Firefly Luciferase Systems: Molecular Mechanisms, Engineering Advances, and Biomedical Applications

Yuyan Wen*

St. Thomas Aquinas Catholic Secondary School, Oakville, ON L6K 2W1, Canada

*Corresponding author's e-mail: chloewen597@gmail.com

Abstract. Firefly luciferase-based systems represent a highly efficient and versatile platform for molecular imaging, biosensing, and functional protein analysis. This study systematically explores the biochemical mechanisms underlying luciferase-catalyzed bioluminescence, highlighting key factors affecting activity, including pH, temperature, and essential cofactors. Advances in luciferin analogues, such as AkaLumine-HCl, have extended emission wavelengths into the near-infrared region, enabling deeper tissue imaging with enhanced sensitivity and reduced autofluorescence. Engineered luciferase mutants demonstrated improved catalytic efficiency and stability, as validated by cellular uptake and in vivo imaging performance. Additionally, luciferase-based assays provide powerful tools for real-time ATP detection, offering insights into cellular energy metabolism and tumor microenvironments. The integration of split-luciferase and BRET systems further expands the application scope to dynamic monitoring of protein–protein interactions with high spatiotemporal resolution. Collectively, these findings deepen our understanding of luciferase's structure–function relationships and pave the way for next-generation bioluminescent probes with improved performance in biomedical research, diagnostics, and environmental monitoring.

Keywords: "Firefly luciferase", "Bioluminescence imaging", "Luciferin analogues"

1 Introduction

In recent years, the molecular mechanism of firefly bioluminescence and its application in molecular imaging have seen systematic advancements, reflecting the close integration of basic research with clinical needs. At the molecular level, firefly bioluminescence is a highly specific enzymatic reaction, in which luciferase catalyzes the oxidative transformation of the luciferin substrate. This reaction exhibits remarkable biochemical features: first, it has a quantum yield exceeding 90%, with an energy conversion efficiency far superior to that of conventional chemiluminescent systems; second, it is strictly dependent on ATP as an energy source, a characteristic that confers unique biological specificity in live-animal imaging [1].

K. Bellitürk et al. (eds.), *Proceedings of the 2025 2nd International Symposium on Agricultural Engineering and Biology (ISAEB 2025)*, Advances in Biological Sciences Research 50,
https://doi.org/10.2991/978-94-6463-910-0_15

However, further studies have revealed that the luminescent properties of this system are regulated by multiple factors. On the molecular scale, the conserved amino acid residues at the active site of luciferase form key catalytic regions essential for enzymatic activity. In terms of environmental factors, the reaction operates optimally at a pH range of 7.5 to 8.0 and is sensitive to temperature changes within 25–30°C, with magnesium ions (Mg^{2+}) required as essential cofactors. While these finely tuned regulatory mechanisms ensure high specificity, they also pose limitations to the stable application of the system under complex biological conditions [2] [3]. This review uniquely integrates recent biochemical and structural insights into luciferase systems, with a focus on their translational potential in tumor imaging, ATP biosensing, and protein interaction analysis.

2 Bioapplications of Luciferase System

2.1 In Vivo Tumor Imaging

The bioluminescence of fireflies represents an efficient cold light phenomenon, wherein approximately 90% of the energy is converted into light energy. The emitted light is typically green, yellow, or blue in color, depending on the specific type of luciferin used and the surrounding environmental conditions. The underlying mechanism of this light emission involves the catalytic action of luciferase on luciferin, in the presence of oxygen and ATP, resulting in the formation of a high-energy intermediate that ultimately releases photons. However, the emission peak of D-luciferin is approximately 562 nm, which limits its penetration through biological tissues, particularly in deeper tissues such as internal organs, thus hindering effective deep-tissue imaging. Moreover, while optimal temperatures can enhance enzyme activity, extreme temperatures inhibit the reaction altogether, further complicating its application [2].

The activity of luciferase is also significantly influenced by variations in pH levels, with different species of fireflies exhibiting distinct optimal pH ranges for maximal enzyme activity. Additionally, the concentrations of oxygen and ATP are crucial determinants of the intensity of light emission, and metal ions such as magnesium can enhance the reaction's efficiency. Conversely, certain heavy metals act as inhibitors of luciferase activity. These various factors contribute to the instability of luciferase in complex biological environments, such as within living organisms, thus limiting its ability to produce reliable and consistent results for detection purposes. Due to its inherent instability, luciferase is predominantly suitable for short-term experimental use, and it fails to support long-term monitoring applications, such as tracking tumor growth over several weeks [4]. This instability also necessitates repeated injections of luciferin substrates during therapeutic applications, as enzyme inactivation occurs, leading to increased costs and risks associated with treatment. Furthermore, as luciferase is commonly sourced from various species, its introduction into animal or human systems may trigger immune responses, accelerate enzyme clearance, and potentially induce inflammation.

In response to these challenges, researchers have adopted innovative, interdisciplinary strategies that integrate advances in chemistry, structural biology, and enzyme engineering. In the realm of chemical modification, design of the luciferin core structure has led to development of novel derivatives of AkaLumine-HCl. The peak emission wavelength of AkaLumine-HCl is 675 nm, which has better deep tissue imaging ability and is suitable for BLI at low doses. X-ray crystallographic analysis reveals that the introduction of rigid moieties, effectively alters the molecular orbital energy distribution in a red-shift of the emission wavelength into the near-infrared region (approximately 677 nm). Spectroscopic characterization further confirms a substantial Stokes shift of up to 115 nm, which significantly reduces interference from tissue autofluorescence. In the field of enzyme engineering, directed evolution techniques have produced mutant luciferases exhibiting markedly enhanced catalytic efficiency along with improved tolerance to physiological temperature fluctuations. The efficacy of these engineered systems is further substantiated by cellular experiments: flow cytometry analysis demonstrates a 2.3-fold increase in cellular uptake of the novel probes compared to conventional systems, while fluorescence imaging visually illustrates their superior intracellular distribution. Collectively, these technological advancements offer a multifaceted solution to the limitations of current bioluminescence imaging systems, paving the way toward more sensitive and reliable applications in deep-tissue imaging and real-time biological monitoring [6].

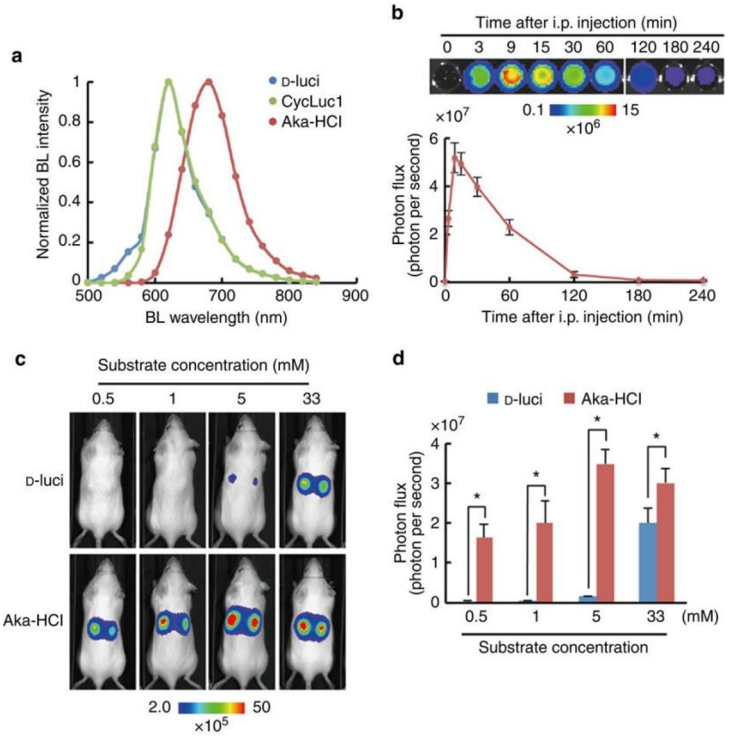

Fig. 1. In vivo bioluminescence imaging using AkaLumine-HCl.

Substrate-dependent emission spectra, serum half-life, and tumor imaging data demonstrate the superior performance of Aka-HCl in deep-tissue bioluminescence imaging. Figure adapted from (Kuchimaru et al., 2016) [6].

The results in animal studies have further substantiated the clinical potential of the newly developed bioluminescent imaging system (Figure 1). In standardized murine models, near-infrared (NIR) probes demonstrated pronounced advantages: in subcutaneous tumor models, the signal-to-noise ratio increased by 7-fold, while in the more challenging orthotopic lung metastasis models, detection sensitivity was enhanced by up to 9-fold. Of particular interest are the dynamic imaging data, which revealed through pharmacokinetic analysis that the plasma half-life of the novel probes was extended to 4 hours, thereby enabling prolonged and continuous monitoring. Tissue distribution studies uncovered another critical feature, showing that probe accumulation in tumor tissues was 5 times higher than in normal tissues—a characteristic likely attributed to the enhanced permeability and retention (EPR) effect. This red-shifted imaging system, developed through multiple rounds of mutagenesis, has not only enabled single-cell detection in deep tissues but also exemplifies how rational protein engineering can overcome the optical limitations of wild-type luciferases. Recent reviews emphasize that such advances—particularly the creation of Akaluc—mark a turning point in luciferase-based imaging by increasing brightness by up to 1000-fold compared to native systems [5].

However, signal attenuation was still clearly observed in certain specialized microenvironments, such as bone tissue, suggesting that further optimization of the probe's physicochemical properties is needed. From the perspective of translational medicine, these findings not only provide valuable directions for improving the technology, but also enhance our understanding of the underlying mechanisms of bioluminescent imaging in living systems. The limitations identified in the current study help to outline future research priorities [3].

2.2 Molecular Activities

The luciferase system, known for its high specificity and sensitivity, has been increasingly applied to the detection of small molecules in real time and under physiological conditions. Recent developments have integrated luciferase with a variety of recognition domains or energy transfer systems to create functional probes capable of monitoring key molecular activities such as energy metabolism and signal transduction.

Since ATP is an essential substrate in the luciferase-catalyzed light-emitting reaction, it naturally serves as a readout molecule in bioluminescent ATP assays. Smirnova et al. reported that luciferase-based fusion constructs—such as those incorporating biotin-streptavidin interactions or immobilized antibody complexes—have been successfully applied for ATP quantification in microbiological, food safety, and environmental monitoring contexts. These systems achieve rapid detection with high sensitivity, often reaching nanomolar levels [7].

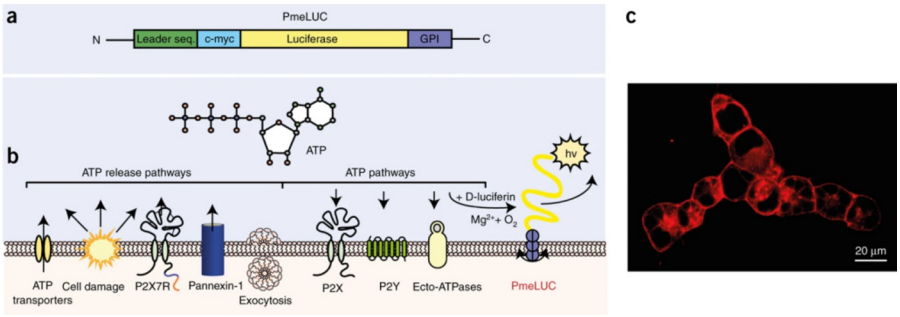

Fig. 2. Structure and function of the pmeLUC system.

Membrane-anchored luciferase enables spatial detection of extracellular ATP released through various cellular pathways. Figure 2 adapted from (Morciano, G., 2017) [8].

However, potential limitations include non-specific ATP sources in complex biological samples, which can lead to false-positive signals. Moreover, the enzyme's sensitivity to environmental conditions such as pH and temperature necessitates careful optimization for stable performance.

2.3 Protein Interactions

Protein-level structural variation and intramolecular interactions within luciferase enzymes have been identified as key regulators of bioluminescent function and evolutionary diversification. Recent studies on cypridinid sea fireflies have demonstrated that mutations at specific amino acid sites in the c-luciferase gene significantly affect emission wavelength (λmax) and light decay rate, providing valuable insights into the structure–function relationship of luciferase proteins [9].

Using RNA sequencing, in vitro protein assays, and spectral analysis, researchers characterized 13 c-luciferase genes from 21 cypridinid species. At least four of these were functionally validated, and multiple amino acid sites were found to be under positive selection. Notably, mutations at residue 160 were shown to influence both λmax and light decay, indicating pleiotropy, where a single genetic change affects multiple phenotypic traits. Such pleiotropic effects may underlie functional trade-offs in bioluminescent signalling [9].

Furthermore, the conformational stability of luciferase proteins under environmental stress plays a critical role in maintaining enzymatic activity. A foundational study by Watkins et al. showed that luciferase activity declined sharply at temperatures well above or below the optimal range, likely due to partial protein denaturation or conformational shifts, which disrupt the active site and reduce light output [2]. This finding underscores the importance of environmental conditions in influencing protein function, especially in imaging applications requiring consistent signal performance.

Together, these studies illustrate how structural variation and environmental sensitivity at the protein level shape the performance and evolution of bioluminescent luciferases. Future work involving site-directed mutagenesis and high-resolution structural

analysis will further elucidate these mechanisms and may support the rational design of improved luciferase-based imaging probes. Figure 3 adapted from (DeLeo, D.M. and H.D. 2021) [9].

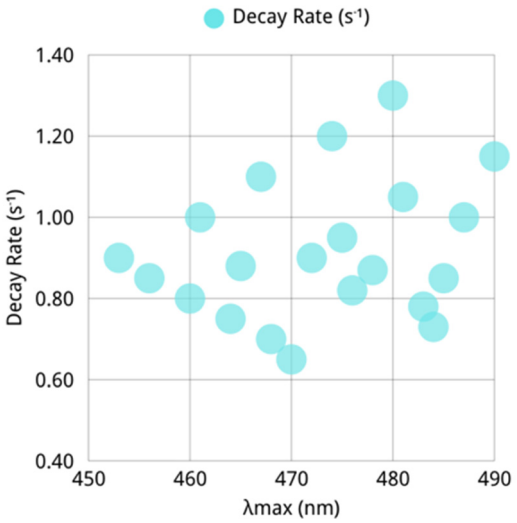

Fig. 3. Relationship between peak emission wavelength (λmax) and light decay rate among cypridinid ostracods. Each point represents a c-luciferase sequence from a distinct species. The distribution illustrates the potential correlation and pleiotropic constraints between emission color and kinetic properties of the luciferase-catalyzed bioluminescence reaction.

3 Future Directions

To further advance the utility of luciferase-based bioluminescent systems, future research should explore several critical directions. One priority is improving the stability and catalytic efficiency of luciferase enzymes under diverse physiological conditions, which is particularly important for prolonged in vivo imaging and clinical diagnostics. This can be addressed through protein engineering strategies such as rational design and site-specific mutagenesis, supported by high-resolution structural analysis. Another promising direction involves the synthesis of novel luciferin analogues with enhanced cell permeability, tenable emission profiles, and reduced background interference. Such efforts will be essential to overcome current limitations in tissue penetration and improve the quantitative accuracy of bioluminescent signals, especially in the red and near-infrared spectral regions. Additionally, further development of ATP biosensors and protein–protein interaction systems—such as split-luciferase and BRET probes—will enhance dynamic cellular analysis and broaden diagnostic utility.

4 Conclusion

In summary, this study highlights the remarkable potential of firefly luciferase-based systems as powerful tools for molecular imaging, biosensing, and functional protein analysis. From their highly specific ATP-dependent enzymatic mechanism to the diverse structural modifications that enable deeper tissue imaging, luciferase-based platforms offer a unique combination of biological compatibility, high sensitivity, and flexible engineering capacity. Advances in luciferin chemistry and enzyme evolution have successfully addressed key limitations such as low tissue penetration and thermal instability, leading to the development of near-infrared probes with enhanced pharmacokinetics and improved in vivo performance. Furthermore, recent research on c-luciferase in sea fireflies reveals that protein-level variation—including pleiotropic and epistatic effects—plays a crucial role in tuning light signal traits and evolutionary adaptation. Collectively, these findings deepen our understanding of the biochemical and structural basis of bioluminescence, while opening new avenues for developing next-generation bioluminescent probes for real-time biological monitoring. Additionally, luciferase-based biosensors have shown significant promise in field-deployable and point-of-care diagnostics, highlighting their versatility in low-resource settings [10].

Furthermore, luciferase-based ATP assays have proven to be indispensable tools for real-time monitoring of cellular energy metabolism, apoptosis, and tumor microenvironment dynamics. By exploiting the strict ATP dependence of the luciferase reaction, these assays provide highly sensitive and rapid detection of ATP fluctuations in living cells and animals, enabling valuable insights into physiological and pathological processes.

In addition, split-luciferase and BRET-based biosensors have expanded the utility of luciferase to include the dynamic analysis of protein–protein interactions with exceptional spatiotemporal resolution. These systems allow non-invasive tracking of signaling events in live cells, facilitating the elucidation of key molecular pathways involved in cancer progression, immune responses, and other complex biological phenomena.

References

1. Smirnova, D.V. and N.N. Ugarova, Firefly luciferase-based fusion proteins and their applications in bioanalysis. Photochemistry and photobiology, 2017. 93(2): p. 436-447.
2. Rabha, M.M., U. Sharma, and A.G. Barua, Light from a firefly at temperatures considerably higher and lower than normal. Scientific Reports, 2021. 11(1): p. 12498.
3. Mezzanotte, L., et al., In vivo molecular bioluminescence imaging: new tools and applications. Trends in biotechnology, 2017. 35(7): p. 640-652.
4. Alsawaftah, N., et al., Bioluminescence imaging applications in cancer: A comprehensive review. IEEE reviews in biomedical engineering, 2020. 14: p. 307-326.
5. Vysotski E S. Bioluminescent and fluorescent proteins: molecular mechanisms and modern applications[J]. International Journal of Molecular Sciences, 2022, 24(1): 281.
6. Kuchimaru, T., et al., A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. Nature communications, 2016. 7(1): p. 11856.

7. Watthaisong, P., et al., Luciferin Synthesis and Pesticide Detection by Luminescence Enzymatic Cascades. Angewandte Chemie International Edition, 2022. 61(16): p. e202116908.
8. Morciano, G., et al., Use of luciferase probes to measure ATP in living cells and animals. Nature Protocols, 2017. 12(8): p. 1542-1562.
9. DeLeo, D.M. and H.D. Bracken-Grissom, Lighting the way: Forces driving the diversification of bioluminescent signalling in sea fireflies. 2021, Wiley Online Library.
10. Calabretta M M, Gregucci D, Martínez-Pérez-Cejuela H, et al. A luciferase mutant with improved brightness and stability for whole-cell bioluminescent biosensors and in vitro biosensing[J]. Biosensors, 2022, 12(9): 742.