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Imaging beyond the proteome

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Abstract

Imaging technologies developed in the early 20th century achieved contrast solely by relying on macroscopic and morphological differences between the tissues of interest and the surrounding tissues. Since then, there has been a movement toward imaging at the cellular and molecular level in order to visualize biological processes. This rapidly growing field is known as molecular imaging. In the last decade, many methodologies for imaging proteins have emerged. However, most of these approaches cannot be extended to imaging beyond the proteome. Here, we highlight some of the recently developed technologies that enable imaging of non-proteinaceous molecules in the cell: lipids, signalling molecules, inorganic ions, glycans, nucleic acids, small-molecule metabolites, and protein post-translational modifications such as phosphorylation and methylation.

Introduction

Molecular imaging is a powerful tool that has enabled the visualisation of biomolecules as they function in their native setting.[1] The ability to monitor biological events in real time at the subcellular level has furthered our understanding of many physiological processes, including protein trafficking, protein localisation, and protein–protein interactions.[2] Arguably, the most widely used set of tools in molecular imaging are fluorescent proteins. The discovery and development of the green fluorescent protein (GFP) by Shimomura, Chalfie, and Tsien, who were awarded the 2008 Nobel Prize in Chemistry for their efforts, has enabled the tagging and imaging of many proteins of interest.[3]

Although imaging of target proteins using fluorescent protein fusions has revolutionised many areas of biology, extension of this strategy to other components of the cell such as glycans, lipids, and nucleic acids has remained challenging. While proteins comprise the largest fraction of biological molecules in the cell, non-proteinaceous biomolecules also play important roles in cell biology.[4] Thus, the ability to directly visualise all the components of the cell would allow for a more comprehensive understanding of cellular biochemistry. Here, we discuss the development of emerging technologies that enable imaging beyond the proteome, namely protein post-translational modifications such as glycosylation, phosphorylation, methylation, and lipidation, as well as other classes of biomolecules, including lipids, glycans, nucleic acids, small-molecule metabolites, and inorganic ions.

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1. Fluorescent proteins

Traditionally, fluorescent proteins have been genetically fused to the gene that encodes the protein of interest in order to produce a chimeric protein that contains the fluorescent protein at its N- or C-terminus.[5] Beyond the now routine use of this strategy to image proteins, several groups have applied fluorescent proteins to image lipids, signalling molecules, and post-translational modifications in the cell. Through protein-small molecule interactions, certain proteins can faithfully report on the location of non-proteinaceous biomolecules in the cell, perhaps most notably many classes of lipids.

1.1 Genetically-encoded probes for imaging membrane lipids

Membrane lipids, such as diacylglycerol, phosphoinositides, and phosphatidylserine, are important regulators of cellular homeostasis and many signal transduction pathways.[6] These lipids are predominantly found in the inner leaflet of the plasma membrane and on the cytosolic face of organelle membranes and are responsible for recruiting a wide variety of proteins and initiating a variety of signalling pathways.[7] Fluorescent protein fusions to the lipid-binding domains of these recruited proteins have been used to study lipid dynamics in live cells using fluorescence microscopy.[8,9] For example, the pleckstrin homology domains of phospholipase C δ and Akt have been used to image the phosphoinositides PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, respectively.[7] Fluorescent protein fusions of other lipid-binding domains have enabled imaging of other phosphoinositides and membrane lipids as well.[7] This methodology has been instrumental in determining the localisation of these important lipids during various biological processes.

1.2 Genetically-encoded and Förster resonance energy transfer (FRET)-based sensors of signalling molecules

In addition to their contributions toward developing the fluorescent protein toolkit, Tsien and co-workers have also developed a FRET-based reporter system for imaging secondary messengers.[3] They were initially interested in visualising cyclic adenosine 3′,5′-monophosphate (cAMP), a small molecule that is involved in intracellular signal transduction. Their first FRET reporter consisted of fluorescein-labeled catalytic subunits of cAMP-dependent protein kinase (PKA) and rhodamine-labeled regulatory subunits of PKA.[10] Upon binding of cAMP, the regulatory subunit of PKA dissociates from the catalytic subunit, thus causing a loss in FRET between the two labels. Despite the utility of this probe, Tsien hoped to replace the small-molecule organic fluorophores with fluorescent proteins in order to enable imaging of other metabolites as well as proteins through genetic fusion.[3] Using mutagenesis, they were able to develop GFP variants with enhanced fluorescence as well as fluorescent proteins of different colors. They discovered that the yellow fluorescent protein (YFP) is a good FRET acceptor for the cyan fluorescent protein (CFP). Following this development, Pozzan and co-workers teamed up with the Tsien laboratory to replace the two fluorophores with CFP and YFP.[11] In their work, they demonstrated that this fluorescent protein FRET-based reporter can be used to image cAMP in stimulated rat cardiac myocytes.[12] More recently, Dyachok *et al.* have developed a FRET-based sensor to measure cAMP dynamics in pancreatic beta cells using ratiometric evanescent wave microscopy.[13,14] Other applications of cAMP imaging probes have been comprehensively reviewed elsewhere.[15]

Tsien and co-workers also developed fluorescent protein FRET-based sensors of Ca^{2+}, one of the most important secondary messengers in cell biology.[16] Dubbed ‘cameleons,’ these indicators consisted of fusions between CFP, the Ca^{2+}-binding protein calmodulin (CaM), the CaM-binding peptide M13, and YFP (Fig. 1).[17–20] Increased levels of Ca^{2+} causes CaM to bind to M13, thus changing the distance between the two fluorescent proteins and causing

an increase in FRET. More recently, an improved CaM-based FRET indicator was used to detect single action potentials in neurons from brain slices and *in vivo*, a feat which was not possible with previously existing Ca^{2+} sensors.[21,22] The success of these probes, however, is limited by both sensitivity and impaired targeting efficiency due to the large size of the chimeric probe.[23] Thus, alternative fluorescent protein-based Ca^{2+} sensors have been developed. One class of second-generation probes, named 'camgaroos,' is based on a single YFP which is pH-sensitive.[19] Another second-generation Ca^{2+} sensor, named 'pericam' because it is a circularly permuted version of YFP, enables ratiometric measurements, or the simultaneous recording of two distinct fluorescent signals that can allow for quantitative readouts.[24] A more recent improvement to this probe known as yellow cameleon-nano was used to image subtle changes in Ca^{2+} dynamics.[25] Imoto and co-workers have developed a high-affinity Ca^{2+} probe composed of a single GFP fused to CaM and M13 known as GCaMP.[26] Others have developed improved versions of GCaMP termed GCaMP2 and GCaMP3. GCaMP2 has been used to image Ca^{2+} in the murine heart[27] and to measure synaptic activity in zebrafish.[28] GCaMP3, a more recent improvement to GCaMP2, is brighter, has a greater dynamic range, and has a higher affinity for Ca^{2+}.[29] Recently, the palette of genetically-encoded sensors for Ca^{2+} was expanded to include improved green and red fluorescent protein indicators.[30]

Fluorescent protein-based FRET reporters for sensing glutamate were also developed by Tsien and co-workers as well as Frommer and co-workers. Glutamate is the major excitatory neurotransmitter in the brain, and monitoring its levels could provide insight into many neurological processes. Okumoto *et al.* have developed a FRET-based sensor consisting of CFP fused to both a clamshell-shaped bacterial glutamate periplasmic binding protein (YbeJ) and YFP (Fig. 1).[31] Hires *et al.* have improved this sensor by optimizing the linker sequences and glutamate affinities to enhance its signal-to-noise ratio.[32,33]

A genetically-encoded fluorescent indicator of intracellular H_2O_2 was developed by Lukyanov and co-workers.[34] H_2O_2 is a reactive oxygen species (ROS) that is speculated to be involved in paracrine signalling.[35] In their design, they fused a bacterial H_2O_2-sensitive transcription factor (OxyR) to a circularly permuted YFP. Cysteine oxidation of the OxyR portion induces a conformational change that changes the emission profile of the fluorescent protein. This probe has been used to image peroxide production during wound healing in zebrafish.[36]

1.3 Genetically-encoded and FRET-based sensors of protein post-translational modifications

Post-translational modifications of proteins are important regulators of protein function.[37] Genetically-encoded FRET probes based on the initial design pioneered by Tsien and co-workers have been constructed for monitoring post-translational modifications such as phosphorylation, methylation, acetylation, and glycosylation (Fig. 1).[38,39]

Protein phosphorylation by protein kinases is key for the activation of numerous signal transduction pathways.[40] FRET-based probes for phosphorylation, which sense kinase activities, consist of CFP and YFP fused to a consensus substrate for the relevant kinase and a binding domain.[38,41] These sensors have been used to detect protein kinase A,[42] protein tyrosine kinase,[43] Akt/protein kinase B,[44,45] and protein kinase C[46] activities. Improved versions of these sensors with better specificity and reversibility have enabled visualisation of protein phosphorylation by the tyrosine kinase SrcA during cell mechanotransduction as well as monitoring of protein kinase A activity during insulin signalling.[47,48] More recently, Kapoor and co-workers have examined the dynamics of protein phosphorylation by aurora B kinase, a key mitotic regulator, using FRET-based sensors in live cells.[49] There have also

been reports of genetically-encoded sensors with enhanced sensitivity and optimized signal-to-noise ratios for fluorescence lifetime imaging.[50,51]

Histone modifications play important roles in regulating gene expression. Ting and co-workers have developed CFP/YFP reporters of histone phosphorylation and methylation, which are important modifications that regulate transcription of nearby genes.[52–54] More recently, changes in histone acetylation were monitored with a FRET-based reporter.[55] Similarly, Mahal and co-workers have developed a FRET-based sensor of protein O-GlcNAcylation, a reversible form of glycosylation of intracellular proteins akin to phosphorylation.[56,57] Development of this probe has allowed for dynamic monitoring of O-GlcNAc modifications in live cells.

2. Small-molecule sensors

Small-molecule indicators can be advantageous relative to genetically-encoded ones because they often exhibit greater dynamic ranges and increased sensitivity.[58] They also tend to exhibit faster response kinetics. However, unlike genetically-encoded sensors, with few exceptions they cannot be localised or specifically targeted to a particular organelle in the cell by fusion to the protein of interest.[16]

Small-molecule fluorophores have been developed for sensing metal ions such as calcium, zinc, copper, and iron. Metal ions are interesting targets for imaging because they play critical roles in cell biology.[59] The design of small-molecule sensors for metal ions is challenging because the probe must be selective for the specified metal over other biologically abundant cations, including those that exist at much higher cellular concentrations such as Na^+, K^+, and Mg^{2+}. Thus, the design of metal-selective probes requires the successful application of principles of coordination chemistry.

2.1 Fluorescent Ca^{2+} indicators

The first small-molecule fluorescent Ca^{2+} sensor was developed by Tsien.[60] In this pioneering work, he replaced the two methylene groups of a well-known Ca^{2+} chelator with two benzene rings that enable the molecule to function as a chromophore (BAPTA, Fig. 2). Since then, many low- and high-affinity dyes for Ca^{2+}-sensing have been developed that emit UV and visible light. Some of these probes include the fluorescein derivatives Fluo-3, developed by Tsien and co-workers, and Fluo-4, a brighter, more photostable derivative of Fluo-3, developed by Molecular Probes (Eugene, OR) (Fig. 2).[58] Indo-1 and Fura-2 are ratiometric dyes that are widely considered the standard for quantitative intracellular Ca^{2+} measurements (Fig. 2).[61] Red-shifted Ca^{2+} indicators based on the rhodamine scaffold, including Rhod-2, have also been developed to minimize autofluorescence from biological samples (Fig. 2).[61,62] Tour *et al.* have combined the concept of a small-molecule sensor of Ca^{2+} and the localisation that the tetracysteine motif and FlAsH/ReAsH reagents provide by developing a biarsenical Ca^{2+} indicator to probe Ca^{2+} levels around specific proteins.[63]

2.2 Small-molecule Zn^{2+} indicators

In the past century, Zn^{2+} has emerged as an important regulator of protein function and signalling.[64–66] It has also been implicated in the pathophysiology of several neurodegenerative disorders, including Alzheimer's disease.[67] Thus, the ability to image Zn^{2+} using small-molecule sensors could elucidate metal ion homeostasis in relation to disease.

Several groups have developed Zn^{2+} sensors that were inspired by analogous probes for Ca^{2+} detection. Gee *et al.* have removed a single acetate arm from the Ca^{2+} chelator of Fluo-3 to create FluoZin-3, which maintains high affinity for Zn^{2+} (Fig. 3).[68] Lippard and

co-workers have developed several classes of fluorescein-based sensors for Zn^{2+} that are modified with a Zn^{2+} chelator on the xanthene core. These include the Zinpyr,[69–73] Zinspy,[74] and Quinozin[75] series of probes (Fig. 3). An improvement to the Zinpyr family of dyes, known as ZPP1, exhibits lower background fluorescence and higher fluorescence turn-on when complexed to zinc.[76] This probe was recently used to image high levels of Zn^{2+} in a mouse model of prostate cancer.[77] Nagano and co-workers have developed fluorescein-based Zn^{2+} sensors that are functionalised at the phenyl ring of the fluorophore-scaffold.[78] These include the ZnAF series (Fig. 3). In addition to fluorescein-based reagents, BODIPY,[79] cyanine,[80,81] and coumarin[82,83] probes for sensing Zn^{2+} in biological systems have also been developed.

Several ratiometric sensors of Zn^{2+} that capitalize on different mechanisms have been synthesized for quantitative measurements of Zn^{2+} concentrations *in vivo*. FuraZin and IndoZin are ratiometric Zn^{2+} sensors derived from the Ca^{2+} probes Fura and Indo, which operate *via* an internal charge transfer mechanism.[84] Nagano and co-workers have also developed the ratiometric Zn^{2+} sensors ZnAF–R1 and –R2, which are based on the Fura scaffold (Fig. 3).[85] Taki *et al.* have developed ratiometric sensors known as the Zinbo series that exploit excited-state intramolecular proton transfer processes in benzoxazole scaffolds.[86] Lippard and co-workers have also developed ratiometric Zn^{2+}-sensors that rely on a two-fluorophore system in which ester-mediated hydrolysis liberates coumarin as an internal standard.[87] In addition to fluorescent sensors, Zn^{2+} sensors that rely on alternative imaging modalities such as magnetic resonance imaging (MRI) have been developed.[88–90]

2.3 Small-molecule Cu^{+} and Fe^{2+}/Fe^{3+} indicators

Oxidative stress is thought to cause neuronal cell damage that can eventually lead to pathologies such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.[91] Redox-active metals such as Cu^{+} and Fe^{2+} have been implicated in causing oxidative stress by producing ROS such as H_2O_2, superoxide ($O_2^{-}\bullet$), and hydroxyl radicals (•OH).[92] Therefore, there has been much interest in imaging not only the ROS themselves (see sections 1.2 and 2.4) but the metal ions as well.

Fluorescence detection of copper using small-molecule sensors is difficult because Cu^{+} is the major active species within the reducing environment of the cytosol; however, this ion can readily disproportionate to Cu^{2+} and Cu^{0}. In addition, both Cu^{+} and Cu^{2+} are capable of quenching the fluorescence of many fluorophores.[64] Currently, there are a few reports of fluorescent Cu^{+} sensors that have been successfully used for visualizing Cu^{+} in live cells. Yang *et al.* have developed a sensor known as CTAP-1 (Fig. 4A), which comprises a pyrazoline dye platform appended to an azatetrathiacrown receptor for metal coordination.[93] A more water-soluble version of this probe, CTAP-2, was recently described.[94] Chang and co-workers have developed a Cu^{+} indicator known as CS1 (Fig. 4A) that combines a BODIPY dye and a thioether metal chelator.[95,96] Since then, a ratiometric fluorescent sensor based on this dye has been described, as well as a mitochondrial targeted version that allows for imaging of mitochondrial pools of copper.[97,98] A second-generation probe with different substituents on the boron center of the BODIPY dye known as CS3 was used in combination with X-ray fluorescence microscopy to image labile copper pools in neurons.[99] Recently, a near-infrared copper sensor, CS790, was reported for imaging copper in a murine model of Wilson disease.[100] Chang and co-workers have also developed an MRI-based sensor for copper.[101,102]

Like copper, the major forms of iron in the cell, Fe^{2+} and Fe^{3+}, also exhibit significant quenching capabilities, and designing fluorogenic sensors of iron is thus difficult as well. As a result, all of the sensors for detecting Fe^{2+}/Fe^{3+} in cellular systems are turn-off probes. For example, calcein (Fig. 4B) is a commercially available fluorophore that uses a metal chelator

for turn-off Fe^{2+} detection.[61] Phen Green SK (Fig. 4B) is another commercial probe that can detect both Fe^{2+} and Fe^{3+}.[61] One drawback of these probes, however, is that they are only partially selective for iron and can detect other metal ions as well.[103] The most specific sensors for Fe^{3+} rely on receptors derived from siderophores, which are small, high-affinity iron chelating compounds secreted by bacteria, that can be conjugated directly onto fluorophores.[61,104,105] A few turn-on sensors for Fe^{3+} have been reported; however, their utility for imaging in cells has not yet been established.[106,107]

2.4 Small-molecule ROS and RNS indicators

As discussed in Section 2.3, there is mounting evidence that ROS are linked to several neurodegenerative diseases.[91] Reactive nitrogen species (RNS) are also thought to cause neuropathological disorders.[108] More recently, ROS and RNS have been implicated as signalling molecules during normal physiological processes.[35,109] The canonical ROS and RNS involved in these processes are H_2O_2 and NO, respectively. Due to space limitation, herein we will discuss only small-molecule fluorescence indicators of H_2O_2 and NO because in-depth reviews on sensors for other ROS and RNS as well as metal-based fluorogenic probes for NO have been written.[110–113]

The traditional indicators for sensing H_2O_2 include dihydrofluorescein (DHFC, Fig. 5A)[114] or dihydrorhodamine (Fig. 5A);[115] however, these dyes suffer from auto-oxidation by the excitation light as well as reactivity with other ROS.[116,117] Chang *et al.* have developed a class of boronate-based fluorescent probes for sensing H_2O_2, which includes their first generation probe Peroxy-fluor-1 (PF1, Fig. 5A).[118] In the presence of H_2O_2, the boronic esters are chemoselectively deprotected to yield a fluorescent molecule. This concept has also been applied to blue- and red-shifted analogs, Peroxyxanthone-1 (PX1) and Peroxyresorufin-1 (PR1).[119] Second-generation probes that are more sensitive and are capable of detecting H_2O_2 at physiologically relevant concentrations have also been developed. These include Peroxycrimson-1 (PC1) and Peroxygreen-1 (PG1) (Fig. 5A).[120] Further, sensors of additional colors and FRET-based ratiometric probes for sensing H_2O_2 have been recently described.[121,122] Hydrogen peroxide sensors that can be targeted to specific organelles for visualizing ROS within subcellular locations have also been developed. These include probes for visualizing H_2O_2 in the mitochondria, nucleus, and endoplasmic reticulum.[123–125] Finally, new hydrogen peroxide sensors have been used to elucidate that redox signalling through Nox2 is essential for the normal growth and proliferation of neural stem cells.[126] These sensors were also used to show that H_2O_2 uptake is mediated by the water channel aquaporin to modulate downstream signalling events.[127]

Most sensors for NO utilize an *o*-phenylenediamine scaffold that is oxidized to the corresponding aryl triazole in the presence of NO and air (Fig. 5B).[109] In this design, the electron-rich diamine quenches the fluorescence of the dye by photoinduced electron transfer. Reaction with NO and O_2 generates an electron-poor triazole that triggers the fluorescence turn-on of the dye. Nagano and co-workers have developed a wide variety of probes based on this design that contain fluorescein (DAF-2),[128] rhodamine (DAR-4),[129] BODIPY (DAMBO and DAMBOO),[130,131] and cyanine (DAC-P, Fig. 5B).[132] This group has also developed fluorescent sensors of NO with increased sensitivity due to longer intracellular retention times.[133] Recently, a variation of the *o*-phenylenediamine scaffold was developed that is based on a quenched rhodamine B spirolactam (Fig. 5B).[134]

3. Chemical reporter strategy

For biomolecules such as glycans, certain protein post-translational modifications, and nucleic acids, detection *via* genetically-encoded reporters such as fluorescent proteins or

small-molecule sensors has largely not been possible. Thus, alternative strategies have been developed.

3.1 Lectins and antibodies for imaging glycans

Glycans are interesting targets for imaging because they play key roles in many dynamic biological processes. For example, cell-surface glycans are known to mediate cell–cell adhesion and communication, as well as host-pathogen interactions.[135] Other examples include embryonic development,[136] leukocyte homing,[137] and cancer cell metastasis.[138] Traditionally, glycans have been detected using antibodies or carbohydrate-binding proteins known as lectins.[139] Though lectins have been widely used for the detection and enrichment of glycoconjugates,[140,141] these glycan-binding proteins generally have low affinities for their targets and require multivalency for high-avidity binding.[142] Other drawbacks include toxicity and tissue impermeability.[143] For these reasons, lectins have limited capabilities for *in vivo* imaging; however, they have been used routinely for imaging glycans *ex vivo* on cultured cells and on tissue samples.[144,145]

Like lectins, antibodies have limited use for *in vivo* imaging; however, there is one report of their use for glycan-specific imaging in mice. In this study, Licha *et al.* imaged a peripheral lymph node endothelial glycan termed sulfoadhesin using the MECA-79 antibody.[146] Though there are a number of monoclonal antibodies against distinct epitopes, it is difficult to generate antibodies against certain epitopes because the synthesis of many glycan structures can be very cumbersome.[147] Thus, applications of antibodies toward *in vivo* imaging are also limited. Furthermore, like lectins, antibodies also suffer from poor tissue access.[148]

3.2 Bioorthogonal chemical reporter strategy for imaging glycans

Though lectins and antibodies have been used to image glycans, they are not ideal for imaging dynamic changes in the glycome, the ensemble of glycans displayed on the cell surface. We have developed an approach for imaging the glycome that enables the visualization of glycan dynamics *in vivo*.[149] Our method is a two-step strategy in which the first step involves the metabolic incorporation of an unnatural monosaccharide into an organism's glycome.[150] This substrate analog contains a reactive group known as a "chemical reporter.' In the second step, the chemical reporter is labelled with an imaging probe *via* a chemoselective reaction.

The requirements for such a chemical reaction are quite rigorous. First, the chemical reporter and its reaction partner must react in a physiological environment (pH 6–8, 37 °C), while simultaneously remaining inert to all of the surrounding functionality in the biological environment. This task is challenging because there are many nucleophilic and electrophilic functional groups found in cells and living organisms. Second, the reaction must not produce any toxic byproducts or cause harm to the biological sample. Finally, the reporter and its complementary probe must have good bioavailability and form a stable bond. Chemical reactions that meet this collection of criteria are known as "bioorthogonal."[151]

Currently, only a handful of reactions possess the quality of bioorthogonality. These reactions include the condensation of ketones and aldehydes with hydrazide or alkoxyamine probes, the Staudinger ligation of triarylphosphines and azides, and Cu-free click chemistry between cyclooctynes and azides (Fig. 6A–B, D). These reactions have been used to label not only proteins but glycans and lipids as well.

Ketones and aldehydes are not truly bioorthogonal because keto and aldehydic metabolites are abundant within cells and in biological fluids. In addition, the pH optima of hydrazone and oxime formation with hydrazide and alkoxyamine groups is 3–4, which precludes their

use *in vivo*.[152] Ketones and aldehydes have been utilised to label proteins and glycans on cell surfaces and in the extracellular environment. These mild electrophiles can form reversible Schiff bases with primary amines such as lysine side chains; however, in water, the equilibrium favors the carbonyl. In contrast, the formation of hydrazones and oximes using hydrazide and aminooxy groups, respectively, are favored in physiological conditions and are quite stable (Fig. 6A). Dawson and co-workers have greatly accelerated both oxime and hydrazone formation *via* the use of a nucleophilic aniline catalyst.[153,154]

We have recognized the utility of ketones and aldehydes as chemical reporters and have used them to label glycans.[155] In this work, Mahal *et al.* reported that an unnatural keto analog of *N*-acetylmannosamine (ManNAc), *N*-levulinoylmannosamine (ManLev), can be metabolized by mammalian cells to the corresponding keto sialic acid (SiaLev) and incorporated within cell-surface glycans (Fig. 7A–B).[155] Sadamoto *et al.* have introduced ketones into bacterial cell walls and labeled the chemical reporters with a hydrazide fluorophore probe.[156]

More recently, the azide has emerged as the chemical reporter of choice. This functional group is unique in that it is truly bioorthogonal to the biological milieu. Only one naturally occurring azido-metabolite, which was isolated from unialgal cultures, has been reported to date.[157] Moreover, azides can undergo highly selective reactions such as the Staudinger ligation with triarylphosphines, the copper-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC), and Cu-free click chemistry with strained alkynes (Fig. 6B–D).[158]

The Staudinger ligation is based on the classic Staudinger reduction, which was developed by Hermann Staudinger in 1919. In this work, Staudinger reported that azides react with triarylphosphines under mild conditions to yield aza-ylide intermediates.[159] In the presence of water or an appropriate electrophile, this intermediate can be hydrolyzed or trapped to yield an amine and the corresponding phosphine oxide.[160] We modified the classic Staudinger reaction by introducing an electrophilic trap onto the phosphine.[161] Termed the Staudinger ligation, this reaction ultimately results in the formation of a covalent amide bond between the azide and phosphine along with oxidation of the phosphine (Fig. 6B). This reaction also proceeds at physiological pH with no apparent toxicity to live cells and whole organisms.[162]

We have shown that an azide-containing analog of ManNAc, termed *N*-azidoacetylmannosamine (ManNAz), can also be metabolically incorporated into cell-surface glycans as the corresponding azido sialic acid (SiaNAz) (Fig. 7).[161] The Staudinger ligation has been used to image these labelled glycans on live cells using phosphine fluorophore conjugates[163] as well as bioluminescent and fluorogenic phosphines.[164,165] Furthermore, we and others have shown that this reaction can be used to label glycans within living animals.[162,166,167]

Paulson and co-workers have shown that natural sialic acids can be oxidized to the corresponding aldehyde using sodium periodate and then subsequently imaged using oxime chemistry with alkoxyamine probes.[168] In addition to metabolic labelling of sialic acid-containing glycans,[169,170] other sectors of the glycome can be metabolically labelled using azido analogs of *N*-acetylglucosamine (β-*O*-GlcNAcylated proteins),[171] *N*-acetylgalactosamine (mucin-type O-linked glycans and β-*O*-GlcNAcylated proteins),[172,173] and fucose (fucosylated glycans) (Fig. 7B).[174,175] Extending the scope of this methodology, which has thus far focused on labelling individual monosaccharides within the larger glycan structure, a recent report described the targeting of the disaccharide LacNAc (Galβ-1,4-GlcNAc).[176]

Azides can also be exploited as 1,3-dipoles in a [3+2] cycloaddition with alkynes to form triazole products (Fig. 6C).[158] Sharpless and co-workers and Meldal and co-workers independently showed that the normally sluggish reaction between azides and acetylenes can be greatly accelerated with the addition of a copper catalyst.[177,178] The copper-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is one of the most popular reactions from a family of "click reactions," defined by their highly selective and modular approach to forming chemical bonds.[179] CuAAC is much faster than the Staudinger ligation; however, the necessary copper catalyst is cytotoxic, although new biocompatible Cu(I) catalysts have been described to reduce toxicity to live cells.[150,180–183] CuAAC has been used for imaging glycans using alkynyl ManNAc analogs as well as azido and alkynyl fucose analogs in fixed cells (Fig. 7B).[175,184,185] In addition, CuAAC was recently used to image lipopolysacccharide (LPS) in bacterial membranes by targeting 3-deoxy-D-mannooctulosonic acid, a specific and essential component of the inner core of LPS.[186]

We have eliminated the need for a toxic copper catalyst by activation of the alkyne *via* an alternative mechanism—ring strain. This reaction is accordingly known as Cu-free click chemistry. Agard *et al.* first demonstrated this concept using a cyclooctyne reagent (OCT) whose kinetics were on par with the Staudinger ligation (Fig. 6D and 8).[187] In order to accelerate the strain-promoted cycloaddition, Baskin *et al.* introduced fluorine atoms in an attempt to lower the LUMO energy of the alkyne (MOFO, DIFO, DIFO2, DIFO3; Fig. 8).[188,189] This reaction has been used to image glycans on live cells,[188] the nematode *C. elegans*,[190] and zebrafish embryos.[191–194] We have also shown that Cu-free click chemistry can be used to label glycans within mice.[195] Second-generation cyclooctynes have been developed, including more water-soluble probes (ALO and DIMAC, Fig. 8)[189,196] as well as faster reagents based on a dibenzocyclooctynyl scaffold that have been utilized for imaging glycans on cells (DIBO, S-DIBO, DIBAC, BARAC, Photo-DIBO, TMDIBO, keto-DIBO; Fig. 8),[197–203] a coumarin-based fluorogenic cyclooctyne,[204] and thiacycloalkynes with improved stability.[205] Several theoretical studies have been published to explain the enhanced reactivity of a number of these cyclooctynes.[206–208]

In addition to Cu-free click chemistry using strained alkynes, several new chemistries using strained alkenes have emerged for labelling proteins in live cells and animals.[209] These include reactions of strained alkenes with azides,[210] tetrazines,[211,212] nitrile oxides,[213] and nitrile imines,[214] as well as photoinducible tetrazole–alkene cycloadditions, known as photo-click chemistry.[215] Recently, a new bioorthogonal reaction between highly strained quadricyclanes and Ni(bis)thiolenes was described that is selective and proceeds readily in aqueous environments.[216] Although these reactions have only been described for targeting proteins thus far, their selectivity and versatility will hopefully encourage their use in imaging beyond the proteome. Applications using multiple bioorthogonal chemistries would ultimately enable simultaneous visualisation of different classes of biomolecules within the same cell.

3.3 Bioorthogonal chemical reporter strategy for imaging lipids

As secondary metabolites and protein post-translational modifications, lipids, like glycans, are not amenable to imaging *via* genetically-encoded reporters. Lipids are attractive targets for imaging because they play important roles in cellular processes such as signal transduction and membrane fusion.[217] In addition, lipidation of proteins regulates the signalling properties, subcellular localisation, trafficking, and activity of many proteins.[218] One approach to imaging lipids in live cells has been the application of the bioorthogonal chemical reporter strategy.[219]

Phospholipids are the major constituents of cell membranes. Those containing the head group choline are important structural components of membranes and play critical roles in

cell signalling.[220] Despite the crucial roles of these lipids, little is known about their localisation and trafficking.[221] Thus, Jao *et al.* have utilized the chemical reporter strategy in order to image their dynamics in cells and tissues. In order to achieve this goal, they synthesized a choline analog that contains a terminal alkyne moiety, which can be detected by using CuAAC (Fig. 9). Using this technology, they were able to examine the kinetics of phospholipid turnover and the distribution of the lipids in cells and in mice.[221] Similarly, Schultz and co-workers have shown that phosphatidic acid analogs that contain either terminal alkynes or cyclooctynes within the lipid tail can be used to image these lipids in cells (Fig. 9).[222]

Lipidation of proteins *via* fatty-acylation and prenylation has been known to greatly affect the specificity and efficiency of signal transduction as well as protein–protein interactions.[218] Several groups have applied the bioorthogonal chemical reporter strategy to image these protein post-translational modifications.[223–225] Zhao and co-workers have used azido analogs of farnesylated proteins in order to profile proteins that are post-translationally modified (Fig. 9).[226,227] Alkynyl farnesol reporters have also been described.[228,229] Several groups have demonstrated that fatty-acylation of eukaryotic proteins by attachment of S-palmitoyl groups and N-myristoyl to cysteine and N-terminal glycine residues, respectively, can be imaged or profiled by employing azido and alkynyl analogs of these lipids and the Staudinger ligation or CuAAC (Fig. 9).[230–236] More recently, a chemical reporter of cholesterylated proteins has also been described.[237]

3.4 Bioorthogonal chemical reporter strategy for imaging nucleic acids

Nucleic acids are traditionally detected by intercalating dyes (*e.g.*, 4′6-diamidino-2-phenylindole), *in situ* hybridization techniques, and metabolic incorporation of nucleoside analogs that are either radiolabelled or detected *via* antibodies. Hybridization approaches include molecular beacons, which have been used for visualizing both DNA and RNA.[238,239] Development of these fluorescent oligonucleotide probes requires the incorporation of fluorescent nucleotides, which can be synthesized using different chemistries.[240] For example, Ju and co-workers have synthesized fluorescent oligonucleotides *via* both CuAAC and the Staudinger ligation for the purposes of DNA sequencing using fluorescence detection.[241,242] Excess fluorophore conjugates of oligonucleotide probes must be washed away to eliminate background signal, so fluorogenic approaches are highly sought after as well. Toward this end, Cai *et al.* have developed a fluorogenic peptide nucleic acid-based probe that is activated by the Staudinger ligation upon DNA hybridization.[243]

Other techniques include metabolic incorporation of deoxynucleoside analogs such as [^{3}H]thymidine ([^{3}H]T) and 5-bromo-2′-deoxyuridine (BrdU) (Fig. 10). [^{3}H]T and BrdU are detected by autoradiography and immunohistochemistry, respectively. Though these analogs have been useful for probing DNA synthesis, both of these techniques are not ideal. Autoradiography can be cumbersome, and BrdU immunostaining can compromise the integrity of the tissue samples.[244] Mitchison and co-workers have synthesized an alkynyl analog of thymidine, 5-ethynyl-2′-deoxyuridine (EdU, Fig. 10), which is readily incorporated into DNA during DNA replication.[245] This chemical reporter can then be detected with CuAAC and has enabled visualization of DNA synthesis in cells and in tissue sections from mice. In addition, this methodology causes much less degradation of biological specimens relative to traditional BrdU immunostaining. Arabinosylated EdU analogs (F-ara-EdU, Fig. 10) and alkynyl cytosine analogs have been reported that were used for imaging DNA in cells and zebrafish.[246,247] More recently, an alkynyl analog of uridine, 5-ethynyluridine (EU, Fig. 10) has been used to image RNA synthesis *in vivo* using an analogous strategy.[248]

4. Label-free imaging methods

4.1 Imaging mass spectrometry

All of the technologies discussed thus far require labels or contrast agents in order to visualise the biological molecules of interest. Imaging mass spectrometry (IMS) is an emerging label-free technique that allows for the visualisation of endogenous proteins, lipids, and small molecules.[249] IMS can be used to create 2D and 3D images of the proteomic or small-molecule content of cells or tissues by combining the measurement capability of mass spectrometers with a surface sampling process.[250] Currently, while there exist many different modes of ionisation, two popular ionisation methods are used to generate images.[251] These techniques include matrix-assisted laser desorption ionisation (MALDI) and secondary ion mass spectrometry (SIMS) IMS. Though IMS has been used for imaging both proteins and other biological molecules, for the purposes of this review, we will focus exclusively on imaging small molecules.

Of these two IMS techniques, MALDI IMS has seen more widespread use for analysing biological samples due to its large mass-to-charge ratio (m/z) range (up to 100 kDa), which is useful for analysing proteomes.[252] Imaging with MALDI mass spectrometry, however, is only capable of achieving spatial resolutions of about 20 μm while SIMS can achieve better than 100 nm with specialised instruments.[253] On the other hand, SIMS has been documented to detect molecules of masses up to only 1 kDa.[250] While many groups have taken advantage of these imaging technologies, a number of laboratories continue to pursue the development of new and improved methodologies, including those that can achieve ionisation under ambient conditions.[254,255]

For analysis by MALDI IMS, biological samples are prepared in a similar manner to protein or peptide samples. Typically, a thin cryosection of tissue is covered with an organic matrix. The sample is then scanned by the mass spectrometer, and spectra are collected over a predefined area. The resulting dataset contains an array of spectra including different signals of measured mass and intensity. The intensity of signal, or combinations of signals, can then be plotted to generate an image over the sample surface.[256] This technique has been used to image many different molecules, including phospholipids.[256] As discussed in Section 3.3, these lipids are involved in cell signalling pathways and have been implicated in diseases such as lipid storage disorders as well as Alzheimer's disease and Down syndrome.[257] McLean *et al.*, Jackson *et al.*, and Rujoi *et al.* have used MALDI IMS to visualise phospholipids in rat brains and mammalian lens tissue.[258–261] A growing number of researchers are applying IMS to the diagnosis of diseases such as cancer by looking at differences in lipid profiles between diseased and healthy tissue.[262–265] In addition to lipids, Dorrestein and co-workers have applied MALDI IMS to imaging secondary metabolites produced by marine cyanobacteria and sponges as well as *Bacillus subtilis* and *Streptomyces coelicolor*.[266–268]

SIMS uses an accelerated primary ion beam to bombard the sample, a process that generates secondary ions that are then detected by a mass spectrometer.[253] Based on instrument design, there are two different approaches to SIMS. The first method, time-of-flight (TOF) SIMS, uses a primary ion beam that is pulsed, and the resulting secondary ions are detected by a TOF mass spectrometer. The second approach, termed dynamic SIMS, uses a continuous primary ion beam, and preselected ions are detected by a magnetic sector mass spectrometer. Specialised instruments have been developed to improve both the sensitivity and spatial resolution of SIMS (*vide infra*).[253] TOF-SIMS has been used to image lipids in cells and tissues.[269–271] TOF-SIMS has also been used to image highly curved membranes during Tetrahymena mating (Fig. 11).[272] In this study, Ostrowski *et al.* demonstrated that

the fusion region contains elevated amounts of phosphatidylethanolamine, a high-curvature lipid.

In the last decade, advancements in instrumentation have greatly improved the utility of SIMS for imaging biological samples. These developments include nanoSIMS, which enables image resolution of a few tens of nanometers.[253] This technique has been applied in tracer studies examining ^{13}C-labeled free fatty acid transport across cell membranes.[273] Boxer and co-workers have used nanoSIMS to image lipid domains within supported lipid bilayers with a lateral resolution of 100 nm.[274] NanoSIMS has also been used to demonstrate that deep-sea anaerobic methane-oxidizing archaea fix nitrogen and cyanide and share the products with sulfate-reducing bacterial symbionts.[275] Lechene *et al.* have developed multiple-isotope imaging mass spectrometry (MIMS), in which multiple isotopes are detected simultaneously, enabling both imaging and quantification of labeled molecules within subcellular compartments.[276] This technique was utilised to test the idea that stem cells retain the older template DNA to insure lifetime genetic stability, a concept known as the immortal strand hypothesis.[277] In this study, the authors showed that the DNA strands are randomly segregated during cell proliferation. Another technical advance includes cluster time-of-flight (TOF)-SIMS, which has been shown to improve the yield of secondary ions produced.[278] This technique utilises heavier primary ions that have improved efficiency for production of secondary ions, thus allowing easier identification of biomolecules. Many groups have demonstrated that cluster TOF-SIMS imaging can be used to image lipids in rodent brains and tissues.[279–283] Finally, Heeren and co-workers have developed matrix-enhanced SIMS (ME-SIMS), which combines the high spatial resolution of SIMS with the sample preparation of MALDI IMS.[284] Like cluster TOF-SIMS, ME-SIMS also increases the molecular ion yield.

4.2 Raman and coherent anti-Stokes Raman scattering (CARS) microscopy

Raman and coherent anti-Stokes Raman scattering (CARS) microscopy are emerging label-free methods for cellular imaging.[285] These two techniques rely on optical signals and have been used to image proteins, lipids, and nucleic acids.[286,287] Xie and co-workers have recently demonstrated that stimulated emission can also be used as a contrast mechanism for microscopy.[288] They have used this technique to image chromoproteins, small-molecule drug distributions *in vivo*, and haemoglobin. For the purposes of this review, we will focus exclusively on Raman and CARS applications for imaging small molecules.

Raman microscopy relies on a phenomenon known as Raman scattering. This effect occurs when the wavelength of scattered light shifts slightly from the original wavelength of light due to excitation of the light-scattering molecules to a vibrationally excited state.[286] Because the shift in wavelength depends strongly on the molecule's structure and chemical environment, the resulting spectra can be used to identify specific molecules in the sample. In Raman microscopy, spectra are obtained from each position in the sample to construct a 2D or 3D image of molecular distribution based on signal intensities. This imaging technique has been used to visualize nucleic acids[289,290] and lipid bodies[291] in live cells. Raman microscopy has also been used to discern malignant tissue from healthy tissue for cancer diagnostic purposes.[292–294] Raman microscopy, however, suffers from several limitations.[295] First, the Raman effect is very weak, and consequently, data acquisition times are long. Second, creation of images requires high-powered lasers and long integration times per pixel, impeding video-rate microscopy. Xie and co-workers have greatly improved the sensitivity of Raman microscopy by developing a new technique known as stimulated Raman scattering (SRS) microscopy, which has been used to visualize lipids in live cells and tissues from mice.[296] Furthermore, *in vivo* imaging was enabled by enhancement of the signal and by increasing the imaging speed to video-rate.[297] Recently, Freudiger *et al.* have

improved the spectral selectivity of SRS, thus allowing the molecular distinction between related molecules by using spectrally tailored excitation.[298]

Stronger vibrational signals can be obtained using CARS microscopy. In CARS, light beams with two different optical frequencies interact with the sample. A strong CARS signal is produced when the difference of the two frequencies matches the vibrational frequency of the molecules. Because CARS is orders of magnitude more sensitive than Raman microscopy, it allows video-rate vibrational imaging. The Reintjes group was the first to use CARS as a contrast mechanism for microsopy.[299] The technique was popularized by Xie and co-workers almost two decades later when they developed a greatly improved method that allowed for higher sensitivity, higher spatial resolution, and three-dimensional sectioning capabilities.[300]

With these improvements, CARS has been used extensively for many *in vitro* and *in vivo* imaging applications. Wurpel *et al.* and Potma *et al.* have used CARS to image lipid vesicles and lipids on supported bilayers.[301,302] Xie and co-workers have also used CARS to image lipids in live cells.[303–305] Others have imaged lipids in infection models,[306,307] mouse brains,[308] and whole organisms such as *C. elegans* (Fig. 12).[309–311] Improvements to CARS include video-rate microscopy applications of imaging lipids in the skin of live mice[312] and frequency modulation CARS, which significantly increases the detection sensitivity of CARS by decreasing the nonresonant background.[313]

Conclusions

Recent advances in molecular imaging techniques have been made possible by fundamental advances in disparate areas of chemistry, spanning from metal-coordination chemistry to biochemistry to spectroscopy. These new methods have enabled the visualisation of virtually every major class of molecule inside living cells. As these methods are further improved and gain wider usage, it is our hope that they will continue to shed light on many biological processes.

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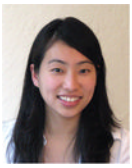
Biographies



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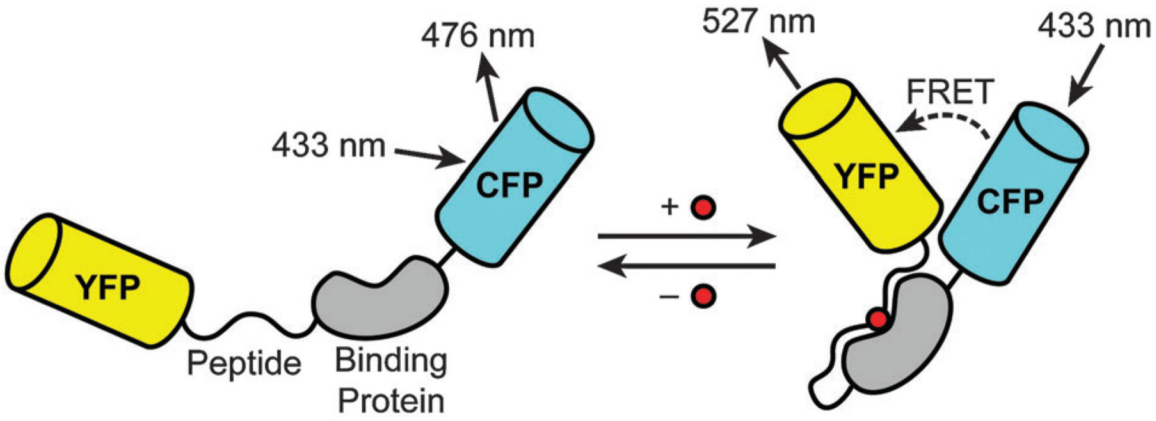


Analyte or PTM (●)	Peptide	Binding Protein	References
Calcium	M13	Calmodulin	16-22, 24-25
Glutamate	YbeJ[a]		31-32
Phosphorylation	Kinase substrate peptide	Phosphoaminoacid binding domains	38-51
Histone methylation	N-terminal region of the histone protein H3	Chromodomains	52
Histone phosphorylation	Histone H3 peptide	14-3-3 protein	53
Histone acetylation	Histone protein H4	Bromodomains	55
β-O-GlcNAc	Casein kinase II substrate peptide	GafD	56

[a]YbeJ is a bacterial glutamate-binding protein that undergoes a significant conformational change after binding glutamate

Fig. 1.
Genetically-encoded fluorescent protein FRET reporters.

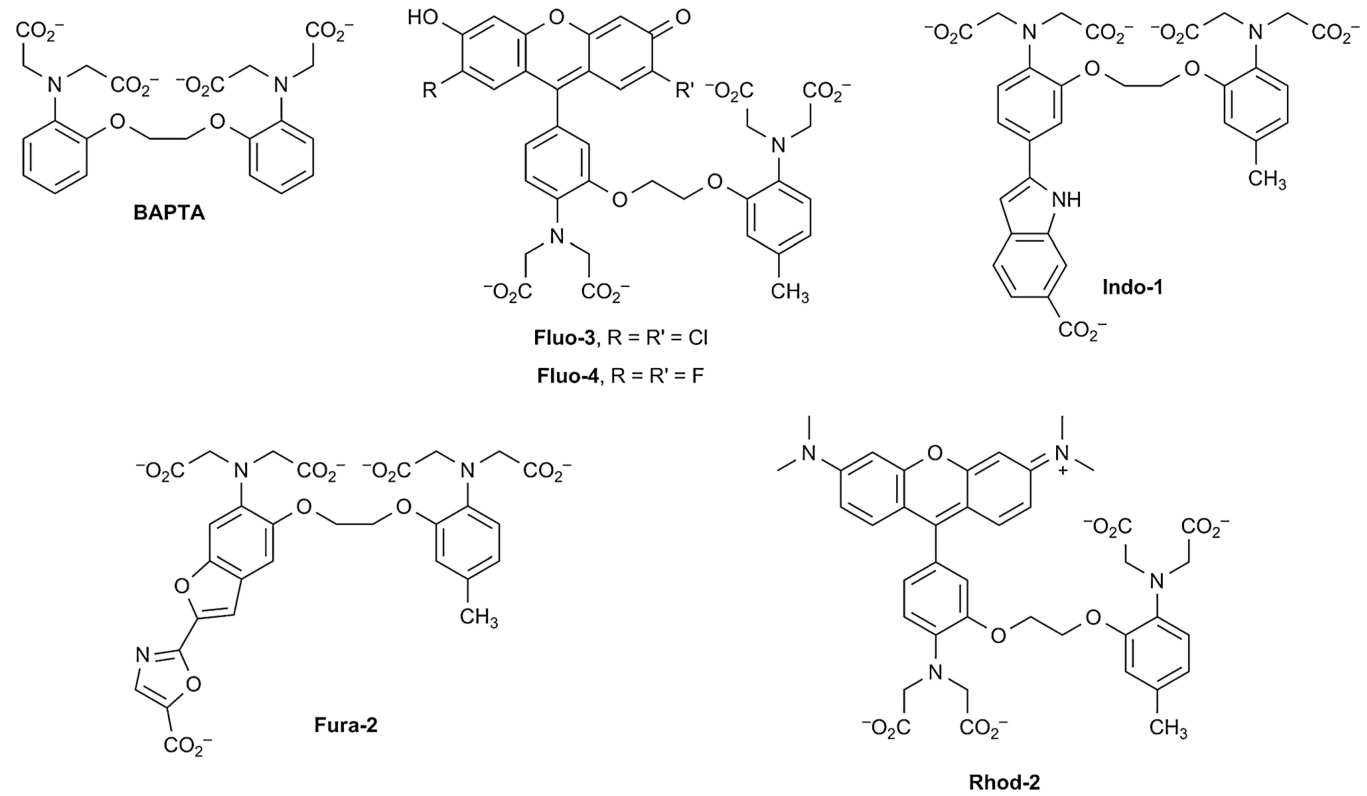


Fig. 2.
Small-molecule fluorescent Ca^{2+} sensors.

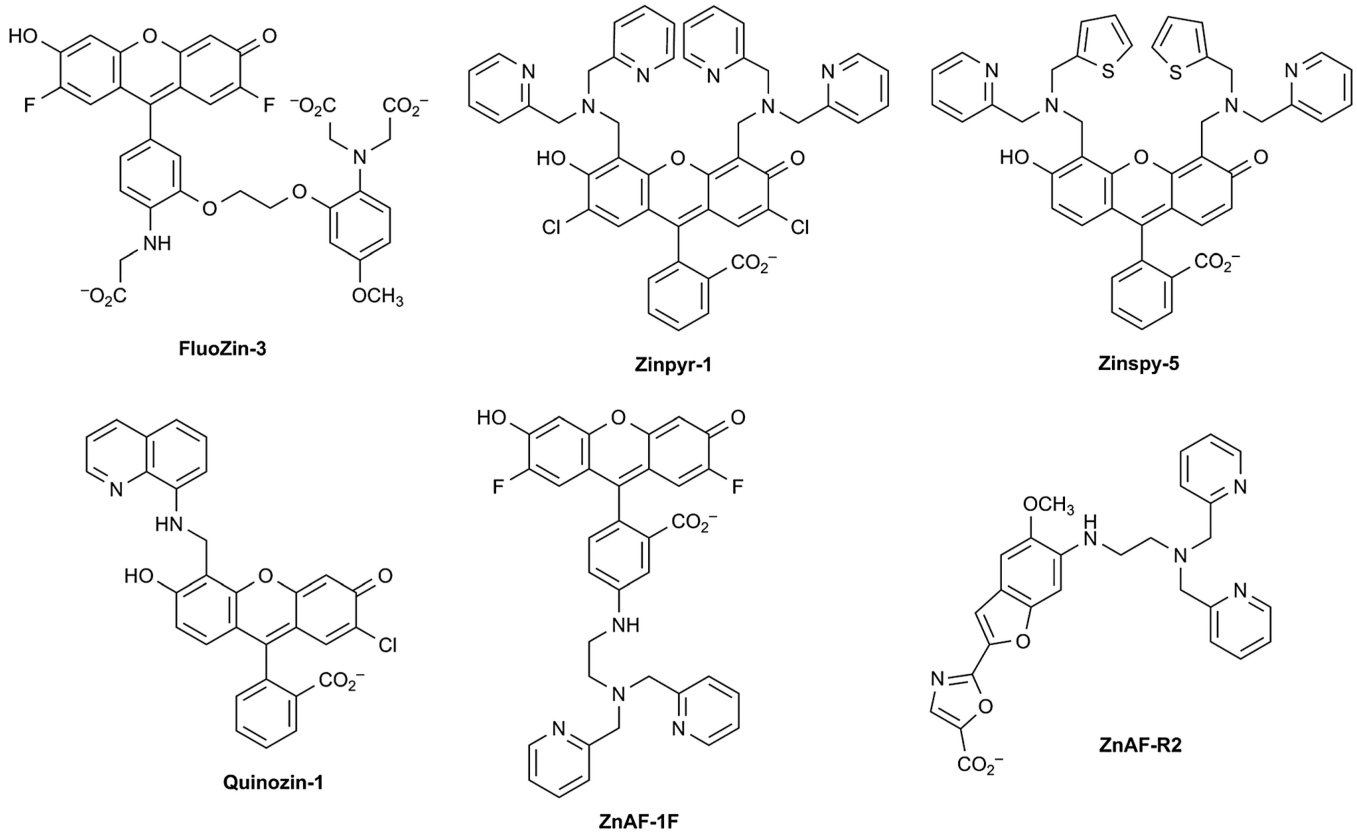


Fig. 3.
Small-molecule fluorescent Zn^{2+} sensors.

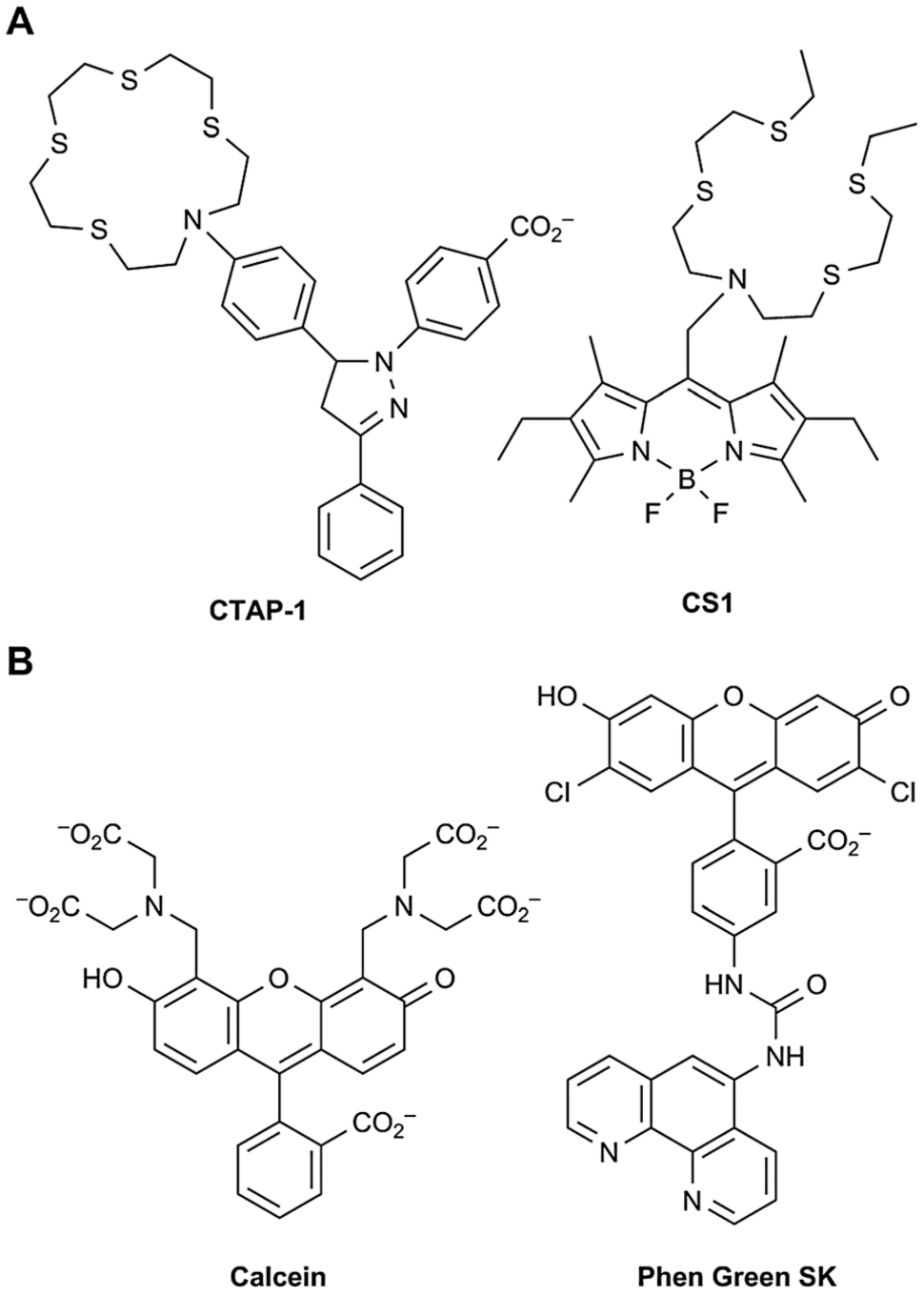


Fig. 4.
Small-molecule fluorescent Cu^{+} and Fe^{2+}/Fe^{3+} sensors.

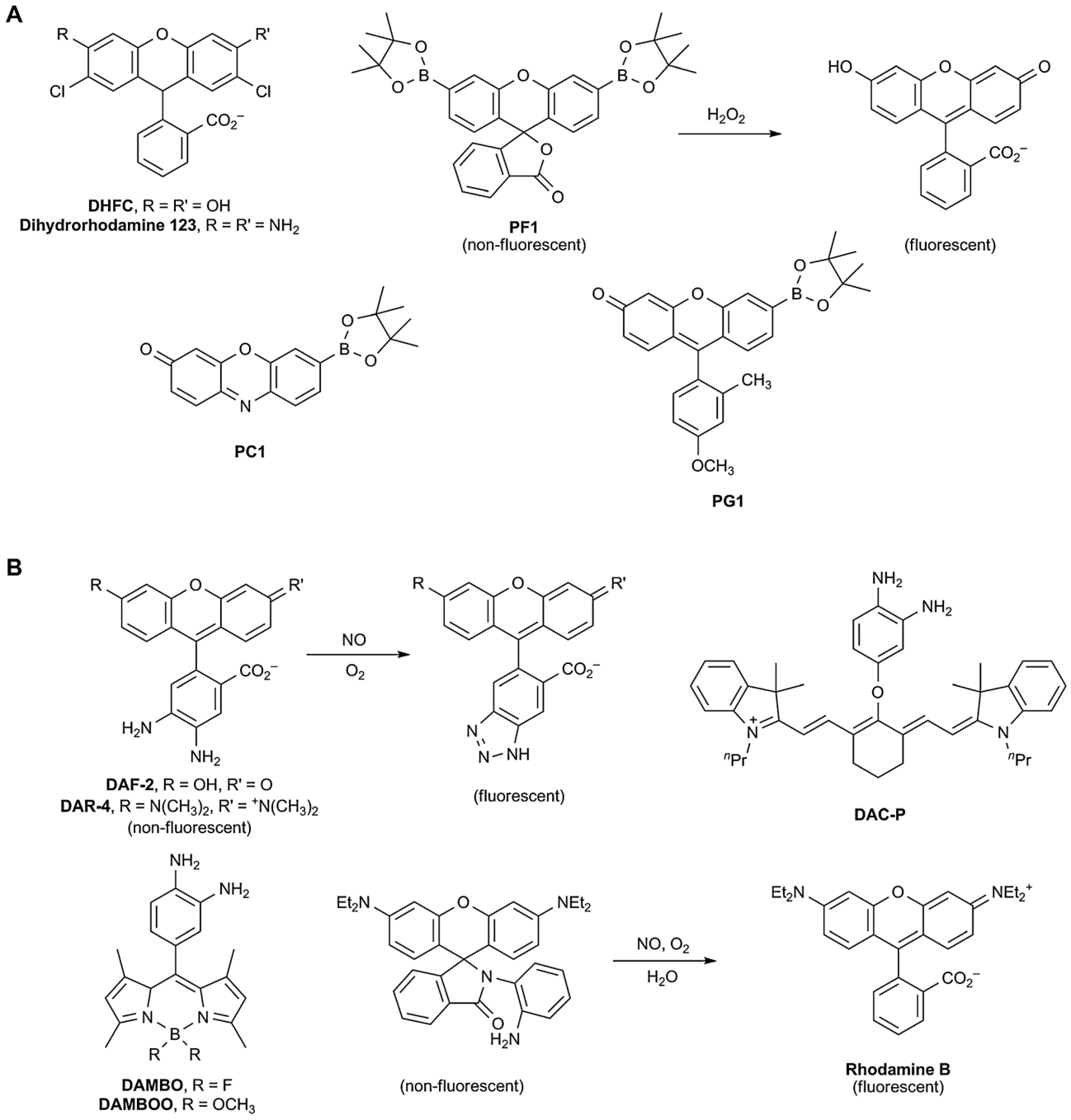


Fig. 5.
Small-molecule fluorescent ROS/RNS sensors. (A) Fluorogenic H_2O_2 probes. (B) Fluorogenic NO probes.

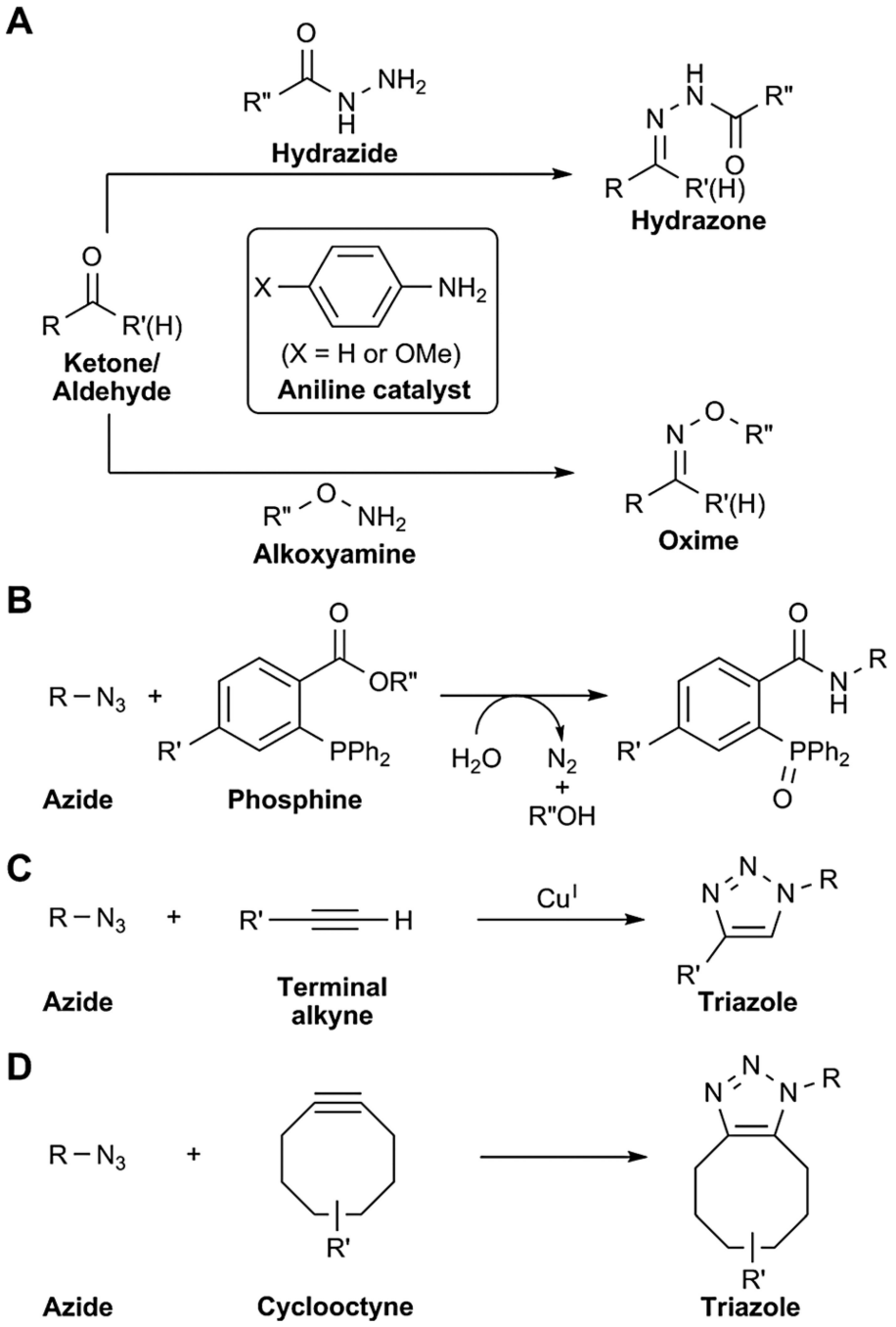


Fig. 6.

Bioorthogonal chemical reactions. (A) Ketone and aldehyde condensation with hydrazides or alkoxyamines. (B) Staudinger ligation between azides and triarylphosphines. (C) Copper-catalysed azidealkyne 1,3-dipolar cycloaddition (CuAAC). (D) Cu-free click chemistry.

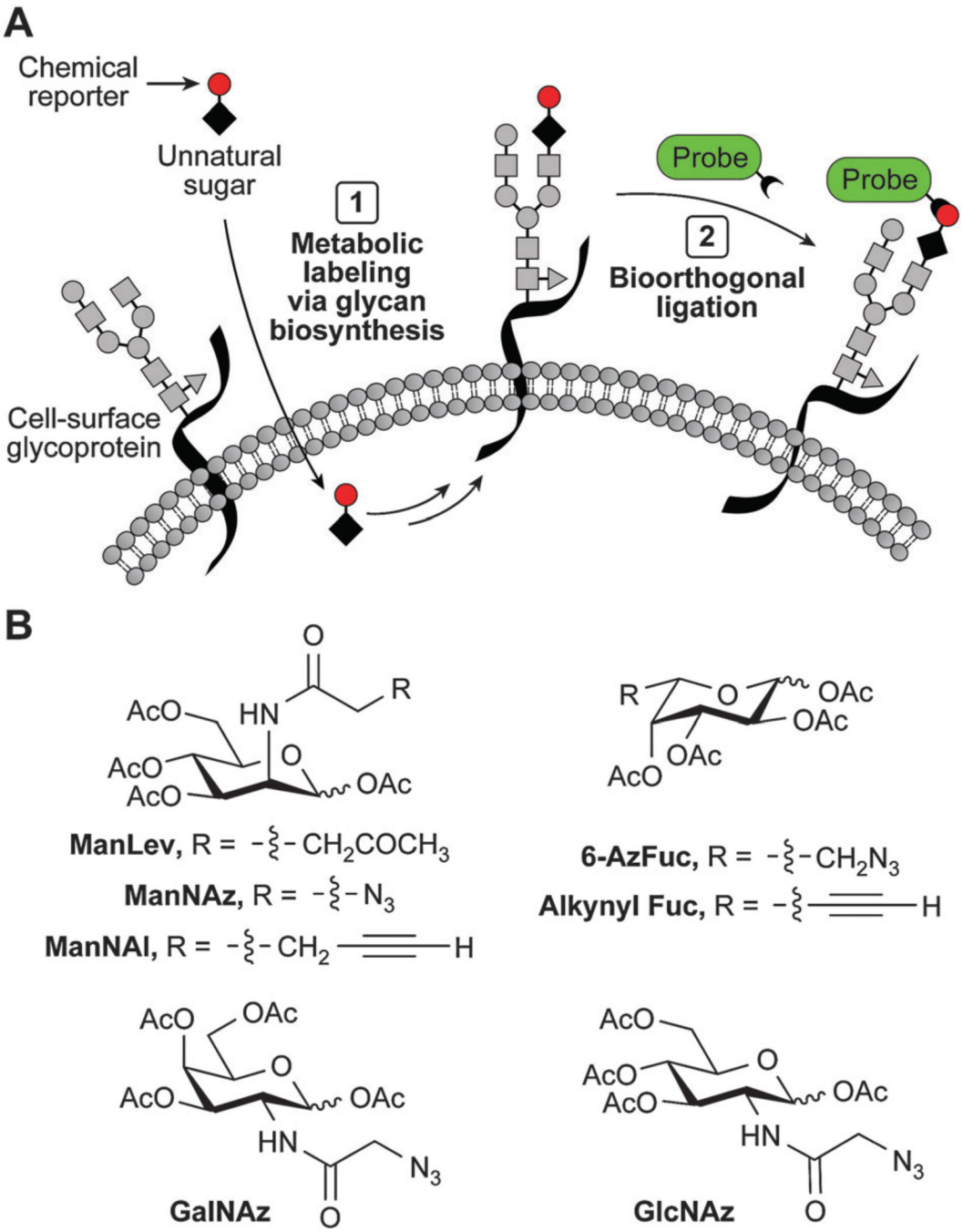


Fig. 7.
Bioorthogonal chemical reporter strategy. (A) Metabolic oligosaccharide engineering. (B) Unnatural sugars.

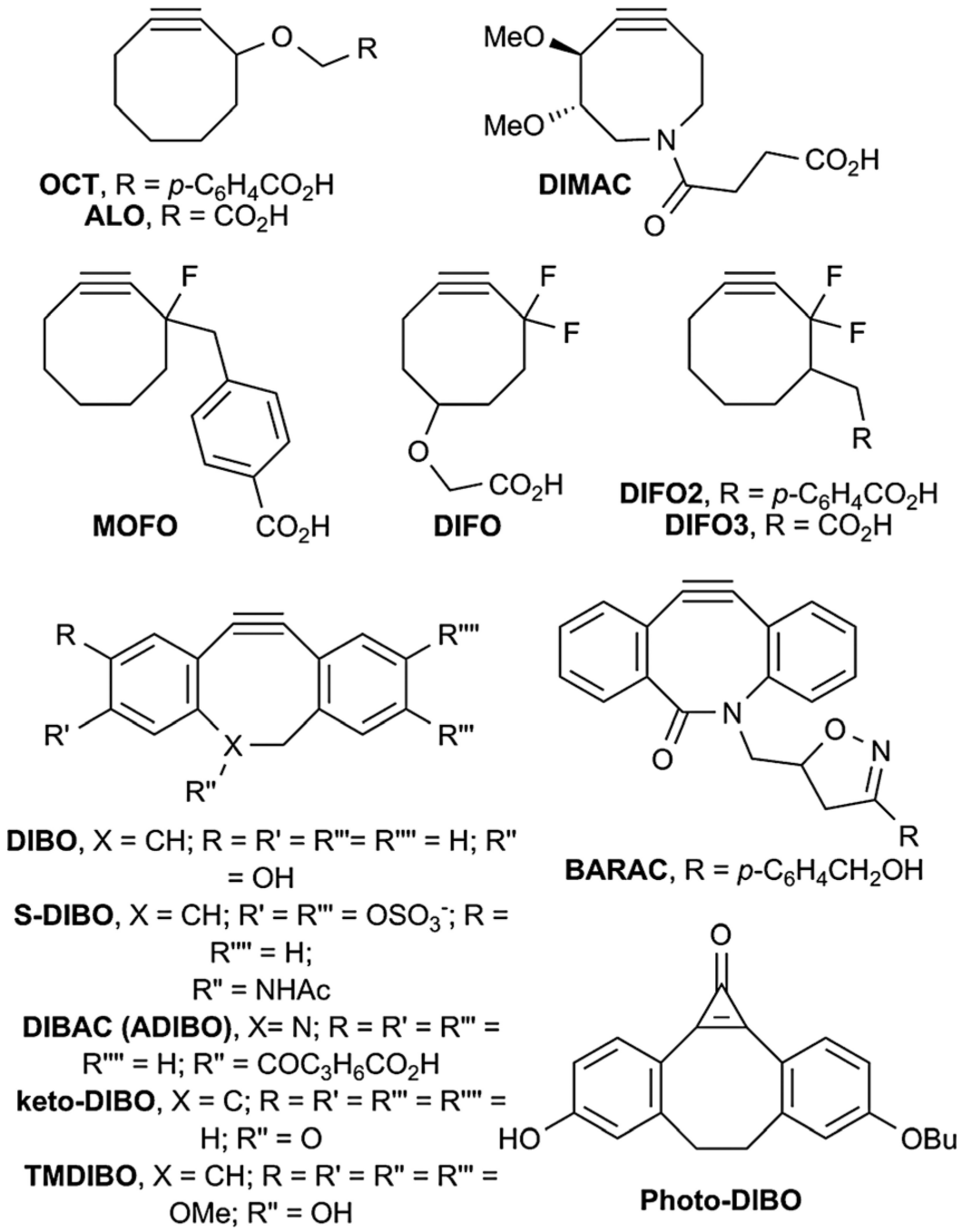


Fig. 8.
Cyclooctyne reagents for Cu-free click chemistry.

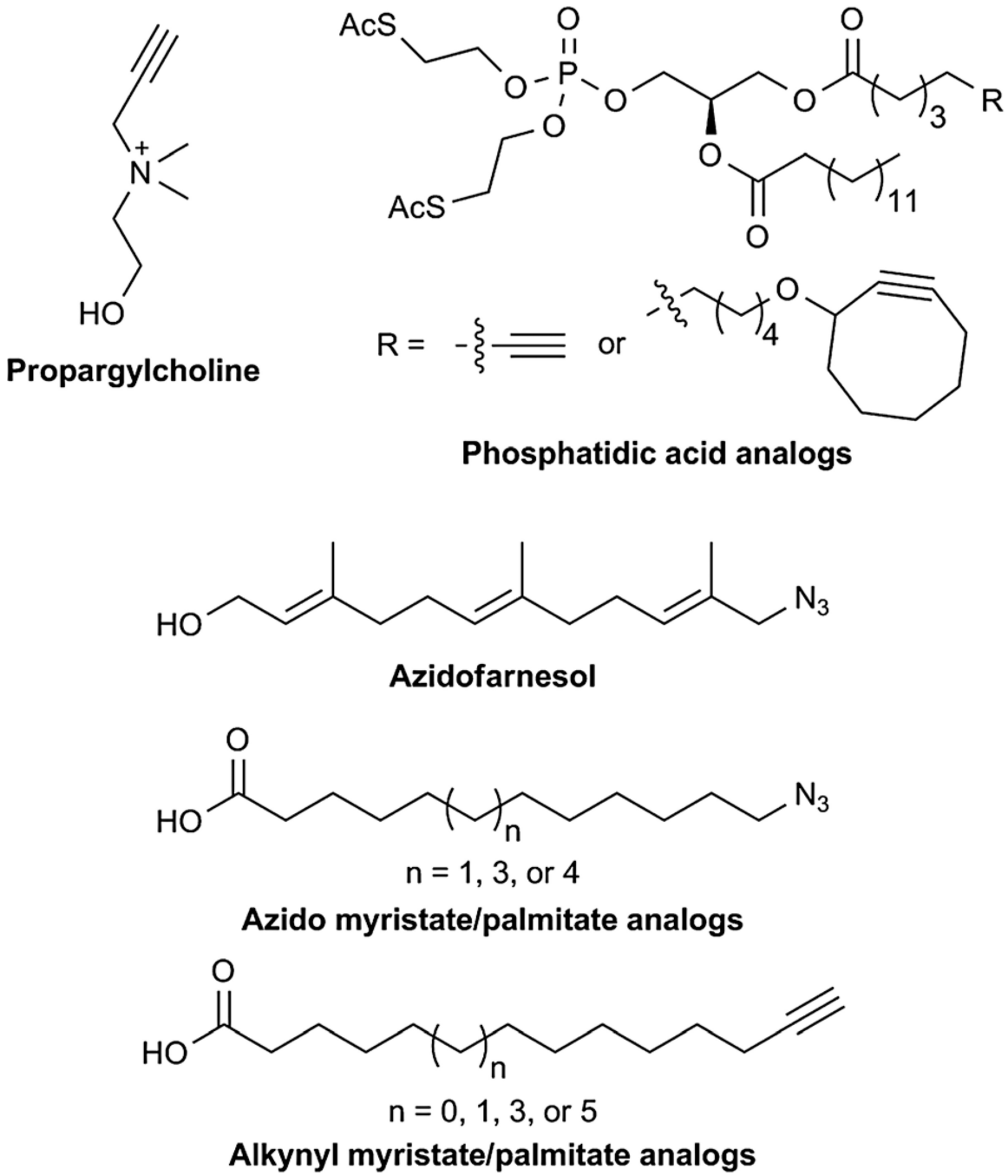
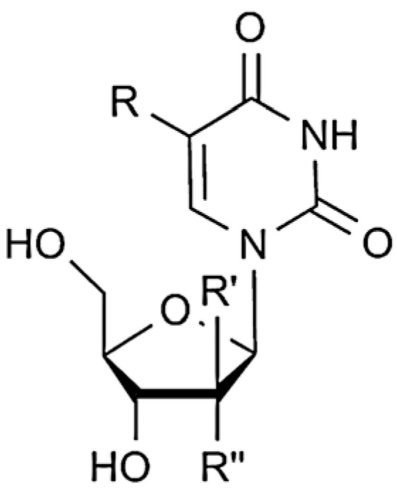


Fig. 9.
Chemical reporters for lipids.



	R	R'	R"
[^{3}H]T	Methyl-(^{3}H)	H	H
BrdU	Br	H	H
EdU	-≡-H	H	H
EU	-≡-H	H	OH
F-ara-EdU	-≡-H	F	H

Fig. 10.
Metabolic labels for nucleic acids.

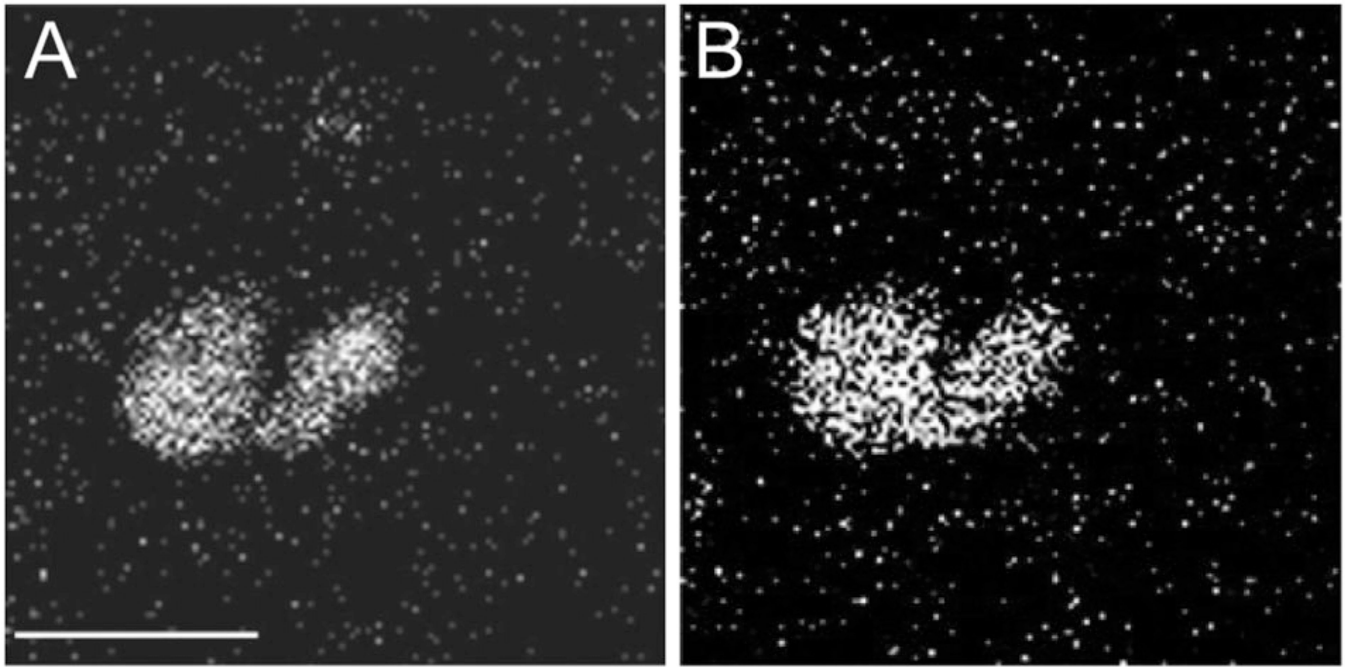


Fig. 11.

Tetrahymena thermophila mating results in lipid heterogeneity at membrane fusion sites. (A) SIMS image for *m*/*z* 184, representative of the phosphocholine headgroup, demonstrating the absence of phosphatidylcholine at the conjugation junction. (B) SIMS image for *m*/*z* 126, representative of the phosphoethanolamine headgroup, demonstrating the presence of phosphatidylethanolamine at the conjugation junction. SIMS images were acquired using a 200 nm beam spot size (B250 nm lateral resolution). Scale bar = 50 μm. From *Science* 2004, **305**, 71. Reprinted with permission from AAAS.

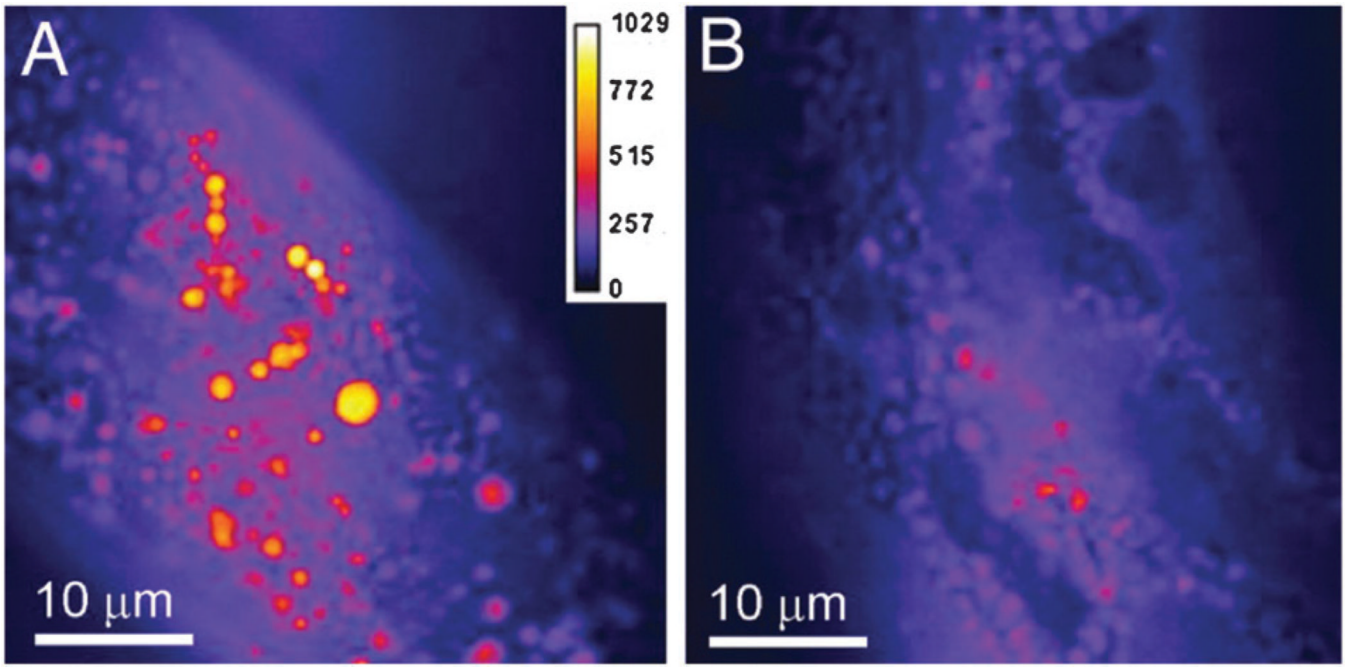


Fig. 12.

CARS imaging of lipid storage in *C. elegans*. CARS microscopy reveals a dramatic difference in lipid droplet densities from wild-type *C. elegans* (A) and a feeding-defective mutant, *pha-3* (B). From *Proc Natl Acad Sci* 2007, **104**, 14658. Reprinted with permission from National Academy of Sciences, U.S.A.