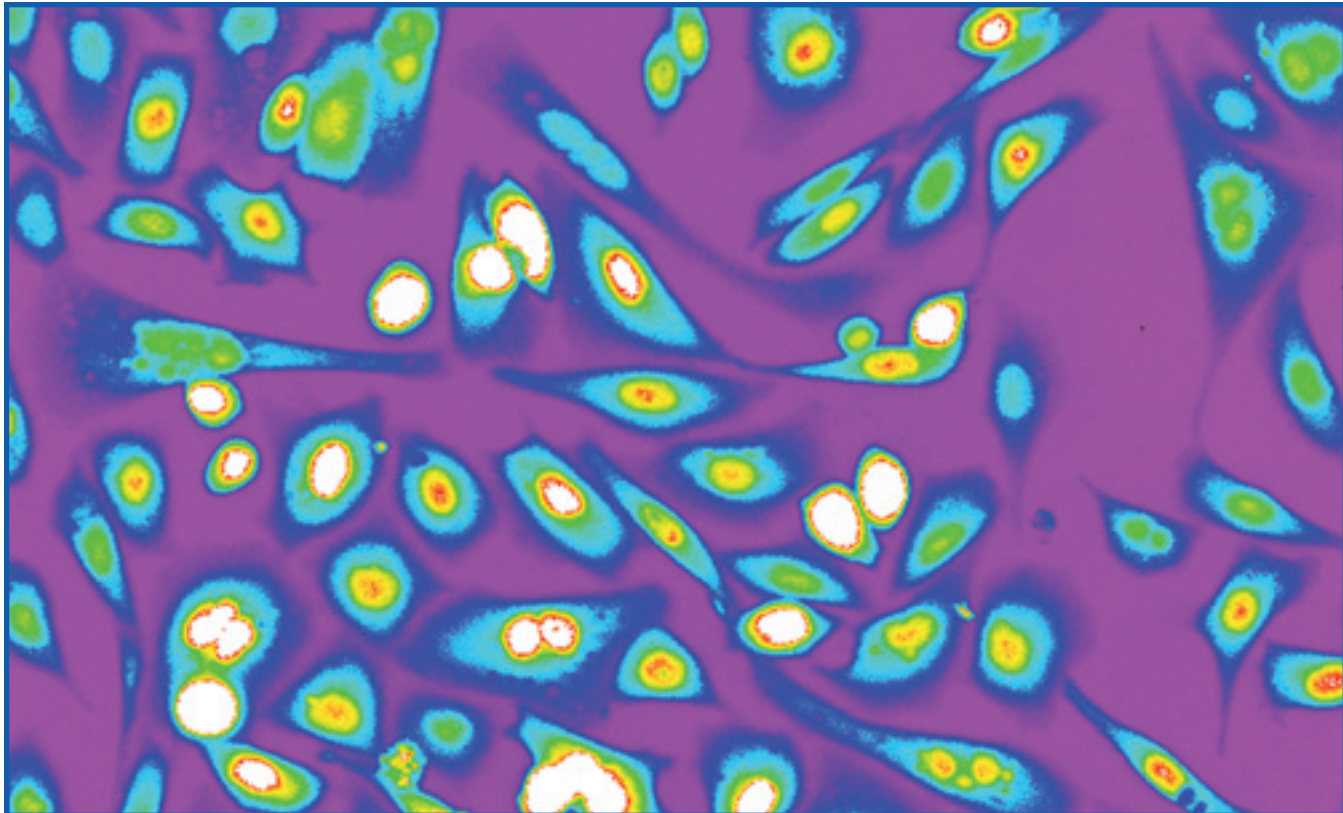


BD Biosciences

Measurement of $[Ca^{2+}]_c$ and Data Classification in ATP-Stimulated Chinese Hamster Ovary (CHO-K1) Cells



Introduction

Analysis of temporal changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) belongs to the most important methods in drug discovery, including G-protein research (Kiselyov *et al.*, 2003), receptor activation, and apoptosis studies. Unlike other second messengers, changes in $[Ca^{2+}]_c$ are often short-lived, show high signal/noise, and are often referred to as the “molecular switch” of a cell in organisms as diverse as nematodes (Mathews *et al.*, 2003), humans and plants (Scrase-Field & Knight, 2003). The ability to measure the temporal aspects of $[Ca^{2+}]_c$ has greatly enhanced the knowledge of signal transduction mechanisms. In drug

research, the kinetic measurement of Ca^{2+} responses is widely performed with plate-reader type systems. These systems make it possible to read multiple wells at the same time and excel in throughput. However, they do not visualize single cells due to their low resolution and hence assume that the cellular population is uniform and shows homogeneous responses. This is not necessarily the case, as shown in many studies from cell cultures (e.g. Suzuki *et al.*, 1991) to isolated tissue (e.g. Ruehlmann *et al.*, 2000). Only microscopy allows the investigation of individual cells but this technique has traditionally had throughput limitations.

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BD Bioscience has developed the BD Pathway™ Bioimager, a sophisticated automated cellular imaging platform for advanced academic research and assay development. The BD Pathway Bioimager uses lamp excitation and can take advantage of the entire visible, near-UV, and near-infrared spectrum for excitation of multiple probes. The system's proprietary confocal disk can be switched into and out of the light path by the user in order to accommodate virtually all imaging needs.

Unlike other systems, the BD Pathway Bioimager moves the objective underneath the plate, thus ensuring optimal sample stability. Loosely attached cells can be imaged as well as those that ordinarily would respond to mechanical stress with an increase in $[Ca^{2+}]_c$ even in absence of a pharmacological stimulus (for example, Arora *et al.*, 1994).

Aim

The aim of this experiment was to investigate the effects of ATP on the Ca^{2+} signaling properties of Chinese Hamster Ovary (CHO) cells and to illustrate the versatility of the BD Pathway Bioimager system.

Methods

Cell Culture

Chinese Hamster Ovary cells (CHO-K1, American Type Culture Collection, CCL-61) were grown and maintained in Minimum Essential Medium, Eagle's, Dulbecco's Modification (DMEM, Biofluids, P104G) containing 10% Fetal Bovine Serum (Gibco, 26140-079), 1% Penicillin/Streptomycin (Biofluids, 303), 1% Non-Essential Amino Acids solution (Biofluids, P332). For experimentation, 7000 cells/well were seeded into 96 well plates (Costar, 3614) and grown overnight at 37°C, 5% CO₂/95% Air.

Dye Loading

Cells were loaded with 2 μM Fluo4/AM and probenecid (1.25mM in 5mM HEPES) (Molecular Probes, F14201) for 30 minutes at 37°C. Cells were washed with HBSS (Hanks Buffered Saline Solution, pH 7.2) to remove excess dye and left to sit for another 10 minutes at room temperature to complete de-esterification of the dye.

These experiments were performed without the use of a nuclear dye.

Imaging

Plates were moved into the environmental chamber of the BD Pathway 855 Bioimager. The drug treatment plate was placed into its holder where it was accessible to the three-dimensional automated dispenser. The dispenser uses disposable tips obviating the need for a wash station and allows the addition of the drug directly above the imaging station and during a time course, without having to move the sample plate. Drugs were added as a bolus in non-contact mode. Although the system is capable of active mixing, it was found unnecessary in this experiment (data not shown). Cells were imaged at 37°C and under 5% CO₂/95% Air.

Unlike some systems, the BD Pathway Bioimager does not have an extra dispensing station and can acquire images while drugs are being added to the well. This is especially effective for fast Ca^{2+} experiments, since moving the plate back and forth from an imaging position to a dispensing position introduces an unacceptable time delay.

The cells were imaged in non-confocal configuration using a 20X U-Apo 340 Objective (Olympus, NA 0.75). Images were binned 2x2.

Images were acquired at 0.69Hz. After the fifth image, the automated, on-board dispenser was programmed to add 10μL of 1mM ATP to the well containing 90μL buffer, which resulted in a final well concentration of 100μM ATP.

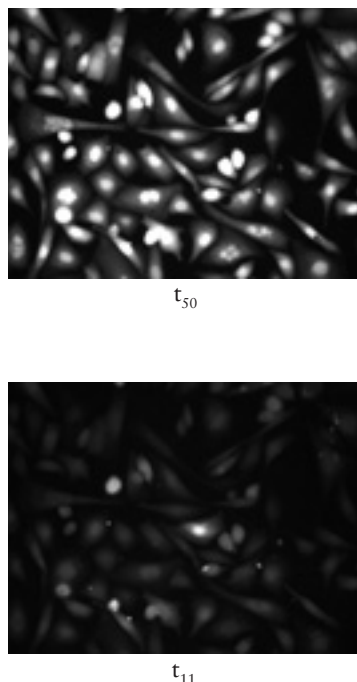


Figure 1 ATP-induced Increase in $[Ca^{2+}]_c$ in CHO-K1 Cells

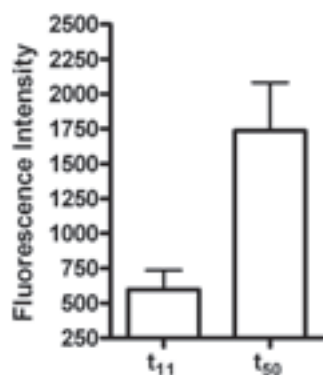


Figure 2 Increase in $[Ca^{2+}]_c$ in Response to ATP in CHO-K1 Cells: Average of all cells in the field of view at t_{11} and at t_{50}

Optimal dispense rate parameters were previously established (data not shown). Images were acquired using Hg lamp illumination and filters appropriate for Fluo-4. Due to the high sensitivity of the Orca ER (Hamamatsu) camera at Gain 100 and the large quantum yield of Fluo-4, an ND1 (10% transmission rate) filter was used to attenuate the excitation intensity. Images were captured with 80ms exposure times and every third image was saved as a 16 bit Tagged Image File Format (.tif) file.

The real-time image acquisition and analysis environment of AttoVision allows image analysis on the fly, so images are saved only as required. For example, in this experiment, to reduce storage requirements, only every third image was saved.

The proportion of images saved per experimental run is fully user-adjustable. If required, the system can save every image for later re-analysis or can save none if storage space is low. In this case, the system only saves the data associated with the Regions of Interest (ROI) which are adjusted prior to the experiment.

All imaging options can be saved in macro-form and re-used as required.

Data Analysis

Data was analyzed using the AttoVision proprietary software for kinetic imaging. The intensity values of each ROI and some associated morphological information were saved into a tab-delimited text file and opened, and analyzed in MicrosoftTM ExcelTM.

Image Preparation

Images are generated in standard 16 bit .tif. For display purposes, images were exported in 24 (3x8) bit format, split by channel and cropped for display. All display preparation was performed in Paint Shop Pro (Jasc Software Inc, MN, USA).

Results

Calcium Signals

CHO-K1 cells showed a robust increase in $[Ca^{2+}]_c$ in response to ATP. Especially striking is the strong nuclear signal compared to that of the cytoplasm. This confirms data from Berridge's group (Thomas *et al.*, 2000) who compared multiple ratio metric and non-ratiometric Ca^{2+} probes. Their findings show that different dyes load into different compartments within the cells. Fluo4 and Fluo3 loaded more into the nuclear region of the cells than Fura2. Although the reason for this is not clear, the non-ratiometric dyes appear to indicate a greater increase in $[Ca^{2+}]_c$ inside the nucleus than in the cytoplasm.

As Figure 1 shows, virtually all of the cells responded to the addition of ATP (100 μ M at t_{21}) with a significant increase in $[Ca^{2+}]_c$. The average fluorescence intensity of the cells shows that the response is highly significant ($p < 0.0005$, $n=58$ cells, mean \pm S.D., as illustrated in Figure 2.) Note that the data shown contains ~200 dark-noise levels.

Average Cellular Fluorescence Intensity

Plate-reader type systems like the FLIPR (Molecular Devices) or the FDSS (Hamamatsu) system excel in their throughput of kinetic $[Ca^{2+}]_c$ experiments and are popular in drug discovery application.

The BD Pathway™ Bioimager can serve as an assay development station for these systems due to its ability to report the average fluorescence intensity of all cells in the field of view (Figure 3).

As discussed above, cellular Ca^{2+} responses can be highly heterogeneous and an analysis of each cell's response illustrates this point. Plate-reader type instruments, however, can not resolve heterogeneity and rely on a homogeneous cell population. In drug screening, weak drug effects often manifest themselves only in discrete sub-populations of cells and an “averaging” approach will reduce their signal/noise, or, in extreme cases will miss the response altogether.

When the average cellular intensity is plotted over the time course as in Figure 3, but this time with error bars indicating the S.D., the curve appears to be quite heterogeneous, especially at the time of drug addition. The traces shown are that of a robust signal change (100 μ M ATP) but still, to detect a putative inhibitor under those noise conditions may be challenging on a plate-reader type system.

Data Classification

AttoVision is able to visualize the source of this heterogeneous response. The screenshot in Figure 5 shows the time course of the individual cells' response on the right, and the end-point image on the left. Although virtually all the cells showed a robust rise in $[Ca^{2+}]_c$ by the end of the experiment (left panel), a number of cells in the field seemed to have “fired” prematurely, that is, before the addition of drug at t_{20} s (see traces on the right).

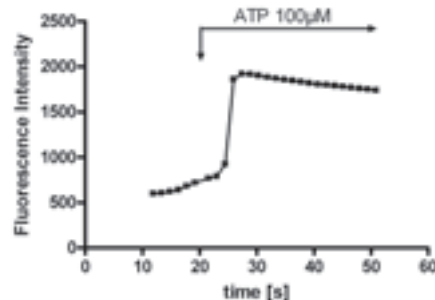


Figure 3 Average Ca^{2+} Response of CHO-K1 Cells to ATP: Shown is the average fluorescence intensity over time of 58 cells.

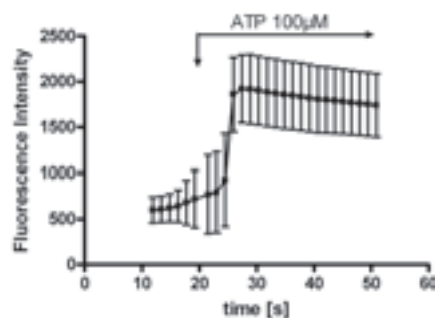


Figure 4 Variability of the Average Ca^{2+} Response of CHO-K1 Cells to ATP

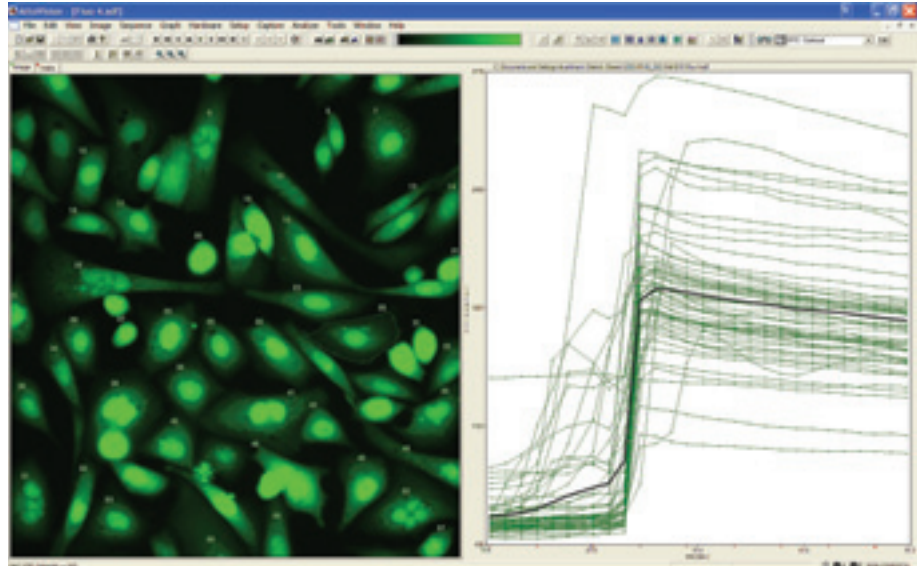


Figure 5 Screenshot of AttoVision: Variability of the average Ca^{2+} response of CHO-K1 cells to ATP

This premature activity may reflect truly spontaneous activity (such as oscillations) or reactive oxygen-species (ROS) induced Ca^{2+} influx (Bielefeldt *et al.* 1997) or store release (Granville *et al.* 2001). Although the reasons for this behavior are intriguing, for the purpose of this experiment, prematurely firing cells represent a distinct subgroup of cells that can be disregarded since they do not participate at all in the actual drug response.

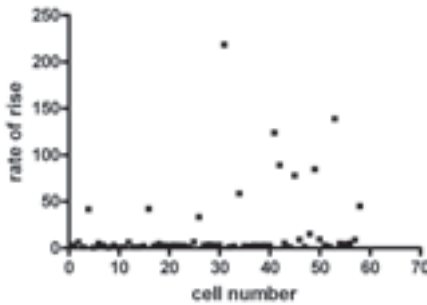


Figure 6 Scatter Plot of the “Rate of Rise” Values of all the Cells in Field of View

AttoVision allows in-depth kinetic analysis of the Ca^{2+} response. To this effect, the time course is partitioned into discrete “treatment zones” (TZ). The first TZ was set from the beginning of the experiment to the time of drug addition ($\sim t_{21}$ s). This TZ contains seven data points which can be analyzed separately from the rest of the experiment. The cells that show premature activity can be identified by their increased Rate of Rise of their $[\text{Ca}^{2+}]_c$ over this period (Figure 6).

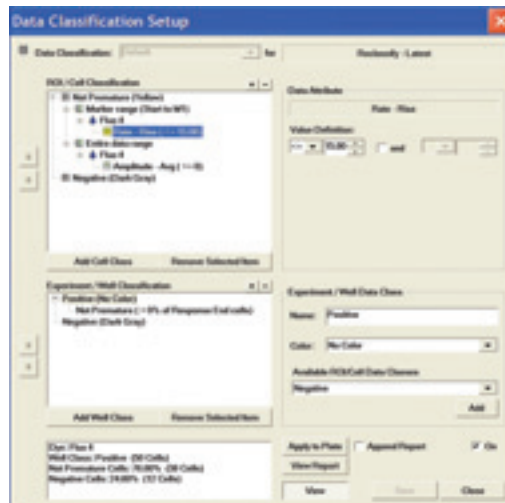


Figure 7 Screenshot of the AttoVision Data Classification Setup Menu.

AttoVision allows the specific exclusion of these cells from the analysis using a graphical user interface. In this example, the rate of rise for TZ1 was set to ≤ 15 allowing for some moderate rise but excluding cells that truly show a response (Figure 7).

The effect of this exclusion is shown in Figure 8. Cells that are retained in the analysis are depicted in green, both in the ROI outline and the traces on the right. Rejected cells are shown in grey.

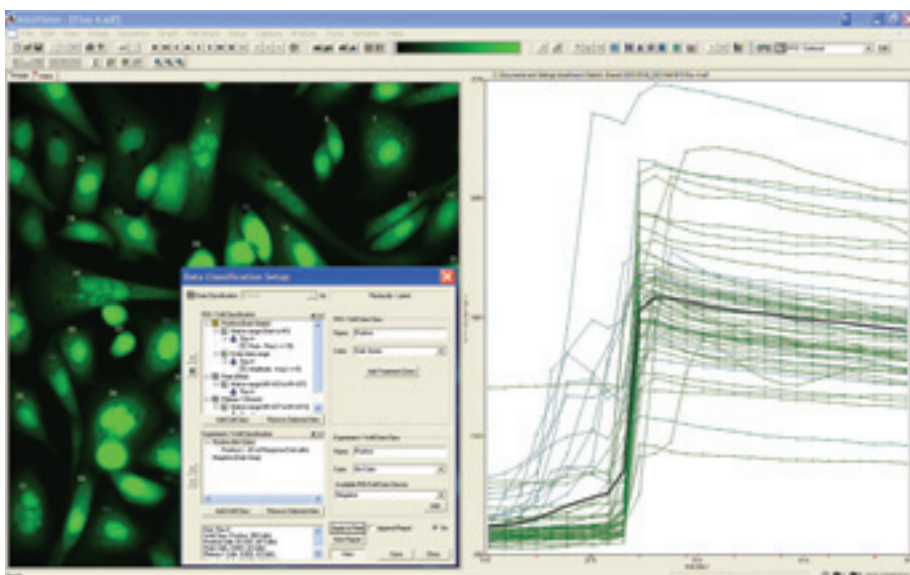


Figure 8 Screenshot of Classified Data

When the so classified data is again plotted as a mean \pm S.D., the resulting figure shows the impact of this calculation (Figure 9).

The effect of the data classification is especially noticeable when compared to Figure 3 and Figure 4. Elimination of prematurely “firing” cells resulted in a more discrete response by the cells on average, basically increasing the usable assay window of the experiment. The remaining high heterogeneity of the $[Ca^{2+}]_c$ plateau is of great interest when discrete subgroups of cells need to be identified. The presented data classification steps did not impact on this data and hence do not skew the results.

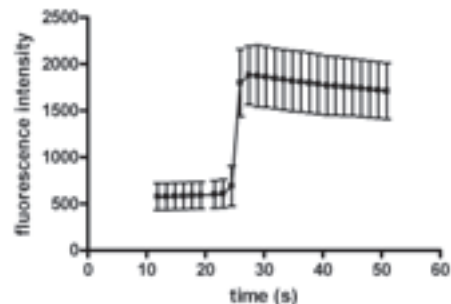


Figure 9 Time Course Data of $[Ca^{2+}]_c$ after Data Classification

Discussion

Fluo-4 - loaded CHO-K1 cells respond with a pronounced increase in $[Ca^{2+}]_c$ when challenged with ATP at 100 μ M. In accordance with published reports, the response is especially noticeable in the nuclear region of the cells due to Fluo-4's chemical properties.

Although the cells are derived from a clonal line, they show a very heterogeneous increase in $[Ca^{2+}]_c$. Again, this is consistent with published reports showing that both cultured cells and cells and organs derived from animals can exhibit very different response patterns to a single agonist. Although not investigated here, the reason could be differential expression of receptors, their coupling efficiency, second messenger cascade proteins (e.g. IP3 channels), or the morphology of the cell (that is, physical size and position of the superficial buffer barrier [Poburko et al. 2004]).

The current experiment was designed to show the flexibility of AttoVision and its data classification routine. As an example, cells were excluded from the analysis if they showed a significant rise of $[Ca^{2+}]_c$ prior to drug addition. Other scenarios of sub-population analysis can be envisaged and the software introduces virtually no limits on the user to differentiate cells either by kinetic or non-kinetic parameters. Although the current data set was a single color experiment, multispectral acquisition allows the classification across several fluorescence markers. For example, a nuclear dye could generate information on apoptosis or cell cycle phase. Those parameters can be multiplexed with the rise in $[Ca^{2+}]_c$ to generate a truly multidimensional data landscape. To aide these complex calculations, BD Bioscience has introduced the BD Image Data Explorer, a sophisticated Add-In for Microsoft™ Excel™.

Although cultured cells show heterogeneity, the real challenge lays in primary cells, transiently transfected or RNA_i knock-down cells. Here, the cell population is by definition heterogeneous and any attempt to analyze kinetic data using a plate-reader type instrument will generate sub-optimal results.

The BD Pathway™ Bioimager with AttoVision and the data analysis suite BD Image Data Explorer are designed for this growing field in biomedical and pharmaceutical research.

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