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Near-Infrared Luciferin Analogs for *In Vivo* Optical Imaging

Ryohei Saito-Moriya, Rika Obata and Shojiro A. Maki

Abstract

The firefly bioluminescence reaction has been exploited for *in vivo* optical imaging in life sciences. To develop highly sensitive bioluminescence imaging technology, many researchers have synthesized luciferin analogs and luciferase mutants. This chapter first discusses synthetic luciferin analogs and their structure–activity relationships at the luminescence wavelength of the firefly bioluminescence reaction. We then discuss the development of luciferin analogs that produce near-infrared (NIR) light. Since NIR light is highly permeable for biological tissues, NIR luciferin analogs might sensitively detect signals from deep biological tissues such as the brain and lungs. Finally, we introduce two NIR luciferin analogs (TokeOni and seMpai) and a newly developed bioluminescence imaging system (AkaBLI). TokeOni can detect single-cell signals in mouse tissue and luminescence signals from marmoset brain, whereas seMpai can detect breast cancer micro-metastasis. Both reagents are valid for *in vivo* bioluminescence imaging with high sensitivity.

Keywords: Firefly bioluminescence, Bioluminescence imaging, Structure–activity relationships, Multicolor, Near-infrared light

1. Introduction

In Japan, watching the light of fireflies has been a summer tradition for over one thousand years. Modern fireflies are known to glow yellow-green, but in ancient times they emitted a dark green luminescence, as confirmed by recent molecular biology techniques [1]. The detailed mechanism of firefly bioluminescence is described in previous chapters. This chapter focuses on synthetic substrates of firefly luciferase, which are employed in firefly bioluminescence imaging (BLI).

In recent biological research, BLI technology has observed biological events *in vivo* [2–8]. For example, in cancer research, BLI has been applied to real-time monitoring of gene expression, cell numbers, and other biological events in transgenic mouse models [9–16]. Our group has developed firefly substrate analogs for use in these research fields.

The firefly bioluminescence reaction proceeds via the oxidation of *D*-luciferin (1, LH2, **Figure 1**) catalyzed by firefly luciferase (Fluc) in the presence of adenosine triphosphate (ATP), Mg^{2+} and O_2 by a two-step reaction. In the first step, LH2 is adenylated with ATP, and is then oxidized by O_2 , forming excited-state oxyluciferin that relaxes to the ground state with yellow-green light emission ($\lambda_{max} = 560$ nm) [17–19]. However, yellow-green light is not able to easily penetrate biological tissues [20], and is useful only for imaging shallow tissues such as subcutaneous tissues. To detect signals from deep tissues such as brain and lung [21], near-infrared (NIR)

Figure 1.

Structures of D-luciferin (**1**, LH2) and aminoluciferin (**2**).

light should be used, as it is highly permeable to biological tissues [20] and is suitable for *in vivo* deep tissue imaging [21]. Recently, many synthetic luciferin analogs have been reported. Our group has synthesized various luciferin analogs and compared them with **1**. By studying the structure–activity relationships of these analogs and *Photinus pyralis* (Ppy) luciferase, we have developed luciferin analogs that produce wide-spectrum light (from blue to red), along with NIR luciferin analogs (AkaLumine, TokeOni, seMpai) for BLI. Our different analogs are described in this chapter.

2. Luciferin analogs of firefly luciferase

Many researchers have synthesized luciferin analogs, and different substrates reacted with luciferases exhibit different luminescence activities [22–24]. Most luciferin analogs are formed by modifying the benzothiazole moiety of **1**. Analogs of **1** were first synthesized by White *et al.* in 1966. They showed that aminoluciferin (**2**, **Figure 1**), in which the hydroxyl group of benzothiazole is replaced with an amino group, can function as a substrate of Fluc and emit red bioluminescence [25].

2.1 Development of luciferin analogs based on LH2

As mentioned above, many luciferin analogs are prepared by modifying the benzothiazole moiety of **1** [22–24]. For instance, *N*-cycloaminoluciferins (**2a–f**, **Figure 2A**) are prepared by cyclizing the NH₂ of **2**. These analogs were reported by two independent groups, who synthesized them by different routes [26, 27] (**Figure 2B–C**). When reacted with Fluc, **2a–f** show longer wavelengths than **1**, probably reflecting the electron donation effect of cycloamine substitutes. Comparing the bioluminescence activities and emission wavelengths of analogs **2e** and **2f** on Fluc and Fluc mutant luciferase R218K, it was found that **2e**/Fluc and **2f**/R218K produced light at 604 and 614 nm, respectively, whereas **2e**/R218K and **2f**/Fluc produced no light [26]. The interaction between the active site of luciferase and the substrate is very critical, indicating that the structures of both reactants play essential controlling roles in luminescence activity.

Miller *et al.* synthesized CycLuc1 (**7a**, **Figure 3**) by fusing *N*-cycloalkylation of **2** with benzothiazole [28]. Analog **7a** exhibited a longer luminescence wavelength on Fluc (599 nm) than **1** on Fluc, and was emitted more intensely than **1** in a *Photuris pennsylvanica* firefly luciferase mutant (Ultra-Glo). The BLI of **7a** detects the signals from deep organs such as brains and lungs [21, 29]. Li *et al.* synthesized CybLuc (**7b**, **Figure 3**) by substituting the hydroxy group of **2** with a cycloamino group. Analog **7b** produced light at 603 nm and its BLI detected the signals from mouse brain [30].

Iwano *et al.* developed luciferin analogs **8a–g** (**Figure 4A**) by substituting the benzothiazole moiety of **1** with a simple benzene ring and extended π -conjugations [31]. Olefins were extended by the Wittig reaction from **10c–d** and **12e–f** as starting

Figure 2.
Structures and synthetic routes of luciferin analogs **2a–f**. (A) *N*-cycloaminoluciferin analogs **2a–f**, (B) the synthetic route reported by Miller *et al.* [28], and (C) the synthetic route reported by Hirano *et al.* [29].

Figure 3.
Structures of CycLuc1 (**7a**) and CybLuc (**7b**).

materials. In this synthesis, hydrolysis was stepwise followed by condensation with *D*-Cys(STrt)-OMe, thiazoline cyclization, and methyl ester deprotection (**Figure 4B**). The obtained analogs **8a–f** produced luminescent colors over a wide range (blue to red) [31]. Among these, AkaLumine (**8e**), which produces light at 675 nm, is a leading compound for NIR luciferin analogs, as described in Section 2.2. Later, analog **8e** was used as a reagent for BLI. In the same paper, 3-hydroxyl analog **8g** (**Figure 4A**) was also synthesized, but this analog produced no light [31]. Therefore, the position of the OH substituent is critical in the firefly bioluminescence reaction.

In contrast, the thiazoline site is rarely modified. Conley *et al.* synthesized a seleno-aminoluciferin analog **13a** (**Figure 5A**) in which the S of the thiazoline ring of **2** was replaced with Se [32], and Ioka *et al.* synthesized O- or C- substituted analogs **13b–c** (**Figure 5A**) [33]. Analog **13a**, which produced light at 600 nm, was synthesized by the cyclization reaction of selenocysteine (**Figure 5B**) [32]. Analog **13b** was obtained by synthesizing an amide **16b** synthesizing an amide from *D*-serine, cyclizing it with diethylaminosulfur trifluoride (DAST), and hydrolyzing it with Amano lipase (**Figure 5C**). Analog **13c** was prepared by coupling with bromothiazole **19** and pyrrolidione carboxylate **21** to form glutamate-linked benzothiazole **16c**, and cyclizing **16c** with trifluoroacetic acid (TFA) (**Figure 5D**). Interestingly, **13c** produces light at 547 nm, whereas **13b** is non-bioluminescence [33] but shows chemiluminescent ability. This result indicates that the thiazoline of **1** is an essential moiety for recognizing the activity site in luciferase.

A **B**

Figure 4.
Structures of **8a–g** (A) and their synthetic routes **8a–f** (B).

2.2 Structure–activity relationships for developing NIR luciferin analogs

Based on these structure–activity relationships, additional luciferin analogs have been designed and synthesized for NIR light production. For example, Anderson *et al.* synthesized iLH2 (**22**, **Figure 6**) by inserting an olefin into the structure of **1**. Analog **22** produced NIR light at 706 nm [34]. However, the luciferase used at that time was a mutant (S284T), and the luminescence wavelength on Fluc was 670 nm. The same authors developed an *in vivo* dual-imaging technique that combines **1** and **22** with two different luciferases. This system can potentially observe new biological events by tracking two processes simultaneously [35]. Hall *et al.* synthesized NH₂–NpLH2 (**23**, **Figure 6**) by extended conjugation of **2**. Analog **23** produced no light with Fluc, but its luminescence wavelength was extended to 743 nm by reaction with CBR2, a mutant luciferase of click beetles (*Pyrophorus plagiophthalmus*) [36]. All of these studies achieved long-wavelength emissions from mutant luciferases, but their luminescence activity is much lower than that of combinations of **1** and wild type *Ppy* luciferase.

Figure 5.
Structures of **13a–c** (A) and their synthetic routes **13a** (B), **13b** (C) and **13c** (D).

Figure 6.
Structures of NIR luciferin analogs **iLH2 (22)** and **NH₂-NpLH2 (23)**.

Meanwhile, Maki's group has developed a number of analogs based on the structure of **8e**, which are expected to produce NIR light. Miura *et al.* formed a mother skeleton by a coupling reaction, and thus synthesized biphenyl analogs **24a–c** (**Figure 7** and **8A**) [37]. Analog **24a** produced light at 675 nm, but the luminescence intensity was weak. Although its conjugation was more extended than in **8e**, the luminescence wavelength of **24a** did not change as that of **8e** (675 nm). This result suggests that the biphenyl moiety rotates and reduces the fluorescent intensity.

Kiyama *et al.* synthesized cyclic amino analogs of **8e** (**25a–d**, **Figure 7**) [38] from 4-fluorobenzaldehyde **32** as the starting material. They replaced the F group with various secondary amines, and conducted the Horner–Wadsworth–Emmons

Figure 7.
Structures of NIR luciferin analogs 24–29.

reaction, condensation and cyclization to obtain the final compounds **25a–d** (**Figure 8B**). Despite containing an electron-donating amino group, **25a–d** produced luminescence at almost the same wavelengths (656–667 nm) as **8e** (668 nm). However, the luminescence intensity of **25a** was approximately four times stronger than that of **8e**. The fluorescence quantum yields of **8e** and these cyclic amino analogs **25a–d** were almost identical, suggesting that the luminescence intensity largely depends on the reactivity with luciferase.

The luminescent wavelength can be lengthened not only by extending the π -conjugations and introducing an electron donate substituent, but also by introducing an allyl group. Kitada *et al.* synthesized allyl analogs **26a–b** (**Figure 7**) by introducing allyl groups into **8c**, **8e** and naphthol analogs **27a–d** (**Figure 7**). The analogs were introduced by two routes: Pd-catalyzed Stille coupling (**Figure 8C**) and Claisen rearrangement (**Figure 8D**) [39]. Although these analogs delivered very low luminescence intensities, their wavelength shift was long (approximately 15–35 nm). As the allyl group itself does not affect the π -conjugations of the substrate structure, it was considered that induce fitting was occurred at the luciferase active site and stabilized the substrate metabolite to lower energy state conformation. To develop a long-wavelength, Kitada *et al.* synthesized NIR analog (**28** in **Figure 7**) by introduced both an electron-donating NMe₂ and an allyl group. When reacted with Fluc, **28** produced NIR light at a sufficiently long-wavelength (705 nm), but the luminescence intensity was only 1.3% of that of **8e**. Although the allyl group extends the luminescent wavelength, it greatly reduces the luminescence intensity, which is a major disadvantage.

The aromatic ring site has also been targeted in the development of potential NIR emitters. Saito *et al.* synthesized three analogs **29a–c** (**Figure 7**) in which the

Figure 8.
Synthetic routes of NIR luciferin analogs 24a–c (A), 25a–d (B), 26a–b (C) and 27a–d (D).

aromatic ring of **8e** was replaced with an *N*-heteroaromatic ring [40]. Interestingly, the luminescence wavelengths of three analogs depended on the positions and numbers of their N atoms; **29a** produced red light at 645 nm, seMpai (**29b**) produced NIR light at 675 nm, and **29c** produced orange light at 625 nm. This result highlights the importance of interactions between the luciferase active site and the N atoms of the heterocycle. Although the luminescence wavelength of all three analogs were shorter than 700 nm, the wavelength was changed with a single atom, suggesting that interaction with the luciferase active site is an important part of molecular design.

3. Solubility enhancement of TokeOni and seMpai (**29b**) for sensitive *in vivo* imaging

As mentioned in the previous section, our research group has developed multicolor luciferin analogs for *in vivo* imaging. The luminescence activities of AkaLumine (**8e**) and seMpai (**29b**) are potentially suitable for BLI. Therefore, the usefulness of these analogs as reagents for *in vivo* BLI was evaluated in further animal experiments.

3.1 Development of AkaBLI (TokeOni and AkaLuc)

Prior to administering the reagent into the animal models, we increased the aqueous solubility of **8e** (which is inherently low) and developed an HCl salt of **8e**, AkaLumine-HCl (Tokeoni) [21]. In ultrapure water, the solubility of Tokeoni was 40 mM, approximately 20 times higher than that of **8e** (2 mM). Therefore, Tokeoni enable to administered to experimental animals with a smaller solution volume and higher concentration than **8e**. Tokeoni/Fluc BLI was performed with significantly higher sensitivity than LH2/Fluc and CycLuc1/Fluc in the lungs [21] and brain [41] of mice. However, as the *in vitro* luminescence intensity of Tokeoni/Fluc was lower than that of LH2/Fluc, it must be improved before imaging large animals such as marmosets.

Accordingly, Iwano *et al.* developed a mutant luciferase AkaLuc specialized for Tokeoni. They developed the artificial bioluminescence system AkaBLI, which combines Tokeoni with AkaLuc [42] (a mutation of 28 amino acid residues on *Ppy* luciferase). The AkaBLI luminescence intensity was approximately 10-fold higher in cells (*in vitro*), 52-fold higher in mouse lungs (*in vivo*), and 1400-fold higher in mouse brain tissue (*in vivo*) than LH2/Fluc luminescence intensity. In addition, AkaBLI detected single-cell signals from mouse lung and to quantified 1–10 cells. For large animal imaging, the authors inserted the AkaLuc gene into an adeno-associated virus (AAV) vector, and introduced the recombinant AVV into the striatal neurons of marmosets. AkaBLI achieved video-rate real-time imaging of marmoset brains.

3.2 BLI with seMpai

seMpai (**29b**) was developed to improve two weak points in Tokeoni: neutral pH and disturbance by hepatic background signals. As Tokeoni is acidic [43], it may cause acidosis when injected; moreover, Tokeoni detects the hepatic background signals, which are not detected by LH2 [43, 44]. Due to the effect of N atom, seMpai was sufficiently soluble for administration to experimental animals and 69 mM was dissolved in phosphate buffered saline (pH 7.4) [40]. In Fluc-expressing lung cancer model mice, the sensitivities of seMpai and Tokeoni were not significantly different [40], but seMpai detected no hepatic background signals and seMpai BLI detected breast cancer micro-metastasis [43]. When repeated with Tokeoni and LH2, this experiment was unsuccessful. Although seMpai/Fluc was less sensitive than AkaBLI for single-cell imaging, its imaging sensitivity could be improved mutant luciferases such as AkaLuc.

Fukuchi *et al.* monitored the expression of brain-derived neurotrophic factor (BDNF) in *Bdnf*-luc transgenic mice with LH2, Tokeoni, and seMpai [45]. Tokeoni achieved the most sensitive BLI, and seMpai and LH2 were comparable. The result probably reflects the different abilities of the compounds to penetrate the blood–brain-barrier. This result also indicates the necessary of evaluating the imaging reagent in terms of both its luminescence activity and pharmacokinetics.

Additionally, biocompatibility such as cytotoxicity of Tokeoni and seMpai has not been reported yet. No acute toxicity or adverse side effects were observed in mice when these compounds were administered at a concentration of 33 mM [40], however, preliminary experiments are recommended when using new analogs, not limited to Tokeoni and seMpai.

4. Conclusion

By investigating the structure–activity relationship of luciferin analogs, researchers have developed various methods for tuning the luminescence

wavelengths of these analogs. However, the intensity of the luminescence is poorly controlled. If the luminescence intensity and structure–activity relationships could be associated by a predictable law, we could synthesize new luciferin analogs with high luminescence intensity, and further develop an imaging technology with greater usefulness than conventional technologies.

For a practical imaging technology, both the luminescent substrate/enzyme activity and the pharmacokinetics are very important. Improving the various properties of the substrates and enzymes will enhance the sensitivity of bioluminescence imaging.

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Conflict of interest

The authors declare no conflict of interest.

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References

- [1] Oba Y, Konishi K, Yano D, Shibata H, Kato D, Shirai T. Resurrecting the ancient glow of the fireflies. *Sci Adv*. 2020;6(49):eabc5705. DOI: 10.1126/sciadv.abc5705
- [2] Contag PR, Olomu IN, Stevenson DK, Contag CH. Bioluminescent indicators in living mammals. *Nat Med*. 1998;4(2):245-7. DOI: 10.1038/nm0298-245h
- [3] Kim J-B, Urban K, Cochran E, Lee S, Ang A, Rice B, et al. Non-invasive detection of a small number of bioluminescent cancer cells in vivo. *PLoS One*. 2010;5(2):e9364. DOI: 10.1371/journal.pone.0009364
- [4] Badr CE, Tannous BA. Bioluminescence imaging: progress and applications. *Trends Biotechnol*. 2011;29(12):624-33. DOI: 10.1016/j.tibtech.2011.06.010
- [5] Kaskova ZM, Tsarkova AS, Yampolsky I V. 1001 lights: luciferins, luciferases, their mechanisms of action and applications in chemical analysis, biology and medicine. *Chem Soc Rev*. 2016;45(21):6048-77. DOI: 10.1039/C6CS00296J
- [6] Love AC, Prescher JA. Seeing (and using) the light: Recent developments in bioluminescence technology. *Cell Chem Biol*. 2020;27(8):904-20. DOI: 10.1016/j.chembiol.2020.07.022
- [7] Chen L, Chen Z, Zheng S, Fan L, Zhu L, Yu J, et al. Study on mechanism of elemene reversing tumor multidrug resistance based on luminescence pharmacokinetics in tumor cells in vitro and in vivo. *RSC Adv*. 2020;10(57):34928-37. DOI: 10.1039/D0RA00184H
- [8] Yamada K, Noguchi K, Kimitsuki K, Kaimori R, Saito N, Komeno T, et al. Reevaluation of the efficacy of favipiravir against rabies virus using in vivo imaging analysis. *Antiviral Res*. 2019;172:104641. DOI: 10.1016/j.antiviral.2019.104641
- [9] Bennett J, Duan D, Engelhardt JF, Maguire AM. Real-time, noninvasive in vivo assessment of adeno-associated virus-mediated retinal transduction. *Invest Ophthalmol Vis Sci*. 1997;38(13):2857-63. PMID: 9418740
- [10] Yanagihara K, Takigahira M, Takeshita F, Komatsu T, Nishio K, Hasegawa F, et al. A photon counting technique for quantitatively evaluating progression of peritoneal tumor dissemination. *Cancer Res*. 2006;66(15):7532-9. DOI: 10.1158/0008-5472.CAN-05-3259
- [11] Nakayama J, Ito E, Fujimoto J, Watanabe S, Semba K. Comparative analysis of gene regulatory networks of highly metastatic breast cancer cells established by orthotopic transplantation and intra-circulation injection. *Int J Oncol*. 2017;50(2):497-504. DOI: 10.3892/ijo.2016.3809
- [12] Kuchimaru T, Kataoka N, Nakagawa K, Isozaki T, Miyabara H, Minegishi M, et al. A reliable murine model of bone metastasis by injecting cancer cells through caudal arteries. *Nat Commun*. 2018;9(1):2981. DOI: 10.1038/s41467-018-05366-3
- [13] Fleiss A, Sarkisyan KS. A brief review of bioluminescent systems (2019). *Curr Genet*. 2019;65(4):877-82. DOI: 10.1007/s00294-019-00951-5
- [14] Manni I, de Latouliere L, Gurtner A, Piaggio G. Transgenic animal models to visualize cancer-related cellular processes by bioluminescence imaging. *Front Pharmacol*. 2019;10(March):1-12. DOI: 10.3389/fphar.2019.00235

- [15] Kuroiwa Y, Nakayama J, Adachi C, Inoue T, Watanabe S, Semba K. Proliferative classification of intracranially injected HER2-positive breast cancer cell lines. *Cancers* (Basel). 2020;12(7):1811. DOI: 10.3390/cancers12071811
- [16] Han Y, Nakayama J, Hayashi Y, Jeong S, Futakuchi M, Ito E, et al. Establishment and characterization of highly osteolytic luminal breast cancer cell lines by intracaudal arterial injection. *Genes to Cells*. 2020;25(2):111-23. DOI: 10.1111/gtc.12743
- [17] Branchini BR, Murtiashaw MH, Magyar RA, Portier NC, Ruggiero MC, Stroh JG. Yellow-green and red firefly bioluminescence from 5,5-dimethyloxyluciferin. *J Am Chem Soc*. 2002;124(10):2112-3. DOI: 10.1021/ja017400m
- [18] Branchini BR, Southworth TL, Murtiashaw MH, Magyar RA, Gonzalez SA, Ruggiero MC, et al. An alternative mechanism of bioluminescence color determination in firefly luciferase. *Biochemistry*. 2004;23(23):7255-62. DOI: 10.1021/bi036175d
- [19] Hirano T, Hasumi Y, Ohtsuka K, Maki S, Niwa H, Yamaji M, et al. Spectroscopic studies of the light-color modulation mechanism of firefly (beetle) bioluminescence. *J Am Chem Soc*. 2009;131(6):2385-96. DOI: 10.1021/ja808836b
- [20] Weissleder R. A clearer vision for in vivo imaging. *Nat Biotechnol*. 2001 (4):316-7. DOI: 10.1038/86684
- [21] Kuchimaru T, Iwano S, Kiyama M, Mitsumata S, Kadonosono T, Niwa H, et al. A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. *Nat Commun*. 2016;7(1):11856. DOI: 10.1038/ncomms11856
- [22] Miller SC, Mofford DM, Adams ST. Lessons learned from luminous luciferins and latent luciferases. *ACS Chem Biol*. 2018;13(7):1734-40. DOI: 10.1021/acscchembio.7b00964
- [23] Podsiadły R, Grzelakowska A, Modrzejewska J, Siarkiewicz P, Słowiński D, Szala M, et al. Recent progress in the synthesis of firefly luciferin derivatives. *Dye Pigment*. 2019;170(February):107627. DOI: 10.1016/j.dyepig.2019.107627
- [24] Li S, Ruan Z, Zhang H, Xu H. Recent achievements of bioluminescence imaging based on firefly luciferin-luciferase system. *Eur J Med Chem*. 2021;211:113111. DOI: 10.1016/j.ejmech.2020.113111
- [25] White EH, Wörther H, Seliger HH, McElroy WD. Amino analogs of firefly luciferin and biological activity thereof. *J Am Chem Soc*. 1966;88(9):2015-9. DOI: 10.1021/ja00961a030
- [26] Sharma DK, Adams ST, Liebmann KL, Miller SC. Rapid access to a broad range of 6'-substituted firefly luciferin analogues reveals surprising emitters and inhibitors. *Org Lett*. 2017;19(21):5836-9. DOI: 10.1021/acs.orglett.7b02806
- [27] Kakiuchi M, Ito S, Kiyama M, Goto F, Matsuhashi T, Yamaji M, et al. Electronic and steric effects of cyclic amino substituents of luciferin analogues on a firefly luciferin-luciferase reaction. *Chem Lett*. 2017;46(8):1090-2. DOI: 10.1246/cl.170361
- [28] Reddy GR, Thompson WC, Miller SC. Robust light emission from cyclic alkylaminoluciferin substrates for firefly luciferase. *J Am Chem Soc*. 2010;132(39):13586-7. DOI: 10.1021/ja104525m
- [29] Evans MS, Chaurette JP, Adams ST, Reddy GR, Paley MA, Aronin N, et al.

A synthetic luciferin improves bioluminescence imaging in live mice. *Nat Methods*. 2014;11(4):393-5. DOI: 10.1038/nmeth.2839

[30] Wu W, Su J, Tang C, Bai H, Ma Z, Zhang T, et al. cybLuc: An effective aminoluciferin derivative for deep bioluminescence imaging. *Anal Chem*. 2017;89(9):4808-16. DOI: 10.1021/acs.analchem.6b03510

[31] Iwano S, Obata R, Miura C, Kiyama M, Hama K, Nakamura M, et al. Development of simple firefly luciferin analogs emitting blue, green, red, and near-infrared biological window light. *Tetrahedron*. 2013;69(19):3847-56. DOI: 10.1016/j.tet.2013.03.050

[32] Conley NR, Dragulescu-Andrasi A, Rao J, Moerner WE. A selenium analogue of firefly D-luciferin with red-shifted bioluminescence emission. *Angew Chemie Int Ed*. 2012;51(14):3350-3. DOI: 10.1002/anie.201105653

[33] Ioka S, Saitoh T, Iwano S, Suzuki K, Maki SA, Miyawaki A, et al. Synthesis of firefly luciferin analogues and evaluation of the luminescent properties. *Chem - A Eur J*. 2016;22(27):9330-7. DOI: 10.1002/chem.201600278

[34] Jathoul AP, Grounds H, Anderson JC, Pule MA. A dual-color far-red to near-infrared firefly luciferin analogue designed for multiparametric bioluminescence imaging. *Angew Chemie Int Ed*. 2014;53(48):13059-63. DOI: 10.1002/anie.201405955

[35] Stowe CL, Burley TA, Allan H, Vinci M, Kramer-Marek G, Ciobota DM, et al. Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin. *eLife*. 2019;8:1-22. DOI: 10.7554/eLife.45801

[36] Hall MP, Woodroffe CC, Wood MG, Que I, van't Root M, Ridwan Y, et al.

Click beetle luciferase mutant and near infrared naphthyl-luciferins for improved bioluminescence imaging. *Nat Commun*. 2018;9(1):132. DOI: 10.1038/s41467-017-02542-9

[37] Miura C, Kiyama M, Iwano S, Ito K, Obata R, Hirano T, et al. Synthesis and luminescence properties of biphenyl-type firefly luciferin analogs with a new, near-infrared light-emitting bioluminophore. *Tetrahedron*. 2013;69(46):9726-34. DOI: 10.1016/j.tet.2013.09.018

[38] Kiyama M, Iwano S, Otsuka S, Lu SW, Obata R, Miyawaki A, et al. Quantum yield improvement of red-light-emitting firefly luciferin analogues for in vivo bioluminescence imaging. *Tetrahedron*. 2018;74(6):652-60. DOI: 10.1016/j.tet.2017.11.051

[39] Kitada N, Saitoh T, Ikeda Y, Iwano S, Obata R, Niwa H, et al. Toward bioluminescence in the near-infrared region: Tuning the emission wavelength of firefly luciferin analogues by allyl substitution. *Tetrahedron Lett*. 2018;59(12):1087-90. DOI: 10.1016/j.tetlet.2018.01.078

[40] Saito R, Kuchimaru T, Higashi S, Lu SW, Kiyama M, Iwano S, et al. Synthesis and luminescence properties of near-infrared N-heterocyclic luciferin analogues for in vivo optical imaging. *Bull Chem Soc Jpn*. 2019;92(3):608-18. DOI: 10.1246/bcsj.20180350

[41] Fukuchi M, Izumi H, Mori H, Kiyama M, Otsuka S, Maki S, et al. Visualizing changes in brain-derived neurotrophic factor (BDNF) expression using bioluminescence imaging in living mice. *Sci Rep*. 2017;7(1):4949. DOI: 10.1038/s41598-017-05297-x

[42] Iwano S, Sugiyama M, Hama H, Watakabe A, Hasegawa N, Kuchimaru T, et al. Single-cell bioluminescence imaging of deep tissue in freely moving animals. *Science*.

2018;359(6378):935-9. DOI: 10.1126/
science.aag1067

[43] Nakayama J, Saito R, Hayashi Y, Kitada N, Tamaki S, Han Y, et al. High sensitivity in vivo imaging of cancer metastasis using a near-infrared luciferin analogue seMpai. *Int J Mol Sci.* 2020;21(21):7896. DOI: 10.3390/ijms21217896

[44] Su Y, Walker JR, Park Y, Smith TP, Liu LX, Hall MP, et al. Novel NanoLuc substrates enable bright two-population bioluminescence imaging in animals. *Nat Methods.* 2020;17(8):852-60. DOI: 10.1038/s41592-020-0889-6

[45] Fukuchi M, Saito R, Maki S, Hagiwara N, Nakajima Y, Mitazaki S, et al. Visualization of activity-regulated BDNF expression in the living mouse brain using non-invasive near-infrared bioluminescence imaging. *Mol Brain.* 2020;13(1):122. DOI: 10.1186/s13041-020-00665-7