Chapter

Biotechnological Advances in Luciferase Enzymes

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Abstract

This chapter explores the history of the bioengineering advances that have been applied to common luciferase enzymes and the improvements that have been accomplished by this work. The primary focus is placed on firefly luciferase (FLuc), *Gaussia* luciferase (GLuc), *Renilla* luciferase (RLuc), *Oplophorus* luciferase (OLuc; NanoLuc), and bacterial luciferase (Lux). Beginning with the cloning and exogenous expression of each enzyme, their step-wise modifications are presented and the new capabilities endowed by each incremental advancement are highlighted. Using the historical basis of this information, the chapter concludes with a prospective on the overall impact these advances have had on scientific research and provides an outlook on what capabilities future advances could unlock.

Keywords: firefly luciferase (FLuc), *Gaussia* luciferase (GLuc), *Renilla* luciferase (RLuc), *Oplophorus* luciferase (OLuc; NanoLuc), bacterial luciferase (Lux), biotechnology

1. Introduction

1.1 Historical perspective on the discovery of luciferase enzymes

The bioluminescent phenotype, which is spread across a variety of different insects, bacteria, fungi, and marine animals, has intrigued mankind since before the dawn of the modern scientific era [1]. The discovery that proteins, which would come to be known as luciferases, were responsible for bioluminescent production can be traced to early experiments by Raphael Dubois, who was able to produce bioluminescence *in situ* by mixing the contents of click beetle abdomens in cold water and extracting the components required for light production [2]. However, it was not until the late 1940s that the first luciferase protein was successfully purified from fireflies [3]. Around that same time, bacterial luciferase was elucidated and successfully expressed *in situ* [4]. However, despite the progress made with these luciferases, it would be some time until biotechnology had advanced to the point where the genes responsible for their expression could be cloned and exogenously expressed, setting off the use of luciferases as tools for scientific discovery [5, 6].

Following the exogenous expression of the previously described firefly and bacterial luciferases, *Renilla* luciferase was isolated from the sea pansy *Renilla reniformis* [7] and *Oplophorus* luciferase was isolated from the deep-sea shrimp, *Oplophorus gracilirostris* [8]. Shortly thereafter, firefly luciferase was successfully

expressed in mammalian cells [9] and it was demonstrated that different luciferases could be used in tandem within a single host if they utilized different luciferin compounds [10]. More recently, *Gaussia* luciferase has been isolated from the marine copepod, *Gaussia princeps* [11], which was a notable discovery because, unlike alternative luciferases, it is naturally secreted and thus could be monitored without needing to sacrifice the host cell during luciferin treatment. Since the discovery of *Gaussia* luciferase there has been rapid development of these enzymes through genetic engineering, but little progress on the introduction of new systems. However, this was recently changed with the introduction of fungal luciferase as a novel luciferase system, which like bacterial luciferase is capable of genetically encoding both the luciferase and luciferin pathway genes to support autobioluminescent production [12].

1.2 Available luciferase systems for biotechnological applications

Of the ~40 different bioluminescent systems known to exist in nature [13], relatively few are available for biotechnological applications. The primary reasons for this are the lack of elucidated functional units, similarities in performance characteristics (such as wavelength output) relative to existing systems, the entrenchment of existing luciferase systems within the literature and as commercially-available products, and the relatively high monetary and time costs required to explore novel systems in depth relative to their ultimate utility as research tools. As a result of these barriers, the luciferases available as research tools are generally limited to those listed in **Table 1**.

1.3 The necessity of engineering luciferase proteins

Despite the variety of different luciferases available, it is impossible to identify just one that could fit the needs of every experimental design. Furthermore, it is unfortunately frequent that no luciferase can be found to fit the needs of a given experiment. As a result, there has been significant effort to engineer the existing luciferase enzymes to improve their functionality, make them easier to use, and expand their utility. This is especially true as the prevalence of luciferase usage has increased in biomedical applications, which rely upon human cellular and small animal model systems that have significantly different physical and biochemical properties relative to the native host organisms from which these proteins were sourced.

These changes in physical properties and the constraints applied by the needs of biomedical research have necessitated that luciferases be modified to express at longer output wavelengths that better penetrate animal tissues or that can be co-expressed with alternative luciferases, to produce light upon exposure to alterative luciferin compounds, to produce altered signal output kinetics that are shorter

Luciferase	Luciferin compound	Output wavelength (nm)
Firefly luciferase (FLuc)	D-luciferin	560
Bacterial luciferase (Lux)	Tetradecanal	490
Renilla luciferase (RLuc)	Coelenterazine	480
Oplophorus luciferase (OLuc)	Coelenterazine	460
Gaussia luciferase (GLuc)	Coelenterazine	470

Table 1.
Common luciferases available for biotechnological applications, their luciferin compound, and their output wavelength.

Technique	Common uses
Mutagenic PCR	Wavelength shifting, thermostability improvement, improve signal output intensity
Rational sequence mutation	Wavelength shifting, altering luciferin compatibility, altering signal output kinetics
Synthetic recapitulation	Enable functionality in alternative hosts, improve expression efficiency, improve ease of use
Codon optimization	Improve expression efficiency
Circular permutation	Thermostability improvement, improve expression efficiency, expand reporter functionality
Alternative luciferin supplementation	Wavelength shifting, altering signal output kinetics
Split luciferase complementation	Alter signal output kinetics, expand reporter functionality

Table 2.
Common approaches for engineering improvements in luciferase functionality.

or longer than their wild-type kinetics, to allow multimeric enzymatic structures to function as monomers, to stabilize or destabilize protein structure within the host, to make expression more efficient, and to increase output intensity so that it is easier to detect the signal. Imparting these changes makes it possible to utilize specialized versions of each luciferase that better fit the experimental needs of the researcher. As the breadth of luciferase usage continues to grow, and as new luciferase systems have been introduced over the years, the lessons learned from these modifications are refined and re-applied in order to continuously unlock new applications and improved functionality.

1.4 Common methods for engineering improvements

To support the need for continued luciferase improvement, a number of techniques have become commonplace for different engineering goals. The most commonly utilized approaches and their common engineering endpoints are shown in **Table 2**. Examples of the use of these techniques can be found in each of the following sections.

2. Firefly and click beetle luciferases

2.1 Background

Firefly luciferase (FLuc) is perhaps the most well-known, well-studied, and widely-used of all the luciferases. It, and its close relatives from click beetles, both function through the ATP-dependent oxidation of reduced D-luciferin (2-(4-hydroxybenzothiazol-2-yl)-2-thiazoline acid) in the presence of magnesium (Mg^{2+}) and molecular oxygen (O_2) to yield carbon dioxide (CO_2), AMP, inorganic pyrophosphate (PP_i), and oxyluciferin. The resulting oxyluciferin is initially produced in an excited state, and as it returns to its ground state energy is released in the form of light. The naturally occurring peak emission wavelength for FLuc (as commonly derived from *Photinus pyralis*) is ~560 nm, while click beetle luciferases, such as those from *Pyrophorus plagiophthalamus* and related species, can produce a variety of wavelengths from 537 to 613 nm depending on their source organism [14, 15].

Although FLuc and click beetle luciferase were among the first luciferases to be studied [16], it was not until the mid-1900s that significant progress was made in understanding the system at a level where it could be experimentally useful. At this time, McElroy successfully extracted firefly luciferase from purified firefly lanterns and determined that ATP was required for bioluminescence [17]. This led to the determination of D-luciferin's structure as 2-(4-hydroxybenzothiazol-2-yl)-2-thiazoline acid and its eventual chemical synthesis [16]. With these pieces in place, chemists were able to isolate oxyluciferin as a purified product of the luminescence reaction and validate its mechanism of action [18]. In 1985, FLuc cDNA was cloned by DeLuca et al. [19]. This provided an alternative to the use of crude extracts of beetles as a source of the luciferase enzyme and opened the door for widespread use in biotechnological applications.

2.2 Initial application and limitations

In its initial incarnation, FLuc was highly useful as a reporter in molecular biology and bioimaging studies and for assaying the presence and quantification of the metabolites that participate in or are connected to the light reaction. The early discovery that ATP concentration was proportional to light intensity in beetle luciferase reactions made this assay the primary method for monitoring the cell's main source of energy. Further entrenching this technology was its exceptional sensitivity. FLuc-based bioluminescent ATP assays display detection capabilities down to 10^{-17} mol [15]. This sensitivity for measuring ATP concentrations has been used in several applications including screening for microbial contamination in food industries, assessing cell viability [20], and assaying enzymes involving ATP generation or degradation [21]. However, ATP concentrations found in living cells (1–10 mM) are generally saturating for FLuc and therefore it cannot be routinely used to assay intracellular ATP content [15]. In a similar vein, FLuc has also been used to assay for the other metabolites that participate in its bioluminescence reaction: CoA, AMP, and PP_i [20].

The major limitation encountered during the use of FLuc or beetle luciferases has been the requirement that the luciferin substrate be exogenously provided for luminescence to occur. To date, there are no bacterial systems for generating luciferin *de novo*, which necessitates chemical synthesis and results in potential storage concerns due to the labile nature of the chemical [18]. Furthermore, this often requires that the host cell harboring the luciferase be lysed to enable substrate uptake, which has prevented its use for reporting real-time expression.

2.3 Engineering improved expression and output

Applications of wild-type beetle luciferases can be limited due to structural and functional stability issues or variations in the specific activity of the enzyme under varying temperatures, pHs, ion concentrations, or inhibitors [22]. For instance, wild-type FLuc protein has a half-life of only 15 minutes at 37°C. This required that more thermostable forms be developed to assay human and small animal model-relevant temperature conditions [23]. Pozzo et al. sought to address this issue by combining amino acid mutations shown to enhance thermostability with other mutations reported to enhance catalytic activity, resulting in an eight amino acid FLuc mutant that exhibited both improved thermostability and brighter luminescence at low luciferin concentrations [24].

Similarly, Fujii et al. produced variants capable of producing 10-fold higher luminescence than the wild-type enzyme by screening a mutant library of FLuc proteins generated by random mutagenesis [25]. Site-directed mutagenesis experiments were then performed based on mutant sequences that produced increased

luminescence. It was observed that the substitution of D436 with a non-bulky amino acid, I423 with a hydrophobic amino acid, and L530 with a positively charged amino acid all increased luminescence intensities relative to the wild-type enzyme. They further demonstrated that combining the mutations at I423, D436, and L530 resulted in an overall increase in affinity and turnover rate for the ATP and D-luciferin substrates that resulted in high amplification of luminescence intensity. Studies like this represent an emerging trend of combining alterations to specific properties of firefly luciferases in order to enhance its overall practical utility.

2.4 Engineering alternative output wavelengths

Engineering wavelength-shifted luciferases has become an intense area of study to enable multi-color assays and improve the efficiency of *in vivo* bioimaging. Due to hemoglobin's absorbance of wavelengths below 600 nm in mammalian tissues, the use of wild-type firefly luciferase is relatively handicapped compared to more red-shifted variants [15]. To overcome this limitation, mutagenic engineering approaches have been successfully used to generate a variety of red-shifted versions [26, 27]. Notable among this group is a variant developed by Branchini et al. containing a S284T mutation. This variant produces a red-shifted output with a peak at 615 nm, a narrow emission bandwidth, and improved kinetic properties [26]. However, this is by no means the only option available. Today, the wide variety of available output wavelengths enables researchers to choose the variant most well suited to their needs, or multiple variants that can be simultaneously triggered upon exposure to D-luciferin.

2.5 Engineering alternative signal kinetics

It has been demonstrated that varying the concentrations of FLuc's substrates (D-luciferin, ATP, etc.) can alter its reaction kinetics. High or saturating concentrations produce flash-type kinetics that result in an intense initial signal followed by a rapid decay, while low concentrations produce glow-type kinetics with a relatively lower initial signal and a slower decay [18]. There are many possible inhibitors that could be responsible for these changes. Under high substrate conditions, byproducts of the reaction such as oxyluciferin and L-AMP can act as tight active-site binding inhibitors preventing enzyme turnover, or inhibitor-based stabilization can increase activity when substrate levels are high enough to compete with the inhibitory compound [14]. Commercial reagents containing micromolar concentrations of components such as pyrophosphate and/or CoASH have been shown to convert FLuc reactions from flash- to glow-type kinetics, possibly due to the breakdown of oxidized luciferin-AMP *via* pyrophosphorolysis and thiolysis into the less potent inhibitors oxidized luciferin and oxidized luciferin-CoA, respectively. These commercial reagents are now widely used to support different experimental needs [14].

Another strategy that has been applied to alter reaction kinetics is the modification of the luciferin substrate. Mofford et al. demonstrated that near-infrared light emission can be increased >10-fold from wild-type FLuc by replacing D-luciferin with synthetic analogues [28]. These synthetic analogues were designed to emit longer wavelength light by incorporating an aminoluciferin scaffold. Nearly all the aminoluciferins tested in their studies resulted in higher total near-IR (695–770 nm) photon flux from live cells under both high- and low-dose conditions. A more recent substrate modification strategy has been to conjugate the luciferin with distinctive functional groups. These so-called "caged" luciferins react when they are cleaved by enzymes or bioactive molecules and subsequently freed [29]. This strategy allows for specified monitoring of biological processes by linking light output to the activity and/or concentration of enzymes or molecules reacting to cleave the caged luciferins.

3. *Renilla* luciferase

3.1 Background

Like FLuc, *Renilla* luciferase (RLuc) is another commonly used bioluminescent reporter. Derived from the sea pansy *Renilla reniformis*, RLuc is a decarboxylating oxidoreductase that uses coelenterazine as its substrate. During its bioluminescent reaction, coelenterazine is converted to coelenteramide in the presence of molecular oxygen, yielding blue light with an emission peak at 480 nm [30]. In addition to their substrate preferences, one other important differentiator between RLuc and FLuc is that the RLuc bioluminescent reaction does not require ATP. It is also significantly less efficient than FLuc and produces a reduced relative light output intensity with a quantum yield of ~7% [31].

The RLuc protein was first purified and characterized in the late 1970s [7]. However, its cDNA sequence was not identified and cloned into *Escherichia coli* until 1991 [31]. Following that accomplishment, the recombinant RLuc protein was quickly expressed in other organisms, including yeast [32], plant [33], and mammalian cells [34] to serve as a gene expression reporter. The successful detection of RLuc bioluminescence from mammalian cells was particularly important because it represented the proof-of-principle demonstration of this enzyme as a reporter target for *in vivo* animal imaging. And indeed, imaging of RLuc activity in living mice was successfully validated just several years later [35]. In this demonstration, Bhaumik and Gambhir showed that intraperitoneally implanted RLuc-expressing cells could be detected following the injection of coelenterazine into the tail-vein [35]. Similarly, when cells were injected *via* the tail-vein, bioluminescent signal could be used to visualize cell trafficking to the liver and lungs. This study also validated that D-luciferin could not be used as a substrate, opening the door for future studies to multiplex RLuc with FLuc as dual-reporters for *in vivo* applications.

3.2 Engineering improved expression and output

The initial limitation for using RLuc as a reporter was its less-than-optimal expression efficiency within mammalian cellular hosts. This limitation was overcome *via* a codon-optimization strategy that modified the RLuc gene sequence while maintaining the wild-type protein sequence. A synthetic humanized version of the luciferase gene that utilizes this strategy, called hRLuc, is now commercially available and has been shown to produce up to several 100-fold higher light output in many mammalian cell lines. Further hampering the expression of RLuc in cell culture and small animal imaging applications was its tendency to be rapidly inactivated upon exposure to animal serum. In its wild-type orientation the half-life of the enzyme under routine experimental conditions ranged from 30 to 60 minutes [36]. An early study by Liu and Escher showed that a single mutation from cysteine to alanine at amino acid 124 (RLucC124A) increased serum resistance, while simultaneously increasing overall light output [37]. Following this study, Loening et al. employed a consensus sequence guided mutagenesis strategy to screen for mutants with improved serum stability [36]. These efforts identified a variant termed RLuc8, which harbored eight substations (A55T/C124A/S130A/L136R/A143M/M185V/M253L/S287L). The RLuc8 variant was shown to be >200-fold more stable in mouse serum than the native protein and displayed an improved half-life of 281 hours. Fortuitously, the RLuc8 mutant also exhibited a 4-fold improvement in brightness. The improved stability and light output characteristics of RLuc8 make it a more favorable reporter than wild-type RLuc for mammalian imaging applications.

3.3 Engineering alternative output wavelengths

Despite the improvements made to increase expression efficiency and output, RLuc's 480 nm output maximum remained problematic for *in vivo* animal imaging applications because it was prone to absorption and attenuation in organs and tissues. This was especially problematic for deep tissue imaging (below subcutaneous layer), where only 3% of the emission spectra could efficiently penetrate animal tissue for detection. Therefore, to improve RLuc's *in vivo* utility, many efforts were undertaken to red-shift its emission spectra. Loening et al. hypothesized that modifying the active site of the luciferase could create a chemical environment favorable to specific coelenteramide species (i.e., the pyrazine anion form) that emit green (535–550 nm) light upon returning from their excited state. To test this hypothesis, they made site-specific mutations at 22 amino acid residues at the predicted active site of RLuc8 and identified red-shifted light emissions (peaks between 493 and 513 nm) in variants with mutations at eight of these locations [38]. Unfortunately, these red-shifted mutants also possessed substantially reduced signal intensities. To restore light output, random mutagenesis was carried out on the red-shifted mutants. This process identified several residues where mutations increased light output or resulted in further red-shifting. Based on these encouraging results, Loening and colleagues performed several more rounds of site-directed mutagenesis and successfully engineered three promising variants RLuc8.6-535, RLuc.6-545, and RLuc8.6-547, which peaked at 535, 545, and 547 nm, respectively, when using coelenterazine as the substrate. All three variants exhibited greater light output than wild-type RLuc, with the most improved, RLuc8.6-535, showing 6-times greater intensity and similar stability to RLuc8. In practice, this translated to roughly a 2.2-fold increase in transmitted signal from the lungs of living mice compared to an equal initial light flux from RLuc8.

In addition to engineering the protein itself, synthetic coelenterazine substrate analogs have also been created to improve light output and/or yield red-shifted emission spectra. The analog coelenterazine-*v* was first shown to shift the emission peak of wild-type RLuc to 513 nm [39] and later demonstrated to yield emission peaks at 570 nm (yellow) and 588 nm (orange) in the RLuc8.6-535 and RLuc8.6-547 variants, respectively [38]. However, this substrate is currently not commercially available due to high background activity and difficulty in purification. Other analogs, such as coelenterazine-*f*, -*h*, and -*e* have been shown to increase signal intensity by 4- to 8-fold relative to coelenterazine in RLuc-expressing mammalian cells *in vitro*, but each has failed to compete with the native coelenterazine in living animal imaging [40]. Despite these setbacks, Nishihara et al. have reported that analogs with ethynyl or styryl group substitutions at the C-6 position significantly increased bioluminescent output and signal stability in RLuc8 and RLuc8.6-535 [41, 42], which suggest that the development of new synthetic coelenterazine analogs will continue to be a promising route for enhancing RLuc functionality.

3.4 Engineering split luciferase applications

Due to its small size (311 amino acids, ~36 kDa) and monomeric orientation, the RLuc protein is an attractive option for use in split luciferase complementation assays aimed at monitoring real-time protein-protein interaction. In an early study attempting to achieve this goal, Paulmurugan and Gambhir [43] created RLuc fragment pairs at two split sites (I223/P224 and G229/K230) and fused the individual fragments to either the MyoD or Id proteins. They then successfully demonstrated that RLuc could properly re-fold and restore luciferase activity upon complementation during MyoD/Id interaction. This study also showed that the split RLuc

reporter signal could be modulated by using an inducible promoter (e.g., NFκB promoter/enhancer) to regulate the expression level of one of the two fragments. The fragment pair based on the G229/K230 split site was later used to characterize interactions between heat shock protein 90 (Hsp90) and the co-chaperone protein Cdc37 [44], between Hsp90 and the Epstein-Barr virus protein kinase GBLF4 [45], and to visualize androgen receptor translocation in the brains of living mice [46]. Kaihara et al. similarly leveraged a variant of RLuc split between S91 and Y92 to demonstrate the recovery of bioluminescent activity during insulin-stimulated protein-protein interactions [47], and Stefen et al. created a split variant using fragments separated between residues 110 and 111 fused to protein kinase A (PKA) regulatory and catalytic subunits to quantify G protein-coupled receptor (GPCR)-induced disassembly of the PKA complex in living cells [48]. These types of split RLuc complementation assays have also been applied to profile protein-protein interactions in the Golgi apparatus *in planta* [49] and to study protein dynamics during chemotaxis in bacteria [50], making it a broadly applicable approach.

4. *Gaussia* luciferase

4.1 Background

Isolated from the marine copepod *Gaussia princeps*, *Gaussia* luciferase (GLuc) is the smallest known luciferase. It is comprised of only 185 amino acids and has a molecular weight of 19.9 kDa. Like RLuc, GLuc catalyzes the oxidative decarboxylation of coelenterazine in an ATP-independent manner to produce blue light with a peak wavelength around 480 nm. Despite this relatively short wavelength, GLuc is one of the brightest luciferases and is capable of generating light output several orders of magnitude higher than FLuc and RLuc [11]. However, unlike FLuc and RLuc, the GLuc protein is naturally secreted from the cells. In biotechnological applications, this allows signal measurements to be performed on culture medium without cell lysis and when using blood or urine samples obtained during animal applications [51, 52]. Its secretory nature also enables unique applications such as monitoring protein processing through the secretory pathway and drug-induced endoplasmic reticulum (ER) stress [53, 54]. It was first isolated and cloned by Bruce and Szent-Gyorgyi in 2001 [55], and since has enjoyed rapid adoption within the research community through a variety of engineered improvements.

4.2 Engineering improved expression and output

To enable improved expression efficiency in biomedical applications, a humanized version of GLuc, hGLuc, was generated *via* codon optimization. This human-optimized variant has been shown to produce 2000-fold higher bioluminescent signal than the wild-type variant when expressed in mammalian cells [11]. In addition to mammalian systems, the GLuc gene sequence has also been codon optimized for efficient expression in the alga *Chlamydomonas reinhardtii* [56], the fungus *Candida albicans* [57], mycobacteria [58], and *Salmonella enterica* [59].

Building on this codon optimization-based approach, which enhances light output by improving protein expression in the host organism without modifying the peptide sequence, mutagenetic approaches have similarly been successfully applied to engineer variants that produce greater signal intensities than the wild-type protein. In one such example, Kim et al. performed site-directed mutagenesis to the hydrophilic core region of GLuc and identified that changing the isoleucine at position 90 to leucine (I90L) was the major contributing factor for improved

signal intensity [60]. The I90L variant produced six times higher light output than the wild-type protein in mammalian cells. Using a directed molecular evolution approach, Degeling et al. also identified a variant (S16K/M43V/V159M) that showed 2-fold enhanced luciferase activity [61].

4.3 Engineering alternative output wavelengths

One limitation of the native GLuc protein is that its relatively blue-shifted emission wavelength is easily absorbed and scattered by pigmented molecules in animal tissues. This limits its utility in *in vivo* animal imaging applications. Several attempts have been made to engineer a red-shift towards increased wavelengths, but these efforts have met with only moderate success. In one notable example, Kim et al. engineered a variant, which they termed Monsta, that harbors four mutations (F89W/I90L/H95E/Y97W) resulting in a shifted peak emission wavelength of 503 nm. This is ~20 nm red-shifted compared to wild-type GLuc [60]. Similarly, several alternative variants (L40P, L40S, and L30S/L40P/M43V) generated by Degeling et al. show 10–15 nm shifts in their emission peaks [61]. Despite the fact that these red-shifted variants have not enjoyed similar success to those of RLuc, GLuc's relatively increased signal strength can often compensate for the loss of signal due to absorption.

4.4 Engineering alternative signal kinetics

Wild-type GLuc catalyzes a flash-type bioluminescent reaction, meaning that the light signal decays rapidly following luciferin exposure. Practically, this necessitates immediate signal reading after substrate addition and thus makes GLuc unsuitable for the majority of high-throughput applications. To overcome this rapid signal decay, researchers have successfully engineered mutants that emit more stable bioluminescence [61–63]. Noticeably, a L30S/L40P/M43V variant has been shown to exhibit glow-type kinetics with only a 20% loss in signal intensity over 10 minutes, compared to the >90% loss in signal intensity after 1 minute from the wild-type enzyme [61]. GLuc mutants such as these have been demonstrated to function in 96- and 384-well plate formats, which effectively allows them to overcome the wild-type kinetic limitations and enables their use in high-throughput assay formats.

4.5 Engineering split luciferase applications

Like RLuc, GLuc's small size (185 amino acids, 19.9 kDa) makes it a good candidate for split luciferase complementation assays. In an early attempt at developing this functionality, Remy and Michnick evaluated the ability of fragment pairs generated from cut sites between amino acids 65–109 of a truncated hGLuc sequence exclusive of the secretion signal to reconstitute luciferase activity upon rejoining [64]. By fusing the respective 5′ and 3′ sequences of the split hGLuc gene to a GCN4 leucine zipper-coding sequence and co-expressing the resulting fusions in HEK293 cells they were able to show that hGLuc activity could be successfully reconstituted by leucine zipper-induced complementation of the split fragments. Their study determined that the optimal split site for complementation was between G93 and E94. This fragment pair has since been further demonstrated to be inducible and reversible, which allows it to function as a highly sensitive tool for quantifying protein-protein interactions in cells and living mice [65]. Similarly, Kim and colleagues also developed a split GLuc variant dissected at Q105 and demonstrated its utility to monitor calcium-induced calmodulin and M13 peptide interaction, phosphorylation of the estrogen receptor, and steroid-receptor binding in living cells [60].

5. *Oplophorus* luciferase

5.1 Background

Oplophorus luciferase (OLuc) is a naturally-secreted luciferase isolated from the decapod *Oplophorus gracilorostris*, a deep-sea shrimp that ejects OLuc from the base of an antennae in a brightly luminous cloud when stimulated. It is one of the more complex luciferase proteins, as it is a 106 kDa heterodimeric tetramer consisting of two regions, each comprised of a 35 and 19 kDa subunit. Like RLuc and GLuc, OLuc uses coelenterazine as a substrate and does not require ATP for functionality [17]. It produces primarily blue light, with a peak emission wavelength of 462 nm. Even in its wild-type form, OLuc possesses robust biochemical and physical characteristics relative to alternative luciferases. It exhibits relatively little change in quantum yield throughout a pH range from 6 to 10, maintains thermostability across a temperature range of 20–50°C, and can still produce observable light output at 70°C [8].

OLuc was first discovered in 1975 [66], and shortly after in 1978 the mechanics of its bioluminescent reaction were identified [8]. Inouye et al. were the first to clone the OLuc cDNAs encoding the 35 and 19 kDa subunit proteins, which led to their discovery that the 19 kDa protein was responsible for catalyzing the luminescent oxidation of coelenterazine. Although this 19 kDa protein was found to be the smallest known protein capable of catalyzing bioluminescence, it was also found to be poorly expressed and unstable without the support of its 35 kDa partner [67].

5.2 Engineering improved expression and output

The need to co-express the 19 and 35 kDa subunits of OLuc made it problematic for routine reporter usage. To overcome this, Hall et al. performed three rounds of mutagenesis on the 19 kDa subunit to produce a novel variant, which they termed NanoLuc (NLuc). This variant showed improved structural stability as well as increased bioluminescent activity and glow-type kinetics with a peak emission wavelength of 460 nm. Furthermore, it was shown that this variant could oxidize an alternative luciferin, furimazine, which resulted in greater light intensity and lower background autoluminescence than when coelenterazine was used. NLuc's 19 kDa size and absence of post-translational modifications made it more agile than FLuc, while its naturally high tolerance to temperature and pH made it more robust. In practice, this NLuc variant was shown to poses 150-fold greater specific activity than either FLuc or RLuc [68]. However, these improvements proved to be a double-edged sword. The high stability and glow-type kinetics made it difficult to employ NLuc for transient reporting activities, while its highly blue-shifted output limited its signal penetration in mammalian cellular applications.

Nonetheless, NLuc's small size and efficient expression make it an excellent choice for studying low-dynamic activities. In one such example, Chen et al. developed a sensitive assay in which NLuc was used to study the activity of deubiquitinating enzymes. In this work, NLuc was fused to the C-terminus of His-tagged ubiquitin that was attached to Ni^{2+} agarose beads. This allowed NLuc to be released as the α-peptide linkages were cleaved so that deubiquitination could be monitored *via* NLuc luminescence [69]. Similarly, Lackner et al. [70] used a CRISPR-Cas9-mediated strategy to tag three cytokine-inducible genes (DACT1, IFIT1, and EGR1) with NLuc. This allowed cytokine-induced upregulation to be measured in HAP1 cells. Under this design, they were able to show that NLuc luminescence correlated strongly with quantitative PCR data, demonstrating that NLuc could reliably be used to monitor gene expression.

5.3 Engineering split and paired luciferase applications

Zhao et al. showed that a split luciferase-based system could be used to monitor protein stability by tracking protein aggregation with NLuc-based luminescence [71]. To accomplish this, they broke NLuc into two fragments, termed N65 and 66C, and demonstrated that, upon interaction, luminescence was modulated by the solubility of the protein fused to the N65 fragment. This property was maintained in both bacterial and mammalian systems, confirming its utility for sensitive detection of protein solubility in a straightforward, high-throughput assay format in living cells.

In addition to these traditional split luciferase applications, NLuc has also been employed for paired luciferase applications that utilize an unfused variant to provide the highest possible light intensity and sensitivity, a destabilized variant with an appended degradation signal (e.g., NLuc-PEST) that allows rapid response to dynamic changes in environment, and a secreted variant (e.g., secNLuc) [17].

6. Bacterial luciferase

6.1 Background

Unlike the monomeric luciferases discussed above, bacterial luciferase (Lux) is a heterodimer of two genes, *luxA* and *luxB*, that must join together to form a functional unit. It is also only one of two systems, along with the fungal system discussed below, that additionally has a known genetic pathway for luciferin synthesis. In the case of bacterial luciferase, this pathway consists of three additional genes, *luxC*, *luxD*, and *luxE*, that work together to produce a long chain fatty aldehyde [72]. In this process, *luxD* transfers an activated fatty acyl group to water, forming a fatty acid. The fatty acid is then passed off to *luxC* and activated *via* the attachment of AMP to create a fatty acyl-AMP. The *luxE* gene finally reduces this fatty acyl-AMP to an aldehyde [72]. The natural aldehyde for this reaction is tetradecanal, however, the luciferase is also capable of functioning with alternative aldehydes as substrates [72]. Along with these genetic components, the system requires two cofactors: oxygen and reduced riboflavin phosphate. When all components of the system are present, bacterial luciferase will produce bioluminescence in an autonomous fashion at a wavelength of 490 nm.

Although this process has been most well-studied in marine bacteria from the *Vibrio* genus, the genetic organization and biochemical underpinnings of the system are consistent across all known bacterial phyla [18]. Due to the complexity of this system relative to its monomeric counterparts, it was not exogenously expressed until the early 1980s. Even then, it was initially utilized through expression of the *luxA* and *luxB* genes as a standalone luciferase [5] before subsequently being employed as a fully functional cassette that was capable of functioning in an autonomous fashion [73]. Shortly after these demonstrations the crystal structure of the bacterial luciferase heterodimer was determined [74], however, this structural knowledge has yet to be leveraged as a means for engineering improved functionality.

6.2 Initial uses and limitations

Because Lux emits its bioluminescent signal without the need for external stimulation, it quickly became a valuable tool for optical imaging. The low hanging fruit for this system was the real-time monitoring of gene expression. This was first

demonstrated by Enbreghet et al. [75], who fused Lux to inducible promoters to study the mechanics of IPTG and arabinose induction in *E. coli*. This proved to be a valuable approach because it allowed samples to be continuously monitored in order to track gene expression dynamics over time. Building upon this work, a variety of instances have been described where Lux has been placed under the control of a promoter with a known inducer to track compound bioavailability. Repeated use of the system for this propose has demonstrated that it is capable of reporting bioavailability in a dose/response fashion [76], which makes it a valuable tool for monitoring contaminant levels in mixed environmental samples. At a higher level, it has been used for *in situ* bacterial monitoring, such as the visualization of bacterial invasion of leaf [77] and root structures [78]. Further, due to the absence of light production from non-bioluminescent species, it was also used to track specific populations of bacteria within mixed communities within unperturbed environments [79].

Despite the advantages offered by avoiding the need for external stimulation concurrent with visualization, Lux was significantly handicapped by its inability to function within eukaryotic cells. Because of this, it was not originally applicable to most modern biotechnological and biomedical applications outside of tracking bacterial infections [80]. Furthermore, as a consequence of encoding both the luciferase and luciferin generation pathways this system required significantly more foreign DNA to be introduced in order to function exogenously. This made the system more difficult to work with at the molecular level; especially before the advent of today's more efficient genetic assembly tools. Similarly, the heterodimeric nature of the luciferase enzyme is more cumbersome than the monomeric orientation of its counterparts. Nonetheless, given its relative advantages over the other systems, it continues to be engineered to overcome these detriments and expand its utility.

6.3 Engineering eukaryotic expression

Although several early attempts were made to enable Lux functionality within eukaryotic hosts, none of these achieved significant success [81–83]. The first major breakthrough came with the expression of the luciferase in *S. cerevisiae* [84]. This achievement was made possible by using luciferase genes from the terrestrial bacterium, *Photorhabdus luminescens*, which showed higher thermal stability than those of marine bacteria, and expressing the individual heterodimer genes from a single promoter using an internal ribosomal entry site (IRES) to link them together. Under this orientation the luciferase was able to properly express within the cell and produce light upon exposure to an n-decanal substrate. This same strategy was then expanded to incorporate the expression of IRES-linked luciferin synthesis pathway genes from dual promoters. When expressed concurrently with the luciferase genes, the cell produced a bioluminescent signal without external stimulation. The functionality of the system was then further improved by shifting the intracellular redox balance to a more reduced state through the introduction of a flavin oxidoreductase gene, *frp*.

Despite this success in *S. cerevisiae*, the direct application of these changes was not sufficient to permit similar bioluminescent production from human cellular hosts. To achieve this, the genes were codon optimized for the human genome and mammalian-optimized IRES elements were employed to improve expression of the downstream genes in human cells [85]. It was also determined that the full pathway could not be expressed from a single promoter using IRES elements, so the luciferin synthesis pathway was encoded on a separate plasmid. This approached allowed for functionality in human cells, but the overall level of bioluminescent production was several orders of magnitude lower than that of alterative bioluminescent systems such as firefly luciferase [86].

6.4 Engineering increased light output

To overcome Lux's low level of bioluminescent output in human cells the orientation of the cassette was subjected to further engineering. It was determined that the use of multiple plasmids was detrimental to achieving high level expression, and that the use of IRES elements was inefficiently expressing the downstream genes in the paired orientation. Therefore, the IRES elements were replaced with viral 2A linker sequences. These sequences were significantly shorter than the IRES sequences they replaced and allowed for each linker region to have a unique genetic code that reduced the chance for unintended recombination events. As a result, the full bacterial luciferase cassette, inclusive of the flavin oxidoreductase component, could be placed under the control of a single promoter and expressed from a single plasmid. This new orientation made it possible to express bacterial luciferase as a single genetic construct similar to what was commonly done with the alternative monomeric, luciferin-requiring luciferase systems. As a result, the bacterial luciferase system could be expressed more easily across a larger number of cell types and was capable of producing an enhanced level of signal output relative to its previous incarnation [87].

In addition to engineering increased expression *via* improved expression efficiency, work has also been performed to alter the peptide sequence of the bacterial luciferase genes to make light output more efficient. Gregor et al. [88] used random mutation to alter the coding sequence of Lux cassette and uncovered a series of 15 mutations that improved light output and thermostability. Of these mutations, six were within the luciferase genes (three each in *luxA* and *luxB*), six were in the luciferin synthesis pathway (with all six located in the *luxC* gene), and three were located in the oxidoreductase gene, *frp*. These mutations resulted in both improved thermotolerance and a ~7 times increase in bioluminescent production relative to the wild-type sequence.

6.5 Engineering improved bioreporter functionality

Just as it has been used extensively as a bioreporter in bacterial species, the engineering of bacterial luciferase to function in eukaryotic cells opened the door to this same functionality under much broader applications. The transition of the Lux cassette to function as a single open reading frame made it possible to replace the constitutive promoter with an inducible promoter and regulate its expression in response to compound bioavailability [87]. However, computational modeling aimed at calculating the metabolism of the required substrates and cofactors for the reaction relative to their intracellular availably suggested that that control of the system should be imparted at the level of the aldehyde recycling pathway, with *luxA* and *luxB* expressed continuously, and *luxC*, *luxD*, and *luxE* placed under the control of the inducible promoter [89]. This model was later proven to be correct when direct comparisons were performed using either single open reading frame constructs where the full cassette was controlled by the inducible promoter, or split cassettes where the luciferase and luciferin pathway genes were switched between inducible and constitutive promoters [90]. Together, these results significantly improved the functionality of the bacterial luciferase system as a bioreporter despite its relative complexity compared the other luciferases.

7. Fungal luciferase

7.1 Background

Fungal luciferase is the most recent luciferase system to be functionally elucidated and made available for biotechnological applications. At the core of

this system is a monomeric luciferase gene, *luz*. In addition to the luciferase, two luciferin synthesis genes: *hisps* and *h3h*, work together as a polyketide synthase and a 3-hydroxybenzoate 6-monooxygenase to supply the required luciferin, 3-hydroxyhispidin. In addition to these genetic components, the reaction also requires molecular oxygen and NAD(P)H as co-factors [91, 92]. When all components of the system are present, it produces a luminescent signal at 520 nm. Like Lux, fungal luciferase is notable in that the genetic sequence of all components required for bioluminescent production is characterized. This allows the fungal luciferase cassette to be genetically encoded and exogenously expressed to produce an autobioluminescent phenotype [12]. However, for this to occur the host organism must either be capable of naturally synthesizing caffeic acid to act as a precursor for luciferin synthesis, or the necessary genes for caffeic acid synthesis must be co-expressed. Under this strategy, it is possible to synthetically assemble a seven gene cassette consisting of the fungal luciferase genes: *luz*, *hisps*, and *h3h*, along with a tyrosine ammonia lyase, two 4-hydroxyphenylacetate 3-monooxygenase components and the 4′-phosphopantetheinyl transferase gene *npgA*, to support caffeic acid synthesis and continuous light production in any host.

7.2 Initial uses and limitations

Unlike the previous luciferases that have been discussed, fungal luciferase has only recently been elucidated as of the time of this chapter. As a result, there have yet to be any reports of its functionality outside of its initial validation [12]. Regardless, the initial characterization of the system provides valuable insights into its functionality and potential limitations. From a practical standpoint, it has been demonstrated that the system can be fully recapitulated in yeast to achieve autobioluminescent signal production. At this time only one luciferin synthesis pathway has been demonstrated, but because genes sourced from alternative organisms are used to enable caffeic acid synthesis in hosts that do not natively support these reactions, it is likely that alterative genes could be substituted for these parts of the pathway.

For more complex hosts, such as human cells, the functionality of the system has been demonstrated only under non-autobioluminescent conditions. In this case, only the luciferase was genetically encoded and the luciferin was exogenously applied. Using this strategy, it has been possible to observe luminescence in cultured human cells, *Xenopus laevis* embryos, and small animal models subcutaneously injected with labeled cells. These demonstrations bode well for the use of the fungal luciferase in the types of experimental designs most commonly associated with traditional luciferase reporters and provide researchers with a novel imaging tool that can be differentiated from alternative luciferases based on its luciferin specificity.

It is currently unknown if the lack of demonstrated autobioluminescent production in hosts outside of yeast is incidental, or if it is the result of metabolic or molecular limitations on the expression of the full cassette within these organisms. One possible explanation is that the required culture temperatures were not compatible with full cassette functionality. It has been shown that fungal luciferase is temperature sensitive and begins to decrease its output signal at temperatures >18°C. Relative light output is halved at room temperature (26°C) and is abolished above 30°C. This is detrimental to the use of this luciferase in human cell culture and small animal model systems, as they will require the maintenance of temperatures above 30°C to avoid the introduction of secondary environmental effects. Similarly, the luciferase is only ~50% efficient at pH 7, which could be detrimental to some experimental designs. The optimal pH is 8, with improved retention of performance at increased pH relative to decreased pH.

7.3 Potential future engineering goals

There are ~100 fungal species that use this luciferase/luciferin pathway for bioluminescent production [93]. It is believed that fungal bioluminescence evolved only once, but that evolutionary pressure led to uneven distribution of the phenotype among species. While this simplifies the system by allowing development to focus on only a single incarnation, it is also potentially limiting in that there are fewer evolutionary cues that can be leveraged as starting points for biotechnological advancement. Nonetheless, this system is clearly in its infancy and will benefit from the copious knowledgebase developed through the engineering of alternative luciferases. It is likely that the primary development target will be overcoming the thermostability issues present in the current incarnation of the system. Beyond this, and similar to Lux, it is likely that investigators will seek to streamline expression of the relatively large cassette size to make it more manageable from a molecular biology standpoint. Once these efforts are achieved, the autobioluminescent nature and somewhat red-shifted output of the fungal system will make it a welcome addition for real-time imaging applications that currently rely on only the bacterial luciferase system.

8. Outlook for future developments

There are ~40 different bioluminescent systems known to exist in nature [13]. However, only seven different families have been well described and only five of the six detailed in this chapter enjoy widespread use [94]. Despite the relative wealth of unexplored systems, relatively few new systems have become available in recent history. Within the last 10 years, the most notable advancements have been the engineering of the bacterial luciferase system to function in eukaryotic organisms and the elucidation of the fungal luciferase genetic pathway. Despite this, the considerable progress of incremental engineering for firefly luciferase and the development of NanoLuc from *Oplophorus* luciferase have provided a clear roadmap for continued progress within the field. Historically, the ability to alter luciferase conformation or luciferin compatibility to enable alternative output wavelengths that better penetrate tissue, allow for multiplexed imaging of multiple luciferases, or pair with fluorescent reporters for BRET applications has enabled new experimental designs that have led to important discoveries. With the emergence of autobioluminescent capabilities from the bacterial and fungal systems, it is likely that the barriers will again be pushed back. These systems will compete with the established luciferases and encourage further development to keep them competitive within an increasingly crowded marketplace. In parallel they can also leverage the decades of previous development in the other luciferases to jumpstart their engineering of alternative output wavelengths, expression kinetics, and luciferin compatibly. Paired with improvements in bioluminescent detection hardware and modern synesthetic biology engineering tools, it is likely that this renewed age of luciferase engineering will continue to expand the application space for bioluminescent imaging and drive further exploration into the untapped potential of underexplored luciferases.

9. Conclusion

There are a variety of different luciferase systems available for biotechnological applications that can help investigators achieve their experimental goals. The high utility afforded by these enzymes is the result of a rich history of engineering that has enabled them to become versatile research tools. Historically, significant shifts

in utility have occurred with the elucidation and introduction of new luciferases, followed by slower, but steady, incremental improvements as they are iteratively engineered to improve their ease of use and expand their functionality. In the context of the historical achievements that have been made with firefly, *Renilla*, *Gaussia*, and *Oplophorus* luciferase, the improvements being made to bacterial luciferase and the recent introduction of fungal luciferase point to promising things to come and give hope that new luciferase systems will continue to be introduced to keep the pace of development strong in the future.

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Conflict of interest

S.R., G.S., and D.C. are board members in the for-profit entity 490 BioTech.

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