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Drug Discovery Assay Tutorial

Live-Cell Luminescent Assays for GPCR Studies

Combination of Sensitive Detection and Real-Time Analysis Expands Applications

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GPCRs represent a major class of drug targets, and the prevalence of these receptors in physiologically important signaling events is well known. Although a number of assay formats already exist for monitoring GPCR function, the development of new technologies that enable increased sensitivity and novel experimental readouts will undoubtedly advance efforts both in academic research and drug discovery.

Promega (www.promega.com) recently developed a biosensor technology platform that enables the intracellular detection of key signaling events. These biosensors are made via fusion of polypeptides or protein domains to mutant forms of luciferase. Once expressed, these sensors detect changes in the concentration of important second messengers or post-translational modifications using a live-cell, nonlytic assay format. The ability to sensitively monitor these changes in real time is a hallmark of the technology.

The GloSensor cAMP Assay is the first commercial application of this technology, where the biosensor consists of a fusion of a cAMP binding domain to a circularly permuted form of firefly luciferase (*Figure 1A*). Upon binding to cAMP, conformational changes in the expressed sensor lead to large increases in light output in the presence of substrate (*Figure 1B*).

The GloSensor cAMP Assay is a sensitive and easy-to-use format for the interrogation of Gs- or Gi-coupled 7-TM receptors. Following preequilibration with substrate, biosensor expressing cells are mixed with compounds, and luminescence is measured using either a kinetic or end-point format. For example, HEK293 cells transiently

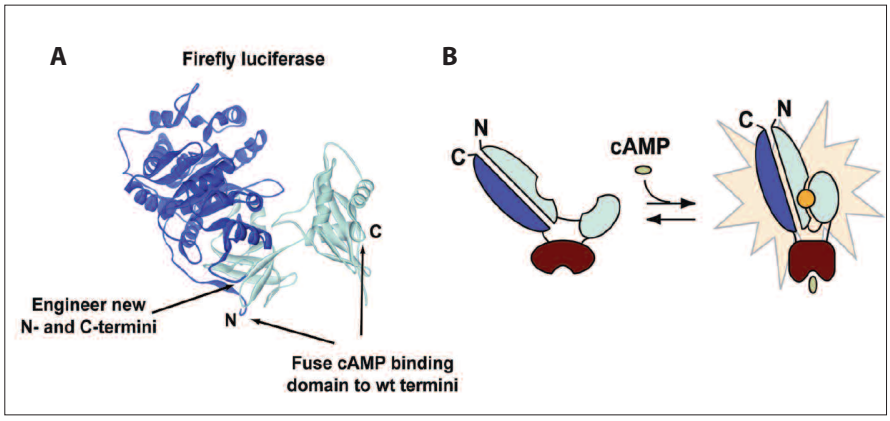

Figure 1. Overview of the GloSensor cAMP Assay: (A) Schematic representation of the biosensor used in the GloSensor cAMP Assay. (B) Protocol for use of the assay in cells-to-compounds or compounds-to-cells format.

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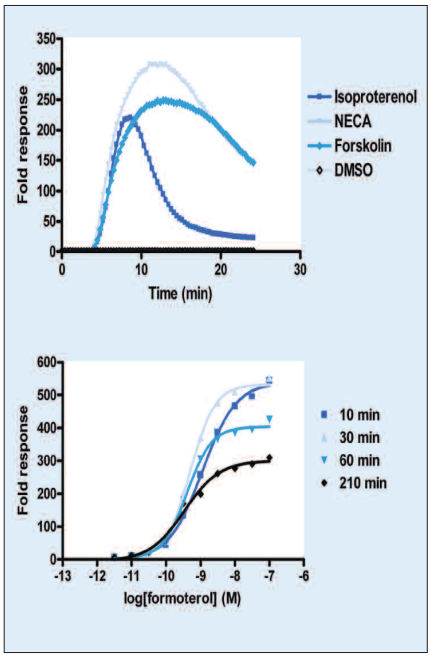

Figure 2. Transient expression of an improved biosensor variant in HEK293 cells and activation of endogenous Gs-coupled receptors: (A) Kinetic measurements at 28°C. (B) Concentration response curves at varying time points following agonist addition for an assay done in the presence of 500 µM IBMX. Isoproterenol and formoterol, β2-adrenergic receptor agonists; NECA, adenosine A2B receptor agonist; forskolin, cell-permeable activator of endogenous adenylate cyclase. n = 1 per dose/trace.

expressing an improved variant of the biosensor were treated with agonists to the endogenous β2-adrenergic or adenosine A2B receptors, and luminescence was monitored continuously for 25 minutes (*Figure 2A*).

The kinetic traces of each response show a rapid initial increase in luminescence that either remains relatively stable or decreases owing to apparent receptor desensitization, with maximal fold increases in light output in excess of 200- or 300-fold within four to eight minutes of compound addition, respectively.

This represents an improved response window over our initial version (approximate 10-fold maximal response under similar conditions), and it demonstrates the useful application of directed evolution toward enhancing the performance of this type of engineered construct.

In related experiments, increases in light output in excess of 500-fold have been detected in the presence of a phosphodiesterase inhibitor, where inclusion

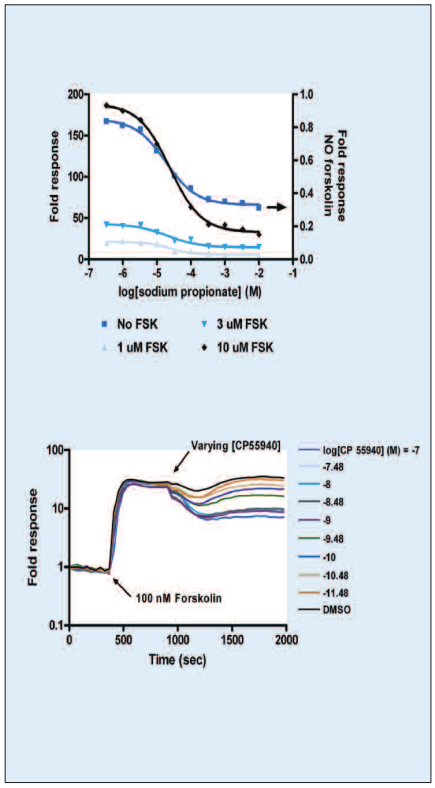

Figure 3. Transient expression of an improved biosensor variant and use in Gi-coupled receptor assays: (A) HEK293T cells stably expressing the FFA3/GPR41 receptor were treated with varying concentrations of agonist followed by 0, 1, 3, or 10 µM forskolin. (B) HEK293T cells stably expressing the cannabinoid CB1 receptor were treated with 100 nM forskolin followed by varying concentrations of CP55940 agonist at 28°C. Cell lines were obtained from Multispan.

of this type of inhibitor can both increase the magnitude of the response and stabilize signals for various applications, including high-throughput screening (*Figure 2B*).

The sensitivity of the assay is also evident when looking at the response of Gi-coupled receptors. For example, HEK293T cells stably expressing the FFA3/GPR41 receptor (**Multispan**) were treated with varying concentrations of sodium propionate agonist for five minutes followed by the addition of fixed concentrations of forskolin.

As shown in *Figure 3A*, similar signal-to-background ratio and EC_{50} values are seen in the presence or absence of forskolin, with accurate EC_{50} determination even in the absence of replicate measurements. Indeed, in separate experiments on HEK293T cells stably expressing the DP2/GPR44 receptor (Multispan), we have obtained Z´ values ≥0.8 in the presence or absence of forskolin following treatment with saturating concentrations of PGD2 agonist.

In a similar fashion, the assay is sensitive enough to monitor the activity of inverse agonists of overexpressed Gs- and Gi-coupled receptors in the absence of additional compounds such as forskolin. It is worth noting that kinetic measurements can also be performed on Gi-coupled receptors, where the activity of individual compounds can be measured in series.

For example, HEK293T cells stably

Advantages of the GloSensor cAMP Assay for High-Throughput Screening and Compound Profiling

- Extremely sensitive—Interrogation of endogenous Gs- and Gi-coupled receptors, direct detection of inverse agonists
- Huge signal-to-background ratio, up to 500-fold increases in light output
- Easily miniaturized assay format, including uHTS
- Low cost in a variety of formats (1-100 µL final assay volume)
- Glow-type bioluminescent readout; no fluorescence-based interference issues
- Enables combination screens for agonists, allosteric modulators and/or antagonists
- Frozen-cell compatible
- Single-cell imaging using luminescence microscopy; whole-animal imaging studies in progress

expressing the cannabinoid CB1 receptor (Multispan) were first treated with 100 nM forskolin, and, following attainment of a stable signal, the cells were treated with varying concentrations of the agonist CP55940 (*Figure 3B*). This type of ordered addition of test compounds can facilitate the study of allosteric modulators of GPCR function, where the activity of each compound can be tested in series.

The sensitivity of the system is again evident in assays performed on both endogenous Gs- and Gi-coupled receptors. As mentioned above, increases in light output in excess of 100-fold are common following activation of endogenous Gs-coupled receptors when assayed using the improved variant.

In several cases, the biosensor assay was able to detect activation of endogenous Gs-coupled receptors where published reports using label-free systems have apparently failed. In addition to stimulatory responses, the assay is sensitive enough to detect activation of endogenous Gi-coupled receptors as well.

The unique characteristics of the assay are particularly well suited for high-throughput screening and drug discovery. For example, the assay's sensitivity facilitates interrogation of endogenous receptors, where there is a growing trend toward use of this type of model system, and it may enable primary screens for inverse agonists.

In addition, the response window of the assay can exceed those of antibody-based approaches that utilize time-resolved fluorescence, especially for Gs-coupled receptors, which may facilitate identification of weak hits and novel lead compounds. Moreover, the luminescent readout avoids problems associated with fluorescence interference of select library compounds, which is still a problem with TRF assays. Additional advantages of the system for high-throughput screening and compound profiling are listed in the *Table*.

In summary, luminescent biosensors offer a unique insight into cellular function, where the combination of sensitive detection and real-time analysis enables novel approaches in both academic research and drug discovery. The first member of the GloSensor platform, a new line of genetically encoded luminescent biosensors that can be used to monitor signal transduction within the context of living cells, is now available. GEN