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Diels-Alder Cycloaddition for Fluorophore Targeting to Specific Proteins inside Living Cells

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Abstract

The inverse-electron-demand Diels-Alder cycloaddition between *trans*-cyclooctenes and tetrazines is biocompatible and exceptionally fast. We utilized this chemistry for site-specific fluorescence labeling of proteins on the cell surface and inside living mammalian cells by a two-step protocol. *E. coli* lipoic acid ligase site-specifically ligates a *trans*-cyclooctene derivative onto a protein of interest in the first step, followed by chemoselective derivatization with a tetrazinefluorophore conjugate in the second step. On the cell surface, this labeling was fluorogenic and highly sensitive. Inside the cell, we achieved specific labeling of cytoskeletal proteins with green and red fluorophores. By incorporating the Diels-Alder cycloaddition, we have broadened the panel of fluorophores that can be targeted by lipoic acid ligase.

Due to their small size and advantageous photophysical properties relative to Green Fluorescent Protein, chemical fluorophores have the potential to be very useful for live cell imaging. An impediment to their widespread use, however, is the difficulty of targeting them to specific cellular proteins. Our lab has developed an approach to the targeting problem called PRIME, for Probe Incorporation Mediated by Enzymes¹⁻³. In PRIME, a mutant of the *E. coli* enzyme lipoic acid ligase (LplA) is used to catalyze the covalent conjugation of chemical probes to recombinant proteins fused to a 13-amino acid LplA acceptor peptide, or LAP, inside living cells. The naturally high sequence-specificity of LplA makes this targeting method highly specific. In comparison to other intracellular post-translational protein labeling methods – SNAP⁴, HaloTag⁵, and FIAsH⁶ – PRIME uniquely offers the combination of a small peptide-directing sequence and high labeling specificity.

PRIME, however, has so far only been demonstrated inside cells with small blue fluorophores based on coumarin^{1;7;8}. To be generally useful, PRIME must be extended to other fluorophores, particularly red-shifted ones with excellent photophysical properties such as tetramethylrhodamine (TMR) and Alexa Fluors. Our experience with engineering the LplA active site to accommodate unnatural substrates^{1;2;7} suggests that major structural redesign would be required for LplA to directly ligate these much larger dyes. An alternative

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Supporting Information. Synthesis and characterization of *trans*-cyclooctenes and tetrazine-fluorophore conjugates. Validation of *trans*-cyclooctene ligation by LplA. Kinetic characterizations. Additional cell imaging experiments and experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

strategy is to use LplA to ligate a functional group handle, which can then be chemoselectively derivatized in the cellular context with fluorophores of interest.

We considered three types of reactions for the chemoselective derivatization: copper-catalyzed azidealkyne cycloadditions (CuAAC)⁹, strain-promoted azidecycloalkyne cycloadditions¹⁰, and inverse-electron-demand Diels-Alder cycloadditions of tetrazines and *trans*-cyclooctenes¹¹. CuAAC is restricted to the cell surface due to its dependence on toxic Cu(I)¹². We previously used PRIME in conjunction with strain-promoted cycloaddition for fluorescent labeling of cell surface proteins². The slow kinetics of this reaction ($k = 10^{-3}$ to $1 \text{ M}^{-1}\text{s}^{-1}$)¹³, however, limited our overall labeling yield and hence the achievable signal-to-noise ratio for imaging. Both CuAAC (k up to $10^4 \text{ M}^{-1}\text{s}^{-1}/\text{M}$ copper)¹⁴ and the Diels-Alder cycloaddition (k up to $10^4 \text{ M}^{-1}\text{s}^{-1}$)¹⁵ are much faster. The Diels-Alder reaction is also compatible in principle with the cell interior, although the only previous demonstration was intracellular labeling of a taxol derivative¹⁶. Due to both its speed and potential for intracellular compatibility, we selected the Diels-Alder cycloaddition for this study (**Figure 1A**).

To utilize this chemistry, we first needed to choose between having LplA ligate the tetrazine or the *trans*-cyclooctene. We noted that the *trans*-cyclooctene moiety would be less bulky and therefore require less re-engineering of LplA. This is because tetrazine itself is unstable in aqueous solution, and must be stabilized by conjugation to one or more aromatic rings¹⁷, making the overall moiety quite large. Additionally, tetrazines quench the fluorescence of some covalently attached fluorophores, until reaction with *trans*-cyclooctene¹⁶. To allow for the possibility of fluorogenic labeling, we opted to conjugate the fluorophore to tetrazine.

Based on our experience, LplA prefers substrates with 3 – 4 linear methylenes linking the carboxylate and the bulky feature¹. We therefore synthesized three *trans*-cyclooctene substrates for LplA: **TCO₁**, **TCO₂**, and **TCO₃**, with structures shown in **Figure 1B** and syntheses enabled by our photochemical flow method (**Scheme 1**)¹⁸. **TCO₁** and **TCO₂** differ only in the length of their aliphatic linkers, while **TCO₃** has a cyclopropane ring fusion, which adds strain and accelerates the cycloaddition up to 160-fold¹⁹. We prepared a panel of LplA mutants and screened for their ability to ligate these three TCOs onto LAP using an HPLC assay (**Table 1**). Not surprisingly, wild-type LplA was unable to ligate any of the three substrates efficiently. Our other LplAs each harbored a single mutation at Trp37, a gate-keeper residue that has given us access to various unnatural substrates in the past^{1;20}. We tested the Trp37→Gly mutant, with the active site maximally enlarged, as well as Trp37→Ile and →Val mutants that carve out a smaller, hydrophobic hole. We found that **TCO₂** scored significantly better than the other probes, and was best paired with the Val mutant (designated ^{W37V}LplA, **Table 1**).

Enzyme-dependent ligation was confirmed by negative controls omitting ATP or ^{W37V}LplA, and by mass spec-trometry (**Supporting Figure 1A** and **1B**). We estimated the Michaelis-Menten k_{cat} of **TCO₂** ligation onto LAP to be $0.34 \pm 0.02 \text{ s}^{-1}$, comparable to the fastest unnatural probe that we have reported to date (aryl azide: $0.31 \pm 0.04 \text{ s}^{-1}$)²⁰, and only 2-fold slower than ligation of the natural substrate lipoic acid by the same enzyme (**Supporting Figure 2**).

We next focused on the design and syntheses of the tetrazine-fluorophore conjugates. The previously reported tetrazine structure **Tz1** (**Figure 1C**) reacts rapidly with *trans*-cyclooctenes, and has been used for small-molecule labeling in the cellular context¹⁶. However, the lack of a second aryl substitution on **Tz1** leaves it susceptible to non-specific reactions with cellular nucleophiles and dienophiles. Following the design of Blackman²¹, we synthesized an alternative 3,6-diaryl-*s*-tetrazine, **Tz2** (**Figure 1C**; synthesis shown in

Supporting Figure 3). Structures closely related to **Tz2** had been shown to be unusually stable toward amines and thiols²¹. The electron withdrawing *p*-trifluoromethyl substituent of **Tz2** augments the reactivity toward *trans*-cyclooctenes.

Both **Tz1** and **Tz2** were conjugated to fluorescein. Upon reaction with excess *trans*-cyclooctene, we measured 13.4- and 16.7-fold increases in fluorescein emission, respectively, in agreement with previous reports describing similar dyes¹⁶ (**Supporting Figure 4**). We also measured second order rate constants for reaction with TCO₂-ligated LAP, and found values of 5000 ± 700 and $380 \pm 40 \text{ M}^{-1} \text{ s}^{-1}$ for **Tz1**-fluorescein and **Tz2**-fluorescein, respectively (**Supporting Figure 5**).

We proceeded to test cell surface fluorescence labeling. Here, nucleophiles are less abundant than inside cells, so we utilized only the faster tetrazine probe, **Tz1**-fluorescein. LAP-tagged low density lipoprotein receptor (LAP-LDL receptor) was expressed in human embryonic kidney 293T cells (HEK cells). We externally supplied $5 \mu\text{M}$ ^{W37V}LpIA, $100 \mu\text{M}$ TCO₂ and ATP for 15 minutes. We rinsed off the excess reagents, then stained the cells with 100 nM **Tz1**-fluorescein for 5 minutes and observed specific labeling on transfected cells after further brief rinsing (**Figure 2A**). Negative controls with ATP omitted, wild-type LpIA, or inactive LAP mutant all eliminated the labeling signal.

Furthermore, we found it possible to perform fluorogenic labeling of LAP-LDL receptor, using 50 nM **Tz1**-fluorescein and without rinsing. Fluorescence signal accumulated specifically on transfected cells, with signal-to-noise ratios saturating after approximately 3 minutes, and with minimal background signal from the surrounding excess **Tz1**-fluorescein (**Figure 2B and 2C**).

To extend cell surface labeling to other colors and cell types, we conjugated **Tz1** to Alexa 647, a brighter fluorophore suitable for single molecule fluorescence detection and super-resolution imaging²². **Tz1**-Alexa 647 was used to label LAP-tagged neuroligin-1 on the surface of rat neurons with high specificity and minimal apparent toxicity (**Figure 2D**).

We directly compared Diels-Alder cycloaddition with two other bioorthogonal labeling chemistries that are compatible with the cell surface: CuAAC and strain-promoted azide-alkyne cycloaddition. We found that under otherwise identical conditions, Diels-Alder cycloaddition gave specific signal at 10 nM of **Tz1**-Alexa 647, while other methods required at least $1 \mu\text{M}$ of dye to achieve a similar signal-to-noise ratio. These results demonstrate that the Diels-Alder cycloaddition is much more sensitive while retaining similar specificity (**Supporting Figure 6A**). Additionally, using an assay of cellular ATP content, we found that labeling by the Diels-Alder cycloaddition was not toxic, in contrast to CuAAC with TBTA ligand²³ (although CuAAC with a new generation ligand, THPTA²⁴, was considerably less toxic), (**Supporting Figure 6B**).

Negative charges on fluorescein and Alexa 647 prevent their tetrazine conjugates from crossing cell membranes. To label intracellular proteins, we first prepared cell-permeable derivatives, **Tz1**- and **Tz2**-fluorescein diacetate. Upon entering the cell interior, endogenous esterases hydrolyze the acetyl groups and release the intact tetrazine-fluorescein conjugate. For initial experiments, we expressed nuclear-localized, LAP-tagged blue fluorescent protein (nuclear LAP-BFP), as well as cytoplasmic ^{W37V}LpIA inside HEK cells. The whole-cell localization of ^{W37V}LpIA versus the nuclear localization of its LAP substrate imposes an additional test of labeling specificity.

We first compared **Tz1**- and **Tz2**-fluorescein diacetate at $10 \mu\text{M}$, $1 \mu\text{M}$, and 100 nM loading concentrations. **Tz1** was expected to generate higher signal because of its higher Diels-Alder

reactivity, but higher background was also expected due to its increased lability. Inside cells, we observed that **Tz1** indeed generated more background fluorescence than **Tz2** at high loading concentrations, but due to its much higher labeling signal, the overall signal-to-noise ratio (**Supporting Figure 7**). We therefore chose **Tz1** over **Tz2** for subsequent intracellular labeling experiments.

We synthesized another cell-permeable, red-shifted fluorophore conjugate, **Tz1**-tetramethylrhodamine (**Tz1**-TMR), and proceeded to optimize the cellular labeling conditions for both this conjugate and **Tz1**-fluorescein diacetate. Following the optimized protocol shown in **Figure 3A**, we observed labeling signal specific to the nuclei of transfected cells, despite the presence of LplA in both the cytosol and the nucleus. Negative controls with **TCO₂** omitted, wild-type LplA, or an inactive LAP mutant abolished labeling signals (**Figure 3B**). We also examined the labeling specificity by lysing cells after **Tz1**-fluorescein diacetate treatment, and imaging the fluorescence of the lysate after gel separation. **Supporting Figure 8** shows that a single protein corresponding to the size of LAP-BFP was selectively labeled over the endogenous proteome.

We were unable to achieve fluorogenic labeling inside cells because high fluorescence signal was observed inside untransfected cells as well as cells free of **TCO₂** treatment, immediately upon loading of both **Tz1**-fluorophore conjugates (data not shown). We were, however, able to wash away off-target dyes after 2 hours. In COS-7 cells, where the required dye washout time was shorter, we also successfully labeled actin filaments (LAP- β -actin) and intermediate filaments (vimentin-LAP) with high specificity (**Figure 3C**). **Supporting Figure 9** shows that actin and vimentin filaments labeled by **Tz1**-TMR co-localized perfectly with filaments detected by immunofluorescence staining in the same cells, indicating that the labeling was specific.

In summary, we have found that the tetrazine-*trans*-cyclooctene Diels-Alder cycloaddition is highly efficient for the fluorescence labeling of cell surface proteins and sufficiently bioorthogonal for labeling of intracellular proteins. We utilized this fast chemistry for the extension of PRIME to a panel of useful fluorophores, including tetramethylrhodamine and Alexa 647, while retaining a level of specificity comparable to direct fluorophore ligation by PRIME¹. This method is generally applicable to different proteins in various cell types.

On the cell surface, we achieved fluorogenic labeling using tetrazine-fluorescein, but failed to accomplish fluorogenic labeling with Alexa 647 because its red-shifted fluorescence emission was not significantly quenched when conjugated to **Tz1** (**Supporting Figure 4B**). Inside the cell, we observed a tradeoff between the reactivity and stability of two different tetrazine structures. We surmise that, while monoaryl-substituted **Tz1** is significantly more reactive than diaryl **Tz2** toward *trans*-cyclooctene, the former is also more prone to cross-reactivity with endogenous nucleophiles or dienophiles. Our study therefore illustrates the need for next-generation tetrazines that are less kinetically hindered by protective substitutions, and more able to quench the fluorescence of red dyes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Uttamapinant C, White KA, Baruah H, Thompson S, Fernandez-Suarez M, Puthenveetil S, Ting AY. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:10914–10919. [PubMed: 20534555]
2. Fernandez-Suarez M, Baruah H, Martinez-Hernandez L, Xie KT, Baskin JM, Bertozzi CR, Ting AY. *Nat. Biotechnol.* 2007; 25:1483–1487. [PubMed: 18059260]
3. Puthenveetil S, Liu DS, White KA, Thompson S, Ting AY. *J. Am. Chem. Soc.* 2009; 131:16430–16438. [PubMed: 19863063]
4. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. *Nat. Biotechnol.* 2003; 21:86–89. [PubMed: 12469133]
5. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohane RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. *ACS Chem. Biol.* 2008; 3:373–382. [PubMed: 18533659]
6. Griffin BA, Adams SR, Tsien RY. *Science.* 1998; 281:269–272. [PubMed: 9657724]
7. Cohen JD, Thompson S, Ting AY. *Biochemistry.* Aug 31.2011 in press.
8. Jin X, Uttamapinant C, Ting AY. *ChemBioChem.* 2011; 12:65–70. [PubMed: 21154801]
9. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG. *J. Am. Chem. Soc.* 2003; 125:3192–3193. [PubMed: 12630856]
10. Agard NJ, Prescher JA, Bertozzi CR. *J. Am. Chem. Soc.* 2005; 127:11196.
11. Blackman ML, Royzen M, Fox JM. *J. Am. Chem. Soc.* 2008; 130:13518–13519. [PubMed: 18798613]
12. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. *Angew. Chem., Int. Ed.* 2002; 41:2596–2599.
13. Agard NJ, Baskin JM, Prescher JA, Lo A, Bertozzi CR. *ACS Chem. Biol.* 2006; 1:644–648. [PubMed: 17175580]
14. Presolski SI, Hong V, Cho SH, Finn MG. *J. Am. Chem. Soc.* 2010; 132:14570–14576. [PubMed: 20863116]
15. Devaraj NK, Upadhyay R, Hatin JB, Hilderbrand SA, Weissleder R. *Angew. Chem., Int. Ed.* 2009; 48:7013–7016.
16. Devaraj NK, Hilderbrand S, Upadhyay R, Mazitschek R, Weissleder R. *Angew. Chem., Int. Ed.* 2010; 49:2869–2872.
17. Balcar J, Chrisam G, Huber FX, Sauer J. *Tetrahedron Lett.* 1983; 24:1481–1484.
18. Royzen M, Yap GPA, Fox JM. *J. Am. Chem. Soc.* 2008; 130:3760–3761. [PubMed: 18321114]
19. Taylor MT, Blackman ML, Dmitrenko O, Fox JM. *J. Am. Chem. Soc.* 2011; 133:9646–9649. [PubMed: 21599005]
20. Baruah H, Puthenveetil S, Choi YA, Shah S, Ting AY. *Angew. Chem., Int. Ed.* 2008; 47:7018–7021.
21. Blackman, ML. Thesis. University of Delaware; Newark, DE: 2011.
22. Jones SA, Shim SH, He J, Zhuang XW. *Nat. Methods.* 2011; 8:499–505. [PubMed: 21552254]
23. Chan TR, Hilgraf R, Sharpless KB, Fokin VV. *Org. Lett.* 2004; 6:2853–2855. [PubMed: 15330631]
24. Hong V, Steinmetz NF, Manchester M, Finn MG. *Bioconjugate Chem.* 2010; 21:1912–1916.

Figure 1.

Two-step, site-specific fluorescence labeling of proteins using lipoic acid ligase (LplA) and Diels-Alder cycloaddition. **(A)** Optimized labeling scheme. In the first step, the Trp37→Val mutant of LplA ligates *trans*-cyclooctene TCO₂ onto LplA acceptor peptide (LAP), which is fused to the protein of interest. In the second step, ligated *trans*-cyclooctene is chemoselectively derivatized with a fluorophore conjugated to Tz1 tetrazine. **(B)** Three *trans*-cyclooctenes synthesized and evaluated in this study. **(C)** Two tetrazines used in this study.

Figure 2.

Cell surface fluorescence labeling using LplA and Diels-Alder cycloaddition. **(A)** HEK 293T cells expressing LAP-tagged low density lipoprotein receptor were treated with purified ^{W37V} LplA and TCO_2 , followed by Tz1-fluorescein. Fluorescein images (yellow) are shown beside overlaid images of DIC and the cyan fluorescent protein transfection marker. Negative controls with ATP omitted, wild-type ligase, and inactive LAP are shown. **(B)** Time-lapse imaging of the same experiment without rinsing away 50 nM Tz1-fluorescein, captured every 30 seconds. **(C)** Signal-to-noise quantification of (B). Noise is defined as fluorescence signal on untransfected cells. Error bars, 2 SEM. **(D)** Rat neurons expressing LAP-tagged neuroligin-1 and green fluorescent protein-tagged Homer1b (Homer1b-GFP, green) were treated with purified ^{W37V} LplA and TCO_2 , followed by Tz1-Alexa 647 (red), then imaged live after brief rinsing. All scale bars, 10 μm .

Figure 3.

Intracellular fluorescence labeling with LplA and Diels-Alder cycloaddition. **(A)** General protocol. **(B)** HEK 293T cells expressing nuclear-localized, LAP-tagged blue fluorescent protein (LAP-BFP) and cytosolic $^{\text{W37V}}$ LplA were labeled as in (A), with 500 nM **Tz1**-fluorescein diacetate (yellow, left panels) or 1 μ M **Tz1**-tetramethylrhodamine (**Tz1**-TMR, red, right panels), followed by 2-hour dye wash-out and imaged live. Negative controls with TCO_2 omitted, wild-type LplA, or inactive LAP also shown. **(C)** COS-7 cells expressing $^{\text{W37V}}$ LplA and LAP-tagged β -actin (left panels), or LAP-tagged vimentin (right panels) were treated as in (A), with 100 nM **Tz1**-fluorescein diacetate (yellow) or 1 μ M **Tz1**-tetramethylrhodamine (red), followed by 1-hour dye washout and imaged live. Scale bars, 10 μ m.

Table 1

Ligation efficiencies of lipoic acid ligase variants with the three *trans*-cyclooctene substrates (TC01-3). The relative abilities of wild-type and mutant ligases to ligate TC01-3 onto LplA acceptor peptide (LAP) were measured by an HPLC assay after 30 min. reaction time. Average, normalized product percentages from triplicate measurements are shown. (--) indicates no detectable product. Errors, ± 1 s. d.

Ligase	Wi Id-type	Trp37→Gly	Trp37→Ile	Trp37→Val
Probe				
TC01	0.5 $\pm$ 0.0	--	0.5 $\pm$ 0.4	--
TC02	2.6 $\pm$ 0.9	52.6 $\pm$ 1.1	77.8 $\pm$ 1.6	100.0 $\pm$ 8.3
TC03	6.5 $\pm$ 1.0	22.6 $\pm$ 2.0	6.0 $\pm$ 0.6	6.6 $\pm$ 0.6