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New label-free technologies provide an unprecedented viewpoint of GPCR signaling, forecasting a flurry of novel screening applications.

Label-free whole-cell assays: expanding the scope of GPCR screening

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A new class of instruments offers an unprecedented combination of label-free detection with exquisite sensitivity to live-cell responses. These instruments can quantify G-protein-coupled receptor (GPCR) signaling through G_s, G_i and G_q pathways and in some cases distinguish G-protein coupling, with sensitivity high enough to detect endogenous receptors. Here, we review emerging data evaluating impedance- and optical-based label-free instruments for GPCR drug discovery. In comparison with traditional GPCR assays, we highlight strengths, weaknesses and future opportunities for label-free biosensors. The ability to qualitatively distinguish G-protein coupling has groundbreaking potential for assessing functional selectivity, a concept that is changing the way GPCR pharmacology is defined and screening strategies are designed.

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Introduction

Cell-based assays have an important role in drug discovery. Designed appropriately, these *in vitro* tests can help predict the effect of chemical agents *in vivo* and can provide relevant biochemical and pharmacological insight that is not possible in a whole animal study. Many detection methods used in cell-based assays have been miniaturized and automated to enable high-throughput screening (HTS) of chemical libraries to seek compounds that induce desired changes in a particular cellular function. Assays for G-protein-coupled receptors (GPCRs) often use functional outputs (e.g. changes in second messenger levels) that can discriminate different pharmacological classes of ligands. These assays have suitable precision to define the structure–activity relationship (SAR) of synthetic ligands generated in a drug hunting campaign. Despite success with these assays, *in vitro* pharmacologists are constantly looking for improvements in cell-based GPCR detection methods to provide greater biological insight and new and broader applications, or those that can be applied to more native environments.

Label-free technologies with the potential to substantially change some aspects of whole-cell assays, including GPCR screening, have emerged within the past few years (reviewed in Refs. [1–5]). These technologies detect changes in cellular features including adhesion and morphology, complex endpoints that are modulated by many different receptors, ion channels and signal transduction pathways. Some label-free instruments have gained particular prominence in

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measuring GPCR function because they can detect the activation of G_s, G_i and G_q signal transduction pathways [6–11]. These readouts are achieved in real-time and have shown exquisite sensitivity to enable detection of endogenous receptor function with output that can be quantified with high precision. In addition, these label-free assays do not require the addition of detection reagents to the cells or expression strategies involving forced G-protein coupling or promiscuous G proteins, thus offering the potential for investigating a more physiological state.

Depending on the cell type and the activating ligand, GPCRs can stimulate different or multiple signal transduction pathways. Initial studies with label-free instruments have demonstrated the potential for detecting and exploring the diversity of these signaling events in a manner not previously possible. Here, we review these findings and label-free GPCR drug discovery applications that have been validated to date. We particularly focus on assay features required to enable SAR studies with label-free instruments and to detect different classes of pharmacological agents. Current limitations and areas not yet investigated in this rapidly evolving field of study are also highlighted.

Detection principles and instruments

Currently available label-free instruments use either an impedance-based biosensor or an optical-based biosensor to detect changes in cell behavior (Fig. 1). The underlying concepts of these biosensors are described below. Detailed information on the technology used in these biosensors has been described previously [12–15].

Impedance-based systems

The use of impedance to measure cellular processes was first reported by Giaever and Keese at the GE Corporation Research and Development Center [16]. In their seminal experiments, fibroblasts cultured on thin-film gold electrodes impeded the flow of a very weak alternating current (0.1 V at 4 kHz). When the impedance was tracked in real-time, it showed a continuous fluctuation that was dependent on both ATP and actin polymerization and was, thus, linked to cellular motion [17]. Impedance measurements were found to be exquisitely sensitive to changes in cell attachment and spreading on the electrodes. Now, it is generally believed that the impedance value is the sum of cellular events that include the relative density of cells over the electrode surface and the relative adherence of these cells (i.e. the distance between the cell surface and the electrodes). Giaever and Keese started Applied Biophysics to commercialize the technology in the form of the Electric Cell-substrate Impedance Sensing (ECIS™) instrument. Applied Biophysics instruments provide a robust platform for a wide range of target applications that measure cellular responses occurring over hours to days including cell attachment, migration and wound healing.

Two other manufacturers, each focusing their platform on different target applications, have launched impedance-based

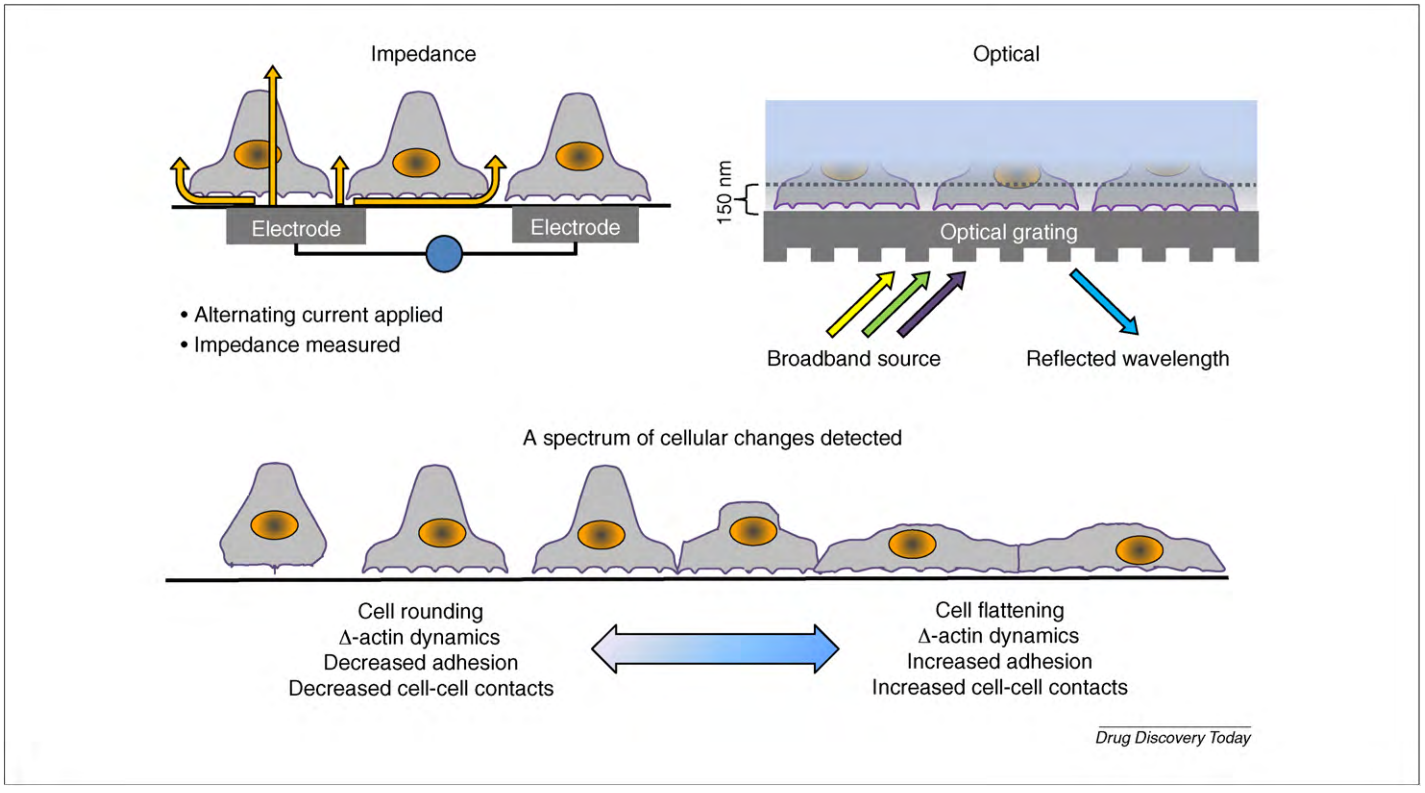

FIGURE 1

Principles of impedance- and optical-based biosensors and their detection of cell behavior. Impedance biosensors (top left) measure the impedance to current flow across two electrodes mounted in the base of the microtiter well. Cells that adhere to the surface of the microtiter well restrict the flow of current between the electrodes. Optical biosensors (top right) measure the wavelength of light reflected by a grating surface at the base of the microtiter well. This process is sensitive to changes in biomass within ~150 nm of the plate surface. Both types of biosensors detect various changes in cellular behavior (bottom). Modulation of these cellular events by GPCR ligands yields qualitative data that provides insight into the pharmacology of the ligand and activation of particular G-protein pathways. The biosensor data can also be quantified to yield potency and efficacy values.

TABLE 1

Characteristics of different label-free instruments

Characteristic	CellKey™	ECIS™	xCELLigence	BIND®	Epic®
Biosensor type	Impedance	Impedance	Impedance	Optical	Optical
Plate formats available	96, 384	8, 16, 96	16, 96	16, 96, 384, 1536	384, 1536
Temperature-controlled plate stage	Yes	No	No	No	Yes
CO_2 environment for longer term reads (hours)	No	Fits inside cell culture incubator	Fits inside cell culture incubator	No	No
On-board liquid handling	96- or 384-well pipetting head	None	None	None	None
Detection modes	Continuous read	Continuous read	Continuous read	Continuous read and interleaved[a]	Continuous read and interleaved[a]
Launch year	2005	1995	2008	2005	2006
Manufacturer	MDS Analytical Technologies	Applied Biophysics	Roche Applied Science and ACEA Bioscience	SRU Biosystems	Corning Inc.
Web site	http://www.cellkey.com	http://www.biophysics.com	http://www.roche-applied-science.com	http://www.srubiosystems.com	http://www.corning.com

[a] Interleaved detection means the ability to rotate assay plates on and off the detector to increase assay throughput.

instruments (Table 1). Like the ECIS instrument, the xCELLigence System (Roche Applied Science and ACEA Biosciences) is built to fit inside cell culture incubators to enable the measurement of cellular responses such as attachment, migration and proliferation that occur over longer time periods, typically hours to days. Both the ECIS and xCELLigence instruments can detect GPCR responses that occur over several hours [6,18–21]. The CellKey instrument (MDS Analytical Technologies) includes features designed for measuring acute cellular responses in higher throughput mode. Specifically, CellKey includes a liquid handling system and offers 96- and 384-well assay formats. CellKey also adds cellular dielectric spectroscopy, whereby impedance values are measured over a wide spectrum of frequencies (1 kHz to 110 MHz) and combined into a signal output through a proprietary transformation [13]. Perhaps because of the use of cellular dielectric spectroscopy and the data transformation, CellKey traces have the unique feature of differential kinetic profiles depending on the particular G-protein class activated (discussed in detail below).

Optical-based systems

Optical biosensors utilize grating surfaces embedded in the bottom of microtiter plates. When illuminated with white or broadband light, these grating surfaces reflect a very narrow band of light characteristic of the refractive index near the grating surface. The refractive index is highly sensitive to changes in the concentration of biomolecules, which results in a change in the wavelength of reflected light. The relative shift in the peak wavelength is proportional to the change in biomass within the penetration depth of the biosensor, which is ~150 nm. Both of the commercially available optical-based instruments, BIND (SRU Biosystems) and Epic (Corning Inc.), were initially developed for detecting the binding of ligands to soluble targets immobilized on the microtiter plate surface. Such biochemical affinity assays are robust and are now firmly established as an important label-free application; however, they generally have limited value for proteins like GPCRs that are not easily extracted from cells and immobilized in a way that retains their native structure.

Success with biochemical binding assays naturally evolved into studying the binding of cells to coated and uncoated microtiter plates [14,22] and, subsequently, aspects of cell morphology [23,24] and GPCR signaling [2,9]. The penetration depth of the biosensor is small relative to the height of adherent cells, so only the bottom portion of the cells is monitored. The cellular phenomenon detected with these biosensors has been called dynamic mass redistribution. Like impedance assays, cellular processes that affect cell shape, adhesion and reorganization of cytoskeletal components involve dynamic mass redistribution. Optical-based assays are less dependent on confluent cell layers than impedance assays, however; this distinction can be important for specialized applications such as primary neurons that grow in sparse networks or for primary cells in limited supply.

Sensitivity and cellular mechanisms

Giaever and Keese calculated that impedance measurements can detect a 1 nm change in vertical motion of cells [25] and thus can register very subtle changes in cell adhesion and morphology, a concept they termed 'cell micromotion'. To put 1 nm in perspective, the plasma membrane is approximately 3 nm in width, and

light microscopy has a detection limit of ~250 nm. Similar data on morphological resolution are not available for the optical biosensors, which prevents a direct comparison of sensitivity. However, it seems reasonable to infer that cell-based assays with optical instruments also offer exquisite sensitivity based on resolution established in biochemical binding assays. When target proteins are immobilized on the surface of an optical biosensor plate, binding of ligands in the 100–300 Da range is detectable. In other words, the optical instruments can detect the relative movement of ~100 Da molecules into the ~150 nm detection zone at the plate surface. For comparison, changes in cell morphology and adhesion involve the redistribution of substantial biomass (e.g. polymerization of 43,000 Da actin monomers) in the 150 nm detection zone of the optical biosensor. Therefore, although how impedance and optical biosensor sensitivities compare in terms of detecting specific cellular events is not yet clear, both technologies have sensitivity that far exceeds traditional methods.

The biochemical pathways that link GPCR activation with cellular endpoints detected with label-free biosensors have not been established by direct testing. G-protein signaling can induce changes in morphology [26–29], and there are well-established links between G_s, G_i, G_q and $G_{12/13}$ signaling pathways and modulation of the actin cytoskeleton [26,30–33]. Upon activation, many GPCRs undergo β-arrestin-mediated receptor internalization, which is an actin-dependent process [34–37]. Furthermore, pretreating cells with actin polymerization inhibitors diminishes both impedance and optical biosensor GPCR responses [11]. This suggests that the actin cytoskeleton is an important downstream integrator of GPCR responses that are detected by the label-free instruments.

Interpreting kinetic profiles

Changes in impedance and refractive index are measured in real time, enabling one to follow the kinetics or the profile of a given response (Fig. 2). For CellKey, the first of these instruments to be aimed at GPCRs, distinct response profiles are observed depending on the G-protein pathway activated. Receptors that activate the G_i pathway produce an increase in impedance; G_q activation causes a transient decrease followed by a larger increase in impedance response, and G_s activation causes a decrease in impedance

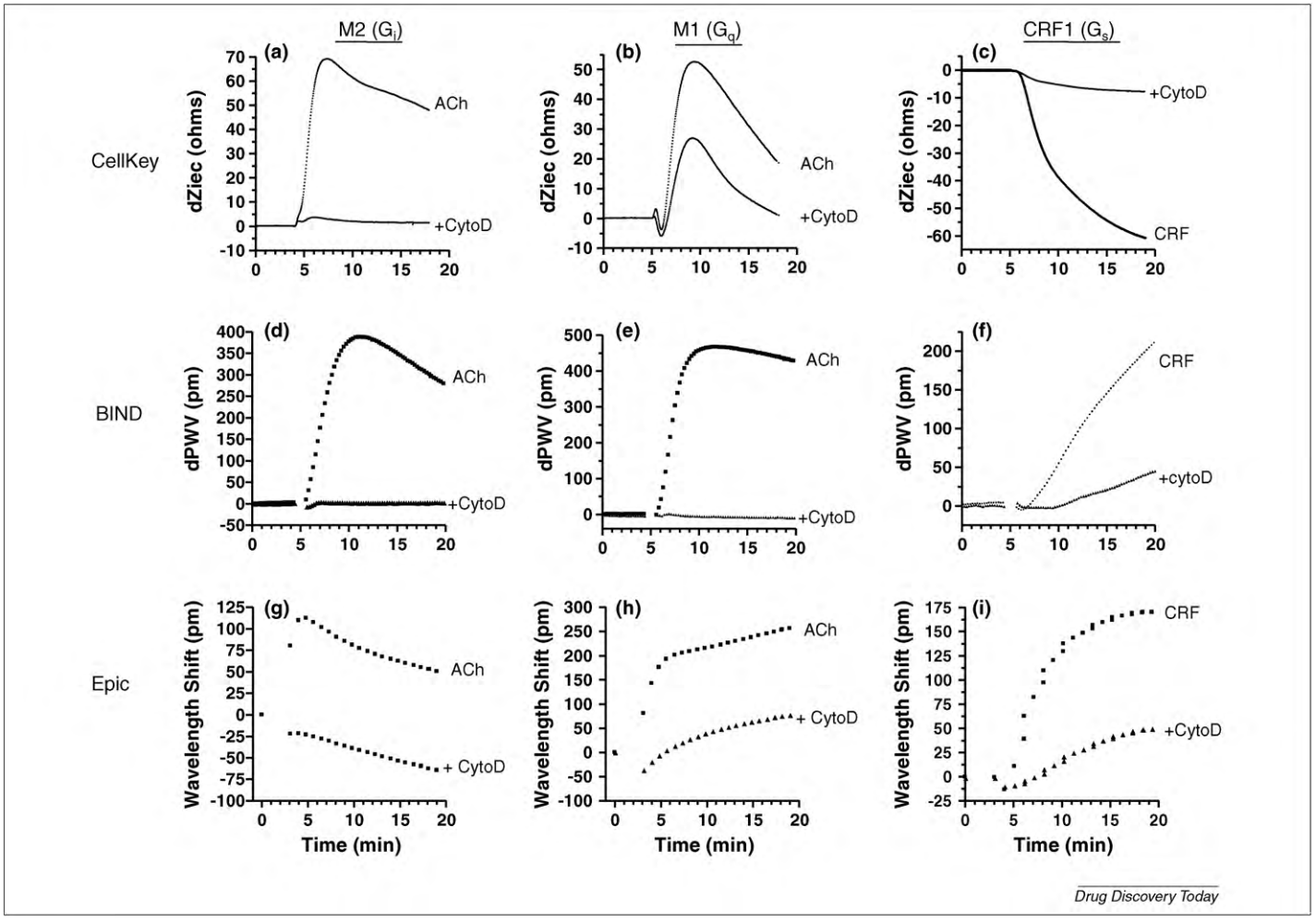

FIGURE 2

GPCR activation profiles measured in different label-free instruments. The agonist temporal response profiles for muscarinic M2, muscarinic M1 and corticotropin-releasing factor (CRF1) receptors were determined in one impedance-based (CellKey) and two optical-based biosensors (BIND and Epic). CHO cells expressing either M2, M1 or CRF1 were treated with an EC80 concentration of acetylcholine (M2 and M1) or CRF1. Each of the agonist response profiles was diminished by pretreating the cells with cytochalasin D, which indicates that cellular features regulated by G_q, G_i and G_s pathways and detected by the label-free biosensors are sensitive to actin microfilament stability. Data reproduced from Ref. [11].

response (Fig. 2). Accumulating evidence from multiple laboratories indicates this pathway-distinguishing power is consistent across a range of GPCRs and cell types [7,8,10,13,38]. The most notable concern identified to date relates to the variability in the transient dip associated with G_q activation. In practice, this variability has minimal impact on the ability to distinguish G_q from G_i because this is typically confirmed with pertussis toxin treatment. There are concerns, however, that impedance profiles are, in fact, merely qualitative correlates with signaling pathways and proper pathway determination requires independent confirmation with pathway-specific agents. Certainly this caveat must be emphasized, yet it also must be balanced against a pragmatic perspective that the ability to discriminate the activation of different G-protein pathways by one instrument is unprecedented and a major advance (specific applications discussed in the section 'Pleiotropic signaling and functional selectivity'). Studies that demonstrate whether selective activation of the $G_{12/13}$ signaling pathway can be detected with an impedance or optical biosensor have not yet been published. GPCRs that activate the $G_{12/13}$ pathway often also activate other G-protein pathways, which results in a more complex signaling environment. It will be important to determine whether pathway deconvolution to discern $G_{12/13}$ signaling is possible with label-free instruments.

The G-protein distinguishing capability revealed for CellKey set the standard for subsequently introduced label-free instruments. For the optical-based instruments, the kinetics are similar to CellKey in a general sense – G_i and G_q peak within 5 min and then begin to decline, whereas G_s has a slower onset that is sustained. However, the specific profiles and the ability to discriminate coupling are different on the optical systems. Most notably, the G_s profile is not consistently inverted relative to G_i (Fig. 2). Furthermore, available data suggest that the profiles are not consistent across cell types. For example, very different profiles were observed with Epic when different cell lines were treated with forskolin to directly activate adenylate cyclase [39]. The optical systems might detect cellular features that vary between cell types and, therefore, produce profiles that reflect cell-type-specific biology. Nonetheless, the optical instruments can detect activation of G_s, G_i and G_q pathways, so once a baseline profile for a given receptor or cell type is well characterized, the profile can be monitored as an indicator of changes in signaling [40].

GPCR signaling measurements in the xCELLigence impedance instrument reveal time courses that generally match the other label-free instruments [6]. Surprisingly, however, the absolute shapes of xCELLigence's response profiles are more consistent with the optical-based instruments than with CellKey. Thus, the response kinetics are similar among all four instruments, but the highly valued ability to distinguish coupling does not align with technology types. Perhaps the various platforms detect similar underlying molecular events, but different profiles result from differences in data transformation. Although this is purely speculative, if it is true then refining data transformation algorithms could enhance pathway-distinguishing capability in future generations of instruments. A detailed comparison of the technologies underlying these instruments and the opportunity for expanded pathway-distinguishing capability is needed but is beyond the scope of this review.

Quantifying kinetic profiles

Biosensor temporal response curves are quantified by subtracting a buffer control from the peak ligand-induced response value. These data are used to generate concentration–response curves and to calculate potency and efficacy values. This approach was used to quantify known agonists and antagonists of the GPCRs described in Fig. 2 using one impedance and two optical-based instruments. As shown in Table 2, the potency value for a single GPCR–ligand pair is similar across the three instrument platforms; this is true for each of the two agonists and two antagonists tested for each receptor. In fact, the largest difference between biosensor potency values seen across this panel of 12 ligand–receptor pairs was 30-fold, whereas most differences were within experimental error. Although the two types of biosensors use different technologies to detect changes in cell behavior, the quantitative outcome for G_s-, G_i- and G_q-coupled GPCRs in Chinese hamster ovary (CHO) cells are remarkably similar. An endogenous G_s-coupled receptor response in U-2 OS cells showed equivalent potency profiles in impedance and optical biosensors [11], which extends the similarities to endogenously expressed receptors that show more native coupling.

TABLE 2

GPCR agonist and antagonist potency values obtained with different label-free instruments

(a)	AChR M2		AChR M1		CRF R1 (G_s)		CRF R1 (G_i)	
	ACh	Carbachol	ACh	Carbachol	CRF	Sauvagine	CRF	Sauvagine
CellKey	7.3 ± 0.2	6.6 ± 0.2	7.8 ± 0.1	6.6 ± 0.1	10.3 ± 0.2	10.5 ± 0.3	6.8 ± 0.2	6.7 ± 0.1
Epic	7.3 ± 0.2	6.5 ± 0.1	7.2 ± 0.2	6.3 ± 0.2	11.7 ± 0.7	11.5 ± 0.6	7.3 ± 0.2	7.2 ± 0.2
BIND	7.1 ± 0.3	6.5 ± 0.3	7.1 ± 0.1	6.1 ± 0.3	10.8 ± 0.2	10.8 ± 0.5	7.8 ± 0.6	7.8 ± 0.6

(b)	AChR M2		AChR M1		CRF R1	
	Scopolamine	Pirenzepine	Scopolamine	Pirenzepine	YP-20	R121919
CellKey	8.1 ± 0.3	5.6 ± 0.4	8.7 ± 0.0	7.3 ± 0.3	6.6 ± 0.2	6.4 ± 0.3
Epic	8.4 ± 0.3	6.0 ± 0.4	9.1 ± 0.0	7.5 ± 0.1	6.6 ± 0.3	5.8 ± 0.6
BIND	8.3 ± 0.3	5.8 ± 0.4	9.0 ± 0.0	7.5 ± 0.2	6.3 ± 0.7	7.2 ± 0.2

The potency and variance for M2, M1 and CRF1 receptor ligands were measured with different label-free instruments. Values represent **(a)** agonist pEC_{50} or **(b)** antagonist pIC_{50} and standard deviation. All three instruments observed biphasic concentration–response curves with the CRFR1 agonists: the potency and variance for the high- and low-affinity phases are shown. The two phases correspond to a high-affinity G_s response and a lower affinity G_i response. Data are modified from Ref. [11].

Assay robustness is frequently defined with a Z' value [41] using the following equation:

$$Z' = 1 - \frac{3\text{SD of sample} + 3\text{SD of control}}{|(\text{mean of sample} - \text{mean of control})|}$$

where sample and control represent the stimulated and basal assay conditions. Assays with $Z' \geq 0.5$ have tight replicate values with wide separation between basal and stimulated signals, attributes that are required for SAR studies. Published GPCR assay Z' values on the Epic [42,43] and CellKey [10,38] are all $\geq$0.5. The label-free assays described in Table 2 also met this criterion. We have had more experience with the CellKey instrument, and for GPCR assays we routinely achieve this high level of assay precision, regardless of the signaling pathway activated by the receptor and the pharmacology being pursued. In summary, the optical and impedance kinetic profiles can be quantified and, where studied, yield equivalent results in terms of potency and data variance.

Comparison with traditional technologies

To validate label-free detection applications for drug discovery, the pharmacology derived from these instruments should align with more traditional measures of GPCR signaling. Ligands that span large potency and efficacy ranges should be used to determine whether (a) the instrument has sufficient dynamic range and sensitivity to discriminate high and low affinity, as well as varying efficacy; (b) the potency values show a similar rank order to that seen with other functional readouts (i.e. independent of the detection system); and (c) the assay precision is suitable to define SAR. If any of these criteria are not met, the technology could be used in some situations (e.g. as a high-throughput detection assay or as a yes/no confirmation screen) but not as a 'SAR-driving assay' that defines the pharmacology of newly synthesized compounds in a drug discovery project.

Few publications comparing optical biosensor data with label-based measures exist to enable the assessment of these points for a particular GPCR. Dodgson *et al.* [42] compared Epic and a Ca^{2+} mobilization assay using nine muscarinic ligands on CHO cells expressing the muscarinic M3 acetylcholine receptor. The two assays had similar standard error values for the test compounds. Three agonists were each approximately tenfold less potent in Epic than in FLIPR, whereas six antagonists that spanned ~1000-fold potency range gave comparable values in both assays. Lee *et al.* [44] tested 12 reference compounds against CHO cells expressing the dopamine D3 or muscarinic M2 receptor. Most of the results agreed with data from cAMP or Ca^{2+} readouts with one exception, and the Epic responses detected weak activity that was not observed with the label-based assays. Although these two studies suggest alignment between optical- and label-based measures of GPCR function, additional studies with different receptors and cell lines are warranted to better understand how closely these readouts overlap and to identify conditions that might cause biased outcomes.

Comparative pharmacology studies using the CellKey impedance instrument and traditional technologies have been published [10,13,38,45]. Most of these studies used small compound sets (4–6 ligands) and did not include assay precision metrics, but each demonstrated similar rank-order potency values between CellKey and Ca^{2+} or cAMP readouts. Stronger comparisons require larger set of ligands covering a wide potency window, but these are typically not commercially available. We solved this limitation by using in-house ligands for a G_i-coupled receptor termed G_iPCR-X for proprietary reasons [38]. This study compared results between a whole-cell label-free assay and a plasma membrane [^{35}S]GTPγS binding assay. The label-free assay potency values for 14 agonists tracked well with the potencies observed in the [^{35}S]GTPγS binding assay (Fig. 3). The relative efficacy values also compared well between the two assays. In addition, 13 antagonists for this receptor also showed a potency correlation, albeit three- to tenfold more potent in CellKey. Data variance in the CellKey assay was modestly better than the [^{35}S]GTPγS assay, the SAR-driving assay for the G_iPCR-X project. Overall, the results with G_iPCR-X demonstrate the ability to meet the SAR-driving assay criteria with a label-free instrument.

To gain a greater breadth of comparison between label-free and label-based assays, EC_{50} values were determined for various receptor–agonist pairs using CellKey and traditional assays (Table 3). These studies were performed using the same cell line across

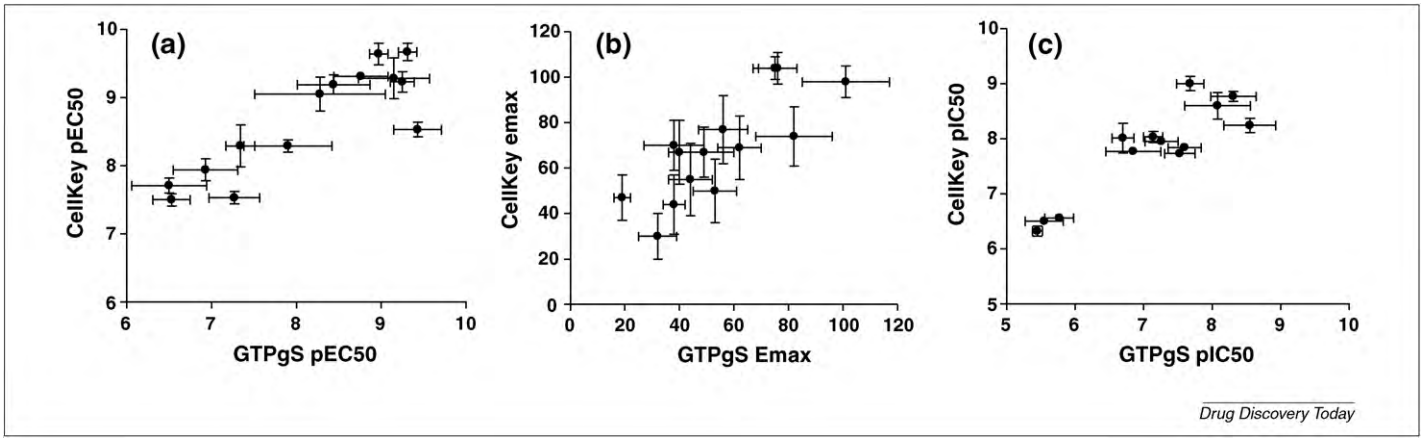

FIGURE 3

Comparing quantitative data from a label-free instrument with a more traditional measure of GPCR function. A collection of known agonists **(a)**, **(b)** and antagonists **(c)** for the G_i-coupled receptor G_iPCR-X were tested in CellKey and the results compared with data from a [^{35}S]GTPγS binding assay. Data reproduced from Ref. [38].

TABLE 3

Comparing agonist EC_{50} values from CellKey and label-based assays[a].

G protein	Receptor	Agonist	CellKey	Comparison assay		Potency ratio
			Potency	Readout	Potency	
$G_{i/o}$	CB1	CP55940	1.6E−09	GTPγS	4.0E−10	0.25
	AChR-M4	ACh	1.5E−08	GTPγS	3.8E−07	26
	MCH-R1	MCH	1.3E−10	GTPγS	1.0E−09	14
	Peptide GPCR (Class A)	Selective synthetic agonist	3.0E−10	GTPγS	7.0E−09	23
	Amine GPCR (Class A)	Native agonist	2.0E−10	GTPγS	1.1E−09	6
	D2	Dopamine	1.0E−09	GTPγS	2.0E−09	2
				cAMP	1.6E−07	160
G_q	NK3	Senktide	5.2E−09	Ca^{2+} flux	7.7E−10	7
	MCH-R1	MCH (+PTX)	2.2E−09	Ca^{2+} flux	1.4E−08	6
	AChR-M1	ACh	6.5E−08	Ca^{2+} flux	1.6E−09	0.02
G_s	CRFR1	CRF	4.0E−12	cAMP	4.0E−10	100
	D1	Dopamine	6.0E−11	cAMP	3.3E−08	550
	D5	Dopamine	1.0E−11	cAMP	2.7E−09	270

[a] Potency ratios are expressed as CellKey: comparison assay.

technologies to enable a direct comparison of potency values. Roughly half of the G_i- and G_q-coupled receptors showed a similar potency with CellKey and a traditional assay (i.e. less than tenfold different), whereas the others showed a 14–50-fold difference in potency. At present, it is unclear why some receptors show a notable potency difference between the functional assays. A more systematic assessment is needed to understand the relative sensitivity of label-free biosensors to certain variables, including receptor expression levels and receptor–effector coupling efficiency.

Three G_s-coupled receptors and one G_i-coupled receptor were tested in CellKey and cAMP assays. All four receptors showed a ≥100-fold more potent response in CellKey, which suggests that very small changes in cAMP concentration are sufficient to evoke cellular behaviors detectable by CellKey. This amplified response is not unique to CellKey: CellKey, Bind and Epic gave similar potency values for G_s agonists (Table 2). In contrast to these results, similar potency values between impedance and cAMP assays were reported for dopamine D3 [45] and D1 receptors [6]. The reason for the differing results is unclear and indicates the need for additional studies to explore the frequency and conditions whereby label-free biosensors give amplified responses compared to cAMP levels. To our knowledge, no comparative studies using optical biosensors and traditional assays with G_s-coupled receptors have been published.

Independent of absolute potency, precision is a crucial factor when choosing a detection method for a SAR-driving assay. Greater assay precision means a small difference in potency between two test compounds is deemed statistically significant. Thus, an assay with good precision is better able to distinguish whether chemical modifications to a compound improve activity. Although the Z' value is a valuable metric of assay quality, it does not define the minimal potency difference between compounds that is statistically significant. Given the importance of this latter metric for assessing SAR, we routinely measure it for different detection methods. Operationally, we strive to deliver SAR-driving assays in which a threefold or smaller difference in potencies between two compounds is statistically significant. Assays in which a fourfold potency difference is the limit of statistical

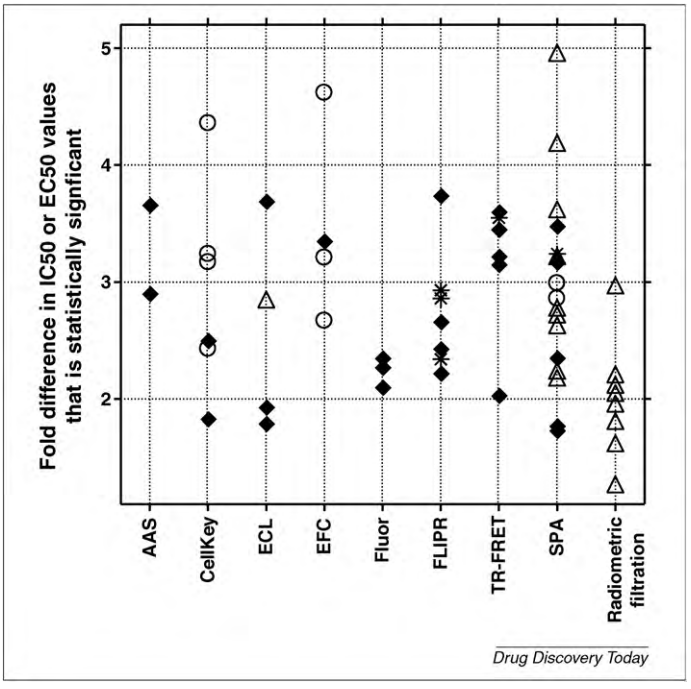

FIGURE 4

Comparing the precision of *in vitro* assays that use label-free or label-dependent detection methods. Each symbol represents an assay developed with one of the nine detection methods listed on the *X*-axis. The precision of each assay was established by testing at least ten compounds ($n = 3$) that spanned a >100-fold potency range and determining the minimal difference in potency values that is statistically significant (with 95% confidence). Assuming that two compounds will be run using n replicates and have the same standard deviation (SD) and normally distributed data, the SD of the difference (SD_{diff}) between the two compounds is sqrt(2)SD/sqrt(n) and the minimal difference in potency that is statistically significant is defined by $2SD_{diff}$. The assays compiled in this figure quantified binding affinity (△) or pharmacology including agonism (○), antagonism (◆) or positive allosteric modulation (✱). Data were derived from whole-cell assays except for SPA and radiometric filtration assays, which used membrane preparations from cultured cells. Abbreviations: AAS, atomic absorption spectroscopy; ECL, electrochemiluminescence; EFC, enzyme fragment complementation; SPA, scintillation proximity assay.

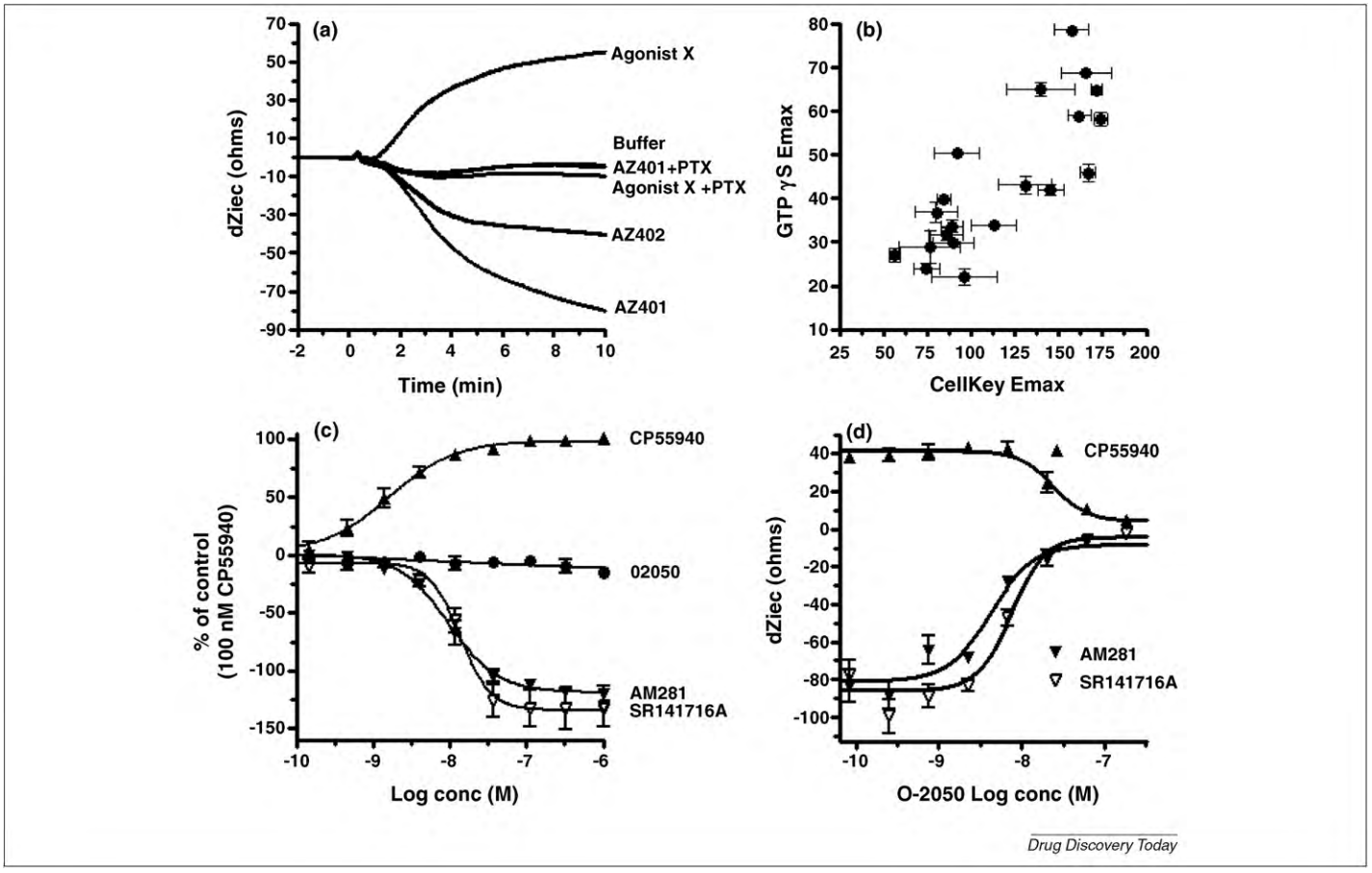

FIGURE 5

Detecting and quantifying inverse agonists using an impedance label-free instrument. **(a)** Representative temporal response profiles generated in CellKey for the native agonist X and two inverse agonists of G_iPCR-X are shown. **(b)** The maximum efficacy determined in CellKey for 20 G_iPCR-X inverse agonists are plotted against data obtained in a [^{35}S]GTPγS binding assay. GTPγS Emax data normalized to the endogenous agonist X set as −100%. Data correspond to the mean ± SD of three experiments each run in triplicate wells. Pearson $r = 0.81$ and $P < 0.001$. **(c)** The temporal response in CellKey for CB1-CHO cells treated with agonist CP55940, neutral antagonist O-2050, and inverse agonists AM281 and SR141716A. Data are expressed as a percentage of response observed with 100 nM CP55940 and represent the mean ± SD of three experiments each conducted on at least triplicate wells. **(d)** Increasing concentrations of neutral antagonist O-2050 can fully inhibit the impedance response to CP55940, AM281 and SR141716A. Data are the mean ± SEM of one representative experiment conducted on quadruplicate wells. In three independent experiments, average pIC_{50}s for O-2050 inhibition of CP55940, SR141716A and AM281 were: 8.0 ± 0.4, 8.3 ± 0.1 and 8.2 ± 0.1, respectively.

significance are marginal for SAR studies, and those requiring a fivefold potency difference to be statistically significant are not suitable. Figure 4 displays the calculated values for different assays using CellKey or traditional label-based readouts. Clearly, assays developed with CellKey are capable of achieving the desired criterion and compare well with the other technologies. This data plus the reported Z' values provide confidence that label-free assays can be used to generate high-quality receptor pharmacology data.

Detecting a spectrum of pharmacology

Whole-cell label-free assays have been validated for routine agonist and antagonist SAR-driving assays, but for maximal impact, broader applications are required. As described below, other pharmacological profiles can also be detected and quantified with these biosensors.

Inverse agonist

Detecting inverse agonists requires a cellular system with sufficient constitutive activity, or 'basal tone', to measure a reduced response. With traditional pharmacology assays, an inverse agonist induces the opposite signaling effect from the native agonist; the same seems to be true with label-free biosensors. As shown in Fig. 5a for G_iPCR-X, the agonist stimulates an increased impedance response in CellKey, whereas inverse agonists AZ402 and AZ401 induce a decrease in impedance. Pretreating the cells with pertussis toxin inhibits these biosensor responses, which confirms they are G_i- and not G_s-mediated. Three publications have described findings consistent with inverse agonist responses, two on reportedly G_q-coupled receptors [6,11] and one with a G_i-coupled receptor [44]. These publications include data from both optical- and impedance-based instruments. To our knowledge, detection of G_s-coupled inverse agonists using label-free biosensors has not been reported.

As with agonists and antagonists, the magnitude of the inverse agonist response can be quantified. For a group of G_iPCR-X inverse agonists, the relative efficacies determined with CellKey agreed with values obtained in the [^{35}S]GTPγS binding assay (Fig. 5b).

Neutral antagonists can be distinguished from inverse agonists. For example, the known CB1 inverse agonists AM281 and

SR141716A gave concentration-dependent decreases in impedance, whereas compound O-2050 had no effect on the impedance response (Fig. 5c). O-2050, however, reduced both the agonist (CP55940) and inverse agonist responses in a concentration-dependent manner (Fig. 5d), consistent with neutral antagonism in this assay.

Allosteric modulators

Developing allosteric modulators of GPCRs as therapeutic agents has gained considerable interest (reviewed in Refs. [46,47]). Functional measures of GPCR activity are used to detect compounds with this mechanism of action because radioligands for modulator site(s) on most GPCRs are not available. As proof of concept, three positive allosteric modulators for the muscarinic M4 receptor were tested in CellKey, and the results were compared to a traditional [^{35}S]GTPγS binding assay [38]. CellKey gave similar rank-order results to the [^{35}S]GTPγS binding assay (potency, efficacy and cooperativity values), but showed approximately twofold greater sensitivity and dynamic range. For example, AZ202 had cooperativity values of 12.5 and 6.5 in CellKey and [^{35}S]GTPγS binding, respectively, and brucine oxide had values of 3.0 and 1.8. Most GPCR modulators have a small cooperativity value; a two- to eightfold shift in agonist EC_{50} is typical, so having an assay that provides a twofold increased signal while maintaining good precision is an advantage. Whether this sensitivity advantage is typical for modulator assays is unknown. Clearly, this is an area with untapped potential that should be investigated further.

Pleiotropic signaling and functional selectivity

The ability to signal through multiple pathways seems to be a ubiquitous feature of GPCRs. Ligands can have different relative effects on each pathway, thereby making the pharmacology observed highly dependent on the assay selected [48]. The complexities of pleiotropic signaling include both challenges and opportunities, but neither has been adequately addressed because of a combination of issues. Technically, traditional assays detect only a single mediator; therefore, evaluating complex responses requires comparing across multiple assays, which typically have different end-points and relative sensitivities. Furthermore, there is a cost – knowledge conundrum – prior knowledge of which combination of pathways to test is needed, but investing resources to obtain this knowledge is difficult to justify without supporting data. In this context, the novel ability of label-free assays to detect multiple G-protein responses in a single assay is a considerable improvement. The ability of CellKey to qualitatively distinguish G-protein coupling provides an approach to address issues related to pleiotropic signaling in a manner not previously possible.

The heavy reliance on overexpressed GPCRs for drug screening lead Kenakin [49] to predict that altered receptor stoichiometry relative to other signaling components would lead to pharmacological anomalies. Consistent with this hypothesis, we have observed a remarkable frequency of switches in G-protein coupling with receptors expressed in different cell lines. In two out of three instances in which both CHO and HEK transfected cell lines were available, each cell line was associated with different G-protein coupling (CB1: G_i in CHO versus G_s/G_i in HEK, Fig. 6; MC4: G_q in CHO versus G_s in HEK [8]). Such switches are not limited to recombinant cell lines; testing several untransfected cell lines for endogenous D1/D5 responses revealed two cell lines with pharmacologically validated responses but coupled through different G proteins [8]. In each case, follow-up literature searches revealed data supporting the observed G-protein switches. This apparently high frequency of coupling changes requires further study. Nevertheless, the current data are sufficient to demonstrate the ease with which unsuspected coupling changes can be identified, and this adds an important technical advance for selecting '*in vivo*-relevant' cell lines for *in vitro* screens.

Perhaps the most tantalizing opportunity in GPCR drug discovery today has its roots in pleiotropic signaling. Functional selectivity, the notion that different ligands can bind the same receptor yet activate different downstream signaling pathways, could be exploited to develop drugs that target only therapeutically relevant pathways. Despite the obvious impact, the industry has been slow to embrace this functional selectivity because of the

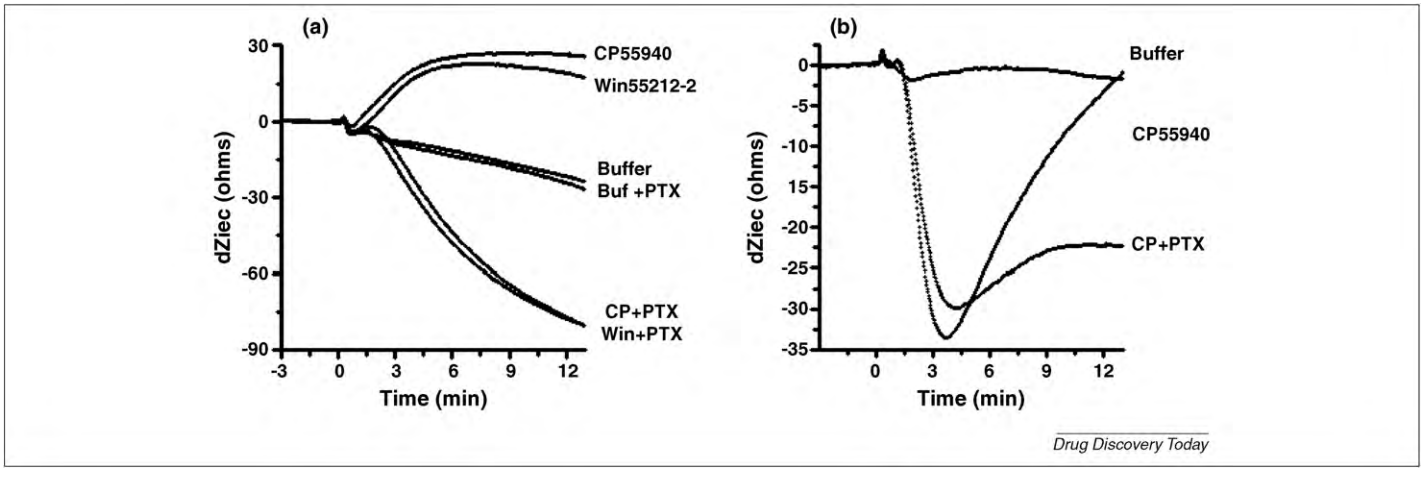

FIGURE 6

Both biased and dual signaling can be detected using the CellKey label-free instrument. **(a)** In CB1-transfected CHO cells, agonists CP55940 and Win55212-2 preferentially induce G_i responses (increased impedance). Pretreating the cells with pertussis toxin to eliminate G_i coupling revealed agonist-induced G_s signaling (decreased impedance). **(b)** In CB1-HEK cells, CP55940 stimulates both G_s and G_i signaling in a temporally distinct manner. G_s signaling (decreased impedance) occurs first, followed by G_i signaling (increased impedance) that can be reduced by pertussis toxin pretreatment. Data are reproduced from Ref. [8].

technical issues outlined above [50]. The label-free biosensors seem to offer potential; however, the paucity of available tool compounds has limited attempts to conclusively demonstrate this capability. For example, all three label-free instruments detected CRF1 signaling through both G_s and G_i, with G_i activation occurring at higher agonist concentration [11] (Table 2). If CRF1 ligands were available that differed in their preferential activation of G_s versus G_i, it seems reasonable to expect that the wide separation in potency between the responses would enable clear detection of this bias by each label-free instrument. Detecting dual G_s and G_i signaling that occurs at similar agonist concentrations is a bit more challenging. In CellKey, where G_s and G_i profiles are distinct, this co-activation results in a novel response profile consistent with merging of the G_s and G_i signatures, as shown with CB1-HEK cells (Fig. 6b). This demonstrates the dynamic nature of response profiles and suggests an agonist with biased activation of one pathway would be revealed by a corresponding change in the response shape. Several natural and synthetic CB1 agonists were tested in this system, but biased signaling was not observed (M.F.P., unpublished data). Fang and Ferrie [40] published response profiles for β_2 adrenergic ligands on A431 cells that they interpreted as being biased agonists, although proof of activating different signaling pathways was not provided. Thus, although not yet formally demonstrated, label-free biosensors represent new technology to help pursue the detection and optimization of functionally selective ligands.

Applications for drug discovery

The advantages of label-free technology can be exploited for different purposes in a drug discovery environment. Because these instruments can detect a functional response from receptors that activate G_s, G_i or G_q signaling pathways, a label-free biosensor can be used as a platform technology to support GPCR drug hunting projects. Some of the more prominent applications are listed below.

HTS. The sensitivity, precision, ease of assay development (once familiar with the technology) and throughput with some label-free instruments are sufficient to enable their use in HTS. The cost of some instruments and consumables (proprietary microtiter plates), however, might limit broad acceptance. Proof of concept was shown by Dodgson *et al*. [42], who performed an HTS for muscarinic M3 agonists using Epic and compared the output to a Ca^{2+} mobilization HTS using the same cell line. One caveat to consider with high-throughput label-free screens is the possibility that signaling through multiple pathways with opposing signals can result in a lack of an overall response, resulting in a false negative outcome. One clear demonstration of this can be seen with the CRF1 receptor, where equal contribution by G_s/G_i coupling would lead to no signal if tested in single concentration mode (CRF 1 μM) in CellKey [11].

Confirmation screen. Because the label-free biosensors use different detection methods to the label-based readouts, technology-based false positives can be eliminated. Whole-cell label-free assays are straightforward to establish and highly versatile, making them an increasingly common alternative for difficult confirmation assays.

SAR-driving assay. Although label-based detection methods for G_s, G_i and G_q signaling can be used successfully as SAR-driving assays, a label-free readout could prove advantageous depending on the available instrumentation and nuances with the target and desired pharmacology. For example, a whole-cell assay for G_i-coupled receptor antagonists is notoriously difficult to achieve with high precision using traditional cAMP detection methods because of the requirement to prestimulate adenylate cyclase with forskolin, inhibit the response with agonist and then measure reversal of the agonist effect with antagonists. The prestimulation step can be eliminated and assay precision improved by using label-free detection. Other methods that have been employed successfully to quantify Gi-coupled receptor antagonists include ERK phosphorylation and [^{35}S]GTPγS membrane binding assay. Our experiences using label-free biosensors for SAR-driving assays with G_i-coupled GPCRs have been overwhelmingly positive.

Selectivity screening. Assessing relative selectivity within and across GPCR families can easily be performed with label-free biosensors, keeping in mind the potential for biased and cell-specific signaling. Access to nonselective ligands enables normalizing the response profile within GPCR families. Verdonk *et al*. [7] used CellKey for broad GPCR profiling, and others have reported results and experiences from selectivity screening exercises [44,45]. Some contract research organizations now offer receptor profiling using label-free biosensors to screen for agonist and antagonist pharmacology.

In vitro translational pharmacology. Because the sensitivity of label-free instruments enables detection of endogenous receptor pharmacology, results derived from recombinant systems (historically often used in HTS) can be compared with non-engineered cells to ensure that pharmacology is reproduced in a more native environment. In addition, screening endogenous receptors with label-free techniques can circumvent proprietary limitations on some recombinantly expressed targets. Comparing pharmacology across species, using recombinant or native cells, to determine whether compounds are suited for progressing to *in vivo* studies in animals is also possible. If a cell type can be acquired from human patients and normal controls, label-free biosensors offer the potential to verify the pharmacology of candidate drugs in a true disease-relevant environment.

Functional selectivity. The therapeutic impact of ligand-specific signaling for GPCR drug discovery could be substantial. The main hurdles to realizing this potential are technical: (i) limited capacity to detect and distinguish signaling changes in large screens and (ii) validating a particular signaling pathway in a disease process. As discussed above, the whole-cell label-free assays are a notable advance with regard to the first point. Success in the identification of pathway-selective tool compounds can be used to assess the second point.

Current unknowns, limitations and future opportunities

The past five years can be viewed as a period of substantial exploration and growth using label-free instruments to measure aspects of cellular biology. In particular, studying GPCR signal transduction with label-free biosensors has provided both insight and practical applications. By applying appropriate biochemical tools and cell biology perspectives, several areas of unexplored biology might reveal additional applications for these instruments. Some examples include the following.

Biochemical mechanisms that underpin biosensor signals. As noted above, many publications have demonstrated a link between GPCR modulation of the actin cytoskeleton and changes in label-free biosensor response. The Rho family of GTPases is a prominent regulator of the actin cytoskeleton; their activity can be modulated by GPCRs via several G-protein pathways (reviewed in Ref. [51]). Modulators of microtubule organization also can affect biosensor signals [12,15,52], and it is highly likely that other cytoskeletal modulators and cellular pathways impact cellular features that are detected by label-free biosensors. Thus, considerable work is needed to define the detailed biochemical pathway or pathways that specifically link(s) a particular GPCR with an increase or decrease in biosensor response. Providing a molecular understanding to the processes that influence the label-free signals will enable greater interpretation of the kinetic response profiles, particularly when more complex responses are observed.

$G_{12/13}$ signaling. As noted above, these G proteins have not been directly tested to determine whether they induce a label-free biosensor response. Detection is highly likely, however, because they activate Rho GTPases, which modulate cytoskeletal proteins to induce stress fiber formation and focal adhesion assembly (reviewed in Ref. [33]). In addition, activated mutants of G_{12} and G_{13} induce changes in cell morphology and growth characteristics [53–55]. Most GPCRs that activate $G_{12/13}$ can also activate one or more of the other classes of G proteins and, therefore, the resulting biosensor signal could represent a composite response. Two publications have demonstrated GPCR-induced impedance responses that were not blocked by inhibitors of G_q or G_i signaling but were blocked with a Rho kinase inhibitor, which suggests the involvement of $G_{12/13}$ in the impedance response [18,56].

β-Arrestin-mediated signaling. Several signaling proteins are recruited by β-arrestin during receptor internalization and thereby induce G-protein-independent signaling (reviewed in Ref. [57]). Antagonism of D2/β-arrestin signaling has been proposed as a clinically important feature of current antipsychotics [58]. Whether the internalization process itself or any of the β-arrestin-dependent signaling events lead to a discernable response with these biosensors is unknown. Some GPCR ligands that induce β-arrestin-mediated internalization but not G-protein signaling have been identified [59]. These might be useful tool compounds to determine whether β-arrestin internalization imparts a biosensor response.

Detecting modulators of G-protein signaling pathways. It should be possible to assess the pharmacology and SAR of compounds that regulate enzymes within GPCR signaling pathways. For targets that reside in large protein families like kinases and phosphodiesterases, however, the potential for detecting nonselective effects should be recognized. This is a concern for any whole-cell functional assay but particularly for label-free biosensors because they are sensitive to perturbations of multiple signaling pathways.

Profiling primary cells. Although deemed a major application for label-free screening, only three proof-of-concept studies have published thus far. Leung *et al.* [10] observed a concentration-dependent effect of IL-8 on human neutrophils that matched potency values seen in chemotaxis assays. This result also demonstrates the ability to detect a label-free response with non-adherent cells. Presumably, the cells settle onto the microtiter plate surface sufficiently close to the electrodes to create a resistance barrier. Ligand-induced responses have also been profiled with human skeletal muscle myoblasts [60] and prostrate stromal and epithelial cells [61].

Effect of adhesion substrates on GPCR responses. Although poly-D-lysine is the default surface coating when growing cells in culture and on microtiter plates, some cell types (particularly primary cells) might require other substrates for optimal adhesion and growth. Both the type of surface coating material and its concentration can influence cell adherence to the biosensor surface and the resulting biosensor response. For example, protein components of the extracellular matrix (ECM), such as fibronectin or laminin, improve adherence of PC12- and NIH3T3 cells compared to nonspecific coatings [21,62]. Some ECM proteins bind in a specific manner to integrins, members of a family of cell surface receptors. The integrin intracellular domain forms a complex with various cytoplasmic proteins and the actin cytoskeleton, and thereby effectively couples the ECM outside the cell with the intracellular cytoarchitecture. Integrin–ECM coupling can induce intracellular signals [63] and might be an important prerequisite for certain morphological changes that are stimulated by GPCRs and detected with the label-free biosensors.

Differences between polarized and nonpolarized cells. Polarized cells form apical and basolateral plasma membranes, each with distinct cytoarchitecture and function. As such, GPCRs expressed by polarized cells might show a different response profile compared with their expression on other cell types. This might be particularly relevant when studying polarized cells with optical biosensors because the optical signal is most heavily influenced by the region closest to the surface of the microtiter plate (i.e. the basolateral membrane).

Signal specificity. When performing label-free biosensor studies, it is important to recognize that the signal is not limited to a biological response from one particular receptor or signaling pathway. Therefore, ensuring that the biosensor profile for a test compound is not due to or influenced by other receptors expressed by the cell is essential. If screening for agonists, a subtype-selective antagonist can be used to confirm specificity of the signal. When using transfected cells to identify a GPCR agonist, untransfected cells serve as a specificity control. When screening for antagonists, a subtype-selective agonist is preferred. Of course, such tools are not always available. In this situation, the potential for detecting ancillary pharmacology should be recognized, especially if screening a random selection of bioactive compounds. Employing RNAi technology can reduce the expression of a particular receptor and thereby demonstrate specificity of the label-free signal. Atienza *et al.* [64] used RNAi to study aspects of cell adhesion measured with an impedance biosensor and demonstrated compatibility of RNAi methods with label-free detection.

Concluding remarks

Impedance- and optical-based instruments introduce two fundamental advances – label-free detection and unparalleled sensitivity. Combining these in a single instrument is at the heart of their exceptional power and versatility. Although the potential extends to a wide range of *in vitro* applications, the impact on GPCR drug discovery is particularly great. Specifically, this versatility and power enables detecting GPCR coupling through different classes of G proteins even when expressed at endogenous levels in the cell.

One important gap that needs to be addressed is the lack of data specifically elucidating the cellular mechanisms that underlie the label-free detection of GPCR function; however, current speculation that label-free GPCR responses are linked to changes in cell morphology is both plausible and consistent with the observation that blocking actin polymerization inhibits both impedance and optical responses. The Epic, BIND and CellKey instruments reveal similar absolute ligand potencies and robust data quality sufficient to support SAR-driving assays. Further comparisons with traditional assays reveal alignments ranging from excellent for G_i to possible hypersensitivity for G_s (as measured by cAMP). The real-time response profiles for the various G-protein pathways have different kinetics that are generally conserved across instruments. Importantly, CellKey traces are more consistent in their ability to distinguish coupling through each G-protein class. This unprecedented feature of CellKey has so far revealed a remarkable frequency with which GPCRs are observed to switch G-protein coupling depending on the host cell type – an important fact for screening. That CellKey profiles are dynamic and can reveal complex, dual coupling suggests perhaps the most exciting opportunity – the ability to detect ligand-specific coupling or functional selectivity. Overall, present evaluation data clearly establish these platforms as destined for broad integration in GPCR drug discovery and suggests the breadth of applications will continue to expand.

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References

1 Minor, L.K. (2008) Label-free cell-based functional assays. *Comb. Chem. High Throughput Screen.* 11, 573–580
2 Fang, Y. *et al.* (2008) Label-free cell-based assays for GPCR screening. *Comb. Chem. High Throughput Screen.* 11, 357–369
3 Cooper, M.A. (2009) Signal transduction profiling using label-free biosensors. *J. Recept. Signal Transduct. Res.* 29, 224–233
4 Rocheville, M. and Jerman, J.C. (2009) 7TM pharmacology measured by label-free: a holistic approach to cell signalling. *Curr. Opin. Pharmacol.* 9, 643–649
5 Xi, B. *et al.* (2008) The application of cell-based label-free technology in drug discovery. *Biotechnol. J.* 3, 484–495
6 Yu, N. *et al.* (2006) Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Anal. Chem.* 78, 35–43
7 Verdonk, E. *et al.* (2006) Cellular dielectric spectroscopy: a label-free comprehensive platform for functional evaluation of endogenous receptors. *Assay Drug Dev. Technol.* 4, 609–619
8 Peters, M.F. and Scott, C.W. (2009) Evaluating cellular impedance assays for detection of GPCR pleiotropic signaling and functional selectivity. *J. Biomol. Screen.* 14, 246–255
9 Fang, Y. *et al.* (2007) Non-invasive optical biosensor for assaying endogenous G protein-coupled receptors in adherent cells. *J. Pharmacol. Toxicol. Methods* 55, 314–322
10 Leung, G. *et al.* (2005) Cellular dielectric spectroscopy: a label-free technology for drug discovery. *JALA* 10, 258–269
11 Peters, M.F. *et al.* (2010) Comparing label-free biosensors for pharmacological screening with cell-based functional assays. *Assay Drug Dev. Technol.* 8, 219–227
12 Solly, K. *et al.* (2004) Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev. Technol.* 2, 363–372
13 Ciambrone, G.J. *et al.* (2004) Cellular dielectric spectroscopy: a powerful new approach to label-free cellular analysis. *J. Biomol. Screen.* 9, 467–480
14 Cunningham, B.T. *et al.* (2004) Label-free assays on the BIND system. *J. Biomol. Screen.* 9, 481–490
15 Fang, Y. *et al.* (2006) Resonant waveguide grating biosensor for living cell sensing. *Biophys. J.* 91, 1925–1940
16 Giaever, I. and Keese, C.R. (1984) Monitoring fibroblast behavior in tissue culture with an applied electric field. *Proc. Natl. Acad. Sci. U. S. A.* 81, 3761–3764
17 Giaever, I. and Keese, C.R. (1991) Micromotion of mammalian cells measured electrically. *Proc. Natl. Acad. Sci. U. S. A.* 88, 7896–7900
18 McLaughlin, J.N. *et al.* (2005) Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *J. Biol. Chem.* 280, 25048–25059
19 Reddy, L. *et al.* (1998) Assessment of rapid morphological changes associated with elevated cAMP levels in human orbital fibroblasts. *Exp. Cell Res.* 245, 360–367
20 Waters, C.M. *et al.* (2006) Cell migration activated by platelet-derived growth factor receptor is blocked by an inverse agonist of the sphingosine 1-phosphate receptor-1. *FASEB J.* 20, 509–511
21 Atienza, J.M. *et al.* (2006) Dynamic and label-free cell-based assays using the real-time cell electronic sensing system. *Assay Drug Dev. Technol.* 4, 597–607
22 Lin, B. *et al.* (2006) A label-free biosensor-based cell attachment assay for characterization of cell surface molecules. *Sens. Actuators B: Chem.* 114, 559–564
23 Fang, Y. *et al.* (2005) Probing cytoskeleton modulation by optical biosensors. *FEBS Lett.* 579, 4175–4180
24 Fang, Y. *et al.* (2006) Cellular functions of cholesterol probed with optical biosensors. *Biochim. Biophys. Acta* 1763, 254–261
25 Giaever, I. and Keese, C.R. (1993) A morphological biosensor for mammalian cells. *Nature* 366, 591–592
26 Lamb, N.J. *et al.* (1988) Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. *J. Cell Biol.* 106, 1955–1971
27 Graler, M.H. *et al.* (2003) The sphingosine 1-phosphate receptor S1P4 regulates cell shape and motility via coupling to Gi and G12/13. *J. Cell. Biochem.* 89, 507–519
28 Davies, S.L. *et al.* (2006) Ca^{2+}-sensing receptor induces Rho kinase-mediated actin stress fiber assembly and altered cell morphology, but not in response to aromatic amino acids. *Am. J. Physiol. Cell Physiol.* 290, C1543–C1551
29 Singh, I. *et al.* (2007) Galphaq-TRPC6-mediated Ca^{2+} entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. *J. Biol. Chem.* 282, 7833–7843
30 Patterson, C.E. *et al.* (2000) Regulation of endothelial barrier function by the cAMP-dependent protein kinase. *Endothelium* 7, 287–308
31 Hirshman, C.A. *et al.* (2001) Actin depolymerization via the beta-adrenoceptor in airway smooth muscle cells: a novel PKA-independent pathway. *Am. J. Physiol. Cell Physiol.* 281, C1468–C1476
32 Gujdar, A. *et al.* (2003) Phorbol ester-induced migration of HepG2 cells is accompanied by intensive stress fibre formation, enhanced integrin expression and transient down-regulation of p21-activated kinase 1. *Cell. Signal.* 15, 307–318
33 Siehler, S. (2009) Regulation of RhoGEF proteins by G(12/13)-coupled receptors. *Br. J. Pharmacol.* 158, 41–49
34 Krilov, L. *et al.* (2008) Glucagon receptor recycling: role of carboxyl terminus, beta-arrestins, and cytoskeleton. *Am. J. Physiol. Cell Physiol.* 295, C1230–C1237
35 Zoudilova, M. *et al.* (2007) Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. *J. Biol. Chem.* 282, 20634–20646
36 McLaughlin, N.J. *et al.* (2006) Platelet-activating factor-induced clathrin-mediated endocytosis requires beta-arrestin-1 recruitment and activation of the p38 MAPK signalosome at the plasma membrane for actin bundle formation. *J. Immunol.* 176, 7039–7050
37 Laroche, G. *et al.* (2005) Involvement of actin in agonist-induced endocytosis of the G protein-coupled receptor for thromboxane A2: overcoming of actin disruption by arrestin-3 but not arrestin-2. *J. Biol. Chem.* 280, 23215–23224
38 Peters, M.F. *et al.* (2007) Evaluation of cellular dielectric spectroscopy, a whole-cell, label-free technology for drug discovery on Gi-coupled GPCRs. *J. Biomol. Screen.* 12, 312–319

39 Tran, E. and Fang, Y. (2009) Label-free optical biosensor for probing integrative role of adenylyl cyclase in G protein-coupled receptor signaling. *J. Recept. Signal Transduct. Res.* 29, 154–162

40 Fang, Y. and Ferrie, A.M. (2008) Label-free optical biosensor for ligand-directed functional selectivity acting on beta(2) adrenoceptor in living cells. *FEBS Lett.* 582, 558–564

41 Zhang, J.H. *et al.* (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4, 67–73

42 Dodgson, K. *et al.* (2009) A 100K well screen for a muscarinic receptor using the Epic (R) label-free system – a reflection on the benefits of the label-free approach to screening seven-transmembrane receptors. *J. Recept. Signal Transduct. Res.* 29, 163–172

43 Tran, E. and Fang, Y. (2008) Duplex label-free G protein-coupled receptor assays for high-throughput screening. *J. Biomol. Screen.* 13, 975–985

44 Lee, P.H. *et al.* (2008) Evaluation of dynamic mass redistribution technology for pharmacological studies of recombinant and endogenously expressed G protein-coupled receptors. *Assay Drug Dev. Technol.* 6, 83–94

45 McGuinness, R.P. *et al.* (2009) Enhanced selectivity screening of GPCR ligands using a label-free cell based assay technology. *Comb. Chem. High Throughput Screen.* 12, 812–823

46 Conn, P.J. *et al.* (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discov.* 8, 41–54

47 Wang, L. *et al.* (2009) Allosteric modulators of G protein-coupled receptors: future therapeutics for complex physiological disorders. *J. Pharmacol. Exp. Ther.* 331, 340–348

48 Kenakin, T.P. (2009) Cellular assays as portals to seven-transmembrane receptor-based drug discovery. *Nat. Rev. Drug Discov.* 8, 617–626

49 Kenakin, T. (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol. Sci.* 18, 456–464

50 Urban, J.D. *et al.* (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J. Pharmacol. Exp. Ther.* 320, 1–13

51 Cotton, M. and Claing, A. (2009) G protein-coupled receptors stimulation and the control of cell migration. *Cell. Signal.* 21, 1045–1053

52 Abassi, Y.A. *et al.* (2009) Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. *Chem. Biol.* 16, 712–723

53 Voyno-Yasenetskaya, T.A. *et al.* (1994) Mutant alpha subunits of G12 and G13 proteins induce neoplastic transformation of Rat-1 fibroblasts. *Oncogene* 9, 2559–2565

54 Yamazaki, J. *et al.* (2008) Lysophosphatidic acid and thrombin receptors require both Gα12 and Gα13 to regulate axonal morphology in hippocampal neurons. *Biol. Pharm. Bull.* 31, 2216–2222

55 Jiang, H. *et al.* (1993) The transforming activity of activated Ga12. *FEBS Lett.* 330, 319–322

56 Meshki, J. *et al.* (2009) Neurokinin 1 receptor mediates membrane blebbing in HEK293 cells through a Rho/Rho-associated coiled-coil kinase-dependent mechanism. *J. Biol. Chem.* 284, 9280–9289

57 Kendall, R.T. and Luttrell, L.M. (2009) Diversity in arrestin function. *Cell. Mol. Life Sci.* 66, 2953–2973

58 Masri, B. *et al.* (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13656–13661

59 Gesty-Palmer, D. *et al.* (2009) A β-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation. *Sci. Transl. Med.* 1, 1–9

60 McGuinness, R. (2009) Label-free cell-based assays bring greater biorelevance to early drug discovery. *Assay Drug Dev. Technol.* 7, 191–193

61 McGuinness, R. (2007) Impedance-based cellular assay technologies: recent advances, future promise. *Curr. Opin. Pharmacol.* 7, 535–540

62 Slaughter, G.E. *et al.* (2004) Improving neuron-to-electrode surface attachment via alkanethiol self-assembly: an alternating current impedance study. *Langmuir* 20, 7189–7200

63 Schwartz, M.A. and Ginsberg, M.H. (2002) Networks and crosstalk: integrin signalling spreads. *Nat. Cell Biol.* 4, E65–E68

64 Atienza, J.M. *et al.* (2005) Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. *J. Biomol. Screen.* 10, 795–805