Towards a high-throughput microfluidic drug discovery platform for the screening of GPCR targets in cells

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Abstract

G-Protein Coupled Receptors (GPCRs) constitute a large protein family of membrane receptors that play an important role in many cellular processes related to diseases in human beings, and so are primary drug targets for 30-50% of the pharmaceutical molecules currently available, accounting for annual revenues in the order of the tens of billions (10^9) of US Dollars. There are about 1000 DNA sequences identified as likely to be GPCRs, with nearly 100 of these confirmed as receptors without any known ligand, and therefore, with great drug discovery potential. Nowadays, the discovery of new drugs targeting GPCRs is done using high throughput screening (HTS) technologies with the activation of receptors being monitored by differences in intracellular calcium. Microfluidics based live cell calcium assays can be performed with low material cost, using smaller volumes of expensive solutions, to present cells with multiple cues that are present in their normal environment.

In this work, an integrated microfluidic device for the screening of GPCR drug targets in cells was conceptualized, with three distinct modules: a microfluidic channel for conducting live cell calcium assays for the screening of GPCR drug targets, a microfluidic gradient generator channel with integrated single cell trapping for performing assays with different concentrations in a single run and integration of hydrogenated amorphous silicon photodiodes with fluorescence filters with the microfluidic channel capable of detecting free calcium concentrations similar to intracellular calcium levels before and after GPCR activation.

Keywords: G-Protein Coupled Receptors, microfluidics, photodiodes, gradient generator.

1. Introduction

G-Protein Coupled Receptors (GPCRs) play an important role in many physiological and disease related processes in human beings and, due to their importance in the regulation of cell activity, are primary drug targets for 30-50% of the pharmaceutical molecules currently available, which account for annual revenues in the order of the tens of billions (10^9) of US Dollars.[1]-[4] They are one of the largest classes of receptors in the human genome, with about 1000 sequences identified as likely to be GPCRs and with nearly 100 of these sequences confirmed as receptors, but without any known ligand.[5] These receptors are active in practically all organ systems, and hence, present broad array of opportunities as therapeutic targets in areas such as cancer, cardiac dysfunction, diabetes, central nervous systems disorders, obesity, inflammation and pain. [6] The discovery of new molecules that have GPCRs as drug targets is currently being performed by high-throughput screening platforms (HTS). In these platforms, millions of different test compounds are being brought into contact with live cells and the response elements of the GPCR’s signaling cascade monitored using fluorescent or luminescent read-outs.[3] The signaling system of GPCR is highly complex and based on three major elements. A GPCR with the ability to couple with a heterotrimeric guanosine-5’-triphosphate (GTP) binding protein (G-protein), a GTP-transferase active G-protein and a second messenger generating enzyme. The general accepted mechanism of GPCRs assumes that the connection of the ligand to the receptor is coupled to the second messenger forming enzyme through the heterotrimeric G-protein. The binding of the ligand to the GPCR causes a change in the receptor conformation that in turn binds and activates the G-protein. The now active form of the G-protein is released from the surface of the receptor, dissociating into its α and βγ subunits. These two subunits will, in turn, activate their specific effectors, leading to the release of second messengers, which are recognized by specific proteins, such as protein kinases, causing their activation and triggering the signaling cascade towards a complex biological event. The G-protein is regenerated through the hydrolysis of the GTP molecule and re-trimerisation of the G-protein to its inactive form.[5] The second messenger releasing enzymes comprise two main groups, with each one being activated or inactivated by different types of G-proteins. The Gαs and Gαi subtypes either activate or inactivate the adenylate cyclase enzyme that converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP),
simultaneously releasing pyrophosphate, whereas other subtypes, namely Gaq and Gao will alternately activate the phosphoinositol phospholipase C enzyme (PLC) which hydrolyses phosphatidylinositol-4,5-biphosphate (PIP2) into sn-1,2 diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). The IP3 molecule binds to an endoplasmic reticulum calcium channel, triggering the release of calcium ions into the cytosol. This process is schematically represented in Figure 1.

**Figure 1** Intracellular calcium release after GPCR activation.

GPCR targeting drugs bind to a receptor and either inhibit (agonist) its action or stimulate (agonist) the receptor to give a biological response characteristic of the drug. Agonists commonly have similar structure to the endogenous ligands of the receptor. The increase of concentration of an agonist cause increased cell activity, until it reaches a maximum, at which point the receptors of the cells for that particular agonist are saturated. Agonists are ligands that inhibit the activation of a receptor by preventing the binding of an agonist.[7] The main GPCR studied in this work was P2Y2, which belongs to the purinergic receptor family and is expressed in many tissues including lung, heart, spleen, kidney, skeletal muscle, liver and epithelia. This receptor plays an important role in regulating ion transport in epithelial cells and can directly couple to PLCβ1 (phospholipase C- β1) via Gaq/11 protein to mediate the production of IP3, second messenger for calcium release from intracellular stores. In terms of activation, P2Y2 is activated almost equipotently by agonists UTP and ATP, while being weakly antagonized by suramin.[7][8]

In the last couple of decades, the field of miniaturization has seen great progress, one of the disciplines that emerged from it being microfluidics. In its simplest form, microfluidics can be defined as the science that deals with liquid flows inside channels at the micrometer scale.[9] The use of microfluidics brings many advantages to a variety of fields, with the possibility of integration, in miniaturizing chips of otherwise very complex assays, reducing costs and time. The costs are reduced by using smaller volume of expensive reagents and through economies of scale, which also provide the possibility of high throughput assays.[10] Using microfluidics, it is possible to conduct live cell assays under a precisely controlled environment, while using minor quantities of expensive chemicals and precious drugs. These microfabricated systems can present cells with multiple cues that are present in their normal environment, including direct cell to cell contact and biochemical and mechanical interactions with ECM proteins.[11], [12] In microfluidics it is possible to generate gradients of proteins, surface properties, and fluid streams containing growth factors, toxin, enzymes, drugs and other important biological molecules are greatly beneficial for biological studies, such as cell-based assays.[13], [14] Microfluidics also provides the opportunity of integration and along with it, the small footprint and low power consumption of integrated systems, which allow the creation of new portable devices capable of performing sophisticated analyses previously only possible in research laboratories.[12] One of the most promising technologies for integration in a microfluidic assays involving fluorescence and chemoluminescence are photodiodes, which are semiconductors capable of converting light into current.[15]

Hydrogenated amorphous silicon photodiodes (a-Si:H) have been used for a variety of different applications in microfluidics, ranging from the detection of chemiluminescent molecules, such as horseradish peroxidase (HRP), for the quantification of proteins or DNA, to the quantification of molecules labeled with fluorescent probes and quantum dots.[16]–[19]

In this work, the characterization of the P2Y2 GPCR in HEK293T is demonstrated through live cell calcium assays using a traditional assay platform (microtiter plates) and a microfluidic chamber channel. The present work also demonstrates a microfluidic channel capable of gradient generation formation through the use of different laminar flow fluxes, while also featuring integrated single cell trapping functionality. The feasibility of using a-Si:H photodiodes with integrated absorption filters in a microfluidic channel as a platform for the detection of Fluo4 stained calcium solutions of similar concentrations to HEK293T intracellular calcium, before and after GPCR activation, is also demonstrated.

### 2. Methods

#### 2.1. Animal cell culture

The HEK 293T cells used for the live cell calcium assays were obtained from working cell banks (3×10^6 cells preserved at -80°C) by thawing followed by DMSO removal, seeding in T75 cell culture flasks using Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (penicillin, streptomycin and Fungizone®) and incubation at 37°C in a 5% CO2 atmosphere until reaching a confluence of 80% (approximately 4 days). After reaching 80% confluence, the non-adhered cells were removed by washing with PBS and the adhered cells detached by incubation, for 3 minutes, with a solution of trypsin-0.05% EDTA. The cells were
then passed to another platform for assaying or to a culture flask (T25 or T75) at an initial density between 0.15×10^6 cells/mL and 0.3×10^6 cells/mL and then grown for 24-48 hours under the same conditions.

2.2. Microtiter plate live cell calcium assays
HEK293T cells were transferred to microtiter plates (Becton-Dickinson) at an initial cell density of 0.15×10^6 cells/mL and incubated for 24h at 37°C in a 5% CO2 atmosphere, using DMEM with 10% FBS and 1% AntiAnt in a total volume of 100 µL per well. The adhered cells were then incubated for 30 minutes at 37°C with 100 µL of Fluo-4 Direct™ prepared in assay buffer (1×HBSS, 20mM HEPES supplemented with 2.5 mM probenecid) and then in the dark for 30 minutes, at room temperature. The compounds to be assayed were prepared fresh and diluted in assay buffer, in a way to achieve the desired concentrations inside the wells, in the range of 10^(-8)-10^(-4) for UTP and 10^(-6)-10^(-3) for Suramin. The agonist live cell calcium assays were done on a fluorescence inverted microscope (Olympus CKX41), with the microtiter plates mounted on the microscope stage and the baseline fluorescence recorded for 20 s. Then 50 µL of the agonist, concentrated 5 times, was injected using an automated pipette and the change in fluorescence recorded until 180 s elapsed. For the antagonist assays the cells baseline fluorescence was recorded for 20 s and then 40 µL of the antagonist, concentrated 6 times, added using an automated pipette. The response of the cells to the antagonist was recorded for 60 s and then 40 µL of the agonist, concentrated 7 times, added to the well and the change in fluorescence recorded until a total of 180 s had passed.

2.3. Hard Mask Fabrication
The fabrication of the microfluidic structure started with the creation of a 2D design in AutoCAD 2014 software. Initially a 200 nm thick layer of aluminum was deposited on top of a glass substrate in a Nordiko 7000 magnetron sputtering system. Then, a 1µm thick positive photoresist layer was spin-coated on the aluminum covered glass substrate. The 2D design was then transferred to the photoresist by exposing it at 442 nm using a Heidelberg DWL II direct write laser lithography equipment. After the photoresist was developed, the aluminum was etched using an aluminum etchant standard mix and the remaining photoresist cleared using acetone. This aluminum hard mask patterned with the desired 2D design works as a mask for the fabrication of a SU-8 photoresist mold.

2.4. Mold Fabrication
A silicon substrate was cleaned with acetone followed by a sonicator bath in Alconox® at 65°C for 20 minutes, then rinsed with IPA and distilled water. The cleaning step was finished in an UVO-cleaner for 15 minutes. A SU-8 photoresist layer was spincoated over the cleaned silicon substrate. The photoresist used varied with the desired height of the SU-8 spincoated layer, for a height of 17 µm SU-8 2015 was used whereas for a height of 60 µm SU-8 50 was chosen. Both formulations of SU-8 were purchased from Microchem and the spincoater was a Laurel WS-650-23. The SU-8 covered silicon substrate is then pre baked at 65°C for 3 min, then soft baked at 95°C for 8 min and finally cooled down at room temperature for 5 min. Then, the hard mask, with the desired patterned design, was placed on top of the silicon substrate with the aluminum side facing the SU-8. The SU-8 was then exposed, through the mask, using an UV lamp which induced the hardening of the exposed photoresist. The SU-8 that was not exposed through the mask was removed from the substrate by developing with a 99% solution of PGMEA, purchased from Sigma-Aldrich. The substrate was hard baked at 150°C for 15 min and then the thickness of the SU-8 mold measured in a profilometer (Tencor Alpha-Step 200). The height of the SU-8 layer varied from 15 to 20 µm for the gradient generator structure and from 50 to 60 µm for the leaf chamber structure. For the gradient generator structure, due to the small traps features in the mask, the mold was done directly on the mask, instead of using a silicon substrate the substrate was the mask by itself.

2.5. PDMS Fabrication
PDMS (SYLGARD 184 silicon elastomer kit) was prepared by mixing the base monomer with curing agent 10:1 parts and degassed in a vacuum chamber. The degassed PDMS was poured over the SU-8 mold and cured in an oven at 70°C for 90-120 min and then peeled off from the mold. The inlet and outlet holes of the structure were done on the PDMS using a 20 ga syringe needle bought from Instech Solomon. A glass slide was cleaned in a solution of Alconox® for 20 min and then for 5 min in a solution of IPA, both steps were done inside a sonicator. The PDMS structure was also cleaned with IPA for 5 min inside a sonicator. Both the PDMS structure and the glass were then rinsed with water and dried with compressed air. For the sealing, the PDMS structure and the glass were then placed inside a UVO-cleaner (Jelight Model 144AX) for 11 min with the area to be sealed facing upwards. After the 11 min elapsed the glass slide was placed on top of the PDMS structure and pressed to form an irreversible seal. The resulting channels were left for 24h before further usage.

2.6. Microfluidic live cell calcium assay
The microfluidic channels were functionalized with ethanol and left overnight at 4°C to remove air bubbles. The channels were then washed with water followed by incubation with Fibronectin (100 µg/mL in H2O) for 2 hours at 37 °C. For the insertion of HEK293T cells, at a concentration of 3×10^6 cells/mL in DMEM, were inserted into the microchannel using infusion pumps (KDS Legato 100), the initial flow rate (Q) set to 50 µL/min and after cells were entering the chamber, it was set to 1.5 µL/min in order to control cell placement inside the channel. When a sufficient amount of cells where inside the channel, the flow was stopped. The microfluidic channels with cells were then incubated for 20 min at 37 °C and in a 5% CO2 atmosphere to allow cell adhesion. Afterwards DMEM
was flowed inside the channel at Q=2 µL/min for 10 min to wash out cell debris and provide fresh cell medium to the cells followed by a 30 min incubation step at 37 °C to allow the cells to adhere to the channel. Then, 250 µL of Fluoro-4 Direct™ prepared in assay buffer (1×HBSS, 20 mM HEPES supplemented with 2.5 mM probenecid) were mixed with 750 µL of DMEM with 10% FBS and 1% AntiAntii in an Eppendorf and then flowed inside the channel at a Q=1.25 µL/min until 8 µL had been inserted. The cell were then incubated for 30 min at 37 °C and afterwards for 30 min in the dark at room temperature. UTP was then injected into the channels (Q=1.25 µL/min) and the P2Y2 GPCR activation was monitored in real-time using fluorescence microscopy. The microscope used was an Olympus, the exposure time was set to 1 s and the gain to 12.

2.7. Gradient Generation with Calibrated Calcium Solutions

The gradient generator channel was functionalized with ethanol and left overnight at 4 °C to remove air bubbles. The channel was then cleaned with water. Both steps were done using the same materials as the microfluidic live cell assay at a flow rate of 0.5 µL/min for 10 minutes. For the gradient generation with calcium solutions, Calcium Calibration Buffer Kit #1, purchased from Life Technologies, was used to prepare solutions with different concentrations of calcium, by mixing CaEGTA with K2EGTA buffer and using, EGTA (ethylene glycol tetraacetic acid) as a chelating agent. Three different concentrations of calcium were prepared in eppendorfs, each totaling 250 µL. Then 2 µL of 1mM Fluo4 pentapotassium salt solution, purchased from Life Technologies, was added to each Eppendorf so that the final Fluo4 concentration in each solution equalled 4 µM. The solutions were then put on three different 1ml syringes, on a support that enables the simultaneous pumping of three solutions at the same time, and then connect the tubing to the adapters on the channels. After the syringes were connected to the inlets, the pump was turned on and the flow rate set to 0.5 µL/min. Images of the trap area of the microfluidic chamber channel and the fluorescence intensity first measured using and Olympus microscope, with the gain set for 12 and exposure time of 5s to video intensity.

2.8. Photodiodes Characterization

The photodiodes used in this work were made of a-Si:H of the p-i-n variety, with a 5000 Å wide i-region and 200 Å p and n regions. The dimensions of the photodiodes were 200 µm by 200 µm and had an integrated absorption filter (Figure 2).

A conventional blue LED with peak emission at 470 nm coupled to a low pass filter (Thorlabs) and a tungsten-halogen lamp (250 W) coupled to a monochromator (McPherson 2035) were used as light sources. The wavelengths used in the lamp-monochromator combo were 494 and 516 nm. The photon flux of the light sources used was measured using a crystalline silicon photodiode (Hamamatsu S1226-5BQ), with the response of the photodiodes to the characterization experiments obtained using a picoammeter (Keithley 237) at room temperature. The photon flux was calculated using Equation 1.

\[ \Phi(\lambda) = \frac{I(\lambda)}{\lambda \times S(\lambda) \times c \times h} \]  

\[ \text{Equation 1} \]

In this equation, \( I(\lambda) \) is the current at a given wavelength, \( \lambda \) the wavelength, \( A \times \) the area of the photodiode, \( c \) the speed of light, \( h \) the Planck’s constant and \( S(\lambda) \) is the responsiveness of the calibrated crystalline silicon photodiode. The Current vs Voltage of the photodiode was also analyzed using the lamp coupled to the monochromator (set to 494 and 516 nm) and the LED with and without filter, setting a range of voltages from -1 to 0 in steps of 0.1 V. In this characterization step, a measurement in the dark was also done. It is important to note that all current measurements were converted to current density, by dividing the current obtained by the area of the photodiode (0.0004 cm²). The characterization of the integrated fluorescence filter and photodiode was also performed, with respect to the suitable wavelengths. For this, external quantum efficiency (EQE) vs. wavelength graph was plotted. The lamp-monochromator combo was used for this experiment. The current for different wavelengths was measured, starting at 600 nm and decreasing to 400 nm with a step of 5 nm. The EQE was calculated using Equation 2.

\[ \text{EQE} = \frac{J}{\Phi \times q} \]  

\[ \text{Equation 2} \]

In this equation, \( J \) is the current density in A.cm² and \( q \) is the electron charge. Using neutral density filters to cut the intensity of the incoming light from the LED or lamp-monochromator combo, the characterization of the response of the photodiodes to different light intensities at the same wavelength was performed. The neutral density filters used ranged from cutting 10-1000 times the original intensity. A calibrated fluorescent calcium experiment was also performed using CaEGTA solutions mixed with K2EGTA and Fluo4 Pentapotassium Salt, purchased from Invitrogen. The solutions at different concentrations were inserted into the microfluidic channel and the fluorescence intensity first measured using and Olympus microscope. Then the microchannels were transported to the optical table and aligned to a working photodiode. Then, after channel alignment, the light source was also aligned to the channel, so that it was directly on top of it. The room light was shut down and the measurements done in the dark, except for the experiment light source, using the picoammeter. An experiment was also done with HEK293T cells inside.
microchannels. In this experiment, the protocol was the same for the microfluidic live cell calcium assay in the microfluidic chamber channel, but instead of using an Olympus microscope to record a video, the channel was placed on top of the photodiode and the current measured during the assay duration.

3. Results and Discussion

3.1. Macroscale

As a starting point towards the characterization of GPCRs in microfluidic devices, the response of a GPCR, the P2Y2 receptor, was characterized using the traditional macroscale platform, microtiter plates, using HEK293T. HEK293T was the chosen cell type because they can be assayed while adhered, have an average doubling time of less than 24h, are commonly used in GPCR characterization assays and express the chosen GPCR P2Y2 endogenously. This receptor was chosen because one of the second messengers in the signaling cascade is cytosolic calcium which can be assayed easily with established protocols.

3.1.1. Agonist Assays

In the agonist live cell assays in microtiter plates, using Fluo4 Direct™, the intracellular calcium concentration was monitored over time. When UTP was added to the well plates, the cells responded by releasing intracellular calcium which translated into increased fluorescence, however, when just assay buffer, without UTP, was added, the cells didn’t respond significantly, as seen in Figure 3. In this figure, it is possible to observe that the response of cells was higher when a concentration of 150 μM was used, compared to 2 μM as the cells were not as fluorescent at the halfway time point, indicating that not as much calcium was released into the cytosol. Also, when no UTP was present, only assay buffer used, the cells didn’t respond, meaning that it was the UTP that triggered the release of calcium inside the cells.

In order to quantify and compare all the UTP concentrations assayed, the assay videos were analyzed in ImageJ software and the fluorescence normalized to the baseline of each assay, consisting of the first 20 s of each video. This normalization is needed because the baseline cell fluorescence varies from well to well. UTP concentrations ranging from 0 to 150 μM were assayed, with the cell fluorescence being monitored for 100 s. As it was assumed that the P2Y2 receptor was saturated when a concentration of 150 μM was used, the value of fluorescence obtained for this concentration was normalized to 100%, since it accounts for the maximum cell fluorescence achieved among all the assays performed. After doing this normalization, a Hill dose-response was plotted.

![Figure 4](image-url) Hill dose-response curve for UTP in microtiter plates. The two set of points represent the same experiments but using different software analysis methods, one where the background of the well plate was removed and another where it was not. The error bars of each point represent the standard error of the mean (SEM). The EC50 values for removing and not removing background were 3.5 μM and 4.2 μM, respectively.

In the UTP dose response curve pictured in Figure 4, it is possible to see that the graph has three distinct phases. The lower plateau, corresponding to the lower concentrations 0 to 10^{-7} M (0.1 μM), is the phase where there is not a significant increase in response, the exponential phase, where there is a great increase in intracellular calcium release, and the upper plateau, where the receptor is most likely saturated and the response stabilizes. It is possible to notice that, although there is a slight variation between the plot with the background removed and the plot without removal, the EC50 (the concentration that produces a response of 50% of the maximum) is about the same. Removing the background the EC50 was 3.5 μM, with a 95% confidence interval falling between 2.7 μM and 4.6 μM, whereas without removing background the EC50 was 4.2 μM, with a 95% confidence interval of 3.4 μM to 5.2 μM. So, both methods of analysis are in the same 95% confidence intervals making the difference not very significant. The removing background method was used because of the microfluidic assays, as removing the background is important, since the concentration of cells is much lower than in microtiter plates. The values of EC50 obtained fit in the range of 1.5-5.8 μM reported in [20] for murine and human P2Y2, however other studies report an EC50 of 0.14 μM, such as [8].
3.1.2. Antagonist Assays

The antagonist used in the assays of this section was Suramin. The cells weren’t expected to respond to the addition of Suramin, because, as an antagonist, it should block the P2Y2 receptor binding of UTP and, subsequently, prevent the release of calcium. However, for high concentrations of Suramin, the cells had a significant response. The UTP concentration used for every antagonist assay was 10 µM, equivalent to the EC80 (effective concentration to achieve 80% of maximal response) for the microtiter plate agonist assays. It was found that high suramin concentrations can cause the release of intracellular calcium, with this effect being enhanced by the presence of calcium on the assay buffer. The response of the cells to suramin was an unexpected result, as in the literature suramin was always depicted as an antagonist to GPCRs that have calcium as a second messenger in the signaling cascade, and never as an agonist or partial agonist. One of the possible explanations to this effect is the large plethora of functions that intracellular calcium can have in cells, not just as a GPCR second messenger. Intracellular calcium can also be released in response to thermal, kinetic stress among others, so possibly the addition of such a high concentration of suramin (1.5 mM) could have triggered a GPCR unrelated stress response. One other possibility is the presence of powder particles on solution, since suramin was prepared by dissolving its powder form on assay buffer.

Because suramin is a weak P2Y2 antagonist, the inhibition is not total, and there is still some response due to UTP on the highest suramin concentration, 1 mM. This suramin concentration was the highest possible to assay, since increasing its concentration from this point only lead to increased cell response from the suramin alone. The IC50 (the antagonist concentration that inhibits 50% of the response) obtained for suramin was high compared with the literature, 342 µM as opposed to the reported 50 µM.[8]

3.2. Microscale

The experiments performed at macroscale, agonist and antagonist assays, were also done at microscale. A microchannel with a chamber for cell adherence and a total volume of 255 nL was used. The channel had a height of 60 µm, a chamber with 1 mm of diameter and two arms coming from each side of the chamber with a width of 200 µm. The channel was first filled with fibronectin, an ECM protein, to facilitate the adherence of the cells to the channels surface. The cells were inserted into the channel and settled inside the chamber.

3.2.1. Agonist Assays

The range of UTP concentrations used for the microfluidic agonist cell assays was similar to the one used in the microtiter plate experiments, 100 µM-0 µM UTP as opposed to 150 µM-0 µM UTP. The reason for this change was the fact that for concentrations below 100 µM, the receptor appeared to be already saturated, so there was no need for trying higher concentrations. Initially, it was thought that the EC50 for the macroscale experiment would be lower than the microscale, as the cells would get a violent burst of UTP after it was dispensed from the pipette with convection being the major mass transfer mode, as opposed to diffusion in the microfluidic channel. A big difference between the two assaying platforms is the concentration of cells being assayed. In the microtiter plates, the microscope view area, 10x objective, would have a higher number of cells, ranging from a few hundreds to almost a thousand of cells, enabling a good estimation of the mean calcium response of a cell population. In the case of the microfluidic channel, the cell concentration was lower. In the microfluidic assays, because the drug was flowed, the way the cells responded to the drugs was slightly different, the cells had different response times and also the fluorescence was of smaller duration, with some cells just showing signal and then quickly returning to their basal fluorescence level.
Figure 7. Cell response to different UTP concentrations at similar time points in the microfluidic chamber. In these assays the cell basal fluorescence was recorded for 10 s, and then the fluorescence shutter closed, as not to bleach the cells. Then, at about 40 s the pump was turned on, with UTP flowing into the channel, and the fluorescence shutter opened 20 s later. The remaining assay time was for the monitoring of the cell response to UTP.

As can be seen in Figure 7, the cell concentrations inside the chamber varied significantly, something not ideal for consistent measurements, as the lower the number of cells caused a larger background area and the cells to appear more fluorescent, since the Fluo4 solution is the same for all assays, with each cell absorbing more fluorophore. Also, due to the flow, the cells can respond to the buffer, as just the slight convection might lead to the release of calcium not directly related to GPCR signaling.

Figure 8. Hill dose-response curve for UTP in the microfluidic chamber. The video data used for this graph was analyzed using the removing background method. The EC50 value for the microfluidic GPCR assay with UTP was 0.24 µM.

The video analysis method where the background is removed was used for microfluidics, the reason for this choice being that for lower cell concentrations, it would be difficult to detect the maximum fluorescence because of the background noise. The EC50 obtained for the microfluidic experiments was one order of magnitude smaller than the one obtained for macroscale, which was between 4.2 and 3.1 µM. The 95% confidence interval for the microfluidic UTP assay, in Figure 8, was from 0.1 to 0.56 µM, so the difference between the two platforms is quite significant. There is no discrete conclusion as for which method has the best EC50 value compared to the literature, because there are values in the 1.5-5.8 µM range and there are values in the 0.14 µM range, with the latter being a value within the 95% confidence interval for the microfluidic assay.[20][8] It is important to note that the percentage of failed assays using microfluidics was quite high, as many technical challenges can arise, and that for a significant portion of this work, the live cell calcium assays in microfluidics weren’t satisfactory, as the cells were not responding.

3.2.2. Antagonist Assays

The methodology adopted for the microfluidic antagonist assays was to mix different concentrations of agonist with the same antagonist concentration. With this approach, named mixed antagonist, instead of obtaining an IC50 value, what would be obtained would be an EC50 with an expected shift to the right, meaning that a higher concentration of UTP would be needed to reach the 50% of maximum response as suramin should block the receptor.

Figure 9. Hill dose-response curves for the mixed antagonist assays in the microfluidic chamber. The video analysis method used was the one where the background was removed. The best-fit values for the EC50, for the assays where a concentration of 250 µM and 150 µM suramin was used, were 0.52 and 0.3 µM, respectively. The dose response for the agonist assay was also plotted for comparison, its EC50 is 0.24 µM.

The best-fit values for the EC50 for the two concentrations of suramin tested were 0.3 µM for 150 µM suramin, with a 95% confidence interval of 2.6×10⁻⁴ to 3414 µM and 0.51 µM for 250 µM suramin, with a 95% confidence interval of 0.01 µM to 18.5 µM. The 95% intervals obtained were very broad meaning that the plotted curve was not the best, however, the best-fit values obtained, even if not incredibly accurate, were what would be expected. There was a shift in the EC50 to higher concentrations of UTP, when higher concentrations of suramin were used. For the agonist experiments where only UTP was used the EC50 obtained was 0.24 µM, when 150 µM of suramin were present in solution the EC50 value rose to 0.3 µM and in the case of 250 µM of suramin the EC50 value was 0.52 µM. This was to be expected because if antagonist...
and agonist are both present in solution there should be some competition to bind to the receptor, and the higher the concentration of the antagonist, the more molecules there would be compared to the agonist, with a greater chance of the antagonist binding to the receptor and preventing the release of intracellular calcium due to GPCR activation.

3.2.3. Single Cell Traps

Single cell trapping experiments were carried out in the gradient generator channel. In the first experiment, with the gradient generator channel, fibronectin was used to promote cell adherence to the channel after the cells were trapped.

![Cells trapped inside the gradient generator channel. A) Experiment with the channel coated with fibronectin. B) Experiment with the channel coated with cell medium (DMEM with 10% FBS and 1% Anti).](image)

In the trapping experiment with fibronectin (Figure 10 A), incubated for 2h, the concentration of cells might have been too high, and after some time, the cells started to cluster together in the trapping area. The fibronectin contributed to this clustering, making the channel stickier and causing the cells to remain in the channel in areas that had no traps, causing blockages. In the experiment without using fibronectin (Figure 10 B), although there was also some clustering, the cell distribution in the channels is homogeneous, with similar concentration on the top, middle and bottom and more traps with only one cell, as evidenced by Figure 11.

![Single cell traps in the experiment without fibronectin.](image)

Despite some clogging problems, the traps seemed to work properly; however, there is room for improvement in the trapping process. The traps should have a smaller gap, such as 4-5 µm instead of 7 µm, as the majority of the cells, more than 90%, didn’t get trapped, and also the channel should have an increased height, as against the current 16-17 µm, so that the velocity inside the channel diminishes, lowering cell shear, and cells can pass above adhered cells, avoiding clogging.

3.2.4. Gradient Generation with Calcium Solutions

Gradient generation was tested using calibrated calcium solutions in the gradient generator channel. For this experiment, different concentrations of CaEGTA were mixed with K$_2$EGTA. The gradient generator’s main principle was the mixing of different concentrations in laminar flow through diffusion. There were three inlets, with each having a different concentration flowed, and, through the contact with flows from the other inlets, a gradient was formed. The testing was done flowing 10 mM CaEGTA, 5 mM CaEGTA with 5 mM K$_2$EGTA and 10mM K$_2$EGTA with 0 mM CaEGTA, these concentrations correspond to 39 µM, 0.15 µM and 0 µM of calcium ions, respectively. The calculation of the concentrations of the outputted gradient was based on [13]. The results of the experiment can be seen in Figure 12.

![Gradient generation using CaEGTA solutions with Fluo4. On the bottom of each channel, on each time point is the fluorescence value of the channel, in random fluorescence units.](image)

The gradient generation worked well, with a distinct fluorescence difference between every channel after 30 minutes and then stabilization of the gradient without significant differences on the fluorescence of the channels. Also, the left channel of the 30 and 45 min time points didn’t fluoresce at all, as its fluorescence value even decreased further compared to time point 0 min. There was a problem in the injection at the beginning of the experiment, with one of the concentrations 10 mM not entering the channel properly, which if done right at the beginning would have stabilized the gradient at the time point of 15 min. To determine the exact time of gradient formation, another experiment was done, this time capturing video instead of taken single photos after a certain time. For this the same microscope and software were used, but an exposure time of 5 s used instead. The time it took for a defined gradient to form was 145 s.
3.3. Photodiode Experiments

3.3.1. Photodiode Characterization

To determine the voltage at which the photodiode experiments should be done, a current density versus voltage experiment was performed. The operating voltage used was 0 V as it offered the greatest difference between the dark current and the other light sources. The efficiency of the integrated filter of the photodiode was also characterized. For this experiment, wavelengths ranging from 400 to 600 nm in intervals of 5 nm were tested and the current density analyzed and converted into external quantum efficiency.

![Figure 13](image.png)

**Figure 13** External quantum efficiency of the photodiode for wavelengths ranging from 400 to 600 nm.

The photodiode integrated absorption filter blocks low wavelength light, wavelengths under 450 nm have a very low current, and because it is an absorption filter there is a steady increase of current with higher wavelengths until a plateau at about 550 nm is reached, where the current is maximal. In Figure 13, the most important thing to consider is the ratio between 494 and 516 nm, as these are the wavelengths that need to be distinguishable. The EQE for 494 nm is 0.0044 and for 516 nm is 0.012, which corresponds to a small EQE ratio of 2.8. Due to the small Stokes shift of Fluo4 and the characteristics of the integrated filter, the EQE difference between the emission (516 nm) and absorption (494 nm) is not very big, meaning that it would be difficult to differentiate between the excitation light and the fluorescence emitted from HEK293T cells.

The filter integrated in the photodiodes is not optimal for this type of cell assays, as the EQE ratio between excitation and emission is low, it is only 2.8, in other studies ratios of about 20 have been reported for amorphous silicon photodiodes. [1] The photon flux for the light sources used was measured with a calibrated crystalline silicon photodiode, and expressed in Table 3.1.

<table>
<thead>
<tr>
<th>Light Source</th>
<th>λ, Wavelength (nm)</th>
<th>Φ, Photo Flux (cm²s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LED</td>
<td>470*</td>
<td>3.85×10¹⁵</td>
</tr>
<tr>
<td>LED with filter</td>
<td>470*</td>
<td>2.80×10¹⁵</td>
</tr>
<tr>
<td>Lamp and monochromator</td>
<td>494</td>
<td>2.51×10¹⁵</td>
</tr>
<tr>
<td>Lamp and monochromator</td>
<td>516</td>
<td>2.81×10¹⁵</td>
</tr>
</tbody>
</table>

The current density obtained from the photodiodes using these light sources with different external filters neutral density filters was also measured and is represented in Figure 14.

![Figure 14](image.png)

**Figure 14** Relationship between the current densities (J) and the incident photon flux (Φ) for different light sources.

It is important to note that all of the points in Figure 14 have a higher current density that the dark current density at the operating voltage (2.5 nA), with the lowest current density obtained for the LED with filter being 14 nA. The fact that all light sources have higher current densities values than the dark is good, meaning that experiments can have good sensitivity. The higher the current densities, the more linear the relationship between the photon flux and current density, as evidenced by the 494 and 516 nm plots.

3.3.2. Calcium Fluorescence Experiments

The measurement of different fluorescence calcium concentrations, using the 200 × 200 µm² a-Si:H photodiodes were done in the microfluidic chamber channel using a LED coupled to a low pass filter and tungsten-halogen lamp coupled with a monochromator at a wavelength of 494 nm. Different concentrations of calcium were prepared mixing CaEGTA and K₂EGTA, with the final solutions having 0, 2, 4 and 10 mM CaEGTA, which equates to 0, 0.038, 0.1 and 39 µM of free calcium. The solutions were analyzed in a fluorescence microscope, as seen in Figure 15, and then analyzed using the a-Si:H photodiodes, as represented in Figure 16.
and the small Stokes shift of the fluorophore being used (Fluo4) in the photodiode experiments; however, with good optimization strategies these problems could be overcome in the future.

References


