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Second-Generation Covalent TMP-Tag for Live Cell Imaging

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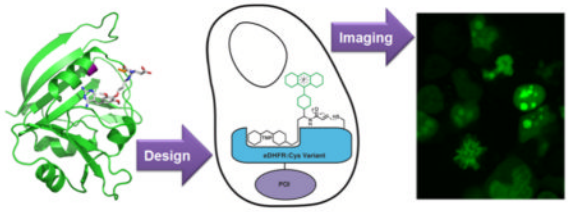
Abstract



Chemical tags are now viable alternatives to fluorescent proteins for labeling proteins in living cells with organic fluorophores that have improved brightness and other specialized properties. Recently, we successfully rendered our TMP-tag covalent with a proximity-induced reaction between the protein tag and the ligand-fluorophore label. This initial design, however, suffered from slow *in vitro* labeling kinetics and limited live cell protein labeling. Thus, here we report a second-generation covalent TMP-tag that has a fast labeling half-life and can readily label a variety of intracellular proteins in living cells. Specifically, we designed an acrylamide-trimethoprim-fluorophore (A-TMP-fluorophore v2.0) electrophile with an optimized linker for fast reaction with a cysteine (Cys) nucleophile engineered just outside the TMP-binding pocket of *Escherichia coli* dihydrofolate reductase (eDHFR) and developed an efficient chemical synthesis for routine production of a variety of A-TMP-probe v2.0 labels. We then screened a panel of eDHFR:Cys variants and identified eDHFR:L28C as having an 8-min half-life for reaction with A-TMP-biotin v2.0 *in vitro*. Finally, we demonstrated live cell imaging of various cellular protein targets with A-TMP-fluorescein, A-TMP-Dapoxyl, and A-TMP-Atto655. With its robustness, this second-generation covalent TMP-tag adds to the limited number of chemical tags that can be used to covalently label intracellular proteins efficiently in living cells. Moreover, the success of this second-generation design further validates proximity-induced reactivity and organic chemistry as tools not only for chemical tag engineering but also more broadly for synthetic biology.

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The authors declare the following competing financial interest(s): V.W.C. and M.P.S. hold patents on the TMP-tag technology, and the technology is licensed and commercialized by Active Motif.

Supporting Information
Model estimation of the optimal linker length between TMP and acrylamide; synthetic procedures and characterization of each compound; vector construction, protein expression, and purification procedure; *in vitro* alkylation protocol and gel pictures; cell culture, transfection and labeling protocol; live cell imaging protocol and in-gel fluorescence scanning/Western blotting protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

INTRODUCTION

Chemical tags are emerging from the proof-of-principle stage to viable reagents for labeling proteins in living cells with fluorophores with high photon outputs and other specialized properties[1–3] to enable experiments difficult or not possible with the fluorescent proteins (FPs).[4] With chemical tags, rather than tagging the target protein with an FP, the target protein is tagged with a polypeptide, which is subsequently labeled with a cell-permeable fluorophore ligand or substrate. Thus, chemical tags combine the advantage of specificity through genetic encoding with a modular organic fluorophore. Chemical tags now in use include the seminal peptide chelator-based FlAsH/ReAsH system,[5] the enzyme suicide substrate-based SNAP/CLIP-tags[6,7] and Halotag,[8] the small-molecule inhibitor-based TMP-tag,[9] and the enzyme-mediated polypeptide labeling-based lipoic acid ligase tag.[10] Exciting recent applications of the chemical tags include single-molecule imaging of spliceosome function in yeast cell extracts,[11] magnetically modulating mammalian cells using decorated iron oxide nanoparticles,[12] imaging LDL receptor oligomerization during endocytosis,[13] and super-resolution imaging of cellular proteins.[14] While new chemical tags are regularly being introduced in the literature,[1–3] our TMP-tag still stands out as one of the few chemical tags able to label intracellular, as opposed to cell-surface, proteins with high selectivity.

With TMP-tag, the target protein is tagged with *Escherichia coli* dihydrofolate reductase (eDHFR) through standard genetic encoding and then labeled by binding to a cell-permeable trimethoprim-fluorophore (TMP-fluorophore) conjugate. eDHFR is an attractive protein tag because it is 18 kD (about two-thirds the size of GFP) and monomeric and thus minimally disrupts biological function of the tagged protein and pathway. With low nanomolar affinities for eDHFR, the TMP-fluorophore conjugates can be used at near stoichimetric quantities to efficiently label tagged cellular proteins, which average a ~1 μM concentration in the cell. At the same time, with >1000-fold selectivity for *E. coli* over mammalian DHFRs, TMP-tag shows minimal background labeling of endogenous proteins and no apparent cellular toxicity in mammalian cell lines. TMP is commercially available and can be readily modified without disrupting binding to eDHFR, facilitating the preparation of a wide variety of TMP analogues.[15] Finally, there is a wealth of biochemical and structural knowledge of the interaction between TMP and eDHFR, which facilitates further engineering of the TMP-tag.[16] Fine tuning of the fluorophore hydrophobicity and linker structure have produced optimized versions of TMP-tag for lower, unspecific, background staining and better cell permeability.[17] On the strength of its robustness, TMP-tag labels have been developed to enable super-resolution imaging[14] and two-photon imaging of cellular proteins,[18] chromophore-assisted laser inactivation (CALI) of components of the focal adhesion complex,[19] and single-molecule imaging of spliceosome assembly.[11]

To provide a more permanent label for advanced applications such as single-molecule tracking or pulse–chase labeling, we recently reported a covalent variant of the TMP-tag based on a proximity-induced reaction between the eDHFR tag and the TMP-fluorophore label.[20] Briefly, a unique Cys nucleophile was engineered just outside the TMP-binding pocket of eDHFR (eDHFR:L28C) in position to react with an acrylamide electrophile installed on the TMP-fluorophore label (acrylamide-TMP-fluorophore, or A-TMP-fluorophore). This design was based on the long-standing use of proximity-induced reactivity[21,22] for the design of covalent inhibitors[23,24] and more recent application to chemical biology tools.[25] Work of Belshaw and co-workers[26,27] led us to believe that the acrylamide electrophile would have the right balance in reactivity, being a sufficiently mild electrophile to minimize nonspecific, background labeling of cellular components, but being reactive enough to undergo a rapid Michael addition upon TMP binding to eDHFR. Our initial design was successful, and we demonstrated that A-TMP-biotin reacted with eDHFR:L28C with a half-life of ~50 min *in vitro* and that A-TMP-fluorophore could

covalently label a nuclear-localized eDHFR fusion protein in live cells with minimal background labeling of other cellular proteins. However, this first-generation covalent TMP-tag was unable to label cytoplasmic proteins tagged with eDHFR:L28C, limiting its utility. We hypothesized that this limited reactivity resulted from the slow half-life with which A-TMP reacted with eDHFR:L28C.

Thus, we sought to design a second-generation covalent TMP-tag with a rapid labeling half-life that would improve its utility for live cell imaging. Previous reports in the literature have shown that the half-life of both covalent inhibitors[24] and chemical biology tools[26] can be improved to a few minutes with optimization of the reaction geometry between the protein nucleophile and the organic electrophile. We present the design and synthesis of an optimized v2.0 A-TMP-probe in conjunction with the rational design and screening of a panel of eDHFR:Cys variants to generate a v2.0 covalent TMP-tag with a rapid reaction half-life. Finally, we challenge the robustness of this v2.0 covalent TMP-tag with live-cell, confocal fluorescence imaging of multiple intracellular proteins in different mammalian cell lines.

RESULTS

Design of the Second-Generation Covalent TMP-Tag

On the basis of the success of our initial covalent TMP-tag, a second-generation covalent TMP-tag was also built around the Cys nucleophile and acrylamide electrophile (Figure 1), while optimizing the positioning of the nucleophile and electrophile to improve the reaction half-life. The Cys nucleophile and acrylamide electrophile had exceeded our expectations for minimal background labeling of endogenous cellular components and minimal cellular toxicity and yet still were able to undergo a fairly rapid binding-induced Michael addition. Previous literature on the design of covalent inhibitors and chemical biology reagents suggested that we could achieve a reaction half-life of a few minutes simply by optimizing the positioning of the Cys side-chain and acrylamide group undergoing the Michael addition.[26] For our initial covalent TMP-tag,[20] we chose a conservative design containing a 21-atom linker between the 4′-OH group of TMP and the reactive β-carbon of the acrylamide functional group to ensure that the acrylamide would be available to react with the Cys nucleophile installed on the surface of eDHFR. Because of this long linker length, it was not surprising that the initial covalent TMP-tag had an *in vitro* labeling reaction half-life of almost 1 h.

We chose to use rational design in combination with screening of a small number of variants to create a covalent TMP-tag with the minimum necessary distance between the Cys nucleophile and the acrylamide electrophile to achieve the desired reduction in reaction half-life. First, molecular modeling was applied to the high-resolution structure of eDHFR[16,28] to identify residues that had solvent-accessible side chains in which the side chain faced the binding pocket to ensure the engineered Cys residue would be accessible to react with the acrylamide electrophile. A model of TMP bound to eDHFR was created by structurally aligning a high-resolution structure of *E. coli* DHFR[28] to a high-resolution structure of TMP bound to *Lactobacillus casei* DHFR.[29] Only residues in close proximity to the binding pocket were selected, since precedent has shown that the closer the residue to the binding pocket, the more rapid the rate of alkylation.[26] Four residues were selected that met this criterion: Ala19, Asn23, Leu28, and Arg52. An approximation was made of the minimum linker length between TMP and the electrophile that would allow proximity-induced covalent labeling to occur upon binding to a mutant eDHFR containing each of these Cys mutants (Table S1). According to the model described above, we envisioned that a 10-atom spacer between the 4′-OH group of TMP and the β-carbon of the acrylamide would enable the electrophile to reach all the four engineered Cys nucleophiles (Figure 2).

Synthesis of the A-TMP-Probe Heterotrimer

Guided by molecular modeling, we designed an A-TMP-probe v2.0 heterotrimer with a 10-atom spacer between the 4′-OH group of TMP and the β-carbon of the acrylamide. We chose aspartic acid as the trifunctional core because amino acid derivatives could provide a convenient protection strategy for the sequential addition of the three different groups. In the proof-of-principle demonstration we chose fluorescein diisobutyrate as the probe, as cell behavior of fluorescein derivatives have been well-studied.[17,32] We chose fluorescein diisobutyrate conjugate due to its higher chemical stability over fluorescein diacetate conjugate as DMF stock solution. Addition of the probe in the final step could be beneficial to the generality of the tag, facilitating the preparation of a variety of A-TMP derivatives with different probe molecules.

The synthetic route of the target molecule is summarized in Scheme 1. H-Asp(OBu-^{t})-OH (**2**), a commercially available aspartic acid derivative, was treated with acryloyl chloride to yield carboxylic acid **3**. Amine **7** was prepared by O-alkylation of TMP phenol **4**[20] with a three-carbon Boc-amino iodide (**5**) followed by TFA deprotection of the Boc group. Coupling of carboxylic acid **3** and amine **7** with EDCI led to *tert*-butyl ester **8**, which was subjected to TFA deprotection to yield carboxylic acid **9**, a key intermediate toward the A-TMP-probe heterotrimer. A PEG linker was incorporated to carboxylic acid **9** by EDCI-mediated coupling with a monoprotected PEG bis-amine (**10**) followed by TFA deprotection of the Boc group. The product, amine **12**, was coupled with protected fluorescein NHS-ester (**13**) and purified by HPLC to yield the final heterotrimer, compound **1**, in pure form. The heterotrimer was prepared from aspartic acid derivative **2** in 3.4% overall yield with the longest linear sequence consisting of seven steps. This modular synthetic plan would allow us to prepare a variety of v2.0 A-TMP-probe molecules. For an illustrative example, A-TMP-biotin v2.0 (**S2**) was also prepared by a similar synthetic plan (Figure S2).

In Vitro Screening of A-TMP v2.0 with eDHFR:Cys Variants

After we obtained the A-TMP-probe v2.0 molecule, we moved to *in vitro* labeling studies to determine the best eDHFR:Cys variant to pair with A-TMP (Figure 3A). We found that the most rapid reaction occurred between eDHFR:L28C and A-TMP-biotin v2.0 among the tested eDHFR variants. Under the tested labeling conditions, the half-life of the labeling reaction was determined to be 8 min in the presence of NADPH.

Evaluation of the reactions between A-TMP V2.0 and eDHFR variants was conducted using purified proteins. The four designed eDHFR:Cys variants eDHFR:A19C, eDHFR:N23C, eDHFR:L28C, and eDHFR:R52C were over-expressed via the T7 promoter using the corresponding *E. coli* expression vectors and purified using Ni-NTA spin columns. The proteins were judged to be more than 95% pure according to Coomassie staining of SDS-PAGE gels. The endogenous cysteines of eDHFR, Cys85, and Cys152 were mutated to serines in these vectors to minimize possible cross reactivity with the engineered Cys nucleophile. A-TMP-biotin v2.0 was used as the tag in the conditional screening due to its better solubility in PBS buffer compared with the hydrophobic protected A-TMP-Fl.

Next, the *in vitro* labeling kinetics were determined using a SDS-PAGE gel shift assay to identify the fastest eDHFR/A-TMP pair (Figure 3B). Following the conditions reported in our previous study,[20] 10 μ M A-TMP-biotin was reacted with 5 μ M eDHFR:Cys variant in PBS buffer, and the reaction mixture was quenched at appropriate intervals with 6X SDS and then subjected to SDS-PAGE. Gel shifts were produced due to covalent modification of the eDHFR:Cys varients, simplifying analysis of the reaction progression as described in Figure 3. We first tested the reaction between eDHFR:L28C and A-TMP-biotin v2.0. The labeling reaction was near quantitative after 3 h, and the time required for 50% labeling was

determined to be 17 min. We then tested the effect of NADPH to this labeling reaction, as NADPH is a native cofactor of eDHFR. In the presence of 50 μ M NADPH, estimated to be the cellular concentration of NADPH,[33,34] the reaction between eDHFR:L28C and A-TMP-biotin v2.0 was accelerated with a half-life of 8 min. Further screening found that all of the eDHFR variants (Figure S5) reacted with A-TMP-biotin, but with significantly different reaction half-lives (Table 1). Intriguingly, NADPH promoted the reaction of A-TMP-biotin v2.0 with eDHFR:L28C, N23C and A19C, but slowed the reaction with eDHFR:R52C, perhaps indicative of complex conformational effects of NADPH binding. Overall, eDHFR:L28C was chosen as the fastest target for the designed second-generation A-TMP-probe molecule, especially in the presence of NADPH. This system would be particularly suitable for intracellular targets because of the abundance of NADPH in the reducing cellular environment.

Protein Labeling in Live Cells with the Second-Generation Covalent TMP-tag

Encouraged by the rapid *in vitro* labeling reaction between eDHFR:L28C and A-TMP-probe v2.0, the selected pair was next evaluated by labeling of cellular proteins. eDHFR:L28C was genetically fused to the C-termini of four different target proteins: histone H2B, Tomm20, α-actinin, and myosin light chain (MLC). Mammalian cell lines expressing the fusion proteins were successfully labeled with A-TMP-fluorescein v2.0 in 10 min and were characterized by both microscopy and in-gel fluorescence analysis. These results demonstrate the generality of the v2.0 covalent TMP-tag for live cell imaging.

At first, we aimed for labeling of an abundant cellular target. We chose histone H2B, an essential nuclear protein which has been intensively investigated, as the first target. HEK 293T cells transiently transfected with plasmids encoding H2B-eDHFR:L28C fusion protein were incubated with 1 μ M A-TMP-Fl v2.0 for 10 min. After staining, cells were washed twice with media and imaged by confocal microscopy. Distinct nucleic distribution of fluorescence was observed in transfected cells, with chromosomal patterns observed in a number of cells (Figure 4A). No significant background cytosol staining was detected. These observations indicated that A-TMP-Fl v2.0 was able to selectively bind to H2B-eDHFR:L28C fusion proteins with rapid kinetics in live cells.

To confirm that the labeling reaction was covalent, HEK 293T cells expressing the H2B-eDHFR:L28C fusion protein were treated with 1 μ M A-TMP-Fl v2.0 for 10 min, 30 min, or 3 h. After staining, cells were lysed and analyzed by SDS-PAGE and in-gel fluorescence (Figure 4B). A major band with a green channel emission was detected as the expected 35 kD H2B-eDHFR:L28C-A-TMP-Fl v2.0 conjugate, while no detectable background binding was observed in nontransfected cells (Figure S6). The labeling products were further confirmed by Western blot analysis using Anti-H2B antibody. Several minor bands with lower molecular weight were also detected in cells expressing the H2B-eDHFR:L28C fusion protein, particularly in longer incubation. These bands are likely to be the degradation products of labeled H2B, as the control experiment showed undetectable background staining of endogenous proteins.

Our next goal was to test the versatility of the second-generation covalent TMP-tag for labeling diffuse cellular protein targets. Tomm20, a mitochondrial localized protein, was chosen as an organelle target in HEK 293T cells. Myosin light chain (MLC) and α-actinin, two cytoplasmic proteins, were chosen as cell skeleton targets in fibroblast cells. Cells expressing eDHFR:L28C fusions were treated with 1 μ M A-TMP-Fl v2.0 for 10 min and then examined using a confocal microscope. In each scenario, H2B-mCherry fusion protein was cotransfected. The fluorescence images indicate that all three eDHFR:L28C fusion proteins could be successfully labeled (Figure 5A). To further characterize the labeling specificity, cells expressing the different eDHFR:L28C fusions were lysed after 10 min

treatment of 1 μ M A-TMP-Fl v2.0. The lysates were subjected to SDS-PAGE and analyzed by in-gel fluorescence with a 488 nm laser. In all cases, a single main fluorescent band of the expected molecular weight was observed (Figure 5B), indicating a rapid labeling with high specificity among all the examined cellular protein targets in different mammalian cell lines.

With A-TMP-Fl v2.0 in hand, we performed labeling experiments to evaluate the performance of this covalent TMP-tag compared to noncovalent TMP-tag (Figure 6). HEK 293T Cells transiently expressing Tomm20-eDHFR and Tomm20-eDHFR:L28C were labeled with TMP-Fl[17] and A-TMP-Fl v2.0 for 10 min, respectively. In both experiments H2B-mCherry was cotransfected as a counter stain. After washing with fresh media, cells were imaged with a confocal microscope. While noncovalent TMP-tag exhibits a similar labeling specificity in live cell imaging experiments with the covalent TMP-tag, the labeling pattern could not be distinguished 12 h after paraformaldehyde fixation. In contrast, the labeling of Tomm20-eDHFR:L28C with A-TMP-Fl v2.0 withstands the fixation protocol, which potentially facilitates the applications of novel microscopic studies requiring long acquisition time.

Finally, we prepared A-TMP-Atto 655 and A-TMP-Dapoxyl to demonstrate the adaptability of second-generation covalent TMP-tag over novel fluorophores for potential advanced imaging applications. Atto 655 has been demonstrated as an ideal organic fluorophore for live-cell super-resolution imaging due to its unique cellular-environment-compatible photo-switching mechanism.[14] After 3 h incubation of 1 μ M A-TMP-Atto 655 with HEK 293T cells transiently expressing H2B-eDHFR:L28C or plasma membrane-targeted eDHFR:L28C (PMLS-eDHFR:L28C),[9] selective labeling could be observed in both cases with confocal microscopy (Figure 5A). Dapoxyl dye, since its invention,[35] has been gaining growing attention due to its large and environmentally sensitive Stokes shift.[36] A-TMP-Dapoxyl was tested in labeling experiments with H2B-eDHFR:L28C as well as plasma membrane targeted eDHFR:L28C. Organelle-specific fluorescence images were obtained using confocal microscopy with a 405 nm excitation laser (Figure 7B). To test the labeling efficiency of the second-generation covalent TMP-tag, HEK 293T cells expressing eDHFR:L28C-6X His were incubated with media containig 1 μ M A-TMP-Dapoxyl. At certain time points, cells were harvested, lysed, analyzed by SDS-PAGE, and blotted with Anti-6X His. According to the band shift, near-quantitative labeling was achieved under the condition of 3 h incubation (Figure 7C). These labeling assays pave the way toward the development of novel biophysical, physiological, and multicolor pulse–chase applications with TMP-tag.

DISCUSSION

Together these results establish that, by improving the design and hence the reaction half-life of our covalent TMP-tag, the v2.0 covalent TMP-tag is now a robust and general reagent for live cell imaging. The covalent TMP-tag design was improved by optimizing the spatial positioning of the Michael addition pair—the engineered Cys residue on eDHFR and the acrylamide conjugated to the A-TMP-probe heterotrimer. While numerous chemical tags have been reported, our v2.0 covalent TMP-tag is one of the few examples that is selective enough to enable high signal-to-noise imaging of intracellular proteins, particularly diffuse, cytoplasmic proteins. Impressively, this speed and selectivity is achieved not from enzyme catalysis, but rather from ligand–receptor binding followed by a proximity-induced organic reaction using a well-chosen mild electrophile. To date, the v2.0 covalent TMP-tag has been successful for a variety of protein targets and mammalian cell lines, and we expect it to be broadly useful to the community for imaging a wide range of proteins in living cells.

From a chemical perspective, proximity-induced reactions offer a combination of reactivity and specificity, which are both critical for protein labeling in live cells. Traditional protein-conjugation reagents such as maleimide electrophiles, which are designed for labeling purified proteins, cannot provide the desired selectivity for labeling proteins within the cell. More recently introduced bio-orthogonal reactions, such as the copperless click reaction[37] or the photoinduced reactions which are triggered by UV-irradiation,[38] require unnatural amino acid incorporation,[39] and are technically demanding and/or damaging to cells. In comparison, the noncovalent eDHFR-TMP interaction specifically accelerates the covalent reaction between the engineered Cys on eDHFR and the acrylamide electrophile. This approach, conceptually resembling the biomolecule-templated organic reactions,[40] expands the scope of bioconjugation reactions as well as synthetic biology.

From an engineering point of view, proximity-induced reactions are facile implements for the development of novel chemical tags. In the case of the covalent TMP-tag, the specificity between ligand (A-TMP-probe) and receptor (eDHFR) is guaranteed by high-affinity enzyme–inhibitor recognition as opposed to heavy-metal chelations (FlAsH/ReAsH)[5] or additional enzyme-catalyzed reactions (PRIME).[10] Significantly, the covalent TMP-tag, which is based on high-affinity binding, exhibits superior specificity and efficiency that enables intracellular protein labeling with minimal background. Using similar approaches, the vast pool of bioactive natural products and hit compounds from combinatorial libraries could be potentially engineered into orthogonal chemical tags based on proximity-induced reactions.[41]

With an *in vitro* labeling half-life of 8 min, the second-generation covalent TMP-tag is significantly improved over our first-generation design.[20] Although the reaction rate is slower than the suicide-substrate-based tags, e.g. SNAP tag,[6] we consider the rate difference of little practical significance given that it typically requires 10 min to over an hour to label proteins in living cells, with uptake of the organic fluorophore considered to be the rate-limiting step. If needed, however, the labeling reaction kinetics could likely be further optimized by either molecular engineering of the small-molecule ligand or directed evolution of eDHFR, or both. Notably, an advantage to a chemical tag based on high-affinity binding is that it does not require the high concentration of ligand–probe conjugate necessary with enzyme-based chemical tags, where K_M's typically range from micromolar to millimolar, leading to high background noise from unbound fluorophore and necessitating extensive washing steps.

The second-generation covalent TMP-tag reported here is seen as a pressing improvement of the TMP-tag toward advanced protein-labeling applications. With its improved labeling kinetics and well-demonstrated cellular behavior, one might be able to track single-protein molecules inside a cell[42] with a fluorophore of high photon count. Moreover, the viability and robustness of the second-generation covalent TMP-tag point the way to multicolor protein labeling using orthogonal chemical tags.

CONCLUSION

By improving the reaction geometry of our covalent TMP-tag, we now have a v2.0 covalent TMP-tag that is a robust cellular reagent. This v2.0 covalent TMP-tag is an important addition to the limited arsenal of orthogonal covalent chemical tags available for multicolor imaging. Because our covalent TMP-tag is based on a modular organic reaction rather than a specific enzyme modification, we expect to be able to more readily build additional features into the covalent TMP-tag and generate new orthogonal tags simply by extending the Michael addition reaction to other drug–receptor pairs. While used here for live cell imaging, the covalent TMP-tag can be used broadly as a biotin–avidin surrogate for *in vitro*

applications or in other applications of chemical dimerizers in live cells. Beyond the utility of proximity-induced reactivity for chemical tag engineering, the excellent reactivity and specificity of the proximity-induced Michael addition in a live cell illustrates the potential of organic chemistry for synthetic biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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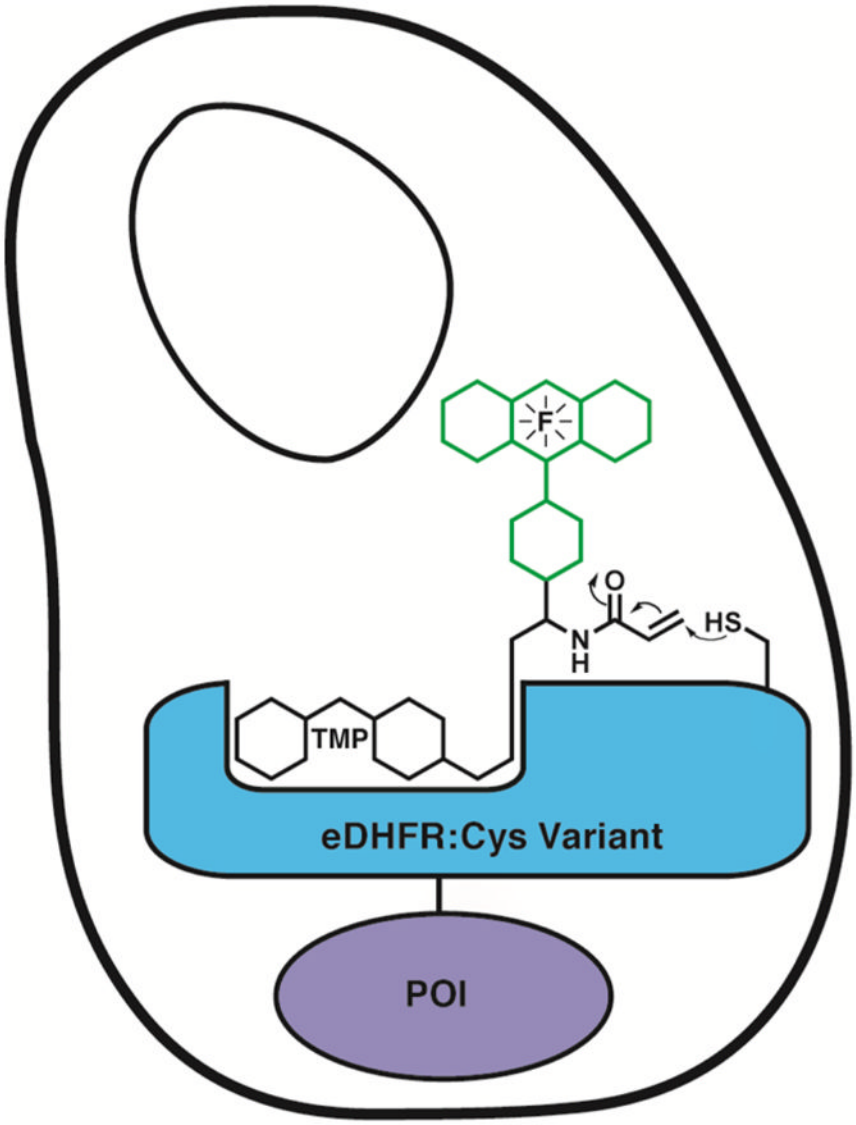


Figure 1.

Schematic representation of the covalent TMP-tag design. Previously, we demonstrated that the noncovalent TMP-tag, which exploits the high-affnity, selective interaction between trimethoprim (TMP) and *E. coli* dihydrofolate reductase (eDHFR), could be rendered covalent by proximity-induced reaction between a Cys residue engineered on the eDHFR surface and a mild acrylamide electrophile installed on the TMP-fluorophore probe. Here, by optimizing the positioning of the Cys nucelophile and the acrylamide electrophile, we achieve rapid covalent labeling of the eDHFR tag by the TMP-fluorophore probe, rendering the covalent acrylamide TMP-tag (A-TMP-tag) a robust reagent for live cell imaging.

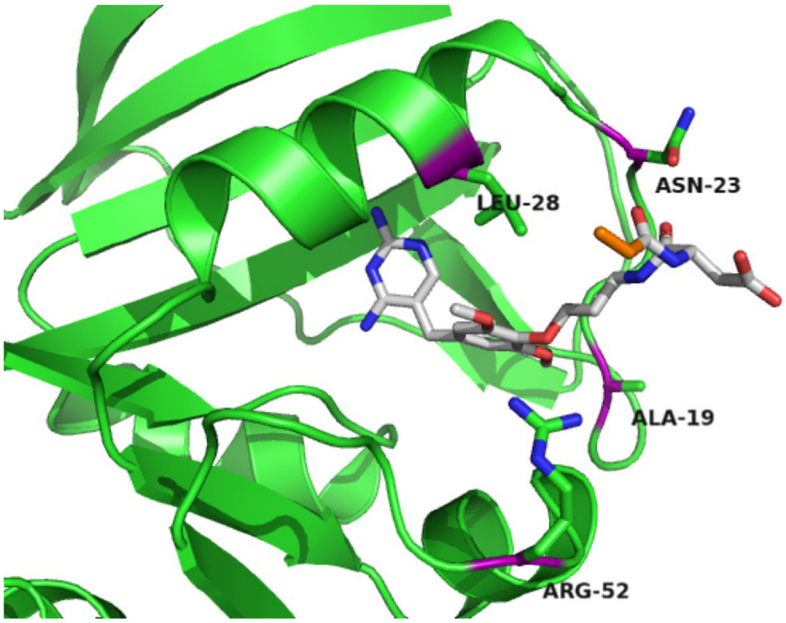


Figure 2.

Design of the optimized, second-generation covalent A-TMP-tag. The acrylamide elecrophile on the TMP-fluorophore probe and the Cys nucleophile on the eDHFR surface were redesigned to bring the two in close proximity to achieve a rapid reaction half-life. Depicted is a cartoon of a second-generation A-TMP molecule (stick representation, electrophile highlighted in orange) with a 10-atom spacer between the TMP ligand bound in the active site of eDHFR (green ribbon diagram) and the acrylamide electrophile with the four residues chosen for mutation to Cys highlighted (stick representation, α carbon highlighted in purple). Since there is no reported high-resolution structure of TMP bound to eDHFR, this model was created by structurally aligning a high-resolution structure of *E. coli* DHFR[28] to a high-resolution structure of TMP bound to *L. casei* DHFR.[29] The acrylamide-TMP structure was built in Maestro[30] and then superimposed on TMP in the eDHFR model. The graphic was prepared using PyMOL.[31].

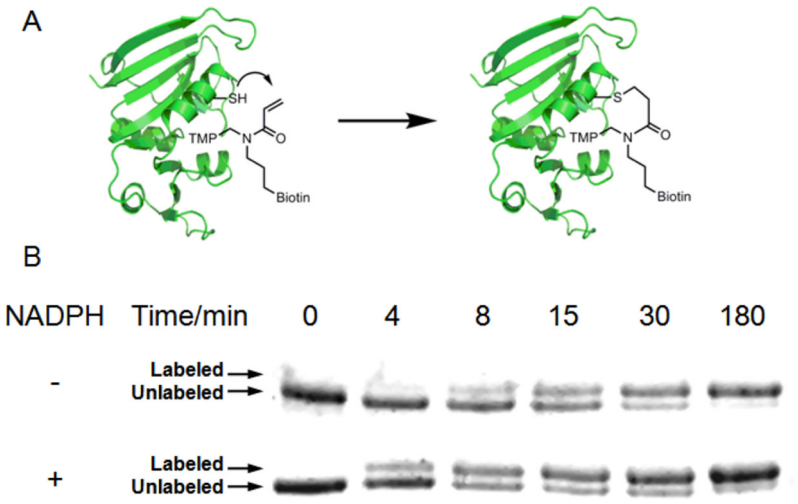


Figure 3.

Determination of the rate of covalent labeling between A-TMP-biotin v2.0 and eDHFR:L28C *in vitro*. (A) Illustrative reaction scheme of the proximity-induced Michael addition of the thiol nucleophile of L28C to the acrylamide electrophile of A-TMP-biotin v2.0. (B) Analysis of the labeling reaction between A-TMP-biotin v2.0 and eDHFR:L28C by SDS-PAGE. Purified eDHFR:L28C (5 μ M) was incubated with A-TMP-biotin v2.0 (10 μ M) in PBS buffer (pH 7.4) with reduced glutathione (1 mM) at 37 °C, with or without NADPH (50 μ M). At proper time points, aliquots (20 μ L) were removed from the reaction mixture, quenched with 6× SDS and boiled for 5 min. The time point aliquots were then analyzed by SDS-PAGE and Coomasie staining. Conveniently, covalent modification of eDHFR:L28C gave rise to a gel shift such that the reaction progress could be readily measured by densitometry analysis of Coomassie stained gels using Image-J. The labeling half-life was determined by linear regression, applying the pseudo-first-order model onto the ratio of the substrate and product. eDHFR:L28C was found to react with A-TMP-biotin v2.0 with a half-life of 8 min at these physiologically relevant conditions.

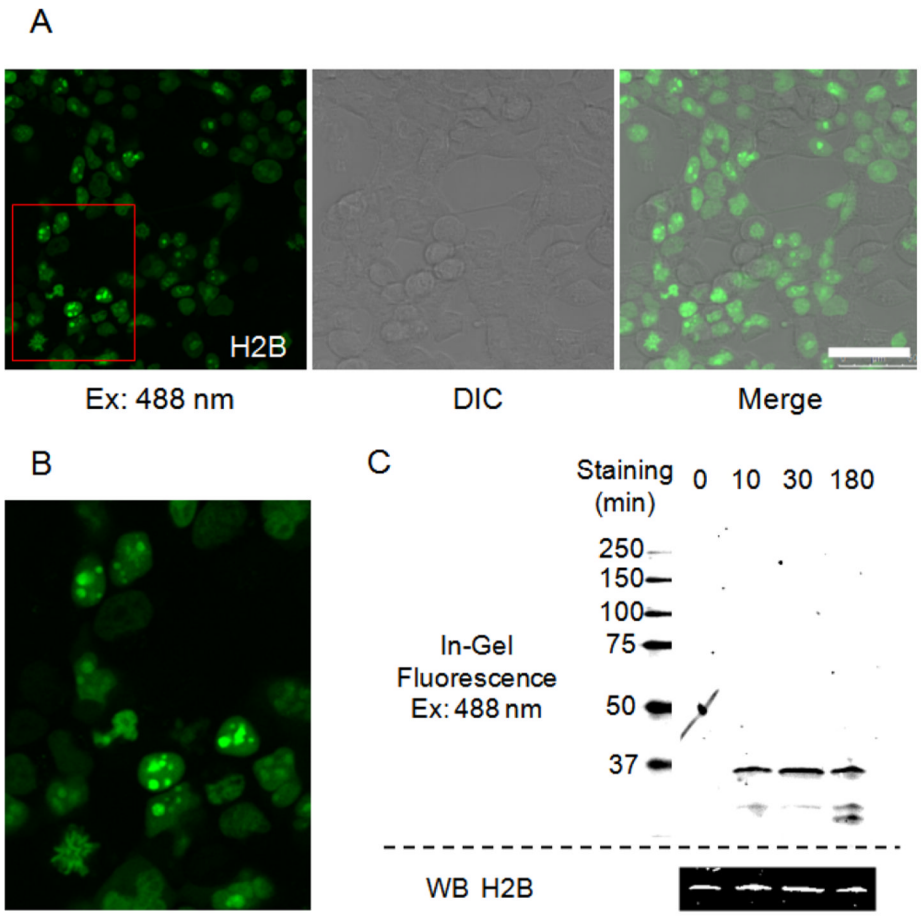


Figure 4.

Labeling of H2B with the covalent A-TMP-fluorescein v2.0 in HEK293T cells. (A) Microscopic evidence of successful labeling of H2B tagged with eDHFR:L28C (H2B-eDHFR:L28C) by A-TMP-fluorescein v2.0 in human embryonic kidney (HEK) 293T cells. HEK293T cells transiently transfected with a vector encoding the H2B-eDHFR:L28C fusion protein were incubated with 1 μ M A-TMP-fluorescein v2.0 in the appropriate media for 10 min, washed twice, and then directly imaged using confocal fluorescence and differential interference contrast (DIC) microscopy. Fluorescein was excited at 488 nm. Scale bars are 50 μ m. (B) Zoom-in view of the fluorescence image shown in (A). (C) In-gel fluorescence and Western blot analysis of the labeling reaction. Labeled HEK 293T cells were lysed and then analyzed by SDS-PAGE and in-gel fluorescence scanning with an excitation laser at 488 nm. Together, these results provide evidence that A-TMP-fluorescein v2.0 labels eDHFR:L28C tagged H2B rapidly, selectively, and covalently.

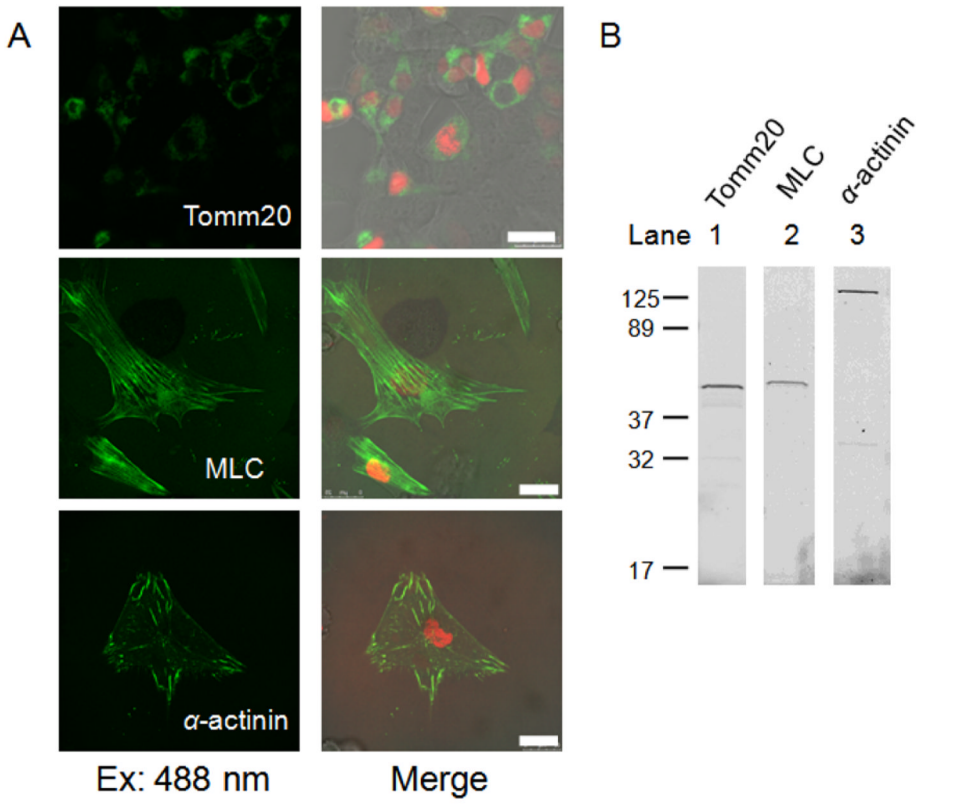


Figure 5.

Labeling of diffused protein targets with covalent A-TMP-Fl v2.0. Three different proteins, Tomm20, MLC, and α-actinin, were successful labeled and imaged in two different mammalian cell lines. (A) Microscopic imaging of A-TMP-tag v2.0 labeling. HEK293T cells (for Tomm20) or mouse embryonic fibroblast (MEF) cells (for MLC and α-actinin) transiently cotransfected with vectors encoding POI-eDHFR:L28C and H2B-mCherry fusion proteins, respectively, were incubated with 1 μ M A-TMP-fluorescein v2.0 in media for 10 min, washed twice with media, and directly imaged using confocal and differential interference contrast (DIC) microscopy. Fluorescein was excited at 488 nm, mCherry was excited at 594 nm. Scale bars are 25 μ m. (B) In-gel fluorescence analysis of A-TMP-Fl v2.0 labeling. The cells transfected with corresponding POI-eDHFR:L28C vectors were harvested after 10 min incubation with 1 μ M A-TMP-fluorescein v2.0, lysed, and analyzed by SDS-PAGE and in-gel fluorescence scanning with an excitation laser at 488 nm. These results show the target versatility of the second-generation covalent TMP-tag for live cell protein labeling.

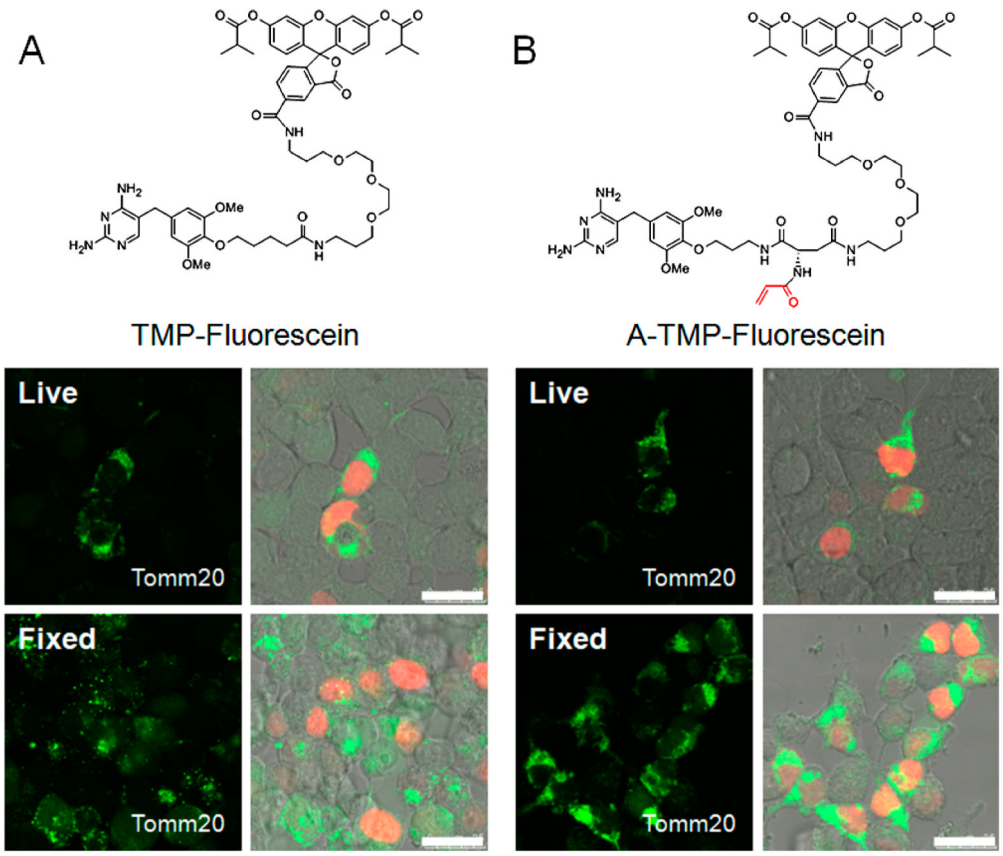


Figure 6.

Comparative studies of labeling with noncovalent TMP-tag and covalent TMP-tag. (A) HEK 293T cells expressing Tomm20-eDHFR were labeled with TMP-Fl as in Figure 5A. After labeling, cells were imaged using confocal microscope (Live panel) or fixed with 4% paraformaldehyde in PBS for 10 min followed by washing with PBS for 12 h before imaged (Fixed panel). (B) HEK 293T cells expressing Tomm20-eDHFR:L28C were labeled with A-TMP-Fl and examined with and without fixation treatment as in (A). Scale bars are 25 μ m.

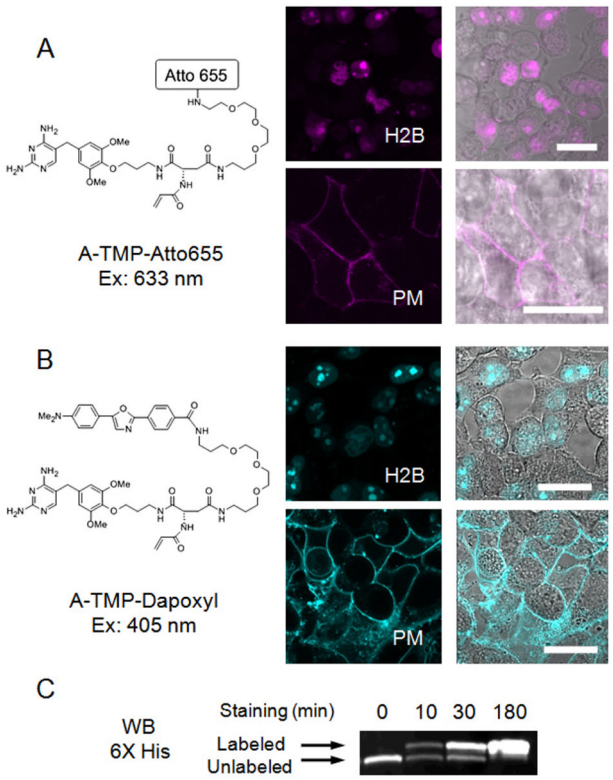
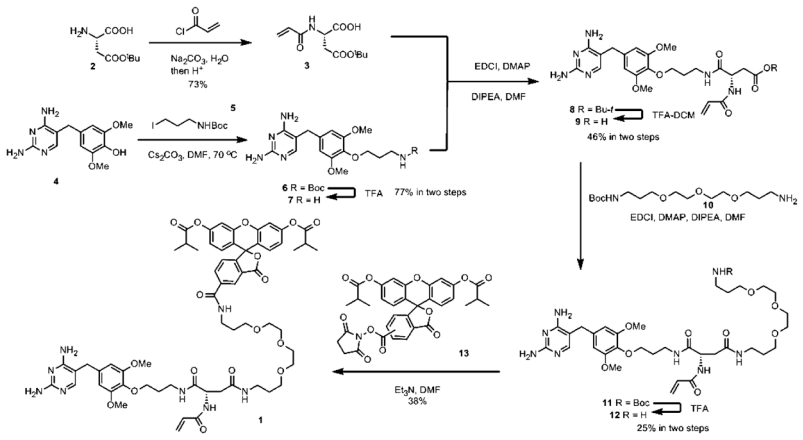


Figure 7.

Labeling of cellular protein targets with A-TMP-Atto655 and A-TMP-Dapoxyl. (A) HEK293T cells transiently expressing H2B-eDHFR:L28C or PMLS-eDHFR:L28C were incubated with 1 μ M A-TMP-Atto 655 in media for 3 h, washed twice, and imaged using confocal and differential interference contrast (DIC) microscopy. Atto 655 was excited at 633 nm. (B) A-TMP-Dapoxyl was tested under the same conditions as in (A), except Dapoxyl was excited at 405 nm. Scale bars are 25 μ m. (C) Western blot analysis of the labeling efficiency. HEK293T cells transiently expressing eDHFR:L28C-6X His were incubated with 1 μ M A-TMP-Dapoxyl in media for 10 min, 30 min, and 3 h before being lysed and analyzed by SDS-PAGE/Western blot with 6X His antibody.



Scheme 1.

Synthesis of Optimized Acrylamide-TMP-Fluorescein Heterotrimer (A-TMP-fluorescein v2.0, compound 1)

Table 1

Reaction Half-Lives [min] of A-TMP-Biotin v2.0 with eDHFR:Cys variants[a]

eDHFR variants		L28C	N23C	A19C	R52C
NADPH	−	17	35	330	130
NADPH	+	8	20	100	220

[a]Purified eDHFR:Cys variant was labeled under the same conditions as in Figure 3B.