Bioluminescence and Its Impact on Bioanalysis

Daniel Scott, Emre Dikici, Mark Ensor, and Sylvia Daunert

Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami, Miami, Florida 33101; email: SDaunert@med.miami.edu

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Abstract

There is an increasing need for versatile yet sensitive labels, posed by the demands for low detection in bioanalysis. Bioluminescent proteins have many desirable characteristics, including the ability to be detected at extremely low concentrations; no background interference from autofluorescent compounds present in samples; and compatibility with many miniaturized platforms, such as lab-on-a-chip and lab-on-a-CD systems. Bioluminescent proteins have found a plethora of analytical applications in intracellular monitoring, genetic regulation and detection, immuno- and binding assays, and whole-cell biosensors, among others. As new bioluminescent organisms are discovered and new bioluminescence proteins are characterized, use of these proteins will continue to dramatically improve our understanding of molecular and cellular events, as well as their applications for detection of environmental and biomedical samples.

1. INTRODUCTION

Bioluminescence is a natural phenomenon that has captivated the attention of mankind throughout history. The Greeks were fascinated with bioluminescence, and Aristotle made detailed observations of bioluminescence in nature. Even earlier accounts of bioluminescence can be found in the legends of a number of ancient civilizations, such as the Polynesians and Siberians (1, 2). Bioluminescence has been at the center of various legends and superstitions, a part of religious beliefs and ceremonies, and more recently, the focus of scientific explorations.

Bioluminescence is the production and emission of light by a living organism. It has been observed in diverse organisms, including insects, bacteria, fish, fungi, plants, and an assortment of marine invertebrates (**Figure 1**). The functions of bioluminescence are just as varied, ranging from defense, predation, and communication to metabolism, in which it acts as a terminal oxidase (3). In general, bioluminescent light is generated by an internal biochemical reaction that generates an excited-state product that, upon relaxation, causes the emission of a photon.

Although bioluminescence has been observed for thousands of years, only relatively recently (approximately within the past 50 years) has the true analytical potential of bioluminescence become appreciated. In 1962, Shimomura et al. (4) discovered and isolated aequorin and green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, which sparked renewed interest in bioluminescence. Later, Chalfie et al. (5) cloned GFP, making it widely available to researchers for a multitude of chemical and biological applications. More recently, Tsien et al. (6) prepared a series of mutants having a broad range of emission wavelengths that resulted in proteins fluorescing in most of the colors of the spectrum. The unprecedented applications of proteins in biomedical

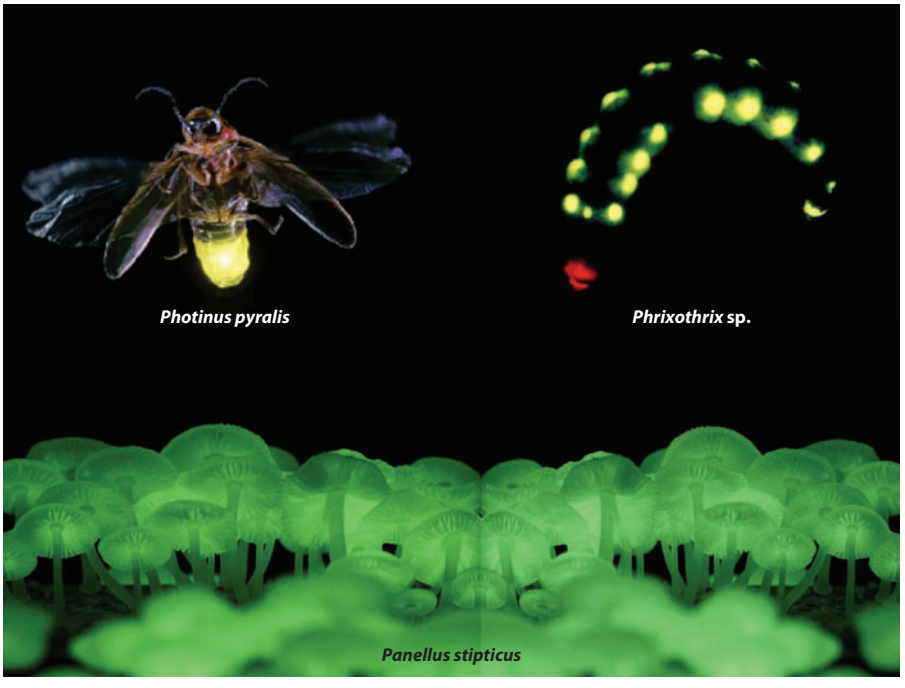

Figure 1

Bioluminescent proteins can be isolated from different organisms, such as *Photinus pyralis*, *Phrixothrix* sp., and *Panellus stipticus*.

research and bioanalysis were recognized in 2008, when Shimomura and his colleagues were awarded the Nobel Prize in Chemistry for the discovery and development of GFP (7).

Improvements in photonics instrumentation have also enabled the use of bioluminescent proteins in numerous detection and imaging applications. Bioluminescence offers marked advantages in this area because it exhibits efficient quantum yields (8). Moreover, given that bioluminescence is caused by a biochemical reaction, there is no need for an external excitation source, which eliminates any background noise associated with scattering from the source as well as from autofluorescent molecules present in the sample (especially in physiological fluids). Therefore, the use of bioluminescent proteins has been especially successful in applications and fields that require high sensitivity and low limits of detection.

Advances in molecular biology have also greatly diversified the potential uses of bioluminescent proteins. They are used as reporters and labels in intracellular ATP and Ca^{2+} detection, DNA and RNA hybridization assays, immunoassays, gene expression, whole-cell biosensors, and bioluminescence resonance energy transfer (BRET) assays, as well as in miniaturized systems such as lab-on-a-chip- and lab-on-a-CD-based instruments.

2. BIOLUMINESCENT PROTEINS

Bioluminescent proteins are typically divided into two subgroups: luciferases and photoproteins. Bioluminescence emission in luciferases arises from an enzyme-substrate reaction in which, in the presence of oxygen, the luciferase oxidizes luciferin (the substrate), thereby forming a product that is in an electronically excited state and whose subsequent relaxation causes the emission of a photon (**Figure 2**) (9). Thus, the amount of light emitted is proportional to the concentration of luciferin.

Photoproteins, however, can emit light in a manner that is proportional to the concentration of the protein present. Such proteins can be considered a very stable form of an enzyme-substrate complex in which the dissociated form is not energetically favorable and, thus, remains complexed and represents the primary light-emitting component (10). Both bioluminescent enzymes and photoproteins have distinct advantages; the preferential use of one type over the other often depends on the application.

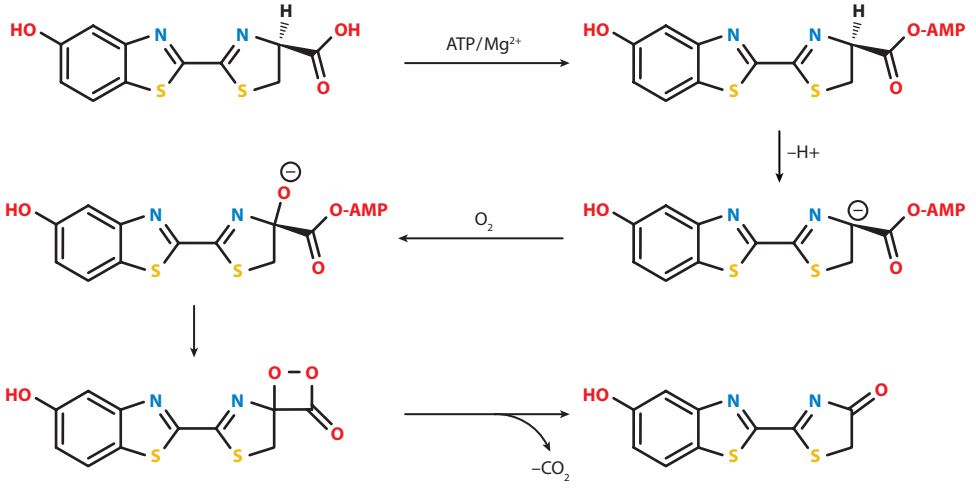

Figure 2

The ATP-catalyzed reaction mechanism that underlies the bioluminescence of firefly luciferase.

As mentioned above, bioluminescence occurs in various terrestrial and marine organisms. In these organisms, bioluminscence has numerous biological functions. These functions include feeding, mating, communication, and concealment from predators (11). Despite the abundance of bioluminescent organisms in the oceans, the number of bioluminescent proteins isolated and characterized from these organisms is surprisingly low, and consequently, their use in analytical applications is still fairly meager. This review focuses on the known proteins and describes their use in bioanalysis.

The most extensively studied bioluminescent proteins are the luciferases found in certain beetles; the most prominent is the firefly luciferase from the Lampyridae family. This group of luciferases from terrestrial insects also includes the railroad worm (Phengodidae family) and click beetle (Elateridae family) luciferases. These luciferases generate bioluminescent light emission by catalyzing a reaction in which oxidation of D-luciferin in the presence of ATP and oxygen results in an excited-state product, oxyluciferin, that emits a photon upon relaxation (12). The bioluminescent light produced by firefly luciferase is observed as a broad band with an emission wavelength that peaks at 560 nm (3). These luciferases are used as labels in ATP assays for estimation of total biomass and the contamination of microorganisms, in cell viability and enzyme assays, and as reporter genes in various cellular and physiological investigations (13).

The most abundant source of bioluminescent organisms, however, is undoubtedly the marine environment. A broad range of marine organisms exhibit bioluminescence (11, 14), but only a few bioluminescent proteins from these organisms have been employed in analytical applications. Among these proteins are the luciferases found in the marine copepod *Gaussia princeps* and in the sea pansy *Renilla reniformis*. These luciferases use coelenterazine as a substrate that, in the presence of molecular oxygen, undergoes an oxidation step that results in an excited form of coelenterazine, coelenteramide, that emits light upon relaxation (14). The wavelengths of emission maxima of the *Renilla* and *Gaussia* luciferases are almost identical at 485 nm and 490 nm, respectively (15). These luciferases have been employed in protein-protein-interaction studies, BRET assays, and whole-cell biosensors, among other applications (16–18).

As with their terrestrial counterparts, marine luciferases are the best-characterized bioluminescent proteins. In the marine bacterial luciferases from *Vibrio fischeri* and *V. harveyi*, the production of light arises from a two-step mechanism; the first step involves a reaction between (*a*) luciferase bound to reduced flavin mononucleotide and (*b*) oxygen to produce a peroxyflavin intermediate. In the second step, this intermediate reacts with an aldehyde luciferin that is endogenous to the bacteria to form a second intermediate, whose decay generates the emission of bioluminescent light at a wavelength of ~490 nm (**Figure 3**) (19). Interestingly, the *lux* cassette gene (*luxCDABE*) is responsible for the coding of both the luciferase and the enzymes that can synthesize the necessary substrates for the bioluminescence reaction; in contrast, the luciferin-synthesizing enzymes of other organisms are controlled by separate genes. The incorporation of the entire *lux* cassette as a reporter gene in biosensor applications results in systems that do not require addition of the external luciferase substrate—in other words, self-reporting systems. Such technology has been employed in the design and development of whole-cell- and protein-based biosensing systems that can monitor, for example, water toxicity, heavy metals, quorum-sensing molecules (20), and organic compounds (21–24).

The photoproteins (*a*) aequorin, found in the jellyfish *Aequorea victoria*, and (*b*) obelin, found in the hydromedusa *Obelia longissima*, differ from the luciferase proteins in terms of their mechanism of bioluminescence emission. Aequorin and obelin are formed by an apoprotein and an imidazopyrazine chromophore, coelenterazine. In these photoproteins, therefore, the amount of bioluminescence generated is proportional to the concentration of the protein. In both proteins, coelenterazine resides in a hydrophobic pocket of the protein and is oxidized in the presence

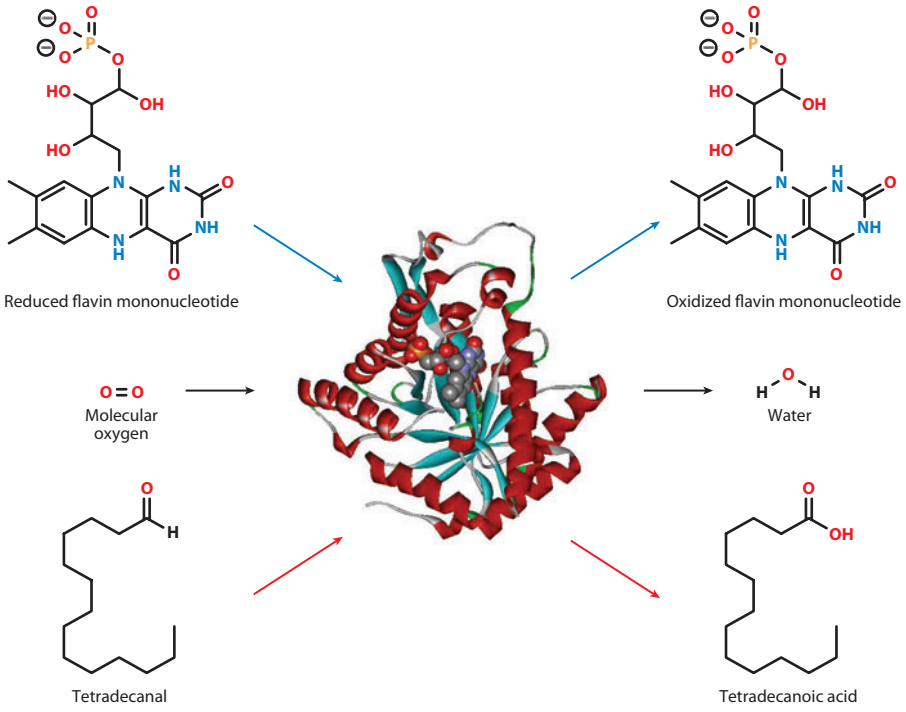

Figure 3

The reaction mechanism that underlies the bioluminescence of bacterial luciferase.

of molecular oxygen to form an excited coelenteramide (**Figure 4**). However, in aequorin, for example, the coelenterazine in the hydroperoxide form is tightly bound in a hydrophobic pocket that does not allow such oxidation. Upon binding Ca^{2+}, the protein undergoes a conformational change that allows the oxidation of coelenterazine and subsequent conversion into a highly unstable dioxetanone intermediate. This intermediate then loses CO_2, yielding an excited enolate anion of coelenteramide that, upon relaxation, produces a flash of light at 469 nm (25, 26). The relaxed coelenteramide remains held by the photoprotein and is released very slowly; if excess coelenterazine is added to the initial solution, the resulting light intensity is directly proportional only to the amount of photoprotein in the sample (25). Because aequorin is a Ca^{2+}-regulated photoprotein, it was initially employed as a probe for the monitoring of intracellular Ca^{2+}, but recently its use has been expanded to a range of other applications, including hybridization and binding assays (10, 27).

3. ANALYTICAL APPLICATIONS OF BIOLUMINESCENCE

As mentioned above, bioluminescent proteins can provide the technology necessary to quantify extremely low concentrations of a desired analyte with the required sensitivity and reproducibility. Unsurprisingly, the use of these proteins recently expanded to include many different detection systems and methods, including assays for Ca^{2+} and ATP, nucleic acid quantification and hybridization assays, and immunoassays, among others. In addition, bioluminescence is also employed in resonance energy transfer and protein-protein-interaction studies.

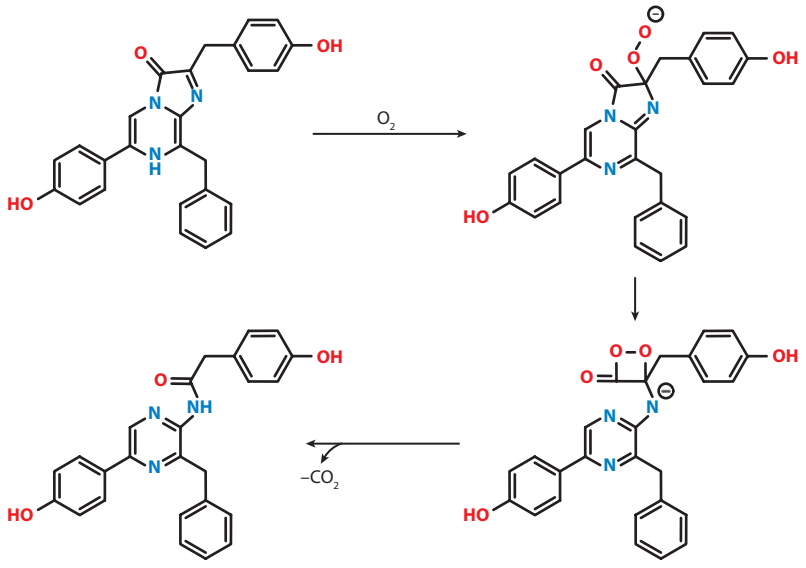

Figure 4

The reaction mechanism that underlies the bioluminescence of aequorin. In the presence of Ca^{2+}, aequorin undergoes a conformational change, oxidizing coelenterazine in the process.

3.1. ATP Assays: Quantifying Energy Production

Many luciferase enzymes require ATP to catalyze the oxidation reaction of the luciferin substrate. In these cases, the absence of ATP blocks the bioluminescence reaction. On the basis of this premise, researchers have developed highly sensitive bioluminescent assays for the detection of ATP. For example, through the use of firefly luciferase, ATP can be measured at concentrations as low as 0.01 fmol (28). Assays in various formats have been implemented. A rapid screening test to determine levels of ATP produced through the contamination of fungi, bacteria, and yeast in food and feeds was developed with firefly luciferase. In this assay, the amount of light emitted directly corresponded to the microbial number (29). Given the increasing prevalence of multidrug-resistant organisms, the ability to quickly screen a number of drugs for their effectiveness against different organisms provides valuable information in the clinical setting (30, 31). Lafond et al. (32) used the bioluminescence ATP assay for the rapid determination of antibiotic susceptibility. They employed the ATP assay in conjunction with adenylate kinase and quinine:NAD(P)H oxidoreductase in a parallel assay to rapidly check for antibiotic resistance, thereby shortening the investigation time to 6 h (24 h of incubation is usually required). In the future, this system could be automated in a microplate format to allow for more high-throughput screening applications. Other areas in which bioluminescence ATP assays have been employed include hygiene (33), cytotoxicity (34), microbiology, drug discovery (35), and enzymatic assays (13).

3.2. Intracellular Calcium Ion Detection: Illuminating Signal Transduction Pathways

Similar to the initial use of luciferase for ATP measurements, the photoprotein aequorin was first employed for the detection of its cofactor, Ca^{2+}. Aequorin was both injected into cells and expressed in vivo to monitor intracellular Ca^{2+} fluctuations in various cellular locations and in

response to different external stimuli (27, 36). Recent publications by Michelini et al. (37) and Villalobos et al. (38) discuss, in detail, the methodologies and the instrumentation required for the imaging of Ca^{2+} in vivo. The determination of Ca^{2+} concentrations in physiological fluids and cells is important because of the role that this ion plays in cellular signaling events. The release of Ca^{2+} can be triggered by the activation of G protein receptors and ion channels through the initiation of the signal transduction mechanism in the cell. It is important to understand the mechanism of action of these receptors and ion channels and their interaction with Ca^{2+} to elucidate signaling pathways, as well as to design and develop new drug targets (39). Thus, as the need for faster, more efficient detection methods capable of high-throughput screening increases, the inherent advantages of bioluminescence become better appreciated. For instance, aequorin was recently used in a high-throughput screening for Ca^{2+} with a microplate reader setup. Menon et al. (40) intracellularly expressed apoaequorin alongside a chimeric G protein and a G protein–coupled dopamine receptor, then used the cells to screen 8,106 compounds as potential drugs to target G protein–coupled receptors and ion channels. Validation of this method and comparison between it and the current high-throughput screening method of choice for the Ca^{2+} ion, a fluorescence-based system, demonstrated that the two techniques perform similarly in terms of the identification of positive hits. However, the bioluminescence-based system has the advantages of (*a*) being more cost-effective; (*b*) having better reproducibility; and (*c*) yielding a decreased number of false positives, most probably due to the lack of interference from autofluorescent compounds and excitation sources.

4. BIOLUMINESCENCE IN GENETICS: THE IMPORTANCE OF DNA DETECTION

Nucleic acids are the building blocks of DNA and RNA, and as such, they play a vital role in the function and replication of living organisms. DNA forms the basis of the genetic code that defines both our genotype and our phenotype. In other words, DNA dictates, for example, what we look like and whether or not we are carriers of a genetic disorder. To determine whether an individual is a carrier of a hereditary disease, it is necessary to establish the presence of harmful mutations in the DNA present in the affected chromosomes and determine their concentrations. Nucleic acids experience changes at extremely low concentrations, which has prompted the development of new technologies based on bioluminescence detection. These methods afford the low detection limits needed for measuring nucleic acids (41, 42). Methods based on luminescence, such as real-time quantitative polymerase chain reaction (PCR), have significantly advanced the field. Briefly, quantitative PCR involves designing an oligonucleotide probe that is specific to the target DNA, then labeling it with both a fluorescent probe and a related quencher molecule. The labeled oligonucleotide anneals to the target DNA, and as the polymerase progresses along the DNA strand it cleaves off the labeled probe. This process increases the distance between the fluorescent probe and the attached quencher, which subsequently increases the observed fluorescence intensity (**Figure 5**). Drawbacks of this method include its cost and the need for preparation and labeling of the specific fluorescent nucleotide probes.

A bioluminescent alternative that utilizes the bioluminescence regenerative cycle has been developed. This method involves quantifying the pyrophosphate molecules released during the polymerization of the nucleic acids by their respective polymerases; thus, these released pyrophoshate molecules directly correspond to the number of target molecules. The released pyrophosphate is converted to ATP by an ATP-sulfurylase enzyme, which can be used by firefly luciferase to generate bioluminescence in the presence of luciferin (**Figure 6**). Additionally, after the ATP is used by the luciferase to emit light, it is regenerated back to a pyrophosphate molecule, thereby

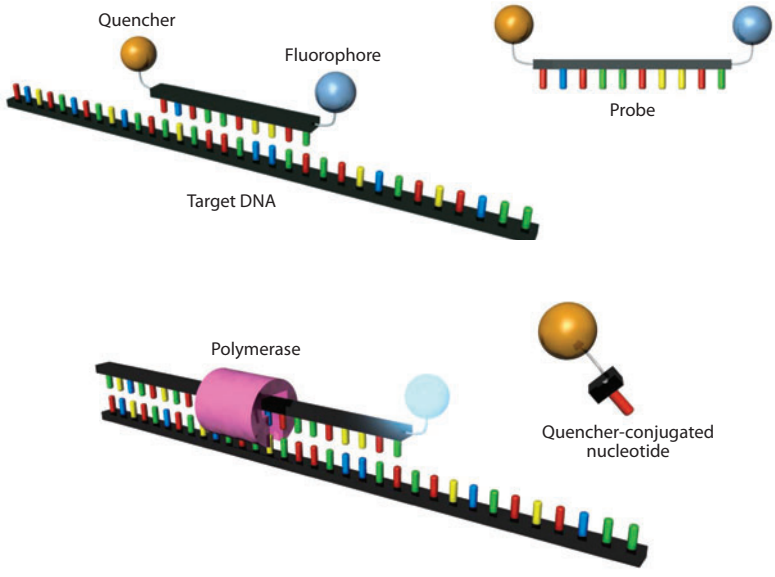

Figure 5

Real-time polymerase chain reaction.

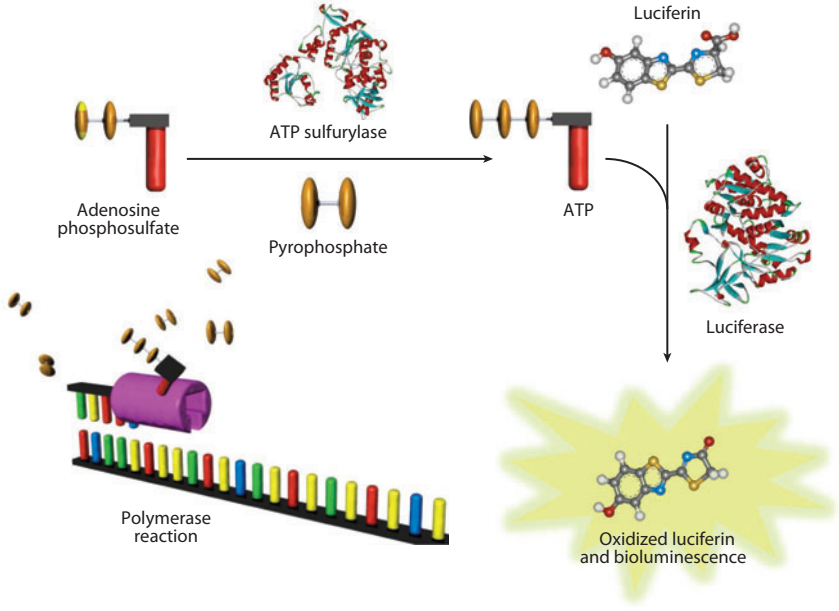

Figure 6

The bioluminescence regenerative cycle.

enabling recycling and amplification of the signal from a single pyrophosphate molecule. At a concentration of 1×10^{-8} M pyrophosphate, the amount of light produced at a given time interval is proportional to the number of nucleic acid molecules in the sample. This translates to a detection limit of 1 amol of DNA in a solution, which may permit applications in assays for gene expression, DNA and RNA quantification, and single-nucleotide polymorphism (SNP) detection (43).

Additionally, following the sequencing of the human genome in 2003, SNPs became a potential marker for disease susceptibility and are now used to monitor drug effectiveness and side effects. Thus, the ability to quickly monitor and recognize SNPs is valuable for applications in clinical medicine, genome drug discovery, and personalized medical treatment (44, 45). A method similar to the above-mentioned bioluminescence regenerative cycle, the bioluminescence pyrophosphate assay, has also been used for SNP detection. In this technique, the pyrophosphate released by DNA polymerization from a specific primer, following polymerization to the target DNA, is converted to ATP by pyruvate phosphate dikinase, which can also be quantified by firefly luciferase bioluminescence. This method identified SNPs in both the *p53* and *Kras* genes (46). Variations in the mannose-binding lectin gene, which are associated with increased susceptibility to infection and autoimmune disorders, have also been identified through the use of aequorin as the reporter in genotyping tests (45).

During the late twentieth century, the popularity of hybridization assays in solution phase increased dramatically. Traditionally, the preferred detection method for such assays was radiolabeling. However, due to the hazardous nature of radiolabels and the extensive waste-disposal procedures that were required, alternative, nonradiometric labels were developed. These novel labels have gradually replaced traditional radiolabels in DNA and RNA detection. As mentioned above, bioluminescence offers great detection sensitivity and is not susceptible to the safety issues posed by radioisotopes (47, 48). In general, a bioluminescent protein attaches to the oligonucleotide of interest, which is complementary to an immobilized or target oligonucleotide sequence. A potential limitation of this method is that the bioluminescent protein becomes inactive following conjugation to the oligonucleotide. However, this limitation can be overcome through the use of site-specific attachment methods. For example, Nagatsugi et al. (49) attached an oligonucleotide to a cysteine of luciferase without adversely affecting luminescence activity or the selectivity of the probe. In another study (50), the photoprotein aequorin was employed for sensitive detection of the malaria parasite *Plasmodium falciparum*. The assay was based on a competition between free-target DNA and an aequorin-labeled DNA molecule. A genetically engineered aequorin molecule was attached to a DNA oligonucleotide through a biotin-streptavidin interaction. The assay achieved detection limits of 3 pg μl^{-1} in human serum; these values fall within the range required for the diagnosis and management of malaria. The most important advantage of this method is that there is no need for PCR amplification of the target DNA; therefore, the assay can be prepackaged and used in a microtiter plate format, which allows faster throughput analysis of a large number of samples. In addition, compared with the currently used light microscopy technique for parasite detection (50), this assay offers the advantages of (*a*) automation and (*b*) utility in developing regions and remote locations.

5. DRIVING THE DETECTION OF BIOMOLECULES TO THE LOWEST LIMITS: HIGHLY SENSITIVE BIOLUMINESCENCE IMMUNO- AND BINDING ASSAYS IN BIOMEDICAL ANALYSIS

An immunoassay measures the concentration of a species of interest within a complex biological matrix by employing biological reagents that display molecular recognition toward each other. To detect a target analyte in a complex biological matrix, the method employed must be exceptionally

selective. In immunoassays, such selectivity is usually achieved through the use of antibodies raised against the analyte of interest, whereas in competitive binding assays, selectivity is provided by a binding partner—often a binding protein or a receptor—that displays high affinity for the analyte ligand. Another factor to consider in analyses in biological matrices is the potential for low concentrations of analyte in the sample, which determines the need for very sensitive labeling strategies. In the early stages of immunoassay development, the required high sensitivity was achieved by employing radionucleotides. The undesirable properties of radioactivity led researchers to search for less dangerous alternatives that employ electrochemical and optical methods. To that end, the number of applications of bioluminescent proteins as sensitive labels in immunoassays has increased dramatically in recent years (8, 47, 51–53). Bioluminescent proteins can be used for both sandwich-type and competitive immunoassays. The sandwich immunoassay consists of an antibody labeled with a bioluminescent protein, which is used to quantify a target analyte bound by a separate antibody attached to a solid support. As the amount of analyte in the solution increases, so does the observed bioluminescent signal, as more labeled antibodies are able to bind to the analyte. In a competitive immunoassay format, the analyte of interest is labeled directly with the bioluminescent protein and competes with free unlabeled analyte in solution for a limited number of analyte-binding sites on the antibody. Thus, the amount of light produced is indirectly proportional to the amount of analyte in a given sample. A large amount of analyte results in fewer free sites on the antibody for binding of the labeled analyte, which in turn yield a smaller bioluminescent signal. In both cases, dose-response or calibration curves can be generated that correlate the amount of bioluminescence signal produced with the concentration of analyte in the sample.

A bioluminescent protein can be conjugated to an analyte or antibody through either chemical or genetic methods. Chemical conjugation conveniently permits the use of a single-protein label for a broad range of applications: One alters only the molecule to which the bioluminescent protein is attached, without having to express and purify a different protein each time. Traditionally, the use of chemical conjugation to label luciferases has been limited due to the resulting loss of luciferase activity. However, conjugation through a biotin-streptavidin-binding pair is effective for maintaining the bioluminescence activity of luciferase. A biotinylated *Cypridina* luciferase was used for a sandwich immunoassay for α-interferon with a linear range of detection of 7.8 to 500 pg ml^{-1}; this assay demonstrated the enzyme's ability to provide sensitive and versatile detection (54). Interestingly, aequorin is more amenable to chemical conjugation than are some other bioluminescent proteins. Although it can be adversely affected by chemical modification, the residual bioluminescence activity still allows for aequorin's effective use as a label in assay development. One such example involves the use of genetically modified aequorin with a unique cysteine; this combination was recently used to chemically label α-fetoprotein antibody, a serological marker of liver cancer. The assay detected α-fetoprotein in the range of 0.02 to 200 ng ml^{-1} (55). Aequorin has been incorporated as a label in many other immunoassays and has led to the development of excellent methods for the determination of various physiologically relevant analytes, such as cortisol, thyroxine, and 6-keto-prostaglandin-F1-α. These results demonstrate the broad applications of aequorin in bioanalysis (56).

The advantages of genetic conjugation include (*a*) the known one-to-one conjugation ratio and (*b*) reproducible, batch-to-batch production of the labeled probe, in which the bioluminescent protein's coding gene is placed in a frame with the target protein or peptide's coding sequence. Thus, following transcription and translation, a single fusion protein consisting of the two proteins is created. An example of such a strategy is the angiotensin II assay, in which an angiotensin II–aequorin fusion protein competes with free angiotensin in human serum, which yields a detection limit of 1 pg ml^{-1} (57). Fusion proteins with obelin and luciferases have also been developed

for applications such as (*a*) streptavidin fusion proteins for biotin conjugations and (*b*) assays for immunoglobulin G and other proteins of interest (52).

6. DESIGNER PROTEINS: SPLIT BIOLUMINESCENT PROTEINS AND MOLECULAR SWITCHES

Proteins can be genetically manipulated via various strategies that encompass fusions, site-directed and random mutagenesis, insertions, deletions, incorporation of unnatural amino acids into the proteins' structures, and even splicing. For the last strategy, a protein is spliced (or split) into two fragments; the splicing usually causes total or significant loss of the protein's original activity, which is regained when the two fragments recombine. The proteins must be able to regain (most of) their original activity either by spontaneously reassembling or by coming into close contact with one another with the help of a second protein. Researchers have utilized these strategies to split bioluminescent proteins into fragments so as to prepare molecular switches, protein complementation, and protein-protein-interaction assays (58–61). In such assays, an external process such as analyte binding or protein interaction is used to reposition the fragments; this process modulates the bioluminescence emission of the protein, which is related to the concentration of analyte and therefore allows quantification or visualization of the event (**Figure 7**). The interaction between the split bioluminescent proteins and other analytes and/or proteins is interesting and versatile: It can inform the design of a plethora of assays by allowing a choice of different methods to split and recombine the proteins.

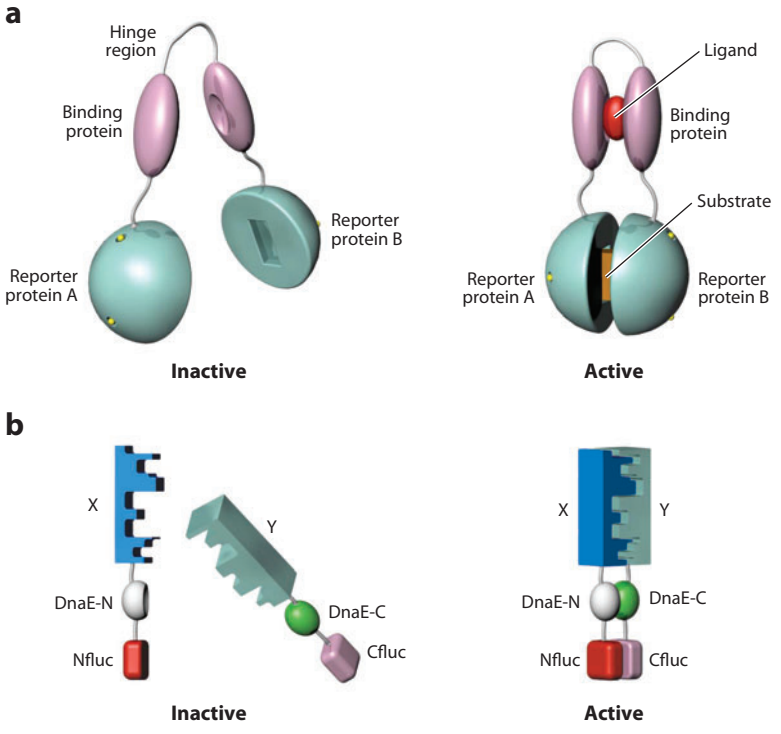

Figure 7

Designer proteins. Schematics of bioluminescent (*a*) molecular switches and (*b*) split complementation assays.

In their natural environment, many proteins require specific interaction and recognition of other protein complexes to be activated or to perform a function. Much remains to be learned about these processes, which are key to cellular functions, energy storage, onset of disease, and so on. Thus, new technologies that can provide insight into these interactions and mechanisms of action of proteins should advance our understanding of cellular events. To that end, protein complementation assays and protein-protein interactions have been developed. An interesting example with a somewhat complicated design involves splitting firefly luciferase and genetically attaching the N and C termini of the luciferase fragments to the N and C termini of the split intein containing DnaE to develop an assay for insulin. These fusion proteins are then linked to a protein of interest, such as insulin receptor substrate 1 (IRS-1), that is attached to the N-terminal luciferase DnaE and its target protein, the Src homology 2 (SH2) domain of phosphoinositol 3-kinase, which is linked to the C-terminal luciferase DnaE. The DnaE intein excises and ligates together the flanking external proteins, in this case the N- and C-terminal luciferase complexes. The insulin-induced phosphorylation of IRS-1 leads to the recognition of the SH2 domain, thereby bringing the DnaE fragments together; subsequently, the luciferase fragments are spliced to reform the bioluminescent luciferase. The corresponding light can then be directly correlated with the concentration of insulin in a dose-dependent manner (62). A similar mechanism with *Renilla* luciferase has further demonstrated the versatility of such systems (63).

The study of protein-protein interactions employing protein splicing is not limited to intein-mediated reassembly, as in the case described above; *Renilla* luciferase has been employed for such studies without intein excision. Specifically, a genetically modified *Renilla* luciferase was split and attached to the Y941 peptide of the aforementioned IRS-1 and N-terminal SH2 domains, which allowed the detection of low (picomolar-to-nanomolar) levels of insulin (64). Small molecule–mediated protein-protein interactions have also been investigated through the use of the rapamycin-mediated interaction between the human FK506-binding protein and the FKBP12-rapamycin-binding domain (65). Given the many potential target proteins for such assays, the number of possible applications is limitless; these applications include high-throughput drug screening and investigation of specific pathways in signaling events, among others.

A molecular switch that employs aequorin as its signal generator was recently reported. This molecular switch differs from protein complementation in that both components of the split bioluminescent proteins are attached to a single recognition protein. The two fragments of aequorin are genetically attached to the termini of the recognition protein that, upon the specific binding of a target analyte, undergoes a conformational change, thereby altering the orientation of the two aequorin fragments. This process creates a so-called on-off switch: The binding of the analytes can turn the bioluminescence either on or off, which allows for quantification of the specific analytes. Investigators (66) developed an aequorin molecular switch for the detection of glucose by inserting the DNA-coding sequence of glucose galactose–binding protein (GBP) into the aequorin coding sequence, yielding a unique fusion protein in which the two fragments of aequorin were attached to the N and C termini of GBP. Upon the selective binding of glucose, GBP underwent a conformational change to bring the two fragments of aequorin into close enough proximity to reassemble the bioluminescence complex, thereby turning the bioluminescence on. The switch showed a selective response to glucose over other sugars, and it responded linearly to physiological levels of glucose (66).

7. SENSING WITH LIVING CELLS: WHOLE-CELL BIOSENSORS BASED ON BIOLUMINESCENT REPORTERS

Protein-based assays and biosensing systems are excellent tools for the detection and quantification of analytes in different types of samples, such as biomedical or environmental toxins (67).

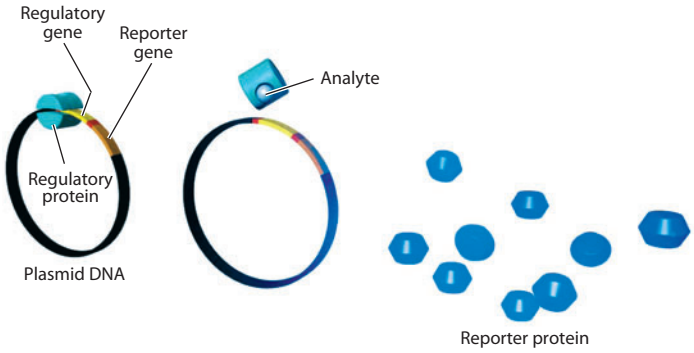

Figure 8

Whole-cell bioluminescent sensors.

A disadvantage of these systems is that they cannot indicate the bioavailability of said toxins, and therefore, researchers cannot obtain information about cytotoxicity, genotoxicity, or the synergistic toxic effects of these environmental pollutants on living systems. Information about bioavailability and the toxins' effect on cellular functions can be obtained only when whole-cell biosensors are employed. Use of such biosensors provides insight into living cells and shows how a particular substance or group of substances influences living organisms (68–70).

In general, bioluminescent whole-cell sensors are based on the principle that either a specific analyte or a group of analytes interacts with living cells that contain a gene for a bioluminescent protein. The interaction between the analytes and the cells turns the bioluminescent property of the cells either on or off, depending on the type of system employed. Systems that employ the "off" approach are typically applied in toxicity assays, in which a naturally bioluminescent species, such as *V. fischeri*, is exposed to a water sample. The value of such toxicity assays, also known as cell death–based assays, is the known sensitivity of *V. fischeri* to various environmental toxins. The presence of a toxin in the water sample can either interfere with the metabolic pathway of the cell or block the expression of luciferase, which is observed as a decrease in the bioluminescence signal generated by the bioluminescent organism. Similar strategies incorporate the *luxCDABE* cassette into different microorganisms, including *Escherichia coli*, *Pseudomonas fluorescens*, and cyanobacteria, which increases the versatility of the whole-cell sensing systems (71). In systems that employ the "on" approach, the expression of a bioluminescent gene, such as the *luxCDABE* cassette, is controlled by a selective promoter or a regulatory gene. Thus, when the target analyte is present, the expression of Lux proteins is turned on to produce the bioluminescent signal (**Figure 8**). The specific DNA promoters allow the cells to be engineered to detect specific toxins, including (*a*) heavy metals such as cadmium, lead, copper, and mercury, among others; (*b*) antibiotics; (*c*) aromatic and other organic compounds; and (*d*) quorum-sensing molecules (20, 72–78). Such biosensing systems have had an important impact on environmental analysis, as well as on drug and cell screening in pharmacological and biomedical applications.

The Achilles' heel of living-cell biosensing systems, which limits their field applications, consists of both these systems' long-term stability and the difficulty of transporting them. To overcome these problems, the Daunert group (79, 80) used sporulating microorganisms as the living cells harboring the chosen sensing system; specifically, the authors utilized sporulating bacteria such as *Bacillus subtilis* and *B. megaterium* to develop sensing systems for arsenic and zinc. Spores can be stored for very long periods of time, which keeps their genetic material intact. Under favorable environmental conditions, spores can be germinated to generate viable and metabolically

active cells over many cycles. In these studies, the authors employed a whole-cell bioluminescent biosensing system, based on reporter-gene strategies, that was capable of detecting arsenite. The data obtained demonstrated that, after either six months of storage or three cycles of sporulation and regermination, the sensing system retains its analytical performance in terms of detection limit, dynamic range, and reproducibility. These findings demonstrated the feasibility of employing spores as a means of long-term storage and for the transportation of whole-cell biosensors (79, 80). Such storage strategies could enable the use of whole-cell sensing systems in on-site and field applications in which the systems may be exposed to harsh or suboptimal conditions.

8. TAKING A CUE FROM NATURE: BIOLUMINESCENCE RESONANCE ENERGY TRANSFER ASSAYS

BRET is a naturally occurring phenomenon that was first discovered in marine organisms (81). Perhaps the best example of a bioluminescent protein emitting light to excite a second protein is that of the pairing between the photoprotein aequorin and GFP. Aequorin and GFP reside in photocytes located in the umbrella of *Aequorea* jellyfish. Under certain circumstances that cause stress to the organism, and in cold, dark depths of the ocean, aequorin emits bioluminescent light, which excites GFP via a radiationless energy transfer. This transfer leads to the now well-known and well-characterized emission typical of green fluorescence. Taking a cue from nature, researchers have designed BRET-based systems in which the energy produced by a bioluminescent protein is transferred from the donor molecule to an acceptor over short distances. Typically, for the transfer to be effective, this distance must be 10 nm, and the bioluminescent donor protein's emission profile must have good spectral overlap with the fluorescent acceptor molecule's absorption profile (15). BRET offers an advantage over fluorescence resonance energy transfer in that it does not require an external excitation source. Therefore, BRET can be used in cells that are photosensitive, as well as in cells that exhibit autofluorescence. The application of BRET also reduces photobleaching problems associated with fluorophores, which arise from their constant bombardment with high-intensity sources. Because the donor and the acceptor must be in close proximity, BRET can be employed for the study of various protein-protein interactions (82–84), receptor-ligand interactions (16), conformational changes (85, 86), and RNA detection and quantification (47) in various applications. The analytical applications of BRET are the subject of recent reviews (15, 87, 88), so we describe them only briefly here.

An interesting recent report (89) described a novel biomineralization approach for the development of a near-infrared (NIR) light-emitting BRET complex. The emission of NIR light is advantageous in deep-tissue imaging; thus, NIR-BRET complexes have a plethora of applications in medicine. Biomolecules have been used for the bottom-up control of inorganic nanostructure synthesis; however, in the proposed biomineralization, the biomolecules function both (*a*) as a template for nanostructure formation and (*b*) as an active participant in the production of NIR light. In this study (89), a mutant *R. reniformis* luciferase acted as a template for the growth of lead sulfide quantum dots; the presence of luciferase was necessary to form a template for the quantum dot configuration. The luciferase activity was preserved during this process, so the addition of coelenterazine light was produced in the NIR region. The purified *R. reniformis* luciferase typically showed an emission maximum of ~480 nm. This emission wavelength was observed in the quantum dot complex; however, the intensity of the emission was significantly reduced compared with that of the luciferase outside of the quantum dot when emission in the NIR region appeared. This finding confirmed the occurrence of a radiationless transfer of energy from the luciferase to the lead sulfide quantum dot, which yielded the NIR emission. This study represents an important

example of coupling between biological and inorganic materials, and it has expanded the use of such hybrid structures into a wide range of fields (89).

9. KILLING TWO (OR MORE) BIRDS WITH ONE STONE: MULTIANALYTE DETECTION

An area in which the use of bioluminescence has been limited, compared with other optical methods such as fluorescence, is multiplex detection. Multiplexing, or the simultaneous detection of multiple analytes within a given sample, has received much attention in recent years.

The most frequently used reporters for multiplex assays are fluorescent compounds. Fluorescent reporters are available throughout the visible-light region, which enables differentiation among signals from several different sources. Many bioluminescent proteins, however, exhibit broad emission profiles in similar regions, impeding the discernment of multiple signals. The use of bioluminescent labels for multiplexing assays would offer advantages for many technologies that require high sensitivity and low detection limits, such as miniaturized devices and high-throughput screening assays. Great progress has recently been made in this area due to extensive mutagenesis studies performed on the bioluminescent proteins aequorin and luciferase, as well as on combinations of proteins. Firefly luciferase natively emits light at a maximum of approximately 560 nm (3). Through site-directed mutagenesis, key amino acids can be altered, which shifts the emission maximum toward the red region at 615 nm and toward the blue region at 549 nm (90). This 66-nm difference in emission maxima enabled the development of a dual-analyte assay. Further investigations into the mechanism underlying this protein's bioluminescence emission revealed that the wavelength of light emitted is determined by the degree of molecular rigidity of the excited-state species, which is affected by the interacting amino acids. This information allowed further tuning of the emission characteristics of luciferase (91). Another promising method of altering the emission maximum through the incorporation of nonnatural amino acids shifts the maximum emission to 603 nm (92).

An interesting approach to multianalyte detection is the so-called self-illuminating quantum dot conjugates. This methodology, developed at the Gambhir laboratory (93–94) at Stanford University, involves the conjugation of different quantum dots to *Renilla* luciferase. The bioluminescence energy transfer between the luciferase and the quantum dots results in different colors of luminescence, which can be used to image cellular events or detect more than one analyte simultaneously.

The deep ocean is full of bioluminescent organisms that either have not been fully studied or cataloged or have yet to be discovered. The growing collection of available bioluminescent proteins with different wavelengths of emission will offer an alternative to the use of genetic engineering to create proteins for multiplex analysis. Ito et al. (95) employed two natural proteins that emit at different wavelengths, namely aequorin and luciferase, to develop a dual-analyte sandwich immunoassay for prostate-specific antigen–α-fetoprotein and prostate-specific antigen–prostatic acid phosphatase pairs. The assay utilized aequorin to label one antibody and firefly luciferase to label the other. The bioluminescence of both proteins was triggered simultaneously by adding Ca^{2+} and D-luciferase to the wells in a microtiter plate. The signal emitted by aequorin was measured first; thereafter, the signal emitted by luciferase was measured. Both measurements were completed in 4 s. The assay demonstrated high sensitivity, and its performance correlated well with that of commercially available detection kits.

The photoprotein obelin has also been spectrally tuned through alterations of the interactions between its amino acids and its chromophore, coelenterazine. Obelin, which natively emits light at 491 nm, was genetically engineered to emit at 493 and 390 nm. Mutations at positions W92F and H22E of the protein sequence yielded the obelin mutant with the 390-nm maximum, and a

Y193F mutation created the 493-nm variant. These proteins were used to label antibodies for the detection of human follicle-stimulating hormone and luteinizing hormone, which are useful in the evaluation and diagnosis of diseases dealing with gonadal function. The light-emitting reactions were triggered by the addition of Ca^{2+}, and the assay achieved detection levels comparable to those obtained with radioimmunoassay techniques (96).

Similarly, investigators have subjected aequorin to extensive genetic modifications, in the form of random and rational mutagenesis, in their search for aequorin variants with a wide range of spectral characteristics. Variations in the chemical structure of aequorin's chromophore, coelenterazine, also play a major role in affecting the mechanism of bioluminescence emission and, ultimately, in altering the wavelength of emission of aequorin. Pairing aequorin with different natural and synthetic coelenterazines is an additional way to tune the spectral characteristics of aequorin (97). A large library of such semisynthetic aequorin variants has been prepared and cataloged; it includes bioluminescence emission wavelengths that range from 448 nm to 519 nm (98). Because the bioluminescence emission of aqueorin has flash-type kinetics, altering the structure of the protein and pairing it with different celenterazines change the decay life of the protein. Wild-type aequorin paired with native coelenterazine typically exhibits flash-type kinetics with a short half-life of ~2 s. In contrast, aequorin mutants paired with the coelenterazine i analog have half-lives ranging from 11.8 to 50.1 s—significantly longer than the half-lives of most other semisynthetic aequorin combinations (98).

The incorporation of nonnatural amino acids into aequorin is yet another method of performing spectral tuning; this technique causes a shift in the emission maximum up to 517 nm (99). In this case, four nonnatural amino acids were site-specifically incorporated into aequorin at amino acid position 82 through the use of a specially designed transfer RNA and an amber codon. The introduction of the nonnatural amino acid at a critical position within the aequorin's hydrophobic pocket at which coelenterazine resides altered the protein's interaction with coelenterazine; as explained above, this alteration caused a shift of the bioluminescence emission wavelength. An array of bioluminescence emissions with diverse spectral characteristics were recorded when the nonnatural aequorin variants were combined with synthetic coelenterazines.

Aequorin variants paired with different coelenterazine analogs have been employed to develop a simultaneous, dual-analyte assay in a single well for two important cardiovascular molecules, 6-keto-prostaglandin-FI-α and angiotensin II. A cysteine-free aequorin mutant was chemically conjugated to 6-keto-prostaglandin-FI-α and genetically fused to angiotensin II. The 6-keto-prostaglandin-FI-α–aequorin complex was paired with the native coelenterazine and yielded a half-life of 0.5 s, whereas the angiotensin II–aequorin construct was paired with coelenterazine i and yielded a half-life of 11.7 s. Two distinct time intervals of 0 to 6 s and 6 to 25 s were used to differentiate the signals, allowing for simultaneous detection (100). The combination of time and wavelength resolution can further increase the potential uses of aequorin in multiplex analysis. To that end, three interleukins (ILs) of interest—IL-1β, IL-6, and IL-8—were genetically conjugated to three different semisynthetic aequorin variants. The IL-1β–aequorin conjugate with coelenterazine f had an emission maximum of 506 nm and a 0.56-s half-life; the IL-6–aequorin conjugate with coelenterazine i had an emission maximum of 507 nm and a 14.21-s half-life; and the IL-8–aequorin conjugate with coelenterazine cp had an emission maximum of 452 nm and a 0.16-s half-life. Three discrete time–wavelength windows were used to discriminate the signals, and a competitive assay integrated onto a microtiter plate was developed. Band-pass filters were used to select a specified wavelength for two photomultiplier tubes, thereby differentiating between the two signals with a short half-life, whereas time intervals were employed to select for the third signal with an extended half-life. This setup achieved detection in the picogram-per-milliliter range, which allowed for detection of the ILs in serum (**Figure 9**) (101).

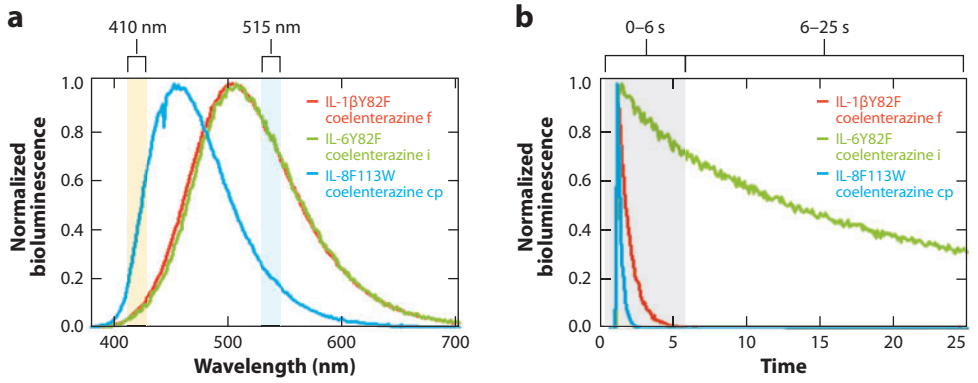

Figure 9

Multianalyte detection of interleukin (IL)-1β, IL-6, and IL-8 through the use of (*a*) spatial and (*b*) temporal resolution of semisynthetic aequorins.

10. CONCLUSIONS

As molecular engineering techniques and instrumentation continue to evolve, so will the use of bioluminescent proteins in analytical applications. Over the past 10 years, the sensitivity of charge-coupled device cameras and other luminescent detectors has greatly improved, allowing these instruments to detect only a few light-emitting molecules (102). This development, along with the miniaturization of instruments, has allowed for the integration of luminescent detectors into miniaturized detection systems such as lab-on-a-chip- and lab-on-a-CD-type devices (103). In response to the push for faster, more portable, and more robust detection capabilities for point-of-care and on-site analysis, these systems will provide many of the desired characteristics. Progress in this area has already been made, as exemplified by many novel technologies. For example, antibody-coated magnetic beads have been used to capture specific bacteria on a microfluidic device, after which the bacteria were indirectly quantified with firefly luciferase (104). Whole-cell biosensors made with engineered *E. coli* cells have been incorporated into lab-on-a-chip setups that can rapidly monitor environmental and water pollutants in samples (105, 106). Firefly luciferase has also been employed in a lab-on-a-chip metabolic profiling system that can detect ATP down to submicromolar concentrations (107). The ability to carry out multiplex detection will further enhance the utility of bioluminescence in miniaturized systems. Thus, as the discovery and characterization of new bioluminescent proteins, as well as the genetic modification of current bioluminescent proteins, continue to expand the spectral diversity and favorable characteristics of these systems, the potential uses of these proteins will increase. In addition, the use of bioluminescent proteins for in vivo imaging has become a cost-effective and sensitive alternative to traditional imaging techniques (108).

Bioluminescence is a fascinating natural phenomenon that, when coupled to modern instrumentation and reagents, yields bioanalytical methods with exquisitely low detection limits. The ever-growing number of reported organisms that are bioluminescent or that interact with bioluminescent organisms in a symbiotic manner is helping us discover new proteins. These new bioluminescent proteins, in turn, can be employed to develop methods that improve our understanding of cellular and physiological events and interactions, help detect previously undetectable molecules, simultaneously identify multiple target molecules, and even help us understand how large marine animals move and behave in the oceans. Finally, recent discoveries of bioluminescent

organisms and plants that live deep in the Antarctic ocean and in rainforests will undoubtedly provide researchers with a myriad of interesting proteins that should find many applications in bioanalysis.

SUMMARY POINTS

1. Bioluminescent proteins offer a feasible alternative to many of the labeling and detection schemes traditionally employed in bioanalysis.
2. The ATP requirement of luciferase bioluminescence allows for extremely sensitive quantification and detection of ATP.
3. Intracellular Ca^{2+} detection with aequorin has greatly enhanced our understanding of cellular signal transduction pathways.
4. The highly sensitive and specific nature of bioluminescent light emission has driven the implementation of bioluminescent proteins in various areas, including nucleic acid analysis, binding and immunoassays, and BRET applications.
5. The bioavailability and toxicity of many toxins and substances of interest have been investigated through the novel use of bioluminescent whole-cell biosensors.

FUTURE ISSUES

1. As the sensitivity and efficiency of detection instrumentation continue to improve, new bioluminescent proteins will continue to become labels of choice for in vivo diagnosis and imaging.
2. Efforts to miniaturize detection systems and to make them more portable will create new opportunities for the use of bioluminescent proteins.
3. The use of bioluminescent sensing systems will improve our understanding of molecular and cellular events.
4. Investigators will continue to design and prepare a range of available bioluminescent labels with different wavelengths and lifetimes, through not only the isolation of new bioluminescent proteins but also the genetic manipulation of currently used bioluminescent proteins.

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