Chapter 14

Label-Free Biosensor Assays in GPCR Screening

Manuel Grundmann and Evi Kostenis

Abstract

About one third of currently marketed drugs target G protein-coupled receptors (GPCRs), which form the largest group of transmembrane proteins in the human proteome. GPCRs are ubiquitously expressed throughout the human body and play a pivotal role in a vast number of physiological and pathophysiological processes. Because of their intriguing complexity, their relevance, and yet unexploited potential in the treatment of diseases, GPCRs are studied intensively by both academic and industrial research labs.

Classical biochemical and molecular biology techniques, including traditional second messenger assays, took biomedical research to the next level and represent the fascinating power of in vitro pharmacology. While extremely efficient in capturing one clearly defined cellular readout, those methods do not authentically portray the events taking place in living cells as a whole; hence the process of drug discovery runs the risk to lose sight of a wider context already in early stages. Label-free cell-based assays hold the promise to overcome these shortcomings by considering cellular processes holistically. If combined with diligent assay adjustments, dynamic mass redistribution (DMR) technology is an excellent tool to investigate GPCR signaling. In this article we aim to provide guidance for scientists seeking for information on how to set up and optimize DMR assays with the objective to establish a knowledge base on deciphering integrated cellular readouts. For this reason we focus on a basic DMR protocol for the investigation of the long-chain fatty acid FFA1 receptor as a model family A GPCR and complement it with information that allow a sophisticated approach to more specialized scientific questions with the use of this comparatively novel method.

Key words Dynamic mass redistribution, DMR, Resonant waveguide grating, RWG, G protein-coupled receptor, Optical biosensor, Label-free, FFA1, Holistic readout, Integrated cell response, Real-time assay, Ligand bias

1 Introduction

Optical label-free biosensors based on resonant waveguide grating (RWG), also known as guided-mode grated resonance, arose in the 1990s and were primarily used to detect binding of target molecules to immobilized receptors or, more generally, to determine interaction between molecular partners [1, 2]. The phenomenon underlying the technique can be described as electromagnetic resonance and explained by a simplified design of the biosensor that

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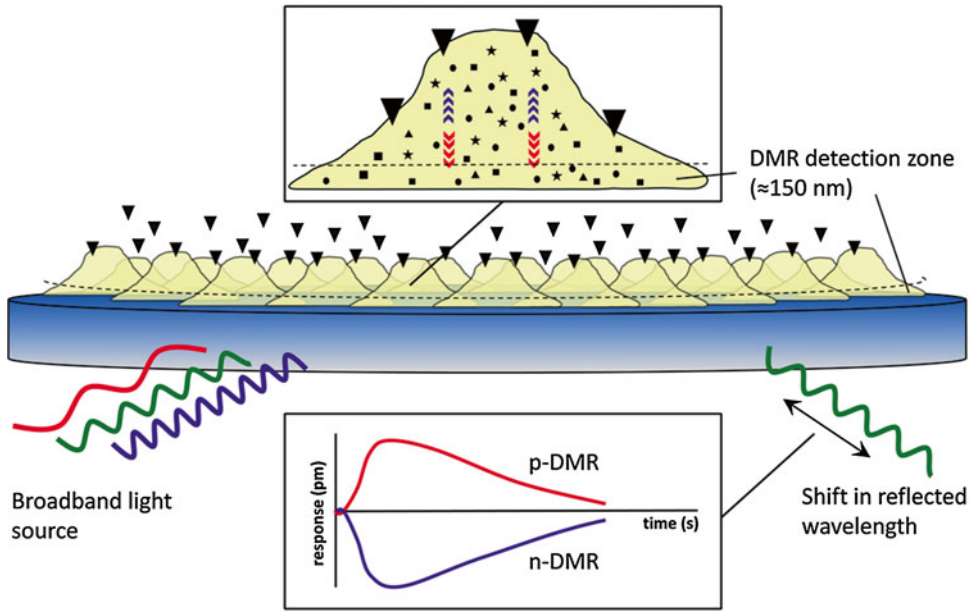

Fig. 1 The structural connection between a planar waveguide and a dielectric diffraction grating allows polarized light to couple in and out when illuminating the sensor under a defined angle. The high efficient coupling wavelength is designated resonance wavelength, whose propagation along the biosensor induces evanescent waves that extend into a zone approximately 150 nm above the sensor. Depending on the optical density of the system (biosensor and adjacent areas above the sensor), a shift of the reflected wavelength (Δpm) can be detected and recorded. In the case of a cell layer above the sensor, changes in optical density can occur by the redistribution of intracellular mass toward or away from the sensor, summarized as dynamic mass redistribution (DMR). Mass movements toward the sensor induce positive wavelength shifts (*p*-DMR), whereas movements away from the surface cause negative shifts of the reflected wavelength (*n*-DMR) at the bottom of the biosensor

contains an optical grating interlocked with a layer of special glass (Fig. 1). More recently, it was recognized that the capabilities of this technology go far beyond analyzing binding events toward a more in-depth understanding of cellular signaling [3–6].

Eukaryotic cells constantly exchange information with their environment. Transmembrane proteins such as G protein-coupled receptors (GPCRs), configured in a unique assembly of signaling pathway components, behave as molecular microprocessors that transduce signals from outside the cell into the cell's interior. For a proper communication, both internally and externally cellular systems need an orchestrated behavior which emerges as morphological transformation. This is often realized by a cytoskeleton-guided transport of molecules. The cellular architecture is controlled by a variety of effector molecules such as calmodulin or protein kinase A, activated by the classical second messenger calcium and cAMP, respectively. Hence, alpha-subunits of heterotrimeric G proteins like $G\alpha_s$, $G\alpha_q$, or $G\alpha_i$ but also $G\alpha_{12/13}$ modulate actin filament

structure within eukaryotic cells. Likewise, small GTPases such as Rho, Rac, or CDC42 and also Gβγ subunits interacting with PH domains (pleckstrin homology domains) are competent to regulate the cytoskeleton [7, 8]. In the field of GPCRs, cell-based assays capturing morphological rearrangement demonstrate the potential of the technology to aid the understanding of receptor biology and compound behavior [9].

The increasing mechanization and automation in drug discovery programs in the past with the aim to accelerate the process from target identification to lead identification raised high hopes for an advent of novel drugs. As a result of an intensified effort to understand the underlying pathophysiology of a given disease, the process of target validation has received much more attention. However, the reductionistic approach of taking a target out of a dysfunctioning context and confining it to a specific readout, often forced by genetic manipulation in an artificial biological environment, enabled researchers to run big screening campaigns testing millions of compounds with hitherto unprecedented efficiency. Despite diligent and rigorous preselection of possible drug candidates in this procedure, high dropout rates in preclinical and clinical stages cannot obscure the fact that these hopes were disappointed. There are various well-argued approaches to that problem [10, 11] and growing consensus about the necessity to investigate compound behavior in a more holistic manner, e.g., reflected in a multiple signaling pathway readout. This becomes even more important when considering the impact of cellular background and altered physiology in disease states on the compound pharmacology and receptor biology [12]. To address the demand for extensive profiling of receptor-modulating ligands, a new approach based on innovative assay designs or combinations of existing assay formats is needed.

The process of drug discovery in particular could benefit from the capabilities of the DMR technique. The label-free and holistic nature of the assay provides more pharmacologically relevant data, thus allows eliminating false-positive results from label-based assays, and improves significance in selectivity screenings. Cellular rearrangement plays a pivotal role as a common cellular phenotype in various diseases such as inflammatory disease, cancer, neurological diseases, and cardiovascular diseases (e.g., cardiac hypertrophy), since many signaling pathways converge at the level of cytoskeleton architecture [13]. Hence, the assay has the potential to detect all cellular events that translate into mass movement, such as (intra) cellular rearrangement or changes in cell morphology, as an integrated cellular response to various inputs. One major advantage of this assay format is the option to investigate test compounds in a native or primary cell setting that is closer to in vivo conditions. Especially the possibility to integrate tissues from patients, reflecting disease relevant conditions holds the promise to stimulate the

field of in vitro translational medicine and thereby addresses the issue of wasting time and money on the production of rather irrelevant data [14].

Furthermore, DMR assays can be automated for HTS purposes or other primary screens [15]. However, the main fields of application most likely are further pharmacological evaluation as an orthogonal assay platform, for example, in hit confirmation or in structure-activity relationship (SAR) studies. By combining the HTS compatibility with the unbiased perspective of dynamic mass redistribution on cell response, the DMR assay facilitates uncovering biased ligands, i.e., functionally selective ligands, more efficiently. A key advantage of this assay is the sensitivity to investigate biased signaling of GPCRs and functional selectivity of their ligands, features that are proposed to deliver more precise therapeutic benefit with reduced amount of side effects [16]. A study including a selection of molecular tool compounds extending the label-free assay allowed the dissection and characterization of all four G protein-mediated pathways and compared them with results from traditional endpoint assays [6].

An interesting possibility opens up by monitoring cellular response in real time. It is becoming increasingly evident that conceiving the spatiotemporal aspect of GPCR signaling solely as one isolated and linear event proves insufficient; in fact, recent data support the idea that signaling via GPCRs rather emerges as multiple signaling waves [17]. Research projects approaching kinetic questions could therefore benefit from this method.

Downside aspects of the technology go along with the unique features of an integrated, holistic view on cellular events. Sometimes referred to as "black box assay," the molecular mechanisms that account for the phenomenon of dynamic mass redistribution remain to be elucidated. As an integrated cell response, unperturbed DMR traces can be composed of on- and off-target effects. It is also possible that positive and negative DMR neutralize each other, and a net null signal would result that can lead to the misinterpretation as an indication of no biological effect. Without the appropriate molecular tools and the effort of different approaches like combining the label-free assay with other biological readouts, it can thus be challenging to decode DMR signatures. Also, high costs of biosensor plates might limit the widespread usability of this technology. Nevertheless, DMR is a powerful, highly versatile, and user-friendly technology platform that is applicable to a vast array of different cell types, ranging from overexpression to native cell systems, and that offers the great advantage of studying target protein behavior in the absence of any labels that may confound protein function. In this protocol, we exemplify the applicability of label-free DMR for analyzing real-time signaling of the long-chain fatty acid FFA1 receptor in different biological contexts.

2 Materials

1. Centrifuge with rotor for microplates.
2. 12-channel pipettor for washing 384-well plates.
3. 8-channel manifold to aspirate liquids from 384-well plates.
4. 75 cm^2 sterile cell culture flasks.
5. Cell line: Flp-In™ T-REx™ 293 cells, stably transfected with the human FFA1 receptor (for more detailed information regarding this cell system, please refer to the manufacturer's manual (http://www.lifetechnologies.com)).
6. Agonists: conjugated linolenic acids (CLA) as long fatty acids and the small molecule TUG-424.
7. FFA1-HEK cell medium: DMEM (Dulbecco's Modified Eagle's Medium), containing 10 % heat-inactivated fetal bovine serum (FBS), 1 % penicillin/streptomycin, 100 µg/mL hygromycin B, and 15 µg/mL blasticidin S.
8. Phosphate buffered saline (PBS): NaCl 8.01 g/L, KCl 0.20 g/L, $Na_2HPO_4 \times 2H_2O$ 1.78 g/L, KH_2PO_4 0.27 g/L, adjusted to pH 7.4 and sterilized.
9. Trypsin/EDTA: 0.05/0.02 % in PBS, sterile filtered.
10. Hank's balanced salt solution (HBSS): $CaCl_2$ 1.4 g/L, $MgCl_2 \times 6H_2O$ 1 g/L, $MgSO_4x7H_2O$ 1 g/L, KCl 4 g/L, KH_2PO_4 0.6 g/l, NaCl 80 g/L, $Na_2HPO_4x7H_2O$ 0.9 g/L, D-glucose 10 g/L.
11. HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid).

2.1 Microplates and Biosensor instruments

1. Corning® Epic® biosensor (Corning).
2. PerkinElmer EnSpire® label-free multimode reader (PerkinElmer).
3. 384-well liquid handling station for compound transfer or for carrying out washing steps.
4. 384-well tips for use with a liquid handling robot.
5. Corning® Epic® biosensor (384-well, fibronectin-coated) microplates (Corning).
6. EnSpire®-LFC (384-well, fibronectin-coated) plate (PerkinElmer).
7. 384-well polypropylene microplate for compound dilutions.

3 Methods

Here, we present our standard protocol for basic investigation of the G protein-coupled receptor FFA1 (free fatty acid receptor 1), formerly described as GPR40 [18, 19], with the help of an optical label-free biosensor platform based on the phenomenon of dynamic mass redistribution (DMR). To this end, we use a HEK293 cell line inducibly expressing the receptor and both conjugated linolenic acids (CLA) as long-chain fatty acids and the selective small molecule TUG-424 as FFA1 agonists [20, 21]. For details about how to apply the DMR technology to decipher GPCR signaling using selective pathway inhibitors, refer to **Note 1**. To study cell lines endogenously expressing the receptor, *see* **Note 2.** In the following steps, all cell culture liquids and the biosensor plate are pre-warmed to 37 °C.

3.1 Preparation of Cells

1. Culture the cells in 75 cm^2 cell culture flasks in 15 mL growth medium. When cells have reached approximately 80 % confluency, aspirate the medium, rinse with 5 mL PBS to remove any residues of growth medium, and aspirate the buffer.
2. Subsequently, detach cells with 1 mL trypsin/EDTA (0.05 %/0.02 %) and count after addition of growth medium to stop trypsin-induced cell detachment.
3. Resuspend in the appropriate amount of growth medium containing 1 μg/mL doxycycline for receptor expression to achieve a cell density of 18,000 cells/30 μL, and seed 30 μL of this suspension into each well of a fibronectin-coated (*see* **Note 3**) 384-well biosensor plate.
4. Spin down the plate for 10 s at 150 × *g* to assure cells settle onto the bottom of each well.
5. Let cells adhere for approximately 18 h at 37 °C, 5 % CO_2. Doxycycline will induce receptor expression during that time (for suspension mode *see* **Note 4**).
6. Make sure that cells have reached confluency (*see* **Note 5**) in the biosensor plate before removing the medium.
7. Wash cells twice with 30 μL HBSS (*see* **Note 6**) supplemented with 20 mM HEPES and the appropriate amount of DMSO if needed (*see* below) using a manifold or a liquid handling station. Make sure to adjust the total volume to 30 μL buffer in each well after the last washing step. Caution: When aspirating the washing buffer, avoid detachment of cells by either direct contact or mechanical disintegration of the cellular monolayer.
8. Keep the plate for at least 1 h at measurement temperature for equilibration purpose before starting the assay, preferably in a temperature-controlled DMR reader (*see* **Note 7**). Extend this time period in case of a starvation step (*see* **Note 8**).

3.2 Preparation of Compounds

1. Prepare the compounds to be tested in HBSS buffer (+20 mM HEPES) (*see* **Note 6**) at fourfold concentration. If a compound is solved in DMSO, make sure to adopt the correct DMSO amount in all concentration steps as well as in the washing buffer to avoid any solvent mismatch-induced DMR signals (*see* **Note 9**) when adding the compound solution to the cells during the assay.
2. Transfer 30 μL of compound solution in each well of a 384-well source plate, e.g., in triplicates. The exact amount depends on the geometry of the compound plate and the technical conditions of the liquid handling system.
3. Ensure to include negative controls (vehicle) as well as reliable positive controls to document cell viability, for example, by addressing endogenous receptors (ATP, PGE_1) or other targets (forskolin, FBS) and those to differentiate between on- and off-target effects (*see* **Note 10**).
4. Equilibrate the compound plate at the same temperature as the biosensor plate to avoid temperature-caused shifts in DMR response (*see* **Note 7**).

3.3 DMR Measurement

1. Start the measurement by recording a baseline read over 300 s. It might be necessary to record for a longer time period until the signal is stable, i.e., no or only weak shift in wavelength is detected (*see* **Note 11**).
2. Add 10 μL of compound solution to the biosensor plate using a liquid handling station.
3. Transfer the plate back to the reader and record DMR immediately after addition to capture rapid cell responses.
4. Monitor cellular response as long as needed—e.g., 1 h—then stop the measurement, save the run, and collect the data for further analysis.

3.4 Data Analysis

1. To illustrate cellular response, plot the wavelength shift in picometer (*y*-axis) against the measurement time (*x*-axis) (e.g., in GraphPad Prism®) with scatter for the respective replicates. Throughout this chapter, mean value + s.e.m. is shown.
2. Subtract the buffer trace (i.e., cell response upon addition of vehicle) from compound-induced traces to obtain baseline-corrected DMR traces (*see* **Note 11**).
3. Quantify the DMR response of agonist substances (for antagonist characterization, refer to **Note 12**) by extracting an appropriate parameter, e.g., max Δpm values in a certain time frame (*see* **Note 13**), and plot it against the concentration of compound to generate concentration-response curves by nonlinear regression to determine agonist potency and efficacy (Fig. 2).

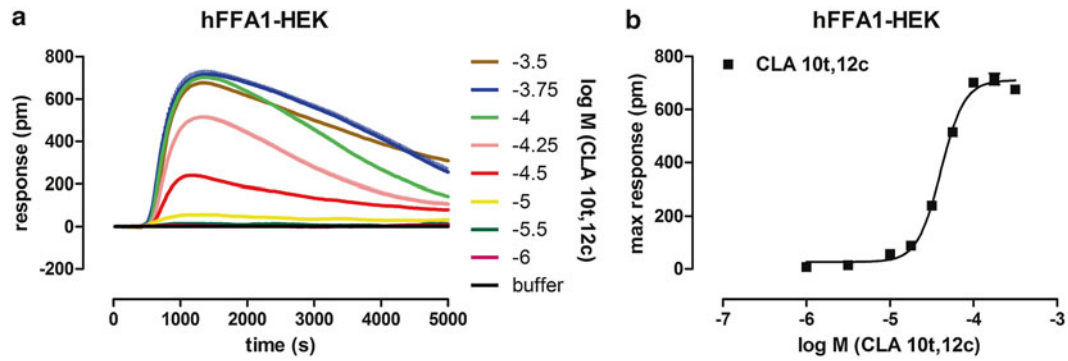

Fig. 2 DMR traces of hFFA1-HEK cells challenged with increasing concentration of the 10t,12c CLA-isomer (**a**). In this case, the maximum Δpm value between 0 and 1,500 s was taken to generate the concentration effect curve (pEC_{50}: 4.39) (**b**). Data from M. Grundmann, redrawn from ref. 20

4 Notes

1. Signal deconvolution: Since most GPCRs do not only signal via one pathway, the holistic readout of a DMR assay enables the researcher to uncover biased compounds, i.e., substances favoring one signaling route over the other. The information-rich DMR trace reflects a signaling fingerprint of the compound-cell interaction. Depending on the research question, it might be relevant to dissect these fingerprints with the help of pathway modulators such as PTX to inhibit $G\alpha_i$ and YM-254890 or FR900359 (=UBO-QIC) to inhibit $G\alpha_q$ proteins [22–24] (http://www.pharmbio.uni-bonn.de/signaltransduktion). The FFA1 receptor is predominantly coupled to $G\alpha_q$ proteins as revealed by FFA1-HEK cells that were pretreated with YM-254890 for 1 h in the biosensor plate before the assay. However, the use of PTX, which is present in the medium during 18–24 h before the assay, also uncovers a substantial contribution of $G\alpha_i$ proteins to the overall cell response upon stimulation with the FFA1 agonist TUG-424 (Fig. 3).
2. Impact of cellular background: As mentioned earlier, the DMR technology entails the advantage to investigate GPCRs in a variety of cell systems including primary, native, and recombinant cell lines. Since the FFA1 receptor is abundantly expressed in pancreatic beta cells, it is possible to use dissociated islets isolated from mouse pancreas and the native rat beta cell line INS-1E endogenously expressing the FFA1 receptor (Fig. 4).
3. Plate coating: Depending on the cellular background, it might be necessary to use extracellular matrix (ECM) or polymer-coated biosensor plates to establish a firm attachment in order to allow for uniform cellular behavior, such as cell movement.

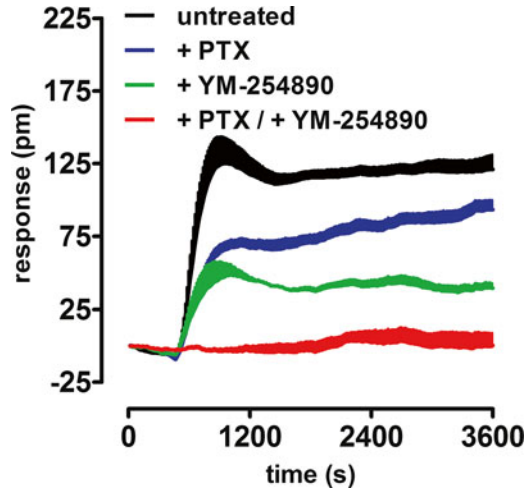

Fig. 3 Dissection of DMR response with pathway inhibitors in FFA1-HEK cells stimulated with 3 μM of FFA1 agonist TUG-424. The overall DMR signal is composed of both $G\alpha_q$ and $G\alpha_i$ proteins as shown by pretreatment with $G\alpha_i$ inhibitor PTX (5 ng/mL) and/or the selective $G\alpha_q$ inhibitor YM-254890 (300 nM), each of which partially reduce the DMR response. The combination of the inhibitors, however, completely abolishes the signal. Redrawn from ref. 6

Matrix coatings are likely to also influence growth, differentiation, and overall cellular function. Preliminary tests and the experience with the particular cell culture will help to decide which coating material and cells will work in harness. Coating or even co-coating plates on your own with the material of your choice is possible, but their suitability has to be determined in each case.

4. Adherent vs. suspension mode: It is also possible to perform DMR experiments in suspension mode, e.g., for nonadherent cells or some primary cells. For this purpose, cells are prepared according to specific protocols for isolation and purification. A higher cell number is required when running the assay in suspension mode (about threefold compared to adherent cells). The appropriate cell number is reconstituted in the assay buffer (e.g., HBSS + 20 mM HEPES), and 30 μL are seeded into each well of a 384-well biosensor plate. The plate is centrifuged subsequently at $150 \times g$ for 10 s to assure that cells are positioned in the bottom part of the well, i.e., in close proximity to the biosensor surface. The subsequent steps correspond to the protocol for adherent cells. Depending on the cell system and the coating, interaction between the cells and the coating material might occur which can be displayed in a transient DMR response. This issue is encountered by an extended incubation time. Note that the DMR traces obtained from suspension mode can differ significantly from those in adherent mode

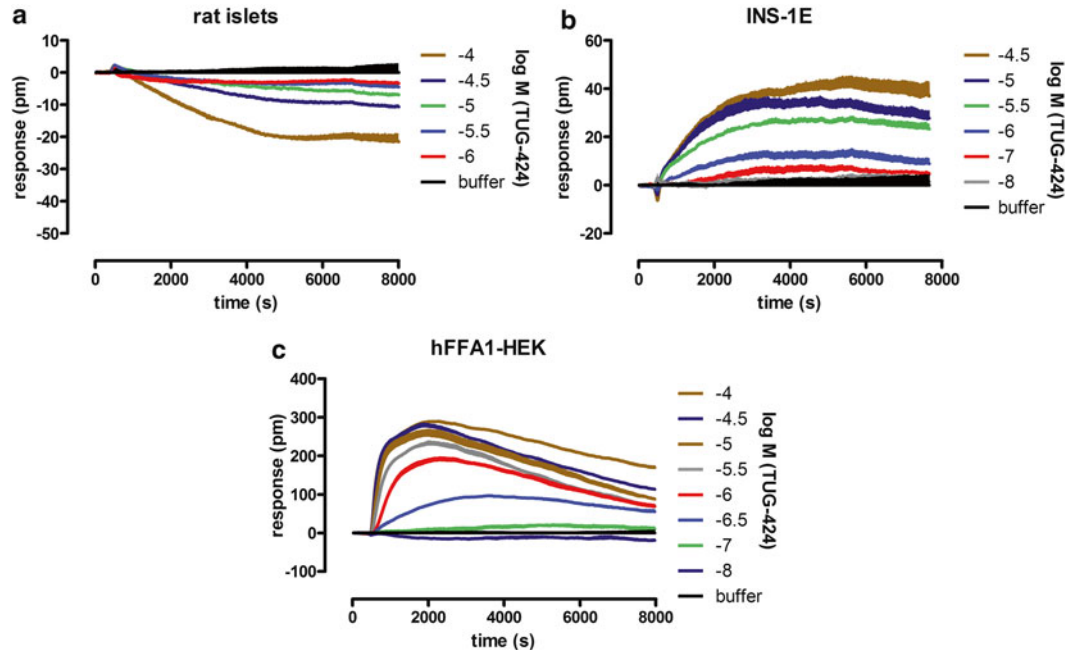

Fig. 4 Primary, native, and recombinant cell lines demonstrate the versatility of the assay platform. Rat islets were dissociated with trypsin and measured 6 days after seeding into the biosensor plate (**a**). INS-1E and FFA1-HEK cells were prepared according to the standard protocol with 30,000 cells/well (INS-1E) (**b**) and 18,000 cells/well (FFA1-HEK) (**c**). The cells were then stimulated with the FFA1 agonist TUG-424 and DMR response was recorded. Different cellular backgrounds can have great impact on the behavior in the assay. Note the different *y*-axis scaling! Data from M. Grundmann, University of Bonn, unpublished observations

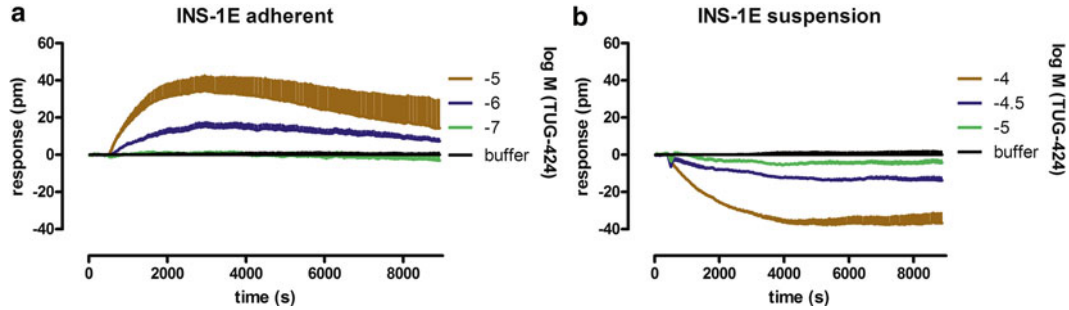

Fig. 5 Rat beta cell line INS-1E endogenously expressing FFA1 and treated with FFA1 agonist TUG-424 shows positive DMR if growing adherent (30,000 cells/well) (**a**) and negative DMR when in suspension mode (90,000 cells/well) (**b**). Data from M. Grundmann, University of Bonn, unpublished observations

(and it might therefore be advisable to choose alternative parameters for data analysis) (Fig. 5).

5. Confluency: Critical steps in optimizing a DMR protocol include growth conditions and cell number in the biosensor plate, because intercellular interaction greatly influences the cell response. As a starting point for assay optimization, always

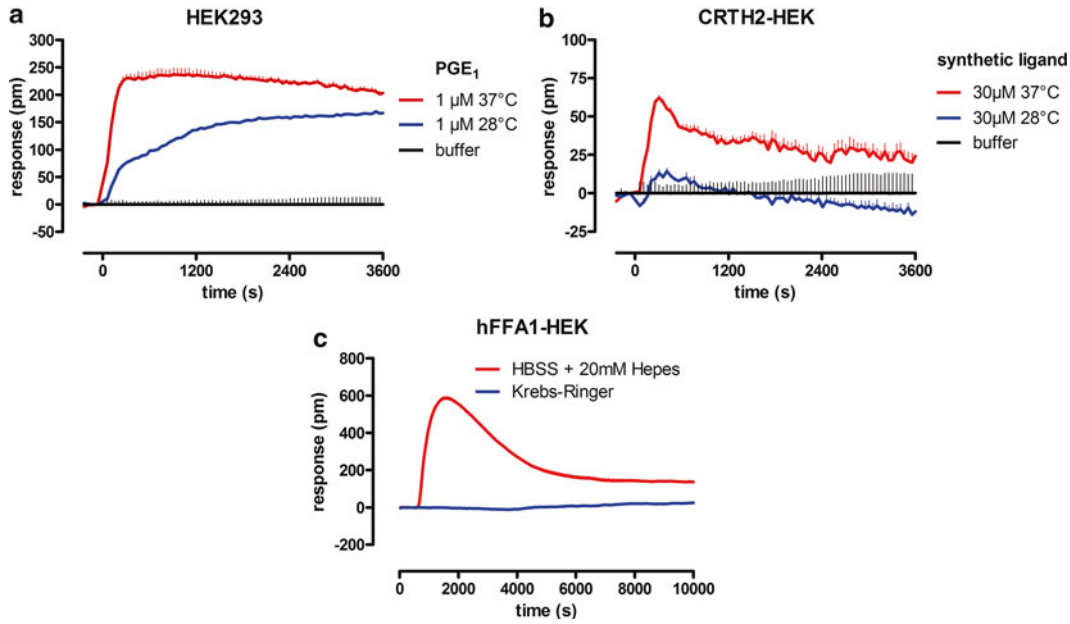

Fig. 6 Influence of temperature and buffer composition on the DMR assay. At higher assay temperatures, DMR signals appear accelerated and with greater amplitude (prostaglandin E1 activating EP2/4 receptors endogenously expressed in HEK293 cells) (**a**). A small molecule CRTH2 agonist induces DMR in HEK cells stably transfected with the CRTH2 receptor at physiological temperature but not at 28°C (**b**). Changing the assay buffer can have great impact on DMR signals by either lowering the cellular responsiveness or limiting the solubility of test compound. Depicted are DMR responses of FFA1-HEK cells stimulated with 100 µM of the 9c,11t CLA-isomer in standard HBSS buffer or Krebs-Ringer buffer (**c**). Data from M. Grundmann and R. Schröder, University of Bonn, unpublished observations

aim for a confluent cellular monolayer with nicely attached cells whose phenotype resembles their usual morphology. To achieve this, it might be necessary to vary growth medium additives, coating material, and especially the cell number.

6. Buffer influence: Changing the assay buffer is possible, but should be made with caution. The system comprising biosensor, cells and compounds might might react differently under modified buffer conditions. Solubility of test compounds can be limited in one buffer and as a result, can greatly affect DMR response (Fig. 6c). However, varying assay buffers is suggested as part of an assay optimization process.
7. Temperature (Fig. 6a, b): Optical density and accordingly the shift in wavelength as the readout of DMR assays are highly sensitive to changes in temperature. It is therefore recommended to assure a uniform temperature throughout the measurement, i.e., minimize temperature differences between test plate and compound plate to be transferred; keep the plates at the same temperature for a sufficient time to ensure temperature equilibration. Otherwise, artifacts such as a wavelength

shift or drift can occur, emerging under negative control conditions such as vehicle addition. Since temperature can be distributed unevenly over the test plate (edge effects), it is recommended to position negative controls randomly over the plate. In addition to system-related effects, temperature has significant impact on overall cellular function as well. Higher temperature generally leads to higher signal amplitude and accelerated kinetics of DMR signals. To some degree, this also implies an extended sensitivity to weak agonists to be detected in a threshold-based screening (Fig. 6b). Note that under different temperature conditions, an expanded cellular repertoire can be displayed that might also complicate interpretation of DMR responses, for example, in signaling pathway decoding.

8. Starvation: Keeping the cells under starvation conditions for varying time periods before the assay (e.g., serum starvation in serum-free medium or in the assay buffer) can also have an impact on cellular response in the DMR assay. Although not generally applicable, especially those experimental setups that suffer from a small assay amplitude may benefit from starvation techniques to gain signal enhancement.
9. DMSO mismatch: DMSO artifacts generally recognized as sharp spikes (up to thousands of Δpm) occur if the DMSO amount in the compound solution is different from that in the assay buffer surrounding the cells. Adjust the DMSO concentration in all assay solutions conscientiously, because the assay is very DMSO sensitive! Avoid DMSO concentrations way above 1.2 %! The DMR technology is less sensitive to other solvents such as ethanol.
10. On- and off-target effects: The holistic nature of the method can make it challenging to differentiate between on- and off-target effects. To trace a given DMR response back to a specific molecular target, it is necessary to include negative controls in the setup. In the described protocol, we could use cells that were not induced with doxycycline and thus do not express our target protein on the cell surface. In the case of primary cells, knockout material would be an appropriate negative control. Another possibility is the use of a target-specific antagonist that is capable of blocking the DMR response of an agonist (*see* **Note 12**). Furthermore—in the absence of suitable pharmacological inhibitors—cross-desensitization or gene silencing methods may be applied to help attribute a DMR signal to a certain cellular event.
11. Buffer control: The course of the buffer signal can give insight into the overall cellular behavior such as detachment from the plate surface as well as temperature effects, which can result in a DMR drift over the time. Normally, these events can be prevented by optimizing cell culture conditions (e.g., cell

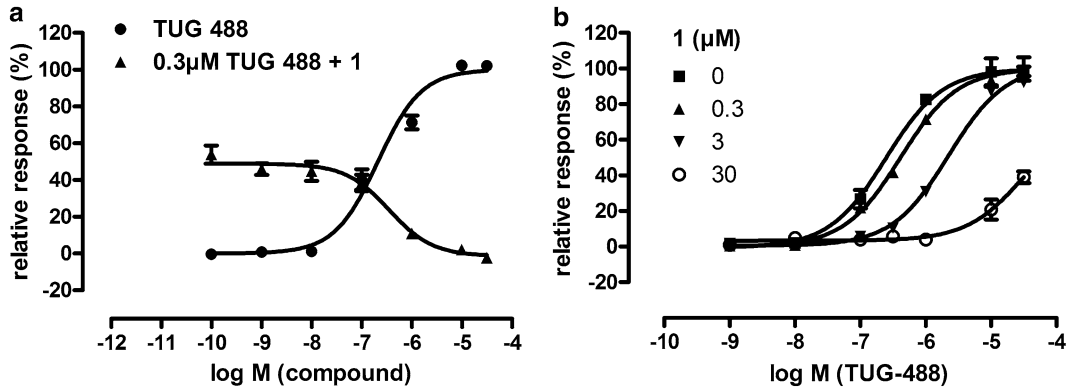

Fig. 7 Analysis of the FFA1 antagonist **1** [26] competing with small molecule FFA1 agonist TUG-488 [27]. A pIC_{50} of 6.45 can be determined for **1** by inhibition curve (**a**); Schild analysis reveals competitive/surmountable antagonism of **1** (**b**). Data from M. Grundmann, unpublished observations

density, plate-coating, media additives, etc.) or measurement options (e.g., temperature equilibration, time, and accuracy).

12. Antagonist mode: The DMR technique is perfectly suited for characterizing antagonists as well. Besides standard determination of IC_{50}-values or the mode of action (competitive, non-competitive, uncompetitive, inverse agonist, partial agonist), it is also possible to describe the kinetic profile of a test substance. Real-time DMR recordings after antagonist addition to agonist-induced cell responses allow the user to gain insight into kinetic features of test compounds or receptors (see, e.g., ref. 25). A basic protocol to determine the mode of action of an antagonist involves a two-step addition. In the first step, after washing the cells, increasing concentrations of antagonist are added, and DMR is recorded to detect any cellular response upon antagonist application. In the second step, after preincubation with antagonist, the agonist is added. After DMR measurement and basic data analysis, an inhibition curve or a Schild regression analysis can be performed to further describe antagonist parameters and mode of action (Fig. 7).
13. Data quantification: Parameters for data quantification range from peak value at a certain time point or within a defined time frame, the area under curve (AUC) between certain time points or steepness of a tangent to the initial DMR trace to capture kinetic compound behavior. In certain cases, the real-time readout enables the researcher to distinguish between multiple phases in cell response. The number, the duration, or the time needed to pass one phase adds to the list of possible quantification parameters. Since the content-rich DMR readout can appear highly complex and multiphasic, we recommend to address the issue of quantification on a case-by-case basis (Fig. 8).

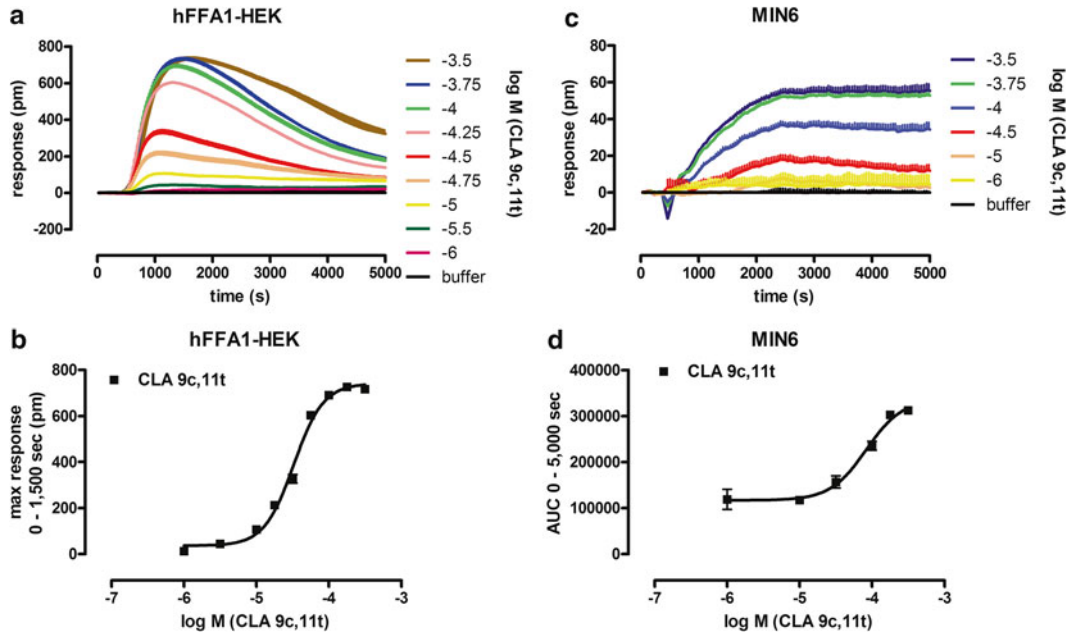

Fig. 8 The parameter used to quantify the cell response depends on the appearance of the DMR traces. One possibility is to calculate the peak Δpm value between 0 and 1,500 s for the HEK cells (18,000 cells/well, pEC_{50}: 4.49) (**a**, **b**) and the AUC between 0 and 5,000 s for the slower response in the MIN6 cell line (20,000 cells/well, pEC_{50}: 4.08) (**c**, **d**). Both cell lines were treated with increasing concentrations of the 9c,11t-isomer of CLA. Data from M. Grundmann, University of Bonn, unpublished observations

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