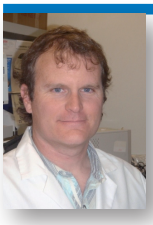
xCELLigence System Real-Time Cell Analyzer

Focus Application
Receptor Activity

Featured Study:
Functional Cell Profiling of Endogenous GPCRs using the xCELLigence System



Jeff Irelan
ACEA Biosciences, Inc., San Diego, USA.

Jonathan H. Morgan
Product Line Business Manager ATCC,
Manassas, VA, USA.

Introduction

G-coupled protein receptors (GPCRs), also known as 7-transmembrane proteins, constitute the single largest class of therapeutic targets for clinical and investigational drugs. There are >300 predicted functional members of this class in the human genome, involved in diverse signaling pathways in a wide array of cells and tissue types. Modulation of GPCR function has proven to have therapeutic benefit in a wide variety of diseases in immunology, neurology, cardiology, and oncology.

New technology for assaying receptor function in a cellular context has greatly increased the identification of novel regulators and reduced attrition rates (defined as failure in preclinical testing/clinical trials) for candidate compounds. GPCR function data obtained from basic research are however sometimes difficult to translate into a disease-relevant context. This is in part due to the fact that traditional cell-based assays use (1) artificial receptor overexpression often in a heterologous or a less biologically relevant cell type, (2) exogenous detection reagents such as a fluorescent labels, (3) invasive treatments such as dye loading and cell lysis, and (4) the generation of data for only single time point rather than continual cell monitoring.

The recent development of more biologically relevant assay systems should significantly improve the success rate of GPCR drug development and translational research (1). The xCELLigence System from Roche Applied Science assesses real-time endogenous GPCR function in disease-relevant cells without using exogenous labels. Cells are seeded onto plates containing microelectrodes, allowing precise measurement of subtle changes in cytoskeletal structure and cellular contraction induced by GPCR activation. GPCRs transmit extracellular signals by binding coupled guanine nucleotide-binding proteins, or G proteins, in the cytoplasm. All major second messenger pathways coupled to G proteins are known to activate some combination of cyclic AMP/protein kinase A, calcium/phospholipase C, β-arrestin/MAPK and the Rho family GTPases, resulting in morphological changes easily detected by cellular impedance recording (2).

In contrast to traditional assays, morphological impedance-based measurements can capture the aggregate effect of multiple signaling pathways. The advantages of assaying endogenous GPCR function include (1) assessing the target receptor at its normal expression level; (2) analyzing the natural interaction of receptors with regulatory partners including homo- or heterodimers; and (3) permitting the native coupling to intracellular G proteins. Use of label-free assay systems also significantly reduces reagent costs, because a single assay can measure all the second messenger pathways a given GPCR activates. Impedance-based real-time kinetic recordings can thus detect all the GPCR responses during the course of the experiment.

In the present study, we show that the xCELLigence System is a sensitive and robust assay for continually measuring endogenous GPCR function. A panel of 43 ligands encompassing 24 therapeutically relevant receptor families (see Table 1) was examined, producing functional GPCR profiles for the commonly used tumor cell lines, HeLa, U2OS, SH-SY5Y and CHO-K1, as well as the disease-relevant primary cells: human vascular endothelial cells and mixed renal primary epithelial cells.

Target Family	Ligand	HeLa	CHOK1	U2OS	SH-SY5Y	MREC	HUVEC
Histamine	Histamine						
Acetylcholine	Carbachol						
Adrenergic	Isoproterenol						
Glutamate	L-Glutamate						
Glutamate	(S)-3,5-DHPG						
Glutamate	(S)-MCPG						
Glutamate	DCGIV						
Glutamate	L-AP4						
Glutamate	LY342495						
Dopamine	Dopamine						
Serotonin	Serotonin						
Serotonin	8-OH-DPAT-HBr						
GABA	GABA						
Prostaglandin	PGE1						
Prostaglandin	PGE2						
Prostaglandin	Iloprost						
Glucagon	Glucagon						
Glucagon	GLP-1						
Glucagon	Exodus-2						
CC Chemokine	RANTES						
CC Chemokine	MIP-3b						
CXC Chemokine	AMD3100						
CXC Chemokine	SDF1a						
Calcitonin	Calcitonin						
Melanocortin	a-MSH						
Bradykinin	Bradykinin						
Ghrelin	Ghrelin						
Cannabinoid	Virodhamine						
Cannabinoid	ACEA						
Endothelin	Endothelin1						
Endothelin	[Ala1,3,11,15]-ET						
Lysophosholipid	LPA						
Lysophosholipid	S1P						
Lysophosholipid	SEW 2871						
Oxytocin	Oxytocin						
Melatonin	Melatonin						
Somatostatin	Somatostatin						
Vasopressin	AVP						
Purinerqic	ATP						
Free Fatty Acid	GW 9508						
Free Fatty Acid	Lauric Acid						
Free Fatty Acid	Acetate						
Free Fatty Acid	Butyrate						

= Max Cell Index above 3X standard deviation of buffer controls

= Min Cell Index below 3X standard deviation of buffer controls

Table 1: GPCR functional profiling summary

Materials and Methods

Cells and culture conditions: Cell lines were obtained from ATCC. HeLa (#CCL-2), U2OS (#HTB-96), SH-SY5Y (#CRL-2266), CHO-K1 (#CCL-61), human vascular endothelial cells (HUVEC; # PCS-100-010 lot # 58570370) and mixed renal primary epithelial cells (MREC; ATCC # PCS-400-012 lot # 58488854) were cultured according to ATCC recommendations, and expanded fewer than ten passages for tumor cell lines, and four passages for primary cell lines.

Assay procedure: All assays were performed in a tissue culture incubator (5% CO_2 at 37°C). Cells were plated in growth media at a seeding density of 1-2 x 10^4 cells per well in duplicate 96 well E-Plates and monitored overnight using the xCELLigence System, during which time cells grew to near confluence. After 18-24 hours in culture, compound dilution plates were prepared. Compounds previously prespotted at 2 µl volume in stock 96 well plates and stored at -20°C were thawed, were diluted by adding 132 µl of assay buffer (1 x HBSS; Sigma H8264; 20mM HEPES, Cellgro 25-060-Cl; 0.1% BSA, Fisher Scientific #BP1600). E-Plates 96 were removed from the station, washed once with assay buffer, and 140 µl assay buffer was added per well using a Biotek MicroFlo Select. E-Plates 96 were returned to the RTCA MP Station for 15 minutes and cells were allowed to equilibrate, and then removed for simultaneous addition of compounds in 10 µl assay buffer using a Beckman Multimek 96, for a final concentration of 10 µM for small molecules and 1 µM for peptides.

Data analysis: Data from each well were normalized to the time just before compound addition using the RTCA Software. For each compound class, data for cells in each well had background values subtracted using the mean value of all cells in control wells containing correspondingly diluted compound solvent, DMSO for small molecules and 0.1% BSA for peptides.

Resulting data were exported to Microsoft Excel for the remaining calculations. A maximum and minimum Cell Index value was identified for cells in each test well, and the average and standard deviation of the mean calculated from replicates (n = 2). For cells in control wells, the standard deviation of the mean of eight wells for each replicate was determined. These values were then averaged for replicates and multiplied by three to calculate the "hit" threshold. For Z factor and EC_{50} calculations, a single time point was identified as optimal for each cell line and compound combination using the kinetic response profile produced by the RTCA MP Instrument.

Results

To assure reproducibility, cells were obtained from a proven source, guaranteeing cell quality and identity. All experiments described were performed on cells obtained from the American Type Tissue Collection (ATCC) cell bank. Cells were maintained according to the recommended conditions after a limited number of passages before assaying.

Endogenous GPCR assay robustness. HeLa cells were tested under various conditions for responses to activators of endogenous GPCRs. In addition, the calcium ionophore, A23187, was tested as a positive control. At low concentrations, A23187 is known to mimic calcium mobilization due to activation of a Gq-coupled GPCR response. This response is useful in developing assay conditions in a variety of cells because it is independent of the expression of GPCRs. The most robust responses were observed in confluent plated cells grown overnight. At this time, growth media were exchanged with an assay buffer commonly used for GPCR assays (see Materials and Methods for assay condition details).

These assay conditions were used to evaluate the responses of several different cell lines to A23187 and to sphingosine 1 phosphate (S1P), an endogenous agonist for the ubiquitously expressed GPCR family, the lysophospholipid or endothelial differentiation gene (EDG) receptors. The Z factor metric, which takes into account both the signal-to-noise ratio and variability of an assay, was used to assess assay robustness (3). Cells in eight replicate wells were treated with S1P at a final concentration of 1 µM or the equivalent concentration of its buffer vehicle (0.1% BSA), and A23187 at a final concentration of 100 nM or the equivalent concentration of its buffer vehicle (DMSO). Cell responses were monitored in real-time at one-minute intervals using the xCELLigence System.

Initial cellular responses were detected within minutes of agonist addition. Maximal responses were observed during the first 30-40 minutes for each cell line (see Figure 1). HeLa cells, as shown, produced a maximal response for A23187 and near-maximal response to S1P approximately 30 minutes after treatment. For this time point, the Z factors were 0.83 and 0.90 for A23187 and S1P, respectively. At least one of the two treatments produced Z factor scores above 0.5 for each cell type tested, indicating a very robust assay (see Table 2). In contrast, the primary renal mixed primary epithelial cells (RMEC), produced a Z factor of approximately 0.25 for these two ligands, indicating a marginally significant assay. Subsequent GPCR screening however revealed that several other GPCRs produced a much more robust response for this cell type.

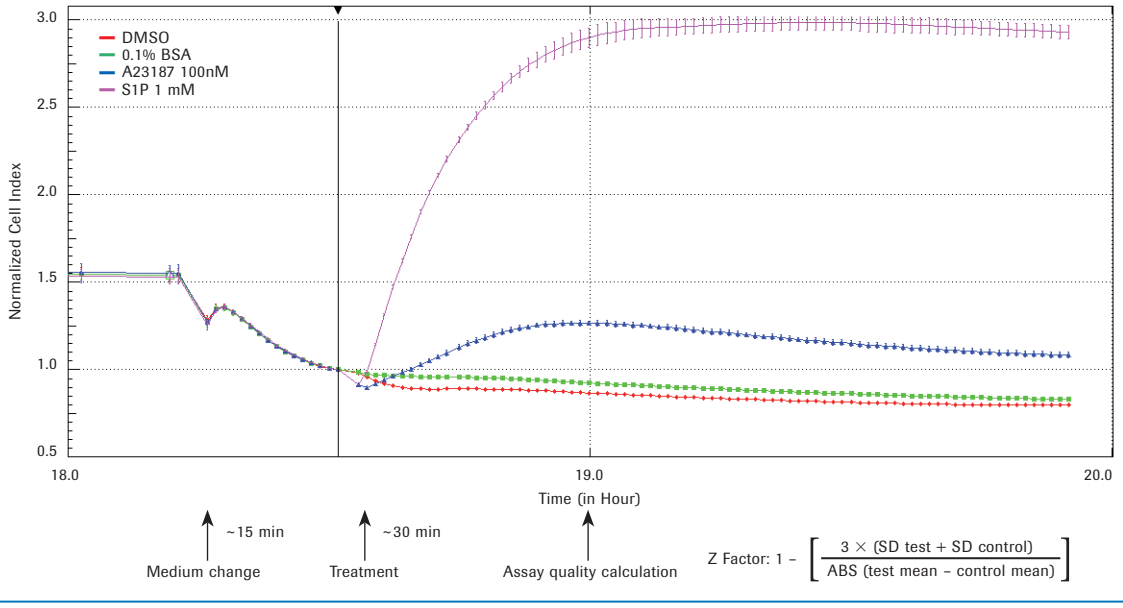


Figure 1: Evaluation of GPCR assay robustness. HeLa cells were seeded at 10,000 cells per well in an E-Plate 96 and grown in the xCELLigence RTCA MP Instrument placed in an incubator overnight. Growth media were then removed, assay buffer added, and after 15 minutes of continuous cell monitoring, compounds that modulate GPCRs were added and cellular responses recorded every minute for an additional 1.5 hours. Time-dependent cell response profiles were obtained for the mean of cells in eight treated culture wells. Error bars represent the standard deviation of the mean. Assay quality was calculated from Z factor values at a single time point 30 minutes after compound addition. DMSO is the vehicle control for A23187; 0.1% BSA for S1P. **SD** = standard deviation, **ABS** = absolute value.

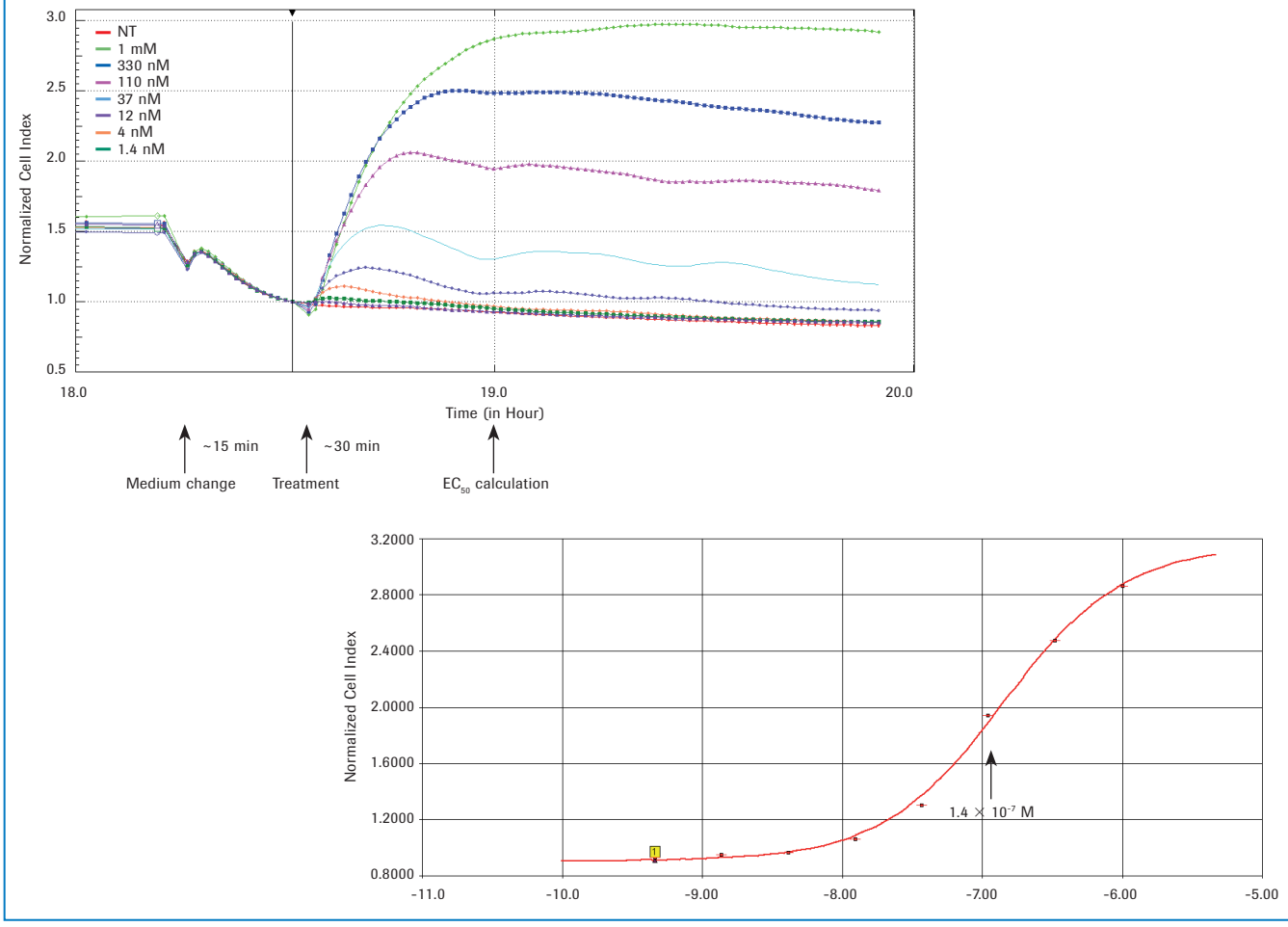


Figure 2: Evaluation of GPCR assay sensitivity. HeLa cells were assayed as shown in Figure 1, and assessed for cell responses to an 8-point dosage treatment series of each compound. Assay sensitivity was determined by calculating EC_{50} values for each compound 30 minutes after compound addition using the RTCA Software.

Endogenous GPCR assay sensitivity. We also used A23187 and S1P to test assay sensitivity, using an eight point dose-response curve (see Figure 2). The concentration of agonist producing 50% maximal response (EC_{50} value) was calculated by the RTCA Software at the same time point used for Z factor determination.

EC_{50} values for each cell line tested are shown in Table 2. Each cell type tested was very sensitive to A23187 treatment, and with the exception of CHO-K1, all cell types were very sensitive to S1P treatment, producing EC_{50} values in the low 10^{-7} to 10^{-9} range. Although CHO-K1 cells express EDG receptors (4), and scored as positive for both S1P and lysophosphatidic acid (LPA) responses in subsequent testing (see below), the dosages used here were apparently not sufficient to saturate the biological response in order to generate an accurate EC_{50}.

Endogenous GPCR functional profiles. After optimizing assay parameters, a panel of 43 small molecule and peptide modulators of therapeutically relevant GPCRs was used to examine functional responses in the cell types used in this study (see Table 1). The 24 receptor families targeted by this panel contain fifty potentially responsive receptors, representing all major GPCR coupling types (Gs, Gi, Gq, $G_{12/13}$). Each of the cell types was tested in duplicate plates using the assay procedure described above.

	S1P	A23187	S1P	A23187
Cells	Z factor	Z factor	EC50	EC50
HeLa	0.90	0.82	$1.4 \times 10^{(-7)}$	$4.8 \times 10^{(-8)}$
CHO-K1	0.56	0.75	$1.0 \times 10^{(-6)}$	$6.2 \times 10^{(-8)}$
U2OS	0.52	0.73	$3.1 \times 10^{(-7)}$	$3.7 \times 10^{(-8)}$
SH-SY5Y	0.16	0.65	$9.8 \times 10^{(-9)}$	$2.5 \times 10^{(-7)}$
MREC	0.25	0.25	$1.1 \times 10^{(-8)}$	$3.4 \times 10^{(-9)}$
HUVEC	0.35	0.51	$1.2 \times 10^{(-8)}$	$4.2 \times 10^{(-7)}$

Table 2: Assay development and evaluation summary

The four tumor cell lines, HeLa, CHO-K1, U2OS and SH-SY5Y, each produced a distinct time-dependent cellular response profile (TCRP) for the panel of compounds (data not shown). We determined the maximal and minimal Cell Index value for each well, irrespective of time point, and plotted the mean and standard deviation of that value (see Figure 3). GPCR functional profiles revealed distinct differences in the increase or decrease in Cell Index values for ligands that elicited responses. For example, HeLa cells exhibited a large number of positive Cell Index changes, while CHO-K1 cells had many more negative Cell Index changes, including GPCRs in the same receptor family, such as the prostanoid receptors activated by PGE1, 2 and Iloprost (see Figure 3). These results indicate that cell background differences can significantly alter the morphological response to the same stimuli, possibly due to differences in G protein coupling, or perhaps due to differences in the downstream signal transduction pathways leading to changes in cell morphology. The overall pattern of Cell Index values also mirrored the relative specificity of the different GPCR ligands. SDF1α , the endogenous agonist for CXCR4, elicited a strong positive Cell Index response only in HeLa cells, whereas calcitonin resulted in a strong negative Cell Index response only in CHO-K1 cells. We also tested the panel of GPCR ligands in two additional tumor cell lines, SH-SY5Y neuroblastoma cells and U2OS osteosarcoma cells, as well as two primary cell types, human vascular endothelial cells (HUVEC) and mixed renal primary epithelial cells (abbreviated here as MREC).

To determine which ligands are active in which cell lines, we compared the maximal and minimal Cell Index response values to the intrinsic variation measured in the eight control wells. Cells in each test well showing a Cell Index value above or below three standard deviations relative to cells in control wells were deemed to be showing an active response. Ligands eliciting such active responses are shown in Figures 3 and 4. A summary of the corresponding endogenous GPCR identified for each cell type is presented in Table 1.

Fourteen different receptor families were identified as actively responsive in one or more of the cell types tested. In many cases, the endogenous ligands used in the present study were capable of activating multiple members of the same receptor family. For example, serotonin is known to activate all of the endogenous serotonin receptors, comprising several subfamilies, some of which have multiple isoforms. In summary, xCELLigence System Cell Index profiling proved to be a robust way to functionally assay fourteen different GPCR families in one or more tumor cell types and the primary cells used in the present study.

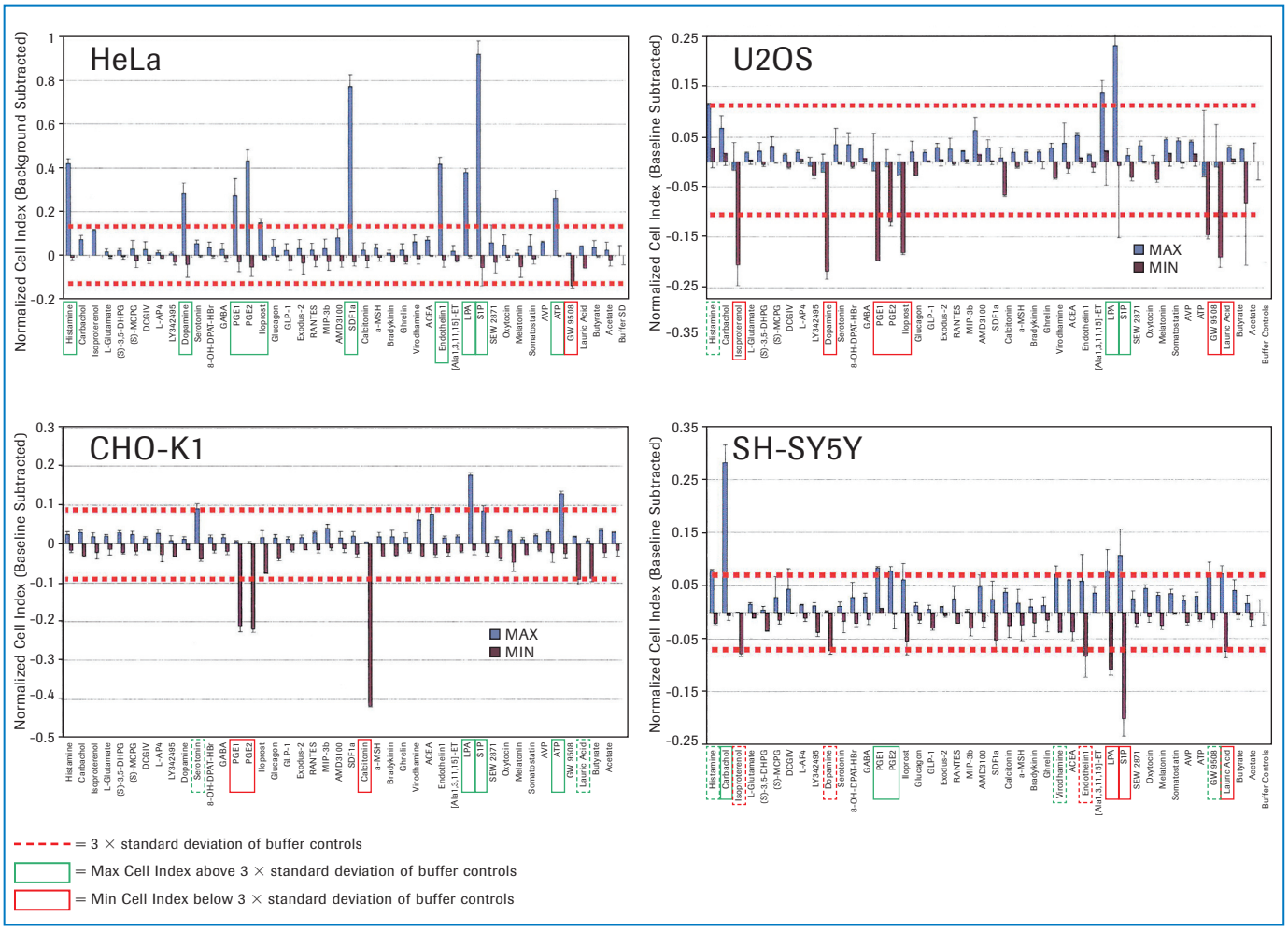


Figure 3: GPCR functional profiling of tumor cell lines. Cells of the indicated type were assayed as shown in Figure 1, and assessed for responses to a panel of 43 GPCR modulators in single wells on replicate plates. Maximal and minimal Cell Index values were determined for each well (see Materials and Methods). Data were normalized by subtracting the mean baseline of the Cell Index values for the set of buffer control wells at each time point using the RTCA Software (see text for details). Cells Index values for cells in control wells are thus zero. Error bars represent the standard deviation of those wells throughout the monitored time window. Replicate Cell Index values for treated cells in wells were plotted as the mean of two replicate plates (n = 2). Error bars represent one standard deviation between replicate Cell Index values. The **red dotted line** represents the value of three standard deviations relative to the control wells. Compounds that clearly produced an increase (**green line**) or decrease (**red line**) in Cell Index values are highlighted by **solid boxes**; those showing statistically insignificant effects (just below the three standard deviation-cutoff) are shown as **dotted boxes**.

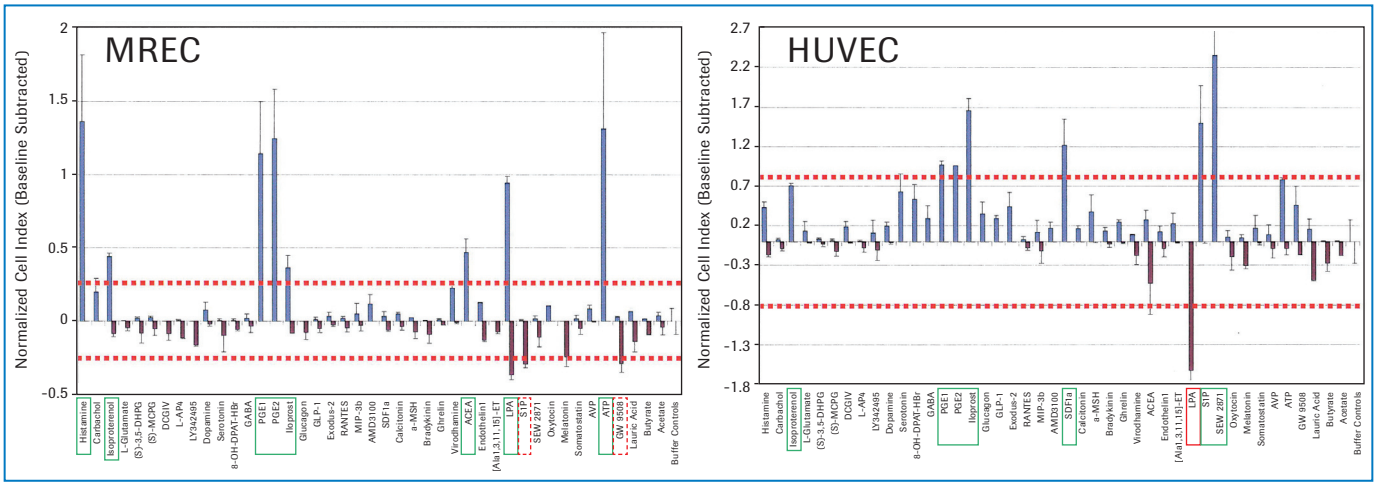


Figure 4: GPCR functional profiling of primary cells. Primary cells of the indicated cell type were assayed as shown in Figure 3.

Discussion

Label-free cell-based assays performed using the xCELLigence System can accelerate the process of drug discovery, by more quickly translating basic research data for possible use in clinical settings. Here we show that the function of a wide variety of GPCRs can be assayed using the xCELLigence System in tumor cell lines and primary cells. Most of the receptor target GPCR families tested produced robust responses greater than three standard deviations above the mean relative to cells in control wells, in one or more of the cell lines used in the present study. Testing a wider variety of cell types in a similar manner should significantly increase the number of endogenous GPCR assays now shown on this list. Some of the ligands tested are known to activate multiple members of the same GPCR family. Additional experiments using selective agonists and antagonists combined with gene expression profiling and siRNA knockdown of individual receptors should allow for identification of the specific receptor subtypes responsible for the morphological changes detected using the xCELLigence System.

Conclusion

- The xCELLigence System can be used to assay the function of a wide variety of endogenous GPCRs.
- These GPCR assays are very sensitive and robust.
- GPCR assays can be performed on both cancer cell lines and primary cells.

References

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Ordering Information

Product	Pack Size	Cat. No.
RTCA Analyzer	1 instrument	05 228 972 001
RTCA MP Station	1 instrument	05 331 625 001
RTCA Control Unit	1 instrument	05 229 014 001
E-Plate 96	6 plates	05 232 368 001
E-Plate 96	6 x 6 plates	05 232 376 001

Intended Use:
For life science research only. Not for use in diagnostic procedures.

Key Words:
Endogenous GPCRs, xCELLigence System, impedance measurement, functional profiling

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