

High-Throughput Two-Color Mitotic Index Assay

Mitotic Count (Validated Assay)

Blueshift Biotechnologies Inc. (BBI) has developed a powerful new screening platform, the **IsoCyte™**, for high throughput multiparametric screening of cells in multi-well plates using laser scatter and fluorescence measurements. Multiparametric analysis of adherent cells has become increasingly important to researchers. BBI has been working closely with the research community to develop a platform with integrated image acquisition and analysis modules with the flexibility important for developing, running, and validating image analysis procedures to enable high throughput assays. The first validated assay co-developed with Active Motif (Carlsbad, CA) was the ToxCOUNT™ Cell Viability Assay kit; a homogeneous cytotoxicity assay. Here we report on our development of a fast and simple mitotic index assay using automated image acquisition and analysis where the mitotic cells are identified by the immunofluorescent detection of phosphorylated histone H3 and DNA content.

Assay Procedure

Cells and Culture Conditions. HeLa cells were cultured in MEM supplemented with 10% FCS. Cells were plated in 96-well, black walled, clear-bottomed polystyrene plates at a density of 2,500 cells/well in 50 μ l of growth medium and incubated overnight at 37°C in a 5% CO₂ incubator. The growth medium was removed and the cells were treated with various concentrations of paclitaxel in 50 μ l of complete medium.

Detection of Mitotic cells by IsoCyte™. After paclitaxel treatment, the cells were fixed, permeabilized and immunostained with a mitotic marker antibody directed against the phosphorylation of Ser10 of histone H3 (phospho-H3). The rabbit anti-phospho histone H3 antibody was a gift from Millipore (Upstate Cat # 06-570). An anti-rabbit Alexa Fluor-488 (AF488, Invitrogen) labeled secondary antibody detected the primary antibody and propidium iodide (PI) was used to identify all nuclei. Whole well images were analyzed on a cell-by-cell basis by identifying cells in the red channel and using the background corrected integrated fluorescence intensity for the green and red channels. The **IsoCyte™** laser scanning platform was setup for 2-channel acquisition with the following band pass filters: Ch1: 510-540nm (green) and Ch3: 600nm long pass (red). The image acquisition was done at 5 x 5 micron sampling.

Results & Discussion

4 h Paclitaxel Treatment

Whole well image results for phospho-H3 (PH3) and PI staining of cells treated for 4 hours with 1 μ M paclitaxel are shown below in **Figure 1**. Image acquisition and automatic mitotic cell quantification by two-color image processing is illustrated.

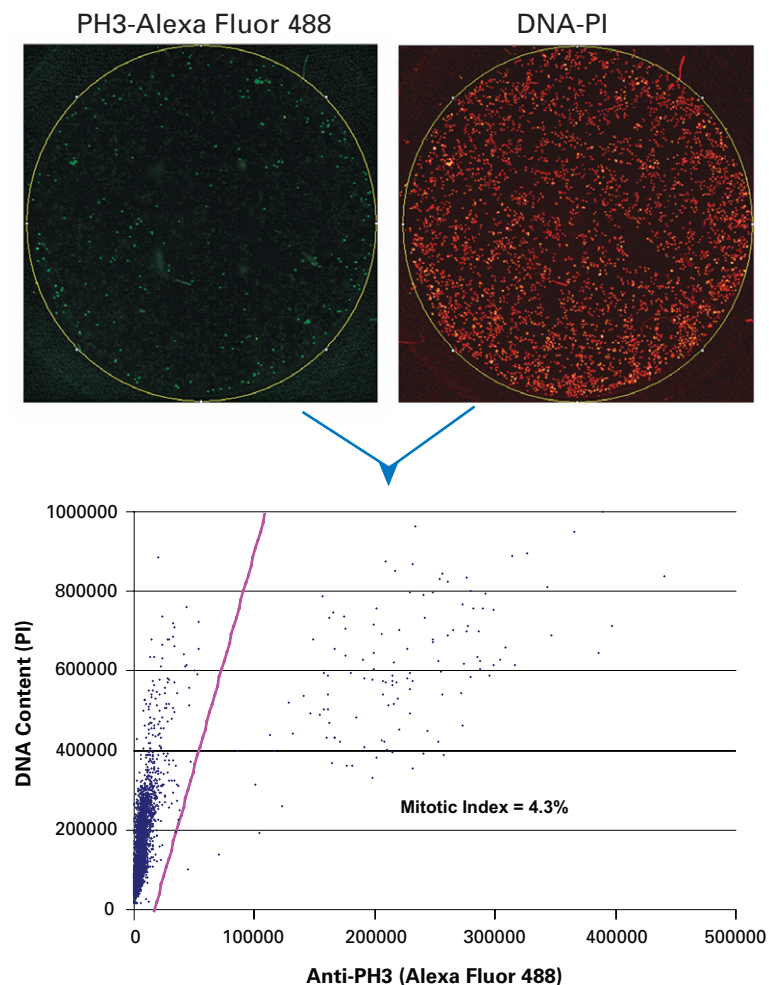


Figure 1. Whole well images of cells treated for 4 hours with 1 μ M paclitaxel. The images are anti phospho-H3 (anti-PH3) stained with AF488 (left) and the nuclear stain PI (right). The bottom 2-parameter dot plot shows anti-PH3 integrated intensity (Ch3) on the x-axis versus the DNA content integrated intensity (Ch1) on the y-axis. The purple dividing line represents the Ch1/Ch3 ratio with an adjustable x-axis intercept that defines the settings used for automatic mitotic index quantitation. The mitotic index (MI) for the sample shown was 4.3%. The MI for control samples versus paclitaxel treated was 0.9 ± 0.2 and 3.4 ± 0.8 percent, respectively (n = 4).

17 h Paclitaxel Treatment

Results for PH3 and PI staining of cells treated for 17 hours with the indicated concentrations of paclitaxel are shown below. Image acquisition and automatic MI quantification by two-color image processing generated the results shown in **Figure 2**. Paclitaxel increased MI in a dose-dependent fashion as expected, with a demonstrated maximum effect. As shown in **Figure 3** the effect of paclitaxel treatment on the cell number demonstrated a reduced number of cells left in the wells. A lower number of cells is believed to contribute to the higher MI error bars observed for the high paclitaxel concentrations (**Figure 2**). The image acquisition was done at 5 x 5 micron sampling with a total scan and analysis time of 4 minutes.

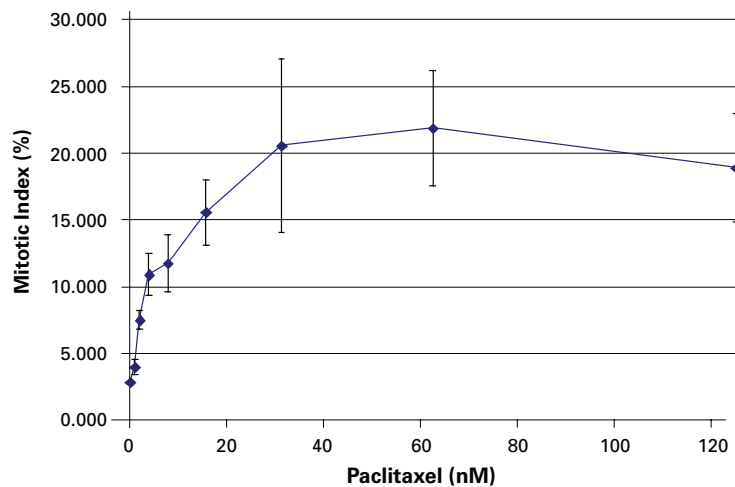


Figure 2. Paclitaxel treatment for 17 hours resulted in a concentration-dependent increase in the number of mitotic cells. Each data point represents n = 4 wells.

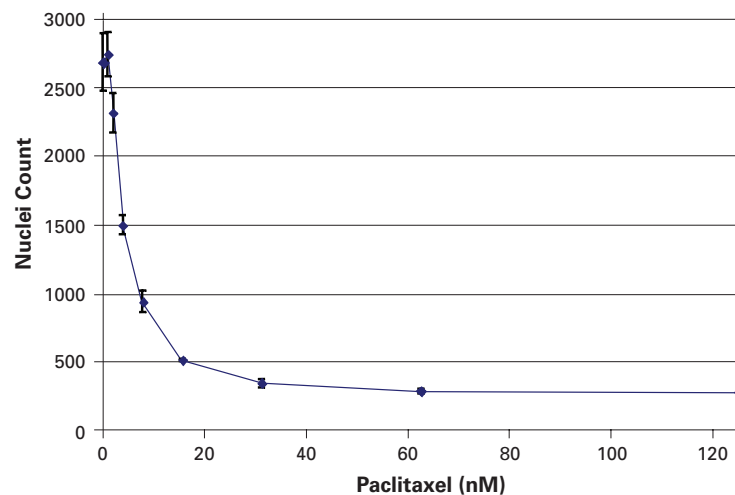


Figure 3. Paclitaxel treatment for 17 hours resulted in a concentration-dependent decrease in the number of cells. Each data point represents n = 4 wells.

17 h Paclitaxel Treatment and Cell Cycle Histograms

Histograms of the fluorescence distribution of the PI stained cells using the Ch3 (PI) integrated intensity were generated using an Excel macro developed by BBI. Results for PI staining of cells treated for 17 hours with the indicated concentrations of paclitaxel are shown in **Figure 4**.

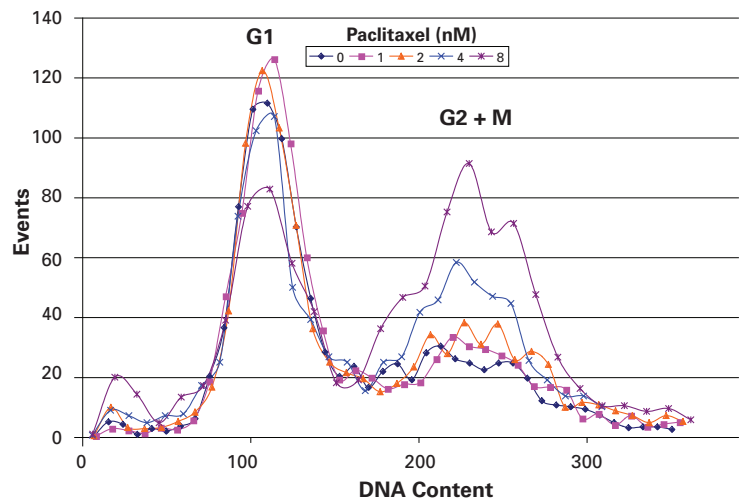


Figure 4. Paclitaxel treatment for 17 hours results in a concentration-dependent increase in the number of mitotic cells and decrease in the number of G1 cells. The DNA content measurement was normalized by setting the G1 peak at approximately 100.

Conclusions

In this report we have shown how the **IsoCyte™** laser scanning platform with its unique optics and electronics has enabled a high throughput 2-color assay for MI quantitation. Paclitaxel (Taxol™) was used as a model drug compound resulting in a late G2/M block and increased MI after 4 and 17 h treatments. Image analysis occurs concurrently with scanning and the results can be viewed immediately afterward. The results are saved as a list file (.csv) enumerating cell-by-cell data for each well and as a summary text file showing the percent mitotic, non-mitotic, number of mitotic and non-mitotic cells in a plate layout format. Each 96-well plate is scanned in 4 minutes at 5 x 5 micron sampling or 2 minutes at 10 x 10 sampling. Significant features of this platform for advancing this assay are:

- Designed to run existing MI assays in a simple high throughput fashion
- Collection optics with a confined detection region that reduces fluorescence background and provides ability to convert this assay to a single step and homogeneous non-wash format
- Whole well and cell-by-cell analysis normalizes measurement to total cell count and compensates for drug-induced loss of cells
- DNA content is measured at the same time allowing for cell-cycle analysis
- The platform is available with an integrated plate handler (Twister II) for walk-away operation (up to 160 plates)

Blueshift Biotechnologies Inc.

245 Santa Ana Court

Sunnyvale, CA 94085

Ph 408.773.1050 | Fax 408.773.1164