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Development and Applications of Bioluminescent and Chemiluminescent Reporters and Biosensors

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Abstract

Although fluorescent reporters and biosensors have become indispensable tools in biological and biomedical fields, fluorescence measurements require external excitation light, thereby limiting their use in thick tissues and live animals. Bioluminescent reporters and biosensors may potentially overcome this hurdle because they use enzyme-catalyzed exothermic biochemical reactions to generate excited-state emitters. This review first introduces the development of bioluminescent reporters, and next, their applications in sensing biological changes in vitro and in vivo as biosensors. Lastly, we discuss chemiluminescent sensors that produce photons in the absence of luciferases. This review aims to explore fundamentals and experimental insights and to emphasize the yet-to-be-reached potential of next-generation luminescent reporters and biosensors.

1. INTRODUCTION

Bioluminescence is a light-producing phenomenon occurring in natural organisms for communication, prey, or defense (1). The light production mechanism of bioluminescence involves a biochemical reaction relying on the oxidation of a substrate (e.g., luciferin) by an enzyme (e.g., luciferase). Unlike fluorescence, bioluminescence measurements do not require incident radiation. The signal essentially glows on a dark background that offers excellent sensitivity over fluorescence where phototoxicity and autofluorescence are usually problematic during sampling. Additionally, fluorescence is not ideally suited for in vivo imaging when scattering and absorbing of excitation photons are serious hurdles. Although it is possible to gain high spatiotemporal resolution with in vivo fluorescence endoscopy, this procedure is often invasive and requires high levels of expertise. Bioluminescence, on the other hand, circumvents most of these issues, acting as an excellent candidate to achieve noninvasive imaging in live animals. Moreover, optogenetic tools, which have been widely used to manipulate or control biological systems, are not always compatible with common fluorescent probes due to spectral overlaps. In this context, bioluminescence has become a highly intensive area of research, and bioluminescent reporters have been employed by biomedical researchers in a wide range of applications, including gene regulation and signaling, protein–protein interactions, drug screening, molecular imaging, cell-based assays, and noninvasive in vivo imaging (2–6).

As bioluminescence suffers from its low-brightness nature largely caused by the slow turnover of luciferases, bioluminescent reporters are mostly used in macroscopic imaging with limited spatiotemporal resolution; however, recent studies have produced brighter bioluminescent tools that have enabled microscopic imaging at a single-cell resolution. To further alter colors of emission while keeping high photon flux, recent advances in engineered bioluminescent reporters start to mirror the development of fluorescent proteins. Compared to the engineering of fluorescent proteins, engineering of novel bioluminescent reporters usually requires expertise in synthetic chemistry, protein engineering, and animal studies. Therefore, this type of work is highly challenging, but meanwhile, there are many strategies for improvement.

Supplemental Material >

This review provides an overview of the field and highlights recent advances in the development of bioluminescent reporters (**Table 1**) and biosensors (**Supplemental Table 1**), with a main focus on bioanalytical and diagnostic applications. Furthermore, it provides a brief summary of

Table 1 Common luciferase–luciferin pairs

Original species	Luciferase	Luciferin	Size (kDa)	λ_{max} nm	Reference
Photinus pyralis	FLuc	D-Luciferin	61	560	10
Photinus pyralis	Akaluc	AkaLumine	61	650	20
Pyrophorus plagiophthalamus	CBR	D-Luciferin	61	615	25
Pyrearinus termitilluminans	ELuc	D-Luciferin	61	538	26
Renilla reniformis	RLuc8	CTZ	36	480	33
Renilla reniformis	RLuc8.6	CTZ	36	535	34
Gaussia princeps	GLuc	CTZ	20	473	42
Oplophorus gracilirostris	NanoLuc	Furimazine	19	460	53
Oplophorus gracilirostris	teLuc	DTZ	19	502	59
Photorhabdus luminescens	iLux	Fatty aldehyde and $FMNH_2$	>200[a]	490	74

[a]The total size of *luxA*, *luxB*, *luxC*, *luxD*, and *luxE*.
Abbreviations: CBR, click beetle red luciferase; CTZ, coelenterazine; DTZ, diphenylterazine; FLuc, firefly luciferase; FMNH2, reduced flavin mononucleotide; GLuc, *Gaussia* luciferase; RLuc, *Renilla* luciferase.

recent advances in chemiluminescent probes that are capable of producing photons in the absence of any luciferase and a perspective on future directions for bioluminescent and chemiluminescent tools.

2. BIOLUMINESCENT REPORTERS

2.1. Development and Applications of D-Luciferin-Consuming Luciferases

In the 1940s to 1960s, the mystery of light emission from the North American firefly *Photinus pyralis* was uncovered by McElroy, his coworkers, and other investigators (7–9). A few decades later, the gene of firefly luciferase (FLuc) was cloned and expressed in mammalian cells (10, 11). The enzyme responsible for bioluminescence, FLuc, is now probably the most widely used luciferase for biological and biotechnological applications. FLuc catalyzes a reaction between its native D-luciferin substrate and adenosine-5′-triphosphate (ATP), yielding AMP-luciferin that is further oxidized by molecular oxygen (O_2) to form an excited state (**Figure 1*a***). This high-energy intermediate releases energy in the form of yellow-green light that peaks at 560 nm, leading to a ground-state product, oxyluciferin (12). FLuc was further codon optimized for mammalian expression (e.g., the *luc2* gene). Recently, consecutive single amino acid deletion mutants of FLuc, ΔFlucs, have been reported for higher activities and lower K_M toward D-luciferin (13). To further improve the thermal and pH stability of FLuc, a chimeric luciferase was developed by fusing the N-terminal domain of FLuc and the C-terminal domain of *Luciola italica* luciferase, thereby resulting in a codon-optimized PLG2 that shows ~threefold higher activity than the original FLuc (14).

Chemists have invested enormous efforts in developing synthetic D-luciferin analogs. For in vivo bioluminescence imaging (BLI), an ideal luciferin should emit near-infrared (NIR) photons to maximize tissue penetration by minimizing tissue absorption and scattering. Although many D-luciferin analogs have been synthesized (15), most red-shifted analogs do not increase photon fluxes. In one study, Moerner and coworkers (16) replaced the sulfur atom in 6-amino-D-luciferin with a selenium atom, leading to 6-aminoseleno-D-luciferin with red-shifted emission at 600 nm and in vivo brightness comparable to or slightly higher than that of 6-amino-D-luciferin. A few other D-luciferin analogs, such as CycLuc1 (17), AkaLumine (18), and CybLuc (19) (**Figure 1*b***), improved the in vivo performance of FLuc. Recently, a reengineered FLuc variant, Akaluc, in the

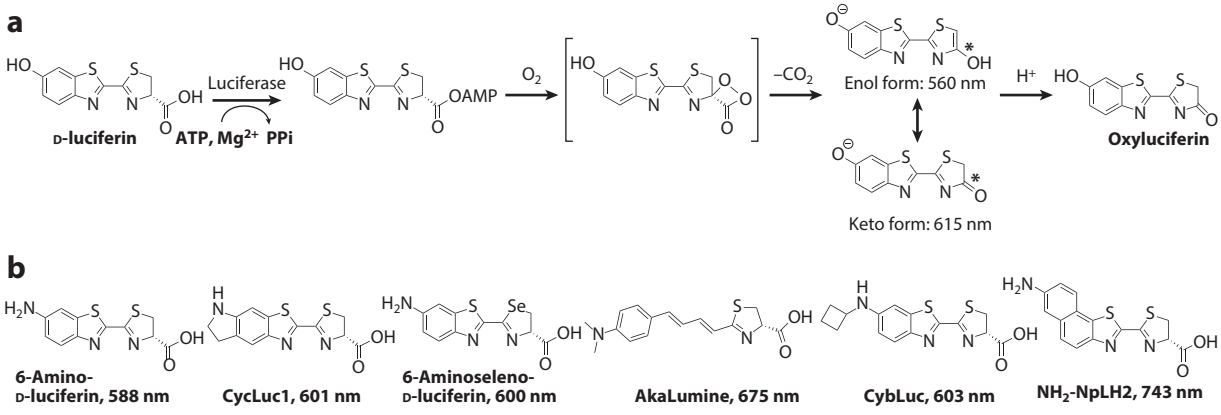


Figure 1

(*a*) Mechanism for bioluminescence emission generated by luciferase-catalyzed oxidation of D-luciferin. (*b*) Representative D-luciferin analogs and their peak emission wavelengths in the presence of firefly luciferase (FLuc).

presence of its synthetic AkaLumine substrate and a sensitive electron-multiplying charge-coupled device camera, was able to highlight single cells in mice (20). Furthermore, Akaluc-AkaLumine has been demonstrated for the labeling of neurons in the brains of freely moving mice and a common marmoset (20).

Effort has also focused on the development of luciferase–luciferin pairs orthogonal to FLuc and D-luciferin. Miller's group (21) identified FLuc mutants (R218K, L286M, and S3411) that have abolished activity toward D-luciferin but remain active toward CycLuc analogs (22). These pairs were further demonstrated for mouse brain imaging. In addition, Prescher and coworkers (23) synthesized a series of sterically modified D-luciferin analogs and screened for FLuc mutants that can discriminate these analogs, resulting in a set of FLuc-derived orthogonal luciferase–luciferin pairs. These new tools enabled sequentially multiplexed BLI in the same live animals (24).

To date, more than 30 D-luciferin-consuming luciferases have been discovered from diverse species. In addition to FLuc, click beetle luciferases from *Pyrophorus plagiophthalamus* and *Pyrearinus termitilluminans*, such as CBR and ELuc, are also popular reporters due to their excellent thermal stability and ability to emit different colors of bioluminescence from 538 nm to 615 nm in the presence of D-luciferin (25). In particular, codon-optimized ELuc from *P. termitilluminans* exhibits ~10-fold stronger signals than FLuc (26). These click beetle luciferase variants together can achieve spectra-resolved multicolor assays (27) and multiplexed in vivo BLI (28) (e.g., monitoring the expression of two genes simultaneously or labeling two different cell types in individual animals). Recently, Hall et al. (29) reported an engineered click beetle luciferase mutant, CBR2opt, which shows maximal emission at 743 nm when paired with NH_2-NpLH2, a synthetic naphthyl-luciferin analog. However, despite the dramatic red-shift, CBR2opt still displayed better in vivo sensitivity in the presence of D-luciferin than NH_2-NpLH2.

2.2. Development and Applications of Coelenterazine-Consuming Luciferases

Coelenterazine (CTZ), harboring an imidazopyrazinone core structure, is probably the most widely presented luciferin in luminous marine organisms, including sea pansies, copepods, squids, shrimps, and jellyfishes (1). The light production mechanism has been proposed as follows: First, the C-2 position of CTZ interacts with O_2 to form a dioxetanone intermediate; next, the intermediate loses CO_2 to give a high-energy, excited-state coelenteramide, from which photons are produced (**Figure 2*a***). It has been suggested that photons may be emitted from different chemical forms of coelenteramide within the enzyme active site (30). For example, the presence of phenolate anion in the excited state may be responsible for emission at ~480 nm.

Unlike FLuc, most CTZ-consuming luciferases do not require additional cofactors, such as ATP. Since the chemical structure of CTZ was identified and CTZ was confirmed as a shared substrate of various marine luciferases, many CTZ analogs have been synthesized (31). Some representative synthetic CTZ analogs, which may lead to unique reactivity, higher brightness, red-shifted emission, or better in vivo performance are shown in **Figure 2*b***. Generally speaking, CTZ-consuming bioluminescent reporters commonly used in laboratories are derived from the sea pansy *Renilla reniformis*, the marine copepod *Gaussia princeps*, or the deep-sea shrimp *Oplophorus gracilirostris*. In the following sections, we describe various options that are currently available in the BLI toolbox.

2.2.1. *Renilla* luciferase and its derivatives.

Renilla luciferase (RLuc) was cloned from *R. reniformis*, which emits at 480 nm in the presence of CTZ (32). Loening et al. (33) reported a RLuc8 variant, which harbors eight amino acid substitutions from RLuc and shows a fourfold enhancement in brightness and a 200-fold enhancement in serum stability. To shift the emission

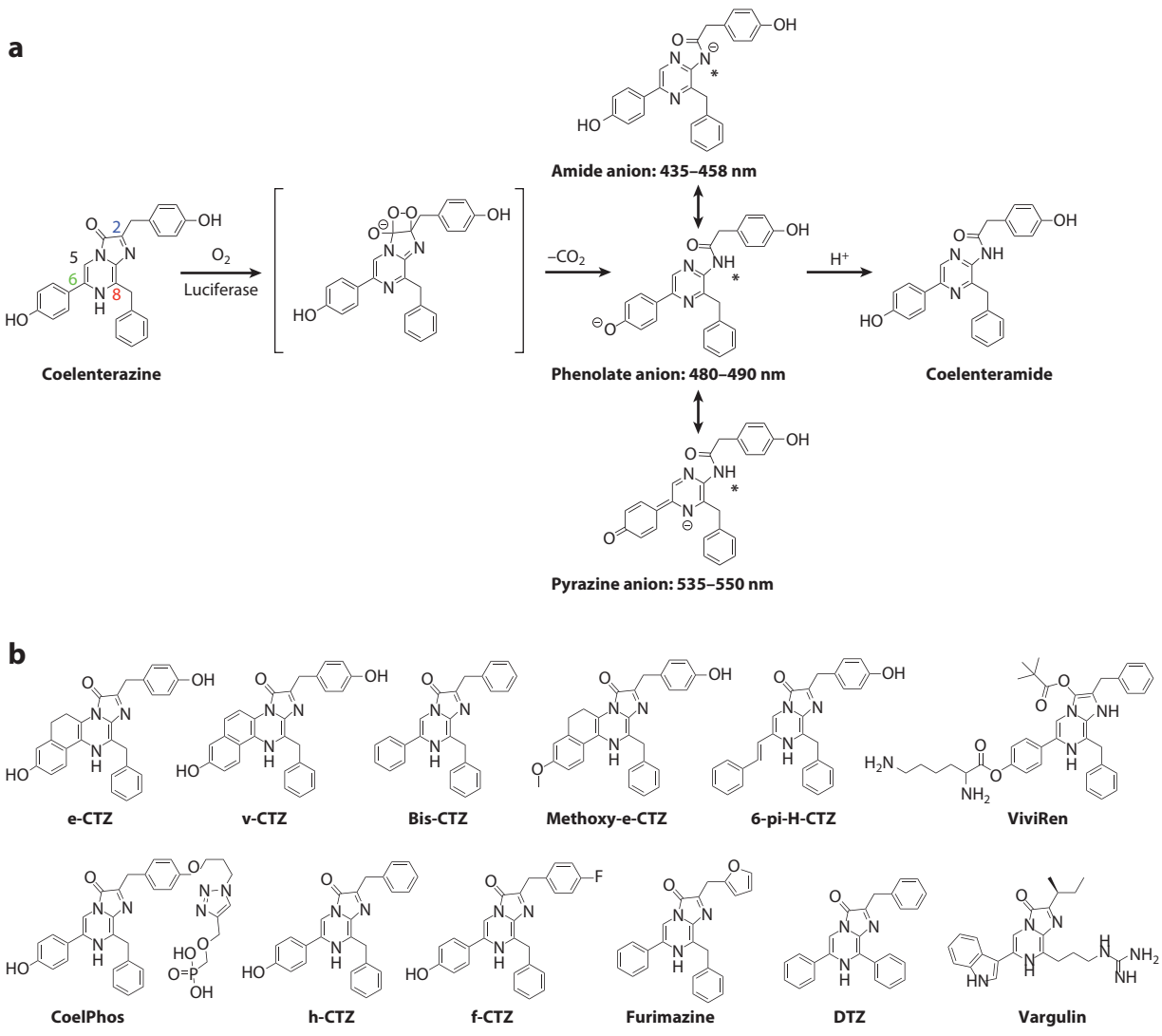


Figure 2

(*a*) Mechanism for luciferase-catalyzed oxidation of coelenterazine (CTZ) and proposed excited-state emitters. (*b*) Representative CTZ analogs, which lead to unique reactivity, higher brightness, red-shifted emission, or better in vivo performance.

of RLuc, its active site was reengineered, leading to a number of new variants with bioluminescence emission spanning from 475 nm to 547 nm in the presence of CTZ (34). In particular, several red-shifted variants, such as RLuc7-521 and RLuc8.6-535, which have emission peaks at 521 nm and 535 nm, respectively, were demonstrated for improved in vitro and in vivo brightness over native RLuc (35). More recently, a super RLuc mutant was reported for improved thermostability and emission at 540 nm when paired with CTZ (36).

Numerous CTZ analogs bearing diverse substitutions at the C-2, C-5, C-6, or C-8 positions have been synthesized mainly to enhance bioluminescence brightness and to red-shift emitting photons (**Figure 2*b***) (31). In particular, the RLuc and e-CTZ pair displays dual emission peaks at 418 nm and 475 nm and is sevenfold brighter than the RLuc and CTZ pair (37). Also, the RLuc8.6

and v-CTZ pair yields red-shifted emission peaking at 588 nm (34). On the other hand, bis-CTZ could blue-shift the bioluminescence of RLuc8 to ~405 nm (37). Similarly, methoxy-e-CTZ and several recently reported π-conjugated substitutions (e.g., 6-pi-H-CTZ; **Figure 2*b***) at the C-6 position cause similar hypsochromic shifts but are approximately tenfold brighter than Bis-CTZ (38). Because the violet emission of RLuc8 paired with methoxy-e-CTZ is well overlapped with the second absorption peak (Soret band) of NIR fluorescent iRFPs, fusion proteins between RLuc8 and iRFPs have been generated for NIR emission via bioluminescence resonance energy transfer (BRET) (39).

To gain sustained light output in vivo, CTZ was chemically modified into temporarily inactive forms. For example, ViviRen (**Figure 2*b***) has two ester protection groups on the C-3 and C-6 positions; after entering cells, they are slowly de-esterified by endogenous esterases. This strategy not only greatly reduces the auto-oxidation of these substrates and slows down their consumption in vivo, but it also leads to improved signals in mouse brains, as demonstrated in an RLuc-labeled brain tumor model (40).

It can be concluded that several RLuc-derived luciferase–luciferin pairs are now available with emissions spanning from ~400 nm to ~590 nm. RLuc has been a very popular bioluminescence reporter in protein- and cell-based assays. Moreover, because the blue emission of RLuc is overlapped with the excitation of bright green fluorescent fluorophores, such as enhanced green fluorescent protein (GFP), RLuc has been widely used as a BRET donor in biosensor development (41).

2.2.2. *Gaussia* luciferase and its derivatives. The cDNA of *Gaussia* luciferase (GLuc) was cloned from the marine copepod *G. princeps* in 2002 (42). GLuc, which is a naturally secreted luciferase, emits flash-type bioluminescence at ~473 nm in the presence of CTZ. Under similar experimental conditions, GLuc is ~100 times brighter than RLuc in mammalian cells (43). To date, a number of GLuc variants have been reported. For example, GLuc4 shows stable light output suitable for high-throughput screening (44). GLuc8990 is approximately tenfold brighter than GLuc and Monsta (a red-shifted mutant of GLuc) and produced a wavelength peak at 503 nm (45). Recently, GLuc has been fused with multiple repeats of an endoplasmic reticulum–targeting sequence, resulting in intracellular retention of GLuc for biosensing and imaging applications (46). Its high brightness and naturally secreted features make GLuc an attractive reporter for real-time ex vivo monitoring of biological processes in cultured cells, or in blood or urine from animals (47).

Interestingly, bright GLuc variants have been used to excite channelrhodopsins and proton pumps to initiate or inhibit neuronal activity (48). The resulting fusions—luminopsins—integrate both chemogenetic and optogenetic concepts and are becoming useful research tools for the interrogation of neuronal circuits and brain functions (49).

In contrast to RLuc, GLuc is not very reactive toward typical synthetic CTZ analogs. Only a cell-impermeable CTZ analog, CoelPhos (**Figure 2*b***), was reported to light up GLuc localized on the cell surface (50). Expanding the substrate scope of GLuc via protein engineering may lead to more bioluminescence colors and a broader range of applications.

2.2.3. *Oplophorus* luciferase and its derivatives. *Oplophorus* luciferase (OLuc) was isolated from deep-sea shrimp *O. gracilirostris* in 1978 (51). A key 19-kDa subunit of OLuc was later cloned, overexpressed, and confirmed for enzymatic activity toward CTZ (52). Recently, the Promega Corporation converted this 19-kDa subunit into NanoLuc, which has 16 additional mutations, and in the presence of a synthetic CTZ analog, furimazine (**Figure 2*b***), it is ~100-fold brighter in vitro than FLuc-D-luciferin (53). Furthermore, Inouye et al. (54) reported that three known CTZ analogs, including h-CTZ, f-CTZ, and bis-CTZ (**Figure 2*b***), when paired with NanoLuc, can

generate two- to threefold stronger bioluminescence than furimazine. The same research group also reported on eKAZ, which has three additional mutations (V44I, A54I, Y138I) from NanoLuc; when CTZ served as the substrate of eKAZ, the bioluminescence brightness was comparable to that of the NanoLuc-furimazine pair (55).

Because of its small size, high brightness, high stability, and successful commercialization, NanoLuc has been quickly adapted by a large number of biomedical researchers (56), outperforming RLuc and FLuc in many aspects (57). NanoLuc has been successfully used to track virus spread in vivo, and the small NanoLuc gene, but not the large FLuc gene, could be integrated into the genome of influenza virus (58). However, a major drawback of NanoLuc is its blue emission peak at ~460 nm, resulting in limited tissue penetration due to strong absorption and scattering of blue photons by biological tissues. To red-shift the bioluminescence of NanoLuc, we recently engineered a teLuc luciferase, which displayed intense bioluminescence peaking at ~502 nm when paired with a synthetic diphenylterazine (DTZ) substrate (**Figure 2*b***) (59). The brightness of the teLuc-DTZ pair is approximately twofold higher than NanoLuc-furimazine. Moreover, because the emission of teLuc-DTZ is quite broad, teLuc-DTZ actually emits 13 times more photons than FLuc-D-luciferin at wavelengths longer than 600 nm. In fact, we were able to gain 5- to 13-fold more signals from teLuc-DTZ compared to the standard FLuc-D-luciferin imaging condition in a hydrodynamic liver transfection mouse model. A recent study has reported novel CTZ analogs that can red-shift the emission of NanoLuc to nearly 600 nm, although their bioluminescence intensities were low, and it remains unclear whether NanoLuc could be further engineered to rescue bioluminescence for these new substrates (60).

Another strategy to red-shift NanoLuc uses BRET. Lin and coworkers (61) fused NanoLuc with a CyOFP fluorescent protein, resulting in a construct named Antares that showed much-improved in vivo bioluminescence. We also developed an Antares2 reporter by genetically fusing teLuc with CyOFP, thereby further enhancing in vivo BLI sensitivity (59). Similarly, Nagai's group (62) genetically fused NanoLuc with a number of fluorescent proteins to generate five different bioluminescence colors for multicolor cellular imaging. Furthermore, NanoLuc has been fused to a SNAP- or Halo-tag and further labeled with various fluorescent dyes (60, 63). Although this semisynthetic strategy could red-shift NanoLuc for far-red or even NIR emission, it requires an additional dye conjugation step, thereby limiting its uses in vivo.

Split luciferases are ideal tools for studying protein–protein interactions via complementary assays. NanoLuc was split into a 1.3-kDa C-terminal fragment and an 18-kDa N-terminal fragment with 190-μM affinity (64). This NanoBiT technology has been utilized to detect the activities of G protein–coupled receptors (GPCRs) (65). Furthermore, taking advantage of this small C-terminal fragment (11 amino acid residues), researchers have integrated the DNA sequence into genomes to label specific genes of interest by using CRISPR/Cas9 without disturbing the functions of endogenous genes (66). When the corresponding 18-kDa N-terminal fragment was coexpressed, a complete NanoLuc could form on tagged proteins to generate bioluminescence signals. NanoLuc has further been split into three fragments, which were individually fused to two antibody variable domain fragments (V_H and V_L) for a sandwich bioluminescence immunoassay (67).

OLuc-derived luciferases, such as NanoLuc and teLuc, clearly exhibit advantageous features over RLuc and other luciferases. Moreover, OLuc-derived luciferases seem to be amenable to both protein and substrate engineering, thereby leading to the flexibility to further tune the color of emission.

2.2.4. Artificial luciferases. Kim et al. (68) created a series of artificial luciferases (ALucs) based on a sequence alignment of several CTZ-consuming copepod luciferases. The resultant ALucs exhibited high thermostability and prolonged kinetics. Moreover, different lineages of ALucs

were evolved, yielding unique substrate preferences (69) and enhanced brightness (70). Recently, Kim's group (71) further reported a group of dye-conjugated CTZ analogs. In particular, ALuc16 showed a minor, far-red emission peak in the presence of a 6-Nile-R-CTZ substrate, and under this condition, ~11% of the total emission was at wavelengths longer than 600 nm.

2.3. Development and Applications of Bacterial Luciferase

Photons from naturally bioluminescent bacteria are caused by a *luxCDABE* operon that encodes a heterodimeric luciferase (*luxA* and *luxB*) and three biosynthetic enzymes (*luxC*, *lucD* and *luxE*) responsible for the production of a long-chain aldehyde substrate. This bioluminescent system can be fully genetically expressed and can glow without the addition of exogenous luciferins. However, it is limited by low brightness, blue emission (490 nm), and dependence of reduced riboflavin phosphate ($FMNH_2$) and long-chain aldehydes. Optimizations have enabled heterologous expression of bacterial luciferase (Lux) to label other bacteria in infectious animal models (72) or to directly label mammalian cells (73). Because the availability of $FMNH_2$ could be limited in cells, an enhanced system, iLux, has been recently reported by coexpressing a flavin mononucleotide (FMN) reductase and introducing additional mutations across the *luxCDABE* operon (74). The resultant bioluminescent system was six- to eightfold brighter than FLuc in single bacterial cells. Furthermore, long-chain aldehydes may be potentially toxic to heterologous hosts, limiting the use of Lux as a common bioluminescent reporter.

2.4. Other Miscellaneous Luciferases

To date, more than a thousand species have been observed for bioluminescence, but most have not been well studied. For example, luciferases cloned from the luminous ostracod, *Cypridina noctiluca* (CLuc) and *Vargula hilgendorfii* (VLuc), use Vargulin (**Figure 2*b***) as their substrate (75), which shares a core imidazopyrazinone structure with CTZ but different substitutions on the C-6 and C-8 positions. This makes CLuc and VLuc evolutionarily unique. Because they do not cross-react with CTZ or D-luciferin, they are suitable for simultaneous dual-luciferase assays and multiplex imaging (76).

Fungi have long been observed to emit light during the night. Research on the chemical basis of fungal bioluminescence suggests that this light production process involves two steps (77). The precursor luciferin is first reduced by an NAD(P)H-dependent enzyme, and next, oxidized by the luciferase. Recent studies demonstrated that the oxidation could yield a high-energy endoperoxide intermediate, and the emission could be tuned from 480 nm to 564 nm by modulating the chemical structures of their substrates (78). This wavelength-tunable feature indicates that fungal luciferases may have great potential as practical bioluminescent reporters. During the writing of this review, a genetically encoded bioluminescent system from fungi was reported (147).

3. BIOLUMINESCENT BIOSENSORS

3.1. Chemically Modified Luciferins for Functional Assays or Imaging

To convert bioluminescent reporters into biosensors that change bioluminescence intensities in response to changes in surrounding environment, luciferins have been chemically modified into labile, inactive structures (i.e., caged luciferins) that can undergo uncaging reactions in the presence of specific molecules or enzymes. Modification at the C-6 position is the most common strategy for D-luciferin (**Figure 3**). For example, this strategy has been applied to monitoring of enzymatic

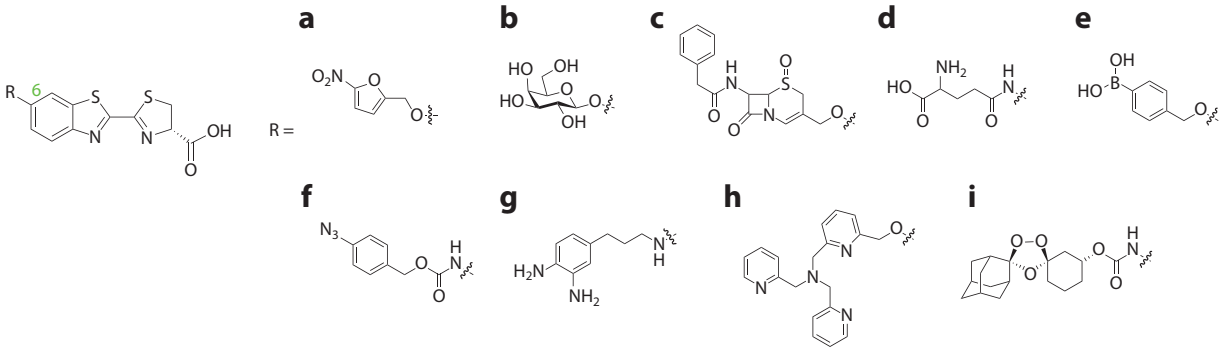


Figure 3

Examples of caged D-luciferins for functional bioluminescence assays or imaging. These prosubstrates are for the detection of (*a*) nitroreductase, (*b*) β-galactosidase, (*c*) β-lactamase, (*d*) γ-glutamyl transpeptidase, (*e*) hydrogen peroxide, (*f*) hydrogen sulfide, (*g*) nitric oxide, (*h*) copper, and (*i*) ferrous.

activities, such as furin (79), nitroreductase (80), β-galactosidase (81), β-lactamase (82), caspase-3 (83), and γ-glutamyl transpeptidase (84). This approach has also been expanded to image metabolites, reactive small molecules, or metal ions, such as hydrogen peroxide (85), hydrogen sulfide (86), hypochlorite (87), nitric oxide (88), fluoride (89), copper (90), and ferrous (91). Recently, Prescher and colleagues (92) developed a caged luciferin that can be selectively activated by enzymes in one cell type, releasing a free luciferin that can be consumed by surrounding luciferase-expressing cells for light production. This example demonstrated the use of bioluminescence to image cell–cell proximity.

To date, fewer studies have been done to develop CTZ derivatives for functional assays or bioimaging, likely because it is more challenging to chemically modify CTZ. In one study, the C-3 carbonyl of CTZ was caged with a β-galactose functional group, resulting in a bGalCoel substrate for the detection of the β-galactosidase activity (93). In addition, several caged CTZ analogs (i.e., prosubstrates) were included in a patent by the Promega Corporation to probe various glycosidase activities and to report metabolically active cells (94).

3.2. BRET–Based Biosensors

Resonance energy transfer (RET) is a photophysical phenomenon describing a donor chromophore at its excited state that may transfer energy to an acceptor chromophore via a nonradiative dipole–dipole coupling process. When the donor is excited by bioluminescent reactions, this phenomenon is called BRET. The BRET efficiency is inversely proportional to the sixth power of the distance between the donor and the acceptor. Therefore, BRET is a useful method for monitoring subtle distance changes between donors and acceptors (95), leading to a large array of applications in monitoring protein–protein interactions, biosensing, and functional imaging (5, 96–97). Previous studies mostly used luciferases as BRET donors and fluorescent proteins as BRET acceptors and fused them with various sensory elements to create diverse bioluminescent biosensors. We highlight some examples below with a focus on applications in in vitro assays, live cells, and animals.

3.2.1. FLuc-based BRET biosensors. Although FLuc is a widely used bioluminescent reporter, it is not a popular choice for the development of BRET-based biosensors. First, FLuc is ATP dependent, making it a natural ATP sensor. For other live-cell or in vivo applications, the

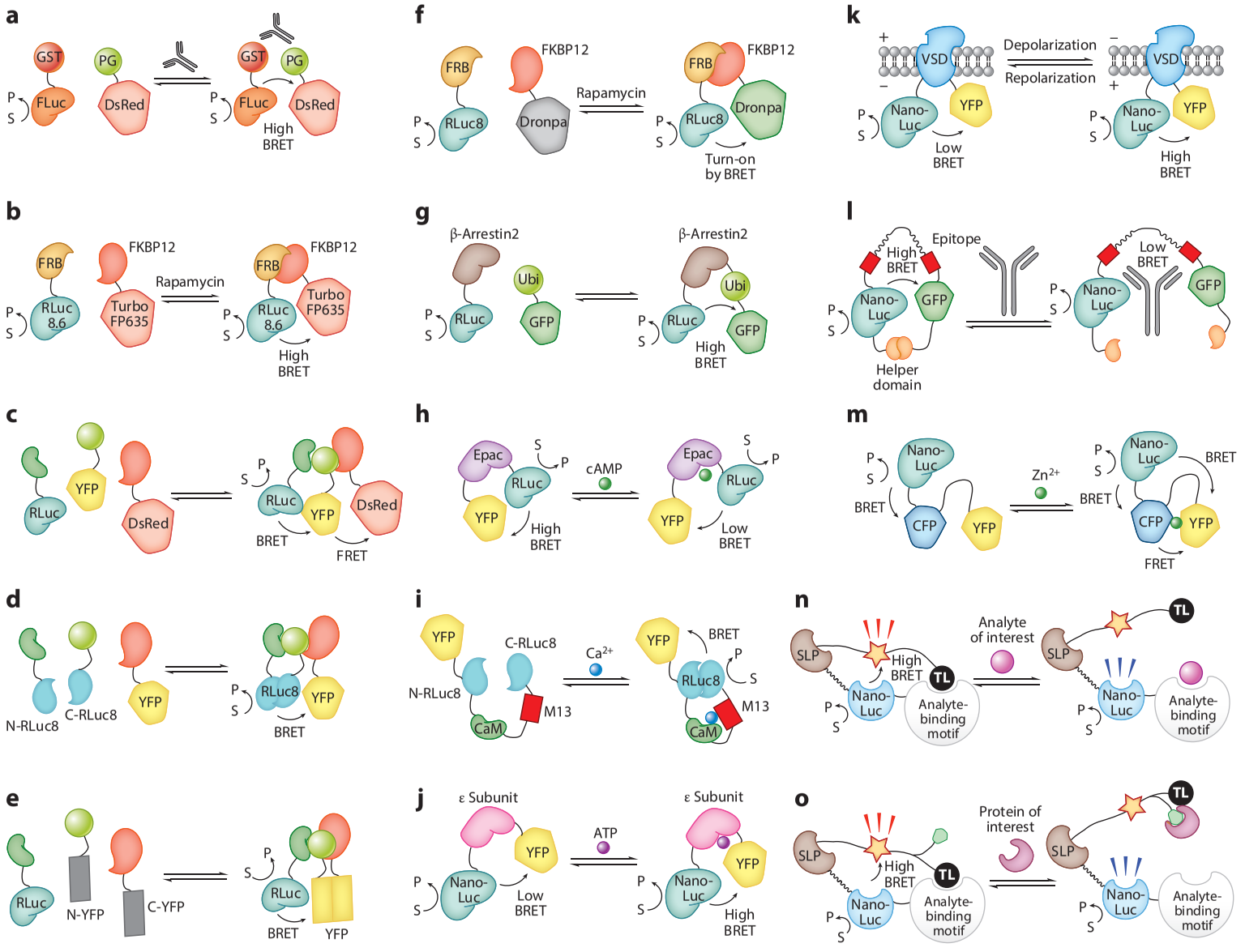
variations in the ATP level might be problematic. Moreover, consumption of cellular ATP by this bioluminescence-generating process would definitely perturb cell physiology, as ATP is not only the most important energy currency in living cells but also a key molecule connecting metabolism to signaling. Furthermore, the bulky size of FLuc (61 kDa) makes the designing of FLuc-based BRET biosensors difficult, because it is hard to predict donor-acceptor distances and relative orientations. Finally, fluorescent proteins that are suitable as BRET acceptors for FLuc are typically not bright (98), and therefore, it is hard to gain sensitized emission from the acceptors. An early study made a GST (glutathione *S*-transferase)–FLuc fusion and a PG (protein G)–DsRed fusion (**Figure 4*a***). The presence of an anti-GST antibody can bring these two fusion proteins in close proximity, leading to increased BRET. This system was used to measure the concentrations of the anti-GST antibody (99). However, the emission overlap between FLuc (emission: 560 nm) and DsRed (excitation: 558 nm, emission: 583 nm) is problematic. To address this issue, another study further tagged cysteine residues on DsRed with an Alexa Fluor 680 dye (excitation: 680 nm, emission: 705 nm), resulting in sequential resonance energy transfer (SRET) for improved spectral separation (100). This strategy has been further developed for probing caspase-3, thrombin, and factor Xa activities (100). In another study, FLuc was combined with quantum dots to form BRET pairs and used to develop protease assays (101).

3.2.2. BRET biosensors based on coelenterazine-consuming luciferases. RLuc and its mutants have been widely used as the BRET donors, resulting in diverse BRET pairs such as RLuc-CTZ and YFP, RLuc-bis-CTZ and GFP, RLuc8-CTZ and mOrange, and RLuc8.6-CTZ and TurboFP635 (102, 103). In one example, a caspase-1 substrate sequence was inserted between RLuc8 and YFP. Therefore, upon caspase cleavage, BRET is disrupted. This sensor has been used as a ratiometric indicator for pro-IL-1β processing (104). Another successful example demonstrated the monitoring of rapamycin-induced FRB-FKBP12 association by fusing RLuc8.6 to FRB and TurboFP635 to FKBP12 (**Figure 4*b***). Rapamycin induces the dimerization of FRB and FKBP12 and subsequently brings RLuc8.6 and TurboFP635 together for increased BRET. Furthermore, the system was used to monitor FRB-FKBP12 dimerization in HT1080 cells trapped in the lungs of living mice by measuring emission at 535 nm and 635 nm (103).

RLuc has also been used to monitor the interactions of three proteins. In one study, a SRET system based on RLuc-bis-CTZ, YFP, and DsRed (**Figure 4*c***) enabled the BRET-FRET (Förster resonance energy transfer) monitoring of three-protein interactions. Moreover, this method has been applied to the identification of neurotransmitter receptor complexes in live cells (105). In another study, RLuc and two complementary fragments of a split YFP were individually fused to partner proteins, leading to a biomolecular fluorescence complementation (BiFC)-BRET method that can also detect the interactions of three proteins (**Figure 4*d***) (106). Similarly, RLuc has been split into two complementary fragments and used for BRET with YFP (**Figure 4*e***) (107). This so-called complemented donor-acceptor RET (CODA-RET) method, which also allowed the identification of heteromeric complexes of three different proteins, has been used to investigate dynamic G protein–associated signaling cascades (107).

To take advantage of both BRET and high-intensity fluorescence imaging, RLuc8 was paired with photoactivable fluorescent protein, Dronpa, to achieve bioluminescence-assisted switching and fluorescence imaging (BASFI) (108). When RLuc8 and Dronpa were brought into close proximity, Dronpa was photoswitched to a fluorescence state by the luminescence generated from RLuc8 in the presence of its substrate (**Figure 4*f***). This process can be detected as increased BRET and the fluorescence turn-on of Dronpa.

Understanding extracellular and intracellular signal transduction is key to unlocking the mystery of cell biology. Ubiquitination is a type of posttranslational modification controlling protein



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Representative BRET-based biosensors and their response mechanisms. (*a*) An immunoassay based on FLuc and DsRed to detect antibody concentrations. (*b*) Monitoring of rapamycin-induced FRB-FKBP12 association with the RLuc8.6 and TurboFP635 pair. (*c*) SRET to detect the interactions of three proteins. (*d*) BRET from a donor luciferase to complementary acceptor fragments (i.e., BiFC-BRET) to detect the interactions of three proteins, and (*e*) BRET from two complementary luciferase fragments to an acceptor fluorescent protein (i.e., CODA-RET) to detect the interactions of three proteins. (*f*) Bioluminescence-assisted switching-on of Dronpa and BRET (i.e., BASFI) to monitor the rapamycin-induced FRB-FKBP12 interaction in both bioluminescence and fluorescence modes. (*g*) Monitoring of β-arrestin2 ubiquitination by using RLuc-β-arrestin2 and GFP-ubiquitin fusion proteins. (*h*) An RLuc-YFP BRET-based cAMP biosensor, CAMYEL. (*i*) A BRET-based Ca^{2+} biosensor, Nano-lantern (Ca^{2+}), via Ca^{2+}-induced reconstitution of RLuc8. (*j*) A NanoLuc-YFP BRET-based ATP biosensor, BTeam. (*k*) A NanoLuc-YFP BRET-based voltage sensor, LOTUS-V. (*l*) LUMABS for highly sensitive detection of antibodies down to the picomolar range. (*m*) A bioluminescent Zn^{2+} biosensor, BLZinCh-3, based on BRET from NanoLuc to CFP and YFP of an existing fluorescent Zn^{2+} biosensor. (*n*) Semisynthetic LUCIDs and (*o*) CLASH sensors for the detection of therapeutic drugs and protein effectors. Abbreviations: BiFC, biomolecular fluorescence complementation; BRET, bioluminescence energy resonance transfer; CaM, calmodulin; CFP, cyan fluorescent protein; CLASH, chemical ligand–associated steric hindrance; Epac, exchange protein activated by cAMP; GFP, green fluorescent protein; GST, glutathione *S*-transferase; LUCIDS, luciferase-based indicators of drugs; LUMABS, luminescent antibody sensors; PG, immunoglobulin G–binding protein G; SLP, self-labeling protein, such as SNAP-, Halo- and CLIP-tags; SRET, sequential resonance energy transfer; TL, tethered ligand; Ubi, ubiquitin; VSD, voltage-sensing domain; YFP, yellow fluorescent protein.

degradation, function, localization, and interaction. Perroy et al. (109) fused RLuc to β-arrestin2 and GFP to ubiquitin to study the ubiquitination dynamics of arrestin2 triggered by GPCRs (**Figure 4*g***). The ubiquitination of arrestin2 brings RLuc and GFP into close proximity and, thus, increases BRET from RLuc to GFP. Monitoring the ratiometric emission of RLuc and GFP enables real-time measurement of ubiquitination in live cells. In another study, a BRET sensor was developed to measure intracellular cAMP, a cyclic nucleotide and an intracellular second messenger (110). In the resultant cAMP sensor (CAMYEL), a cAMP-binding Epac (exchange protein activated by cAMP) domain was inserted between RLuc and YFP (**Figure 4*h***). cAMP binds to Epac and triggers a conformational change to modulate the BRET from RLuc to YFP and, thus, the determined BRET ratios can be used as optical readouts for live-cell cAMP levels. A similar approach has been applied to create BRET-based biosensors for cGMP (111), Ca^{2+} (112), and ATP (113). In 2012, Nagai's group (114) reported a chimera of RLuc8 and Venus, named Nano-lantern, which exhibited tenfold increased brightness compared with RLuc8 alone and allowed the real-time BLI of tumors in freely moving mice. By inserting different sensory domains into the N- and C-terminal domains of a split RLuc8 in Nano-lantern, three sensors for Ca^{2+}, ATP, or cAMP were derived (**Figure 4*i***). These sensors were combined with optogenetic tools, such as halorhodopsin and channelrhodopsin-2, for live-cell imaging of rat hippocampal neurons (114).

Since the invention of NanoLuc, which is much brighter than RLuc, many NanoLuc-based biosensors have been developed, mirroring the development processes of RLuc-based biosensors. For example, following the design of CAMYEL, NanoLuc-based Ca^{2+} and ATP sensors have been created. In particular, a Ca^{2+} sensor, CalfluxVTN, exhibits a larger dynamic range than Nano-lantern (Ca^{2+}) and can work compatibly with optogenetic actuators in rat hippocampal neurons (115). Furthermore, an ATP sensor, BTeam (**Figure 4*j***), which is a fusion protein of both NanoLuc, an ATP-binding ε subunit, and YFP, was developed and used to determine intracellular ATP concentrations (113). Moreover, NanoLuc replaced RLuc8 in Nano-lantern, followed by linker optimization, yielding a number of BRET-based, high-affinity Ca^{2+} indicators for multicolor BLI of Ca^{2+} in multiple subcellular organelles of living cells (116). In addition, a BRET-based ratiometric voltage indicator, LOTUS-V (**Figure 4*k***), showed robust signal-to-noise ratios. In its design, NanoLuc and YFP were fused to the two termini of a voltage-sensing domain, and voltage-induced structural changes in the voltage-sensing domain alter the BRET from NanoLuc to YFP. LOTUS-V is free from external excitation and thus provides an excellent way to monitor

neuronal activities in combination with optogenetic actuators that typically overlap with GFP-based voltage sensors (117). The low phototoxicity of bioluminescent indicators also makes them ideal for long-term imaging.

Antibodies are important immunological markers for infectious and immunological diseases (118). A series of NanoLuc-based biosensors, called LUMABS (luminescent antibody sensors), were developed for fast detection of antibodies in blood plasma (119). All LUMABS sensors share the similar design that comprises NanoLuc, mNeonGreen, two antibody-binding epitopes, and a pair of weakly dimerizing helper domains that bring together NanoLuc and mNeonGreen for high BRET (**Figure 4*l***). Upon the binding of an antibody to the epitopes, the interaction between the two helper domains is disrupted, resulting in a large decrease of BRET and a color change from green to blue. This sensor has been demonstrated for the detection of picomolar antibodies in blood plasma. This general strategy has been adapted for the detection of other antibodies, such as trastuzumab (anti-HER2), obinutuzumab, rituximab (anti-CD20), and cetuximab (anti-EGFR), providing a potential alternative assay for monitoring therapeutic drugs in patients (120). Another strategy introduced an unnatural amino acid, *p*-azidophenylalanine, via genetic code expansion to replace the epitope of the original LUMABS. The incorporated *p*-azidophenylalanine can be further site-specifically coupled with dinitrophenol or creatinine through click chemistry and become small-molecule epitopes to detect dinitrophenol- or creatinine-recognized antibodies (121).

Although many biosensors based on FRET have been developed (122), it is typically not possible to obtain responsive BRET sensors by simply replacing FRET donors with luciferases. Fortunately, additional strategies have been developed to simplify the conversion of existing FRET biosensors into BRET biosensors (123). In one example, NanoLuc was fused to existing FRET-based Zn^{2+} biosensors eCALWY (124) and eZinCh-2 (125), leading to dual BRET and FRET sensors for quantitative detection of intracellular Zn^{2+} (**Figure 4*m***) (126). The bioluminescent reaction of NanoLuc was used to excite CFP and YFP while the efficiencies of the RET processes were Zn^{2+} dependent. Because the sensors have two chromophores that compete with the bioluminescent light source, introducing a chromophore-silencing mutation into CFP yielded a BRET biosensor with improved responsiveness. Similarly, NanoLuc has been fused to GCaMP6s and thus gives LUCI-GECO1 for monitoring Ca^{2+} dynamics in live cells in the bioluminescence mode (127).

In addition to biosensors that are based on BRET between luciferases and fluorescent proteins, a series of semisynthetic biosensors based on BRET between luciferases and synthetic dyes were developed by Johnsson and coworkers (128) (**Figure 4*n***). These so-called luciferase-based indicators of drugs (LUCIDs) generally comprise a BRET donor luciferase (e.g., NanoLuc paired with furimazine), an analyte-binding domain, and a self-labeling protein through which a synthetic, fluorophore-containing intramolecular tether could be linked. The corresponding intramolecular tethers contain a motif for selective labeling reactions with self-labeling proteins, such as SNAP- (129), Halo- (130), and CLIP- (131) tags, a competitive ligand for the analyte-binding domain, and a RET acceptor fluorophore (e.g., Cy3). Initially, the competitive ligand would interact with the analyte-binding domain to form a closed state that keeps the RET acceptor in close proximity to the RET donor, leading to high BRET. In the presence of an analyte of interest, the analyte would compete with the tethered competitive ligand, leading to an open state in which BRET from the acceptor to the donor becomes low. By monitoring BRET, the concentration of the analyte can be quantitatively determined. To date, a number of LUCIDs have been reported, showing excellent dynamic range and sensitivity toward various therapeutic drugs (132), such as methotrexate, tacrolimus, sirolimus, cyclosporine A, topiramate, and digoxin. For this application, bioluminescence-based biosensors outperformed similar fluorescence-based biosensors because

blood serum is highly absorptive and autofluorescent. LUCIDs can be readily modified and tuned for quantitative measurements of molecules other than drugs. In one example, the analyte-binding domain of LUCIDs was substituted with antigen-binding fragments of antibodies (133). This strategy provides a shortcut to make bioluminescent biosensors for diverse antigens, and its potential application could lead to point-of-care diagnostics. In another study, LUCIDs were modified into the so-called CLASH (chemical ligand–associated steric hindrance) biosensors (134), which contained two exclusive ligands on the intramolecular tether (**Figure 4*o***). One ligand still has affinity with the analyte-binding domain, but the other ligand can bind to a specific effector protein, which increases the overall steric hindrance and prevents the interaction of the first ligand with the analyte-binding domain. CLASH allows an unrelated effector to control the function of a designed protein and demonstrates a general approach to the analysis of protein targets. Recently, a LUCID variant, LUPIN (luciferase-based photocatalysis induced via nucleic acid template) has been demonstrated to promote photocatalysis and facilitate a ruthenium-based uncaging reaction; thus, it releases a specific inhibitor to control enzyme function (135).

4. LUCIFERASE-INDEPENDENT CHEMILUMINESCENT BIOSENSORS

Chemiluminescence, a process similar to bioluminescence, can generate light from chemical reactions in the absence of luciferases. Recent development of chemiluminescent reactions has resulted in a few choices for chemiluminescent imaging in live cells and organisms (136). Luminol, oxalate esters, and sterically stabilized 1,2-dioxetane are common scaffolds that have been utilized in developing chemiluminescent biosensors. For example, adamantylidene–dioxetane (Schaap's dioxetane) has been linked to various analyte-responsive protecting groups, and particular analytes could trigger the removal of the protecting groups and initiate electron exchange luminescence at ~470 nm (**Figure 5*a***). Recently, Schaap's dioxetane has been systematically modified. By introducing an electron-withdrawing acrylic group, a 3,000-fold increase in chemiluminescence intensity was observed, and the resultant highly emissive Schaap's dioxetane works well in aqueous, physiologically relevant conditions (137). Along these lines, chemiluminescent imaging agents (**Figure 5*b*** and ***c***) that are responsive to singlet oxygen (138), hydrogen peroxide (139), peroxynitrite (140), hydrogen sulfide (141), and enzymes such as β-galactosidase (142), nitroreductase (143) and cathepsin B (144), have been developed. In particular, it is now possible to shift the emission of these chemiluminescent probes to the NIR range (139) (**Figure 5*d***), suggesting a bright future for chemiluminescent imaging in vivo.

5. SUMMARY AND PERSPECTIVES

A large collection of luciferins and luciferases is now available for diverse applications from in vitro to in vivo. At the cellular level, it is now possible to use multiple orthogonal bioluminescent reporters to track multiple targets. The recent development of highly bright luciferases such as NanoLuc and teLuc also opened the door for cellular and subcellular bioluminescence microscopy. At the organism level, BLI has been one of the best options for in vivo imaging. The FLuc-D-luciferin pair has already been broadly used, and the recently developed AkaLuc-AkaLumine and Antareas2-DTZ pairs are new benchmarks for in vivo BLI.

CTZ-consuming bioluminescent reporters have several intrinsic advantages, such as small size, high turnover, high brightness, high stability, and no need for cofactors such as ATP. For live-cell and animal imaging, ATP-dependent luciferases may consume a significant portion of intracellular ATP and disturb normal physiology. Moreover, in blood plasma, urine, and other biological fluids where the ATP level is low, ATP-dependent luciferases would not emit signals. On the other hand,

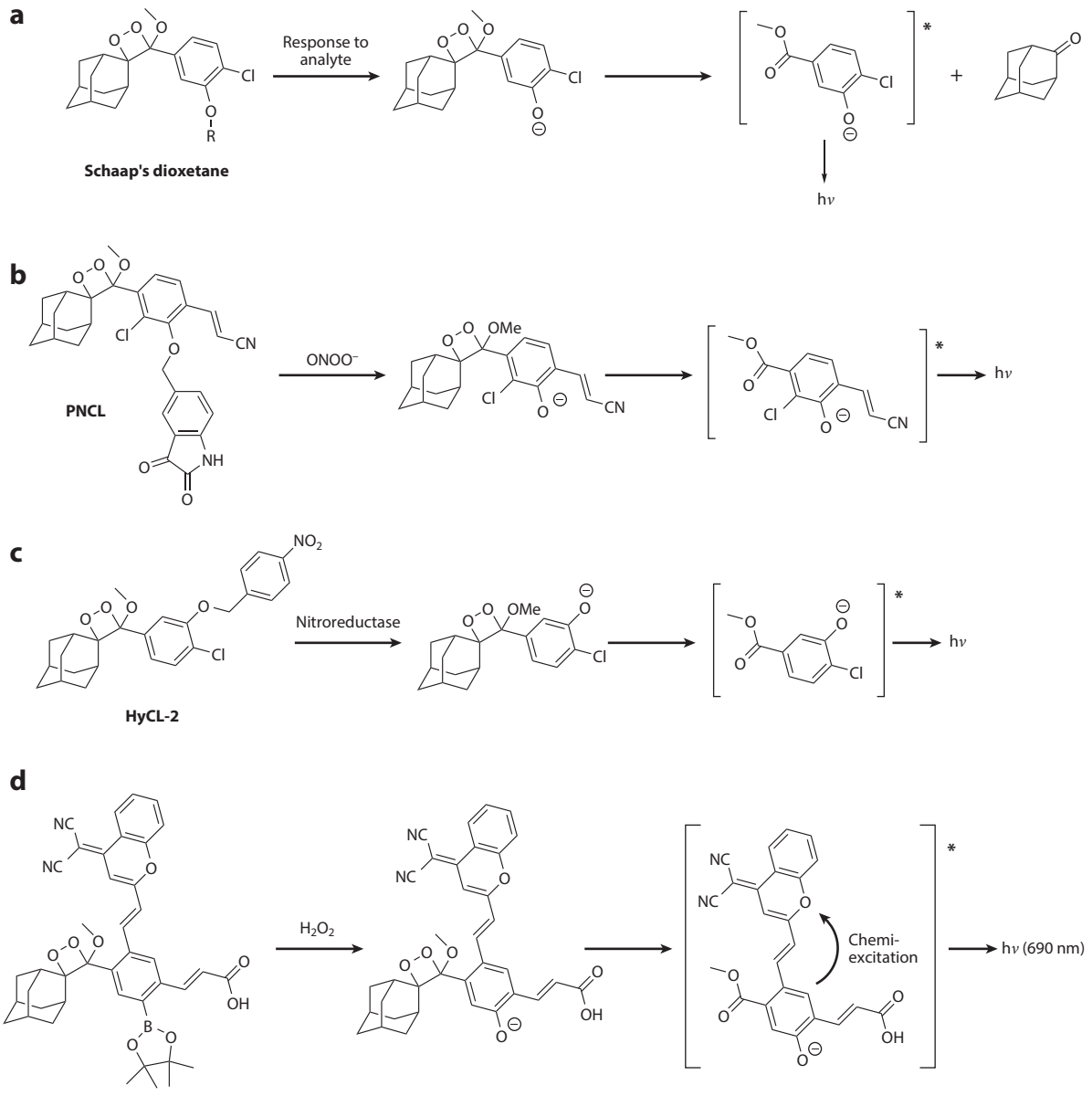


Figure 5

Representative chemical structures and mechanisms for chemiluminescent biosensors. (*a*) Schaap's dioxetane and a general mechanism for its derived chemiluminescent biosensors. (*b*) PNCL, a chemiluminescent biosensor for peroxynitrite ($ONOO^-$). (*c*) HyCL-2, a chemiluminescent biosensor for nitroreductase. (*d*) A chemiluminescent biosensor for hydrogen peroxide (H_2O_2) with near-infrared emission.

CTZ-consuming bioluminescent reporters still have serious limitations. First, the peak emission of these reporters has not yet reached the far-red and NIR spectral region. Also, the biodistribution of CTZ seems to depend on the route of injection (e.g., intravenous or intraperitoneal) (145). Moreover, the slow auto-oxidation of CTZ analogs in biological fluid (146) may limit their use in

detection of very low-abundance targets. We expect that future studies will provide novel CTZ analogs that have better solubility, bioavailability, stability, and drastically red-shifted emission. Reengineering of luciferases for these new substrates may lead to novel luciferase–luciferin pairs that further enhance in vivo BLI sensitivity by a few orders of magnitude.

The current BLI toolbox has limited options for multicolor in vivo BLI because short-wavelength photons are essentially scattered and absorbed by tissues. By combining efforts on engineering various luciferase–luciferin pairs, we imagine that two orthogonal luciferase–luciferin pairs with emission at the far-red and NIR spectral range (e.g., ~650 nm and ~750 nm) may be developed to achieve simultaneous, multicolor in vivo BLI.

The applications of current bioluminescent biosensors are mostly limited to in vitro or in live cells. For in vivo BLI, a large response dynamic range is required. Moreover, further red-shifted emission is highly preferred. We expect that biosensors based on far-red and NIR bioluminescent reporters would perform much better in vivo, and more work in this direction is warranted.

The availability of various bioluminescent reporters and their derived biosensors has greatly facilitated our understanding of biology. It is expected that more reporters and biosensors will become available to facilitate biological discoveries. Moreover, with the assistance of gene-editing techniques, the applications of genetically encoded bioluminescent reporters and biosensors will quickly expand by developing bioluminescent cell lines and transgenic animals to fit diverse applications.

DISCLOSURE STATEMENT

H.W.A. and H.W.Y. are coinventors of a patent filed by the Regents of the University of California, titled "Red-Shifted Luciferase-Luciferin Pairs for Enhanced Bioluminescence."

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