Introduction

One strength of the field of biophysics is the ability to see the interactions between species many orders of magnitude smaller than that which may be detected with the human eye. Nearly every tool in the imaging toolbox involves the injection of energy into a system and observing the resulting reflection, scatter, or emission of that energy. For instance, the current choice in most cellular imaging, fluorescence microscopy, uses a specific wavelength of light to excite molecules known to absorb light and then observe the emission of light of a longer wavelength, or lower energy, when these fluorophores returns to their ground states. In contrast, the subject of this paper, a class of enzymes called luciferases, catalyze the oxidation of certain chemicals, called luciferins, (literally, “light bearers”) resulting in the release of a photon of light (1). This process is of interest to the biophysical chemistry community due to the physical phenomena underlining the chemical process as well as its inherent promise as a tool for cellular imaging and even for single molecule studies by producing light for imaging in situ.

Biophysics of the Reaction catalyzed by Luciferases

Luciferases are thought to have evolved in a convergent manner several times over many classes of fauna (2). The substrates and enzymes utilized in the light-producing reactions are diverse and share very little homology. However, the general principles in the generation of bioluminescence is remarkably well-preserved across the various organisms and may be summarized schematically as follows:

Luciferin + O_2 → Oxyluciferin + light

Where an excited state of the product is initially produced and the need to come back down to a ground state, results in the release of a photon of light. Most systems utilize additional cofactors such as NADH, ATP and use a two-step mechanism:
luciferin + ATP → luciferyl adenylate + PP_i
luciferyl adenylate + O_2 → oxyluciferin + AMP + light (3)

These types of reactions are characterized by a term called quantum yield. This is a measure of the efficiency of the bioluminescent reaction. The equation used is:

$$\Phi = \frac{\# \text{ reactant molecules decomposed}}{\# \text{ photons absorbed}}.$$  

The limit of this function is one, indicating that every single excitation of the luciferin molecule results in the production of a photon of light. In the case of luciferases, the quantum yield achieves this theoretical limit within the
error of the measurement, untrue of most other photochemical reactions (4). In order to successfully emit its photon of light, the excited oxyluciferin species must take a specific electronic pathway to the ground state. The deep hydrophobic active site of most luciferases is proposed to protect the excited species and help ensure that each excited molecule successfully produces its associated photon (5). The high and predictable quantum yield makes luciferase catalyzed reactions desirable targets for quantifying the yield of the reaction, particularly in the case monitoring the consumption of the ATP cofactor in firefly luciferases (6).

Both the kinetics and thermodynamics of luciferases have been heavily studied. Luciferases obey Michaelis-Menten kinetics. Both the stability of the luciferase-luciferin complex, as well and the velocity of the enzyme relate inversely with temperature. The binding of luciferin to firefly luciferin ranges from 15-25 μM over 280-290 K, while $V_{\text{max}}$ drops one hundred-fifty percent over the same range (7). A study of the interactions of anesthetics with luciferases (one of the few soluble proteins which are sensitive to these drugs) determined the changes in thermodynamic properties associated with the hydrophobic anesthetics in aqueous buffer to firefly luciferase show favorable $\Delta G^\circ$ and $\Delta H^\circ$ values across the board for six different anesthetics while only a few have positive $\Delta S^\circ$. Additionally, changes in the thermodynamic state variables, show $\Delta G^\circ$ depends solely on $\Delta H^\circ$. Both of these observations suggest that the binding is an enthalpically driven process (8). Understanding the process of substrate binding has been essential in tuning the system for molecular imaging.

Modulation of Bioluminescence

After the identification and characterization of a biological reaction, the next step in developing that reaction as a chemical and biological tool, is its optimization for its intended assay. Green fluorescent proteins are now available in a myriad of colors and forms optimized for different applications (9). Similar efforts are underway for luciferases. The natural range for $\lambda_{\text{max}}$ emission lies between 450 and 590 nm, or from deep blue to yellow in the visible range, with blues and yellow greens being most frequent (10). Emitters of longer wavelength, or “red-shifted” are desirable for use in biological systems due to the tendency of bio macromolecules, notably any that contain a heme prosthetic group, to absorb blue and green light (11). Changes of one or two key amino acids in firefly luciferase yields shifts in maximum wavelength of twenty-five nanometers into the red region. These mutations result in a slightly different form of the excited oxyluciferin intermediate (a keto form vs. the typical enolate) (12). Other studies that have promoted the bioengineering of useful red-shifted luciferases demonstrated that

Shifts in maximum emission wavelength generated by point mutation. Taken from Branchini et al. 1999.
wild type luciferases normally hold the luciferin substrate in a rigid pocket, preventing energy loss prior to the oxyluciferin’s return to the ground state. Mutations which open up and loosen the confirmation of the hydrophobic binding site allow some fraction of the energy to escape before light is emitted, yielding lower energy light, sometimes approaching the infrared-region. The incorporation of unnatural amino acids have further extended the range of color available, shifting \( \lambda_{\text{max}} \) an incredible forty-four nanometers (14). The luciferase system highlights the tendency in biological systems for certain small changes to yield large differences in effects. The understanding of the general mechanism of tuning emission wavelength has been essential in developing bioluminescence as a molecular imaging tool.

**Applications of Luciferase in Biophysical Assays**

The firefly luciferase has been optimized for expression in mammalian cells. The ability, coupled with technology allowing for its fusion in the genome to other genes and proteins of interest makes it a desirable tool in the molecular toolkit. Unlike fluorescence, luciferase enzymes are not subject to photo bleaching and will continue to produce light as long as they are supplied with their luciferin, allowing for a temporal resolution not available in most other assays (15). The time scale for investigation is particularly useful in vivo where mouse models of many human diseases may be visualized over the course of the development of the disease, such as the recruitment of T-cells to sites of infection or tumors (16). A vast variety of cellular processes have been probed with a variety of luciferases. The ability to express more than one protein under the control of a given promoter allows luciferase to act as a reporter of gene expression when the luciferase encoding gene is inserted downstream of the gene of interest (17).

Aequorin, isolated from the same jellyfish that naturally express green fluorescent protein (GFP), has proved especially useful. It is considered a photoprotein, rather than a bioluminescent one, due to the fact it is the only system identified that does not require the oxidation of its substrate because the luciferin coelenterazine is covalently linked as a prosthetic group (18). Its calcium sensitivity has been harnessed in order to visualize the flux of calcium levels in the skeletal muscle fibers of frogs (19).

One of the most useful applications of bioluminescence arises out of its natural occurrence. Aequorin and GFP are expressed co-locally in jellyfish and the blue light emitted by the Aequorin luciferin is absorbed by the GFP that then emits the characteristic green light. This process is called bioluminescence resonance energy transfer (BRET) and has been utilized extensively to elucidate cellular processes. Fluorescent molecules often give a brighter signal than bioluminescent ones, but the ability to illuminate the area of interest in animals allows the fluorescent molecules to be used most efficiently.
In the figure above, the researchers have taken advantage of the way bioluminescence and fluorescence complement each other as techniques. A gene that up-regulates vascularation in tumors was observed by a firefly luciferase tag in mice injected with cancer stem cells in both a wild type form as well as a mice in which this gene has been removed. In the pseudo color image on the left, the bioluminescence is much less bright in the knockout mouse. The bioluminescent signal guided the excision of tissue from the animals shown on the left; this material was then investigated by fluorescence microscopy on the right where the knockout mice have visibly fewer cancerous cells (20).

A current trend in biophysical research is the desire to follow the movements and transformations of individual molecules in the cell. GFP and quantum dots have both been utilized to do this but bioluminescence may be used to optimize the system. Proteins can be used as scaffolds to help form quantum dots; however until now, the function of the protein has been purely structural. Recently, firefly luciferases were used to create PbS quantum dots; the light emitted from the enzyme was then used to do BRET providing the first example of a protein that adds functionality to an existing fluorescent quantum dot system (21). Bioluminescence provides a very useful assay in and of itself allowing for the determination of ATP stores, visualizing pathologies in mouse models and as a genetic marker, but it is most powerful when coupled to other biophysical tools such as knockout studies and fluorescence as demonstrated in the aforementioned applications.

**Conclusion**

Luciferase enzymes have earned considerable interest in their own inherent biophysical attributes. The real value of this protein system, however, is contained in its promise as a means towards greater understanding of other biological systems. The uses highlighted above only scratch the surface of the types of studies completed using bioluminescent proteins. Notable examples out of the scope of this paper include membrane association of luciferase to look at T-cells and studies on the regulation of protein stability, both relying on luciferases’ special native signal. Studies ranging from single molecules all the way up to an image of molecular processes occurring within whole animals just begin to tap the richness of possible diversity of applications of the luciferase system.
References

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