Fitness Factors for Bioorthogonal Chemical Probes

Yulin Tian[†] and Qing Lin*

Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260-3000, United States

qinglin@buffalo.edu

Abstract

Bioorthogonal chemistry has offered an invaluable reactivity-based tool to chemical biology owing to its exquisite specificity in tagging a diverse set of biomolecules in their native environment. Despite tremendous progress in the field over the last decade, designing a suitable bioorthogonal chemical probe to investigate a given biological system remains a challenge. In this perspective, we put forward a series of fitness factors that can be used to assess the performance of bioorthogonal chemical probes. The consideration of these criteria should encourage continuous innovation in bioorthogonal probe development as well as enhance the quality of biological data.

Key words

Bioorthogonal Chemistry: A subfield of chemistry in which chemical reactions are carried out inside a living cell with no interference to its native biochemical processes. A common goal of bioorthogonal chemistry is to modify a biomolecule of interest in its native environment with a chemical in order to track its localization or regulate its function.

Chemical Reporters: Modified chemical building blocks of cells including amino acids, nucleosides, lipids, sugars, and cofactors containing a bioorthogonal chemical handle. A chemical reporter needs to be biochemically introduced into biomolecules for functional studies.

Bioorthogonal Chemical Probes: A class of chemical compounds comprising of a bioorthogonal reactive group and a biophysical/biochemical probe. The reaction of bioorthogonal chemical probe with its cognate reaction partner pre-installed in a biomolecule of interest allows selective attachment of the biophysical/biochemical probe to the biomolecule *in vivo* for functional studies.

Fitness Factors: The intrinsic molecular properties that determine the suitability of chemical probes for an interested biological system. For reactivity-based probes, the reactivity and selectivity represent two additional classes of fitness factors, in addition to those known to the binding-based small molecule probes.

Sequence Tags: An oligomeric structure exhibiting sequence-dependent chemical reactivity. The sequence tags are commonly identified through a biological selection experiment.

Fluorogenicity: The fluorescence associated with a chemical transformation. A fluorogenic probe exhibits enhanced fluorescence from either un-quenching or de novo synthesis.

Steric shielding effect: A type of steric effect in which the sterically bulky groups are used to control molecular conformation, which in turn regulate the reactivity of a bioorthogonal reagent.

Biomolecular incorporation: The incorporation of chemical reporters into biomolecular structures in cellular systems. Depending on the type of biomolecules involved, other terms such as genetic code expansion, metabolic engineering, and metabolic labeling may be used.

■ Introduction

Owning to their exquisite chemo-selectivity, bioorthogonal chemical probes enable covalent modification of the biomolecules and subsequent studies of their dynamics and function in their native environment.[1-3] Unlike the binding-based small-molecule probes, the reactivity-based bioorthogonal chemical probes require a pair of reaction partners: one as a chemical reporter to be installed into a biomolecule of interest through appropriate biochemical processes, and the other as a biophysical probe carrying the cognate reactive motif. To study the underlying biological process, both reaction partners need to be stable in biological milieu, bioavailable to reach their targets, mutually reactive yet inert to other molecules in cells and tissues.[4] The development of these biocompatible chemo-selective probes has allowed high-precision manipulation of biomolecules in living systems. Inspired by the wide adoption of fitness factors for the binding-based small-molecule probes,[5] we envision that a parallel set of fitness factors should be considered in contemplating the use of the reactivity-based probes in any biological system in order to generate the high-quality and reproducible biological data.

Early reports of bioorthogonal probes have focused on the discovery and optimization of the bioorthogonal reactions that permit selective ligation of a pair of reactants in biological systems. Later studies have greatly expanded the scope of bioorthogonal chemistry to include in situ assembly of bioactive compounds for drug discovery,[6] and bioorthogonal "click to release" of drugs,[7] signaling molecules,[8] and caged enzymes.[9] In this Perspective, we decide to focus on the bioorthogonal ligation probes because of their unique ability to interrogate biomolecular function in native cellular environment. In this context, while a large number of such reactions have become available, the design of reactivity-based probes for a specific biological system remains a challenge for the

following reasons. First, the efficiency of incorporating chemical reporters into the targeted biomolecules is highly variable and needs to be assessed directly. Second, the biological function of the probe-tagged biomolecules needs to be verified using appropriate assays. In this perspective, we propose four classes of fitness factors comprised of reactivity, selectivity, physicochemical properties, and biological context that the developers and users alike should consider when they design the bioorthogonal chemical probes (Figure 1). We present some selected examples to illustrate how optimization of these fitness factors can lead to their successful use in probing specific biological systems. Through the lens of fitness factors, we hope the discussions presented here will not only help the developer design better chemical tools but also assist the users in choosing appropriate bioorthogonal chemical probes for their specific biological systems.

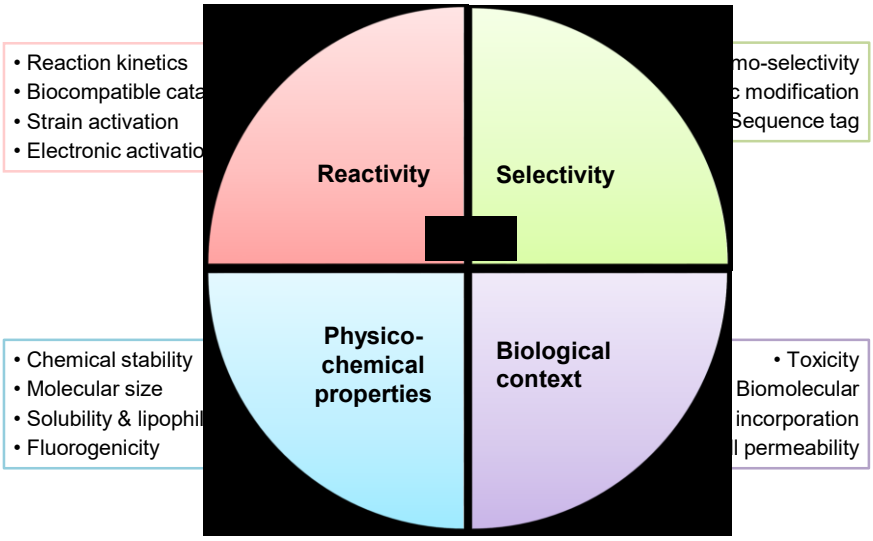

Figure 1. Fitness factors for bioorthogonal chemical probes.

■ Reactivity

Reactivity is without question one of the most important fitness factors to consider in the design of bioorthogonal chemical probes.[10] Since the majority of bioorthogonal reactions belong to

bimolecular ligation, the reaction rate is proportional to the second-order rate constant, k_2, and the concentrations of each reactant. With a higher rate constant, a more significant amount of product is generated at any given time. Moreover, the shortened exposure and the lower probe concentration reduce the undesired side effects such as toxicity and nonspecific reactions. A high second-order rate constant is also imperative for interrogation of fast biological processes involving low-abundance target molecules such as transmembrane receptors.

In optimizing reactivity, one strategy is to activate substrates through ring strain. For example, the tetrazine–*trans*-cyclooctene (TCO) ligation reported by Fox and co-workers represents the fastest bioorthogonal reaction known to date, with the second-order rate constant approaching 10^5 M^{-1} s^{-1}.[11, 12] Other strained alkenes and alkynes such as (*E*)-bicyclo[6.1.0]non-4-ene (sTCO),[13] bicyclononyne (BCN),[14] and spiro[2.3]hex-1-ene (Sph)[15] also undergo fast cycloadditions with tetrazines (Figure 2a).[16] On the other hand, substrate electronic tuning offers another way to improve reaction kinetics (Figure 2b). For example, 3,6-dipyridyl-*S*-tetrazine (DpTz) carrying the electron-withdrawing pyridyl groups offers a fast reaction with TCO.[17] Conversely, the electron-rich vinylboronic acid represents a robust and stable reaction partner for DpTz (Figure 2b).[18] For the tetrazole-based photoclick chemistry, substitution of 2,5-diaryltetrazoles with the electron-donating groups has been shown to accelerate the cycloaddition reaction by raising the HOMO energy of the *in situ* generated nitrile imines (Figure 2b).[19] For the sydnone–alkyne click chemistry, the electrophilic substitution of the sydnone ring with fluorine increased the second-order rate constant to 10^4 M^{-1} s^{-1} (Figure 2b).[20]

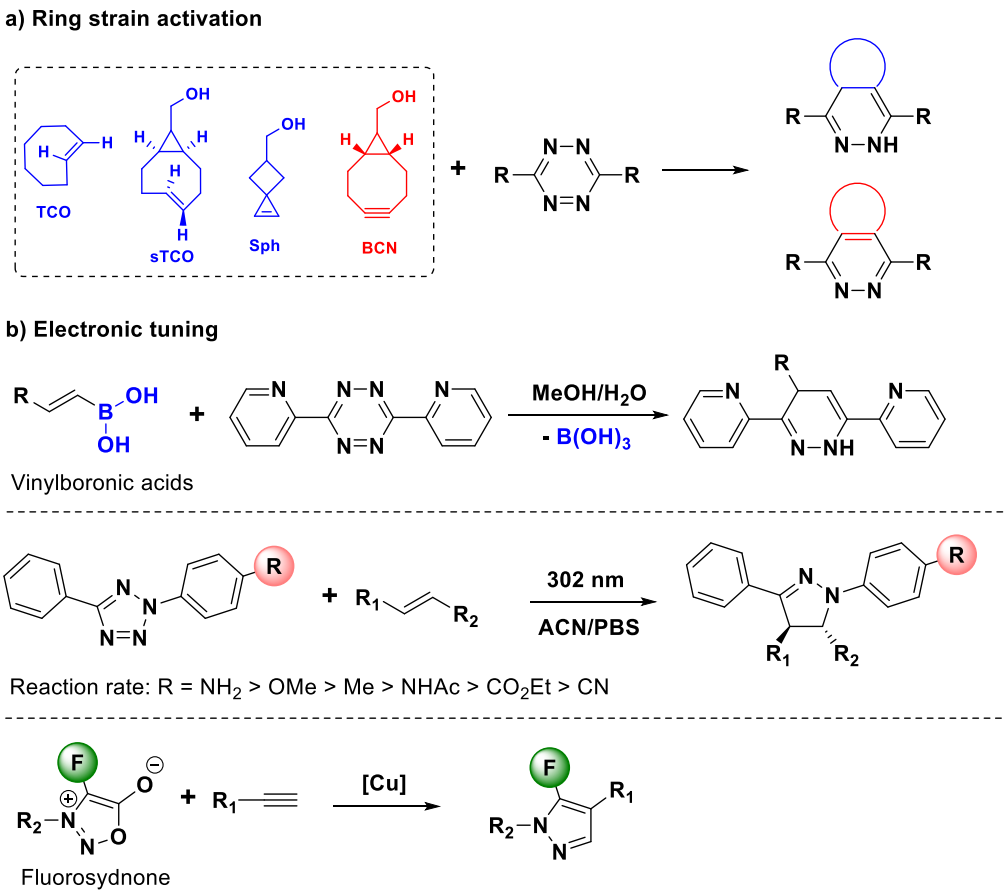

Figure 2. Bioorthogonal chemical probes with optimized reactivity. (**a**) Ring strain activation to improve the reactivity. (**b**) Electronic tuning to improve reactivity.

■ Selectivity

The complexity of intracellular chemical space demands exquisite selectivity on the part of bioorthogonal probes, particularly against abundant biological nucleophiles and electrophiles. Since side reactions shift reactants away from the desired bioorthogonal reaction pathway, the reactivity optimization should not come at the expense of probe stability and thus selectivity. For example, the fast-reacting tetrazines with strong electron-withdrawing substituents also exhibited lower stability due to increased hydrolysis of the tetrazine scaffold.[21]

In improving probe selectivity, one strategy exploits the differential sensitivity toward the

sterically bulky groups in bioorthogonal cycloaddition reactions. For example, the Bertozzi group reported the use of 3,3,6,6-tetramethylthiacycloheptyne[22] (TMTH), a motif first reported by Krebs and Kimling in 1970, for strain-promoted click chemistry (Figure 3a). The placement of four methyl groups adjacent to the triple bond effectively blocks the competing thiol-yne reaction with minimum effect on the desired cycloaddition reaction. On the other hand, the small bioorthogonal chemical reporter, cyclopropene, is susceptible to nucleophilic attack by biological thiols as well as polymerization *via* ene reaction.[23] To improve selectivity, the Devaraj group designed a methyl-substituted cyclopropene that is stable in aqueous solution and elicits strong fluorescence upon reaction with the tetrazine-fluorophore probes suitable for live-cell imaging (Figure 3a).[24] Similarly, to improve the stability of cyclopropenone, an attractive motif for bioorthogonal ligation with phosphines,[25] the Prescher group designed the dialkyl-substituted cyclopropenones that possess steric bulk to prevent the competing nucleophilic thiol attack (Figure 3a).[26] In our work, to direct the photogenerated, highly reactive nitrile imine away from the competing nucleophilic addition side reaction, the di-ortho-*N*-Boc-pyrrole-substituted diphenyltetrazole was designed for selective photoclick chemistry with a strained alkene in live cells (Figure 3a).[27]

Another strategy involves placing the reactive motif in a unique sequence environment, affording sequence tags with higher selectivity than the reactive motif alone. To this end, a number of cysteine-containing sequence tags have been developed, including the tetracysteine tag (CCPGCC) for fluorogenic ligation reactions with biarsenical FlAsH[28] and ReAsH,[29] the π-clamp for biocompatible cysteine *S*-perfluoroarylation,[30] the CX10R7-tag for cysteine–2-cyanobenzothiazole ligation,[31] and the DBCO-tag for site-selective cysteine–cyclooctyne conjugation[32] (Figure 3b). It is noted that the π-clamp and the DBCO-tag-mediated ligation reactions have not been demonstrated in

living cells. Nevertheless, these sequence tags illustrated the potential in expanding the chemical space of bioorthogonal reporters to include the naturally occurring structural motifs.

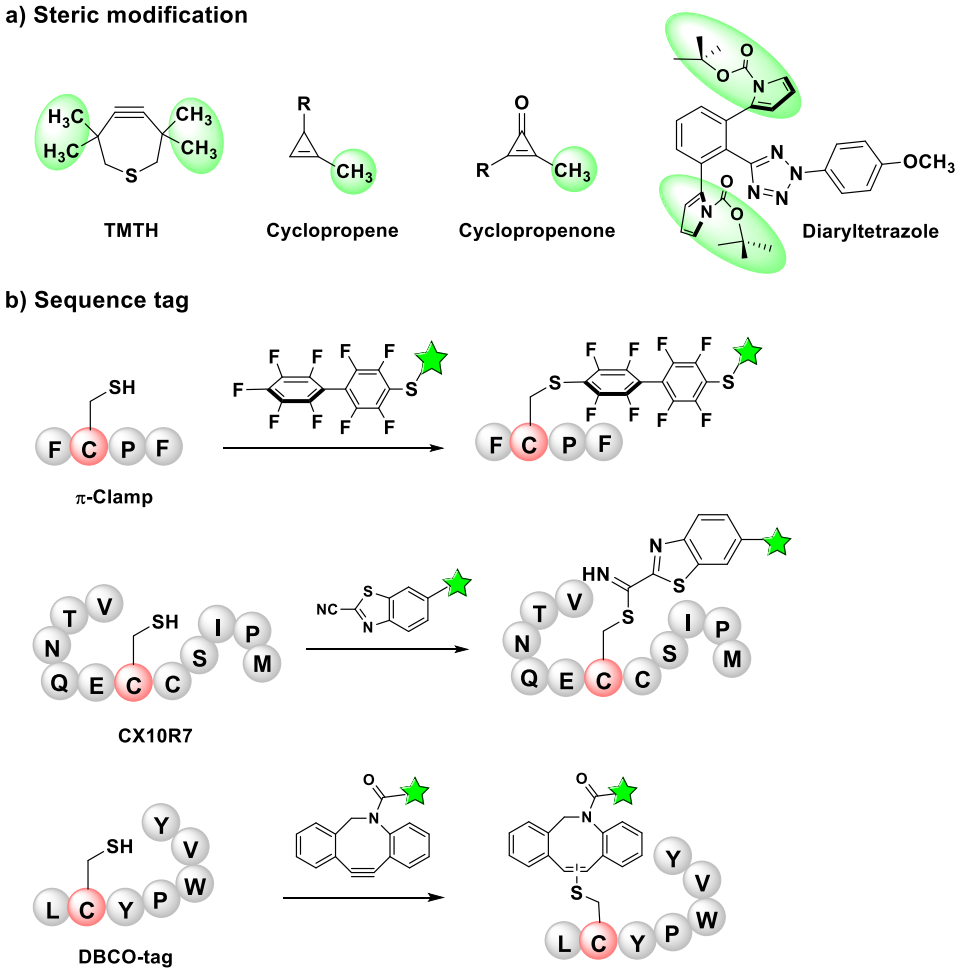

Figure 3. Bioorthogonal chemical probes with improved selectivity. (**a**) Steric modification to improve selectivity. (**b**) Sequence-specific peptide tags for site-selective bioconjugation.

■ Physicochemical properties

The physicochemical properties of bioorthogonal reaction partners, including molecular size, chemical stability, aqueous solubility, lipophilicity, and fluorogenic properties, have not attracted as much attention as reactivity and selectivity in designing bioorthogonal chemical probes. However, their importance cannot be overstated as non-optimized physicochemical properties may compromise

the performance of bioorthogonal probes in a biological system and generate the experimental data that are difficult to interpret.

Molecular size

Small bioorthogonal reporters are highly desirable because they are less likely to perturb the native function of a biomolecule of interest. Indeed, smaller functional groups, such as diazo,[33] azides, alkynes, and alkenes, were frequently employed as chemical reporters, whereas their larger reaction partners, including tetrazines, tetrazoles, and other heterocycles, were used as reagents for their subsequent functionalization. For example, despite its lower overall reactivity than TCO, the smaller cyclopropene and its derivatives have been exploited as chemical reporters in chemical proteomic studies, allowing identification and imaging of the newly synthesized proteins at discrete development stages in Drosophila through the use of tetrazine ligation.[34]

Chemical stability

The reactivity and stability are intrinsic, inseparable properties of any chemical structure, representing the "two sides of a coin." As such, it is generally challenging to find a balance in designing bioorthogonal chemical probes with the right combination of reactivity and stability. To realize the full potential of bioorthogonal chemical probes in living systems, we need to understand both the advantages and practical limitations of each bioorthogonal reactant pair. For example, in TCO-mediated tetrazine ligation, TCO is known to undergo the thiol-catalyzed isomerization to cis-cyclooctene,[12] which can lead to the incomplete reaction despite the use of an excess amount of tetrazines. To address the stability limitation, Fox and coworkers designed a dioxolane-fused trans-cyclooctene (d-TCO), which displayed higher reactivity than TCO and showed no appreciable

isomerization or decomposition in human serum at room temperature after four days.[35] Therefore, the suitability of bioorthogonal chemical probes in any specific application setting, including buffer conditions and biological environments with a high concentration of glutathione, needs to be carefully evaluated.

Aqueous solubility and lipophilicity

While aqueous solubility was rarely discussed in bioorthogonal reaction development, it is impossible to overemphasize its importance because poor solubility often leads to probe precipitation and causes nonspecific binding to cellular structures, resulting in reduced reaction efficiency and increased background signal. To increase aqueous solubility, polar functional groups such as carboxylic acid, alcohol, and sulfate can be added to the probe structures.[36] Alternatively, the carbon-to-heteroatom substitution can be performed to enhance aqueous solubility without a marked increase in molecular weight. For example, the oxygen placement at C5-position of TCO led to the design of oxoTCO, which exhibited greater hydrophilicity, improved stability, and faster reaction kinetics in tetrazine ligation.[37] Notably, there was no correlation detected between the rate constant, k_2, and the lipophilicity of the cyclooctyne derivatives in the strain-promoted azide-alkyne cycloaddition reaction.[38] When a chemical reporter is imbedded in a biomolecule, however, it is crucial to assess how the reporter interacts with its surrounding environment. A hydrophobic local environment may reduce the accessibility of a lipophilic chemical reporter toward its reaction partner and as a result, deactivate the chemical reporter. In this context, sometimes it might be advantageous to use less reactive but more accessible, hydrophilic chemical reporter to achieve greater reaction efficiency.[16]

Fluorogenicity

Fluorogenic probes are attractive in fluorescence-based studies of biomolecular dynamics in living systems because they become highly fluorescent only after the bioorthogonal ligation reaction. Three types of fluorogenic probes have been described in the literature (Figure 4).[39] The first involves internal fluorescence quenching through photoinduced electron transfer (PeT). An excellent example is that the weakly fluorescent azide-modified fluorophores were converted to the highly fluorescent triazole-substituted fluorophores after copper-catalyzed click chemistry (Figure 4a).[40, 41] The second type involves fluorescence quenching by a bioorthogonal functional group such as tetrazine *via* through-bond energy transfer (TBET). For instance, a fluorescence turn-on ratio of 1600 was observed for a BODIPY-conjugated tetrazine probe after its reaction with TCO[42] (Figure 4b). The third type involves de novo synthesis of fluorophores from the bioorthogonal reactant pairs. One prominent example is the formation of fluorescent pyrazolines from the photoinduced tetrazole-alkene cycloaddition reaction[43] (Figure 4c). Similarly, tetrazines are also known to react with certain alkenes such as styrene to produce fluorescent 1,4-dihydropyridazine products.[44]

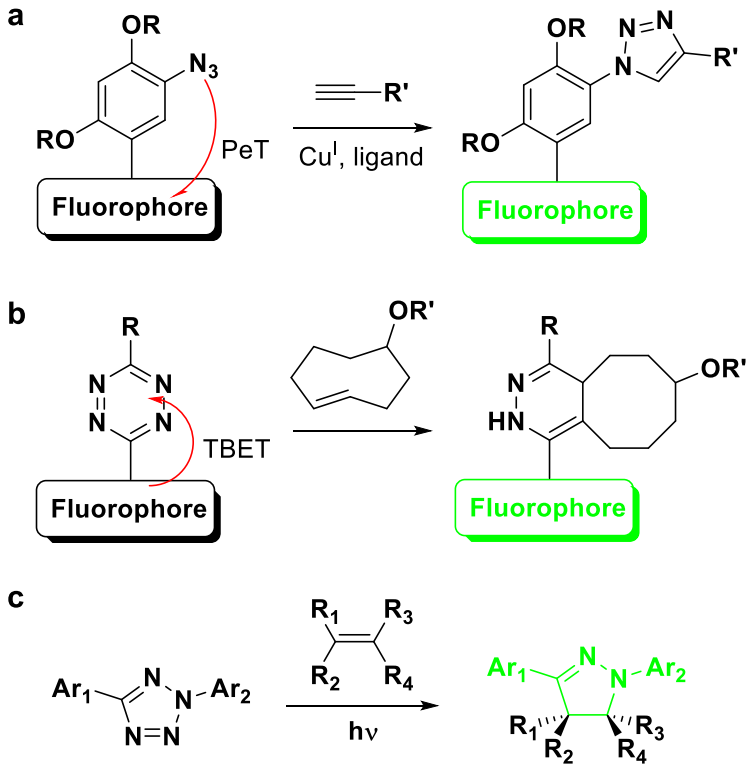

Figure 4. Representative fluorogenic bioorthogonal reactions. (**a**) Fluorogenic azide probes. (**b**) Fluorogenic tetrazine probes. (**c**) Fluorogenic tetrazole probes for de novo fluorophore synthesis.

■ Biological context

Depending on the biological application context, additional fitness factors may be considered. Generally speaking, for robust interrogation of cellular systems, bioorthogonal probes need to be non-toxic to cells, readily incorporated into biomolecules in a site-specific manner, cell-permeable, and easy to be washed off after the reaction so that biological measurements can be carried out.

Toxicity

A prerequisite in applying bioorthogonal probes to living systems is that the reagents are not toxic to cells. Indeed, most bioorthogonal reagents can be safely employed in a concentration range of μM to mM. One notable exception is copper ion used in the copper-catalyzed azide–alkyne

cycloaddition (CuAAC) reaction[45] because of its role in catalyzing the formation of reactive oxygen species.[46] In reducing this toxicity, one way is to use the preformed copper-chelated azides, which decreases the concentration of the copper ion used (*vide infra*).[47] Alternatively, the copper-free strain-promoted azide−alkyne cycloaddition (SPAAC) reaction was developed, which obviates the need for the copper ion.[48]

Biomolecular incorporation

Installation of a bioorthogonal reporter into biomolecules such as proteins and lipids with exquisite specificity represents the first step in successful bioorthogonal labeling and subsequent studies of the biomolecule of interest. For protein targets, one powerful approach involves the genetic code expansion that exploits substrate promiscuity of some aminoacyl-tRNA synthetases for installation of non-canonical amino acids with new chemical functionalities into specific locations within a protein.[49] In particular, pyrrolysyl-tRNA synthetase (PylRS) and its many variants represent the most commonly used synthetases for charging lysine and phenylalanine analogs carrying the chemical reporters.[50] The advances in rapidly evolving new PylRS variants and other aminoacyl-tRNA synthetases have enabled site-specific incorporation of a large number of bioorthogonal reporters including azide, alkene, alkyne, tetrazines, and tetrazoles into the protein of interest.[51] Similarly, a diverse set of chemically functionalized monosaccharides including GalNAc, fucose, and GlcNAc carrying an azide, alkyne, diazo, or cyclopropene reporter have been successfully incorporated into the glycans through either metabolic labeling or chemoenzymatic reactions in living cells and organisms.[52] Subsequent bioorthogonal reactions have allowed in vivo imaging of glycan dynamics[53] as well as mass spectrometry analysis of low-abundance glycoproteins and their signal-dependent changes.[54] For lipid targets, the successful design of a growing list of fatty

acid, isoprenoid, and other lipid probes carrying azide and alkyne functionalities[55] have allowed proteomic analysis of protein lipidation in cell lysates,[56] visualization of lipid localization and trafficking,[57] and elucidation of substrate specificity of lipid transferases such as DHHC-PATs.[58]

Cell permeability

When target molecules are present on the cell surface, the lack of cell permeability of a bioorthogonal probe could be advantageous as it prevents probes from perturbing intracellular signaling. For example, DpTz is a tetrazine reagent commonly used for cell surface labeling due to its membrane impermeability.[59] However, when target molecules reside inside the cell, bioorthogonal probes need to cross the cell membrane to access their targets. In general, the charge-neutral bioorthogonal probes such as mono-substituted tetrazines have shown excellent cell permeability suitable for reactions with intracellular targets.[60] With respect to fluorophore selection, the charged fluorophores such as cyanine dyes were frequently used in cell surface labeling,[27] whereas the charge-neutral fluorophores including coumarin, BODIPY and fluorescein-diacetate were used in intracellular protein labeling.[60] To achieve high signal-to-noise ratio, excess bioorthogonal probes need to be washed off from intracellular space after the reaction. To this end, Chang and co-workers reported that the probe washability could be predicted based on the following probe properties: lipophilicity, water solubility, and charged van der Waals surface area.[61] The background-free probes should possess adequate lipophilicity, high water-solubility, and moderate negative surface charge. Three molecular descriptors were found to be necessary and sufficient in predicting reversible cell permeability and washability with the optimal values shown in parentheses: Slog*P* for lipophilicity (1 ~ 4), Log*S* for water solubility (-2 ~ -6), and Q_VSA_FNEG for negatively charged van der Waals surface area (0.15 ~ 0.35).

■ Examples of optimized bioorthogonal probes

Chelate-containing probe for CuAAC

While the CuAAC reaction has been used extensively in bioconjugation due to its excellent reaction kinetics and selectivity, the cytotoxicity of Cu(I) ion diminishes its appeal in live-cell studies.[45] To meet this challenge, the Ting group reported the use of the picolyl azide in a chelation-assisted CuAAC reaction.[47] The chelation eliminates the need for external Cu(I) ligands and decreases the Cu(I) concentration required for the reaction (Figure 5a). In demonstrating the chelation-assisted CuAAC was suitable for live-cell study, the PRIME (probe incorporation mediated by enzymes) method was employed in which picolyl azide was added to the LAP (LplA acceptor peptide) side chain using a lipoic acid ligase (LplA) mutant. In HEK cells expressing the LAP-tagged cyan fluorescent protein (CFP) or neurexin-1β, treatment with picolyl azide followed by Alexa Fluor 647 conjugated alkyne resulted in site-specific fluorescent labeling of the target protein with fluorescence intensity 25-fold higher than the non-chelating azide (Figure 5b). In neuronal culture experiments, the picolyl azide probe also reduced the Cu(I) ion concentration to levels minimally toxic to neurons without decreases in signal intensity.

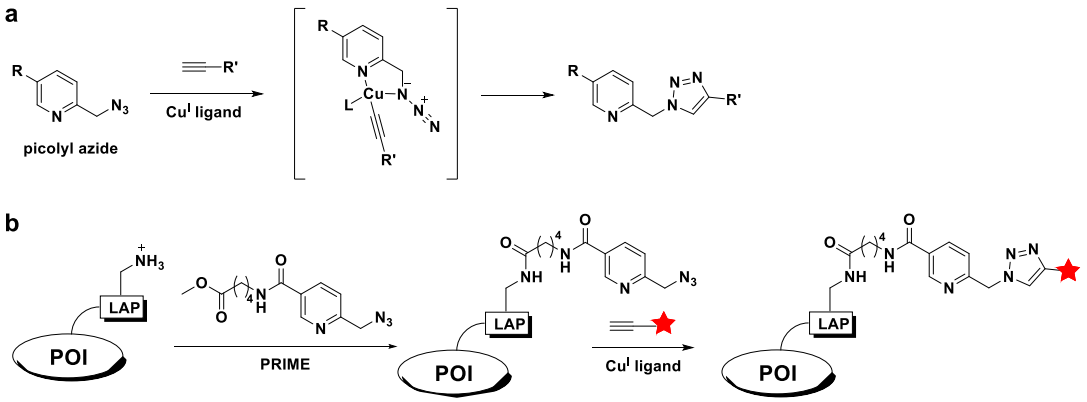

Figure 5. Design of a copper-chelating azide for cell-compatible site-specific protein labeling. (**a**)

Scheme for the chelation-assisted CuAAC reaction. (**b**) Introduction of picolyl azide into a protein of interest (POI) *via* PRIME and subsequent bioorthogonal labeling with AF647-conjugated alkyne *via* the chelation-assisted CuAAC reaction.

Ag·sTCO probe for tetrazine ligation

Following the seminal report of bioorthogonal tetrazine–TCO ligation, the Fox group designed a cyclopropane-fused TCO named sTCO that adopts a high-energy "half-chair" conformation and reacts with DpTz 19 times faster than TCO.[13] While it showed excellent stability in human serum, sTCO was found to isomerize to the unreactive *cis*-cyclooctene form in the presence of high concentration of thiols. When presented in specific organelles using the HaloTag-mediated organelle targeting strategy, sTCO displayed high stability in the ER, but low stability in the nucleus. To address these shortcomings, Johnson and co-workers discovered that the readily prepared Ag-sTCO complex showed improved stability during storage and excellent bioorthogonal protein labeling efficiency in live mammalian cells (Figure 6a).[62] After Ag-sTCO complex was conjugated with the HaloTag-fused histone 2B (H2B) in HeLa cells, highly selective fluorescent labeling of H2B was achieved through tetrazine ligation with a cell-permeable mono-substituted TAMRA-tetrazine probe (Figure 6b). It is noteworthy that the stabilization effect is temporary as the Ag-sTCO complex dissociates instantaneously when it encounters NaCl present in high concentration in cell culture medium.[63]

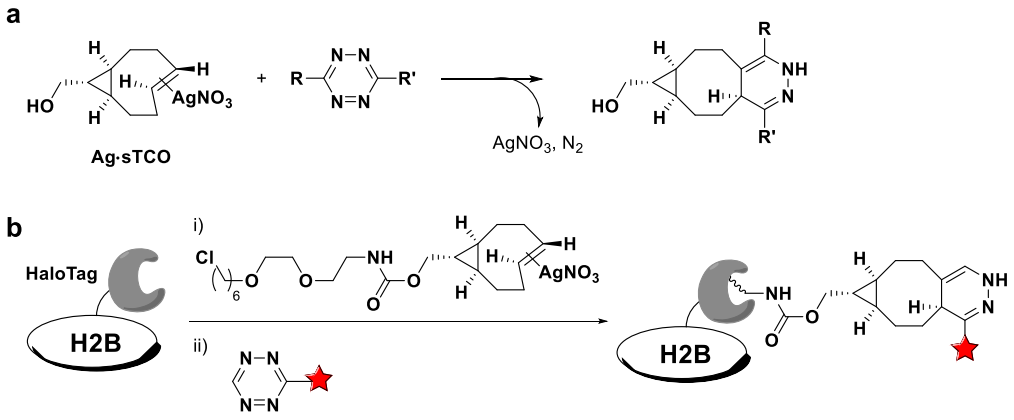

Figure 6. Bioorthogonal labeling *via* silver-stabilized sTCO-tetrazine ligation. (**a**) Reaction scheme. (**b**) Introduction of the silver-stabilized sTCO into histone 2B (H2B) using HaloTag technology followed by fluorescent labeling *via* tetrazine ligation.

Steric-shielded tetrazole probe for photoclick reaction

For the tetrazole-based photoclick chemistry, the photogenerated nitrile imine manifests both 1,3-dipole and electrophile characters, leading to the formation of thiol and water addition products when a suitable dipolarophile is absent. To harness nitrile imine reactivity for the cycloaddition reaction, our group recently discovered that a pendant group, *N*-Boc-pyrrole, situated at the *ortho* positions of C-aryl ring drastically extends the half-life of the photogenerated nitrile imine in the aqueous medium to 102 s, owing to the steric shielding effect. As a result, the nucleophilic addition reactions were suppressed while the desired cycloaddition reaction remained robust (with a k_2 value of 2800 ± 200 M^{-1} s^{-1} toward spiro[2.3]hex-1-ene or Sph) (Figure 7a).[27] When the genetically encodable Sph-lysine was introduced site-specifically at the extracellular loop 3 (ECL3) region of glucagon receptor (GCGR) expressed in HEK293T cells, fast bioorthogonal labeling (< 1 min) of GCGR with the Cy5-conjugated sterically shielded tetrazole probe was accomplished with 89% yield (Figure 7b). Owing to its superior reactivity and selectivity, this type of steric-shielded tetrazole

probe offers an exciting opportunity to probe the conformational dynamics of GPCRs in a native cellular environment.

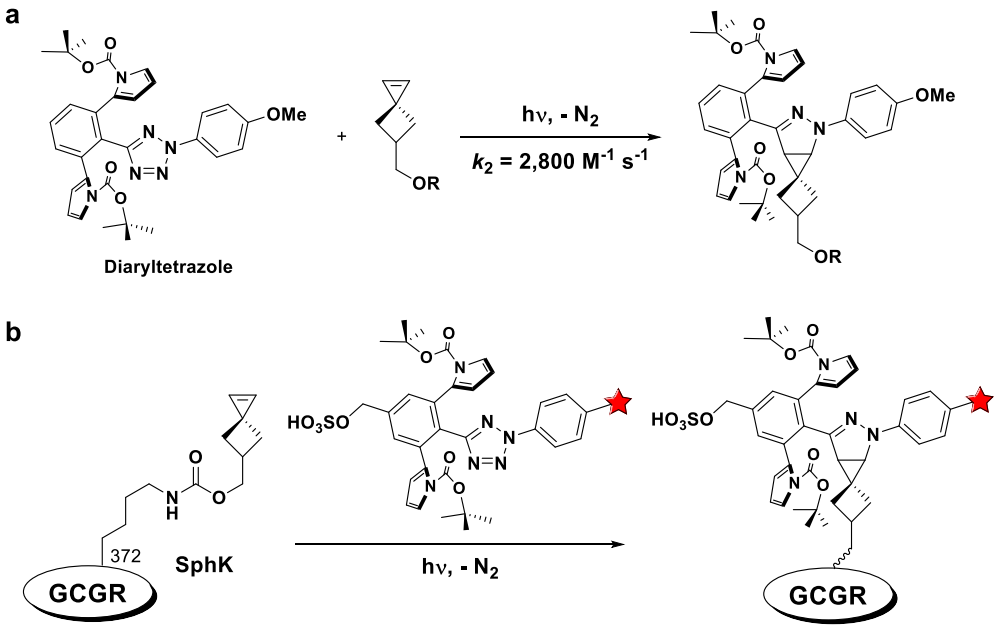

Figure 7. A sterically shielded tetrazole probe with improved selectivity. (**a**) Scheme of photoinduced tetrazole-Sph cycloaddition reaction. (**b**) Bioorthogonal fluorescent labeling of the SphK-encoded GCGR in HEK 293T cells with a Cy5-conjugated sterically shielded tetrazole.

■ Summary

Bioorthogonal chemical probes have been increasingly utilized in the studies of biomolecular dynamics and function in living systems over the last decade. While the repertoire of bioorthogonal reactions has been steadily growing, the design of bioorthogonal chemical probes with optimal properties for a specific biological system remains a challenge. In this perspective, we put forward a series of fitness factors for bioorthogonal chemical probes based on reactivity, selectivity, physicochemical properties, and biological context that need to be optimized concurrently during the probe development and subsequent applications. It is noteworthy that the fitness factors enumerated

here are interdependent as changes to one fitness factor will inevitably lead to the changes to others.

While ideal bioorthogonal chemical probes should possess fast reaction kinetics, high selectivity toward the biomolecule of interest without cross-reactivity with other biomolecules, optimal physicochemical properties, and excellent compatibility with the biological system, in practice it is not necessary to have all fitness factors fully refined before we can contemplate their use in a biological experiment. For example, the nonspecific effects of an incompletely optimized bioorthogonal chemical probe can be delineated using a control probe that is structurally analogous but lacks the necessary reactive moiety, similar to the chirality inversion in applying less-selective binding-based small-molecule probes. Furthermore, while it is tempting to prescribe allowable values or "rules of thumb" for the specific fitness factor, the current data set in the literature are incomplete and in many cases conflicting because the use of different solvent systems, buffers, culture media, and cell lines prevents data comparisons across different experimental systems. Nevertheless, the field could benefit tremendously if a standard set of in vitro and cell-based experiments can be performed for bioorthogonal chemical probes in the future before they undergo specialization and optimization for applications in a specific biological system.

AUTHOR INFORMATION

Corresponding Author

* E-mail: qinglin@buffalo.edu.

ORCID

Yulin Tian: 0000-0002-2355-380X

Qing Lin: 0000-0002-9196-5718

Present Address

†Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China

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■ Reference

(1) Sletten, E. M., and Bertozzi, C. R. (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality, *Angew. Chem., Int. Ed. 48*, 6974-6998.

(2) Ramil, C. P., and Lin, Q. (2013) Bioorthogonal chemistry: strategies and recent developments, *Chem. Comm. 49*, 11007-11022.

(3) Devaraj, N. K. (2018) The Future of Bioorthogonal Chemistry, *ACS Cent. Sci. 4*, 952-959.

(4) Patterson, D. M., Nazarova, L. A., and Prescher, J. A. (2014) Finding the right (bioorthogonal) chemistry, *ACS Chem. Biol. 9*, 592-605.

(5) Workman, P., and Collins, I. (2010) Probing the probes: fitness factors for small molecule tools, *Chem. Biol. 17*, 561-577.

(6) Lewis, W. G., Green, L. G., Grynszpan, F., Radić, Z., Carlier, P. R., Taylor, P., Finn, M. G., and Sharpless, K. B. (2002) Click Chemistry In Situ: Acetylcholinesterase as a Reaction

Vessel for the Selective Assembly of a Femtomolar Inhibitor from an Array of Building Blocks, *Angew. Chem., Int. Ed. 41*, 1053-1057.

(7) Versteegen, R. M., Rossin, R., ten Hoeve, W., Janssen, H. M., and Robillard, M. S. (2013) Click to Release: Instantaneous Doxorubicin Elimination upon Tetrazine Ligation, *Angew. Chem., Int. Ed. 52*, 14112-14116.

(8) Ji, X., Pan, Z., Yu, B., De La Cruz, L. K., Zheng, Y., Ke, B., and Wang, B. (2019) Click and release: bioorthogonal approaches to "on-demand" activation of prodrugs, *Chem. Soc. Rev. 48*, 1077-1094.

(9) Li, J., and Chen, P. R. (2016) Development and application of bond cleavage reactions in bioorthogonal chemistry, *Nature Chemical Biology 12*, 129-137.

(10) Lang, K., and Chin, J. W. (2014) Bioorthogonal reactions for labeling proteins, *ACS Chem. Biol. 9*, 16-20.

(11) Blackman, M. L., Royzen, M., and Fox, J. M. (2008) Tetrazine ligation: fast bioconjugation based on inverse-electron-demand Diels-Alder reactivity, *J. Am. Chem. Soc. 130*, 13518-13519.

(12) Rossin, R., van den Bosch, S. M., Ten Hoeve, W., Carvelli, M., Versteegen, R. M., Lub, J., and Robillard, M. S. (2013) Highly reactive trans-cyclooctene tags with improved stability for Diels-Alder chemistry in living systems, *Bioconjug. Chem. 24*, 1210-1217.

(13) Taylor, M. T., Blackman, M. L., Dmitrenko, O., and Fox, J. M. (2011) Design and synthesis of highly reactive dienophiles for the tetrazine-trans-cyclooctene ligation, *J. Am. Chem. Soc. 133*, 9646-9649.

(14) Dommerholt, J., Schmidt, S., Temming, R., Hendriks, L. J., Rutjes, F. P., van Hest, J. C., Lefeber, D. J., Friedl, P., and van Delft, F. L. (2010) Readily accessible bicyclononynes for

bioorthogonal labeling and three-dimensional imaging of living cells, *Angew. Chem., Int. Ed. 49*, 9422-9425.

(15) Yu, Z., and Lin, Q. (2014) Design of spiro[2.3]hex-1-ene, a genetically encodable double-strained alkene for superfast photoclick chemistry, *J. Am. Chem. Soc. 136*, 4153-4156.

(16) Ramil, C. P., Dong, M., An, P., Lewandowski, T. M., Yu, Z., Miller, L. J., and Lin, Q. (2017) Spirohexene-Tetrazine Ligation Enables Bioorthogonal Labeling of Class B G Protein-Coupled Receptors in Live Cells, *J. Am. Chem. Soc. 139*, 13376−13386.

(17) Oliveira, B. L., Guo, Z., and Bernardes, G. J. L. (2017) Inverse electron demand Diels-Alder reactions in chemical biology, *Chem. Soc. Rev. 46*, 4895-4950.

(18) Eising, S., Lelivelt, F., and Bonger, K. M. (2016) Vinylboronic Acids as Fast Reacting, Synthetically Accessible, and Stable Bioorthogonal Reactants in the Carboni-Lindsey Reaction, *Angew. Chem., Int. Ed. 55*, 12243-12247.

(19) Wang, Y., Song, W., Hu, W. J., and Lin, Q. (2009) Fast alkene functionalization in vivo by Photoclick chemistry: HOMO lifting of nitrile imine dipoles, *Angew. Chem., Int. Ed. 48*, 5330-5333.

(20) Liu, H., Audisio, D., Plougastel, L., Decuypere, E., Buisson, D. A., Koniev, O., Kolodych, S., Wagner, A., Elhabiri, M., Krzyczmonik, A., Forsback, S., Solin, O., Gouverneur, V., and Taran, F. (2016) Ultrafast Click Chemistry with Fluorosydnones, *Angew. Chem., Int. Ed. 55*, 12073-12077.

(21) Karver, M. R., Weissleder, R., and Hilderbrand, S. A. (2011) Synthesis and evaluation of a series of 1,2,4,5-tetrazines for bioorthogonal conjugation, *Bioconjug. Chem. 22*, 2263-2270.

(22) de Almeida, G., Sletten, E. M., Nakamura, H., Palaniappan, K. K., and Bertozzi, C. R. (2012) Thiacycloalkynes for copper-free click chemistry, *Angew. Chem., Int. Ed. 51*, 2443-2447.

(23) Matsuura, M., Saikawa, Y., Inui, K., Nakae, K., Igarashi, M., Hashimoto, K., and Nakata, M. (2009) Identification of the toxic trigger in mushroom poisoning, *Nat. Chem. Biol. 5*, 465-467.

(24) Yang, J., Seckute, J., Cole, C. M., and Devaraj, N. K. (2012) Live-cell imaging of cyclopropene tags with fluorogenic tetrazine cycloadditions, *Angew. Chem., Int. Ed. 51*, 7476-7479.

(25) Shih, H. W., and Prescher, J. A. (2015) A Bioorthogonal Ligation of Cyclopropenones Mediated by Triarylphosphines, *J. Am. Chem. Soc. 137*, 10036-10039.

(26) Row, R. D., Shih, H. W., Alexander, A. T., Mehl, R. A., and Prescher, J. A. (2017) Cyclopropenones for Metabolic Targeting and Sequential Bioorthogonal Labeling, *J. Am. Chem. Soc. 139*, 7370–7375.

(27) An, P., Lewandowski, T. M., Erbay, T. G., Liu, P., and Lin, Q. (2018) Sterically Shielded, Stabilized Nitrile Imine for Rapid Bioorthogonal Protein Labeling in Live Cells, *J. Am. Chem. Soc. 140*, 4860-4868.

(28) Griffin, B. A., Adams, S. R., and Tsien, R. Y. (1998) Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells, *Science 281*, 269-272.

(29) Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., Llopis, J., and Tsien, R. Y. (2002) New Biarsenical Ligands and Tetracysteine Motifs for Protein Labeling in Vitro and in Vivo: Synthesis and Biological Applications, *J. Am. Chem. Soc. 124*, 6063-6076.

(30) Zhang, C., Welborn, M., Zhu, T., Yang, N. J., Santos, M. S., Van Voorhis, T., and Pentelute, B. L. (2016) π-Clamp-mediated cysteine conjugation, *Nat. Chem. 8*, 120-128.

(31) Ramil, C. P., An, P., Yu, Z., and Lin, Q. (2016) Sequence-Specific 2-Cyanobenzothiazole

Ligation, *J. Am. Chem. Soc. 138*, 5499-5502.

(32) Zhang, C., Dai, P., Vinogradov, A. A., Gates, Z. P., and Pentelute, B. L. (2018) Site-Selective Cysteine-Cyclooctyne Conjugation, *Angew. Chem., Int. Ed. 57*, 6459-6463.

(33) Andersen, K. A., Aronoff, M. R., McGrath, N. A., and Raines, R. T. (2015) Diazo Groups Endure Metabolism and Enable Chemoselectivity in Cellulo, *J. Am. Chem. Soc. 137*, 2412-2415.

(34) Elliott, T. S., Townsley, F. M., Bianco, A., Ernst, R. J., Sachdeva, A., Elsasser, S. J., Davis, L., Lang, K., Pisa, R., Greiss, S., Lilley, K. S., and Chin, J. W. (2014) Proteome labeling and protein identification in specific tissues and at specific developmental stages in an animal, *Nat. Biotechnol. 32*, 465-472.

(35) Darko, A., Wallace, S., Dmitrenko, O., Machovina, M. M., Mehl, R. A., Chin, J. W., and Fox, J. M. (2014) Conformationally strained trans-cyclooctene with improved stability and excellent reactivity in tetrazine ligation, *Chem. Sci. 5*, 3770-3776.

(36) Yu, Z., Ohulchanskyy, T. Y., An, P., Prasad, P. N., and Lin, Q. (2013) Fluorogenic, two-photon-triggered photoclick chemistry in live mammalian cells, *J. Am. Chem. Soc. 135*, 16766-16769.

(37) Lambert, W. D., Scinto, S. L., Dmitrenko, O., Boyd, S. J., Magboo, R., Mehl, R. A., Chin, J. W., Fox, J. M., and Wallace, S. (2017) Computationally guided discovery of a reactive, hydrophilic trans-5-oxocene dienophile for bioorthogonal labeling, *Organic & biomolecular chemistry 15*, 6640-6644.

(38) Debets, M. F., van Berkel, S. S., Dommerholt, J., Dirks, A. T., Rutjes, F. P., and van Delft, F. L. (2011) Bioconjugation with strained alkenes and alkynes, *Acc. Chem. Res. 44*, 805-815.

(39) Shieh, P., and Bertozzi, C. R. (2014) Design strategies for bioorthogonal smart probes, *Org.*

Biomol. Chem. 12, 9307-9320.

(40) Shieh, P., Hangauer, M. J., and Bertozzi, C. R. (2012) Fluorogenic azidofluoresceins for biological imaging, *J. Am. Chem. Soc. 134*, 17428-17431.

(41) Shieh, P., Dien, V. T., Beahm, B. J., Castellano, J. M., Wyss-Coray, T., and Bertozzi, C. R. (2015) CalFluors: A Universal Motif for Fluorogenic Azide Probes across the Visible Spectrum, *J. Am. Chem. Soc. 137*, 7145-7151.

(42) Carlson, J. C., Meimetis, L. G., Hilderbrand, S. A., and Weissleder, R. (2013) BODIPY-tetrazine derivatives as superbright bioorthogonal turn-on probes, *Angew. Chem., Int. Ed. 52*, 6917-6920.

(43) Song, W., Wang, Y., Qu, J., Madden, M. M., and Lin, Q. (2008) A photoinducible 1,3-dipolar cycloaddition reaction for rapid, selective modification of tetrazole-containing proteins, *Angew. Chem., Int. Ed. 47*, 2832-2835.

(44) Shang, X., Song, X., Faller, C., Lai, R., Li, H., Cerny, R., Niu, W., and Guo, J. (2017) Fluorogenic protein labeling using a genetically encoded unstrained alkene, *Chem. Sci. 8*, 1141-1145.

(45) Hong, V., Steinmetz, N. F., Manchester, M., and Finn, M. G. (2010) Labeling Live Cells by Copper-Catalyzed Alkyne-Azide Click Chemistry, *Bioconjug. Chem. 21*, 1912–1916.

(46) Kennedy, D. C., McKay, C. S., Legault, M. C., Danielson, D. C., Blake, J. A., Pegoraro, A. F., Stolow, A., Mester, Z., and Pezacki, J. P. (2011) Cellular consequences of copper complexes used to catalyze bioorthogonal click reactions, *J. Am. Chem. Soc. 133*, 17993-18001.

(47) Uttamapinant, C., Tangpeerachaikul, A., Grecian, S., Clarke, S., Singh, U., Slade, P., Gee, K. R., and Ting, A. Y. (2012) Fast, cell-compatible click chemistry with copper-chelating azides for biomolecular labeling, *Angew. Chem., Int. Ed. 51*, 5852-5856.

(48) Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems, *J. Am. Chem. Soc. 126*, 15046-15047.

(49) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code, *Annu. Rev. Biochem. 79*, 413-444.

(50) Wan, W., Tharp, J. M., and Liu, W. R. (2014) Pyrrolysyl-tRNA synthetase: an ordinary enzyme but an outstanding genetic code expansion tool, *Biochim. Biophys. Acta 1844*, 1059-1070.

(51) Lang, K., and Chin, J. W. (2014) Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins, *Chem. Rev. 114*, 4764-4806.

(52) Palaniappan, K. K., and Bertozzi, C. R. (2016) Chemical Glycoproteomics, *Chem. Rev. 116*, 14277-14306.

(53) Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008) In vivo imaging of membrane-associated glycans in developing zebrafish, *Science 320*, 664-667.

(54) Rouhanifard, S. H., Nordstrøm, L. U., Zheng, T., and Wu, P. (2013) Chemical probing of glycans in cells and organisms, *Chem. Soc. Rev. 42*, 4284-4296.

(55) Hang, H. C., Wilson, J. P., and Charron, G. (2011) Bioorthogonal chemical reporters for analyzing protein lipidation and lipid trafficking, *Acc. Chem. Res. 44*, 699-708.

(56) Peng, T., Thinon, E., and Hang, H. C. (2016) Proteomic analysis of fatty-acylated proteins, *Curr. Opin. Chem. Biol. 30*, 77-86.

(57) Bumpus, T. W., and Baskin, J. M. (2018) Greasing the Wheels of Lipid Biology with Chemical Tools, *Trends Biochem. Sci. 43*, 970-983.

(58) Zheng, B., DeRan, M., Li, X., Liao, X., Fukata, M., and Wu, X. (2013) 2-Bromopalmitate

analogues as activity-based probes to explore palmitoyl acyltransferases, *J. Am. Chem. Soc. 135*, 7082-7085.

(59) Lang, K., Davis, L., Wallace, S., Mahesh, M., Cox, D. J., Blackman, M. L., Fox, J. M., and Chin, J. W. (2012) Genetic Encoding of bicyclononynes and trans-cyclooctenes for site-specific protein labeling in vitro and in live mammalian cells via rapid fluorogenic Diels-Alder reactions, *J. Am. Chem. Soc. 134*, 10317-10320.

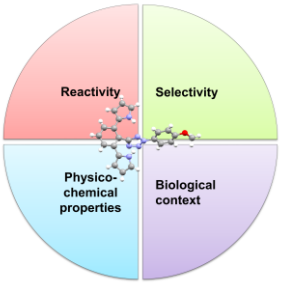
(60) Peng, T., and Hang, H. C. (2016) Site-Specific Bioorthogonal Labeling for Fluorescence Imaging of Intracellular Proteins in Living Cells, *J. Am. Chem. Soc. 138*, 14423-14433.

(61) Alamudi, S. H., Satapathy, R., Kim, J., Su, D., Ren, H., Das, R., Hu, L., Alvarado-Martinez, E., Lee, J. Y., Hoppmann, C., Pena-Cabrera, E., Ha, H. H., Park, H. S., Wang, L., and Chang, Y. T. (2016) Development of background-free tame fluorescent probes for intracellular live cell imaging, *Nat. Commun. 7*, 11964.

(62) Murrey, H. E., Judkins, J. C., Am Ende, C. W., Ballard, T. E., Fang, Y., Riccardi, K., Di, L., Guilmette, E. R., Schwartz, J. W., Fox, J. M., and Johnson, D. S. (2015) Systematic Evaluation of Bioorthogonal Reactions in Live Cells with Clickable HaloTag Ligands: Implications for Intracellular Imaging, *J. Am. Chem. Soc. 137*, 11461-11475.

(63) Fang, Y., Judkins, J. C., Boyd, S. J., am Ende, C. W., Rohlfing, K., Huang, Z., Xie, Y., Johnson, D. S., and Fox, J. M. (2019) Studies on the stability and stabilization of trans-cyclooctenes through radical inhibition and silver (I) metal complexation, *Tetrahedron 75*, 4307-4317.

TOC Figure



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