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Mechanistic pharmacology, new developments

Label-free receptor assays

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Label-free biosensors offer integrated, kinetic and multi-parametric measures of receptor biology and ligand pharmacology in whole cells. Being highly sensitive and pathway-unbiased, label-free receptor assays can be used to probe the systems cell biology including pleiotropic signaling of receptors, and to characterize the functional selectivity and phenotypic pharmacology of ligand molecules. These assays provide a new dimension for elucidating receptor biology and for facilitating drug discovery.

Introduction

Historically, receptor signaling was viewed as a linear cascade [1]. As a result, point-of-contact based measurements, such as alterations in intracellular cyclic AMP (cAMP), Ca²⁺ mobilization, protein trafficking and phosphorylation of downstream kinases, have become the most commonly used strategy in receptor biology research and drug discovery campaigns [2,3]. The quest to discover the full complement of cell signaling components has led to identification of various activators, effectors, enzymes and substrates involved in receptor signaling, many (if not all) of which work in concert to guide pivotal intracellular processes [4]. The integration of distinct signaling pathways dedicates how signals propagate within the cell and ultimately how the cell responds to environmental cues [5,6]. Pharmacological assays now in development, such as label-free receptor assays, are making it possible to pathway-unbiasedly monitor cell signaling both spatially and temporally [7,8]. As cells use sophisticated regulatory mechanisms to ensure an appropriate integrative response upon stimulation, assays based on integrated readouts as promised by label-free receptor assays are naturally suited

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to sense the complexity of receptor signaling and ligand pharmacology. Together with high sensitivity, the pathway-unbiased nature permits the label-free receptor assay a single platform for recording a wide array of receptor signaling in native cells including primary cells.

Label-free receptor assays

Biosensor systems

Central to label-free receptor assays is the biosensor that is capable of converting a cellular response into a quantifiable signal [9]. Label-free biosensors including surface plasmon resonance [10] and its derivatives [11] were initially developed as an analytical tool for biomolecular interaction analysis. Soon after birth these biosensors were also explored to sense cellular activity; but early attempts were mostly limited to cell adhesion [12,13] and cytotoxicity [14]. With sophisticated engineering and advanced assay designs, the new generation biosensor systems such as Cellkey™ (MDS Analytical Technologies), xCELLigence (Acea Biosciences), BIND (SRU Biosystems) and Epic® (Corning Inc.) systems have made it possible to robustly probe receptor signaling, particularly in the microtiter plate format which is the standard footprint for large scale research and drug discovery. The detailed descriptions of these systems can be found in the websites of these vendors (see the *Links*) and are not reviewed here.

Both CellKey™ [15] and xCELLigence [16] systems exploit a microelectrode array electrical biosensor to probe changes in impedance of a cell layer. Sinusoidal voltages that are swept through a range of frequencies in continuous wave

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mode are used to generate electric fields between the electrodes, which are impeded by the presence of cells.

Both SRU BIND [17] and Epic[®] [18] systems use a nano-grating waveguide biosensor to characterize cellular responses. Lights that cover a range of wavelengths are used to generate a diffraction grating coupled waveguide resonance, thus leading to a surface-bound electromagnetic wave, which is perturbed by the presence of cells [18].

Biosensor signals

Electrical biosensor uses a sophisticated algorithm to determine and separate two types of electrical currents, extracellular and transcellular currents [15]. This is because of the fact that the cellular plasma membrane acts as an insulating barrier directing the current to flow between or beneath the cells. The flow of both currents is termed impedance. The cellular impedance is a measure of changes in the electrical conductivity or permeability of the cell layer, and the extracellular current is more robust than the transcellular current. Since the impedance is mostly a function of ionic environment surrounding or within the cells, the electric biosensor is largely sensitive to, and thus biased toward, changes in cellular morphology and ionic redistribution.

Optical biosensor mostly tracks changes in the central wavelength or angle (i.e. resonance wavelength or angle, respectively) of the biosensor resonant spectrum [8,18]. For resonant waveguide grating (RWG) systems such as Epic[®] and SRU BIND, the resonance wavelength is a function of the local refractive index at or near the sensor surface, which is mostly proportional to the mass density and distribution of biomaterials within the cells. Receptor signaling often involves protein trafficking, microfilament remodeling, cell adhesion alterations and morphological changes of cells, all of which can lead to significant dynamic mass redistribution (DMR) [8]. Such redistribution is not random; instead, it is tightly regulated and is often dynamic both spatially and temporally. The biosensor simply acts as a noninvasive monitor to record the DMR in real time. Since most optical biosensors use long-wavelength light for illumination, these biosensors are believed to be, or close to be, truly noninvasive. However, since most optical biosensors including SPR and RWG have limited detection volume because of the short penetration depth (~200 nm) of the surface-bound evanescent wave, the cellular responses measured are often biased toward cellular changes within the bottom portion of cells [8].

Assay formats

Cell signaling propagates in several sequential waves, from the early responses including receptor activation, second messengers and protein trafficking, to the intermediate responses including *de novo* synthesis, exocytosis and gene expression alterations and to the late responses such as cell proliferation and survival. Label-free receptor assays are non-

invasive in general, thus permitting multiple assay formats to track the entire course of cell signaling in real time. The two common assay formats are costimulation and sequential stimulation assays [19]. Most label-free receptor assays are to probe the early processes of cell signaling.

Label-free receptor assays for receptor biology

Cell signaling by membrane receptors is a coordinated relay of messages derived from environmental cues to intracellular effectors. It is evident that sophisticated regulatory mechanisms are utilized for cells to encode, process and integrate the information received. Common to label-free receptor assays is to record an integrated signal in real time of whole cells. Such integrated measurements often reflect the complexity of receptor signaling, permitting the study of the systems cell biology of receptors, the cross-talks between receptors, and the integration of receptor signaling.

Systems cell biology of receptors

The quest to discover the full complement of receptor signaling components has led to identification of various activators, effectors, enzymes and substrates. Pharmacological tools including small molecules and interference RNA have become widely available, thus making it possible to selectively perturb the cellular activities of many signaling proteins downstream of a receptor. Assays that measure point-of-contacts in the complexed signaling network are also useful for orthogonal confirmation. Thus, it is feasible to study the systems cell biology of receptors using an integrated and complex readout of whole cells as promised by label-free receptor assays.

The DMR assay using Epic[®] system was the first label-free receptor assay used to map the signaling and its network interactions of several receptors including epidermal growth factor (EGF) receptor [20]. The EGFR is one of the most well-studied receptor tyrosine kinases. EGF binds to the receptor and stimulates its intrinsic protein-tyrosine kinase activity, initiating signal transduction that principally involves the MAPK cascades, Akt signaling, STAT activation and the PLC γ pathway. Using the EGF DMR signal in A431 cells as a readout, we found that EGFR signaling is cellular status dependent – quiescent cells respond more robustly to EGF than proliferating cells. The modulation profiles of an array of known modulators also linked several targets and cellular processes to the EGFR signaling. The EGFR signaling was found to require its intrinsic tyrosine kinase activity and to be mostly originated from the internalized receptors. The EGFR signaling also led to actin remodeling, dynamin- and clathrin-dependent receptor internalization and MEK pathway-mediated cell detachment (possibly via FAK).

A recent cellular impedance study for the EGFR in Cos7 cell, using the xCELLigence system, confirmed the ability of label-free receptor assays to probe the systems cell biology of the EGFR [21]. In this study, the authors claimed that the

impedance signal is a direct result of growth factor-induced morphological changes, and can be used as a quantitative readout for receptor tyrosine kinase activity in Cos7 cells. Both EGF and insulin-induced impedance signals were found to be correlated with the morphological changes of cells and levels of receptor autophosphorylation measured using fluorescent microscopy and enzyme-linked immunosorbent assay, respectively.

However, since the EGFR signaling consists of hundreds (if not thousands) of components which often display different temporal dynamics and spatial gradients [4,5], it will be naturally difficult to map out the full complement of signaling components using label-free receptor assays, and thus to fully comprehend the origin of the biosensor signal of the receptor. For this reason, label-free receptor assays are often referred to 'black box' [22,23]. However, the abovementioned studies suggest that it is quite promising for these assays to identify many important nodes in the receptor signaling network (Fig. 1).

Pleiotropic signaling of receptors

G-protein-coupled receptors (GPCRs) have been and continue to be one of the richest families of drug targets. There are at least two key drivers for this. The first driver is the increasing numbers of orphan receptors being deorphanized, some of which have implications for human diseases. Examples are GPR3 for Alzheimer's disease [24] and GPR40 for diabetes [25]. The second driver is associated with the recent realization that GPCRs are competent to elicit a rich array of cell signaling pathways (i.e. pleiotropic signaling) [7,26], and ligands might give operational biases to activate the receptor [27]. These pathway-biased ligands might open new revenues for drug discovery.

Label-free offers new means in discovering new receptor behaviors. Our recent DMR assays for endogenous bradykinin B₂ receptor in A431 cells showed that the receptor mediates signaling via at least dual pathways – G_{αs}- and G_{αq}-mediated signaling; and remarkably, the two signaling pathways counter-regulate each other [28]. Kostenis and her colleagues [29] have found, using DMR assays and classical endpoint assays, that CRTH2 receptor utilizes its C-tail domain to silence its own signaling, and the C terminus does not encode G protein specificity determinants. A CRTH2 mutant receptor lacking this domain displays paradoxically enhanced G_{αi} and ERK1/2 activation.

Signaling compartmentalization and cross-talks

Cell signaling specificity requires that signaling molecules encounter their intracellular interactants in the right place and at the right time. Cell surface receptors including GPCRs might preferentially locate within some cholesterol and sphingolipid-enriched microdomains. For example, the endogenous B₂ receptor in A431 might be associated with

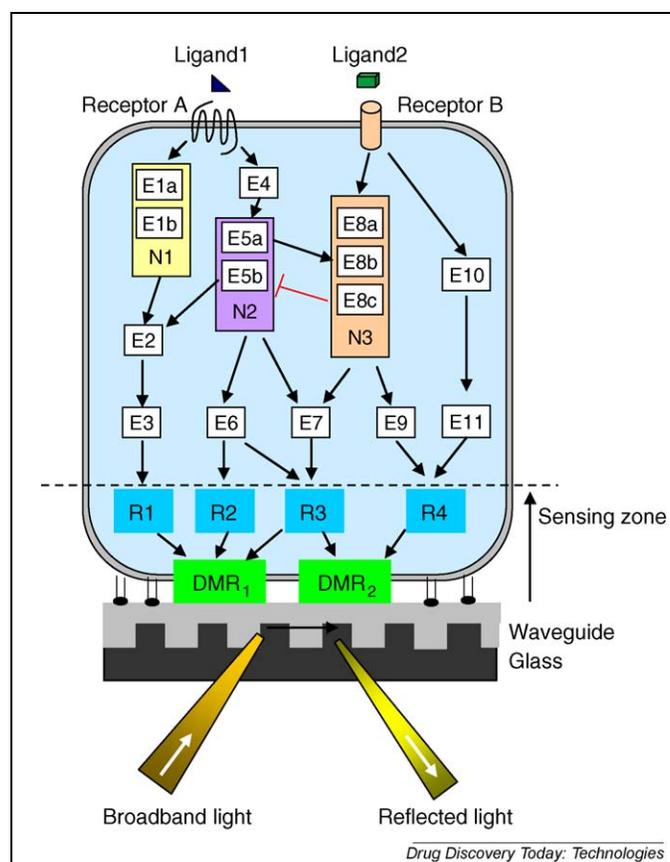


Figure 1. Schematic depiction of the ability of label-free receptor assays to detect crucial nodes in the signaling network of a receptor. Cells employ regulatory and compensatory pathways to fine tune their decision-making process. It is highly possible for label-free receptor assays to identify at least some of these important signaling nodes. For example, the binding of agonist 1 to the receptor A leads to two major pathways: the first one consists effectors E1a, E1b, E2, E3, leading to response R1, while another consists of E4, E5a, E5b, E6 and E7, leading to responses R2 and R3. The integration of the responses R1, R2 and R3 when occurred within the sensing zone of the biosensor leads to the DMR₁. The effectors E1a and E1b form the crucial node N1, while E5a and E5b form another crucial node N2. Similarly, the activation of the receptor B by ligand 2 leads to activation of two pathways: the first one consists effectors E8a, E8b, E8c, E7 and E9, leading to the responses R2 and R3, while the second one consists of the effectors E10 and E11, leading to the response R4. The integration of R3 and R4 when occurred within the sensing zone of the biosensor leads to the DMR₂. E8a, E8b and E8c form a crucial node N3 for the receptor B signaling.

such microdomains, since its signaling was found to be sensitive to cholesterol depletion [28].

Compartmentalization of enzymes in proximity to substrates is another means of spatially restricting cell signaling events [30]. As a result, distinct signaling pathways might undertake spatially different routes. For this reason, the activation of different pathways could lead to distinct biosensor signals, and the biosensor signals obtained can be used as 'signatures' to identify pathways being activated [15,16,31]. Using the DMR assays, we [32] found that the activation of endogenous G_q-coupled histamine H₁ receptor

in A431 led to a G_q -type DMR, while the activation of endogenous G_s -coupled β_2 -adrenergic receptor (β_2 -AR) in the same cell led to a G_s -type DMR. The coactivation of both receptors by epinephrine and histamine led to a DMR signal that is close to the sum of both histamine and epinephrine DMR signals. This result suggests that both G_q - and G_s -mediated signals undergo spatially distinct routes at the complex pathway level.

Cells use various regulatory and compensatory pathways to fine tune cellular responses. It is common for distinct receptors to cross-talk at multiple levels, including receptor oligomerization [33], interactions of intracellular signal transduction cascades [34,35] and phosphorylation of receptors and regulatory proteins by kinases [36]. The cross-talk ensures the information exchange between pathways and allows the cells to make appropriate responses. The recent DMR study of A431 cells [32] showed that histamine slightly attenuated the epinephrine response, while epinephrine partially attenuated the histamine response. This result indicates that there are cross-talks between the epinephrine- and histamine-mediated signaling. Label-free receptor desensitization assays [37] showed that in A431 cells, the forskolin pretreatment heterologously desensitized G_{α_s} signaling, partially attenuated G_{α_q} signaling, but had complicate impacts on G_{α_i} signaling. This result suggests that adenylyl cyclase acts as an effective integrator for GPCR signaling.

Label-free receptor assays for ligand pharmacology

Central to drug development is the pharmacological characterization of drug molecules. Because of the rich behavior of receptors and the heterogeneity of signaling components in different cell types, drug molecules can have complicated pharmacology, including pathway-biased activity (i.e. functional selectivity), phenotypic pharmacology and polypharmacology. The possibility to have multiple efficacies for a given ligand makes it difficult in practice to systematically represent the ligand efficacy using pathway-biased approaches, because of the large number of possible signaling outputs [2,3]. The ability to detect the integrated response of whole cells by endogenous receptors empowers the label-free assays to classify drug molecules in new dimensions [7,31,38].

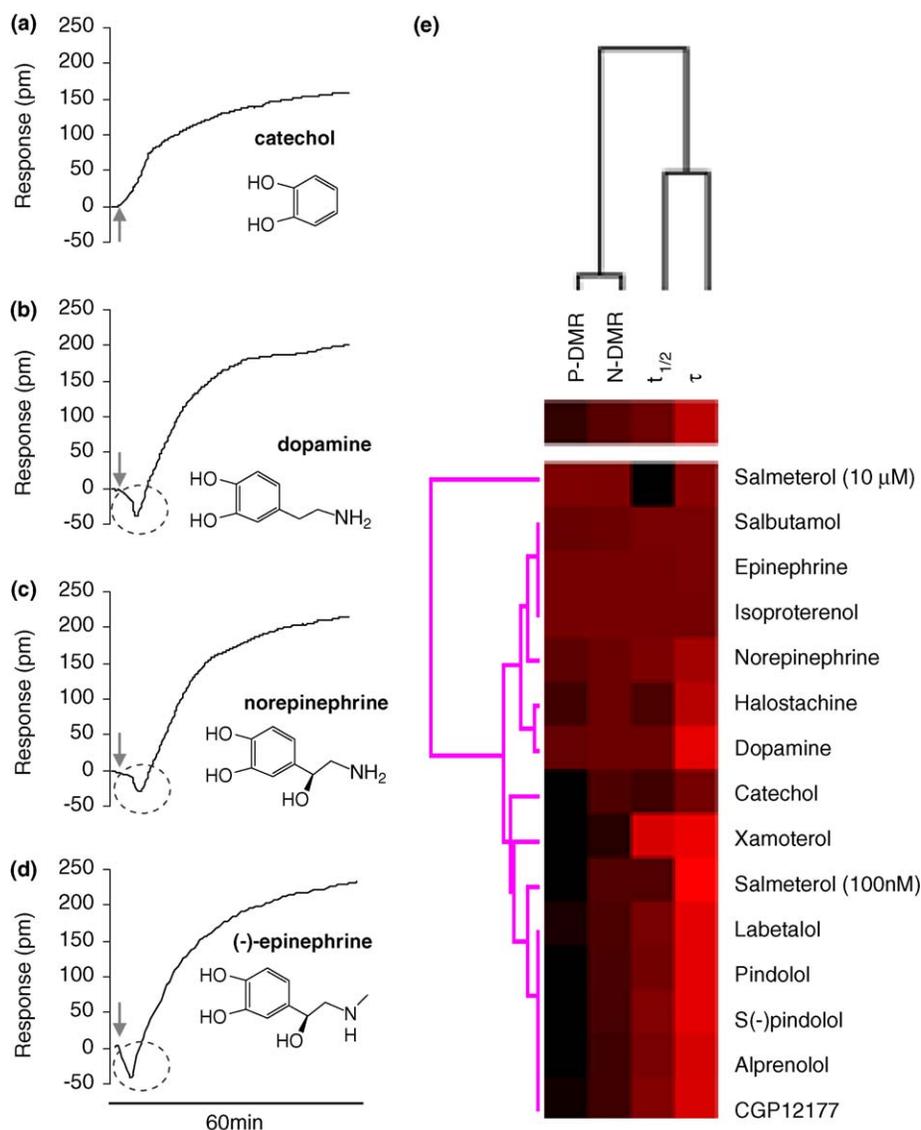
Ligand-directed functional selectivity

The quest to fully characterize the pharmacological activity of drug molecules with a wide spectrum of point-of-contact and phenotypic assays has led to the discovery of novel pathway-biased activity of many ligands for increasing numbers of GPCRs. A classical example is the beta blocker propranolol. Propranolol was recently identified as an inverse agonist for G_{α_s} pathway, and also a β -arrestin-dependent extracellular signal regulated kinase (ERK) agonist [39]. These pathway-biased activities might contribute to the complex therapeutic profiles of drug molecules.

Label-free receptor assays allow a greater array of changes in the receptor to be detected. Using the DMR assays, a panel of β_2 -AR ligands was characterized in quiescent A431 cells [38]. Multi-parameter analysis revealed unique patterns in the characteristics of their corresponding DMR signals (Fig. 2). Full agonists such as epinephrine and isoproterenol gave rise to a DMR with maximum amplitudes, fast transition time but slow kinetics for the P-DMR event. In comparison, partial agonists such as catechol and halostachine led to a DMR signal with smaller amplitudes, slightly slower transition time but faster kinetics for the P-DMR event. Similarity analysis suggests that these parameters can be used to categorize the agonism activity of these molecules (Fig. 2). Interestingly, the partial agonists catechol and pindolol bind to different portions of the binding pocket of the β_2 -AR, triggering largely independent signaling. This is consistent with results obtained using both theoretical and biophysical approaches. Protein ensemble theory predicts a range of receptor active states, each of which could have its own signaling preference [40]. Biophysical studies suggest that for the β_2 -AR ligand binding and receptor activation is a kinetically and conformationally complex process, and agonist binding and receptor conformational changes occur through a sequence of conformational intermediates [41,42].

The most notable finding from this study is the dual efficacies of the long-acting agonist salmeterol. Salmeterol resulted in a biphasic dose-dependent response, leading to two well-separated EC_{50} values. Salmeterol binds not only to the active site (Ser-204, Ser-207 and Asp-113) of the β_2 -AR but also to its exosite, and results in a slow internalization of the β_2 -AR [43]. Salmeterol was known to have dual efficacies: a very weak partial agonist for producing an effective interaction between the receptor and β -arrestin 2, and a full agonist of cAMP accumulation in C2C12 cells stably expressing the β_2 -AR [44]. In human kidney embryonic 293 cells, despite stimulating GRK-mediated receptor phosphorylation after 30 min to an extent similar to those observed with agonists of high intrinsic efficacy such as epinephrine, salmeterol of 50 nM did not induce significant β_2 -AR internalization and was incapable of stimulating the translocation of green fluorescent protein-arrestin 2 chimera proteins to the cell surface [45].

Allosteric modulators that edit the behavior of the receptor toward agonists can also provide basis for functional selectivity. For example, neurokinin normally triggers G_s and G_q signaling via NK1 receptor. The cobinding with neurokinin of allosteric modulator LP1805 to the receptor led to *enhanced* G_q response and *antagonism* of G_s activation [46]. Label-free receptor assays have also been used to characterize allosteric modulators for metabotropic glutamate receptor 7 [47], and to examine a novel class of ligands that can simultaneously bind to both allosteric and conserved orthosteric sites of the same muscarinic receptor [48].



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Figure 2. Characteristics of β_2 -AR agonist DMR signals of quiescent A431 cells. **(a)–(d)** Representative DMR signals and structures of a panel of β_2 -AR agonists: (a) catechol of 500 μ M; (b) dopamine of 32 μ M; (c) norepinephrine of 100 nM; and (d) (-)-epinephrine of 8 nM; each at the saturating concentration. **(e)** The heat map classification of β_2 -AR agonist pharmacology based on the characteristics of their corresponding DMR signals. Data used were from Ref. [36]. The heat map was generated using the Euclidean hierarchical cluster analysis (Ref. [57]), after all DMR parameters were normalized to the epinephrine response. Data suggest that the first subgroup consists of full agonists and strong partial agonists including isoproterenol, epinephrine, norepinephrine and salbutamol, while the second group consists of partial agonists including halostachine and dopamine. The third group consists of the beta-blockers with weak partial agonism activity, including labetalol, pindolol, S(-)-pindolol, alprenolol, CGP12177 and to certain extent, salmeterol of 100 nM. The weak agonist catechol and the partial agonist xamoterol are between the second and third group. Salmeterol of 10 μ M leads to very unique DMR that is similar but not identical to the full agonists.

Protease activator receptor-1 (PAR1) plays important roles in regulating the permeability of the endothelial cell barrier at the blood–tissue interface. Thrombin activates PAR1 by cleavage of its amino terminus to unmask a tethered ligand [49]. Synthetic PAR-activating peptides (PAR-APs), corresponding to the first five or six amino acids of the tethered ligand sequences, can directly activate PAR1 [50]. Using an electric biosensor cellular assay, Hamm and her colleagues [51] had found that thrombin increased the permeability of

the monolayer of a human dermal microvascular endothelial cell line HMEC-1 primarily via Rho kinase pathway. Furthermore, thrombin gave rise to relatively lower potency in activating Ca^{2+} mobilization. However, the opposite order of activation was observed for the two PAR-AP agonists, SFLLRN-amide and TFLLRNKPKDK. A mathematic numerical analysis suggests that the PAR-AP agonists alter receptor/G protein binding to favor G_{α_q} activation over $G_{\alpha_{12/13}}$ by ~ 800 -fold.

Phenotypic pharmacology

The cellular environment can influence, sometime determine, the functional responses of receptors, thus to large extent, define drug actions. The cellular-context-dependent drug actions are also viewed as phenotypic pharmacology. The cellular factors that could influence drug actions include the receptor expression level, the diversity in receptor conformations and organizations and the heterogeneity in expression and organization of cytosolic interactants. There are many examples in literature that a given receptor might prefer different signaling pathways when presented in different cellular backgrounds [52,53]. Recently, Peters and Scott [54] used cellular impedance assays to characterize several receptors in different cell backgrounds, and found that melanocortin-4 receptor (MC4R) can trigger either $G_{\alpha s}$ or $G_{\alpha q}$ coupling in a cellular-context-dependent manner. In HEK-MC4R cells MC4R preferred $G_{\alpha s}$ coupling, while in CHO-MC4R cells it favored $G_{\alpha q}$ coupling.

Conclusion

The evolution of pharmacological assays has led to the discovery of a wide array of cellular behavior of receptors and drug molecules. Label-free receptor assays are emerging as a powerful assay platform to study receptor signaling, and to elucidate crucial nodes of receptor signaling networks. Label-free receptors also offer new dimensions to characterize and categorize drug molecules. With recent advancements in biosensor instrumentation such as Epic[®] system, high throughput screening becomes a reality for the activity assessments of drug molecules acting on native cells including primary cells. The next generation biosensor systems will have higher sensitivity and spatial resolution in directions perpendicular and/or parallel to the sensor surface [55,56]. It will be possible to characterize receptor biology and ligand pharmacology in single cells, and in mixed populations of cells such as tissue cells, reprogramed cells and unpurified primary cells. Together with smarter assay design and development of novel methodologies, label-free receptor assays will provide new perspectives in cell biology and drug pharmacology.

Related articles

- Fang, Y. *et al.* (2006) Resonant waveguide grating biosensor for living cell sensing. *Biophys. J.* 91, 1925–1940
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- For Epic[®] system: http://www.corning.com/lifesciences/us_canada/en/whats_new/epic_system.aspx
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Acknowledgement

This work is supported partially by National Institutes of Health Grant 5U54MH084691.

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