Point of VIEW

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# Click Imaging of Biochemical Processes in Living Systems

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**ABSTRACT** Understanding the function of biomolecules is crucially dependent on observing the dynamics of their biosynthesis, distribution, and metabolism in their native environment at the cellular and organismal levels rather than their behavior in the isolated samples. Small bioorthogonal functional groups that cause minimal, if any, perturbations of the native structure and function of the target molecule under physiological conditions and react selectively with an appropriately derivatized probe could function as chemical handles that allow selective visualization of the target molecule in the complex biological milieu. Small and generally unreactive, organic azides are ideally suited for this task: they can be carried unnoticed through multiple biosynthetic steps only to be revealed, when desired, by action of a suitably derivatized visualization label.

Chemists and biologists have learned reasonably well to study even the most complex biological molecules when they are isolated, but how could one get a molecular-level glimpse of the chemical processes as they unfold in living systems? After all, understanding the function of specific proteins, glyco- and lipo-conjugates, and their metabolites requires observing the dynamics of their biosynthesis, distribution, and metabolism in their native environment (i.e., in as close to real-time living conditions as possible) rather than their behavior in the isolated (read dead) samples. Clearly, dissecting the intricate chemical networks of processes that occur in living organisms—or at least living cells—is not a trivial task. Enter bioorthogonal reporters. These are small and biologically inert functional groups that are incorporated in the target biomolecule at the genetic or post-translational level by the cell's native biochemical pathways. They remain invisible and can be carried unnoticed through multiple biosynthetic steps until they are revealed *via* a selective chemical transformation. Because of their stability and very narrow reactivity profiles, organic azides are perhaps best suited to function as small chemical reporters. In a recent paper, Bertozzi and coworkers (*1*) describe a new reagent, 3,3-difluorocyclooctyne (DIFO), for addressing organic azides metabolically incorporated in the cell surface glycans. DIFO is stable to biological nucleophiles and reacts with organic azides rapidly, irreversibly, and selectively without a catalyst.

Although methods for introduction of biopolymer-based reporter tags (GFP is a prime example) into proteins at the genetic level have been developed and used prolifically for cellular imaging and protein tracking, the repertoire of chemical transformations that allow selective labeling of proteins, nucleic acids, and glyco- and lipo-conjugates with small-molecule-based tags has remained quite limited. This is not surprising given the aqueous milieu and the diversity of chemical functionality present in living organisms. To be useful for studies of biological systems at the molecular level, functional groups used for tagging (i) have to be small and readily incorporated into the target of interest without significantly perturbing its function; (ii) have to remain "invisible" to the cellular biochemical machinery and unreactive with water and ubiquitously present endogenous nucleophiles, such as amines and thiols; and (iii) should be addressable in a rapid and selective fashion so they can be revealed, when required, by using a selective and irreversible chemical reaction with a visualization label containing a complementary reactive center. Organic azides, because of their uniquely narrow reactivity profiles, are among the most useful chemical reporters.

Although they have been used in synthesis for >100 years, the utility of organic azides has been often limited to the facile introduction of the amino group into organic molecules. Other facets of their unique reactivity, especially in biological environments, remained largely unexplored until relatively

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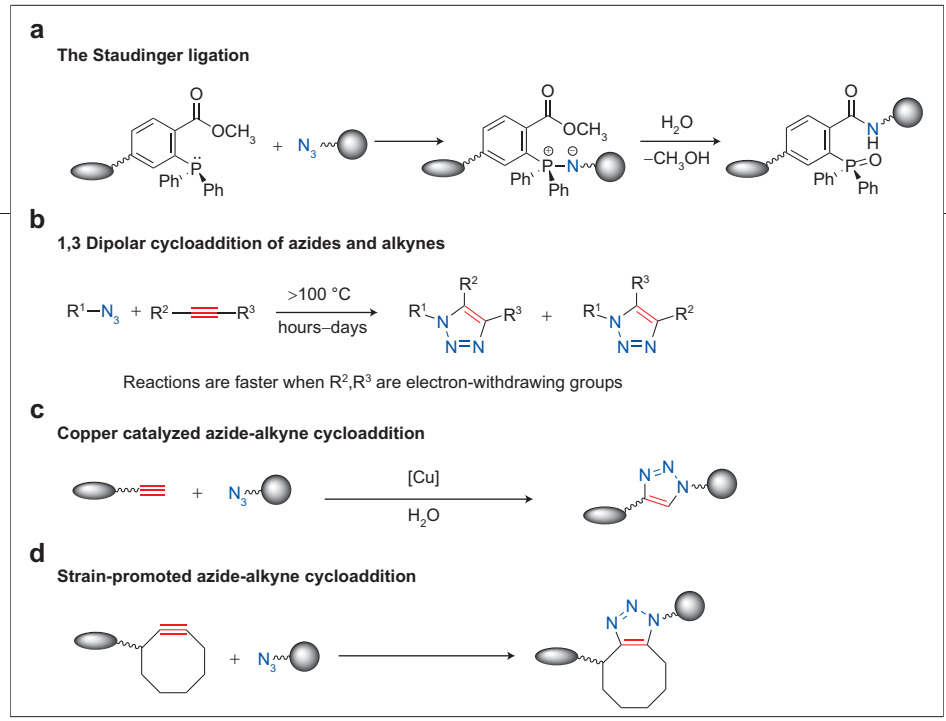


**Figure 1. Chemical transformations used to address azides in biological systems. a) Modified Staudinger ligation takes advantage of the selective reactivity of phosphines with azides. The reaction has been used prolifically in labeling of glycans and proteins, both *in vitro* and *in vivo*. However, it requires a large excess of phosphine to attain useful reaction rates, and phosphine reagents are prone to oxidation. b–d) 1,3-Dipolar cycloaddition of azides and alkynes. Although exquisitely selective, the thermal reaction (b) is very slow and cannot be used in bioconjugations. c) CuAAC is very selective and efficient, but it requires addition of a copper catalyst, which may not be compatible with live cells. d) Strain-promoted cycloaddition does not require a catalyst, but its rate is not sufficient for tracking fast biological processes.**

recent years. In the pioneering work on the development of small-molecule chemical reporters, Bertozzi recognized their nearly bioorthogonal properties and the ease of introduction into the biological molecules and elegantly exploited their reaction with phosphines (the modified Staudinger ligation, Figure 1, panel a) in metabolic oligosaccharide engineering studies (*2, 3*). Around the same time, Sharpless and colleagues (*4*) defined "click chemistry" as a set of "near-perfect" bond-forming reactions useful for rapid assembly of molecules with desired function. These transformations are easy to perform, give rise to their intended products in very high yields with little or no byproducts, work well under many conditions (usually especially well in water), and are unaffected by the nature of the groups being connected to each other. The "click" moniker is meant to signify that with the use of these methods, joining molecular fragments is as easy as "clicking" together the two pieces of a buckle. The buckle works no matter what is attached to it, as long as its two pieces can reach each other, and the components of the buckle can make a connection only with each other. The potential of organic azides as highly energetic yet very selective functional groups in organic synthesis was highlighted, and their dipolar cycloaddition with alkynes was placed among the reactions fulfilling the "click" criteria.

The fundamental thermal reaction, involving terminal or internal alkynes (Figure 1, panel b, 1,3-dipolar azide-alkyne cycloaddition, AAC), has been known for more than a century (the first 1,2,3-triazole was synthesized by Michael from phenyl azide and diethyl acetylene-dicarboxylate in 1893) (*5*) and has been most thoroughly investigated by Huisgen and others in the 1950s–1970s in the course of their studies of the family of 1,3-dipolar cycloaddition reactions (*6, 7*). The process is strongly thermodynamically favored ($\Delta H^o = -45$to $-55$ kcal/mol) because of the high-energy nature of the two reaction components, but it has a relatively high kinetic barrier (~26 kcal/mol for methyl azide and propyne (*8*)), thus rendering the reaction very slow at RT for unactivated reactants. Copper catalysts (Figure 1, panel c) accelerate the reaction of azides with terminal alkynes (*9, 10*) by $\sim 10^7$ relative to the uncatalyzed version (*8*), making it conveniently fast at and even below RT. The reaction is unaffected by water and by most organic and inorganic functional groups, therefore all but eliminating the need for protecting group chemistry (*11*). The 1,2,3-triazole heterocycle has the advantageous properties of high chemical stability (in general, being inert to severe hydrolytic, oxidizing, and reducing conditions, even at high temperature), strong dipole moment (4.8–5.6 D), aromatic character, and hydrogen bond accepting ability (*12, 13*). Thus, it can interact productively in several ways with biological molecules and can serve as a hydrolytically stable replacement for the amide linkage. Compatibility of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction with a broad range of functional groups and reaction conditions (*14–17*) has made it broadly useful across the chemical disciplines, and its applications include the synthesis of biologically active compounds, the preparation of conjugates to proteins and polynucleotides, the synthesis of dyes, the elaboration of known polymers and the synthesis of new ones, the creation of responsive materials, and the covalent attachment of desired structures to surfaces (*18–22*). CuAAC has become the most widely used click reaction discovered so far (unsurprisingly, it is often called "click chemistry", whereas it is in fact only a representative of the "click" family of reactions).

Although CuAAC has been used in a range of bioconjugation applications for modification of virus particles (*14*) and whole *Escherichia coli* cells (*15, 16, 23*) and labeling of proteins from murine tissue

lysates (*17, 24*), it has not been employed directly in living cells because of the cytotoxicity and attendant bioregulation of copper. In 2004, Bertozzi and coworkers demonstrated the utility of cyclooctyne-containing probes (Figure 1, panel d) in catalyst-free bioconjugations (*25*). Cyclooctyne is sufficiently stable but is known to react with azides rapidly because of the ring strain (*26*). However, the rate of the reactions of cyclooctyne probes with azides is similar to that of the Staudinger ligation and is lower than the rate of CuAAC (*27*) to make it useful for studies of fast processes. Now, the same researchers boosted the reactivity of the cyclooctyne by incorporating two strongly electron-withdrawing fluorine atoms adjacent to the triple bond. As reported in the Bertozzi paper (*1*), the reactions of DIFO probes with aliphatic azides were 17–63-fold faster than the corresponding Staudinger ligations, allowing efficient labeling of the azide-containing proteins in $<1$ h ([DIFO] $= 25\ \mu$M).

The authors then turned to labeling of live mammalian cells that contained azidosialic acid residues in their cell surface glycans (Figure 2). The cell's own biosynthetic pathways are used to display azidosialosides at the cell surface using the now well-established metabolic engineering procedure, whereupon cells are fed a suitably labeled azidosugar precursor, such as peracetylated *N*-azidoacetyl mannosamine (*28*). Short of the synthesis of the DIFO label itself, the subsequent labeling protocol developed by Bertozzi and her team is experimentally simple and involves incubation of azide-labeled cells with 25–100 $\mu$M of the biotinylated DIFO label for 1 h. Flow cytometry analysis indicated excellent labeling efficiency, superior to both Staudinger ligation with phosphine probes and nonfluorinated cyclooctyne labels. Furthermore, the DIFO-based labels also performed well in the time-lapse imaging studies of glycans trafficking inside the cells, easily the most demanding test of this new labeling methodology. To be useful for probing fast biological processes, such as trafficking of glycans between cell membrane and intracellular compartments, the labeling reaction has to be exceptionally selective, biochemically benign, and fast. DIFO-based reagents appear to meet these criteria, acting as stealthy, yet exquisitely reactive probes ca-

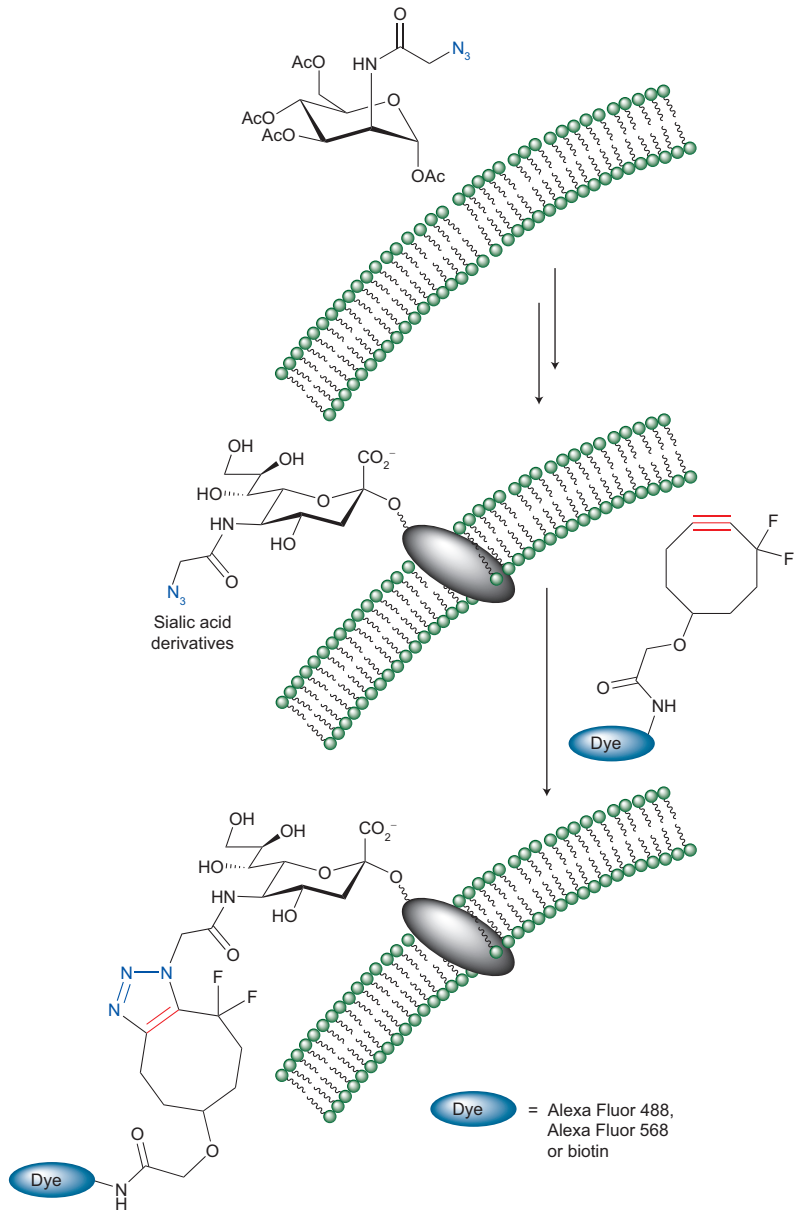


**Figure 2. DIFO probes incorporate two activating features in the alkyne component, ring strain and electron-withdrawing fluorine substituents, making them much more reactive than their nonfluorinated parent cyclooctyne and allowing fast labeling of cell surface glycans containing azide groups.**

pable of tracking the fate of azide-containing conjugates. After incubation of the cells with Alexa Fluor 488-DIFO conjugate for only 1 min, sufficient incorporation of the label occurred and surface glycans could be visualized by fluorescence microscopy. Images taken at 15 min intervals revealed the subtleties of the glycan transport from the cell membrane to the intracellular compartments, showing that internalization of these particular cell-surface messengers was complete in ~30 min.

The new bioconjugation protocol devised by Bertozzi and coworkers is an impressive addition to the still small family of chemical transformations that can be used to selectively address the azide functionality in biological systems. DIFO-based labeling is noticeably more efficient than the widely used Staudinger ligation and is devoid of the problems associated with phosphine labels. It also does not require a copper catalyst, which may limit the utility of the CuAAC when following the dynamics of biomolecules in the living cells and whole organisms by a covalent attachment of a trackable label is the goal. Clearly, it is not limited to studies of glycans and should find immediate use in bioconjugation experiments involving proteins, nucleic acids, lipoconjugates, and their metabolites.

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