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Site-Specific Protein Modification Using Lipoic Acid Ligase and Bis-Aryl Hydrazone Formation

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A screen of Trp37 mutants of *Escherichia coli* lipoic acid ligase (LplA) revealed enzymes capable of ligating an aryl-aldehyde or aryl-hydrazine substrate to LplA's 13-residue acceptor peptide. Once site-specifically attached to recombinant proteins fused to this peptide, aryl-aldehydes could be chemoselectively derivatized with hydrazine-probe conjugates, and aryl-hydrazines could be derivatized in an analogous manner with aldehyde-probe conjugates. Such two-step labeling was demonstrated for AlexaFluor568 targeting to monovalent streptavidin in vitro, and to neurexin-1β on the surface of living mammalian cells. To further highlight this technique, we labeled the low-density lipoprotein receptor on the surface of live cells with fluorescent phycoerythrin protein to allow single-molecule imaging and tracking over time.

Introduction

Chemoselective ligation chemistries are important tools for the detection, analysis, and perturbation of proteins in order to elucidate structure and function. For this reason, numerous biocompatible versions of such chemistries have been developed, including the Staudinger ligation,[1] copper-catalyzed 3+2 azide–alkyne cycloaddition,[2] strain-promoted azide–alkyne cycloaddition,[3] and native chemical ligation.[4] When these reactions are performed on proteins, the ability to conjugate in a site-specific manner can become crucial, because protein function is intimately linked to structure, and conjugation at the wrong site can reduce activity, block interactions with other proteins, or even cause structural destabilization. Therefore, these biocompatible ligation reactions have been augmented through combination with site-specific protein-labeling techniques. For example, our laboratory showed that the alkyl azide partner of the strain-promoted 3+2 cycloaddition reaction could be site-specifically introduced onto a peptide tag fused to recombinant cellular proteins through the action of *Escherichia coli* lipoic acid ligase (LplA).[5,6] Thereafter, the protein-linked alkyl azide could be chemoselectively derivatized with cyclooctyne–fluorophore conjugates. Similarly, unnatural amino acid mutagenesis has been used to introduce azides[7] and ketones[8] into proteins, for chemoselective derivatization by alkyne– and aminoxy–probe conjugates.

The goal of this work is to couple a powerful biocompatible ligation reaction, aniline-catalyzed bis-aryl hydrazone formation,[9] with our LplA-based site-specific protein-labeling methodology. Bis-aryl hydrazone formation between an aromatic aldehyde and a 6-hydrazinopyridyl moiety is one of the fastest chemoselective ligation reactions, with a rate constant in the range of 10^2 to $10^3\,M^{-1}s^{-1}$,[9] compared to 10^{-4} to $10^{-2}\,M^{-1}s^{-1}$ for the Staudinger ligation[1] and 10^{-3} to $1\,M^{-1}s^{-1}$ for strain-promoted azide–alkyne cycloaddition.[3,5] The resulting covalent adduct is highly stable at physiological pH, although, usefully, the linkage can be reversed when desired by the addition of aniline and hydroxylamine.[10] The formation kinetics of the bis-aryl hydrazone bond are 20 to 900 times higher than those of conventional hydrazone/oxime bonds between alkyl aldehydes/ketones and alkyl hydrazide or aminooxy probes.[9,11] Studies have shown that treatment with 10 mM aniline for 90 min (to catalyze oxime formation) is not toxic to living mammalian cells.[12] Despite all these attractive features, no methods have been described for the site-specific introduction of bis-aryl hydrazone reaction partners into full-length proteins, either in vitro or within complex environments such as cell lysates or living cells.

Results and Discussion

E. coli LplA catalyzes highly sequence-specific lipoic acid conjugation to a 13-residue recognition sequence called LAP (LplA acceptor peptide).[13] We have previously shown that mutation of the lipoic acid binding pocket can confer the ability to ligate a range of unnatural substrate structures, including 7-hydroxycoumarin,[14] an aryl azide photo-crosslinker,[15] and *trans*-cyclooctene.[16] To test if mutants of LplA could accept aryl aldehyde and aryl hydrazine substrates, we synthesized the two structures shown in Figure 1 A, in addition to analogues with one methylene fewer. These four substrates were screened against wild-type LplA and the seven mutants shown in Figure 1 B. We have previously found that W37, which is located at the end of the lipoic acid binding tunnel, acts as a "gatekeeper" residue, and its mutation allows LplA to accept substrates whose size and shape differ greatly from those of lipoic acid. We tested a small panel of W37 mutants that have previously shown activity for unnatural probe ligation.[14,16,17] No

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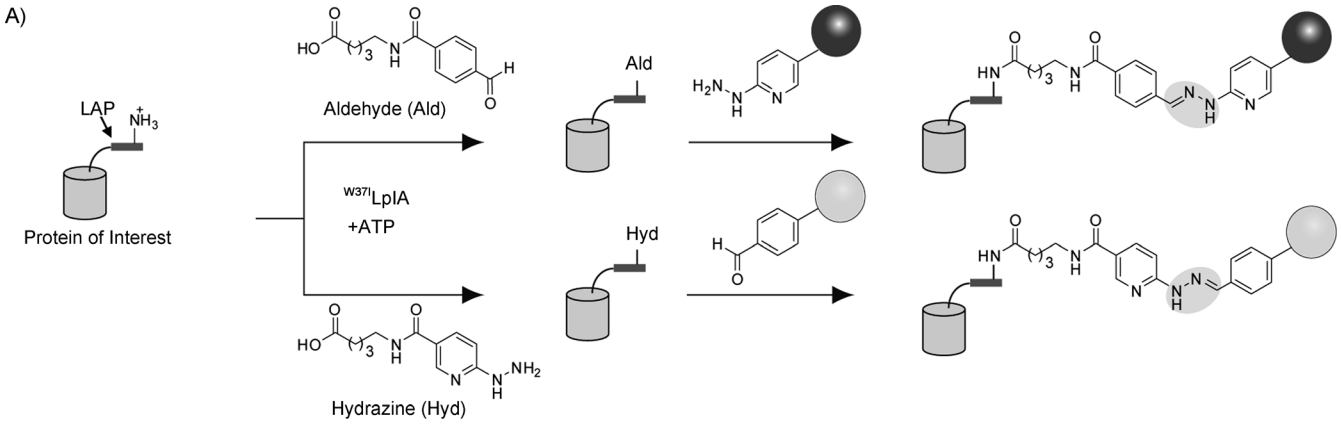


B)

Ald

Mutant	% Conversion
W37I	61.8 ± 11.3 *
W37V	56.8 ± 2.0 *
W37T	51.6 ± 3.0
W37L	19.9 ± 4.3
W37C	11.8 ± 6.2
W37A	2.8 ± 0.8
W37G	7.8 ± 1.5
WT	0.8 ± 0.3

10 minute reactions
* 5 minute reactions

Hyd

Mutant	% Conversion
W37I	49.9 ± 9.6
W37V	43.5 ± 0.5
W37T	33.1 ± 1.2
W37L	20.2 ± 0.6
W37C	9.9 ± 2.5
W37A	N.D.
W37G	1.0 ± 0.0
WT	0.9 ± 1.2

60 minute reactions

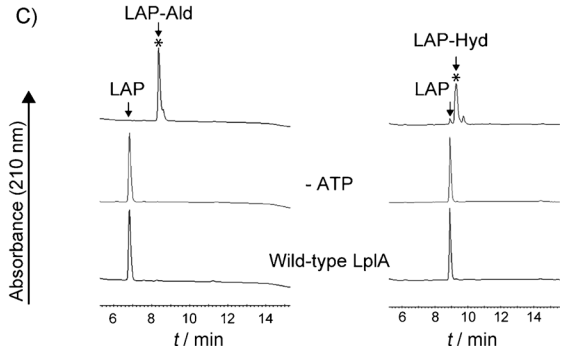


Figure 1. Screening for aldehyde and hydrazine ligases. A) Scheme for using LplA to conjugate an aldehyde or hydrazine functional group to LAP fusion proteins (LAP = GFEIDKVWYDLDA[13]). After ligation, the aldehyde or hydrazine moiety can be chemoselectively derivatized with fluorophores, photo-crosslinkers, or even proteins (represented by spheres). The linkage is a hydrazone (highlighted in gray) that is stabilized by conjugation to the neighboring aromatic rings. B) Wild-type (WT) LplA and seven W37 point mutants were screened against the aldehyde (Ald) and hydrazine (Hyd) substrates shown in (A), each at 500 μM. Percentage conversion of LAP peptide to the LAP–product conjugate is reported in the right columns. Data are represented as mean ± standard deviation. N.D., not detected. Representative traces are shown in Figure S1. C) HPLC traces showing complete ligation of Ald (left) and Hyd (right) to LAP catalyzed by ^{W37I}LplA with extended reaction times of 70 and 120 min, respectively. Negative controls are shown with ATP omitted (middle traces) and ^{W37I}LplA replaced by wild-type LplA (bottom traces). Starred peaks were analyzed by mass spectrometry in Figure S1.

activity was detected with any of the LplA mutants with the shorter aldehyde and hydrazine substrates (data not shown). However, the longer aryl aldehyde ("Ald") shown in Figure 1A was recognized and ligated to the LAP peptide by several of the W37 mutants, with ^{W37I}LplA having the highest activity (Figure 1B). When using 1 μM ^{W37I}LplA, 500 μM Ald probe, and 150 μM LAP peptide, the reaction proceeded to 62% completion in 5 min (Figure 1B).

We found that the aryl hydrazine ("Hyd") probe was also ligated by many of the LplA mutants, but not as efficiently as the aryl aldehyde. Interestingly, the relative activities of the W37 mutants for the Hyd probe were similar to those for the Ald probe, with ^{W37I}LplA again having the highest activity. However, the overall activity with the Hyd probe was lower than that for the Ald probe, reacting to 50% completion in 60 min. We determined the k_{cat} values for ^{W37I}LplA-catalyzed attachment of the Ald and Hyd probes to LAP peptide to be 0.33 ± 0.01 and 0.021 ± 0.003 s^{-1}, respectively (Supporting Information). Both ligations required ATP and could not be catalyzed by wild-type LplA (Figure 1C). The identities of the product peaks were confirmed by mass spectrometry (Figure S1 in the Supporting Information).

In vitro protein labeling through LplA and bis-aryl hydrazone coupling

We proceeded to test whether our LplA-mediated protein-tagging method could be used for the specific modification of proteins in vitro. We turned first to streptavidin, a protein used ubiquitously in biotechnology due to its extremely high affinity and specificity for the small molecule biotin. The ability to form site-specific conjugates of streptavidin to reporters such as fluorophores, enzymes (e.g., horseradish peroxidase, alkaline phosphatase) and phycoerthyrin could be extremely beneficial for enhancing activity and hence performance in applications ranging from ELISA and western blotting to live-cell imaging.

We prepared streptavidin protein displaying a single LAP tag by utilizing our previously described monovalent streptavidin technology.[18] Monovalent streptavidin is prepared by refolding one equivalent of wild-type streptavidin ("alive", A) with three equivalents of "dead" (non-biotin-binding, D) streptavidin (Figure 2A). The resulting mixture of heterotetramers is then purified by gradient nickel affinity chromatography to isolate the species with exactly one wild-type subunit and three dead subunits, that is, a single biotin binding site within a tetrameric

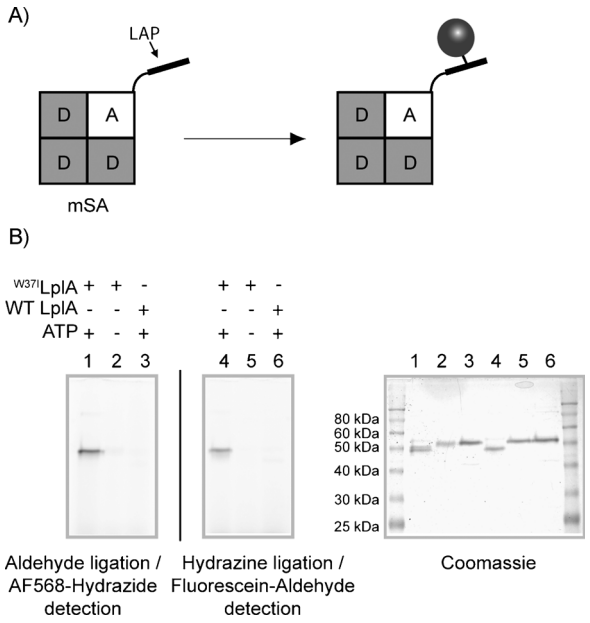


Figure 2. Site-specific fluorophore conjugation to monovalent streptavidin (mSA). A) Overview of site-specific labeling of mSA. mSA consists of three "dead" subunits and one "alive" subunit that has a single N-terminal LAP fusion. This allows site-specific conjugation of mSA to a single fluorophore (sphere). B) mSA with a single LAP tag was labeled with ^{W37I}LplA and 100 μM of either Ald substrate (lanes 1–3) or Hyd substrate (lanes 4–6). The conjugation reactions were analyzed by SDS-PAGE and in-gel fluorescence in which lanes 1–3 were derivatized with AlexaFluor568–Hyd, while lanes 4–6 were derivatized with fluorescein–Ald. Negative controls were performed with ATP omitted from the ligation reaction (lanes 2 and 5), or ^{W37I}LplA replaced by wild-type LplA (lanes 3 and 6). Coomassie staining of the same samples is shown at right. mSA–AlexaFluor568 conjugated as in lane 1 was used for cell-surface protein labeling in Figure S2.

protein. We genetically fused the 13-residue LAP tag to the N terminus of the wild-type subunit. Therefore, the resulting purified monovalent streptavidin (mSA) had a single LAP tag on the functional biotin-binding subunit of the tetrameric protein.

Labeling with ^{W37I}LplA was performed with either Ald or Hyd substrate for 1 hour. After labeling, the crude mixtures were combined with either AlexaFluor568–hydrazide (AF568–Hyd) or fluorescein-aldehyde to selectively derivatize Ald or Hyd, respectively. Reactions were performed overnight in the presence of 20 mM aniline catalyst at pH 5.0 and at room temperature. Figure 2B shows the specific conjugation of AF568–Hyd to Ald-functionalized mSA–LAP, and fluorescein-aldehyde to Hyd-functionalized mSA–LAP. Importantly, negative controls with ATP omitted from the first step, or wild-type LplA used in place of ^{W37I}LplA, showed no labeling.

To test if these site-specific mSA–LAP–fluorophore conjugates were active, we used them to label and image biotinylated cell-surface proteins. In Figure S2, human embryonic kidney (HEK) cells were transfected with plasmids for acceptor peptide (AP)-tagged low-density lipoprotein receptor (LDLR) and endoplasmic reticulum (ER)-targeted biotin ligase. Previous work has shown that such conditions result in site-specific biotinylation of the AP tag in the ER lumen by biotin ligase.[19] These cells were then treated with the mSA–LAP–AlexaFluor568 conjugate prepared as in Figure 2. Specific fluorescence labeling is seen on the surface of transfected cells expressing AP-LDLR and the nuclear yellow fluorescent protein (YFP) transfection marker. Labeling is not seen when the AP tag is mutated, excess biotin is added to quench mSA, or cells are not transfected. Hence Figure S2 demonstrates that the mSA–fluorophore conjugate prepared by LplA and bis-aryl hydrazone formation is functional for live-cell labeling and imaging.

To illustrate generality, we performed similar labeling of two other proteins. One was alkaline phosphatase, an enzyme frequently attached to antibodies and streptavidin and used to generate a chromogenic signal in ELISA assays. We prepared a LAP fusion to the N terminus of alkaline phosphatase, labeled with LplA and Ald, and then derivatized it with fluorescein-Hyd. Figure S3 shows that this labeling was effective and dependent on ATP. The second protein we labeled was E2p, a 9-kDa domain of pyruvate dehydrogenase, one of LplA's natural protein substrates in *E. coli*.[20] Figure S3 shows successful conjugation of fluorescein-Ald to Hyd-labeled E2p protein, as well as the reverse scheme.

A major benefit of the LplA protein labeling strategy is the exceptional sequence specificity of LplA. Hence, we explored the ability of our two-step labeling protocol to specifically conjugate fluorophores to LAP in complex mixtures containing thousands of competing proteins. Figure S4 shows a labeling experiment with a LAP–YFP fusion in the presence of mammalian cell lysate. AlexaFluor568 and fluorescein are conjugated to LAP–YFP only, and not to any endogenous mammalian proteins. Negative controls with LAP–YFP omitted or wild-type LplA in place of ^{W37I}LplA show no labeling.

Cell-surface protein labeling with LplA and bis-aryl hydrazone formation

We next tested our labeling protocol on living mammalian cells. This context tests both the specificity of our labeling scheme and its biocompatibility. We co-transfected HEK cells with expression plasmids for LAP4.2-neurexin-1β and a nuclear YFP transfection marker (Figure 3). Neurexin-1β is a transmembrane protein with an extracellular N terminus that functions in synaptic adhesion. LAP4.2 (sequence given in the Experimental Section)[13] is a less hydrophobic variant of LAP that frequently gives better surface targeting than the original LAP sequence. Labeling was performed with ^{W37I}LplA, ATP, and 100 μM Ald for 45 min at 37 °C. Reagents were washed away, and then 100 μM AF568–Hyd was added together with 10 mM aniline for 30 min at 4 °C. Cells were washed and immediately imaged. Figure 3 shows that cell-surface labeling was specific to transfected cells expressing LAP4.2-neurexin-1β. Negative controls with wild-type LplA, ATP omitted, or a LAP containing an alanine mutation showed no labeling.

Cell-surface protein labeling with phycoerythrin and single-molecule imaging

Single-molecule imaging is a powerful way to study protein trafficking in cells without losing information through ensem-

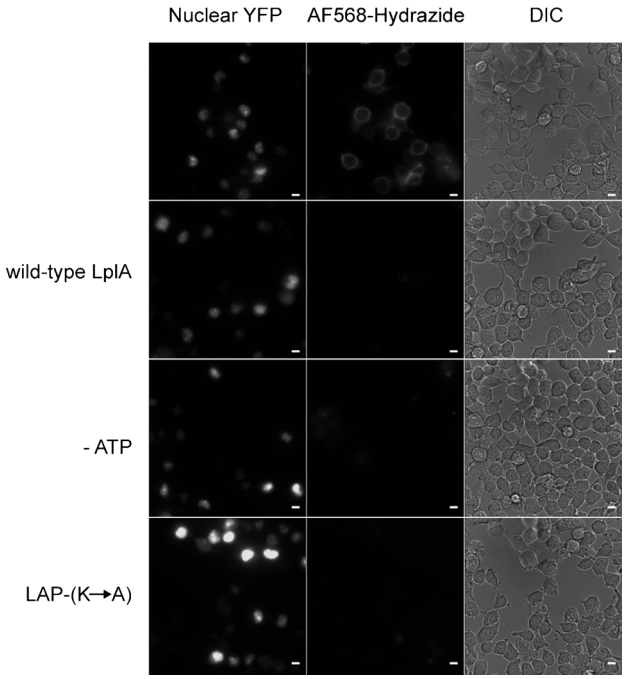


Figure 3. Fluorophore labeling of cell-surface proteins. HEK cells expressing LAP4.2–neurexin-1β were labeled with ^{W37I}LplA and Ald substrate for 45 min, washed, then treated with AlexaFluor568–Hyd in the presence of 10 mM aniline for 30 min, before imaging (top row). Nuclear YFP is a co-transfection marker. Negative controls were performed with wild-type LplA, ATP omitted, or an alanine mutation in LAP4.2. Scale bars = 10 μm.

ble averaging. Single-molecule imaging in the cellular context requires fluorophores that are exceptionally bright and photostable. Quantum dots have excellent photophysical properties, but commercial versions are very large and multivalent.[19] Small organic dyes such as the AlexaFluors and cyanine dyes are much dimmer and require intense illumination to achieve reasonable signal-to-noise ratios at the single-molecule level. Under these conditions, photobleaching occurs rapidly and prevents single-molecule tracking for longer than a few minutes or even seconds.[21]

For biotechnological applications requiring extreme fluorophore brightness, such as fluorescence-activated cell sorting (FACS), phycoerythrin has been used as a much brighter alternative to organic dyes and a smaller and less expensive alternative to QDs. R-phycoerythrin (PE) is a 240-kDa protein with a disk shape (11×6 nm), containing 34 embedded phycobilin-type chromophores. It is usually obtained by purification from red algae.[22] With an extinction coefficient (ε) of $2.0\times10^6\,M^{-1}cm^{-1}$ at 566 nm, and a quantum yield (QY) of 0.85, it is more than 25 times brighter than AlexaFluor568 ($\varepsilon = 91\,300\,M^{-1}cm^{-1}$ at 568 nm; QY = 0.69), which emits at the same wavelength.

PE has rarely been explored as a reagent for single-molecule imaging. Previously, Irvine et al. used PE for single-timepoint imaging of single peptide molecules binding to major histocompatibility complex (MHC) on the surface of antigen-presenting cells.[23] We wished to explore the use of our LplA method to target PE to specific cell-surface proteins, and to image them at the single-molecule level. As PE can only practically be added to cells at low-micromolar concentrations, it is essential that it be targeted by using a method with an extremely high second-order rate constant. For instance, calculations show that the yield would be $<1\%$ when using a targeting method with a rate constant of $\sim0.1\,M^{-1}s^{-1}$, such as azide–azadibenzocyclooctyne cycloaddition,[5] after 1 h of labeling. With its extremely fast kinetics and cell compatibility, the bis-aryl hydrazone conjugation is ideal for this application.

We prepared HEK cells expressing LAP4.2-neurexin-1β as in Figure 3, and labeled them with the Hyd probe by using ^{W37I}LplA. After labeling, cells were washed and treated with 20 mM aniline and PE modified with 4-formylbenzamide (PE-Ald). After 45 min, the cells were washed and imaged. As shown in Figure 4A, clear labeling was observed in transfected cells. No labeling was seen in negative controls (wild-type LplA, without ATP, or with an alanine mutation in LAP4.2).

To perform single-molecule imaging with PE, we prepared COS7 cells expressing LAP4.2–LDLR on their surfaces. LDLR is a constitutively internalized receptor that promotes the plasma clearance of LDL particles through clathrin-mediated endocytosis. A single-molecule imaging platform for LDLR based on our hydrazine-labeling technique could potentially provide insight into the mechanisms of LDLR targeting to clathrin-coated pits. We labeled the LDLR by using our Hyd probe, followed by treatment with 20 mM aniline and PE-Ald. As shown in Figure 4B, total internal reflection fluorescence (TIRF) microscopy reveals individual labeled LDLR molecules as single diffraction-limited spots on the cell surface. To confirm that the labeled spots were indeed single receptors and not aggregates, we compared the intensity distribution of >2900 spots on cells to individual PE molecules randomly distributed on glass slides. Similar distributions were seen (Figure S5). The labeled receptors are also dynamic, as shown in time-lapse experiments with images captured at a rate of 1 fps over 60 s (see Movie S1 in the Supporting Information). The brightness of the PE molecules offers high signal-to-background ratios unmatched by organic fluorophores, and photobleaching is reduced because of the lower laser intensity required for illumination.

Conclusion

In summary, LplA provides a general method for targeting small-molecule probes with extremely high specificity to proteins in vitro, in lysate, and in living cells. Bis-aryl hydrazone formation is an extremely fast and biocompatible ligation reaction. By combining these two technologies in this study, we have developed a method to prepare protein–small molecule and protein–protein conjugates with high specificity and great facility. We have demonstrated the methodology on monovalent streptavidin, alkaline phosphatase, YFP, LDL receptor, and neurexin-1β by preparing conjugates to AlexaFluor568, fluorescein, and the extremely bright fluorescent protein phycoerythrin.

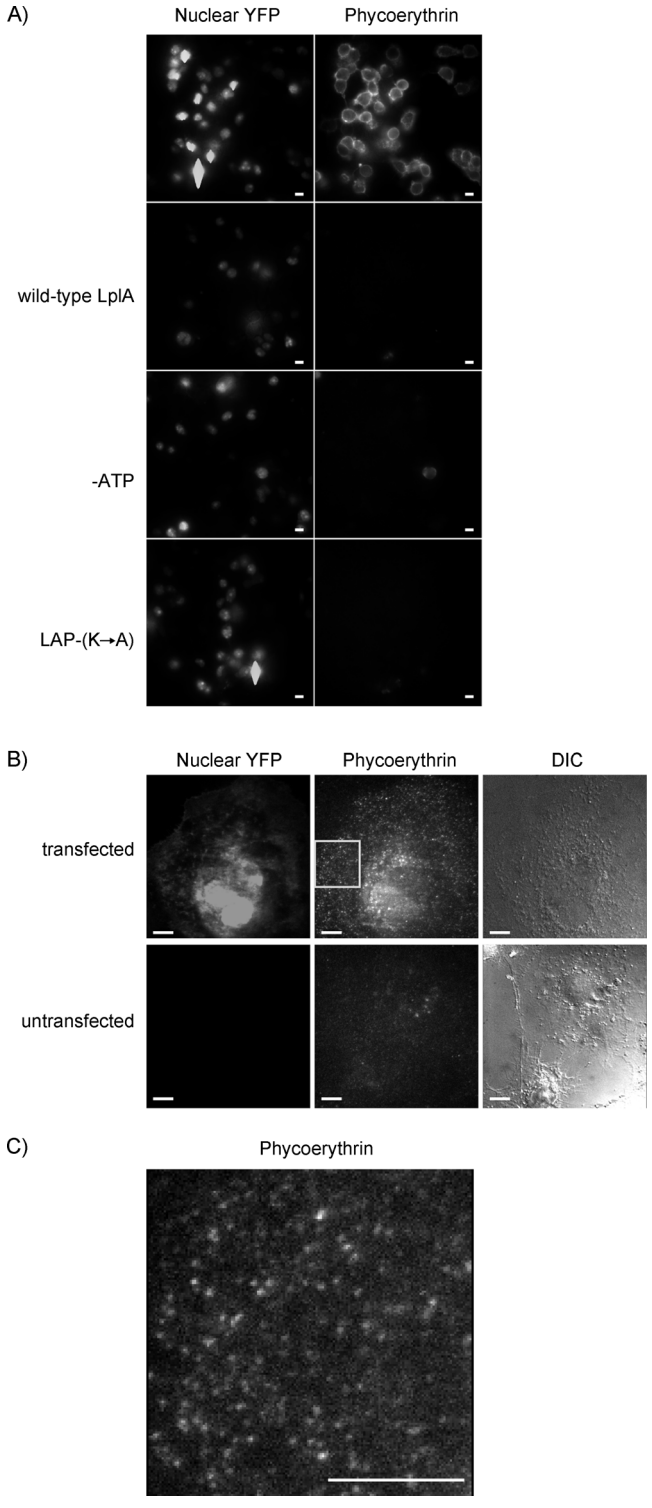
Presently, several methods exist to incorporate the reaction partners for conventional hydrazone/oxime formation, such as alkyl aldehydes by formylglycine generating enzyme (FGE)[24,25]

or ketones through unnatural amino acid mutagenesis.[8] In comparison to these methods, our LplA-based labeling takes advantage of the enhanced kinetics and stability of bis-aryl hydrazone formation, and we show that the same LplA mutant can target both the aryl aldehyde reaction partner and the hydrazinopyridine reaction partner.

Although we have demonstrated specific labeling on the surface of live cells, we note that expansion of this methodology to the cell interior is likely to be complicated by the presence of endogenous aldehydes.

This study expands the panel of probes that can be ligated by LplA mutants for the specific labeling of proteins. In comparison to lipoic acid ligation by wild-type LplA ($k_{cat} = 0.22\ s^{-1}$), and 7-hydroxycoumarin ligation by ^{W37V}LplA ($k_{cat} = 0.019\ s^{-1}$), the measured k_{cat} for Ald ligation ($0.33 \pm .01\ s^{-1}$) is extremely rapid and among the best for an unnatural probe/LplA mutant pair.[14] The hydrophobic nature of the substrate recognition could partially explain the tenfold greater activity of Ald versus Hyd, as the polar nature of the hydrazine might interfere with binding.

We envision using this method to prepare improved conjugates of streptavidin and antibodies to reporters, particularly enzyme reporters such as peroxidase and alkaline phosphatase, in which nonspecific chemical conjugation could block their active sites and reduce activity. Such reagents could lead to improved sensitivity and reproducibility for ELISAs, western blots, and immunofluorescence staining. Finally, we note that our method showcases the use of phycoerythrin for single-molecule imaging of specific proteins in the context of live cells. We believe this should be generalizable and provide an alternative to small organic dyes (due to increased brightness) and QDs (due to smaller size and lower cost).



Experimental Section

Plasmids: For expression of His_6-tagged LplA in *E. coli*, we used the LplA-pYJF16 plasmid.[14] The cloning of LAP-streptavidin-pET21a for bacterial expression is described in the Supporting Information. For expression of LAP fusion proteins in mammalian cells, we used LAP4.2-neurexin-1β-pNICE[14] and LAP4.2-LDLR-pcDNA4.[27] Mammalian expression plasmids for BirA-ER,[28] AP-LDLR,[29] and H2B-YFP[19] have been described previously.

In vitro screening for Ald and Hyd ligation activity (Figure 1)**:** Ligation reactions were assembled as follows: purified LplA mutant (1 μM),[14] synthetic LAP peptide (GFEIDKVWYDLDA; 150 μM), ATP (5 mM), either Ald or Hyd probe (500 μM), magnesium acetate (5 mM), and Na_2HPO_4 (pH 7.2, 25 mM) in a total volume of 20 μL. Reactions were incubated for 5–60 min at 30 °C and then quenched with ethylenediamine tetraacetate (EDTA) to a final concentration of 45 mM. Samples were diluted to a total volume of 80 μL in conjugation buffer (10 mM Na_2HPO_4, 3.2 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 5.0) and analyzed on a Varian Prostar HPLC by using a reversed-phase C18 Microsorb-MV 100 column

Figure 4. Site-specific protein labeling with phycoerythrin on the surface of living mammalian cells. A) HEK cells expressing LAP4.2–neurexin-1β were labeled with ^{W37I}LplA and Hyd substrate for 45 min, washed, then treated with 3 μM phycoerythrin–Ald in the presence of 10 mM aniline for 45 min, before confocal imaging. Negative controls are shown with wild-type LplA, ATP omitted, and an alanine mutation in LAP. Nuclear YFP was a co-transfection marker. B) COS7 cells expressing LAP4.2–LDL receptor were labeled as in (A), except that a lower concentration of phycoerythrin–Ald (0.3 μM) was used, and the cells were imaged in TIRF mode. Nuclear YFP is a transfection marker. Images of a transfected (top) and untransfected (bottom) cell are shown. C) Enlarged view of the boxed area in (B) showing individual phycoerythrin particles conjugated to LAP4.2–neurexin-1β on the COS7 cell surface. A time-lapse movie showing the labeled cell-surface LDL receptors over a period of 1 min is shown in the Supporting Information. All scale bars = 10 μm.

(250×4.6 mm). Chromatograms were recorded at 210 nm. For analysis of the aldehyde ligation reaction we used a 10 min gradient of 30–60% acetonitrile in water with 0.1% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. For analysis of the hydrazine ligation reaction, a gradient of 25–60% of the same solvents over 14 min was used. Percent conversions were calculated by dividing the product peak area by the sum of (product + starting material) peak areas. Reactions were performed in triplicate (Ald) or duplicate (Hyd), and the average values are shown. Reactions in Figure 1C were performed under the above conditions with a reaction time of 70 min for Ald and 120 min for Hyd.

LAP–monovalent streptavidin expression and purification: Monovalent streptavidin containing a single LAP tag fused to the N terminus of the "alive" subunit was expressed and purified as previously described.[28] Briefly, the alive (LAP-tagged, His_6-tagged) and dead (untagged) subunits of streptavidin were expressed separately in *E. coli*. The inclusion bodies were solubilized, and the alive and dead proteins were combined in a 3:1 ratio. After refolding to obtain a statistical mixture, monovalent streptavidin containing exactly one alive subunit and three dead subunits was purified by gradient nickel affinity chromatography. Monovalency was confirmed by using a DNA gel-shift assay. LAP–mSA was mixed with 250 bp biotinylated DNA at 1:1 and 10:1 molar ratios and run on a 1.5% agarose gel. A band corresponding to binding of a single biotinylated DNA was observed. In comparison, wild-type streptavidin under the same conditions binds between 1–4 biotinylated DNA molecules.

In vitro labeling of LAP fusion proteins (Figure 2)**:** Reaction mixtures were assembled from LAP–mSA (2 μM), ^{W37I}LplA (500 nM), ATP (5 mM), either Ald or Hyd (100 μM), magnesium acetate (5 mM), and Na_2HPO_4 (pH 7.2, 25 mM) in a total volume of 20 μL. The mixtures were incubated at room temperature for 1 h. Each reaction mixture was then diluted to a volume of 500 μL with phosphate-buffered saline (PBS), and the buffer was adjusted to pH 5 by using HCl. Thereafter, the solution was concentrated to ~30 μL in an ultrafiltration concentrator with a MWCO of 5 kDa (Vivaspin 500, GE Healthcare). This was repeated twice in order to fully exchange the buffer and eliminate excess probe. Conjugation was then performed by adding aniline (20 mM) and either AlexaFluor568-hydrazide (200 μM, Invitrogen) or fluorescein-aldehyde (4FB-PEG3-fluorescein, Solulink; 200 μM). Reaction mixtures were incubated overnight and analyzed on 10% SDS-PAGE gel. In-gel fluorescence imaging was performed by using a Fujifilm FLA-9000.

Mammalian cell culture: HEK and COS-7 cells were cultured in minimum essential medium (MEM, Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Morningside, QLD, Australia). Cells were maintained at 37 °C under 5% CO_2. For imaging, HEK cells were grown on glass coverslips pre-treated with fibronectin (50 μg mL^{-1}, Millipore) to increase their adherence. COS-7 cells were grown in LabTek II chambered coverglass system eight-well plates.

Microscopy: Cells were imaged in Dulbecco's phosphate-buffered saline (DPBS) at room temperature. The confocal images in Figures 3 and 4A were collected on a Zeiss AxioObserver.Z1 microscope with a 40× oil-immersion objective and 2.5× Optovar. The images were collected in confocal mode by using a Yokogawa spinning disk confocal head with a Quad-band notch dichroic mirror (405/488/568/647 nm). YFP (491 nm laser, 528/38 emission filter), AlexaFluor568/Phycoerythrin (561 nm laser, 617/73 emission filter), and Normarski-type DIC images were collected by using a Cascade II:512 camera and Slidebook software (Intelligent Imaging Innovations, Denver, CO, USA). Fluorescence images in each experiment were normalized to the same intensity range.

TIRF images (Figure 4) were acquired on the same microscope by using a TIRF slider. YFP (491 nm laser excitation, 525/30 emission filter, 502 nm dichroic mirror), Alexa Fluor 568/Phycoerythrin (561 nm laser excitation, 605/30 emission filter, 585 nm dichroic mirror), and Normarski-type DIC images were collected at 100× magnification by using Slidebook software (Intelligent Imaging Innovations). Digital images (16-bit) were obtained with a cooled EMCCD camera (QuantEM:512SC, Photometrics, Tucson, AZ, USA) with exposure times between 50 and 200 ms.

Cell-surface labeling (Figures 3 and 4)**:** An alternative peptide sequence called LAP4.2 (GFEIDKVWHDFPA)[13] was used for neurexin-1β and LDLR in order to boost cell surface expression levels. For Figure 3, HEK cells were transfected with 200 ng LAP4.2-neurexin-1β and 200 ng H2B-YFP co-transfection marker plasmid per 0.95 cm^2 cells at ~70% confluency by using Lipofectamine 2000 (Invitrogen). The growth medium was removed 15 h after transfection, and the cells were washed with DPBS (3×) with 0.5% casein. Casein was added to DPBS for all washing and labeling steps as a blocking agent and was required to reduce nonspecific sticking of the probes. The cells were then labeled by applying Ald probe (100 μM), ^{W37I}LplA (1 μM), ATP (1 mM), and $Mg(OAc)_2$ (5 mM) in DPBS with 0.5% casein at 37 °C for 45 min. Cells were then washed with DPBS (3×) with 0.5% casein and treated with aniline (10 mM) and 100 μM AlexaFluor568–hydrazide (100 μM) at 4 °C for 30 min. Cells were washed an additional three times and imaged live. The cell-surface labeling in Figure 4A was performed in the same fashion with the following changes: labeling was done by using Hyd probe for 45 min at room temperature, and the fluorophore conjugation was done by using PE-Ald (4FB-R PE, 3 μM, Solulink) for 45 min at 4 °C. Labeling for Figure 4B was performed as described for Figure 4A except that COS-7 cells were transfected with LAP4.2-LDLR (200 ng) and H2b-YFP (100 ng) co-transfection marker, Hyd probe (20 μM) was used in the initial labeling, and PE-Ald (0.3 μM) with aniline (20 mM) for 45 min was used for the fluorophore conjugation.

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