Label-free Profiling of Ligands for Endogenous GPCRs Using a Cell-Based High Throughput Screening Technology

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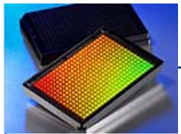
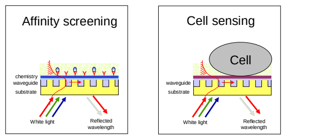


Abstract

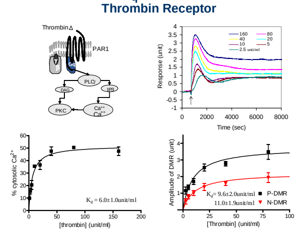
G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors and the largest class of drug targets. The activation of GPCRs is known to lead to the dynamic translocation of multiple signaling molecules or molecular assemblies during its signaling cycle, and in many cases cytoskelatal reorganization. Such a movement and/or reorganization results in dynamic redistribution of cellular contents, equivalent to dynamic mass redistribution (DMR), which can be monitored online in living cells using Corning® Epic™ system – a label-free and non-invasive biosensor system that is centered around resonant waveguide grating (RWG) biosensors. The resultant DMR signal offers a novel and functional optical signature for studying GPCR signaling and screening GPCR drug compounds. The theoretical considerations and assay principles are first described. The unique optical signatures for over 20 endogenous G_q, G_s and G_i-coupled receptors in A431 human epidermoid carcinoma cells are presented. As exampled in studying the signaling pathways and network interactions of endogenous GPCRs, as well as screening GPCR ligands using both kinetic and endpoint measurements, Corning® Epic™ system is an easily scaleable biosensor HTS platform for G_q, G_s and G_i GPCR drug discovery and deorphanization.

Cell-Based Assays on the Epic™ System:
Theory of Operation

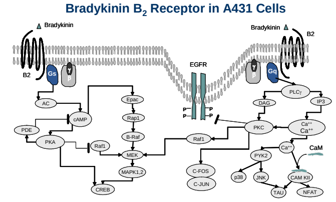
- Evanescent waveguide sensor
- Resonant coupling of light into a waveguide using a diffraction grating
- Waveguide formed of four layers, each with different index of refraction
- Monitor changes in reflected wavelength or angle (proportional to local mass redistribution)
- Only monitor the bottom portion of cells:
  - Evanescent wave has limited penetration into cell monolayer on the sensor surface - depth/sensing volume (~120nm)



Signature of G_q-Coupled Receptors:
Thrombin Receptor



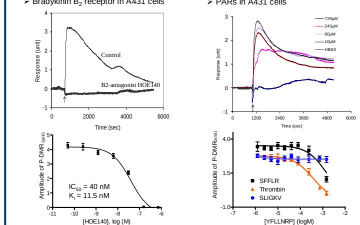
Mapping Signaling Network Interactions:
Bradykinin B_2 Receptor in A431 Cells



Neves et al. *Science* 2002, 296, 1636.
Graness et al. *Biochem. J.* 2000, 347, 441-7
Liebmann et al. *Biochem. J.* 1996, 313, 109-118.

Compound Potency

- Bradykinin B_2 receptor in A431 cells
- PARs in A431 cells



Cell-Based Assays on the Epic™ System:
Dynamic Mass Redistribution (DMR)

The spatial and temporal redistribution in local mass density and amount in the vicinity of the sensor surface

- cellular targets or molecular assemblies moving into or out of the sensing volume of the optical biosensor

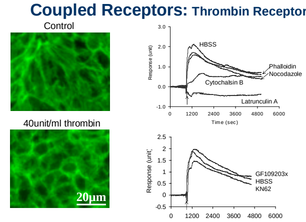
"There's Plenty of Room at the Bottom"
-- Richard P. Feynman

List And Profiles of Agonists Examined for Known *Endogenous GPCRs* in A431

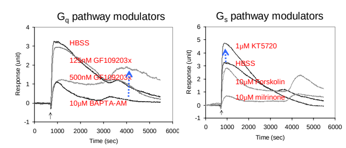
Receptor	Signaling	Agonist	K_d (Epic™)*	K_d(Fluo-3)	Reference
A1 (ADORA1)	G_i	Adenosine-amine-cogener	3.7±1.1μM	N.A.	Khoo, H. E. *et al.* (1997) *J. Biochem. Mol. Biol. Biophys.* 1, 109-116.
A2A (ADORA2A), A2B (ADORA2B)	G_s	NECA	72.4±28.0nM	N.A.	Khoo, H. E. *et al.* (1997) *J. Biochem. Mol. Biol. Biophys.* 1, 109-116.
β2 (ADRB2)	G_s	Epinephrine	5.0±1.0nM	N.A.	Delavier-Klutchko, C. *et al.* (1984) *FEBS Lett.* 169, 151-155.
B2 (BDKRB2)	G_q	bradykinin	6.5±1.6nM	10.0±2.8nM	Liebmann, C. *et al.* (1996) *Biochem. J.* 313, 109-118.
BB1 (BRS1)	G_q	Bombesin	N.D.	N.D.	Gorbulev, V. *et al.* (1994) *FEBS Lett.* 340, 260-264.
H1 (HRH1)	G_q	HTMT	45.4±14.2nM		Tilly, B.C. *et al.* (1990) *J. Cell Biol.* 110, 1211-1215.
H2 (HRH2)	G_s			N.A.	
LPA1	G_i	LPA			Ohta, H. *et al.*(2003) *Mol. Pharmacol.* 64, 994-1005.
LPA2, LPA3	G_q		N.D.	524±164nM	
P2Y1 (low), P2Y4, P2Y6	G_q	ATP	2.2±0.6μM	7.0±1.7μM	van Baal, J. *et al.* (2005) *J. Biol. Chem.* 280, 9870-9878.
P2Y2	G_q				Greig, A.V.H. *et al.* (2003) *J. Invest. Dermatol.* 121, 315-327.
P2Y11	G_q/G_s				van Baal, J. *et al.* (2005) *J. Biol. Chem.* 280, 9870-9878.
PAR1	G_q/ $G_{12/13}$	Thrombin SFFLR-amide	9.6±2.0unit/ml 1.9±0.1μM	6.0±1.0unit/ml 5.0±0.4μM	van Baal, J. *et al.* (2005) *J. Biol. Chem.* 280, 9870-9878.
PAR2	G_q	Trypsin GLIGLR-amide GLIGKV-amide	98.0±27.8nM 2.3±0.6μM 6.1±1.0μM	45.7±5.8nM 2.5±0.3μM 3.8±0.4μM	Iwakiri, K. *et al.* (2004) *J. Invest. Dermatol.* 122, 937-944.

* The K_d value was determined based on the amplitude of P-DMR signal as a function of agonist concentration

The Nature of DMR Mediated Through G_q-Coupled Receptors: Thrombin Receptor

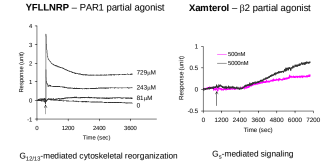


Mapping Signaling Network Interactions:
Bradykinin B_2 Receptor in A431 Cells

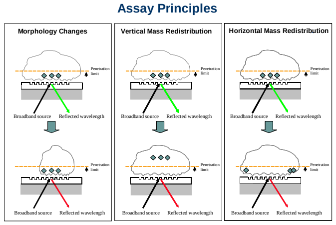


- B_2 receptor in A431 uses dual signaling pathways: G_s and G_q.
- The G_s and G_q signaling can cross-regulate each other.

Partial Agonist-Mediated GPCR Activation



Cell-Based Assays on the Epic™ System:
Assay Principles

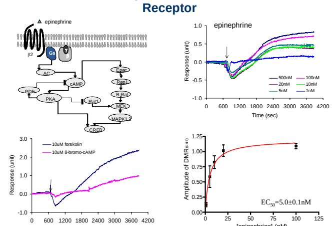


List of Agonists Examined for GPCRs That Are Unknown for Their Endogenous Level in A431

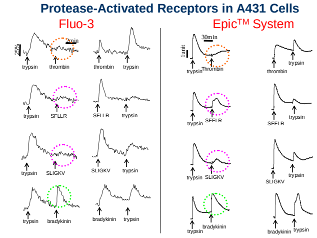
Receptor	Signaling	Agonist	Receptor	Signaling	Agonist
M1, M3,M5	Gq	Oxotremorine M	GALR2	Gq	galanin
M2, M4*	Gi	oxotremorine M	GALR2	Gq	galanin
α1A (ADRA1A)	Gq	oxymetazoline	GCGR	Gs	glucagon
α2A, B,C (ADRA2A,B,C)	Gi	UK14304, Clonidine	Y1,2,4,5,6 (NPY1,2,4,5,6R)	Gi	neuropeptide Y
β1 (ADRB1)	Gs	Xamterol	MC1,2,3,4,5R	Gs	β-MSH, α-MSH
AT1 (AGTR1)	Gq/Gi	angiotensin II	MOTR	Gi?	motilin
BB2,3 (BRS2,3)	Gq	Bombesin	NTSR1	Gq	Neurotensin, neuromedin N
CB1,2 (CNR1,2)	Gi	Anandamide	μ (OPRM1)	Gi/o	dynorphin A, DAMGO
CXCR1	Gi	IL-8	κ (OPRK1)	Gi/o	dynorphin A
CXCR3	Gi	IP-10	ORL1 (OPRL1)	Gi/o	nociceptin
D1 (DRD1)	Gs	A-77636, SKF38392	5-$HT_{1E/F}$ (HTR1E/F)	Gi/o	BRL54443
D5 (DRD5)	Gs	SKF38393	NK1 (TACR1)	Gq	substance P
ET-A,B (EDNRA,B)	Gq	endothelin-1	NK2 (TACR2)	Gq	neurokinin A
APJR (AGTRL1)	?	apelin 1-17	UT2 (GRP14)	Gq	urotensin II
GALR1,3	Gi/o	galanin	V1a, V1b	Gq	vasopressin

* Signatures were obtained with Epic™ system.

Signature of G_s-Coupled Receptors: β2 Receptor

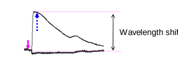


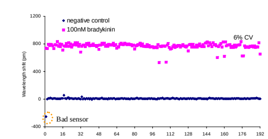
Receptor Activation and Desensitization:
Protease-Activated Receptors in A431 Cells



High-Throughput GPCR Cell-Based Assays:
End-Point Measurements

- End-point measurements
- Offline solution addition
- 384well Epic™ plate
- 5 minutes assay





Publications

Fang, Y., Li, G., and Peng, J. (2005) Optical biosensor provides insights for bradykinin B_2 receptor signaling in A431 cells. *FEBS Lett.* 579, 6365-6374.

Fang, Y., Ferrie, A.M., Fontaine, N.H., and Yuen, P.K. (2005) Characteristics of dynamic mass redistribution of EGF receptor signaling in living cells measured with label free optical biosensors. *Anal. Chem.* 77, 5720-5725.

Fang, Y., Ferrie, A.M., and Li, G. (2005) Probing cytoskeleton modulation by optical biosensors. *FEBS Lett.* 579, 4175-4180.

Fang, Y., Ferrie, A.M., Fontaine, N.H., and Yuen, P.K. (2005) Optical biosensors for monitoring dynamic mass redistribution in living cells mediated by epidermal growth factor receptor activation. *Proceedings of IEEE Engineering in Medicine and Biology, 361 (pp1-4).*

Fang, Y., Ferrie, A.M., Li, G., and Fontaine, N.H. (2006) Cellular functions of cholesterol probed with optical biosensors. *In Press.*

Fang, Y., Ferrie, A.M., and Fontaine, N.H. (2006) Resonant waveguide grating biosensors for living cell sensing. Submitted.

Fang, Y., and Ferrie, A.M. (2006) Signaling of protease-activated receptors in A431 cells: dynamic mass redistribution and regulation by cholesterol. *Submitted.*

Dynamic Mass Redistribution in Cell Biology:
Location, Location, Location – Signaling and Trafficking

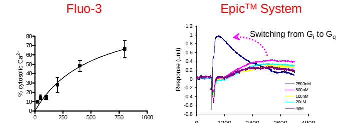
- Cellular targets are highly organized and spatially restricted
- The relocalization of proteins and molecular assemblies is fundamental to:
  - cell signaling by enabling precise control in its amplitude, duration and kinetics
  - cell functions – migration, invasion, growth, cycling, differentiation, survival, and death

There are many cellular events that lead to mass redistribution

- **Protein target or substrate recruitment**
  - to the nucleus
  - to the membrane
  - to the cytosol
  - throughout recycling pathways
  - to or from other organelles
  - uptake from extracellular space
- **Endocytosis and recycling**
- **Exocytosis**
- **Apoptosis**
- **Cytoskeleton rearrangement**

Hudder, A., et al. (2003) *Mol. Cell. Biol.* **23**, 9318-9326
Fang, Y., et al. (2005) *FEBS Lett.* **579**, 4175-4180
Kholodenko, B.N. (2003) *J. Exper. Biol.* **206**, 2073-2082.

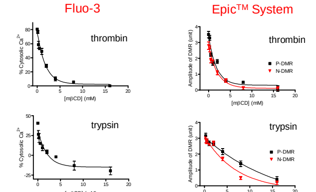
Switching of GPCR Signaling:
$LPA_{1,2,3}$ Receptors in A431 Cells



- G_q signaling occurs within the range of LPA doses examined.
- The DMR signals exhibited a switching from primarily G_i at low LPA doses to G_q at high LPA doses.

Ohta, H. *et al.*(2003) *Mol. Pharmacol.* 64, 994-1005.

Regulation of GPCR Signaling by Cholesterol:
Cholesterol Depletion with Methyl-β-cyclodextrin



Conclusions

- Epic™ System is also a scalable biosensor HTS platform for ***biochemical assays***.
- Epic™ System is a scalable biosensor HTS platform for ***cell-based G protein-coupled receptors drug discovery and deorphanization***.
- The Epic™ biosensor provides novel and unique signatures for all G_q-, G_s-, and G_i-coupled receptors.
  - Label-free
  - Standard SBS 96 well and 384 well microplate format
  - Endogenous GPCRs
  - Real physiological conditions
  - Multiplexing
  - High information content:
    - kinetics, efficacy/potency, signaling pathways, network interactions, signaling regulation, oligomerization, cross-communications, …