# Genetically Encoded Multispectral Labeling of Proteins with Polyfluorophores on a DNA Backbone

Vijay Singh, Shenliang Wang,[†] and Eric T. Kool*

Department of Chemistry, Stanford University, Stanford, California 94305-5080, United States

ⓢ Supporting Information

**ABSTRACT:** Genetically encoded methods for protein conjugation are of high importance as biological tools. Here we describe the development of a new class of dyes for genetically encoded tagging that add new capabilities for protein reporting and detection via HaloTag methodology. Oligodeoxyfluorosides (ODFs) are short DNA-like oligomers in which the natural nucleic acid bases are replaced by interacting fluorescent chromophores, yielding a broad range of emission colors using a single excitation wavelength. We describe the development of an alkyl halide dehalogenase-compatible chloroalkane linker phosphoramidite derivative that enables the rapid automated synthesis of many possible dyes for protein conjugation. Experiments to test the enzymatic self-conjugation of nine different DNA-like dyes to proteins with HaloTag domains in vitro were performed, and the data confirmed the rapid and efficient covalent labeling of the proteins. Notably, a number of the ODF dyes were found to increase in brightness or change color upon protein conjugation. Tests in mammalian cellular settings revealed that the dyes are functional in multiple cellular contexts, both on the cell surface and within the cytoplasm, allowing protein localization to be imaged in live cells by epifluorescence and laser confocal microscopy.
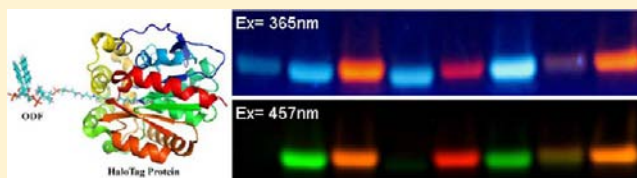
## INTRODUCTION

Elucidating the movements, locations, interactions, and chemical microenvironments of proteins inside living cells is crucial for a detailed understanding of biomolecular mechanisms and cellular functions. The study of protein interactions and trafficking has been revolutionized by the application of genetically encoded fluorescent proteins, which are available in multiple colors for labeling of separate species.[1] More recently, the strategy of small-molecule fluorescent labeling of genetically encoded proteins has become prominent;[2−5] this approach can offer the advantage of time resolution of labeling, as the dye can be added at any time during the cell cycle or during organismal development. Most small-molecule approaches take advantage of enzyme mechanisms to covalently attach an appropriate substrate to an engineered protein domain; prominent examples make use of enzymes such as dihydrofolate reductase,[6] O6-alkylguanine alkyltransferase,[7,8] β-lactamase,[9] and lipoic acid ligase.[10] Among the most widely used approaches is the HaloTag method, which requires only the conjugation of a simple haloalkane moiety to the desired label.[11] The original haloalkane dehalogenase is a bacterial enzyme that removes halides from aliphatic hydrocarbons by a nucleophilic displacement mechanism and forms a covalent ester bond between the haloalkane and Asp106 in the enzyme.[12] A critical mutation in the catalytic active site (H272F) in the HaloTag variant renders the covalent ester bond stable toward hydrolysis.[11] The engineered HaloTag domain is 34 kDa in size and is readily coexpressed as a chimera with arbitrary proteins of interest using commercially available

vectors. Standard small-molecule fluorophores are available in haloalkyl-derivatized form to label proteins of interest.[13]

The recent rapid growth of fluorescence instrumentation and techniques for biomolecular analysis and imaging has highlighted a need for new optical capabilities in fluorescent labels. Labels that can be physicochemically switched, can act as sensors, or are sensitive to the environment are all under study, but to date, few examples[14,15] are available for genetically encoded tagging. Another missing capability is multispectral emission, which refers to sets of differently colored dyes that share a common excitation wavelength. This property enables simultaneous real-time tracking of multiple labeled species even in rapidly moving systems[16,17] and simplifies the necessary equipment since only a single filter set is needed for imaging. One potential fluorophore class that exhibits this multispectral behavior is inorganic quantum dots, which can be excited in the UV and exhibit size-tunable emissions. Although they can be conjugated to proteins,[18] difficulties in uniform chemical modification and intracellular delivery, along with their large size and cytotoxicity, can place limits on their applications in cellular settings.[18b,19] In general, biological research would benefit from having small, discrete organic labels that exhibit some of these new capabilities, and the ability to employ them in genetically encoded tagging would be broadly useful.

We have undertaken a program to develop a broad class of fluorescent labels using the modular design of DNA.[20,21] The DNA bases in these short oligomeric dyes (termed
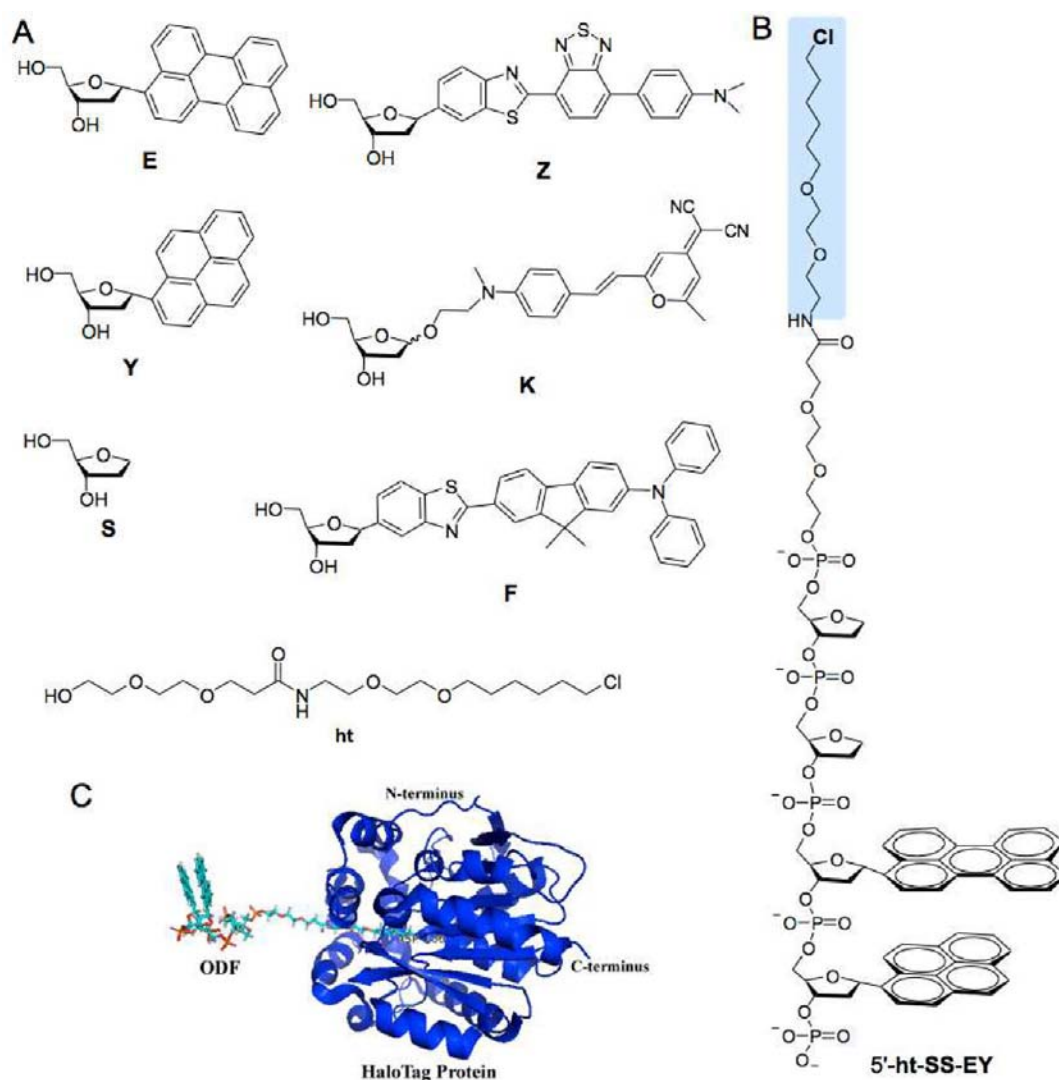
**Figure 1.** Structures in this study. (A) Monomers used for making chloroalkyl-ODF−HaloTag ligands. (B) Structure of a typical ODF−HaloTag ligand with the sequence of 5′-htS₂EY (the haloalkyl group is marked in blue). (C) Illustration of the conjugate formed from the engineered dehalogenase enzyme and an ODF ligand (5′-htS₂EY).

oligodeoxyfluorosides, or ODFs) are replaced by fluorophores; the phosphodiester backbone in ODFs confers aqueous solubility and acts as a scaffold to hold the fluorophores close, promoting complex electronic interactions. The modular structure of ODFs, composed of sequences of fluorophore monomers, facilitates the rapid construction of thousands of dyes with distinct, selectable optical properties[20,22] and enables rapid automated synthesis on a DNA synthesizer. Multiple forms of energy and excitation transfer such as Förster resonance energy transfer, exciplex, excimer, H-dimer, and other mechanisms have been observed, yielding dyes with extraordinarily large Stokes shifts, high quantum yields, and long fluorescence lifetimes. A broad spectrum of ODFs can be excited at a single wavelength, which offers the possibility of real-time multicolor application in biological systems.[17] ODFs have also been identified or designed that exhibit fluorescence changes in response to light exposure,[22] specific small molecules,[23] or enzyme activities.[24]

To date, ODFs have only been conjugated to proteins (antibodies in the reported case) nonspecifically via click reactions to functionalized lysine residues.[25] ODFs chemically resemble DNA, and one might therefore make use of

methodologies for protein conjugation that have been developed for DNA itself;[26−29] however, to our knowledge, DNA has yet to be conjugated to proteins via the haloalkane dehalogenase approach. In the present work, we have developed a general strategy for genetically encoded labeling of proteins with ODF fluorophores (and potentially with DNA as well) that employs the HaloTag haloalkane dehalogenase enzyme. We have adapted this technology for covalent tethering of ODF fluorescent dyes directly to proteins in vitro as well as to proteins of interest expressed in live cells and have also applied it to multispectral cellular imaging.

## ■ EXPERIMENTAL SECTION

**Synthesis of Halolinker Phosphoramidite B8.** The chlorolinker phosphoramidite derivative **B8** was prepared after coupling of precursors **A4** and **B5** (see Scheme 1), which were derived from 2-(2-aminoethoxy)ethanol and diethylene glycol, respectively. The N-hydroxysuccinimide (NHS) ester-mediated coupling product (**B6**) was further converted in two steps to the desired phosphoramidite derivative, which is suitable for automated DNA synthesis. Synthesis details and characterization data are given in the Supporting Information (SI).

**Scheme 1. Synthesis of B8[a]**
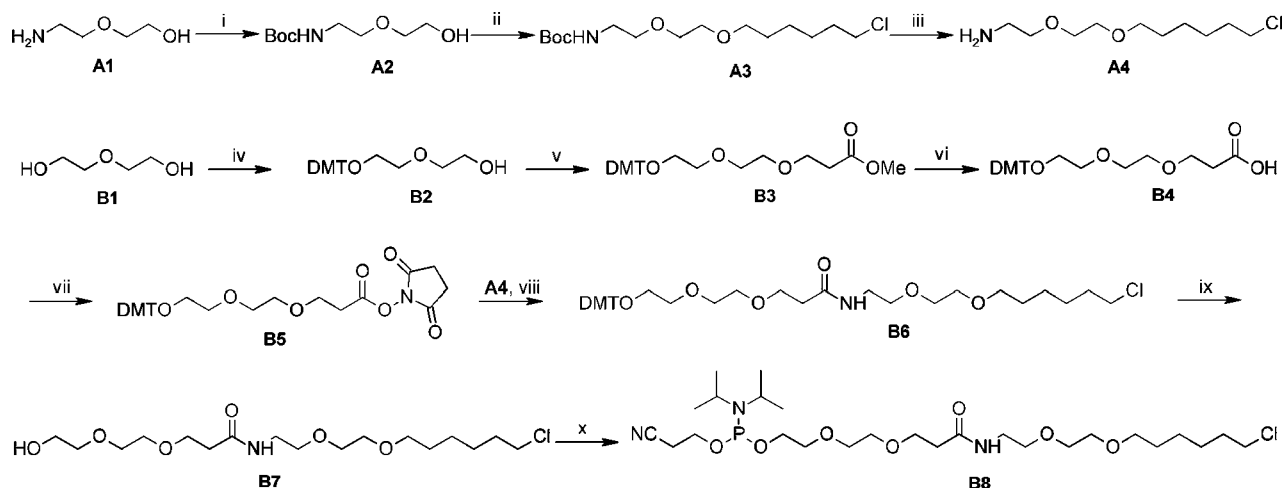


[a]Reagents and conditions: (i) Boc$_2$O, anhyd. EtOH, 0 °C to room temperature (rt), 2 h, 99%. (ii) NaH, 6-chloro-1-iodohexane, THF/DMF, 0 °C to rt, overnight (o/n), 69%. (iii) TFA, CH$_2$Cl$_2$, 0 °C to rt, 2 h, 81%. (iv) DMT-Cl, Et$_3$N, CH$_2$Cl$_2$, rt, 6 h, 67%. (v) Methyl acrylate, NaH, THF, 0 °C, 1 h, 75%. (vi) LiOH, MeOH/H$_2$O, rt, 2 h, 93%. (vii) N-Hydroxysuccinimide, DCC, CH$_2$Cl$_2$, 0 °C to rt, o/n, 95%. (viii) DIPEA, CH$_2$Cl$_2$, rt, o/n, 82%. (ix) AcOH, H$_2$O, rt, 2 h, 68%. (x) DIPEA, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, CH$_2$Cl$_2$, 0 °C, 45 min, 98%.

**Synthesis of Fluorescent Monomer F.** Bromo-substituted (diphenylamino)fluorenylbenzothiazole dye **6** was prepared from dibromofluorene as outlined in Scheme 2. This was coupled to a common *tert*-butyldiphenylsilyl (TBDPS)-protected dehydrotetrahydrofuran derivative of *d*-deoxyribose via Pd-mediated Heck chemistry to yield **F** (compound **7**). Details of the synthetic methods as well as NMR and mass spectrometry (MS) characterization data are given in the SI.

**Synthesis of Fluorescent ODF–HaloTag Ligands.** ODF–HaloTag ligands were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer using 3′-phosphate controlled pore glass (CPG) columns on a 1 μmol scale with the DMT-off method (DMT = 5′-dimethoxytrityl). Coupling of each monomer used standard 3′-to-5′ cyanoethyl phosphoramidite chemistry with an extended coupling time (999 s). The oligomers were cleaved from the CPG resin and deprotected by overnight incubation with 0.05 M K$_2$CO$_3$ in methanol. The purification was carried out utilizing a Shimadzu series HPLC with an Alltech C5 column and acetonitrile and triethylammonium acetate (TEAA) buffer (100 mM, pH 7.2) as eluents. The identities of the ODF–HaloTag ligands were confirmed by absorption spectroscopy and matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) MS analysis. See the SI for details.

**Construction and Expression of HaloTag Fusion Protein Vectors.** The vector encoding α-tubulin–HaloTag fusion protein was constructed by inserting the α-tubulin gene between the *Nco*I and *Bam*HI sites of commercially available HaloTag plasmid pFN21A (Promega, G2821), a mammalian expression vector. The plasmid encoding cell surface–HaloTag fusion protein was obtained from Dr. S. Gambhir (Stanford University) and was constructed by inserting the HaloTag protein gene into the pDisplay vector (Invitrogen). The resulting plasmids were then transformed into Top-10 bacterial cells using a standard heat-shock method. The transfected cells were propagated in LB medium at 37 °C. Isolated plasmids were characterized by agarose DNA gel and DNA sequencing analysis. See the SI for details.

**Optical and Microscopy Studies.** Absorption measurements were carried out on a Varian Cary 100 Bio UV–vis spectrophotometer. Fluorescence emission spectra were measured on a Jobin Yvon-Spex Fluorolog 3 spectrophotometer by exciting ODFs at 344 nm and collecting the emission between 365 and 750 nm. The fluorescence emission spectra of protein–ODF conjugates were performed on a FLEXstation II-384 fluorescent plate reader. Cellular imaging was performed on a Leica sp5 confocal microscope with a PL APO 63× oil objective. During imaging, HeLa cells were in phenol-free Dulbecco's modified Eagle's medium (DMEM). The ODFs were excited at 405 nm with an argon laser source.

**Cell Culture, Transfection, and Labeling.** HeLa cells were cultured in DMEM with glutamine (Gibco no. 11995) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Pen/Strep). All of the cells were maintained under 5% CO$_2$ at 37 °C. For live-cell imaging, cells were plated in Lab-Tek eight-well chambered coverglass slides (Nunc 155409) 24 h before transfection. Thereafter, HeLa cells were transfected with HaloTag fusion plasmids (2.0 μg of DNA per well) using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's protocol. After transfection, the cells were incubated in growth medium for 48 h. The protein labeling was performed by incubating HeLa cells in 200 μL of growth medium (DMEM without FBS and Pen/Strep) containing 5.0 μM ODF–HaloTag ligand for 15 min at 37 °C. After the labeling procedure, the staining medium was removed, and the cells were washed two times with phosphate-buffered saline (PBS), after which a final washing was performed by incubating the cells in phenol-free DMEM at 37 °C for 30 min. The medium was replaced with fresh phenol-free medium before imaging.

## ■ RESULTS

**Design and Synthesis of ODF–HaloTag Substrates.** To prepare ODF fluorescent dyes as possible substrates for the HaloTag dehalogenase, we required a haloalkane linker that could be placed at the end of an ODF sequence. Probably the most straightforward way to conjugate DNA is to incorporate the conjugate during DNA synthesis; with this in mind, we designed a new phosphoramidite reagent (**B8**) that contains a haloalkane moiety (designated **ht**) consisting of a chlorohexyl group at the terminus of a longer linker (Figure 1). The synthesis of the chlorolinker phosphoramidite reagent **B8** was efficient and straightforward (Scheme 1; also see the SI): the amino-functionalized chlorolinker **A4**, which was derived from 2-(2-aminoethoxy)ethanol (**A1**) in two steps, was then coupled with the DMT-protected NHS ester of diethylene glycol propionic acid (**B5**). Two routine steps subsequently yielded the desired phosphoramidite reagent **B8**.

Having the functional group in hand, we then prepared a test set of ODF dyes with a range of emission colors. The fluorescent building blocks (previously described monomers **E**,

**Scheme 2. Synthesis of Green-Emitting Monomer F (Compound 7)**[a]
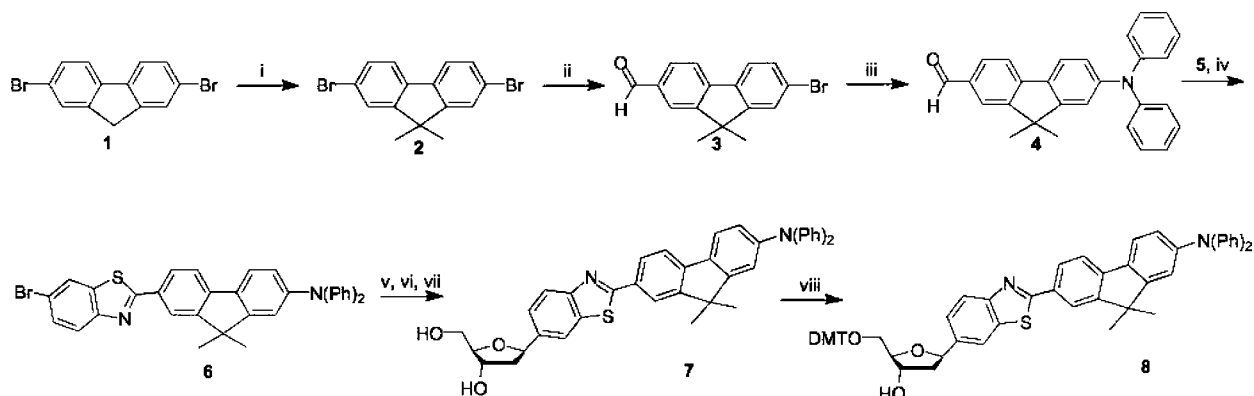


[a]Reagents and conditions: (i) MeI, KOH, KI, DMSO, rt, 24 h, 97%. (ii) $n$BuLi, DMF, THF, −78 °C, 1 + 2 h, 90%. (iii) Diphenylamine, $Cs_2CO_3$, Pd(OAc)$_2$, $t$-Bu$_3$P, toluene, reflux, 24 h, 67%. (iv) 2-Amino-5-bromobenzenethiol (**5**), TsOH, toluene, reflux, 2 days, 80%. (v) Cy$_2$NMe, 3′-$O$-TBDPS-1,2-dehydro-2-deoxy-$d$-ribofuranose, Pd($t$-Bu$_3$P)$_2$, Bu$_4$NBr, dioxane, 90 °C, 36 h. (vi) TBAF, AcOH, THF, 0 °C, 1 h, 25% (three steps). (viii) Na(OAc)$_3$BH, HOAc, CH$_3$CN, THF, −10 °C, 1 h. (viii) DMT-Cl, pyridine, DIPEA, rt, 3 h, 82%.
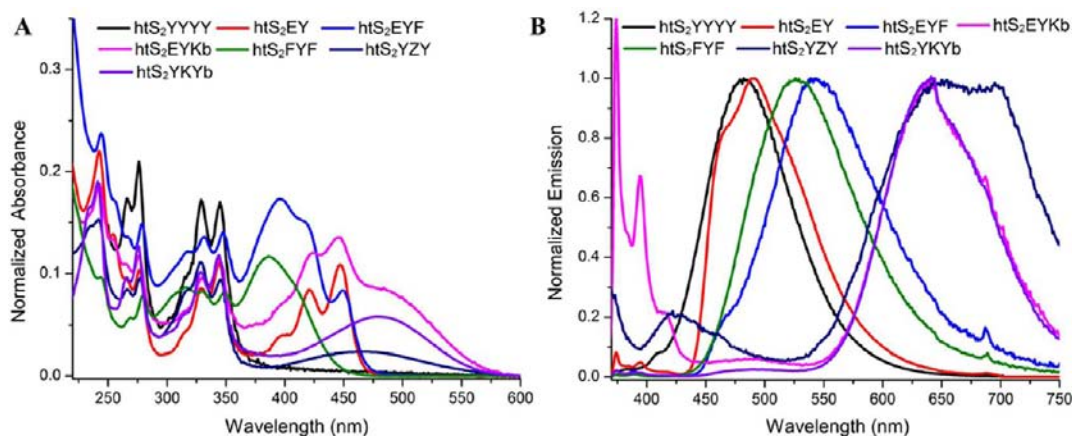


**Figure 2.** Spectra of ODF−HaloTag ligands: (A) absorption spectra; (B) normalized fluorescence emission spectra. Conditions: 2.0 $\mu$M ODF−HaloTag ligand in PBS ($\lambda_{ex}$ = 344 nm).

**Y, K,** and **Z**)[20,25,30,31] (Figure 1) were synthesized according to literature procedures. To include more opportunities for green emission in the ODF−HaloTag ligands, we synthesized one new green-emitting monomer, **F** (Figure 1), following the synthetic procedure shown in Scheme 2. Monomer **F** absorbs maximally at 393 nm and emits fluorescence at 495 nm with a quantum yield of 0.37 (Figure S1 in the SI). In addition to the five fluorescent monomers, we also added a commercially available tetrahydrofuran (THF) spacer (**S**) to increase both the water solubility of the ODF and the distance between the ODF and the protein, to avoid unfavorable interactions that might hinder reaction.

A set of seven different ODF sequences containing varied combinations of the five fluorescent monomers was chosen as possible HaloTag substrates. The chloroalkyl-ODF HaloTag lignds were prepared on a DNA synthesizer using 3′-phosphate-ON CPG columns (see the SI). After HPLC purification, they were characterized by MALDI−TOF MS (Table S1 in the SI) as well as by their absorption and emission spectra. The ODF monomers were used in their anomerically pure forms, except for monomer **K**, which was a mixture of $\alpha$- and $\beta$-anomers as reported previously.[20a] By HPLC we were able to separate the two anomers of **K** in the ODFs that contained it (htS$_2$EYK and htS$_2$YKY); these were studied

separately in further experiments (see below). The absorption spectra of the nine ODF−HaloTag ligands showed diverse absorption profiles, but they all had strong absorption at 344 nm (Figure 2a). As a result, we used this wavelength for fluorophore excitation in subsequent fluorescence analyses.

The fluorescence spectra of the ODF−HaloTag ligands show emission across the full visible spectrum (from 360 to 750 nm) with the single 344 nm excitation (Figure 2b; also see the SI). Upon comparison of the photophysical properties of the two anomers of **K**-containing ODF−HaloTag ligands, we found, not surprisingly, that the two anomers of htS$_2$YKY (htS$_2$YKYa and htS$_2$YKYb) have essentially identical absorption and fluorescence emission properties (Figure S2). However, the anomers of htS$_2$EYK (htS$_2$EYKa and htS$_2$EYKb) behaved differently: while their absorption profiles were identical, their emission properties were different (Figure S2). Among all nine ODF−HaloTag ligands, htS$_2$EY had the highest quantum yield (0.65), and htS$_2$YYYY displayed the longest fluorescence lifetime (7 and 42 ns; see Table S1). We also tested the photostability of the dyes in the absence of antifade reagents (Figure S17). Two were more rapidly bleached than fluorescein (the dyes containing monomer **F**); one showed stability approximately equal to that of fluorescein (htS$_2$YYYY); and three dyes (those containing chromophores EY, EYK, and

YKY) were exceptionally stable, with resistance to photobleaching as least as good as that of the stable commercial dye Alexa Fluor 350.

**HaloTag Fusion Protein Expression and Labeling.** For in vitro protein labeling experiments, we constructed a vector encoding glutathione S-transferase (GST)−HaloTag fusion protein (see the SI and Figure S3). The fusion protein was expressed in a KRX *Escherichia coli* bacterial strain, and the overexpression of fusion protein was achieved by overnight incubation of bacterial culture in the presence of 0.05% rhamnose. The GST−HaloTag fusion protein was purified by passing cell lysate through GST affinity resin and subsequent elution with 10 mM glutathione. In addition to the GST−HaloTag fusion protein, we also obtained the HaloTag protein alone by cleaving a TEV protease linker between the domains. The identities and purities of the two proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray ionization (ESI) mass spectrometry (Figures S4−S6).

To test initially whether a chloroalkyl-substituted ODF could be functional in HaloTag labeling, we separately incubated GST−HaloTag fusion protein and HaloTag protein in the presence of 5.0 $\mu$M chloroalkyl-ODF htS$_2$EY in PBS for 30 min. The formation of a covalent bond between ODF and the protein was confirmed for both proteins by the presence of fluorescence signals specifically in the protein treated with ODF−HaloTag ligand after separation on SDS-PAGE gels (Figure S4). Thereafter, the efficiency of labeling was investigated by performing ODF-concentration-dependent and reaction-time-dependent experiments. Those data are shown in the SI; the results confirmed the need for at least equimolar amounts of ODF for a given amount of protein for labeling as expected (Figure S7). The time-dependent experiments revealed complete labeling within 5 min using low-micromolar concentrations of chloroalkyl-ODF and protein (Figure S8).

We then proceeded to test the general applicability of ODFs in protein labeling, treating GST−HaloTag fusion protein as well as Halotag protein alone (ca. 2.0 $\mu$M) separately with the nine synthesized ODF ligands (4.0 $\mu$M each). The labeled proteins were then resolved and analyzed by SDS-PAGE. The fluorescence image of the gel, which was visualized with excitation at 365 nm, showed that multicolored protein labeling can be achieved by using ODF fluorescent dyes (Figure 3). Multispectral emission was also observed upon excitation at 457 nm (which corresponds to another absorption peak common to several of the ODFs), but different colors were obtained (Figure S9). Comparing the gel fluorescence intensity of free ODF−HaloTag ligands with the protein-conjugated ODFs, we found that several of the ODFs (htS$_2$YYYY, htS$_2$EY, htS$_2$EYF, and htS$_2$YZY) showed apparent lighting-up responses upon conjugation to protein, and some of the ODFs (htS$_2$YKY and htS$_2$EYK) changed their emission color upon protein conjugation (Figures 3 and S9). Interestingly, we also observed that the anomers of htS$_2$EYK (htS$_2$EYKa and htS$_2$EYKb) displayed similar colors before protein conjugation but were clearly different in hue after protein conjugation (Figure 3A, lanes 8 and 9). This was reproducible and was seen for both proteins.

**Characterization of Protein−ODF Conjugates.** The multicolor protein gel observations indicated that the fluorescence properties of some ODF−HaloTag ligands were affected by the change in their local environment upon protein
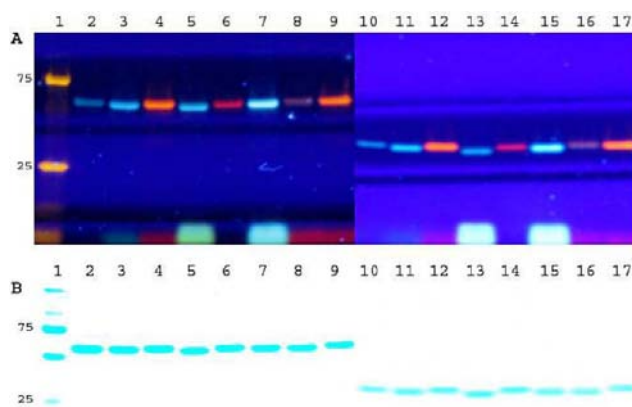


**Figure 3.** Multispectral labeling of GST−HaloTag fusion protein (lanes 2−9) and HaloTag protein (lane 10−17) with ODF−HaloTag ligands: (A) fluorescence image ($\lambda_{ex}$ = 365 nm); (B) Coomassie blue staining. Lane 1, marker; lanes 2 and 10, htS$_2$YYYY; lanes 3 and 11, htS$_2$EY; lanes 4 and 12, htS$_2$YKY; lanes 5 and 13, htS$_2$FYF; lanes 6 and 14, htS$_2$YZY; lanes 7 and 15, htS$_2$EYF; lanes 8 and 16, htS$_2$EYKa; lanes 9 and 17, htS$_2$EYKb.

conjugation. To explore this in more detail, we prepared protein−ODF conjugates on larger scale and compared their optical properties with those of unbound ODF−HaloTag ligands at known concentrations by fluorescence spectrometry (Figures 4, S10, and S11). The data showed that the fluorescence intensities of several of the ODF−HaloTag ligands were enhanced significantly upon conjugation with protein (Figures 4 and S10). The greatest increases occurred with the dyes htS$_2$EYKb (2.9-fold) and htS$_2$YZY (2.7-fold).

In addition, the emission maximum of the htS$_2$EYF ligand was shifted markedly toward the blue (by 42 nm) upon protein conjugation along with a ca. 2-fold enhancement in brightness (Figure S10). As observed on the gel, the isomers of htS$_2$EYK (htS$_2$EYKa and htS$_2$EYKb) both showed marked spectral changes upon protein conjugation. The "b" isomer, which exhibited one main emission band, yielded a strong light-up signal, while the "a" isomer, which initially exhibited two nearly equal peaks, shifted in color substantially: the 480 nm peak decreased in intensity while the 620 nm peak increased strongly, yielding a 2.5-fold change in the peak-height ratio (Figure S10).

**Cellular Labeling and Imaging.** To test the application of ODFs in cellular imaging of proteins, we expressed HaloTag fusion proteins in HeLa cells and then treated the cells with chloroalkane-ODFs to achieve labeling. Initially, we expressed a cell-surface protein [platelet-derived growth factor receptor transmembrane domain (PDGFR-TM)] fused with the HaloTag domain. Forty-eight hours post-transfection, the cells were labeled by incubating them for 15 min in growth medium containing 5.0 $\mu$M ODF−HaloTag ligands. No cell-uptake reagents were used, and excess dye was removed by exchanging the medium. We used the htS$_2$EY ODF−HaloTag ligand for cyan color, htS$_2$FYF for green labeling, and htS$_2$YKY for red color in separate experiments. The presence of fluorescence on the surface of each HeLa cell expressing cell-surface protein showed apparent labeling of cell-surface protein with ODF−HaloTag ligands (Figures 5a−c and S15). Control cells lacking the HaloTagged fusion protein showed lower fluorescence on the cell surface and a small amount of
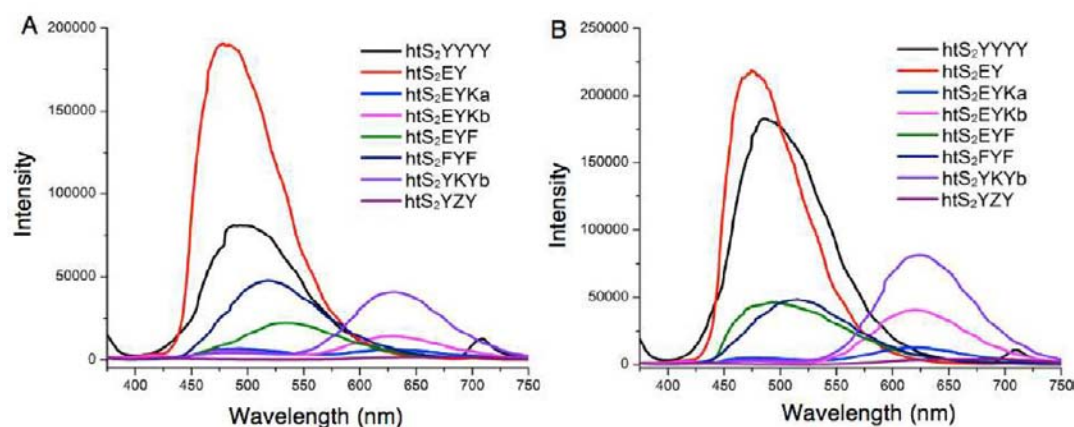
**Figure 4.** Fluorescence emission spectra of (A) ODF ligands alone and (B) protein−ODF conjugates. ODF−HaloTag ligands were 1.0 μM in PBS; protein−ODF conjugates were prepared from 1.0 μM ODF and 2.5 μM HaloTag protein in PBS for 30 min at 37 °C. $\lambda_{ex}$ = 344 nm.
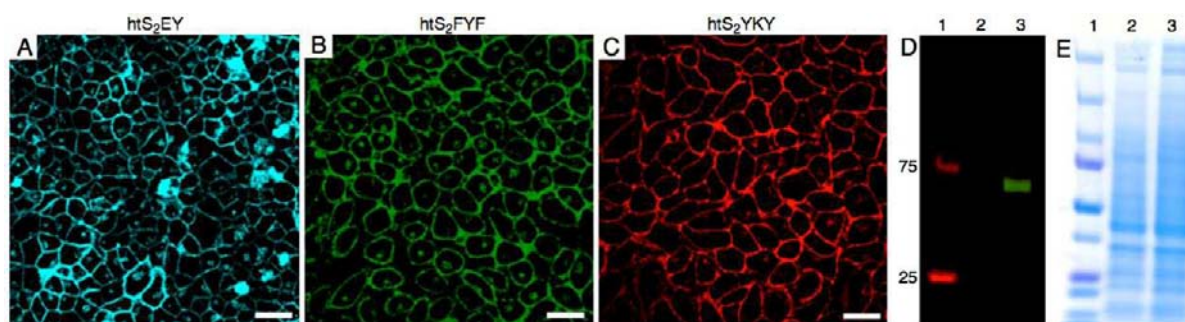


**Figure 5.** (A−C) Imaging of cell-surface protein in live HeLa cells with ODF−HaloTag ligands by confocal microscopy ($\lambda_{ex}$ = 405 nm; scale bars denote ca. 20 μm). (D, E) SDS PAGE gel of cell extracts showing labeling of 66.8 kDa cell-surface protein: (D) fluorescence scan; (E) Coomassie blue staining. Lane 1, size marker; lane 2, control HeLa cells; lane 3, HeLa cells expressing cell-surface protein.
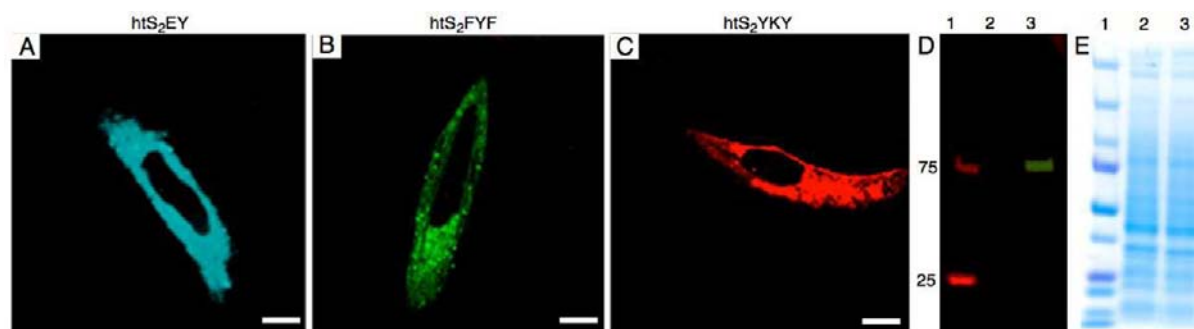


**Figure 6.** (A−C) Imaging of α-tubulin in live HeLa cells after labeling of cytoplasmic α-tubulin−HaloTag fusion protein with three different chloroalkyl-ODF ligands ($\lambda_{ex}$ = 405 nm; scale bars denote ca. 5 μm). (D, E) SDS-PAGE gel of cell extracts showing labeling of the 88.5 kDa fusion protein: (D) fluorescence scan; (E) Coomassie blue staining. Lane 1, size marker; lane 2, control HeLa cells; lane 3, HeLa cells expressing α-tubulin fusion protein.

fluorescence in the cytoplasm after dye treatment (Figure S12). The formation of stable covalent bonds between cell-surface protein and ODFs was confirmed by protein gel analysis. For that, the labeled HeLa cells expressing cell-surface protein as well as control cells were lysed and the protein was resolved by SDS-PAGE (Figure 5D). The presence of a fluorescence band at 66.8 kDa in the cells expressing fusion protein and not in the control cells confirmed the labeling of cell-surface fusion protein with ODF HaloTag ligands.

Next, we attempted to label a cytoplasmic protein with chloroalkyl-ODF labels. To achieve this, HeLa cells were transfected with a fusion vector encoding α-tubulin−HaloTag

fusion protein. After transfection and incubation (48 h), the fusion protein was labeled with cyan, green, or red ODF HaloTag ligand (5.0 μM, 60 min), and the labeled cells were then imaged under a confocal microscope. The presence of fluorescence in the entire cytoplasmic region of HeLa cells expressing the α-tubulin fusion protein indicated labeling of cytoplasmic protein by the ODF−HaloTag ligands (Figure 6; Figure S16 shows Z stacks). The labeling resolution and efficiency were similar to those achieved using a commercial tetramethylrhodamine (TMR)−HaloTag dye (Figure S13). Similar to the prior cell-surface protein labeling experiments, the formation of covalent bonds between ODF−HaloTag

ligands and cytoplasmic protein was established by the presence of a fluorescent band at 88.5 kDa (corresponding to the molecular weight of the fusion protein) in the cell lysate of HeLa cells expressing this protein (Figure 6D). The fluorescent band was absent in the lysate of control HeLa cells treated with the dyes.

The above experiments demonstrated that ODFs can be employed in genetically encoded protein labeling both on the surface and in the interior of cells. Since ODFs are capable of emitting a broad range of colors across the visible spectrum with single-wavelength excitation, they offer the capability of simultaneously visualizing two or more proteins located in different cellular compartments. To test this, we expressed cytoplasmic $\alpha$-tubulin in HeLa cells and labeled it with htS$_2$EY (cyan). Thereafter, the same cells were transfected with a vector encoding cell-surface fusion protein, which was then labeled (after an expression time of 48 h) with htS$_2$YKY (red). Confocal imaging clearly demonstrated the distinguishable labeling of the cell-surface and cytoplasmic proteins with two different ODF colors using a single 405 nm laser excitation line (see Figure 7). Lower fluorescence was observed in the
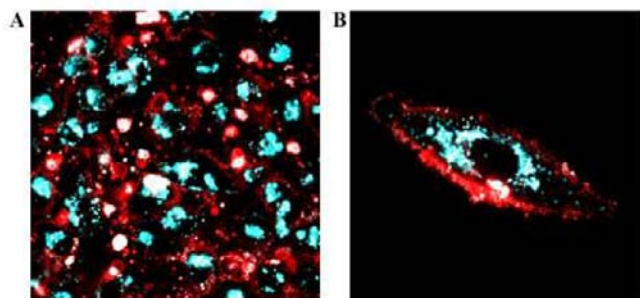


**Figure 7.** Live HeLa cell imaging showing two-color, single-excitation protein labeling. Cytoplasmic $\alpha$-tubulin was first expressed and labeled with the cyan ODF HaloTag ligand htS$_2$EY, and cell-surface protein was then expressed and (48 h later) labeled with red htS$_2$YKY. Laser confocal imaging was carried out in phenol-free growth medium ($\lambda_{ex}$ = 405 nm). (A) Multiple-cell view. (B) Closeup of a single cell.

cytoplasm during the dual-labeling experiment than during the cytoplasmic protein labeling experiments described above, which was expected because the continuous division of HeLa cells during the incubation time diluted the initially labeled cytoplasmic protein.

## ■ DISCUSSION

Our experiments showed rapid and high-yielding conjugation of HaloTag domains by ODF dyes using the chloroalkane linker developed here. The reactions appeared to proceed to apparently 100% yield in vitro and were complete in less than 5 min. The experiments confirmed that the haloalkane dehalogenase domain can accept organic substrates larger than conventional small-molecule dyes and that the multiple negative charges of the ODFs do not present a significant barrier to reaction. Examination of the structure of the chloroalkane recognition channel of the enzyme[13] suggested that a chain longer than ca. 15 Å should be sufficient to place the conjugated species outside the enzyme into solution. With the current linker, the length is ca. 27 Å in extended conformation, allowing ample distance for the ODF to reside in solution once conjugated.

Interestingly, some of the ODFs tested here changed their fluorescence emission properties upon conjugation. Most prominent were the dyes htS$_2$EYKb and htS$_2$YZY, whose brightness increased 2.9- and 2.7-fold respectively, and htS$_2$EYF, which was blue-shifted (from 530 to 488 nm) upon HaloTag conjugation. Also noteworthy is the ratiometric response observed in the dye htS$_2$EYKa. The changes suggest that although the ODF moieties could extend into solution given the chain length, they may (at least in these cases) interact with the nearby protein surface, resulting in these changes. This does not appear to be a general effect of protein–ODF conjugation; for example, several ODF dyes conjugated to antibodies previously showed little spectral change.[25] In a practical sense, however, such emission changes could be useful by yielding enhanced brightness upon application and in distinguishing conjugated dyes from nonconjugated ones.

The current experiments established facile cell-surface labeling by ODFs that are HaloTagged to chimeric cell-surface proteins, with labeling complete in less than 15 min. More surprising is the finding that intracellular proteins in intact cells can be tagged by ODFs as well, despite their multiple negative charges. We previously observed that ODFs are taken up by cells in a charge-dependent fashion, with shorter sequences (having fewer charges) entering cells more readily.[32] For cases that are slower to enter cells, the use of cationic lipid delivery agents provides effective uptake, although it should be noted that no lipid delivery agents were used here. We previously observed that the addition of hydrophobic structure to an ODF appears to enhance uptake without the aid of cationic lipids,[24,32] consistent with previous studies of oligonucleotides.[33] In the present case, it seems likely that the added haloalkane linker increases the hydrophobicity of the ODFs substantially, which may well enhance cellular uptake. Controlled studies would be needed to confirm this, but in the practical sense we observed no difficulties labeling an intracellular protein with the current ODF ligands. This establishes the first successful strategy for intracellular labeling with multispectral dyes; although quantum dots also have this useful optical property and can be adapted to HaloTag conjugation,[18] they have been applied only to extracellular labeling, possibly because of limitations in cellular uptake.

In addition to its possible favorable effects on uptake, the chloroalkane reactive group is notably stable here. The chloroalkane phosphoramidite reagent used here is easily handled during its synthesis and is stable toward DNA synthesis, deprotection, and purification chemistries. This not only makes it trivial to conjugate ODF dyes to proteins but also should make it possible more generally to conjugate DNAs or RNA oligonucleotides via their 5′-termini to proteins as well. Such nucleic acid–protein conjugates can have many uses in analyte detection, arraying methodologies, and nanostructure assembly.[34−36]

The current experiments have demonstrated successful HaloTagging for several different ODF dyes with distinct colors. Our results show no strong differences among the sequences in efficiency of tagging, suggesting that the chloroalkane-ODFs are essentially modular. This implies that any of thousands of possible ODF dyes having wide-ranging excitation and emission properties[20] might be employed in the same way. Moreover, since different ODFs have been developed recently not only for static fluorescence emission but also for sensing,[22,24,37] the results suggest the future possibility of genetically encoded tagging of proteins of interest

with sensors of small molecules or reporters of enzyme activities. More work will be needed to explore this possibility.

## ■ ASSOCIATED CONTENT

### ⓢ Supporting Information

Experimental details, synthetic procedures, protein expression and labeling procedures, and NMR spectral data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

**Corresponding Author**

kool@stanford.edu

**Present Address**

†Siemens Healthcare Diagnostics, 333 Coney St., East Walpole, MA 02032.

**Notes**

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

## ■ REFERENCES

(1) Giepmans, B. N.; Adams, S. R.; Ellisman, M. H.; Tsien, R. Y. *Science* **2006**, *312*, 217−224.

(2) Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063−6076.

(3) Miller, L. W.; Cornish, V. W. *Curr. Opin. Chem. Biol.* **2005**, *9*, 1−6.

(4) Gronemeyer, T.; Godin, G.; Johnsson, K. *Curr. Opin. Biotechnol.* **2005**, *16*, 453−458.

(5) Chen, I.; Ting, A. *Curr. Opin. Biotechnol.* **2005**, *16*, 35−40.

(6) Miller, L. W.; Cai, Y.; Sheetz, M. P.; Cornish, V. W. *Nat. Methods* **2005**, *2*, 255−257.

(7) Keppler, A.; Pick, H.; Arrivoli, C.; Vogel, H.; Johnsson, K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9955−9959.

(8) Gautier, A.; Juillerat, A.; Heinis, C.; Corrêa, I. R., Jr.; Kindermann, M.; Beaufils, F.; Johnsson, K. *Chem. Biol.* **2008**, *15*, 128−136.

(9) Watanabe, S.; Mizukami, S.; Hori, Y.; Kikuchi, K. *Bioconjugate Chem.* **2010**, *21*, 2320−2326.

(10) Uttamapinant, C.; White, K. A.; Baruah, H.; Thompson, S.; Fernández-Suárez, M.; Puthenveetil, S.; Ting, A. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10914−10919.

(11) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ACS Chem. Biol.* **2008**, *3*, 373−382.

(12) Newman, J.; Peat, T. S.; Richard, R.; Kan, L.; Swanson, P. E.; Affholter, J. A.; Holmes, I. H.; Schindeler, J. F.; Unkefer, C. J.; Terwilliger, T. C. *Biochemistry* **1999**, *38*, 16105−16114.

(13) (a) Los, G. V.; Wood, K. *Methods Mol. Biol.* **2007**, *356*, 195−208. (b) Kosaka, N.; Ogawa, M.; Choyke, P. L.; Karassina, N.; Corona, C.; McDougall, M.; Lynch, D. T.; Hoyt, C. C.; Levenson, R. M.; Los, G. V.; Kobayashi, H. *Bioconjugate Chem.* **2009**, *20*, 1367−1374. (c) http://www.promega.com/products/protein-expression-and-mass-spectrometry/protein-labeling-and-detection/halotag-technology-products/halotag-fluorescent-ligands (accessed Jan 18, 2013).

(14) Maurel, D.; Banala, S.; Laroche, T.; Johnsson, K. *ACS Chem. Biol.* **2010**, *5*, 507−516.

(15) Lee, H. L.; Lord, S. J.; Iwanaga, S.; Zhan, K.; Xie, H.; Williams, J. C.; Wang, H.; Bowman, G. R.; Goley, E. D.; Shapiro, L.; Twieg, R. J.; Rao, J.; Moerner, W. E. *J. Am. Chem. Soc.* **2010**, *132*, 15099−16101.

(16) Serge, A.; Bertaux, N.; Rigneault, H.; Marguet, D. *Nat. Methods* **2008**, *5*, 687−694.

(17) Wang, S.; Guo, J.; Ono, T.; Kool, E. T. *Angew. Chem., Int. Ed.* **2012**, *51*, 7176−7180.

(18) (a) Zhang, Y.; So, M. K.; Loening, A. M.; Yao, H.; Gambhir, S. S.; Rao, J. *Angew. Chem., Int. Ed.* **2006**, *45*, 4936−4940. (b) So, M.; Yao, H.; Rao, J. *Biochem. Biophys. Res. Commun.* **2008**, *374*, 419−423.

(19) (a) Resch-Genger, U.; Grabolle, M.; Cavaliere-Jaricot, S.; Nitschke, R.; Nann, T. *Nat. Methods* **2008**, *5*, 763−775. (b) Smith, A. M.; Nie, S. *Nat. Biotechnol.* **2009**, *27*, 732−733. (c) Byers, R. J.; Hitchman, E. R. *Prog. Histochem. Cytochem.* **2011**, *45*, 201−237.

(20) (a) Teo, Y. N.; Wilson, J. N.; Kool, E. T. *J. Am. Chem. Soc.* **2009**, *131*, 3923−3933. (b) Gao, J.; Strässler, C.; Tahmassebi, D. C.; Kool, E. T. *J. Am. Chem. Soc.* **2002**, *124*, 11590−11591.

(21) Wilson, J. N.; Kool, E. T. *Org. Biomol. Chem.* **2006**, *4*, 4265−4274.

(22) Gao, J.; Watanabe, S.; Kool, E. T. *J. Am. Chem. Soc.* **2004**, *126*, 12748−12749.

(23) Samain, F.; Ghosh, S.; Teo, Y. N.; Kool, E. T. *Angew. Chem., Int. Ed.* **2010**, *49*, 7025−7029.

(24) Dai, N.; Teo, Y. N.; Kool, E. T. *Chem. Commun.* **2010**, *46*, 1221−1223.

(25) Guo, J.; Wang, S.; Dai, N.; Teo, Y. N.; Kool, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 3493−3498.

(26) Niemeyer, C. M.; Adler, M.; Gao, S.; Chi, L. *Bioconjugate Chem.* **2001**, *12*, 364−371.

(27) Jongsma, M. A.; Litjens, R. H. G. M. *Proteomics* **2006**, *6*, 2650−2655.

(28) Duckworth, B. P.; Chen, Y.; Wollack, J. W.; Sham, Y.; Mueller, J. D.; Taton, T. A.; Distefano, M. D. *Angew. Chem., Int. Ed.* **2007**, *46*, 8819−8822.

(29) Shimada, J.; Maruyama, T.; Hosogi, T.; Tominaga, J.; Kamiya, N.; Goto, M. *Biotechnol. Lett.* **2008**, *30*, 2001−2006.

(30) Chaudhuri, N. C.; Ren, R. X.; Kool, E. T. *Synlett* **1997**, 341−347.

(31) Strässler, C.; Davis, N. E.; Kool, E. T. *Helv. Chim. Acta* **1999**, *82*, 2160−2171.

(32) Teo, Y. N. Ph.D. Thesis, Stanford University, Stanford, CA, August 2010.

(33) Wolfrum, C.; Shi, S.; Jayaprakash, K. N.; Jayaraman, M.; Wang, G.; Pandey, R. K.; Rajeev, K. G.; Nakayama, T.; Charrise, K.; Ndungo, E. M.; Zimmermann, T.; Koteliansky, V.; Manoharan, M.; Stoffel, M. *Nat. Biotechnol.* **2007**, *25*, 1149−1157.

(34) Kazane, S. A.; Sok, D.; Cho, E. H.; Uson, M. L.; Kuhn, P.; Schultz, P. G.; Smider, V. V. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 3731−3736.

(35) Niemeyer, C. M.; Sano, T.; Smith, C. L.; Cantor, C. R. *Nucleic Acids Res.* **1994**, *22*, 5530−5539.

(36) Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2010**, *49*, 1200−1216.

(37) Tan, S. S.; Kool, E. T. *J. Am. Chem. Soc.* **2011**, *133*, 2664−2671.