A Duplexed Calcium and β-Arrestin Assay Protocol for Human Histamine H1 Receptor PathHunter CHO-K1 HRH1 β-Arrestin Cell Line

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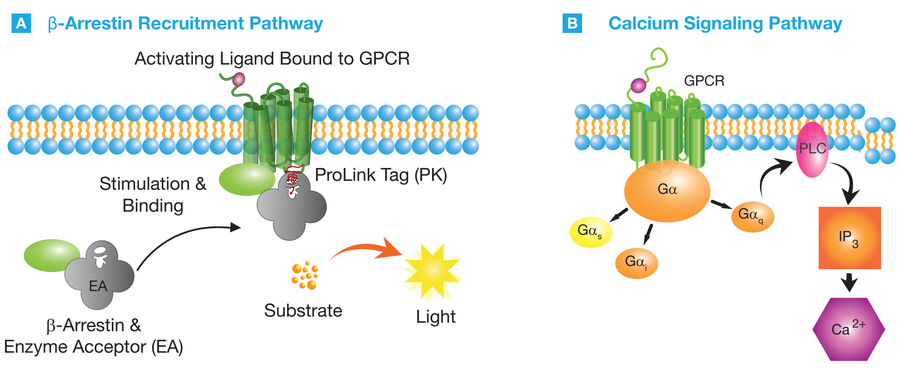
Abstract

The screening and functional characterization of novel ligands for G-protein-coupled receptors (GPCRs) remains a key activity in drug discovery process. Furthermore, an increasing awareness of the subtleties of receptor signaling and potential for ligand bias requires multiple endpoints to be monitored. Efforts to reduce costs and improve efficiency also remain a key consideration. In this poster, we describe efforts to develop a duplexed assay for the Histamine H1 receptor employing the DiscoverX PathHunter® β-Arrestin clonal cell line. We have adapted existing protocols such that from a single plate we can monitor calcium signaling with fluorescent dyes sensitive to intracellular Ca^{2+} ion flux in real time, followed by the recruitment of β-arrestin as an endpoint assay. Protocol development will be discussed and successful implementation of the final assay conditions will be exemplified with a characterization of H1 agonists and antagonist pharmacology.

GPCR-Mediated β-Arrestin and Calcium Signaling Dual Assay Principle



GPCR-mediated A. β-arrestin recruitment and B. Ca^{2+} signaling pathways in the CHO Histamine H1 PathHunter β-Arrestin cell line.

Methods

Cell Culture

PathHunter CHO-K1 HRH1 β-Arrestin cell line (Part Number 93-0503C2) co-expressing the ProLink™ (PK) tagged GPCR and the Enzyme Acceptor (EA) tagged β-arrestin-2, were used. Cells were maintained in AssayComplete™ Media as per recommendations for the cell line. One day before running the assay, 20,000 cells were plated in 200 µL into each well of a 96-well black, clear-bottom µClear® culture microplate. These assay plates were incubated for 18 - 24 hours in 5% CO_2 incubator at 37°C .

Calcium Signaling Assay

The Fluo-4 AM dye solution was prepared as per manufacturer's protocol.

Test compounds, agonists or antagonists (10 mM stock solution) were diluted in AssayComplete Cell Plating (CP) 2 Reagent or in 1 X HBSS-Ca^{2+}/Mg^{2+} and 20 mM HEPES buffer (pH 7.4).

Cell culture medium was removed and replaced with 100 µL of Fluo-4 dye solution. Cells were then incubated for 30 - 45 minutes in 5% CO_2 incubator at 37°C . Cells were washed with 200 µL of the 1 X HBSS Buffer to remove excess dye and re-suspended in 80 µL of CP 2 Reagent. At this step, 10 µL of 10 X antagonist solution (antagonist mode) or 10 µL of CP 2 Reagent (agonist mode) were added to each well. Plates were incubated in the dark for 30 minutes at RT for esterase activity and antagonist binding.

The plate was placed in the FDSS/µCell (Hamamatsu). 10 µL of 10 X agonist solution (Histamine at 300 nM final concentration) were injected (3 mm height, aspiration rate at 40 µL/s) at a dispense rate of 10 µL/s).

Signal Detection and Data Analysis

Fluorescence was measured for 90 seconds (1 lecture/s), binning at 2 X 2, exposure time 200 ms (sensitivity 2), Ex 480/Em 540.

Responses were calculated as the difference between maximum and minimum fluorescence counts ($F_{max} - F_{min}$). Results were normalized and were presented as a % of maximal response of histamine agonist.

β-Arrestin Assay

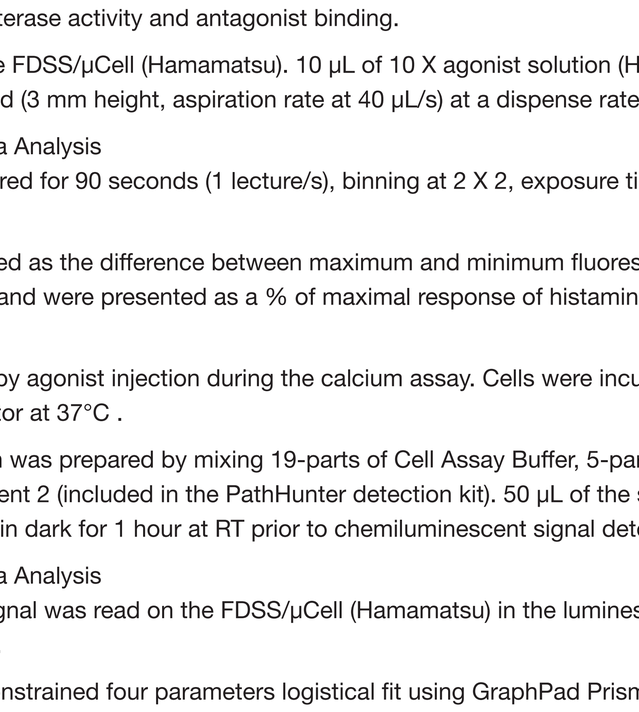
β-arrestin assay is started by agonist injection during the calcium assay. Cells were incubated for additional 90 minutes in 5% CO_2 incubator at 37°C .

Working Detection Solution was prepared by mixing 19-parts of Cell Assay Buffer, 5-parts of Substrate Reagent 1, and 1-part Substrate Reagent 2 (included in the PathHunter detection kit). 50 µL of the solution was added to the assay plate and incubated in dark for 1 hour at RT prior to chemiluminescent signal detection.

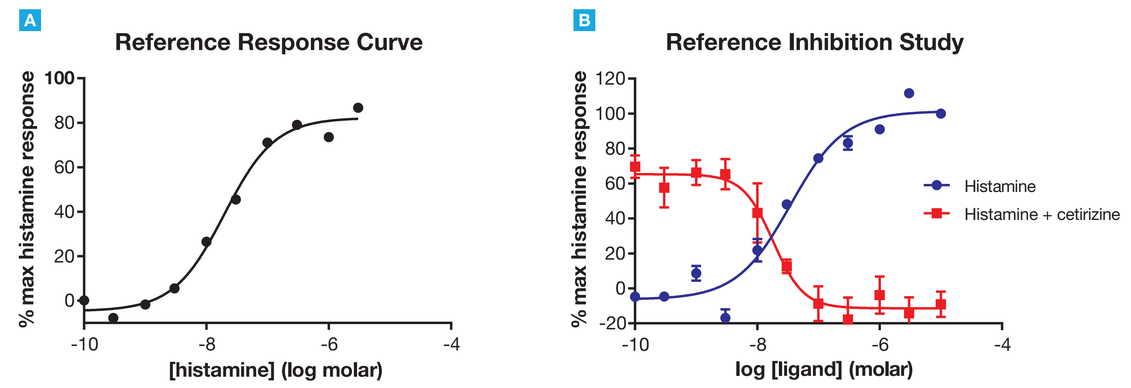
Signal Detection and Data Analysis

The chemiluminescent signal was read on the FDSS/µCell (Hamamatsu) in the luminescence mode: 10 samples, 10 s interval, sensitivity 5.

All curves fitted using unconstrained four parameters logistical fit using GraphPad Prism software.

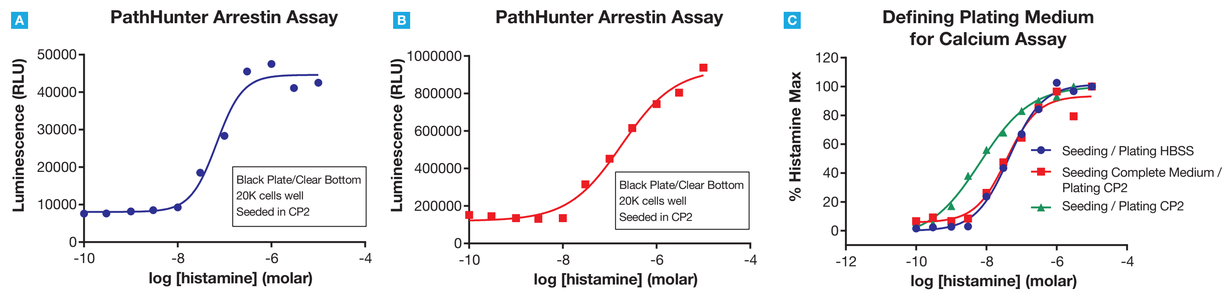


Reference Calcium Signaling with CHO Histamine H1 PathHunter Cell Line



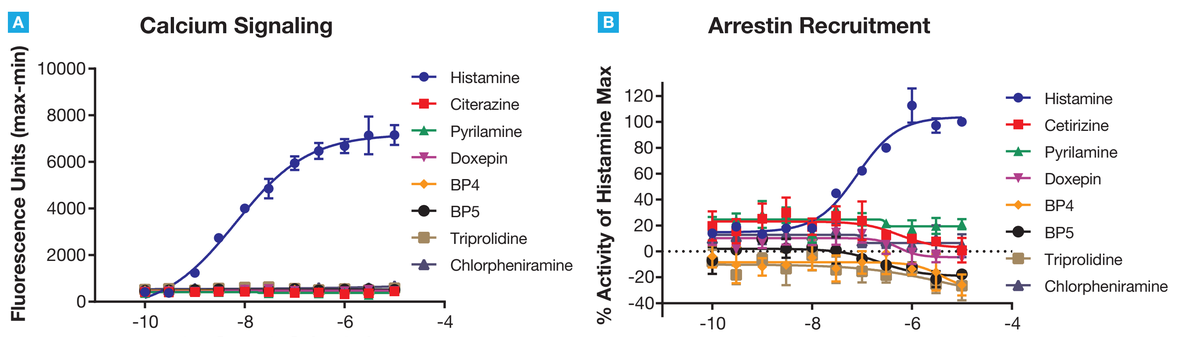
A. Histamine concentration responses curves and B. Cetirizine inhibition study using standard calcium signaling protocol on a FDSS/µCell Reader (Hamamatsu).

Black Plates Are Suitable for β-Arrestin Recruitment Assay on Standard Luminometers



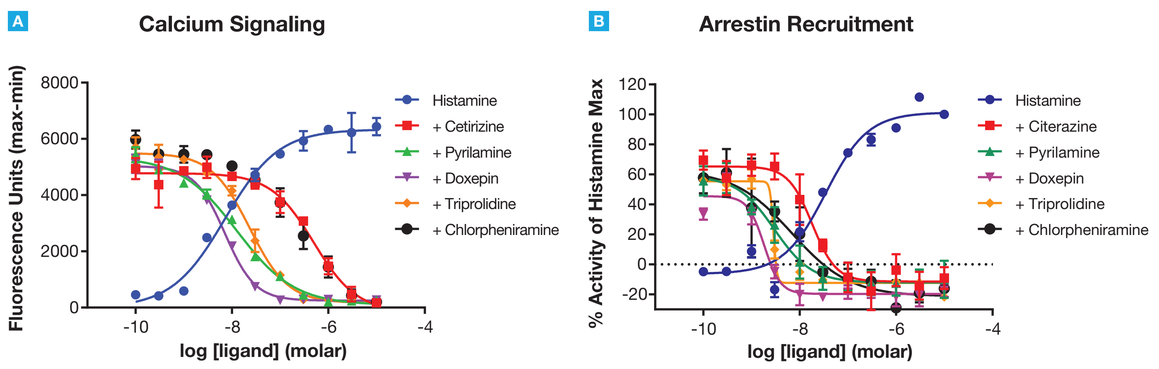
Optimizing the assay conditions to facilitate dual readouts from a single well. Cells were seeded in black/clear bottom TC-treated plates and assays read in standard luminometers A. FDSS/µCell Reader (Hamamatsu) and B. Microbeta² Microplate reader (Perkin Elmer) C. Appraisal of seeding and dye loading buffers for calcium assays. Histamine concentration response curves were compared in standard loading conditions to using CP 2 for both seeding and loading in CP 2 to enable compatibility with the β-arrestin recruitment assay protocol. CP 2 can be used to support a calcium signaling study with Fluo-4 dye, and the same plate and culture conditions are compatible with β-arrestin recruitment measurement.

Study of Histamine H1 Agonist Pharmacology Using Dual Protocol



Agonist potency and rank order was assessed using the dual protocol with both A. Calcium signaling and B. β-arrestin recruitment, read on a FDSS/µCell. This dataset is also summarized in the table below.

Study of Histamine H1 Antagonist Pharmacology Using Dual Protocol



Study of Histamine H1 antagonist pharmacology using dual protocol. Antagonist potency and rank was assessed using the dual protocol with both A. Calcium signaling and B. β-arrestin recruitment, read on FDSS/µCell. Antagonists were pre-incubated with cells before addition of EC_{80} of histamine. This dataset is also summarized in the table below.

Potency Ranking of GPCR Modulators for Calcium Signal and β-Arrestin Pathways

Different Assays on Human Histamine H1 Receptor

	3H Mepyramine binding BioProjet HEK-hu-H1	GTPγ35S binding BioProjet HEK293-hu-H1				Calcium Flux										β-Arrestin		
						Native HUVEC				BioProjet HEK293-hu-H1				DiscoverX CHO-K1-hH1				DiscoverX CHO-K1-hH1		
	Ki (nM)	Agonist/inverse agonist EC_{50} (nM)	i.a	Kb (nM) against HA		Agonist EC_{50} (nM)	i.a	Kb (nM) against HA	i.a	Agonist EC_{50} (nM)	i.a	Kb (nM) against HA	i.a	Agonist EC_{50} (nM)	i.a	Kb (nM) against HA	i.a	EC_{50}(nM)	i.a	Kb (nM) against HA
HA	4400	30000	1			1737	1			7.7	1			7.2	1			110	1	
Cetirizine	12.6			21	0							0.9	0	Inactive		75.2	0	Inactive	-0.1	2.2
Pyrilamine	0.2			5.6	0			0.6	0.0					Inactive		1.5	0	Inactive	0.0	0.3
Doxepin	0.1			0.2	0									Inactive		1	0	Inactive	-0.2	0.2
Triprolidine	2.2			15.4	0									Inactive		0.5	0	Inactive	-0.2	0.25
Chlorpheniramine	3.8			9.6	0							7.6	0	Inactive		12.4	0	Inactive	-0.1	0.9
BP4	0.4			2.6	0			7	0					Inactive		6.4	0	Inactive	-0.1	0.6
BP5	348			Inactive	0			Inactive	0					Inactive		Inactive	0	Inactive	0.0	Inactive

Summary

- β-arrestin cell lines expressing H1 receptor provided robust responses by detecting HA-induced β-arrestin recruitment
- To measure both fluorescence (Ca^{2+}) and luminescence (β-arrestin), use of black clear-bottom microplate as well as CP 2 plating medium is highly recommended
- Single-plate dual Ca^{2+} and β-arrestin assay have been implemented for hH1R expressing β-arrestin cell line: Strong fluorescence (calcium) as well luminescence (β-arrestin) signals have been recorded
- Histamine H1 antagonists profiling can be obtained regarding β-arrestin recruitment and calcium mobilization
- Implementation of single plate dual assay in other β-arrestin cell lines expressing receptors coupled to calcium signaling (e.g. CHO-K1-H3, CHO-K1 ADRA1B, U2OS HTR2A) may be of interest

