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New Applications of GPCR Analysis using BD ACT*One*™ Technology

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Abstract

BD ACT*One*™ is the only high-throughput G-Protein Coupled Receptor (GPCR) screening technology that can directly measure the intracellular changes of the secondary messenger cyclic AMP (cAMP) in living cells, in real-time. It uses a proprietary modified cyclic nucleotide-gated (CNG) channel, which is co-localized with adenylate cyclase at the plasma membrane, as a biosensor of cAMP activity. Using BD 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.

The intensity of signal increase caused by GPCR activation is directly related to the receptor number on cell surface. Antibody based cAMP assays sometimes have difficulties detecting signals from endogenous GPCRs because of the low expression level. Using BD ACT*One* assay, we were able to detect activities of some endogenous Gs coupled receptors in HEK293 cells that have not been reported in literature. In addition, we have also detected weak Gs coupled activity of a GPCR that was widely considered to be only linked to Gq coupled pathway. BD ACT*One* technology, in combination with BD™ Membrane Potential Assay Kit and BD™ Calcium Assay Kits, also enables us to analyze Gq, Gs and Gi coupled receptors in one platform without artificial coupling the receptors to promiscuous Gα subunits.

Introduction

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 Gs-coupled receptors activates plasma membrane-bound adenylate cyclases that synthesize cAMP, while stimulation of Gi-coupled receptors inhibits adenylate cyclases.

The BD ACT*One*™ cAMP assay is performed using cell lines that express an exogenous cyclic nucleotide-gated (CNG) channel (Figure 1). The CNG channel is colocalized with adenylate cyclases on 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. It provides information on real time intracellular cAMP changes and is highly sensitive. By combining kinetic and endpoint readouts, we are able to capture and analyze transient responses from endogenous GPCRs and weak responses caused by weak Gs or Gi coupled GPCR activities. Since the BD ACT*One* assay is a live cell assay, the same well of cells can be objected to multiple measurements. For example, in the same well, Gq, Gs and Gi coupled activities can be measured using BD™ Calcium Assay Kit and the same GPCR can be analyzed multiple times by washing off ligands after each measurement. The BD ACT*One* provides a useful tool for GPCR de-orphanization.

Conclusion

1. BD ACTOne™ assay is a homogeneous assay for real time cAMP measurement in live cells.
2. BD ACTOne can detect endogenous GPCRs and weak Gs coupled receptors in HEK293 cells.
3. The same well of cells can be objected to multiple measurements.
4. BD ACTOne can be used to analyze partial agonists of Gi coupled receptors.
5. A simple platform can be developed to de-orphanize GPCRs by combining BD ACTOne assay with Gq coupled calcium flux assay using BD calcium assay kit.

References

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2. Rich, T. C., et.al. (2001a) J. Gen. Physiol. 118:63–78.
3. Kaupp, U. B. & Seifert, R. (2002) Physiol. Rev. 82(3):769–824.
4. Li, J. & Lester, H. A. (1999) Mol. Pharmacol. 55(5):873–882.
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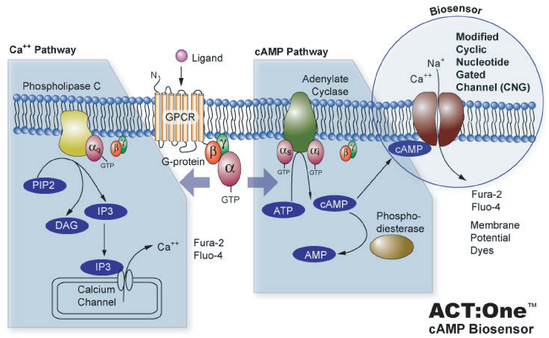
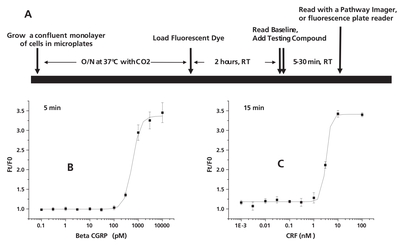
6. An S, Cutler G, Zhao JJ, Huang SG, Tian H, Li W, Liang L, Rich M, Bakleh A, Du J, Chen JL, Dai K. (2001) Proc Natl Acad Sci U S A. 98(13):7576-81.

1 Overview of BD ACTOne™ Technology



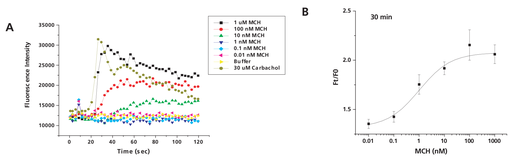
BD™ ActOne uses a modified cyclic nucleotide-gated channel (CNG) selective for cAMP as a biosensor to monitor local intracellular cAMP concentrations. The CNG biosensor enables a physiological cAMP assay in living cells by signaling through calcium or membrane potential (MP) responsive fluorescent dyes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

2 BD ACT*One*™ detects Endogenous GPCRs of HEK293 with High Sensitivity



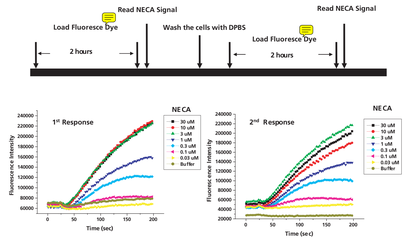
Panel A: The flow chart of BD ACT*One*™ assay with endpoint readout using BD membrane potential dye. **Panel B** and **Panel C**: BD ACT*One* parental cells (HEK-293 cells stably transfected with CNG, Cat. No. 80200-200) were stimulated with various concentrations of Beta-CGRP (B) or CRF(C). The signals were detected with BD ACT*One* endpoint assay.

3 BD ACT*One*™ Detects Weak Gs Activity of MCH2R



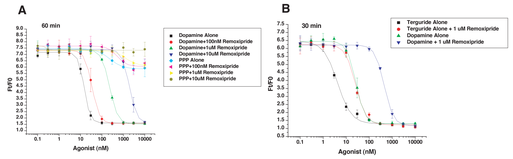
Panel A: HEK-293 cells stably expressing CNG channels and MCH2R were plated on a 96-well plate. On the second day, BD PBX calcium assay kit was loaded into each well and incubated at 37°C for one hour. Different concentrations of MCH were added into each well after baseline reading using a FlexStation. The responses via Gq coupled pathway have been observed as expected. Carbachol (30 uM) was used as the positive control. **Panel B**: Same cells as in the panel A were plated on a 384-well plate. On the second day, BD ACT*One* MP dye was loaded and incubate at room temperature for 2 hours. BD ACT*One* endpoint assay was performed with different concentrations of MCH and a weak Gs coupled activity was observed.

4 Repetitive Activation of the Same GPCR After Washing off the Ligand



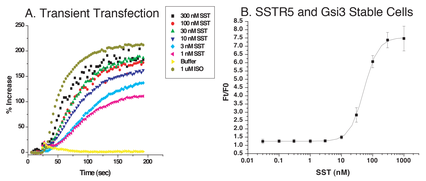
HEK-293 cells stably expressing CNG channels were plated on a 96-well plate. Next day, BD ACT*One*™ MP dye was loaded and incubated at room temperature for 2 hours. Kinetic assay was performed with different concentrations of NECA. Afterwards, the cells were washed with 1XDPBS three times. Fresh culture medium was added back into each well. BD ACT*One* MP dye was loaded and incubated at room temperature for additional 2 hours. Then, the 2nd kinetic assay was performed with different concentrations of NECA again.

5 Partial Agonist Assays with Gi coupled receptor DRD2



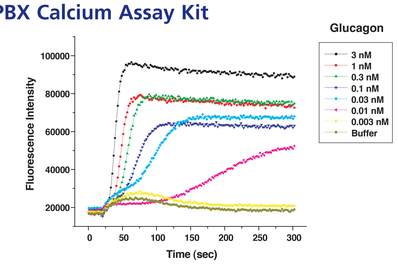
BD ACT*One*™ cells expressing DRD2 (Cat. No. 80300-206) were plated on 384-well plates. On the second day, BD ACT*One* MP dye was loaded into each well and the plates were kept at room temperature for 2 hours. The cells were first incubated with DPBS or different concentrations of remoxipride for 25 min. Then the mixtures of 300 nM Iso with different conentrations of partial agonist was added into each well. The dose reponse curves were ploted using ratio of Ft/F0 (Ft represented fluorescence intensity 30 min or 60 min after agonist addition, F0 represented fluorescence intensity before compound addition).(A) Dose response curves of S (-)-PPP and dopamine in the presence or absence of remoxipride. (B) Dose response curves of terguride and dopamine in the presence or absence of remoxipride.

6 Detection of SSTR5 Signaling by Artificially Coupling Gi to Gs Pathway through Gsi Chimera



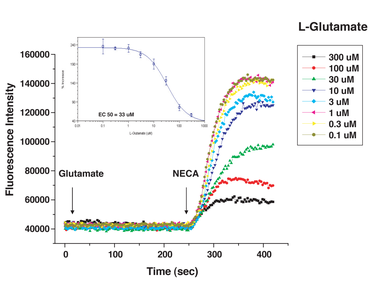
Panel A: Expression vectors containing somatostatin receptor 5 (SSTR5) and Gsi3* genes were co-transfected into HEK-293 cells stably expressing CNG channels. Next day, the transfected cells were seeded on a 96-well plate overnight. BD ACT*One* kinetic assay was performed with different concentrations of somatostatin (SST). Isoproterenol (1 μM) was used as a positive control. **Panel B:** HEK-293 cells stably expressing CNG channels, SSTR5 and Gsi3 were seeded on a 384-well plate. On the 2nd day, ACTOne endpoint assay was performed with different concentrations of SST and the dose response curve was plotted. (*Gsi is a chimeric G protein in which the last 5 amino acids of Gs protein are replaced by 5 amino acids of Gi3 protein)

7 Analysis of Gs-Coupled GPCR Using BD PBX Calcium Assay Kit



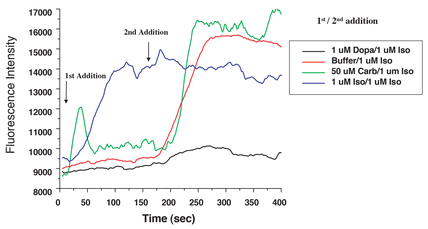
HEK-293 cells stably expressing CNG channels and Glucagon receptor (Cat. No. 80200-217) were seeded on a 96-well plate. Next day, cells were loaded with BD PBX calcium assay kit at 37°C for 1 hour. Different concentrations of glucagon were prepared in HBSS buffer containing 10mM CaCl2. Kinetic assay was then performed on FlexStation.

8 Analysis of Gi-Coupled GPCR Using BD PBX Calcium Assay Kit



HEK-293 cells stably expressing CNG channels and metabotropic glutamate receptor 8 (Cat. No. 80300-239) were seeded on a 96-well plate. On the 2nd day, cells were loaded with BD PBX calcium assay kit at 37oC for 1 hour. Different concentrations of L-glutamate were prepared in HBSS buffer and 5 X 200 uM NECA was prepared in HBSS buffer containing 15 mM CaCl2. Kinetic assay was then performed on FlexStation as indicated.

9 Functional Assay of Gq, Gs and Gi GPCRs in One Platform



The cell line in the assay was HEK293 containing recombinant CNG and DRD2 (Gi). It also contains endogenous muscarinic receptor M1 (Gq) and β2-adrenergic receptor (Gs). The cells were first stimulated with buffer, Gq (carbachol), Gs (isoproterenol) or Gi (dopamine) agonist. Gs and Gq responses were observed. 150 seconds later, the cells were stimulated with 1μM Isoproterenol. The wells previously stimulated with buffer and Gq agonist responded to isoproterenol, while the wells stimulated with Gi and Gs agonists first responded poorly to isoproterenol.