

# Label-Free Cell-Based Assays for GPCR Screening

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**Abstract:** G protein-coupled receptors (GPCRs) have been proven to be the largest family of druggable targets in the human genome. Given the importance of GPCRs as drug targets and the de-orphanization of novel targets, GPCRs are likely to remain the frequent targets of many drug discovery programs. With recent advances in instrumentation and understanding of cellular mechanisms for the signals measured, biosensor-centered label-free cell assay technologies become a very active area for GPCR screening. This article reviews the principles and potential of current label-free cell assay technologies in GPCR drug discovery.

**Keywords:** G protein-coupled receptor, optical biosensor, resonant waveguide grating biosensor, electrical biosensor, dynamic mass redistribution, impedance, high throughput screening.

G-protein-coupled receptors (GPCRs) are the largest family of cell surface receptors that share common structural motif – seven  $\alpha$ -helical transmembrane-spanning domains joined by intra- and extracellular loops [1]. GPCRs are expressed in virtually all tissues, with distinct expression patterns in different cell systems [2]. The extracellular ligands for GPCRs are diverse, including biogenic amines, amino acids, ions, small peptides, proteins, and bioactive lipids [3]. This diversity of GPCR activators underscores the physiological importance of this receptor class – GPCRs control a wide variety of physiological processes, such as neurotransmission, chemotaxis, inflammation, and cell proliferation. It is no surprise that GPCRs have been implicated in almost every major disease class, including asthma, cancer, and inflammatory and cardiovascular diseases [4-6].

Given their importance in health and disease together with their ability for therapeutic intervention by small molecule drugs, GPCRs represent the largest and most successful class of druggable targets in the human genome [7-10]. It is estimated that the human genome encodes as many as 1,000 GPCRs, of which ~400 are non-chemosensory receptors, predicted to bind endogenous ligands. Many of these (~150) are classified as orphan GPCRs for which their cognate ligands or biological functions are unknown. De-orphanization of these receptors could bring novel therapeutic targets and opportunities to the industry [11, 12], in light of the enormous therapeutic success of this class of drug targets. The future potential becomes even much clearer when one considers that approximately 50% of all clinically available drugs are active, directly or indirectly, on this family of receptors, yet these drugs are active only on small percentages of all known GPCRs [7].

Continued success in GPCR drug discovery and development has seen an evolution in assay technologies and methodologies. Assays for GPCR screening can be broadly

classified into whole cell assays and cell-free assays. Cell-based assays provide a highly accurate representation of cellular behavior in response to stimulation. Compared to the data obtained using cell-free assays, direct measurements of compound-modulated GPCR functions in cell systems offer far more useful information, such as the action, mode and mechanisms of compounds under conditions more closely resembling the physiological environment. In large measure, these benefits have helped drive the increasing use of whole cell systems for drug screening and testing in the last decade [13-15]. Given the availability of various comprehensive reviews of assay technologies for GPCR screening [16-25], this article is focused primarily on recent advances in label-free cell-based assays that make use of biosensor technologies for GPCR screening.

## GPCR SIGNALING

GPCRs participate in a wide array of cell signaling pathways, primarily mediated through their coupled G proteins [26, 27]. GPCR signaling is encoded by the spatial and temporal flux of downstream signaling networks, which are tightly controlled by intracellular signaling and regulatory machineries [28]. The consensus models describing GPCR signaling assume a receptor as a functional monomeric entity interacting through its specific intracellular domains with a single G protein, once stabilized in its active conformation(s) by agonist binding. The binding of a ligand to a GPCR results in changes in the conformation of the receptor [29, 30]. The receptor activation in turn leads to the activation of an associated G protein heterotrimer through the GTP-GDP exchange on  $G_{\alpha}$  subunit. The activated G protein then modulates the activity of several intracellular enzymes, which in turn control the production of several key intracellular second messengers such as cyclic AMP (cAMP), cGMP,  $Ca^{2+}$ , inositol triphosphate, and arachidonic acid. These second messengers then act on several downstream targets including ion channels and kinases that regulate gene transcription and cell functions. For example,  $G_s$ -coupled receptor signaling proceeds through sequential activation of the receptor, G protein, and adenylyl cyclase (AC) at the plasma membrane, and increased accumulation of a diffusible second messenger

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cAMP, and activation of cAMP-activated protein kinase (protein kinase A, PKA). This pathway governs multiple cellular machineries, including ion channels, transcription factors, cytoskeletal proteins, and metabolic enzymes. Furthermore, common to almost all GPCRs is rapid attenuation of the receptor responsiveness upon agonist stimulation (known as desensitization), followed by receptor resensitization after removal of agonist [31-33].

A growing body of evidence suggests the prevalence of GPCR dimers (or higher order oligomers), which can form among identical or different receptors [34-38]. It has been reported that oligomerization of some receptors is modulated by agonists [39, 40]. However, many studies suggest that GPCR oligomerization is constitutive [41]. For example, some receptors may be synthesized as an oligomeric unit [42]; and oligomerization of some receptors is required for their appropriate cell surface expression [43]. Biophysical [44], bioinformatics [45] and evolutionary trace analysis [46] suggests that interactions between receptors in a dimeric assembly likely involve conformational changes at the dimer interface. The receptor oligomerization has been shown to have effects on ligand binding, receptor activation, desensitization and trafficking, as well as receptor signaling [34-38]. However, much remains to be elucidated about the formation, regulation, and functional and physiological consequences of receptor oligomerization, particularly under endogenous cell systems [47]. Nonetheless, the possibility of

receptor oligomerization in the plasma membrane greatly diversifies their pharmacological and physiological properties, and has implications for pharmacological interventions.

### LABEL-DEPENDENT CELL-BASED ASSAYS

An important aspect of GPCR signaling is that it consists of a series of spatial and temporal events. Each discrete cellular event exhibits its own characteristics in terms of kinetics, dynamics, amplitude and location, depending on the cellular context of the cell system studied. For example, the kinetics of cellular events mediated through receptor activation greatly differs, ranging from milliseconds (e.g., GPCR conformational changes), tens of seconds (e.g.,  $\text{Ca}^{2+}$  flux), minutes (e.g., cytoskeletal modulation, morphological changes), to hours (e.g., change in gene transcription) [23, 48-50]. A ligand-induced cellular event could also have distinct dynamics (e.g., cycling or oscillation) [51]. In the context of assays, virtually every single GPCR signaling event has been utilized as the basis of various assay technologies. Table 1 summarizes common cell-based assays widely used for GPCR screening [52]. Since most of these cell-based assays require a certain degree of engineering, manipulation, or labeling, these assays are referred to as label-dependent cell-based assays.

*Assays to measure changes in second messenger* – GPCRs transmit signals mainly through their coupled G proteins, and lead to changes in intracellular levels of second

**Table 1. Comparison of Various Cell-Based Assays for GPCR Screening**

Cellular Event Measured	Technology Variants	Applicability to GPCRs/Ligands
Change in intracellular cAMP	TRF (DELIFA) EFC TR-FRET cAMP-gated $\text{Ca}^{2+}$ ion channel	$G_s/G_i$
Change in intracellular calcium	Fluorescent dyes (FLIPR) Aequorin	$G_q/G_{16}$
Change in reporter gene activity	$\beta$ -Lactamase(intact cells) Luciferase (fixed cells) $\beta$ -Galactosidase(fixed cells) Nitroreductase (intact cells)	$G_i$ (NFAT) $G_s/G_i$ (CRE)
Protein-protein interaction	FRET BRET TR-FRET EFC	Variable
Trafficking	$\beta$ -Arrestin-GFP GPCR-GFP GPCR/CypHer5-antibody	Universal Universal Universal
Integrated cellular response	Optical biosensor Electrical biosensor	Universal Universal
Phenotype	Melanophore phenotype	$G_s/G_i$

\*DELIFA — dissociation enhanced lanthanide fluoroimmunoassay; TRF — time-resolved fluorometry; EFC — enzyme fragment complementation; FRET — fluorescence resonance energy transfer; TR-FRET — time-resolved fluorescence resonance energy transfer; BRET — bioluminescence resonance energy transfer; GFP — green fluorescent protein; cAMP — 3',5'-cyclic adenosine mono-phosphate; FLIPR — fluorometric imaging plate reader.

messengers such as cAMP, inositol triphosphate, and  $\text{Ca}^{2+}$ . Direct measurement of intracellular levels of these messengers upon stimulation is a popular functional assay format for GPCR screening in mammalian cells. Examples are highly sensitive calcium flux assays such as FLIPR (fluorometric imaging plate reader) and Aequorin assays for receptors coupled to  $G_q$  proteins or promiscuously coupled to  $G_{15/16}$  proteins. For cAMP assays, changes in intracellular cAMP, induced by the activation of  $G_s$  or  $G_i$ -coupled receptors, are generally detected by the competition between cellular cAMP and a labeled form of cAMP for binding to an anti-cAMP antibody [23]. Alternatively, a modified rat olfactory cyclic nucleotide gated (CNG) channel, which is engineered such that it enhances the cAMP binding affinity but reduces the cGMP binding affinity, is used as a cAMP biosensor for  $G_s$  and  $G_i$  GPCR screening (BD ACTOne, BD Biosciences, Rockville, MD).

**Assays to measure protein-protein interaction** – The recent realization that GPCRs potentially function as homo-oligomeric and hetero-oligomeric complexes [34-47] has given rise to rationale to the development of assays based on protein-protein interactions. These assays are useful not only for elucidating the nature of receptor interaction within the oligomeric complexes, but also for monitoring ligand-induced reorientation of an existing oligomer, particularly relative to its bound G protein subunits. These assays have also been used to study distinct protein interactions during GPCR signaling cycle, including the interactions of ligand-activated receptor with its coupled G proteins, or other regulatory proteins such as GRKs and beta-arrestins.

These assays typically utilize either resonance energy transfer (RET) [36, 38, 41] or protein fragment complementation [54-56]. The RET is a non-radiative transfer of energy between a donor and an acceptor when they are in proximity, with an efficiency that varies inversely with the sixth power of the distance between the two molecules. The donor moiety can either be a fluorophore (FRET) or a bioluminescent enzyme that emits light upon oxidation of a substrate (BRET). In both cases, the acceptor is a fluorophore. The changes in RET in response to stimuli offers an effective means for real-time monitoring of dynamic protein-protein interactions that are involved in GPCR activation and regulation in cells.

Alternatively, protein fragment complementation assays utilize a pair of recombinantly engineered and inactive fragments of either an enzyme (e.g.,  $\beta$ -galactosidase [54]) or a fluorescent protein (e.g., green fluorescent protein (GFP) [55,56]), each being recombinantly attached to a protein. When the receptor activation leads to the interaction between the two proteins having the complementary fragments, this interaction drives the functional complementation of the protein mutant fragments. GPCR activation is measured directly by quantifying the restored activity of the enzyme or fluorescence of the fluorescent protein. Since protein fragment complementation is an amplified signal detection system, exquisite detection sensitivity is achieved, permitting analysis of protein interactions at concentrations approximating their normal levels of expression. Although protein interaction-driven functional complementation of 2 split fragments is reversible, the rate of association of the two yellow fluorescent protein (YFP) fragments fused to Fos and Jun has

been reported to be much slower ( $t_{1/2}$  of ~60sec) than the subunit exchange rate of Fos-Jun heterodimers ( $t_{1/2}$  of ~10 ec) [56]. Such slow kinetics is mainly due to relatively weak affinity between the 2 split YFP fragments, and is typically slower than the reorientation of emitter/acceptor fluorescent proteins. Thus, cautious is warranted to interpret the kinetics of protein-protein interactions measured using protein fragment complementation assays.

**Assays to measure protein trafficking** – GPCRs modulate diverse physiological signaling pathways by virtue of changes in receptor activation and inactivation states. Functional changes in receptor state lead to dynamic trafficking of the receptor [57, 58] and many of its downstream signaling molecules (e.g., arrestins [59], protein kinase C [51]) at various stages of GPCR signaling cycle. Direct visualization of protein trafficking provides the basis for high-content screening [26]. Since this technology requires no prior knowledge of the interacting G protein, it is well suited for screening orphan GPCRs. However, these trafficking assays suffer drawbacks associated with the fact that some agonists, potentially partial agonists, could stimulate receptor signaling without causing the desensitization seen when the receptor is stimulated by its native ligand [60]. For example, morphine, a partial agonist, exhibits relatively poor activity to cause internalization of  $\mu$ -opioid receptor [61].

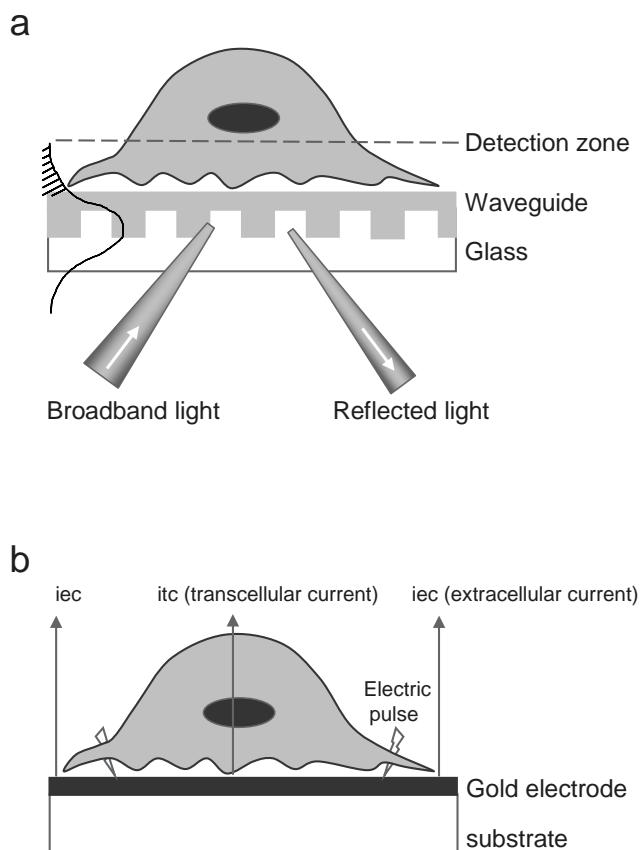
**Assays to measure changes in gene reporter activity** – GPCR activation is well known to alter gene transcription. Several genes contain elements responsive to second messengers induced by the activation of GPCRs, thus providing the basis for reporter gene assays [18,23]. Modulation of intracellular cAMP by  $G_s$  or  $G_i$  GPCRs is detected with a reporter gene, whose transcription is regulated by the transcription factor cAMP response-element binding protein (CREB) binding to upstream cAMP response elements (CREs). Calcium flux, mobilized by  $G_q$ -coupled receptors, is detected using a reporter gene, whose transcription is regulated by the calcium-sensitive AP1 (activator protein 1) or NFAT (nuclear factor of activated T cells) elements. Commonly used reporter genes include  $\beta$ -galactosidase, GFP, luciferase, and  $\beta$ -lactamase.

**Assays to measure changes in phenotype** – Receptor activation ultimately leads to changes in cell phenotype. One example is the melanophore technology (Arena Pharmaceuticals, San Diego, CA), which uses GPCR targets expressed in frog skin cells containing a pigment that is highly sensitive to changing levels of cAMP [3, 16]. The pigment disperses throughout the cell and causes the cell to appear black when the intracellular cAMP level increases; conversely, when the cAMP level decreases, the pigment aggregates at the center of the cell, causing the cell to appear clear.

## LABEL-FREE CELL-BASED ASSAYS

Label-free cell-based assays generally employ a biosensor to monitor ligand-induced responses in living cells. A biosensor typically utilizes a transducer such as an optical, electrical, calorimetric, acoustic, or magnetic transducer, to convert a molecular recognition event or a ligand-induced change in a cell layer into a quantifiable signal. These label-free biosensors are commonly used for molecular interaction analysis, which involves characterizing how molecular complexes form and disassociate over time. Many comprehen-

sive reviews are available for different aspects of these biosensors for bio-molecular interaction analysis [62-66]. This section highlights the applications of these biosensors for whole cell sensing, particularly for functional GPCR screening. Fig. 1 highlights two types of biosensors that are currently used as the basis for label-free cell-based assays – resonant waveguide grating (RWG) biosensors [67] and electrical biosensors [68-70].



**Fig. (1).** Principles of two types of biosensors for living cell sensing. **(a)** A RWG biosensor for monitoring ligand-induced dynamic mass redistribution in living cells. Cells are directly cultured onto the surface of a biosensor. The biosensor consists of a glass substrate, a waveguide thin film within which a grating structure is embedded. Only the mass redistribution within the bottom portion of cells is directly measured. **(b)** An electric biosensor for monitoring the ionic environment surrounding the biosensor and the cells. Cells are cultured on the surface of a biosensor having arrayed gold microelectrodes. Both flows of extracellular (iec) and transcellular (itc) current are measured, and a low AC voltage at variable frequencies is applied to the cell layer.

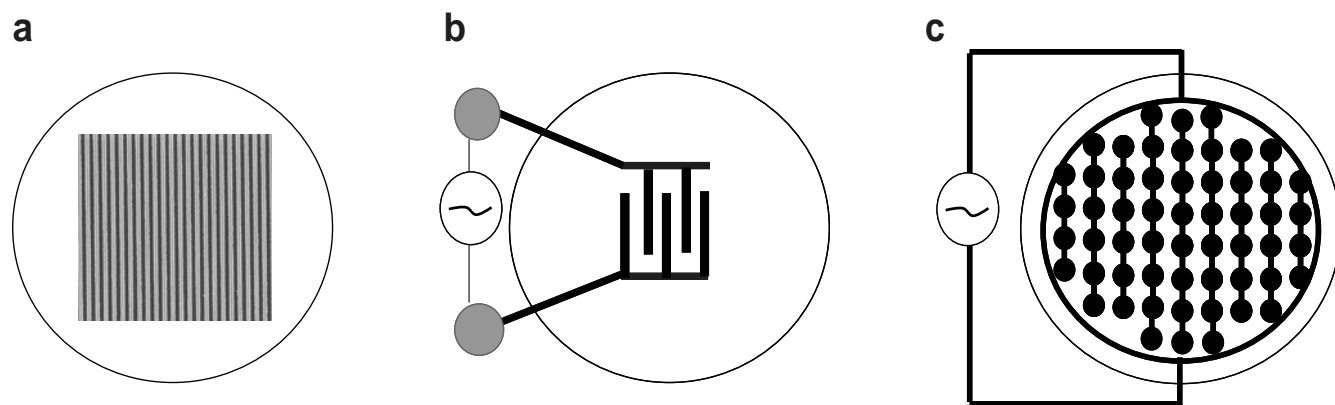
**RWG biosensors and systems** – An RWG biosensor consists of a substrate (e.g., glass), a waveguide thin film with an embedded grating structure, and a cell layer (Fig. 1a). The RWG biosensor utilizes the resonant coupling of light into a waveguide by means of a diffraction grating, leading to total internal reflection at the solution-surface interface, which in turn creates an electromagnetic field at the interface. This electromagnetic field is evanescent in nature [71], meaning

that it decays exponentially from the sensor surface; the distance at which it decays to  $1/e$  ( $e$  is a numerical constant that is equal to 2.71828) of its initial value is known as the penetration and is a function of the design of a particular RWG biosensor, but is typically on the order of  $\sim 200\text{nm}$  [72]. This type of biosensor exploits such evanescent wave to characterize ligand-induced alterations of a cell layer at or near the sensor surface [73].

RWG instruments can be subdivided into systems based on angle-shift or wavelength-shift measurements [67]. In a wavelength-shift measurement, polarized light covering a range of incident wavelengths with a constant angle is used to illuminate the waveguide; light at specific wavelengths is coupled into and propagates along the waveguide. Alternatively, in angle-shift instruments, the sensor is illuminated with monochromatic light and the angle at which light is resonantly coupled is measured. The resonance conditions are influenced by the physical properties of the cell layer that contacts with the surface of a biosensor (e.g., cell confluency, adhesion and status such as proliferating or quiescent states). When a ligand or an analyte interacts with a cellular target (e.g., a GPCR, a kinase) in living cells, any change in local refractive index within the cell layer can be detected as a shift in resonant angle (or wavelength) [74-77].

The Corning<sup>®</sup> Epic<sup>®</sup> system uses RWG biosensors for label-free biochemical or cell-based assays (Corning Inc., Corning, NY). The Epic<sup>®</sup> System consists of a RWG plate reader and Society for Biomolecular Screening (SBS) standard microtiter plates (Fig. 2a) [78]. The detector system in the plate reader exploits integrated fiber optics to measure shifts in wavelength of the incident light, as a result of ligand-induced changes in cells. A series of illumination/detection heads are arranged in a linear fashion, so that reflection spectra are collected simultaneously from each well within a column of a 384-well microplate. The whole plate is scanned so that each sensor can be addressed multiple times, and each column is addressed in sequence. The wavelengths of the incident light are collected and used for analysis. A temperature-controlling unit is built in the instrument to minimize spurious shifts in the incident wavelength due to any temperature fluctuations.

**Electrical biosensors and systems** – Electrical biosensors consist of a substrate (e.g., plastic), an electrode, and a cell layer (Fig. 1b). In this electrical detection method, cells are cultured on small gold electrodes arrayed onto a substrate, and the system's electrical impedance is followed with time. The impedance is a measure of changes in electrical conductivity of the cell layer. Typically, a small constant voltage at a fixed frequency or varied frequencies is applied to the electrode or electrode array, and the electrical current through the circuit is monitored over time. The ligand-induced change in electrical current provides a measure of cell response. The application of impedance measurements for whole cell sensing was first realized in 1984 [79]. Since then, impedance-based measurements have been applied to study a wide range of cellular events, including cell adhesion and spreading [80], cell micromotion [81], cell morphological changes [82], and cell death [83, 84]. Classical impedance systems suffer from high assay variability due to use of a small detection electrode and a large reference electrode [84]. To overcome such variability, the latest generation of



**Fig. (2).** Schematic drawings showing the surface configurations of three types of biosensor-embedded microplates used for label-free cell-based assays. **(a)** A well of a 384-well Epic<sup>®</sup> RWG biosensor microplate. The inset shows a scanning electronic microscopic graph of a portion of the biosensor. **(b)** A well of a 96-well CellKey gold electrode-embedded microplate. **(c)** A well of a 96-well RT-CES gold electrode-embedded microplate.

systems, such as CellKey system (MDS Sciex, South San Francisco, CA) and RT-CES (ACEA Biosciences Inc., San Diego, CA), utilize an integrated circuit having a microelectrode array.

The CellKey system consists of an environmentally controlled impedance measurement system, a 96-well electrode-embedded microtiter plate, an onboard 96-well fluidics, and custom acquisition and analysis software [68]. The cells are seeded in the culture wells; each well has an integrated electrode array (Fig. 2b). The system operates using a small-amplitude alternating voltage at 24 frequencies, from 1 KHz to 10 MHz. The resultant current is measured at an update rate of 2 sec. The system is thermally regulated and experiments can be conducted between 28°C and 37°C. A 96-well head fluid delivery device handles fluid additions and exchanges onboard.

The RT-CES system is composed of four main components: electronic microtiter plates (E-Plate<sup>™</sup>), E-Plate station, electronic analyzer, and a monitoring system for data acquisition and display [69]. The electronic analyser sends and receives the electronic signals. The E-Plate station is placed inside a tissue culture incubator. The E-Plate station comes in three throughput varieties: a 16x station for running six 16-well E-Plates at a time, a single 96-well E-Plate station, and the Mult-E-Plate<sup>™</sup> station, which can accommodate up to six 96-well E-Plates at a time. The cells are seeded in E-Plates, which are integrated with microelectronic sensor arrays (Fig. 2c). The system operates at a low-voltage (less than 20 mV) AC signal at multiple frequencies.

**Optical signals of GPCR activation with RWG biosensor** – Cells are dynamic objects with relatively large dimensions – typically tens of microns. RWG biosensors enable detection of ligand-induced changes within the bottom portion of cells, determined by the penetration depth of the evanescent wave. Furthermore, the spatial resolution of an optical biosensor is determined by the spot size (~100 microns) of the incident light source. Thus, a highly confluent cell layer is generally used in order to achieve optimal assay results; and the sensor configuration can be viewed as a three-layer waveguide composite, consisting of a substrate, waveguide thin film and a cell layer. Following a 3-layer

waveguide biosensor theory [70] in combination with cellular biophysics [85, 86], we found that for whole-cell sensing, a ligand-induced change in effective refractive index, the detected signal  $\Delta N$ , is governed by [73]:

$$\begin{aligned} \Delta N &= S(C)\Delta n_c \\ &= S(C)\alpha d \sum_i \Delta C_i \left[ e^{-\frac{-z_i}{\Delta Z_c}} - e^{-\frac{-z_{i+1}}{\Delta Z_c}} \right] \end{aligned} \quad (1)$$

where  $S(C)$  is the system sensitivity to the cell layer, and  $\Delta n_c$  is the ligand-induced change in local refractive index of the cell layer sensed by the biosensor.  $\Delta Z_c$  is the penetration depth into the cell layer,  $\alpha$  is the specific refractive index increment (about 0.18/mL/g for proteins),  $z_i$  is the distance where the mass redistribution occurs, and  $d$  is an imaginary thickness of a slice within the cell layer. Here the cell layer is divided into an equal-spaced slice in the vertical direction. We assumed that the detected signal is, to first order, directly proportional to the change in refractive index of the bottom portion of cell layer  $\Delta n_c$ . The  $\Delta n_c$  is directly proportional to changes in local concentration of cellular targets or molecular assemblies within the sensing volume, given that the refractive index of a given volume within cells is largely determined by the concentrations of bio-molecules, mainly proteins [85, 86]. A weighted factor  $\exp(-z_i/\Delta Z_c)$  is taken into account for a change in local protein concentration occurring, considering the exponentially decaying nature of the evanescent wave. Thus, the detected signal is a sum of mass redistribution occurring at distinct distances away from the sensor surface, each with unequal contribution to the overall response. Eq. 1 suggests that the detected signal with an RWG biosensor is sensitive primarily to the vertical mass redistribution, as a result of any change in local protein concentration and where and when it occurs. The detected signal is often referred to as a dynamic mass redistribution (DMR) signal.

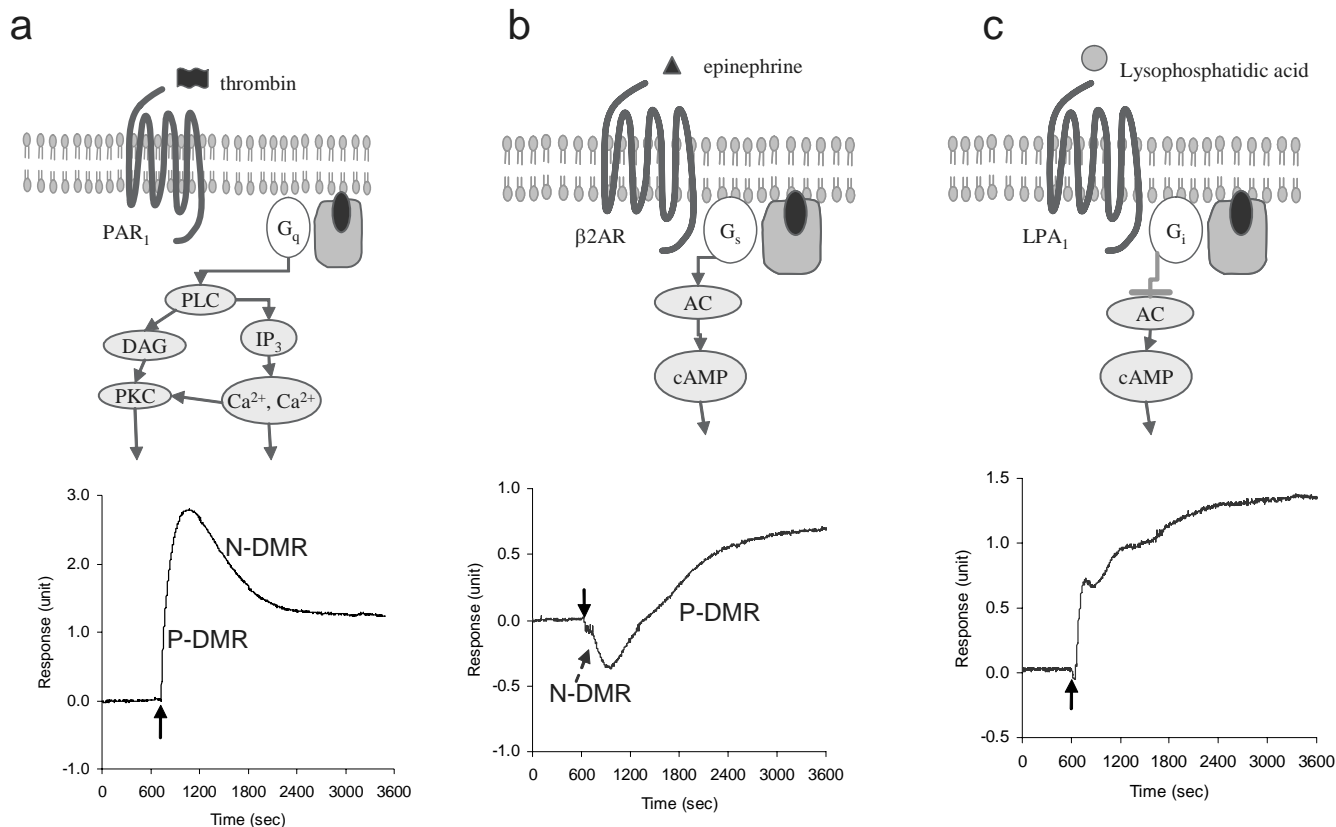
GPCR activation leads to a series of spatial and temporal events, including ligand binding, receptor activation, protein recruitment, receptor internalization and recycling, second messenger alternation, cytoskeletal remodeling, gene expression, and cell adhesion changes, to name but a few. Each cellular event has its own characteristics regarding its kinet-

ics, duration, amplitude, and mass movement. Thus it is reasonably assumed that these cellular events may contribute differently to the overall DMR signal, depending on the location where they occur. Using a panel of agonists targeting a variety of GPCRs, we identified three classes of DMR signals in human epidermoid carcinoma A431 cell, which reflect the signaling pathways mediated [87] (Fig. 3). Since each is correlated with the activation of a class of GPCRs depending on the G protein with which the receptor is coupled, the DMR signals obtained were named  $G_q$ -,  $G_s$ - and  $G_i$ -DMR signals, respectively. Each class of DMR signals exhibits distinct kinetic and dynamic characteristics, reflecting the unique signaling integration mediated through different classes of GPCRs. Interestingly,  $G_q$ -type DMR signal appears to be rapid, whereas  $G_s$ -type DMR signal is comparatively slow. Our recent chemical biology and orthogonal fluorescence imaging studies suggest that  $G_q$ -DMR signal is downstream of  $Ca^{2+}$  mobilization [88], whereas  $G_s$ -DMR signal is downstream of cAMP accumulation [89; and unpublished data].

Following classical receptor biology, a numerical analysis suggests that protein translocation and receptor internalization are two primary resources for the DMR signatures observed for  $G_q$ -coupled receptor signaling [73]. Unique to  $G_q$ -coupled receptor signaling is the dramatic translocation of its signaling components, including several protein kinase C (PKC) isoforms, GPCR kinases (GRKs),  $\beta$ -arrestin, phosphatidylinositol phosphate (PIP)-binding proteins, and di-

acylglycerol (DAG)-binding proteins, to name but a few [51,90]. The protein trafficking mediated by the activation of  $G_q$ -coupled receptors is relatively rapid and become obvious shortly after agonist stimulation [51], leading to a rapid  $G_q$ -type DMR signal. On the other hand, we have found that an increase in cell adhesion is the major contributor to the positive-DMR (P-DMR) event in the  $\beta_2$ AR ( $\beta_2$  adrenergic receptor) signal induced by epinephrine [89, unpublished data] (Fig. 3b). The occurrence of the initial negative-DMR (N-DMR) event reflects the fact that the majority of downstream signaling components directly involved in the  $\beta_2$ AR signaling complexes, with the exception (thus far) of AKAPs (A-kinase anchoring protein) and  $\beta$ -arrestins, are already compartmentalized at or near the cell membrane [91-94]. Thus, for  $G_s$ -coupled receptor the recruitment of intracellular targets to the activated receptors is much less pronounced than  $G_q$ -coupled receptor signaling, and is overwhelmed by other cellular events leading to the decrease in local mass density in cells. Our recent confocal fluorescence imaging studies suggest that the increase in cell adhesion appears to be evident 10 min. after the activation of  $G_s$ -coupled receptors, consistent with the slow P-DMR event of the  $G_s$ -DMR signals (unpublished data).

To date, we have found that both  $G_q$  and  $G_s$ -DMR signals are quite universal across multiple cell lines tested, although their fine features differ, reflecting the important roles of cellular context in GPCR signaling [78, 87, unpublished data]. Our current understanding is incomplete for the  $G_i$ -



**Fig. (3).** The GPCR signaling and its DMR signal. (a)  $G_q$ -signaling and its DMR signal (CHO cells responding to 40unit/ml thrombin). (b)  $G_s$ -signaling and its DMR signal (A431 cells responding to 25 nM epinephrine). (c)  $G_i$ -signaling and its DMR signal (A431 responding to 200nM LPA). The broken arrows indicate the time when the agonist solution is introduced (Reproduced with permission from ref. [87], Copyright Elsevier, 2007).

DMR signals, which exhibit strong dependence on the cellular context (unpublished data).

**Bioimpedance signals of GPCR activation** – In a typical impedance-based cell assay, cells are brought into contact with a gold electrode arrayed on the bottom of culture wells. The total impedance of the sensor system is determined primarily by the ion environment surrounding the biosensor [70, 95]. Under application of an electrical field, the ions undergo field-directed movement and concentration gradient-driven diffusion. For whole cell sensing, the total electrical impedance has four components: the resistance of the electrolyte solution, the impedance of the cell, the impedance at the electrode/solution interface, and the impedance at the electrode/cell interface. In addition, the impedance of a cell comprises two components – the resistance and the reactance. The conductive characteristics of cellular ionic strength provide the resistive component, whereas the cell membranes, acting as imperfect capacitors, contribute a frequency-dependent reactive component [96, 97]. Thus, the total impedance is a function of many factors, including cell viability, cell confluency, cell numbers, cell morphology, degree of cell adhesion, ionic environment, the water content within the cells, and the detection frequency.

In the RT-CES system, a percentage of a small voltage applied is coupled into the cell interior. Such signals applied to cells are believed to be much smaller than the resting membrane potential of a typical mammalian cell and thus present minimal or no disturbance to cell function. The RT-CES system measures changes in impedance and displays it as a parameter called cell index. The cell index is calculated according to the formula [95].

$$CI = \max_{i=1,\dots,N} \left( \frac{R_{cell}(f_i)}{R_0(f_i)} - 1 \right) \quad (2)$$

where N is the number of frequency points at which the impedance is measured (e.g., N=3 for 10 kHz, 25 kHz, and 50 kHz), and  $R_0(f)$  and  $R_{cell}(f)$  are the frequency electrode resistance without cells or with cells present in the wells, respectively.

In the CellKey system, a change in sensor system's impedance is attributed to a change in complex impedance ( $\Delta Z$  or  $dZ$ ) of a cell layer that occurs in response to re-

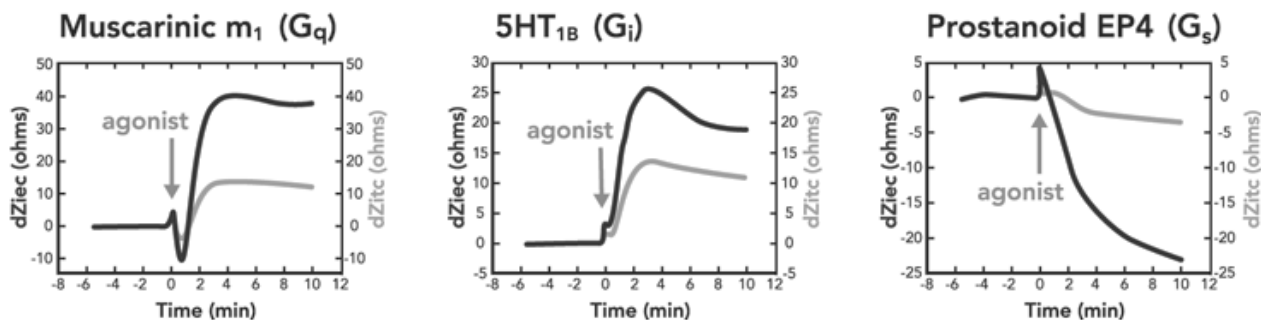
ceptor stimulation [68]. At low frequencies, the small voltage applied induces extracellular currents (iec) that pass around individual cells in the layer. However, the conduction currents through cell membrane due to ion channels may also be important at low measurement frequencies [97]. At high frequencies, they induce transcellular currents (itc) that penetrate the cellular membrane (Fig. 1b). The ratio of the applied voltage to the measured current for each well is its impedance ( $Z$ ) as described by Ohm's law.

When cells are exposed to a stimulus, such as a receptor ligand, signal transduction events are activated that lead to complex cellular events that cause changes in cell adherence, cell shape and volume, and cell-to-cell interaction. These cellular changes individually or collectively affect the flow of extracellular and transcellular current, and therefore, affect the magnitude and characteristics of the measured impedance. Fig. 4 shows three types of impedance signals mediated through the activation of three classes of GPCRs, depending on the G protein with which the receptor is coupled [68, 98]. The profiles are obtained using CellKey system. Similar profiles were also recorded using the RT-CES system [99]. It is believed that these impedance signals are due to the different effects on the actin cytoskeleton that affect the cellular parameters measured by impedance, in response to the activation of different classes of GPCRs. It has been shown that activation of  $G_q$  [100, 101] and  $G_i$  GPCRs [102, 103] leads to increased actin polymerization, while stimulation of  $G_s$  GPCRs leads to actin depolymerization [104].

#### LABEL-FREE CELL-BASED ASSAYS FOR GPCR SCREENING

**Receptor panning** – Label-free cell-based assays are capable of monitoring the activity of different classes of GPCRs, offering a universal assay platform for assaying endogenously expressed and functionally active receptors in a cell system. Panning of a GPCR ligand library, known to activate many families of GPCRs, would allow one to reliably map endogenous receptors in a cell system [87].

It is also known that more than one family member of many GPCR families are often endogenously expressed in a single cell system, such as purinergic P2Y receptors in A431 cells as well as human embryonic kidney (HEK) cells. In addition, a ligand including naturally occurring agonists



**Fig. (4).** The impedance signals of  $G_q$ ,  $G_i$  and  $G_s$  GPCRs. The profiles are obtained in Chinese ovary hamster (CHO) cells stably expressing rat muscarinic receptor subtype 1 (CHO $m_1$ ) using the CellKey system. The agonists are carbachol ( $M_1$ ), 5-hydroxytryptamine (5HT $_{1B}$ ) and prostaglandin E2 (prostanoid EP $_4$ ). Both 5HT $_{1B}$  and EP $_4$  receptors are endogenously expressed (Reproduced with permission from ref. [70], Copyright Elsevier, 2005).

could have cross-activity, but often different efficacies, to activate distinct members of a receptor family. Since the biosensor measures an integrated cellular response [67, 73], a ligand-induced biosensor signal may represent the signaling mediated through the activation of multiple receptors. Determining the efficacies (i.e.,  $EC_{50}$  and the maximal responses) of panels of non-selective and selective agonists against the receptor family should enable the assessment of which family member is dominated in the cell system, wherein multiple family members are co-endogenously expressed [87]. The cell system- or tissue-specific functional activity of a GPCR might indicate its physiological roles, or hold potential for the side-effect evaluation of a drug candidate. The ability of receptor panning at both the cell system and receptor family levels allows one to easily screen disease-relevant cell types, and enables more physiologically relevant selectivity and specificity screens.

Using Corning® Epic® System, we have found that in A431 cells multiple families of GPCRs are endogenously expressed and active [87]. Moreover, at least three types of GPCRs,  $G_q$ -,  $G_s$ - and  $G_i$ -coupled receptors, co-exist in A431 cells. Interestingly, the maximal amplitudes of the DMR signals are found to be well correlated with the maximal increase in intracellular  $Ca^{2+}$  level induced by several  $G_q$ -coupled receptor agonists, as well as the expression pattern of these receptors, indicating that the ligand-induced DMR signal is sensitive to the expression level of its cognate receptor.

Researchers at MDS Sciex also have applied the CellKey system to identify functional endogenous receptors in cells commonly used in drug discovery, such as HEK293, and U-2 OS cells [68, 98]. Receptor panning has resulted in the identification of many functionally active, differently coupled endogenous receptors, some of which have not been previously documented in the literature.

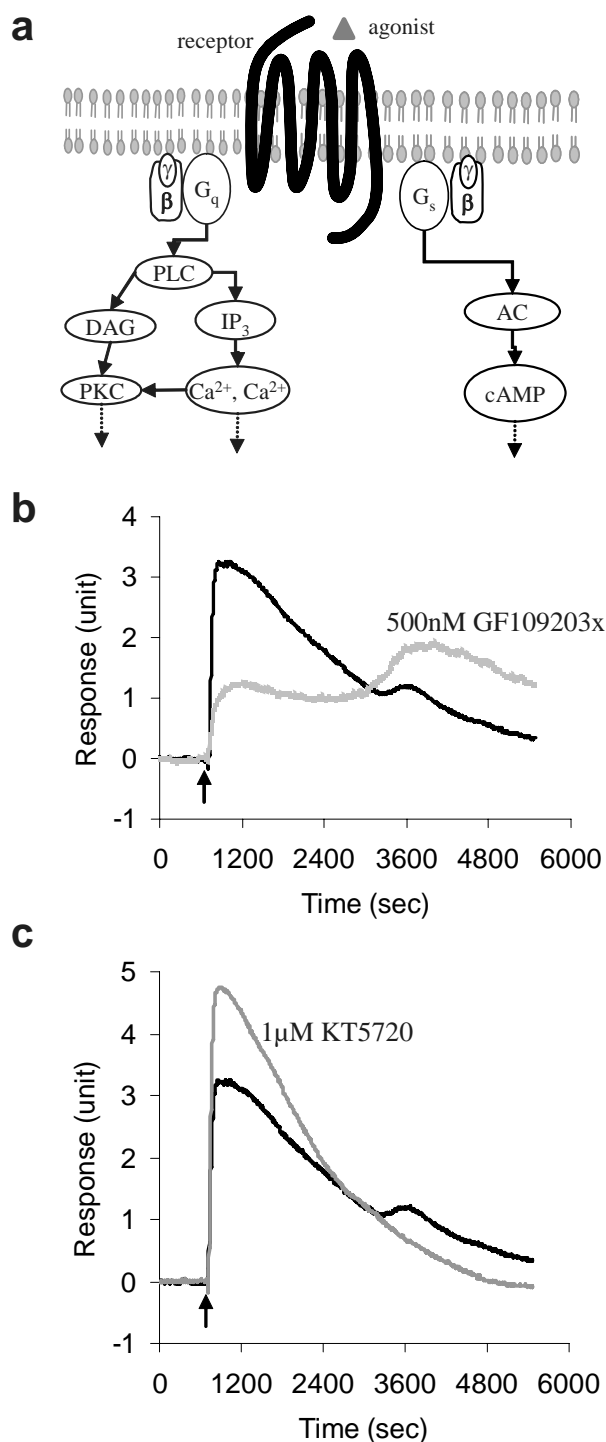
**Systems cell biology studies of GPCR signaling** – Cells rely on highly dynamic network interactions in their decision-making processes. An exogenous signal typically affects the functionality of specific target(s), thus resulting in the execution of various cellular machineries in order for the cell to accommodate such a signal. Much information is available for the signaling of GPCRs *via* either G protein-dependent or independent mechanisms [26, 27]. However, as a result of the complexity involved, the network interactions of GPCR signaling cascades are still far from being completely understood. Label-free cell assay technologies typically measure an integrated cellular response, such as DMR signals obtained with RWG biosensors, or the impedance signals obtained with electrical biosensors. We have shown that the DMR signal is a novel, quantifiable and global representation signature for cell signaling [75, 88, 89, 105]. The DMR signal mediated through a receptor is an integrated cellular response that reflects the native signaling pathways mediated through either a receptor, an unknown receptor, a receptor in different cellular contexts, or even the same receptor by different ligands. Thus, these biosensors enable systems cell biology studies of GPCR signaling.

Combined with chemical biology, cell biology and biophysical approaches, we have studied the systems cell biol-

ogy of GPCR signaling using RWG biosensors [88, 89]. Chemical biology, which uses chemicals for intervention of a specific cell-signaling component, has become popular in addressing biological questions. This is made possible by the identification of a great number of modulators that specifically control the activities of many different types of cellular targets. We have adopted this approach to map the signaling and network interactions mediated through a receptor, including the endogenous  $\beta_2$ ARs [89] and the bradykinin  $B_2$  receptors [88] in A431 cells. For example, stimulation with bradykinin of quiescent A431, obtained by continuous culturing using 0.1% fetal bovine serum for 20 hrs after the cells reach ~90% confluency, resulted in a dose-dependent and saturable DMR response, which can be inhibited by a  $B_2$  specific antagonist HOE140 in a dose-dependent manner, suggested that the DMR signal is a direct result of  $B_2$  receptor activation. The sensitivity of the DMR signal to cholesterol depletion by methyl- $\beta$ -cyclodextrin argued that  $B_2$  receptor signaling is dependent on the integrity of lipid rafts; disruption of these microdomains hinders the  $B_2$  signaling. Modulations of several important intracellular targets with specific inhibitors suggested that  $B_2$  receptor activation results in signaling *via* at least dual pathways –  $G_s$ - and  $G_q$ -mediated signaling, and the two signaling pathways counter-regulate each other (Fig. 5). Several critical downstream targets including PKC, PKA, dynamin, and epidermal growth factor receptor had been identified to involve in  $B_2$  receptor signaling. These findings suggest that the RWG biosensor appears enable the analysis of GPCR signaling.

Similarly, impedance-based biosensors have been used for studying complex signal transduction of GPCRs, including human melanin-concentrating hormone receptor 1 (MCHR1) expressed heterologously in CHO and U2-OS cells and endogenously in MOLT4 cells. The biosensor enables a dose-dependent differentiation of pertussis toxin-sensitive and  $Ca^{2+}$ -coupled events simultaneously [68].

**Ligand pharmacological profiling** – GPCR ligands display broad variations in their power to prompt receptor activation [106-109]. This intrinsic ligand/receptor property, known as efficacy, displays a wide range, including full agonism, partial agonism and inverse agonism [110]. The efficacy of a ligand is usually determined by the extent of the ligand-induced optimal receptor configuration for G protein activation [29, 30]. However, mounting evidences suggest that cell-specific parameters, such as the ratio of active to inactive receptor species, the rate constant for G protein activation, and expression levels of receptors and G proteins, are also important in influencing ligand agonism [111]. Consequently, affinity and efficacy are often considered as properties that have independent and distinct structural requirements. It is quite common that the efficacy of a specific agonist can greatly vary, depending on which event is measured. Agonists such as dopamine that result in the formation of intermediate activation states act as a strong partial agonist for the  $\beta_2$ AR promoting interactions with  $G_{s\alpha}$ , but is only marginally effective at promoting the  $\beta_2$ AR internalization [112]. These findings suggest that the efficacy of a ligand, instead of being linear (i.e., controlling receptor activation also controls the full spectrum of GPCR behaviors such as desensitization, internalization, and phosphorylation) should be viewed as “collateral” [113].



**Fig. (5).** RWG biosensors enable the analysis of complex GPCR signaling. (a) The dual signaling pathways of endogenous bradykinin B<sub>2</sub> receptors in A431 cells. (b,c) The modulation profiles of the bradykinin-induced DMR signal by GF109203x or KT5720, respectively. The quiescent A431 cells were pretreated with either compound for about 1 hr, before the stimulation with bradykinin at 16nM. GF 109203x is a PKC inhibitor, where KT 5720 is a PKA inhibitor. The black curves in (b,c) represent the bradykinin response without any pretreatment. The solid arrows in (b,c) indicate the time when bradykinin is introduced (Reproduced with permission from ref. [88]).

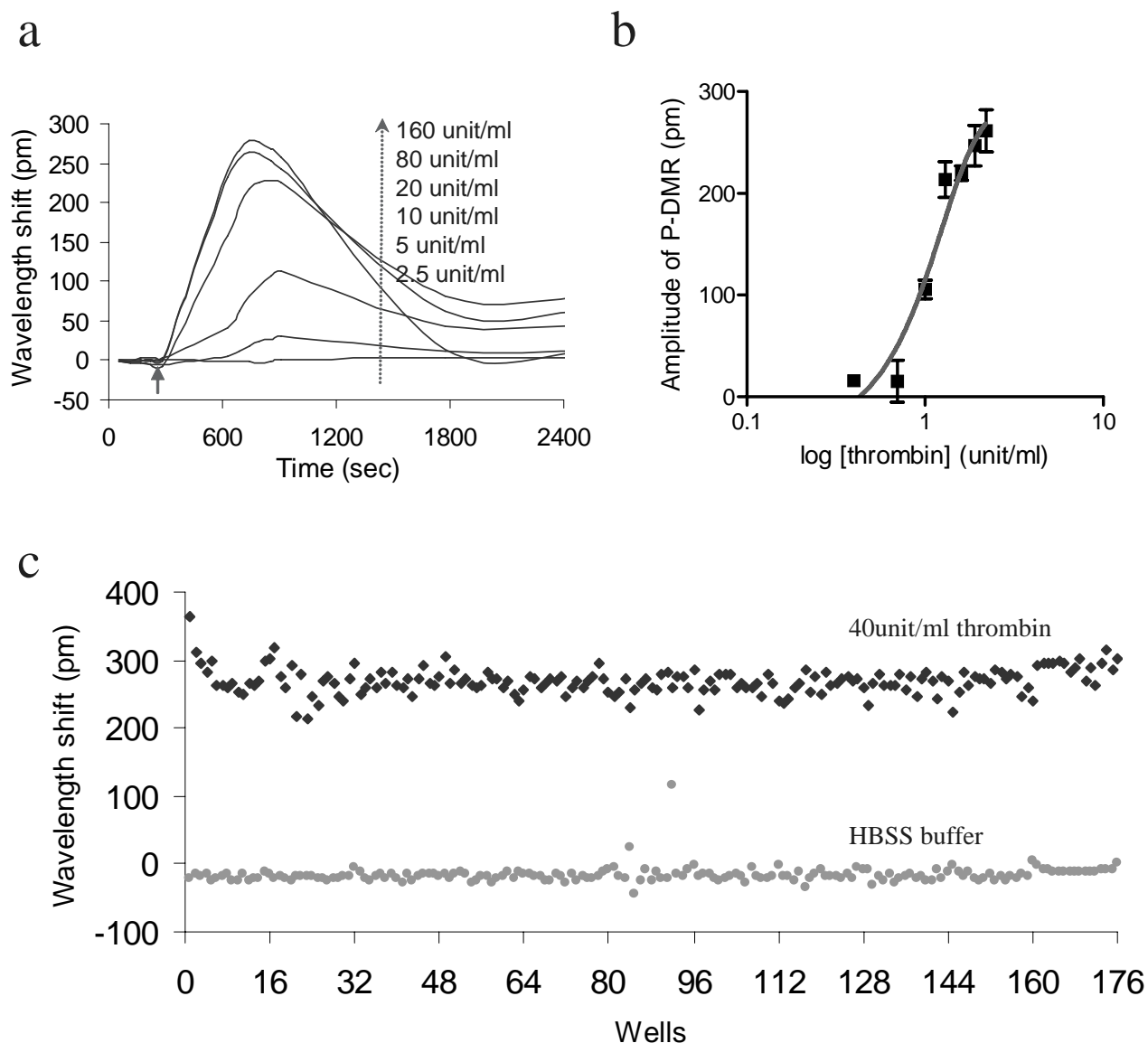
The non-invasive and manipulation-free feature of biosensor-based cell assays, particularly optical biosensors, provides a powerful means to generate pharmacologically relevant data. Biosensor-based cell assays offer a universal assay platform for conducting pharmacological evaluation (hit confirmation, potency ranking, efficacy analysis, selectivity analysis, Schild analysis) of ligands across a spectrum of receptors and cell types irrespective of the coupling mechanism.

Using Epic<sup>®</sup> system, we have examined a broad spectrum of GPCR ligands for a number of receptors. The biosensor can distinguish full agonists, partial agonists, antagonists, and allosteric modulators in a single assay [89; unpublished data]. Interestingly, salbutamol, a weak binder to β<sub>2</sub>AR [114], acts as a full agonist with high efficacy (~nanomolar) to mediate a G<sub>s</sub>-type DMR signal in A431 cells, suggesting that the efficacy of GPCR agonists is indeed collateral. These results suggest that RWG biosensors enable the study of receptor biology and ligand pharmacology in living cells under physiologically relevant conditions. However, since the DMR signal is an integrated cellular response consisting of contributions of many cellular events involving dynamic redistribution of cellular matters within the bottom portion of the cells, biosensor-based assays can be susceptible to interference from off-target effects. This could be complicated by the fact that multiple subclasses of receptors are expressed in a given type of cells, and an agonist could have cross-activity among these subclass members of receptors. For example, we had observed a dose-dependent switching from a G<sub>i</sub>-type to a G<sub>q</sub>-type DMR signal in A431 cells in response to lysophosphatidic acid (LPA) stimulation [87]. In A431, RT-PCR studies have shown that A431 cells endogenously express three family members of the LPA receptors: LPA<sub>1</sub>, LPA<sub>2</sub> and LPA<sub>3</sub> [115]. LPA<sub>1</sub> is mainly a G<sub>i</sub>-coupled receptor, where the other two are G<sub>q</sub>-coupled receptors. Such DMR signal switching induced by increasing LPA doses supports the hypothesis that at low doses, LPA preferably activates the LPA<sub>1</sub> receptor that leads to G<sub>i</sub>-signaling. When the concentration of LPA increases, the endogenous LPA<sub>2</sub> and LPA<sub>3</sub> receptors become activated, leading to G<sub>q</sub>-signaling. This is consistent with Fluo-3 assays, which show that high doses of LPA are required to induce Ca<sup>2+</sup> mobilization. Alternatively, since either of LPA receptors can couple to more than one type of G proteins, such DMR signal switching observed may reflect the possibility of dose-dependent switching of the coupling of a LPA receptor to different G proteins through an unknown mechanism. Previously, the switching of the coupling of β<sub>2</sub>-AR from G<sub>s</sub> to G<sub>i</sub> proteins was found to be mediated by protein kinase A [116].

Recently, the CellKey system was also used for examining activation of endogenously expressed receptors in primary cells [68], providing a technique with results that are more predictive of the efficacy and safety of drug compounds acting on GPCRs *in vivo*. The ability to generate pharmacology on native receptors expressed in their native cells allows the scientist to bring more relevant biology to the drug discovery process and has the potential to identify compounds that would be otherwise undetectable or misjudged in artificial systems.

**High throughput screening** – Among the three label-free cell assay systems discussed, only the Epic<sup>®</sup> System permits high throughput screening (HTS) with 384-well SBS compatible microplates. The Epic<sup>®</sup> System is designed to operate either as a standalone workstation, or integrated with external liquid handling devices and others instruments. This ability to integrate with existing lab automation equipment enables the system to be used in HTS for effectively conducting compound screening. Through this process one can rapidly identify active compounds, which modulate the activity of a GPCR of interest. Based on the overall dynamics and well-characterized kinetics of the DMR signal induced by a particular ligand, one can readily develop endpoint meas-

urements for HTS applications. Recently we have demonstrated that the Epic<sup>®</sup> System is amenable to high throughput screening against endogenously expressed GPCRs. Examples include the bradykinin B<sub>2</sub> receptors and the β<sub>2</sub>ARs in A431 cells, and the protease activated receptor subtype 1 (PAR<sub>1</sub>) in Chinese hamster ovary (CHO) cells [78]. Based on the kinetics of agonist-mediated optical signals, two time points, one being before and another being 5 min. after the stimulation, were chosen to develop high throughput screening assays for both B<sub>2</sub> receptors in A431 and PAR<sub>1</sub> in CHO cells. Fig. 6a shows dose-dependent kinetic responses of A431 cells upon stimulation with thrombin. Thrombin mediates an apparently saturable G<sub>q</sub>-type DMR signal in CHO



**Fig. (6).** The Epic<sup>®</sup> System enables HTS GPCR screening. (a) The kinetic response of CHO cells in response to thrombin at different doses. The solid arrow indicates the time when thrombin is introduced. (b) The wavelength shift, obtained using endpoint measurements, as a function of thrombin concentration. (c) The wavelength shift, obtained using endpoint measurements, as a function of wells. Here in a 384-well plate having CHO cells at ~90% confluency, 11 columns were stimulated with thrombin, the second set of 11 columns were treated with the assay buffer vehicle only (1xHBSS), while the rest (2 columns) were stimulated with thrombin at different doses (Reproduced with permission from ref. [64], Copyright Elsevier, 2006).

cells. The amplitude of P-DMR signal, as measured using the endpoint measurements and shown in Fig. 6b, also appear to be saturable to thrombin concentration, leading to an EC<sub>50</sub> of 8.3unit/ml. The biosensor-based thrombin assays using A431 cells are robust, as shown in Fig. 6c. The Z-factor was found to be around 0.70 [78]. Results suggest that such endpoint measurements enable not only high throughput screening of compounds using endogenous GPCRs, but also determination of agonist efficacies and potencies.

**Hits to leads** – Once a hit is selected from primary screening, the action of the hit on a cell system can be systematically studied using label-free cell assay technologies. Dose responses can be generated to calculate the efficacy or potency of the hits in a cell system or several cell systems, which more closely resemble the native environment. In addition, the biosensor-based cell assays can be used as an alternative means for orthogonal testing or secondary screening, because of their ability to non-invasively study receptor biology and ligand pharmacology in a more physiologically relevant environment.

The identification of an appropriate lead structure is a key step in drug discovery. Since biosensors provide an integrated and physiologically related cellular response, they are well suited for testing a series of analogous compounds to define quantitative structure-activity relationship. The hits or leads can be further optimized such that they only selectively modulate a specific receptor conformation, an effector function of a hetero-oligomeric complex, or a specific pathway or a state of cells. The ensemble of activated states is thought to be the molecular basis underlying agonist-dependent trafficking (i.e., functional selectivity) [113]. For example, impedance biosensors have been used for hit confirmation against human dopamine D2L agonists. A total of 48 representative compounds were evaluated for their selectivity and potency to D2L. Compound agonist activities were tested on four different cell lines, each over-expressing a different human dopamine receptor subtype, which is coupled to a different signaling pathway [D1 (Gs), D2L (Gi), D4.4 (Gi), and D5 (Gs)]. Results show that dopamine served as a non-selective agonist to all receptors, whereas quinpirole was identified as a D2L-selective agonist.

## CHOICE OF ASSAY PLATFORMS FOR GPCR SCREENING

Because of the unpredictability in the intracellular signaling mediated by synthetic compounds, a single compound could behave as an agonist or antagonist, depending on the signaling events measured; and the lack of efficacy on a given event measured does not guarantee a lack of receptor activation [12, 13, 111]. Thus, a rational GPCR screen should not rely on a single assay; rather, an integrated approach should be employed to measure a multitude of signaling events. Technologies that are independent of cell signaling pathway(s) should be advantageous, given the recent findings that GPCR activation also leads to G protein-independent signaling under many circumstances [26, 27, 117].

The ability to discriminate the efficacies of different agonists is also a factor to be considered and is dependent on the receptor expression level and coupling efficiency [111]. Unlike conventional label-dependent cell assays that measure a

specific cellular event, biosensor-based cell assays measure an integrated cellular response. Thus, these biosensor-based cell assays are well suited for screening agonists, particularly partial agonists. Recently we have shown that CGP 12177, a previously classified  $\beta$ -adrenergic receptor-selective antagonist [118], serves as a highly potent partial agonist to endogenous  $\beta_2$ ARs in A431 cells. This ability is extremely attractive, since many other technologies require cell systems in which the receptor is expressed at higher levels. Such high expression may lead to a mistaken assessment of a partial agonist as a full agonist, particularly when a reporter-gene assay is used [23]. The reporter gene assay generally has a great number of amplification steps, which might improve the apparent coupling efficiency. Furthermore, identification of partial agonists could prove beneficial to a project if tolerance is observed with chronic dosing of a full agonist.

Biosensor-based cell assays allow one to monitor activation of endogenous receptors in a more physiologically relevant environment. Such non-invasive capability leads to a more attractive representation of ligand pharmacology. Most GPCR cell assays utilize certain engineering or manipulations of cells in order to achieve the desired sensitivity for robust and reliable detection [119-121]. In these artificial systems, the potency or efficacy of a ligand might be altered, due to the high expression level of the receptor or the interference of certain manipulations (e.g., GFP-tagging, or the act of transfection) on the cellular physiology of the targets [109].  $\alpha_{2A}$ -adrenergic receptor ( $\alpha_{2A}$ AR) is commonly believed to couple to G<sub>ai</sub> proteins, leading to the inhibition of adenylate cyclase activity. In a native HEL 92.1.7 cells,  $\alpha_{2A}$ AR ligand levomed is an inverse agonist, causing an increase in cAMP production [122]. However, in transfected PC10 fibroblasts levomed is found to be a positive agonist, causing an inhibition of cAMP production [123].

In addition, biosensor-based cell assays bypass the need of any sort of fluorescent labels, which are otherwise widely used for detection in many conventional assays. Such ability could significantly improve the data quality of GPCR screens, since the interference of cell components, labels or compounds having fluorescence is minimized [121].

It is expected that like other functional assays, label-free cell-based assays are also prone to false positives – compounds that do not act on the receptor [23, 67]. False positives that act on the downstream signaling cascade can easily be removed by further evaluation in an equivalent assay that uses different cell systems. Those compounds that are found to act on the receptor also need further characterization for their ligand-binding properties to confirm whether they are competitive or allosteric modulators.

## CONCLUSION

Biosensor-based cell assays enable the measurement of multiple classes of GPCRs simultaneously from a single assay, providing a universal yet highly sensitive platform for GPCR screens. Conversely, multiple conventional assays are required to evaluate all major GPCR classes; most of these assays require prior knowledge of the signaling transduction of the receptors of interest. Given the broad applicability for non-invasively assaying endogenous GPCRs in both kinetic and HTS formats, biosensor-based cell assays have a bright future and should further strengthen the role of GPCRs in

drug development. The advent of high throughput screening instruments and further understanding of the signals obtained will accelerate acceptance of label-free cell-based assays in new areas of drug discovery, for which physiologically relevant representation of GPCR drug candidates is important and valuable.

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