Application Note

Rapid Cell Signaling

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Characterizing Calcium Mobilization Using Kinetic Live Cell Imaging

Author

Joe Clayton, PhD and
Rebecca Mongeon, PhD
Agilent Technologies, Inc.

Abstract

Ca^{2+} acts as an important second messenger in diverse signaling pathways, including G protein-coupled receptors. Characterizing these pathways requires the ability to detect rapid changes in intracellular Ca^{2+} levels with high temporal resolution. This application note describes a live cell imaging-based approach to quantify Ca^{2+} flux kinetics using an Agilent BioTek Lionheart FX and Fluo-4 Ca^{2+} indicator dye that delivers sub-second resolution and a large assay window.

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 G_s and G_q subfamily of α-subunits triggers the release of intracellular calcium stores into the cytoplasm, which propagates the signal by regulating calcium-dependent proteins.[3]

Fluo-4 calcium indicators have been used extensively for detecting changes in calcium levels. In the presence of calcium, Fluo-4 fluorescence intensity is increased, potentially providing a valuable tool to detect GPCR activation. However, applications involving certain cell lines and receptors have been limited by the relatively weak fluorescent signal produced by Fluo-4 calcium indicators. This application note describes a live cell imaging assay to detect calcium-dependent Fluo-4 fluorescence using the Agilent BioTek Lionheart FX automated microscope and HeLa cells expressing endogenous P2Y GPCRs. In-line dispense tips enable the addition of agonist with continuous monitoring of cell response. This method provides sensitive detection and characterization of intracellular calcium flux with sub-second temporal resolution.

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. The environmental control cover provides incubation to 40 °C and effective containment for CO_2/O_2 control. The humidity chamber and reagent injector add a greater level of environment optimization for live cell imaging workflows. Agilent BioTek Gen5 software provides automated image capture and analysis, plus annotation and movie maker functions. Gen5 offers ease and simplicity across a broad range of live and fixed cell applications, including perfusion assays. Augmented microscopy is the combination of all of these features in one compact system.

Figure 1. Agilent BioTek Lionheart FX automated microscope with dual-reagent injector module.

Fluorescent labels

Fluo-4 is a calcium indicator that exhibits increased fluorescence at 469/525 (GFP) in the presence of calcium. Fluo-4 AM by Thermo Fisher Scientific (Waltham, MA) is an acetoxymethyl ester derivative of Fluo-4 that can permeate cell membranes. Once the Fluo-4 AM molecule is inside the cell, nonspecific esterases cleave the lipophilic blocking groups, forming a charged compound that is less likely to leak out of cells.

Cell preparation

HeLa 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. Cells were seeded overnight at 15,000 cells per well (100 µL) in Corning 3904 black-sided clear-bottom plates. Approximately 24 hours later, cell media was replaced with 100 µL of Advanced DMEM containing 4 µM Fluo-4 AM. Following one hour of incubation at room temperature protected from light, media was removed and wells were washed with 100 µL of Dulbeccos phosphate-buffered saline (DPBS). Wash was then removed and replaced with 100 µL fresh DPBS for imaging.

Imaging

Images were acquired using a 4x objective at a rate of 3 frames per second. Cells were imaged for a short period to determine baseline fluorescence prior to addition of ATP agonist. In-line injectors were used to dispense 20 µL of either DPBS (control) or DPBS plus 60 µM ATP (10 µM final) into the wells, and cells were imaged for an additional 30 seconds to monitor response.

Image preprocessing

Background fluorescence was reduced by applying preprocessing to images prior to analysis (Table 1, Figure 2).

Table 1. Preprocessing parameters for GFP images.

Single Channel Fast Kinetic Image Processing	
Image Set	GFP
Background	Dark
Rolling Ball Diameter	Automatic
Image Smoothing Strength	0 cycles of 3 x 3 average filter

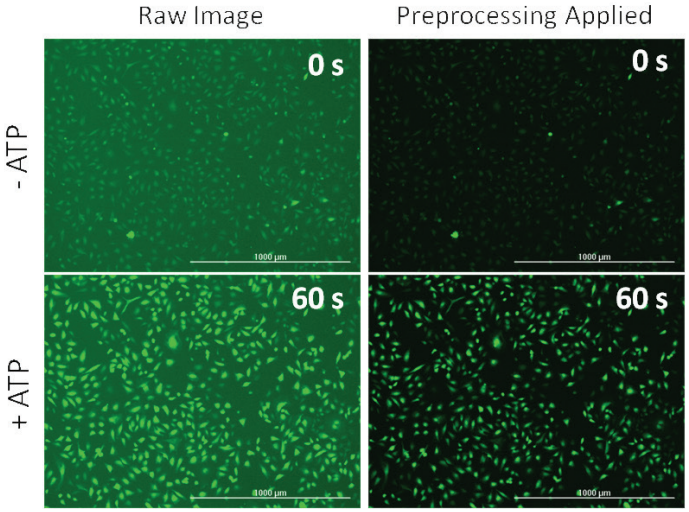

Figure 2. Preprocessing of images reduces background fluorescence. Representative images showing unprocessed images before and after addition of ATP on the left and corresponding images with preprocessing applied on the right.

Identification of cells with threshold mask improves detection of GPCR activation

The Gen5 object masking feature enables identification of cells within the imaging field. This feature can be used to apply a mask around cells by setting the threshold just below the Fluo-4 AM fluorescence generated from the baseline intracellular calcium (Table 2).

Table 2. Cellular analysis parameters for generating a mask around cells. Setting a fluorescent threshold within the GFP channel improves ability to detect changes in Fluo-4 fluorescence.

Single Channel Fast Kinetic Cellular Analysis Parameters	
Channel	GFP
Threshold	3,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimum Object Size	10 µm
Maximum Object Size	300 µm
Include Primary Edge Objects	Checked
Analyze Entire Image	Checked
Advanced Analysis Parameters	
Rolling Ball Diameter	50 µm
Image Smoothing Strength	1 cycle of 3 × 3 average filter
Evaluate Background On	20% of lowest pixels
Primary Mask	Use threshold mask

Calcium-dependent changes in Fluo-4 AM fluorescence can then be measured within these defined cellular areas (Figure 3).

In addition to aggregate cellular analysis, individual cell calcium flux can be investigated through the Gen5 object tracking module. The Gen5 object tracking module identifies, enumerates, and tracks individual cells through a kinetic image series. Object tracking can be applied to rapid calcium kinetic analysis to investigate the mean fluorescence response profiles of individual cells (Table 3).

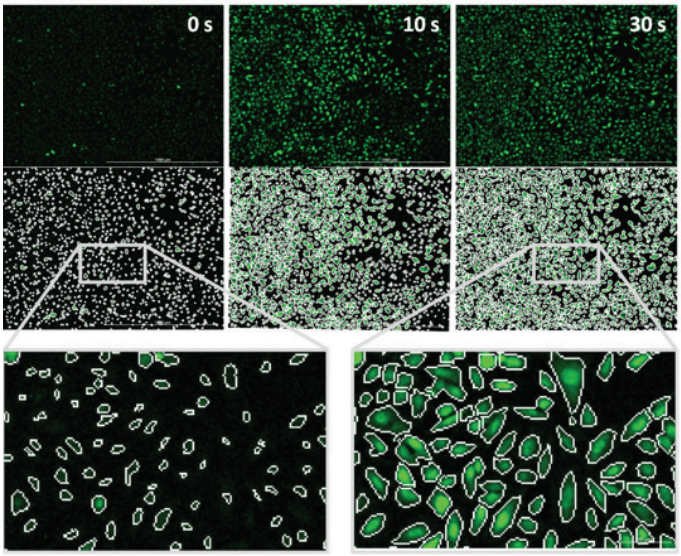

Figure 3. The masking tool enables measuring changes in fluorescence only within Fluo-4 containing cells. Baseline level of Fluo-4 fluorescence is low pre-addition of ATP agonist (time = 0 s). After ATP addition, the cellular mask expands as Fluo-4 AM fluorescence increases with calcium mobilization within the cell (time = 10 s), until maximum fluorescence is reached (time = 30 s). Enlarged images (bottom) highlight masking around cells pre- and post-addition of ATP.

Table 3. Cellular analysis parameters for the Agilent BioTek Gen5 cell tracking module used for the analysis presented in Figure 5. Search radius was minimized due to the expectation that cell position would remain essentially stable over this kinetic timeframe (~ 30 s). Minimum lifetime chosen here reflects the total number of images (frames) captured in this kinetic series, so only cells tracked from beginning to end will be included in the analysis. Values used should be optimized according to each specific experimental design.

Object Tracking Module Analysis Parameters	
Channel	GFP
Threshold	Auto
Background	Dark
Auto Threshold Slider Value	88
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Track Objects	Checked
Minimum Object Size	10 µm
Maximum Object Size	100 µm
Include Primary Edge Objects	Checked
Analyze Entire Image	Checked
Trajectory Options	
Search Radius	20 µm
Minimum Lifetime	100 frames
Advanced Analysis Parameters	
Background Flattening	Unchecked
Image Smoothing Strength	1 cycle of 3 x 3 average filter
Primary Mask	Use threshold mask

Results and discussion

Detecting GPCR activation within cell population

ATP-induced activation of endogenously expressed P2Y receptors was measured in HeLa cells. Intracellular calcium mobilization was detected by monitoring Fluo-4 AM fluorescence using kinetic live cell imaging. Image preprocessing reduced background fluorescence, improving detection and analysis of calcium flux. An increase in intracellular calcium was detected approximately 3 seconds after addition of ATP (10 µM final).

Peak calcium mobilization for the entire field of cells was reached 13 seconds post ATP addition (Video 1). Mean fluorescence increased three-fold following addition of ATP. However, the object masking tool improved the assay window considerably by eliminating background fluorescence from the analysis. Using this method, a seven-fold increase in total Fluo-4 AM fluorescence resulted from the addition of ATP (Figure 4). Relative fluorescence for both analysis methods was calculated by dividing each time point by the initial fluorescence intensity.

Characterizing kinetics of calcium flux in individual cells

TIn addition to the population cell analysis shown in Figure 4, further analysis was performed through the Gen5 software object tracking module to automatically track and display the calcium responses of individual cells (Figure 5). Using object tracking, average Fluo-4 intensity was reported over time for hundreds of cells, and individual calcium responses were observed with high temporal resolution. Overlays of individual cell responses can be chosen by highlighting multiple cells of interest, as shown in Figure 5. The resulting GPCR activation profiles can be used to compare cellular responses under different conditions or between different cell types.

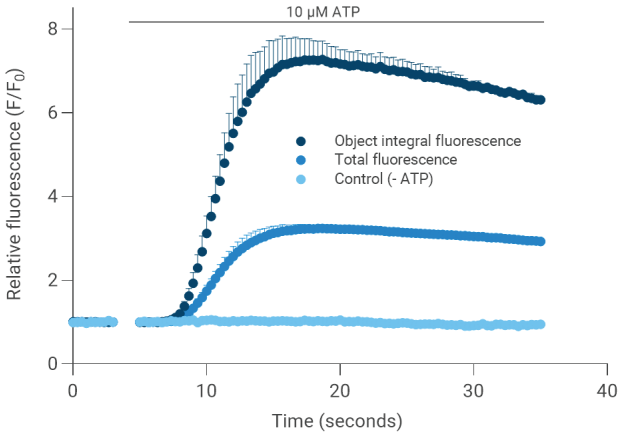

Figure 4. Time course of calcium mobilization following activation of P2Y receptor by ATP. Calcium-dependent Fluo-4 fluorescence was measured using preprocessed images that were collected at a rate of three frames per second. Following an initial baseline imaging period of three seconds, ATP (10 µM final) was automatically added using in-line injectors and responses imaged for an additional 30 seconds. Object integral fluorescence following ATP addition exhibited a seven-fold increase over baseline. Evaluating receptor activation using total fluorescence resulted in a three-fold increase over baseline.

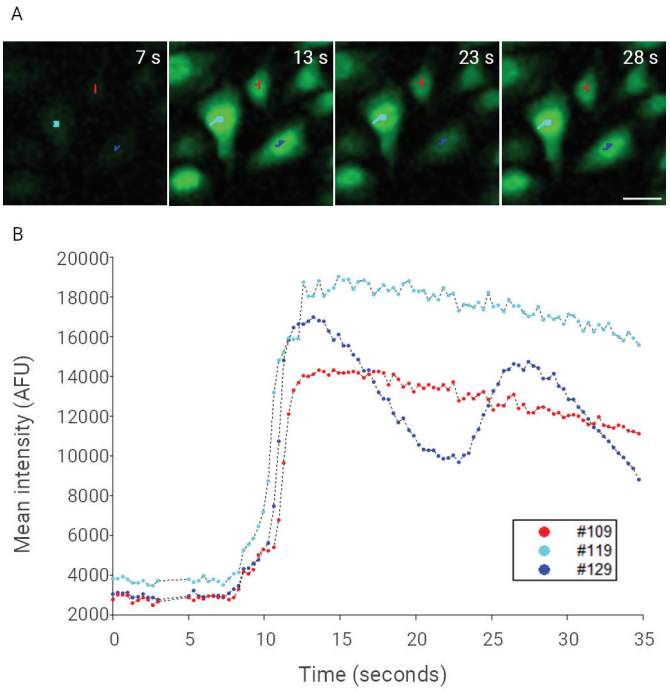

Figure 5. Single cell calcium mobilization kinetics with Agilent BioTek Gen5 software object tracking analysis. (A) A subregion of an example image series containing three HeLa cells captured at 4x magnification. Images at four representative timepoints are shown with time indicated. Cell centers are color-coded and correspond to the traces shown in panel B. Cell boundary mask overlays are omitted for clarity. Scale bar corresponds to 30 µm. (B) Automated object tracking analysis plots of the mean intensity of each cell selected in the image. Two of the example cell responses to 10 µM ATP application are characterized by a single peak calcium rise (teal and red), while the third cell demonstrates repetitive calcium transients (dark blue).

Conclusion

Monitoring calcium flux is an important method for characterizing GPCR activation. This application note demonstrates the benefits of a live cell imaging-based approach to measuring calcium-dependent Fluo-4 AM fluorescence using the Agilent BioTek Lionheart FX automated microscope and Agilent BioTek Gen5 microplate reader and imager software. In-line reagent injectors allow the addition of GPCR agonist with continuous monitoring of intracellular calcium levels. The sub-second image capture rate enables high temporal resolution for characterizing the rapid kinetics of calcium release following Gs- and Gq-coupled receptor activation. 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. Isolation of individual cells for analysis using the Gen5 object tracking module can be used to generate detailed profiles of GPCR kinetics. The automated image capture and image analysis methods detailed above can readily be modified to suit a variety of cell types and receptors. Additionally, Fluo-4 AM was selected as the calcium indicator dye for this study because it is widely accessible to researchers. However, the Lionheart FX system can be used to image intracellular calcium mobilization using a variety of calcium-dependent dyes and molecular sensors. Furthermore, this approach allows the characterization of other calcium-dependent processes, including studies involving cardiomyocyte contraction in response to mobilization of sarcomeric calcium stores.

References

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RA44909.567974537

Printed in the USA, January 10, 2023
5994-2523EN

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