In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag

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The introduction of green fluorescent protein and its variants (GFPs) has allowed protein analysis at the level of the cell. Now, chemical methods are needed to label proteins *in vivo* with a wider variety of functionalities so that mechanistic questions about protein function in the complex cellular environment can be addressed. Here we demonstrate that trimethoprim derivatives can be used to selectively tag *Escherichia coli* dihydrofolate reductase (eDHFR) fusion proteins in wild-type mammalian cells with minimal background and fast kinetics.

GFPs have proven to be invaluable tools for cell biology, allowing for selective protein labeling and fluorescent microscopic imaging of protein distribution in living cells. The next step is to use imaging technologies to understand protein biochemistry inside the cell—how the complex cellular environment affects, for example, the lifetime of protein interactions. Because the GFP fluorophore is formed by amino acids in the protein core, however, it has proven difficult to engineer a well-behaved red variant of GFP, much less to modify the spectral properties of GFP for more sophisticated applications[1]. Thus, beginning with the report of fluorescein-based biarsenical dye FlAsH in 1998 (ref. 2), researchers have sought to develop chemical methods to selectively label proteins with small-molecule probes *in vivo*[3]. The recent use of ligand-receptor pairs to label proteins combines the advantages of genetic encoding to provide selectivity (the receptor) and synthetic chemistry to allow precise manipulation of the probe properties (the ligand-probe conjugate; **Fig. 1a**)[4–8]. Each of the chemical labeling approaches reported thus far, however, has one or more substantial limitations: use of a cell line in which the endogenous receptor has been knocked out; high background labeling; or a half-life for labeling of several hours or more. Addressing each of these limitations, we report here that 2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine (trimethoprim or TMP) can be used to selectively label eDHFR fusion proteins in wild-type mammalian cell lines with low background and fast kinetics. Demonstrating the advantages of this chemical labeling method, we show that TMP/eDHFR can replace GFP to provide an improved, transient screen for small interfering RNA (siRNA) gene silencing.

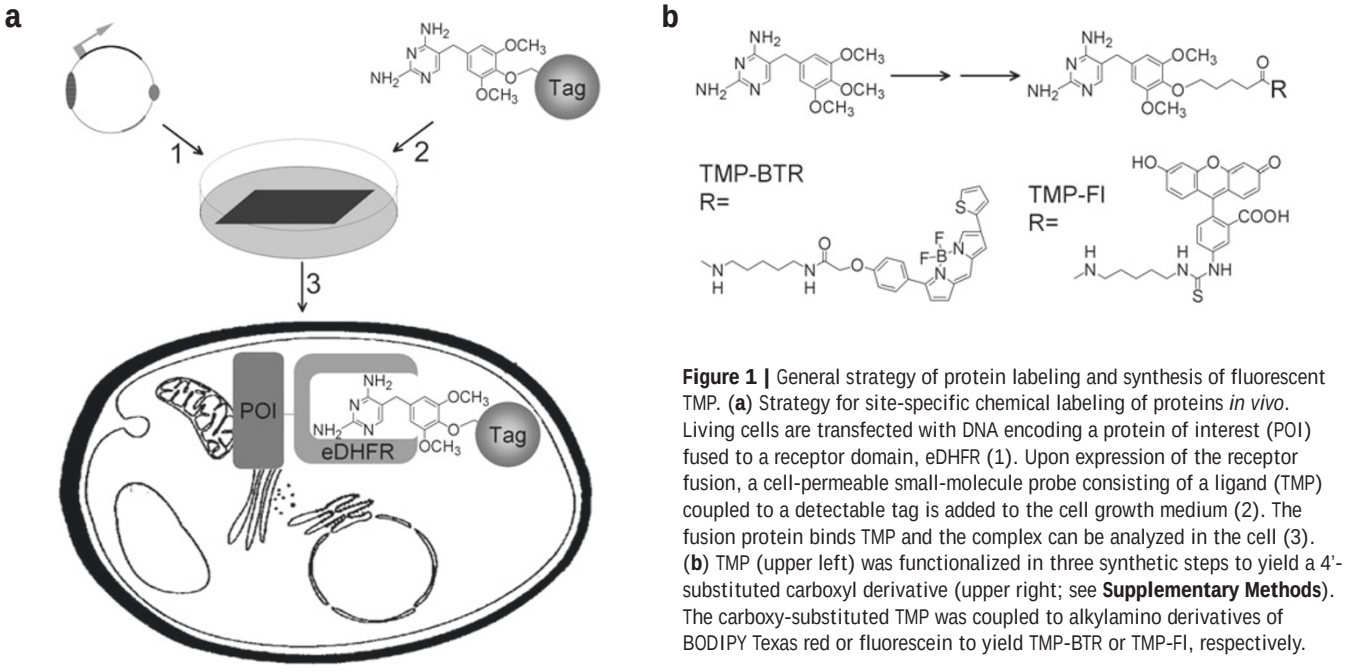

Figure 1 | General strategy of protein labeling and synthesis of fluorescent TMP. (**a**) Strategy for site-specific chemical labeling of proteins *in vivo*. Living cells are transfected with DNA encoding a protein of interest (POI) fused to a receptor domain, eDHFR (1). Upon expression of the receptor fusion, a cell-permeable small-molecule probe consisting of a ligand (TMP) coupled to a detectable tag is added to the cell growth medium (2). The fusion protein binds TMP and the complex can be analyzed in the cell (3). (**b**) TMP (upper left) was functionalized in three synthetic steps to yield a 4'-substituted carboxyl derivative (upper right; see **Supplementary Methods**). The carboxy-substituted TMP was coupled to alkylamino derivatives of BODIPY Texas red or fluorescein to yield TMP-BTR or TMP-FI, respectively.

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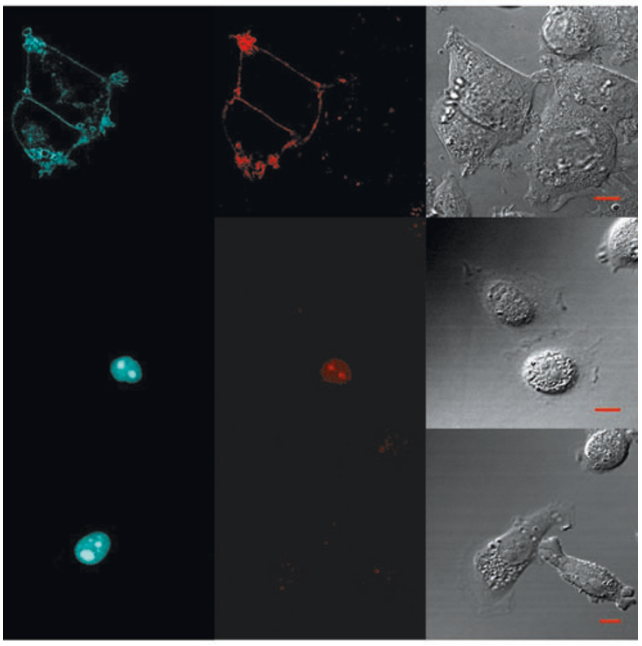

Figure 2 | Selective chemical labeling of subcellularly targeted eDHFR in wild-type CHO cells. (**a–c**) Confocal micrographs: left column, excitation at 458 nm; middle column, excitation at 568 nm; right column, differential image contrast. (**a**) CHO cells transiently cotransfected with DNA encoding plasma membrane–targeted eDHFR and CFP. Images taken 5 min after addition of growth medium containing 10 nM TMP-BTR. (**b**) CHO cells transiently cotransfected with DNA encoding nucleus-targeted eDHFR and CFP. Images taken 15 min after addition of growth medium containing 10 nM TMP-BTR. (**c**) CHO cells transfected as in **b**. Incubated in medium containing 1 μM TMP for 15 min, then medium containing TMP-BTR was added to a final concentration of 100 nM. Images taken after 30 min show no nuclear staining because eDHFR binding sites are blocked with TMP.

We chose TMP and eDHFR as a ligand-receptor pair for general-purpose protein labeling because TMP selectively inhibits eDHFR, because eDHFR is small (~18 kDa) and monomeric (by contrast, GFP is 27 kDa), and because TMP can be easily derivatized without substantially disrupting its binding to eDHFR[9]. TMP has high affinity (K_I = ~1 nM) for eDHFR, but substantially lower affinity (K_I = ~4 μM) for a mammalian form of this receptor (SR-1 rodent lymphoma DHFR)[10]. Building on prior work with fluorescent conjugates of methotrexate and methylbenzoprim[11–13], we had been able to specifically label eDHFR fusion proteins expressed in DHFR knockout Chinese hamster ovary (CHO) cells with fluorescent methotrexate[8]. Given the favorable characteristics of TMP, we reasoned that TMP derivatives could be used to label eDHFR fusion proteins in mammalian cells with little or no background resulting from binding to endogenous DHFR. We synthesized a 4′ carboxy–substituted TMP derivative in three steps (**Supplementary Methods** online), and then we coupled the carboxyl-substituted TMP to commercially available alkyl-amino derivatives of BODIPY Texas red or fluorescein, yielding TMP-BTR and TMP-Fl, respectively (**Fig. 1b**). Using a fluorescence polarization binding assay, we determined that TMP-Fl binds to eDHFR with a dissociation constant of 32 ± 3 nM (**Supplementary Methods** and **Supplementary Fig. 1** online).

We detected selective binding of TMP-BTR to eDHFR fusion proteins expressed in wild-type CHO cells using confocal microscopy. Expression vectors that targeted eDHFR to either the plasma membrane or the nucleus were used (see **Supplementary Methods**).

eDHFR was targeted to the plasma membrane by fusing to its N terminus the 10-amino-acid myristoylation/palmitoylation sequence from Lyn (MGCIKSKGKD)[8], and this receptor was targeted to the nucleus by encoding eDHFR with a C-terminal fusion of three copies of the canonical simian virus 40 large T-antigen nuclear localization sequence (DPKKKRKV)[14]. As controls, we prepared analogous expression vectors encoding cyan fluorescent protein (CFP) with the same plasma membrane– or nuclear-targeting sequences. We then transiently cotransfected CHO cells with either equal parts plasma membrane–targeted eDHFR and plasma membrane–targeted CFP DNA, or nucleus-targeted eDHFR and nucleus-targeted CFP DNA. Approximately 24 h after transfection, the cells were incubated with a low concentration (10 nM) of TMP-BTR and imaged using a laser scanning confocal microscope (**Fig. 2a,b**).

Unambiguous labeling of plasma membrane– or nucleus-targeted eDHFR was achieved in CHO cells within minutes of exposure to nanomolar concentrations of TMP-BTR in the growth medium. Only cells that exhibited localized CFP fluorescence showed corresponding TMP-BTR fluorescence. Because the small-molecule concentration needed for labeling is so low, the TMP-BTR–labeled eDHFR fusion proteins were detectable when TMP-BTR was present in the growth medium (**Fig. 2a,b**). A control experiment established that the red fluorescent signal was dependent on the specific labeling of the eDHFR fusion proteins with TMP-BTR. Preincubation of CHO cells expressing nucleus-targeted eDHFR in medium containing 1 μM TMP and subsequent incubation in medium containing 100 nM TMP-BTR resulted in no nuclear labeling (**Fig. 2c**).

To better evaluate the performance of the TMP-eDHFR labeling system, we used TMP-Fl to label eDHFR fused to a cytosolic protein: puromycin N-acetyl transferase (Puro^R-eDHFR; see

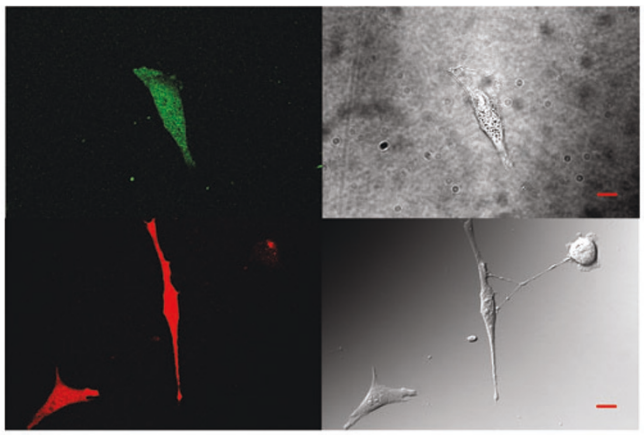

Figure 3 | Selective chemical labeling of eDHFR fusion proteins in MEF cells. (**a**,**b**) Confocal micrographs: left column, excitation at 488 nm (**a**), excitation 568 nm (**b**); right column, differential image contrast. (**a**) MEF cells transfected with pSIREN RetroQ vector modified to express a puromycin N-acetyl transferase–eDHFR fusion protein. Cells were incubated in 10 μM TMP-BTR for 2 h, washed 3× with PBS and imaged 30 min after washout. (**b**) MEF cells transfected as in **a**, and incubated in medium containing 1.7 μg/ml puromycin for 72 h. Cells were then incubated in 50 nM TMP-BTR for 15 min, washed 3× with PBS and imaged. Bar, 10 μm.

Supplementary Methods). Upon expression of Puro^R-eDHFR in mouse embryonic fibroblast (MEF) cells and labeling with TMP-Fl (10 μM) for 2 h, we could clearly detect transfected cells (**Fig. 3a**: an analogous GFP fusion was made, and expression in fibroblasts yielded a similar phenotype; see **Supplementary Fig. 2** online). Perhaps unsurprisingly, a higher concentration of TMP-Fl was required for labeling than TMP-BTR, most likely because of the greater hydrophilicity of fluorescein relative to BODIPY Texas red (**Fig. 1b**). Thus, we expect the dye-loading kinetics to vary with the label attached to TMP. To determine the longevity of the TMP-Fl-eDHFR interaction, we followed the decrease in the fluorescent signal relative to background for a fibroblast cell expressing Puro^R-eDHFR. The signal decreased approximately 13% per hour and was still visible 2.5 h after washout (**Supplementary Methods** and **Supplementary Fig. 3** online).

The imaging experiments show that derivatized TMP can be used to selectively tag an eDHFR fusion protein in wild-type mammalian cells with minimal background. Thus, the TMP-eDHFR system should be adaptable to many biological applications. One immediate, broadly useful application is siRNA gene silencing[15]. Now GFP is used as the marker to screen for successful siRNA transfectants, but the use of GFP precludes fluorescent studies of siRNA-modified cells in that wavelength range. Because the TMP-BTR conjugate can be washed out at the end of an experiment, the TMP-eDHFR labeling system could be used in place of GFP to provide a transient label for successful siRNA transfectants. We first modified a commercially available siRNA expression plasmid (pSIREN RetroQ; BDBiosciences) by cloning a siRNA target sequence derived from the coding region of the β-actin gene into the multiple cloning site under control of the human U6 promoter (**Supplementary Methods**). We then cloned eDHFR DNA C-terminally to the puromycin N-acetyl transferase (under control of a separate PGK promoter). Approximately 24 h after transfection with the modified siRNA expression plasmid, we exposed MEF cells to TMP-BTR and examined them for fluorescence. Transfected cells were clearly distinguishable by their fluorescent phenotype, and based on the fluorescent cell count, the transfection efficiency was 12%. Transfected cells were then exposed to puromycin (1.7 μg/ml) for ~72 h, and the dye-loading and microscopy protocol was repeated. Fluorescent cells comprised ~50% of the puromycin-treated population, indicating that successful transfectants have the fluorescent phenotype, and the TMP-BTR label could be washed out of the transfected cells (**Fig. 3b**).

Here we have shown that TMP conjugates can be used to label proteins tagged with eDHFR with fast kinetics and minimal background in wild-type mammalian cell lines. Thus, TMP and eDHFR should provide an immediate alternative to GFP for more sophisticated imaging experiments. The ease of synthesis of TMP derivatives should allow the introduction of fluorophores or other labels optimized for fluorescence resonance energy transfer, chromophore assisted light inactivation of proteins[16], photoaffinity labeling or other *in vivo* bioassays. We also envision the development of chemically modified TMP derivatives that bind specifically to mutated forms of eDHFR[17], thereby allowing the simultaneous labeling of multiple protein targets in a single cell.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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