Characterizing GPCR Activation Using Automated Live-Cell Imaging

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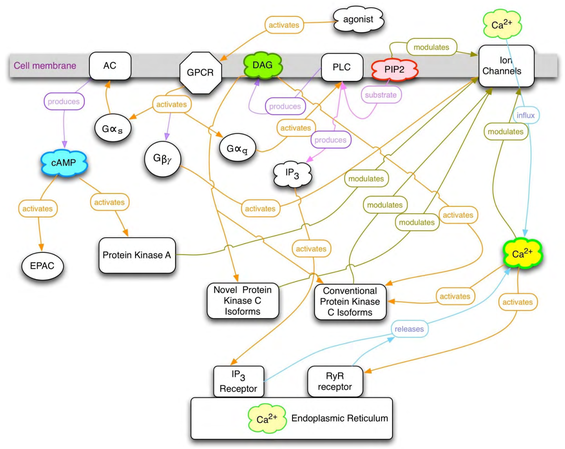
Introduction

G protein coupled receptor (GPCR)-mediated pathways are critical for cells to respond to intercellular and environmental cues and are a major focus of drug discovery efforts, particularly for cancer treatment. The molecules that activate GPCRs, and the resulting signaling cascades triggered by associated G proteins, are diverse. Fluorescent dyes and biosensors can be used to monitor changes in second messenger levels, including Ca^{2+} and cyclic AMP (cAMP), in response to GPCR activation. Here we describe a live-cell imaging based approach to detect GPCR activation using the Lionheart FX automated imager and Gen5 image analysis software. This method provides a large assay window and improved sensitivity over methods relying on total fluorescence intensity measurements. Dual in-line dispense tips enable addition of GPCR agonists with continuous monitoring of cellular response. Additionally, an image capture rate of up 20 frames per second enables characterization of rapid GPCR kinetics.



Schematic diagram of G-Protein Coupled Receptor (GPCR) Signaling Pathways.

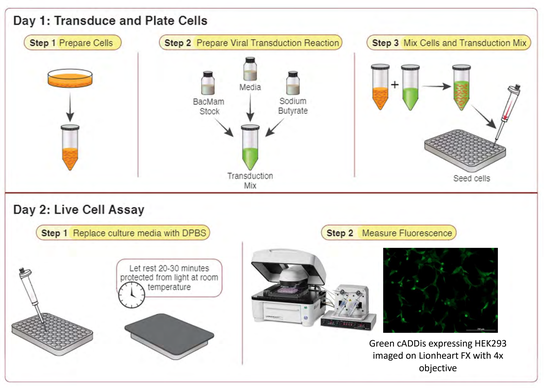
Instrumentation

Lionheart™ FX Automated Live Cell Imager with Augmented Microscopy™

- **All inclusive microscopy system:** Optimized for live cell imaging with brightfield, color brightfield, phase contrast and fluorescence channels. Up to 100x air and oil immersion magnification
- **Up to 20 fps image capture and dual in-line reagent injectors:** Enables characterization of rapid cellular events and addition of reagents with uninterrupted monitoring of cellular response
- **Integrated environmental control:** Incubation up to 40 °C with CO_2/O_2 and available humidity control provides optimal conditions for long-term imaging of live cells
- **Powerful Gen5™ 3.0 Imager software:** Automated image capture, processing, and analysis tools, including dual masking for cell counting and subpopulation analysis, plus annotation and movie maker functions

Methods

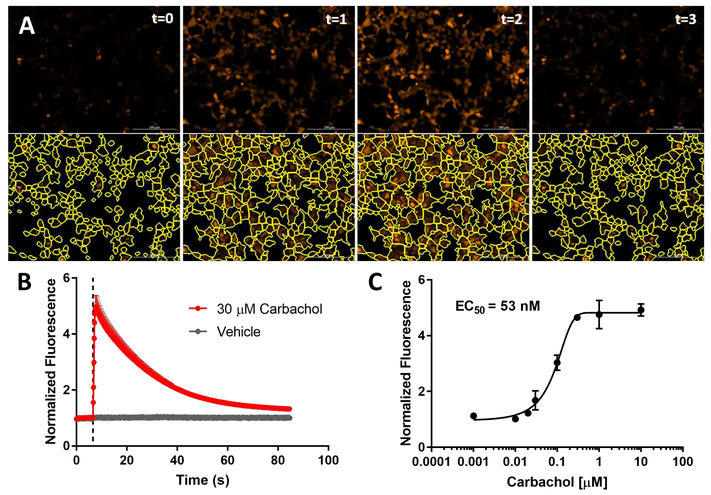
Live cell imaging of GPCR activity using Montana Molecular biosensors



Green cADDis expressing HEK293 imaged on Lionheart FX with 4x objective

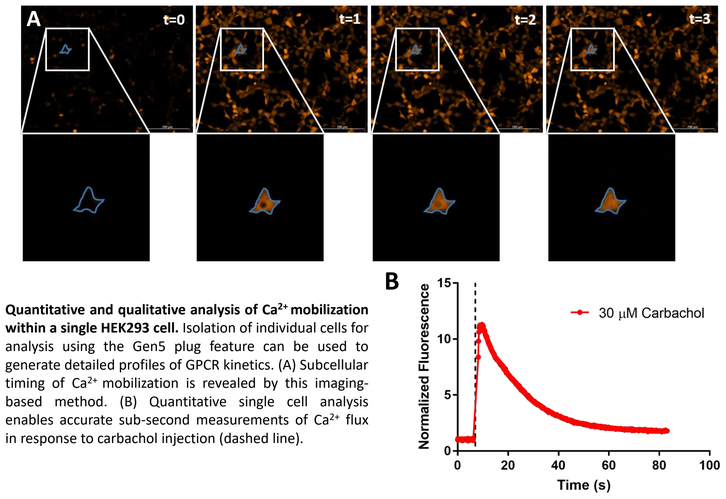
Detecting rapid Ca^{2+} mobilization with R-GECO biosensor

Monitoring intracellular Ca^{2+} release in response to G_q-coupled hM1 receptor activation



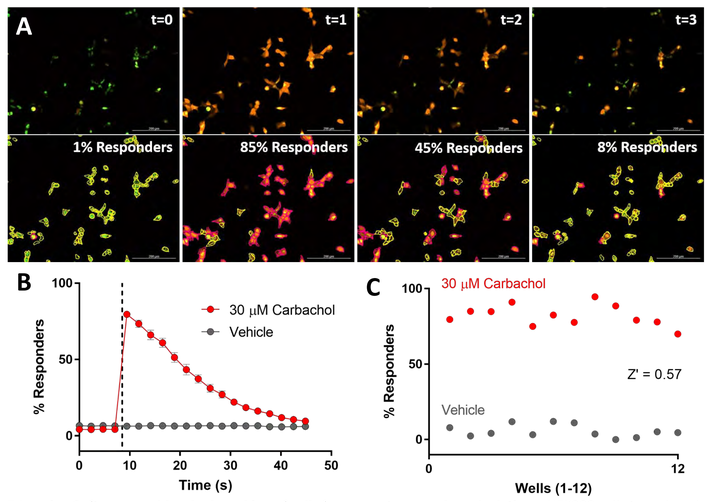
Quantifying activation of G_q-coupled hM1 receptors in HEK293. (A) Image panel of HEK293 expressing red upward R-GECO sensor and hM1 receptor (top) with Gen5 placed masks around cells containing R-GECO fluorescence above a determined threshold (bottom). R-GECO fluorescence – which increases with increasing levels of Ca^{2+} – is initially low at baseline (t=0). Stimulation of G_q-coupled hM1 receptors by injection of 30 µM (final) carbachol causes intracellular mobilization of Ca^{2+} and a corresponding rapid increase in R-GECO fluorescence (t=1-2), followed by a gradual decrease in cytoplasmic Ca^{2+} levels to near baseline (t=3). Images were captured at 10 fps for 85 seconds. (B) Kinetic profile of R-GECO object sum integral fluorescence (F/F^0, n=8) in response to G_q-coupled hM1 receptor activation by 30 µM carbachol (dashed line). (C) Carbachol dose response curve (F/F^0, n=4 per concentration) with calculated EC_{50} value.

Characterizing kinetics of hM1 stimulation-induced calcium flux within individual cells



Quantitative and qualitative analysis of Ca^{2+} mobilization within a single HEK293 cell. Isolation of individual cells for analysis using the Gen5 plug feature can be used to generate detailed profiles of GPCR kinetics. (A) Subcellular timing of Ca^{2+} mobilization is revealed by this imaging-based method. (B) Quantitative single cell analysis enables accurate sub-second measurements of Ca^{2+} flux in response to carbachol injection (dashed line).

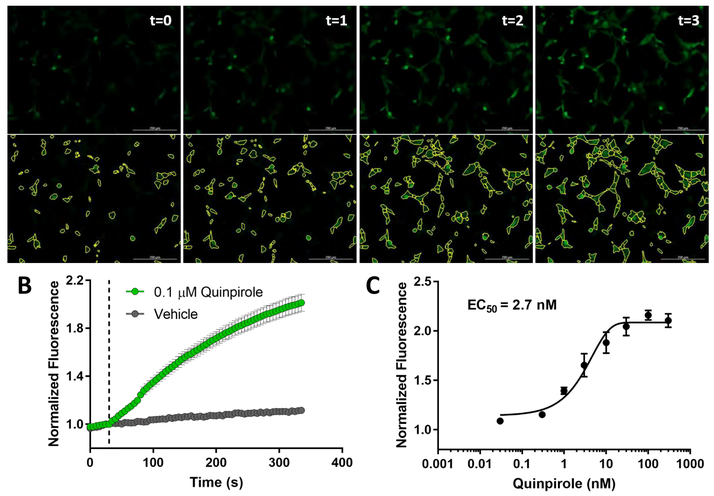
Quantifying G_q-coupled hM1 activation by percent responder using dual masking



Gen 5 Imaging Software enables dual masking of cells for percent responder calculations. HEK293 cells transduced with R-GECO and hM1 muscarinic acetylcholine receptors with a nuclear localized GFP tag were stimulated with 30 µM carbachol and imaged for 45 seconds at 0.5 fps. (A) Primary object masks were placed around the nuclei of each GFP positive cell to generate a total count of transduced cells. A secondary mask was then placed around the primary mask based on a determined R-GECO fluorescence threshold value to generate a count of responder cells. (B) Kinetic analysis of percent responders over time provides a sensitive and (C) robust assay for measuring GPCR activation.

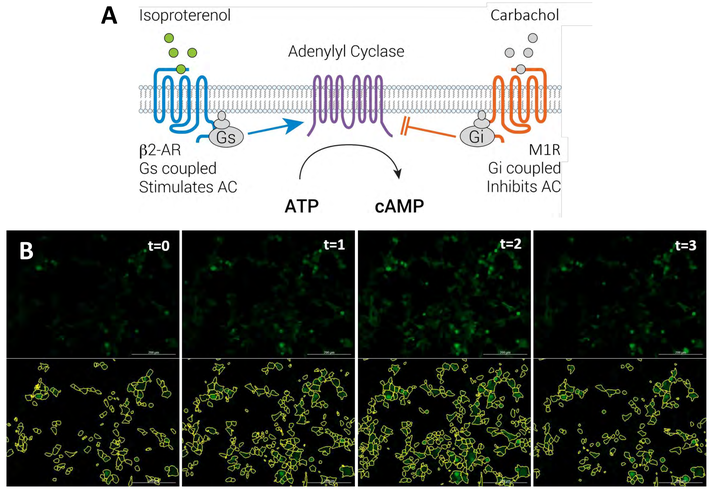
Monitoring cAMP levels in real time with cADDis biosensor

Robust detection of G_i-mediated decrease in cAMP levels



Quantifying activation of G_i-coupled hD2 receptors in HEK293. *(A) Image panel of HEK293 expressing green downward cADDis sensor and hD2 receptor over time (top) with Gen5 placed masks around cells containing cADDis fluorescence above a determined threshold (bottom). cADDis fluorescence – which increases with decreasing levels of cAMP – is initially low due to high cAMP levels at baseline (t=0). Stimulation of G_i-coupled hD2 receptors by injection of 0.1 µM (final) quinpirole causes cADDis fluorescence to steadily increase over time as cAMP levels decrease (t=1-3). Images were capture at 0.2 fps for 330 seconds. (B)* Kinetic profile of cADDis object sum integral fluorescence (F/F^0, n=8) in response to G_i-coupled hD2 receptor activation by *0.1 µM quinpirole (dashed line). (C) Quinpirole dose response curve (F/F^0, n=4 per concentration) with calculated EC_{50} value.*

Characterizing interactions between G_i- and G_s-coupled regulation of cAMP levels



Dual reagent injectors enable characterizing G_i and G_s interactions through the sequential addition of M1R and β2 receptor agonists. HEK293 cells expressing green cADDis, M1 receptor, and endogenous β2 adrenergic receptor. (A) G_s-coupled β2AR and G_i-coupled M1R act antagonistically to regulate adenylyl cyclase activity. (B) cADDis fluorescence is low at t=0 due to high baseline levels of cAMP. Addition of 30 µM (final) carbachol stimulates G_i activity which decreases cAMP and increases cADDis signal (t=1-2). However, cADDis fluorescence is quickly reduce back to near baseline by stimulation of G_s-coupled β2AR with 1 µM (final) isoproterenol (t=3). (C) Quantification of cADDis object sum integral fluorescence (F/F^0, n=6) over time in response to G_i- and G_s-coupled receptor activation (dashed lines).

Conclusions

- **Together, the Lionheart FX Automated Live Cell Imager and Montana Molecular biosensors provide a versatile and robust system for detecting biologically relevant GPCR signaling**
- **Up to 20 fps image capture and dual in-line reagent injectors allow for uninterrupted monitoring of rapid cellular responses including Ca^{2+} flux and G_s/G_i-dependent regulation of cAMP production**
- **Imaging-based approach to detecting GPCR activation enables detailed characterization of single cell kinetic profiles and percent responder measurements**
- **96-well format and automated image capture and analysis increases GPCR assay productivity and reproducibility**