Using the Promega GloSensor™ cAMP technology on the FLIPR® Tetra system for live cell G_i- and G_s- coupled GPCR second messenger assays

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Abstract

Detection of G_{s-} and G_i-coupled GPCR second messenger signal activity has been traditionally accomplished using endpoint assays such as radioactive binding or cAMP assays that require cell lysis. These assays measure activity at a single point in the pathway and do not provide valuable kinetic information. Another option utilizes forced-coupling of G_s- and G_i-GPCRs to $G_{\alpha 16}$ followed by fluorescence detection of calcium flux upon receptor activation. However, this assay is also sub-optimal as it does not signal through the more biorelevant cAMP pathway. In this poster we demonstrate the use of the modified luminescent firefly luciferase-based Promega GloSensor™ cAMP Assay on the FLIPR® Tetra system to enable detection of cAMP mediated G_s- and G_i-coupled GPCR activity in a true kinetic assay. These GPCR subtypes can now be evaluated in a live cell assay that measures changes in intracellular cAMP concentration, the relevant second messenger mechanism. We demonstrate endogenous receptor activity in CHO-K1 and HEK-293 cell lines stably expressing the GloSensor plasmid. In addition, transfected G_s- and G_i-coupled receptor activity will be measured from cell lines with stably transfected GPCR receptors and transient transfection of the GloSensor plasmid. We also show that multiplexing G_q- and G_s-coupled GPCR assays is possible using the GloSensor cAMP assay and FLIPR® Calcium 5 Kit in the same well. Combined with the GloSensor cAMP Assay, the FLIPR® Tetra system delivers the complete solution for kinetic screening of the major classes of GPCR subtypes.

About the GloSensor™ cAMP Assay

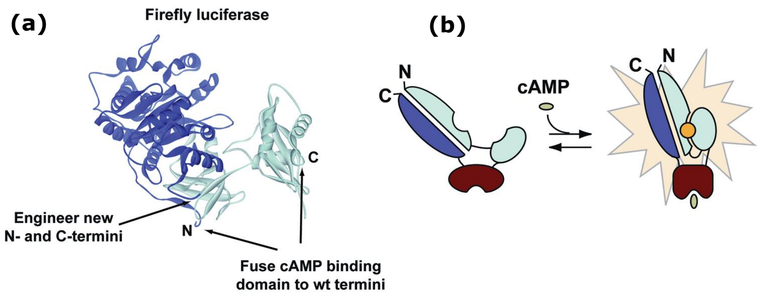


Figure 1. a) The GloSensor cAMP Assay was created by fusion of a cAMP binding domain to the wild-type N- and C-termini of native firefly luciferase. In the absence of cAMP, the genetically modified luciferase containing the cAMP binding domain is in the inactive state. b) Upon binding to cAMP, conformational changes in the cAMP binding domain likely determine the increased luminescence in the activated state that can be detected in living cells on the FLIPR® Tetra system. Both forms of luciferase are represented in the presence of luciferase substrate.

About the FLIPR® Tetra System

- Sensitive dual readout ICCD camera provides optimal luminescence and fluorescence detection
- FLIPR® Tetra system enables kinetic cAMP and Ca^{2+} detection (plus many others) in live cell assays
- Scalable assay throughput: 96-, 384- and 1536-well plate formats, easily integrated with automation

Materials and Methods

About the Cells: GloSensor cAMP HEK-293 stable (Cat# E1261, Promega) and Rat Y2R/GloSensor cAMP 23F CHO-K1 double stable cell lines were provided by Promega R&D, Dopamine D_4 stable HEK-293T cell line (Cat# C1338) was provided by Multispan, Inc., Hayward, CA.

Plasmid: GloSensor assay plasmid p22F cAMP (Cat# E2301) was generously provided by Promega Inc.

GloSensor Assay Reagents:
Plating medium: 90% CO_2-independent medium (Cat# 18045, Life Technologies), 10% FBS.
GloSensor cAMP Reagent (Cat# E1291, Promega)
Equilibration medium: Plating medium (above) and 2% or 5% GloSensor cAMP Reagent
Compounds: diluted in HBSS + 20 mM HEPES and added during kinetic read on the FLIPR® Tetra system at 5X final concentration.

Calcium Flux Assay Reagents:
Dye loading medium was made in 50% GloSensor plating medium and 50% Hanks HBSS + 20 mM HEPES and FLIPR® Calcium 5 Assay Kit (Catalog# R8186, Molecular Devices LLC)

Multiplexed assay reagent:
2% final volume GloSensor reagent was added to the to the Calcium 5 assay kit loading buffer and dye.

Stable GloSensor cAMP cell line assay:
Cells were plated overnight in 384-well black wall clear bottom plates. Two hours prior to assay, culture media was removed and cells were incubated for 1 hour @ 37° C in 5% CO_2 and 1 hour @ room temperature in equilibration media containing GloSensor cAMP reagent. HEK cells were incubated in equilibration media containing 2% GloSensor cAMP reagent. CHO-K1 cell lines were incubated 5% reagent. 5X compound was added by the FLIPR® Tetra system during kinetic read. Exposures were taken every 10 or 30 sec. for 10-25 min.

Transient transfection GloSensor cAMP assay:
Cultured cells were lifted from flasks and incubated with pGloSensor cAMP plasmid using an optimized Fugene HD transfection reagent protocol (Cat# 0409705001, Roche). Transfected cells were plated in 384-well black wall clear bottom plates for two days prior to assay. After this point, the stable cell assay protocol was followed. Data were exported from FLIPR ScreenWorks® software to GraphPad Prism for analysis.

G_q- and G_s- coupled GPCR Multiplexing experiments:
GloSensor cAMP-22F HEK 293 cells were plated following the protocol for the stable GloSensor cAMP cell line assay. The cells were incubated in both Calcium 5 Kit and GloSensor reagent for 1hr @ 37ºC and 1 hr @ RT. On the FLIPR® Tetra instrument, carbachol was added to stimulate the G_q-coupled muscarinic M_3 receptor at the same time as isoproterenol was added to stimulate the G_s-coupled β_2 adrenoceptor. During addition, the FLIPR® Tetra instrument first read fluorescence for 3 min. to measure G_q mediated calcium flux. After the fluorescence read was complete, a separate protocol was run to read luminescence for 10 min. after addition of isoproterenol to stimulate the cAMP response.

Results

Endogenous β_2 Adrenoceptor Agonist Response (GloSensor cAMP 22F HEK-293 Cells)

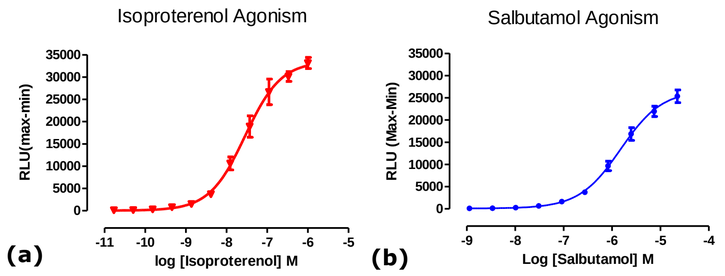
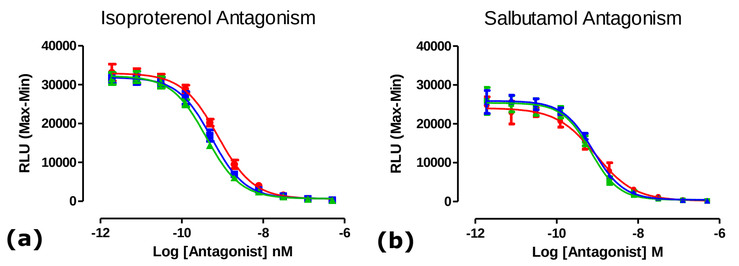


Figure 2. Endogenous G_s-coupled cAMP response in HEK-293 cells stimulated by isoproterenol and the partial agonist salbutamol. Consistent with partial agonism, salbutamol gave a smaller response. In this experiment, flexibility of the assay was shown by substituting HBSS buffer + 20mM HEPES and 1% BSA for the equilibration media. Calculated EC_{50} values for the assay are (a) 29 nM for isoproterenol and (b) 1.5 μM for salbutamol with Z' > 0.7

Endogenous β_2 Adrenoceptor Antagonist Response (GloSensor cAMP 22F HEK-293 cells)



	Propranolol	Pindolol	Alprenolol
IC_{50} nM	2	1.2	1.5
Z at IC_{50}	0.86	0.88	0.84

	Propranolol	Pindolol	Alprenolol
IC_{50} nM	0.91	0.69	0.78
Z at IC_{50}	0.74	0.74	0.84

Figure 3. Antagonism of isoproterenol and salbutamol response by three known inhibitors. (a) Inhibition of EC_{80} isoproterenol response. (b) Inhibition of EC_{80} salbutamol response. The robust signal window enables Z factors > 0.7.

Transfected Dopamine D_4 Receptor in HEK-293 cells (Transiently Expressed GloSensor cAMP-22F)

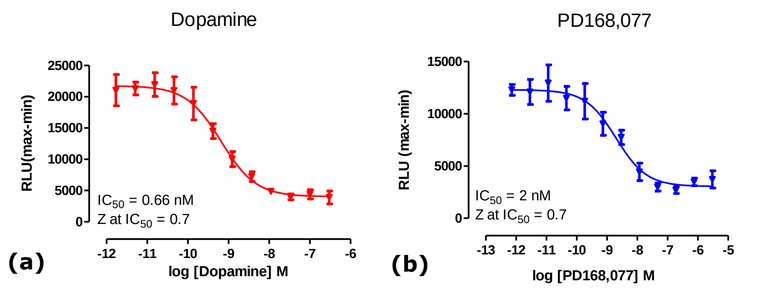


Figure 4. HEK-293 cells over expressing G_i-coupled Dopamine D_4 receptor from Multispan, Inc. were transiently transfected with GloSensor cAMP-22F plasmid. Ligand was added on-line to the wells, followed by a 5 min. incubation. The FLIPR® Tetra system then added 10 μM forskolin to stimulate cAMP production. Inhibition of forskolin mediated cAMP production by (a) Dopamine and (b) the D_4 receptor specific compound, PD168,077.

Transfected Rat Y2 Receptor in CHO-K1 cells (Stably Expressed GloSensor cAMP-23F)

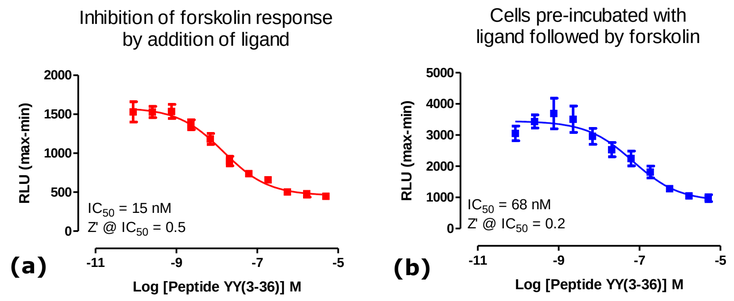


Figure 5. G_i-coupled receptor activity results in a reduction in signal correlated with a reduction in cAMP production. Here we compared the addition order of forskolin. (a) 10 μM forskolin addition followed 15 min. later by addition of agonist Peptide YY(3-36) on the FLIPR® Tetra system. (b) Peptide YY(3-36) was added 15 min. prior to addition of 10 μM forskolin. Both methods illustrate a reduction in signal related to inhibited cAMP production.

Multiplexing endogenous G_q- and G_s-Coupled GPCRs (Stably Expressed GloSensor cAMP-22F)

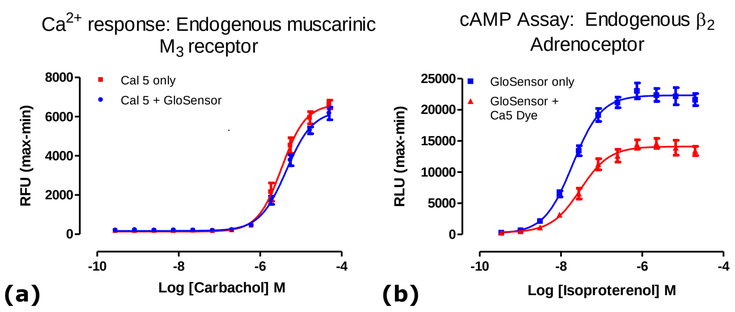


Figure 6. Stable cAMP-22F HEK-293 cells were incubated with both FLIPR® Calcium 5 Kit and 2% GloSensor cAMP Assay reagent for 1 hour @ 37ºC followed by 1 hour @ RT. (a) The GloSensor reagent did not interfere with the fluorescent calcium read, the EC_{50} for carbachol was ~4 μM in both cases. (b) There was a small reduction in the signal window of the luminescent GloSensor assay using this method. Again, there was no effect on agonist EC_{50} estimates (25 nM vs. 30 nM). Further assay optimisation may reduce this apparent reduction in signal

Summary

- Working together, the GloSensor™ cAMP assay and the FLIPR® Tetra system enable live cell kinetic HTS screening for G_i- and G_s-coupled GPCRs.
- We have shown assay development flexibility using GloSensor cAMP cell lines and GloSensor cAMP plasmids.
- Use of the FLIPR® Tetra with GloSensor cAMP assay enables kinetic measurement of G_i- and G_s-coupled receptor signalling not possible using endpoint assays on standard plate readers.
- Multiplexing G_q- and G_s-coupled GPCR assays is demonstrated using the GloSensor cAMP and FLIPR® Calcium 5 Assay reagents

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