

Characterizing Gs- and Gi-Dependent Regulation of cAMP Levels in Real Time with cADDis Biosensor and Automated Live-Cell Imaging

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Abstract

The second messenger cAMP is involved in diverse cellular processes. The Gs and Gi family of proteins regulate the cAMP-dependent signaling pathway in response to associated G protein coupled receptor (GPCR) activation. In this study, kinetic live-cell imaging was used to monitor cAMP levels in HEK293 cells expressing a cAMP biosensor while stimulating D2, M2R, and β 2 receptors. Furthermore, automated image analysis was used to characterize Gs- and Gi-dependent changes in cAMP levels over time and determine pharmacological dose-responses.

Introduction

GPCR-mediated pathways are critical for cells to respond to intercellular and environmental cues and are a major focus of drug discovery efforts.^{1,2} Stimulation of GPCRs results in complex and overlapping signaling cascades involving associated G proteins and regulation of second messenger levels³ (Figure 1).

Activation of the G_s family of proteins stimulates production of the second messenger cAMP by adenylyl cyclase (AC). Elevated levels of cAMP, in turn, propagates the signaling cascade by activating cAMP-dependent protein kinases. In contrast, activation of the G_i family of proteins inhibits AC activity, decreasing cAMP levels. The interplay of these two arms of the cAMP-dependent pathway modulates cellular communication.⁴

The cADDis biosensor from Montana Molecular provides an alternative to measuring cAMP accumulation in cell lysates that is capable of measuring the dynamic interaction between G_s and G_i signaling without the use of forskolin.⁵ This application note describes an imaging-based approach to measure changes in cAMP levels over time in live cells using the Agilent BioTek Lionheart FX automated microscope and the cADDis biosensor. Agilent BioTek Gen5 automated image processing and analysis tools deliver a large assay window and improved sensitivity over methods relying on total fluorescence intensity measurements. Dual in-line dispense tips and rapid image capture rates support sequential addition of G_s- and G_i-coupled receptor agonists for detailed characterization of cAMP-dependent signaling pathways.

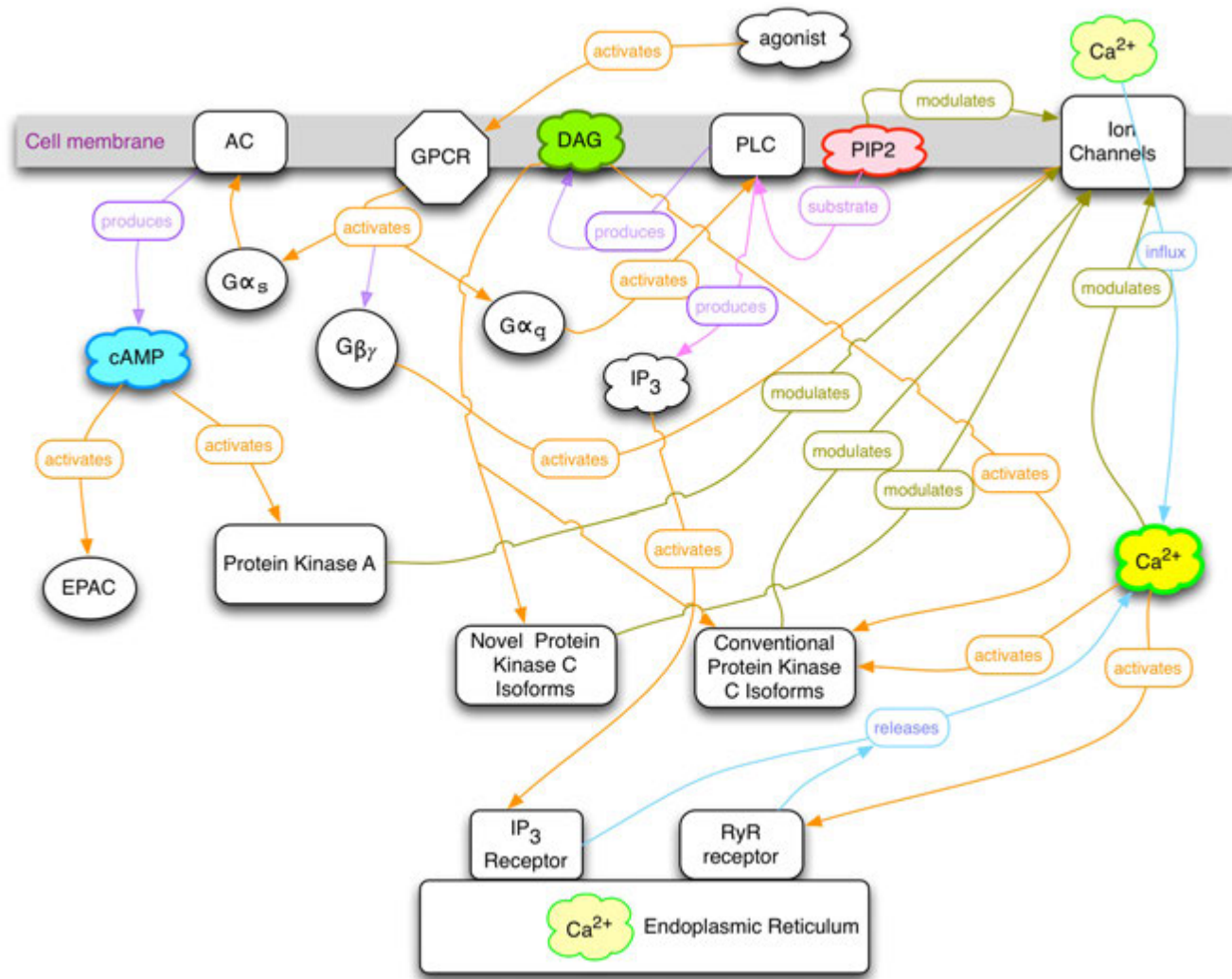


Figure 1. Schematic diagram of G-protein coupled receptor (GPCR) signaling pathways.

Materials and methods

Agilent BioTek Lionheart FX automated microscope

The Lionheart FX automated microscope with augmented microscopy is an integrated microscopy system, optimized for live-cell imaging with up to 100x air and oil immersion magnification. Brightfield, color brightfield, phase contrast, high contrast brightfield, and fluorescence channels offer maximum support for a wide range of imaging applications. A unique environmental control cover provides incubation to 40 °C and effective containment for CO₂/O₂ control. The available humidity chamber and dual reagent injector add a greater level of environment optimization for live-cell imaging workflows. Gen5 software provides automated image capture and analysis for a broad range of live and fixed cell applications.



Figure 2. Agilent BioTek Lionheart FX automated microscope with dual reagent injector module and CO₂/O₂ controller.

Montana Molecular cADDis biosensor

Montana Molecular offers a range of fluorescent biosensors for studying GPCR activation. The biosensor mechanism is based on circularly permuted fluorescent proteins that bind to a specific second messenger. The downward cyclic AMP Difference Detector *in situ* (cADDis) green biosensor exhibits decreased fluorescent intensity in response to increased levels of intracellular cAMP. Constitutively active Gs increases steady-state levels of cAMP for Gi studies, eliminating the need for forskolin. cADDis can be combined with the Ca²⁺ and DAG sensors for simultaneous pathway readouts. The BacMam delivery system enables consistent and controllable expression in a wide variety of cell types including primary cells and iPSCs.

Transduction and cell plating

HEK293 were cultured in Advanced DMEM with 10% fetal bovine serum and penicillin-streptomycin in 5% CO₂ at 37 °C. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluence. Cell transductions were done following the Montana Molecular protocol with volumes optimized for cell density and viral titer, and desired number of samples (Figure 3). Briefly, the viral transduction reaction (125 µL of cADDis sensor, 3 µL of 500 mM sodium butyrate, 25 µL of receptor (D2 or M2), 25 µL of constitutively active Gs, and 72 µL of Advanced DMEM plus 10% FBS and pen/strep) was added to 3.5 mL of a 70,000 cells/mL Advanced DMEM cell suspension. After mixing gently, 150 µL of mix was seeded per well in a Costar 3904 96-well microplate, which was then covered with aluminum foil to protect from light and incubated in a cell culture hood for 30 minutes. Cells were then transferred to a 37 °C incubator under normal cell growth conditions for 24 hours to ensure optimal sensor expression.

Imaging procedure

In preparation for imaging, culture media was replaced with 100 µL of room temperature DPBS, and cells were allowed to acclimate at room temperature for 20 minutes while protected from light. The plate was then transferred to a Lionheart FX automated microscope with aligned reagent injectors primed with Dulbecco's Phosphate Buffered Saline (DPBS) plus 6x final concentration of agonist or DPBS alone.

Experiments were performed at room temperature using the GFP 469/525 LED/filter cube set and 4x objective. Focus was maintained using laser autofocus. Exposure settings were optimized to visualize cADDis expressing cells pre-excitation, while low enough to accommodate a considerable increase in fluorescence over time. Exposure settings were as follows: LED 9; Integration time 100 ms; Gain 0.6. Images were acquired at 0.2 frames per second (FPS) for 330 seconds. Addition of reagents was carried out by the dual reagent injectors, dispensing 20 µL via angled injection tips at a rate of 225 µL/sec.

Image processing and cellular analysis

Image preprocessing was applied to images with auto settings. The Gen5 object-masking feature enables identification of cells within the imaging field. This feature was used to apply a mask around cells by setting the threshold just below the baseline cADDis fluorescence. Recommended cellular analysis settings are contained in Table 1. Threshold values will vary depending on exposure settings and biosensor expression levels. Object sum integral values were used for all data reduction steps.

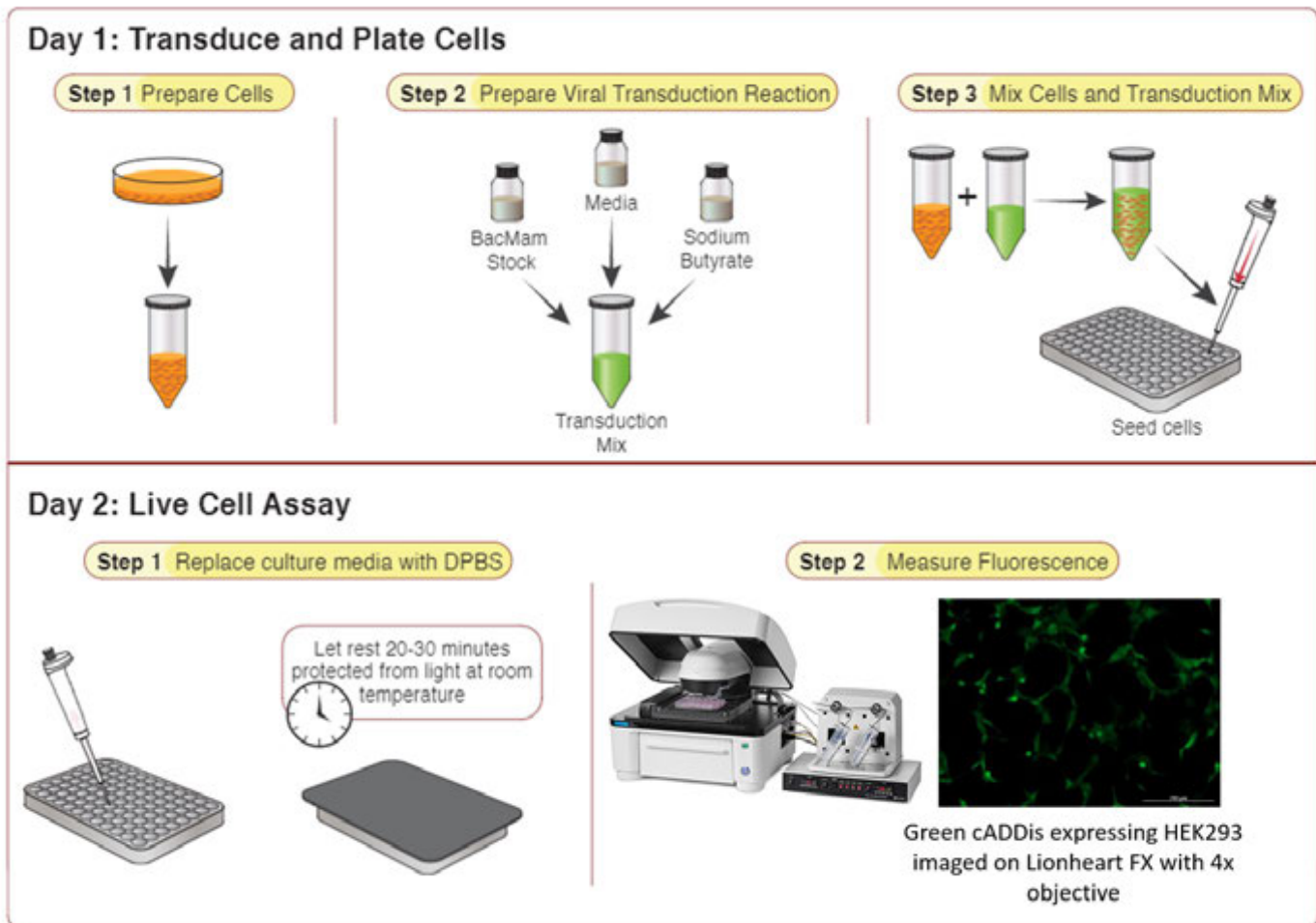


Figure 3. Montana Molecular protocol for biosensor cell transduction.

Table 1. Image preprocessing and cellular analysis parameters for kinetic profile of receptor activation. Preprocessing and object masks reduce background, resulting in improved analysis and a larger assay window.

Image Preprocessing		Advanced Detection Options	
Image Set	GFP	Rolling Ball Diameter	Automatic
Background	Dark	Image Smoothing Strength	1 cycle of 3 × 3 average filter
Rolling Ball Diameter	Automatic	Evaluate Background On	5%
Image Smoothing	0 cycles	Primary Mask	Use threshold mask
Cellular Analysis Parameters			
Channel	Tsf[GFP]		
Threshold	7,000		
Background	Darkq		
Split Touching Objects	Checked		
Fill Holes in Mask	Checked		
Minimum Object Size	5 μm		
Maximum Object Size	1,000 μm		
Include Primary Edge Objects	Checked		
Analyze Entire Image	Checked		

Results and discussion

Quantifying activation of Gi-coupled D2 receptors in HEK293

HEK293 cells expressing the downward cADDi biosensor, D2 receptors, and constitutively active Gs, exhibited low cADDi fluorescence at baseline due to high cAMP levels. Stimulation of Gi-coupled D2 receptors by the addition of 0.1 μM quinpirole resulted in an increase in cADDi fluorescence

within 5 seconds, with levels plateauing near the end of the 330-second time course. A kinetic profile was generated using normalized cADDi fluorescence measurements (F/F_0) for a range of quinpirole concentrations. Normalized fluorescence levels at $t = 300$ seconds were used to generate a quinpirole dose response curve, resulting in a calculated EC_{50} value of 2.7 nM (Figure 4).

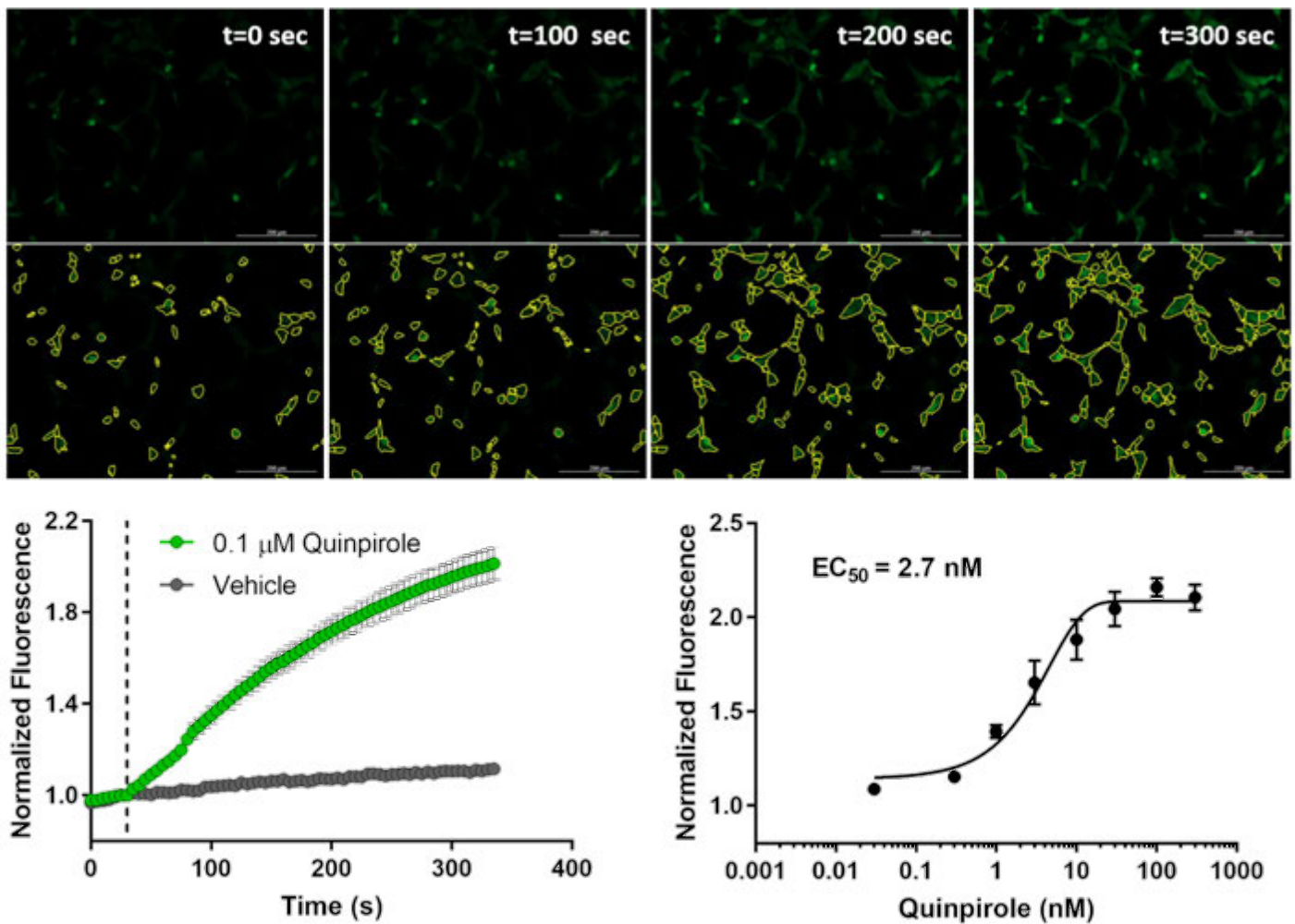


Figure 4. Quantifying activation of Gi-coupled D2 receptors in HEK293. (A) Image time course of HEK293 expressing green downward cADDi sensor, D2 receptor, and constitutively active Gs (top) with Agilent BioTek Gen5 placed masks around cells expressing cADDi fluorescence above a minimum threshold (bottom). cADDi fluorescence, which increases with decreasing levels of cAMP, is initially low due to high cAMP levels at baseline ($t = 0$). Stimulation of Gi-coupled hD2 receptors by injection of 0.1 μM (final) quinpirole causes cADDi fluorescence to steadily increase over time as cAMP levels decrease ($t = 1-3$). Images were captured at 0.2 fps for 330 seconds. (B) Kinetic profile of cADDi object sum integral fluorescence (F/F_0 , $n = 8$) in response to Gi-coupled D2 receptor activation by injection of 0.1 μM quinpirole or DPBS (dashed line). (C) Quinpirole dose response curve (F/F_0 at $t = 300$ seconds, $n = 4$ per concentration) with calculated EC_{50} value.

Characterizing interactions between Gi- and Gs-coupled regulation of cAMP levels

Kinetic monitoring of cAMP levels in live cells reveals the dynamic interaction between Gi- and Gs-dependent pathways. HEK293 cells expressing endogenous β_2 adrenergic receptor (β_2 AR) were transduced with green cADDis, M2 receptor (M2R), and constitutively active Gs. Activated Gs-coupled β_2 AR promotes adenylyl cyclase activity, whereas activated Gi-coupled M2R inhibits cAMP production (Figure 5).

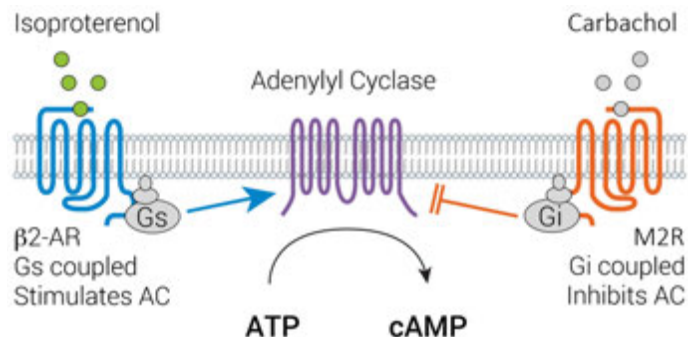


Figure 5. Regulation of adenylyl cyclase activity by Gs- and Gi-dependent pathways. Gs-coupled β_2 AR and Gi-coupled M2R act antagonistically to regulate cAMP levels.

Expression of constitutively active Gs results in high cellular levels of cAMP, causing cADDis fluorescence to be low at baseline. Addition of 30 μ M (final) carbachol to the system stimulates the Gi pathway, leading to a sustained increase in cADDis fluorescence. However, stimulation of Gs-coupled β_2 AR with 1 μ M (final) isoproterenol quickly reduced cADDis fluorescence intensity to near baseline by overcoming Gi-dependent inhibition of adenylyl cyclase (Figure 6A). A profile of normalized cADDis fluorescence over time was used to quantify the interaction between Gi- and Gs-mediated signaling. Excitation of Gi led to a detectable increase in cADDis fluorescence within 10 seconds of carbachol addition that began to level off at approximately 2.5 fold above baseline within 300 seconds. Subsequent addition of 1 μ M isoproterenol rapidly reduced cADDis fluorescence back to near baseline levels within 100 seconds (Figure 6B).

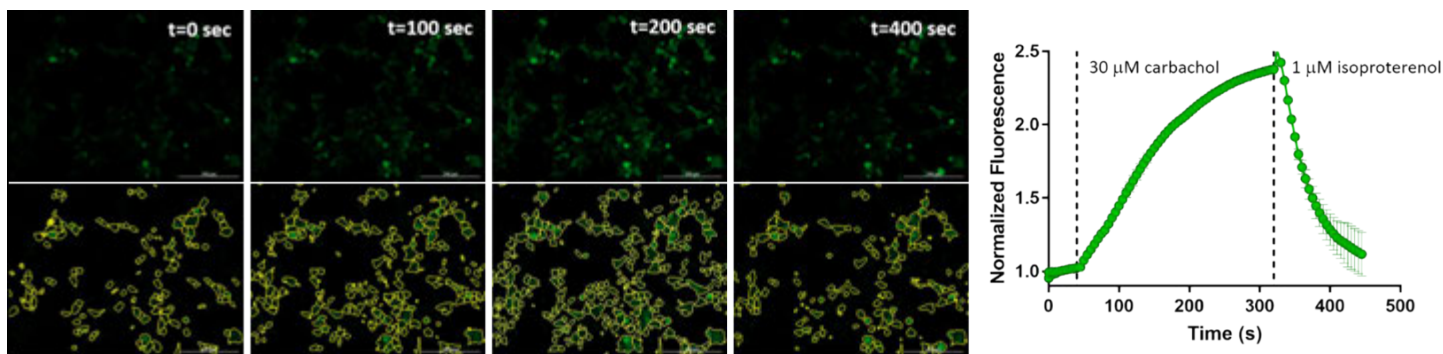


Figure 6. Dual reagent injectors enable characterizing Gi and Gs interactions through the sequential addition of M1R and β_2 receptor agonists. (A) Image time course of HEK293 expressing green downward cADDis sensor, M2 receptor, and constitutively active Gs (top panel) with Agilent BioTek Gen5 placed masks around cells exhibiting cADDis fluorescence above a minimum threshold (bottom panel). cADDis fluorescence is initially low due to high cAMP levels at baseline (t = 0). Addition of 30 μ M (final) carbachol stimulates Gi activity, which decreases cAMP and increases cADDis signal (t = 1-2). However, cADDis fluorescence is quickly reduced back to near baseline by stimulation of Gs-coupled endogenous β_2 AR with 1 μ M (final) isoproterenol (t = 3). (B) Profile of cADDis object sum integral fluorescence (F/F₀, n = 6) over time in response to Gi- and Gs-coupled receptor activation (dashed lines).

Conclusion

This study demonstrated the ability to conduct robust quantitative analysis on the cAMP-dependent signaling pathway using an Agilent BioTek Lionheart FX automated microscope and the cADDis biosensor. Image-based monitoring of cAMP levels in live cells enables sensitive detection and characterization of GPCR activation in real time, including the interaction between Gi and Gs signaling pathways. While some cAMP accumulation assays rely on the diterpene forskolin because of its ability to amplify the effects of Gs on adenylyl cyclases, the use of forskolin in the assays described above is not necessary or recommended due to the disruptive effects on biological signaling.⁶⁻⁸

Although all of the experiments described here were conducted on the Lionheart FX, each of these assays can also be conducted on an Agilent BioTek Cytation cell imaging multimode reader. The rapid image capture rates and dual reagent injectors available with these instruments enable uninterrupted monitoring of rapid cellular responses. Automated Agilent BioTek Gen5 image processing and cellular analysis tools greatly reduce background fluorescence, providing a large assay window and improved flexibility and sensitivity over methods relying on total fluorescence intensity measurements.

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