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Review Article (Invited)

Current advances in the development of bioluminescent probes toward spatiotemporal trans-scale imaging

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Bioluminescence imaging has recently attracted great attention as a highly sensitive and non-invasive analytical method. However, weak signal and low chemical stability of the luciferin are conventional drawbacks of bioluminescence imaging. In this review article, we describe the recent progress on the development and applications of bioluminescent probes for overcoming the aforementioned limitations, thereby enabling spatiotemporal trans-scale imaging. The detailed molecular design for manipulation of their luminescent properties and functions enabled a variety of applications, including *in vivo* deep tissue imaging, long-term imaging, and chemical sensor.

Key words: bioluminescence, *in vivo* imaging, long-term imaging, biochemical probe

Significance

This review discusses how traditional bioluminescent systems can be modified to impart desired properties and functions for bioimaging, such as longer emission wavelengths, chemical stability, and target responsiveness, and what applications can be realized with the developed probes.

Introduction

Bioluminescence is the luminescence produced from the oxidation reaction of the substrate luciferin catalyzed by the enzyme luciferase (luciferin–luciferase reaction: L-L reaction) [1]. Bioluminescence is characterized by its high luminescence quantum yield and extremely low heat generation. In recent years, bioimaging using bioluminescence has become widely used along with fluorescence imaging in various applications such as gene expression in cells [2], protein–protein interactions [3,4], tracking cancer cells and immune cells *in vivo* [5,6], and viral infections [7]. Bioluminescence imaging does not require any external light source, thus avoiding autofluorescence and high signal-to-noise (S/N) ratio. The lack of damage to the organism by the excitation light makes this technique useful for light-sensitive cells or for long-term imaging [8,9]. While bioluminescence imaging offers high-sensitivity analysis, it has some drawbacks including the signal intensity dependence on concentration and a weaker signal compared to fluorescence. Nevertheless, recent improvements in detector sensitivity and luciferases are overcoming these limitations [10–12]. For example, a bioluminescence resonance energy transfer (BRET) mechanism contributes to increasing the sensitivity and enables

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quantitative analysis in bioluminescence imaging [13]. BRET is an energy transfer from a bioluminescent substrate (donor) to a fluorescent substance (acceptor) when both are in the vicinity of each other. By using an acceptor with a higher quantum yield than the donor and enhancing BRET efficiency, it is possible to enhance luminescence efficiency while simultaneously achieving longer wavelengths [14,15]. The BRET-type sensor protein enables quantitative analysis by measuring the ratio of the intensity of the donor's emission to that of the acceptor's fluorescence [16–18]. While the BRET-based approach improves the luminescence properties by combining the original luciferase with a fluorescent protein, another approach involves modifying the luciferase or the luciferin itself. NanoLuc (NLuc) is an artificial luciferase developed by Hall and co-workers in 2012. They modified *Oplophorus* luciferase (OLuc) to produce more stable and brighter luminescence. NLuc paired with its synthetic substrate furimazine (FMZ) emits light about 150 times more intensely than *Renilla* luciferase (RLuc)/coelenterazine (CTZ) system. In addition to the high stability against pH and heat, the molecular weight of NLuc (19 kDa) is much smaller than that of RLuc (36 kDa) and Firefly luciferase (FLuc) (61 kDa), leading to various biological applications (Figure 1) [19,20]. With these advancements, the applications of bioluminescence to *in vitro*/*vivo* bioassays are becoming more and more widespread. However, luciferin must be externally provided, and it has not been able to achieve long-term imaging due to the bottleneck of its short half-life in blood and substrate instability. Autonomous bioluminescent mammalian cells [21] and plants [22] have been developed using bacterial and fungal bioluminescence systems, respectively. However, their application to bioluminescence imaging is limited. Moreover, there is a limited variety of luciferins emitting in near-infrared (NIR) region, known as the "bio-optical window" ranging from 650 to 900 nm. Few have demonstrated significant results in live cells or under biological conditions. These issues restricted the sensitivity and spatiotemporal resolution for both *in vitro* and *in vivo* imaging. This review introduces bioluminescent probes for *in vitro*/*vivo* imaging, including those developed by our group.

Bioluminescence Systems for *in vivo* Imaging

While bioluminescence imaging is characterized high S/N ratio and non-invasiveness, the short emission wavelength of bioluminescent systems poses a challenge for *in vivo* applications. NIR region is desirable for imaging because of its high tissue permeability and low tissue damage. Unfortunately, the emission wavelengths of the popular bioluminescence systems, FLuc/D-luciferin (D-Luc), RLuc/CTZ, and NLuc/FMZ are 560–620 nm, 480 nm, and 460 nm, respectively (Figure 1). Brighter and red-shifted bioluminescence is necessary for observing deep tissues and achieving higher sensitivity [23]. This chapter describes the studies on the application of these bioluminescence systems for *in vivo* imaging, primarily by lengthening the luminescence wavelength.

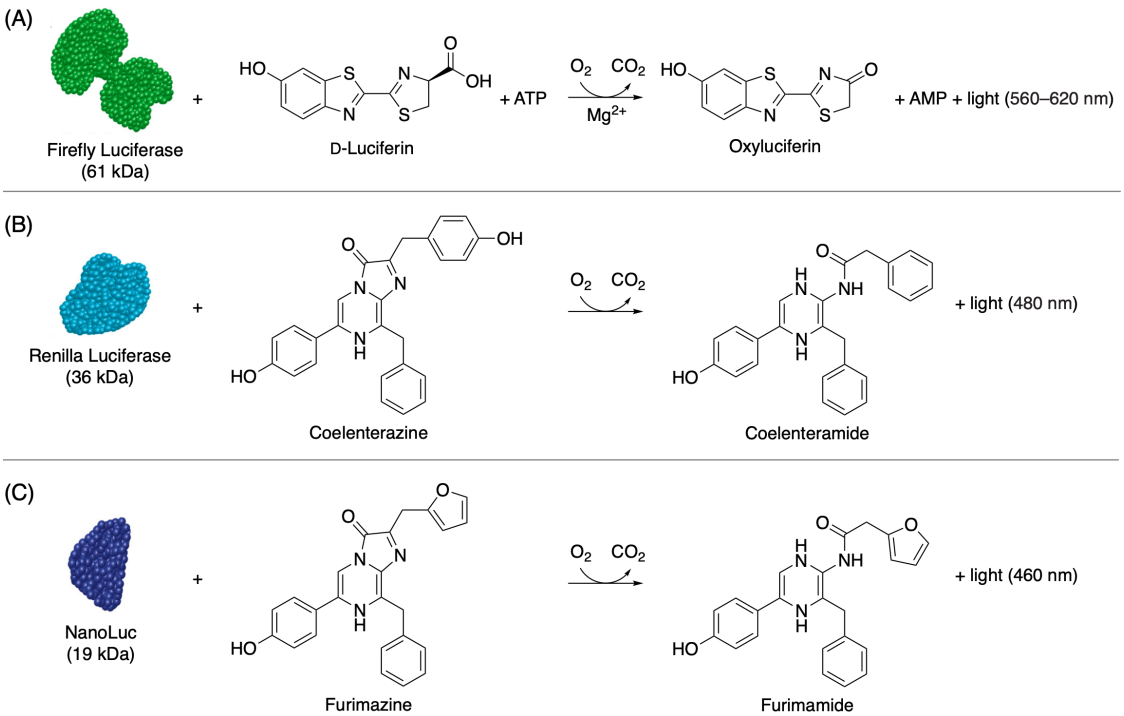

Figure 1 (A) FLuc/D-Luc, (B) RLuc/CTZ, and (C) NLuc/FMZ bioluminescence. Reproduced with permission from England, C. G., Ehlerding, E. B., Cai, W. Bioconjug. Chem. 27, 1175–1187 (2016) [19]. Copyright 2016 American Chemical Society.

FLuc/D-Luc system is often used in bioimaging due to its high quantum yield and the stability of D-Luc [24]. The substrate tolerance of FLuc has allowed structural modifications for longer emission wavelength [25]. Nagano and co-workers demonstrated that NIR luminescence can be achieved by BRET from the substrate D-aminoluciferin to the NIR dye combined via a linker [26]. D-Luc derivatives with emission wavelength above 700 nm have been synthesized by elongating the π-conjugated system, but their emissions were weaker than the native D-Luc in *in vivo* imaging [27,28]. Maki and co-workers developed the substrate AkaLumine, and Miyawaki's group produced its optimized enzyme Akaluc. AkaLumine possesses an (*p*-aminophenyl)butadiene moiety instead of the benzothiazole ring of D-Luc. Akaluc/AkaLumine system (AkaBLI) glows at NIR region (650–677 nm), and AkaLumine showed good tissue permeability. These features have led to successful single-cell imaging in the mouse body using AkaBLI [29]. Our group embarked on the development of substrates with further longer emission wavelength by modifying the structure of the AkaLumine [30]. Six NIRLucs with cyclic amine moieties, replacing dimethylamino group, were synthesized, applying the structural modification method for rhodamine dyes (Figure 2A) [31]. Each NIRLuc was recognized by FLuc and showed emission at 680–690 nm, which is longer than that of AkaLumine, presumably due to the increased electron-donating ability of the dialkylamino group resulting from the inhibition of C–N bond rotation (Figure 2B). Among them, NIRLuc2 showed longer half-life in blood (17.8 min) than AkaLumine (7.9 min). Moreover, its photon flux was approximately 7-fold (16-fold with an NIR filter) compared to D-Luc in mice bearing subcutaneous tumor, and its luminescent activity was equivalent to that of AkaLumine (Figure 2C). We also investigated the changes in luminescence properties of the four luciferin scaffolds when replacing the dimethylamino group with an azetidine moiety [32]. It has been known that azetidine substitution of a fluorescent dye improves its fluorescence quantum yield. Azetidine-substituted D-Luc-type substrate displayed 2.3-fold increase in the fluorescence quantum yield in buffer compared to the corresponding dimethylamino analog. This substrate showed enhanced bioluminescence properties while maintaining enzyme affinity and cell membrane permeability. This improvement is attributed to the suppression of the twisted intramolecular charge transfer (TICT) state formation as reported for fluorescent dyes. On the other hand, azetidine substituion of AkaLumine-type luciferin did not improve the fluorescence quantum yield and resulted in a decrease in bioluminescence intensity. Thus, it was quantitatively evaluated that the effect of azetidine substitution to fluorescence and bioluminescence properties varies depending on the luciferin scaffold. Increasing the fluorescence quantum yield of luciferin was shown to be an effective strategy for improving its luminescence properties.

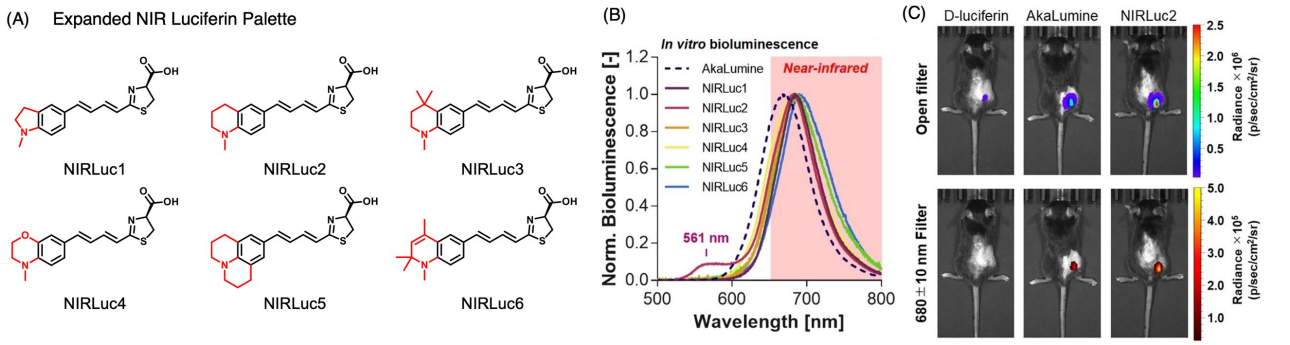

Figure 2 (A) The molecular designes of NIRLucs based on the AkaLumine scaffold. (B) Bioluminescence emission spectra of Akalumine and the NIRLucs with native FLuc. (C) Bioluminescence images of C67BL/6j mice bearing subcutaneous tumors of B16-F10-FLuc cells with open filter (upper) of NIR (680 ± 10 nm) filter (bottom) recorded 15 min after intravenous injection of luciferins. Reproduced with permission from Ikeda, Y., Nomoto, T., Hiruta, Y., Nishiyama, N., Citterio, D. Anal. Chem. 92, 4235–4243 (2020) [30]. Copyright 2020 American Chemical Society.

Prescher's group developed chemically diverse D-Luc analogs and screened the FLuc mutant which specifically process each analog using computational algorithms [33]. Among them, two luciferase–luciferin pairs, which work orthogonally to each other, were identified. Luminescence occurs only when the luciferin contacts the complementary luciferase. Stepwise multicomponent imagings using these pairs were successfully performed in cells and in mice. Prescher and co-workers developed also NIR-emitting D-Luc analogs containing coumarin ring and the corresponding luciferins and achieved multicomponent imaging jointly using other bioluminescent systems [34,35].

Several luciferases for D-Luc other than FLuc have been found from beetles, and the luminescence wavelength varies according to the environment in the catalytic pocket of the luciferase. D-Luc paired with the luciferase of Brazilian click beetle (*Pyrearinus termitilluminans*), called Emerald luciferase (Eluc), produces bright luminescence at 538 nm. Ozawa and co-workers developed a mutant of Eluc, which exhibit a greatly red-shifted luminescence (626 nm) [36].

The RLuc/CTZ system has the advantage of not requiring cofactors, ATP and magnesium ion [37]. However, the emission wavelength is shorter than that of FLuc/D-Luc. This section focuses on lengthening the emission wavelength through luciferase modification. In 2007, Gambhir and co-workers developed RLuc8.6 series, further improved variants from the engineered enzyme RLuc8, exhibiting higher stability and luminescence brightness than native RLuc [38]. In particular, RLuc8.6-535 demonstrated a luminescence activity about six times higher than that of RLuc and a maximum emission wavelength of 535 nm, approximately 55 nm longer than the original RLuc emission system. Furthermore, wide range of emission wavelengths from 400 to 600 nm was achieved by combination with several CTZ derivatives. Maki and co-workers achieved further red-shifted emission by jointly using RLuc8.6-535 and π-extended CTZ derivatives [39]. The BRET mechanism has also been utilized for longer emission wavelengths of RLuc systems [14]. Employing eBAF-Y, a fusion of RLuc8.6 and fluorescent protein EYFP, the emission wavelength was 525 nm through BRET from RLuc8.6 to EYFP. Nagai and co-workers fabricated Nano-lantern series consisting of modified RLucs and fluorescent proteins, mTurquoise2, mVenus, and mKusabiraOrange2, and achieved wavelength extensions to 470, 530, and 560 nm, respectively [40,41]. BRET-type fusion proteins often provide more brilliant signal than the original luminescent system due to efficient energy transfer and high quantum yields. Thus, they are useful not only for wavelength shift but also for signal enhancement. Kim and co-workers aimed to develop a novel system that produces bright NIR luminescence by optimizing both substrate and enzyme. A CTZ derivative BBlue2.3 strongly glowed at around 400 nm, which has a large overlap with the excitation spectrum of NIR fluorescent protein iRFP [42]. The linker sequence between RLuc8.6-535SG and iRFP was optimized for efficient BRET. The combination of BBlue2.3 and iRFP-RLuc8.6-535SG produced approximately 1.7 times brighter signal in NIR region in cell compared to when using CTZ. This system was applied to the imagings of model mice bearing subcutaneous or lung cancer. Compared to the RLuc8.6-535SG without BRET, iRFP-RLuc8.6-535SG could detect tumors with 3-fold greater sensitivity, indicating its usefulness for deep tissue imaging.

As mentioned above, the NLuc/FMZ system has the features of stronger luminescence and smaller molecular weight of the enzyme NLuc than the RLuc/CTZ system. Nagai and co-workers developed fused NLuc with various fluorescent proteins, called enhanced Nano-lanterns, and performed multicolor imaging of cellular organelles in the 475–585 nm wavelength range using them [43]. By fusing NLuc with the orange fluorescent protein CyOFP, a bright bioluminescent reporter Antares was developed [44]. However, the low solubility and bioavailability of FMZ have limited the luminescence brightness of Antares for *in vivo* imaging. Kirkland and Lin developed more water-soluble FMZ derivative, hydrofurimazine (HFz), which enables higher volume dosing to mice [45]. Antares/HFz system gave the similar level of luminescence to Akaluc/AkaLumine in mouse liver. They next focused on fluorine substitution for further improvement of *in vivo* performance of the substrates. The difluorinated analog of HFz bearing amino group (fluorofurimazine: FFz) exhibited even higher intensity of light both *in vitro* and *in vivo*. They succeeded in dual visualization of tumor size and CAR-T cells within the same mice parallel using Antares/FFz and Akaluc/AkaLumine systems. The same group subsequently found the difluorinated FMZ analog with improved performance for the brain imaging, cephalofurimazine (CFz) [46]. Only a few examples of bioluminescence imagings in the central nervous system have ever been reported, because of the insufficient blood–brain barrier (BBB) permeability of the conventional luciferase substrates. CFz with Antares compared favorably with Akaluc/AkaLumine in the brightness in the brain imaging of transgenic mouse. The applications of this system to non-invasive *in vivo* imaging of mice were also achieved. In addition to the approach using Antares, the development of NLuc mutant has also been investigated for longer emission wavelength. Ai and co-workers reported an engineered NLuc variant, QLuc, and its substrate QTZ, a CTZ analog with a quinoline ring at C-8 position [47]. The emission peak of QLuc/QTZ system stood at 585 nm, about 130 nm of bathochromic shift compared to NLuc/FMZ. This system was applied to deep-tissue imaging of mice liver and *in vivo* immunobioluminescence imaging using the fusion of single-chain variable antibody fragment (scFv) and QLuc.

Bioluminescent Probes for Long-term Imaging

Based on a certain substrate specificity of luciferase, the introduction of a protective group to the corresponding substrate should inhibit the enzymatic oxidation reaction owing to the change in molecular structure or reduced reactivity with oxygen, thus preventing luminescence. When this protective group (“cage”) is removed, the luminescence reaction occurs by returning to the original substrate. Such a protected bioluminescent substrate is called “caged luciferin” and has been made use of assays and imaging [48–50].

The applications of caged luciferins to improve luminescence persistence and chemical stability are presented in this section. Imidazopyrazinone-type luciferins such as CTZ and FMZ are prone to undergo oxidative degradation in aqueous solution or serum even in the absence of enzyme, which leads autoluminescence causing decrease of S/N ratio [51–53]. Moreover, the rapid L-L reaction of RLuc/CTZ and related bioluminescent system results in the quick decay of luminescence and makes their application to long-term imaging difficult. Therefore, caged CTZ-type luciferins, whose C-3 carbonyl oxygen (reactive site) was masked, have been developed for the suppression of autoluminescence and longer luminescence duration [54,55]. Gambnir and co-workers protected the bisdeoxy analog of CTZ (diphenylterazine: DTZ)

as ester or acyloxymethyl ether, which is cleaved by intracellular hydrolases [56]. Luminescence measurements in GFP-RLuc8-expressing cells showed that the luminescence behavior varied depending on the protecting group. The signal half-life of DTZ is about 5 min, whereas caged DTZ with the bulky protecting group is more than 1 h. Li and co-workers screened various protective groups for 8-thio derivative of DTZ [57]. Three of the tested substrates sustained luminescence in cells for over 24 h, in contrast to luminescence of the original 8-thio-DTZ decreased by 80% in 10 min. In addition, one of these compounds has been successfully used for long-term *in vivo* imaging. Ai and co-workers designed and synthesized a caged DTZ possessing a negatively charged carboxylate moiety in the protecting group (ETZ) for improving hydrophilicity [58]. They also prepared a similar caged DTZ bearing dimethylamino group (C3-DMA-DTZ), which has positive charge at physiological pH. When applied to *in vivo* imaging of mice brain, the behavior of C3-DMA-DTZ was much worse than DTZ despite having caged moiety. Contrary to that, ETZ showed good blood retention avoiding efflux by BBB and persistent bioluminescence, and its total luminescence signal during the experiment was approximately 4-fold higher than DTZ. On the other hand, it was difficult to ensure both luminescence duration and brightness, and no single-cell imaging has been achieved using caged CTZ derivatives. Hence, we applied the caging strategy to NLuc/FMZ with high emission intensity [59]. C-3 carbonyl of FMZ was protected with three acyl groups and *tert*-butoxycarbonyl (Boc) group (Figure 3A). All caged FMZs exhibited higher stability in ethanol than FMZ. Acyl-protected FMZ was more stable in buffer solution, as the protecting group became bulkier. In contrast, Boc-FMZ was found to be quickly deprotected by water. The rate of deprotection can be adjusted by the protecting group, thus allowing the substrate to be selected according to the purpose of imaging. In cells, Boc-FMZ and Pivaloyl-FMZ continued to emit light for 4- and 6-fold longer than FMZ, respectively. They were utilized for single-cell imaging in microscopy and allowed long time observations with high S/N ratio (Figure 3B, C). We further expanded the caged FMZ derivatives by introducing much bulkier acyl group, 1-adamantanecarbonyl group, at the C-3 carbonyl position of FMZ and FMZ-OH (hydrofurimazine). Ad-FMZ and Ad-FMZ-OH (Figure 4A) surpassed the signal duration of the live-cell imaging at the single-cell level. Ozawa and co-workers previously developed the probe for quantitative evaluation of myogenesis, which is consist of split FLuc fragments combined with split DnaE inteins [60]. Cell fusion during myogenesis results bioluminescence in the cytosol due to the reconstitution of FLuc. This probe was applied to the screening myogenesis-promoting compounds. Based on its probe design, the split NanoKAZ-based bioluminescence assay system was constructed. By combining Ad-FMZ-OH and the assay system, the continuous monitoring of myocyte fusion, which is one of the actual cellular events, for more than 24 h with single-cell resolution was achieved (Figure 4B) [61]. As described above, caging technology contributes to expand the possibilities of imaging applications of bioluminescent systems, especially those based on CTZ-type substrates, by altering the chemical properties and luminescence behavior of original luciferins.

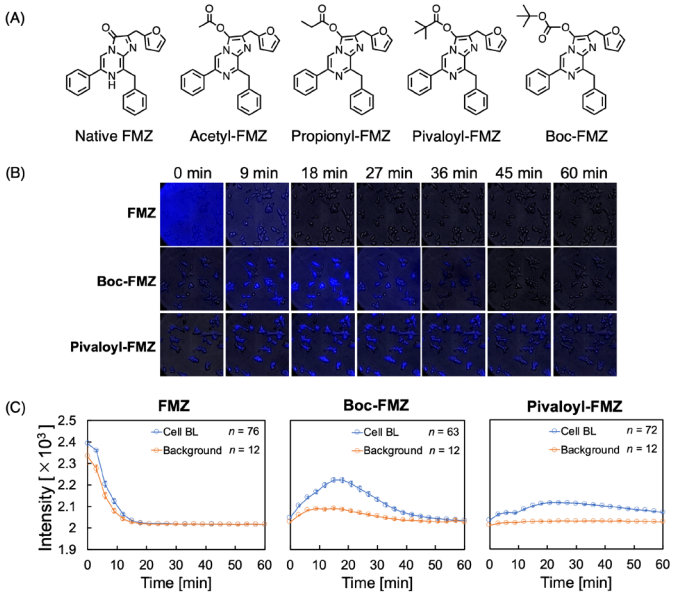

Figure 3 (A) Molecular structures of caged FMZ analogues. (B) Single cell bioluminescence imaging with FMZ and its caged analogues in HEK293T cells stably expressing NLuc. (c) Time-dependent bioluminescence intensity extracted from single cell imaging data. Cell bioluminescence and background represent the average bioluminescence intensities inside and outside of cells, respectively. Error bars represent means ± SEM. Reproduced with permission from Mizui, Y., Eguchi, M., Tanaka, M., Ikeda, Y., Yoshimura, H., Ozawa, T., et al. Org. Biomol. Chem. 19, 579–586 (2021) [59]. Copyright 2021 Royal Society of Chemistry.

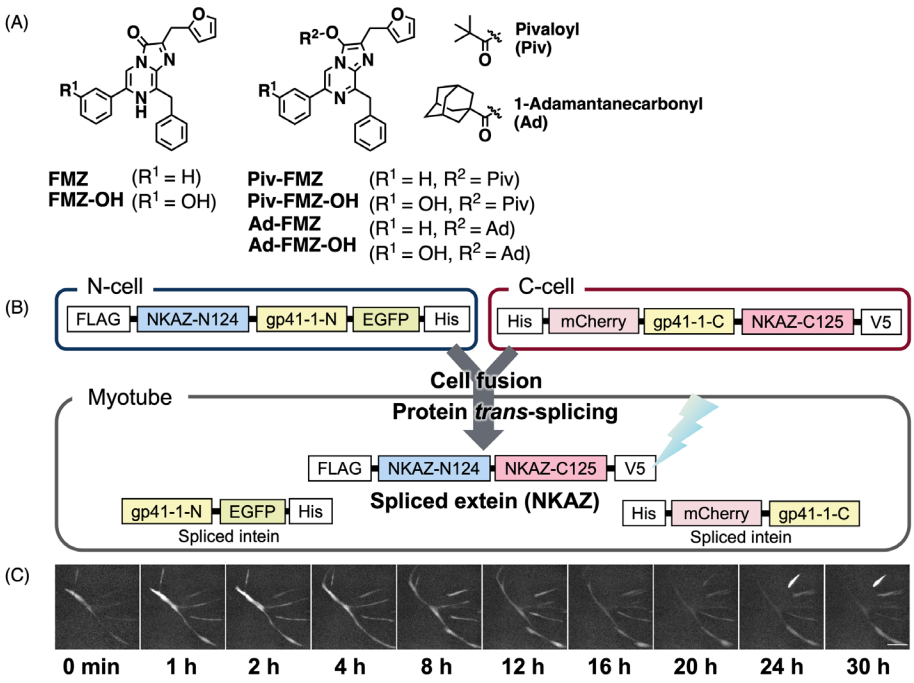

Figure 4 (A) Chemical structures of the caged FMZ derivatives. (B) Schematic image of the expression vectors for N- and C-probes and the mechanism of split NanoKAZ-based cell fusion assay. (C) Bioluminescence imaging of cell fusion of N- and C-cells after Ad-FMZ-OH injection. Reproduced with permission from Orioka, M., Eguchi, M., Mizui, Y., Ikeda, Y., Sakama, A., Li, Q., et al. Bioconjug. Chem. 33, 496–504 (2022) [61]. Copyright 2022 American Chemical Society.

Bioluminescent Probes for Specific Target

This section describes the uses of bioluminescent systems as probes to detect or quantify the chemical species of interest. First, structurally modified luciferins with sensor function are introduced. A type of such bioluminescent probes is caged luciferin with a protecting group that is removed by specific response to the target. Only in the presence of the target, the original luciferin is released, and light emission occurs. Such probes make it possible that the observe and quantify the target based on luminescence signal. Bioluminescent probes for various biological materials have been reported, including hydrogen peroxide (H_2O_2) [62], enzymes [63,64], copper ion [65], and iron ion [66].

Our group developed bioluminescent probes targeting biothiols, such as cysteine and glutathione. Simple and sensitive method for detection of biothiols has been required because they are related to serious diseases including diabetes [67]. Caged D-Luc probe "SEluc-1" was designed and synthesized by introducing a thiol-labile sulfinate moiety via a self-immolative linker at the phenolic hydroxy group [68]. This probe emitted light quantitatively reflecting intracellular thiol concentration in FLuc-expressing COS-7 cells. When oxidative stress was applied by addition of H_2O_2 or incubation in glucose-free medium, a decrease in bioluminescence intensity linked to that in the intracellular concentration of thiols was observed. Our group next worked on the development of a CTZ-type probe for *in vivo* detection of biothiols, aiming at combined use with BRET-type fusion protein [69]. Methoxy-CTZ-Methoxy (MCM) as the parent luciferin and acryloyl group as thiol-reactive site were employed. The synthesized probe AMCM detected biothiols with high sensitivity like SEluc-1. The combination of AMCM with iRFP713-RLuc8.6-535 realized NIR luminescence and deep-tissue imaging of mice.

Caging D-Luc-derived substrates by esterification of their carboxy group with conventional self-immolative benzyl linker was difficult because the resulting ester is readily hydrolyzed. Chan and co-workers invented α-isopropylbenzyl (Ph^{i}Pr) ester that resists hydrolysis under acidic/basic conditions or by esterase [70]. They developed a nitroreductase-responsive NIR probe by masking NIR luciferin BL660 with *p*-nitro variant of Ph^{i}Pr group.

As shown above, caged luciferin-based probes are useful for the observation of target substances in cells and *in vivo*. In addition, probes for the detecting specific enzyme were reported using complexes of D-Luc encapsulated in the polymer micelle, which cleaves in response to the target enzyme [71,72]. Such non-covalent cage methods are expected to simplify probe design and enable long-term observation.

Next, imparting sensor functions to a luciferase is described. NLuc is often employed in BRET-type sensors in combination with fluorescent proteins, quantum dots, and fluorescent dyes because its high brightness and small size favor efficient BRET [73–76]. LUMABS (LUMinescent AntiBody Sensor) is a ratiometric sensor protein that incorporates epitopes (antibody recognition sites) and helper domains into a BRET pair consisting of NLuc and fluorescent protein mNeonGreen [77]. The luminescence color of LUMABS changes as BRET is turned off and on in the presence and absence of antibody, respectively. LUMABS has applied to point-of-care testing (POCT)-oriented analytical devices for measurement of antibody levels in whole blood [78–80]. The signal detection with a digital camera or more user-friendly smartphone camera was possible. Nagai and co-workers developed bioluminescent indicators for bilirubin [81] and thrombin [82] based on NLuc and created diagnostic tools that use a smartphone camera. There are also examples of fusion protein probes other than BRET sensors. Ozawa's group recently reported an RNA probe using split NLuc [83]. This probe is composed of two split NLuc fragments each bound to an RNA-binding domain. Upon binding to the target RNA, the fragments approach each other and reconstitute NLuc, resulting in luminescence (Figure 5A). The intracellular distribution of endogenous β-actin mRNA was spatiotemporally visualized with this probe (Figure 5B). Furthermore, the detailed localization of β-actin mRNA in primary hippocampal neurons was also observed. As described above, the functionalization of luciferase by the combination of other proteins enables the creation of probes that cannot be achieved by modification of small-molecule substrates, thereby expanding the possibilities of bioluminescent systems.

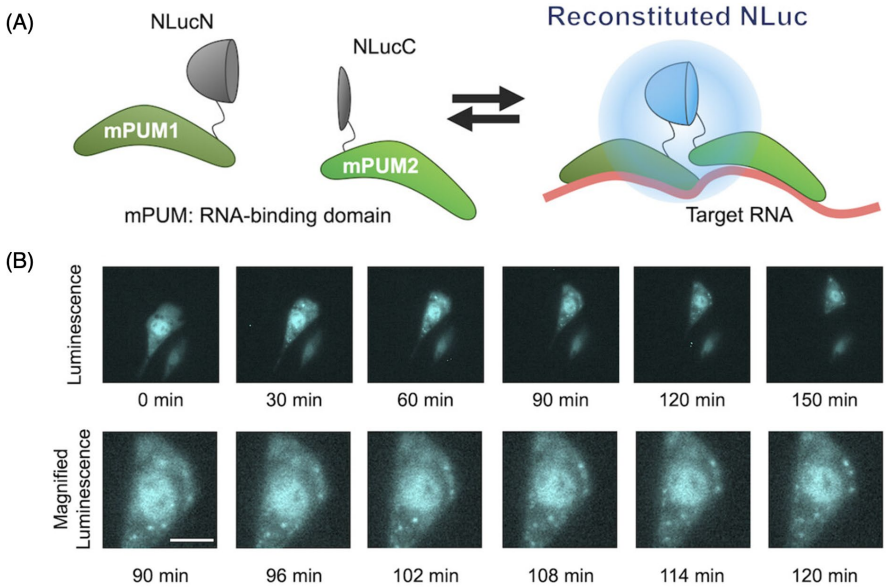

Figure 5 (A) Schematic of the mechanism for visualizing a target RNA with an NLuc luciferase-based RNA probe. (B) Single cell time-lapse imaging of the probe by luminescence microscopy. Luminescence images of the NIH3T3 cell expressing the probes. Reproduced with permission from Eguchi, M., Yoshimura, H., Ueda, Y., Ozawa, T. ACS Sens. 8, 4055–4063 (2023) [83]. Copyright 2023 American Chemical Society.

Toward Further Applications of Bioluminescent Probes

The basic properties of bioluminescent systems have so far been evaluated exclusively using systems in which only the major components (luciferin, luciferase, and cofactor) are present. The influence of other molecules on the L-L reaction has not been well considered. The condensate which is formed by liquid–liquid phase separation (LLPS) of biomolecules, called "membrane-less organelle" (MLO), has been recently proposed to be a spatiotemporally controlled site for enzyme reactions in cells. Nishihara and co-workers investigated in detail how high-density condition of biomolecules affects the bioluminescence of FLuc/D-Luc using peptide-based coacervates mimicking MLO [84]. The results suggested the mechanisms by which the coacervate promotes the L-L reaction. It was demonstrated that coacervate increases the bioluminescence quantum yield of the FLuc/D-Luc system by more than 10%. Elucidation and control of the intracellular regulatory mechanisms of the L-L reaction by biomolecule condensates will contribute to the creation of bioluminescent systems with high-intensity luminescence.

In general, luciferin generates luminescence in the presence of luciferase. Nevertheless, there are known luciferins, such as CTZ, which also undergo luminescence reaction catalyzed by non-luminescent proteins like serum albumin. Hence, it was suggested that luciferin alone could be used to detect a certain biological protein. Nishihara and co-workers recently

developed a luciferin, named HuLumino**1**, which emits light specifically in the presence of human serum albumin (HSA) through structural optimization of CTZ derivatives [85]. Kim and co-workers designed a CTZ analog to selectively responds to HSA and bovine serum albumin (BSA) and found that the emission wavelength changes with the type of serum albumin [86]. These luciferins have enabled specific and quantitative detection of HSA and BSA without luciferin expression. Furthermore, Nishihara and Kurita synthesized caged HuLumino**1** equipping the protecting group which is selectively cleaved by copper (I) ion. This caged luciferin was applied to luciferase-independent measurement of copper ion level in human serum [87]. While revising the current Review Article, the selective pseudo-luciferase activity of the SARS-CoV-2 spike protein for *Cypyidina* luciferin was reported, which have the potential to be used for the quantitative analysis of SARS-CoV-2 [88]. Such a luciferase-free bioluminescent system realizes rapid and simple analysis, not requiring any sample pretreatment and complicated biochemical protocols for luciferase expression.

The emission range of most conventional fluorescent and bioluminescent probes was in the visible to NIR region (400–900 nm), but in recent years, the use of the second NIR (NIR-II: 1000–1700 nm) region with higher biopermeability has been advancing rapidly, especially for fluorescence imaging [89]. This wavelength range enables high-resolution imaging of deeper tissues. Also for bioluminescence imaging, NIR-II region is becoming available through the use of BRET and multistep fluorescence resonance energy transfer (FRET) [90]. Although new bioluminescent probes are still being developed, small molecule probes, which have been the main focus of this review, are considered to have limitations for bioimaging [91]. For example, their short half-lives in blood due to hepatic metabolism and renal excretion hinder their applications for long-term imaging in mice. Furthermore, it remains challenging to selectively deliver a sufficient amount of probe to the site to be visualized. Although there is a high demand for brain imaging probes, BBB-permeable structures are limited. It is possible to overcome these problems by introducing a new functional moiety, however it makes the probe preparation complicated and may impart luminescent properties. Further applications of bioluminescence imaging are anticipated by a technique to overcome these problems, for example, the establishment of a delivery system for luciferin into the site to visualize.

Conclusion

In this review, we described the design, development, and application of bioluminescent probes, mainly taking our research as examples. The molecular designs of luciferin and caged luciferin to meet the need for imaging tools were mainly addressed. High-resolution bioluminescence imaging of deep tissue becomes possible by designing the luciferin molecule and the BRET/FRET system so that the emission wavelength reaches the NIR region. Not only the modification of luciferin alone, but also the functionalization of luciferase by fusion with other proteins can enhance the utility of bioluminescent systems. Luciferin-free bioluminescent systems have also been developed. These recent progresses are expanding the scope of applications of bioluminescence, such as the continuous imaging of life phenomenon at the single-cell level over a day, the video-rate non-invasive imaging in freely moving mouse, and the rapid and convenient assay system for POCT, especially for biomacromolecules, which could not be detected by the conventional small molecule-based probe. By exploring new methodologies beyond the mere development of luciferase and luciferin pairs, the potential of bioluminescence chemistry is expected to broaden even further.

Conflict of Interest

There are no conflicts to declare.

Author Contributions

Akihiro Sakama: writing – original draft. Mariko Orioka: writing – original draft. Yuki Hiruta: writing – review & editing.

Data Availability

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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