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Engineering Bioluminescent Proteins: Expanding their Analytical Potential

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Synopsis

Bioluminescence has been observed in nature since the dawn of time, but now, scientists are harnessing it for analytical applications. Laura Rowe, Emre Dikici, and Sylvia Daunert of the University of Kentucky describe the origins of bioluminescent proteins and explore their uses in the modern chemistry laboratory. The cover features spectra of bioluminescent light superimposed on an image of jellyfish, which are a common source of bioluminescent proteins. Images courtesy of Emre Dikici and Shutterstock.

Bioluminescent proteins are used in a plethora of analytical methods, from ultrasensitive assay development to the in vivo imaging of cellular processes. This article reviews the most pertinent current bioluminescent-protein-based technologies and suggests the future direction of this vein of research. (To listen to a podcast about this feature, please go to the *Analytical Chemistry* multimedia page at pubs.acs.org/page/ancham/audio/index.html.)

Since the dawn of civilization, humans have shown enormous interest in exploring the world surrounding us. Our curious nature, fueled by a desire to both understand and control natural phenomena, has spurred the development of techniques and tools that constitute the foundation of today's analytical chemistry. In the past 100 years, analytical science has progressed from crude techniques such as filtration and distillation to highly sophisticated techniques such as atomic force spectroscopy, surface plasmon resonance, and chemometrics-based signal deconvolution algorithms. With these developments, our ability to observe and analyze has progressed from the macroscopic world visible to the naked eye to the microscopic domain, which must be magnified with optical lenses, and now to the nanometer scale and beyond—a feat that has piqued the interest of engineers, physicists, biologists, and chemists alike. Observing and quantifying events at these miniscule dimensions present new challenges and require a diverse array of specialized tools. In that regard, light-emitting proteins are invaluable for detection and imaging, as well as for revealing the properties of these nanoscale environments.

Light has inspired many cultural superstitions: early Polynesians, Scandinavians, and ancient seafarers all wove tales and mythologies about the lights and fires they beheld over water and in fields and mountains. Because they were unable to rationally explain these illuminations, they attributed the mysterious lights to machinations of the gods. Many notable philosophers and explorers, from Aristotle to Christopher Columbus, also observed "cold lights"—what we know now as bioluminescence. As logic took hold in the age of reason, scientists such as Robert Boyle and Charles Darwin attempted to rationalize the existence and purpose of bioluminescent

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phenomena. Today, we recognize bioluminescence as the production and emission of light by a living organism. An internal reaction converts chemical energy into light—a reaction almost always catalyzed by a protein.

A large variety of bioluminescent proteins of many biological origins and evolutionary functions have been studied, and their reaction mechanisms, substrates, and bioluminescent properties vary widely. Researchers group bioluminescent proteins into two major categories: photoproteins and luciferases (Figure 1). Photoproteins are bioluminescent proteins that are capable of emitting light in proportion to the concentration of protein, whereas in luciferase-catalyzed reactions, the amount of light emitted is proportional to the concentration of the substrate luciferin (1). Although a multitude of bioluminescent proteins have been discovered, relatively few have been studied thoroughly, and fewer still have been isolated and cloned for analytical applications. Thus, this article will focus primarily on proteins that have led to significant advances in analytical technology: Ca^{2+}-regulated photoproteins that use coelenterazine as a substrate and luciferases. Moreover, the number of papers published on photoproteins or luciferases in the last ten years is enormous; a complete review would no doubt fill a large book. Therefore, the goal of this article is to introduce the current analytical uses and evolving future of bioluminescent proteins. More in-depth sources for specific experimental techniques and protocols can be found elsewhere (2,3).

THE ORIGINS AND CHEMISTRY OF GLOWING PROTEINS

In nature, proteins are the building blocks of life, and organisms use them for both simple tasks such as constructing architectural scaffolds and complex tasks such as creating and tuning illumination. Such illumination methods fall into two broad categories: fluorescence and bioluminescence, both of which use proteins to emit and manipulate light. [link url="http://dx.doi.org/10.1021/ac802613w" title="Fluorescent-Protein-Based Biosensors: Modulation of Energy Transfer as a Design Principle"] Fluorescent proteins [end link] (FPs) tune light available from the surrounding environment whereas bioluminescent proteins create light by converting chemical bond energy to light energy. Both of these types of proteins are enormously useful for analytical applications and have been employed as highly sensitive labels in assay development, as reporters of gene expression, and as imaging agents, among other uses. Their importance to analytical investigations was confirmed when the 2008 Nobel Prize in Chemistry was awarded to Osamu Shimomura, Martin Chalfie, and Roger Tsien for the discovery and development of GFP (4).

The dominant sources of light-emitting proteins—available in a rainbow of colors—are marine organisms such as corals, fish, bacteria, and jellyfish and insects such as fireflies and click beetles. Though some FPs, such as those from corals, acquire their excitatory energy from the sun, many deep-dwelling organisms employ a bioluminescent protein as a source of excitatory light for fluorescence. For example, the jellyfish *Aequorea victoria* uses the bioluminescent protein aequorin to excite GFP. After the bioluminescence reaction occurs in *Aequorea victoria*, the electrons of FPs are promoted into an excited electronic state by a radiationless energy transfer and then relax when they emit light. Bioluminescent proteins, on the other hand, create light from the energy derived from an oxidation reaction. This self-illumination allows bioluminescent proteins to achieve ultrasensitive detection limits for analytical applications without an external excitation source (5).

Although all bioluminescent proteins emit light as a result of an oxidation reaction, the specific reaction that takes place in Ca^{2+}-regulated photoproteins differs from that of luciferases. For example, the photoprotein aequorin tightly—but not covalently—binds its substrate coelenterazine. In the absence of Ca^{2+}, the photoprotein cannot oxidize coelenterazine. However, upon addition of Ca^{2+}, the enzyme changes its conformation and catalyzes the

oxidation, producing coelenteramide and CO_2 (Figure 2A). Coelenteramide is produced in an excited state and upon relaxation to the ground state, emits a flash of blue light. After the reaction, the removal of spent coelenteramide and the incorporation of fresh coelenterazine is time consuming. Therefore, the light intensity of photoprotein-based bioluminescence is dependent on the concentration of the photoprotein itself. After equimolar saturation of photoprotein to coelenterazine, the concentration of the substrate does not affect the bioluminescence intensity (6).

On the other hand, in the case of firefly bioluminescence, the oxidation of the substrate, luciferin, is catalyzed by luciferase and requires ATP and molecular oxygen. The reaction yields a highly unstable, singlet-excited compound, oxyluciferin. This excited-state compound also emits light upon relaxation to the ground level. However, this reaction takes place in two steps: the first step involves the adenylation of luciferin followed by the oxidation of this adenyl–luciferin complex. In the second step, the luciferin is activated and the luciferase acts as an oxygenase on the adenyl-activated luciferin to produce oxyluciferin and CO_2 (Figure 2B). Upon completion of this reaction, the enzyme releases the oxyluciferin and is ready to catalyze a new reaction immediately. Therefore, the light intensity of a luciferase reaction is dependent on the concentration of luciferin, and the concentration of the enzyme does not affect the bioluminescence intensity (7). Regardless of these structural and mechanistic differences, both of these types of bioluminescent proteins have been manipulated extensively with recombinant DNA methods and employed ubiquitously for assays, sensors, and more recently, for in vivo and in situ imaging.

ANALYTE DETECTION

One of the first and most well-known uses of photoproteins is the in vivo detection of Ca^{2+} using aequorin. Ca^{2+} is an important regulator of cellular function, and knowledge of its flux and concentration is integral to understanding cellular physiology. Aequorin was initially injected into cells for these studies, but following its isolation and cloning, researchers found it much easier to express recombinant aequorin within cells (8). Because the aequorin does not emit light until its coelenterazine chromophore is present, it is easy to temporally control Ca^{2+} concentration detection by simply adding coelenterazine, (which will passively diffuse into the cell) when a reading of the cellular Ca^{2+} levels is desired. Luciferases have also been used extensively for in vivo detection. Because the bioluminescent reaction of luciferases is initiated by the ATP-activated oxidation of benzothiazolic luciferin, these bioluminescent proteins can be used to track and quantify ATP levels both in vivo and in vitro (9). Genetically-encoded peptide or protein tags within aequorin or luciferase that target specific organelles also can aid in determining Ca^{2+} and ATP levels in discrete cellular locations.

Both photoproteins and luciferases have also been used extensively as reporters or labels in binding assays. As previously mentioned, the self-illumination of bioluminescent proteins allows them to achieve very low detection limits. This is a distinct advantage when an analyte is present in very low concentrations or when an analytical technique is being miniaturized—for example, for high-throughput screening or micro total analysis systems such as lab-on-a-chip or lab-on-a-CD platforms (10). In addition, bioluminescent labels offer sensitivity and ease of use and are a safer alternative than some other methods of detection (such as radiolabels). Because of these advantages, photoproteins and luciferases have been employed in a large breadth of binding assays; there are too many to cover in detail here. For a more thorough discussion and specific examples see two recent reviews (5,11).

In general, these binding assays are ELISA-type, homogeneous, and DNA-hybridization assays and whole cell sensing systems. The ELISA-type assays use a bioluminescent protein that is conjugated to the analyte of interest by either chemical or genetic methods. This labeled analyte

competes with the free analyte present in the sample for a limited number of antibody binding sites; the antibody reacts with the sample solution either in its free form or immobilized on a solid substrate (for example, polystyrene beads, microtiter plates, magnetic microspheres, or portable paper strips). When using immobilized antibody, the greater the free analyte present in the sample, the lower the bioluminescent signal detected when the substrates required for light emission are added to the immobilized labeled complex. Many diverse, ELISA-type assays that use photoproteins have been developed for biologically important molecules: for example, aequorin-based assays for thyroxine, angiotensin II, cortisol, and 6-keto-prostaglandin-F1-alpha. These target molecules must be sensitively detected in biological fluids, making a bioluminescent label preferable to many other labeling alternatives (2). Luciferases have also been used as labels in ELISA-type assays for a variety of analytes, although luciferase bioluminescence has been used more in whole cell and reporter gene assays in recent years (12).

Bioluminescent-protein-based DNA hybridization assays are similar to the ELISA-type assays, except the bioluminescent protein is attached to a DNA sequence that is complementary to an immobilized DNA sequence. Christopoulos and colleagues have employed photoprotein-based DNA hybridization methods for digoxigenin and prostate specific antigen mRNA assays, for a quantitative PCR technique, and for the detection of transgenes and single nucleotide polymorphisms (13).

Bioluminescent-based homogeneous assays simplify detection by circumventing the immobilization, incubation, and washing steps that are required in both the ELISA and DNA hybridization assays. In these cases, the binding of the analyte causes a detectable physical change, such as a change in the intensity of the bioluminescence generated by the photoprotein. Aequorin-based homogeneous assays have been developed for HIV proteases, thyroxine, biotin, and cortisol, and luciferase-based homogeneous assays have been developed for oxidases, proteases, and proteosome activity (2,14,15). Luciferases have also been employed along with a fluorescent partner in homogeneous protein–protein interaction (PPI) assays based on bioluminescence resonance energy transfer (BRET) (16,17).

As demonstrated by the aforementioned examples, bioluminescent assays can detect a variety of molecules, hormones, proteases, and oligonucleotides. Some of these assays have approached attomolar detection limits and have therefore also been incorporated onto miniaturized microfluidic detection platforms (18). Luciferases in particular are used extensively for mass-market contamination-type assays and in bio-threat detection devices (10). Luciferase detects ATP (which indicates the presence of cells) with such sensitivity that luciferase-based bioluminescence assays have been developed for the analysis of biomass in seawater and water treatment plants; as hygiene monitors in hospitals and food and beverage institutions; for cell viability studies (10); and as reporters for transformation and transfection, gene expression, and promoter assays (10). Luciferase-based bioluminescence has also been employed extensively in whole cell sensing systems and biosensors for a variety of environmental pollutants.

Whole cell assays have the benefit of detecting only the bio-available portion of pollutants and can quantify the synergistic effects that multiple toxins may have on living organisms. These are very important factors to consider when determining the actual impact of pollution, targeting and tracking bioremediation efforts, and designing sustainable environmental protection policies. The most famous commercial examples are likely the Microtox toxicity kits, which test for contamination in drinking water. The Microtox system uses the live bacteria *Vibrio fischerii*, which naturally expresses bioluminescence through its luxCDABE gene cassette (19). A broad range of environmental toxins in water kill *V. fischerii*, and thus as toxin levels increase, bioluminescence decreases.

This bacteria-based toxicity assay has been adapted to other organisms to analyze non-aqueous media. The luxCDABE cassette has been transformed into *E. coli*, cyanobacterium, and multiple fungi species; firefly luciferase and GFP replace the original bacterial luciferase in some cases (20). Fusing selective promoters and regulatory genes to the luxCDABE cassette has further increased the specificity of this type of assay. Bioluminescent whole cell sensing systems are now available for a wide range of specific chemicals, including heavy metals, carcinogens, genotoxins, and organic compounds (such as polychlorinated biphenyls) (21,22, 23).

The current trend in this vein of bioluminescence-based research lies in developing methods of immobilizing and integrating these whole cell systems onto biosensor platforms (for example, sol-gel and agar encapsulation or immobilization onto optical fibers and into biochips and labs-on-a-CD), creating high-throughput arrays, and increasing the stability and shelf life of these cell lines (for example, freeze and vacuum drying, immobilization in biocompatible polymers, and sporulation) (24,25). Advantages of bioluminescent whole cell assays are their speed, affordability, portability, and robustness. Effective storage and immobilization of these cells will enhance the last two advantages even further.

TUNING THE LIGHT

Despite the inherent advantages that bioluminescent proteins possess, they suffer from a significant drawback: they are not nearly as diverse and versatile as FPs. Most lacking is the availability of proteins with different bioluminescent wavelengths of emission. Multiple colors of bioluminescent proteins would allow these labels to be used in multi-analyte single-well experiments and in multimodal ex vivo and in vivo imaging, for example. Since GFP was first discovered, researchers have successfully mutated it and the *Anthozoa*-derived FPs to create a vast array of colors, pH stabilities, half-lives, aggregation tendencies, reduced cytotoxicities, and other desired properties, greatly expanding the FP's range of analytical applications. Researchers now can "pick and choose" the FPs that are most suited for a given study. But bioluminescent proteins have not undergone the same intensive mutagenesis, and as a result, there are few color varieties available. However, this limitation is being gradually overcome by recent mutagenesis studies on both aequorin and luciferase. The goal of these studies is primarily to tune the emission light of bioluminescent proteins commonly used in labeling. Other targeted outcomes include increasing stability and activity, reducing cross-reactivity and spectral width, and altering the decay kinetics of wild-type bioluminescent proteins.

Luciferases have undergone significant cloning and mutagenesis manipulation. A luciferase commonly used in analytical applications is firefly luciferase (26), which emits a yellow–green light (λ_{em} = 557 nm) with glow-type kinetics and a broad emission band. Various firefly luciferases have been mutated to shift their emission wavelengths toward the red region of the spectrum (λ_{em} = 615 nm) (27). Recently, glycosylated tyrosine residues were incorporated into the firefly luciferase, resulting in an 18-nm red-shift in its emission spectrum (30). To meet the goal of increasing spectral diversity, the search for novel luciferases from newly discovered organisms is also underway. For example, the luciferase from the railroad worm is both red-emitting and has a narrower emission band than firefly luciferase (28), whereas the luciferase from the click beetle is significantly blue-shifted and more stable (29). Powerful bioluminescent-based methods, such as a triple-color reporter cell-based assay (31), have already been developed with this palette of new luciferases.

Similar work has been performed with the Ca^{2+}-regulated photoproteins aequorin (λ_{em} = 471 nm) and obelin (λ_{em} = 491 nm). These studies used a two-pronged approach: altering structure of coelenterazine and mutating the amino acid sequence of the apoprotein itself. Altering certain functional groups of coelenterazine retained bioluminescent activity while

concomitantly shifting the wavelength of emission (32). Mutations near the active site of aequorin and obelin shifted the wavelength of emission maxima (33,34). A combination of apoprotein mutations and chromophore analogues resulted in even farther shifts. Cysteine modifications and active site mutations synergistically caused the largest red-shift in photoprotein mutants to date (λ_{em} = 518 nm) (Figure 3) (35).

SPLITTING THE PROTEINS

As analytical chemists we strive for quantitative and qualitative methods that satisfy critical performance requirements, such as selectivity, sensitivity, and reproducibility. Bioluminescent proteins paired with a selective recognition element for a particular analyte satisfy these desired requirements in many instances. Traditionally, the recognition element was attached to the bioluminescent label at the C- or N-terminus, at specific amino acids within the protein sequence, or via biotin–streptavidin or hapten–antihapten binding. However, the current frontier of bioluminescence-based analysis lies in molecular switches and split complementation assays. These divide the bioluminescent label into two separate components, which reassemble and form a light-emitting product in response to the binding of a specific analyte to a recognition element that has been inserted in or attached to the split bioluminescent label (Figure 4). The strength of these assays lies not only in the surprisingly strong tendency of bioluminescent proteins to form their native and active structures, but also in the assays' applicability in imaging and in vivo studies. Bioluminescent and fluorescent molecular switches and split complementation assays combine the versatility of rational bioengineering with the detection capability of light-emitting proteins. These chimeric, designer molecules can be constructed for particular purposes such as development of nanosensors and devices, allosteric enzyme creation, and stimuli-responsive nanobiomaterial construction (36).

These molecular switches have been created with aequorin by inserting a recognition protein into the structure that undergoes a significant conformational change upon binding an analyte. For example, the DNA sequence coding for the glucose binding protein (GBP) was inserted into the aequorin sequence; upon binding of glucose, the GBP portion underwent a significant conformational change, which brought the two halves of the aequorin molecule close enough together to form a bioluminescent product. Thus, this molecular switch functioned as an "on–off" switch for detecting glucose between 1.0×10^{-7} and 1.0×10^{-2} M, which is well within the physiologically relevant range of 2–20 mM. The switch was selective for glucose over several significant mono- and disaccharide molecules (37).

In contrast to molecular switches, split complementation assays divide a bioluminescent protein completely into two noncovalent halves and fuse recognition elements and fusion moieties to the termini of each of these halves. Advantageously, the two halves of the bioluminescent proteins used in these assays do not freely associate with one another in solution and can thus be adapted for the detection of PPIs. The human genome encodes for a set number of proteins, but the structural and mechanistic roles of these proteins are very often activated and regulated by their interactions with one another or with DNA, for example. The study of the vast array of physiological mechanisms therefore relies on efficient detection and trapping of PPIs. Light-emitting proteins are very attractive for PPI research because of their visualization advantages; bioluminescent reporters are also non-toxic, which further promotes their use for in vivo studies.

Toward this end, Umezawa and colleagues split firefly luciferase into two segments and genetically fused these halves to complementary sections of the split intein DnaE and a known phosphorylation-mediated PPI pair (IRS-1 and SH2N) (38). One half was fused with both the N-terminus of DnaE intein and IRS-1, whereas the other half was fused with the C-terminus of DnaE intein and the IRS-1 PPI partner, SH2N (Figure 4). When the IRS-1 and SH2N pair

came in close proximity to one another after phosphorylation, the two halves of the luciferase reassociated. Simultaneously, the two halves of the DnaE intein were brought close enough together that the intein self-spliced, forming a covalent bond—the IRS-1–SH2N PPI could essentially be "frozen" in time. The researchers successfully tracked in vivo phosphorylation of the IRS-1 and SH2N domains in response to insulin stimulation in eukaryotic cells using this system.

Intein splicing is advantageous when the interaction between two proteins is relatively weak and transient, but bioluminescence-based split complementation assays without inteins, such as a *Renilla* luciferase system (39), also have been developed. *Renilla* luciferase has been used extensively in imaging of live cells and animals because it has long light-emission kinetics and is not responsive to ATP. For this system, the PPI between two known interaction partners, Y941 peptide and the N-terminal SH2 domain, was successfully detected and tracked following insulin injection into Chinese hamster ovary cells using the same method described for the luciferase split complementation assay.

As previously stated, bioluminescent protein switches and split complementation assays have great potential for tracking PPIs in vivo and for imaging in vivo and in situ. In the past decade, advances in the sensitivity of CCD cameras have allowed for the ascent in use of bioluminescence imaging in several biomedical applications; long-term, longitudinal studies of live animals are the most prevalent current applications. *Renilla* and firefly luciferase are used in these in situ studies because of their long light-emission kinetics and easy expression in mammalian cells. Examples of bioluminescence imaging with these proteins include studying tumor progression and charting anticancer therapeutic effectiveness in mouse models, cellular circadian regulation, and bacterial and viral pathogenesis both in vivo and in situ (40,41,42).

FUTURE PERSPECTIVES

A great deal of potential lies in developing bioluminescent proteins to satisfy the demands imposed by new frontiers in analytical research. Obvious examples include expanding the type, sensitivity, and multianalyte capability of bioluminescence-based assays; developing additional bioluminescence-based biosensors and increasing the range and versatility of bioluminescent-protein-based reporter systems; BRET and PPI detection methods; and in vivo and in situ imaging applications. The discovery of new and the spectral tuning of currently known bioluminescent proteins will increase their spectral diversity and optimize important properties such as robustness and expression capability in a variety of cell types. Molecular switches and split complementation assays will continue to allow for the detection of PPIs and additional in vivo bioluminescence methods.

As these well-established bioluminescence techniques continue to expand, even more applications likely will be created. For example, using bioluminescence to understand the progression of bacterial infection has recently been receiving increased attention (see sidebar). Several diseases are regulated by the quorum sensing of bacteria. Quorum sensing is the specific release of molecules by bacteria when a certain bacterial density is reached; these molecules can trigger the bacteria to function as a more aggressive (and often destructive) population. Bioluminescence, especially that of *Vibrio fischerii*, has been used extensively to elucidate these complex communication patterns among bacterial populations (43). Moreover, researchers are currently employing bioluminescence to more fully understand, control, and treat bacterial diseases such as Crohn's disease (44). Future promise lies in using these light-emitting proteins to more fully understand bacterial communication and establish effective methods of blocking that communication, which would in turn allow more successful disease management.

Bioluminescent proteins illuminate the intricate dance of life as it unfolds at the cellular level. Without doubt, much like our ancestors used bioluminescent foxfire torches and firefly lamps to light their way through dark and unknown territories, researchers will continue to harness the power of these proteins to help reveal the mystery of the world around us and the depth of life itself.

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Biography

Laura Rowe received her Ph.D. from the University of Kentucky and is currently a postdoctoral research associate at the University of Cambridge (U.K.), Department of Chemical Engineering and Biotechnology. Her research focuses on manipulating biological moieties in order to create novel analytical methods and devices. She is interested in engineering bioluminescent and fluorescent proteins and in developing biomimetic ligands for glycobiololgy applications. Emre Dikici is a postdoctoral associate at the University of Kentucky, Department of Chemistry. He is interested in luminescent proteins, designing and developing new fluorescent labels for protein labeling, and biofuel cells. Professor Sylvia Daunert is the Gill Eminent Professor of Chemistry at the University of Kentucky. Dr. Daunert's research interests lie in the area of bioanalytical chemistry. More specifically, she is interested in the use of recombinant DNA technology to design molecular diagnostic tools, biosensors, biofuel cells, and smart biomaterials for responsive drug delivery systems.

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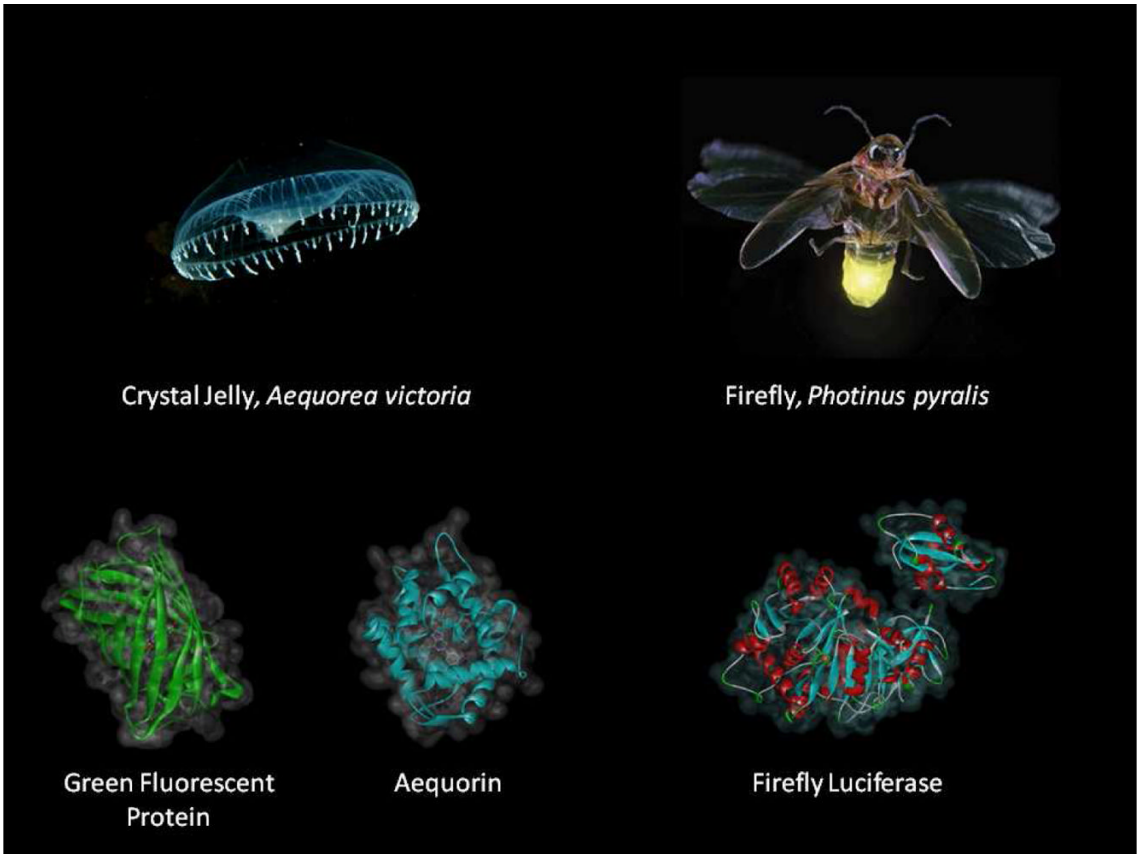

Figure 1.
The popular bioluminescent reporter proteins aequorin and firefly luciferase and the fluorescent protein GFP are isolated from the bioluminescent organisms *Aequorea victoria* and *Photinus pyralis*. The protein structures are visualized from the X-ray crystallography data (Aequorin: 1EJ3 (46), GFP: 1EMA (47), Luciferase: 1LCI (48)) using DSViewer Pro 4.0.

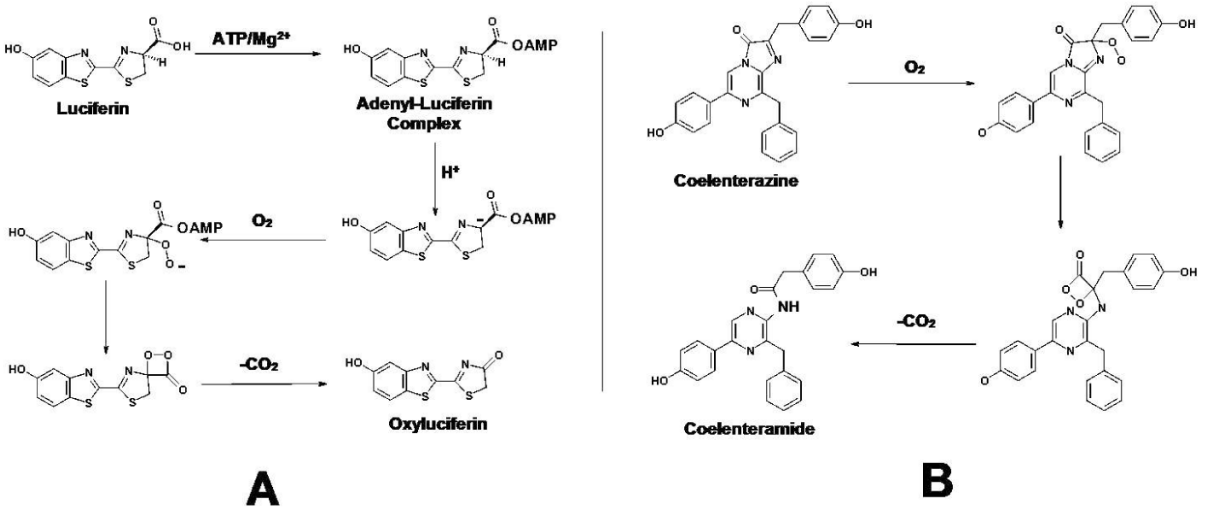

Figure 2.

A) Ca^{2+} causes a conformational change in photoproteins, which destablizes the peroxi-coelenterazine that is formed when coelenterazine is incorporated into the photoprotein. The destabilization leads to cyclization, decarboxylation, and emission of CO_2 and bioluminescent light. B) In the case of luciferases, ATP and Mg^{2+} cause the adenylation of luciferin. This is followed by the luciferase-catalyzed oxidation, cyclization, and decarboxylation of the adenyl–luciferin complex, resulting in the emission of CO_2 and bioluminescent light.

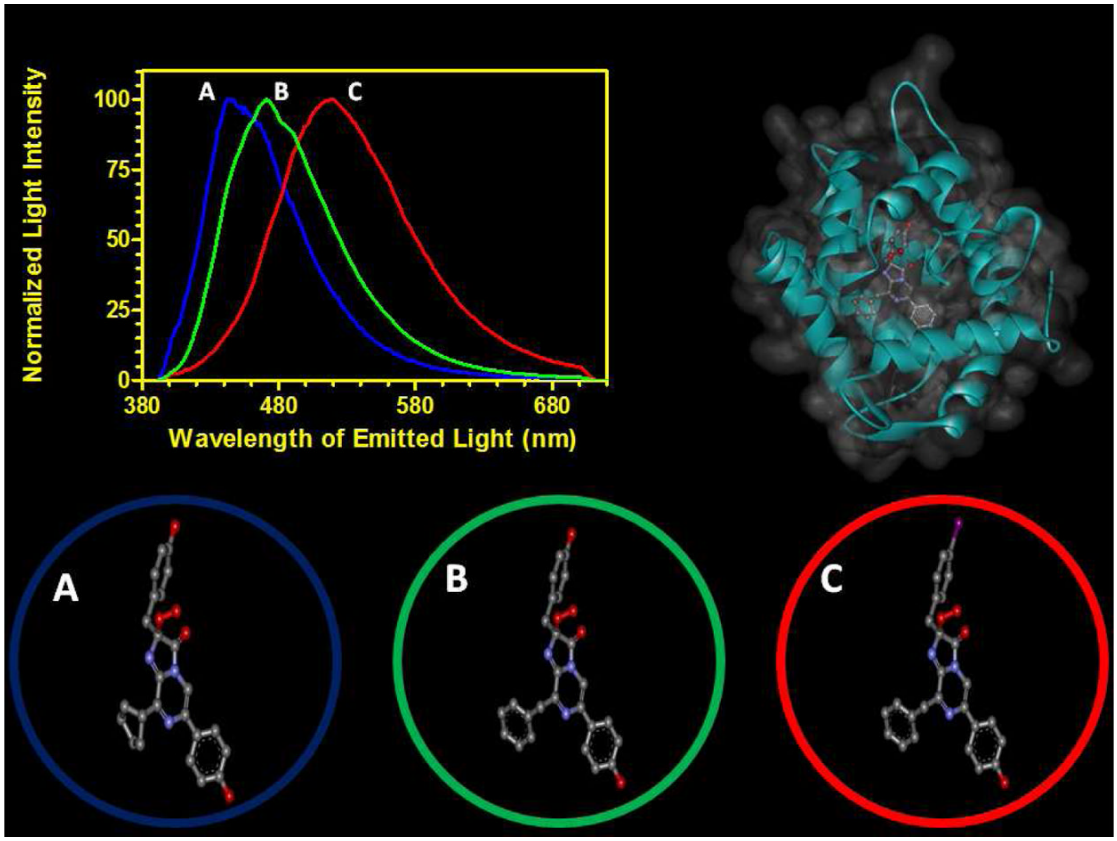

Figure 3.

Spectral tuning of aequorin. Different coelenterazine analogues can be incorporated into the native aequorin to alter the color of bioluminescence emitted. A, B, and C represent the structure and bioluminescence emission spectra of three coelenterazine analogues. Spectrum A is native aequorin with coelenterazine *hcp*; B is native aequorin with native coelenterazine; C is aequorin mutant Y82F with coelenterazine *i.* The chemical structures below the spectra contain carbon (grey), oxygen (red), nitrogen (blue), and iodine (purple). The protein structures are visualized from the X-ray crystallography data (Aequorin:1EJ3 (46)) using DSViewer Pro 4.0.

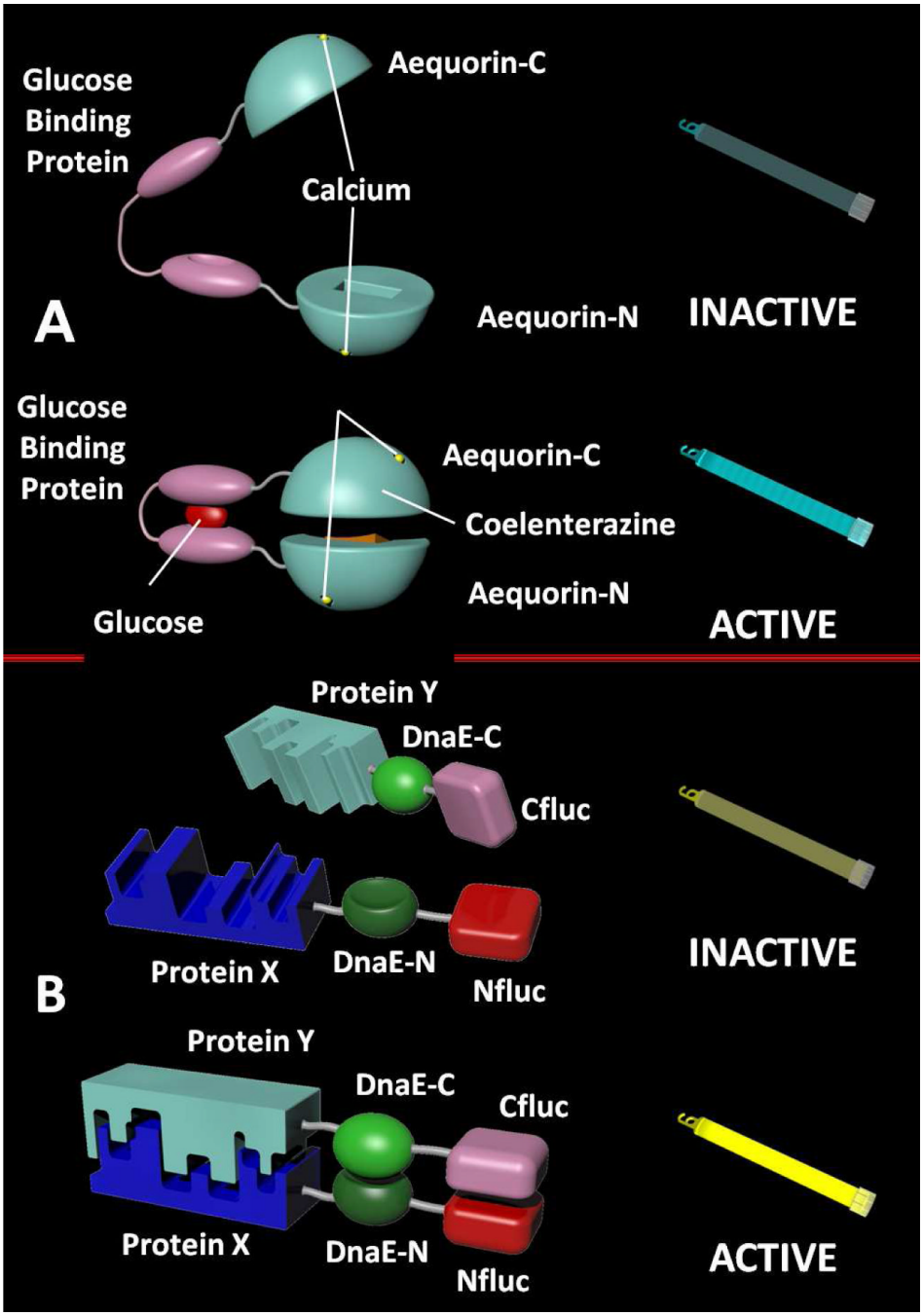

Figure 4.

Schematics of bioluminescent split complementation assays and molecular switches. A) Molecular switch. Aequorin is split into two sections and fused together with GBP. GBP assumes a closed conformation upon binding glucose, bringing the two halves of aequorin close enough together to reconstitute the bioluminescent signal, which is observed following the addition of coelenterazine and Ca^{2+}. B) Split complementation assay. Firefly luciferase is split into two halves. The Cfluc half is fused with half of the split intein (DnaE-C) and one partner of a PPI pair, Protein Y. The other half of the luciferase, Nfluc, is fused to the remaining half of the split intein (DnaE-N) and Protein X, which interacts with Protein Y. The association of the two PPI partners in vitro or in vivo brings the two halves of the luciferase close enough

together to reconstitute bioluminescence following luciferin addition. The close proximity allows the two halves of the split intein to self-splice and form a covalent bond, thereby freezing the PPI complex together.