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Perspective

Autonomous Bioluminescence Systems: From Molecular Mechanisms to Emerging Applications

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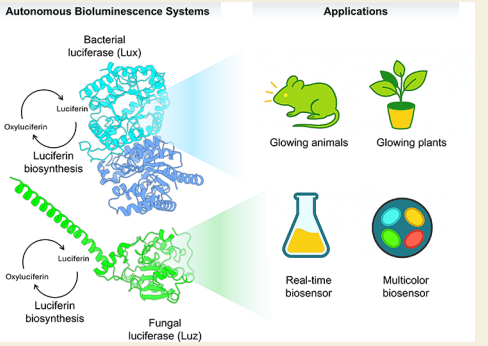
ABSTRACT: Autonomous bioluminescence systems—genetically encoded platforms that integrate luciferase enzymes with complete substrate biosynthetic pathways—have emerged as transformative tools for real-time, noninvasive imaging in living systems. Unlike conventional substrate-dependent bioluminescence, these systems provide continuous light emission without external substrates, enabling long-term monitoring with minimal phototoxicity compared to the use of fluorescence. Here, we present a critical perspective on recent advances in the two best-characterized autonomous systems: bacterial and fungal bioluminescence systems. We assess their molecular mechanisms, protein engineering strategies, and emerging applications in single-cell imaging, multicolor biosensing, and whole-organism monitoring. By comparing their strengths and limitations, we highlight persistent challenges, such as low quantum yield in bacterial bioluminescence and substrate availability constraints in fungal bioluminescence, and discuss strategies to address them—including AI-guided mutagenesis, *de novo* protein design, and metabolic pathway optimization. We conclude by outlining application-driven design targets for the next generation of autonomous bioluminescent systems in biomedical research, environmental monitoring, and synthetic biology.



KEYWORDS: *bioluminescence, autonomous bioluminescence systems, bacterial luciferase (lux), fungal luciferase (luz), biosensors, bioimaging*

INTRODUCTION

Bioluminescence—the emission of light through enzymatic oxidation of a substrate (luciferin) by an enzyme (luciferase)[1]—occurs across diverse organisms, from bacteria and fungi to marine invertebrates and insects. Emission wavelengths span approximately 400−700 nm, yet the majority of natural systems emit in the blue−green region, with relatively few examples in yellow and red.[2] The molecular diversity of luciferases and luciferins has long inspired their adoption in biological research, particularly for sensitive, low-background imaging.[3]

All bioluminescent reactions are catalyzed by luciferase enzymes, which may be encoded by a single gene or multiple genes. For example, bacterial luciferase is encoded by two genes, *luxA* and *luxB*, while firefly luciferase is encoded by a single gene, *FLuc*.[1] Bioluminescence classification is primarily based on the type of luciferin substrate used, as many luciferases share the same luciferin. Coelenterazine, for instance, is used by various marine organisms and emits blue (450−550 nm) light via *Renilla*,[4] *Gaussia*,[5] and NanoLuc[6] luciferases. D-Luciferin,[7] used by firefly and click beetle luciferases, produces yellow (540 nm) to orange-red light (590 nm). Tetrapyrrole-based luciferins[8] emit blue light (470 nm) in dinoflagellates luciferase, and *Cypridina* luciferin[9] emits blue light (460 nm) through *Cypridina* luciferase. Bacterial luciferase utilizes reduced flavin mononucleotide ($FMNH_2$) and tetradecanal to emit blue-green light (480−500 nm).[10] Lastly, fungal luciferase uses 3-hydroxyhispidin as its substrate to emit green light (530 nm).[11]

In conventional bioluminescence imaging (BLI), the luciferase is genetically expressed, but luciferin must be supplied exogenously (*e.g.*, D-luciferin, coelenterazine, or *Cypridina* luciferin). As a result, signal kinetics, duration, and experimental burden are tightly dependent on substrate dosing and delivery including repeated administration, variable tissue penetration, and pharmacokinetics.[12,13] In contrast, autonomous bioluminescence systems are fully genetically encoded: cells express both the luciferase and the enzymes required for luciferin biosynthesis, enabling continuous, exogenous luciferin supply independent light emission. In practice, such autonomy allows longitudinal and real-time readout without external

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Table 1. Comparison of Bacterial (Lux) and Fungal (Luz) Autonomous Bioluminescence Systems

year	breakthrough	system/host	key advance	impact	refs
2018	*ilux* (enhanced Lux operon)	bacteria	∼7× brighter lux operon, improved substrate flux	demonstrated long-term autonomous imaging in bacteria	15
2018	FBP1 (fungal bioluminescence pathway 1)	yeast and mammalian cells	full pathway of fungal bioluminescence system	enable long-term autonomous imaging in yeast with FBP1	11
2019	co Lux	mammalian cells	codon-optimized Lux and Frp expression	single mammalian cells autonomous bioluminescence imaging	17
2021	BRET-Lux fusions	bacteria, mammalian, and plant	fusion of luciferase with fluorescent acceptor (Venus) to shift emission and boost light	enhanced spectral flexibility and brightness via energy transfer	86
2022	Cryo-EM of LuxC−LuxE	bacterial enzymes	structural elucidation of fatty acid reductase complex	informed rational protein engineering	21
2022	*ilux2*	bacteria	integration of Lux mutants into chromosomes for stable expression and brightness gains	enables durable and bright autobioluminescent bacterial strains for long-term use	22
2020	plant with FBP1	plant	engineered fungal bioluminescence pathway in plant expression	self-sustained luminescence plant that is visible to the naked eye	20,63
2023	eFBP (enhanced fungal bioluminescence pathway)	plant	metabolic engineering of fungal luciferin biosynthesis by introducing coumaroyl shikimate/quinate 30-hydroxylase (C3′H) gene	enhanced brightness of luminescent plants up to 3×10^{11} photons/min/cm^2	25
2023	FBP2/3 (optimized fungal bioluminescence pathway)	yeast, plant, and mammalian cells	engineered fungal pathway with much higher brightness	stronger autoluminescence across diverse hosts	24
2024	A hybrid FBP	yeast, plant, and mammalian cells	A hybrid pathway of FBP with new hispidin biosynthesis genes (type III polyketide synthases)	reduce the size of HispS gene (5.1 kbp) to 1.2 kbp (PpASCL)	23
2024	nano-lanternX (NLX) (multicolor Lux)	bacteria, mammalian, and plant	designed 5 spectrally distinct autonomous reporters via BRET-Lux	multiplexed, substrate-free spectral imaging across diverse hosts	18
2025	transgenic autobioluminescent mouse	mammalian whole organism	chromosomal insertion of full bacterial lux genes enabling *in vivo* autonomous bioluminescence	first mammal with substrate-free bioluminescence for animal imaging	91

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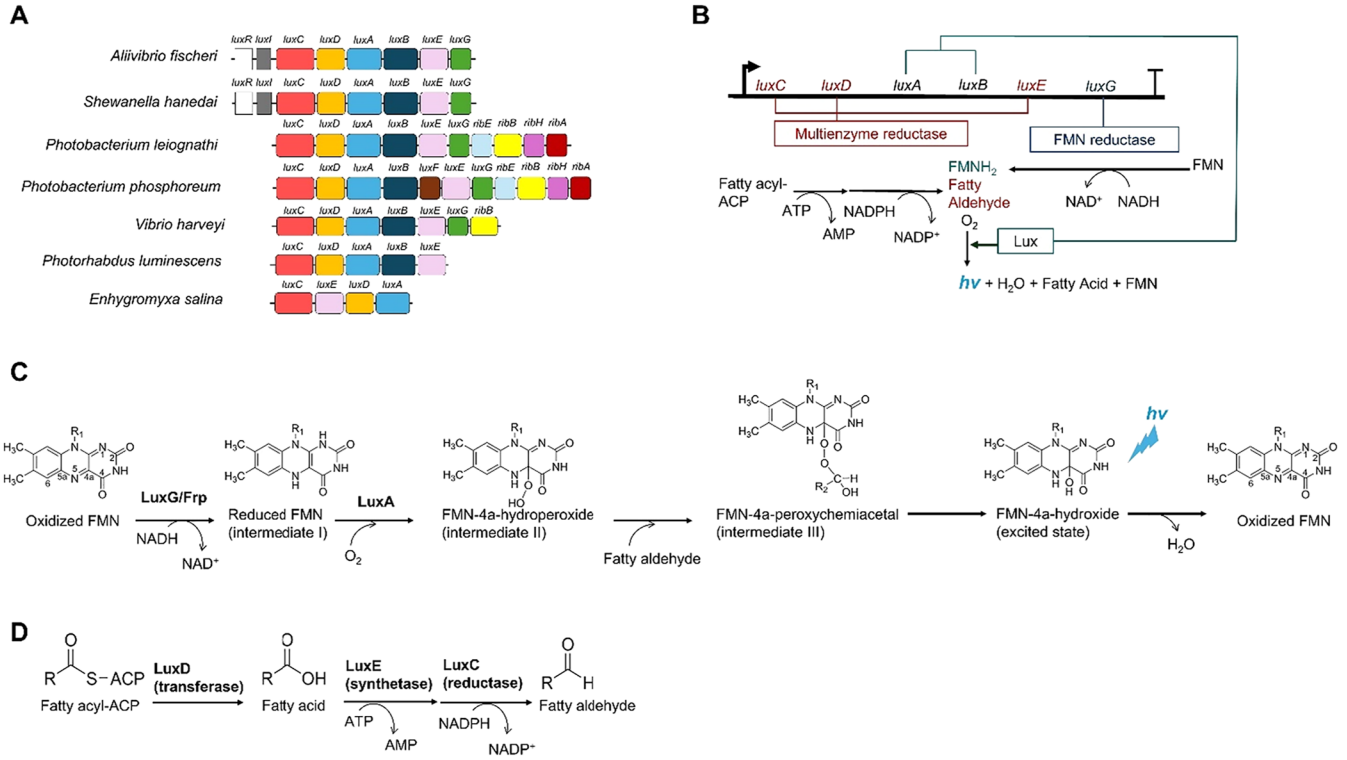

Figure 1. Bacterial bioluminescence reactions pathway. (A) Bacterial bioluminescence genes order of bioluminescent bacterial strains. (B) Overall bacterial bioluminescence reaction. (C) Catalytic mechanism and intermediates of bacterial bioluminescence reactions. (D) Fatty aldehyde reaction pathway of bacterial bioluminescence pathway.

reagents, while introducing design trade-offs such as metabolic load, pathway balance, and spectral constraints.[11,14] Collectively, these properties make autonomous systems particularly attractive for persistent imaging and whole-cell bioreporters where reagent-free operation provides a clear advantage.

Currently, only two bioluminescence systems, the bacterial (Lux) and the fungal (Luz), have been fully elucidated with respect to both luciferase and the endogenous biosynthesis of luciferin.[11,14,15] In other systems (*e.g.*, firefly, Coelenterazine-dependent systems, *Cypridina* luciferin-based systems) luciferases are well known, but luciferin biosynthesis remains unresolved.[16] The Lux system, comprising *luxCDABE* operon and associated genes for flavin reduction, naturally functions in prokaryotic hosts and has been adapted for mammalian and plant cells through codon optimization and metabolic support.[14,17,18] The Luz system, discovered more recently in fungi, integrates into the plant shikimate pathway to produce its luciferin, enabling striking, naked-eye−visible luminescence in transgenic plants.[19,20]

In the past five years, structural insights (*e.g.*, cryo-EM of LuxC−LuxE),[21] improved Lux operons (*ilux*/*ilux2*),[15,22] mammalian implementations (co Lux),[17] multicolor Lux (Nano-lanternX/NLX),[18] and pathway optimization of Luz[23−25] have reshaped the field and expanded autonomous BLI across organisms (Table 1). Despite these advances, both systems face persistent challenges: Lux suffers from low quantum yield[26] and limited spectral range, while Luz is constrained by substrate availability outside plants[11] and by limited structural data for protein engineering. Several complementary strategies have been developed to mitigate these constraints. These including optimized Lux operons and engineered Luz variants generated through directed mutagenesis,[15,22,24] enhancement of luminescence quantum yield via BRET-based approaches,[18] cofactor supply engineering to increase $FMNH_2$ and NAD(P)H availability,[27] efforts to improve substrate pathways to support Luz activity outside plants,[24,28] and protein design guided by artificial intelligence (AI) tools such as AlphaFold3.[29,30] This Perspective critically evaluates recent developments in both systems, compares their relative merits, and outlines targeted strategies to address these challenges. By integrating molecular insights with application-driven goals, we aim to provide a roadmap for the next generation of autonomous bioluminescence technologies.

MOLECULAR MECHANISM OF BACTERIAL BIOLUMINESCENCE

Bioluminescent bacteria possess conserved gene clusters encoding the proteins responsible for light emissions. These genes are typically organized under a single promoter in the conserved order *luxCDABE*.[14] The lux operon has undergone evolutionary changes through vertical gene transfer, gene duplication and deletion, and gene acquisition. In some lineages, such as *Enhygromyxa salina*, the operon has lost *luxB*, resulting in a simplified *luxCDAE*.[31] In other cases, duplication of *luxB* has led to the incorporation of an additional gene, *luxF*, into the operon that specifically binds 6-(3′-(*R*)-myristyl)-flavin mononucleotide (myrFMN) instead of $FMNH_2$.[32] Marine bioluminescent bacteria often contain *luxG*, which encodes a flavin reductase, whereas terrestrial species like *Photorhabdus luminescens* lack *luxG* and instead rely on *fre* to encode flavin reductase[33,34] (Figure 1A). The genes *luxA* and *luxB* encode the heterodimeric luciferase, while *luxC*, *luxD*, and *luxE* encode the fatty acid reductase complex required for luciferin synthesis[14,33] (Figure 1B).

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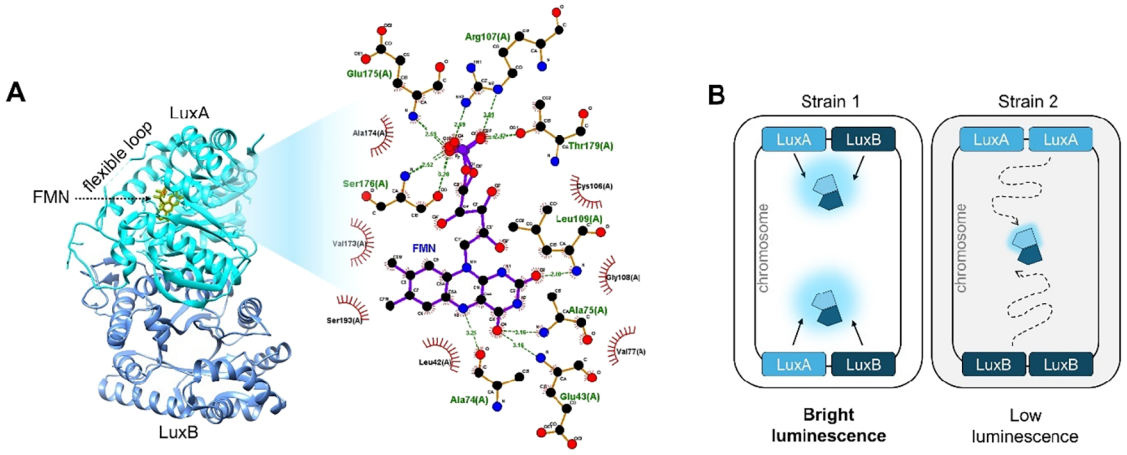

Figure 2. Structure of bacterial luciferase and functions. (A) Structure of LuxAB with residues around the FMN binding site (PDB: 3FGC). (B) The relation of LuxA and LuxB distance integrated into the chromosome on luminescence intensity.

LuxAB catalyzes the monooxygenation of a long-chain fatty aldehyde to its corresponding fatty acid, using $FMNH_2$ as a cosubstrate.[14,33] Tetradecanal (C14) is considered the natural substrate of bacterial luciferase; however, long-chain fatty aldehydes ranging from C9 to C16 can also serve as potential substrates.[10] In the luminescence reaction, binding $FMNH_2$ (Intermediate I) by the enzyme is followed by interactions with O_2 to form a flavin-4a-hydroperoxide (Intermediate II). This intermediate is unstable in the absence of long-chain fatty aldehydes and leads to intermediate III (FMN-4a-peroxychemiacetal) after the binding of long-chain fatty aldehyde and luciferase-bond intermediate II.[35] The monooxygenation of intermediate III forms the excited state of FMN-4a-hydroxide, which serves as a bioluminophore.[36] The excited state relaxes to the ground state and releases free energy as light and byproduct fatty acids (R_2-COOH). After the release of one water molecule, the ground state of FMN-4a-hydroxide was oxidized to FMN[37] (Figure 1C). The quantum yield for the reaction has been estimated at 0.1−0.2 photons.[26]

In luciferin-related biosynthesis, both $FMNH_2$ and long-chain fatty aldehydes are produced through energy-dependent enzymatic processes. $FMNH_2$ is generated by flavin reductases, which utilize NADH and/or NADPH as electron donors.[38,39] $FMNH_2$ is then transferred to luciferase via a free diffusion mechanism.[40] The gene encoding the flavin reductase varies among bioluminescent species.[34] For instance, in *Vibrio harveyi*, *Vibrio fischeri,* and *Photobacterium leiognathi*, $FMNH_2$ production is mediated by *frp* or *luxG*. In contrast, *P. luminescens* and heterologous systems such as *Escherichia coli* rely on endogenous *fre* to encode flavin reductase.[34] Structurally, *fre* from *E. coli* and *luxG* from *V. harveyi* share approximately 40% sequence identity.[41] Three enzymes are required for the biosynthesis of long-chain fatty aldehydes (Figure 1D). First, the transferase (encoded by *luxD*) converts acyl-ACP or acyl-CoA into free fatty acids.[42−44] Next, the synthetase (encoded by *luxE*) activates the free fatty acid in an ATP-dependent manner, forming an acyl-AMP intermediate that is covalently attached to Cys362 of LuxE.[45] Finally, the reductase (encoded by *luxC*) reduces this intermediate using NADPH, resulting in the formation and release of the fatty aldehyde product.[43,46]

Structurally, bacterial luciferases form heterodimeric proteins with the α- and β-subunits. The α subunit (LuxA) (40 kDa) contains an active site, whereas the β subunit (LuxB) (36 kDa) stabilizes LuxA.[47] Both LuxA and LuxB have a TIM (β/α)8 barrel folding structure, which shares approximately 30% sequence identity and more than 95 of the 350 amino acids conserved.[48,49] In the crystal structure of *V. harveyi* luciferase (PDB: 3FGC), FMN binds to the active site of LuxA in the side chains of Leu42, Glu43, Ala74, Ala75, Val77, Cys106, Arg107, Leu109, Tyr110, Agr125, Val173, Ala174, Glu175, Ser176, Thr179, and Trp192 (Figure 2A). The major difference between LuxA and LuxB is that LuxA contains a 29-residue flexible loop near the active site cavity. This flexible loop protected the intermediate reaction from bulk solvent exposure.[48] The interaction between LuxA and LuxB is mediated by four conserved helix bundles at the subunit interface, involving both hydrophobic interactions and hydrogen bonding. Tyr151 of LuxB plays a critical role in this intersubunit association, serving as a key residue for stabilizing the LuxA−LuxB complex.[48,49] Mutational analyses have shown that substitutions such as LuxB-Tyr151Lys and LuxB-Tyr151Trp significantly reduce quantum yield without affecting subunit association or dissociation.[49]

The *in vivo* assembly of LuxA and LuxB requires ribosome-associated exposure of key structural elements at the dimerization interface of LuxB. The trigger factor (TF) chaperone facilitates subunit interaction by preventing premature association of LuxA with nascent LuxB and vice versa.[50,51] Furthermore, dimerization efficiency is enhanced when the luxA and luxB genes are integrated in close proximity within the genome; in contrast, greater intergenic distance results in a marked reduction in luminescence, with activity decreasing to approximately 60%[50] (Figure 2B). LuxA alone is incapable of producing luminescence without the presence of LuxB. Efforts to generate a functional monomeric luciferase have thus far involved fusing LuxA and LuxB, rather than eliminating LuxB entirely.[52−55] However, a recent report describes an exception in *E. salina* LuxA, where LuxA alone appears to produce luminescence in the absence of LuxB. This *E. salina* LuxA forms a homodimer and utilizes short-chain fatty aldehydes (C4−C9) as substrates, in contrast to the canonical Lux system, which requires long-chain fatty aldehydes. Despite this unique activity, the resulting luminescence is substantially dimmer—several orders of magnitude lower—than that of the *P. luminescens* LuxAB complex.[31]

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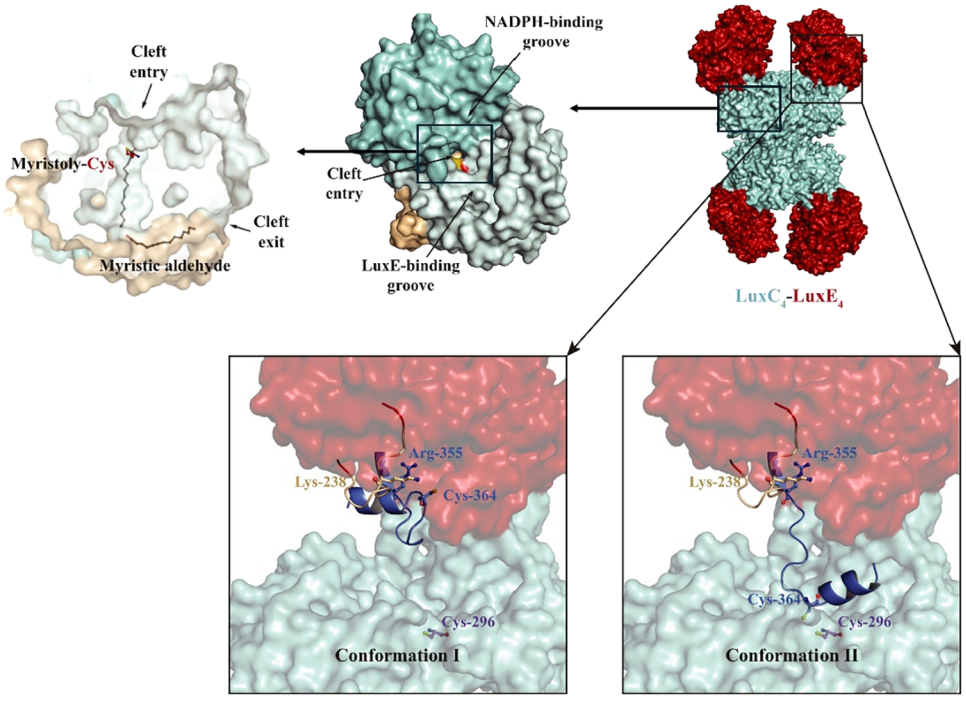

Figure 3. Structure of LuxC-LuxE complex with catalytic site and flexible conformation (I and II) of LuxE to LuxC complex. Reproduced from ref 21. Available under a CC-BY-NC-ND 4.0 license. Copyright 2022 Tian et al.

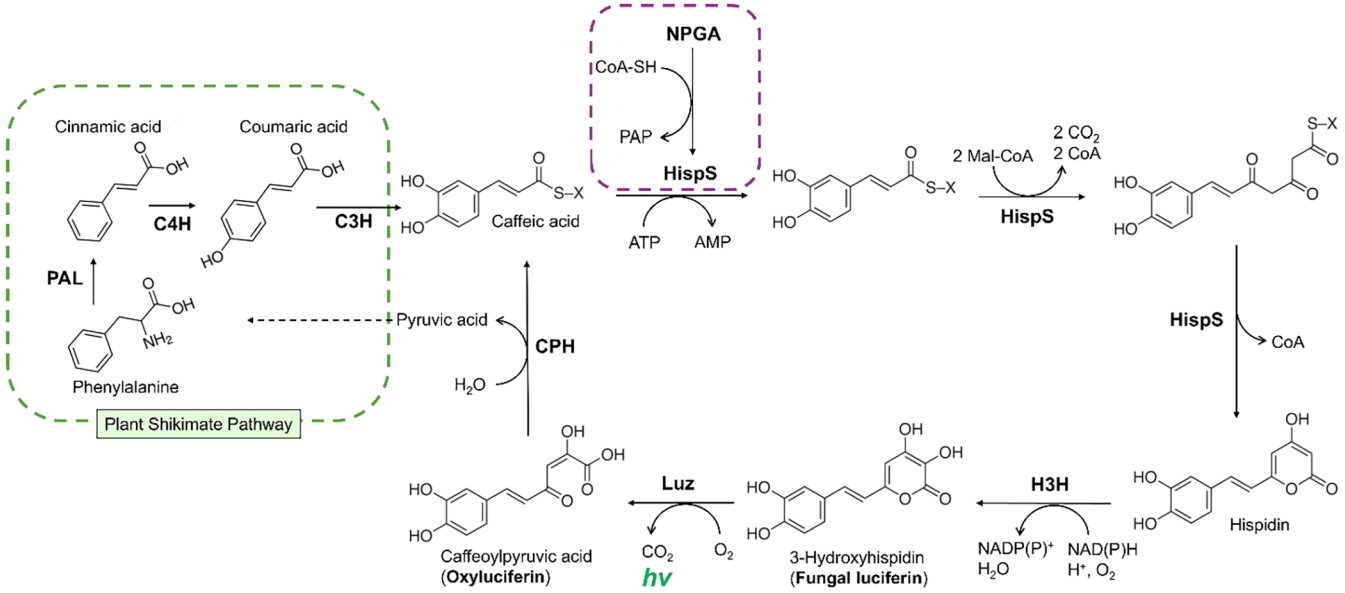

Figure 4. Fungal bioluminescence reaction pathway.

A recent cryo-EM study of the fatty acid reductase components in the bacterial bioluminescence system—LuxC and LuxE—has revealed that LuxC and LuxE form a tetrameric complex to catalyze the production of fatty aldehydes using ATP and NADPH. The interaction between LuxE and LuxC is dynamic, mediated primarily by weak nonpolar interactions, resulting in the formation of a $LuxC_4$–$LuxE_4$ complex[21] (Figure 3). The catalytic domain of LuxC features a key cysteine residue (Cys296) located at the entrance of a deep cleft between the cofactor-binding and catalytic domains. Mutation of this residue (C296A) abolishes the enzyme's activity by preventing covalent binding of the substrate to LuxC.[21] The study also demonstrated that NADPH acts as a direct cosubstrate, playing a critical role in cleaving the thioester bond and facilitating the final step in fatty aldehyde production.

The rate of light emission in the Lux system (B) can be modeled mathematically, as reported in the development of *ilux2*.[22] Here, F denotes $FMNH_2$, A aldehyde, O molecular oxygen, and L luciferase.

$$B = \frac{[F][A][O][L]}{(2 + [A])\cdot(1 + [O])\cdot(1 + [F] + [A])} \quad (1)$$

As shown in eq 1, bioluminescence increases less than proportionally with substrate concentrations. When both

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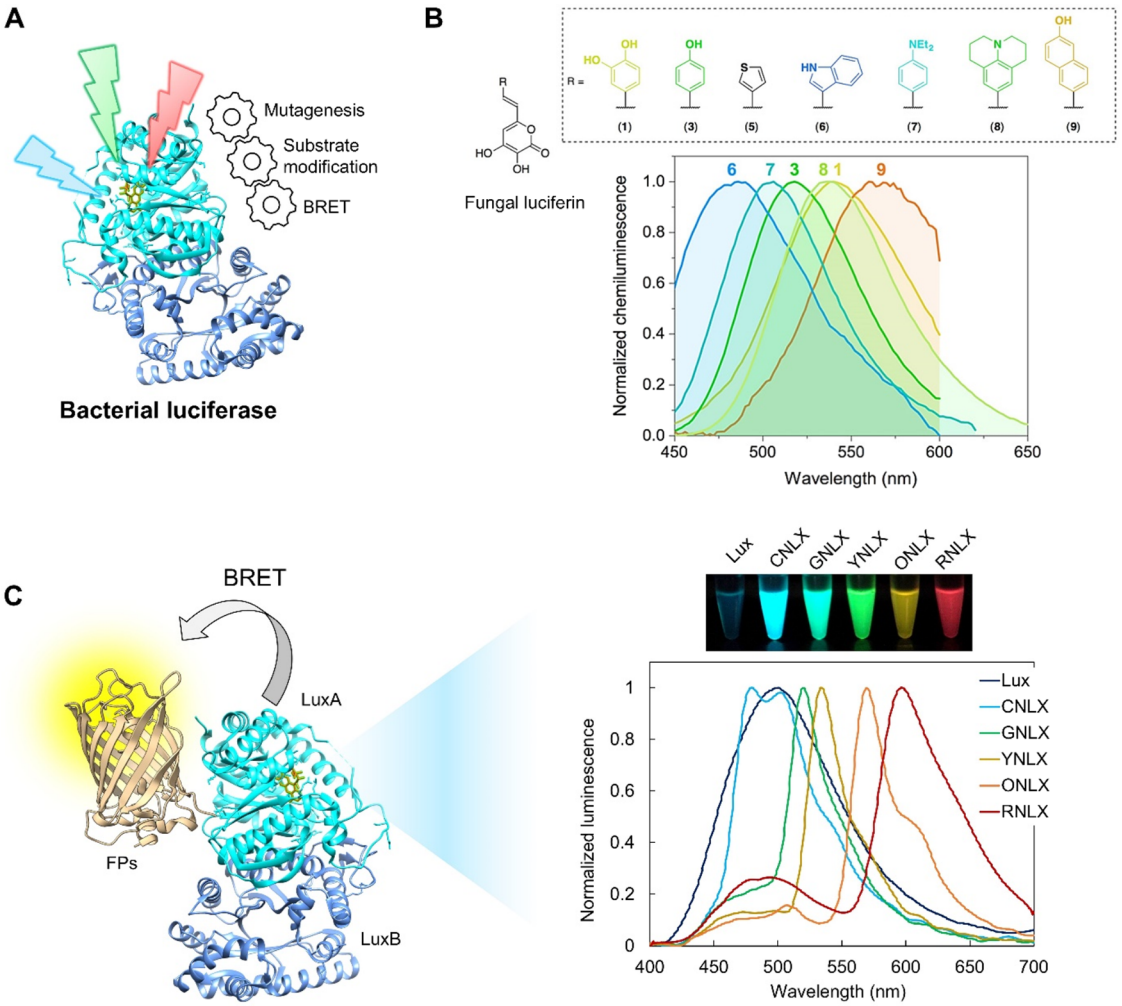

Figure 5. Color modulation of autonomous bioluminescence systems. (A) Strategy to shift the emission wavelength of autonomous bioluminescent proteins. (B) Modification of fungal luciferin to shift the emission wavelength. Reproduced from ref 66. Available under a CC-BY-NC 4.0 license. Copyright 2017 Kaskova et al. (C) Color modulation through BRET strategy. LuxA fuses with several fluorescent proteins to generate multicolor Lux (Nano-lanternX) from cyan to red variants. Reproduced from ref 18. Available under a CC-BY-NC-ND 4.0 license. Copyright 2024 Kusuma et al.

$FMNH_2$ and aldehyde are present in high concentrations, the luciferase becomes rate-limiting for the overall reaction. Sustained light emission therefore requires adequate intracellular supplies of ATP, NADPH, and FMN.[22] Fatty acids activation is tightly coupled to their reduction, with a stoichiometry of one fatty acid reduced to aldehyde and one NADPH oxidized for every ATP converted to AMP.[56] In *E. coli*, steady-state intracellular concentrations are approximately 1310 μM ATP, 560 μM NADPH, and 88 μM FMN.[57] Based on previous mathematical modeling,[58] the production of one LU of Lux bioluminescence (2.2×10^{10} photons s^{-1}) requires ~0.02 μM ATP and 4.36 μM NADPH, together with ~13.9 μM aldehyde and ~1.14 μM $FMNH_2$. These values indicate that the aldehyde−NADPH regeneration cycle constitutes the principal metabolic burden of the Lux system, whereas the ATP cost per photon is comparatively minor.[58,59]

Attempts to enhance Lux brightness have included codon optimization for mammalian expression (co Lux),[17] introduction of exogenous flavin reductase (Frp),[15,17] and a directed evolution of the *luxCDABE* operon (*ilux* and *ilux2*).[15,22] These strategies have improved performance in both bacterial and eukaryotic hosts, but further gains will likely require rational design informed by structural and computational approaches.

FUNGAL BIOLUMINESCENCE SYSTEM

The genes encoding the fungal bioluminescence pathway are relatively recent discoveries compared to those of bacterial bioluminescence.[11,60] Green light emission (~520 nm) was first confirmed from luciferase−luciferin extracts of*Mycena luxaeterna* in 2009.[61] Subsequent studies revealed that the fungal bioluminescence system utilizes a single gene to encode the luciferase enzyme.[62] In 2018, the complete biosynthetic cycle of the fungal bioluminescence pathway was elucidated from *Neonothopanus nambi*, identifying four key genes responsible for constituting an autonomous bioluminescence system.[11] This autonomous system integrates four essential enzymes (Figure 4):

1. Hispidin synthase (HispS)—converts caffeic acid to hispidin;
2. Hispidin-3-hydroxylase (H3H)—hydroxylates hispidin to produce the luciferin 3-hydroxyhispidin;
3. Luciferase (Luz)—oxidizes 3-hydroxyhispidin to generate oxyluciferin and emit green light (~520 nm);

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4. Caffeylpyruvate hydrolase (CPH)—recycles oxyluciferin back to caffeic acid, completing the cycle.

A fifth auxiliary enzyme, phosphopantetheinyl transferase (NPGA), post-translationally activates HispS, ensuring efficient luciferin production.[63] In plant expression systems, caffeic acid biosynthesis is driven by the native shikimate pathway,[20,64,65] which is also responsible for producing lignin and flavonoids. Phenylalanine, a key precursor, is converted to cinnamic acid by phenylalanine ammonia-lyase (PAL). Cinnamic acid is subsequently hydroxylated to p-coumaric acid by cinnamic acid 4-hydroxylase (C4H) and further converted to caffeic acid by p-coumaric acid 3-hydroxylase (C3H). Recent studies have shown that overexpression of C3H significantly enhances the brightness of the fungal bioluminescence system.[25]

Structurally, Luz protein from *N. nambi* (nnLuz) consists of 267 amino acids and has a molecular mass of approximately 28.5 kDa.[11] In contrast to bacterial LuxAB, nnLuz is relatively insoluble and possesses a predicted N-terminal transmembrane helix.[66] Recombinant nnLuz exhibits optimal enzymatic activity at around pH 8.0 and moderate temperatures, with a marked decline in activity above 30 °C.[11] As the crystal structure of nnLuz has not yet been resolved, the precise binding site for 3-hydroxyhispidin within the active site remains unknown, limiting rational protein engineering.[11] Nevertheless, stability improvements have been achieved through consensus mutagenesis (*e.g.*, I3S, N4T, F11L, I63T, T99P, T192S, and A199P).[24]

COLOR MODULATION OF AUTONOMOUS BIOLUMINESCENCE SYSTEM

Tuning the emission spectrum of autonomous bioluminescence systems expands their utility in multiplexed imaging, biosensing, and deep-tissue applications.[18] Three principal strategies have been applied to Lux and Luz systems (Figure 5A):

Luciferase Active-Site Mutagenesis

In Lux, mutations around the isoalloxazine-binding site of FMN can alter the emission peak. For example, LuxA-D113N red-shifts emission from 490 to 507 nm while LuxA-C106V induces an 8−10 nm shift.[67,68] A triple mutant (C106V/A75G/V173A) yields a 15 nm shift but reduces $FMNH_2$ affinity, lowering brightness.[69,70] These examples illustrate a common trade-off between spectral shift and luminescence intensity

Chemical Modification of Luciferin

In the fungal system, color modulation has been achieved by synthesizing analogs of 3-hydroxyhispidin with modified α-pyrone rings[66] (Figure 5B). This approach has produced five distinct colors *in vitro*, ranging from blue, green to orange, without altering the luciferase protein.

To date, modifying the α-pyrone ring of 3-hydroxyhispidin *in vivo* remains a major challenge, as no natural enzymatic reaction capable of efficiently performing such modifications has been identified.[66] While administration of synthetic analogs is feasible—for example, hispidin injections have been shown to induce transient luminescence in plants—this approach renders the system nonautonomous, as exogenous substrates must be supplied.[11,20] Within the fungal pathway, oxyluciferin is hydrolyzed enzymatically to regenerate caffeic acid, which is recycled through the styrylpyrone pathway to form hispidin; engineering analogous recycling loops for modified substrates would be substantially more complex.[71] Looking forward, AI-driven protein design tools (*e.g.*, ESM-3,[72] Pinal,[73] RFdiffusion[74]) may help identify or create enzymes capable of tailoring the α-pyrone ring *in vivo*, potentially enabling autonomous multicolor fungal bioluminescence in plants.

Resonance Energy Transfer (RET) to Fluorescent Proteins

Bioluminescence resonance energy transfer (BRET) can shift emission spectra by transferring energy from luciferase to a fluorescent protein acceptor.[75,76] Natural examples occur in *Photobacterium* species, where LuxAB associates with yellow fluorescent protein (YFP)[77] or lumazine protein (LumP),[78,79] producing yellow or blue-shifted emissions, respectively. The primary mechanism of energy transfer in these systems is BRET, and it has been suggested that long-chain fatty aldehydes facilitate the association between luciferase and the fluorescent protein.[80] However, the use of *Photobacterium* YFP and LumP as acceptor proteins is sensitive to environmental conditions, such as temperature and pH, which can alter the apparent color of Lux luminescence.[81,82]

To improve BRET efficiency, genetically encoded BRET-based luciferases have been developed by fusing *Renilla* luciferase or NanoLuc to various fluorescent proteins (FPs), resulting in the Nanolantern series.[83−85] Inspired by these systems, engineered fusions of LuxA[18] or LuxB[86] to fluorescent proteins have yielded multicolor autonomous systems such as Nano-lanternX (NLX),[18] spanning cyan to red variants. BRET efficiency is highly dependent on donor−acceptor distance and orientation, making rational linker design critical (Figure 5C).

While Lux has demonstrated *in vivo* multicolor imaging in bacteria, mammalian cells, and plants through the NLX platform, fungal systems have not yet achieved equivalent flexibility.[66] Two major bottlenecks remain: the limited structural information available for Luz and the complexity of synthesizing luciferin analogs *in vivo*.[66] However, a recent study addressed these challenges by enhancing BRET efficiency in nnLuz through an engineering approach that fused fluorescent proteins directly into the luciferase sequence.[29] Using an insertion−deletion strategy guided by AlphaFold[30] structural predictions, Morozov et al. identified flexible loop regions near residues 167 and 188 of nnLuz that tolerated the insertion of fluorescent proteins such as mScarlet-I3 and mKate2.[29] These fusions successfully red-shifted the emission spectrum beyond green, demonstrating that structure-guided FP insertion can expand the spectral range of fungal autonomous bioluminescence systems.

AUTONOMOUS BIOLUMINESCENCE IMAGING APPLICATIONS

Bioluminescence imaging (BLI) offers inherent advantages over fluorescence imaging by eliminating the need for external excitation light, thereby reducing background noise, minimizing phototoxicity, and enabling sensitive detection in opaque tissues.[83,84,87] Autonomous bioluminescent systems extend these advantages by continuously generating their luciferin substrate *in situ*, allowing long-term monitoring without repeated luciferin addition.[15]

Bacterial bioluminescent proteins (Lux), which possess autonomous bioluminescence capabilities, still produce relatively low light intensity compared to nonautonomous bioluminescent systems such as NanoLuc or firefly luciferase

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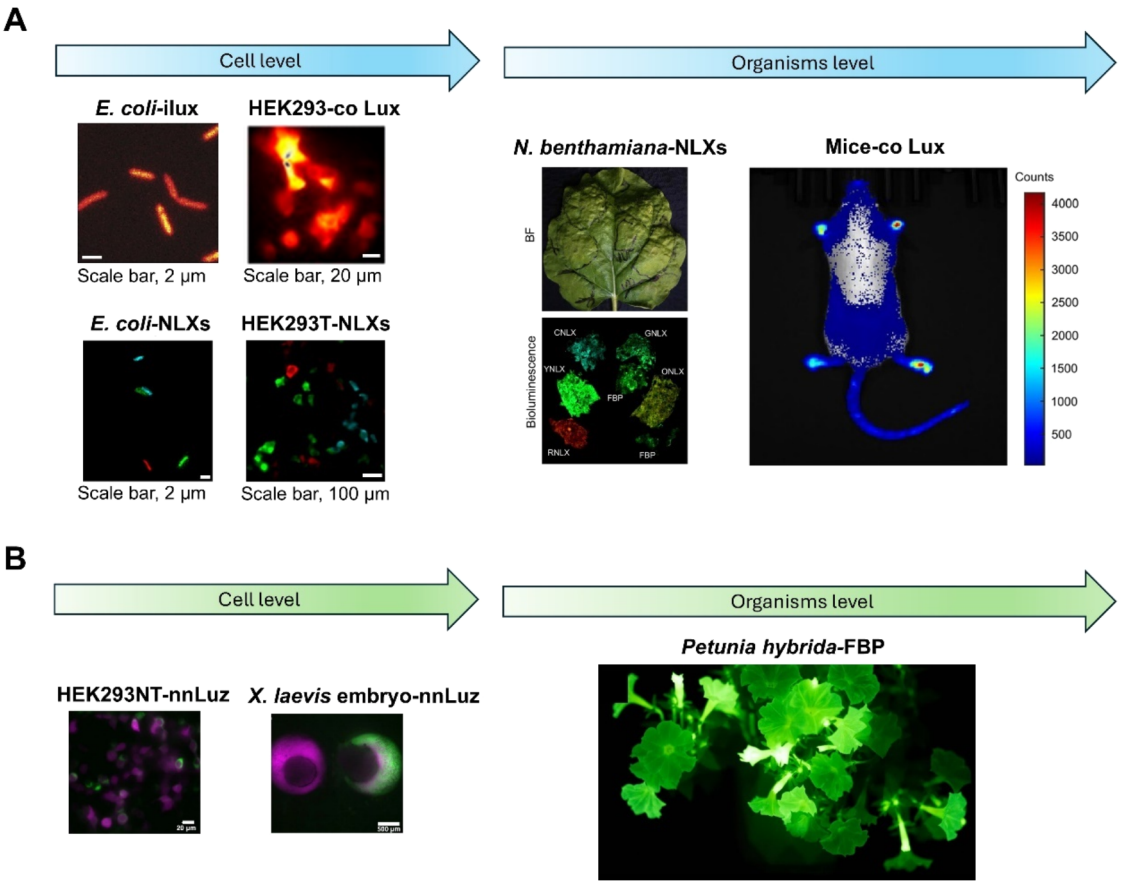

Figure 6. Autonomous bioluminescence imaging in living cells and organisms. (A) Expression of Lux variants in single color[15,17] and multiplexed imaging[18] in cell level, plant,[18] and mice.[91] Reproduced from ref 15,17,18. All images are available under a CC-BY-NC-ND 4.0 license. Copyright 2018 Gregor et al., 2019 Gregor et al., and 2024 Kusuma et al. Reproduced from ref 91. Available under a CC-BY-NC 4.0 license. Copyright 2025 Kiszka et al. (B) Expression of Luz variants in cell levels to plants.[11,24] Reproduced from ref 11. Available under a CC-BY-NC-ND 4.0 license. Copyright 2018 Kotlobay et al. Reproduced from ref 24. Available under a CC-BY 4.0 license. Copyright 2024 Shakova et al.

(Fluc), particularly for single-cell imaging.[15,18] To enhance Lux signal output, error-prone mutagenesis has been applied to the entire *luxCDABE* operon and *Frp* gene, resulting in the development of *ilux*.[15] *ilux* enables long-term bioluminescence imaging of single *E. coli* cells using a bioluminescence microscope (Figure 6A). The improved luminescence intensity is comparable to that of Fluc, with the added benefit of stable signal output. Further rounds of mutagenesis led to *ilux2*,[22] which, when chromosomally integrated in *E. coli*, showed a 7-fold increase in brightness compared to *ilux*.

However, when Lux is expressed in eukaryotic systems such as mammalian cells, it typically generates dim luminescence, posing a challenge for single-cell imaging.[17] To address this, human codon optimization has been employed to enhance the codon adaptation index (CAI) for efficient expression in mammalian cells.[17,88,89] Higher CAI values are critical for improving Lux-based luminescence; for example, the previously reported codon-optimized version, $pCMV_{lux}$[89] (CAI: 0.74), exhibited lower luminescence compared to co Lux[17] (CAI: 0.95). In addition, the introduction of flavin reductase (Frp) is essential, as mammalian cells lack an endogenous equivalent, unlike *E. coli*. co Lux has been successfully applied in mammalian systems such as HEK293 cells, enabling long-term luminescence imaging[17] (Figure 6A).

ilux and co Lux are well suited for single-cell imaging in bacterial and mammalian systems, respectively. Multiplexed imaging using Lux can be achieved through NLX,[18] a multicolor variant of Lux, which enables successful multiplexed imaging of single *E. coli* cells. Furthermore, by incorporating NLX into the co Lux system, autonomous multicolor bioluminescence imaging has also been realized in mammalian cells (Figure 6A). At the organismal level, the Lux system has been successfully expressed in *Nicotiana benthamiana* leaves using NLXs,[18] in *Caenorhabditis elegans* through the AMBER construct (a fusion of the Ciona voltage-sensing domain with LuxAB),[90] and in the first transgenic mice engineered to express co Lux[91] (Figure 6A).

In fungal luciferase systems, autonomous bioluminescence imaging in bacterial and mammalian cells shows limited performance due to the lack of endogenous caffeic acid biosynthesis.[11,23,24] Although single-cell imaging has been demonstrated in mammalian cells[11] (Figure 6B), bioluminescence emission requires either the external addition of fungal luciferin[11] or supplementation with micromolar concentrations of caffeic acid[24] in cells expressing the complete fungal bioluminescence pathway (FBP). At the organismal level, the FBP performs exceptionally well in plant hosts.[20,23−25] Since plants naturally produce caffeic acid, autonomous bioluminescence signals can be detected by the naked eye in various plant species[24] (Figure 6B). Despite these successes, significant improvements in brightness, spectral range, and

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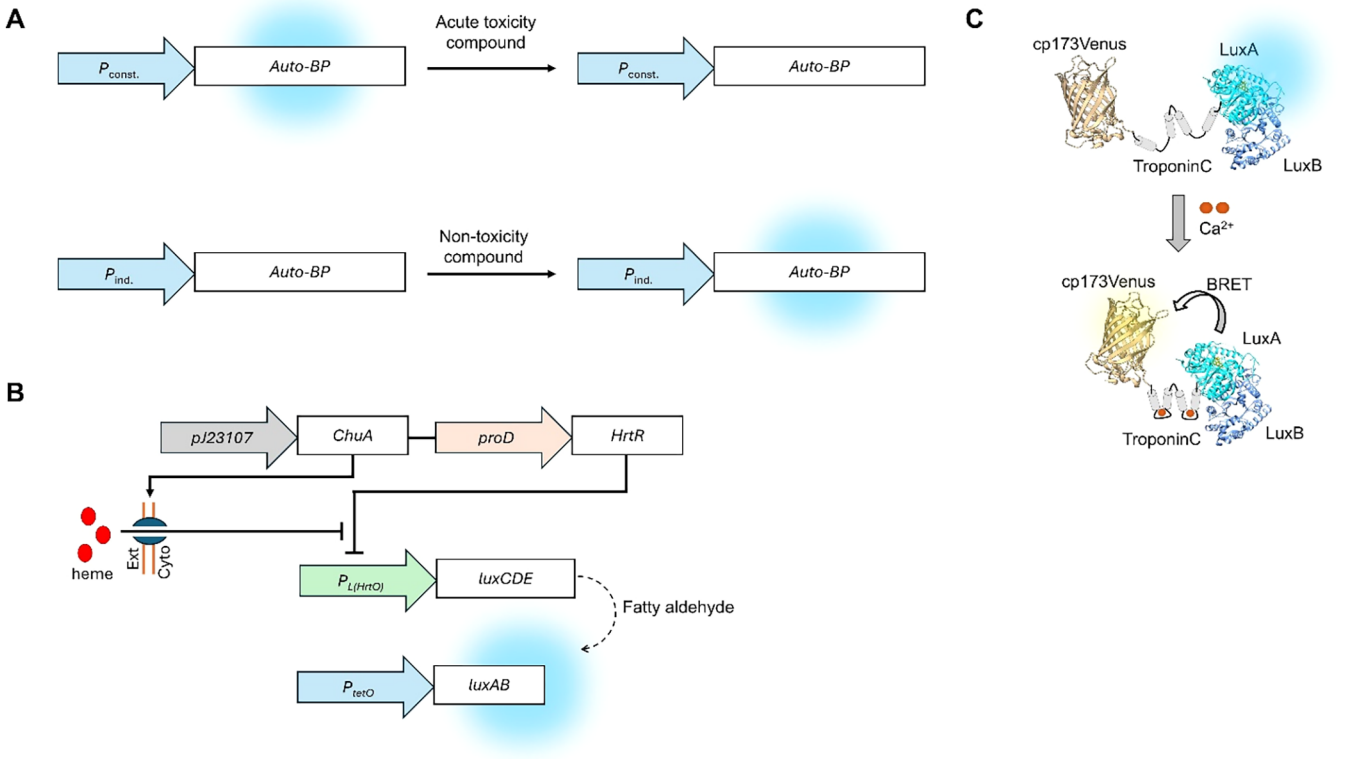

Figure 7. Type of autonomous bioluminescence assay. (A) "Turn-off" (top)- and "turn-on" (bottom)-type assays. $P_{const.}$ and P_{ind} represent constitutive and inducible promoters, respectively. Auto-BP is an autonomous bioluminescence pathway cassette. (B) Genetic circuit of "turn-on" assay based on Lux biosensor. (C) Ratiometric assay using a bioluminescent donor and a fluorescent acceptor. Reproduced from ref 18. Available under a CC-BY-NC-ND 4.0 license. Copyright 2024 Kusuma et al.

tissue penetration depth are needed for broader adoption, particularly in mammalian biomedical research.[11]

AUTONOMOUS BIOLUMINESCENCE SYSTEMS AS BIOREPORTER APPLICATIONS

Autonomous bioluminescence systems are uniquely suited for whole-cell biosensing because they allow continuous, reagent-free monitoring of cellular responses.[17,18,88,91,92] Unlike protein-based assays, whole-cell bioreporters capture integrated physiological effects, including cytotoxicity, metabolic stress, and regulatory network activity.[22] Based on changes in light emission, bioreporter formats can be broadly categorized into turn-off,[22] turn-on,[93] and ratiometric types.[18] (Figure 7).

Turn-Off Assays

Autobioluminescence systems consume cellular energy for luciferin biosynthesis, even though the luciferase itself is ATP-independent.[22] As a result, compounds or pollutants that disrupt ATP or NADPH production can influence overall luminescence intensity.[15,22] In "turn-off" type assays, acute toxicants or antibiotics cause a rapid decrease in luminescence before cell death occurs (Figure 7A). For example, *E. coli* expressing *luxCDABE* without antibiotic resistance shows blinking bioluminescence before the signal is eventually lost following prolonged incubation with kanamycin.[15] Coexpression with the fluorescent ATP biosensor QUEEN-2m[94] has revealed that the loss of bioluminescence under kanamycin treatment is accompanied by a corresponding decrease in intracellular ATP levels.[15] In addition, *ilux2* has been applied not only to study antibiotic resistance but also in food safety research. By integrating the lux operon into the *E. coli* chromosome, it stably emits luminescence, enabling long-term monitoring of bacterial behavior under different food storage conditions.[22]

Turn-On Assays

"Turn-on" type assays represent the most common application of autobioluminescent systems[95−98] (Figure 7A). These assays utilize specific promoters or regulatory regions that respond to inducible compounds or molecules to drive bioluminescence as a readout.[93] Naturally, in some bioluminescent bacteria—such as *Aliivibrio fischeri*—luminescence is regulated through a phenomenon known as quorum sensing, which is linked to cell population density.[99] As illustrated in Figure 1A, the *luxI* gene encodes an enzyme that synthesizes the autoinducer N-acyl homoserine lactone (AHL).[34,99] Upon reaching a threshold concentration, AHL binds to the *luxR* transcriptional regulator, activating the lux operon and triggering bioluminescence.[99] This native quorum sensing mechanism is valuable for studying bacterial population dynamics, biofilm formation, and the emergence of antibiotic resistance.[99−101]

The self-sufficient property of the "turn-on" type assays makes them highly promising for use as whole-cell biosensors for detecting specific metabolites and compounds.[92] In Lux-based assays, a variety of specific promoters—such as *recN, sbmC, sulA, dinI, alkA, grpE, soxS*, and *ibpA*—have been fused to the Lux operon for applications including genotoxicity screening and monitoring of SOS response, heat shock, and oxidative stress responses.[95,102] For the detection of metal ions and metalloids, specific promoters and regulatory elements have been coupled with the Lux operon, including *arsD* for

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Table 2. Comparison of Bacterial (Lux) and Fungal (Luz) Autonomous Bioluminescence Systems

feature	bacterial (Lux)	fungal (Luz)	refs
emission peak	blue-green (~480−500 nm)	green (~520 nm)	11,15
quantum yield	~0.1−0.2 photons per reaction	estimated similar or slightly lower (exact QY not yet established)	26,66
genetic components	*luxCDABE* operon ± *luxG*/*frp*; 5−6 genes	HispS, H3H, Luz, CPH ± NPGA; 4−5 genes	14,15,20
substrate biosynthesis	requires $FMNH_2$ and long-chain fatty aldehyde (*e.g.*, tetradecanal)	requires 3-hydroxyhispidin (from caffeic acid via shikimate pathway)	15,20
endogenous substrate availability	complete pathway in bacteria; in eukaryotes requires $FMNH_2$ supplementation (Frp)	in plants: caffeic acid via native pathway; in bacteria/mammals: needs supplementation	17,20
brightness in native host	moderate; improved variants (*ilux*, co Lux) increase brightness	high in plants; low in bacteria/mammals without supplementation	15,17,20
host range (demonstrated)	bacteria, mammalian cells, *C. elegans*, plants, transgenic mice	plants (*e.g.*, *N. benthamiana, Arabidopsis*), limited in bacteria/mammals	18,24,90,91
color tunability	active-site mutations, BRET with FPs, substrate analogs	primarily via luciferin analogs; limited protein engineering	18,66
strengths	fully genetically encoded in bacteria; multicolor (NLX); ratiometric biosensing possible	bright, sustained luminescence in plants; compatible with visible imaging	18,24
limitations	low brightness in eukaryotes; metabolic burden; limited red-shift	substrate limitation in nonplants; lack of structural data; few ratiometric tools	18,24
key recent advances	codon optimization (co Lux), high-brightness mutants (*ilux2*), multicolor NLX	pathway elucidation, substrate analog−based color variants, C3H overexpression in plants	17,18,22,24,25

arsenic,[103] *cop*A for silver, gold, and copper ions,[96,104] *merR* for mercury,[105] *zntR* and *pbr* for Zn^{2+} and Pb^{2+}, respectively.[106]

In biological and biomedical applications, Lux has also been fused with circadian-related promoters. For example, in *Bacillus subtilis*, Lux has been linked to the *ytvA* and *kinC* genes,[107] and in cyanobacteria (*Synechococcus* strains) to the *kaiABC* circadian genes.[108] Although circadian rhythms in mammalian cells are typically monitored using firefly luciferase (FLuc),[109] a human codon-optimized Lux offers a viable alternative for autonomous detection. Not only circadian-related promoter but also the Lux operon has been fused to lipopolysaccharide (LPS) gene cluster promoters of bacterial pathogens such as *Yersinia enterocolitica* serotype O:3 (YeO3), enabling real-time monitoring of virulence gene expression and trafficking both *in vitro* and *in vivo*.[110] Notably, codon-optimized Lux has been successfully applied to monitor induced pluripotent stem cell (iPSC) activity via the *NANOG* promoter,[98] as well as β-catenin[18] in HEK293T cells using Wnt-responsive promoter elements. In addition, codon-optimized Lux has been employed in drug discovery as a rapid and high-throughput biosensor. Application includes screening of androgen agonist compounds through androgen receptor (AR)-mediated transcriptional activation,[97] monitoring the cytotoxic effects of doxorubicin exposure in various mammalian cell lines,[98] and the identification of inhibitors targeting the NF-κB-signaling pathway.[93]

The "turn-on" assay based on lux has been expanded beyond single-promoter, single-signal formats to include synthetic gene circuits. One notable example is a genetic circuit designed for heme detection in urine (Figure 7B).[92,111] To minimize leaky expression while maintaining strong luminescence output and dynamic range, four promoters were strategically deployed: (1) *luxAB* was constitutively expressed under the P_{tetO} promoter on a low-copy plasmid; (2) *luxCDE* was placed under the heme-sensitive promoter $P_{L(HrtO)}$ on a high-copy plasmid; (3) the outer-membrane transporter *ChuA*, responsible for heme uptake, was constitutively expressed from the pJ23107 promoter on a medium-copy plasmid; and (4) the heme-responsive transcriptional repressor HrtR, which regulates $P_{L(HrtO)}$, was expressed under the proD promoter. Upon exposure to varying heme concentrations, the system produced significant bioluminescence signals, demonstrating a robust and sensitive platform for noninvasive detection of heme in biological samples.[92]

In fungal luciferase (Luz)-based "turn-on" assays, successful applications have been demonstrated using specific promoters in plants. For example, Luz has been used to monitor gene expression driven by flower-specific promoters such as *ODORANT1*, as well as hormone-responsive promoters including *At-RAB18* (abscisic acid/ABA), *WRKY70* (salicylic acid), and *ORCA3* (jasmonic acid) during drug treatments and pathogen attacks.[63,112] Overall, both Lux and Luz systems offer valuable platforms for real-time, self-luminescent "turn-on" assays that eliminate the need for external luciferin addition.

Ratiometric Assays

Ratiometric signal−based assays rely on the ratio of bioluminescence to an acceptor signal, typically from a fluorescent protein[18,85] (Figure 7C). Among autobioluminescent systems, only the Lux system has been engineered to provide ratiometric output, for example, by fusing calcium-binding proteins such as Troponin C (TnC) to respond to intracellular calcium levels.[18] This ratiometric approach enables more accurate signal interpretation by minimizing variability caused by metabolic fluctuations affecting luminescence intensity.[18] While both Lux and Luz systems have proven adaptable, limitations remain in sensitivity, host range, and quantitative robustness.[18,20] Addressing these will expand their utility in environmental monitoring, biotechnology, and biomedical diagnostics.

COMPARATIVE OVERVIEW OF BACTERIAL (LUX) AND FUNGAL (LUZ) BIOLUMINESCENCE SYSTEMS

To contextualize the strengths, limitations, and potential applications of bacterial and fungal autonomous bioluminescence, we summarize key features side-by-side (Table 2). This comparison highlights fundamental biochemical differences, practical performance metrics, and engineering opportunities for each platform.

OUTLOOK AND FUTURE PERSPECTIVE

Autonomous bioluminescence systems have transitioned from biological curiosities to versatile, genetically encodable tools

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Table 3. Critical Summary of Autonomous Bioluminescence Systems

feature	key advances	limitations	future opportunities	refs
bacterial autonomous bioluminescence	functional reconstruction of *luxCDABE* operons across diverse hosts, with metabolic support from *luxG* or *frp*.	low quantum yield (~0.1−0.2) limits the detection of rapid and low-intensity events.	AI-guided mutagenesis and *de novo* design to enhance catalytic efficiency and reaction kinetics.	14,18,21,26,114
	Cryo-EM elucidation of the LuxC−LuxE complex, revealing the structural basis of fatty acid reduction.	metabolic burden from long-chain aldehyde synthesis may impair host growth.	spectral expansion from blue-green toward near-infrared for deep-tissue imaging.	
			modularized size-reduced gene cassettes for delivery via viral vectors and other compact systems	
fungal autonomous bioluminescence	complete pathway elucidation in *N. nambi*, enabling autonomous luminescence in plants.	in nonplant hosts, caffeic acid availability severely limits brightness.	structural determination of Luz to guide targeted protein engineering.	11,24,25
	exploitation of the plant shikimate pathway for endogenous caffeic acid production.	lack of crystal structure for Luz hampers rational mutagenesis and spectral tuning.	engineering caffeic acid biosynthesis pathways into nonplant hosts.	
			integration of Luz-based reporters into plant synthetic biology for spatiotemporal monitoring of gene expression and physiology.	
color modulation of autonomous bioluminescence	Lux emission tuning through active-site mutations and BRET fusions with fluorescent proteins.	fungal multicolor emission demonstrated only *in vitro*, with limited *in vivo* validation.	use of AI-based structural prediction and molecular docking to optimize BRET donor−acceptor configurations.	18,66,114
	in vitro generation of distinct fungal emission colors via luciferin analogs.	RET efficiency strongly depends on precise spatial arrangement, complicating design.	*in vivo* compatible synthesis of fungal luciferin analogs for multicolor plant imaging.	
	development of nano-lanternX (NLX) for multicolor autonomous imaging across hosts.			
autonomous bioluminescence imaging applications	*ilux* and *ilux2* enable single-cell bacterial imaging with brightness comparable to conventional luciferases.	Lux brightness in mammalian systems remains insufficient for high-speed or deep-tissue imaging.	engineering Lux for increased photon output and faster emission kinetics for dynamic process monitoring.	18,22,24,29
	co Lux supports long-term luminescence in mammalian cells, with multicolor capability via NLX.	fungal system's performance is restricted outside plant hosts due to caffeic acid limitation.	introducing caffeic acid biosynthesis pathways into nonplant hosts to enable robust Luz imaging.	
	fungal pathway achieves naked-eye−visible, substrate-free imaging in transgenic plants.	high-resolution imaging often requires long exposure times, limiting temporal resolution.	developing hybrid Lux−Luz systems or chimeric pathways to combine complementary strengths.	
bioreporter applications	Lux-based reporters developed for diverse targets: genotoxicity, oxidative stress, metals, quorum sensing, circadian rhythms, and signaling pathways.	autonomous bioluminescent metabolic burden can reduce sensitivity in long-term assays.	metabolic streamlining of Lux and Luz for stable, low-burden long-term sensing.	18,24,29
	ratiometric Lux designs (*e.g.*, NLX-calcium sensors) improve quantitative reliability.	Luz applications remain plant-centric, with limited cross-host adaptability.	expanding Luz reporter applications to nonplant systems via precursor pathway engineering.	
	Luz-based reporters in plants enable hormone and developmental pathway monitoring without substrate addition.	quantitative accuracy in fluctuating metabolic states requires normalization strategies.	developing multiplexed, autonomous, multiparameter biosensors for real-time environmental and biomedical monitoring.	
			developing hybrid Lux−Luz systems or chimeric pathways to combine complementary strengths.	

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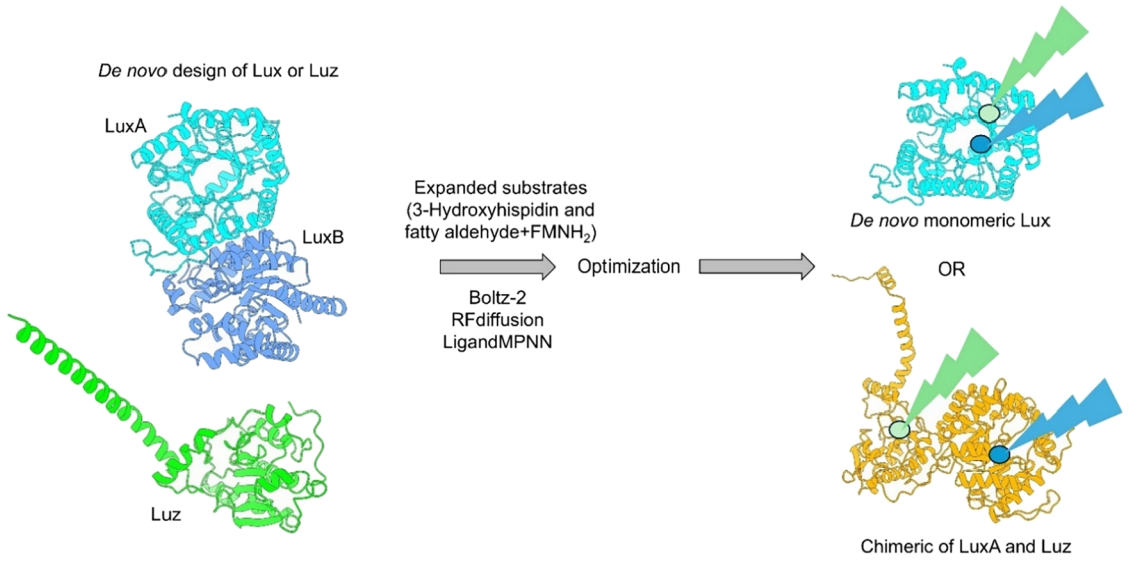

Figure 8. Hybrid lux-luz system generation by leveraging AI approach. Protein design models were generated by Alphafold3.[30]

for imaging, sensing, and synthetic biology.[18,20,90,91] Their defining advantage—the ability to produce sustained light emission without exogenous substrate—has enabled long-term, noninvasive monitoring in diverse contexts, from microbial physiology to plant development and mammalian cell signaling.[20,91] To conclude this Perspective, we provide a critical summary of recent advances, current limitations, and future opportunities of autonomous bioluminescence systems for clarity, accessibility, and overall readability (Table 3).

Despite their potential, next-generation systems must overcome persistent limitations. For Lux, the primary bottlenecks are low quantum yield (~0.1−0.2),[26] restricted spectral range (blue-green), and significant metabolic burden from long-chain aldehyde biosynthesis.[18] In eukaryotes, limited $FMNH_2$ availability further constrains brightness.[17] For Luz, while plant performance is exceptional, substrate availability (caffeic acid) is a major barrier in nonplant hosts, and the absence of high-resolution structural data limits rational protein engineering.[11]

Looking forward, several strategies are poised to transform the field:

1. *Structure-guided protein engineering and AI-driven design.* Recent cryo-EM and homology modeling of Lux components open the door to targeted active-site redesign to enhance catalytic turnover, substrate affinity, and quantum yield.[21] AI-guided mutagenesis[113] and *de novo* luciferase design promise to accelerate this process, with early successes such as LuxSit luciferase,[114] which represents a synthetic, nonautonomous bioluminescent platform. At the molecular level, a hybrid system could be realized by coupling the efficient substrate-synthesis modules of one pathway with the luciferase of the other. For example, the fungal Luz luciferase could, in principle, be engineered to accept bacterial aldehyde-derived intermediates as alternative substrates, thereby leveraging the Lux pathway's robust aldehyde-generating machinery, and vice versa. However, achieving such cross-compatibility remains a significant challenge. In combination with recent advances in AI-driven de novo protein design, including emerging tools such as Boltz-2,[115] BindCraft,[116] and earlier frameworks like RFdiffusion[117] and LigandMPNN,[117] this approach may become feasible in the near future (Figure 8).
2. *Metabolic pathway optimization.* Streamlining aldehyde and $FMNH_2$ production in Lux systems can reduce metabolic burden and improve stability in long-term applications.[27] In Luz, introducing caffeic acid biosynthesis into nonplant hosts will broaden applicability.[29]
3. *Spectral expansion and multiplexing.* Combining active-site engineering, luciferin analog synthesis, and BRET-based designs can extend emission into the yellow−red and near-infrared ranges, enabling deep-tissue imaging.[18,29] Near-infrared (NIR) wavelengths are particularly advantageous because biological tissues exhibit reduced light absorption and scattering in this range, allowing photons to penetrate deeper and improving imaging sensitivity in whole organisms.[118] Parallel development of orthogonal color channels will facilitate multiparameter sensing.
4. *Application-driven design targets.* For biomedical research: near-infrared Lux/Luz variants with >10× current brightness and millisecond-scale temporal resolution.[18,29] For environmental monitoring: low-burden Lux systems operable for months in field conditions.[119] For synthetic biology: modular gene cassettes <10 kb for viral or organelle-targeted delivery.[23]

By integrating these strategies, autonomous bioluminescence systems could evolve into general-purpose, plug-and-play modules for biological monitoring, enabling a paradigm shift toward truly continuous, *in situ*, multiscale observation across the life sciences.[17,18,24,91,98]

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The manuscript draft was written by S.H.K. through contribution from M.H. and edited by T.N. CRediT: **Subhan Hadi Kusuma** writing - original draft; **Takeharu Nagai** writing - review & editing.

Notes

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■ ABBERVIATIONS

Auto-BP, autonomous bioluminescence pathway; BRET, bioluminescence resonance energy transfer; FBP, fungal bioluminescence pathway

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