Cell Death Analysis in Flow Cytometry

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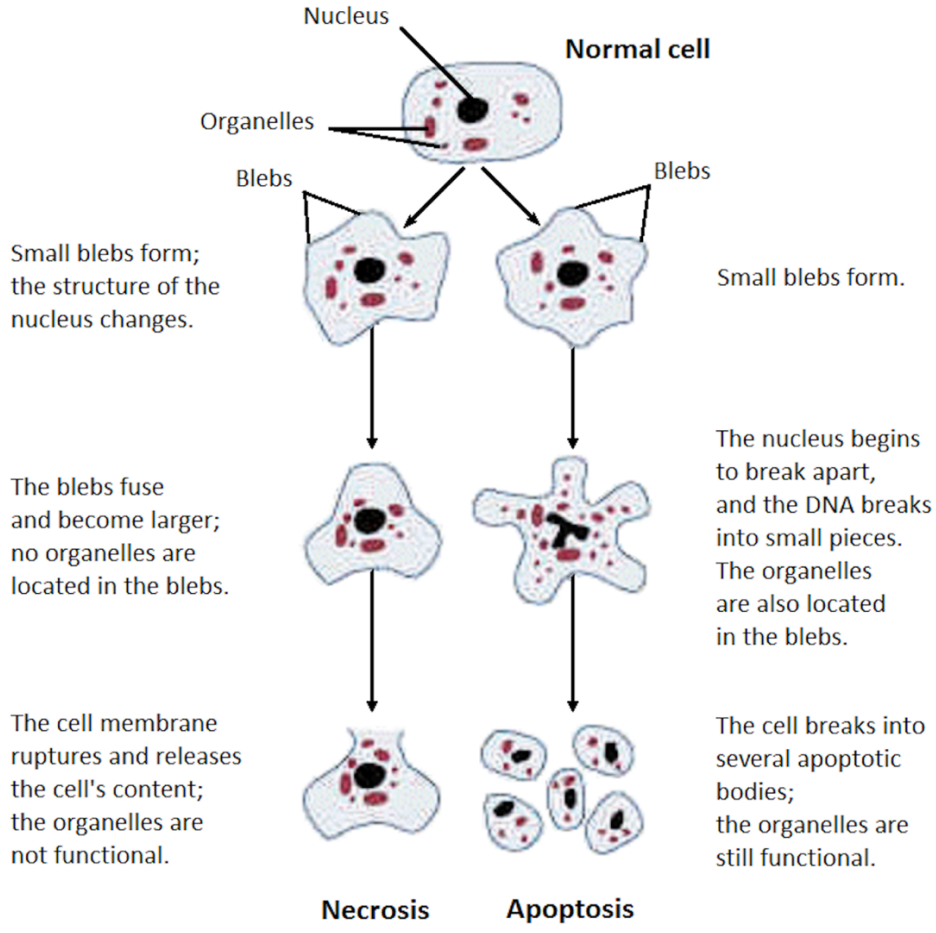
Date: May 2020

Cell death can be divided into 4 groups:

1. **Apoptosis:** Programmed and coordinated cell death
2. **Autophagy:** A rare type of programmed cell death (1)
3. **Necroptosis:** A viral defense mechanism, allowing the cell to undergo "cellular suicide" in a caspase-independent fashion in the presence of viral caspase inhibitors to restrict virus replication.
4. **Necrosis:** Cell death along with degradation of tissue by hydrolytic enzymes

Difference between Necrosis and Apoptosis (figure from Charles Goodlett - ResearchGate.net)



FEATURE	APOPTOSIS	AUTOPHAGY	NECROSIS
Definition	Programmed and coordinated enzyme mediated cell death	A rare form of programmed cell death	Cell death along with degradation of tissue by hydrolytic enzymes.
Causative agents	Physiological and pathological processes	Caused by nutrient stress e.g. insulin lack	Hypoxia, toxins
Morphology	1. No inflammatory reaction 2. Death of single cells 3. Cell shrinkage 4. Many blebs on cell membrane 5. Apoptotic bodies 6. Chromatin condensation 7. Phagocytosis of apoptotic cell	1. No inflammatory reaction 2. Death of single cells 3. Vacuolization 4. Loss of integration 5. Some enlargement of Golgi and ER 6. No chromatin condensation 7. No phagocytosis	1. Inflammatory reaction always present 2. Death of many adjacent cells 3. Cell swelling initially 4. Membrane disruption 5. Damaged organelles 6. Nuclear disruption 7. Phagocytosis of cells 8. Cell lysis
Size of cell	Shrunken cell due to loss of cytoplasm from cytoplasmic buds that pinch off and become apoptotic bodies	Extensive vacuolization of cytoplasm	Intracellular swelling due to Sodium-containing water entering the cell. (dysfunctional Na/K ATPase pump)
Enzymes involved	Initiator caspases, Executioner caspases (protease, endonuclease) No leak of lysosomes		Phospholipase, protease, endonuclease Leak of lysosomes
Genes involved	BCL-2(anti -apoptosis), BAX (proapoptotic), BAK (proapoptotic) DNA cleavage		NONE No DNA cleavage
Role	Physiologic functions, pathologic functions (e.g. removal of misfolded proteins, removal of neutrophils in acute inflammation)		Usually associated with a pathologic process

Ref. neurones.co.uk, researchgate.net, science direct.com

Apoptosis, things to bear in mind before you start:

- Apoptosis is a very variable process depending of cell type. Always use more than one method for detecting apoptosis.
- A good way to know your cell type is to take pictures along the process.
- If your cells produce extensive blebbing a non-flow method would perhaps be better
- Remember that flow cytometry is a snapshot of the process.
- Timepoints of the kinetic process is crucial for a reliable result and also a drug dosage curve.
- Centrifugation, vigorous pipetting or rough treatment can destroy the apoptotic cells. Never use vortex.
- Density gradient can hide apoptotic cells due to a change in density caused by chromatin and cytoplasm condensation.
- If you have adherent cell be aware that apoptotic cells round up and detach the surface they are growing on, collect them, don't wash them off.
- Remember to have good positive (e.g. Doxorubin or Saponin treated cells) and negative controls and a live cell sample.

Tools:

Membrane staining

For many of you the first choice will probably be **Annexin V**. It is not the easiest staining. Here are some tips/tricks.

- Remember to have Ca^{++} and Mg^{++} present all the way through the staining procedure.
- Quickly analyze on the flow cytometer after staining.
- You can't fix your cells.
- Some cells have more Phosphatidyl serine on the surface naturally and will therefore look positive for Annexin V although they are living cells and not in apoptosis.
- Necrotic cells are also positive for Annexin V.

Another membrane dye is **F2N125** (Thermo Fisher Scientific):

- The dye is excited by the violet laser
- 5 min labeling of your cells - no wash
- Independent of trypsin and cell scrape of your cells
- The fluorescence shift from orange to green during apoptosis

Mitochondrial changes

These dyes can be used to measure mitochondrial membrane potential to identify an early apoptosis event. Apoptosis is an energy driven process and demands mitochondria.

- JC-1
- TMRE
- Mitospy® dyes
- MitoView® dyes
- MITO-ID® dyes
- MitoTracker® dyes

Caspase Activity

These dyes can be used to measure caspase activation to mark intermediate apoptosis.

- NucView® dyes
- FLICA® dyes

Amine reactive dyes

Binds to protein in the membrane disrupted cell
Live cells will also bind the dyes but much less than dead cells

- Viobility™ dyes
- Zombie™ dyes
- Live-or-Dye™
- VivaFix™ dyes
- Ghost Dyes™
- Horizon Brilliant™ dyes
- eFlour ® dyes
- Live/Dead™ dyes

Enzyme Activated Dyes

- Enzyme activated dyes can be applied to stain living cells without killing them.
- These dyes are often weakly or non-fluorescent until activated by an enzyme reaction.
- They freely pass through living cell membranes, where they are converted into membrane-impermeant, fluorescent reaction products.
- The dyes are retained in living cells through several generations, and can be transferred to daughter cells, making them useful for tracking cell movement and proliferation as well.
  However, because these dyes require activation by active enzymes, they are typically not recommended for fixation and permeabilization experiments.

	CellTracker dye	Common filter set	Ex/Em (nm)	Quantity	Cat. No.
CellTracker Blue CMAC (7-amino-4-Chloromethylcoumarin)	CMAC	DAPI	353/466	5 mg	C2110
CellTracker Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin)	Blue CMF_2HC	DAPI	371/464	5 mg	C12881
CellTracker Violet BMQC (2,3,6,7-tetrahydro-9-bromomethyl-1H,5H-quinolizino(9,1-gh)coumarin)	Violet BMQC	DAPI	415/516	5 x 0.1 mg	C10094
CellTracker Green CMFDA (5-chloromethylfluorescein diacetate)	Green CMFDA	FITC	492/517	20 x 50 µg	C7025
CellTracker Orange CMRA	Orange CMRA	TRITC	548/576	20 x 50 µg	C34551
CellTracker CM-DiI	CM-DiI	TRITC	553/570	20 x 50 µg	C7000
CellTracker Red CMTPX	CMTPX	Rhodamine	577/602	20 x 50 µg	C34552
CellTracker Deep Red	Deep Red	Cy5	630/650	20 x 15 µg	C34565

Link to CellTracker : https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/cell-tracing-tracking-and-morphology/cell-tracking

Membrane Permeability

These dyes can be used to measure membrane permeability during intermediate apoptosis.

DNA-binding dyes

Dye	Fixed Cells	Ex	Em
DAPI	No	358	461
Hoechst (33258)	No	352	461
Hoechst (33342)	No	350	461
SYTOX® Blue	No	444	480
SYTOX® Green	No	504	523
SYTOX® Orange	No	547	570
SYTOX® AAdvanced	No	546	647
SYTOX® Red	No	640	658
TO-PRO®-1	No	515	531
TO-PRO®-3	No	642	661
TOTO®-1	No	514	533
TOTO®-3	No	642	660
Ethidium Monoazide Bromide	No	504	600
Ethidium Bromide	No	210/285	605
Propidium Iodide	No	488	617
7-AAD	No	543	647
DRAQ5™	No	647	681
DRAQ7™	No	633	695
Helix NP™ NIR	No	640	660
RedDot™1	No	662	694
RedDot™2	No	665	695
YO-PRO™-1	No	491	509
YO-PRO™-3	No	612	631
LDS 751	Yes	543	712

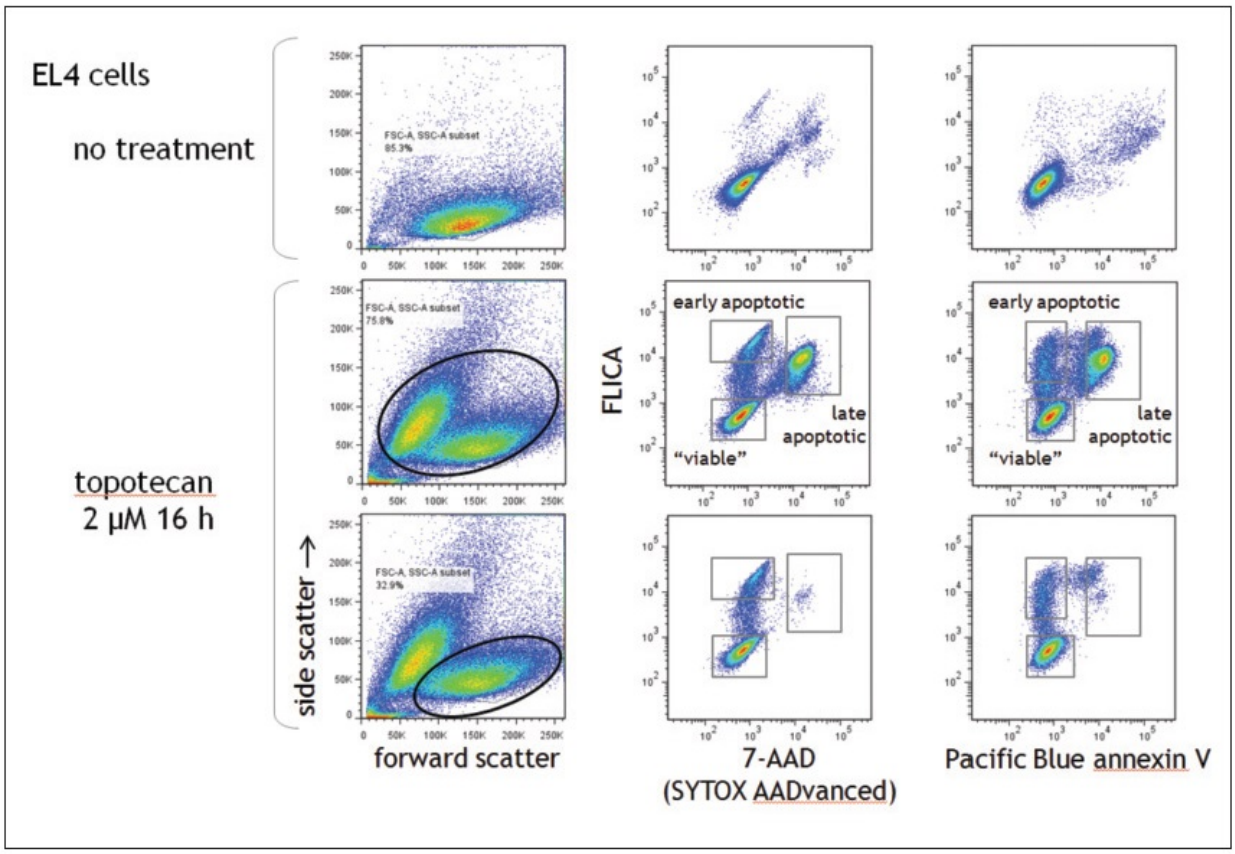


Figure 1 The progression of apoptosis can be studied by combining multiple assays into one analysis using multiparametric flow cytometry

Figure from: https://www.ddw-online.com/drug-discovery/p274231-cell-death:-discriminating-between-apoptosis--necrosis---autophagy.html

1. (https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.22312)