Applying label-free dynamic mass redistribution technology to frame signaling of G protein–coupled receptors noninvasively in living cells

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Label-free dynamic mass redistribution (DMR) is a cutting-edge assay technology that enables real-time detection of integrated cellular responses in living cells. It relies on detection of refractive index alterations on biosensor-coated microplates that originate from stimulus-induced changes in the total biomass proximal to the sensor surface. Here we describe a detailed protocol to apply DMR technology to frame functional behavior of G protein–coupled receptors that are traditionally examined with end point assays on the basis of detection of individual second messengers, such as cAMP, Ca^{2+} or inositol phosphates. The method can be readily adapted across diverse cellular backgrounds (adherent or suspension), including primary human cells. Real-time recordings can be performed in 384-well microtiter plates and be completed in 2 h, or they can be extended to several hours depending on the biological question to be addressed. The entire procedure, including cell harvesting and DMR detection, takes 1–2 d.

INTRODUCTION

G protein–coupled receptors (GPCRs) are the largest transmembrane protein family in the human genome and the most commonly targeted receptor class for therapeutic intervention. A number of blockbuster drugs act by modulation of GPCRs, which—at present—occupy about 50% market share of medical therapeutics[1]. It has become apparent during recent years that signaling of this receptor family is far more complex than previously anticipated and that a single receptor may possess a number of different active conformations that enable recruitment of some, while simultaneously disrupting other signaling components[2–4]. Despite the increasing complexity of GPCR-triggered cellular responses, biochemical assays that are widely used for discovery of GPCR-targeted tool compounds focus on single-functional end points, such as production of intracellular Ca^{2+} or inositol phosphate for Gq-linked receptors and cAMP increase or decrease for $G\alpha_s$- and $G\alpha_i$-coupled receptors, respectively, rather than on integrated cellular responses. In addition, as it has become apparent that GPCRs are likely to modulate more than one signaling event at a time, and that these events may be highly interconnected, analysis of receptor function should move to another level and study the complex ways in which biochemical signaling pathways are integrated into larger signaling networks in whole cells[5,6].

Label-free whole-cell assays for live-cell sensing

It is possible to record such integrated live-cell responses in whole cells by applying one of the innovative label-free technology platforms that have emerged recently and that are either impedance-based (CellKey, MDS Analytical Technologies; xCelligence, ACEA Bioscience and Roche Applied Science; ECIS, Applied Biophysics) or optical-based (Epic, Corning; BIND, SRU Biosystems; Enspire, PerkinElmer)[7–11]. For detailed information on the technologies used in these biosensors please refer to specialized reading[12–14]. In principle, any of these label-free instruments would be suitable for detection of integrated cell responses. In this protocol, we apply the Corning Epic label-free dynamic mass redistribution (DMR) technology, the suitability of which for detection of holistic cellular responses has been previously shown by our laboratory[15–19] and others[20,21].

Label-free DMR technology

The principle of DMR detection is illustrated in **Figure 1**. DMR refers to cellular processes that occur when biomolecules change their localization within a cell. A number of cellular behaviors are associated with mass relocation, such as protein trafficking, morphological changes, cytoskeletal rearrangement, receptor internalization or adhesion, although the exact details underlying label-free responses are not yet resolved. DMR can be detected by passing polarized broadband light through the bottom portion of cells grown on microtiter plates in which grating surfaces are embedded and by recording a change of the wavelength of outgoing light (detailed in **Fig. 1**). Cellular DMR is then transformed into a single optical output that collates all these processes and therefore represents a cumulative response overlay of entire living cells, which should offer a far more comprehensive pharmacological estimate of drug effects as compared with traditional end point assays[8,9,18]. An overview of the general DMR assay procedure is given in **Figure 2**.

We have previously shown that DMR is able to capture activation of receptors linked to all four G protein families ($G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$)[18]. As mass relocation is not a random optical traces derived from stimulation of GPCRs that couple to different effector pathways may even be indicative of defined signaling events[18]. However, we will also provide examples in this protocol that mechanistic assumptions should not be based on visual inspection of optical traces but rather on rigorous signal deconvolution.

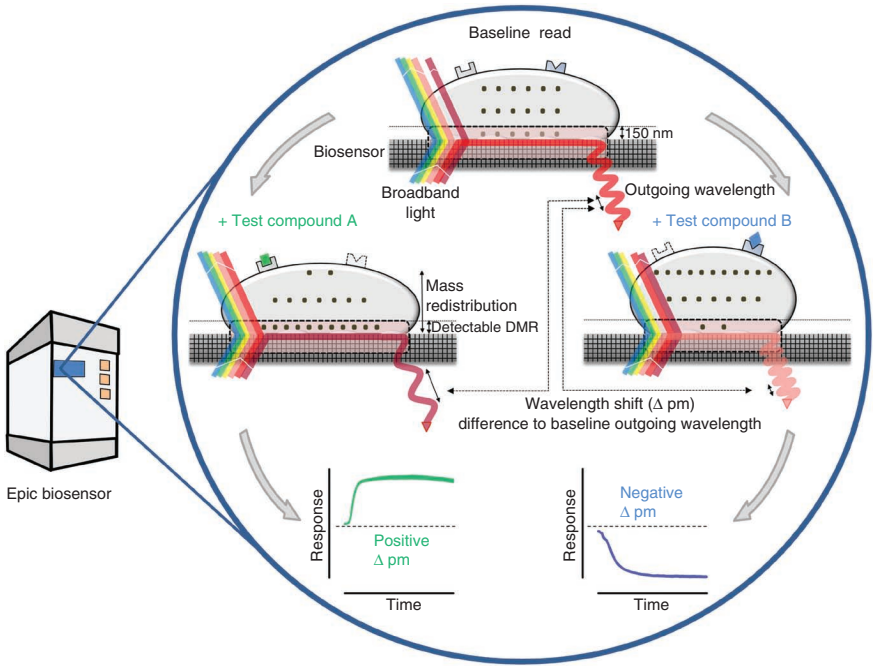

Figure 1 | Principle of DMR detection. Cells are seeded into 384-well biosensor microplates and are then placed into the DMR-reader. In the baseline read, polarized broadband light, covering a range of wavelengths, illuminates the bottom of the microplate in which a resonant waveguide-grating (RWG) biosensor is embedded[7,13]. This grating interacts with the cell layer or biomass on its surface, thereby forming an optical system capable of propagating and reflecting the specific wavelength that is in resonance to the system, whereas the other wavelengths are transmitted. The propagated light generates an evanescent wave that penetrates the bottom portion of the cell layer with a penetration depth of 150 nm into the cell ('defined as sensing volume' of the biosensor), and the outgoing wavelength (resonant wavelength) is recorded and normalized to zero. Resonance of this optical system to a specific wavelength depends on the OD of the cell layer on top of the RWG, and it therefore achieves sensitivity to alterations in the refractive index. On addition of a test compound, the OD of the cell layer changes near the biosensor within the bottom region of the cells because of a redistribution of intracellular particles and/or a change of cell shape. This results in a shift of outgoing (resonant) wavelength relative to the baseline value and is recorded in picometers (Δ pm). Test compound A increases the OD in the sensing volume of the biosensor resulting in an increase of the outgoing light's wavelength, ultimately leading to positive Δ pm and upward deflection of the 'Δ pm versus time' signal. Test compound B decreases the OD in the 'sensing volume' resulting in a negative 'Δ pm versus time' signal.

Diversity of cell lines suitable for DMR measurements and potential applications of the method

DMR recordings of nonadherent cells. As recording of DMR is noninvasive, the procedure is in principle applicable to any living cell type, provided that sufficient DMR is generated upon receptor stimulation. Indeed, concentration-dependent temporal DMR activity profiles have been recorded for a number of GPCRs expressed in various immortalized and recombinant[7,15,18–21] as well as primary[18] cell systems that adhere to the biosensor microplates. Notably, application of DMR technology is not restricted to cells that firmly adhere to the microplates, but is also suited to study receptor functionality in suspension cells, as exemplified in **Figure 3** for a $G\alpha_i$ response observed with nicotinic acid in primary human neutrophils. The ability to directly monitor engagement of the $G\alpha_{i/o}$ signaling pathway in a human primary cell is particularly noteworthy for the benefits over the currently used cAMP inhibition assays. The latter do not monitor $G\alpha_{i/o}$ activation directly but require prior stimulation of adenylyl cyclase with forskolin to then measure a reduction of this response by a $G\alpha_{i/o}$ GPCR stimulus and often require tedious optimization of the time window ideally suited for quantification of the $G\alpha_{i/o}$ response (see ref. 22 and **Fig. 3**). DMR, in contrast, offers a direct measure of $G\alpha_{i/o}$-coupled GPCR activation without the need to pharmacologically manipulate the adenylyl cyclase–cAMP module to probe for $G\alpha_{i/o}$ activity.

DMR recordings of detached cells in suspension. It may also be of relevance to note that DMR can be applied to study adherent cells in suspension. This is exemplified for the free fatty acid receptor FFA1/GPR40 stably expressed in a human embryonic kidney (HEK293) cell background. FFA1 engages $G\alpha_{i/o}$ and $G\alpha_q$ signaling pathways[18] and retains both its pharmacological and its signaling profile, irrespective of whether cells are grown to confluence on biosensor microplates or gently spun down as suspension cells before application of a receptor agonist (**Fig. 4**). The ability to analyze adherent cells in suspension may be relevant if assay timing is critical, since suspension cells can be analyzed immediately after their detachment or purification as opposed to adherent cells that require time to form confluent monolayers prior to DMR detection. Application of DMR technology to record receptor functionality in suspension cells is described in detail in this protocol.

Evaluation of antagonist activity in real time. Not only do DMR assays allow unique insights into the mode of action of agonists, but also their noninvasive nature may equally well provide a more comprehensive picture of pharmacological antagonist behavior. Traditional end point assays require antagonists to either be preincubated or be coapplied with the agonists. This procedure allows determination of antagonist potency and their mode of action (competitive versus noncompetitive), but no direct insight into the temporal aspects of receptor inhibition can be achieved. DMR technology, in contrast, offers the option to visualize antagonist action in real time by adding antagonists onto agonist-treated cells (**Fig. 5**) and—by doing so—captures their full temporal profiles. It is therefore conceivable that label-free DMR may be exploited to even distinguish between antagonists that hold different kinetic profiles.

Additional applications for label-free technologies

Label-free DMR is not restricted to detection of GPCR signaling. In principle, any biological phenomenon associated with micromotion of cells and redistribution of intracellular biomolecules should cause DMR. For example, label-free platforms are known to be susceptible to signaling of receptor tyrosine kinases[23–25] and

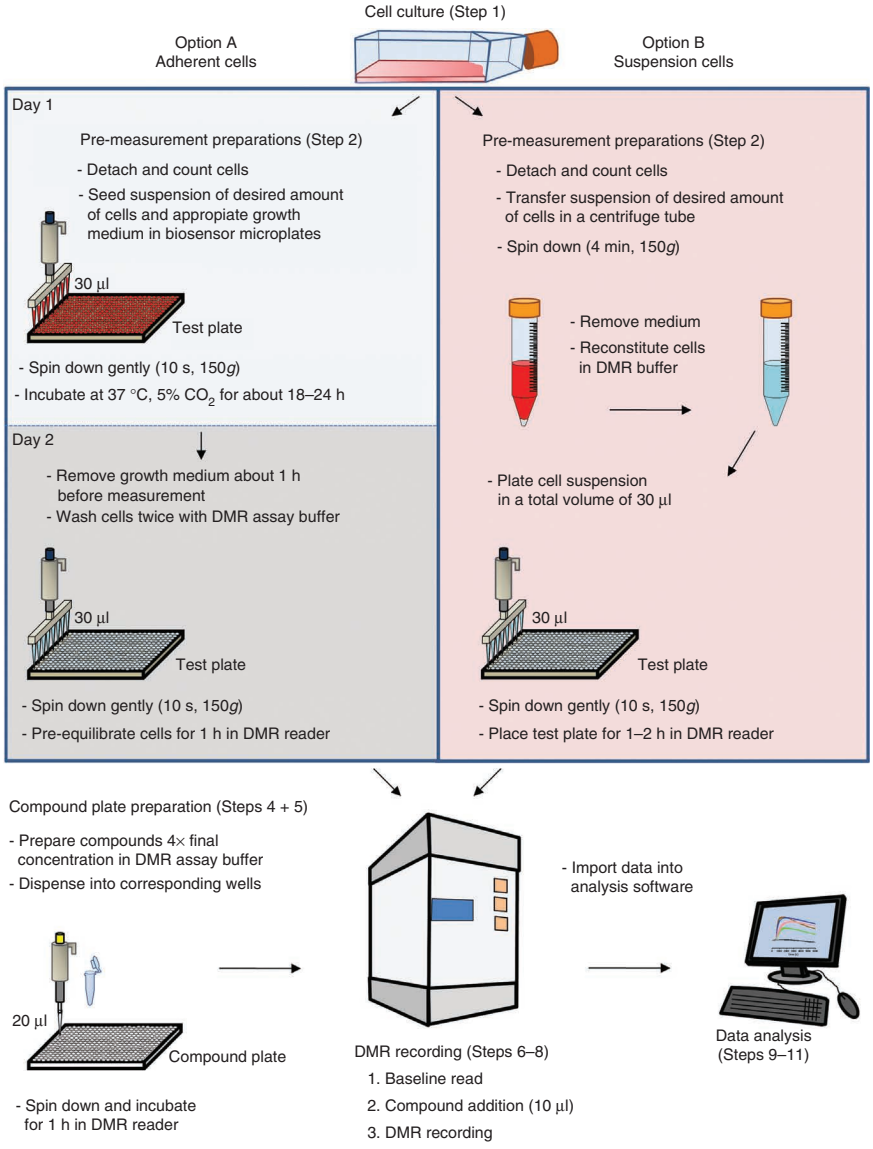

Figure 2 | Schematic diagram illustrating the major steps of the DMR assay procedure.

can also sense activation of intracellular enzymes[26] or processes such as cardiomyocyte contractility (http://www.srubiosystems.com/applications/Cytotoxicity.html), migration and cell proliferation[14,27,28]. Several of these applications are, however, still in their infancies and the authors wish to note that they have exclusively applied label-free DMR technology to study signaling of cell surface GPCRs.

Limitations and challenges of label-free DMR detection

Neutral net responses (zero signatures) despite biological ligand activity. Label-free is a detection platform that translates a variety of cellular processes into a single optical function and therefore represents a cumulative overlay of multiple signaling phenomena occurring within a single population of cells. If engagement of individual signaling pathways by a defined stimulus leads to both positive and negative DMR, weak or even neutral net responses may be obtained that lead to underestimation of biologically active molecules. Nevertheless, pathway blockers would help to unmask such hidden pathway activation.

Interpretation of label-free results. A second limitation is closely linked to the 'black-box' nature of label-free responses, although the ability of label-free to discern signaling mechanisms is frequently stated[29]. It is important to keep in mind that activity profiles of compounds vary in a cellular context-dependent manner[18]; therefore, mechanistic conclusions must not be drawn from visual inspection of optical response profiles without taking the cell type under study into account. In fact, rigorous signal deconvolution should be applied if response profiles are to be interpreted in terms of signaling routes within a cell (see ref. 18 and the ANTICIPATED RESULTS section of this protocol for more details on the necessity of signal deconvolution).

Specificity of response. Although high sensitivity for detection of signaling by receptors expressed at endogenous levels is deemed a big advantage of label-free, the lack of appropriate pharmacological tools to examine the specificity and nature of response may be perceived as limitations. Receptor antagonists and pathway inhibitors (if available), and also gene silencing, may be required to interpret the origin of label-free responses, i.e., signals may not be easily amenable to simple deconvolution but require a well-equipped label-free toolbox. We wish to remark, however, that whole-cell sensing of integrated cellular activity without deconvolution must also be highly valued for the possibility of probing pharmacology in the context of whole biological system's efficacy.

Comparison of label-free DMR with traditional end-point assays

It is frequently stated that ligand pharmacology determined with label-free assays should align with that determined in traditional second messenger or biochemical assays[11] and that label-free technology needs to be validated by comparison with these more conventional assays. However, the fact that label-free detects cumulative cell responses rather than individual vertical signaling events raises doubt that such alignment should be posited. In fact, differences in whole-cell efficacy or potency for ligands would not only be suggested, but also rather distinctly predicted. For this reason, we recommend not contrasting traditional end point assays with newly developed label-free assays, but rather using a combination of both in parallel to complement each other.

Experimental design

Label-free DMR is a straightforward approach to record live-cell responses in a wide range of cellular backgrounds[7,9–11,13,15–18]. The purpose of this protocol is to provide detailed information on how to apply DMR in a step-by-step manner to recombinant and

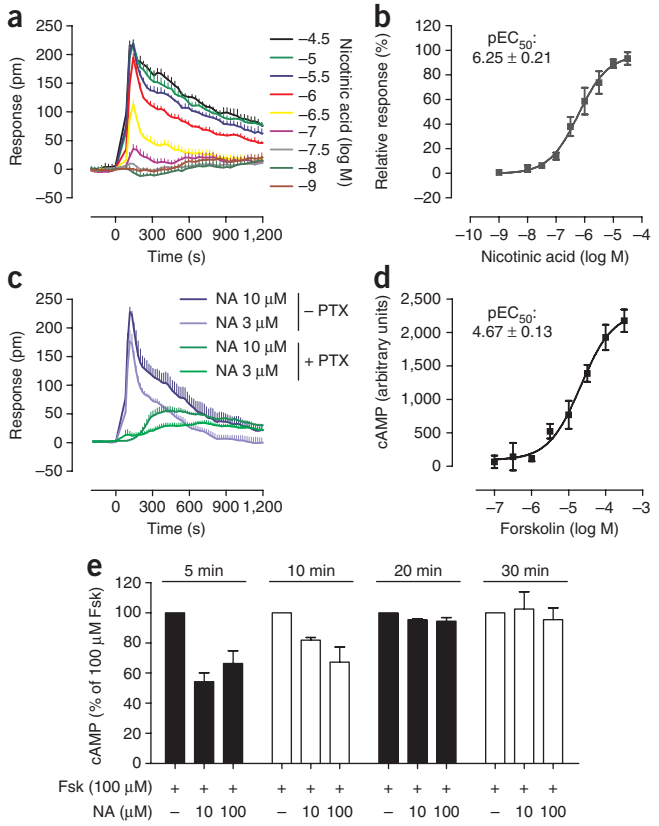

Figure 3 | $G\alpha_{i/o}$ GPCR activity can be assayed directly in primary human neutrophils. (**a**) Mature blood neutrophils were isolated from human peripheral blood of healthy donors by Ficoll-Hypopaque centrifugation according to standard protocols[33]. All donors provided written and informed consent and the study was approved by the Ethics Committee of the University of Bonn. Cells were seeded into fibronectin-coated Epic 384-well microplates (60,000 cells per well) and exposed to nicotinic acid (NA), a compound known to activate $G\alpha_{i/o}$ signaling in human neutrophils[22]; DMR was recorded as a measure of cellular activity. BSA (0.1%, wt/vol) was included in the assay buffer for DMR measurement of primary neutrophils. (**a**–**c**) NA-induced DMR is concentration-dependent (representative optical traces (**a**); concentration-effect curve and half-maximal response (pEC_{50} value) generated from response peak maxima of four individual experiments (**b**)) and mediated by a $G\alpha_{i/o}$-sensitive GPCR, as it is diminished in neutrophils pretreated for 1 h with 3 µg ml^{-1} of the $G\alpha_{i/o}$ inhibitor pertussis toxin (PTX) (**c**). (**d**) To determine NA-mediated inhibition of forskolin (Fsk)-stimulated cAMP production, a Fsk concentration-effect relationship needs to be established prior to performing cAMP inhibition assays. (**e**) NA decreases intracellular cAMP levels in mature blood neutrophils, but detection of $G\alpha_{i/o}$ activity is critically dependent on the time interval of agonist exposure: NA-mediated reduction of cAMP can be detected after 5 and 10 min of agonist exposure, but is hardly visible after 20 or 30 min. This is as opposed to DMR, which offers real-time detection of nicotinic acid activity without the need to identify an optimal time window for the assay and without the need to pharmacologically manipulate the neutrophils with Fsk. Shown are means ± s.e.m. of three experiments each performed in triplicates. Detection of cAMP levels was performed using the LANCE Ultra cAMP-Kit (12,000 cells per well) according to the manufacturer's instructions.

primary, including suspension, cells, as well as highlight potential problems and provide solutions.

Ligands to pharmacologically modulate GPCRs and their associated signaling pathways. As a pathway-unbiased platform to sense GPCR activation, there is no limit to the application of potential GPCR stimuli: neurotransmitters, hormones, lipids, nucleotides, chemokines, synthetic small molecules, and so on. In principle, any GPCR-activating ligand may be examined in these phenotypic response assays, either alone (one-step assay) or in conjunction with a specific receptor inhibitor (two-step assay). Receptor agonists used in this protocol include nicotinic acid (**Fig. 3**), the neurotransmitter acetylcholine (**Figs. 5** and **6**), and the bronchodilator orciprenaline (**Fig. 6**), among several others.

One-step and two-step DMR assays. Activating ligands are routinely examined using one-step assays, i.e., DMR is monitored on application of a single chemical stimulus. One-step assays are also appropriate to determine integrated system responses on co-stimulation with more than one ligand, to determine receptor antagonism under non-equilibrium conditions or to judge whether pathway inhibitors/receptor antagonists may be endowed with

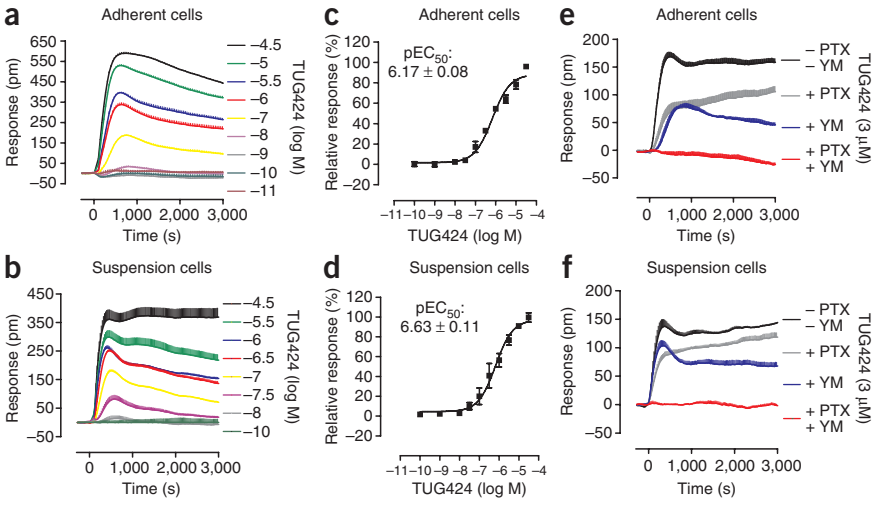

Figure 4 | The FFA1 receptor has an identical signaling profile and pharmacology in both adherent and suspension cells. (**a**,**b**) Flp-In T-REx FFA1-HEK cells were seeded onto fibronectin-coated 384-well microplates at a density of 15,000 cells per well and cultured for 24 h to reach confluent monolayers (**a**) or were collected from a tissue culture flask, washed two times in assay buffer and sedimented by centrifugation at a density of 45,000 cells per well as described in this protocol (**b**). Cells were then stimulated with the small-molecule FFA1–selective agonist TUG424 (ref. 30) and DMR was recorded for 3,000 s. (**c**,**d**) Concentration-effect curves and half-maximal responses (pEC_{50} values) resulting from the DMR traces in **a** and **b**. Curves were generated using the maximum DMR peaks between 0 and 1,000 s on ligand addition. Data are mean ± s.e.m. of at least three independent experiments. (**e**,**f**) The summation of FFA1 signaling pathways is deconvoluted with the signaling pathway inhibitors pertussis toxin (PTX, 5 ng ml^{-1}) and YM (300 nM). FFA1-triggered DMR is partly sensitive to pretreatment with YM and PTX, but completely blunted in the presence of a combination of both inhibitors. (**a**,**b**,**e**,**f**) Depicted are representative response traces (means + s.e.m.) of at least three independent experiments.

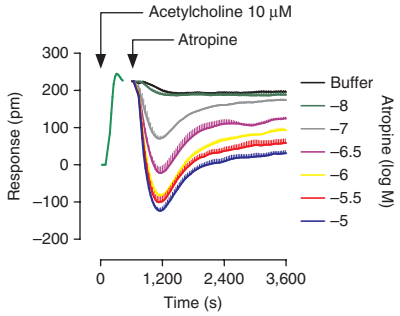

Figure 5 | Determination of the dynamic nature of agonist and antagonist action in DMR assays. Flp-In CHO-M2 cells (12,500 cells per well, stably expressing the muscarinic M2 receptor) were exposed to 10 µM of acetylcholine and receptor-mediated DMR was recorded for 600 s followed by application of varying concentrations of antagonist/inverse agonist or the corresponding solvent as control. Compound application is indicated by arrows. Atropine inhibition of acetylcholine-induced DMR is concentration-dependent (pIC_{50} value, i.e., the half-maximal inhibitory concentration, was 6.97 ± 0.45, n = 3, representative traces are shown).

unappreciated activities that may arise from influences on cellular processes beyond the target of interest. Two-step assays are advisable for determining antagonism under equilibrium conditions, but also to observe antagonism in real time as depicted in **Figure 5** of this protocol, or to assess the influence of pathway inhibitors on agonist-mediated responses (**Fig. 4e**,**f**, inhibition of FFA1 activity with the Gq selective inhibitor YM).

Data evaluation. Label-free DMR assays interpret biological activity of molecules by displaying ligand-mediated changes of cell behavior as refractive index alterations (Δ picometer (pm) values, **Fig. 1**). As these are observed in real time, integrated response profiles of whole cells can be followed kinetically. If real-time kinetic profiles (Δ pm versus time values) are altered in a ligand-concentration-dependent manner, compound activities can be quantified using standard pharmacological parameters such as potency (location along the concentration axis, expressed as pEC_{50} value, i.e., the −log concentration of agonist required to induce a half-maximal response) or efficacy (magnitude of maximal response). Conversion of optical response profiles into concentration-effect curves using standard curve fitting procedures is exemplified in **Figure 6** for receptors engaging distinct intracellular signaling pathways.

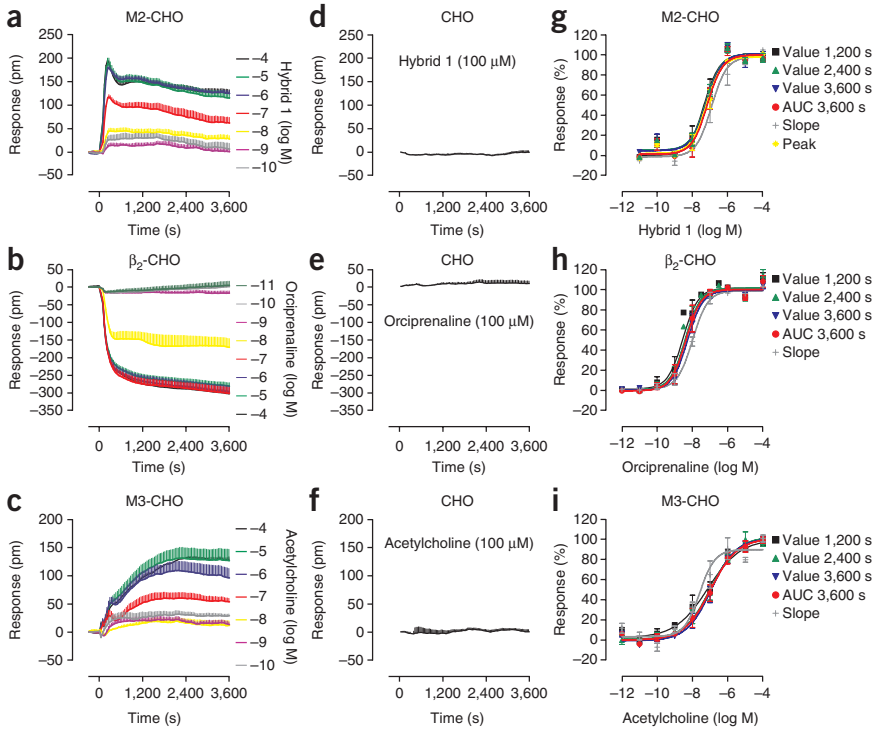

Figure 6 | Dynamic mass redistribution analysis of three prototypical GPCRs in recombinant CHO cells and deduction of concentration-effect curves from DMR signals. (**a**–**c**) Recordings in CHO cells (12,500 cells per well) stably transfected with M2-, β2AR or M3-receptor cDNAs and stimulated with the indicated concentrations of the corresponding agonists (M2, hybrid 1; β_2, orciprenaline; M3, acetylcholine). (**d**–**f**) Inactivity of the indicated ligands in native CHO cells. (**g**–**i**) Concentration-effect curves resulting from different read-outs (value 1,200, 2,400, 3,600 s, response at the indicated time point; AUC, area under the response curve; slope, steepness of a tangent to the origin of the signal; peak, maximum response). pEC_{50}-values (the –log concentrations at which agonists cause half-maximal responses) are: M2: value 1,200 s: 7.35 ± 0.22, value 2,400 s: 7.30 ± 0.11, value 3,600 s: 7.30 ± 0.10, AUC 300–3,600 s: 7.15 ± 0.21, slope: 6.94 ± 0.22, peak: 7.21 ± 0.27; β_2: value 1,200 s: 8.52 ± 0.30, value 2,400 s: 8.37 ± 0.23, value 3,600 s: 8.28 ± 0.24, AUC 300–3,600 s: 8.35 ± 0.24, slope: 8.02 ± 0.06; M3: value 1,200 s: 7.12 ± 0.19, value 2,400 s: 7.00 ± 0.18, value 3,600 s: 6.93 ± 0.20, AUC 300–3,600 s: 7.00 ± 0.20, slope: 7.31 ± 0.21. All data are mean values (± s.e.m.) of at least three independent experiments carried out in triplicates or quadruplicates.

MATERIALS

REAGENTS

- DMSO (cell culture grade, AppliChem, cat. no. 3672.0100) **! CAUTION** The solution is toxic; protect eyes and skin.
- PTX (pertussis toxin, Sigma-Aldrich, cat. no. P2980, supplied as solution) **! CAUTION** It is toxic; protect eyes and skin.
- CTX (cholera toxin, Sigma-Aldrich, cat. no. C8052, supplied as solid) **! CAUTION** It is toxic; protect eyes and skin and do not inhale dust.
- Forskolin (Tocris, cat. no. 1099) **! CAUTION** It is harmful; protect eyes and skin and do not inhale dust.
- BSA (Sigma-Aldrich, cat. no. A6003); store undiluted at 4 °C.**! CAUTION** It is harmful; protect eyes and skin and do not inhale dust.
- YM-254890 (Astellas Pharma) **! CAUTION** It is toxic; protect eyes and skin.
- $AlCl_3$ (Merck, cat. no. 801081) **! CAUTION** It is harmful; protect eyes and skin and do not inhale dust.

- NaF (Merck, cat. no.106441) ! CAUTION It is toxic; protect eyes and skin and do not inhale dust.
- HBSS (Hank's balanced salt solution, Invitrogen, cat. no. 14025)
- HEPES (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, AppliChem, cat. no. A3268) ! CAUTION It is harmful; protect eyes and skin and do not inhale dust.
- Cell culture reagents (according to the cells of interest and their respective culture conditions)
- Appropriate cell lines (recombinant, native, primary, adherent or suspension)
- Nicotinic acid (Carl Roth, cat. no. 3815.1) ! CAUTION It is harmful; protect eyes and skin and do not inhale dust.
- Acetylcholine (acetylcholine-iodide, Sigma, cat. no. A7000) ! CAUTION It is an irritant; protect eyes and skin and do not inhale dust.
- Orciprenaline (metaproterenol-hemisulfate, Sigma, cat. no. M2398) ! CAUTION It is harmful; protect eyes and skin and do not inhale dust.

EQUIPMENT

▲ CRITICAL Note that the procedure described in this protocol is carried out on the Corning Epic label-free detection platform with integrated robotic liquid handling, which is able to transfer 384 samples simultaneously from a compound source plate to a cell microplate within the Epic reader. Seeding of cells and all washing steps are carried out manually by using an 8-channel electronic pipettor and an 8-channel manifold connected to a standard cell culture aspiration device.

- Corning Epic biosensor (Corning) or alternatively PerkinElmer EnSpire label-free multimode reader (PerkinElmer), which contains the Epic optical biosensor technology
- Corning Epic biosensor microplates, 384-well, uncoated, Corning, cat. no. 5040; fibronectin-coated, cat. no. 5042; synthetic surface-coated, cat. no. 5048
- EnSpire-LFC plates, 384-well, uncoated, PerkinElmer, cat. no. 6057408; fibronectin-coated, cat. no. 6057428
- 384-well format preracked tips for use with a liquid-handling robot (for the Epic reader with the on-board liquid-handling device we use: CyBio, cat. no. 3800-25-513-N)
- 384-well polypropylene microplates for compound dilutions (e.g., Corning, cat. no. 3657)
- Optional: 384-well format liquid-handling device and/or plate washer
- 8- or 16-channel pipettor for dispensing into and washing 384-well plates
- 8- or 16-channel manifold to aspirate liquids from 384-well microplates (e.g., Popper & Sons, cat. no. 3389)
- Centrifuge equipped with rotors for microplates (e.g., Eppendorf 5810, Eppendorf)
- LANCE Ultra cAMP-Kit (PerkinElmer)
- GraphPad Prism software

REAGENT SETUP

PTX Supplied as solution, store at −20 °C for no longer than 1 year. Prepare working dilution in the desired buffer or the appropriate cell culture medium immediately before use. ! CAUTION It is toxic; protect eyes and skin.

CTX Supplied as solid. Reconstitute CTX in sterile, purified water and store at 4 °C for no longer than 1 year. Prepare dilution in the desired buffer or the appropriate cell culture medium fresh before use. ! CAUTION It is toxic; protect eyes and skin and do not inhale dust.

Forskolin Prepare stock (10 or 100 mM) in DMSO and store at −20 °C for no longer than 2 months. Make up a fresh working solution for each assay. ! CAUTION Harmful; protect eyes and skin and do not inhale dust.

BSA Prepare a fresh working solution before use, e.g., 0.1% (wt/vol) in DMR assay buffer. ! CAUTION Harmful; protect eyes and skin and do not inhale dust.

DMR assay buffer Supplement commercial HBSS with a sterile HEPES solution (1 M in purified water, pH 7.2) to a final concentration of 20 mM HEPES in HBSS. DMR assay buffer can be stored at 4 °C for several weeks. ▲ CRITICAL DMSO (and potentially other solvents) may induce bulk refraction index differences leading to artificial peaks. To avoid this, we recommend determining the highest DMSO concentration that will be added during the assay (e.g., by compound addition) and adjusting the DMR assay buffer used for preassay cell washing, as well as all compound dilutions to the same percentage of DMSO. In general, we recommend testing solvents used to reconstitute test compounds regarding their effect on the DMR responses. Potentially, many other buffers may be similarly suitable, but we recommend varying the assay buffer only if DMR responses appear poor or if it is desired to examine buffer influence on DMR responses. BSA may be added to the buffer to improve the DMR signal (we recommend 0.1% (wt/vol) BSA); however, keep in mind that addition of BSA may also lead to ligand depletion.

Aluminum fluoride solution To generate a 1.2 mM AlF_4^- solution, mix equal amounts of 2.4 mM $AlCl_3$ and 80 mM NaF, each dissolved in DMR assay buffer. $AlCl_3$ and NaF solutions can be stored at −20 °C for several weeks. We recommend freshly preparing AlF_4^- solutions on every assay day. ▲ CRITICAL Note that AlF_4^- is a pan–G protein activator and may help to investigate G protein origins of response traces.

Cell culture Cells can be cultured in the appropriate growth medium before the DMR assay.

EQUIPMENT SETUP

Handling of biosensor microplates Do not touch the underside of biosensor microplates with your fingers and do not touch the bottom of the wells with the pipette tips. If plate washing is performed manually, we recommend using a manifold to aspirate liquids. The manifold must be adjusted to not touch the bottom of the wells and to leave a defined liquid volume after aspiration in the wells (we use a remaining volume of 10 µl).

Baseline read and measurement period Generally, we perform a 300-s baseline read and monitor ligand-mediated DMR for a period of at least 1 h. Adjust these times to your specific requirements.

Because of the noninvasive nature of the assay and the absence of components that may affect cell viability, such as fluorescent dyes or any other labels, DMR can be recorded over many hours. It will depend on each user to define the time intervals optimally suited to repeat DMR detections in each well and also the total time period required to best capture the system's biological responses. Although DMR assays can also be performed in an end point mode (i.e., detection of DMR at a specific time after ligand addition), we recommend capturing traces kinetically over defined time periods to avoid overlooking substances that may induce unexpected response profiles and to extract the maximum amount of biological information.

Because temperature differences may affect the DMR registration, avoid temperature variations of more than 1 °C during the assay period. We routinely conduct measurements at 28 °C, but higher or lower temperatures (e.g., 20–30 °C) are also appropriate.

PROCEDURE

Cell culture

1| Culture cells by using your established growth medium and appropriate culture conditions.

Pre-measurement preparations

2| DMR can be monitored in adherent cells (option A) or in suspension cells (option B). To achieve an optimal assay window, we recommend using confluent cell layers for both options, because the biosensor measures an averaged response of the cell population.

(A) Preparing adherent cells for DMR measurement ● TIMING **18–24 h**

(i) Detach and collect the desired cells according to your established method.

(ii) Seed cells into Epic 384-well biosensor microplates in the appropriate growth medium using a multichannel pipette at a density sufficient to achieve confluence after 18–24 h. Typically, the cell number for adherent cells is about 10,000–20,000 cells per well. For HEK293 and CHO cells stably transfected to express a receptor of interest, we recommend plating cells in a volume of 30 µl per well at a density of 15,000 and 12,500 cells per well, respectively. For HEK cells, which detach easily from the plate during cell washing, use fibronectin-coated plates; for CHO cells surface-coated plates are not necessary. If desired, increase the plating volume to about 60 µl per well.
▲ **CRITICAL STEP** The exact number of cells to be plated will have to be determined for each cell line and depends on many factors such as cell size and growth rate, the surface of the biosensor microplate (uncoated, fibronectin-coated or synthetic surface) and the transfection procedure.

(iii) Centrifuge the microplate briefly for 1–10 s (130–150*g*) to allow the cells to settle onto the base of each well.

(iv) Maintain cells (mammalian cells) in biosensor microplates at 37 °C, 5% CO_2 for about 18–24 h to allow the cells to fully adhere to the plate and grow to confluency before DMR detection.

(v) Remove growth medium carefully from cells by aspiration to avoid dislodging of cells. Wash cells twice with 30–50 µl per well of DMR assay buffer (supplemented with DMSO if appropriate; see REAGENT SETUP), to obtain comparable conditions for each DMR measurement. After the last washing step, leave a total volume of 30 µl DMR assay buffer in each well. Keep cells in assay buffer for at least 60 min at the same temperature as used in the subsequent measurement (Step 6) to allow temperature equilibration (we routinely perform DMR measurements at ~28 °C).
▲ **CRITICAL STEP** As optical density (OD) is temperature-dependent, differences may impact the DMR response.

(B) Preparing suspension cells for DMR measurement ● TIMING 3–5 h

(i) Prepare suspension cells either by detaching adherent cells or by purification according to your standard protocol, and reconstitute suspension cells in DMR assay buffer supplemented with DMSO, if appropriate (please see REAGENT SETUP for details).

(ii) Plate the desired amount of cells in a total volume of 30 µl of DMR assay buffer into each well. Note that DMR detection in suspension cells requires a higher cell number as compared with adherent cells. We recommend increasing the cell numbers by about threefold (30,000–60,000 cells per well) when reading DMR on suspension cells.
▲ **CRITICAL STEP** The exact cell number to be plated has to be determined for each cell line.

(iii) Centrifuge cells briefly at ambient temperature (20–24 °C) and low speed (100–150*g*, 1–10 s) to allow the cells to settle to the bottom of the microplates. FFA1-HEK (**Fig. 4b**,**d**,**f**), for example, were detached, resuspended in assay buffer at a density of 45,000 cells per well and centrifuged (130*g*, 10 s).
▲ **CRITICAL STEP** In principle, any suspension cell type may be used but sedimentation speed and cell number may have to be optimized depending on cell size and viability after forced sedimentation. It may also be possible to allow cells to settle for a defined period of time to cover the microplate bottom; however, spontaneous sedimentation requires longer time periods and may not be advisable for cells with a critically short life span.

(iv) Keep suspension cells for 60 min at the same temperature as used in the subsequent measurement (Step 6) to allow for temperature equilibration.
▲ **CRITICAL STEP** As OD is temperature-dependent, differences may impact the DMR response.

3| (Optional) If pretreatment of cells with pharmacological inhibitors/toxins/signaling pathway modulators is desired, add these agents at appropriate times before DMR detection. Always include vehicle-treated samples for control purposes in parallel. For example, receptor antagonists may be added 10–60 min before agonist addition, whereas PTX or CTX are typically included for 18–24 h before DMR detection (PTX: 5–100 ng ml^{-1}; CTX: 100–250 ng ml^{-1}) by application of a dilution in cell culture medium to the cells of interest. Note that as the action of PTX and CTX is irreversible, these toxins should not be replenished after washing (adherent cells) or plating (suspension cells). PTX and CTX can also be applied at much higher concentrations for shorter periods of time, such as in primary cells with short lifetimes and/or in suspension cells.
▲ **CRITICAL STEP** Make sure that cells retain viability if short-term exposure to toxins in high concentrations is required.
▲ **CRITICAL STEP** Examine pathway inhibitors for any influence on the baseline signal, as label-free detection is susceptible to perturbations of multiple signaling pathways. To do so, do not simply preincubate cells with the respective pathway inhibitor but monitor potential DMR changes on inhibitor application as described in Steps 6–8. We generally exclude those inhibitors from mechanistic DMR studies that induce heavy effects on cellular DMR when applied alone.

4| Prepare compounds (the desired receptor ligands or pharmacological inhibitors, see EXPERIMENTAL DESIGN section for further information) at 4× final concentration in DMR assay buffer and dispense these solutions into the corresponding wells of the compound source plate. When using a liquid-handling robot, a volume of at least 20 µl per well may be advisable (consider the respective manufacturer's recommendations), but always prepare at least 10 µl more compound than needed in each well of the compound source plate (we typically transfer 10 µl per well of 4× compound from the compound source

plate onto the cell plate (which already contains 30 µl per well DMR assay buffer) in Step 7). If compounds are dissolved in DMSO, make sure that the compound dilutions and the DMR assay buffer are matched exactly with respect to DMSO content to avoid solvent-induced DMR peaks (see REAGENT SETUP and **Supplementary Fig. 1** for DMSO-mediated DMR traces).
▲ **CRITICAL STEP** You may instead prepare compound dilutions before washing (adherent cells, Step 2A(v)) or plating (suspension cells, Step 2B(ii)).
▲ **CRITICAL STEP** Although DMSO mismatch may be tolerated to a certain degree, depending on the nature of expression system, we always recommend adjusting DMSO amounts in compound dilutions and in the washing buffer.

5| Incubate compound microplates to achieve temperature equilibration according to the assay conditions used for the subsequent measurement (Step 6).
▲ **CRITICAL STEP** We routinely perform DMR measurements at 28 °C, but, in principle, other temperatures below and above are equally suitable for DMR detection. Always ensure that compound and cell plates are equilibrated to identical temperatures and that temperature does not vary during the assay.

DMR measurement ● TIMING 1–2 h (or longer if desired)
6| Start the measurement by clicking the 'Start' button to initiate the program. Initially obtain a baseline read (300–400 s are sufficient) to define a zero level and to ensure that the signal is stable. Unstimulated cells attached to the sensor surface typically give a zero-DMR response that defines the baseline signal.

7| Add compound solutions and solvent controls from Step 5 to the cell plate from Step 6 (we typically transfer 10 µl per well of 4× compound from the compound source plate onto the cell plate using the inbuilt liquid-handling robot) and monitor DMR for at least 1 h. One, two or more compound additions are possible (see EXPERIMENTAL DESIGN).
▲ **CRITICAL STEP** DMR readings may be obtained over extended periods of time, but they must be obtained within the time frame allowed to ensure cell viability.
▲ **CRITICAL STEP** Use untransfected cells as specificity controls for receptor agonists if agonists are tested on receptor-transfected cells. If agonists are tested on native or primary cells, include subtype-selective (if available) antagonists or use silencing RNA delivery or cells from knockout animals to verify specificity of the label-free signal.
? TROUBLESHOOTING

8| Repeat compound addition and DMR recording as described in Step 6 if a two-step protocol is applied.
▲ **CRITICAL STEP** Both agonists and antagonists can be added in this second step and monitored for either real-time activation or real-time inhibition profiles.

Data analysis ● TIMING 1–2 h (or more)
9| Import raw data ('Δ pm versus time' values) into a data analysis software such as GraphPad Prism and compute 'Δ pm versus time' mean values with scatter for the respective replicates.

10| Perform a baseline-correction, i.e., subtract the kinetic profile obtained upon buffer addition (Step 6) from each individual ligand-induced signal to compute net 'Δ pm versus time' curves.

11| Quantify the DMR response traces using parameters of interest such as peak Δ pm or area under 'Δ pm versus time curve' if desired and use these values to generate a concentration-effect curve by nonlinear regression using GraphPad Prism (as shown in **Figs. 3a**,**b** and **4a**–**d**, or **Fig. 6**).

12| As cells do not die during the assay procedure, one may decide to entirely reuse the cell plate after careful washing with 30–50 µl of assay buffer per wash. Note that the authors of this protocol have not reused cells for repeated DMR detection. This may be feasible in principle, and it could potentially be applied to receptor desensitization studies.

? TROUBLESHOOTING
In principle, label-free detection is broadly applicable to a diverse range of cells (recombinant, native, primary, adherent and suspension) and straightforward to carry out. Still, it is possible that no or negligible DMR is observed on ligand addition. Please refer to **Table 1** for troubleshooting advice.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	No or insufficient DMR signal	Cells detach during the washing procedure or on ligand addition	Consider switching from uncoated to fibronectin or synthetic-surface plates
		Incorrect number of cells	Adjust the cell number according to the growth rate of cells in order to achieve confluent monolayers for adherent cells and sufficient packing of wells with suspension cells
		Plate surface is not ideal for detection of cell functionality	Compare DMR traces on plates with different surface coating
		Cell type is unsuitable for label-free detection	Vary cellular background for recombinantly expressed proteins
		Inappropriate culture conditions before DMR detection	Culture cells in starvation medium using standard starvation techniques (such as serum starvation) before the assay, but do not initiate starvation before firm attachment of cells
		More than one pathway is activated at the same time with identical kinetics for both positive and negative DMRs (and hence, neutral net responses)	Use pathway affecting agents to unmask activation of hidden signaling pathways
	Spiky DMR peaks with large amplitudes (in pm)	Check whether assay buffer on cells and compound solutions match with respect to concentrations of DMSO or other solvents	Determine the highest DMSO (or other solvent) concentration, which is added during compound addition and adjust DMSO content of washing/assay buffer accordingly

● TIMING

Step 1, Cell culture: variable
Step 2, Pre-measurement preparations: up to 24 h for adherent cells; 3–5 h for suspension cells.
Step 3 (Optional): Variable
Steps 4 and 5: Variable, depending on the number, concentrations and chemical properties to be examined, but compound source plates may be prepared while temperature equilibration in Step 2 takes place.
Steps 6 and 7, DMR measurement: variable; may last 60 min or may be extended to several hours, if system pharmacological data are to be obtained or if different types of compounds (activators and inhibitors) are examined for their real-time profiles in the same wells.
Steps 9–11, Data analysis: variable; may require 1–2 additional h or more, particularly if separate regions of interest of kinetic profiles are utilized for derivation of pharmacological measures of potency or efficacy.

ANTICIPATED RESULTS

Typical DMR experiment on cells engineered to overexpress GPCRs

A typical DMR experiment using CHO cells that overexpress the $G\alpha_{i/o}$-sensitive muscarinic M2 receptor, the $G\alpha_q$-linked muscarinic M3 receptor and the $G\alpha_s$-coupled adrenergic β_2 receptor (β2AR) is shown in **Figure 6**. On stimulation of cells with the respective agonist ligands, characteristic optical recordings were obtained that were concentration- and time-dependent and varied with the primary signaling pathway of each receptor (**Fig. 6a–c**). The absence of optical responses on native CHO cells for all three ligands (**Fig. 6d–f**) demonstrates receptor specificity of the DMR signals in the recombinant cell lines.

Conversion of optical traces into concentration-effect curves

Analysis of optical traces and their conversion into concentration-effect curves may at first glance appear demanding. This is because they may be rather complex and sometimes multiphasic in nature, may differ in amplitude, shape and signal direction over time and may be both downward- and upward-deflected for a single stimulus (e.g., **Fig. 6a**). Such observations bear significance for data evaluation as it is a priori not clear how best to extract pharmacological parameters from

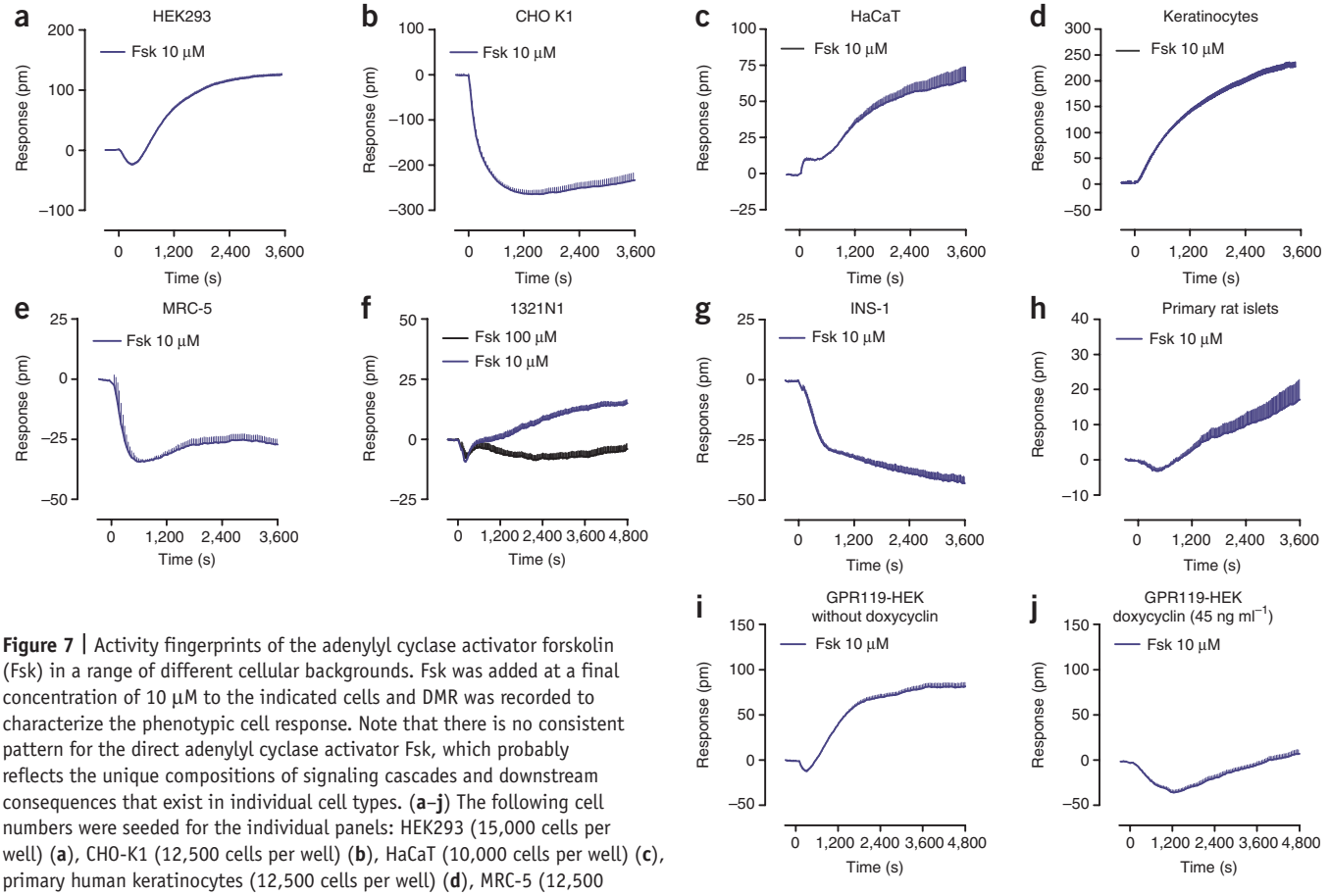

Figure 7 | Activity fingerprints of the adenylyl cyclase activator forskolin (Fsk) in a range of different cellular backgrounds. Fsk was added at a final concentration of 10 μM to the indicated cells and DMR was recorded to characterize the phenotypic cell response. Note that there is no consistent pattern for the direct adenylyl cyclase activator Fsk, which probably reflects the unique compositions of signaling cascades and downstream consequences that exist in individual cell types. (**a**–**j**) The following cell numbers were seeded for the individual panels: HEK293 (15,000 cells per well) (**a**), CHO-K1 (12,500 cells per well) (**b**), HaCaT (10,000 cells per well) (**c**), primary human keratinocytes (12,500 cells per well) (**d**), MRC-5 (12,500 cells per well) (**e**), 1321N1 (15,000 cells per well) (**f**), INS-1 (30,000 cells per well) (**g**), primary rat islets (15,000 cells per well) (**h**), GPR119-HEK (15,000 cells per well) (**i**,**j**). (**i**,**j**) GPR119-HEK cells express the constitutively active Gs-linked GPR119 in an inducible (doxycyline-dependent) manner. In **i**, no doxycyclin was added; in **j**, receptor expression was induced with the indicated amount of doxycyclin. Note the characteristic change of Fsk trace when the cellular context is altered (low versus high endogenous cAMP production). Shown are representative data (means + s.e.m.) of at least three independent experiments each performed in triplicates.

multiphasic traces and it might be anticipated that pharmacological estimates for ligand potency (pEC_{50} values) or efficacy (maximal response) would depend on the time point or part of response profile chosen to derive these pharmacological parameters (peak DMR, DMR after defined time intervals, area under the curve over a selected or the entire time interval, ascending or descending slopes of separate regions of interest). **Figure 6g–i** illustrates different modes of data analysis to convert the kinetic profiles into concentration-effect relationships for each receptor. For the three examples presented here, all modes of quantitative data evaluation lead to essentially identical potency estimates for the activating ligands (**Fig. 6g–i**). Nevertheless, we advise researchers to test and compare different modes of quantitative data evaluation for individual trace profiles, as, in fact, distinct cellular phenomena may be masked in individual parts of the information-rich traces.

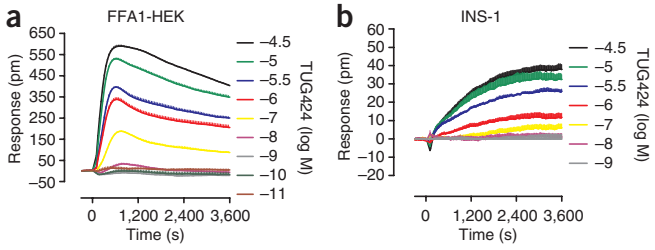

Figure 8 | Activity profiles of a selective small-molecule FFA1 agonist are not consistent across diverse cellular backgrounds. (**a**,**b**) FFA1-HEK and rat insulinoma INS-1 cells were plated (15,000 cells per well in **a**; 30,000 cells per well in **b**) and DMR was recorded on stimulation with the small-molecule agonist TUG424 (ref. 30) as a measure of FFA1 receptor activity. It is apparent that FFA1 traces are not universal across different cell lines, but exhibit strong cellular context-dependency and very noticeable differences in signal amplitudes and kinetics. Depicted are optical traces (means + s.e.m.) representative for at least three such experiments.

DMR biosensor signals are cellular context-dependent

As optical traces derived from stimulation of GPCRs that couple to different effector pathways can be distinguished by signal amplitude and duration, it is tempting to link phenotypic cell responses to defined receptor coupling mechanisms simply by visual inspection. Although the overall logic of signaling circuits and the function of

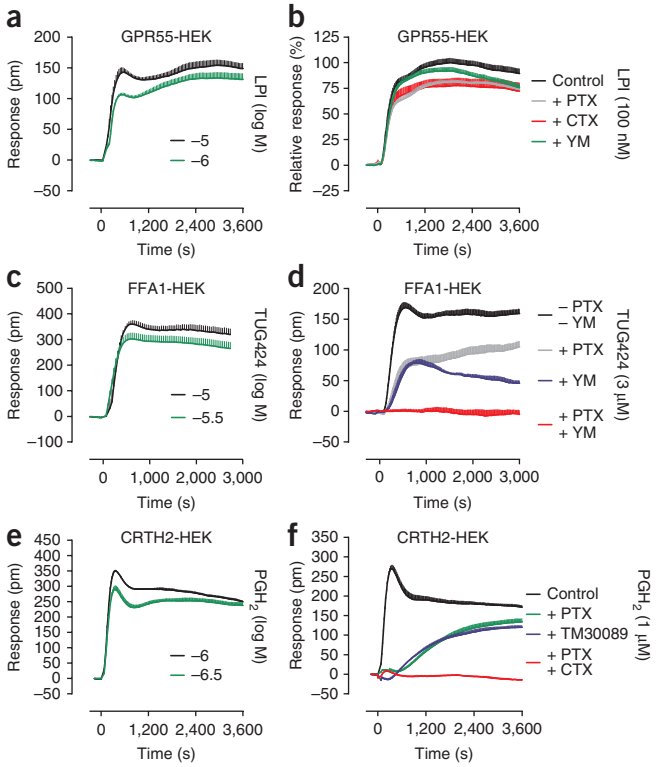

Figure 9 | Signaling profiles for a given cell type do not necessarily represent signaling pathway indicators. (**a**) GPR55-HEK cells (10,000 cells per well) were stimulated with the indicated concentrations of lysophosphatidylinositol (LPI) and receptor activation is recorded over time. (**b**) GPR55 traces originating from engagement of $G\alpha_{13}$ proteins[18] are insensitive to pretreatment of cells with the signaling pathway modulators PTX, CTX and YM, masking $G\alpha_{i/o}$-, $G\alpha_s$- and $G\alpha_q$-activation, respectively. (**c**) The $G\alpha_q/G\alpha_i$-sensitive receptor FFA1 is stimulated with the selective small-molecule agonist TUG424. (**d**) FFA1 signaling is partially sensitive to pretreatment with PTX and YM, but completely abolished in the presence of a combination of both inhibitors. (**e**) Activity profiles of prostaglandin H_2 (PGH_2) in HEK293 cells stably expressing the $G\alpha_i$-coupled CRTH2 receptor. (**f**) For PGH_2, it is important to recognize that the signal is derived from a biological response to three receptors (CRTH2 and prostanoid EP2 and EP4, respectively) and two signaling pathways ($G\alpha_{i/o}$ and $G\alpha_s$). Accordingly, PGH2-mediated traces are partially sensitive to pretreatment with the $G\alpha_{i/o}$ inhibitor PTX (green trace) as well as TM30089 (ref. 31; CAY10471), a selective antagonist for the $G\alpha_i$-coupled CRTH2 receptor (blue trace). PGH_2 responses were completely abolished when both $G\alpha_{i/o}$ and $G\alpha_s$ signaling was prevented with a combination of PTX and CTX, respectively (red traces). Shown are representative data (means + s.e.m.) of at least three independent experiments, each performed in triplicate.

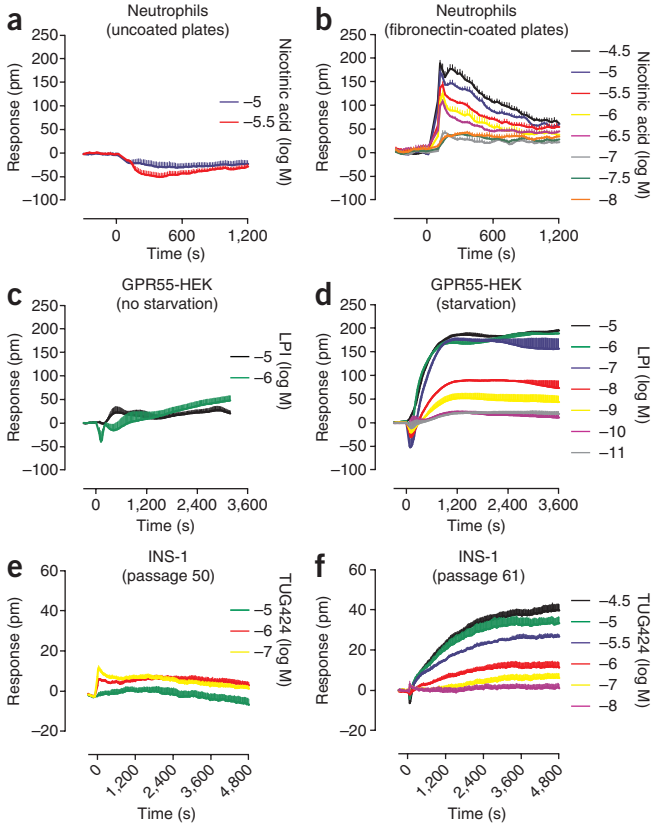

Figure 10 | Selected DMR experiments that required troubleshooting to obtain meaningful results. (**a**,**b**) Nicotinic acid–mediated stimulation of DMR in neutrophils purified from human blood is barely detectable on uncoated biosensor microplates (**a**), but distinct activation profiles can be measured on fibronectin-coated microplates (**b**). Neutrophils (60,000 cells per well) were purified from human blood using standard procedures[33] and then processed as described for suspension cells in this protocol. (**c**,**d**) HEK293 cells stably expressing GPR55 were seeded at a density of 10,000 cells per well. 0.1% (vol/vol) BSA was included in the assay buffer for DMR measurement of GPR55-HEK cells and temporal response profiles for the GPR55 agonist lysophosphatidylinositol (LPI) were recorded in the absence (**c**) and presence (**d**) of starvation medium. Note that GPR55 activation was recorded only when cells were serum-starved for 18–24 h prior to functional DMR detection. (**e**,**f**) Functionality of the free fatty acid receptor FFA1 can be detected in the rat immortalized insulin-producing INS-1 cell line (15,000 cells per well) at a high (**f**) but not at a low passage number (**e**) after challenging with the selective small-molecule FFA1 agonist TUG424 (ref. 30). Successful DMR experiments were performed with INS-1 cells from passages 55–80.

individual components may be preserved across different cellular backgrounds, individual components as well as their interaction partners are likely to differ in the relative stoichiometry in a cellular context-dependent manner. This is illustrated for the adenylyl cyclase–activating agent forskolin when applied to a variety of different immortalized and primary cells that all contain the adenylyl cyclase–cAMP-PKA module yet differ in shape of the optical signal (**Fig. 7**). Optical traces induced by the small-molecule FFA1-agonist TUG424 (ref. 30) also hold different shape characteristics depending on the cellular context in which the long-chain free fatty acid receptor FFA1 is expressed (**Fig. 8**). As a consequence, signature profiles must not generally be linked to defined pathways; rather, they must be determined *de novo* for each cellular background.

Signaling profiles for a given cell type do not necessarily represent signaling pathway indicators

Even signatures obtained within a defined cellular context should not be interpreted on the basis of historical data and linked to defined signaling events, but rather be subjected to signal deconvolution if mechanistic information is to be extracted. This is illustrated for three optical traces that share very similar signal amplitudes and duration yet originate from

three distinct signaling events within the same cell line (**Fig. 9**). Nonetheless, for a cell line overexpressing a given receptor, signature profiles may be established with reference ligands, and variations of such profiles in the presence of distinct ligands acting through the same receptor may be viewed as indicative of pharmacological alteration of cellular signaling activity.

Lack of DMR response on ligand addition

Although label-free DMR technology is an easy-to- adopt, user-friendly and broadly applicable assay platform, individual cell lines and receptors may turn out to be more challenging to handle than anticipated, and no or insignificant DMR amplitudes may be observed when starting to establish the protocol. We offer a number of general recommendations, such as varying microplate surface coating, cell number, assay buffer or culture conditions prior to the DMR assay; furthermore, here we present three specific examples from experiments that required troubleshooting to obtain meaningful results (**Fig. 10**). However, we wish to remark that there is no general recipe to obtain meaningful results; each individual cell line or receptor needs to be explored on a case-by-case basis to achieve analyzable data.

Note: Supplementary information is available via the HTML version of this article.

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