Brightening up Biology: Advances in Luciferase Systems for *in Vivo* Imaging

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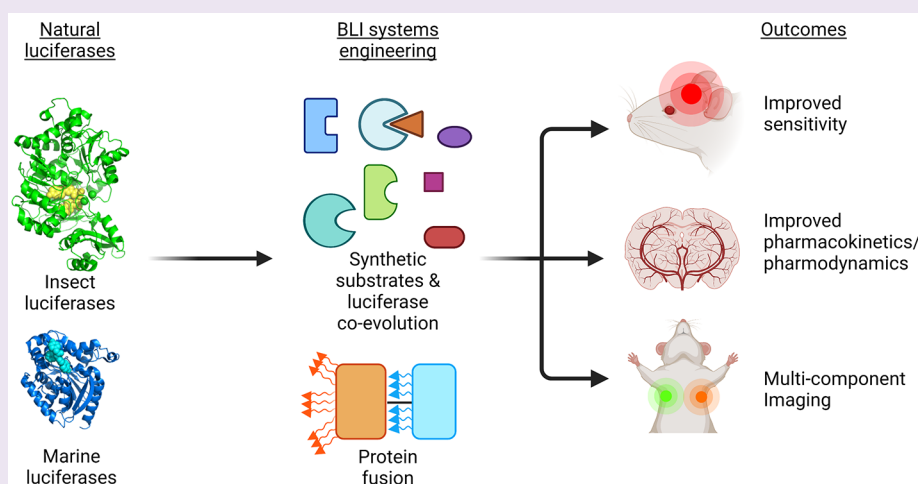
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ABSTRACT: Bioluminescence imaging (BLI) using luciferase reporters is an indispensable method for the noninvasive visualization of cell populations and biochemical events in living animals. BLI is widely performed with preclinical rodent models to understand disease processes and evaluate potential cell- or gene-based therapies. However, *in vivo* BLI remains constrained by low photon production and tissue attenuation, limiting the sensitivity of reporting from small numbers of cells in deep locations and hindering its application to larger animal models. This Review highlights recent advances in the development of luciferase systems that improve the sensitivity of *in vivo* BLI and discusses the expanding array of biological applications.

INTRODUCTION

Bioluminescence imaging (BLI) with luciferase reporters is a highly valuable technique for tracking genetically labeled cell populations and monitoring molecular processes *in vivo*. In its simplest form, BLI detects the presence of labeled cells at a particular location over time, which can reveal changes in cellular proliferation or survival throughout development, disease, or therapy.[1] In a more specific scheme, the expression of luciferase reporters can be made responsive to biochemical signals, allowing BLI to track these signals over time.[2] A major advantage of BLI is that it can be performed longitudinally and noninvasively. Other benefits of BLI include the lack of an intrinsic bioluminescence signal in animals allowing for high sensitivity and specificity, its relative simplicity and afford-ability, and the ability to perform simultaneous whole-body imaging of model animals such as mice.[3−5]

To perform BLI, cells are genetically engineered to express a luciferase enzyme that reacts with an exogenously delivered substrate to produce light, which is then detected by a camera[6] (Figure 1A). The most thoroughly studied luciferases are those isolated from insects, with the *Photinus pyralis* firefly luciferase (FLuc) being the most widely used. In insect luciferases, oxidation of the substrate D-luciferin in the presence of oxygen, ATP, and Mg^{2+} produces yellow light with an emission peak at 578 nm at 25 °C.[7,8] Other insect luciferases that are used for BLI include those from the firefly *Luciola italica* and the click beetle *Pyrophorus plagiophthalamus.* A larger number of known luciferases, however, originate from marine organisms and produce blue light (emission peak 450−500 nm) from the oxidation of coelenterazine (CTZ) in the absence of cofactors other than oxygen. Commonly used CTZ-dependent systems
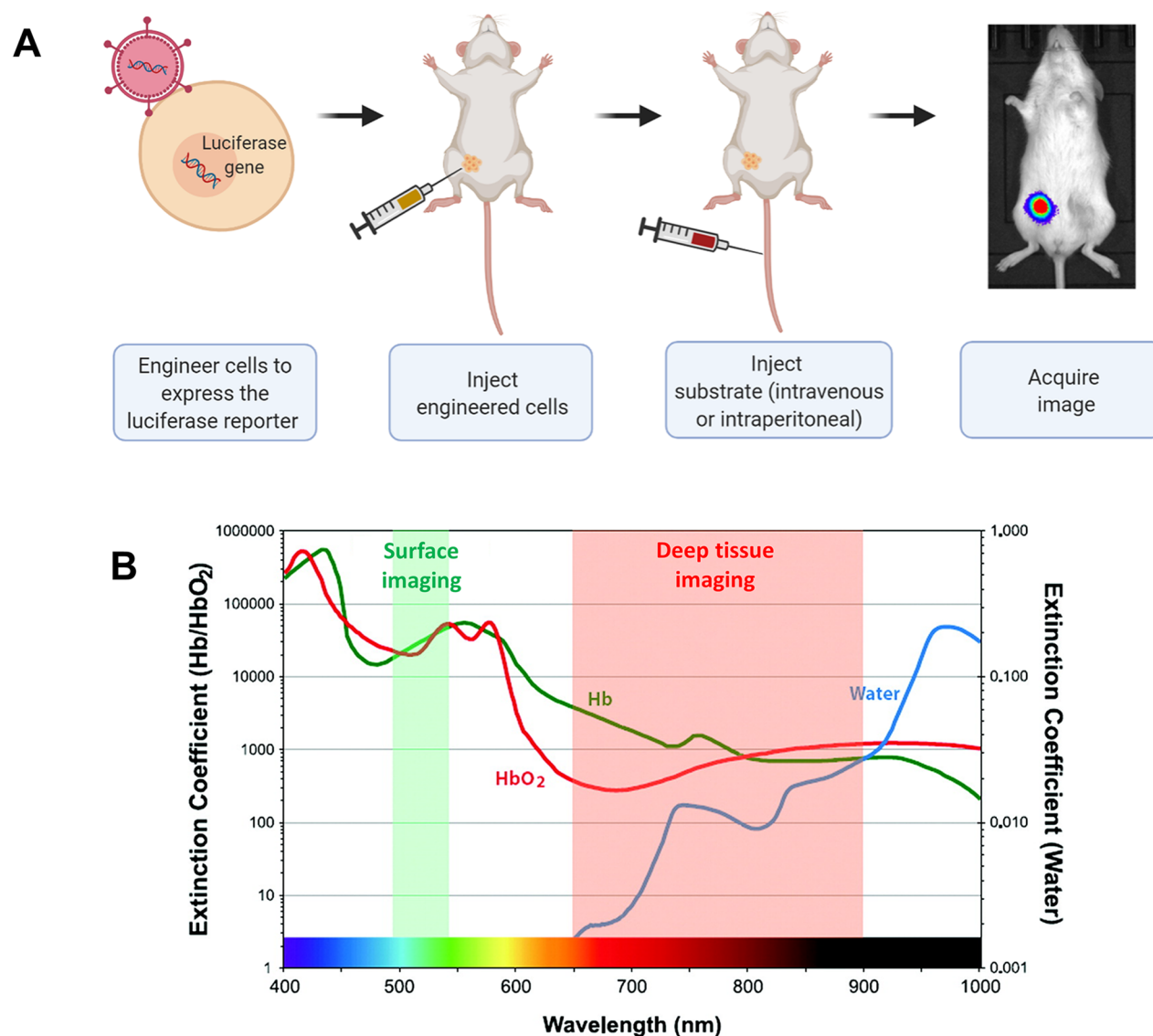
Figure 1. *In vivo* bioluminescence imaging (BLI) of engineered cells. (A) Cells are first genetically engineered to express the luciferase, then transplanted into the animal. The appropriate substrate is injected, and the emitted light is captured to generate an image. (B) Extinction coefficient value of water, oxyhemoglobin (HbO$_2$), and deoxyhemoglobin (Hb) for wavelengths in the visible to near-infrared region. Adapted with permission from Kobayashi et al.[10] Copyright 2010 American Chemical Society.

include Renilla (RLuc), Gaussia (GLuc), and NanoLuc luciferases.[9]

BLI detection favors superficially located cells due to absorption and scattering of light by tissue. In mammalian tissues, hemoglobin impairs transmission of wavelengths below 600 nm, which partially or completely overlaps with the emission spectra of most natural luciferases discovered to date.[8] Therefore, to improve the detection of sparse cell populations at greater tissue depths, it would be advantageous to develop luciferases with red-shifted emission spectra (Figure 1B). Alternatively, luciferases with higher specific activity or higher levels of expression (through more efficient translation or slower degradation) would improve limits of detection for small numbers of cells but alone would not reduce depth-dependent attenuation. Additionally, many current substrates possess uneven biodistribution within mice. For example, intravenously injected ^{14}C−D-luciferin showed early high uptake in the kidney and liver, late predominant uptake in

the bladder, and limited access to the brain.[11] Substrate solubility and stability are also limiting factors of CTZ and its analogs.[12]

Due to these limitations, significant efforts have been invested in the development of improved BLI luciferase/luciferin pairs for improved monitoring of cellular processes. Here, we summarize recent advances in luciferase systems aimed to improve *in vivo* sensitivity in mammalian model systems, and we highlight novel biological applications of the expanding BLI toolbox.

■ BLI SYSTEMS ENGINEERING

With the goal of improving detection sensitivity from deep locations in mammalian subjects, multiple efforts have attempted to mutate luciferases to increase catalytic activity or to red-shift emission (Table 1). One of the earliest examples of wavelength shifting comes from the S284T mutant of FLuc, PpeRS, discovered by random mutagenesis to create a ~50 nm

Table 1. Engineered Luciferase/Luciferin Pairs with Demonstrated *in Vivo* Applications

| category	luciferase(s)	substrate(s)	emission peak	advantages	disadvantages	refs
firefly luciferase (FLuc)	FLuc	D-luciferin	578 nm	effective at shallow tissue imaging; low background emission	poor substrate blood-brain barrier permeability; ATP-dependent (as are all FLuc-based systems)	7, 8, 54
	FLuc	aminoluciferins (e.g., CycLuc1, cybLuc)	up to 591 nm	superior to D-luciferin at several-fold lower dose; improved blood–brain barrier permeability; more sustained signal	substrate solubility may be lower than D-luciferin	22, 23, 55
	FLuc	near-infrared (NIR) luciferins	up to 690 nm	longer half-life in blood and higher affinity to FLuc		32
	Akaluc	AkaLumine-hydrochloride (AkaLumine-HCl)	650 nm	capable of single-cell detection in deep tissues; demonstrated video-rate deep-brain imaging in marmosets	background hepatic signal; potential toxicity due to acidosis	27, 30
	Akaluc	seMpai	675 nm	no background hepatic signal	lower brightness and affinity to Akaluc compared to AkaLumine-HCl	31
click beetle luciferase	FLuc mutants (x5, x5 S284T)	infraluciferin	up to 706 nm	produces distinct bioluminescent colors with various mutants		33, 56
	click beetle mutants (CBR2, CBG2)	NH_2–NpLH2	up to 730 nm	produces distinct bioluminescent colors with various mutants		34, 57
Renilla luciferase (RLuc)	RLuc	CTZ	482 nm	ATP-independent (as are all CTZ-based systems)	poor tissue penetration due to blue light emission; high substrate autoluminescence; rapid inactivation in serum	36, 58
	RLuc	ViviRen	482 nm	reduced autoluminescence compared to CTZ	poor tissue penetration due to blue light emission	39
	RLuc8	CTZ	487 nm	4-fold brighter *in vitro* and 200-fold more resistant to inactivation in serum than RLuc	poor tissue penetration due to blue light emission	15
	RLuc8.6−535	CTZ	535 nm	2.6-fold brighter than RLuc8 *in vivo*		16
NanoLuc	NanoLuc	furimazine, hydrofurimazine (HFz), fluorofurimazine (FFz)	460 nm	small size and high structural stability; 9-fold brighter with FFz than furimazine	poor tissue penetration due to blue light emission	44, 59
	Antares	furimazine, HFz, FFz	584 nm	13-fold brighter than FLuc/D-luciferin at equivalent dose *in vivo*		30, 53
	teLuc	diphenylterazine (DTZ)	502 nm	54-fold brighter than FLuc/D-luciferin at equivalent dose *in vivo*		45
	Antares2	DTZ	584 nm	35−90% signal increase over teLuc/DTZ *in vivo*; enhanced substrate solubility compared to DTZ	substrate solubility is lower than D-luciferin	45
	LumiLuc	8pyDTZ	525 nm	3-fold brighter than Akaluc/AkaLumine-HCl *in vivo*	substrate solubility is lower than D-luciferin	46
	LumiScarlet	8pyDTZ	600 nm	3-fold brighter than LumiLuc/8pyDTZ *in vivo*		46
Gaussia luciferase (GLuc)	GLuc	CTZ	480 nm	secreted into circulation	poor tissue penetration due to blue light emission; high substrate autoluminescence; rapidly decaying signal	60
	GLuc variants (e.g., I90L, 8990, Monsta)	CTZ	up to 503 nm	up to 7-fold brighter than native GLuc *in vivo*; more sustained signal	up to only 1.7% of photons >600 nm	61
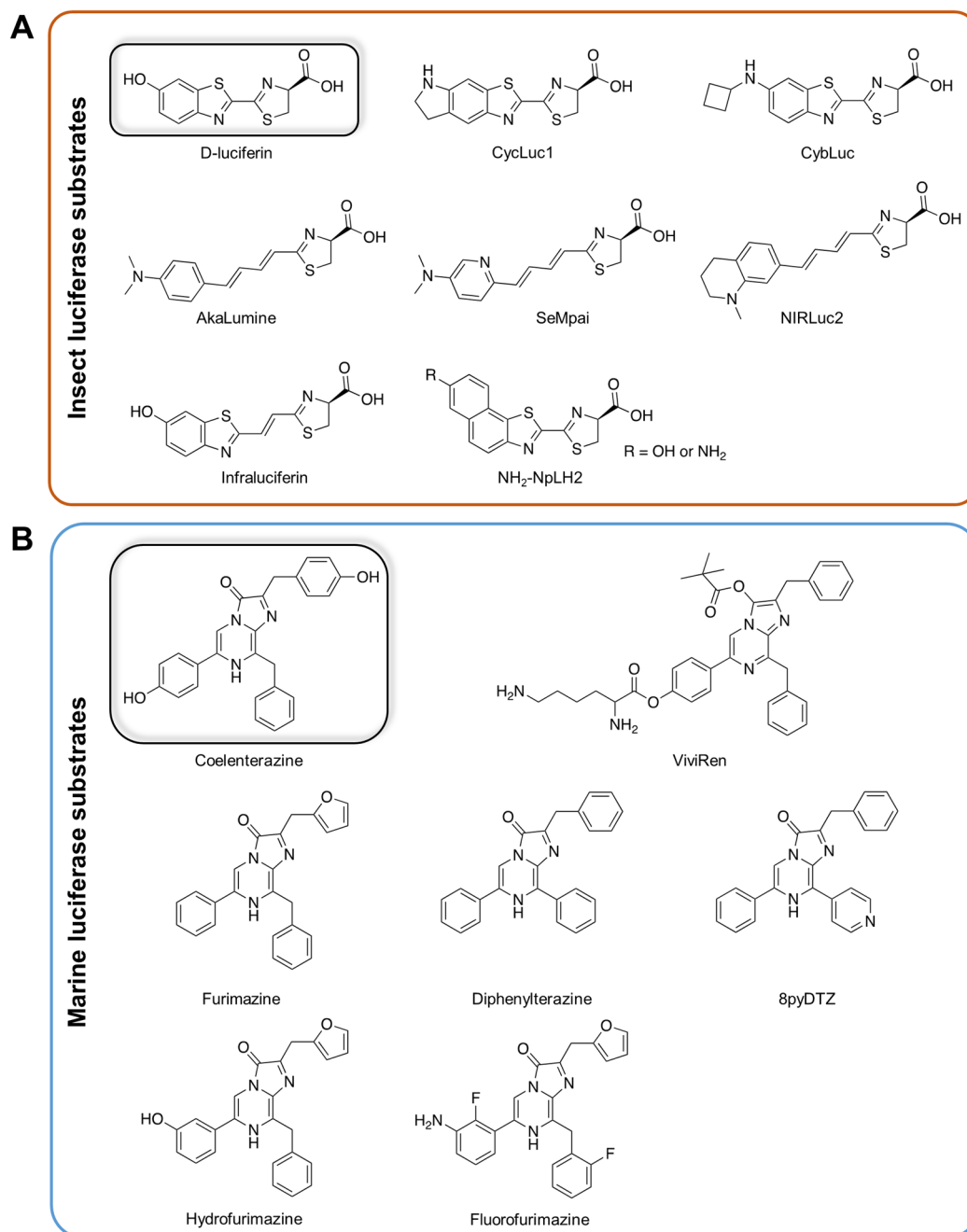
Figure 2. Chemical structures of native luciferins and their analogs: (A) Native insect luciferase substrate D-luciferin and its analogs. (B) Native marine luciferase substrate coelenterazine and its analogs.

red-shift in the emission peak.[13] However, the brightness of PpeRS *in vivo* is not significantly increased, suggesting the mutation merely eliminates the production of green photons rather than increasing the output of red photons. A similar observation of selective loss of green emission without an increase in red emission was made with a Y257A mutation in *Luciola* firefly luciferase, which also produced a ~50 nm red-shift.[14] Efforts on RLuc engineering have been relatively more successful in emission shifting. The RLuc8 variant, consisting of eight mutations, displays a 4-fold increase in brightness,[15] while RLuc8.6−535 exhibits a 50 nm peak wavelength shift to 535 nm and 2.6-fold brighter emission than RLuc8 at a tissue depth of 1−2 mm.[16] This suggests that the increased

brightness of RLuc8.6−535 is indeed due to an increased production of red photons, as desired. However, the emission spectrum of RLuc8.6−535 lies nearly completely below 600 nm, reducing the sensitivity of imaging by RLuc8.6−535 to lower than that of FLuc.

Taken together, these early studies suggest that mutagenesis of insect and marine luciferases alone may not be sufficient to dramatically improve *in vivo* performance of these BLI systems. Thus, additional strategies, such as engineering of luciferase substrates with improved emission characteristics or dramatically red-shifting emission using bioluminescence resonance energy transfer (BRET), may be necessary.

Synthetic Substrate Analogs. An orthogonal approach to modifying bioluminescence properties, and one that can overcome wavelength limitations inherent to D-luciferin or coelenterazine, is to chemically alter the substrate (Figure 2). Substrate engineering is often then accompanied by enzyme engineering to ensure maximal optimization of the system. For the firefly luciferase system, it has been discovered that emission color is primarily determined by the charge distribution on the oxyluciferin product.[17] Various methods to redistribute the electronic charge, such as replacing substituent groups or extending conjugation on the luciferin, have led to the synthesis of novel luciferin analogs capable of red-shifting the emission light.[18,19] Efforts have also focused on modifying luciferins to enhance the circulation time, water solubility, and membrane permeability of luciferins for deep tissue imaging.[20,21]

A major group of D-luciferin analogs is based on amino-luciferins. The first aminoluciferin analog was developed in 1966 by replacing the 6′-hydroxyl group of D-luciferin with an amino group, resulting in a peak emission shift to 590 nm and a ∼10-fold increased affinity for FLuc.[22] Research focused on optimizing analogs for *in vivo* imaging led to the development of the cyclic alkylaminoluciferin CycLuc1. When injected at a dose 20-fold lower than D-luciferin, CycLuc1 can still lead to brighter signal and achieve sensitive brain imaging in FLuc-expressing mice.[23,24] While wild-type FLuc is promiscuous, Adams et al. later engineered some FLuc mutants that exhibit 40−50-fold selectivity for aminoluciferins (including CycLuc1) over D-luciferin in mouse brains.[25] Notably, when paired with aminoluciferins, the engineered FLuc variants did not exhibit increased brightness, indicating the primary cause of the selectivity is likely a loss of utilization of D-luciferin rather than increased utilization of the synthetic aminoluciferins. Recently, Wu et al. investigated a series of novel N-cycloalkylaminolu-ciferins called cyaLucs and reported one analog, cybLuc, with improved sensitivity *in vivo*. At maximum doses (100 mM for D-luciferin and 10 mM for CybLuc), CybLuc provided 7-fold brighter bioluminescence in the mouse brain, which suggests that cybLuc can efficiently traverse the blood−brain barrier and has higher accessibility to deep brain tissues. In addition, the cybLuc signal plateaued for more than 30 min, whereas the D-luciferin signal began to drop after only 5 min, suggesting cybLuc can be used in applications requiring long-time observation *in vivo*.

Recent efforts have succeeded in further shifting bio-luminescence into the near-infrared (NIR) region. Kuchimaru et al. synthesized a novel luciferin analog, AkaLumine-hydrochloride (AkaLumine-HCl), by replacing the benzothia-zole structure in D-luciferin with a dimethylaniline moiety and extending the π-conjugated system.[26] When paired with native FLuc, AkaLumine-HCl emits NIR light peaking at 677 nm and possesses a more favorable biodistribution. Iwano et al. further refined this system by engineering FLuc through successive rounds of mutagenesis to optimize pairing with AkaLumine-HCl, and their work yielded the novel luciferase Akaluc.[27] Imaging with Akaluc/AkaLumine-HCl exhibited up to 1000 times brighter emissions *in vivo* compared to FLuc/D-luciferin and was capable of single-cell detection in deep lung tissues in mice. Additionally, improved BLI in the brain owing to efficient blood−brain-barrier penetration of AkaLumine-HCl enabled video-rate imaging of striatal neurons in marmosets, a significant milestone in *in vivo* BLI. Later, the superiority of Akaluc over FLuc was recaptured in the mouse brain.[28]

However, it was questioned by another study when applied in the imaging fate of cell therapy outside the brain, although in this research D-luciferin was administered at a dose 6-fold higher than common usage.[29] Some potential drawbacks of AkaLumine-HCl include the background hepatic signal[30,31] and noticeable toxicity to skin and heart,[29,31] likely due to the acidity in AkaLumine-HCl solution. A structurally similar luciferin analog, seMpai, does not produce a detectable hepatic signal when paired with Akaluc *in vivo* and may be more suitable for sensitive imaging of the liver, although at the cost of lower brightness compared to AkaLumine-HCl.[31]

In 2020, Ikeda et al. further expanded the palette of NIR-emitting substrates with the development of NIR luciferins (NIRLucs).[32] Using the structure of AkaLumine as a framework, they synthesized a series of novel luciferins via a ring fusion strategy and reached peak wavelengths up to 690 nm when paired with FLuc. One candidate, NIRLuc2, was tested *in vivo*. Although NIRLuc2 was dimmer than AkaLumine *in vitro*, it was comparable to AkaLumine in a subcutaneous tumor model which might be due to its improved pharmacokinetics properties (although the dose was questionably maintained at 5 mM and intravenous injection was adopted instead of intraperitoneal). More sustained bioluminescence was also achieved as a result of longer half-life in blood and higher affinity to FLuc.

Other NIR luciferins include the beetle luciferin analog infraluciferin, with an emission peak at 708 nm.[33] Moreover, infraluciferin produces distinct bioluminescent colors with various luciferase mutants and therefore may be useful for imaging multiple cell populations or biochemical processes within the same animal (see the Multicomponent BLI section). Similarly, the naphthyl-based luciferin NH_2−NpLH2 can be paired with the mutant click beetle red luciferase (CBR2) to produce NIR emission (730 nm), with potential applications in multicomponent imaging.[34]

Investigation into novel CTZ analogs that enhance bio-luminescence in animals has also been a wide field of study. However, this has been much more challenging due to the blue emission of native CTZ systems.[35] It should also be noted that due to its unstable imidazopyridine structure, CTZ can undergo enzyme-independent oxidation, resulting in back-ground autoluminescence.[36] Substitution at the C-2, C-5, C-6, and C-8 positions of the imidazopyrazinone core has yielded enhancements in bioluminescence properties, with changes at the C-2 position modifying the reaction rate and the C-6/C-8 positions influencing the emission spectra.[37,38] One notable CTZ analog developed by Promega Corporation, ViviRen, exhibited up to a 10-fold higher signal *in vivo* than CTZ when paired with RLuc, while showing reduced autoluminescence.[39] Recent studies and overviews covering the progress of the chemical synthesis and structure−activity studies of CTZ analogs exist for interested readers.[37,40−43]

In 2012, Promega Corporation synthesized furimazine, a CTZ analog containing a furan group at the C-2 position that could pair with NanoLuc, an engineered luciferase derived from the small luciferase subunit (19 kDa) of the deep sea shrimp *Oplophorus gracilirostris*.[44] The NanoLuc/furimazine pair produced ∼150-fold greater bioluminescence *in vitro* compared to RLuc/CTZ, with a slightly blue-shifted emission wavelength (460 nm). Because of its brightness, small size, and high stability, NanoLuc was quickly commercialized for widespread use in cell-based assays; however, its blue emission still limited *in vivo* applications. Yeh et al. were successful in
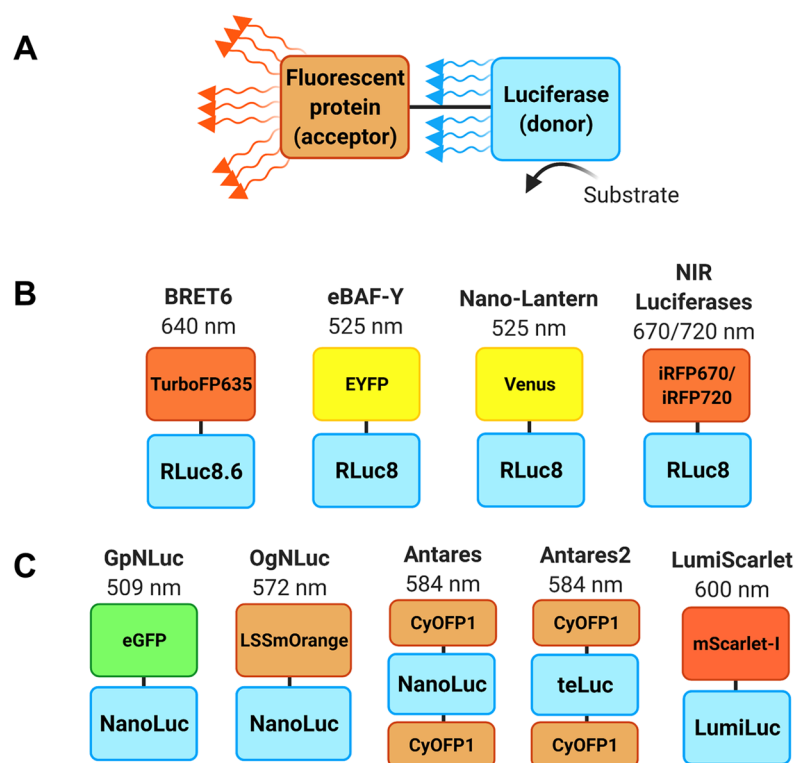
Figure 3. Bioluminescence resonance energy transfer (BRET)-based luciferase systems: (A) A luciferase donor reacts with its substrate to produce light which is then absorbed by a fused fluorescent protein and re-emitted at a longer wavelength. (B) RLuc-based BRET systems and (C) NanoLuc-based BRET systems and their respective peak emission wavelengths.

red-shifting its emission by engineering it to pair with the CTZ analog diphenylterazine (DTZ), resulting in the teLuc luciferase with an emission peak at 502 nm.[45] Imaging in deep tissues of mice with teLuc/DTZ generated ∼54-fold brighter emission than FLuc/D-luciferin at an equivalent dose. Yeh and colleagues then synthesized 8pyDTZ, an analog with 4- to 14-fold enhanced solubility.[46] They then performed directed evolution of teLuc to optimize pairing with 8pyDTZ, resulting in the LumiLuc luciferase which also exhibited a shifted emission peak at 525 nm. LumiLuc/8pyDTZ produced ∼3-fold higher photon flux than Akaluc/AkaLumine-HCl from superficial implants in mice.

BRET-Based Luciferase Systems. In nature, multiple marine organisms have evolved bright luciferase-fluorescent protein systems that utilize the phenomenon of BRET. In BRET, the excited luciferase-bound substrate transfers its energy to an acceptor chromophore through a nonradiative dipole–dipole coupling mechanism, of which the efficiency is dependent on the spectral overlap and intermolecular distance between the two molecules (Figure 3A). The resulting emission light from the acceptor chromophore exhibits a red-shifted spectrum and overall higher quantum yield. Inspired by nature, multiple engineering efforts on marine luciferases have focused on using BRET to red-shift emission and enhance bioluminescence brightness.

Most of the early examples of BRET-based reporters were based on RLuc or its enhanced variants (Figure 3B). BRET6, a TurboFP635-RLuc8.6 fusion protein, enabled direct imaging of drug-mediated protein–protein interaction in deep tissues of living mice.[47] eBAF-Y, consisting of enhanced yellow fluorescent protein (EYFP) and RLuc8, displayed 26-fold bioluminescence enhancement over RLuc and 3-fold over

RLuc8.[48] In a more recent study, by replacing EYFP with Venus and extensively optimizing the unstructured linker, Saito et al. developed an enhanced BRET-based reporter termed Nano-Lantern with a 2.9 times brighter emission than eBAF-Y. Notably, the enhanced signal enabled video rate (30 Hz) imaging of luciferase-expressing tumors in freely moving unshaved mice.[49] In another interesting example, RLuc8 was fused with iRFP670 or iRFP720, phytochrome-based NIR fluorescent proteins that have an excitation peak at around 380 nm, to create reporters with NIR emission.[50] Compared to NIR fluorescent *in vivo* imaging, NIR BLI provided 10-fold increased sensitivity, enabling detection of as low as 10^4 tail vein-injected MTLn3 tumor cells in the lungs of CFW mice. However, fluorescent proteins alone may still be beneficial when substrate delivery is an issue or in hypoxic regions for oxygen-dependent luciferases.

Ever since its development, NanoLuc has become a popular donor for engineering novel BRET-based reporters due to its superior catalytic efficiency over RLuc variants (Figure 3C). By replacing RLuc8 in Nano-Lantern with NanoLuc, a series of enhanced Nano-Lanterns (eNL) were reported.[51] Specifically, fluorescent proteins mTurquoise2, mNeonGreen, Venus, mKOκ, and tdTomato were chosen as BRET acceptors to generate cyan (CeNL, 475 nm), green (GeNL, 520 nm), yellow (YeNL, 530 nm), orange (OeNL, 565 nm), and red (ReNL, 585 nm) eNLs. Among them, CeNL and GeNL showed a roughly 2-fold increased total luminescence intensity compared to NanoLuc, while YeNL and OeNL were 4- or 1.8-fold brighter than their Nano-Lantern counterparts YNL and ONL. Meanwhile, in an independent study, two other chimeric fusion reporter proteins termed LumiFluors were developed using a similar design.[52] The green LumiFluor, eGFP-NanoLuc
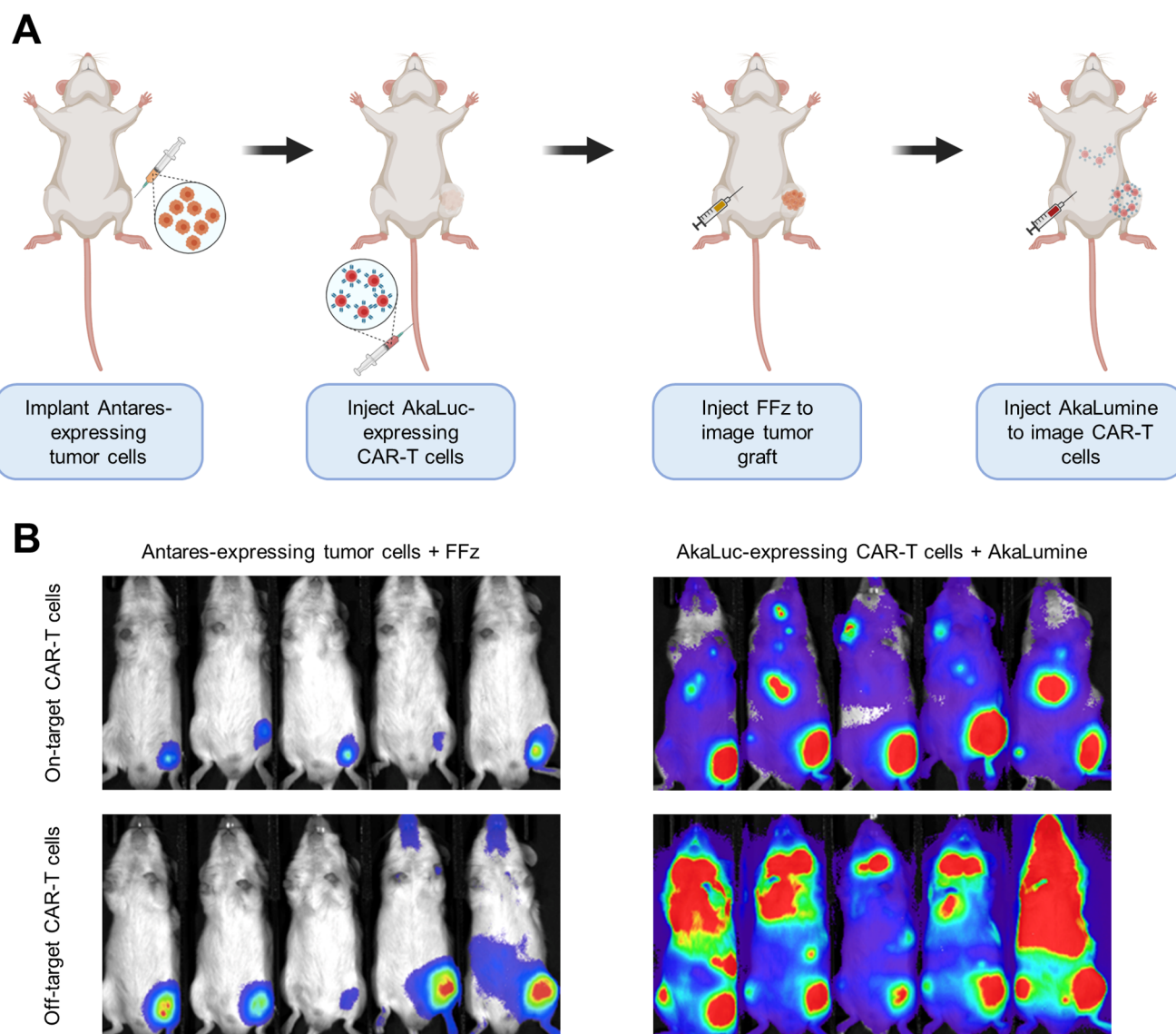
Figure 4. Example of sensitive multicomponent BLI enabled by state-of-the-art bioluminescent systems: (A) Scheme of experimental design for *in vivo* tracking of both Antares-expressing tumor cells and AkaLuc-expressing CAR-T cells. (B) Representative BLI images showing the change in tumor size and the localization of expanded CAR-T cells. Adapted with permission from Su et al.[30] Copyright 2020 Springer Nature.

(GpNLuc), displayed 45-fold more luminescence than YNL *in vitro*. In *NOD/SCID* mice, following a tail vein injection of 500 000 GpNLuc-expressing tumor cells, colonization could easily be detected in the lungs. The orange LumiFluor, LSSmOrange-NanoLuc (OgNLuc), was even several fold brighter than GpNLuc for imaging allografts of mouse lymphoma cells in Albino C57Bl/6 mice. Given their similar signal intensity *in vitro*, this improvement *in vivo* supports the strategy of red-shifting luciferases for enhanced tissue penetration.

While most BRET systems exploit one copy of the fluorescent protein as the acceptor, introducing multiple copies may further enhance the BRET efficiency. On the basis of this hypothesis, two copies of CyOFP1, a bright cyan-excitable orange fluorescent protein, were fused to both the N- and C-termini of NanoLuc to create a highly sensitive bioluminescent reporter called Antares.[53] When compared to the spectrally similar bioluminescent protein ONL, Antares

produced 20-fold more photons across all wavelengths and 28-fold more above 600 nm *in vitro*.

Other engineered luciferases based on NanoLuc, such as teLuc and LumiLuc mentioned above, were also exploited as donors in BRET systems. Compared to NanoLuc/furimazine, the emission of teLuc/DTZ peaking at 502 nm overlaps better with the absorbance of CyOFP1 in Antares. Thus, NanoLuc in Antares was replaced by teLuc to generate Antares2, which consistently exhibits a 584 nm emission peak. In BALB/c mice, when luciferase-coding plasmids were hydrodynamically delivered to have luciferases expressed in the liver, and equimolar doses of luciferase substrates were administered intraperitoneally, Antares2 exhibited an approximately 35–90% signal increase over teLuc and Antares.[45] For the BRET system that utilizes LumiLuc/8pyDTZ, which is further red-shifted from teLuc-DTZ, a recently reported red fluorescent protein mScarlet-I was adopted instead as the BRET acceptor.[46] The resulting fusion protein mScarlet-I-LumiLuc, named LumiScarlet, has an emission peak at 600 nm and has

51% of its emitted photons above 600 nm. When applied to image luciferase-expressing HeLa cells trapped in the lungs of NU/J mice with intravenously injected substrates, LumiScarlet-8pyDTZ gave a comparable signal to that from Akaluc with a comparable amount of intravenous AkaLumine-HCl, and 3-fold brighter than that from LumiLuc/8pyDTZ.

However, the brightness of Antares or LumiScarlet with the more convenient intraperitoneal injection of furimazine or 8pyDTZ does not reach the brightness attainable by AkaLuc with intraperitoneal AkaLumine delivery.[30,45] Two NanoLuc substrates, hydrofurimazine (HFz) and fluorofurimazine (FFz), with a higher aqueous solubility that can be injected into higher total amounts were recently reported.[30] When coupled with Antares, these novel substrates enabled comparable or even brighter bioluminescence *in vivo* compared to AkaLuc/AkaLumine at limiting doses for each substrate.

■ BLI SYSTEMS APPLICATION—MULTICOMPONENT BLI

In many biological studies, the need to observe different cellular events within the same animal requires the cells of interest to be tagged with multiple luciferases. Applications include investigations into the endogenous expression patterns of two distinct genes, tracking of the differentiation/activation status of individual cells by regulating the expression of each luciferase with state-dependent promoters and the trafficking of two separate cell populations such as therapeutic cells (e.g., cytotoxic T cells) and diseased cells (e.g., cancer cells). Unlike fluorescence imaging where the cells of interest can be visualized by simply switching a filter, multicomponent BLI requires more complex methods which can pose limitations on the detection sensitivity and accuracy.

One option is to use substrate-resolved luciferases, where the injection of a single substrate is used to activate the respective luciferase. This requires tagging of luciferases with minimal cross-reactivity with the other substrate(s), also known as orthogonal luciferase-luciferin systems. The dual-BLI proof-of-concept was first demonstrated in 2002 by Bhaumik and Gambhir, who subcutaneously implanted C6 rat glioma cells expressing either RLuc or FLuc into single mice, followed by their sequential visualization using CTZ and D-luciferin, respectively.[35] Since then, several other groups have used dual-BLI to visualize distinct molecular events or cell populations *in vivo*, using other luciferase pairs such as FLuc/D-luciferin and GLuc/CTZ,[62] and FLuc/D-luciferin and NanoLuc/furimazine.[63] A triple-BLI system has also been demonstrated using FLuc/D-luciferin, GLuc/CTZ, and the codon-optimized luciferase from *Vargula hilgendorfii* (Vluc) with its substrate vargulin.[64] Prescher's group has expanded the toolbox of multicomponent BLI by synthesizing novel orthogonal luciferase—luciferin systems based on FLuc/D-luciferin system,[65,66] including a class of π-extended luciferins enabling red-shifted emission.[67] The challenge with these systems, however, remains their low sensitivity *in vivo*, limiting their detection capability in deep tissues.

The low sensitivity issue could be resolved with the state-of-the-art bioluminescent reporters described above. The AkaLuc/AkaLumine pair has been used orthogonally with Antares/fluorofurimazine. With intraperitoneally administered FFz or AkaLumine, the expansion or recession of engrafted Antares-expressing MG63.3 osteosarcoma tumor and the circulation and localization of intravenously injected CAR-T cells could be sensitively visualized within the same NSG mice,

over a period of 30 days[30] (Figure 4). This pair of luciferase and luciferins for dual-BLI of two cell populations is the most sensitive reported to date.

Another limitation with substrate-resolved luciferases is the need for the signal from the previous luciferase to clear, which can take hours to days. A solution to this issue was recently developed by Rathbun et al., which relies on substrate unmixing algorithms: substrates are administered in rapid succession, beginning with the dimmest substrate, so that the residual signal from the previous luciferase becomes part of the background noise of the next.[68] The set of images can then be used to deconvolute individual substrate signatures. Using this technique, Yao et al. demonstrated for the first time multicomponent imaging of four luciferase-luciferin pairs *in vitro*.[67] In 2021, Moroz et al. demonstrated quadruple-BLI *in vivo* with a slightly different approach, using light wavelength-blocked filters to separate signals.[69] They multiplexed RLuc, CBRLuc, CBGLuc, and a new reporter named membrane-anchored Cypridina luciferase (maCLuc), which is paired with the vargulin substrate, to visualize a metastatic tumor model.

With these developments, interpretation of substrate-resolved multicomponent BLI can still be biased by differences in substrate kinetics and biodistribution. Another way to perform multicomponent BLI is through spectrally resolved luciferase pairs, whereby the injection of a single substrate produces distinct emission spectra with multiple luciferases which can be separated using spectral unmixing algorithms. This often employs green- and red-shifted mutant luciferases to minimize overlap in emission spectra.[13] Mezzanotte et al. demonstrated *in vivo* dual-BLI of human embryonic kidney cells expressing green CBG99 (λ_{max} = 537 nm) and red PpyRE8 (λ_{max} = 618 nm) luciferases.[70] They achieved efficient spectral unmixing and sensitive imaging of distinct cell populations injected subcutaneously in mice. However, imaging in deeper tissues can be hampered by greater attenuation of the lower wavelength light which would introduce bias into quantitative comparisons. Recently, Aswendt et al. developed an improved dual-color BLI system with the further mutated x5r/x5g pair showing better separated spectra and higher photon flux with the ability to accurately predict cell population ratios from quantitative unmixing results.[71] The x5r/x5g pair can also be coupled with infraluciferin for red-shifted dual-color imaging.[56] In 2021, Zambito et al. introduced a further improved NIR dual-BLI system using the substrate NH_2−NpLH2 paired with novel click beetle mutants CBG2 (λ_{max} = 660 nm) and CBR2 (λ_{max} = 730 nm).[57] They showed sensitive deep tissue imaging in a lung model and were able to spectrally resolve and quantify the emissions. Furthermore, the versatility of the system was demonstrated with *in vivo* dual-BLI of CBG2/NH_2−NpLH2 and Akaluc/Akalumine-HCl.

■ TECHNOLOGICAL ADVANCEMENTS

In addition to the engineering of luciferase systems at the chemical level, developments in camera technologies and imaging methods have advanced the capabilities of *in vivo* BLI (Table 2). In brief, these imaging systems consist of a light-tight chamber in which the subject is placed and a cooled charge-coupled device (CCD) camera which collects emitted photons. The sensitivity of imaging systems has been greatly improved through the development of intensified CCD (ICCD) and electron multiplying CCD (EMCCD) cameras. This increased sensitivity has subsequently decreased acquis-

Table 2. Summary of Key Advancements in Instrumentation and Imaging Methods for *in Vivo* BLI

year	technological advancement	ref
2001	first commercial system designed for BLI of small animals (IVIS, Xenogen Corporation)	75
2003	first bioluminescence tomography (BLT) prototype developed, enabling integration with CT for quantitative 3D localization of signals	76
2006	development of spectral unmixing algorithms for separating signals from multiple BLI reporters	77
2008	new imaging system developed for video-rate imaging of nonanaesthetized and freely moving mice	72
2012	first time multiple BLI reporter genes were imaged *in vivo* using BLT	78
2013	development of a 3D BLI method based on multiple rotating cameras for accelerated data acquisition and improved signal quantification	79
2018	video-rate imaging of Akaluc-expressing striatal neurons in nonanaesthetized marmosets using a MIIS system (Molecular Devices Japan)	27
2020	*in vivo* BLI in the second near-infrared region (1000−1700 nm) achieved using a InGaAs CCD camera (Princeton Instruments, NIRvana-LN)	80

ition times from several minutes to milliseconds, enabling video-rate imaging of unanesthetized animals.[27,49,72] In addition, development of bioluminescence tomography (BLT) that incorporates CT/MRI information has aided anatomical localization in three dimensions.[73,74] Traditional CCD cameras are blue sensitive, but newer cameras have been optimized for higher quantum efficiency at wavelengths >600 nm, which is especially useful for imaging NIR-emitting luciferases.[75] In summary, technological breakthroughs that complement chemical engineering of bioluminescent systems is expected to improve imaging sensitivity and expand future BLI applications.

CONCLUSIONS

Over the past few decades BLI has emerged as a powerful tool for preclinical visualization of cellular and molecular processes *in vivo*. The high signal-to-noise ratio, direct relationship of signal to cell viability, and ability to survey processes on a whole-animal scale offers an unparalleled approach to non-invasive imaging. However, the attenuation of light by tissue raises a pressing need for the development of novel red-shifted luciferases and luciferins that can enable sensitive, deep-tissue imaging. Recent advancements in protein engineering and substrate synthesis have pushed the limits of detection, and the expanding toolbox of multicomponent BLI has improved imaging of complex cellular interactions.

The *in vivo* behavior of BLI systems remains incompletely understood. For substrates, *in vivo* evaluation should not only characterize bioluminescence output from one location but should also address pharmacodynamics and pharmacokinetics since these substrates act like drugs *in vivo*.[21] An ideal synthetic luciferin should have its organ/tissue biodistribution clearly characterized,[11] cause little to no toxicity or behavioral abnormality to the injected animal[30] or have its safe dosage established, and be free of background bioluminescence caused by auto-oxidation.[30,31] For D-luciferin, distribution has been assessed with radioactive tracers, but this has yet to be performed for other substrates. We expect animal testing of advanced luciferase systems to be an area of growing interest as the field of BLI continues to expand.

Another interesting future development is in the combination of the most sensitive BLI reporters with other imaging methods. For example, a multimodal system consisting of a BLI reporter for ease of use and affordability with either an MRI reporter for high 3D resolution or a PET reporter for high 3D sensitivity may overcome limitations inherent to each modality.

In conclusion, we envision further development of luciferase/luciferin systems, along with *in vivo* testing and incorporation with other imaging reporters, to be a growing and exciting area as we continue to challenge the limits of *in vivo* imaging.

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Notes

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KEYWORDS

Bioluminescence imaging (BLI), an imaging technique that allows the visualization of biochemical events and labeled cells via the detection of light emitted by enzyme-catalyzed reactions; **Luciferase**, an enzyme that produces bioluminescence by oxidizing a compatible substrate; **Luciferin**, the substrate oxidized by a luciferase to produce bioluminescence; **Near-infrared luciferins (NIRLucs)**, a class of luciferins emitting light in the near-infrared range (∼650−900 nm), enabling higher light penetration at deep tissues; **Bioluminescence resonance energy transfer (BRET)**, an energy transfer event occurring between an excited luciferase-bound substrate and an acceptor fluorescent protein; the emitted wavelength of a BRET system is higher than that of the donor luciferase; **Dual-BLI**, BLI of a pair of orthogonal luciferases and luciferins, allowing the tracking of independent bio-

chemical or cellular events; **Substrate-resolved luciferases**, a pair of luciferases that are independently activated following administration of the appropriate substrate; **Spectrally resolved luciferases**, a pair of luciferases that utilize the same substrate but produce distinct emission spectra; their activity is separated using spectral unmixing algorithms.

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