Title: A flow-cytometric method for continuous measurement of intracellular Ca^{2+} concentration.

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Abstract:
Alterations in intracellular Ca^{2+} concentration are amongst the most rapid responses to a variety of stimuli in mammalian cells. In the nervous system in particular, responses occur within nanoseconds. A major challenge in intracellular Ca^{2+} analysis is to achieve measurements within this very fast time frame. To date, the dynamic intracellular Ca^{2+} concentration has been monitored by confocal microscope, plate based assays and spectrofluorometry, although there are issues with the number of cells analysed or gaps in recording due to the addition of compounds, with significant loss of detail of a rapid Ca^{2+} response.

The new generation of flow cytometers (such as Accuri C6) resolves this problem by allowing the addition of test compounds with continuous monitoring of thousands of cells, providing a method for highly accurate dynamic Ca^{2+} measurements.

This system was tested with commonly used Ca^{2+} modulating agents in C6 glioma cells. Thapsigargin (TG), a blocker of Ca^{2+} uptake into the endoplasmic reticulum (ER), causes a significant increase in the intracellular calcium concentration via ER emptying followed by Ca^{2+} entry via store operated Ca^{2+} channels (SOCC). This well established pathway can be partially inhibited by 2-APB, a blocker of SOCC. Both the increase with TG alone and the partial increase when co-incubated with 2-APB were observed with continuous recording along with calibration curves using an Accuri C6 flow cytometer.

With these new cytometers dynamic Ca^{2+} concentration measurement becomes extremely accessible and accurate, while also providing extensive and valuable data regarding population health and responsiveness.

Introduction:
Alteration in intracellular calcium concentration is one of the most common second messengers now known in mammalian cells. It has been shown to play a critical role in long established mechanisms such as action potential generation [1,2], and has been implicated in a wide variety of responses and regulatory pathways including the initiation of apoptosis [3], the alteration of cell surface protein expression [4] and in response to mechanical stress on the plasma membrane [5]. The vast majority of these responses are extremely rapid, occurring in nanoseconds. Therefore if the full implications of the change in intracellular calcium are to be elucidated, a method for extremely rapid and accurate intracellular calcium determination is required.
Methods currently in use for intracellular calcium determination include confocal microscopy, plate based assays and spectrofluorometry. While confocal microscopy examines in great detail the intracellular calcium dynamics over time, there may be as little as 10 cells per field and therefore much of the response of the population is unrecorded. Plate based assays overcome this issue by recording the response of the entire population, however this is an overall average and thus were there to be subpopulations of cells with varying responses, these vital details would be lost. The same is true of spectrofluorometry.

Flow cytometry allows for cell by cell analysis of a population, however due to the commonly used pressurised system, addition of test compounds is impossible without a break in the recording. Due to the speed of calcium alterations, essential information regarding the nature of the response is lost.

However, the new generation of flow cytometers such as Accuri C6, resolve this problem by operating via peristaltic pump, as opposed to a pressurised system, thus allowing the addition of test compounds. Consequently continuous monitoring of thousands of cells is possible, providing a method for highly accurate dynamic Ca$^{2+}$ measurements of the entire population.

The Accuri C6 was used in a test system (see below) for determination of changes in intracellular calcium in C6 glioma cells. The success of these preliminary experiments indicate that the system will be ideal for expansion of our research programme to determination of changes in intracellular calcium in primary cultures of astrocytes and neurones in addition to cell lines. Experiments planned include a determination of changes in intracellular calcium in neuronal cells by novel amphetamine-type drugs, which may be linked to the toxic properties of these compounds, and analysis of receptor-mediated changes in intracellular calcium in astrocytes that controls expression and functional activity of neurotransmitter transporter proteins that protect cells from damage of the type observed in specific neurodegenerative disorders. It is anticipated that the results of this research will yield new information on calcium-mediated regulatory mechanisms that will form the basis for future investigations in the field of neuroscience.

**Experimental Design and Methods:**
To test this system, C6 glioma cells were exposed to a number of different calcium modifying agents. Thapsigargin (TG) inhibits the SERCA pump therefore blocking uptake of calcium into the endoplasmic reticulum (ER), resulting in a significant increase in the intracellular calcium concentration via ER emptying followed by Ca$^{2+}$ entry via store operated Ca$^{2+}$ channels (SOCC). 2-APB truncates this well established pathway by inhibiting SOCC, and so a partial increase in calcium is observed due to the initial release from the ER. A23187, a calcium ionophore and EGTA, a calcium chelator were used as positive and negative controls for the experiment.

C6 glioma cells were placed into Eppendorf tubes at 1 x 10$^6$/ml and loaded with 3 μM Fluo-4™ at 37°C in complete DMEM for 20 min. Post incubation, cells were washed 3 times with Ca$^{2+}$/Mg$^{2+}$ PBS (0.1mM CaCl and 1mM MgCl)
using centrifugation, (1min @ 1700rpm). Cells were then resuspended in Ca\textsuperscript{2+}/Mg\textsuperscript{2+} PBS. Fluorescence was filtered through the 585/40 band pass filter and collected in FL2. Baseline calcium levels were recorded for 60 sec on the Accuri C6 and were followed by the addition of 10 μM TG or 50 μM 2-APB followed by 10 μM TG. At the end of each test, 2.5 μM A23187 was added to each sample, followed by 40 mM EGTA to provide calibration curves for each sample.

Dot plots of continuous calcium dynamics are shown in figure 1, and clearly indicate the absence of gaps due to addition of the compounds.

![Figure 1: Fluorescence of Fluo-4 and Forward Scatter versus time.](image_url)

**References**


