

Fluorogenic Reactions in Chemical Biology: Seeing Chemistry in Cells

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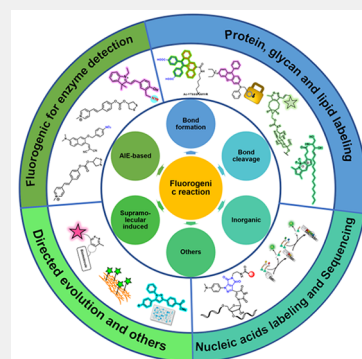
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ABSTRACT: Among the recent development of click chemistry and bioorthogonal chemistry, fluorogenic reactions occupy a unique place in that fluorescence is generated from nonfluorescent reactants, thereby rendering them highly useful and convenient in no-wash live-cell imaging. This topic was extensively reviewed in 2010 by Wang et al. (*Chem. Soc. Rev.* **2010**, 39, 1233–1239) and in 2014 by Lin et al. (*Curr. Opin. Chem. Biol.* **2014**, 21, 89–95). This review presents a comprehensive and up-to-date overview on the fluorogenic reactions in the past decade. The reactions are classified into four major categories on the basis of the mechanisms of fluorescence generation. Representative examples of each type are discussed briefly in terms of structure, mechanism, and advantages. We describe the latest applications of fluorogenic reactions in chemical biology. In the end, future opportunities and challenges in this field are tentatively proposed.

KEYWORDS: Bioorthogonal chemistry, Click chemistry, Fluorogenic reaction, Bond formation, Bond cleavage, Aggregation-induced emission, Nuclear acid-labeling, Protein-labeling



1. INTRODUCTION

In October 2022, Professors Carolyn R. Bertozzi, Morten Meldal, and K. Barry Sharpless were awarded the Nobel Prize for Chemistry in recognition of the creation of click chemistry and bioorthogonal chemistry. Click chemistry and bioorthogonal chemistry involve simple and fast chemical reactions that occur within living organisms without disrupting normal biological function, which enables selective reactions in the highly complex biological environments of living organisms.

Fluorogenic reactions occupy a unique place among rich click chemistry and bioorthogonal chemistry. Basically, non- or weakly fluorescent starting materials react to generate fluorescent molecules, thereby resulting in highly useful and convenient no-wash live-cell imaging. This topic was extensively reviewed in 2010 by Wang et al.¹ and 2014 Lin et al.²

Notably, bond cleavage-based fluorogenic reactions were comprehensively reviewed in two contributions from Chen et al. in 2016 and 2021.^{3,4} In 2019, Chang et al. extensively reviewed the activity-based sensing (ABS) methods using organometallic palladacycle fluorogenic probes.^{5–7} In 2021, Kim et al. reviewed tetrazine-based fluorogenic probes.⁸

As of this paper by the end of 2022, prominent research advances have occurred in the important area of fluorogenic reactions. Generally, these reactions are classified into two types.

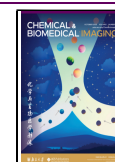
- (1) The first reaction occurs when one of the starting materials contains a dye that is quenched by a certain mechanism, and the formation of the products restores the fluorescence. For example, in 2004, Wang et al. and Fahrni et al.^{9,10} reported the first fluorogenic CuAAC reactions to produce fluorescent products on the basis of nonfluorescent coumarin derivatives. Naturally, one of the starting materials was limited by the selection of quenched dyes. In most cases, the fluorescence of the original dyes was restored by a bond cleavage reaction.
- (2) The second reaction creates a new structure that emits fluorescence. For example, in 2008, Lin et al.¹¹ reported the generation of strongly fluorescent pyrazoline cycloadducts from tetrazole–alkene cycloaddition reactions. This was the first report of tetrazole–alkene photoclick chemistry as a new bioorthogonal reaction for biological applications, which established the suitability of photoclick chemistry for *in vivo* protein labeling. In most cases, the fluorescence comes from a bond-forming reaction.

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Another example of this reaction is the aggregation-induced emission (AIE)-based fluorogenic and supramolecular interaction-induced fluorogenic reaction.

In this review, we grouped fluorogenic reactions into fluorogenic bond formation reactions, fluorogenic bond cleavage reactions, and several other types, including aggregation-induced fluorogenic reactions, as shown in Figure 1.

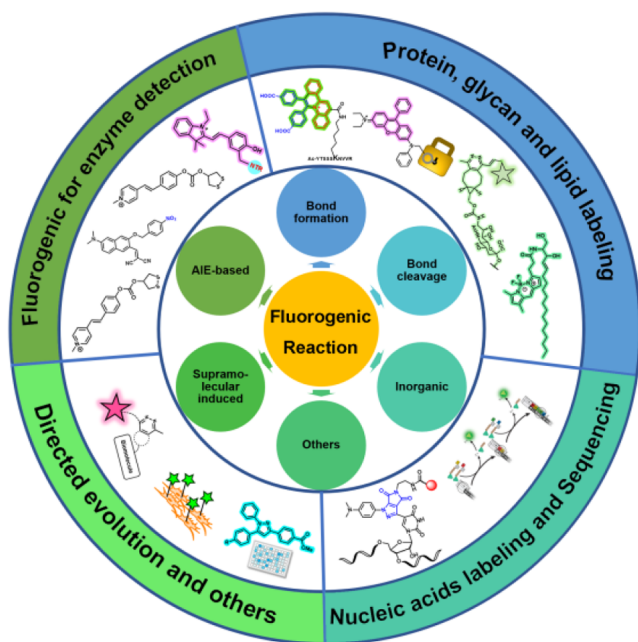


Figure 1. Fluorogenic reactions and their applications in chemical biology.

2. FLUOROGENIC BOND FORMATION

2.1. Copper-Catalyzed Click Reactions

Cu-catalyzed azide–alkyne cycloaddition (CuAAC) reactions have become an influential tool in the field of chemical biology. In recent years, these fluorogenic CuAAC reactions have greatly developed at numerous sites.

In 2012, Bertozzi et al.¹² proposed fluorogenic azido fluoresceins (**2-2**) for no-wash imaging of cells upon a copper-catalyzed click reaction. With the support of density functional theory calculations, they identified potential azide-functionalized fluorogenic probes (**2-2**) by predicting the fluorescence quantum yield. The strategy provided a good guideline for identifying other fluorogenic probes (Figure 2a).

In 2012, Ting et al.¹³ reported a quick and biocompatible Cu-catalyzed azide–alkyne cycloaddition (CuAAC) click reaction with copper-chelating azides (**2-4**), copper ligands, and lower copper concentrations. During the *in vitro* analysis phase, a fluorogenic reaction was used to measure CuAAC reaction time courses (Figure 2b).

In 2014, Wong et al.¹⁴ identified a novel fluorogenic probe, **2-6**, which had a higher signal-to-noise ratio than previously reported probes. The probe was utilized to selectively label alkyne-tagged proteins *in vitro* and was suitable for visualizing the localization of alkyne-functionalized glycoconjugates in cultured cells at extremely low concentrations. Moreover, the cells were lysed and analyzed by sodium dodecyl sulfate–

polyacrylamide gel electrophoresis (SDS–PAGE) utilizing **2-6** for direct in-gel detection of alkyne-labeled glycoproteins. The probe was conducive to promoting a better understanding of biological processes (Figure 2c).

In 2015, for the first time, Binder et al.¹⁵ designed a mechanochemically triggered click catalyst, which could activate a CuAAC reaction by ultrasound or force in solution and in the solid polymer matrix. A fluorogenic click reaction was utilized to quantify the activation of the catalyst (Figure 2d).

In 2018, Zimmerman et al.¹⁶ reported copper-containing single-chain nanoparticles that could dramatically accelerate fluorogenic CuAAC reactions with high selectivity. The increasing efficiency was due to an enzyme-like substrate binding process. The strategy offered a new method of catalyst design for chemical biology (Figure 3a).

In 2019, Gerwick et al.¹⁷ showed the utility of a fluorogenic probe combined with genome mining for the discovery and characterization of six new lipopeptides from the cyanobacterium *Moorea producens* ASI16Jul14-2 (Figure 3b).

In 2022, Chang et al.¹⁸ presented the first example of the identification of an Fe^{II}/αKG-dependent hydroxylase as a candidate for reaction pathway engineering and conversion into a halogenase. Active halogenases were discovered from a fluorogenic screening platform (Figure 3c).

In addition to usual fluorogenic CuAAC click reactions, in 2018, Taran et al.¹⁹ designed a fluorogenic Cu-catalyzed sydnone–alkyne cycloaddition (CuSAC) click reaction for biological imaging. A series of sydnone–coumarins (**2-21**) were combined with diverse alkynes (**2-22**) to identify potential clickable turn-on probes (Figure 3d).

2.2. Copper-Free Click Reactions

2.2.1. Inverse Electron-Demand Diels–Alder Reactions (iEDDA). The rapid advances in inverse electron-demand Diels–Alder (iEDDA) fluorogenic reactions have led to a series of new applications, such as labeling nucleic acids and proteins and live cell imaging.

In 2012, Lemke et al.²⁰ reported a series of unnatural amino acids for an iEDDA fluorogenic reaction with tetrazines (**2-25**) to label proteins. The reaction showed orthogonality to the strain-promoted azide–alkyne cycloaddition (SPAAC) reaction (Figure 4a).

In 2015, Kath-Schorr et al.²¹ described the development and application of a general strategy where an unnatural ribonucleoside triphosphate Nor-UB containing a norbornene component (**2-27**) reacts with tetrazine derivatives (**2-28**) to allow for posttranscriptional functionalization via a fluorogenic iEDDA reaction (Figure 4b).

In 2017, Vrabel et al.²² showed the development of a fluorogenic cycloaddition of **2-30** with **2-31**, which provides a tool for rapid cell labeling (Figure 4c).

In 2021, Jiang et al.²³ reported organelle-enriched probes that comprise a pair of tetrazine (**2-33**) and *trans*-cyclooctene (**2-34**) probes separately enriched in the plasma membrane or lysosomes and mitochondria designed to directly visualize autophagic and endocytic fluxes with high specificity and high contrast via a fluorogenic click reaction. The design provides insight into the roles of autophagic or endocytic flux in regulating the expression of immunotherapeutic targets (Figure 4d).

In 2022, Luedtke et al.²⁴ described the rational development of a probe for imaging nucleosidic alkene groups (PINK) (**2-**

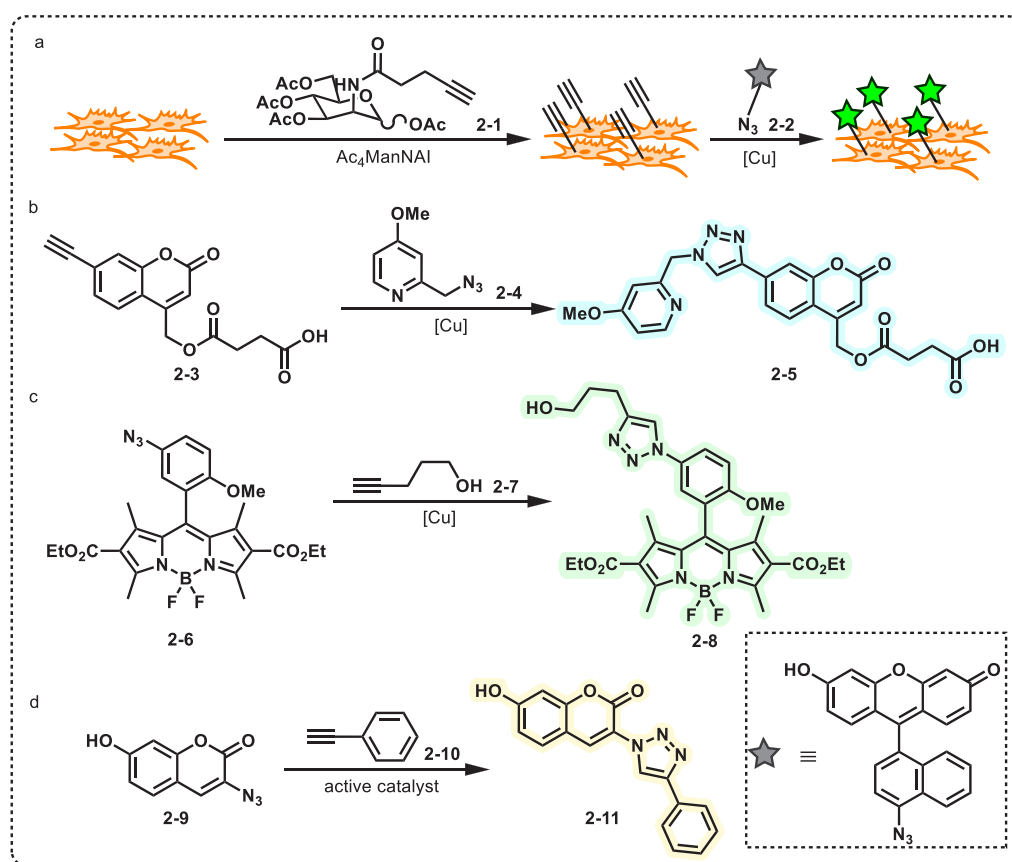


Figure 2. Copper-catalyzed fluorogenic click reactions (I): (a) No-wash labeling trial; adapted with permission from ref 12. Copyright 2012 American Chemical Society. (b) Use of 7-ethynylcoumarin (2-3) to detect the reaction process; adapted with permission from ref 13. Copyright 2014 Wiley. (c) Reaction of 4-pentyn-1-ol (2-7) and azido-BODIPY (2-6); adapted with permission from ref 14. Copyright 2014 American Chemical Society. (d) Reaction of phenylacetylene (2-10) with coumarin dye (2-9) triggered by mechanical force. Adapted with permission from ref 15. Copyright 2015 Wiley.

37) by taking advantage of a kinetic and fluorogenic enhancement strategy and its application to function as a fluorogenic intercalating agent for the first time undergoing iEDDA reactions with nucleic acids in living cells (Figure 4e).

2.2.2. Strain Promoted Azide–Alkyne Cycloaddition Reactions (SPAAC). Mushrooming growth of SPAAC fluorogenic reactions has been studied in recent years and has promoted the progress of biological imaging. In 2011, for the first time, Bertozzi et al.²⁵ reported a SPAAC fluorogenic click reaction. The design strategies embodied in coumarin-conjugated cyclooctyne (2-39) provided a platform for generating fluorogenic probes (Figure 5a).

In 2012, Boons et al.²⁶ showed that a novel SPAAC fluorogenic reaction was designed via cycloadditions of dibenzocyclooctyne (2-42) and azido-containing compounds (2-43). The cycloaddition products exhibited a large Stokes shift, over 1000-fold in brightness, and the 2-42 probe was successfully utilized in protein labeling (Figure 5b).

In 2015, Wnuk et al.²⁷ reported a fluorogenic click reaction of 5-azidouracil and 2- or 8-azidoadenine nucleosides, as well as 8-azido-ATP, with various types of cyclooctynes (2-46) and its application to direct imaging in living MCF-7 cancer cells (Figure 5c).

In 2016, Kele et al.²⁸ described an imaginative double-clicking bis-azide fluorogenic probe (2-49) that underwent double SPAAC reactions with bis-cyclooctynylated peptide

sequences (2-48). The new probe had the potential to be used for self-labeling peptide tags (Figure 5d).

2.2.3. Strain-Promoted Sydnone–Alkyne Cycloaddition Reactions (SPSAC). SPSAC fluorogenic reactions are rapidly increasing in utilization and have further provided chemical biology with an alternative approach for accelerating probe development.

In 2018, Friscourt et al.²⁹ reported a SPSAC fluorogenic click reaction that exhibited captivating photophysical properties and expanded the toolbox of chemical biology (Figure 6a).

In the same year, Friscourt et al.³⁰ developed sydnone-modified coumarins (2-54) as fluorogenic click reagents for cycloadditions with cyclooctynes (2-55). Fluorogenic reagents were utilized in the application of protein labeling in no-wash conditions (Figure 6b).

In 2019, Taran et al.³¹ reported sydnone-based turn-on fluorogenic probes (2-57) for cell imaging and wash-free protein labeling. The novel probes exhibited high reactivity toward cyclooctynes (2-58) with enhanced kinetic and fluorogenic properties (Figure 6c).

2.3. Photoclick Reactions

Photoclick chemistry has emerged as a powerful tool used in several research fields, from medicinal chemistry and biology to material sciences. Lin and Gaunt et al. reviewed the developments in the optimization of this chemistry and summarized the recent advances in the development of photoclick reactions and their applications in chemical biology

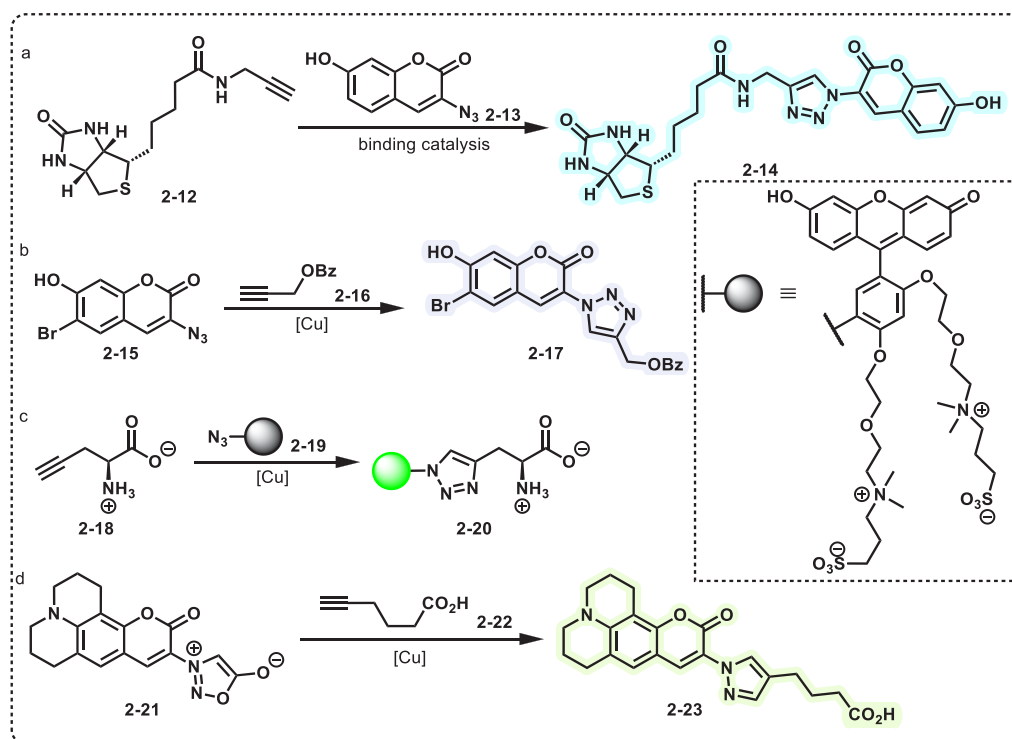


Figure 3. Copper-catalyzed fluorogenic click reactions (II): (a) Reaction catalyzed selectively via single-chain nanoparticles; adapted with permission from ref 16. Copyright 2018 American Chemical Society. (b) Testing of the bromoazidocoumarin (2-15) probe with terminal alkynes (2-16); adapted with permission from ref 17. Copyright 2019 Wiley. (c) Screening library variants via a fluorogenic probe; adapted with permission from ref 18. Copyright 2022 Nature. (d) Copper-catalyzed sydnone–alkyne cycloaddition reaction; adapted with permission from ref 19. Copyright 2018 Royal Society of Chemistry.

and materials science.^{2,32} Next, we will summarize the photoclick chemistry applied in inducing fluorogenic reactions in recent years.

In 2018, Zhang et al.³³ reported a visible-light-triggered bioorthogonal reaction, which was the photocycloaddition of 9,10-phenanthrenequinone with electron-rich alkenes to form fluorogenic [4 + 2] cycloadducts (Figure 7a). The bioorthogonal functionality in this fluorogenic reaction did not cross-react with alkynes and electron-deficient alkenes, such as monomethyl fumarate.

In 2020, Adronov et al.³⁴ reported a visible-light-functionalized conjugated (2-63), which contained a 9,10-phenanthrenequinone moiety in the backbone. This polymer backbone 2-63 could react with electron-rich vinyl ethers via visible-light-mediated photoclick chemistry. The emission properties of the polymer backbone produced a significant change after coupling to polyethylene glycol vinyl ether by photoclick reaction. (Figure 7b).

Another fluorophore-forming bioorthogonal reaction is the photoinducible 1,3-dipolar cycloaddition reaction between an alkene and a disubstituted tetrazole. This reaction was initially reported in 1967 using benzene as the solvent.³⁵ In an effort to improve the fluorogenic property of the alkene–tetrazole reaction, in 2017, Guo et al.³⁶ used an aromatic alkene (styrene) as the dipolarophile; after reacting with compound 2-66 (Figure 8a), an over 30-fold improvement in the quantum yield of the reaction product was achieved, which was significantly higher than that of reactions using aliphatic terminal alkenes as the dipolarophile. In 2019, Zhang et al.³⁷ designed and synthesized a series of coumarin-fused tetrazole

probes (2-68) and demonstrated its fluorogenic labeling ability of DNA under 302 nm UV light activation (Figure 8b).

In 2019, Barner-Kowollik et al.³⁸ prepared fluorescent microspheres (2-72) by using a precipitation polymerization technique (Figure 8c). Well-defined random polystyrene based on copolymers was synthesized and then functionalized with either a tetrazole or acrylate moiety. Upon exposure to 300 nm UV irradiation, the two precursor copolymers underwent a nitrile-imine-mediated tetrazole-ene cycloaddition cross-linking reaction, which yielded fluorescent microparticles.

In the same year, Shi et al.³⁹ demonstrated that small gold nanoparticles (AuNPs) (23 nm) containing both 2,5-diphenyltetrazole (2-73) and methacrylic acid (2-74) on their surfaces could form covalently cross-linked aggregates upon laser irradiation. *In vitro* studies indicated that the light-triggered assembly shifted the surface plasmon resonance of AuNPs significantly to the near-infrared (NIR) regions, which, as a consequence, effectively enhanced the efficacy of photothermal therapy for 4T1 breast cancer cells (Figure 8d). In addition to the tetrazole–alkene cycloaddition reactions using tailed alkenes as dipolarophiles, other similar reactions were developed; these included the intramolecular tetrazole–alkene cycloaddition reaction and the reaction between maleimide-functionalized compounds and tetrazole. These reactions also exhibited good application prospects in chemical biology.

In 2018, Lin et al.⁴⁰ synthesized a panel of sterically shielded tetrazoles with different *N*-aryl groups in the photoinduced tetrazole–alkene cycloaddition reaction (Figure 9a). It was found that an increase in the HOMO energy of the corresponding nitrileimines led to a faster cycloaddition

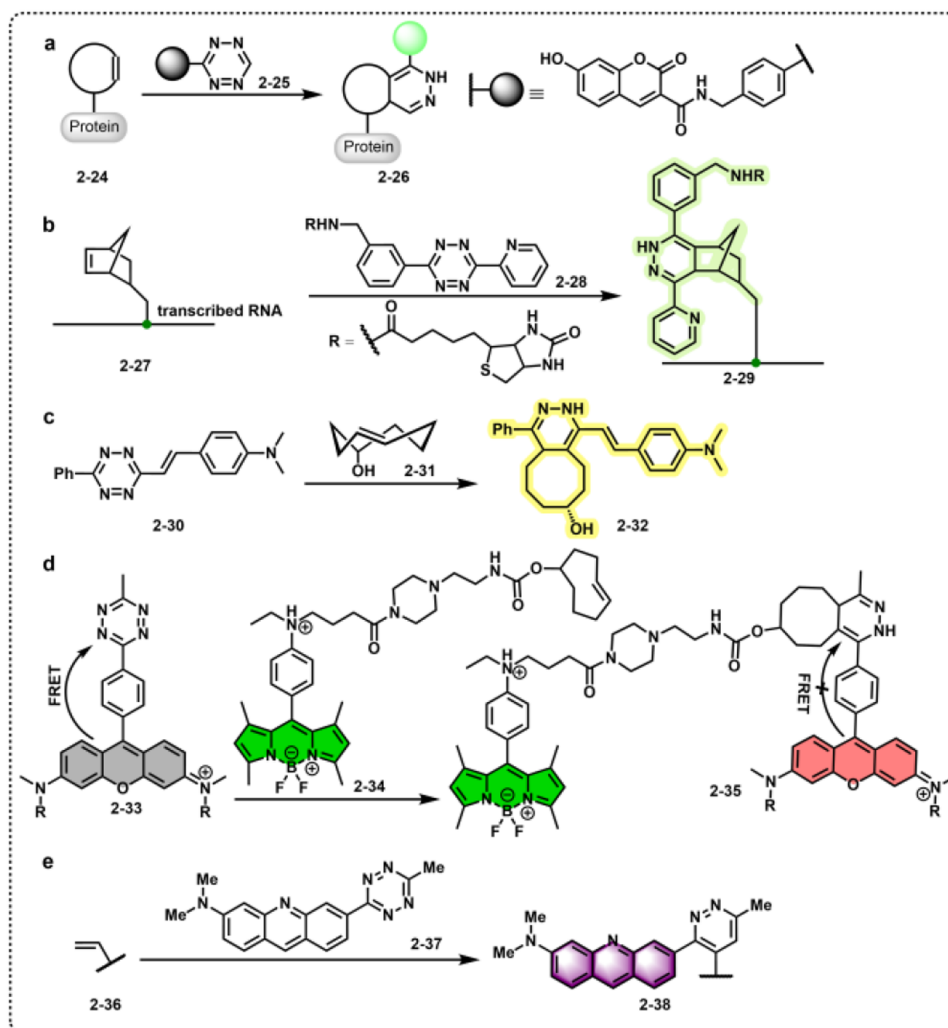


Figure 4. Inverse electron-demand Diels–Alder fluorogenic reactions: (a) *E. coli* cultures reacting with coumarin tetrazine (2-26); adapted with permission from ref 20. Copyright 2012 Wiley. (b) Reaction of *meta*-substituted tetrazine (2-28) with the unnatural nucleobase Nor-UB (2-27); adapted with permission from ref 21. Copyright 2015 Royal Society of Chemistry. (c) Reaction of the axial TCO isomer (2-31) and dipyrityltetrazine (2-30); adapted with permission from ref 22. Copyright 2017 Wiley. (d) Clicking of organelle-enriched probes strategy involves designing a pair of TCO (2-34) and tetrazine (2-33) probes; adapted with permission from ref 23. Copyright 2021 Royal Society of Chemistry. (e) Reaction between 2-37 and a vinyl-modified nucleotide (2-36); adapted with permission from ref 24. Copyright 2022 Wiley.

reaction, along with a red shift in the fluorescence emission of the pyrazoline cycloadduct.

In 2020, Wagenknecht et al.⁴¹ developed a bromoaryl-tetrazole-modified uridine (2-79), which could act as RNA building block for bioorthogonal, light-activated, and post-synthetic modification with commercially available fluorescent dyes, including sulfo-Cy3 (Cy3), AlexaFluor555 (AF555) and AlexaFluor647 (AF647). By irradiation with 300 nm LED at internal and terminal positions of presynthesized RNA with maleimide-conjugated fluorophores (2-80), the fluorescent emission increased, which was attributed to the formation of a fluorescent pyrazoline moiety (Figure 9b). The fluorescence of the photoclick reaction was significantly enhanced by energy transfer between the pyrazoline as the reaction products and the photoclicked dyes as the strong emitter, which are important for chemical biology and fluorescent imaging of RNA in cells.

In 2020, Zhang et al.⁴² developed a strategy to spatiotemporally label sialic acids with a near-infrared light-activated upconverting nanoprobe 2-83. With this photo-

clickable nanoprobe and a stable *N*-alkene-D-mannosamine (2-82), metabolically synthesized alkene sialic acids on the cell surface were labeled and imaged in real time through fluorogenic cycloaddition (Figure 9c).

In 2021, Huang et al.⁴³ designed an *in situ*, real-time fluorescence imaging system that targeted mitochondria and lysosomes in a spatiotemporally controllable manner. Upon irradiation, the pyrazoline fluorophore was generated *in situ* by the intramolecular tetrazole–alkene cycloaddition reaction (Figure 9d). This strategy exhibited features such as fast response, high efficiency, strong fluorescence intensity without background, and superior stability.

In 2022, An et al.⁴⁴ developed aryl azide-tetrazole probes for the photoactivatable fluorogenic azide–alkyne click (PFAAC) reaction in which the aryl azide–tetrazole probes were not photoactivatable fluorogenic themselves, but after the click reaction, the triazole products were prefluorophores that could be activated by light (Figure 9e). Therefore, in PFAAC strategy, fluorogenic probes could be activated by two orthogonal events: the azide–alkyne click reaction and light,

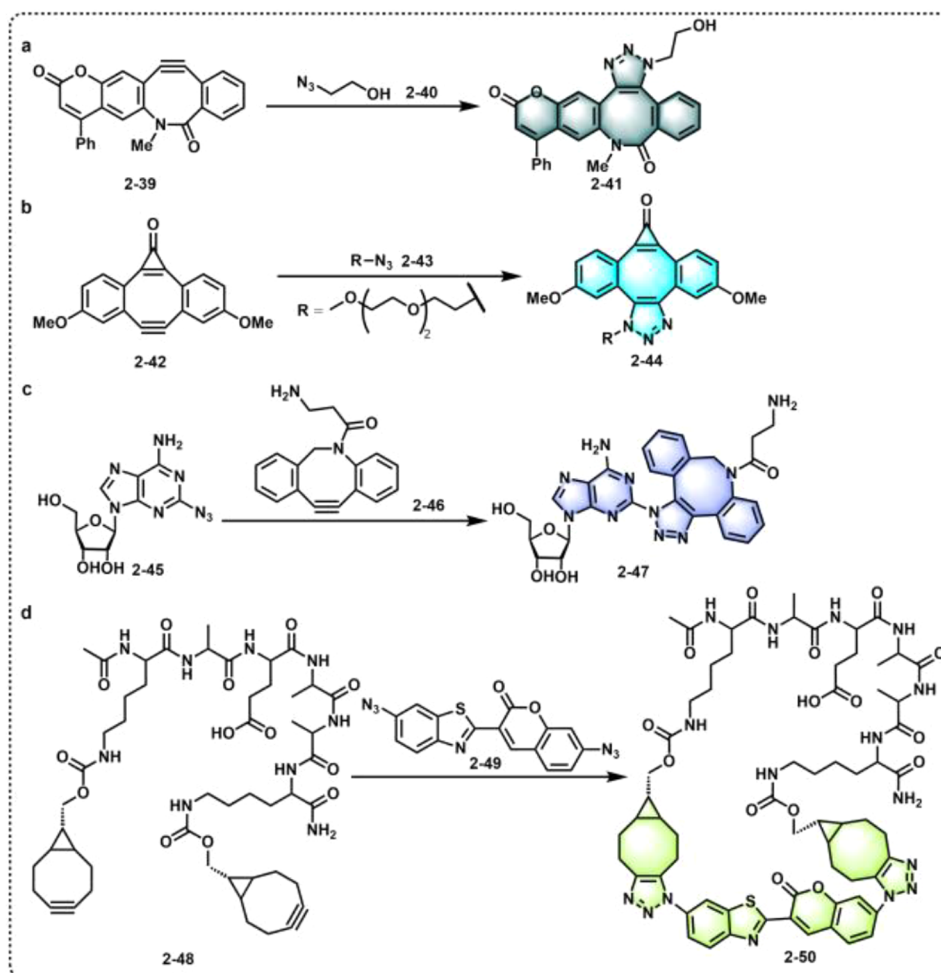


Figure 5. Strain-promoted azide–alkyne cycloaddition fluorogenic reactions: (a) Coumarin-conjugated cyclooctyne (2-39) reacting with 2-azidoethanol (2-40); adapted with permission from ref 25. Copyright 2011 American Chemical Society. (b) The reaction between azide (2-43) and the 2-42 probe; adapted with permission from ref 26. Copyright 2012 American Chemical Society. (c) Click reaction of dibenzyl cyclooctyne (2-46) with 2-azidoadenosine (2-45); adapted with permission from ref 27. Copyright 2015 American Chemical Society. (d) Reaction of the target peptide (2-48) and bis-azide (2-49) probe; adapted with permission from ref 28. Copyright 2016 Wiley.

which leads to spatiotemporal resolution and a high signal-to-noise ratio.

2.4. Other Bioorthogonal Fluorogenic Reactions

Except for the fluorogenic reactions mentioned above, there are many other types of bioorthogonal reactions, such as strain-promoted alkyne-diazo cycloaddition (SPADC), strain-promoted iminosydnone–cycloalkyne cycloaddition (SPICC), and *o*-dione and furan-2(3*H*)-one cycloaddition (DFC). These reactions have made great contributions to the field of bioimaging.

In 2015, Boons et al.⁴⁵ investigated a fluorogenic SPADC reaction that exhibited a greater than 10 000-fold enhancement in brightness and an approximately 160-fold increase in fluorescence quantum yield. The result that diazo-tagged proteins were successfully labeled with probes 2-52 provided biomolecule labeling possibilities (Figure 10a).

In 2016, Meier et al.⁴⁶ studied a bioorthogonal fluorogenic reaction for oncometabolite detection via a 1,3-dipolar cycloaddition of nitrileimines (2-93) and fumarate (2-94). The integration of metabolite reactivity with bioorthogonal chemistry provided a fresh concept for applications in areas such as biological diagnosis and imaging (Figure 10b).

In 2019, Tian et al.⁴⁷ designed fluorogenic cycloaddition reactions between nitrile oxide and alkene (2-97). Additionally, 4-oxime-1,8-naphthalimide (2-96) was developed as a bioorthogonal turn-on probe showing a 5- to 13-fold enhancement of fluorescence (Figure 10c).

In 2020, Taran et al.⁴⁸ reported a fluorogenic cleavable linker—iminosydnone (2-99)—that enabled the double turn-on click and release SPICC click reactions for biological imaging. The new-type probe had latent applications in the drug delivery field (Figure 10d).

In 2022, Zhang et al.⁴⁹ developed an innovative *o*-dione and furan-2(3*H*)-one cycloaddition (DFC) bioorthogonal reaction, which could be combined with iEDDA and SPAAC reactions for mutually orthogonal labeling of different proteins or live cells without cross-talk. In the study of fluorescence labeling of live cells, *o*-dione-TAMRA (2-103) could quickly label cells with furan-2(3*H*)-one (2-102) attached in a fluorogenic way (Figure 10e).

2.5. Other Bond Formation Fluorogenic Reactions

In addition to the click-based fluorogenic reactions mentioned above, some other bond formation reactions could also induce fluorogenic effects, such as the Michael addition reaction of thiolate with acrylate, bond formations mediated by a heavy-

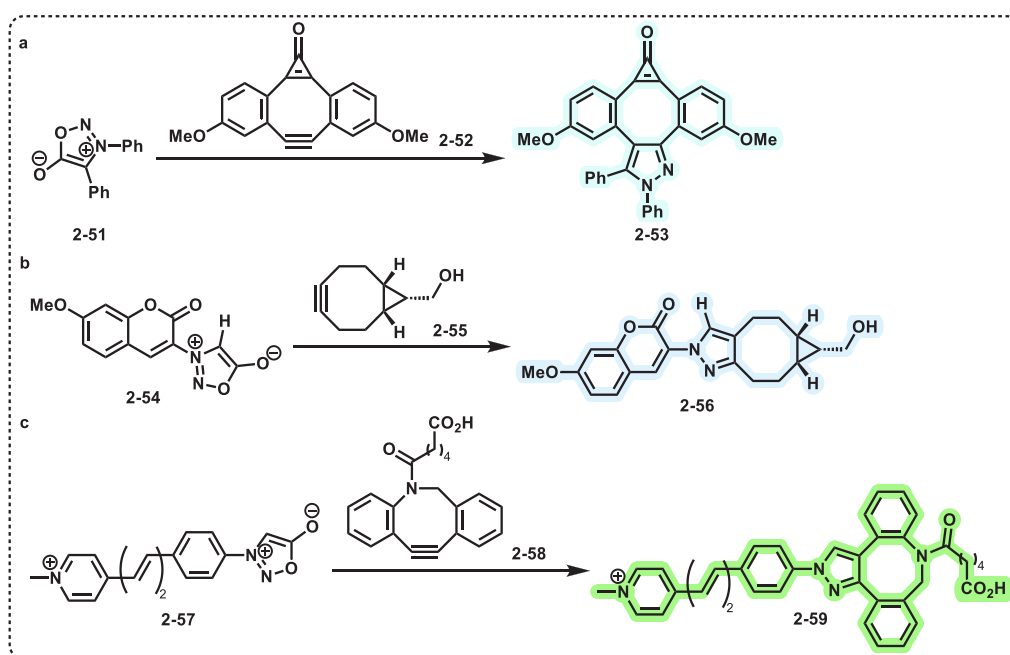


Figure 6. Strain-promoted sydnone–alkyne cycloaddition fluorogenic reactions: (a) Reacting sydnone (2-51) with fluorogenic dibenzocyclooctyne (2-52); adapted with permission from ref 29. Copyright 2018 American Chemical Society. (b) Reaction between sydnone-modified coumarin (2-54) and 2-55; adapted with permission from ref 30. Copyright 2018 American Chemical Society. (c) Reaction of sydnone (2-57) with 2-58; adapted with permission from ref 31. Copyright 2019 Royal Society of Chemistry.

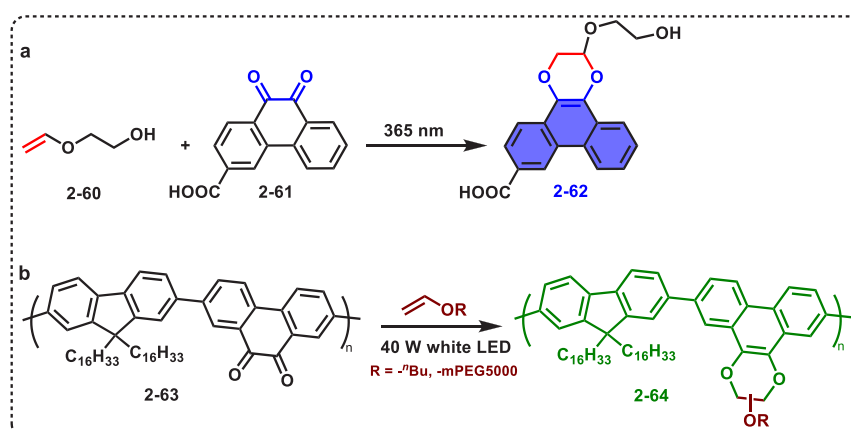


Figure 7. (a) Visible-light-triggered bioorthogonal photoclick cycloaddition of 2-60 and 2-61. Adapted with permission from ref 33. Copyright 2018 American Chemical Society. (b) Visible-light-mediated photoclick coupling between 2-63 and vinyl ether derivatives. Adapted with permission from ref 34. Copyright 2020 American Chemical Society.

atom removal process, and regioselective activation and cyclization of cyclopropanones.

In 2010, Herrmann et al.⁵⁰ demonstrated a conceptual approach for generating a strong fluorescence signal through chemical bond formation mediated by a heavy-atom removal process (Figure 11a). Since the internal heavy-atom effect is suppressed in the iodine removal process, and the probability of the S1 → T1 intersystem crossing (ISC) is reduced, a strong fluorescence signal is generated. Given this, this method enables the fluorogenic detection of DNA and shows exceptional quantum yield and low limits.

In 2022, Cosa et al.⁵¹ reported a chemically tuned fluorogenic electrophile 2-107. Consisting of a lipophilic BODIPY fluorophore tethered to an electrophilic cyanoacrylate warhead, probe 2-107 remained nonemissive because of internal conversion along the cyanoacrylate moiety. Inter-

mittent fluorescence occurred following thiolate Michael addition to the probe, which was then followed by a retro-Michael reaction (Figure 11b). This design enabled long-term super-resolved imaging of live cells by preventing fluorescent product accumulation and background increase while preserving the pool of the probe.

In 2022, Prescher et al.⁵² developed a fluorogenic reaction featuring cyclopropanone reporters and phosphines. As shown in Figure 11c, the transformation involved regioselective activation and cyclization of cyclopropanones to form coumarin products (2-110). With optimal probes, the reaction provided a significant signal turn-on.

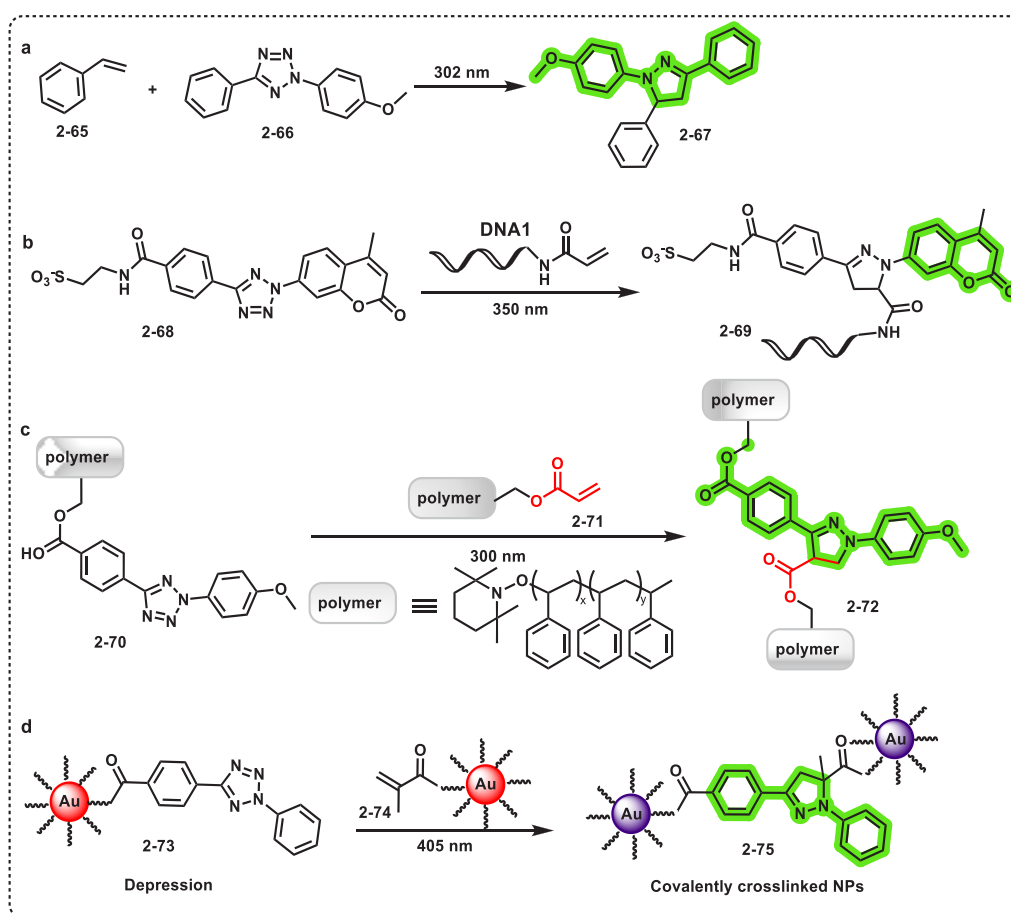


Figure 8. Fluorogenic reaction between tetrazole and vinyl derivatives: (a) With styrene and tetrazole; adapted with permission from ref 36. Copyright 2017 American Chemical Society. (b) Structures of photoclickable tetrazoles and pyrazoline cycloadduct with DNA1; adapted with permission from ref 37. Copyright 2019 American Chemical Society. (c) Synthesis of fluorescent microspheres from functionalized cross-linkable polymer precursors; adapted with permission from ref 38. Copyright 2019 Royal Society of Chemistry. (d) Fabrication and light-triggered cross-coupling of tm-AuNPs; adapted with permission from ref 39. Copyright 2019 Wiley.

3. FLUOROGENIC BOND CLEAVAGE REACTIONS

3.1. Photoinduced Bond Cleavage Reactions

In parallel with the development of ligation reactions, a series of biocompatible reduction, deprotection, or decaging reactions have emerged in recent years; these reactions share a common feature where the functional groups on reactants undergo bond cleavage to generate the final products. Light-induced fluorescence enhancement after an uncaging process is usually achieved by modifying a chemical function directly responsible for the fluorescent signal; these include the aromatic hydroxyl or amino groups of the usual fluorophores, such as fluorescein, rhodamine, Q-rhodamine, resorufin, and hydroxycoumarin derivatives. Most of these fluorescent derivatives use *o*-nitrobenzyl (*o*-NB) derivatives as photo-removable protecting groups.⁵³ *o*-NB caging groups have also been used on TokyoGreen derivatives, which are fluorescein compounds with improved fluorescent properties.

In 2010, Schultz et al.⁵⁴ developed a photoactivatable green fluorescent protein (GFP) appropriate for use as a molecular marker or in super-resolution imaging using nonnatural amino acid mutagenesis with *o*-NB tyrosine, whose fluorescence was activated on the same time scale as proteins previously used for these applications. The *o*-NB group likely deactivated the excited fluorophore through photon-induced electron transfer. Irradiation at 365 nm was sufficient to remove the *o*-NB group

and restore fluorescence to (3-3) (Figure 12a). The mechanism of photoactivation did not require the conservation of any other residues; thus, it should be useful for producing a variety of fluorescent proteins ranging from GFP to red fluorescent protein (RFP). Finally, unlike previous photo-activatable proteins, the dark form of cGFP is practically nonfluorescent at any wavelength, which frees up the rest of the visible spectrum for other uses.

In 2015, Raymo et al.⁵⁵ designed molecular guests with photoactivatable fluorescence for self-assembling amphiphilic polymer supramolecular host nanoparticles and demonstrated that the activation of the fluorescent cargo under optical control permitted the tracking of nanocarrier translocation across hydrogel matrices with the sequential acquisition of fluorescence images. These photoresponsive compounds combined a boron dipyrromethene (BODIPY) chromophore and a photocleavable oxazine within their covalent skeleton. Under illumination at an appropriate activation wavelength, the oxazine ring irreversibly cleaved to cause the adjacent BODIPY to fragment in conjugation with an indole heterocycle (Figure 12b). This structural transformation bathochromically shifted the BODIPY absorption and permitted the selective excitation of the photochemical product with concomitant fluorescence. In fact, these operating principles enabled the photoactivation of BODIPY fluorescence with large brightness and infinite contrast.

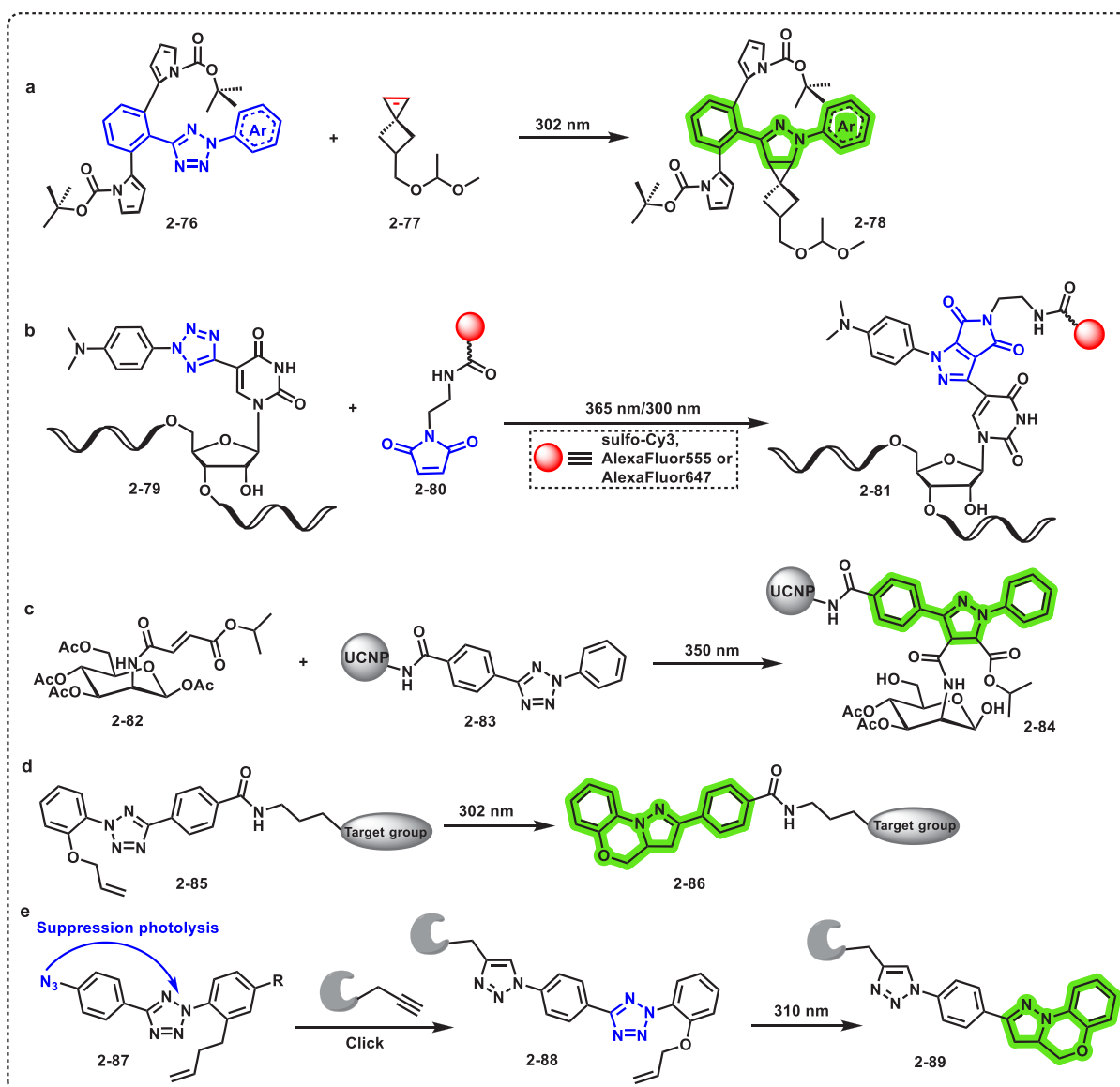


Figure 9. (a) Chemical structures of the photoinduced cycloaddition of tetrazoles **2-76** with spiro[2.3]hex-1-ene **2-77**; adapted with permission from ref 40. Copyright 2018 Royal Society of Chemistry. (b) Photoclick reaction between tetrazoles-modified single-stranded RNA uridine **2-79** and maleimide-conjugated dye **2-80**; adapted with permission from ref 41. Copyright 2020 Multidisciplinary Digital Publishing Institute (MDPI). (c) Fluorogenic reaction scheme of Ac4ManNIPFA **2-82** with Tz-UCNP **2-83**; adapted with permission from ref 42. Copyright 2020 Royal Society of Chemistry. (d) Schematic diagram of photoactivatable fluorescent probes **2-85** for super-resolution imaging of the targeting organelle; adapted with permission from ref 43. Copyright 2021 Royal Society of Chemistry. (e) Chemical structures and transformation of photoactivatable fluorogenic azide–alkyne click reaction; adapted with permission from ref 44. Copyright 2022 Wiley.

In 2020, Chan et al.⁵⁶ developed the first photoactivatable donor for formaldehyde (FA). Alkylated silicon-based fluorophores are nonfluorescent because of the perturbed electronic push–pull system, as well as donor-photoinduced electron transfer (dPeT) quenching from the proximal nitro group. Irradiation with light-induced cleavage of the photocage produced an unstable hemiacetal intermediate that spontaneously collapsed to generate FA and the corresponding dye (Figure 12c). Their optimized photoactivatable donor (**3-6**) was equipped with a fluorescence readout that enabled monitoring of FA release with a concomitant 139-fold fluorescence enhancement. Application of (**3-6**) revealed the concentration range necessary for arresting wound healing in live cells. This was the first report where a photoactivatable donor for any analyte was used to quantify intracellular release.

3.2. Metal-Induced Bond Cleavage Reactions

Transition-metal-induced bioorthogonal cleavage reactions are highly efficient and widely studied reactions.

In 2010, Shin et al.⁵⁷ developed a reaction-based turn-on fluorescent probe for palladium species. An *O*-propargylated fluorescein sensed several palladium species in the oxidation states of 0, +2, and +4 with no additional reagents through a depropargylation reaction, which provided a turn-on-type fluorescence change (Figure 13a). This probe was used for the fluorescent imaging of palladium chloride in a living species.

In 2014, inspired by the palladium-mediated chemical decaging strategy, Chen et al. pioneered a universal protein activation technology *in vitro* and *in vivo*.⁵⁸ As shown in Figure 14a, this was achieved by rational design and catalyst screening

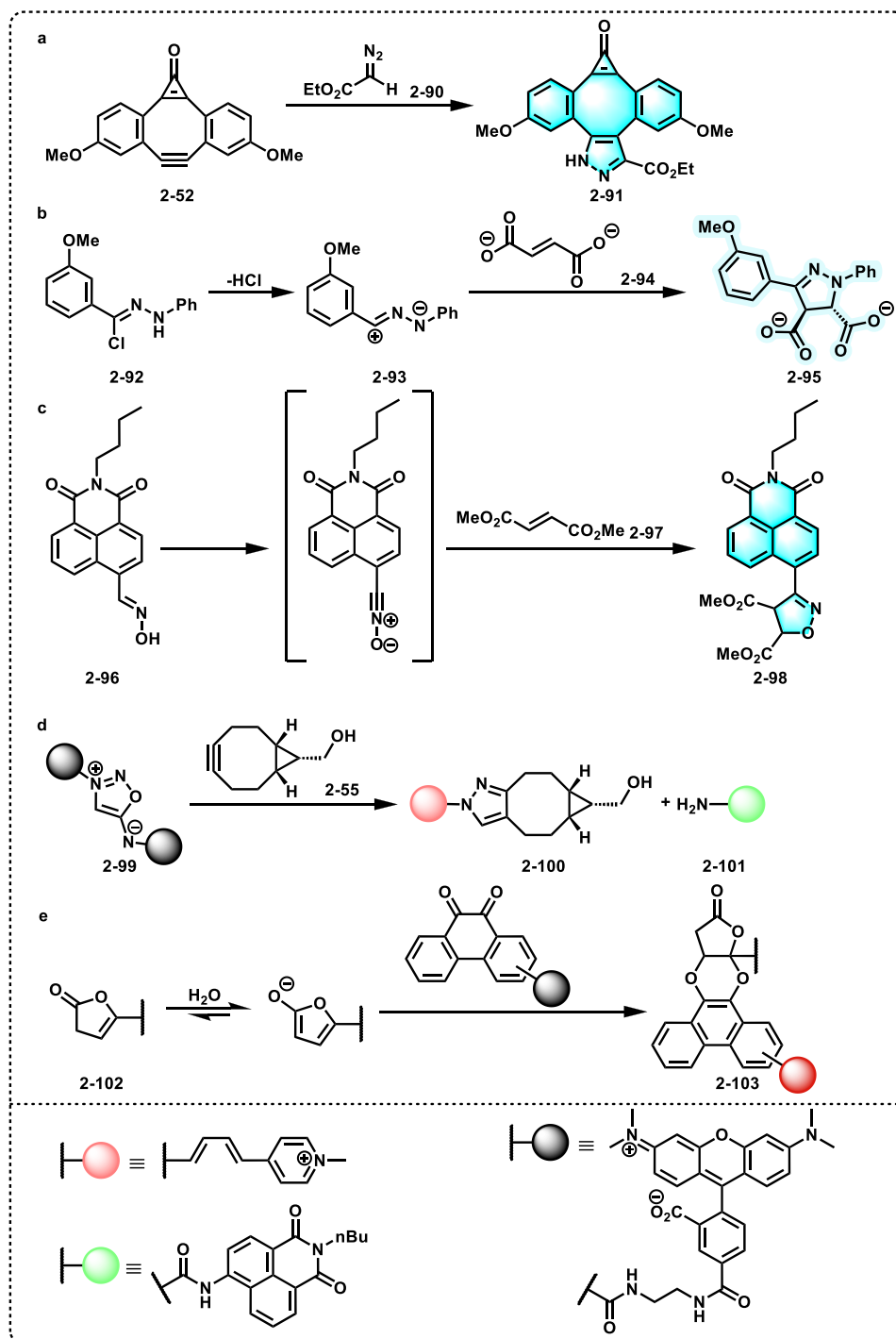


Figure 10. Other fluorogenic click reactions: (a) Reaction of diazoacetate (2-90) with 2-52; adapted with permission from ref 45. Copyright 2015 Wiley. (b) Bioorthogonal [3 + 2] cycloaddition for fumarate (2-94) detection; adapted with permission from ref 46. Copyright 2016 American Chemical Society. (c) Design of a fluorogenic probe with 4-oxime-1,8-naphthalimide (2-96); adapted with permission from ref 47. Copyright 2019 Royal Society of Chemistry. (d) Click and release reaction entailing cyclooctyne (2-55) and iminosydnone (2-99); adapted with permission from ref 48. Copyright 2020 Royal Society of Chemistry. (e) Fluorescent mark through the DFC reaction; adapted with permission from ref 49. Copyright 2022 Wiley.

for a biocompatible protection group/catalyst pair on fluorogenic small molecule reporters (from 3-15-1 and 3-15-2 to 3-16), as well as on intact fluorescent proteins.

In 2016, Chen et al.⁵⁹ further evaluated the reactivity of different alkenes to cage phenol groups using fluorescent coumarin analogues as reporter groups. By extending the Pd-triggered decaging reaction to uncage the phenol group on

Tyr, they could control the activity of Tyr-dependent proteins (Figure 13b).

In 2006, Meggers et al.⁶⁰ reported the first Ru-mediated deallyloxycarbonyl (allyl carbamate) reaction in live cells for the activation of a fluorescent dye. They synthesized this cage fluorophore protected by a diallyloxycarbonyl group (3-15-1), which is almost nonfluorescent and stable in the presence of E.

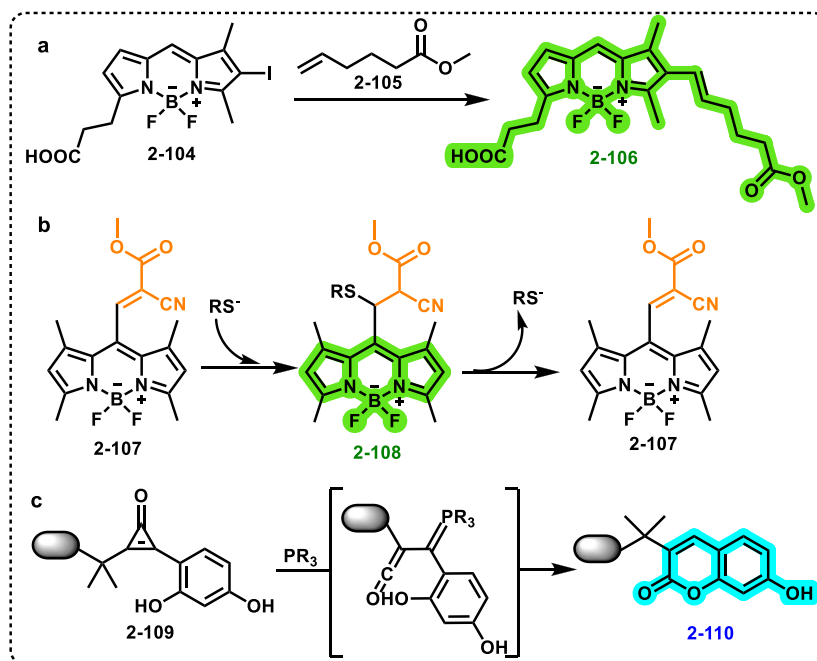


Figure 11. (a) Structures of iodinated BODIPY probe 2-104 and fluorescent Heck-coupling product 2-106; adapted with permission from ref 50. Copyright 2010 American Chemical Society. (b) Reaction scheme for the reversible fluorogenic electrophile 2-107; adapted with permission from ref 51. Copyright 2022 American Chemical Society. (c) Selective reaction of fluorogenic cyclopropanones with phosphine nucleophiles to form fluorescent hydroxycoumarins; adapted with permission from ref 52. Copyright 2022 American Chemical Society.

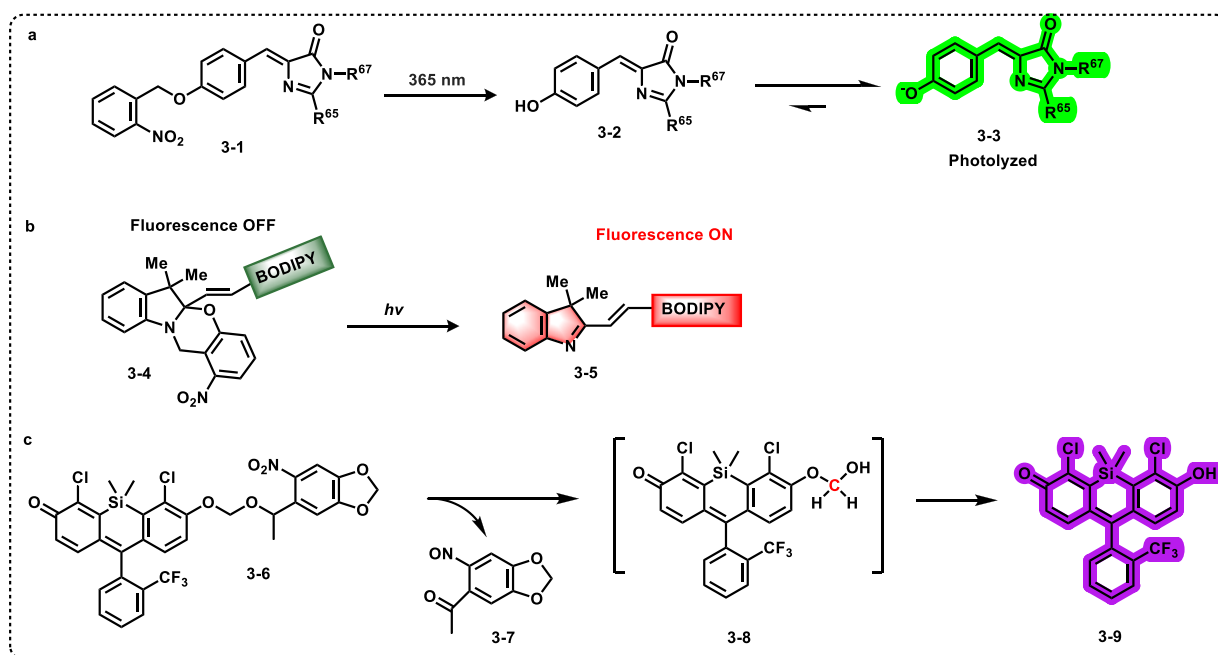


Figure 12. (a) Uncaging of the nonfluorescent (3-1) via UV photolysis to generate a fluorescent protein; adapted with permission from ref 54. Copyright 2010 Wiley. (b) Under illumination at an appropriate activation wavelength, cleavage of the oxazine ring causes the adjacent BODIPY to fragment in conjugation with an indole heterocycle; adapted with permission from ref 55. Copyright 2015 American Chemical Society. (c) Mechanism of light-mediated FA release; adapted with permission from ref 56. Copyright 2020 American Chemical Society.

coli extracts and, upon deprotection of the allyl carbamate, releases an intense green fluorescence (Figure 14a) that can be used as a tool for studying ruthenium-induced cleavage of allyl carbamates in living mammalian cells. In 2013, using the same fluorogen, Finn et al.⁶¹ developed a copper-sensitive fluorescent probe (3-15-2) that under typical aqueous CuAAC conditions cleaves both alkynyl carbamate groups

(Figure 14a), thereby causing an 1800-fold increase in fluorescence.

In 2017, Santamaría et al.⁶² proposed a bioorthogonal uncaging strategy triggered by heterogeneous gold catalysis to incubate [Au]-resins with the nonfluorescent compound (3-15-3), which released intense fluorescence upon O-depropargylation (Figure 14a), and this solid-supported catalytic

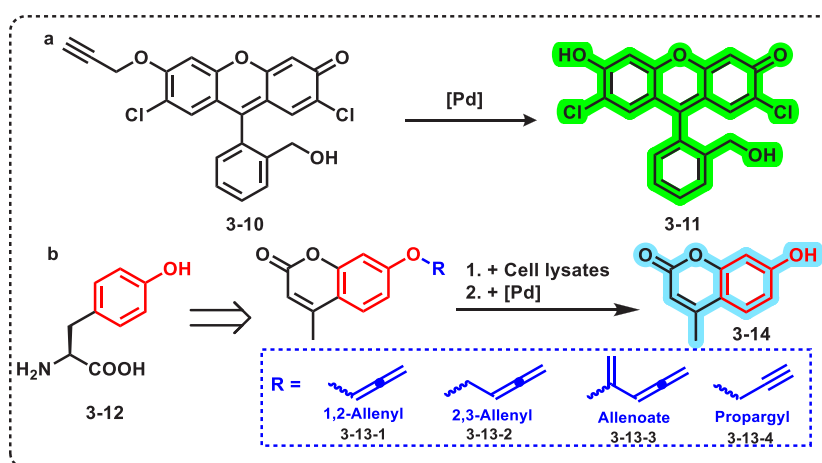


Figure 13. (a) Cleavage of *N*-Poc-protected fluorophore (3-10) in the presence of palladium chloride; adapted with permission from ref 57. Copyright 2010 Royal Society of Chemistry. (b) Coumarin-based assay for examining the bioorthogonality and cleavage efficiency of different allene-based caging moieties for chemical rescue of Tyr; adapted with permission from ref 59. Copyright 2016 American Chemical Society.

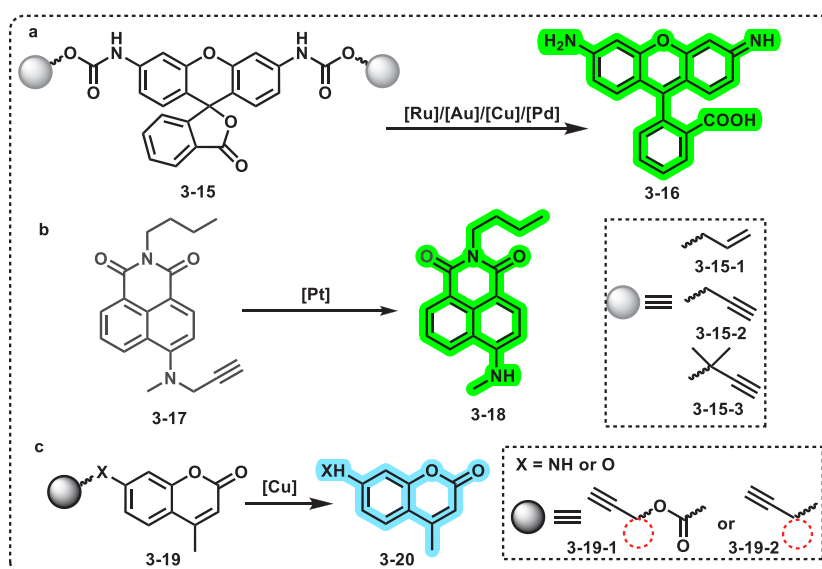


Figure 14. (a) Cleavage of *N*-Poc-protected fluorophore (3-15) in the presence of various metal catalysts; adapted with permission from ref 58. Copyright 2014 Nature. Adapted with permission from ref 60. Copyright 2006 Wiley. Adapted with permission from ref 61. Copyright 2013 American Chemical Society. Adapted with permission from ref 62. Copyright 2017 Wiley. (b) Use of the naphthalimide-based fluorogenic probes to study the cleavage efficiency of the platinum reaction for decaging alkyne-containing molecules; adapted with permission from ref 64. Copyright 2020 American Chemical Society. (c) Coumarin-based fluorogenic assay for examining the bioorthogonality and cleavage efficiency of different dsProc and metal catalyst combinations; adapted with permission from ref 63. Copyright 2019 American Chemical Society.

system achieved the first locally controlled release of a fluorescent dye in the zebrafish brain.

By further systematic survey of transition metals in catalyzing the bioorthogonal cleavage reactions, Chen et al.⁶³ employed Cu(I)-BTAA/dsProc and Cu(I)-BTAA/dsPra pairs as a “traceless linker” strategy to construct cleavable antibody–drug conjugates in 2019. As shown in Figure 14c, fluorogenic coumarins were employed as a high-throughput screening platform to systematically survey the transition-metal-catalyzed bioorthogonal depropargylation reaction. This strategy could temporally and reversibly control protein and cell conjugations and hold the traceless release of ADCs on tumor sites, as well as alter or remove targeting elements on the cell surface on demand.

In 2020, Bernardes et al.⁶⁴ presented a decomposition reaction of alkynes with platinum complexes, such as K_2PtCl_4

and $Pt(NH_3)_2Cl_2$ (CisPt) that could release secondary amines from other stable tertiary amines, which was carried out via a platinum-mediated intramolecular cyclization mechanism. This reaction was later extended and applied to the *N*-propynyl group (Figure 14b) with comparable efficiency to the palladium-mediated deprotonation reaction.

3.3. Enzyme and Organic Small-Molecule-Induced Bond Cleavage Reactions

Organic small-molecule-triggered bioorthogonal cleavage reactions have attracted much attention because of their high biocompatibility, fast reaction rate, and robustness.

Cathepsin B (CTB) is a lysosomal protease that has been recognized as a promising biomarker for many malignant tumors, and accurate detection of its activity is important in the early diagnosis of cancers and predicting metastasis. In 2016, Zhang et al.⁶⁵ reported a lysosome-targeting fluorogenic

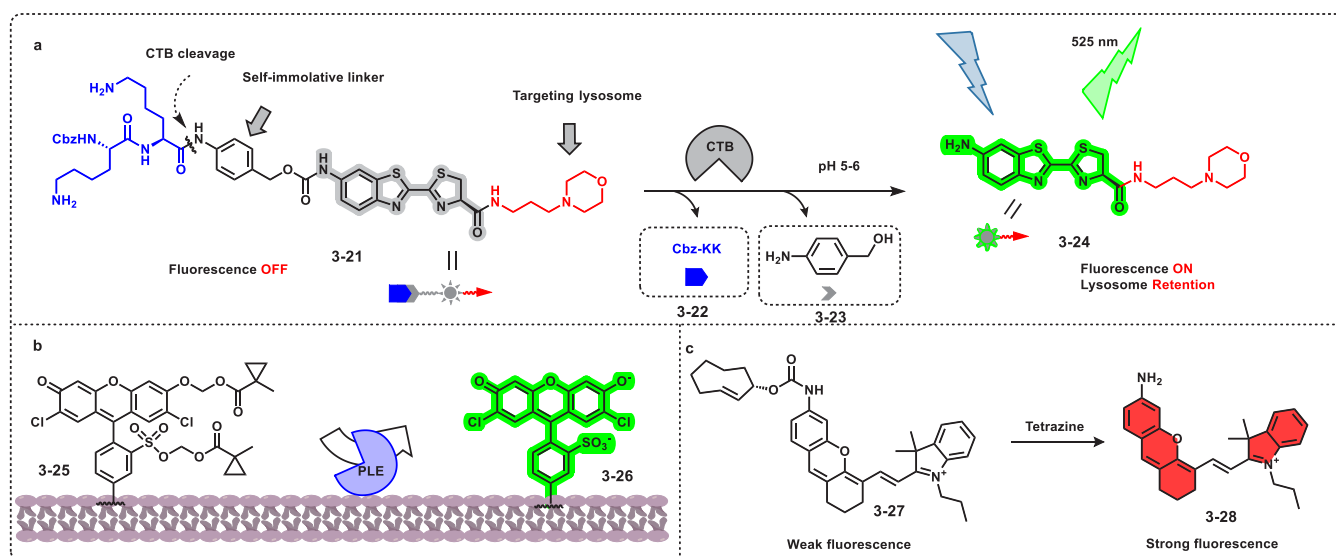


Figure 15. (a) Chemical structure of (3-21) and the proposed chemical conversion in response to CTB. Adapted with permission from ref 65. Copyright 2016 American Chemical Society. (b) Scheme of neuron-specific targeting with ester/esterase pairs. Adapted with permission from ref 66. Copyright 2017 American Chemical Society. (c) The structure of (3-28) and its activation via a tetrazine-mediated cleavage reaction. Adapted with permission from ref 67. Copyright 2020 Wiley.

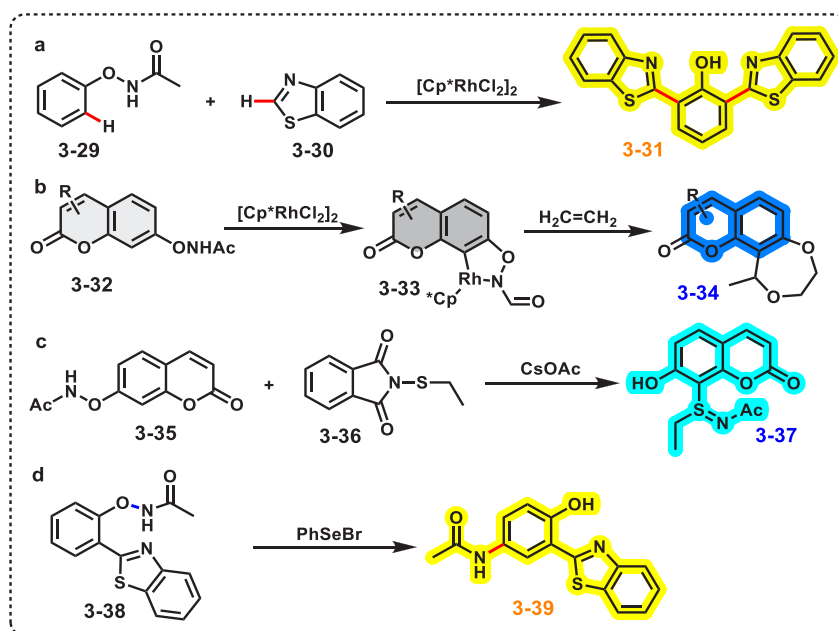


Figure 16. (a) Route to fluorescent bis-heteroarylated hybrid product 3-31. Adapted with permission from ref 68. Copyright 2017 Royal Society of Chemistry. (b) Fluorogenic detection of ethylene by a Rh^{III} -coumarin complex (3-33). Adapted with permission from ref 69. Copyright 2021 Wiley. (c) Reaction of 3-35 with 3-36 in aqueous conditions. Adapted with permission from ref 70. Copyright 2017 Nature. (d) Se-catalyzed para-amination of the phenol derivative 3-39. Adapted with permission from ref 71. Copyright 2018 Nature.

small molecule probe for the fluorescence imaging of lysosomal CTB in living cancer cells by incorporating a CTB-recognitive peptide substrate Cbz-Lys-Lys-*p*-aminobenzyl alcohol and a lysosome-locating group morpholine (Figure 15a). They demonstrated that the probe could be efficiently activated by CTB to generate an ~ 73 -fold enhancement in fluorescence in an acidic lysosomal environment (pH 4.5–6.0), thereby providing for high sensitivity and specificity to detect CTB. This study highlighted the potential of using a lysosome-targeting group to design a sensitive and specific fluorogenic probe for fluorescence imaging of lysosomal CTB in living cells.

In 2017, Miller et al.⁶⁶ presented a method to target voltage-sensitive fluorescent dyes to specified cells using an enzyme-catalyzed fluorogenic reaction on cell surfaces. The dye/enzyme hybrids were composed of a photoinduced electron transfer (PeT)-based fluorescent voltage indicator and a complementary enzyme expressed on the cell surface. The hydrolytically stable ether could be removed by the action of porcine liver esterase (PLE) to reveal the bright unmodified VoltageFluor (Figure 15b). The action of the exogenous enzyme on the dye resulted in fluorogenic activation of the dye, which enabled fast voltage imaging in defined neurons

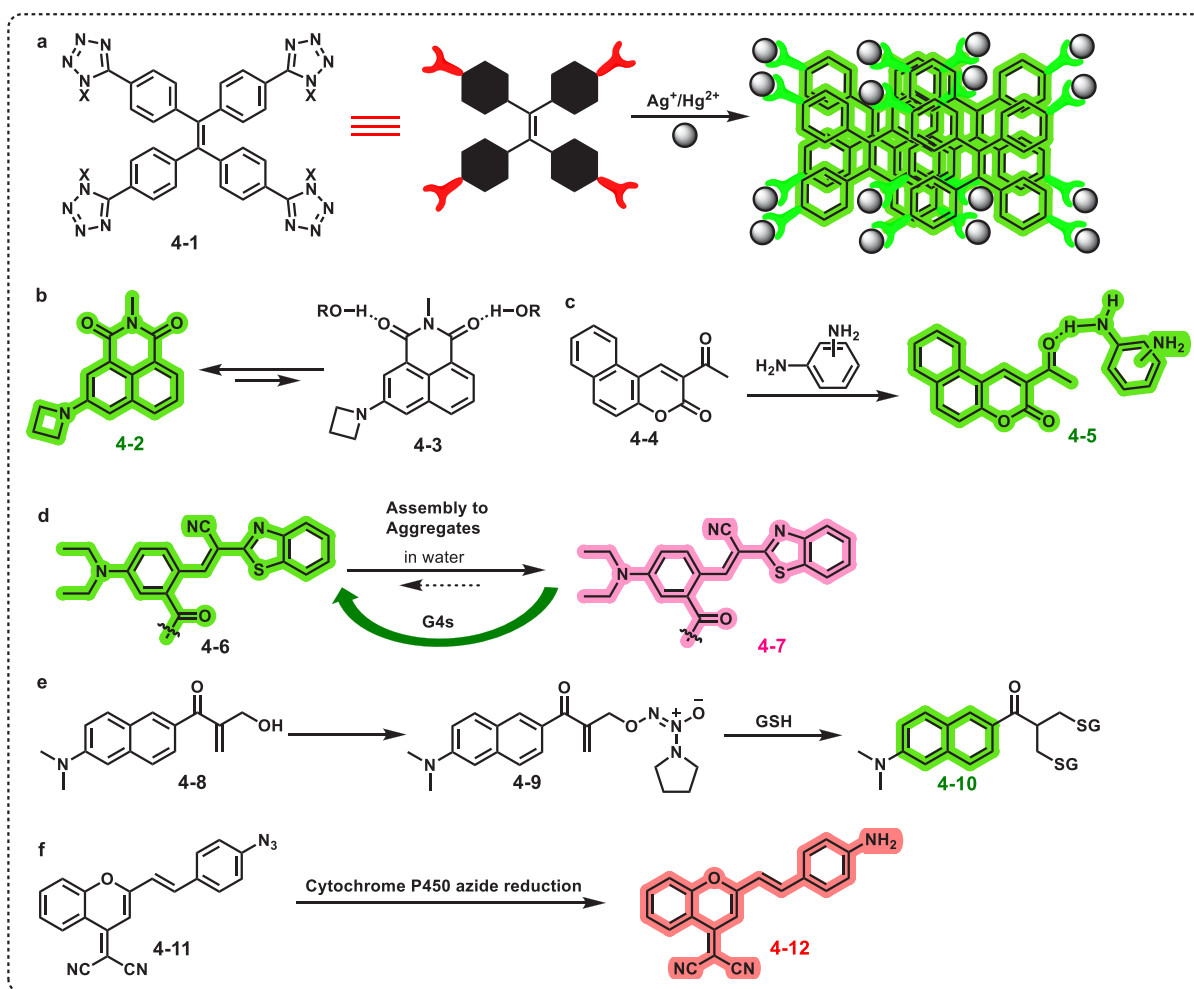


Figure 17. (a) Probe 4-1 and the proposed fluorogenic $\text{Hg}^{2+}/\text{Ag}^{+}$ detection via a tetrazolate– $\text{Hg}^{2+}/\text{Ag}^{+}$ complexation-triggered AIE process; adapted with permission from ref 72. Copyright 2018 Wiley. Adapted with permission from ref 73. Copyright 2022 Wiley. (b) Mechanism of 4-3 for LD fluorogenic and dynamic imaging; adapted with permission from ref 76. Copyright 2021 Wiley. (c) Interaction between the probe (4-4) and phenylenediamine isomers. Adapted with permission from ref 77. Copyright 2022 Iranian Chemical Society. (d) 4-6 self-assembles in water to emit red emission and 4-7 interacts with G4 DNAs to trigger the disassembly of aggregates into green-emissive monomeric dyes 4-6. Adapted with permission from ref 78. Copyright 2023 Wiley. (e) Response of 4-8 to GSH. Adapted with permission from ref 81. Copyright 2022 American Chemical Society. (f) Reduction of the nonfluorescent azide 4-11 to produce the corresponding fluorescent amine 4-12. Adapted with permission from ref 80. Copyright 2017 American Chemical Society.

with sensitivity surpassing that of genetically encoded approaches.

The bioorthogonal inverse electron-demand Diels–Alder (iEDDA) cleavage reaction between tetrazine and *trans*-cyclooctene (TCO) is a powerful way to control the release of bioactive agents and imaging probes. In 2020, Bernardes et al.⁶⁷ developed a fluorogenic NIR probe with emission at 720 nm by protecting the amine group on (3-28) with TCO (Figure 15c). This NIR fluorescence imaging technique was used in living cells with an instantaneous turn-on signal in mitochondria with no need for washing. With sequential systemic administration of TZ@SWCNTs and tHCA in living mice with xenograft tumors, tetrazine-triggered fluorogenic imaging resulted in higher tumor selectivity over liver and kidneys compared with direct fluorophore delivery.

3.4. PhO-N Bond Cleavage Reactions

In 2017, Zhao et al.⁶⁸ developed a unified strategy for cross-dehydrogenative coupling reactions between arenes and heteroarenes (Figure 16a). Internal and external oxidation could be controlled using N–O bond cleavage or a silver

oxidant. Mono- and rarely reported bis-arylated phenol derivatives of different oxidation states were prepared in one step. Application research showed that the products exhibited excellent fluorogenic properties.

In 2021, by using a PhONHAc-derived organometallic intermediate, Ye et al. reported a Rh^{III} -based, activity-based sensing, fluorogenic probe 3-33 for monitoring ethylene, as shown in Figure 16b. Probe 3-33 could act as a real-time fluorescent sensor to detect ethylene concentration in CHO-K1 cells, tobacco, and *Arabidopsis* leaves.⁶⁹

In 2017, inspired by the ergothioneine biosynthesis catalyzed by EgtB, Zhao's group developed a bioinspired strategy for the synthesis of *ortho*-sulfiliminyl phenols by using an internally oxidizing O–N bond as a directing group under mild conditions.⁷⁰ This efficient method enabled the simultaneous construction of C–S and S=N bonds and applied to the *in situ* formation of fluorogenic phospholipid membranes (Figure 16c).

In 2018, they reported a Se-catalyzed *para*-amination of phenols.⁷¹ A catalytic amount of phenylselenenyl bromide

smoothly converted *N*-aryloxyacetamides to *N*-acetyl-*p*-aminophenols. However, when the para position was substituted, dearomatized 4,4-disubstituted cyclodienone products were obtained (Figure 16d). Further property research showed that the obtained products 3-39 exhibited significant AIE behavior, and this reaction also caused fluorescence conversion from “turn-off” to “turn-on.”

4. OTHER FLUOROGENIC REACTIONS

4.1. AIE-Based Fluorogenic Reactions

In 2018, Tang et al.⁷² reported a novel water-soluble fluorogenic Ag⁺ probe (4-1); the sensing mechanism was based on an aggregation-induced emission (AIE) process driven by tetrazole Ag⁺ interactions (Figure 17a). Fluorogenic sensing could substitute for chromogenic reactions, thereby leading to a new fluorescence silver staining method. This staining method provided sensitive detection of total proteins in polyacrylamide gels with a broad linear dynamic range.

In addition, in 2022, Tang et al.⁷³ further reported that the AIE-active probe (4-1) could also ultrasensitively detect heavy metal mercury ions (Hg²⁺) directly in water and showed great selectivity for competitive cations (Figure 17a). Moreover, by embedding 4-1 in a hydrophilic poly(vinyl alcohol) substrate, the resulting hydrogel film enabled *in situ* detection of Hg²⁺ with a laser-induced fluorescence analysis setup in an aqueous environment.

In 2022, Tian et al.⁷⁴ reported a series of tetrazine-modified tetraphenylenes (TPEs) as bioorthogonally activated AIE fluorogenic probes. The tetrazine group could quench the fluorescence and AIE features of TPEs through the through-bond energy transfer (TBET) mechanism. The fluorescence and AIE features could be activated by converting tetrazine to pyridazine via the inverse electron-demand Diels–Alder (iEDDA) reaction. In addition, these AIE fluorogenic probes were successfully applied to mitochondria-specific bioorthogonal imaging in live cells under no-wash conditions.

In 2022, Kim et al.⁷⁵ synthesized an AIE-type fluorescence probe for endoplasmic reticulum (ER) detection in the cell by the click reaction between azide-modified glibenclamide and an alkyne-containing indolizine core skeleton. Aru68 accumulated inside the ER and induced the AIE process through both intramolecular charge transfer (ICT) and restriction of molecular motion (RIM) mechanisms.

4.2. Supramolecular-Interaction-Induced Fluorogenic Reactions and Others

In 2021, Xu et al.⁷⁶ reported a fluorescence probe (4-3) for imaging lipid droplet dynamics using structured illumination microscopy (SIM). Probe 4-3 enabled wash-free imaging of lipid droplet (LDs) because of a hydrogen-bond sensitive fluorogenic response, as shown in Figure 17b. The replacement of photobleached 4-3 by intact probe molecules outside the LDs ensured the long-term stability of the fluorescence imaging. In addition, 4-3 was successfully applied for tracing various types of LD dynamics by super-resolution microscopy, including growth and shrinkage, heterogeneity and composition variations, coalescence, and organelle interactions.

In 2022, Padmini et al.⁷⁷ synthesized a chromogenic- and fluorogenic-active coumarin-based chemodosimeter (4-4). Probe 4-4 selectively detected phenylenediamine isomers in the presence of competitive species and showed excellent fluorogenic emission for *ortho*-phenylenediamine (565 nm) and *para*-phenylenediamine (462 nm) after the interaction

with phenylenediamine isomers through hydrogen bond interactions (Figure 17c).

In 2023, Kim et al.⁷⁸ constructed fluorogenic probe 4-7 (as shown in Figure 17d) by conjugating the excimer-forming dye with a peptide sequence (L-Arg-L-Gly-glutaric acid), which could selectively light up parallel G4 DNA over antiparallel topologies. The probe 4-7 showed red excimer-emitting nanoaggregates in aqueous media; its specific binding to G4s triggered its disassembly into rigidified monomeric dyes, which led to a dramatic fluorescence enhancement.

In 2022, Gao et al.⁷⁹ constructed a self-reporting bioorthogonal prodrug activation system using fluorescence emission to interpret prodrug activation events. In designed reactive oxygen species (ROS)-instructed supramolecular assemblies, the bioorthogonal reaction handle of tetrazine had a dual role as a fluorescence quencher and prodrug activator. In addition, the inverse electron-demand Diels–Alder (iEDDA) reaction between TCO and tetrazine (Tz) enabled the liberation of the active drug and fluorescence simultaneously.

In 2017, Hammond et al.⁸⁰ reported a fluorogenic probe 4-11 for the detection of depleted oxygen levels (hypoxia). Azide-based 4-11 was able to image hypoxia in a range of human cancer cell lines. In addition, the experimental results indicated that the azide group was a new bioreductive functionality that could be used in prodrugs and dyes, as shown in Figure 17f.

In 2022, Fang et al.⁸¹ reported a versatile thiol-triggered fluorogenic release system using Baylis–Hillman (BH) adducts as a general template (Figure 17e). Common functional groups in a molecule of interest (MOI) were readily integrated into the template to generate target compounds, which were activated by thiols with nearly quantitative release efficiency and a high amplitude increase in the fluorescence signal.

5. INORGANIC-CLUSTER-BASED FLUOROGENIC REACTIONS

5.1. Noble Metal (Au and Ag) Nanoclusters

Metal nanoclusters (NCs) occur between atoms and small nanoparticles (less than 2 nm) and usually only comprise several to dozens of atoms; however, they can sometimes contain hundreds of metal atoms.⁸² The diameter of metal NCs is comparable with the Fermi wavelength of the electrons in the metal, which results in the splitting of successive energy bands into discrete energy levels and, thus, renders metal NCs' attractive characteristics, including unique optical, electrical, electrochemical, magnetic, and chemical properties.⁸³

With the development of preparation methods, metal nanoparticles have been applied to fluorescence detection and biomedicine and have become popular in the fields of chemiluminescence (CL) and electrochemiluminescence (ECL).

5.1.1. Luminescence Mechanism of Au NCs and Au NC Composites. AuNCs are considered as a promising ECL material because of their low toxicity, high stability, good water solubility, easy preparation, and biocompatibility. In 2011, Chen et al.⁸⁴ illustrated the ECL emission of bovine serum albumin protected by triethylamine as a coreactant and its use in Pb²⁺ detection potential applications. A strong ECL signal was observed on the Pt electrode.

Shortly thereafter, the ECL behavior of the cathodic coreactants on the indium tin oxide (ITO) electrode was

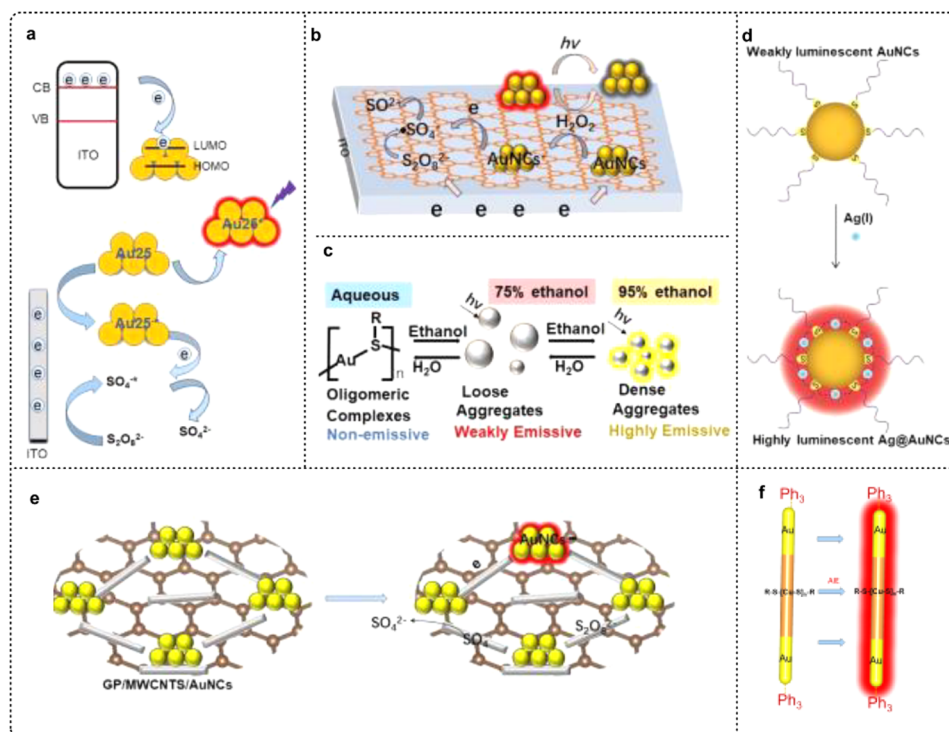


Figure 18. (a) Luminous mechanism of BSA@Au₂₅ NCs. Adapted with permission from ref 85. Copyright 2011 American Chemical Society. (b) Luminescence of graphene/AuNCs. Adapted with permission from ref 86. Copyright 2011 Royal Society of Chemistry. (c) Strong emission of Au(I)-thiol complexes. Adapted with permission from ref 87. Copyright 2012 American Chemical Society. (d) Highly luminescent Ag@AuNCs. Adapted with permission from ref 89. Copyright 2013 Royal Society of Chemistry. (e) Strong emission of Au(I)-thiol complexes. Adapted with permission from ref 88. Copyright 2014 Elsevier. (f) Luminescence of the Au-Cu nanocluster. The yellow patterns in all the figures are gold atoms. Adapted with permission from ref 90. Copyright 2016 Wiley.

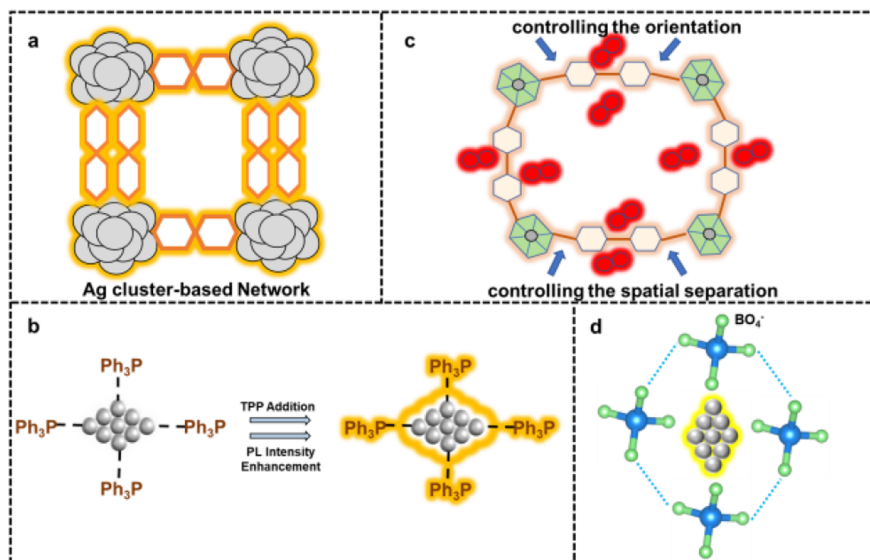


Figure 19. (a) Rigid metal-organic backbone with fluorescent switch. Adapted with permission from ref 92. Copyright 2017 Nature. (b) Luminescence of the Ag₂₉ nanocluster. Adapted with permission from ref 93. Copyright 2018 Royal Society of Chemistry. (c) Luminescence of silver thionanoclusters. Adapted with permission from ref 94. Copyright 2019 American Chemical Society. (d) Ag quantum cluster luminescence. The gray patterns in all the figures are silver atoms. Adapted with permission from ref 95. Copyright 2022 Wiley.

observed using K₂S₂O₈ as a coreactant.⁸⁵ The possible ECL mechanism was attributed to the effective electron transfer from the conduction band of the excited ITO to the Au₂₅NCs, as shown in Figure 18a. In addition, this ECL system has a good selectivity for the detection of dopamine. The ECL

increased linearly with increasing dopamine concentration in the range of 2.5 to 47.5 μM.

In the same year, Zhu's group⁸⁶ illustrated the *in situ* preparation of dispersible graphene/AuNCs on the basis of bilayer graphene as a template. The ECL strength of graphene/Au nanocomposites was greater than that of BSA@Au

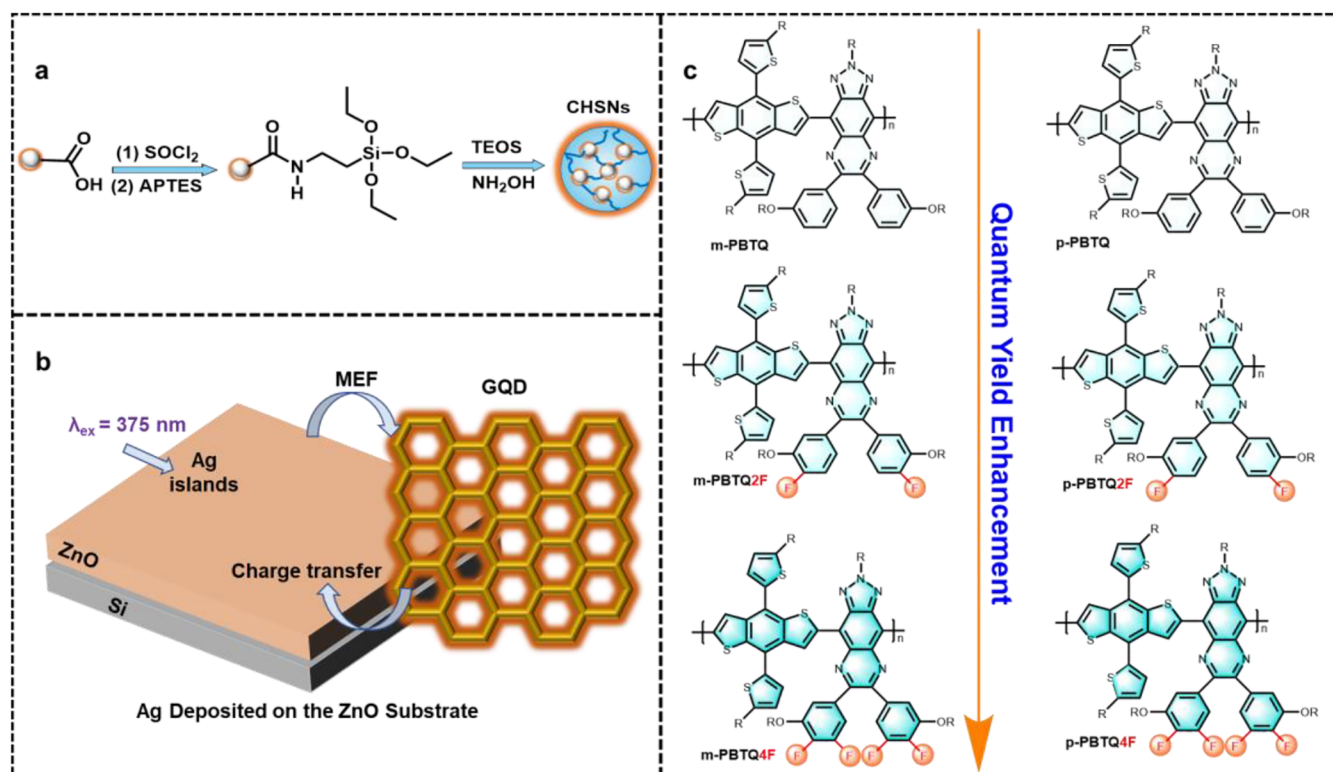


Figure 20. (a) Super fluorescence of heterogeneous silica nanospheres. Adapted with permission from ref 99. Copyright 2016 Wiley. (b) Blue-emitting graphene quantum dots and green-emitting graphene oxide quantum dots. Adapted with permission from ref 100. Copyright 2018 American Chemical Society. (c) Fluorescence of yields polymer dots. Adapted with permission from ref 101. Copyright 2020 Wiley.

nanocomposites, and the graphene/AuNCs hybrids were expected to construct hydrogen peroxide biosensors in clinical analysis (Figure 18b).

In 2012, Xie's group⁸⁷ illustrated an interesting finding of Au(I)–thiolate complexes: strong luminescence emission caused by the AIE mechanism (Figure 18c). The AIE property of the complexes was then used to develop a simple one-pot synthesis of highly luminescent Au–thiolate NCs with a quantum yield of ~15%.

In 2014, Liu's group⁸⁸ illustrated that a BSA@Au NC-Si nanoparticle composite and a water-soluble graphene/MWCNT/AuNC hybrid were designed and used for ECL detection of H₂O₂ and phenolic compounds, respectively (Figure 18e).

In 2013, Xie's group⁸⁹ illustrated that the introduction of Ag(I) ions to bridge and fix the small Au(I)–thiolate motifs on the weakly luminescent Au₁₈(SG)₁₄ surface led to the formation of larger Au(I)/Ag(0)–thiol motifs on the initial nanocluster surface. The assembled Ag(I)@Au₁₈(SG)₁₄ alloy nanoclusters were strongly luminescent (Figure 18d).

In 2016, Zhu's group⁹⁰ designed a method to activate the restriction of intramolecular rotation (RIR) on the basis of a golden cudgel (Au–Cu nanocluster). In their work, the active Cu complexes acted as surface ligands and were aggregated by the introduction of an inert Au kernel. The assembled Au–Cu nanocluster exhibited greatly improved PL intensity by activating the RIR process and then boosting the AIE of these Cu(I)–SR complexes (Figure 18f).

Quantum clusters have quantum effects, plasma resonance properties, inherent biocompatibility, and ease of functionalization, thereby providing their suitability as contrast agents for fluorescence (FL) imaging. Therefore, fluorescence imaging of

gold quantum clusters has been explored. In 2020, Kircher and colleagues⁹¹ illustrated the use of a one-step procedure modulated by α -LA to synthesize ultrasmall Au quantum clusters (AuQCs) with fluorescence emissions.

5.1.2. AgNC and AgNC Composites as Luminophores.

Compared with AuNCs, the Ag NC studies are limited. In 2017, Zang and co-workers⁹² showed that they grafted Ag(I) sulfide/chalcogenide/chalcogenide clusters onto adaptable bridging ligands, thereby enabling them to connect to each other and form a rigid metal–organic backbone by controlling the spatial quantum yield (12.1%). An ultrafast dual-function fluorescent switch (<1 S) was also achieved (Figure 19a).

In 2018, Zhu et al.⁹³ showed that by adding PPh₃ into a solution of the Ag₂₉ nanocluster the PL intensity was significantly enhanced (13-fold enhancement, quantum yield from 0.9% to 11.7%) because of the restricted PPh₃ dissociation–aggregation process (Figure 19b).

In 2019, Bakr et al.⁹⁴ synthesized silver thionanoclusters to form a one- and two-dimensional structural framework composed of bipyridine-linked nanocluster nodes; by controlling for the difference in the size of individual silver atoms in the nanocluster, their optical properties and thermal stability were adjusted (Figure 19c).

In 2022, Fan's group⁹⁵ showed Ag quantum cluster luminescence. A fully inorganic Ag quantum cluster that was stable in borate glass was prepared with excellent chemical stability, adjustable PL emission, and high quantum yield. The PL properties of the AgQCs responded significantly to changes in the embedding environment and exhibited significant switching from fluorescence to phosphorescence (F–P) (Figure 19d).

5.2. Metalloid-Based Fluorogenic Reactions

Carbon dots (CDs) and silicon nanomaterials have attracted much attention as emerging fluorescent materials in recent years.

5.2.1. CD-Based Fluorogenic Reactions. CDs, a class of zero-dimensional carbon nanomaterials with significant fluorescence performance, were first discovered in 2004 during the preparation of single-walled carbon nanotubes.⁹⁶ In 2007, carbon dots were described in detail as zero-dimensional carbon nanomaterials with diameters of 2–10 nm.⁹⁷ CDs showed excellent water solubility and biocompatibility, good stability, and unique optical properties; because of these qualities, CDs were of research interest to the academic community and had good application prospects in biological imaging.

The chemical structure and chemical/physical properties of fluorescent carbon nanoparticles are very similar to those of graphene oxide. They usually have carbonaceous graphitic cores of <10 nm with varying degrees of oxidation.⁹⁸ Specifically, carbon dots include graphene quantum dots, carbon nanodots, and polymer dots.

In 2016, Pang et al.⁹⁹ showed superbright carbon nanodot hybrid silica nanospheres (CSNs) synthesized by the Stöber process of silane-functionalized CDs. The fluorescence of the carbon nanodots converged strongly. The brightness of the CSN was approximately 3800 times brighter than that of individual carbon nanodots. In addition to its high brightness and low cytotoxicity, CSN showed its potential application in cell labeling (Figure 20a).

In 2018, Jeon et al.¹⁰⁰ demonstrated the plasma metal-enhanced fluorescence properties of blue-emitting graphene quantum dots and green-emitting graphene oxide quantum dots using fluorescence lifetime imaging microscopy (Figure 20b).

In 2020, Wu et al.¹⁰¹ showed a fluorination strategy for the development of semiconductor polymers with high brightness second near-infrared region (NIR-II) detectors (Figure 20c). The tetrafluorination reaction yielded polymer dots (Pdots) with a fluorescence quantum yield of 3.2%, which was more than a 3-fold increase over the unfluorinated counterpart. The fluorescence enhancement was attributed to the nanoscale fluorescence effect in the Pdots, which maintained the planarity of the molecule and minimized the structural distortion between the excited and ground states, thus reducing the nonradiative relaxation.

5.2.2. Silicon-Based Fluorogenic Reactions. In the past decade, we have achieved immense accomplishments in the field of silicon materials. Among them, fluorescent silicon nanomaterials have attracted considerable attention because of their unique advantages, including strong fluorescence coupled with robust photostability, rich resource support, low cost, industrial maturity, and good biocompatibility. Extensive efforts have been devoted to developing effective methods for the synthesis and functionalization of fluorescent silicon nanomaterials with different nanostructures, thereby facilitating the promotion of this promising material for myriad optical applications.

In 2018, label-free multiplexed photoluminescent silicon nanoprobe (PLSN-probe) were illustrated by Venkatarishnan et al.¹⁰² By altering the intrinsic nonphotoluminescent silicon substrate, the PLSN-probe demonstrated not only unique optical properties that could be used for bioimaging but

also cell-selective uptake that could distinguish and diagnose HeLa and fibroblasts (Figure 21a).

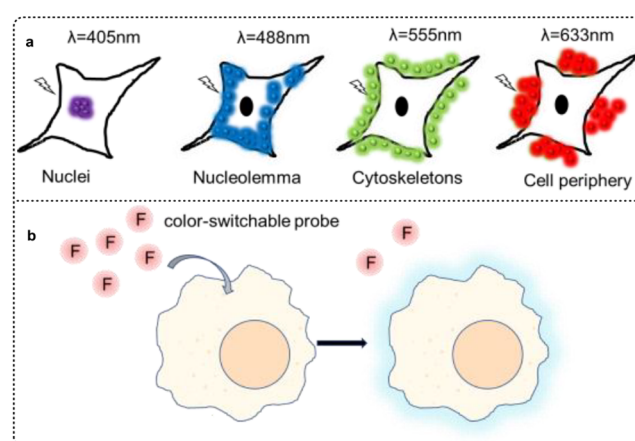


Figure 21. (a) Photoluminescent silicon nanoprobe (PLSN-probe). Adapted with permission from ref 102. Copyright 2018 Wiley. (b) Aldehyde-based silicon nanocrystals for cellular imaging applications. Adapted with permission from ref 103. Copyright 2022 American Chemical Society.

In 2022, Yang's group produced pioneering color-switchable probes (CSPs) for *in situ* cell imaging using aldehyde-functionalized silicon nanocrystals (SiNCs) that quickly switched their intrinsic photoluminescence from red to blue when interacting with amino acids in live cells¹⁰³ (Figure 21b).

6. APPLICATIONS OF FLUOROGENIC REACTIONS IN CHEMICAL BIOLOGY

Fluorogenic reactions offer several advantages, such as easy preparation, bioorthogonality of starting materials, and very mild reaction conditions. Because of these advantages, fluorogenic reactions are ideal reactions for chemical biology applications. Additionally, after the fluorogenic reaction, a very strong fluorescence signal can be obtained; the starting materials do not need to be fluorescent or weakly fluorescent, which is of particular interest for *in vivo* labeling in bioconjugation, such as labeling and sequencing of nuclear acids^{104–107} and labeling of proteins,¹⁰⁸ lipids,^{109–111} glycans, and others.¹¹² In addition, enzyme-triggered bioorthogonal cleavage fluorogenic reactions have attracted wide attention because of their high biocompatibility, fast reaction rate, and robustness.^{113–120} These fluorogenic reactions can also be applied in detecting and acting as no-washing probes for fluorescent imaging.^{121,122}

6.1. Fluorogenic Reactions for DNA/RNA Labeling and Sequencing

In 2015, He and Yi et al.¹²³ presented a bisulfite-free method for cyclization-enabled C-to-T transition of 5-formylcytosine (fC), which could detect whole-genome fC signals in mouse embryonic stem cells at a single-base resolution. This method was achieved by selectively modifying fC-containing genomic DNA with 1,3-indandione and then coupling a cleavable biotin to the 1,3-indandione-labeled fC via click reaction (Figure 22a). This bisulfite-free method could have wider applications in epigenome sequencing. In addition, in 2019, Kikuchi et al.¹²⁴ reviewed their efforts to develop fluorescent probes for detecting histone deacetylase activity and DNA methylation in living cells, including, for example, an AIE and intramolecular

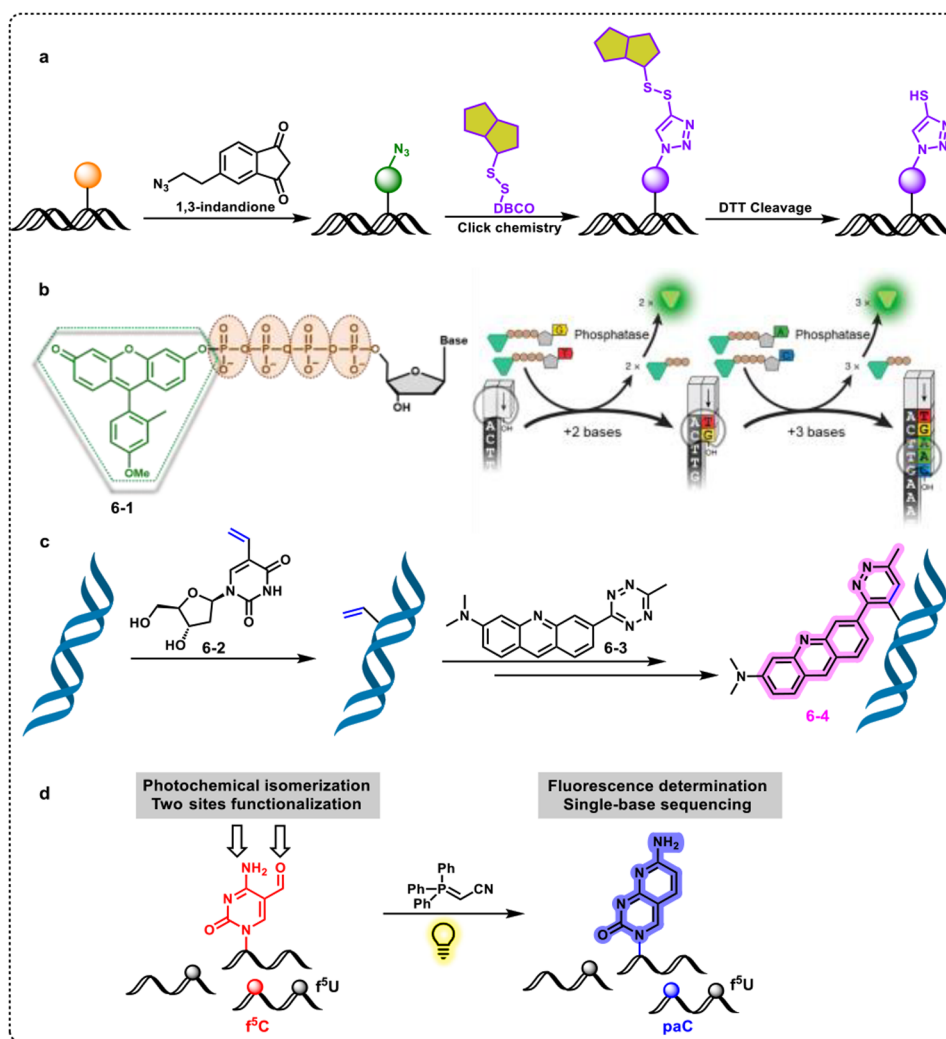


Figure 22. (a) Schematic diagram of cyclization-enabled C-to-T transition of f^5C . Adapted with permission from ref 123. Copyright 2015 Nature. (b) Chemical structure of a Tokyo Green (TG)-terminated fluorogenic nucleotide substrate 6-1 and its sequencing procession to DNA. Adapted with permission from ref 125. Copyright 2017 Nature. (c) Inverse electron-demand Diels–Alder reaction process of acridine–tetrazine conjugate 6-3 with DNA containing 5-vinyl-2'-deoxyuridine (6-2). Adapted with permission from ref 126. Copyright 2022 Wiley. (d) Chemical labeling processing of 5-formylcytidine reagents with variable substituted Wittig. Adapted with permission from ref 127. Copyright 2022 Wiley.

transesterification-based fluorescent probe that could detect histone deacetylase activity and a hybrid probe that was created by using a protein labeling system to visual methylated DNA in living cells and verify its dynamics during cell division.

In 2017, Huang et al.¹²⁵ pioneered a strategy for DNA sequencing, ECC sequencing, that could greatly improve sequencing accuracy and read length using a dual-based flowgram combined with fluorogenic SBS chemistry. The ECC sequencing approach enabled a mixture of two types of nucleotide substrates to be introduced into each reaction cycle, as shown in Figure 22b.

In 2022, Luedtke et al.¹²⁶ reported a dual enhancement strategy for nucleic-acid-templated reactions, which utilized a fluorogenic intercalating agent capable of undergoing inverse electron-demand Diels–Alder reactions with DNA containing 5-vinyl-2'-deoxyuridine (6-2) or RNA containing 5-vinyl-uridine. Reversible high-affinity intercalation of an acridine–tetrazine conjugate 6-3 increased the reaction rate of tetrazine–alkene on duplex DNA compared with the non-templated reaction. Additionally, loss of tetrazine–acridine

fluorescence quenching rendered the reaction highly fluorogenic and detectable under no-wash conditions (Figure 22c).

In 2022, Cheng et al.¹²⁷ developed a detection strategy on the basis of the fluorescence signal of the cyclization product 4,5-pyridin-2-amine-cytidine (paC), as shown in Figure 22d. The results clearly identified f^5C with a limit of detection of 0.58 nM. This method altered the hydrogen bonding activities of f^5C and modulated its reverse transcription signature in its sequencing profile. By using this strategy, named paC-Seq, f^5C could be detected with a single-base resolution from tRNA^{Met} segments with high accuracy.

6.2. Fluorogenic Reactions for Protein-Labeling

In 2020, Ding and Xie et al.¹²⁸ reported a tetrazole-functionalized photoclick hydrogel (6-5) that exhibited high photocapture protein efficiency under ultraviolet light conditions (Figure 23a). Combined with the single-cell immunoblotting method, use of this photoactive gel enabled simultaneous monitoring of subtle protein expression level changes in ≈ 2000 individual cells that could be concealed using conventional techniques.

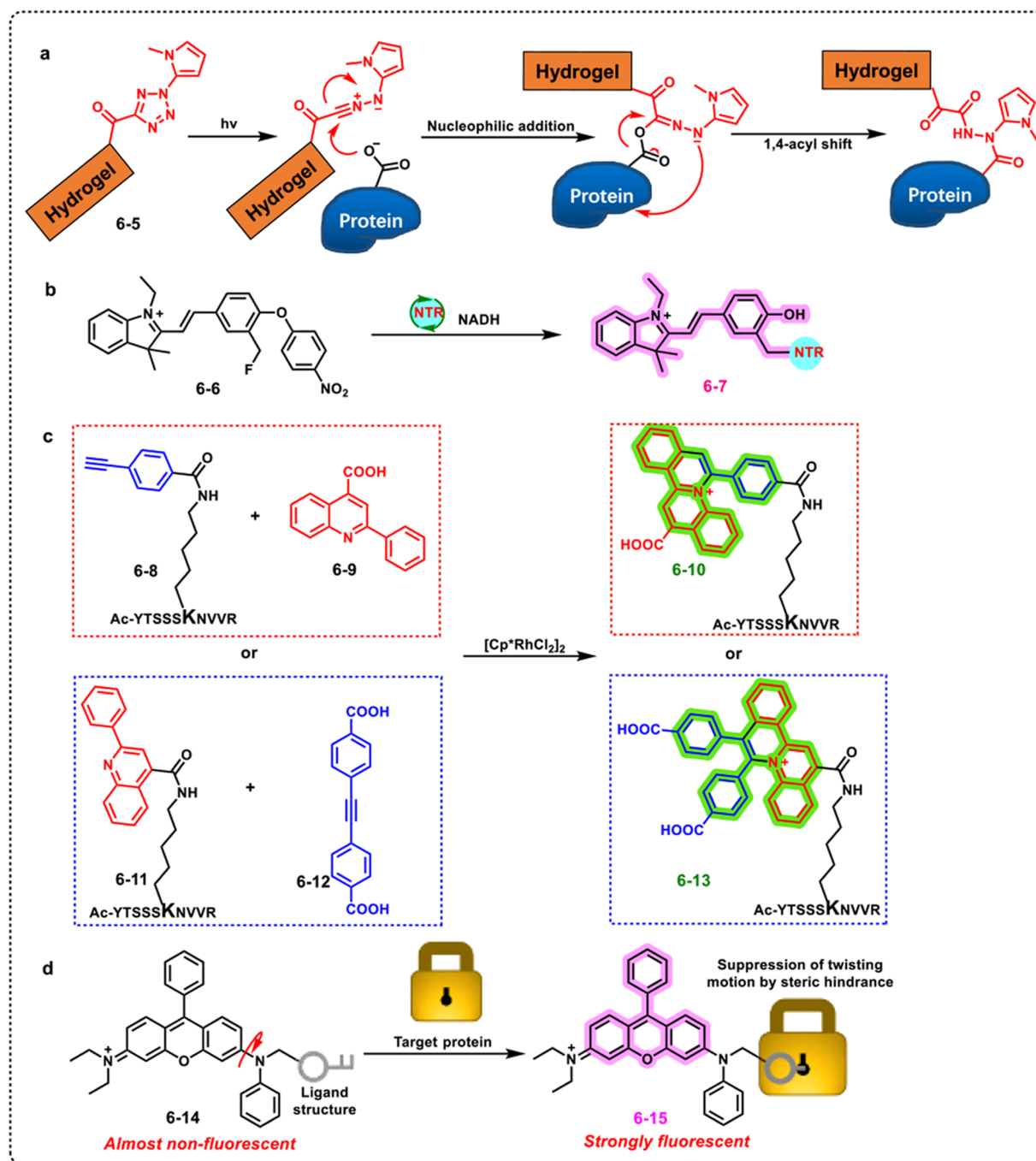


Figure 23. (a) Photoclick cycloaddition reaction of mPyTC (marked in red) on gel 6-5, which immobilizes proteins. Adapted with permission from ref 128. Copyright 2020 Wiley. (b) Chemical structure of probe 6-6 and fluorogenic production 6-7 after the reaction with NADH and NTR. Adapted with permission from ref 129. Copyright 2022 American Chemical Society. (c) Synthesis of quinolinizinium-labeled lysozyme (6-10 and 6-13) through two different pathways. Adapted with permission from ref 130. Copyright 2022 American Chemical Society. (d) Strategies for fluorogenic probe (6-14) for tag proteins. Adapted with permission from ref 131. Copyright 2022 American Chemical Society.

In 2022, Ge et al.¹²⁹ reported a nitroreductase (NTR) activatable, fluorogenic, mitochondrial localizable, quinone methide proximity-based protein labeling probe 6-6 (Figure 23b). The NTR probe not only provided a mitochondrial localizable and fluorogenic response but also achieved permanent retention at the site of activation with an enhanced spatial resolution to improve the detection sensitivity even after cell fixation.

In 2022, Wong et al.¹³⁰ developed a strategy for fluorescent turn-on ligation targeting alkyne- and quinoline-linked

peptides and proteins using the [Cp*RhCl₂]₂ catalyst. This bioconjugation strategy provided stable fluorescent quinoliniziniums under mild reaction conditions with good to excellent conversion. Moreover, native proteins, such as lysozyme, were successfully labeled with fluorescent quinolinizinium and subjected to in-gel fluorescence imaging (Figure 23c).

In 2022, Urano et al.¹³¹ established a new molecular design strategy to rationally develop activatable fluorescent probes 6-14, which exhibited a fluorescence off/on change in response to target biomolecules (as shown in Figure 23d), by controlling

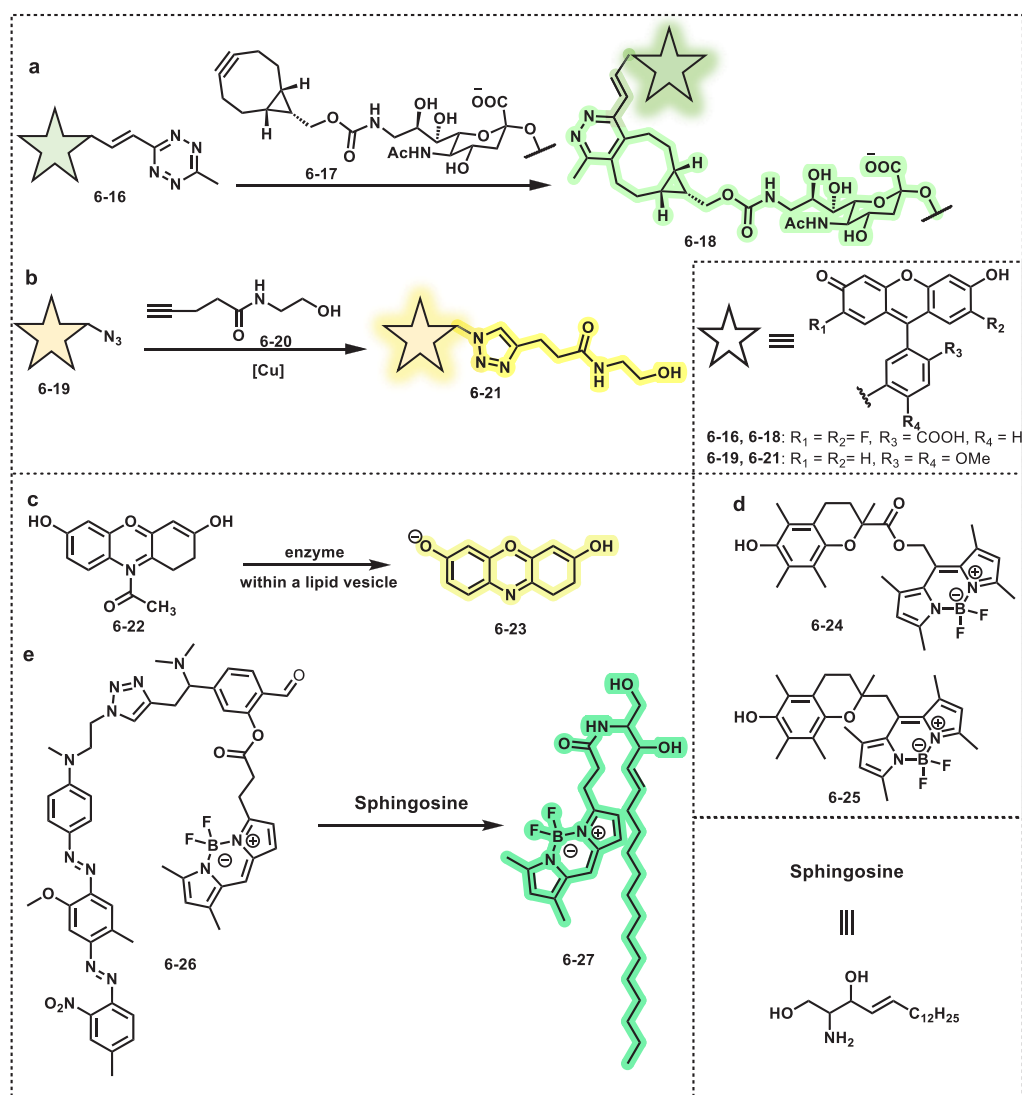


Figure 24. Fluorogenic reactions for glycans imaging. (a) Incorporation of a bicyclononyne-functionalized sialic acid derivative into glycans followed by a fluorogenic reaction. Adapted with permission from ref 134. Copyright 2015 Wiley. (b) CalFluor probes activated by fluorogenic chemistry and lipid-related fluorogenic probes. Adapted with permission from ref 135. Copyright 2015 American Chemical Society. (c) A fluorogenic enzymatic reaction, which happened within a lipid vesicle. (d) Two α -tocopherol-based fluorogenic probes for peroxyl radical reactions in liposomes response. Adapted with permission from ref 139. Copyright 2012 American Chemical Society. (e) The fluorogenic probes for sphingosine detection. Adapted with permission from ref 140. Copyright 2020 American Chemical Society.

the twisted intramolecular charge transfer (TICT) process. To illustrate and validate this TICT-based design strategy, it was used to develop practical fluorogenic probes for HaloTag and SNAP-tag.

6.3. Fluorogenic Reactions for Glycan and Lipid-Labeling

Glycans mediate various essential biological processes. Therefore, glycans imaging has an irreplaceable role in chemical biology. In recent years, excellent studies on glycans imaging via fluorogenic reactions have been reported.^{112,132,133}

In 2015, Bertozzi et al.¹³⁴ reported the metabolic incorporation of a cyclooctyne-functionalized sialic acid in combination with the utilization of a fluorogenic probe, which allowed for the imaging of zebrafish glycans during embryogenesis for the first time. The work breaks a new path for further study in chemical biology (Figure 24a). In the same year, Bertozzi et al.¹³⁵ developed fluorogenic probes called click-activated luminogenic fluorophores (CalFluors) on the

basis of photoinduced electron transfer (PeT). The probe can be used to visualize glycans *in vivo* during zebrafish growth (Figure 24b).

In 2020, Wong et al.¹³⁶ designed a fluorogenic terbium(III) complex probe that is capable of labeling and imaging glycans in living cells and zebrafish. The research makes it possible to time-resolvedly label and image biomolecules.

In 2022, Godula et al.¹³⁷ demonstrated a productive and adjustable approach for generating neoproteoglycan conjugates by merging glycosaminoglycan chains and a protein scaffold via a reactive fluorogenic linker. The use of a fluorogenic linker gives the green light to monitor the conjugation reaction.

Lipid-related fluorogenic reactions occupy a crucial position in biomolecules imaging. In 2017, Klymchenko et al.¹⁰⁹ reviewed environment-sensitive fluorogenic and solvatochromic probes that can help with figuring out the properties of lipid membranes. Recently, Klymchenko et al.¹¹⁰ reviewed

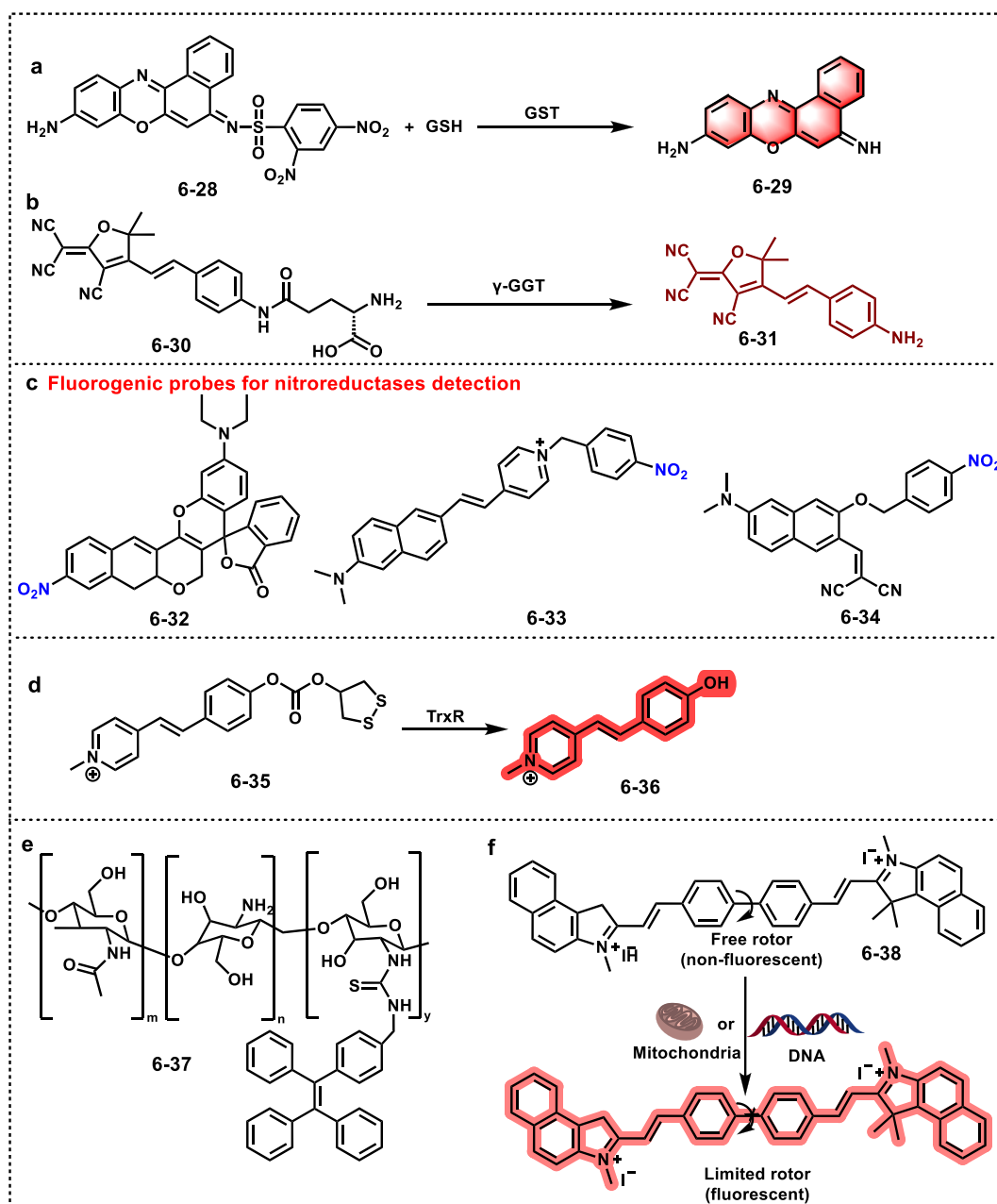


Figure 25. Different enzyme-induced fluorogenic reactions: (a) Fluorogenic substrate, GST-catalyzed reaction with GSH; adapted with permission from ref 141. Copyright 2011 American Chemical Society. (b) Recognition mechanism of probe 6-30 to γ -GGT; adapted with permission from ref 142. Copyright 2018 Wiley. (c) Different fluorogenic probes for NTRs detection; adapted with permission from ref 145. Copyright 2022 Elsevier. Adapted with permission from ref 146. Copyright 2022 Elsevier. Adapted with permission from ref 147. Copyright 2022 Wiley. (d) Response process of probe 6-35 for TrxR; adapted with permission from ref 148. Copyright 2022 American Chemical Society. (e) Bioconjugate of TPE and Chitosan; adapted with permission from ref 157. Copyright 2013 American Chemical Society. (f) Structure of MMP tracking rotor 6-38 and proposed mechanism; adapted with permission from ref 159. Copyright 2019 Wiley.

numerous fluorogenic probes for lipid membranes. Additionally, there have been some relevant reports in recent years.

In 2012, Haran et al.¹³⁸ developed a fluorogenic enzymatic reaction in which individual enzyme molecules are trapped in lipid vesicles as catalysts (Figure 24c). The relevant results imply that enzymes have evolved the ways to correlate fluctuations at different sites. In the same year, Cosa et al.¹³⁹ displayed a remarkable method to study in the important role of lipid diversity in affecting the relative antioxidant dynamics in model lipid membranes with fluorogenic α -tocopherol analogues (Figure 24d).

On the basis of the importance of sphingolipids in biology, many ways to label sphingolipids and metabolites have been reported and reviewed.¹¹¹ In 2020, Devaraj et al.¹⁴⁰ first reported a fluorogenic probe (6-26) for the detection of the significant signaling lipid sphingosine in cultured mammalian cells (Figure 24e). The strategy has shown a promising outlook for the diagnosis of lipid storage disorders.

6.4. Different Enzyme-Induced Fluorogenic Reactions

Enzyme-triggered bioorthogonal cleavage reactions have attracted wide attention because of their high biocompatibility, fast reaction rate, and robustness. Various enzymes have been

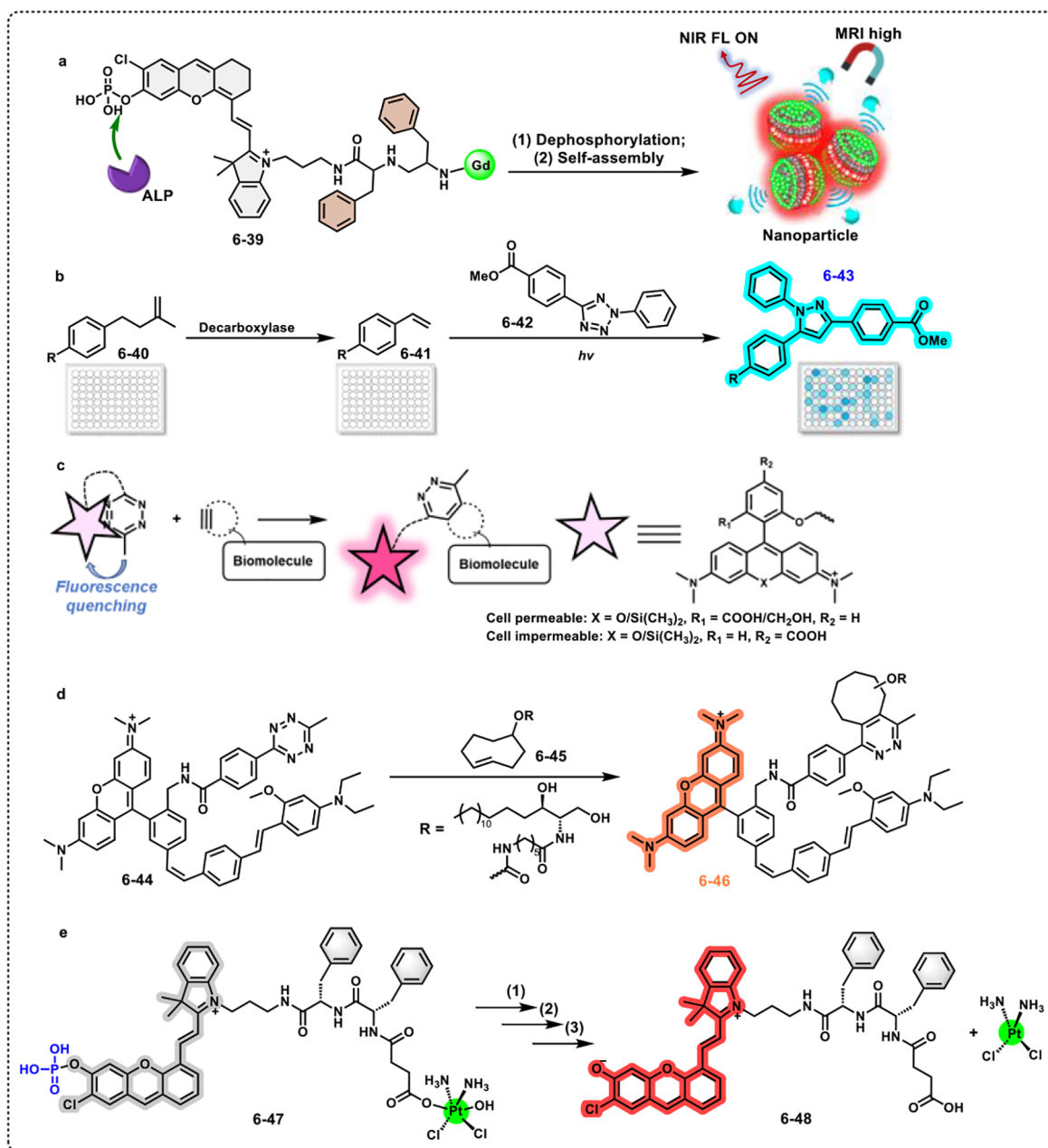


Figure 26. (a) Chemical structure of **6-39** and proposed ALP-mediated fluorogenic reaction and *in situ* self-assembly of **6-39** into NPs. Adapted with permission from ref 161. Copyright 2019 American Chemical Society. (b). Photoclick-chemistry-based high-throughput screening assay to interrogate mutagenesis libraries of the decarboxylase OleT for enzyme variants with improved selectivity for aromatic substrates. Adapted with permission from ref 164. Copyright 2021 American Chemical Society. (c) Tetrazine dyes can be conjugated to dienophile-modified biomolecules accompanied by an increase in fluorescence intensity. Adapted with permission from ref 164. Copyright 2021 American Chemical Society. (d) Structures of the **6-44** and **6-45** for targeted delivery of RhoVRs to organelles. Adapted with permission from ref 165. Copyright 2022 American Chemical Society. (e). Chemical structure of **6-47** and (1) proposed ALP-triggered dephosphorylation and (2) *in situ* self-assembly into Pt^{IV} NPs, followed by (3) GSH-triggered disassembly to release cisplatin and **6-48**. Adapted with permission from ref 166. Copyright 2023 Nature.

developed to specifically cleave protective groups preinstalled on target molecules. Fluorescence-based optical probes targeting enzymes can be used to monitor living biological processes in real time.

Glutathione transferases (GSTs) are phase II detoxification enzymes that catalyze glutathione (GSH) binding activity, glutathione peroxidase (GPx) and isoenzyme activity, steroid hormone and prostaglandin biosynthesis, and regulation of signaling pathways. GSTs play an important role in intracellular protection against environmental and oxidative stress

and have also been implicated in cellular resistance to drugs. In 2011, Morgenstern et al.¹⁴¹ synthesized substrates (**6-28**) by introducing electrophile sulfonamides into fluorescent molecules containing amino groups, which released free fluorophores (**6-29**) (and 1-glutathione-2,4-dinitrobenzene + SO_2) after GST-catalyzed cleavage of dinitrobenzenesulfonamides (Figure 25a). The authors described a general strategy for generating fluorescent GST substrates and provided the most sensitive assay for GST described to date.

γ -Glutamyl transpeptidase (γ -GGT) is called a membrane-associated enzyme, which plays an important role in many physiological and pathological processes, such as regulating oxidation and reduction metabolism, drug resistance, and diabetes. As a typical biomarker, the overexpression of γ -GGT enzyme is related to the growth and progress of ovarian cancer, cervical cancer, and other cancers. In 2018, Peng et al.¹⁴² developed an enzyme-triggered fluorescent probe **6-30** to detect γ -GGT enzyme *in vitro* and *in vivo* (Figure 25b). They installed a 2-dicyanomethane-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) derivative by introducing γ -glutamine group as a recognition unit, and the released amino group restored the intramolecular charge transfer (ICT) of molecule **6-31** with a significant far-red fluorescence signal upon γ -GGT enzyme triggering, with a supersensitive and rapid response. Furthermore, in 2020, Xie's group reported an activatable self-immobilized near-infrared probe (**6-32**) by combining quinone methide and a fluorogenic enzyme substrate for the imaging of GGT activity *in vitro* and *in vivo*.¹⁴³

Ubiquitin-specific proteases (USPs) are the largest class of the deubiquitinating enzyme (DUB) family, and USP16 is a key regulator of hematopoietic stem cell differentiation and is involved in mitosis and stem cell self-renewal. In 2019, Ova et al.¹¹⁵ developed an active molecular probe (ABP) and a fluorescent substrate that is highly selective for DUB USP16. This ABP has a rhodamine portion attached to the N-terminal end, which enables rapid readout for analysis, while the propargyl portion reacts covalently and captures the targeted DUB to achieve selectivity for USP16.

Nitroreductases (NTRs) are widely found in bacterial genera and to a lesser extent in eukaryotic cells, hypoxic tumor cells, and tumor tissues, and are widely used to evaluate the tumor microenvironment. To date, a number of optical probes have been reported for the detection of NTRs. In 2018, Hu et al.¹⁴⁴ designed and synthesized an NTR-enhanced magnetic resonance contrast agent probe, which can easily detect NTR activity *in vitro* and in *E. coli*. In the presence of NADH, NTR can selectively reduce the *para*-nitrobenzyl portion of the probe to form Gd-DOTA. In 2022, Shen et al.¹⁴⁵ reported an NTR-activated NIR detector (Figure 25c, **6-32**) with good selectivity and sensitivity. Upon reaction with NTR, the probe exhibited a significant fluorescence switching response at 740 nm. Shortly thereafter, Li et al.¹⁴⁶ designed a mitochondria-targeted two-photon fluorescent probe consisting of *N,N*-dimethylnaphthalen-2-amine, pyridine salt, and *para*-nitrophenyl (Figure 25c, **6-33**). Restricted by intramolecular rotation, the fluorescence intensity of the probe was significantly enhanced with increasing viscosity. Additionally, the probe can be applied to the specific imaging of NTR in zebrafish. In the same year, Yao's group¹⁴⁷ introduced a series of two-photon (TP) small molecule fluorescent probes capable of sensitively detecting NTR activity in a variety of biological samples. Probe **6-34** has excellent TP-excited fluorescence properties, and with excellent TP tissue penetration imaging properties, **6-34** has been used for detecting endogenous NTR activity from liver lysates and cardia tissue of cancer patients.

In addition, thioredoxin reductase (TrxR), a crucial antioxidant enzyme, has been applied for rapid imaging *in vivo*. In 2022, Zhou's group¹⁴⁸ developed a rapid mitochondrial probe with the ability to penetrate the blood–brain barrier and inactivate TrxR2 in a cellular Parkinson's disease model (Figure 25d, **6-35**).

6.5. Fluorogenic Reactions in Cellular Compartments

Some chemical substances, enzymes, and biomacromolecules play important roles in life activities. These substances are transported in organelles and also carry out various biochemical reactions. Therefore, the detection and imaging of these substances in organisms and different organelles has important guiding significance for researchers to explore the unknown world of life sciences.^{149–156}

In 2013, Tang et al.¹⁵⁷ synthesized a novel fluorescent probe by attaching TPE to a chitosan (CS) chain (Figure 25e, **6-37**). The TPE-CS bioconjugate exhibited unique AIE behavior. The internalized AIE aggregates were stored in the cellular compartments, and the other cell lines in the culture systems were not contaminated, which allowed a specific cell line to be distinguished from other unstained cell lines and be used as long-term cell tracers.

In 2018, Belousov et al.¹⁵⁸ reported a gene-encoding fluorescent probe, which exhibited various fluorescence intensities in different cellular compartments. This probe can be utilized for pH dynamics imaging of mitochondria in living neurons and quantitative pH measurements in zebrafish embryos.

In 2019, Wang et al.¹⁵⁹ reported a spatial-dependent fluorescent molecular rotor **6-38** that can track mitochondrial membrane potential (MMP) status through different limited rotations to produce fluorescence (Figure 25f). The cationic probe **6-38** accumulated in mitochondria at normal MMP state but accumulated in the nucleus under vanished MMP condition. When MMP status decreased, the probe could be presented in both the mitochondria and nuclei.

In 2020, Graham and Johnston et al.¹⁶⁰ reported a localization sensor composed of a quenched substrate SNAP-tag. This probe could be used to track the DNA complexes from endosomes into the cytosol and nucleus and quantitatively track localization of materials in cells.

6.6. Fluorogenic Reactions for Directed Evolution and Others

In 2019, Ye et al.¹⁶¹ designed a small-molecule-based activatable NIR fluorescence and magnetic resonance bimodal probes by integrating a fluorogenic reaction into ALP enzyme-responsive and *in situ* self-assembly, as shown in Figure 26a, which could be applied to map orthotopic liver tumor margins in intraoperative mice, thereby allowing for real-time image-guided resection of liver tumors.

Enzymatic oxidative decarboxylation is an upcoming reaction; however, efficient screening methods for the directed evolution of decarboxylases are lacking. In 2008, Lin et al.¹⁶² used a simple plate reader assay for the detection of small terminal alkenes, and they fluorescently labeled a noncanonical amino acid containing a terminal olefin moiety (*O*-allyl-tyrosine) using diaryltetrazoles by forming a fluorescent cycloadduct. Because of this work, in 2021, Schwaneberg et al.¹⁶³ developed a photoclick chemistry-based high-throughput screening assay to interrogate mutagenesis libraries of the decarboxylase OleT for enzyme variants with improved selectivity for aromatic substrates (Figure 26b). The simple handling and convenient fluorescent read-out enabled a high-throughput screening of comprehensive decarboxylase variant libraries within hours. This study had potential implications for the directed evolution of OleT and other decarboxylases.

The main challenge in the development of fluorogenic probes for bioorthogonal chemistry is to accommodate both

high fluorogenicity upon target binding and favorable properties for live-cell imaging in one molecular structure. In 2021, Wombacher et al.¹⁶⁴ reported close proximity tetrazine–dye conjugates that enabled highly efficient quenching of rhodamines and SiRs. The tetrazine–dye conjugates exhibited excellent properties for the targeting of unnatural amino acids and enabled extra and intracellular multicolor wash-free labeling (Figure 26c). Finally, they demonstrated that bioorthogonal labeling with HDyes (“Heidelberg Dyes”) enabled live-cell stimulated emission depletion (STED), as well as super-resolution optical fluctuation imaging.

Electrical potentials across biological membranes, including those that form membrane-bound organelles, regulate and initiate a host of physiological processes. However, organelle membrane potentials remain relatively underexplored. To address the challenge of monitoring changes in organelle membrane potential, Miller et al.¹⁶⁵ reported a new small molecule sensor (6-44) capable of targeting subcellular membranes in 2022, as shown in Figure 26d. The sensor was capable of targeting the endoplasmic reticulum (ER) of living cells and showing the membrane potential fluctuations of this organelle in the intact cells.

Recently, fluorogenic reaction also enabled a stimuli-responsive cisplatin prodrug (6-48) that could undergo alkaline phosphatase-triggered *in situ* self-assembly and intracellular glutathione-triggered disassembly process, which elicits the burst release of cisplatin in tumor cells (Figure 26e).¹⁶⁶

CONCLUSIONS AND PERSPECTIVES

Fluorogenic reactions are one of the most useful classes of biocompatible reactions in chemical biology. In this review, we surveyed the related literature published in the past 10 years and classified the relevant fluorogenic reactions into fluorogenic bond formation reactions, fluorogenic bond cleavage reactions, and several other types, including aggregation-induced fluorogenic reactions, as shown in Figure 1.

Despite tremendous potential, we are only beginning to realize some of the practical applications of fluorogenic reactions, as mentioned in Section 6. However, there are still some challenges that need to be addressed to proceed with effective applications in the biotech industry.

Some of the challenges are the development of new reaction types, the generation of novel fluorescent structures, and diverse fluorescent properties. Recently, because of a fast-growing body of knowledge in organometallic chemistry, highly reactive biocompatible organometallic “intermediates” have been designed as novel bioconjugation reagents for protein labeling. Pioneered by three groups of Buchwald, Pentelute, and Chang, organometallic fluorogenic reactions might represent a promising direction in chemical biology.^{7,167,168}

Another challenge is the use of more energy sources to generate fluorescence. Besides chemical and photochemical energy, we can image mechanical, electrochemical, and more possibilities. Even though there are many difficulties in the development of new fluorogenic reactions, this emerging field will most likely generate many novel applications in the future.

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Notes

The authors declare no competing financial interest.

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VOCABULARY

bioorthogonal chemistry: a class of high-yielding chemical reactions that proceed rapidly and selectively in biological environments without side reactions toward endogenous functional groups

click chemistry: a synthetic philosophy inspired by nature in terms of its efficiency, selectivity, and simplicity

quantum cluster: a cluster smaller than nanoclusters and with quantum effects
chemiluminescence: excitation luminescence using energy generated by chemical reactions
electrochemiluminescence: a specific chemiluminescence reaction triggered by electrochemistry on the electrode surface

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