Label-Free Dynamic Mass Redistribution and Bio-Impedance Methods for Drug Discovery

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Label-free biosensors are increasingly employed in drug discovery. Cell-based biosensors provide valuable insights into the biological consequences of exposing cells and tissues to chemical agents and the underlying molecular mechanisms associated with these effects. Optical biosensors based on the detection of dynamic mass redistribution (DMR) and impedance biosensors using cellular dielectric spectroscopy (CDS) capture changes of the cytoskeleton of living cells in real time. Because signal transduction correlates with changes in cell morphology, DMR and CDS biosensors are exquisitely suited for recording integrated cell responses in an unbiased, yet pathway-specific manner without the use of labels that may interfere with cell function. Described in this unit are several experimental approaches utilizing optical label-free system capturing dynamic mass redistribution (DMR) in living cells (Epic System) and an impedance-based CDS technology (CellKey). In addition, potential pitfalls associated with these assays and alternative approaches for overcoming such technical challenges are discussed.

Keywords: label-free • biosensor • DMR • CDS • phenotypic assay

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INTRODUCTION

The early drug discovery process is moving from an isolated target approach back to more complex pharmacological settings that have greater pathophysiological relevance. Native, intact systems are now increasingly being used in the early stages of the research process to increase biological significance and decrease the rate of attrition for clinical candidates (Eder, Sedrani, & Wiesmann, 2014; Mullard, 2015; Vincent et al., 2015). Higher costs, additional need for investigator time and expertise, low throughput, lack of insight into molecular mechanisms, and increased regulatory demands argue against the exclusive use of ex vivo and in vivo models in early-phase drug discovery. While the simplicity and efficiency of artificial cell systems make them an indispensable component of a drug discovery program, assays utilizing complex biological systems are more predictive of responses in humans and therefore of greater value in bridging the gap between pre-clinical and clinical development (van der Greef & McBurney, 2005). A number of reasons have been proposed to explain why the increase in the research and development costs in the drug industry do not correlate with an increase in new drug approvals (Scannell, Blanckley, Boldon, & Warrington, 2012). “Molecular reductionism” has been identified as a key component of this problem. This experimental approach was driven by the misconception that the drug discovery process would be more predictable, efficient, and less costly if the initial stages focused solely on target-based high-throughput

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screening using well defined biologic environments, such as artificial cell systems (Scannell et al., 2012). The recent resurgence of phenotypic drug screening using primary or stem-cell-derived cells, e.g., inducible pluripotent stem cells (iPSC), suggests a reversion to utilizing analysis of more complex biological systems for identifying and selecting drug candidates (Mullard, 2015; Scannell et al., 2012; Wagner, 2016; Zheng, Thorne, & McKew, 2013). Label-free phenotypic readouts capturing whole-cell responses that may be used on a wide range of cell types, including primary cells, lend themselves to use in screening compounds *in situ* in tissue preparations that are more physiologically relevant than artificial cell systems (Kenakin, 2010, 2016; Schröder et al., 2010).

The development of novel technologies has always been a driving force in science. For instance, sophisticated molecular and genetic engineering techniques, such as CRISPR-Cas9 and tissue engineering technologies, have made it possible to access cell and tissue material possessing properties of therapeutic relevance while taking advantage of the efficiencies associated with the use of cell cultures (Fellmann, Gowen, Lin, Doudna, & Corn, 2017). The generation of genetically modified isogenic human cell lines and of animal models as a source of primary cells and tissues has accelerated significantly with CRISPR-Cas9 gene-editing technology (Schumann et al., 2015). Human cell gene knock-out, knock-down, and activation have proven to be useful techniques for generating animal models of genetic diseases and for identifying promising drug targets in CRISPR-Cas9-based screening systems (Konermann et al., 2015; Qi et al., 2013; Shalem et al., 2014). Moreover, iPSC techniques, as well as 3-D culture advances, allow for the creation of organoids with more physiologically relevant functional and genetic properties compared to artificial cell systems (Fatehullah, Tan, & Barker, 2016). In combination with recent developments in gene editing, such techniques provide new disease-relevant in vitro models that can be used in identifying patient-specific therapies (Matano et al., 2015).

Traditional in vitro pharmacology relies heavily on the ability to overexpress drug targets in artificial cell systems and on the use of native cell systems that may be limited in their target repertoire. In addition, the conventional in vitro drug screening assay utilizes labeled agents to quantify binding or signaling events in isolated cells or cellular components. In contrast, ex vivo and in vivo pharmacological approaches assess compound interactions and effects in intact organs or organisms. However, the border between in vitro and in vivo systems is beginning to blur because of the use of in vivo–like material (native and primary cells, engineered tissues, etc.) in traditional in vitro assays. Hence, new in vitro pharmacology tools could yield drug screening systems with a great predictive value and thereby facilitate the transfer of basic research findings to the clinic.

The continued development of label-free assays addresses this issue. Early applications of label-free technologies were limited to the detection of a compound-target interaction or diagnostic interaction assays, such as for studying antigen-antibody binding (Sang et al., 2016; Wu & Li, 2015). This approach was extended to cell-based applications where label-free assays were used to investigate compound-cell interactions and provide functional insights into the effects of the test agent. Currently, both optical and impedance-based biosensors are utilized for such cell-based assay systems. Optical biosensors that are based on resonant waveguide grating (RWG) detect mass redistribution in living cells, whereas electrical biosensors measure the change in cellular dielectric properties, i.e., the cellular impedance (Fang, 2014; Peters, Vaillancourt, Heroux, Valiquette, & Scott, 2010).

Corning's Epic System and the label-free module in PerkinElmer's multimode EnSpire and EnSight readers are both based on an optical RWG biosensor and allow for measurements in multi-well formats, e.g., 384-well plates (Fang, Ferrie, Fontaine, Mauro, & Balakrishnan, 2006). With the RWG biosensor, broadband light illuminates the bottom of

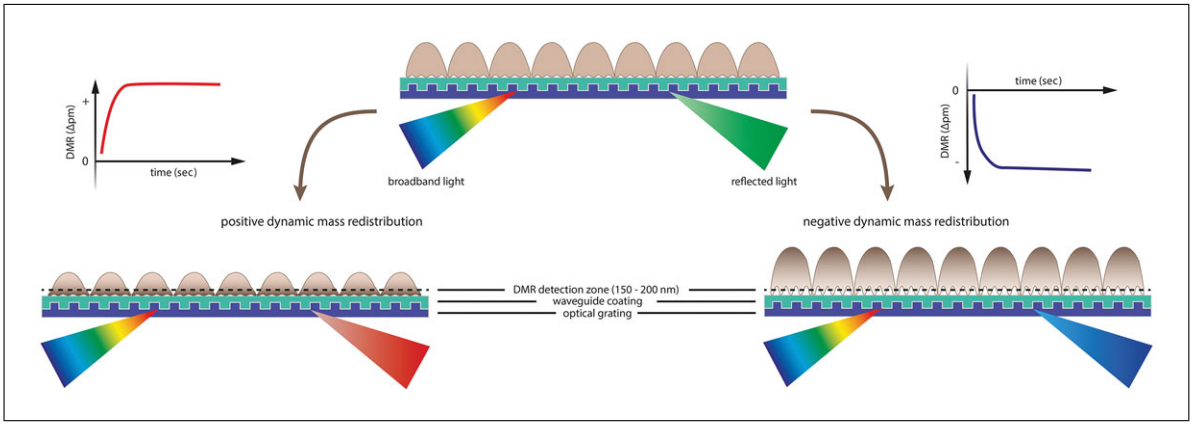

Figure 9.24.1 Measurement principle of a dynamic mass redistribution (DMR) experiment. Broadband light illuminates the bottom of the biosensor (incorporating a resonant waveguide grating) and induces an energy field in the DMR detection zone whose strength drops off exponentially away from the sensor. Under baseline conditions (upper middle panel), cells are in proximity to the biosensor and their refractive index determines the reflected wavelength that is ultimately measured. If cells undergo morphological changes, e.g., due to a signaling event, refractive indices above the sensor either increase (lower left side) or decrease (lower right side). Accordingly, the reflected wavelength is longer or shorter, respectively. The shift of the measured wavelength is plotted against the recording time and can show either positive (upper left side) or negative DMR (upper right side).

the sensor and a single wavelength is reflected which corresponds to an evanescent field that penetrates 150 to 200 nm into the cell layer. Changes in the refractive index within this field—and therefore in the cell layer—affect the reflected wavelength measured by the instrument (Fig. 9.24.1). Morphological rearrangement of the cells on the sensor, denoted as dynamic mass redistribution (DMR; Fang, Ferrie, Fontaine, & Yuen, 2005), induces either a positive or negative shift in the reflected wavelength (Δpm), depending on the direction of mass movement in relation to the sensor. Increases and decreases in mass above the biosensor induce positive and negative wavelength shifts, respectively (Fig. 9.24.1). The DMR measurement is viewed as a universal cellular readout because signal-transduction events are linked tightly to changes in cell morphology (Meyers, Craig, & Odde, 2006).

Another label-free readout for cellular applications is the CellKey System (Molecular Devices), which is based on the phenomenon of cellular dielectric spectroscopy (CDS). With this type of biosensor, the electrodes are embedded into the bottom of a 384-well cell plate, to which alternating low voltages with a broad range of frequencies (1 kHz to 110 MHz) are applied (Leung et al., 2005). Low frequencies induce extracellular current (dZiec), whereas high frequencies lead to transcellular current (dZitc). While dZiec values are more sensitive to shape changes beneath or between cells, dZitc values respond more to changes in cell confluency, because transcellular current is greatly affected by cell membrane capacitance. The different frequencies allow for the determination of cell permeability and capacitance. This makes it theoretically possible to extract additional information on changes in cell morphology depending on the applied frequency. Thus, CDS detects modifications in the impedance of a cellular matrix that are essentially guided by morphological changes (Fig. 9.24.2). Accordingly, both CDS and DMR provide readouts of signal-transduction events occurring in living cells (Peters et al., 2007; Verdonk et al., 2006).

An advantage of both the optical and impedance-based systems is the absence of labels, such as radioactive atoms or fluorescent moieties, that are used in traditional assay technologies but which can yield artifactual data by interfering with cellular functions themselves. Native or primary cells with endogenous target expression and more physiologically relevant behavior are compatible with label-free assays, and therefore more

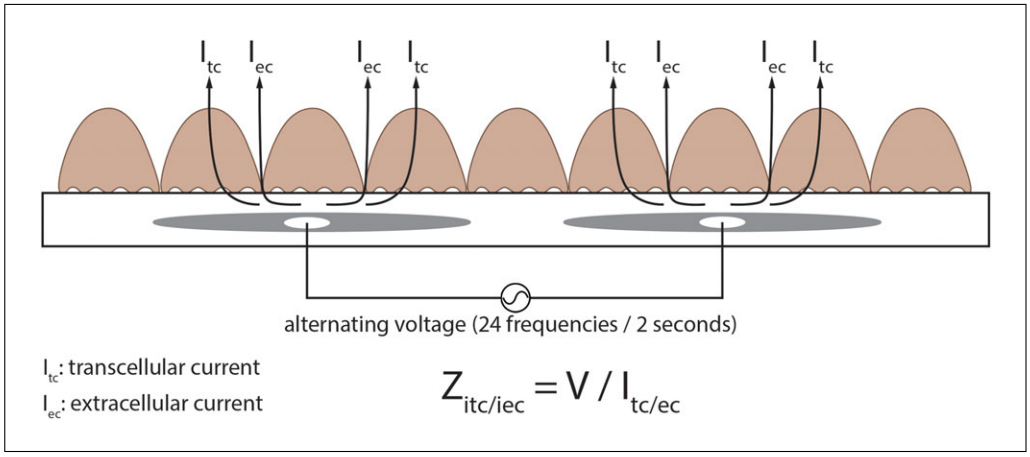

Figure 9.24.2 Measurement principle of a cellular dielectric spectroscopy (CDS) bio-impedance experiment. Alternating voltages are applied to electrodes that are incorporated into the CDS assay plate. In this system, a cellular layer on the electrical biosensor has a characteristic impedance that is defined as the ratio of the applied voltage (V) and the induced electrical current (I). Electric current flows around or between cells (I_{ec}) and through cells (I_{tc}). The resulting impedance Z_{iec} or Z_{itc} can be differentiated by the applied frequency of the voltage (see text). A change in cell shape, volume, or cell-cell contacts, e.g., as a consequence of a signaling event, entails a change in the impedance (dZ), which is measured by the instrument.

reliably provide meaningful and predictive data than assays utilizing labels for signal detection (Grundmann & Kostenis, 2015b; Schröder et al., 2010). However, the lack of labels sometimes complicates data interpretation, making it necessary to perform additional experiments to decode the label-free cellular fingerprint (see Critical Parameters and Troubleshooting). Because signal transduction translates into morphological alterations in the cellular structure, DMR and CDS are able to recognize signaling events by sensing whole-cell responses at the level of cellular shape change. As neither DMR nor CDS-based readouts are biased toward a specific signaling event, they are capable of detecting any cell activation irrespective of the investigated target as long as the signaling event translates into a cytoskeletal rearrangement (Du et al., 2009; Schröder et al., 2010; Sun et al., 2014; Tran & Fang, 2009; Zaytseva et al., 2013). Among the many possible drug targets, G protein–coupled receptors (GPCRs) have been investigated most extensively using label-free methods. Therefore, GPCRs will serve as the model targets for describing the use of DMR and CDS techniques in this unit (Arthofer et al., 2016; Blättermann et al., 2012; Grundmann et al., 2016; Hennen et al., 2013; Peters & Scott, 2009; Schröder et al., 2010; Scott & Peters, 2010; Verdonk et al., 2006). GPCR signaling is pleiotropic and may follow canonical and non-canonical pathways. However, the notion that a receptor does not always engage the entire spectrum of its assigned signaling routes but rather regulates the extent to which a pathway is activated, e.g., by the binding of distinct ligands, is referred to as functional selectivity or signaling bias. Pleiotropic signaling is not limited to GPCRs; like functional selectivity, canonical and non-canonical signaling are also characteristics of other important pharmacological targets (Kim et al., 2017; Valbuena & Lerma, 2016). Label-free technologies are universal cell sensors, as they capture cellular reactions regardless of the trigger or pathway. These detectable events include phenotypic behaviors such as shape change, migration, proliferation, adherence, or cytotoxicity. However, because signaling from one target might be detected more robustly by one label-free method than signaling from another target, the suitability of individual label-free technologies must be determined empirically in every case.

The components of compound-evoked cellular signaling events are mirrored in their DMR or CDS signature. These components serve as starting points for the exploration

and deconvolution of integrated cell responses with the help of pharmacological tools (e.g., pathway-specific inhibitors) or biological engineering (e.g., mutational or genetic ablation of key signaling proteins), or in combination with more traditional techniques (e.g., second messenger or reporter assays).

Conceptually, the unbiased nature of the label-free measurement technology allows for the identification of unknown compound properties on subordinate pathways as on-target effects, as well as those that are unrelated to an interaction with the intended target (off-target effects). The information-rich fingerprints of DMR and CDS readouts, in combination with the compatibility of label-free methods with a variety of cell systems, provide insights into cell type-dependent pharmacological phenotypes and enable sophisticated compound profiling throughout the drug discovery process.

The DMR and CDS assays can be established as real-time or conventional endpoint assays. They are easy to develop and master because no labels or specific messengers need to be validated and quantified (Grundmann & Kostenis, 2015b; Schröder et al., 2011). This makes them useful as efficient, high-throughput screening assays, as well as a means for detailed pharmacological profiling. Label-free technologies can be used in drug discovery for target validation, for fragment-based screening, for hit identification and validation, for hit-to-lead and lead optimization, for secondary screening, or as orthogonal assays for compound evaluation. Moreover, DMR and CDS can be employed to detect off-target effects, allow for compound and target validation in a native tissue environment, and monitor changes in the profile of a chemical class or individual compound following modifications in the chemical structure.

Label-free assays such as DMR and CDS assays are occasionally referred to as "black box assays" because of the uncertainties associated with data interpretation. Indeed, DMR and CDS provide less defined readouts than label assays, which is why the label-free approach should not be employed in isolation. The results generated by such assays are most interpretable when considered together with data obtained using more conventional assays. Decoding of the cellular signaling fingerprints generated by label-free assays can serve as a valuable information source for signal-transduction research. Historically, most first-in-class and best-in-class drugs were originally identified using "black box assays" wherein the underlying cellular mechanism of action was unknown (Scannell et al., 2012). Label-free black box assays that lend themselves to use on intact human cells revive the use of phenotypic screening for the discovery of effective and safe drug candidates (Kenakin, 2009).

Detailed below are several protocols involving the use of DMR and CDS assays. Basic Protocol 1 describes a simple means for studying the response of adherent cells using the DMR assay, while Alternate Protocol 1 is a variation on this procedure to measure activation of cells in suspension. Alternate Protocol 2 describes a basic antagonist characterization using the DMR assay, whereas Basic Protocol 2 contains advice on how to organize and conduct a simple CDS experiment to investigate cell response using the same cell system employed in Basic Protocol 1.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

BASIC PROTOCOL 1

STANDARD DYNAMIC MASS REDISTRIBUTION ASSAY (DMR) ASSAY

Provided below is a basic DMR experimental protocol that can be employed for virtually any cell-based, label-free investigation and that can serve as the starting point for more sophisticated applications. Described is the preparation of cultured cells, their seeding onto the biosensor, the DMR experiment itself, and data analysis. The system used for this example is HEK293 cells expressing the free fatty acid receptor FFA2.

Materials

HEK293 cells expressing the FFA2 receptor (FFA2-HEK; contact Evi Kostenis at *kostenis@uni-bonn.de* for cell line requests) growing in T-75 (75-cm^2) flasks
Complete growth medium (see recipe)
Phosphate-buffered saline (PBS; see recipe)
0.05% (w/v) trypsin/0.02% (w/v) EDTA (e.g., Gibco, Thermo Fisher Scientific)
Assay buffer (see recipe)
1 M propionic acid stock solution, aqueous (e.g., Sigma-Aldrich)
Controls (see annotation to step 14, below)

384-well, fibronectin-coated biosensor plate (e.g., Corning Cell Assay Plate 5042)
Multichannel and/or repeat pipettors
Multichannel manifold attached to cell culture vacuum pump (e.g., from Socorex, see Fig. 9.24.3; alternatively, use a liquid handling station that can be used for washing protocols, e.g., PerkinElmer JANUS automated workstation)
Centrifuge
DMR reader (e.g., Corning Epic BT reader or PerkinElmer EnSight)
384-well storage plates (e.g., Corning Costar 3657)

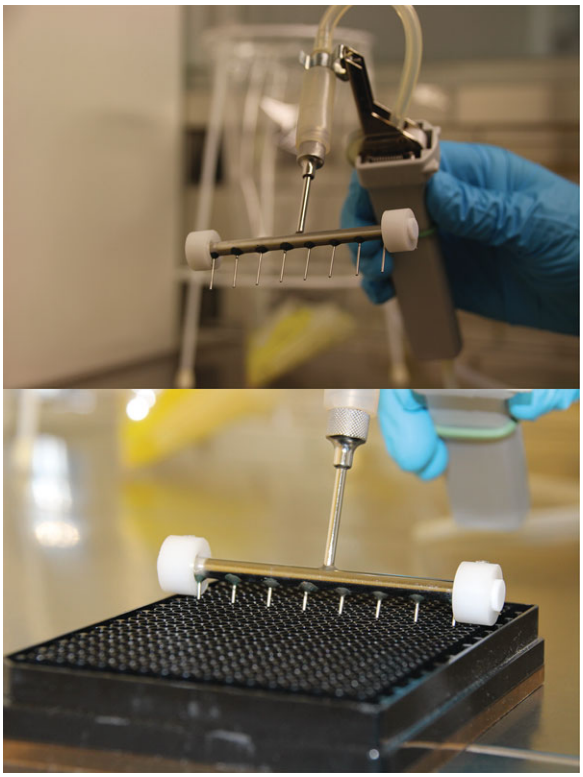

Figure 9.24.3 Suction device. 8-channel manifold attached to cell culture vacuum pump (upper panel) and process of liquid aspiration from DMR biosensor plate (lower panel).

Liquid-handling station capable of transferring all 384-well formats at the same time (e.g., CyBi-SELMA, Analytik Jena)

Incubator for label-free readers without an on-board temperature control unit (e.g., from Memmert)

PC workstation for processing label-free data running e.g., EpicImager (Corning) or Kaleido Data Acquisition and Analysis Software (PerkinElmer), Microsoft Excel, and GraphPad Prism

Additional reagents and equipment for cell culture including trypsinization and cell counting (Phelan & May, 2016)

Prepare cells

Protocols for basic cell culture techniques can be found in Phelan & May (2015).

1. Aspirate medium from cultured FFA2-HEK293 cells growing in a T-75 flask.
2. Carefully rinse the cells with 5 ml warm PBS solution and then aspirate.
3. Add 1 ml trypsin/EDTA solution and incubate for 2 min at 37°C.

   Incubation time for trypsinization varies among cell lines. For those cells that must not be treated with trypsin, use EGTA or EDTA instead (see Troubleshooting).

4. Gently swirl the flask to detach cells.

   Do not bump the flask, as this may adversely affect cellular integrity, subsequent growth, and/or cell attachment. Natural morphology is a critical determinant of cell shape detected by label-free biosensor assays.

5. Quickly add 9 ml complete growth medium to stop the reaction, and resuspend the cells by carefully pipetting them up and down.

   Avoid air bubbles and harsh handling, as this will possibly damage cells and reduce their attachment to the biosensor.

6. Count the number of cells in an aliquot of the suspension and adjust the volume with complete growth medium to achieve a dilution of 500,000 cells/ml (i.e., 15,000 cells per 30 µl).

   Measuring in triplicate has proved to be adequate. However, depending on the data variability, and to increase statistical power, it may be necessary to increase the number of measurement replicates. In all cases, measurements must be in duplicate at least.

Prepare biosensor plate

7. Seed 30 µl of the cell suspension into a warm 384-well biosensor plate using a multichannel pipettor.

   When seeding by hand, position the multichannel pipettor at a 45° angle or steeper on each well and inject the suspension at medium to high velocity to ensure proper settlement. Automated cell seeding may also be performed using the JANUS automated workstation or the FlexDrop Precision Reagent Dispenser (PerkinElmer).

8. Centrifuge the cell suspension samples 10 sec at 130 × *g*, room temperature, and allow the cells to attach overnight at 37°C in a 5% CO_2 incubator.

   Seed the cells in a timely manner and spin them down quickly to ensure consistency of cell density on the biosensor. Seeding a whole 384–well plate should be completed within ~10 min.

9. On the following day, aspirate 20 µl of medium from each well using a manifold adjusted to leave 10 µl volume in each well (see Fig. 9.24.3).

10. Wash the cells at least twice with 30 µl of prewarmed assay buffer. Using the manifold, aspirate the supernatant between washing steps.

    While increasing the number of washing steps possibly results in less well-to-well variability, cell detachment may occur if washing is too extensive. Generally, two washing steps are sufficient for obtaining good results.

11. Spin down cell samples for 10 sec at 130 × *g*, room temperature, after the final washing step and add 10 µl assay buffer to each well.

12. Centrifuge again as in step 11 and incubate the plate at 37°C for at least 1 hr to equilibrate, preferably in the prewarmed DMR reader.

    As the necessary equilibration time can vary greatly from cell line to cell line, the precise time must be established empirically for each prior to initiating the experiment. To this end, record basal dynamic mass redistribution for at least 1 hr after washing and observe when the DMR signal remains unchanged, i.e., no net change in reflected wavelength (baseline).

Prepare test compound(s)

13. Prepare a dilution series of the agonist (propionic acid) in assay buffer.

    Because during the DMR experiment 10 µl from the storage plate is transferred onto the biosensor plate, which already contains 20 µl, the compound solution must be three times greater than the final desired concentration.

    Make certain to cover the needed range for an entire concentration-response curve using full logarithmic steps to capture a possible multiphasic curve. Use half-logarithmic (or less) steps around the agonist EC_{50} concentration to more precisely characterize the curve.

14. Include positive and negative controls, such as activators of a generic cellular target and assay buffer, respectively.

    Suitable positive controls for HEK293 cells are ATP, carbachol, or isoproterenol, which act at GPCRs, or EGF and insulin for GPCR-independent receptor stimuli. The direct adenylyl cyclase activator forskolin is useful as a positive control that acts downstream of membrane receptors and G proteins. Triplicates should be placed at different plate positions to equalize any possible effects of position on the plate, e.g., edge effects. This especially applies to negative controls (assay buffer) because all DMR events will be corrected by the negative control response.

15. Transfer 30 µl of compound solution into the appropriate wells of the storage plate using a repeating pipettor and equilibrate at 37°C, preferably in a liquid-handling robot such as the CyBi-SELMA that has been placed in a pre-warmed incubator.

Perform measurements

16. Once a stable assay plate DMR signal is achieved, i.e., there is no net change in the reflected wavelength, begin the measurement by recording a baseline (pre-addition run) for 3 to 5 min.

    Depending of the specific requirements, use an appropriate temporal resolution. In this example, a resolution of 1 data point per 15 sec is sufficient.

17. Pause the measurement and transfer 10 µl of compound solution from the storage plate into the assay plate using the liquid-handling station.

    Use an addition speed of 5 µl/sec without mixing after addition. Make certain not to touch the cell layer while adding compound, to avoid disturbing cellular integrity. Use a blowout volume of 5 µl to ensure complete compound transfer.

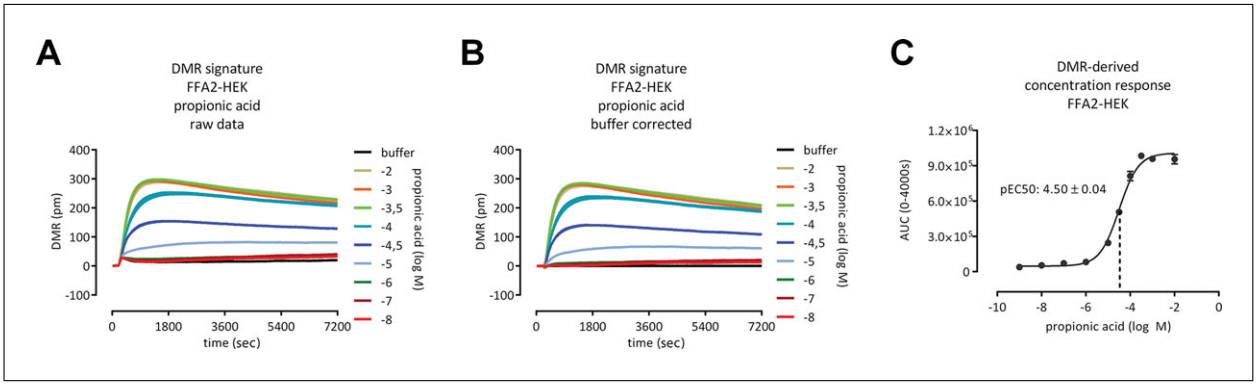

Figure 9.24.4 Agonist characterization in the DMR assay. (**A**) Raw DMR signatures of HEK293 cells expressing the FFA2 receptor stimulated with increasing concentrations of the endogenous agonist propionic acid (C3). Cells react with a dose-dependent positive DMR to agonist treatment. Note that the buffer control also induces a nonspecific and small (picometer) shift. (**B**) Real-time DMR signatures after buffer correction of data from (A). Buffer values were subtracted from all raw DMR signatures. (**C**) Area under the curve (AUC) values taken from 0 to 4000 sec of the DMR signatures from (B) plotted against the agonist concentration result in a concentration-response curve of propionic acid at the FFA2 receptor and allow the calculation of agonist parameter such as potency and efficacy.

18. Resume measurement immediately after compound addition and record the DMR for at least 1 hr (post-addition run).

    While measurement times are not fixed, it is generally advisable to observe DMR for more than 1 hr. Depending on the aim of the study, it may be best to use longer or shorter time periods.

19. Stop the measurement and import the raw data into the analyzer software.

Compute results

20. Name, save and export the data into an accessible data format, e.g., MS Excel.

21. Import the data into GraphPad Prism software for data analysis.

22. Setup an *x-y* table containing the appropriate number of replicates (triplicates in this example). Insert the timeline in seconds as *x* data and the DMR values as the *y* data in the correct sub-columns.

23. Either leave data uncorrected to view cell response to buffer addition (Fig. 9.24.4A) or subtract baseline values from compound data to generate buffer-corrected results (Fig. 9.24.3B).

24. Generate a concentration-response curve plot of either the maximum DMR value from within an appropriate time frame or the area under the curve (AUC) against the logarithmic agonist concentrations (Fig. 9.24.4C).

    Depending on the response kinetics and the parameter being examined, use either a short (fast cell activation) or long timeframe (for a delayed or sustained cell activation; Grundmann et al., 2016).

25. Compute the EC_{50} value (potency) and maximum response (efficacy) from a non-linear regression curve fit of the plotted data (Fig. 9.24.4C).

DMR ASSAY WITH CELL SUSPENSIONS

ALTERNATE PROTOCOL 1

The suspension mode is an alternative approach if the standard adherent DMR assay protocol yields unsatisfactory results. Sometimes the cell system is unsuitable for the standard (adherent) DMR assay protocol, possibly because the cells are unable to grow in adherent culture or fail to reconstitute in uniform monolayers. This assay mode is typically used to study cells that grow under suspension conditions, such as blood cells.

However, adherent cells may be investigated with this approach as well, especially if they do not reach confluency under standard protocol conditions or tend to grow in aggregates that prevent the build-up of a cellular monolayer covering most of the biosensor surface. Conducting DMR assays in suspension mode can overcome such problems. Despite the lack of extensive contact area between cells and the biosensor surface, the DMR of suspension cells that are in close proximity to the biosensor (~150 to 200 nm) can be captured. For this reason, this protocol requires a thorough centrifugation of the assay plate containing the suspended cells. Allow cells to settle during the equilibration time.

For materials, see Basic Protocol 1.

1. Perform steps 1 to 5 of Basic Protocol 1.
2. Count the number of cells in a measured aliquot of the suspension (Phelan & May, 2015) and centrifuge the desired number of cells for 4 min at 130 × *g*, room temperature.

   As a rough guide, use approximately three times the number of cells compared to the standard DMR protocol in the suspension mode. It has been found that 50,000 HEK293 cells per well generally yield good results. However, the precise cell number depends on cell type and size and must be determined for each case.
3. Aspirate the supernatant and resuspend the cells in assay buffer (HBSS containing 20 mM HEPES).
4. Seed 20 µl of cell suspension onto the assay plate and centrifuge for at least 30 sec at 130 × *g*, room temperature.
5. Equilibrate that samples at 37°C in the DMR reader until a stable baseline is attained.

   Equilibration time may vary greatly among adherent and suspension cells. Avoid excessive agitation, as cells must be allowed to settle on the biosensor surface.
6. Proceed with steps 13 to 25 of the standard DMR protocol (Basic Protocol 1).

ALTERNATE PROTOCOL 2

ANTAGONIST-MODE DMR ASSAY

Provided below are the steps taken to analyze the effects of an antagonist. This procedure is based on the standard DMR protocol (Basic Protocol 1) detailed above.

Additional Materials *(also see Basic Protocol 1)*

At least two ligands (antagonist, e.g. CATPB or GLPG0974, and an established agonist, e.g., propionic acid, acetic acid, 4-CMTB)
Two 384-well storage plates (e.g., Corning Costar 3657)

Prepare cells and compounds

1. Perform steps 1 to 12 of Basic Protocol 1.
2. Prepare a series of dilutions of the antagonist to be tested in assay buffer at 4× final concentration.

   Also include buffer without antagonist as an infinite dilution step.
3. Transfer 30 µl of each dilution of antagonist solution in triplicate to storage plate 1 and incubate at 37°C.
4. Prepare a single 4× concentration of agonist, at around the EC_{80} concentration (i.e., the concentration that evokes 80% of the maximal response), along with standard control stimuli (see Basic Protocol 1).

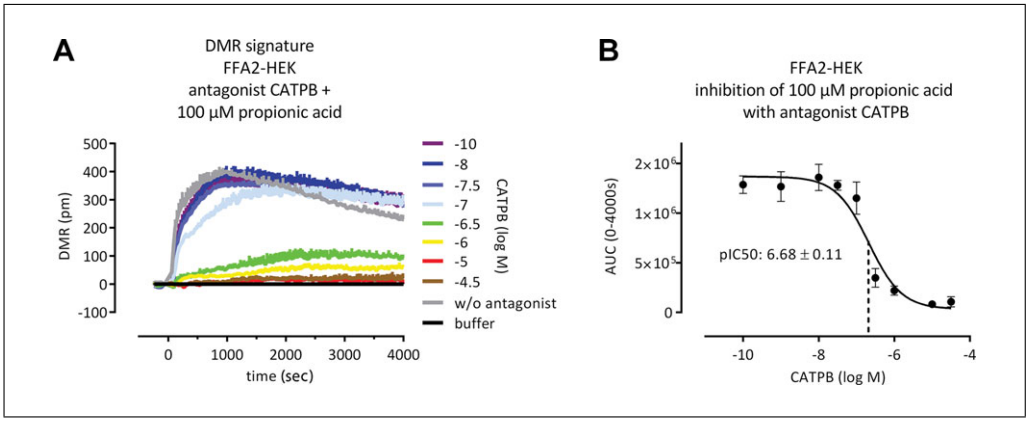

Figure 9.24.5 Basic antagonist characterization by the DMR assay. (**A**) Agonist (C3)–induced DMR signatures of HEK293 cells expressing the FFA2 receptor and pretreated (30 min) with increasing concentrations of the FFA2-selective antagonist CATPB. Note that only the cell response to the second addition (agonist) is depicted. (**B**) Inhibition curve of CATPB based on area under the curve (AUC) values from 0 to 4,000 sec from the DMR data in (A).

5. Transfer the agonist preparation in triplicate to storage plate 2 at 30 µl/well and incubate at 37°C.

Perform measurements

6. Set up the DMR reader with the 384-well assay plate. Once a stable DMR signal with the assay plate has been attained, begin the measurement by recording a baseline (pre-addition run) for 3 to 5 min.

   Use a temporal resolution that meets the aim of the study. In this example, a resolution of 1 data point per 15 sec is sufficient for accumulating the desired data.

7. Pause the measurement and add 10 µl of the contents of each well of storage plate 1 to the corresponding well of the assay plate (first addition).

8. Return to the DMR reader and read the plate for at least 30 min to ensure equilibration between the antagonist and target site.

   The incubation time must be adjusted depending on the kinetics of compound-induced cell response. An intrinsic agonist activity of the inhibitor, e.g., inverse or partial agonism, can be monitored in this step. Wait until a stable (no net change) DMR response is attained.

9. Pause the DMR measurement and add 10 µl of the contents of each well of storage plate 2 to the corresponding well of the assay plate (second addition, Fig. 9.24.5A).

10. Return immediately to the DMR reader and resume monitoring for the predetermined period of time.

11. Follow steps 19 to 23 of the standard DMR protocol (Basic Protocol 1).

Perform calculations

12. Identify and indicate first and second additions in the analysis software.

13. Calculate the concentration-response relationship by plotting either maximum DMR values or area under the curve (AUC) from the second addition against the log of the antagonist concentrations (Fig. 9.24.5B).

14. Perform a nonlinear regression curve fit and compute the IC_{50} value (concentration of the antagonist needed to inhibit half of the agonist response) and minimal response values for the antagonist (e.g., residual agonist activity in the presence of a saturating concentration of the antagonist) (Fig. 9.24.5B).

BASIC PROTOCOL 2

STANDARD BIO-IMPEDANCE (CDS) ASSAY PROTOCOL

Described in this section is a procedure for evaluating agonist characteristics using a CellKey system bio-impedance (CDS) assay. An impedance-based analysis of propionic acid, an endogenous FFA2 agonist, provides data for a direct comparison between the CDS and DMR results.

Materials

0.1 mg/ml poly-D-lysine (PDL)
Phosphate-buffered saline (PBS; see recipe)
HEK293 cells expressing the FFA2 receptor (FFA2-HEK; see Grundmann 2016) growing in T-75 (75-cm^2) flasks
0.05% (w/v) trypsin/0.02% (w/v) EDTA (e.g., Gibco, Thermo Fisher Scientific)
Complete growth medium (see recipe)
Assay buffer (see recipe)
1 M propionic acid stock solution, aqueous (e.g., Sigma-Aldrich)
Controls (see annotation to step 15, below)

384-well cellular dielectric spectroscopy biosensor plate (e.g., CellKey Assay Plate, Molecular Devices)
Multichannel and/or repeat pipettors
Multichannel manifold attached to cell culture vacuum pump (e.g., from Socorex, see Fig. 9.24.3; alternatively, use a liquid handling station that can be used for washing protocols, e.g., PerkinElmer JANUS automated workstation)
Centrifuge
384-well storage plate (e.g., Corning Costar 3657)
Bio-impedance CDS reader (e.g., Molecular Devices CellKey System)
PC workstation to process label-free data (e.g., Molecular Devices CellKey 384 Software, Microsoft Excel, GraphPad Prism)

Additional reagents and equipment for cell culture including trypsinization and cell counting (Phelan & May, 2016)

Prepare cells

Protocols for basic cell culture techniques can be found in Phelan & May (2015).

1. For HEK293 cells, coat the surface of the CellKey assay plate to ensure cell attachment and growth. Add 10 µl of aqueous 0.1 mg/ml poly-D-lysine (PDL) to each well, centrifuge 30 sec at 200 × *g*, room temperature, and incubate for at least 30 min at 37°C.

   Incubation overnight is also possible.

2. Aspirate PDL and wash twice with 30 µl PBS.

3. Aspirate medium from cultured FFA2-HEK293 cells contained in a T-75 flask.

4. Carefully rinse the cells with 5 ml warm PBS and aspirate.

5. Add 1 ml trypsin/EDTA solution and incubate for 2 min at 37°C.

   Incubation time for trypsinization varies among cell lines. For those cells that must not be treated with trypsin use EGTA or EDTA instead (see Troubleshooting).

6. Gently swirl the flask to detach cells.

   Do not bump the flask, as this may adversely affect cellular integrity, subsequent growth, and/or cell attachment. Natural morphology is a critical determinant of cell shape that is detected by label-free biosensor assays.

7. Quickly add 9 ml complete growth medium and resuspend the cells by carefully pipetting up and down.

   Avoid air bubbles and harsh handling, as this may damage cells and diminish cell attachment to the biosensor.

8. Count the number of cells in an aliquot of the suspension and adjust the concentration to 400,000 cells/ml (i.e., 12,000 cells per 30 µl) with complete growth medium.

   Triplicate measurements have proven to be appropriate.

Prepare biosensor plate

9. Seed 30 µl of cell suspension per well of a warm 384-well biosensor plate using a multichannel pipettor.

   When seeding by hand, position the multichannel pipettor at a 45° angle or steeper on each well and inject at medium to high velocity to ensure proper settling of the cell suspension. Automated cell seeding can also be performed using the JANUS automated workstation or the FlexDrop Precision Reagent Dispenser (PerkinElmer).

10. Centrifuge the cell suspension samples 30 sec at 200 × *g*, room temperature, and allow the cells to attach overnight at 37°C in a 5% CO_2 incubator.

    To avoid medium- or air-bubble gaps in the cellular monolayer, add another 10 µl of medium and centrifuge again after the plate is incubated and cells begin to attach.

11. On the following day, aspirate 25 µl of medium from each well using a manifold adjusted to leave a 5-µl volume in each well (Fig. 9.24.3).

12. Wash the cells at least three times with 30 µl assay buffer pre-warmed to 37°C. Aspirate the supernatant between wash steps using the manifold (Fig. 9.24.3).

    Thorough washing enhances assay precision and the measurement window.

13. After the last washing step, add 25 µl of the assay buffer and centrifuge 30 sec at 200 × *g*, room temperature.

14. Incubate the assay plate at 37°C.

Prepare test compounds

15. Prepare the agonist dilution series in full and/or half-log steps in assay buffer.

    Include positive, negative, and buffer controls, such as agonists known to stimulate endogenously expressed receptors (P2Y), compounds known to act through receptor-independent targets (forskolin), and a vehicle (assay buffer only) control.

16. Transfer 30 µl of agonist solution into the appropriate wells of the storage plate.

Perform measurements

17. After initialization and reaching the impedance measurement temperature (ZMS) of the CellKey Instrument, load both the assay and storage plate along with a 384-tips rack.

18. Enter and save a one-addition protocol using the CellKey 384 software.

    The following settings are recommended:

    Initial cell plate well volume: 30.0 µl
    Compound addition volume: 10.0 µl
    Dispense offset: 0.0 mm
    Dispense height: 2.4 mm
    Dispense rate: 3.5 µl

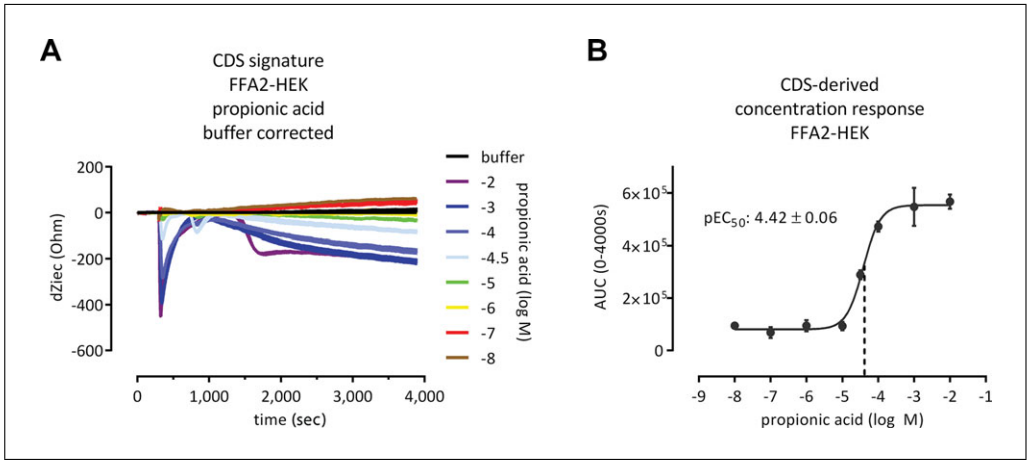

Figure 9.24.6 Agonist characterization in the CDS bio-impedance assay. (**A**) Real-time impedance recording of HEK293 cells expressing the FFA2 receptor stimulated with increasing concentrations of the endogenous agonist propionic acid (C3). Note that only changes in impedance based on extracellular current (dZiec) are depicted. (**B**) Area under the curve (AUC) values from the entire measurement duration were plotted against logarithmic C3 concentrations to obtain a concentration-response curve.

Mix cycles: 0
Equilibration duration: 00:00 (mm:ss)
Baseline measurement duration: 05:00 (mm:ss)
Compound addition time: 05:00 (mm:ss)
Post-addition measurement duration: 01:00:00 (hh:mm:ss)
Experimental Temperature Set Point: 37°C
Update interval: 4 sec.

19. Run the protocol.

    The compound addition will now be performed by the CellKey instrument.

20. Export the results as 'Kinetic Data – dZiec' (impedance based on extracellular current).

    It is possible to open the exported data in MS Excel and save it as an `.xlsx` *file for further processing.*

21. Transfer relevant data (timeline and impedance values) into an analysis program such as GraphPad Prism in an *x-y* table format.

22. Identify the buffer values and perform a buffer correction for each data point.

23. Plot the kinetic data for inspection (Fig. 9.24.6A), select the most appropriate quantification method (max values, area under curve, steepness), and process the results accordingly (e.g., cumulative signaling by analyzing AUC response; Fig. 9.24.6B).

REAGENTS AND SOLUTIONS

Use deionized, distilled water for all recipes.

Assay buffer

140 mg/liter $CaCl_2$
100 mg/liter $MgCl_2$
100 mg/liter $MgSO_4$
400 mg/liter KCl

continued

60 mg/liter KH_2PO_4
350 mg/liter $NaHCO_3$
8 g/liter NaCl
48 mg/liter Na_2HPO_4
1 g/liter D-glucose
4.7 g/liter HEPES Store up to 2 months at room temperature

Composition: HBSS plus 20 mM HEPES.

Complete growth medium

Dulbecco´s Modified Eagles Medium (DMEM, e.g., Invitrogen, Thermo Fisher Scientific) containing:
10% (v/v) fetal bovine serum (e.g., PAN Biotech)
100 U/ml penicillin and 100 mg/ml streptomycin (added from 100× pen/strep stock, e.g., Gibco, ThermoFisher)
Store up to 3 months at 4ºC

Phosphate-buffered saline (PBS)

144 mg/liter KH_2PO_4
795 mg/liter $Na_2HPO_4 \cdot 7H_2O$
9 g/liter NaCl
Store up to 6 months at room temperature

COMMENTARY

Background Information

Optical biosensors employed for studying cellular processes in a label-free environment are for the most part designed to detect a resonance phenomenon that occurs at the biosensor surface. In surface plasmon resonance (SPR) systems (e.g., Biacore), changes in the refraction index on the sensor surface alter the properties of the reflected wavelength (angle/intensity). These optical biosensors were therefore first used as mass detectors. Subsequently, initial applications were focused on the detection and characterization of (bio)molecular binding events (Schuck, 1997). Introduction of waveguide gratings into the biosensor opened the field for cell-based applications (Fang et al., 2006). These systems rely on the resonance of the coupled waveguide and the reflected wavelength (Corning Epic System, PerkinElmer Enspire/EnSight). In contrast to SPR, changes in the refractive index in the evanescent field lead to changes in the reflected wavelength instead of changes in angle/intensity (Fig. 9.24.1). The implementation of resonant waveguide gratings allows for the real-time monitoring of dynamic mass redistribution (DMR) in living cells. DMR-based instruments also serve as platforms for the study of (cell-free) biomolecular interaction. Indeed, this application preceded cell-based approaches (Wu et al., 2006).

Another optical biosensor is based on biolayer interferometry (BLI), which measures the interference pattern of white light that is reflected from two surfaces. Interaction of molecules or particles with the surface alters the interference pattern (bound versus unbound states) which can be detected by BLI. Instruments based on microfluidic (bScreen, Berthold Technologies) or static design (ForteBio Octet, Pall) are available (Concepcion et al., 2009; Kahsai et al., 2016; Verzijl, Riedl, Parren, & Gerritsen, 2017; *http://www.berthold.com*).

Acoustic wave–based biosensors, such as surface acoustic wave (SAW) biosensors and the quartz crystal microbalance (QCM), are other label-free techniques that resemble SPR in application but differ in the fundamental measurement principle (acoustic versus optical) (Fogel, Limson, & Seshia, 2016).

Another label-free biosensor currently being employed in a cell-based application is based on dielectric spectroscopy (ECIS system, Applied Biophysics; xCELLigance, Acea Biosciences; and CellKey, Molecular Devices; Grundmann et al., 2016; Peters et al., 2007; Verdonk et al., 2006). Changes in the impedance of a cellular monolayer are measured as an holistic response after cell stimulation (Fig. 9.24.2). Although DMR and CDS assays are more commonly used in combination with living cells for

drug research, other label-free technologies—primarily used as biomolecular interaction assays—were adapted for cell-based applications as well. For instance, surface plasmon resonance (SPR) and acoustic wave quartz crystal microbalance (QCM) biosensors have been employed to detect whole cell activation (Chen, Obinata, & Izumi, 2010; Wang et al., 2011; Yanase et al., 2014). In addition, biolayer interferometry (BLI) was shown to capture holistic cell responses of living cells in real time mediated by dynamic mass redistribution (Verzijl et al., 2017). The ability of cells to react to a morphological change is essential for detection with all label-free techniques. Because cell shape change is the common measurement parameter of the cell-based label-free assays, it is unknown whether certain techniques are better suited to detect a particular signaling event and its source than others. DMR, for example, is excellent for studying G protein signaling whether the signaling network is activated from surface receptors or intracellularly by interaction with effector proteins (Blättermann et al., 2012; Grundmann & Kostenis, 2015a; Schrage et al., 2015; Schröder et al., 2010). However, it is also suited to detect cell responses mediated by tyrosine kinase signaling, ion channel modulation, or cell adhesion (Du et al., 2009; Sun et al., 2014; Zaytseva et al., 2013). Because DMR and CDS access the same phenotypic behavior, i.e., cell shape change, it is not surprising that CDS detects G protein signaling with comparable high accuracy and sensitivity to DMR (Grundmann et al., 2016; Hennen et al., 2013). Genuine and unprocessed signaling fingerprints derived from CDS and DMR records, however, differ in temporal resolution of FFA2 receptor-mediated cell activation (Grundmann et al., 2016). Knowledge about the coupling efficiency of distinct signaling events to an eventual cell shape change is still incomplete and further investigation is required to improve these techniques and their use. It will be interesting to learn whether label-free readouts other than RWG-based DMR and CDS-based impedance sensors can provide additional information about the biology and pharmacology of cellular processes that are not based on cytoskeletal rearrangement.

Critical Parameters and Troubleshooting

Because minute changes in the range of the evanescent field (150 to 200 nm) above the biosensor are detected by DMR-based biosensors, the density and distribution of the cells on the sensor surface are critical technical issues. A homogenous cellular monolayer is advantageous and generally yields both high measurement windows and more robust results. Conversely, low or inconsistent confluency of the cell layer on the biosensor is the most common reason for poor signal quality. However, because cell-cell contact has a substantial influence on the responsiveness of living cells, it is recommended—if not essential—to determine the correct cell density on the biosensor for each experimental setup and for every different cell type to be tested. To this end, different cell numbers should be seeded and evaluated to optimize the assay window (Aplin, Howe, Alahari, & Juliano, 1998; Grundmann & Kostenis, 2015b; Lieb et al., 2016; Schröder et al., 2011). Microscopic control of cell confluency in the well after an overnight incubation can serve as a reference. It should be between 85% and 100%. In this context, the imaging function of the EnSight (PerkinElmer) multimode reader can be set up to automatically determine cell confluency for each well separately. Diligent resuspension before seeding prevents aggregates that hinder confluency on the sensor.

Detachment in routine cell culture usually involves the use of trypsin. The proteolytic activity of trypsin, however, can impair normal cell physiology and recovery by degrading membrane proteins or other cellular constituents. In this case, removal of residual proteins by a physiological saline like PBS or HBSS followed by incubation with EDTA or EGTA should be attempted. While EDTA is a relatively nonselective chelator of divalent cations, EGTA binds Ca^{2+} ions with higher affinity than other cations. These differing characteristics are exploited, for instance, when investigating beta cell or pancreatic islet cell dissociation, as the removal of zinc ions by EDTA can negatively affect insulin synthesis, secretion, and signaling (Bosco et al., 2010). It is therefore important to be well informed regarding the biochemical and physiological properties of the cell system being investigated.

Problems pertaining to cell attachment and growth can sometimes be circumvented by experimenting with different coatings. Extracellular matrices such as collagen, fibronectin, poly-lysine, or poly-ornithine are often used as a surface coating for cellular assays. Biosensor plates can be purchased pre-coated, or coating can be applied manually at lower cost. Extracellular matrices can have a great impact on cell adherence and motility and can influence

changes in cell morphology. As cytoskeleton rearrangement is the most critical biological parameter affecting DMR and CDS impedance measurements, label-free data can differ from one surface coating to another. Therefore, an evaluation of the impact of different coating materials on cellular behavior should be performed. Slow-growing cell types that aggregate when seeded at higher densities might benefit from an extended growth time on the biosensor plate. For that, cells can be seeded and grown in the assay plate for several days until the a cellular monolayer is formed.

If problems persist with more demanding cells that make it impossible to obtain measurements from a confluent monolayer, consideration should be given to performing the assay in suspension mode. One must keep in mind that cell density has an even higher impact on the signal quality of assays in suspension mode compared to the adherent protocol. For this reason, greater attention should be paid to the evaluation of cell number.

Low assay windows can result from many factors, two of which are poor target expression and insufficient biologically active material on the biosensor. Depending on the aim of the experiment, amplification of target expression or coupling efficiency of the target to the cellular signaling machinery is an effective but artificial manipulation, and is often neither possible nor desired. Occasionally, enhancing cell responses to an external stimulus by withdrawal of growth factors (FBS) from the growth medium or replacement of the growth medium with assay buffer (starvation) produces sensitized cells with larger signal amplitude. However, the extent of withdrawal and time of starvation need be determined for the cell type under investigation (Arthofer et al., 2016; Schröder et al., 2010, 2011).

While negative DMR responses can be indicative of a biological response to signal transduction, an extensive drop in picometer shifts of untreated or buffer treated cells is generally a sign of cell detachment and a decline in cell viability. Systematic troubleshooting of general cell parameters, such as cell passage, nutrient factors (starvation time and conditions), temperature, effect of toxic compounds, or incompatibility with the surface coating, should be performed.

Changes in the index of refraction correlate to changes in the reflected wavelength and thus to the DMR response. Artifacts from compound addition can arise if the test agent is diluted in a vehicle with significantly higher or lower refractive index, e.g., DMSO. Ethanol does not have this effect. This is why solvents with different refractive indices like DMSO must be adjusted with care in the washing buffer as well as in the compound dilutions to minimize mismatches of refractive indices.

Nonspecific DMR effects caused by direct interaction between added agents and the biosensor are sometimes observed. For example, bovine serum albumin (BSA), which is often a component of test compound preparations, evokes positive DMR responses on fibronectin-coated and uncoated biosensors. These effects can be misinterpreted as a cell or target-specific response. Careful adjustment of the concentrations of BSA or similar substances with high binding capabilities in the assay buffer, washing solution, and compound dilutions is recommended to avoid artifacts resulting from such nonspecific interactions. Caution also needs to be exercised if crude biological samples are analyzed in a mass/refraction-sensitive label-free system.

Temperature is another critical determinant of the refractive index and the resonance wavelength, and therefore of the measurement windows. Maintaining a constant and homogenous temperature throughout the measurement period is critical because temperature fluctuations can cause irregular DMR shifts that resemble a cellular response to a stimulus. In particular, the addition of compound solutions with different temperatures can cause artifacts that may overlay the target-specific cell response. It is recommended that the storage plate harboring the compound solutions be allowed to equilibrate to measurement temperature. Temperature is an influential factor for overall signal strength as well as for the information content of the biosensor signal. Besides the physical impact on the measurement principle, temperature affects cell behavior. For example, receptor internalization and receptor trafficking are highly temperature sensitive. In some cases, it is advisable to perform the assay in a temperature-controlled environment such as in an external incubator (Corning Epic System, Applied BioPhysics ECIS system, and Acea Biosciences xCELLigence Systems) or with an on-board thermostatic measurement chamber (PerkinElmer EnSight, Molecular Devices CellKey).

Inasmuch as the label-free methods rely on an intact sensor (surface), it is crucial the sensor be protected from soiling and scratches. Gloves should always be worn when handling

the biosensor plates and care taken to avoid touching the bottom side of the plate.

DMR and CDS-based assays provide real-time kinetics of cell response upon stimulation with signaling-inducing compounds. Because the response is unique for each test agent, the response is considered a cellular fingerprint. The fingerprint typically comprises an initial increase or decrease in DMR and impedance, respectively, followed by either a monophasic return to a constant signal level in the simplest case or multiple phases of different directions and deflections in a more complex scenario. In comparison to more traditional endpoints, the measured response is not a simple yes or no. Depending on whether curve steepness, peak height, or area under the curve is taken as the prime measure, the response calculation will yield different results. This raises a question as to what response parameter and level represents the "real" cell response (Grundmann & Kostenis, 2015b; Schröder et al., 2011). Even though the temporal shape of the signature itself is an abundant source of information on drug mechanisms (Grundmann et al., 2016), it is possible to consider certain label-free signals as the cell response. However, it should be kept in mind that integrated assay systems mirror the complete pharmacological repertoire of a compound's ability to alter cellular behavior and serve as a direct readout for compound efficacy at the whole-cell level. The notion that certain ligands induce only a subset of all possible signaling-competent conformations upon receptor binding, denoted as functional selectivity or signaling bias, indicates that efficacy has multiple dimensions, as does a cell response (Kenakin, 2009). Integrated assay systems therefore reflect all dimensions of efficacy, including the temporal dimension, in one synoptic signature. Data analysis approaches for label-free assays based solely on area under the curve or peak height yield only intermediary and surrogate, although invaluable, measures of the compound's complete pharmacological actions.

Anticipated Results

Only minimal changes in label-free responses (DMR or impedance) should occur (stable baseline) before addition of a test agent (see short time frame before compound addition in Figs. 9.24.4A,B, 9.24.5A, and 9.24.6A). Upon compound addition, a picometer shift or impedance change should result depending on the nature of the added ligand (e.g., see increase at 280 sec on Fig. 9.24.4A,B). Agonist potency and efficacy can be derived from concentration-response curves calculated with respect to the area under the curve for a defined time frame (Figs. 9.24.4C and 9.24.6B). Both directions of response might indicate the activity of the compound, and cannot automatically be attributed to features such as agonism or antagonism (Schröder et al., 2010). Positive and negative DMR signatures, for instance, simply reflect a total net increase or decrease of mass above the biosensor. They cannot be generalized to discrete signaling pathways that are activated or deactivated by agonist or antagonists. Label-free readouts allow direct access to cell type–dependent pharmacology. That is, cell responses vary depending on the cellular background, and serve as platforms for dissecting signaling networks (Grundmann & Kostenis, 2015b; Schröder et al., 2010). It is possible, however, to determine antagonist potency by demonstrating a concentration-dependent inhibition of antagonist-induced DMR response (Fig. 9.24.5). Although cell-based DMR and impedance data are integrated readouts that reflect the ability of a compound to alter the cellular architecture, it is not possible to directly assign signatures to distinct signaling events *per se* without further signal deconvolution. The negative initial peak response to stimulation of FFA2 cells (Fig. 9.24.6A), for instance, is not mediated by activation of a different signaling pathway downstream of the receptor compared to the delayed cell response, but is rather a result of a sequential receptor activation to trigger a temporally biased cell response (Grundmann et al., 2016). Likewise, a net null response does not exclude activity, as test agent-induced positive and negative signals might cancel each other out.

Minor assay artifacts after compound addition are not a matter of concern if they do not overlay compound-target effects.

Time Considerations

Cell culture/seeding of the cells takes 30-60 min for a single assay plate depending on number of different cell lines.

Cell washing requires 15 to 30 min.

Equilibration of the assay plate will take 60 min.

Compound preparation takes approximately 30 min.

While lengths of baseline and final read(s) are variable, they typically require approximately 5 and 60 min, respectively. Time for data export and analyzing is approximately 30 min, with more sophisticated analyses requiring considerably longer.

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