

## 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 capability of researchers to understand the molecular interactions leading to cell death and apoptosis.

One such interaction involves the phosphorylated state of the histone  $\gamma$ -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  $\gamma$ -H2AX in response to double-strand DNA damage. Phosphorylation reaches half its maximum between 1-3 minutes after DNA damage occurs, and hundreds to several thousand molecules of  $\gamma$ -H2AX are present per double-strand break. This antibody is unique in only detecting phosphorylated histones at sites of double-stranded DNA breaks. ([www.Trevigen.com](http://www.Trevigen.com))

## Experimental Objective

We employ the iCyte™ Imaging Cytometer (CompuCyte Corporation) to analyze phosphorylated histone H2AX expression and DNA content and to dissect the effects of the DNA topoisomerase 1 inhibitor camptothecin, which is known to prevent the re-ligation of DNA strand breaks, on the cell cycle of a cultured cell line.

## Materials

### Cell Line

CHO cells were originally obtained from the American Type Culture Collection (ATCC) and were kept in continuous culture according to the provider's recommendations.

### Reagents

1. Trypsin, Sigma cat. no. T3924
2. Dulbecco's Minimal Essential Media, ATCC cat. no 30-2006
3. Fetal Bovine Serum, Sigma cat. no. F4135
4. Penicillin/Streptomycin, Sigma cat. no. P4333
5. Camptothecin, Sigma cat. no. C9911
6. Phosphate Buffered Saline (PBS), Sigma cat. no. D8537
7. Ethanol Fixative, Sigma cat. no. 2858
8. Bovine Serum Albumin, Sigma, cat. no. A7030
9. Phosphorylated histone H2AX antibody, Trevigen cat. no. 4411-PC-100
10. Alexa 488 goat anti-rabbit, Molecular Probes cat. no. A11034
11. Propidium iodide, Sigma cat. no. D8537
12. RNase, Sigma cat. no. R5503

### Sample Carrier

96-well plastic microtiter plate, Whatman cat no. 7716-2380

### Cell Media

Prepare Cell Media as follows: Dulbecco's Minimal Essential Media, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin

### Blocking Solution

Prepare blocking solution as follows: 1% BSA in PBS

**Instrumentation**

iCyte™ Imaging Cytometer, CompuCyte Corp., utilizing iCyte™ Application Software version 2.6.1 and iBrowser™ Data Integration Software version 2.6.1

**Experimental Method****CHO Cell Plate Preparation**

1. Trypsinize exponentially growing CHO cells and wash with cell media.
2. Centrifuge the cells and re-suspend to a cell density of  $2.5 \times 10^4$  cells per ml.
3. Add 100  $\mu$ l of the CHO cell suspension to each well of a 96-well plastic bottom microtiter plate.
4. Incubate microtiter plates at 37°C for at least 7 hours to allow cells to attach to the well.

**Technical Hint**

Although not required, optical quality plastic bottom microtiter plates produce better results and are strongly recommended.

**Cell Treatment**

5. Dilute camptothecin to 0.6  $\mu$ M in media.
6. Dilute the 0.6  $\mu$ M camptothecin solution two-fold serially to 2.3 nM.
7. Add 100  $\mu$ l of the camptothecin solutions to the test wells of the plate, starting with the most concentrated solution (0.6  $\mu$ M) in column 1 to the least concentrated (2.3 nM) in column 9.
8. Place 100  $\mu$ l of media in Control wells (columns 10-12).
9. Incubate plates overnight at 37°C.

**Cell Staining****Antibody Preparation**

10. Remove plates from the incubator and remove liquid from the wells.
11. Wash wells 3 times with 200  $\mu$ l of PBS.
12. Fix cells by adding 200  $\mu$ l of ethanol to each well and incubate for 30 minutes at room temperature.
13. Remove ethanol from the wells.
14. Wash cells twice with 200  $\mu$ l of PBS.
15. Add blocking solution to all wells and incubate at room temperature for at least 45 minutes. Decant blocking solution.
16. Prepare phosphorylated histone H2AX antibody at 1:100 in PBS.
17. Add 50  $\mu$ l of diluted H2AX antibody to all wells.
18. Cover the plate and incubate at room temperature in the dark for one hour.

**Technical Hint**

Washing wells between steps helps to reduce background by removing unbound antibody.

Plates should be blocked with 1% BSA or other blocking agent to prevent non-specific binding of antibody.

For best results, incubation times for antibodies should not exceed 1 hour.

**Antibody Staining**

19. Wash cells twice with 200  $\mu$ l of Blocking Solution.
20. Alexa 488 goat anti-rabbit is used as the developing reagent. Prepare it at a 1:200 dilution in PBS.
21. Add 50  $\mu$ l of developing reagent to all wells and incubate at room temperature in the dark for 30 minutes.
22. Wash wells 3 times with PBS.

### Counterstaining

23. Counterstain cells by adding 100 µl of 5 µg/ml propidium iodide and 200 µg/ml RNase in PBS to each well and incubate at room temperature in the dark for 30 minutes.
24. Decant the PI/RNase solution from the wells and add 200 µl of fresh 5 µg/ml PI.

**Technical Hint**

Keep at least 200 µl of fluid in the wells to minimize meniscus effects which will induce variability in images.

### Plate Setup

Figure 1 shows a typical microtiter plate with the Control wells to the right and the drug-treated wells to the left.

	1	2	3	4	5	6	7	8	9	10	11	12
A											C	
B											o	
C											n	
D	Decreasing drug concentration from col. 1 to col. 9										t	
E											r	
F											o	
G											l	
H											s	

**Figure 1: Typical microtiter plate setup for toxicological studies**

**Technical Hint**

To use a different sample carrier, select an alternative carrier in the *Instrument > General* dialogue box. To define a new type of sample carrier, go to *Administration > Manage Carrier Types > New*. For information about accessing the Administration menu see the *iCyte User Guide*.

## Sample Analysis

### Introduction

Primary contouring is performed using the Crimson channel, which identifies the PI (DNA) staining. The Phospho-histone H2AX, which identifies double-stranded DNA breaks, is labeled with Alexa Fluor 488 and is detected in the Green channel. Scatter provides a visual aid for changes in cell morphology. For this type of analysis, the iGeneration analysis workflow is broken down into five parts:

- (1) Initial Set-up
- (2) Scan Settings Set-up (Test Scans)
- (3) Analysis Scan Set-up
- (4) Analysis Scan (Scan & Save)
- (5) Data Analysis

The detailed steps for each of these parts are described below.

### Initial Set-up

The Initial Setup defines the specimen carrier and specific cytometer hardware settings required for the scan.

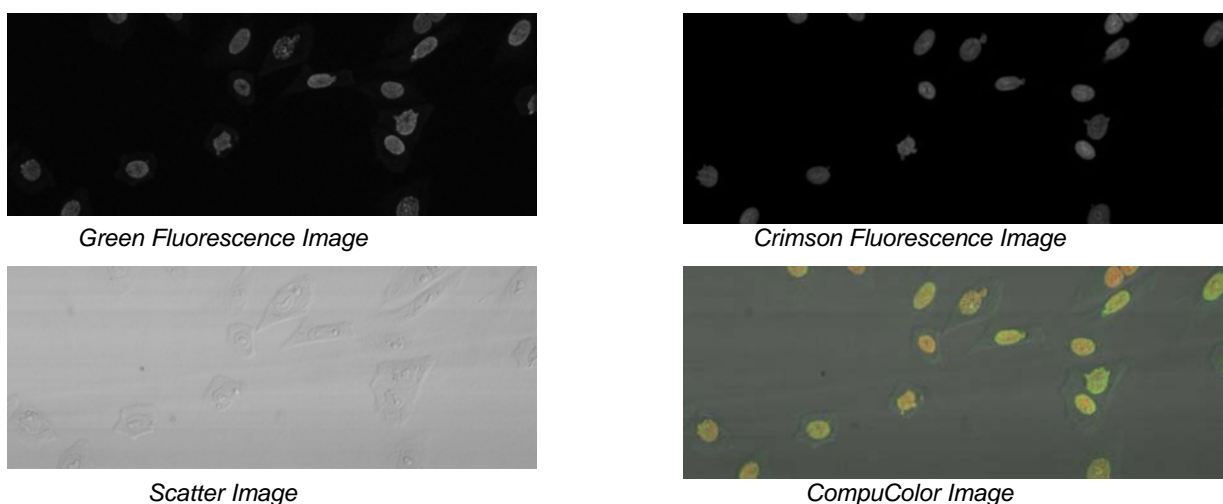
1. Select the appropriate type of microtiter plate.
2. Set the instrument settings for the Cytometer hardware as detailed in Figure 2.

Laser	Argon, 5 mW
Channels	Green (H2AX)
	Crimson (PI)
	Scatter
Objective	40x
X Step Size	0.5 microns

**Figure 2: Instrument settings**

### Scan Settings Set-up (Test Scans)

3. Select a control well that is untreated with the drug.
4. Select a scan area of 6 (wide) x 6 (high).
5. Set the detectors to the appropriate setting based on a test scan:
  - a. Scan a single field and observe the Crimson scan image in the Scan View window. Move the cursor over several nuclei to display the pixel values.
  - b. Adjust the Detector Voltage % to produce pixel values of about 5000 for the dim nuclei. Bright spots within the nuclei may have values ranging up to about 10,000. Avoid having too many areas within cells with saturated (16,384) pixel values.
  - c. Adjust the Detector Offset value to achieve a background (areas with no nuclei) pixel value below 500.
  - d. Observe the Green scan image in the Scan View window. Move the cursor over several nuclei to display the pixel values. With the H2AX antibody, the nuclei should have pixel values of about 2500. It is expected that there will be bright spots within the nuclei with pixel values ranging up to 10,000 – 12,000.
  - e. Adjust the Detector Offset value to achieve a background (areas with no nuclei) pixel value below 500.
  - f. Observe the Scatter scan image in the Scan View window. Move the cursor over several nuclei to display the pixel values. Adjust the Scatter Detector Gain to give pixel values of about 10,000.
  - g. Observe the scanned images for all channels. Images should appear similar to those shown in Figure 3.



**Figure 3: Scanned images of cells with proper PMT settings**

- Set the primary segmentation parameters to achieve optimal segmentation of events. Suggested values are shown in Figure 4 below. Perform rescans after making any setting changes and observe the effect.

Segmentation Primary	Crimson (PI)
Threshold	3000
Minimum Area	5 $\mu^2$
Added Pixels	5
Background	Dynamic, applied to all channels

**Figure 4: Contouring settings**

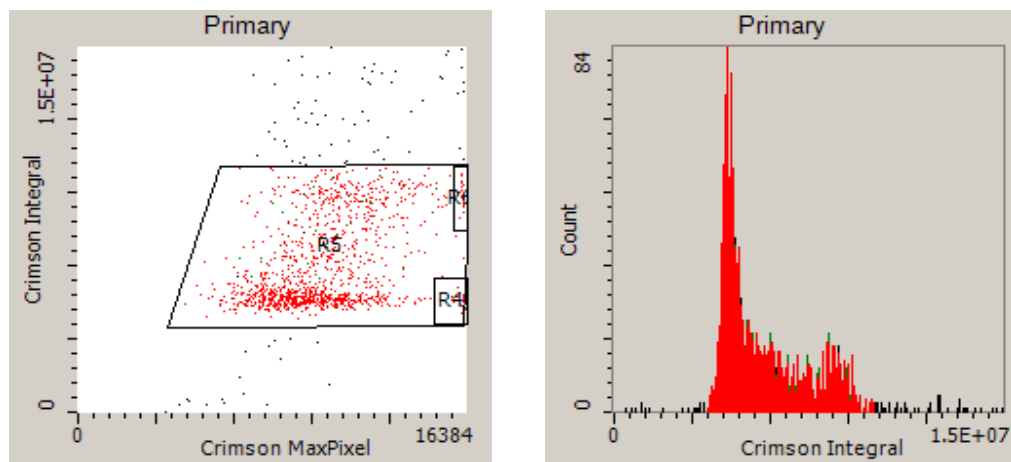
**Technical Hint**

To prevent prolonged exposure of fluorochromes to laser light while performing initial Test Scans, set the initial scan area larger than the final scan area.

To help reduce background from high levels of ambient light, place a dark cloth over the specimen carrier while scanning.

**Analysis Scan Set-up**

- Define the following Scattergrams and Histograms:
  - Density Plot: Green (H2AX) Integral (x) vs. Crimson (PI) Integral (y)
  - Green (H2AX) Integral (x) vs. Crimson (PI) Integral (y)
  - Crimson (PI) Max Pixel (x) vs. Crimson (PI) integral (y)
  - X Position (x) vs. Y Position (y)
  - Crimson (PI) Integral Histogram
  - Green (H2AX) Integral Histogram
- Start a Test Scan. When the first scan field image appears in the Scan View window, select Draw Event Contour to view the cellular segmentation.
- Open the Adjust window and adjust the Threshold value in the Primary Contour Tab so that event contours are drawn around the individual cells. After changes are made to the Threshold value, select Refresh Contours to see the effect on the cells.
- Allow iCyte to scan several scan areas within the well to test both the PMT and Threshold settings. When both are properly adjusted, the Crimson Max Pixel vs. Crimson Integral Scattergram and the Crimson Integral Histogram should be similar to the ones shown in Figure 5.



**Figure 5: Expected Crimson Max Pixel and Crimson Integral (DNA) distributions when PMT and contours are set properly**

### Data Acquisition

11. Set the Data Options to Save Raw Data as JPEG, all Scan Field Images for all detectors and the Well Image.
12. When the Well Features Option is selected, features related to the scan are highlighted for further analysis in the iBrowser software. Those features selected are shown in Figure 6.

Component	Feature	Channel	Stat	Region	Min	Max	Valid
Primary	Area		Mean	None	0	500	True
Primary	Integral	Crimson	Mean	None	0	10000000	True
Primary	Integral	Green	Mean	None	10000	7000000	True
Primary			Count	R5	0	1000	True
Primary			Count	R6	0	20	True
Primary			Count	R4	0	20	True
Primary			Count	R1	0	500	True
Primary			Count	R3	0	500	True

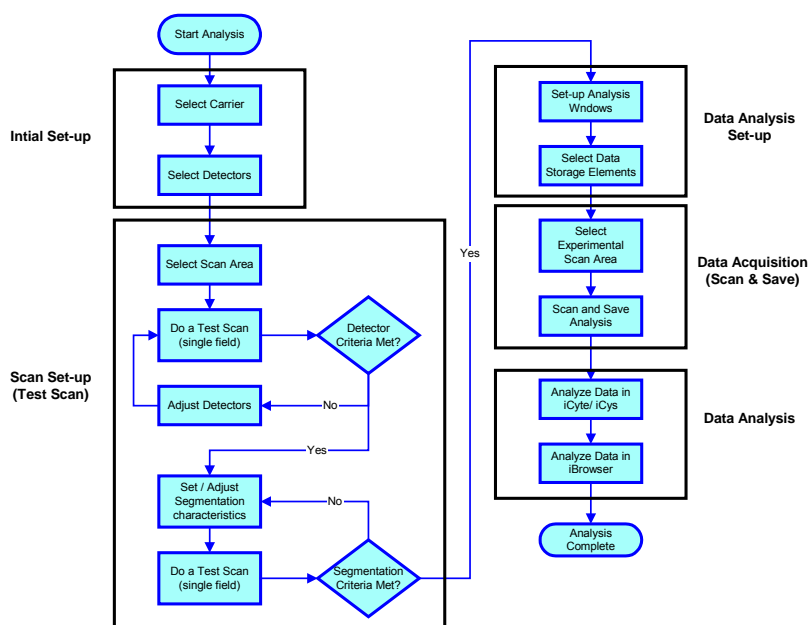
**Figure 6: Selected Well Features**

13. Set the scan area within the well to 6 (wide) x 6 (high).
14. Select the appropriate wells for analysis.
15. Select Scan and Save. The system will begin to scan the plate and save the data.

### Data Analysis

16. Upon completion of the scan, the data are analyzed using tools in the iGeneration application, CompuCyte's iBrowser™ Data Integration Software and in Microsoft® Excel. The data analysis is described in detail along with the presentation of results in the next section.

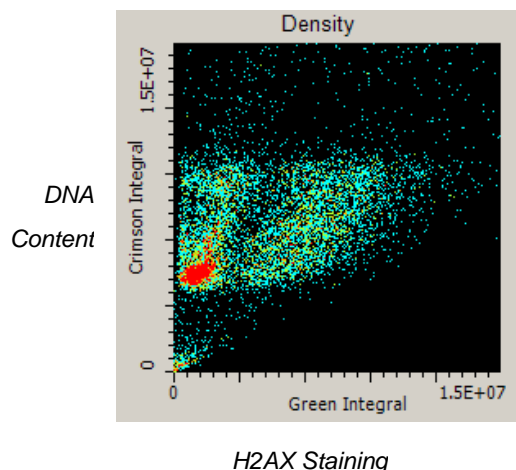
The Analysis workflow described above is summarized in the diagram in Figure 7.



**Figure 7: iGeneration Work Flow**

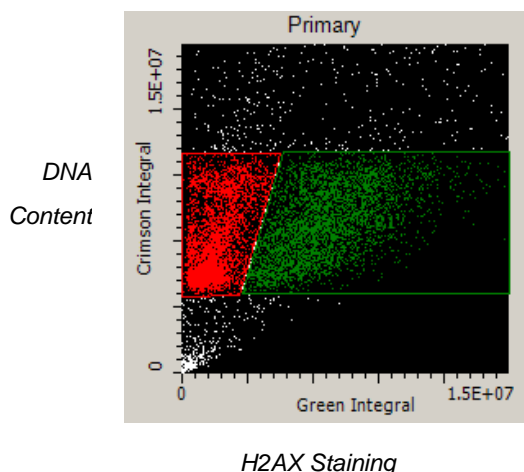
### Results

After the analysis is completed, a density plot showing the H2AX staining vs. the DNA content is plotted for all wells in the analysis. Two main clusters of events are apparent, corresponding to H2AX-positive cells and H2AX-negative cells. See Figure 8.



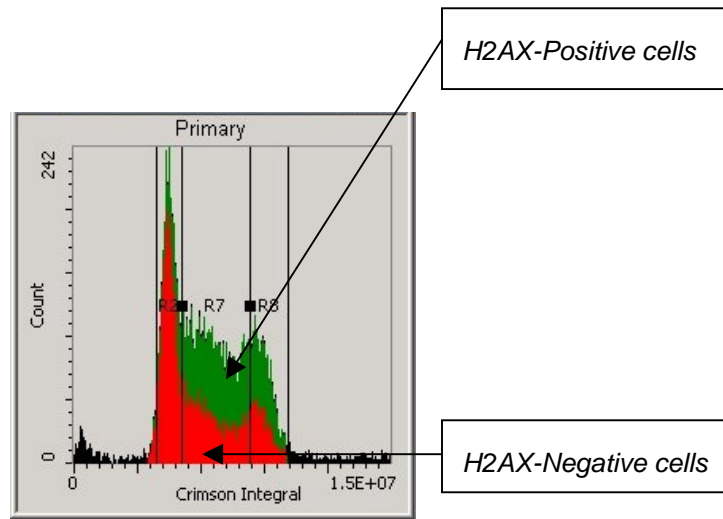
**Figure 8: Density Plot showing expression of H2AX staining and DNA Content**

In a scattergram with the same axes and scales, regions are drawn around the two main clusters. Region 1 is color-coded green, and is drawn around the H2AX-positive cells. Region 2 is color-coded red, and is drawn around the H2AX-negative cells, as shown in Figure 9. Cells above the regions are multiple cells, and cells below the regions are apoptotic bodies and other cellular debris.



**Figure 9: Scattergram showing color coding of H2AX-negative and -positive cells**

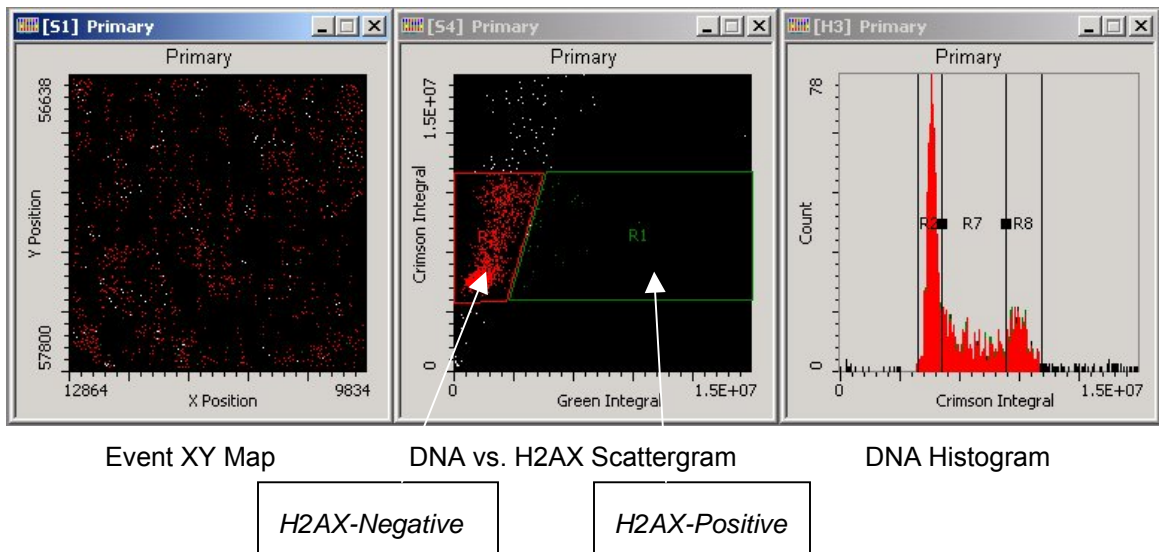
When colors for a region are set in a window, the colors for the events are carried through to all of the windows for the event type. In Figure 10, the red histogram shows the H2AX-negative cells, and their DNA distribution is typical for exponentially growing cells. The H2AX-positive cells, shown in green, display a buildup of events in the S and G2 phases.



**Figure 10: Colored histogram showing H2AX-negative and -positive cells**

**Control Well**

Figure 11 shows the analysis of a control well. The middle window is a scattergram showing the measured green fluorescence of the phosphorylated H2AX antibody (Green Integral) vs. the total DNA content (Crimson 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. Control well histograms should show two peaks, the first peak (G1) should rise sharply from the background while the second (G2) peak should be smaller and have a fluorescence intensity approximately equal to two times the G1 peak.

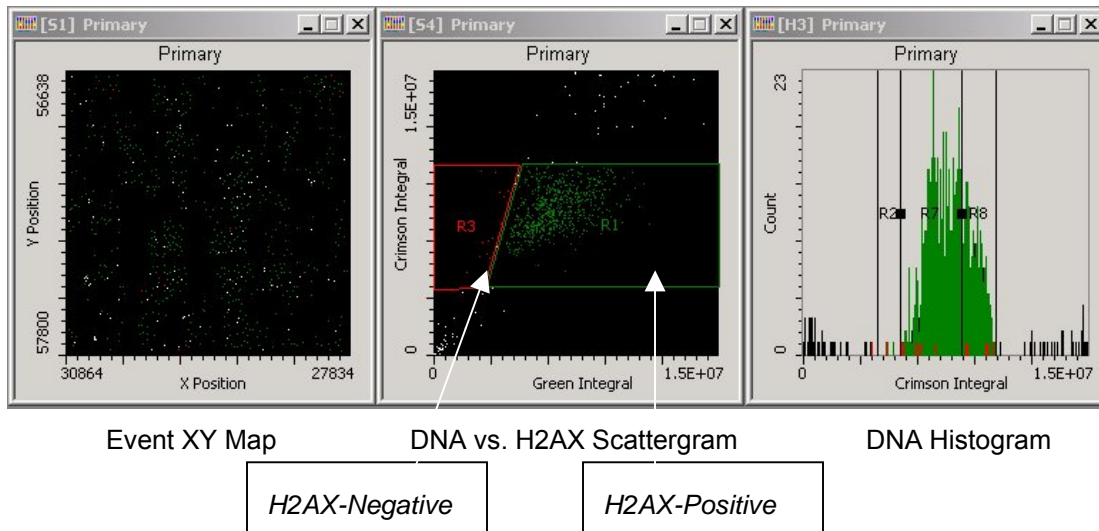


**Figure 11: Analysis of control well**

**Camptothecin-Treated Well**

Figure 12 shows a well treated with a low dosage of camptothecin. Compared to the previously shown control, the density of the cells in the 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 (Crimson Integral), the distribution is altered, reflecting a block of the cells in the S-phase

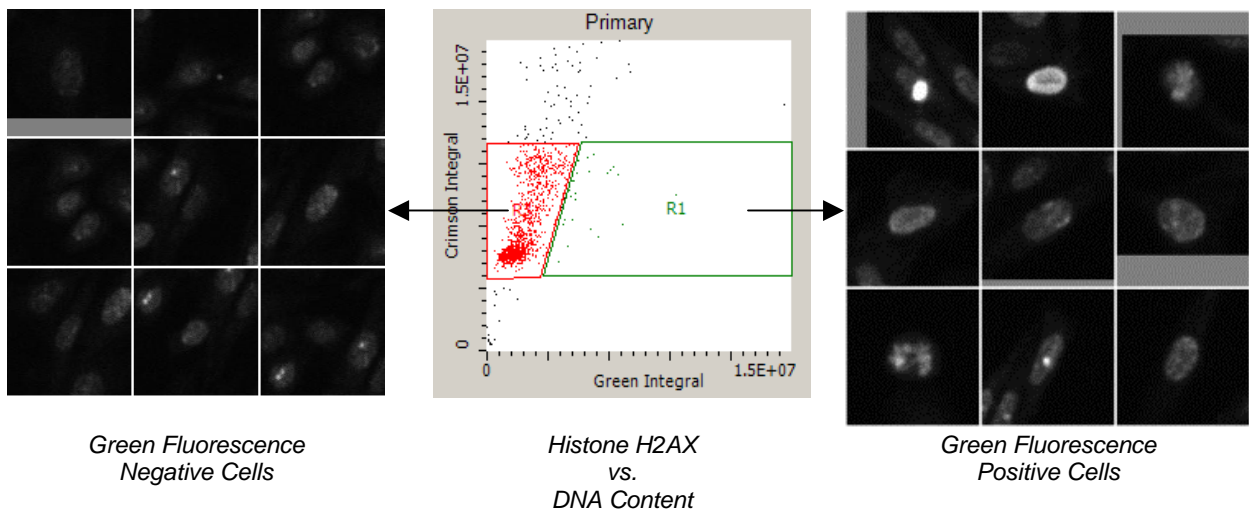
portion of the cell cycle. In the images, the lower cell density is apparent, as is the change in the cellular morphology and a marked increase in green fluorescence.



**Figure 12: Analysis of a camptothecin-treated well**

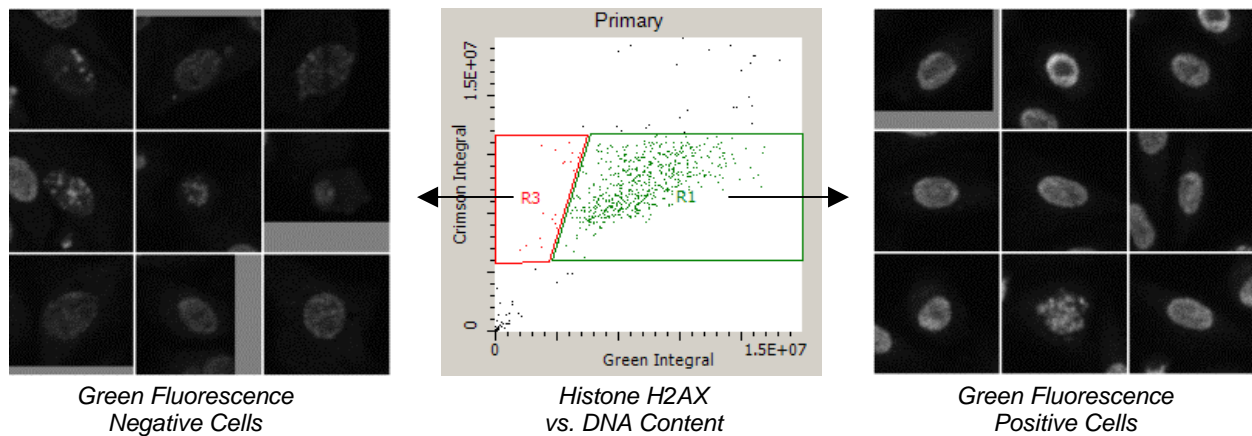
### Galleries of Relocated Cells

Galleries of images of representative cells from within a region can be automatically obtained. In the control well shown in Figure 13, the majority of the cells are negative for histone H2AX. Images of the cells relocated from the red region show some spot-like background staining. Although they are few in number, there are some cells in the H2AX positive region.



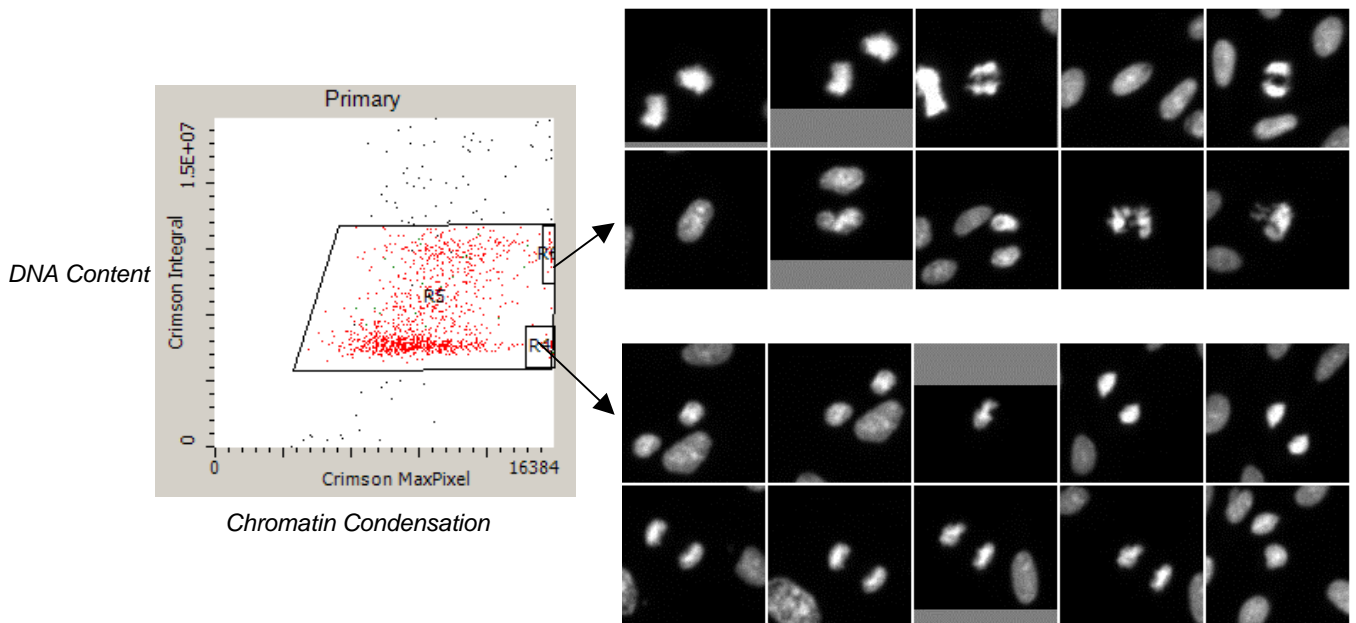
**Figure 13: Gallery of control well showing mostly H2AX-negative cells**

In this camptothecin-treated well (Figure 14) the majority of the cells are positive and fall in the green region. The images of relocated cells from this region show smooth, even staining in most of the cells. Images of the cells relocated from the red region show some spot-like background and some dim specific staining.



**Figure 14: Gallery of cells from camptothecin-treated wells showing positive green fluorescence**

Additionally, mitotic cell count information is obtained in the analysis. In Figure 15, a scattergram is defined plotting chromatin condensation vs. DNA content, and a region is drawn around the single-cell population. Two smaller regions are drawn at the areas of the scattergram where mitotic cells are expected to occur, before and after cytokinesis. Relocated gallery images confirm the presence of the mitotic cells, as evidenced by the morphology of the relocated cells in the center of each image.



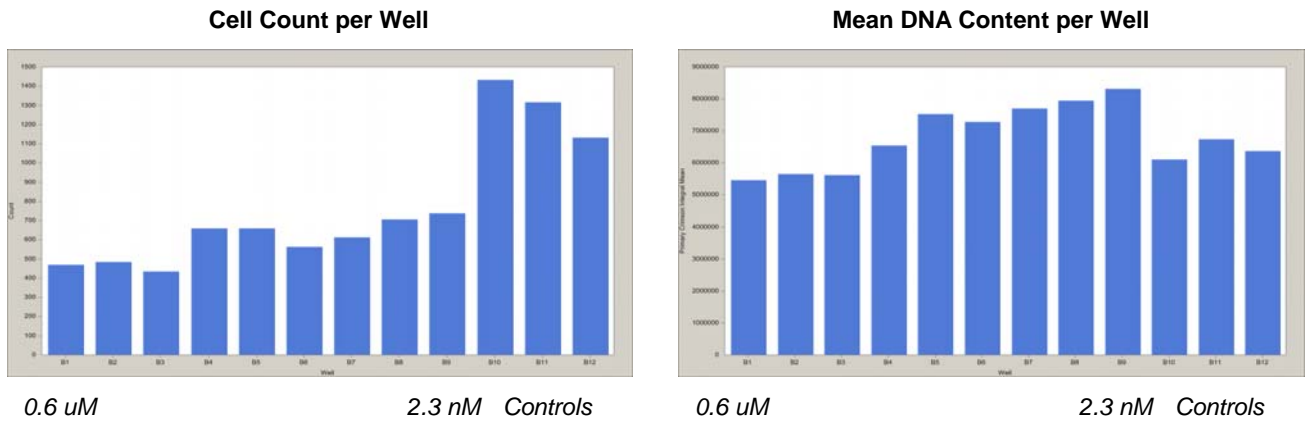
**Figure 15: Galleries of mitotic cells from the analysis**

**iBrowser Analysis**

Histograms from the analysis are output as part of the data stored for each analysis. The iBrowser Data Integration Software allows display of the data for an experiment as a whole, as well as statistical analysis.

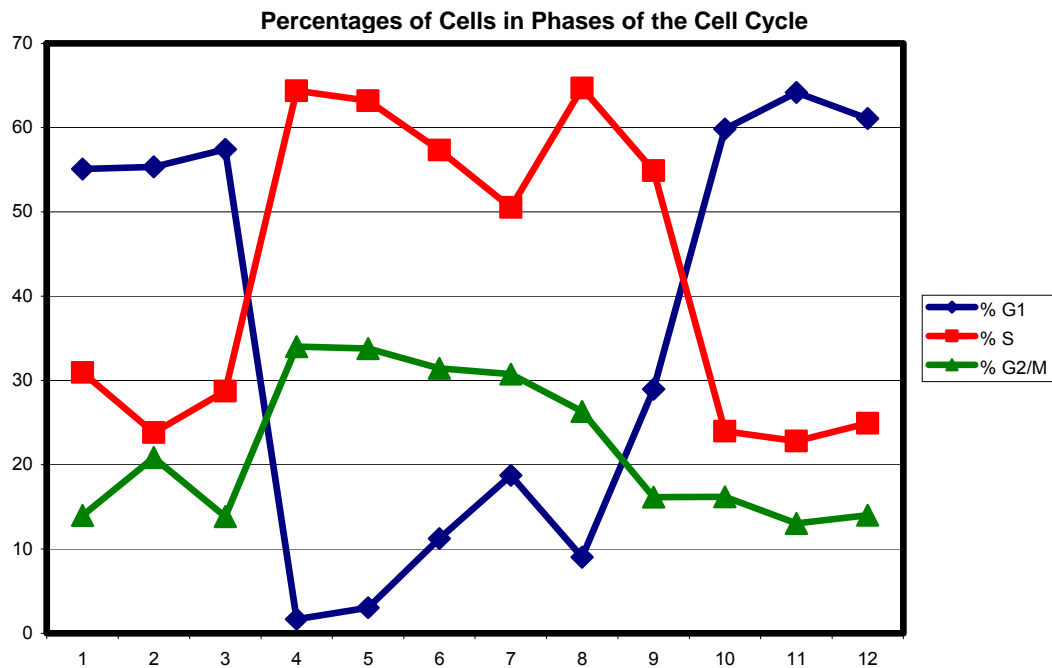


of proliferation of cells when they are blocked in the cell cycle. The Mean DNA Content Per Well histogram reflects the blockage of the cells in S-phase in the low dosage of the camptothecin treatment.



**Figure 17: iBrowser Well Feature histograms showing decreased cell count and increased mean DNA content in drug-treated wells**

Well feature data can also be formatted in iBrowser for export to third-party software packages, such as Microsoft Excel, for further analysis. The percentages of cells falling into the different phases of the cell cycle are shown in the graph in Figure 18. The differential response of the cells is clearly seen as the red plot of the cells in S-phase, which increases dramatically at the low dosage of the drugs.



**Figure 18: Well Feature data analyses plotted in Excel, showing the percentage of cells falling into the various stages of the cell cycle**

### Summary

In this Application Protocol, immuno-fluorescence staining is combined with quantitative DNA analysis to analyze the effects of a drug on a cell line using the iCyte™ Automated Imaging Cytometer and associated software.

The antibody used was specific for phosphorylated histone H2AX, a protein that surrounds sites of double-stranded DNA breakage. The topoisomerase 1 inhibitor camptothecin, a compound known to cause double-stranded DNA breaks in DNA replicating cells, was used as the test drug.

Differential effects were seen in response to increasing drug concentrations. At lower drug concentrations, the cell populations were blocked in the S phase of the cell cycle and there was a corresponding increase in the mean DNA content of the cells. At the same time, there was a decrease in the cell count 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.

At higher concentrations of camptothecin, there was a decrease in the average DNA content of the cells, and a corresponding reduction in the amount of staining with the H2AX antibody.

The biological significance of the differential staining is still under investigation, but reports have shown that the accumulation of DNA strand breaks leads to cell death by caspase-3 activation and subsequent apoptosis. The accumulation of cells in the G1 phase of the cell cycle may be a step in an alternative pathway of cell death by necrosis.

### Features Summary

Camptothecin is a good example of a drug that has different effects on the cell cycle depending on its concentration. To demonstrate these effects, we utilized the following iCyte features:

Feature	Use during this study
Multi-channel Analysis	Simultaneous collection of green and red fluorescence and morphological images and data
Automated Analysis	Data collection at multiple test points to obtain continuous dosage responses
Integral Feature	Collection of precise stoichiometric DNA content data to allow correlation of drug effects with position in the cell cycle
Max Pixel Feature	Measurement of chromatin condensation to isolate mitotic cells
Population Analysis	Correlation of Green (H2AX) and Long Red (DNA) events
Well Features	Characterization of the well itself, such as the cell count in the region scanned, the average DNA content and the event area
Relocation	Presentation of representative images allowing identification of morphological changes within specific populations
Kolmogorov-Smirnov Test	Statistical method for highlighting difference in DNA content between control samples and drug-treated samples