Cell-Based Assays for G-Protein Independent Signaling Using Arrestin Binding and Endocytosis

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Abstract

GPCR activation results in G-protein dependent events such as second messenger signaling, as well as initiation of a host of cellular responses that regulate function and activate alternative pathways that are independent of G-protein activation. Recent studies have highlighted the importance of G-protein independent signaling in normal and pathogenic tissue physiology. Two of the well-characterized G-protein independent events are Arrestin recruitment and endocytosis. Quantitatively examining these pathways in detail can aid in defining compound function and lead to the discovery of novel compounds with unique attributes. Previously, DiscoveRx has developed a system to monitor protein-protein interactions in live cells using enzyme fragment complementation (EFC). More recently, we have adapted this methodology to monitor the fate of activated and internalized GPCRs as they traffic through the endosomal compartment using a simple chemiluminescent detection method that is amenable to high-throughput screening. Here we present the development of the system and the application to a wide range of GPCRs. Case studies demonstrating compound-specific internalization and recycling patterns of multiple receptors including the human delta opioid receptor (OPRD1) will be discussed.

PathHunter GPCR Internalization Systems

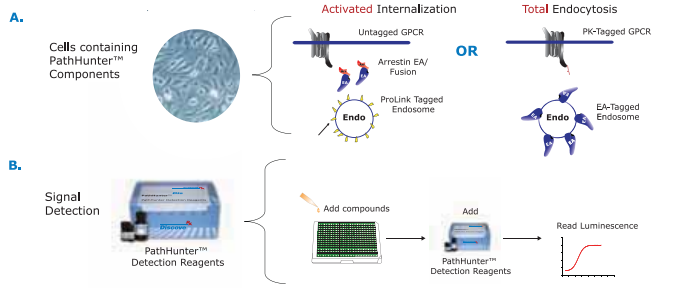

Figure 1. PathHunter GPCR Internalization Assays provide a novel, non-imaging approach for measuring GPCR activation and internalization in live cells. The PathHunter systems combine engineered clonal cell lines validated for stable GPCR receptor, tagged endosome and tagged β-Arrestin (in the case of the Activated Internalization System). This system combines a simple, one-step addition protocol and standard chemiluminescent detection and is ideally suited for 96-, 384- or 1536-well compound screening.

Activated GPCR Internalization Format

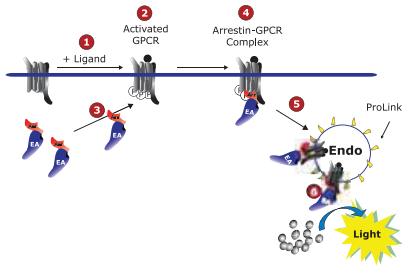

Figure 2. In the PathHunter GPCR Internalization Assays, the small enzyme fragment of β-galactosidase (ProLink™) is localized to the surface of cellular endosomes and the larger, complementing enzyme fragment (termed Enzyme Acceptor or EA) is fused to β-Arrestin. Stimulation of the receptor results in Arrestin binding to the activated GPCR, internalization of the receptor and trafficking to cellular endosomes resulting in enzyme complementation and an increase in activity that is easily measured using PathHunter Detection Reagents.

Internalization Technology is Broadly Applicable

Agonist 384-well Protocol

- Seed 5,000 cells in 20 µL optimized cell plating reagent*
- Incubate Overnight @ 37°C
- Induce cells with 5 µL of Agonist
- Incubate for 3 hr @ RT (19 - 25°C)
- Add 13 µL PathHunter™ Detection Reagent
- Incubate for 1 hr @ RT
- Read Chemiluminescent Signal

* *Cell Plating Reagent is specific to each GPCR cell line*

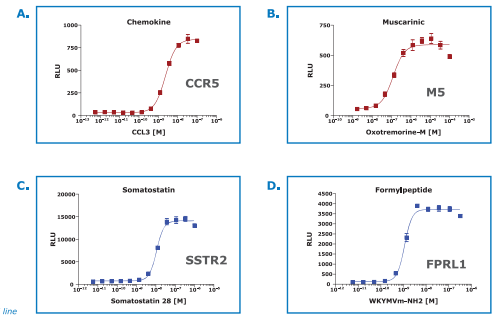

Figure 3. Cell expressing the native, untagged **(A)** Chemokine CCR5, **(B)** Choninergic Muscarinic CHRM5, **(C)** Somatostatin SSTR2, and **(D)** Formylpeptide FPRL1 were treated with increasing concentrations of control agonist and assayed using PathHunter Detection Reagents. Together, this data demonstrates that the PathHunter Activated GPCR Internalization Technology is broadly applicable to a large number of GPCR families.

Available Activated GPCR Internalization Targets

Family	Target	Coupling	S:B	EC_{50}	Family	Target	Coupling	S:B	EC_{50}	Family	Target	Coupling	S:B	EC_{50}
Adrenergic	ADRA1A	Gq	2.1	136 nM	Cannabinoid	CNR1	Gi	6.9	13 uM	Motilin	MLNR	Gq	2.7	6.8 nM
Apelin	AGTRL1	Gi	26.8	0.94 nM	Corticotropin-releasing factor	CRHR1	Gs	20.6	28 nM	Neuromedin U	NMU1R	Gq	4.9	19.9 nM
Vasopressin & oxytocin	AVPR1B	Gq	2.3	10.3 uM						Neuropeptide Y	NPY2R	Gi	3.1	14.6 nM
Vasopressin & oxytocin	AVPR2	Gs	21.6	6.8 nM	Chemokine	CXCR1	Gi	13.8	6.1 nM	Neurotensin	NTSR1	Gq	47	1.4 nM
Bradykinin	BDKRB2	Gq	37.7	29 nM	Chemokine	CXCR2	Gi	11.4	1.5 nM	Opioid	OPRD1	Gi	3.9	1.3 nM
Anaphylatoxin	C5L2		4	2.5 nM	Chemokine	CXCR3	Gi	7	67 nM	Opioid	OPRM1	Gi	4.8	193 nM
Anaphylatoxin	C5R1	Gi	28.9	2 nM	Chemokine	CXCR6	Gi	4	17 nM	Vasopressin & oxytocin	OXTR	Gi & Gq	26.1	2.1 nM
Cholecystokinin	CCKAR	Gq	3.3	4.2 nM	Dopamine	DRD1	Gs	3.7	21.9 uM	P2Y	P2RY11	Gs & Gq	3.6	8.1 uM
Cholecystokinin	CCKBR	Gq	14.5	0.2 nM	Lysophospholipid	EDG3	Gi	4	102 nM	P2Y	P2RY2	Gq	32.4	1.5 uM
Chemokine	CCR1	Gi	4.6	8.6 nM	Lysophospholipid	EDG5	Gs	15.6	1 uM	Prokineticin	PROKR2	Gs	5.7	77 nM
Chemokine	CCR2	Gi	2.7	1.7 nM	Protease activated	F2RL1	Gi & Gq	7	8 uM	Platelet-activating factor	PTAFR	Gi & Gq	15.2	28.8 nM
Chemokine	CCR5	Gi	32.4	2.6 nM	Protease activated	F2RL3	Gi & Gq	4.6	182 uM	Glucagon	SCTR	Gs	14.3	23.8 nM
Chemokine	CCR6	Gi	2.4	15.9 nM	Formylpeptide	FPRL1	Gi	33.7	1.1 nM	Somatostatin	SSTR2	Gi	23.1	10.8 nM
Chemokine	CCR7	Gi	19.6	56.7 nM	Glucagon	GLP1R	Gs	3.7	5.6 nM	Tachykinin	TACR1	Gs & Gq	33.2	3.31 nM
Chemokine	CCR8	Gi	1.6	74 nM	Glucagon	GLP2R	Gs	3.5	28 nM	Tachykinin	TACR2	Gs & Gq	4.5	381 nM
Acetylcholine	CHRM1	Gq	3	416 nM	Class A Orphan	GPR1		5.3	2.6 nM	Tachykinin	TACR3	Gs & Gq	16.6	5.3 nM
Acetylcholine	CHRM2	Gi	8.2	224 nM	Bombesin	GRPR	Gq	22	3.9 nM	Urotensin	UTR2	Gq	5.4	0.83 nM
Acetylcholine	CHRM5	Gq	3	3.9 uM	Orexin	HCRTR1	Gq	16.9	51 nM	VIP & PACAP	VIPR1	Gs	16.2	92.2 nM
Chemokine	CMKLR1	Gi	3.7	2.6 nM	Orexin	HCRTR2	Gi & Gq	18.7	27.5 nM	VIP & PACAP	VIPR2	Gs	30.5	71 nM
Chemokine	CMKOR1		12.7	101 nM	Serotonin	HTR2C	Gq	2.2	75.3 nM					

Uncovering Biased Ligands using Complete GPCR Porfolio

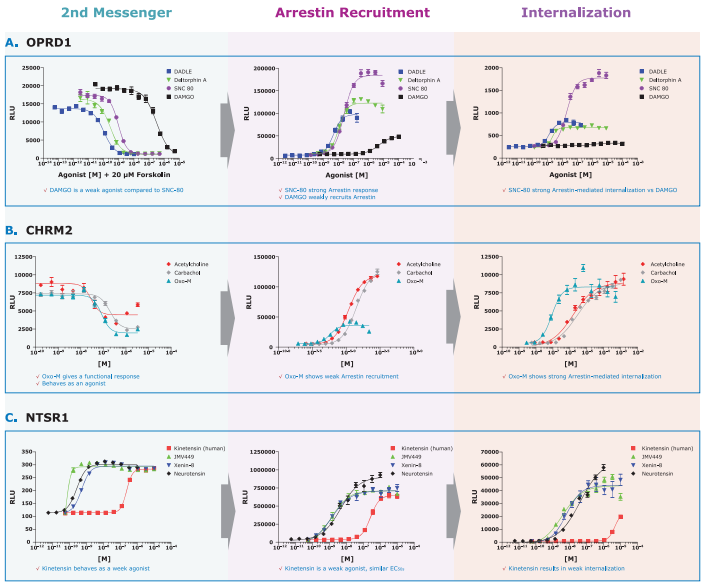

Figure 4. Cell expressing the **(A)** Opioid Receptor Delta (OPRD1), **(B)** Cholinergic Muscarinic M2 Receptor CHRM2, and **(C)** Neurotensin NTSR1 Receptor were treated with increasing concentrations of agonist. The left panels show the second messenger response (cAMP or Calcium). The middle panels show the PathHunter Arrestin recruitment response. The right panels show the Arrestin-mediated internalization response. In all cases, compound pharmacology differences were observed across the different assay platforms.

Total GPCR Endocytosis Assay Validation

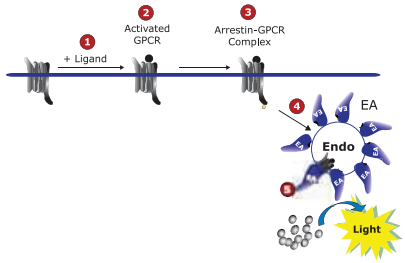

Figure 5. In the PathHunter Total GPCR Endocytosis Assays, the large portion of β-galactosidase (termed Enzyme Acceptor or EA) is localized exclusively to the surface of cellular endosomes and the smaller, complementing enzyme fragment is fused to the GPCR of interest. Stimulation of the target receptor results in internalization of the receptor and trafficking to cellular endosomes. This action forces complementation of the two enzyme fragments, resulting in an increase in enzyme activity that is easily measured using chemiluminescent PathHunter Detection Reagents.

Quantitative Measure of Total Endocytosed GPCRs

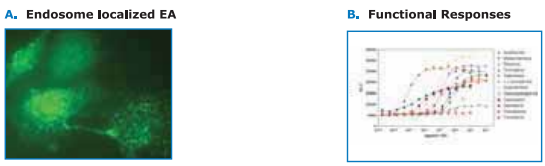

Figure 6. **(A)** An anti-EA monoclonal antibody was used to detect EA-tagged intracellular endosomes in the PathHunter ADRB2 Total Endocytosis cell line. **(B)** This same cell line was incubated with increasing concentrations of known agonists for the ADRB2 receptor.

Summary

- PathHunter GPCR Internalization Assays extend the utility of DiscoveRx's proprietary EFC technology, allowing direct and quantitative measurements of internalized GPCRs localized to intracellular endosomes.
  - ***Activated GPCR Internalization Assays*** utilize an untagged GPCR and give a direct measure of activated and internalized receptor
  - ***Total Endocytosis Assays*** give a direct measure of total endocytosed receptor, both arrestin-dependent and independent
- Unlike other imaging or antibody-based internalization technologies, PathHunter assays are simple, non-radioactive chemiluminescent assays that are amenable to high throughput screening.
- These novel, functional cell-based assays are ideal of studying receptor activation kinetics, identifying novel inhibitors or confirming compound pharmacology following a primary screen.
- Importantly, the availability of the same GPCR target in multiple pathway read-outs such as second messenger, β-Arrestin recruitment and GPCR internalization allows for a more thorough and complete understanding of receptor biology and the potential discovery of novel ligands that are biased toward one signaling pathway over another.

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