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Fluorescent labelling in living cells

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The labelling of proteins with green fluorescent protein enabled the visualization of proteins in living cells for the first time. Since then, much progress has been made in the field. Modern strategies allow the labelling of proteins in live cells through a range of specialized methods with sophisticated chemical probes that show enhanced photophysical properties compared to fluorescent proteins. This review briefly summarizes recent advances in the field of fluorescent chemical protein labelling *inside* living cells and illustrates key aspects on the requirements and advantages of each given method.

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Introduction

Labelling of proteins inside living cells is of tremendous importance in the characterization of protein dynamics, mobility and function and thus in the understanding of biological processes. Labelling of proteins *in vivo* is not a new area, with proteins being expressed as fusion constructs with fluorescent proteins such as GFP to monitor their cellular localization as far back as 1995 [1]. The creation of fusion proteins with GFP has been tremendously useful to study protein localization, movement, interactions and function inside living cells. However, labelling with GFP has significant drawbacks as proteins fused to the fluorescent protein can have altered localization and, due to its considerable size, altered function as well. More sophisticated strategies for the fluorescent labelling of proteins in living systems are being developed constantly towards obtaining methods that are fast, selective and minimally disruptive to the living system.

Chemical labelling of proteins is more versatile than labelling via fusion proteins and opens the possibility of labelling with a wide array of functionalities, most notably fluorescence, with bright organic dyes of a broad range of colours. Countless techniques for chemical protein labelling exist and are discussed broadly in several great review articles [2,3••]. In this article, only techniques that can be applied to the labelling of proteins inside living cells are compared and contrasted in order to guide the reader to identify the optimal labelling strategy for a given scenario.

The specific chemical labelling of proteins inside living cells poses a special challenge, as labelling reactions have to run under mild conditions in the cell interior and be very selective for the target protein. As such, very specialized techniques exist, ranging from the labelling of target proteins via entire protein domains, over the usage of specialized peptide tags to the labelling of endogenous proteins in living systems (see Table 1 for an overview).

Protein domain recognition strategies

Proteins have the ability to show high affinity binding to natural as well as synthetic ligands. This can be of use in protein labelling, wherein the protein of interest is fused to a protein showing high affinity for a ligand that is itself labelled. The small ligands can penetrate cells and bind the corresponding protein inside the cell, in turn leading to the indirect labelling of the protein of interest as part of the fusion protein. Labelling of a protein with ligand-binding or self-labelling enzymatic protein domain brings along a substantial increase in size that can potentially be disruptive to the protein's localization and function but in turn promises high specificity and reaction rate with a great variety of labels. Selected labelling methods using protein domains are shown in Figure 1.

Protein–ligand-interactions

The FK506 binding protein (FKBP) binds a number of natural and synthetic compounds with high affinity thus rendering them good candidates for such a labelling method—however, FKBP ligands can also interact with endogenous FKBPs. FKBP' is a mutant of the FK506 binding protein that binds the synthetic ligand SLF' with a more than 1000-fold selectivity in comparison to the wildtype protein [4]. This interaction can be used to label

Table 1

Labelling strategies for the fluorescent labelling of proteins inside living cells discussed in this article. FKBP' = FK506 binding protein mutant; eDHFR = *E. coli* dihydrofolate reductase; TMP = trimethoprim; Halo = haloalkane dehalogenase; PYP = photoactive yellow protein; Ni^{2+}-NTA = nickel-nitrilotriacetic acid; LplA = lipoic acid ligase; DnaE = DNA polymerase III; UAA = unnatural amino acids; LDT = ligand-directed chemistry

Labelling technique	One-step or two-step labelling	Covalent or non-covalent	Size of genetic tag	Modification site
Protein domain-based labelling strategies				
FKBP' – SLF'	One-step	Non-covalent	12 kDa	N- or C-terminus
eDHFR – TMP	One-step or two-step	Non-covalent or covalent	18 kDa	N- or C-terminus
SNAP tag, CLIP tag	One-step or two-step	Covalent	20 kDa	N- or C-terminus
Halo tag	One-step or two-step	Covalent	33 kDa	N- or C-terminus
PYP	One-step or two-step	Covalent	14 kDa	N- or C-terminus
Peptide-based labelling strategies				
His tag – Ni^{2+} – NTA	One-step	Non-covalent	10 amino acids	N- or C-terminus
D4 tag – DpaTyr	One-step	Non-covalent or covalent	4–12 amino acids	N- or C-terminus
Tetracysteine (CCXXCC)	One-step	Covalent	6 amino acids	N- or C-terminus or internal
dC10α	One-step	Covalent	22 amino acids	N- or C-terminus
Sortase A	Two-step	Covalent	6 amino acids	C-terminus
LplA	One-step or two-step	Covalent	13 amino acids	N- or C-terminus or internal
Cell-penetrating DnaE inteins	One-step or two-step	Covalent	~11 kDa depending on choice of intein pair	C-terminus
Endogenous protein labelling strategies				
UAA	One-step	Covalent	1 amino acid	N- or C-terminus, or internal
LDT	One-step	Covalent	–	Internal (proximal to ligand binding site)

proteins of interest in live mammalian cells, by expressing the protein of interest as a fusion to FKBP' and then applying a fluorescently labelled cell-permeable SLF', which leads to labelling of the FKBP' fusion protein inside living cells [5].

Trimethoprim (TMP) is an inhibitor of the 18 kDa *Escherichia coli* dihydrofolate reductase (eDHFR). TMP shows high affinity towards eDHFR that is not substantially disrupted by derivatisation of TMP while showing much lower affinity for mammalian analogues of the enzyme. Because of this, it is possible to visualize fusion proteins expressed with eDHFR in mammalian cells with the help of fluorescently labelled TMP that can penetrate cells and bind eDHFR [6].

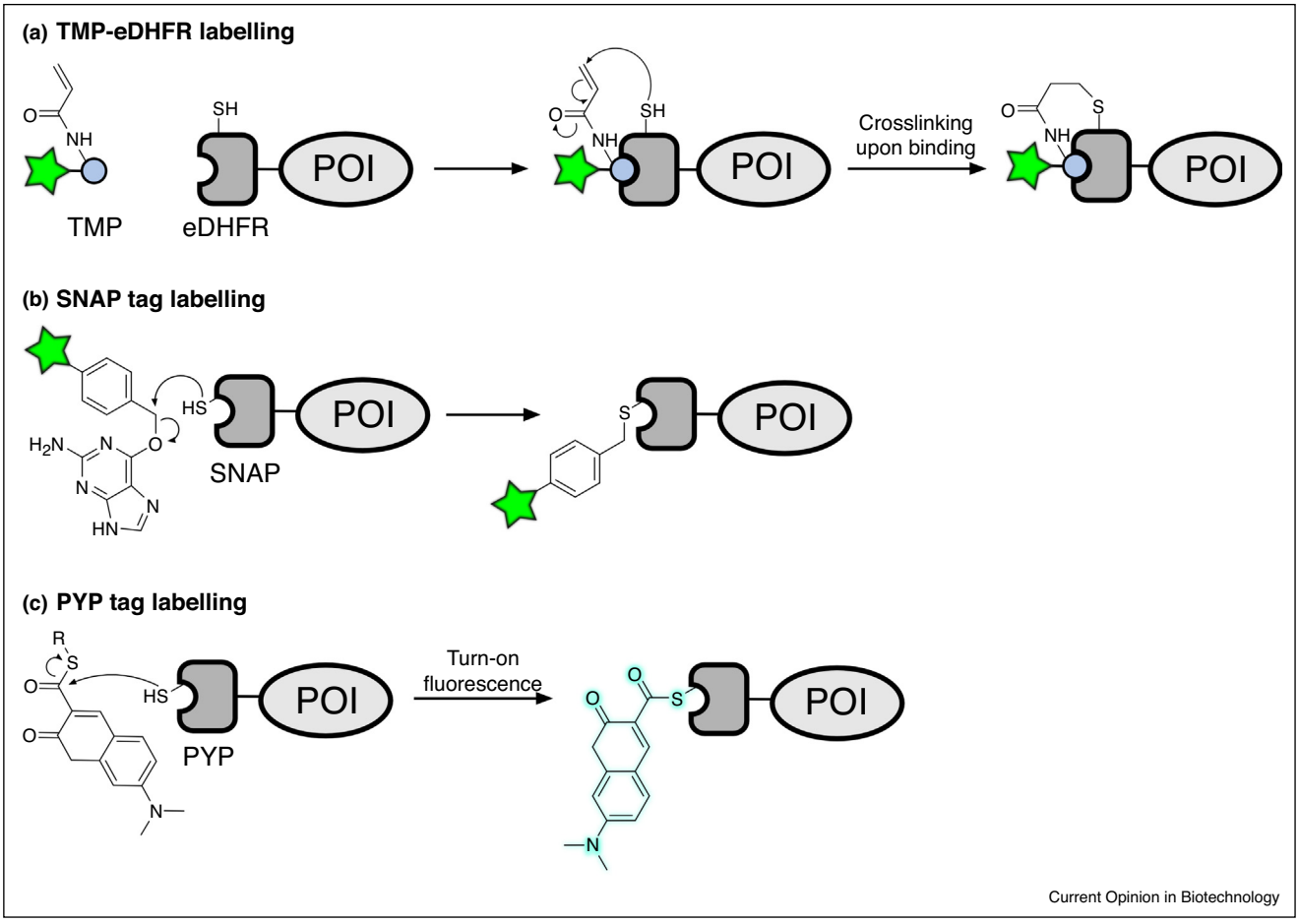
Since its inception, this system has seen substantial improvements. Recent approaches utilize the introduction of a reactive cysteine residue in the eDHFR protein near the TMP binding site while the fluorescent TMP label is outfitted with an acrylamide electrophile leading to the formation of a covalent bond between the two thus making the labelling essentially irreversible (Figure 1a) [7]. A more modular approach has been developed as well, in which the TMP carries an azide label that is then covalently attached to the protein of interest via the TMP-eDHFR interaction inside living cells before it is addressed in a second step with dibenzocyclooctyne (DBCO) or bicyclononyne (BCN) carrying probes [8].

Self-labelling protein domains

The 20 kDa large DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) catalyzes the attachment of O^6-alkylguanine or O^6-benzylguanine or a derivative to a cysteine residue on the enzyme. As a fusion to a protein of interest, AGT (marketed as SNAP tag) can catalyze the labelling of the fusion construct with a fluorescent, cell permeable derivative of O^6-benzylguanine (Figure 1b) [9]. An orthogonal system, the CLIP tag, has also been developed that utilizes derivatives of O^6-benzylcytosine instead [10]. Because of the orthogonality of SNAP and CLIP, they can be used together in double labelling approaches such as pulse-chase experiments [10] or as split-tags that activate and become fluorescently labelled upon protein–protein interaction, allowing the individual monitoring of proteins after they dissociate [11]. The Halo tag (33 kDa) is another example of a self-labelling enzyme that was engineered from the bacterial enzyme haloalkane dehalogenase to attach a haloalkane derivative to a reactive aspartate residue in the enzyme's active center [12].

One issue with self-labelling enzymes is that washing steps are often required to remove the unbound fluorophore from the cells in order to avoid a high background signal, which complicates labelling experiments with a quick readout. Therefore, labels have been developed for SNAP, CLIP and Halo tags that turn on fluorescence upon labelling, making it possible to image without a

Figure 1



Protein domain-based labelling strategies. **(a)** Labelled trimethoprim (TMP) binds the *E. coli* dihydrofolate reductase (eDHFR). Through a mutated cysteine on eDHFR and a chloroacetamide moiety on the TMP, a covalent bond is formed. **(b)** The O^6-alkylguanine-DNA alkyltransferase (marketed as SNAP tag) catalyzes the transfer of an O^6-benzylguanine derivative carrying a label to the SNAP tag. **(c)** The photoactive yellow protein (PYP) tag catalyzes the attachment of a 4-hydroxycinnamic acid derivative that turns on fluorescence upon binding to the hydrophobic interior of the PYP, enabling no-wash labelling.

washing step [13•] even if the labelling itself is quite slow and the background is high.

The more recently developed PYP (photoactive yellow protein)-tag, based on a protein derived from purple bacteria, is smaller in size than the SNAP-, CLIP- and Halo-tags (14 kDa) and can form a stable thioester bond with derivatives of 4-hydroxycinnamic acid [14]. In order to develop a rapid staining method with low background even without washing, fluorescent probes were designed for the PYP-tag that show only low fluorescence in the polar environment of the cell interior but much higher fluorescence in the low-polar environment of the PYP-tag binding pocket (Figure 1c). Thereby it was possible to complete the labelling of intracellular proteins without washing after only 6 min [15].

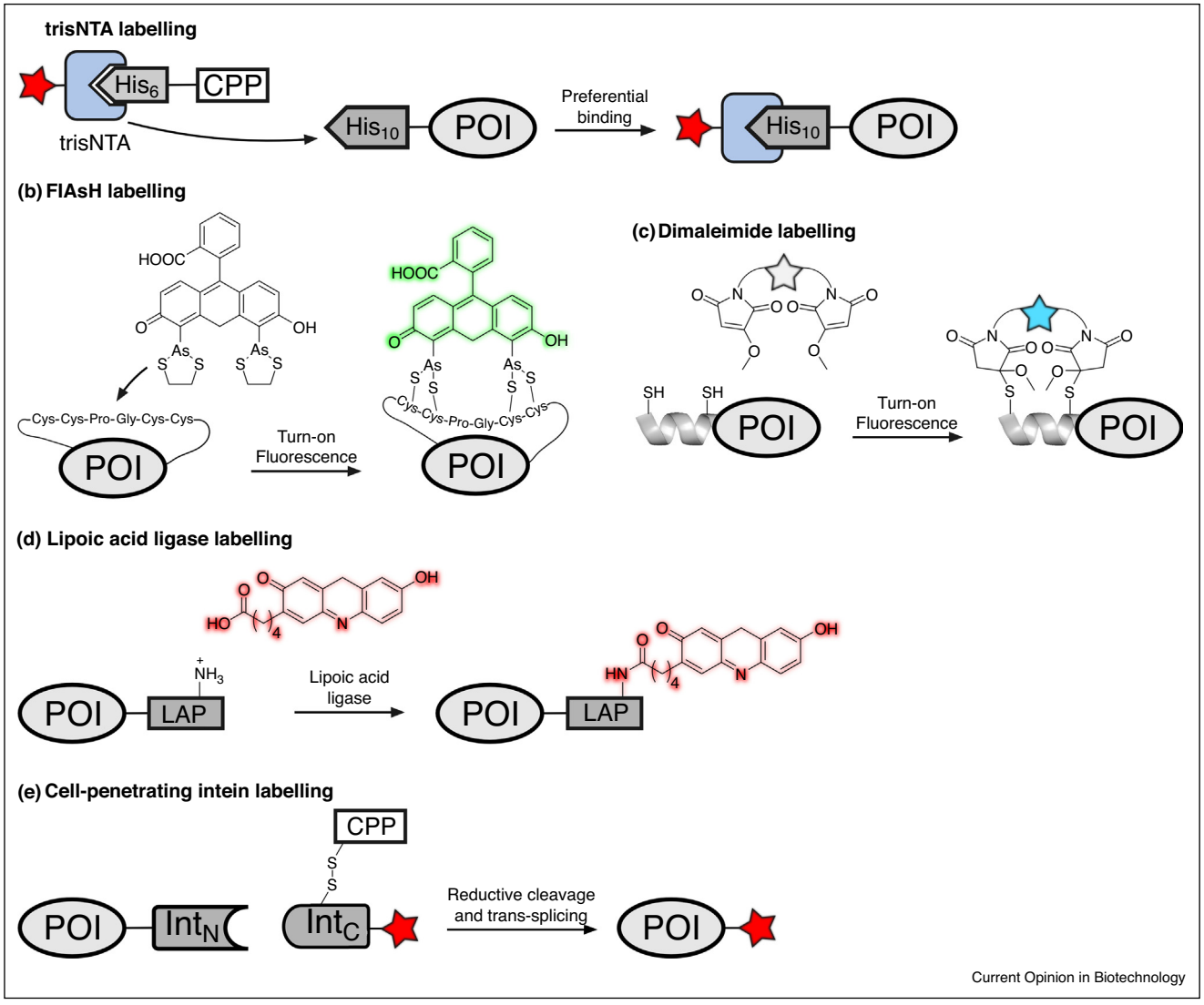
Peptide recognition strategies

Peptides, due to their smaller size, promise to be less invasive than entire protein domains when attached to a protein of interest. However, techniques utilizing an affinity peptide as a recognition motif to label a target protein are often less selective and lead to lower specificity of the labelling. Selected labelling strategies are shown in Figure 2.

Metal-ion-affinity

Metal chelation capabilities of peptide tags such as the Ni^{2+}-chelation of the poly-histidine-tag in combination with *N*-nitrilotriacetic acid (NTA) are commonly used for affinity chromatography of proteins. It can also be used as a tool for protein labelling with the advantage that only a small peptide tag is used that is only minimally disruptive

Figure 2



Peptide-based labelling strategies. **(a)** Labelled trisNTA will be cotransported into cells by a His_6-tagged cell-penetrating peptide (CPP). Once there, the trisNTA preferentially binds to the His_{10}-tagged protein. **(b)** The fluorescein arsenical helix binder (FlAsH) can penetrate cells and bind tetracysteine motifs, turning on fluorescence upon binding. **(c)** The dimaleimide fluorophore binds selectively to the dC10α-tag and then turns on fluorescence. **(d)** Lipoic acid ligase catalyzes the transfer of a fluorescent lipoic acid derivative on to the lipoic acid acceptor peptide (LAP). **(e)** An intein fused to a CPP via a disulfide linkage can penetrate cells. The CPP is cleaved off through reduction and trans-splicing occurs, leaving behind a minimally labelled protein.

to protein function. The strong and specific interaction between NTA and the His tag has been applied to the non-covalent fluorescent labelling of his-tagged membrane proteins on the surface of live cells using an NTA-chromophore conjugate [16].

More recently, this technique has also been applied to the labelling of poly-histidine-tagged proteins inside living cells through the usage of a cell-penetrating multivalent NTA complex. For this, the cell-penetrating TAT (transactivator of transcription)-peptide was synthesized with an *N*-terminal His_6-tag used in combination with a trivalent and fluorescently labelled NTA (trisNTA). Upon binding of the cell-penetrating peptide to the trisNTA, the complex can enter the cells, where the trisNTA will preferentially bind the His_{10} tagged protein of interest (due to the 10-fold higher binding affinity of NTA to the longer tag) resulting in non-covalently labelling (Figure 2a) [17•].

The tetra-aspartate (D4 tag, DDDD) is an alternative to the His tag and can be labelled through the use of zinc complexes, such as the DpaTyr complex based on 2,2′-dipicolylamine on a tyrosine scaffold. This has been used

in the non-covalent labelling of membrane proteins on living cells using a triply repeated D4 tag [18]. It has also been applied to the covalent labelling of proteins inside live *E. coli* cells, for which the D4 tag is outfitted with a cysteine while the DpaTyr label carries an N-α-chloroacetyl moiety, leading to the formation of a covalent bond upon binding [19].

Self-labelling-tags

FlAsH (fluorescein arsenical helix binder) is a fluorescent chemical label that allows the specific modification of tetracysteine (CCXXCC) motifs in proteins. First reported in 1998 by Roger Tsien, it was the first notable alternative to tagging proteins with GFP. The peptide tag is short and due to its small size and labelling occurring on the amino acid side chains, it can be introduced at any position in a protein instead of just the termini.

The FlAsH label itself is also small (<1 kDa), therefore cell-permeable and can be used to specifically label proteins inside living cells (Figure 2b) [20]. A range of different biarsenic dyes have been created that vastly increase the range of applications for the system, for example allowing pulse-chase experiments [21], or monitoring protein dynamics or folding inside living cells [22]. Nevertheless, FlAsH and similar biarsenic compounds show high background labelling as well as cell toxicity, requiring the addition of 1,2-ethandiol and thus making them less-than-ideal labels for experiments inside living cells.

Another prominent tag is the fluorette tag. It binds a TexasRed fluorophore and through a reactive cysteine residue can form a covalent bond with an *N*-α-chloroacetamide group on the fluorophore, enabling one-step labelling of proteins in live bacterial cells that are tagged with the genetically encodable tag [23]. The fluorette tag is small and the labelling is specific and rapid, although it is limited to the TexasRed fluorophore.

The more recently developed dC10α-tag represents a self-labelling peptide tag with an alpha-helical structure presenting two cysteine residues, one on each end of the helix. The corresponding label contains a fluorophore and two distinct maleimide functionalities quenching the fluorescence through photoinduced electron transfer (PeT) until both maleimides have undergone thiol addition to the dC10α-tag (Figure 2c) [24]. In order to prevent unspecific additions of the maleimide reagent to glutathione or other free thiols in the cell interior, the reactivity of the maleimide was attenuated using a methoxy substituent on the maleimide which decreases unspecific reactions while maintaining high reaction rate with the two reactive cysteines in the dC10α-tag. The authors selectively label actin in the cytosol and histone H2B in the nucleus of live cells with only minor unspecific labelling [24].

Enzyme catalyzed label transfer

Sortase A is a transpeptidase enzyme that requires an LPXTG recognition sequence on the *N*-terminal fragment and an oligoglycine sequence on the *C*-terminal fragment and catalyzes the formation of a peptide bond between the two fragments [25]. Bioconjugation using sortase A is versatile and can be used to attach a variety of functionalities to proteins. However, because of the peptidic nature of the recognition sequences, sortase A is mostly limited to protein labelling *in vitro*, but has been used for protein labelling on live cell-surfaces [26] and even in the ligation of GFP to proteins of interest in various cellular compartments inside live mammalian cells [27].

An engineered variant of sortase A that increases the activity while eliminating the need for calcium in the reaction was now used in the labelling of proteins inside living *E. coli* cells with substrate mimics containing a bioorthogonal handle [28]. For this, the sortase A variant is simply coexpressed with the protein of interest and the substrate mimic containing an azide functionality is added to the cells. After cell lysis, the clarified lysate was treated with an azide-reactive dye and gel electrophoresis could show that only the protein of interest was labelled to a high degree. This study could also enable two-step labelling of proteins inside mammalian cells in the future.

The *E. coli* enzyme lipoic acid ligase (LplA) recognizes a short peptide sequence and catalyzes the ligation of lipoic acid onto proteins important for the *E. coli* metabolism [29]. The enzyme is somewhat promiscuous and can be used to ligate an azide derivative to an engineered acceptor peptide sequence, enabling two-step labelling with cyclo-octyne carrying probes on the cell surface of living cells [30]. More recently, the LplA has been engineered to directly accept a coumarin derivative as substrate and thereby label the acceptor peptide fluorescently in one step [31]. A coumarin derivative carrying two acetoxymethyl (AM) groups to mask the negative charges on the molecule was able to enter mammalian cells where the AM protecting groups are removed by endogenous esterases. The molecule then serves as substrate for the LplA and a 13 amino acid long peptide sequence fused to the protein of interest can be labelled in the cytosol and nucleus of living cells. The method reportedly shows higher specificity and lower toxicity than arsenic-based probes while keeping the tag length short [31]. The usage of coumarin is not ideal in cellular contexts as the wavelength required to excite the fluorophore is harsh on cells. Through computational design it was now also possible to create a LplA mutant that is able to instead ligate a red fluorophore (Figure 2d) [32•].

Split inteins are a useful tools for protein engineering and labelling, because during protein trans splicing (PTS) the

intein fragment is excised leaving behind a protein with only a small peptide sequence. The naturally split DNA polymerase III (DnaE) intein pair from *Nostoc punctiforme* (Npu) has an exceptionally high reaction rate and is extraordinarily robust, showing reactivity at temperatures between 6 and 37°C, with different extein sequences and in the presence of up to 6 M urea [33]. Because of its natural robustness, the DnaE intein pair has previously been used to label proteins on the surface of live cells with synthetic moieties containing bioorthogonal handles in a two-step labelling strategy [34].

The *C*-terminal fragment of the DnaE intein in combination with the *N*-terminal fragment of a split intein from the cyanobacterium *Anabaena variabilis* (Ava) has now also been applied to the labelling of histones inside live cells. For this, the *C*-terminal intein sequence including the desired cargo were fused to a cell-penetrating peptide (CPP) via a disulfide bond [35••]. The construct enters the cell and the CPP is removed by reductive cleavage of the disulfide. The cargo diffuses into the nucleus and can then undergo trans-splicing with the target protein tagged with the *N*-terminal intein sequence, leading to the excision of the intein and leaving behind a labelled histone protein (Figure 2e). This was used to semi-synthetically generate a native histone modification inside live cells and evaluate its epigenetic effect [35••].

Endogenous protein labelling strategies

Strategies that allow labelling of proteins without the use of an affinity tag are the most modern approaches and perhaps the most elegant.

Unnatural amino acids

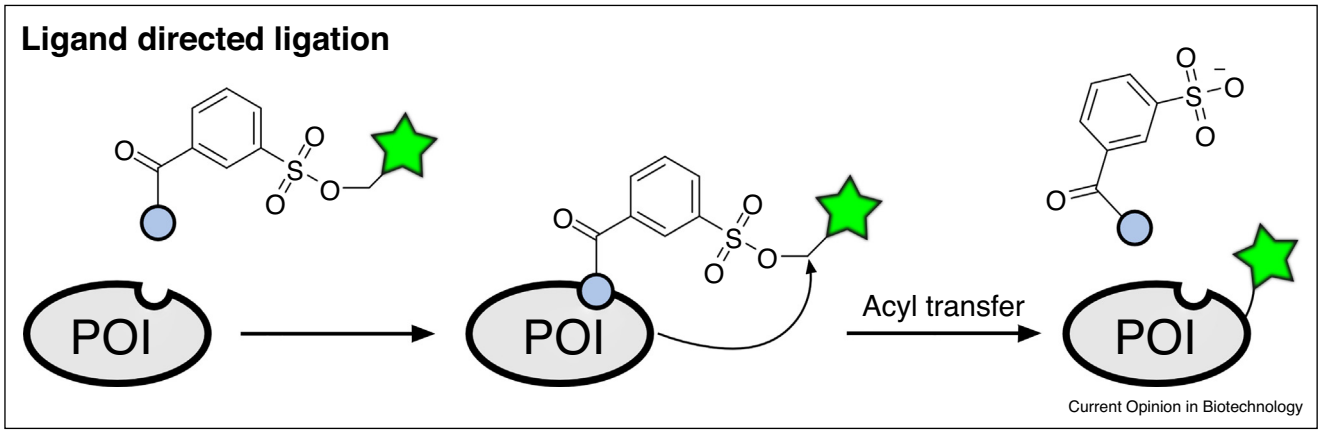
Unnatural amino acids (UAAs) allow the introduction of a bioorthogonal handle onto a protein with only a single amino acid change as opposed to a peptide or a protein domain. A variety of bioorthogonal handles for labelling can be introduced using a range of unnatural amino acids discussed in detail in recent review articles [36,37]. However, despite some recent advances, the usage of unnatural amino acids requires extensive engineering of the living system in question and can still often lead to low yields of labelled protein.

Ligand-directed ligation

Ligand-directed tosyl chemistry (LDT-chemistry) can be used to modify an endogenous protein at a specific site. This is done through the usage of a ligand bearing a tosyl ester. The ligand binds the target protein at a specific site and through a nucleophilic attack from a nearby amino acid side-chain will label the target protein at a residue proximal to the binding site. The label is removed from the ligand, which can then liberate the binding site and leave a functional, labelled protein behind (Figure 3) [38]. The technique has already been used more extensively *in vitro* and on cell surface proteins [39], where it has now also been used in a bimolecular fluorescence quenching and recovery (BFQR) assay to identify modulators of a cell-surface receptor [40•]. In living cells, the technique has recently been applied to the labelling of native FKBP12 in order to characterize the protein's interaction with the FKBP-rapamycin-binding domain from mTOR (FRB) [41].

Because the technique does not require a protein or peptide tag it is possibly less invasive than other labelling methods, and it allows the labelling of proteins on a site near the active center instead of the termini of the protein. However, LDT-chemistry requires extensive knowledge of the system in question as the ligand molecules have to be carefully designed. The reaction also does not go to completion and LDT-chemistry is slow: in

Figure 3



Ligand directed ligation. A ligand derivatised with a label via a tosylate linker binds the protein of interest at the active site and labels a nearby nucleophilic residue.

the case of FKBP12 incubation with the ligand *in vitro* at 37°C shows a labelling yield of 70% after 48 hours [41].

Faster ligand-directed labelling approaches are being developed [42,43] but they still cannot match other labelling methods in speed.

Conclusion

The labelling of proteins inside living cells has made unprecedented advances in recent years. Flexible chemical labelling of proteins offers great advantages over the labelling with fluorescent proteins, with sophisticated probes being developed for fluorescence imaging, NMR and mass spectrometry.

Yet, the rule of thumb that the smaller the tag, the lower the specificity of the labelling reaction still often holds true, and even a label as minimal as a His tag can perturb a proteins structure or function [44]. LDT-chemistry can circumvent this problem, but suffers from its own set of problems, most prominently the amount of labour required and the low labelling rate. Therefore, work remains to be done when it comes to creating quick, stable, versatile and specific labelling strategies that do not lead to the perturbation of the living systems in question.

Conflict of interest

There is no conflict of interest relating to this article.

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References and recommended reading

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

- • of special interest
- •• of outstanding interest

1. Rizzuto R, Brini M, Pizzo P, Murgia M, Pozzan T: **Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells**. *Curr Biol* 1995, **5**:635-642.

2. Schumacher D, Hackenberger CP: **More than add-on: chemoselective reactions for the synthesis of functional peptides and proteins**. *Curr Opin Chem Biol* 2014, **22**:62-69.

3. •• Chen X, Wu YW: **Selective chemical labeling of proteins**. *Org Biomol Chem* 2016, **14**:5417-5439.
A comprehensive overview of the chemistries and corresponding labelling strategies that are available for bioorthogonal, chemical labelling of proteins in different contexts.

4. Clackson T, Yang W, Rozamus LW, Hatada M, Amara JF, Rollins CT, Stevenson LF, Magari SR, Wood SA, Courage NL, Lu X, Cerasoli F Jr, Gilman M, Holt DA: **Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity**. *Proc Natl Acad Sci U S A* 1998, **95**:10437-10442.

5. Marks KM, Braun PD, Nolan GP: **A general approach for chemical labeling and rapid, spatially controlled protein inactivation**. *Proc Natl Acad Sci U S A* 2004, **101**:9982-9987.

6. Miller LW, Cai Y, Sheetz MP, Cornish VW: **In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag**. *Nat Methods* 2005, **2**:255-257.

7. Gallagher SS, Sable JE, Sheetz MP, Cornish VW: **An in vivo covalent TMP-tag based on proximity-induced reactivity**. *ACS Chem Biol* 2009, **4**:547-556.

8. Chen X, Li F, Wu YW: **Chemical labeling of intracellular proteins via affinity conjugation and strain-promoted cycloadditions in live cells**. *Chem Commun (Camb)* 2015, **51**:16537-16540.

9. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K: **A general method for the covalent labeling of fusion proteins with small molecules in vivo**. *Nat Biotechnol* 2003, **21**:86-89.

10. Gautier A, Juillerat A, Heinis C, Corrêa IR Jr, Kindermann M, Beaufils F, Johnsson K: **An engineered protein tag for multiprotein labeling in living cells**. *Chem Biol* 2008, **15**:128-136.

11. Mie M, Naoki T, Kobatake E: **Development of a split SNAP-CLIP double labeling system for tracking proteins following dissociation from protein–protein complexes in living cells**. *Anal Chem* 2016, **88**:8166-8171.

12. Los GV, Wood K: **The HaloTag: a novel technology for cell imaging and protein analysis**. *Methods Mol Biol* 2007, **356**:195-208.

13. • Lukinavičius G, Umezawa K, Olivier N, Honigmann A, Yang G, Plass T, Mueller V, Reymond L, Corrêa IR Jr, Luo ZG, Schultz C, Lemke EA, Heppenstall P, Eggeling C, Manley S, Johnsson K: **A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins**. *Nat Chem* 2013, **5**:132-139.
The authors develop a near-infrared fluorophore that has ideal characteristics for *in vivo* high resolution imaging with its high permeability, brightness and stability. They go on to apply this fluorophore using different labelling techniques in various cells and tissues.

14. Hori Y, Ueno H, Mizukami S, Kikuchi K: **Photoactive yellow protein-based protein labeling system with turn-on fluorescence intensity**. *J Am Chem Soc* 2009, **131**:16610-16611.

15. Hori Y, Norinobu T, Sato M, Arita K, Shirakawa M, Kikuchi K: **Development of fluorogenic probes for quick no-wash live-cell imaging of intracellular proteins**. *J Am Chem Soc* 2013, **135**:12360-12365.

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

17. • Wieneke R, Labòria N, Rajan M, Kollmannsperger A, Natale F, Cardoso MC, Tampé R: **Live-cell targeting of his-tagged proteins by multivalent *N*-nitrilotriacetic acid carrier complexes**. *J Am Chem Soc* 2014, **136**:13975-13978.
The authors demonstrate a potent labelling strategy that should be immediately applicable to a great variety of proteins as the only requirement for its usage is a His-tag.

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

19. Nonaka H, Tsukiji S, Ojida A, Hamachi I: **Non-enzymatic covalent protein labeling using a reactive tag**. *J Am Chem Soc* 2007, **129**:15777-15779.

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

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

22. Gelman H, Wirth AJ, Gruebele M: **ReAsH as a quantitative probe of in-cell protein dynamics**. *Biochemistry* 2016, **55**:1968-1976.

23. Sunbul M, Nacheva L, Jäschke A: **Proximity-induced covalent labeling of proteins with a reactive fluorophore-binding peptide tag**. *Bioconjug Chem* 2015, **26**:1466-1469.

24. Chen Y, Clouthier CM, Tsao K, Strmiskova M, Lachance H, Keillor JW: **Coumarin-based fluorogenic probes for no-wash protein labelling**. *Angew Chem Int Ed Engl* 2014, **53**:13785-13788.

25. Mazmanian SK, Liu G, Ton-That H, Schneewind O: ***Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall**. *Science* 1999, **285**:760-763.

26. Tanaka T, Yamamoto T, Tsukiji S, Nagamune T: **Site-specific protein modification on living cells catalyzed by sortase**. *Chembiochem* 2008, **9**:802-807.

27. Strijbis K, Spooner E, Ploegh HL: **Protein ligation in living cells using sortase**. *Traffic* 2012, **13**:780-789.

28. Glasgow JE, Salit ML, Cochran JR: **In vivo site-specific protein tagging with diverse amines using an engineered sortase variant**. *J Am Chem Soc* 2016, **138**:7496-7499.

29. Cronan JE, Zhao X, Jiang Y: **Function, attachment and synthesis of lipoic acid in *Escherichia coli***. *Adv Microb Physiol* 2005, **50**:103-146.

30. Fernández-Suárez M, Baruah H, Martínez-Hernández L, Xie KT, Baskin JM, Bertozzi CR, Ting AY: **Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes**. *Nat Biotechnol* 2007, **25**:1483-1487.

31. Uttamapinant C, White KA, Baruah H, Thompson S, Fernández-Suárez M, Puthenveetil S, Ting AY: **A fluorophore ligase for site-specific protein labeling inside living cells**. *Proc Natl Acad Sci U S A* 2010, **107**:10914-10919.

32. • Liu DS, Nivón LG, Richter F, Goldman PJ, Deerinck TJ, Yao JZ, Richardson D, Phipps WS, Ye AZ, Ellisman MH, Drennan CL, Baker D, Ting AY: **Computational design of a red fluorophore ligase for site-specific protein labeling in living cells**. *Proc Natl Acad Sci U S A* 2014, **111**:E4551-E4559.

The article offers great insights on the work done to generate an enzyme mutant that can ligate a substrate of choice to a peptide tag. The authors then apply the ligase in different cell types and compare their labelling method with other, commonly used methods.

33. Zettler J, Schütz V, Mootz HD: **The naturally split Npu DnaE intein exhibits an extraordinarily high rate in the protein trans-splicing reaction**. *FEBS Lett* 2009, **583**:909-914.

34. Schütz V, Mootz HD: **Click-tag and amine-tag: chemical tag approaches for efficient protein labeling in vitro and on live cells using the naturally split Npu DnaE intein**. *Angew Chem Int Ed Engl* 2014, **53**:4113-4117.

35. •• David Y, Vila-Perelló M, Verma S, Muir TW: **Chemical tagging and customizing of cellular chromatin states using ultrafast trans-splicing inteins**. *Nat Chem* 2015, **7**:394-402.

The authors propose a labelling strategy that is minimally disruptive as it leaves behind a protein with only a minimal label through intein trans-splicing. The authors demonstrate their strategy with different labels and characterize the outcome in detail.

36. Kim CH, Axup JY, Schultz PG: **Protein conjugation with genetically encoded unnatural amino acids**. *Curr Opin Chem Biol* 2013, **17**:412-419.

37. Ramil CP, Lin Q: **Bioorthogonal chemistry: strategies and recent developments**. *Chem Commun (Camb)* 2013, **49**:11007-11022.

38. Tsukiji S, Miyagawa M, Takaoka Y, Tamura T, Hamachi I: **Ligand-directed tosyl chemistry for protein labeling in vivo**. *Nat Chem Biol* 2009, **5**:341-343.

39. Wang H, Koshi Y, Minato D, Nonaka H, Kiyonaka S, Mori Y, Tsukiji S, Hamachi I: **Chemical cell-surface receptor engineering using affinity-guided, multivalent organocatalysts**. *J Am Chem Soc* 2011, **133**:12220-12228.

40. • Yamaura K, Kiyonaka S, Numata T, Inoue R, Hamachi I: **Discovery of allosteric modulators for GABAA receptors by ligand-directed chemistry**. *Nat Chem Biol* 2016, **12**:822-830.

A great example of what is possible using ligand-directed ligation of a fluorescent reporter to a target protein of interest and subsequent evaluation.

41. Tamura T, Kioi Y, Miki T, Tsukiji S, Hamachi I: **Fluorophore labeling of native FKBP12 by ligand-directed tosyl chemistry allows detection of its molecular interactions in vitro and in living cells**. *J Am Chem Soc* 2013, **135**:6782-6785.

42. Fujishima SH, Yasui R, Miki T, Ojida A, Hamachi I: **Ligand-directed acyl imidazole chemistry for labeling of membrane-bound proteins on live cells**. *J Am Chem Soc* 2012, **134**:3961-3964.

43. Yamaguchi T, Asanuma M, Nakanishi S, Saito Y, Okazaki M, Dodo K, Sodeoka M: **Turn-ON fluorescent affinity labeling using a small bifunctional *O*-nitrobenzoxadiazole unit**. *Chem Sci* 2014, **5**:1021-1029.

44. Chant A, Kraemer-Pecore CM, Watkin R, Kneale GG: **Attachment of a histidine tag to the minimal zinc finger protein of the *Aspergillus nidulans* gene regulatory protein AreA causes a conformational change at the DNA-binding site**. *Protein Expr Purif* 2005, **39**:152-159.