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### **Abstract**

The *in vitro* micronucleus assay is routinely performed to detect clastogenic and other chromosomal damage as a result of test samples being exposed to toxic substances. Typically it is performed by manual scoring of the test slides by microscope observation, often using cytochalasin B to identify cells that have completed DNA replication and are able to allow drug effects to manifest themselves.

We investigated if DNA content per cell, the nuclear area, the number of cells per test well, expression of apoptotic markers, combined with the micronucleus count would be a suitable replacement for the cytochalasin B cell blockage method.

CHO cells were seeded in microtiter wells, incubated with test substances (etoposide, mitomycin C) in increasing dosages, with and without cytochalasin B, fixed *in situ*, and stained with DNA specific dyes, and a protein marker. The plates were analyzed with the iCyte Automated Imaging Cytometer (CompuCyte, Cambridge, MA). Dosage response curves were obtained for multiple cellular factors.

In experiments without cytochalasin B, control wells are characterized by an exponential cell cycle distribution, less than .5% micronuclei, and normal nuclear morphology. With an increase in drug concentration we saw a progression of formed micronuclei (up to 12%), blockage of cells in S-phase in the cell cycle, and a decrease in cell count. An increase in nuclear area was observed at the highest dosages.

In cytochalasin B treated experiments, control wells exhibited a large proportion of binucleate cells. Increases in drug dosages were accompanied by increased amounts of micronuclei, a decrease in the percentage of binucleate and total number of and the appearance of sub-G2 phase cells in the cell cycle distributions. A moderate increase in nuclear area was registered.

Both methods gave equivalent results in terms of the dosage presenting the maximum micronuclei, however, the non-cytochalasin B treated sets had a higher signal to noise level, indicating greater sensitivity.

For further information on this and other LSC applications, contact [techsupport@compucyte.com](mailto:techsupport@compucyte.com)

## ***Drugs Tested***

### **Etoposide**

- Antitumor agent that complexes with topoisomerase II and DNA to enhance double-strand and single-strand cleavage of DNA and reversibly inhibit re-ligation. Blocks the cell cycle in in S-phase and G2-phase of the cell cycle; induces apoptosis in normal and tumor cell lines
- Starting dose=50uM, serial dilute 1:2

### **Cyclophosphamide**

- S-phase dependent clastogen
- It cross-links DNA, causes strand breakage, and induces mutations. Its clinical activity is associated with a decrease in aldehyde dehydrogenase 1 (ALDH1) activity.
- Starting dose high=50ug/ml and low=1 ug/ml, serial dilute 1:2

### **Actinomycin D**

- An antineoplastic antibiotic that inhibits cell proliferation by forming a stable complex with DNA and blocking the movement of RNA polymerase which interferes with DNA-dependent RNA synthesis. Induces apoptosis. Potent antitumor agent.
- Starting dose high=50ug/ml and low=1 ug/ml, serial dilute 1:2

### **Vincristine Sulfate Salt**

- Plant alkaloid that inhibits microtubule assembly by binding tubulin and inducing coiled spiral aggregate formation. Arrests cell cycle in G2/M-phase by blocking mitotic spindle formation.
- Starting dose high=20ug/ml and low= 0.10ug/ml, serial dilute 1:2

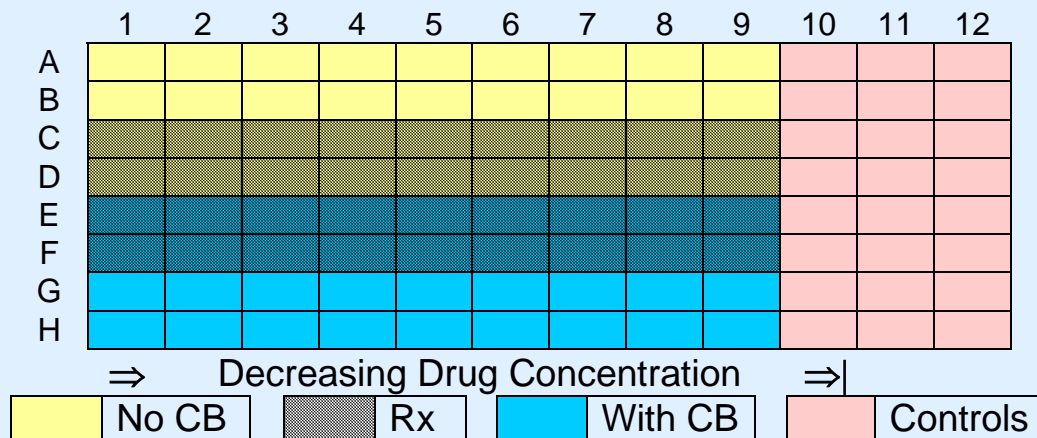
### **Cisplatin**

- cis-Diammineplatinum(II) dichloride
- Forms adducts with the DNA dinucleotide d(pGpG), inducing intrastrand crosslinks.
- Starting dose high=0.5 mg/ml and low=2 ug/ml, serial dilute 1:2

### Materials and Methods

CHO cells were obtained from the ATTC and kept in continuous culture.

For experiments, cells were seeded into the wells of 96-well optical plastic microtiter plates (Whatman) at empirically determined densities, and allowed to stabilize overnight. They were then exposed to the test substances for 7 hours. Columns 10 – 12 of the plate were control wells, and columns 9 to 1 had increasing amounts of the test substance. Rows A and B, and G and H were untreated control rows. One half of the plates, rows E to H, were exposed to cytochalasin B, and the cells were incubated overnight. The cells were then fixed with ethanol, and stained with FITC (Molecular Probes) to label the protein of cells. Propidium iodide (Molecular Probes) and RNase (Sigma) were used to stoichiometrically label the DNA of the cells.

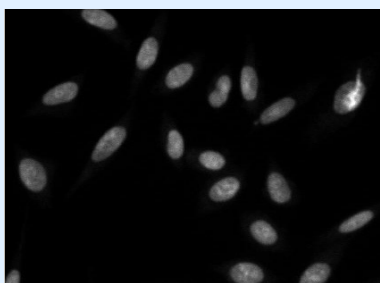


Experiments were analyzed on the iCyte™ Imaging Cytometer (CompuCyte, Cambridge, MA).

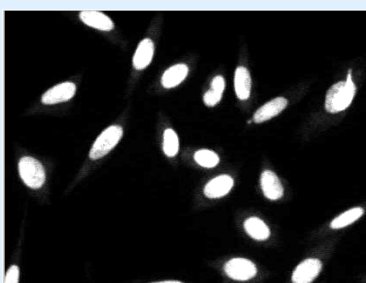
Argon ion laser( 488 nm) excitation was used to excite the fluorescent dyes. Signal detection by photomultiplier tubes was as follows: Channel 2 –Green: FITC staining of the cytoplasm. Channel 3 – Orange: Optical summation of the FITC signal and some of the PI signal; Channel 4 – Propidium Iodide staining of the DNA set for stoichiometric cell cycle analysis. In addition, virtual channels were used to create masks of the nuclear staining for nucleus and micronucleus segmentation.

## Segmentation Strategy 1

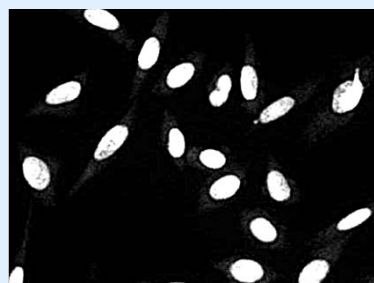
In this segmentation strategy, the green and orange channel images are summed and used to obtain an image of the cells cytoplasm that is used as the primary identification of events. The crimson scan image is obtained and used for quantitative DNA content analysis. Binary masks of the DNA staining are created as a separate channel, and segmented as sub-events. Image processing watershed algorithms are employed to dissect closely spaced nuclei and micronuclei.



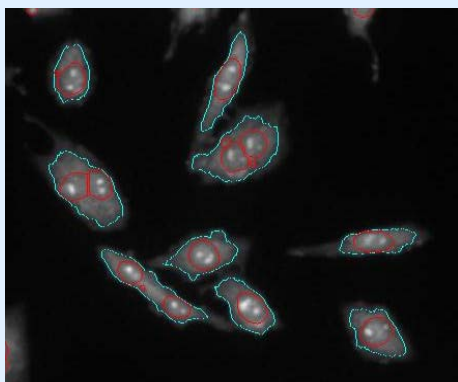
*DNA scan field used for stoichiometric analysis*



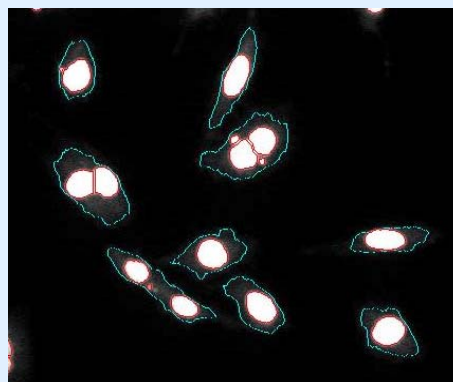
*Saturated scan field used for morphological evaluation*



*Sharpened scan field to highlight small events*



*The cyan contours are automatically drawn around cells based on the staining of the cytoplasm.*



*The red contours are drawn around the nuclei based on the DNA staining.*

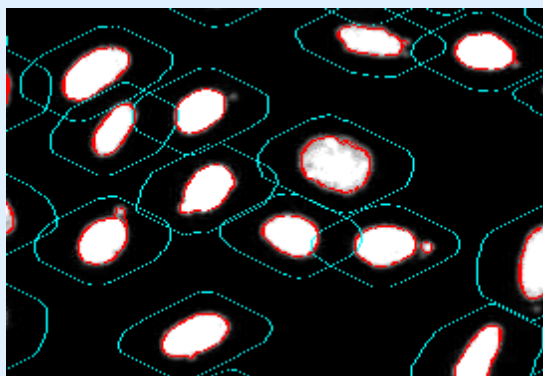
## Segmentation Strategy 2

In the course the second round of experiments, the loss of cytoplasmic staining was noted in some samples, so an alternative segmentation strategy was devised. In this analysis strategy, primary segmentation was done on saturated images of the cell nuclei. The area was expanded by 20 pixels, and is represented by the cyan contour. Secondary segmentation was also done on the saturated nuclei, but at a higher threshold level. The secondary contours were not expanded, and are drawn in red.

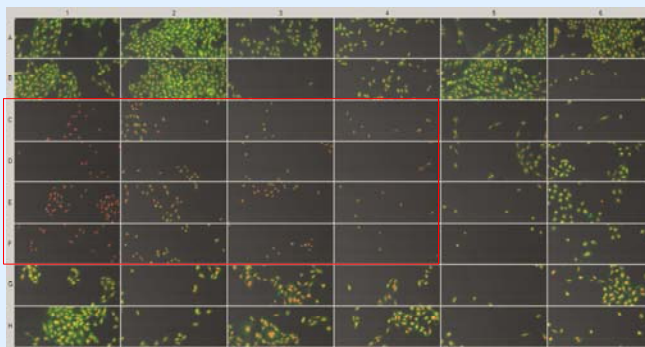
The method is also more tolerant of closely spaced cells. There is a strong possibility that the integration area may encroach over a neighboring cell, and that a particular micronuclei may be counted more than once. The probability of this happening is independent of the amount of micronuclei, and thus it is an unbiased error.

It is also possible that the cytoplasm of the cells may extend past the integration contour, and some micronuclei may be missed. Again, this error applies to both positive and negative samples.

Other experimental errors occur in both methods, including the caveat that if a cell extends beyond the boundary of the scan area, it is not counted. Again, this is a non-biased error.



*Peripheral segmentation method*



*Cisplatin induced loss of  
cellular cytoplasm*

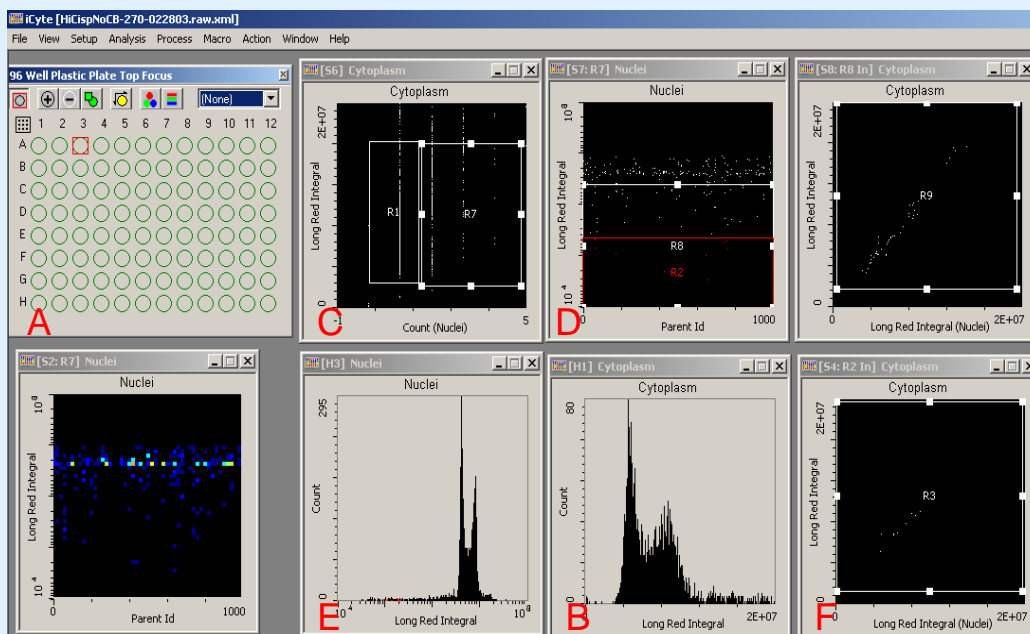
## Analysis Strategy

Laser Scanning Cytometry (LSC) technology from CompuCyte is based on obtaining many features for each of many thousands of cells. Techniques for this kind of population analysis have been developed and include entities such as scattergrams (dot plots) histograms, and two-dimensional histograms (density plots). One parameter histograms showing frequency distributions are common, either on logarithmic or linear scales. There is the ability to draw regions around specific populations of interest, and then these regions can be used as the source for subsequent data displays, or as the source for image data.

Similar analysis paradigms are employed for both segmentation strategies, with slight modifications made to accommodate the use of the Cytochalasin B in the first gating strategy.

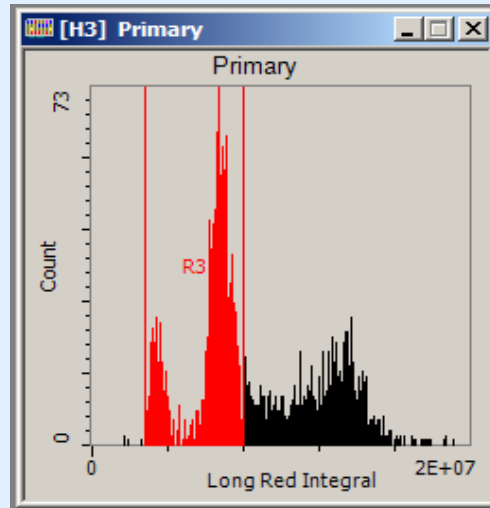
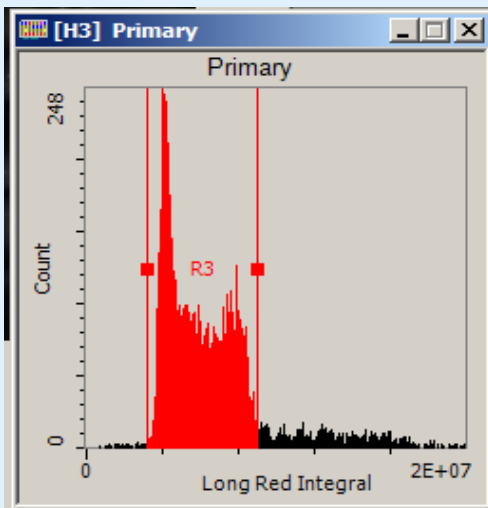
The basic workflow of the assay as shown in the screen is as follows:

- Window A is an icon showing the current analysis well on the plate.
- B is the DNA content histogram of the well.
- C plots the DNA content vs. the number of nuclei per cell. Regions are drawn to separate mononucleate vs. binucleate cells.
- D is a plot of the DNA content of the nuclei vs. their parent cell ID. The regions are drawn to identify cells with damaged or micronuclei (colored red).
- E is a histogram of the DNA content of the nuclei. Note the logarithmic scale. The micronuclei are colored red.
- F is a scattergram that reconverts the nuclei data to cellular data. This region is used to quantify the number of cells that have micronuclei.



***Analysis Strategy, continued***

The first step is to use a gating region to restrict further analysis to cells that have the proper ploidy level for the analysis. In general, this is between the 2N and the 4N ploidy values. Segmented events with higher ploidy levels are generally multiple cell events.

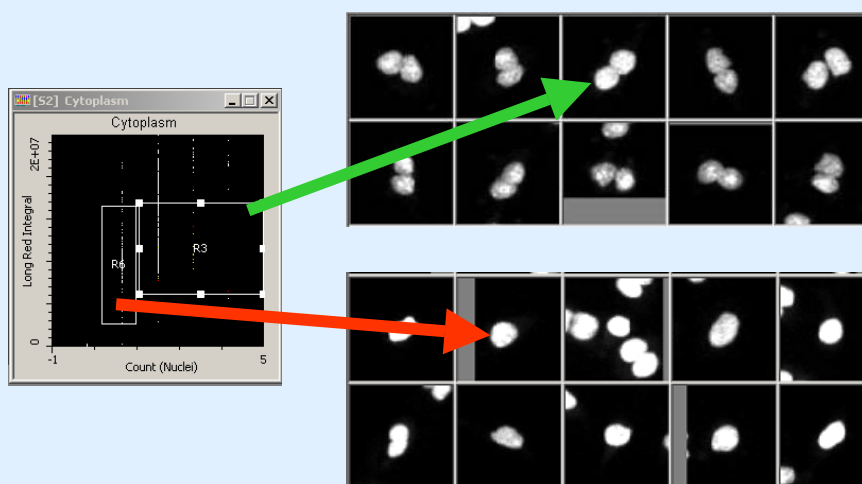


***Single vs. Multinucleated Cells Are Identified***

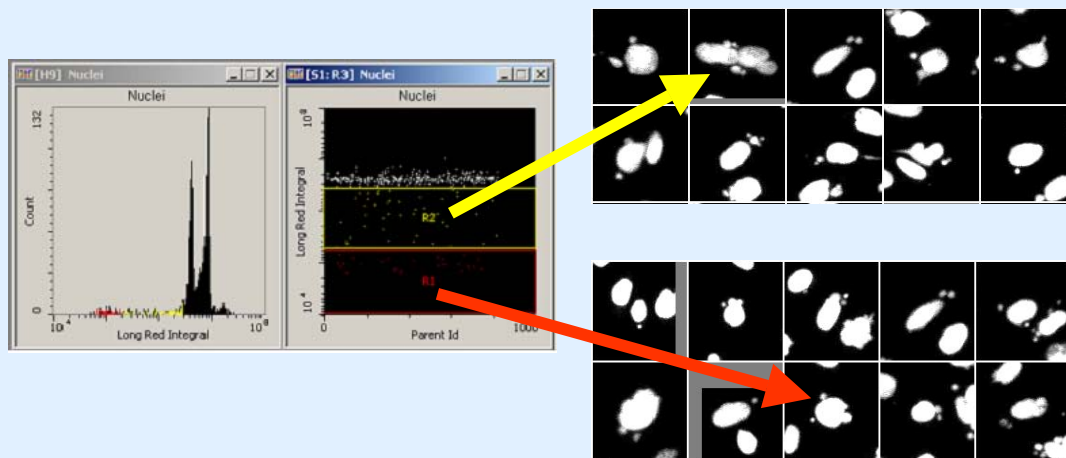
### *Analysis Strategy, continued*

#### *DNA Content is Used to Characterize The Size of Aberrant Nuclei*

The second step is to display the number of nuclei found for each event. This separates single from binucleate cells, as well as cells containing micronuclei. In non-CB assays, the cells with two or more nuclei are potential micronuclei bearing cells. In CB assays, cells with three or more nuclei are potential micronuclei bearing cells.



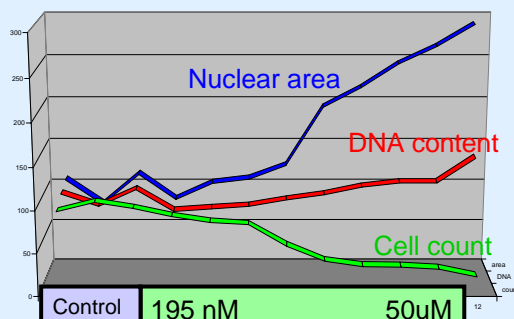
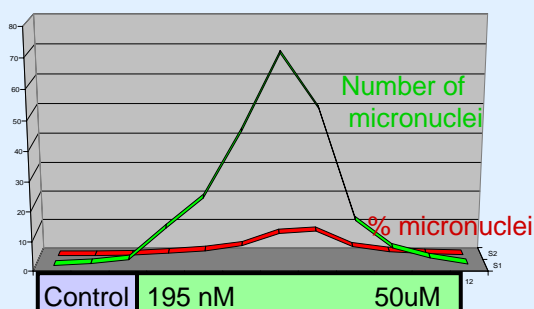
The third step is to characterize the individual nuclei for each parent cell based on the DNA content. Adjustable criteria are used to identify micronuclei.



## Etoposide Results

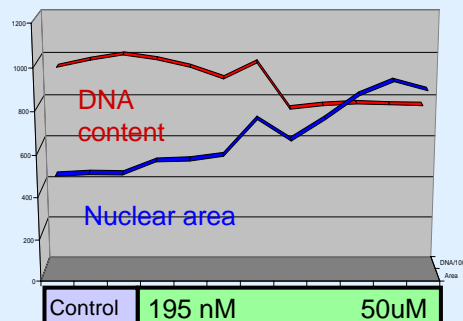
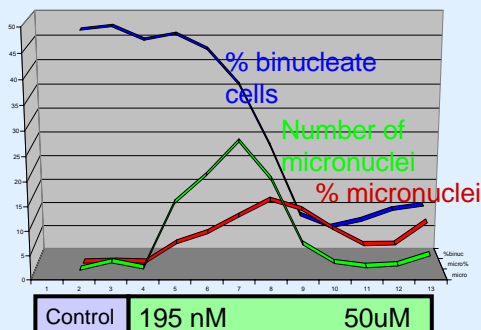
### Etoposide Effects Without Cytochalasin B

In the initial rounds of experiments involving etoposide, micronuclei were automatically identified, and were found to peak at the mid range of the dosages used. Other features regarding the cell populations were noticeable at higher dosages, such as a marked increase in the nuclear area, an increase in the DNA content as the cells are being blocked in S phase, and a decrease in the cell numbers per scanned area per well.

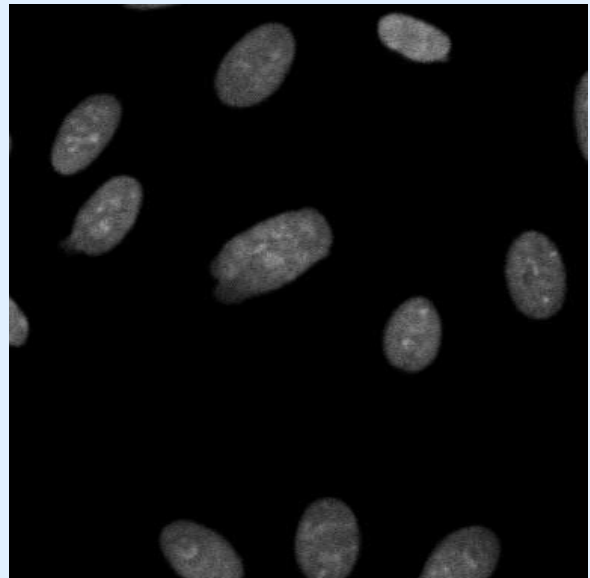
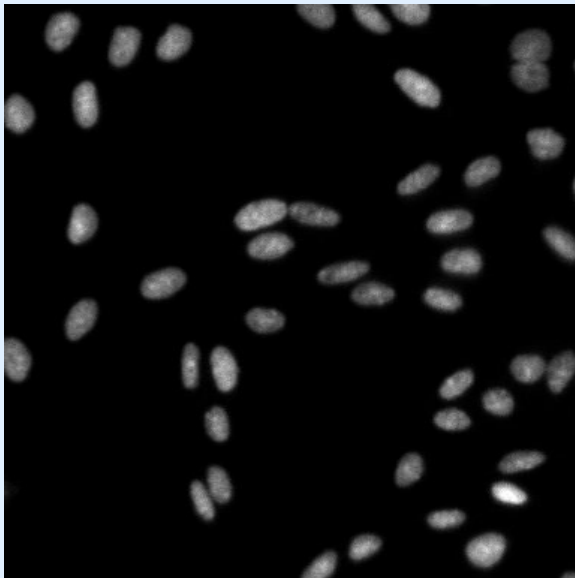


### Etoposide Effects With Cytochalasin B

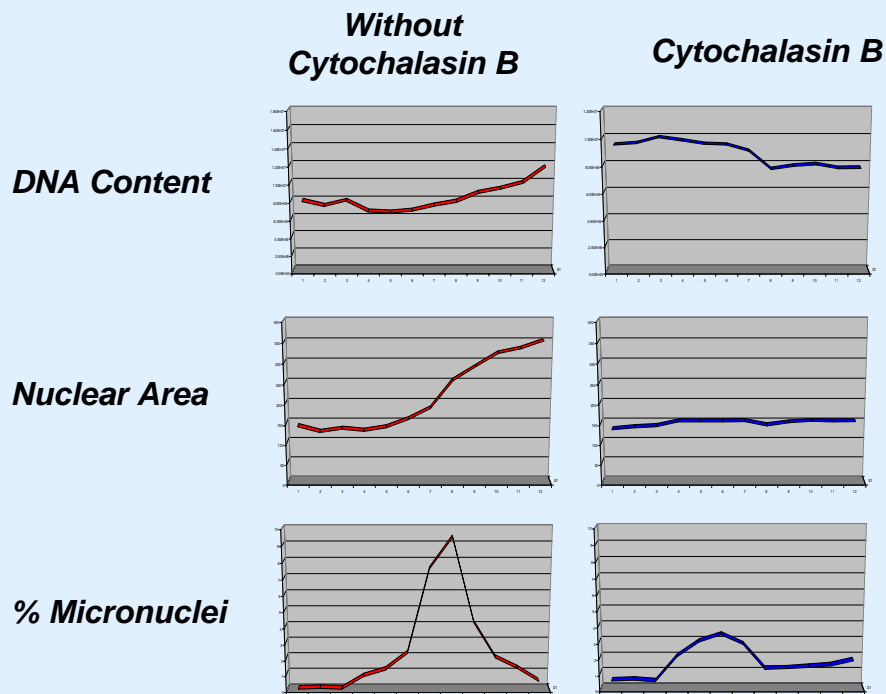
In Cytochalasin B treated wells, the percentage of micronucleated binucleate cells peaks at approximately the same dosage, although there is an increase in the absolute numbers of found micronuclei. Concurrently, this is the beginning in a sharp decrease in the number of binucleate cells found. This is indicative of cells being blocked in S phase and not progressing far enough in the cell cycle to be affected by the CB. The mean DNA level goes down from the starting level, contrasting sharply to the untreated cells.



***Etoposide Results, continued***



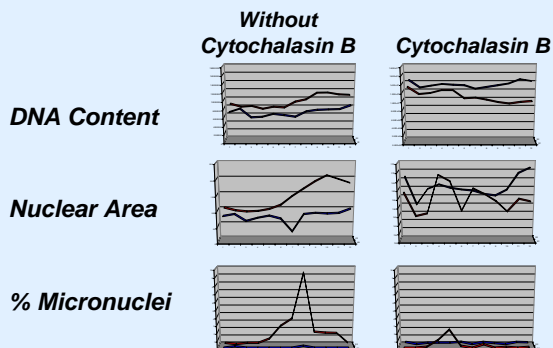
*Enlargement of the cell nuclei was particularly evident in etoposide treated samples.*



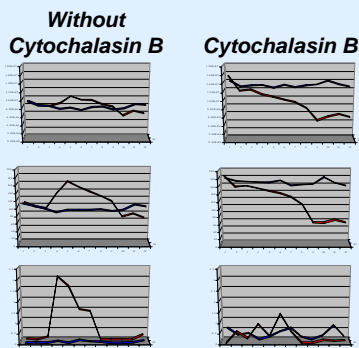
### Testing a Variety of Substances

These are graphs of the most informative dosage for the panel of test substances. Data was gathered and graphed for both treated (Red) and control (Blue) cells.

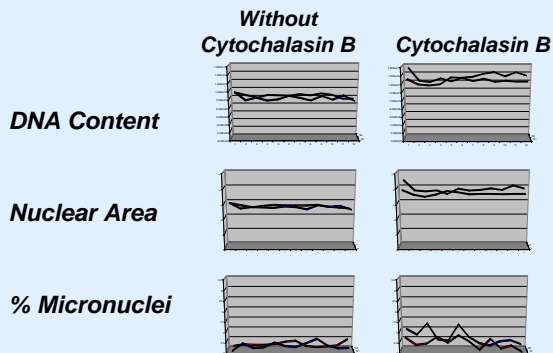
#### Etoposide



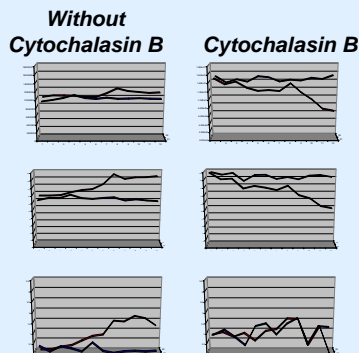
#### Cisplatin Low Dosage



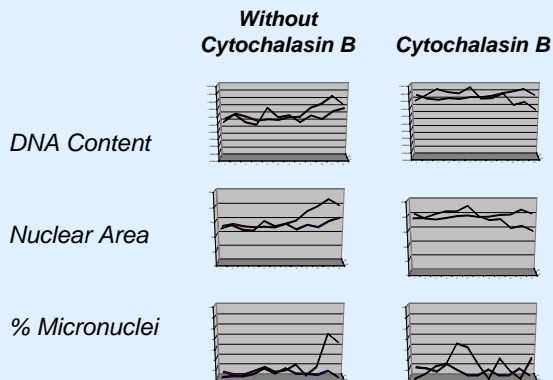
#### Cyclophosphamide High Dosage



#### Actinomycin High Dosage



#### Vincristine Sulfate Salt High Dosage



### Summary Table

The summary table highlights conditions that were visually determined to demonstrate an effect on the cell populations. The presence of micronuclei is accompanied by other changes.

	Micronucleus	DNA Content	Nuclear Area
<b>Etoposide High</b>	Well defined 9% peak	Increase from 6	Strong increase (doubling)
With CB	Well defined 3% peak	decrease from 5	no change
<b>Cyclophosphamide High</b>	not discernable back < .5%	no change	no change
With CB	not discernable back 1.2%	no change	no change
<b>Cyclophosphamide Low</b>	not discernable back < .5%	no change	no change
With CB	not discernable back .8%	no change	no change (noisy)
<b>Actinomycin D High</b>	gradual increase to 1.5% hi	moderate increase	gradual increase levels off
With CB	not discernable, high backgrd.	high decrease	continuous decrease
<b>Actinomycin D Low</b>	not discernable	no change	slight increase
With CB	not discernable	slight decrease	slight decrease
<b>Vincristine sulfate High</b>	2% peak	increase	increase
With CB	Not discernable	decrease	decrease
<b>Vincristine sulfate Low</b>	not discernable	no change	no change
With CB	2.50%	no change	no change
<b>Cisplatin High</b>	not discernable	no change	no effect
With CB	Possible peak of 3%	no change	no effect
<b>Cisplatin Low</b>	Well defined 3% at low range	increase then decrease	Increase then decrease
With CB	not resolved	decrease	decrease

## Discussion

Automated scanning and analysis of cells grown and treated and stained in microtiter plates was done, and micronuclei were detected with some of the drugs tested. Etoposide gave the highest percentage of micronuclei, with discernable increases over background seen in cisplatin, vincristine and actinomycin. Although the percentages were low, around 2%, images of detected micronuclei were used to verify the identification.

In this system, there were substantially lower numbers of micronuclei found in the cytochalasin B samples.

In non-drug controls, there is a different starting base value for the average amount of DNA content per cell in untreated (NCB) and cytochalasin B treated (CB) samples. In NCB samples, the average value is based on the average of the cycling cells in the population, so it will be at a level higher than the diploid level for the test population. In the CB samples, the starting value is at a level equivalent to the tetraploid value for the cells. The NCB cells are in an open ended experimental mode, while the CB cells are at a fixed end point, i.e. on S-phase division.

If the cell cycle is perturbed by a blocking agent, i.e. camptothecin, an agent known to block cells in S-phase, the effect on the NCB cells will be an **increase** in the average DNA level per cell, as there is a depletion of G1 level cells from the population. In CB treated cells, there will be a **decrease** in the average DNA value, as cells failed to reach the cytochalasin induced cell cycle block.

Blockage in the cell cycle was usually accompanied by changes in the nuclear area.

When micronuclei were present in samples above control levels, there were usually accompanying changes in the DNA content and also the nuclear area of the cells. These changes usually occurred at higher dosages of the test drugs than those that exhibited the peak micronuclei formation. Other cellular markers, such as the amount of cytoplasmic staining, and the number of cells present in the scan areas also give information about the toxicity of the compounds. Together, these data present a panel of markers for the toxicity of a compound, with the clastogenic effects measured by the presence of micronuclei.

The purpose of adding cytochalasin B to micronucleus assays is to ensure that the cells undergoing the test have undergone DNA synthesis, and are thus eligible to manifest clastogenic effects. In effect, it is a morphological indicator of the cell cycle status. In laser scanning cytometry, the status of the cell cycle is measured directly by the DNA content, and also indirectly by the nuclear area and other factors. This panel of markers gives the same information as the cytochalasin B does in the manual methods.

In this study, the automated analysis found fewer micronuclei in the Cytochalasin B treated samples, and there was often a higher background level associated with them. Essentially, there was less sensitivity. Since cell cycle information is obtained by other features, it appears that for automated analysis, it is preferable not to use cytochalasin B.