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Review Article

Label-Free Biosensors for Cell Biology

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Label-free biosensors for studying cell biology have finally come of age. Recent developments have advanced the biosensors from low throughput and high maintenance research tools to high throughput and low maintenance screening platforms. In parallel, the biosensors have evolved from an analytical tool solely for molecular interaction analysis to powerful platforms for studying cell biology at the whole cell level. This paper presents historical development, detection principles, and applications in cell biology of label-free biosensors. Future perspectives are also discussed.

1. Introduction

The cell is the functional basic unit of life. The ability of examining living cells is crucial to cell biology. In the past several decades, advances in molecular biology have made it a routine laboratory practice to manipulate a cellular target in living cells. Gene expression can be used to increase the amount of a specific protein in cells, while interference RNA can be used to suppress or eliminate a specific protein, and mutagenesis to alter the structure and functions of a particular protein, so that the functional consequences of the target protein can be studied [1, 2]. In parallel, analytical techniques have also been advanced to meet the increasing demands in characterizing molecules in living cells with high temporal and spatial resolutions, as well as with high throughput [3, 4]. Although these molecular assays only measure independent molecules one at a time, they have made it possible to identify various activators, effectors, enzymes, and substrates for many important cellular processes including signaling [5]. Thus, these assays have been dominating cell biology studies nowadays. However, since signaling proteins mostly operate through a large and complex network to direct the propagation of signals within a cell and ultimately to determine how the cell responds to environmental cues [6, 7], there are increasing demands in technologies that not only allow one to investigate cellular responses at the whole cell and cell systems level, but also enable mechanistic delineation. Label-free biosensors fulfill these needs by measuring integrated and phenotypic responses of whole cells with high temporal resolutions [8, 9]. Further, these biosensors enable noninvasive and highly sensitive measurements of many different cellular responses, ranging from cell adhesion to cell barrier functions, signaling, infection, migration, proliferation and death, and differentiation (Figure 1), part of which are topics of this paper.

2. Label-Free Biosensors

Label-free biosensors generally use a transducer to convert a stimulus-induced cellular response into a quantifiable signal (i.e., biosensor signal) [9]. Depending on the nature of transducers, label-free biosensors used for whole cell sensing are mostly divided into optical- and electric-based (Figure 2). It is worth noting that there are many other types of biosensors currently under development. These include atomic force microscopy for measuring biomechanics of cells [10, 11], Raman imaging for measuring the production and organization of unsaturated fatty molecules in cells [12, 13], and whispering-gallery-mode biosensors [14] and resonant mirrors [15] for biosensing. Since these biosensors have limited throughput for whole cell sensing at the present time, they are excluded in this paper.

Optical biosensors include surface plasmon resonance (SPR) and resonant waveguide grating (RWG), both of which use a surface bound evanescent wave to characterize alterations in local refractive index at the sensor surface. SPR

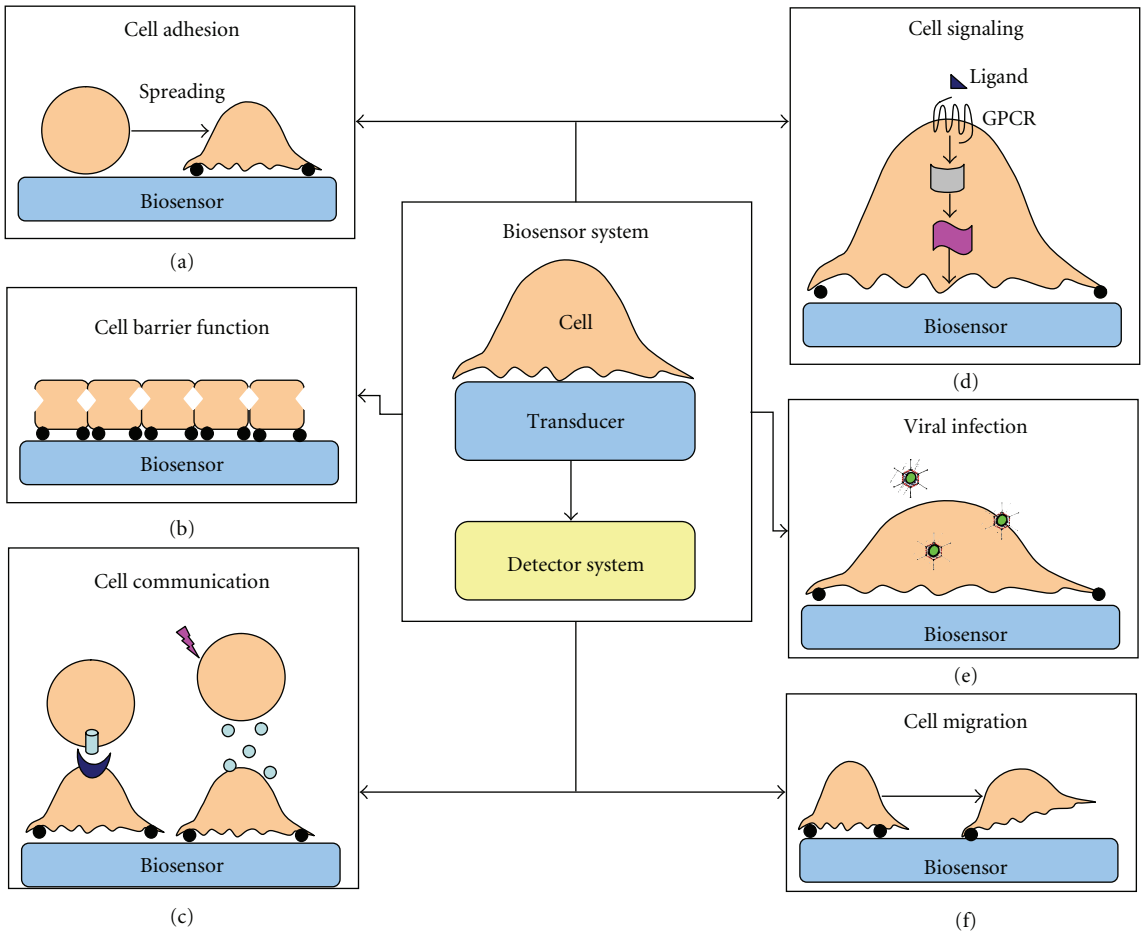

Figure 1: Label-free biosensor and its uses for cell biology. (a) Cell adhesion to a surface; (b) cell barrier functions and regulation; (c) cell-to-cell communication via direct interactions or chemical communication; (d) cell signaling via the receptor activation by an agonist; (e) viral infection; and (f) cell migration.

employs light excited surface plasmon polaritons (SPPs) to detect the adsorption of biomolecules onto a metallic surface (typically gold or silver) [16] (Figure 2(a)). The SPP is a surface-bound electromagnetic wave arising from the interaction of light with mobile surface chargers in a metal [17, 18]. The waves propagate along the interface between materials with negative and positive permittivities (e.g., the metal/dielectric interface), leading to an electromagnetic field that is primarily present in and decays evanescently into the dielectric medium due to increased damping in the metal [19]. Biacore (now GE Healthcare) first introduced a SPR instrument for biomolecular interaction analysis to the market in 1990 [20]. Because of its ability to measure the binding affinity and kinetics of an interaction, SPR is often referred to affinity-based biosensors. Recently SPR imaging has become a reality [21], and localized SPR also has started gaining attractions [22]. However, SPR is still limited to low throughput in processing different samples today. Commercial products include SPR series from GE Healthcare and SPR imager from GWC instruments and others (Figure 2(a)).

RWG uses a leaky mode nanograting waveguide structure to couple light into the waveguide thin film via diffraction, so an evanescent wave is generated (Figure 2(b)). RWG is also named grating coupler, or photonic crystal biosensor. Resonant anomalies in periodic structures were first reported in 1902 [23, 24]. Only until 1980s, a surface bound and waveguide guided mode resonance was achieved using grating couplers and used for chemical sensing by Teifenthaler and Lukosz [25, 26]. Similar to SPR, RWG also employs an evanescent wave for detection, and thus, was initially developed for biomolecular interaction analysis [27, 28]. In recent years, large-scale fabrication, together with new biosensor and instrument designs as well as advanced assay protocols, has made RWG system the first commercial platform for high throughput biochemical and cell-based assays [9, 29–38]. Commercial products include Epic system from Corning Inc., EnSpire multimodal reader containing Epic technology from PerkinElmer, and BIND system from SRU BioSystems (Figure 2(b)).

Electric biosensors use a low electrolyte impedance interface to detect the impedance of a cell layer under electric fields generated with sinusoidal voltages [38, 39]. Under the electric fields the cellular plasma membrane acts as an insulating barrier directing the current to flow between or beneath the cells, leading to extracellular and transcellular

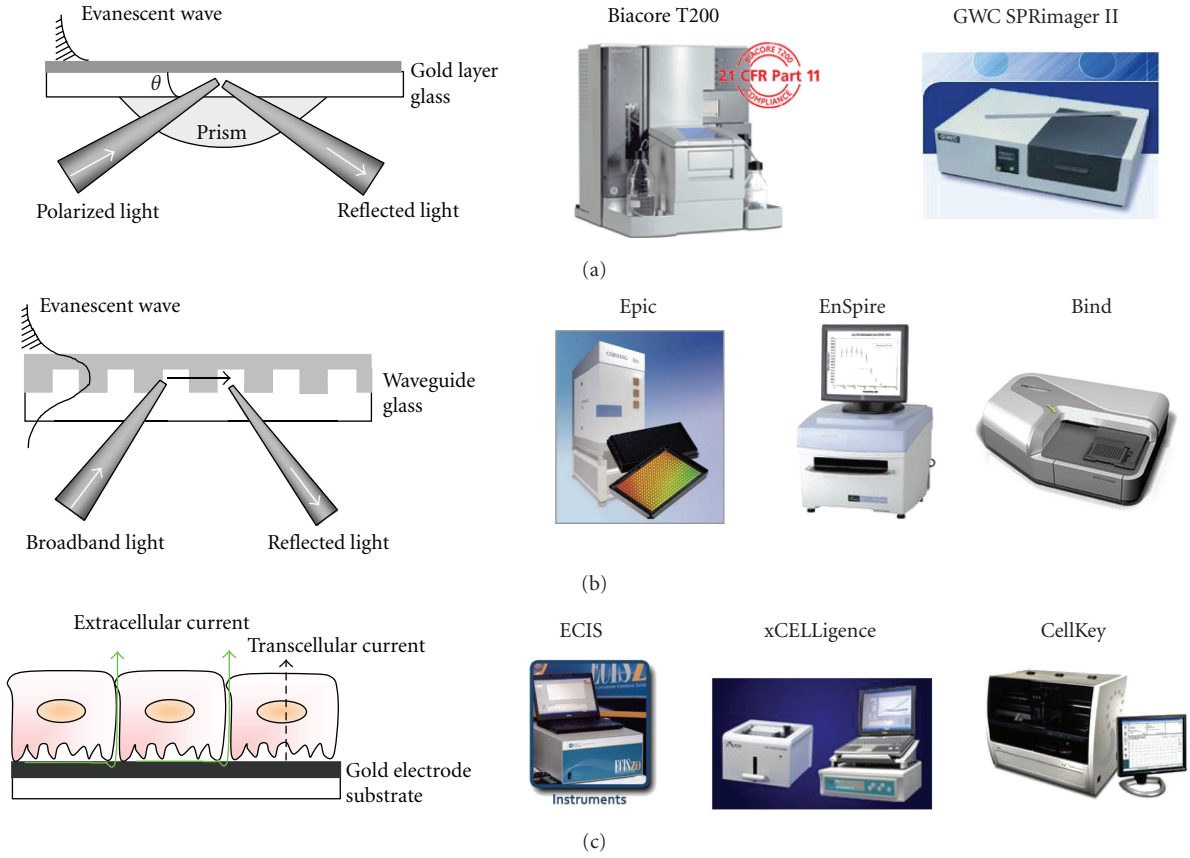

Figure 2: Principles and commercial instruments of three distinct types of label-free biosensors. (a) Surface plasmon resonance, which uses light excited surface plasmon polaritons to sense whole cells. Biacore SPR T200 and GWC SPRImagerII are two examples of commercial products. (b) Resonant waveguide grating, which uses leaky mode nanograting waveguide structure to generate an evanescent wave to sensor whole cell responses. Epic, EnSpire, and BIND are three commercially available products. (c) Electric biosensor, which uses a low electrolyte impedance interface to sense whole cell responses. ECIS, xCELLigence, and CellKey are three commercial products.

currents, respectively (Figure 2(c)). The extracellular current is mostly due to the intercellular conduction, while the transcellular current is a result of the control of cell-membrane capacitance. The extracellular current can be separated from the transcellular current using sophisticated algorithms and is more robust than the transcellular current. ECIS (Electric cell-substrate impedance sensing) instruments from Applied BioPhysics were the first commercial impedance systems for cell-based assays [40, 41]. Newer systems use sophisticated algorithms to record and process impedance signals, leading to improved signal to noise ratios [42]. Commercial products include ECIS systems from Applied BioPhysics, xCELLigence (Real Time Cell Electric Sensing; RT-CES) from Roche/Acea Biosciences and Cellkey (Cellular Dielectric Spectroscopy; CDS) from Molecular Devices (Figure 2(c)).

The first generation biosensor systems can only measure a few samples at a time. SPR was limited up to 4 individual channels for parallel measurements and also required microfluidics for sample delivery. The first ECIS system measured the impedance of living cells cultured on small electrodes up to 16-well plate [41]. The current generation systems are targeting moderate to high throughput screening (HTS), which requires highly reproducible data collection and straightforward data analysis. The user experience is the top priority of these products; thus, innovative instrument designs, assay protocols, and data analysis software have made these systems low maintenance screening platforms [9, 32].

The current biosensor systems differ greatly in measurements. For optical biosensors, the cellular responses are often referred to dynamic mass redistribution (DMR) [8, 9]. This is because the local refractive index is mostly proportional to the mass density at the sensor surface; thus, a change in local refractive index (i.e., the detected signal) reflects the redistribution of cellular matter within the sensing volume of the biosensor. Due to the relatively short penetration depth (~200 nm) of the evanescent waves, both SPR and RWG measure the DMR originated from the bottom portion of cells. However, for electric biosensors, the cellular responses are often referred to impedance signal, which is sensitive to ionic movement and cell morphological changes [9, 41].

The current biosensor systems differ greatly in instrument configurations. All biosensor systems are standalone readers, except for EnSpire which is a benchtop multimodal microtiter plate reader containing Epic label-free technology in addition to label technologies. All biosensor systems are benchtop instruments targeting low to moderate screening

markets, except for Epic system which is specifically designed for HTS laboratories. Although there are somewhat differences in spatial and temporal resolutions, all biosensor systems provide an averaged response of a population of cells. It is worth noting that due to relatively low volume in manufacturing as well as being in early phase of development and adoption of these technologies, all label-free biosensors are considered to be moderate or high in cost.

3. Cell Adhesion

Cell adhesion refers to the binding of a cell to a surface, extracellular matrix (ECM) or another cell. Almost all of the early works related to label-free whole cell sensing are centered on cell adhesion (Figure 1(a)). This is no surprise partly because cell adhesion often leads to great alterations of local environment at the sensor surface, and partly because cell adhesion is important to the survival and functions of tissue cells.

In a landmark paper of the ECIS, Giaever and Keese [40] investigated the behavior of two fibroblast cell lines on gold electrodes under an alternating electric field at 4000 Hz. Results showed that the adhesion and spreading of these cells had a marked effect on the impedance of the biosensor system. Further, the impedance after cell adhesion fluctuated with time and was sensitive to the presence of an actin inhibitor, cytochalasin B. Later, they found that electric biosensor can detect cell micromotions down to the nanometer level [43]. Thus, they concluded that electronic biosensor is a morphological biosensor for living cells [41].

The ECM onto which cells are harbored is part of environmental cues for regulating the dynamic behaviors of cells. Focal adhesion complex and podosome are commonly formed during the adhesion of cells to a surface and ECM. The focal adhesion complex is a specific attachment site where the cell attaches to the underlying ECM or to cell-surface molecules on neighboring cells via the interaction with integrin receptors in the plasma membrane. The podosome is a cell-matrix adhesion complex that functions in the cell adhesion events associated with cell motility and cell spreading. Label-free biosensors have been used to investigate the adhesion and spreading of distinct types of cells on various surfaces including distinct ECM proteins [44–48] and self-assembled monolayers (SAMs) presenting ligands for integrins [49, 50].

Cell adhesion mechanisms are dependent on the types of cells and ECM. Wegener et al. [45] applied the ECIS to study the adhesion and spreading of Madin Darby Canine kidney (MDCK) epithelial cells and found that distinct mechanisms regulate the cell adhesion on different ECM coatings-cell adhesion on laminin was primarily mediated by the binding of a glycolipid, Forssman antigen, while cell adhesion on fibronectin was mostly due to the interaction with integrin receptors. Luong et al. [48] found that the adhesion of a human rhabdomyosarcoma cell line RDX2C2 to collagen- or laminin-coated gold electrodes increased in the cells transfected with $\alpha 2\beta 1$ integrin. However, on fibronectin the cell adhesion appears to be optimal; the expression of $\alpha 2\beta 1$ integrin had little impact on the cell adhesion degree, but the deletion of its $\alpha 2$ cytoplasmic domain resulted in marked decrease in cell adhesion. This $\alpha 2\beta 1$ mutant was believed to lead to dysregulated recruitment to focal adhesion complexes that mediate the binding of the cells to fibronectin.

Since ECM proteins are macromolecules with multiple binding sites for cell surface integrins, it is highly possible that multiple mechanisms are involved in cell adhesion process. Thus, SAMs presenting a specific integrin binding motif would be advantageous to study cell adhesion. Roberts et al. [49] applied SPR to study the adhesion of bovine capillary endothelial cells on SAMs of alkanethiolates on gold. The SAMs obtained contain a mixture of arginine-glycine-aspartate (RGD) and oligo(ethylene glycol) moieties. RGD is a tripeptide that promotes cell adhesion by binding to cell surface integrin receptors, and oligo(ethylene glycol) moieties resist nonbiospecific adsorption of cells. The attachment and subsequent soluble GRGDSP-induced detachment of cells suggest that RGD alone is sufficient for adhesion and survival of the cells over 24 h.

Cell adhesion to substrates is an active and dynamic process. Characteristics of cell adhesion can be studied in details using label-free biosensors because they allow noninvasive and real-time quantitation of entire cell adhesion process [51–53]. In the first paper describing the use of RWG biosensor for studying cell adhesion, Ramsden et al. found that cell adhesion follows a biphasic process: an initial passive sedimentation followed by active spreading [51]. Using infrared SPR (IR-SPR) which provides an extended penetration depth, Yashunsky et al. found that MDCK epithelial cells underwent a multiphase cell adhesion and proliferation process, starting from initial contact with the substrate to cell spreading, to formation of intercellular contacts, to cell clustering, and finally to the formation of a continuous cell monolayer [52].

An important feature of cell adhesion is the cell-substrate separation distance. Lo et al. applied the ECIS to measure changes in averaged cell-substrate separation in response to an upward magnetic force [53]. The magnetic force was controlled by the position and the number of permanent magnets, applying an average 320 or 560 pN per cell after collagen-coated ferric oxide beads attached to integrin receptors in the dorsal surfaces of osteoblast-like ROS 17/2.8 cells. The average distance between the basal cell surface and substrate was found to be sensitive to temperature; the distance was estimated to be about 84, 45, and 38 nm at temperatures of 4°, 22°, and 37°C, respectively. The cell-substrate distance was also sensitive to external magnetic force; an increased force led to an increased separation distance; and at 22°C the force-induced changes were 11 and 21 nm for 320 and 560 pN, respectively. The authors further estimated that the spring constant of individual adhesion bonds is from about 10^{-3} to 10^{-1} pN nm^{-1}.

The ability of cells to recognize, interact, and respond to environmental signals, including ECM components, is central to many biological processes including inflammation and organogenesis. Thus, it is no surprise to see that various effectors influencing cell adhesion and spreading process have been extensively investigated using label-free biosensors. These effectors include biosensor surface chemistry

[44–51], temperature [53], biosensor surface roughness [54], cell numbers [55], cell types [56], and expression of specific proteins such as integrins [48], cyclooxygenase and lipoxygenase [57], and small molecules that modulate cellular targets important to cell adhesion [8, 58]. Using high throughput RWG we examined the ability of small molecules to modulate cell adhesion process. Using human skin cancerous cell line A431 as a model, RWG measurements showed that vincristine, a plant alkaloid that inhibits microtubule assembly by binding to tubulin proteins, significantly reduced the cell adhesion degree and the kinetics of cell spreading. This study opens possibility for HT screening of cell adhesion-modulating small molecules.

The adhesion of cells to the ECM is a complex and dynamic process involving biological signaling processes. The cell surface integrins often bind to ligands in the ECM substratum and transduce signals through their intracellular domains, thus regulating diverse functions of cells. Label-free biosensors may offer insights about the cell signaling during the cell adhesion process. Using a reverse waveguide configuration that allows multidepth sensing, Horvath et al. showed that the adhesion of fibroblast cells results in inhomogeneity in refractive index within the distinct layers of the cells perpendicular to the biosensor surface [58], possibly due to the consequence of cell signaling during the adhesion process.

Interactions with the ECM shape the signaling and functions of many types of cells and receptors. Further, distinct ECM coatings have been used in a wide array of substrates for characterizing receptor biology, and for assaying and screening drug molecules. Thus, elucidating the impacts of surface chemistry on receptor biology and ligand pharmacology is important to improve the quality of screening assays and hits identified. Recently, we applied RWG to systematically study the influence of distinct ECM coatings on the signaling of endogenous purinergic P2Y receptors in human embryonic kidney HEK293 cells [59]. Purinergic 2Y (P2Y) receptors are a family of G protein-coupled receptors (GPCRs) whose natural agonists are nucleotides including ATP, ADP, UTP, UDP, and UDP-glucose. The label-free receptor assays showed that the potency and efficacy of P2Y agonists were sensitive to ECM coatings. Compared to those on the tissue culture treated surfaces, fibronectin coating increased the potency of all agonists, while gelatin had little impact. Further, fibronectin, collagen IV and gelatin all generally increased the biosensor signal amplitudes of all P2Y agonists.

4. Cell Barrier Functions

Label-free biosensors have found applications in characterizing cell barrier functions including blood-brain barrier (BBB) and epithelial cell barriers (Figure 1(b)). The BBB is the regulated interface between peripheral circulation and central nervous system (CNS) [60]. Endothelial cells line cerebral microvessels and form the BBB. The BBB controls the exchange of molecules between blood and CNS, thus maintaining the homeostasis of the brain microenvironment that is crucial to neuronal signaling. The BBB works together with astrocytes, pericytes, neurons, and the ECM to form a neurovascular unit that is essential for the health and function of the CNS. Further, the BBB often limits *in vivo* efficacy of many drug candidate molecules that are designed to target diseases associated with the CNS such as malignant primary or metastatic brain tumors [61].

A hallmark of the BBB is its intrinsic and high electrical resistance because the BBB consists of capillary endothelial cells that are connected together with continuous tight junctions [62]. The permeability of the BBB is tightly regulated via a vital and complex process involving intracellular signaling and rearrangement of tight junction proteins. Upon stimulation with exogenous signals and substances, the BBB undergoes remodeling, leading to a change in transendothelial permeability. Thus, measuring the permeability of the BBB can offer insights about its integrity and regulation mechanisms. To date, transepithelial electrical resistance (TEER or TER) is the most popular technique to measure the functions of the BBB *in vitro* [63]. Electric biosensors are also suited to measure the functions of *in vitro* endothelia cell model systems, due to their sensitivity to ionic movement and ability to separate extracellular resistance from transcellular resistance [9, 39].

Thrombin is a potent stimulus for endothelium-dependent vasodilatation and is a natural agonist to thrombin receptor (protease-activated receptor-1; PAR1). Thrombin cleaves the amino terminus of the PAR1 to unmask a tethered ligand, which, in turn, binds intramolecularly to and activates the receptor. Thrombin was found to cause the formation of the intercellular gap, leading to decrease in impendence via a protein kinase C inhibitor-sensitive manner when both bovine pulmonary microvessel endothelial cells and bovine pulmonary artery endothelial cells were tested with the ECIS [64].

PAR1 is known to mediate signaling via multiple pathways. Thus, it is possible that multiple pathways govern the thrombin-induced permeability of endothelia cells [65–67]. McLaughlin et al. compared the functional consequences of the PAR1 activation induced by thrombin and PAR activating peptides [65]. Results showed that the potency (EC_{50}: 0.1 nM) for thrombin to cause the increased endothelial monolayer permeability obtained using the ECIS was higher than that to cause mobilization of intracellular calcium (EC_{50}: 1.7 nM). However, the opposite order of activation was observed for the agonist peptides (SFLLRN-$CONH_2$ or TFLLRNKPDK). Further, only PAR1 activation affected barrier function, which is mostly via $G_{\alpha 12/13}$-mediated signaling, instead of $G_{\alpha q}$-mediated signaling. However, for human umbilical vein endothelial cells (HUVECs), Wang et al. [66] found that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a mediator of thrombin-stimulated increases in permeability of the cell monolayer. CaMKIIδ6 isoform is the predominant CaMKII isoform expressed in the HUVEC. Thrombin potently and maximally increased CaMKIIδ6 activation, which, in turn, activates RhoA. siRNA targeting endogenous CaMKIIδ suppressed expression of the kinase by >80% and significantly inhibited 2.5 nM thrombin-induced increases in monolayer permeability assessed by the ECIS. Further, Rho kinase inhibition strongly suppressed

thrombin-induced HUVEC hyperpermeability, but inhibiting ERK1/2 activation had no effect. Interestingly, the relative contribution of the CaMKIIδ6/RhoA pathway(s) diminished with increasing thrombin doses, indicating recruitment of alternative signaling pathways that regulate the endothelial barrier dysfunction.

The measurement of cell barrier functions with the ECIS is complicated by the presence of multiple types of resistance including cell-cell, cell-matrix, and transcellular resistances [68–70]. Generally, cell-to-cell gaps mainly affect the total resistance value, while cell-to-substrate gaps mainly affect total capacitance value. Effectors that modulate the components of resistance of endothelial cells include cell types and confluency [68, 71], endogenous and exogenous extracellular matrices [71], the presence of exogenous molecules [68, 72, 73], and the substrate [74, 75]. For confluent cultured HUVEC cells, an ECIS measurement suggests that histamine led to a rapid decrease in transendothelial resistance mostly via decreases in cell-cell resistance, and the restoration of resistance was initiated by first increase in cell-matrix resistance, followed by increase in cell-cell resistance [66]. However, histamine led to increased resistance in subconfluent HUVECs in which there was limited or no cell-cell contact. Together, these results suggest that it is possible to deconvolute the molecular mechanisms that regulate the cell barrier functions.

For investigating cell barrier functions, distinct biosensors can offer complementary insights how cell barrier functions are regulated. Because of the short penetration depth or sensing volume, optical biosensors can directly resolve cell-matrix interactions, but cannot directly resolve cell-cell interactions. In contrast, electrical biosensors provide an aggregated measurement that integrates cell-cell and cell-matrix interactions, which can be separated using mathematical modeling [69, 70].

5. Cell-to-Cell Communication

Label-free biosensors are flexible in assay conditions and formats [9]. Together with real-time kinetics, label-free biosensors offer an alternative means to study cell communication (Figure 1(c)). Cell-to-cell communication is essential for multicellular organisms. Cells that are connected through gap junctions can communicate rapidly with each other by passing electrical current or through the diffusion of small second messengers such as cyclic AMP and inositol 1-, 4-, 5-trisphosphate (InsP3). Sriram et al. [76] used the ECIS to study the effect of ovarian cancer cells on the permeability of a confluent pleural mesothelial cell (PMC) monolayer. Results showed that ovarian cancer cells adhered to the PMC monolayer, which, in turn, induced a localized dysfunction of the PMC barrier.

In the case of chemical communication, one cell upon activation releases a stimulus, which diffuses to a target cell that has receptors for the stimulus. The binding of the stimulus activates the receptor, leading to cell signaling in the target cells. Treeratanapiboon et al. [77] applied the ECIS to study the effect of membrane-associated malarin antigen-activated human peripheral blood mononuclear cells (PBMCs) on the integrity of porcine brain capillary endothelial cells (PBCEC). Results showed that the antigens obtained from lysed Plasmodium falciparum schizont-infected erythrocytes caused the PBMC to secrete tumor necrosis factor alpha, which, in turn, led to the breakdown of the endothelia PBCEC monolayer, possibly via disruption of tight junction complexes.

The human immune system enables the destruction of dangerous microbes with great precision via specific targeting of immune cells to sites of infection. Central to the defense mechanism is the interaction of cells with adhesion molecules involved in migration and invasion. Kataoka et al. [78] used the ECIS to study the interaction of monocytes with endothelial cells. By combining AFM with the ECIS, they found that the interaction of monocytic THP-1 cells with the interlukin-1β-stimulated HUVEC monolayer caused a decrease in adhesion to the substrate and an increase in deformability of the endothelial cells. A recent RT-CES study showed that adhesion of human monoblastic cell line U937 cells to endothelial cells was sensitive the presence of lipopolysaccharide [79].

Critical to human immune defense mechanisms is the effector-cell-mediated killing of target cells [80]. For example, natural killer (NK) cell-mediated cytotoxicity requires cell-to-cell contact, which is mediated by the pairwise recognition between multiple receptors present on the surfaces of effector and target cells. The NK cells are considered the major cytotoxic effector cells for innate immunity that can recognize and kill malignantly transformed and infected cells. Glamann and Hansen [81] utilized real-time cell electrical sensing (RT-CES) to detect the interactions between natural killer (NK) cells in suspension and adherent breast cancer cells MCF7 cultured on the electrode biosensor surface. Results showed that NK cells caused apoptosis of MCF7 cells, depending on the NK cell-to-target cell ratio.

6. Cell Signaling

Cell signaling is a tightly regulated process to direct the information flow and ultimately control cellular responses once the cell receives exogenous signals (Figure 1(d)). Signaling by membrane receptors begins with the activation of receptors, followed by generation of intracellular messengers. These messengers then engage various effectors to activate diverse cellular responses including microfilament remodeling, protein trafficking, and alterations in cell adhesion and gene expression. Molecular assays have led to identification of many protein components of various signaling pathways, and high-resolution imaging have resolved many cellular events downstream the activation of a receptor. However, the use of label-free biosensors for studying cell signaling was sparse in the literature before 2004 [64]. Since 2004, two important developments had made label-free a versatile technology for cell signaling study. First, high throughput label-free systems became a reality [29, 42, 82–87], so it became possible to study receptor signaling in native cells without any labels at an unprecedented scale. Second, it

was finally realized that a biosensor signal arising from the activation of a receptor is an integrated response that faithfully reflects the signaling pathways downstream the receptor activation [8, 83, 85, 86]. This led to subsequent adoption of chemical biology for pathway deconvolution of receptor signaling [85, 86]. These developments have turned label-free a morphological biosensor into a systems cell biology biosensor [9, 29].

6.1. G Protein-Coupled Receptors. GPCRs are the largest gene families in the human genome and are the leading molecular target class against which the drugs are designed. GPCRs transmit an enormous number and variety of exogenous signals including light, odorants, neurotransmitters, hormones, and proteases. These exogenous ligands bind to a receptor, and induce a conformational change in the receptor that is then transmitted through the membrane to activate the heterotrimeric GTP-binding proteins (G proteins). The G proteins function as the transducers to relay information to different signaling pathways such as the cyclic AMP and InsP3/diacylglycerol signaling pathways. Since 2005, label-free cellular assays have attracted much attention in molecular delineation of receptor biology and ligand pharmacology for many GPCRs [8, 42, 59, 62, 65, 85–120]. Many GPCRs in distinct cell backgrounds have been examined using label-free cellular assays (Tables 1 and 2). These receptors are either endogenously expressed in native cells including primary cells, or stably or transitly expressed in various cell lines.

Label-free profiling of endogenous receptors in native cells had led to discover "signatures" of distinct classes of GPCRs, depending on the G protein with which the receptor is coupled [9, 42, 86, 88, 92]. Although it holds great promise in a given cell background and for receptors which lead to a single G protein-mediated pathway, the concept of "signature" quickly yielded to "phenotypic response" or "systems cell biology readout" [9, 29, 99, 115, 121]. This is because label-free signals often reflect the cellular background-dependent and receptor-specific complexity in receptor signaling.

Label-free characterization of many GPCRs in various cell backgrounds has led to discovery of novel pathways downstream a receptor [9, 86, 96, 101, 105, 115], and also led to high-resolution classification of distinct ligands acting on a specific receptor [9, 65, 96, 100, 101, 108, 109, 119, 120]. These receptors include bradykinin B2 receptor, protease activated receptor-1 (PAR1) and -2 (PAR2), lysophosphatidic acid (LPA) receptors, histamine H1 receptor, adenosine A2B receptor, β2-adrenergic receptor, purinergic P2Y receptors P2Y1, P2Y2, P2Y4, and P2Y11, sphingosine-1 phosphate (S1P) receptors, vasoactive intestinal peptide (VIP) receptor VPAC1, vasopressin V1a receptor, serotonin 5HT1A receptor, dopamine D1, D2, D3, and D5 receptors, muscarinic M1, M2, M3, and M4 receptors, cannabinoid CB1 and CB2 receptors,, pituitary adenylate cyclase-activating polypeptide receptor (PACAP1), chemokine CXCR2 receptor, free fatty acid receptor-1, 2 and 3 (GPR40, GPR43, and GPR41, respectively), metabotropic glutamate receptor 1 (mGluR1) and 7 (mGluR7), prostaglandin EP2 and EP4 receptors, GPR55, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), corticotropin releasing hormone receptor 1 (CRF), melanocortin receptor-4 (MC4R), mu and delta opioid receptors, and GPR35.

6.2. Receptor Tyrosine Kinases. Receptor tyrosine kinases (RTKs) are a family of cell surface growth factor receptors with an intrinsic, ligand-regulated tyrosine-kinase activity. Epidermal growth factor receptor (EGFR) is one of the most well-studied RTKs. EGFR is a single membrane-spanning protein with an N-terminal extracellular ligand-binding domain and a C-terminal region that has a kinase domain and numerous tyrosine docking sites participating signaling. EGF binds to the receptor and stimulates its intrinsic protein-tyrosine kinase activity, initiating signal transduction that principally involves multiple pathways, including MAPK, STAT, and the PLCγ pathways. RWG was the first label-free biosensor used to characterize and deconvolute the pathways of EGFR in native A431 cells [39, 83, 85]. This study was based on chemical intervention of the EGF-induced DMR signal to map out the pathways downstream the EGFR activation (Figure 3). This study had led to a hypothesis that label-free signals arising from the activation of a receptor is an integrative readout of systems cell biology. Follow-up studies of EGFR signaling with different label-free technologies [122–127] confirmed such a hypothesis.

6.3. Ion Channels. Ion channels control the electrical properties of neurons and other excitable cells by selectively allowing ions to flow through the plasma membrane. These receptors transduce the information into channel opening, leading to marked amplification of the signal via conducting large amounts of charge. Such an amplification makes these receptors effective transducers of sensory information. Ion channels are often modified by signaling proteins and molecules to regulate neuronal excitability and other cell functions. Label-free cellular assays hold promise to follow in real-time the pathways downstream the open and close of ion channels. Such an ability overcomes the poor resolution of traditional assays to examine the interaction between channels and regulatory proteins in living cells. Using DMR assays enabled by RWG biosensor, Fleming and Kaczmarek found that the activation of endogenous Gq-coupled receptors in HEK-293 cells was significantly modified by the presence of a sodium-activated potassium channel, Slack-B [110]. Recently, Pänke et al. also showed that electric biosensor is also feasible to characterize transient receptor potential (TRP) ion channels including TRP1 [128]. TRP channels are nonselective ion channels permeable to cations including Na^+, Ca^{2+}, and Mg^{2+}. The TRP channels are involved in many Ca^{2+}-mediated cell functions and implicated in inflammation.

6.4. Immunoreceptors. Immunoglobulin E (IgE) is one of immunoglobulins produced by the immune system, and the one most associated with allergies. Allergic individuals exposed to minute quantities of allergen often experience an immediate response, which is due to the permanent sensitization of mucosal mast cells by allergen-specific IgE antibodies bound to their high-affinity receptor (FcεRI). The IgE-mediated mast cell activation includes two important

Table 1: Receptors, cell lines, and technologies and key points of the studies related to the use of label-free cellular assays for GPCRs.

Receptors	Cells	Biosensors	Key findings	Ref
PAR2 Bradykinin B2	A431	DMR	Biosensor signal is originated from DMR	[8]
Adenosine A2B Bradykinin B2 β2-adrenergic	A431	DMR	Similarity analysis segregates ligands into clusters	[38]
EP4 H1 LPA receptors P2Y1			DMR signatures of distinct classes of GPCRs	[92]
PAR1 PAR2 S1P receptors VPAC1			Integrative roles of adenylyl cyclases in GPCRs	[93]
P2Y1/2/11	HEK293	DMR	ECM coatings impact receptor signaling	[59]
LPA receptors	Porcine brain endothelial cells	ECIS	LPA increases tight junction permeability	[62]
PAR1	Primary endothelial cells	ECIS	Thrombin promotes the formation of intercellular gaps	[64]
PAR1	HMEC-1	ECIS	Functional selectivity of PAR1 agonists	[65]
Bradykinin B2	A431	DMR	Systems cell biology of B2 receptor	[86]
PAR1	A431	DMR	HTS compatibility test	[87]
Endogenous receptors	HeLa U-937 U2OS TE671	CDS	Receptor panning	[88] [89]
LPA receptors S1P receptors	Rabit corneal epithelial cell Rabit corneal endothelial cells	ECIS	The role of Gi signaling in cell monolayer permeability	[90]
Histamine H1 Vasopressin V1a 5-HT1A D1	CHO-H1 1321-N1-V1a CHO-5HT1A CHO-D1	RT-CES	Impedance signals were correlated with morphological changes	[91]
PAR1 PAR2	A431	DMR	Receptor cross-desensitization	[94]
Dopamine D2S Muscarinic M4 Dopamine D5	CHO-D2S CHO-M4 CHO-D5	CDS	Ligand pharmacology characterization	[95]
Muscarinic M1 Melanocortin MC4 Cannabinoid CB1 Cannabinoid CB2	CHO-M1 CHO-MC4 HEK-MC4 CHO-CB1 CHO-CB2	CDS	Ligand-directed functional selectivity GPCR pleiotropic signaling	[96]
Histamine H1 β2-AR	A431	DMR	Duplexed receptor assays for HT screening	[97]

events: cell sensitization resulting from IgE binding to the FcεRI receptor and cell activation triggered by allergen-mediated oligomerization of membrane-bound IgE. Abassi et al. used the RT-CES to characterize IgE-mediated activation of RBL-2H3 mast and found that the impedance results were correlated with morphological dynamics and mediator release [129].

Hide and his colleagues reported a series of papers related to the use of SPR for characterizing the activation of RBL-2H3 mast cell and found that SPR detects the downstream events of active PKCβ in antigen-stimulated mast cells [130–133]. The RBL-2H3 mast cells overexpressing dominant-negative spleen tyrosine kinase or src-like adaptor protein led to a suppressed SPR signal arising from the mast cell activation. Likewise, expression of dominant-negative linker for activation of T cells and Grb2-related adaptor protein led to almost complete suppression of the antigen-induced SPR signal. Overexpression of protein kinase C

Table 2: Receptors, cell lines, and technologies and key points of the studies related to the use of label-free cellular assays for GPCRs.

Receptors	Cells	Biosensor	Key findings	Ref
Dopamine D3 Muscarinic M1	CHO-D3 CHO-M1	DMR	Ligand pharmacology characterization	[98]
β2-AR	A431	DMR	Systems cell biology of the β2AR Ligand-directed functional selectivity Ligand-directed desensitization	[99] [100] [101]
PACAP1	TM3	RT-CES	PACAP agonists suppress the proliferation of immature mouse Leydig cell line TM3	[102]
CXCR2	NIH-3T3-CXCR2	ECIS	The role of CXCR2 in cell transformation	[103]
Muscarinic M2	CHO-M2	DMR	Novel dualsteric M2 agonists	[104] [105]
Muscarinic M1	CHO-M1	DMR	HT screening identified novel M1 ligands	[106]
GPR40/FFA1 GPR41/FFA3 GPR43/FFA2	1321N1-GPR40 HEK-GPR43 HEK-GPR41 HEK-GPR40	DMR	Discovery of potent and selective agonists for FFA receptors	[107] [108] [109]
Endogenous muscarinic receptor	HEK-293	DMR	GPCR activation modulates Slack ion channel activity	[110]
Cannabinoid CB2 mGluR1	CHO-CB2 CHO-mGluR1	RT- CES	Ligand pharmacology characterization	[111]
Prostaglandin EP2	C6G-EP2 HCT15-EP2	DMR	Compound nanoparticles act as allosteric potentiators	[112]
mGluR7	HEK-mGluR7	DMR	Negative allosteric modulators	[113]
GPR55	HEK-GPR55	DMR	GPR55 pleiotropic signaling	[114]
Muscarinic M2 β2-AR Muscarinic M3 GPR55 CRTH2 EP2/3 GPR40 EP receptors EP receptors	CHO-M2 CHO-β2AR CHO-M3 HEK-GPR55 HEK-CRTH2 HEK-EP2/3 HEK-GPR40 HaCaT Keratinocytes	DMR	Pathway deconvolution GPCR pleiotropic signaling Novel pathways for M3	[115]
Muscarinic M1 Muscarinic M2 CRF MC4R	CHO-M1 CHO-M2 CHO-CRF CHO-MC4R	CDS BIND DMR	Label-free reader comparison	[116]
CRTH2	HEK-CRTH2	DMR	Novel function of CRTH2 C-terminal	[117]
Mu opioid Cannabinoid CB1 Cannabinoid CB2 Delta opioid	CHO-MOR CHO-CB1 CHO-CB2 CHO-DOR	DMR	Pathway deconvolution	[118]
PAR1	A549	DMR	Ligand-directed functional selectivity on receptor trafficking	[119]
GPR35	HT29	DMR	Discovery of tyrphostins as GPR35 agonists	[120]

(PKCs), apart from PKCβ, showed a reduced SPR signal in response to antigen stimulation, while knockdown PKCβ with interference RNA suppressed the antigen-induced signal. These results indicate that the activation of multiple kinases in the PKC pathway is determinative in the antigen-induced SPR signal of mast cells.

7. Viral Infection

Viral infections provoke an immune response that normally leads to elimination of the infecting virus. However, certain viruses including those causing AIDS evade human immune responses and result in chronic infections. Cytopathic effect

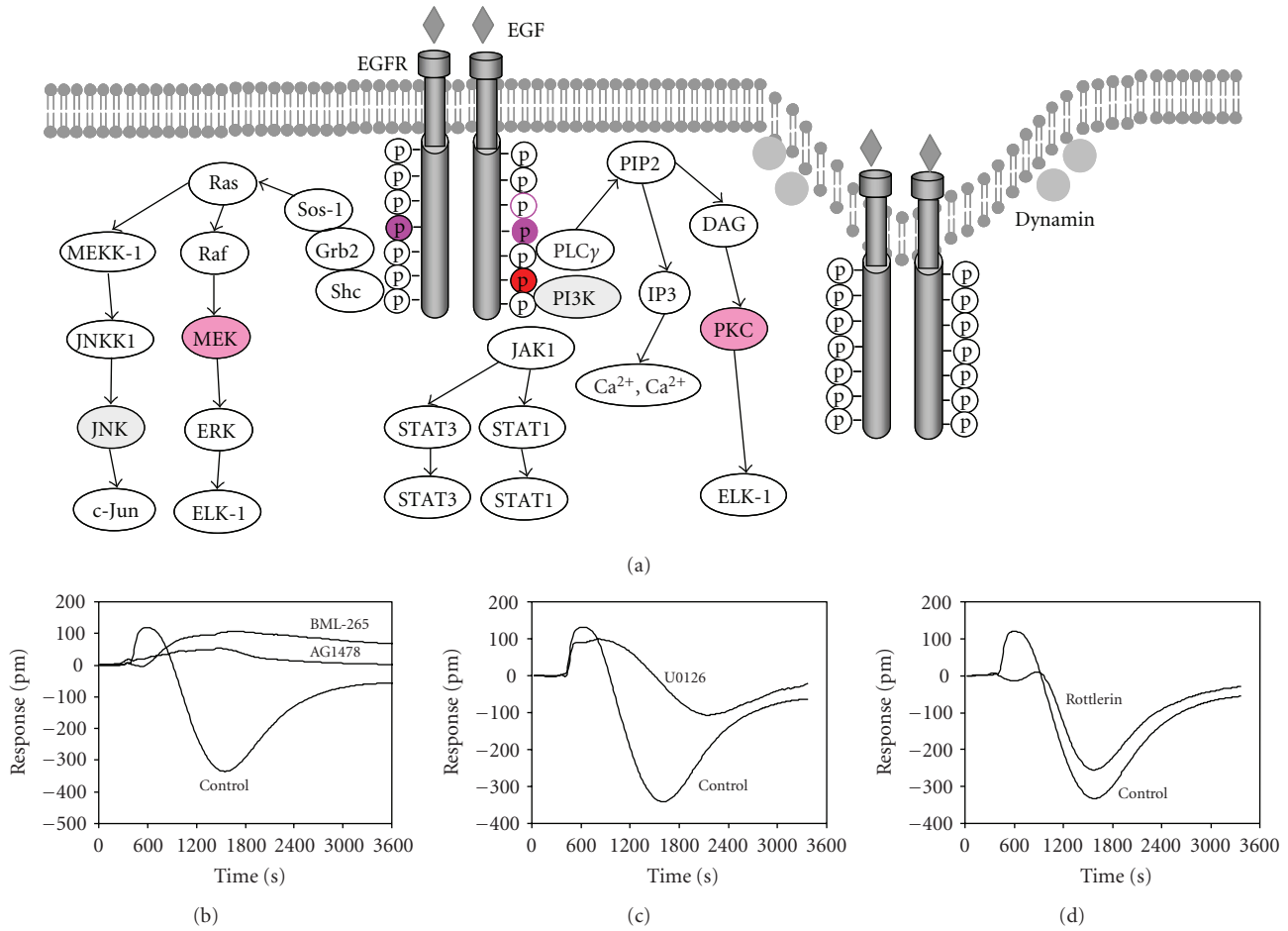

Figure 3: The DMR signal arising from epidermal growth factor (EGF) activated EGFR in native A431 cells is a systems cell biology readout of the EGFR. (a) Schematic drawing of EGFR signaling. (b, c, d) The sensitivity of the EGF DMR signal to different modulators, the two EGFR tyrosine kinase inhibitors AG1478 and BML-265 (b), the MEK1/2 inhibitor U0126 (c) and the PKC kinase inhibitor rottlerin (d). The control is the EGF response of cells pretreated with the assay vehicle only. In all experiments, EGF was at 32 nM, whereas the rest compounds used to pretreat the cells were at 10 μM.

(CPE) due to virus infection in cell culture has been used as *in vitro* model systems to study viral infection and screen molecules that inhibit the viral infection. However, the CPE has long been difficult to quantify. The ability to work with native cells makes label-free an attractive means to real-time-monitor the viral infection process (Figure 1(e)). The ECIS has been explored to monitor the progression of CPE due to influenza A virus infection [134]. Recently, Owens et al. used the DMR assays to monitor the infection process of HeLa cells with two different human rhinovirus strains, HRV14 and HRV16 [135]. Results showed that both virus strains triggered a virus titer-dependent DMR signal, which is correlated with multiple phases of viral infection, starting from early signaling mediated by viral entry to viral replication, and finally cell apoptosis. This study also showed that it is possible to screen inhibitors that modulate distinct processes of viral infection. Jia et al. also showed that DMR assays with Epic system enabled high throughput screening of inhibitors that block the cytopathic effect induced by influenza virus (A/Udorn/72, H3N2) [136].

Cocaine is a suspected cofactor in human-immunodeficiency-virus- (HIV-) associated dementia. However, it is unknown how cocaine influences HIV infection. Fiala et al. used the ECIS to study the mechanism by which cocaine increases HIV-1 invasion through brain microvascular endothelial cells (BMVECs) [137]. Results showed that cocaine treatment of BMVECs disrupts intercellular junctions and induces cell ruffling, and also alters the location patterns of virus once entered the cells. This study suggests that the toxicity of cocaine for the blood-brain barrier may lead to increased virus neuroinvasion and neurovascular complications of cocaine abuse.

Recombinant viral vectors are widely used in genetic manipulation of living cells. However, the impact of these vectors on cell biology is largely unknown. Using the ECIS, Müller et al. found that adenoviral transfection vector (Ad5-derivate) dose dependently caused the apoptosis of porcine ileal epithelial cell line IPI-2I [138]. This study suggests that label-free is an attractive alterative to determine minimal nontoxic doses for viral vector-based transfection study.

8. Label-Free versus Label-Based Cellular Assays

The quest to discover the full complement of cell signaling components has made label-based cellular assays the mainstream technology in cell biology. Label technologies can provide high spatial resolutions to resolve the location, trafficking, and organization of single signaling molecules within a specific pathway. Multicolor molecular assays can further investigate the interactions among distinct signaling molecules and the functional consequences of the invention of a cellular target with a molecule. However, the molecular assays often give rise to low temporal resolution, are weak in resolving cell-surface biology, and provide a linear measure of cell signaling.

Label-free cellular assays are complementary to label-based technologies. First, in contrast to label technologies which are biased towards a single pathway and/or a single molecule, label-free offers integrated and systems cell biology readouts of cell signaling. This allows one to study the integration of cell signaling in native cells, to map out signaling pathways downstream receptor activation with wide pathway coverage, and to greatly differentiate the on-target pharmacology of drug molecules acting on a single target receptor [139]. Second, in comparison with the relatively poor dynamic resolution of label technologies, label-free provides a real-time kinetic measurement of cell signaling with high temporal resolutions and high sensitivity. This allows one to track the entire process of diverse cell signaling and cellular processes in native cells. Third, in contrast to label technologies that often require modifications or even destruction of live cells, label-free is noninvasive without the need of any cellular manipulations. This allows one to design distinct assay formats, as well as to integrate label-free with other technologies, so different aspects of receptor signaling and drug pharmacology can be studied. For example, adoption of microfluidics enables one to control the duration of receptor activation, so that comparison of label-free signals under sustained stimulation conditions with those under pulse stimulation conditions can differentiate the routes of signal propagation after receptor activation, as well as the long acting agonism or antagonism of drug molecules [9, 101, 119, 140, 141]. However, unlike label technologies, label-free lacks intracellular spatial resolution to resolve many important cellular processes, including the location and organization of signaling molecules, intracellular trafficking, metabolism, and cytoskeletal remodeling. Thus, it is important to know what the hypotheses is being tested so the appropriate technologies can be used.

9. Conclusion Remarks

Advances in label-free biosensors, particularly high throughput screening platforms and adoption of chemical biology tools in label-free cellular assays, have made them indispensable platforms in cell biology studies. Today, label-free biosensors have found applications in a wide array of cellular processes ranging from cell adhesion to cell barrier functions, receptor signaling, and viral infection. The ever increasing use of label-free cellular assays for studying various targets including GPCRs, RTKs, ion channels, and immunoreceptors have been witnessed in the increased numbers of published literature in recent years. Novel insights about the integration of cell signaling, the complexity of receptor signaling pathways, and the modes of action of drug molecules have been obtained. New generation label-free currently under development will have better spatial resolutions, so that cell signaling can be studied at the single cell level [142–145]. Development of novel methodologies for data analysis [9, 38, 146] will further advance label-free to become a *de facto* technology in cell biology.

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