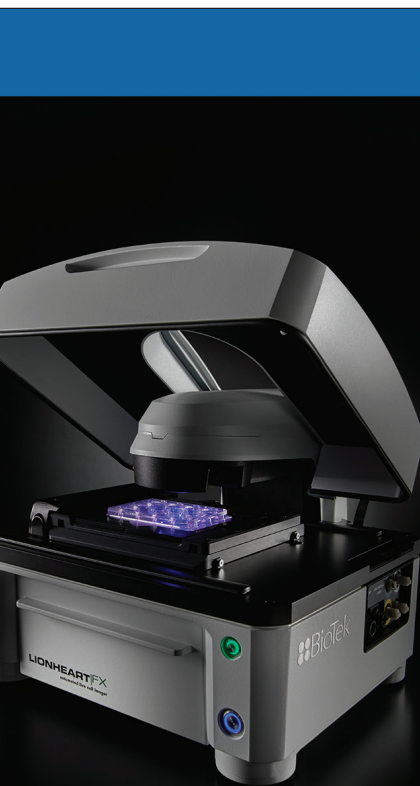


Characterizing Calcium Mobilization using Kinetic Live Cell Imaging

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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. Here we describe a live cell imaging based approach to quantify Ca^{2+} flux kinetics using the 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 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].

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. Here we describe a live-cell imaging assay to detect calcium-dependent Fluo-4 fluorescence using the Lionheart™ FX Automated

Live Cell Imager and HeLa cells expressing endogenous P2Y GPCRs. In-line dispense tips enable 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

Lionheart™ FX Automated Live Cell Imager

Lionheart FX Automated Live Cell Imager 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 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. Augmented Microscopy is the combination of all of these features in one compact system.

Key Words:

Calcium Signaling
GPCR Activation
Fluo-4 AM
Calcium Imaging



Figure 1. Lionheart™ FX Automated Live Cell Imager 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 ThermoFisher 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% CO₂ 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 Advanced DMEM containing 4 µM Fluo-4 AM. Following one hour incubation at room temperature protected from light, media was removed and wells were washed with 100 µL 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 base-line 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).

Single Channel Fast Kinetic Image Processing

Image set	GFP
Background	Dark
Rolling Ball Diameter	Automatic
Image Smoothing Strength	0 cycles of 3x3 average filter

Table 1. Preprocessing parameters for GFP images.

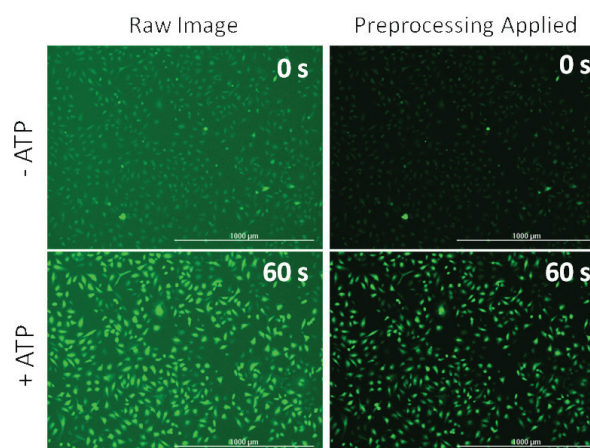


Figure 2. Preprocessing of images reduces background fluorescence. Representative images showing unprocessed images before and after addition of ATP on the left and corresponding images with preprocessing applied on the right.

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 utilized 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).

Single Channel Fast Kinetic Cellular Analysis Parameters

Channel	GFP
Threshold	3000
Background	Dark
Split touching objects	Checked
Fill holes in masks	Checked
Min. Object Size	10 µm
Max. 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 3x3 average filter
Evaluate Background On	20% of lowest pixels
Primary Mask	Use threshold mask

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.

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

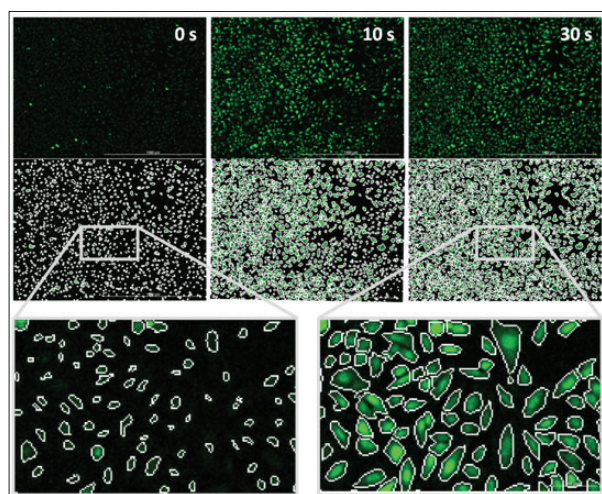


Figure 3. The masking tool enables measuring changes in fluorescence only within Fluo-4 containing cells. Baseline level of Fluo-4 fluorescence is low pre-addition of ATP agonist (time = 0 s). After ATP addition, the cellular mask expands as Fluo-4 AM fluorescence increases with calcium mobilization 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.

Results

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 μ M 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.

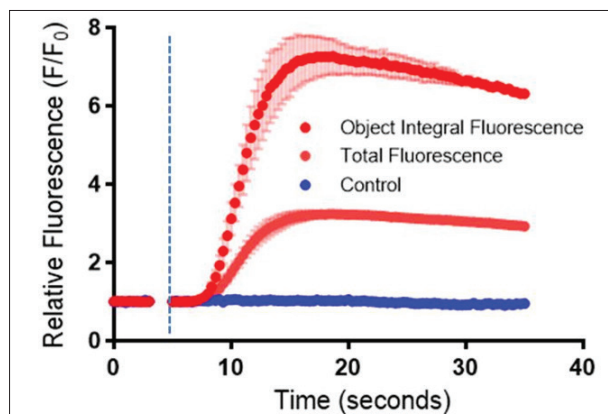


Figure 4. Time course of calcium mobilization following activation of P2Y receptor by ATP. Calcium-dependent Fluo-4 fluorescence was measured using preprocessed images that were collected at a rate of 3 frames per second. ATP (10 μ M final) was added using in-line dispenser at $t = 5$ seconds (dashed line). 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.

Characterizing Kinetics of Calcium Flux in Individual Cells

The plug tool can be used to define regions of interest for analysis. By setting the plug around a single cell within the population, the kinetics of GPCR activation and resulting calcium flux can be characterized for individual cells (Figure 5). Using this method, the rate of intracellular calcium release and the interval between spikes in calcium levels can be defined with high temporal resolution. The resulting GPCR activation profiles can be used to compare cellular responses under different conditions or between different cell types.

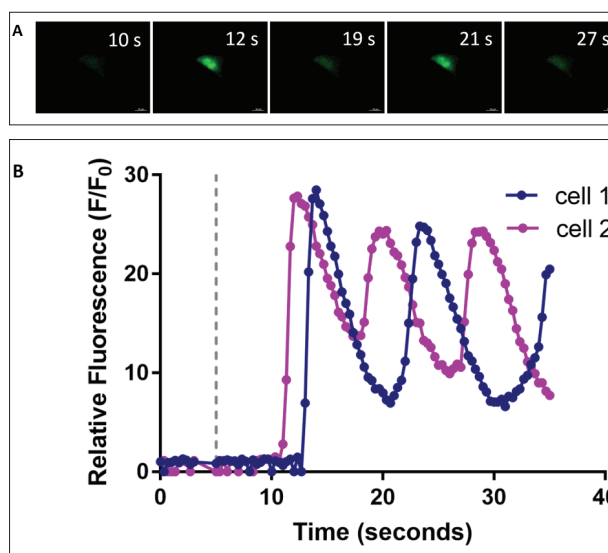


Figure 5. Single Cell Kinetics of GPCR Activation. Two cells were selected from a representative experiment and the relative object integral fluorescence was measured over time to characterize GPCR activation. (A) Images of plug set around cell 2 at five time points. (B) Cells 1 and 2 exhibited similar activation profiles. A rapid spike in calcium mobilization occurred 5-7 seconds post ATP addition (10 μ M final) (dashed line), followed by a gradual decline in Fluo-4 fluorescence over 6-8 seconds before calcium levels spiked again.

Conclusions

Monitoring calcium flux is an important method for characterizing GPCR activation. Here we have demonstrated the benefits of a live-cell imaging based approach to measuring calcium dependent Fluo-4 AM fluorescence using the Lionheart™ FX Automated Live Cell Imager and Gen™ 5 Microplate Reader and Imager Software. In-line reagent injectors allow for 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 plug feature can be used to generate detailed profiles of GPCR kinetics. The automated image capture and image analysis methods detailed above can be readily 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 employed to image intracellular calcium mobilization using a variety of calcium-dependent dyes and molecular sensors. Furthermore, this approach allows for 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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