VIP Live-Cell Imaging

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A FRET-Based Fluorogenic Phosphine for Live-Cell Imaging with the Staudinger Ligation**

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Fluorescent labeling is a central tool for studying localization, trafficking, and expression levels of biomolecules in live cells. Biologists routinely rely on this information to assist in the study of cellular processes. While fluorescent fusion proteins and other genetically encoded tags have been used to image specific proteins in live cells,[1] analogous labeling techniques have not been available for imaging biomolecules not directly encoded by the genome, including glycans, lipids, and other metabolites. As a result, a lag in cell-based studies of these molecules has occurred despite their documented importance in many essential biological processes.[2]

The metabolic labeling technique can be used to introduce bioorthogonal chemical reporters into cellular biomolecules without genetic manipulation.[3] Subsequent treatment with an exogenously delivered probe allows direct tagging of the target biomolecule. The most versatile bioorthogonal chemical reporter is the azide. Metabolic labeling of biopolymers with azido amino acids,[4] sugars,[5] lipids,[6] and cofactors[7] have all been realized in live cells. Once in place, the azides can be reacted with triaryl phosphines by the Staudinger ligation[8] and alkynes by either a copper-catalyzed [3+2] cycloaddition (i.e., click chemistry)[9] or a strain-promoted [3+2] cycloaddition.[10] While the copper catalyst required for click chemistry has been reported to be toxic to cells in some cases,[4a,10c] the Staudinger ligation reagents have no apparent toxicity,[11] rendering it attractive for studies with live cells or organisms.

Fluorescent phosphine probes have been used for direct imaging of various azide-bearing biomolecules with the Staudinger ligation in cell-free environments.[12] Recently, we applied phosphine-based dyes to image azides on the surface of live cells.[13] Notably, significant labeling above background levels could only be achieved using a highly negatively charged fluorophore; other fluorophores suffered from nonspecific cell binding and, accordingly, high background labeling and low sensitivity. This finding underscores the major challenge posed by direct fluorescence imaging approaches: how to minimize background fluorescence to increase the signal-to-noise ratio.

An ideal labeling reagent would remain nonfluorescent until bound to its target. This "fluorogenic" principle has been widely employed for nucleic acid detection[14] and enzyme activity assays.[15] We previously explored phosphine–coumarin analogues in which the lone pair of electrons on phosphorus quenched the fluorophore to which it was directly attached.[16] During the Staudinger ligation, oxidation to the phosphine oxide enhanced fluorescence by 60-fold. However, phosphines are prone to nonspecific air oxidation as well, a side reaction that produced high background fluorescence in cell-imaging experiments. More recently, fluorogenic naphthalimide and coumarin dyes have been designed to label azide- or alkyne-modified biopolymers using click chemistry.[5c,17] While suitable for fixed cells, the toxicity of the

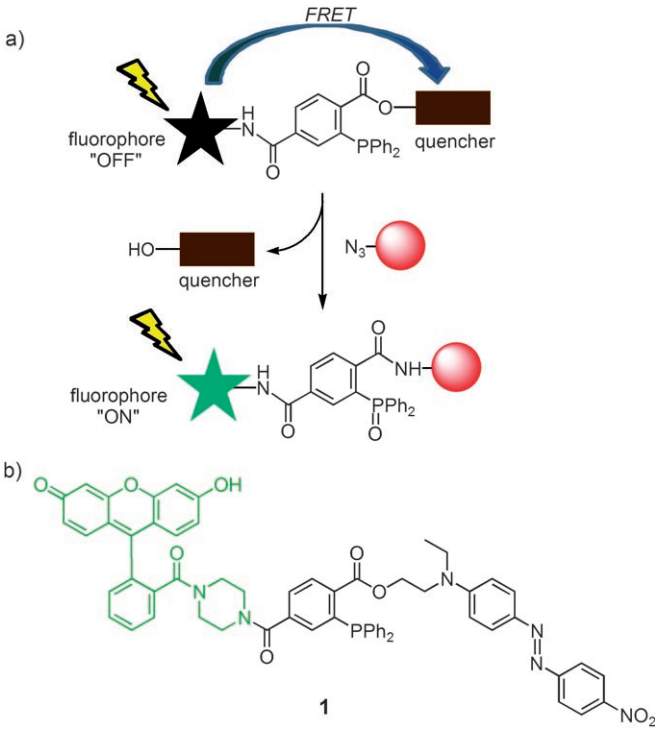

Figure 1. A FRET-based fluorogenic phosphine for live-cell imaging. a) The design of a quenched phosphine–fluorophore that is activated upon Staudinger ligation with azides. b) Compound **1**, possessing fluorescein (green) and disperse red 1 (dark red) moieties.

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copper reagent precludes the use of such dyes for live-cell imaging.

Here, we report the design of a fluorogenic phosphine reagent that can image azides on live cells with minimal background. The reagent, compound **1** (Figure 1), comprises a phosphine-tethered fluorophore moiety that is quenched intramolecularly by an ester-linked fluorescence resonance energy transfer (FRET) quencher, disperse red 1.[18] Staudinger ligation of compound **1** with azides results in cleavage of the ester and concomitant unquenching.

Nonspecific phosphine oxidation should not interfere with the FRET quenching efficiency; hence, this design overcomes the significant shortcoming of our previously described fluorogenic phosphine. As a fluorescein analogue, compound **1** also benefits from spectral properties that are better suited for live-cell imaging than earlier coumarin and naphthalimide dyes.

The synthesis of compound **1** is described in detail in the Supporting Information and is outlined in Scheme 1. Briefly, carboxylic acid **2**[19] was protected to yield *tert*-butyl ester **3**. Subsequent mild saponification of the methyl ester provided compound **4**, which was converted to triaryl phosphine **5** by palladium cross-coupling with diphenylphosphine. Esterification with commercially available disperse red 1 gave **6**, which was then deprotected to afford acid **7**. Coupling of fluorescein derivative **8**[13] with **7** yielded compound **1**.

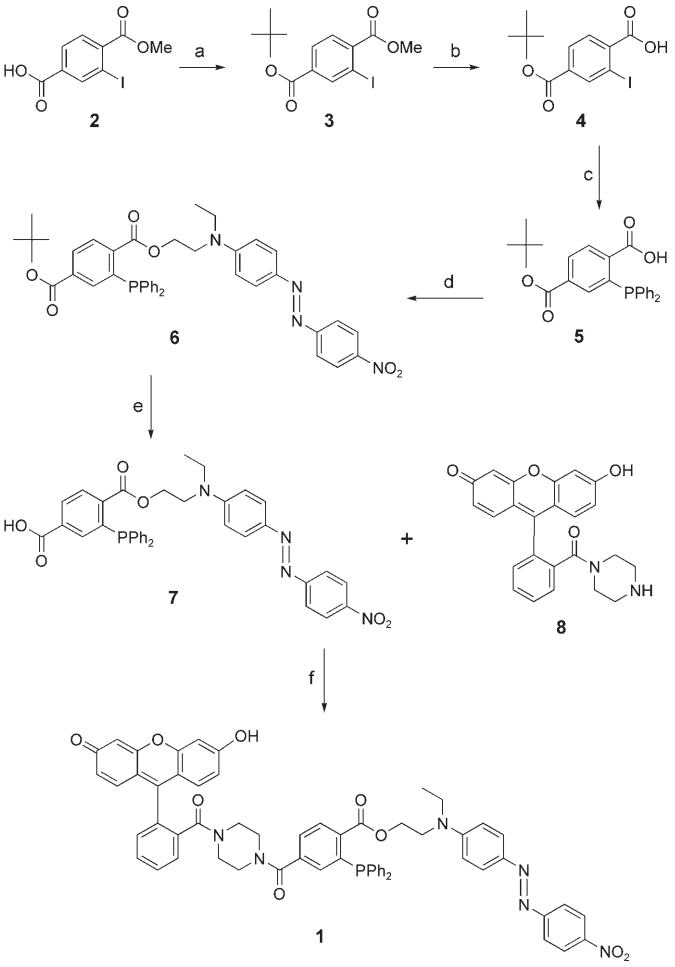
A model reaction of **1** and benzyl azide was performed in aqueous KH_2PO_4 (10 mM)/acetonitrile (1:1) (Scheme 2). The Staudinger ligation to form **9** occurred with an apparent second-order rate constant of $0.0038 \pm 0.0008\ M^{-1}s^{-1}$. As expected based on previous kinetic and mechanistic studies,[20] replacing the methyl ester of earlier Staudinger ligation reagents with the disperse red 1 ester did not affect the reaction rate.

We next measured the photophysical parameters of **1** and its ligation product **9** (Table 1). Also, the phosphine oxide derived from **1** (referred to as **1**-oxide, see the Supporting Information) was synthesized and analyzed. Importantly, **1** and **1**-oxide were found to be essentially nonfluorescent (quantum yields for both were < 0.01). Therefore, this FRET-based fluorogenic phosphine will not suffer from background fluorescence in the event of nonspecific phosphine oxidation. In contrast to **1** and **1**-oxide, Staudinger ligation product **9** was strongly fluorescent, with a quantum yield of 0.64 ± 0.02, reflecting an increase in fluorescence quantum yield relative to **1** of at least 170-fold. From these data, it is clear that **1** exhibits very efficient intramolecular FRET quenching and is unquenched upon Staudinger ligation with an azide.

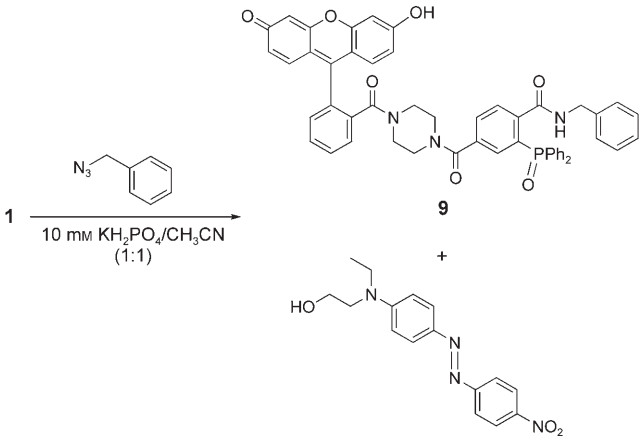
Compound **1** was next tested with an azide-modified protein (Figure 2). Recombinant murine dihydrofolate reductase (mDHFR) containing azidohomoalanine in place of native methionine residues,[19] as well as native mDHFR as a control, were incubated with 12.5 μM **1** for 20 h at room temperature. The crude reaction mixtures were analyzed by SDS-PAGE, and the gel was imaged by fluorescence, revealing azide-specific labeling with no detectable background fluorescence.

Compound **1** was then employed to label azides displayed on live cells. Chinese hamster ovary (CHO) cells were incubated with peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz) for three days in order to introduce *N*-α-azidoacetyl sialic acid (SiaNAz) into their cell surface, secreted, and Golgi-resident glycans.[8,11a] The Ac_4ManNAz-treated CHO cells were incubated with 25 μM **1** for 8 h at 37 °C and subsequently analyzed by flow cytometry. Robust fluorescent labeling was observed for cells treated with both Ac_4ManNAz and **1** (Figure 3). By contrast, control cells lacking azides but treated with **1** displayed minimal fluorescence. Importantly, we did not observe any nonspecific ester hydrolysis by cellular esterases that would liberate the quencher prematurely and create unwanted background fluorescence.

Finally, we evaluated compound **1** for live-cell imaging by fluorescence microscopy. HeLa cells were treated with Ac_4ManNAz for 40 h, rinsed, and then incubated with 50 μM



Scheme 1. Synthesis of phosphine **1**. Reagents and conditions: a) *t*BuOH, DMAP (0.5 equiv), EDAC, CH_2Cl_2, 84%; b) LiOH (1.5 equiv), MeOH/H_2O (3:1), 94%; c) $HPPh_2$, K_2CO_3, $Pd(OAc)_2$ (0.3 mol%), CH_3CN, reflux, 72%; d) disperse red 1, DMAP (0.1 equiv), DCC, CH_3CN (83%); e) TFA (26 equiv), TES (5 equiv), CH_2Cl_2 (87%); f) **8**, HATU, DIPEA, DMF (65%). DCC = *N*,*N*′-dicyclohexylcarbodiimide, DIPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, EDAC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TES = triethylsilane, TFA = trifluoroacetic acid.



Scheme 2. Model Staudinger ligation.

Table 1: Photophysical parameters of phosphine probes.

Probe	ε [$M^{-1}cm^{-1}$][a]	λ_{abs} [nm][a]	λ_{em} [nm][a,b]	Φ_F[a–c]
1	6600 ± 200	505	515	0.00372 ± 0.00005
1-oxide	18800 ± 300	505	520	0.00521 ± 0.00004
9	44000 ± 1000	501	520	0.64 ± 0.02

[a] Measured in phosphate-buffered saline (PBS), pH 7.0. [b] λ_{ex} = 470 nm. [c] Relative quantum yields of fluorescence (Φ_F) measured using fluorescein as the standard.

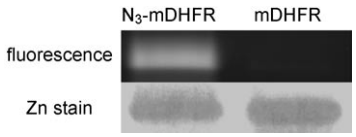


Figure 2. Labeling of azido-mDHFR with compound **1**. Purified azido-mDHFR (left lane) and native mDHFR (right lane) were labeled with 12.5 μM **1** for 20 h at RT in PBS under denaturing conditions. The crude reaction mixtures were separated by SDS-PAGE and the gel was analyzed by fluorescence imaging (top row) and by Zn stain to reveal total protein content (bottom row).

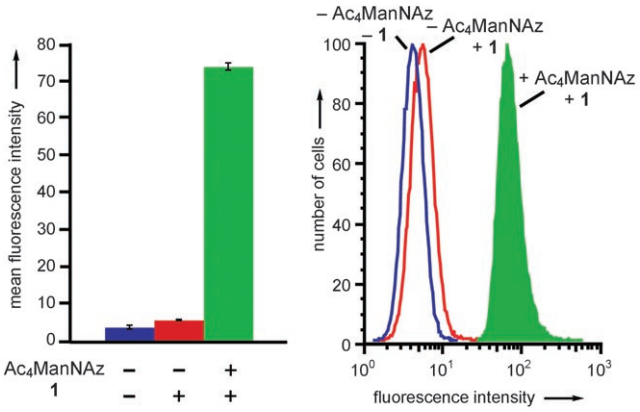


Figure 3. Flow cytometry analysis of live Ac_4ManNAz-treated CHO cells labeled by phosphine **1**. Cells were treated with Ac_4ManNAz for three days and then phosphine **1** for 8 h. Control cells were either untreated or treated with phosphine **1** alone. Error bars represent the standard deviation of the mean for triplicate measurements.

1 for 8 h at 37 °C. Bright cell-surface labeling was observed for cells displaying azides (Figure 4a), with essentially no background labeling observed for cells lacking azides (Figure 4b). HeLa cells bearing SiaNAz residues also demonstrated intracellular labeling that colocalized with a live-cell Golgi marker (Figure 4a, top row), as well as a Golgi-protein-specific antibody (Figure 4a, bottom row). Because **1** was shown to be live-cell impermeant in other assays (data not shown), the Golgi labeling observed in this experiment likely reflects the internalization of labeled cell-surface glycans rather than direct labeling of Golgi-resident azides. In fact, we recently observed this phenomenon with difluorinated cyclooctyne imaging reagents as well.[10d] Also, **1** was shown to be nontoxic to the cells by exclusion of propidium iodide, a reagent that selectively stains the nuclei of dead cells (Figure 4). Additionally, lack of increased staining relative to untreated cells with early apoptosis marker Annexin V confirmed that **1** is not cytotoxic (see Figure S2 in the

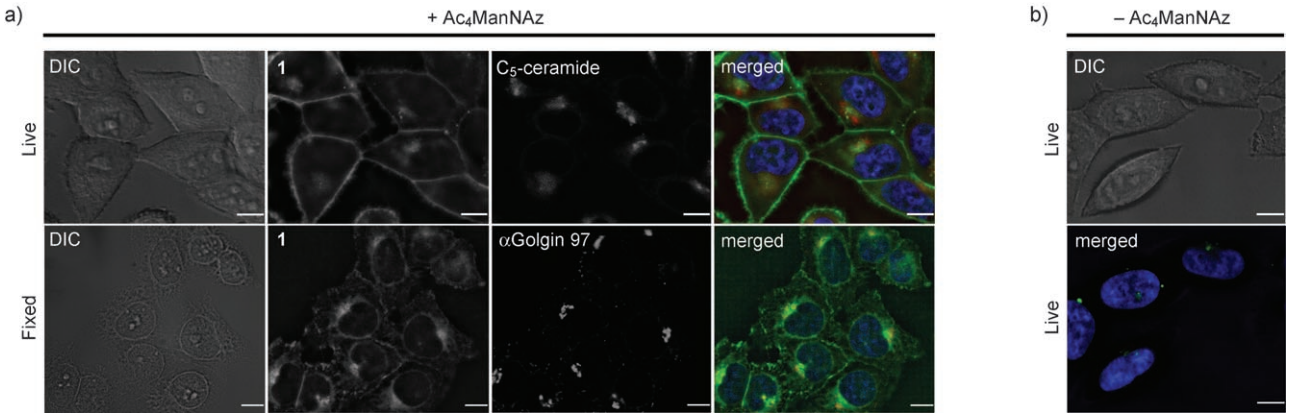


Figure 4. Fluorescence microscopic images of Ac_4ManNAz-treated HeLa cells labeled with phosphine **1**. a) Top row: Live HeLa cells treated with Ac_4ManNAz, **1** (FITC channel), and the live-cell Golgi marker BODIPY TR C_5-ceramide (Cy3 channel); bottom row: HeLa cells treated with Ac_4ManNAz, **1**, then fixed, permeabilized, and treated with anti-Golgin 97 mouse mAb and goat anti-mouse IgG-Alexa Fluor 647 (Cy5 channel). b) Live HeLa cells treated with **1** only. All cells were treated while alive with nuclear stain Hoechst 33342 (DAPI channel) and viability stain propidium iodide (Cy3 and Cy5 channels). The lack of nuclear fluorescence in Cy3 and Cy5 channels indicates propidium iodide exclusion from cells. The scale bars represent 10 μm.

Supporting Information). The observed cell-surface turnover during labeling and imaging, coupled with the demonstrated cell viability, underscore the suitability of compound **1** for imaging dynamic cellular events without perturbing normal cellular behavior.

In conclusion, the azide is rapidly gaining popularity as a chemical reporter group for biomolecules and posttranslational modifications. The ability to visualize this functional group in live cells with compound **1** provides a new avenue for probing the cellular dynamics, localization, and regulation of labeled biomolecules. Further, the design strategy outlined in Figure 1 a can accommodate numerous fluorophores and complementary quenchers, enabling extension to multicolor imaging. Future research will include the design of cell-permeant variants of this reagent likely utilizing fluorophores and quenchers with improved cell permeability, prodrug masking strategies, or carrier delivery systems. We anticipate applications to the study of protein glycosylation, lipidation, and de novo protein biosynthesis, in both live cells and organisms.

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