The unique peristaltic sample delivery system of the CytoFLEX analyzer enables optimized measurements of transient changes in intracellular calcium in cells following agonist activation

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- How to set up a flow cytometeric assay to measure changes in cellular of calcium
- How to adapt the CytoFLEX flow cytometer to make real-time measurements for cellular assays
- About a rapid screening method of calcium flux by flow cytometry

Principal of the Technique

Generation of fluorescent antibody or genetic labels to identify hormone and neurotransmitter receptor activity can be difficult and time consuming. A useful alternative is recording physiological changes in response to agonist binding its cognate receptor, many of which are G-protein coupled. When an agonist binds a G-protein coupled receptor, it triggers a quick cascade of events that often results in a transient release of calcium from intracellular stores. Alterations in transient intracellular calcium ($[Ca^{2+}]_i$) levels have been used previously in flow cytometry to identify functional receptor expression in cellular subpopulations [2], here we show that peristaltic sample delivery of the new CytoFLEX analyzer is particularly well suited to agonist-based calcium studies. Using the ester based, green fluorescent calcium indicator, Fluo-4 AM (Life Technologies), $[Ca^{2+}]_i$ changes were measured in HEK-293 cells in response to ATP stimulation. Simple plumbing modifications to the CytoFLEX allowed easier access to the sample tube for agonist application; further modifications were made to implement a "stop time" technique. By using response to agonist as our physiological criteria, we have fundamentally enabled receptor identification and conclusively demonstrated its functionality.

Materials and Methods

Calcium indicator and cell loading

5 x 10^6 HEK-293 cells were dissociated and re-suspended in 1.6 mL Dulbecco's phosphate-buffered saline (DPBS) containing 3 µM Fluo-4 AM [1]. The cells were incubated at room temperature in the dark for 20 minutes. Following incubation, cells were spun down and resuspended in 2 mL DPBS. 10 mM ATP was freshly prepared in DPBS and added directly to the cells during the experiment to reach a final concentration of 100 µM.

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CytoFLEX Configuration

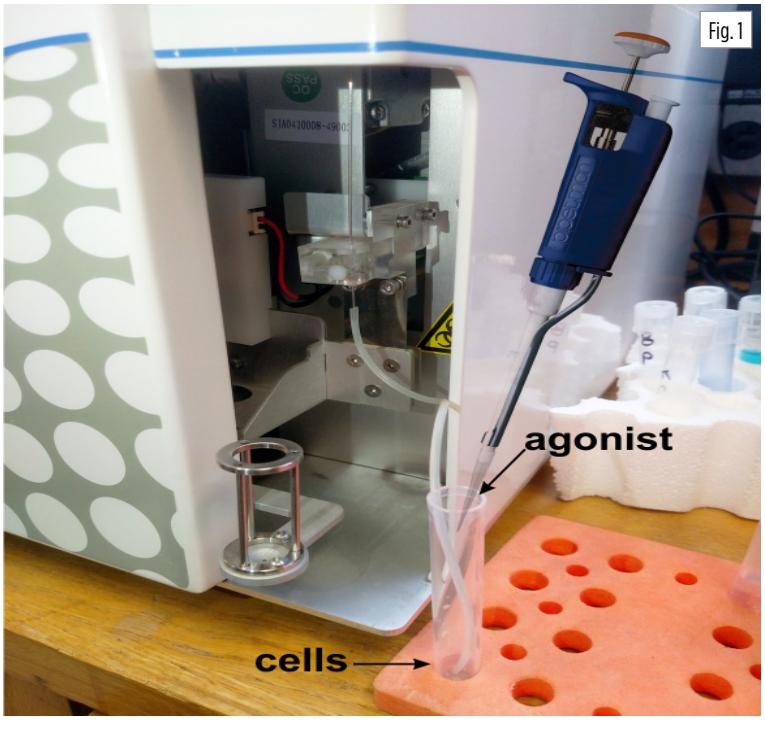
Laser WL	405nm					488nm					638nm		
Detectors	1	2	3	4	5	1	2	3	4	5	1	2	3
Fluorchomes						Fluo-4							

Parts

Vendor	Description	Part No.
Instech Laboratories, Inc.	0.30 mm ID silicone tubing	BTSIL-025
Instech Laboratories, Inc.	3-Way Y connector	SCY25

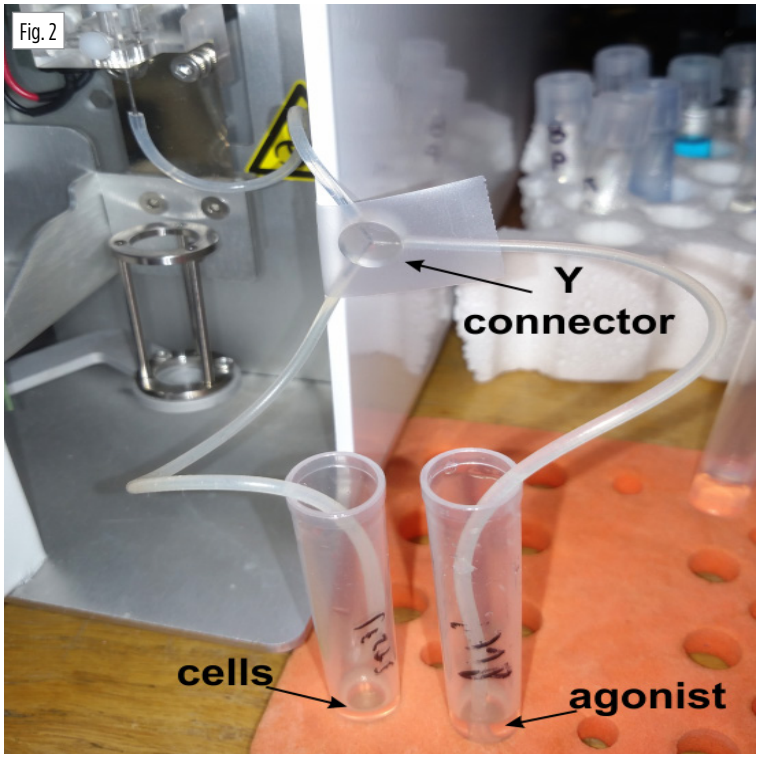
Time course setup

Modifications were made to the CytoFLEX unit configuration in order to be able to detect time sensitive data response. The modification and procedure have not been validated by Beckman Coulter. Howard Hughes Medical Institute, and the Department of Pathology and Cell biology at Columbia University Medical Center have found this to be a viable modification and methodology. A 16 cm long piece of silicon tubing with 0.30 mm internal diameter (ID) was placed onto the end of the CytoFLEX sample pick up probe. This additional tubing permitted easier access to the sample tube enabling agonist addition to the cell suspension during sample analysis. The CytoFLEX sample delivery was set to manual mode in order to implement these modifications. Additionally, this modification permitted the sample tube to be placed in a rack beside the instrument where agonist could be added without disturbing sample flow (Fig. 1).



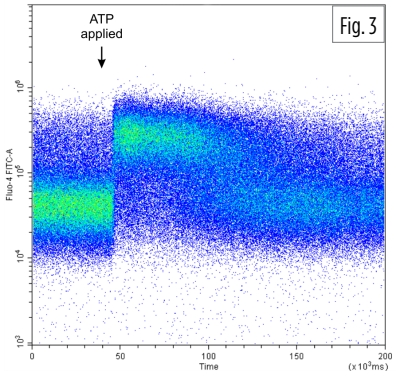
Stop time setup

A 'y' connector was constructed by imbedding 3 different 15 mm lengths of 26 gauge stainless steel tubing into a Plexiglas disk (a similar 'y' connector can be purchased directly from Instech laboratories, Inc.). One of these ports was connected to the sample probe with a 9 cm length of 0.30 mm ID silicon tubing [2]. Two additional pieces of silicon tubing were connected from the other two ports of the 'y' to the cell suspension and the agonist tubes respectively (Fig. 2). This setup created an environment where each analyzed cell had equal exposure time to agonist. The exposure time could be adjusted to capture the peak $[Ca^{2+}]_i$ transient by changing the length of the tubing that goes from the 'y' to the sample probe and by changing the peristaltic pump rate (differential pressure).

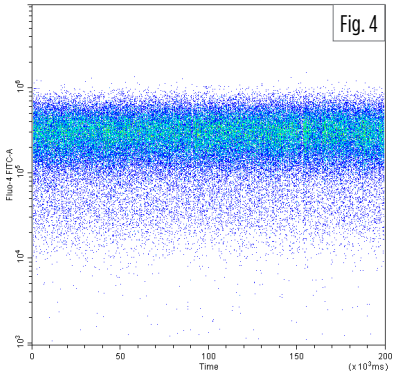


Results

When ATP was applied directly to the HEK-293 cell suspension using the setup in Fig. 1, the transient $[Ca^{2+}]_i$ shift was observed in a plot of time vs. Fluo-4 fluorescence. ATP application response was easily observed as untreated cells continue through the system and treated cells enter the system (Fig.3). The transient $[Ca^{2+}]_i$ shift starts to decrease significantly in approximately 25 seconds and returns to baseline levels over the next 90 seconds. Differential pressure was set to manual mode and set to approximately 75 % of maximum to achieve a short delay between agonist exposure and analysis.



When ATP was applied to the suspension of HEK-293 cells using the 'y' connector to create a fixed exposure time to agonist, virtually all of the detected cells were in the high $[Ca^{2+}]_i$ range (Fig. 4). This optimized "stop time" method allowed cells to maintain their peak $[Ca^{2+}]_i$ change during interrogation due to the cells having equal exposure to ATP. This method increases the sensitivity of detecting and analyzing small sub-populations of cells with the ATP receptor when compared to simply adding the ATP into the tube of cells (Fig. 3).



Discussion

The efficacy of the CytoFLEX peristaltic pump

Our laboratory has been performing $[Ca^{2+}]_i$ assays for many years using flow cytometers with pressurized sample delivery systems. These systems require a break in data collection because the sample pressure has to be stopped to add the agonist. It requires high dexterity to break the seal, add the agonist, and boost sample pressure to get it flowing before ability to detect the response decays. The peristaltic pump on the CytoFLEX maintains a continuous flow of cells and allows direct agonist addition to the open tube, enabling a more complete capture of the time course of receptor activation and deactivation of the cells. In some flow cytometers with large pressurized sample chambers it would be impossible to restart the cell flow quickly enough after agonist addition to detect a response.

Time course response of $[Ca^{2+}]_i$

There is valuable information in the time course of a Ca^{2+} response when investigating receptors on an unknown cell population. The rapidly decaying $[Ca^{2+}]_i$ response after agonist activation is typical of G-protein coupled receptors. A long and sustained $[Ca^{2+}]_i$ increase would be more typical of a receptor activating calcium permeable channels. With some agonists and cells both receptor types may be present and monitored in the data.

Continuous analysis of cells at a set time point of agonist exposure

The "stop time" method that locks agonist exposure time permits the continuous collection of data from cells at their peak $[Ca^{2+}]_i$ change in response to agonist (Figs. 2, 4). This controlled environment supports a more accurate assay for calculating percent of responsive cells and detecting small sub-populations of responsive cells that would normally be undetectable in the time course method.
This difference is illustrated in Figure 5 which displays data collected over a 200 second time course for each sample.

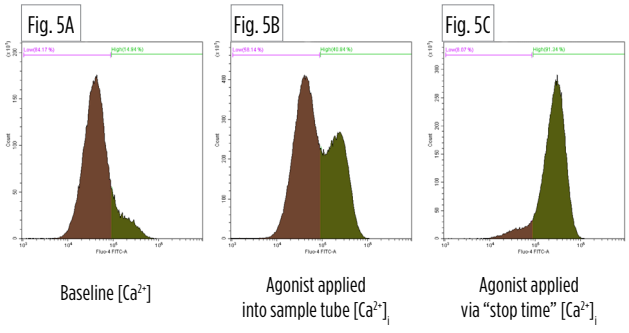


Figure 5A shows a histogram of cell number as a function of fluorescence representing $[Ca^{2+}]_i$ with no ATP added. A small portion of the total cell population has high $[Ca^{2+}]_i$, shown in green. In Figure 5B, ATP was applied as in Figures 1 and 3. There is a clear increase in the population of cells with elevated $[Ca^{2+}]_i$ collected. However, when ATP was applied using the "stop time" method shown in Figures 2 and 4, it becomes clear (Figure 5C) that the majority of cells have elevated $[Ca^{2+}]_i$ and thus are sensitive to ATP. Therefore, the "stop time" method, when optimized for the peak calcium response, is a more accurate test for measuring percent positive cells in a population.

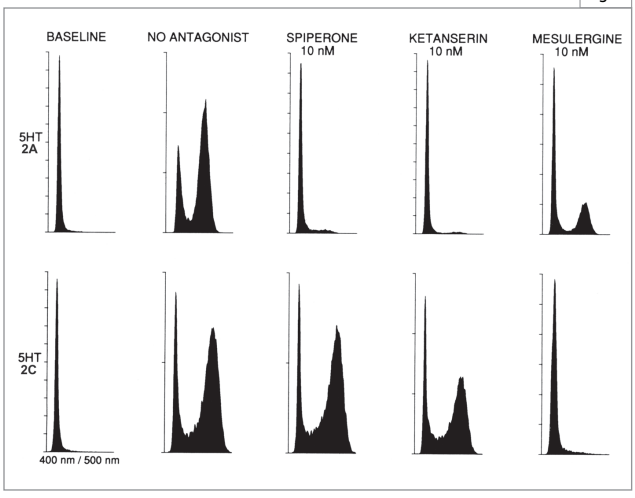
Applications

Identifying receptor subtypes

G-protein coupled receptors often have a variety of subfamilies within each receptor family. These subtypes can be identified by their different pharmacological profiles. CytoFLEX can be used to identify the specific subtypes of G-protein coupled receptor present on cells by testing the cell sample pre-incubated with selective antagonists for specific receptor subtypes. By evaluating which antagonists affect $[Ca^{2+}]_i$ responses to agonist, the receptor subtype can be determined.

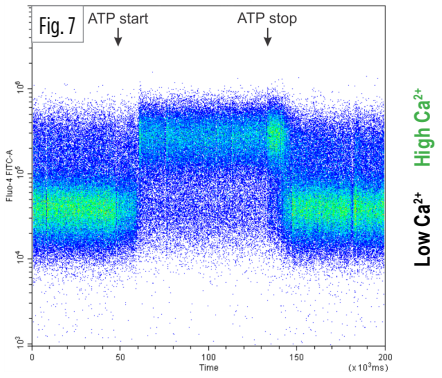
This is demonstrated in Figure 6 showing data previously acquired in this laboratory using the "stop time" method to distinguish the subtype of serotonin receptor transfected into a cell line [3].

Fig. 6



CytoFLEX, a rapid analysis screening tool

It was noted while experimenting with the CytoFLEX and the "stop time" method of ATP activation that we could clamp off the agonist pick up line and quickly see a return to normal $[Ca^{2+}]_i$ levels while the cells continued to run and analysis continued (Fig. 7). The ease of switching sample or agonist tubes while the analysis continues adds flexibility to the system and could be the basis of a fast screening method. Incorporating 96 well plate sampling with the plate loader would add even more efficiency. These simple fluidics changes create a sensitive, efficient and quick, screening assay for functional G-protein coupled receptors on cells.



References

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2. Schieren I, MacDermott A (1988 Nov). "Flow cytometric identification and purification of cells by ligand-induced changes in intracellular calcium". J Neurosci Methods 26(1):35-44.
3. Schieren I, MacDermott AB (1992), Isolation and characterization of transfected cell lines expressing neurotransmitter receptors using a calcium dye and flow cytometry, Wood JN, ed, Neuronal Cell Lines, A practical Approach, Oxford University Press, Oxford. P. 161-179

Modifications described in the document have not been validated by Beckman Coulter Life Sciences.

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