

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 microscopy, plate-based assays, spectrofluorometry, and flow cytometry, although there are issues with the number of cells analyzed 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 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-aminoethoxydiphenyl borate (2-APB), a blocker of SOCC. Both the increase with TG alone and the partial increase when coincubated 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. © 2010 International Society for Advancement of Cytometry

• Key terms

continuous calcium measurement; calcium flux; Ca^{2+} ; live cells; kinetic; flow cytometry; Accuri C6

AN alteration in intracellular calcium concentration is one of the most common second messenger responses now known in mammalian cells. These responses have been shown to play a critical role in long established mechanisms such as action potential generation in electrically excitable cells (1,2) and have been implicated in a wide variety of intracellular signaling and regulatory pathways, including initiation of apoptosis (3), alteration of cell surface protein expression (4), and response to mechanical stress on the plasma membrane (5). The vast majority of changes in intracellular calcium is extremely rapid and occurs within a nanosecond timescale. Therefore, if the full implications of a change in the intracellular calcium concentration are to be understood, then a protocol for extremely rapid and sensitive intracellular calcium determination is required.

Methods currently in use for intracellular calcium determination include confocal microscopy, plate-based assays, spectrofluorometry, and flow cytometry. Although confocal microscopy examines in great detail the intracellular calcium dynamics over time, the number of cells examined per field is low, and therefore, much of the response throughout a population of cells may be unrecorded. Plate-based assays overcome this issue by recording the response of the entire population; how-

ever, this is an overall average and thus, if there were subpopulations of cells with varying responses, these vital details would be lost. The same is true of spectrofluorometry.

The major advantage of flow cytometry is that it allows identification and analysis of individual cells within a population based on their light-scatter profile and selective responsiveness to specific stimuli. A range of methodologies have been developed for analysis of changes in intracellular calcium concentration in cell populations using cell-permeable calcium-sensitive fluorescent dyes (6–11). Most flow cytometers have a pressurized system for aspirating the sample in which the tubes must be sealed, and therefore, there is no opportunity for continuous addition of test compounds to the cell suspension. It is therefore necessary to pause cell aspiration into the flow cytometer whilst compounds under study are added (stop-flow method). The accuracy and reproducibility of results requires that aspiration is paused for as short a time as possible and that the pause time should be the same in each experiment. In experiments designed to measure changes in intracellular calcium essential information may be lost due to the speed of the responses generated, particularly in neuronal cells. An alternative approach is to use a system such as the Time Zero System (Cytex) that is compatible with FACStar, FACStar Plus, EPICs, Elite, and FACS Vantage flow cytometers and which provides rapid addition of reagents to a sample, thus minimizing time loss due to sample addition (10).

The new generation of flow cytometers, such as Accuri C6, resolve the problem of addition of test compounds by operating via a peristaltic pump, which allows the use of open tubes, such as Eppendorfs, and straightforward addition of test compounds to the cell suspension, as shown in Figure 1. Consequently, continuous monitoring of thousands of cells is possible, providing a new method for dynamic Ca^{2+} measurements of the entire population.

To test this new system, rat C6 glioblastoma cells were exposed to a number of different calcium modifying agents and changes in the intracellular calcium concentration recorded. Thapsigargin (TG) inhibits the sarco(endo)plasmic reticulum calcium-ATPase (SERCA) pump, therefore blocking uptake of calcium into the endoplasmic reticulum (ER), resulting in a significant increase in the intracellular calcium concentration that occurs via ER emptying, followed by Ca^{2+} entry by store-operated Ca^{2+} channels (SOCC) on the plasma membrane (12,13). 2-aminoethoxydiphenyl borate (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 (14). For comparative purposes, the calcium ionophore, A23187 (15), was used as positive control for influx of extracellular calcium in these experiments.

MATERIALS AND METHODS

Cell Culture

C6 glioma cells (ATCC cat. no. CCL-107TM) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine (2 mM) in T75 cell culture flasks (Greiner Bio-



Figure 1. Addition of cells or compounds using Eppendorf tubes during sample analysis in an Accuri C6.

One, Cruinn Diagnostics, Dublin) at 37°C in a humidified atmosphere of 5% CO_2 , 95% air. Cells were grown as a monolayer and routinely passaged twice weekly. All cell culture products were obtained from Gibco, Bio Sciences, Dublin unless otherwise indicated.

Flow Cytometry

C6 glioma cells were trypsinized, washed, and placed in Eppendorf tubes at $1 \times 10^6/\text{ml}$ and incubated with 3 μM Fluo-4-acetoxymethyl ester (Fluo-4 AM; Molecular Probes, Eugene OR) in 3% dimethylsulfoxide (DMSO; final concentration of DMSO in each assay, 0.06%) at 37°C in complete DMEM for 20 min. Fluo-4 AM is a high-affinity calcium indicator with an λ_{ex} 470–490 nm and an λ_{em} 520–540 nm. After incubation, cells were washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ PBS (0.1 mM CaCl_2 and 1 mM MgCl_2 ; Sigma-Aldrich, Ireland) by centrifugation (1 min at 300g). Cells were then resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ PBS. The fluorescence in each sample was analyzed using the 530/30 filter. Although the ideal filter is the 530/30, in some cases, the signal was too bright and we had to use the 585/40 for measuring the signal as based on the spectral diagrams, about 10% of the signal can be detected using this filter. This fact suggested that a better titration of the reagent is necessary in order to get a more robust and sensitive measurement. Baseline calcium levels were recorded for 60 sec on the Accuri C6 and were followed by the addition of 2.5 μM A23187 (in DMSO; Sigma-Aldrich Ireland), 10 μM

Beckman Coulter Cyan ADP:

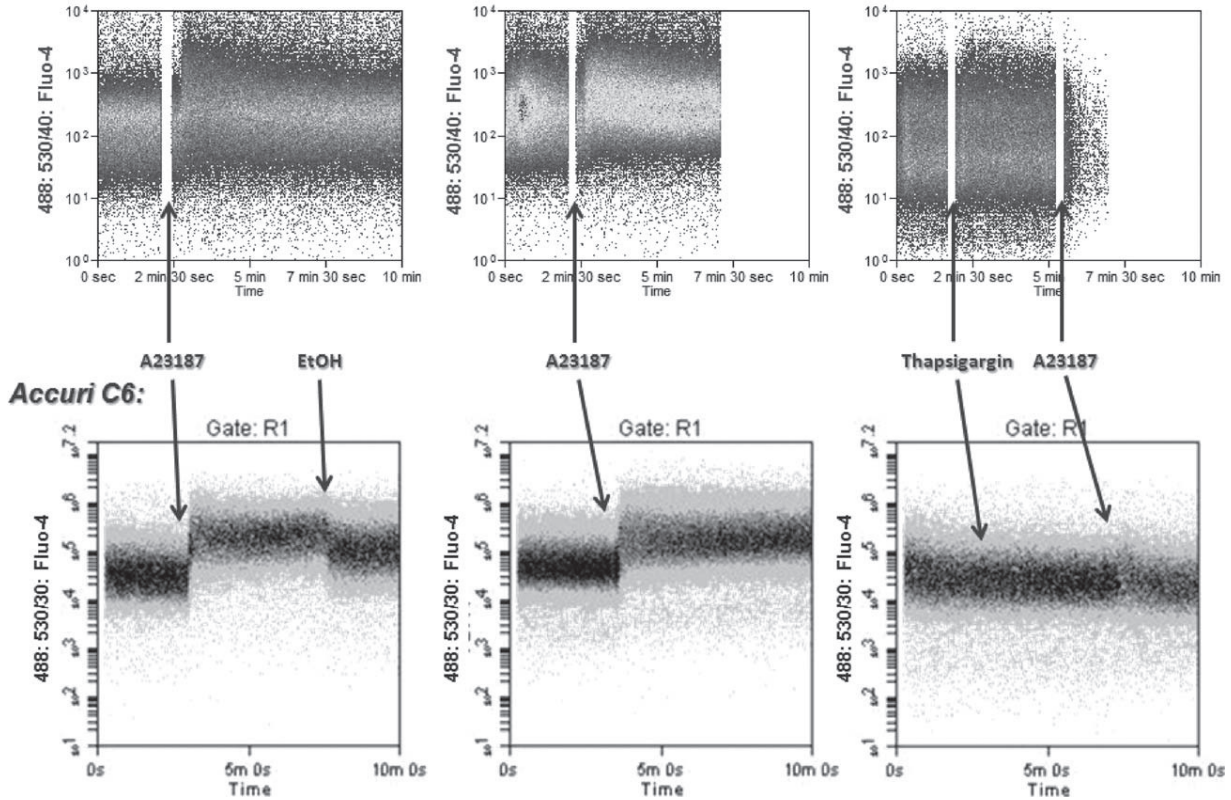


Figure 2. Comparative cytograms of fluorescence of Fluo-4 versus time displaying the addition of various control compounds (Ionophore A23187, ethanol, and Thapsigargin). Gated data using the gating strategy shown in Figure 4. Upper cytograms correspond to the data obtained in a Beckman Coulter Cyan ADP using the stop-flow methodology. Lower cytograms represent the data obtained using a replicate of the previous samples adding the same compounds. Comparable data was obtained in both methods, although no gaps are observed during the acquisition with the Accuri C6.

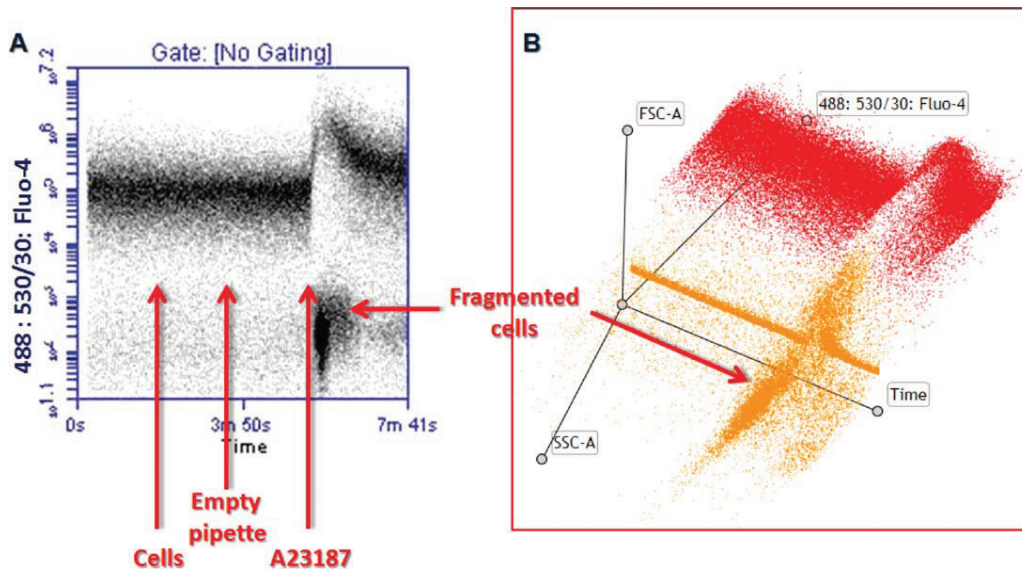


Figure 3. A: Dot plot of ungated data of fluorescence of Fluo-4 versus time displaying the addition of various control compounds. No changes were observed after the addition of fresh sample or an empty pipette. After the addition of A23187, an increase in the number of events was observed (fragmented cells). **B:** The same sample reanalyzed with Kaluza software. The radial display shows a clear difference on FSC and SSC after the addition of the ionophore. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

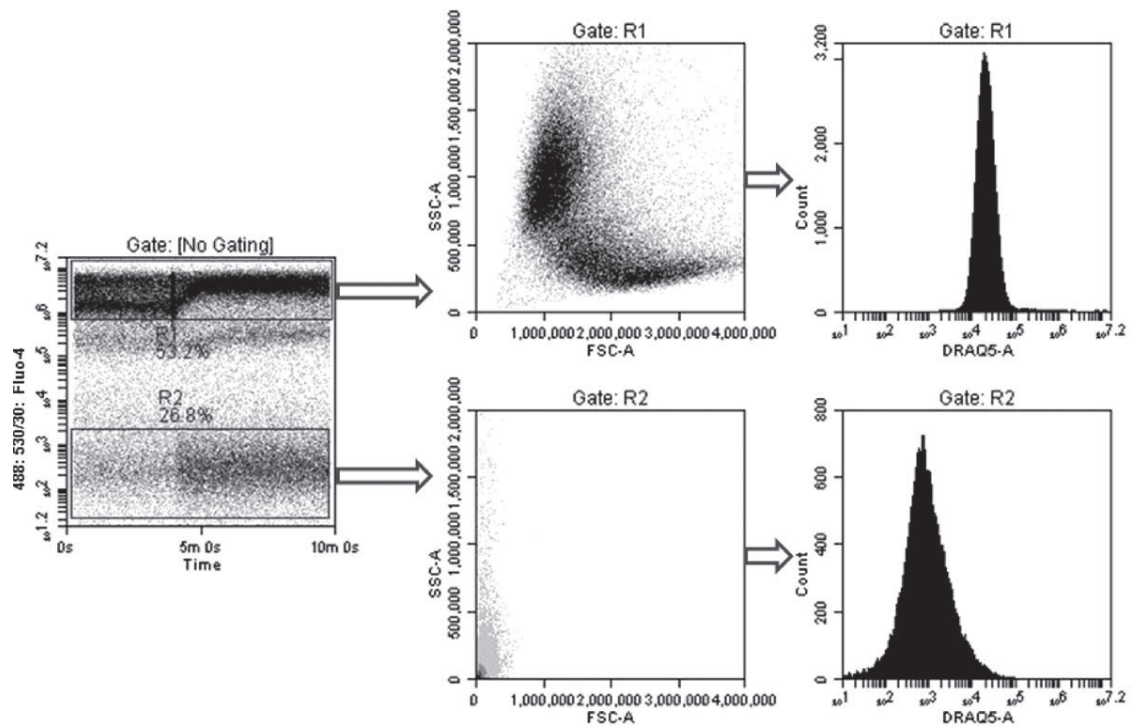


Figure 4. Low fluorescent events have very low DRAQ-5 fluorescence and their distribution in FSC versus SSC is clearly different to the high fluorescent events. This confirms that these events correspond to fragmented cells.

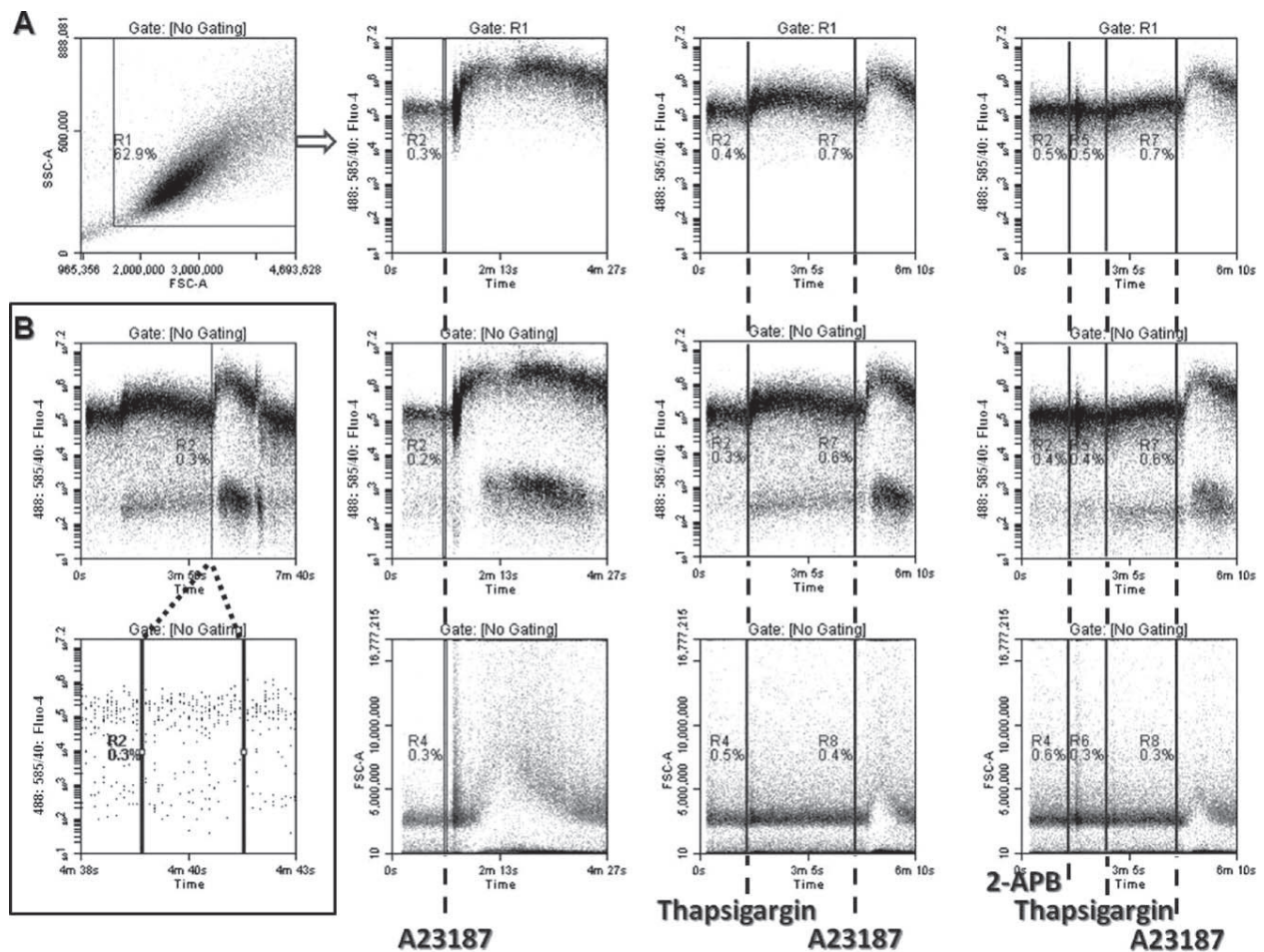


Figure 5. A: Cytoprams of fluorescence of Fluo-4 (585/40) and forward scatter versus time. Addition of compounds is indicated by dashed lines. Upper panel indicates the changes produced on fluorescence of cells by gating on forward and side scatter (Region 1), middle panel indicates the fluorescence of the ungated population, and the lower panel indicates the changes on size. **B:** Addition times can be accurately displayed by using the CFlow Plus zoom tool.

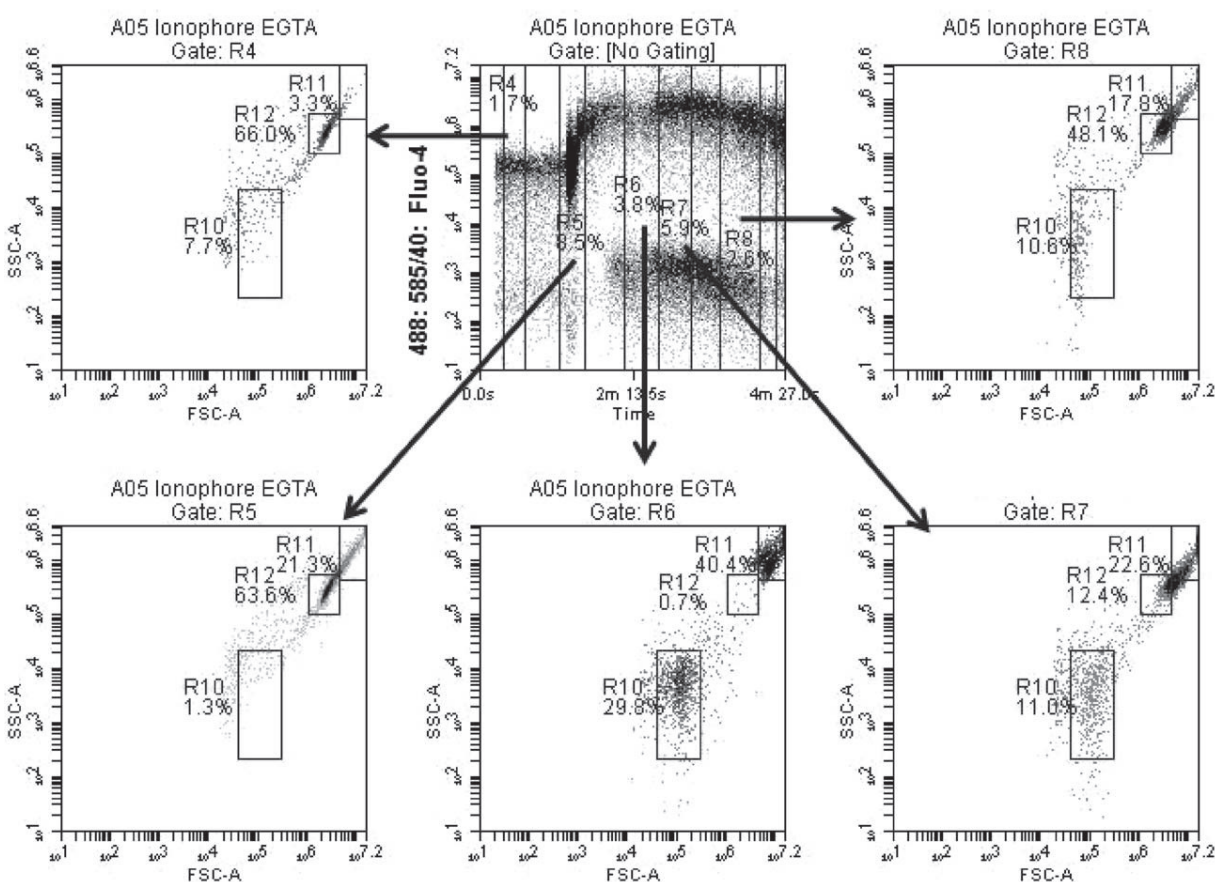


Figure 6. Changes on calcium signal can induce changes in FSC and SSC. Different scatter plots of the same sample, gated at different time points, show these changes. An increase in the number of fragments can be also observed.

TG (in DMSO; Sigma-Aldrich Ireland), or 50 μM 2-APB (in DMSO; Tocris, Avon, UK) followed by 10 μM TG. All compounds were added using a gel loading pipette tip (STARLAB, Switzerland), allowing for the addition of test compounds and mixing of the sample with ease. At the end of each test, 2.5 μM A23187 was added to each sample as a positive control.

To confirm the obtained data, samples were analyzed in parallel in an Accuri C6 using this new methodology and in a Beckman Coulter Cyan ADP using the stop-flow method (Fig. 2). One of the main disadvantages of the stop-flow method is the lack of information during the first seconds after the addition of the compounds; as in some cases, the major changes are produced in a few seconds.

To ensure that there was no interference in data collection during the addition and mixing of compounds, certain controls were performed. Air bubbles were introduced into the sample with an empty pipette, and the cell suspension was removed or added or mixed with the sample. None of the above induced changes in the baseline fluorescence, as shown in Figure 3. Finally, 5 μM DRAQ-5 (Biostatus, UK) was added to the cell suspension and analyzed (675LP filter) in order to guarantee that our data included only determinations of fluorescence in cells, as DRAQ-5 is a DNA-specific dye (Fig. 4).

For the analysis of the time delay between the addition of the compounds and its analysis in the flow cell, calibration beads with different fluorescence and sizes were added at different time points. These time points were selected and marked with a region, as soon as the data of the first set of beads is shown at that region, the next set of beads is added.

RESULTS AND DISCUSSION

Dot plots of continuous calcium dynamics post exposure to various compounds are shown in Figure 5 and clearly indicate the absence of gaps due to addition of the compounds.

All responses seen in the tests described earlier were consistent with results expected post exposure to the calcium-modifying agents. A23187, a calcium ionophore, induced a large increase in intracellular calcium and thus Fluo-4 AM fluorescence. TG, a commonly used tool for emptying intracellular ER stores that then triggers an influx of extracellular calcium via SOCC (12), also significantly increased fluorescence, an effect that was partially blocked by the addition of 2-APB. A notable increase in fluorescence was also observed on addition of A23187 at the end of the experiment.

In calcium studies, exclusion of dead cells in flow cytometric analysis is frequently achieved by gating out cells that show no fluorescence as can be seen, for example, in the

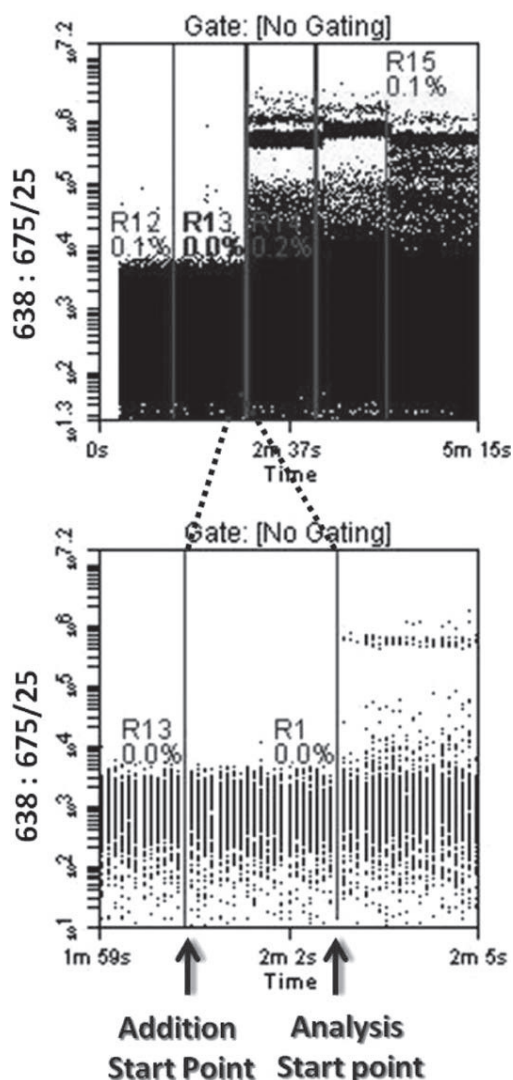


Figure 7. Illustration of the time-lag between addition of test compound to the Eppendorf tube and aspiration into the flow cytometer (less than 2 sec)

case of Fluo-3 (16). Although fragmented cells can be recognized by scatter signal (Figs. 3 and 6), we used DRAQ5 as an alternative method for detection of such events. In Figure 4, we show that events with the lowest fluorescence of Fluo-4 AM correspond to those events with the lowest scatter signal and lowest signal of DRAQ5.

It is noted that samples that were not treated with 2-APB and TG had a greater increase in intracellular calcium concentration, higher scatter signal, and a greater number of fragments compared with the treated samples (Figs. 5 and 6).

Because of the design in the Accuri C6, flow cytometry has become a considerably cheaper and easy to use tool in monitoring the responses of live cells to a variety of stimuli. By recording the response of the whole cell population, it is now possible to identify any subpopulation of cells that may respond differently to the main population. This can be extre-

mely useful in experiments where there are multiple cell types in one sample which may have differing responses to the same stimulus, as, for example, in the recent experiments on whole blood calcium measurements by Schepers et al. (17). Second, this advantage can be exploited for the identification of varying degrees of calcium responsiveness within a single population of cells (18). Third, this method considerably improves the accuracy of the data, as any dead or fragmented cells can be removed from the population, as shown in Figures 3–6. Alternatively, if required, the response of these subpopulations can also be determined and their contribution to the overall response assessed.

With these new cytometers, dynamic Ca^{2+} concentration measurement becomes extremely accessible, while also providing extensive and valuable data regarding population health and responsiveness. Comparative measurements of intracellular calcium using the Accuri C6 and a system such as the Cytex time-zero module would provide additional insights into the scope of the Accuri C6. It is apparent that a comparable time delay of less than 2 sec between addition of test reagent and laser intercept point can be achieved by the peristaltic pump-based system in the Accuri C6 (Fig. 7) and a pressure-based-system that use a time-zero module (for example, Ref. 10).

This method that we have developed for use with the Accuri C6 could be applied to any other flow cytometer with a peristaltic pump that allows continuous cell aspiration from an open sample tube or vial. Equally, this approach could be applied to any one of a number of live cell imaging platforms such as pH, reactive oxygen, nitrogen species, mitochondrial membrane potential, and/or nanoparticle uptake.

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