Detecting Apoptosis by Flow Cytometry

Where are we after 30 years?

Bill Telford, Ph.D.

NCI Flow Cytometry Core Laboratory
National Cancer Institute
National Institutes of Health

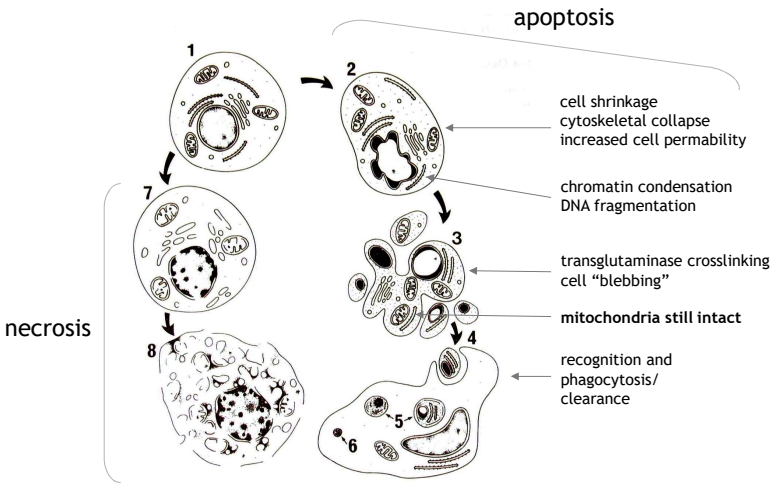
Annual Course in Flow Cytometry
- Research Methods and Applications -

The University of New Mexico

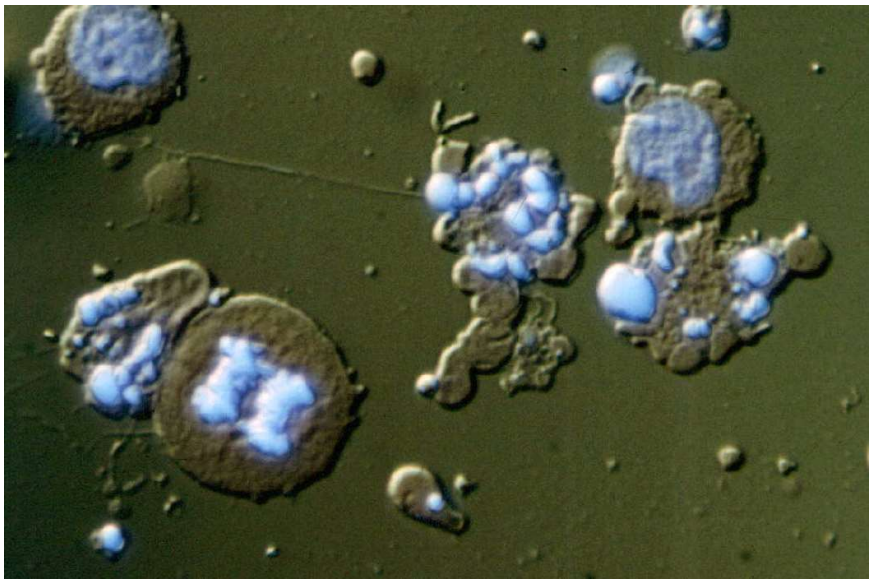
Center for Biomedical Engineering

2019

Apoptosis was first identified as a distinct morphological phenomenon in the 1960s (and probably earlier), and was well-accepted as an important regulatory process by the 1970s…



From Kerr, J.F.R., J Pathology 105, 13-20, 1971 (!)

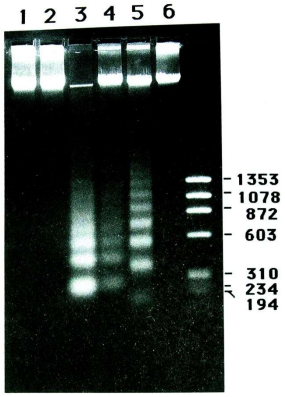


From Z. Darzynkiewicz

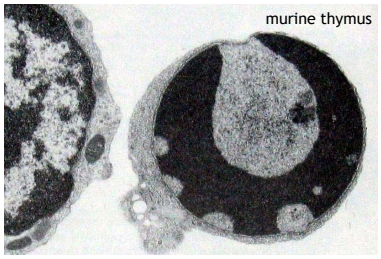
MOLT-4 cells treated with etoposide

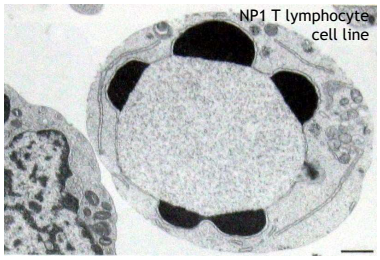
Much of the early work on immune cell apoptosis was done in the murine thymus…

Electrophoresis of DNA fragments or light/electron microscopy



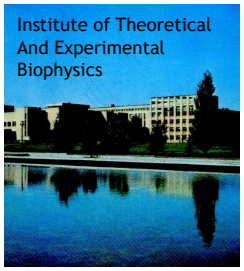
Not a single cell assay





A single cell assay, but low throughput…

Pushchino, Serphkov region, Russia



РАДИОБИОЛОГИЯ

т. XXVI 1986 вып. 6

УДК 577.391; 611.42

ОПРЕДЕЛЕНИЕ РАДИОЧУВСТВИТЕЛЬНОСТИ ИНТЕРФАЗНО ГИБНУЩИХ КЛЕТОК ТИМУСА, СЕЛЕЗЕНКИ И КОСТНОГО МОЗГА КРЫС МЕТОДОМ ПРОТОЧНОЙ ЦИТОМЕТРИИ

УМАНСКИЙ С. Р., АФАНАСЬЕВ В. Н., КОРОЛЬ Б. А., ПЕЧАТНИКОВ В. А.

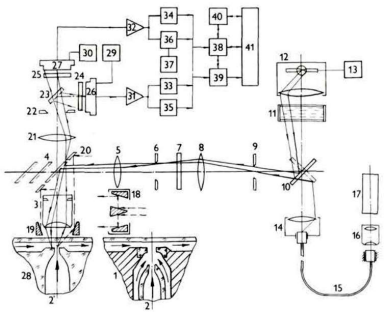


Fig. 1. Block-scheme of flow cytometer LAKS-1

gamma irradiated rat thymus, spleen and BM

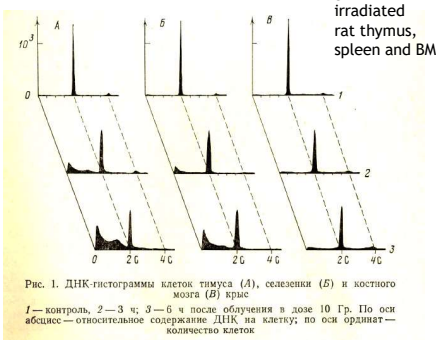


Рис. 1. ДНК-гистограммы клеток тимуса (*А*), селезенки (*Б*) и костного мозга (*В*) крыс

1 — контроль, *2* — 3 ч; *3* — 6 ч после облучения в дозе 10 Гр. По оси абсцисс — относительное содержание ДНК на клетку; по оси ординат — количество клеток

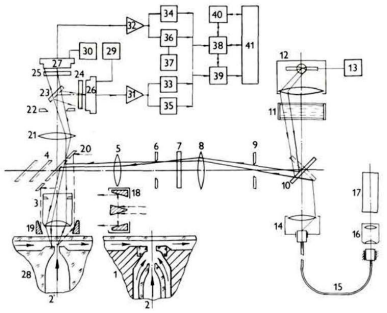
Hoechst 33258

Radiobiologiia. 1986 Nov-Dec;26(6):728-32. Russian.

FLOW CYTOMETRY USED TO STUDY INTERPHASE DEATH OF THYMUS, SPLEEN AND BONE MARROW CELLS OF IRRADIATED RATS

UMANSKY S. R., AFANASIEV V. N., KOROL' B. A., PECHATNIKOV V. A.

Institute of Biological Physics, USSR Academy of Sciences, Pushchino



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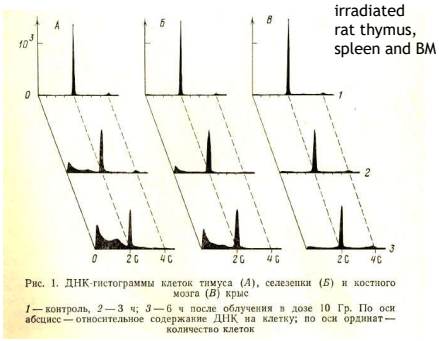

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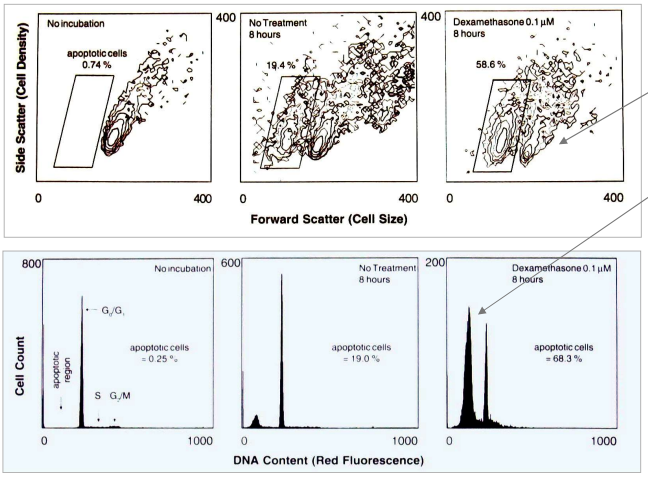
Hoechst 33258

Flow cytometry assays for apoptosis are now 30 years old…

The earliest flow cytometry assays for apoptosis analyzed changes in **forward and side scatter**, and **DNA fragmentation / loss** following ethanol treatment. Unlike earlier assays, flow cytometry analyzed apoptosis in **individual cells**.



changes in light scatter

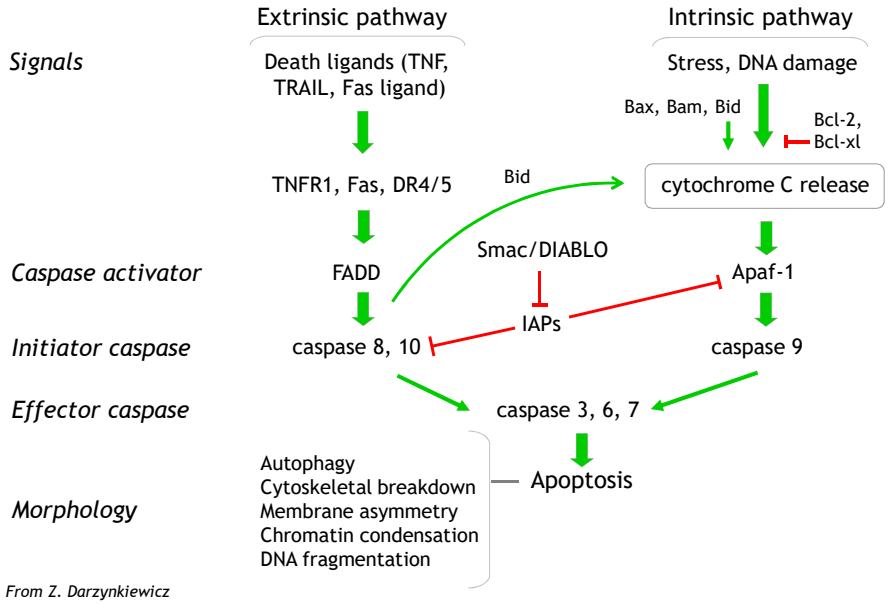
the "sub-G0/G1" peak

Apoptosis measurement in *individual* cells (not lysates)

Analysis on an Ortho Cytofluorograph 50-H (!)

From Telford et al., Applied Fluorescence Technology 4, 12-17 (1992)

Signal transduction of apoptosis



From Z. Darzynkiewicz

Types of assays…

Flow cytometry assays now target almost every stage of apoptosis, from the earliest mitochondrial changes to caspase activation, membrane changes and DNA damage.

	characteristic	flow cytometry assay
"early" apoptotic events	Cell volume fluctuations	Minor changes in scatter
	Cytochrome C release	Cytochrome C release assay
	Changes in cell membrane potential	Membrane potential probes
	Mitochondrial potential changes	Mitochondrial potential probes
	Signaling events (bcl-2, Bax, etc.)	Bax translocation
	Initiator (proximal) caspase activation (1,9,10,8)	Fluorgenic caspase substrates PhiPhiLux FLICA CellEvent Green
	Effector (distal) caspase activation (3,6,7)	Immunolabeling of active caspases
"late" apoptotic events	Organelle changes	Organelle-specific probes
	PS membrane "flipping"	Annexin V, structure-specific plasma membrane probes
	Transglutaminase crosslinking	
	Changes in chromatin organization	Immunolabeling of histones and histone associated proteins
	DNA strand breaks	TUNEL assays
	Membrane "blebbing"	Major changes in scatter
	Global chromatin damage	Loss of DNA dye binding
	Loss of membrane permeability	

Take-home lessons…

Apoptosis is a highly variable process. There is a lot of variation in the apoptotic process and phenotype between cell types, and even the same cell type at different levels of activation or differentiation.

You therefore need to find the best method for measuring apoptosis for your particular cell system. Don't just choose a method at random!

Never use only one assay for apoptosis. And combine multiple assays wherever possible! Always measure cell death using several different methods, *preferably in the same sample*. Multiparametric flow cytometry is ideal for this. Combine biochemical and morphological assays when possible. The *process* of apoptosis can be observed.

Let your assay not only *measure* cell death, but *characterize* it as well. You can learn interesting things about your cells and your system.

Take pictures! Visualizing the cells is important and very educational! Many new options in image cytometry make this possible.

Using DNA dyes and viability probes as a starting point for apoptosis assays

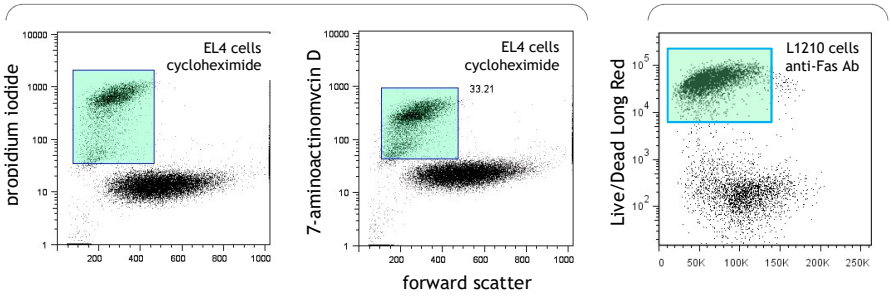
DNA dyes used as viability probes (like PI, 7-AAD, DAPI, Hoechst 33258 and the SYTOX dyes), and covalent viability probes like Live/Dead (Thermo Fisher) and the Zombie dyes (BioLegend) are great starting points for building an apoptosis assay.

Alone, they are not enough to "prove" apoptosis, but they make essential counter-labels for assays like annexin V and caspase substrates.

Most DNA dyes are NOT fixable – covalent viability probes ARE.

DNA binding dyes

covalent viability probe

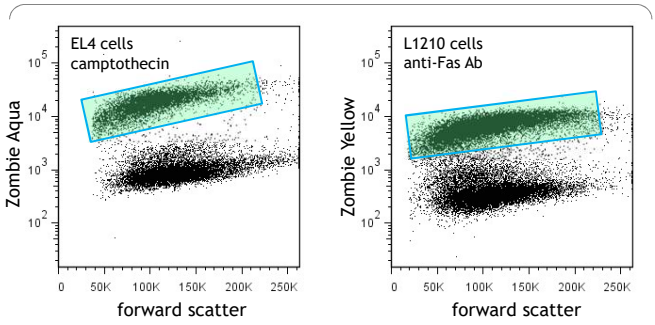


Using DNA dyes and viability probes as a starting point for apoptosis assays

The **Live/Dead dyes** (Thermo Fisher Scientific) come in a variety of colors. Pick one with minimal spectral overlap into your apoptosis assays. Near IR (red laser excited) and Violet, Aqua and Yellow (violet laser excited) are good choices.

The BioLegend **Zombie dyes** and the BD **Horizon Fixable Viability Stains** work by the same principle, and are also available in a variety of colors.

covalent viability probes



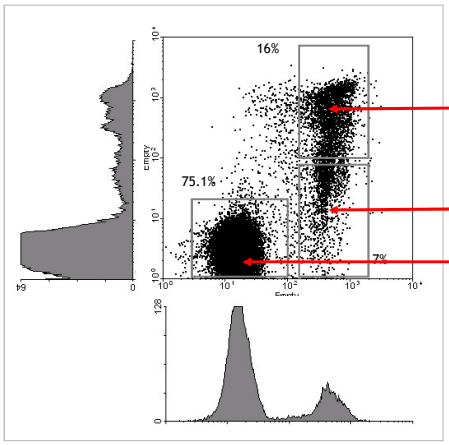
Combining multiple apoptosis assays

Annexin V and a DNA binding dye is an excellent example of combining two assays into a multiparametric method.

EL4 cells
actinomycin D
4 hours

PS "flipping" occurs prior to 7-AAD permeability

At least two stages of apoptotic death are being measured here.



annexin V+ PI+
(late apoptotic/ necrotic)

annexin V+ PI-
"early apoptotic"

"viable" cells

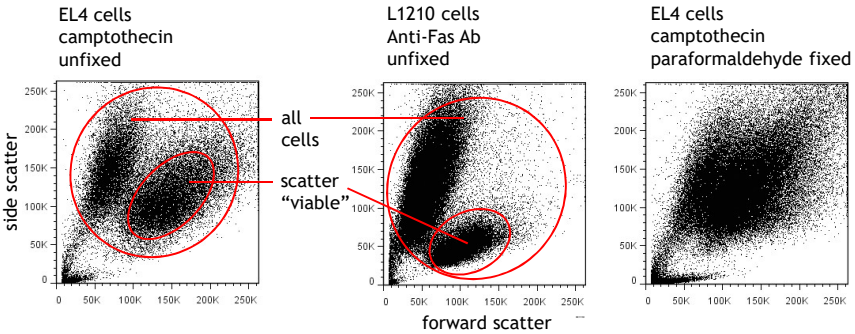
Interpreting forward and side scatter

Many cell types show a distinctive loss of forward scatter and increase in side scatter during apoptosis. However, this is not always the case.

Fixation can also blur the distinction between live and dead cells.

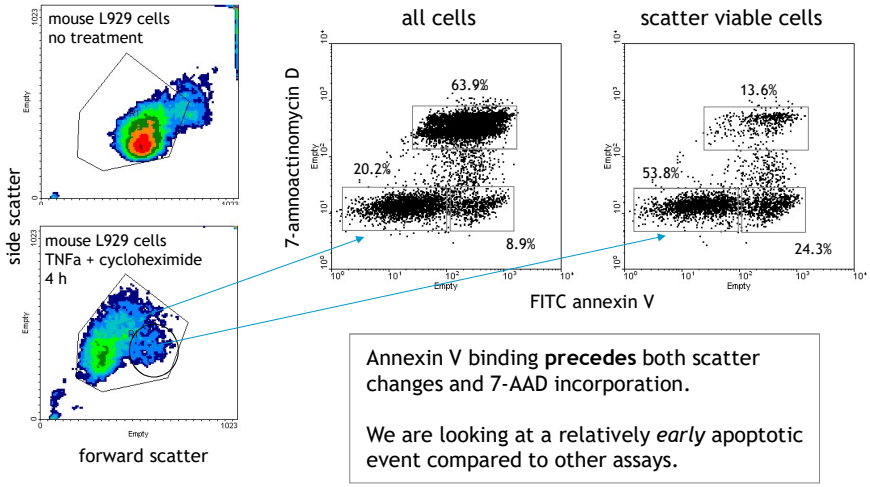
Gate carefully! We recommend that you gate on both “viable” and “non-viable” cells, but look at the apoptotic phenotype in the scatter ”viable” cells only as well.

You will be able to see the earliest stages of apoptosis.



Analysis of scatter “viable” cells

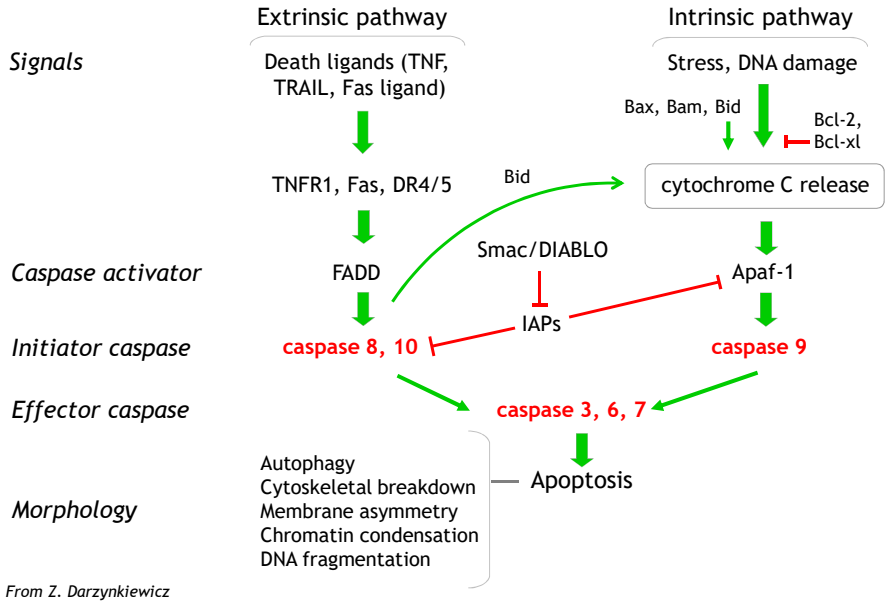
With scatter, four parameters are being measured here. Combining assays allows the progression of apoptosis to be studied, and provides a much richer picture than any one assay can give.



Annexin V binding **precedes** both scatter changes and 7-AAD incorporation.

We are looking at a relatively *early* apoptotic event compared to other assays.

Signal transduction of apoptosis



From Z. Darzynkiewicz

Caspase substrates

Caspase substrate peptides coupled to a fluorochrome, and in some cases a reactive group.

Three major types…

FLICA	Fluorochrome-labeled inhibitors of caspases
PhiPhiLux	Exciton-based fluorogenic caspase substrates
CellEvent Green NucView 488	Substrate-immobilized DNA binding dye

All can be used to analyze endogenous caspase activity in unfixed cells.

All have advantages and limitations.

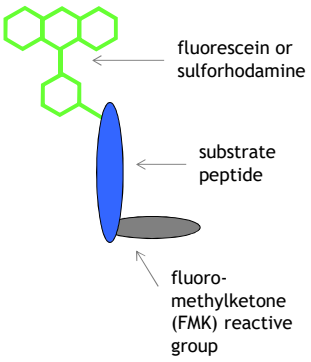
FLICA substrates

Available from a number of commercial sources

Immunochemistry Technologies FLICA
Thermo Fisher Life Tech Vybrant FAM and Image-iT

Consists of a caspase consensus substrate peptide flanked by a fluorochrome molecule and a fluoromethylketone (FMK) reactive group.

Complex is always fluorescent.



Unfixed cells are incubated with the substrate, which interacts with active caspase binding domains. The FMK regions then crosslinks to amine residues at the caspase activation site. *FLICA reagents are inhibitors, since they crosslink and inactivate caspases.*

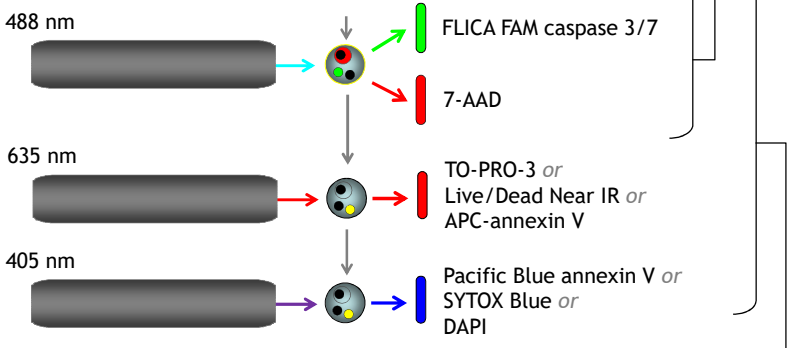
The unreacted substrate is then washed out. Cells can be analyzed as is, or fixed for later analysis.

Available in fluorescein, sulforhodamine 101 and Cy5 (FLICA 660) conjugates. *In vivo* form available (FLIVA). Available in different caspase specificities.

Building multicolor apoptosis assays

Building multicolor apoptosis assays is like building any multicolor assay…

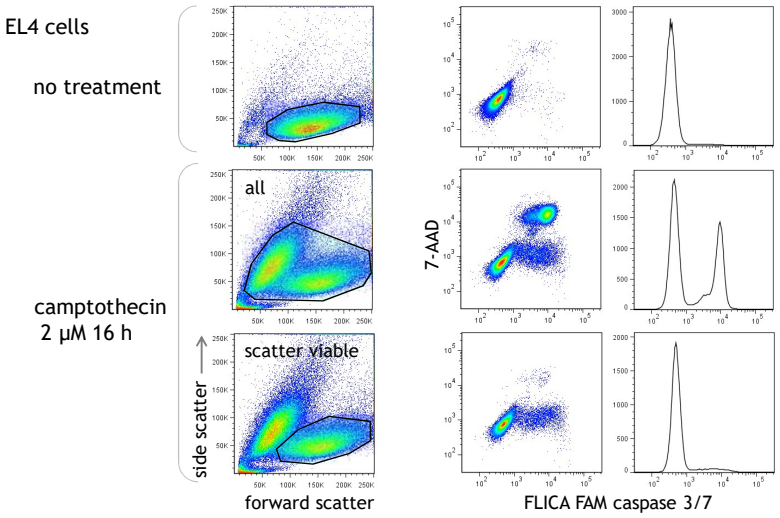
Choose fluorescent probes with good spectral separation, minimizing compensation.



If using a multi-laser instrument, distribute the probes across all lasers, also minimizing compensation.

FLICA detection of apoptosis-associated caspase activation

The FLICA reagents can (and should) be combined with a DNA permeability dye, like propidium iodide or 7-AAD.



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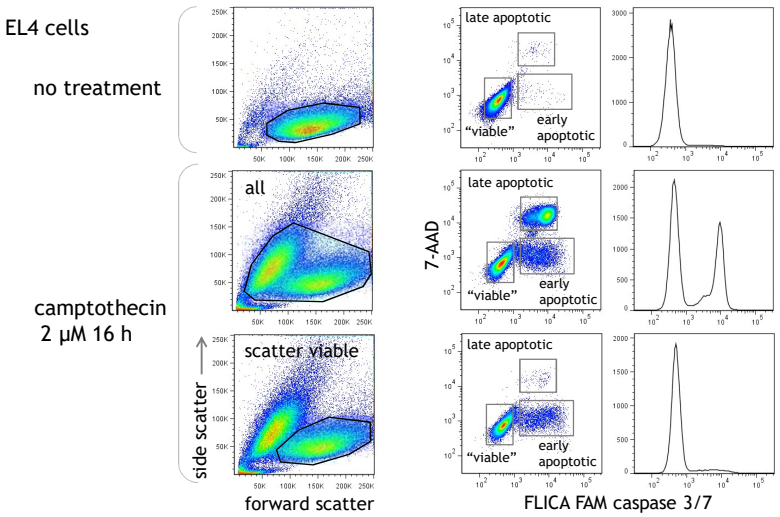
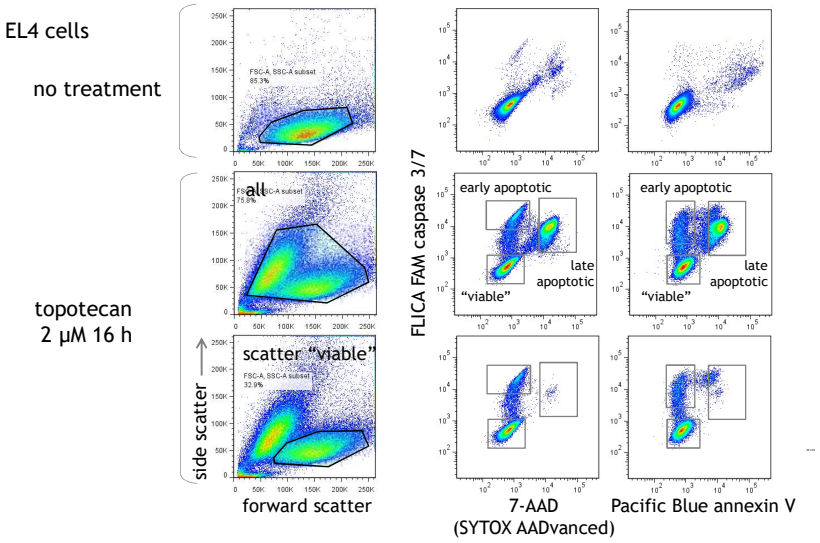


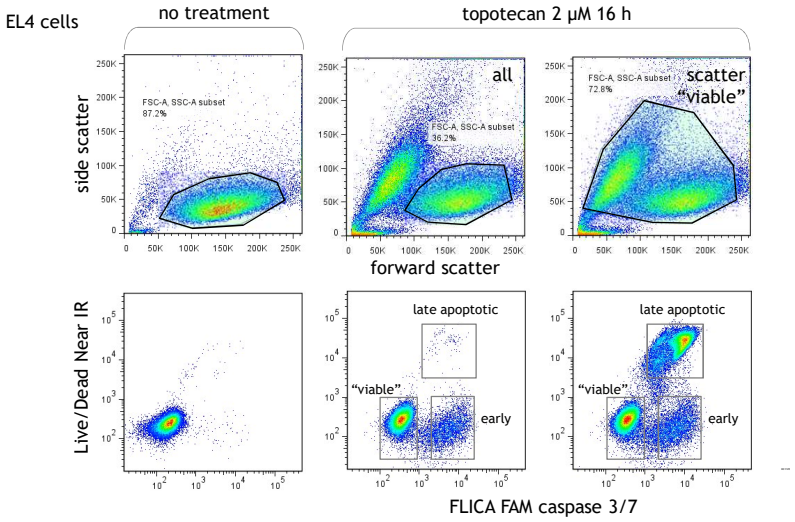
FLICA detection of apoptosis-associated caspase activation

The FLICA reagents can be combined with annexin V and a DNA dye like 7-AAD for a powerful multiparametric apoptosis assay. Cells should not be fixed.



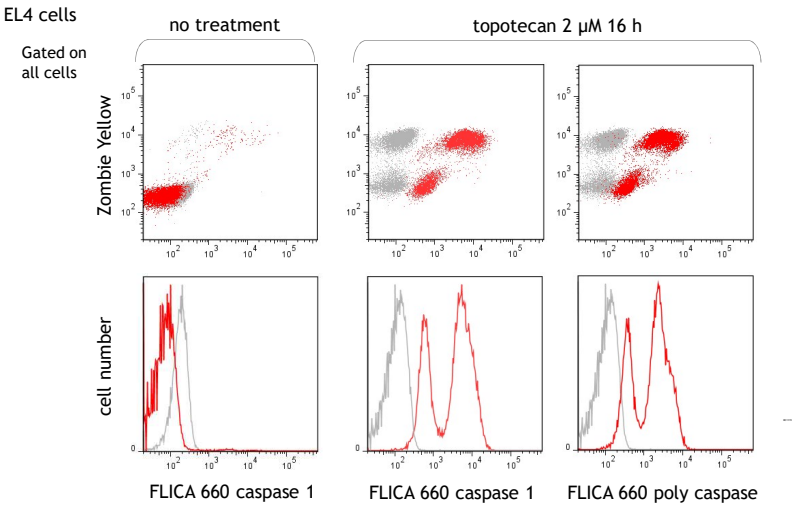
FLICA detection of apoptosis-associated caspase activation

The FLICA reagents can also be combined with covalent viability dyes like Live/Dead. **Cells can be fixed under these circumstances.**



FLICA 660

A red-excited, red-emitting FLICA reagent (FLICA 660) is available from Immunochemistry Technologies, Inc.



FLICA substrates

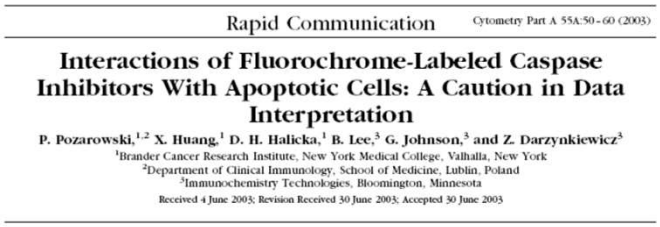
Advantages

FLICA substrates covalently crosslink to the active site, so location of caspase activity is retained.

Fixed and permeablized cells can be analyzed at a later time.

Issues

FLICA substrates have been found to bind non-specifically to intracellular sites with no caspase activity (FMK problems).



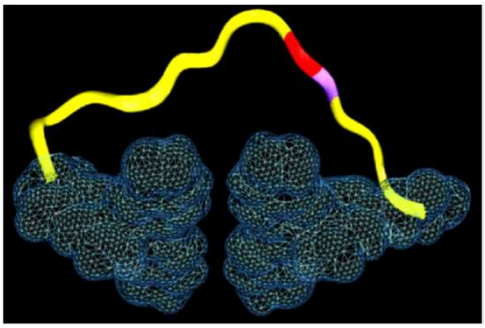
Rapid Communication Cytometry Part A 55A:50–60 (2003)

Interactions of Fluorochrome-Labeled Caspase Inhibitors With Apoptotic Cells: A Caution in Data Interpretation

P. Pozarowski,[1,2] X. Huang,[1] D. H. Halicka,[1] B. Lee,[3] G. Johnson,[3] and Z. Darzynkiewicz[3]
[1]Brander Cancer Research Institute, New York Medical College, Valhalla, New York
[2]Department of Clinical Immunology, School of Medicine, Lublin, Poland
[3]Immunochemistry Technologies, Bloomington, Minnesota

Received 4 June 2003; Revision Received 30 June 2003; Accepted 30 June 2003

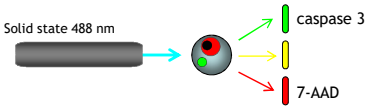
PhiPhiLux™ caspase substrates



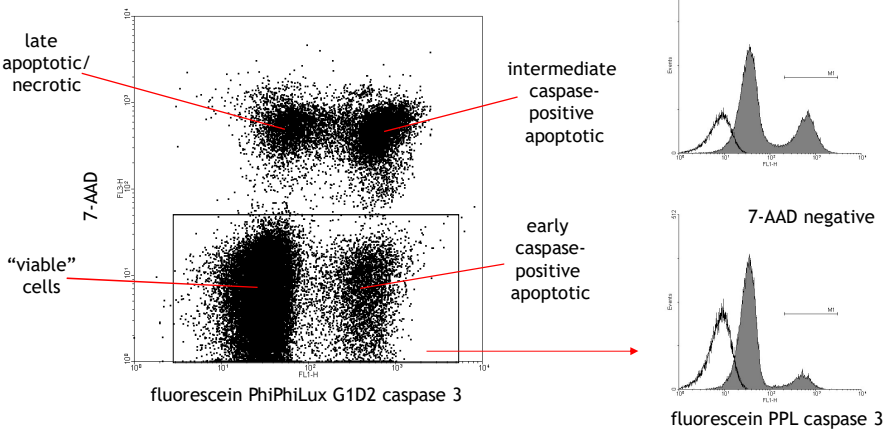
Oncoimmunin, Inc.

- peptide backbone containing enzyme consensus cleavage site (DEVD for caspase 3/7)
- fluorochrome molecules attached to terminal ends of the peptide
- peptide backbone modified to bring fluorochrome molecules into close steric proximity, resulting in fluorescent quenching
- complex is relatively non-fluorescent when uncleaved
- cleavage of the consensus site "frees" the fluorochromes, which then fluoresce
- cells are not permeablized or fixed following substrate incubation, but are analyzed immediately – *PhiPhiLux does not inactivate caspase, and is not an inhibitor*
- conjugated with fluorescein-, rhodamine and Cy5-like fluorochromes

PhiPhiLux caspase substrates



Like annexin V, PhiPhiLux can (and should) be combined with a DNA binding dye (PI or 7-AAD are fine).



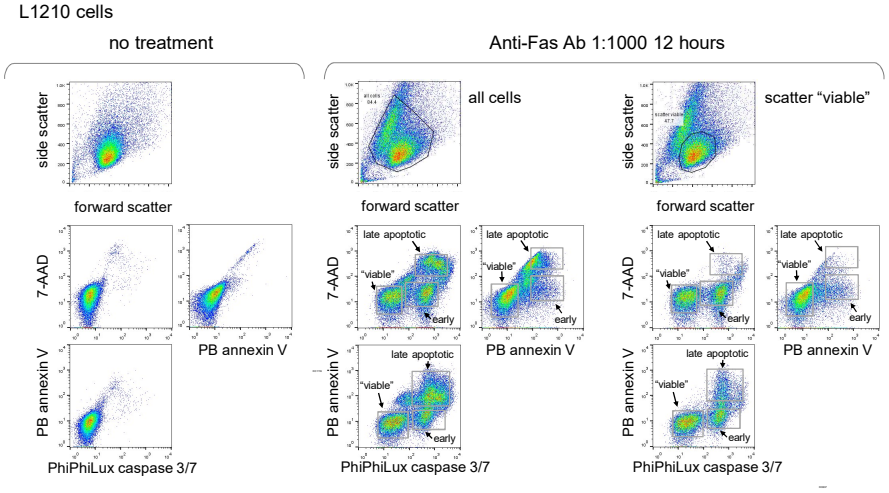
fluorescein PhiPhiLux G1D2 caspase 3

fluorescein PPL caspase 3

EL4 cells cycloheximide 50 µg/ml 4 hr

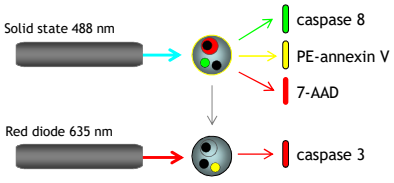
PhiPhiLux caspase substrates

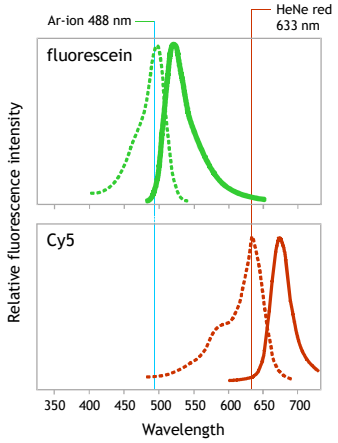
Like FLICA, PhiPhiLux can be readily combined with annexin V and a DNA binding dye, or a covalent viability probe. Unlike FLICA, PhiPhiLux is **not fixable.**



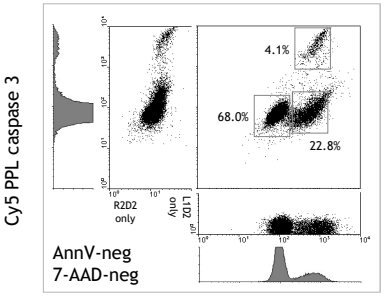
Detection of two distinct caspase activities by flow cytometry

Simultaneous labeling of apoptotic EL4 cells with fluorescein and Cy5 caspase 3 and 8 substrates



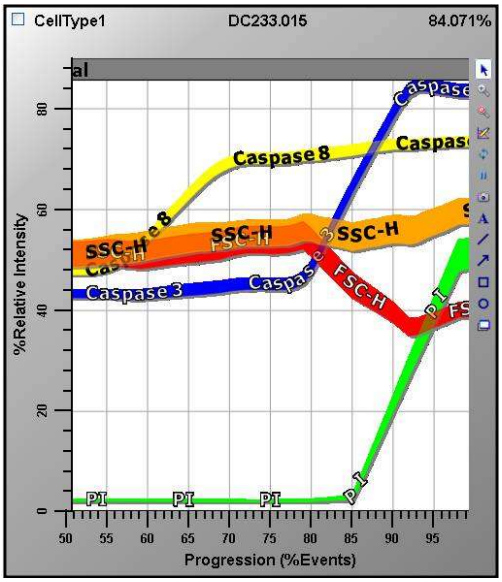


caspase 8 (pro-caspase 3) → caspase 3



From Telford, W.G., Komoriya, A. and Packard, B.Z. (2004) *Methods in Molecular Biology* Volume 263, Flow Cytometry Protocols, pp. 141-159.

Gemstone™ probability state modeling of caspase activation



Simultaneous labeling of apoptotic EL4 cells with fluorescein and Cy5 caspase 3 and 8 substrates

Our "knowns" are forward and side scatter, propidium iodide membrane permeability and caspase 3.

Layering in caspase 8, we can see that it comes up well before caspase 3.

Gemstone analysis by C. Bruce Bagwell, Verity Software House

PhiPhiLux caspase substrates

Advantages

They are relatively non-fluorescent prior to cleavage, making for lower backgrounds. Caspases remain active, since the substrate does not crosslink and inactivate the enzyme.

Specificity studies demonstrate relatively good specificity for target caspases.

Issues

They do not covalently bind to the site of activity – analysis and localization studies must be done quickly. Not fixable.

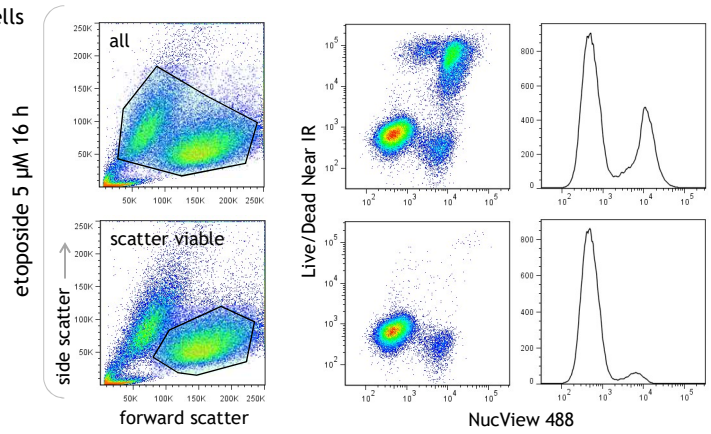
The cleaved form will diffuse out of the cell over time.

NucView 488™ (Biotium)
CellEvent Green™ (Thermo Fisher Scientific)

A substrate complex that binds to DNA upon enzyme cleavage.

Loaded into viable cells – in the presence of caspase, the complex is cleaved and the dye can bind to nuclear DNA. **Not fixable.**

EL4 cells

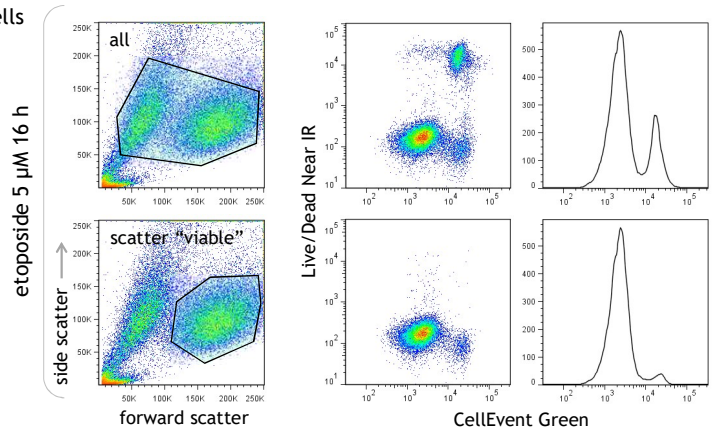


CellEvent Green™ (Thermo Fisher Scientific)

A substrate complex that binds to DNA upon enzyme cleavage.

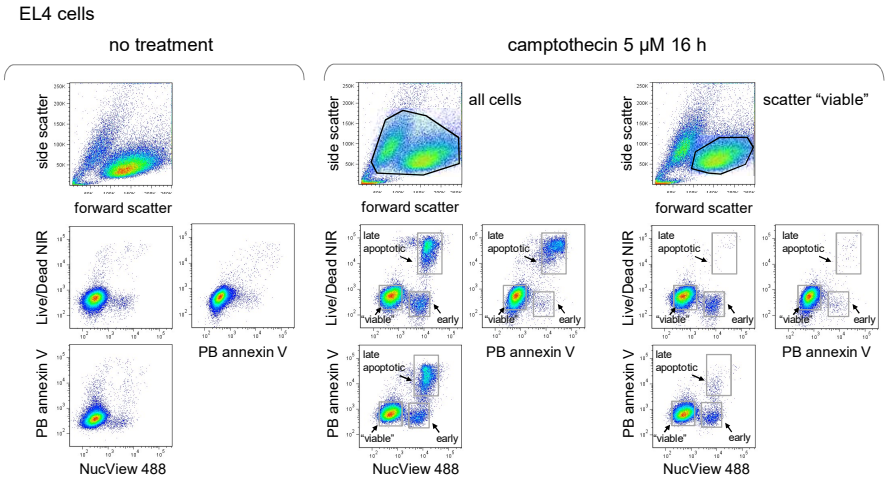
Loaded into viable cells – in the presence of caspase, the complex is cleaved and the dye can bind to nuclear DNA. **Not fixable.**

EL4 cells



NucView 488™ (Biotium)

Like FLICA and PhiPhiLux, NucView 488 can be combined with annexin V and a DNA binding dye or covalent viability probe.



NucView 488™
CellEvent Green™

Advantages

Rapid incorporation and labeling, and washing not required.

Other caspase specificities and fluorochromes coming soon.

Issues

They do not covalently bind to the site of activity – analysis and localization studies must be done quickly. Not fixable. Not site-specific.

About all caspase substrates…

No synthetic substrate is *completely* specific for its target enzyme.

Cell permeability is never total.

Can caspases be activated in circumstances other than apoptosis?

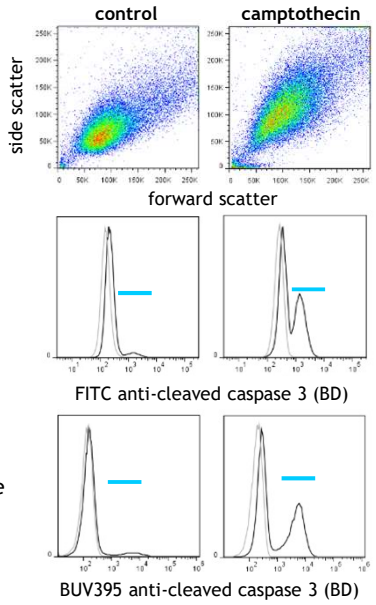
Immunodetection of cleaved caspase 3 by flow cytometry

Several monoclonal antibodies against the cleaved active form of caspase 3 are available (BD Biosciences rabbit monoclonal and Cell Signaling Technologies polyclonal rabbit) conjugated to several fluorochromes and biotin.

These antibodies can label apoptotic cells in *fixed* cell preparations.

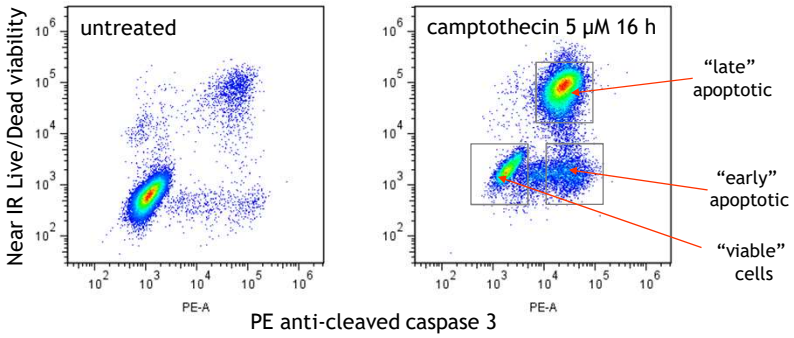
Requires paraformaldehyde fixation and detergent treatment.

Saponin-based methods of the type used for intracellular cytokine analysis work well too.



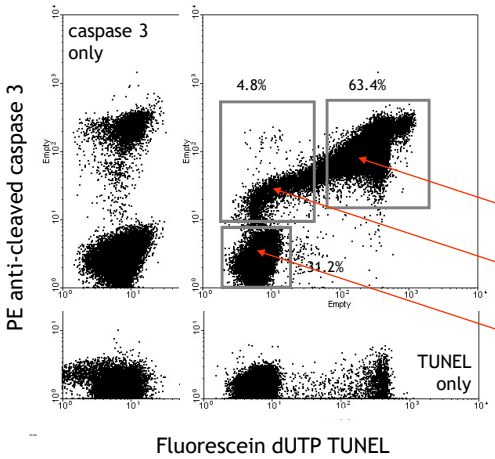
Immunodetection of cleaved caspase 3 and Live/Dead labeling

Since caspase 3 immunolabeling requires fixation permeablization, you can combine it with a fixable Live/Dead assay in place of a DNA binding dye. Cells should be labeled with the Live/Dead reagent prior to fixation.



Immunodetection of cleaved caspase 3 and TUNEL

Since caspase 3 immunolabeling requires permeablization, you can combine it with a TUNEL assay for an even better multidimensional picture of apoptosis *in fixed cells*.



Cells were TUNEL labeled, then caspase labeled.

As expected, caspase 3 comes up first, but only slightly precedes DNA fragmentation.

"late" caspase 3 -positive
DNA fragmentation -positive

"early" caspase 3 -positive
DNA fragmentation -negative

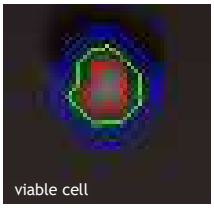
"viable" cells

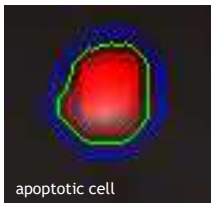
Apoptosis and image cytometry

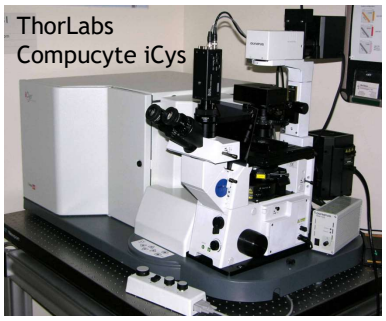
Visualizing apoptotic cells is an excellent idea. Why?

- Apoptosis is highly variable and pleiotropic. Imaging can give verification that apoptosis is occurring, and characterize it.
- Imaging gives additional analysis options (like pixel-by-pixel analysis) that are useful for apoptotic analysis.
- Imaging allows analysis of adherent cells without removal of the cells from their substrate.

Many options now exist for performing *image cytometry,* where cytometric data and correlated cell images can be collected simultaneously.

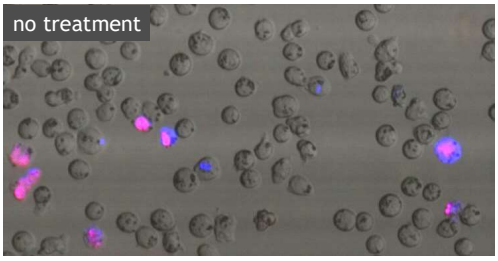


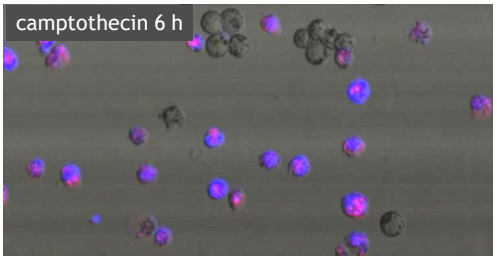




Apoptosis and image cytometry

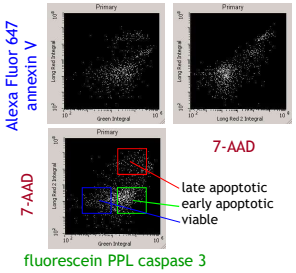
Compucyte iCys analysis of apoptotic EL4 cells



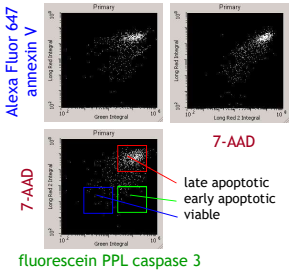


Compucyte iCys field scans

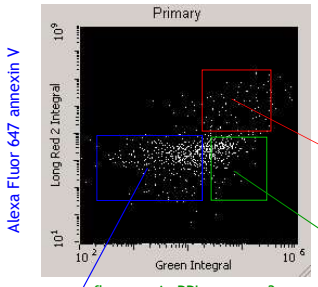
no treatment



camptothecin 6 h



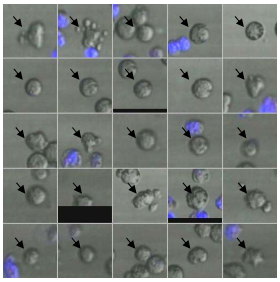
Apoptotic cell analysis with laser scanning cytometry



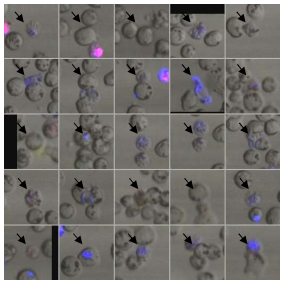
Direct correlation between the cytometric data and the imagery (relocation analysis).

Morphological analysis using light scatter or absorption.

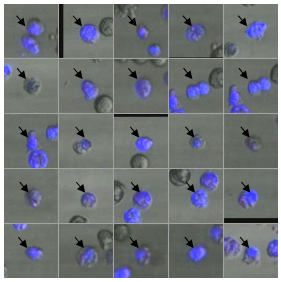
viable



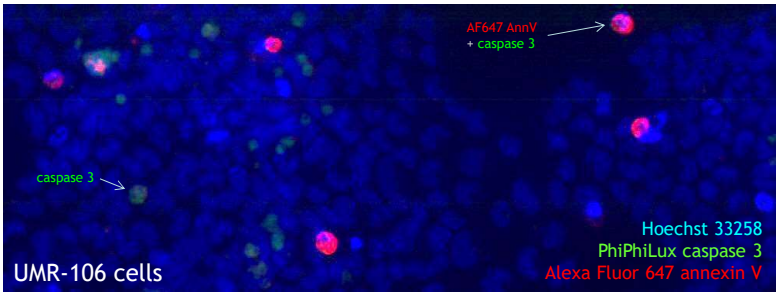
early



late



Discrimination of adherent apoptotic cells by image cytometry

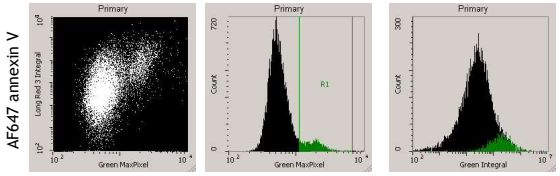


Compucyte iCys field scan

Image cytometry can analyze site-specific fluorescence from images, improving sensitivity. Trypsin or accutase detachment, which can "muddy" apoptotic labels, is not necessary.

Apoptotic cells "round up" and can be lost from the growth substrate.

Max Pixel versus total Integral



PPL caspase 3 Max Pixel | Max Pixel | Integral

PPL caspase 3

Imaging cytometers

A sampling of commercial imaging cytometry systems.

Perkin-Elmer Opera

Nexcelom Celigo

GE Life Sciences IN Cell 2200

Perkin-Elmer Operetta

Yokogawa CQ1

GE Life Sciences IN Cell 6000

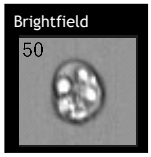
TTP LabTech Explorer series

Thermo Fisher Cellomics VTI

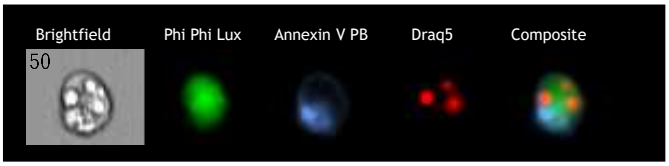
Analyzing apoptosis on the Amnis ImageStream

A stream-based scanning cytometry system like the Amnis ImageStream or FlowSight similarly is an another excellent way to combine cytometric and morphological analysis.

Again, direct correlation between cytometry and imagery.



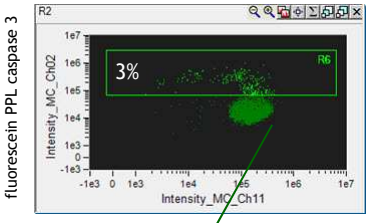
Daudi cells induced with camptothecin

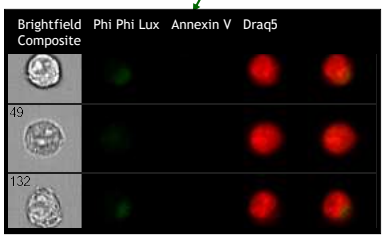


Data from Brian Hall and Tad George, Amnis

Analyzing apoptosis on the Amnis ImageStream

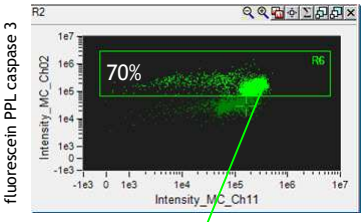
untreated

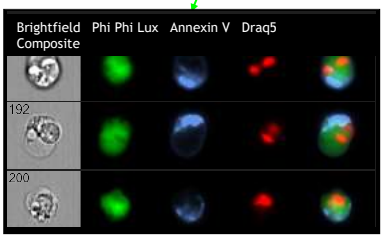




Images of live cells

camptothecin 6 h

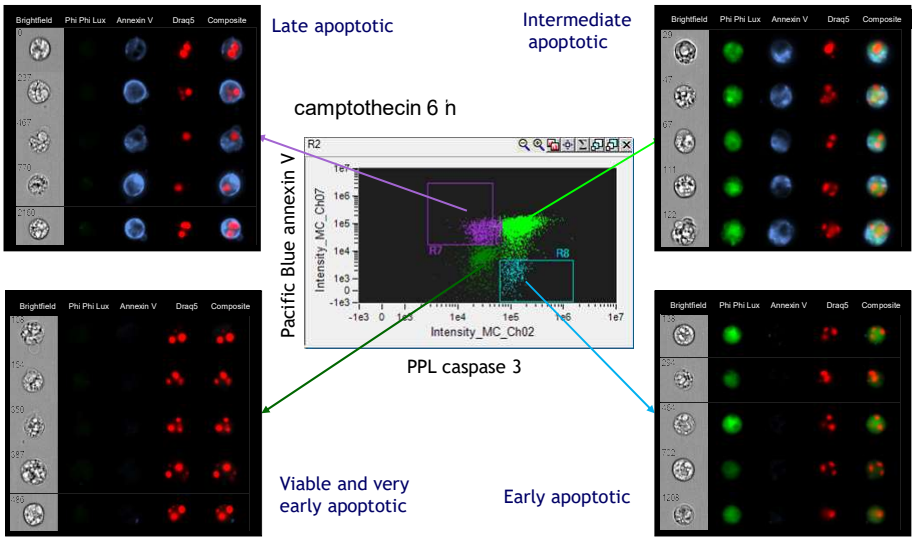




Images of PhiPhiLux positives

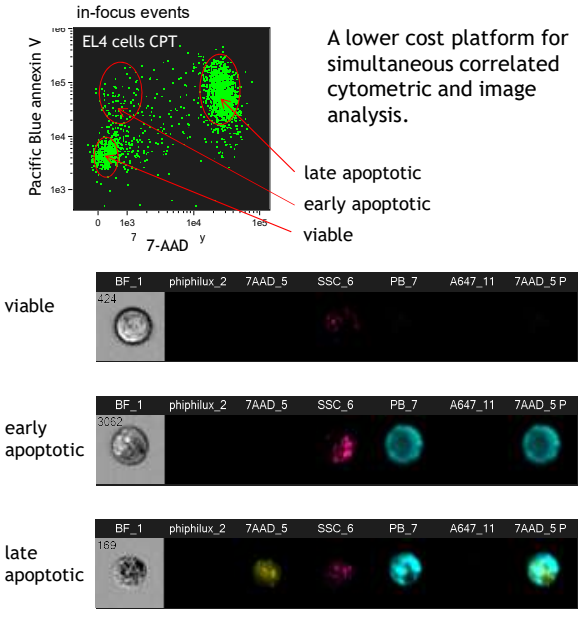
Data from Brian Hall and Tad George, Amnis

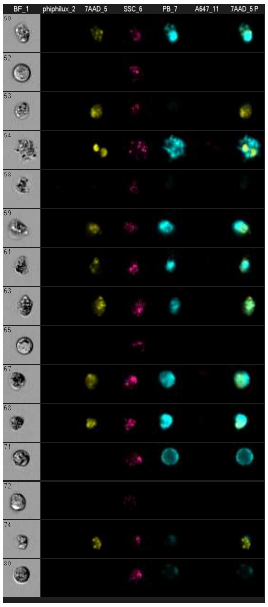
Analyzing apoptosis on the Amnis ImageStream



Data from Brian Hall and Tad George, Amnis

Analyzing apoptosis on the Amnis FlowSight





Data from Darin Fogg, Amnis

Types of assays…

Flow cytometry assays now target almost every stage of apoptosis, from the earliest mitochondrial changes to caspase activation, membrane changes and DNA damage.

	characteristic	flow cytometry assay
"early" apoptotic events	Cell volume fluctuations	Minor changes in scatter
	Cytochrome C release	Cytochrome C release assay
	Changes in cell membrane potential	Membrane potential probes
	Mitochondrial potential changes	Mitochondrial potential probes
	Signaling events (bcl-2, Bax, etc.)	Bax translocation
	Initiator (proximal) caspase activation (1,9,10,8)	Fluorgenic caspase substrates PhiPhiLux FLICA CellEvent Green
	Effector (distal) caspase activation (3,6,7)	Immunolabeling of active caspases
"late" apoptotic events	Organelle changes	Organelle-specific probes
	PS membrane "flipping"	Annexin V, structure-specific plasma membrane probes
	Transglutaminase crosslinking	
	Changes in chromatin organization	Immunolabeling of histones and histone associated proteins
	DNA strand breaks	TUNEL assays
	Membrane "blebbing"	Major changes in scatter
	Global chromatin damage	Loss of DNA dye binding
	Loss of membrane permeability	

So what assay is best for my application?

That depends on your application. What question are you asking?

Example: Yes-no viability screening of a new drug. Many samples, high throughput.

Fixed assays ("sub-G0/G1", TUNEL, FLICA, caspase immunolabeling) will be the most practical for large numbers of samples where they will have to sit for a while prior to analysis. Simple is probably better, although a two-parameter assay is best to exclude necrotic cells.

Example: Analyzing ability of lymphocytes to undergo apoptosis in a signal transduction knockout mouse model. Small numbers of samples.

A different question. You don't just want to quantify apoptosis, you want to *characterize it*, especially at the signaling level. A combination of "viable" and fixed cell assays to analyze caspases, caspase targets and other apoptotic signaling molecules. Multiple assays within a single sample will give the most information.

Don't limit yourself to one method!
Combine whenever possible.

So what assay is best for my cells?

Apoptosis is a highly variable process. Determine what assays work best for your cell system. Understand how your cells undergo apoptosis and design your detection method accordingly.

Example: EL4 cells treated with cycloheximide (transcriptional inhibitor)

- Strong caspase 3 expression
- High levels of DNA strand breaks
- No blebbing (annexin V binding strong)

Example: MCF-7 cells with ellipticine (topoisomerase II inhibitor)

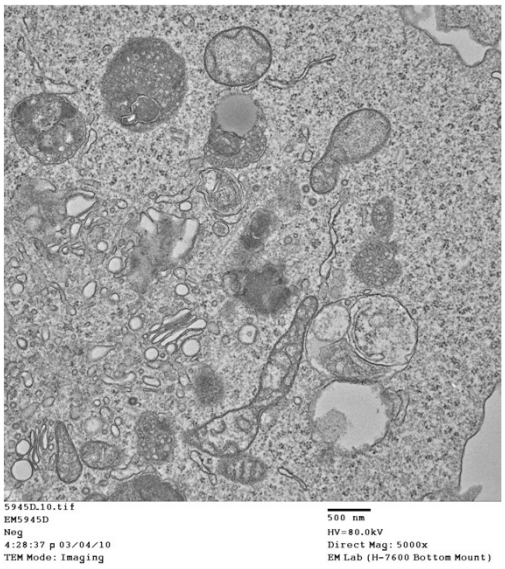
- Undetectable levels of caspase 3 expression
- Activation of caspase 8 and 9
- Blebbing ("sub-G0/G1" peak detection and annexin V detection problematic)
- High levels of DNA strand breaks

Don't limit yourself to one method!
Combine whenever possible.

Autophagy

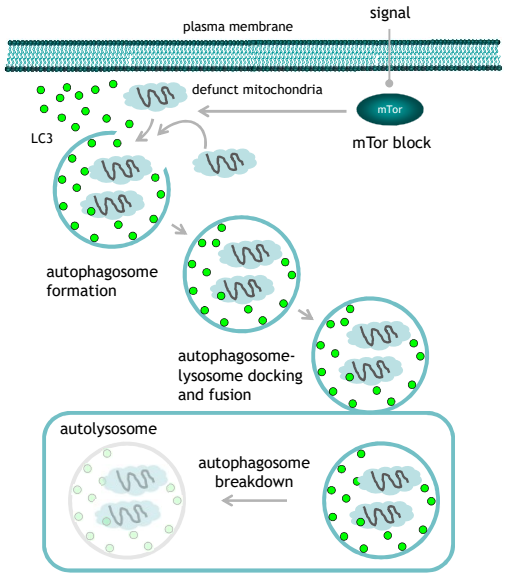
Active elimination of intracellular organelles by endocytosis and proteolysis, through the formation of autophagosomes.

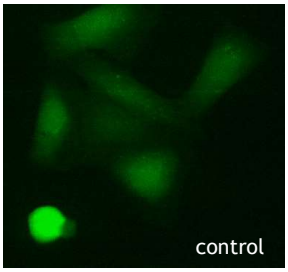
Originally thought to be primarily a pathway for apoptosis, but now believed to play a critical role in maintaining cellular homeostasis and survival, particularly during cell stress.

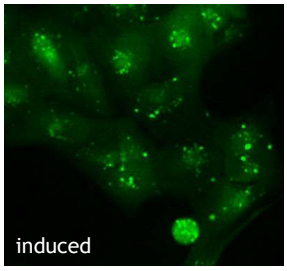


From Shoba Amarnath, ETIB-NCI-NIH

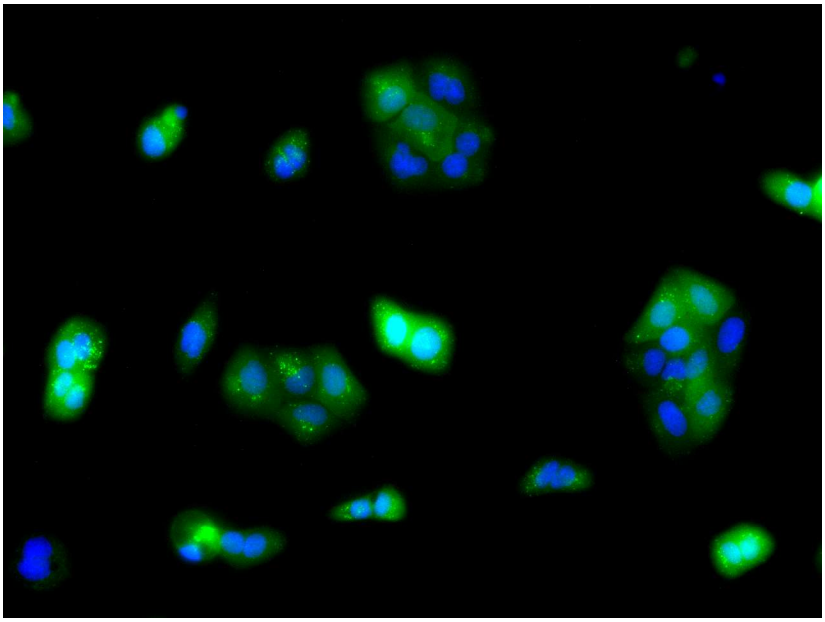
Autophagy







U2OS cells expressing GFP-LC3
EMD Millipore



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