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Design, Synthesis, and Application of the Trimethoprim-Based Chemical Tag for Live-Cell Imaging

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ABSTRACT

Over the past decade, chemical tags have been developed to complement the use of fluorescent proteins in live-cell imaging. Chemical tags retain the specificity of protein labeling achieved with fluorescent proteins through genetic encoding, but provide smaller, more robust tags and modular use of organic fluorophores with high photon output and tailored functionalities. The trimethoprim-based chemical tag (TMP-tag) was initially developed based on the high affinity interaction between *E. coli* dihydrofolate reductase and the antibiotic trimethoprim and was subsequently rendered covalent and fluorogenic via proximity-induced protein labeling reactions. To date, the TMP-tag is one of the few chemical tags that enable intracellular protein labeling and high-resolution live-cell imaging. Here we describe the general design, chemical synthesis, and application of TMP-tag for live-cell imaging. Alternate protocols for synthesizing and using the covalent and the fluorogenic TMP-tags are also included. *Curr. Protoc. Chem. Biol.* 5:131-155 © 2013 by John Wiley & Sons, Inc.

Keywords: chemical tag • fluorescence microscopy • live-cell imaging • protein label

INTRODUCTION

Over the past two decades, the development of fluorescent proteins (FPs) as selective, genetic protein tags has revolutionized the way protein function and dynamics is studied in intact cells (Chalfie et al., 1994; Heim et al., 1994). The original green fluorescent protein (GFP) from *A. victoria* is a 238-amino acid protein, which, upon folding, spontaneously forms a fluorescent chromophore by rearrangement and oxidation of Ser, Tyr, and Gly residues in the core of the eleven-stranded β-barrel (Ormo et al., 1996; Tsien, 1998). Since these original reports, naturally occurring and engineered FPs have been routinely used to observe the timing and location of protein expression in living cells, often providing significant mechanistic insight (Tsien, 1998; Giepmans et al., 2006; Shaner et al., 2007). Although FPs continue to be invaluable tools for cell biology, they have limitations for more sophisticated biophysical and mechanistic studies. Thus, chemical tags have been developed as an alternative for labeling proteins with modular organic fluorophores directly in living cells, opening the possibility of labeling proteins with fluorophores that have high photon output and/or special photophysical properties (Jing and Cornish, 2011).

With chemical tags, the protein is tagged with a polypeptide, which is subsequently modified with an organic fluorophore (Griffin et al., 1998; Keppler et al., 2003; Fernandez-Suarez et al., 2008). The trimethoprim-based chemical tag (TMP-tag, Fig. 1A) relies on the high-affinity interaction between *E. coli* dihydrofolatereductase (eDHFR) and the folate analog trimethoprim (TMP) (Miller et al., 2005; Calloway et al., 2007). In brief, the target protein is tagged with eDHFR, which binds TMP with high affinity (~1 nM K_D) and selectivity (affinities for mammalian DHFRs are $K_D > 1$ μM) (Roth et al.,

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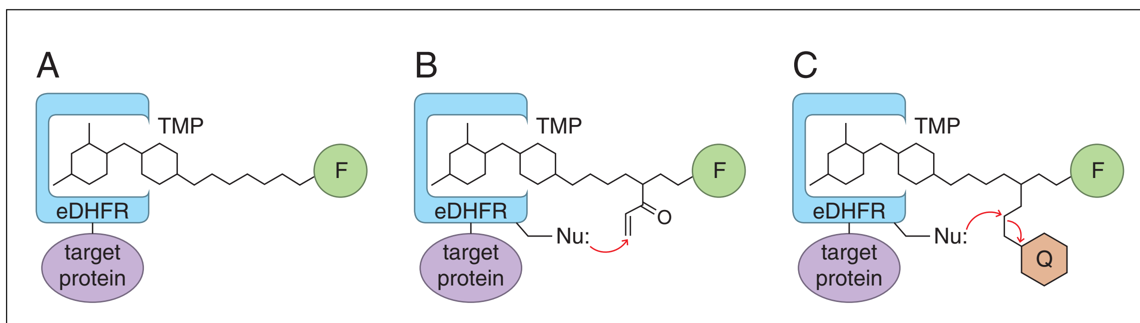

Figure 1 TMP-tags. (**A**) Noncovalent TMP-tag is based on the high-affinity interaction between *E. coli* dihydrofolate reductase (eDHFR) and the antibiotic trimethoprim (TMP). The target protein (purple) is tagged with eDHFR (blue) and then labeled with a cell-permeable TMP-fluorophore (F) heterodimer. (**B**) The TMP-tag is rendered covalent by installing a nucleophilic amino acid near the binding pocket to react with a latent electrophile (acrylamide) when TMP binds to eDHFR. (**C**) The fluorogenic TMP-tag centers a TMP-quencher (Q) –fluorophore (F) heterotrimer. When TMP binds to eDHFR, the nucleophilic amino acid near the binding pocket initiated a proximity-induced S_N2 reaction that cleaves the electrophile attached to the quencher and thus switches on the fluorophore.

1981; Baccanari et al., 1982). Organic fluorophores can be conjugated to TMP with only minor perturbation of the high-affinity binding to eDHFR (Miller et al., 2005). Among numerous chemical tags reported in the past decade, the TMP-tag is one of the few that can label proteins with high efficiency and selectivity, both in vitro and inside of live cells.

To complement the use of the original noncovalent TMP-tag, a covalent variant was engineered by installing a unique Cys residue on eDHFR in position to react with an acrylamide electrophile added to the TMP-probe molecule (Fig. 1B) (Gallagher et al., 2009). Upon optimization, the second-generation covalent TMP-tag undergoes rapid, quantitative covalent labeling (in vivo $t_{1/2}$ ∼8 min) and enables imaging of various intracellular proteins with distinct subcellular localization (Chen et al., 2012). Moreover, the covalent TMP-tag has also been demonstrated to label cellular proteins with a series of fluorophores of different colors and enable specific labeling in both live and fixed cells. See Strategic Planning for a discussion of advantages of the various types of covalent versus noncovalent tags.

The TMP-tag has also recently been rendered fluorogenic (also covalently linked to the target protein) via a proximity-induced S_N2 reaction initiated by the same Cys nucleophile on eDHFR, replacing an electrophilic leaving group attached to a quencher in the TMP-quencher-fluorophore heterotrimer (Fig. 1C) (Jing and Cornish, in press). In vitro characterization confirmed the covalent labeling and high specificity and efficiency of fluorescence switching, with in vitro $t_{1/2}$ of ∼10 min and 20-fold fluorescence enhancement. In live-cell imaging experiments the fluorogenic TMP-tag displays significantly reduced background fluorescence from unbound or nonspecifically bound fluorophores and thus increases the signal-to-background ratio, which is crucial for high-resolution imaging. Notably, the fluorogenic TMP-tag achieved the demanding goal of not only labeling highly abundant, localized intracellular proteins, but also less abundant, more dynamic cytoplasmic proteins.

The TMP-tag labels intracellular proteins with modular synthetic probes, enabling sophisticated biophysical experiments that are difficult or impossible with FPs. The TMP-tag has been successfully applied for super-resolution imaging of histone dynamics in live cells (Wombacher et al., 2010); single-molecule detection of spliceosome assembly in yeast cell extract (Hoskins et al., 2011); and chromophore-assisted laser inactivation of intracellular proteins (Cai et al., 2010). Beyond fluorescence microscopy, the TMP-tag has also been used to construct chemically induced protein dimers and has been

conjugated to magnetic nanoparticles for protein and cell manipulation (Czlapinski et al., 2008; Long et al., 2011). Several examples of biological applications are discussed in the Commentary.

In this unit we present the general design, as well as detailed protocols for synthesizing and using the TMP-tag for live-cell imaging. In the Strategic Planning section a generic workflow is laid out with focus on choosing the appropriate TMP-tag for specific biophysical applications. The Basic Protocol then describes the synthesis and application of the noncovalent TMP-tag with a far-red fluorescent probe. Two variations, the synthesis and application of covalent and fluorogenic TMP-tags with different fluorophores, are provided in Alternate Protocols. Since the TMP-tag is modular, these protocols can be readily adapted to incorporate virtually any synthetic probe. Examples of biological applications of TMP-tag along with potential variations on critical parameters are covered in the Commentary section.

STRATEGIC PLANNING

Technically, labeling a target protein with the TMP-tag is very similar to labeling with FPs (Fig. 2): a plasmid encoding a fusion target protein with eDHFR is constructed using standard molecular cloning techniques and is then introduced into the desired cell. The transfected cells are briefly incubated with the TMP-fluorophore conjugate, which diffuses into cells and is then specifically bound by or conjugated to the eDHFR tag. Together, the TMP-tag retains the specificity of protein labeling achieved with FPs through genetic encoding, but provides a slightly smaller, more robust tag and allows modular use of organic fluorophores with high photon output and tailored functionalities.

Choice of TMP-Tags

Three types of TMP-tags have been developed to enable selective protein labeling both in vitro and inside of living cells: the noncovalent TMP-tag, the covalent TMP-tag, and the covalent fluorogenic TMP-tag. Choice of the appropriate form of TMP-tag depends on the specific biological application.

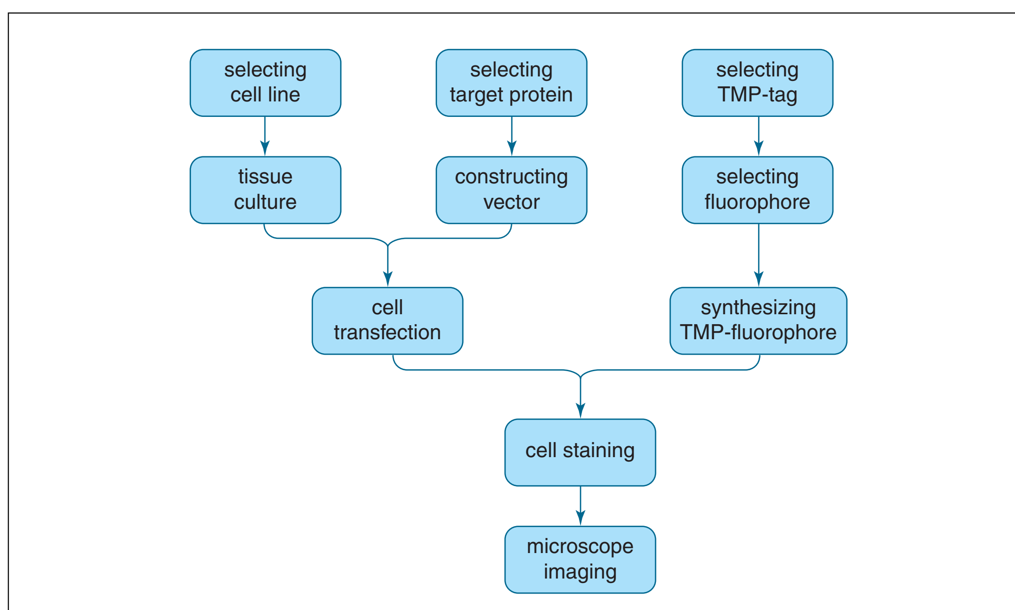

Figure 2 General workflow of live-cell imaging with TMP-tag.

To tag a protein using the noncovalent TMP-tag, the target protein is fused to wild-type eDHFR, which binds TMP-fluorophore via noncovalent interaction (Fig. 1A). The advantage of noncovalent TMP-tag is that the TMP-fluorophore is straightforward to prepare and optimize. Important for adoption by laboratories not specializing in organic synthesis, some TMP-fluorophore heterodimers are commercially available from Active Motif. The noncovalent TMP-tag has been demonstrated to be a robust reagent for a variety of live cell imaging applications (see Commentary for examples). Nevertheless, because the TMP-fluorophore slowly dissociates from eDHFR (dissociation $t_{1/2} \sim$ tens of minutes), the fluorescence signal from a noncovalent TMP-tag gradually diminishes after washout. Therefore, to monitor a target protein in live cells for a long period of time (>24 hr), it is recommended to use the covalent TMP-tag instead. Additionally, the noncovalent TMP-tag is not compatible with cell fixation or other treatment that may interrupt the TMP/eDHFR interaction.

Creation of the covalent TMP-tag relies on the proximity-induced Michael addition between the eDHFR:L28C variant and the acrylamide-TMP-fluorophore heterotrimer (A-TMP-fluorophore). After A-TMP-fluorophore binds to eDHFR:L28C, the Cys nucleophile specifically attacks the acrylamide group to create a covalent bond (Fig. 1B). The covalent TMP-tag is especially favorable for applications that require permanent labeling, such as time-lapse imaging, pulse-chase experiments, pull-down assays, and fixed-cell imaging. On the other hand, the covalent TMP-tag with high reactivity is currently not commercially available and several extra steps are required to prepare both A-TMP-fluorophore and eDHFR:L28C vector, compared to the noncovalent TMP-tag.

The fluorogenic TMP-tag centers a trimeric TMP-quencher-fluorophore molecule (TMP-Q-fluorophore) with the quencher attached to an electrophilic leaving group that, upon TMP binding to eDHFR:L28C, would be cleaved by the Cys nucleophile (Fig. 1C). The fluorogenic TMP-tag provides a covalent label with reduced background fluorescence, which is particularly useful for high-resolution live-cell imaging. Significantly, the fluorogenic design minimizes nonspecific background staining of cellular compartments often observed for the positively charged, high photon-output fluorophores (see following section). For maximal efficiency and signal-to-background, the fluorogenic TMP-tag needs to be carefully designed and optimized. Additionally, the fluorogenic TMP-tag tends to hydrolyze in alkaline conditions and thus requires specific precautions during synthesis and handling (see Alternate Protocol 2 for details).

Choice of Fluorophores

Although hundreds of organic fluorophores have been developed for in vitro biophysical studies, very few of them are suitable for live-cell imaging. Generally, a robust fluorophore for high-resolution live-cell imaging should have (1) sharp excitation and emission spectra in the visible or infra-red (IR) region; (2) high photon output (the total number of photons emitted by a fluorophore before photo-bleaching); (3) photo-physical properties compatible with the cellular environment; and (4) excellent cell permeability and, perhaps even more important although less well understood, specific behavior once inside the cell, i.e., not binding to cellular components or partitioning to lipid-rich or charged compartments. For most commercially available fluorophores, photo-physical data such as excitation and emission spectra, extinction coefficient and quantum yield are available in literature (Lavis and Raines, 2008; Dempsey et al., 2011) or from suppliers. The cell permeability of a dye and its behavior inside cells, on the other hand, usually needs to be evaluated practically (Longmire et al., 2008).

An ideal fluorophore for live-cell imaging should have sharp excitation and emission spectra in the visible to IR region with a large Stokes shift (>20 nm). Ultraviolet (UV)

light can be used for excitation but may cause photo-damage to cells. Far-red and IR fluorophores are favorable for deep tissue imaging. However, some commercial fluorescence microscopes do not have built-in IR lasers and detectors and thus need to be customized for fluorophores with λ_{ex} exceeding 700 nm. For multi-color imaging it is desirable to use a set of spectrally resolvable fluorophores. If spectral crosstalk is unavoidable, a multispectral imaging fluorescence microscope can help to distinguish fluorophores with overlapping excitation and emission spectra while potentially compromising temporal and/or spatial resolution.

A high photon output dye is crucial for high-resolution live-cell imaging, especially when labeling low-abundance cytosolic proteins. Unfortunately, rigorous and systematic evaluations of total photon output under conditions compatible with live-cell imaging have only been reported for very few synthetic fluorophores (Kasper et al., 2007). In practice, most organic fluorophores are characterized in terms of the extinction coefficient (ε), the quantum yield (QY), photon flux (number of photons emitted during a given period of time), and photo-stability (measured as survival rate after a certain period of time or photo-bleaching decay constant). For most commercially available fluorophores, ε and QY values are available from suppliers. The photon flux and photo-stability data, on the other hand, strongly depend on the experimental setup (laser power, buffer, O_2 concentration, etc.) and therefore are only comparable if two dyes are measured under exactly the same conditions (Dempsey et al., 2011). In general, the cyanine dyes (e.g., Cy3, Cy5, Alexa647), rhodamines (e.g., Rhodamine 6G, Tetramethylrhodamine, TexasRed), and oxazine dyes (e.g., Atto655, Atto680) are considered to be among the brightest and most photo-stable fluorophores, while some organic fluorophores, e.g., fluorescein, may have remarkable ε and QY but are prone to photo-bleaching, and thus are not recommended for single-molecule imaging or particle tracking.

Unlike photo-physical parameters, there is no well-established measurement for cell permeability or dye behavior inside of cells, and these properties vary significantly between cell lines. Generally, a small hydrophobic probe with positive charge or no net charge under physiological conditions is potentially cell permeable. Therefore, it is recommended to transform the carboxyl groups to esters that can be enzymatically cleaved inside live cells (e.g., see Alternate Protocol 1). Notably, a hydrophobic probe with positive charge may be readily cell permeable, but tends to aggregate and bind to subcellular compartments and thus develop a high level of background fluorescence. In such case, it is suggested to use the fluorogenic TMP-tag that minimizes nonspecific staining.

Finally, the photo-physical properties and biological activities of a fluorophore may be significantly influenced by the chemical tag, target protein, and cell line used. For a particular biological application, a trial-and-error process may be necessary to find the best chemical tag-dye combination. Therefore, it is recommended to start with TMP-tags that have been proven to label intracellular proteins with high signal-to-background (see Table 1). When trying a new fluorophore, a general guide for optimization is provided in the Commentary.

Construction of Target Protein-eDHFR Fusion

The eDHFR label can be fused to target protein on either N- or C-terminus, whichever does not interrupt the function and dynamics of the target protein. A flexible linker such as Gly- and Ser-rich sequence $(GSG)_n$ is usually placed between the eDHFR and target protein. The vector DNA encoding the target protein-eDHFR fusion can be constructed using standard molecular cloning techniques as outlined below. The eDHFR gene is available from multiple commercial sources (e.g., Addgene, cat. no. 20214; Active Motif, cat. no. 34001).

Cell Transfection

A variety of commercial reagent kits are available for both transient and stable transfection of mammalian cells. For a particular cell line, the transfection protocol needs to be individually optimized following the manufacturer's instruction. We recommend FuGene HD transfection kit for HEK 293T, HeLa, and COS7 cells, while for NIH 3T3, MEF, and CV1 cells electroporation usually yields much higher transfection efficiency than transfection reagents. A plasmid encoding nuclear-localized GFP (such as H2B-EGFP) is a useful control for optimizing the transfection protocol. Generally speaking, once a transfection protocol is optimized for a certain cell line, it should allow various kinds of plasmids to be introduced. When labeling cytosolic proteins with the TMP-tag it is also recommended to co-transfect the cells with H2B-EGFP or H2B-mCherry, whichever is spectrally resolvable with the TMP-tag, to monitor the transfection efficiency. The transfection reagents do not discriminate plasmids with different sequences. If the target protein-eDHFR and H2B-FP are encoded by plasmids with similar sizes (5 to 10 kb), controlled by the same promoter (e.g., CMV promoter), they are expected to be expressed at similar levels.

BASIC PROTOCOL

SYNTHESIS AND APPLICATION OF NONCOVALENT TMP-TAG FOR LIVE-CELL IMAGING

This protocol describes the synthesis of noncovalent TMP-Atto655 (compound **1**), the construction of an H2B-eDHFR vector, and live cell imaging in HEK 293T cells. The chemical synthesis is summarized in Figure 3. The general procedures also apply to other fluorophores, target proteins and cell lines, potentially with some modification in details, as discussed in the Strategic Planning and Commentary sections.

Materials

Hydrobromic acid (HBr)
Trimethoprim (Sigma-Aldrich, cat. no. T7883)
Sodium hydroxide (NaOH)
Millipore-purified water
Ammonium hydroxide (28% in water, w/w)
Anhydrous *N*,*N*-dimethylformamide (DMF) in sure-seal bottle
Potassium carbonate (K_2CO_3)
Ethyl 5-bromovalerate
Compressed Ar
Brine (saturated NaCl water solution)
Ethyl acetate
Methanol (MeOH) for chromatography
Dichloromethane (DCM) for chromatography
Silica gel
Deuterated methanol (CD_3OD); used to take NMR spectra
Hydrochloric acid (HCl)
Deuterated dimethyl sulfoxide (DMSO-d6); used to take NMR spectra
Propane flame
Boc-1-amino-4,7,10-trioxa-13-tridecanamine (BocNH-PEG-NH_2), CAS: 194920-62-2
Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)
N,N-Diisopropylethylamine (DIEA)
Trifluoroacetic acid (TFA)
Atto655-NHS ester (Sigma-Aldrich, cat. no. 76245)
Acetonitrile (MeCN) for HPLC
Dimethyl sulfoxide (DMSO)

Phosphate-buffered saline (PBS; see recipe)
PCR primers:
Forward: 5′ACGTCACCGGTCGCCACCATGGTGGGTTCTGGTGGTTCTGG TATCAGTCTGATTGCGGCG3′
Reverse: 5′ACGTCGCGGCCGCTTTAGTGATGGTGATGGTGATGCCGCCG CTCCAGAATCT3′
H2B-GFP vector (Addgene, cat. no. 11680)
*Age*I and *Not*I restriction endonucleases
Agarose for electrophoresis
QIAquick Gel Extraction kit (Qiagen, cat. no. 28704)
T4 DNA ligase
E. coli (TG1) competent cells (Lucigen, cat. no. 60502)
LB agar plates with 50 mg/liter kanamycin
Plasmid mini-prep and maxi-prep kits (Qiagen, cat. nos. 27106 and 12663)
HEK 293T cells
Dulbecco's modified Eagle medium (DMEM; Invitrogen, cat. no. 11995)
Fetal bovine serum (FBS)
100× penicillin/streptomycin solution
Cell transfection kit (e.g., Lipofectamine, Invitrogen; XtrmeGene, Roche; Fugene HD, Promega) including:
Transfection reagent
Trypsin (0.25%, EDTA)
LB media with 50 mg/liter Kanamycin
Anhydrous sodium sulfate (Na_2SO_4)

50- to 500-ml round-bottomed flasks and rubber septa
Water-cooled condensation tube
Hot plate magnetic stirrer with contact thermometer oil bath
Ice bath
Fritted filter funnel
pH papers
Rotary evaporator
Columns for chromatography
Electronic oven
Dual nitrogen-vacuum manifold with vacuum pump
Gel electrophoresis apparatus and power supply
37°C *E.coli* incubator
37°C incubator supplied with 5% CO_2
10-cm petri dishes for tissue culture
8-well chambered coverslip
Fluorescence microscope
Imaging acquiring and processing software (e.g., LAS AF software)

NOTE: Appropriate personal protective equipment (e.g., gloves, laboratory coats, goggles, and fume hoods) must be used for all synthetic work.

Synthesize TMP-Atto655 conjugate

*Synthesize intermediate **2** TMP-OH*

1. To a 500 ml two-neck round-bottomed flask equipped with water-cooled condenser and a thermometer, add 214 ml (48% w/w in water) HBr followed by 17.15 g (60 mmol) trimethoprim. Heat the reaction using a hot plate magnetic stirrer with contact thermometer oil bath. Reflux the solution under air for 20 min while maintaining the reaction temperature at 95°C, and then transfer the flask into an ice bath and partially neutralize the solution by carefully adding 51 ml (50% w/w) NaOH

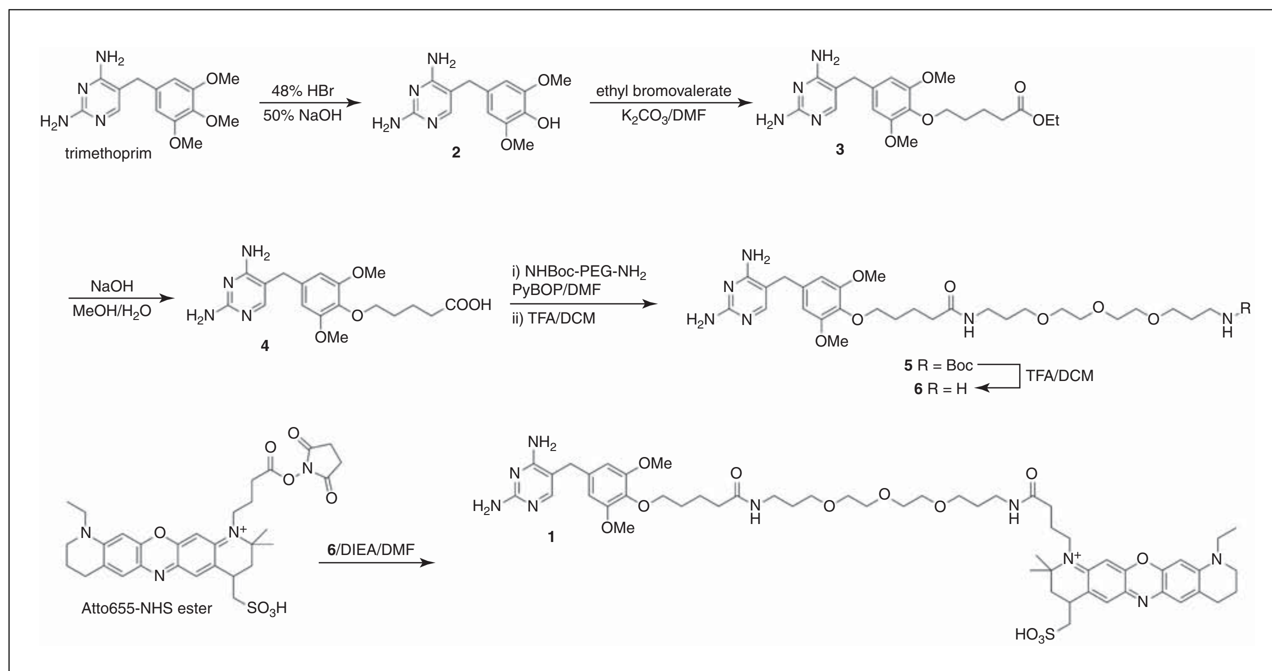

Figure 3 Synthetic route of compound **1** TMP-Atto655.

while actively stirring. Stop the agitation and allow the solution to cool to room temperature and then place overnight at 4°C; this should result in beige needle-like crystals.

It is crucial to control the reaction condition so that trimethoprim is not over-hydrolyzed. The key factors are reaction temperature (95°C), reaction time (<20 min), and pH for recrystallization (7.0). Multiple rounds of recrystallization may be necessary to obtain pure product. Impurities can be readily detected by NMR. Only two singlet peaks (δ = 7.51, 1H; 6.63, 2H) should show up in the aromatic region for pure product. Extra aromatic protons may indicate by-products.

The 48% HBr and 50% NaOH solutions are extremely corrosive and must be handled and prepared with extra caution. Do not store 50% NaOH solution in glass container for long-term storage. The neutralization of HBr by NaOH is highly exothermic and must be performed carefully with the reaction mixture actively stirred in an ice bath.

2. Filter the solution using a fritted filter funnel and collect crystals. Then recrystallize by dissolving in a minimal amount of cold Millipore-purified water, which is then neutralized to pH 7 with ammonium hydroxide (28% in water, w/w). Chill the solution at 4°C and filter the solution using a fritted filter funnel. Collect the crystal to obtain compound **2**.

*Compound **2**: Expected 9.130 g, 33.0 mmol, 56 % yield). ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 7.51 (s, 1H), 6.63 (s, 2H), 3.85 (s, 6H), 3.65 (s, 2H). HSMS (FAB+) m/z Calcd. for $C_{13}H_{16}O_3N_4$ $[M+H]^+$: 277.1222. Found: 277.1312.*

*Synthesize intermediate **3** TMP-COOEt*

3. In a 100-ml round-bottomed flask, dissolve 1.381 g (5 mmol) intermediate **2** in 15 ml DMF and then add 3.46 g (25 mmol) K_2CO_3 and 1.58 ml (10 mmol) ethyl 5-bromovalerate.

The TMP-OH and ethyl 5-bromovalerate will dissolve in DMF while the K_2CO_3 only partially dissolves, resulting in a white suspension.

4. Heat the reaction mixture in a 70°C oil bath under Ar for 5 hr while stirring, during which the solution may turn yellow or slightly brown.

5. After cooling to room temperature, extract the reaction mixture with 100 ml brine and ethyl acetate (3 × 40 ml). Combine the organic phase; dry over Na_2SO_4 and remove ethyl acetate by rotary evaporation.

6. Purify the crude product by flash chromatography (MeOH/DCM, 1:10 v/v over silica gel) to yield compound **3.**

   *Compound **3**: Expected 1.1 g, 2 mmol, 54% yield; (R_f = 0.48 in MeOH/DCM, 1:10 v/v). ^1H NMR (400 MHz, CD_3OD, 25°C): δ = 7.50 (s, 1H), 6.51 (s, 2H), 4.11 (q, J = 9.6 Hz, 2H), 3.89 (t, J = 8.0 Hz, 2H) 3.77 (s, 6 H), 3.63 (s, 2H), 2.39 (t, J = 8.8 Hz, 2H), 1.86 – 1.65 (m, 4H), 1.23 (t, J = 9.6 Hz, 3H). MS (FAB+) m/z Calcd. for $C_{20}H_{28}O_5N_4$ $[M+H]^+$: 405.21. Found: 405.31.*

*Synthesize intermediate **4** TMP-COOH*

7. In a 100-ml round-bottomed flask, dissolve 404 mg (1 mmol) compound **3** in 25 ml methanol, and then add NaOH in water (1 N, estimated volume 3 ml) to this solution until a small amount of beige precipitate is formed.

8. Following the formation of the precipitate, stir the reaction mixture under air for 4 hr and the precipitate should disappear.

9. Titrate the reaction mixture to pH 4 with HCl (1 N, estimated volume 3 ml) and beige crystals will form from solution. Filter the crystal and wash with 10 ml brine and 20 ml water (cooled at 4°C) to yield compound **4**.

   Compound 4: Expected 290 mg, 0.77 mmol, 77%). ^1H NMR (DMSO-d6): δ = 7.51 (s, 1 H), 6.54 (s, 2 H), 3.77 (t, J = 8.0 Hz, 2H), 3.70 (s, 6H), 3.51 (s, 2H), 2.26 (t, J = 9.2 Hz, 2H), 1.67 – 1.62 (m, 4H). MS (FAB+) m/z Calcd. for $C_{18}H_{24}O_5N_4$ $[M+H]^+$: 377.17. Found: 377.42.

*Synthesize intermediate **6** TMP-PEG-NH_2*

10. Prior to the synthesis, bake a 25-ml round-bottomed flask at 95°C for 48 hr in an electronic oven. Right before setting up the reaction, further dry the flask by propane flame.

11. Add 56 mg (0.15 mmol) compound **4**, 96 mg (0.3 mmol, 2 equiv.) BocNH-PEG-NH_2 and 155 mg (0.3 mmol, 2 equiv.) PyBOP to the hot flask and put under vacuum.

12. After the flask cools to room temperature (after approximately 1 hr), add 10 ml DMF and 130 μl (0.745 mmol, 5 equiv.) DIEA to the flask under Ar. Stir the reaction mixture overnight at room temperature and then remove solvent by rotary evaporation and purify the crude product by flash chromatography (MeOH/DCM, 1:30 over silica gel) to yield compound **5** (R_f = 0.33 in 15:1 v/v DCM/MeOH).

13. Deprotect the Boc group by adding TFA in DCM (50%, v/v), stir at room temperature for 1 hr, and then remove solvent by rotary evaporation.

   *The deprotection reaction is quantitative so that the resulting compound **6** (expected 31 mg, 36 % yield in 2 steps) is used for the next step without further purification.*

   *^1H NMR for compound **5** (400 MHz, CD_3OD, 25°C): δ = 7.24 (s, 1H), 6.56 (s, 2H), 3.93 (m, 2H), 3.81 (s, 6H), 3.52 (s, 2H), 3.52–3.50 (m, 12 H), 3.26 (t, J = 6.8 Hz, 2 H), 3.12 (t, J = 6.4 Hz), 2.26 (t, J = 7.2 Hz, 2 H), 1.80 – 1.70 (m, 8 H), 1.43 (s, 9 H). HRMS (FAB+) m/z Calcd. for compound **5** $C_{33}H_{54}O_9N_6$ $[M+H]^+$: 679.40. Found: 679.40.*

*Synthesize compound **1** TMP-Atto655*

14. In a 25-ml round-bottomed flask, mix 2.3 mg (3.9 μmol) compound **6** with 1 mg (1.6 μmol) Atto655-NHS ester and 2.8 μl (16 μmol) DIEA in 1 ml DMF. Stir the reaction mixture overnight under Ar and remove solvent with rotary evaporation.

15. Purify the crude product (~5 mg) by reverse-phase HPLC to yield compound **1** (expected 1 mg, 0.95 μmol, 57 %). HPLC conditions for C18 semi-prep column: start with 10:90 (v/v) acetonitrile:water, gradient elution for 80 min, end with 60:40 (v/v) acetonitrile:water. Retention time: 33 to 34 min.

    ^{1}H NMR (400 MHz, CD_3OD, 25°C) δ = 7.79 (d, J = 1.7 Hz, 1H), 7.47 (d, J = 1.5 Hz, 1H), 7.24 (s, 1H), 7.07 (s, 1H), 6.94 (s, 1H), 6.45 (s, 2H), 3.82 (t, J = 6.2 Hz, 1H), 3.77 (m, 2H), 3.7 (s, 6H), 3.67 – 3.53 (m, 18H), 3.51 (m, 2H), 3.23 (m, 2H), 3.06 – 2.90 (m, 4H), 2.52 (dd, J = 13.6, 4.4 Hz, 1H), 2.45 – 2.36 (m, 2H), 2.24 (t, J = 7.4 Hz, 2H), 2.06 (m, 4H), 1.85 – 1.70 (m, 8H), 1.70 – 1.61 (m, 2H), 1.52 (s, 3H), 1.43 (s, 3H), 1.38 (t, J = 7.2 Hz, 7H). MS (FAB+) m/z Calcd. for $C_{55}H_{78}O_{12}N_9S$ $[M]^+$: 1088.55. Found: 1089.15.

16. Dissolve the final product TMP-Atto655 in DMF or DMSO (initially use 100 μl solvent for 1 mg HPLC-purified product) and determine the concentration by measuring absorbance at 663 nm. The extinction coefficient of Atto655 at 663 nm $\varepsilon_{663} = 1.25 \times 10^5 mol^{-1}cm^{-1}$, measured in PBS. It is recommended to prepare a stock solution in DMF or DMSO with a concentration of 1 to 5 mM.

    The DMSO is less toxic to cells compared to DMF. However, DMF has a low boiling point so the compound dissolved in DMF can be more easily recovered by rotary evaporation.

    The TMP-Atto655 stock solution in DMF or DMSO can be stored at −80°C for long-term storage or −20°C for short-term storage (up to 6 months). It is recommended to use amber glass vials sealed by Parafilm. Do not store the compound in aqueous solution.

Construct H2B-eDHFR vector

This is a general workflow for constructing the H2B-eDHFR plasmid using standard molecular cloning technology. For detailed technique, please refer to related protocols (Bloch, 2001; Kramer and Coen, 2001; Voytas, 2001; Lohman et al., 2011).

17. Amplify the eDHFR gene via PCR.

    Primers:
    Forward: 5′ACGTCACCGGTCGCCACCATGGTGGGTTCTGGTGGTTCTG GTATCAGTCTGATTGCGGCG3′ (*Age*I);
    Reverse: 5′ACGTCGCGGCCGCTTTAGTGATGGTGATGGTGATGCCGCCG CTCCAGAATCT3′ (*Not*I).

    The eDHFR gene is commercially available from Active Motif (LigandLink pLL-1 kit, cat. no. 34001) and Addgene (DHFR-myc, cat. no. 20214).

18. Digest the H2B-GFP vector and the amplified eDHFR gene with *Age*I and *Not*I restriction endonucleases. Purify the digested DNA by agarose gel electrophoresis and recover DNA from the agarose gel using a gel extraction kit.

    The expected length for the digested H2B-GFP vector is 5 kb; the eDHFR gene is 500 bp.

19. Ligate the digested vector and eDHFR gene using T4 DNA ligase. Then transform *E. coli* by electroporation with the ligated vector and plate the cells on LB-Kanamycin plates.

20. Amplify the H2B-eDHFR plasmid and confirm the construction by sequencing (Genewiz) the open reading frame region using CMV forward primer (5′ CGCAAATGGGCGGTAGGCGTG 3′). Make sure there is no stop codon or frame shift between H2B and eDHFR.

21. Perform an endo-free plasmid maxi-prep to obtain plasmid in high concentration (~1 μg/μl) for mammalian cell transfection.

Stain the cells and prepare the samples

22. Culture HEK 293T cells in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. Maintain all cells in 10-cm petri dishes under 5% CO_2 at 37°C.

    For standard procedure of tissue culture, see Xu (2005).

23. One day prior to transfection, suspend the cells by treating with trypsin (1 ml/75-cm^2 cell culture) and reseed in 8-well chambered coverslip.

    The recommended cell density is ~30,000 cells/cm^2.

24. Transfect the cells by adding the mixture of transfection reagent (included in the transfection kits) and DNA (prepared in step 21) into the cell culture (prepared in step 23). Incubate for 12 to 24 hr at 37°C.

    Several commercially available cell transfection kits are known to be highly efficient for HEK 293T cells, including the lipofectamine (Invitrogen), the XtrmeGene (Roche), and the Fugene HD (Promega). Follow the manufacturer's instruction to optimize transfection conditions.

25. Prior to cell staining, dilute the TMP-Atto655 to 2 μM in supplemented medium (see step 22). Prepare ~100 μl TMP-Atto655 in medium for every 1 cm^2 cell culture.

26. Remove the old medium with transfection reagent from the cell culture by aspiration. Gently add the medium containing 2 μM TMP-Atto655 to cells. Make sure the solution covers the bottom of the chamber. Incubate for 1 hr at 37°C. It is recommended to stain the cells at 50% to 80% confluency.

    The HEK 293T cells do not adhere tightly to the coverslip. Therefore, it is recommended to add and remove media very gently to avoid washing off the cells.

27. Remove the TMP-Atto655 solution by aspiration. Wash the cells by adding 200 μl supplemented medium (see step 22) for every 1 cm^2 cell culture and incubate for 10 min at 37°C.

28. Remove the washing medium and repeat step 27. Leave 200 μl medium for every 1 cm^2 cell culture. The cells are now ready for imaging.

    It is crucial to keep the cells sterilized during the whole procedure. Contamination with bacteria is harmful to the cells and may introduce nonspecific fluorescence because the TMP-tag binds to E. coli endogenous DHFR with high affinity.

Perform fluorescence imaging

This is a general guideline for imaging nuclear-localized eDHFR/TMP-Atto655 using the LEICA TCS SP5 confocal microscope with a HCX PL APO CS 20x 0.70 DRY UV objective or a HCX PL APO CS 100× 1.46 OIL objective. The TMP-tag can also be imaged with various confocal, epi-fluorescence and total internal reflection fluorescence (TIRF) microscopes; variations on the procedures may apply. Refer to the manufacturer's direction for details setting up and using the microscope.

29. Turn on the thermostat and CO_2 valve on the CO_2 chamber installed on the microscope.

    HEK 293T cells can survive at room temperature for a couple of hours without CO_2 supply. However, to maintain healthy cells for sophisticated cell biology experiments, it is very important to maintain the temperature (37°C) and CO_2 concentration (5%) during imaging.

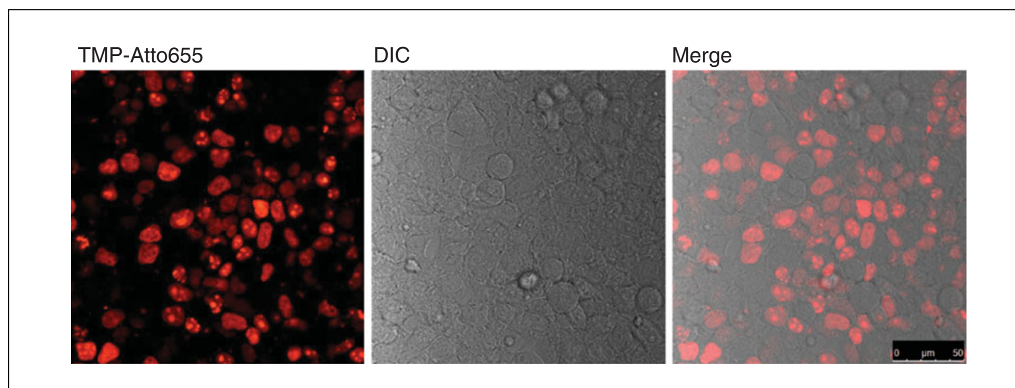

Figure 4 Live-cell imaging of H2B labeled with noncovalent TMP-tag. The left image shows expected result of fluorescence imaging of H2B in live HEK 293T cells using TMP-Atto655. The middle image is from differential interference contrast (DIC) channel. The right image is obtained by merging the Atto655 channel with the DIC channel. Scale bar is 50 μm.

30. Turn on the laser cooling system, the laser, the Hg lamp, the microscope, and then start the LAS AF software.
31. Start from 20× dry objective and focus on cells using white light and eyepiece.
32. On the software, select 633-nm He-Ne laser and set the photon multiplier tube (PMT) to collect emission light from 660 to 750 nm. Also, activate the differential interference contrast (DIC) channel.
33. In the fast scanning mode (400 Hz), fine-tune the focus, the laser intensity, and the PMT gain voltage until a clear image is obtained in both far-red fluorescence and DIC channels.
34. Acquire high-resolution images by switching to slow scanning (10 Hz) mode and/or using 100× oil immersion objective.
35. Process the fluorescence images using the LAS AF software or other image processing software.

    See Figure 4 for representative images.

ALTERNATE PROTOCOL 1

SYNTHESIS AND APPLICATION OF COVALENT TMP-TAG FOR LIVE-CELL IMAGING

This protocol describes the synthesis of covalent A-TMP-fluorescein (compound **14** in Fig. 5), the construction of H2B-eDHFR:L28C vector, and live-cell imaging in HEK 293T cells. The chemical synthesis is summarized in Figure 5. An example of anticipated images is displayed in Figure 6. The general procedures also apply to other fluorophores, target proteins and cell lines, potentially with some modification in details, as discussed in the Strategic Planning and Commentary sections.

Additional Materials *(also see Basic Protocol)*

L-Aspartic acid 4-*tert*-butyl ester (CAS no. 3057-74-7)
Sodium carbonate
Acryloyl chloride
Sodium bisulfate ($NaHSO_4$)
Compound **2** TMP-OH (from the Basic Protocol, step 2)
tert-butyl *N*-(3-iodopropyl)carbamate (CAS no. 167479-01-8)
Cesium carbonate

Reverse-phase C18 silica gel

1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDCI)
4-Dimethylaminopyridine (DMAP)
6-Carboxyfluorescein *N*-hydroxysuccinimide ester (6-carboxyfluorescein-NHS ester; CAS no. 92557-81-8)
Triethylamine
Pyridine
Isobutyric anhydride
Toluene

Synthesize A-TMP-fluorescein heterotrimer

*Synthesize compound **7***

1. In a 100-ml round-bottomed flask, chill a solution of L-aspartic acid 4-*tert*-butyl ester (1.00 g, 5.28 mmol) and sodium carbonate (840 mg, 7.93 mmol) in 20 ml water in an ice bath.

2. Add 502 mg (449 µl, 5.55 mmol) acryloyl chloride dropwise to this ice-cold solution and subsequently remove the ice bath to warm the reaction mixture to room temperature and stir for another 1 hr. Adjust the pH of the solution to 2.0 by addition of 1 M $NaHSO_4$ aqueous solution.

3. Extract the mixture with ethyl acetate (2 × 100 ml) and combine the organic layers.

4. Dry the organic layer over anhydrous Na_2SO_4 and remove solvent by rotary evaporation to yield compound **7**.

   *Compound **7**: Expected 0.93 g, 3.85 mmol, 73%. Rf = 0.82 in MeOH) as a colorless oil. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 6.80 (d, J = 7.5 Hz, 1 H); 6.35 (dd, J = 16.8 Hz, 1.2 Hz, 1 H); 6.16 (dd, J = 16.8 Hz, 10.2 Hz, 1 H); 5.75 (dd, J = 10.2 Hz, 1.2 Hz, 1 H); 4.89(m, 1 H); 3.02 (dd, J = 17.1 Hz, 4.2 Hz, 1 H); 2.78 (dd, J = 17.1 Hz, 5.4 Hz, 1 H); 1.44 (s, 9 H). HRMS (FAB+) m/z Calcd. for $C_{11}H_{18}O_5N$ $[M+H]^+$: 244.1185. Found: 244.1181.*

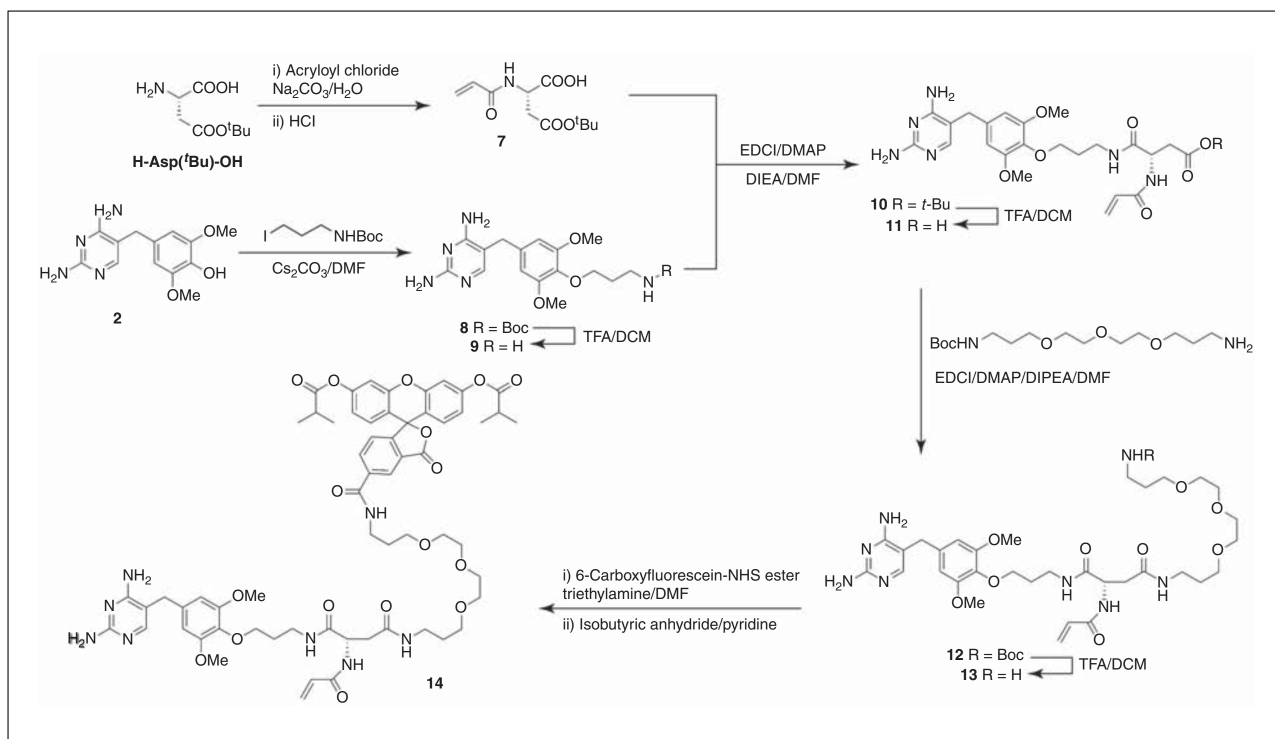

Figure 5 Synthetic route of compound **14** A-TMP-fluorescein.

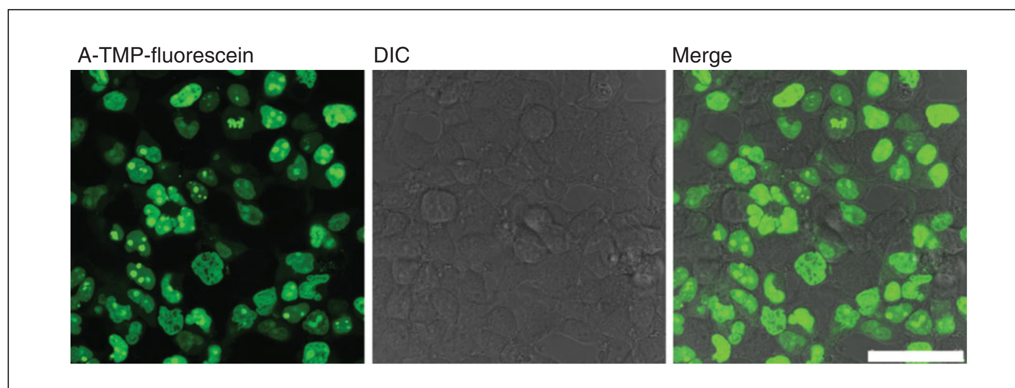

Figure 6 Live-cell imaging of H2B labeled with covalent TMP-tag. The left image shows expected result of fluorescence imaging of H2B in live HEK 293T cells using A-TMP-fluorescein. The middle image is from differential interference contrast (DIC) channel. The right image is obtained by merging the fluorescein channel with the DIC channel. Scale bar is 50 μm.

*Synthesize compound **9** TMP-NH_2*

5. In a 100-ml round-bottomed flask, dissolve 600 mg (2.16 mmol) compound **2** TMP-OH, 618 mg (2.16 mmol) *tert*-butyl *N*-(3-iodopropyl)carbamate, and 1.41 g (4.32 mmol) cesium carbonate in 30 ml anhydrous DMF.

6. Heat the reaction mixture to 70°C and stir for 5 hr under Ar, followed by the removal of solvent by rotary evaporation.

7. Purify the crude product by column chromatography with silica gel (eluant: 9:1 v/v DCM/MeOH) to yield compound **8** ($R_f = 0.33$ in 9:1 v/v DCM/MeOH).

8. Deprotect compound **8** by directly dissolving in 5 ml TFA and 5 ml DCM while stirring at room temperature. After 4 hr, remove the TFA by rotary evaporation to yield compound **9** in the form of its TFA salt (expected 715 mg, 1.66 mmol, 77%) as colorless oil.

   ^{1}H NMR (300 MHz, CD_3OD) δ ppm: 7.28 (s, 1 H); 6.64 (s, 2 H); 4.12 (t, J = 5.4 Hz, 2 H); 3.87 (s, 6 H); 3.70 (s, 2 H); 3.30 (t, J = 6.0 Hz, 2 H); 2.08 (m, 2 H). HRMS (FAB+) m/z Calcd. for $C_{16}H_{24}O_3N_5$ $[M+H]^+$: 334.1879. Found: 334.1883.

*Synthesize compound **11** A-TMP-COOH*

9. Prior to the synthesis, bake a 50-ml round-bottomed flask for 48 hr at 95°C. Immediately before setting up the reaction, further dry the flask by propane flame.

10. Add 187 mg (0.77 mmol) compound **7**, 271 mg (0.63 mmol, in the form of TFA salt) compound **9**, 39 mg (0.30 mmol) DMAP, and 241 mg (1.26 mmol) EDCI to the hot flask and put under vacuum.

11. After the flask cools to room temperature (after approximately 1 hr), add 10 ml DMF and 384 μl (2.00 mmol) DIEA to the flask under Ar. Stir the reaction mixture for 12 hr at room temperature and remove solvent by rotary evaporation.

12. Purify the crude product by silica gel flash chromatography (9:1 v/v DCM/MeOH) to yield the coupling product compound **10** ($R_f = 0.43$ in 6:1 v/v DCM/MeOH).

13. Dissolve compound **10** in 20 ml 1:1 (v/v) TFA-DCM and stir for 4 hr at room temperature.

14. Remove the solvent by rotary evaporation and further purify the deprotection product by column chromatography on silica gel (7:3 v/v DCM/MeOH) to yield compound **11** (expected 127 mg, 253 μmol, 46%) as a colorless oil.

^{1}H NMR (300 MHz, CD_3OD) δ ppm: 7.25 (s, 1 H); 6.57 (s, 2 H); 6.24 (dd, J = 17.1 Hz, 9.0 Hz, 1 H); 6.16 (dd, J = 17.1 Hz, 3.0 Hz, 1 H); 5.61 (dd, J = 9.0 Hz, 3.0 Hz, 1 H); 4.80 (m, 1 H); 3.98 (m, 2 H); 3.82 (s, 6 H); 3.68 (s, 2 H); 3.46 (t, J = 6.0 Hz, 2 H); 2.87 (dd, J = 16.8 Hz, 6.0 Hz, 1 H); 2.72 (dd, J = 16.8 Hz, 7.5 Hz, 1 H); 1.88 (m, 2 H). HRMS (FAB+) m/z Calcd. for $C_{23}H_{31}O_7N_6$ $[M+H]^+$: 503.2254. Found: 503.2273.

*Synthesize compound **13** A-TMP-NH_2*

15. Dissolve 40 mg (80 µmol) compound **11**, 38 mg (119 µmol) NHBoc-PEG-NH_2, and 5 mg (40 µmol) DMAP in 10 ml anhydrous DMF and then add 31 mg (160 µmol) EDCI and 70 µl (0.4 mmol) DIEA at room temperature. Stir the reaction mixture for 12 hr at room temperature before concentrating by rotary evaporation.

16. Purify the crude product by silica gel flash chromatography (9:1 v/v DCM/MeOH) to yield compound **12** (R_f = 0.33 in 3:1 v/v DCM/MeOH).

17. Dissolve compound **12** in 5 ml TFA and 5 ml DCM and stir for 4 hr at room temperature.

18. Remove the solvent by rotary evaporation and further purify the crude product by column chromatography on reverse-phase C18 silica gel with 10% acetonitrile in water to yield compound **13**.

    Compound 13: Expected 16 mg, 20 µmol, in the form of TFA salt, 25% yield) as a white solid.^{1}H NMR (400 MHz, CD_3OD) δ ppm: 7.25 (s, 1 H); 6.57 (s, 2 H); 6.22 (dd, J = 17.2 Hz, 9.6 Hz, 1 H); 6.15 (dd, J = 17.2 Hz, 2.8 Hz, 1 H); 5.61 (dd, J = 9.6 Hz, 2.8 Hz, 1 H); 4.83 (m, 1 H); 3.96 (m, 2 H); 3.81 (s, 6 H); 3.69-3.62 (m, 10 H); 3.59-3.55(m, 2 H); 3.49 (t, J = 6.0 Hz, 2 H); 3.48-3.40 (m, 2 H); 3.24 (dt, J = 7.2 Hz, 3.2 Hz, 2 H); 3.10 (t, J = 6.4 Hz, 2 H); 2.76 (dd, J = 14.8 Hz, 5.6 Hz, 1 H); 2.58 (dd, J = 14.8 Hz, 7.6 Hz, 1 H); 1.92 (m, 2 H); 1.87 (m, 2 H); 1.73 (m, 2 H). HRMS (FAB+) m/z Calcd. for $C_{33}H_{53}O_9N_8$ $[M+H]^+$: 705.3936. Found: 705.3917.

*Synthesize compound **14** A-TMP-fluorescein*

19. In a 25-ml round-bottomed flask, dissolve 3.0 mg (3.7 µmol) compound **13** and 3.1 mg (6.5 µmol) 6-carboxyfluorescein-NHS ester in 1 ml anhydrous DMF and then add 5 µl (36 µmol) triethylamine. Stir the reaction mixture at room temperature for 12 hr before concentrating by rotary evaporation.

20. Redissolve the crude product in 0.40 ml pyridine and add 62 µl (0.37 mmol) isobutyric anhydride. Stir the reaction mixture at room temperature for 2 hr, during which the color and fluorescence will disappear.

21. Add 20 µl water to the mixture and incubate for 5 min at room temperature.

22. Remove the solvent by rotary evaporation. Add 10 ml toluene to the crude product and evaporate by rotary evaporation to remove residual water.

23. Finally, purify the reaction residue (∼10 mg) by reverse-phase HPLC to yield compound **14** (expected 1.7 mg, 1.4 µmol, 38%). Recommended HPLC condition for C18 semi-preparative column: starting with 33:67 (v/v) acetonitrile:water, gradient elution for 50 min, end with 50:50 (v/v) acetonitrile:water. Retention time: 42 to 43 min.

    ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 8.46 (d, J = 0.8 Hz, 1 H), 8.22 (dd, J = 8.0 Hz, 1.6 Hz, 1 H); 7.38(d, J = 8.0 Hz, 1 H); 7.23 (s, 1 H); 7.19 (m, 2 H); 6.90 (m, 4 H); 6.22 (dd, J = 16.8 Hz, 9.6 Hz, 1 H); 6.14 (dd, J = 16.8 Hz, 2.4 Hz, 1 H); 5.59 (dd, J = 9.6 Hz, 2.4 Hz, 1 H); 4.78 (t, J = 6.4 Hz, 1 H); 3.94 (t, J = 5.6 Hz, 2 H); 3.79 (s, 6 H); 3.66-3.60 (m, 10 H); 3.55-3.52 (m, 4 H); 3.47-3.40 (m, 4 H); 3.20 (m, 2 H); 2.85 (m, 2 H); 2.71 (dd, J = 15.2 Hz, 6.0 Hz, 1 H); 2.60 (dd, J = 15.2 Hz, 7.2 Hz, 1 H); 1.92 (m, 2 H); 1.83 (m, 2 H); 1.68 (m, 2 H); 1.30 (d, J = 7.2 Hz, 12 H). MS (FAB+) m/z Calcd. for $C_{62}H_{75}O_{17}N_8$ $[M+H]^+$: 1203.53. Found: 1203.63.

24. Dissolve the final product A-TMP-fluorescein in DMF or DMSO and determine the concentration by measuring absorbance at 285 nm. Refer to step 16 in the Basic Protocol for more technical details.

    *The extinction coefficient of molecule **14** at 285 nm $\varepsilon_{285} = 1.35 \times 10^4 mol^{-1} cm^{-1}$, measured in DMF. It is recommended to make a 1 to 5 mM stock solution in DMF or DMSO.*

Construct H2B-eDHFR vector

This is a general workflow for constructing the H2B-eDHFR:L28C plasmid using standard site-directed mutagenesis (SDM) technology. For the detailed technique, see Kunkel (2001).

25. Amplify the H2B-eDHFR plasmid by PCR using mutagenesis primers 5′- GGA ACC TGC CTG CCG ATT GCG CAT GGT TTA AAC GCA AC -3′ and 5′- GTT GCG TTT AAA CCA TGC GCA ATC GGC AGG CAG GTT CC-3′.

    There are numerous commercially available SDM kits. PCR conditions should be optimized according to the kit manufacturer's instructions.

26. Digest the mutagenesis PCR product with *Dpn*I.

    All methylated DNA (e.g., wild-type plasmid DNA) will be fragmented, leaving only the mutated DNA.

27. Transform *E. coli* with the mutated vector by electroporation and grow on LB-kanamycin plates.

28. Amplify the H2B-eDHFR:L28C plasmid and confirm the mutation by sequencing the open reading frame region using CMV forward primer.

29. Prepare endo-free Maxi-prep of plasmid (~1 μg/μl) for mammalian cell transfection.

Stain the cells and perform fluorescence imaging

30. Transfect HEK 293T cells with plasmids encoding H2B-eDHFR:L28C and stain with A-TMP-fluorescein for live-cell imaging with confocal microscope.

    The procedures are the same as with TMP-Atto655 in the Basic Protocol (see steps 22 to 35), with the following variations: (1) since the protected fluorescein is highly cell-permeable, HEK 293T cells only need to be stained for 5 min before proceeding to washing and imaging; (2) for fluorescence imaging, select 488-nm Ar laser and set the photon multiplier tube (PMT) to collect emission light from 520 to 600 nm.

    See Figure 6 for representative results.

ALTERNATE PROTOCOL 2

SYNTHESIS AND APPLICATION OF FLUOROGENIC TMP-TAG FOR LIVE-CELL IMAGING

This protocol describes the synthesis of fluorogenic TMP-Q-Atto520 (compound **23** in Fig. 8) and live-cell imaging in HEK 293T cells. The chemical synthesis is summarized in Figure 7 and Figure 8. An example of expected image is displayed in Figure 9. The general procedures also apply to other fluorophores, target proteins and cell lines, potentially with some modification in details, as discussed in the Strategic Planning and Commentary sections.

Additional Materials *(also see Basic Protocol)*

Compound **9** TMP-NH_2 (from Alternate Protocol 1, step 8)
N-Boc-propargylglycine
Hydroxybenzotriazole (HOBt)
1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDCI)

Atto520-NHS ester
N-(3-aminopropyl)-*N*-methylaniline
Di-*tert*-butyl dicarbonate
Fast Corinth V salt
Sodium carbonate (Na_2CO_3)
3-(Chlorosulfonyl)benzoyl chloride
2-Azidoethanol
Ascorbic acid
$CuSO_4 \cdot 5H_2O$

Synthesize TMP-Q-Atto520 heterotrimer

*Synthesize compound **16** TMP-alkyne*

1. In a 25-ml round-bottomed flask, dissolve 192 mg (0.4 mmol, in the form of TFA salt) compound **9**, 85.3 mg (0.4 mmol) *N*-Boc-propargylglycine, 16.2 mg (0.12 mmol) HOBt, and 230 mg (1.2 mmol) EDCI in 3 ml anhydrous DMF at room temperature.

2. Add 871 µl (4 mmol) DIEA and stir the reaction under Ar for 12 hr at room temperature. Concentrate by rotary evaporation.

3. Purify the crude product by silica gel flash chromatography (1:6 v/v methanol:DCM) to yield the coupling product compound **15** (R_f = 0.45 v/v in 1:6 methanol: DCM, v/v).

4. Dissolve compound **15** in 20 ml 1:1 (v/v) TFA-DCM and stir for 4 hr at room temperature.

5. Remove the solvent by rotary evaporation to yield compound **16** (expected 140 mg as TFA salt, 257 µmol, 64% yield in two steps) as a colorless solid. (R_f = 0.05 in 1:6 methanol:DCM).

   *Compound **15** ^1H NMR (400 MHz, CD_3OD) δ ppm: 7.66 (s, 1H), 6.39 (s, 2H), 5.43 (d, J = 8 Hz, 1H), 5.06 (s, 1H), 4.29 (q, J = 8, 6.5 Hz, 1H), 4.03 (m, 2H), 3.80 (s, 6H), 3.64 (S, 2H), 3.53 (m, 2H), 2.74 (d, J = 17.1 Hz, 1H), 2.59 (m, 1H), 1.99 (m, 2H), 1.91 (m, 2H), 1.37 (s, 9H). Compound **16** MS (FAB+) m/z Calcd. for $C_{21}H_{28}O_6N_4$ $[M+H]^+$: 429.22. Found: 429.81.*

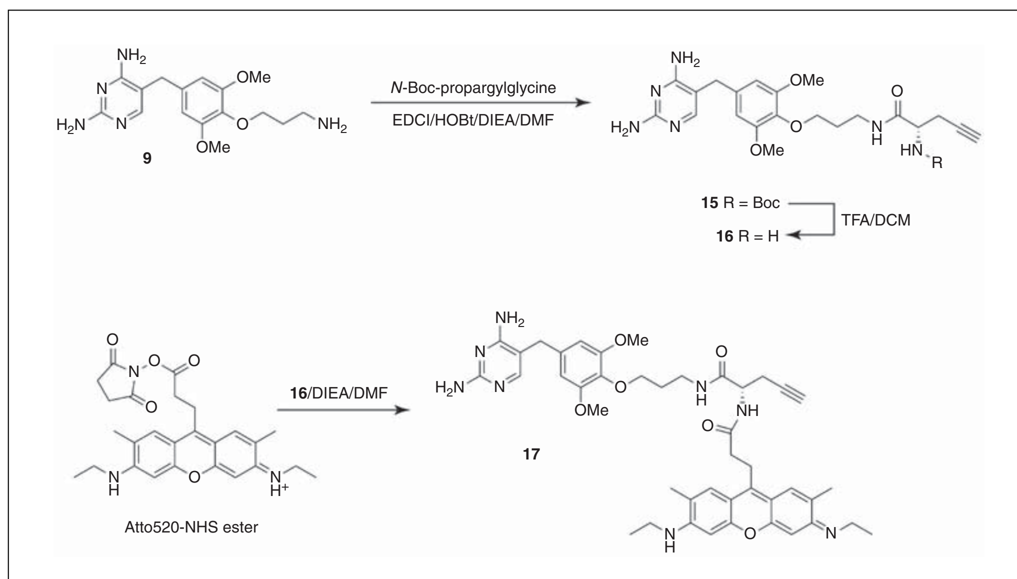

Figure 7 Synthetic route of compound **17**.

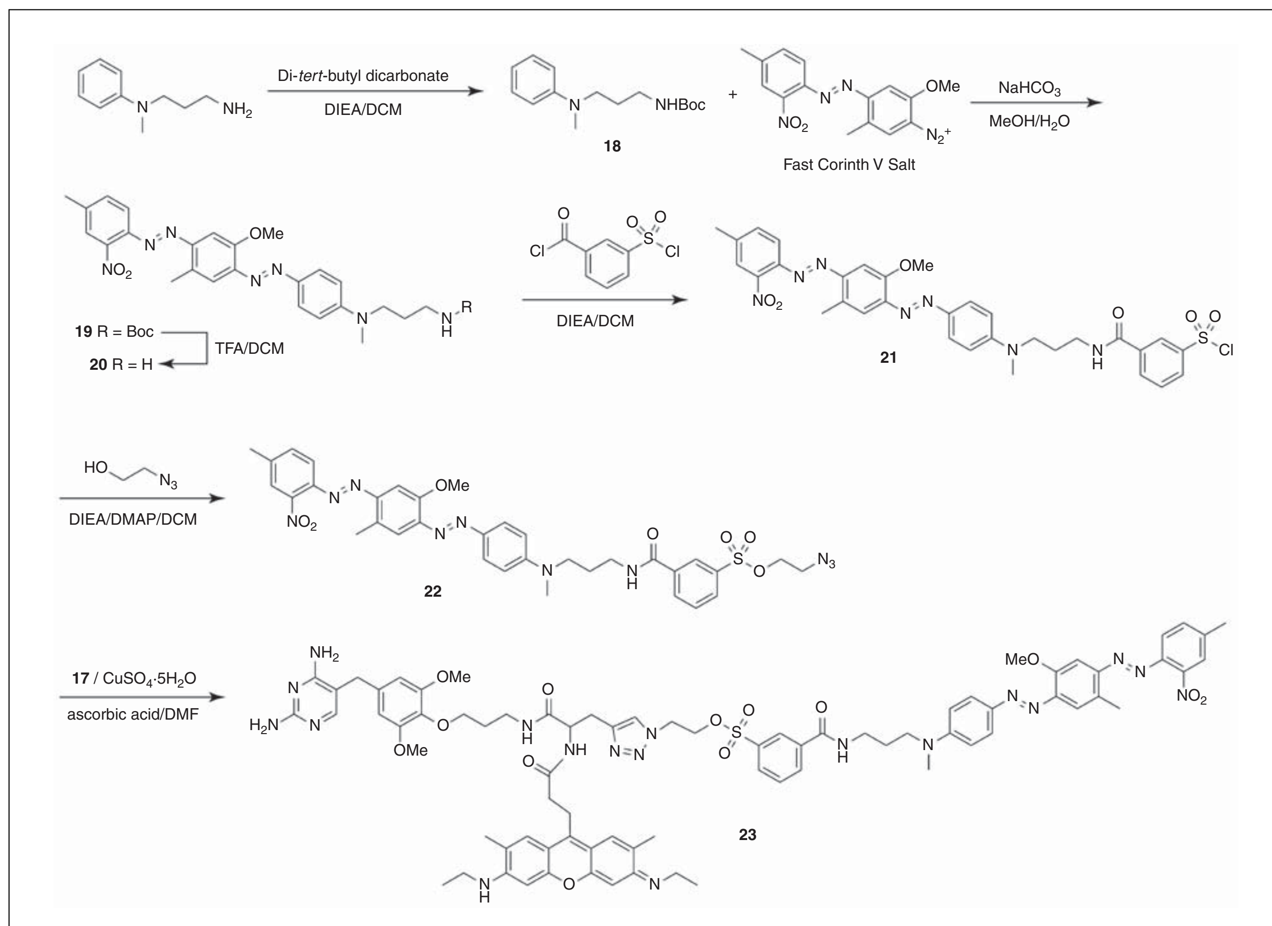

Figure 8 Synthetic route of compound **23** TMP-Q-Atto520.

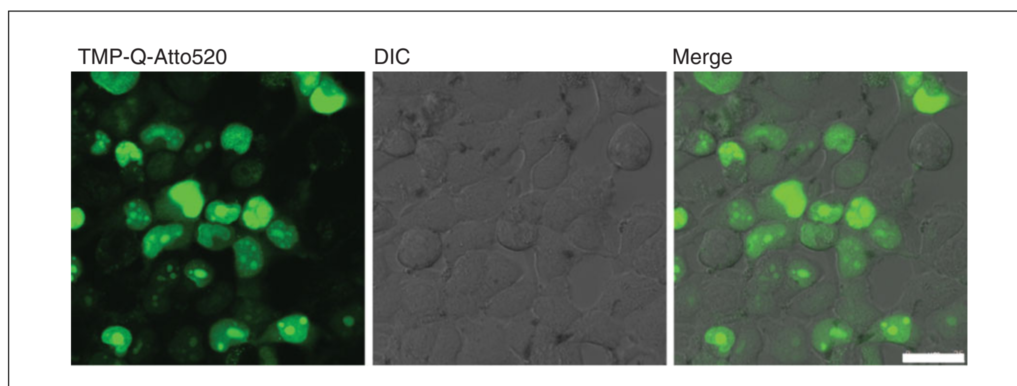

Figure 9 Live-cell imaging of H2B labeled with fluorogenic TMP-tag. The left image shows expected result of fluorescence imaging of H2B in live HEK 293T cells using TMP-Q-Atto520. The middle image is from differential interference contrast (DIC) channel. The right image is obtained by merging the fluorescein channel with the DIC channel. Scale bar is 25 µm.

*Synthesize compound **17** TMP-alkyne-Atto520*

6. Dissolve 7 mg (13 µmol) compound **16** and 5.0 mg (8.9 µmol) Atto520-NHS ester in 0.5 ml anhydrous DMF in a round-bottomed flask and then add 15 µl (89 µmol) DIEA.

7. Stir the mixture under Ar at room temperature for 12 hr before concentrating by rotary evaporation.

8. Purify the reaction residue by reverse-phase HPLC to yield compound **17**.

Compound 17: Expected 6 mg as TFA salt, 6.7 μmol, 76% yield). ^1H NMR (400 MHz, CD_3OD) δ ppm: 7.91 (s, 2H), 7.23 (s, 1H), 6.85 (s, 2H), 6.56 (s, 2H), 4.40 (dd, J = 7.2, 4.8 Hz, 1H), 3.98 (t, J = 5.9 Hz, 2H), 3.81 (s, 6H), 3.73 (m, 2H), 3.65 (s, 2H), 3.52 (q, J = 7.2 Hz, 6H), 3.22 (m, 2H), 2.87 (s, 1H), 2.67 (m, 2H), 2.52 (m, 2H), 2.35 (s, 6H), 2.30 (m, 1H), 1.87 (p, J = 6.3 Hz, 2H), 1.33 (m, 6H). HRMS (FAB+) m/z Calcd. for $C_{43}H_{52}O_8N_8[M+H]^+$: 777.41. Found: 777.41.

Recommended HPLC conditions for C18 column: start with 10:90 (v/v) acetonitrile:water, gradient elution for 80 min, end with 60:40 (v/v) acetonitrile:water. Retention time: 40 to 41 min.

*Synthesize compound **18***

9. In a 100-ml round-bottomed flask, dissolve 1 ml (6.09 mmol) *N*-(3-aminopropyl)-*N*-methylaniline in 10 ml DCM and then add 2.1 ml (12.18 mmol) DIEA and 7 ml (30.45 mmol) Di-*tert*-butyl dicarbonate to the solution dropwise.

10. Stir the reaction under Ar for 20 min. Remove solvent by rotary evaporation.

11. Purify the product by flash column chromatography on silica gel (1:5 v/v ethyl acetate:hexane) to yield compound **18**.

*Compound **18**: Expected 1.6 g, 6.05 mmol, 99% yield as a colorless oil. $R_f = 0.35$ in 1:5 (v/v) ethyl acetate:hexane. ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 7.23 (m, 2H), 6.70 (m, 3H), 4.56 (s, 1H), 3.40 – 3.32 (t, J = 7.2 Hz, 2H), 3.17 (q, J = 6.8 Hz, 2H), 2.92 (s, 3H), 1.77 (p, J = 7.0 Hz, 2H), 1.45 (s, 9H). MS (FAB+) m/z Calcd. for $C_{15}H_{24}O_2N_2$ $[M+H]^+$:265.18. Found: 265.22.*

The amine group on N-(3-aminopropyl)-N-methylaniline must be protected so that the next step–the azo coupling—can proceed smoothly without significant side reactions.

*Synthesize compound **20** BHQ1-NH_2*

12. In a 100-ml round-bottomed flask, suspend 472 mg (dye content 90%, 1.02 mmol) Fast Corinth V Salt in 8 ml water and chill to 0°C in an ice bath.

13. In another container, dissolve 600 mg (2.27 mmol) compound **18** in 8 ml methanol and add 6 ml 5% (w/w) Na_2CO_3 aqueous solution (5 g Na_2CO_3 dissolved in 95 ml Millipore-purified water). The compound **18** solution will turn milky. Add the compound **18** suspension dropwise to the Fast Corinth V Salt suspension while stirring in an ice bath. Continue stirring the reaction mixture for another 2 hr during which the color should change from orange to dark red.

14. Filter the reaction mixture using a fritted filter funnel to obtain crude product as dark red solid and wash three times, each time with 10 ml cold water and then dry under vacuum.

15. Purify the crude product by column chromatography on silica gel (4:6 v/v ethyl acetate:hexane) to yield compound **19** as a dark red solid.

16. Deprotect the Boc group by dissolving compound **19** in 10 ml of 30% (v/v) TFA in DCM and stir for 1 hr. The color will change from red to dark blue.

17. Remove the solvent by rotary evaporation to obtain compound **20**.

*Compound **20**: Expected 360 mg as TFA salt, 0.61 mmol, 60% yield in 2 steps as dark purple solid. $R_f = 0.3$ in 1:2 ethyl acetate:hexane. Compound **19** ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 7.91 (m, 2H), 7.77 (s, 1H), 7.66 (d, J = 8.0 Hz, 2H), 7.58 (s, 1H), 7.47 (m, 2H), 7.40 (s, 1H), 6.4 (m, 2H), 4.57 (s, 1H), 4.02 (s, 3H), 3.49 (t, J = 7.3 Hz, 2H), 3.20 (q, J = 6.7 Hz, 2H), 3.08 (s, 3H), 2.70 (s, 3H), 2.51 (s, 3H), 1.84 (p, J = 7.1 Hz, 2H), 1.46 (s, 9H). Compound **20** MS (FAB+) m/z Calcd. for $C_{25}H_{29}O_7N_3$ $[M+H]^+$: 476.23. Found: 476.58.*

*Synthesize compound **21***

18. In a 50-ml round-bottomed flask dissolve compound **20** (TFA salt, 100 mg, 0.173 mmol) in 3 ml anhydrous DCM and chill to 0°C in an ice bath.

19. Add 55 µl (0.346 mmol) 3-(chlorosulfonyl)benzoyl chloride to the solution, followed by 0.3 ml (1.73 mmol) DIEA. Stir the reaction mixture in an ice bath under Ar for 15 min, and then warm to room temperature and stir for another 1 hr.

20. Collect the crude product by evaporating the solvent and then purify by column chromatography on silica gel (7:3 v/v ethyl acetate:hexane) to yield compound **21**.

    *Compound **21**: Expected 99 mg, 0.145 mmol, 84% yield as a dark red solid. $R_f = 0.42$ in 2:1 ethyl acetate:hexane, v/v. ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 8.38 (t, J = 1.8 Hz, 1H), 8.16 (ddd, J = 8.0, 2.0, 1.1 Hz, 1H), 8.10 (dt, J = 8.0, 1.4 Hz, 1H), 7.91 (m, 2H), 7.70 (m, 2H), 7.66 (d, J = 8.2 Hz, 1H), 7.6 (s, 1H), 7.47 (ddd, J = 8.1, 1.8, 0.9 Hz, 1H), 7.39 (s, 1H), 6.78 (d, J = 8.8 Hz, 2H), 4.01 (s, 3H), 3.59 (m, 4H), 3.10 (s, 3H), 2.70 (s, 3H), 2.51 (s, 3H), 2.03 (p, J = 7.2 Hz, 3H). MS (FAB+) m/z Calcd. for $C_{32}H_{32}ClO_7N_6S$ $[M+H]^+$: 678.18. Found: 660.27 (hydrolysis product).*

    For this reaction, it is very important to keep all the solvents (DCM, ethyl acetate and hexane) and containers dry. It is highly recommended to take anhydrous DCM and DIEA from sure-seal bottles using oven-dried glass syringes and needles.

    Add 3-(chlorosulfonyl)benzoyl chloride and DIEA dropwise to the reaction mixture and monitor the temperature by using a thermometer or touching the flask by hand. Make sure the reaction temperature is kept near 0°C while adding the reagents. High temperature may promote side reaction in which the BHQ1-NH_2 reacts with 3-(chlorosulfonyl)benzoyl chloride on the sulfonyl chloride side.

*Synthesize compound **22** BHQ1-azide*

21. In a 50-ml round-bottomed flask, dissolve 99 mg (0.146 mmol) compound **20** and 18 mg (0.164 mmol) DMAP in 2 ml anhydrous DCM and then add 25 µl (0.292 mmol) 2-azidoethanol followed by 128 µl (0.73 mmol) DIEA. Stir the reaction under Ar at room temperature for 4 hr.

22. After removing the solvent by rotary evaporation, purify the crude product by column chromatography on silica gel (100% ethyl acetate) to yield compound **22**.

    *Compound **22**: Expected 65 mg, 89 µmol, 61% yield as a dark red solid. $R_f = 0.58$ in 100% ethyl acetate. ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 8.30 (s, 1H), 8.11 (m, 1H), 8.07 (m, 1H), 7.93 (m, 3H), 7.71 (m, 2H), 7.66 (m, 2H), 7.58 (s, 1H), 7.48 (m, 1H), 7.39 (s, 1H), 4.24 (t, J = 4.8 Hz, 2H), 4.02 (s, 3H), 3.78 (t, J = 5.0 Hz, 1H), 3.59 (m, 4H), 3.50 (t, J = 4.8 Hz, 2H), 3.12 (s, 3H), 2.70 (s, 3H), 2.52 (s, 3H), 2.02 (m, 2H). MS (FAB+) m/z Calcd. for $C_{34}H_{36}O_{10}N_7S$ $[M+H]^+$: 729.25. Found: 729.30.*

*Synthesize compound **23** TMP-Q-Atto520*

23. Transfer compound **22** (TFA salt, 6 mg, 6.7 µmol) and compound **17** (9 mg, 12.3 µmol) to a 10-ml round-bottomed flask and dissolve in 0.2 ml DMF.

24. Add 0.68 mg (3.86 µmol) ascorbic acid and 0.19 mg (0.771 µmol) $CuSO_4 \cdot 5H_2O$ and stir the reaction mixture under Ar at room temperature for 24 hr.

    If it is difficult to weigh and transfer the small amount of ascorbic acid and $CuSO_4 \cdot 5H_2O$, first dissolve these reagents in DMF to make 0.1 M solutions and then transfer to the reaction mixture using a syringe.

25. After removing the solvent by rotary evaporation, purify the product by HPLC to yield compound **23**.

    *Compound **23**: Expected 1.3 mg, 0.86 µmol, 11% yield as a dark purple solid, $R_f = 0.72$ in 1:4 methanol:DCM with 0.5% acetic acid (v/v). ^1H NMR (400 MHz, CD_3OD) δ ppm:*

8.86 (t, J = 5.7 Hz, 1H), 8.24 – 8.14 (m, 3H), 8.05 (t, J = 5.6 Hz, 1H), 7.93 (m, 1H), 7.79 (dd, J = 1.8, 0.9 Hz, 1H), 7.73 – 7.58 (m, 7H), 7.28 (d, J = 0.8 Hz, 1H), 7.22 (d, J = 1.1 Hz, 1H), 7.17 (s, 1H), 6.61 (s, 2H), 6.56 (s, 2H), 4.66 – 4.56 (m, 3H), 4.50 (m, 2H), 3.92 (m, 2H), 3.86 (s, 3H), 3.80 (s, 6H), 3.63 (s, 2H), 3.62 – 3.56 (m, 3H), 3.55 – 3.50 (m, 2H), 3.38 (q, J = 7.2 Hz, 4H), 3.33 (d, J = 1.6 Hz, 2H), 3.11 (s, 3H), 3.06 (dd, J = 14.9, 5.7 Hz, 1H), 2.90 (dd, J = 14.9, 8.3 Hz, 1H), 2.57 (s, 3H), 2.54 (s, 3H), 2.18 (s, 6H), 2.01 (m, 2H), 1.82 (m, 2H), 1.29 (t, J = 7.2 Hz, 6H). MS (ESI) m/z Calcd. for $C_{77}H_{88}O_{18}N_3S$ *[M+H]*$^+$*: 1506.66. Found: 1506.5.*

Recommended HPLC conditions for C18 column: start with 30:70 (v/v) acetonitrile:water, gradient elution for 80 min, end with 80:20 (v/v) acetonitrile:water. Retention time: 49 to 51 min.

26. Dissolve the final product TMP-Q-Atto520 in DMF or DMSO and determine the concentration by measuring absorbance at 580 nm.

The extinction coefficient of molecule 23 at 580 nm is $\varepsilon_{580} = 1.97 \times 10^4 mol^{-1} cm^{-1}$*, measured in DMF. It is recommended to make a 1 to 5 mM stock solution in DMF of DMSO.*

Stain the cells and perform fluorescence imaging

27. Transfect HEK 293T cells with plasmids encoding H2B-eDHFR:L28C and stain with TMP-Q-Atto520 for live-cell confocal imaging.

The procedures of cell staining and fluorescence imaging with TMP-Q-Atto520 are the same as with TMP-Atto655 in the Basic Protocol (see steps 22 to 35), with variations: (1) HEK 293T cells need to be stained for 3 hr before proceeding to washing and imaging; (2) for fluorescence imaging, select 488-nm Ar laser and set the photon multiplier tube (PMT) to collect emission light from 520 to 600 nm.

See Figure 9 for representative results.

REAGENTS AND SOLUTIONS

Use deionized, distilled water for all solutions and protocol steps.

PBS, 1×

Dissolve the following in 800 ml deionized water:
8 g of NaCl (final concentration 137 mM)
0.2 g of KCl (final concentration 2.7 mM)
1.44 g of Na_2HPO_4 (final concentration 10.0 mM)
0.24 g of KH_2PO_4 (final concentration 2.0 mM)
Adjust pH to 7.4 using 1 N HCl or 1 N NaOH
Adjust volume to 1 liter with additional deionized water
Sterilize by autoclaving
Store up to 2 years at room temperature

COMMENTARY

Background Information

Since the first report in 2005, the TMP-tag has undergone transition from proof-of-principle studies of imaging nuclear-localized proteins to real-world biophysical experiments both in vitro and inside of living cells, especially in circumstances that are difficult or not otherwise possible with FPs. Meanwhile, the palette of TMP-fluorophores has been expanded from fluorescein-based green and red dyes to include high photon output cyanine dyes, a far-red photo-switching Atto655, a two-photon fluorophore BC575, and lanthanide probes (summarized in Table 1). Significantly, the advantage of TMP-tags for sophisticated biophysical experiments has been verified by several recent reports, including single-molecule (SM) imaging of spliceosome assembly in yeast cell extracts; chromophore-aided laser inactivation (CALI) of localized myosin light chain in live MEF cells; and super-resolution imaging of nucleosomes in live HeLa cells.

Table 1 TMP-Tags with Various Fluorophores for Live Cell Imaging

Chemical tag	Fluorophore	Protein of interest	Reference
Noncovalent TMP-tag	BODIPY Texas red	Plasma membrane (inner surface); cell nucleus	Miller et al. (2005)
	Fluorescein (protected)	Myosin light chain (MLC); cell nucleus	Miller et al. (2005); Cai et al. (2010)
	Hexachlorofluorescein (protected)	Myosin light chain kinase; α-tubulin	Calloway et al. (2007)
	Atto655	Histone protein 2B (H2B)	Wombacher et al. (2010)
	Cy3	Spliceosome in yeast cell extract	Hoskins et al. (2011)
	Terbium complex	Plasma membrane (outer surface)	Rajapakse et al. (2009)
Covalent TMP-tag	Fluorescein (protected)	H2B; TOMM20; MLC, α-actinine	Gallagher et al. (2009); Chen et al. (2012)
	Dapoxyl	H2B; plasma membrane	Chen et al. (2012)
	Atto655	H2B; plasma membrane	Shaner et al. (2007)
Fluorogenic (and covalent) TMP-tag	Fluorescein (protected)	H2B; plasma membrane (inner surface)	C. Jing and V.W. Cornish (in press)
	Atto520	H2B; TOMM20; MLC, α-actinine	C. Jing and V.W. Cornish (in press)

In 2011, the TMP-tag was exploited in combination with the SNAP-tag to enable single-molecule imaging of the spliceosome in yeast cell extracts, an experiment not possible with the lower photon-output FPs (Hoskins et al., 2011). Despite intense interest in understanding the mechanism by which pre-mRNAs are correctly spliced to mature mRNAs, the spliceosome is difficult to study because it is a complex 2-3 MD machinery of proteins and RNAs and cannot be reconstituted in vitro from purified components. The TMP- and SNAP-tags allowed pairs of the small nuclear ribonucleoprotein (snRNP) components of the spliceosome to be labeled directly in cell extracts with high photon output dyes and imaged as they assembled on individual pre-mRNAs.

Likewise, the high photon output TMP-tags are beginning to impact “super-resolution” (SR) imaging technologies that break the diffraction barrier to allow fluorescence imaging at the nanometer-length scale of natural proteins. Stochastic SR imaging technologies, including PALM (photo-activatable localization microscopy) and STORM (stochastic optical reconstruction microscopy) hinge on photo-switchable fluorophores with high photon output that enable the locations of subsets of the total fluorophore population to be determined precisely over time. Until recently, SR imaging has typically relied on either photo-activatable FPs, which have limited photon output and palettes, or antibodies conjugated with organic fluorophores, which offer higher resolution and many more colors, but are incompatible with live cells (Jones et al., 2011; Klein et al., 2011). Chemical tags have the potential to combine the advantages of these two contrasting labeling methodologies—they are genetically encoded and thus compatible with live cells, and they are modular and thus allow use of photo-switchable high photon output organic fluorophores. Bringing this potential to reality, in 2010, building on the discovery by Sauer et al. that the reducing environment of the cell catalyzes reversible photo-switching of high photon-output Atto organic fluorophores at time scales well suited for direct STORM (dSTORM) imaging (Heilemann et al., 2009), we demonstrated dynamic dSTORM imaging of H2B labeled with TMP-Atto655 (Wombacher et al., 2010). Significantly, we achieved exceptional spatial (~20 nm) and temporal (~10 sec) resolutions, which are not possible with FPs.

Besides single-molecule and SR imaging, chemical tags with organic fluorophores also have advantages over FPs for numerous biophysical studies. For example, chromophore-assisted laser inactivation (CALI) is a useful

technique for selective protein inactivation in which the dye molecules produce reactive oxygen species (ROS) upon laser irradiation, thus damaging the tagged proteins with spatial and temporal control. Small-molecule dyes are shown to be more efficient ROS generators than FPs probably because the chromophore in an FP is buried in a β-barrel, which hinders the interaction between the fluorophore and ROS. In a representative study, the eDHFR/TMP-fluorescein tag has been used to image and manipulate myosin-II via CALI (Cai et al., 2010). Local inactivation of myosin-II disrupts the continuity of the actomyosin-II network and results in decrease of cytoplasmic coherence, verifying that non-muscle myosin-II contraction of cytoplasmic actin filaments establishes a coherent cytoskeletal network that transmits force across the cytoplasm.

Finally, the TMP-tag is not limited to traditional fluorescence imaging. The modular design allows for conjugation of a great variety of synthetic molecules, including magnetic nanoparticles for manipulating proteins and live cells (Long et al., 2011); synthetic peptides for small molecule-induced protein ligation (Ando et al., 2007); and dexamethasone as chemical inducer of protein dimerization (Czlapinski et al., 2008).

Critical Parameters

As outlined in the Strategic Planning section, the TMP-tag should be carefully designed and optimized based on specific requirements of the biophysical experiment. Potential variations on aforementioned protocols are listed below:

Fluorophores. For live-cell imaging, it is crucial to choose fluorophores with both exceptional photo-physical properties and favorable biological activities. The three TMP-tags described in the protocols: TMP-Atto655, A-TMP-fluorescein (protected), and TMP-Q-Atto520, have all been demonstrated to enable live-cell imaging of various intracellular proteins. When using other fluorophores it is recommended to select those with minimal negative charges (in the form of carboxyl groups or sulfonates). If the fluorophore has multiple negative charges, the regular cell staining protocol may not be highly efficient and some special techniques may be necessary to introduce the TMP-fluorophore conjugate into live cells. These techniques may include electroporation, cell scraping, and induced pinocytosis.

For in vitro experiments or imaging cell surface proteins, however, the TMP-fluorophore does not need to be highly cell-permeable or well behaved (i.e., not aggregating, not staining nonspecific biomolecules, and not partitioning into lipid-rich compartments) inside of cells. In such cases, any of a large number of cyanine dyes and Alexa dyes may be good choices because of their outstanding brightness and photo-stability.

Linkers. The linker between TMP and the fluorophore may impact solubility and cell permeability of the molecule, and is therefore also important for live-cell imaging. Previous studies have shown that PEG linker is favored over methylene linker for mammalian cells (Calloway et al., 2007). In contrast, the methylene linker works slightly better than PEG linker in yeast cells (C. Jing and V.W. Cornish, in press).

Target proteins. eDHFR is about two-thirds the size of FPs. Therefore, most target proteins that can be labeled by FPs without compromising function and dynamics should also be compatible with the TMP-tag. eDHFR is a monomeric, well-behaved, rapidly folding protein that can be expressed well both on the surface and inside of live cells. For in vitro studies, both eDHFR and eDHFR:L28C variant can be overexpressed in *E. coli* and purified using standard protein purification techniques.

Cell staining condition. The cell staining conditions may vary for different cell lines and target proteins. Generally, for noncovalent and covalent TMP-tags, 0.5 to 5 μM of dye staining for 5 to 60 min at 37°C should efficiently label most intracellular proteins. For fluorogenic TMP-tags, the analogous parameters are 1 to 10 μM of dye staining for 0.5 to 3 hr.

Optimization of the fluorogenic TMP-tag. Previous studies have found that for fluorogenic TMP-tags, different fluorophores may pair with different eDHFR:Cys variants to reach maximal labeling efficiency and fluorescence enhancement (C. Jing and V.W. Cornish, in press). For example, TMP-Q-Atto520 works best with eDHFR:L28C, while TMP-Q-fluorescein prefers eDHFR:P25C. Therefore, when using a fluorophore other than Atto520 or fluorescein, it is suggested to determine the appropriate eDHFR:Cys mutant by in vitro fluorometric measurements. Potential eDHFR:Cys candidates include P25C; L28C; A29C; N23C; K32C; R52C, and P55C. Additionally, BHQ1 effectively quenches fluorophores emitting in the green-yellow region (480 to 580 nm). When using a red-shifted fluorophore, a red quencher such as BHQ3 or QSY21 may be used instead of BHQ1.

Table 2 Troubleshooting

Step	Problem	Possible reason	Suggested solution
Basic Protocol, step 2	Cannot obtain pure product by recrystallization	The hydrolysis reaction was not well controlled	Carefully control the reaction temperature and time. Purify the product by silica column, if necessary.
Basic Protocol, step 12	Cannot obtain pure product by silica column	The unreacted BocNH-PEG-NH_2 eluted together with the product	Refine the intermediate TMP-PEG-NHBoc by silica column. If the purity is still not sufficient, proceed to the deprotection step and purify the final compound **5** by HPLC.
Alternate Protocol 1, step 4	The product compound **7** is not pure	The reaction temperature was not well controlled	Make sure the reaction mixture is kept at 0°C. If necessary, purify the product compound **7** by reverse-phase chromatography.
Alternate Protocol 1, step 18	Cannot obtain pure compound **13** by reverse-phase chromatography	The unreacted BocNH-PEG-NH_2 eluted together with the product	Refine compound 13 by HPLC
Alternate Protocol 2, steps 18-25	No reaction or product is not pure	Tosylate group hydrolyzed	Use anhydrous solvent and reagents. Dry all containers, stir bars, syringes, and needles in a 95°C oven prior to use. Keep the intermediates cool and dry. If the intermediates and products are stored in refrigerators or freezers, warm them to room temperature before opening the container.

Troubleshooting

See Table 2 for troubleshooting information.

Anticipated Results

Representative results for chemical synthesis and fluorescence imaging are provided in the corresponding protocols.

Time Considerations

For the Basic Protocol, the chemical synthesis will take 10 to 15 days. For Alternate Protocols 1 and 2, the chemical synthesis is anticipated to take 20 to 25 days. The vector construction and plasmid maxi-prep will take 3 to 10 days. The cell transfection, staining, and imaging is anticipated to take 3 days with cells in culture.

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