

Automated Fluorescence Imaging of GPCR Dependent Second-Messenger Systems

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Fluorescent Biosensors for Live Cell Discovery

Abstract

Cell signaling involves an integrated, multi-faceted series of second-messenger systems that act in concert to transfer cell surface receptor mediated signals to a wide variety of intracellular processes. G-Protein Coupled Receptors (GPCRs) are the most notable initiators of cell signaling cascades and are the targets of a large amount of the drug discovery effort. Drug discovery screening of compound libraries relies on the ability to rapidly make assay determinations on large numbers of samples. At the same time, phenotypic information is also desired to assess the true cellular response. Towards that end, having multiple sensors capable of simultaneously detecting different signaling components of the GPCR pathways are of particular importance.

Here we describe the use of a combination microplate reader and imager to detect changes in second messenger levels using a series of genetically encoded fluorescent sensors. The multimode microplate reader is capable of digital microscopy and conventional microplate detection. New genetically-encoded biosensors for diacylglycerol (DAG), PIP₂ phospholipid, Ca²⁺, and cyclic adenosine monophosphate (cAMP) change fluorescence in response to changes in analyte concentration, and can be multiplexed in a single live cell assay [1,2,3]. This combination of reader/imager and second messenger biosensor has the capability of making both rapid analytical determinations, as well as phenotypic assessments in live cells.

Coordinated GPCR receptor and phospholipase C (PLC) activation were assessed using green and red fluorescent sensors for DAG, PIP₂, cAMP and Ca²⁺ in a multiplexed format. A number of sensors capable of either a greater "upward" or a lesser "downward" amount of fluorescence in the presence of their specific analyte were tested. For example, the addition of carbachol to HEK293 cells transfected with "Upward DAG" green plasmid resulted in a rapid increase in green fluorescence, with fluorescence levels returning to baseline within 15 seconds from the addition of stimuli, while the response of the "DownwardDAG" green sensor is a loss of fluorescence that is regained over time. Object cell counting of green cell fluorescence demonstrates a 4-fold increase in the green positive cell number with an upward calcium specific probe. Counting analysis of green and red fluorescence multiplexed reactions tracking DAG against a red control results in a 15% increase in the Green/Red ratio, while the use of a Downward Green DAG probe in conjunction with an Upward Red Ca²⁺ probe resulted in a 50% decrease in the Green/Red ratio with carbachol stimulation. Image and analytical analysis of results will be presented.

GPCR Signal Pathway

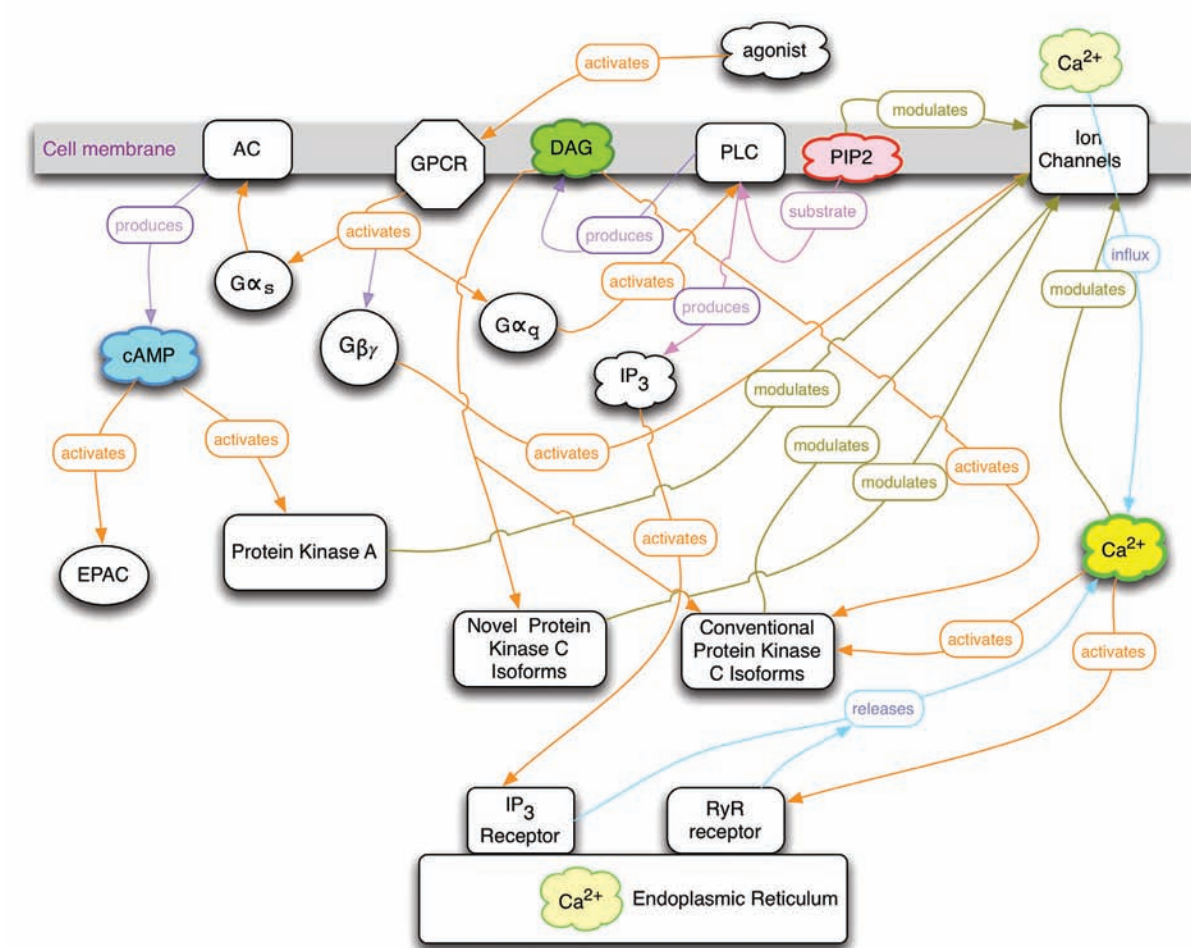


Figure 2 – Schematic diagram of a G Protein-Coupled Receptor (GPCR) Signal Pathway. The activated receptor catalyzes the activation of the heterotrimeric Gq protein. The Gq activates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5 biphosphate (PIP₂) to produce both diacylglycerol (DAG) and inositol1,4,5-triphosphate (IP₃). The IP₃ triggers the release of Calcium (Ca²⁺) from intracellular stores, and the combination of raised intracellular Ca²⁺ and DAG activates conventional protein kinase C enzymes with a multitude of downstream targets.

Assay Process

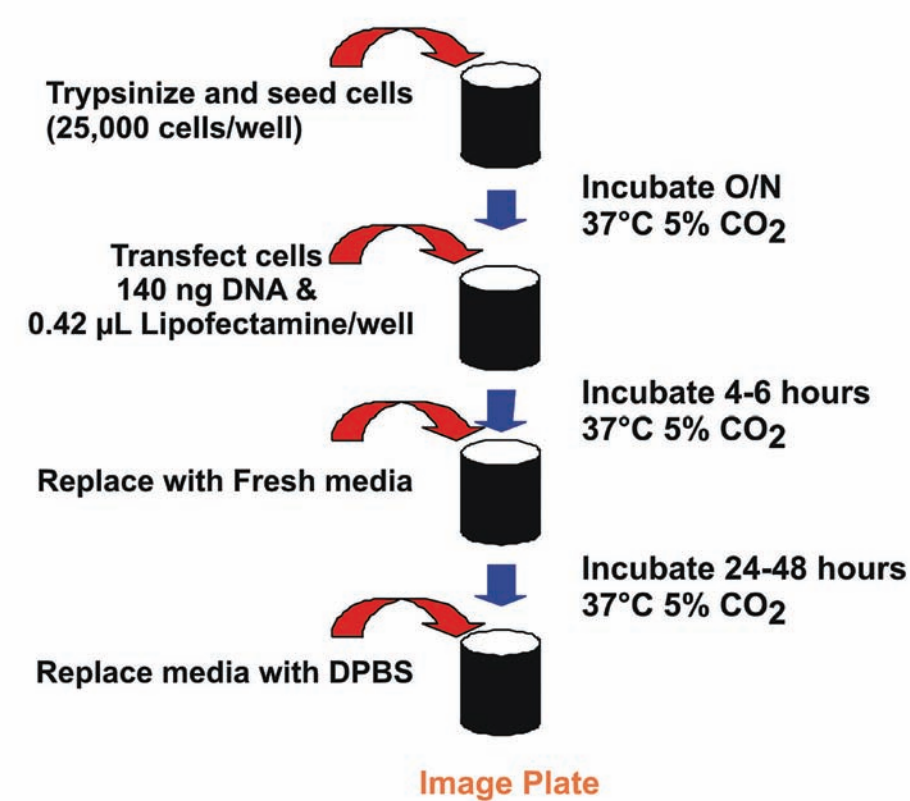


Figure 1 – Assay Process Prior to GPCR Pathway Analysis. Cells are seeded onto glass or plastic bottom plates and transiently transfected with the receptor and second messenger biosensor of interest. Transfected cells are used experimentally 24-48 hours after transfection to allow for adequate expression of the introduced genetic elements.

Materials and Methods

Cell Culture

HEK293T cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in 5% CO₂. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For cell transfection, cells were plated at a density of 25,000 cells per well in 100 µL growth medium.

Cell Transfection

For each transfection (i.e. single well of a 96-well plate) 140 ng plasmid DNA (100 ng biosensor DNA and 40 ng receptor) was diluted to 25 µL in Opti-MEM. In addition, 0.42 µL of Lipofectamine was diluted to 25 µL Opti-MEM and the two solutions mixed and added to the cells. Cells were incubated with this mixture for 4-6 hours and the mixture and growth media replaced with fresh medium. Cells were allowed to grow for 24-48 hours prior to imaging. Immediately prior to imaging, growth medium was replaced with 1X Dulbecco's phosphate-buffered saline (DPBS).

Imaging

Transfected cultures were imaged using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) with GFP and Texas Red light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the Texas Red light cube uses a 586/15 excitation and 647/57 emission filters.

Image Analysis

Digital image data was analyzed for mean fluorescence intensity as well as object cell counting using Gen5™ Data Analysis Software. Gen5 defines contiguous regions or areas that are outlined by designated threshold intensity value, as well as minimum and maximum size limits. These regions are counted as "objects" or "cells". Mean fluorescence intensity values are plotted as a fold change from the initial value (ΔF/F), while the change in cell number is plotted.

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Green Downward DAG & R-GECO Ca²⁺

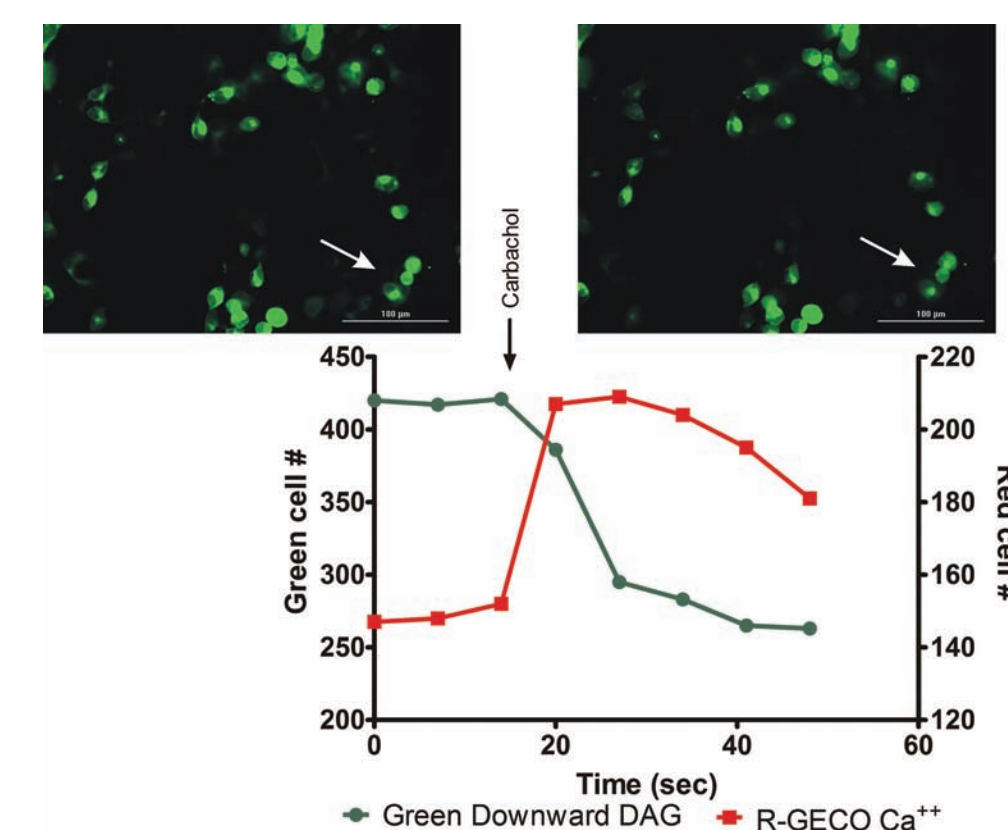


Figure 5 – Green Downward DAG1 and R-GECO2 Biosensor fluorescence. HEK293 cells cotransfected with Green Downward DAG and R-GECO biosensors were imaged kinetically at 4x and 20x using a GFP and Texas red light cubes with a Cytation 3 Cell Imaging Multi-Mode Reader. The change in cell number of cells positive for each 4x image was plotted against time for both colors. Addition of carbachol is indicated by black arrow. Green and red images of cells before and after carbachol addition are shown. White arrows indicate cells with obvious changes in fluorescence with stimulation.

cAMP Sensors

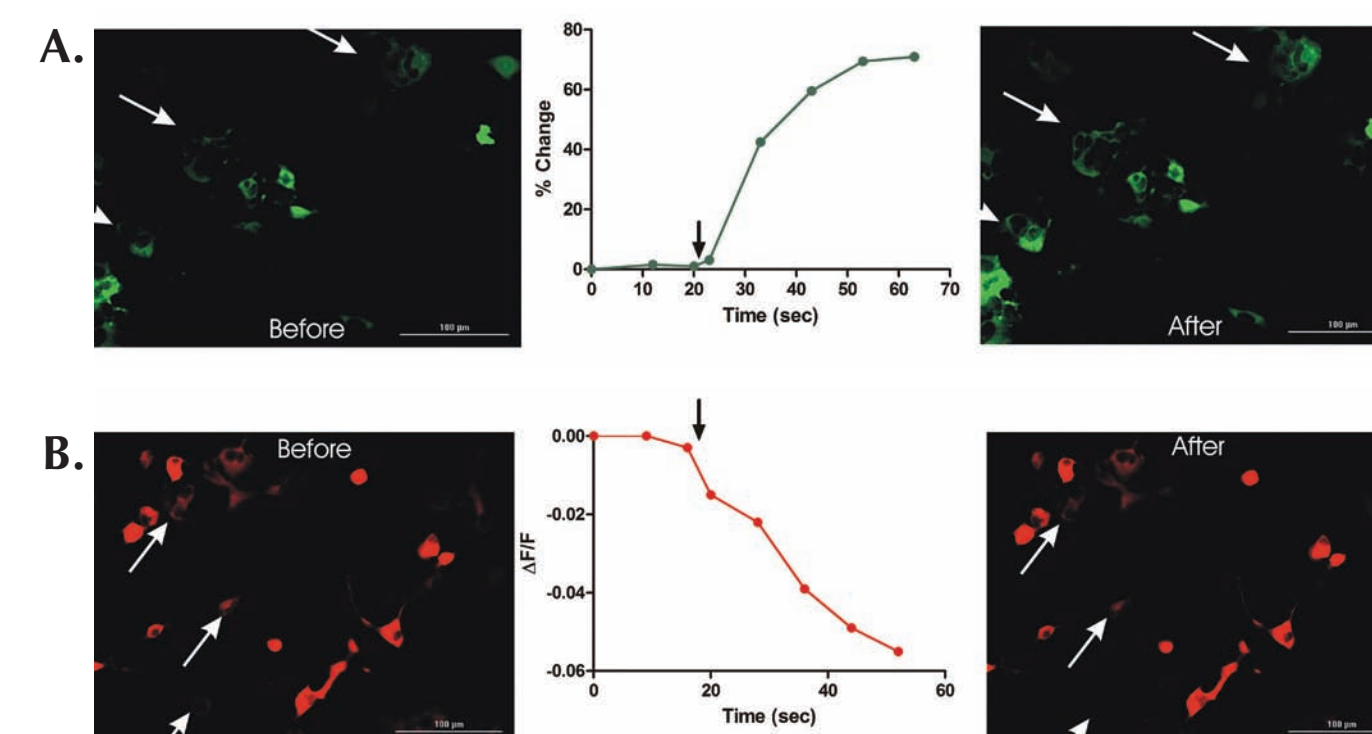


Figure 6 – Green Upward cAMP and Red Downward cAMP Sensor fluorescence. HEK293 cells transfected with (A) Green Upward cAMP or (B) Red Downward cAMP sensors were imaged kinetically at 20x using a GFP and Texas red light cubes respectively with a Cytation 3 Cell Imaging Multi-Mode Reader. The fold change in fluorescence mean of each image was plotted against time for both colors. Addition of carbachol is indicated by black arrow. Green and red images of cells before and after carbachol addition are shown. White arrows indicate cells with obvious changes in fluorescence with stimulation.

G-GECO Ca²⁺ Sensor

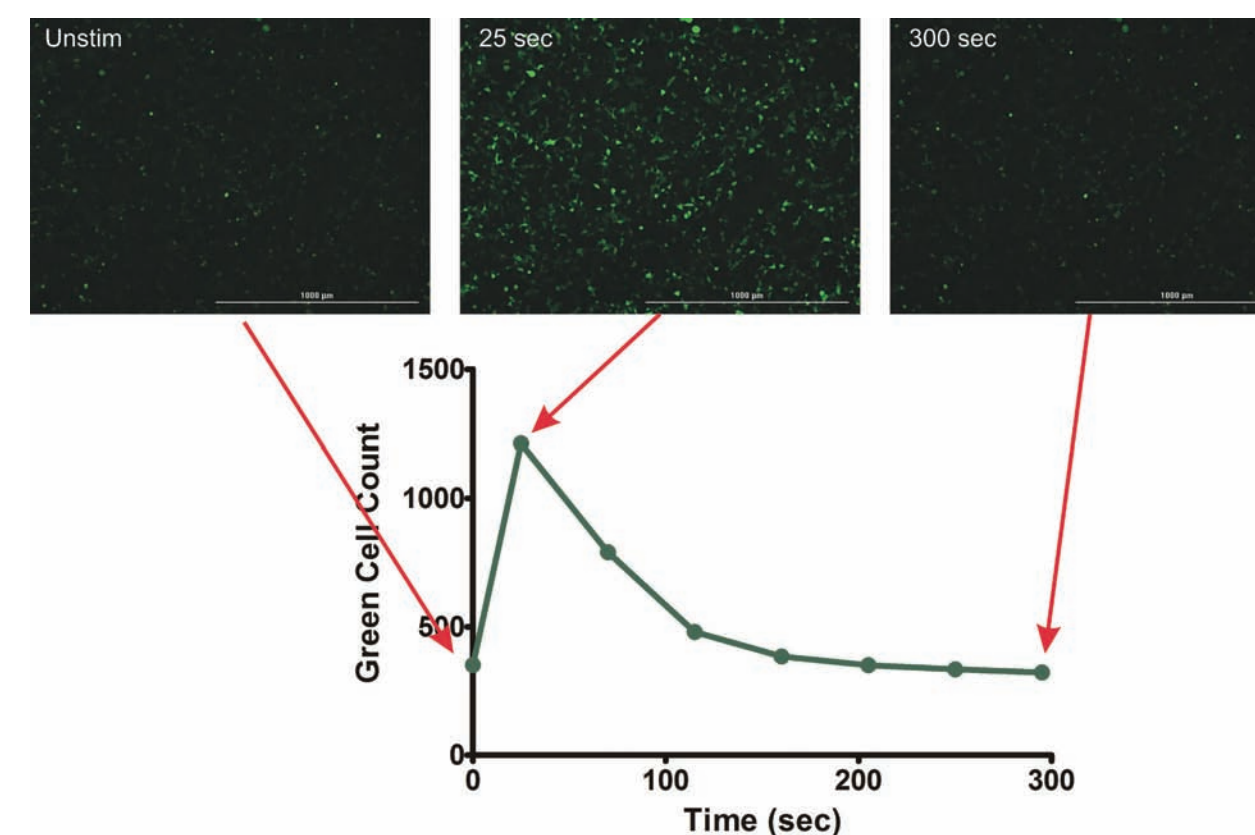


Figure 7 – Stimulation of G-GECO Ca²⁺ Sensor. Calcium flux in HEK293 cells transfected with G-GECO Ca²⁺ sensor [2] were stimulated with carbachol and green fluorescence monitored kinetically by imaging using a Cytation 3 Cell Imaging Multi-Mode Reader. Automated cell analysis with Gen5 Data Analysis Software determined cell count of Green positive cells. Images at indicated times points are also presented.

Green Upward DAG & R-GECO Ca²⁺

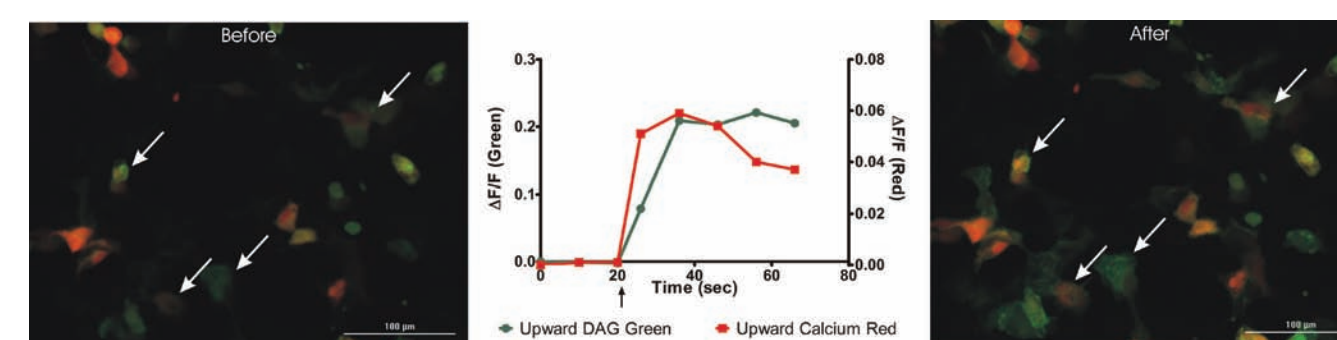


Figure 8 – Green Upward DAG and R-GECO Ca²⁺ Sensor fluorescence. HEK293 cells cotransfected with Green Upward DAG and R-GECO Ca²⁺ sensors were imaged kinetically at 20x using a GFP and Texas red light cubes with a Cytation 3 Cell Imaging Multi-Mode Reader. The fold change in fluorescence mean of each image was plotted against time for both colors. Addition of carbachol is indicated by black arrow. Overlaid green and red images of cells before and after carbachol addition are shown. White arrows indicate cells with obvious changes in fluorescence with stimulation.

Repeatability

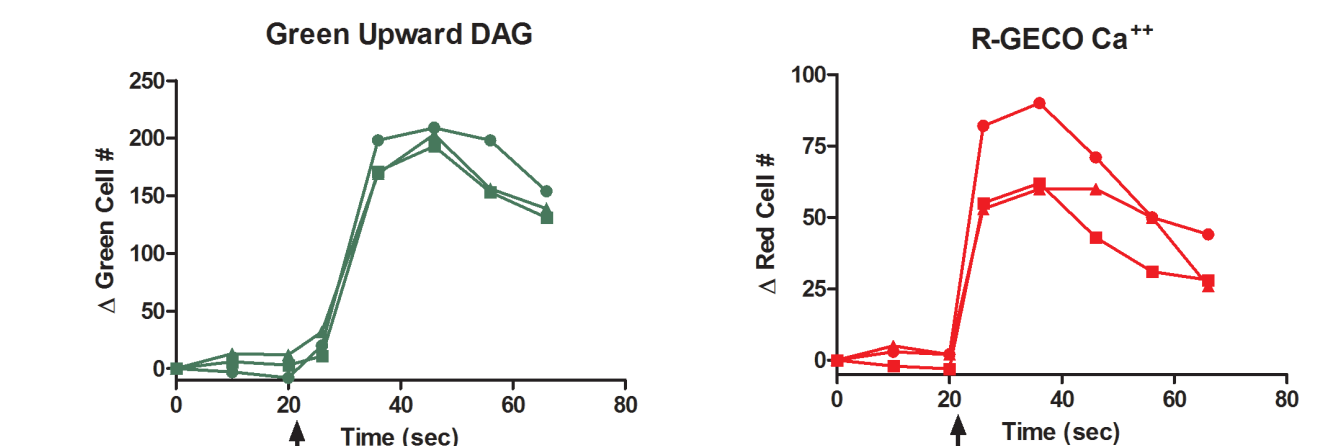


Figure 9 – Repeatability of GPCR sensors. HEK293 cells cotransfected with both Green Upward DAG and R-GECO Ca²⁺ sensors were stimulated with carbachol (arrow). Cells from multiple wells were imaged before and after stimulation. Cellular analysis of the images to determine cells positive for green and red signals was performed. Data represents the change from the initial read in the number of positive cells.

Green Upward DAG & RFP Control

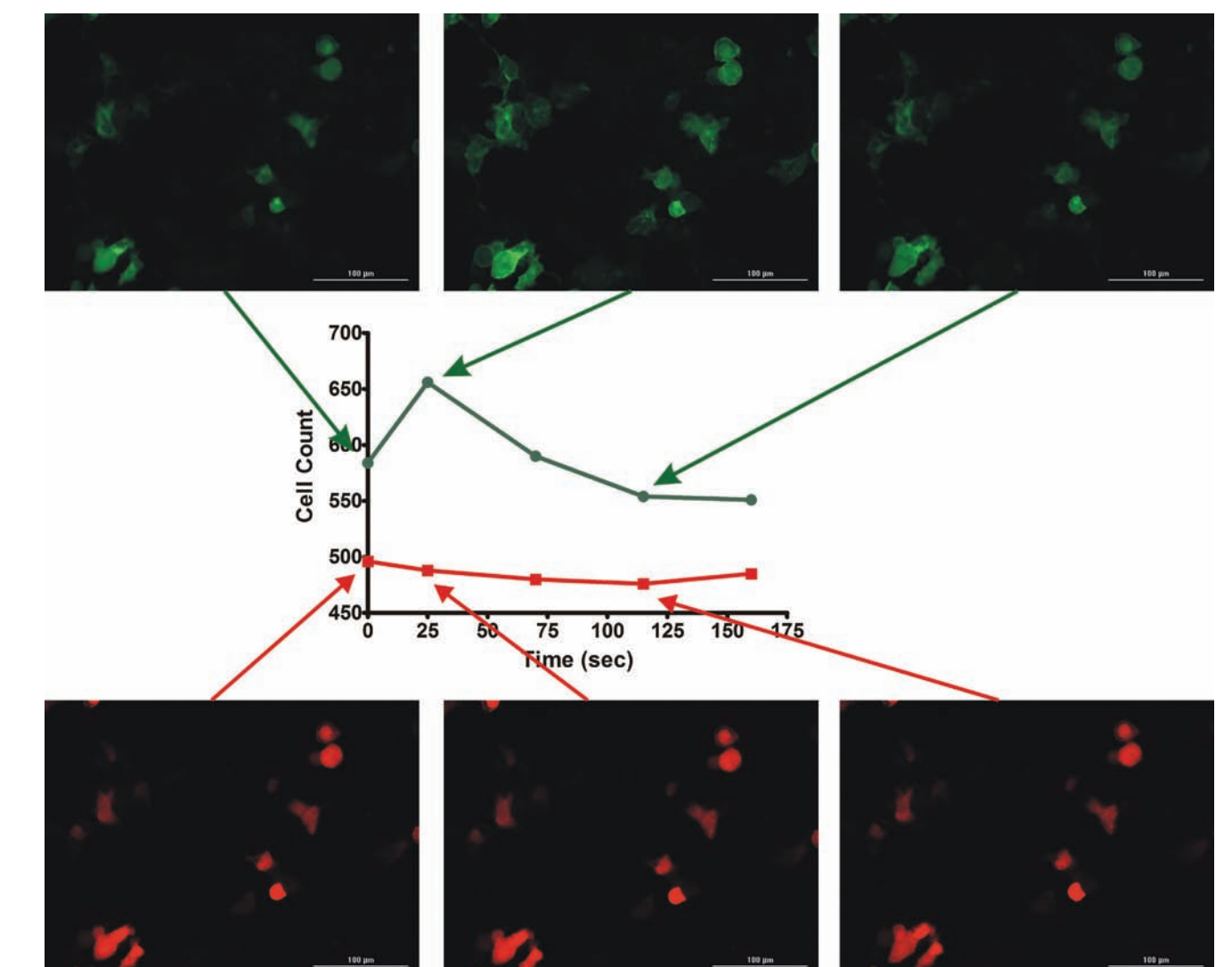


Figure 10 – Coexpression of Green Upward DAG sensor with RFP control. HEK293 cells transfected with both Green Upward DAG and RFP control were stimulated with carbachol (arrow). Cells were imaged over time using either a 4x or a 20x objective. Cellular analysis of the 4x images to determine cells positive for green was performed. 20x images at similar times points depict typical fluorescence images. White arrow indicate cells with obvious fluorescence change over time.

Red Downward PIP₂

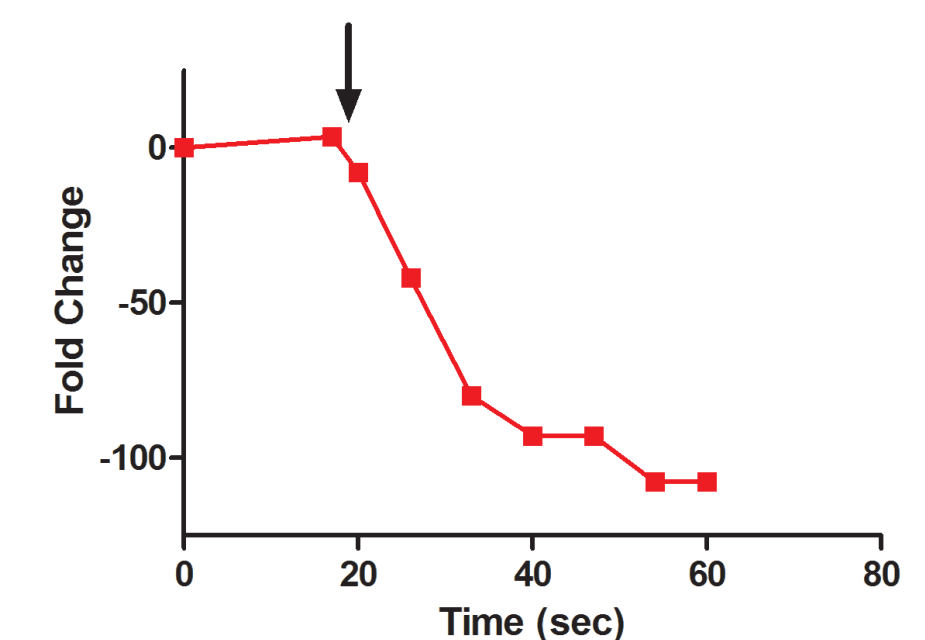


Figure 11 – Quantitation of Red Downward PIP₂ Sensor Response. HEK293 cells transfected with Red Downward PIP₂ sensor were stimulated with carbachol (arrow). Cells were imaged over time using a 4x objective. Cellular analysis of the 4x images to determine cells positive for red was performed. The fold change in cell number was plotted over time.

Conclusions

- Cytation 3 Cell Imaging Multi-Mode Reader can be used to perform multiplexed fluorescent assays of GPCR activated second-messengers in live cells
 - Combination of reader/imager
 - Rapid analytical determinations and phenotypic imaging of live cells
- Cytation 3 has a number of features that enable live cell imaging
 - Auto-focus and Auto-exposure
 - Multiple color Imaging capabilities
 - Gas Controller and Temperature Control allow long term studies and time lapse videos
- Quantitative Image Analysis Using Gen5 Data Analysis Software
 - Mean signal change
 - Population analysis
 - Allows for easy assay development
- New Generation Live Cell Fluorescent Sensors
 - Genetically encoded
 - Multiple options
 - Upward or Downward changing sensors
 - Different colors
- Multiplexed Assays
 - Allows for internal controls
 - Assess multiple Second-messengers simultaneously
 - Unambiguous readout for a particular pathway
 - Improve assay reliability
 - Provide relative ratios of activity with different signaling components