Engineering Luciferase Enzymes and Substrates for Novel Assay Capabilities

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ABSTRACT

In the development of HTS as a central paradigm of drug discovery, fluorescent reporter molecules have generally been adopted as the favored signal transducer. Nevertheless, luminescence has maintained a prominent position among certain methodologies, most notably genetic reporters. Recently, there has been growing partiality for luminescent assays across a broader range of applications due to their sensitivity, extensive linearity, and robustness to library compounds and complex biological samples. This trend has been fostered by development several new assay designs for diverse targets such as kinases, cytochrome p450's, proteases, apoptosis, and cytotoxicity. This review addresses recent progress made in the use of bioluminescent assays for drug discovery, highlighting new detection capabilities brought about by engineering luciferase enzymes and substrates. In reporter gene applications, modified luciferases have provided greatly improved expression efficiency in mammalian cells, improved responsiveness to changes of transcriptional rate, and increased the magnitude of the reporter response. Highly stabilized luciferase mutants have enabled new assays strategies for high-throughput screening based on detection of ATP and luciferin. Assays based on ATP support rapid analysis of cell metabolism and enzymatic processes coupled to ATP hydrolysis. Although luciferin is found natively only in luminous beetles, coupled assays have been designed using modified forms of luciferin requiring the action of second enzyme to yield luminescence. Due to the very low inherent background and protection of the photon-emitter afforded by the enzyme, bioluminescent assays often outperform the analogous fluorescent assays for analyses performed in multiwell plates.

Keywords: bioluminescence, luciferase, luciferin, fluorescence, drug discovery, high-throughput screening

1. PHOTOMETRIC ASSAYS IN DRUG DISCOVERY

Because pharmaceutical companies are not yet cleaver enough to create novel drugs through rational design alone, they instead rely on various strategies for searching through large collections of drug-like molecules for those having the desired medicinal properties. The search is usually done one molecule at a time, and requires a biological system that mimics the clinical conditions of interest. A typical collection of compounds, referred to as a compound library, may contain between 10,000 to over a million distinct structures, so screening these libraries for the desired properties in a reasonable period of time requires that the assays be quite rapid, typically averaging less than 5 seconds per assay. To achieve ultra high-throughput screening, generally considered greater than 100,000 assays per day, the time required per sample cannot exceed tenths of a second.

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However, speed alone is not sufficient. Successful candidate molecules within a library are almost certainly rare, so they can be overlooked in a screen due to the combined effect of statistical noise in the assay and the overwhelming number of compounds that lack the desired traits. Thus, assay accuracy is also essential. Finally, since these compound libraries are expensive, and only a small amount of each compound is generally available, the assays should be highly sensitive.

Fundamentally, an assay is a means for translating a biomolecular effect into an observable parameter. Although theoretically there are many strategies by which this could be achieved, in practice, assays capable of delivering the speed, accuracy and sensitivity necessary for effective screening are based on photon production. Assays based on photon absorption are also commonly used, but these are usually much less sensitive. Photon production is realized primarily through fluorescence and chemiluminescence. Both processes yield photons as a consequence of energy transitions from excited-state molecular orbitals to lower energy orbitals. However, they differ in how the excited-state orbitals are created. In fluorescence, the excited states are created by absorption of light, whereas in chemiluminescence the excited states are the product of exothermic chemical reactions.

This distinction in how these excited states are created greatly affects the character of the photometric assay. Most obvious is that fluorescence-based assays tend to be much brighter, since the photon used to create the excited states can be pumped into a sample at a very high rate. In chemiluminescence, the chemical reactions required to generate excited states usually proceed at a much lower rate, and so yield a lower rate of photon emission. The greater brightness of fluorescence would appear to correlate with better assay sensitivity, but this is commonly not the case. Assay sensitivity is determined by a statistical analysis of signal relative to noise, where the signal represents a sample measurement minus the background measurement. The limitation of fluorescence is that it also tends to have much higher backgrounds.

This is primarily because fluorometers must discriminate between the very high influx of photons entering into the sample and the much smaller emission of photons from the analytical fluorophores. This is done largely by optical filtration, since emitted photons are longer wavelength than excitation photons; and by geometry, since the emitted photons typically travel a different path than the excitation photons. But optical filters are not perfect in their ability to differentiate between wavelengths, and photons can also change direction through scattering. Chemiluminescence has the advantage that, since photons are not required to create the excited states, they do not constitute an inherent background when measuring photon efflux from a sample.

Fluorescence can also be limited by the presence of interfering fluorophores within the samples. This is especially problematic in biological samples, which can be replete with a variety of heterocyclic compounds typically in concentration much above the analytical fluorophores of interest. The problem is minimized in relatively simple samples, such as purified proteins. But in drug discovery, living cells are increasingly used for high-throughput screening as tractable models of the human organism. Unfortunately, cells are enormously complex in their chemical constitutions, which among other difficulties exhibit substantial inherent fluorescence. The screen of the compound library is also inherently complex since, although each assay sample may contain only one or a few compounds, the data

set from which the drug leads are sifted is cumulated from many thousands of compounds. These compounds also may present problems with fluorescence interference since drug-like molecules typically have heterocyclic structures.

Concerns about the sensitivity of fluorescent assays on living cells may seem misplaced given the wealth of exquisite fluorescent images available revealing intricate subcellular architecture. It seems paradoxical, if we expect chemiluminescent assays to offer higher sensitivity, that images of cells using chemiluminescence are uncommon. But for these applications, brightness counts because the optics required for imaging cellular structures is relatively inefficient for light gathering. Thus, the low background inherent in chemiluminescence is of little advantage since it is usually far below the detection capabilities of imaging devices. Furthermore, imaging is largely a matter of edge detection, which has different signal-to-noise characteristics that simply detecting the presence of an analyte. Edge detection relies heavily on signal strength and suffers less from uniform background noise.

Thus, for image analysis of microscopic structures, fluorescence is almost universally preferred over chemiluminescence. But in macroscopic measurements requiring accurate quantification with high sensitivity, chemiluminescent assays often outperform analogous assays based on fluorescence. Such macroscopic measurements are the foundation for most high-throughput screening, which relies heavily on the use of multiwell plates, typically 96-, 384- or 1536-well plates, to measure a single parameter in a large number of samples as quickly as possible. Although assays based on either fluorescence or chemilumienscence can yield high sample throughput, chemiluminescence is less hinder by photon contamination as a requirement of creating the excited-state orbitals, or from the chemical compositions of the samples and compound libraries. The limitation of chemiluminescence in high-throughput screening has been largely the lack available assay methods. Fluorescence has been more commonly used due to its greater familiarity and easier implementation into a new assay designs. But new capabilities in chemiluminescence, particularly in bioluminescence, are now changing this.

2. PHOTOMETRIC ASSAYS USING BIOLUMINESCENCE

Bioluminescence is a form of chemiluminescence that has come about through natural evolution. Although most people are aware of bioluminescence primarily through the nighttime displays of fireflies, there are many distinct classes of bioluminescence derived through separate evolutionary histories. These classes are widely divergent in their chemical properties, but nevertheless hold few distinguishing features in common. Most important of these is that they are all based on the interaction of an enzyme, luciferase, with a luminescent substrate, luciferin. The luciferases that have been used most widely in high-throughput screening are beetle luciferases (including firefly luciferase), Renilla luciferase, and aequorin. The beetle luciferases have been the most versatile of this group, and the number of new applications is rapidly expanding. Renilla luciferase has been used primarily for reporter gene applications, although this too is recently expanding. Aequorin has been used almost exclusively for monitoring intracellular calcium concentrations.

Firefly luciferase, the paradigm for the beetle luciferases, is a monomeric enzyme of 61 kD that requires no post-translational modifications for activity. It acts by first combining beetle luciferin with adenosine triphosphate (ATP), to form luciferyl-AMP as an enzyme-bound intermediate. This intermediate then reacts with O_2 to create another enzyme-

bound intermediate, oxyluciferin with high-energy orbitals. The energy transition to the ground state orbital yields a photon and oxyluciferin as reaction products (Figure 1).

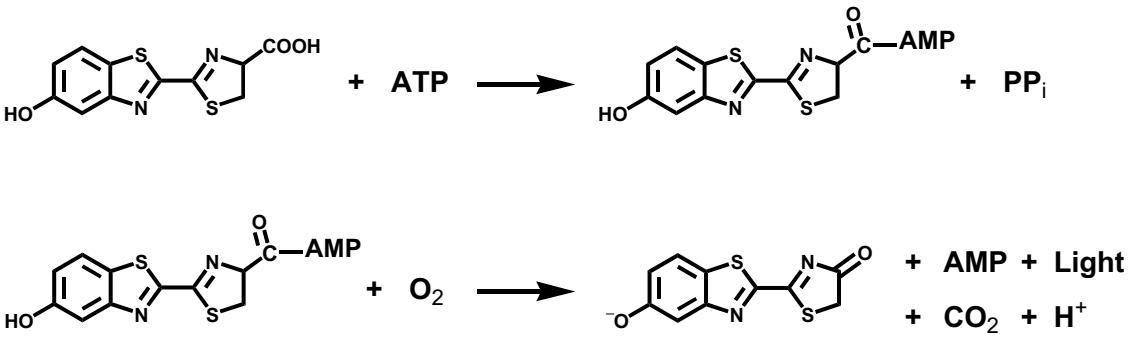

Figure 1. Luminescent reaction of beetle luciferases. Luciferin (upper left) undergoes a two-step reaction to form the photon-emitter, oxyluciferin (lower right). All steps, including light emission, occur within the protected environment of the enzyme active site.

Achieving efficient chemiluminescence is not a trivial matter, as evident by the relative paucity of this phenomenon in our daily experiences. The large energy transitions required for visible luminescence generally are disfavored over smaller ones that dissipate energy as heat. A major mechanism for this dissipation is through interactions with surrounding molecules, especially with water molecules in aqueous solutions. Because of these interactions, chemiluminescence tends to be strongly dependent on environmental conditions. Chemiluminescent assays are often designed to incorporate hydrophobic compounds, such as micelles, to protect the excited state from the water; or rely on energy transfer to fluorophores that are less sensitive to solvent quenching. The other difficulty with chemiluminescence is efficient coupling of the reaction pathway to the creation of excited-state orbitals.

While most chemiluminescence has relied on the ingenuity of creative chemists, bioluminescence instead has relied on the processes of natural evolution. As luminous organisms through the eons were selected by the brightness of their light, their luciferases have evolved both to maximize chemical coupling for creating the excited states, and to protect the excited states from water. In firefly luciferase, it appears that the enzyme excludes water by wrapping around the substrate, so that the excited-state reaction products are completely secluded from water. The enzyme structures shows two domains connected by a single polypeptide which may act as a hinge. It is likely that the substrates bind between the domains, causing them to close together like a lid onto a box. The enzyme would thus act as an insulator between the excited state and the environment around it. This strongly contrasts with synthetic forms of chemiluminescence, where the excited states are exposed to the solvent. In effect, a distinctive feature of bioluminescence is that the luciferase serves as a box that both generates and protects the excited states.

The challenge for designing bioluminescent assays is harnessing this efficient light-emitting chemistry into analytical methodologies. Most commonly this is done by holding the concentrations of all components in the

luminescent reaction constant, except for one that is allowed to vary in correlation to a biomolecular process of interest. When configured properly, the light intensity is directly proportional to the variable component, thus coupling an observable parameter to the process. In high-throughput assays using firefly luciferase, the variable component may be ATP, luciferin, or the enzyme itself. Because of the very low backgrounds in bioluminescence, the linear range of this proportionality can be enormous, typically extending 10^4 to 10^8 fold over the concentration of the variable component.

3. ASSAYS OF LUCIFERASE CONCENTRATION

Firefly luciferase is best known in high-throughput screening for its applications as a genetic reporter. Many cellular events of relevance to drug discovery can be associated with a genetic regulatory element. By coupling the regulatory element to expression of the luciferase gene, typically by placing the regulatory element just upstream of a gene encoding the luciferase, the cellular event may be made readily detectable by a luminescent signal. A common example is monitoring the activity of G-protein coupled receptors (GPCR's) in the cellular membrane by using cAMP-responsive elements (CRE's) positioned upstream of a luciferase gene. Activation of receptors coupled to Gαs proteins causes in increase in intracellular cAMP, which in turn activates PKA to phosphorylate CREB. The luciferase concentration within cells is increased when phorphorylated CREB is bound to the CRE sequence, causing an increase in the transcription rate of the luciferase gene.

The intracellular luciferase is typically quantified by adding a buffered solution containing detergent to lyse the cells, and luciferase substrates to initiate the luminescent reaction. The luminescence in this reaction will slowly decay due to side reactions causing irreversible inactivation of the enzyme. The nature of these side reactions is not well understood, but they are probably due to the formation of damaging free radicals. To maintain a steady luminescence over an extended period of time, ranging from minutes to hours, it is often necessary to inhibit the luminescent reaction to various degrees. This reduces the rate of luminescence decay to where it will not interfere over the time required for measuring multiple samples. However, even under these conditions, the luciferase may be quantified down to 10^{-18} moles per sample, which corresponds to roughly 10 molecules per cell. These assays are convenient for high-throughput applications because sample processing is not necessary prior to adding the reagent. Simply add and read.

One of the disadvantages commonly associated with reporter genes is their relatively slow response to dynamic cellular events. Reporter expression is usually correlated to changes in the transcriptional rate of the reporter gene, which can vary quickly in response to cellular physiology. Yet, because the reporter enzymes have a finite lifetime within cells before being degraded, the net population of reporter enzymes constitutes a collection of molecules created at different times. As the transcriptional rate associated with each reporter enzyme may be different, depending on when the reporter molecule was created, the net population represents also a collection of corresponding transcription rates. Hence, upon assaying the net population of reporter enzymes contained in a cell, the result represent the cumulative, or average transcription rate over the lifetime of all the reporter molecules in the population. This "time averaging" of the transcriptional rate tend to dampen the apparent responsiveness of the reporter to rapidly changing conditions.

Reducing the lifetime of the reporter within the cell, thus effectively reducing the time averaging window, can minimize this effect. This can be achieved by adding protein and mRNA degradation sequences to the reporter gene.

Firefly luciferase genes containing such degradation sequences have been shown to respond much more quickly to environmental conditions, such as the action of agonist or antagonists to cellular receptors (Figure 2). In high-throughput screening, such destabilized genes should increase the dynamics response of the assay, reduce the time require for assay, and minimize the occurrence of secondary effect that may arise from the prolonged incubation of cells with the library compounds.

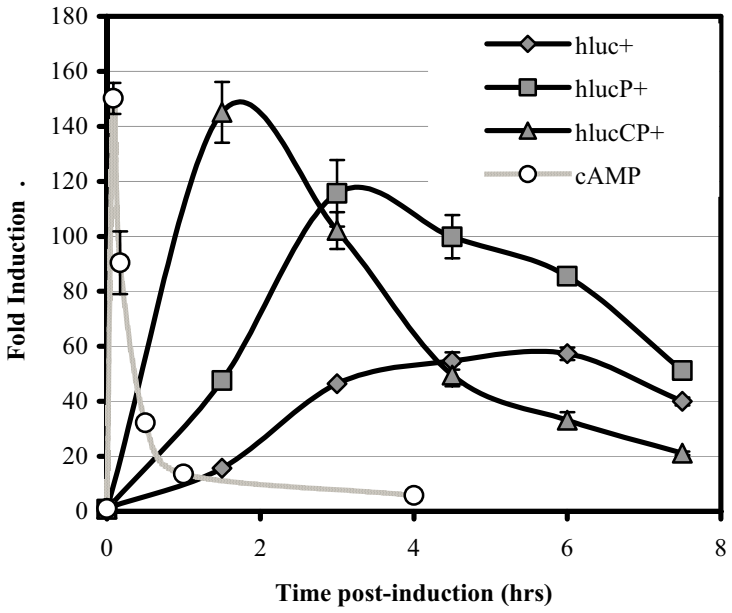

Figure 2. Luciferase genes containing degradation sequences are more responsive. Inclusions of a single degradation sequence (hlucP+) or multiple degradation sequences (hlucCP+) reveal faster and relatively larger responses than the parental luciferase (*h*luc+) to a transient pulse of intracellular cAMP. Intracellular cAMP was induced by activation of cellular GPCR's in cultured d293 cells upon addition of 1 μM isoproterenol hydrochloride; the luciferase genes were coupled to cAMP Response Elements (CRE's). Intracellular cAMP was quantitated using an enzyme-linked immunoassay.

Ideally, the time averaging effect could be entirely eliminated by reducing the reporter lifetime to zero. Unfortunately, this would also mean that no reporter enzyme accumulates within the cell and, thus, no signal would be detectable in the assay. Reducing reporter lifetime increases dynamic response, but also reduces signal intensity. However, the inherently high sensitivity provided by bioluminescent reporters makes this strategy feasible. Because most high-throughput screens using luciferase are done in conditions of excess signal strength, the assay dynamics may be improved without sacrificing sensitivity.

4. ASSAYS OF ATP CONCENTRATION

Firefly luciferase has long been known for it utility in measuring adenosine triphosphate, especially as a means for detecting microbial cells. Nonetheless, the application of bioluminescent ATP assays in high-throughput screening

has been relatively recent, most commonly for rapidly screening cytotoxicity in compound libraries. Because it is the major conduit for metabolic energy, ATP serves as a good indicator of cell viability. Particularly in mammalian cells, ATP concentrations are homeostatically maintained through a balance of generation and consumption pathways. The generation pathways require the coordinated interaction of many enzymes through a complex intracellular architecture, which are readily disrupted upon cell death. But the cellular ATPase of the consumption pathways may act independently, and thus continue to consume available ATP even after cell death. Accordingly, cell death is accompanied by an almost instantaneous drop in intracellular ATP, and thus a loss luminescence when assayed using luciferase.

The most convenient bioluminescent assays for ATP work through a simple mix-and-read format, analogous to reporter gene assays. The assay reagent both extracts ATP from the cells and converts it to a luminescent signal. The results correlate well with the widely used tetrazolium assays (e.g., MTS, MTT, and XTT) that indicate cell viability through detection of intracellular redox activity, which is also closely associated with cellular metabolism. However, the bioluminescent assay can detect about 100-fold fewer cells (to less than 10 cells) than tetrazolium-based assays, and the assay is much faster (5 minutes compared to several hours). The preferred bioluminescent assays are designed to emit stable luminescence lasting for several hours.

One of the challenges for designing an assay reagent for stable luminescence is the need to inactivate the endogenous cellular ATPases. If these ATPases are not inactivated upon cell lysis, they will continue consuming the ATP released from the cells, causing the luminescence to be prematurely extinguished. However, because cellular ATPases constitutes diverse enzyme classes with little in common other than their reactivity with ATP, the inactivating components of the assay reagents must be broadly targeted to the mechanisms for ATP consumption, or be generally disruptive to enzyme activity regardless of function. However, this presents a conflict in the reagent formulation, as luciferase is also an enzyme with ATPase activity. To resolve this, a highly stabilized form of luciferase has been created using directed evolution. This enzyme, trademarked UltraGlo, is resistant to broad-spectrum ATPase inhibitors, thus allowing protection of the extracted ATP without compromising the luminescent reaction.

Bioluminescent assays for ATP can also be used for quantifying the activity of enzyme that either consume or generate ATP. Of primary interest to drug discovery are assays for kinase activity. Most kinase assays are designed for detecting the phosphorylation of a kinase substrate, usually a specific peptide sequence. For example, immunoassays directed toward phosphorylated amino acids are commonly used. Because these assay strategies are associated with the substrate specificity of the kinases, different classes of kinases necessitate separately configured assays. However, as all kinases utilize ATP, a single assay design based on ATP consumption can be applied nearly universally. Bioluminescent ATP assays are particularly useful for screening kinase inhibitors, since luminescence will be highest in those samples exhibiting the greatest amount of inhibition. The assay is also useful when kinase activity is not directed toward a peptide, for example, assays of saccharide or lipid kinases. However, this universality of this assay method also imposes a restriction that the kinase activity be free of other potential ATPases, since the assay cannot selectively differentiate the source of the ATP consumption. Thus, for example, this assay is not suitable for assaying specific kinase activities within a cell lysate, unless these activities dominate over the other ATPase activities found within the cells.

5. ASSAYS OF LUCIFERIN CONCENTRATION

In a manner similar to bioluminescent ATP assays, luciferases may also be used to quantify luciferin. However, unlike ATP, luciferin metabolism is not naturally coupled to mammalian cell physiology or enzyme catalysis. Although it is has a relatively simple chemical structure, beetle luciferin is biochemically unique among living systems, being found only in luminous beetles. This provides an opportunity for using luciferin as a probe of specific biochemical activities even when incorporated into complex compositions. To couple luciferin concentrations to the activity of a specific enzyme requires development of a "pro-luciferin" that can be acted upon by that enzyme. A pro-luciferin is a molecule than cannot support luminescence directly when combined with luciferase, but that can be converted into luciferin through the catalytic processing of a second enzyme. By this means, the luminescent signal of an assay becomes dependent on this second enzyme.

This approach has been useful for developing a series of sensitive assays for cytochrome p450 activities. Cytochrome p450's are importance in drug discovery as they play an important role in how drugs are metabolized within the body. These enzymes, found predominately in the liver, modify the structures of xenobiotic molecules largely through oxidative cleavage reactions, with different members having catalytic specificities towards different molecular structures. A common means for rapidly assessing their activity is by adding a fluorogenic molecule, which though the oxidative cleavage becomes a more efficient fluorophore. The same strategy can be applied to luciferin that has been modified with a cleavable group, such as 6'-O-methyl. Upon incubation with specific cytochrome p450's, the 6'-O-methyl-luciferin is converted to luciferin, which can be sensitively detected using luciferase. Luminescent assays based on enzymatic conversions of pro-luciferins are found to be typically 10- to 100-fold more sensitive than the comparable fluorogenic assays.

Often, it is feasible to combine the pro-luciferin with all the components necessary to support luminescence into a single reagent. Thus, when this reagent is added to a sample, luminescence is generated instantaneously as the pro-luciferin is converted into luciferin. An example of this has been developed for assaying apoptosis in cells using DEVD-6'-aminoluciferin, a pro-luciferin specific for protease cleavage by caspase-3. Although the cleavage reaction yields aminoluciferin, this luciferin analog also serves as a luminogenic substrate of luciferase. When added to a sample containing caspase-3, the concentration of aminoluciferin rises until the rate of the cleavage reaction matches the rate of the luciferase reaction. A constant luminescent signal is thus produced in this steady-state reaction, where the light intensity is proportional to the level of the caspase-3 activity.

Such coupled reactions, where the generation of luciferin is directly linked with light production through luciferase, differ kinetically from fluorogenic assays of enzyme activity. In the coupled assay, the steady-state reaction gives a nearly instantaneous indication of enzyme activity. An increase or drop in enzyme activity is reflected rapidly in a change in the steady state. Fluorogenic assays instead are based on the accumulation of fluorophore over an extended period of incubation. Because the coupled luminescent represents only an instant in time, whereas the fluorogenic assay draws on an extended incubation, it would appear that the fluorescent methods would be more sensitive. As before, however, this is typically not the case due to the very low background of the luminescent method. In the case of caspase-3 assays, a highly sensitive fluorogenic assay is based on cleavage of $DEVD_2$-rhodamine. This assay is directly

comparable to the assay using DEVD-6'-aminoluciferin, as both methods are based on the cleavage of the same tetrapeptide from the chromophore. Nevertheless, the coupled luminescent assay shows greater than 10-fold improvement in sensitivity (Figure 3).

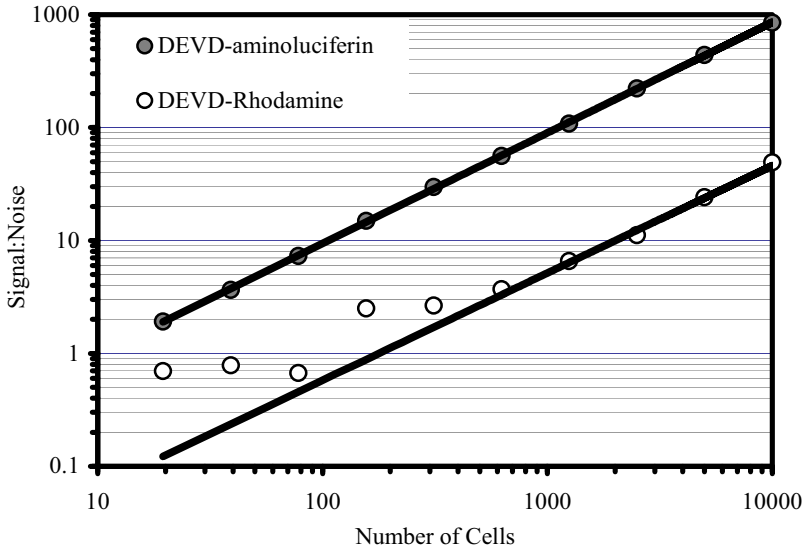

Figure 3. Comparison of bioluminescent and fluorescent assays for caspase-3 activity. Assays were performed on Jurkat cells induced to apoptosis by addition of staurophorin to the growth medium. Assay reagents based on DEVD-aminoluciferin (closed symbols) or DEVD-rhodamine (open symbols) were incubated with cells for one hour prior to measurement. The higher signal:noise ratio reveals that the bioluminescent assay was greater than 10-fold more sensitive than the fluorescent assay

6. CONCLUSION

For creating highly sensitive assays in drug discovery, the dominant method by far has been fluorescence. Yet, fluorescence does not necessarily lend the best performance, and typically has lower sensitivity than chemiluminescent methods. Due to the ability to provide very low backgrounds, chemiluminescence has been known for having very high sensitivity. But most forms of chemiluminescence are also quite sensitive to environmental conditions, and thus their robustness in complex system has been limited. An exception to this has been bioluminescence, which due to the protected environment created by the active site of the enzyme, can yield efficient and reliable signals under biologically relevant conditions.

The benefits of bioluminescence have been widely recognized for applications of genetic reporters. Recent advances have further improved this technology, such as improved response times of the genetics. But these benefits have not generally been extended to other types of biomolecular assays until recently. With the development of highly stabilized luciferases, new assay reagents that incorporate luciferase as a component of the formulation have become feasible. These reagents are characterized by being robust, highly sensitive, and providing extensive linearity. It is expected that as more assay designs become available, bioluminescence will increasingly become a method of choice in pharmaceutical research.