Title
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Journal
Nature reviews. Methods primers, 1(1)

ISSN
2662-8449

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Publication Date
2021

DOI
10.1038/s43586-021-00028-z

Peer reviewed

Published in final edited form as:

Nat Rev Methods Primers. 2021 ; 1: . doi:10.1038/s43586-021-00028-z.

Bioorthogonal chemistry

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Abstract

Bioorthogonal chemistry represents a class of high-yielding chemical reactions that proceed rapidly and selectively in biological environments without side reactions towards endogenous functional groups. Rooted in the principles of physical organic chemistry, bioorthogonal reactions are intrinsically selective transformations not commonly found in biology. Key reactions include native chemical ligation and the Staudinger ligation, copper-catalysed azide–alkyne cycloaddition,

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Introduction (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Experimentation (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Results (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Applications (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Reproducibility and data deposition (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Limitations and optimizations (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Outlook (J.M.F., S.L.S., D.A.B., R.H., W.L., S.S.N., M.X., C.W.a.E., M.G.F., K.L., Q.L., J.P.P., J.A.P. and M.S.R.); Overview of the Primer (J.M.F.).

Competing interests

W.L. and C.W.a.E. are employees of Pfizer Inc. M.S.R. is an employee and shareholder of Tagworks Pharmaceuticals. S.L.S., D.A.B., R.H., S.S.N., M.X., M.G.F., K.L., Q.L., J.P.P., J.A.P. and J.M.F. declare no competing interests.

strain-promoted [3 + 2] reactions, tetrazine ligation, metal-catalysed coupling reactions, oxime and hydrazone ligations as well as photoinducible bioorthogonal reactions. Bioorthogonal chemistry has significant overlap with the broader field of ‘click chemistry’ — high-yielding reactions that are wide in scope and simple to perform, as recently exemplified by sulfuryl fluoride exchange chemistry. The underlying mechanisms of these transformations and their optimal conditions are described in this Primer, followed by discussion of how bioorthogonal chemistry has become essential to the fields of biomedical imaging, medicinal chemistry, protein synthesis, polymer science, materials science and surface science. The applications of bioorthogonal chemistry are diverse and include genetic code expansion and metabolic engineering, drug target identification, antibody–drug conjugation and drug delivery. This Primer describes standards for reproducibility and data deposition, outlines how current limitations are driving new research directions and discusses new opportunities for applying bioorthogonal chemistry to emerging problems in biology and biomedicine.

Bioorthogonal chemistry encompasses a class of high-yielding rapid and selective chemical reactions that proceed in biological environments, with little or no reactivity towards endogenous functional groups. Rooted in the principles of physical organic chemistry and classic organic reactivity, bioorthogonal reactions are intrinsically selective transformations not commonly found in biology. Chemical tools to study biological processes with molecular detail are foundational to modern science. The advent of recombinant protein expression enabled tracking protein dynamics in living systems using fluorescent proteins and antibodies[1,2]. These tools are often essential for studying intricate protein systems, but can be limited by their large size. Common genetic tags such as fluorescent proteins can disrupt protein function and trafficking, and for these same reasons they cannot be easily translated to non-protein biomolecules such as glycans, lipids and nucleic acids. An expanded set of biomolecules can be tagged via bioorthogonal chemistry. The classes of reactions include native chemical ligation and the Staudinger ligation, copper-catalysed azide–alkyne cycloaddition, strain-promoted [3 + 2] reactions, tetrazine ligation, metal-catalysed coupling reactions, oxime and hydrazone ligations as well as photoinducible bioorthogonal reactions (Fig. 1). Bioorthogonal reactions are intrinsically chemoselective, and therefore do not require proximity effects to achieve site-selective labelling. Also, bioorthogonal chemistry must readily proceed in aqueous environments at biocompatible pH and temperature, and be non-toxic under these conditions. Another consideration is the reaction rate[3], as it is generally advantageous for reactions to proceed rapidly at the low concentrations required for many biological experiments. The size of reaction partners is an intrinsically important parameter as the native function of many targets can be sensitive to bulky chemical groups.

Chemoselective

A chemical reaction that is selective for a certain functional group even in the presence of differing functional groups. Reaction partners in bioorthogonal chemistry are chemoselective for each other, even in biological settings.

Bioorthogonal chemistry enables probing biological systems through selective covalent bond formations that minimally disrupt the system(s) being studied. These reactions have been applied broadly both in cellular systems as well as in living animals; the growing list of applications includes probe construction, biomedical imaging, medicinal chemistry, polymer science and materials science as well as surface science. Similar to the broadening of applications enabled by bioorthogonal chemistry, the toolbox of chemical reactions has grown to meet the diverse needs of the chemical biology community.

Click chemistry.

Bioorthogonal chemistry has significant overlap with the broader field of click chemistry, which is defined by high-yielding and modular reactions that are wide in scope, simple to perform and generate only inert by-products[4,5]. Click chemistry also encompasses reactions that are not strictly bioorthogonal (as defined above) as these include selective reactions of functional groups commonly found in biological molecules. For example, alkylations and conjugate addition reactions of cysteines are classic approaches to achieving residue-selective protein modification, and the ‘thiol–ene’ reaction between unactivated alkenes and free thiols has become a broadly used tool across a range of applications, including organic synthesis, polymer science and materials science[6].

Click chemistry

A concept, coined by K. B. Sharpless and colleagues, describing bond-forming reactions that are thermodynamically driven, highly selective and reliable, and proceed in water without toxic by-products. Click reactions are often, but not strictly, bioorthogonal.

Scope.

Within the scope of this Primer are transformations where both reaction partners are not commonly found in biology and are the sources for chemoselectivity. This type of approach is complementary to strategies in which genetically programmed molecular recognition is responsible for selectivity, where effective chemistry is often still required, but the biological system is an active player and, thus, is at least somewhat perturbed by the modification(s). Here, examples include the use of reversible covalent reactions to modify specific protein sequences such as with genetically encoded tetracysteine motifs[7]. Another approach uses enzymatic modifications of proteins with encoded peptide sequences as a method of creating covalent bonds to exogenous molecules with high selectivity, enabling site-selective modifications at the carboxy terminus, amino terminus or internal positions[8]. Glycosyltransferases have been used for selective chemoenzymatic modification of glycans[9]. Directed evolution has been used to develop fusion proteins for covalent self-labelling with probe molecules[10]. These genetically programmed transformations represent powerful approaches to covalent bond formation, but they differ from bioorthogonal approaches where chemoselectivity derives directly from the reaction partners.

In this Primer, the scope of bioorthogonal chemistries examined includes the early developments such as native chemical ligation, oxime ligation and the Staudinger

ligation, which all exemplified how reaction design and mechanistic insight can guide the development of new chemical tools to study biology. With improved rates and minimally sized reactants, the copper-catalysed cycloaddition between azides and alkynes to form triazoles has been transformative for numerous scientific disciplines including bioorthogonal chemistry. Using ring strain to enhance reactivity is a classic concept in organic chemistry that has proven especially powerful in the area of bioorthogonal chemistry. Initially demonstrated with azides, the [3 + 2] cycloadditions of cyclooctyne derivatives with 1,3-dipoles have emerged as broadly important bioorthogonal reactions. Inverse electron-demand Diels–Alder (IEDDA) reactions of strained alkenes with tetrazines are also facilitated by the release of ring strain, and the reactions of *trans*-cyclooctene (TCO) represent the fastest bioorthogonal reactions developed to date. Spatio-temporal control over bioorthogonal chemistry can be achieved through the application of light through photoinduced processes that liberate reactive nitrile imine dipolarophiles or cycloalkynes. Metal-catalysed cross-coupling and ruthenium-mediated olefin metathesis have also emerged as complementary tools that can function in complex biological settings.

Here, we describe the range of reactions that encompass the bioorthogonal chemistry toolbox (Experimentation) and highlight the broad range of applications that implement this chemistry from site-specific protein labelling both in vitro and in vivo, as well as polymers and materials sciences (Applications). We discuss how handling these highly reactive reagents should be treated to ensure their use across diverse sets of applications (Reproducibility and data deposition). Finally, we examine the current limitations of bioorthogonal chemistry, the exciting areas of development in this research area (Limitations and optimizations) and what to anticipate in the next decade (Outlook).

Experimentation

The underlying mechanisms of key reactions in the bioorthogonal chemistry toolbox and their ideal operating conditions are discussed in this section, including native chemical ligation, oxime and hydrazone ligations, the Staudinger ligation, copper-catalysed azide–alkyne cycloaddition, strain-promoted [3 + 2] reactions, tetrazine ligation, metal-catalysed coupling reactions and photoinducible bioorthogonal reactions.

Native chemical ligation.

For chemical protein synthesis and bioconjugation, a significant body of work has focused on selective reactivity at the protein N terminus. Grounded in classic work by Wieland and pioneered by Kent, Dawson and Muir, native chemical ligation involves the ligation of a peptide bearing a C-terminal thioester with a second peptide with an N-terminal cysteine[11–14] (Fig. 2a). An initial thiol–thioester exchange reaction at the N terminus is key to the chemoselectivity of native chemical ligation, providing a transient thioester intermediate that undergoes an intramolecular *S,N*-acyl transfer to afford a native peptide bond to cysteine. As thioester exchange is the rate-determining step, native chemical ligation can be accelerated by nucleophilic catalysts including thiols or imidazole[12,15]. Subsequent to ligation, desulfurization reactions can serve to convert the internal cysteine into alanine[16,17]. Using selenocysteine and peptide selenoesters has enabled accelerating

these ligation reaction rates, and selenocysteines can function as alanine surrogates in complex target synthesis as deselenization reactions can proceed in the presence of unprotected cysteine and methionine residues[18,19]. Expressed protein ligation is another variant of native chemical ligation where the fragment containing the C-terminal thioester is created from a recombinant protein fused to an intein[20]. These methods are important tools for the synthesis and semi-synthesis of proteins that are too large to make by automated peptide synthesis alone and also provide a way to create proteins bearing site-specific post-translational modifications (for example, glycoproteins) and unnatural amino acids (UAAs).

Nucleophilic catalysts

Electron-rich additives that increase the reaction rate for certain polar bioorthogonal chemistries. in general, the most effective catalysts target the rate-limiting step for a given transformation. Catalysts must also adhere to the same strict requirements as bioorthogonal reagents (non-toxic, chemoselective and so on) if used in a biological context.

Post-translational modifications

Chemical transformations that occur on reactive side chains (such as lysine, serine and cysteine) of proteins. The identity of the modification can drastically affect the function and activity of the target protein. The modifications can be installed enzymatically or can occur spontaneously in solution.

Numerous techniques complementary to native chemical ligation for the formation of amide bonds have been developed, including serine–threonine ligation, which involves a C-terminal *o*-formylphenolate reacting with an N-terminal serine or threonine[21,22]. Also, the α-ketoacid–hydroxylamine (KAHA) ligation involves a C-terminal ketoacid group reacting with N-terminal hydroxylamine functionality and the KAT ligation between *O*-carbamoylhydroxylamines and potassium acyltrifluoroborates[23,24]. Other approaches for the selective modification at a protein's N terminus include the direct condensation of N-terminal serine[25], cysteine[26] and tryptophan[27] side-chain nucleophiles with aldehydes to form cyclic products. Inspired by luciferin biosynthesis, N-terminal cysteine can also rapidly react with 2-cyanobenzothiazole derivatives in vitro or on the surface of live cells[28]. Transamination reactions with pyridoxal-5′-phosphate[29] or Rapoport's salt[30] convert the N terminus into a ketone or aldehyde capable of oxime formation. One-step N-terminal conjugation can be also achieved with 2-pyridinecarboxaldehyde derivatives to form a cyclic imidazolidinone product[31]. In combination with solid-phase peptide synthesis, native chemical ligation methods and variants that form native amide bonds have found extensive applications in the chemical synthesis and semi-synthesis of peptides and proteins[12,13].

Solid-phase peptide synthesis

Amino acids are iteratively coupled from the carboxy terminus to the amino terminus on a solid support. Protecting group strategies ensure that only one amide bond is formed at a time, without oligomerization or cross-reactivity with reactive side chains. After

cleavage from the solid support, peptides are typically purified through high-performance liquid chromatography.

Oxime/hydrazone ligation chemistry.

The formation of oximes and hydrazones from the condensation of carbonyls with hydrazines and alkoxylamines, respectively, represents some of the oldest reactions applied to biological ligation applications. Carbonyl groups were some of the first extrinsic functional groups explored as reacting partners for bioorthogonal applications[32] (Fig. 2b). As products of condensation, hydrazones and oximes display higher stability towards hydrolysis than imines owing to the stabilizing effect of the heteroatom adjacent to the sp^2 nitrogen atom; with oximes being more stable to hydrolysis than hydrazones[33]. Under physiological conditions, and especially in acidic environments (pH 5–7), however, these ligation reactions can be reversible, a feature that can be tuned for the release of desired cargo[34–36]. At neutral pH, these reactions proceed with a second-order rate constant (k_2) of 0.01 M^{-1} 2 s^{-1}, slower than most commonly employed bioorthogonal cycloaddition reactions[37,38]. Initial findings showed improved reaction rates with lower pH conditions (pH 4–6) or the use of aniline as a catalyst, through reactive Schiff base formation[39–43]. Improvements in reaction rates were further achieved at neutral pH through application of substituted anilines such as 5-methoxyanthranilic, 3,5-diaminobenzoic and 2-aminobenzenephosphonic acids, with enhancements of reaction rates of up to 40 times compared with the original aniline catalysts[44,45]. Amine buffer systems have also been recently shown to promote condensation with fast rates[46].

Schiff base

A subclass of imine compounds characterized by a carbon–nitrogen double bond, with a general formula of $R^1R^2C{=}NR^3$, where R^3 is not a hydrogen atom. They often arise from the condensation reaction between an amine and a carbonyl, and are classified as secondary ketimines or aldimines.

The Pictet–Spengler ligation is a more hydro-lytically stable variation of oxime ligation between indolyl-substituted nucleophiles and aldehydes[27], and variations using hydrazine nucleophiles have also been reported[47]. Carbonyls have been traditionally incorporated into biomolecules via oxidative cleavage of vicinal diols in sugar moieties using sodium periodate, allowing facile modification of glycoproteins through sialic acid oxidation[48,49]. Oxidative cleavage of 1,2-amino alcohols, including N-terminal serine and threonine, is an analogous strategy[50]. Nucleic acids can also be functionalized through sodium periodate oxidation of 3′-ribonucleotides to yield dialdehydes[51]. The use of carbonyl-bearing UAAs and nucleic acids also allows for the site-selective incorporation of modification sites[52–54]. Comparatively, fewer methods have been developed for the incorporation of alkoxyamine and hydrazine nucleophiles, mostly limited to synthetic nucleic acids, through the use of phosphoramidites to modify 5′-end ribonucleotides and appropriately functionalized nucleobases. Although improvements to reaction rates and hydrolytic stability have been achieved, these ligations are best suited to in vitro, cell surface labelling, and to biological

ligation applications owing to their innate reversibility and potential cross-reactivity with carbonyl-bearing metabolites[27,47–54]. However, the recent development of new catalysts and more selective reacting partners has allowed for more specialized applications with reaction rates as fast as 0.1 M^{-1} s^{-1} (REFS[44,45]).

Phosphine-based transformations.

The Staudinger ligation — a reaction between triarylphosphines and organic azides — exploits the mild electrophilicity of organic azides and their propensity to react with mild nucleophiles[55,56]. Phosphine–azide chemistry was first reported by Staudinger in the context of azide reduction[57]. The reaction produced iminophosphorane intermediates that hydrolysed in water to give amine and phosphine oxide products. The relative simplicity of the Staudinger reaction — and the virtual absence of triarylphosphines and azides in biological settings — provided an ideal platform for biocompatible reaction development. Bertozzi and co-workers built upon this foundation to create a, now iconic, bioorthogonal transformation[58]. A methyl ester was installed on the triarylphosphine core, which served as an electrophilic trap for the iminophosphorane intermediate, circumventing hydrolysis and driving formation of a ligated adduct. This variant of the Staudinger reaction was termed the Staudinger ligation (or the Staudinger–Bertozzi ligation[59]). The Staudinger ligation was the first bioorthogonal transformation to be widely used in cells and living systems[60] (Fig. 3a). The two reactants — organic azides and phosphines — are both bioavailable and biocompatible. Azides also rank among the smallest bioorthogonal functional groups, making them a go-to choice for many applications. Additionally, phosphines can be outfitted with various probes for detection in biological environments and have been used to image, retrieve and profile biomolecules[56]. Initially used for cell surface glycan labelling[58], the Staudinger ligation has since been used to target proteins[61–63], nucleic acids[64] and other biomolecules[65–67]. Fluorogenic phosphines and azides[68,69] as well as more water-soluble probes have enabled additional studies[70,71]. The Staudinger ligation is one of the few bioorthogonal reactions that can be used in rodent models[72,73]. Although versatile, the ligation still ranks among the slowest bioorthogonal chemistries[74] (Fig. 3a), which inspired the pursuit of more rapid reactions and drove many early advances in bioorthogonal cycloadditions as described below.

The Staudinger ligation also spurred the development of other phosphine-based transformations. The most well known feature phosphines with alternative electrophilic traps (for example, thioesters)[75,76]. Some of these variants react with azides to form amide bonds with concomitant release of the phosphine probe. Such ‘traceless’ versions of the ligation were developed contemporaneously by Raines and Bertozzi[77,78] and have seen application in peptide and protein synthesis[56,79,80]. Alternative phosphine nucleophiles, including phosphite[81] and phosphonite[82] probes, have been used to functionalize peptides and proteins. These reagents react with azides to form covalent adducts under physiological conditions.

Bioorthogonal phosphines have also been used for ligation chemistries with electrophiles other than azides. Recent examples include Michael-type reactions with α,β-unsaturated amides[83,84] and cyclopropenone derivatives[85–88].

Azide–alkyne cycloaddition.

The azide and terminal alkyne functional groups are very small and lack the ability to associate with other molecules by anything more stable than weak dipolar interactions. Accordingly, they often minimally perturb the functional properties of the molecules to which they are attached. Azides and alkynes are largely unreactive with most other molecules in biological systems, and so can move through cells and organisms intact, only to be revealed when provided with the right conditions or catalysts for their reaction.

The Huisgen 1,3-dipolar cycloaddition of organic azides and alkynes, giving 1,2,3-triazoles, is usually much too slow to be of use in biological applications. The introduction of copper(I) catalysts, reported independently by the groups of Meldal[89] and Sharpless[90], transformed the process into one of wide use by accelerating triazole formation up to 100 million-fold (Fig. 3b). Copper(I)-catalysed azide–alkyne cycloaddition (CuAAC) enables the exclusive formation of 1,4-disubstituted triazoles, which are good mimics of native peptide-based *trans*-amide connectors, in terms of size and geometry, and are far more stable than amides to thermal and chemical cleavage. The CuAAC reaction is modular, compatible with aqueous conditions, usually free of by-products and user-friendly, all characteristics that enable successful click reactions[4]. Its mechanism is stepwise, proceeding through an easily formed binuclear copper acetylide intermediate[91,92]. A wide range of catalysts have been employed, including simple copper(I) salts[90], small-molecule copper-binding ligands[92,93] and heterogeneous, polymeric and nano-structured materials of different kinds[94–96]. The development of CuAAC has driven many applications in the realms of organic synthesis, polymer functionalization, drug discovery and chemical biology.

Although a few examples exist of terminal alkyne biosynthetic pathways in microorganisms and fungi[97,98], naturally occurring azides are exceedingly rare. Proteins, lipids, oligonucleotides and cell surface moieties can be labelled with azides or alkynes through benign chemical transformations or via metabolic incorporation of labelled metabolites, allowing their conjugation to an orthogonally labelled partner. The use of CuAAC for bioorthogonal bioconjugation was largely enabled by the development of water-soluble copper-binding ligands, which allowed users to address the most striking limitation of CuAAC for biology: the copper-mediated formation of reactive oxygen species[99–101]. These ligands stabilize the required copper(I) oxidation state and provide access to coordination sites on the metal, accelerating the reaction while minimizing copper-mediated oxidative stress. Ligands with notable characteristics such as the ability to scavenge reactive oxygen species[99–101] or to enhance local concentrations of reactive partners by combining substrate and copper-binding moieties[102–107] have further allowed CuAAC to be adapted for more demanding biological applications such as the labelling of living cellular surfaces[108], labelling tagged enzymes or other biomolecules in complex cellular mixtures[109], fast click reactions inside cellular compartments[110,111] and versatile dye labelling of a wide variety of cellular structures for super-resolution expansion microscopy[112].

Reactive oxygen species

Highly reactive forms of oxygen involved in diverse cellular signalling processes, and tightly regulated in cells. For bioorthogonal chemistry, reactive oxygen species arise from

the oxidation of copper(i) to copper(ii) in water, which generates damaging superoxide or hydroxyl radicals. An accumulation of reactive oxygen species damages nucleic acids, proteins and lipids, and is cytotoxic.

A few limitations remain in the application of CuAAC to bioconjugation. First, copper is highly regulated in cells, and tightly bound by a network of interacting proteins and chaperones; thus, the best copper-binding ligands for CuAAC cannot retain the metal ion when encountering endogenous copper-binding proteins[113]. In addition, thiols — abundant in the form of protein cysteines and intracellular glutathione — bind copper tightly, and thereby inactivate catalysts and trigger copper-mediated oxidative stress[114]. A systematic evaluation of copper-mediated toxicities in various human cell lines illustrates the practical consequences of these phenomena[114]. Copper-free variants of CuAAC, detailed below, were developed in part to address these limitations, but suffer from reduced reaction rates, susceptibility to unproductive side reactions or use of sterically larger connecting groups that can compromise function. Thus, fully biocompatible accelerating ligands for CuAAC, able to rapidly catalyse intracellular reactions without inducing biological stress and exchanging copper with endogenous biomolecules, await discovery.

Strain-promoted [3 + 2] cycloadditions.

Strained cyclooctynes were introduced as an alternative to CuAAC to circumvent copper catalysts in various applications[115]. Cyclooctynes are the most commonly used substrates for strain-promoted azide–alkyne cycloadditions (SPAAC; as shown in Fig. 4a), with rates that are rapid enough to be used in living systems without the need for a catalyst[116,117]. Many different cyclooctynes have been developed specifically for use in SPAAC reactions, including a difluorinated cyclooctyne (DIFO)[116,118,119] (Fig. 4a). However, early versions of cyclooctynes (OCT; Fig. 4a) suffered from slow reaction rates and poor aqueous solubility. These limitations spurred the development of alternative scaffolds that enabled rates as fast as 1 M^{-1} s^{-1}, including bicyclic derivatives (for example, bicyclo[2.1.0]nonyne (BCN))[120] (Fig. 4a), benzannulated cyclooctynes (for example, biarylazacyclooctynone (BARAC) and dibenzocyclooctyne (DIBO or DBCO))[121–124] (Fig. 4a) and more hydrophilic variants[125–127]. More highly strained cycloheptynes have also been used for SPAAC reactions to achieve further rate acceleration (for example, 3,3,6,6-tetramethylthiaheptyne (TMTH))[128–130] (Fig. 4a). Fluorogenic cyclooctynes serve to both conjugate and introduce reaction-dependent fluorescence read-outs for SPAAC reactions[131,132]. Distortion/interaction modelling has also been a useful tool for tuning the reactivity of strained alkyne systems[133–135]. Overall, there are many options for using SPAAC chemistry to report on different biological events or to connect components, making this bioorthogonal chemistry a popular choice for in situ and in vivo applications.

Although SPAAC reactions are both versatile and robust, dipoles other than azide have been found to react with faster rates in the analogous cycloaddition reactions, which can be critical for the success of bioorthogonal reactions in living systems. Different dipoles also offer additional sites for stereoelectronic tuning of their reactivity. As an example, strain-promoted alkyne–nitrone cycloaddition reactions involve nitrone dipoles that are faster and have three sites of substitution available for reaction tuning[136–139]. Other dipoles that

react with cyclooctynes include diazo compounds and nitrile oxides[140–143]. Strain-promoted sydnone–alkyne cycloadditions are also versatile and rapid alternatives to SPAAC reactions, which have been applied in positron-emission tomography imaging using ^{18}F-labelled antibodies, where fast reaction rates are required[144–146]. The strain-driven quadricyclane (QC) ligation involving nickel bis(dithiolene) derivatives offers another unique tool for bioorthogonal bond creation[147]. Taken together, these examples illustrate the large and growing number of possibilities for fast and efficient bioorthogonal chemistry utilizing strained alkynes with different dipoles as reaction partners.

Inverse electron-demand Diels–Alder reactions.

The bioorthogonal tetrazine ligation refers to the reaction between a 1,2,4,5-tetrazine and an alkene or alkyne dienophile via a sequence of [4 + 2]/retro [4 + 2] cycloaddition to provide a dihydropyridazine or pyridazine conjugate (Fig. 4b). Tetrazine ligation is an IEDDA reaction that is notable for rapid kinetics without the need for catalysis, which yields nitrogen gas as the only by-product. Tetrazines initially found utility in organic synthesis[148,149] and were noted for their rapid kinetics in reactions with strained dienophiles[150].

Dienophile

An alkene or alkyne that reacts with a conjugated diene in [4 + 2] cycloadditions. Diels–Alder cycloadditions are enabled by electron-poor dienophiles and electron-rich dienes. Conversely, inverse electron-demand Diels–Alder reactions occur between electron-rich dienophiles and electron-poor dienes.

In 2008, the bioorthogonal reactions of tetrazines with strained alkenes were first described[151–153], including the reactions with derivatives of TCO and norbornene by the groups of Fox and Weissleder, respectively[151,152]. There has since been a growing diversity of chemical richness for both tetrazines and their dienophile reaction partners for the tuning rate, stability and design of ‘minimal’ reporter molecules. As an IEDDA reaction, the tetrazine ligation is accelerated by electron-withdrawing groups on the tetrazine.

Tetrazines are most often prepared through condensation of Pinner salts or nitriles with hydrazine and facilitated by Lewis acidic, thiol or sulfur catalysts[154–156]. Milder protocols have been developed for directly introducing intact tetrazine groups via palladium-catalysed coupling reactions of aryl halides or aryl boronic acids[157,158]. Carboxylic esters also serve as a handle for the preparation of unsymmetrical and monosubstituted tetrazines[159].

A range of dienophiles have been developed for tetrazine ligation. TCO can be prepared by flow photochemistry[160,161], and variants have been designed that combine with tetrazines in the most rapid bioorthogonal reactions known — with $k_2 = 10^4$–10^6 M^{-1} s^{-1} under aqueous conditions[162,163] — with applications that extend to radiochemistry, in vivo chemistry, cell imaging and click to release chemistry. Highly strained cyclooctynes such as BCN also react rapidly ($k_2 = 10^2$–10^3 M^{-1} s^{-1}) with tetrazines, and produce aromatic conjugates that can simplify workflows where defined stereochemistry is needed[164]. Cyclopropenes ($k_2 = 1$–10^4 M^{-1} s^{-1}) are the smallest class of ring-strained dienophiles, offering a complementary approach when size and stability are important, such as for genetic encoding

and metabolic incorporation[165,166]. Norbornene retains good kinetics at a low cost and has proven an advantage for scale-up and materials applications. Small dienophiles such as cyclobutenes, azetidines, unstrained olefins and isonitriles also react efficiently with tetrazines but typically require large excess of one reagent owing to slower kinetics[167–171]. Similarly, triazines can serve as surrogates for tetrazines that offer advantages of smaller size and greater stability[172].

Another useful feature of tetrazine ligation is that many tetrazine–fluorophore conjugates are fluorogenic. Resonant energy transfer to the tetrazine reduces fluorescence, but upon reaction with a dienophile the fluorescence is 'switched' on with up to an 11,000-fold increase in fluorescence intensity[173–175]. Fluorogenic tetrazine–fluorophores have found particularly strong use in the field of live-cell microscopy, and the area is actively growing with a focus on improving the signal to noise ratio that can result from background tetrazine reactivity in the intracellular environment[176,177]. External cues for temporal control of the tetrazine ligation have been developed for photoactivatable cyclooctynes or redox activation of dihydrotetrazine to tetrazines using electrochemistry, photocatalysts or enzymes[178–180]. This toolkit of dienes and dienophiles has enabled an exciting range of applications as further detailed throughout the applications section below.

Photoinducible bioorthogonal chemistry.

In contrast to thermal processes, light-triggered reactions often display higher yields and selectivity without the need for transition metal-mediated catalysis. The first example of using light to initiate selective reactions efficiently in biological systems is a photoinduced 1,3-dipolar cycloaddition reaction between a 2,5-diaryltetrazole and an alkene, often referred to as photoclick chemistry[181] (Fig. 5a). This chemistry was based on the seminal work by Huisgen, who reported outstanding reactivity of the transient nitrile imine dipole generated photochemically from 2,5-diphenyltetrazoles in benzene[182]. Milder conditions using a handheld low-powered UV lamp[183] facilitated the rapid development of tetrazole photoclick chemistry for bioorthogonal protein modifications in vitro[184] and inside bacterial cells[185]. Mechanistically, the reaction proceeds through a photoinduced cycloreversion to exude N_2 and generate a highly reactive nitrile imine followed by [3 + 2] cycloaddition with an alkene dipolarophile. A prominent feature of tetrazole photoclick chemistry is that the pyrazoline adducts are fluorescent, making the reaction fluorogenic[184].

Photoclick chemistry

Click chemistry in which reactions that are initiated using light as an external stimuli. Photoclick reactions can use light sources ranging from short-wavelength to near-IR light and allow for spatial and temporal control of reactions.

2,5-Diaryltetrazoles can be prepared from cycloadditions of azides with nitriles, condensations of phenylsulfonyl hydrazide with arene diazonium salts or copper-catalysed regioselective *N*-arylation of 5-aryl-2*H*-tetrazoles with either phenyl iodonium salts or aryl boronic acids[186]. As the rate of cycloaddition is dictated primarily by the HOMO (dipole)–LUMO (dipolarophile) energy gap, electron-rich tetrazoles[187] and electron-deficient alkenes

such as acrylamide[188] and fumarate[189] are privileged substrates for tetrazole photoclick chemistry. Because of these electronic requirements, tetrazole photoclick chemistry is mutually exclusive to the tetrazine ligation[190]. Tetrazoles bearing the steric shielding groups show improved reaction selectivity[191]. For enhanced spatio-temporal control, photoactivation of tetrazoles can be achieved using a 405-nm laser[192] and a near-IR femtosecond laser[193] by modifying tetrazole structures as well as combining a near-IR laser with nanoparticles capable of two-photon upconversion[194].

HOMO

(Highest occupied molecular orbital). A molecule's highest energy molecular orbital containing an electron pair.

LUMO

(Lowest unoccupied molecular orbital). A molecule's lowest energy molecular orbital not containing an electron. The energies of HOMO and LUMO are related to the reactivity of the molecule and the energy difference between the HOMO and LUMO is termed the HOMO–LUMO gap.

Two-photon upconversion

A molecule is excited from the ground state (S_0) to the second excited singlet state (S_2) by simultaneous absorption of two photons, via a virtual state. A photon with frequency greater than those of the absorbed photons is emitted upon relaxation from the excited state, that is, two-photon upconversion.

For increased stability in biological systems, strained alkenes such as 3,3-disubstituted cyclopropene[195], spiro[2.3]hex-1-ene (REF.[196]) and water-soluble azaspiro[2.3]hex-1-ene (REF.[197]) have been developed for tetrazole photoclick chemistry with robust reaction kinetics ($k_2 = 10^2$–10^4 M^{-1} s^{-1}). The alkene-based chemical reporters, along with the optimized tetrazole reagents, have allowed bioorthogonal labelling of membrane proteins[191], DNA[198], RNA[199] and glycans[200]. The fluorogenic property has been exploited in the design of photoactivatable fluorescent probes for cellular proteins[201] and the detection of oncometabolites[202].

The spatio-temporal control afforded by photoinduction can be infused with SPAAC. For example, a dibenzocyclopropenone undergoes decarbonylation upon photoirradiation at 350 nm to generate dibenzocyclooctyne, which then reacts rapidly with azides to form the cycloadducts[203] (Fig. 5a). The utility of this dibenzocyclopropenone-based photoclick chemistry was demonstrated through successful glycan labelling[203] as well as surface immobilization[204]. More recently, a cyclopropenone-caged dibenzoannulated bicyclo[6.1.0] nonyne probe was applied in conjugation with tetrazine ligation using irradiation at 365 nm (REF.[179]). Spirocyclic aminocyclopropenes can be protected with either light or enzyme labile groups and uncaged for controlled reactions with tetrazines[205]. The photoclick

chemistry toolbox also includes hetero-Diels–Alder reactions based on 2-naphthoquinone-3-methide (REF.[206]), *o*-quinodimethane[207] and 9,10-phenanthrenequinone[208], light-induced tetrazine ligation[180] and diarylsydnone–alkene photoligation[209].

Palladium-catalysed and ruthenium-catalysed bioorthogonal reactions.

The power of palladium-catalysed cross-coupling reactions has been harnessed for biomolecular functionalization in vitro and in living cells (Fig. 5b). Early efforts uncovered conditions with limited reaction efficiency and biocompatibility[210–213], including high catalyst loading, high temperature and the presence of organic co-solvent, but further developments have led to the discoveries of novel catalyst systems and additives that enable efficient palladium-mediated cross-coupling reactions with a broader substrate scope and utility in biological systems[214,215].

In homogeneous catalysis, the palladium–2-amino-4,6-dihydroxypyrimidine catalyst allowed fast and high-yielding Suzuki–Miyaura cross-coupling between proteins bearing *p*-iodobenzylcysteine and various aryl and vinyl-boronic acids in phosphate buffer[216]. The palladium–2-dimethylamino-4,6-dihydroxypyrimidine catalyst enabled copper-free Sonogashira cross-coupling between homopropargylglycine-encoded proteins and aryl iodides in aqueous media as well as inside *Escherichia coli*[217]. The aryl–palladium(II) complexes from decarboxylative palladation of substituted benzoic acid derivatives enabled functionalization of a styrene-modified protein with biotin and a cyanine dye via a Heck-type mechanism[218]. In heterogeneous catalysis, polystyrene microspheres encapsulating Pd^0 allowed cytosolic uptake of the palladium catalyst and subsequent in situ synthesis of a fluorescent dye via Suzuki–Miyaura cross-coupling inside HeLa cells[219]. The main advantage of using the palladium-encapsulated microspheres is that the palladium catalysts stay inside cells to perform intracellular chemistry for an extended period without posing a toxicity risk.

Homogeneous catalysis

The catalyst and reaction mixture are in the same phase.

Heterogeneous catalysis

The catalyst and reaction mixture are in a different phase.

Obtaining kinetic parameters for these cross-coupling reactions is challenging owing to the complex reaction mechanisms involved and the excess amount of palladium catalysts used. In cases where stable palladium catalysts were used, the apparent k_2 values were determined to be 0.011 M^{-1} s^{-1} for Suzuki–Miyaura cross-coupling[220] and 5.2 M^{-1} s^{-1} for copper-free Sonogashira cross-coupling[221]. By placing an alkyne reporter in the middle of a selected protein sequence environment to facilitate recruitment of the palladium complex, faster reactions were observed for Sonogashira cross-coupling with apparent k_2 values as high as 1.3×10^4 M^{-1} s^{-1} (REF.[222]).

Palladium-mediated cross-coupling reactions have been used for modifying proteins with fluorophores[218,223], polyethylene glycol[224] and carbohydrates[224] in vitro, on the surface of *E. coli*[225], inside bacterial cells[217] and on the mammalian cell surface[220–222], as well as for modifying DNA[226]. As palladium is an abiotic transition metal, the toxicity[225] and cell permeability of palladium complexes need to be carefully monitored in cellular applications. Also, non-specific sequestration of palladium catalysts by biomolecules[227] may further decrease the cross-coupling reaction efficiency.

Ruthenium-mediated cross-metathesis of olefins has also been adopted for bioorthogonal chemistry (Fig. 5b). As ruthenium alkylidenes are highly reactive and prone to decomposition in protic solvents, the use of allyl chalcogen-based privileged substrates for accelerated cross-metathesis[228], $MgCl_2$ as a competitive, hard Lewis acid and *tert*-butanol as co-solvent has proven crucial. This unique reactivity was harnessed for modifying proteins bearing allyl chalcogen tags such as *S*-allylcysteine[229] and Se-allyl selenocysteine[230] in vitro using the Hoveyda–Grubbs second-generation catalyst.Kinetic studies[231] showed that Se-allyl-selenocysteine is ten times more reactive towards allyl alcohol than *S*-allyl-cysteine, with an apparent k_2 value of 0.3 M^{-1} s^{-1}. The development of a genetically encoded allylsulfide reporter, *S*-allylhomocysteine, further enhanced the utility of this chemistry[232]. It remains to be seen whether this reaction is sufficiently robust for cellular applications.

Lewis acid

A chemical species that can accept a pair of non-bonding electrons.

Safety.

Bioorthogonal reagents are designed to react quickly. Accordingly, the energetic properties of any bioorthogonal reagent that is purchased or prepared should be carefully considered prior to use. Safety analysis is especially important for nitrogen-rich compounds including azides, diazo compounds, tetrazines and tetrazoles, or compounds with other high-energy functional groups (for example, alkynes and nitro groups). For reagents that are synthesized, the safety of all synthetic intermediates should be critically evaluated even for compounds already known in the literature. Some common starting materials for bioorthogonal chemistry also have documented safety concerns. For example, sodium azide (used to prepare reagents for CuAAC and SPAAC) is incompatible with acids and halogenated solvents such as dichloromethane[233], and anhydrous hydrazine (often used to prepare tetrazines) is highly toxic, energetic and not available for purchase in all countries[234].

For nitrogen-rich bioorthogonal reagents, it is generally observed that the safety profile improves with an increasing carbon to nitrogen ratio. One rule of thumb is to avoid compounds that do not have at least six carbons (or other atoms of about the same size) per high-energy functional group (such as azide, diazo, nitro and so on)[4]. However, there are exceptions[235], and therefore testing safety properties is recommended for any compound with a high-energy functional group. Differential scanning calorimetry (DSC) represents a simple and informative initial measurement of energetic safety that requires only milligrams of material. In DSC, heat flow associated with phase transitions is measured as a function of

time and temperature. To obtain accurate data, DSC analyses of energetic compounds must be performed in sealed pans rated for pressure. Energetic compounds display an exothermic decomposition that can be analysed for onset temperature, decomposition energy and rate of decomposition. Based on these DSC data, the Yoshida correlation can also be used to inform whether to further test for explosivity or impact sensitivity of the material[236]. Therefore, DSC provides a good starting point for understanding the overall energetics of a material[237], and to help guide the reaction scale and safety precautions (for example, blast shields) that should be employed[238].

Differential scanning calorimetry

(DSC). A technique that measures heat flow rates to determine phase transitions and quantitative heats of decomposition of a compound of interest. DSC requires only milligram quantities of a sample and provides a rapid measurement of the thermal properties of a compound.

Yoshida correlation

The impact sensitivity and explosive propagation properties of compounds can be derived from differential scanning calorimetry data.

Applications

Bioorthogonal chemistry is rich in chemical diversity and offers a palette of tools for many applications. Live-cell imaging has been revolutionized by bioorthogonal reactions and these chemistries have been essential for genetic code expansion and detecting chemical reporters following their metabolic incorporation into target biomolecules[239]. Activity-based protein profiling (ABPP) utilizes advancements in reaction and probe design for understanding protein function in complex biological systems[240]. Applications of bioorthogonal chemistry in polymer and materials science and surface chemistry have expanded significantly, including fabrication of three-dimensional (3D) networks, hydrogels for live cells and drug-delivery strategies[241]. Advances in reaction chemistry have not only been used for bond-forming reactions but also bond-breaking ‘decaging’ reactions for release or activation of biomolecules or small molecules[242]. Together, the tools available for bioorthogonal chemistry have provided a diverse set of methods that are impacting multidisciplinary research and are expanding to medicinal applications.

Metabolic engineering.

Metabolic engineering is a tactic used to install non-natural functional groups into target biomolecules using the cell’s own enzymatic machinery. Metabolites outfitted with bioorthogonal functional groups (chemical reporters) can hijack biosynthetic pathways and be incorporated into target biomolecules[239]. Once installed, the reporters are detected via bioorthogonal ligation with complementary reagents[243–245] (Fig. 6a). This two-step approach (the chemical reporter strategy) has been routinely used to image and profile proteins[61], glycans[246,247] and other biomolecules[248,249] in cells and other

environments[250–252] (Fig. 6b). Importantly, the chemical reporter strategy provides a mechanism to examine structures that are difficult to study via genetic manipulation, such as lipids[67,253], small-molecule cofactors[254] and other secondary metabolites[255].

The most widely used chemical reporters are the azide and alkyne. These motifs are among the smallest and most stable bioorthogonal functional groups, and they are tolerated by a wide variety of cellular enzymes[256,257]. Azides and alkynes can also be readily appended to biosynthetic precursors for metabolic labelling[258]. Typical detection chemistries include CuAAC (primarily in the context of fixed or lysed samples) or SPAAC (in the case of live cells)[259,260]. Other bioorthogonal functional groups can be employed for metabolic engineering, although smaller functional groups are the most useful. Ketones[261] and cyclopropenes[165,262] are included in this group. Importantly, these motifs (and their ligation chemistries) are orthogonal to typical azide/alkyne reactions, enabling multicomponent detection.

Metabolic labelling strategies — although straightforward — are not often selective for a single target. The metabolite employed can be incorporated into multiple biomolecules, depending on the enzymatic pathways employed, because some metabolites are common to multiple biosynthetic pathways. Additional modifications to probes and pathways are necessary to label specific cell populations or individual biomolecules[263–265]. For example, cell-selective labelling can be achieved using targeted delivery strategies or caged metabolites that are released in response to specific enzymes. Caged probes have been primarily used to label cancer cell targets[264,266], although other cell and tissue types can be labelled. Biosynthetic enzymes can also be engineered to selectively process non-natural metabolites[267–269], and are the underlying strategy for site-specific protein tagging/genetic code expansion technologies with bioorthogonal amino acids.

Genetic code expansion.

Metabolic engineering approaches, such as residue-specific incorporation of UAAs bearing bioorthogonal side chains, allow globally modifying proteins and targeting proteomes of entire cells or organisms[270], but genetic code expansion approaches enable the site-specific genetic encoding of UAAs to modify proteins with medicinal chemistry-like precision[271,272]. Genetic code expansion relies on the use of an orthogonal aminoacyl-tRNA synthetase (aaRS)–tRNA pair to direct co-translational UAA incorporation in response to an amber stop codon introduced at a chosen position in a gene of interest. Orthogonality refers to mutual and exclusive recognition of an orthogonal aaRS–tRNA pair for charging the UAA in the host of choice without interfering with endogenous aaRS, tRNAs and natural amino acids. In the past 20 years, numerous orthogonal aaRS–tRNA pairs have been developed for incorporation of UAAs with bioorthogonal functionalities[271] to site-specifically modify and label proteins in bacteria, yeast, mammalian cells and multicellular model organisms (Fig. 6c). This strategy offers a modular approach for installing diverse probes with a single genetic system and removes limitations that the translational machinery may place on the size of probes. The most common and useful bioorthogonal UAAs that can be encoded include keto, azide, terminal alkyne, strained alkyne/alkene and tetrazine functionalities and have been used for site-specific protein conjugation through oxime

ligation, Staudinger ligation, CuAAC, SPAAC, photoclick and IEDDA reactions[271,273]. Many of these approaches have been used for generating in vitro defined protein conjugates such as PEGylated and biotinylated proteins, antibody–drug conjugates (ADCs) and proteins labelled with spectroscopic probes such as fluorophores and spin labels. An important recent advance has been the encoding of IEDDA reaction partners[274], such as strained alkenes/alkynes[164,275,276] and tetrazines[179,277,278] that permit rapid and selective labelling in live cells (both on the cell surface and intracellularly; shown in Fig. 6d) and have allowed researchers to visualize and image proteins in their native environment[176,275,279–281] and to monitor protein turnover[282]. Site-specific incorporation of two distinct bioorthogonal UAAs into the same protein using further engineered translational machineries has enabled selective dual protein labelling for applications such as Förster resonance energy transfer studies on protein folding[283,284]. Furthermore, the exquisite selectivity and fast rate (Fig. 4b) of the IEDDA reaction between a site-specifically incorporated cyclooctyne-bearing UAA and a tetrazine–inhibitor conjugate has been used to selectively inhibit a target enzyme within live cells[285]. Introducing a photoisomerizable linker into tetrazine–inhibitor conjugate has allowed reversible switching of enzyme activity with light in living mammalian cells[285].

Cell imaging.

Bioorthogonal chemistry has proven a powerful and versatile approach for imaging various processes in living cells. Strategies for cell imaging that allow for direct attachment of reporter molecules to an expanded set of important biomolecules are free of conventional restraints imposed by genetically fused tags, and benefit from a researcher's choice of chemistry for their particular application(s). Many bioorthogonal reactions have been used for cell imaging, but some are more well suited than others. For example, CuAAC and SPAAC have been used in bacteria to image peptidoglycan remodelling during cell division[286] (Fig. 6e), and SPAAC has been used to image differential expression of different glycosylated proteins within cells and transparent model organisms such as zebrafish[246] and *Caenorhabditis elegans*[287]. Strain-promoted alkyne–nitrone cycloaddition reactions have been used to image external growth factor receptors[136], and for duplex labelling to image different bacterial species simultaneously[139]. In combination with genetic code expansion to site-specifically incorporate strained dienophile-bearing UAAs, the tetrazine ligation has enabled labelling and imaging of cell surface and cytoskeletal proteins by super-resolution microscopy[176,279,288,289] (Fig. 6f). Furthermore, the tetrazine ligation has been used to image and enrich diverse proteomes in bacteria[290], mammalian cells[290], *Drosophila melanogaster*[291] and the mouse brain[292] in a temporally and spatially resolved fashion. Aside from being spatially permissive and applicable to different types of biomolecule, bioorthogonal chemistry strategies enable the use of newer and advanced imaging agents.

A suite of organic fluorophores covering the entire visible spectrum can be chosen for super-resolution microscopy techniques. In addition, some bioorthogonal reagents and fluorophore pairs are capable of turn-on fluorescence to enhance the signal to noise ratio[132,174,201,293–295]. Depending on the application, the turn-on approach has enabled 'no-wash' imaging strategies[175]. The groups of Bertozzi and Tsien combined bioorthogonal chemistry with electron microscopy in creative ways to perform super-resolution imaging of non-protein biomolecules. Selected dyes could be used to image cells by fluorescence,

and then these cells were fixed and the dyes used to photooxidize the electron microscopy stain diaminobenzidine (DAB) to create contrast for electron microscopy imaging[296] (Fig. 6d). This approach led to unprecedented resolution in images of subcellular localization of DNA, RNA, lipids and metabolites. Another complementary technique is bioorthogonal Raman spectroscopy, where spectroscopically bioorthogonal Raman tags, such as alkynes, azides and nitriles, are directly engineered into biological targets in live cells[297]. Taken together, these bioorthogonal chemistry approaches provide a wide variety of possibilities for molecule-specific imaging of cells with different imaging modalities.

Pharmaceutical applications.

Bioorthogonal chemistry has found widespread use in drug discovery programmes, including applications in target identification (for example, phenotypic screening), target engagement and selectivity profiling of lead compounds[298]. An immensely valuable method to evaluate these directions has been ABPP, which assesses protein functionality in complex physiological systems[299–301]. As compared with assessing mRNA or total protein levels, ABPP differentiates active proteins from inactive proteins resulting from zymogens, post-translationally modified and/or inhibitor-bound forms. This method is dependent on an activity-based probe that consists of a chemical ‘warhead’ (for example, an electrophile), designed to covalently react in an activity-dependent manner, attached via a linker to a reporter group that allows for downstream analysis of labelled proteins by in-gel fluorescence, western blot and/or mass spectrometry-based proteomics that are attached to each other with a linker[299,300]. Relevant applications of ABPP include identification of activity in different cancer cell lines[302], characterization of aberrant enzymatic activity in aggressive cancer cells[303] and illumination of previously uncharacterized protein targets[302–305].

Zymogens

inactive forms of an enzyme. The enzyme takes its active form following a natural biochemical process such as cleavage, hydrolysis or post-translational modification.

When evaluating reversible small molecules as ligands across the proteome, a probe can be designed for photoaffinity labelling[301,306,307]. In addition to a protein binding element, photoaffinity probes possess a photo-reactive group, such as benzophenone, phenyl azide or diazirine. Upon irradiation with UV light, these groups form reactive species, each by a unique chemical mechanism, to generate covalent bonds with proximal amino acid residues[306,307]. Similar to ABPP, a reporter group is necessary for identification. Photoaffinity labelling has been useful for finding protein targets in phenotypic screens[306], as well as off-target interactions that can lead to undesired toxicological outcomes, and discovering novel protein–protein interaction partners as exemplified by the interaction of IFITM3 with γ-secretase[308,309].

Common tagging groups for activity-based and photoaffinity probes include biotin and various fluorophores; however, the incorporation of these large reporter groups can negatively impact cellular permeability and localization. Towards this end, less perturbative

CuAAC chemistry handles (for example, azide or alkyne) have been incorporated to improve the physiochemical properties of the probes[240,310,311]. Copper-free clickable handles have also been utilized in activity-based probes to increase experimental throughput and avoid copper removal steps that can result in protein loss[312]. These bioorthogonal probes have been useful for screening compound libraries in the development of selective inhibitors[305,313] and for assessing target engagement and proteome selectivity for small-molecule drug candidates[314–316].

Bioorthogonal chemistry handles also grant modularity during a proteomic workflow for both ABPP and photoaffinity labelling (Fig. 7a). For example, cleavable linkers and/or isotopic reporters can be incorporated to assess sites of engagement and/or perform quantitative analysis[317,318]. A variant of ABPP employs a generally reactive warhead, such as cysteine-reactive iodoacetamide appended to an alkyne handle, to discover novel ligandable hotspots in the proteome[319,320]. These ligands can provide useful handles for developing novel small-molecule inhibitors or for other therapeutic modalities such as targeted protein degradation[321].

Targeted protein degradation

A technique used for targeting specific proteins for degradation within a cell. Commonly, hetero-bifunctional small-molecule compounds are used for targeting the protein of interest to an E3 ubiquitin ligase protein. This facilitates the polyubiquination and subsequent degradation of the targeted protein.

An additional area of interest in drug discovery that has benefited from bioorthogonal chemistry is the development of ADCs. ADCs combine a potent small-molecule drug and a highly selective antibody for delivery to an antigen-specific cell type[322]. This approach has been utilized primarily in oncology indications to avoid toxic effects to normal tissue cells by targeting the cytotoxic molecule directly to cancerous tissues. Bioorthogonal chemistry has played a pivotal role in linking the small molecule to the antibody[322]. Traditionally, this linkage occurs via acylation of lysine residues by *N*-hydroxysuccinimide esters or alkylation of cysteines by maleimide; however, heterogeneous labelling of the antibodies has led to the exploration of alternative chemistries, including the development of site-selective small-molecule conjugation strategies that exploit the incorporation of UAAs, followed by bioorthogonal conjugation reactions such as SPAAC or oxime condensation[323].

Bioorthogonal uncaging strategies.

Successes in the rapidly evolving field of bioorthogonal conjugation inspired the development of bioorthogonal cleavage approaches to release or activate small molecules and biomolecules[242] (Fig. 7b). Most cleavage reactions were derived from their bioorthogonal bond-forming counterparts, typically resulting in conjugation intermediates designed to decompose and release a payload. By initially targeting and later releasing a bioactive molecule, bioorthogonal uncaging represents a way to achieve spatio-temporal control over cargo delivery in biological systems. In addition to the parameters that are key

for successful bioorthogonal conjugation (reactivity and stability), the release rate and the nature of the group being released are important factors when choosing a cleavage reaction.

The first examples used the Staudinger ligation and Staudinger reaction to achieve, respectively, the elimination of a carbamate from the part originating from the triphenylphosophine[324] or an azide to amine reduction[71,325], optionally followed by further release of a self-immolative linker to liberate the payload. Similarly, cycloaddition of TCO with organic azide affords an unstable product that rearranges to liberate the corresponding amine[326]. The well-known SPAAC between cyclooctyne and azide has not yet been transformed into a dissociative reaction, but the conjugation between cyclooctyne and sydnones has led to efficiently cleavable iminosydnones[144]. Also, hydroxyl-functionalized cyclooctyne was used to release an amide-linked payload from a tetrazine following cycloaddition and intramolecular cyclization[327]. The IEDDA between tetrazines and dienophiles has proven to be a fertile ground for the development of several tetrazine-triggered cleavage reactions, such as the cleavage of allylic-substituted TCO (IEDDA pyridazine elimination)[328], vinyl ethers[329–331], 3-isocyanopropyls[332] and carbamate-linked benzonorbornadienes[333]. From this series, allylic-substituted TCO has the highest reactivity[328], mirroring the reactivity differences between the click conjugation reactions. Recently, click conjugation reactivity was increased by three orders of magnitude by the development of a new IEDDA pyridazine elimination mechanism where the roles are reversed, with highly reactive TCO triggering payload release from a tetrazine linker[334]. Isonitriles were shown to cleave this tetrazine linker through a slightly different mechanism[335]. Finally, the well-known fluoride-mediated removal of the *tert*-butyldimethylsilyl (TBS) protecting group in organic synthesis was recently reworked into an effective bioorthogonal cleavage reaction by using imaging agent-derived Phe-BF_3 as the fluoride source[336].

Self-immolative linker

A class of linker that, when exposed to a certain trigger, is designed to break the payload connecting bonds via an intramolecular process.

Bradley and co-workers have used transition metals as triggers for bioorthogonal cleavage reactions[219]. The amine-protecting allyloxycarbonyl (alloc) group can be catalytically removed in physiological conditions by ruthenium and palladium complexes[337,338]. Pd^0 nanoparticles and palladium complexes encapsulated in nanoparticles afforded selective alloc removal in cells and mice[219]. Likewise, the amine-protecting propargyl group has been catalytically removed by palladium[338], copper[339] and gold[340] complexes as well as metallic palladium[341]. Hydroxyl groups were caged with an allene moiety and uncaged with palladium catalysts[342]. Although the use of metals or metal complexes may pose challenges in living systems in view of long-term stability and biocompatibility, their catalytic nature offers compelling opportunities, for example in prodrug activation.

Prodrug

A pharmacologically inactive precursor compound that is converted into an active drug through in vivo chemical modification achieved via metabolic/enzymatic processes. Prodrugs are employed to improve pharmacokinetic properties (absorption, distribution, metabolism and elimination) and pharmacodynamics properties (selectivity, reduction of adverse effects) of the active drug molecule.

In vivo chemistry.

A central development in bioorthogonal chemistry has been the enormous reaction rate increase as new transformations are developed. As the reactivity increased, so did the application scope, leading to today's use in a wide range of fields. Higher reactivity enables equimolar reagent stoichiometry, lower concentrations and shorter reaction times, and therefore allows demanding applications such as those found in medicine.

The Staudinger ligation and SPAAC were successfully used for the detection of metabolically engineered tissues in mice[343], but the reaction kinetics ($k_2 \sim 10^{-3}$–10^{1} M^{-1} s^{-1}) required a high dose of secondary reagent (such as triarylphosphines and cyclooctynes, respectively) to achieve detectable binding, limiting the application scope. Nevertheless, there are examples of medical applications, such as SPAAC-mediated clearing of azide-containing warfarin[344] and cardiac homing of endogenous stem cells[345].

The rate constant for the tetrazine ligation ($k_2 \sim 10^{5}$ M^{-1} s^{-1}) is on a par with biomolecule association rates, opening up applications where chemistry substitutes for, or works in conjunction with, biology, at the same timescale and the same low concentrations. It was shown to be fast enough to be used for pre-targeted radioimmunoimaging, which involved treating tumour-bearing mice with TCO-tagged monoclonal antibody followed a few days later by administration of an equimolar amount of a fast-clearing radiolabelled tetrazine probe and its reaction with the tumour-bound monoclonal antibody[346–348] (Fig. 7c). This approach has since been markedly improved by the development of tags with improved reactivity and stability[349], pharmacokinetics[350], the introduction of bioorthogonal monoclonal antibody clearing agents[351,352] and the development of a wide range of probes for imaging[353,354] and for radiotherapy[355,356], together affording strongly increased tumour to normal tissue ratios. As a result, the tetrazine ligation is now an established method for companion diagnostic imaging of monoclonal antibodies and pre-targeted radioimmunotherapy in live animals.

Mirroring its parent reaction, the high reactivity of the IEDDA pyridazine elimination reaction has led to widespread utility[328], applications of which include unmasking of TCO-containing ADCs[357,358] (Fig. 7d), prodrugs[359], proteins[360] and peptide antigens[361]. Contrary to the imaging and radiotherapy applications, in cleavage applications the tetrazine is typically not radiolabelled and can be dosed in excess to achieve complete activation yields, as demonstrated for the activation of a tumour-bound ADC by a tetrazine administered intravenously in a second step[357,358]. Other approaches centre on the pre-administration of the tetrazine[362,363], such as the intratumoural injection of a

tetrazine followed by intravenous administration of a TCO prodrug, local capture and drug activation[359]. The IEDDA between liposome-encapsulated tetrazine and vinyl ether prodrug afforded an enhanced permeability and retention-mediated drug release in tumours[364], and the iminosydnone release reaction was used to trigger fragmentation of and payload release from a tumour-bound micelle[344]. Likewise, nanoparticle-bound drugs were efficiently activated inside cancer cells in vivo using the desilylation reaction[336]. Finally, nanoparticle-encapsulated palladium complexes have been successfully used for on-tumour prodrug activation of alloc-caged doxorubicin as well as its micelle-encapsulated analogue[365,366].

Most applications of in vivo chemistry have been mainly performed in mice. Bioorthogonal uncaging using the TCO–tetrazine pyridazine elimination has recently entered phase I clinical evaluation as a method of breast cancer chemotherapy[367]. Future clinical studies are expected to be fuelled by very promising results in several of the imaging, radiotherapy and drug delivery studies.

Polymer and materials science.

Bioorthogonal reactions have emerged as enabling tools in the areas of polymer science, materials science and surface science. In the field of polymer modification, CuAAC has been impactful with applications extending to the synthesis and functionalization of essentially every polymer class, including adhesives, block and multiblock copolymers, brush and comb copolymers as well as star, hyperbranched and dendronized polymers[281,368–371]. Bioorthogonal chemistry can also be used to create high molecular weight polymers through polymer–polymer couplings[372]. The tetrazine ligation has been used to investigate defects in polymer network formation[373]. Bioorthogonal reactions have also been used as tools for the construction of monodisperse macromolecules including dendrimers[374] and sequence-specific polymers[375]. Here, the sequential application of two mutually orthogonal reactions can be used to produce well-defined macromolecules. For example, CuAAC in combination with sulfuryl fluoride exchange (SuFEx) chemistry (BOX 1) has been applied to the preparation of sequence-specific polymers[376], and SPAAC in combination with tetrazine ligation has been used to produce dendrimer scaffolds attached to antibodies that can be used to amplify the signal in pre-targeted positron-emission tomography imaging[377].

Sequence-specific polymers

Macromolecules that are monodisperse with defined monomer sequences or block sequences. This requires controlled, sequential addition of subunits using highly efficient bond-forming chemical reactions. Naturally occurring examples of sequence-specific macromolecules include DNA, RNA and protein.

Multivalency is a ubiquitous phenomenon in biology involving the simultaneous binding of multiple copies of a ligand displayed on one surface to complementary binders or receptors on another surface. Bioorthogonal chemistry has been used broadly to enable the multivalent display of designer ligands based on naturally occurring biomacromolecules. For example, CuAAC has been used to label the outer and inner surfaces of viral particles[378,379], and

has been a widely used tool for the creation of multivalent glycomaterials, glycopolymers and glycosurfaces[380]. Bioorthogonal reactions are also important to the functionalization of surfaces as well as nanoparticles[381]. CuAAC emerged as an early tool for electrode and surface functionalization[382], and, more recently, SPAAC, tetrazine ligation and other bioorthogonal reactions have become important tools for the selective attachment of proteins and other biomolecules to surfaces[383]. Beyond surface modification, bioorthogonal chemistry also enables the creation of 3D networks, with applications that include the development of hydrogel supports for cell culture applications[241]. Here, copper-free methods including SPAAC and tetrazine ligation are proving especially useful for the assembly of 3D scaffolds that display biological ligands capable of supporting tissue-like structures[384,385].

Materials construction (and deconstruction) in response to external stimulus is a field of increasing importance in various applications, and bioorthogonal reactions allow for creative applications of molecular connectivity in tailored circumstances or environments. Examples include photoinducible ligations, which have proven highly useful for the activation and patterning of surfaces and 3D materials, such as for biomolecular attachment in 3D scaffolds[386,387]. The tetrazole photoclick chemistry has also been employed in materials science for preparation of photodegradable supramolecular hydrogels[388], grafting polymers onto silicon and cellulose surfaces[389], and synthesis of polymeric networks[390]. Thiol–ene and thiol–yne chemistry has so far been the technique of choice for light-based 3D printing, such as for interesting hydrogel materials[391]. The inherent reversibility of properly designed thiol–ene connections has also been exploited for controllable disassembly[392]. Similarly, the rapid kinetics of tetrazine ligation can be used for further advantage in materials assembly through interfacial processes that can be used for attaching ultrasound microbubbles to cell surfaces[393], as a method for modulating cell–cell adhesion[394] and as a method for the creation of molecularly patterned fibres[395], core-shell particles[396] and hydrogel channels[397] or shapes[398] capable of directing cell behaviour in three dimensions.

Reproducibility and data deposition

The characterization of new bioorthogonal reagents and small-molecule conjugates follows the standards of the field of organic chemistry. At a minimum, new compounds are generally characterized by ^{1}H and ^{13}C NMR spectroscopy as well as elemental analysis and/or high-resolution mass spectrometry. Additional characterization by NMR (probing other nuclei, 2D NMR experiments as well as measuring the nuclear Overhauser effect), Fourier transform infrared spectroscopy, chromatography (for example, high-performance liquid chromatography or gas chromatography) and X-ray crystallography are also routinely performed to support structural assignments. In general, copies of spectral data along with spectral peak listings are submitted for peer review and made available as supporting information by the journal at the time of publication. Additionally, X-ray crystallographic data are typically deposited in the Cambridge Structural Database. Where possible, kinetic rate constants should be measured under purely aqueous conditions to enable rate comparisons across different reaction types and, where applicable, the effect of the catalyst concentration on the rate should also be indicated. One of the hallmarks of bioorthogonal chemistry is that reactions are reliable and successful under a wide range of conditions.

However, the high reactivity associated with the most reactive bioorthogonal substrates can also require additional considerations to verify reagent quality prior to use. It is generally advisable to store reagents under refrigeration and to periodically validate reagent quality by spectroscopic (such as NMR) or chromatographic (such as high-performance liquid chromatography) analysis.

Additionally, reagent stability should be evaluated under physiological conditions. Valuable methods include studying the reagent in the biological milieu of interest (for example, blood, serum, cell lysate) as well as in the presence of glutathione at 10 mM, which approximates the thiol's intracellular concentration. In addition to rigorous analytical chemical characterization, bioorthogonal reactions should be characterized on labelled biomolecules whenever possible. Mass spectrometry as well as quantification by high-performance liquid chromatography or gel electrophoresis should be routinely used for deducing reaction conversions and yields. To validate bioorthogonal reaction efficiency in live cells, it is generally useful to quantify labelling using fluorescent probes and in-gel fluorescence. Mass spectrometry can be used to validate overexpressed protein targets in bacteria. In mammalian cells, it is useful to validate specificity through labelling of recombinant fluorescent proteins with fluorescent probes, where co-localization of fluorescence from the protein and probe can be used to provide a measure of the specificity and efficacy of labelling.

Limitations and optimizations

The impact of bioorthogonal chemistry on biology and biomedical research has been facilitated by the increasing availability of commercial reagents to the wider scientific community, and a growing number of companies now specialize in reagents for bioorthogonal and click chemistry. An increased variety of bioorthogonal reagents in formats that are ready to use for bioconjugation and applications including fluorescent imaging, radiochemistry and proteomics would be beneficial for researchers using these tools. There is a continued need to develop improved reagents that display rapid kinetics while exhibiting high stability towards extended incubation in the cellular or in vivo environment. There are opportunities for synthetic chemists to further impact the scientific community by creating new and improved bioorthogonal reagents.

The expanding catalogue of bioorthogonal reactions has brought the associated challenge of deciding how to choose a bioorthogonal reaction for a given application. As pointed out above, different reactions in the bioorthogonal toolbox have associated strengths and limitations, and it is advisable for practitioners to begin by prioritizing the characteristics that are most important for the application. With small and readily available reaction partners and fast rates under catalytic conditions, CuAAC has been applied across a remarkable range of applications in vitro and in extracellular and cell surface settings, but, as noted above, is typically less well suited for intracellular labelling applications. For intracellular and in vivo applications where fast reactivity is required, the kinetics of tetrazine ligation can enable fast labelling even at sub-micromolar concentrations. For tetrazine ligation, a consideration is that both reaction partners are relatively bulky, especially for TCO dienophiles, which are also the most reactive. Cycloalkyne [3 + 2]

cycloadditions also function efficiently in the intracellular environment, with the advantage of using azides and other small dipoles as one reaction partner. However, the kinetics are generally less rapid than tetrazine ligation and require concentrations in the high micromolar to millimolar range. Native chemical ligation serves as the standard for the conjugation of large peptidic or protein fragments using native amide linkages but is relegated to in vitro applications. The Staudinger ligation offers an alternative for the formation of amide bonds in cellular or in vivo settings, but can be limited by bulky reagents and relatively slow kinetics. Oxime, palladium-catalysed and ruthenium-catalysed reactions offer unique new functional group handles in bioorthogonal chemistry applications. With some exceptions, these tools are generally best suited for applications in vitro and in the extracellular environment. Photoclick chemistry has proven to be a powerful tool for exercising spatio-temporal control over bioorthogonal reactivity in varied settings, including live-cell applications. Continued efforts to reduce the size of photoinducible groups and red-shift activation wavelengths will enable a wider range of applications. Finally, the development of cleavage reactions with increased reactivity and release kinetics would provide researchers with a wider set of uncaging tools.

Outlook

Bioorthogonal chemistry has emerged as an enabling method for the construction of molecules with complexity and in contexts that were previously unthinkable. The impact of bioorthogonal reactivity has been far-reaching with applications spanning protein synthesis, drug discovery, in vivo chemistry and polymer science. Moving forwards, bioorthogonal chemistry will continue to develop into a tool capable of delivering new drugs, creating new materials and answering biological questions that cannot be addressed by modern molecular biology tools (such as GFP and CRISPR–Cas9) or small-molecule ligands.

Despite tremendous progress in the development and application of bioorthogonal chemistry, there is much work to be done. Although all bioorthogonal groups are much lower in molecular weight than directly expressed protein tags, bioorthogonal linkers still represent a perturbation to the biological system under study, and bulky derivatives may not be accepted by the enzymatic machinery used to incorporate a tag into a biological target. There is still a need for new reagents that are small, safe, stable and capable of rapid kinetics, with high value for reagents that work efficiently inside living cells and animals. Initial efforts to minimize the size and improve the physiological properties of bioorthogonal groups have been successful, yet few classes of bioorthogonal reactions can produce native biological bonds such as amides. New classes of reactions capable of producing native covalent bonds with rapid kinetics in living systems would be valuable tools for chemical biology. The continued advancement of ‘orthogonal bioorthogonal’ reactions[399], which enable the simultaneous use of multiple bioorthogonal reactant pairs in living systems, offers another level of control over the biological system under study and is valuable. Furthermore, bioorthogonal covalent reactions that can be reversed using benign triggers would be ideal tools for studying and manipulating biological systems.

Biological imaging has been advanced through the development of methods that couple bioorthogonal chemistry to imaging in living systems. Innovations in the development of

fluorogenic bioorthogonal reactions as well as in vivo radiochemistry have emerged as powerful imaging tools from the cellular to the whole animal level[346–356]. Complementary approaches are needed that will continue to push the limits of detection by ameliorating non-specific interactions or side reactions that can decrease signal to noise ratios, and thereby enable new frontiers in imaging, including the live-cell imaging of low abundance biomolecular targets (such as endogenous proteins), and in the development of in vivo approaches for diagnosing, monitoring and staging human diseases in the clinic. These developments will benefit from ongoing efforts in synthesizing sophisticated dyes and fluorophores with custom-designed physical properties to illuminate individual biomolecules in complex biological systems.

Chemical techniques for site-specific attachment of bioorthogonal handles onto biomacromolecules have made tremendous advancements in recent years. Recombinant methods to install uniquely reactive peptide tags have simplified site-specific incorporation with broad substrate scope onto protein targets. But these methods do suffer from incomplete conversions and require high reagent concentrations, which results in limited in vivo use. Genetic code expansion has also been instrumental for installing bioorthogonal handles with amino acid precision devoid of extra peptide tags. However, extensive re-engineering of machinery is often necessary to accommodate new substrates. Simple methods for quantitative, non-invasive, highly pure, site-specific attachment of small molecules to proteins in a living system with broad substrate scope are highly desirable, and represent an idealized protein conjugation method to strive for. Substantial effort has been focused on protein conjugation strategies; expanding these strategies to other biomacromolecules and complex metabolites remains largely unexplored and a critical area for newer methodology.

Photoclick chemistry has also provided a powerful method for the study of cellular processes with spatio-temporal precision. Moving forwards, the control offered by photoinducible bioorthogonal chemistry may enable real-time imaging of individual biological molecule dynamics and provide a tool for elucidating the biomolecule function in signalling pathways. The development of photoinducible bioorthogonal chemistry based on tissue-penetrant near-IR light[180] may offer the ability to induce rapid reactivity in vivo, providing a new level of precision for studying pathogenesis in living animals.

From the intersection of organic chemistry and biology, bioorthogonal chemistry has emerged as an essential way to construct biological molecules and to study them in their native environment. With ever-increasing momentum, bioorthogonal chemistry is enabling research in biology and biomedicine and is influencing scientists across the spectrum of disciplines through a broad range of applications.

Acknowledgements

J.A.P. acknowledges support over the years from the Alfred P. Sloan Foundation, the Camille & Henry Dreyfus Foundation and the National Institutes of Health (NIH) (R01 GM126226). J.M.F. acknowledges support from the NIH (R01 GM132460), National Science Foundation (NSF) (DMR 1809612 and 2011824) and Pfizer. Q.L. acknowledges support from the NIH (R35 GM130307) and NSF (CHE-1904558). K.L acknowledges support from the German Science Foundation DFG through programmes SFB1035 and SPP1623.

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Box 1 |

Sulfuryl fluoride exchange chemistry

Sulfuryl fluoride exchange (SuFEx) chemistry represents a class of biocompatible click reactions described in 2014 by Sharpless and colleagues, who identified sulfuryl fluorides (RSO_2F) and related species as having balanced reactivity well matched to bioorthogonal applications[5]. Sulfur(VI) fluorides are compatible with water and are potently activated by acid-mediated stabilization of fluoride as a leaving group in aqueous media, the H···F interaction being the strongest known hydrogen bond[400]. Sulfuryl fluorides and related nitrogen-containing groups (such as sulfuramidimidoyl fluorides)[401] are thereby proving to be quite selective in biomolecular labelling promoted by initial non-covalent interactions. In the formation and modification of polymeric materials, activation of the S–F bond by silyl reagents has proven especially useful. Thus, the combination of building blocks containing S–F and Si–O bonds, when addressed by the right nucleophilic catalysts, gives very high yields of S–O linkages, thermodynamically driven by the concomitant formation of ultra-stable Si–F by-products[402]. The term ‘exchange’ in the SuFEx moniker is meant to emphasize the fact that both the departing fluoride and the entering alkoxide/aryloxide must be appropriately activated in order for the reaction to proceed, and that thermodynamic factors governing the swap of S–F for S–O bonds are more balanced than is typical for other bioorthogonal ligations. As the most recently recognized click reaction, SuFEx chemistry is only now becoming recognized as an especially useful tool for numerous biological applications[403], and relevant reagents for SuFEx chemistry are becoming increasingly available[404,405].

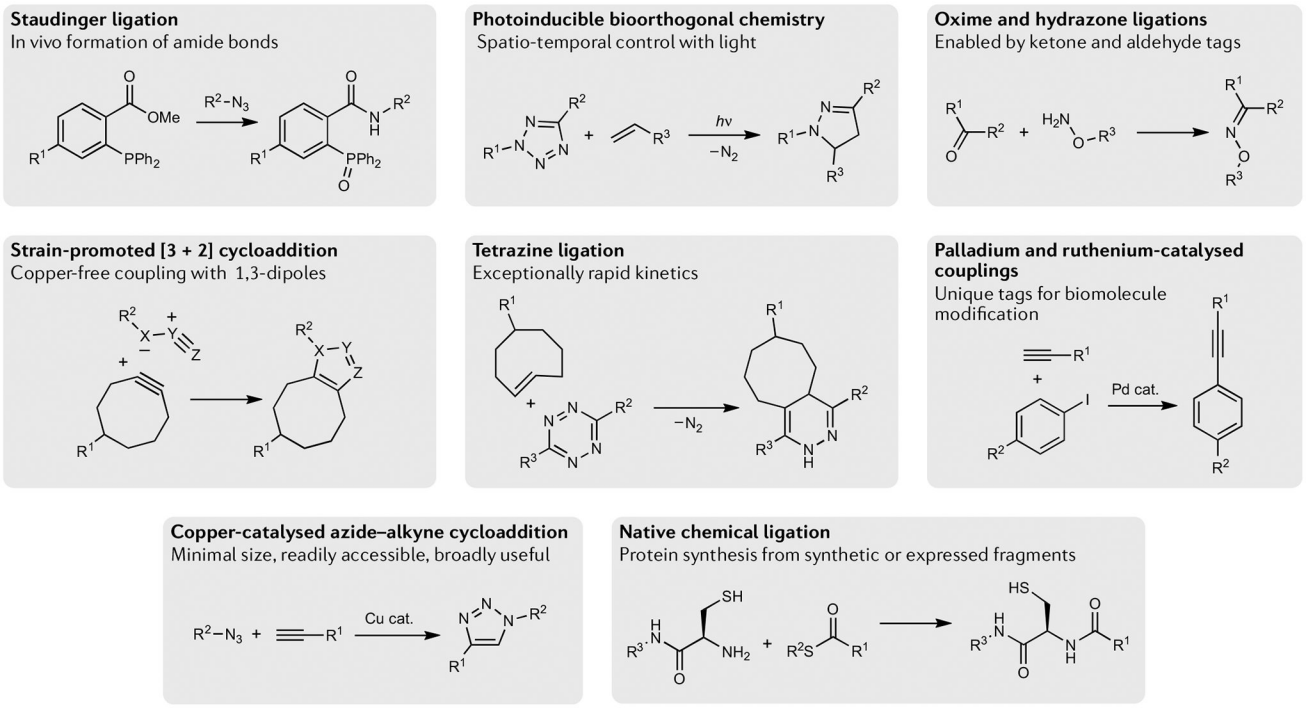

Fig. 1 |. Different classes of bioorthogonal reactions.

The broad range of bioorthogonal reactions with their associated reactants, key reagents, products and key feature(s) are highlighted here.

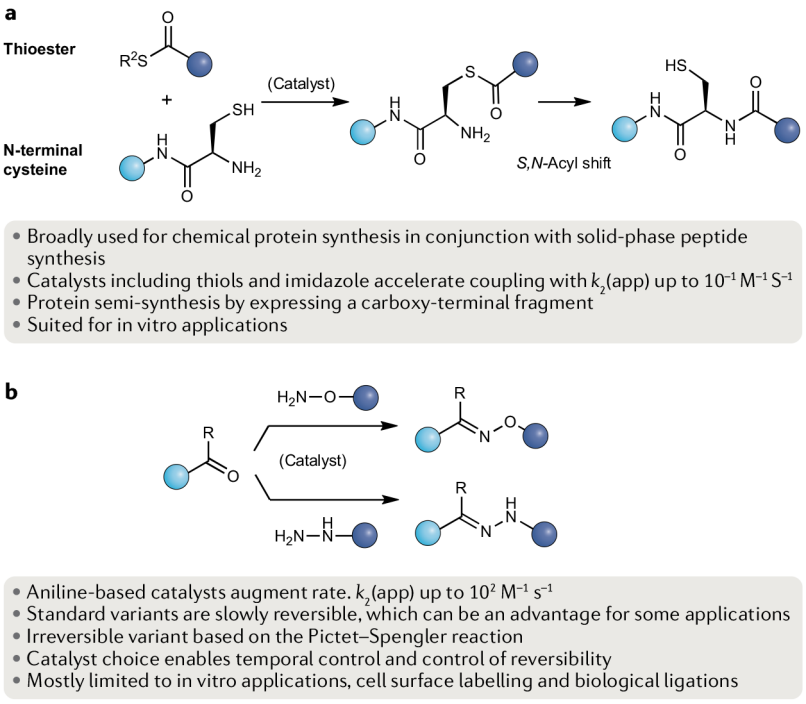

Fig. 2 |. The native chemical ligation and oxime and hydrazine ligations.

a | Native chemical ligation is enabled by a catalysed reaction between a thioester and an amino-terminal cysteine residue to afford a native amide bond through a thioester intermediate that undergoes an *S*,*N*-acyl shift. **b** | Using aniline-based catalysts, the oxime and hydrazone ligations occur between a carbonyl group with a hydroxylamine or a hydrazine, respectively. k_2, second-order rate constant.

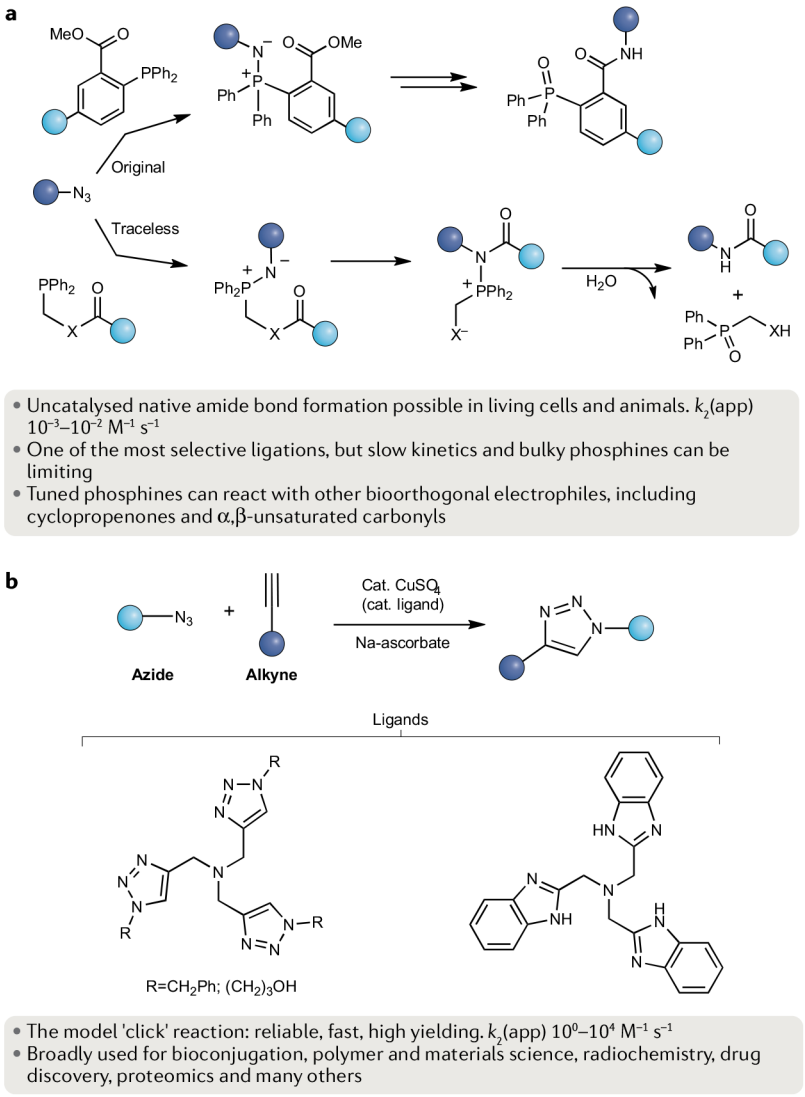

Fig. 3 |. The Staudinger ligation types and the copper-catalysed azide–alkyne reaction.
a | Both the original and traceless Staudinger ligations enable native amide bond formation between an azide and a carbonyl group using triaryl phosphines to afford the product and a phosphine oxide as a by-product. **b** | The model ‘click reaction’ between an azide and an alkyne enabled by use of a copper-based catalysis route to afford a triazole product. Ligand examples are provided. k_2, second-order rate constant.

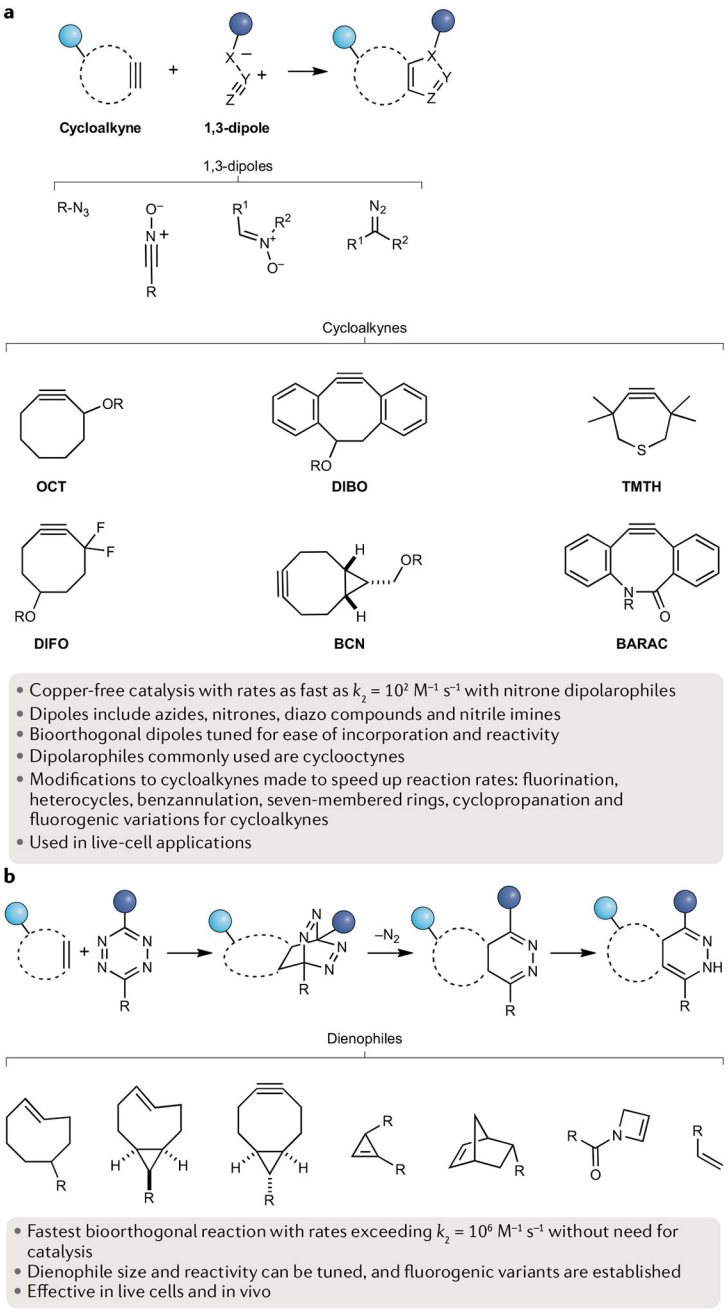

Fig. 4 |. Cycloaddition-based bioorthogonal chemical reaction types.

a | Strained alkynes enable copper-free catalysis of cycloaddition to dipoles such as azides. **b** | The fastest biorthogonal reaction involves the inverse-electron demand [4 + 2] addition between a tetrazine and a dienophile. BARAC, biarylazacyclooctynone; BCN, bicyclo[2.1.0]nonyne; DIBO, dibenzocyclooctyne; DIFO, difluorinated cyclooctyne; k_2, second-order rate constant; OCT, cyclooctyne; TMTH, 3,3,6,6-tetramethylthiaheptyne.

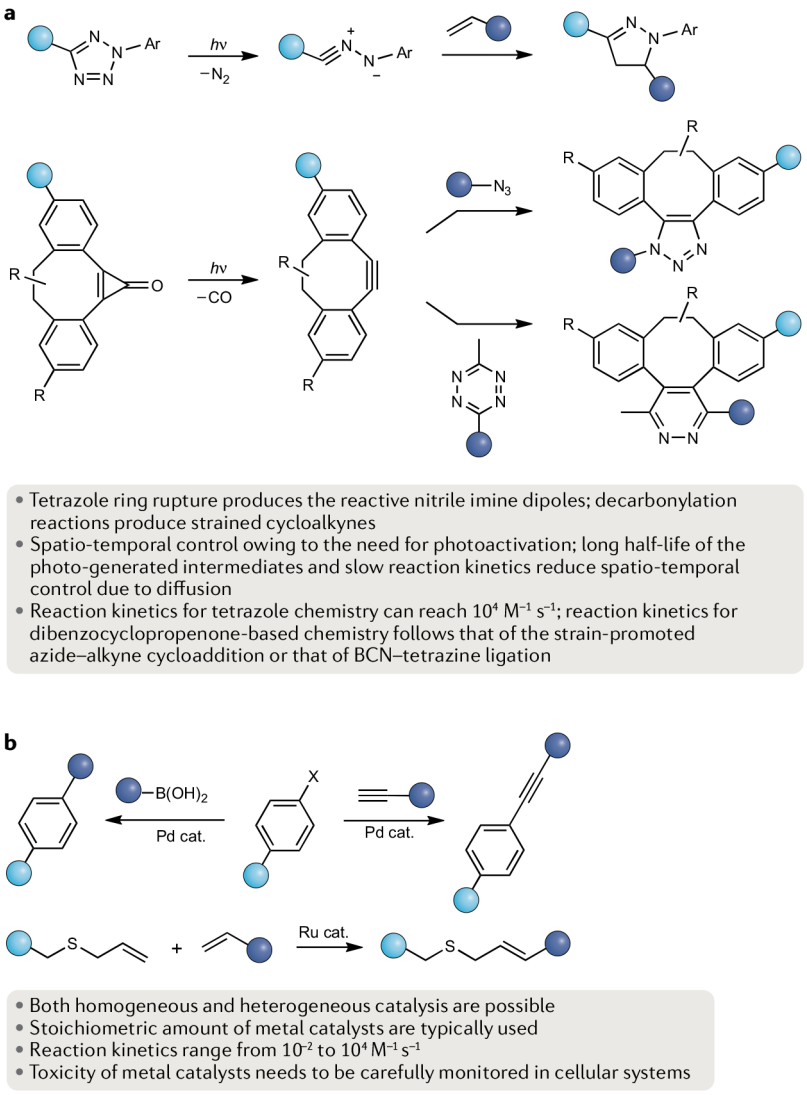

Fig. 5 |. Light-activated click chemistry and metal-catalysed coupling reactions.
a | Two examples of photoclick chemistry: a photoinduced tetrazole-alkene 1,3-dipolar cycloaddition reaction to generate a pyrazoline adduct (top) and a photo-triggered alkyne-azide cycloaddition reaction (bottom). The cycloalkyne is masked in the dibenzocyclopropenone form. **b** | Two examples of palladium-catalysed reactions: Suzuki–Miyaura cross-coupling and copper-free Sonogashira cross-coupling (top left and right, respectively), and ruthenium-mediated olefin cross-metathesis (bottom) involving the use of allyl chalcogen-based privileged substrates. BCN, bicyclo[2.1.0]nonyne.

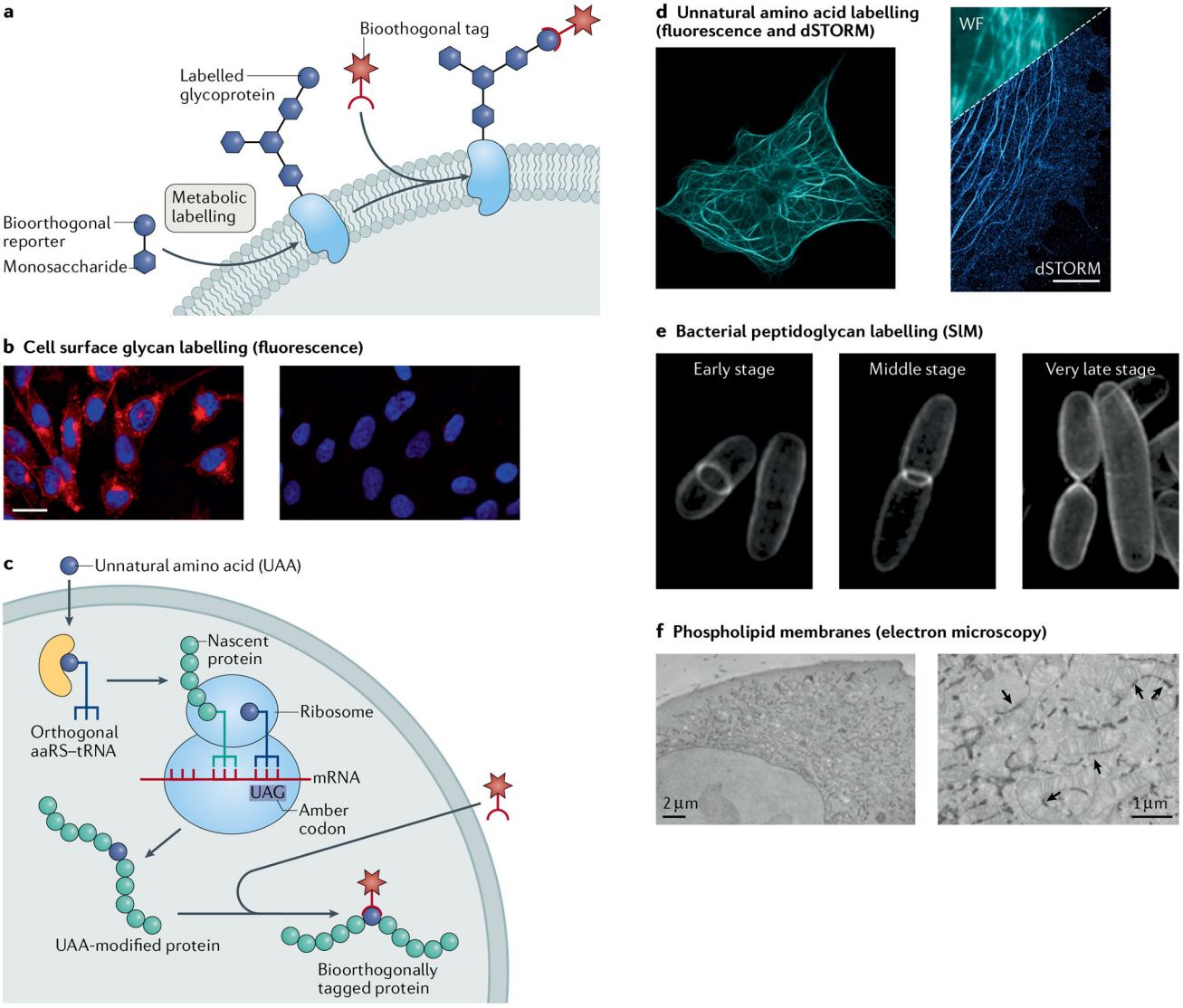

Fig. 6 |. Applications for labelling different molecule types in cells.

a | Model for metabolic engineering for cell labelling and imaging. **b** | Fluorescence microscopy of CHO cells incubated in the presence (left) or absence (right) of peracetylated *N*-azidoacetylmannosamine ($Ac_4ManNAz$) and labelled with a fluorophore by the Staudinger ligation. **c** | Model for genetic code expansion as a strategy for cell labelling and imaging. **d** | Fluorescence and direct stochastic optical reconstruction microscopy (dSTORM) super-resolution images of COS-7 cells where microtubule-associated protein was encoded with an unnatural *trans*-cyclooctene (TCO) amino acid and tetrazine ligation was used to attach a microscopy dye. **e** | Structured illumination microscopy (SIM) images of *Escherichia coli*, where *N*-azidoacetyl-muramic acid (NAM) was metabolically incorporated into the bacterial peptidoglycan and fluorophore-labelled by copper(I)-catalysed azide–alkyne cycloaddition (CuAAC). **f** | Electron microscopy images of HeLa cells, where azido-choline was metabolically incorporated, and cyclooctyne/azide click chemistry was used to conjugate electron microscopy imaging agents. The

arrows indicate sites of endoplasmic reticulum–mitochondria contacts. aaRS, aminoacyl-tRNA synthetase; WF, widefield image. Images in panel **b** adapted with permission from REF.[252], ACS. Images in panel **d** reprinted from REF.[176], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Images in panel **e** reprinted from REF.[286], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Images in panel **f** reprinted from REF.[296], Springer Nature Limited.

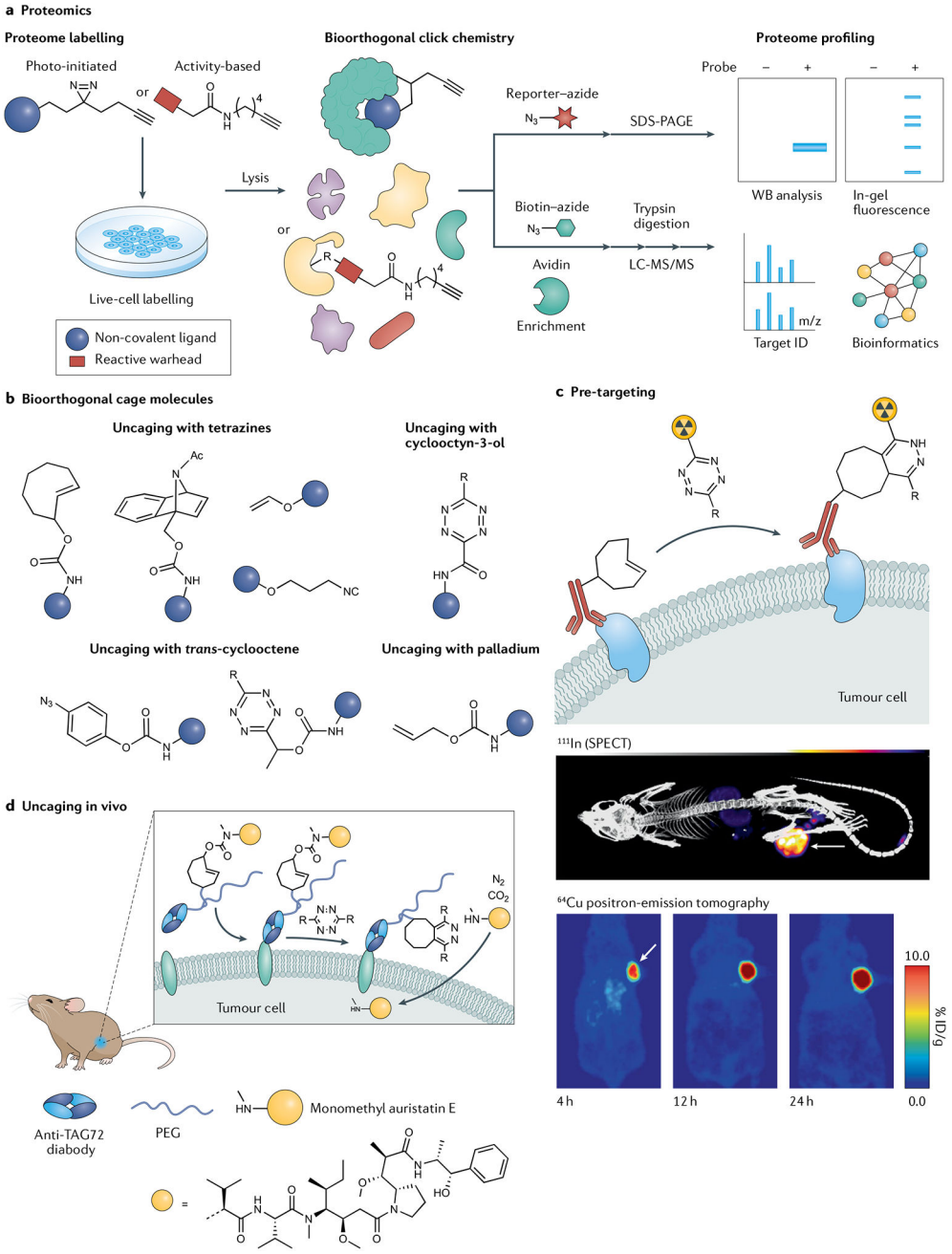

Fig. 7 |. Examples of biorthogonal chemistry applications in vitro and in vivo.

a | Enrichment strategies for proteome labelling enabled by the copper-catalysed azide–alkyne reaction. **b** | Examples of bioorthogonal cage molecules. **c** | The tetrazine ligation as a strategy for pre-targeted radiochemical imaging of cancer. **d** | Uncaging small-molecule cargo has been applied at tumour sites in animal models. LC-MS/MS, liquid chromatography with tandem mass spectrometry; SPECT, single-photon emission computed tomography; WB, western blot. Middle image in panel **c** originally published in REF.[351], JNM. Rossin, R., Läppchen, T., Van Den Bosch, S. M., Laforest, R. & Robillard, M. S. Diels–Alder reaction for tumor pretargeting: in vivo chemistry can boost tumor radiation

dose compared with directly labeled antibody. *J. Nucl. Med.* **54**, 1989–1995 (2013). © SNMMI. Bottom image in panel **c** adapted with permission from REF.[354], ACS. Panel **d** adapted from REF.[357], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).