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Technology Review

Bioluminescent Assays for High-Throughput Screening

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Abstract: In the development of high throughput screening (HTS) as a central paradigm of drug discovery, fluorescence has generally been adopted as the favored methodology. Nevertheless, luminescence has maintained a prominent position among certain assay formats, most notably genetic reporters. Recently, there has been growing partiality for luminescent assays across a wider range of applications due to their sensitivity, broad linearity, and robustness to library compounds and complex biological samples. This trend has been fostered by the development of several new assay designs for diverse targets such as kinases, cytochrome p450s, proteases, apoptosis, and cytotoxicity. This review addresses recent progress made in the use of bioluminescent assays for HTS, highlighting new detection capabilities brought about by engineering luciferase genes, enzymes, and substrates. In genetic reporter applications, modifications to the luciferase genes have improved assay sensitivity by substantially increasing expression efficiency and enhanced response dynamics by reducing expression lifetime. The performance of assays based on detection of ATP and luciferin has been enhanced by modifications to the luciferase enzyme that increase its chemical and physical stability. Detection of ATP allows rapid analysis of cell metabolism and enzymatic processes coupled to ATP metabolism. Because luciferins are not naturally associated with mammalian physiology, assays for luciferin detection utilize synthetic derivatives designed to yield luminescence only when coupled with specific target enzymes. Finally, new methods for modulating the specific activity of luciferases are leading to the development of intracellular biosensors for dynamic detection of physiological processes.

Introduction

HTS OF COMPOUND LIBRARIES against pharmacological targets is a key strategy in contemporary drug discovery. To generate meaningful data with the required throughput, high-quality assay methods are needed for translating specific biomolecular phenomena into observable parameters. Typically this is achieved through measurements of radioactivity, photon absorption, or photon emission. Although the scintillation proximity assay and other radioactive methods have been successfully applied in HTS, they have become less popular primarily because of the burden of handling radioactive materials and the availability of nonradioactive alternatives. Assays based on photon absorption are commonly used, but they impose limits on assay design and miniaturization since they typically provide low sensitivity. Photon emission is by far the dominant assay methodology due to its broad adaptability to biological targets and automation methods, and its ability to deliver the speed, accuracy, and sensitivity necessary for successful HTS campaigns.[1]

Photon emission is achieved primarily through fluorescence and chemiluminescence (which includes bioluminescence). Although both processes create photons through energy transitions from excited states to their cor-

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ABBREVIATIONS: cAMP, cyclic adenosine monophosphate; CRE, cyclic adenosine monophosphate response element; GPCR, G-protein-coupled receptor; HEK293, human embryonic kidney 293; NFAT, nuclear factor of activated T cells.

responding ground states, they differ in how the excited states are generated. In fluorescence, the energy needed for producing excited states is gained through the absorption of light, whereas in chemiluminescence, the energy results from exothermic chemical reactions (Fig. 1). This difference fundamentally affects assay characteristics and performance. Fluorescent assays tend to be much brighter since the photons generating the excited states can be introduced into a sample at a very high rate. However, this high influx of photons also leads to higher background, which may influence assay sensitivity accordingly. In contrast, although chemiluminescent assays typically yield lower light intensities because of the slower rate by which excited states can be created, assay backgrounds are virtually absent since no photons need to be introduced into the samples. This relationship between brightness and background has significant consequences for their suitability in HTS assays.

In cellular microscopy, optics requirements for resolving subcellular structures impose restrictions on the efficiency of light collection. A similar situation applies for cell analysis by flow cytometry. Under these circumstances, because limits on photon detection are dictated by the instrumentation, brightness becomes the dominant consideration for good performance. Consequently, fluorescence is almost universally preferred over chemiluminescence. However, because more photons can be collected from macroscopic samples, reducing background associated with the assay method becomes more important. This is the case for assays performed in multiwell plates, where chemiluminescent assays often outperform analogous fluorescent assays.[2,3] The low background inherent in chemiluminescence allows for a better signal-to-noise ratio and thus better assay sensitivity. Such macroscopic measurements are the foundation for most HTS methods, which rely heavily on the use of multiwell plates to rapidly measure a single parameter across a large number of samples. Furthermore, fluorescent assays can also be affected by the presence of interfering fluorophores within the biological samples or in the library compounds, whereas chemiluminescence is less hindered.

Although assays based on either fluorescence or chemiluminescence can yield high sample throughput, fluorescence has been used more commonly because of its broader familiarity and easier implementation into new assay designs. The main obstacle to the use of chemiluminescence in HTS has been the lack of available assay methods. But, new capabilities in chemiluminescence, particularly in bioluminescence, are now changing this. This review will focus on the expanding scope of bioluminescence technologies available for HTS and provide some perspectives on future developments.

Bioluminescence

Bioluminescence is a special form of chemiluminescence found in living organisms. As a class, it is characterized as being an enzyme-catalyzed process, where the high efficiency of photon emission is derived through natural evolution. The enzymes are called luciferases, and the photon-emitting substrates are luciferins. Bioluminescent chemistries have evolved from multiple independent origins, and thus comprise many distinct molecular structures. Of the many natural forms, only three have been widely used in HTS: firefly luciferase, *Renilla* luciferase, and aequorin.

Firefly (*Photinus pyralis*) luciferase is a monomeric enzyme of 61 kDa that requires no posttranslational modifications for activity.[4] It acts by first combining beetle luciferin with ATP, to form luciferyl-AMP as an enzyme-bound intermediate. This intermediate reacts with O_2 to create another bound intermediate, oxyluciferin, in a high-energy state. The subsequent energy transition to the ground state yields yellow-green light with a spectral maximum of 560 nm (Fig. 2).

Renilla luciferase from the sea pansy *Renilla reniformis* is a 36-kDa monomeric enzyme that catalyzes the oxidation of coelenterazine to yield coelenteramide and blue light with a spectral maximum of 480 nm.[5] It has been used primarily as a co-reporter in conjunction with firefly luciferase. For HTS, it is used in a dual luciferase format to provide an internal control for identifying aber-

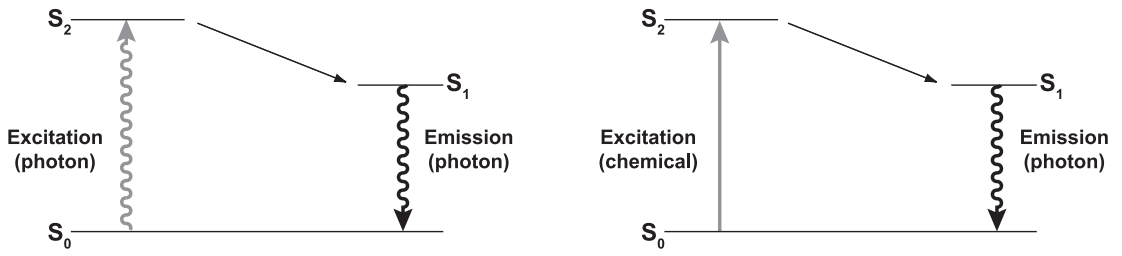

FIG. 1. Comparison of fluorescence (**left**) to luminescence (**right**). S_0, ground state; S_1, excited state after vibrational relaxation; S_2, excited state.

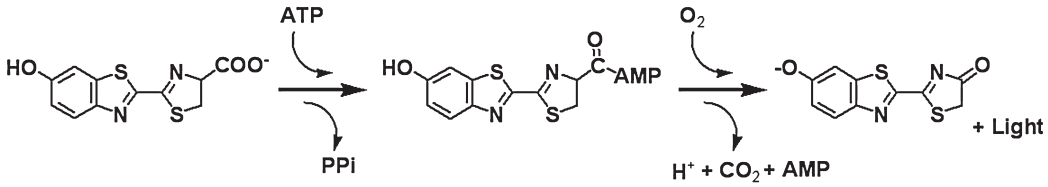

FIG. 2. Chemical reaction catalyzed by firefly luciferase. PPi, inorganic pyrophosphate.

rant sample data, or to multiplex with firefly luciferase to simultaneously monitor two distinct cellular pathways.[6–8]

Aequorin from the jelly fish *Aequorea victoria* is a calcium-sensitive photoprotein and has been used almost exclusively for monitoring intracellular calcium concentrations.[9] Apoaequorin and its cofactor coelenterazine form active aequorin in the presence of molecular oxygen. Upon binding of a calcium ion, aequorin undergoes a conformational change resulting in the oxidation of coelanterazine and emission of a blue light flash. The aequorin assay has been adapted for HTS of G-protein-coupled receptor (GPCR) modulators since receptor activity can often be linked with mobilization of intracellular calcium.[10]

Other luciferases that have been introduced as candidates for genetic reporters include click beetle, *Gaussia*, *Metridia*, and *Vargula* luciferases.[11–14] Despite some of the unique features reported for these luciferases, they are not generally regarded as offering significant advantages over the widely used firefly and *Renilla* luciferases. Since firefly luciferase has been the benchmark and the most versatile luciferase for HTS assays, this review will focus on the range of applications developed from this luminescent chemistry.

As a general approach, light intensity of firefly bioluminescence is correlated to the chemical concentrations of the reaction components. When configured properly, the light intensity can be used to associate an observable parameter with a molecular process. 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 with the process of interest. Depending on the assay design, the variable component may be ATP, luciferin, or the enzyme itself. Examples of bioluminescent assays designed for HTS are shown in Fig. 3. The linear range of these assays can be enormous because of the very low backgrounds in bioluminescence, typically extending four to eight orders of magnitude across the concentration of the variable component. Most recently, new assay formats have been developed based on modulating the specific activity of luciferase through specific molecular interactions directly with the enzyme structure (luciferase biosensors [see last section of this review]).

Assays of Luciferase Concentration (Genetic Reporters)

In HTS applications, luciferase has been best known as a genetic reporter. Many cellular events of relevance to drug discovery can be associated with regulation of gene transcription. By coupling the operative regulatory elements to expression of a luciferase gene, typically by placing the regulatory element just upstream of a gene encoding luciferase, the cellular event can be readily detected by a luminescent signal. Luciferase assays have been successfully developed for numerous GPCRs and nuclear receptors, which represent two major drug target classes.[15–18]

For GPCR assays, the activity of a receptor coupled to Gαs protein is monitored by using a cyclic adenosine monophosphate (cAMP) response element (CRE) positioned upstream of a luciferase gene. Activation of the receptor causes an increase in intracellular cAMP, which in turn activates protein kinase A to phosphorylate CRE binding protein. The luciferase concentration within cells is increased when phosphorylated CRE binding protein is bound to the CRE sequence, causing an increase in the transcription rate of the luciferase gene. Similarly, other GPCRs can be monitored by using response elements for nuclear factor of activated T cells (NFAT), activator protein 1, and serum response element. For nuclear receptor assays, the luciferase reporter can be placed downstream of a promoter containing a hormone response element, or several repeats of a Gal4 binding sequence and a mammalian promoter. Binding of the nuclear receptor in the vicinity of the promoter activates luciferase gene expression. Luciferase reporter assays configured for HTS provide simple and efficient measurements of cellular physiology without sacrificing data quality.

Genetic reporter assays benefit from the signal amplification afforded by intracellular signal transduction and gene transcription. To achieve good data quality and minimize the risk of false screening hits, it is important to control for artifacts that may arise from this biological mechanism. For example, in GPCR assays, reporter expression can be prone to interference from cytotoxic compounds, leading to false-positive hits during antagonist screening. Incorporation of an internal control (*e.g.*, *Re-*

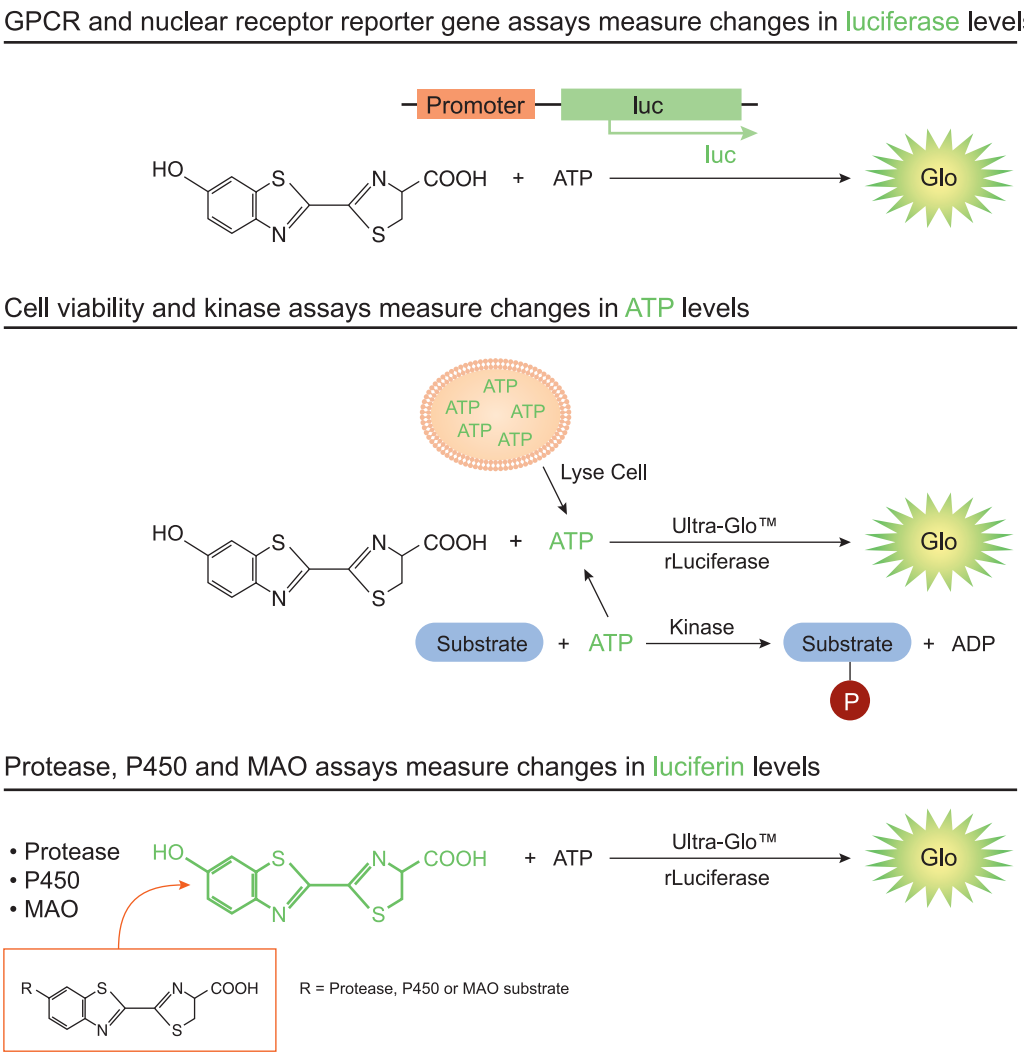

FIG. 3. Bioluminescent assays for HTS. luc, firefly luciferase gene; Ultra-Glo™ rLuciferase, stable recombinant firefly luciferase (Promega, Madison, WI); MAO, monoamine oxidase.

nilla luciferase) can help mitigate these false hits and improve data quality. However, introducing both reporters into the cell on the same plasmid backbone can result in aberrant expression due to cross interference between promoters and response elements (authors' unpublished data).

An alternative strategy for generating dual luciferase assays was developed based on a standard two-plasmid approach (Fig. 4). One plasmid features both a firefly luciferase gene regulated by the response element of interest (*e.g.*, CRE or NFAT) and a hygromycin-selectable marker. The second plasmid expresses the target GPCR

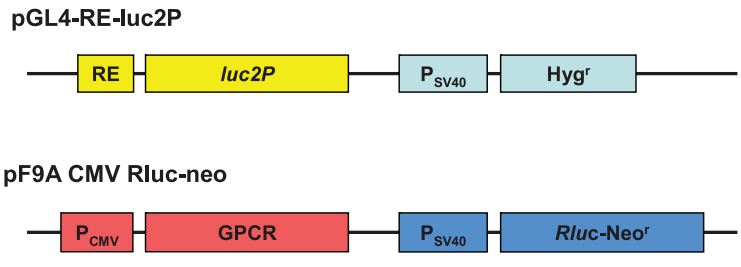

FIG. 4. A diagram of two plasmids involved in the dual-luciferase assay of GPCRs. RE, response element/promoter; *luc2P*, destabilized and optimized firefly luciferase with the -Pro-Glu-Ser-Thr (PEST) sequence; P_{SV40}, simian virus 40 promoter; Hyg^r, hygromycin resistance gene; P_{CMV}, cytomegalovirus promoter; Rluc-Neo^r, *Renilla* luciferase and neomycin resistance gene fusion. PEST sequences are associated with rapidly degraded proteins.

(*e.g.*, muscarinic receptor) and a *Renilla* luciferase-neomycin–selectable marker fusion protein. HTS assays can be configured through either stable or transient transfection of the above plasmids. A dual-luciferase reagent can then be used to measure signals of both firefly and *Renilla* luciferases in the same sample. By this method, potentially problematic samples can be readily identified by resultant deviations in the *Renilla* luciferase internal control signal (Fig. 5). For example, a decrease of *Renilla* luciferase signal is often associated with compound toxicity. Ratiometric normalization of the two signals also reduces sample variation to further improve data precision (*i.e.*, higher *Z* values).

Reporter genes are typically used to monitor changes in transcriptional rate, which can vary quickly in response to a specific cellular event. Yet, because 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. Therefore, the resultant luminescent signal represents the cumulative, or average, transcription rate over the lifetime of all reporter molecules in the population. This "time averaging" of the transcriptional rate tends to dampen the apparent responsiveness of the reporter to rapidly changing conditions. Therefore, proteins such as green fluorescent protein that have a long maturation time and lifetime in cells are not well suited for use as genetic reporters. Responsiveness can be improved by reducing the lifetime of the reporter within the cell, and thus effectively reducing the time averaging window. This can be achieved by adding protein 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 agonists or antagonists on cellular receptors (Fig. 6). Deployment of such destabilized genes in HTS can lead to increased response dynamics, reduced assay time, and minimized secondary effects that may arise from the prolonged incubation of cells with screening compounds.

The trend in HTS towards miniaturation to reduce consumption of costly screening compounds by using high-density multiwell plates (*e.g.*, 1,536- or 3,456-well format) also demands adoption of highly sensitive assay methods. In principle, the sensitivity of reporter assays could be improved by either increasing the expression efficiency of the reporter gene or enhancing performance of the assay reagent. The native firefly and *Renilla* luciferase genes have undergone multiple rounds of sequence optimization, resulting in improved versions having much higher expression levels. In some instances, expression has been increased by several hundred-fold in mammalian cells. These optimized genes are particularly useful for reporters designed to respond rapidly to transcriptional dynamics. Reducing the lifetime of a reporter also reduces its intracellular accumulation, thereby diminishing the net signal generated in a sample. Luciferase reporters are well suited for this strategy because of

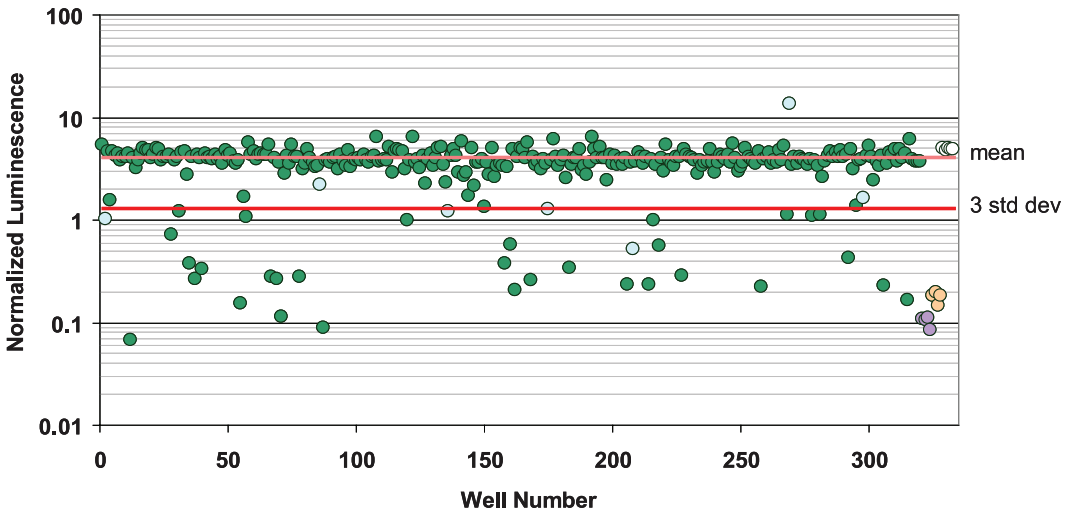

FIG. 5. Multiplexing with internal control reporter improves HTS quality. The library of pharmacologically active compounds (LOPAC) was screened for dopamine receptor D1 (Drd1) antagonists using double stable HEK293 cells expressing CRE-luc2P and Drd1. The Drd1 agonist, dopamine, was added at a concentration of 5 μM followed by each LOPAC compound at 10 μM. Data are displayed as the ratio of firefly luminescence divided by *Renilla* luminescence. Points below the mean − 3 standard deviation (std dev) are considered hits. Points marked by ● indicate samples with abnormal *Renilla* luminescence and therefore potential false hits; ○, controls with only dopamine; ●, controls with dopamine plus a known antagonist, SCH23390; ●, controls without any compounds.

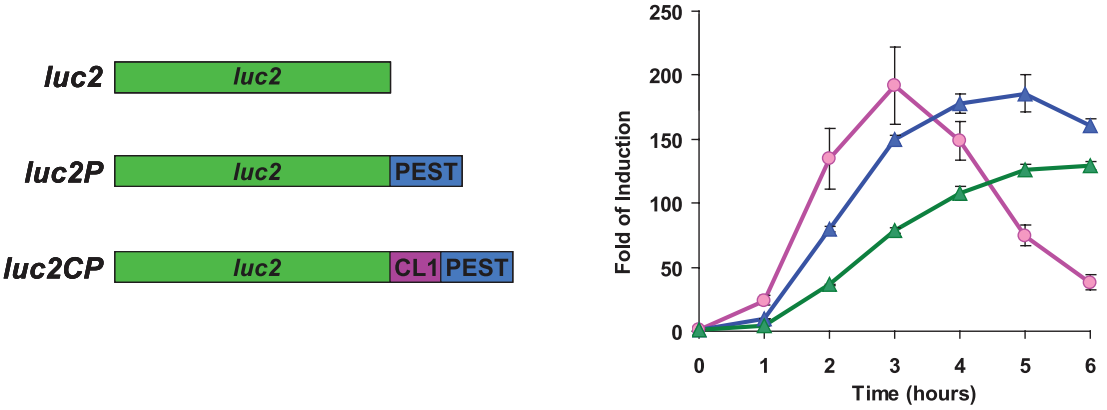

FIG. 6. Destabilized luciferases increase the response and reduce the time to maximum induction: *luc2*, optimized firefly luciferase (green line); *luc2P*, firefly luciferase with -Pro-Glu-Ser-Thr (PEST) sequence (blue line); *lucCP*, firefly luciferase with CL1 and PEST sequences (purple line). These luciferases were used to monitor the CRE response. An HEK293 cell line expressing endogenous β2-adrenergic receptor and stably transfected with a CRE-luc construct was induced with 1 μM isoproterenol/100 μM Ro-20-1724, and samples were harvested every hour for quantifying luminescence.

their inherently high assay sensitivity, which provides sufficient detection capacity to easily tolerate this loss in signal. Nonetheless, the expression-optimized luciferase genes further enhance the utility of this approach.

Achieving greater light intensity from the assay reagent is made difficult because of an inherent trade-off with the stability of the luminescent signal. The luminescent reaction causes an apparently unavoidable inactivation of the enzyme, possibly due to the formation of damaging free radicals. Since the luminescent decay is coupled to catalysis, it can be reduced by similarly reducing catalytic turnover. Thus, to maintain a steady luminescent signal over an extended period of time, ranging from minutes to hours, it is often necessary to inhibit the luminescent reaction to various degrees. This decreases the rate of decay to where it will not significantly diminish the level of luminescence over the time required for measuring multiple samples. Moreover, even under these reduced kinetic conditions, the luciferase may be quantified down to 10^{-18} moles per sample, which corresponds to approximately 10 molecules per cell.

Although most cell-based HTS campaigns have been conducted using standard mammalian cell lines (*e.g.*, human embryonic kidney 293 [HEK293] or Chinese hamster ovary [CHO]) it may often be desirable to use more biologically relevant samples such as primary cells. Luciferase reporter genes can be readily delivered to a variety of cells using approaches such as viral vectors, chemical transfection, or electroporation transfection.[19,20] Their applications for HTS are expected to grow in the future.

Assays of ATP Concentration

Firefly luciferase has long been known for its utility in measuring ATP, especially as a method for detecting microbial cells. As a component of the reagent composition, the firefly luciferase must provide consistent catalytic activity to enable reliable performance.[21] However, low stability of enzymes purified from native sources had limited its applicability for HTS. To resolve this problem, a highly stabilized form of luciferase was created using optimization strategies such as directed evolution.[22] The resulting modified luciferase is resistant to a broad spectrum of detergents and ATPase inhibitors. This stabilized luciferase enables the configuration of reagent formulations having simple "Add & Read" formats analogous to the luciferase reporter assays.[23,24]

In HTS applications, bioluminescent ATP assays have been most widely used to screen for cytotoxicity in compound libraries.[23,25] ATP serves as a good indicator of cell viability because it is the major conduit for metabolic energy. ATP concentrations are homeostatically maintained through a balance of generative and consumptive pathways. The generation pathways require the coordinated interaction of many enzymes through a complex intracellular architecture, which are readily disrupted upon cell death. But, cellular ATPases of the consumption pathways may act independently and thus continue to consume available ATP even after cell death. Accordingly, the concentration of intracellular ATP drops instantaneously upon cell death with a corresponding loss of luminescence when assayed using luciferase.

An optimally designed reagent should integrate three essential capabilities into the formulation: rapid and efficient ATP extraction from the mammalian cells, inhibition of the endogenous intracellular ATPases, and generation of a stable luminescent signal. The data from these assays correlate well with those from assays utilizing tetrazolium dyes, which are commonly used to indicate cellular metabolism through intracellular redox activity.

However, the bioluminescent assays are about 100-fold more sensitive, being able to detect fewer than 10 cells per sample. Furthermore, these assays are also much faster, requiring only about 5 min compared to several hours necessary to detect conversion of the tetrazolium dyes.[26,27]

Bioluminescent ATP assays can also be used for quantifying the activity of enzymes that either consume or generate ATP. Assays for kinase activity are of primary interest to drug discovery.[28–30] Most kinase assays are designed to detect 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 consume ATP in the phosphorylation reaction, a single assay method for detecting ATP depletion 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 directed toward substrates other than peptides, for example, saccharide or lipid kinases. However, the universality of this method requires that the kinase activity be free of other potential ATPases since the assay cannot selectively differentiate the source of the ATP consumption. Thus, ATP depletion is not suitable for assaying specific kinase activities within a cell lysate, unless these activities dominate over the other ATPase activities found in the cells.

Based on the same principle, bioluminescent assays can be developed for other enzymes, small molecules or cellular processes that can be linked to ATP generation or consumption, for example, an assay for cAMP that was linked through the allosteric activation of protein kinase A.[31]

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. Despite its relatively simple chemical structure, beetle luciferin is biochemically unique among living systems and is 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 that cannot support luminescence directly when combined with luciferase, but that can be converted into luciferin through catalytic processing by a second enzyme. By this means, the luminescent signal of an assay becomes dependent on this second enzyme.

The pro-luciferin approach has been useful for developing a series of sensitive assays for cytochrome p450 activities.[3] Cytochrome p450s are important in drug discovery as they play an essential 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 reactions, with different isozymes having catalytic specificities toward different molecular structures. A common method for rapidly assessing their activities is through the use fluorogenic molecules that become fluorescent upon reaction with a cytochrome p450. An analogous strategy can be applied to luciferins that have been modified to contain a cleavable group, such as 6′-*O*-methyl. Upon incubation with specific cytochrome p450s, 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 typically found to be 10–100-fold more sensitive than the comparable fluorogenic assays. As the demands increase for early assessments of toxicology profiles for lead compounds, these HTS-compatible p450 assays provide additional tools for drug discovery.

It is often feasible to combine the pro-luciferin with all 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 and caspase-7 (Fig. 7).[32] Although the cleavage reaction yields aminoluciferin, this luciferin analog also serves as a luminogenic substrate of luciferase. When DEVD-6′-aminoluciferin is added to a sample containing caspase-3 or caspase-7, 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 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. In contrast, fluorogenic assays are based on the accumulation of fluorophore over an extended period of incubation. Because the coupled luminescent assay represents only an instant in time, whereas the fluorogenic assay draws on an extended in-

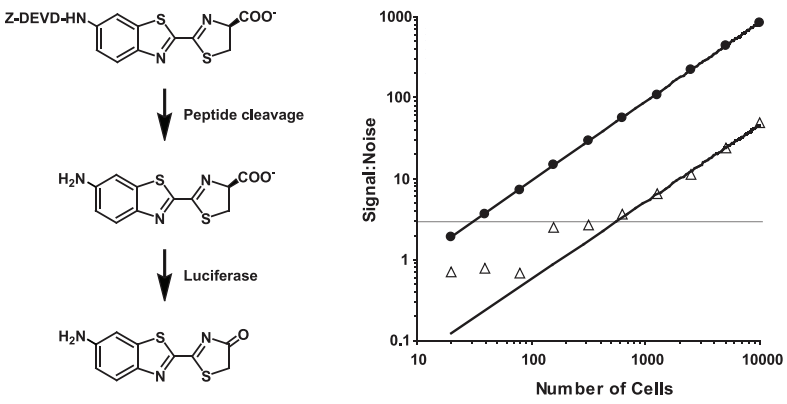

FIG. 7. A comparison of bioluminescent and fluorescent measurements of caspase-3/7 activity: Z-DEVD-aminoluciferin (●); Z-DEVD-rhodamine110 (△). The assays were run simultaneously using the same anti-FAS-treated Jurkat cells serially diluted in 96-well plates. After addition of the substrates, readings were taken at 1 h. Results were plotted as Signal:Noise (mean signal − mean background/standard deviation of background). Background signals were determined from wells containing culture medium without cells. The limit of detection is defined as the number of cells giving a Signal:Noise ratio ≥3 (black horizontal line). Reprinted with permission from O'Brien *et al*.[32]

cubation, it would be reasonable to assume that the fluorescent methods would be more sensitive. As already noted, however, this is typically not true because of the very low background of the luminescent method. In the case of caspase-3/7, a highly sensitive fluorogenic assay based on cleavage of $DEVD_2$-rhodamine is available. 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 (Fig. 7).[32]

Similar assays have been developed for a number of proteases including caspase-8, caspase-9, dipeptidyl peptidase IV, calpain, and proteasome. It is worth noting that some of these reagents were used directly on zebrafish embryos in 96-well microplates to monitor caspase activities in accessing the effects of radiation.[33]

Based on the same principle, bioluminescent assays can be developed for other enzymes, small molecules, or cellular processes that can be linked to the generation of substrates for luciferase. More examples include assays for other drug metabolism enzymes such as monoamine oxidase and glutathione *S*-transferase.[34–36]

Assays of Luciferase Activity (Biosensors)

In contrast to ATP and luciferin, luciferase can be linked to light output not only through concentration, but also through modulation of its enzymatic activity. This could provide the basis for a new approach to bioluminescent assays developed for a range of molecular interactions or cellular processes. Early attempts yielded relatively limited performance unsuitable for HTS applications. However, recent progress in protein engineering of luciferases has allowed for novel sensing capabilities to be configured. Because of their potential sensitivity and dynamic range, these new luciferase biosensors may

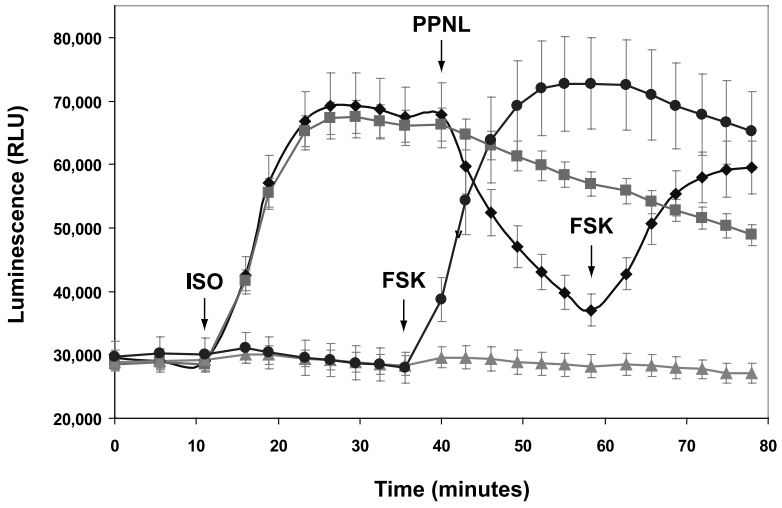

FIG. 8. Real-time monitoring of intracellular cAMP using a luciferase biosensor. HEK293 cells transiently expressing a chimeric protein of a circularly permuted luciferase and a cAMP-binding domain from protein kinase A were mixed with 5 m*M* luciferin and incubated at 37°C for 2 h. Following incubation, cells were aliquoted to a 96-well plate (1×10^5 cells per well) and equilibrated at room temperature for 0.5 h. Isoproterenol (ISO; 10 μM), propranolol (PPNL; 10 μM), or forskolin (FSK; 10 μM) was added at the time points indicated by the arrows. Luminescence was measured in relative light units (RLU) from living cells using a GloMax™ Luminometer (Promega) (24 replicates per condition). ISO and PPNL are agonist and antagonist, respectively, to the endogenous β2-adrenergic receptor. FSK is an activator of adenylate cyclase, which increases cellular cAMP levels.

prove to be more amenable to HTS methods than their analogous counterparts using fluorescent resonance energy transfer technologies.

In one example, a cAMP sensor was developed by incorporating a cAMP-binding domain from protein kinase A into a circularly permuted firefly luciferase. The resulting chimeric protein functions as an intracellular biosensor for cAMP when expressed in mammalian cells (Fig. 8). Treating the cells with forskolin, an activator for adenylate cyclase, showed a titratable increase of the luminescent signal with an compound concentration where 50% effectiveness (EC50) is seen of 0.35 μM. At maximum induction the signal was sixfold over background. The intracellular luminescence was measurable with good precision in a 384-well format, as evidenced by a Z' value of 0.8. More research will be necessary to further characterize and optimize the capabilities of this technology for HTS.

Conclusions

To date, the dominant methodology for developing assays in the drug discovery field has been fluorescence. Yet, fluorescence does not necessarily yield the best performance and typically has lower sensitivity than chemiluminescence. On the other hand, chemiluminescent assays tend to be susceptible to environmental conditions, and thus their robustness in complex systems can be limited. An exception to this has been bioluminescence, where photon emission is located within the well-protected environment of the enzymatic active site. As a consequence, these enzymes can yield efficient and reliable signals under biologically relevant conditions. The benefits of bioluminescent enzymes as genetic reporters have been widely recognized for many years. Recent advances have yielded further improvements to this technology, such as increased expression levels and response dynamics to cellular events. Yet, these enhancements have only recently been extended to other types of biomolecular assays. 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 robust and highly sensitive and provide broad linearity. It is expected that as more assay designs become available, bioluminescence will increasingly become a method of choice for HTS.

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