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Optimized Fluorescent Trimethoprim Derivatives for in vivo Protein Labeling

Nathaniel T. Calloway,[a] Michael Choob,[b] Ana Sanz,[b] Michael P. Sheetz,[c] Lawrence W. Miller,*[d] and Virginia W. Cornish*[e]

The combined technologies of optical microscopy and selective probes allow for real-time analysis of protein function in living cells. Synthetic chemistry offers a means to develop specific, protein-targeted probes that exhibit greater optical and chemical functionality than the widely used fluorescent proteins. Here we describe pharmacokinetically optimized, fluorescent trimethoprim (TMP) analogues that can be used to specifically label recombinant proteins fused to E. coli *dihydrofolate reductase (eDHFR) in living, wild-type mammalian cells. These improved fluorescent tags exhibited high specificity and fast labeling kinetics, and they could be detected at a high signal-to-noise ratio by using fluorescence microscopy and fluorescence-activated cell sorting (FACS). We also show that fluorescent TMP–eDHFR complexes are complements to green fluorescent protein (GFP) for two-color protein labeling experiments in cells.*

Introduction

Green fluorescent protein and its variants (GFPs) have proven invaluable to the biological sciences since they allow the direct dynamic visualization of proteins in living mammalian cells by using fluorescence microscopy.[1] The spectral properties of GFPs are inherently limited, however, because the chemistry of the fluorophore is linked to the tertiary structure of the protein.[2] While mutagenesis has led to a number of enhancements, the GFPs currently available still have limitations that affect their use as biological markers and indicators. For example, it has proven difficult to engineer a monomeric, bright, red-shifted variant.[3] By contrast, small-molecule fluorophores are easily modified by using synthetic methods, and there has been tremendous effort to develop probes for biological analysis.[4] Consequently, there have been efforts to develop small-molecule fluorescent tags that will label proteins in vivo with the same selectivity afforded by GFPs.[5]

Chemical labeling of fusion proteins has the advantage that the selectivity of labeling is genetically encoded, but the fluorescent properties of the ligand can be modified synthetically. Beginning with the bis-arsenical fluorescein-based FlAsH (fluorescein arsenical hairpin binder) ligand,[6] researchers have developed a variety of intracellular protein-labeling strategies. These include noncovalent binding of a small-molecule inhibitor by an enzyme,[7] or covalent linkage of a small molecule to an enzyme.[8] We previously demonstrated the feasibility of using antifolates methotrexate and trimethoprim (TMP) and *E. coli* dihydrofolate reductase (eDHFR) as ligand–receptor pairs for in vivo imaging.[9] The TMP–eDHFR pair is particularly advantageous because the interaction is orthogonal to mammalian systems. When added to cell-culture medium, fluorescent TMP derivatives diffuse into cells and bind selectively to eDHFR fusion proteins (Figure 1). TMP binds much more tightly to eDHFR ($K_I \approx 10^{-9}$ M) than to mammalian forms of DHFR ($K_I >$ 10^{-6} M).[10] Thus, the use of TMP–eDHFR does not require a knock-out or otherwise modified cell line. TMP is readily derivatized with fluorophores without disrupting the interaction

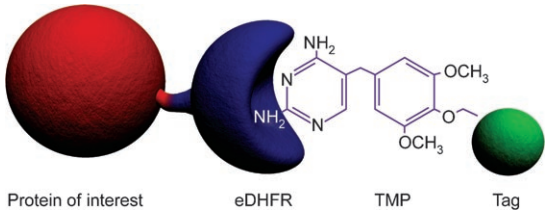

Figure 1. Schematic representation of the TMP–eDHFR labeling system in which TMP that is covalently attached to a fluorescent tag is bound by a chimeric fusion of eDHFR. When fluorescent TMP is added to culture medium, it rapidly diffuses into mammalian cells. The orthogonal TMP–eDHFR interaction engenders specific fluorescent labeling of the protein of interest.

[a] *N. T. Calloway*
Department of Chemistry and Chemical Biology, Cornell University
Ithaca, NY 14853 (USA)

[b] *Dr. M. Choob, Dr. A. Sanz*
Active Motif, Inc.
1914 Palomar Oaks Way, Suite 150, Carlsbad, CA 92008 (USA)

[c] *Prof. M. P. Sheetz*
Department of Biological Sciences, Columbia University
1244 Amsterdam Avenue, New York, NY 10027 (USA)

[d] *Prof. L. W. Miller*
Department of Chemistry, University of Illinois at Chicago
845 W. Taylor St., MC 111, Chicago, IL 60607 (USA)
Fax: (+1) 312-996-0431
E-mail: lwm2006@uic.edu

[e] *Prof. V. W. Cornish*
Department of Chemistry, Columbia University
3000 Broadway, MC 3111, New York, NY 10027 (USA)
Fax: (+1) 212-932-1289
E-mail: vc114@columbia.edu

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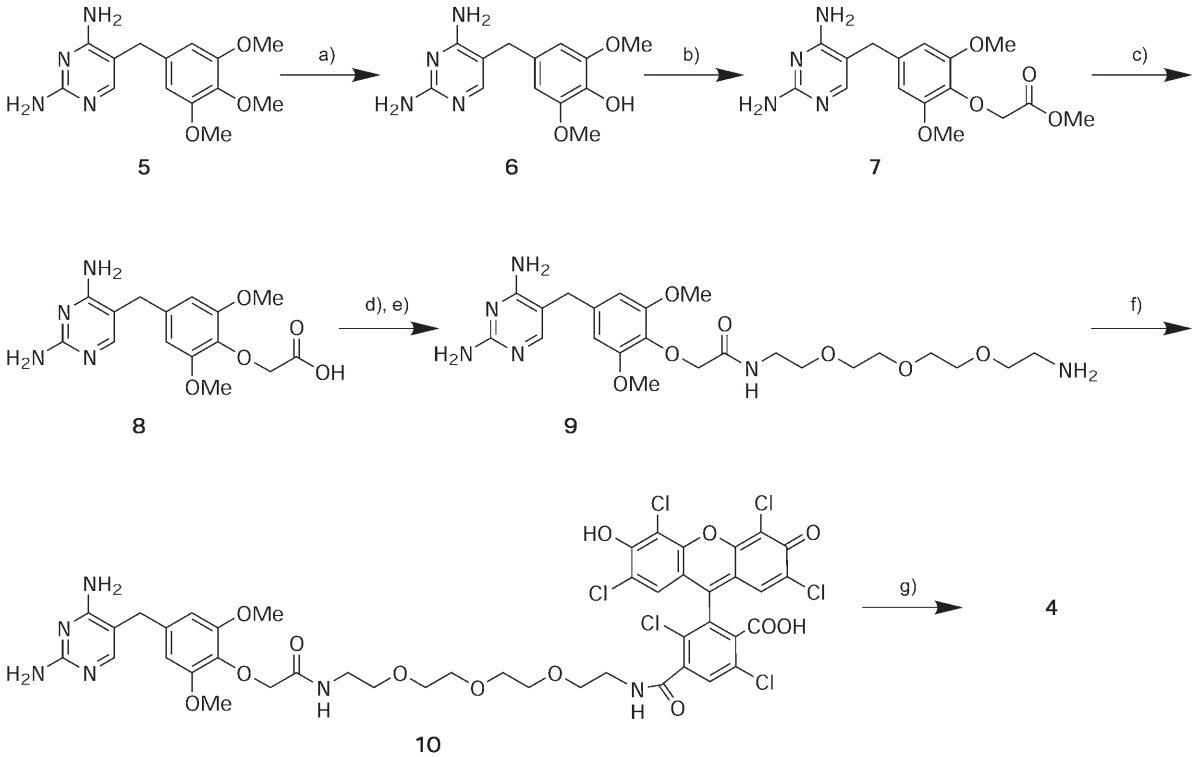
with eDHFR. The initial attempts to derivatize TMP conjugates for fluorescent labeling resulted in dyes capable of specifically labeling eDHFR fusions localized to a particular subcellular region. However, the labeling of diffuse cytosolic proteins proved difficult due to a high degree of background staining. Additionally, the dyes appeared to nonspecifically aggregate within the cell; this caused a speckled background. It was hypothesized that these limitations were intrinsic chemical properties of the dyes, and could be alleviated by varying the structures of the TMP–dye conjugates. In this paper, we present a new generation of optimized TMP–fluorophore conjugates that label intracellular eDHFR fusion proteins with fast kinetics, and that can be detected with high signal-to-noise ratio. We also show that an orange fluorescent TMP conjugate can be used with GFP for multicolor labeling experiments.

Results and Discussion

Design of improved TMP-linked fluorophores

We sought to develop fluorescent TMP derivatives that fulfilled three criteria: 1) red-shifted absorption and emission spectra that could be resolved from the commonly used GFP; 2) fast (<1 h) labeling kinetics; and 3) no nonspecific background staining. Furthermore, we wanted probes that were sufficiently bright so as to allow for detection of diffuse cytosolic proteins. We chemically linked a variety of dyes to TMP, including fluorescein, rhodamine, and cyanine derivatives. When we screened these conjugates microscopically for their ability to stain living mammalian cells, we found that many were either completely cell impermeable (e.g., chromeon 642—a phosphonated cyanine derivative) or aggregated in subcellular structures, and yielded a nonspecific, punctate staining pattern (e.g., tetramethylrhodamine; data not shown). We hypothesized that uncharged, hydrophobic TMP–fluorophore conjugates would readily diffuse across the cell membrane, and yield rapid labeling of intracellular eDHFR fusion proteins when added to cell-growth medium. While hydrophobicity is a necessary criterion for membrane permeability, very hydrophobic fluorescent probes are known to aggregate in intracellular lipid vesicles (Molecular Probes, Inc., personal communication). We ultimately chose fluorescein and hexachlorofluorescein as the most promising dyes for this study. Hexachlorofluorescein has red-shifted absorption ($\lambda_{abs}=533$ nm) and emission ($\lambda_{em}=560$ nm) spectra relative to GFP. In order to increase the cell permeability of the dyes, cleavable hydrophobic protecting groups were placed on the fluorophore in the form of both acetyl (not reported) and *iso*-butyryl esters. We linked the fluorescein derivatives to TMP with a hydrophilic tetraethylene glycol (TEG) linker to both prevent interference with TMP binding, and to minimize excessive lipid partitioning.

The synthesis and characterization of dyes **1** and **2** were reported previously.[9] In addition, two dyes, **3** and **4**, were synthesized by using the same methodology of successive amide couplings, but with the aforementioned improvements to their chemical structure (Scheme 1). The new dyes proved to be more hydrophilic than **2** due to the addition of the TEG linker and the lack of the highly hydrophobic BODIPY fluorophore. Moreover, due to the *iso*-butyryl protecting groups, the dyes lacked the isolated charge of the deprotonated carboxyl group



Scheme 1. Synthesis of TMP-linked fluorophore, **4**: a) 48% HBr in H_2O; b) methyl bromoacetate, DBU; c) NaOH, MeOH; d) PyBOP, diaminotetraethylene glycol, BOC_2O; e) TFA, $MeCl_2$; f) hexachlorofluorescein–NHS ester, DMF; g) *iso*-butyric anhydride, pyridine.

that is characteristic of fluorescein derivatives in aqeuous medium at physiological pH (e.g., dye **1**). The structural modifications incorporated into dyes **3** and **4** allowed for greatly improved, in vivo protein-labeling performance, as detailed below.

Fluorescent labeling of nucleus-localized eDHFR in mammalian cells

When Chinese hamster ovary (CHO) or mouse embryonic fibroblast (MEF) cells were transfected with DNA that encoded eDHFR fused to three copies of the simian large T antigen nuclear-localization sequence (eDHFR–NLS), and incubated with any one of four fluorescent TMP derivatives, distinct nuclear staining was observed (Figure 2). This fluorescent phenotype was identical to that observed for an analogous nucleus-targeted cyan fluorescent protein (CFP; see the Supporting Information for images of cells that expressed fluorescent fusion proteins analogous to the eDHFR fusions described here).[9] Because of this clear, reproducible phenotype, eDHFR–NLS was used as the benchmark for determining the optimal dye-loading conditions for the individual cell lines. The TEG-linked TMP derivatives yielded distinct nuclear staining with virtually no fluorescence in nonexpressing cells or in the extranuclear region of expressing cells, when the dyes were used under optimal conditions (Figure 2C and D). The results for the TEG-linked dyes are in contrast with those observed with **1** or **2** (Figure 2A and B). Those dyes showed low levels of staining with high background and frequent nonspecific staining. Dye **1** labeled eDHFR–NLS when it was added to cell-growth medium at high concentrations (5 mM) and incubated with the cells for 2 h. This result suggests that **1** is relatively cell-imper-

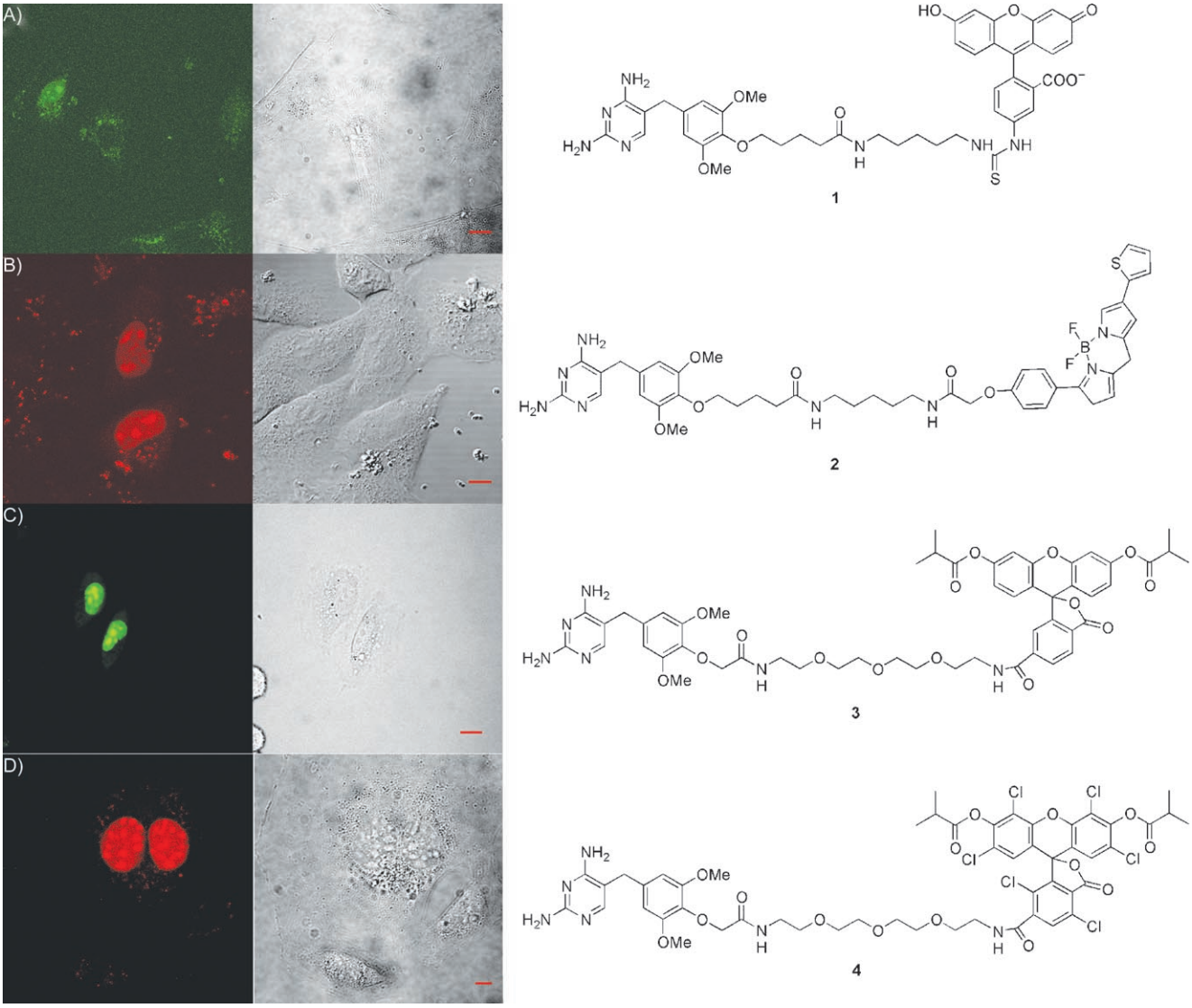


Figure 2. Fluorescence and differential interference contrast (DIC) images from eDHFR–NLS expressing cells labeled with the indicated compounds: A) MEF cells labeled with **1** (5 μM, 2 h, 37 °C) showing low levels of labeling and speckled background; B) CHO cells labeled with **2** (10 nM, 30 min, 37 °C) showing a high degree of background staining; C) CHO cells labeled with **3** modified with a tetraethyleneglycol linker and protected fluorophore, stained for 1 h at 37 °C with 2 μM dye; D) MEF cells labeled with **4** modified with similar structural changes; cells were stained for 10 min at 37 °C with 0.4 μM dye; red bar: 10 μm.

meable. By contrast, dye **2** required low (10 nM) concentrations and short incubation time (30 min) to effectively stain the nucleus. This suggests that dye **2** is very membrane permeable, most likely due to its hydrophobicity. However, **2** also yielded a nonspecific, punctate staining pattern in both expressing and nonexpressing cells. This might be due to the partitioning of hydrophobic dye **2** to intracellular lipid bodies.

One factor that is well known to decrease cell-membrane permeability of a molecule is the presence of a localized chemical charge. Charged groups increase polarity and thereby limit solubility in the plasma membrane. Therefore, we chose to mask the charge of the fluorescein moieties in the TEG-linked dyes with protecting groups, which rendered the dyes nonfluorescent but dramatically increased their cell permeability. Fluorescein dyes are commonly acetylated to increase cell permeability, and they are believed to be hydrolyzed by nonspecific, intracellular esterases.[4] However, acetylated forms of fluorescein spontaneously hydrolyze at physiological pH.[11] Therefore, we prepared *iso*-butyryl esterified forms of the fluorescein dyes, reasoning that the *iso*-butryryl group would be more resistant to ambient hydrolysis due to steric occlusion of the carbonyl, which increases the effective concentration of protected dye available to the cell. Another structural modification that we incorporated was to link TMP to the protected fluorescein with a TEG linker. This was done to increase the solubility of the ligand–probe conjugates and minimize any unwanted lipid partitioning. We observed that the *iso*-butyryl-protected, TEG-linked, TMP-fluorescein derivatives labeled eDHFR–NLS when they were added to cell-growth medium at low concentrations and incubated with the cells for short time periods (Figure 2C and D).

Labeling eDHFR fused to full-length proteins

While **1** and **2** were capable of staining eDHFR–NLS, albeit poorly, it was difficult to microscopically detect specific labeling of various full-length proteins fused to eDHFR. More generally, only those constructs with a concentrated, subcellular localization of over-expressed protein, like eDHFR–NLS, could be stained with these dyes. Therefore, we assessed the ability of the improved dyes to label eDHFR fused to α-tubulin and myosin light chain kinase—two fusion proteins that could not be labeled with **1** or **2**. When tested with either dye **3** or **4**, cells that expressed α-tubulin–eDHFR and myosin light-chain kinase–eDHFR (MLCK–eDHFR) were successfully stained and showed fluorescent phenotypes consistent with their known biochemistry and subcellular localizations.

Cells that expressed α-tubulin–eDHFR showed fluorescence throughout the cytosol and were similar in appearance to cells that expressed α-tubulin–GFP under the same growth conditions (Figure 3C and D; see the Supporting Information for images of cells that expressed α-tubulin–GFP). It is known that a large proportion of α-tubulin remains cytosolic when overexpressed in mammalian cells (as high as 40% for CHO cells).[12] Cells that expressed MLCK–eDHFR showed a fluorescence pattern that was consistent with localization on actin filaments, as also shown by MLCK–GFP (Figure 3A and B and Supporting Information).[13–15] These results demonstrate the efficient and specific labeling of the individual eDHFR fusion constructs in every case with both dyes.

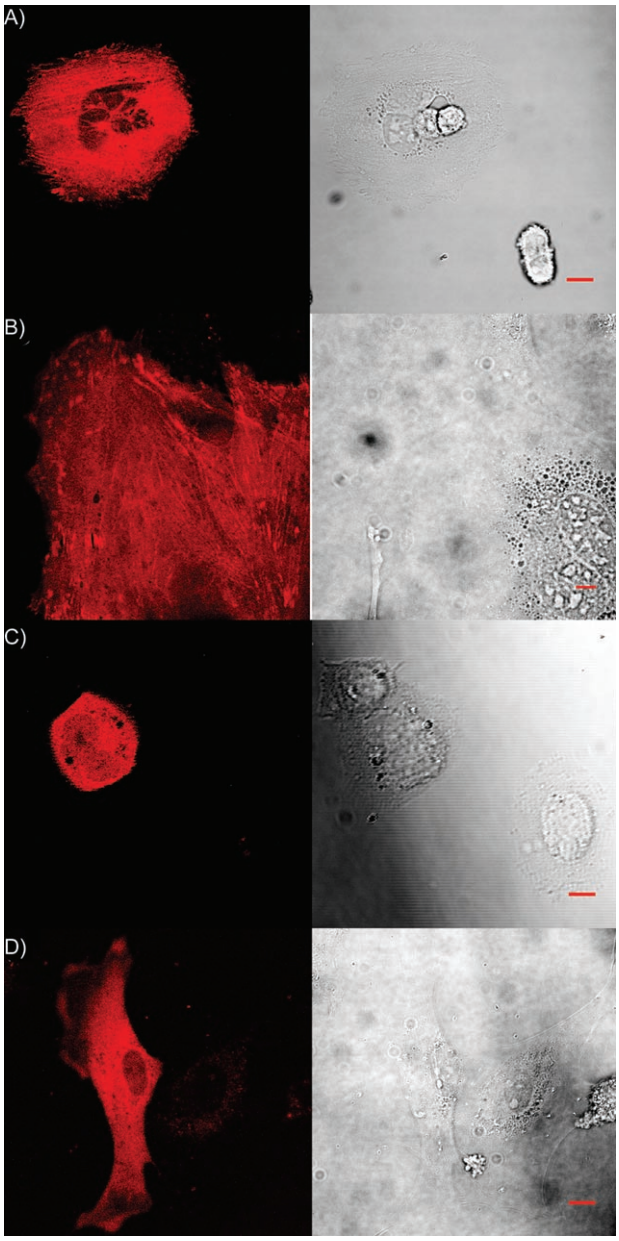


Figure 3. Fluorescence and DIC images of cells labeled with **4** at the indicated concentrations and incubation times: A) CHO cells expressing MLCK–eDHFR: 2 mM dye, 1 h incubation at 37 °C; B) MEF cells expressing MLCK–eDHFR: 0.4 mM dye, 10 min incubation at 37 °C; C) CHO cells expressing α-tubulin–eDHFR: 2 mM dye, 1 h incubation at 37 °C; D) MEF cells expressing α-tubulin–eDHFR: 0.4 mM dye, 10 min incubation at 37 °C. Note, in (A) and (B) the appearance of the actin network and regions of increased MLCK concentration on these filaments; red bar: 10 μm.

Optimal labeling conditions are dependent on cell line

When the eDHFR constructs were stained in MEF cells, it was found that a much lower concentration of dye and shorter incubation time were required to achieve a high degree of

specific labeling than when the same dye and construct were used in CHO cells. We found that MEF cells only required incubation with 400 nM of dye for 10 min to produce efficient staining. Higher dye concentrations and longer incubation times led to very high background fluorescence in expressing and nonexpressing cells. However, CHO cells could withstand higher concentrations (up to 4 μM) for up to two hours without significant background staining. In this case longer incubation times and dye concentrations increased specific labeling. For the purposes of microscopy, however, shorter times and lower concentrations were more than sufficient for adequate signal-to-noise ratios. Based on a crude kinetic analysis, these results could be mechanistically explained as the difference between the flux of ester-protected dye into the cell, and the flux of free, deprotected dye out of the cell. Previously, organic anion transporters were implicated in the cell-type dependent efflux of fluorescent dyes.[16] Cells that can efficiently eliminate excess dye from their cytoplasm will quickly equilibrate with the wash medium, and have low background fluorescence. In particular, CHO cells have shown efficient organic anion efflux.[17] However, in cell types with inefficient efflux, care must be taken to avoid accumulation of excess dye in the cytoplasm because it might become kinetically trapped, which results in high background fluorescence.

TMP–hexachlorofluorescein is a complement to GFP for multicolor labeling

It is often desirable to simultaneously image two or more spectrally resolved, fluorescently labeled proteins. Recognizing that thousands of GFP fusion protein expression plasmids have been prepared, we wanted to show that **4**–eDHFR- and GFP-labeled proteins could be simultaneously imaged in the same cell. When U2OS cells (human osteosarcoma cell line) were co-transfected with DNA encoding a nucleus-localized GFP protein and eDHFR fused to a plasma membrane localization signal sequence, and labeled with **4**, the differently colored fusion proteins could be imaged simultaneously (Figure 4). These experiments demonstrate that GFP and **4** bound to eDHFR, are complementary protein labels for multicolor tagging applications.

Labeling of eDHFR fusion proteins is fast and stable, but reversible

When cells were labeled with these dyes at concentrations optimized for the specific cell type, their specific fluorescent phenotype was clearly observable with little reduction in the signal-to-noise ratio after dye washout. Cells were observed to maintain a high degree of specific labeling, and were suitable for fluorescence imaging more than 3 h after dye-loaded growth medium was removed. However, if the cells are treated with a high concentration of unlabeled TMP (10 μM) the fluorescence in expressing cells decreases dramatically. Within 10 min, specific labeling

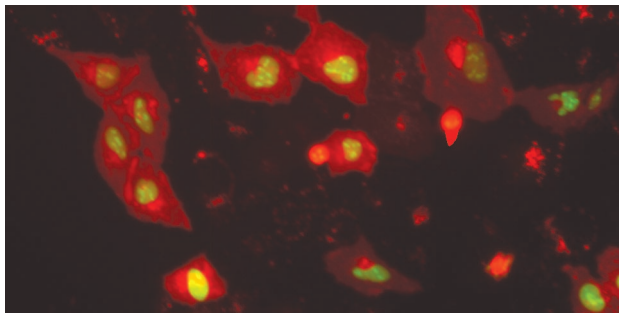


Figure 4. Multicolor applications of eDHFR fusion proteins. Overlay of epifluorescence micrograph showing U2OS cells that expressed nucleus-localized GFP (excitation 450–490 nm, emission 500–550 nm) and membrane-targeted eDHFR labeled with **4** (excitation 530–560 nm, emission 573–648 nm). Cells were incubated with 2 mM **4** for 1 hour at 37 °C, washed with PBS, and analyzed.

was indiscernible. This result is evidence that the observed fluorescent phenotypes were due to the specific interaction of the TMP-linked fluorophores and eDHFR fusion proteins.

In addition to observing the labeling microscopically, fluorescence activated cell sorting (FACS) was employed to measure the rate of dye uptake and stability of the fluorescence after washout (Figure 5). To measure the signal-to-noise ratio for eDHFR–NLS labeled with dye **3** in CHO cells, FACS analysis was performed at a dye concentration found to yield a high degree of specific labeling during imaging (2 μM). Fluorescent cells were counted as a function of time and fluorescence. During the course of the experiment, two separate populations of cells quickly emerged. The majority of cells (~90%) became fluorescent very slowly, while a minority of cells (~10%) became significantly more fluorescent by 26-fold (measured at the final time point). This minority of cells was not seen when nontransfected cells were used. This result indicates that these cells represent the same population of eDHFR–NLS expressing

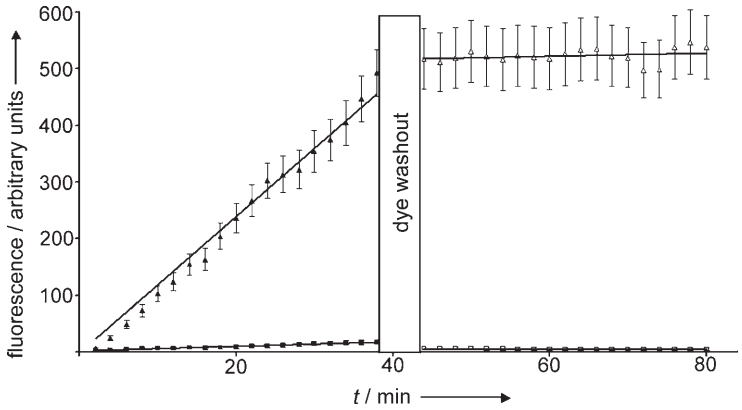


Figure 5. Flow-cytometry data from a single experiment for eDHFR–NLS expressing CHO cells labeled with **3** (1 μM). The graph shows fluorescence intensity during dye uptake by expressing (▲) and nonexpressing (■) cells, and fluorescence intensity after washing of expressing (△) and nonexpressing (□) cells; error bars indicate standard deviations about the mean intensity of each time point. The standard deviations for both sets of data for nonexpressing cells are about three units. Note the decrease in the fluorescence of nonexpressing cells after washing while the fluorescence of expressing cells remained constant for the remainder of the experiment.

cells as seen under the microscope. The rate at which the fluorescence of the transfected cells increased was constant throughout the 40 min time period. Also, consistent with microscope experiments, when these same cells were centrifuged and resuspended to remove extracellular dye, the level of fluorescence in the eDHFR–NLS expressing cells was constant within error for the course of the experiment. However, the fluorescence of the majority of nontransfected cells steadily decreased, which increased the signal-to-noise ratio to 96-fold. These data show the high level and stability of the staining after washout.

Conclusions

Here we have shown that bifunctional, TMP-linked, ester-protected fluorescein derivatives can be used to quickly and effectively label eDHFR fusion proteins in different wild-type mammalian cell lines. The fluorescent phenotypes observed in stained cells were due exclusively to the orthogonal, high-affinity interaction between TMP and eDHFR. Cells were stained efficiently when incubated for less than 1 h in growth medium that contained modest concentrations of the dyes, and the specific, fluorescent labeling was stable for more than 3 h. The absorption and emission spectra of **4** were red-shifted relative to GFP, and this dye should prove useful for simultaneous two-color labeling of eDHFR and CFP or GFP fusion proteins.

The ability to quickly and stably label fusion proteins with fluorophores or other small molecules simply by adding the probe to cell-growth medium makes a host of cell-biology applications possible. For example, the linkage of TMP to single-molecule detection-compatible fluorophores would be very useful for in vivo single-molecule microscopy because GFP is prone to photobleaching.[18] Additionally, one can envision linking TMP to pH, calcium, or other biochemical sensors as a means of providing specific organelle targeting and cell retention. As we have shown here, the development of effective, extracellularly administered protein labels requires careful optimization of the probe's pharmacokinetic properties. This work can serve to guide future development of targeted probes for in vivo functional protein studies.

Experimental Section

Synthesis of fluorescent TMP ligands: Fluorescein–NHS, hexachlorofluorescein–NHS, and BODIPY–Texas red–NHS were obtained from Molecular Probes. Diamino-tetraethylene glycol was obtained from Molecular Biosciences. All other materials and reagents were obtained from Sigma–Aldrich.

Four fluorescent-TMP conjugates were synthesized (Figure 2): TMP with an alkyl linker to fluorescein (**1**), TMP with an alkyl linker to BODIPY–Texas Red (**2**), TMP with a tetraethylene glycol linker to *iso*-butyryl protected fluorescein (**3**), and TMP with a tetraethylene glycol linker to *iso*-butyryl protected hexachlorofluorescein (**4**). Dyes **1** and **2** were synthesized as previously reported.[9a] Dyes **3** and **4** were synthesized in the same manner, as follows (details correspond to synthesis of **4**, as illustrated in Scheme 1).

Synthesis of compound 6: Trimethoprim (**5**; 17.15 g, M_W: 290) was added to a round-bottomed flask that contained HBr (214 mL, 48%) and refluxed at 95 °C. The solution was stirred under air for 20 min, and the temperature was maintained with an internal temperature probe. The solution was then partially neutralized with NaOH (51 mL, 50%, *w/w*). Agitation was stopped and the solution was allowed to cool to room temperature and then placed at 4 °C, overnight; this resulted in beige needle-like crystals. The crystals were filtered from solution and recrystalized by being dissolved in a minimal amount of cold H_2O, which was then neutralized to pH 7 with NH_2OH. The solution was chilled at 4 °C, and the resulting pure crystals were filtered to yield **6** (9.130 g, 33.0 mmol, 55.8%). ^{1}H NMR (400 MHz, CD_3OD, 25 °C): $\delta = 7.51$ (s, 1H, ArH), 6.63 (s, 2H, ArH), 3.85 (s, 6H, $ArOCH_3$), 3.65 ($ArCH_2Ar$).

Synthesis of compound 7: Compound **6** (1.381 g, M_W: 276.29, 5 mmol) was dissolved in DMSO (15 mL) and DBU (5.5 mmol) was added. After TMP–OH was completely dissolved, the solution turned a deep red and methyl bromoacetate (5.5 mmol) was added. The solution was incubated for 30 min at room temperature, and the solution became green. Water (50 mL) was added and the product was extracted with EtOAc (4×50 mL). The combined EtOAc solution was washed with water (100 mL), dried over $MgSO_4$, and evaporated. The residue was resuspended in a small amount of methanol (20%, *v/v*) in dichloromethane, and precipitated by addition of ether/pentane (~100 mL, 4:1, *v/v*), filtered, washed with ether, and dried to yield **7** (697 mg, 2 mmol, 40%). ^{1}H NMR (400 MHz, CD_3OD, 25 °C): $\delta = 7.54$ (s, 1H, ArH), 6.57 (s, 2H, ArH), 4.56 (s, 2H, $ArOCH_2$), 3.83 (s, 6H, $ArOCH_3$), 3.81 (s, 3H, $COOCH_3$), 3.69 ($ArCH_2Ar$).

Synthesis of compound 8: Compound **7** (348 mg, M_W: 348.35, 1 mmol) was dissolved in methanol (25 mL). This solution was then diluted with NaOH in water (50%, *w/w*) until a small amount of beige precipitate was formed. The solution was then stirred under air for 4 h and the precipitate disappeared. The reaction mixture was then titrated to pH 4 with HCl (1 N), the beige crystals formed were filtered from solution, and washed with brine and water to yield **8** (334 mg, 1 mmol, quant). 1H NMR (CD_3OD): $\delta = 7.68$ (s, 1H, ArH), 6.76 (s, 2H, ArH), 4.47 (s, 2H, $ArOCH_2$), 3.93 (s, 6H, $ArOCH_3$), 3.87 ($ArCH_2Ar$).

Synthesis of compound 9: Prior to the synthesis, a round-bottomed flask was baked at 175 °C for 48 h and then flamed. Subsequently, **8** (50 mg, M_W: 334.33, 0.149 mmol) and PyBOP (155 mg, 0.299 mmol, 2 equiv) were added to the hot flask and put under vacuum. After the flask had cooled (after approximately 1 h), DMF (10 mL) and diamino-tetraethylene glycol (TEG; 143 g, 0.745 mmol, 5 equiv) were added to the flask under N_2. The mixture was stirred overnight and then cooled to 0 °C. BOC anhydride (488 μL, 1.49 mmol, 10 equiv) was added, and the reaction was allowed to progress until CO_2 production ceased. The solvent was removed by rotary evaporation and the mixture of crude products were separated with flash chromatography ($MeOH/MeCl_2$, 1:30 over SiO_2). The BOC group was deprotected from the crude product after the mixture was stirred in TFA in $MeCl_2$ (50%, *v/v*) for 5 h. The reaction was monitored by TLC. Deprotection yielded the pure compound **9** (27 mg, 0.053 mmol, 36%). ^{1}H NMR (400 MHz, CD_3OD, 25 °C): $\delta =$ 7.29 (s, 1H, ArH), 6.65 (s, 2H, ArH), 4.44 (s, 2H, $ArOCH_2$), 3.90 (s, 6H, $ArOCH_3$), 3.73–3.69 (m, 18H, $ArCH_2Ar$+TEG linker H).

Synthesis of compound 10: Compound **9** (5.0 mg, M_W: 508.57, 9.8 μmol) was mixed with hexachlorofluorescein–NHS ester (4.5 mg, 1 equiv) in DMF (1 mL). The mixture was stirred overnight under N_2 in a round-bottomed flask, and subsequently purified by

HPCL to yield **10** (3.1 mg, 2.9 μmol, 29%). ^{1}H NMR (400 MHz, CD_3OD, 25 °C): $\delta = 7.75$ (s, 1H, ArH), 7.27 (s, 1H, ArH), 7.20 (s, 2H, ArH), 6.61 (s, 2H, ArH), 4.43 (s, 2H, $ArOCH_2$), 3.86 (s, 6H, $ArOCH_3$), 3.67–3.46 (m, 18H, $ArCH_2Ar$+TEG linker H).

Synthesis of compound 4: Compound **10** (5 mg, M_W: 1073.54, 4.7 μmol) was dissolved in pyridine (0.4 mL). *iso*-Butyric anhydride (0.3 mmol) was added and the mixture was incubated at room temperature for 0.5–1 h during which time the color and fluorescence disappeared. Water (20 μL) was added and the mixture was evaporated twice with toluene after 5–10 min incubation. The residue was washed twice with ether/pentane (1:4, *v/v*), and dried in vaccuo, overnight. The product was then purified by using a 2.54 cm pipette column (15:1, $MeCl_2$/MeOH over SiO_2) to produce the colorless oil, **4** (4 mg, 3.2 μmol, 70%, M_W: 1213.7). ^{1}H NMR (400 MHz, CD_3OD, 25 °C): $\delta = 7.88$ (s, 1H, ArH), 7.40 (s, 1H, ArH), 7.31 (s, 2H, ArH), 6.60 (s, 2H, ArH), 4.42 (s, 2H, $ArOCH_2$), 3.85 (s, 6H, $ArOCH_3$), 3.68 (s, 2H, $ArCH_2Ar$), 3.59–3.47 (m, 16H, TEG linker H).

ESI-MS (*m/z*) calcd for **3** $[C_{52}H_{59}N_6O_{15}]^+$: 1008.1; found: 1007.9. ESI-MS (*m/z*) calcd for **4** $[C_{52}H_{53}Cl_6N_6O_{15}]^+$: 1214.7; found: 1214.8.

TMP linked to either fluorescein (**3**) or hexachlorofluorescein (**4**) was stored in methanol (3.5 mM and 4.0 mM, respectively).

Construction of eDHFR fusion proteins

Myosin light chain kinase–eDHFR plasmid: The gene encoding eDHFR was subcloned from plasmid pMONDHFR to MLCK–GFP, to generate MLCK–DHFR. The plasmid MLCK–GFP was prepared by subcloning the DNA for the full-length avian form of MLCK into the *Eco*RI–*Bam*HI sites of pEGFP-N1(Clontech).[14] A 518 bp *Bam*HI–*Not*I fragment encoding eDHFR was prepared with PCR from pMONDHFR by using the primers 5′-GGATCC TGG AAT GAT CAG TCT GAT TGC GGC GTT AG (*Bam*HI, coding strand) and 3′-GCG GCC-GCT TAC CGC CGC TCC AGA ATC TC (*Not*I, noncoding strand). This fragment was inserted between the *Bam*HI and *Not*I sites in MLCK–GFP to give MLCK–eDHFR. Upon transfection into mammalian cells, MLCK–eDHFR expressed the fusion protein MLCK–GDPGM–eDHFR.

α-Tubulin–eDHFR plasmid (pLM1298): The gene encoding eDHFR was subcloned from plasmid pMONDHFR to pEGFP–TUB (Clontech), to generate pLM1298. A 539 bp *Nhe*I–*Xho*I fragment encoding eDHFR with an N-terminal Kozak sequence and valine in the second position was prepared with PCR by using pMONDHFR as template, together with the primers 5′-GCA TAC GTC GCT ACG GC-TAC C GGT CGC CAC CA GGT GAT CAG TCT GAT TGC GGC (*Nhe*I, coding strand) and 3′-GCA TAC GTC CTC GAG ATC TGA GTC CCG ACC GCC-GCT CCA GAA ATC (*Xho*I, noncoding strand). This fragment was inserted between the *Nhe*I and *Xho*I sites in pEGFP–TUB to give pLM1298. Upon transfection into mammalian cells, pLM1298 expressed the fusion protein eDHFR–SGLRSRV–α-tubulin.

GFP–eDHFR plasmid (pGFP–eDHFR): The gene encoding eDHFR was subcloned from plasmid pMONDHFR to pEGFP–TUB, to generate pGFP–eDHFR. A 572 bp *Xho*I–*Bam*HI fragment encoding eDHFR was prepared with PCR by using pMONDHFR as template, together with the primers 5′-GCA TAC GTC CTC GAG TGG GCT TCG GCA TCA-GTC TGA TTG CGG C (*Xho*I, coding strand) and 3′-GCA TAC GGA TCC-TCA CTT GTC GTC GTC CTT GTA GTC CCG CCG CTC CAG AAT C (*Bam*HI, noncoding strand). This fragment was inserted between the *Xho*I and *Bam*HI sites in pEGFP–TUB to give pGFP–eDHFR. Upon transfection into mammalian cells, pGFP–eDHFR expressed the fusion protein GFP–SGLRSRVGSG–eDHFR–DYKDDDK. Plasmid pLM1302, which expressed nuclear-localized eDHFR, and pLM1208, which expressed plasma membrane-localized eDHFR were described previously.[9a]

Fluorescent labeling of eDHFR fusion constructs: CHO or MEF cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS; 10%, Gibco), L-glutamine (2 mM), penicillin (100 IU μL^{-1}), streptomycin (100 mg mL^{-1}), HEPES (15 mM), and incubated in a humidified atmosphere at 37 °C and 5% CO_2. Cells (ca. 80000) were seeded into 6-well plates, and transient transfection was performed by using FuGene 6 reagent according to the manufacturer's protocol (Roche; 1 μg DNA per 3 μL FuGene 6) when they were 80% confluent. After 12–24 h, approximately 50000 cells were placed onto 22 mm^2 glass coverslips. For imaging CHO cells, one of the dyes was diluted (1–2 μM) in culture medium, and incubated with the cells for 1 h at 37 °C. For imaging MEF cells, one of the dyes was diluted (200–400 μM) in culture medium and incubated with the cells for 10 min at 37 °C. In both cases, this was followed by washing the cells three times with media.

Images were collected by using an Olympus IX81 inverted microscope with a FV500 laser scanning confocal module that was fitted with a 60X Plan Fluor oil immersion objective (1.4 numerical aperture). Excitation of the dyes was carried out either by using 488 nm, 514 nm, or 543 nm lines, and filters/dichroics appropriate for imaging fluorescein ($\lambda_{em} = 514$ nm), hexachlorofluorescein ($\lambda_{em} = 560$ nm), or Texas Red ($\lambda_{em} = 588$ nm). The width of the confocal aperture, laser intensity, and detection sensitivity were adjusted to give optimal resolution of detail in the images with minimal photobleaching. The microscope stage was maintained at 37 °C throughout the experiment.

For multicolor labeling experiments, U2OS cells were cultured in DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 IU μL^{-1}), streptomycin (100 mg mL^{-1}), HEPES (15 mM), and incubated in a humidified atmosphere at 37 °C and 5% CO_2. Cells were transfected with DNA that encoded GFP–NLS and plasma membrane targeted eDHFR (pLM1208) as described above. Cells were incubated with **4** (2 μM) for 2 h, washed with phosphate buffered saline (PBS), and imaged with a Nikon TS100 epifluorescence microscope equipped with a 20X objective. GFP fluorescence was collected by using 450–490 nm excitation and 500–550 nm emission filters. For compound **4** fluorescence was collected by using 530–560 nm excitation and 573–648 nm emission filters. Images were processed by using Spot Advanced 4.0.9 software.

Fluorescence cell sorting of eDHFR–NLS expressing cells: For fluorescence microscopy, CHO cells were cultured as described above. The cells were then trypsinized and passaged into culture medium. While in suspension, the cells were treated with dye **3** (2 μM), and immediately put onto a Becton–Dickinson FACSCalibur flow cytometer set at the low-flow-rate setting (150–200 cells per s). The fluorescence from fluorescein was continuously monitored for 40 min. The same tube of cells was centrifuged at 1000 *g* for 5 min. The pellet was resuspended in dye-free culture medium, put back onto the flow cytometer, and monitored for 40 min. The time resolved FACS data was reduced by using CellQuest software.

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