Site-specific protein labeling using PRIME and chelation-assisted click chemistry

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This protocol describes an efficient method to site-specifically label cell-surface or purified proteins with chemical probes in two steps: probe incorporation mediated by enzymes (PRIME) followed by chelation-assisted copper-catalyzed azide-alkyne cycloaddition (CuAAC). In the PRIME step, *Escherichia coli* lipoic acid ligase (LplA) site-specifically attaches a picolyl azide (pAz) derivative to a 13-aa recognition sequence that has been genetically fused onto the protein of interest. Proteins bearing pAz are chemoselectively derivatized with an alkyne-probe conjugate by chelation-assisted CuAAC in the second step. We describe herein the optimized protocols to synthesize pAz to perform PRIME labeling and to achieve CuAAC derivatization of pAz on live cells, fixed cells and purified proteins. Reagent preparations, including synthesis of pAz probes and expression of LplA, take 12 d, whereas the procedure for performing site-specific pAz ligation and CuAAC on cells or on purified proteins takes 40 min–3 h.

INTRODUCTION

PRIME is a versatile tool for site-specific tagging of proteins with chemical probes that possess useful biophysical properties such as fluorescence or photo-cross-linking ability[1–3], enabling functional studies of a given protein *in vitro* or in cells. The central component of the PRIME method is an engineered mutant of *E. coli* LplA that catalyzes the covalent tagging of the desired probe onto a specific lysine residue within a 13-aa recognition sequence called the LplA acceptor peptide (LAP), which is genetically fused to the protein of interest. LplA's high specificity for the single lysine in LAP ensures that there is no labeling of other proteins that are present in the labeling environment (such as other cellular proteins), nor is there any labeling of other sites within the LAP fusion protein. Probe targeting can be accomplished in a single step if the probe is small enough to fit into the engineered small-molecule binding pocket of LplA. The fluorescent probes coumarin[2], Pacific Blue[4], aminocoumarin[5] and resorufin (D.S.L. *et al.*, unpublished results), as well as the photo-cross-linker aryl azide[6], have been targeted in this way. Larger fluorophores and probes that cannot fit into the LplA active site—including most green and red organic fluorophores and quantum dots (QDs)—must be targeted in two steps: first, by using LplA to ligate a functional group handle to LAP, and second, by using bio-orthogonal ligation chemistry[1,3,7] or a protein-ligand binding interaction[8] to target the probe to the functional group handle.

As the labeling efficiency of two-step PRIME depends not only on LplA ligation kinetics but also on the kinetics of the derivatization chemistry, the best schemes use fast derivatization reactions such as the inverse-electron-demand Diels-Alder cycloaddition[3], chelation-assisted CuAAC[1] and HaloTag-mediated labeling[8]. Among our two-step labeling schemes, PRIME with Halotag is most suited for QD targeting to cell-surface proteins because of HaloTag's superior labeling kinetics over all bio-orthogonal chemistries (QDs can only be practically supplied for cell labeling at low-nanomolar concentrations), whereas PRIME with Diels-Alder is our best protocol for labeling intracellular proteins with diverse organic probes. For site-specific labeling of cell-surface proteins or purified proteins with small molecules, which is the focus of this protocol, PRIME with chelation-assisted CuAAC is preferred over PRIME with HaloTag or Diels-Alder chemistry. This is because HaloTag adds undesired steric bulk (35 kDa) to the protein of interest, which could disrupt its function, and *trans*-cyclooctene and tetrazine reagents for Diels-Alder are not as synthetically accessible as reagents for CuAAC.

In two-step labeling with PRIME and chelation-assisted CuAAC, a copper-chelating pAz molecule is used as the LplA substrate (**Fig. 1a**). pAz ligation onto LAP-tagged cell-surface proteins can be accomplished in two distinct ways (**Fig. 1b**). The first option is to add purified LplA mutant (^{W37V}LplA) to the cell culture medium along with the pAz probe and ATP[1]. This protocol tags the cell-surface pool of a LAP fusion protein selectively, not labeling intracellular subpopulations in the endoplasmic reticulum (ER) or Golgi. The second option is to co-express with the LAP fusion construct an LplA mutant targeted to the cell's ER. For this option, we use a quadruple mutant of LplA shown to have higher activity in the secretory pathway than the simple ^{W37V}LplA mutant. This construct is called ^{W37A,T57I,F147L,H267R}LplA or ^{AILR}LplA (ref. 9).

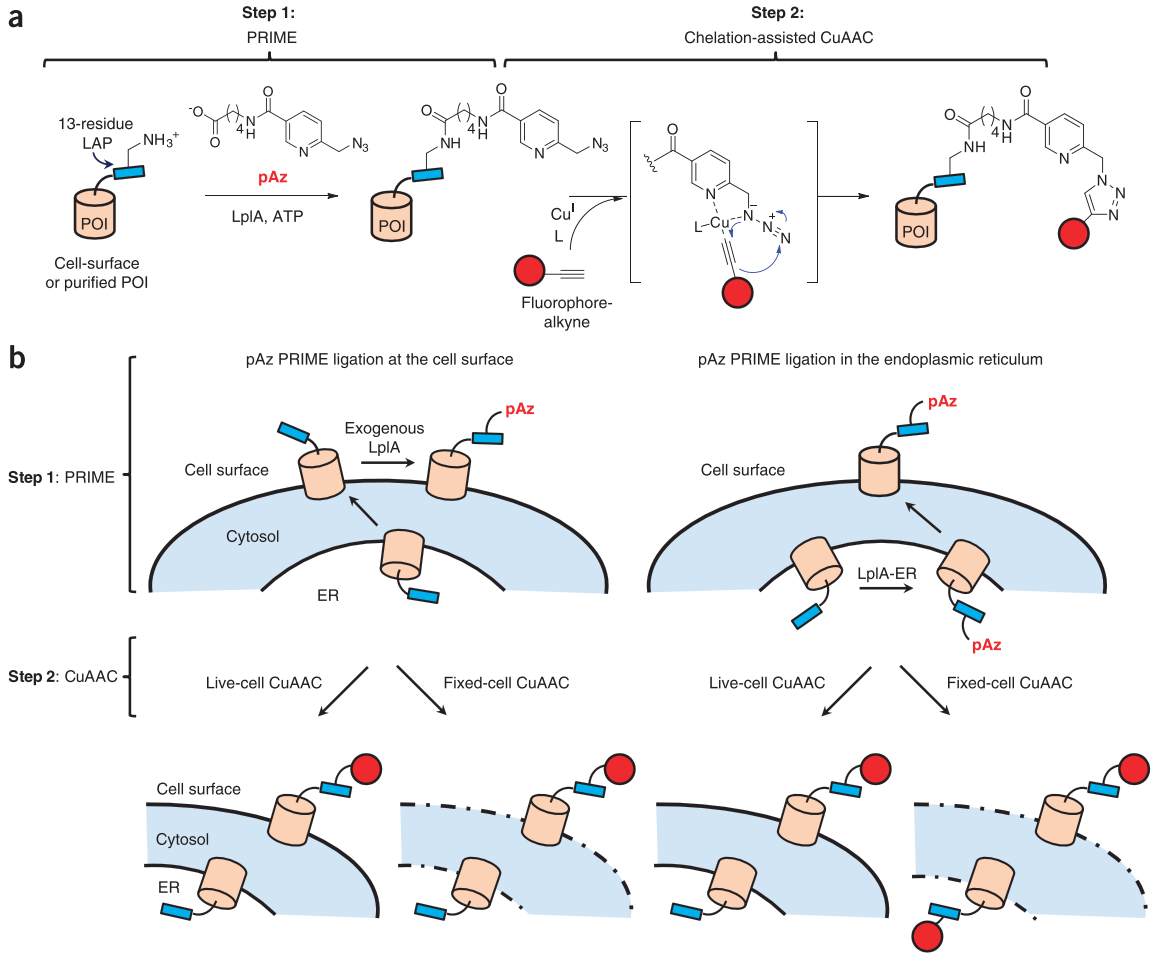
Once pAz is ligated to LAP, it is chemoselectively derivatized with alkyne-probe conjugates via chelation-assisted CuAAC. This variant of CuAAC is faster (as a result of increased local copper concentration induced by the picolyl moiety) and is more cell compatible (because of the lower concentration requirement for toxic copper) than conventional CuAAC using alkyl azides[1].

Because pAz is charged, it does not efficiently cross the plasma membrane of living cells. Consequently, for the PRIME labeling option using ER-expressed ^{AILR}LplA (**Fig. 1b**), it is necessary to protect pAz as an acetoxymethyl (AM) ester (**Fig. 2**) so that it can access LplA in the ER. Once inside the cell, the AM ester is cleaved by endogenous cellular esterases, releasing the parent pAz. Before unmasking, pAz-acetoxymethyl ester (pAz-AM) itself does not act as an LplA substrate, because a free carboxylate is required for conjugation of the probe to LAP.

Both PRIME and CuAAC labeling steps are efficient. PRIME ligation at the cell surface with pAz reaches ~80% completion after 20 min using purified ^{W37V}LplA[1]. For chelation-assisted CuAAC performed *in vitro*, we achieved complete conversion to product in <5 min in the presence of 10–40 μM copper and a Cu^{I}-stabilizing ligand Tris-(hydroxypropyltriazolylmethyl)amine (THPTA)[10], in contrast to a <40% conversion with conventional CuAAC under the same conditions. On cells, chelation-assisted CuAAC increased labeling yields by 2.7- to 25-fold compared with conventional CuAAC[1]. We estimate the overall two-step labeling yields for both cell-surface proteins and purified proteins to be >70% using 30–60-min labeling protocols. As much less cytotoxic copper (10–100 μM, compared with low-millimolar amounts in traditional CuAAC) can be used to achieve similar or better reaction rates, chelation-assisted CuAAC is inherently less toxic than conventional CuAAC[1].

Applications of the method

Two-step PRIME-CuAAC labeling has been applied to fluorophore tagging of a variety of cell-surface and purified proteins,



Protein pool(s) labeled	Cell-surface pool	Cell-surface pool	Cell-surface pool	Cell-surface pool and within secretory pathway
Advantages	Live-cell imaging; high-labeling sensitivity	Highest labeling sensitivity; no CuAAC toxicity	No need to express and purify LplA; avoid use of ATP; live-cell imaging	No need to express and purify LplA; avoid ATP; no CuAAC toxicity
Disadvantages	Residual CuAAC toxicity	Fixed-cell imaging	Residual CuAAC toxicity; pAz ligation in the ER is ~4-fold less sensitive than pAz ligation with exogenous LplA	Fixed-cell imaging; less sensitive than pAz ligation with exogeous LplA

Figure 1 | Site-specific protein labeling via PRIME and chelation-assisted CuAAC[1]. (**a**) The two-step labeling scheme. In the first step, an engineered PRIME ligase (Trp37→Val mutant of lipoic acid ligase or LplA) covalently attaches a copper-chelating picolyl azide derivative (pAz) onto LplA's acceptor peptide (LAP), which is genetically fused to a cell-surface or purified protein of interest (POI). In the second step, pAz-modified proteins are chemoselectively derivatized with a terminal alkyne-probe conjugate (red circle) by chelation-assisted CuAAC. Cu^{I} is generated *in-situ* from 10–100 μM $Cu^{II}SO_4$ and 2.5 mM sodium ascorbate. Ligand (L) represents Cu^{I}-stabilizing ligands, such as THPTA[10], BTTAA[27] or TBTA[28]. The LAP sequence is GFEIDKVWYDLDA[24] (lysine labeling site underlined). (**b**) Four different configurations for PRIME-CuAAC labeling. PRIME ligation of pAz can be performed at the cell surface (left), with application of exogenous LplA enzyme to the cell medium. Alternatively, pAz ligation can be performed in the cell's secretory pathway (right), using ligase expressed in the ER. Thereafter, CuAAC derivatization of pAz-modified proteins can be performed on live cells or after cell fixation. Key features of each labeling configuration are listed in the table below.

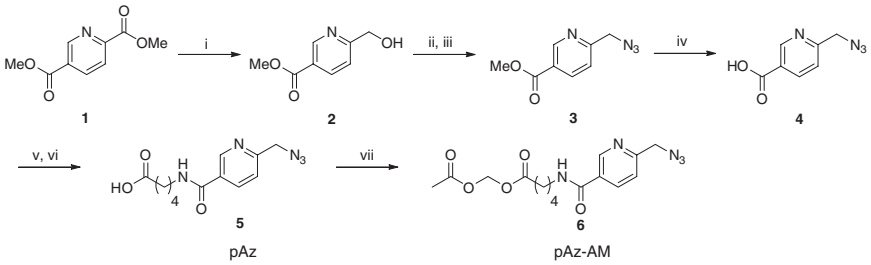


Figure 2 | Synthesis of pAz and pAz-AM reagents for PRIME labeling. The synthesis begins with commercially available dimethyl 2,5-pyridine dicarboxylate and proceeds through six steps to give pAz. pAz-AM is made from pAz in one additional step. (i) $NaBH_4$, $CaCl_2$, THF/MeOH; (ii) TsCl (*p*-toluenesulfonyl chloride), TEA (triethylamine), CH_2Cl_2; (iii) NaN_3, THF; (iv) LiOH, MeOH/H_2O; (v) *N,N'*-disuccinimidyl carbonate, TEA, DMF; (vi) 5-aminovaleric acid, TEA, DMF; and (vii) bromomethyl acetate, TEA, DMF.

including neurexin, neuroligin and kinesin. The labeling is highly specific in all eukaryotic cell types we have tested, including yeast, human cell lines and rat neurons. Versatile chemical probe targeting to cell-surface proteins can be useful for diverse applications, such as single-molecule tracking of receptor motion[11], super-resolution imaging of cell-surface adhesion proteins and receptors[12,13] and imaging assays that probe the fate and properties of the surface subpopulation of proteins[14] (as opposed to the total or intracellular protein pool).

PRIME-CuAAC can also be adapted not only for imaging specific cellular proteins but also for monitoring protein-protein interactions. In a separate study, we have used interaction-dependent PRIME-CuAAC tagging to visualize trans-cellular neurexin-neuroligin interactions in cultured human embryonic kidney (HEK) 293T cells and rat neurons[9].

Apart from its pairing with PRIME, chelation-assisted CuAAC is by itself useful for a variety of detection assays. For example, we have shown that it can be used to detect proteins and RNA metabolically labeled in live cells with azide analogs[1].

Comparison with other protein-labeling methods

Two-step PRIME-CuAAC labeling offers several advantages over existing site-specific protein-labeling methods. First, the total label size is small (13 aa for LAP plus the fluorophore of interest joined via a stable triazole linker as in **Fig. 1a**), ensuring minimal interference with the protein of interest, unlike the much larger GFP[15], SNAP[16] and HaloTag[17] labels (which range between 20 kDa and 35 kDa). Second, the label is monomeric and will not induce artificial cross-linking like wild-type streptavidin[18] and antibodies[19]. Third, both labeling steps are kinetically efficient, and therefore these steps can be performed quickly and with low concentrations of precious reagents without sacrificing yield. This contrasts with strain-promoted azide-alkyne cycloaddition-based labeling[7] and the aliphatic aldehyde tag[20,21], for example, whose much slower second-order rate constants result in lower labeling yields even when much higher probe concentrations are used. Fourth, labeling is highly specific, not only because of the inherent specificity of LplA-mediated ligation and CuAAC but also because the reaction partners (LAP, pAz and alkyne) are biologically inert until reaction catalysts (LplA, then copper) are supplied. This contrasts with components in many protein-labeling methods (e.g., FlAsH[22]) and bioorthogonal ligation chemistries[3,23] that have side reactions with endogenous biomolecules. Finally, even though PRIME-CuAAC labeling requires tailored small-molecule reagents, the reagents are straightforward to synthesize or commercially available and are stable during long-term storage.

Limitations

At 13 aa, the LAP tag can still interfere with the function or trafficking of proteins to which it is fused. If possible, one should perform control experiments to ensure proper functioning of the tagged recombinant protein. For example, we confirmed that LAP-tagged postsynaptic adhesion protein neuroligin-1 traffics correctly to the postsynapse, via colocalization analysis with a postsynaptic protein marker fused to a fluorescent protein[1,9], and that its binding to its adhesion partner neurexin is not impaired by the introduction of a labeling tag.

One should also ensure that overexpression of the tagged protein does not alter its function. If needed, expression levels of the protein can be tuned by using tunable promoters or promoters of different strengths, or by generating stable cell lines.

Even at a copper concentration of 10–40 μM, CuAAC can still be somewhat toxic to cells and damaging to proteins. We have included measures to ensure maximal cell viability when performing CuAAC, including the use of the best Cu^{I}-stabilizing ligands available, immediate sequestration of copper ions after labeling (via use of the cell-compatible copper chelator bathocuproin sulfonate) and the addition of the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) to quench reactive oxygen species. By using the optimized protocol presented here, we show that chelation-assisted CuAAC is as nontoxic as copper-free strain-promoted azide-alkyne cycloaddition and can be performed on delicate cultured rat neurons[1]. As CuAAC toxicity directly correlates with the amount of copper used and the labeling duration, one should finely tune these two parameters to obtain a balance between maximal signal and minimal disruption of cellular and protein function.

In its current form, the two-step PRIME-CuAAC process is not applicable to the labeling of intracellular proteins. Although pAz ligation can occur effectively inside the cytosol, it is currently not possible to perform CuAAC inside living cells for many reasons. First, $Cu^{I/II}$ would need to be delivered across the cell membrane. Second, once inside, glutathione in the cytosol would likely reduce any Cu^{II} species to the CuAAC-competent Cu^{I} species, but a major problem would be the sequestration of Cu^{I} by the same thiol. Third, cell-protective Cu^{I} ligands such as THPTA and bis[(tertbutyltriazoyl)methyl]-[(2-carboxymethyltriazoyl)methyl]-amine (BTTAA) must also be delivered across the cell membrane.

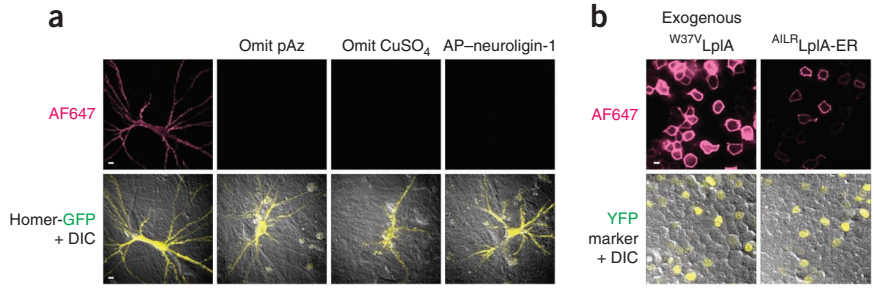


Figure 3 | Cell-surface protein labeling via PRIME and chelation-assisted CuAAC. (**a**) Demonstration of labeling specificity. Rat hippocampal neurons (at 14 d *in vitro*) expressing LAP–neuroligin-1 (LAP on the extracellular N terminus) and Homer1b-GFP were labeled live with 10 μM ^{W37V}LplA, 200 μM pAz and 1 mM ATP for 20 min at 37 °C. After two rounds of washing, cells were fixed using formaldehyde and blocked with 0.5% (wt/vol) casein. CuAAC was performed using 1 mM $CuSO_4$, 100 μM TBTA, 2.5 mM sodium ascorbate and 5 μM AF647-alkyne for 1 h at room temperature. Negative controls are shown with pAz omitted during the PRIME step, $CuSO_4$ omitted during CuAAC or AP-tagged neuroligin-1 replacing LAP-neuroligin-1 (AP = acceptor peptide for biotin ligase[29] rather than LplA). (**b**) Comparison of PRIME ligation of pAz at the cell surface versus within the ER. HEK cells expressing LAP–neurexin-1β (LAP on the extracellular N terminus), ^{AILR}LplA-ER and histone 2B-YFP (a transfection marker) were labeled with exogenous ^{W37V}LplA as in **a** (left), or with 100 μM pAz-AM for 1 h at 37 °C (right). Thereafter, CuAAC was performed on the live HEK cells using 50 μM $CuSO_4$, 300 μM BTTAA, 2.5 mM sodium ascorbate and 20 μM AF647-alkyne for 5 min at room temperature. Quantification shows that the AF647 signal is ~4.6-fold higher in the case of exogenous labeling. DIC, differential interference contrast. Scale bars, 10 μm.

Designing LAP-tagged cell-surface proteins

LAP sequence. The most kinetically efficient LAP, called LAP2 (GFEIDKVWYDLDA)[24], generally works best and should be tested first as a fusion tag to the protein of choice. An alternative LAP sequence, called LAP4.2 (GFEIDKVWHDFPA)[24], has also performed well in our hands, especially when fused to cell-surface proteins. In the case that expression, surface trafficking or labeling of a LAP2-tagged protein is problematic, one should test LAP4.2 as a possible remedy, along with other strategies such as changing the LAP fusion site or inserting a linker to increase flexibility around the LAP sequence. To increase labeling signal, tandem LAPs (such as 2× or 3× LAP) can also be used, although one caveat is that there might be a decrease in surface trafficking of the fusion protein as a result of the larger, more disordered tag.

LAP fusion site. LplA can catalyze probe ligation onto LAP at the N terminus, C terminus or in an internal loop of a protein. For tagging of cell-surface proteins as described in this protocol, the LAP sequence must be on the extracellular or luminal face of the protein. If needed, include a few glycines or serines on either side of the LAP sequence to separate LAP from the folded protein domain and to make LAP more sterically accessible to LplA.

Probe synthesis. A synthetic route to access the pAz substrate for LplA (structure **5**) is shown in **Figure 2**. To access other pAz derivatives such as pAz-fluorophore conjugates, one can use the succinimidyl ester of 6-azidomethylnicotinic acid **4** to react with an amine of choice. Many alkyne-fluorophore conjugates can be purchased (for example, from Life Technologies or Sigma-Aldrich) or synthesized in a single step via amide coupling between a commercially available fluorophore-succinimidyl ester and an amino-alkyne such as propargylamine[1].

Design of labeling experiments. In the initial protein-labeling tests (whether at the cell surface or *in vitro*), it is best to include negative controls (omit LplA, omit pAz, omit copper or introduce a Lys→Ala point mutation in LAP; **Fig. 3a**) in order to ensure that the labeling signal is specific and results from the activity of LplA and the chemistry of CuAAC. When designing the protein construct, it is very helpful to insert a short epitope tag (for example, hemagglutinin (HA), FLAG, *c*-Myc or V5), in addition to the LAP tag, so that one can verify expression of the fusion protein by immunofluorescence staining or western blotting, independent of PRIME-CuAAC labeling.

To selectively label the cell-surface pool of a protein with PRIME-CuAAC labeling, at least one of the labeling steps must be performed on live cells with membrane-impermeant reagents, so that the intracellular protein pool will not be labeled. If PRIME ligation with pAz is performed on live cells with exogenously supplied ^{W37V}LplA, the CuAAC derivatization step is flexible: it can be performed on living cells to enable real-time tracking of surface proteins, or it can be performed after cells are fixed and permeabilized. For the purpose of detecting protein localization, the fixed-cell CuAAC protocol should be considered, as it provides more sensitive detection than live-cell CuAAC (because more-forcing CuAAC reaction conditions, such as higher copper concentrations, can be used), and any residual toxicity from CuAAC becomes irrelevant. If PRIME ligation of pAz is performed with ^{AILR}LplA-ER, intracellular pools of the protein will also be tagged

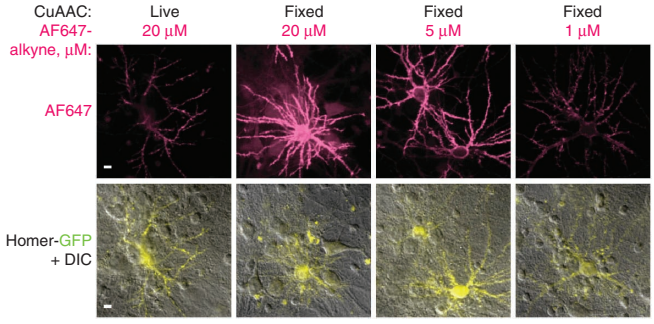


Figure 4 | Comparison of live-cell and fixed-cell CuAAC labeling protocols. Rat hippocampal neurons were transfected with LAP-neuroligin-1 and Homer1b-GFP and labeled live with pAz as in **Figure 3a**. Then, cells were either labeled live by CuAAC using 50 μM $CuSO_4$, 250 μM BTTAA, 2.5 mM sodium ascorbate, 100 μM TEMPOL and 20 μM AF647-alkyne for 5 min, or they were fixed. For fixed cells, CuAAC was performed as described in **Figure 3a**, except that the AF647-alkyne concentration was varied (1, 5 or 20 μM). All AF647 images are shown with the same intensity thresholds. For fixed-cell CuAAC, higher labeling signals were obtained, as well as higher background due to nonspecific binding of AF647-alkyne. DIC, differential interference contrast. Scale bars, 10 μm.

with pAz in addition to the surface pool. It is thus necessary to use live-cell CuAAC (copper ions are not membrane-permeant) after ER-pAz ligation if one wants to detect only the surface pool of the protein.

For labeling of the surface pool of a protein, we generally recommend using purified ^{W37V}LplA over ^{AILR}LplA-ER as the former is approximately four- to fivefold more sensitive than the latter (**Fig. 3b**). ^{AILR}LplA-ER is technically simpler and might be advantageous in certain biological applications, such as labeling and imaging in tissue slices, in which protein delivery may be a concern. Performing pAz ligation in the ER also eliminates the need to add ATP during labeling, which is a preferred practice for neuron cultures as ATP can activate purinergic receptors in neurons and cause excitotoxicity[25].

When performing live-cell CuAAC on hardy cell lines (for example, HEK 293T, HeLa and COS-7 cells), one can omit the cell-protective reagents TEMPOL and bathocuproin sulfonate without affecting labeling efficiency. One should not omit these reagents when working with more delicate cells such as neurons.

The choice of fluorophore-alkyne and its labeling concentration matters for both live-cell and fixed-cell CuAAC. For live-cell CuAAC, choose fluorophores that are not membrane-permeable (such as the Alexa Fluor (AF) dyes, almost all of which are polysulfonated) to avoid intracellular uptake. For fixed-cell CuAAC, it is also best to choose polysulfonated, hydrophilic fluorophores in order to minimize nonspecific sticking to fixed cells. A higher fluorophore-alkyne concentration results not only in faster CuAAC labeling but also in greater nonspecific sticking. Ideally, one should perform a titration of dye concentrations to identify conditions that give a maximal signal-to-noise ratio. An example of cell-surface protein labeling with both the live-cell and fixed-cell CuAAC protocols, at different fluorophore-alkyne concentrations, is shown in **Figure 4**. In this example, 5 μM AF647-alkyne for fixed-cell CuAAC provides the highest signal-to-noise ratio.

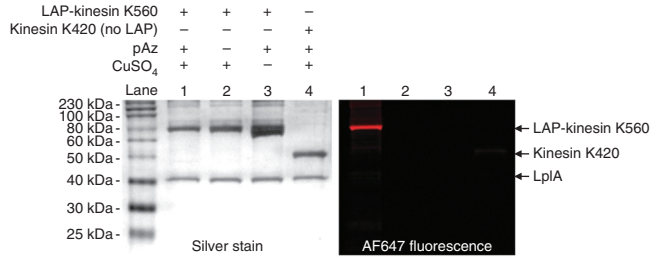


Figure 5 | *In vitro* protein labeling with PRIME and chelation-assisted CuAAC. LAP-tagged kinesin K560 (ref. 30) (at 10 μM) was labeled with 5 μM ^{W37V}LplA, 100 μM pAz and 500 μM ATP for 1 h at room temperature. Excess small-molecule reagents were then removed by centrifugation through a dialysis membrane. CuAAC labeling was performed with 200 μM $CuSO_4$, 1 mM BTTAA ligand, 2.5 mM sodium ascorbate and 20 μM AF647-alkyne for 30 min at room temperature. The reaction was quenched with EDTA (final concentration 20 mM) and then analyzed with 12% (wt/vol) SDS-PAGE. Negative controls are shown with pAz omitted during the PRIME step (lane 2), $CuSO_4$ omitted during CuAAC (lane 3) or kinesin K420 (no LAP) replacing LAP-kinesin K560 (lane 4). The left gel shows total protein (silver stain), and the right gel shows AF647 fluorescence. Kinesin K560 and kinesin K420 are the 560-aa and 420-aa N-terminal fragments of human kinesin, respectively.

For labeling purified proteins (**Fig. 5**), we describe the expression and purification of two LplA variants: hexahistidine (His_6)-tagged ^{W37V}LplA (His_6-^{W37V}LplA) and tagless ^{W37V}LplA. The choice of which enzyme to use depends on how one wishes to purify proteins after PRIME labeling. For example, it is helpful to use tagless LplA to label a His_6-tagged protein so that the latter can be easily separated from LplA after labeling. In contrast, His_6-tagged LplA can be used to label a protein with an orthogonal purification tag (such as FLAG or GST).

MATERIALS

REAGENTS

Reagents for chemical synthesis

- Dimethyl 2,5-pyridine dicarboxylate (Alfa Aesar, cat. no. A17250)
- Sodium borohydride ($NaBH_4$; Sigma-Aldrich, cat. no. 241-004-4)
- Calcium chloride, anhydrous ($CaCl_2$; Alfa Aesar, cat. no. 12316)
- Sodium azide (Alfa Aesar, cat. no. 14314) ! **CAUTION** Sodium azide is toxic and potentially explosive.
- *p*-Toluenesulfonyl chloride (Sigma-Aldrich, cat. no. 89730)
- Triethylamine (Sigma-Aldrich, cat. no. T0886)
- Lithium hydroxide monohydrate (LiOH; Sigma-Aldrich, cat. no. 43171)
- *N,N′*-disuccinimidyl carbonate (Sigma-Aldrich, cat. no. 43720)
- 5-Aminovaleric acid (Alfa Aesar, cat. no. B24788)
- Bromomethyl acetate (Sigma-Aldrich, cat. no. 303208)
- Sodium sulfate (Sigma-Aldrich, cat. no. 239313)
- Silica gel, 150 Å (200–425 mesh; Sigma-Aldrich, cat. no. 236810)
- Methanol, puriss, absolute, over molecular sieve (Sigma-Aldrich, cat. no. 66542)
- Hexanes, ACS reagent grade (Sigma-Aldrich, cat. no. 178918)
- Ethyl acetate, ACS reagent grade (Sigma-Aldrich, cat. no. 319902)
- Dichloromethane, puriss, absolute, over molecular sieve (Sigma-Aldrich, cat. no. 66749)
- Chloroform, ACS reagent grade (Sigma-Aldrich, cat. no. 437581)
- *N,N*-Dimethylformamide (DMF), puriss, absolute, over molecular sieve (Sigma-Aldrich, cat. no. 40228)
- Tetrahydrofuran (THF), ACS reagent grade (Sigma-Aldrich, cat. no. 676764)

Reagents for LplA expression in *E. coli*

- pYFJ16-His_6-^{W37V}LplA plasmid, available from Addgene (ID no. 34838)
- pYFJ16-His_6-ENLYFQG-^{W37V}LplA plasmid, available from Addgene. Note that ENLYFQG is a 7-aa recognition sequence for the Tobacco Etch Virus (TEV) protease[26]
- *E. coli* BL21 (DE3) pLysS chemically competent cells (Novagen)
- Luria-Broth (LB) medium and LB-ampicillin plates (ampicillin 100 μg ml^{-1})
- Ampicillin, sodium salt (Amresco) (see Reagent Setup)
- Isopropyl-β-D-thiogalactopyranoside (Calbiochem) (see Reagent Setup)
- Bacterial protein extraction reagent (B-PER) for bacterial lysis (Pierce)
- Phenylmethylsulfonylfluoride (PMSF; Amresco) (see Reagent Setup) ! **CAUTION** PMSF is harmful.
- Protease inhibitor cocktail (Sigma-Aldrich) ▲ **CRITICAL** The protease inhibitor must be EDTA-free, as EDTA can remove nickel from the nickel-affinity column. Store it at −20 °C.
- Nickel-nitrilotriacetic (NTA) acid agarose resin (Qiagen)
- Nickel-NTA binding buffer (see Reagent Setup)
- Nickel-NTA wash buffer (see Reagent Setup)
- Nickel-NTA elution buffer (see Reagent Setup)
- Imidazole, 1 M
- DTT
- LplA dialysis buffer (see Reagent Setup)
- AcTEV protease (Life Technologies, cat. no. 12575)

- TEV protease cleavage buffer, 20× (Life Technologies)
- Standard reagents for protein gel electrophoresis

Reagents for mammalian cell–labeling experiments

- HEK 293T cells (ATCC) or mammalian cell type of interest. The protocol has been shown to work in HEK cells and in dissociated rat neuron cultures
- Human fibronectin (Millipore), for promotion of adherence of HEK cells to glass coverslips
- pDisplay-LAP2-CFP-TM plasmid, available from Addgene (ID no. 34842)
- pcDNA4-^{AILR}LplA-ER, available on request from the Ting laboratory
- Standard media for mammalian cell culture
- Standard reagents for DNA cloning
- Lipofectamine 2000 (Life Technologies, cat. no. 11668) or other transfection reagent of choice
- Picolyl azide (pAz, no commercial vendor, synthetic procedure given in Step 1 of the PROCEDURE)
- pAz-AM (no commercial vendor, synthetic procedure given in Step 1 of the PROCEDURE)
- ^{W37V}LplA (expression and purification protocols given in **Box 1**)
- Adenosine 5′-triphosphate disodium salt hydrate (ATP, Sigma-Aldrich)
- Magnesium acetate heptahydrate (Alfa Aesar, cat. no. 11596)
- Dulbecco's PBS (DPBS), no calcium or magnesium (Life Technologies)
- BSA (Amresco, cat. no. 0332)
- BSA blocking buffer (see Reagent Setup)
- Casein, vitamin free (MP Biomedicals, cat. no. 904520)
- Casein blocking buffer (see Reagent Setup)
- Tyrode's buffer (for use with cultured neurons; see Reagent Setup)
- Copper(II) sulfate pentahydrate ($CuSO_4$; Mallinckrodt chemicals, cat. no. 4752-10)
- Sodium ascorbate (Spectrum, cat. no. S1349)
- Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, Sigma-Aldrich, cat. no. 678937).
- Tris-(hydroxypropyltriazolylmethyl)amine (THPTA, no commercial vendor, synthesize according to Hong *et al.*[10])
- Bis[(tertbutyltriazoyl)methyl]-[(2-carboxymethyltriazoyl)methyl]-amine (BTTAA, no commercial vendor, synthesize according to Besanceney-Webler *et al.*[27])
- TEMPOL (Calbiochem, cat. no. 581500). Store it at −20 °C
- AF647-alkyne, triethylammonium salt (Life Technologies, cat. no. A10278). Alkyne conjugates to AF488, AF594 and AF555 are also commercially available from Life Technologies
- Bathocuproin sulfonate (Alfa Aesar, cat. no. B22550-MD)
- Formaldehyde solution in water, 10% (vol/vol), methanol-free (Polysciences, cat. no. 04018) **! CAUTION** Formaldehyde is carcinogenic, toxic, irritant and lachrymatory. Handle it in a well-ventilated fume hood while wearing protective gear. Wash your hands after using formaldehyde.
- Glycerol
- NaCl
- DMSO
- NaOH
- KCl
- $MgCl_2$
- $NaH_2PO_4{\cdot}H_2O$
- Glucose
- HEPES

EQUIPMENT

Equipment for chemical syntheses

- Weighing balance
- Spatulas
- Weighing paper
- Syringes and needles
- Fume hood
- Magnetic stir plate
- N_2 gas and a silicone oil–filled bubbler
- Round-bottomed flasks
- Teflon-coated magnetic stir bars
- Graduated cylinders
- Erlenmeyer flasks
- Separatory funnels
- Pasteur pipettes
- SiO_2 thin-layer chromatography (TLC) plate and spotter
- TLC developing chamber
- Tweezers
- UV handheld lamp
- Heat gun
- Columns for flash chromatography
- Rotary evaporator
- Vacuum pump
- Electric oven capable of heating up to 130 °C
- Access to ^{1}H- and ^{13}C nuclear magnetic resonance (NMR) spectrometers

Equipment for LplA expression in *E. coli*

- Standard equipment for bacterial cell culture
- Standard equipment for protein gel electrophoresis
- Spectrophotometer to measure A_{600} to monitor bacterial growth
- Floor centrifuge, capable of spinning down 1 liter of bacterial culture at 8,000*g*
- Centrifuge tubes for spinning liter cultures
- Microcentrifuge tubes (Eppendorf)
- Motorized pipetter (Drummond)
- Poly-Prep protein chromatography column (Bio-Rad)
- Snakeskin dialysis tubing, 10-kDa cutoff (Thermo Scientific, cat. no. 68100)
- Stir plate and large stir bar
- Access to a temperature-controlled (4 °C) room or cabinet

Equipment for mammalian cell–labeling experiments

- Standard equipment for mammalian cell culture
- Bottle-top filters for sterile filtration of reagents
- Sterile 48-well cell culture plates
- Thick glass coverslips, 22 mm × 22 mm, no. 1 or no. 1.5
- Tweezers
- Fluorescence microscope, with filter sets appropriate to the relevant fluorophore

REAGENT SETUP

Ampicillin Dissolve ampicillin at 100 mg ml^{-1} in water and filter-sterilize it. Store it at −20 °C for >6 months.

PMSF Make a 100 mM solution in isopropanol (100× stock). **! CAUTION** PMSF is harmful. Store the reagent at −20 °C for >6 months.

Isopropyl-β-D-thiogalactopyranoside Dilute the stock (1,000×) to 100 mg ml^{-1} in water before use. Store 1,000× stock at −20 °C for >6 months.

^{W37V}LplA ^{W37V}LplA is expressed from pYFJ16-His$_6$-^{W37V}LplA or pYFJ16-His$_6$-ENLYFQG-^{W37V}LplA in *E. coli* as described in **Box 1**. LplA protein aliquots can be stored at −80 °C for >2 years without loss of activity.

Nickel-NTA binding buffer Prepare Nickel-NTA binding buffer by mixing 50 mM Tris base and 300 mM NaCl; adjust the pH to 7.8 with 1 M HCl. Store the buffer at 4 °C for >6 months.

Nickel-NTA wash buffer and elution buffer Add 1 M imidazole in nickel-NTA binding buffer, and adjust the pH to 7.8 with 5 M HCl. Dilute the imidazole stock solution in nickel-NTA binding buffer to prepare nickel-NTA wash buffer (imidazole concentration of 10 mM) and nickel-NTA elution buffer (imidazole concentration of 100 mM).

LplA dialysis buffer Freshly prepare LplA dialysis buffer by mixing 20 mM Tris base, 1 mM DTT and 10% (vol/vol) glycerol. Adjust the pH to 7.5 with 1 M HCl. **▲ CRITICAL** Freshly add DTT before dialysis.

TEV protease cleavage buffer (minus DTT), 20× Prepare 20× TEV protease cleavage buffer by mixing 1 M Tris and 10 mM EDTA. Adjust the pH to 8.0 with 1 M HCl.

TEV protease cleavage buffer Dilute 20× TEV buffer and add DTT to prepare a solution of 50 mM Tris (pH 8.0), 0.5 mM EDTA and 1 mM DTT. **▲ CRITICAL** Add DTT fresh before dialysis.

pAz Make a 20 mM stock solution of pAz in water. Store it at −80 °C for > 1 year.

pAz-AM Make a 100 mM stock solution of pAz-AM in DMSO. Store it at −80 °C for >6 months.

ATP Make single-use 100 mM aliquots (100× stock) of ATP, adjust the pH to 7.5 with 1 M NaOH and store them at −80 °C for >6 months. **▲ CRITICAL** Avoid freeze-thaw cycles. Adjust pH carefully to ensure that the pH does not exceed 8.0, at which point ATP will be rapidly hydrolyzed.

Magnesium acetate heptahydrate Make a 500 mM stock solution of magnesium acetate heptahydrate in water. Store the solution at 4 °C for >1 year.

BSA blocking buffer Make 3% (wt/vol) BSA solution in DPBS and filter-sterilize the solution. Store the buffer at 4 °C for up to 1 week.

Casein blocking buffer Make an ~0.5% (wt/vol) casein solution in DPBS. As casein is not very soluble in DPBS, we first microwave DPBS, add casein, and then stir it at 40–50 °C for 30 min. Insoluble casein is then removed via

Box 1 | ^{W37V}LplA expression and purification ● TIMING 4–5 d

If you are planning to perform exogenous cellular labeling with pAz (Step 2A of the PROCEDURE) or labeling of a purified protein, then you need to express and purify ^{W37V}LplA. If pAz ligation will be performed with ER-targeted ^{AILR}LplA (Step 2B of the PROCEDURE), this procedure is not required. Modules A and B describe the expression and purification of His$_6$-^{W37V}LplA. These same steps could also be performed to prepare His$_6$-ENLYFQG-^{W37V}LplA. Module C describes how to remove the His-tag from His$_6$-ENLYFQG-^{W37V}LplA and purify the resulting tagless ^{W37V}LplA. TEV protease-mediated cleavage of the ENLYFQG sequence removes His$_6$-ENLYFQ (cleavage occurs between glutamine and glycine) from ^{W37V}LplA. Thereafter, the reaction mixture is run through nickel-NTA resin, which selectively retains His$_6$-ENLYFQ peptide and His$_6$-AcTEV protease, and allows tagless ^{W37V}LplA to flow through.

(A) Expression of His$_6$-^{W37V}LplA in *E. coli*

(i) Transform chemically competent BL21 (DE3) pLysS *E. coli* with pYFJ16-^{W37V}LplA, and plate it on an LB-ampicillin agar plate. Incubate the plate at 37 °C overnight.

(ii) Pick one colony from the plate and inoculate it into 5 ml of LB-ampicillin (LB with 100 μg ml^{-1} ampicillin). Shake the flask at 220 r.p.m. at 37 °C overnight.

(iii) To make an expression culture, dilute the 5-ml overnight culture into 500 ml of LB-ampicillin in a 2-liter culture flask. Shake the culture flask at 37 °C until the A_{600} value reaches 0.5.

▲ CRITICAL STEP For maximal LplA protein yield, it is better to induce expression when the A_{600} value is below 0.7. Once the A_{600} value reaches 0.1, the cell density should double every 20 min.

(iv) Move the culture flask to a room-temperature shaker and allow the flask to cool down to room temperature for ~5 min.

(v) Add isopropyl-β-D-thiogalactopyranoside to a final concentration of 100 μg ml^{-1}.

(vi) Shake the culture flask at room temperature overnight (~8 h).

▲ CRITICAL STEP The lower culture temperature during induction greatly improves the folding efficiency of ^{W37V}LplA.

(vii) Collect bacteria by centrifuging the culture at 5,000*g* for 10 min at 4 °C. Discard the supernatant via decanting. Keep the pellet on ice.

■ PAUSE POINT The bacterial cell pellet can be stored at −80 °C for at least 3 months.

(B) Purification of His$_6$-^{W37V}LplA on a nickel-affinity column

▲ CRITICAL Always keep the cell pellet, cell lysate and any LplA-containing solutions on ice to minimize LplA aggregation and precipitation.

(i) Lyse bacteria using B-PER. First, mix 10 ml of B-PER with 50 μl of protease inhibitor cocktail and 50 μl of 100 mM PMSF. Transfer the mixture to the pellet-containing culture bottle. Completely resuspend the pellet in B-PER using pipetting or vortexing. Add an additional 10 ml of B-PER (also with protease inhibitor cocktail and PMSF).

(ii) Gently agitate the homogenous cell suspension for 10 min at 4 °C on a motorized shaker.

(iii) Centrifuge the cell suspension at 10,000*g* for 5 min at 4 °C. Collect the supernatant in a 50-ml conical tube as the cell lysate.

(iv) Prepare the nickel-NTA resin. Load a Poly-Prep column with ~1 ml (packed volume) of nickel-NTA agarose resin, and then wash the resin with five column-volumes of ice-cold nickel-binding buffer by allowing the buffer to flow through the resin.

▲ CRITICAL STEP Only use gravity-driven flow. Do not apply extra pressure.

(v) Add ~20 ml of ice-cold nickel-binding buffer to the cell lysate, and then transfer the washed nickel-NTA resin to the same tube. Gently agitate the tube via shaking for 20 min at 4 °C to ensure His-tagged protein binding to the resin.

(vi) Add the cell lysate/resin mixture to a Poly-Prep column and let the lysate flow through. Avoid letting the column run dry.

(vii) Wash the resin with ten column-volumes of nickel-NTA wash buffer to remove non-specifically bound proteins.

(viii) Elute LplA from the column using 500-μl aliquots of nickel-NTA elution buffer. Collect each 500-μl fraction, and check for the presence of protein in the fractions by measuring the A_{280} value. Pool the positive fractions.

▲ CRITICAL STEP ^{W37V}LplA can precipitate if its eluted concentration is too high (>5 mg ml^{-1}). It is best not to use imidazole concentration higher than 100 mM during elution.

(ix) Dialyze ^{W37V}LplA twice, each time for >6 h, against at least 1,000-fold ice-cold LplA dialysis buffer at 4 °C.

(x) Measure the LplA concentration using standard protein concentration measurement assays, such as the bicinchoninic acid assay.

▲ CRITICAL STEP Ensure that the protein concentration measurement assay of choice is not affected by the presence of 1 mM DTT in the dialysis buffer. The bicinchoninic acid assay is compatible with 1 mM DTT.

(xi) Confirm the purity of ^{W37V}LplA using protein gel electrophoretic analysis. The molecular weight of LplA is 39 kDa.

(xii) Aliquot LplA and store it at −80 °C.

■ PAUSE POINT ^{W37V}LplA is stable at −80 °C for >2 years.

(C) Preparation of tagless ^{W37V}LplA from His$_6$-ENLYFQG-^{W37V}LplA

(i) Express and purify His$_6$-ENLYFQG-^{W37V}LplA as described in steps A and B above.

(ii) After nickel-NTA purification, dialyze the protein aliquots in LplA dialysis buffer twice, each time for >6 h, against at least 1,000-fold ice-cold buffer at 4 °C.

(iii) Further dialyze LplA twice in TEV protease cleavage buffer, each time for >6 h, against at least 1,000-fold ice-cold buffer at 4 °C. After dialysis, measure the LplA concentration to make sure that it does not exceed 5 mg ml^{-1}; make dilutions if necessary. We typically work with a 2–3 mg ml^{-1} solution during the protease cleavage step.

▲ CRITICAL STEP Do not directly dialyze LplA into TEV protease cleavage buffer, which does not contain glycerol and can cause LplA to precipitate.

Box 1 | (continued)

(iv) To perform protease-mediated cleavage, add AcTEV protease to the LplA solution. We generally use ~10 units of the protease per 60 µg of LplA (~3 times less protease than recommended by Life Technologies).
(v) Incubate the reaction at 4 °C for 36–48 h.
(vi) Prepare nickel-NTA resin by loading the resin onto a column, and then wash the resin with five column volumes of ice-cold nickel-binding buffer. We use ~1 ml (packed volume) of resin per ~5 mg of protein.
(vii) Add the LplA/protease reaction directly to the column and collect the flow-through.
(viii) Wash the resin once using one column-volume of ice-cold nickel-binding buffer. Collect the flow-through.
(ix) Check for the presence of protein in the flow-through fractions using A_{280}. Pool the positive fractions.
(x) Dialyze ^{W37V}LplA twice, each time for >6 h, against at least 1,000-fold LplA dialysis buffer at 4 °C.
(xi) Measure the concentration and confirm the purity of LplA as described in step B above. Divide LplA into aliquots and store them at −80 °C.
■ **PAUSE POINT** ^{W37V}LplA is stable at −80 °C for >2 years.

centrifugation (at 10,000*g* for 10 min at 4 °C). The supernatant is collected via decanting without disturbing the pellet and is then filter sterilized. Store the buffer at −80 °C for >6 months.
Tyrode's buffer Prepare Tyrode's buffer by mixing 145 mM NaCl, 1.25 mM $CaCl_2$, 3 mM KCl, 1.25 mM $MgCl_2$, 0.5 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM glucose and 10 mM HEPES. Adjust the pH to 7.4 with 1 M HCl and filter-sterilize the buffer. Store it at 4 °C for >6 months.
$CuSO_4$ Make a 20 mM stock solution of $CuSO_4$ in water. Store the solution at 4 °C for >1 year.
Sodium ascorbate Make a 100 mM stock solution of sodium ascorbate in water. ▲ **CRITICAL** Freshly prepare the stock solution right before use. Discard unused portions.
TBTA Make a 1.7 mM stock solution of TBTA in 20% (vol/vol) DMSO in *tert*-butanol. Store the solution at −20 °C for >6 months.
THPTA Make a 50 mM stock solution of THPTA in water. Store the solution at −80 °C for >1 year.
BTTAA No commercial supplier. Prepare a 50 mM stock solution of BTTAA in water. Store the solution at −80 °C for >1 year.
TEMPOL Make a 100 mM stock solution of TEMPOL in water. ▲ **CRITICAL** Freshly prepare the stock solution right before use. Discard unused portions.
AF647-alkyne Make a 20–100 mM stock solution of AF647-alkyne in DMSO. In general, aim to make a DMSO probe stock that is at least 1,000× more concentrated than the final probe concentration used for labeling. Store the solution at −80 °C for >1 year.
Bathocuproin sulfonate Make 500 mM stock solution of bathocuproin sulfonate in water. The solution can be stored at −20 °C for >6 months without evidence of decomposition.
Formaldehyde fixative solution Prepare 4% (vol/vol) formaldehyde solution in DPBS. Store the solution at 4 °C for up to 2 weeks.
Formaldehyde fixative solution for cultured neurons Assemble the fixative solution as described below, and adjust the final volume with water and the final pH to 7.4. Store the fixative solution in aliquots at −20 °C. Right before use, thaw appropriate portions of the fixative at 37 °C. Discard the unused portions. ! **CAUTION** Formaldehyde is carcinogenic, toxic, irritating and lachrymatory. Handle formaldehyde in a well-ventilated fume hood while wearing protective gear. Wash your hands after using formaldehyde.

Component	Stock concentration	Final concentration
Formaldehyde	10% (vol/vol)	4% (vol/vol)
PIPES, pH 7.0	500 mM	60 mM
HEPES, pH 7.0	500 mM	25 mM
EGTA, pH 8.0	500 mM	10 mM
$MgCl_2$	1 M	2 mM
Sucrose	1.2 M	120 mM

EQUIPMENT SETUP
Glassware and stir bars Glassware and stir bars used for tosylation, succinimidyl ester formation, AM ester formation and amide coupling reactions should be dried before use in an oven or by flame drying. ▲ **CRITICAL** These reactions are conducted using anhydrous solvents under inert atmosphere. The presence of moisture in the reaction vessels from improper drying can cause unwanted hydrolysis of the reagents or desired products.
Glass coverslips for cell plating Cut no. 1 (22 × 22 mm) or no. 1.5 coverslips by hand into squares of 7 × 7 mm using a diamond glass cutter. The cut coverslips are sterilized by irradiation under a UV lamp in the cell culture hood for at least 1 h. Mammalian cells are then grown on these cut coverslips placed in a 48-well plate. ▲ **CRITICAL** We often perform cell-labeling experiments in a 48-well plate format to conserve precious labeling reagents. In each well of a 48-well plate, only ~150 µl of the labeling solution is needed. For imaging, we transfer the coverslip to an imaging dish with a no. 1 glass bottom.

PROCEDURE

Syntheses of pAz and pAz-AM ester

▲ **CRITICAL** All reactions are carried out under slightly positive pressure of dry nitrogen and stirred with a Teflon-coated magnetic stir bar.

1| Depending on whether you are planning to label the cell-surface protein exogenously (using exogenous ^{W37V}LplA) or endogenously (using ^{AILR}LplA-ER), you should prepare either pAz or pAz-AM. To label purified proteins, use pAz. To prepare pAz, perform modules A–D (Steps 1A–1D); to prepare pAz-AM, perform modules A–E (Steps 1A–1E).

(A) Synthesis of methyl 6-(hydroxymethyl)nicotinate 2 ● **TIMING 6 h**

(i) Weigh 10.0 g of dimethyl 2,5-pyridine dicarboxylate (51.2 mmol) and 22.8 g of anhydrous $CaCl_2$ (205.4 mmol), and then transfer both to a 1-liter round-bottom flask equipped with an ice bath. Transfer 100 ml of anhydrous THF and 200 ml of anhydrous methanol to the flask and stir the slurry mixture. Allow the mixture to cool to 0 °C for 5–10 min.

(ii) Weigh 3.9 g of $NaBH_4$ (103.1 mmol), and then transfer it in portions to the reaction flask. Stir the reaction at 0 °C for ~2 h.

(iii) Quench excess $NaBH_4$ by adding 100 ml of ice-cold water to the reaction mixture.

(iv) (Optional) Concentrate the reaction mixture using rotary evaporation until the total volume is reduced to ~150 ml. This makes the subsequent extraction step easier to handle.

(v) Extract the reaction mixture four times with chloroform (70 ml for each extraction). Combined organic extract is washed twice with water, dried with sodium sulfate and concentrated using rotary evaporation to obtain methyl 6-(hydroxymethyl)nicotinate **2** as a white solid (4.5 g, 52% yield). R_f = 0.27 (1:1 hexanes/ethyl acetate).

▲ **CRITICAL STEP** The aqueous wash step is crucial, as it removes a polar by-product (caused by over-reduction of two esters instead of one) without the need for a subsequent chromatographic purification step.

■ **PAUSE POINT** Methyl 6-(hydroxymethyl)nicotinate can be stored at −20 °C for >1 year.

(B) Synthesis of methyl 6-(azidomethyl)nicotinate 3 ● **TIMING 32 h**

(i) Weigh 4.0 g of methyl 6-(hydroxymethyl)nicotinate **2** (23.9 mmol), transfer to a 500-ml round-bottom flask and dissolve it in 240 ml of anhydrous dichloromethane.

(ii) Add 13.4 ml of anhydrous triethylamine (95.7 mmol) and 6.8 g of *p*-toluenesulfonyl chloride (35.9 mmol) consecutively to the reaction flask. Stir the reaction at room temperature (21 °C) for 3 h.

(iii) Remove dichloromethane from the reaction mixture using rotary evaporation.

! **CAUTION** Ensure that dichloromethane is completely removed, as it can react with sodium azide used in the subsequent step to form potentially explosive diazidomethane.

(iv) Dissolve the resulting residue in 240 ml of THF.

(v) Add 15 g of sodium azide (230.9 mmol). Stir the reaction at room temperature for 24 h.

▲ **CRITICAL STEP** Note that the R_f of methyl 6-(azidomethyl)nicotinate **3** is almost identical to that of the tosylate intermediate, making reaction monitoring by TLC difficult. If needed, use ^{1}H NMR to monitor the reaction progress instead.

(vi) Reduce the volume of the reaction mixture to ~60 ml through rotary evaporation.

! **CAUTION** Do not use heat during rotary evaporation. Although sodium azide is not explosive except when heated near its decomposition temperature (300 °C), heating should generally be avoided when working with it.

(vii) Dilute the resulting residue with ethyl acetate and water. Further extract the aqueous layer three times with ethyl acetate (70 ml each time). Wash the combined organic extract with brine solution, and then dry it over Na_2SO_4 and concentrate using rotary evaporation.

(viii) Purify the resulting residue by flash chromatography on silica, using isocratic 4:1 hexanes:ethyl acetate, to afford methyl 6-(azidomethyl)nicotinate **3** as a light-yellow solid (3.26 g, 71% yield). R_f = 0.79 (1:1 hexanes/ethyl acetate).

■ **PAUSE POINT** Store methyl 6-(azidomethyl)nicotinate at −20 °C for >1 year.

(C) Synthesis of 6-azidomethylnicotinic acid 4 ● **TIMING 4 h**

(i) Transfer 1.32 g of methyl 6-(azidomethyl)nicotinate **3** to a 500-ml round-bottom flask and dissolve it in 68 ml of methanol.

(ii) Add 20.5 ml of 1.0 M solution of LiOH in water (20.5 mmol). Stir the mixture at room temperature for 25 min.

▲ **CRITICAL STEP** Take care not to prolong the LiOH hydrolysis step, as the azido group can be labile under aqueous basic conditions.

(iii) Add ~700 µl of acetic acid to the reaction mixture and concentrate the crude mixture using rotary evaporation.

(iv) Purify the resulting residue by flash chromatography on silica, using isocratic ethyl acetate + 1% (vol/vol) acetic acid, to obtain 6-azidomethylnicotinic acid **4** as a yellow solid (1.12 g, 92% yield). R_f = 0.35 (ethyl acetate + 1% acetic acid).

■ **PAUSE POINT** Store 6-azidomethylnicotinic acid at −20 °C for >1 year.

(D) Synthesis of 5-(6-(azidomethyl)nicotinamido)pentanoic acid 5 or pAz ● **TIMING 12 h**

(i) Transfer 300 mg of 6-azidomethylnicotinic acid **4** (1.68 mmol) to a 100-ml round-bottom flask, and then dissolve it in 5 ml of anhydrous DMF.

(ii) Add 353 µl of anhydrous triethylamine (2.53 mmol) and 650 mg of *N,N'*-disuccinimidyl carbonate (2.53 mmol). Stir the reaction at room temperature for 3 h.

(iii) Dilute the reaction mixture with chloroform and water. Further extract the aqueous layer three times with chloroform (20 ml each time). Wash the combined organic extract with brine solution, dry it over Na_2SO_4 and concentrate it using rotary evaporation.

(iv) Purify the resulting residue by flash chromatography on silica, using isocratic 1:1 hexanes:ethyl acetate, to afford the succinimidyl ester of 6-azidomethylnicotinic acid (166 mg, 36% yield). R_f = 0.67 in 9:1 chloroform:methanol.

(v) Dissolve 166 mg of the succinimidyl ester of 6-azidomethylnicotinic acid (0.36 mmol) in 1.75 ml of anhydrous DMF.

(vi) Add 150 µl of anhydrous triethylamine (1.08 mmol) and 127 mg of 5-aminovaleric acid (1.08 mmol). Stir the reaction at room temperature for 3 h.

(vii) Concentrate the reaction mixture using rotary evaporation.

(viii) Purify pAz by either normal-phase purification on a hand-packed silica column (Step 1D(ix)) or by reverse-phase purification using HPLC (Step 1D(x)). Purification on a hand-packed silica column is suitable for large-scale purifications, whereas HPLC purification affords the product with higher purity.

(ix) Dissolve the crude reaction mixture in a minimum volume of 90:5:5 ethyl acetate:methanol:acetic acid. Load the resulting mixture directly on to a silica gel column equilibrated with ethyl acetate + 1% (vol/vol) acetic acid. Elute the product using isocratic ethyl acetate + 1% (vol/vol) acetic acid to afford the product as light yellow oil (131 mg, 93% yield).

▲ **CRITICAL STEP** ^{1}H-NMR analysis shows that pAz purified according to Step 1D(ix) co-elutes with *N*-hydroxysuccinimide (δ 2.69 in CD_3OD, singlet).

(x) For an alternative HPLC purification, use a Varian Prostar 210 HPLC equipped with an Agilent 325 UV-vis dual-wavelength detector, an Agilent 440-LC fraction collector and a Microsorb C18 column (Varian, 5-µm particle size, dimensions of 21 mm × 250 mm). We use a 0–10% (vol/vol) acetonitrile in water gradient over 30 min at a 10 ml min^{-1} flow rate. pAz elutes at 29–30 min. Collect the desired fractions, remove acetonitrile using rotary evaporation, and then flash-freeze and lyophilize the resulting aqueous solution. pAz purified this way appears as a pale yellow solid, and it does not contain the *N*-hydroxysuccinimide contaminant.

■ **PAUSE POINT** Store pAz at −20 °C for >1 year.

(E) Synthesis of AM 5-(6-(azidomethyl)pyridine-3-carboxamido)pentanoate 6 or pAz-AM ester ● **TIMING 5 h**

(i) Transfer 60 mg of pAz **5** (0.216 mmol) to a 5-ml round-bottom flask and dissolve it in 1.2 ml of anhydrous DMF.

(ii) Add 60 µl of anhydrous triethylamine (0.432 mmol) and 42 µl of bromomethyl acetate (0.432 mmol). Stir the reaction at room temperature for 1 h.

(iii) Dilute the reaction mixture with chloroform (10 ml) and water (10 ml). Further extract the aqueous layer three times with chloroform (10 ml each time). Wash the combined organic extract with brine solution, dry it over Na_2SO_4 and concentrate using rotary evaporation.

(iv) Purify the resulting residue by flash chromatography on silica, using isocratic 1:2 hexanes:ethyl acetate, to afford the AM ester of pAz **6** as a pale yellow solid (35 mg, 46% yield). R_f = 0.37 (1:1 hexanes/ethyl acetate).

■ **PAUSE POINT** Store pAz-AM ester at −80 °C for >6 months.

pAz ligation onto cell-surface proteins with LplA

2| pAz ligation can be performed exogenously with purified ^{W37V}LplA (option A) or endogenously with ^{AILR}LplA-ER (option B). Exogenous pAz ligation with ^{W37V}LplA is our recommended protocol for most cell-surface protein–labeling experiments as it is more sensitive than pAz ligation in the ER. Users should only consider using ^{AILR}LplA-ER if it is known that: the 39 kDa LplA might have problems accessing the labeling site on the protein of interest at the cell surface, whereas such hindrance is not present if the labeling is performed within the ER, or the reagents required for exogenous labeling such as ATP are toxic to the cell type of interest. Relating to the toxicity concern, note that pAz-AM needed for ER labeling is not water soluble and has to be supplied as a DMSO stock, which increases the toxicity of the ER labeling procedure. pAz needed for exogenous LplA labeling, in contrast, is water soluble. Note that this step is not required for labeling of purified proteins; users wanting to do this should proceed to **Box 2**.

(A) pAz ligation of cell-surface proteins using exogenous ^{W37V}LplA ● **TIMING 2 d (i,ii); 15 min–1 h (iii,iv)**

(i) Plate the cells onto 7-mm × 7-mm coverslips in a 48-well plate. For HEK cells, we coat coverslips with human fibronectin (50 µg ml^{-1}) according to the manufacturer's instructions, and we generally plate ~8,000 cells in 250 µl of medium per well. This cell density should result in cells that are 80% confluent ~24 h after plating, which is optimal for Lipofectamine transfection.

(ii) Transfect the cells with an expression plasmid for the LAP-tagged cell-surface protein. For transient transfection using Lipofectamine 2000, we typically use 200–600 ng of DNA, and maximal expression is often reached at ~24 h after transfection. For easy identification of transfected cells during imaging, we recommend co-expressing a fluorescent protein marker.

(iii) To perform pAz ligation, add 10 µM ^{W37V}LplA, 200 µM pAz, 1 mM ATP and 5 mM magnesium acetate heptahydrate in regular cell growth medium for 15–60 min either at room temperature or at 37 °C. If other labeling medium such as PBS or calcium-free DPBS is preferred, add 3% (wt/vol) BSA to the solution to prevent nonspecific sticking of labeling reagents to the cell surface.

Box 2 | Labeling of purified proteins with pAz and CuAAC ● TIMING 3–4 h

LAP-tagged proteins can be recombinantly expressed and purified using standard techniques appropriate for a given protein. We have successfully performed both pAz ligation and CuAAC labeling steps on ~10 μM purified protein solution using the protocol provided below. For protein samples more concentrated than 50 μM, use more pAz such that pAz concentration is at least twice the concentration of the protein. It is also permissible to add more LplA in the labeling solution; up to 20 mol% with respect to the amount of the LAP-tagged protein should be more than adequate. ATP and magnesium acetate heptahydrate are already provided in large excess in the protocol below and need no adjustment for labeling of proteins of up to 100 μM.

▲ **CRITICAL STEP** If the target LAP-tagged protein is His_6-tagged, it is best to use tagless ^{W37V}LplA here so the target protein can be purified after labeling using nickel affinity column. If the target protein is not His_6-tagged, you can use His_6-^{W37V}LplA and elute the desired target protein from the nickel-NTA resin while His_6-LplA remains bound to the resin.

pAz ligation

1. To label a 10 μM purified protein solution, add 1 μM ^{W37V}LplA, 50 μM pAz, 500 μM ATP, and 2.5 mM magnesium acetate heptahydrate (concentrations given are the final concentrations in the labeling solution) to the protein solution for 15–60 min at room temperature.

▲ **CRITICAL STEP** pAz ligation is compatible with standard buffers such as phosphate and HEPES, but avoid using buffers containing high concentrations of Ca^{2+} or lipoic acid during labeling. Also, LplA has a pI of 5.8 and will precipitate near that pH.

2. To remove small-molecule reagents (pAz and ATP), perform more than five rounds of centrifugal washing using a Centricon centrifugal filter unit with an appropriate molecular weight cutoff. Wash with more than 10× volume of the target protein–friendly buffer during each wash step. Gel filtration, such as with NAP resins, can also be used to remove small molecule reagents. It might not be necessary to perform this small-molecule purification step if you plan to purify your target protein using affinity capture.

3. To remove LplA, use a nickel affinity column. For labeling with a His_6-tagged target protein and a nontagged LplA, use nickel-NTA resin to bind to the target protein and wash away LplA. For labeling with a non-His_6-tagged protein and a His_6-tagged LplA, use nickel-NTA resin to bind and remove LplA.

Reaction with CuAAC

4. Refer to Presolski *et al.*[31], which includes a detailed protocol on how to perform CuAAC on biomolecules, including proteins, *in vitro*. Pay attention to many of their recommendations with respect to how to perform optimal CuAAC, such as which buffers are compatible with CuAAC. We have used 200 μM $CuSO_4$, 1 mM BTTAA (THPTA can likewise be used), 20 μM AF647-alkyne, 2.5 mM sodium ascorbate and 100 μM TEMPOL to label ~10 μM LAP-kinesin for 30 min at room temperature; we observed that labeling signal was already saturated after 30 min of CuAAC.

Estimation of *in vitro* protein labeling yield

5. After excess fluorophore is removed from the protein sample via gel filtration, dialysis or centrifugal washing, one can estimate the overall labeling yield on the protein by measuring the dye-to-protein molar ratio. Measure the A_{280} value and the maximal absorbance at a particular wavelength for each fluorophore (for example, A_{650} for AF647) to determine the molar concentration of the protein and the fluorophore in a given sample, respectively. To calculate molar concentrations, find out the extinction coefficients (ε) of the protein and the fluorophore. If the value of ε of a protein is not known in the literature or cannot be determined experimentally, one can roughly estimate ε from the protein's primary sequence using computational means (for example, ProtParam tool from ExPASy, which estimates ε on the basis of the cysteine, tryptophan and tyrosine present in a given protein). As fluorophores also absorb at 280 nm, one must apply correction factors for the portion of the A_{280} value that is contributed by the fluorophore. Information about fluorophores' extinction coefficients and A_{280} correction factors, as well as more details on dye-to-protein ratio calculation, can be obtained from the websites of common fluorophore manufacturers and vendors.

▲ **CRITICAL STEP** Do not use any buffer that contains high Ca^{2+} (such as Ca^{2+}-containing DPBS or Tyrode's buffer) during labeling, as Ca^{2+} inhibits LplA. If serum-containing medium is used as a labeling buffer, check that the lipoic acid content in the serum is not too high (not more than 5 μM). Lipoic acid is a natural, more efficient substrate for LplA than pAz and will reduce the labeling yield of pAz.

(iv) Wash the cells at least twice with cell growth medium to remove labeling agents before proceeding to the CuAAC derivatization step.

▲ **CRITICAL STEP** For cultured neurons, ensure that the neurons are not exposed to air for longer than 2–3 s during reagent addition or wash steps. You can use two pipettes to simultaneously add and withdraw solution from the wells.

(B) pAz ligation of cell-surface proteins using ^{AILR}LplA-ER ● TIMING 2 d (i); 1.5–4.5 h (ii,iii)

(i) After cell plating, co-transfect the cells with expression plasmids for the LAP-tagged cell-surface protein and ^{AILR}LplA-ER (pcDNA4-^{AILR}LplA-ER). For transfection using Lipofectamine 2000, we typically use 200–600 ng of each plasmid DNA. You can use a fluorescent co-transfection marker if desired. Wait 18–24 h before performing labeling.

(ii) To perform pAz ligation in the ER, add 25–100 μM pAz-AM in serum-free DMEM for 1–4 h at 37 °C.

▲ **CRITICAL STEP** Do not use serum-containing medium, as trace amount of esterases present in the serum can hydrolyze pAz-AM and prevent its cell entry.

(iii) To remove excess pAz-AM from the cells, wash the cells with cell growth medium three times over 15 min at 37 °C.

? TROUBLESHOOTING

CuAAC derivatization of pAz-tagged cell-surface proteins

3| There are two ways to detect pAz on LAP-tagged cell-surface proteins: live-cell CuAAC (option A) or fixed-cell CuAAC (option B). The fixed-cell CuAAC protocol can be used when one wants to completely avoid residual toxicity of live-cell CuAAC. As illustrated in **Figure 1b**, pAz-labeled cells from either Step 1A or Step 1B can be used for either CuAAC procedure.

(A) pAz derivatization on live cells using CuAAC ● TIMING 10–20 min

(i) Mix $CuSO_4$ solution with either THPTA or BTTAA solution at stock concentrations in an Eppendorf tube. Use a volume of each such that the final concentration of $CuSO_4$ is 40–100 μM and THPTA or BTTAA is 200–500 μM (recommended concentrations) after dilution (Step 3A(iii) below). THPTA and BTTAA are used at 5 molar equivalents with respect to $CuSO_4$.
▲ **CRITICAL STEP** Use the lowest $CuSO_4$ concentration needed to obtain adequate signal, as cytotoxicity is proportional to $CuSO_4$ concentration. It is possible to lower the $CuSO_4$ concentration below 40 μM if the protein target is overexpressed. We do not recommend using $CuSO_4$ at a concentration of more than 100 μM.
▲ **CRITICAL STEP** THPTA and BTTAA are interchangeable in most cases of cell-surface protein labeling, but we observed lower toxicity when CuAAC is performed on cultured neurons with BTTAA. BTTAA also yields slightly higher labeling signal for cell-surface protein labeling[1].
▲ **CRITICAL STEP** Do not use water-insoluble ligands such as TBTA in a live-cell labeling experiment.

(ii) Add sodium ascorbate, then TEMPOL at stock concentrations such that the final concentration of sodium ascorbate is 2.5 mM and TEMPOL is 100 μM after dilution (Step 3A(iii) below). Incubate the mixture at room temperature for 5–10 min. Keep the tube cap closed during incubation.
▲ **CRITICAL STEP** The 5- to 10-min incubation step is crucial, as it allows THPTA/BTTAA and TEMPOL to quench reactive oxygen species that are generated as by-products of the CuAAC reaction.

(iii) Dilute the mixture in DPBS. To label cultured neurons, use Tyrode's buffer.
▲ **CRITICAL STEP** Do not use MEM, DMEM, serum-supplemented medium, BSA-containing buffer or other protein-containing buffer as a labeling buffer. High amine or protein content in these buffers can sequester copper ions and reduce labeling yields.

(iv) Add 5–20 μM fluorophore-alkyne to the labeling solution. A dye-alkyne concentration higher than 20 μM (up to 100 μM) can be used to increase labeling sensitivity and decrease labeling time. Ensure that the fluorophore-alkyne stock solution is sufficiently concentrated such that no more than 0.1% (vol/vol) DMSO is added to the cells.

(v) Add the complete labeling solution to the cells for 1–10 min at room temperature. Five minutes is a good starting point for optimizing the labeling time. Labeling can be performed at lower temperature to reduce endocytosis, but labeling yield will decrease. Keep the labeled cells from air and light exposure by carrying out the reaction in a foil-wrapped, lidded plate.

(vi) After labeling, add regular cell growth medium containing 500 μM bathocuproin sulfonate to the cells for 30 s to remove copper from the cells.
▲ **CRITICAL STEP** This bathocuproin sulfonate wash step helps reduce toxicity as copper is immediately removed after labeling.

(vii) Wash the cells twice more with regular cell growth medium.

(B) pAz derivatization in fixed cells using CuAAC ● TIMING 1.5–3 h

(i) Fix the cells following the advice in **Box 3**. Fixed cells should be blocked with casein blocking buffer for at least 1 h at room temperature. Alternatively, block the cells with casein at 4 °C overnight.

(ii) Mix $CuSO_4$ solution with TBTA solution at stock concentrations in an Eppendorf tube. We recommend final concentrations of 1 mM $CuSO_4$ and 100 μM TBTA after dilution (Step 3B(iv) below).

Box 3 | Cell fixation (optional) ● TIMING 1 h

Cells can be fixed after the pAz ligation step (after Step 2) if one wants to perform CuAAC after cell fixation to maximize labeling sensitivity, or after both labeling steps are performed (after Step 3) to preserve cells for subsequent imaging experiments.

(A) Cell fixation after pAz ligation step

(i) If you want to detect the surface pool of the cell-surface protein, you can fix the cells using formaldehyde without additional membrane permeabilization or protein precipitation steps. Glutaraldehyde can also be used, but do not use a reducing agent such as $NaBH_4$ to quench glutaraldehyde, as pAz will also be reduced. If you want to access the intracellular pool of the cell-surface protein, fix the cells and use methanol to precipitate proteins and permeabilize the membrane. It is best to avoid detergents, as they can extract cell-surface proteins from the cell membrane.

(B) Cell fixation after CuAAC step

(i) Use standard fixation methods with formaldehyde or methanol. Glutaraldehyde can also be used, but do not use a reducing agent such as $NaBH_4$ to quench glutaraldehyde, as many fluorophores can be reduced and destroyed.

▲ CRITICAL STEP Do not use THPTA or BTTAA for fixed-cell CuAAC labeling. Fixed-cell CuAAC requires the use of a highly concentrated protein-blocking reagent such as casein, which is incompatible with THPTA- and BTTAA-based CuAAC.

(iii) Add 2.5 mM sodium ascorbate to the mixture. Incubate the mixture at room temperature for 5–10 min. Keep the tube cap closed during incubation.

(iv) Dilute the mixture in casein blocking buffer. If casein is not available, BSA blocking buffer can be used as an inferior substitute.

(v) Add 1–5 μM fluorophore-alkyne to the labeling solution.

▲ CRITICAL STEP The use of a higher dye-alkyne concentration can lead to high nonspecific sticking background from excess off-target dye. It is better to use a lower dye-alkyne concentration with a longer labeling time to increase labeling sensitivity.

(vi) Add the complete labeling solution to the cells for 30–120 min at room temperature. One hour is a good starting point for optimizing the labeling time. Reduce air and light exposure to the labeled cells by carrying out the reaction in a foil-wrapped, lidded plate.

(vii) After labeling, wash the cells three times with casein blocking buffer over 15 min.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Cell toxicity	Toxicity from the pAz ligation step	Reduce the ATP concentration, which can activate purinoreceptors in neurons and cause excitotoxicity. Perform labeling at 37 °C instead of room temperature. Use CO_2-independent medium for labeling at room temperature outside of an incubator. Especially for neurons, ensure that they are never left dry throughout labeling
3	Low or no labeling	Purified ^{W37V}LplA is not active	Check to see that there is no enzyme precipitate; re-express and purify if necessary. Use a positive control LAP construct (available on the Ting laboratory's Addgene site; http://www.addgene.org/Alice_Ting) and see whether labeling works on the positive control
		Poor transfection	Check for expression of a co-transfection fluorescence marker. Compare transfection efficiency obtained with that in literature reports; obtain a new batch of transfection reagents if they do not perform well
		The LAP-tagged protein is not trafficked properly to the cell surface	Check for surface expression of the protein using an antibody against an extracellular epitope. Perform immunofluorescence staining to visualize the total pool of protein and see what percentage is trafficked to the surface. Check the literature for precedents of successful surface trafficking of the target protein upon fusion to an epitope tag or a fluorescent protein
		LAP is not accessible to LplA	Use more enzyme, more pAz and longer labeling time to see whether LAP can be labeled at all. Perform lipoic acid ligation followed by immunostaining with a lipoic acid–specific antibody[24] as a positive control to confirm that LAP is recognized by LplA. Change the LAP location within the protein or introduce flexible linkers if needed
		Degraded reagents	Do not use re-thawed ATP and ^{W37V}LplA. We have observed that ligands such as THPTA perform poorly if left at room temperature for a long time, probably as a result of degradation. Make fresh sodium ascorbate solution
	High labeling background with live-cell CuAAC	Poor cell health	Use milder CuAAC conditions: less copper and shorter labeling time. Ensure that the supplements that preserve cell health (sodium ascorbate, TEMPOL and bathocuproin sulfonate) are good. Sodium ascorbate and TEMPOL solutions should be freshly made
	High labeling background with fixed-cell CuAAC	Nonspecific sticking of fluorophore alkynes	Poor blocking reagent is often the main reason for probe sticking. Test different batches of the blocking reagents to compare their efficiency. Use casein instead of BSA when possible
	Cell toxicity	Toxicity from the CuAAC step	Reduce copper concentration and labeling time, and compensate for loss in labeling yield by increasing the dye-alkyne concentration. Ensure that the supplements that preserve cell health (sodium ascorbate, TEMPOL and bathocuproin sulfonate) are good. Sodium ascorbate and TEMPOL solutions should be freshly made

● TIMING

Step 1A (module A), synthesis of 6-hydroxymethyl-nicotinic acid methyl ester **2**: 6 h
Step 1B (module B), synthesis of methyl 6-(azidomethyl)nicotinate, intermediate **3**: 32 h
Step 1C (module C), synthesis of 6-azidomethylnicotinic acid **4**: 4 h
Step 1D (module D), synthesis of pAz **5**: 12 h
Step 1E (module E), synthesis of pAz-AM ester **6**: 5 h
Step 2A, pAz ligation of cell-surface proteins using exogenous ^{W37V}LplA: 2 d (i,ii); 15 min–1 h (iii,iv)
Step 2B, pAz ligation of cell-surface proteins using ^{AILR}LplA-ER: 2 d (i); 1.5–4.5 h (ii,iii)
Step 3A, pAz derivatization on live cells using CuAAC: 10–20 min
Step 3B, pAz derivatization in fixed cells using CuAAC: 1.5–3 h
Box 1, ^{W37V}LplA expression and purification: 4–5 d
Box 2, Labeling of purified proteins with pAz and CuAAC: 3–4 h
Box 3, (optional) cell fixation: 1 h

ANTICIPATED RESULTS

Analytical data

6-Hydroxymethyl-nicotinic acid methyl ester, intermediate 2

^{1}H NMR (500 MHz, $CDCl_3$): 9.16, (d, 1H, J = 2.0 Hz), 8.29 (dd, 1H, J = 2.0, 8.5 Hz), 7.36 (d, 1H, J = 8.5 Hz), 4.83 (s, 2H), 3.96 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): 165.7, 163.7, 149.9, 138.3, 125.2, 120.4, 64.4, 52.7. HR-ESI-MS: $[M+H]^+$ m/z 168.0655 calculated, 168.0655 observed.

Methyl 6-(azidomethyl)nicotinate, intermediate 3

^{1}H NMR (500 MHz, $CDCl_3$): 9.18 (d, 1H, J = 2.0 Hz), 8.32 (dd, 1H, J = 8.5, 2.0 Hz), 7.44 (d,1H, J = 8.5 Hz), 4.56 (s, 2H), 3.95 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 165.7, 160.3, 151.6, 138.4, 125.5, 121.6, 55.7, 52.7. HR-ESI-MS: $[M+H]^+$ m/z 193.0726 calculated, 193.0733 observed.

6-Azidomethylnicotinic acid, intermediate 4

^{1}H NMR (400 MHz, CD_3OD): 9.10 (dd, J = 2.1, 0.8 Hz), 8.39 (dd, 1H, J = 8.1, 2.1 Hz), 7.57 (dd, 1H, J = 8.1, 0.8 Hz), 4.59 (s, 2H). ^{13}C NMR (100 MHz, CD_3OD): 167.7, 161.3, 151.5, 139.9, 127.6, 123.3, 56.0. HR-ESI-MS: $[M+H]^+$ m/z 179.0569 calculated, 179.0563 observed.

pAz 5, (5-(6-(azidomethyl)nicotinamido)pentanoic acid)

^{1}H NMR (500 MHz, D_2O): 8.83 (s, 1H), 8.18 (d, 1H, J = 8.5 Hz), 7.59 (d, 1H, J = 8 Hz), 4.62 (s, 2H), 3.42 (m, 2H), 2.32 (m, 2H), 1.65 (m, 4H). ^{13}C NMR (100 MHz, CD_3OD): 177.7, 167.6, 160.0, 149.2, 137.7, 131.1, 123.3, 55.9, 40.7, 34.7, 29.8, 23.5. HR-ESI-MS: $[M+H]^+$ m/z 278.1248 calculated, 278.1264 observed.

pAz-AM ester 6, (AM 5-(6-(azidomethyl)pyridine-3-carboxamido)pentanoate)

^{1}H NMR (400 MHz, $CDCl_3$) 8.95 (d, 1H, J = 1.7 Hz), 8.14 (dd, 1H, J = 2.3, 8.1 Hz), 7.43 (d, 1H, J = 8.4), 5.72 (s, 2H), 4.54 (s, 2H), 3.47 (m, 2H), 2.43 (t, 2H, J = 6.8), 2.09 (s, 3H), 1.70 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): 172.5, 169.9, 165.4, 158.8, 147.9, 136.4, 129.7, 121.8, 79.5, 55.5, 39.8, 33.5, 28.9, 21.7, 21.0. HR-ESI-MS: $[M+H]^+$ m/z 350.1459 calculated, 350.1449 observed.

LplA expression

The typical yield of ^{W37V}LplA from a 500-ml culture of bacteria is 20–40 mg of protein.

Cell-surface protein labeling

Typical labeling results with negative controls for pAz ligation and CuAAC derivatization of cell-surface proteins in neurons are shown in **Figure 3a**. A comparison of labeling signal obtained from pAz ligation using exogenous LplA versus ER-pAz ligation is shown in **Figure 3b**. A comparison of labeling signal obtained from live-cell CuAAC versus fixed-cell CuAAC is shown in **Figure 4**. The overall cell-surface labeling yield from pAz ligation with exogenous ^{W37V}LplA followed by live-cell CuAAC is shown in **Supplementary Figure 1**.

In vitro protein labeling

The specificity of pAz and CuAAC labeling on LAP-tagged kinesin is shown in **Figure 5**. By using the labeling protocols provided in **Box 2**, we have obtained a dye-to-protein ratio of ~0.7 on LAP-kinesin with AF647-alkyne.

Note: Supplementary information is available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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Corrigendum: Site-specific protein labeling using PRIME and chelation-assisted click chemistry

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In the version of this article initially published, Katharine A. White, Scott Grecian, Scott Clarke and Kyle R. Gee were not present in the author list. The error has been corrected in the HTML and PDF versions of the article. The author contributions section and competing financial interests statement have also been modified to include information relevant to these authors.