Application Note

Rapid Cell Signaling

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Automated Imaging Assay for Characterizing Ca^{2+} Flux with R-GECO Biosensor

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Abstract

GPCR activation was kinetically monitored using a Ca^{2+} biosensor that was transfected along with the human muscarinic M1 receptor into HEK 293 cells. Rapid Ca^{2+} flux was evident upon carbachol stimulation that peaked 1 second after injection, followed by a decay back to baseline fluorescence over 90 seconds. Imaging also allowed the monitoring of single cells within the cell population in the field of view where differences in kinetics can be assessed. Suitable assay performance was achieved using a % Responders readout (% of cells responding to carbachol stimulation) that provided z' values above 0.5 and consistent pharmacology.

Introduction

Mobilization of intracellular calcium stores following G protein coupled receptor (GPCR) activation is critical for cells to respond to intercellular and environmental cues. There are over 800 GPCRs identified in humans, each sharing a common structure in which the external N-terminus connects to the internal C-terminus by seven transmembrane-spanning segments.[1] Ligand binding at the N-terminus causes a conformational change in the receptor that initiates a signaling cascade. GPCR-mediated pathways are a major focus of drug discovery efforts, particularly for cancer treatment.[2]

The signaling molecules that activate GPCRs, and the functional consequence of receptor activation, are diverse. Binding of a signal molecule to a GPCR activates the associated trimeric GTP-binding protein (G protein). G proteins are comprised of an α-subunit, which binds guanine nucleotide and hydrolyzes GTP to GDP when activated, and a β- and γ-subunit complex. Different subtypes of each G protein subunit can be brought together to achieve diverse functional outputs. Activation of the Gs and Gq sub-family of α-subunits triggers the release of intracellular calcium stores into the cytoplasm, which propagates the signal by regulating calcium-dependent proteins.[3]

Montana Molecular offers a range of fluorescent biosensors for studying GPCR activation. The biosensors are transfected into live cells using BacMam delivery, providing simple assay preparation as viral particles are added directly to cells, then seeded in microplates. The biosensor mechanism is based on circularly permuted fluorescent proteins that binds to a specific second messenger. The red fluorescent, genetically encoded Ca^{2+} indicator (R-GECO) increases in fluorescence intensity in response to increasing levels of intracellular calcium.

The Agilent BioTek Lionheart FX and Agilent BioTek Cytation automated imagers provide a powerful and convenient system to characterize Ca^{2+} flux using R-GECO. This automated imaging-based approach enables sensitive detection of intracellular calcium release and detailed characterization of GPCR kinetics. Automated Agilent BioTek Gen5 image preprocessing and cellular analysis tools greatly reduce background fluorescence, providing a large assay window and improved sensitivity over methods relying on total fluorescence intensity measurements. Dual reagent injectors and image capture rates of up to 20 frames/second enable uninterrupted monitoring of rapid cellular events and characterization of complex signaling pathways.

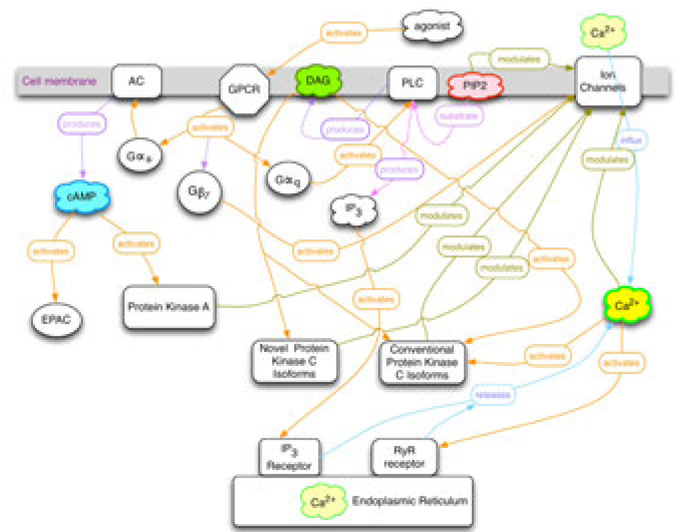

Figure 1. Schematic diagram of G-Protein coupled receptor (GPCR) signaling pathways.

Materials and methods

Agilent BioTek Lionheart FX automated microscope

Lionheart FX automated microscope with Augmented Microscopy is an all-inclusive microscopy system, optimized for live-cell imaging with up to 100x air and oil immersion magnification. Brightfield, color brightfield, phase contrast 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 CO_2/O_2 control. The available humidity chamber and reagent injector add a greater level of environment optimization for long term live-cell imaging workflows. Gen5 3.0 software provides automated image capture and analysis, plus annotation and movie maker functions. Gen5 3.0 offers ease and simplicity across a broad range of live and fixed cell applications, including perfusion assays.

Figure 2. Agilent BioTek Lionheart FX automated microscope with dual reagent injector module.

Montana Molecular R-GECO biosensor

The red fluorescent, genetically encoded Ca^{2+} indicator (R-GECO) increases in fluorescent intensity in response to increasing levels of intracellular calcium.[4] The optimal R-GECO excitation wavelength is 590 nm and the emission wavelength spans 600 to 700 nm. R-GECO can be paired with other GPCR biosensors from Montana Molecular, including cADDis, a green fluorescent cAMP sensor, or the green fluorescent upward and downward diacylglycerol (DAG) sensors to simultaneously detect both Ca^{2+} and cAMP or Ca^{2+} and DAG in living cells. The BacMam delivery system enables consistent and controllable expression in a wide variety of cell types including iPSC-derived cardiomyocytes.

Transduction and cell plating

HEK293 were cultured in Advanced DMEM with 10% fetal bovine serum and penicillin-streptomycin in 5% CO_2 at 37 °C. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluence. Cell transfections were done following Montana Molecular protocol with volumes optimized for cell density and viral titer, and desired number of samples. Briefly, viral transduction reaction (20 µL of R-GECO sensor, 0.6 mL of 500 mM sodium butyrate, 5 µL of hM1 receptor, and 24.4 µL of Advanced DMEM plus 10% FBS and pen/strep) was added to 700 µL of a 450,000 cells/mL Advanced DMEMcell 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 tissue culture hood for 30 minutes. Cells were then transferred to a 37 °C incubator under normal cell growth conditions for 40 hours to ensure optimal sensor expression.

Image 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 an Agilent BioTek Lionheart FX automated microscope with aligned reagent injectors primed with Dulbecco's Phosphate Buffered Saline (DPBS) plus 6x final concentration of Carbachol or DPBS alone.

Experiments were performed at room temperature using the RFP 531/593 LED filter cube set and 10x objective. Focus was maintained using laser autofocus. Exposure settings were optimized to visualize R-GECO expressing cells pre-excitation, while low enough to accommodate a considerable increase in fluorescence over time. Briefly, exposure settings were as follows: LED 10; Integration time 100 ms; Gain 7.3. A 10 FPS capture rate was used for characterization of receptor kinetics, and 0.5 FPS capture rate for percent responder experiments involving dual-channel acquisition. Images were acquired for a total of 65 seconds with 20 µL of either DPBS plus carbachol or vehicle alone dispensed at time equals 5 seconds.

Cellular analysis: kinetic profile of receptor activation

Image preprocessing was applied to images with auto settings. The Agilent BioTek 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 R-GECO fluorescence. Recommended cellular analysis settings are contained in Table 1. Threshold values will vary depending on exposure settings and biosensor expression levels.

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.

Imaging Preprocessing	
Image Set	RFP
Background	Dark
Rolling Ball Diameter	Automatic
Image Smoothing	0 cycles
Primary Mask	
Channel	Tsf[RFP]
Threshold	3,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Mask	Checked
Minimum Object Size	10 µm
Maximum Object Size	1,000 µm
Include Primary Edge Objects	Checked
Analyze Entire Image	Checked
Advanced Detection Options	
Rolling Ball Diameter	Automatic
Image Smoothing	1 cycle of 3 × 3 average filter
Evaluate Background On	5%
Primary Mask	Use threshold mask

Cellular analysis: percent responders

The Gen5 dual masking tools provide the ability to conduct a variety of automated quantitative analysis, including determining the percentage of total cells that respond to a given treatment. Using this feature, a primary mask is set around a nuclear marker, such as the nuclear GFP marker included with the hM1 construct, to generate total cell counts. A secondary mask that expands out around the primary mask within the RFP channel is then set to determine total RFP fluorescence for each cell over time. A subpopulation of responder cells is then defined as having total fluorescence levels above a threshold that is two standard deviations above the mean of negative control cells. The percent responders for each condition is calculated by taking the ratio of responder cells to the total number of cells. Representative Gen5 cellular analysis settings for determining percent responders are included in Table 2, although appropriate threshold values will vary depending on experimental conditions.

Table 2. Image preprocessing and cellular analysis parameters for determining percent responders. Preprocessing settings from Table 1 were applied to GFP and RFP channels.

Primary Mask	
Channel	Tsf[GFP]
Threshold	500
Background	Dark
Split Touching Objects	Checked
Fill Holes in Mask	Checked
Minimum Object Size	10 µm
Maximum Object Size	100 µm
Include Primary Edge Objects	Checked
Analyze Entire Image	Checked
Advanced Detection Options	
Rolling Ball Diameter	Automatic
Image Smoothing	1 cycle of 3 × 3 average filter
Evaluate Background On	5%
Primary Mask	Use threshold mask
Secondary Mask	
Channel	Tsf[RFP]
Measure within a Secondary Mask	Including primary mask and expand 100 µm
Threshold	3,000
Method	Propagate mask
Fill Holes in the Mask	Checked
Image Smoothing	1 cycle of 3 × 3 average filter

Results and discussion

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

R-GECO fluorescence, which increases with increasing levels of Ca^{2+}, is initially low at baseline. Stimulation of G_q-coupled hM1 receptors by injection of 30 µM (final) carbachol causes rapid intracellular mobilization of Ca^{2+} and a corresponding rapid increase in R-GECO fluorescence within a population of HEK293 cells. Object masks were placed around R-GECO expressing cells at each timepoint (Figure 3A). Kinetic profiles of intracellular calcium mobilization were generated using object integral fluorescence values over time normalized to mean baseline fluorescence levels (T/T_0). Maximum cellular response (5-fold increase in R-GECO fluorescence) was reached on average one second after agonist injection, followed by a gradual decrease in R-GECO fluorescence levels back to near baseline over approximately 70 seconds (Figure 3B). The increase in R-GECO fluorescence resulting from carbachol addition was determined for a range of concentrations (1 nM to 30 µM) and an EC_{50} value of 53 nM was calculated from the resulting dose-response curve (Figure 3C).

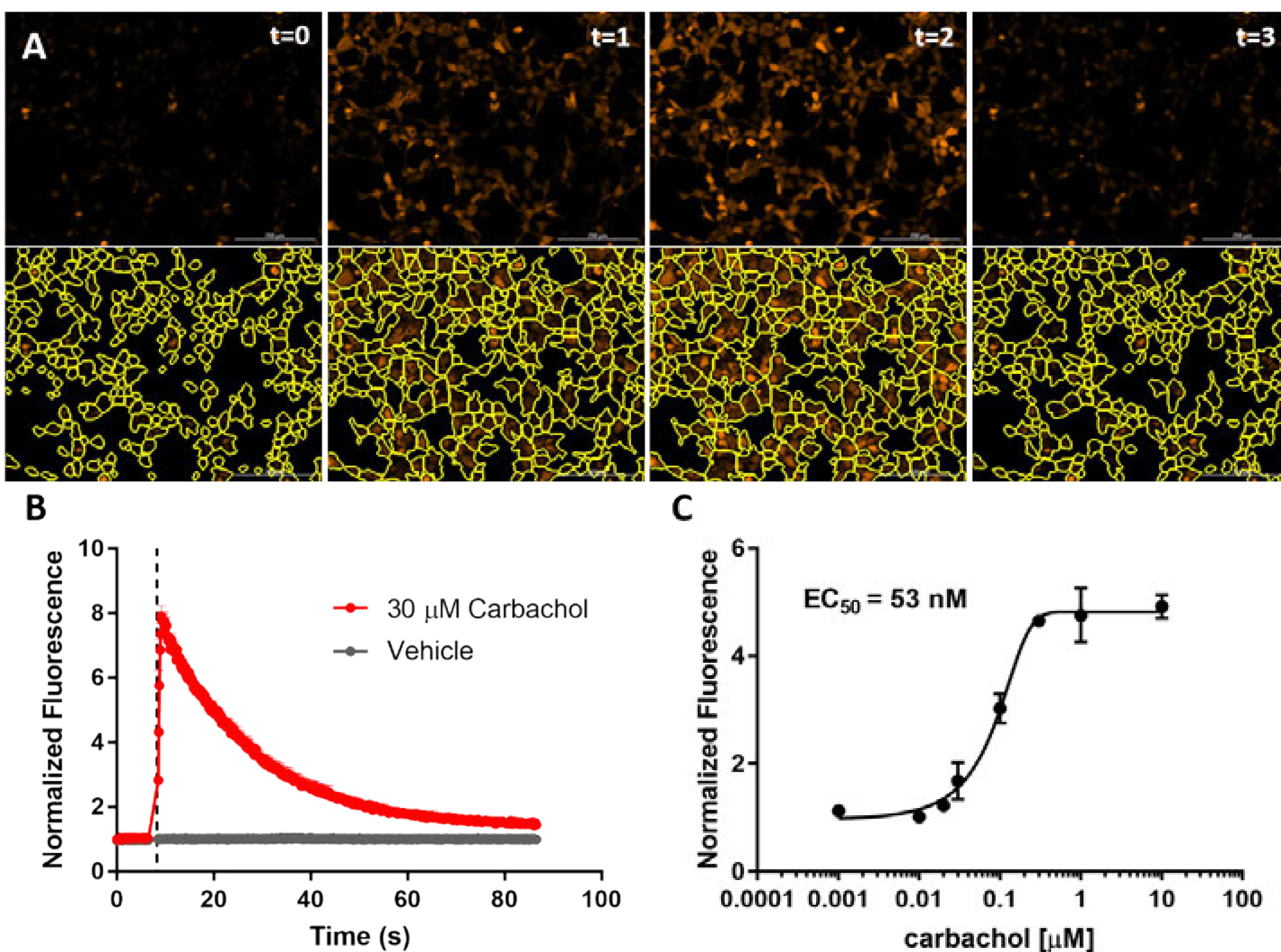

Figure 3. Monitoring intracellular Ca^{2+} release within a cell population over time following G_q-coupled hM1 receptor activation. (A) Image panel of HEK293 expressing red upward R-GECO sensor and hM1 receptor (top row) with Agilent BioTek Gen5 placed masks around cells containing R-GECO fluorescence above a determined threshold (bottom row). T = 0, 1, 2, and 3 equals 0, 0.4, 0.9, and 50 seconds post injection, respectively. (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 addition (dashed line). (C) Carbachol dose-response curve (F/F_0, n = 4 per concentration) with calculated EC_{50} value (t = 0.9 seconds post carbachol injection).

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

The Gen5 plug feature was used to measure Ca^{2+} mobilization within individual cells (Figure 4A), providing a detailed kinetic profile of GPCR activation following agonist addition (Figure 4B). Additionally, this imaging-based method revealed subcellular differences in the timing of Ca^{2+} release, in which an increase in R-GECO fluorescence within the cytoplasm was observed prior to the nucleus.

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

Gen5 dual masking was used to determine the number of cells that were activated by addition of 30 µM carbachol. 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 in the RFP and GFP channels every two seconds. Primary object masks were placed around the nucleus of each GFP positive cell to generate a total count of cells expressing the hM1 receptor. A secondary mask within the RFP channel was then used to generate a count of responder cells that had an increase in R-GECO fluorescence above a set threshold (Figure 5A). This automated imaging-based assay provides a unique and robust method for evaluating the ability of compounds to activate GPCR pathways (Figures 5B and 5C).

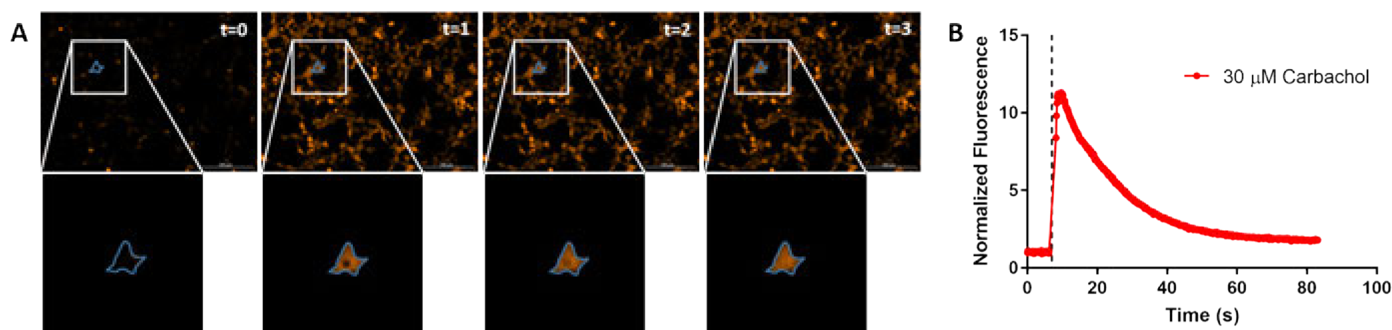

Figure 4. Quantitative and qualitative analysis of Ca^{2+} mobilization within a single HEK293 cell. Isolation of individual cells for analysis using the Agilent BioTek Gen5 plug feature can be used to generate detailed profiles of GPCR kinetics. (A) The plug tool isolates individual cells of interest to measure Ca^{2+} flux over time, revealing subcellular differences in the timing of calcium release within different regions of the cell. T = 0, 1, 2, and 3 equals 0, 0.4, 0.9, and 50 seconds post injection, respectively. (B) Quantitative single cell analysis enables accurate sub-second measurements of Ca^{2+} flux in response to carbachol injection (dashed line).

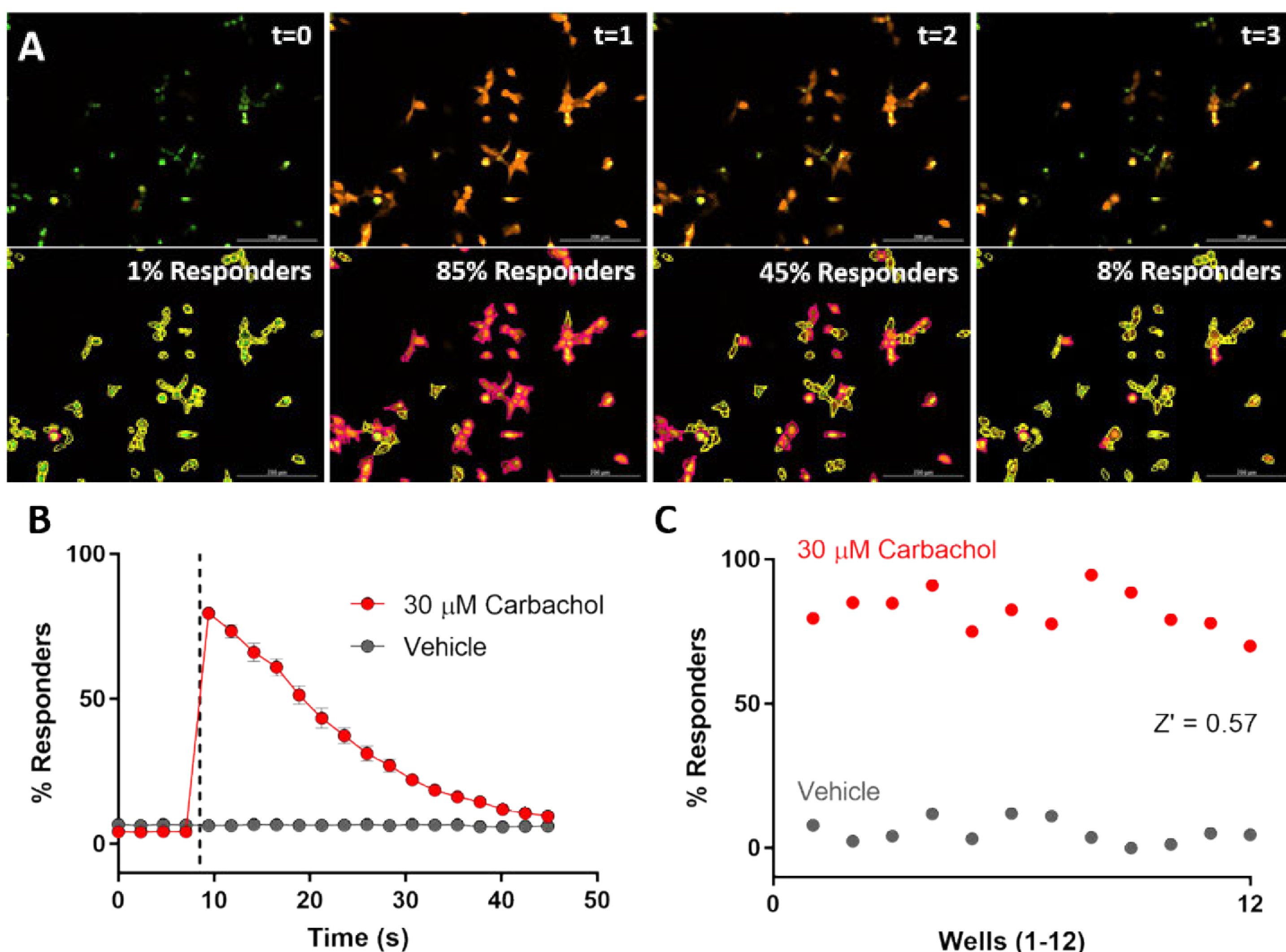

Figure 5. Agilent BioTek Gen 5 imaging software enables dual masking of cells for percent responder calculations. HEK293 cells transduced with R-GECO and hM1 muscarinic acetylcholine receptors with a nuclear localized GFP tag were stimulated with 30 µM carbachol and imaged every 2 seconds in the RFP and GFP channels. (A) Primary object masks were placed around the nucleus of each GFP positive cell. A secondary mask was used to measure RFP fluorescence. T = 0, 1, 2, and 3 equals 0, 0.4, 0.9, and 50 seconds post injection, respectively. (B) Kinetic analysis of percent responders over time provides a sensitive and (C) robust assay for measuring GPCR activation (Z' = 0.57).

Conclusion

The Agilent BioTek Lionheart FX and Cytation automated imagers are powerful platforms to conduct diverse GPCR-related applications, including detecting Ca^{2+} flux with the R-GECO Ca^{2+} biosensor from Montana Molecular. The rapid image capture rates and dual in-line reagent injectors available with these instruments enable uninterrupted monitoring of rapid cellular responses. Agilent BioTek Gen5 image preprocessing and cellular analysis tools greatly reduce background fluorescence, providing a large assay window and improved sensitivity over methods relying on total fluorescence intensity measurements. GPCR activation can be quantified using total cellular fluorescence within a population or percent responder measurements. Isolation of individual cells for analysis using the Gen5 plug feature provides detailed profiles of GPCR kinetics. The 96-well format and automated image capture and analysis increases GPCR assay productivity and reproducibility.

Together with the R-GECO Ca^{2+} biosensor, the Agilent BioTek Lionheart FX and Cytation imaging systems provide a sensitive and robust solution for calcium mobilization studies.

References

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