



Analysis of Proliferation and Apoptosis Using the EIDAQ™ 100 High Throughput Microscopy System

This application note describes the measurement of proliferation and apoptosis on the EIDAQ™ 100 High Throughput Microscopy (HTM) system from Q3DM. Quantitative fluorescence and in-focus images enable accurate nuclear boundaries to be defined. Combined with Q3DM's proprietary dynamic image segmentation, this leads to precise delineation of distinct cell cycle phases and apoptosis from a simple DNA stain. For accurate reading/measurement, the EIDAQ™ 100 automatically images populations of cells, defines cell morphology and measures the amount of specific fluorescent signal in each cell. The EIDAQ™ 100 is a new High Throughput Microscopy system that delivers accurate, quantitative, imaging, and analysis of cell populations, at high speeds, directly from slides or microtiter plates.

Background and Significance

The rates of cell renewal and cell death in culture are important assay endpoints in the discovery of candidate drugs for cancer, inflammation, tissue regeneration or degeneration. This includes immune system disorders, wound healing, and neurodegenerative disease. Effective drugs can act by modulating the balance between cell proliferation and apoptosis, a form of programmed cell death. By measuring cell cycle kinetics at the same time as apoptosis, the mechanism of action of candidate drugs can be elucidated, and potential side effects and toxicity can be identified early in the discovery process.

The basis for these assays is the change in DNA content of the cell as well as the organization of the DNA in the cell. The degree of condensation or packing of the DNA into the nucleus and eventually into chromatin fibers and chromosomes can be measured by local fluorescence intensity. The power of image-based DNA fluorometry is that image data can discriminate M from G2 cells based on fluorescence intensity distribution measurements. In contrast, in flow cytometry, M and G2 phase cell are indistinguishable using DNA content as the sole parameter of measurement. CytoShop™ software automatically delineates the boundary of the nucleus and measures the distribution of fluorescence intensity between pixels in that region. The accuracy of this measurement is critically dependent on marking the exact nuclear boundary and quantifying fluorescence intensity. Q3DM core technologies of image segmentation and lamp stabilization enable rapid, reproducible, and highly cost-effective assays for the analysis of cell proliferation and apoptosis.

Cell cycle analysis by nuclear DNA content and condensation

When cells are stimulated to enter the cell cycle, DNA is replicated or synthesized; this is denoted as S phase. The nuclear DNA content gradually increases until it reaches twice the diploid amount and the cell then enters the G2 phase of the cell cycle. At the subsequent onset of mitosis, known as M phase, the replicated DNA has to distribute equally between the two daughter cells. This is achieved by condensing the DNA into chromosomes and moving them to opposite ends of the mitotic spindle. The DNA stains Hoechst 33243 and 4',6'-Diamidino-2-phenylindole (DAPI) bind stoichiometrically to DNA and can therefore be used to quantify cellular DNA content by measuring the integrated intensity of that dye in the nucleus. The DNA

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content of cells in a population is conventionally expressed as a histogram with the integrated fluorescence intensity plotted on a log scale. Curve fitting programs designed for flow cytometry can then be applied to calculate proportions of cells in G1, S and G2 plus M phases.

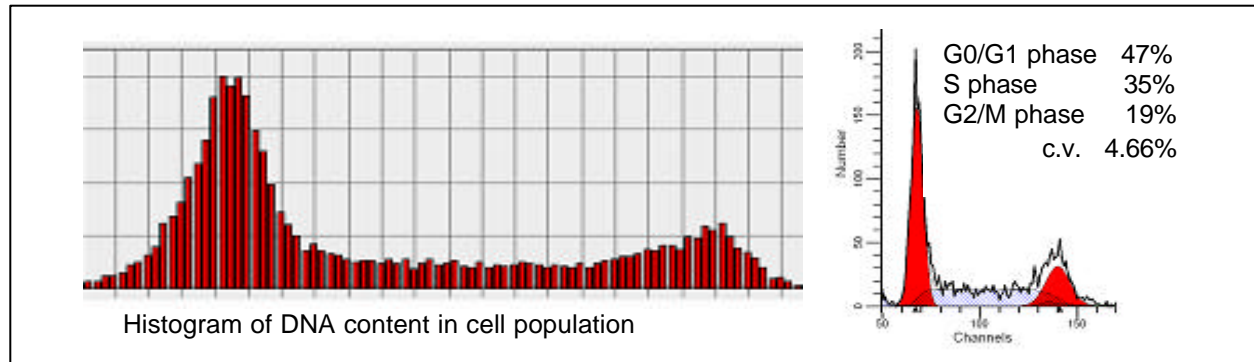


Figure 1. Histogram of DNA content generated using CytoShopTM software using the metric integrated intensity of fluorescence in the nucleus plotted on a log scale on the X axis (left). Cell cycle analysis using Modfit LTTM v3.0, Verity Software House, Topsham, ME (right).

Experimental Results and Automation

Single color images of DAPI stained cells were automatically acquired using the EIDAQTM HTM system and segmented using CytoShopTM software. Using a 20X 0.5NA objective lens, a matrix of 20 by 20 image fields yielded a database with 16,353 individual cells. Each cell had associated pixel intensity data corresponding to some 50 absolute, distribution, or order statistics-based metrics. Figure 2 shows cell montages derived from gates drawn on the scatterplot. On the left, daughter cells with G1 DNA content are distinguished from metaphases and anaphases with G2 DNA content, shown on the right. To validate selection criteria for any "gate" or subpopulation of cells, CytoShopTM software provides a gallery of cell images corresponding to a population defined on any parameter, or multiple parameters. This feature ensures accurate cell cycle and data analysis.

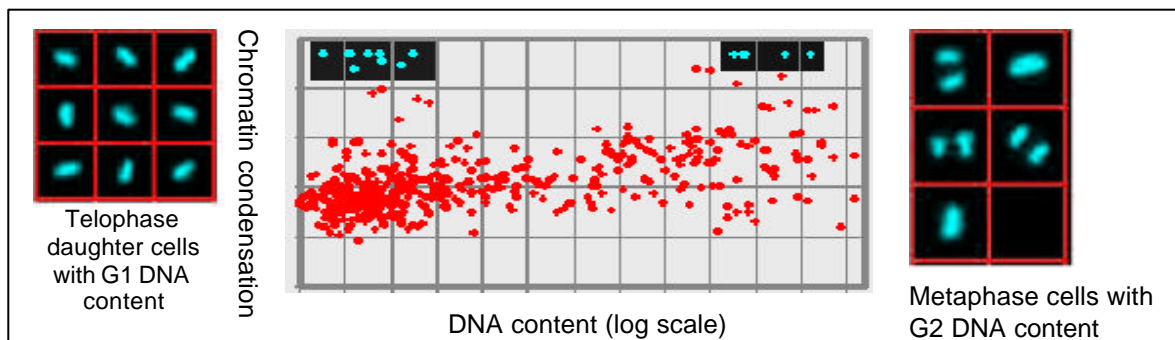


Figure 2. Scatterplot and cell montages corresponding to daughter cells and metaphases generated by CytoShopTM software. The integrated intensity values of all the pixels in the nuclear mask was used as a measure of DNA content and plotted along the X axis. Chromatin condensed

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into chromosomes is detected by high values of the fluorescence intensity at the maximum 5% of pixels for each that nucleus plotted on the Y axis.

Analysis of apoptosis by nuclear DNA content and condensation

When cells undergo apoptosis, the chromatin condenses and the nucleus becomes fragmented, while the plasma membrane stays intact. In some cell types, nuclear fragmentation leads to a sub-G1 DNA content, but in other cell types apoptotic cells retain their DNA, but can be detected by extreme chromatin condensation in shrunken nuclei. Thus a stoichiometric DNA stain can be used with image-based metrics to simultaneously discriminate cell cycle stages and apoptosis. Eventually, apoptotic cells detach from the monolayer and so the cell number decreases.

Experimental Results and Automation

Human carcinoma cell lines A549 (lung), HT-29 (colon), and RKO (colon), along with CMV210 genetically modified mouse fibroblasts grown in a 96-well glass bottomed microtiter plate, were treated with cis-platinum in varying doses and stained with Hoechst 33243 at 1 $\mu\text{g}/\text{ml}$. The EIDAQ™ 100 HTM system imaged 12 images per well using UV excitation and 400nm long pass emission filters. Cell-by-cell databases were constructed for each well through automated image segmentation and fluorescent intensity measurement. The CytoShop™ software enabled straightforward well-by-well image visualization and associated cell-by-cell measurement capabilities.

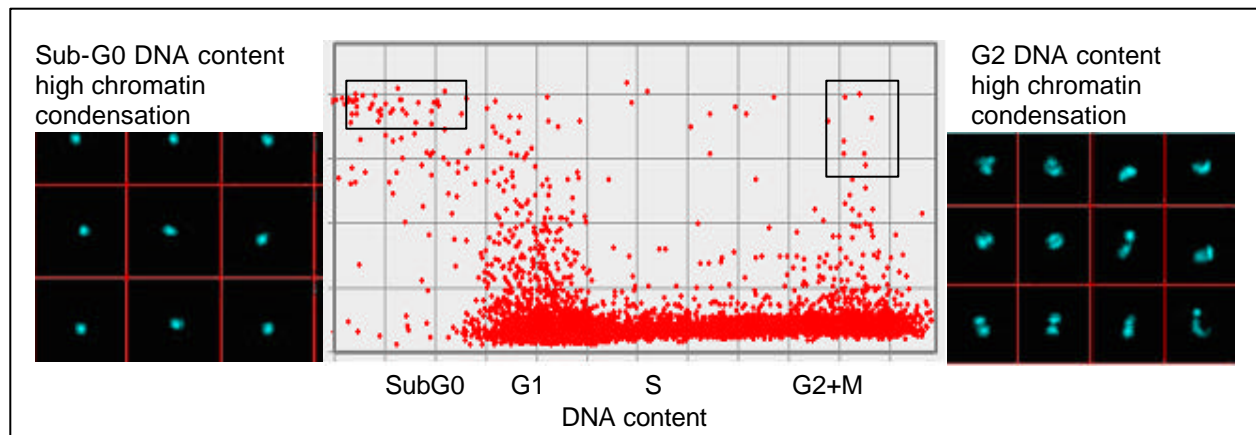


Figure 3. Scatterplot and cell montages corresponding to apoptotic nuclei and metaphase/anaphase. The integrated intensity of the nuclear stain is plotted on the X axis and the fluorescence intensity of the brightest 5% of pixels in the nucleus is plotted on the Y axis. As in Figure 2, this is a measure of chromatin condensation.

From this initial interrogation, “drilling down” from the plate response to the population of cells within an individual well, it was determined that DNA content was a valid metric for measuring apoptosis. The effect of decreasing the dose is clearly evident in the comparison of histograms shown alongside representative image data in Figure 4. The top series of images are from wells treated with 50 μM and the lower series from wells treated with 6 μM cis-platinum. The shift in DNA content to the right indicates less apoptosis and more cells

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entering cycle at the lower dose. These data are supported further by the characteristic appearance of nuclear blebbing of apoptosis at the higher dose and the metaphase plates and anaphases observed at the lower dose.

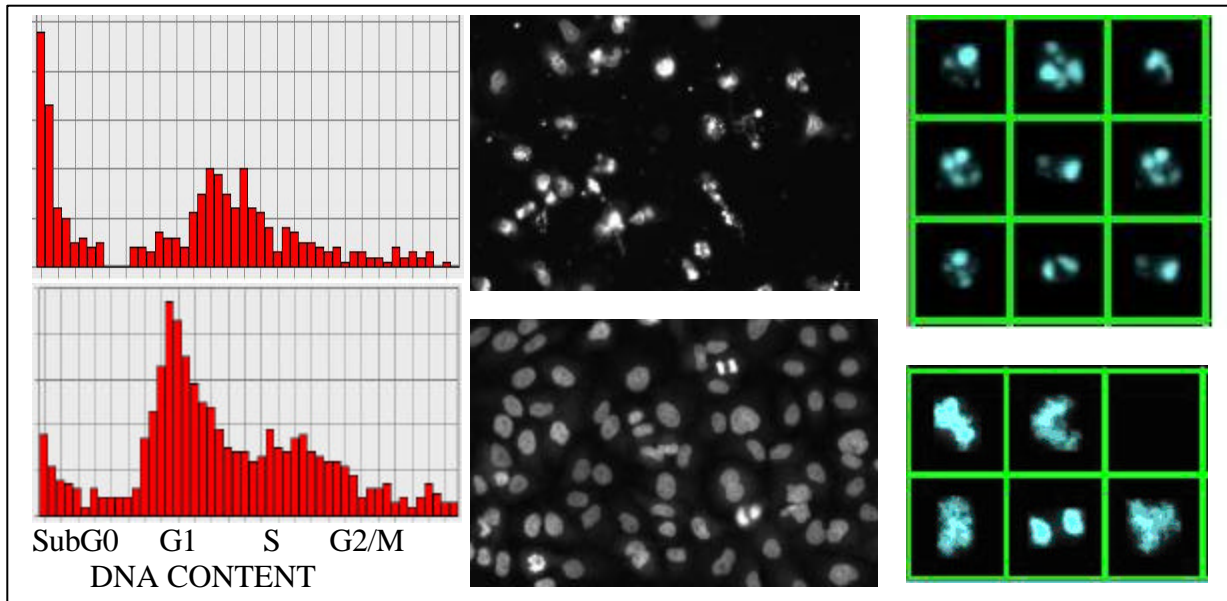


Figure 4. Representative histograms of DNA content with corresponding image fields (20X 0.5 N.A.) and cell montages representing apoptotic cells with sub G0 DNA content and high chromatin condensation (above) Metaphases/anaphases with G2 DNA content and high chromatin condensation are shown in the lower images.

CytoShop™ also calculates cell number for each well along with well averages, standard deviations and standard errors for each metric. These results can be instantly displayed on a wellplate schematic and trends easily identified using a color gradient display tool. Figure 5 shows the trends for the four cell lines plated in duplicate rows with a dose response decreasing from left to right. In this example, the color gradient reflects DNA content averaged over all the cells in a well. The middle four rows show a dose response for the selected metric. The numerical values corresponding to this data can be easily accessed from the database, plotted using CytoShop™, or exported to enterprise-wide software.

Interestingly, different kinetics of cytostatic or cytotoxic activity are suggested by the four cell lines. When evaluating cell number or DNA content, differential responses can be observed. Specifically, Figure 5 shows that the cell line A549 showed a dramatic decrease in cell number (blue datapoints) but only modest changes in DNA content (red datapoints). These data suggest that cis-platinum acted either as a cytostatic agent, or that apoptosis occurred so rapidly that cells have already detached from the plate. In this case, time course experiments can readily resolve the exact mechanism of action. In contrast, HT-29 showed a decrease in DNA content consistent with the nuclear fragmentation characteristic of intermediate stages of apoptosis while the total cell number did not change in the dose range studied. Finally, the colon carcinoma cell line RKO demonstrated resistance to cis-platinum as measured by both cell number and DNA content.

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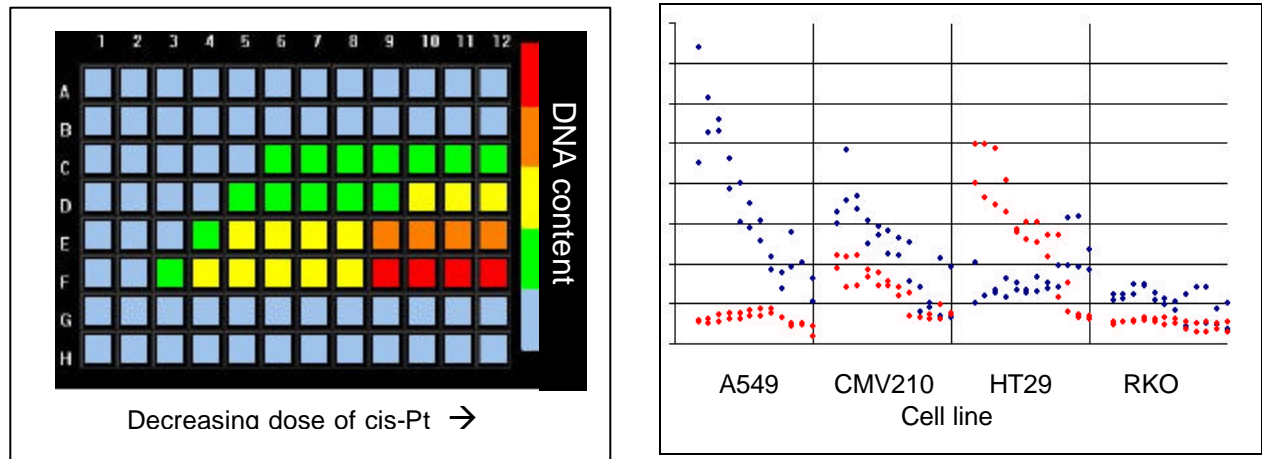


Figure 5. Wellplate schematic showing easy identification of trends using CytoShopTM color thermometer read-out (left). In this image the cellular DNA content is calculated from the integrated grayscale intensity in the nuclear mask for each cell, and the mean for each well is displayed. Graph showing the effect of increasing cis-platinum dose (X axis log scale) on cell number (blue datapoints) and DNA content (red datapoints) for four cell lines treated with cis-platinum (right). Duplicate wells in adjacent rows are evaluated for each dose.

Conclusion

DNA fluorometry using the EIDAQTM 100 HTM system is a powerful tool for the analysis of proliferation and apoptosis. The combined power of the EIDAQTM 100 and a single inexpensive reagent (DAPI or Hoechst) results in a rapid, robust and cost-effective screening assay.

Alternatively, DNA content and distribution can be combined with other biosensors to simultaneously measure proliferation and apoptosis as part of a multiparametric analysis. The unparalleled combination of core technologies in the EIDAQTM 100 provide accurate DNA image cytometry critically dependent on quantitative, in-focus high-resolution images and exact image segmentation to define nuclear boundaries. In addition, the CytoShopTM software allows easy assay development with novel cell types by enabling the user to visualize the actual cells corresponding to a population metric.

The EIDAQTM 100 automated High Throughput Microscopy (HTM) system from Q3DM Inc. delivers an unmatched combination of speed, accuracy and detail to quantitative imaging and analysis of cell populations. The EIDAQTM 100 is used to accelerate drug discovery, for clinical diagnostics, and in basic research.