High Content Compatible Fast Frame Rate Imaging of GPCR mediated Calcium Signaling

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1 Introduction

G-protein coupled receptors (GPCRs) are the largest family of plasma membrane receptors. They are activated by a huge variety of different molecules outside the cell and activate intracellular signal transduction pathways, which ultimately lead to different cellular responses, such as the increase of intracellular calcium concentration. Dysregulation of GPCR signaling, particularly fluctuations in calcium homeostasis signaling, has been implicated in different diseases, e.g. cancer and Alzheimer's disease. Currently about 34% of all FDA approved drugs target GPCRs indicating their importance for therapeutic treatments.-. Thus, *in vitro* cellular GPCR measurements are considered a hallmark assay to understand the kinetics and mechanisms of actions to identify new drugs. Here we show how to analyze fast changes in intracellular calcium levels upon GPCR stimulation and inhibition using a fluorescence imaging assay on the Opera Phenix® Plus high-content screening system. Compounds are added using the on-board liquid handling module which transfers an agonist solution from a compound plate to the assay plate. Acquisition frame rates of up to 100 frames per second allow the monitoring of rapid changes in intracellular calcium concentration and dedicated image analysis tools of Harmony® software enable quantification of information from these image sequences.

2 Assay Preparation

Cell Culture and Staining
CHO-K1 AequoScreen® Histamine H1 cells were seeded at 3.5E4 cells per well in 150 µl growth medium in a CellCarrier-96 Ultra plate. After overnight incubation the medium was removed, and cells were stained for 30 minutes with 50 µl of 1 µM Cal-520® in growth medium. Then the calcium dye staining solution was replaced by 150 µl of fresh growth medium and incubated for a further 30 minutes in the incubator prior to imaging. For the antagonist assay, the staining solution additionally contained various concentrations of the inhibitor pyrilamine. After 30 minutes the staining solution was replaced with 150 µl of fresh medium containing pyrilamine at the same concentrations.

Table 1: Materials

Cells	CHO-K1 AequoScreen® Histamine H1 (PerkinElmer #ES-390-
Growth Medium	Hams F12 (Carl Roth #9108.1)
Calcium Dye	Cal-520®, AM (AAT Bioquest; Biomol #ABD-21130)
Agonist	Histamine (Sigma #H7250)
Antagonist	Pyrilamine (Sigma #P5514)
Buffer	DPBS without MgCl2 and CaCl2 (Sigma #D8537)
Microplate (Assay Plate)	CellCarrier-96 Ultra, Tissue Culture Treated (PerkinElmer #6055302)
Microplate (Compound Plate)	StorPlate-96, V-bottom (PerkinElmer #6008290)
Microplate Seal for Compound Plate	Aluminium Sealing Foil (Porvair #229572)
Pipettor Tips	200 µL Clear Roborack (PerkinElmer #6000681)
Imaging Instrument	Opera Phenix Plus System with Liquid Handling Option (PerkinElmer #HH14001000)

Preparation of Compound Storage Plates
For the agonist assay 100 µl per well of different histamine concentrations, 40 µM - 0.4 nM in DPBS were provided in a StorPlate-96V (four replicates) and for the antagonist assay 100 µl per well of 400 nM histamine solution in DPBS. To minimize evaporation, compound storage plates were heat-sealed.

Automated Onboard Liquid Handling and Image Acquisition
Compound and assay microplates were transferred to the Opera Phenix Plus system to equilibrated temperature, CO2, and regulated relative humidity. Images were acquired in non-confocal mode using a 20x water immersion objective lens. The FITC channel was used with 10 ms exposure time to measure one plane within a time series measurement in well-repeat mode. Each well was processed with two consecutive image acquisition sequences with one pipetting step between the two sequences. The first sequence included five time points with 1 fps followed by one automated pipetting step transferring 50 µl compound solution into the appropriate well of the assay plate. The fast kinetic image acquisition of the second sequence was synchronized with the start of the compound transfer procedure, at 68 fps for a total of 400 timepoints.

3 Agonist Assay

The GPCR activation was kinetically monitored using histamine as agonist compound. The fluorescent dye Cal-520® crosses the cell membrane and then binds to the intracellular released calcium. Upon agonist stimulation, intracellular calcium release will correlate with increase fluorescence signal. Various concentrations of the agonist histamine were transferred with the onboard liquid handling module from the compound plate to the assay plate. With increasing histamine concentration, rising Cal-520 fluorescence was detected. Correspondingly, the maximum Cal-520 fluorescence intensity is detected at an earlier timepoint with increasing histamine concentration (Figure 1).

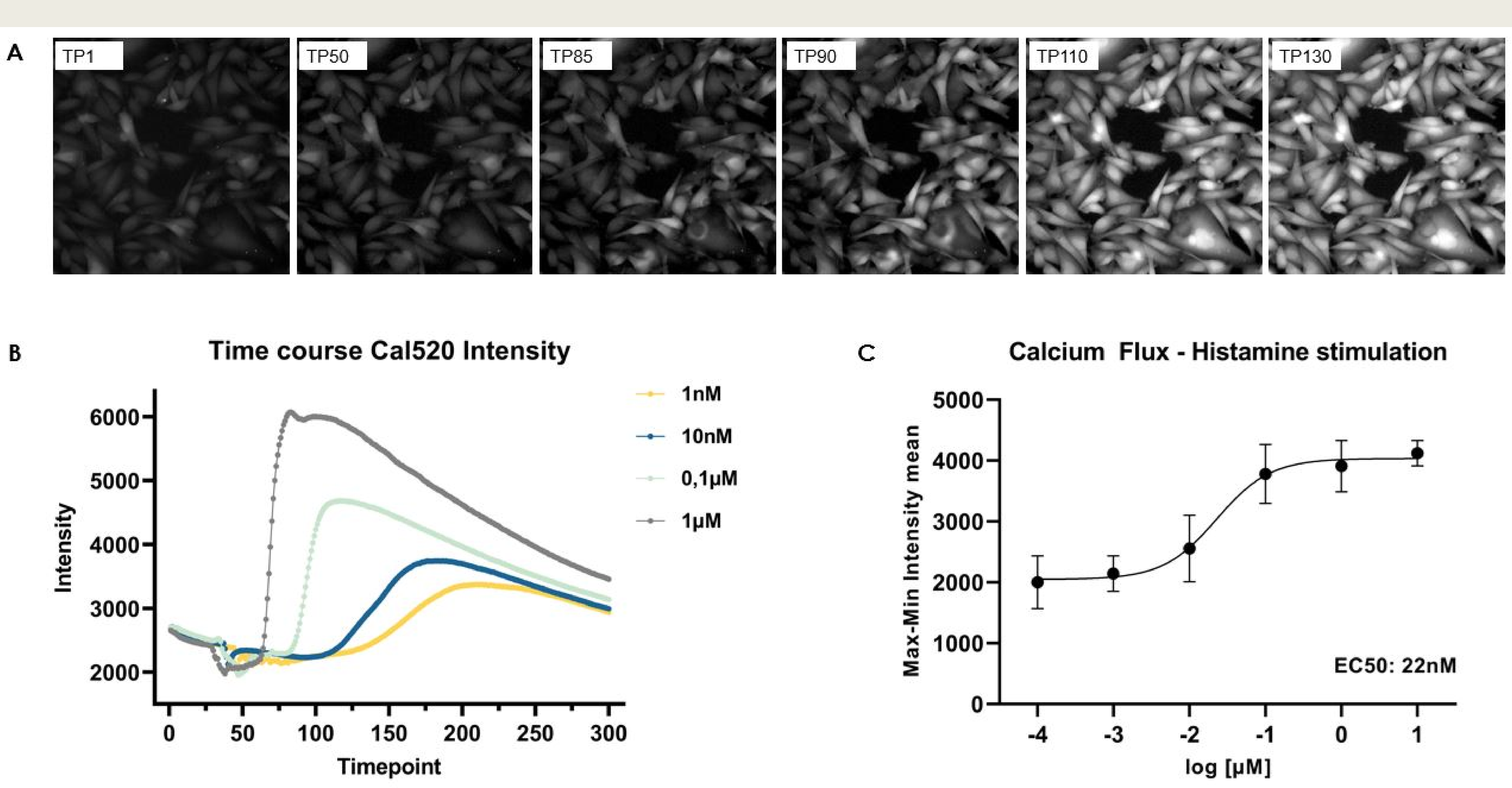

Figure 1. Monitoring calcium release over time following histamine H1 receptor activation. (A) Sample images showing a histamine dependent increase in Cal-520 fluorescence intensity. TP1 (t=0) is the first time point of the first sequence before pipetting. TP 50, 85, 90, 110 and 130 correspond to 0.64, 1.16, 1.23, 1.52 and 1.82 seconds after histamine addition. (B) Mean Cal-520 fluorescence intensity in cells over time before and after addition of histamine. With increasing histamine concentration, the response of calcium release accelerates and the maximum of the mean fluorescence intensity in cells increases. (C) Calculation of the EC_{50} value of histamine induced calcium release in cells using the mean max-min value (n=4, error bars = standard deviation).

4 Single Cell Analysis

To analyze histamine induced changes, fluorescence intensity was calculated for individual cells over the whole time series. The maximum and minimum fluorescence intensity value of each cell was determined. The maximum calcium signal corresponds to the difference of the maximum and minimum value (max-min). The calculated EC50 value of the max-min value was 22 nM for histamine (Figure 1).

The analysis of individual objects can provide more detailed information than whole-population data. It allows, for example, the identification of heterogeneous subpopulations of low and high responders, or false positives (Figure 2).

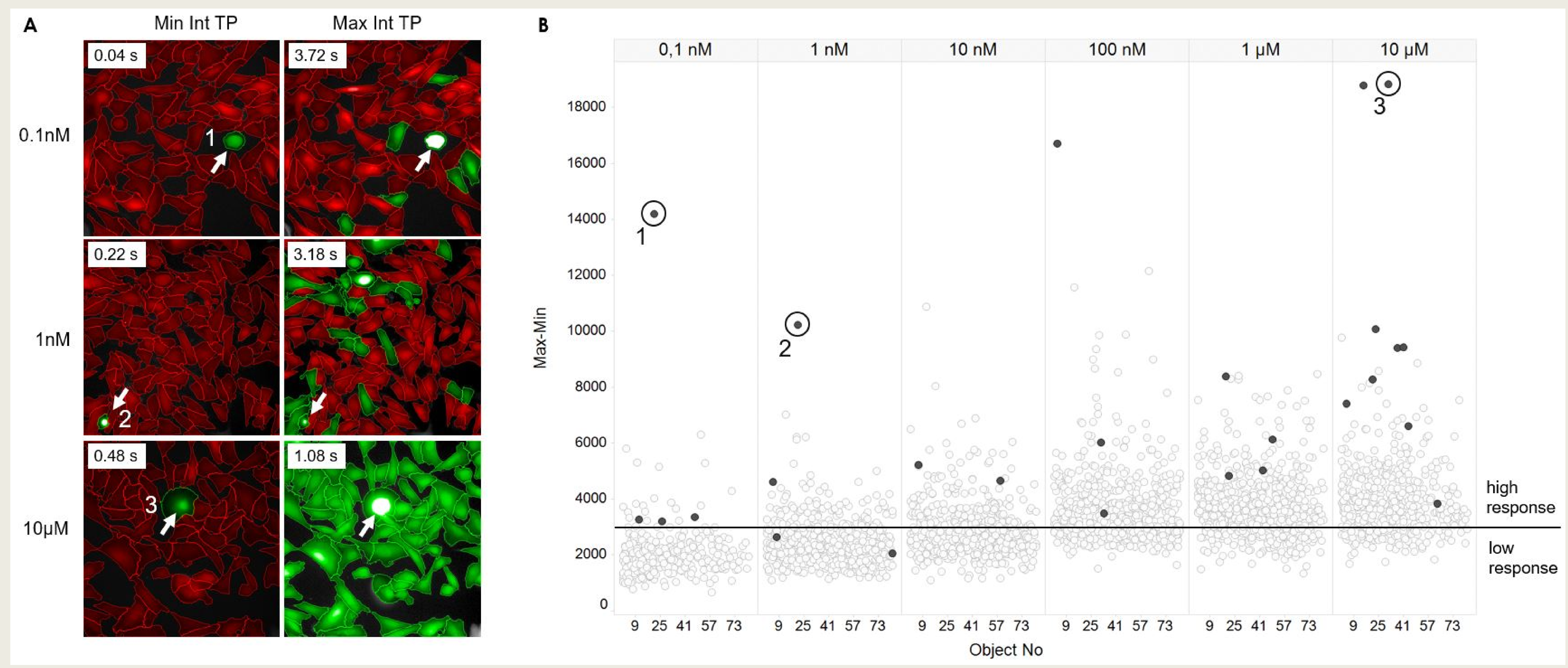

Figure 2. Analysis of individual cells. (A) Image panels show the respective time points with the minimum (left) and maximum (right) Cal-520 intensity. Single objects were identified and grouped into two populations of cells with either a low or high Cal-520 fluorescence intensity. The baseline established threshold was 2x the average minimum value of all cells. Cells below this threshold are pseudocolored red and above this threshold, green. With increasing histamine concentrations, the number of high intensity cells increases; and the timepoint to reach a maximum response decreases from 3.72 s at 0.1 nM histamine to 1.08 s at 10 µM histamine. In the minimum time point images (left panel) three high intensity cells were identified. (B) The calcium flux response for individual cells is shown. Each spot represents the maximum intensity of one cell over the time course. This corresponds to the difference of the maximum and minimum intensity values (max-min). The max-min values increase with increasing histamine concentrations. Cells with a high minimum value (2x of the average minimum values of all cells) are marked as dark spots in the graph. Among the high responders a few cells with extremely high response could be identified. These outliers, with max-min values around 5x higher than the threshold, are cells with a high minimum value at the same time (highlighted with dark spots). In the images (marked as 1-3) these outliers can be identified as "round cells". Furthermore, for the cell outlined in 2, a decrease in fluorescence intensity over time was identified. Therefore, the analysis of single cell responses allows the identification of false positive and negative results.

5 Antagonist assay

The GPCR activation was kinetically monitored using histamine as an agonist compound and pyrilamine as an antagonist. Cells were incubated with pyrilamine in various concentrations together with Cal-520 for one hour before GPCR activation with histamine. The cells were then treated with 100 nM histamine (four times EC_{50} determined in agonist assay above). The presence of pyrilamine lead to a dose-dependent decrease of the calcium release. As for the agonist assay, the difference between the maximum and minimum fluorescence intensity values of each single cell was determined. The EC_{50} for the pyrilamine inhibition of the calcium release was calculated as 117 nM (Figure 3).

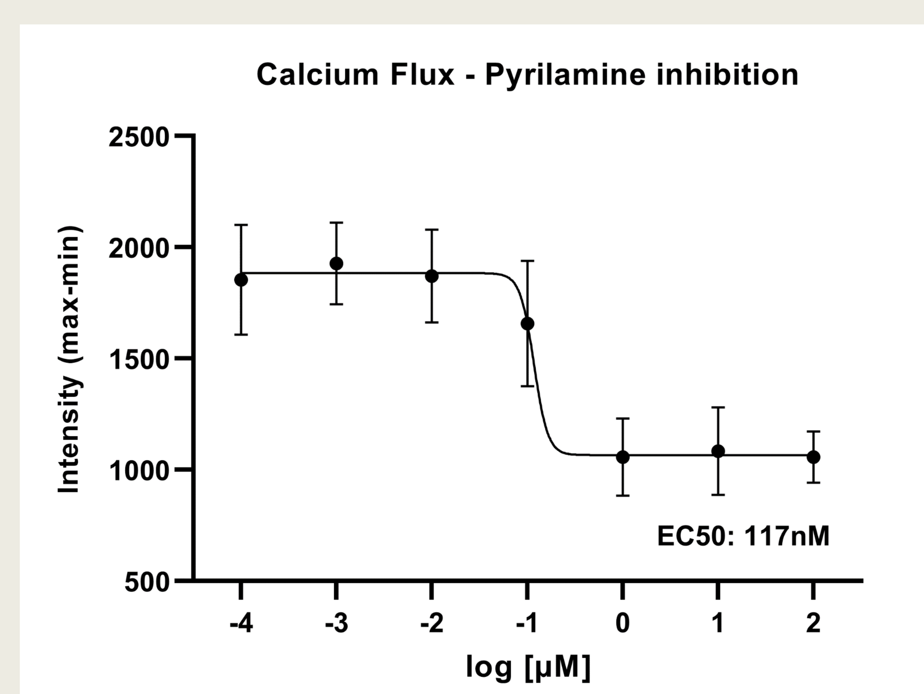

Figure 3. Pyrilamine inhibits histamine-mediated calcium release. Cells were pre-incubated with pyrilamine one hour prior to GPCR stimulation with 100 nM histamine. With increasing pyrilamine concentration, the Cal-520 fluorescence signal decreases. The calcium flux responses of single cells were calculated as the difference between the maximum and minimum fluorescence intensity values of each cell over the time course. The calculated EC_{50} for the pyrilamine inhibition of the calcium release was 117 nM (n=4 wells, error bars=standard deviation).

6 Summary

Imaging the kinetic changes of GPCR-mediated calcium release requires synchronized high frame-rate imaging with compound addition. The Opera Phenix Plus system offers a tip-based liquid handling module which enables fast-response assays in which cell responses occur within milliseconds to seconds after compound addition. The compounds can either be transferred from a compound plate or from a small or large reservoir and a total of two pipetting steps per experiment can be defined. The system can pipette into either 96-well or 384-well microplates. For 96-well plates, 1-200 µl, and for 384-well plates, 1-25 µl can be transferred with a single pipetting step. Furthermore, the dose-dependent increase in intracellular calcium release upon GPCR activation can be easily quantified using the Harmony software image analysis tools. The cell-based analysis of calcium release allows robust data to be extracted even from a heterogeneous population owing to the ability to classify each cell as non-, low- or a high-responder. With frame rates of up to 100 frames per second, synchronized compound addition and the analysis tools of Harmony software, the Opera Phenix Plus System will enable any of your fast kinetic high-content screening applications.

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