Chapter 7

Screening G Protein-Coupled Receptors: Measurement of Intracellular Calcium Using the Fluorometric Imaging Plate Reader

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Abstract

G protein-coupled receptors (GPCRs) are the target of approximately 40% of all approved drugs and continue to represent a significant portion of drug discovery portfolios across the pharmaceutical industry. As a result, GPCRs are the focus of many high-throughput screening (HTS) campaigns. Historically, ligand-binding assays were used to identify compounds that targeted GPCRs. Current GPCR drug discovery efforts have moved toward the utilization of functional cell-based assays for HTS. Many of these assays monitor the accumulation of a second messenger such as cAMP or calcium in response to GPCR activation. Calcium stores are released from the endoplasmic reticulum when $G_{\alpha q}$-coupled GPCRs are activated. Although $G_{\alpha i}$- and $G_{\alpha s}$-coupled receptors do not normally result in this mobilization of intracellular calcium, they can often be engineered to do so by expressing a promiscuous or a chimeric G_{α}protein, which couples to the calcium pathway. Thus calcium mobilization is a readout that can theoretically be used to assess activation of all GPCRs. The fluorometric imaging plate reader (FLIPR) has facilitated the ability to monitor calcium mobilization in the HTS setting. This assay format allows one to monitor activation and inhibition of a GPCR in a single assay and has been one of the most heavily utilized formats for screening GPCRs.

Key words: GPCR, FLIPR, Heterotrimeric G proteins, Calcium mobilization, HTS.

1. Introduction

In the early days of GPCR drug discovery, there was limited choice with respect to assay formats and researchers relied primarily on radioligand-binding assays. The advances in understanding GPCR biology coupled with the introduction of new assay technologies have permitted the implementation of functional cell-based assays

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in HTS campaigns *(1)*. Perhaps the most widely used functional assay for screening GPCRs has been the measurement of calcium mobilization. This assay is applicable for $G_{\alpha q}$-coupled GPCRs because this signaling pathway culminates in the release of calcium stores from the endoplasmic reticulum into the cytosol. $G_{\alpha i}$- and $G_{\alpha s}$-coupled GPCRs do not normally signal through this pathway, but they can be engineered to do so by expressing chimeric *(2, 3)* or promiscuous *(4)* G_{α} proteins. The basic principle of the calcium mobilization assay is to load cells with a calcium-sensitive dye, which fluoresces when bound to calcium; therefore, an increased fluorescence signal is indicative of activation of the target GPCR.

The utilization of this assay in HTS applications was enabled by the development of the FLIPR (Molecular Devices Corporation, Sunnyvale, CA) in the mid-1990s *(5)*. Other instruments with similar capabilities such as the FDSS (Hamamatsu Corporation, Hamamatsu City, Japan) and the CellLux (Perkin Elmer, Waltham, MA) have been released in recent years. The latest FLIPR model, FLIPR$^{TETRA™}$, has an integrated 96-, 384-, or 1536-well pipettor and is able to accommodate multiple reagent reservoirs allowing one to perform multiple additions to the assay plate. The earlier FLIPR models used an argon ion laser, but the FLIPR$^{TETRA™}$ uses LEDs to excite the microtiter plate. The image of each well is captured simultaneously by a cooled charge-coupled device (CCD) camera, which is capable of updating images once per second. This is critical for measurement of the rapid calcium response that is typically observed for GPCRs.

The kinetic data obtained on the FLIPR essentially provides a fingerprint for activation of the target GPCR. It enables one to extract more information regarding the activity of a test compound as compared to a single-read endpoint assay. The kinetic profile of an agonist can vary slightly depending on the GPCR and/or the cell background. The agonist-induced increase in intracellular calcium may be transient and return to baseline levels relatively quickly (**Fig. 7.1A**). Alternatively, in some instances, the level of intracellular calcium remains elevated for an extended period of time following agonist treatment (**Fig. 7.1B**). Knowing what the kinetic profile of a true agonist looks like can help the researcher distinguish between compounds that have agonist activity and those that are likely false positives (**Fig. 7.1C,D**). The most obvious false positive is a fluorescent compound that elicits a very distinct kinetic profile characterized by an extremely rapid increase in fluorescence signal, which remains relatively unchanged over time (**Fig. 7.1D**).

The experimental workflow for a calcium mobilization assay on the FLIPR is shown in **Fig. 7.2**. Briefly, cells expressing the GPCR of interest are plated the day prior to the assay, the cells are incubated with a cell-permeable calcium-sensitive dye and then the plate is placed on the FLIPR for the assay. Here we describe the

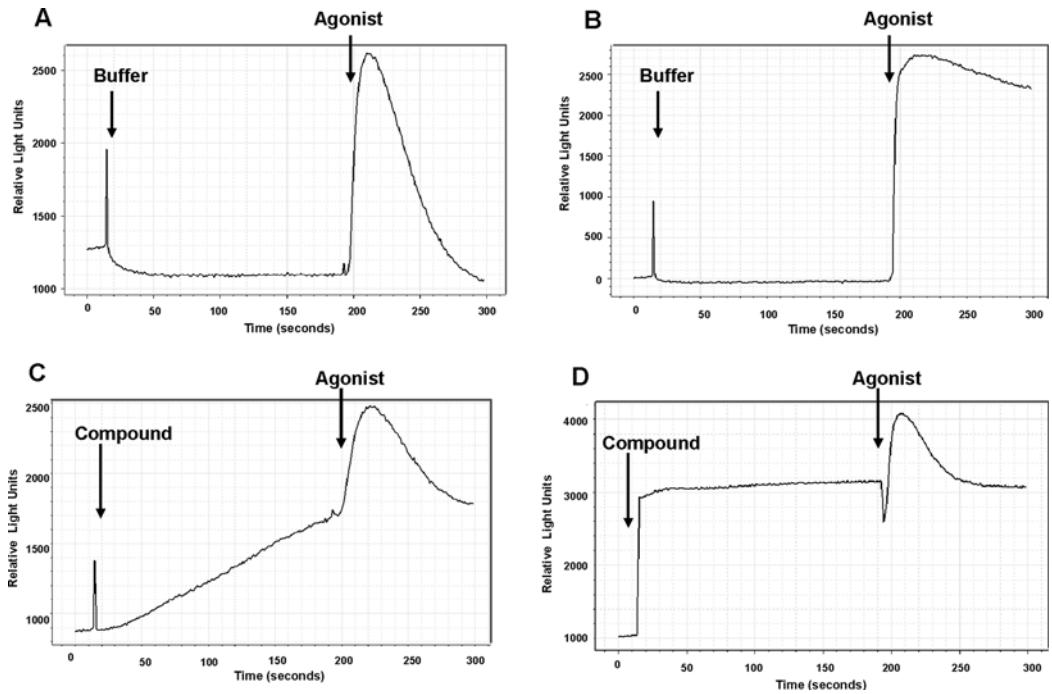

Fig. 7.1. **Representative kinetic profiles of a calcium mobilization assay**. CHO-K1 cells stably expressing a promiscuous G_{α} protein and a $G_{\alpha s}$-coupled GPCR were subjected to a calcium mobilization assay using a standard two-addition format performed on the FLIPR$^{TETRA^{®}}$. The first addition was either assay buffer (**A** and **B**) or test compound (**C** and **D**). The fluorescence signal was captured for 3 minutes and then a known agonist was added to the cell plate (**A**–**D**) and the fluorescence signal was monitored for an additional 2 minutes. (**A**) A representative kinetic profile of a GPCR that exhibits a transient increase in intracellular calcium upon treatment with an agonist. (**B**) An example of a GPCR that has a prolonged response to agonist. (**C**) An example of a compound that results in an increase in intracellular calcium, but with different kinetics than a known agonist. (**D**) A representative kinetic profile of a fluorescent compound. The sharp spike in fluorescence signal at the beginning of the kinetic profiles in **A**–**C** is an artifact of the clear FLIPR tips entering the well prior to the first addition.

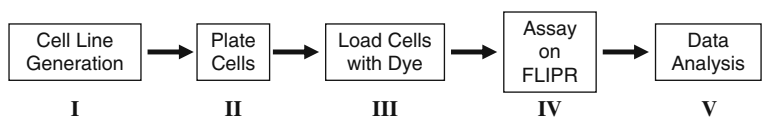

Fig. 7.2. Experimental flow scheme for a calcium mobilization assay on FLIPR.

method for a two-addition calcium mobilization assay on the FLIPR$^{TETRA^{TM}}$ for a $G_{\alpha s}$-coupled GPCR that was stably coexpressed with the promiscuous G protein, $G_{\alpha 16}$, in CHO-K1 cells. Recommendations regarding alternative conditions and parameters that may be applicable to other GPCRs and/or cell lines are also discussed.

2. Materials

2.1. Cell Culture

1. Cell culture medium for CHO-K1 cells: Ham's F-12 (Mediatech Inc., Manassas, VA) supplemented with 10% characterized fetal bovine serum (FBS, Hyclone, Ogden, UT).
2. Solution of trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.53 mM) from Mediatech, Inc.
3. Phosphate-buffered saline (PBS) from Mediatech, Inc.
4. T175 tissue culture flasks (Greiner Bio-One, Monroe, NC).
5. 384-well black-walled, clear-bottom tissue culture-treated microtiter plates (Greiner Bio-One).
6. Multidrop (Thermo Scientific, Waltham, MA) or equivalent liquid dispenser for 384-well format.
7. FuGENE^{TM} 6 transfection reagent from Roche (Indianapolis, IN).

2.2. Calcium Mobilization Assay

1. Assay buffer: 20 mM HEPES, 11.1 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM NaCl, 5 mM probenecid, and adjust pH to 7.4. Store at 4°C and warm to room temperature before use.
2. 500 mM probenecid (MP Biomedicals, Solon, OH) made in 1 N NaOH and stored at room temperature.
3. Fluo-4/AM cell permeant from Invitrogen (Carlsbad, CA) is solubilized to a 1 mM stock solution with 100% DMSO by sonicating for 10 minutes. This stock solution is stable at –20°C.
4. The 2 μM Fluo-4/AM dye-loading solution is prepared on the day of assay as follows. Mix the necessary volume of 1 mM Fluo-4/AM with an equal volume of 20% Pluronic F-127 in DMSO (Invitrogen). Add this solution to the appropriate amount of assay buffer. The final dye-loading solution consists of 2 μM Fluo-4/AM and 0.04% Pluronic F-127 in assay buffer and should not be stored and used the next day.
5. 384-well clear FLIPR tips from Axygen (Union City, CA).

3. Methods

The method presented here was used to enable a calcium mobilization assay for a family of $G_{\alpha s}$-coupled GPCR using the FLIPR-$^{TETRA TM}$. The signaling of this GPCR was redirected to the calcium mobilization pathway by generating a stable cell line

(*see* **Note 4.1, item 1**) that coexpresses the GPCR and $G_{\alpha 16}$(*see* **Note 4.1, item 2**). Chinese hamster ovary (CHO-K1) cells (ATCC, Manassas, VA) were used for these studies. These cells are adherent, but nonadherent cells can also be used for these types of assays (*see* **Note 4.1, item 3**).

3.1. Cell Line Generation

Several methods exist to introduce the target GPCR and G_{α} proteins (if needed) into cells. The most commonly used methods for transfection include calcium phosphate, electroporation, or newer lipid transfection reagents such as FuGENE™ 6. The example presented here utilizes FuGENE™ 6.

1. The optimal DNA:FuGENE™ 6 ratio is determined by performing several small-scale transfections in which the concentration of a plasmid encoding green fluorescent protein (GFP) and FuGENE™ 6 is varied. The GFP signal is monitored and used to determine the DNA:FuGENE™ 6 ratio for optimal transfection efficiency.
2. Seed cells in a 6-well plate at a density of 1×10^4cells per well in 3 mL of growth medium for 24 hours prior to transfection. Maintain cells in an incubator set at 37°C/5% CO_2. Transfect cells with 6 µL of FuGENE™ 6 + 1.5 µg of DNA according to the manufacturer's instructions (*see* **Note 4.1, item 4**).
3. Harvest cells 48 hours posttransfection and seed a T175 flask containing growth medium supplemented with the appropriate concentration of antibiotic encoded by the transfected plasmid. Maintain cells under antibiotic selection and monitor for the formation of drug-resistant colonies.
4. Harvest the cells once colonies have developed. This cell population is called the stable "pool." A clonal line is isolated by single-cell sorting cells into a 96-well plate with the selection media (*see* **Note 4.1, item 5**). Other methods, such as seeding cells into 100 mm dishes and isolating colonies with cloning rings, may also be used. Wells are monitored over 2 weeks and wells with single colonies are harvested for testing and expansion. Each colony is tested in the FLIPR for correct response to agonist. Those clones with a positive response are chosen for further characterization with known agonists and antagonists.
5. Once a clonal cell line is chosen, expand the cells and make a liquid nitrogen cell bank for long-term storage. Cells are frozen at a density of 1×10^7cells/mL in 90% FBS/10% DMSO.
6. Cells are maintained in culture by splitting every 3 days with a seeding density of 3×10^6cells in a T175 flask (*see* **Note 4.1, item 6**).

3.2. Cell Plating

1. Cells for the calcium mobilization assay should be in exponential growth for optimal response.
2. Harvest cells with trypsin–EDTA (*see* **Note 4.2, item 1**).
3. Seed cells in a 384-well black-walled, clear-bottom plate (*see* **Note 4.2, item 2**) at a density of 10,000 cells per well (*see* **Note 4.2, item 3**) in 50 µL of growth medium.
4. Leave plates in a single layer at room temperature for at least 60 minutes (*see* **Note 4.2, item 4**).
5. Incubate plates overnight in a 37°C/5% CO_2 incubator.

3.3. Loading Cells with Dye

1. Prepare dye-loading solution (2 µM Fluo-4/AM, 0.04% Pluronic F-127 in assay buffer) (*see* **Note 4.3, items 1–2**).
2. Completely remove media from the cell plate (*see* **Note 4.3, item 3**). This can be done by manually inverting the plate and flicking the media out of the plate or by using a plate washer to aspirate the media from the wells (*see* **Note 4.3, item 4**).
3. Add 20 µL per well of the dye-loading solution and place the plate into a 27°C incubator in a single layer for 60 minutes (*see* **Note 4.3, item 5**).
4. After 60 minutes, remove dye and replace with 20 µL per well of assay buffer (*see* **Note 4.3, item 6**).

3.4. Assay on FLIPR

These instructions are for an assay performed on the FLIPR$^{TETRA™}$ instrument in 384-well format using LED excitation at 470–495 nm and emission at 515–575 nm. These instructions can be adapted to other instruments that can read fluorescent signals in real time and have liquid handling capabilities such as those mentioned in **Section1**.

Prior to running the assay, a protocol file needs to be written on the FLIPR. The protocol file tells the instrument where the plates are located on the deck, the type of plates being used, the type of pipettor head (96, 384, or 1536), the excitation and emission wavelengths, and the height and speed for the integrated pipettor (*see* **Note 4.4, item 1**). The protocol also defines the length of the assay, when to add the reagents, and the frequency that images are captured during the run.

The protocol for the example presented here consists of two additions to the cell plate (*see* **Note 4.4**) and has the following settings:

a. Ten reads are collected with a 1-second read interval prior to the first addition.

b. First addition: add 20 µL of compound. The dispense speed is 20 µL/second and the height is 20 µL. Images are collected at 1-second interval for 3 minutes.

c. Second addition: add 20 μL of agonist with a dispense speed of 20 μL/second and a height of 40 μL. Images are collected at 1-second interval for 2 minutes.

1. After dye loading is complete, place the cell plate in the read position on the FLIPR$^{TETRA^{TM}}$. Wait for 5 minutes before running the assay to allow the cells to stabilize. Reading the plate immediately after dispensing buffer will result in a drift in the response.
2. Place the reservoirs containing the reagents to be added to the plate in the appropriate positions on the FLIPR. The reservoir for the first addition consists of compound at twice the desired final concentration. The reservoir for the second addition contains agonist at three times its EC_{80} concentration.
3. Place pipet tips in tip-loading position on the FLIPR (*see* **Note 4.4, item 2**).
4. Perform a signal test to determine the dye-loading efficiency of the cells and the variability across the plate. Set the LED camera gain to 100 and the excitation intensity at 70 with the CCD camera exposure held constant at 0.4 seconds. The CCD camera takes a picture of the entire plate and the images are converted to a numerical readout called relative fluorescent units (RFU). The maximal saturation of the LEDs in the FLIPR$^{TETRA^{TM}}$ is reported to be 9000 RFU; however, the authors' experience is that the saturation limit is 6000 RFU. Above 6000 RFU a saw-tooth pattern is visible in the data, which can result in false positives. Once the signal test is performed, the gain and excitation should be adjusted so that the average RFU across the plate is 1000. In addition to viewing the RFUs, it is possible to see the image of the plate. The image is extremely helpful in identifying smudges or lint that may be causing high or low RFU values in specific wells. It also helps to visualize the cell monolayer and patterns resulting from cell plating, dye, or buffer additions. The overall %CV for a 384-well plate under optimal culturing and assaying conditions should be $<10\%$.
5. Once the signal test is complete and the camera gain and the excitation intensity are set, the run can be initiated.

3.5. Data Analysis

The software provided with the FLIPR instrument is used to reduce and export the data for additional analysis.

1. Apply spatial uniformity correction to the plate (*see* **Note 4.5, item 1**).
2. Define the time sequences for the first and second response as 20–180 seconds and 200–300 seconds, respectively. These sequences are termed "time cuts" (*see* **Note 4.5, item 2**).

3. Identify the maximal signal over each time cut using the FLIPR software and export the data as a statistics file.
4. This data can then undergo additional analysis such as log–concentration response curves using graphing applications such as GraphPad Prism® (San Diego, CA).

4. Notes

The method provided here describes the assay for one GPCR and the conditions will not necessarily apply to all GPCRs. The parameters as presented here may require some modifications in order to obtain an optimized assay for other GPCRs. In this section we describe the parameters that are often examined to deliver an optimized assay.

4.1. Cell Line Generation

1. A stable cell line expressing the target GPCR and promiscuous or chimeric G_α protein (where applicable) is not always required. Transiently transfected cells have been successfully used in calcium mobilization assays performed on the FLIPR *(6, 7)*.
2. The redirection of $G_{\alpha i}$- or $G_{\alpha s}$-coupled GPCRs to the calcium mobilization pathway by chimeric *(2, 3)* or promiscuous *(4)* $G_\alpha$proteins may not be without consequence. Alteration of ligand pharmacology in terms of efficacy and potency has been reported for some GPCRs that have been redirected to the calcium pathway *(4, 8, 9)*.
3. The development of no-wash calcium dyes (*see* **Note 4.3**) has significantly simplified the protocol for suspension cells in the calcium mobilization assay *(10, 11)*.
4. When both the target GPCR and a G_α protein need to be transfected into cells, one can either cotransfect both plasmids simultaneously or transfect them in series. The authors have had success with the latter method. Typically the G_α protein is transfected and a stable pool of cells is generated, which is then transfected with the target GPCR and stable clones are isolated and screened for response. In this instance, it is critical that the plasmids used encode different antibiotic resistance.
5. The pool of stably transfected cells may provide an acceptable assay, thus eliminating the need to screen and select a clonal line. However, due to the cell-to-cell variability of plasmid transfections, a better assay is generally obtained by generating a single-cell clonal line. The authors have had success using retroviral gene transduction to create stable cell lines

that perform well in this assay without isolating a clone. This is attributed to less variability in the expression level of the transduced GPCR across the cell population than is observed with plasmid transfections.

6. It may be possible to use cells directly from frozen stocks rather than continuously culturing cells for the assay. This approach has been successful for a number of laboratories *(12, 13)*, but it needs to be tested on a case-by-case basis. If the cells are amenable to use directly from frozen stocks, it can ease the burden of continuously culturing large amounts of cells during an HTS campaign. Moreover, if a large single batch of cells is frozen in aliquots for a screen, it may improve the day-to-day variability in response that is typically observed for cell-based assays.

4.2. Cell Plating

1. Some GPCRs contain trypsin-sensitive sites in their extracellular domain, resulting in a decreased response when the cells are harvested using trypsin. In these cases an enzyme-free dissociation buffer is recommended for harvesting the cells.
2. Nonadherent cells or cells that are not strongly adherent, such as HEK293 cells, may perform better on plates coated with agents such as poly-D-lysine, collagen, or fibronectin.
3. The optimal cell density needs to be determined for each cell line used. This should be done by plating an entire plate at a single density and examining the variability across the plate. Adjustments in the cell density can often overcome variability issues such as edge effects. Cell densities typically fall in the range of 10,000–25,000 cells per well.
4. Incubation of newly seeded plates at room temperature prior to placing them in a 37°C/CO_2incubator has been reported to reduce edge effects *(14)*.

4.3. Loading Cells with Dye

The example presented here used Fluo-4/AM dye; however, additional cell-permeable calcium-sensitive dyes are available, including Calcium Green-1 (Molecular Probes, Leiden, Netherlands) and Fluo-3/AM (Molecular Probes). Kits utilizing no-wash dyes are also available and have the advantage of eliminating the need to replace the dye solution with assay buffer prior to the assay. These no-wash dyes include a quenching agent in the formulation thereby reducing the background fluorescence caused by the extracellular dye. Such kits include Screen Quest^{TM} Fluo-8/NW Calcium Assay Kit (ABD Bioquest, Sunnyvale, CA), BD^{TM} Calcium Assay Kit (BD Biosciences, Rockville, MD), Fluo-4/NW Calcium Assay Kit (Invitrogen, Carlsbad, CA), and FLIPR Calcium 3 or Calcium 4 Assay Kits (Molecular Devices, Sunnyvale, CA). The decision of which dye to use should be determined experimentally as the optimal dye can vary depending on the GPCR and/or the cell type *(15)*.

More recently the use of photoproteins, such as aequorin *(16, 17)* and Photina® *(18)*, have been widely adopted to monitor intracellular calcium levels. These proteins enzymatically generate a luminescent signal upon elevation of intracellular calcium and may offer a larger signal-to-background ratio than the calcium sensitive dyes. Plate readers capable of reading flash luminescence include FLIPR^3, FLIPR^{TETRA}, LumiLux® (Perkin Elmer, Waltham, MA), and CyBi®-Lumax flash HT (CyBio, Jena, Germany).

1. The optimal concentration of dye will vary depending on the cells and the dye used.
2. Some cell lines require the inclusion of the anion transporter inhibitor probenecid in the assay buffer to prevent the efflux of dye from the cells during the experiment. CHO cells are an example of a cell line that requires probenecid during and after the dye-loading step.
3. It is very important to completely remove all media from the wells prior to dye loading the cells. Residual media in the well can result in poor assay performance in terms of both response and variability.
4. Maintenance of the integrity of the cell monolayer is important because the FLIPR is designed to collect fluorescence from the bottom of the well. This will result in improved variability and robustness of the assay.
5. The incubation temperature and time for dye loading are cell line dependent and will need to be determined on a case-by-case basis. The authors' experience has been that most cells are compatible with dye loading at room temperature; however, some cells may perform better when the dye loading is performed at an elevated temperature such as 37°C. Typical time for dye loading is 1–2 hours.
6. When using dyes that are not classified as "no-wash" such as Fluo-3/AM or Fluo-4/AM, it is necessary to remove the dye and replace it with assay buffer prior to performing the assay on the FLIPR. Many protocols include one or more iterations of washing following removal of the dye. This can reduce the performance of the assay and increase variability due to the disruption of the cell monolayer during the washing process. The authors' experience is that additional washing steps are not required and simply exchanging the dye with assay buffer generally suffices.

4.4. Assay on FLIPR

The FLIPR is capable of performing more than one reagent addition to the cell plate. This allows one to design an assay that can detect both agonism and antagonism in the same experiment. The assay consists of two reagent additions to the cell plate (**Fig.7.3A**). First, compound is added and then after a defined period of time, a known agonist is added to the cell plate. A submaximal

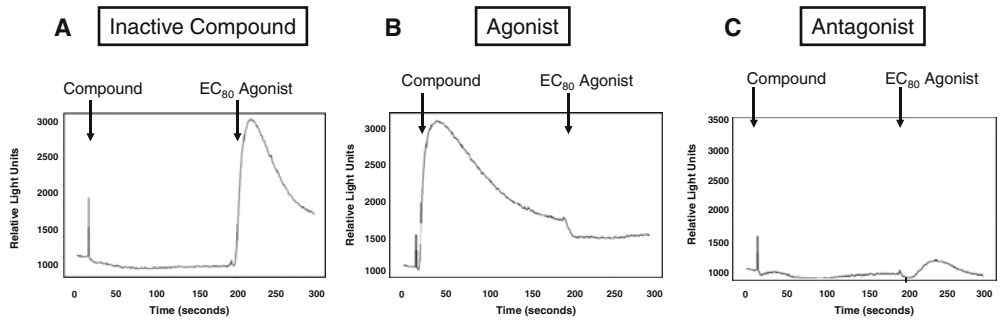

Fig. 7.3. **Two-addition FLIPR assay for detection of agonists and antagonists**. CHO-K1 cells expressing a promiscuous G_{α} protein and a $G_{\alpha s}$-coupled GPCR were assayed using a standard two-addition calcium mobilization assay in 384-well format on the FLIPR$^{TETRA®}$. The first addition consisted of test compound and the fluorescence signal was captured for 3 minutes. Then an EC_{80} concentration of a known agonist was added to the cell plate and the fluorescence signal was monitored for an additional 2 minutes. (**A**) The kinetic profile of an inactive compound that does not elicit an increase in fluorescence signal and that does not alter the response to the agonist. (**B**) The kinetic profile of a compound that exhibits agonist activity. This compound induces an increase in fluorescence upon addition to the cells and reduces the subsequent response to the known agonist due to receptor desensitization. (**C**) The kinetic profile of a compound with antagonist activity. This compound does not result in an increase in fluorescence, but does reduce the subsequent agonist-induced response. The sharp spike in fluorescence signal at the beginning of the kinetic profiles is an artifact of the clear FLIPR tips entering the well prior to the first addition.

concentration of agonist is used, typically an agonist concentration corresponding to the EC_{80}–EC_{90}. The agonist activity of the compound is monitored following the first addition and is indicated by an increased fluorescent signal (**Fig. 7.3B**). The antagonist activity of the compound is assessed after the second addition. An antagonist results in a reduced response elicited by the addition of the known agonist (**Fig. 7.3C**). Note that many GPCRs desensitize in which case any compounds that exhibit agonism will fail to respond to the second addition of the known agonist. Thus, these compounds will appear to be antagonists as well as agonists. Care needs to be taken to exclude these compounds from follow-up as antagonists.

1. The FLIPR is designed to collect fluorescence from the bottom of the well, so it is important to maintain the integrity of the monolayer of cells. This will improve the variability and robustness of the assay. The dispense speed and height of the integrated pipettor on the FLIPR should be adjusted to minimize disruption of the cells while maintaining ample mixing of the reagent that is added to the well.
2. The use of clear pipet tips on the FLIPR may result in a spike of fluorescence signal as the tips enter the well during reagent additions to the cell plate. This can be observed in the kinetic profiles shown in **Figures 7.1** and **7.3**. This artifact can be eliminated by using black pipet tips.

4.5. Data Analysis

The authors have found that the ability to visualize the kinetic profiles at the time of data review is very valuable. This allows one to quickly identify instances of desensitization of the receptor by a compound with agonist activity. In addition, false positives such as fluorescent compounds or compounds that elicit a strange kinetic profile can be quickly identified and eliminated from follow-up (**Fig. 7.1C–D**). This can expedite the postscreening hit assessment phase.

1. A number of data processing methods exist in the FLIPR software and are used to compensate for variations in data due to variations in cell density, fluorescent intensity, dye loading, or other variability associated with the assay run. The data processing algorithms include, but are not limited to, spatial uniformity correction, subtract bias, response over baseline, negative control correction, and positive control

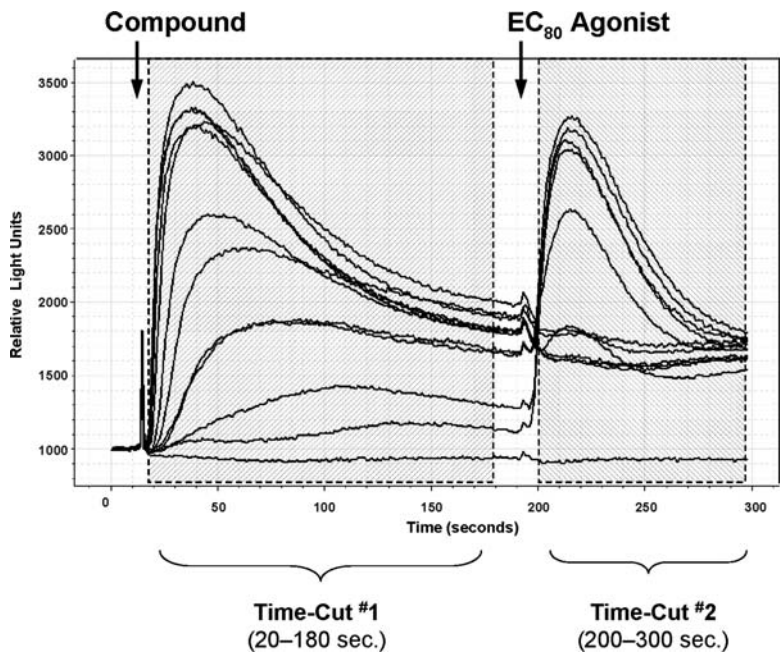

Fig. 7.4. **Time-cut determination for data analysis.** CHO-K1 cells expressing a promiscuous G_{α} protein and a $G_{\alpha s}$-coupled GPCR were assayed using a standard two-addition calcium mobilization assay in 384-well format on the FLIPR$^{TETRA®}$. The first addition consisted of varying concentrations of test compound with known agonist activity and the fluorescence signal was captured for 3 minutes. Then an EC_{80} concentration of agonist was added to the cell plate and the fluorescence signal was monitored for an additional 2 minutes. The time cuts chosen for the first read (20–180 seconds) and the second read (200–300 seconds) are indicated by the hatched boxes. The maximum signal within each time cut was used for subsequent data analysis. The sharp spike in fluorescence signal at the beginning of the kinetic profiles is an artifact of the clear FLIPR tips entering the well.

scaling. Generally, spatial uniformity correction is used when the cell type, cell density, and dye-loading conditions are the same for the entire plate. If different cell densities, cell lines, dye types, or dye concentrations are used in the same plate, then response over baseline can be used.

2. The term "time cut" refers to the time sequence over which the data are analyzed. In a dual-addition assay as presented here, two time cuts are defined. The first consists of a segment of time following the initial addition of compound and is used to monitor for agonist activity. The second is after the agonist is added and is used to measure antagonist activity of the compound. When a fluorescence spike is observed due to the use of clear pipet tips (*see* **Note 4.4, item 2**), it is important that the time sequences used for analyzing data begin after the spike (**Fig.7.4**). Also, for many GPCRs, not only does the magnitude of response decline with decreasing concentrations of agonist, but the peak of the response may also be delayed (**Fig.7.4**). In these cases, a time cut that is too narrow may capture the peak of the response to high agonist concentrations, but miss the peak response with lower concentrations of agonist. Therefore, the authors recommend using a time cut that spans the entire time following each reagent addition.

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