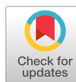

Copper-free click chemistry for dynamic *in vivo* imaging

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Dynamic imaging of proteins in live cells is routinely performed by using genetically encoded reporters, an approach that cannot be extended to other classes of biomolecules such as glycans and lipids. Here, we report a Cu-free variant of click chemistry that can label these biomolecules rapidly and selectively in living systems, overcoming the intrinsic toxicity of the canonical Cu-catalyzed reaction. The critical reagent, a substituted cyclooctyne, possesses ring strain and electron-withdrawing fluorine substituents that together promote the [3 + 2] dipolar cycloaddition with azides installed metabolically into biomolecules. This Cu-free click reaction possesses comparable kinetics to the Cu-catalyzed reaction and proceeds within minutes on live cells with no apparent toxicity. With this technique, we studied the dynamics of glycan trafficking and identified a population of sialoglycoconjugates with unexpectedly rapid internalization kinetics.

azide | bioorthogonal reaction | cyclooctyne glycan trafficking | molecular imaging

Imaging biomolecules within living systems requires the means to distinguish the target from the surrounding components by using a spectroscopic probe. For proteins, genetically encoded tags such as GFP are routinely used (1). In cases where less structurally perturbing labels are required, the target protein can be endowed with a peptide sequence that directs the chemical (2, 3) or enzymatic (4) attachment of small-molecule probes. Although proteins continue to be central targets of cellular imaging, there is growing interest in visualizing biomolecules that are not so amenable to genetic modification. For example, glycans and lipids, which often are posttranslational modifications but also have their own independent functions, cannot be imaged with protein-specific methods. Thus, although these biomolecules have been studied *in vitro* and in static systems, their dynamic behavior in living cells is not well defined.

The bioorthogonal chemical reporter strategy may offer an avenue for labeling and visualizing biomolecules *in vivo*, without the requirement of genetic manipulation. In this approach, the cell's metabolic machinery is used to install a bioorthogonal functional group into target biomolecules, which is then covalently labeled in a second step with a probe (5). The azide is the most widely used chemical reporter because of its small size, metabolic stability, and lack of reactivity with natural biofunctionality. Azides have been introduced into glycans, lipids, and proteins in living cells (5), and in the case of glycans in living animals (6), with minimal physiological perturbation.

Metabolic labeling with azides primes the target biomolecule for visualization by covalent attachment of an imaging probe. For dynamic *in vivo* imaging, the covalent reaction must be fast (i.e., on the time scale of cellular processes) and not harmful to the cell. Unfortunately, the existing azide-specific reactions, the Staudinger ligation (7) and the Cu-catalyzed azide–alkyne cycloaddition (8, 9), fall short. The Staudinger ligation, which forms an amide bond between the azide and an ester-derivatized phosphine, suffers from slow reaction kinetics and competing oxidation of the phosphine reagents (10). Although highly biocompatible in cells and living animals, this reaction cannot be used to detect low-abundance species or to visualize rapid biological processes on the minute time scale.

By contrast, the Cu-catalyzed azide–alkyne cycloaddition, often referred to as click chemistry, allows for high-sensitivity detection of azides. Developed by Sharpless and coworkers (8) and Meldal and colleagues (9), this transformation forms a triazole from an azide and a terminal alkyne, which is activated in the reaction by a Cu catalyst (Fig. 1*A*). Click chemistry has prevailed in applications where toxicity is irrelevant, such as in probing enzyme activities in cell lysates (11) or visualizing biomolecules in fixed cells (12, 13). However, dynamic processes in living systems are inaccessible to click chemistry because the reaction requires a cytotoxic Cu catalyst.

Results and Discussion

Design, Synthesis, and Kinetic Evaluation of a Difluorinated Cyclooctyne (DIFO) for Cu-Free Click Chemistry. We sought to design a bioorthogonal reaction for dynamic cellular imaging that combines the biocompatibility of the Staudinger ligation with the fast reaction kinetics of click chemistry. One approach is to identify an alternative means of activating alkynes for [3 + 2] cycloaddition with azides. We previously explored the use of ring strain, demonstrating that cyclooctynes, the smallest of the stable cycloalkynes, can achieve bioorthogonal labeling of azides (14). Although the sensitivity of this strain-promoted cycloaddition was no better than that of the Staudinger ligation, the cyclooctyne probes demonstrated no cellular toxicity and offered a platform for further reaction development using the principles of physical organic chemistry (15).

To increase the sensitivity of the cyclooctynes for azide detection, we combined two powerful rate-enhancing features, ring strain and electron-withdrawing groups, into the design of Cu-free click reagent **1** (Fig. 1 *B* and *C*). We chose the difluoromethylene moiety as an electron-withdrawing group because of its synthetic accessibility and its inertness in biological systems (16). Importantly, this group does not create an electrophilic Michael acceptor capable of alkylating biological nucleophiles, as would a π-conjugated substituent such as a carbonyl group. The synthetic route for DIFO **1**, hereafter termed DIFO, is

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Abbreviations: DIFO, difluorinated cyclooctyne; DHFR, dihydrofolate reductase; SiaNAz, azido sialic acid; $Ac_4ManNAz$ peracetylated, *N*-azidoacetylmannosamine; $Ac_4ManNAc$, peracetylated *N*-acetylmannosamine.

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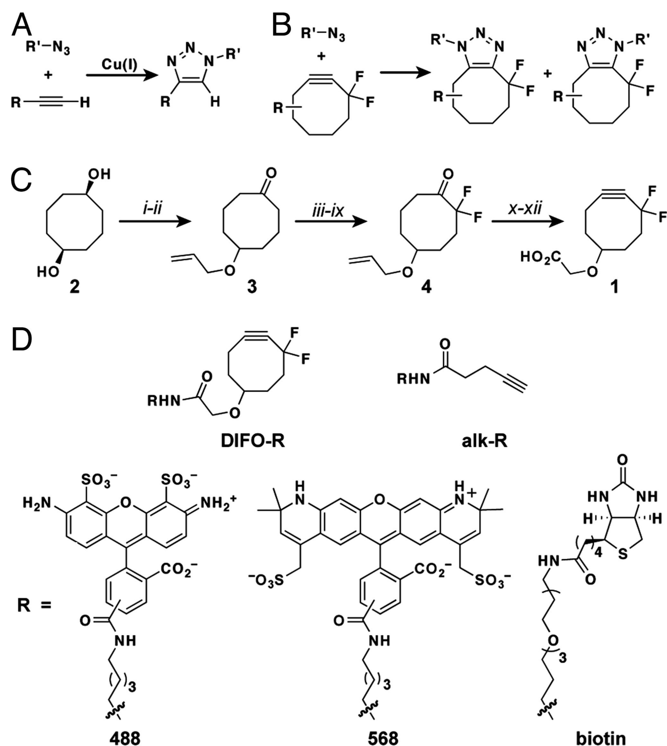

Fig. 1. Design and synthesis of Cu-free click chemistry reagents. (*A*) The copper-catalyzed azide–alkyne cycloaddition. (*B*) The Cu-free click reaction of azides and DIFOs. (*C*) (*i*) Sodium hydride, allyl bromide, 52%. (*ii*) Pyridinium chlorochromate, 91%. (*iii*) Lithium hexamethyldisilazide (LHMDS), chlorotriethylsilane (TESCl), 91%. (*iv*) Selectfluor, 95%. (*v–vii*). Cat. LHMDS, 95%. (*viii*) Potassium hexamethyldisilazide (KHMDS), TESCl, 97%. (*ix*) Selectfluor, 74%. (*x*) Cat. $RuCl_3$, $NaIO_4$, 96%. (*xi*) KHMDS, *N*-phenylbis(trifluoromethanesulfonamide), 46%. (*xii*) Lithium diisopropylamide, 11%. (*D*) Derivatives of DIFO and a linear alkyne (alk) containing Alexa Fluor 488, Alexa Fluor 568, or biotin.

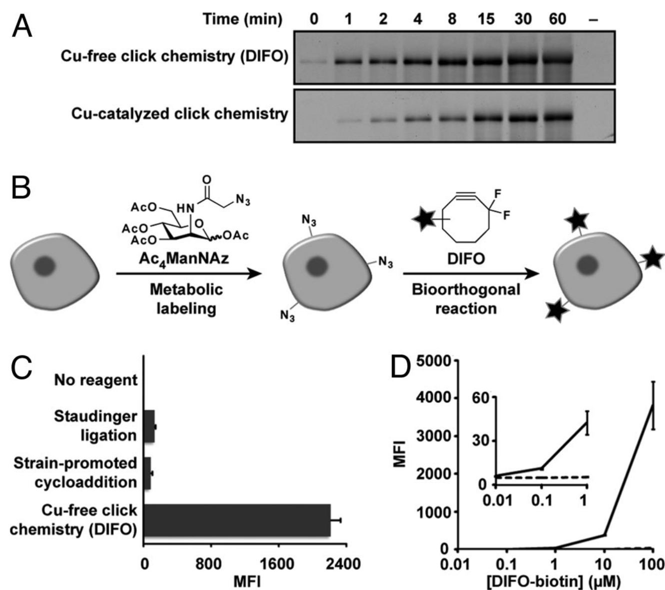

Fig. 2. Comparison of Cu-free click chemistry with existing bioorthogonal ligations. (*A*) Reactions of 10 ng of azidohomoalanine-labeled DHFR with 25 μM DIFO-488 or alk-488 were allowed to proceed for the time indicated. Reactions with alk-488 were performed as described in ref. 26. A negative control reaction (−) using 10 ng of azide-free DHFR was allowed to proceed for 60 min. (*B*) Schematic for metabolic labeling and detection of cell-surface glycans using $Ac_4ManNAz$ and DIFO-based reagents. (*C* and *D*) Flow cytometry plots of labeling experiment described in *B* using Jurkat cells. (*C*) Cells were labeled for 1 h with 100 μM biotinylated derivatives of a phosphine (Staudinger ligation) (20), a nonfluorinated cyclooctyne (strain-promoted cycloaddition) (14), and DIFO (Cu-free click chemistry). In all cases, control cells (incubated in azido sugar-free medium but carried through an identical labeling procedure) displayed mean fluorescence intensity (MFI, arbitrary units) values <20. (*D*) Cells were labeled for 1 h with 10 nM–100 μM DIFO-biotin. Error bars represent the standard deviation of three replicate experiments. Solid line, + $Ac_4ManNAz$; dashed line, − $Ac_4ManNAz$.

shown in Fig. 1*C* and supporting information (SI) Schemes 1–3 and SI Figs. 5–8. Kinetic analysis with benzyl azide gave a second-order rate constant of 7.6×10^{-2} $M^{-1}{\cdot}s^{-1}$, 17–63 times greater than the rate constants for the Staudinger ligation or any of the previously reported strain-promoted cycloadditions (15) (*SI Materials and Methods* and SI Fig. 9). Despite its enhanced reactivity with azides, DIFO and its derivatives (Fig. 1*D*) were found to be stable in aqueous solutions and in the presence of 2-mercaptoethanol, suggesting that DIFO-based reagents will not cross-react with biological nucleophiles.

Comparative *in Vitro* Labeling Efficiencies of Cu-Free and Cu-Catalyzed Click Chemistries on Azido Proteins. We sought to compare the Cu-free click reaction by using DIFO with Cu-catalyzed click chemistry. Because a direct comparison in model reactions is complicated by the difference in reaction order of the two processes (17), we compared the two reactions under pseudo first-order conditions by using an azide-labeled protein as the limiting reagent. Derivatives of DIFO and a terminal alkyne conjugated to Alexa Fluor 488 (DIFO-488 and alk-488, Fig. 1*D*) were reacted with recombinant dihydrofolate reductase (DHFR) bearing the unnatural amino acid azidohomoalanine (18). Both the Cu-free and Cu-dependent reactions appeared to reach saturation within the first hour (Fig. 2*A* and SI Fig. 10), and neither reaction showed labeling of natural DHFR, underscoring the selectivity of both reactions. Thus, in this setting, DIFO demonstrates similar sensitivity for azide detection to Cu-catalyzed click chemistry, in stark contrast to all reported phosphine and cyclooctyne reagents (15).

Comparative Labeling of DIFO with Other Reagents for Bioorthogonal Ligation of Azido Glycans on Live Cell Surfaces. We next compared DIFO to other azide-specific reagents in the context of live cell labeling. Jurkat cells bearing azido sialic acid (SiaNAz) residues within their cell-surface glycans (19) were labeled with biotinylated derivatives of DIFO (DIFO-biotin, Fig. 1*D*), a phosphine (20), or a nonfluorinated cyclooctyne (14). As quantified by flow cytometry, labeling with DIFO-biotin was a factor of 20 greater than that observed with any other compound tested (Fig. 2*C*) and was detected at nanomolar concentrations of the DIFO-biotin conjugate (Fig. 2*D*). At all concentrations, DIFO-based probes showed no cellular toxicity as assessed by morphology and propidium iodide staining (SI Fig. 11). Thus, DIFO combines the high sensitivity of Cu-catalyzed click chemistry with the biocompatibility of the Staudinger ligation and the strain-promoted cycloaddition.

Time-Lapse Imaging of Cell-Surface Glycans in Live Cells. Although glycans, lipids, and other posttranslational modifications are key players in dynamic biological processes, all previous applications of bioorthogonal reactions to their *in vivo* labeling have been restricted to static images. We therefore sought to evaluate the ability of Cu-free click chemistry to image rapid biochemical changes in living cells. To this end, we were drawn to glycosylation, a notoriously complex and dynamic posttranslational modification (21). Cell-surface glycans are known to continuously recycle to the Golgi apparatus and the lysosome for enzymatic processing. However, various studies have reported conflicting kinetics of glycan endocytosis and relative organelle

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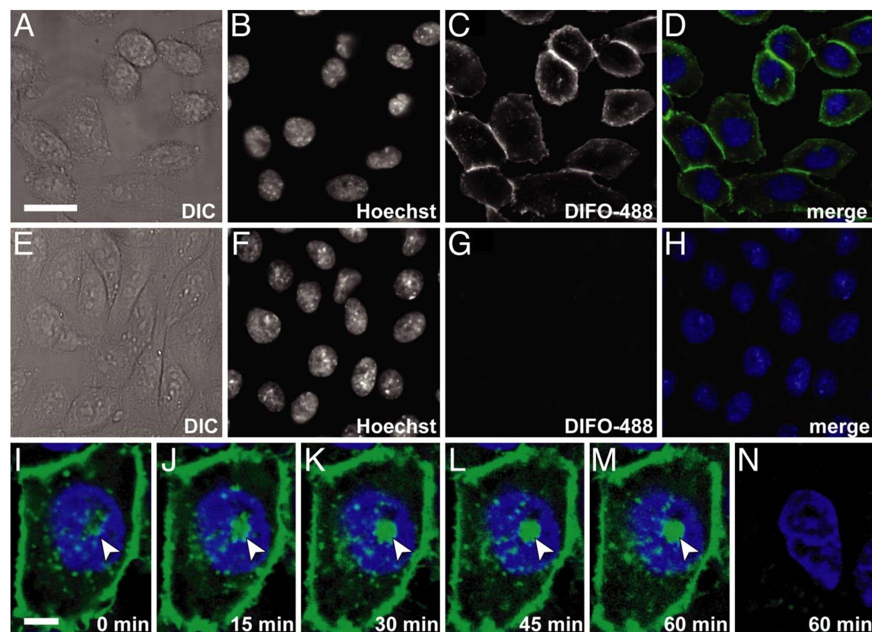

Fig. 3. Time-lapse imaging of glycan trafficking using an Alexa Fluor 488 derivative of DIFO. (*A–H*) CHO cells were incubated with 100 μM $Ac_4ManNAz$ (*A–D*) or 100 μM $Ac_4ManNAc$ as a negative control (*E–H*) for 3 days and subsequently labeled with 100 μM DIFO-488 at 37°C for 1 min. (*I–N*) Time-lapse imaging of a single cell from the previous experiment over 1 h at 25°C (*I–M*, $Ac_4ManNAz$; *N*, $Ac_4ManNAc$).

partitioning, possibly because of the use of indirect analytical tools or different cell lines (22–24).

To achieve dynamic imaging with Cu-free click chemistry, detectable labeling of glycans with fluorescent DIFO probes must occur on the minute time scale. We incubated Chinese hamster ovary (CHO) cells bearing SiaNAz residues within their cell-surface glycans (19) with DIFO-488 for various periods of time and at various concentrations. After only 1 min of labeling, the azide-bearing cell-surface glycans were readily imaged by using DIFO-488, with negligible background fluorescence (Fig. 3 *A–H* and SI Fig. 11).

We then performed time-lapse imaging to monitor the trafficking of the labeled cell-surface glycans. Within the first hour, we observed the appearance of a strong, punctate intracellular fluorescence signal that colocalized with transferrin uptake to endosomes (25) and a Golgi marker (Fig. 3 *I–N* and SI Fig. 11). This signal's intensity increased over the first 30 min and then reached saturation, suggesting a half-life of ≈15 min for internalization of a subset of cell-surface sialoglycoconjugates.

Dynamic Multicolor Imaging of Glycan Internalization and Trafficking. To monitor longer-term glycan trafficking patterns, we used a two-color, time-resolved labeling experiment with DIFO-488 and a red-shifted analog, DIFO-568. CHO cells bearing SiaNAz residues were labeled with DIFO-488 over 1 h, washed, incubated for 23 h with peracetylated *N*-azidoacetylmannosamine ($Ac_4ManNAz$) (19) to install additional SiaNAz residues, and then labeled a second time with DIFO-568. The DIFO-488 fluorescence was identical to the labeling pattern observed in the 1-h time-lapse imaging experiment (Fig. 4 *A* and *B*). However, after 24 h, the DIFO-488-labeled population had trafficked almost entirely to the lysosomes, whereas the more recently synthesized DIFO-568-labeled population mirrored the subcellular distribution of the 1-h labeling experiment (Fig. 4 *C–E* and SI Fig. 11). These results (*i*) suggest that the labeling protocol does not significantly perturb glycan trafficking on the time scale of the experiment and (*ii*) provide a platform for evaluating the kinetics of glycan internalization and subcellular partitioning to the endosomal, Golgi, and lysosomal compartments on the minute, hour, and day time scales.

Conclusion

The portability of azide labeling and Cu-free click chemistry should enable applications in many areas of glycobiology. For example, direct imaging of glycan trafficking under conditions of cell stimulation or pharmacological intervention can be performed in cells, tissues, or even whole organisms. Indeed, as a preliminary step toward this goal, we demonstrated that Cu-free click chemistry using DIFO proceeds selectively inside a living mouse (SI Fig. 12). More broadly, other metabolites, posttranslational modifications (5), enzyme activities (26), and site-specifically labeled proteins (4, 27) can be monitored in real time and in living systems with Cu-free click chemistry.

Materials and Methods

Chemical Synthesis. Details describing the synthesis of all compounds can be found in *SI Materials and Methods* and SI Schemes 1–3.

Kinetic Evaluation of [3 + 2] Cycloaddition Between DIFO and Benzyl Azide. Kinetic evaluation was monitored by ^{1}H-NMR as described (14). Details can be found in *SI Materials and Methods* and SI Fig. 9.

Comparative Labeling of Azido Proteins by Cu-Free and Cu-Catalyzed Click Chemistries. Recombinant murine DHFR was expressed with (azido-DHFR) or without (DHFR) replacement of its methionine residues by azidohomoalanine as described (18), and stock solutions were normalized to 0.1 mg/ml in PBS in 1% SDS. Stock solutions were made of DIFO-488 and alk-488 in PBS (1 mM), $CuSO_4$ in water (20 mM), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in water (20 mM), and Tris-triazolyl ligand (TBTA) (28) (1.7 mM in 4:1 *t*-butanol: DMSO). Reactions were performed in 10-μl volumes at final concentrations of the following reagents: 1 μg/ml azido-DHFR or DHFR, 25 μM DIFO-488 or alk-488, 0.04% SDS, and 1× PBS, pH 7.4. In addition, in accordance with conditions optimized by Speers and

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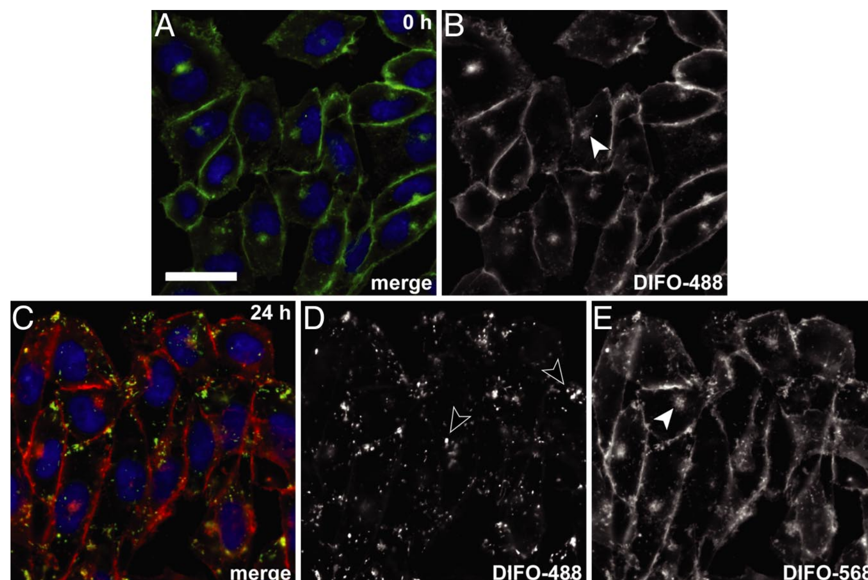

Fig. 4. Multicolor, dynamic imaging of glycan trafficking using Alexa Fluor derivatives of DIFO. CHO cells were grown for 2 days in 100 μM Ac_4ManNAz (*A–E*) or 100 μM Ac_4ManNAc (data not shown) and labeled for 1 h at 37°C with 10 μM DIFO-488 (t = 0 h, *A* and *B*). The cells were returned to medium supplemented with the appropriate sugar for 23 h and then labeled for 1 h at 37°C with 10 μM DIFO-568 (t = 24 h, *C–E*). Labeling in the Golgi apparatus and endosomes (filled arrowheads) and lysosomes (open arrowheads) was confirmed in colocalization experiments with known markers (SI Fig. 9).

Cravatt (26), the Cu-catalyzed reactions with alk-488 contained $CuSO_4$ (1 mM), TCEP (1 mM), and TBTA (100 μM), such that the final concentrations of *t*-butanol and DMSO were 4.8% and 1.2%, respectively. The order of addition was as follows: (*i*) Cu-free click chemistry, PBS, azido-DHFR or DHFR, and DIFO-488 and (*ii*) Cu-catalyzed click chemistry, PBS, azido-DHFR or DHFR, alk-488, TCEP, ligand, and $CuSO_4$. Reactions were started by addition of the final reagent, briefly vortexed, and allowed to sit in the dark at room temperature for 0–60 min. Reactions were quenched by the sequential addition of 1 μl of a solution of 2-azidoethanol in water (500 mM) and 11 μl of a solution of urea in water (8 M) for final concentrations of 23 mM and 4 M, respectively, followed by brief vortexing and an additional hour in the dark at room temperature. (For t = 0 min, the 2-azidoethanol and urea were added to the reaction mixture after the protein was added, followed by brief vortexing, and before the other reagents.) SDS protein loading buffer containing 2-mercaptoethanol (4×, 7.3 μl) was added, and the samples were heated at 100°C for 10 min and loaded onto 12% Bis-Tris Criterion polyacrylamide gel (Bio-Rad, Hercules, CA). After electrophoresis, gels were rinsed in a destain solution (5:4:1 water/methanol/acetic acid) overnight (in the dark), and fluorescence intensities were measured by using a Typhoon 9410 (GE Healthcare). Equal protein loading was confirmed by using silver stain (Bio-Rad) as shown in SI Fig. 10.

Cell Culture. Jurkat (human T cell lymphoma) and CHO cells were maintained in a 5% CO_2, water-saturated atmosphere and grown in RPMI medium 1640 (Jurkat) or F12 (CHO) medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cell densities were maintained between 1×10^5 and 1.6×10^6 cells per ml.

Cell-Surface Labeling of Azido Glycans on Jurkat Cells with Biotinylated Conjugates. Jurkat cells were incubated for 1–3 days in untreated medium or medium containing 25 μM Ac_4ManNAz. The cells then were distributed into a 96-well V-bottom tissue culture plate, pelleted (2,500 × g, 3 min), and washed twice with 200 μl of labeling buffer (PBS, pH 7.4, containing 1% FCS). Cells then were incubated with DIFO-biotin or a similar biotinylated analog of a nonfluorinated cyclooctyne (14) or a triaryl phosphine capable of Staudinger ligation (20) in labeling buffer for 1 h at room temperature at various concentrations (10 nM to 100 μM) with dilutions made from a 2.5 mM stock in 7:3 PBS/dimethylformamide. After incubation, cells were pelleted, washed twice with labeling buffer, and resuspended in the same buffer containing FITC-avidin (1:500 dilution of the Sigma stock). After a 15-min incubation on ice (in the dark), the cells were washed once with 200 μl of labeling buffer, incubated with FITC-avidin for an additional 15 min on ice, washed twice with 200 μl of cold labeling buffer, and then diluted to a volume of 400 μl for flow cytometry analysis.

Cell-Surface Labeling of Azido Glycans on CHO Cells and Imaging by Fluorescence Microscopy. CHO cells were incubated for 2 days in medium containing 100 μM Ac_4ManNAz or peracetylated *N*-acetylmannosamine (Ac_4ManNAc) in an eight-well LabTek II chambered cover glass (Nunc). The medium was gently aspirated, and the cells were washed three times with 600 μl of complete medium. The cells then were treated with a solution of DIFO-488, DIFO-568, or DIFO-647, diluted from a 1 mM stock solution in PBS (pH 7.4), in medium for varying times at varying concentrations and temperatures (see SI Fig. 11 for details). For long time-course labeling studies, the cells were washed with 600 μl of medium three times after the labeling reaction and returned to medium containing 100 μM Ac_4ManNAz or Ac_4ManNAc until the next labeling reaction. Immediately before imaging, the cells were treated with Hoechst 33342 dye to stain the nucleus (1:1,000 dilution in medium of a 1 mg/ml stock solution in DMSO) for 1 min at room temperature, washed twice with 600 μl of medium, and imaged. Optimized conditions for exclusive cell-surface labeling were (*i*) 100 μM DIFO-488 for 1 min in medium prewarmed to 37°C or (*ii*) 100 μM DIFO-488 for 1 h in medium at 4°C. Optimized conditions for cell-surface and Golgi/endosomal labeling were 10 μM DIFO-488 for 1 h in medium at 37°C. For experiments with intracellular organelle markers for lysosomes (LysoTracker Red; Invitrogen, Carlsbad, CA) and Golgi apparatus (BODIPY TR ceramide, Invitrogen), instructions provided by the manufacturer were used. For ex-

periments with transferrin, cells were labeled with 50 μg/ml Alexa Fluor 647-conjugated human transferrin (Invitrogen) for 30 min at 37°C and washed twice with 600 μl of medium. Propidium iodide, used to stain for cell viability, was applied (1:3,000 dilution in medium of a 1 mg/ml stock solution in water for 3 min at room temperature) immediately before imaging, after application of Hoechst dye; the cells then were washed twice with 600 μl of medium and imaged.

***In Vivo* Labeling of Azido Glycans in Mice.** Azido sugar labeling experiments and *in vivo* bioorthogonal ligation using FLAG conjugates were performed essentially as described in ref. 6. Details can be found in *SI Materials and Methods* and SI Fig. 12.

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