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Chemical reporters for biological discovery

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Abstract

Functional tools are needed to understand complex biological systems. Here we review how chemical reporters in conjunction with bioorthogonal labeling methods can be used to image and retrieve nucleic acids, proteins, glycans, lipids and other metabolites *in vitro*, in cells as well as in whole organisms. By tagging these biomolecules, researchers can now monitor their dynamics in living systems and discover specific substrates of cellular pathways. These advances in chemical biology are thus providing important tools to characterize biological pathways and are poised to facilitate our understanding of human diseases.

Introduction

The genomic revolution has offered unprecedented opportunities to understand basic biology and human disease. With increasingly high-throughput and cost-effective sequencing methods, the genomes of many organisms and mutations associated with human diseases have been annotated. In parallel, large-scale gene deletion and overexpression studies as well as protein-protein interaction screens have uncovered complex signaling networks. These studies have not only revealed the genetic basis for many fundamental biological processes but have also presented new possibilities for personalized medicine. Nonetheless, significant challenges lay ahead for translating genetically encoded information into biological function and therapeutic development. At the nucleic acid level, many biological phenotypes are controlled by multiple genes that may not be readily apparent by perturbation of individual genes or alleles. Furthermore, the phenotype of interest may only be transient or controlled by epigenetic mechanisms via chromatin modifications or small RNAs not evident by reading the genetic code. To complicate matters further, the genomes of most organisms encode protein families with overlapping biochemical activities, which are often spatially and temporally regulated by specific interactions with other factors inside of cells or have cell-type specific functions in animals. Beyond nucleic acids and proteins, glycans, lipids and other small molecule cofactors are also important regulators of cellular function because they modify specific substrates that are often directly coupled to metabolism. Biological signaling pathways are therefore often not linear and typically comprise more complex positive and negative feedback networks to amplify or shutdown specific phenotypes. This biological complexity demands a better understanding of the chemistry inside cells and animals so we can decode their genomes and dissect their interactions with the environment. As new technologies have accelerated genomics, here we highlight advances in bioorthogonal chemical reporters that have enabled

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the imaging and functional analysis of nucleic acids, proteins, glycans, lipids and other metabolites in biology.

The advances in bioorthogonal chemistry have inspired a series of azide-, alkyne- and alkene-functionalized metabolites as chemical reporters for nucleic acids, proteins, glycans, lipids as well as other biomolecules (Box 1, Fig. 1)^{1,2}. Key to this bioorthogonal labeling strategy is the ability of native or engineered enzymes to accept these functionalized chemical reporters. This two-step approach allows the visualization and biochemical analysis of endogenous biomolecules that provides several important advantages for biological studies. First, the separation of metabolic or enzymatic incorporation and detection decouples sterically-demanding fluorophores or affinity tags that often interfere with biological activity from chemical reporters. Second, in comparison to protein-based reagents (e.g. antibodies), chemical reporters provide direct detection of the modified substrates and are relatively independent of adjacent functional groups, which may confound the specificity of protein reagents. Lastly, the ability to pulse-label specific populations of molecules in cells with chemical reporters provides an important means to monitor dynamics akin to classic radioactive tracers, but enables more sensitive detection and allows the direct identification of labeled targets. Since newly synthesized molecules can be separated from the steady state population in this manner, chemical reporters are excellent tools for monitoring the dynamics of biomolecules. The diversity of modifications on proteins and nucleic acids makes it difficult to track specific subsets independently. Therefore, specific chemical reporters provide important reagents to isolate distinct populations of biomolecules away from the vast chemical complexity of the cell. Bioorthogonal chemistry has also facilitated the analysis of small molecule interactions with proteins for biochemical target identification and activity-based protein profiling of chemical probes, which is beyond the scope of this review and is summarized elsewhere³. Herein we review the currently available chemical reporters and highlight how they can be applied to monitor temporal changes in cellular activity (Fig. 2a), profile different cell types, states or mutations (Fig. 2b) and identify targets of specific enzymes (Fig. 2c). We close with an outlook on the future challenges and developments that should expand the utility of bioorthogonal chemical reporters to even more complex biological settings *in vivo*.

Chemical reporters for nucleic acids

Gene expression arrays and deep-sequencing methods provide important profiles of steady state DNA and RNA expression, but understanding the rate of nucleic acid synthesis, degradation and modifications is crucial for elucidating their function in different biological contexts. The modification of nucleosides with alkynes has afforded chemical reporters of DNA and RNA synthesis. For example, 5-ethynyl-2'-deoxyuridine (EdU)⁴ and 5-ethynyluridine (EU)⁵ can be metabolized by mammalian cells and incorporated into DNA and RNA for bioorthogonal detection and pulse-chase experiments, respectively (Fig. 3a,b). These nucleic acid reporters function in animals and provide sensitive reagents to compare DNA and RNA turnover in different tissues using fluorescence. A fluorinated arabinosyl-EdU reporter has also been developed to improve the sensitivity of DNA labeling with reduced cytotoxicity compared to EdU (Fig. 3c)⁶. In addition to uridine derivatives, 5-ethynyl-2'-deoxycytosine (EdC)⁷ and N-6-propargyladenosine (N6pA)⁸ can be utilized by mammalian cells to label DNA and RNA, respectively (Fig. 3d,e). N6pA labeling also allows the pulse-chase analysis of RNA polyadenylation⁸. More recently, Raman spectroscopy has been employed to directly image EdU labeling of DNA in mammalian cells by taking advantage of the unique vibrational signature of alkynes⁹. To analyze nucleic acid modifications, a specific glycosyltransferase can be used to install 6-azido-glucose onto 5-hydroxymethylcytosine (5-hmc) marks in DNA for subsequent bioorthogonal detection (Fig. 3f)¹⁰. This chemoenzymatic detection of 5-hmc has provided a particularly important

method for characterizing the mechanism of DNA oxidation and demethylation catalyzed by Tet1–3 enzymes in eukaryotic cells^{11,12}. These studies highlight the utility of chemical reporters for analyzing nucleic acid turnover and modifications that should facilitate future studies on epigenetic mechanisms.

Chemical reporters for protein turnover

Azide- and alkyne-bearing amino acids allow the co-translational labeling of newly synthesized proteins and provide a direct measure of the translational activity in cells (Fig. 4). Two strategies for non-canonical amino acid incorporation have been developed that enable site-specific or residue-selective labeling of proteins. The site-specific approach allows the incorporation of a non-canonical amino acid at a predetermined position in a protein of interest using amber suppression¹³. This method is based on engineering an orthogonal triad of tRNA, aminoacyl-tRNA-synthetase (aaRS) and non-canonical amino acid, which allows the site-specific installation of designer amino acids on individual proteins in bacteria, yeast and mammalian cells¹³ as well as whole animals such as worms¹⁴ and flies¹⁵. For example, the pyrrolysine (Pyl) system has enabled site-specific incorporation of amino acid reporters such as *trans*-cyclooctene-Lys or BCN-Lys into mammalian cells for fluorescence imaging of proteins after Diels-Alder ligation with tetrazine-functionalized dyes in living cells (Fig. 4a)¹⁶. Alternatively, azide and alkyne amino acids such as azidohomoalanine (AHA) and homopropargylglycine (HPG) can replace methionine (Met) for residue-selective incorporation into newly synthesized proteins (Fig. 4b)¹⁷. AHA and HPG are readily taken up and utilized by the wild type methionyl-tRNA-synthetase (metRS) in prokaryotic and eukaryotic cells, enabling the analysis of newly synthesized proteins by bioorthogonal labeling with fluorescence and affinity probes¹⁷. The availability of two uniquely reactive orientations (azide versus alkyne) also makes it possible to label and visualize two distinct protein populations with AHA and HPG, respectively¹⁷. In contrast to metabolic labeling with isotopically labeled amino acids, AHA and HPG labeled proteins can be bioorthogonally reacted with affinity tags and selectively recovered from total cell lysates for mass spectrometry-based protein identification. In addition, O-propargyl-puromycin (OP-puro) (Fig. 4c), an alkyne analog of the covalent translational inhibitor puromycin, can be used to label nascent polypeptide chains in mammalian cell culture as well as in mice¹⁸. In contrast to chemical reporters that are based on amino acid frequency, one OP-puro is attached to each nascent polypeptide, which could facilitate comparative studies of protein synthesis rates in the future.

The ability to differentiate newly synthesized proteins from pre-existing proteins with AHA or HPG has already begun to reveal important biological insights. For example, AHA pulse labeling can be used to enrich newly synthesized nucleosomes¹⁹. Nucleosome turnover potentially erases histone marks and serves as an important epigenetic mechanism. However, previous studies measured histone H3.3 incorporation as a surrogate for nucleosome turnover or used overexpressed histone transgenes to follow nucleosome dynamics. In contrast, AHA allows pulse labeling of endogenous histones and provides an affinity handle for enrichment and subsequent analysis of nucleosome-associated DNA¹⁹. This approach, termed CATCH-IT, provides better temporal resolution compared to previous histone replacement methods and has revealed more rapid turnover of nucleosomes at active genes, specific epigenetic marks, and replication origins¹⁹. AHA labeling also provides a useful means to differentiate secreted proteins from abundant proteins in serum, which in combination with stable isotope labeling with amino acids in cell culture (SILAC) has enabled quantitative analysis of mammalian secretomes²⁰. The union of AHA labeling and SILAC has also facilitated the global analysis of newly synthesized proteins during T cell activation²¹. Spatial and dynamic regulation of protein synthesis is also particularly important in neurons, as localized translation is believed to shape axon growth, guidance and

regeneration in the brain for synaptic plasticity and memory. To explore local translation in neurons, AHA and HPG labeling can be used to visualize specific sites and measure rates of protein synthesis in primary neurons after bioorthogonal labeling with fluorescent dyes and quantum dots²². Another application of AHA labeling in primary neuronal cultures revealed localized protein synthesis adjacent to the transmembrane receptor DCC²³. These studies show how bioorthogonal amino acid reporters provide valuable tools for monitoring discrete protein populations and imaging protein synthesis at high resolution.

Cell-selective labeling of proteomes

Studying cells in their native environment and interaction with other cell types is crucial in many areas of biology. Methods for cell-selective proteome labeling would therefore be valuable for dissecting the activity of individual cells in heterogeneous populations. While AHA and HPG do not provide a means to separate proteomes of individual cell types, metRS mutants (metRS*) can facilitate the incorporation of the otherwise translationally inactive amino acid reporters such as azidonorleucine (ANL)^{17,24}. Since ANL incorporation is dependent on metRS* activity, ANL can be selectively directed to certain cell types by expression of metRS*²⁴. For example, bacterial cells expressing metRS* can be selectively labeled with ANL in the presence of mammalian cells²⁴. To differentiate bacterial proteins from host proteins during infection, 2-aminooctynoic acid (AOA), can also be activated by metRS* to selectively label the Gram-negative bacterial pathogen *Salmonella typhimurium* inside macrophages²⁵. Notably, AOA enabled the proteomic analysis of *S. typhimurium* proteins that are differentially expressed during infection of host cells²⁵. In addition to these Met reporters, an alkyne phenylalanine (Phe) analog, *para*-ethynylphenylalanine (PEP) can also function as a cell-selective amino acid reporter in combination with a phenylalanine-tRNA-synthetase mutant (A294G of *pheS*)²⁶. Amino acids such as Phe are present in higher frequencies than Met and the possible combination of these reporters may allow greater coverage of proteomes or even selective labeling of two different proteomes. These proteome selective labeling systems should facilitate the discovery and characterization of bacterial proteins that are uniquely expressed or secreted in mixed microbial communities like biofilms and host-microbe interactions. An extension of these strategies to eukaryotic systems might also allow selective labeling of individual cell types in multicellular organisms in the future.

Chemical reporters for posttranslational modifications

Chemical reporters have also been developed for diverse protein modifications in bacteria and eukaryotes. Since protein modifications can be sub-stoichiometric, dynamically regulated and further elaborated, these biomolecules can be especially difficult to analyze.

Glycosylation

The covalent attachment of glycans to proteins is one of the most ubiquitous and complex forms of protein modification in biology. Glycans decorate the exterior of eukaryotic cells and mediate a wide range of interactions with soluble extracellular factors, neighboring cells and microbes. Additionally, glycosylation plays an important role inside cells by regulating protein trafficking, turnover and signal transduction. Indeed, the complex roles of glycans and the challenges associated with their detection was a major driving force for the development of the Staudinger ligation (Fig. 1b) and the first azide-glycan reporter, N-azidoacetyl-D-mannosamine (ManNAz, Fig. 5a), for sialic acid biosynthesis²⁷. This landmark study has inspired the development of many other azide- and alkyne-functionalized chemical reporters, including monosaccharide reporters for mucin-type O-linked glycoproteins (Fig. 5b), fucosylated glycans (Fig. 5c), and intracellular O-GlcNAc modified proteins (Fig. 5d), which have improved the biochemical detection and imaging of

these glycans²⁸. The metabolic incorporation of chemical reporters into cell surface glycans can also be used to select cell populations with aberrant glycosylation levels²⁹. An early example of this strategy using a ketone-functionalized sialic acid precursor and hydrazide labeling revealed mutations in glycan biosynthetic enzymes associated with congenital human diseases such as sialuria²⁹. Furthermore, these glycan reporters in combination with activated cyclooctyne probes have now enabled the dynamic imaging of cell surface glycans in live developing zebrafish embryos and in worms³⁰, which opens the door for differential analysis of glycan biosynthesis in specific tissues and cell types *in vivo*. Glycan chemical reporters have also been incorporated into bacteria^{31–33} as well as plants³⁴ that should afford new tools for functional studies in these organisms.

Metabolic and chemoenzymatic chemical reporters are providing new insight into O-GlcNAc modification of cytoplasmic and nuclear proteins. O-GlcNAcylation of serine (Ser) and threonine (Thr) residues is dynamic and regulated form of intracellular glycosylation that is emerging as an important indicator of cellular metabolism and stress in diseases such as diabetes, neurodegeneration and cancer³⁵. Initial studies demonstrated that N-azidoacetyl-D-glucosamine (GlcNAz) could be utilized by the hexosamine salvage pathway and incorporated into O-GlcNAc modified proteins in mammalian cells³⁶. More recently, an alkyne-modified GlcNAc analog (GlcNAIk, Fig. 5d) has enabled more sensitive bioorthogonal detection as well as greater proteomic coverage of O-GlcNAcylation and revealed glycosylation of the ubiquitin ligases such as NEDD4³⁷. Alternatively, O-GlcNAc-modified proteins in tissue/cell lysates can also be detected using a chemoenzymatic method using a ketone or azide-bearing sugar nucleotide reporter in conjunction with an engineered β -1,4-galactosyltransferase *in vitro* (Fig. 5e)³⁸. The enzymatically tagged O-GlcNAc-modified proteins can be evaluated by fluorescence, mass-shift western blotting and quantitative proteomics after bioorthogonal labeling³⁸. This method has revealed both differential dynamics of O-GlcNAcylation in mammalian cells³⁹ and O-GlcNAc regulation of CREB-mediated gene expression in neurons⁴⁰. Azide- and alkyne-sugar nucleotide reporters have also been useful for *in vitro* profiling other glycosyltransferase substrate specificities⁴¹ as well as *in vivo* imaging of glycans⁴². These studies showcase the utility of chemical reporters for monitoring glycan biosynthesis, glycoproteomics and even imaging of glycans *in vivo*.

Lipidation

Lipid modifications of proteins modulate their membrane affinity, localization and trafficking for cell signaling⁴³. Even though some forms of protein lipidation can now be predicted based on conserved amino acid motifs⁴³, direct biochemical detection of lipidated proteins is still required for functional studies and discovery of unannotated substrates in different cell types and physiological states. As specific antibodies for lipid modifications have been difficult to generate, bioorthogonal chemical reporters have been especially valuable for the detection, discovery and characterization of lipidated proteins and their regulatory mechanisms⁴⁴.

Protein S-prenylation is a prominent form of lipidation in mammalian cells that encompasses S-farnesylation or geranylgeranylation of cysteine (Cys) residues on CaaX or CC motifs, respectively⁴³. These isoprenoid modifications typically occur at the C-terminus of proteins such as small GTPases and is crucial for their membrane targeting and activity in cells⁴³. To improve biochemical analysis of protein S-prenylation, azide⁴⁵ and alkyne^{46,47} isoprenoid reporters have been developed that allow more sensitive bioorthogonal detection and enrichment of S-prenylated proteins in mammalian cells. Indeed, proteomic analysis of alkynyl-farnesol (alk-FOH) (Fig. 6c or Fig. 6 – **is fine with me**) modified proteins in macrophages has enabled large-scale profiling of known and unpredicted S-prenylated

proteins and revealed that isoform-specific S-farnesylation is crucial for the membrane targeting and antiviral activity of zinc-finger antiviral protein (ZAP)⁴⁸.

Azide and alkyne fatty acids can also be used to monitor fatty-acylated substrates in bacteria and eukaryotes⁴⁴. Myristoylation of N-terminal glycine (Gly) residues by N-myristoyltransferases (NMTs) occurs primarily cotranslationally and is readily labeled by myristic acid reporters in mammalian cells (Fig. 6a)⁴³. Interestingly, the application of N-myristoylation reporter labeling has revealed a unique proteolytic activity of a secreted bacterial effector IpaJ on N-myristoylated host proteins that contributes to the virulence of bacterial pathogen *Shigella flexneri*⁴⁹. In contrast to N-terminal myristoylation, S-palmitoylation of Cys residues is not readily predicted by primary amino acid sequence, can be reversible, regulated by a family of palmitoyltransferases with the conserved Asp-His-His-Cys-motif (DHHC-PATs) and thus particularly challenging to analyze biochemically⁵⁰. Metabolic labeling with alkyne fatty acid reporters (Fig. 6b) in combination with bioorthogonal detection has largely solved this problem, enabling rapid visualization and proteomic analysis of S-palmitoylated proteins⁴⁴. Notably, bioorthogonal palmitoylome profiling has increased the diversity of S-palmitoylated proteins in mammalian cells^{51–54} and even revealed new fatty-acylated proteins such as IFITM3 in innate immunity⁵³. Fatty acid reporters have also been especially useful for monitoring protein S-palmitoylation dynamics. For example, tandem pulse-chase labeling of T cells with protein synthesis and palmitoylation reporters provides a robust method for quantifying S-acylation dynamics relative to protein turnover⁵⁵. The application of this method revealed accelerated depalmitoylation of signaling kinases such as Lck upon T cell activation⁵⁵. In addition, the large-scale analysis of S-palmitoylation dynamics using bioorthogonal labeling and SILAC showed that many proteins are stably fatty-acylated while other proteins involved in cell signaling undergo rapid S-acylation cycles⁵⁶. The application of fatty acid reporters to model systems such as *Schizosaccharomyces pombe* (fission yeast) has also afforded new opportunities to investigate S-palmitoylation mechanisms⁵⁷. Palmitoylome profiling of wild type *S. pombe* and DHHC-PAT deletion strains and additional functional studies has revealed that quantitative expression levels of a single DHHC-PAT can control S-palmitoylation of specific protein substrates and help drive major cellular transitions such as meiotic entry in fission yeast⁵⁷. In addition cytoplasmic proteins, secreted proteins are also lipid-modified and readily labeled by chemical reporters as highlighted by fatty acid and cholesterol reporter labeling of Wnt⁵⁸ and sonic hedgehog proteins (Fig. 6d)⁵⁹, respectively. *In vitro* biochemical studies and fatty acid reporter labeling experiments in mammalian cells has also shown that long chain-fatty acylation of Lys side chains can be removed by Sirt6, a sirtuin-family deacylase previously thought to function only acetylated Lys⁶⁰, and modulate the secretion of proinflammatory cytokines such as TNF- α . These chemical reporters can also be used monitoring the lipid metabolism and trafficking in mammalian cells^{61–64}. As lipids can be difficult to tag and purify, these challenges have motivated the development of substituted cyclopropanes as another small bioorthogonal tag⁶² and the application cobalt-affinity matrices for the capture and enrichment of alkynyl-lipids⁶³.

Lipid reporters also allow labeling of bacterial proteins⁶⁵. Protein lipidation in bacteria involves the installation of a diacylglycerol group onto N-terminal Cys residue within a well-defined lipobox-motif⁶⁶. Although algorithms are available for the prediction of canonical bacterial lipoproteins⁶⁶, many lipidated proteins in bacteria are poorly characterized and may exist in other forms. In this regard, the application of fatty acid reporters to bacteria allows both the rapid profiling of canonical bacterial lipoproteins as well as the discovery of unconventionally lipidated proteins in bacteria⁶⁵. These applications of bioorthogonal lipid reporters are thus revealing new fatty-acylated proteins and their mechanisms of regulation in variety important biological systems.

Acylation

The acylation of Lys residues plays important roles in epigenetic regulation of transcription, signaling and metabolism⁶⁷. With the increase of Lys acetylated proteins from recent proteomic studies in mammalian cells and bacteria⁶⁷, characterizing what substrates are regulated by specific Lys acetyltransferases (KATs) and deacetylases (KDACs) in various signaling pathways is a major challenge. In this regard, alkyne analogs of acetate and acetyl-CoA such as sodium 4-pentynoate and 4-pentynoyl-CoA can function as chemical reporters of Lys acetylation in cells and *in vitro*, respectively (Fig. 7a). Metabolic labeling of mammalian cells with 4-pentynoate readily labels known Lys acetylated proteins such as histones and allows simple fluorescence profiling of acetylated proteins in different cell types⁶⁸. In addition, 4-pentynoyl-CoA is utilized by the KAT p300 for *in vitro* bioorthogonal profiling of candidate protein substrates in cell lysates⁶⁹. As acetylation also occurs on the N-terminus of proteins⁷⁰ and Ser/Thr residues⁷¹, acetylation reporters should also facilitate the analysis of these modifications. Other forms of Lys acylation such as propionylation, butyrylation, malonylation, succinylation and crotonylation have also been recently described⁶⁷, which may also benefit from specific bioorthogonal reporters to characterize labeled proteins and activity of regulatory enzymes. Indeed, a chemical reporter for protein malonylation has been recently reported⁷².

Methylation

The modification of Lys or arginine (Arg) residues with one or more methyl groups are important posttranslational modifications in chromatin biology and other cellular pathways⁷³. The general cofactor for most cellular methylation reactions is S-adenosyl-L-methionine (SAM), from which the methyl group is transferred onto proteins, nucleic acids or other substrates. The development of bioorthogonal methylation reporters has therefore attracted considerable interest. Initial studies demonstrated that DNA methyltransferases were able to transfer allylic or propargylic groups from SAM reporters onto DNA substrates⁷⁴. AdoEnYn (Fig. 7b) was then developed as a SAM reporter for site-selective labeling of protein methyltransferase substrates such as histone H3⁷⁵. Lys methyltransferase mutants that can utilize (*E*)-hex-2-en-5-ynyl-SAM (Hey-SAM) have also been described for bioorthogonal profiling of protein methylation⁷⁶. In addition to Lys methyltransferases, a double mutant of protein Arg methyltransferase PRMT1 can utilize 4-propargyloxy-but-2-enyl-SAM (Pob-SAM) and label potential target proteins in cell lysates⁷⁷. Interestingly, the expression of engineered SAM synthetase and methyltransferase capable of utilizing an alkynyl-Met reporter in mammalian cells has allowed metabolic labeling of histones and subsequent click chemistry-based chromatin enrichment and parallel DNA sequencing⁷⁸. While thiol-based SAM reporters are functional, these reagents can be unstable and non-specifically label substrates. To address this limitation, a selenium-based SAM reporter has been developed that is more stable and can be readily utilized by both Lys and Arg methyltransferases *in vitro*^{79,80}. Improved methylation chemical reporters in combination with bioorthogonal enrichment methods should facilitate the identification of specific methyltransferase substrates in the future.

ADP-Ribosylation

Proteins can be modified with mono- and poly-ADP-ribose on a range of different residues⁸¹. This modification was initially described as the virulence mechanism for a number of different bacterial toxins, but it is now appreciated as a prevalent protein modification in eukaryotes as well⁸¹. Mono-ADP-ribosylation is carried out by a set of mono-ADP-ribosyltransferases (MARTs) that utilize the cofactor nicotinamide adenine dinucleotide (NAD), from which the ADP-ribose moiety is transferred onto the protein substrate. In eukaryotes, the mono-ADP-ribose modification can be extended by poly-ADP-

ribosylpolymerases (PARPs) to form poly-ADP-ribosylated proteins. The growing list of ADP-ribosylation substrates has motivated the development of chemical reporters to characterize the specificity and functions of the PARPs⁸². For example, 8-alkyne-NAD and 6-alkyne-NAD (Fig. 7c) can function as chemical reporters for diphtheria toxin (DT), a well-characterized MART that modifies elongation factor eEF-2⁸². The analysis of these chemical reporters with PARP-1 and Tankyrase-1, revealed that 6-alkyne-NAD was most effective for monitoring the ADP-ribosylation of p53 and TRF-1⁸². Incubation of cell lysates with recombinant PARP-1 and 6-alkyne-NAD enabled bioorthogonal detection and enrichment of poly-ADP-ribosylated proteins, demonstrating the utility of these reagents for ADP-ribosylation analysis.

AMPylation

Though originally discovered in the context of glutamine synthetase regulation in *E. coli*, AMPylation (adenylylation) has become more widely appreciated since the discovery of secreted bacterial effector enzymes that modify host small GTPases during infection⁸³. AMPylation describes the enzymatic transfer of an adenosine monophosphate (AMP) from ATP onto hydroxyl protein side chains. The conserved protein domains (fic domain, adenylyl transferase domain) responsible for the AMPylation activity are widely present among prokaryotes and eukaryotes, suggesting a ubiquitous role for this PTM⁸⁴. Recent studies have also revealed specific phosphoesterases that can remove the AMP moiety, suggesting that this modification can be dynamic and regulated^{85,86}. The re-emergence and abundance of potential AMPylation enzymes has motivated the development of specific antibodies, mass spectrometry detection methods as well as an AMPylation reporter⁸⁷. Modification of ATP at the N⁶-position with a propargyl group provides an alkyne-decorated AMPylation reporter (N⁶pATP) (Fig. 7d) that can be enzymatically transferred onto specific sites of cognate protein substrates by all known classes of AMPylation enzymes⁸⁷. Furthermore, N⁶pATP can be used to identify AMPylation substrates at endogenous levels in cell lysates⁸⁷, which should facilitate the characterization of AMPylated proteins in diverse organisms.

Phosphorylation

The reversible addition of phosphate onto Ser, Thr or tyrosine (Tyr) residues on proteins are perhaps the best studied PTMs for which specific antibodies and quantitative proteomic methods have been developed⁸⁸. Nonetheless, the large number of phosphoproteins and regulatory enzymes (kinases and phosphatases) with potentially overlapping biochemical activity makes dissecting phosphorylation-dependent signaling pathways still very challenging. Robust biochemical methods for directly monitoring enzyme-specific phosphorylation events are needed. The development of adenosine triphosphate (ATP) analogs and complementary kinase mutants has allowed the direct identification of enzyme-specific protein substrates⁸⁹. To distinguish kinase-specific phosphorylation events from existing phosphoproteins in cell lysates, N⁶benzyl-ATP γ S and mutant kinases were employed to selectively phosphorylate specific protein substrates⁹⁰. The enzymatically installed phosphorothiolate group could then be selectively alkylated with a nitrobenzyl-hapten to yield a semisynthetic epitope for antibody-based detection (Fig. 7e)⁹⁰ or covalently captured and oxidatively cleaved⁹¹ for identification of kinase-specific substrates. Although this system does not utilize alkyne- or azide-functionalized reagents, this two-step labeling protocol is itself bioorthogonal and showcases the utility of bump-hole approaches for identifying enzyme-specific substrates in systems with overlapping biochemical activity.

Oxidation

The oxidation of amino acid side chains can result in significant rearrangements of protein structure as in the case of the green fluorescent protein (GFP) or more subtle changes such as hydroxylation of amino acid residues. These subtle PTMs are perhaps the most challenging for bioorthogonal labeling approaches. Nonetheless, specific chemical reagents have been identified that enable bioorthogonal detection of some protein oxidation reactions. For example, the functionalization of dimedone with an alkyne enabled the selective labeling of sulfenic acid modified proteins in cells and subsequent bioorthogonal detection and proteomic analysis (Fig. 7f)⁹². Interestingly, stimulation of the epidermal growth factor receptor (EGFR) results in elevated hydrogen peroxide levels which in turn oxidizes Cys797 of EGFR to enhance its tyrosine kinase activity⁹². These observations suggest an interesting positive feedback loop to amplify EGFR-dependent signaling⁹². By taking advantage of unique chemical reactivity, bioorthogonal reporters can be developed for specific forms of protein oxidation and reveal previously unappreciated mechanisms of cell signaling.

Summary and Future Outlook

Bioorthogonal chemical reporters are providing powerful tools to track biomolecules in living systems and are illuminating new aspects of biology. While the past decade has seen significant progress in bioorthogonal reactions (Fig. 1)^{1,2} and specific chemical reporters highlighted here, major advances are still needed to dissect complex biological systems with these chemical tools. Amongst the currently available bioorthogonal ligation reactions (Fig. 1), no single method is ideal for all imaging and proteomic applications. So researchers need to choose the labeling method and reagents that best suits their biological interests. For example, CuAAC with biotinylated affinity tags have been highly effective for bioorthogonal proteomic studies with cell lysates, but not ideal for live cell imaging studies due to potential metal toxicity. Metal-free ligation methods are beginning to address the challenges of live intracellular and *in vivo* imaging applications^{2,93,94}, but more selective bioorthogonal reactions are still needed that can integrate membrane-permeable imaging reagents with bright fluorogenic properties and minimal non-specific binding. The development of sensitive imaging methods that target unique spectroscopic signatures of chemical reporters or isotopic labels should also provide new opportunities for *in vivo* imaging studies^{9,95}. On the chemical reporter end, a better understanding of metabolism or interconversion of chemical reporters *in vivo* is necessary to fully understand what biological events these reagents actually report upon. As with protein tagging technologies, the modification of cellular metabolites with reactive groups could interfere with their metabolism, the activity of intermediate enzymes, or the function of labeled substrates. More detailed analyses of chemical reporter metabolism and protein targets is therefore needed, which may in turn yield more specific reagents to selectively monitor key enzymatic pathways and also avoid potential perturbation of native signaling pathways. Many of the cofactor-based reporters only work with some enzymes and would benefit from engineered enzymes for *in vitro* activity studies, and the development of caged precursors for experiments in living cells. The integration of selective targeting methods such as enzyme-mediated uncaging⁹⁶ or liposome delivery⁹⁷ should also facilitate the cell-type specific analysis of chemical reporters *in vivo*. These two-component systems for bioorthogonal chemical reporters could then be used *in vivo* for enzyme- or cell-specific imaging and proteomics studies. By virtue of their specific and dynamic incorporation into biomolecules, chemical reporters can provide snapshots of the chemistry inside cells that integrates the activity of metabolic pathways and specific enzymes for functional studies. Further developments and applications of bioorthogonal labeling methods and chemical reporters should help translate the information encoded in the growing number of sequenced genomes

into biological function and begin to uncover key mechanisms of human diseases for therapeutic intervention.

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Box 1

The last decade has seen the remarkable development of highly selective chemical reactions that have allowed specific labeling of molecules in diverse biological contexts (Fig. 1)^{1,2}. This two-step labeling and detection strategy involves metabolic or enzymatic installation of a chemical reporter that can be selectively reacted with functionalized probes for the visualization or identification of target molecules (Fig. 1a). Central to this chemical labeling strategy is the introduction of azide, alkyne and activated alkene groups as abiotic, minimally perturbing functionalities onto small molecules and their selective covalent reaction with activated phosphine-, alkyne- or azide-modified as well as tetrazine probes (Fig. 1). Since these functional groups are foreign to living organisms and are uniquely reactive with each other, this class of chemical reactions has been described as bioorthogonal^{1,2}. The first example of these bioorthogonal reactions was the Staudinger ligation (Fig. 1b)²⁷, which demonstrated that alkyl-azides can react with ester-functionalized triphenylphosphine reagents to form covalent adducts in aqueous solution and on the surface of mammalian cells²⁷ as well as in living animals². Alternatively, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is a highly chemoselective reaction between alkyl-azides with terminal alkynes that form stable triazole products (Fig. 1c)⁹⁸⁻¹⁰⁰. This prototypic click chemistry reaction is widely used in chemical synthesis and bioorthogonal labeling since the azide and alkyne groups can be used in either orientation, are relatively stable, and are readily accessible. The cytotoxicity of Cu(I) can preclude *in vivo* applications, but the development of activated cyclooctyne reagents has circumvented this limitation and has enabled imaging of bioorthogonal chemical reporters in living organisms (Fig. 1d)². In addition to azide-alkyne cycloadditions, the development of tetrazine reagents has enabled selective and rapid bioorthogonal labeling of activated alkynes and alkenes through the Diels-Alder reaction (Fig. 1e)^{93,94}.

Figure 1.

Bioorthogonal labeling of biomolecules. a) Bioorthogonal chemical reporters and chemoselective reactions allow covalent labeling of biomolecules with detection or affinity tags for imaging or proteomics applications, respectively. b) Staudinger ligation allows labeling of alkyl-azides with triarylphosphine reagents. c) Cu(I)-catalyzed cycloaddition between alkyl-azides and terminal alkynes affords triazole adducts, which can operate in both orientations. d) Activated cyclooctynes can react with alkyl-azides for Cu-free labeling. e) Tetrazine reagents can undergo selective and rapid Diels-Alder reactions with activated alkenes such as *trans*-cyclooctenes.

Figure 2.

Applications of bioorthogonal chemical reporters. a) Pulse-labeling of isolated cells or organisms with a chemical reporter leads to the metabolic incorporation of the reporter into targeted biomolecules. Subsequent labeling of cells with the native metabolites or differentially labeled reporter enables the monitoring of biomolecule turnover rates. b) Pulse-labeling of two distinct cellular populations or organisms allows the comparative analysis of their metabolically labeled biomolecule pools. c) The application of chemical reporters in cell lysates, with purified substrates, or in combination with substrate arrays allows for enzyme-specific substrate detection.

Figure 3. Chemical reporters for nucleic acid synthesis and modifications. a) 5-ethynyl-2'-deoxyuridine (EdU) is incorporated into DNA. b) 5-ethynyluridine (EU) is incorporated into RNA. c) 5-ethynyl-2'-deoxyfluorouridine (F-ara-EdU) is incorporated into DNA. d) 5-ethynyl-2'-deoxycytosine (EdC) is incorporated into DNA. e) N-6-propargyladenosine (N6pA) is incorporated into RNA and into mRNA polyadenylation tails. f) The DNA modification 5-hydroxymethylcytosine (5-hmC) can be enzymatically labeled with 6-azido-glucose for subsequent bioorthogonal detection.

Figure 4.

Amino acid reporters for site- and residue-selective labeling of proteins. a) Analogs of the amino acid pyrrolysine (Pyl) with *trans*-cyclooctene (*trans*-ø) or strained-cyclooctyne (bicyclo[6.1.0]non-4-yn-9-ylmethanol, BCN) groups can be site-selectively incorporated into individual proteins through the expression of orthogonal aminoacyl-tRNA-synthetase and tRNA. b) Analogs of methionine (Met) can be selectively incorporated throughout the bacterial or mammalian proteome to install an alkyne (homopropargylglycine, HPG) or azide (azidohomoalanine, AHA) group. c) O-propargyl-puromycin (OP-puro) covalently labels the C-terminus of nascent peptides.

Figure 5.

Glycan chemical reporters. a) Peracetylated N-azidoacetyl-D-mannosamine ($Ac_4ManNAz$) is metabolically incorporated into sialic acid glycans. b) Peracetylated N-azidoacetyl-D-galactosamine ($Ac_4GalNAz$) is metabolically incorporated into mucin type O-linked glycans. c) Peracetylated 6-ethynyl-L-fucose (Ac_46 -ethynyl-L-fucose) is metabolically incorporated into fucosylated proteins. d) Peracetylated N-alkynylacetyl-D-glucosamine ($Ac_4GlcNAk$) is metabolically incorporated into O-GlcNAc modified proteins. e) O-GlcNAc modified proteins can be chemoenzymatically labeled using a β -1,4-galactosyltransferase that transfers GalNAz onto O-GlcNAc modified proteins. OGT, O-GlcNAc transferase.

Figure 6.

Protein lipidation chemical reporters. a,b) Alkynyl-fatty acid reporters of different chain length enable selective metabolic labeling of fatty-acylated proteins. c) Alk-FOH allows metabolic labeling of S-farnesylated and S-geranylgeranylated proteins in mammalian cells. d) An azide analog of cholesterol can be metabolically incorporated onto the C-terminus of sonic hedgehog. NMT, N-myristoyltransferase. FT, farnesyltransferase.

Figure 7.

Chemical reporters for other posttranslational modifications. a) Sodium-4-pentynoate allows metabolic labeling of acetylated proteins, while 4-pentynoyl-CoA can be used for *in vitro* analysis of acetyltransferase substrates. b) AdoEnYn and other SAM-based reporters have been developed to profile methyltransferase substrates. c) 6-alkynyl-NAD is a chemical reporter for mono- and poly-ADP-ribosylation. d) N-6-propargyl-ATP serves as a chemical reporter for protein AMPylation. e) N-6-benzyl-ATP γ S serves as a substrate for engineered kinases and allows selective modification with a nitrobenzyl-hapten for antibody detection as well as enrichment for proteomic studies. f) The oxidative modification of cysteines to

sulfenic acid can be monitored by reaction with an alkynyl-dimedone reagent for subsequent bioorthogonal detection of protein sulfenation.