nature biotechnology

Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes

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Live cell imaging is a powerful method to study protein dynamics at the cell surface, but conventional imaging probes are bulky, or interfere with protein function[1,2], or dissociate from proteins after internalization[3,4]. Here, we report technology for covalent, specific tagging of cellular proteins with chemical probes. Through rational design, we redirected a microbial lipoic acid ligase (LplA)[5] to specifically attach an alkyl azide onto an engineered LplA acceptor peptide (LAP). The alkyl azide was then selectively derivatized with cyclo-octyne[6] conjugates to various probes. We labeled LAP fusion proteins expressed in living mammalian cells with Cy3, Alexa Fluor 568 and biotin. We also combined LplA labeling with our previous biotin ligase labeling[7,8], to simultaneously image the dynamics of two different receptors, coexpressed in the same cell. Our methodology should provide general access to biochemical and imaging studies of cell surface proteins, using small fluorophores introduced via a short peptide tag.

Fluorescent labeling of cell surface proteins enables imaging of the trafficking and function of receptors, channels and transporters. Many protein labeling methods have been developed in recent years[9], but none currently allows the covalent attachment of small fluorophores onto cell surface proteins modified only by a small peptide tag, with short labeling times and with extremely high specificity over a wide range of expression levels and labeling conditions. To address this shortcoming, we developed a protein labeling technique based on the *Escherichia coli* enzyme LplA[5]. In *E. coli*, LplA catalyzes the ATP-dependent covalent ligation of lipoic acid to one of three proteins involved in oxidative metabolism (E2p, E2o and H-protein[5]) (**Fig. 1a**, top). LplA naturally exhibits extremely high sequence-specificity, but previous work showing that the enzyme accepts octanoic acid, 6-thio-octanoic acid and selenolipoic acid in place of lipoic acid[5] suggests that the small-molecule binding site has considerable plasticity. To harness LplA for fluorescent labeling, we reengineered the system in three stages. First, through synthesis and testing of ten different substrate analogs, we discovered an alkyl azide substrate that can be efficiently used by wild-type LplA in place of lipoic acid. Once ligated to the target protein, the azide functional group can be selectively derivatized with any fluorescent probe conjugated to a cyclo-octyne reaction partner[6] (**Fig. 1a**). Second, to create a minimally invasive tag to direct the ligation of the alkyl azide, we engineered, through iterative cycles of rational design, a 22-amino-acid replacement for LplA's natural protein substrates, which can be fused to the N or C terminus of any protein of interest. Third, we tested the specificity of LplA in mammalian cells and found no background labeling of endogenous proteins.

For the first stage of LplA engineering, we considered a range of small-molecule structures to replace lipoic acid. Direct ligation of a fluorophore would offer a simpler and shorter labeling procedure, but incorporation of a 'functional group handle' is more feasible due to the small size of the lipoate binding pocket, and it provides greater versatility for subsequent incorporation of probes with diverse structures. Many functional group handles have been used in chemical biology, including ketones, organic azides and alkynes[10]. Organic azides are the most suitable for live cell applications, because the azide group is both abiotic and nontoxic in animals and can be selectively derivatized under physiological conditions (without any added metals or cofactors) with cyclo-octynes, which are also unnatural[6]. To test if LplA could accept an azide substrate in place of lipoic acid, we synthesized a panel of alkyl azide carboxylic acids of varying lengths (**Supplementary Fig. 1** and **Supplementary Methods** online), and tested them for ligation onto a 9-kDa lipoyl domain derived from the full-length E2p protein[11], using a high-performance liquid chromatography (HPLC) assay. We also synthesized a series of alkyne carboxylic acids as additional probes of the lipoate binding pocket (**Supplementary Fig. 1** online). All probes were incorporated by LplA to some degree, but the efficiency of ligation exhibited a clear length-dependence, with azide 7 giving the fastest kinetics (**Fig. 1b**). **Figure 1c** shows the HPLC trace associated with azide 7 ligation to E2p, in addition to negative control reactions with LplA or ATP omitted. We collected the product peak (starred) from the top trace and analyzed it by mass spectrometry, which confirmed that one molecule of azide 7 had been site-specifically conjugated to E2p (**Supplementary Fig. 2** online). We also measured the kinetics of azide 7 ligation to E2p (**Supplementary Fig. 2** and **Supplementary Methods** online), and compared the values to those of lipoic acid ligation. The k_{cat} values were only slightly different ($0.111 \pm 0.003\ s^{-1}$ versus $0.253 \pm 0.003\ s^{-1}$) but the K_m increased 75- or 30-fold for azide

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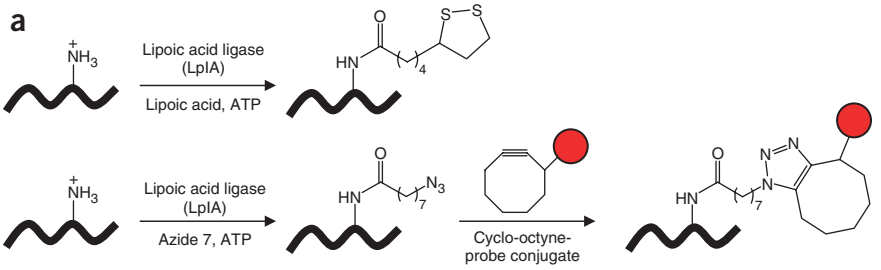
Received 5 July; accepted 23 October; published online 2 December 2007; doi:10.1038/nbt1355



b

Substrate		% Conversion
Lipoic acid		100 ± 3
Azide	$n = 5$	14.9 ± 0.6
	$n = 6$	11.3 ± 0.4
	$n = 7$	55.4 ± 5.7
	$n = 8$	10.2 ± 0.4
	$n = 9$	4.57 ± 0.11
	$n = 10$	3.28 ± 0.17
Alkyne	$n = 4$	3.59 ± 0.23
	$n = 5$	3.15 ± 0.19
	$n = 7$	6.37 ± 0.15
	$n = 8$	5.15 ± 0.23

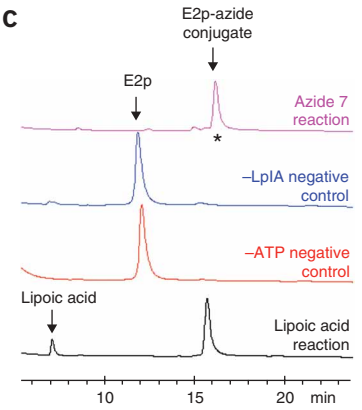


Figure 1 Redirecting LplA for site-specific protein labeling with fluorescent probes. (**a**) Natural reaction catalyzed by LplA (top), and scheme for LplA-catalyzed fluorescent tagging in cells (bottom). Instead of lipoic acid, LplA ligates an alkyl azide to a lysine side-chain within a peptide recognition sequence. The azide is then selectively functionalized with a cyclo-octyne conjugate to a probe of interest (red circle), to give a triazole adduct. (**b**) Comparison of alkyl azide and alkyne substrates of LplA. Conversions are given relative to lipoic acid, which is normalized to 100%. (**c**) HPLC assay showing the ligation of the azide 7 substrate to E2p protein. The starred peak was analyzed by mass spectrometry (**Supplementary Fig. 2**).

7 (127 ± 11 μM) compared to lipoic acid (1.7 μM[5] or 4.5 μM[12]). As seen below, it is straightforward and nontoxic to provide azide 7 at concentrations >127 μM for live cell labeling, thus maximizing the rate of ligation.

For the second stage of engineering, we wished to design a peptide substrate for LplA to replace the known protein substrates. It was necessary for the peptide to be fully transposable (recognized when fused to the N- or C-terminal ends of any protein) and to be modified by LplA with similar efficiency to the natural protein substrates. We accomplished this through multiple rounds of rational design (**Supplementary Fig. 3** online). A major challenge was that E2p presents the lysine modification site at the tip of a sharp hairpin turn[13], a conformation that is difficult to recapitulate in a peptide. Nevertheless, we designed an initial panel of seven peptides by analyzing lipoate acceptor proteins from different species, as well as structurally related biotin acceptor proteins. Peptides that were active in the initial screen were then improved through site-directed mutagenesis and tested for recognition at either terminus of a model protein. The final 22-amino-acid sequence, called the LplA acceptor peptide (LAP), had a k_{cat} of 0.048 ± 0.001 s^{-1} (data not shown), only 2.3-fold lower than the corresponding k_{cat} for full-length E2p (**Supplementary Fig. 2** online).

Our third task was to assess the specificity of LplA in mammalian cells. To do this, we created a LAP fusion to cyan fluorescent protein (CFP), and expressed it in human embryonic kidney (HEK) cells. HEK lysates were then labeled with LplA, azide 7 and ATP, and the ligated azide was detected by western blot analysis, after functionalization with a FLAG peptide through the Staudinger ligation[14]. In the presence of thousands of mammalian proteins in the cell lysate, only LAP-CFP is labeled by LplA (**Fig. 2**). The expression level of LAP-CFP is so low that it cannot be seen above that of endogenous proteins in the Coomassie-stained gel. Negative controls with LplA replaced by a catalytically inactive mutant, or LAP-CFP replaced by an alanine point mutant at the lysine modification site, show that labeling depends on the presence of LplA and an intact LAP sequence. This experiment and the live cell labeling experiments described below demonstrate that LplA is a remarkably specific enzyme at the cell surface, and possibly within the cytosol as well.

To test in live cells our newly engineered small-molecule and peptide substrates for LplA, we first created an artificial construct by fusing LAP to CFP, and then fusing this in turn to the extracellular side of the transmembrane (TM) domain of the platelet-derived growth factor receptor (**Fig. 3**, top). We also synthesized conjugates of our previously reported mono-fluorinated cyclo-octyne[6] (OCT) to two bright, red-emitting and membrane-impermeant fluorophores, Alexa Fluor 568 and Cy3 (**Supplementary Fig. 4** and **Supplementary Methods** online).

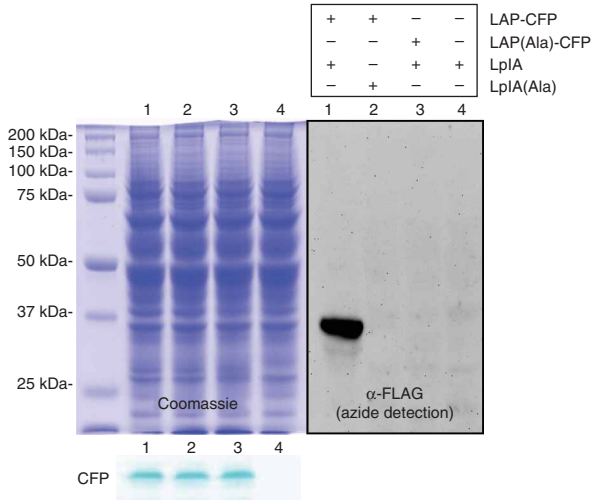


Figure 2 LplA labels the LAP peptide without modifying endogenous mammalian proteins. Lysates from HEK cells expressing a LAP fusion to CFP were labeled *in vitro* with LplA and azide 7. The azide was derivatized with phosphine-FLAG by the Staudinger ligation[14], and the FLAG epitope was detected by blotting with an anti-FLAG antibody. Controls are shown with LAP-CFP replaced by its alanine point mutant (lane 3), or with LplA replaced by its catalytically inactive Lys133Ala mutant (lane 2). Coomassie staining demonstrates equal loading in all lanes. Fluorescence visualization of CFP demonstrates equal expression levels of the LAP fusion in lanes 1–3.

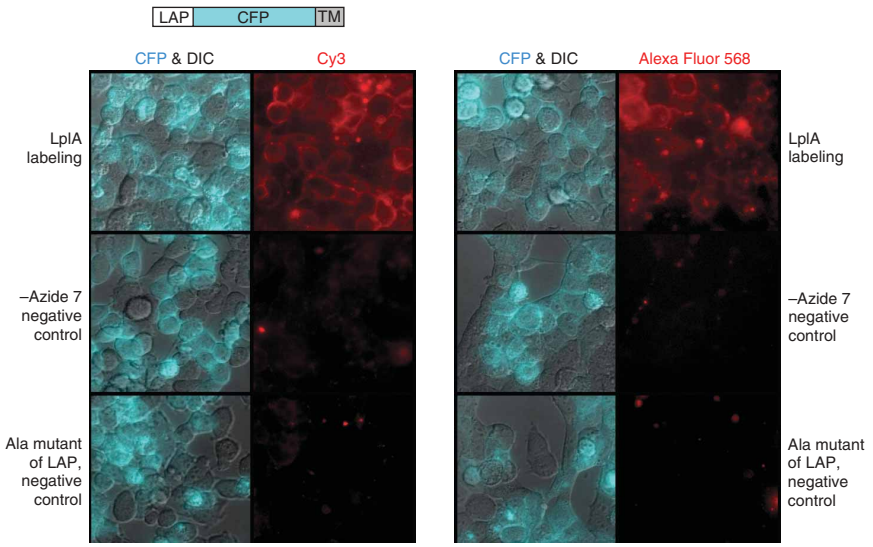


Figure 3 Site-specific labeling of LAP fusion proteins with fluorophores. A LAP-CFP fusion was targeted to the cell surface using a transmembrane (TM) domain. Cell-surface LAP was first labeled with azide 7 by LplA, and the azide was then derivatized with a cyclo-octyne probe conjugated to Cy3 (left) or Alexa Fluor 568 (right). Live cell images of the introduced fluorophores are shown to the right of the merged CFP and DIC images, which highlight the transfected cells. Negative controls with azide 7 omitted from the labeling reaction, or with the LAP-CFP-TM replaced by its alanine point mutant are shown.

Labeling was accomplished by expressing LAP-CFP-TM in HEK cells, and adding 350 μM azide 7 in the presence of LplA for 1 h, followed by one of the fluorophore-OCT conjugates for 20 min. Transfected cells (indicated by CFP fluorescence) were labeled with Alexa Fluor 568 or Cy3, whereas neighboring untransfected cells in the same field of view were not labeled (**Fig. 3**). Interestingly, labeling with Alexa Fluor 568 generated higher background than Cy3 labeling, because of faster nonspecific internalization of the probe. We performed additional negative controls with omission of azide 7 or replacement of LAP-CFP-TM by its alanine mutant, and observed no labeling in either case. Unlike sodium azide, organic azides, such as the clinically approved drug AZT[15], are not known to be toxic to cells, but we nevertheless examined the effect of 24-h exposure to azide 7 on mitochondrial respiration, and found no effect at concentrations <750 μM (data not shown).

We also compared the speed, sensitivity and specificity of LplA labeling to two other previously described peptide-based labeling methods (**Supplementary Fig. 5** online). Biotin ligase (BirA)/ketone tagging makes use of a ketone isostere of biotin, which can be functionalized with hydrazide conjugates after enzymatic ligation to proteins fused to a 15-amino-acid 'acceptor peptide' (AP)[16]. Transglutaminase labeling involves enzyme-mediated attachment of cadaverine-functionalized fluorophores to a glutamine-containing peptide recognition sequence[17]. For the comparison experiments, we used LplA to label LAP-CFP-TM with azide 7, followed by OCT-biotin, and followed by streptavidin-Alexa Fluor 568 to detect the biotin (**Supplementary Fig. 5** and **Supplementary Methods** online). A total labeling time of only 20 min was required for all three steps, and we obtained a signal-to-background ratio $\geq$3:1. In contrast, BirA/ketone labeling of an analogous AP-CFP-TM construct with a biotin-hydrazide compound followed by streptavidin detection required 2 h and 15 min to achieve a similar signal-to-background ratio. We also quantified the sensitivity of LplA labeling using the wedge method[18] and determined that cells expressing as little as 5 μM LAP-CFP-TM could be specifically labeled with OCT-biotin, with a signal-to-background ratio $\geq$3:1 (**Supplementary Methods**). Similar experiments demonstrated that LplA was also superior to transglutaminase, particularly in labeling specificity at higher enzyme concentrations (**Supplementary Fig. 5** online).

To illustrate the use of LplA labeling for imaging biological receptors, we created a LAP fusion to the low-density lipoprotein receptor (LDLR), which functions in the uptake of cholesterol in peripheral tissues of the body[19]. We established that LAP-LDLR could be labeled with OCT-Cy3 or OCT-biotin in HEK cells, even when expressed at levels matching those of endogenous LDLRs (data not shown). For many imaging studies, it is desirable to visualize two different receptors at once in the same cell to compare their distributions or trafficking patterns. To develop this capability, we investigated the compatibility of LplA labeling with BirA/streptavidin targeting. Unlike BirA/ketone labeling, BirA/streptavidin targeting[7,8] makes use of site-specific biotin ligation onto AP-tagged proteins, followed by recognition with streptavidin-fluorophore conjugates. Whereas the use of streptavidin increases the total size of the label, the femtomolar affinity of the biotin-streptavidin interaction makes this labeling approach much faster and much more sensitive than BirA/ketone labeling[7].

E. coli LplA and biotin ligase are mechanistically related, and their natural acceptor proteins share some structural and sequence overlap[20]. However, the engineered LAP and AP sequences are dissimilar, as are the azide 7 and biotin structures. To test the orthogonality of these two labeling methods, we prepared separate dishes of HEK cells expressing LAP-LDLR (with a GFP tag to serve as a transfection marker), or AP-EGFR (AP fused to the extracellular N terminus of the epidermal growth factor (EGF) receptor[16]) together with a CFP transfection marker (**Supplementary Methods**). After 16–24 h of expression, the cells were replated together in a single dish. We performed labeling by first adding a mixture of LplA, BirA, azide 7, biotin and ATP to the cells. Thereafter, OCT-Cy3 was added to derivatize the azide, and streptavidin was added to detect the biotin. Cells expressing LAP-LDLR were selectively labeled with Cy3, whereas cells expressing AP-EGFR were selectively labeled with streptavidin (**Supplementary Fig. 6** online). The same results were obtained using LAP-LDLR in combination with an AP-tagged receptor for ephrinA3 (AP-EphA3). Thus, simultaneous labeling of cells with LplA and BirA is possible, without sacrificing the high specificity of each system.

We then used this two-color labeling protocol to image LAP- and AP-fused receptors coexpressed within the same cell. EGFR and EphA3 are both known to function in cell migration[21,22], and thus we carried out imaging on cells migrating toward an artificial wound. HEK cells were cotransfected with either LAP-LDLR and AP-EGFR, or LAP-LDLR and AP-EphA3. After 16–24 h of expression, the confluent cells were wounded with a pipette tip. We allowed the wound to partially close over 12–18 h, and then performed simultaneous labeling with Cy3 and Alexa Fluor 488 conjugated to monovalent streptavidin[8]. Cy3-labeled LDLR was evenly distributed on the surface of the HEK cells, whereas Alexa Fluor 488-labeled EGFR and EphA3

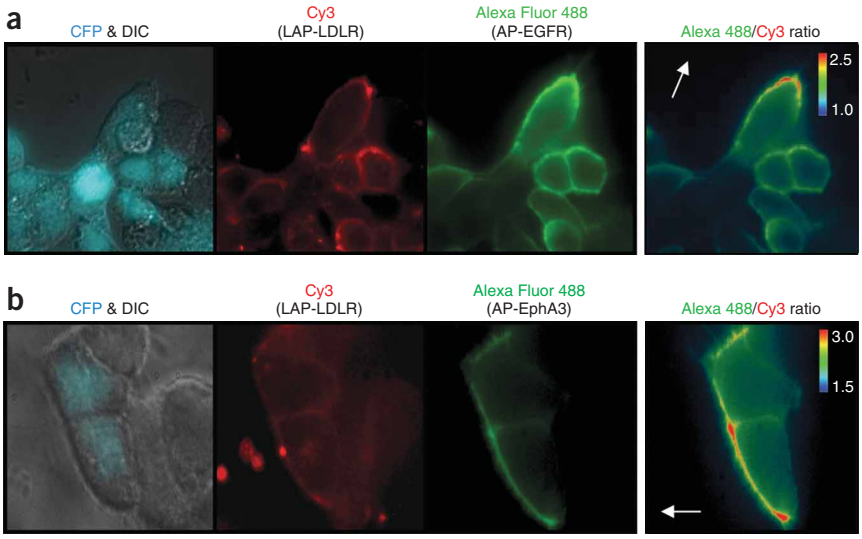


Figure 4 Simultaneous labeling and imaging of two receptors in polarized cells in a wound healing assay. (**a**,**b**) HEK cells coexpressing a LAP-LDLR fusion and either AP-EGFR (**a**) or AP-EphA3 (**b**) were labeled during wound healing by first treating with LplA, BirA, azide 7 and biotin, followed by OCT-Cy3 to derivatize the azide, followed by monovalent streptavidin-Alexa Fluor 488 (ref. 8) to detect the biotin. The Cy3 images show the nonpolarized distribution of surface LAP-LDLR. The Alexa Fluor 488 images show the polarized distribution of AP-EGFR (**a**) and AP-EphA3 (**b**) at the wound edge. CFP is a transfection marker. The images on the far right depict the intensity ratios of Alexa Fluor 488 and Cy3. The white arrows point toward the wound.

were both asymmetrically concentrated at the leading edge of the polarized cells (**Fig. 4**). The same patterns were also observed when the LAP and AP tags were swapped (AP-LDLR and LAP-EGFR), suggesting that the localization patterns do not reflect artifacts of AP and LAP tagging (data not shown).

Whereas the polarization of AP-EGFR to the leading edge of migrating cells is an expected result, and has previously been observed using antibody labeling[23], the pattern of AP-EphA3 staining is surprising. Previous work has shown that *trans* interactions between EphA3 and ephrin ligand expressed on the surface of contacting cells play a role in developmental cell migration[24] and tumor invasion[25]. However, it is unclear what role unliganded EphA3 should play in migratory processes. Our observation of EphA3 accumulation at the free, leading edge of polarized cells suggests that unactivated EphA3 may function in cell signaling, or that EphA3 may be constitutively linked to the actin cytoskeleton.

In summary, we have developed a technology for labeling cell surface proteins fused to a 22-amino-acid recognition sequence for *E. coli* LplA. Small, non-cross-linking probes such as Cy3, Alexa Fluor and biotin can be conjugated site-specifically and covalently to the LAP peptide in as little as 20 min. An important feature of our technique is its generality; potentially a wide variety of cell surface proteins in diverse cell types can be labeled with a wide range of chemical probes linked to cyclo-octynes.

Many new protein labeling methods have been developed in recent years[9], and a survey of these techniques reveals that a general trade-off exists between labeling specificity and tag size. Protein-based tags, such as SNAP/AGT[26] are generally more specific than peptide tags, such as FlAsH[27]. However, protein tags have greater potential to interfere with protein folding, trafficking and activity, as GFP often does[28,29]. We and others (for instance, with Sfp/AcpS labeling methodology[30]) have tried to bridge the requirements of small tag size and high labeling specificity, by making use of enzyme ligases. By capitalizing on the intrinsic sequence-specificity of enzymes such as biotin ligase and LplA, we can achieve highly specific probe conjugation, without sacrificing the small size of the directing tag.

In previous work with BirA, a ketone isostere of biotin could be accepted[16], but compounds with more dissimilar structures, such as alkyne and azide derivatives of biotin, were not recognized by BirA due to structural requirements of the biotin binding pocket. In contrast, LplA exhibits much more relaxed specificity for its small-molecule substrate, while maintaining extremely high specificity for its protein or peptide substrate[5]. This property allows us to harness LplA for unnatural ligation reactions. The next important challenges will be to extend this technology to labeling of intracellular protein targets, and to reengineer LplA for one-step ligation of fluorophore or photoaffinity probes.

We also used LplA in combination with biotin ligase to differentially label two receptors expressed in the same cell. Many problems in receptor biology would benefit from simultaneous imaging of two or more different proteins in the same living cell, instead of separate experiments involving one-color labeling of each receptor. The combination of LplA and BirA tagging, which can be performed simultaneously due to the orthogonality of the reaction components, provides access to such experiments.

METHODS

***In vitro* LplA activity assays.** LplA reactions contained 2 μM LplA, 200 μM E2p, 350 μM probe, 1 mM ATP, 2 mM magnesium acetate and 25 mM sodium phosphate pH 7.0. Reactions were incubated at 30 °C for 30 min, and then quenched with EDTA (final concentration 50 mM). Conversion to product was determined by HPLC on a C_{18} reverse-phase column with a 40–57% gradient of acetonitrile in water with 0.1% trifluoroacetic acid over 20 min (flow rate 1.0 ml/min). Unmodified E2p had a retention time of ~12 min, whereas E2p-probe conjugates eluted at 15–18 min. Percent conversion to product was calculated from the ratio of the E2p-probe peak area to the sum of (E2p + E2p-probe) peak areas. All measurements were performed in triplicate.

LplA specificity test on mammalian lysate. Human embryonic kidney (HEK) 293T cells were transfected with LAP-CFP-pcDNA3 plasmid using Lipofectamine 2000 (1 μg DNA/well of a 6-well plate). Lysates were generated 48 h later by hypotonic lysis to minimize protease release, as follows. Cells were lifted from the plates, concentrated by centrifugation and resuspended in 1 mM HEPES pH 7.5, 5 mM magnesium chloride, 1 mM phenylmethylsulphonyl fluoride and protease inhibitor cocktail (Calbiochem). After incubation at 4 °C for 10 min, the cells were lysed by vigorous vortexing for 2 min at 21 °C. Crude lysate was clarified by centrifugation, and stored at −80 °C. Lysate was labeled by incubating at 30 °C for 10 h with 25 mM sodium phosphate pH 7.0, 1 μM LplA, 250 μM azide 7, 1 mM ATP and 4 mM magnesium acetate. Thereafter, Staudinger ligation was performed by adding FLAG-phosphine[14] to a final concentration of 500 μM, and incubating at 30 °C for 16 h. Each reaction sample was then divided into thirds. The first third was analyzed by 12% SDS-PAGE followed by western blotting with anti-FLAG(M2)-peroxidase antibody conjugate (Sigma, 1:1,000 dilution). The second sample was analyzed by 12% SDS-PAGE followed by Coomassie staining. The last third was analyzed by 12% SDS-PAGE without boiling the samples, to prevent unfolding of CFP, and in-gel fluorescence was visualized on a Storm 860 instrument (Amersham).

Live cell labeling with fluorescent probes. HEK 293T cells were transfected with the LAP-CFP-TM expression plasmid using Lipofectamine 2000. After 36–48 h at 37 °C, the cells were washed twice with fresh growth medium (Dulbecco's Modified Eagle's Medium with 10% FBS and 1% penicillin/streptomycin). Enzymatic ligation of azide 7 was performed in complete growth medium with 10 μM LplA, 350 μM azide 7, 1 mM ATP and 5 mM magnesium acetate for 60 min at 32 °C. Cells were rinsed three times with growth medium, and incubated for 20 min at 21 °C with 200–400 μM OCT-Cy3 or 100–200 μM OCT-Alexa Fluor 568. Thereafter, the cells were washed once with growth medium, twice with a 1% BSA solution in Dulbecco's Phosphate-Buffered Saline (DPBS) pH 7.4, and twice more with DPBS alone. Labeled cells were imaged in the same buffer on a Zeiss Axiovert 200M inverted epifluorescence microscope using a 40× oil-immersion lens. CFP (420/20 excitation, 450 dichroic, 475/40 emission), Cy3 and Alexa Fluor 568 (560/20 excitation, 585 dichroic, 605/30 emission) and differential interference contrast (DIC) images (630/10 emission) were collected and analyzed using Slidebook software (Intelligent Imaging Innovations). Fluorescence images were normalized to the same intensity range. Acquisition times ranged from 10–250 ms.

Two-color live cell labeling with LplA and biotin ligase. HEK 239T cells were cotransfected with the LAP-LDLR and AP-EGFR[16] plasmids in a 1:2 ratio, or with the LAP-LDLR and AP-EphA3 plasmids in a 2:1 ratio. The cells were wounded with a pipette tip 24 h after transfection and allowed to heal over 16–24 h. For labeling, cells were washed twice with complete growth medium, and then incubated with 5 μM BirA, 10 μM LplA, 50 μM biotin, 350 μM azide 7, 1 mM ATP and 5 mM magnesium acetate for 60 min at 32 °C. Cells were then rinsed three times with growth medium, and incubated for 20 min at 21 °C with 200–400 μM OCT-Cy3. Biotin was detected by staining with 50 μg/ml monovalent streptavidin-Alexa Fluor 488 (ref. 8) for 10 min at 4 °C. The cells were washed once with ice-cold 1% BSA in DPBS pH 7.4, then twice with ice-cold DPBS, before imaging in the same buffer using the configuration described above. The Alexa Fluor 488 filter set was 495/20 excitation, 515 dichroic, 530/30 emission.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS
The authors thank Mark Howarth, John Cronan, Irwin Chen, Chi-Wang Lin, Robin Prince and Martin Lackmann for their assistance and advice. This work was supported by the National Institutes of Health (R01 GM072670-01 to A.Y.T. and GM58867 to C.R.B.), the Sloan Foundation, the Dreyfus Foundation, a La Caixa Foundation predoctoral fellowship (to M.F.-S.), and National Science Foundation and National Science Defense and Engineering predoctoral fellowships (to J.M.B.).

AUTHOR CONTRIBUTIONS
M.F.-S., H.B., L.M.-H. and A.Y.T. designed the research; M.F.-S., H.B., L.M.-H. and K.T.X. performed the research; J.M.B. and C.R.B. provided cyclo-octyne starting material; M.F.-S., H.B. and A.Y.T. analyzed data; M.F.-S. and A.Y.T. wrote the paper.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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