A practical guide for detecting apoptosis by flow cytometry

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Apoptosis

"Active" or intentional cell death, in the context of day-to-day maintenance of homeostasis of a tissue or organ system, or an outside agent (like a drug)

Process of apoptosis is aimed at getting rid of the cell cleanly, without bystander damage, with the final goal of cell removal by phagocytosis

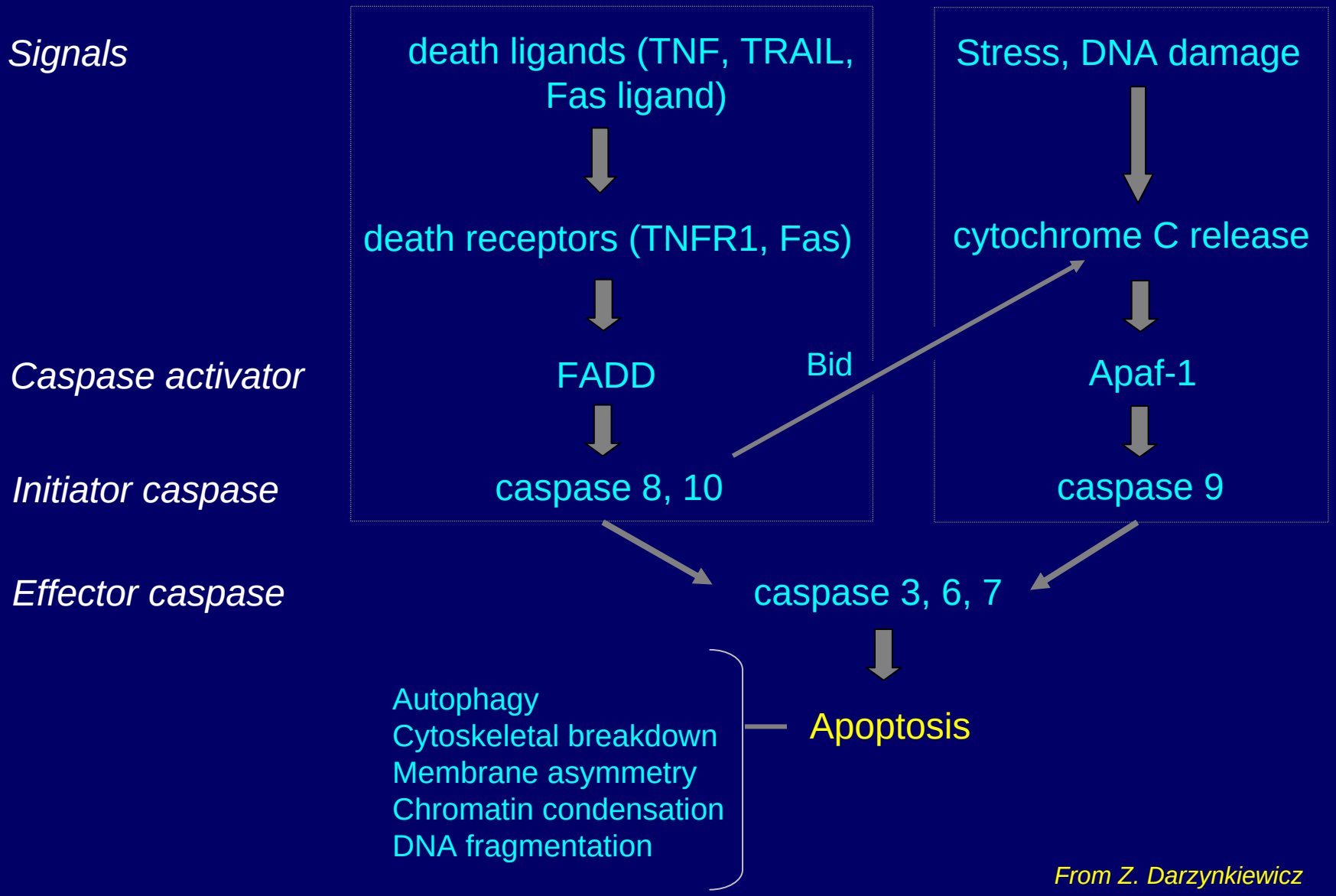
Programmed cell death

Apoptosis in the context of embryonic development. Although a special case of apoptosis, many of the signaling and effector mechanisms are shared.

Necrosis

Accidental cell death, often caused by drugs, toxins or inflammation. No clean resolution. But not always mutually exclusive from apoptosis.

Signal transduction of apoptosis



From Z. Darzynkiewicz

Take-home lessons…

Apoptosis is a highly pleiotropic 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.

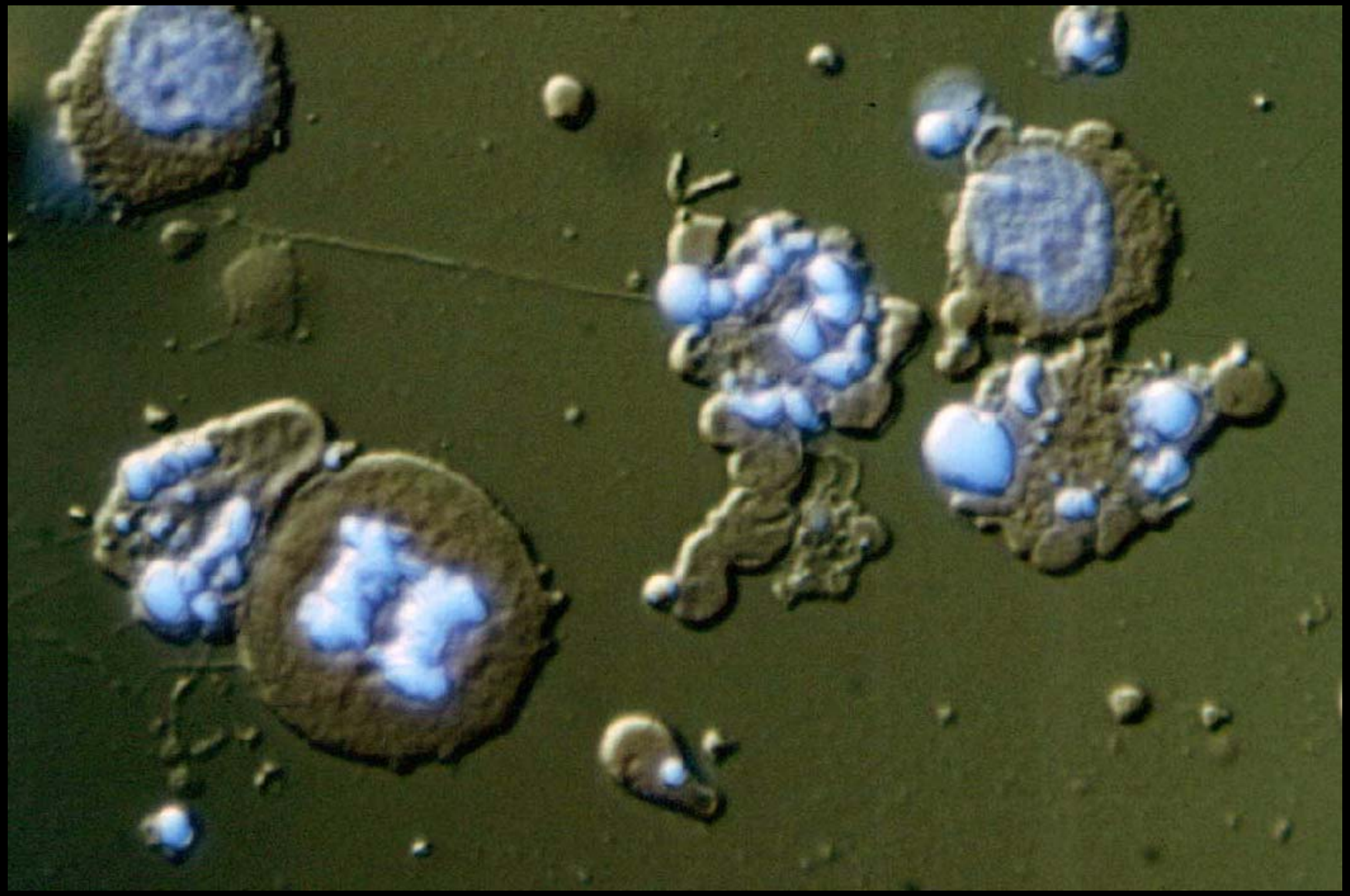
Never use only one assay for apoptosis. Always measure cell death using several different methods, *preferably in the same sample*. Multiparametric flow cytometry is ideal for this.

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 makes this possible.

Types of assays…

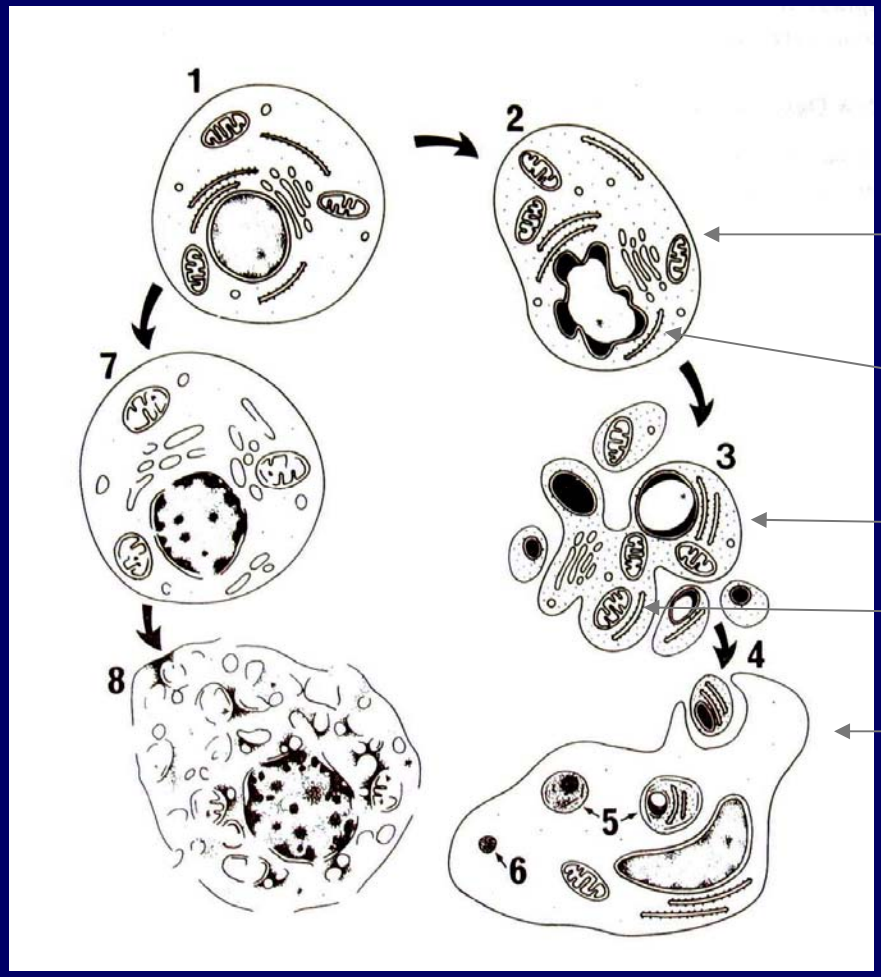
	unfixed "viable" cells	fixed permeablized cells
"early" apoptotic events	Cell volume fluctuations	Cytochrome C release
	Changes in cell membrane potential	
	Mitochondrial potential changes	
	Signaling events (bcl-2, Bax, etc.)	
	Initiator (proximal) caspase activation (1,9,10,8)	Initator (proximal) caspase activation
	Effector (distal) caspase activation (3,6,7)	Effector (distal) caspase activation
		Changes in chromatin organization (histones)
"late" apoptotic events	Organelle changes	
	PS membrane "flipping"	Early DNA strand breaks
	Transglutaminase crosslinking	
	Membrane "blebbing"	
	Loss of membrane permeability	Global chromatin damage



From Z. Darzynkiewicz

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…

apoptosis



cell shrinkage
cytoskeletal collapse
increased cell permability

chromatin condensation
DNA fragmentation

transglutaminase crosslinking
cell “blebbing”

mitochondria still intact

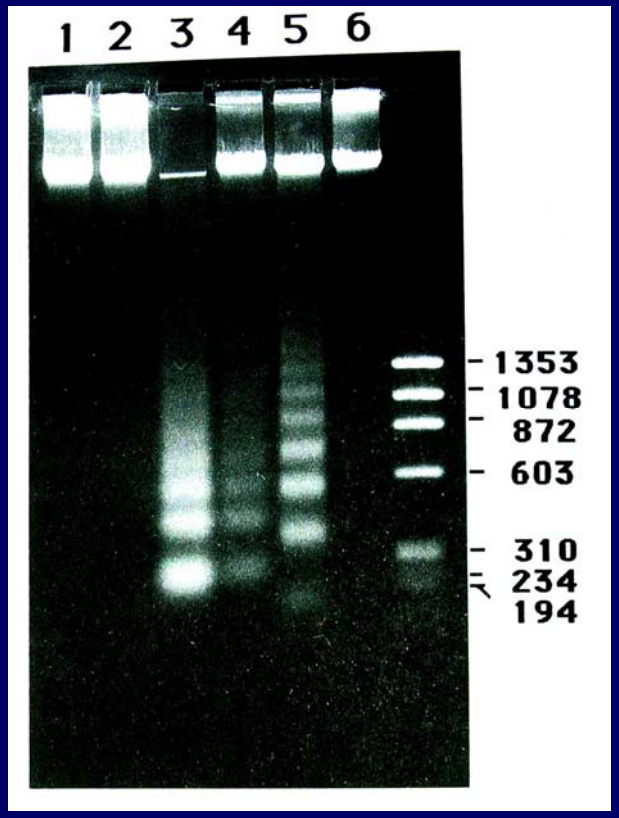
recognition and
phagocytosis/
clearance

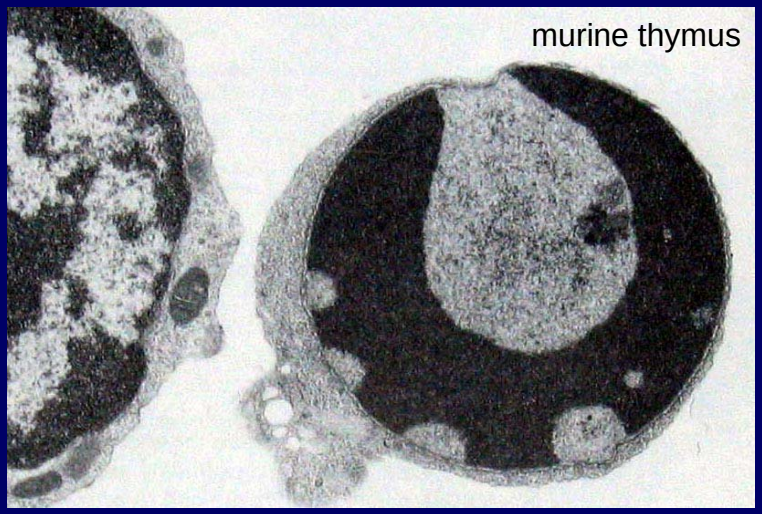
necrosis

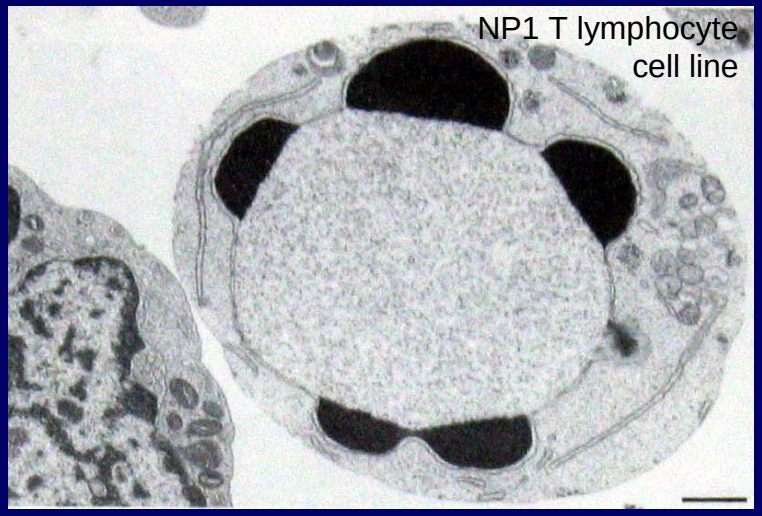
From Kerr, J.F.R., J Pathology 105, 13-20, 1971 (!)

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

Electrophoresis of DNA fragments or light/electron microscopy







Cell shrinkage and chromatin condensation ought to be visible by flow cytometry…

Cell shrinkage and cytoskeletal collapse should be detectable by a change in **forward and/or side scatter**

Chromatin condensation and DNA fragmentation should be detectable by changes in **binding properties of DNA binding dyes** (like propidium iodide)

Afanasev VN et al. (1986) **Flow cytometry and biochemical analysis of DNA degradation characteristic of two types of cell death**. FEBS Letters 194, 347.

Compton MM et al. (1988) **Analysis of glucocorticoid actions on rat thymocyte deoxyribonucleic acid by fluorescence activated flow cytometry**. Endocrinology 122, 2158.

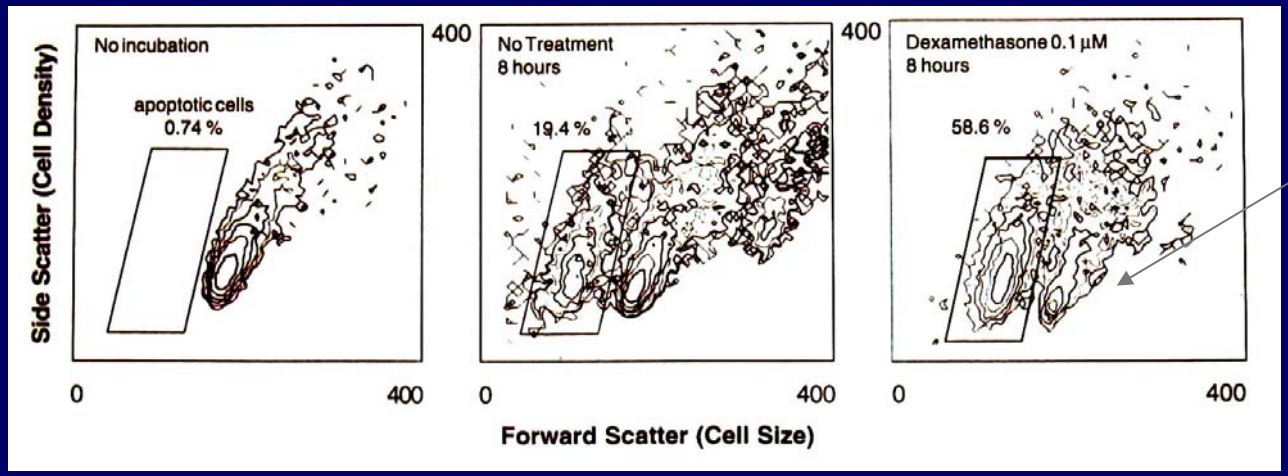
Prosperi E et al. (1990) **Nuclease-induced DNA structural changes assessed by flow cytometry with the intercalating dye propidium iodide.** Cytometry 12, 323.

Telford et al. (1991) **Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flow cytometry.** *Cell Proliferation* 24, 447-459

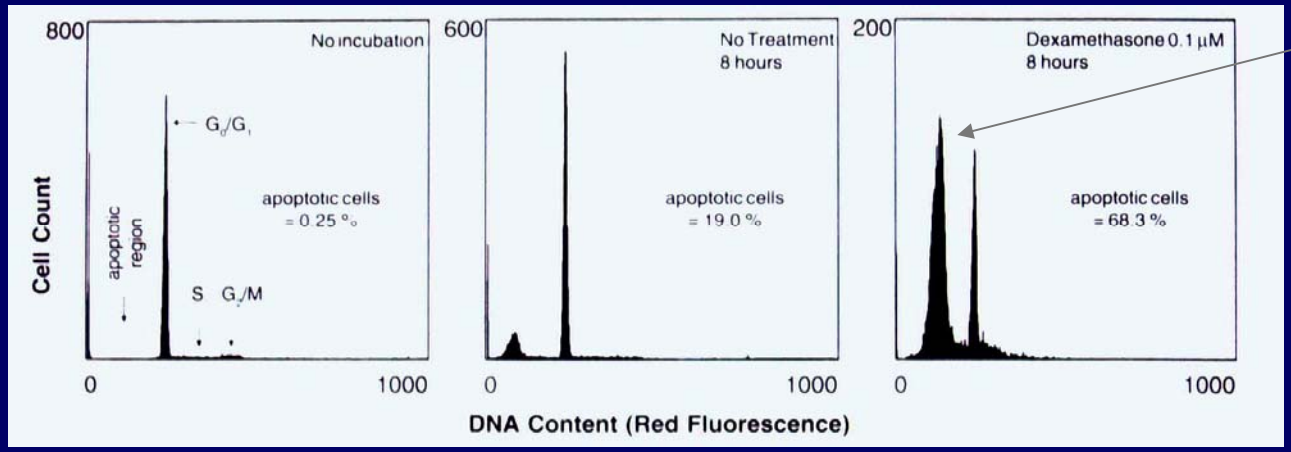
Walker PR et al. (1991) **Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes.**

Cell shrinkage and chromatin condensation by flow cytometry

Cells were usually **ethanol-treated** (to permit loss of fragmented DNA) and labeled with cell cycle appropriate concentrations of a **DNA binding dye** (usually propidium iodide)



changes in light scatter



the "sub-G0/G1" peak

Analysis on an Ortho Cytofluorograph 50-H (!)

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

Problems with measuring apoptosis by “sub-G0/G1” measurement

Despite its relative “antiquity”, measurement of apoptosis by loss of DNA from permeabilized cells remains a popular technique for measuring cell death.

Why? Cheap and easy (unlike some apoptotic assays!)

→ **However… it has some significant problems.**

When we measured cell death this way 15 years ago, most of us had the good fortune to be measuring apoptosis in mouse thymocytes, where most of the cells are in G0/G1.

Thymocytes all die in a relatively uniform morphological manner (especially when you hit them with glucocorticoids or irradiation).

However, cycling cells (activated lymphocytes, tumor cells, etc.) present A MUCH different picture.

Their uncompacted chromatin and tendency to “bleb” causes far greater DNA loss following apoptosis – the apoptotic “peak” tends to be a smear at the low threshold of instrument sensitivity.

Problems with measuring apoptosis by “sub-G0/G1” measurement

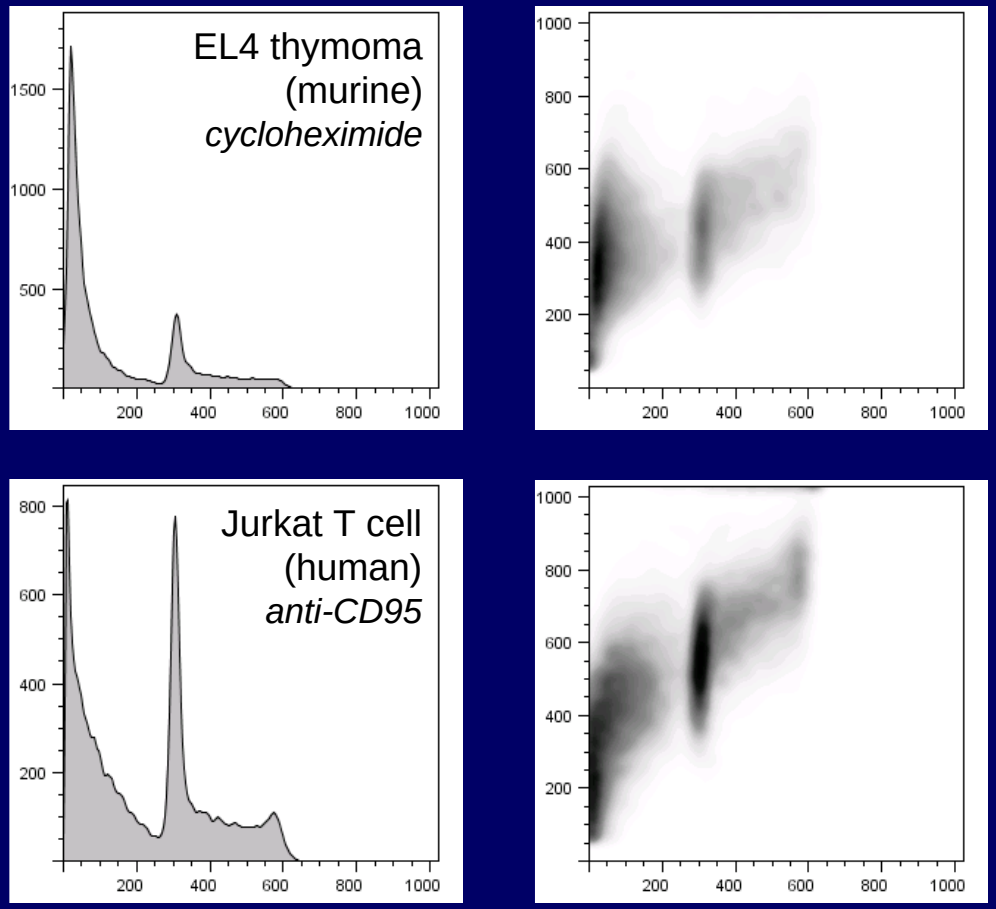
“Sub-G0/G1” analysis measures a very late-stage cell death phenotype.

Many cell types yield not a discrete peak, but a smear that extends to the minimum sensitivity threshold of the instrument.

Peak may consist of…

Apoptotic cells
Necrotic cells and debris
Blebs (cytotoxic drugs)
Other debris

No longer measuring per-cell apoptosis.

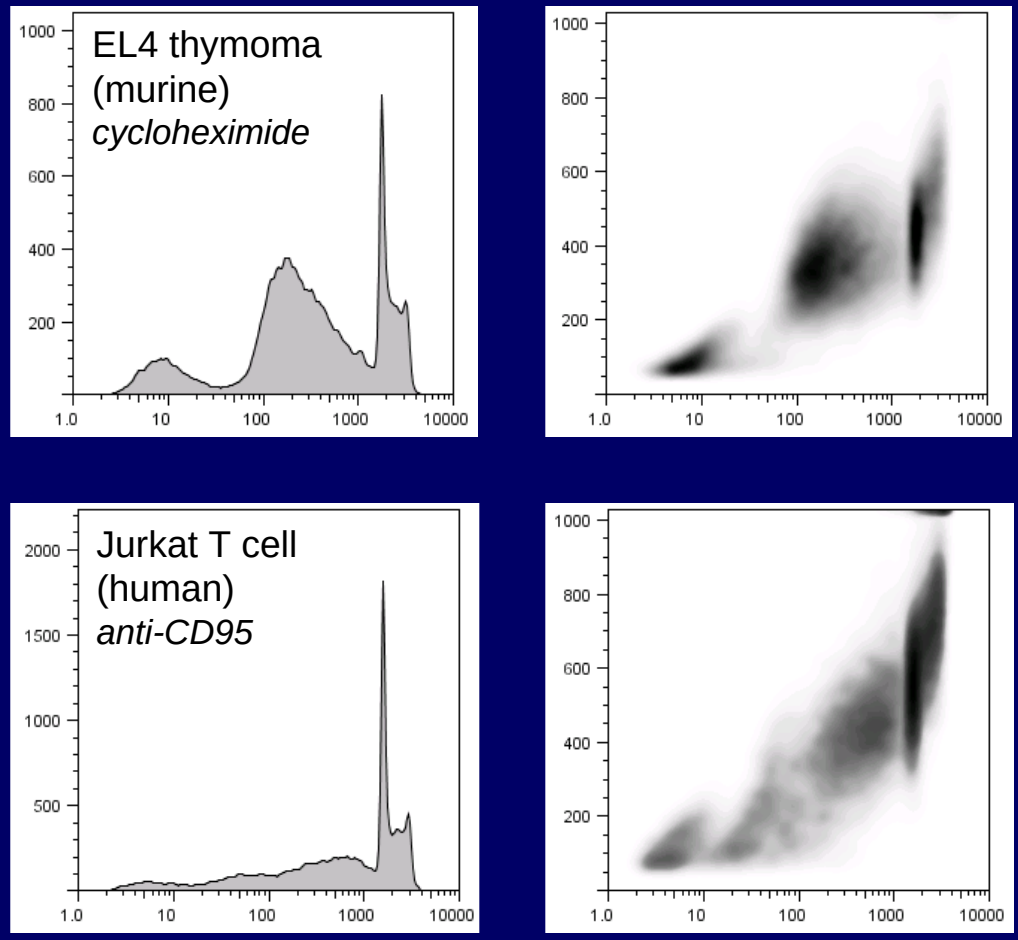


Inappropriate analysis of the “sub-G0/G1” population

Analysis of DNA cell cycle on a log scale can sometimes resolve the true apoptotic cells, but use this method with caution.

As a rule, an apoptotic cell region should be no less than 5-10% of the G1 peak.

Below this is likely debris, blebs and other subcellular objects.



Some other approaches to sub-G0/G1 analysis

- Using high-molarity buffers to control the DNA extrusion level in some cell types (Darzynkiewicz)

- Using cell-permeable DNA binding dyes in unfixed, “viable” cells
  - Hoechst 33342, DRAQ5
  - DyeCycle Violet and Orange (Molecular Probes Invitrogen)
  - *Probably reflects chromatin condensation and loss of high-level organization, rather than DNA loss*

So what to do?

- Approach “sub-G0/G1” quantitation with great caution!
- It does not work well for many cell types.
- Log analysis of “sub-G0/G1” is only valid IF you exclude debris and blebs. This is not always easy and can be somewhat arbitrary.

Take-home advice…

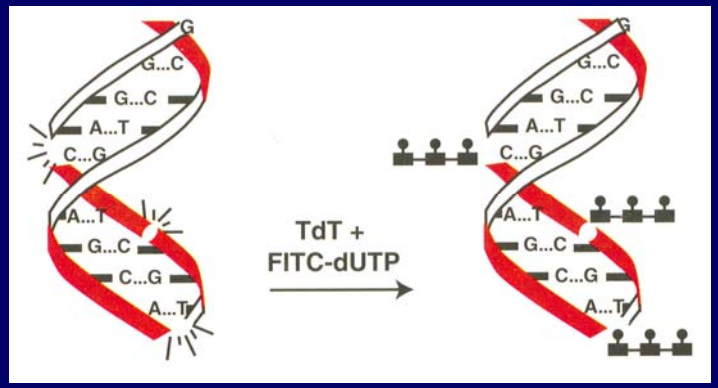
Unless you are analyzing a cell type that gives a good “sub-G0/G1” region, it is best to use this assay for a preliminary, qualitative assessment of apoptosis *only*.

Follow it up with better techniques!

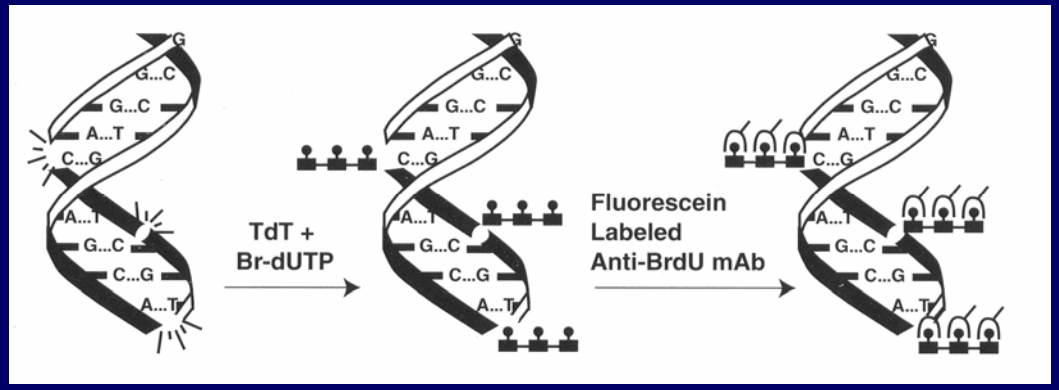
TUNEL assay

Detection of early DNA strand breaks by enzymatically incorporating fluorochrome-labeled nucleotides at the site of the break

> Tdt-dependent incorporation of directly tagged nucleotides (fluorescein or biotin)



> Incorporation of BrdU-tagged nucleotides, followed by anti-BrdU antibody secondary labeling (other amplification systems also used)



Can provide some signal amplification

TUNEL assay

Works well for many cell types.

Cells must be fixed.

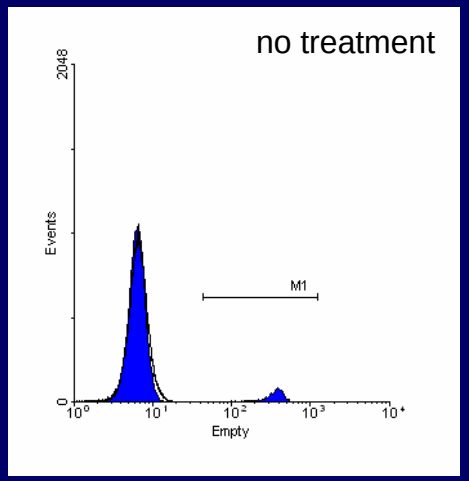
Identifies DNA damage at an earlier stage than “sub-G0/G1” techniques.

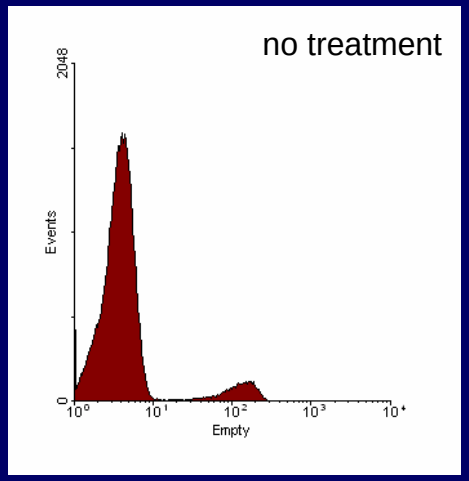
Many kits available – most work well, although kits with signal amplification (biotin-avidin, digoxigenin) may be better for cell types with low levels of DNA cleavage.

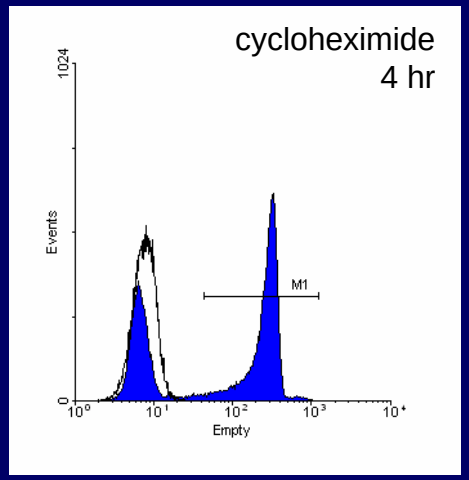
Long-term storage (one week) possible.

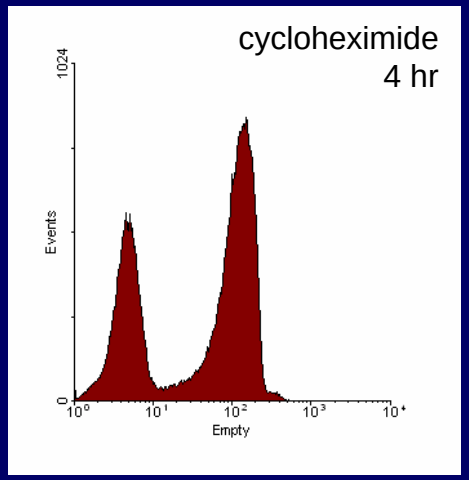
Compatible with other assays requiring fixed cells (such as DNA cell cycle, intracellular proteins).

EL4 cells







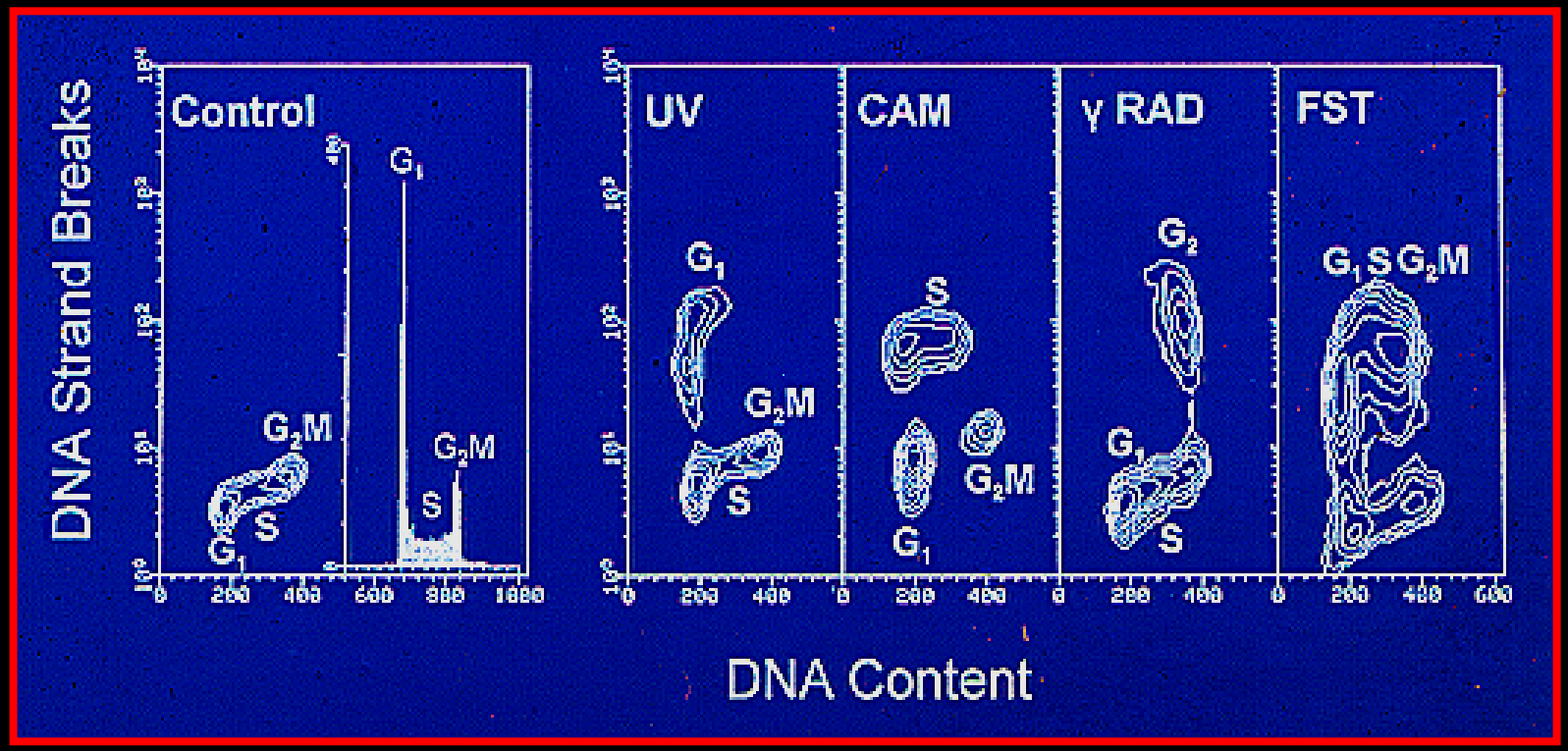


Biotin-dUTP and Alexa Fluor 488 streptavidin

Biotin-dUTP and Alexa Fluor 647 streptavidin

Cell cycle specific apoptosis using combined TUNEL and cell cycle analysis

Cell cycle phase specificity of apoptosis-induced DNA strand breaks is revealed by the bivariate DNA content versus strand breaks analysis



From Z. Darzynkiewicz

Analysis of cell permeability using DNA binding dyes

- Add a DNA binding dye at low concentration to unfixed cells.
- “Viable” cells will exclude the dye.
- Tends to detect cells in an advanced stage of apoptosis, and allow detects necrotic cells.

Not sufficient as an assay for apoptosis alone, but a great adjunct technique with other viability assays involving unfixed cells.

Allows exclusion of very late apoptotic and necrotic cells.

Many, *many* DNA dyes out there.

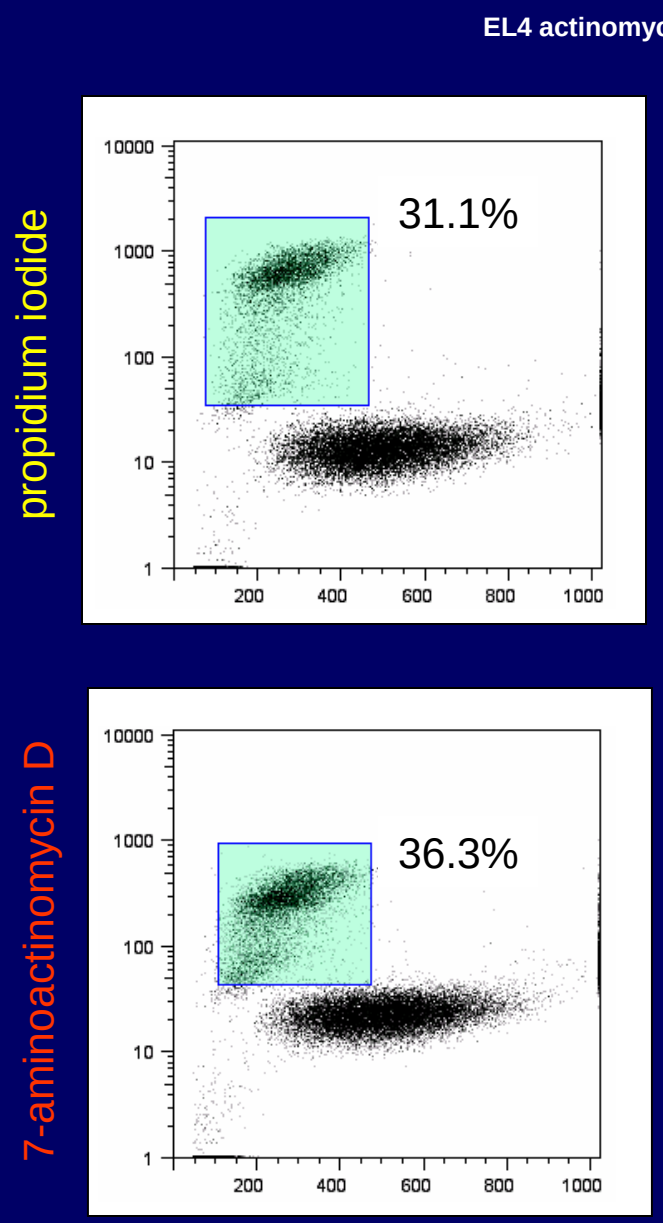
Your choice will be influence mainly by **membrane permeability** and **spectral characteristics**.

Analysis of cell permeability using DNA binding dyes

Propidium iodide is already a common viability reagent for fluorescent immuno-phenotyping and is compatible with most flow cytometers. It is relatively **cell-impermeant.**

7-aminoactinomycin D is also Compatible with most cytometers (longer emission than PI) and is more cell-permeant than PI. It will therefore detect apoptotic cells at an earlier stage of membrane integrity loss.

Not a stand-alone method for apoptosis detection, but a critical adjunct technique

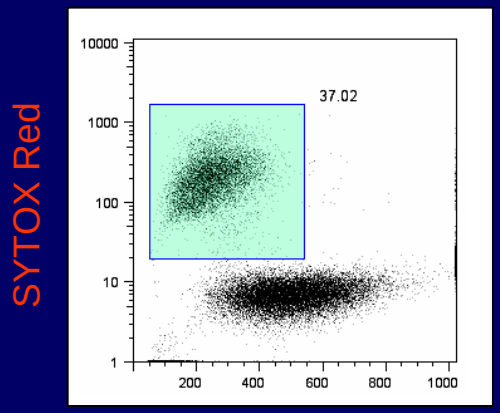


Other useful DNA binding dyes

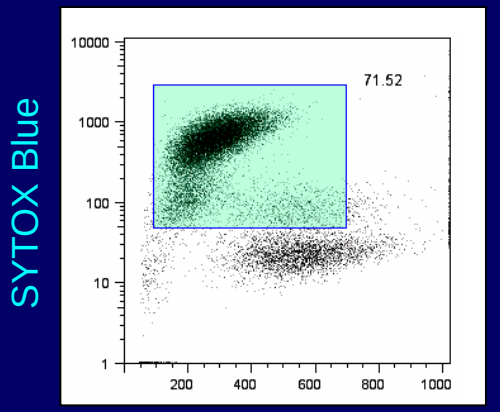
SYTOX Blue is a relatively new violet-excited DNA binding dye that is more cell-ermeable than either PI or 7-AAD. It works well with instruments equipped with violet laser diodes, and has little spectral interference with other probes.

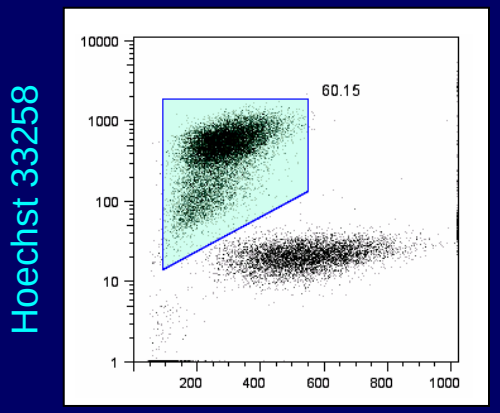
The Hoechst dyes can also make good viability probes for cytometers with UV lasers. Hoechst 33342 is highly cell-permeable, sometimes making it difficult to separate viable from apoptotic cells. Hoechst 33258 may therefore be more useful for apoptotic discrimination.

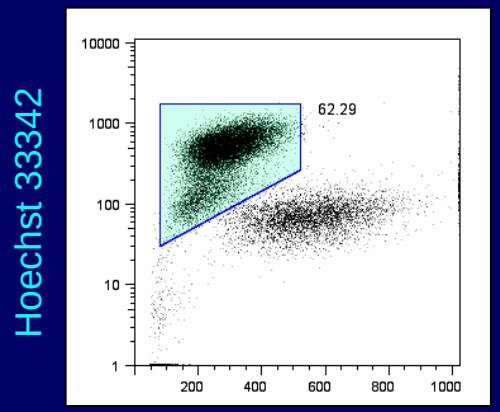
SYTOX Red is a red-excited red-emitting DNA binding dye, very useful for cytometers with red lasers. Permeability seems similar to 7-AAD.



EL4 actinomycin D 6 h







“Live-dead” assays for viability

Usually consist of…

- one or more **DNA binding dyes** (propidium iodide, ethidium homodimer, etc) to detect dead cells
- and an **enzyme or metabolic substrate** to identify “viable” cells
  - fluorescein diacetate
  - calcein

  ] esterase activity
  - C_{12}-resazurin ⟶ resorufin] redox
- some formats for post-assay fixation (for biohazardous samples)

Advantages and Issues

- Rapid – some formats are useful for high-throughput systems
- Detects late-stage apoptosis – not much mechanistic information
- Apoptotic cells can often still exhibit the “viable” phenotype such as esterase and mitochondrial activity

Annexin V binding to apoptotic cells

During apoptosis, phosphatidyl serine (PS) lipid residues “flip” from inside the lipid bilayer to the outside.

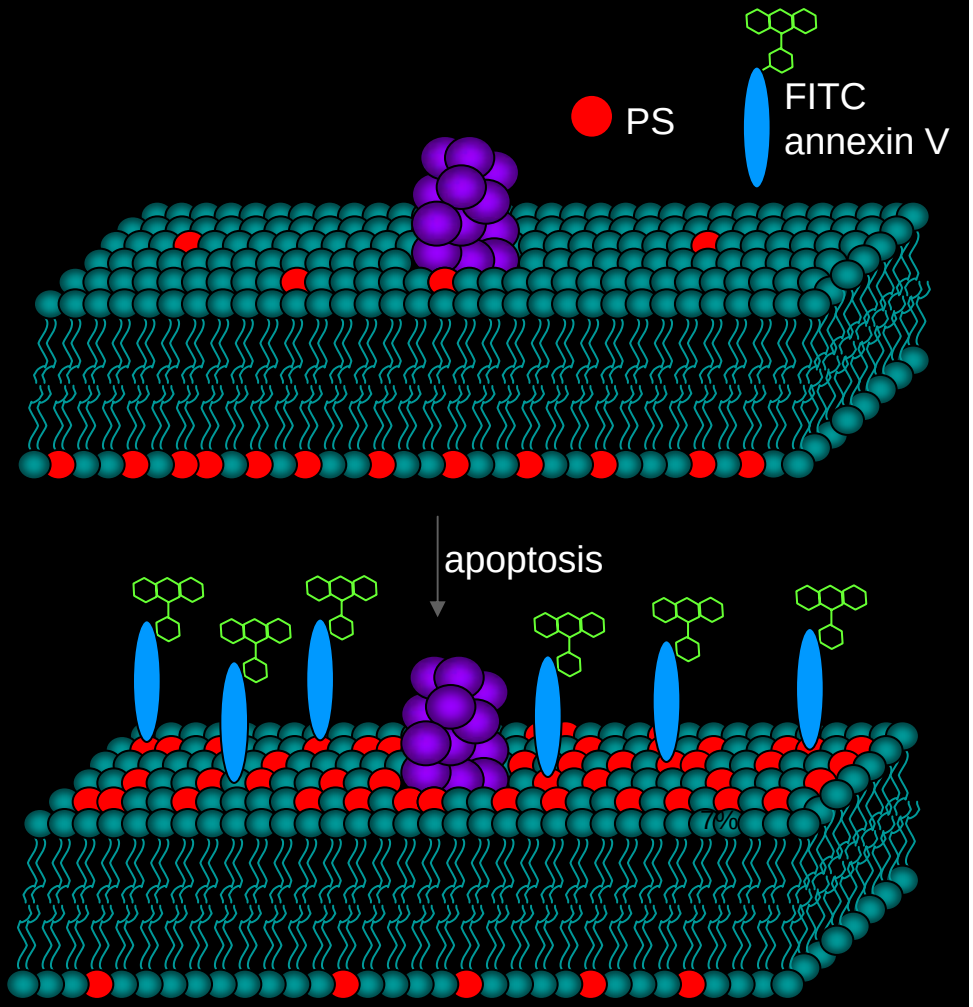
This lipid “flipping” can be detected using the PS binding protein annexin V coupled to a fluorochrome.

This assay requires “viable” cells (i.e. unfixed) and is best done with a DNA binding dye counterlabel (such as propidium iodide or 7-AAD).

Easy assay, but cells must be analyzed soon (within one hour of labeling).

Many kits available. However, annexin V reagents can be bought separately and used with any buffer, as long as it contains calcium and magnesium (a required cofactor).

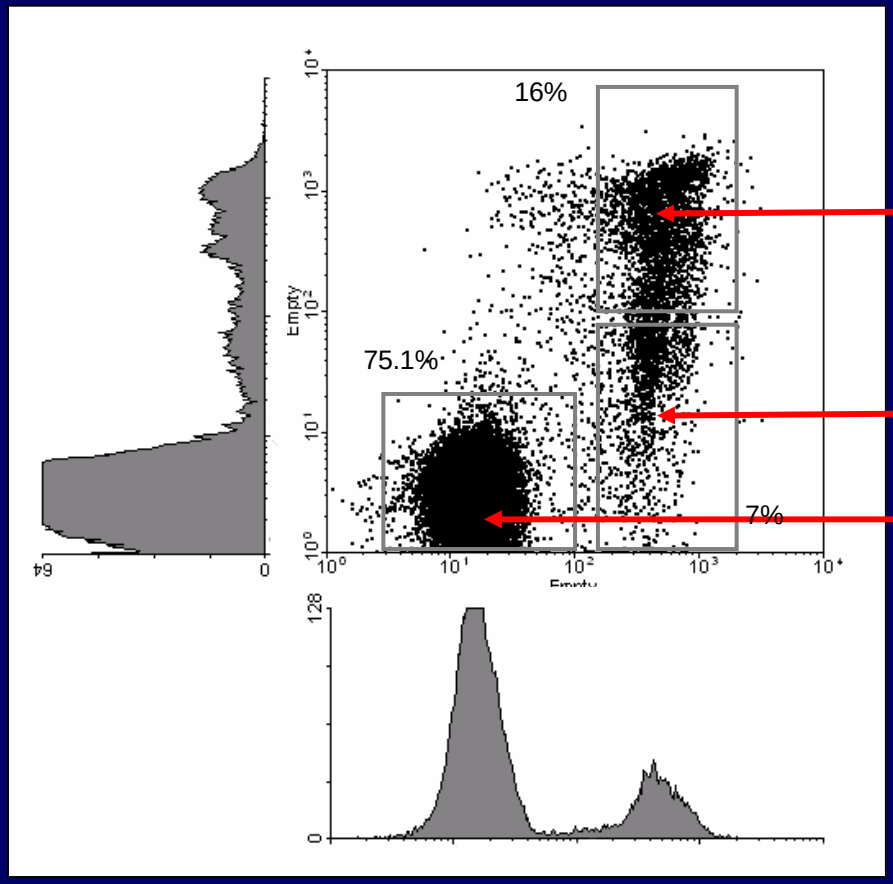
Many fluorochromes available (FITC, PE, PE-Cy5, APC, Cy5, Pacific Blue, biotin, etc.).



Annexin V binding to apoptotic cells

Always use annexin V in combination with a DNA binding dye (such as propidium iodide) to distinguish late apoptotic and necrotic cells

EL4 cells
actinomycin D
4 hours



annexin V+ PI+
(late apoptotic/ necrotic)

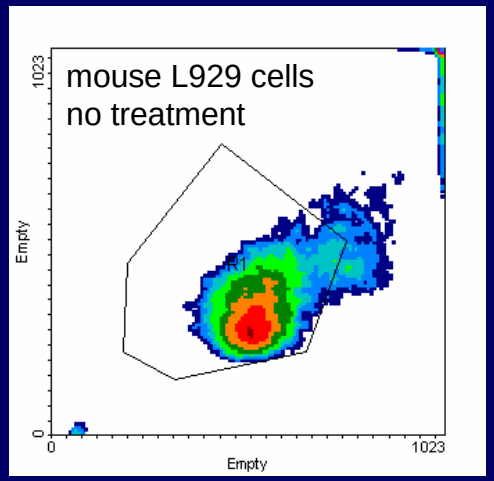
annexin V+ PI-
"early apoptotic"

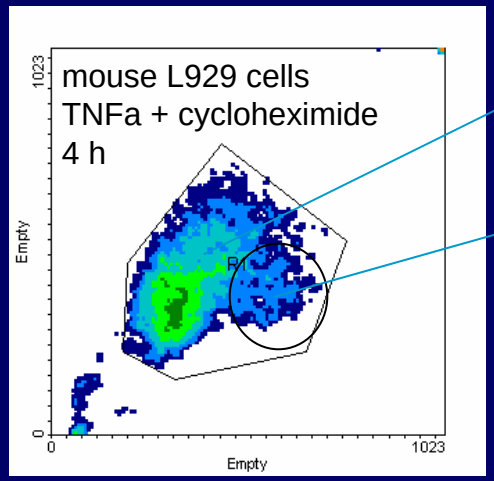
"viable" cells

PS "flipping" occurs prior to 7-AAD permeability

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

Annexin V binding to apoptotic cells



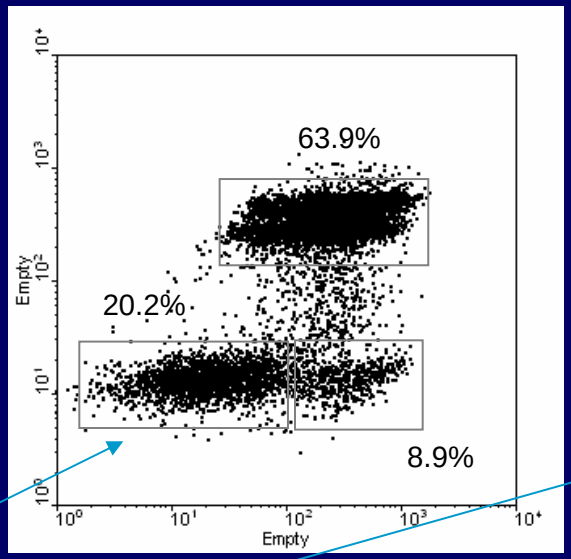


side scatter

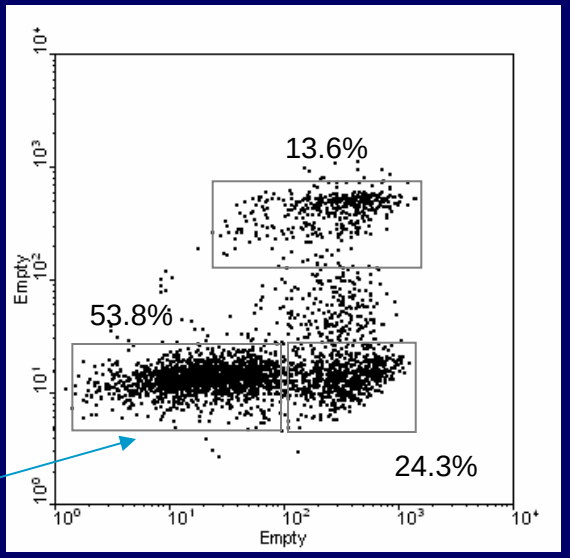
forward scatter

7-actinomycin D

all cells



scatter “viable” cells



FITC annexin V

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

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

Annexin V binding to apoptotic cells

Critical parameters

Calcium and **magnesium** are required for annexin V binding to phosphatidylserine (PS) residues. Binding is reversible, so divalent cations much be present throughout the *entire assay*.

Analysis must be carried out quickly following labeling.

Some cells (i.e. megakaryocytes, platelets, some myeloid lineage cells) can have large amounts of PS on their surface. PBMCs coated with platelets, therefore, may appear to be annexin V positive.

In permeable cells (advanced apoptotic or necrotic), annexin V can label the inner membrane leaflet.

Adherent cells removed with trypsin or mechanical scraping can “flip” their PS residues independent of apoptosis.

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)

NucView 488 (subtrate-immobilized DNA binding dye)

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

All have advantages and limitations.

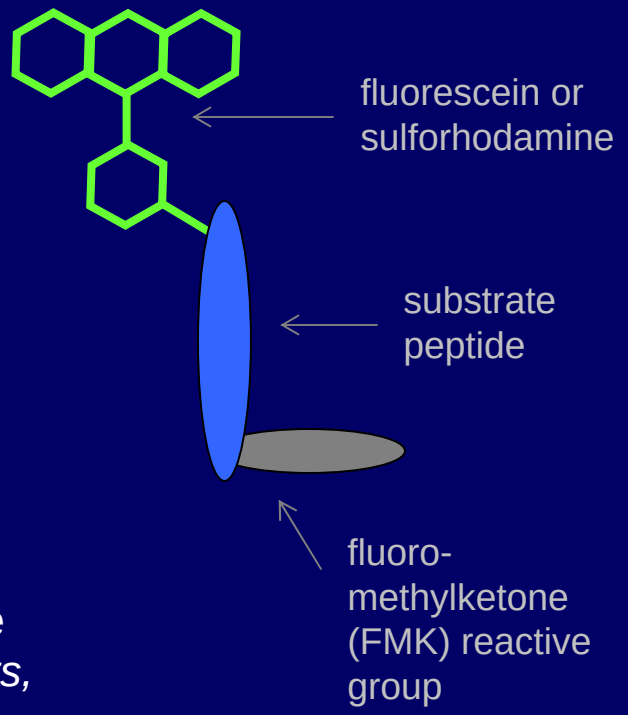
FLICA substrates

Available from a number of commercial sources.

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

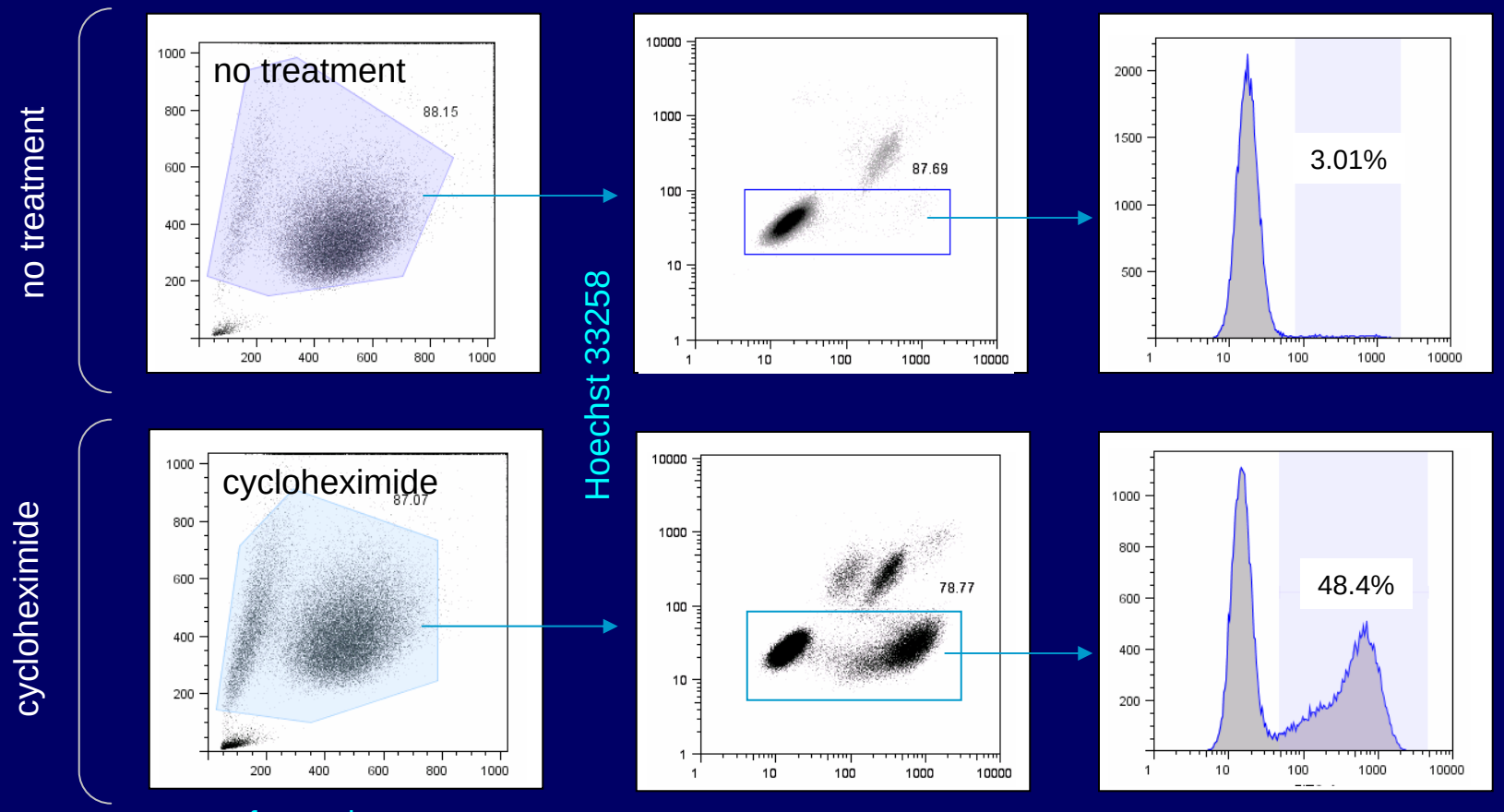
Available in fluorescein and sulforhodamine 101 (SR or “Magic Red”) conjugates. Fluorescein best for flow, unless you have a green or green-yellow laser.

Coumarin-based FLICA reagents are often used in microplate-based caspase assays but are not very useful for cytometry.

FLICA detection of apoptosis-associated caspase activation

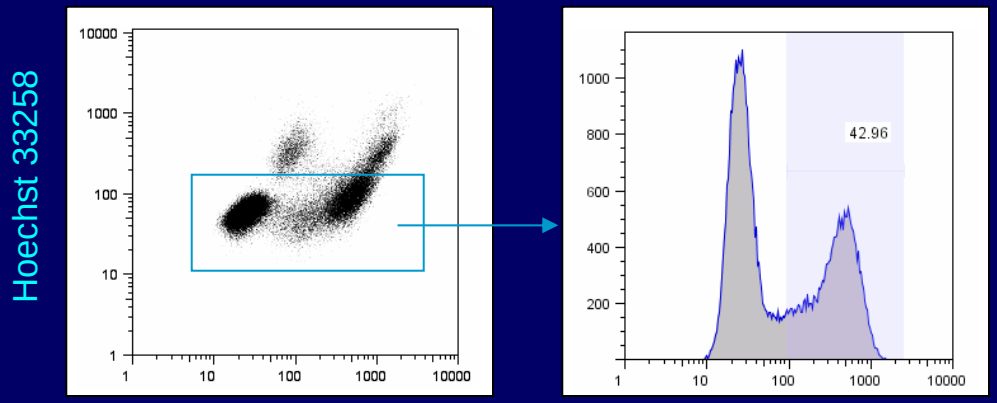
EL4 cells treated with cycloheximide

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



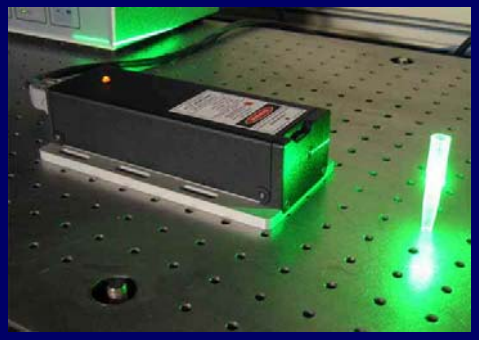
FLICA detection of apoptosis-associated caspase activation

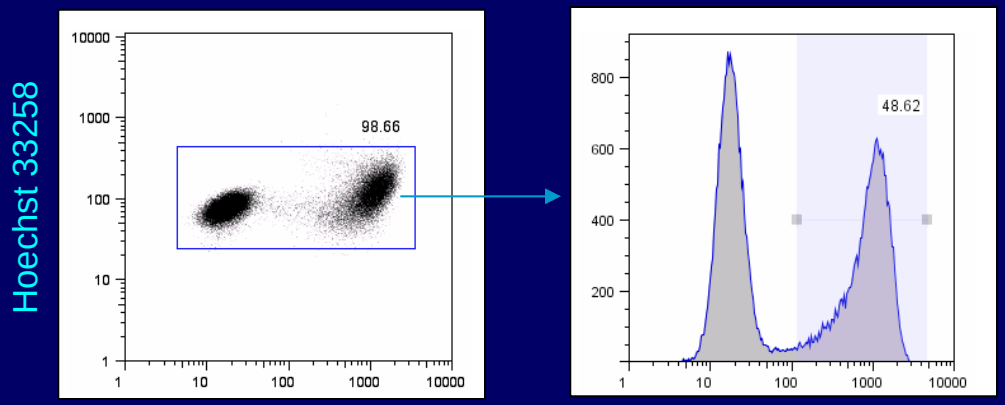
FLICA substrates are available for other caspases, such as caspase 8.



FLICA FL caspase **8**

Sulforhodamine 101 conjugated FLICA reagents are useful for microscopy, or on cytometers equipped with green or green-yellow lasers





FLICA sulforhodamine 101 caspase **3/7**

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 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]
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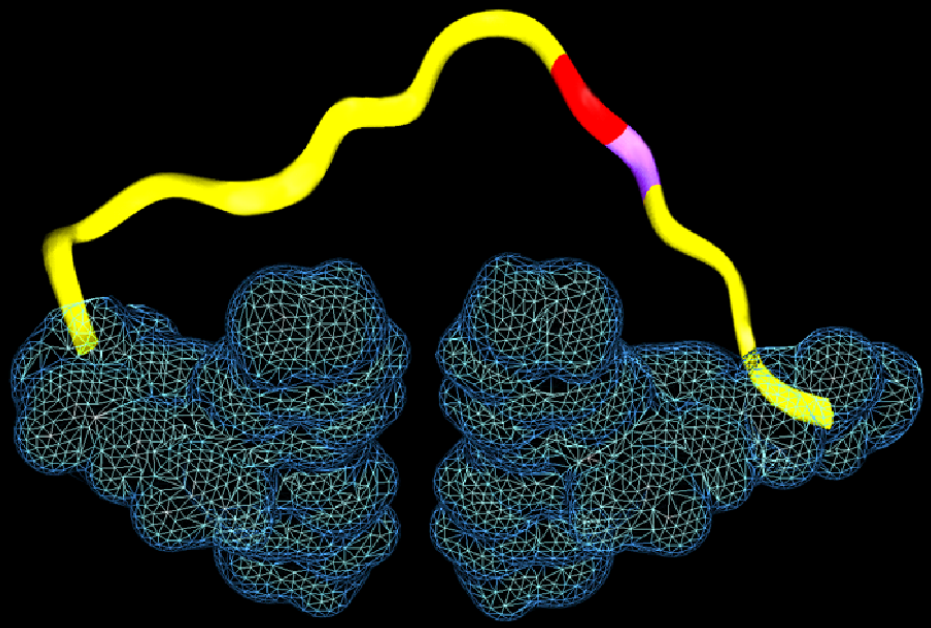
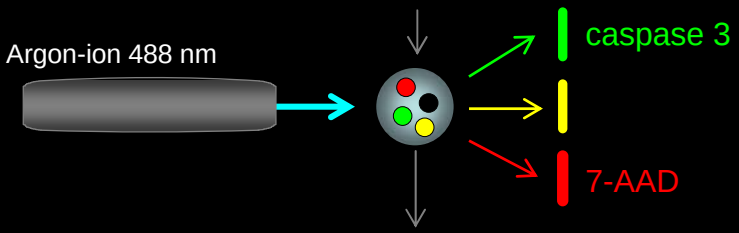
PhiPhiLux caspase substrates

Oncoimmunin, Inc.

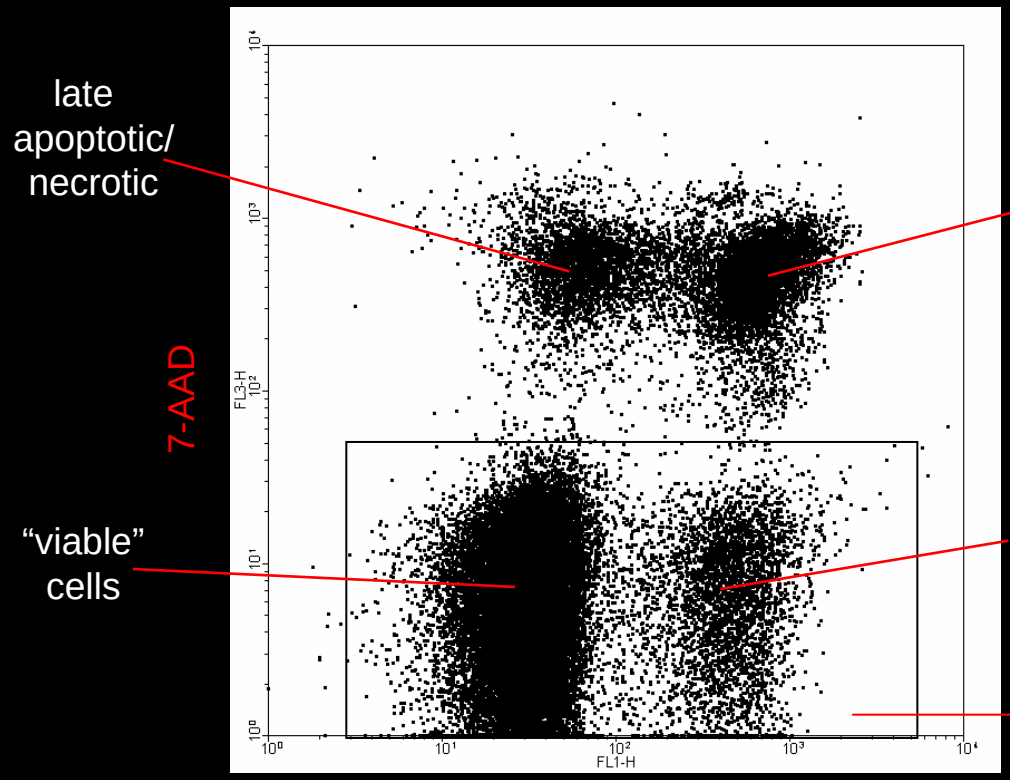


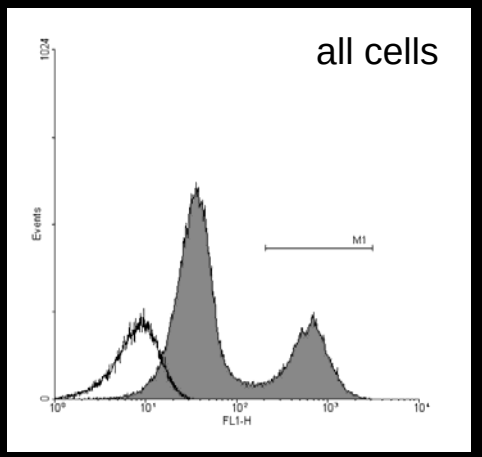
- peptide backbone containing enzyme consensus cleavage site (DEVD for caspase 3/7)
- fluorochrome molecules attached to terminal ends of the peptide
- peptide backbone modified to bring fluorochrome molecules into close steric proximity, resulting in fluorescent quenching
- complex is relatively non-fluorescent when uncleaved
- cleavage of the consensus site “frees” the fluorochrome molecules, 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

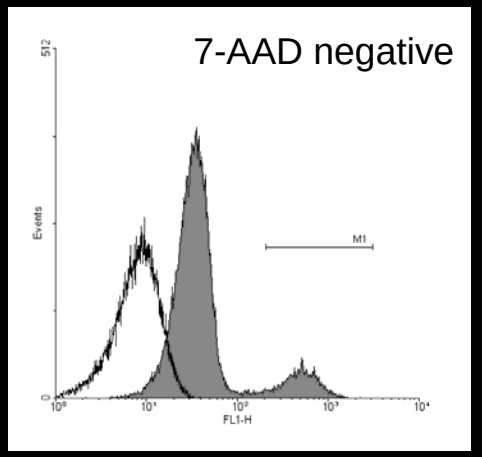
PhiPhiLux caspase substrates



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







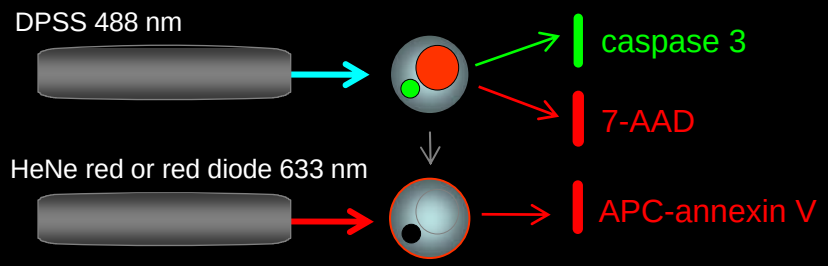
fluorescein PPL caspase 3

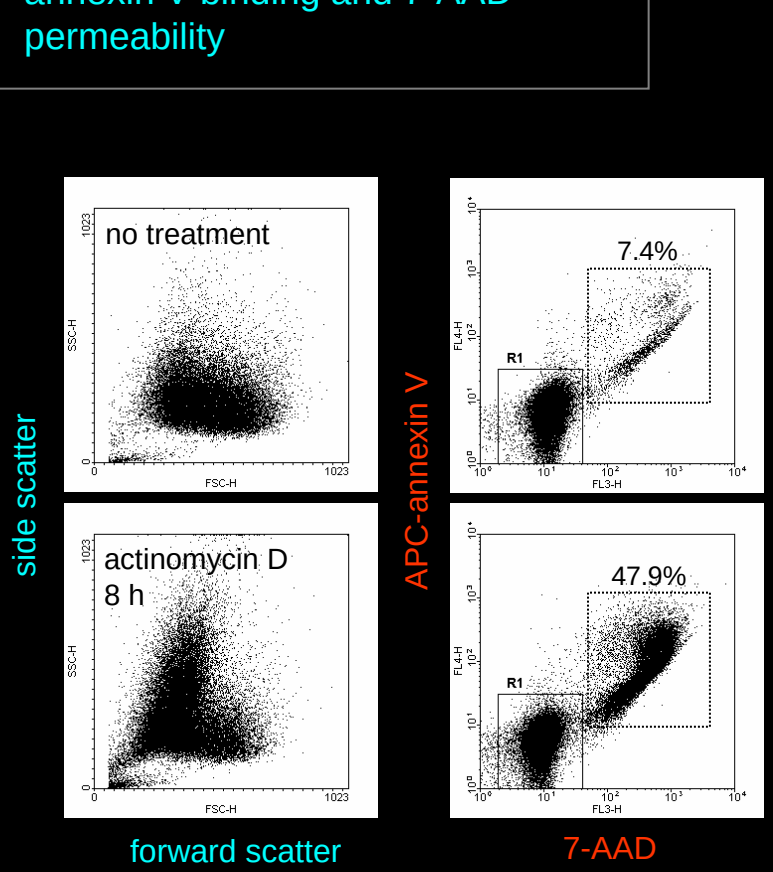
EL4 cells cycloheximide 50 µg/ml 4 hr

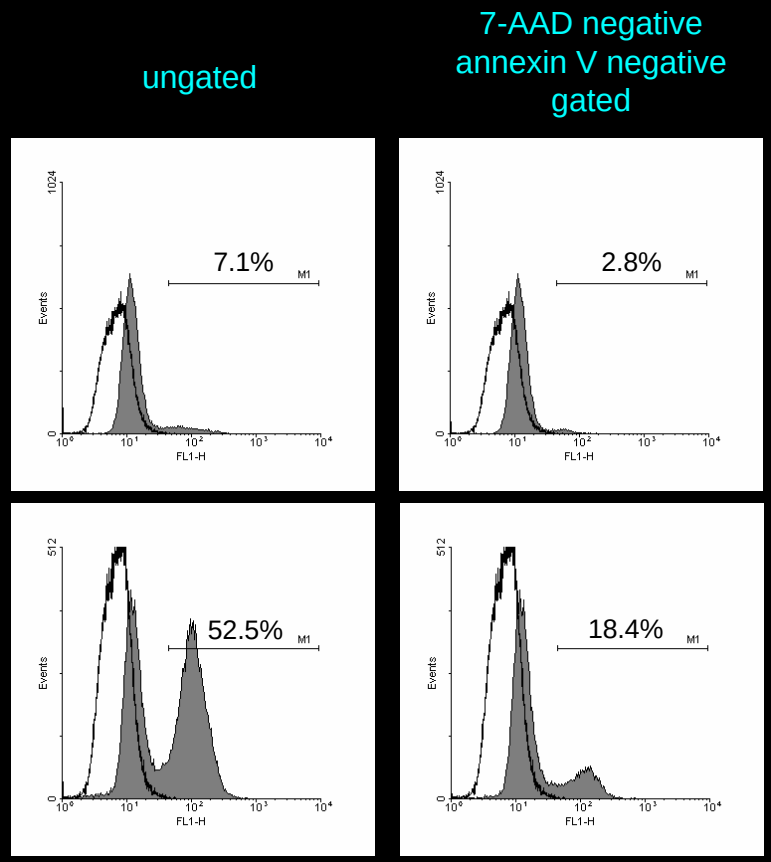
PhiPhiLux caspase substrates

EL4 cells labeled with fluorescein PhiPhiLux, APC-conjugated annexin V and 7-AAD

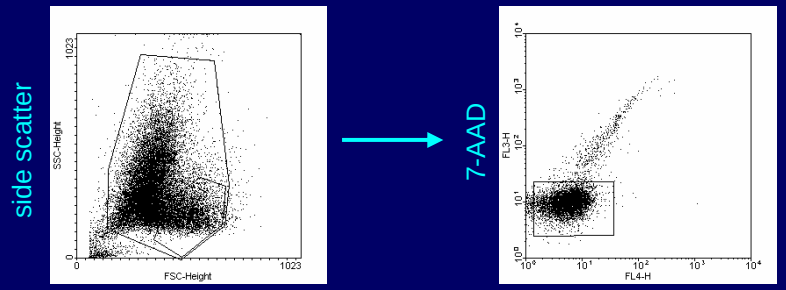
Caspase activation is *early* (before annexin V binding and 7-AAD permeability







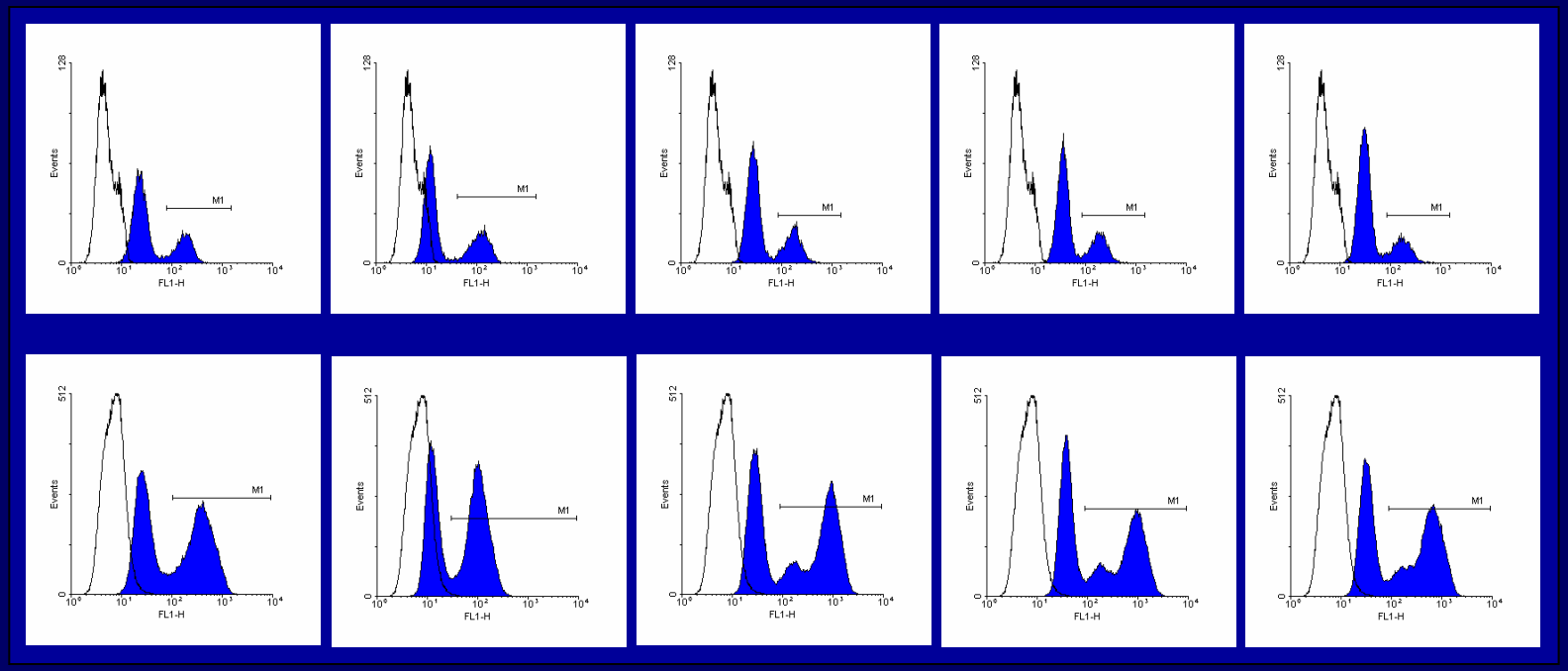
PhiPhiLux substrates for other caspases



Like FLICA, any consensus domain can be inserted into the PhiPhiLux complex, allowing detection of a variety of caspases.

scatter viable
7-AAD neg
annexin V neg

all cells

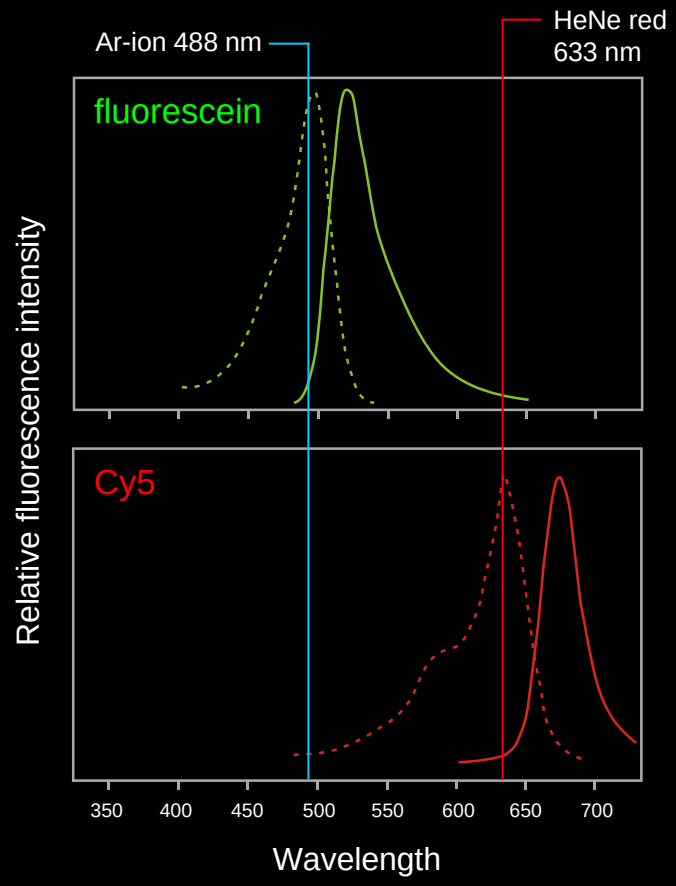


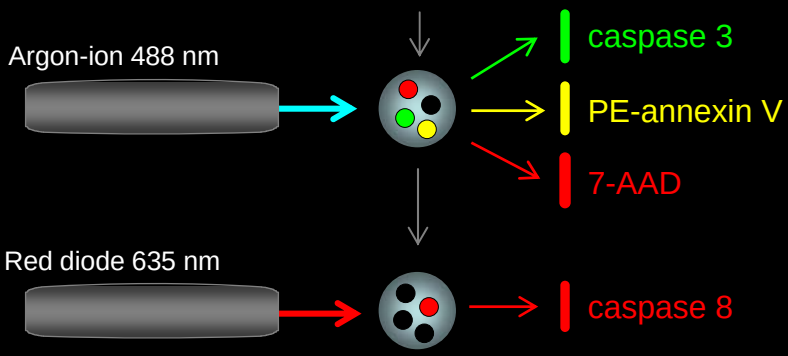
FL-caspase **1** FL-caspase **3** FL-caspase **6** FL-caspase **8** FL-caspase **9**

EL4 cells cycloheximide 50 µg/ml 4 hr

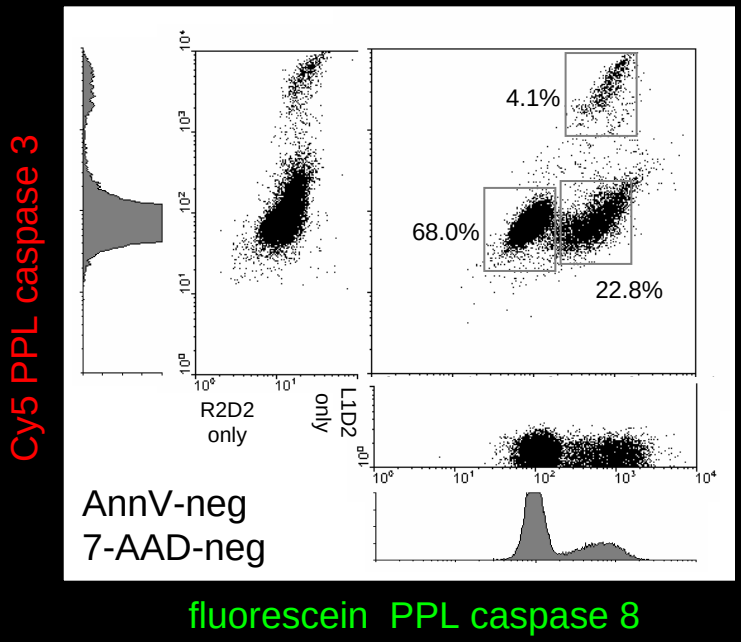
Detection of two distinct caspase activities by flow cytometry

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



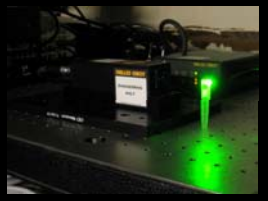


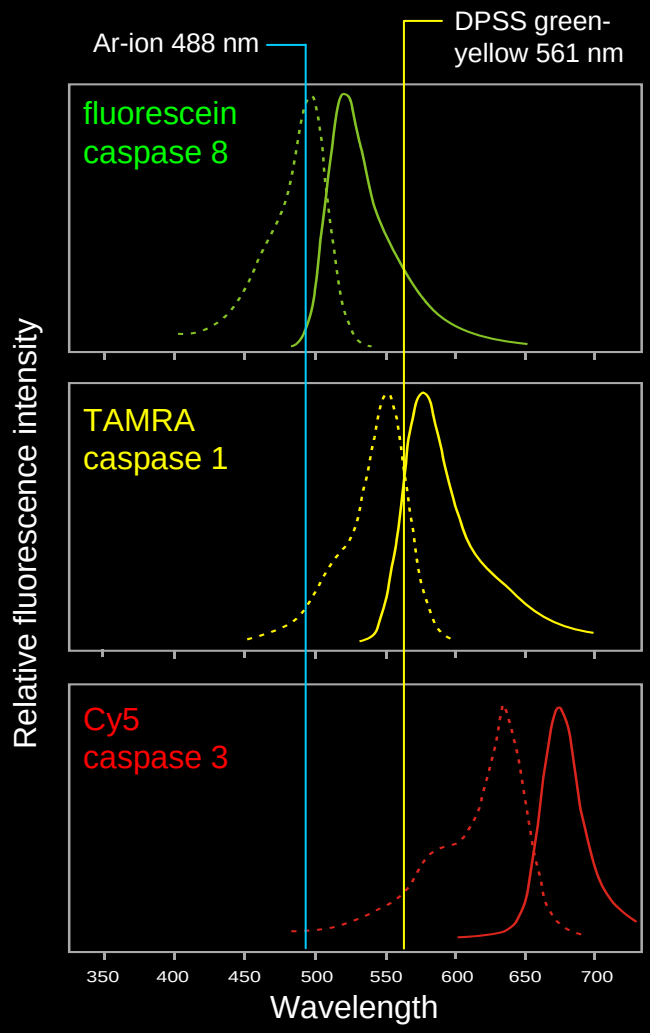
caspase 8 (pro-caspase 3) → caspase 3

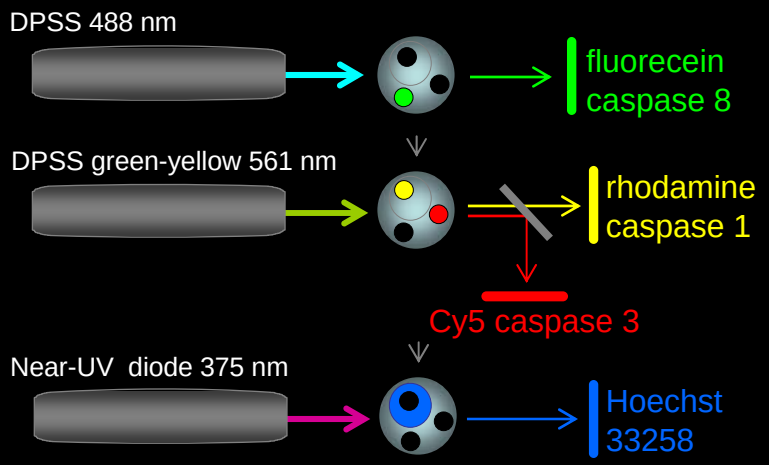


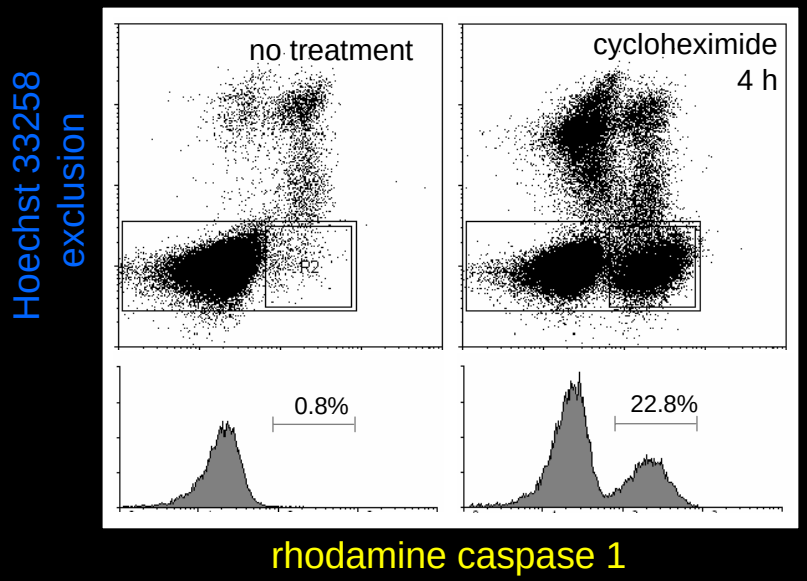
From Telford, W.G., Komoriya, A. and Packard, B.Z. (2004) *Methods in Molecular Biology* Volume 263, Flow Cytometry Protocols, pp. 141-159.

Detection of three distinct caspase activities by flow cytometry



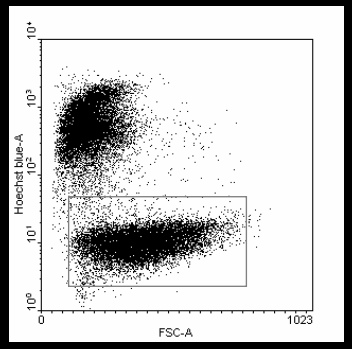






DPSS 561 nm excitation should permit simultaneous excitation of rhodamine and Cy5 labeled substrates.

Detection of three distinct caspase activities by flow cytometry



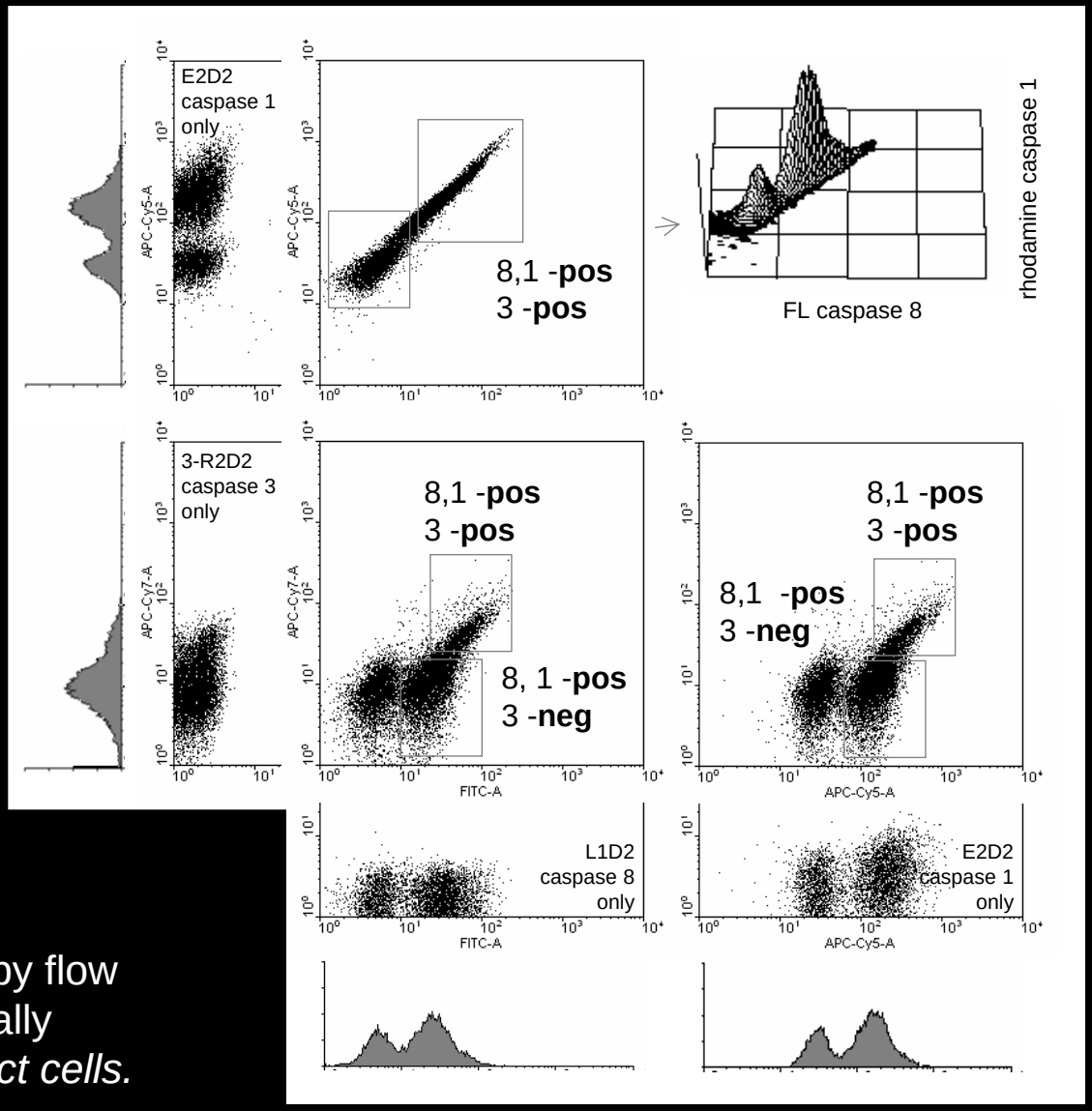
Hoechst 33258

forward scatter

caspase 1
caspase 8
(pro-caspase 3)
caspase 3

rhodamine caspase 1

Cy5 caspase 3



FL caspase 8

rhodamine caspase 1

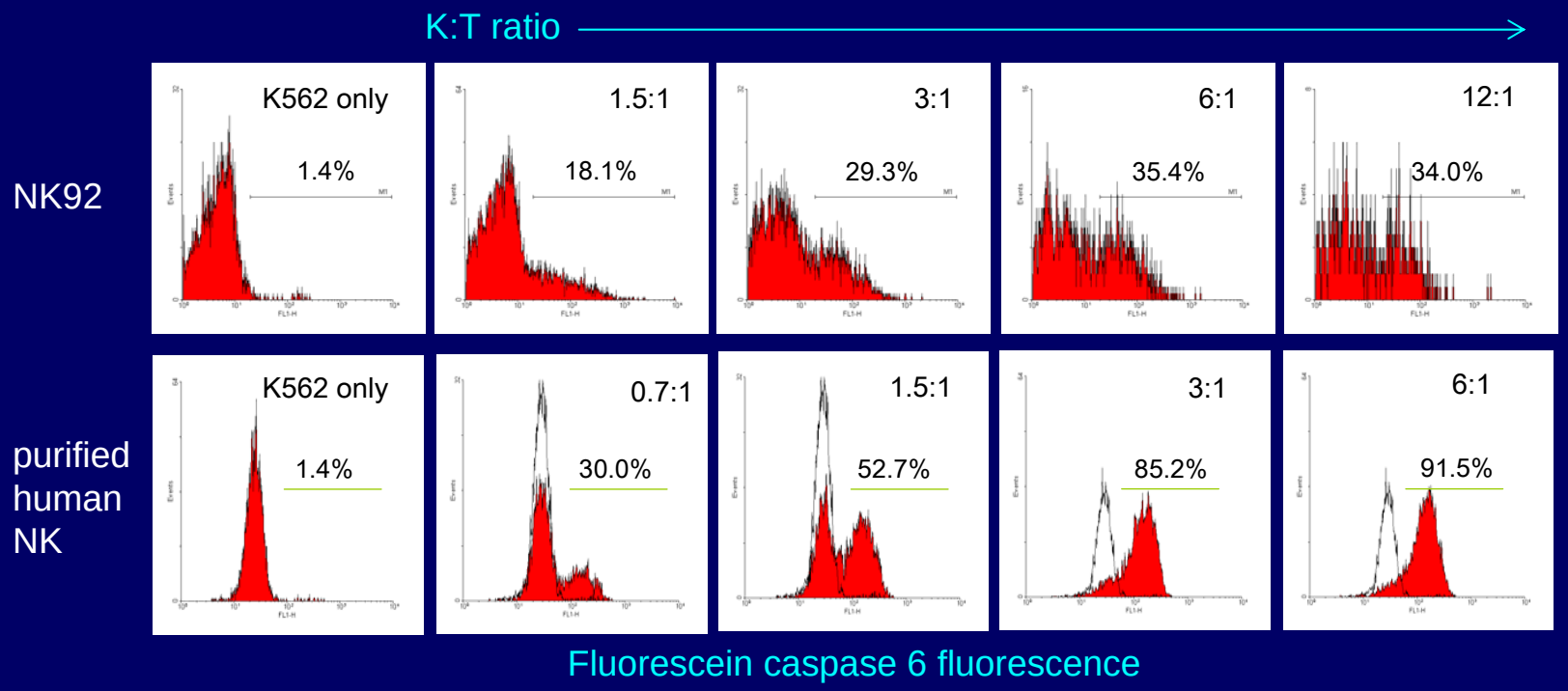
Analyzing multiple substrates by flow cytometry allows us to temporally map caspase activation *in intact cells.*

This is no longer just an apoptosis assay, but is defining how apoptosis occurs.

Cytotoxicity assay based on activation of caspase 6

Caspase 6 activation can be used as an assay for CTL or NK mediated cytotoxicity ("CyToxiLux")

Suspension cultures of NK92 cell line or purified human NK cell effectors and K562 target cells incubated for 1 hour following fluorescein-PPL caspase 6 substrate target cell loading and PKH26 effector cell membrane tagging.



Samples provided by Dr. Elena Kovalenko, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, RUSSIA

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.

Issues

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

About all caspase substrates…

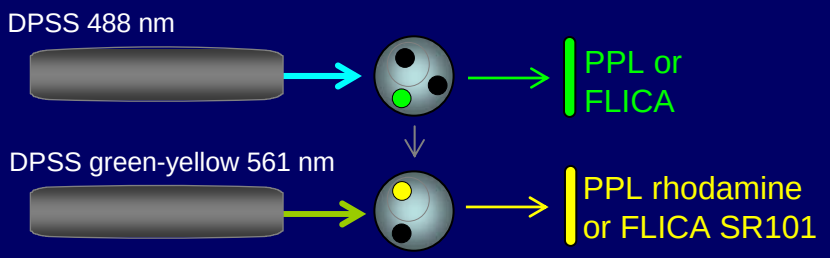
No synthetic substrate is *completely* specific for its target enzyme.

Cell permeability is never total.

Can caspases be activated in circumstances other than apoptosis?

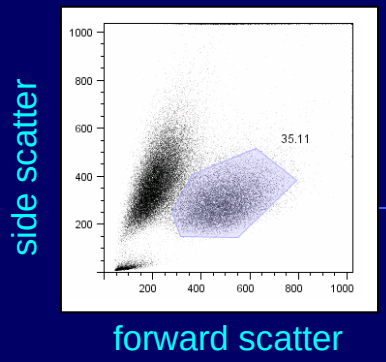
Do PhiPhiLux and FLICA agree with one another?

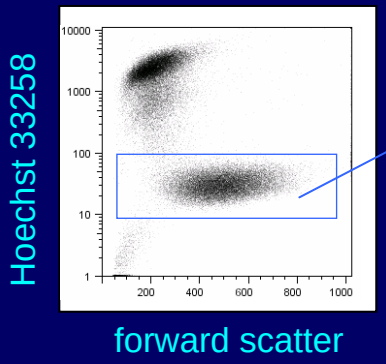
Co-labeling with PhiPhiLux and FLICA *directed against the same caspase*

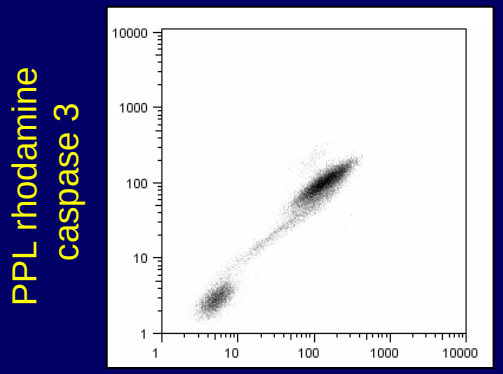


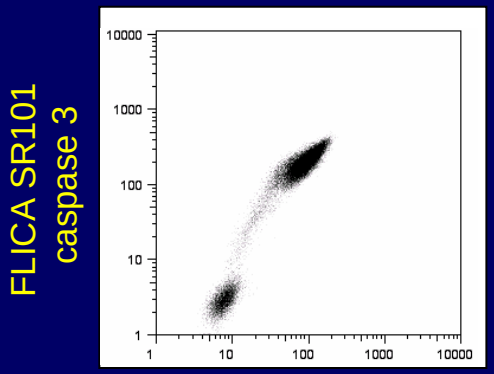
PhiPhiLux (non-inhibitory)

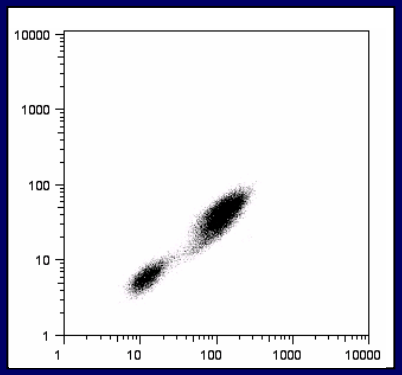
FLICA (inhibitory)











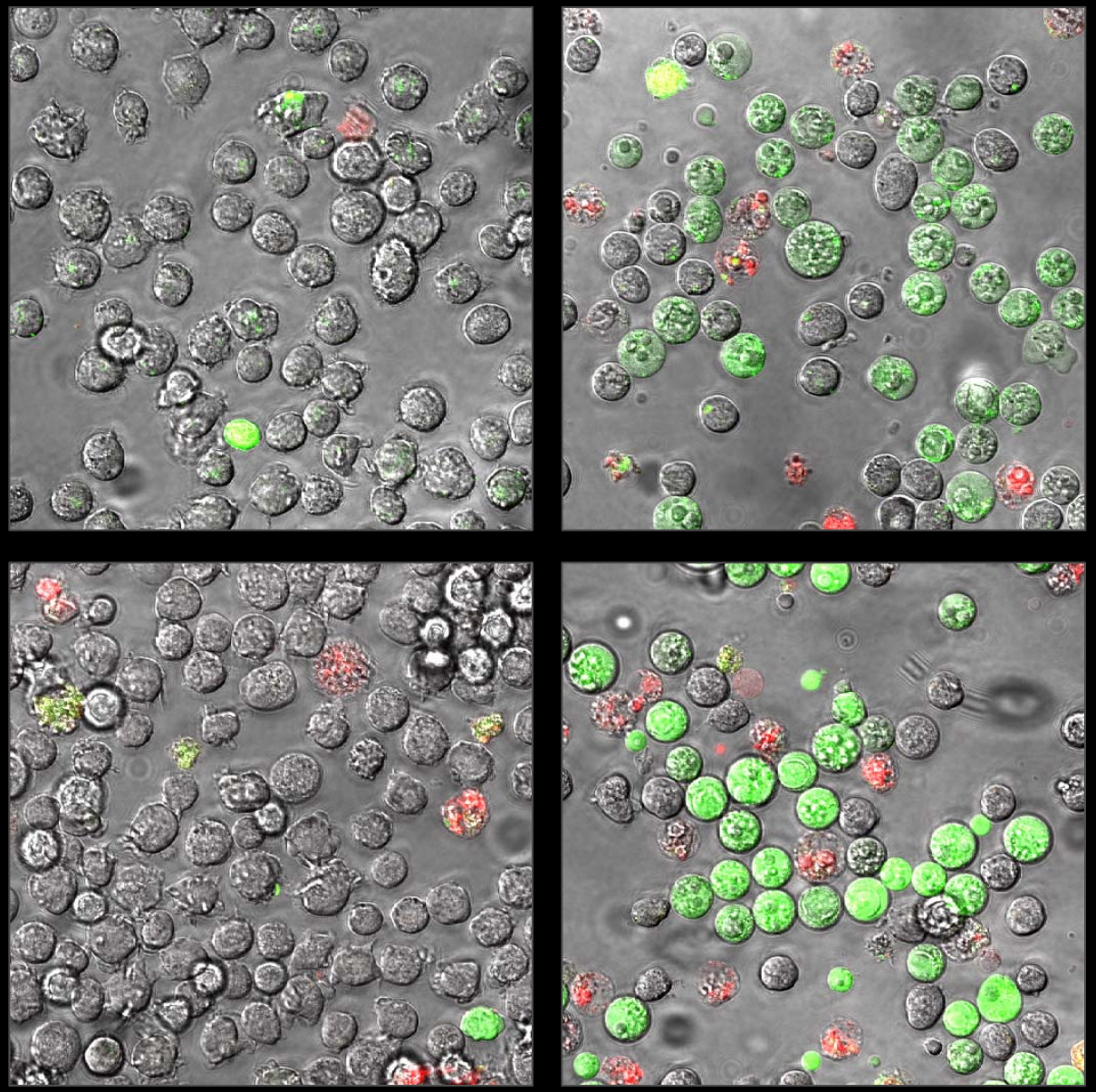
Caspase substrate specificity

control

staurosporin

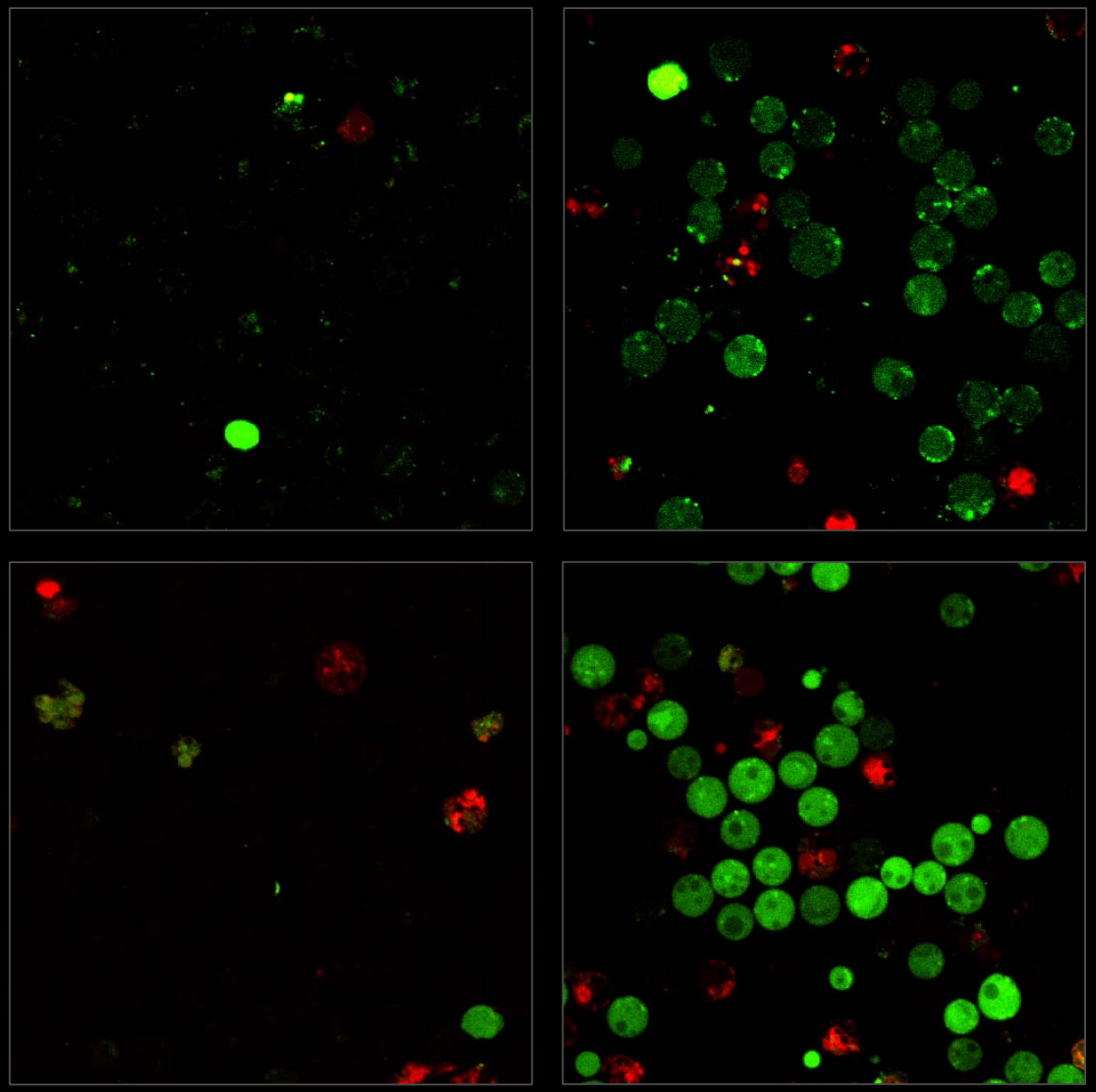
PhiPhiLux

FLICA



Jurkat T cells

Caspase substrate specificity



Caspase substrate specificity

Jurkat T cells
staurosporin 1 µM 4 h

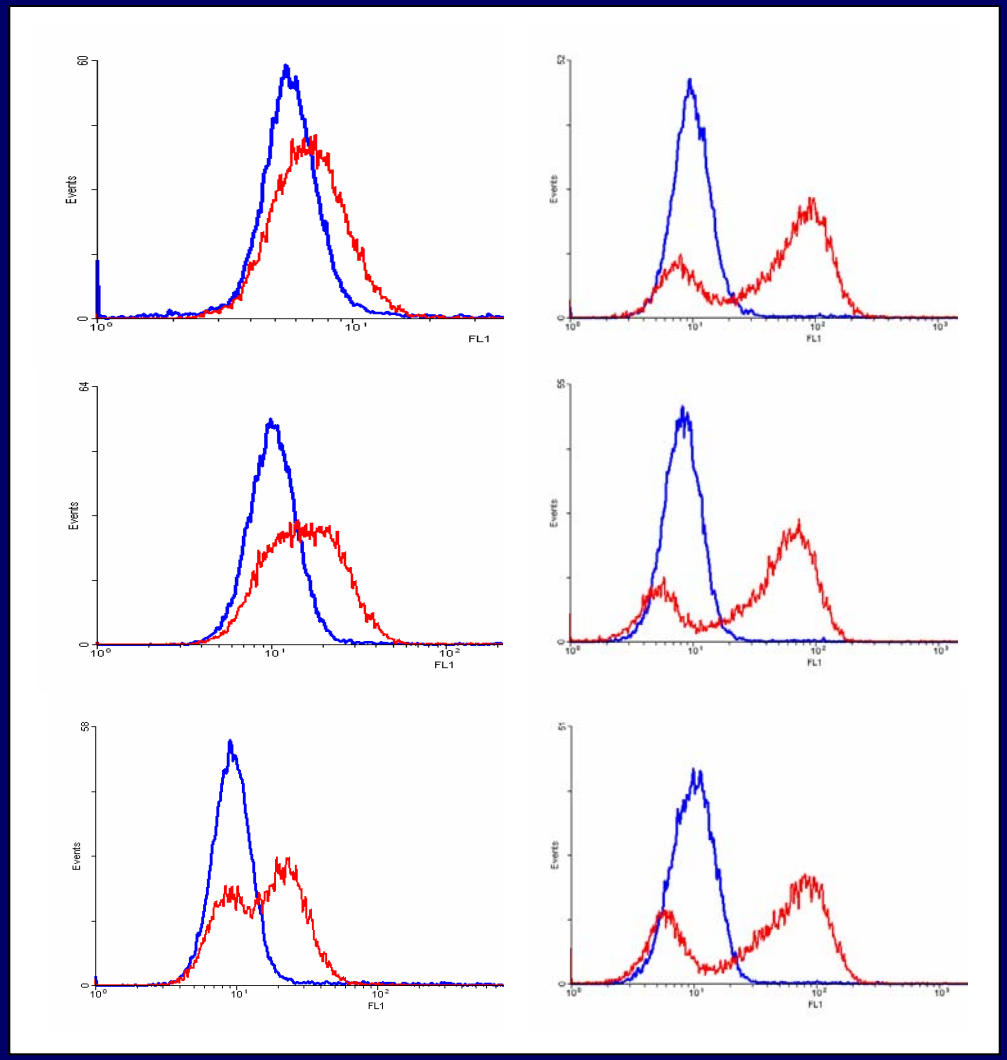
PhiPhiLux FLICA

time of substrate incubation

10 min

20 min

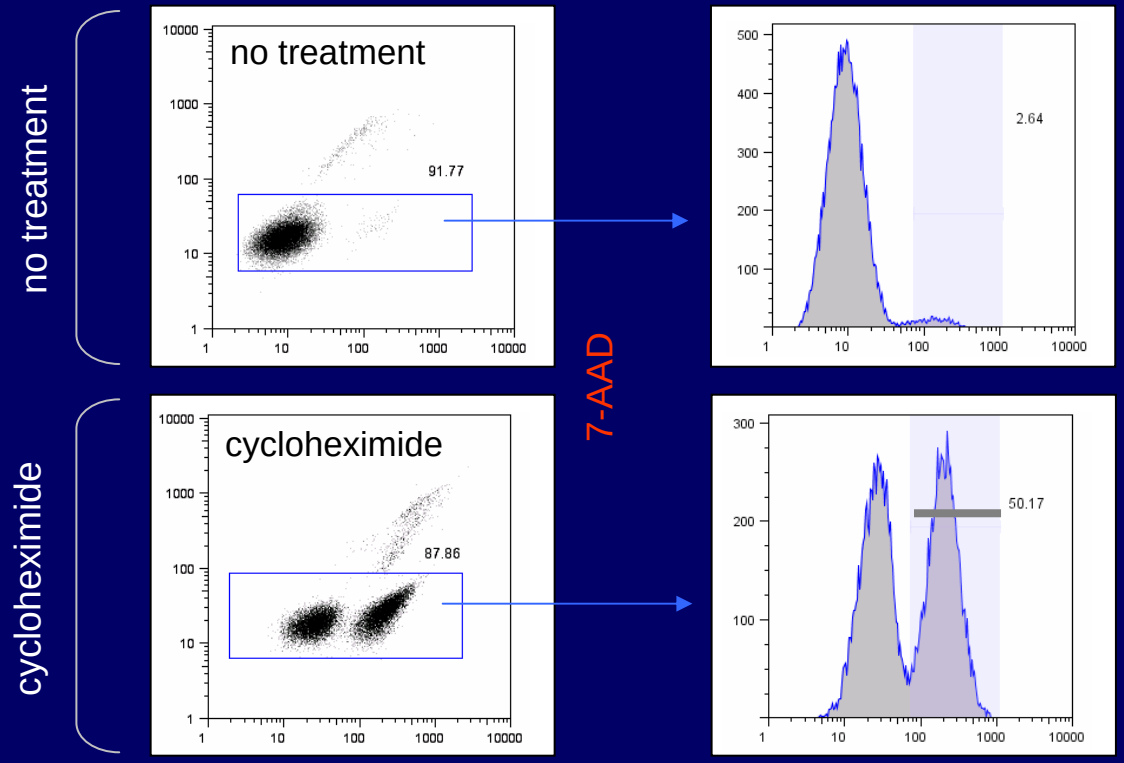
40 min



FLICA binds to apoptotic cells almost immediately following addition, suggesting that caspase-dependent Interactions may not be the primary mechanism of FLICA labeling of apoptotic cells

NucView 488

- A substrate complex that binds to DNA upon enzyme cleavage (Biotium, Inc.)
- Loaded into viable cells – in the presence of caspase, the complex is cleaved and the dye can bind to nuclear DNA



Seems to be very rapid for loading (15 minutes Incubation time)

Can be fixed like FLICA

No site specificity – probably better for rapid screening

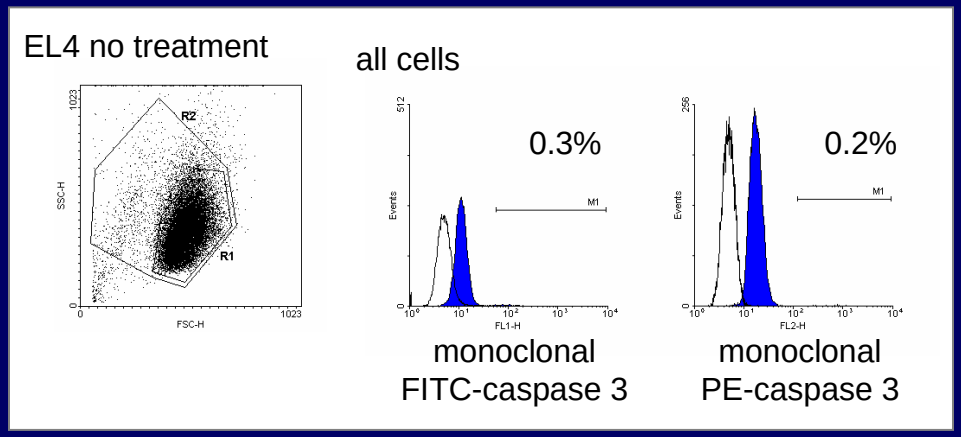
Immunodetection of cleaved caspase 3 by flow cytometry

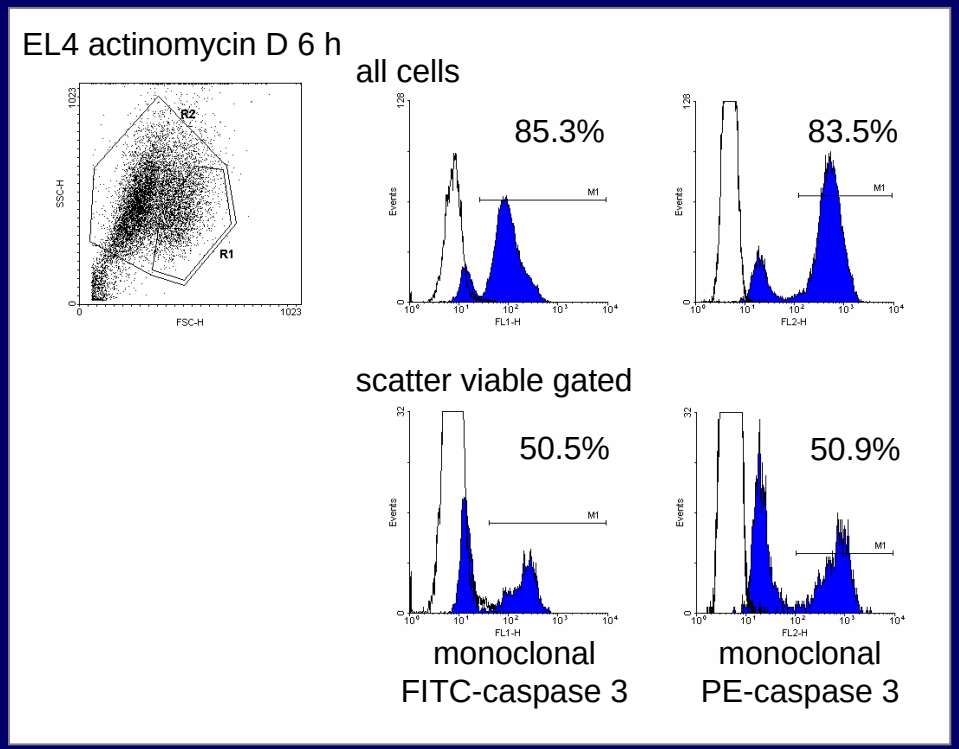
Several monoclonal antibodies against the cleaved form of caspase 3 are available (BD Biosciences rabbit monoclonal in this case) conjugated to several fluorochromes and biotin.

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

Requires paraformaldehyde fixation and detergent treatment.

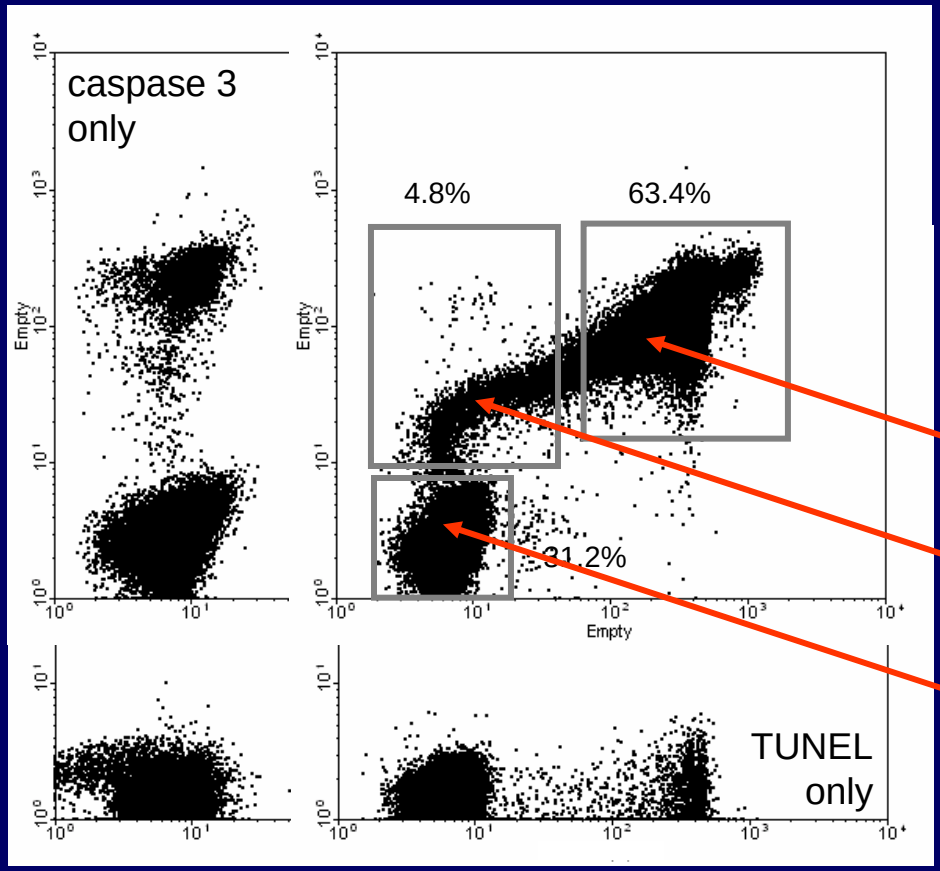
Saponin-based methods work well too.





Caspase 3 immunolabeling combined with 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

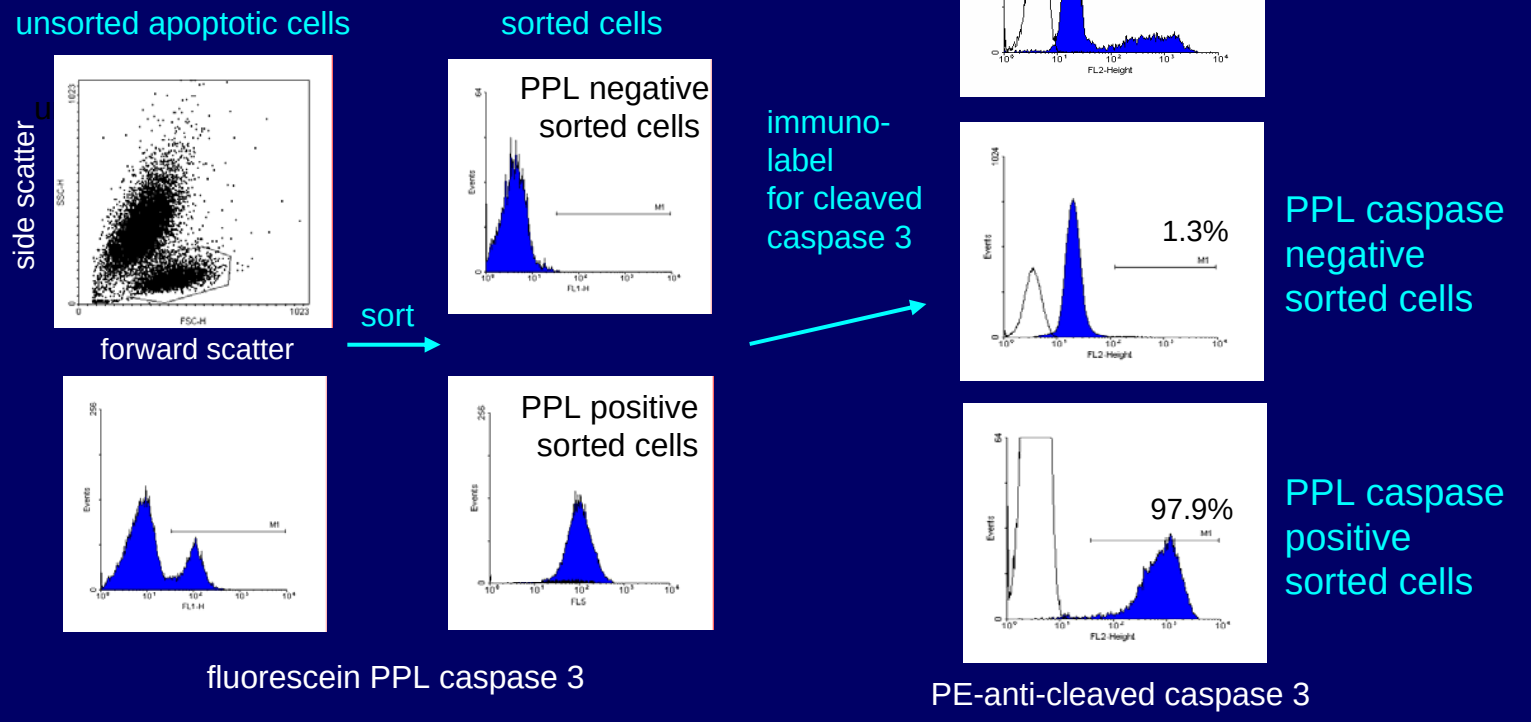
"early" caspase 3 -positive
DNA fragmentation -negative

"viable" cells

Do substrate and immunolabeling agree with one another?

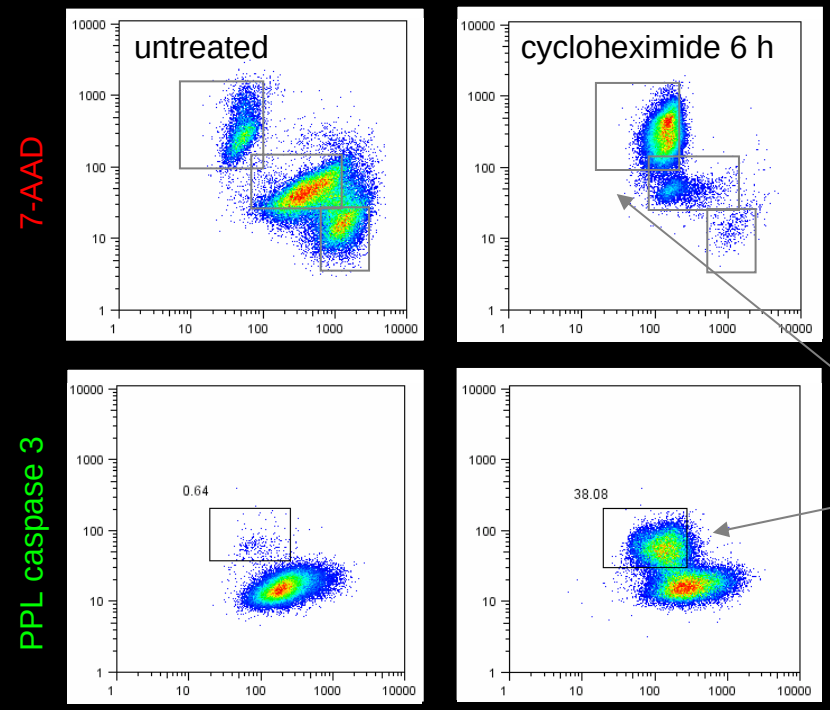
PhiPhiLux and caspase 3 immunolabeling?

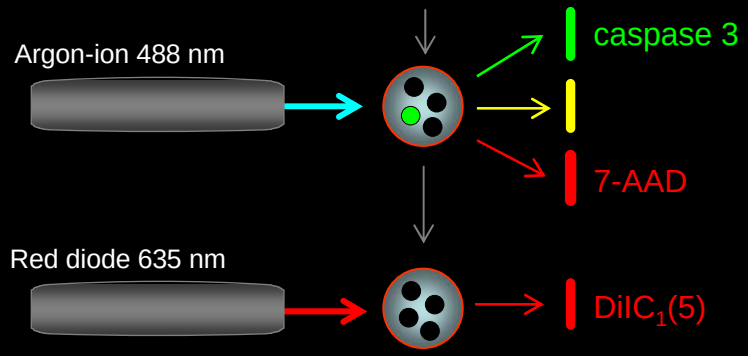
Apoptotic EL4 cells were labeled with PPL and sorted Into **caspase-negative** and **caspase-positive** fractions.



Caspase activity measured by PhiPhiLux substrate cleavage and by immunolabeling correspond very precisely.

Mitochondrial probes for analyzing apoptosis





$DiIC_1(5)$ (a monomeric cyanin membrane potential probe)

Gross changes in mitochondrial membrane potential precede loss of membrane permeability, and even caspase activation

Rhodamine 123
$DiOC_6(3)$] nanomolar concentrations
$DiIC_1(5)$]
MitoTracker Red CMXRos
JC-1 and JC-9
Calcein/Co mitochondrial pore assay

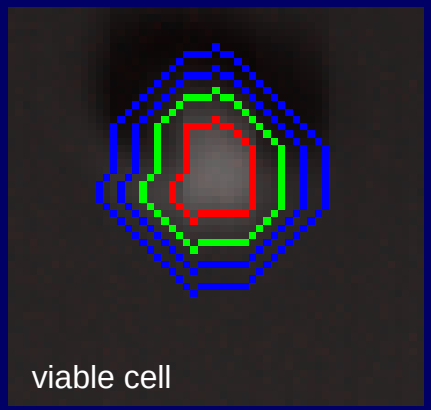
Useful for elucidating the role of mitochondria in initiating and regulating apoptosis, but difficult for routine use.

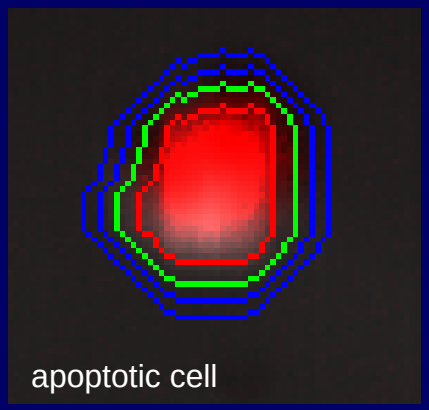
Apoptosis and image cytometry

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.

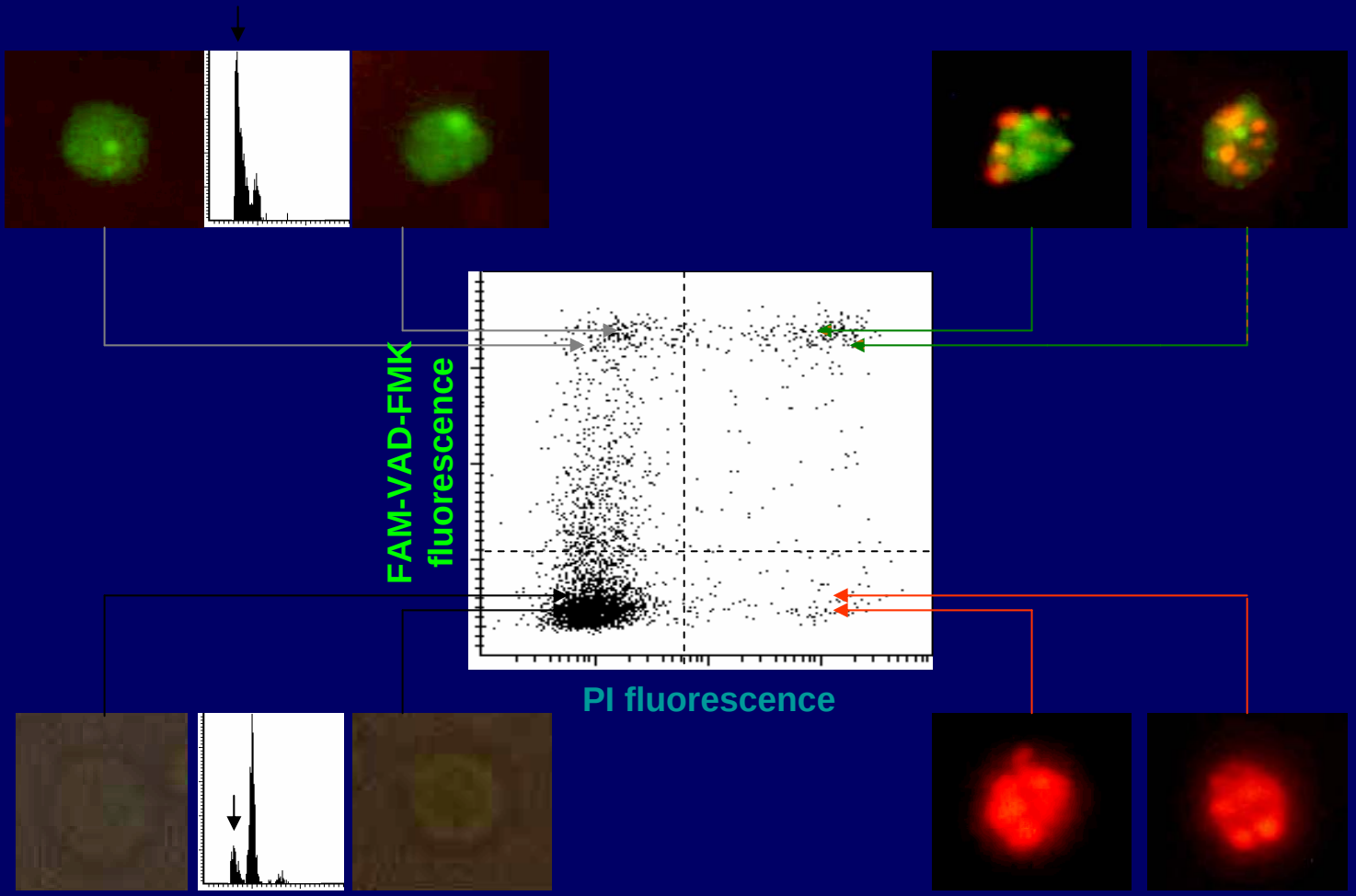
Many options now exist for performing *image cytometry*, where cytometric data and correlated cell images can be collected simultaneously. Many laboratories prefer this technology for the analysis of apoptotic cells (particularly for adherent cells).


viable cell


apoptotic cell

Apoptotic cell analysis with laser scanning cytometry

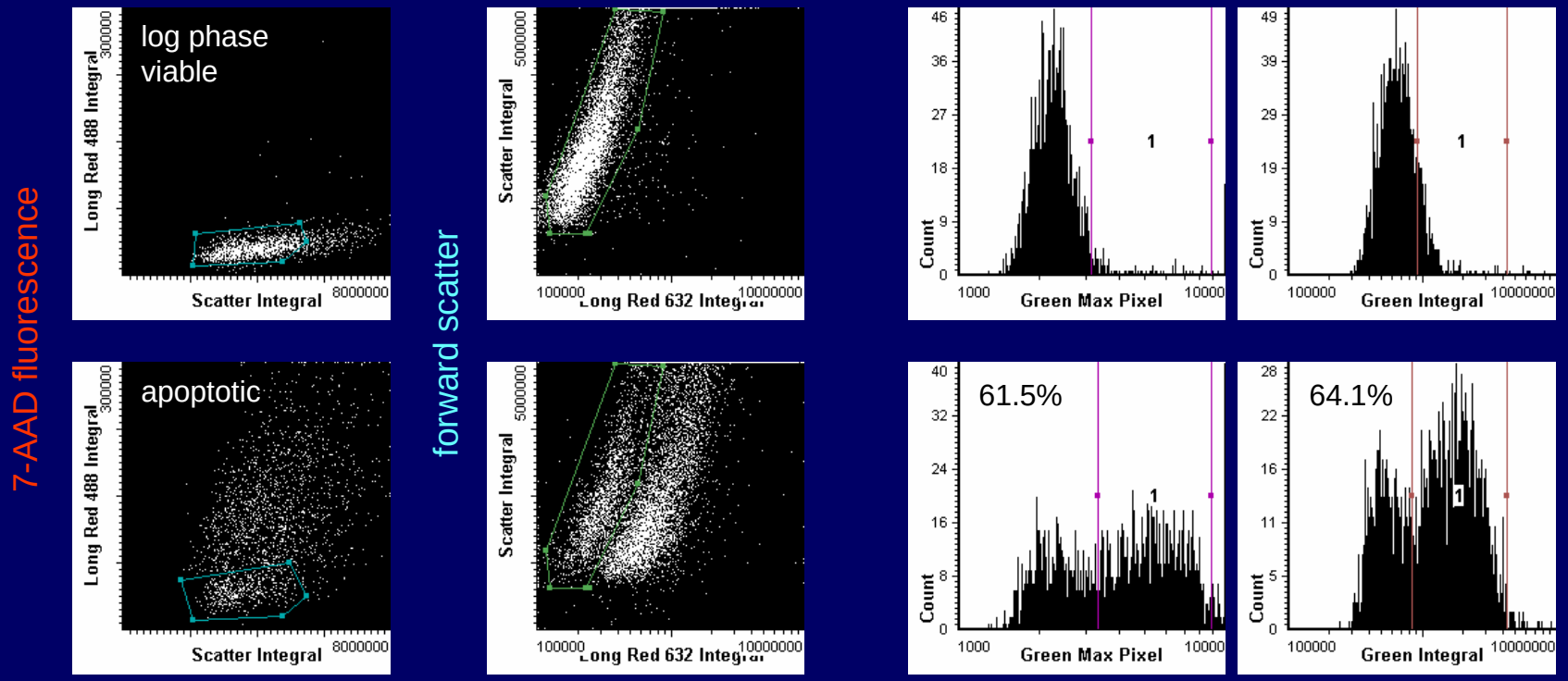
Concurrent cell staining with FAM-VAD-FMK and PI



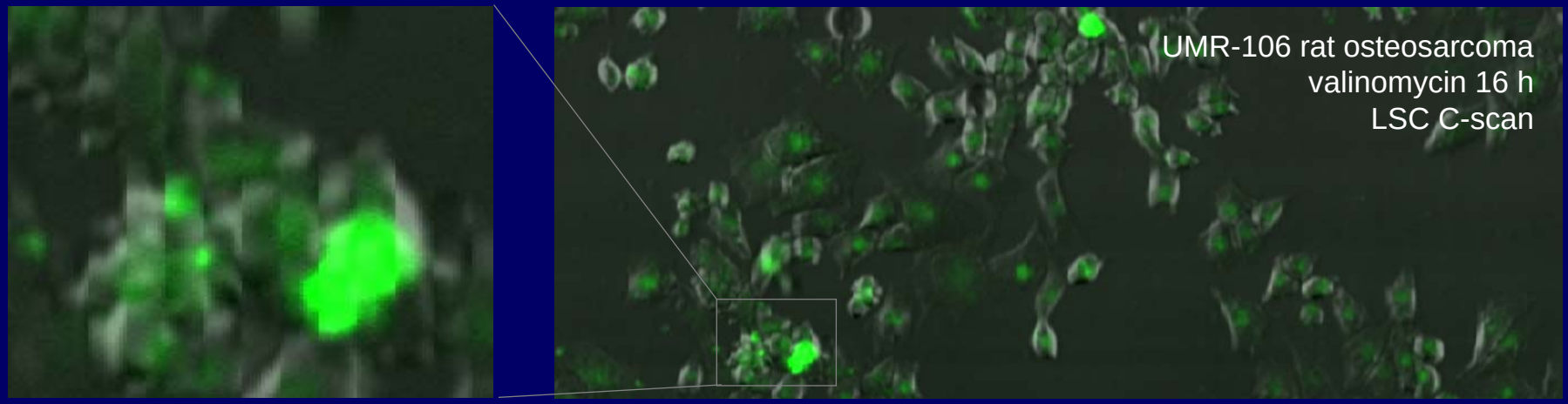
From Z. Darzynkiewicz

Apoptotic cell analysis with laser scanning cytometry

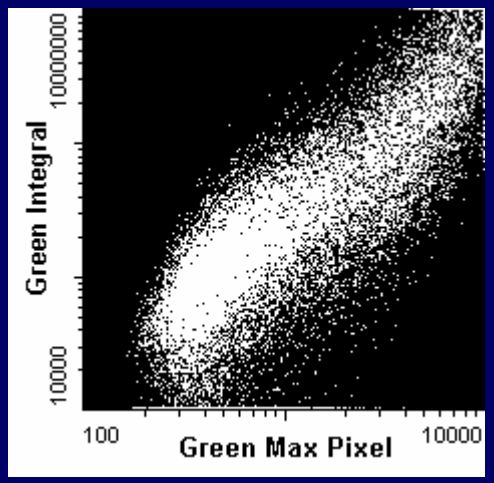
Viable and apoptotic EL4 cells
labeled with PPL caspase 3 (fluorescein),
APC-annexin V and 7-AAD



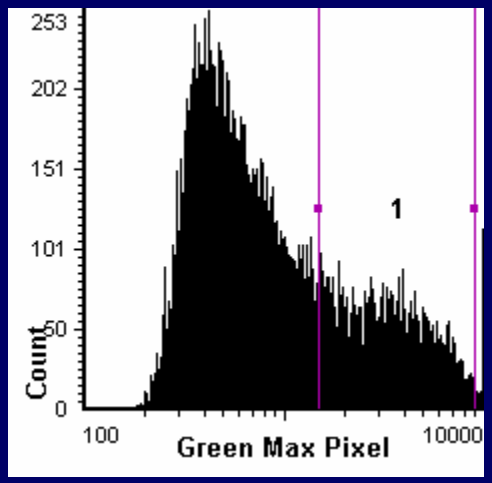
Apoptotic cell analysis with laser scanning cytometry



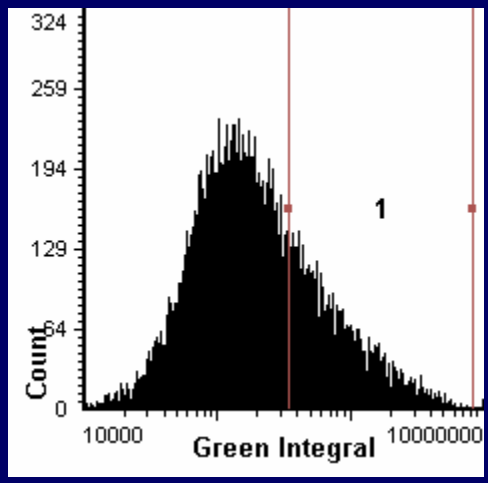
Max pixel analysis gives better resolution of caspase activation than total fluorescence integration



PPL-caspase 3
Max Pixel fluorescence



Max Pixel fluorescence



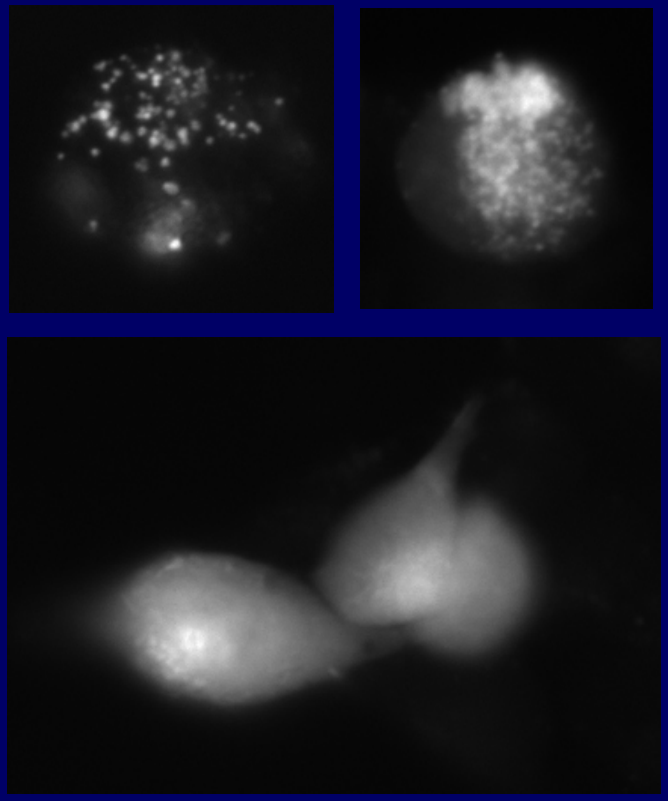
Integral fluorescence

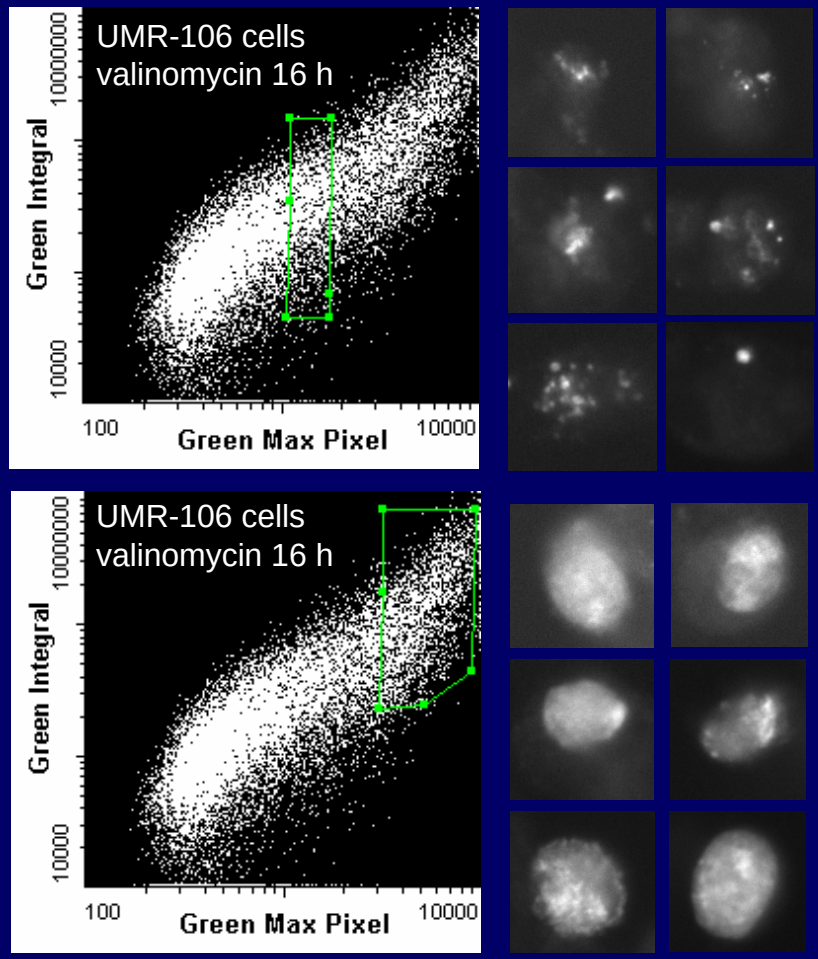
PPL-caspase 3 fluorescence

Apoptotic cell analysis with laser scanning cytometry

Max pixel analysis is not only more sensitive, but can identify early caspase activation loci within the cell.

UMR-106 cells
valinomycin 16 h
epifluorescence images





fluorescein PPL caspase 3 Integral

fluorescein PPL caspase 3
Max Pixel

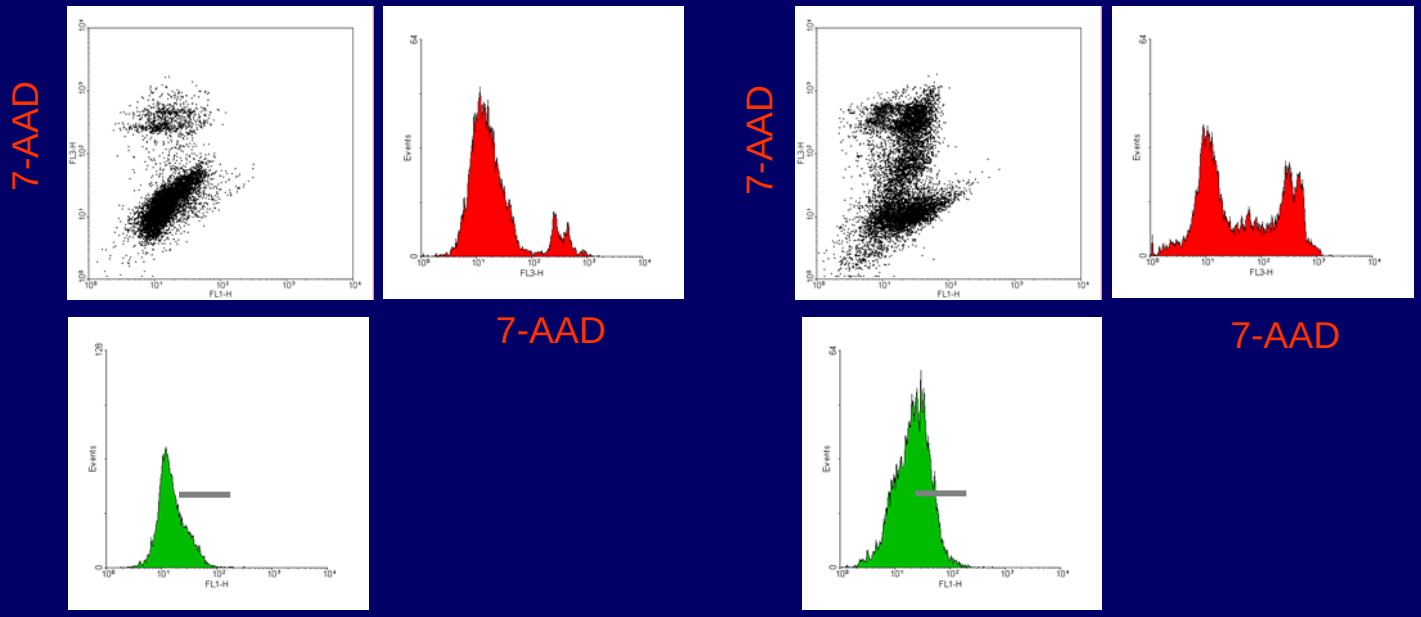
Discrimination of adherent apoptotic cells on the flow cytometer

L929 mouse fibroblasts
TNFα + cycloheximide 6 h

In this experiment, it is difficult to clearly distinguish caspase positive fibroblasts by flow cytometry.

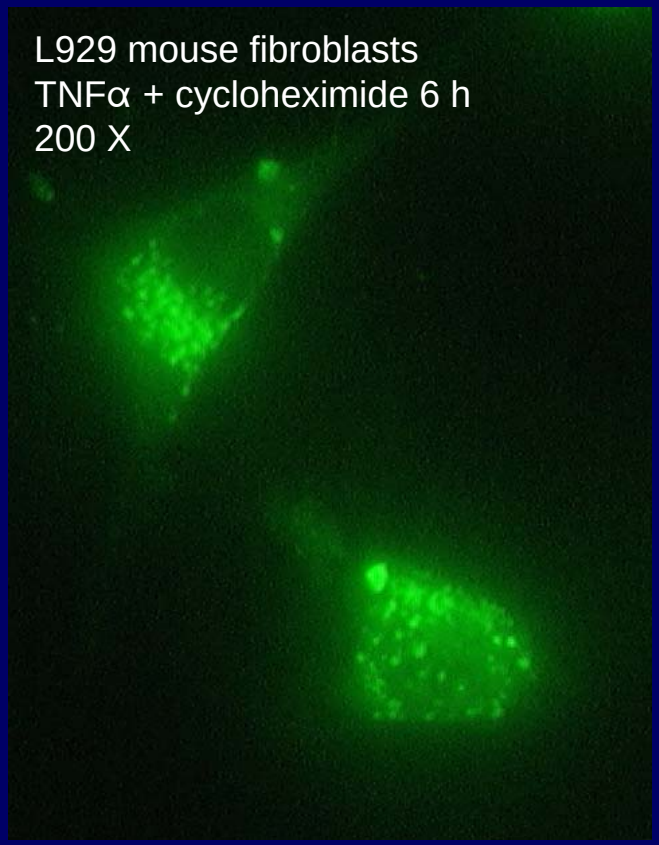
no treatment

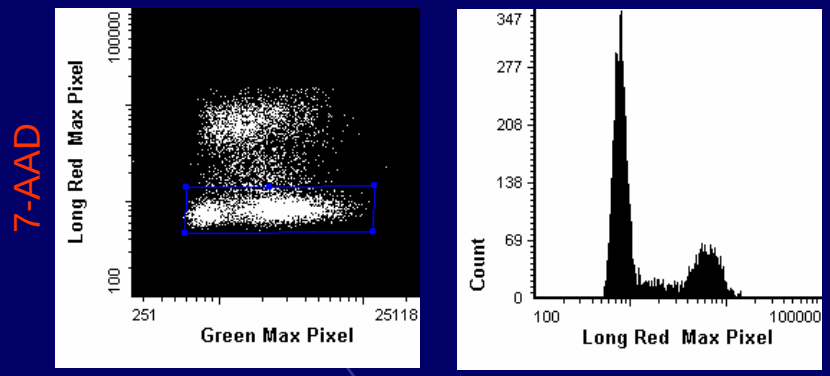
TNFα + cycloheximide



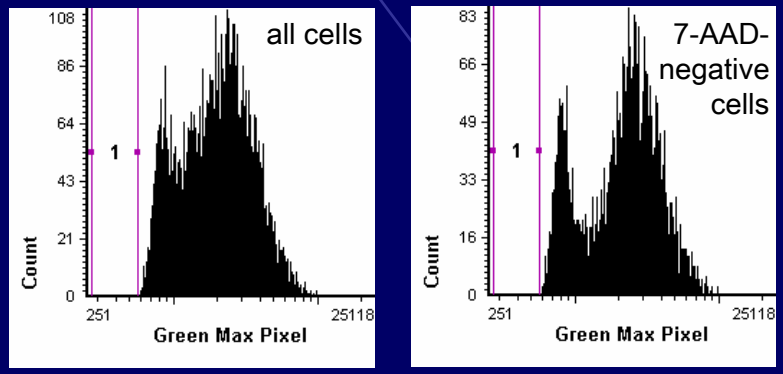
Removal of adherent cells for traditional flow cytometry can disrupt or induce apoptotic markers and make apoptotic cell measurement.

Discrimination of apoptotic cells on the laser scanning cytometer





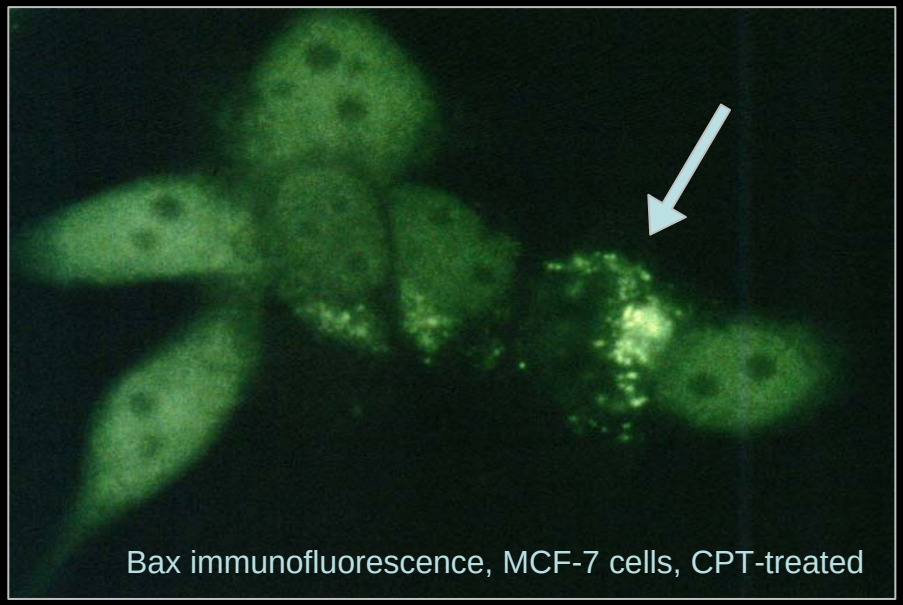
fluorescein PPL caspase 3 7-AAD

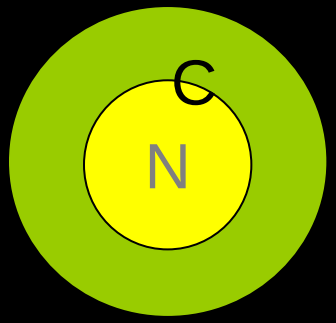


fluorescein PPL caspase 3

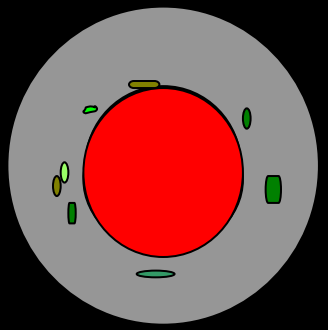
Removal of adherent cells for traditional flow cytometry can disrupt or induce apoptotic markers and make apoptotic cell measurement. Laser scanning cytometry allows measurement of apoptosis without disrupting the cell phenotype.

Bax translocation to mitochondria

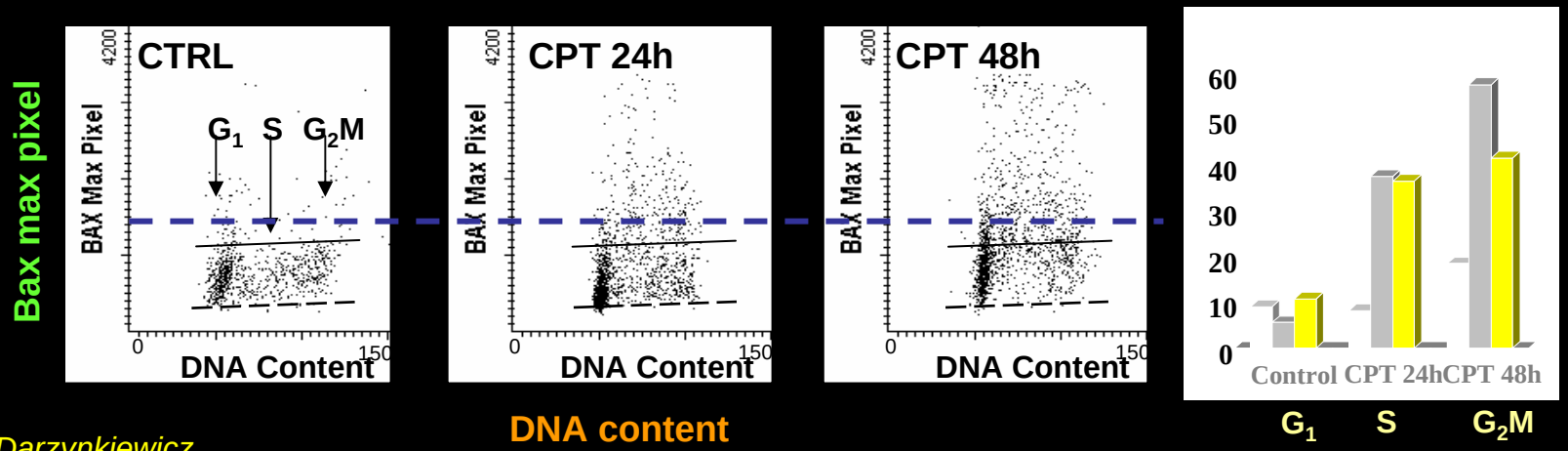




Bax in cytoplasm and in nucleus (diffuse, weak)



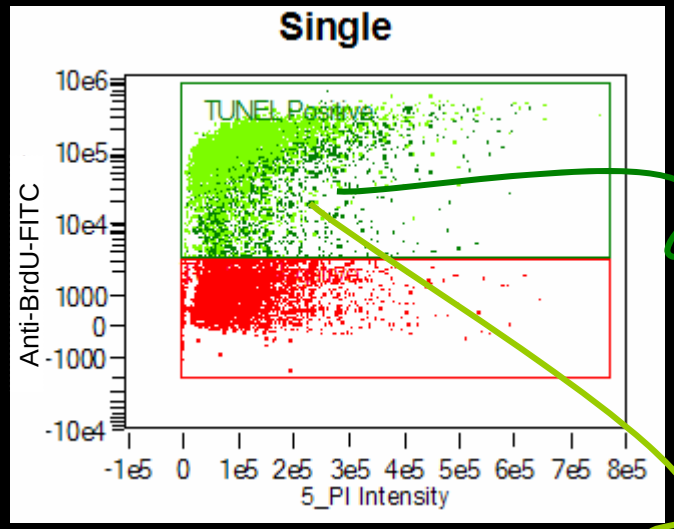
Bax in mitochondria (punctate, strong)



From Z. Darzynkiewicz

Analyzing apoptosis on the Amnis ImageStream

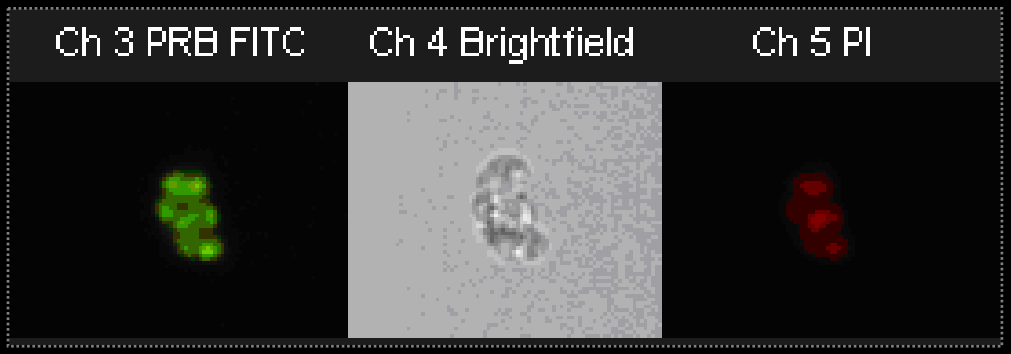
Data from David Coder, Amnis



BrdU-FITC

propidium iodide

False positive



True positive

Population Statistics

Population	Count	% Gated
Single	20613	100
TUNEL Positive & Single	6521	31.6
TUNEL Negative & Single	14092	68.4
TUNEL True Positive & Single	4310	20.9

Types of assays…

	unfixed "viable" cells	fixed permeablized cells
"early" apoptotic events	Cell volume fluctuations	Cytochrome C release
	Changes in cell membrane potential	
	Mitochondrial potential changes	
	Signaling events (bcl-2, Bax, etc.)	
	Initiator (proximal) caspase activation (1,9,10,8)	Initator (proximal) caspase activation
	Effector (distal) caspase activation (3,6,7)	Effector (distal) caspase activation
		Changes in chromatin organization (histones)
"late" apoptotic events	Organelle changes	
	PS membrane "flipping"	Early DNA strand breaks
	Transglutaminase crosslinking	
	Membrane "blebbing"	
	Loss of membrane permeability	Global chromatin damage

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!

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 methods whenever possible to more thoroughly characterize your apoptotic system.

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Visit us at http://home.ncifcrf.gov/ccr/flowcore/index.htm

Apoptosis Laboratory

Dual annexin V and DNA dye exclusion

PhiPhiLux and FLICA caspase detection

TUNEL and anti-active caspase immunolabeling

All genres of instrumentation, *particularly image cytometry*

Instrumentation provided by…

Compucyte Corporation
Guava Technologies
Amnis
Beckman-Coulter

Reagents provided by…

Invitrogen Molecular Probes
Oncoimmunin
Trevigen