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The development of label-free cellular assays for drug discovery

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Introduction: The need to improve drug research and development productivity continues to drive innovation in pharmacological assays. Technologies that can leverage the advantages of both molecular and phenotypic assays would hold great promise for discovery of new medicines.

Areas covered: This article briefly reviews current label-free platforms for cell-based assays and is primarily focused on fundamental aspects of these assays using dynamic mass redistribution technology as an example. The article also presents strategies for relating label-free profiles to molecular modes of actions of drugs.

Expert opinion: Emerging evidence suggests that label-free cellular assays are phenotypic in nature, yet permit molecular mechanistic deconvolution. Together with unique competency in throughput, sensitivity and pathway coverages, label-free cellular assays allow users to screen drugs against endogenous receptors in native cells (including disease relevant primary cells) and determine the molecular modes of action of drug molecules. However, there are challenges for label-free in both basic research and drug discovery: the deconvolution of the cellular and molecular mechanisms for the biosensor signatures of receptor–drug interactions, new methodologies for data analysis and the development of new biosensor technologies. These challenges will need to be met for the wide adoption of these assays in drug discovery.

Keywords: cell-based assays, dynamic mass redistribution, electric biosensor, impedance, label-free, ligand-directed functional selectivity, molecular modes of action, receptor, resonant waveguide grating

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1. Introduction

Pharmacological assays are essential to drug discovery and development process. With advances in understanding of the molecular basis of diseases and the quest to fully characterize drug pharmacology have expanded the numbers of pharmacological assays to screen molecules for modifying the activity of druggable targets. These assays generally fall into phenotypic and target-based molecular approaches. In the era of target-centric drug discovery, first-in-class new medicines are discovered more by phenotypic screening than by target-based molecular assays. A recent analysis of new molecular entities and new biologics that were approved by the US Food and Drug Administration between 1999 and 2008 [1] suggests that out of the 259 approved agents, 75 were first-in-class drugs with new molecular modes of actions (MMOAs), 164 were follower drugs and 20 were imaging agents. Further analysis of 50 first-in-class small-molecule drugs showed that 28 were discovered by phenotypic screens, 5 were developed as synthetic versions of natural substances and 17 were discovered by target-based approaches.

Molecular assays measure a specific signaling pathway and/or molecule to infer the functional consequences of drugs acting through a target receptor, while phenotypic assays examine a specific physiological or cellular phenotype to

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Article highlights.

- The decline in pharmaceutical productivity continues to drive innovations in pharmacological assays. Phenotypic screen is more productive in discovering the first-in-class new medicines than target-based approaches. However, disadvantages are apparent for phenotypic assays – it is impracticable to guide medicinal chemistry without prior knowledge of the molecular mode of action of lead drug molecules.
- Pharmacological assays that are phenotypic in nature yet are amenable to mechanistic deconvolution would hold great promise in discovering new molecular medicines, particularly for unexploited biological mechanisms.
- Label-free has been widely adopted in drug discovery, particularly for characterization of biomolecular interactions.
- Following the promises of label-free biochemical assays to discover molecules with all binding modes, label-free cellular assays have been developed to screen and characterize a wide variety of molecular modes of action of drug molecules in native cellular environments.
- Understanding the fundamentals of the biosensor signatures of drug–receptor interactions is crucial to successful adoption of label-free cell-based assays in drug discovery, ranging from target identification, lead identification and optimization, and drug selection for *in vivo* testing.
- Label-free translates receptor–drug interactions into biosensor signatures in cells, which are novel phenotypic responses and are capable of mechanistic delineation of receptor biology and drug pharmacology, with the help of chemical biology and molecular biology approaches.
- Methods to relate the biosensor signatures of drugs to specific molecular modes of action have been developed. Continuous improvements will further advance label-free and accelerate the adoption of label-free in both basic research and drug discovery

This box summarizes key points contained in the article.

determine the biological functions of drug molecules [2]. Molecular assays have benefits from being compatible to high-throughput screening (HTS) and from being able to guide medicinal chemistry [3]. However, molecular assays often use artificial systems and are mostly biased to a specific MMOA, contributing to the poor correlation between *in vitro* testing and therapeutic impacts [4]. On the other hand, phenotypic assays directly measure the activity of drug molecules in physiologically relevant cells or cell systems, and such an activity might be linked to therapeutic impact for a given disease state [5,6]. However, phenotypic assays often led to physiological or cellular phenotypes that are too complex to derive mechanistic descriptions of drug actions, thus impracticable to optimize drug molecules [1]. Innovative assays that can bridge the gap between molecular and phenotypic assays would be advantageous.

Label-free biochemical assays have been shown to detect multiple types of binding interactions of distinct molecules (e.g., orthosteric and allosteric ligands) to a receptor of interest [7]. The wide adoption of these assays in drug discovery has motivated the development of label-free cellular assays for screening and investigating a wide variety of MMOAs of drugs in native cellular environments [8-11]. The present article reviews current generation of label-free platforms for HTS-compatible cellular assays and discusses key aspects of how to determine MMOAs of drugs using these assays.

2. Label-free technologies for cell-based assays

Label-free cellular assays use a biosensor to detect a drug molecule-induced cellular response [10]. In principle, diverse types of biosensors can be developed and used for cell-based assays by measuring specific physical properties of living cells ranging from size to morphology, membrane capacitance, mechanical and hydrodynamic properties, organization of biomolecules within cells or adhesion patterns of cells on a surface. Recently, two types of biosensors, electric biosensors based on impedance [12,13] and optical biosensors based on dynamic mass redistribution (DMR) [14-16], have been emerging as leading platforms for label-free cellular assays (**Figure 1**). Optical biosensors translate a cellular response into a DMR signal, which is often recorded as the shift in central resonant wavelength (in picometer, pm) as a function of time. Optical biosensors are considered to be noninvasive, due to the use of relatively long-wavelength light for illumination of the biosensor, but not the cell [15]. The electric biosensors use a low electrolyte impedance interface to convert a cellular response into an impedance signal, which is obtained under electric fields generated with sinusoidal voltages. Electric biosensors are considered to be minimally invasive [13].

Current generation label-free biosensor platforms offer a range of throughputs for cell-based assays. The major platforms include Epic® system from Corning Inc. [17,18], EnSpire™ multimodal reader containing Epic technology from PerkinElmer [19], BIND™ systems from SRU BioSystems [20,21], ECIS™ (electric cell–substrate impedance sensing) systems from Applied BioPhysics [22], xCELLigence™ (real-time cell electric sensing; RT-CES) systems from Roche/Acea Biosciences [23,24], and Cellkey™ (Cellular Dielectric Spectroscopy; CDS) systems from Molecular Devices [25,26]. Almost all instrument providers offer multiple instruments with different throughputs for different applications, and the specifications of these instruments can be found in the Web sites of corresponding vendors. The principles, detection schemes and performance specifications of these platforms have been comprehensively reviewed in literature [9-12,18,21,24,26] and thus are not described in details in this review.

Label-free biosensors provide a noninvasive means to investigate cellular responses at the whole-cell and cell-system

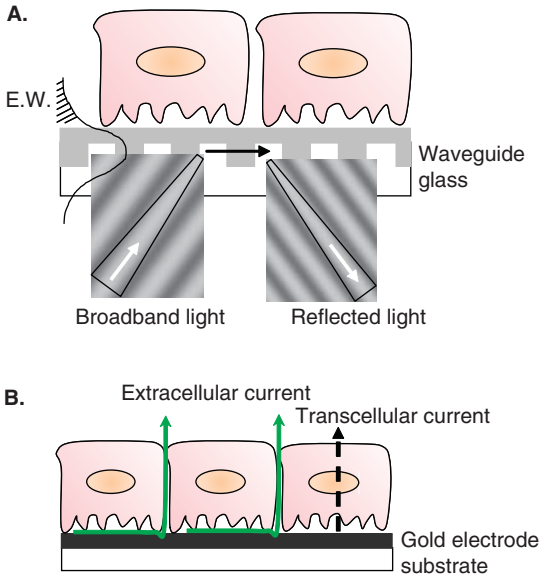

Figure 1. Principles of label-free platforms. A. Resonant waveguide grating, which uses leaky mode nanograting waveguide structure to generate an evanescent wave (E.W.) to sense whole-cell responses. **B**. Electric biosensor, which uses a low electrolyte impedance interface to sense whole-cell responses.

level with high temporal resolutions. With advances in biosensor design and instrumentation has expanded the applications of label-free biosensors to examine a diverse array of cellular processes, including cell adhesion [12,13,27,28], cell barrier functions [29-31], proliferation and toxicity [32,33], cell–cell communication [34,35], cell differentiation [36], cell migration [37,38], muscle cell hypertrophy [39,40], cardiomyocyte beating [41] and viral infection [42,43]. Furthermore, label-free whole-cell assays are highly sensitive and have wide pathway coverage, so it is possible to investigate a wide range of endogenous receptors in native cells including disease-relevant primary cells. Together with gene manipulation techniques (e.g., gene transfection, RNA interference and gene deletion) and pharmacological tools (e.g., agonists, antagonists, allosteric modulators and pathway modulators), label-free whole-cell assays have been used to characterize a diverse array of receptors, including G-protein-coupled receptors (GPCRs) [11,16,44-46], kinases [15,47,48], ion channels [49,50] and immunoreceptors [51,52]. Given that many of these applications have been reviewed in literature, the present article is focused on the fundamental aspects of label-free whole-cell receptor assays and discusses the strategies to relate label-free profiles to MMOAs of drug molecules. Furthermore, considering the differences in biophysical mechanism underlying the measurements as well as in biosensor output signal obtained using different technologies, DMR will be used as an equivalent example for the purposes of this review.

3. Label-free biosensors translate receptor–drug interactions into dynamic signatures

Profiling both endogenous and recombinant receptors using label-free biosensors has discovered that *a dynamic signature is associated with a specific receptor–ligand interaction in a given cell background* [15,24,26,44,45]. The biosensor signature is a real-time kinetic response. It typically begins with a baseline, followed by a kinetic response that tracks the entire time course of receptor signaling. To effectively separate receptor signaling-mediated responses from the background, the biosensor signature of a receptor–drug interaction is generally obtained after the cells form a monolayer [46]. This is partly because cell culturing onto a biosensor surface typically undergoes a dynamic and multistep process, including cell adhesion, spreading and proliferation, each of which gives rise to distinct background responses. Once the cells reach high confluency, a steady baseline is often achieved. The steady baseline lasts long enough so that it is possible to collect a biosensor signature that solely reflects receptor signaling. The common use of cell monolayer in label-free cellular assays is also due to the fact that current biosensors all measure an averaged response from a population of cells. Higher confluency generally leads to higher and more reproducible signal [53]. For certain receptors (e.g., receptor tyrosine kinases), synchronization of the cells into a specific state (e.g., quiescent state via contact inhibition) is important to obtain robust signals [15,54]. It is worthy noting that the high coverage of cells on a biosensor surface is good but not necessary for obtaining reproducible biosensor signals.

The kinetic response arising from a receptor–ligand interaction consists of at least one event, which can be positive and/or negative relative to the baseline. The kinetic response is often saturable and the dose response is monophasic, given that the ligand is specific to the receptor in the cell. **Figure 2** shows DMR characteristics of a newly identified GPR35 agonist tyrphostin-51 to activate endogenous GPR35 in HT29 cells. GPR35 is a poorly characterized orphan GPCR. The tyrphostin-51 dose response fits well with one-phase sigmoidal nonlinear regression, leading to an EC_{50} of 0.11 ± 0.03 μM [55]. Tyrphostin-51 was found to cause internalization of GPR35, phosphorylation of extracellular signal-regulated kinase (ERK) and translocation of β-arrestin via GPR35. Furthermore, the tyrphostin-51 DMR can be blocked by a known GPR35 antagonist.

However, for certain ligands, the dose response can be biphasic, leading to dual efficacy. A biphasic dose response may be related to dual modes of action of the ligand acting at a receptor; that is, the ligand at low doses is biased to a specific pathway, but at higher doses, the ligand activates a broader range of pathways [56]. DMR profiling of salmeterol using A431 led to identification of a biphasic dose response, resulting in two well-separated EC_{50} values (**Figure 3**) [57]. Salmeterol is a β_2-adrenergic receptor (β_2-AR)-specific partial

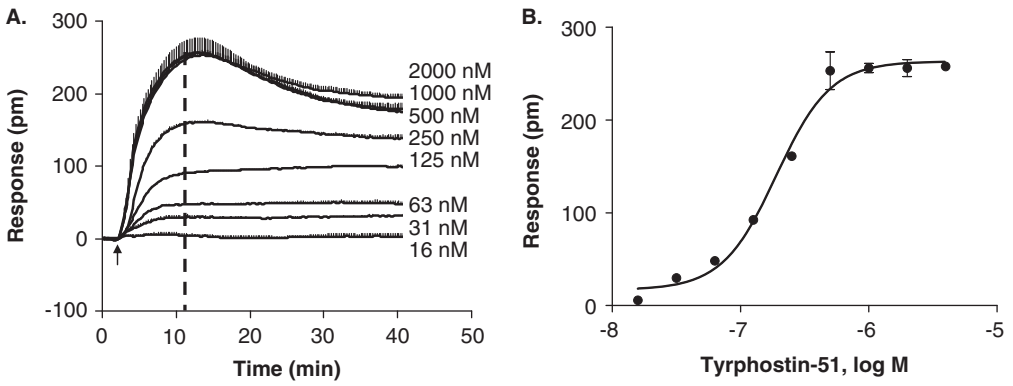

Figure 2. Characteristics of the dynamic mass redistribution (DMR) signal induced by tyrphostin-51 in HT29 cells. A. The real-time kinetics of DMR signals. **B**. The DMR amplitudes at 8 min post-stimulation (indicated by the dot line) as a function of tyrphostin-51 doses.

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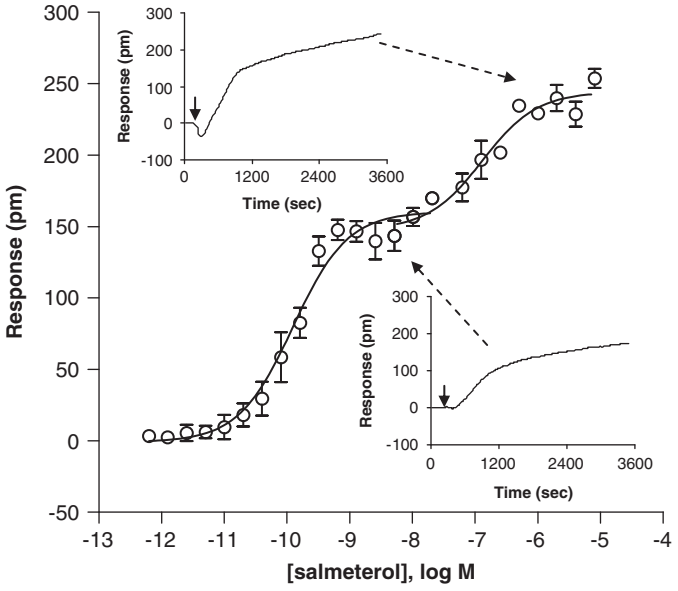

Figure 3. The biphasic dose response of salmeterol in quiescent A431 cells. The positive dynamic mass redistribution (P-DMR) amplitudes (50 min post-stimulation) as a function of salmeterol doses. The inset showed two representative DMRs induced by salmeterol of 10 and 1000 nM, respectively.

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agonist and has been shown to have dual efficacies in C2C12 cells stably expressing the β_2-AR: a weak partial agonist for producing the interaction between the receptor and β-arrestin 2, but a full agonist for cAMP accumulation [58].

Alternatively, a biphasic response may be associated with the existence of different receptor states such as functional monomers and oligomers [59]. The ligand may have different potency to activate distinct receptor populations. The third possibility is that the ligand is able to activate more than one cellular target with different potency. It is well known that many, if not all, of ligands exhibit polypharmacology – the ability to bind to more than one targets [60]. In this case, when the modification of the activity of distinct targets by the same ligand leads to the activation of distinct pathways, it often results in distinct signatures at different dose ranges. This indicates that the biosensor profiles alone should not be used to distinguish polypharmacology from functional selectivity of a ligand. Detailed pharmacological characterization, together with specificity determination, is necessary to separate polypharmacology from functional selectivity of a ligand. **Figure 4** shows the dose–response of tyramine in A431. Tyramine appears to activate another unknown receptor beside the β_2-AR [57].

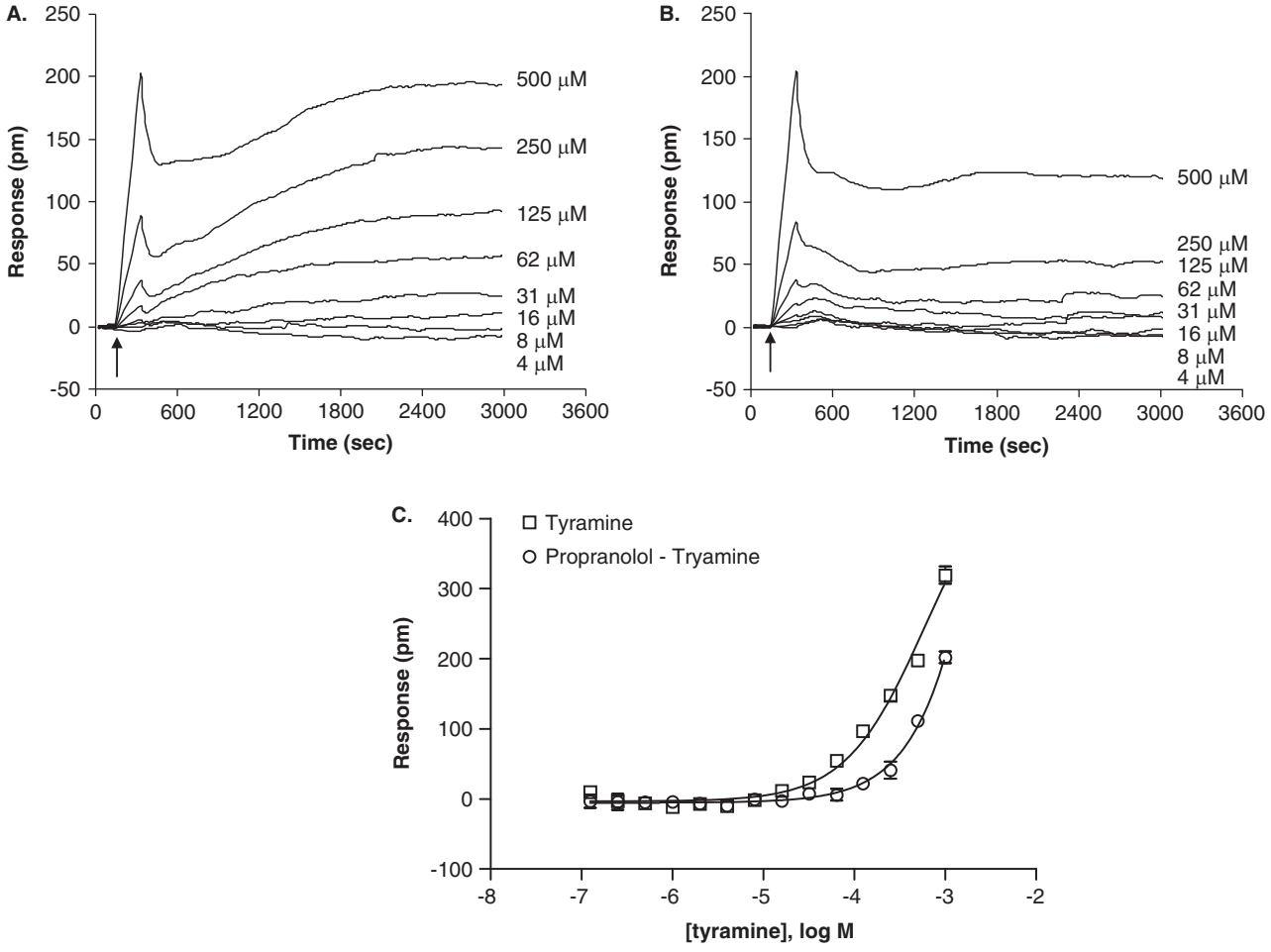

Figure 4. The dose dynamic mass redistribution (DMR) response of tyramine in quiescent A431 cells. A. DMR in the cells without any pretreatment. **B** DMR in the cells after pretreated with 1 μM propranolol for 1 h. **(C)** The DMR amplitude at 50 min post-simulation is plotted as a function of tyramine doses.
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These observations suggest that label-free cellular assays mirror the complexity of ligand pharmacology. Complex pharmacology is quite literally a fact of life. Assay technologies that can monitor and reflect that complexity are powerful. Label-free cellular assays are uniquely sensitive to the complexities of receptor-mediated signal transduction at the whole-cell level and, as such, inform the process of drug discovery in ways that other assay technologies cannot.

4. The biosensor signature of a receptor is ligand dependent

Label-free cellular profiling using an expanded panel of ligands for a specific receptor has shown that *the biosensor signature arising from the activation of a receptor is ligand dependent*. Compared with conventional single endpoint-based assays, label-free cell-based assays promise to track receptor behaviors with wider pathway coverage [10]. The pathway-unbiased nature in measurement of label-free assays offers a new dimension in deciphering the complexity of drug pharmacology. **Figure 5** shows characteristic DMR signals of quiescent A431 cells in response to a panel of β_2-AR ligands, which fall into four distinct types of DMR [57,61]. The first type of DMR is biphasic – a small negative DMR (N-DMR) with short duration, followed by a greater positive DMR (P-DMR) with longer duration. Ligands that result in DMR in this category are full and strong partial agonists including isoproterenol, epinephrine, norepinephrine, formoterol and salbutamol. The second type of DMR consists of a net-zero DMR with a short duration, followed by a P-DMR. Ligands in this category are partial agonists including pindolol, labetalol, alprenolol, catechol, halostachine and xamoterol. The two β-blockers, propranolol and carvedilol, lead to a DMR distinct from other β_2-AR agonists (**Figure 5**) [61]. Both ligands exhibit a relatively low potency to trigger DMR, considering their high binding affinity to the receptor. Propranolol has been identified as an inverse agonist for $G_{\alpha s}$ pathway [62], and

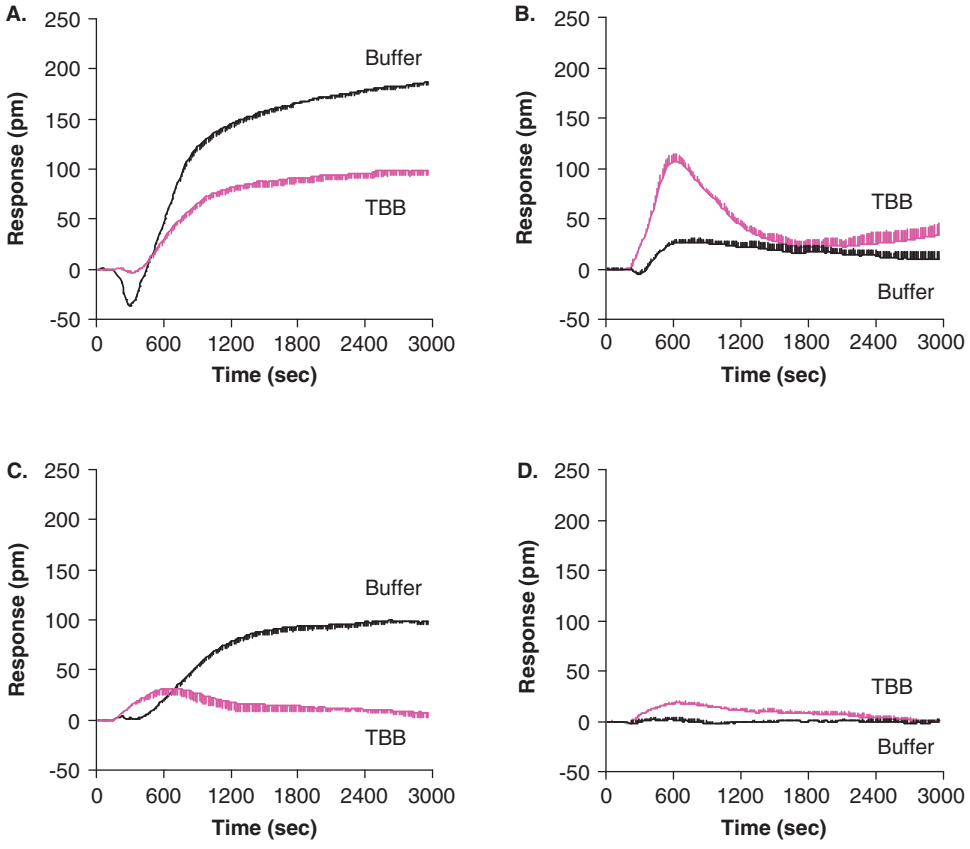

Figure 5. Characteristic dynamic mass redistribution (DMR) and their sensitivity to the CKII inhibition of a panel of the β_2-AR ligands. (A) Formoterol; (B) S-(-)-propranolol; (C) pindolol and (D) nadolol. The cells pretreated with the buffer vehicle (Buffer) and the CKII inhibitor TBB (10 μM TBB) were stimulated with the ligands, each at 10 μM.

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both β-blockers are less potent agonists for β-arrestin-dependent phosphorylation of ERK [62,63]. Lastly, antagonists such as betaxolol and nadolol lead to a net-zero DMR.

These DMR characteristics are in line with the increasing experimental examples that many ligands, acting at a single receptor including the β_2-AR, display functional selectivity – the ability of distinct ligands to differentially activate one of the vectorial pathways mediated through a receptor [64]. Such a ligand-directed functional selectivity or biased agonism is often observed in recombinant or heterologous systems, based on measurements of many downstream signaling molecules and/or events including second messenger generation, β-arrestin translocation, phosphorylation of the receptor and other signaling proteins, and receptor internalization [65]. The biased agonism is often inferred from the relative potency and efficacy to modify one signaling molecule/event over another. As a result, many ligands may give rise to assay readout-dependent potency and efficacy, suggesting that the efficacy for many ligands is collateral [66]. The biased agonism is believed to be originated from a library of active states of a receptor, each of which could have its own signaling preference. This was postulated by protein ensemble theory [67] and has been experimentally confirmed by many biophysics studies including recent X-ray structure analysis [68,69] and energy landscape modeling [70]. Biased agonism was once thought to be an artifact of recombinant technology. Label-free cellular assays have now amply demonstrated that biased agonism is a natural cellular control mechanism. The ability to discover biased agonism in native cells underscores the higher sensitivity of label-free cellular assays to endogenous states of receptor activity and accessibility to greater biorelevance, compared with conventional endpoint assays. Furthermore, the integrative nature in measurement, together with HTS capability, makes label-free cellular assays indispensable tools to tackle complex biological problems and to provide biorelevant information in a matter of minutes.

5. The biosensor signature of a receptor is cellular background dependent

Label-free profiling of many receptors in different cells has revealed that *the biosensor signature arising from a receptor–ligand interaction is cellular background dependent.* Cellular contexts play important roles in cell decision-making process including cell signaling. The functional responses of

drug molecules can be sensitive to many factors, including receptor expression level, dynamics in receptor conformations and organizations, and heterogeneity in expression and organization of cytosolic interactants [71,72]. Such a cellular context-dependent drug action is often described as phenotypic pharmacology.

The phenotypic signature can be observed when a receptor is preferentially coupled with different signaling proteins in different types of cells. This is because a receptor may be able to couple with multiple signaling proteins [73], and distinct types of cells have different expression and/or organizational patterns of these proteins [71,72]. A recent study using cellular impedance assays has shown that melanocortin-4 receptor (MC4R) favors $G_{\alpha s}$ signaling pathway in HEK-293 cells, but it prefers $G_{\alpha q}$ signaling pathway in Chinese hamster ovary (CHO) cells [74]. The MC4R is stably expressed in both cell lines, although the relative expression level is unknown.

The cell context-dependent signature for a given GPCR–ligand interaction may also be observed in different types of cells, even when the receptor is coupled with identical G protein(s). This is because distinct types of cells may have different signaling machineries. The activation of endogenous β_2-AR in A431 results in a biphasic DMR signal [57], but the stably expressed β_2-AR in CHO cells led to a single-phase N-DMR [75].

It has also become apparent that the phenotypic signature of a receptor–ligand interaction can occur in a specific cell, when the cellular background is preconditioned to distinct states. The DMR arising from the activation of endogenous epidermal growth factor (EGF) receptor in A431 was found to be dependent on cell culture conditions – the quiescent A431 responds to EGF with a much more robust DMR than the proliferative cells do [15,76]. Furthermore, the biosensor signal can be sensitive to the basal activity of signaling cascade proteins. The DMR arising from a panel of β_2-AR ligands was found to be sensitive to the pretreatment of A431 cells with a Casein kinase II (CKII) inhibitor – the inhibition of basal CKII activity suppressed the DMR induced by full and partial agonists, but potentiated DMR induced by propranolol and carvedilol [61] (**Figure 5**). Finally, the biosensor signal can be sensitive to surface chemistry. The surface chemistry is part of environmental cues that can influence the functional responses of receptors. Profiling of endogenous P2Y receptors in HEK293 cultured on distinct extracellular matrix (ECM)-coated surfaces has revealed that interactions with the ECM coatings shape the signaling of endogenous P2Y receptors [77]. The activation of endogenous $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ mediates signaling via primarily G_q pathway. ATP and ATPγS also led to G_s-mediated signaling, possibly via $P2Y_{11}$. Compared with those on tissue culture-treated surfaces, fibronectin coating increased the potency of all P2Y agonists examined, while gelatin had little impact.

Together, these observations ascertain that label-free cellular assays are phenotypic in nature. Phenotypic profiling using label-free cellular assays permits discovery of many, if not all, modes of action of ligand molecules for a receptor. Furthermore, the ability of label-free cellular assays to examine a wide range of cell types including disease-relevant cells makes it possible to fully comprehend the impact of cell context on drug pharmacology, which, in turn, is likely to provide previously unrealized opportunities for discovering new drug molecules with greater specificity. However, the common occurring of phenotypic responses obtained using label-free cellular assays leads to a caveat; that is, it would complicate medicinal chemistry optimization process.

6. The biosensor signature is a readout for the systems cell biology of receptor signaling

Realization that a receptor can display rich behaviors in different cell backgrounds and its biosensor signature reflects the signaling pathways being activated has made it apparent that *a label-free profile arising from a specific receptor–ligand interaction should be viewed as a 'phenotypic response' or 'systems cell biology readout,' rather than a 'signature'* [10,75,78]. The systems cell biology readout refers to an integrated kinetic response that reflects cell decision-making process as a result of interactions within an integrated and interacting network of genes, proteins and myriad metabolic reactions [79].

Common to all label-free biosensors for cell-based assays is that they measure an integrated response in real time. The DMR signal obtained using optical biosensors is originated from alterations in local refractive index at the sensor surface [80]. Amass evidence suggest that the information the cells received is processed and encoded into complex temporal and spatial patterns of phosphorylation and topological relocation of signaling proteins [81,82]. The precise temporal control and spatial targeting of activated signal transducers are critical to many pivotal cellular decisions, such as protein trafficking, cytoskeletal reorganization, cell death and survival [83,84]. Many spatial and temporal events during receptor signaling can lead to relocation of cellular matter, resulting in redistribution of local mass density in a given volume that is directly correlated with changes in local refractive index [85]. Integration of these events when occurring within the biosensor sensing volume (~200 nm) gives rise to a DMR signal recorded by optical biosensors [80]. Notable cellular events contributing to the DMR is protein trafficking, microfilament remodeling, cell adhesion alterations and morphological changes of cells, although exact cellular mechanisms are dependent on receptors and cell backgrounds.

The impedance signal measured using electric biosensor is originated from changes in electrical currents beneath or between the cells. The extracellular current between the cells is mostly due to the intercellular conduction, while the transcellular current beneath the cell is a result of the control of cell-membrane capacitance [9]. The extracellular current is more robust than the transcellular current. The morphological changes and ionic redistribution are two leading components of impedance signals [10,86].

Because the biosensor signature of a specific receptor–ligand interaction represents a cumulative response at whole-cell level, it has been postulated that label-free can offer a unique means to follow receptor signaling in real time with much wider pathway coverage than traditional endpoint assays [10,80]. Pathway deconvolution analysis has confirmed such a hypothesis. Pathway deconvolution is made possible by the discovery of various activators, effectors, enzymes and substrates for many receptor signaling pathways, by the availability of many probe molecules to selectively modify the activity of signaling proteins, and by the feasibility to incorporate molecular assays with label-free profiling. The DMR arising from the activation of EGF receptor in A431 has been shown to be due to the activation of multiple pathways, including receptor internalization, PI3K, MAPK and PKC pathways [15,76]. The temporal information of distinct pathways has also been identified – the PKC pathway is encoded in early DMR response, while the receptor internalization, MAPK pathway and decrease in cell adhesion collectively contribute to the late N-DMR event.

The ability for mechanistic deconvolution has made it possible to use label-free for discovering novel signaling pathways for several receptors, and novel MMOAs of drug molecules. Notable examples are the discovery of dual ($G_{\alpha s}$- and $G_{\alpha q}$) signaling pathways for endogenous bradykinin B_2 receptor in A431 cells [16], of a novel intramolecular mechanism to silence its own signaling for CRTH2 receptor [87], of the functional selectivity of thrombin receptor agonists [30], of the differential coupling of the MC4 receptor with different G proteins in distinct cell background [74] and of new GPR35 agonists [55], of the connection between GPCR signaling network and nucleotide metabolism [88], and of the unique pharmacology of 'dualistic' agonists for muscarinic M2 receptor [89]. The dualistic agonists represent a novel class of agonists that consist of both orthogonal and allosteric binding moieties.

7. Strategies to relate biosensor signatures to drug pharmacology

Label-free is often considered to be a black box, due to lack of effective means to relate the biosensor signature of a drug molecule to its pharmacology, as well as of deep understanding of the molecular mechanisms that underline biosensor signals [10]. Since the birth of label-free cellular assays, a common means for data analysis is that the real-time response obtained is converted to endpoint measurements [15,46]. The signal amplitude of a specific biosensor event is the most commonly used parameter to infer drug actions. From a practical perspective, such a strategy makes sense since it reduces data complexity and fits well with HTS procedures. However, an obvious disadvantage is that this strategy downplays the promise of label-free as a multidimensional readout for studying the systems cell biology of receptor signaling as well as the systems cell pharmacology of drug molecules [10,78].

It has been realized that multidimensional analysis of label-free profiles is useful to relate a kinetic parameter to a specific signaling event. For the EGF-induced DMR in A431, three parameters were extracted and used as the basis to determine the readout-dependent potency of EGF [15]. These parameters are the amplitude and decaying kinetics of its N-DMR event and the transition time from its early P-DMR to late N-DMR events. Results showed that EGF gave rise to three distinct potencies based on the kinetic parameters measured, suggesting that distinct molecular mechanisms govern different DMR events.

Similarity analysis is a powerful means to determine the relationships (that is, similarity or distances) among different biological responses, particularly for large sets of biological data such as gene expression data [90]. Similarity analysis of the DMR arising from a panel of β_2-AR ligands using multiple kinetic parameters leads to different clusters, and ligands in each subcluster appear to share similar MMOAs [10,57]. Notably, ligands that cause receptor internalization led to a slower P-DMR than those do not. These studies showed that multiparameter analysis is useful to relate the biosensor signals to specific MMOAs of drugs. However, a caveat of such an approach is that it could be difficult to accurately determine the kinetics of a specific event due to lack of reliable mathematic models to describe the event. Furthermore, understanding the molecular mechanisms that govern a specific parameter is necessary, but may be hard to obtain, for such analysis.

An alternative, but possibly more practical and powerful, means to relate biosensor signatures to receptor biology and drug pharmacology is similarity analysis of the entire kinetic responses themselves. A biosensor signature can be simply considered a poly-dimensional coordinate at discrete time points, that is, a temporal series of biosensor output signals [61,76]. Thus, it can be directly used as a basis for similarity analysis. Similarity analysis of the DMR arising from a panel of GPCR agonists in A431 has shown that it is possible to relate the DMR signals of distinct receptor–ligand pairs to specific pathways [76]. Two subclusters were evident at relatively low resolution for 17 agonists that resulted in detectable DMR in A431. These agonists are specific to different endogenous GPCRs. The two subclusters appear to be separated based on G protein to which their respective receptor is coupled. The first subcluster includes all agonists that activate $G_{\alpha s}$ pathway, while the second subcluster includes the remaining agonists that primarily activates $G_{\alpha q}$ pathway.

Similarity analysis of the whole time course impedance signals has been used as a strategy to screen molecules that influence cell proliferation [91]. In this study, a library of 119,595 compounds was screened at a final concentration of 17.8 μM using H1993 cells. The impedance signal of each compound was recorded in real time for overnight. Similarity analysis of entire kinetics responses identified 1149 hit compounds that share a biosensor signal similar to those induced by well-known antimitotic agents including paclitaxel. Counter

screen using A549 cells led to confirmation of 239 hits, 125 of which were co-clustered with paclitaxel. Sixteen hits were further examined and confirmed to be relatively potent antimitotic agents, although exact MMOAs were unknown.

Similarity analysis of the biosensor signatures of compounds in a specific cell offers an attractive means to identify interesting hit compounds that share similar biosensor signature. However, this approach is, to a large extent, equivalent to traditional phenotypic screen such as cell death assays or high content imaging. Like phenotypic screen results, the hit compounds within a subcluster that lead to similar biosensor signals may not be associated with a specific signaling pathway and/or MMOA, particularly when a large compound library is screened. This is at least partly because of common occurrence of compensatory pathways in cell decision process (e.g., cell cycle arrest, apoptosis, survival). Nonetheless, label-free cellular assays permit multiple means to relate label-free profiles to drug pharmacology. A reductionist's approach may be sufficient for HTS and hit identification, while multiparameter analysis may be optimal for medicinal chemistry optimization and functional characterization of lead molecules, and entire kinetic response-based analysis fully comprehends the rich texture of label-free and may be the method of choice for discovering novel MMOAs of drug molecules.

8. Label-free integrative pharmacology on-target: a possible means to link *in vitro* results with *in vivo* indications

Prioritization of drug candidate molecules for *in vivo* testing is a critical step in drug discovery and development. The current process is as much as art as a process [4]. To improve the efficiency of lead selection process, biological fingerprints based on binding profiles [92], gene expression profiles [93,94], cellular phenotypic effects [95], side effects [96] and chemical structures [97,98] can be produced and used for comparative pharmacology analysis. However, these approaches generally have poor resolution for assessing the on-target pharmacology of a drug, the functional consequences of the drug binding to a specific target.

Amassing evidence has shown that a drug molecule can give rise to an assay readout-dependent efficacy and potency (that is, pluridimensional efficacy) [99,100]. Conventional molecular assays have revealed in great details the *in vitro* pharmacological characteristics of many β-AR drugs [100-102]. However, these *in vitro* profiles alone cannot explain the diverse *in vivo* indications of β-AR drugs, which include the treatment and management of cardiovascular conditions, migraine, ophthalmic disorders, asthma, cardiac decompensation, anaphylaxis, sepsis and premature labor [61]. It is apparent that neither all β-blockers behave equally for treating various heart diseases nor are all β_2-agonists effective in the management of asthma.

As the first attempt, we have developed a label-free integrative pharmacology on-target (iPOT) approach recently [61]. This comparative pharmacology approach is centered on similarity analysis of DMR profiles of β-AR drugs obtained in a model cell line that has been pre-exposed with a wide variety of probe chemicals. The iPOT uses various probe molecules to intervene specific pathways downstream receptor signaling, so that the pathway biased activity, if any, of drugs can be systematically surveyed. After translating DMR profiles into multidimensional coordinates, similarity is analyzed to categorize drugs into distinct clusters. Using β-drugs approved by the US Food and Drug Administration as a model, we profiled the DMR of all drugs using A431 cells under 12 assay conditions (**Figure 6**). The iPOT profiling gave rise to a clustering pattern that seems to be correlated well with their *in vivo* indications. Carvedilol was found to be co-clustered with three isomers of propranolol. A small but positive clinical trial using carvedilol for the prophylactic treatment of migraine has been reported [103]. Furthermore, terbutaline was co-clustered with ritodrine and phenylephrine. Both ritodrine and terbutaline are widely prescribed β-mimetics to arrest or prevent premature labor [104]. The iPOT effectively integrates the system-based, ligand-directed and kinetics-dependent biased activities of the drugs, thus providing an interesting possibility to select lead drug molecules for *in vivo* testing.

9. Conclusions

With new developments in HTS-compatible biosensor instruments has come the adoption of chemical biology approaches to delineate the molecular and cellular mechanisms of receptor signaling at system biology level [76]. Label-free biosensors have been evolving from a tool for biochemical interaction analysis to indispensable cellular phenotypic assays. The landscape for the applications of label-free has been dramatically expanded. A wide array of cellular processes ranging from cell adhesion to cell barrier functions, cell migration, cell differentiation, receptor signaling, viral infection, cell proliferation and death can be studied. Label-free has been shown to offer unprecedented advantages to screen new molecules for numerous receptors and to discover missing links in receptor signaling. Label-free cellular assays can be adopted in many steps in drug discovery and development process, including target identification, primary screening, secondary counter screening, medicinal chemistry optimization and drug selection for *in vivo* testing.

10. Expert opinion

There is an increasing gap between investment in drug research and development and new molecular entities being approved, suggesting that therapeutic innovation has become more challenging [105,106]. The decline in pharmaceutical productivity continues to drive innovations in assay technologies, particularly in areas in which the risk of failure is high due to

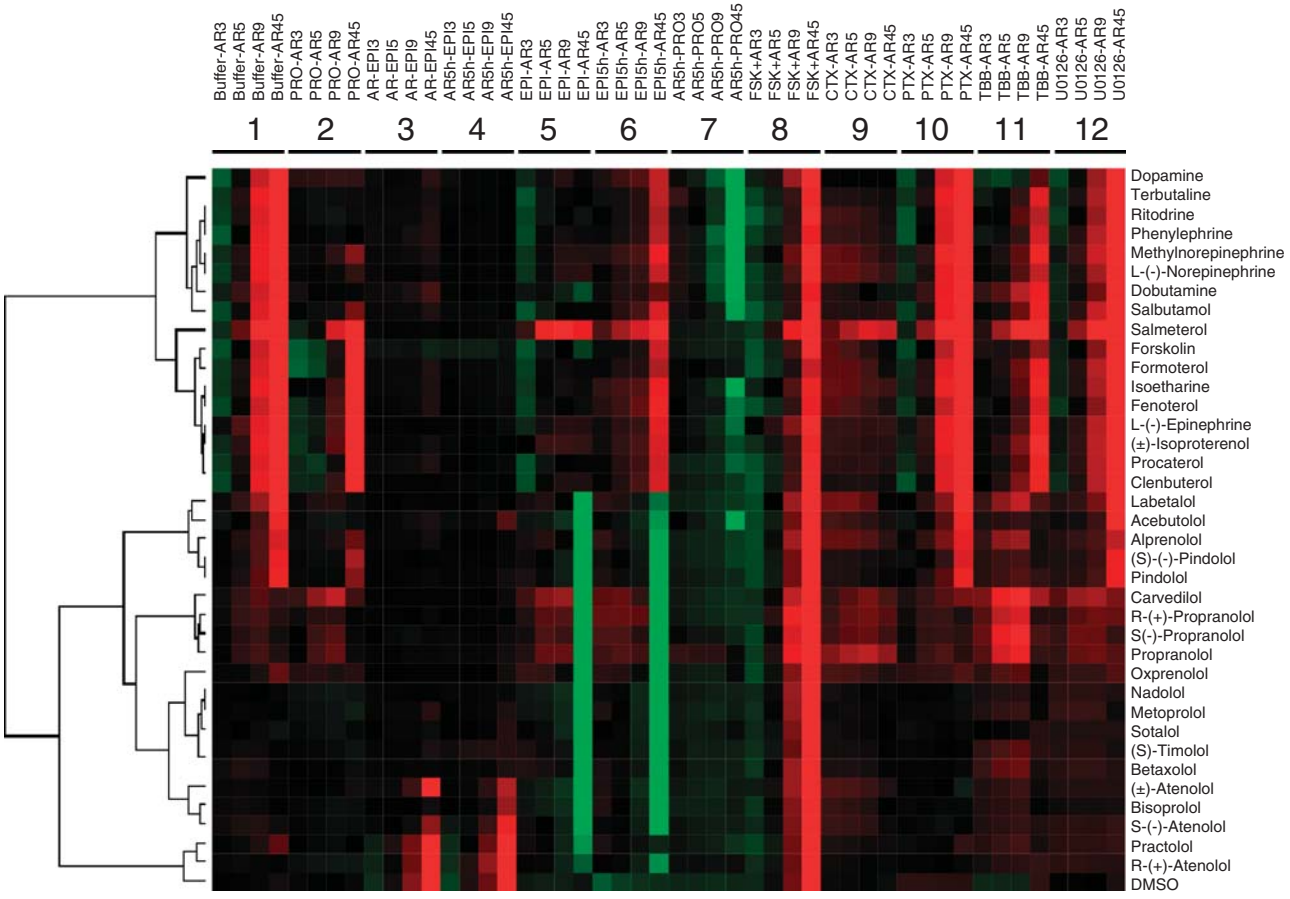

Figure 6. Dynamic mass redistribution (DMR) heat map of clinically available β-drugs. This heat map was obtained using DMR profiling of the drugs under 12 conditions, followed by similarity analysis using the Ward hierarchical clustering algorithm and Euclidean distance metrics. The 12 DMR used for analysis were a drug-induced DMR in the cells pretreated with the assay vehicle for 1 h (Buffer – AR), 1 μM propranolol for 1 h (PRO – AR), 5 nM EPI for 1 h (EPI – AR), 5 nM EPI for 5 h (EPI5h – AR), 400 ng/ml CTx for overnight (CTx – AR), 100 ng/ml PTx for overnight (PTx – AR), 10 μM TBB for 1 h (TBB – AR),and 10 μM U0126 for 1 h (U-126 – AR). In addition, we also included an EPI-induced DMR in the cells pretreated with 10 μM β-drugs for 1 h (AR – EPI) and for 5 h (AR5h – EPI); an PRO-induced DMR in the cells pretreated with 10 μM β-drugs for 5 h (AR5h – PRO); and a forskolin (FSK)-induced DMR in the presence of β-drugs (FSK + AR). For each DMR, the responses at the four distinct time points (3, 5, 9, and 45 min poststimulation) were used as the basis for similarity analysis. These responses were color coded: green, negative value; red, positive value; black, zero value.

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unexploited biological mechanisms. Molecular assays screen drug molecules based on a predetermined hypothesis, but such a hypothesis may not be relevant to the pathogenesis of a disease. Phenotypic assays are often limited in throughput and are not practicable for drug optimization without prior knowledge of MMOAs. With the ability to probe wide pathway coverage in a single assay and to mechanistically delineate drug pharmacology has turned label-free cellular assays into a promising platform for drug discovery.

However, the wide adoption of label-free in both basic research and drug discovery relies on three important developments. First, deconvolution of the cellular and molecular mechanisms for the biosensor signatures of receptor–drug interactions is necessary to advance label-free assays. Genetic manipulation (e.g., gene knock-in, knock-down and depletion) of signaling proteins, in conjunction with chemical biology, needs to be applied to label-free assays, in order to identify and ascertain the linkage between a biosensor parameter and a specific cellular and molecular mechanism. Furthermore, the linkage between a DMR parameter and a specific clinical feature of drug molecules needs to be established.

Second, new methodologies for data analysis are required to further advance label-free. Current reductionist's approach makes label-free an endpoint assay, thus limiting the power of label-free. Similarity analysis based on the entire kinetic responses offers an attractive means to determine specific modes of action of drug molecules. However, algorithms for similarity analysis need to be further optimized, given that the

label-free data generally have great numerical dimensions, as well as our understanding of specific biological mechanisms determining the similarity and differences of drug-induced responses are still largely unknown [61,107].

Third, new biosensor technologies need to be developed. Current label-free cellular assays employ a static and sustained stimulation scheme to determine drug pharmacology and receptor biology [107]. This makes it difficult to accurately measure certain cellular processes, wherein the duration and termination of stimulation are crucial. Label-free under microfluidics offer precise control of drug exposure time, so that it is possible to differentiate the binding reaction-related functional consequences of drug molecules [107] and to investigate new aspects of receptor biology including ligand occupancy-dependent signaling and receptor desensitization and resensitization process at the whole-cell level [107-109]. Label-free imaging of single cells is also possible due to new developments in instrumentation and biosensor design [56,110,111]. Further, multi-depth sensing also holds great promise to expand the reach of label-free [112,113]. Current biosensors are generally sensitive to biological events that take place at the sensor surface. Multi-depth sensing of cell layer is important for probing biological processes that are deep inside the cells (e.g., nuclear receptor signaling).

Declaration of interest:

Y Fang is an employee and stockholder of Corning Inc. Corning Inc. has no role in the preparation of the manuscript and in study design/data analysis/statistical inputs/review of drafts/writing of the article/identification of papers for inclusion/any other form of input.

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