Continuous Measurement of Intracellular Calcium on the BD Accuri™ C6 Flow Cytometer

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White Paper

Contents

Abstract

Calcium flux mediates many important cellular functions, and the calcium cation (Ca^{2+}) serves as a secondary messenger to control many cellular processes. Changes to intracellular Ca^{2+} levels occur rapidly—in some cases within nanoseconds of stimulation—and obtaining accurate data on these changes is a significant research challenge. Dynamic intracellular Ca^{2+} concentration has been monitored by optical microscopy, plate-based assays, spectrofluorometry, electrophysiology, and flow cytometry. Each method has its advantages and disadvantages. Flow cytometry, for example, can identify and analyze individual cells within a population, but the "stop-flow" method often required to add test compounds leaves gaps in recording.

The BD Accuri™ C6 personal flow cytometer supports a new "continuous-flow" method that allows the addition of test compounds with continuous, gap-free monitoring of thousands of cells. We tested this system with the Ca^{2+} modulating agents thapsigargin (TG) and 2-aminoethoxydiphenyl borate (2-APB) in C6 glioma and SH-SY5Y neuronal cells.

The expected effects of TG and 2-APB, along with A23187 (the Ca^{2+} ionophore) and EGTA and BAPTA-AM (Ca^{2+} chelators), were observed with continuous, gap-free recording on a BD Accuri C6. This new continuous-flow method can make dynamic Ca^{2+} concentration measurement accessible and accurate, providing valuable data about population health and responsiveness.

Introduction

Calcium is an important second messenger that mediates many important cellular functions, including muscle contraction, secretion, metabolism, neuronal excitability, cell proliferation, sperm motility and fertilization, and apoptosis and necrosis. The signals are turned on when the cell picks up Ca^{2+} from the external medium or releases it from stores in intracellular organelles. Ca^{2+} pumps are used to turn the signals off.[1,2,3]

Changes to intracellular Ca^{2+} levels in response to stimuli take place extremely quickly in mammalian cells. In the nervous system, responses occur within nanoseconds.[4] It is a significant research challenge to obtain accurate data on the dynamics of intracellular calcium within this rapid time frame.

Many methods have been employed in the quest to monitor dynamic intracellular Ca^{2+} concentration. Most of these methods utilize fluorescent calcium indicator dyes and include optical microscopy, plate-based assays, spectrofluorometry, electrophysiology, and flow cytometry. Each method has its advantages and disadvantages. For example, confocal laser scanning microscopy, two-photon excitation laser scanning microscopy, and other optical microscopy techniques can examine Ca^{2+} dynamics at the subcellular level, and even record the spatial movement of Ca^{2+} within cells.[5] Used with fluorescent dyes, these imaging techniques can depict in elegant detail the propagation of waves of Ca^{2+} release and mitochondrial membrane potential ($\Delta\Psi_m$) depolarization through the cell.[2] However, these optical methods can focus only on a small number of cells per field, and may miss responses in the larger cell population.

Figure 1. Adding test compounds on the BD Accuri C6.

Cells or compounds can easily be added to Eppendorf tubes without interrupting sample analysis on the BD Accuri C6.

The unique advantage of flow cytometry is the identification and analysis of individual cells and different subpopulations within a sample, using cell-permeable, calcium-sensitive fluorescent dyes and antibodies to cell marker proteins. Most flow cytometers, however, use a pressurized fluidics system in which the tubes must be sealed. To add test compounds to the cell suspension, a "stop-flow" method must be used in which sampling is paused, the sample tube opened, the agonist added, and the tube resealed. This technique leaves a gap or blind spot in data collection that may fail to capture essential changes in Ca^{2+} levels.

The BD Accuri C6 personal flow cytometer, in contrast, employs non-pressurized peristaltic pumps in an open fluidics system. The use of open tubes, such as Eppendorf tubes, allows convenient addition of test compounds to the cell suspension without interruption during sampling (Figure 1). This "continuous-flow" method enables non-stop monitoring of thousands of cells and accurate dynamic Ca^{2+} measurement of the entire population.

Table 1. Methods of measuring intracellular calcium.

Method	Advantages	Disadvantages
Electrophysiology	Can measure neuronal excitability.	Limited to small populations of cells. Requires specialized training and equipment. Does not work for all cells.
Plate-based imaging	Useful for High Throughput Screening (HTS).	Requires specialized training and equipment. Cannot see details or subpopulations of cells.
Bioimaging/fluorescence microscopy	Can see subpopulations of cells and cellular subcomponents.	Limited to small populations of cells. Requires specialized training and equipment.
Flow cytometry on the BD Accuri C6	Can look at large populations and subpopulations of cells.	Cannot look at subcellular components. Requires single cells.

To test the continuous-flow method on the BD Accuri C6, we exposed rat C6 glioblastoma cells and human neuronal SH-SY5Y cells to various calcium modifying agents, and recorded changes in intracellular calcium concentration. The cellular pathways are shown in Figure 2.

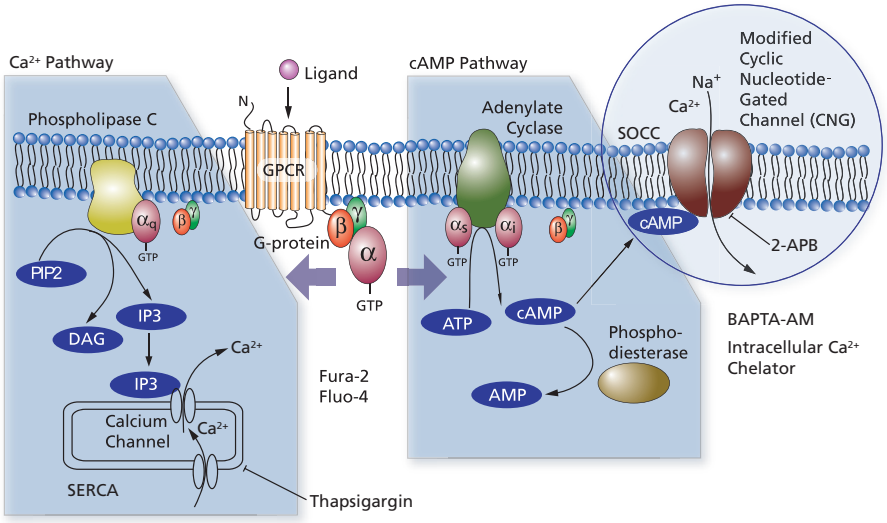

Figure 2. Intracellular calcium pathways.

Thapsigargin (TG) inhibits the SERCA pump, blocking uptake of calcium into the endoplasmic reticulum (ER) and resulting in a significant increase in intracellular calcium concentration. This takes place in a two-step process: first, the ER empties; then, extracellular Ca^{2+} enters via store-operated Ca^{2+} channels (SOCC). 2-aminoethoxydiphenyl borate (2-APB) truncates this well established pathway by inhibiting SOCC, so that the presence of both TG and 2-APB results in a partial increase in calcium, due to the initial release from the ER. The calcium ionophore A23187 served as a positive control for the influx of extracellular Ca^{2+} in these experiments, while EGTA and BAPTA-AM (chelators of extracellular and intracellular calcium, respectively) served as negative controls.

Methods

Instruments

Cytometer data was acquired using a BD Accuri C6 flow cytometer system, adding test substances directly into Eppendorf sample tubes during acquisition. The instrument was set up using Spherotech 8-peak beads. Application settings were applied and compensation performed prior to each experiment using single-stained controls. BD Accuri software was used for acquisition, analysis, and calculation of cell counts.

For comparison purposes, samples were also acquired and analyzed in parallel using a Beckman Coulter CyAn® ADP flow cytometer system, adding test substances using the stop-flow method.

Cell culture

Rat C6 glioma cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine (2 mM) in T75 cell culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were grown as a monolayer and routinely passaged twice weekly.

Human neuronal SH-SY5Y cells (European Collection of Cell Cultures, ECACC) were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin, L-glutamine (2 mM) and 1% L-non-essential amino acids (L-NEAA).

Flow cytometry

C6 glioma cells or SH-SY5Y cells were trypsinized, washed, placed in Eppendorf tubes at 1 x 10^6/mL, and incubated with 3 µM of Fluo-4™ AM (Molecular Probes) in 3% DMSO at 37°C in complete DMEM for 20 minutes. Fluo-4 AM is a high-affinity calcium indicator with λ_{ex} = 470–490 nm and λ_{em} = 520–540 nm. After incubation, cells were washed three times with Ca^{2+}/Mg^{2+} PBS (Sigma-Aldrich) by centrifugation (1 min at 300*g* or 1,700 rpm), and resuspended in Ca^{2+}/Mg^{2+} PBS.

Fluorescence data was collected and analyzed on the BD Accuri C6 in FL1 using a 530/30 BP filter or, when the signal was too bright, a 585/40 BP filter, which can detect about 10% of the signal. (An attenuation filter, such as Cat. No. 653173 or 653172, could also be used to reduce an overly bright signal.)

Baseline calcium levels were recorded for 60 seconds on the BD Accuri C6 and were followed by the addition of 10 µM of TG (Sigma-Aldrich), 2.5 µM of A23187 (Sigma-Aldrich), or 50 µM of 2-APB (Tocris) followed by 10 µM of TG. All compounds were added and mixed using a gel loading pipet tip. At the end of each test, 2.5 µM of A23187 was added to each sample as a positive control. In some experiments, 40 µM of EGTA (Invitrogen) or 10 µM of BAPTA-AM (Sigma-Aldrich) was added as a negative control or to provide calibration curves.

To ensure that the addition and mixing of compounds did not interfere with data collection, additional control conditions were added. Fresh sample was removed or added or mixed with the cell suspension, and air bubbles were introduced into the sample with an empty pipet.

To analyze the time delay between addition of the test compounds and their appearance in the flow cell, calibration beads were added at different time points, which were selected and marked with a region. As soon as the data from the first set of beads appeared in the region, the next set of beads was added.

Confocal microscopy

For comparison, intracellular calcium concentration was measured ratiometrically by confocal microscopy, in a procedure described elsewhere.[7] Briefly, C6 glioma cells were seeded into glass-bottom culture dishes at a density of 0.25 x 10^6 cells per dish and grown at 37°C in a humidified atmosphere in a CO2 incubator until 60–75% confluent. Cells were loaded with two high-affinity, cell-permeant calcium indicators, 3 µM of Fluo-3™ AM (Molecular Probes) and 3 µM of Fura Red™ AM (Molecular Probes), in Ca^{2+}/Mg^{2+} PBS for 1 hour at 37°C. Fluo-3 AM has λ_{ex} = 470–490 nm and λ_{em} = 520–540 nm, while Fura Red AM has λ_{ex} = 450–500 nm and λ_{em} ~660 nm.

The indicators were removed and the cells were washed with Ca^{2+}/Mg^{2+} PBS, followed by another three washes in Ca^{2+}/Mg^{2+} PBS. Using a Carl Zeiss LSM 510 UVMETA confocal microscope, the sample was excited at 488 nm, and fluorescence emissions were recorded simultaneously at 525 and 660 nm. The culture was scanned for 30 seconds to determine a baseline prior to exposure to 10 µM of TG. Frames of 256 x 256 pixels were taken, and the pinhole diameter was kept constant. By this method, 20 to 30 live cells were analyzed in three independent determinations.

Results

Imaging by confocal microscopy

To provide comparative data, 20 to 30 live C6 glioma cells were imaged using confocal microscopy before and after the addition of TG. Fluorescence analysis clearly showed a rapid increase in Ca^{2+} (as shown by fluorescence changes), a plateau, and a more gradual increase (Figure 3).

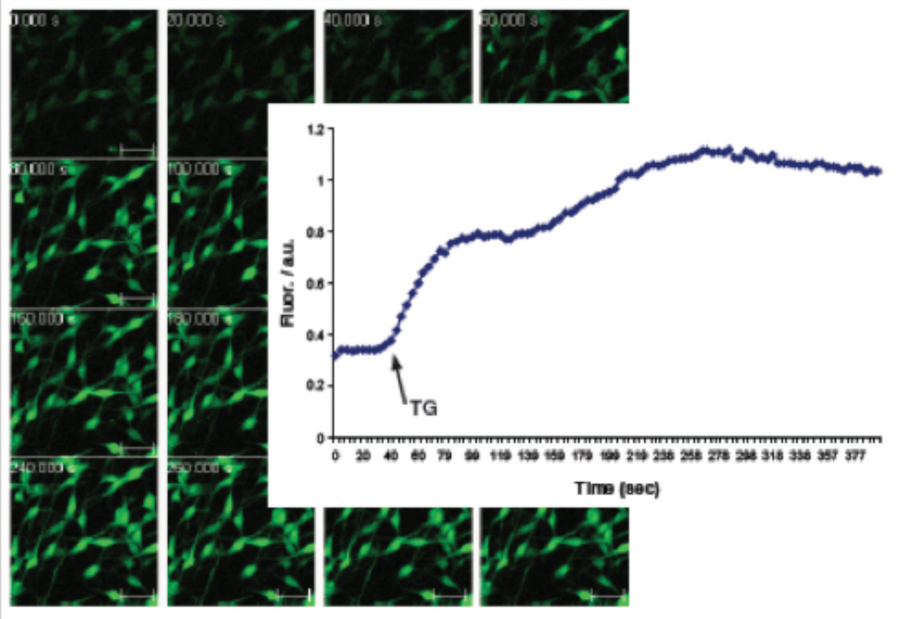

Figure 3. Confocal imaging of live cells.

Comparative data obtained from imaging 20 to 30 live C6 glioma cells by confocal microscopy. The plot shows the fluorescence ratio of the dyes (Fluo-3 AM and Fura Red AM) over time. Data from Vines et al, 2009, reproduced courtesy of the authors.

Controls for sample handling

To ensure that changes in intracellular Ca^{2+} were not due to extraneous elements of the procedure, fresh sample was removed or added or mixed with the cell suspension, and air bubbles were introduced into the sample with an empty pipet. None of these procedures affected baseline fluorescence (Figure 4).

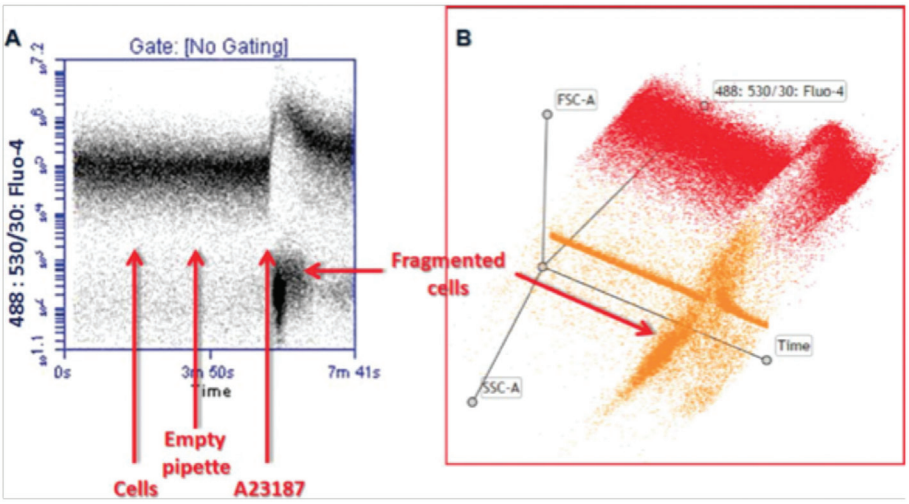

Figure 4. Controls for sample handling.

A. Dot plot of ungated Fluo-4 fluorescence vs time, showing the addition of various control compounds to C6 glioma cells during acquisition. No changes were observed after the addition of fresh sample or air using an empty pipet. After the addition of A23187 (Ca^{2+} ionophore), increases in both fluorescence and the number of events were observed, representing fragmented cells.
B. The same sample was reanalyzed with Kaluza® software, showing that the ionophore had a clear effect on FSC and SSC. Data from Vines et al, 2010, reproduced courtesy of the authors and Cytometry Part A.

Continuous-flow vs stop-flow method

Figure 5 shows comparative C6 glioma cell data using the stop-flow method on the Beckman Coulter CyAn ADP vs the continuous-flow method on the BD Accuri C6. The stop-flow data shows clear time gaps, corresponding to the first few seconds after compounds are added. This is crucial because, in some cases, significant changes are produced within those few seconds.

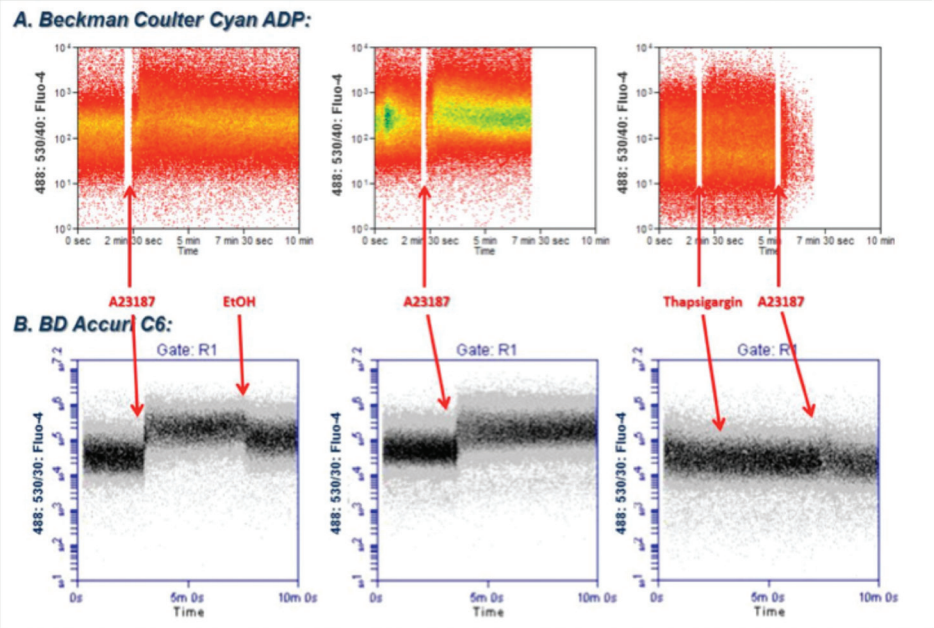

Figure 5. Comparison of continuous-flow vs stop-flow method.

Comparative cytograms of Fluo-4 fluorescence of C6 glioma cells over time, showing the effects of adding control and test compounds (ionophore A23187, ethanol, and thapsigargin). Events are gated on high Fluo-4 fluorescence to exclude fragments. ***A.*** *Upper cytograms show data obtained on a Beckman Coulter CyAn ADP using the stop-flow method, showing time gaps when compounds were added.* ***B.*** *Lower cytograms show data obtained on a BD Accuri C6, adding compounds in open Eppendorf tubes without interrupting sample acquisition. No time gaps were observed. Except for the gaps, comparable data were obtained by both methods. Data from Vines et al, 2010, reproduced courtesy of the authors and* Cytometry Part A.

Effects of test compounds on C6 glioma cells

Figure 6 shows the effects of adding the test compounds to the C6 glioma cells by the continuous-flow method, measured without interruption on the BD Accuri C6. A23187, a calcium ionophore, caused a substantial increase in intracellular calcium as measured by Fluo-4 AM fluorescence. TG, which empties intracellular ER stores, again significantly increased fluorescence by triggering an influx of extracellular calcium via SOCC.

This effect was largely blocked by the addition of 2-APB, which inhibits SOCC. Samples that were treated with both 2-APB and TG (rightmost plots) had a smaller increase in intracellular calcium concentration, lower scatter signal, and fewer fragments compared with the treated samples. However, adding A23187 at the end of the experiment once more increased fluorescence.

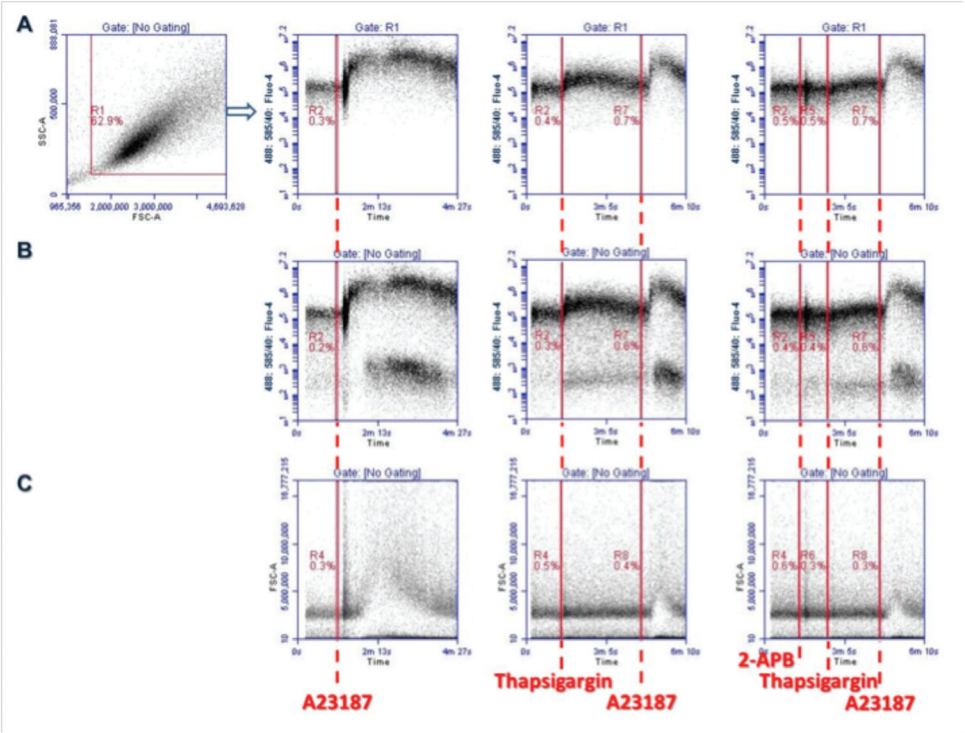

Figure 6. Effects of adding test compounds on C6 glioma cells.

Cytograms of Fluo-4 fluorescence and forward scatter of C6 glioma cells over time, measured on the BD Accuri C6 with the 585/40 BP filter. Dashed lines indicate addition of test compounds. ***A.*** *The upper panel shows the changes in cellular fluorescence, excluding fragments by gating on forward and side scatter (R1).* ***B.*** *The middle panel shows the fluorescence of the ungated population; fragments show lower fluorescence.* ***C.*** *The lower panel shows forward scatter of the ungated population, indicating changes in size.* ***Results:*** *Both A23187 and TG increased Ca^{2+} levels rapidly after addition. The effect of TG was mediated by prior addition of 2-APB. Only A23187 resulted in an increase in forward scatter, signifying larger particles. Data from Vines et al, 2010, reproduced courtesy of the authors and* Cytometry Part A.

Effects of test compounds on SH-SY5Y neuronal cells

Figure 7 shows the effects of adding the test compounds to the SH-SY5Y human neuronal cells by the continuous-flow method, measured without interruption on the BD Accuri C6. An unspecified drug, the calcium ionophore A23187, and the extracellular calcium chelator EGTA were sequentially added to cultured SH-SY5Y cells in the presence of calcium (TG), the intracellular calcium chelator BAPTA-AM, or in the absence of calcium (EGTA).

In the presence of calcium, the drug provoked a momentary spike in intracellular calcium, which might have been missed using the stop-flow method. As expected, A23187 caused a lasting increase in Ca^{2+} concentration, and EGTA reduced it. The presence of BAPTA-AM or the absence of calcium negated the effects of the drug and moderated the effects of A23187 and EGTA.

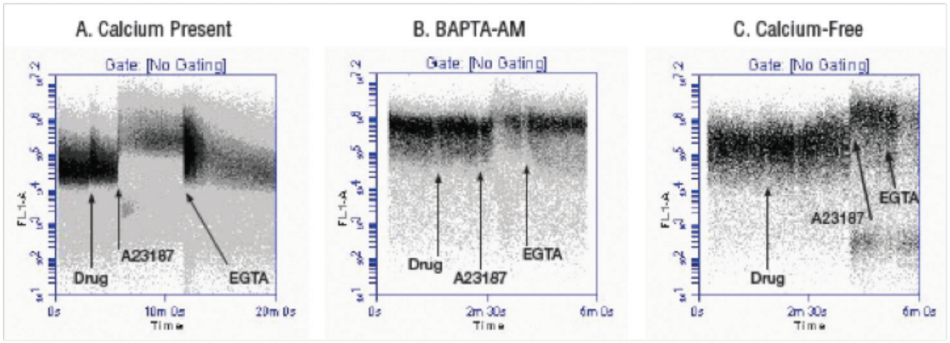

Figure 7. Effects of adding test compounds on SH-SY5Y neuronal cells.

Cytograms of Fluo-4 fluorescence of SH-SY5Y neuronal cells over time, measured on the BD Accuri C6 in the FL1 channel. Arrows indicate addition of test compounds. ***A.*** *Test compounds added in the presence of calcium.* ***B.*** *Test compounds added in the presence of BAPTA-AM, the intracellular calcium chelator.* ***C.*** *Test compounds added in the absence of calcium.* ***Results:*** *All test compounds affected Ca^{2+} levels in the presence of calcium. Both BAPTA-AM and the absence of calcium negated or moderated these effects. Data from Vines et al, 2009, reproduced courtesy of the authors.*

Time delay

Calibration beads were added to the sample to measure the time delay between addition and analysis. The BD Accuri software's Zoom tool was used to focus on a 6-second time frame (Figure 8). The first evidence of increased fluorescence reached the flow cell and was clearly visible less than 2 seconds after adding the test compound.

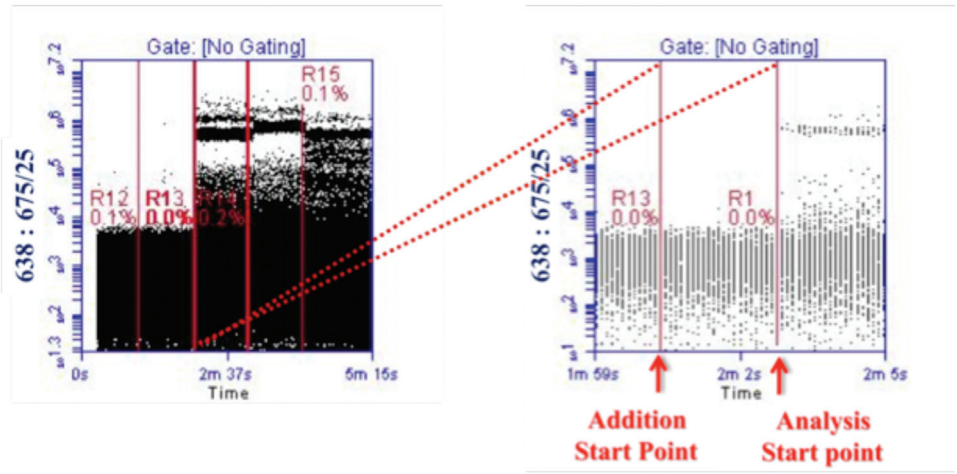

Figure 8. Time delay between addition and analysis.

Zooming in on the interval between initial addition of the test compound to the Eppendorf tube and its aspiration into the flow cell (creating a visible effect) shows a lag of less than 2 seconds. Data from Vines et al, 2010 (poster), reproduced courtesy of the authors.

Discussion

The unique fluidics design of the BD Accuri C6 allows researchers to add test compounds to a running sample and monitor the effects continuously. The resulting data offers insight into cellular responses that can occur within nanoseconds and would be missed by other methods.

Using the continuous-flow method, flow cytometry can provide an extremely sensitive view into cellular dynamics. For example, in a separate experiment, we analyzed changes in cell size and calcium fluorescence across several time segments (Figure 9).[4] A Fluo-4 plot showed a marked increase in Ca^{2+} concentration in the R5 time gate (top center panel). By time gate R6 (bottom center panel), roughly a minute later, analysis of forward and side scatter showed a significant increase in both larger cells (R11) and fragments (R10), which gradually decreased toward baseline in time gates R7 and R8.

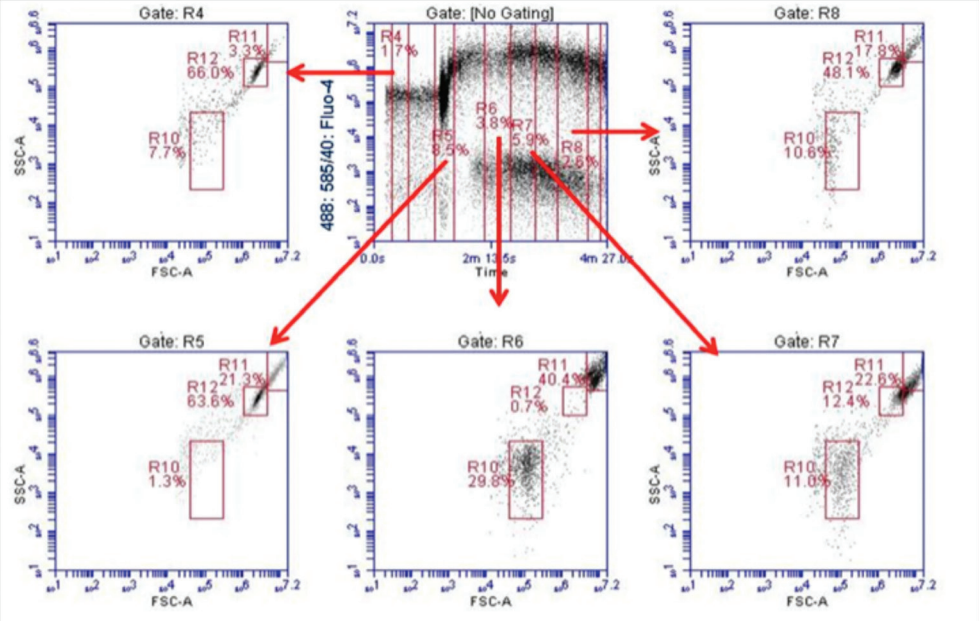

Figure 9. Changes in scatter over time.

A Fluo-4 fluorescence plot over time (top center) shows an increase in calcium signal. Scatter plots of the same sample, gated at different time points, show the effects on FSC and SSC. Both larger cells (R11) and fragments (R10) increased after the calcium spike. Data from Vines et al, 2010, reproduced courtesy of the authors and Cytometry Part A.

The ability of BD Accuri software to zoom in on particular data regions or time frames multiplies the sensitivity of the continuous-flow method. The Zoom tool's usefulness in focusing on a narrow time frame is demonstrated in Figure 8.

Although not shown in the current experiments, the BD Accuri C6 sets no effective limit on the amount of time a sample can be monitored. Sample and test compounds could theoretically be added to an Eppendorf tube and analyzed over a continuous period, until the event capacity of a data well (one million events) is reached.

The continuous-flow method makes the BD Accuri C6 an affordable, easy-to-use tool for monitoring the responses of live cells to a variety of stimuli. By recording the response of the entire cell population, researchers can identify subpopulations of cells that respond differently than the main population. This can be extremely useful in experiments in which different cell types, or even different cells within the same population, may respond differently to the same stimulus.[8,9]

The method also improves data accuracy, since any dead or fragmented cells can be identified and removed from the population (Figures 4, 6, and 9). Alternatively, their responses to the stimuli can be determined independently.

Finally, use of the continuous-flow method on the BD Accuri C6 could be applied to many other research areas that routinely measure dynamic live cell response, such as pH, reactive oxygen and nitrogen species, mitochondrial membrane potential, and nanoparticle uptake.

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