Published in final edited form as:

Nat Biotechnol. 2007 December ; 25(12): 1483–1487. doi:10.1038/nbt1355.

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

Marta Fernández-Suárez¹, Hemanta Baruah¹, Laura Martínez-Hernández¹, Kathleen T. Xie¹, Jeremy M. Baskin², Carolyn R. Bertozzi^{2,5}, and Alice Y. Ting¹

¹Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, Massachusetts 02139, USA

²Department of Chemistry, University of California, Berkeley, California 94720, USA

³Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA

⁴Howard Hughes Medical Institute, University of California, Berkeley, California 94720, USA

⁵The Molecular Foundry, Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

Live cell imaging is a powerful method for studying protein dynamics at the cell surface, but conventional probes, such as antibodies and fluorescent ligands, are bulky, interfere with protein function^{1,2}, or dissociate after internalization^{3,4}. To overcome these limitations, we developed a method to covalently tag any cell surface protein with any chemical probe with remarkable specificity. Through rational design, we re-directed a microbial lipoic acid ligase (LplA)⁵ to specifically ligate an alkyl azide to an engineered LplA acceptor peptide (LAP) tag. The alkyl azide is then selectively derivatized with a cyclooctyne⁶ conjugated to any probe of interest. We demonstrate the utility of this method by first labeling LAP fusion proteins expressed on the surface of living mammalian cells with Cy3, Alexa Fluor 568, and biotin. Next, we combined LAP-tagging with our previously reported tagging method^{7,8} to simultaneously monitor the dynamics of two receptors, co-expressed in the same cell, with different fluorophores. Using a wound-healing assay, we found that while the LDL receptor maintains a uniform distribution on the cell surface, the ephrin receptor EphA3 is polarized to the leading edge. 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 years⁹, but none currently allows the covalent attachment of small fluorophores of any structure 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 new protein labeling method based on the *E. coli* enzyme lipoic acid ligase (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,

Correspondence to: Alice Y. Ting.

Correspondence and requests for materials should be addressed to Alice Y. Ting (ating@mit.edu).

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.X. performed the research; J.M.B. and C.R.B. contributed novel reagents; 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/>.

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

E2o, and H-protein⁵) (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 acid⁵ suggest that the small-molecule binding site has considerable plasticity. To harness LplA for fluorescent labeling, we re-engineered 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 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 cyclooctyne reaction partner⁶ (Figure 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 the mammalian cell context 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 provides greater versatility for subsequent incorporation of probes of any structure. Many functional group handles have been used in chemical biology, including ketones, organic azides, and alkynes¹⁰. Organic azides are the most suitable for live cell applications, because the azide group is both abiotic and non-toxic in animals and can be selectively derivatized under physiological conditions (without any added metals or cofactors) with cyclooctynes, which are also unnatural⁶. 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 tested them for ligation onto a 9 kDa lipoyl domain derived from the full-length E2p protein¹¹ (abbreviated “E2p”) using an HPLC assay. As additional probes of the lipoate binding pocket we also synthesized a series of alkyne carboxylic acids (Supplementary Fig. 1). Figure 1b shows that 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. 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 online), and compared the values to those of lipoic acid ligation. The k_{cat} values were only slightly different ($0.111 \pm 0.003 \text{ s}^{-1}$ vs $0.253 \pm 0.003 \text{ s}^{-1}$) but the K_{m} increased 75- or 30- fold for azide **7** ($127 \pm 11 \text{ }\mu\text{M}$) compared to lipoic acid ($1.7 \text{ }\mu\text{M}$ ⁵ or $4.5 \text{ }\mu\text{M}$ ¹²). As seen below, however, it is straightforward and non-toxic to provide azide **7** at concentrations higher than $127 \text{ }\mu\text{M}$ 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 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 recognized by LplA with similar efficiency to the natural protein substrates. As described in Supplementary Figure 3 online, we accomplished this through multiple rounds of rational design. A major challenge was presented by the fact that E2p presents the lysine modification site at the tip of a sharp hairpin turn¹³, a conformation that is difficult to recapitulate in a peptide. Nevertheless, we designed an initial panel of 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 $0.048 \pm 0.001 \text{ s}^{-1}$, only 2.3-fold lower than the corresponding k_{cat} for full-length E2p.

Our third task was to assess the specificity of LplA in the mammalian cell context. 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, after functionalization with a FLAG peptide via the Staudinger ligation¹⁴. Figure 2 shows that in the presence of thousands of mammalian proteins in lysate, only LAP-CFP is labeled by LplA. The expression level of LAP-CFP is so low that it cannot be seen above 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 our newly engineered small molecule and peptide substrates for LplA in the live cell context, 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 PDGF receptor (Fig. 3, top). We also synthesized conjugates of our previously reported mono-fluorinated cyclooctyne⁶ (OCT) to two bright, red-emitting, and membrane-impermeant fluorophores, Alexa Fluor 568 and Cy3 (Supplementary Fig. 4 online).

To perform labeling, LAP-CFP-TM was expressed in HEK cells, and 350 μ M azide **7** was added in the presence of LplA for 1 hour, followed by one of the fluorophore-OCT conjugates for 20 minutes. The live cell images in Figure 3 show that transfected cells (indicated by CFP fluorescence) are labeled with Alexa Fluor 568 or Cy3, while neighboring untransfected cells in the same field of view are not labeled. Interestingly, labeling with Alexa Fluor 568 generated higher background than Cy3 labeling, due to faster non-specific 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 AZT¹⁵ are not known to be toxic to cells, but we nevertheless examined the effect of 24-hour exposure to azide **7** on mitochondrial respiration, and found no effect at concentrations less than 750 μ M (data not shown).

We also compared the speed, sensitivity, and specificity of LplA labeling to two other peptide-based labeling methods previously described by our lab (Supplementary Fig. 5 online). Biotin ligase (BirA)/ketone tagging makes use of a ketone isostere of biotin, which can be functionalized with hydrazide conjugates to label proteins fused to a 15-amino acid “acceptor peptide” (AP)¹⁶. Transglutaminase labeling attaches cadaverine-functionalized fluorophores to a glutamine-containing peptide recognition sequence¹⁷. 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 online). A total labeling time of only 20 minutes was required for all three steps, in order to achieve 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 hours and 15 minutes to achieve a similar signal to background ratio. We also quantified the sensitivity of LplA labeling using the wedge method¹⁸ and determined that cells expressing as little as 5 μ M LAP-CFP-TM could be specifically labeled with OCT-biotin, with a signal to background ratio $\geq 3:1$. Similar experiments, described in Supplementary Figure 5 online, demonstrate that LplA is also superior to transglutaminase, particularly in terms of labeling specificity under a wide range of conditions.

To illustrate the use of LplA labeling for imaging actual 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 body¹⁹, and we established that LAP-LDLR could be labeled with

OCT-Cy3 or OCT-biotin in HEK cells, even when expressed at levels matching endogenous LDLR (data not shown). We then wished to image LAP-LDLR in the context of a biological assay. For many imaging studies, it is desirable to visualize two different receptors at once in the same cell, in order to compare their distribution 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. While 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 labeling⁷.

E. coli LplA and biotin ligase are mechanistically related, and their natural acceptor proteins share some structural and sequence overlap²⁰. 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 EGF receptor¹⁶) together with a CFP transfection marker. After 16-24 hours of expression, the cells were re-plated 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. Supplementary Figure 6 online shows that cells expressing LAP-LDLR were selectively labeled with Cy3, while cells expressing AP-EGFR were selectively labeled with streptavidin. 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 extremely high specificity of each system.

We then used this two-color labeling protocol to image LAP- and AP-fused receptors co-expressed within the same cell. EGF receptor and EphA3 are both known to function in cell migration^{21,22}, and thus we performed imaging on cells migrating toward an artificial wound. HEK cells were co-transfected with either LAP-LDLR and AP-EGFR, or LAP-LDLR and AP-EphA3. After 16-24 hours of expression, the confluent cells were wounded with a pipet tip. We allowed the wound to partially close over 12-18 hours, and then performed simultaneous labeling with Cy3, and Alexa Fluor 488 conjugated to monovalent streptavidin⁸. Figure 4 shows that Cy3-labeled LDLR was evenly distributed on the surface of the HEK cells, whereas Alexa Fluor 488-labeled EGFR and EphA3 were both asymmetrically concentrated at the leading edge of the polarized cells. 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 labeling (data not shown).

While the polarization of AP-EGFR to the leading edge of migrating cells was expected, and has previously been observed using antibody detection²³, 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 migration²⁴ and tumor invasion²⁵. However, it is unclear that unliganded EphA3 should function in migratory processes. Our observation of EphA3 accumulation at the free, leading edge of polarized cells suggests that unactivated EphA3 may play a role in cell signaling, or that EphA3 may be constitutively linked to the actin cytoskeleton.

In summary, we have developed new methodology for labeling cell surface proteins fused to a 22-amino acid recognition sequence for *E. coli* LplA. Small, noncrosslinking probes such as Cy3, Alexa Fluor, and biotin can be site-specifically and covalently conjugated to the LAP peptide in as little as 20 minutes. An important feature of our methodology is its generality;

any cell surface protein in any cell type can be labeled with any chemical moiety that can be functionalized with a cyclooctyne.

Many new protein labeling methods have been developed in recent years⁹, 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/AGT²⁶ generally give higher labeling specificity than peptide tags, such as FAsH²⁷. However, protein tags have greater potential to interfere with protein folding, trafficking, and activity, as GFP often does^{28,29}. We and others (for example, ACP/PCP labeling methodology³⁰) 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, highly specific probe conjugation can be achieved, without sacrificing the small size of the directing tag.

In previous work with BirA, we found that a ketone isostere of biotin could be accepted¹⁶, but not compounds with more dissimilar structures, such as alkyne and azide derivatives of biotin, due to the 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 substrate⁵. This property allowed us to harness LplA for unnatural ligation reactions in this study. Important next challenges will be to extend this methodology to labeling of intracellular protein targets and to re-engineer LplA for one-step ligation of fluorophore or photoaffinity probes.

We also used LplA in combination with biotin ligase to image two different receptors 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 labeling reaction components, will provide access to such experiments.

Methods

In vitro LplA activity assays

LplA reactions contained 2 μ M LplA, 200 μ M E2p, 350 μ M probe, 1 mM ATP, 2 mM magnesium acetate, and 25 mM sodium phosphate pH 7.0. Reactions were incubated at 30 °C for 30 minutes, and then quenched with EDTA (final concentration 50 mM). Conversion to product was determined by HPLC on a C₁₈ reverse-phase column with a 40-57% gradient of acetonitrile in water with 0.1% trifluoroacetic acid over 20 minutes (flow rate 1.0 mL/minute). Unmodified E2p had a retention time of ~12 minutes while E2p-probe conjugates eluted at 15-18 minutes. 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 μ g DNA/well of a 6-well plate). Lysates were generated 48 hours 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 minutes, the cells were lysed by vigorous vortexing for 2 minutes at 21 °C. Crude lysate was clarified by centrifugation, and stored at -80 °C. Lysate was labeled by incubating at 30 °C for 10 hours with 25 mM sodium phosphate pH 7.0, 1 μ M LplA, 250 μ M azide **7**, 1 mM ATP, and 4 mM magnesium acetate. Thereafter,

Staudinger ligation was performed by adding FLAG-phosphine¹⁴ to a final concentration of 500 μ M, and incubating at 30 °C for 16 hours. 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, 1:1000 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, in order 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 hours at 37 °C, the cells were washed twice with fresh growth media (Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin/streptomycin). Enzymatic ligation of azide **7** was performed in complete growth media with 10 μ M LpIA, 350 μ M azide **7**, 1 mM ATP, and 5 mM magnesium acetate for 60 minutes at 32 °C. Cells were rinsed three times with growth media, and incubated for 20 minutes at 21 °C with 200-400 μ M OCT-Cy3 or 100-200 μ M OCT-Alexa Fluor 568. Thereafter, the cells were washed once with growth media, twice with a 1% bovine serum albumin (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 40x oil-immersion lens. CFP (420/20 excitation, 450 dichroic, 475/40 emission), Cy3 and Alexa Fluor 568 (560/20 excitation, 585 dichroic, 605/30) 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 milliseconds.

Two-color live cell labeling with LpIA and biotin ligase

HEK 239T cells were co-transfected with the LAP-LDLR and AP-EGFR¹⁶ plasmids in a 1:2 ratio, or with the LAP-LDLR and AP-EphA3 (a gift from M. Lackmann, Monash University) plasmids in a 2:1 ratio. 24 hours after transfection, the cells were wounded with a pipet tip and allowed to heal over 16-24 hours. For labeling, cells were washed twice with complete growth media, and then incubated with 5 μ M BirA, 10 μ M LpIA, 50 μ M biotin, 350 μ M azide **7**, 1 mM ATP, and 5 mM magnesium acetate for 60 minutes at 32 °C. Cells were then rinsed three times with growth media, and incubated for 20 minutes at 21 °C with 200-400 μ M OCT-Cy3. Biotin was detected by staining with 50 μ g/mL monovalent streptavidin-Alexa Fluor 488⁸ for 10 minutes 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank Mark Howarth, John Cronan, Irwin Chen, Chi-Wang Lin, and Martin Lackmann for their assistance and advice. This work was supported by the National Institutes of Health (R01 GM072670-01), the Sloan Foundation, the Dreyfus Foundation, a La Caixa Foundation pre-doctoral fellowship (to M.F.S.), and a National Science Foundation pre-doctoral fellowship (to J.M.B.).

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a

b

Substrate		% Conversion
Lipoic acid		100 ± 3
<div style="text-align: center;"> </div>	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
<div style="text-align: center;"> </div>	n = 4	3.59 ± 0.23
	n = 5	3.15 ± 0.19
	n = 7	6.37 ± 0.15
	n = 8	5.15 ± 0.23

c

Figure 1. Re-directing 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 sidechain within a peptide recognition sequence. The azide is then selectively functionalized with a cyclooctyne-probe conjugate (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 in Supplementary Figure 2 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 via the Staudinger ligation¹⁴, 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 LpIA, and the introduced azide was then labeled with a cyclooctyne 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.

Figure 4. Simultaneous labeling and imaging of two receptors in polarized cells in a wound healing assay

HEK cells co-expressing 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 488⁸ to detect the biotin. The Cy3 images show the non-polarized 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.