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TUTORIAL REVIEW

Label-free cell-based assays using photonic crystal optical biosensors

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Biosensor technologies that have been primarily used in the past for characterizing biomolecular interactions are now being used to develop new approaches for performing cell-based assays. Biosensors monitor cell attachment to a transducer surface, and thus provide information that is fundamentally different from that provided by microscopy, as the sensor is capable of monitoring temporal evolution of integrin–surface interactions that are difficult to measure by other means. Label-free biosensor technologies are especially advantageous for monitoring the behavior of cells because they do not require stains that typically result in cell death, and are not subject to effects such as photobleaching. As a result, cells can be quantitatively monitored in their culture environment over an extended period of time while processes such as proliferation, apoptosis, cytotoxicity, chemotaxis, ion channel activation, and membrane-bound protein activation are modulated by the introduction of a variety of chemical or biological stimuli. This review describes the application of photonic crystal optical biosensor microplates to a variety of cell-based assays. Detection instruments for photonic crystals measure the aggregate behavior

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of large cell populations, or, using recently developed biosensor imaging detection, independent monitoring of individual cells. These technological developments offer the ability to perform assays with a limited number of available cells for applications such as high throughput screening with primary cells or stem cells.

Introduction

The pharmaceutical research process relies heavily upon high throughput screening methods as a means for characterizing biomolecular interactions and the effects of chemical compounds that can selectively inhibit or enhance interaction pathways that occur due to disease. However, because the function of many protein targets depends upon their incorporation into larger structures, such as cell membranes, simple biomolecule-based assays are not always sufficient for understanding the effect that a potential drug will play in terms that are physiologically relevant. For example, activation of membrane-bound proteins is well known to modulate cell adhesion to surfaces and to control the interaction between cells within clusters.[1] Likewise, important physiological processes such as cell invasion[2] and chemotaxis[3] are impossible to model by biochemical assays alone. In addition, because of the high cost of drug failures due to unanticipated toxic side effects, it is greatly preferable to use cell-based assays as a means for testing the cytotoxic effects of chemical compounds on cells from a wide variety of healthy and diseased cells as a screening tool before moving forward with animal testing or clinical trials. Because processes such as adhesion, chemotaxis, proliferation, cytotoxicity, and apoptosis can only be observed using cells, it has become increasingly important to develop assay technologies that can rapidly and quantitatively measure the effects of chemical compounds on cells with high throughput.[4–6] Due to the cost of maintaining cell lines in the laboratory and the low rate of proliferation of some cell types, it is also important for assays to use a low number of cells, and to use procedures that may allow a set of cells to be measured multiple times without destroying them. In some cases, particularly for research, it is desirable to observe individual cells or cell clusters.

To address this need, several assay techniques and technologies have been developed to perform cell-based assays. For example, colored and fluorescent dyes are routinely used to selectively label cell components to facilitate visual observation with a microscope. Cell staining enables differentiation between live and dead cells, observation of DNA condensation, visualization of structural components, and, using fluorescent resonant energy transfer, measurement of intracellular protein–protein interactions. While staining is used for microscopy of a small number of cells, stain-based assays are also used to measure the "bulk" status of cell populations. For example, the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is commonly used to measure the percent cell survival for cytotoxicity studies through the production of colored chemical product,[7] while the caspase-3 activity assay is used to indirectly measure apoptosis in a cell population.[8] Due to the importance of cell membrane-associated processes in drug discovery, high throughput fluorescence polarization assays have been developed for measuring the activity of ion channels, while the most common method for studying the interaction of drugs with G protein coupled receptors (GPCRs) is the Fluorescent Imaging Plate Reader (FLIPR) assay.[9]

One of the main drawbacks of cell-based assays that rely upon labels is the effect of the label upon the cells. Many fluorescent or colorimetric dyes and stains are cytotoxic, resulting in the ability to perform only a single measurement upon a single group of cells. Due to cytotoxicity, cells must be sacrificed for each step of a multi-step experiment, requiring an overabundance of cells to be obtained and maintained for complex experiments, particularly assays that are used to study the cell behavior over a period of several days. As complex experimental design is more commonly used to study the effects of cell environment, drug concentration, surface type, and growth factor concentration upon cells, it is becoming increasingly important to make more effective use of primary cells, stem cells, and cell lines that are difficult to propagate. Fluorescent dyes are prone to the effects of photobleaching, which makes long term quantitative study of a cell population especially difficult, as the measured intensity is highly dependent upon illumination intensity, accumulated exposure time, temperature, and pH.

In order to address the challenges inherent in label-based cell assays, label-free biosensor technologies have recently been demonstrated as effective tools for measuring an increasingly diverse range of cell processes. Biosensors generally involve some kind of transducer surface that is able to generate an electrical or optical signal when cells interact with the surface in particular ways. The biosensor output generally depends upon some intrinsic property of the cell (such as its electrical impedance or dielectric permittivity) in order to perform a measurement that translates into a determination of the number of cells in contact with the transducer, or more subtle measurement of the distribution of focal adhesion points with the transducer. A transducer may be prepared with a biomolecular surface that can be used to selectively capture specific cell populations through interaction with proteins that are expressed on the outer surface of cells, or the surface may be prepared with thin coatings of the extracellular matrix material to facilitate attachment. In order to be useful tools for cell-based pharmaceutical screening, the transducer must be compatible with long-term operation in cell media at temperatures commonly used for incubation.

Early biosensor technologies, such as Surface Plasmon Resonance (SPR), were first applied mainly to biomolecular assays within narrow flow cells that were incompatible with the introduction of cells with similar dimensions.[10,11] More recently, biosensor-based systems have been introduced in which the sensors are incorporated into standard format 96-well, 384-well, and 1536-well plastic microplates for greater compatibility with automated fluid dispensing systems and liquid handling that is most commonly used for cell incubation.[12–15] Biosensors with the ability to measure cell attachment to arrays of electrodes have been used to measure cells through their electrical conductivity.[16]

In this review, we focus on the application of optical biosensors based upon photonic crystals (PCs) to a variety of cell-based assays. In contrast to electrical impedance-based biosensors, optical biosensors measure the kinetic adsorption of

biomolecular materials through their greater dielectric permittivity at optical wavelengths compared to water.[17] While optical biosensors have been used for many years for kinetic quantification of protein–protein interactions with sensitivity sufficient for measuring small molecule adsorption, all the materials that a cell comprises, such as membrane components, nuclei, mitochondria, and cytoplasm, also contain molecules that have a greater dielectric permittivity than water. It has been recognized for some time that adsorption of cells within the surface evanescent field region of an optical biosensor will generate an easily measurable output.[18–20] Due to the available resolution of optical biosensors, they have been finding applications in which they are used to measure more subtle effects than simply quantifying the number of attached cells, as the "strength" of cell attachment to a surface can be modulated in a variety of ways. Also unique to optical biosensors is the ability to measure the strength of cell attachment as a function of spatial position on the transducer surface, using an appropriate detection instrument.[21–23] Imaging-based biosensor detection enables label-free observation of cell populations over a long period of time to quantify chemotaxis, proliferation, differentiation, and apoptosis. Recently, label-free biosensor imaging tools have become available that enable close-up study of a single cell or cell cluster. Sufficient image resolution is now available that imaging biosensor detection is capable of monitoring the evolution of focal adhesions.

The advantages of PC-based optical biosensors for cell-based assays are compelling. As a label-free technology, cell attachment to the sensor is measured without the use of dyes and stains, so the same population of cells can be measured many times over the course of several days without disturbing them in their culture medium. The detected output signal is highly quantitative, providing measurements that are repeatable between sensors, instruments, or laboratories without effects such as photobleaching. PC biosensors are fabricated from plastic materials and incorporated into the bottom surface of standard microplates, but require only low intensity illumination with infrared light from beneath the plate for performing a measurement, so no electrical or physical contact between the sensor and the detection system occurs. In contrast to SPR, the PC biosensor strictly limits lateral propagation of light, enabling imaging-based detection with resolution sufficient for measuring variation of cell adhesion within a single cell. PC biosensor imaging provides information that is fundamentally different from that provided by an optical microscope, as the sensor responds to local variation in cell *attachment* to the transducer surface. The sensor can be prepared with a variety of surface states (such as matrix coatings, antibodies, and peptides) and thus can be used as a tool for measuring how cell attachment to surfaces is modulated by drugs, growth factors, or other environmental factors.

In this review, we describe the operation of the PC biosensor and its associated detection instrumentation. Using a single PC biosensor design, separate detection instruments for quantifying ensemble attachment of cell populations, and biosensor-based imaging are discussed. We also describe several exemplary applications of the technology. While measurement of surface-modulated cell attachment,[20] cytotoxicity,[24] and apoptosis[18] has been recently published, the review focuses upon more recent demonstrations that are of broad interest in the field of drug discovery and high throughput screening. These will include detection of cardiomyocyte cytotoxicity, and quantification of subtle cell–sensor interactions, including detection of GPCR activation, ion channel activation, and chemotaxis. The use of the label-free imaging capability will be used to demonstrate detection of stem cell differentiation and measurement of focal adhesions to the biosensor surface.

Photonic crystal biosensor and detection systems

Previous publications have described disposable microplate-based optical biosensors based on the unique properties of PCs.[25–29] Like other optical biosensors, including those utilized in SPR, PC biosensors detect biomolecular or cell interactions on the surface of a transducer through changes in dielectric permittivity with respect to the liquid media. A PC is composed of a periodic arrangement of dielectric material that effectively prevents propagation of light at specific wavelengths and directions. When illuminated with white light, appropriately configured PCs are able to reflect a single wavelength, whose value is dependent on the local density of adsorbed biomolecules or cells (Fig. 1). Association of cells with the sensor surface modulates the peak wavelength value (PWV) of the reflected light, allowing for detection of binding by a shift in the PWV. PC biosensors incorporated into standard format 96, 384, or 1536-well microplates have been used to detect antibody–antigen, small molecule–protein, and whole cell–protein interactions on the biosensor surface without the use of fluorescent labels.[25]

The resonant reflected PWV of the sensor can be measured by illuminating the PC at normal incidence with a broad band of wavelengths, and collecting the reflected light with a spectrometer. Illumination and collection may be performed to gather single measurements over an illuminated spot, or an imaging spectrometer can be used to generate a spatial image of the resonant reflected wavelength. Image-based detection with high spatial resolution is enabled by the PC structure, which is designed to cut off lateral propagation of the resonant wavelength, thus eliminating pixel-to-pixel optical crosstalk. The sensors are inexpensively fabricated on sheets of plastic film, and are compatible with cell culture methods and standard microplate handling procedures.

The SRU Biosystems BIND "Reader" detection instrument (Fig. 1b) utilizes 8 parallel detection heads, each capable of reading a single biosensor microplate well. The optical system casts a ~0.6 mm diameter spot of light from an infrared light emitting diode against the sensor surface from below the microplate, and the reflected light is gathered into a spectrometer, which records the resonant reflection spectrum, while software fits the spectrum to a mathematical model to determine the PWV. A single measurement of 8 probes takes ~20 ms to gather the reflected light, and an entire 96-well biosensor microplate can be read within ~5 s. The measurement may be repeated to gather kinetic information. The entire instrument may be placed inside and continuously operated within a conventional CO_2 incubator, to enable long-term studies of cells at controlled elevated temperature. Through operation of negative control wells in parallel with "active" wells, the effects of refractive index

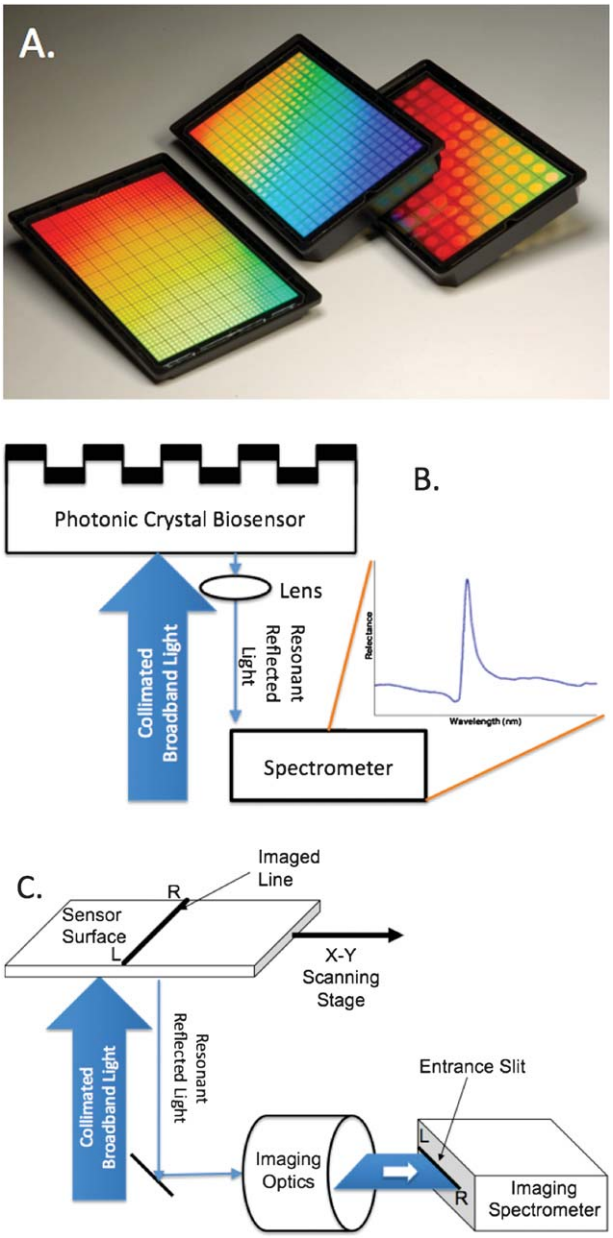

Fig. 1 (A) PC biosensor microplates. (B) Optical fiber-based detection instrument illuminates a 0.6 mm diameter region on the PC surface from below with light from an LED and collects the reflected light with the resonant wavelength into a spectrometer. (C) Schematic of the imaging detection system using a white light lamp for illumination using free-space optics and an imaging spectrometer to gather the resonant reflection.

sensitivity to added buffer solutions are subtracted to report net PWV shift values.

The imaging detection instrument referred to as the BIND Scanner, shown schematically in Fig. 1c, illuminates the sensor surface at near-normal incidence with a light emitting diode with output in the 840–890 nm wavelength band, and a line from the sensor surface is imaged into the entrance slit of an imaging spectrometer. The reflected spectrum of each pixel across the image line is gathered. A PWV image is constructed by sequential scanning of the sensor across the imaged line region in small increments. The detection instrument measures changes in the resonant reflected PWV of the biosensor surface as the detected output on a pixel-by-pixel basis that can generate images of PWV with a spatial resolution as low as $3.75 \times 3.75\ \mu m^2$. Detection of cell attachment to the sensor requires measuring a *shift* in PWV, so the sensor surface is scanned twice: once before and once after cells have been immobilized on the surface. The two images are aligned and subtracted mathematically to determine the difference in PWVs as detected by the sensor. Because the PWV is only increased for pixels in which a cell has been attached to the sensor, the imaging instrument measures the density of cell binding as a function of position within the microplate well. Because the measurement does not harm the cells, the PWV image may be periodically re-scanned to track cell activity over extended periods. The imaging detection system provides a two-dimensional, spatial representation of interactions that occur between cells and the biosensor surface. Label-free cell attachment images can reveal subcellular morphological changes underlying the quantitative PWV shifts measured as cells respond to specific stimuli. Moreover, the instrument provides a measure of response on a pixel-by-pixel basis and at high resolution can be used to monitor individual cellular responses. This feature has enabled the development of label-free applications for use with primary cultured cells making use of new advancements in low cell number and heterogeneous population assays.

The following sections briefly describe several exemplary cell-based assays that have been performed using PC biosensor microplates and the associated detection instruments. The first set of applications involve initial attachment of a high cell density to the PC surface that is followed by the measurement of small positive or negative changes in attachment as specific classes of outer surface-exposed receptors are activated (GPCR, Receptor Tyrosine Kinases (RTKs), ion channels) or cell adhesion processes are modulated by exposure to drugs. The second set of applications take advantage of the capabilities of the imaging detection instrument that enables visualization and quantification of the responses of individual cells. The imaging capability allows high sensitivity measurements to be obtained using a small number of cells, and is thus applied to the study of primary cells and stem cells. The imaging system is capable of separately tracking the response of cells within a heterogeneous population, monitoring chemotaxis, and observing stem cell differentiation.

Receptor activation assays

Activation of a wide array of cell surface receptor classes has been shown to elicit acute responses from cells already attached to optical biosensors. Depending on the receptor class and downstream signaling pathways activated, ligand responses can be manifested as negative or positive PWV shifts which can serve as the basis for quantitative assays. Thus, agonist activation of cell surface receptor classes such as GPCRs and Receptor Tyrosine Kinases (RTKs) leads to changes in cell adhesion to optical biosensors that can be monitored in real-time. These agonist-induced PWV shifts can be easily detected in cells with recombinant receptor expression but are also measurable as endogenous receptor responses in cell lines or in primary cultured preparations. Whereas cell adhesion signals accumulate over

hours following cell addition to biosensor plates, acute stimulation of cells attached to optical biosensors leads to rapid PWV changes that tend to occur over the span of minutes. These PWV responses can be robust (up to 1 nm wavelength shifts in some cases) and can be readily quantified and used as the basis for cell-based screening assays. Furthermore, the real-time kinetic PWV changes that can be monitored following ligand activation often reveal additional information specific to downstream signaling pathways engaged by the cell surface receptor. It is important to consider label-free cellular responses to specific agonists as integrated readouts of a multitude of signal transduction pathways providing the advantage of not being restricted to a particular signaling mechanism. Instead, these kinetic response profiles become "signatures" that can subsequently be used to profile cellular responses and yet can also be dissected with specific antagonists to detail the contributing signaling pathways.

GPCRs

GPCRs have been perhaps the most comprehensively assayed class of cell surface receptors to date on optical biosensors.[30,31] GPCRs are cell surface proteins that span the membrane seven times and bind ligands on their extracellular domains with high affinity and specificity. Once activated by cognate ligands, GPCRs interact with specific intracellular signal transduction proteins, including heterotrimeric G-proteins, that each elicits distinct signaling pathways and biological endpoints. When cells expressing GPCRs are activated by their corresponding agonists, distinct kinetic ΔPWV responses are measured that are particular to the G-protein subunits that are engaged (Fig. 2). For example, in HEK293 cells agonists that activate G_q-coupled receptors elicit positive PWV shifts that reach a maximum at approximately 20 minutes following ligand addition and then plateau thereafter. In contrast, G_i-coupled agonists induce a transient positive PWV shift that then returns to baseline within approximately 20–30 minutes. These kinetic activation profiles display concentration-dependence that can be translated with the appropriate software tools into accurate EC_{50} values at any given time point.

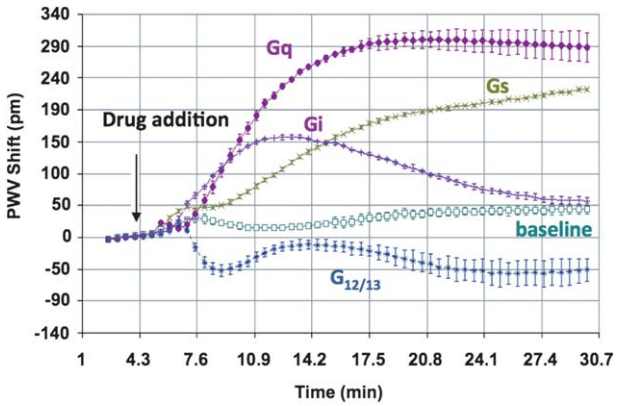

Fig. 2 Endogenous GPCRs in HEK293 cells were incubated with a variety of known natural ligands and BIND responses measured over time. GPCR stimulation results in a variety of BIND kinetic responses which fall into one of four signatures, each of which can be attributed to activation of specific Gα pathways. Data provided by Jason Brown, GSK Neurosciences CEDD.

The molecular mechanisms that contribute to the kinetic signature for any given response can be elucidated to the extent that specific inhibitors are available. For example, on PC biosensors the $G_{12/13}$-coupled endogenous response in HEK293 cells induced by sphingosine-1-phosphate (S1P) is characterized by two distinct PWV shifts: an initial negative response followed by a rebounding positive response. Pre-treatment of cells with an inhibitor of Rho-kinase (a $G_{12/13}$-coupled signaling enzyme) ablates the initial negative PWV shift but not the subsequent positive PWV shift. In contrast, the G_i-specific inhibitor, pertussis toxin, has the opposite effect: a more substantial negative PWV shift is observed and the subsequent positive PWV shift is suppressed. These data reveal two distinct mechanisms involved in the propagation of S1P signaling. Each pathway can be studied separately using selective inhibitors and concentration–response curves for the inhibitors generated within specific regions of the kinetic profiles (Fig. 3). Thus, PC biosensors provide the opportunity to detect dual coupling mechanisms of a particular stimulus using a single assay.

Receptor tyrosine kinases

Activation of cell surface RTKs also leads to changes in the interaction of stimulated cells with the surface of PC biosensors that can be readily measured. In contrast to the extensive body of work involving optical label-free detection of GPCR responses, comparatively little has been studied with respect to RTK activation. The majority of work on RTKs and optical biosensors has come from studies involving activation of the epidermal growth factor receptor (EGFR). When A431 cells, human epithelial carcinoma cells expressing extremely high levels of EGFR, are grown on optical biosensors and then stimulated with EGF the kinetic response profile is characterized by a transient positive PWV shift.[32] The same signature response can be detected in the UPCI-37B squamous cell carcinoma of the head and neck (SCCHN) cell line[33] as well as in the human embryonic kidney cell line, HEK293 (SRU, unpublished results). As with EGFR activation, stimulation of endogenous platelet-derived growth factor or insulin receptors (other members of the RTK superfamily) in HEK293 or Chinese hamster ovary (CHO) cells, respectively, also induces transient positive PWV shifts that reach peak levels approximately 5–10 minutes following agonist addition and then subside thereafter (SRU, unpublished results). Interestingly, an entirely distinct kinetic response to EGF is detected in the A549 lung cancer cell line, indicating that EGFR displays cell type-dependent responses on optical biosensors.[33] The downstream signaling pathways that contribute to EGFR-mediated PWV changes also appear to be cell-specific. Whereas the Ras/MAP kinase signaling pathway is the critical contributor to EGFR-mediated effects in A431 cells, it is the PI3 kinase pathway that is the significant component of signaling changes to optical biosensors in UPCI-37B cells.[32,33] Thus, PC biosensors can be used to measure RTK responses and also to help elucidate signaling pathways involved in transducing downstream signals.

Ion channels

The activation of ion channels by changes in electrical potential or by ligands that directly bind the channel leads to the passage of

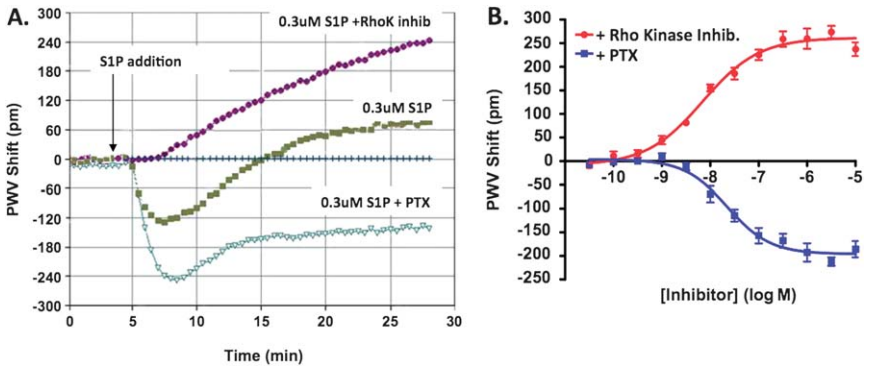

Fig. 3 Sphingosine-1-phosphate (S1P) activation of endogenous receptors in HEK293 cells is shown to occur *via* multiple signaling pathways in a concentration-dependent fashion. (A) Inhibition with a Rho-kinase inhibitor or pertussis toxin results in suppression of different components of the kinetic response signature on BIND. PWVs are baselined to reference wells in the same plate containing untreated cells. (B) Each pathway can be studied separately and quantitatively using selective inhibitors. Data provided by Jason Brown, GSK Neurosciences CEDD.

specific ions that trigger subsequent intracellular signaling pathways. In the case of ligand-gated ion channels, activation of the channel results in robust effects on biosensors that can be used as the basis for functional screening assays. The transient receptor potential (TRP) channels are ligand-gated, non-selective cationic channels through which calcium ions can enter cells. The TRPV1 channel mediates responses to pain stimuli and is a highly attractive target for drug discovery efforts. Historically, however, the isolation and development of drugs targeting ion channels have been difficult. The recent success in using optical biosensors to measure ligand-gated ion channel activity provides an exciting opportunity to utilize the high-throughput capacity of these screening platforms to further drug discovery efforts in this area.

The activation of TRPV1 channels by capsaicin, a pain-inducing stimulus, leads to rapid and robust concentration-dependent negative PWV shifts (−1000 pm) that manifest within seconds of ligand addition and plateau at approximately 25–30 minutes (Fig. 4). The mechanisms that govern the adhesion and/or morphology changes which, in turn, result in negative PWV shifts are currently not well understood. Yet, the capsaicin-mediated responses can be blocked by established TRPV1 antagonists, indicating a link between channel activity and changes in cell contact with the underlying sensor. Furthermore, the mechanism of action for TRPV1 inhibitors can be elucidated by performing classical competitive *vs.* non-competitive antagonist studies. Increasing concentrations of the antagonist, SB566791, leads to a progressive rightward shift in the EC_{50} of capsaicin, indicative of a classical competitive antagonist mechanism of action. In contrast, Ruthenium Red reduces the maximal response of capsaicin in a concentration-dependent manner without significant effects on EC_{50}, a hallmark of non-competitive antagonists (Fig. 4). A further validation for the use of PC biosensors in the assaying of ligand-gated ion channels comes from a comparison of antagonist activities with orthogonal measurements which reveal a linear 1 : 1 relationship with highly comparable IC_{50} values. Thus, microplate-based PC biosensors provide a high-throughput screening alternative for ligand-gated ion channels.

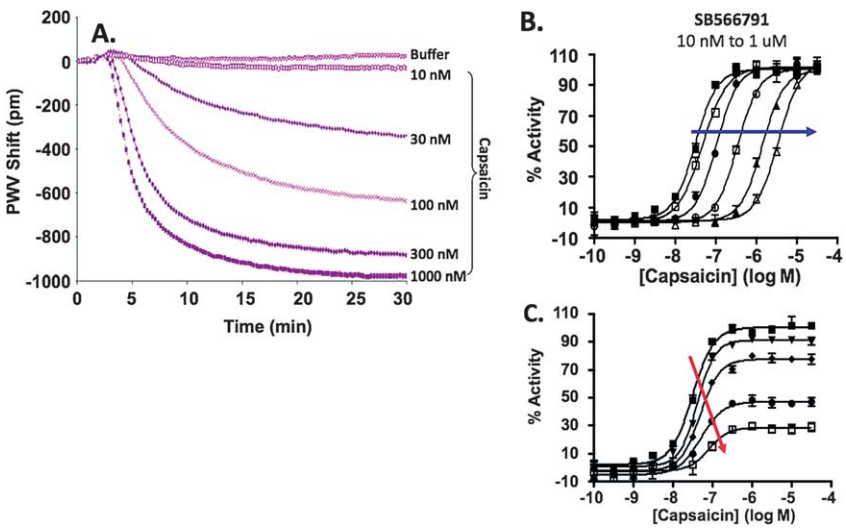

Fig. 4 Ligand-gated ion channel assays on optical biosensors. (A) Capsaicin stimulates a robust and sustained cellular response in CHO cells expressing recombinant TRPV1 in a concentration-dependent manner. Detection of different modes of action by TRPV1 inhibitors: competitive (B) *vs.* non-competitive (C) antagonists. Data provided by Shephali Trivedi, Astrazeneca.

Cellular adhesion assays

Cell adhesion is defined as the binding of a cell to a surface, extracellular matrix (ECM) or another cell *via* cell surface adhesion molecules such as selectins, integrins, and cadherins. Although critical to normal physiology, cell adhesion is also central to the etiology and pathogenesis of many diseases, including inflammatory diseases and oncology. Since the attachment of cells to the surface of PC biosensors produces a significant PWV shift, it is no surprise that cell adhesion assays have been successfully developed. Biosensors can be coated with a variety of different ECM proteins to promote cell attachment. Fibronectin, laminin, collagen, and poly-D-lysine are among the coatings that can individually or in combination promote robust cell attachment signals. The PWV shift measured in response to cell adhesion is directly proportional to the number of cells attaching to the biosensor and thus quantitative when used as an assay readout. Cell attachment signals can be monitored in real-time to obtain a kinetic measurement of cell adhesion or as an endpoint assay in the context of high throughput screening of compound libraries.

Adhesion assays are perhaps most useful when specific cell adhesion molecules (CAMs) that mediate intercellular interactions can be interrogated. Towards that end, specific CAMs can be coated directly onto the sensor or presented as fusions to epitope tags that can be captured *via* tag-specific antibodies. Cells that express the corresponding binding partner to the surface-captured CAM can then be assayed for adhesion activity. For example, a fusion of VCAM to the IgG-binding (zz) domain of protein A can be captured on a PC biosensor by previously coating a TiO_2 surface with IgG (Fig. 5). The high affinity interaction of IgG for the zz domain results in surface capture and presentation of VCAM on the biosensor. J6 cells which express α4β1, a cell surface integrin that binds VCAM, can be shown to adhere to the biosensor with cation-dependence, representative of proper cell adhesion activity. Furthermore, this interaction can be disrupted with antibodies that block CAM/α4β1 binding thus validating the assay for antagonist screens. To the extent that CAMs can be captured and presented in functionally active conformations on a biosensor surface, label-free cell adhesion assays present great promise as a high-throughput platform for robust pharmacological screens.

Cytotoxicity assays

Millions of dollars are spent and lost on drug development every year as drugs fall out of pharmaceutical company pipelines due to *in vivo* toxicity that is not adequately predicted with *in vitro* assays. Label-free optical biosensors provide a high throughput assay platform for cytotoxicity screening with data outputs that are rich in information. The profile of cell death on PC biosensors is typically manifested as a significant negative shift in PWV as cell bodies are reduced in size and lose their strength of attachment with the surface. When cell death is measured in real-time, toxins that work *via* different mechanisms of action display distinct kinetic ΔPWV profiles. For example, when HeLa cells are exposed to toxins that are either detergents, DNA-damaging agents, selective estrogen receptor modulators (SERMs), or protein synthesis inhibitors, four distinct kinetic profiles are detected (Fig. 6). Additional toxins within these mechanistic categories display similar profiles (data not shown). Similar effects have previously been observed on other label-free platforms that utilize electrical impedance as an assay readout.[34] Thus, in addition to providing a robust readout that supports cytotoxicity screening, label-free assays with optical biosensors contribute additional information on the mechanism of action that provides further value in the area of compound profiling.

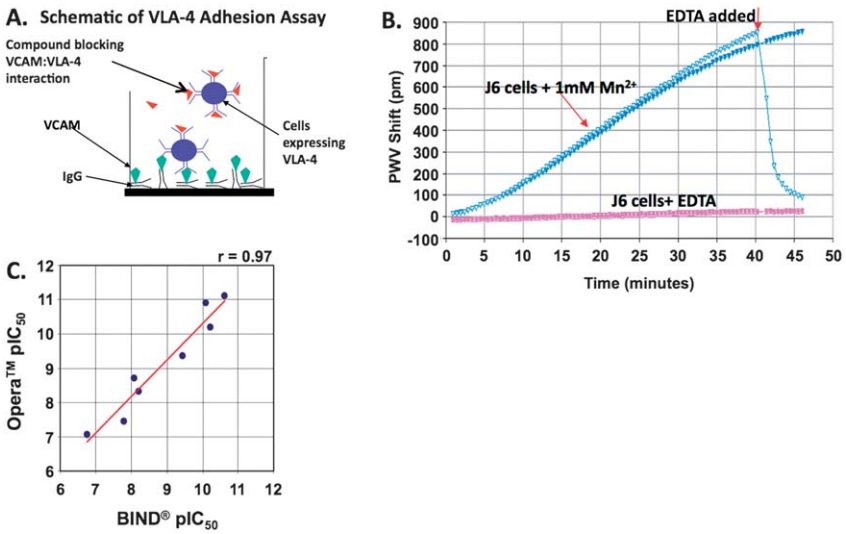

Fig. 5 Cell adhesion assay to measure specific cell adhesion molecule/integrin binding. (A) A specific cell adhesion molecule (*e.g.* VCAM) can be captured/coated directly onto optical biosensors. Following a block to prevent non-specific binding, the specific binding of cells expressing a cell surface integrin (*e.g.* VLA-4) can be measured and used for the basis of antagonist screens. (B) The binding of J6 cells expressing VLA-4 to VCAM on the sensor surface can be observed in real-time and shown to be dependent on divalent cations by chelation with EDTA either during or after binding. (C) Neutralizing activity of VLA-4/VCAM inhibitors correlates well between assays run on the optical-based label-free system (SRU BIND) and high content screening platform (Perkin-Elmer Opera). Data courtesy of Phil Green, GlaxoSmithKline.

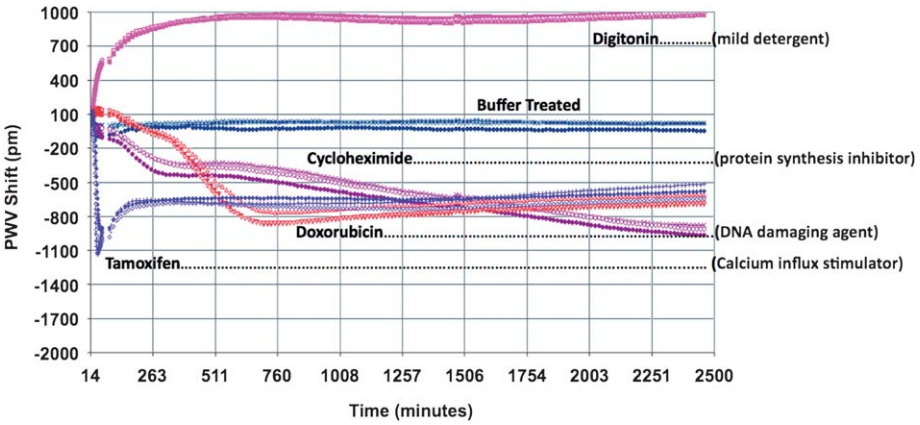

Fig. 6 Cytotoxic compounds generate distinct BIND kinetic profiles, depending on their mechanism of action. HeLa or HEK293 cells were plated on BIND CA2 biosensors at 20k cells per well and allowed to attach overnight. The next day, cells were treated with compounds (100 μM) representing different mechanisms of cytotoxicity. Each compound produced concentration-dependent responses (not shown) and distinct BIND Profiles.

Recently, the availability of embryonic stem (ES) cell-derived and induced pluripotent stem (iPS) cell-derived cardiomyocytes and hepatocytes has begun to revolutionize the pharmaceutical industry's approach to *in vitro* cytotoxicity screening. The availability of these biorelevant cells, in particular human iPS-derived cells, has enabled the concept of moderate throughput and more predictive cytotoxicity screening assays farther upstream in the drug discovery process. When seeded in culture, murine ES cell-derived cardiomyocytes develop a beating phenotype consistent with cardiomyocytes dispersed from primary cultured cells. The same beating phenotype can be observed when ES cell-derived cardiomyocytes are grown on optical biosensors and, consistent with normal growth properties, the beating becomes more synchronous and stronger over time. When label-free measurements are captured at a high sampling rate, cardiomyocyte beating can be detected on as oscillations of positive to negative PWV shifts that accurately reflect the rate of beating (Fig. 7). Moreover, the effect of compounds that elicit changes in either the rate or amplitude of beating can be measured by monitoring the frequency or *Y*-axis spread of positive-to-negative PWV shifts, respectively. Thus, in addition to scoring cell death, cardiotoxicity assays on optical biosensors can also detail the deleterious effects on contractility that compounds, such as hERG channel modifiers, often produce in the heart (Fig. 7). Given the high throughput capabilities of microplate-based biosensor systems, it is now possible to consider secondary screens for toxicity with biorelevant cells to filter out toxic compounds from primary screen hits. Thus, these assays will serve as high value, early stage *in vitro* predictors of *in vivo* toxicity, and will hopefully assist in reducing late-stage attrition in drug development pipelines.

Low cell number assays

When cells are seeded at low density, assay signal strength generally becomes compromised as the signal : background ratio

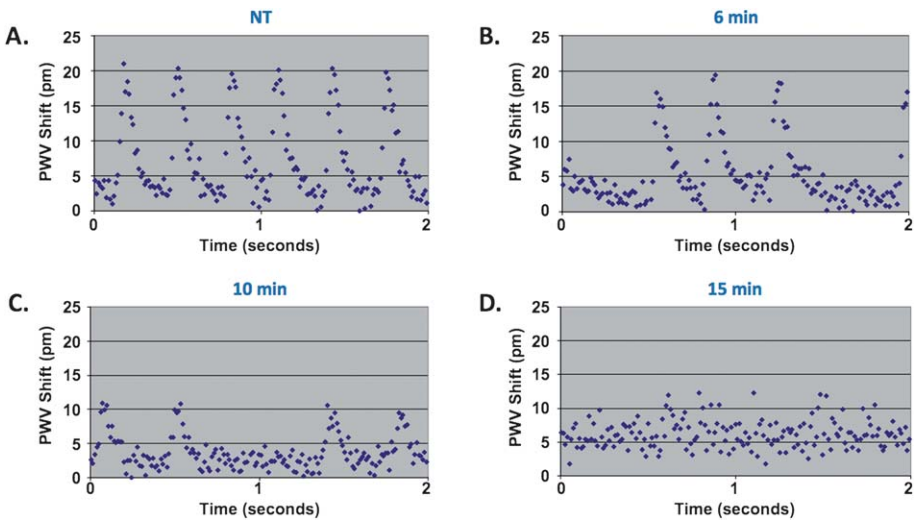

Fig. 7 Measurement of beating cardiomyocytes on optical biosensors. Murine embryonic stem cell-derived cardiomyocytes (Cor.At; Lonza/Axiogenesis) were grown on optical biosensors and measured for wavelength value changes over two second intervals at 80 Hz. (A) Untreated cardiomyocytes induce oscillations of positive-to-negative wavelength changes that correspond to the frequency and amplitude of beating. (B–D) The same well treated with 10 μM amitriptyline and then measured for two second intervals after the indicated time of treatment reveals an effect on frequency, synchronicity, and amplitude over time.

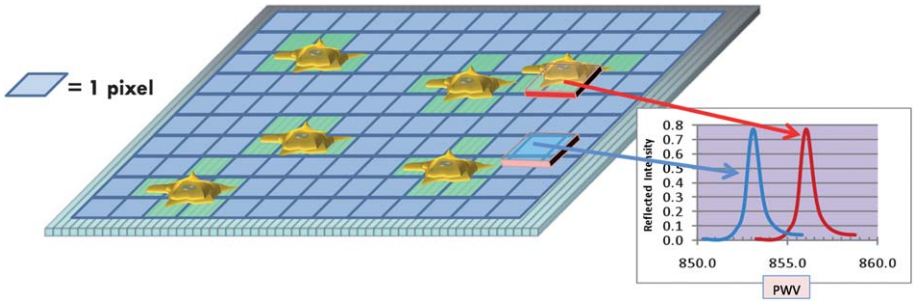

Fig. 8 Schematic diagram of cell finding on a high resolution BIND Scanner. A baseline reading is taken on an empty biosensor generating a PWV for every pixel. Following cell seeding, a software masking algorithm identifies the pixel location of attached cells based on PWV changes. Changes in the cellular response or cell number can then be monitored over time as the PWVs change only within the "mask". Pixels not engaged by cells, which would otherwise "dilute" a quantitative analysis, are ignored during the response measurement enabling assays to be run at low cell density.

reduces below a detection threshold. This is no different with the use of PC biosensors for cell-based applications as the PWV shifts measured across an area of interest become "diluted" by background space not occupied by cells. At low resolution many optical-based cell-based assays lose their sensitivity at cell densities below 2500–5000 cells per well in a standard 384-well microtitre plate. In contrast, using the imaging detection system, wavelength spectra are acquired for each individual pixel. When cells are seeded at low density, masking algorithms can be used to identify the positions within the area of interest where cells have made contact with the biosensors based on an attachment signal, *i.e.*, contiguous pixels generating PWV shifts (Fig. 8). When a subsequent stimulus is added, any cellular responses manifest as additional PWV shifts can be recorded and quantified within the previously identified cellular "mask", effectively ignoring the background spaces in the well devoid of cells. Applying newly developed masking algorithms for the imaging detection instrument, cellular responses can now be measured with as few as 250 cells per well in a 384-well optical biosensor plate (Fig. 9) and, in certain assays (see below), with under 100 cells per well, of significant interest with primary cultured cell applications where cell numbers are typically limited.

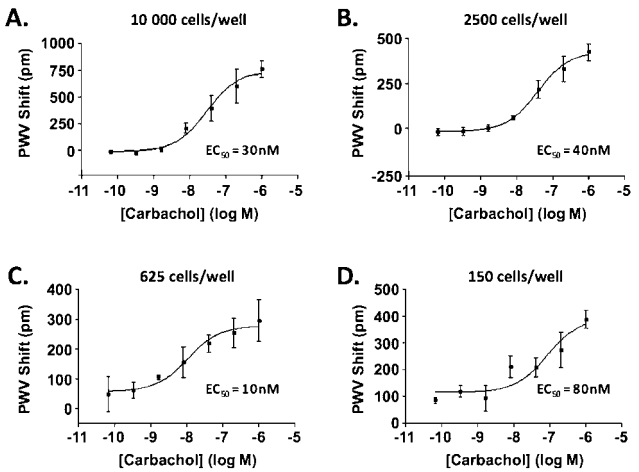

Fig. 9 Low density cellular responses measured with a BIND Scanner. (A–D) RBL cells expressing recombinant M5 muscarinic acetylcholine receptors were seeded at the indicated cell densities and stimulated with various concentrations of the M5 agonist, carbachol. Cell positions from BIND Scanner images were identified by masking algorithms and the response to carbachol quantified only within those pixels, thus enabling robust dose–response profiles down to ~150 cells per well in a 384-well plate.

Heterogeneous cell populations

Another significant challenge in assays with primary cultured cells derives from the heterogeneous nature of these preparations wherein it is often the response within a subset of cell type(s) that is the desired readout. High content imaging assays can be very useful in this regard with fluorescence readouts through the use of cell type-specific antibodies or reporter constructs. The advantage of using primary cultured cells in cell-based assays *in vitro*, however, is in the predictive value that more biologically relevant cell types can provide for translational activity *in vivo*. Thus, avoiding labels such as those used in fluorescence-based assays can provide an even greater measure of biological relevance.

High resolution, label-free detection of cells cultured on PC biosensors provides the opportunity to measure PWV changes that occur within individual cells. To the extent that unique PWV "signatures" can be defined for distinct cell types, responses from subpopulations of cells can be quantified. For example, distinct cell types might attach with differential strength to ECM-coated sensors, thus making it possible to distinguish and bucket subpopulations of cells based on attachment signals prior to challenging with a particular stimulus. Alternatively, the size or morphology of cells defined by their attachment PWV signal could be used to establish criteria for distinguishing cell types. Finally, a biologic profiling approach could be taken to identify distinct cell types that respond differentially to ligands based on PWV responses. Once a label-free phenotype can be defined for a given cell type, wavelength shifts can be quantified within those defined pixels and responses measured for a specific cell population within a heterogenous mix. For example, cardiomyocytes derived from murine ES cells and primary cultured human hepatocytes display distinct size and attachment phenotypes when measured with the imaging detection system. When these two cell types are mixed together the differential cytotoxic responses of these cells to doxorubicin can be measured independently by quantifying the response in the small/weakly attached population (cardiomyocytes) or in the large/strongly attached population (hepatocytes) (Fig. 10). The IC_{50} of each cell type to doxorubicin matches that measured for each cell when

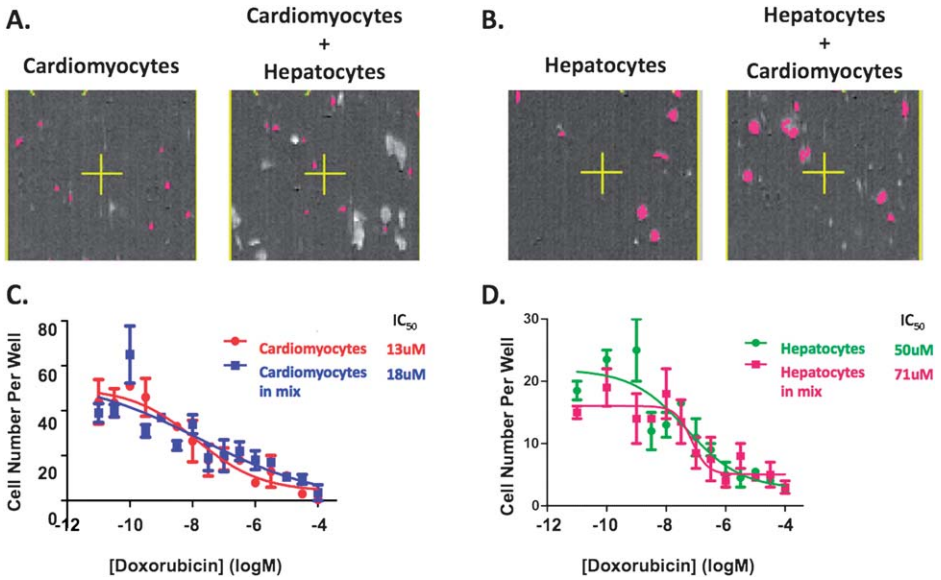

Fig. 10 Mixed hepto- and cardiotoxicity assay with a high resolution BIND Scanner. (A) Cardiomyocytes (colored pink) identified on a BIND Scanner in mix by thresholding on smaller cell size and weaker attachment signal to the sensor than hepatocytes. (B) Hepatocytes (colored pink) identified in mix by thresholding on larger cell size and stronger attachment signal to the sensor than cardiomyocytes. (C and D) The response (IC_{50}) to doxorubicin of cardiomyocytes and hepatocytes in a mixed culture matches that measured for cells seeded independently.

cultured independently. Thus, the imaging detection system can be used to measure responses of subpopulations of cells within a heterogeneous mix, as is typically the case when working with primary cultured cells.

Stem cell differentiation assays

Pluripotent stem cells are defined by their potential to differentiate into multiple cell types depending on intrinsic and extrinsic factors. For example, mesenchymal stem cells (MSCs) are multipotent cells capable of self-renewal that can differentiate into several cell types, including osteoblasts, chondrocytes and adipocytes. In response to acute tissue trauma, MSCs are thought to sense gradients of and migrate towards chemokines released from the injury site where they can repopulate healthy tissue-specific cells. Thus, MSCs have demonstrated significant clinical potential and yet high-throughput approaches for screening compounds that affect MSC differentiation are limited. Given the potential of monitoring individual cellular responses with high resolution measurements on PC biosensors, there is a great promise for establishing label-free stem cell differentiation assays with this technology.

Using the imaging detection system, high throughput assays have recently been developed for monitoring MSC differentiation into bone-producing osteoblasts (Fig. 11). MSCs can be readily propagated on extracellular matrix-coated, 384-well PC biosensors and, following propagation with a differentiation cocktail, display an osteoblast-like phenotype based on several observations. First, biosensor wells in which MSCs have been differentiated initially stain positive for collagen deposition followed by mineralization, consistent with normal bone formation. Second, scanning electron microscopy indicates the secretion and deposition of material clusters that obscure the sensor grating in biosensor wells of differentiated cells. Finally, energy dispersive X-ray (EDS) analysis indicates the presence of both calcium and phosphorus in the secreted material, the defining elements present in bone.

Label-free imaging measurements reveal the appearance of significant PWV shifts as differentiating osteoblasts (less than 50 cells per well) secrete collagen and mineralized deposits onto the sensor surface (Fig. 11). The PWV signal is cumulative over a full differentiation time course and results in wavelength shifts of greater than 30 nm. Interestingly, the label-free readout is more sensitive than traditional mineralization staining as the PWV shifts precede Alizarin Red staining by 3–4 days. Since the assay does not involve any endpoint measurements the biosensor plates can be read as often as desired; ΔPWV signals can be quantified and full differentiation time courses can be derived from a single well of cells. Moreover, the effects of small molecules and/or RNAi reagents that modulate the time course of MSC–osteoblast differentiation can be easily detected by monitoring changes in the ΔPWV kinetic profile. The robust signal and significant throughput capacity of the MSC–osteoblast assay provide a unique opportunity for screening small molecule or RNAi libraries to identify effectors or molecular targets involved in MSC self-renewal, differentiation, or de-differentiation. Additional studies are required to assess the utility of high resolution, label-free assays for measuring other differentiation pathways of these and other stem cell populations.

Chemotaxis assays

Cell migration in response to environmental stimuli is central to a broad range of physiological processes, including immune responses, wound healing, and stem cell homing. In some cases, excessive cell migration can contribute to disease pathologies, including inflammatory diseases and tumor metastasis. Drug discovery efforts for inhibitors of cell migration are hampered by

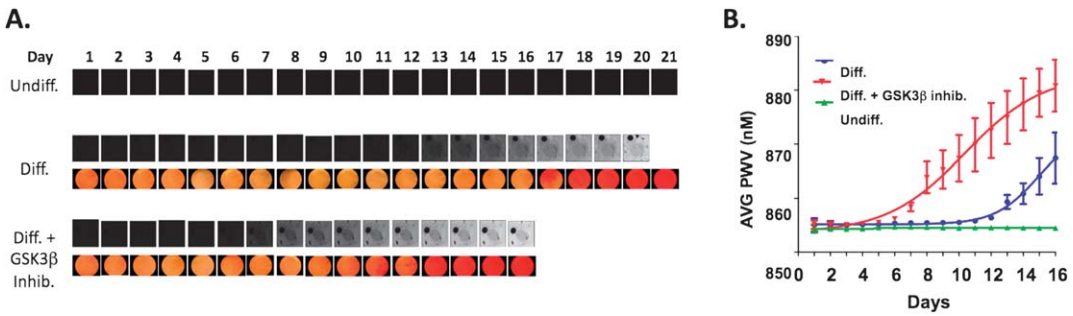

Fig. 11 High throughput mesenchymal stem cell–osteoblast differentiation assay. (A and B) Rat MSCs (Invitrogen) were seeded in 384-well biosensors at 100 cells per well and treated with osteoblast differentiation media. Daily images were acquired on the BIND Scanner and baselined to the day 0 cell attachment signal. A gradual and robust PWV shift (~25 nM) is detected as bone-like minerals are deposited on the sensor surface, as indicated by Alizarin Red staining of parallel wells. An inhibitor of glycogen synthase kinase 3 expedites MSC–osteoblast differentiation consistent with previous reports. Note: BIND images shown are from single wells imaged on multiple days; Alizarin Red requires one well per day as an endpoint staining assay.

the lack of high throughput assays to enable primary screening campaigns in functionally relevant cell types. Most existing assays for measuring chemotaxis involve the use of transwell filters that establish an upper reservoir for cell addition and a lower reservoir for chemokine presentation. As cells in the upper chamber detect chemokine that passes through the filter they initiate migration towards the point of highest chemokine concentration—in the bottom chamber. Transwell assays are currently limited in throughput to 96-well plates and typically require the use of high starting cell densities given the tendency for cells to become caught in the filter frit. Thus, high-throughput screening for effectors of cell migration remains a significant challenge in the pharmaceutical industry.

Label-free cell migration assays are being developed that eliminate the need for transwells and which take advantage of robust cell adhesion signals on PC biosensors as the assay readout (Fig. 12). One label-free chemotaxis assay in development at SRU Biosystems measures the migration of cells through an artificial basement membrane and onto the biosensor surface from which a gradient of chemokine is released. In a distinct assay configuration, cells can also be seeded directly onto the sensor, coated with a layer of collagen and then stimulated with chemokine in the bath media above. Cells detect a concentration gradient of chemokine above and are driven to weaken their interaction with the underlying biosensor, measured as a quantifiable negative PWV signal. Co-culture migration assays can also be developed to support the screening of factors that modulate transmigration of one cell type across another. In particular, considerable interest exists for *in vitro* assays that measure the migration of cells across an endothelial cell layer as occurs, for example, during stem cell homing or tumor metastasis. Label-free assays can be established wherein endothelial cells are grown directly onto the sensor and the migration of a distinct cell type across the endothelial cell layer measured as a subsequent positive PWV cell attachment signal. These optical-based, label-free chemotaxis assays can be run in 96-, 384- or 1536-well formats and, with masking algorithms developed for the imaging detection instrument allow for low cell density cell signals (of less than 500 cells per well) to be accurately quantified. Thus, label-free chemotaxis assays can be implemented in

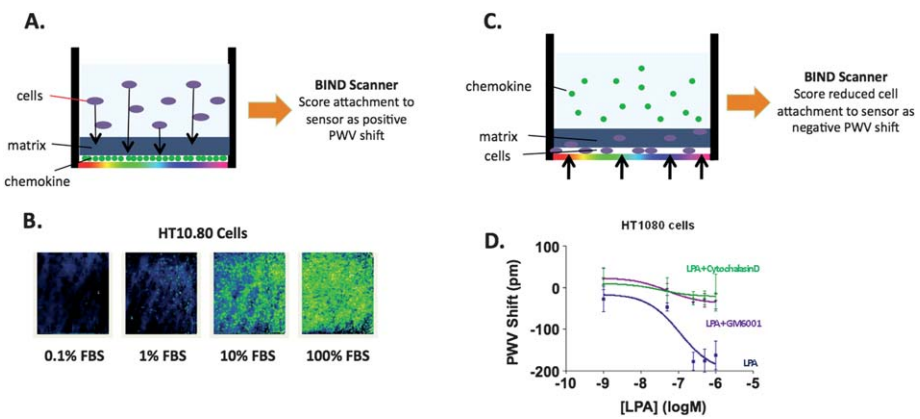

Fig. 12 A and C) Schematic diagrams illustrating the BIND cell migration assays in development. In one configuration (A), optical biosensors are coated with a chemoattractant, layered with a matrix, and then incubated with cells in solution above. (B) HT10.80 cells respond to increasing concentration of fetal bovine serum coated on the sensor by migrating toward and attaching to the biosensor. In another configuration (C), cells are seeded directly onto optical biosensors then layered with matrix and chemoattractant provided in solution above. (D) Ht10.80 cells respond to lysophosphatidic acid (LPA) presented in solution above by reducing their attachment signal on the sensor manifest as a negative PWV shift. The concentration-dependent effect is blocked by inhibitors of actin polymerization (cytochalasin D) or collagenase (GM6001).

a variety of formats with cell populations that are limited in supply and hold a great promise for enabling high throughput screens with biorelevant cell types.

Conclusion

This review has described a variety of cell-based assay applications that are enabled by the unique capabilities of PC label-free biosensors. The incorporation of biosensors into a microplate format, and the ability to place the entire detection instrument within a conventional CO_2 incubator allow long term, label-free assays that monitor modulation of cell attachment to the sensor surface. Two distinct biosensor detection instruments were described that are capable of either ensemble measurement of a large number of cells within a ~0.6 mm diameter illuminated area, or imaging detection that is capable of measuring the response of individual cells. A microscope-based label-free imaging system with 0.6 × 0.6 μm pixel resolution has been recently demonstrated that is capable of measuring the distribution of cell attachment within individual cells[35,36] that promises to push the capabilities of PC biosensors even further. Biosensors offer a quantitative and broadly applicable tool for studying cell attachment and cellular responses to a wide variety of chemical or environmental stimuli.

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