

Introduction

Many new technologies and methodologies are being developed to study the pathways of cell death and apoptosis in cellular models. New instrumentation platforms, fluorescent stains, and antibodies specific for molecules involved in the apoptotic cascade of events are advancing the capabilities of researchers to understand the molecular interactions leading to cell death and apoptosis.

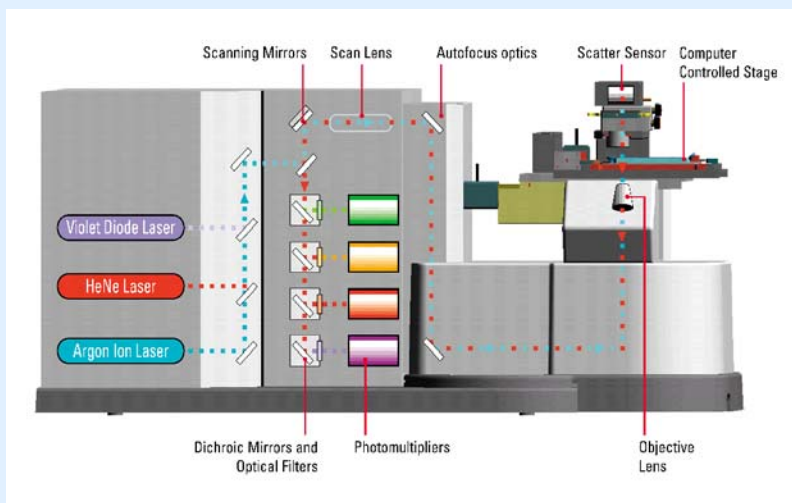
One such interaction involves the phosphorylated state of the histone H2AX molecule.

“ Histone H2AX is a 14 kDa ubiquitous member of the H2A histone family that contains an evolutionarily conserved SQ motif at the C-terminus in eukaryotes. Serine 139 within this motif becomes rapidly phosphorylated to yield a form known as γ -H2AX in response to double-strand DNA damage. Phosphorylation reaches its half maximum between 1-3 minutes after DNA damage occurs, and hundreds to several thousand molecules of γ -H2AX are present per double-strand break. This antibody is unique in only detecting phosphorylated histones at sites of double-strand DNA breaks.” (www.Trevigen.com)

In this report, we employ the newly developed iCytete™ Imaging Cytometer (CompuCytete Corporation) to combine the analysis of phosphorylated histone H2AX antibody labeling with propidium iodide based DNA staining to dissect the effects of the DNA topoisomerase inhibitor camptothecin, which blocks cells in the G2 phase of the cell cycle.

For further information on this and other LSC applications, contact techsupport@compucyte.com

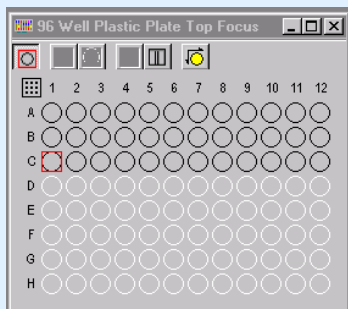
Experimental Methods



iCytete Imaging Cytometer

The iCytete Imaging Cytometer is an analytical cytometer with relevant applications in the areas of pharmaceutical and toxicological screening. It employs an inverted format to enable scanning of microtiter plates or microscope slides. Three excitation lasers are available to excite fluorescent dyes. Multiple photomultiplier tubes and a photodiode detector scan, image, and extract measurements for each of many thousands of segmented events in a typical analysis.

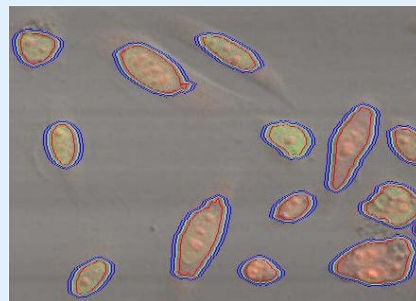
Experimental Methods cont.



- Phosphorylated histone H2AX antibody was obtained from Trevigen, Gaithersburg, MD.
- CHO cells were seeded in a 96 well plastic plate.
- Decreasing concentrations of camptothecin were added in columns 12 to 4 in two-fold dilutions starting at 6 nM (well 12) decreasing to 0.023 pM (well 4)
- Columns 1-3 were untreated controls.
- Cultures were incubated overnight.
- Cells were fixed with EtOH.
- Cells were stained at antibody concentrations of 0 (row A), 1:100 (row B) and 1:250 (row C) for one hour, washed three times and reacted with developing reagent.
- Alexa 488 goat anti Rabbit (Molecular Probes) was used as the developing reagent.
- Cells were counterstained with 5 ug/ml propidium iodide and RNase.

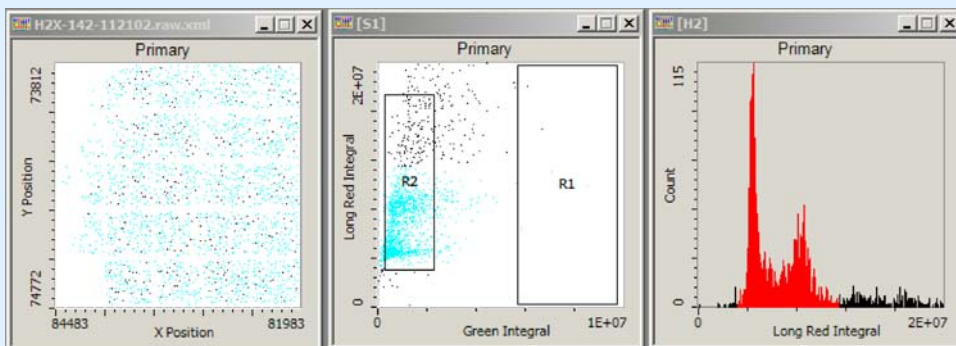
Cell Segmentation

Areas of the sample are scanned and memory arrays of detector measurements are obtained for each of the detectors being employed. These arrays are analogous to digital images, and image processing techniques are employed to segment, or identify and characterize events of interest. For this application primary segmentation was performed around the nuclei of cells (red and green contours). Additional contours shown in blue indicate the area used to define the local background of each event enabling accurate quantification of cellular constituents.



Segmented Events

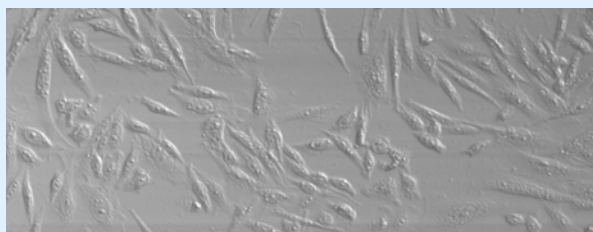
Control Scan – No Camptothecin



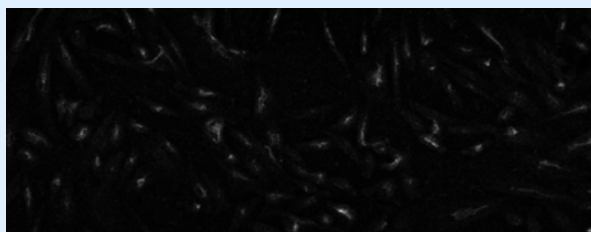
Elements involved in the analysis of a control sample are shown above. The left scattergram above shows the location in the well of cells found in the analysis. In this analysis, a 5 by 5 matrix of scan fields was employed.

The middle window is a scattergram showing the measured green fluorescence of the phospho H2AX antibody (Green Integral) vs. the total DNA content (Long Red Integral). Two regions, R1 and R2, define the populations that are positive and negative for the antibody. The right window is a histogram of the DNA distribution of the sample. This is a typical distribution for an exponentially growing cell culture.

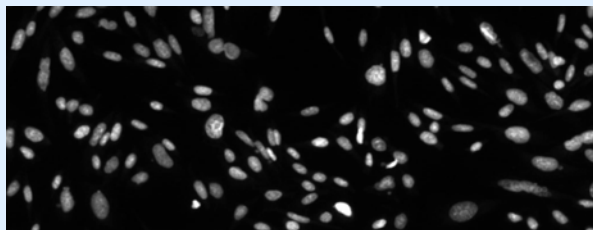
The figures below are the first scan fields from one of the control wells, showing laser scatter (a proprietary brightfield imaging technique), the green fluorescence, the red fluorescence and the combined color image of the other three detectors, known as CompuColor.



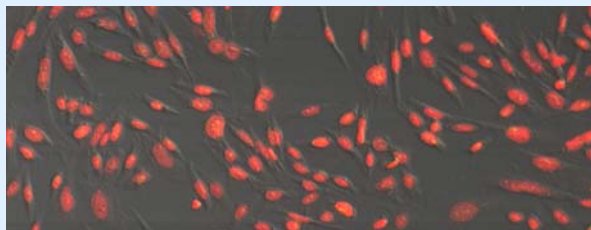
Laser Scatter



Green Fluorescence

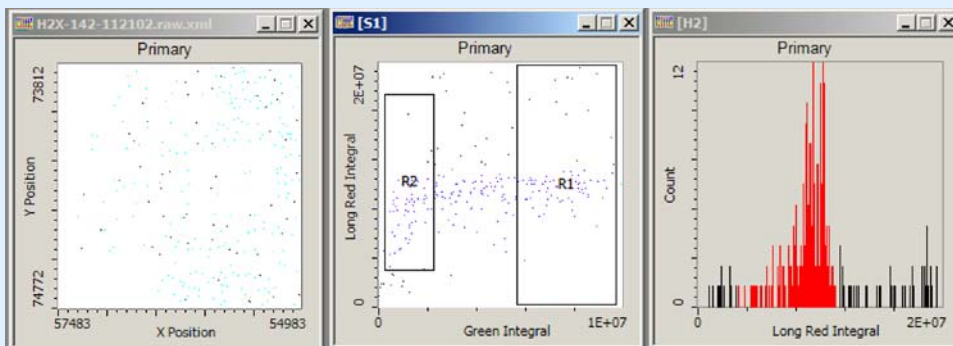


Red Fluorescence

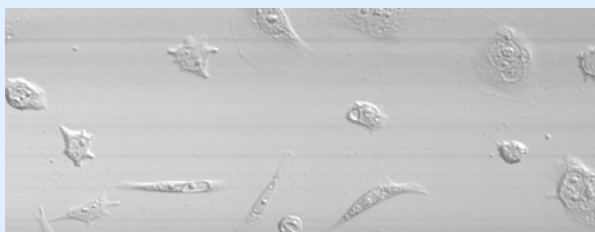


CompuColor

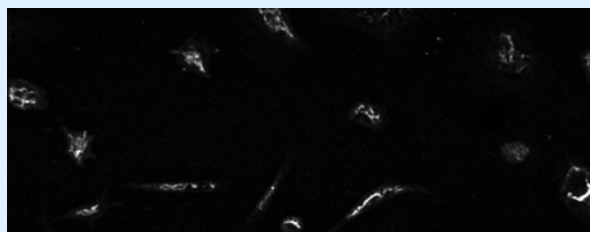
Scan of Camptothecin-Treated Well



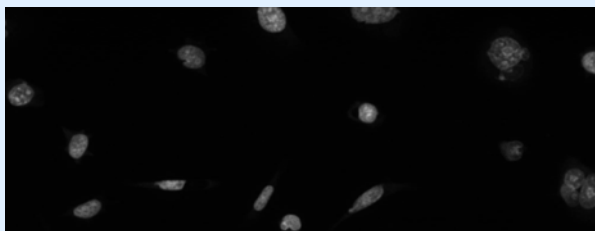
This well was treated with a low dosage of camptothecin. Compared to the previously shown control, the density of the cells in the upper x-y map is much lower. Green fluorescence is increased in the middle scattergram, showing an increase in cells that are positive for the antibody. In the DNA histogram (long red integral), the distribution is altered, reflecting a block of the cells in the G2 portion of the cell cycle. In the images, the lower cell density is apparent as well as the change in the cellular morphology, and a marked increase in the green fluorescence.



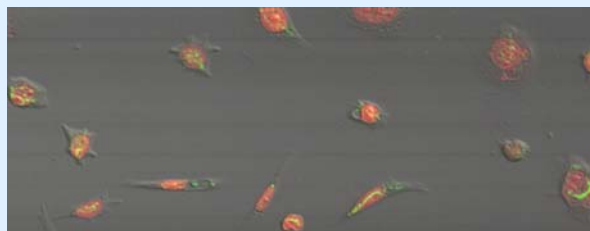
Laser Scatter



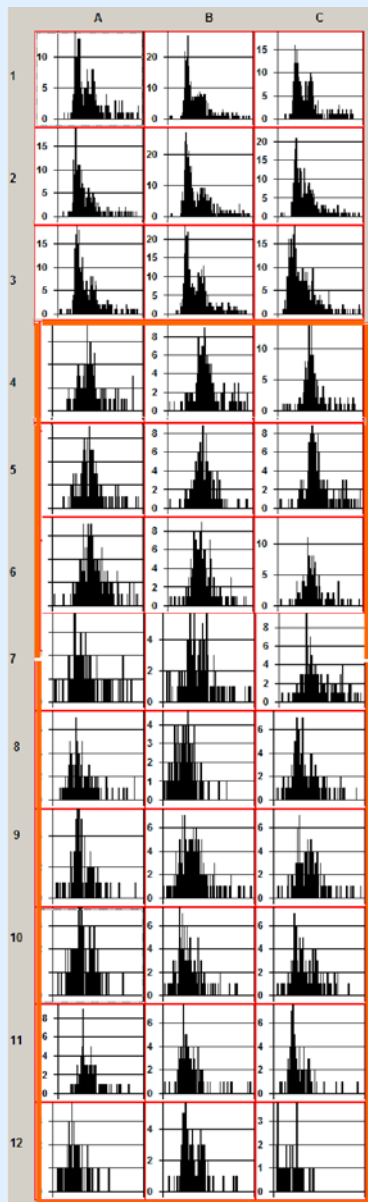
Green Fluorescence



Red Fluorescence



CompuColor

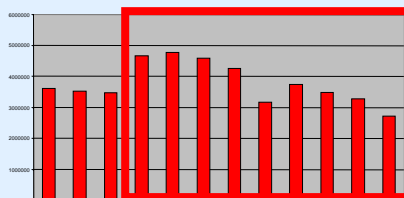


The figure on the left displays the DNA histograms from an analysis. The red box is drawn around the wells that were treated with the drug. At higher concentrations, the distributions contain fewer cells and thus have a more ragged appearance. This is due to necrosis and subsequent cell death and loss.

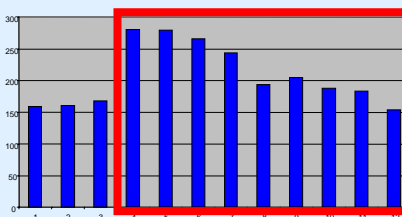
Below are graphs illustrating "well features" for the analysis. The mean DNA content per cell per well reflects the blockage in the G2 phase at the lower concentrations of the drug (area inside the box below).

The mean nuclear area per cell per well is displayed in the middle graph, and this is concordant with the total DNA content.

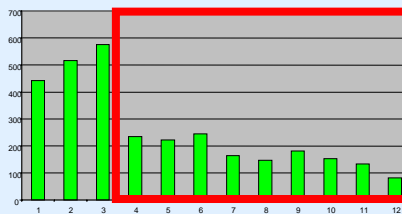
The lower graph shows the cell count per well, and here we see the loss of cells in the treated wells.



DNA content per cell



Nuclear area

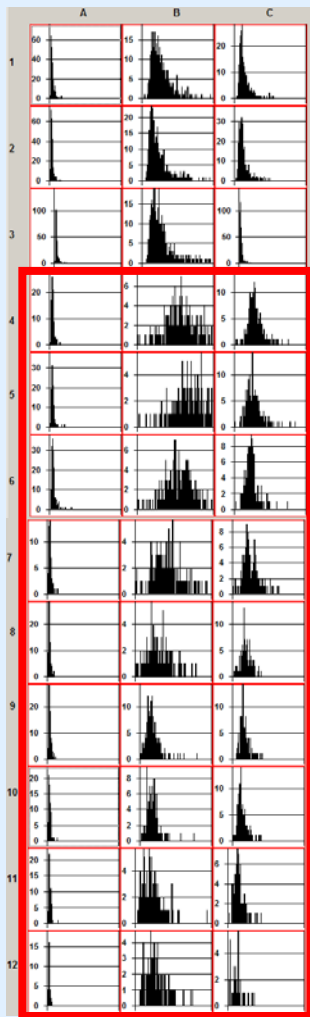


Count per well scan region

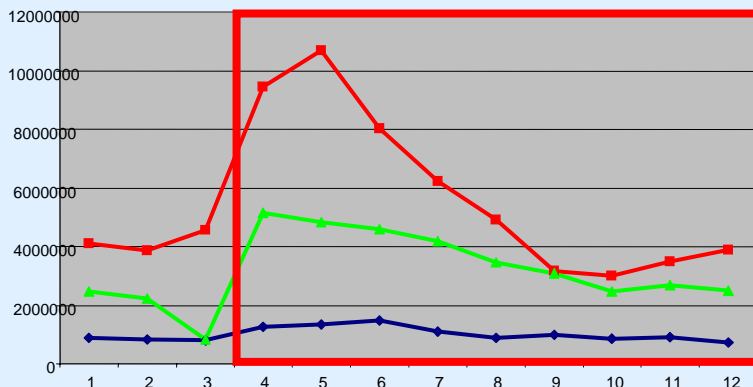
Quantitative analysis of the anti-phosphorylated Histone H2AX staining

The left figure shows the histograms of the green phosphorylated H2AX antibody staining. Column A is a negative control with no primary antibody. The middle and right columns are the high and low concentrations of the antibody, respectively, and as before, the campototecin-treated samples are within the box.

On the right is a graph of the mean green fluorescence for each well, with the control shown in blue, the high antibody concentration in red, and the intermediate value in green. The red border outlines the wells treated with the varying concentration of the drug. The expression of the green fluorescence is highest at the low levels of campototecin, and decreases at the higher levels, in a pattern similar to that observed for the DNA content increase and the nuclear area.



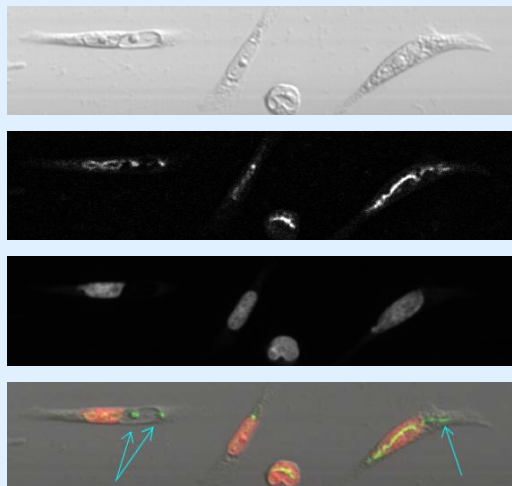
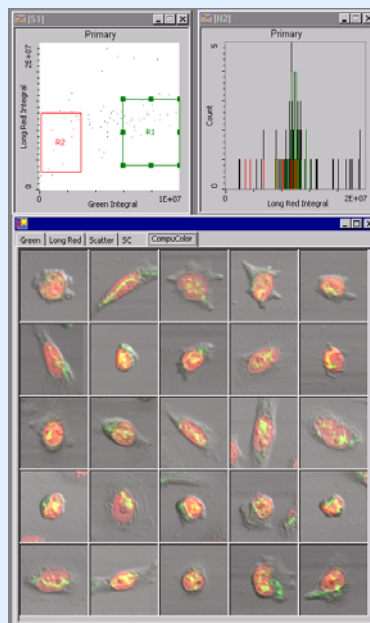
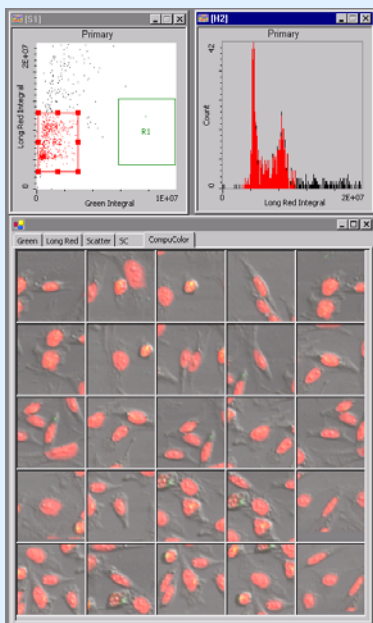
Phospho H2AX antibody staining



Galleries of Relocated Cells

One of the capabilities of the iCyte technology is the ability to produce images of the cells which are displayed in the analytical population data. On the left, region R2 is drawn around the main population of cells in an untreated control sample. A gallery of images of the first 25 cells that fall within the region is displayed.

On the right, data from a low concentration of camptothecin is shown. Here, region R1 is used to define the events of interest. This region is drawn around cells that are positive for the antibody staining. Their morphology, with many blebs in the cytoplasm, is constant with apoptotic cells.



Extra-Nuclear Staining

One of the observations made in the course of this experiment is the presence of cytoplasmic staining in some cells, particularly in low concentrations of the camptothecin (figure on left). One possibility is that the staining arises from the presence of mitochondrial DNA; further study is needed.

Summary

Phosphorylation of the histone H2AX molecule is associated with double-stranded DNA breakage. In this report, an antibody specific to phosphorylated histone H2AX, a protein associated with double stranded DNA breakage, was used in conjunction with quantitative cell cycle analysis and automated image acquisition and feature extraction techniques to examine the effects of camptothecin on a cultured cell line.

Differential effects were seen in response to increasing drug concentrations. At lower drug concentrations, the cell populations were blocked in the G2 phase of the cell cycle, and there was a corresponding increase in the nuclear area of the cells, consistent with a blockage in G2. At the same time, there was a decrease in the cell number per well, a result of the lack of newly divided cells entering the cell population, and also presumably because of cell death. At these same concentrations of camptothecin, there was a corresponding high level of expression of the H2AX antibody, indicating the presence of the strand breaks, a hallmark of apoptosis. The cellular morphology of the cells was also consistent with apoptosis, with a morphological change from spindle shaped, to a more rounded form with cytoplasmic blebbing.

At higher concentrations of the camptothecin, there was no longer the block of the cells in the G2 phase of the cell cycle, and there was a corresponding lowering of the amount of staining with the H2Ax antibody. There continued to be a decrease in cell numbers. These observations are consistent with cell death being caused by a necrotic pathway.

Together, these combination of analytical features allows high content cellular analysis, and in this case it is possible to segregate apoptotic from necrotic cellular pathways.