Multi-parametric high content cellular analysis of cell cycle progression and cleavage of caspase-3 during camptothecin-induced apoptosis

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Abstract

High content cellular analysis was used to examine the cleavage of caspase-3 in response to camptothecin-induced apoptosis in CHO cells. Cells were seeded in microtiter plates and incubated with varied concentrations of camptothecin. After fixation, cells were stained with an antibody specific for cleaved caspase-3 (Cleaved Caspase-3 (Asp175) Antibody, Cell Signaling Technology, Inc. #9661) and Alexa 488 anti-rabbit antibody (Molecular Probes). This caspase-3 cleavage-specific antibody detects only the 17kDa C-terminal fragment generated by caspase-mediated proteolytic digestion adjacent to Asp175. Cells were counterstained with propidium iodide and analyzed using the iCyte™ Imaging Cytometer (CompuCyte Corporation). Fluorescence was captured using photomultiplier tubes with appropriate filters for green and long red fluorescence. Laser light scatter was obtained simultaneously for bright field visualization.

Segmentation of cellular events was done based on nuclear fluorescence measurements. For each nucleus, DNA content, chromatin condensation, and nuclear area were obtained. Caspase-3 cleavage was evaluated within the area covered by the nucleus of the cells, in a perinuclear torus, and also separately segmented. Cell counts and other population statistical values were acquired in the analysis. The results show that DNA staining was an effective measure of camptothecin-induced changes on cell cycle distribution. Surprisingly, low doses of campothecin result in the greatest changes in cell cycle progression, with a complete block of the cells in S-phase and G2. Nuclear area increased with the drug concentration and cell counts decreased. As expected, cleavage of caspase-3 increased with camptothecin dosage. Further, cleaved caspase-3 localization changed from diffusely cytoplasmic to perinuclear. Bright field image galleries of the cells confirmed morphological changes of the cells associated with induction of apoptosis.

The utilization of activation-state specific, including cleavage-specific and phospho-specific antibodies in combination with high content cellular imaging enables robust, multi-parametric quantification of cellular events, including changes in signaling pathway activation, protein modification and distribution, and cellular morphology.

Introduction

Apoptosis is a highly regulated process of programmed cell death that culminates in the condensation of chromatin, the degradation of chromosomal DNA, and eventual removal of damaged cells from a healthy organism. Caspases, central regulators of the apoptotic program, are a conserved family of cysteine acid proteases that exist as dormant zymogens until activated by an apoptotic stimuli. This leads to a cascade of caspase cleavage, resulting in the eventual proteolysis of key proteins involved in the regulation of cellular/nuclear structure and the metabolism of DNA (depicted in Figure 2). Caspase-3 is a key executioner of apoptosis as it cleaves a number of downstream proteins including α-Fodrin, Lamin A, and PARP.

Cell Signaling Technology, Inc. (CST, Beverly MA) is a premier content provider for drug discovery and basic research applications, specializing in the development of activation-state specific antibodies. We have used the Cleaved Caspase-3 (Asp175) Antibody (#9661) with the iCyte™ Imaging Cytometer (CompuCyte Corporation, Cambridge MA) to examine caspase-3 cleavage and subcellular localization following camptothecin treatment. Further, the iCyte™ permits the simultaneous acquisition of cellular morphology data, cell cycle progression, and the automatic capture of cellular events as defined by user set segmentation parameters.

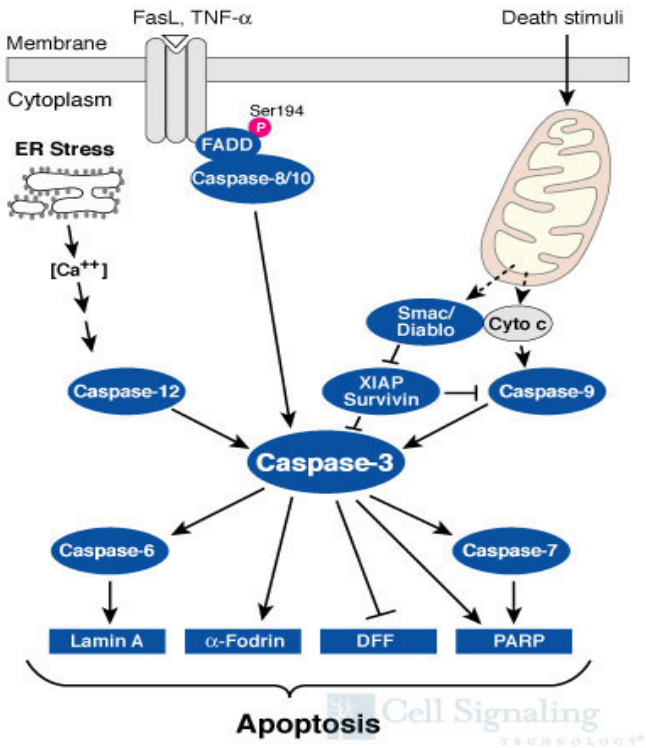


Figure 2. Caspase Signaling.

Materials and Methods

Cell Signaling Technology, Inc.
CompuCyte Corporation

Cleaved Caspase-3 (Asp175) Antibody (#9661)
iCyte™ Imaging Cytometer
Alexa 488 Anti-rabbit (Molecular Probes)

Briefly, CHO cells were seeded in 96-well plates and treated with varied concentrations of camptothecin. 24 hours later, cells were fixed with ethanol and stained with three concentrations of Cell Signaling Technology, Inc. (CST) #9661, Cleaved Caspase-3 (Asp175) Antibody. Alexa 488 goat Anti-Rabbit was used as a secondary reagent. Cells were counterstained with propidium iodide and RNAse. Plates were analyzed on the iCyte™ Imaging Cytometer (Figure 1A). Cells were analyzed on the basis of DNA content and localization of cleaved caspase-3, as visualized by the CST antibody #9661 which recognizes only cleaved, active caspase-3. Further, the iCyte™ enables rapid segmentation and quantification of nuclear, perinuclear, and cytoplasmic cleaved caspase-3 staining (Figure 1C).

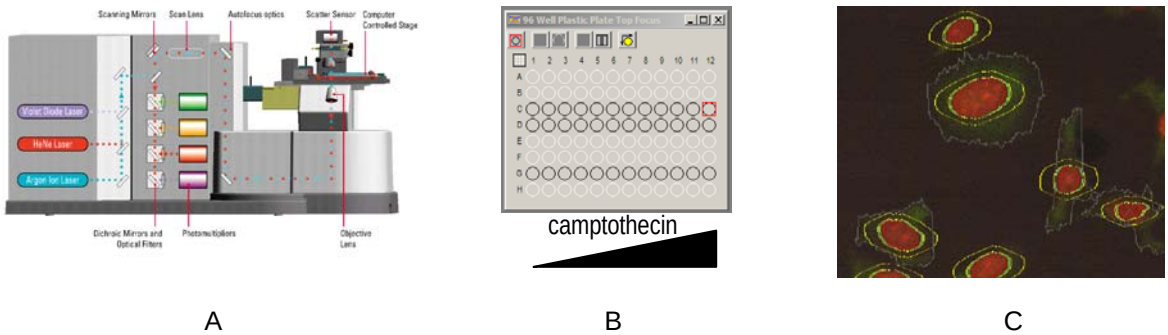
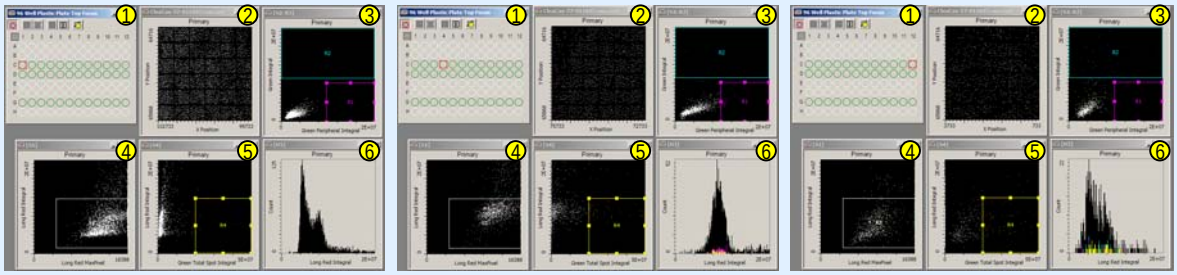


Figure 1. Overview of experimental design. A, The iCyte™ Imaging Cytometer, a new high content screening platform. Uses up to three excitation lasers and multiple photomultiplier tubes to scan, image, and extract morphometric and proteomic features from cellular samples in multiplex. B, Experimental overview. Wells to be selected for analysis are highlighted in gray. Row C, D, and G contain varied antibody dilutions. Columns 1 to 3 are untreated. Columns 4 through 12 are treated with increasing doses of camptothecin. C, Multiple settings that permit high content contouring of cellular samples. Primary segmentation was done around the nucleus (stained with propidium iodide in red and highlighted by green contour). Perinuclear staining was quantified using a torus around the nucleus (yellow contour). Grey contour highlights the boundary of the cytoplasm as defined by extent of green fluorescence.



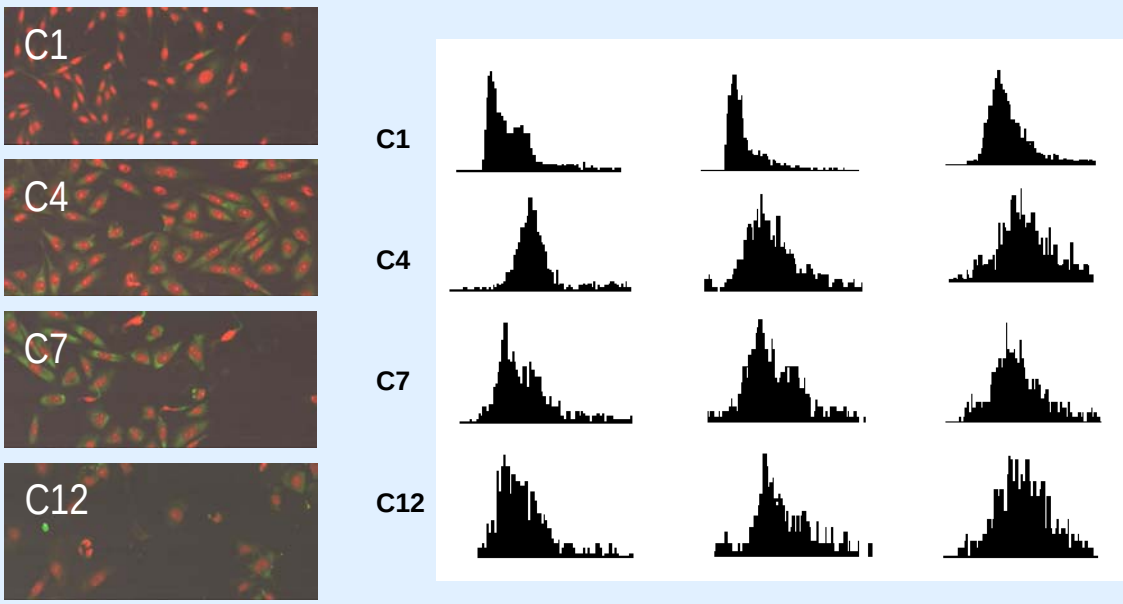


Figure 3. iCyte™ parametric analysis of camptothecin treated CHO cells. A. Low magnification overview of wells C1, C4, C7, and C12 demonstrating induction of caspase-3 cleavage (wells C4 and C7) and increase in perinuclear staining. Brightly stained cell in well C12 is nuclear staining of cleaved caspase-3. B, Analysis of untreated cells (well C1, at left), low dose camptothecin (well C4, center), and high dose camptothecin (well C12, right). Demonstrates Figure B1 gives well position. Figure B2 is an overview of a 6x6 section of the well highlighted in B1 showing location of each cell in the well. Note decrease in cell number with camptothecin treatment Figure B3 demonstrates increases in perinuclear staining in well C4. B6 highlights DNA distribution which shows S phase arrest after camptothecin treatment in well C4. Figure 3C demonstrates quantification of cell cycle arrest and changes in localization of cleaved caspase 3 (Y axis: cell count; X axis: intensity).

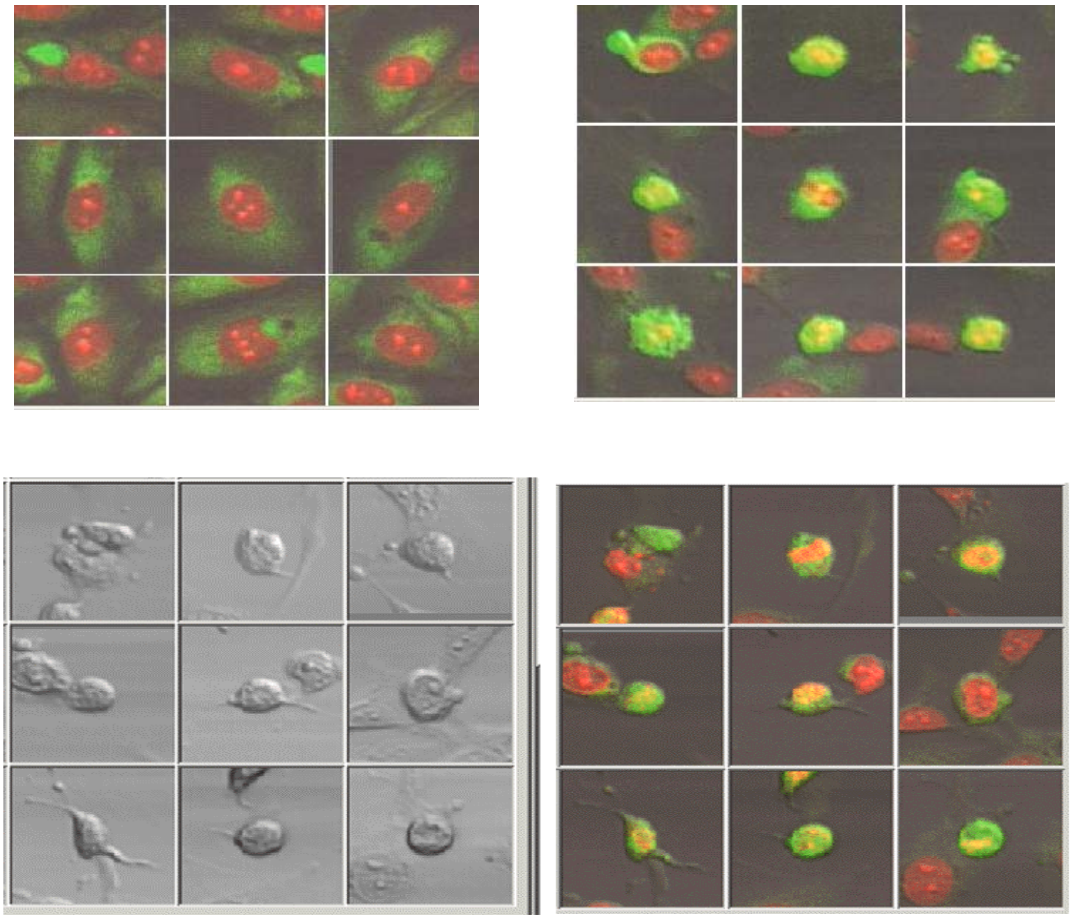


Figure 4. Gallery images automatically acquired by iCyte™ with user defined segmentation requirements. A, Intermediate and high doses of camptothecin result in differential localization of cleaved caspase-3, likely indicative of different stages of apoptotic program. Intermediate dose of camptothecin causes cytoplasmic cleavage of caspase-3. Note perinuclear localization. High doses of camptothecin results in densely staining nuclear cleaved caspase-3. B, Laser scatter (left) and fluorescent gallery images demonstrating morphological changes associated with apoptosis.

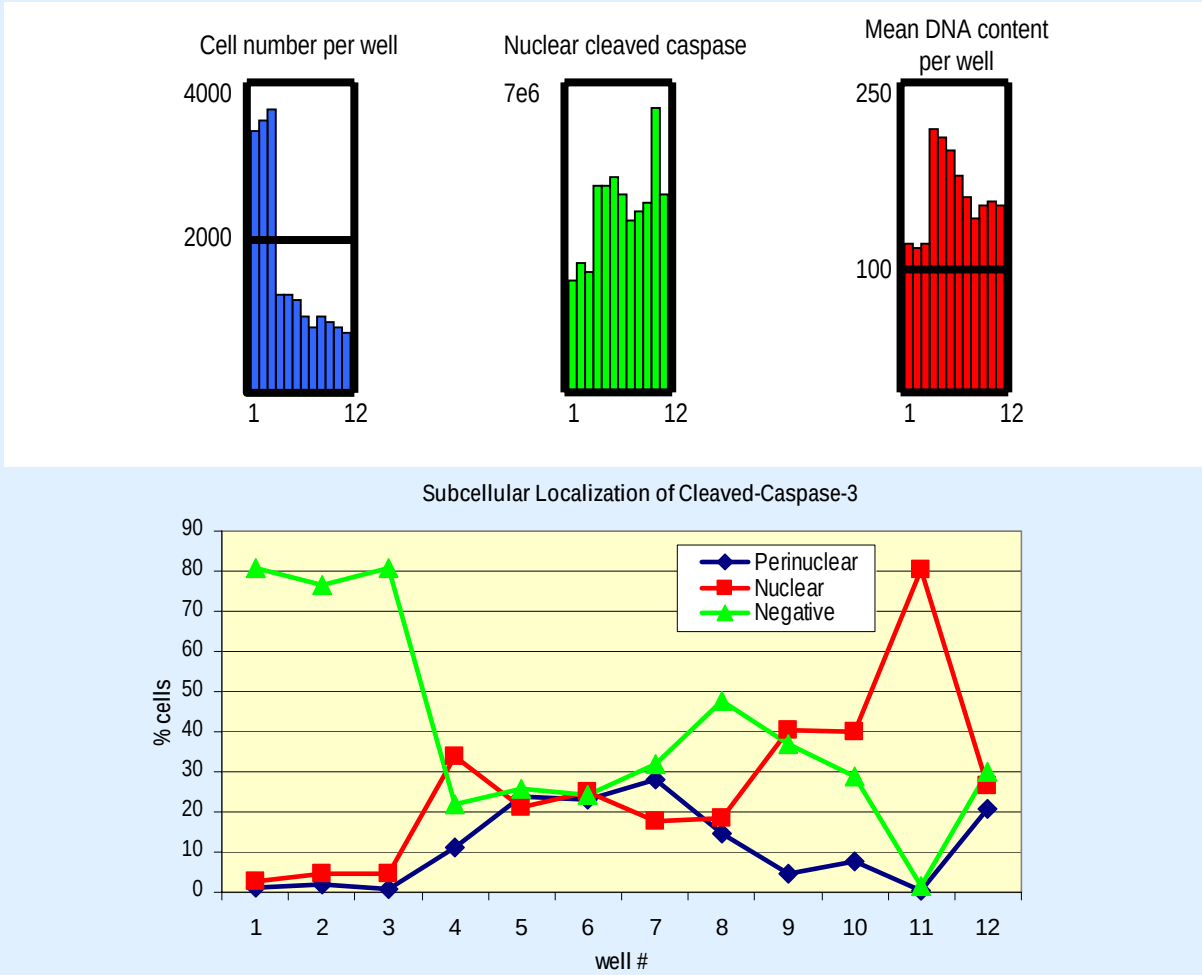


Figure 5. General data trends. Top: Well summary of cell number, nuclear staining of cleaved caspase-3, and mean DNA content. Decreases in cell count corroborate increases in nuclear cleaved caspase 3 and changes in DNA content associated with S phase arrest. Bottom: Changes in subcellular localization of cleaved caspase-3. As shown in green, increases in capsase-3 cleavage occurs in wells 4-12 (increases in camptothecin), as expected. Low dose camptothecin (wells 5-9) demonstrate perinuclear staining of caspase-3 while at high doses, capsase-3 staining is predominantly nuclear.

Summary

Activation-state specific, including cleavage-specific and phospho-specific antibodies used in combination with high content cellular imaging enables robust, multi-parametric quantification of cellular events, including protein modification, localization, and changes in cellular morphology.