

Automated imaging-based technique for monitoring of Ca²⁺ and DAG biosensors enables detailed characterization of GPCR kinetics



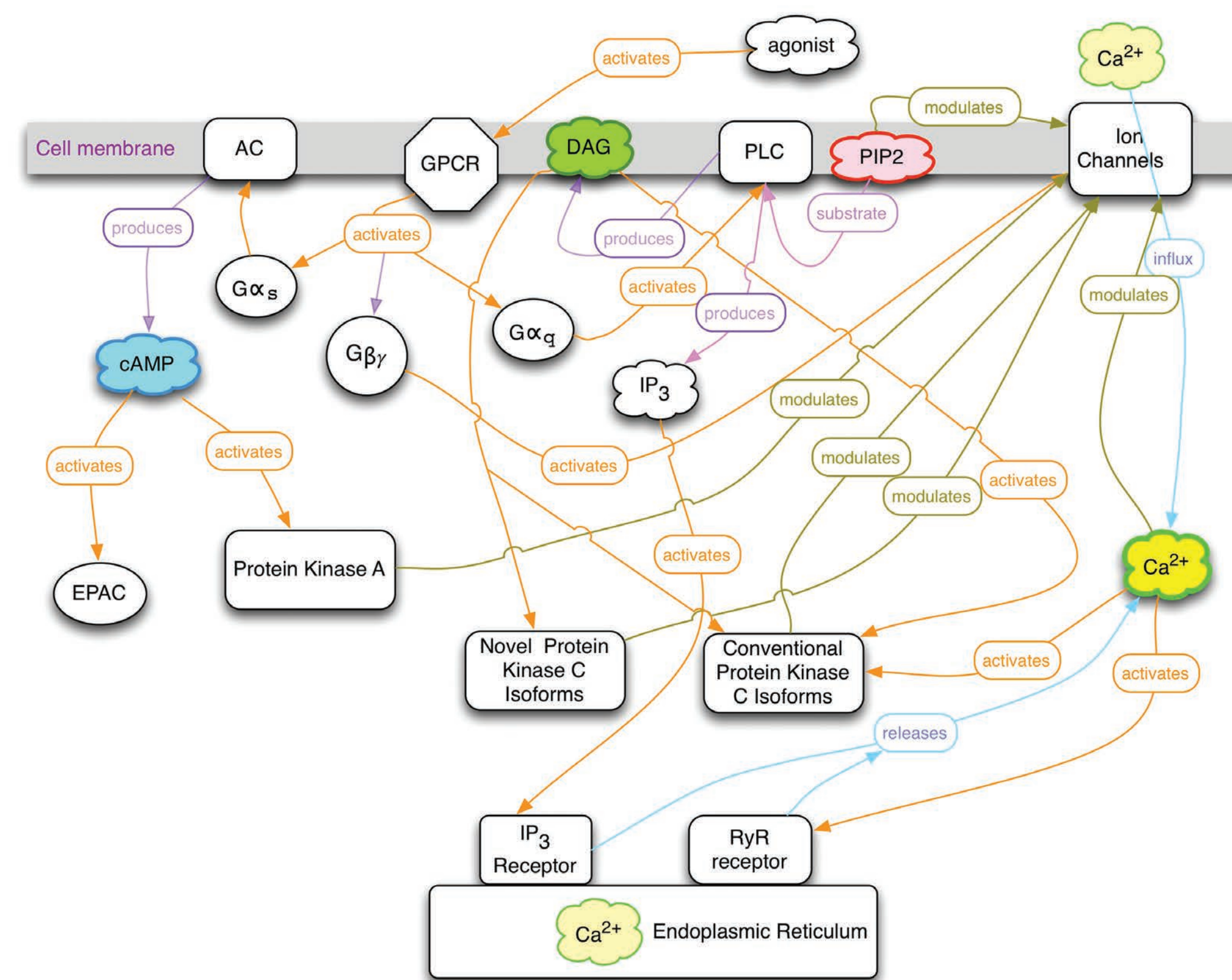
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Introduction

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. The molecules that activate GPCRs, and the resulting signaling cascades triggered by associated G proteins, are diverse. Activation of the G_s sub-family of G proteins increases phospholipase C (PLC) activity, converting phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG), both of which function as second messengers. IP₃ triggers release of intracellular Ca²⁺ stores which regulate calcium-dependent proteins, while membrane-bound DAG activates the signal cascade via protein kinase C.

Here we describe an automated imaging-based approach to characterize the IP₃/DAG signaling pathway using multiplexed R-GECO Ca²⁺ and DAG fluorescent biosensors from Montana Molecular. This method produces detailed kinetic profiles of Ca²⁺ flux and DAG levels following activation of G_s-coupled receptors within the same population of cells. Background subtraction and image analysis tools enable detection of changes in Ca²⁺ and DAG levels that are more sensitive than techniques relying on bulk fluorescence measurements. Additionally, this method enables single-cell analysis for detailed characterization of response dynamics and subcellular effects.



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

BioTek Instrumentation

Lionheart™ FX Automated Microscope 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 frames per second (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 CO₂/O₂ and available humidity control provides optimal conditions for long-term imaging of live cells.

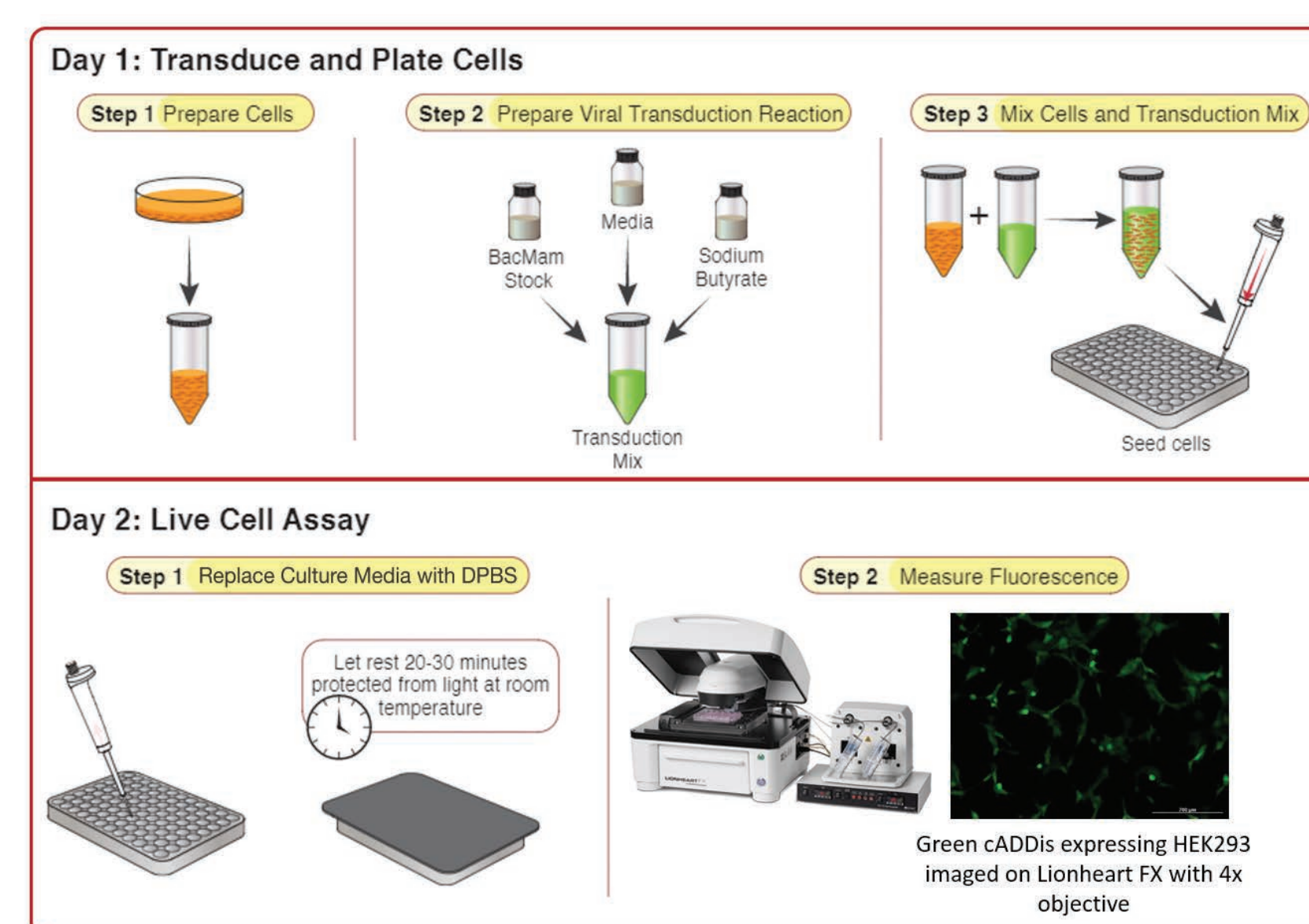
Powerful Gen5™ 3.0 Microplate Reader and Imager

Software: Automated image capture, processing, and analysis tools, including dual masking for cell counting and subpopulation analysis, plus annotation and movie maker functions.



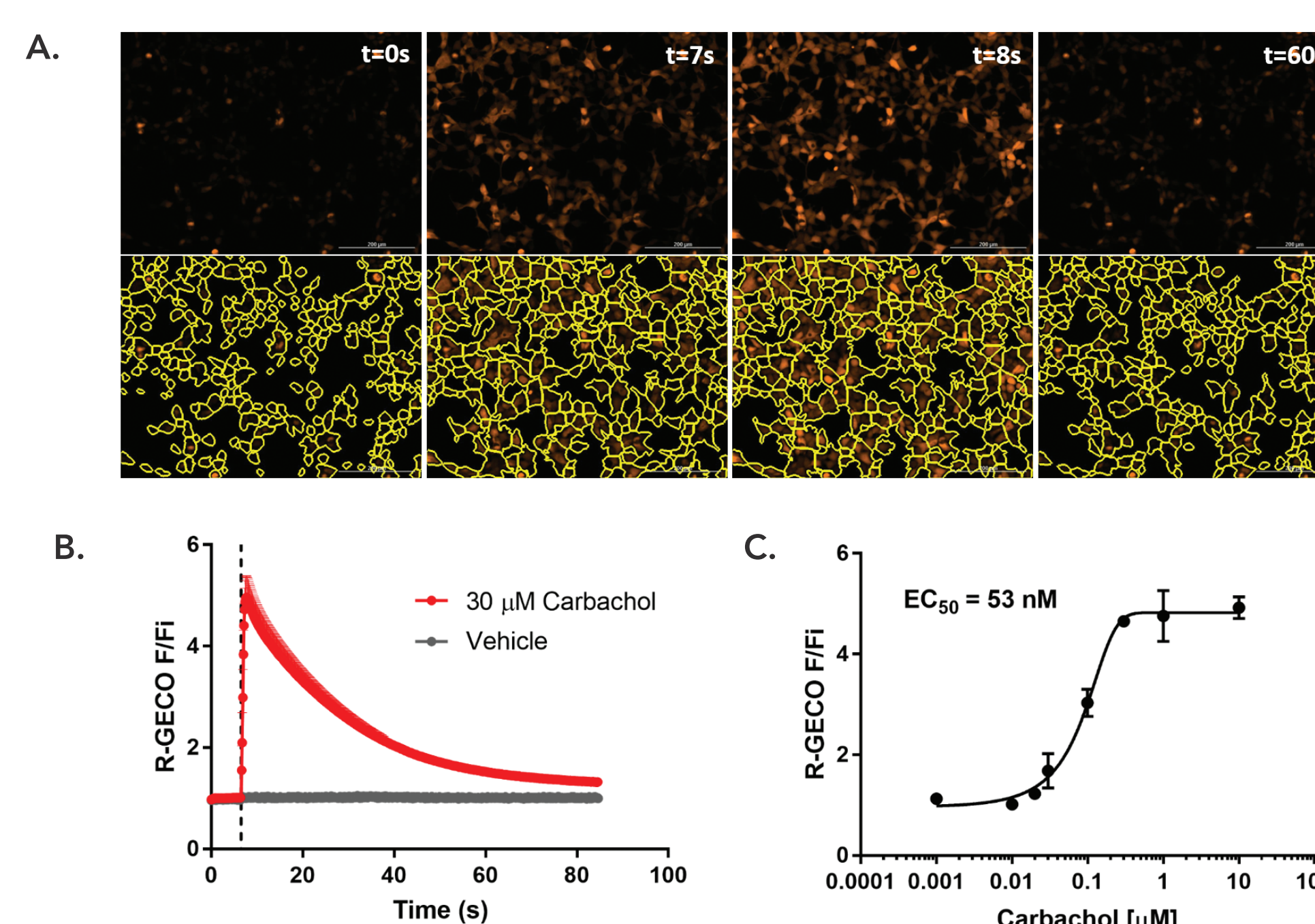
Methods

Live cell imaging of GPCR activity using Montana Molecular biosensors



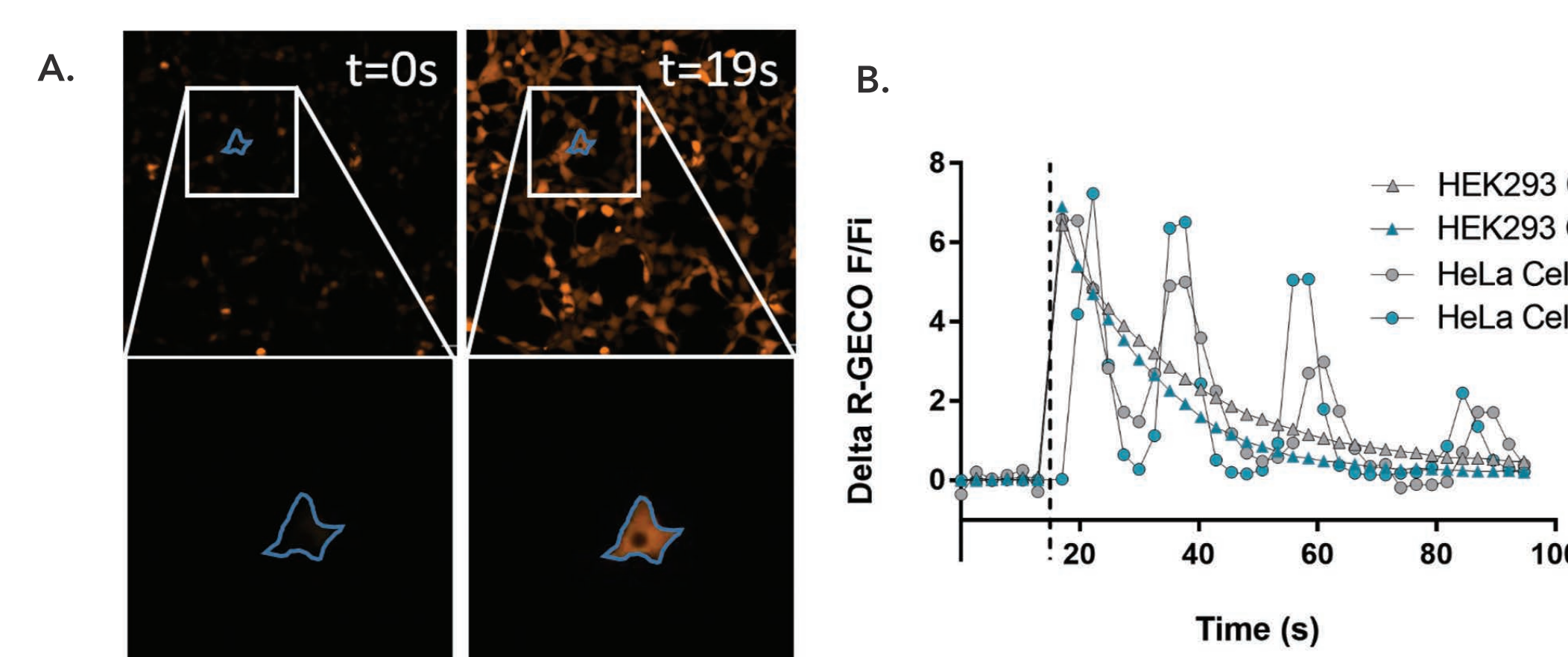
Detecting Rapid Ca²⁺ Mobilization with R-GECO Biosensor

Monitoring intracellular Ca²⁺ release in response to G_s-coupled hM1 receptor activation



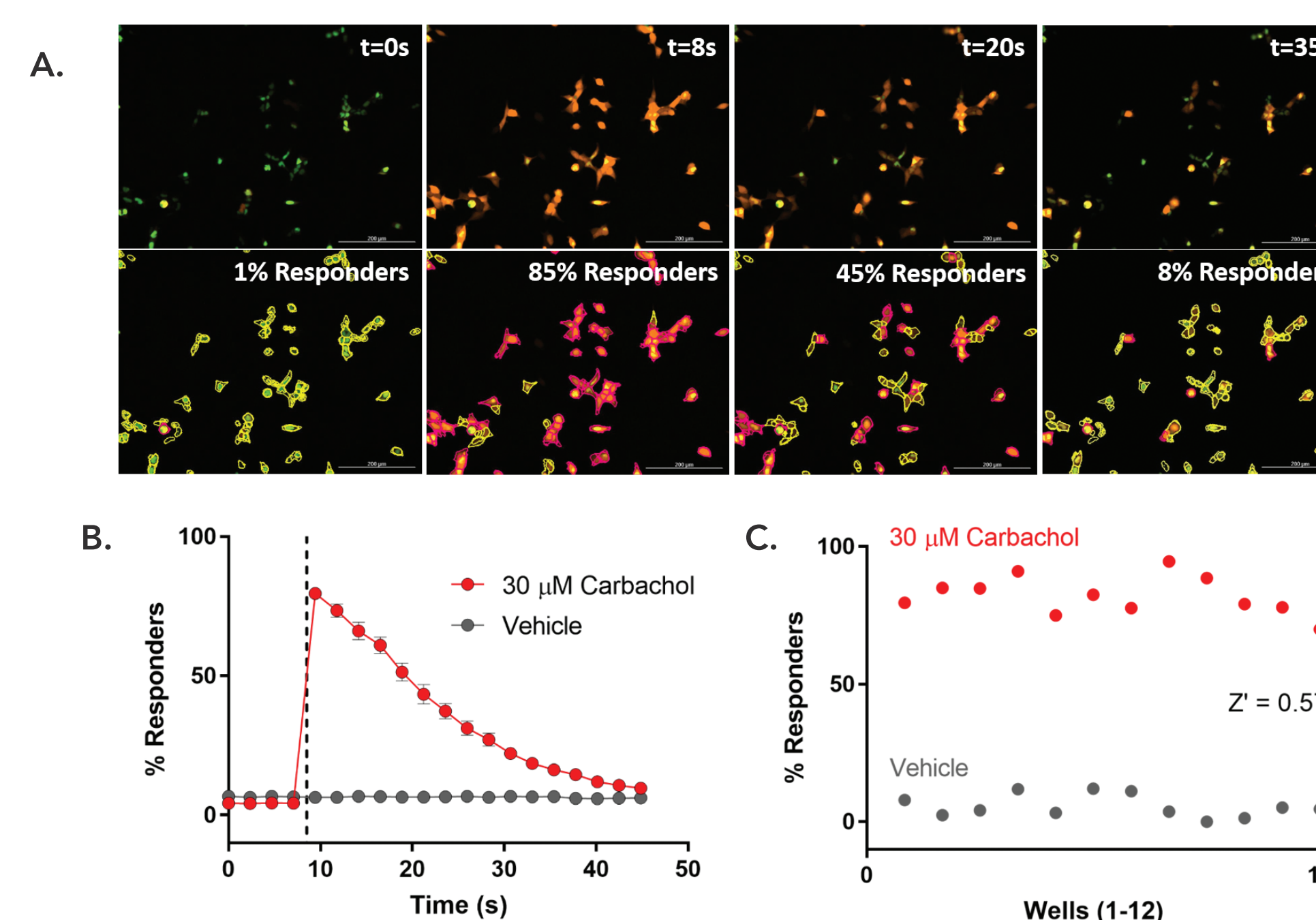
Quantifying activation of G_s-coupled hM1 receptors in HEK293. HEK293 expressing R-GECO and hM1 muscarinic acetylcholine receptors were imaged with a 10x objective for 85 seconds at 10 fps. **(A)** Image panel of HEK293 cells (top) with masks placed by Gen5 software around cells containing R-GECO fluorescence above a determined threshold (bottom). R-GECO fluorescence, which increases with increasing levels of Ca²⁺, is initially low at baseline. Stimulation of G_s-coupled hM1 receptors by injection of 30 μM (final) carbachol causes intracellular mobilization of Ca²⁺ and a corresponding rapid increase in R-GECO fluorescence, followed by a gradual decrease in cytoplasmic Ca²⁺ levels to near baseline. **(B)** Kinetic profile of R-GECO object sum integral fluorescence (n=8) in response to G_s-coupled hM1 receptor activation by 30 μM carbachol (dashed line). **(C)** Carbachol dose response curve (n=4 per concentration) with calculated EC₅₀ value.

Single-cell analysis reveals cell-type dependent differences in calcium flux dynamics



Quantitative and qualitative analysis of Ca²⁺ mobilization within individual cells reveals unique properties. Isolation of individual cells for analysis using the Gen5 plug feature can be used to generate detailed profiles of GPCR kinetics. HEK293 and HeLa expressing R-GECO were imaged with a 10x objective for 95 seconds at 2 fps. **(A)** Images of HEK293 with image plug (outlined in blue) pre- and post-stimulation reveal unique characteristics of Ca²⁺ mobilization. **(B)** Quantitative single cell analysis enables accurate sub-second measurements of Ca²⁺ flux in response to carbachol injection (dashed line). HEK293 exhibit a single calcium flux event followed by a gradual return to baseline. In contrast, HeLa exhibit multiple calcium flux events of decreasing intensity approximately 20 seconds apart.

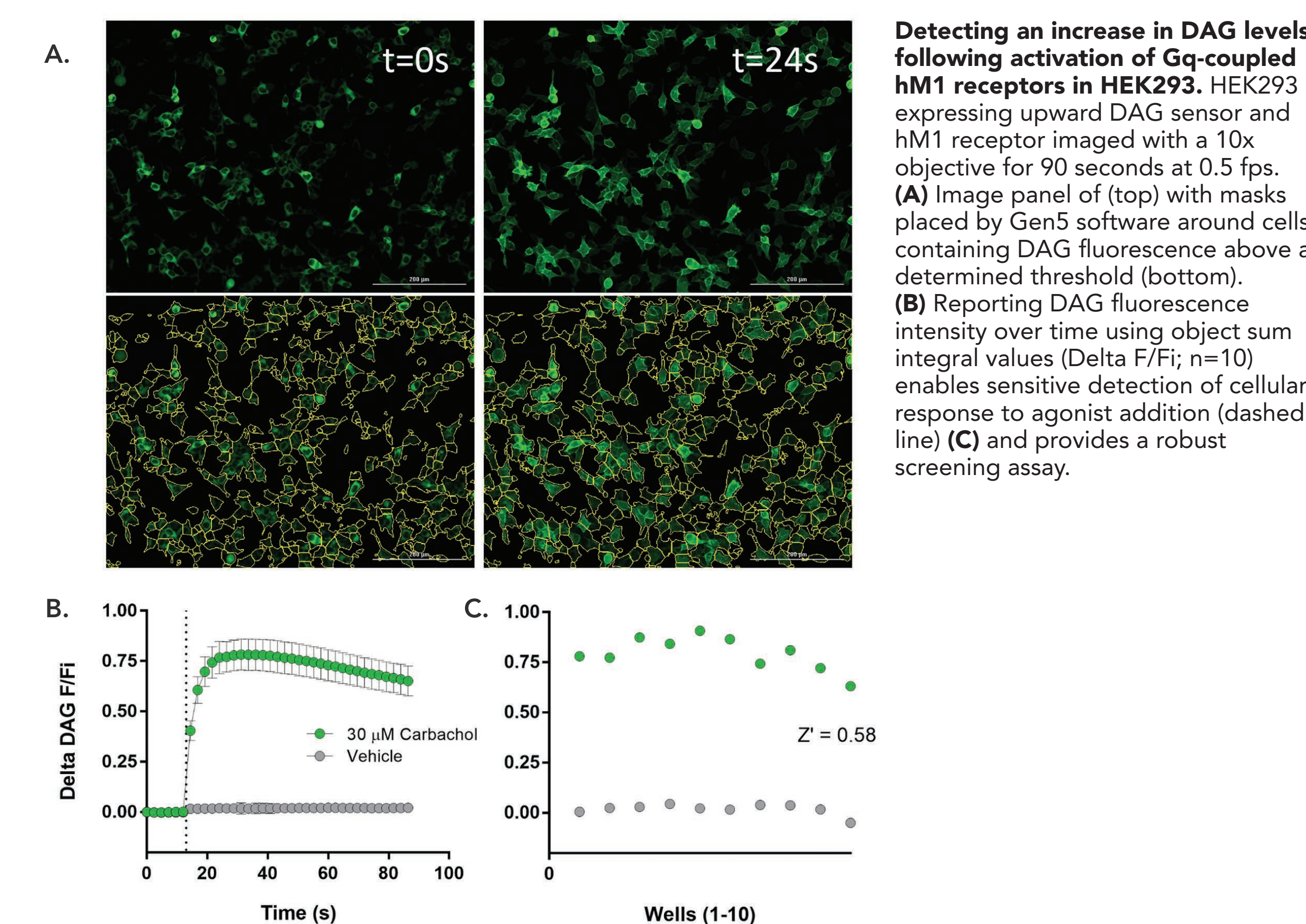
Quantifying G_s-coupled hM1 activation by percent responder using dual masking



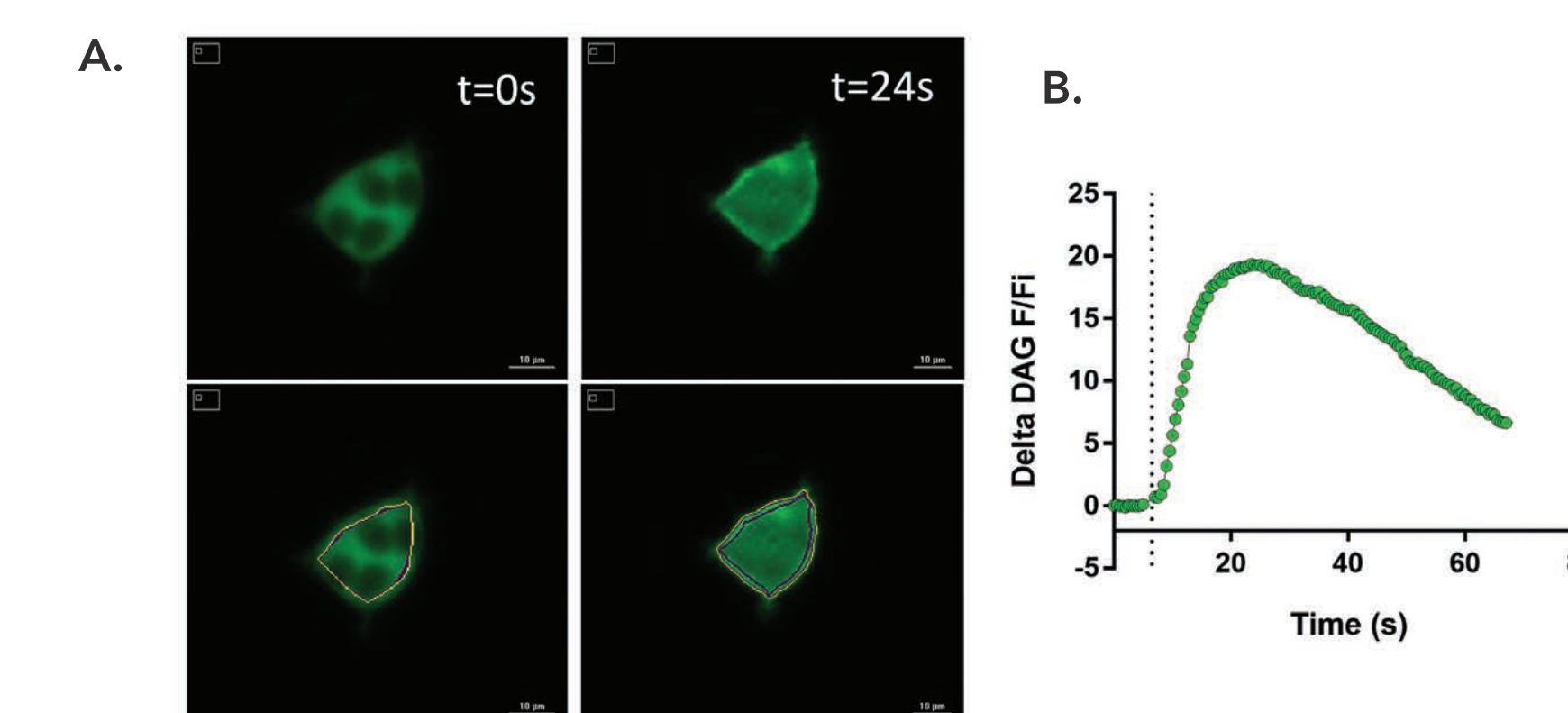
Gen5 Microplate Reader and Imager Software enables dual masking of cells for percent responder calculations. HEK293 cells expressing R-GECO and hM1 receptors with a nuclear localized GFP tag were stimulated with 30 μM carbachol and imaged with a 10x objective for 45 seconds at 0.5 fps. **(A)** Primary 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 DAG Levels in Real Time with Detailed Analysis

Sensitive and robust detection of G_s-mediated increase in DAG levels

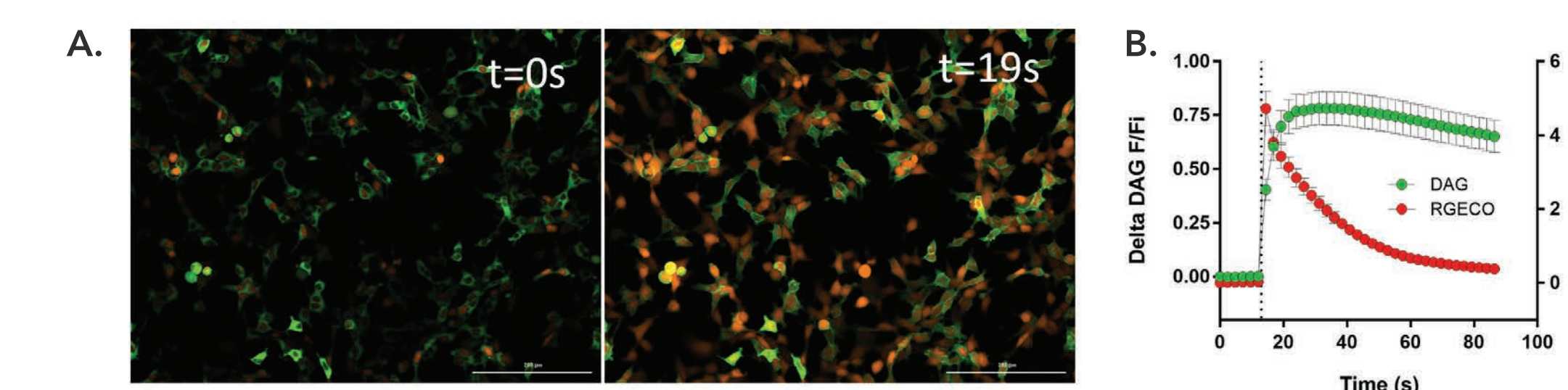


Gen5 image analysis tools enable unique characterization of DAG fluorescence at the cell membrane



Gen5 dual masking enables measuring DAG fluorescence at the cell periphery over time. HEK293 cells expressing upward DAG were imaged with a 20x objective for 70 seconds at 2 fps. **(A)** Individual cells were isolated from the field using the Gen5 plug tool for subcellular analysis. Primary mask (purple) was used to define cellular region excluding 4 μm from perimeter. The secondary mask (yellow) expands out from the primary mask to include peripheral regions with DAG fluorescence above a set threshold. **(B)** Profile of membrane-bound DAG over time reveals a pronounced increase in fluorescence in this region following addition of 30 μM carbachol (dashed line).

Multiplexing DAG and R-GECO biosensors for simultaneous second messenger readout



Co-expressing DAG and R-GECO biosensors enables monitoring of both arms of the IP₃/DAG pathway within the same cells. HEK293 cells expressing green upward DAG, red upward R-GECO, and hM1 receptors were imaged with a 10x objective for 90 seconds at 0.5 fps. **(A)** Images of cells pre- and post-stimulation with 30 μM carbachol. **(B)** Profile of object sum integral DAG and R-GECO fluorescence over time. Both biosensors exhibit a rapid increase in fluorescence following addition of 30 μM carbachol (dashed line) (n=10).

Conclusions

- Together, the Lionheart FX Automated Microscope 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 the IP₃/DAG signaling pathway.
- 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.