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Mammalian-Based Bioreporter Targets: Protein Expression for Bioluminescent and Fluorescent Detection in the Mammalian Cellular Background

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1. Introduction

While originally utilized primarily in prokaryotic organisms, reporter systems such as green fluorescent protein (GFP) and its variants, substrate dependent luciferase systems such as beetle and marine luciferase proteins, and substrate independent luciferase systems such as the bacterial luciferase gene cassette have now become the standards for imaging in the mammalian cellular background as well (Fig. 1). This has occurred in part because the use of cultured mammalian cells or small animal models has increased steadily over time in order to obtain more relevant human proxies for the measurement of cellular processes and bioavailability of biomedically relevant compounds of interest. However, the expression and detection of these reporter systems in eukaryotic models presents unique challenges not encountered in their prokaryotic counterparts.

The differences in gene expression and cellular compartmentalization between prokaryotic and eukaryotic cells represent the major obstacles for the efficient expression of these and other reporter systems at the cellular level, but once the line has been crossed from expression in single cells to expression in multicellular organisms, these problems can be compounded by the increases in absorption and scattering intrinsic to whole animal imaging. As a result, much consideration must be given to the experimental design associated with bioluminescent or fluorescent detection from mammalian cells. The type of system employed, whether it be cell culture or whole animal, the depth of imaging, the relevant time period available for data collection, and even the ability to distinguish multiple reporter systems from within the same tissue must be understood and acknowledged prior to beginning any experiment.

To better prepare for selection of the most appropriate reporter protein for the detection of a bioluminescent or fluorescent signal from mammalian tissue, this chapter will highlight and compare the utility of the most commonly available reporter systems as reported in the current literature. Specifically, the chapter will focus on the green fluorescent protein (GFP) and its color shifted variants, D-luciferin based luciferase proteins (both from the firefly and from click beetles), coelenterazine based luciferase proteins (those from the *Renilla* and *Gaussia* genera), and the bacterial luciferase gene cassette (*lux*). A short background of the major reporter proteins will be given that explains the biochemical requirements of each, as

well as the physical properties that make them unique (emission wavelength, quantum yield, etc.). These properties will be considered in relation to how they influence the ability to detect the resulting bioluminescent or fluorescent signal using commercially available equipment.

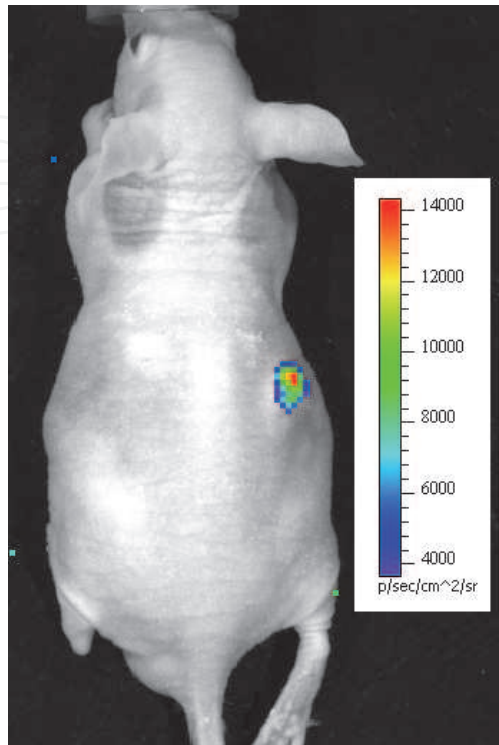


Fig. 1. Bioluminescent detection from a small animal model.

The luminescent (as shown here from cells expressing human codon-optimized bacterial luciferase genes) or fluorescent signals of a reporter cell line can be detected through the tissue of a living small animal host, allowing for localization of the cell population and estimation of its size without the need to sacrifice the host.

To provide a better understanding of the function of each of the reporter systems, relevant examples will be cited that illustrate the common use of each reporter system, as well as novel examples that show how each can be adapted to function under unique circumstances based on their biochemical requirements and physical emission properties. The relative strengths and weaknesses of each of the considered reporter systems will also be discussed, with an eye towards their role in imaging cellular processes at the level of cell culture imaging, near surface detection through tissue in small animal models, and deep tissue (beyond subcutaneous) imaging in small animal models. The overall goal is to present a fair representation of the potential uses of each of the chosen reporter systems to allow for selection of the most appropriate system for a given experimental design.

2. Imaging concerns in biological tissues

There are additional concerns when performing data collection from within a living medium that must be considered in addition to the traditional focus on experimental efficiency. The detection of a fluorescent or luminescent signal from within a tissue sample can be dependent on multiple factors, such as the total flux of photons capable of being

produced by the reporter, the population size of reporter cells that are introduced into the sample, and the location of those reporter cells within the tissue sample itself (Troy et al., 2004). Subsequently, the visualization of the reporter signal is dependent on the absorption and scattering of that signal prior to its detection. One method for overcoming these detrimental conditions is to alter the emission wavelength of the reporter signal. Increasing the wavelength can serve to both reduce the amount of scattering and decrease absorption. This is possible because the majority of photon absorption is the result of signal interaction with endogenous chromophoric material within the cell. By moving to a longer, more red-shifted emission wavelength, where the level of absorption within tissue is lower, it is possible to detect a greater amount of signal intensity than would be possible from an identical reporter with a lower, more blue-shifted emission wavelength (Chance et al., 1998). Because of this, it is paramount to consider the emission wavelength of a given reporter system, along with the other desired attributes of that reporter, prior to experimental design. For example, the bioluminescent signal from the bacterial bioluminescence (*lux*) reaction is produced at 490 nm. This is relatively blue-shifted as compared to the firefly luciferase (Luc)-based bioluminescent probes that display their peak luminescent signal at 560 nm. The shorter wavelength of the *lux*-based signal has a greater chance of becoming attenuated within the tissue and therefore may not be as easily detected if it is used in deeper tissue applications such as intraperitoneal or intraorganellar injections into a small animal host. To overcome the disadvantage of increased attenuation due to the shorter, blue-shifted emission wavelength, similar detection levels using the *lux* reporter would require a longer integration time than would be expected when using the longer wavelength Luc reporter following subcutaneous injection.

It is important to remember that these effects are not specific to bioluminescent reporters and hold true when working with fluorescent reporter proteins as well. However, when introducing a fluorescent reporter system into the mammalian cellular environment, one must take into account the effect that the excitation wavelength will have on overall detectability. This is because the presence of the excitation signal can result in production of high levels of background autofluorescence under small animal imaging conditions, due to the presence of chromophoric material within the mammalian cell (Choy et al., 2003; Troy et al., 2004). This can result in difficulty distinguishing the reporter excitation signal from the background noise if the two are produced at similar wavelengths. Unlike a bioluminescent system, which does not require an excitation light signal, increasing the duration of this signal can lead to a reduction of measureable signal due to the combined results of photobleaching of the reactive photocenter of the reporter protein and the associated increase in background noise from extended excitation of endogenous chromophoric cellular material. For this reason, the photostability of a particular fluorescent protein must be considered in addition to the general concerns of efficiency and brightness that should be weighed prior to selection of any reporter protein, fluorescent or bioluminescent, when designing any experiment.

3. Green fluorescent protein (GFP)

3.1 Introduction

While the green fluorescent protein (GFP) is not the only fluorescent protein target used for visualization in the mammalian cellular background, it is certainly the most well known. Widespread familiarity with this reporter, coupled with its longstanding use in both

prokaryotic and eukaryotic organisms, is perhaps the major impetus that drives investigators to select it as a target for biomarker visualization. The namesake 504 nm emission signal of GFP is relatively low (Patterson et al., 1997) in the green spectrum of visible light, making it a less than ideal candidate for high levels of fluorescent penetration through mammalian tissue (Chance et al., 1998). This disadvantage has been at least partially overcome by the introduction of mutated versions of the GFP protein that have been engineered to fluoresce at higher wavelengths where penetration is greater (Tsien, 1998; Zimmer, 2002). In addition, fluorescent proteins have since been introduced that successfully increase the emission wavelength of the fluorescent signal to a fully red-shifted wavelength for more efficient detection through tissue. This was accomplished first with the introduction of the monomeric red fluorescent protein (mRFP1), derived from the red fluorescent protein of *Discosoma* sp. (Campbell, R. et al., 2002). Further engineering was then performed to develop more efficient variants such as the popular mCherry and tdTomato proteins in use today (Shaner et al., 2004). Despite these advances in fluorescent reporter technology, GFP remains in high use, either in conjunction with or independent of these alternate reporter systems. When used properly, it can be an excellent reporter system for imaging in the mammalian cellular environment and serves as an excellent model for the function of fluorescent proteins in general.

3.2 GFP structure

Wild type GFP is composed of a single polypeptide consisting of 328 amino acids (Tsien, 1998). The mature protein forms an 11-stranded β -barrel that is roughly twice as long as it is wide (diameter of 24 Å and height of 42 Å) (Zimmer, 2002). The only exception to the β -sheet motif is the formation of two short α -helices between the 7th and 8th β -strands. These two α -helical sections act as lids to cover the open ends of the cylinder (Phillips, 1997) and support the formation of the fluorophore (Tsien, 1998). This 11-stranded β -sheet conformation is very unique and has been termed the β -can. It is hypothesized that the tight, almost seamless, structure imparted by the β -can formation is what gives the GFP protein such a high level of resistance to denaturation by heat and chemical denaturants (Ward et al., 1982).

The historical view of the mechanism suggests the fluorophore is autocatalytically formed post-translationally from the side chains of residues 65 – 67 (Phillips, 1997). Following folding into a native conformation, the carbonyl of Ser 65 undergoes a nucleophilic attack from the amide of Gly 67 leading to formation of an imidazolinone. Oxygen then dehydrogenates the α - β bond of Tyr 66 to bring its aromatic side chain into conjugation with the imidazolinone, allowing for absorbance and fluorescence to occur (Tsien, 1998). More recently this mechanism has been revisited and it has been proposed that the position of backbone residues plays a greater role than initially thought (Fig. 2). It is hypothesized that the tight β -can structure holds the residues forming the fluorophore into position, allowing Arg 96 to initiate the acid base reactions required to form an intermediate that is stabilized by Glu 222 even though it is in an energetically unfavorable state. This intermediate is then oxidized to the highly stable aromatic imidazolone and the fluorophore becomes active (Barondeau et al., 2003). It is the formation and oxidation of the fluorophore that is the limiting step in the expression of a mature GFP protein, with the process taking as little as 45

minutes following protein synthesis in optimized protein constructs (Crameri et al., 1996) or as long as 4 hours in the wild type variant (Heim et al., 1994).

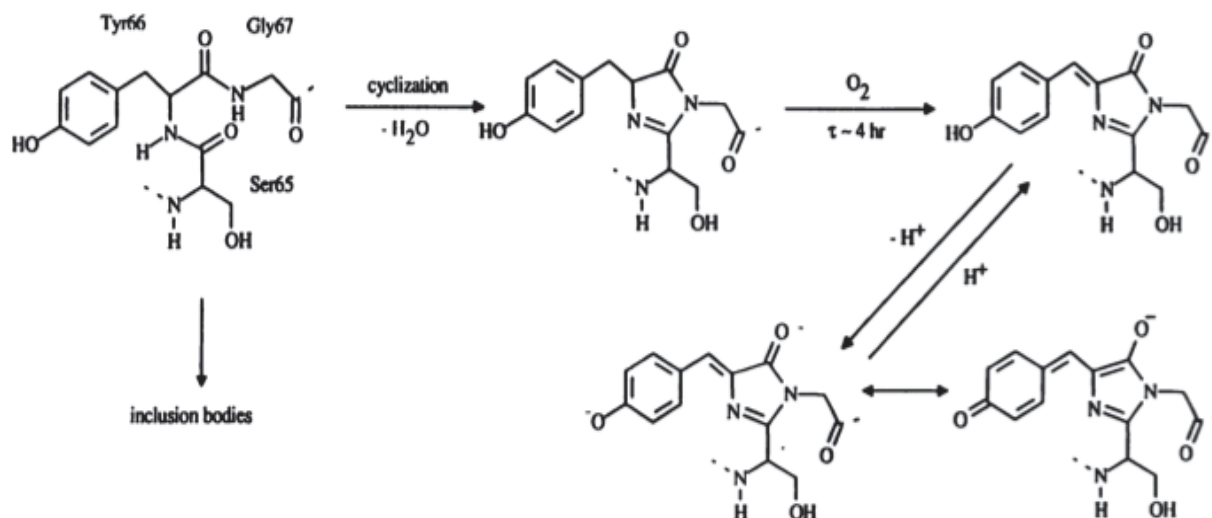


Fig. 2. The proposed biosynthetic scheme for the chromophore of GFP.

The freshly translated protein (upper left) could be trapped by inclusion bodies or remain soluble and nonfluorescent (upper center) until oxidation by O_2 , which could dehydrogenate Tyr 66 to form the fluorophore (upper right). The protonated and deprotonated species (upper and lower right) may be responsible for the 394 and 470 to 475 nm excitation peaks, respectively. The excited state of phenols are much more acidic than their ground states, so that emission would come only from the deprotonated species. Originally published in (Heim et al., 1994). Copyright by the National Academy of Sciences.

While this Ser-Tyr-Gly triplet is common among known proteins, it alone is not sufficient to cause formation of a fluorophore. What makes the sequence unique in GFP is a combination of steric positioning and acid/base chemistry with the surrounding residues. It is an absolute requirement that glycine be present in position 67, as no functional GFP mutants have been isolated with any other residue at this position (Phillips, 1997). The freedom allowed by the short side chain of glycine allows for proper positioning of the fluorophore so that it can properly interact with the surrounding residues (Zimmer, 2002). These interactions provide immobilization of the fluorophore allowing for resonance stabilization under excited state conditions (Phillips, 1997).

It is possible for the native GFP protein to exist as either a monomer or a dimer, with variant fluorescent signatures in either state (Phillips, 1997). This is in contrast to homologous proteins such as GFP from *Renilla reniformis*, which is an obligate dimer (Tsien, 1998). It is generally accepted that whether or not GFP is isolated as a dimer or a monomer is dependent on the isolation conditions and not the result of any *in vivo* influences (Palm et al., 1997) and that the dissociation constant for the dimer is $100 \mu\text{M}$ (Phillips, 1997). Dimerization is localized to the hydrophobic surface formed by the Ala 206, Leu 221, and Phe 223 residues and bolstered by a host of other hydrophilic contacts (Tsien, 1998). When these contacts are made the structure of the fluorophore can be altered, presumptively because its tight immobilization relative to the backbone associated atoms of neighboring residues forces it to change orientation following bond rearrangement (Wu et al., 1996).

3.3 GFP mechanism of action

The wild type GFP protein is able to absorb light at two different wavelengths. A minor peak occurs at 475 nm with the major peak at 397 nm. Regardless of which excitation wavelength is used, emission occurs only at 504 nm (Patterson et al., 1997). The different absorption peaks have been attributed to varying protonation states of the fluorophore, with the neutral state corresponding to the major absorption peak at 397 nm and the anionic form contributing to the minor peak at 475 nm (Niwa et al., 1996). The large shift between the major absorption peak at 397 nm and the emission at 504 nm can be attributed to an excited state proton transfer from the side chain of the Tyr 66 residues of the fluorophore (Chattoraj et al., 1996) to the carboxylate oxygen of Glu 222 (Zimmer, 2002).

Based on this interconversion of the fluorophore, a three state model of photoisomerization has been put forward to explain the chemical basis for shifts in absorption. This model states that excitation of the neutral state fluorophore can cause conversion to the anionic form via an intermediate (Chattoraj et al., 1996). The intermediate is structurally similar to the neutral form of the fluorophore, but has become deprotonated at the phenol group of Tyr 66 (Zimmer, 2002). Excitation of the anionic form is capable of directly emitting fluorescence, while the neutral state must necessarily convert into an excited form of this intermediate prior to emission (Jung et al., 2005). While it is possible for the neutral form to convert to the anionic form following excitation, this is not the most favorable reaction. The majority of excited, neutral fluorophores will convert briefly to the intermediate state, where fluorescence will occur followed by reversion back to the neutral state (Chattoraj et al., 1996). Interconversion between the neutral and anionic states is possible, but requires both proton transfer and conformational change to occur (Zimmer, 2002). Similarly, the majority of anionic fluorophores will revert to the ground state following fluorescent emission, but could instead undergo a conformational change to the intermediate state and then continue on to adopt a neutral charge state (Chattoraj et al., 1996).

In a wild type population, GFP contains a 6:1 ratio of neutral to anionic fluorophores (Tsien, 1998), explaining why the major absorption peak is found at 397 nm. However, upon extended UV illumination this peak will begin to decrease and the minor peak will increase (Cubitt et al., 1995). This behavior corresponds to the photoisomerization of the neutral fluorophore form responsible for the major absorption peak being converted into the anionic form as discussed above. While the photoisomerization characteristics of GFP can prove problematic for quantification, they do allow for the study of protein movement by excitation with intense UV light at 397 nm followed by excitation at 475 nm in order to track the movement of the photoisomerized fluorophores (Yokoe & Meyer, 1996).

3.4 Color shifted GFP variants

Following the discovery of GFP it was quickly proven that amino acid substitutions were capable of altering its fluorescent characteristics. Since that time, versions of GFP have been developed that fold more efficiently at higher temperatures (Cramer et al., 1996), avoid dimerization at high concentration (Zacharias et al., 2002), or fluoresce in the blue (Heim et al., 1994), cyan (Hein & Tsien, 1996), or yellow (Ormo et al., 1996) wavelengths. The history and development of these variants has been reviewed extensively in the past (Tsien, 1998; Zimmer, 2002), and they can now all be classified by dividing the known variants into seven classes based on spectral characteristics. When applied in concert, these variants of the GFP

protein have given researchers the ability to use multiple GFP-based reporters in the same environment at the same time, improving the usefulness and range of this already dynamic protein.

3.5 Red-shifted fluorescent reporter proteins for the mammalian environment

To further red-shift fluorescent emission wavelengths beyond that of the GFP variants, a fluorescent protein from an entirely new organism was used as the starting point. This protein was the red fluorescent protein (dsRed) from the *Discosoma* corals. dsRed is a 26 kDa protein that has an excitation wavelength of 558 nm and a resulting emission wavelength of 583 nm. It is capable of producing fluorescence with a quantum yield of 0.24 (Matz et al., 1999) but acts as a tetramer under wild-type conditions, making it problematic for use as an efficient reporter in its native state. To overcome the problems associated with its tetrameric quaternary structure, dsRed was engineered to functionally express fluorescence as a monomer (Campbell, R. et al., 2002) and then further refined through a process of directed evolution to produce the popular mCherry protein. While mCherry is only 27% as bright as the original dsRed protein, it has improved photostability and red-shifted excitation and emission wavelengths at 587 nm and 610 nm respectively, which allow it to function more efficiently in the mammalian cellular background (Shaner et al., 2004). These red-shifted fluorescent reporter proteins are only a few examples of the type of improved fluorescent reporter proteins that have been developed for use in mammalian imaging, but are representative of the type of mutations that must be engineered to develop additional fluorescent reporters for this unique type of imaging.

3.6 Examples of use as a mammalian biosensor

3.6.1 Steady state imaging

Steady state imaging is the classical hallmark of mammalian visualization. This process begins with transfecting the gene encoding the fluorescent reporter into a cell line. If the researcher is primarily concerned with intracellular processes, this may be all that is required. Once the reporter protein is being expressed within the host cell, its presence can be visualized using fluorescent microscopy following excitation at the appropriate wavelength. If the goal is to determine the location or population size of the cells within a small animal model, the transfected line must first be introduced and then allowed to propagate within the host until it reaches a level capable of being detected through the native host tissue. This growth period can take several days depending on the size of the initial cellular inoculum. When these types of experiments are performed, it is most important to take into consideration the wavelength and brightness of the reporter protein used to ensure efficient performance. For example, when attempting to localize a single protein within a cell, the GFP protein can be fused to the protein of interest, and then quickly and easily visualized under fluorescence microscopy. This is the strategy that was taken by Barak et al. when they developed β -Arrestin 2/GFP fusions that were used to significantly improve detection of G-protein coupled receptor activation. Expression of the GFP-fused proteins allowed the investigators to visualize how the conjugate responded to ligand mediated receptor activation using confocal microscopy in real-time using living cells (Barak et al., 1997). This work has been instrumental in monitoring G-coupled protein receptor activation, which represents the single most important target to date for drug development and medical therapy. However, while GFP provided an excellent target for

detection in single cells, when the goal is localization of a tumor cell population within a mouse model, a reporter such as mCherry should be considered because it would allow for emission in a more red-shifted wavelength, thereby improving the signal penetration through the additional host tissue and allowing for easier detection than would be expected from GFP.

3.6.2 Multi-reporter imaging

In its most basic form, multi-reporter imaging is simply an extension of steady state imaging following introduction of two or more reporter proteins. Under these conditions, the genes for the reporter proteins are introduced into the cell and, following expression, they are exposed to their respective excitation signals in a stepwise manner. The resulting emission signals can be differentiated either temporally from the staggered excitation signal applications or simultaneously based on their differential wavelength characteristics. In this type of approach the most important consideration should be the overlap of the chosen reporter excitation and emission wavelengths. Care must be used to select groupings of reporters that do not have overlapping emission and excitation signals. If multiple reporters have similar excitation wavelengths, there will be no way to separate their expression times since they will be triggered simultaneously. Likewise, if multiple reporters share overlapping emission wavelengths, it may not be possible to differentiate their locations unless they have disparate excitation wavelengths and their expression is controlled temporally. Finally, caution must be used to ensure the emission wavelength of one of the reporters is not within the excitation range of a simultaneously expressed reporter. Under these conditions the two signals cannot be differentiated and the initial signal can be partially or completely consumed during energy transfer to the second reporter in a process referred to as fluorescence resonance energy transfer (FRET).

In some cases, this type of FRET is the desired outcome. FRET is often used to boost the overall fluorescent output of the reporter system by taking advantage of a high penetration excitation wavelength of one reporter, and the resulting increased emission properties of a second. Alternatively, FRET systems can be used to visualize the interaction of proteins within a cell. By creating fusions between two proteins of interest and fluorescent proteins such as GFP and its blue-shifted variant, it is possible to visualize when the two proteins of interest are interacting at a resolution greater than that achievable using traditional optical microscopy. This is possible because these reporters display overlapping emission and excitation wavelengths, thereby allowing investigators to determine where the fused proteins are interacting by using only a single excitation wavelength and reading the opposite partner's excitation wavelength (Day, 1998).

3.6.3 Measuring changes in cellular health

There have been many examples of how fluorescent proteins can be used to monitor changes in cellular health, with an extensive review of these studies having been presented previously (Aguilera et al., 2006). In general, the methodology behind these types of experiments is fairly straightforward. The cell line of interest is first transfected with a fluorescent reporter protein, then, following determination of the baseline level of fluorescence, the cells are treated with a chemical or compound of interest and changes in fluorescent activity are monitored over time. Any resulting decrease in the fluorescent signal

relative to untreated control cells indicates a reduction in cellular health. These types of experiments are beneficial because they can present a simple, high throughput method for screening large numbers of compounds prior to beginning more in depth analysis.

3.7 Summary of advantages and disadvantages

| Advantages and Disadvantages of Fluorescent Reporters | |
|---|---|
| Advantages | Disadvantages |
| Diverse range of colors | Potentially high levels of background fluorescence upon excitation signal |
| Quantitative correlation between signal strength and cell numbers | Can be subject to photobleaching, preventing repeated imaging |
| No requirement for addition of exogenous substrate chemical | Non-genetic system leads to diffusion during cellular division |
| Noninvasive | Photoexcitation can cause tissue damage at low wavelengths |
| Can be used in combination for multiple labeling | |

Table 1. Advantages and Disadvantages of Fluorescent Reporter Use in Mammalian Imaging

4. Luciferase proteins that require exogenous substrate addition

4.1 Introduction

At the most basic level, a luciferase protein can be defined as any protein that, upon binding to its required substrate, produces a luminescent signal as a product of the ensuing enzymatic reaction. The discovery that this signal could be generated without the requirement for introduction of an excitatory light signal has been a mainstay of the biomedical imaging community because it allows for visualization without increased production of unwanted background fluorescence. However, unlike fluorescent reporter systems, the majority of luciferase systems require the addition of an exogenous chemical substrate to elicit their bioluminescent production. While there are many different types of luciferase proteins that have been isolated to date, there are predominantly two main categories that are in common use today: luciferase proteins that utilize D-luciferin as a substrate and luciferase proteins that utilize coelenterazine as a substrate. While these two classes of luciferase proteins utilize different substrates and therefore different mechanisms of action, the end result of the reaction for each results in the production of a luminescent signal in the visible range that can often be detected at lower levels than their fluorescent counterparts due to the lack of endogenous bioluminescent production in mammalian tissue (Close, D., Xu et al., 2010). While this advantage is obvious, it is important to note that the injection of required substrate, be it either D-luciferin or coelenterazine, entails the introduction of additional concerns over the efficiency of substrate injection, the quality of

the substrate being used, the rate of substrate uptake and clearing in the subject tissue, and even the cost of the substrate itself. Although each of these factors could have deleterious effects on the outcome of a particular experiment, they have not prevented the luciferase requiring proteins from becoming the most popular method for visualization in mammalian tissues because of their ease of use and high signal quality.

4.2 Luciferase proteins that utilize D-luciferin as an exogenous substrate

The chemical 2-(4-hydroxybenzothiazol-2-yl)-2-thiazoline acid is more commonly referred to simply as D-luciferin (White, E.H. et al., 1961) and is the substrate utilized by the majority of terrestrial bioluminescent organisms. The majority of these organisms are from the order Coleoptera and are best represented by the common North American firefly *Photinus pyralis* and the click beetles (Fraga, 2008). Historically, research has focused on determining the structure and mechanism of action of the firefly luciferase protein (Luc) as a model for substrate-dependent luminescent production, and recent discoveries have indicated that this mechanism is similar among all luciferase proteins that use D-luciferin as a substrate (Wood, Lam, & McElroy, 1989). Since its discovery, the Luc protein has grown into the most widely used of the bioluminescent reporter systems in mammalian imaging and therefore understanding its function is vital to interpreting the majority of published results on the subject.

4.2.1 Firefly luciferase structure

Luc is a monomeric protein composed of 550 amino acid residues with a molecular weight of 62 kDa (Conti et al., 1996). Originating from a eukaryotic organism, the genomic DNA encoding the Luc protein is comprised of seven exons and six introns that must be spliced out prior to translation in order to form the mature product (de Wet et al., 1986). The primary sequence of Luc shares extensive sequence similarity with the acyl-CoA ligases and this homology has been exploited to determine the location of the active site as well as the binding sites for its required co-factors (Conti et al., 1996). One interesting feature of the protein sequence is a C-terminal tag that directs it to the peroxisome (Viviani, 2002), although it does have some functional features of a membrane protein such as a tendency to associate with phospholipids (Ugarova, 1989).

The Luc protein can be divided into two major domains. The N-terminal domain is by far the larger of the two and comprises the first 436 residues. The C-terminal domain is formed from residues 440 to 550, and is linked to the N-terminal domain by a 4 residue long flexible loop. The large N-terminal domain is rich in secondary structure and is home to an antiparallel β -barrel and two β -sheets that are flanked by α -helices. While physically smaller, the C-terminal domain also contains a mix of secondary structures including two short antiparallel β -strands and a three-stranded mixed β -sheet associated with three α -helices arranged in an $\alpha + \beta$ structure. There are four short connecting regions in the crystal structure that are too disordered to interpret, however, these regions are all exposed to solvent and represent some of the most conserved residues in homologous proteins so their exact position is unlikely to effect structure-based predictions (Conti et al., 1996).

The N-terminal β -barrel is distorted into three distinct faces because of its interactions with the surrounding structures. Two of the three faces are formed by three-stranded antiparallel β -sheets, while the third is comprised of two strands of the neighboring major β -sheet and the disordered region connecting them. Because of this close interaction between the β -

barrel and the two major β -sheets, concave depressions are formed on the surface of the protein in a “Y” shape. The two major β -sheets in the N-terminal domain are each composed of eight strands with a core of parallel strands joined to α -helices running antiparallel on either side of the sheet to form a five-layered α - β - α - β - α tertiary structure. One sheet consists of five parallel and three antiparallel β -strands with six associated helices with all but the last helix being formed from a continuous section of the polypeptide chain, while the other is split between six parallel and two antiparallel strands and six helices and is formed from two non-contiguous portions of the polypeptide (Conti et al., 1996).

There is a wide cleft between the N and C-terminal domains that is bridged by residues 436 to 440. Although the crystal structure does not include bound substrates, all of the invariant residues from the related adenylate-forming enzymes are located on the opposing faces of this cleft, sparking the hypothesis that the domains re-arranged following substrate binding (Conti et al., 1996). This hypothesis has been bolstered by the recent crystallization and X-ray analysis of the related luciferase protein from the Japanese firefly *Luciola cruciata*. This structure was obtained in the presence of bound substrates and shows a much closer association between the two domains (Nakatsu et al., 2006). It has even been proposed that the C-terminal domain changes orientation multiple times to carry out different steps in the luminescent reaction (Branchini, B.R. et al., 2005).

In order to perform its luminescent reaction, Luc must bind with the luciferin, ATP-Mg²⁺, and oxygen (Hastings, J et al., 1953). Despite the widespread use of Luc, the actual binding sites for these components have yet to be determined conclusively. There are two current models put forth by Ugarova (Sandalova & Ugarova, 1999) and Branchini (Branchini, B. et al., 1998) that propose similar active site interactions. Both models suggest that residues Arg 218, His 245-Phe 247, Ala 313-Gly 320, and Lys 529 form the binding site for D-luciferin, with a hydrophobic surface being contributed directly by Ala 313, Ala 348, Ile 351, and Phe 247. The models differ in the importance of Arg 218, with Branchini suggesting that it interacts directly with luciferin phenolate (Branchini, B. et al., 1998) and Ugarova proposing that this interaction occurs with Arg 337. These models were bolstered by the *Luciola* crystal structure which showed association at Phe 249, Thr 253, Leu 286, Glu 311-Ser 314, Arg 337-Tyr 340, and Ala 348 (Nakatsu et al., 2006). Unfortunately, confirmation of these active site models and the determination of the exact binding locations will have to wait until a crystal structure is published showing Luc bound to its substrates.

4.2.2 Firefly luciferase mechanism of action

The Luc protein catalyzes the oxidation of the reduced D-luciferin in the presence of ATP-Mg²⁺ and oxygen to generate CO₂, AMP, PP_i, oxyluciferin, and yellow-green light at a wavelength of 562 nm. It is important to note that D-luciferin is a chiral molecule, and while both the D and L forms can bind to Luc and participate in adenylation reactions, only the D form is capable of continuing on in the reaction to generate light (Fraga, 2008). This reaction was originally reported to occur with a quantum yield of 0.88 (Seliger & McElroy, 1960), but has since been shown to actually achieve a quantum yield closer to only 0.41 (Ando et al., 2007). Because of this high quantum yield, the reaction is well suited to use as a reporter with as few as 10⁻¹⁹ mol of luciferase (2.4 X 10⁵ molecules) able to produce a light signal capable of being detected (Gould & Subramani, 1988).

It has been known since the early 1950's that the chemical reaction underlying firefly luminescence is a two-step process that first requires adenylation of D-luciferin followed by oxidation and the production of light (Hastings, J et al., 1953). Prior to the initiation of the reaction, the Luc protein must first bind to D-luciferin. However, at this time it is not yet capable of undergoing oxidation or producing light. The first step in the generation of light is the adenylation of the bound D-luciferin with the release of pyrophosphate (Ugarova, 1989). The function of this adenylation is to increase the acidity of the C4 proton of the thiazoline ring on D-luciferin. This allows for removal of a proton from C4 causing formation of a carbanion (McCapra, F. et al., 1968). This carbanion is then attacked by oxygen, displacing AMP and driving the formation of a cyclic peroxide with an associated carbonyl group (a dioxetanone ring). As the bonds supporting this structure collapse, it becomes decarboxylated, releasing CO₂ and forming an excited state of oxyluciferin in either the enol or keto form (Fig. 3) (Ugarova, 1989).

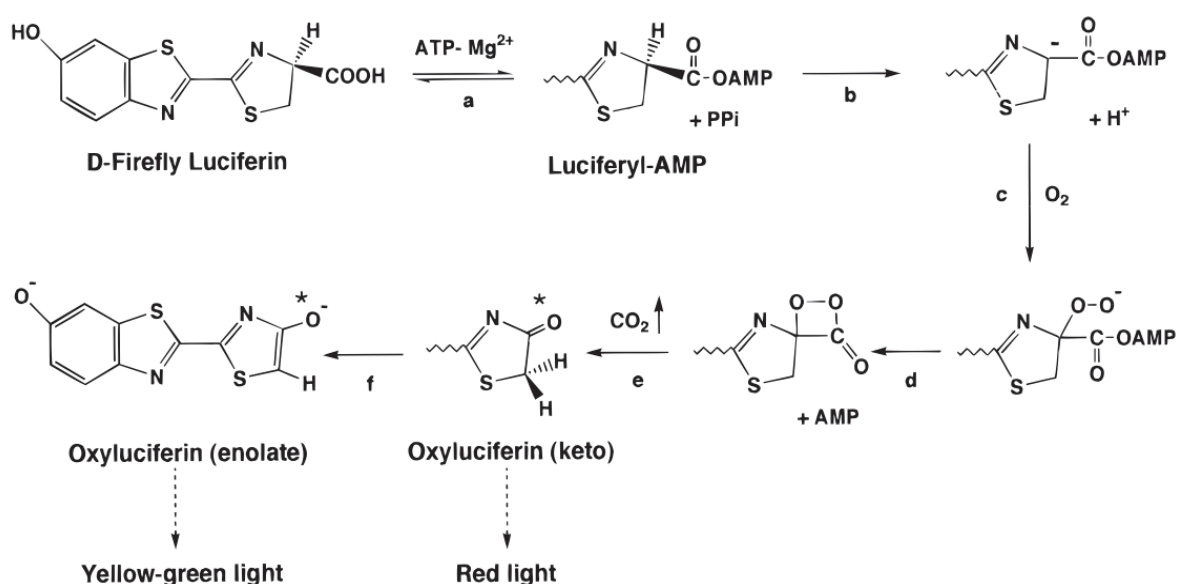


Fig. 3. The firefly luciferase bioluminescent reaction.

The luciferase protein holds the reduced luciferin to allow for adenylation (a). This process is followed by a deprotonation reaction that leads to the formation of a carbanion (b) and attack by oxygen (c), driving the formation of a cyclic intermediate (d). As this intermediate decays, carbon dioxide is released, forming the excited state luciferin in either the keto (e) or enolate (f) form. Used with permission from (Branchini, B. et al., 1998).

The kinetics of this reaction can be altered by varying the concentration of the substrates, with low concentrations (in the nM range) showing steady light production and high concentrations (μM range) producing a bright flash followed by decay to 5–10% of the maximum (DeLuca, Marlene et al., 1979). There are multiple possible inhibitory compounds that could be responsible for the kinetic profile generated under high substrate concentrations. It has previously been shown that even though oxyluciferin is a natural product of the luciferase reaction, it is capable of remaining bound as an inhibitor to enzymatic turnover (Denburg et al., 1969). The same was found to be true of another potential byproduct, L-AMP, which can account for up to 16% of the product formed during

the luminescent reaction (Fontes et al., 1998). This may, in part, explain how the addition of CoA to the luminescent reaction can result in improved performance. When CoA is added during the initial steps of the reaction it prevents the fast signal decay normally observed, and when it is added following this decay it can promote re-initiation of the flash kinetics. This can be attributed to CoA's interaction with L-AMP to form L-CoA, resulting in turnover of the Luc enzyme and reoccurrence of the luminescent reaction (Airth et al., 1958).

4.2.3 Click beetle luciferase proteins

While the Luc protein from *Photinus pyralis* is the most extensively studied of the D-luciferin utilizing enzymes, it is certainly not the only example from within this order of organisms. The insects represent a large related group of bioluminescent organisms, with over 2500 species reported to be capable of generating light (Viviani, 2002). While the vast majority of these luminescent reactions remain unstudied, the main exception is in the order Coleoptera (beetles) where systems have been characterized for the click beetles (Fraga, 2008). The main advantage of the click beetle luciferase proteins are that they are available in a wider array of colors than the firefly Luc protein. Despite these differences in emission wavelength, the substrates and mechanism of action are similar to that of the more well characterized Luc system, allowing for easy substitution with the Luc system if the need arises. Another advantage of the alternate color availability of the click beetle luciferases is that they can be used in conjunction with the Luc system and imaged simultaneously if a means of differentiating the individual emission wavelengths is available.

While it was originally believed that the different colors of the click beetle luciferase proteins were the result of divergent luciferase structures, this was shown not to be the case when the sequences of four luciferase genes from *Pyrophorus plagiophthalmus* with four different emission spectra were sequenced and found that they shared up to 99% amino acid identity (Wood, Lam, Seliger et al., 1989). There are currently three mechanisms that have been proposed to explain the multiple bioluminescent colorations: the active site polarity hypothesis (DeLuca, M, 1969), the tautomerization hypothesis (White, E. & Branchini, 1975), and the geometry hypothesis (McCapra, F., Gilfoyle, DJ., Young, DW., Church, NJ., Spencer P., 1994). The active site polarity hypothesis is based on the idea that the wavelength of light produced is related to the microenvironment surrounding the luminescent protein during the reaction. In non-polar solvents the spectrum is shifted towards blue and in polar solvents it is more red-shifted. It is questionable, however, if polarity fluctuations can account for large scale changes like those that have been observed in *P. plagiophthalmus*. The tautomerization hypothesis states that the wavelength of light produced is dependent on if either the enol or keto form of the luciferin is formed during the course of the reaction. A recent study has reported that by altering the substrate of the reaction, the keto form of the luciferin can produce either red or green light, making this hypothesis unlikely. Finally, the geometry hypothesis suggests that the geometry of the excited state oxyluciferin is responsible for determining the emission wavelength. In a 90° conformation it would achieve its lowest energy state and red light would be produced, whereas in the planar conformation it would be in its highest energy state and green light would be produced. Intermediate colors would be the result of geometries between these two extremes (Viviani, 2002).

4.2.4 Summary of advantages and disadvantages

| Advantages and Disadvantages of the D-luciferin Utilizing Luciferase Proteins | |
|---|--|
| Advantages | Disadvantages |
| <p>High sensitivity and low signal-to-noise ratio</p> <p>Quantitative correlation between signal strength and cell numbers</p> <p>Low background in animal tissues</p> <p>Variations of firefly luciferase (stabilized and red-shifted) and click beetle luciferases (red and green) are available</p> <p>Different colors allow multi-component monitoring</p> | <p>Requires exogenous luciferin addition</p> <p>Fast consumption of luciferin can lead to unstable signal</p> <p>ATP and oxygen dependent</p> <p>Currently not practical for large animal models</p> |

Table 2. Advantages and Disadvantages of Using D-luciferin Utilizing Luciferase Proteins in the Mammalian Cellular Environment

4.3 Luciferase proteins that utilize coelenterazine as an exogenous substrate

While the D-luciferin utilizing Luc system may be the most popular for mammalian imaging experiments, it is the coelenterazine utilizing luciferase proteins that are the most widely occurring. In nature there are examples of these types of luciferase proteins in cnidarians, copepods, chaetognaths, ctenophores, decapod shrimps, mysid shrimps, radiolarians, and some fish taxa as well (Greer & Szalay, 2002). The coelenterazine substrate has the chemical structure of 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzylimidazo-[1,2-a]pyrazin-3-(7H)-one (Bhaumik & Gambhir, 2002), and under its native function is bound to an associated protein to prevent availability to the luciferase. The strength of this bond is dependent on changes in calcium dynamics within the host cell, with increases leading to the detachment and subsequent availability of the substrate to participate in the bioluminescent reaction (Anderson et al., 1974). This system has been adapted, however, so that when the luciferase protein is expressed in a host cell, the coelenterazine substrate can be supplied exogenously, triggering the production of light without the need for changes in intracellular calcium levels. The primary example of a coelenterazine utilizing reporter is the luciferase from the sea pansy *Renilla reniformis* (RLuc). This protein interacts with its coelenterazine substrate to produce bioluminescence at 480 nm (Bhaumik & Gambhir, 2002). Because this wavelength is relatively blue-shifted compared to the D-luciferin luciferase utilizing proteins and because the two reporters require dissimilar substrates for activation, RLuc can be used either as a stand-alone reporter system or in conjunction with the Luc variants to simultaneously image multiple locations within the host. This multi-functionality has led to an increase in the popularity of RLuc for mammalian imaging in recent years.

4.3.1 *Renilla* luciferase structure

Unlike the previously discussed luciferin proteins, those that utilize coelenterazine as a substrate have not been found to display high levels of structural similarity, even when originating from within the same family. This most likely indicates that they are predominantly the result of individual evolutionary events (Loening et al., 2007). The structure of the RLuc gene from *Renilla reniformis* will be given as an example because it is the most laboratory relevant of the coelenterazine utilizing luciferase proteins, but caution should be used when attempting to interpret the associated mechanism of action with alternate luciferase proteins without first determining their structural discrepancies.

The RLuc protein is a 37 kDa enzyme comprised of 311 amino acids that exists as a monomer in solution. Crystal structures of the RLuc protein exist (both with and without bound substrate) at a resolution of 1.4 Å, however, these were constructed using a modified version of the protein that included 8 amino acid mutations (Loening et al., 2007). These mutations were included because they allow for more efficient expression as compared to the native enzyme and have not been shown to have a deleterious effect on bioluminescent production (Loening et al., 2006). The overall structure of the RLuc enzyme can be broken down into two domains. The core domain takes the form of an α/β -hydrolase fold (Loening et al., 2007), a structure composed of 8 β -sheets connected by α -helices. This structure is common to hydrolytic enzymes and is known to contain a catalytic triad that is responsible for carrying out their associated enzymatic reaction (Ollis et al., 1992). The cap domain is located above the core domain and consists of the residues from 146 to 330, which make up the region between α -helix "D" and β -sheet "6" (Loening et al., 2007).

The N terminal region of the protein is believed to exhibit a flexible conformation in solution, with the initial 10-15 residues capable of wrapping around the remainder of the protein towards the presumptive enzymatic pocket. However, it is not believed that these residues are absolutely required for securing the bound substrate or for proper steric positioning. To illustrate this point, RLuc proteins that have had the first 14 residues removed are still capable of producing more than 25% of their original activity. It is believed instead that a 10 amino acid flexible region corresponding to residues 153-163 within the cap domain is responsible for these actions (Loening et al., 2007). This is consistent with previously characterized, structurally similar enzymes and therefore more likely to be the case (Schanstra & Janssen, 1996).

The active site is believed to center around the catalytic triad, which is composed of the amino acids Asp 120, Glu 144, and His 285. This placement is consistent with that of other known α/β -hydrolase proteins, with the nucleophile (Asp 120) located immediately after the fifth β -sheet (Loening et al., 2006). This area is known as the "nucleophile elbow" and follows the general sequence pattern of Gly-X-(nucleophile)-X-Gly (Heikinheimo et al., 1999). In RLuc these residues are Gly 118-His 119-Asp 120-Trp 121-Gly 122. Further evidence that this is indeed the location of the active site was gathered by mutational analysis which showed that the mutations most detrimental to enzyme function occurred either in one of the three proposed catalytic triad residues or in Asn 53, Trp 121, or Pro 220, three residues that reside in the rear of the proposed active site pocket. This pocket is surrounded by a ring of hydrophobic and aromatic residues such as isoleucine, valine, phenylalanine, and tryptophan that are believed to aid in the orientation and binding of the coelenterazine substrate.

4.3.2 *Renilla* luciferase mechanism of action

In the *Renilla* luciferase bioluminescent reaction the luciferin (coelenterazine) undergoes oxidative decarboxylation in the presence of oxygen to produce CO₂, the oxidized oxyluciferin, and light at a wavelength of 480 nm (Hart et al., 1978). Under native conditions this reaction takes place within specialized subcellular compartments called lumisomes, however, during the course of mammalian expression the protein will be located wherever the gene is targeted using common sequence tags. Activation is also simplified during mammalian expression. Unlike under native conditions when the coelenterazine substrate would be trapped by an associated binding protein until changes in local calcium concentration gradients triggered its release, making it available for binding by the RLuc protein (Anderson et al., 1974), during exogenous expression these associated binding proteins are not natively present, and therefore the injection of coelenterazine is all that is required to elicit a bioluminescent response.

The coelenterazine substrate can be thought of as containing three complex reaction sites that each serve a purpose during binding and subsequent oxidation following interaction with the RLuc protein. The first domain (R1) is a *p*-hydroxy-phenyl group, the second (R2) is a benzyl ring, and the third (R3) is a *p*-hydroxy-benzyl ring. While the exact binding locations of each region of the substrate has not been confirmed, docking simulations have suggested potential locations that can be used to support the current hypothesis for the RLuc mechanism of action. These simulations suggest that the R1 group binds in a position where it is accessible to the catalytic triad of Asp 120, Glu 144 and His 285, possibly by stabilization due to interaction between the hydroxyl of the R1 group and Asn 53 of the RLuc protein. Further stabilization would be provided by interaction of the R3 domain with the Thr 184 residue (Woo et al., 2008).

Once the substrate has been bound and localized to the active site of RLuc, the chemical reaction occurs that produces the telltale bioluminescent signal. This reaction appears to be similar to the chemical reaction that occurs in other coelenterazine utilizing luciferase proteins such as aequorin despite their structural differences (Anderson et al., 1974). Once bound to RLuc, oxygen attaches at C2 resulting in the formation of a hydroperoxide. This hydroperoxide then becomes deprotonated (presumably through interaction with the catalytic triad) and the resulting negative charge on the hydroperoxide then undergoes a nucleophilic attack on C3 of coelenterazine to irreversibly form a dioxetanone intermediate. It is this cyclization that then provides the energy required to drive the production of light from the overall reaction (Vysotski & Lee, 2004). As the bonds between newly cyclized oxygens collapse the peroxide is released as CO₂ and the excited, anionic state of coelenterazine is formed. As this form decays a photon is released, and finally the fully oxidized luciferin is formed and released (Hart et al., 1978).

4.3.3 *Gaussia* luciferase

Gaussia luciferase (GLuc) represents an interesting example of a coelenterazine utilizing luciferase protein that is naturally secreted from the cell. GLuc is a small 19.9 kDa protein consisting of only 185 amino acids that, in the presence of its substrate coelenterazine, will produce a bioluminescent signal with a peak at 480 nm similar to RLuc. However, GLuc has some interesting properties that set it apart from RLuc as an imaging target in the mammalian environment. The most unique difference is that the GLuc protein can be encoded to either remain in the cell or be naturally excreted depending on the presence or

absence of an included signal peptide. This property allows the resulting luminescent signal to be used either for localization within a cell or for facile high throughput screening using spent cell culture media without the need to disturb the cells via exposure to coelenterazine. In addition to the excretable nature of the GLuc protein, it has also been shown to produce a brighter bioluminescent signal than its RLuc counterpart following substrate exposure (Tannous et al., 2005). This means that the same 480 nm bioluminescent signal can be achieved as during use with RLuc, but less of the luciferase protein is required to generate the same level of signal. Therefore GLuc, without its associated excretory signal peptide, may be a suitable alternative to RLuc if imaging is required at extremely low cell population sizes. While there are other coelenterazine utilizing luciferase proteins available, the advantages and utility of GLuc make it the main counterpart to RLuc for laboratory use today.

4.3.4 Summary of advantages and disadvantages

| Advantages and Disadvantages of Coelenterazine Utilizing Luciferase Proteins | |
|---|---|
| Advantages | Disadvantages |
| <p>High sensitivity</p> <p>Quantitative correlation between signal strength and cell numbers</p> <p>Stabilized and red-shifted <i>Renilla</i> luciferase are available</p> <p>Secretion of <i>Gaussia</i> luciferase allows for subject-independent bioluminescence measurement</p> | <p>Requires exogenous coelenterazine addition</p> <p>Low anatomic resolution</p> <p>Increased background due to oxidation of coelenterazine by serum</p> <p>Oxygen dependent</p> <p>Fast consumption of coelenterazine can lead to unstable signal</p> <p>Currently not practical for large animal models</p> |

Table 3. Advantages and Disadvantages of Using Coelenterazine Utilizing Luciferase Proteins in the Mammalian Cellular Environment

4.4 Examples of use as a mammalian biosensor

4.4.1 Steady state imaging

Steady state imaging using substrate requiring bioluminescent protein reporters is performed in a similar fashion to imaging using fluorescent reporter proteins, only with the injection of the substrate chemical performed in place of stimulation with an excitation wavelength. The main advantage offered by the use of the bioluminescent systems is that the injection of substrate does not create background luminescence because there are no native

bioluminescent proteins in the mammalian tissue. This allows researchers to achieve detection with much smaller cell population sizes when using bioluminescent reporter systems. The most common use of steady state imaging using these types of reporter systems has been for the study of tumorigenesis and evaluation of tumor treatment. For example, Kim and colleagues have demonstrated this advantage with the newest generation of these reporters designed for tumor detection. These investigators were able to inject codon-optimized FLuc containing 4T1 mouse mammary tumor cells subcutaneously and then image single bioluminescent cells at a background ratio of 6:1 (Kim et al., 2010). This experiment effectively demonstrates how substrate utilizing reporters can be used to continuously monitor cancer development from a single cell all the way to complete tumor formation.

4.4.2 Multi-component bioluminescent imaging

Because the substrate requiring bioluminescent reporter systems are dependent on activation by a specific substrate, commonly either D-luciferin or coelenterazine, it is possible to use one luciferase of each type simultaneously in the same host. To trigger bioluminescent production from an individual reporter protein, its specific substrate is added. This design elicits luminescent production from the target while not activating the alternate bioluminescent reporter. This type of experimental design allows for localization of multiple cellular groups from within a single cell or host animal. It is also possible to use a bioluminescent reporter in conjunction with an associated fluorescent reporter in a manner similar to FRET, only in this case the original luminescent signal is bioluminescent in nature and not fluorescent. This type of experiment is referred to as bioluminescence resonance energy transfer (BRET) and has been used by Angers et. al. to demonstrate the presence of G-protein coupled receptor dimers on the surface of living cells. By tagging a subset of β_2 -adrenergic receptor proteins with RLuc and a subset with the red-shifted variant of green fluorescent protein, YFP, it was possible to detect both a luminescent and fluorescent signal in cells expressing both variants, but no fluorescent signal in cells expressing only YFP since no fluorescent excitation signal was used (Angers et al., 2000).

4.4.3 Overall tumor load imaging

The naturally secreted nature of the GLuc protein has lead to interesting advances whereby it can be used to monitor overall tumor burden in small animal models without the requirement of directly imaging the host animal. This has been demonstrated by Chung and colleagues who induced bioluminescence from blood samples of host animals suffering from tumors that had been tagged with the gene for expression of GLuc. Since the GLuc protein was secreted into the blood it was possible to correlate bioluminescence of the blood sample with overall tumor load without ever having to introduce the coelenterazine substrate to the animal. This process was capable of reporting on tumors at lower levels than would have been possible using traditional steady state tumor imaging, and was capable of reporting on the dynamics of tumor growth in response to treatment (Chung et al., 2009).

4.5 Concerns related to substrate injection route

When working with luciferase proteins that utilize an exogenous substrate in small animal models, it will be necessary to introduce the requisite substrate through injection. However, the chosen route of substrate injection can have influential effects on the emission of a

luminescent signal. As a result, although logistical concerns may be most pertinent to consideration for investigators, the method of injection should be considered in light of the proposed objectives of any study (Inoue et al., 2009). The three most common substrate injection routes are intraperitoneal, intravenous, and subcutaneous. Each results in the introduction of the substrate in a unique manner and, although each should elicit bioluminescent production of an expressed reporter protein, they will all do so on different time scales and with different expression kinetics. It is therefore important to have a basic understanding of the resulting luminescent profiles of each type of injection prior to determining which is best suited to an individual experimental design.

4.5.1 Intraperitoneal injection of substrate

The appeal of intraperitoneal injection for the majority of researchers is its convenience, however, following this route of injection the substrate must absorb across the peritoneum before reaching the luciferase expressing cell populations. Any variations in the rate of absorption can lead to variations in the resulting luminescent signal. These variations, even when subtle, can increase the difficulty of reproducing the luminescent results (Keyaerts et al., 2008). In addition, investigator error can lead to injection into the bowel, causing a weak or non-existent luminescent signal that can be confused with a negative result (Baba et al., 2007). Because of the associated diffusion, intraperitoneal injection produces lower peak luminescence levels than alternate injection techniques when inducing light production in subcutaneous tumor models, however, it has been found that it can also overestimate tumor size when used to induce luminescence from intraperitoneal or spleen-localized tumors, due to direct contact between the luciferin and the luciferase expressing cells (Inoue et al., 2009). The greater availability of the luciferin to the luciferase containing cells increases the amount of bioluminescent output by allowing them greater access to their luciferin without being limited by diffusion through non-luciferase containing tissue. This increases the influx of the luciferin compound into the cell due to the resulting increased concentration gradient.

4.5.2 Intravenous injection of substrate

Intravenous injection can be used to systematically profuse a test subject with D-luciferin or coelenterazine. It is also a facile method for exposing multiple tissue locations to the substrate on relatively similar timescales. Because the administration of the luciferin is systemic, it allows for lower doses to be administered to achieve similar luminescence intensities as would be seen using alternate injection routes (Keyaerts et al., 2008), however, studies using radio-labeled D-luciferin have indicated that the uptake rate of intravenously injected substrate is slower in the gastrointestinal organs, pancreas, and spleen than would be achieved using intraperitoneal injection (Lee et al., 2003). It is also important to note that when intravenous injection is used, the resulting luminescent signal is often of a much shorter duration than would be observed using alternate injection routes (Inoue et al., 2009).

4.5.3 Subcutaneous injection of substrate

Subcutaneous injection is often used as an alternative to intraperitoneal injection in order to avoid the signal attenuation shortcomings of the intravenous injection route. Bryant et al. (Bryant et al., 2008) have demonstrated that repeated subcutaneous injection of luciferin can

provide a simple and accurate model for monitoring brain tumor growth in rats, and though there is concern that repeated injection could cause excessive tissue damage, it has been demonstrated that the repeated subcutaneous injection of D-luciferin or coelenterazine into an animal model results in minimal injection site damage while providing researchers with bioluminescent signals that correlate well with intraperitoneal substrate injection luminescent profiles, albeit with a longer lag time prior to reaching tumor models in the intraperitoneal space (Inoue et al., 2009).

5. The bacterial luciferase proteins

5.1 Introduction

Luminescent bacteria are the most abundant and widely distributed of the light emitting organisms on earth and can be found in both aquatic (freshwater and marine) and terrestrial environments. Despite the diverse nature of bacterial bioluminescence, the majority of these organisms are classified into three genera: *Vibrio*, *Photobacterium*, and *Photorhabdus*. Of these, only those from *Photorhabdus* have been discovered in terrestrial habitats (Meighen, 1991) and developed into reporters capable of functioning within the mammalian cellular environment (Close, D, Patterson et al., 2010). It is the terrestrial nature of the bacterial luciferase (*lux*) genes from *Photorhabdus* that made them suitable for adoption and use in mammalian tissues. The *lux* genes from the *Vibrio* and *Photobacterium* genera are marine in nature, and as such their protein products have been naturally adapted to function at lower ambient temperatures than those required for mammalian expression. However, even with their propensity to function efficiently at 37°C, the *Photorhabdus lux* genes required extensive modification to carry out the bioluminescent reaction in a non-bacterial host cell. Natively, the *lux* gene cassette consists of 5 genes organized sequentially in a single operon in the form *luxCDABE*. The *luxA* and *luxB* gene products form the heterodimeric luciferase enzyme, and the *luxD*, *luxC* and *luxE* gene products form a transferase, a synthase, and a reductase respectively, that work together to produce and regenerate the required myristyl aldehyde co-substrate from endogenous myristyl groups. Because the substrates required by the *luxAB* heterodimer enzyme consist only of oxygen, FMNH₂, and the aldehyde that is formed by the *luxCDE* genes, this system has the unique ability to produce bioluminescence without the addition of exogenous substrate addition (Meighen, 1991). However, unlike the native, uncompartimentalized bacterial cellular environment, the mammalian intracellular environment does not contain high enough levels of reduced FMNH₂ to support efficient bioluminescent production. To alleviate this problem, a sixth *lux* gene must be co-expressed that is not present in all bacterial species. This sixth gene, *frp*, encodes an NAD(P)H:flavin reductase that helps to cycle endogenous FMN into the required FMNH₂ co-substrate (Close, D, Patterson et al., 2010).

To function properly within a mammalian host cell, the 5 *lux* genes, as well as an additional flavin reductase gene (*frp*), must be expressed simultaneously and at high levels. To accommodate these requirements the genes must be codon-optimized to the human codon preference and their expression linked via internal ribosomal entry elements or similar promoter independent intervening sequences. This allows for the relatively normalized levels of expression while reducing the overall amount of foreign DNA that must be introduced and maintained in the host genome. When expressed under these conditions,

the *lux* genes are capable of producing a luminescent signal in the mammalian host cell at 490 nm without the need for any external stimulus (Close, D, Patterson et al., 2010). Although limited due to their relatively low luminescent yield compared to the luciferase-dependent reporter systems and blue-shifted luminescent signal, the unique ability of substrate-free luminescent production makes the Lux system a user friendly and attractive alternative to the D-luciferin or coelenterazine utilizing systems.

5.2 Bacterial luciferase structure

The functional bacterial luciferase enzyme is a heterodimer with a molecular weight of 77 kDa. The individual α and β subunits are the products of the *luxA* and *luxB* genes respectively, and have molecular weights of 40 and 37 kDa. The two subunits appear to be the result of a gene duplication event owing to an approximately 30% amino acid sequence identity (Meighen, 1991). All previously characterized bacterial luciferases appear to be homologous and catalyze the same reaction, however, the majority of research has centered on the luciferase from the marine bacterium *Vibrio harveyi*, so the structure described in this review will be based on the protein from that organism along with its conventional numbering system.

Individually the α and β subunits of the luciferase heterodimer formed by the *luxA* and *luxB* genes are capable of producing a very weak bioluminescent signal, but dimerization is required for the reaction to proceed at biologically relevant levels (Choi et al., 1995). This finding, along with the similarities in structure between the two subunits would tend to implicate the dimer interface as the active site, however, the single active site has been proposed to exist only within the α subunit (Baldwin et al., 1995). Indeed, a recent crystal structure shows the oxidized FMN substrate bound to the α subunit only (Campbell, Z.T. et al., 2009).

Both of the α and β subunits have similar overall conformations, and assemble into a single-domain eight-stranded β/α barrel motif (also known as a TIM barrel after the first identified protein with that structure, triose-phosphate isomerase). The interiors of these barrels are packed with hydrophobic residues, as would be expected to aid in folding, while the N-terminal residues, which are exposed to solvent, contain hydrophilic residues. The C-terminal ends are hydrophobic, but are protected from solvent access by the presence of two antiparallel α -helices. The dimerization of the two subunits is mediated by a parallel four helix bundle centered on a pseudo two-fold axis of symmetry as it relates to the α and β subunit orientation. This region is highly populated with glycines and alanines, which allows for close contact between the two helical bundles. The majority of binding force is provided by van der Waals interactions across the 2150 Å² surface area, but twenty-two proposed hydrogen bonds, as well as forty-five water-mediated intersubunit hydrogen bonds and a series of hydrophobic interactions also aid in attachment (Fisher et al., 1996).

The active site is most probably a large, open cavity on the α subunit that is open to solvent at the C-terminal end of the barrel structure proximal to the β subunit. Crystal structures of the enzyme with an associated flavin show that it is bound here with the isoalloxazine ring in a planar conformation. The ribitol portion of the flavin extends away at an $\sim 45^\circ$ angle while the phosphate is stabilized by the side chains of Arg 107, Arg 125, Glu 175, Ser 176, Thr 179, and the backbone amide of Glu 175. The isoalloxazine ring is held in place through

backbone contacts with Glu 175 and Phe 6 and the ribitol interactions cannot be clearly defined as occurring directly with the protein or being mediated by co-bound water molecules, but they can be localized to individual residues. The carbonyl oxygen at C2 of the ribitol hydrogen bonds with backbone amide hydrogen of Tyr 110, the nitrogen at position three forms a hydrogen bond with the backbone carbonyl oxygen of Glu 43, while the carbonyl oxygen at C4 hydrogen bonds to either the backbone amide proton or the enol form of the backbone carbonyl oxygen of Ala 75. It is likely, but as of yet unproven, that the aldehyde binding location is adjacent to the benzenoid portion of the isoalloxane ring because of its proximity to the FMN binding site, size, and abundance of tryptophan and phenylalanine residues (Campbell, Z.T. et al., 2009).

5.3 Bacterial luciferase mechanism of action

When the bacterial luciferase enzyme is supplied with oxygen, FMNH₂, and a long chain aliphatic aldehyde it is able to produce light at a wavelength of 490 nm. The natural aldehyde for this reaction is believed to be tetradecanal, however, the enzyme is capable of functioning with alternative aldehydes as substrates (Meighen, 1991). The first step in the generation of light from these substrates is the binding of FMNH₂ by the luciferase enzyme and until recently its active site on the enzyme was not known. It has recently been confirmed that FMNH₂ binds on the α subunit in a large valley on the C-terminal end of the β -barrel structure (Campbell, Z.T. et al., 2009). The nature of the interactions between FMNH₂ and the amino acid residues in this area is discussed in the structure section above.

In order for the reaction to proceed the luciferase must undergo a conformational change following FMNH₂ attachment. This movement is primarily expressed in a short section of residues known as the protease liable region: a section of 29 amino acids residing on a disordered region of the α subunit joining α -helix α 7a to β -strand β 7a. The majority of residues in this sequence are unique to the α subunit and have long been implicated in the luminescent mechanism (Baldwin et al., 1995). Following attachment of FMNH₂ this region becomes more ordered and is stabilized by an intersubunit interaction between Phe 272 of the α subunit and Tyr 115 of the β subunit. This conformational change has been theorized to stabilize the α subunit in a conformation favorable for the luciferase reaction to occur (Campbell, Z.T. et al., 2009).

NMR studies have suggested that FMNH₂ binds to the enzyme in its anionic state (FMNH⁻) (Vervoort et al., 1986). With the flavin bound to the enzyme, molecular oxygen then binds to the C4a atom to form an intermediate 4a-hydroperoxy-5-hydroflavin (Nemtseva & Kudryasheva, 2007). It is important to note that this important C4a atom was determined to be in close proximity to a reactive thiol from the side chain of Cys 106 on the α subunit (Campbell, Z.T. et al., 2009), a residue that has long been hypothesized to play a role in the luminescent reaction, but since has been proven to be non-reactive through mutational analysis (Baldwin et al., 1987).

It has been shown, however, that C4a is the central atom for the luciferase reaction and, following establishment of the hydroperoxide there, it is capable of interaction with the aldehyde substrate via its oxygen molecule to form a peroxyhemiacetal group. This complex then undergoes a transformation (through an unknown intermediate or series of intermediates) to an excited state generally accepted to be a luciferase-bound 4a-hydroxy-5-hydroflavin mononucleotide, which then decays to give oxidized FMN, a corresponding

aliphatic acid, and light (Fig. 4) (Nemtseva & Kudryasheva, 2007). There have classically been many theories proposed to explain the exact process required for light emission that continue to expand today as technology for detecting the intermediate complexes has improved (Hastings, JW & Neelson, 1977; Nemtseva & Kudryasheva, 2007).

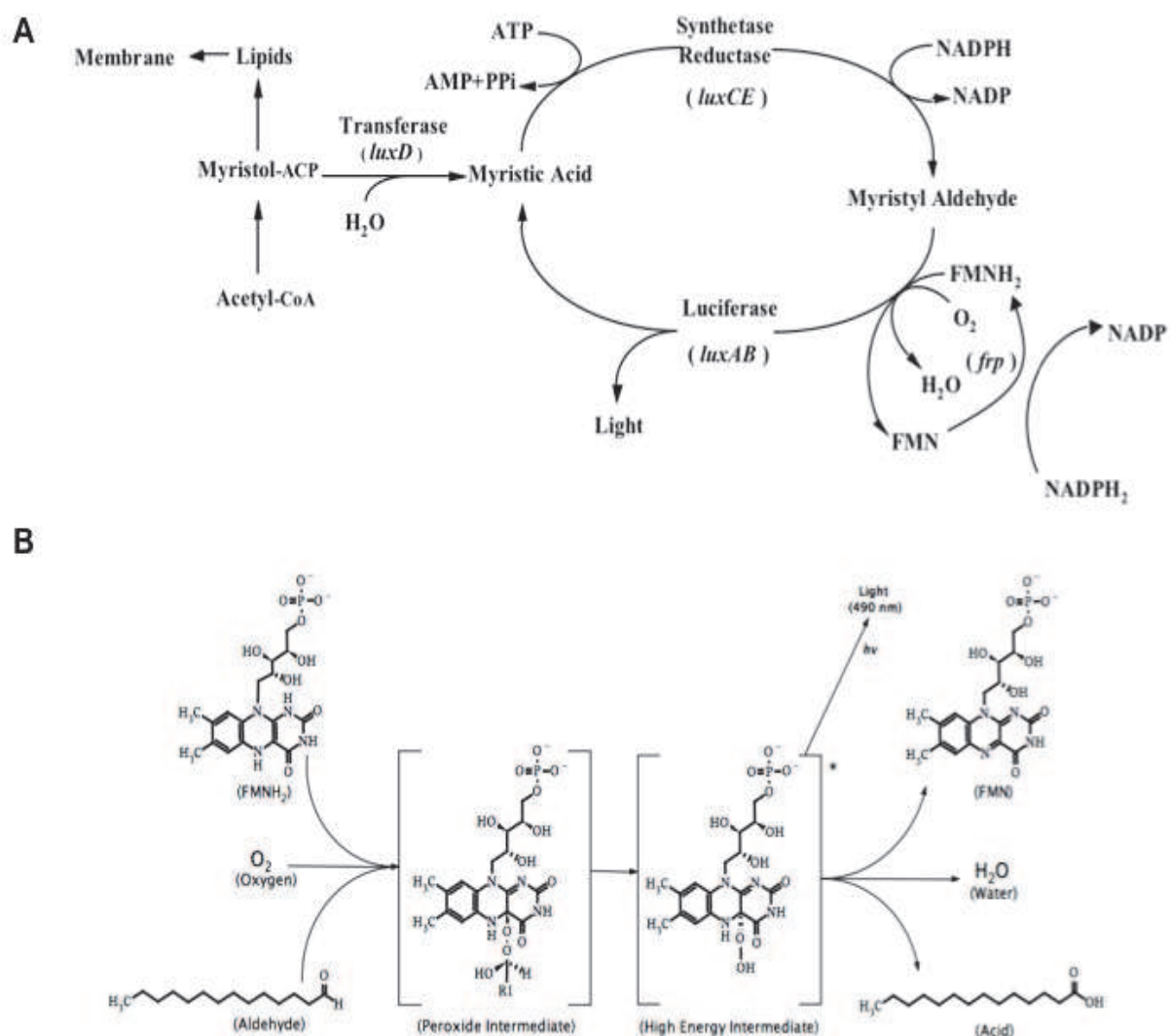


Fig. 4. Bioluminescent reaction catalyzed by the bacterial luciferase genes. A) The luciferase is formed from a heterodimer of the *luxA* and *luxB* gene products. The aliphatic aldehyde is supplied and regenerated by the products of the *luxC*, *luxD*, and *luxE* genes. The required oxygen and reduced riboflavin phosphate substrates are scavenged from endogenous metabolic processes, however, the flavin reductase gene (*frp*) aids in reduced flavin turnover rates in some species. B) The production of light, catalyzed by the products of the *luxAB* genes, results from the decay of a high energy intermediate (R1 = C₁₃H₂₇).

5.4 Use as a mammalian biosensor

Bacterial luciferase is the newest of the bioluminescent reporter proteins to be demonstrated for use with mammalian tissues. As a result, there have not been extensive publications on

its use under these conditions. The initial reports, however, have been promising, with *lux*-containing cells capable of being used for steady state imaging both in culture and in small animal models (Close, D, Patterson et al., 2010). If the *lux* cassette genes undergo widespread adoption there is no reason to believe they will not become capable of functioning in roles similar to the substrate requiring bioluminescent reporter proteins. The main drawback of the *lux* genes for function in the mammalian cellular background has been their low signal strength. As a result, they may not be as well suited for small population size cellular imaging or deep tissue imaging, where their weak signal may be attenuated prior to detection. However, it is important to keep in mind that as this reporter system becomes more common it will be subjected to optimization in a process similar to the other common reporter systems. If this is the case the utility of the *lux* reporter system should continue to increase with time.

5.5 Summary of advantages and disadvantages

| Advantages and Disadvantages of the Bacterial Luciferase Gene Cassette | |
|---|---|
| Advantages | Disadvantages |
| High sensitivity and low signal-to-noise ratio | Bioluminescence at 490 nm prone to absorption in animal tissues |
| Quantitative correlation between signal strength and cell numbers | Low anatomic resolution |
| Fully autonomous system, no requirement for addition of exogenous substrate | NADPH and oxygen dependent |
| Noninvasive | Not as bright as other luciferases |
| Stable signal | Currently not practical for large animal models |
| Rapid detection permitting real-time monitoring | Short history of use |

Table 4. Advantages and Disadvantages of Using the Bacterial Luciferase Gene Cassette in the Mammalian Cellular Environment

6. Conclusions

This chapter has presented only the most basic and widely used of the mammalian reporter proteins and is by no means exhaustive. It is important to recognize that there is no universally recognized optimal reporter system and that the choice of a reporter target should be made in light of the specific demands of each experimental design. Each reporter system has its own advantages and disadvantages, and each can be adapted to work under multiple imaging scenarios. The constant introduction of improved reporter protein targets

and modifications to existing reporter proteins suggest that the future of imaging in mammalian tissues should be bright for years to come.

7. Acknowledgments

Portions of this review reflecting work by the authors was supported by the National Science Foundation Division of Chemical, Bioengineering, Environmental, and Transport Systems (CBET) under award number CBET-0853780, the National Institutes of Health, National Cancer Institute, Cancer Imaging Program, award number CA127745-01, the University of Tennessee Research Foundation Technology Maturation Funding program, and the Army Defense University Research Instrumentation Program.

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Biosensors for Health, Environment and Biosecurity

Edited by Prof. Pier Andrea Serra

ISBN 978-953-307-443-6

Hard cover, 540 pages

Publisher InTech

Published online 19, July, 2011

Published in print edition July, 2011

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 16 different countries. The book consists of 24 chapters written by 76 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

How to reference

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Steven Ripp, Gary Saylor and Dan Close (2011). Mammalian-Based Bioreporter Targets: Protein Expression for Bioluminescent and Fluorescent Detection in the Mammalian Cellular Background, Biosensors for Health, Environment and Biosecurity, Prof. Pier Andrea Serra (Ed.), ISBN: 978-953-307-443-6, InTech, Available from: <http://www.intechopen.com/books/biosensors-for-health-environment-and-biosecurity/mammalian-based-bioreporter-targets-protein-expression-for-bioluminescent-and-fluorescent-detection->

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