Flow Cytometry Analysis of Mitotic Index Data from the IsoCyte™ DL Laser Scanning Cytometer

Michael Sjaastad, Jayne Hesley, and Jestine Ho
Molecular Devices, Inc., Sunnyvale, CA

Abstract

Flow cytometry has been a well adopted technology for monitoring multi-parametric phenotypes of single cells in laboratory research and development since its introduction in 1968. The principle of flow cytometry uses a beam of light (usually laser light) of a single wavelength directed onto a hydro-dynamically focused stream of fluid to quantitate components or structural features of fluorescently tagged cells. Fluorescent antibodies are often used to report the densities of specific surface receptors, and thus to distinguish subpopulations of differentiated cell types, including cells expressing a transgene. The main limitations of flow cytometry in high throughput screening are the requirements to use a large number of cells, cumbersome cell preparation, and slow data acquisition. When acquiring data in flow cytometry, millions of cells must be available to perform one 96 well plate experiment. Since flow cytometers acquire data one well at a time, a 96-well microplate takes over 30 minute to read. Here, we present a novel technology that uses laser illumination of cells and sensitive fluorescence detection to achieve final, analyzed results from an entire microplate in less than 5 minutes. The IsoCyte™ Dual Laser Scanning Cytometer is a high throughput platform for screening of cells in 6-1536 well microplates using fluorescence measurements.

Introduction

Cells were cultured in a 96-well microplate, fixed, stained and scanned. Paclitaxel (Taxol™), a compound that prevents mitotic spindle assembly resulting in a late G2/M block, was used to perturb mitosis. Within each well, the cells in mitosis (mitotic index) were specifically labeled using a primary antibody to detect the phosphorylation of Ser10 of histone H3 (phospho-H3). A Chromeon-488 (Ch-488) labeled secondary antibody was bound to the primary antibody attached to cells arrested in mitosis. Propidium Iodide (PI) was used to identify all nuclei for normalizing results to cell count within a well.

The IsoCyte™ DL was configured with 488 nm and 640 nm lasers although only the 488 nm excitation was used for this assay. All cells within each well are imaged in the green and red emission channels and analyzed simultaneously in this validated assay. Final results are presented within 4 minutes/plate (regardless of well density). In addition to fast acquisition, the IsoCyte software can export data to flow cytometry standard (fcs, version 3.0) format. The principal goal of the flow cytometry standard is to provide a uniform file format, allowing files created by one type of acquisition hardware and software to be analyzed by another type (*Cytometry*, 1990) of software.

The IsoCyte™ DL Laser Scanning Cytometer

Materials and Methods

- U2OS (adherent human osteosarcoma) cells
- Mitotic Index Assay Kit - fluorescent (Active Motif P/N 18020) containing paclitaxel, primary antibody against phosphohistone-H3, Chromeo-488 labeled secondary antibody, propidium iodide, and RNAse A
- MEM culture media + 10% FBS and Penicillin
- Clear-bottom black 96 well microplates (BD Falcon P/N 353948)
- IsoCyte DL Laser Scanning Cytometer (Molecular Devices, Inc.)

U2OS cells were seeded at 2,500 cells/well in 96-well black walled, clear-bottomed polystyrene plates and cultured overnight in MEM supplemented with 10% FBS overnight. The medium was removed and wells were treated with 50 uL of a paclitaxel dilution series for 20 hours of exposure at 37°C in a 5% CO2 incubator. After paclitaxel treatment, the cells were fixed and permeabilized with ice cold methanol, immunostained with Chromeon-488 labeled anti-phosphohistone-3, and, finally, the nuclei were stained by PI/RNAse A treatment. A wash step is unnecessary prior to scanning the plate due to IsoCyte's confined detection region. The IsoCyte was set up using the 488 nm excitation laser and 2-channel acquisition with the following emission filters: Ch1: 510-540nm (green) and Ch3: 600nm long pass (red). The image acquisition was done at 5 x 5 micron resolution. Whole well images were processed to identify all cells in the red channel. IsoCyte's image processing software utilizes standard thresholding algorithms for separating cells from background as well as using particle filters such as area, intensity, and splitting functions. Data was saved as a whole plate file for viewing images in IsoCyte software and a flow cytometry standard (fcs) file was generated for each well. The fcs files were imported into a flow cytometry analysis software package from De Novo software called FCS Express v.3. The ratio of integrated fluorescence intensity for the green and red channels was used to identify populations of mitotic and non-mitotic cells.

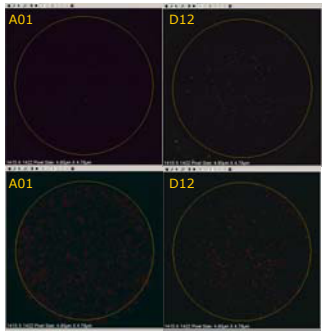

Figure 1. An example of whole well images. Few of the untreated cells on the left are labeled with green Ch-488 as compared with paclitaxel treated cells on the right. The total number of cells in the well is determined by the red PI stained nuclei. (note: region of interest is chosen to be slightly smaller than the actual well diameter)

Results

Data imported from fcs files into flow cytometry software was plotted as 2D histograms, or dot plots, of red intensity vs. green intensity. Objects were binned as mitotic or not by applying a floating quadrant. Mitotic cells were in the top right quadrant and non-mitotic were in top left.

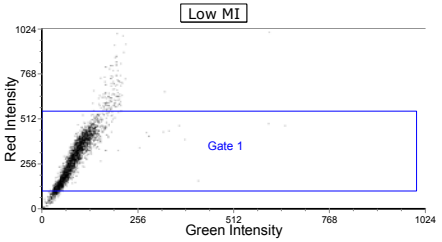

Figure 2. FCS data was analyzed in FCS Express by De Novo software. A simple rectangular gate was first applied to omit outliers from analysis.

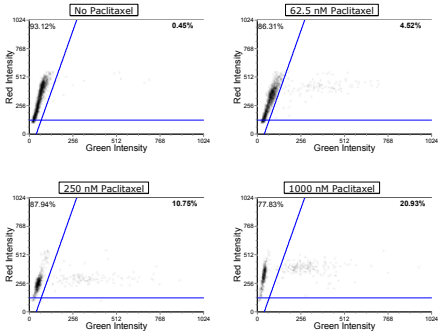

Figure 3. Dot plots from cells treated with various levels of Paclitaxel, which arrests cells in late stage mitosis, can be compared. Mitotic cells fall in the top right quadrant (positive for red and green).

After 20 hours with Paclitaxel treatment, cell death was noted at the higher doses of drug. Dead cells detach and are lost in wash steps, resulting in fewer total cells imaged. An accurate measure of cells in mitosis was accomplished by normalization via percentage calculation based on the total cell count, determined by PI staining of the nuclei.

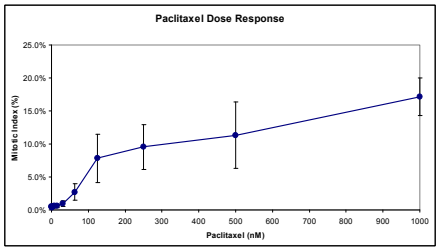

Figure 4. 20 hour Paclitaxel dose response in U2OS cells as determined using IsoCyte for acquisition and image processing and FCS Express for data analysis.

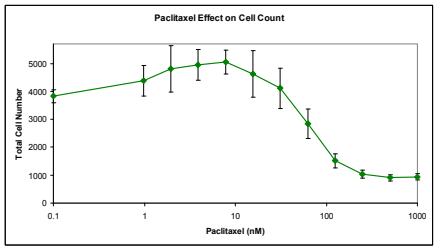

Figure 5. Wells with no or low concentration of Paclitaxel contained greater than 4,000 cells/well (n=6 at each concentration). Starting at 62.5 nM, Paclitaxel had an increasingly toxic effect. Roughly 1000 cells were still measured at the highest doses.

Summary

This study shows that the IsoCyte™ DL Laser Scanning Cytometer acquires quality cell based data from microplates and saves it in output files compatible with flow cytometry analysis software in <5 minutes. Importantly, scan time is independent of plate format, significantly increasing potential throughput of cytometry analysis. The whole well and cell-by-cell analysis normalizes measurement to total cell count and compensates for drug-induced loss of cells. The IsoCyte™ laser scanning technology is suitable for primary screening of cell-based assays. The IsoCyte DL enables streamlined assay development and a high-speed acquisition option for cytometry. The ability to export data to fcs format provides ease of use and a solution for comparing cell-based assays to traditional flow cytometry. The IsoCyte has been integrated with several robotic systems providing a complete solution for a variety of high throughput cell-based assays for research and drug screening.

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