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Quantitative assesment of apoptotic events using image and flow cytometry

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Abstract

Cell death by apoptosis is a complex, tightly regulated process in which a cell orchestrates its own destruction in response to specific internal or external stimuli. Dysregulation of apoptosis can lead to various physical disorders such as cancer and autoimmunity.
Information about apoptosis is commonly obtained either by flow cytometry or fluorescence microscopy. Flow cytometry provides quantitative information for thousands of cells, but does not allow for visualization of the cells. In contrast, fluorescence microscopy provides visual information, but does not allow for easy quantitative measurements of large cell populations. Image based cytometry bridges the gap between these two technologies and allows for simultaneous quantitative analysis and visualization of thousands of cells.

We have employed an image based cytometer, NucleoCounter NC-3000, for quantifying different events in the apoptotic process. Compared to flow cytometric analyses (BD LSRII), NC-3000 demonstrated accurate and precise determination of phosphatidylserine translocation, Caspase 3/7 activation and depolarization of the mitochondrial membrane.

Introduction

Cells respond to apoptotic signals by initiating intracellular processes that result in characteristic physiological changes. Among these changes are externalization of phosphatidylserine to the cell surface, depolarization of the mitochondrial membrane, activation of proteases, compaction and fragmentation of nuclear chromatin, loss of cell membrane integrity, and cell shrinkage.

We have examined three different markers commonly used for detection of apoptotic cells:

1. Phosphatidylserine translocation - using FITC conjugated Annexin V peptide
2. Depolarization of the mitochondrial membrane - using JC-1 dye
3. Activation of Caspase 3/7 - using FAM-conjugated DEVDFMK peptide

Methods

Cell Preparation:
Cell lines: U2OS (human osteosarcoma cell line, ECACC #92022711), CHO (Chinese hamster ovary cell line, ECACC #85050302) and Jurkat (human leukemia T cell line, ATCC #CRL-2570) cells.
Suspension cell line (Jurkat) was grown to a density of 5x10^5 cells/ml in RPMI + 6% FCS. The cell sample was divided into 6 T-flasks and half of the flasks were supplemented with 10 µM camptothecin (CPT). After 16 hours of incubation the CPT-treated and untreated cells were harvested and stained as described below. Each of the samples were analysed in duplicates. Adherent cell lines (U2OS and CHO) were grown to 90% confluency in RPMI + 6% FCS. Cells were harvested by trypsination and the samples were divided into 6 T-flasks. The T-flasks were incubated approximately 24 hours and at 75% confluency half of the T-flasks (3 for each cell line) were supplemented with 10 µM camptothecin (CPT). After further 16 hours of incubation CPT-treated and untreated cells were harvested and stained as described below. Each of the samples were analysed in duplicates.

Cell Staining:
Cells were labelled using, respectively, the FITC Annexin V kit from Biotium (#29001), the FAM-FLICA™ Caspases 3/7 Assay Kit from Immunochemistry (#94), and solutions 7 + 8 from Chemometec (JC-1, #910-3007 + DAPI, #910-3008).

Instrumentation:
Parallel analyses were performed on identical samples by traditional flow cytometry and image cytometry. Standard flow cytometry was performed using a BD LSRII cytometer (BD Biosciences) configured with 405, 488 and 635 nm lasers. 10.000 single cells were acquired. For the annexin V and caspase assays the fluorescence in the FITC and PE-Texas Red channels was collected. For measuring the mitochondrial potential the fluorescence in the FITC and PE channels was collected. Image cytometry was performed using a NucleoCounter NC-3000 (Chemometec). 10.000 single cells were acquired using either the “Annexin V Assay”, the “Caspase Assay” or the “Mitochondrial Potential Assay”. For more details please refer to www.chemometec.com

Data analysis:
To facilitate a direct comparison data obtained with the two different cytometers were exported in FCS format and analyzed using FlowJo, version 7.6.5 (Treestar). Each data point in the graphs shown below represents the average of 6 samples (three independent samples analysed in duplicate).

Results

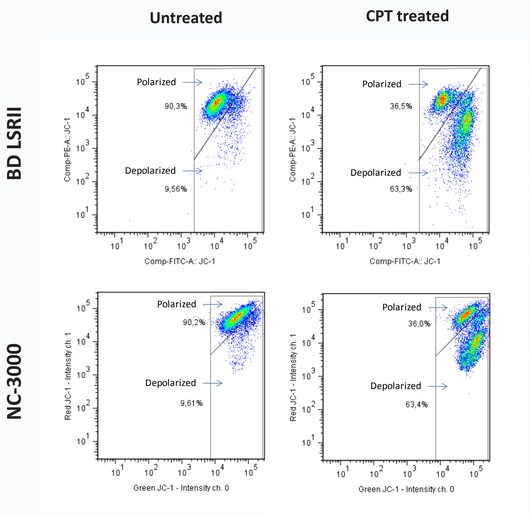

Fig. 1. Representative examples of scatter plots obtained from BD LSRII and NC-3000
Jurkat cells were grown in the absence (untreated) or in the presence of 10 µM camptothecin (CPT treated) for 16 hours. Cells were stained with JC-1 and analyzed by flow cytometry (upper row) and image cytometry (lower row). Acquired data were exported to FlowJo.

The fractions of polarized and depolarized cells obtained with the image cytometer are similar to those acquired with the flow cytometer.

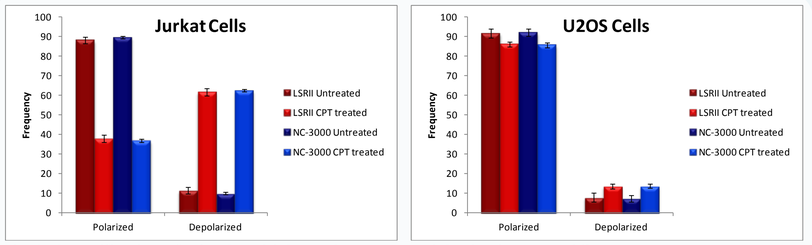

Fig. 2. Quantification of mitochondrial membrane potential using image and flow cytometry.
Exponentially growing cells (untreated) and camptothecin treated cells (CPT treated) were stained with JC-1 and analyzed by flow cytometry (reddish columns) and image cytometry (bluish columns). Columns depict the percentage of the population with, respectively, polarized and depolarized mitochondrial membranes. Each column represents the mean of 6 samples (three independent samples analyzed in duplicate). Standard deviation is indicated by an error bar.

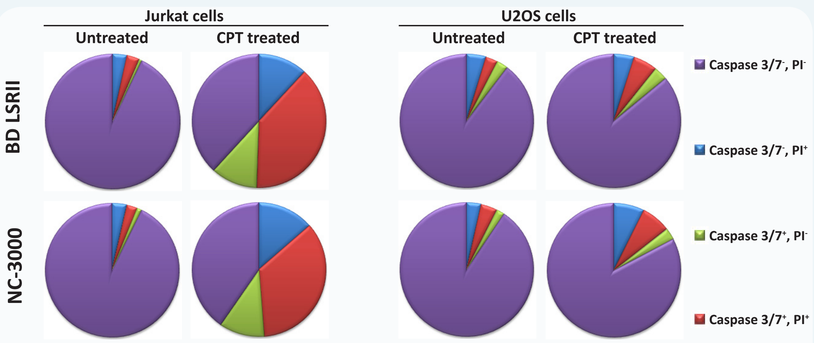

Fig. 3. Quantification of caspase activity using image and flow cytometry.
Exponentially growing cells (untreated) and camptothecin treated cells (CPT treated) were labeled with FAM-DEVDFMK (a FLICA probe which binds to activated Caspase 3/7), Hoechst-33342 and propidium iodide (PI). The fraction of caspase positive and PI positive cells was determined by flow cytometry (upper panel) and image cytometry (lower panel). Each pie represents the mean of 6 samples (three independent samples analyzed in duplicate)

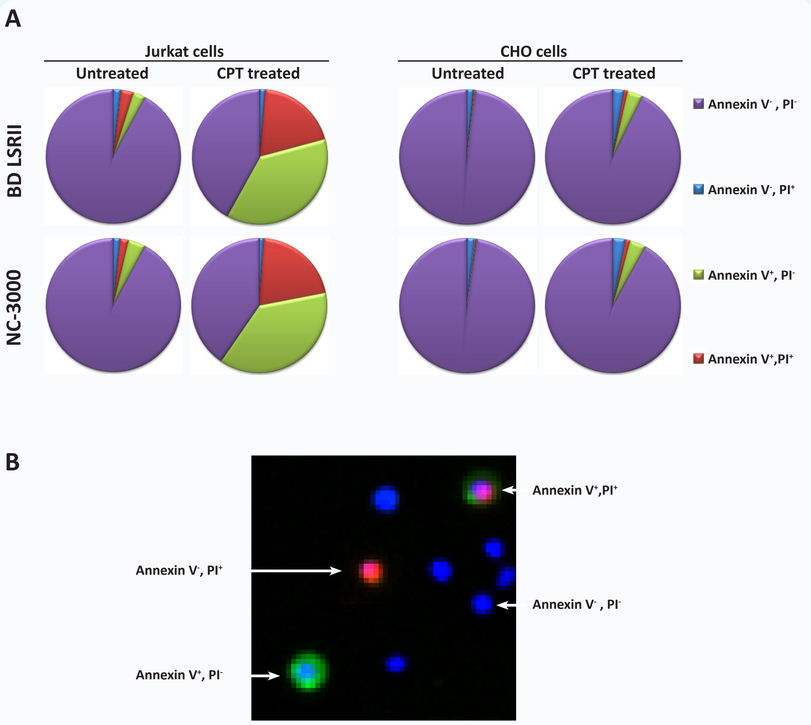

Fig. 3. Comparison of image and flow cytometric determination of phosphatidylserine translocation
Exponentially growing cells (untreated) and camptothecin treated cells (CPT treated) were labeled with FITC-Annexin V (a peptide binding externalized phosphatidylserine), Hoechst-33342 and propidium iodide (PI).
A) The fraction of Annexin V positive and PI positive cells was determined by flow cytometry (upper panel) and image cytometry (lower panel). Each pie represents the mean of 6 samples (three independent samples analyzed in duplicate).
B) Micrograph of CPT treated CHO cells. Micrograph was produced by superimposition of images captured in, respectively, the blue (Hoeschst-33342), green (FITC-Annexin V) and red (PI) channels of NC-3000. Arrows indicate the four different populations present in the samples. The optical magnification of NC-3000 is 2X.

Conclusions

Currently, flow cytometry stands as the gold standard for gathering quantitative information about large cell populations. In this study, we have compared an image cytometer, NucleoCounter NC-3000, and a flow cytometer, BD LSRII, with respect to quantifying different events in the apoptotic process. In the comparison study we have measured phosphatidylserine externalization, collaps of the mitochondrial membrane potential and activation of Caspase 3/7, all of which are well-recognized markers for apoptotic cells. For all three markers NC-3000 was accurate and precise for quantification of apoptotic cells when compared with BD LSRII. Thus, we found a high degree of concordance between the fractions of apoptotic cells measured by the two cytometric systems.

In summary, the NC-3000 image cytometer presents a new technology that allows for accurate and quantitative investigations of subtle alterations in cell homeostasis in large populations. In contrast to conventionel flow cytometry NC-3000 enables the user to validate the sample by visual inspection of the individual cells.