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Analysis of G_i-Coupled Receptors with BD™ ACT*One* Technology

Jianming Lu, Isabel Llorente, Yong Yao, Paul Lee and Xiao Li
BD Biosciences, Bioimaging Systems, 15010 Broschart Road, Rockville, MD 20850 USA

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Abstract

BD™ ACTOne is the only real-time high-throughput G-protein coupled receptor (GPCR) screening technology that can directly measure the intracellular concentration of the secondary messenger cyclic AMP (cAMP) in living cells. It uses a proprietary modified cyclic nucleotide-gated (CNG) channel—which colocalizes with adenylate cyclase at the plasma membrane—as a biosensor of cAMP activity. Using ACT*One*, we are able to detect the subcellular cAMP concentration changes directly caused by GPCR activation. Real-time kinetic readouts minimize artifacts and provide greater content and more statistically relevant data.

High-throughput screening has proved more difficult for G_i-coupled receptors than G_s- and G_q-coupled receptors. We have developed a homogenous assay using ACT*One* technology to monitor the activities of G_i-coupled receptors in living cells. The assay is capable of detecting both agonist and antagonist activities in either a kinetic or end-point mode, and the large assay window is very amenable to high-throughput screening.

Introduction

G-protein coupled receptors (GPCRs) are known to play a crucial role in the development and progression of major diseases such as cardiovascular, respiratory, gastrointestinal, neurological, psychiatric, metabolic, and endocrinological disorders. Approximately 70% of GPCRs signal through the cAMP pathway. Stimulation of G_s-coupled receptors activates plasma membrane-bound adenylate cyclases that synthesize cAMP, while stimulation of G_i-coupled receptors inhibits adenylate cyclases. Screening G_i-coupled receptor targets by measuring down-regulation of cAMP has been severely limited due to the lack of an accurate and sensitive technology for high-throughput screening (HTS). The BD™ ACT*One* cAMP assay provides information on real-time intracellular cAMP changes and is highly sensitive. The assay is performed using cell lines that express an exogenous cyclic nucleotide-gated (CNG) channel (Figure 1). The CNG channel colocalizes with adenylate cyclases on the plasma membrane and opens when the cAMP level near the plasma membrane increases, resulting in ion flux and cell membrane depolarization. The influx of cations through the CNG channel can be quantified using fluorescent ion indicators or membrane potential (MP) dyes. The assay can be kinetic, using a BD™ Pathway HT Imager or a Fluorometric Imaging Plate Reader system (FLIPR) able to detect very small amounts of material or end-point, using a high-content analysis instrument such as the Pathway HT Imager. These are simple, homogenous assays with only one or two steps (Figure 2).

Conclusion

1. The BD™ ACT*One* cAMP assay is a real-time cAMP assay.
2. The ACT*One* assay can be performed homogenously on living cells.
3. The assay is very sensitive and precise.
4. It provides either kinetic or end-point data.
5. It detects agonists and antagonists for both G_s- and G_i-GPCRs.

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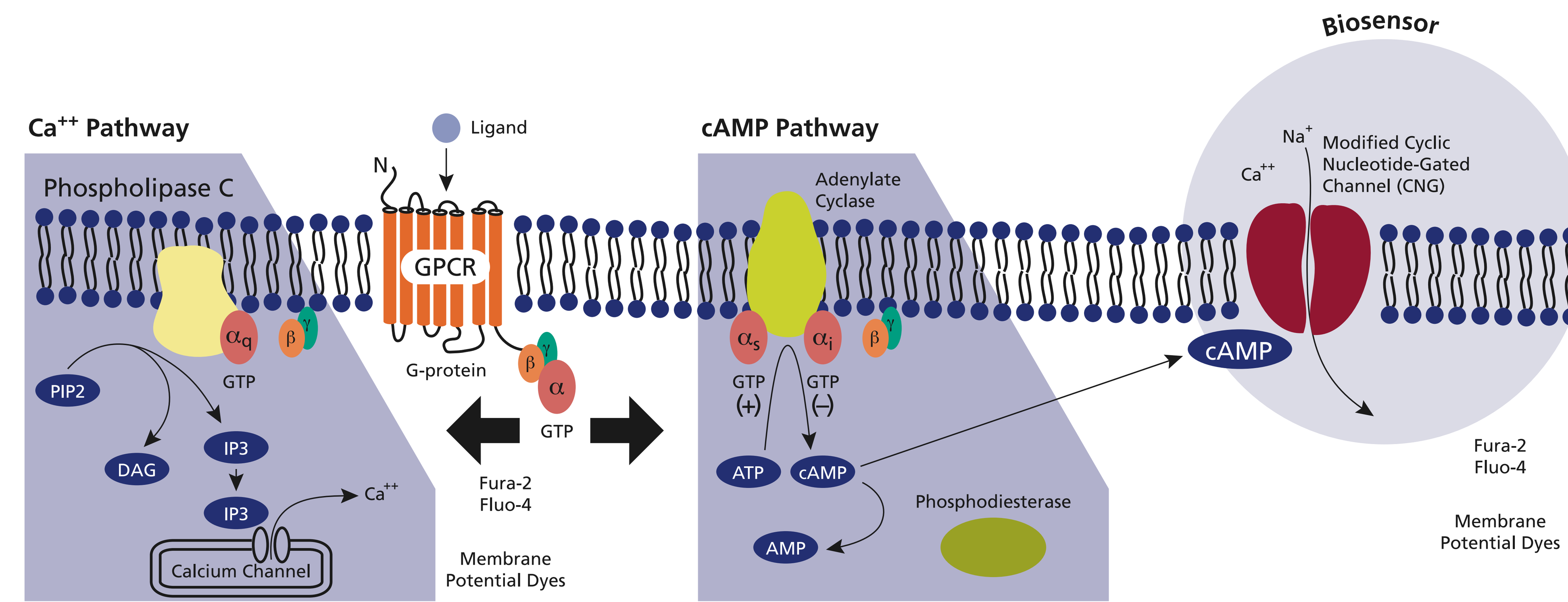
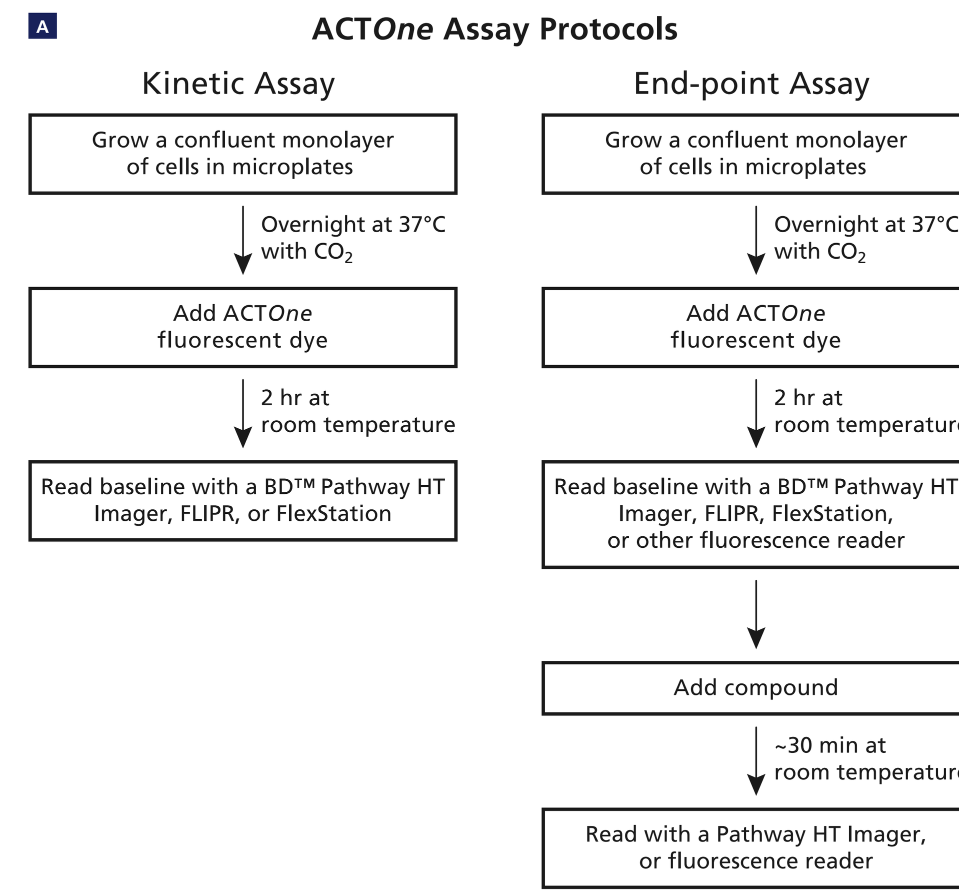
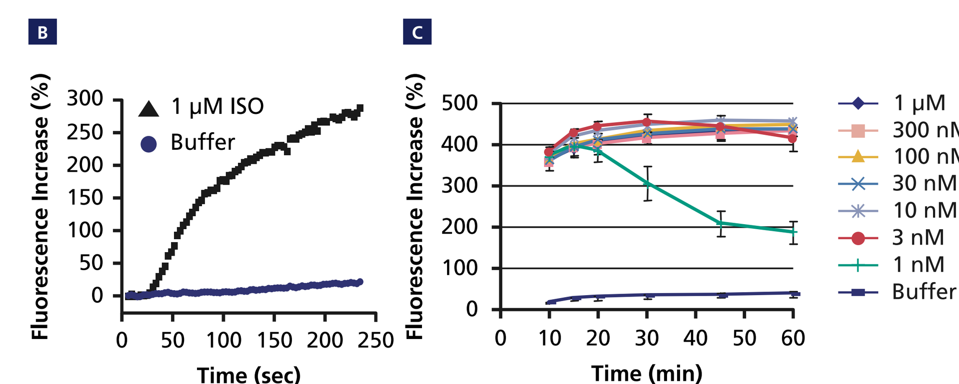
1 Overview of BD™ ACT*One* technology



BD ACT*One* technology uses a modified cyclic nucleotide-gated channel (CNG) selective for cAMP as a biosensor to monitor local intracellular cAMP concentrations. The CNG biosensor enables a physiological cAMP assay in living cells by signaling through calcium or membrane potential responsive fluorescent dyes to measure cAMP levels generated upon activation of adenylate cyclase.

2 The fast response and stable signal of BD™ ACT*One* technology enables both kinetic and end-point assays





Panel A. The BD ACT*One* kinetic and end-point assays are simple, homogeneous one- and two-step protocols utilizing a BD ACT*One* fluorescent dye. **Panel B.** In a BD ACT*One* kinetic assay, HEK 293H cells containing β2-adrenergic receptor, a G_s-coupled GPCR, were stimulated with 1 µM isoproterenol (ISO). The resulting increase in the intracellular cAMP level was detected within several seconds. **Panel C.** The signal from a BD ACT*One* end-point assay of HEK 293H cells stimulated with varying concentrations of ISO lasted for more than one hour.

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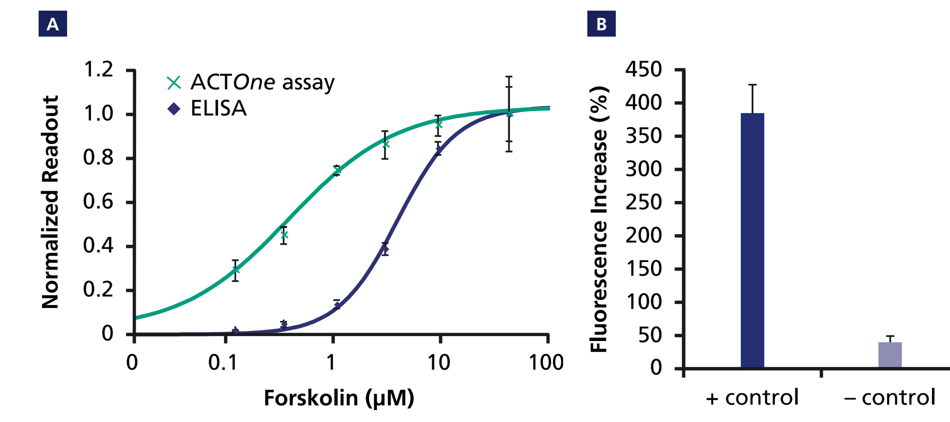
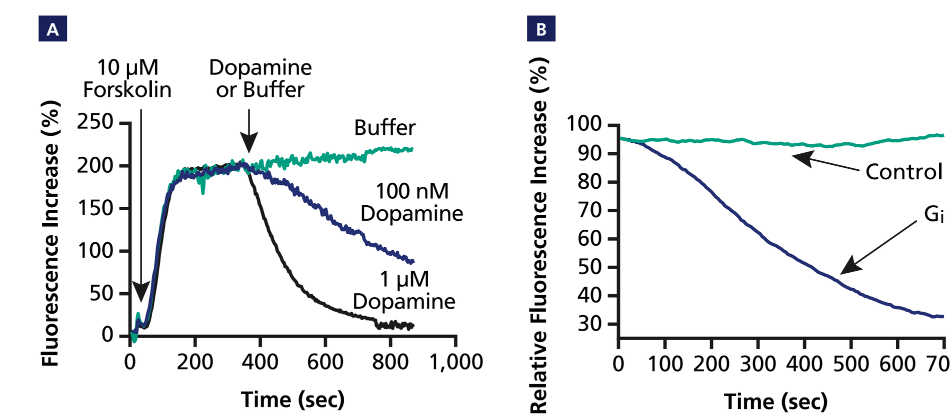
3 The BD™ ACT*One* assay has an excellent sensitivity profile and a high signal-to-noise ratio



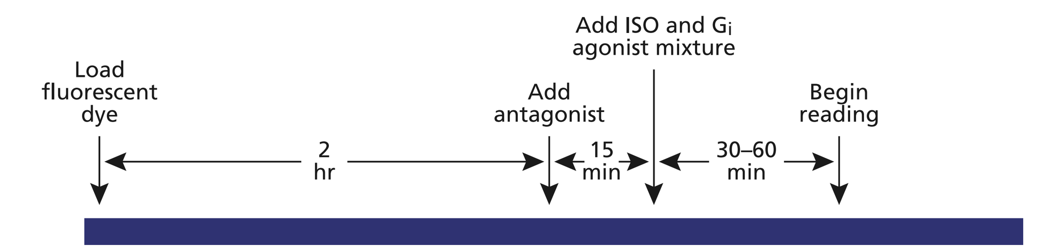
Panel A. In HEK 293H cells stimulated with forskolin, an end-point assay using BD ACT*One* technology was compared with an ELISA assay. A low concentration of forskolin induced a significantly larger readout in the BD ACT*One* assay. The EC_{50} value for forskolin was 0.4 µM using the BD ACT*One* assay, compared to 4.0 µM for the ELISA assay. **Panel B.** End-point assays were performed on HEK 293H cells stably expressing CNG channels. The data were collected from approximately 300 plates assayed on different days. The positive control contained 10 µM NECA, an adenosine A2b receptor agonist, and the negative control contained buffer only. The data displays a high signal-to-noise ratio, with fluorescence values of 385 ± 33 RFU for the positive control and 40 ± 7 RFU for the negative control (CV = 7% and Z' ≥ 0.65).

4 BD™ ACT*One* kinetic assays of G_i-coupled GPCRs allow analysis of real-time cAMP changes



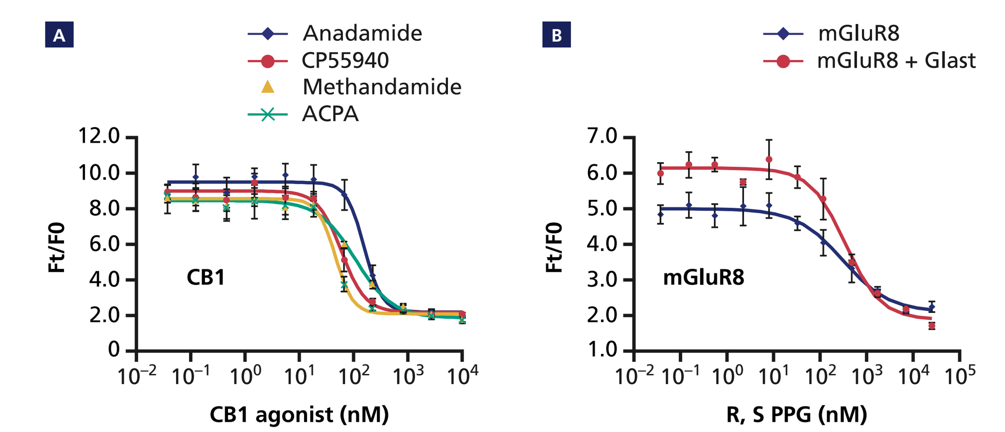
Panel A. A BD ACT*One* kinetic assay was performed with HEK 293H cells stably expressing CNG channels and dopamine receptor D2. After 20 sec, forskolin was added to a final concentration of 10 µM. Two different concentrations of dopamine (1 µM & 100 nM) or a buffer control were added after 400 sec. **Panel B.** A BD ACT*One* kinetic assay was performed with HEK 293H cells stably expressing CNG channels (Control) or CNG channels with somatostatin receptor 5 (G_i). The cells were first stimulated with 5 µM forskolin (final concentration) for 6 min (off-line) and then the signals were recorded on a FlexStation scanning fluorometer (Molecular Devices). After 20 sec of recording, somatostatin was added to a final concentration of 300 nM.

5 The BD™ ACT*One* end-point assay procedure for G_i-coupled GPCRs



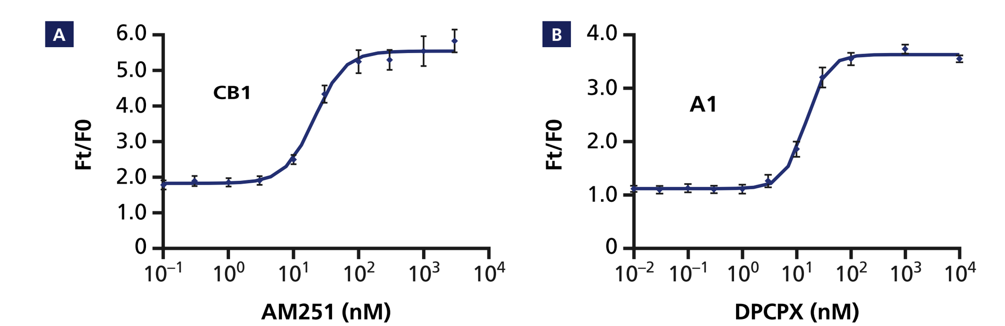
A BD ACT*One* end-point assay may be used to identify both agonist and antagonist activities of G_i-GPCRs. In each case, isoproterenol (ISO) is used to increase the cAMP level.

6 BD™ ACT*One* end-point assays of G_i-coupled GPCR agonists



Two G_i-GPCR stable cell lines were tested with agonists using the BD ACT*One* end-point assay. In each assay, 300 nM of isoproterenol was used. The agonists tested with each G_i-GPCR were able to lower the fluorescence signal dramatically. The cell lines tested contained the following receptors: cannabinoid C1 receptor (CB1 in **Panel A**) and metabotropic glutamate receptor 8 (mGluR8 in **Panel B**). A significant difference in the fluorescence signal was observed when the assay was carried out using a version of the mGluR8-containing cell line that co-expresses a glutamate transporter gene, Glast.

7 BD™ ACT*One* end-point assays of G_i-coupled GPCR antagonists



Panel A. A BD ACT*One* end-point assay of G_i-GPCR antagonists was performed on a HEK 293H cell line stably expressing CNG channels and cannabinoid C1 receptor (CB1). Varying concentrations of the CB1 antagonist AM251 were incubated with the cells for 15 min and then 300 nM isoproterenol and 1 µM CP55940 (a CB1 specific agonist) were added. **Panel B.** A BD ACT*One* end-point assay was performed on a HEK 293H cell line expressing CNG channels and adenosine A1 receptor (A1). Varying concentrations of the adenosine receptor antagonist DPCPX were incubated with the cells for 15 min and then 1 µM isoproterenol and 10 nM R-PIA (an A1 specific agonist) were added.

Table I: BD™ ACT*One* G_i Stable Cell Lines

Available G_i Stable Cell Lines	G_i Stable Cell Lines in Progress
Adenosine A1 receptor (A1)	Glutamate receptor, metabotropic 4
Adenosine A3 receptor	Glutamate receptor, metabotropic 7
Cannabinoid receptor 1 (CB1)	Somatostatin receptor 1
Dopamine receptor D2 (DRD2)	Cannabinoid receptor 2
Glutamate receptor, metabotropic 8 (mGluR8)	Chemokine (C-C motif) receptor 5
Melanin concentrating hormone 1 receptor (MCHR1)	Dopamine receptor D3, isoform short
Somatostatin receptor 5 (SSTR5)	Histamine H4 receptor
Opioid Receptor, Kappa 1	

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