cells with multiple fluorophores, Biotechniques 38 (2005) 945–952.

[24] Y. Sekine-Aizawa, R.L. Huganir, Imaging of receptor trafficking by using α-bungarotoxin-binding-site-tagged receptors, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 17114–17119.

[25] 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, K.V. Wood, HaloTag: a novel protein labeling technology for cell imaging and protein analysis, ACS Chem. Biol. 3 (2008) 373–382.

[26] M.K. So, H. Yao, J. Rao, HaloTag protein-mediated specific labeling of living cells with quantum dots, Biochem. Biophys. Res. Commun. 374 (2008) 419–423.

[27] J. Schroder, H. Benink, M. Dyba, G.V. Los, In vivo labeling method using a genetic construct for nanoscale resolution microscopy, Biophys. J. 96 (2009) L1–3.

[28] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, A general method for the covalent labeling of fusion proteins with small molecules in vivo, Nat. Biotechnol. 21 (2003) 86–89.

[29] D. Maurel, L. Comps-Agrar, C. Brock, M.L. Rives, E. Bourrier, M.A. Ayoub, H. Bazin, N. Tinel, T. Durroux, L. Prezeau, E. Trinquet, J.P. Pin, Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization, Nat. Methods 5 (2008) 561–567.

[30] R. Bonasio, C.V. Carman, E. Kim, P.T. Sage, K.R. Love, T.R. Mempel, T.A. Springer, U.H. von Andrian, Specific and covalent labeling of a membrane protein with organic fluorochromes and quantum dots, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 14753–14758.

[31] J.R. Litowski, R.S. Hodges, Designing heterodimeric two-stranded α-helical coiled-coils. Effects of hydrophobicity and α-helical propensity on protein folding, stability, and specificity, J. Biol. Chem. 277 (2002) 37272–37279.

[32] Y. Yano, A. Yano, S. Oishi, Y. Sugimoto, G. Tsujimoto, N. Fujii, K. Matsuzaki, Coiled-coil tag–probe system for quick labeling of membrane receptors in living cell, ACS Chem. Biol. 3 (2008) 341–345.

[33] K.M. Marks, M. Rosinov, G.P. Nolan, In vivo targeting of organic calcium sensors via

genetically selected peptides, Chem. Biol. 11 (2004) 347–356.

[34] B.A. Griffin, S.R. Adams, R.Y. Tsien, Specific covalent labeling of recombinant protein molecules inside live cells, Science 281 (1998) 269–272.

[35] B.R. Martin, B.N. Giepmans, S.R. Adams, R.Y. Tsien, Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity, Nat. Biotechnol. 23 (2005) 1308–1314.

[36] F. Madani, J. Lind, P. Damberg, S.R. Adams, R.Y. Tsien, A.O. Graslund, Hairpin structure of a biarsenical-tetracysteine motif determined by NMR spectroscopy, J. Am. Chem. Soc. 131 (2009) 4613–4615.

[37] B.A. Griffin, S.R. Adams, J. Jones, R.Y. Tsien, Fluorescent labeling of recombinant proteins in living cells with FlAsH, Methods Enzymol. 327 (2000) 565–578.

[38] S.R. Adams, R.Y. Tsien, Preparation of the membrane-permeant biarsenicals FlAsH-EDT$_2$ and ReAsH-EDT$_2$ for fluorescent labeling of tetracysteine-tagged proteins, Nat. Protoc. 3 (2008) 1527–1534.

[39] A. Zurn, U. Zabel, J.P. Vilardaga, H. Schindelin, M.J. Lohse, C. Hoffmann, Fluorescence resonance energy transfer analysis of $\alpha_{2a}$-adrenergic receptor activation reveals distinct agonist-specific conformational changes, Mol. Pharmacol. 75 (2009) 534–541.

[40] C.T. Hauser, R.Y. Tsien, A hexahistidine-Zn$^{2+}$-dye label reveals STIM1 surface exposure,

Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 3693–3697.

[41] A. Ojida, K. Honda, D. Shinmi, S. Kiyonaka, Y. Mori, I. Hamachi, Oligo-Asp tag/Zn(II) complex probe as a new pair for labeling and fluorescence imaging of proteins, J. Am. Chem. Soc. 128 (2006) 10452–10459.

[42] H. Nonaka, S. Tsukiji, A. Ojida, I. Hamachi, Non-enzymatic covalent protein labeling using a reactive tag, J. Am. Chem. Soc. 129 (2007) 15777–15779.

[43] E.G. Guignet, R. Hovius, H. Vogel, Reversible site-selective labeling of membrane proteins in live cells, Nat. Biotechnol. 22 (2004) 440–444.

[44] S. Lata, M. Gavutis, R. Tampe, J. Piehler, Specific and stable fluorescence labeling of histidine-tagged proteins for dissecting multi-protein complex formation, J. Am. Chem. Soc. 128 (2006) 2365–2372.

[45] V. Roullier, S. Clarke, C. You, F. Pinaud, G.G. Gouzer, D. Schaible, V. Marchi-Artzner, J. Piehler, M. Dahan, High-affinity labeling and tracking of individual histidine-tagged proteins in live cells using $Ni^{2+}$ tris-nitrilotriacetic acid quantum dot conjugates, Nano Lett. 9 (2009) 1228–1234.

[46] C.R. Goldsmith, J. Jaworski, M. Sheng, S.J. Lippard, Selective labeling of extracellular proteins containing polyhistidine sequences by a fluorescein-nitrilotriacetic acid conjugate, J. Am. Chem. Soc. 128 (2006) 418–419.

[47] K. Salnikow, A. Zhitkovich, Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium, Chem. Res. Toxicol. 21 (2008) 28–44.

[48] N. George, H. Pick, H. Vogel, N. Johnsson, K. Johnsson, Specific labeling of cell surface proteins with chemically diverse compounds, J. Am. Chem. Soc. 126 (2004) 8896–8887.

[49] J. Yin, F. Liu, X. Li, C.T. Walsh, Labeling proteins with small molecules by site-specific posttranslational modification, J. Am. Chem. Soc. 126 (2004) 7754–7755.

[50] B.H. Meyer, J.M. Segura, K.L. Martinez, R. Hovius, N. George, K. Johnsson, H. Vogel, FRET imaging reveals that functional neurokinin-1 receptors are monomeric and reside in membrane microdomains of live cells, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 2138–2143.

[51] V. Jacquier, M. Prummer, J.M. Segura, H. Pick, H. Vogel, Visualizing odorant receptor trafficking in living cells down to the single-molecule level, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 14325–14330.

[52] Z. Zhou, P. Cironi, A.J. Lin, Y. Xu, S. Hrvatin, D.E. Golan, P.A. Silver, C.T. Walsh, J. Yin, Genetically encoded short peptide tags for orthogonal protein labeling by Sfp and AcpS phosphopantetheinyl transferases, ACS Chem. Biol. 2 (2007) 337–346.

[53] I. Chen, M. Howarth, W. Lin, A.Y. Ting, Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase, Nat. Methods 2 (2005) 99–104.

[54] M. Fernandez-Suarez, H. Baruah, L. Martinez-Hernandez, K.T. Xie, J.M. Baskin, C.R. Bertozzi, A.Y. Ting, Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes, Nat. Biotechnol. 25 (2007) 1483–1487.

[55] M.W. Popp, J.M. Antos, G.M. Grotenbreg, E. Spooner, H.L. Ploegh, Sortagging: a versatile method for protein labeling, Nat. Chem. Biol. 3 (2007) 707–708.

[56] T. Tanaka, T. Yamamoto, S. Tsukiji, T. Nagamune, Site-specific protein modification on living cells catalyzed by Sortase, Chembiochem 9 (2008) 802–807.

[57] C.W. Lin, A.Y. Ting, Transglutaminase-catalyzed site-specific conjugation of small-molecule probes to proteins in vitro and on the surface of living cells, J. Am. Chem. Soc. 128 (2006) 4542–4543.

[58] T.L. Halo, J. Appelbaum, E.M. Hobert, D.M. Balkin, A. Schepartz, Selective recognition of protein tetraserine motifs with a cell-permeable, pro-fluorescent bis-boronic acid, J. Am. Chem. Soc. 131 (2009) 438–439.

[59] B.R. Sculimbrene, B. Imperiali, Lanthanide-binding tags as luminescent probes for studying protein interactions, J. Am. Chem. Soc. 128 (2006) 7346–7352.

[60] W. Sandtner, F. Bezanilla, A.M. Correa, In vivo measurement of intramolecular distances

using genetically encoded reporters, Biophys. J. 93 (2007) L45–47.

[61] W. Wang, J.K. Takimoto, G.V. Louie, T.J. Baiga, J.P. Noel, K.F. Lee, P.A. Slesinger, L. Wang, Genetically encoding unnatural amino acids for cellular and neuronal studies, Nat. Neurosci. 10 (2007) 1063–1072.

[62] V. Muralidharan, T.W. Muir, Protein ligation: an enabling technology for the biophysical analysis of proteins, Nat. Methods 3 (2006) 429–438.

Figure legends

Fig. 1   Dynamic behavior of membrane proteins in living cells detectable by fluorescence imaging.   (A) Endocytotic internalization.   Following stimulation with an agonist and/or spontaneously, membrane proteins translocate from the cell surface to intracellular vesicles via the endocytotic machinery.   A recycling of proteins to the surface also occurs.   (B) Partitioning into specific membrane domains.   Following activation and/or interaction with other proteins, membrane proteins laterally redistribute among domains with a distinct lipid composition.   The partitioning to a specific domain can be detected by fluorescence resonance energy transfer (FRET) from a fluorophore attached to a protein (green) to an adequate fluorescent marker for the domain (red) and/or colocalization of the two.   The existence and detailed characteristics of lipid nanodomains are still under debate.   (C) Interprotein interactions.   Stimulation of membrane proteins facilitates or suppresses protein–protein interactions.   FRET from the donor fluorophore (green) attached to one protein to the acceptor fluorophore (red) attached to the other interacting protein can detect the interactions.   (D) Conformational changes.   Following ligand stimulation and/or interaction with other proteins, the protein structure changes.   Double labeling of a protein with a FRET donor and an acceptor enables detection of the structural change.

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Fig. 2   Principles of tag–probe labeling.   (A) Protein–ligand interaction.   A protein tag

fused to a target protein is labeled with a ligand conjugated to a fluorophore.   (A') If the

ligand is a peptide, it can be used as a tag that is labeled with the protein probe.   (B)

Peptide–peptide interaction.   Two peptides that form a tight heterodimer are used as a

combination of a tag fused to the target protein and a probe conjugated with a fluorophore.

(C) Peptide–fluorophore interaction.   A polypeptide that directly binds a fluorophore is used

as a tag.   (D) Metal chelation.   A tag and a probe are cooperative chelators for a metal ion.

The tag–metal–probe motif is often multimerized to obtain sufficient binding affinity.   (E)

Enzymatic reaction.   A specific site of a tag sequence (substrate 1) is covalently modified

with a probe (substrate 2) by an enzymatic reaction.


Fig. 3   Coiled-coil labeling.   (A) Helical wheel representation of the E3/ K3 coiled-coil

heterodimer.   White and black arrows indicate hydrophobic and electrostatic interactions

respectively.   (B) Pulse-chase experiments for the internalization of $\beta_2$-adrenergic receptors

($\beta_2$AR) in response to receptor stimulation.   CHO cells expressing E3-$\beta_2$AR were labeled

with tetramethylrhodamine-K3 (TMR-K3) (60 nM) for 2 min, and then incubated with the

agonist isoproterenol (10 µM) for 5 min.   After the cells were washed with PBS, 20 nM

fluorescein-K4 (FL-K4) was added to label the receptors remaining on the cell surface and the cells were observed.   The TMR (internalized receptor) and FL images are merged in the lower right panel.   Reproduced from reference [32] with permission.

Fig. 4   Constructs of the $\alpha_{2A}$-adrenergic receptor for the detection of conformational changes by FRET.   For all constructs, the donor fluorophore CFP was positioned at the very C terminus of the amino acid sequence.   The positions of the different FlAsh-binding sites in the third intracellular loop are marked in white.   The numbers denote the amino acid segments that were replaced by the binding motif "FLNCCPGMEP."   Positions 246 to 257 represent the construct I3-N, positions 297 to 308, I3-M, and positions 350 to 361, I3-C. Modified from reference [39] with permission.

Table 1 Various tag–probe labeling methods

| principle | labeling system | tag | probe | probe structure | available fluorophores | approx. size | cofactor/enzyme | affinity | typical labeling condition | notes | references |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein-ligand interaction | LigandLink™ | E. coli dihydrofolate reductase (eDHFR) | trimethoprim | | arbitrary | 18 kDa | No | 1 nM | 10 nM, 15 min | commercial, intracellular labeling | 20 |
| | FKBP12 | FKBP12 (F36V) | SLF' | | arbitrary (structure-dependent [22]) | 12 kDa | No | 0.1 nM | 1 μM, 30 min | intracellular labeling | 21,22 |
| | BTX | WRYYESSLEPYPD | α-bungatoroxin | - | arbitrary | 8 kDa | No | 14–60 nM | 1 μM, 15 min | | 23,24 |
| | HaloTag™ | Haloalkane dehalogenase (modified) | chloroalkane | | arbitrary | 33 kDa | No | covalent | 5 μM, 15 min | commercial, intracellular labeling | 25–27 |
| | SNAP-tag™ | hAGT | benzilguanine | | arbitrary | 20 kDa | No | covalent | 5 μM, 1 h | commercial, intracellular labeling | 28,29 |
| | Cutinase | cutinase | p-nitrophenyl phosphonate | | arbitrary | 22 kDa | No | covalent | 1μM, 30 min | insert is possible | 30 |
| Peptide–peptide interaction | Coiled-coil | EIAALKE EIAALKE EIAALKE | KIAALEK KIAALEK KIAALEK KIAALEK | - | arbitrary | 6 kDa | No | 6 nM (K4), 64 nM (K3) | 20 nM, 1 min | | 32 |
| Peptide–fluorophore | TR binding peptide | GGGSKVILFEGPAGRWTWPEISEGAPGSKVILFEGGPG | xanthene core of Texas Red | - | Texas Red, X-rhodamine | 3.5 kDa | No | 80 pM (as phage) | 1 μM, 30 min | intracellular labeling | 33 |
| Metal chelation | Biarcenical-tetracycteine | FLNCCPGCCMEP | biarsenical fluorophores | | various | 2 kDa | EDT | covalent | 0.5 μM, 1h | commercial, intracellular labeling, toxicity of As | 11, 34–39 |
| | HisZiFit | HHHHHH | 2-pyridylsulfoneamide | | HisZiFit | 1 kDa | $Zn^{2+}$ | 40 nM | 100 nM, 1min | | 40 |
| | Oligo-Asp/$Zn^{2+}$ complex | DDDDGDDDDGDDDD | Dpa Tyr | | arbitrary | 3 kDa | $Zn^{2+}$, DDDD | <55 nM | 20 μM, 5 min | covalent labeling is also available | 41,42 |
| | NTA-His | HHHHHH | NTA | | quenchers, dichlorofluorescein, quantum dot | 1 kDa | $Ni^{2+}$ | 1–4 μM | 15 μM, 1 min | quenching by $Ni^{2+}$, toxicity of $Ni^{2+}$ | 43–46 |
| Enzymatic reaction | ACP-tag™, MCP-tag™ | acyl carrier protein | coenzyme A | | arbitrary | 9 kDa | PPTase | covalent | 5 μM, 40 min | commercial, shorter tags are availabe | 48–52 |
| | BirA labeling | GLNDIFEAQKIEWHE | biotin | | ketone reactive | 2 kDa | biotin ligase, ATP | covalent | 1 mM, 60 min | two-step labeling | 53 |
| | LplA labeling | DEVLVEIETDKAVLEVPGGEEE | lipoic acid | | azide reactive | 3 kDa | lipoic acid ligase, ATP | covalent | 250 μM, 15 min | two-step labeling | 54 |
| | Sortagging | LPETG | GGGGG | | arbitrary | 2 kDa | sortase | covalent | 100 μM, 10 min | limited to C-terminus labeling | 55,56 |
| | TGase labeling | PKPQQFM | cadaverine | | arbitrary | 2 kDa | transglutaminase, $Ca^{2+}$ | covalent | 400 μM, 25 min | | 57 |

Table 1

**Figure 1**

**A** extracellular
cytosol
endosome

**B** FRET

**C** FRET

**D** FRET

**Figure 2**

**A** protein tag — ligand probe — target protein

**A'** peptide ligand tag — protein probe

**B** peptide tag − + peptide probe

**C** peptide tag — fluorophore probe

**D** chelator peptide tag — metal — chelator probe

**E** substrate-1 peptide/protein tag — substrate-2 probe — enzyme

**Figure 3**

**Figure 4**