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Light-Up “Channel Dyes” for Haloalkane-Based Protein Labeling in Vitro and in Bacterial Cells

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Abstract

We describe a novel molecular strategy for engendering a strong light-up signal in fluorescence tagging of the genetically encoded HaloTag protein domain. We designed a set of haloalkane-derivatized dyes having twisted internal charge transfer (TICT) structures potentially narrow enough to partially fit into the enzyme’s haloalkane-binding channel. Testing a range of short chain lengths revealed a number of active dyes, with seven carbons yielding optimum light-up signal. The dimethylaminostilbazolium chloroheptyl dye (**1d**) yields a 27-fold fluorescence emission enhancement ($\lambda_{\text{ex}} = 535 \text{ nm}$; $\text{Em}_{(\text{max})} = 616 \text{ nm}$) upon reaction with the protein. The control compound with standard 12-atom linkage shows less efficient signaling, consistent with our channel-binding hypothesis. For emission further to the red, we also prepared a chloroheptyl naphthalene-based dye; compound **2** emits at 653 nm with strong fluorescence enhancement upon reaction with the HaloTag domain. The two dyes (**1d**, **2**) were successfully tested in wash-free imaging of protein localization in bacteria, using a HaloTag fusion of the filamenting temperature-sensitive mutant Z (FtsZ) protein in *Escherichia coli* (*E. coli*). The new dye conjugates are inexpensive and easily synthesized enzyme substrates with low background and large Stokes shifts, offering substantial benefits over known fluorescent substrates for the HaloTag enzyme.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.6b00613. General methods, synthetic procedures, and spectra (PDF)

Genetically encoded protein labeling methods are widely employed in chemistry and biology. For example, fluorescent proteins are broadly used for imaging to determine protein location and trafficking, and are being built into numerous and varied tools for the analysis of location, concentration, interactions, and conformation changes in cells.^{1,2}

More recently, post-expression small-molecule tagging methods have gained widespread attention; these methods typically work by expression of the fusion of a modified enzyme with the protein of interest.³ Addition of a cell-permeable small molecule substrate allows for reaction with the protein and covalent bond formation with it. Relative to standard fluorescent proteins, these newer methods offer special utility due to the temporal control that they offer, as small molecule substrates can be added at any desired time to cells expressing the fusion protein. Multiple distinct classes of enzymes have been engineered to make use of this approach.⁴⁻⁷

Among these genetically encoded small-molecule labeling approaches, the HaloTag strategy is especially appealing because of its rapid reaction and the simplicity of the reactive substrates.⁸ The HaloTag domain is an engineered *Rhodococcus* haloalkane dehalogenase enzyme that reacts to displace a terminal chloride in simple straight-chain alkane substrates. The reactive nucleophile, a carboxylate group, exists at the end of a narrow, straight channel extending 15 Å inside the enzyme.⁸ Desired tagging groups, such as biotin or fluorescent labels, are invariably placed at the end of unbranched chains ca. 15 Å long to allow access of the terminal electrophilic carbon to this nucleophile. At the opposite end of this long chain, the appended labels remain outside the protein, presumably residing in solution. Several commercial substrates with this architecture are available, and new ones are being developed in the recent literature.⁹⁻¹²

The temporal control of these small-molecule tagging strategies allows for a range of experiments that would not be possible with standard fluorescent proteins. However, for intracellular fluorescent labeling, there still remains one important time-based limitation of this approach: namely, the need to wash away the excess unreacted label. Such washing steps can easily add 30 min or more to the methodology prior to making cellular measurements; this extra time makes it impossible to measure biological processes on this or shorter time scales. Recently, a number of fluorogenic “lighting up” substrates were developed for some protein tags in order to remove the need for washing steps.¹³⁻¹⁶ The best-performing fluorogenic substrates have incorporated environmentally sensitive dyes or FRET probes into their designs. Notably, Liu et al. developed a SNAP-tag substrate that exhibits a 280-fold increase in fluorescence upon reaction with the enzyme, due to the hydrophobicity of the active site pocket.¹⁷ Naganbabu et al. were able to develop probes using a FRET strategy based on the malachite green/fluorogen activating protein pair, with

66- and 417-fold light-up responses and Stokes shifts larger than 250 nm.¹⁸ For the HaloTag enzyme, there exist several mentions in the literature of light-up substrates. In the first, Lukinavičius et al. report a silicon-rhodamine probe used in super-resolution microscopy that exhibits a 6-fold increase in fluorescence upon reaction with the HaloTag domain.¹⁹ In the most recent study, the fluorescence increase was 12-fold for the best performing dye, but the imaging studies were performed with washing steps.²⁰

It is important to have multiple strategies for genetically encoded light-up labeling, because it is often of interest to follow more than one species simultaneously, or to perform multiple time-encoded measurements. Thus, it would be useful to have better-performing strategies for HaloTag labeling. Here we report a new and simple approach to the design of fluorogenic substrates for the HaloTag domain. In this strategy (Figure 1), we have used linear dyes with constrained structures that are designed to fit within the narrow haloalkane binding channel of this enzyme. As our platform, we have adopted TICT dyes; their freely rotating bonds and internal donor–acceptor structure give them very low quantum yields in solution. Once bound inside the narrow protein channel, we expected that their bond rotation should be restricted, which would be expected to result in an increase in quantum yield and brightness, and in a red shift in emission. However, prior to these experiments it was unclear whether such aromatic molecules could fit within this narrow alkane channel, and to what degree the fluorescence would be affected.

To test this strategy, we prepared a series of dimethylaminostilbazolium dyes with varied lengths of haloalkane chains appended. These were readily synthesized in two steps from commercial starting materials (see details in the Supporting Information file). Haloalkane chain lengths of these new potential substrates were designed to be considerably shorter than the standard HaloTag >12-atom linker, thus requiring the dye to enter the channel if reaction with the chloro-substituted carbon were to occur.^{8,21} Our linkers ranged from 4 to 8 carbons (**1a–1e**, Figure 1), and total molecule lengths ranged from 10 to 18 Å. Thus, the shortest dyes, once reacted, are expected to reside entirely within the protein's channel, and the longest ones should allow only the terminal part of the dye to protrude from the enzyme.

We then reacted this series of compounds with the 62 kDa fusion protein of Glutathione-S-Transferase-HaloTag (GST-HT) in vitro, measuring reaction progress by SDS-PAGE (Figures 2 and S5). Surprisingly, even the compounds with the shortest chains did in fact react with this enzyme, yielding fully tagged protein after 120 min, while the fastest-reacting chain lengths yielded half reaction in less than 5 min. Images of the gels clearly show that the band traveling with the expected molecular weight of GST-HT is fluorescently labeled in all cases, establishing that the dyes remain associated with the protein, as expected for the covalent ester linkage (see Figures S4 and S5).

Next, we evaluated the optical properties of the dyes covalently attached to the enzyme as compared to the unreacted dye substrates. Results of spectroscopic measurements showed that the free dyes absorb maximally near 452 nm and have emission at ca. 607 nm with low quantum yields in aqueous buffer ($\Phi_f = 0.002$ or lower; see Table 1). Reaction with the enzyme greatly changes the dyes' photophysical properties (Figure 3 and Table 2). Absorption maxima generally shift to ca. 535 nm, a pronounced redshift of ca. 90 nm,

consistent with the bound dye having constrained rotations and increased conjugation relative to the free dye.²² Excitation of the labeled proteins at 535 nm yields emission maxima ca. 618 nm. It is also noteworthy that the Stokes shifts of the bound dyes **1a–1e** are rather large (75–135 nm; see Table 2), which should facilitate cellular protein analysis without interference from the excitation light. By contrast, commercial Oregon Green and TMR dyes for the HaloTag domain have Stokes shifts of only 20–30 nm⁹ and exhibit little or no light-up effect upon labeling.

Importantly, the Channel Dyes undergo large increases in fluorescence emission intensity upon reaction with the HaloTag protein. The emission enhancements vary from 3-fold (four carbons, **1a**) to 27-fold (seven carbons, compound **1d**). This is consistent with the notion that binding inside the channel restricts bond rotations and diminishes quenching by internal charge transfer in the chromophore. This degree of increase is large enough to be useful in wash-free intracellular labeling in *E. coli* (see below). For comparison, we synthesized **3**, a dimethylaminostilbazolium dye with the conventional, longer HaloTag linker (Figure 4 and SI). As expected, upon reaction of **3** with the enzyme, its degree of fluorescence enhancement is considerably less than that of the Channel Dyes, presumably because the chromophore is extended outside the alkane binding channel, allowing facile bond rotations (Figure S7). To test the hypothesis that the TICT state is the major fluorescence quenching mechanism in solution, fluorescence spectra of **1d** were measured in water/glycerol mixtures of increasing viscosity. As the proportion of glycerol in the mixture increased, fluorescence intensity of **1d** also increased, consistent with suppression of TICT by restriction of bond rotation as a light-up mechanism for these dyes (Figure S8).

Having established that a chain length of seven carbons in dye **1d** allows for efficient light-up response with the HaloTag domain, we then synthesized and tested a more conjugated chromophore structure with this same chain length (Figure 4) with the aim of increasing the emission wavelength further to the red. Synthetic methods and characterization are given in the Supporting Information. Naphthyl dye **2** effectively labeled the HaloTag domain in vitro, yielding a red-shifted emission maximum of 653 nm with enhancement of 6-fold in brightness upon reaction (Table 2).

To test whether the Channel Dyes could perform in a complex cellular environment, we evaluated compounds **1d** and **2** in labeling FtsZ, the bacterial tubulin homologue that is essential for cytokinesis.²³ At the site of cell fission in *E. coli*, polymeric filaments of FtsZ form a ring-like structure, termed the Z-ring.²³ *E. coli* cells were transformed with vectors encoding either FtsZ with HaloTag domain at C-terminus, or the HaloTag domain alone. After induction of protein expression, cells were incubated with dye **1d** or **2** for 1 h, pelleted, and then suspended in PBS pH 7.4. Results were imaged by epifluorescence microscopy (Figure 5). A diffuse staining pattern was seen when cells expressing the HaloTag domain alone were incubated with the dyes (Figure 5A,C). In contrast, distinct, characteristic localized fluorescent bands were seen in the cells expressing FtsZ-HaloTag (Figure 5B,D). The filamentous cell morphology and strong FtsZ banding pattern of the cells are consistent with previous studies of FtsZ overexpression.^{24,25} Importantly, FtsZ labeling with **1d** and **2** is effective even without a washing step (Figures 6 and S9). After only 5 min of reaction with **1d**, fluorescent Z-rings were clearly visible above background staining, with labeling

contrast increasing over the 60 min studied; to our knowledge, this is the most rapid demonstration of fluorogenic signal generation in living cells using the HaloTag methodology. Comparable staining contrast at longer wavelength was apparent after 10 min of reaction with naphthyl dye **2**.

Our data show that Channel Dyes are effective in light-up labeling of HaloTag fusion domains in vitro and in bacteria. The current designs show stronger light-up labeling responses than any previous HaloTag label, while having the added benefits of simplicity, low cost, and ease of synthesis. Indeed, the synthesis of these ligands may be shorter than that of any other existing ligand for the HaloTag protein, fluorescent or otherwise. Because cationic aromatic dyes tend to show strong mitochondrial localization in mammalian cell applications,²⁶ the current dye designs are not suitable for localized protein labeling in mammalian cell applications, and would require other environmentally sensitive chromophore designs for that application. On the other hand, for protein localization studies in bacteria, as well as for genetic selection experiments in bacteria, the current designs are highly useful, and work is underway in these areas. Their strong fluorogenic response and rapid reaction makes them potentially broadly useful in the study of localization, concentration, and trafficking of bacterial proteins. They are also expected to facilitate new time-dependent biological experiments with considerably shorter time resolution than was previously feasible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Wiedenmann, J., D'Angelo, C., Nienhaus, GU. Fluorescent Proteins: Nature's Colorful Gifts for Live Cell Imaging. In: Jung, G., editor. Fluorescent Proteins II. Springer; Berlin, Heidelberg: 2011. p. 3-33.
2. Newman RH, Zhang J. The design and application of genetically encodable biosensors based on fluorescent proteins. *Methods Mol Biol.* 2014; 1071:1–16. [PubMed: 24052376]
3. Wang Z, Ding X, Li S, Shi J, Li Y. Engineered fluorescence tags for in vivo protein labelling. *RSC Adv.* 2014; 4:7235–7245.
4. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat Biotechnol.* 2002; 21:86–89. [PubMed: 12469133]
5. Gautier A, Juillerat A, Heinis C, Corrêa IR Jr, Kindermann M, Beaufils F, Johnsson K. An engineered protein tag for multiprotein labeling in living cells. *Chem Biol.* 2008; 15:128–136. [PubMed: 18291317]
6. Miller LW, Cai Y, Sheetz MP, Cornish VW. In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag. *Nat Methods.* 2005; 2:255–257. [PubMed: 15782216]
7. Fernández-Suárez M, Baruah H, Martínez-Hernández L, Xie KT, Baskin JM, Bertozzi CR, Ting AY. Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes. *Nat Biotechnol.* 2007; 25:1483–1487. [PubMed: 18059260]

8. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, et al. HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis. *ACS Chem Biol*. 2008; 3:373–382. [PubMed: 18533659]
9. [Accessed Oct 20, 2016] HaloTag Fluorescent Ligands. www.promega.com/products/protein-expression/protein-labeling-and-detection/halotag-technology-products/halotag-fluorescent-ligands/
10. Buckley DL, Raina K, Darricarrere N, Hines J, Gustafson JL, Smith IE, Miah AH, Harling JD, Crews CM. HaloPROTACS: Use of Small Molecule PROTACs to Induce Degradation of HaloTag Fusion Proteins. *ACS Chem Biol*. 2015; 10:1831–1837. [PubMed: 26070106]
11. Best M, Porth I, Hauke S, Braun F, Hertzen DP, Wombacher R. Protein-specific localization of a rhodamine-based calcium-sensor in living cells. *Org Biomol Chem*. 2016; 14:5606–5611. [PubMed: 27072883]
12. Murrey HE, Judkins JC, am Ende CW, Ballard TE, Fang Y, Riccardi K, Di L, Guilmette ER, Schwartz JW, Fox JM, et al. Systematic Evaluation of Bioorthogonal Reactions in Live Cells with Clickable HaloTag Ligands: Implications for Intracellular Imaging. *J Am Chem Soc*. 2015; 137:11461–11475. [PubMed: 26270632]
13. Hori Y, Ueno H, Mizukami S, Kikuchi K. Photoactive Yellow Protein-Based Protein Labeling System with Turn-On Fluorescence Intensity. *J Am Chem Soc*. 2009; 131:16610–16611. [PubMed: 19877615]
14. Watanabe S, Mizukami S, Hori Y, Kikuchi K. Multicolor Protein Labeling in Living Cells Using Mutant β -Lactamase-Tag Technology. *Bioconjugate Chem*. 2010; 21:2320–2326.
15. Sun X, Zhang A, Baker B, Sun L, Howard A, Buswell J, Maurel D, Masharina A, Johnsson K, Noren CJ, et al. Development of SNAP-Tag Fluorogenic Probes for Wash-Free Fluorescence Imaging. *ChemBioChem*. 2011; 12:2217–2226. [PubMed: 21793150]
16. Prifti E, Reymond L, Umebayashi M, Hovius R, Riezman H, Johnsson K. A Fluorogenic Probe for SNAP-Tagged Plasma Membrane Proteins Based on the Solvatochromic Molecule Nile Red. *ACS Chem Biol*. 2014; 9:606–612. [PubMed: 24471525]
17. Liu TK, Hsieh PY, Zhuang YD, Hsia CY, Huang CL, Lai HP, Lin HS, Chen IC, Hsu HY, Tan KT. A Rapid SNAP-Tag Fluorogenic Probe Based on an Environment-Sensitive Fluorophore for No-Wash Live Cell Imaging. *ACS Chem Biol*. 2014; 9:2359–2365. [PubMed: 25105835]
18. Naganbabu M, Perkins LA, Wang Y, Kurish J, Schmidt BF, Bruchez MP. Multiexcitation Fluorogenic Labeling of Surface, Intracellular, and Total Protein Pools in Living Cells. *Bioconjugate Chem*. 2016; 27:1525–1531.
19. Lukinavičius G, Umezawa K, Olivier N, Honigsmann A, Yang G, Plass T, Mueller V, Reymond L, Correa IR Jr, Luo ZG, et al. A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nat Chem*. 2013; 5:132–139. [PubMed: 23344448]
20. Butkevich AN, Mitronova GY, Sidenstein SC, Klocke JL, Kamin D, Meineke DNH, D'Este E, Kraemer PT, Danzl JG, Belov VN, et al. Fluorescent Rhodamines and Fluorogenic Carbopyronines for Super-Resolution STED Microscopy in Living Cells. *Angew Chem, Int Ed*. 2016; 55:3290–3294.
21. Newman J, Peat TS, Richard R, Kan L, Swanson PE, Affholter JA, Holmes IH, Schindler JF, Unkefer CJ, Terwilliger TC. Haloalkane Dehalogenases: Structure of a Rhodococcus Enzyme. *Biochemistry*. 1999; 38:16105–16114. [PubMed: 10587433]
22. Grabowski ZR, Rotkiewicz K, Rettig W. Structural Changes Accompanying Intramolecular Electron Transfer: Focus on Twisted Intramolecular Charge-Transfer States and Structures. *Chem Rev*. 2003; 103:3899–4032. [PubMed: 14531716]
23. Haeusser DP, Margolin W. Splitsville: structural and functional insights into the dynamic bacterial Z ring. *Nat Rev Microbiol*. 2016; 14:305–319. [PubMed: 27040757]
24. Ward JE, Lutkenhaus J. Overproduction of FtsZ induces minicell formation in *E. coli*. *Cell*. 1985; 42:941–949. [PubMed: 2996784]
25. Ma X, Ehrhardt DW, Margolin W. Colocalization of cell division proteins FtsZ and FtsA to cytoskeletal structures in living *Escherichia coli* cells by using green fluorescent protein. *Proc Natl Acad Sci U S A*. 1996; 93:12998–13003. [PubMed: 8917533]
26. Xu Z, Xu L. Fluorescent probes for the selective detection of chemical species inside mitochondria. *Chem Commun*. 2016; 52:1094–1119.

Figure 1.

A. Standard HaloTag substrates have long, narrow linkers to fit into the enzyme's binding channel. The dyes reported in this study have very short linkers, requiring the narrow dye itself to enter the channel. B. Structures of the Channel Dyes tested.

Figure 2. Reaction time course of Channel Dyes **1a–e**, and conventional-linker dye **3** with GST-HT fusion protein. Conditions: 1 μ M GST-HT, 5 μ M dye, PBS, 37 °C.

Figure 3.

A. Emission spectra of free Channel Dyes and Channel Dyes after reaction with GST-HT. B. Fluorogenic nature of the Channel Dyes.

Figure 4.
Additional dyes studied: Naphthyl Channel Dye **2** and control TICT dye with conventional HaloTag linker, **3**.

Figure 5.

Fluorescence images for bacterial HaloTag labeling with Channel Dyes **1d** and **2**. Labeling conditions: 10 μM dye in lysogeny broth, 37 $^{\circ}\text{C}$, 1 h. Cells were resuspended in PBS for imaging by epifluorescence microscopy ($\lambda_{\text{ex}} = 540 \text{ nm}$). Scale bars (5 μM) are shown.

Figure 6. Wash-free labeling of FtsZ-HT fusion protein in *E. coli* with 10 μM Channel Dye **1d** in lysogeny broth, imaged as in Figure 5 ($\lambda_{\text{ex}} = 540 \text{ nm}$). Scale bars (5 μM) are shown.

Table 1Optical Data for Free (Dark) Channel Dyes in Aqueous Buffer^a

Dye	Abs _{max} (nm)	ϵ (L·mol ⁻¹ cm ⁻¹)	Em _{max} ^b (nm)	Φ_f ^c
1a	455	3.92×10^4	607	0.0012
1b	449	2.41×10^4	608	0.0011
1c	453	3.11×10^4	606	0.0013
1d	449	2.76×10^4	606	0.0012
1e	455	2.45×10^4	606	0.0021
2	416	0.99×10^4	641	0.0005

^aConditions: PBS, pH 7.4.^bExcitation at Abs_{max}^cError in Φ_f estimated to be $\pm 10\%$ based on triplicate measurement of **1d**.

Table 2Optical Data for Channel Dyes Bound to the Halotag Enzyme^a

Dye	Abs _{max} (nm)	Em _{max} ^b (nm)	Φ _{fl}	Fold Increase	Stokes Shift (nm)
1a	455	590	nd	3.4	135
1b	540	618	nd	12	78
1c	535	610	nd	8.3	75
1d	535	616	0.159	27	81
1e	535	620	nd	19	85
2	480	653	nd	5.7	173

^aReaction conditions: 10 μM dye, 30 μM GST-HT, 37 °C, 5 h.^bExcitation of **1a–e** at 535 nm. Excitation of **2** at 475 nm.