

Automated Laser Scanning Imaging Cytometry in Apoptotic Pathway Characterization

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Abstract

The past two years have witnessed significant progress in the development of laser scanning cytometry (LSC), which represents a new trend in a cell-based screening for the purpose of new cancer drug development. The solid phase approach enables stoichiometric quantification of molecular labels in combination with morphological image analysis to compartmentalize the location of dyes within cells and determine even subtle morphological changes. Two novel assay technologies were utilized on the LSC platform (CompuCyte Corporation, MA). Multiple cell-based readouts were obtained simultaneously, including DNA content, nuclear area, chromatin condensation, DNA strand breaks utilizing phosphorylated H2AX antibody (Trevigen, MD) and caspase-3 cleavage (Cell Signaling, MA).

In microtiter well assays, features were reported for each of the wells, including the cell count and distribution, important in detecting cell loss due to apoptosis or necrosis. Software algorithms have been developed that treat the entire experiment as the data base, instead of individual samples. The morphometric capabilities of LSC were used to detect and measure translocation of Bax from cytosol to mitochondria and to correlate it with overall Bax and Bcl-2 abundance in the cell, and with cell cycle phase. Activation of NF-kappa B, the event triggering cell survival response mechanisms, was also assayed by measuring its translocation to the nucleus. The capability to relocate cells for imaging was critical to discriminate between atypical apoptosis and necrosis and to distinguish macrophages that phagocytized apoptotic bodies from apoptotic cells. Another unique capability of LSC allowed us to correlate events of apoptosis detected in real time in live cells with the events that can be detected only after cell fixation.

A powerful strategy to investigate apoptotic mechanisms was developed using Fluorescent Resonance Energy Transfer (FRET)-detection. Recent development of FRET-based biosensors that have both cyan and yellow fluorescent proteins (CFP and YFP) linked to the same construct made this approach feasible. Experience with studying gene expression in single cells versus cell populations has shown that even homogenous cell populations possess a high degree of cell-to-cell-variability. Many single-cell recordings and the resulting statistics are needed to analyze the apoptosis response patterns. Such large numbers of cells (hundreds or preferably thousands) can be easily monitored using biosensors and LSC technology. One of the most conserved features of apoptotic response is the activation of caspases. The powerful family of FRET-biosensors is based on CFP-YFP molecules connected by peptide linker with caspase cleavage sites. In the absence of apoptotic stimuli, the cells transiently or stably transfected with FRET-caspase sensor shows a high level of FRET that decreases after specific proteases disrupt the cleavage site in the peptide linker. The discovery that novel cancer therapeutics may be able to activate caspases and overcome tumor growth represents a possible approach to effective tumor treatment if used together with conventional therapies.

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

Introduction

Apoptosis is an evolutionary, conservative, cellular process that plays an important role during organism development and is one of the major mechanisms of tissue homeostasis. Because of its contribution to the pathophysiology of cancer, it became a focus pathway in anti-tumor drug development. Among the key biochemical mediators of apoptosis is a group of proteolytic enzymes known as caspases, which cleave specific target proteins with either structural, regulatory, or housekeeping functions (Thornberry, Lazebnik, 1998).

Unfortunately, methodology for monitoring apoptotic activity in the context of cell-based model systems is often limited to analyzing downstream biochemical events (i.e. DNA fragmentation, western blotting, etc).

The ability to visualize and test apoptosis by quantifying cell cycle dynamics provides a powerful approach for investigation of regulation and functioning of the apoptotic pathways. Cell count, size, fluorescence intensity, nuclear area, chromatin condensation, etc., are most relevant and useful parameters. Quantification of biological reactions in large cell quantities, however, requires automation of data acquisition and complex statistical evaluation of data. Laser scanning cytometry technology (LSC) allows automated processing of thousands of cells and correlation of cytometric data with morphological information.

New classes of antibodies are emerging that recognize not only presence of specific molecules, but also their functional state; adding results from these antibodies to the automated analysis paradigm increases the understanding of the sequence of the events in the apoptotic pathway.

Advances in molecular biology have brought the ability to engineer new cellular organisms that can be used as model systems. We will show how a biomarker construct is applied to give a sensitive marker for early activation of a caspase, one of the key events in the apoptotic cascade.

Laser Scanning Cytometry

Laser scanning cytometry (LSC) was developed as a means to apply the tenets of flow cytometry—such as the quantification of multiple fluorescence signals for each of many thousand cells in a population—to samples affixed to a solid substrate. LSC was originally designed around an upright microscope, relying on the microscope optics for cellular visualization. Recently, two new inverted-format versions of the instrument have been released for automated (iCyte™) and interactive (iCys™) data analysis.

The inverted format enables analysis in new carrier types such as microtiter plates for higher experimental throughput, and plates and chamber slides for live -cell analysis. New computer software paradigms and strategies allow the hardware to take advantage of new developments in biological research.

Laser scanning cytometers are equipped with up to three lasers and four photomultiplier fluorescence detectors, and a light scatter detector.

For each of the excitation lasers, scanned images (C-scans) are obtained for each of the detectors being employed. In addition, C-scans can be mathematically or logically combined to perform new virtual C-scans. These C-scans are segmented using image- or stereological-processing techniques, and relevant feature data is calculated and stored for large numbers of cells or elements. Images are stored, and population data analysis techniques (using scattergrams, histograms, multi-tiered gating and mathematical processing) are used to characterize cellular populations. Well features are collected to characterize the populations in individual wells of microtiter plates.

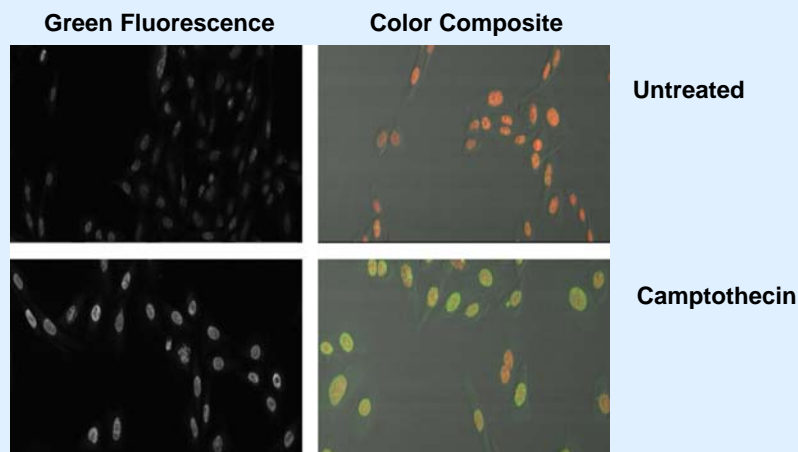


iCyte™ Automated Imaging Cytometer

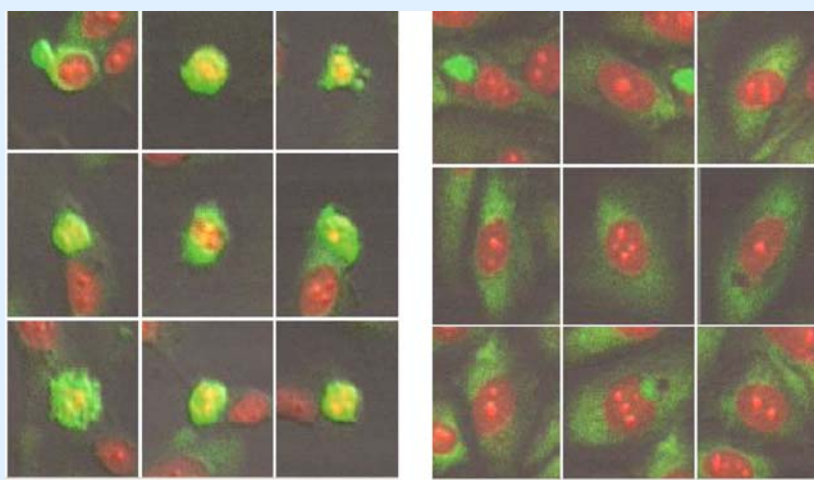
| Source | Scan Fields | Segmentation | Analysis | Well features |
|-----------|-------------|--------------|----------|---------------|
| Argon 488 | | | | |
| Diode 406 | | | | |
| HeNe 633 | | | | |

Fluorescent Antibody Technology

New fluorescent-based probes continue to increase the complexity of analysis of cellular and molecular functions. One of these is anti-sera to the phosphorylated form of the histone, H2AX (Trevigen, MD). The histone is phosphorylated around the sites of ds DNA strand breaks in a temporal fashion.



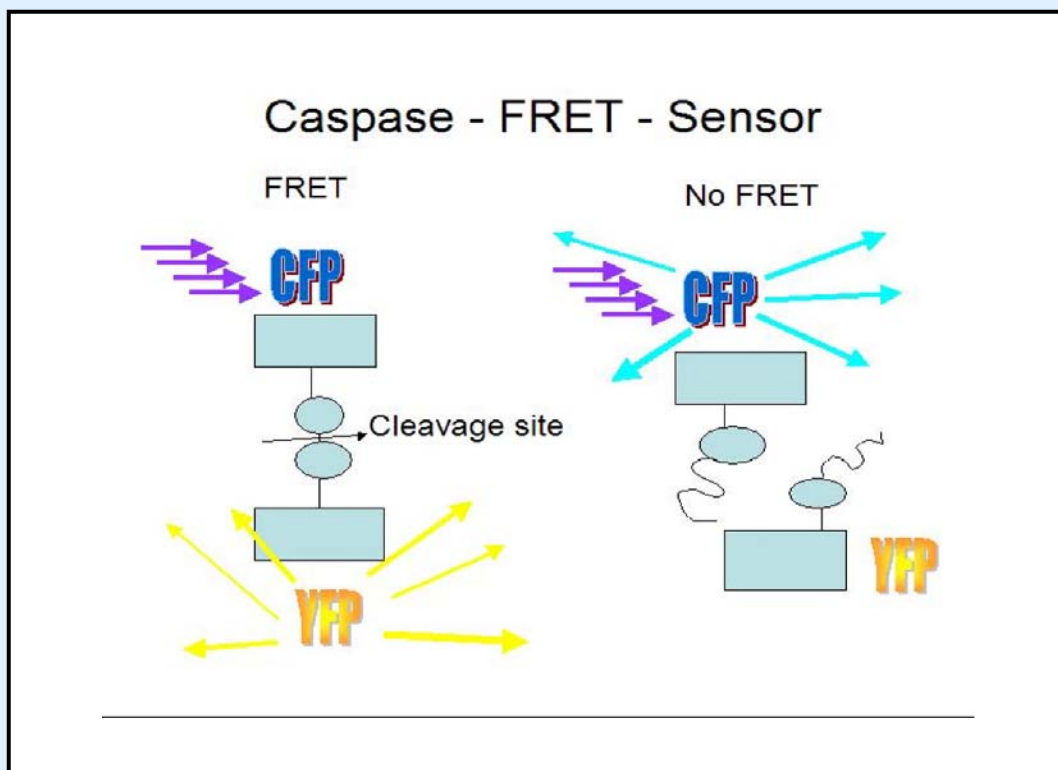
In the figures above, FITC is used to develop the green fluorescence in untreated and treated cells. Note that there is a background level of DNA strand breaks in unperturbed growing cells, breaks that are normally repaired by topoisomerases. Inhibition of the topoisomerase activity causes an accumulation of the strand breaks, and bright fluorescence staining.



Monoclonal antibodies that are specific for the cleaved or activated form of caspases are now available. This antibody, specific for cleaved caspase 3 (Cell Signaling, Beverly, MA), shows staining that originates in the cytoplasm of cells soon after apoptosis induction. At higher dosages, the activated caspases surround the nucleus as the cells round up in the apoptotic process. Laser scanning analysis is able to differentiate and compartmentalize the various modes of staining.

Fluorescent Resonance Energy Transfer (FRET)

The caspase 3 biosensor (see methods) employs FRET between CFP and YFP. In inactivated caspases, the CFP and YFP are in sufficiently close proximity. When a CFP molecule is excited by violet light, the expected fluorescence is shunted to the YFP molecule, and yellow fluorescence is emitted. When the caspase molecule is cleaved, the FRET chain is broken, and the excited CFP molecule emits cyan fluorescence.



Materials and Methods

Cell Lines. Human promyelocytic leukemic HL-60, adherent Chinese hamster ovary CHO, and adherent HeLa cells were obtained from ATCC and kept in continuous culture as recommended.

Antibodies. Antisera to phosphorylated Histone Gamma H2AX was obtained from Trevigen (Gaithersburg, MD). Monoclonal antibody to cleaved caspase 3 was obtained from Cell Signaling Technology (Bedford, MA).

FRET probes. Construction of the recombinant FRET sensor CFP-DEVD-YFP was described previously (Luo et al., 2001). Shortly, a mammalian construct containing the CFP-YFP fusion gene was generated by cloning a PCR amplified EYFP into the EcoRI and BamHI sites of the vector pECFP-C1 (Clontech). The FRET probe, named caspase sensor 3, was generated by modifying an amino acid linker region with DEVD-cleavage sequence. The FRET probe named caspase sensor 8 was produced by inserting in linker region tandem IETD-cleavage sequence.

Generation of GFP derivative cell lines. HeLa cells stably transfected with caspase-3 sensor were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 500 ng/ml G418 at 37°C in an atmosphere of 5% CO₂ in air. K562 cells were transiently transfected with caspase-8 FRET-sensor. A murine T-cell hybridoma mcc9 was created using standard fusion from a T cell clone that recognizes aa 88-103 of moth cytochrome c peptide presented by the murine MHC class II molecule I-Ek and retrovirally transduced with CFP, YFP or GFP-expressing viruses (used for compensation purposes and setups)

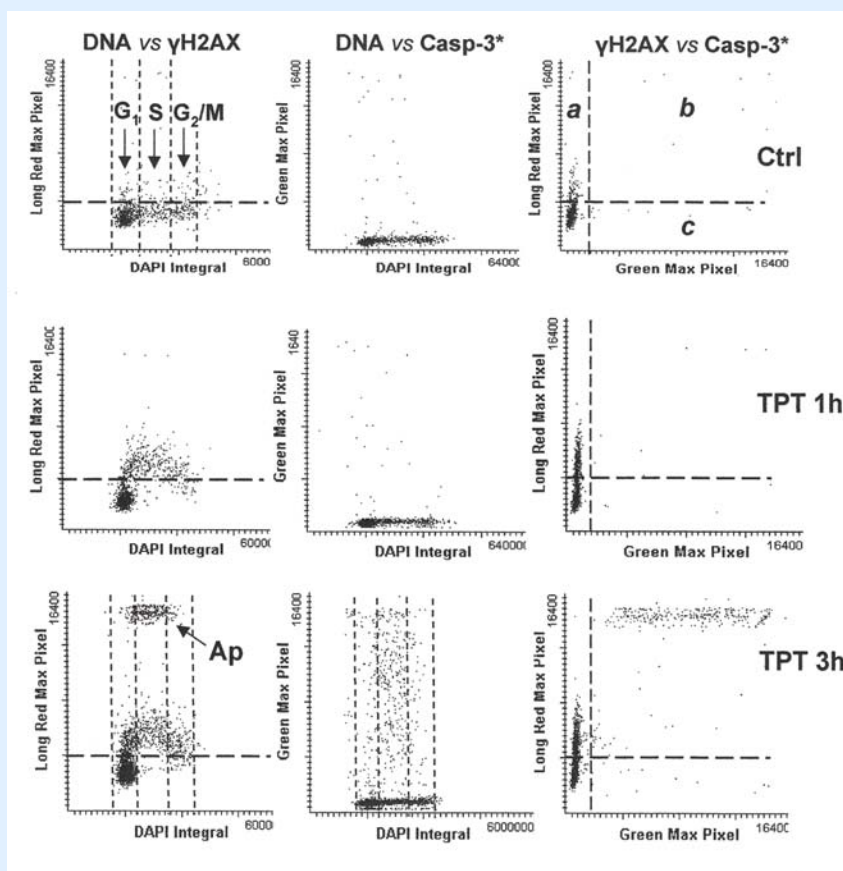
Cell Treatments. To induce apoptosis, the cultures were treated with various concentrations of staurosporine (STS), etoposide (Trevigen), camptothecin and topotecan (Merck) as described in the legends to the figures. To inhibit caspase activation, the cell cultures were treated with the broad-spectrum caspase inhibitor Z-Val-Ala-Asp (O-methyl)-fluoromethyl ketone (Z-VAD-fmk) (Kamiya Biochemicals). Control cultures were treated with the equivalent amounts of dimethylsulfoxide (DMSO, Sigma) that was used for preparation of stock solutions of the drugs.

Fluorescence measurement: LSC. Cellular green (cleaved caspase 3), far red (histone H2AX) and blue (DAPI) fluorescence emission was measured simultaneously in the same cells on the laser scanning cytometer (LSC, CompuC^{yte}), utilizing standard filter settings; fluorescence was excited with 488-nm argon ion, helium neon, and violet diode lasers, respectively. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3,000 cells were measured per sample.

Fluorescence measurement: iC^{yte}. For immunostaining and DNA content experiments, cells were grown in microtiter well plates, treated with test substances typically overnight, ethanol fixed, and stained with propidium iodide for DNA content and specified antibodies. Analysis was done on an iC^{yte} Imaging cytometer using standard laser and filter combinations. For FRET measurements, violet laser excitation was multiplexed with blue laser excitation, and filter cubes optimal for GFP and YFP fluorescence detection were used. Analysis was done on live cells.

Results from Antibody Studies

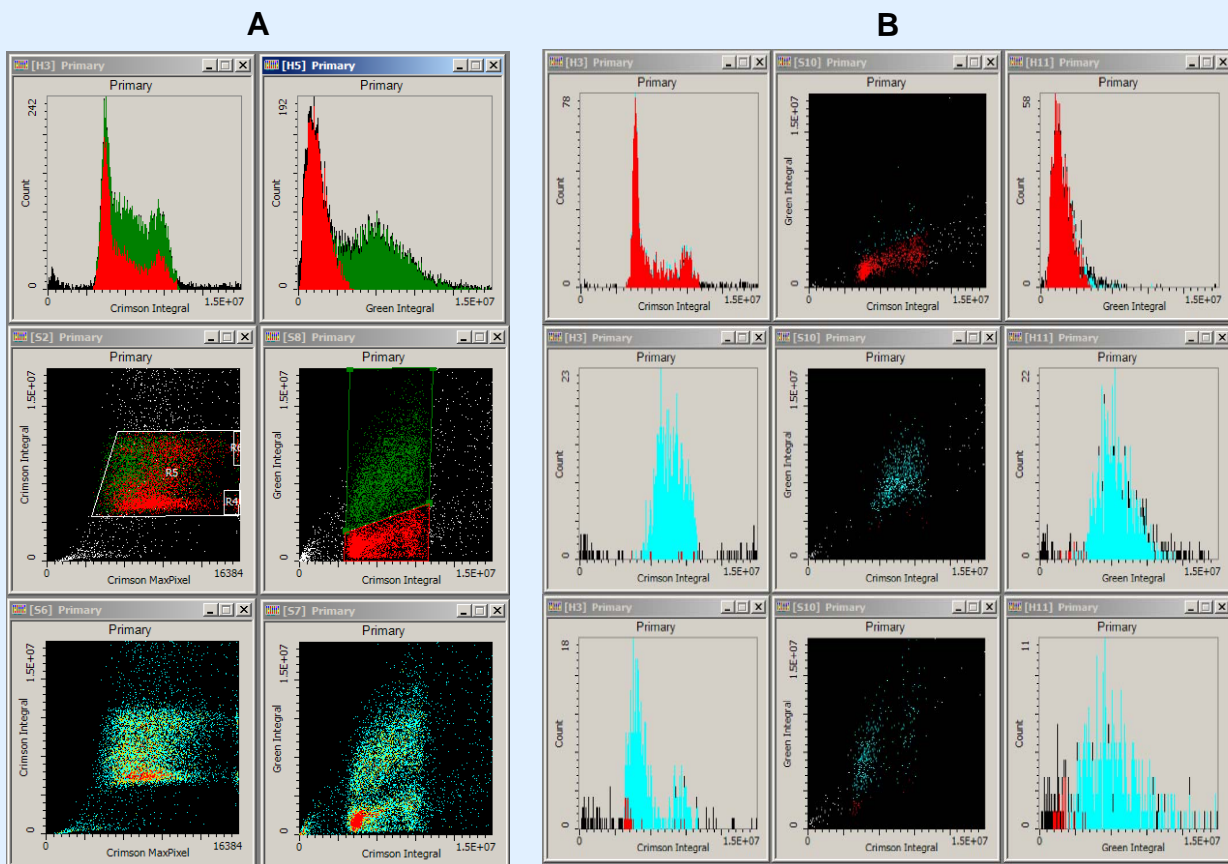
In studies of HL60 cells, both DNA strand breaks and apoptosis are triggered by compounds such as the topoisomerase inhibitor topotecan (TPT) in an ordered sequence. In column A, DAPI-stained DNA fluorescence is plotted against Alexafluor 647-labeled phosho H2AX antibody. In column B, the DNA content is plotted against Alexafluor 488-labeled cleaved caspase 3 antibody staining, and in column C, the H2AX labeled DNA strand breaks are plotted against the cleaved caspase 3 staining.



After one hour of treatment, DNA strand breaks become evident at a low level of staining, and the activated caspase remains at background levels. This staining is attributed to naturally occurring strand breaks that normally would have been repaired, but are prevented from doing so by the TPT. After 3 hours, a population of cells exhibits both high levels of Histone H2AX staining as well as cleaved caspase 3 staining. These cells originate primarily in S-phase of the cell cycle, and are cells that are undergoing additional apoptosis-induced DNA strand breaks. Later in the process, the nuclei become fragmented and lose H2AX staining.

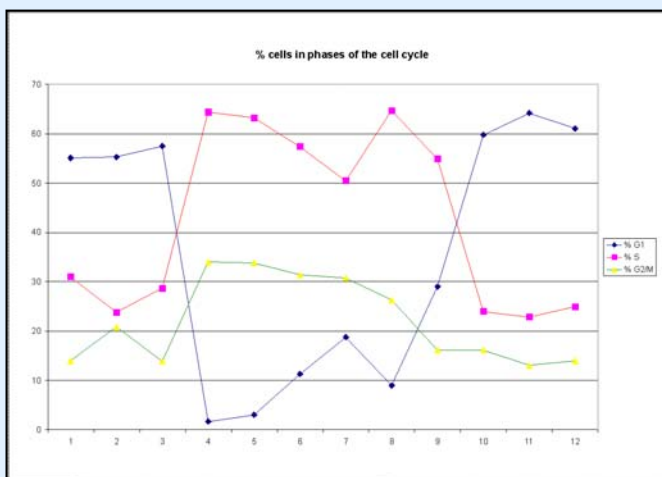
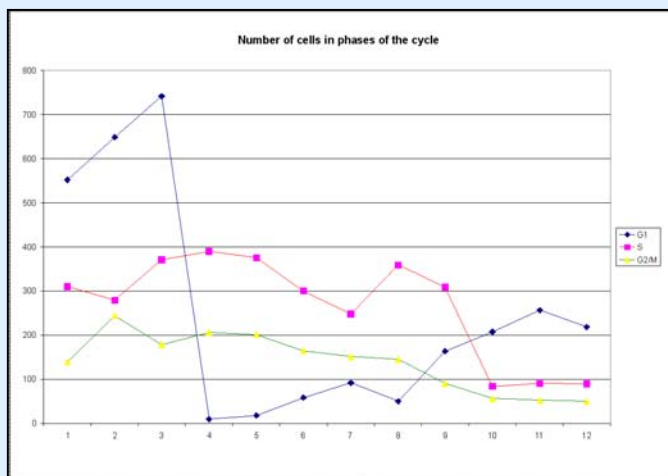
Results from Antibody Studies, continued

In the adherent CHO cell line model, the induction of apoptosis by camptothecin occurs at a much slower rate. At 36 hours, dose response studies show effects on the cell cycle and the induction of DNA strand breaks. In the composite display of all dosages (panel A), H2AX positive and negative cells are clearly demarcated, and are color coded red and green in the histogram displays. In panel B, in untreated cells (top row) there is a normal cell cycle distribution for exponentially growing cells. At a low dosage of camptothecin, (middle row) the cells are blocked in the S-phase of the cell cycle. DNA strand breaks are also evident in S-phase cells. At a high dosage (bottom panel), the cells are blocked at the G1/S and S/G2 checkpoints of the cell cycle.



Results from Antibody Studies, continued

In the dose response curves, the number and percentages of cells in the different phases of the cell cycle is shown, and what is apparent is that there is a significant loss of cells in the drug-treated wells. The cell cycle region percentages suggest that at high dosages of the camptothecin, the cell cycle distribution returns to normal values. This forms the basis for a reasonable hypothesis that at high dosages of the drug, cells became apoptotic, detached from the substrate, and were not accounted for in the analysis.



Results from Antibody Studies, continued

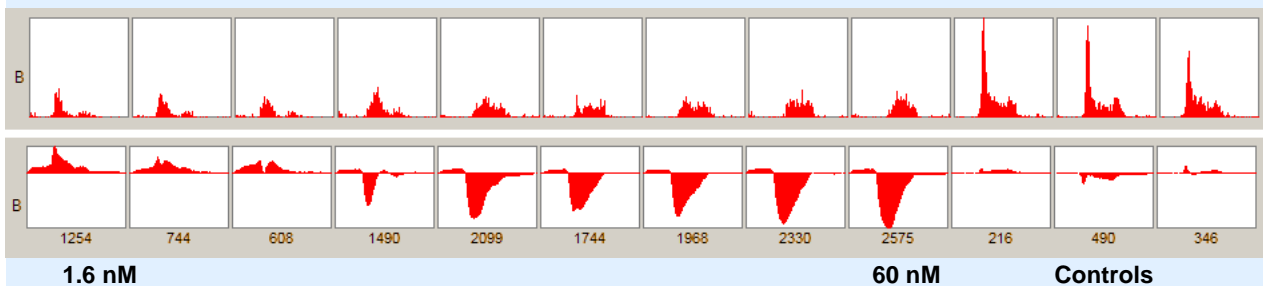
One of the newly developed analysis techniques is the modification of the Kolmogorov-Smirnov statistical test to display histograms of the difference between a histogram and its defined control. A numerical value corresponding to the maximum difference is a reported statistic for the assay.

Relevant histograms from a camptothecin dose response study are shown along with the derived Kolmogorov-Smirnoff differential plots below them. Controls are in the 3 right wells, and dosages of CPT increase from right to left. Histograms and analysis are shown for propidium iodide DNA staining in panel A, and for FITC labeled phosphorylated H2AX antibody in panel B.

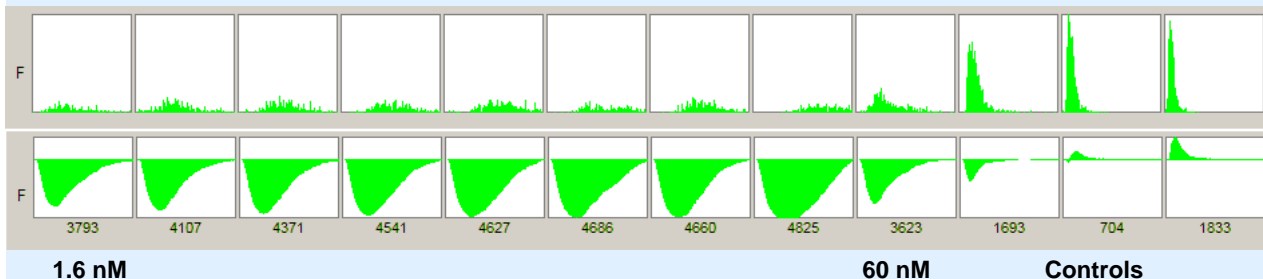
In the DNA histograms, even at the lowest dosage of CPT, there is a decided difference from the control samples – a reflection of the blockage of cells in S-phase. At the higher dosages, the differences change from being a down-going difference to an up-going difference. The KS analysis implies that these are not normally cycling cells, and they have been affected by the drug treatment.

All of the CPT treated samples show a large KS difference in the analysis, with a maximal response at the lower dosages.

DNA Content

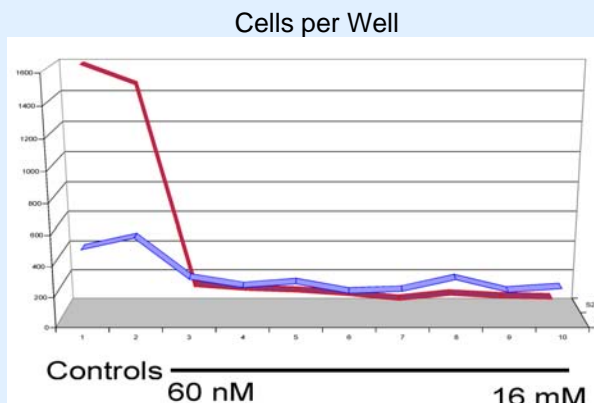
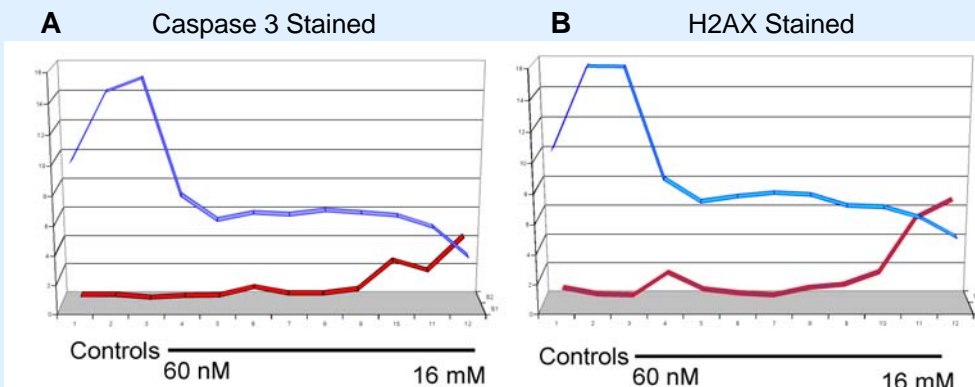
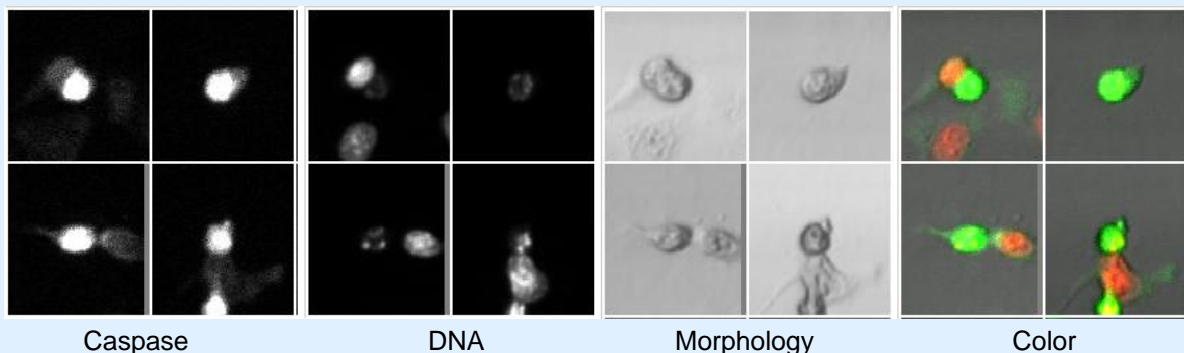


Phosphorylated H2AX



Differentiating Between the Effects of Loss of Proliferation and Cell Loss to Apoptosis

In a CPT titration using cells stained with the cleaved caspase 3 antibody, cells exhibiting the apoptotic morphology characterized by intense caspase staining around the nucleus, rounded morphology, and fragmented nuclei were automatically identified and plotted in chart A (blue) and compared to the cell count (red, different scale). They appear at the highest dosages of the CPT. In a H2AX-stained titration (chart B), apoptotic bodies were quantified with similar results.



Results from CFP/YFP FRET Studies

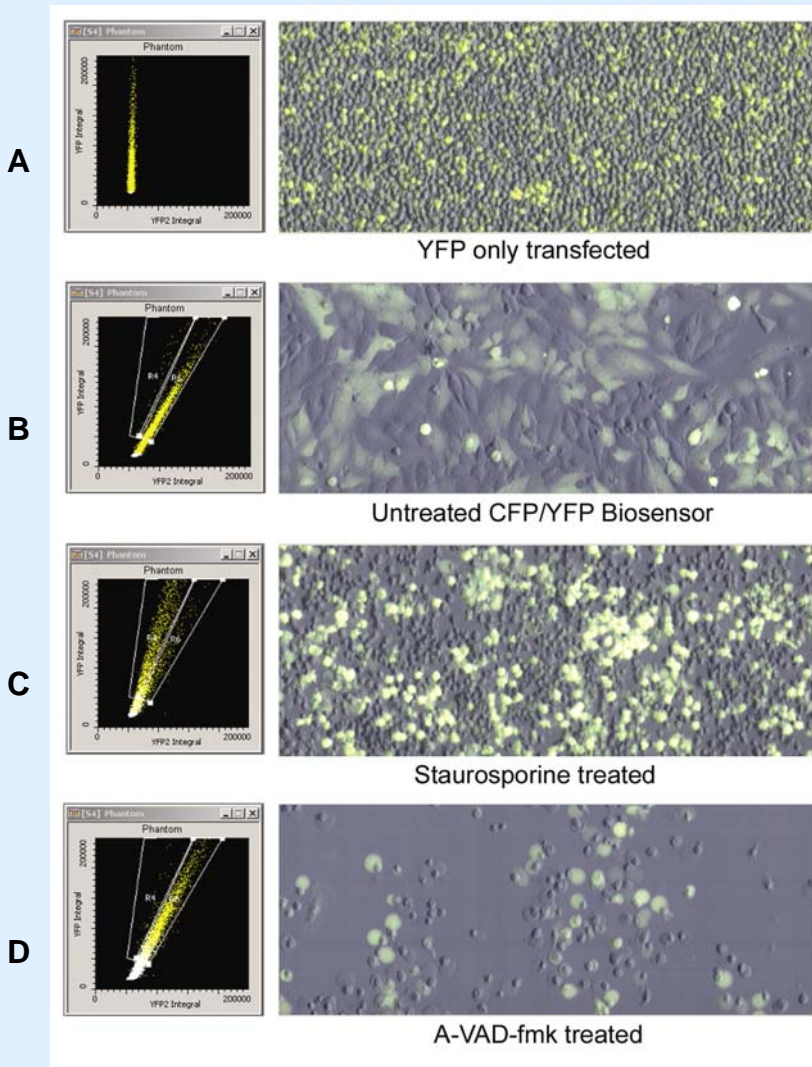
This data presents the characterization of FRET in iCyte technology. Each data pair shows a scattergram of the violet-excited vs. blue-excited yellow fluorescence and a composite color laser scan image.

Panel A shows the murine hybridoma transfected with YFP only. There is fluorescence when excited by the blue laser, but not when the excitation is from the violet laser, resulting in a vertical slope. This is consistent with the spectral properties of YFP.

Panel B shows data from the HeLa cells transfected with the CFP/YFP biomarker construct, and then analyzed untreated. The slope of the distribution is indicative of the maximum amount of FRET that is possible from this construct. Reference region 6 was drawn around the main cluster of sampling events.

In **Panel C**, the cells were treated with staurosporine to induce apoptosis. The slope of the distribution decreases, indicating an abrogation in the amount of FRET as the caspase 3 molecules are cleaved.

In **Panel D**, cells were treated with the caspase inhibitor Z-VAD-fmk before treatment with staurosporine. The data points fall primarily in region 6 as they did in the untreated cells, indicating protection from the caspase cleavage. The morphology of the cells indicates that they are no longer alive, succumbing to an alternative apoptotic pathway.

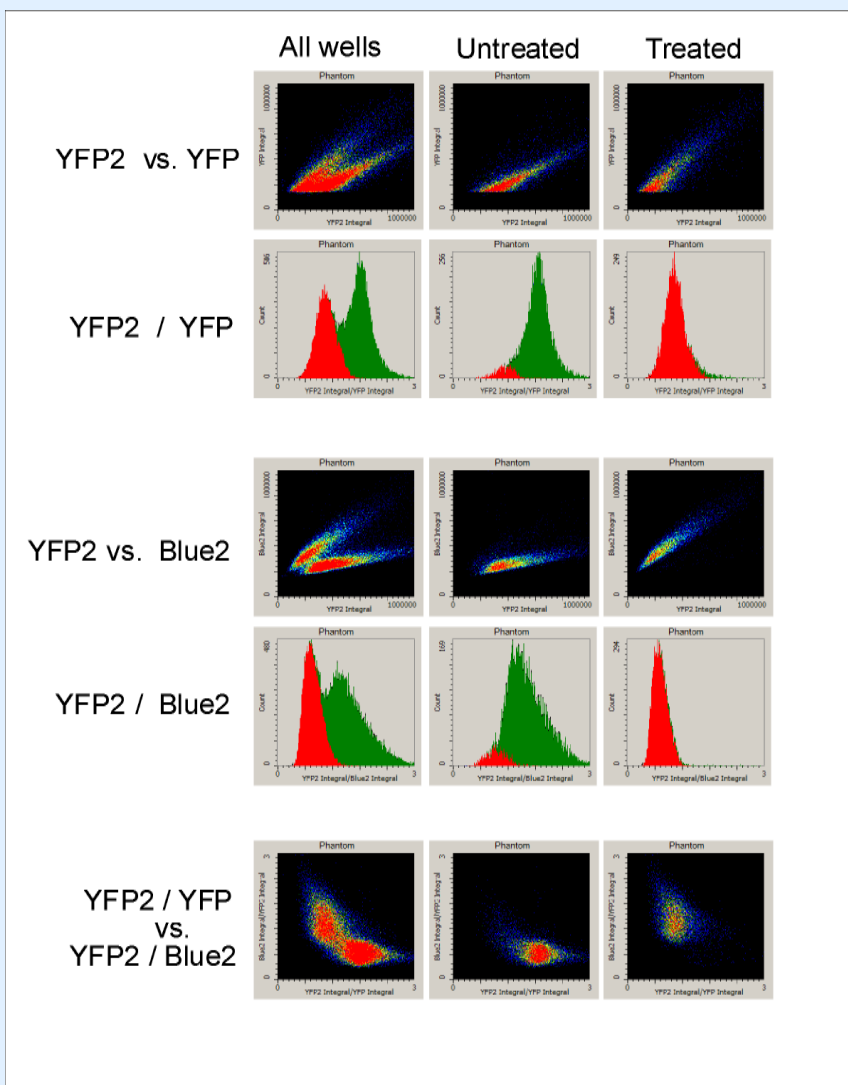


Results from CFP/YFP FRET Studies, continued

In the upper set of panels, violet-excited yellow fluorescence is compared to blue-excited yellow fluorescence. Two clusters of events are discernable, corresponding to live and apoptotic cell populations. The blue excited fluorescence is a measure of the amount of YFP in the cells, and the violet excited blue fluorescence is a combination of yellow fluorescence from the CFP as well FRET from random interactions between cleaved CFP and YFP molecules. The same data is shown in histograms, where the ratio of the two yellow fluorescences is plotted as a frequency distribution. For reference purposes, live and apoptotic cells are color coded green and red.

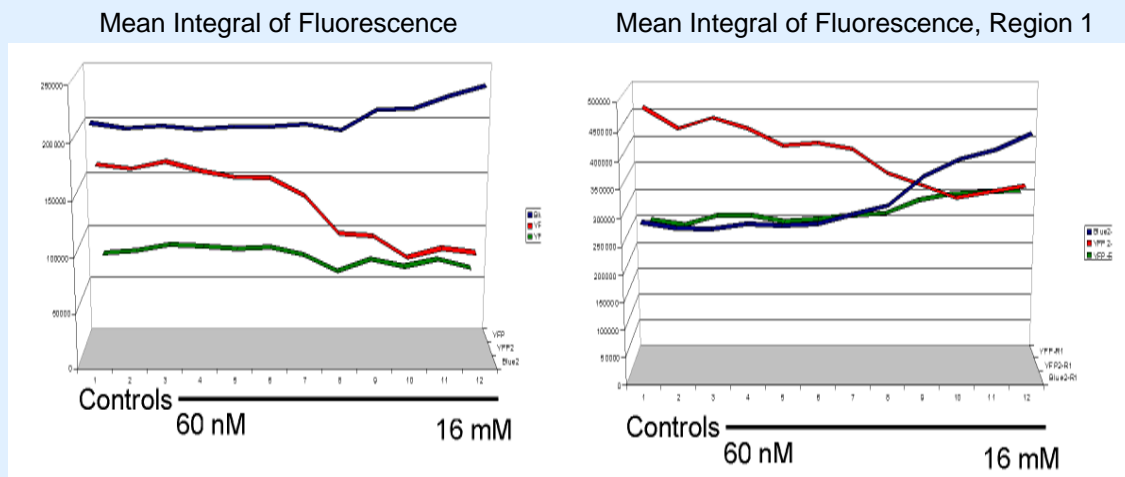
In the middle panels, the same data elements are displayed showing the violet excited yellow fluorescence versus the violet excited blue fluorescence. FRET induces an increase in the yellow fluorescence and a corresponding decrease in the blue fluorescence. The histogram shows the ratios of the two parameters. Note the similarity to the upper histograms in the proportions of the red to green elements, especially in the untreated cells, indicating the presence of spontaneous apoptotic cells in the untreated population.

The lower set of scattergrams shows the results of plotting the two ratios used above vs. each other. This presentation method gives the best resolution between the two populations.

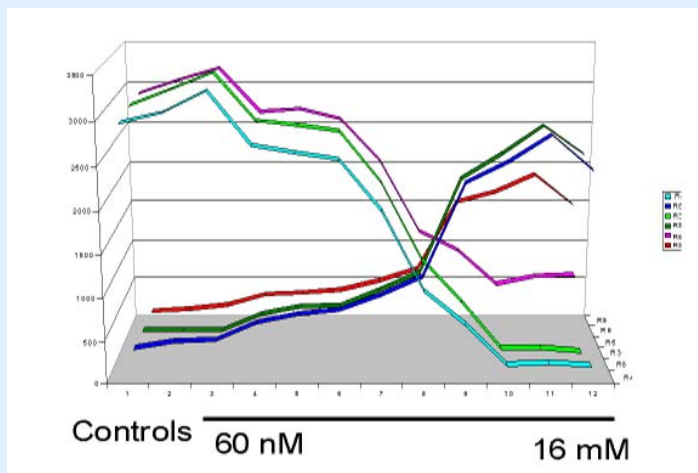


FRET Analysis Strategies

These graphs show the mean integral fluorescence for the sampling elements in a Staurosporine titration of HeLa cells transfected with the caspase-3 biomarker construct. The graph on the left shows all of the elements, and the graph on the right is restricted to elements that exhibit some degree of yellow fluorescence. The blue trace is violet-excited blue fluorescence (Blue2); the red traced is blue-excited yellow fluorescence (YFP2), and the green trace is blue-excited yellow fluorescence (YFP). Both plots show a decrease in the YFP2 signal and an increase in the Blue2 in a dose-related manner, reflecting the loss of FRET as the caspases are cleaved.

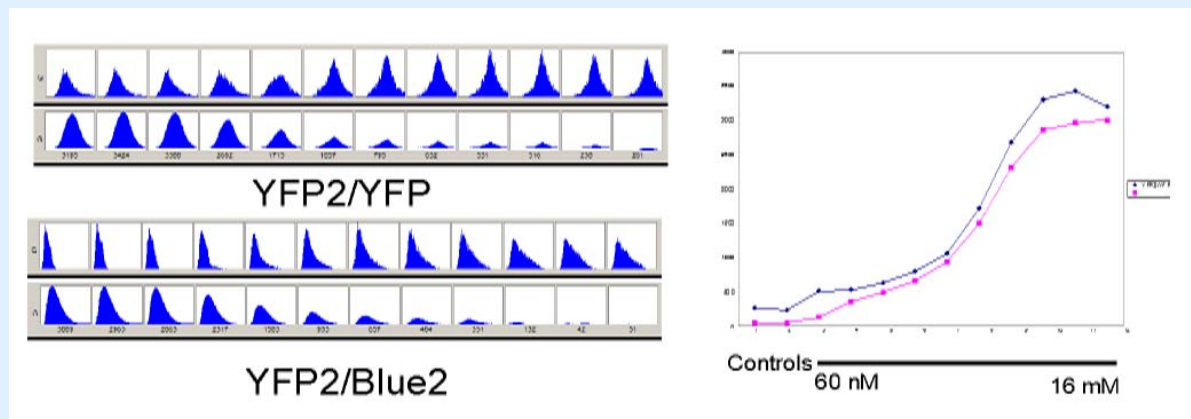


In this analysis strategy, regions were defined around population clusters in scattergrams plotting the YFP2 vs. YFP, the YFP2 vs. Blue2 and the ratio scattergram of YFP2/YFP vs. YFP2/Blue2 fluorescences. The regions were drawn around FRET positive and negative sampling elements (see page 11), and the percentages of the elements within the clusters are plotted. For this construct, all three methods gave similar results showing the dose response to the staurosporine.

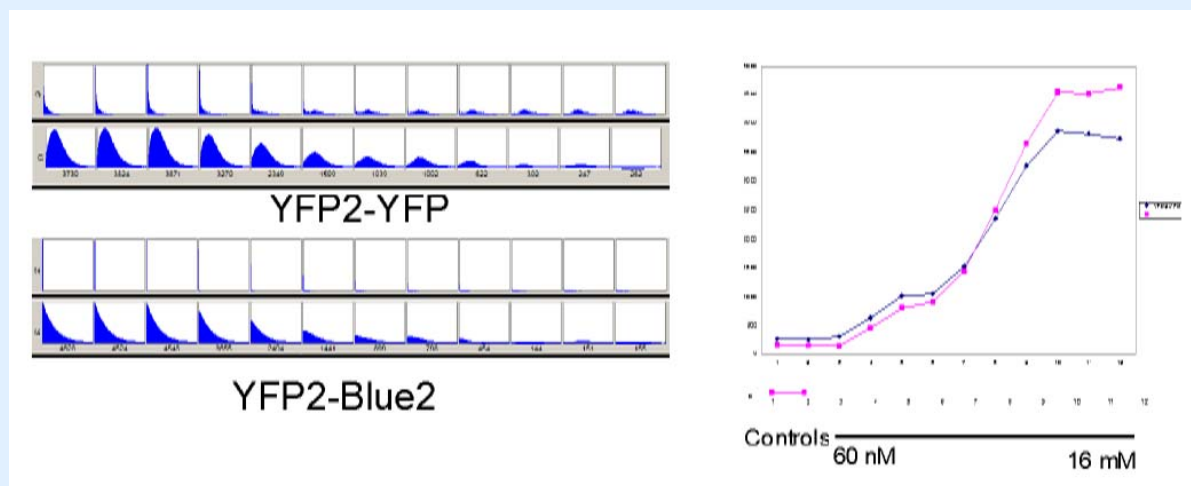


FRET Analysis Strategies, continued

In this analysis strategy, histograms of the ratios of the key fluorescence combinations are compared and quantified using the modified version of the Kolmogorov-Smirnov test. In each group on the left there are the original histograms and the histograms of the D-values for a row of samples in a staurosporine titration. These histograms show the difference between the test histogram and the control, and in this case the control is an untreated sample exhibiting FRET. The differences represent the loss of FRET in the system. The maximal D value is used to quantify this loss of FRET. On the right, the maximum D-values are plotted for the fluorescence combinations showing the dose response to the staurosporine.



In this final analysis strategy, virtual channels were defined where the scan images of the key fluorescence colors were subtracted to give a new image approximating the amount of FRET in the samples. For example, the YFP2 image was subtracted from YFP image and the Blue2 image was subtracted from the YFP2 image. Stereological segmentation was applied, and histograms were obtained. Kolmogorov-Smirnov analysis was done as above, and similar results were obtained from this method as were from the ratio histogram analysis.

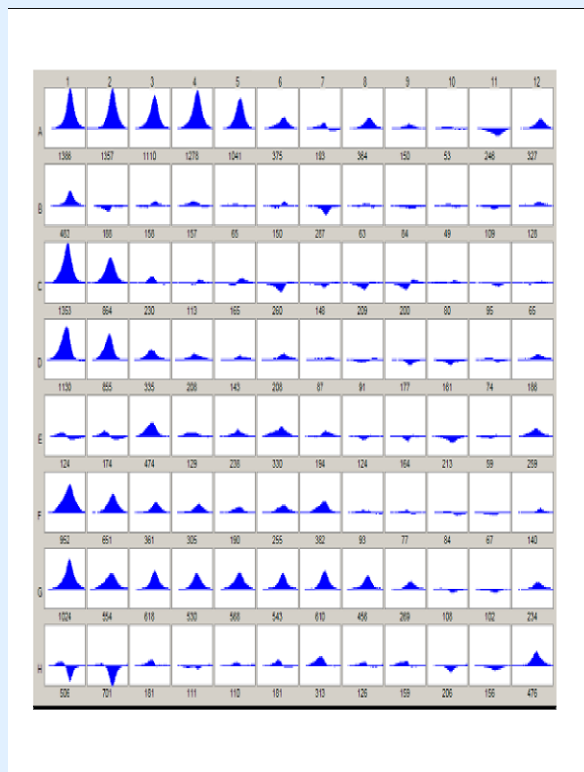
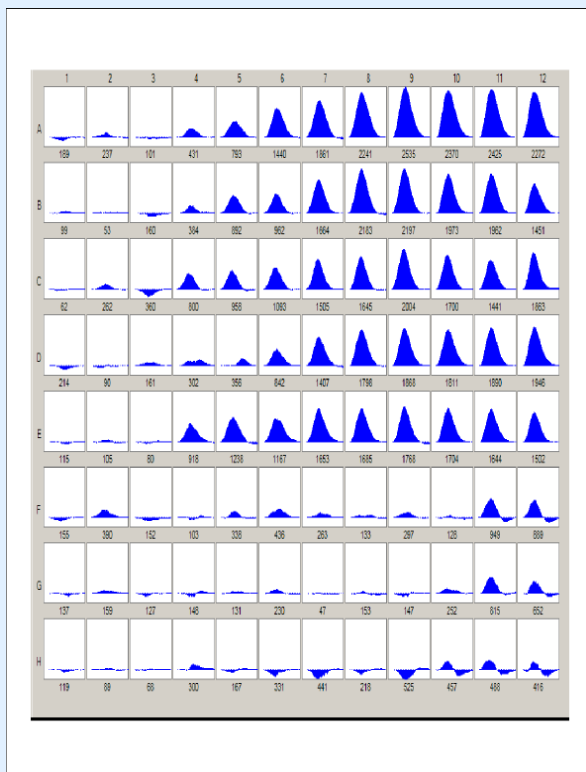


Evaluation Methods

One of the benefits of the caspase 3 biosensor method is that an assay can be performed with minimal requirements for sample preparation since the reporter molecules are inherent in the test cell line. The fluorescence evaluation method is robust and independent of the cell number, automated analysis of the results is done. Thus large numbers of wells in microtiter plates can be analyzed in a short time period.

A

B



Panel A shows an experiment where five rows of a plate were replicates of a staurosporine titration, and three rows were blank.

Panel B shows an experiment where different test substances were applied to the different rows. Note that for each drug tested, a titration response is obtained instead of just a single readout.

Conclusions

We utilized a newly developed laser scanning imaging cytometer capable of morphometric and cytometric quantitative automated analysis to explore different modes of cell damage and death in response to topoisomerase inhibitors. A combination of biochemical, immunological and molecular reporters were used, targeting DNA related features, cell population features, histone H2AX phosphorylation, and caspase 3 activation.

Suspension-grown human promyelocytic leukemic cell line HL 60 data showed that early DNA strand break-related events preceded caspase-3 cleavage and apoptotic death in a time frame of one to three hours. The adherent CHO cell line gave similar results over a time frame of 48 hours. Analysis in microtiter plates allows higher experimental throughputs, and thus larger numbers of experimental test points can be obtained. Population demographics were employed to discern the differences between lack of proliferation and loss of cells to apoptotic degradation. The adherent cells have the advantage that they can be fixed and studied *in situ*, allowing more detailed study of morphometric features.

A rapid and reliable laser scanning cytometry strategy employing FRET-sensors for caspase activation was developed for the first time for high-throughput 96-well plate analysis. Caspase-3 and caspase-8 activities were measured as a function of FRET between CFP- and YFP-molecules linked by an amino acid bridge with DEVD- or IETD tandem cleavage site. FRET fluorescence intensity was maximal in control HeLa or K562 cells transfected with uncleaved FRET constructs. FRET intensity had decreased concentration-dependently or time-dependently upon cleavage of FRET-caspase sensors by activated caspase-3 in response to treatment with staurosporine or etoposide. Pre-treatment with pan-caspase inhibitor z-VAD-fmk abrogated caspase-3 activation and change in FRET intensity. However, modified morphology and loss of attachment of cells that were pre-treated with pan-caspase inhibitor suggested necrotic rather than apoptotic outcome.

The new biomarker system is particularly well suited for automated analysis because the expression of the biomarkers is inherent to the stably transfected cell line. The stereological sampling techniques employed are tolerant of variations in cell number, including overgrowth of the culture wells, thus promoting robust assays.

Apoptosis characterization based on traditional antibody-labeled methods combined with cell cycle characterization, as well as approaches using new GFP-based FRET-sensors, can be successfully performed using laser scanning cytometry. Benefits of such experimental models include the automated processing of thousands cells and the possibility to link morphological information with cytometric data.

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