

**Introduction**

Apoptosis, or programmed cell death, is a desired goal when administering chemotherapeutic agents. Apoptosis can be initiated via a number of cellular pathways, including DNA damage or interference with mitochondrial membrane potential functionality. Analysis with iGeneration instrumentation allows simultaneous evaluation of multiple apoptotic pathways and cell death. While this experiment is performed using the iCyte™ Automated Imaging Cytometer, it may also be performed on other iGeneration instrumentation.

**Experimental Objective**

In this experiment, we seek to determine the ability of experimental compounds to cause cell death through apoptotic pathways initiated by mitochondrial dysfunction. The topoisomerase inhibitors camptothecin (CPT) and etoposide (ETP), compounds known to induce apoptosis by interfering with repairs to DNA damage, are used as control compounds. Cell features examined include DNA content, mitochondrial membrane potential, plasma membrane integrity and nuclear membrane integrity.

**Materials**

**Cell Line**

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

**Reagents**

<i>Reagent</i>	<i>Source</i>
1. Trypsin EDTA solution	Sigma cat. no. T394
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. Propidium iodide, 100 µg/ml stock	Sigma cat. no. P4170
8. Hoechst 33342, 10 mg/ml stock	Molecular Probes cat. no. H3571
9. Mitoshift™ Mitochondrial Potential Assay, 1 mM stock	Trevigen, cat. no. 6305-100-K
10. YO-PRO®-1, 100 mg/ml stock	Molecular Probes cat. no. Y3603

**Sample Carrier**

96-well glass microtiter plate, Whatman cat. no. 7716-2375

**Cell Media**

Dulbecco's Minimal Essential Media, 10% Fetal bovine serum, 1% Penicillin/Streptomycin

**Instrumentation**

iCyte™ Automated Imaging Cytometer, CompuCyte Corporation, utilizing iCyte™ Application Software version 2.6.1 and iBrowser™ Data Integration Software version 2.6.1

**Additional Analysis Tools**

Microsoft® Excel

**Experimental Method**

Test cells are grown in microtiter plates, treated with a test panel of drugs, and stained with dyes that have characteristics described in Figure 1.

Stain	Target	Excitation	Emission
Hoechst 33342	DNA in live cells	405 nm	Blue
Mitoshift	Mitochondrial potential	488 nm	Orange
YO-PRO-1	Apoptosis via membrane permeability	488 nm	Green
Propidium Iodide	Cell death via nuclear membrane permeability	488 nm	Long red

**Figure 1: Characteristics of dyes used in this live cell study**

**Plate preparation**

1. Detach exponentially growing CHO cells from the plates using trypsin, and wash into growth media.
2. Centrifuge and re-suspend the cells to a cell density of  $2.5 \times 10^4$  cells per ml.
3. Add 100 µl of the CHO cell suspension to each well of a 96-well glass-bottom microtiter plate.

**Technical Hint**

*Use glass-bottom plates when using violet-laser excited dyes to prevent absorption of the laser light by plastic plates.*

4. Incubate microtiter plates at 37°C for at least 7 hours to allow cells to attach to the wells.



- Incubate plates at 37°C overnight.

**Cell Staining**

- Prepare a Live Cell Stain Cocktail by diluting the stock dye solution in growth media to the final working concentration as shown in Figure 4.

Stain	Stock Concentration	Dilution	Working Concentration
Hoechst 33342	10 mg/ml	1:1000	10 µg/ml
Mitoshift	1 mM	1:10,000	0.1 µM
YO-PRO-1	10 mg/ml	1:1000	10 µg/ml
Propidium Iodide	100 µg/ml	1:100	1 µg/ml

**Figure 4: Working concentrations of dyes for this analysis**

**Technical Hint**

*Keep the concentration of Hoechst 33342 under 10 µg/ml. Higher dye concentrations may cause the dye to precipitate.*

*Use only the recommended dosage of Mitoshift. Too much will cause high background signal.*

- Remove plates from the incubator and remove the liquid from the wells.
- Add 100 µl of the Live Cell Stain Cocktail to each well and incubate the plate at 37°C for a minimum of 30 minutes.

**Technical Hint**

*The plate must incubate for a minimum of 30 minutes to allow the Hoechst dye to enter the cell nuclei.*

**Sample Analysis**

**Introduction**

Cells are contoured using the Blue2 channel, which identifies the Hoechst (DNA) staining. The Mitoshift is measured using Peripheral Contouring with the Orange channel. Apoptotic cells are measured with the Green (YO-PRO-1) channel.

For this type of analysis, the iGeneration analysis workflow is broken down into five parts:

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

**Initial Set-up**

1. Select the appropriate type of microtiter well plate.
2. Set the iCyte hardware settings as indicated in Figure 5.

<b><i>First pass scan</i></b>	
Laser	Argon, 5 mW
Channels	Green (YO-PRO-1)
	Orange (Mitoshift)
	Crimson (PI)
	Scatter
<b><i>Second pass scan</i></b>	
Laser	Violet
Channel	Blue2 (Hoechst)
Objective	40x
X Step Size	0.5 microns

**Figure 5: Instrument Settings**

3. Place the plate on the instrument.

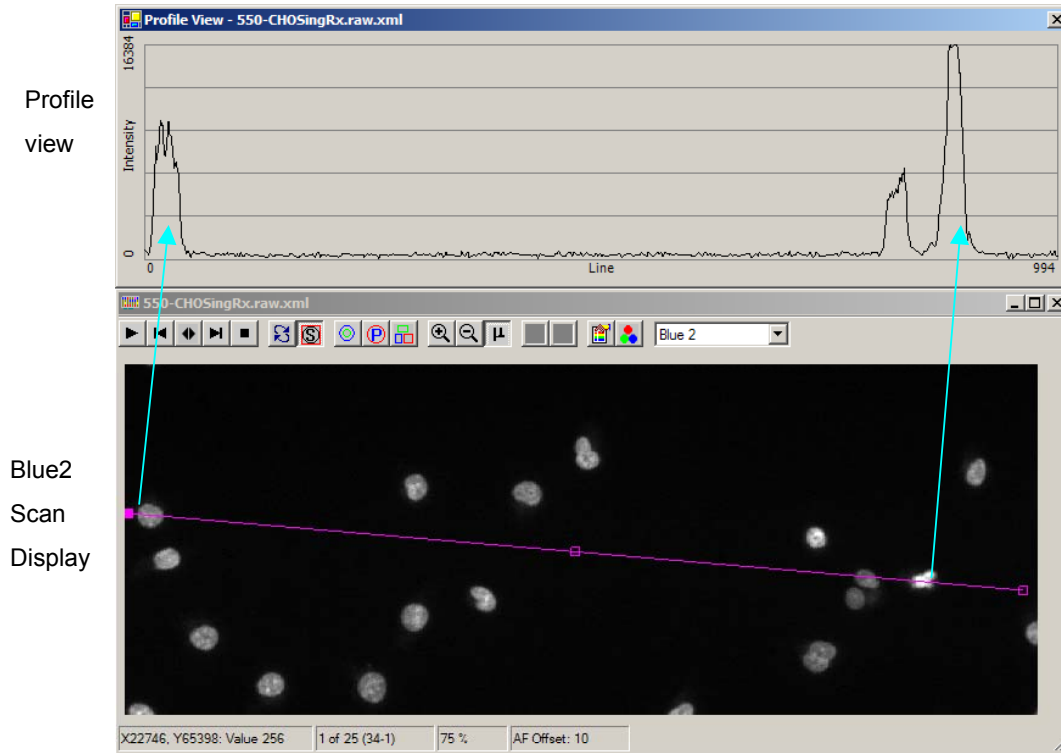
**Scan Set-up**

4. Select a scan area of 5 (wide) x 5 (high).
5. Set the Blue2, Orange and Scatter detectors to the appropriate settings based on a test scan of live control cells.

*Set Blue2Channel:*

- a. Scan a single field in one of the untreated control wells and observe the Blue2 scan image in the Scan View. Move the cursor over several nuclei to display the pixel values.
- b. Adjust the Detector Voltage % to produce pixel values for the dim nuclei of about 5000. 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.

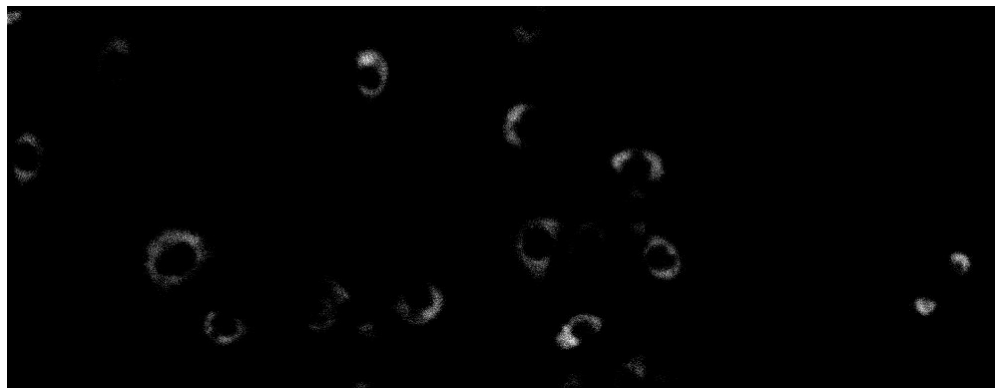
- d. Alternatively, use the Profile function to obtain a scan line through selected cells to estimate the intensity values of the nuclei (Figure 6). Make adjustments based on the nucleus and background readings.



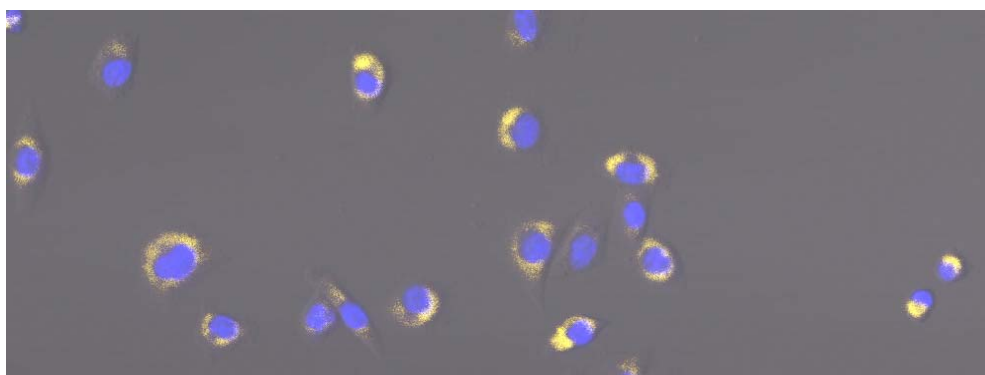
**Figure 6: Profile view of Scan View**

*Set the Orange Channel:*

- e. Select the Orange Fluorescence Scan View. Specific staining of the Mitoshift should be visible as crescents or a halo surrounding the cell nuclei.
- f. Adjust the Orange photomultiplier voltage to give values for live-cell mitochondria up to about 10,000. Adjust the Orange Detector Offset value to achieve a background pixel value below 500. Live-cell mitochondrial staining is shown in Figure 7.



Orange detector showing mitochondrial staining



CompuColor image showing mitochondrial and DNA (blue) staining

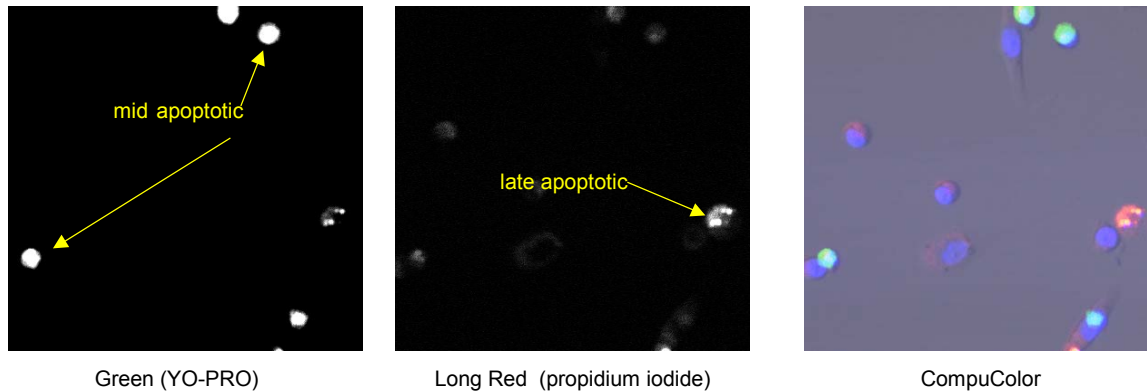
**Figure 7: Orange Detector and CompuColor scanned images**

*Set the Scatter Channel:*

- g. Observe the Scatter scan image in the Scan View. Move the cursor over the background field to display the pixel values. Adjust the Scatter detector gain to give pixel values of about 10,000.
- h. Set the Green and Long Red detectors to the appropriate setting, based on a test scan of wells containing apoptotic cells. Apoptotic cells should be present in wells treated with high dosages of camptothecin or etoposide.

*Set the Green Channel:*

- i. Scan a scan field and observe the Green Fluorescence Scan View image. Although they may be rare, early apoptotic cells (shown as green) should be present, and should exhibit a round morphology as seen in Figure 8. If necessary, advance the scan fields to obtain images of apoptotic cells.



**Figure 8: Scanned Images of early and late apoptotic cells discriminated by YO-PRO and propidium iodide staining**

- j. Adjust the Green PMT so that the green apoptotic cells are close to saturation.

*Set the Long Red Channel:*

- k. Observe the Long Red Fluorescence Scan View. Late apoptotic cells should stain brightly with the propidium iodide and exhibit dense, fragmented nuclei as seen in Figure 8. Advance the scan fields if necessary to find late apoptotic cells.
- l. Adjust the Long Red PMT so that the apoptotic cells are just below saturation.

**Technical Hint**

*YO-PRO-1 will also be detected in the Orange channel. If mitochondrial function of apoptotic cells is of interest, the YO-PRO should be eliminated from the experiment.*

*Annexin V labeled with FITC or Alexa Fluor 488 can be used instead of YO-PRO to label mid-apoptotic cells.*

*Once the detector settings have been optimized, the entire scan can be performed and data saved to a raw data file. The saved data can be used for setting subsequent segmentation and analysis criteria.*

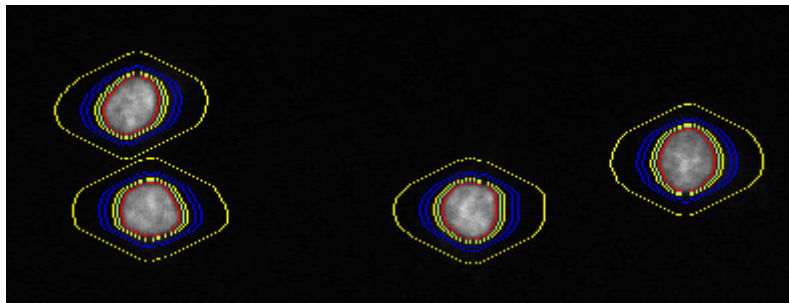
**Segmentation Settings**

- 6. Set the segmentation parameters to achieve optimal segmentation of events. Suggested values are shown in Figure 9 below. Perform rescans after making any setting changes and observe the effect.

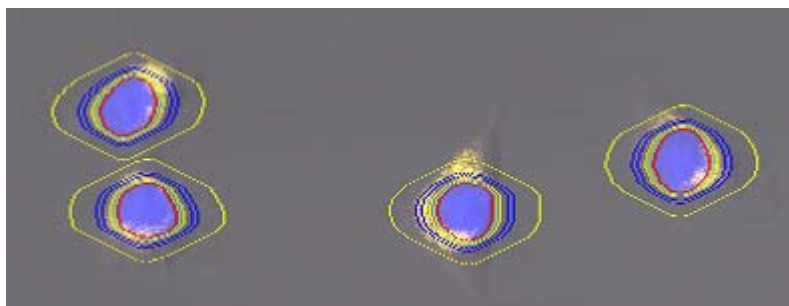
Segmentation Primary	Blue2 (Hoechst)
Threshold	3000
Minimum Area	5 $\mu^2$
Added Pixels	5
Peripheral Contour	Range 2-28
Background	Dynamic, applied to Blue2 channel Distance 4, width 3

**Figure 9: Contouring parameters for this analysis**

- Start a test scan and select the Blue2 channel. When the first scan field image appears in the Scan View window, select Draw Event Contour to view the cellular segmentation. The contours should look similar to those shown in Figure 10, below. In particular, the Red Integration contour should tightly follow the outlines of the nuclei. If the contours are within the nuclei, the segmentation threshold is too high; if they are “loose” or meandering, then the threshold value is too low.



Blue fluorescence - DNA



CompuColor

**Figure 10: Contoured cellular events, Blue2 channel and CompuColor**

- If the contours are not appropriate, open the Adjust window and adjust the Threshold value in the Primary Contour Tab either higher or lower, so that event contours are drawn

around the individual nuclei. Then select the Refresh Contours command to see the effect.

Observe the scan field with CompuColor. Note how the yellow peripheral contours capture a sampling of the mitochondrial fluorescence. Make adjustments as necessary to the peripheral contour settings to optimize sampling of the cytoplasm. In general, increasing the distance away from the cell increases the size of the cytoplasmic area sampled; however, it also introduces greater error as the peripheral contours overlap onto neighboring cells.

**Data Analysis Set-up**

9. Set up the Scattergram and Histogram windows in the iCyte software application as listed in Figure 11.

Title	Segmentation	X Axis	Y Axis	Scale	Regions
DNA content vs. Area	Nuclear	Blue 2 Integral	Area	1.5 E07 400	R1
Chromatin condensation vs. DNA content	Nuclear	Blue Max Pixel	Blue 2 Integral	16384 1.5 E 07	
DNA content	Nuclear	Blue 2 Integral		1.5 E 07	
Cartesian Map	Nuclear	X position	Y position	Normalize	
Nuclear orange vs. cytoplasmic Mitoshift	Nuclear	Orange Integral	Orange Peripheral Integral	2E07 1E07	
Membrane integrity vs. cytoplasmic Mitoshift	Nuclear	Green Integral	Orange Peripheral Integral	5E06 1E07	R2 R3 R4 R5
Mitochondrial staining vs. DNA content	Nuclear	Orange Peripheral Integral	Blue 2 Integral	1 E 07 1.5 E 07	

**Figure 11: Scattergram and Histogram definitions**

The DNA Content vs. Area scattergram is used as an initial gating window. A region is defined around the main cell population to limit the analysis to single cells. See Figure 12A.

The Chromatin Condensation vs. DNA Content scattergram enables separation of mitotic cells from interphase cells. It also can be used to monitor the pyknotic condensation of chromatin in apoptotic cells.

The DNA Content histogram gives the cell cycle status of the cell population. See Figure 12B.

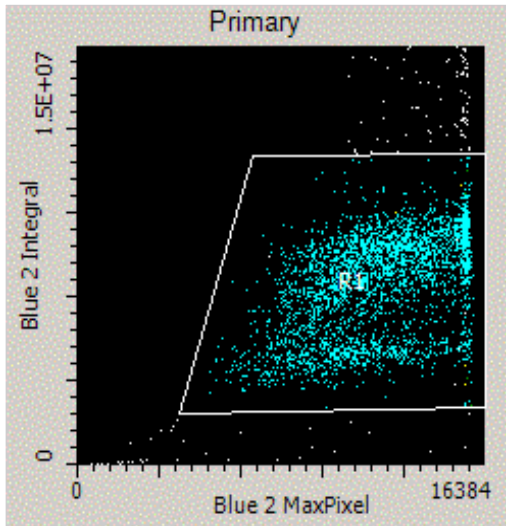


Figure 12A: Chromatin condensation vs DNA content

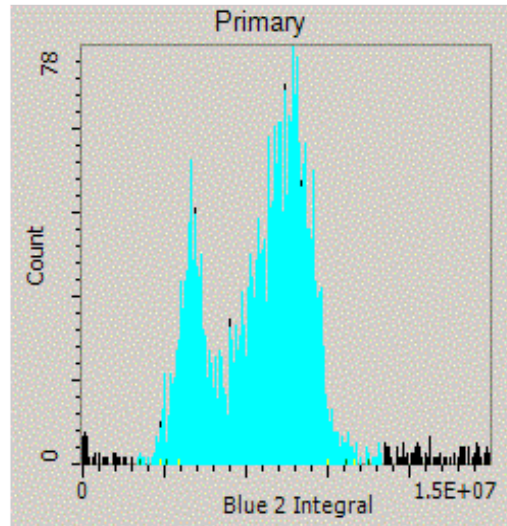


Figure 12B: DNA content

**Figure 12: Cell cycle-related analysis windows**

Cartesian Position Maps are scattergrams that show the location of cells in the scan areas. The maps can be of a single well, giving information about the density and distribution of cells in the well. (See Figure 13A.) They can also display a group of wells, as seen in Figure 13B. (In this case, an entire row was selected. The aspect ratio is distorted to fill the scattergram box.)

The maps can also be set to display data from an entire plate, as shown in Figure 13C. While the density of the dots is too high to see data on individual cells, the overall color provides general information about the cellular makeup of the well.

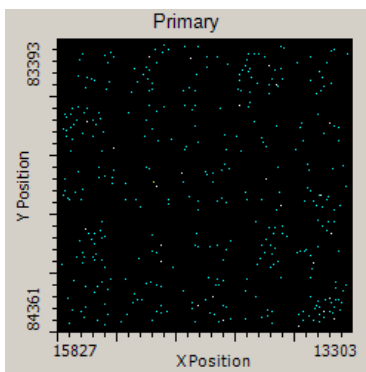


Figure 13A: Single well

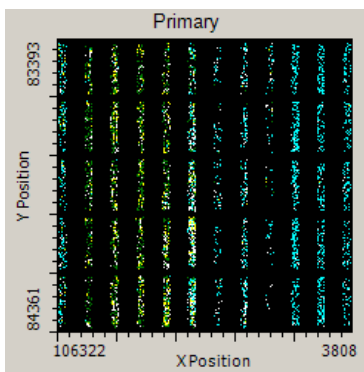


Figure 13B: Single row

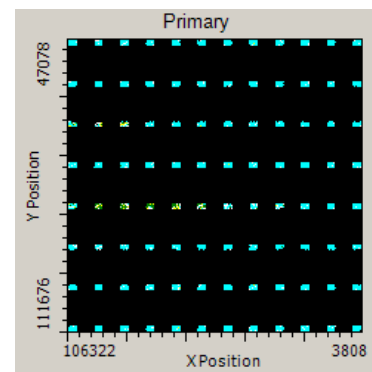


Figure 13C: Entire plate

**Figure 13: Cartesian Position Maps of a single well (A) single row (B) and entire plate (C)**

The Nuclear Orange Fluorescence vs. Cytoplasmic (peripheral) Orange Fluorescence scattergram is used to compartmentalize the Mitoshift fluorescence as shown in Figure 14A.

The Membrane Integrity vs. Cytoplasmic Mitoshift Staining scattergram, Figure 14B, relates the YO-PRO measured membrane permeability to the mitochondrial staining.

The Mitochondrial Staining vs. DNA Content scattergram, Figure 14C, is used to obtain information about the DNA content of live and apoptotic cells.

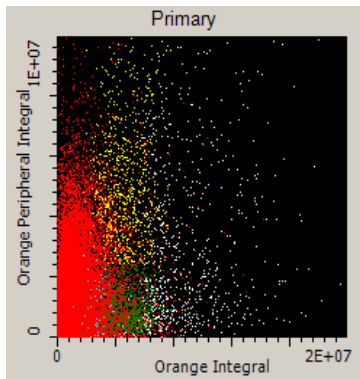


Figure 14A: Nuclear Orange Staining vs. Cytoplasmic Orange Staining

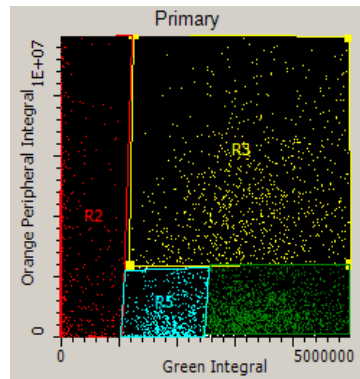


Figure 14B: Membrane integrity vs. cytoplasm

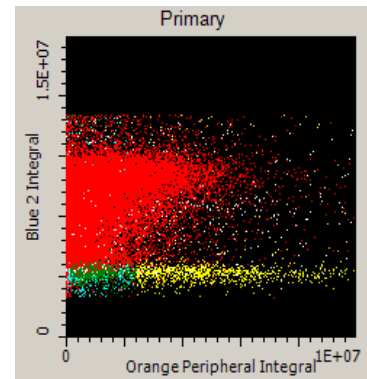


Figure 14C: Mitochondrial staining vs. DNA content

### **Figure 14: Relationships between nuclear and mitochondrial staining**

10. Histograms and scattergrams that are defined in the workspace are exported as data elements for access by the iBrowser™ Data Integration software and other analysis programs.
11. Well features need to be specifically defined to be included in the data output file. For this analysis the well features selected are listed in Figure 15.
12. It is not always possible to set optimal regions before analysis. Regions can be set based on best estimates and previous experimental knowledge, and used to reserve locations in the Well Features file. The regions can be adjusted after the initial analysis, either by rescanning raw data files or by using the population event data.

Component	Feature	Channel	Stat	Region
Primary			Count	
Primary	Area		Mean	
Primary	Integral	Green	Mean	R1
Primary	Integral	Long Red	Mean	R1
Primary	Integral	Blue 2	Mean	R1
Primary	Integral	Orange	Mean	R1
Primary	Peripheral integral	Orange	Mean	R1
Primary	Peripheral Max	Orange	Mean	R1
Primary			Count	R1
Primary			Count	R2
Primary			Count	R3
Primary			Count	R4
Primary			Count	R5

**Figure 15: Well Features for this analysis**

**Data Acquisition**

13. Set the scan-related settings in the Carrier window:
  - a. The entire 96-well plate is to be scanned.
  - b. A 5 x 5 matrix of scan fields is used, giving a scanned area of 2.5 x 1 mm.

**Control Wells**

Before proceeding with data acquisition, a sampling of the control wells must be evaluated.

*Negative Controls:*

DNA distributions in the control wells should have the following characteristics:

- The base line should be low.
- There should be a sharp rise of the G1 peak.
- There should be a distinct G2 peak at about twice the intensity of the G1 peak.

The actual shape of the distribution will depend on the growth conditions of the cell line at the time of testing. In the example shown in Figure 12B, the cells appear to be recovering from a cell cycle block induced by the culture conditions.

It is most important that the control wells have similar DNA distributions. There should be clear separation of the single-cell population from the background, with minimal cellular aggregates or cellular debris.

The cell density should be sufficient for adequate statistical sampling of the number of scan fields set (500 – 1000 cells). If the density is too low, additional scan fields can be added, but this will increase the scanning time.

*Positive Controls:*

Wells treated with compounds with known effects at the test dosages (for example, camptothecin blockage of cells in S-phase of the cell cycle at the lower dosages used in this study) should be scanned to verify the expected results.

**Acquiring Data**

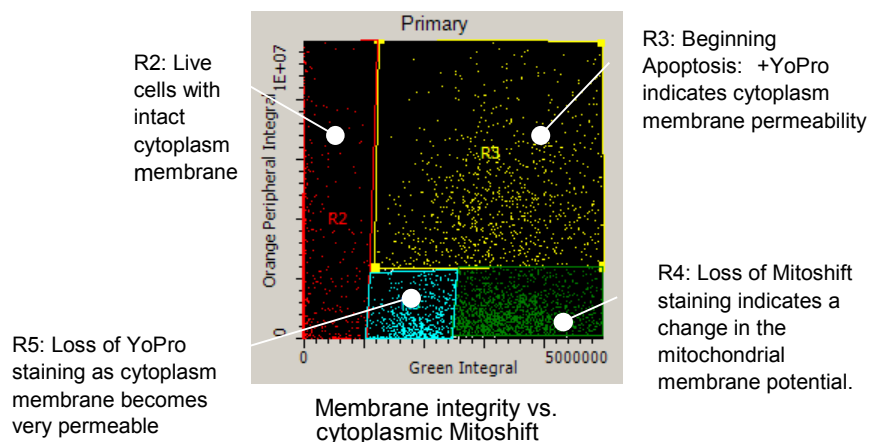
13. Select Scan and Save to acquire data. Saving the raw data files allows re-analysis and experimental verification.

**Data Analysis**

**Identification of Apoptotic Cells**

The Membrane Integrity vs. Cytoplasmic Mitoshift scattergram (Figure 16 below) shows the relationship between the Mitoshift and YO-PRO-1 dyes, allowing monitoring of the stages of apoptosis in relation to the test compound dosages.

- Cells with no YO-PRO-1 staining, *i.e.*, intact plasma membranes, are live and are defined by a region that is colored red (R2). Many of them exhibit high cytoplasmic Mitoshift staining.
- As cells progress through the apoptosis process, they become permeable to the YO-PRO-1 before they lose their Mitoshift staining. A region is drawn around these cells and colored yellow (R3).
- The next stage in the apoptotic pathway is loss of mitochondrial staining potential, while still expressing high levels of the green YO-PRO-1 staining. A region around these cells is colored green (R4).
- As cells continue their apoptosis, their membranes become very permeable and the cells lose the YO-PRO-1 staining. A region is drawn around these cells and colored cyan (R5).



**Figure 16: Relationship between Mitoshift and YO-PRO-1 dyes**

On the Cartesian Position Map for the entire plate (Figure 17A), the apoptotic cells are primarily located at the high-dosage locations on two rows (enclosed within the yellow boxes), corresponding to two of the mitochondrial-specific drugs, Drug E and Drug C. The plot of the single row treated with Drug E (Figure 17B) shows the dosage-related induction of apoptosis. The Chromatin Condensation vs. DNA Content scattergram (Figure 17C) shows that the apoptotic population has a sub-G1 DNA content.

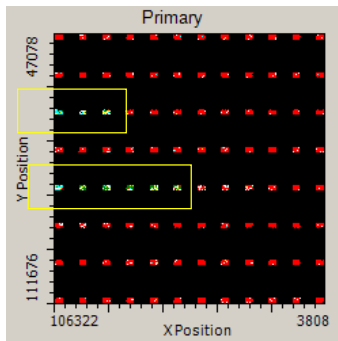


Figure 17A: Cartesian Plate Map

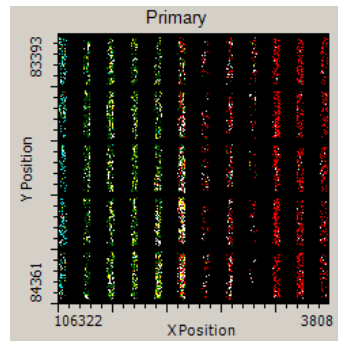


Figure 17B: Cartesian Row Map

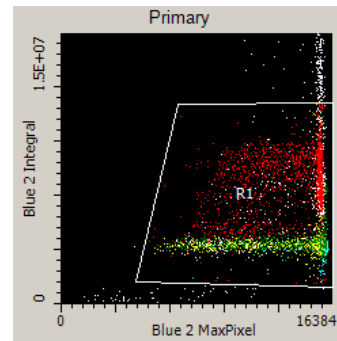
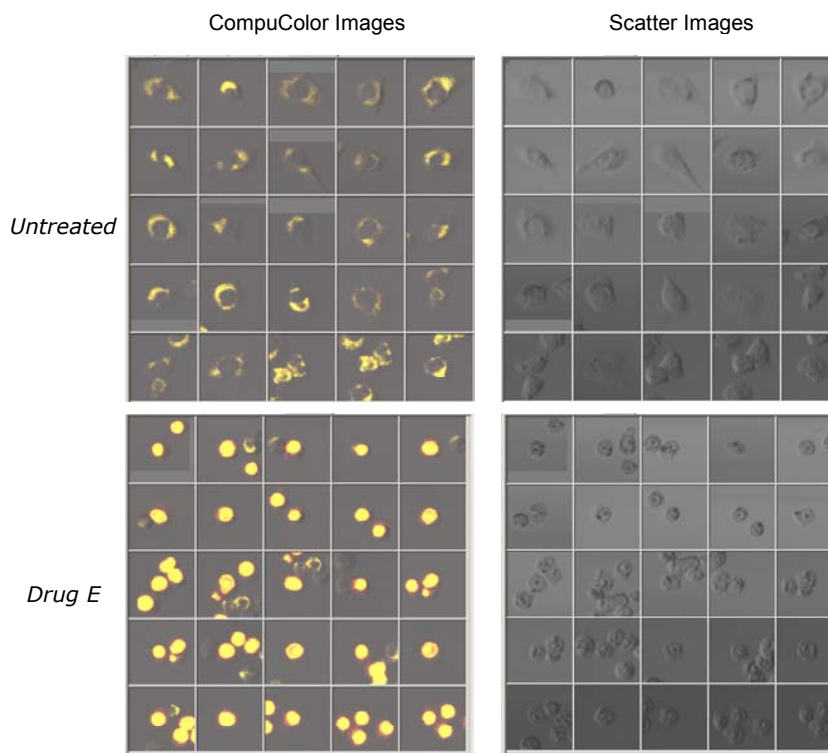


Figure 17C: Chromatin Condensation vs. DNA Content

**Figure 17: Characterization of YO-PRO-1 Positive cells**

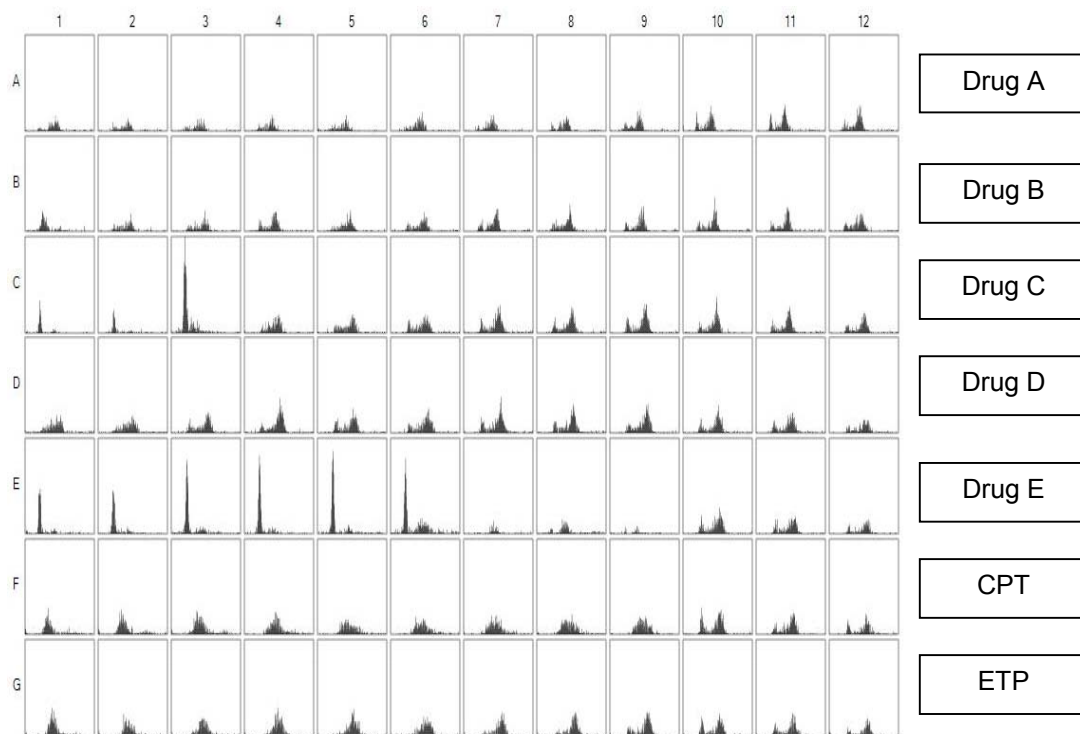
Within the iCyte software application, representative images of any of the identified cell populations can be obtained, either by rescanning or from reanalysis of the raw data files. In this experiment, cells from untreated wells have mitochondrial staining that is seen as bright halos around the nucleus (Figure 18, CompuColor images). The laser scatter images of the cells show a flattened morphology. When cells from the apoptotic regions of a high-dosage well treated with Drug E are viewed, the orange staining of the cells is no longer peri-nuclear. The visualized staining is a combination of the orange fluorescence of the Mitoshift and overlap of the YO-PRO dye into the Orange channel. The rounded morphology of the cells, as seen in both the fluorescence and scatter images, also suggests that they are apoptotic.



**Figure 18: Galleries showing change in mitochondrial staining patterns and cell morphology**

### iBrowser Analysis

The iBrowser™ Data Integration Software can also display and analyze histogram data. Figure 19 shows the DNA histograms from an analysis of the plate. The most pronounced feature is the block of cells in the G1 phase of the cell cycle at high dosages of Drug C and Drug E. CPT and ETP exhibit variable dosage blocks centered in the S-phase portion of the cycle.



**Figure 19: DNA analysis of drug-treated cells**

The same data set is analyzed by a modified version of the Kolmogorov-Smirnov test in the iBrowser (Figure 20). For each row, histograms are compared to the average of their non-treated control wells. The test reports a derived D-value histogram, showing the net differences between the samples and the controls. The strong effects of Drug C and Drug E, as well as the highest dosage of Drug B, are evident, as are the varied effects of the CPT and the ETP.

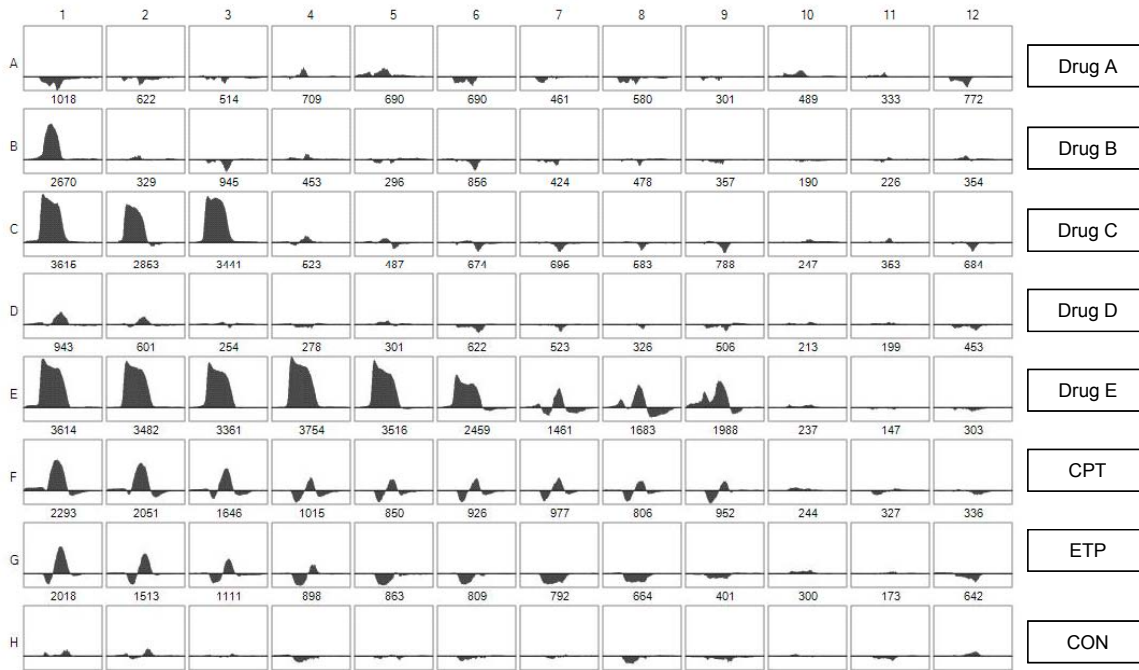
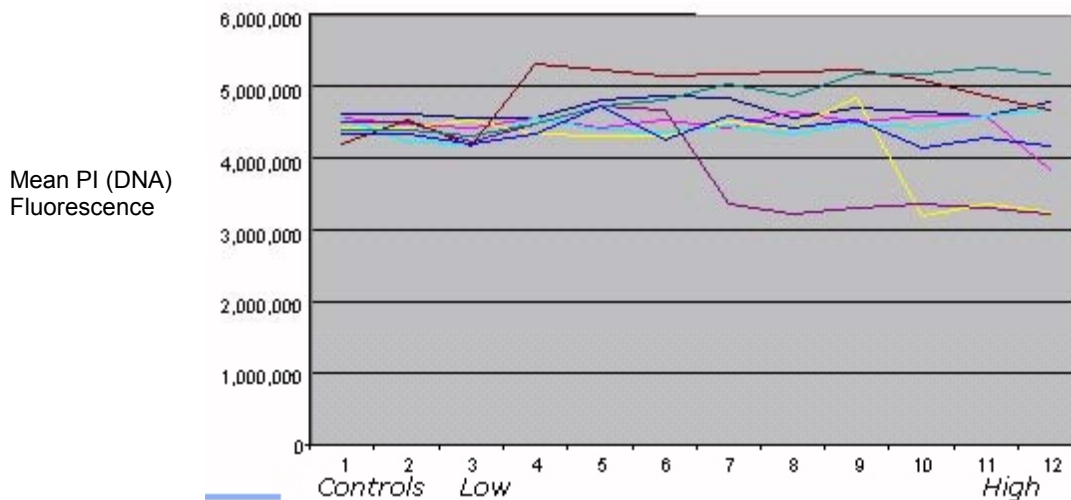


Figure 20: Kolmogorov-Smirnov Test D-Values

**Analysis with Additional Tools**

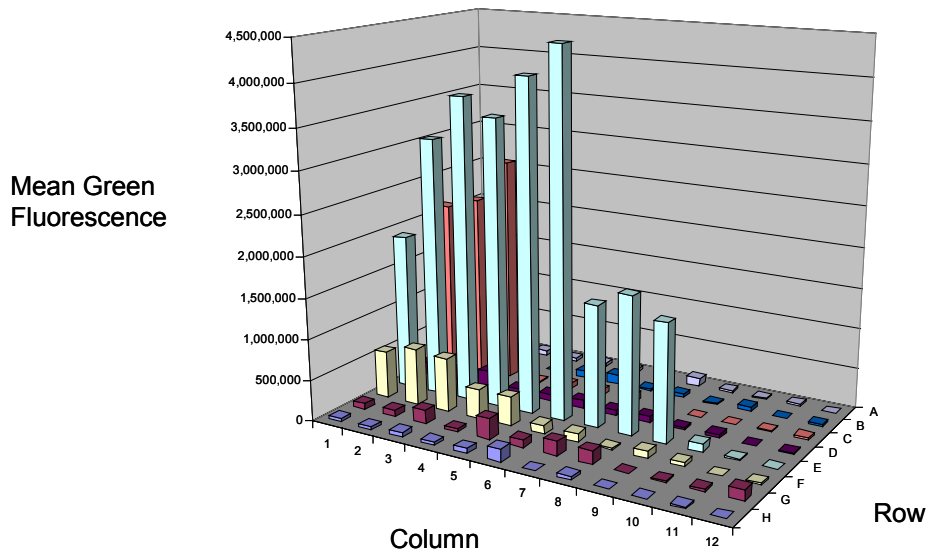
Data for the mean DNA content of the wells was exported into Excel (Microsoft). Here, the ability to simultaneously plot numerous dosage-response curves allows comparison of the cell-cycle effects. The mean DNA content per well is plotted for the entire plate in Figure 21.

Decreases in the mean DNA content are evident for the mitochondrial specific drugs previously noted, reflecting the cells being blocked in the G1 phase of the cell cycle. In contrast, the CPT, and at higher dosages the ETP, show higher mean DNA values reflecting the blockages in S-phase of the cycle.



**Figure 21: Excel Graph showing mean DNA content of drug-treated cells**

Other well features can be formatted by the iBrowser for display in Excel. In Figure 22, the mean green fluorescence, indicative of the YO-PRO staining, is plotted for all wells in the analysis, using the plate format. The rows with the active mitochondrial drugs are easily separated from the background. Any of the saved well features can be similarly plotted, or can be exported to other software databases for integration into existing analysis protocols.



**Figure 22: Excel chart showing green (YO-PRO-1) fluorescence for entire plate**

Summary

Cell lines serve as useful experimental model systems in toxicological studies. When they are stained with homogenous dyes and analyzed in the live state, they remain as close as possible to their natural physiological state. In this protocol, cell lines were treated with a panel of experimental drugs that are believed to trigger apoptosis through mitochondrial pathways.

A four-color homogenous staining cocktail was used to characterize the DNA content, mitochondrial activity, plasma membrane and nuclear membrane integrity of test cells in microtiter plates.

Cell cycle information provided evidence that some of the experimental drugs were blocking cells at the G1 phase of the cell cycle, in contrast to the blockage in S phase with the control drugs. Membrane permeability as evidenced by the YO-PRO staining was seen in the same test populations.

Mitochondrial membrane potential was assessable in live and early apoptotic cells, but as cells went into the later stages of apoptosis, spectral overlap from the YO-PRO dye interfered with the mitochondrial evaluation. Different populations of cells were definable based on the combined Mitoshift and YO-PRO data, and the transition of cells to different populations is drug dosage dependent.

The ability to analyze large numbers of cellular samples in an automated fashion permits use of dose-response curves to detect differential drug-induced responses. Multiple data analysis tools are available at different levels, including the iGeneration application program and the iBrowser™ Data Integration Software. Additionally, data export to third-party software applications is available to enable dissection of often-complex drug effects.

**Features Summary**

This experiment employs the following features available on iGeneration instruments:

<b>Feature</b>	<b>Use during this study</b>
Inverted format	Live-cell scanning in microwells
Violet laser	Excitation of traditional "UV" excited dyes
Multi-channel Analysis	Simultaneous collection of 4 colors of fluorescence and morphological scatter images
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 stage in the cell cycle
Max Pixel Feature	Increased Max Pixel values for apoptotic cell nuclei
Peripheral Integral	Quantification of mitochondrial staining around the cell nuclei
Population Analysis	Correlation of multiple fluorescence colors
Well Features	Characterization of the well itself (such as cell count in the scanned region or average DNA content and event area).
Relocation	Presentation of representative images to allow identification of morphological changes within specific populations
Kolmogorov-Smirnov Test	Highlighting differences in DNA content between control samples and drug-treated samples