

Chapter 8

Engineering Luciferases for Assays and Imaging

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8.1 Introduction

Luciferases are a group of oxidizing enzymes that produce light as a by-product of the chemical reaction that they catalyze. This phenomenon has been observed throughout history, with Aristotle (384–332 BC) writing the first detailed descriptions of bioluminescence (or “cold light”) arising from both marine sources and insects [1]. Within the last several decades, bioluminescence has gained rapid prominence in biomedical experimentation. Luciferases have enabled scientists to shed light (both literally and figuratively) on fundamental biological processes and design analytical tests for a host of salient molecules [2]. However, due to the evolution of luciferases to suit specific applications in the natural world, their properties are not always synchronous with the needs imposed by biomedical research. Two of the most prevalent applications of luciferase, pyrosequencing and molecular imaging, require luciferases that are thermostable and emit redshifted wavelengths, respectively. The vast majority of wild-type luciferases, though, does not possess these attributes, and has thus been subject to modification. Due to their utility in biomedical applications, a substantial amount of effort has been spent within the scientific community to manipulate these and other properties of luciferases through protein engineering. What follows is an attempt to introduce the motivation for modifying luciferases, various techniques used for engineering of these enzymes, and highlights on the most relevant successes.

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8.2 Major Categories of Luciferases

A comprehensive review of luciferases is outside the scope of this chapter. What follows is an abbreviated discussion of classes of luciferases and specific luciferases that are currently commercially available and/or have undergone significant protein engineering. The most salient details for these enzymes are collected in Table 8.1. For more extensive reviews of luciferases in general, please see any one of the several excellent reviews [3, 4].

8.2.1 Beetle Luciferases

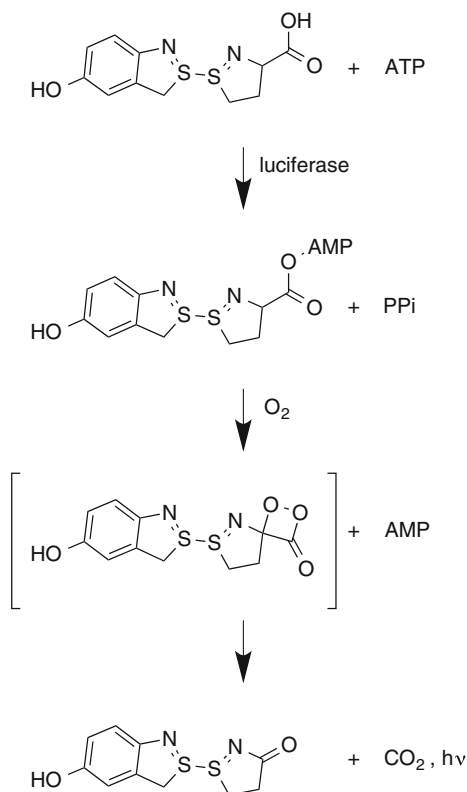
Terrestrial organisms have included luciferases in their evolutionary trajectory for a wide variety of applications, including defense, courtship, and the attraction of prey. Within the terrestrial luciferases, the best studied and by far the most commonly used in biomedical applications are the “beetle luciferases” found in organisms within the order *Elateroidea*. Although many beetle luciferases have been identified, including those from the click beetle (*Pyrophorus plagiophthalmus*) and the railroad worm (*Phrixothrix hirtus*), the most well-known and widely studied is that from the North American firefly, *Photinus pyralis*. The *P. pyralis* luciferase (generally referred to as firefly luciferase = FLuc) is a 62-kDa globular protein and can be thought of as the prototype for the beetle luciferases. The beetle luciferases are homologous, consistent with them evolving only once during the history of this planet. They all catalyze the identical enzymatic reaction, producing light by oxidizing their substrate D-luciferin in the presence of cofactors Mg^{2+} , O_2 , and ATP, but they can demonstrate slight differences in their emission wavelength. The beetle luciferases have been the focus of many engineering pursuits, mainly due to their earlier discovery, relatively redshifted emission compared to other well-studied luciferases, and their necessity for ATP, a central element in cellular metabolism, as a cofactor.

The oxidation of D-luciferin by beetle luciferases is a two-step enzymatic process (Fig. 8.1) [5]. The first step involves the reaction of luciferin and ATP to form luciferyl-adenylate. This step is similar to the one performed by many fatty acyl-CoA synthetases, and some evidence suggests that beetle luciferases are evolutionarily connected to these enzymes [6]. The second step of the reaction involves oxidation of luciferyl-adenylate by molecular oxygen to form an intermediate structure, which spontaneously breaks down into oxyluciferin and carbon dioxide. The energy released by the destruction of the chemical bond takes the form of a photon, and thus bioluminescence is produced.

Table 8.1 The most widely used luciferases in biomedical research are listed below according to their species of origin

Species	Peak wavelength (nm)	Size (kDa)	Native substrate	Co-factors	Native subcellular localization	Reference
<i>Photinus pyralis</i>	560	62	D-luciferin	ATP, O ₂	Intracellular (peroxisomes)	[91]
<i>Phrixothrix hirtus</i>	622	61	D-luciferin	ATP, O ₂	Intracellular (peroxisomes)	[92]
<i>Pyrophorus plagiophthalmus</i>	593	60	D-luciferin	ATP, O ₂	Intracellular (peroxisomes)	[93]
<i>Renilla reniformis</i>	480	36	Coelenterazine	O ₂	Intracellular (lumisomes)	[94]
<i>Gaussia princeps</i>	470	20	Coelenterazine	O ₂	Secreted	[95]
<i>Metridia longa</i>	480	24	Coelenterazine	O ₂	Secreted	[96]
<i>Oplophorus gracilirostris</i>	455	106 (heterotetramer)	Coelenterazine	O ₂	Secreted	[97]
<i>Vargula hilgendorffii</i>	460	68	Cypridina luciferin	O ₂	Secreted	[98]
<i>Cypridina noctiluca</i>	465	61	Cypridina luciferin	O ₂	Secreted	[18]
<i>Pyrocystis lunula</i>	474	140	Dinoflagellate luciferin	ATP, O ₂	Intracellular (microsources)	[19]
<i>Vibrio harveyi</i>	490	77 (heterodimer)	RCHO	FMNH ₂ , O ₂	Intracellular	[20]

Fig. 8.1 The oxidation of luciferin by beetle luciferases is a two-step process that is initiated by the formation of a luciferyl-adenylate intermediate. This chemical is then oxidized in the presence of molecular oxygen and light is generated as a by-product



8.2.2 *Coelenterazine Luciferases*

A variety of luciferin/luciferase systems also exist in marine environments. These marine luciferases most commonly emit light with a blue wavelength peak (~ 480 nm), likely reflecting the end result of selective pressure originating from the preferential transmittance of blue light by ocean water [7]. Within the marine luciferases, the best studied and the only ones in widespread biomedical utilization are those that utilize the substrate coelenterazine (or analogs of coelenterazine).

Coelenterazine is an imidazolopyrazine common in the marine food chain. In contrast to the beetle luciferases, coelenterazine-utilizing luciferases have evolved multiple times from a variety of different precursor enzymes to utilize this same substrate. This likely reflects the relatively simple chemistry and low kinetic barrier of the coelenterazine degradation scheme in comparison to D-luciferin. Although some specific coelenterazine luciferases may require cofactors, in general, these luciferases require only coelenterazine and oxygen, producing coelenteramide, carbon dioxide, and a blue wavelength photon of light. The ease with which a new enzyme can arise that catalyzes this reaction is exemplified by the observation that even albumin catalyzes the degradation of coelenterazine at a low

level [8]. This multiplicity is advantageous for protein engineering as there exists a number of very different starting points for developing novel luciferases.

The well-known coelenterazine luciferases can be categorized into three classes, luciferases from the genus *Renilla* (e.g., *Renilla reniformis* luciferase), copepod luciferases such as those from the family *Metridinidae* (e.g., *Gaussia princeps*, *Metridia longa*), and more recently the luciferase from the decapod *Oplophorus gracilirostris*. These different luciferases also demonstrate variable levels of specificity to coelenterazine versus analogs of coelenterazine, with *G. princeps* luciferase being the most specific, and *O. gracilirostris* luciferase being the least [9].

The coelenterazine luciferase most widely studied is that from the soft coral *R. reniformis*. This luciferase is often simply called “*Renilla* luciferase” (RLuc), although there are other species within the *Renilla* genus that have had their luciferases isolated (e.g., *Renilla mülleri* luciferase). *Renilla* luciferase is a 36-kDa intracellular monomeric protein that is efficiently expressed in a variety of bacterial and mammalian expression systems [10].

The second most studied coelenterazine luciferase is the luciferase from the copepod *G. princeps*, generally referred to simply as *Gaussia* luciferase (GLuc). GLuc is a 20-kDa protein, and similarly to the other copepod luciferases, is a secreted protein that harbors multiple disulfide bonds. These disulfide bonds have made copepod luciferases challenging to express in functional form in bacterial expression systems, although success has been obtained with insect [11] and cell-free systems [12]. The primary sequence of copepod luciferases contains two similar functional domains [13]. In the case of GLuc, these domains have demonstrated an ability to catalyze coelenterazine degradation independently, albeit with greatly decreased activity [14]. A potential limitation of the copepod luciferases to keep in mind is that their enzymatic action appears to be cooperative [15] and therefore light output is nonlinearly related to substrate concentration. However, in most assays, substrate concentration is relatively constant and luciferase concentration is the variable being measured, so this positive cooperative effect is rarely noticeable.

The coelenterazine luciferase from the decapod *O. gracilirostris* (OLuc) is a secreted enzyme complex, consisting of a heteromeric structure containing two 35-kDa and two 19-kDa catalytic subunits [16]. This complexity has previously limited its application to biomedical research. Recently, a monomeric luciferase (termed NanoLuc) has been derived by protein engineering from the 19-kDa OLuc catalytic subunit [17]. This OLuc variant should be more applicable to biomedical applications and is described in more detail later in this chapter.

8.2.3 Other Marine Luciferases

Additional marine luciferases have been studied, but have found much more limited use in biological assays and have not been extensively engineered. Two luciferases from Cypridina ostracods have been cloned, one from *Vargula*

hilgendorffii, and one from *Cypridina noctiluca*. These *Cypridina* luciferases emit blue light when degrading their substrate Vargulin. Given their unique substrate, they can be multiplexed in experimental use with D-luciferin and/or coelenterazine-utilizing luciferases. Although highly stable, these proteins are relatively large (61 kDa), are secreted enzymes, contain a total of 17 disulfide bonds [18], and their substrate vargulin is relatively unstable, all factors that would make the *Cypridina* luciferases a more challenging starting point for protein engineering than the coelenterazine or Beetle luciferases.

Several marine dinoflagellates express a luciferase, most likely used for quorum sensing. This luciferase is relatively large at ~ 140 kDa, and emits blue wavelengths. These properties have prevented a wide adoption of this luciferase, but its unique substrate provides the possibility for multiplexing with other luciferases. A 46-kDa active fragment of dinoflagellate luciferase has been developed and utilized as a reporter in mammalian cells [19]. This truncated dinoflagellate luciferase could be combined with firefly and *Renilla* luciferases, for example, to monitor the expression of three different genes.

8.2.4 Bacterial Luciferase

Luciferase-expressing bacteria can be found in a variety of ecosystems. Most possess symbiotic relationships with other organisms, providing a means for communication, attraction of prey, or defense to the host in exchange for nutrients. The bacterial luciferase is actually a cassette of five genes, two of which are responsible for substrate oxidation leading to bioluminescence, and three that synthesize the substrate from common biomolecules. Due to this level of complexity, the bacterial luciferase is seldom used in biomedical research, although efforts have been made to adapt it for the creation of autonomously luminescent cells [20]. This achievement is attractive for certain longitudinal studies of specific cellular populations because it obviates the necessity for repeated administration of exogenous luciferase substrate.

8.3 Current Biomedical Uses

Due to the intrinsic difficulties of translating optical technologies into a clinical setting (i.e., suboptimal wavelengths, necessity for exogenous genetic/proteinaceous material), luciferases have a limited role in the medical setting. They are widely used, however, in biomedical research, and are gaining ground in the area of diagnostic testing. One technique in particular named luciferase immunoprecipitation system (LIPS) has demonstrated considerable advantages over similar systems that use fluorescent or colorimetric indicators. The LIPS procedure is very similar to enzyme-linked immunosorbent assay (ELISA), with the exception that

an antibody-luciferase fusion protein is used for the detection of antigens in assays. Compared to similar immunodetection procedures, LIPS has proven to be more sensitive and specific [21]. As with other applications that will be discussed below, the use of luciferases for immunodiagnostics requires that the enzymes maintain stability over time and in some cases at elevated temperatures. Thus, engineering thermostable luciferases is crucial for the success of these techniques.

One of the biggest commercial successes of the beetle luciferases is pyrosequencing. This is a DNA sequencing technique that is predicated on three enzymatic reactions. The first reaction occurs during *de novo* DNA synthesis by DNA polymerase. This reaction produces an inorganic pyrophosphate molecule, which can then be converted into ATP by ATP sulfurylase (the second reaction). The final reaction is described in more detail in the previous section and involves the use of this ATP molecule by a beetle luciferase to produce a bioluminescent signal. Therefore, each nucleotide successfully integrated into the growing complementary chain of the template DNA can be quantitatively detected by light emission. Pyrosequencing has been shown to be quicker and simpler than other sequencing techniques and has become the method of choice for many sequencing needs [22]. As DNA polymerization is generally optimal at elevated temperatures, thermostable luciferases are absolutely necessary for this application, and many luciferase engineering studies have been performed to produce more pyrosequencing-friendly beetle luciferase variants.

Additionally, luciferases are used extensively in basic laboratory biomedical research as reporter genes. Traditionally, certain genes whose protein products are detectable by an optical or radiological device (e.g., green fluorescent protein, beta-galactosidase, thymidine kinase) have been used as surrogates to report on a genetic activity of interest. Often this takes the form of driving the expression of a reporter gene with the promoter of the gene of interest. In this manner, the transcriptional activation of a gene can be interrogated using convenient and fast readouts. Luciferases have proven to hold a special niche in this regard as they are often more sensitive than other reporter genes and generally translate better to small-animal *in vivo* studies. Moreover, due to the technique known as split-protein complementation, discussed in greater detail in a subsequent section, luciferases have been engineered to report on much more than gene expression. Recent studies have seen the design of luciferases that can report on small molecule kinase inhibition, DNA methylation, and caspase activity [23–25]. These studies require luciferases that have been specially engineered to only produce bioluminescence after these molecular events have occurred and demonstrate the degree of creativity and ingenuity currently being applied to luciferase engineering.

Small-animal *in vivo* imaging represents the final main application of engineered luciferases. This application is in many ways an extension of reporter gene assays, but imposes additional constraints upon the characteristics of the luciferase used. It generally favors the use of luciferases whose wavelengths have been shifted toward the red end of the electromagnetic spectrum due to the optical window of mammalian tissue (discussed in greater detail in a subsequent section).

These engineered luciferases have contributed greatly to our ability to translate cellular studies into more physiologically relevant models and have provided a potent tool for interrogating biological processes in a living system.

8.4 Rationale for Protein Engineering of Luciferases

Although there are a number of reasons for performing luciferase protein engineering, a common reason is that the native luciferin/luciferase system is not sufficiently robust for consistent use in a variety of laboratory solutions, cellular compartments, or temperatures. Each luciferin/luciferase system has evolved under the selective pressures of its native environment. For instance, *Renilla* luciferase evolved to work in a salt water creature, in small membrane-bound particles called lumisomes, associated with a green fluorescent protein, and at ocean temperatures [26]. Thus, it stands to reason that mutations could be readily identified to improve its performance and stability as a monomeric protein in laboratory solutions at room or mammalian body temperature.

Additionally, luciferases are relatively “easy” to engineer compared to other enzymes because their functional properties make mutants readily identifiable. Large-scale assays can be performed utilizing common bioluminescence imaging systems, or even the human eye for sufficiently bright luciferases [27].

8.5 Designing Selection Strategies for Protein Engineering of Luciferases

An important philosophical point in the protein engineering of luciferases is the definition of what constitutes “better.” Much as beauty is in the eye of the beholder, what constitutes a “better” luciferase is entirely dependent on the end application goal. Most comparisons between luciferases in the literature are made with respect to which is “brighter” in a particular assay condition, with little or no consideration given for the robustness of the improvement when the assay is performed in different solutions/cellular compartments/temperatures or the actual enzyme kinetics. For instance, a kinetically slow enzyme could look “better” than a fast enzyme if the fast enzyme degrades the majority of the applied luciferin before brightness is measured. A secreted luciferase may perform “better” than a nonsecreted luciferase in the context of a cell culture when the entire cell culture dish including media is assessed, but the loss of association between the cell of origin and the location of bioluminescence will limit utility of a secreted luciferase in small-animal bioluminescence imaging.

A closely related point is that many comparisons are based on assays (such as cell culture assays) in which the bioluminescence signal is not normalized to the

total amount of luciferase. This gives an advantage to luciferases that are more stable, as more of the luciferase will accumulate over the course of the experiment, and the experimental condition utilizing the more stable luciferase will appear to be “brighter” even though both luciferases may have the same light output per enzyme. Given the end goal of the assay, this stability may or may not be desired. Increased luciferase stability impedes the ability of an assay to monitor transient fluctuations in luciferase expression, and it takes longer to reach a steady state of luciferase activity within the cell. As discussed in a following section, some luciferase engineering has been directed into making the luciferase mRNA and/or protein less stable for this very reason.

This all leads to the first law of directed evolution: “you get what you select for.” The improved luciferase/luciferin combinations derived under artificial selective forces in the laboratory are being improved with respect to activity in a specific test. Screens must be designed carefully, or contain controls, to ensure that the mutations picked up are being selected for the desired end application goals. As an example, consider a random mutagenesis screen of a luciferase in *E. coli*. If the selection method is simply which colonies are brightest the screen will select for a number of different properties: increased thermostability at the incubation temperature, improved codon utilization for bacterial expression, removal of bacterial protease sites, improved folding in the bacterial expression system, better matching of the emission spectrum to the sensitivity profile of the detector, etc. Some of these “improvements” may be detrimental if the luciferase is utilized in a different system such as mammalian cells. If a more targeted goal is desired, such as faster enzyme kinetics, a more sophisticated screening system must be employed.

8.6 Methods for Protein Engineering of Luciferases

The techniques and theories driving luciferase engineering are not substantially different from those used to modify any other protein, but luciferase’s functional capacity has made it a facile model for experimentation, and its prominence in biomedical research has made it an attractive target.

Luciferase engineering can be broadly divided into two categories. The first category is similar to canonical protein engineering, in which changes made to the primary sequence affect the enzyme’s intrinsic properties (e.g., specific activity, thermostability, emission wavelength).

The second category is more prominent, although not exclusive, to reporter proteins such as the luciferases, and involves the dissection or interruption of the protein’s primary sequence into two distinct domains. The purpose of alterations in this second category is to prevent the enzyme from oxidizing its substrate in the absence of some other biomolecule, thus serving as an analyte detector.

Within each category, various strategies have been employed to introduce mutations. Random mutagenesis has been applied to luciferase engineering with

substantial success [28–33]. Different techniques have been used to induce random mutations, although the most common is error-prone PCR. In theory, this approach yields an unbiased distribution of mutants across the coding region of the protein, effectively maximizing the variable space. In practice, certain codons are more susceptible to mutagenesis than others, and the altered residues are often less randomly distributed than expected. Nevertheless, this technique yields a prodigious number of mutated enzymes that can be subjected to high throughput screens. In the case of luciferase engineering, the screens generally take the form of bioluminescent output under various constraints. For example, one screen used by several investigators to identify thermostable variants involves the incubation of luciferase-expressing bacterial colonies at an elevated temperature followed by application of the luminescent substrate and identification of the brightest colonies. Constructs are then sequenced to identify the mutations contributing to the new phenotype.

The other main technique for introducing mutations into luciferase is site-directed mutagenesis. Unlike random mutagenesis, this method is usually based on a specific hypothesis regarding how a certain mutation or mutation site will affect the enzyme's properties [10]. Site-directed mutations are most often initiated by structural or sequence analysis and can be far more efficient than random approaches. For instance, in the generation of the RLuc variant RLuc8 [10], only ~30 single-mutation RLuc variants were screened to generate a variant that was fourfold brighter and two orders of magnitude more stable.

Combinatorial mutagenesis is a variation on both of these methods for introducing mutations. This semi-rational method entails the random incorporation of a subset of site-specific mutations. Although this method has been used sparingly for luciferase engineering [28], its results are encouraging and may see wider use in the future.

8.6.1 Structure-Based Versus Sequence-Based

Sequence homology between the luciferase proteins of various species has driven many of the studies that seek to enhance these enzymes' properties. This has been a motivating impulse for those seeking to induce bathochromatic shifts in the beetle luciferases, as well as groups interested in improving protein stability in both beetle and coelenterazine luciferases, although the precise rationale in each case is slightly different. To provoke changes in peak wavelength of the beetle luciferases, sequence homology is often assessed in an attempt to graft the attributes of one luciferase onto another.

Sequence homology has also been used with great success for the creation of more stable luciferase enzymes [10, 34, 35], although for this application, homology is assessed between multiple species. One of the guiding principles of protein evolution is that conservation is driven either by stability or function. By selectively mutating residues within a luciferase so that they conform to a

consensus sequence, one is choosing candidate mutations that have already been screened by nature to be tolerated or even preferred within the context of the proteins' fold and are much less likely to be deleterious to the protein than a residue picked at random. This consensus mutagenesis approach requires that a number of homologous proteins already exist in the sequence database that, while being similar enough to allow a valid alignment, are evolutionarily distinct enough that a bias toward stabilizing mutations can be identified.

Structural considerations have also played a role in luciferase engineering. Different tactics have been used in this respect, including mutagenesis of residues involved in the active site, introduction of cysteine residues to form disulfide bridges, and mutagenesis of solvent-exposed residues [28, 36–38]. These studies use publicly available crystal structures of a handful of luciferases to develop biochemical hypotheses regarding the contribution of individual amino acids to stability and wavelength emission.

8.6.2 Codon Optimization

Although not strictly protein engineering as the primary sequence of the protein is conserved, evaluation of codon usage is often a useful initial step in improving expression levels of luciferase proteins. Several studies have generated codon-optimized luciferase genes, primarily for mammalian expression, to help improve transcriptional activity and mRNA stability [39–42]. This procedure is not specific for luciferases and is generally performed when attempting to generate robust expression of a nonmammalian gene in a mammalian system.

8.6.3 Protein Truncation/Extension

Certain studies have manipulated the stability or intracellular compartmentalization of luciferases by truncating or appending additional residues to the primary sequence. Again, this technique is not specific to luciferase engineering and has been applied to many other proteins.

In general, these modifications are used to alter properties of the luciferase that are of importance when used as a reporter gene (discussed in further detail later). Adding the PEST sequence or ubiquitinylation-prone sequences to the luciferase protein has the general effect of reporting on cellular dynamics and intracellular protein transport [43–45]. Removal of N-terminal signal peptide sequences or adding transmembrane domains have been utilized to maintain association of a normally secreted protein to its cell of origin [41, 46].

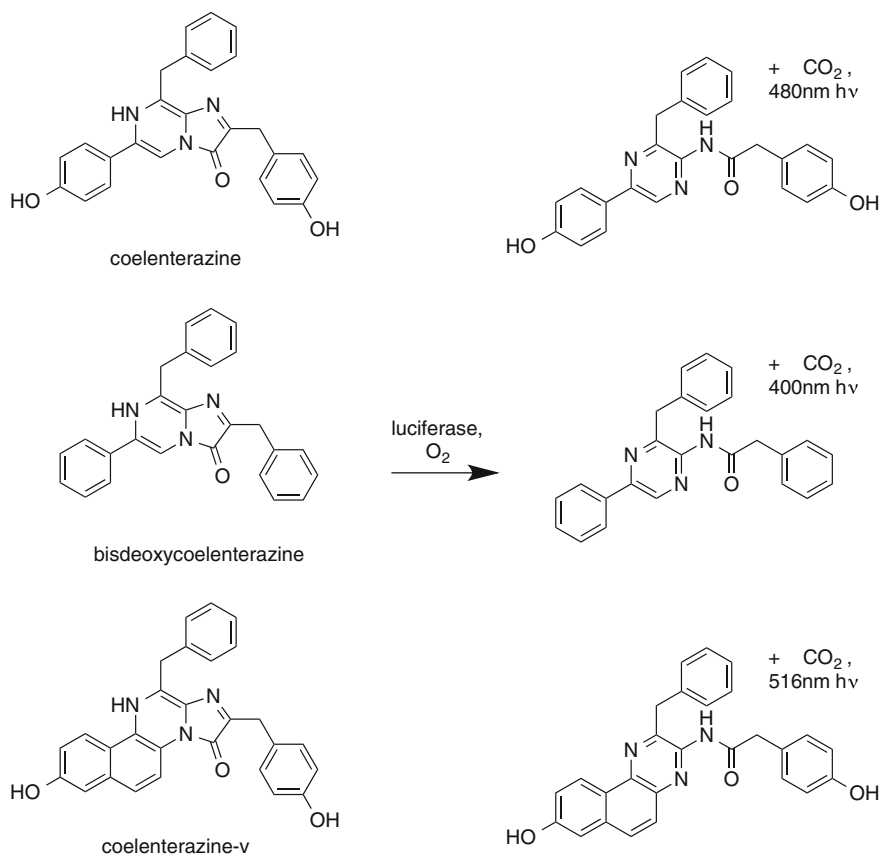


Fig. 8.2 Native coelenterazine (*top*) and two derivatives, bisdeoxycoelenterazine (*middle*) and coelenterazine-v (*bottom*), are all oxidized by *Renilla* luciferase. The wavelengths of the light produced by the oxidation of each substrate, however, are significantly different [27, 28, 88–90]

8.6.4 Substrate Alteration

An in-depth discussion of chemical modifications to luciferase substrates, chiefly D-luciferin and coelenterazine, is beyond the scope of a chapter focused on protein engineering of luciferases. However, it is important to note that an equally large body of work has reported on the successful design of modified luciferase substrates that have the potential to alter emission wavelength when oxidized, or prevent oxidation prior to conversion by a molecule of interest. Thus, equivalent results in terms of red shifts and molecular-sensing have been achieved by engineering of the luciferase substrates rather than the luciferases themselves [28, 47, 48].

It bears mentioning, that a factor to consider when comparing between different substrates is the autoluminescence rate of the luciferin under the assay conditions employed. For instance, the coelenterazine analog coelenterazine-v (Fig. 8.2)

generates an order of magnitude higher background autoluminescence than coelenterazine [47]. Even if a luciferase is brighter with coelenterazine-v, the increase in brightness is unlikely to make up for the order of magnitude decrease in sensitivity for conditions in which very small amounts of luciferase are present.

8.7 Examples of Luciferase Protein Engineering

8.7.1 Intensity Improvements

Although only a single study in the literature has specifically sought to enhance the intensity of beetle luciferase, many mutants have demonstrated increased light output as a result of stabilizing or color-shifting mutations. Further complicating the issue of comparing mutants between studies is the number of variables involved in measuring luminescent output (e.g., luciferin concentration, ATP concentration, acquisition time, photomultiplier tube wavelength sensitivity). For the purposes of this chapter, activity values relative to the mutant's parenteral luciferase are reported when possible, as this accounts for variations in luminescence measurements. Using this metric, the brightest, or most intense, mutant described to date is a triple mutant of the *P. pyralis* luciferase (I423L, D436G, L530R), which demonstrated a 12-fold increase in intensity over wild type [32]. Each mutation was discovered independently by random mutagenesis coupled with screening of bacterial cell lysates, and then all three mutations were combined to produce the final version. Also of note in this category of engineered luciferases is a report of a double mutant of the *P. hirtus* luciferase (I212L, N351K), yielding a 9.8-fold improvement in intensity over wild type [34]. These amino acid alterations were introduced by site-specific mutagenesis based on sequence homology to stabilizing mutations previously characterized in the luciferases of *Luciola cruciata* and *P. pyralis*. While even this mutant is much less bright compared to its compatriots in the luciferase gallery due to the low intrinsic activity of wild-type *P. hirtus*, this study is of particular interest because of the long peak emission wavelength of *P. hirtus*, an attribute which is examined in the following section.

Similar to the case for beetle luciferases, while a number of studies have generated "improved" coelenterazine luciferases, improvements in light output have generally been due to improvements in stability and therefore the amount of functional protein, rather than mutations that improve the quantum yield or kinetics of the enzyme. Utilizing a consensus sequence approach, an M185V mutation was identified for *Renilla* luciferase that led to a threefold increase in light output arising from improvements in both quantum yield and kinetics [10]. A random mutagenesis screen identified the mutations K189V and V267I that led to a threefold to fourfold improvement in light output compared to native *Renilla* luciferase [49]. These improvements were attributed to a combination of improved stability, improved kinetics, and decreased levels of substrate inhibition. These

mutation sites had previously been studied with other amino acid substitutions based either on a consensus mutation approach (V267) or due to their proximity to the active pocket (K189), but these other substitutions at the same sites did not yield improvements [27].

For the copepod luciferases, recognition that the N-terminal domain of *M. longa* was not homologous to *Gaussia* luciferase led to an N-terminal-truncated *M. longa* variant that demonstrated sixfold to tenfold improvements in light output compared to the native luciferase, albeit with decreased thermal stability [50]. A semi-rational mutagenesis strategy was performed on *Gaussia* luciferase by targeting hydrophobic regions presumed to constitute the enzymatic pocket, and substituting in other hydrophobic amino acids [51]. Screening of the resultant single-mutation variants and combining the beneficial mutations, led to a 4-mutation variant termed “Monsta” that exhibited ~six-fold greater light output than GLuc, with this improvement due to improved folding, turnover rate and quantum yield.

8.7.2 Stability Alterations

One of the major biomedical applications of the beetle luciferases is pyrosequencing. This technology is predicated on the necessity for ATP in order for beetle luciferases to oxidize D-luciferin. However, like most proteins produced by mesophilic organisms, beetle luciferases are unstable at elevated temperatures. Thus, a need exists for this particular application (among others) for the production of thermostable beetle luciferase mutants. A number of different groups have tackled this problem with varying techniques and results. Similar to the discussion of evaluating luciferase intensity, it is often difficult to compare reports of luciferase stability from one study to the next for two reasons: (1) the enzyme’s activity is highly dependent on environmental (i.e., buffer) conditions, and (2) different studies report stability at different temperatures (thus, a mutant that is highly stable at 37 °C may degrade quickly at 50 °C, but conversely, a mutant with good stability at 50 °C may not be as robust as others at 37 °C). What follows is a brief discussion of some of the most stable mutants described for discrete temperatures, and the methods used to engineer them.

One of the earliest, and still among the most successful, attempts at creating a thermostable beetle luciferase was carried out by Hall and colleagues on the luciferase from *Photuris pennsylvanica* [52]. This study used error-prone PCR to create random mutations in the luciferase gene, followed by cloning into a bacterial expression vector. Transformed bacteria were then plated and exposed to elevated temperatures prior to treatment with D-luciferin. Bioluminescent capture of the bacterial plates revealed individual colonies that expressed luciferase with stabilizing mutations. Remarkably, they were able to identify one mutant that retained activity after 5 h at 65 °C. The exact mutations of this enzyme, however, are unknown to the general scientific community, as this mutant remains a proprietary entity. A highly stable variant of the *P. pyralis* luciferase has been

described with a half-life at 37 °C of 11.5 h (T214A, A215L, I232A, F295L, E354K) [53, 54]. These mutations were discovered independently by random mutagenesis and screens similar to the one described above, with the site-directed combination of all 5 producing a synergistic effect. Of the reported mutants evaluated at 42 °C, an octuple mutant of the luciferase from *Luciola mingrelica* (S118C, C146S, R211L, T213S, A217V, E356K, S364C) has a half-life at that temperature of nearly 10 h [31]. This mutant was discovered by directed evolution, in which 4 rounds of random mutagenesis were employed, using the most thermostable mutant identified from each round as the basis for further mutagenesis. In contrast to the studies described above, a structure-based approach was used to engineer a double mutant of *L. mingrelica* luciferase (G216N, A217L) with a greater than 4.5 h half-life at 45 °C [35]. Using the known stabilizing, but activity-compromising, mutation A217L, these investigators altered residues with spatial proximity to 217 according to the crystal structure of the protein, in the hopes of producing a mutant that was both stable and highly active.

This subset of luciferase engineering has seen the use of several methods that are not as widely used as those previously discussed. Of note is one study that identified an undecuple mutant of the *P. pyralis* luciferase (F14A, L35Q, M118L, N138G, V182K, A215L, F295L, E354R, V366A, S420T, F465R) with a half-life of more than 72 h at 40 °C using combinatorial consensus mutagenesis [55]. This technique involves construction of a consensus sequence by aligning multiple proteins (in this case, all sequenced beetle luciferases) and identifying the residues at which the sequence of interest deviates from the consensus. Primers are then designed to mutate the wild-type sequence to the consensus sequence. In contrast to more canonical site-directed mutagenesis, however, combinatorial consensus mutagenesis incorporates these selected mutations at random, and then a screen is employed to select for mutants possessing the desired property (in this case thermostability).

Another unique method of engineering thermostability into a beetle luciferase was accomplished by creating disulfide bonds by mutating certain residues to cysteines. This attempt was made based on the observations that most proteins exposed to harsh extracellular conditions contain at least one disulfide bond. Using the crystal structure of the luciferase from *P. pyralis* and in silico analysis of putative disulfide sites, several mutations were made by site-directed mutagenesis [56]. Although the most stable mutant identified (A296C, A326C) is not as robust as some of the others discussed in this section, this study warrants mention as an alternative method for luciferase engineering with respect to thermostability [57].

One final technique that deserves inclusion in a discussion of engineering beetle luciferase thermostability is a site-directed mutagenesis of trypsin-cleavable sites within the primary sequence of the luciferase from *P. pyralis* [58]. The sites selected for mutagenesis were chosen from previous empirical studies coupled with interspecies luciferase sequence homology to exclude from alteration residues thought to be necessary for enzymatic activity. The most successful mutant, R337Q, demonstrated substantial resistance to trypsin hydrolysis and improved thermodynamic stability over wild type.

Several publications have focused on improving the stability of *Renilla* luciferase (RLuc). An early publication examining the effects of cysteines on stability for a secreted form of RLuc found that a C124A mutation was \sim six-fold more resistant to inactivation in their assay [59]. Although the authors postulated this was due to reduced inadvertent disulfide bond formation, the stability improvement could also be explained based on C124A being a mutation toward the consensus sequence [27]. A later publication performing a consensus-sequence-based approach on this RLuc/C124A variant identified an additional six stabilizing mutations that when combined generated an RLuc variant (RLuc8) that was 200-fold more resistant to inactivation in murine serum than the native luciferase and four-fold brighter on a per-enzyme basis [10]. Little attention has been paid to increasing the stability of the secreted copepod luciferases, as these already demonstrate impressive stability to pH conditions and heat shock [60].

8.7.3 Emission Wavelength Shifts

In the field of small-animal bioluminescence imaging, wavelengths in the visible red region of the electromagnetic spectrum reign supreme. This is due to the relative optical window for mammalian tissue that exists between 600 nm (below which wavelengths are absorbed predominantly by hemoglobin) and 1,200 nm (above which wavelengths are absorbed by water). For luciferase-based imaging, this means that despite the greater activity of the *P. pyralis* luciferase (557-nm peak wavelength), the number of photons that reach the surface of the subject being imaged could be fewer than those emanating from less active, but more redshifted luciferase such as *P. hirtus* (623-nm peak wavelength). Unfortunately, the *P. hirtus* luciferase is especially heat-labile and enzymatically slow, making it a poor choice for most molecular imaging applications.

Among the most successful attempts at engineering redshifted beetle luciferase enzymes is a single mutant of the luciferase from *Lampyrus turkestanicus* (S284T) that emitted a peak wavelength of 618 nm (compared to 555 nm of the wild-type enzyme) [61]. This mutation was based on sequence homology to a redshifted mutant of *P. pyralis* (S284T) with a similarly impressive bathochromatic shift (615-nm peak wavelength) identified by random mutagenesis [62]. The most redshifted variant of the *P. pyralis* luciferase described to date is a nonuple mutant referred to as Ppy RE9 (T214A, A215L, I232A, S284T, F295L, R330G, I351V, E354I, and F465R, 617-nm peak wavelength) that also uses the redshifted single-mutant S284T as a point of departure [40]. Ppy RE9 was designed by incorporating the stabilizing mutations T214A, I232A, and F295L, identified by random mutagenesis [63], A215L, based on homology to a stabilized mutant of the *L. cruciata* luciferase identified by random mutagenesis [64], the stabilizing mutation E354I, identified by random mutagenesis [29], and stabilizing mutations I351V and F465R, identified by selective mutagenesis of solvent-exposed amino acid residues determined by crystal structure analysis [38]. The redshifted mutation R330G was

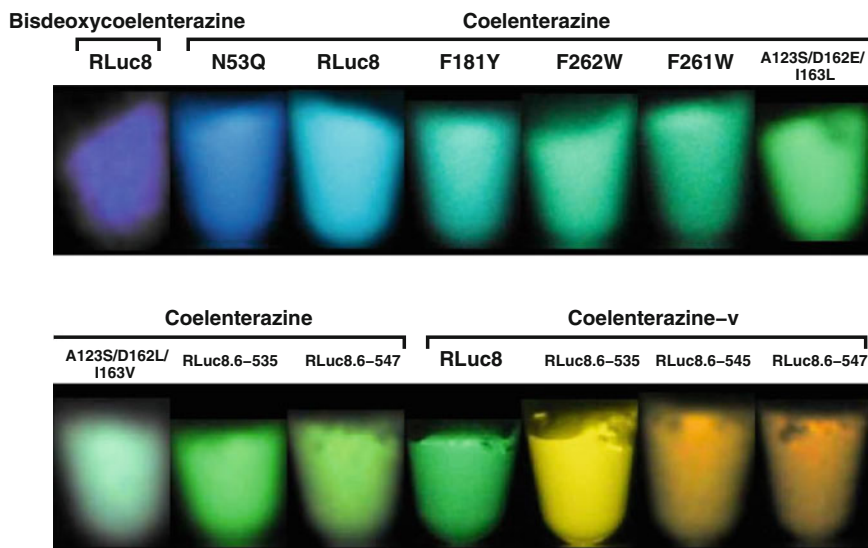


Fig. 8.3 A range of emission spectra can be achieved with *Renilla* luciferase via mutation of the luciferase and/or alteration of the substrate. The specific mutations utilized here have been previously published in the literature [10, 27, 28]

identified by performing random mutagenesis on this stabilized mutant and highlights the interconnectivity of luciferase attributes. A consistent theme running throughout the luciferase engineering literature is that mutations that enhance one attribute of the enzyme often come at the cost of another. Case in point, the authors of the Ppy RE9 study describe how the incorporation of the R330G mutation in a less stable version of the *P. pyralis* luciferase had significantly destabilizing effects.

While the most successful examples of shifting the peak wavelengths of beetle luciferases have been achieved by random mutagenesis as described above, similarly encouraging studies have resulted from site-directed mutagenesis based on sequence homology to red light-emitting luciferases such as *P. hirtus*. Such studies have yielded a 608-nm-emitting *P. pyralis* luciferase (K356 insertion [65]) and a 616-nm-emitting *L. turkestanicus* (R353 insertion [66]).

An extensive study performed on *Renilla* luciferase variants utilizing a combination of random mutagenesis with screening for color shifts and structural-based semi-rational mutagenesis, led to *Renilla* luciferase variants with redshifts up to 66 nm from the native luciferase (Fig. 8.3) [28]. The variant titled RLuc8.6-535 was felt to be the most useful by the authors and demonstrated improved properties compared to their previously described RLuc8 variant, with comparable stability to RLuc8, as well as a sixfold increased light output on a per-enzyme basis and a 49-nm redshift in comparison to RLuc. When combined with the redshifted substrate analog coelenterazine-v, the redshifts were found to be additive, with the combined total redshift being 89 nm in comparison to the native

luciferase/native substrate combination. A subsequent protein engineering study was performed to generate a redshifted RLuc variant without the increased stability that would be more appropriate for assessing transient changes in gene expression [67]. The result, RLuc7–521, demonstrated equivalent stability to the native luciferase, a 60 % improvement in light output on a per-enzyme basis, and a 40-nm redshift. When combined with coelenterazine-v, a total redshift of 73 nm was achieved with respect to the native luciferase/native substrate combination. Comparable mutations to those utilized to generate RLuc8.6–535 have been incorporated into *R. mulleri* luciferase to yield equivalent redshifts in its emission spectrum [47].

No studies have been performed on copepod luciferases to specifically alter the emission spectrum, but the 4-mutation variant of *Gaussia* luciferase Monsta (previously discussed in the intensity section) was found to have a 33-nm redshift with respect to native GLuc [51].

8.7.4 Codon Optimization

As mentioned previously, although codon optimization is not technically protein engineering, it is such a common first step for improving protein expression that we have included examples of codon optimization here. Although these optimizations are targeted toward improved protein expression in mammalian systems, the resultant codon utilization is generally improved for bacterial expression as well.

The firefly luciferase sequences that are most widely used have been extensively modified with respect to their codon utilization. One of the first and most notable changes to the gene's nucleotide sequence resulted in a codon usage profile specific for mammalian expression and the removal of a peroxisome-targeting sequence present in the wild-type luciferase sequence [68]. This version, known as luc+, was further improved by changing the gene's sequence to remove transcription factor binding sites and renamed luc2 [69]. This version of the firefly luciferase gene is present in Promega's widely used pGL4 reporter gene vectors.

Similar to the case for firefly luciferase, the most commonly used marine luciferases have also undergone codon optimization, and these codon-optimized forms have been used as the genetic starting point for most protein engineering that has followed. Alterations of the DNA sequence of *Renilla* luciferase for improved mammalian codon utilization and removal of binding sites for mammalian transcription factors resulted in 10- to 100-fold signal improvements in signal in a mammalian expression assay [42]. Alteration of the DNA sequence of *Gaussia* luciferase (GLuc) for improved mammalian codon utilization reported a 1,000-fold improvement in signal for a mammalian expression assay [41].

8.7.5 Cellular Localization

Adding intracellular localization signals to luciferases has been performed to alter their intracellular kinetics. Versions of firefly luciferase, *Renilla* luciferase, and NanoLuc have all been generated as fusion proteins with the 41 amino acid PEST degradation sequence to shorten intracellular half-life. In the case of FLuc, this reduces the intracellular half-life from 3.5 h to 0.8 h [45]. Similarly, this modification reduces the intracellular half-life of NanoLuc from >6 h to 20 min [17].

As discussed previously, secreted luciferases are problematic for small-animal bioluminescence imaging due to the decoupling of bioluminescence signal from the location of protein expression, and work has been performed to transform these secreted luciferases into forms that will remain in proximity to their cell of origin. While removal of the signal peptide of *Gaussia* luciferase decreased levels of secreted luciferase, it did not increase levels of cellular-associated luciferase [41]. Addition of a KDEL sequence to GLuc in an attempt to divert transit of the expressed protein to the endoplasmic reticulum resulted in increased luminescence in cell lysates, but did not alter the cell-associated signal in unlysed cells [41]. Addition of a CD8 transmembrane domain to the C-terminus of GLuc, to generate a variant termed extGLuc, was successful in retaining the expressed luciferase to the cell surface [46]. This approach has an advantage compared to the use of a cytoplasmically retained luciferase (such as RLuc), in that an extracellular luciferase obviates the need for the substrate to traverse the cell membrane and therefore removes confounding effects caused by MDR1 P-glycoprotein-mediated efflux of coelenterazine [70]. However, a carefully performed *in vivo* comparison experiment between FLuc, RLuc, and extGLuc involving viral luciferase expression in the mouse liver found extGLuc signal to be undetectable [71]. This finding reiterates the concern for a lack of robustness in many engineered luciferases and the need to confirm the applicability of a given luciferase for each specific biomedical use.

8.7.6 Luciferase-GFP Fusions

In nature, *Renilla* luciferase is normally associated with a green fluorescent protein (*Renilla* GFP). In this context, the energy released from substrate degradation is not directly released as a photon of light from the luciferase, but in a process termed bioluminescence resonance energy transfer (BRET), the energy is used instead to excite the adjacent fluorescent protein and then released as a photon of light from the fluorophore. In *Renilla*, the combination of luciferase and GFP leads to improved light output/quantum yield compared to the luciferase on its own [72]. In a similar manner, enhanced yellow fluorescent protein (EYFP) was combined as a fusion protein with RLuc8 to generate a luminescent protein (eBAF-Y) that was 26-fold brighter than RLuc in mammalian cell culture conditions [73].

In this fashion, Saito et al. generated a *Renilla* luciferase-fluorescent protein fusion protein with even greater light output [74]. They first subjected RLuc8 to a random mutagenesis screen and identified an S257G mutation that yielded a small improvement in activity (note though that S257G has previously been described as a destabilizing mutation in the context of native RLuc [10]). They then combined this new luciferase with the yellow fluorescent protein variant “Venus,” to generate a luminescent protein they termed “Nanolantern.” Nanolantern demonstrated a ~five-fold improvement in light output compared to RLuc8, a ~three-fold improvement in light output compared to eBAF-Y, and a redshifted emission spectrum (peak ~530 nm) consistent with the principle light emitter in Nanolantern being the fluorescent protein. This improvement in light output may arise from improved quantum yield of the BRET system compared to the luciferase, and/or fluorescent protein-induced conformational changes to the luciferase that improve the enzymatic properties of the luciferase. The authors went on to demonstrate the utility of Nanolantern for imaging intracellular structures in living cells, for video-rate bioluminescence imaging in a small-animal model, and for the construction of Ca²⁺ and ATP sensors.

8.7.7 *NanoLuc*

As a prime example and excellent summary of many of the techniques stated above, Hall and colleagues performed extensive mutagenesis and protein engineering on the secreted decapod luciferase from the deep-sea shrimp *O. gracilirostris* (OLuc), turning it into a small, highly active, monomeric, intracellular luciferase called NanoLuc [17]. OLuc is a heterotetramer containing two 35-kDa and two 19-kDa subunits, with the luciferase activity associated with the smaller 19-kDa subunits. A consensus approach to semi-rational mutagenesis was not available due to the lack of similar proteins in the protein databases. Based on fold homology to intracellular lipid binding proteins, an N166R mutation was introduced into the smaller 19-kDa subunit that led to ~three-fold increased luminescence intensity. They then performed a single round of random mutagenesis, uncovering eight additional beneficial mutations that, when combined, led to a 30,000-fold increase in light output in their assay. Random mutagenesis was then performed on this 9-mutation variant of the 19-kDa OLuc subunit and the variants were subjected to a library of coelenterazine analogs. From this screen, they identified a number of additional beneficial mutations, as well as a coelenterazine analog (titled furimazine) that showed both decreased background autoluminescence and increased luminescence relative to coelenterazine in the presence of their variants. They combined these beneficial mutations into a 14-mutation variant and then performed another random mutagenesis screen with furimazine as the substrate. Incorporating the additional beneficial mutations, the final product was a 16-mutation variant of the 19-kDa OLuc subunit that the authors termed “NanoLuc.” In a final step, they performed codon optimization for mammalian

cell expression. Based on western blot analysis, the superiority of NanoLuc compared to OLuc was credited to improved stability and protein expression, rather than enzymatic improvements in the luciferase. Gel permeation chromatography demonstrated the monomeric nature of NanoLuc, and thermal and pH stability were confirmed. NanoLuc (with furimazine) demonstrated ~ 150 -fold more luminescence per enzyme than either FLuc (with D-luciferin) or RLuc (with coelenterazine) under glow-type assay conditions. Given NanoLuc's recent development at the time of this publication, its robustness has yet to be confirmed in the scientific literature, but it appears quite promising for cell culture experiments. Given its narrow blueshifted emission peak (460 nm) compared to RLuc and FLuc, very few photons will be generated within the optical window for mammalian tissue (above 600 nm). As such, its suitability of bioluminescence imaging in small-animal models remains to be determined.

8.7.8 *Split-Lucs*

As alluded to previously, a prominent area of luciferase engineering is related to a technique known as split-protein complementation. This phenomenon has been described in only a handful of other enzymes (of which the fluorescent protein GFP is probably the most prominent). It revolves around the observation that the luciferase can be split into two separate polypeptides, which by themselves possess little to no luminescent activity, but when sufficiently colocalized, can perform the bioluminescent reaction without reestablishing a covalent peptide bond between the two entities. This property has been exploited extensively to image protein-protein interactions, most commonly by creating gene fusion constructs between the split halves and a pair of interacting proteins. It has been adapted to encompass imaging of protein-DNA, protein-RNA, and DNA-DNA interactions, as well as protein phosphorylation, small molecule inhibition, nutrient sensing, viral pathogenesis, and protease activity.

As it applies to the discussion of luciferase engineering, a handful of studies have attempted to determine the most optimal split site within the luciferase enzyme. For most applications, this entails choosing a site that renders two fragments with little to no activity, but retain the ability to reconstitute as much of the intact enzyme's bioluminescence as possible when colocalized. The first report of split luciferase complementation, and the one which has served as a basis for almost all subsequent investigations on split beetle luciferases, used the crystal structure of the *P. pyralis* luciferase to estimate the most optimal split site [75]. The investigators split the protein within the flexible linker connecting the disparate N- and C-terminal domains (437/438). This split site was used as a starting point for a different approach performed several years later. In the subsequent study, a library of semi-random, and frequently overlapping, luciferase fragments were fused to the rapamycin-inducible interacting protein pair of FRB (rapamycin-binding domain of mTOR) and FKBP (FK506-binding protein 12) and

then expressed in bacteria [76]. When the bacteria were exposed to rapamycin, FRB and FKBP were induced to interact with one another, bringing the split luciferase halves into proximity with one another and facilitating luciferin oxidation. This experimental design allowed the authors to both screen against self-complementing pairs by omitting colonies that luminesced in the absence of rapamycin, and screen for pairs whose complementation resulted in the highest levels of photon emission by selecting the brightest colonies in the presence of rapamycin. The study revealed slightly overlapping luciferase halves (416/398) with more favorable properties than the original split luciferases. Another study using a similar experimental design but a different library revealed a potentially more favorable split site of 398/394 [77].

In contrast to the intermolecular interaction screen discussed above, a split site for the *P. plagiophthalmus* luciferase was determined by intramolecular inducible folding [78]. The split luciferases in this study were evaluated by inserting the androgen receptor ligand binding domain and its dihydrotestosterone (DHT)-inducible peptide-binding partner at various sites with the luciferase gene. Then, in a fashion similar to the previous studies, individual colonies were screened in the presence and absence of DHT to determine the most optimal split site (439/440). A split site for the luciferase from *Pyrearinus termitilluminans* (415/394) was found using a semirational approach in which overlapping fragments were systematically evaluated by altering the split site with single amino acid resolution [79]. While the strategy employed for this evaluation was similar to the ones described above, the study represents the first time that a luciferase split site had been so rigorously characterized.

A number of split coelenterazine luciferases appropriate for protein complementation assays have also been developed. Split *Renilla* luciferases were initially developed without structural information to guide split selection sites [80, 81], but more recent publications have selected split sites derived from homology-based [82] or crystallographic structural information [83]. A number of split *Gaussia* luciferases have also been developed. Due to an absence of homology or structural information for the copepod luciferases, these split GLuc have been developed by primary sequence analysis including predictions of unstructured regions [84] and hydrophobicity [85].

8.7.9 Chemical Sensitivity/Altered Substrate Specificity

Independent of the luciferases engineered for sensitivity to small molecules via split-protein complementation design, several variants have been constructed that have intrinsic sensitivity to various chemicals due to mutations in their primary sequence. Often, this involves altering the enzymatic specificity of luciferase. Due to the extensive investigations of luciferase enzymology and the availability of high resolution crystal structures, the engineering of luciferase with altered chemical sensitivities is generally a very rational endeavor. Aminoluciferin is a

derivative of D-luciferin in which the hydroxyl group of luciferin is replaced with an amino group. This enables more facile functionalization of the substrate and is often used as a starting material for substrate engineering studies. However as a synthetic substrate, beetle luciferases do not oxidize aminoluciferin as efficiently as they do D-luciferin. This problem was rectified by performing saturation mutagenesis on active site residues of the luciferase from *P. pyralis*, as determined by the crystal structure, followed by screening bacterial lysates in the presence of aminoluciferin and its derivatives [86]. A library of mutants with improved specificity for a variety of aminoluciferin derivatives were discovered.

A random approach was taken for engineering the luciferase from *L. lateralis* to be less sensitive to the detergent benzalkonium chloride (BAC). BAC is used in certain protocols for the extraction of intracellular ATP from mammalian cells. Since ATP is a necessary cofactor of luciferase, it can be quantified by bioluminescence detection. Unfortunately, BAC can interfere with luciferin oxidation, so a random mutagenesis approach was taken, followed by a screen of bacterial lysates, to identify a BAC-resistant double mutant (A217L, E490K) [87].

8.8 Conclusion

The field of luciferase engineering has seen a wave of extraordinary advances in recent years, though by no means has it reached its apogee. Because of its functional capacity, altering the properties of luciferase is a relatively straightforward endeavor. Thus, luciferase engineering will remain a topic of interest to those concerned not only with the native proteins' biochemistry, but also to investigators seeking to synthesize biomolecules with novel capacities. As we tried to demonstrate in this chapter, luciferase engineering is limited largely by the creativity and effectiveness of the screen. Similar to other fields such as phage display and catalytic RNA, in which a large pool of variants must be sifted through to find the most beneficial changes, luciferase engineering has few procedural bounds and relies on the cleverness of the investigator to produce appreciable results.

Due to this fundamental reliance on the screen, however, the results of luciferase engineering are not necessarily transferable from one application to another. The selection of a luciferase remains an unavoidably empirical exercise. The luciferase that proves to be optimal for detecting bacterial contamination in the field may demonstrate little utility when assessing lymphocyte activation in a living animal and vice versa. Therefore, the reader should be cautioned not to assume that the examples cited herein can serve every need. And though several of the mutants described above may perform well across many platforms, we hope that the studies enumerated in this chapter can serve as guideposts for the development of a panoply of new mutants, each exquisitely tuned for a precise application.

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