Caractérisation et méthodes d'études de la mort cellulaire par cytométrie en flux

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EA7270 - Equipe 'Biochimie du Peroxysome, inflammation et Métabolisme Lipidique'

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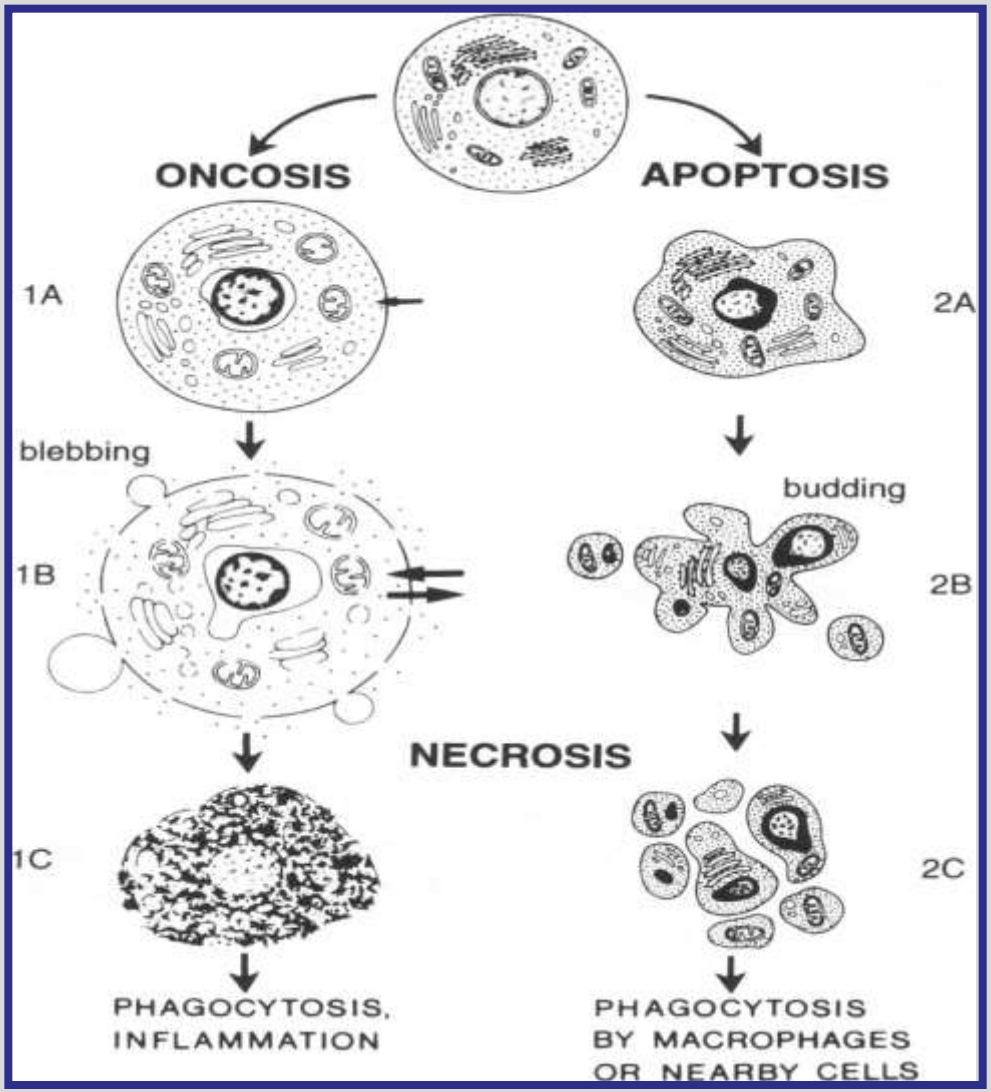
Rabat, 29 novembre 2018

CytoMagh 2018



Two pathways of cell death leading to necrosis and apoptosis. At the top is shown a normal cell. 1A: Swelling. 1B: Vacuolization, blebbing, and increased permeability. 1C: Necrotic changes. ie, coagulation, shrinkage, and karyolysis. 2A: Shrinkage and pyknosis. 2B: Budding and karyorhexis. 2C: Necrotic changes, ie, breakup into a cluster of apoptotic bodies.

Majno G & Joris I Am J Pathol 1995, 146: 3 - 15.

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ORGANELLES INVOLVED IN CELL DEATH

Organelles	*Apoptosis*	*Autophagy*	*Necrosis / Necroptosis*
- Mitochondria	+	+ (mitophagy)	+
- Lysosomes	+ / -	+	+ / -
- Endoplasmic-reticulum (ER)	+ / -	+	+ / -
- peroxisome	?	+ (pexophagy)	?

Bröker LE et al. Clin Cancer Res 2005, 11: 3155-3162.

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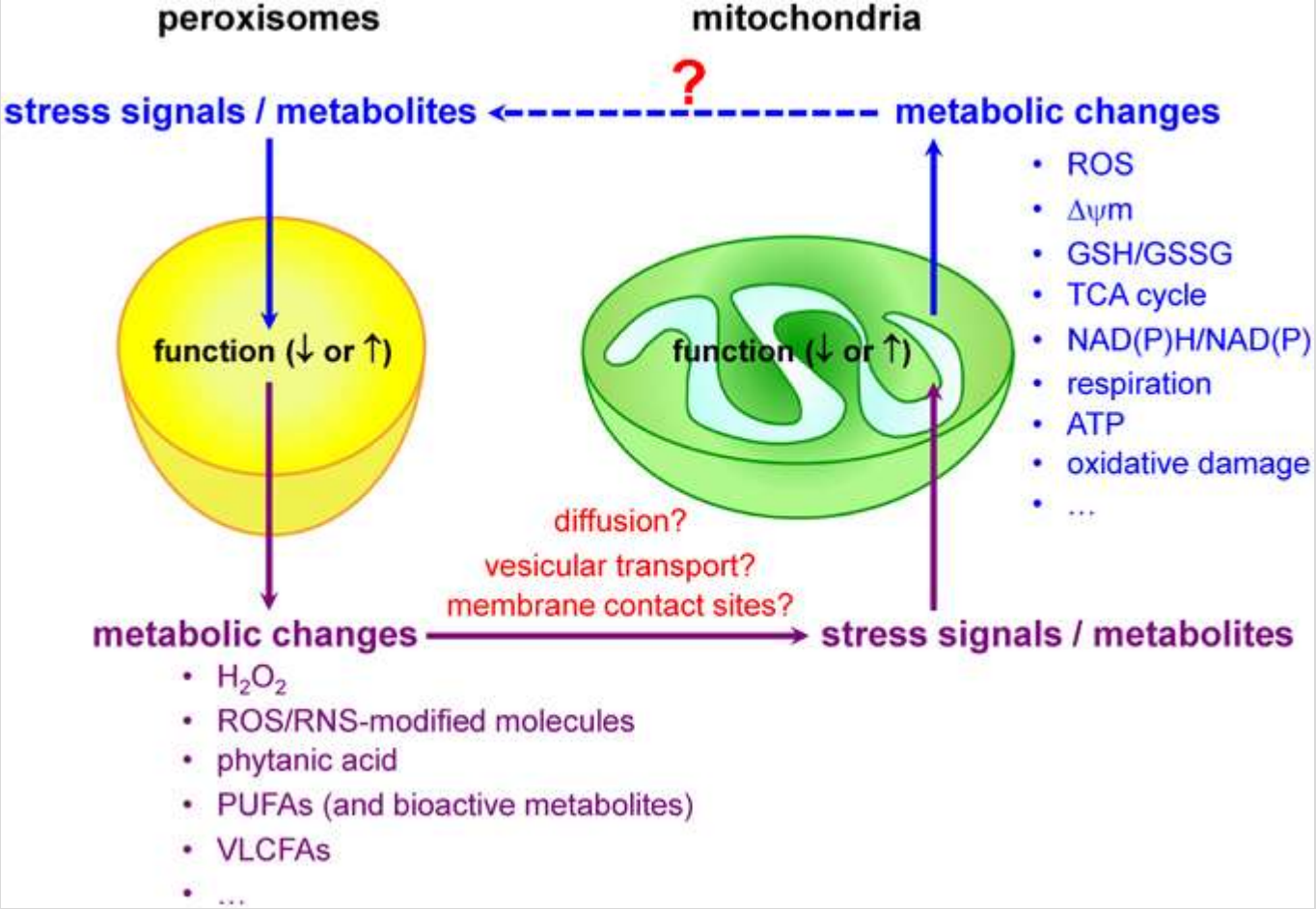
Relations peroxysome / mitochondrie

Implication dans le contrôle de l'équilibre RedOx et l'activation de la mort cellulaire



Lismont C, Nordgren M, Van Veldhoven PP, Fransen M. Front Cell Dev Biol. 2015, 27;3:35.

Cell Death (initial concept)

Apoptosis
apoptotic morphology

Necrosis
necrotic morphology

Active programmed cell death

Caspase-dependent cell death

- Mitochondrial pathway
(Associated or not with reticulum stress)

- Death receptor pathway

Passive unprogrammed cell death

Classical /Canonical Necrosis

Cell Death Independent of Caspases

Kitanaka C & Kuchino Y Cell Death Differ 1999, 6: 508-515.

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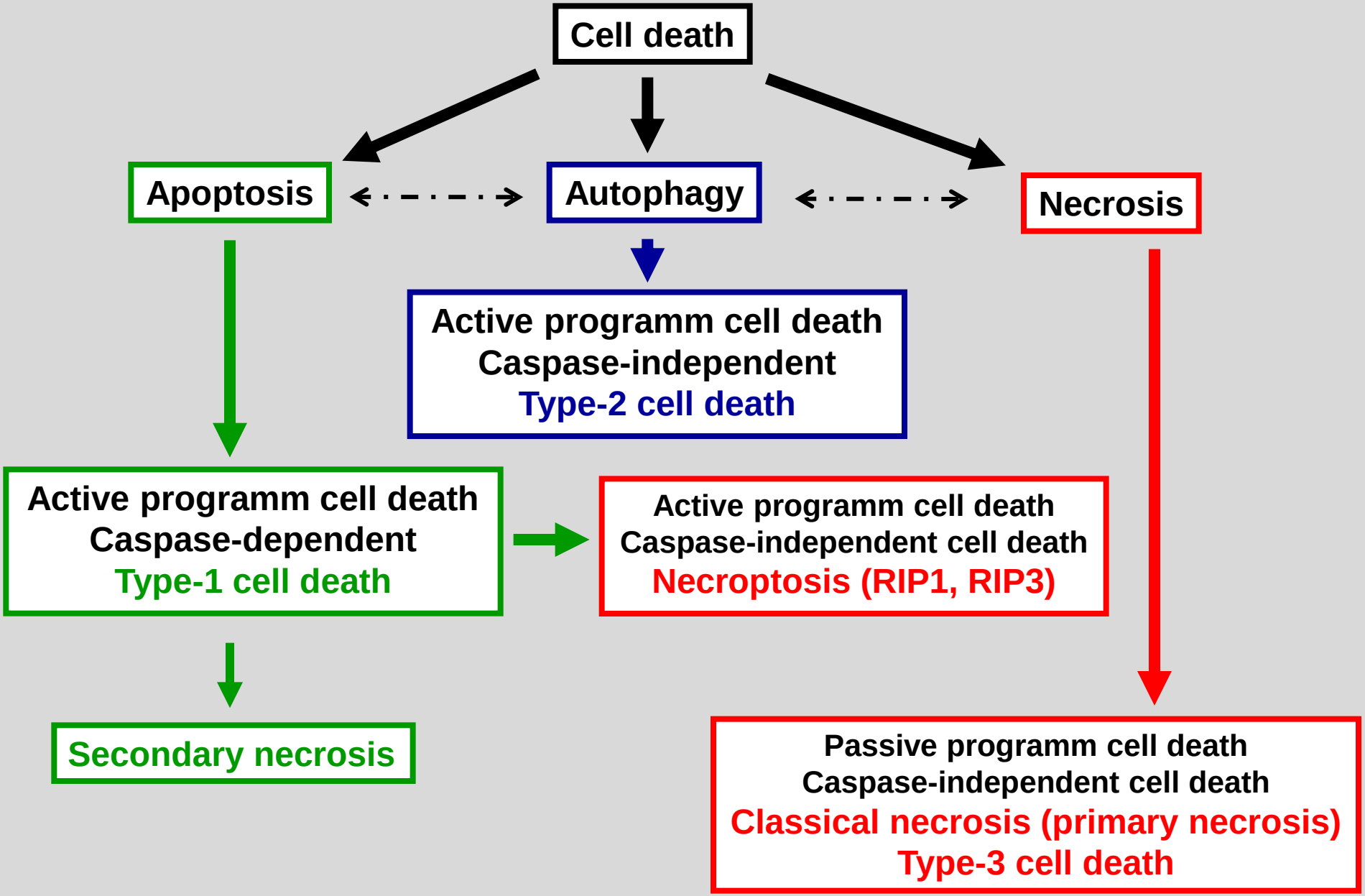

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AVAILABLE METHODS ALLOWING THE CHARACTERIZATION OF APOPTOSIS, NECROSIS / NECROPOTOSIS, AND AUTOPHAGY

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FUNCTIONNAL CRITERIA ASSOCIATED WITH APOPTOSIS, NECROSIS, AND NECROPTOSIS

- **Microscopy, flow cytometry, biochemistry**

- enhanced permeability of cytoplasmic membrane (trypan blue, fluorescent probes, LDH)

- externalization of phosphatidylserine

double staining with AnnexinV /propidium iodide (PI) (or aminoactinomycine D (AAD)) to distinguish between normal (AnnexinV$^-$/PI $^-$), necrotic (AnnexinV$^+$/PI$^+$), and apoptotic (AnnexinV$^+$/PI$^-$) cells

- Coloration SYTO16 - IP

- Sub-G1 peak (not present in normal and necrotic cells)

- loss of transmembrane mitochondrial potential: numerous fluorescent probes available. Currently, DioC_6(3) and JC1 are the most reliable.

- FLICA (fluorochromes labeled inhibitors of caspases): *in situ* identification of activated caspases (does not permit to distinguish between necrosis and caspase-independent cell death)

- TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling): *in situ* identification of internucleosomal DNA fragmentation

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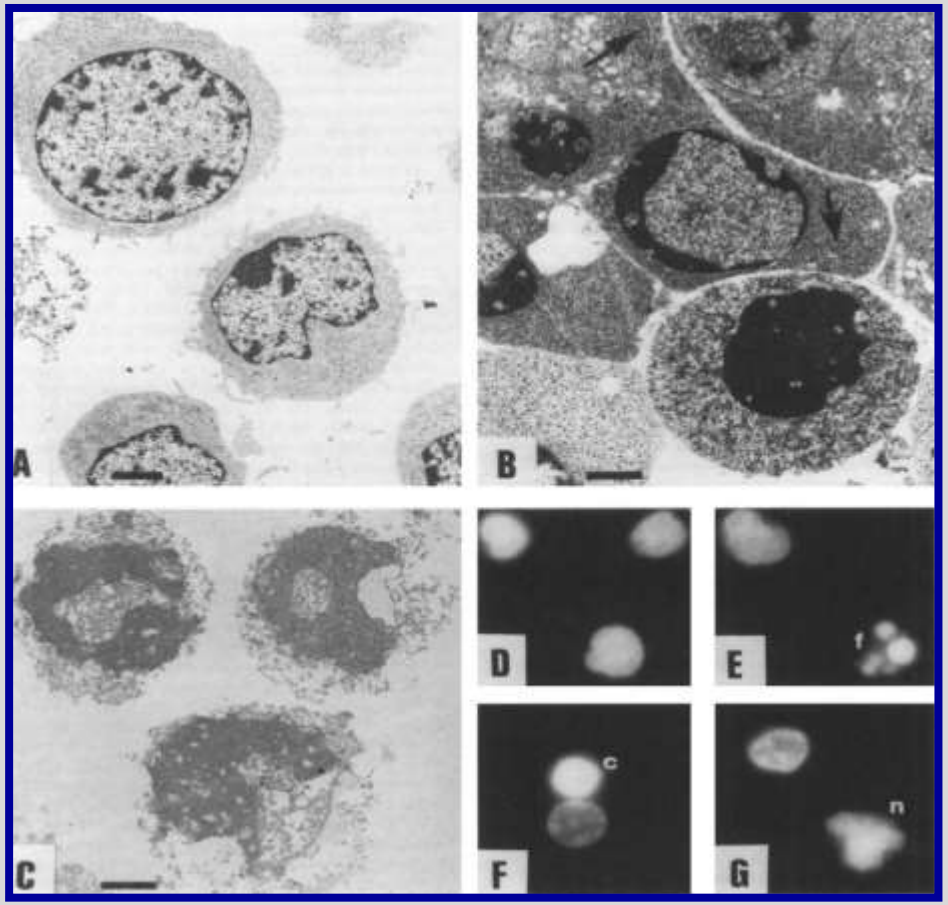
APOPTOSIS / NECROSIS: MORPHOLOGICAL CRITERIA



Electron microscopy A: Control. B: Cells treated by VP-16; apoptotic cells with condensed and perinuclear chromatin are observed, cytoplasmic and nuclear membrane integrity are preserved as well as morphology of mitochondria (arrow). C: Cells treated by NaN3; necrotic cells are characterized by a loss of integrity of cytoplasmic and nuclear membranes, degradation of cytoplasm and chromatin.

Fluorescence microscopy after staining with Hoechst 33342 D: Control cells. Nuclei show regular contours. E: Cells treated by VP-16; cell with fragmented (f) nucleus. F: Cells treated by VP-16; cell with condensed (c) nucleus. G: Necrotic (n) cells observed under treatment by NaN3;
the nucleus is diffuse and irregular. (***Lizard G et al. Cytometry 1995, 21: 275-283***)

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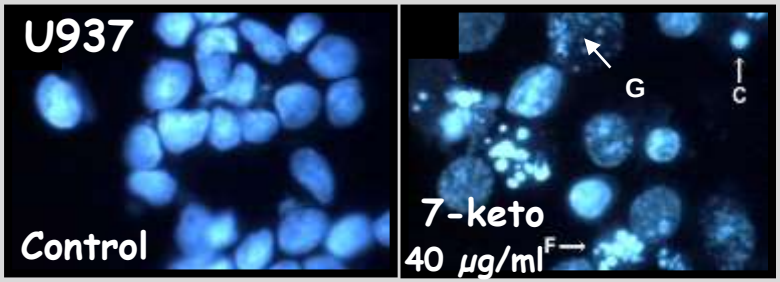
MORPHOLOGICAL CRITERIA ASSOCIATED WITH APOPTOSIS, NECROSIS AND NECROPTOSIS

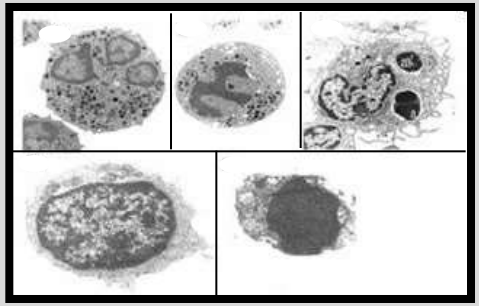
*** Phase contrast microscopy: loss of refringence; loss or not of cell adhesion?**

*** Brightfield microscopy: after staining with GIEMSA,…**

*** Fluorescence microscopy: Hoechst staining allows to easily distinguish between normal, necrotic and apoptotic cells (nuclear criteria)**



*** Transmission electron microscopy (nuclear criteria)**



*** Flow cytometry: changes of light scatter properties *on non-fixed cells* (FSC ↓ ; SSC more or less ↑)**

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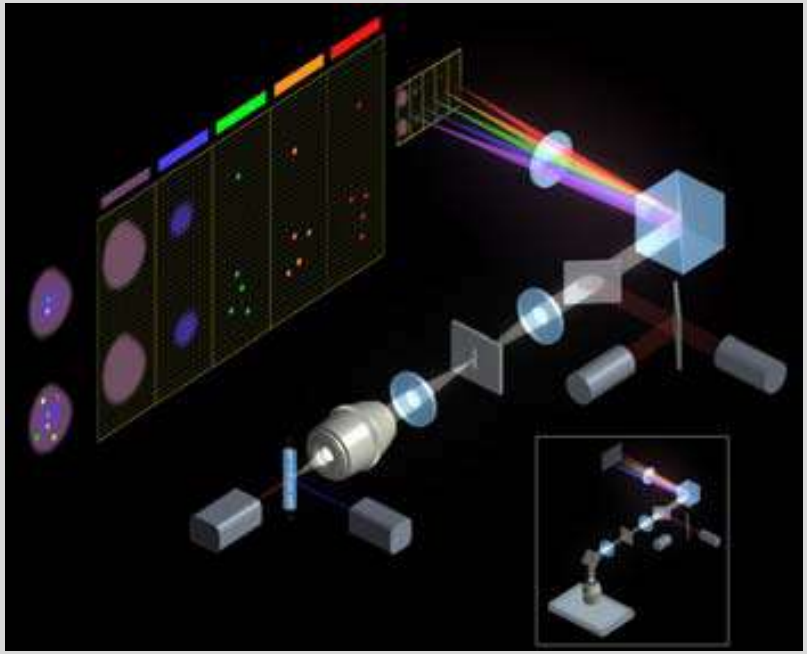
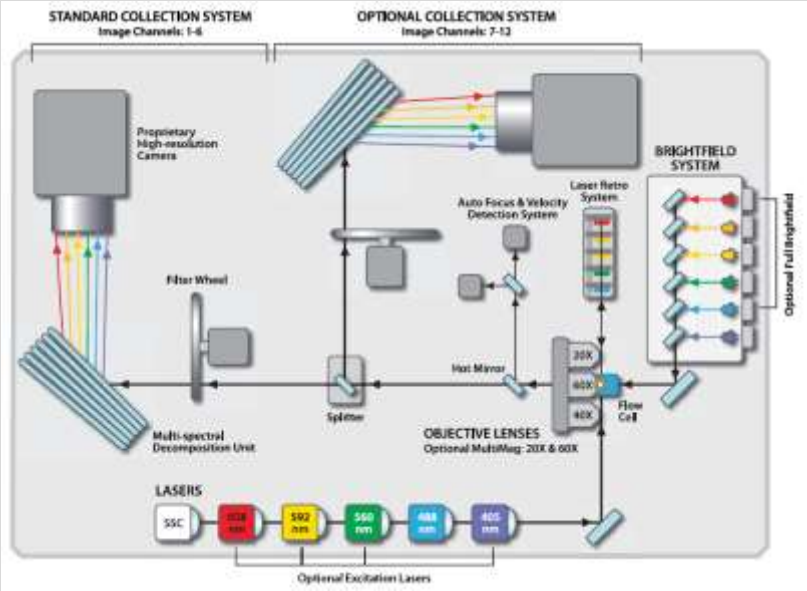
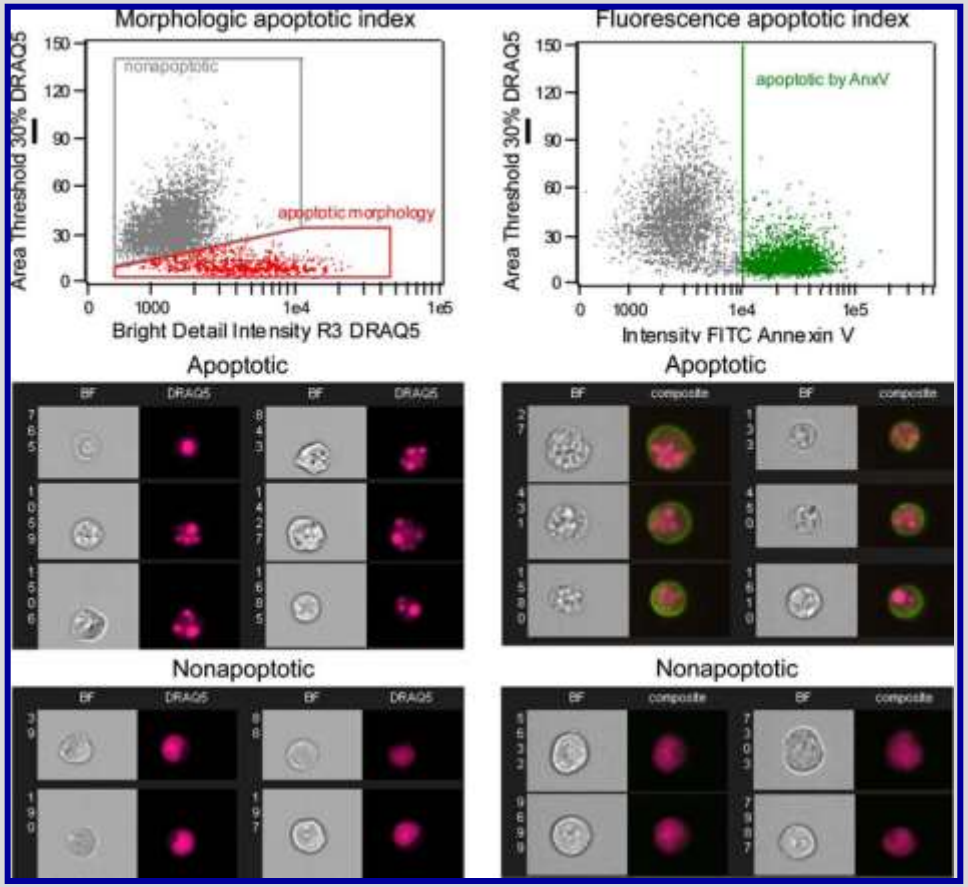
Annexin V-FITC / PI (Amnis technology)



http://www.amnis.com





Henery S et al. Apoptosis 2008, 13: 1054-1063

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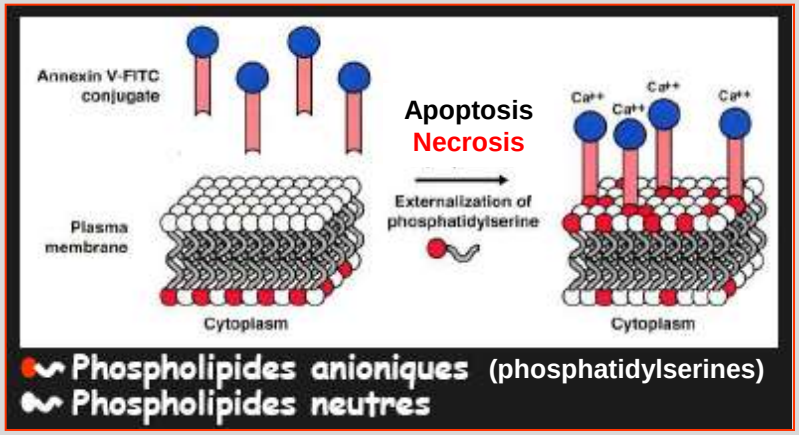
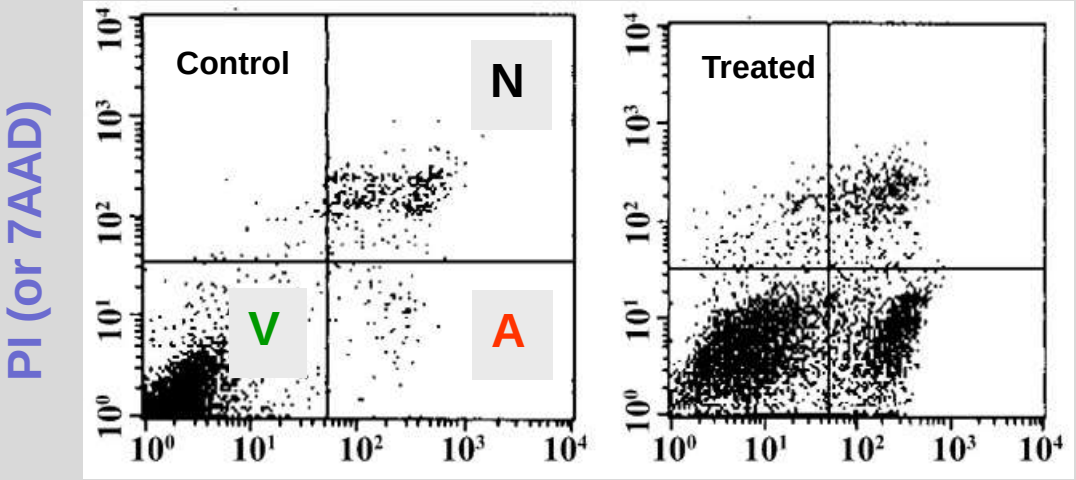
Phosphatidylserine externalization : Annexin V-FITC / PI test



Cells	Annexine V	PI
Viable (**V**)	−	−
Apoptotic (**A**)	+	−
Necrotic (**N**)	+	+



PI: propidium iodide
7 AAD: 7 amino actinomycin D

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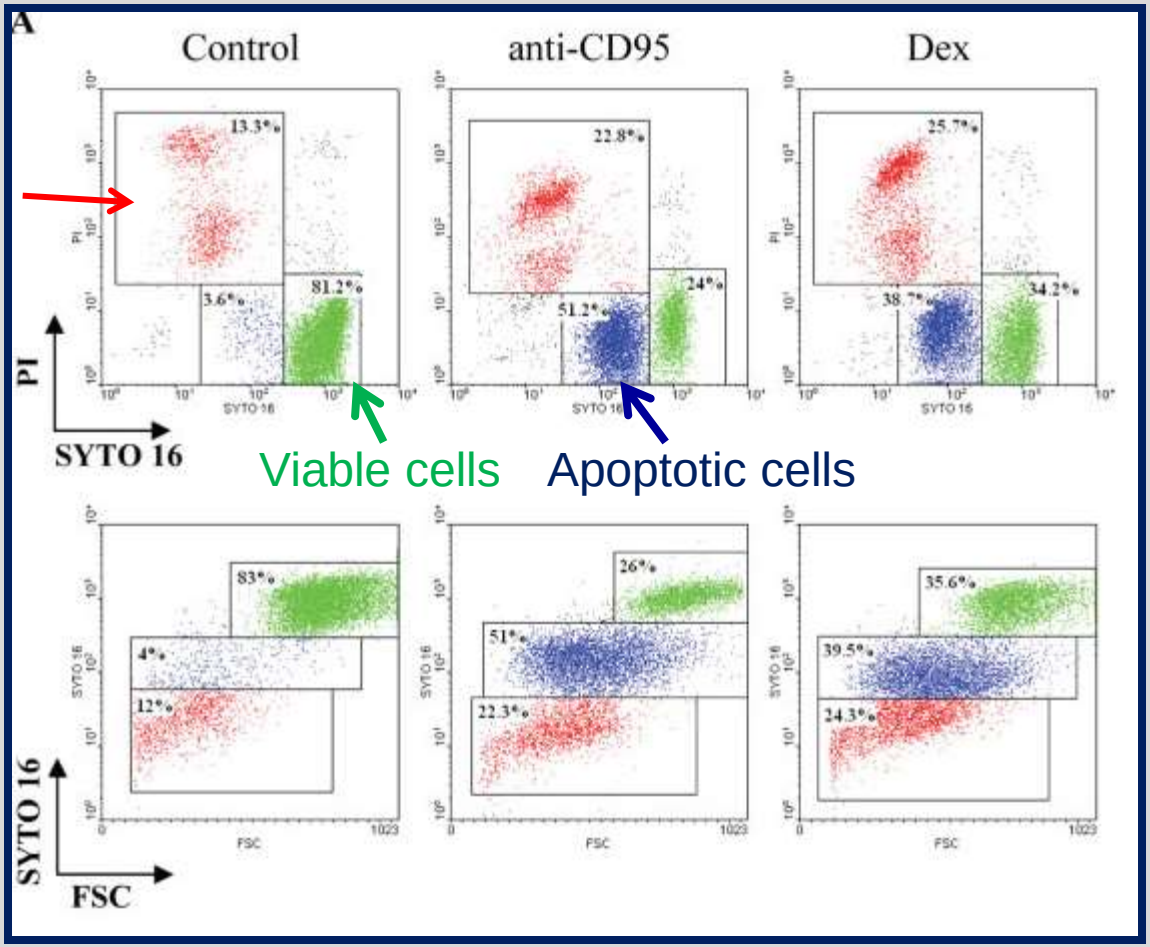
Towards an Understanding of Apoptosis Detection by SYTO Dyes

Donald Wlodkowic,1* Joanna Skommer,1 and Jukka Pelkonen1,2
1Institute of Clinical Sciences, Department of Clinical Microbiology, University of Kuopio, Kuopio, Finland
2Department of Clinical Microbiology, Kuopio University Hospital, Kuopio, Finland

Cytometry Part A 71A:61–72 (2007)

Necrotic cells



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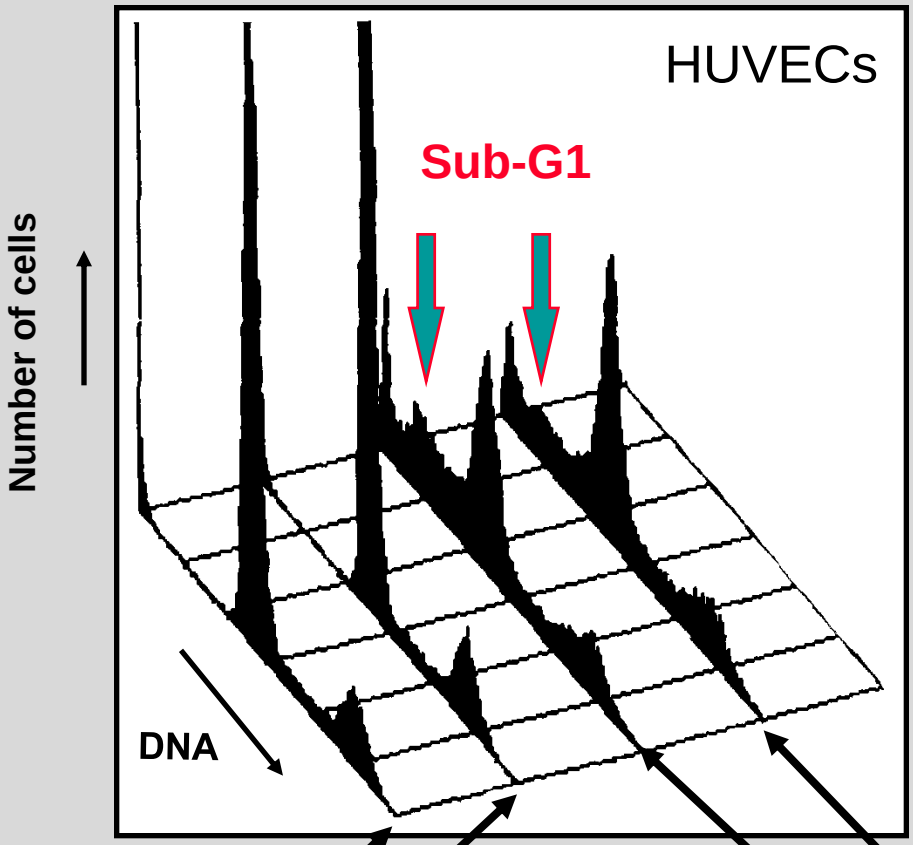
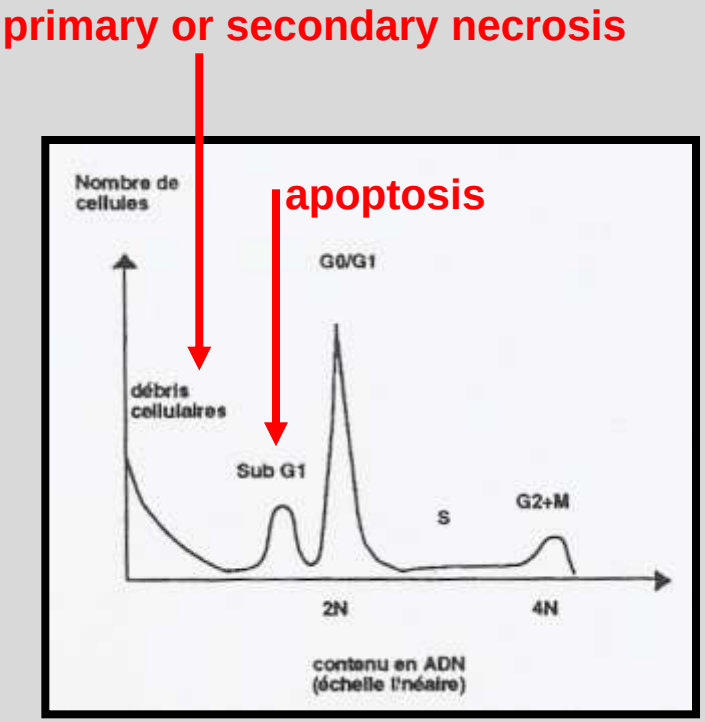
Sub-G1 (FCM)





control

cholesterol

7-ketocholesterol

7ß-hydroxycholesterol

Lizard G et al. Am J Pathol 1996, 148: 1625-1638

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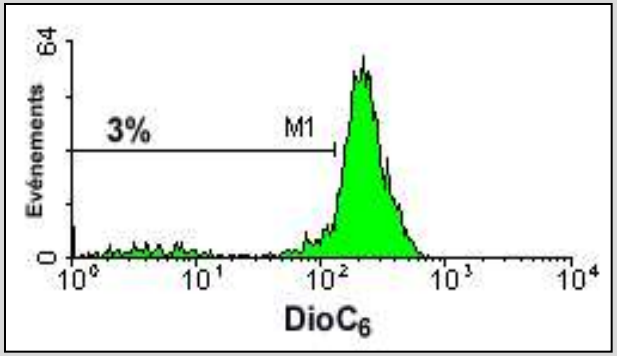
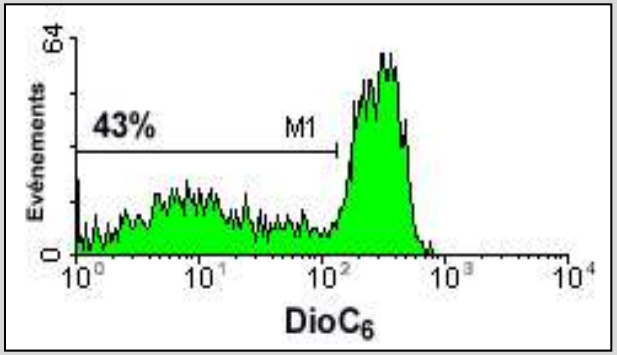
Mitochondrial potential (FCM)

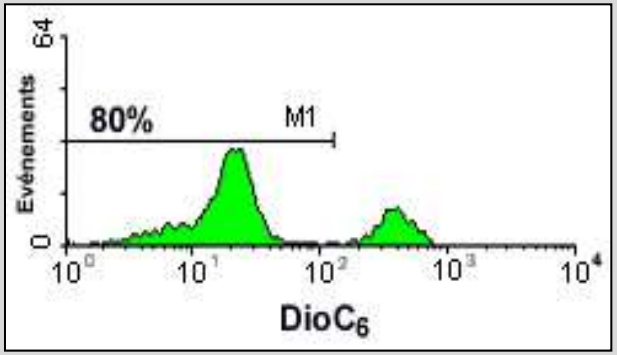
Control

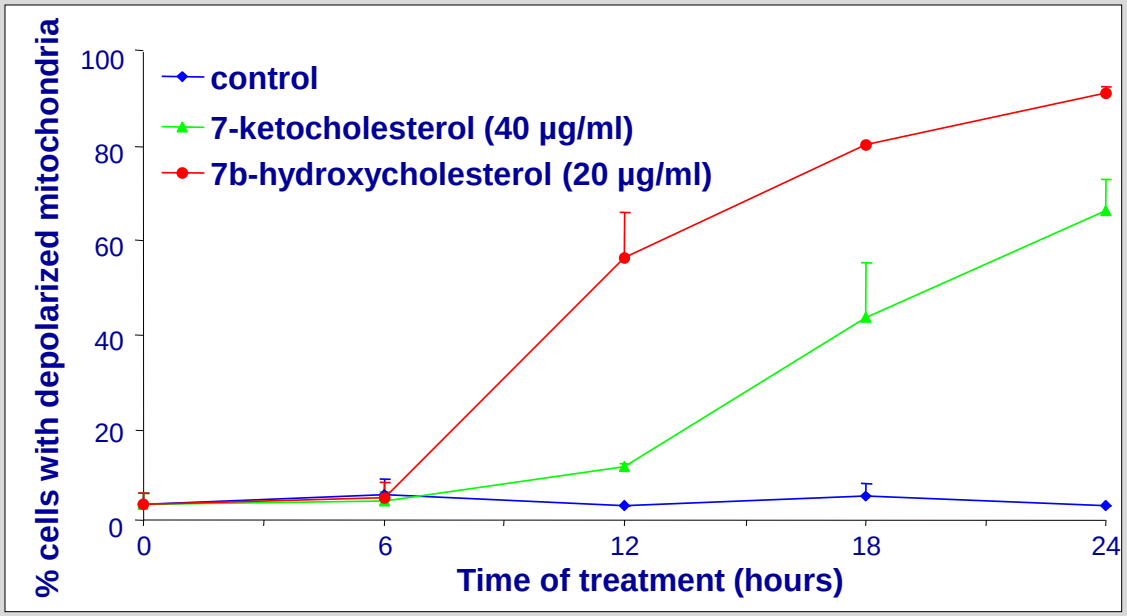


7-ketocholesterol



7β-hydroxycholesterol





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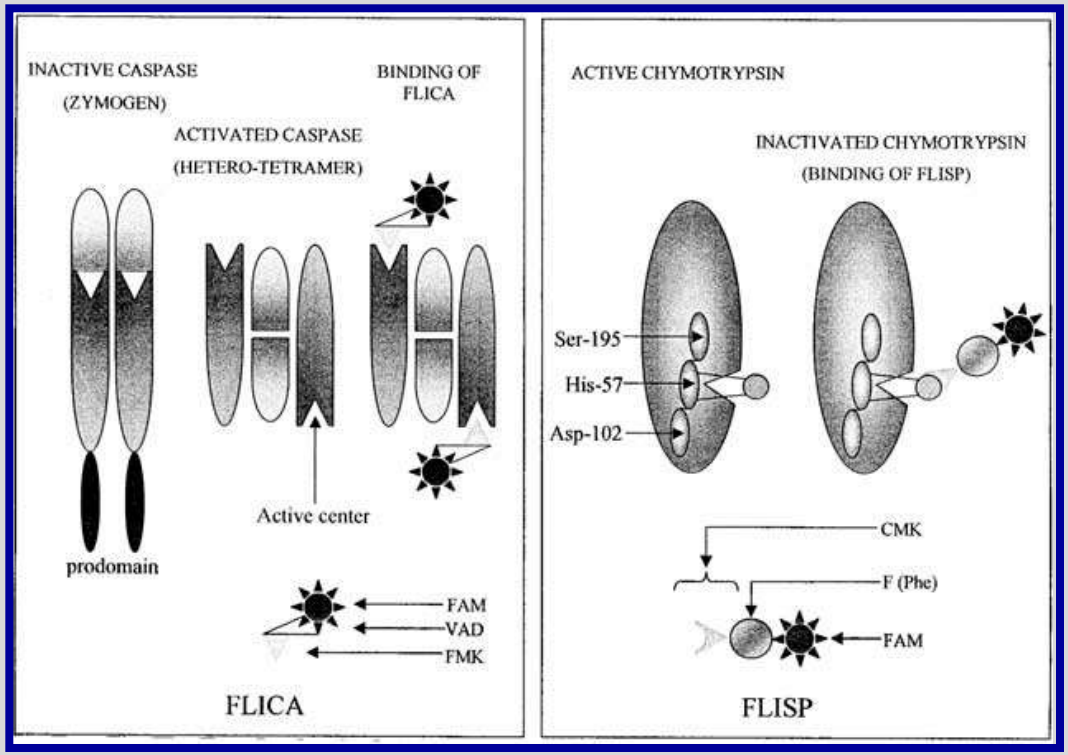
FLICA / FLISP (Microscopy, Flow Cytometry)



Grabarek J & Darzynkiewicz Z Exp Hematol 2002, 30: 982-989

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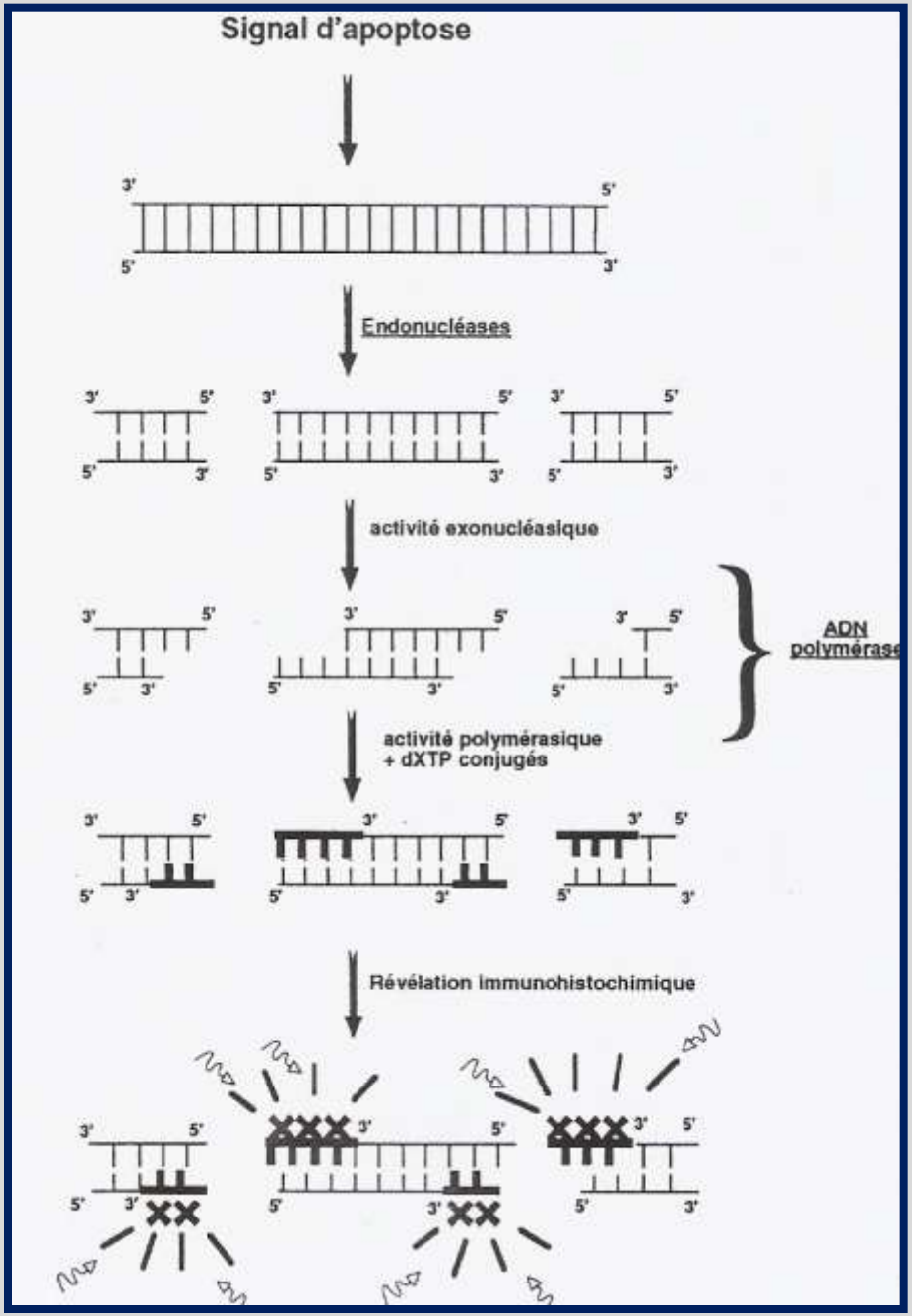
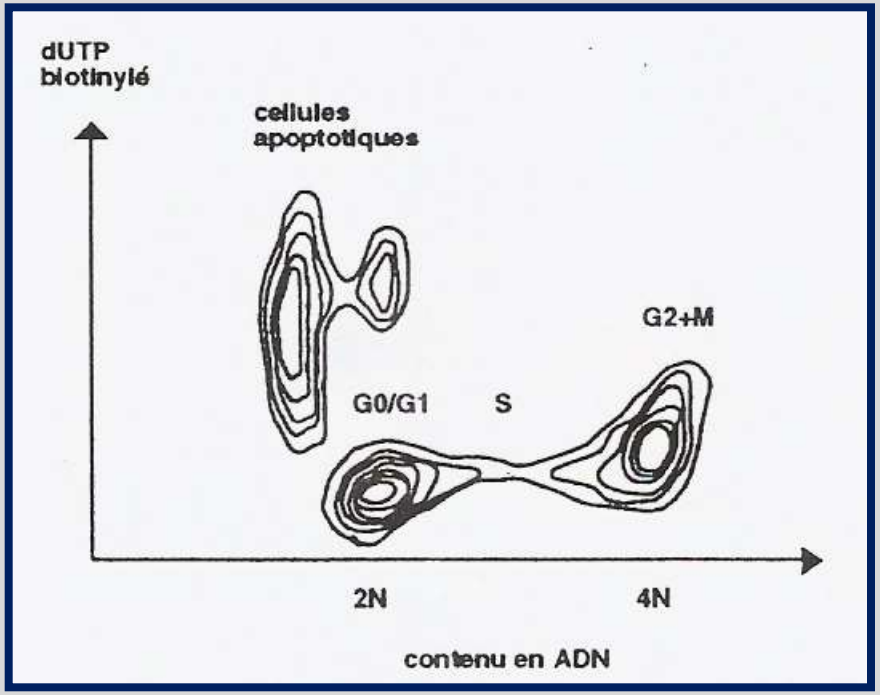


TUNEL (Flow Cytometry)



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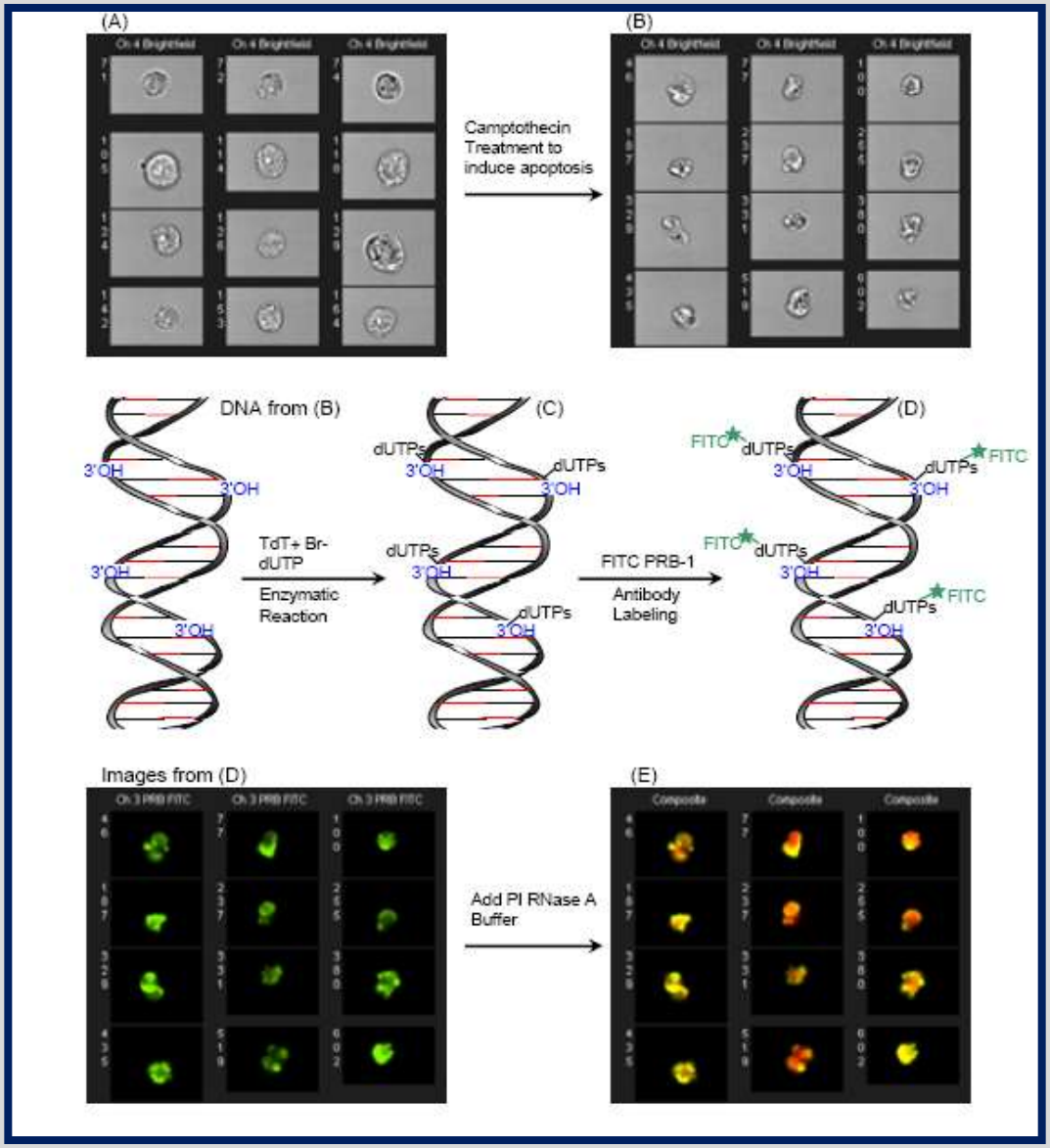
TUNEL
(Amnis technology)



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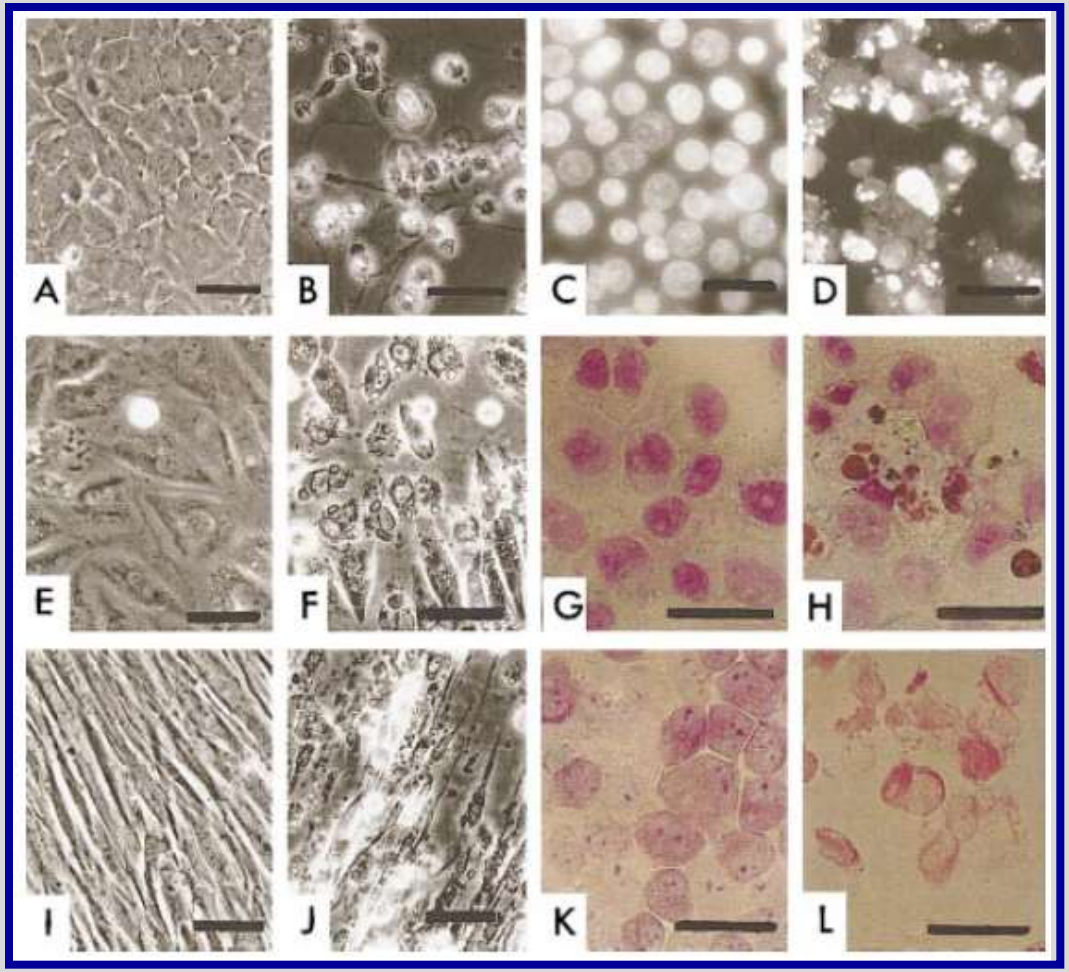
Phase contrast microscopy, Hoechst 33342, GIEMSA, TUNEL



Apoptosis = TUNEL positive cells
Necrosis = TUNEL negative cells

Lizard G et al., Arterioscler Thromb Vasc Biol 1999, 19: 1190-2000

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BIOCHEMICAL CRITERIA ASSOCIATED WITH APOPTOSIS, NECROSIS, AND NECROPTOSIS

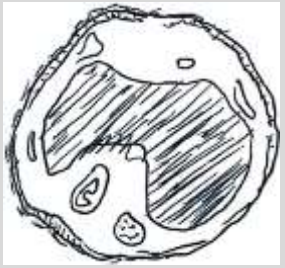
* Analysis of the DNA fragmentation pattern on agarose gel
  - Apoptosis: DNA ladder, multiple of 150-200 base pairs (internucleosomal DNA fragmentation)
  - Necrosis: no DNA ladder, smears

* Enzymatic activities (LDH)

* Electrophoresis on acrylamide gel and Western Blotting

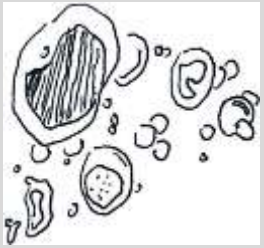
Stimuli apoptotique : génération de céramides (détection par immunofluorescence)

Cellule normale

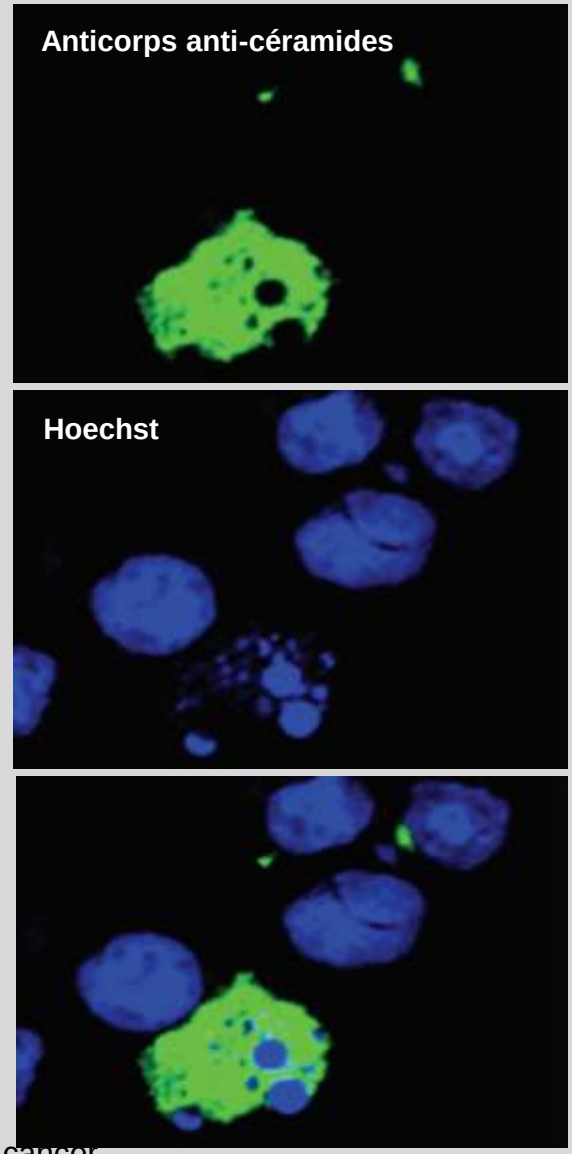


Cellule pré-apoptotique

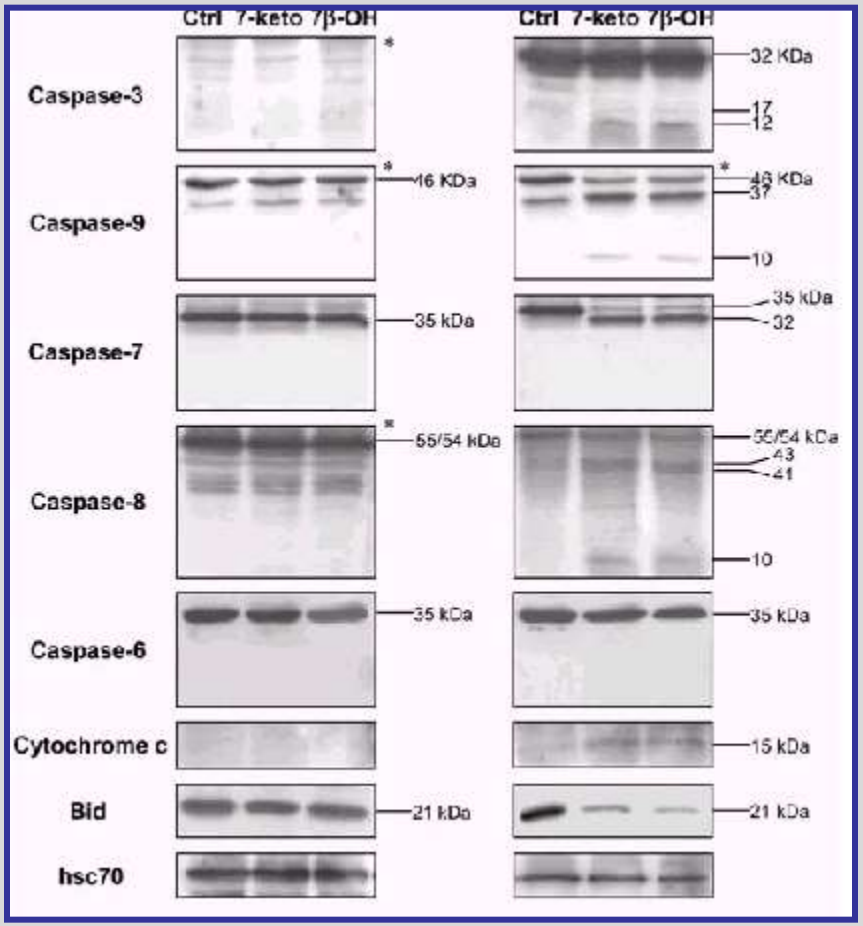
Cellule apoptotique

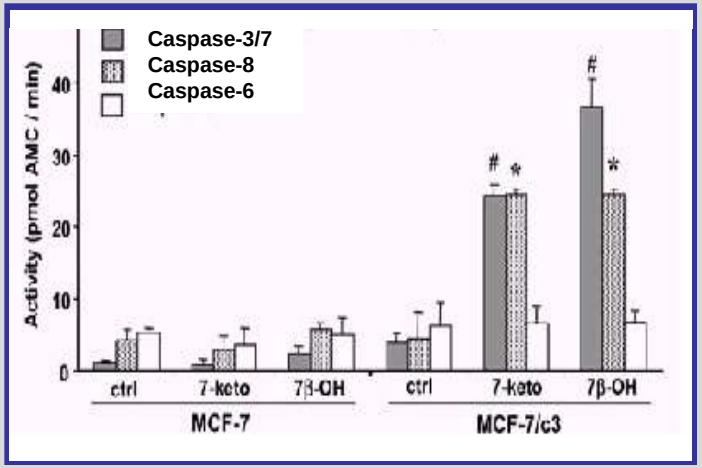


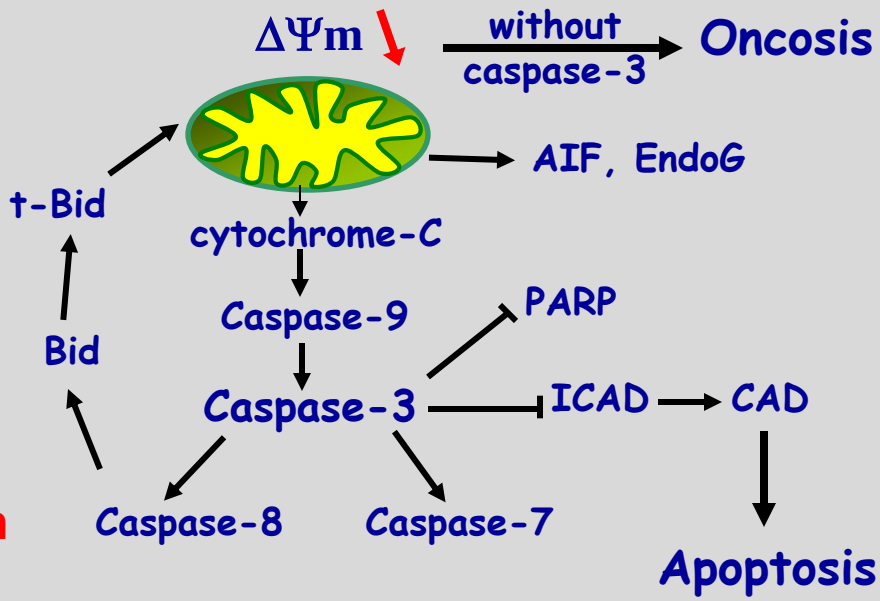
Expression de céramides



Huang WC, Chen CL, Lin YS, Lin CF. Apoptotic sphingolipid ceramide in cancer therapy.







Necrosis/Oncosis = no caspase activation

Prunet C et al. Cell Death Differ, 2001; J Biochem Mol Toxicol, 2005

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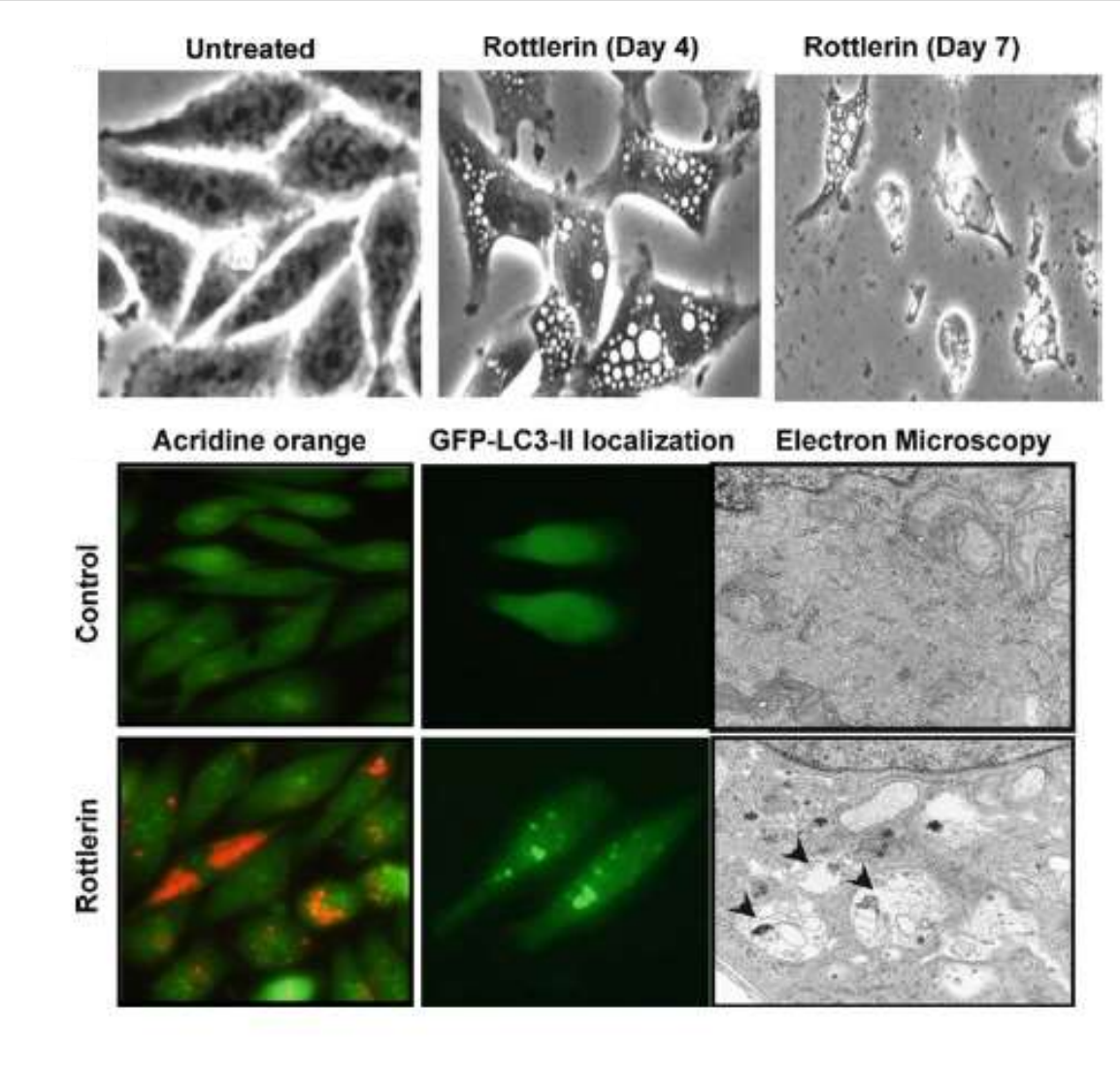
CHARACTERIZATION OF AUTOPHAGY: 'VISUAL' CRITERIA



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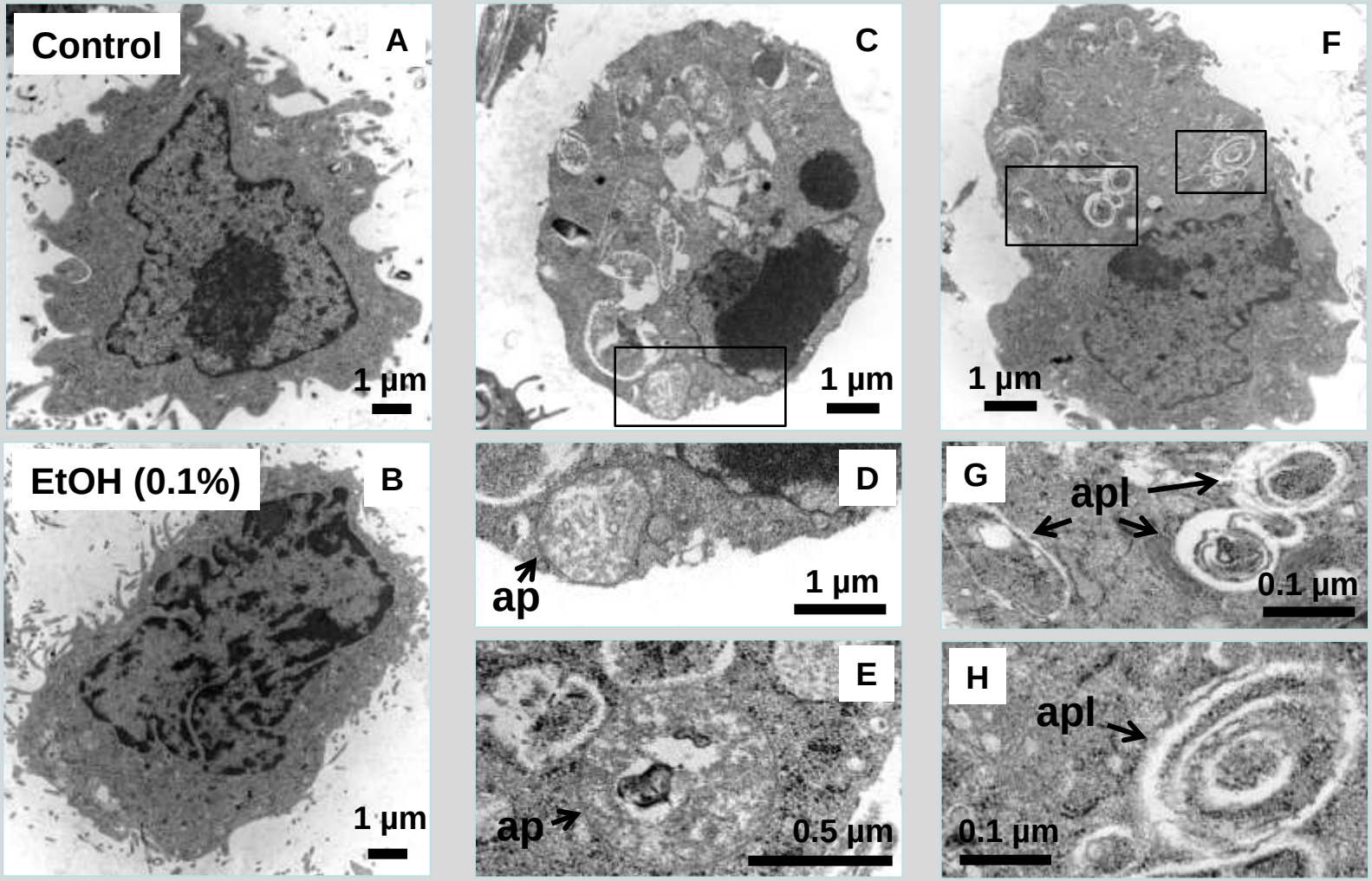
CHARACTERIZATION OF AUTOPHAGY:
ULTRASTRUCTURAL CRITERIA – Transmission Electron Microscopy

7KC (50 µM, 24 h)



ap: autophagosome; **apl**: autophagolysosome

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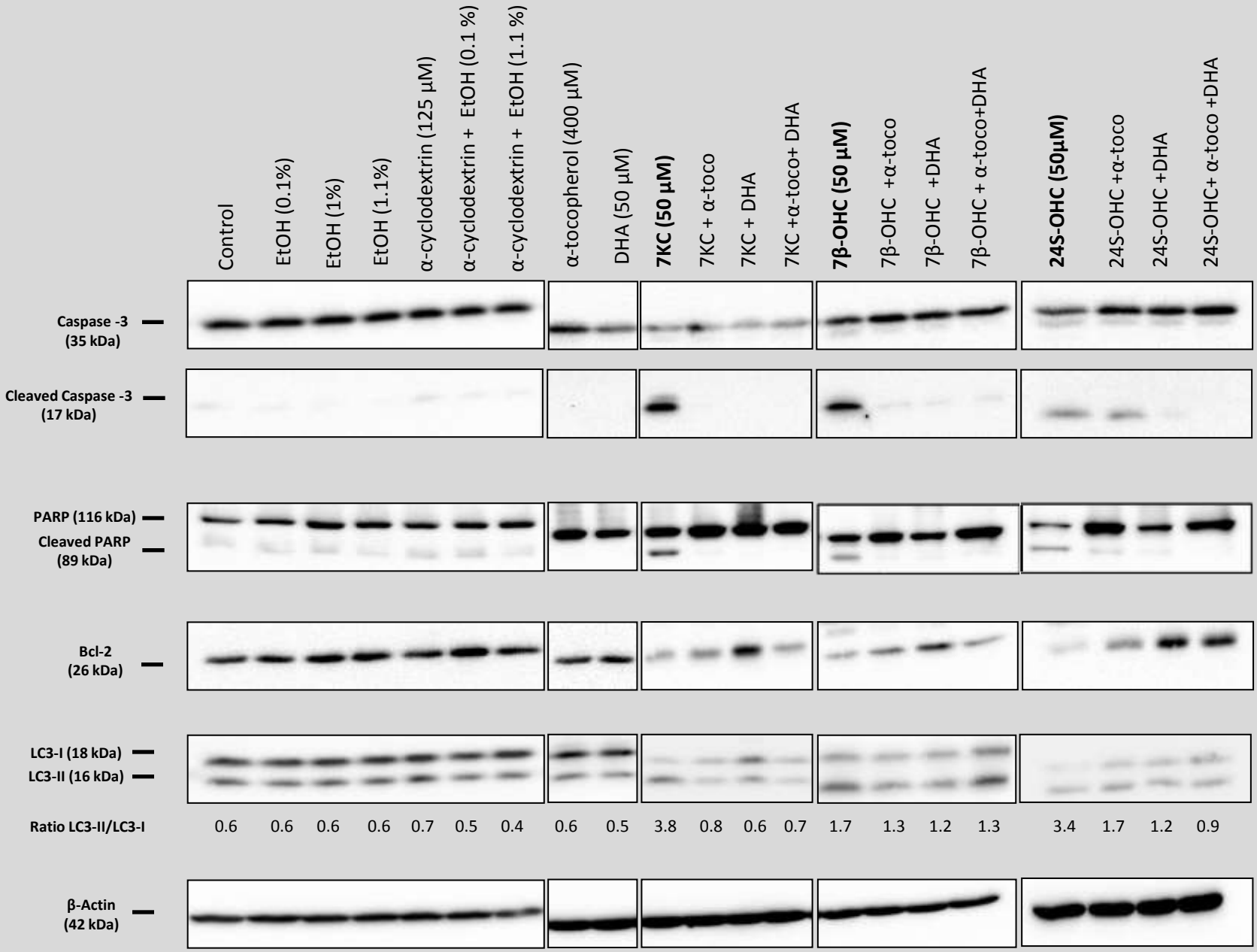
CHARACTERIZATION OF AUTOPHAGY: BIOCHEMICAL CRITERIA
Murine oligodendrocytes (158N) - autophagy and apoptosis (oxiapoptophagy)



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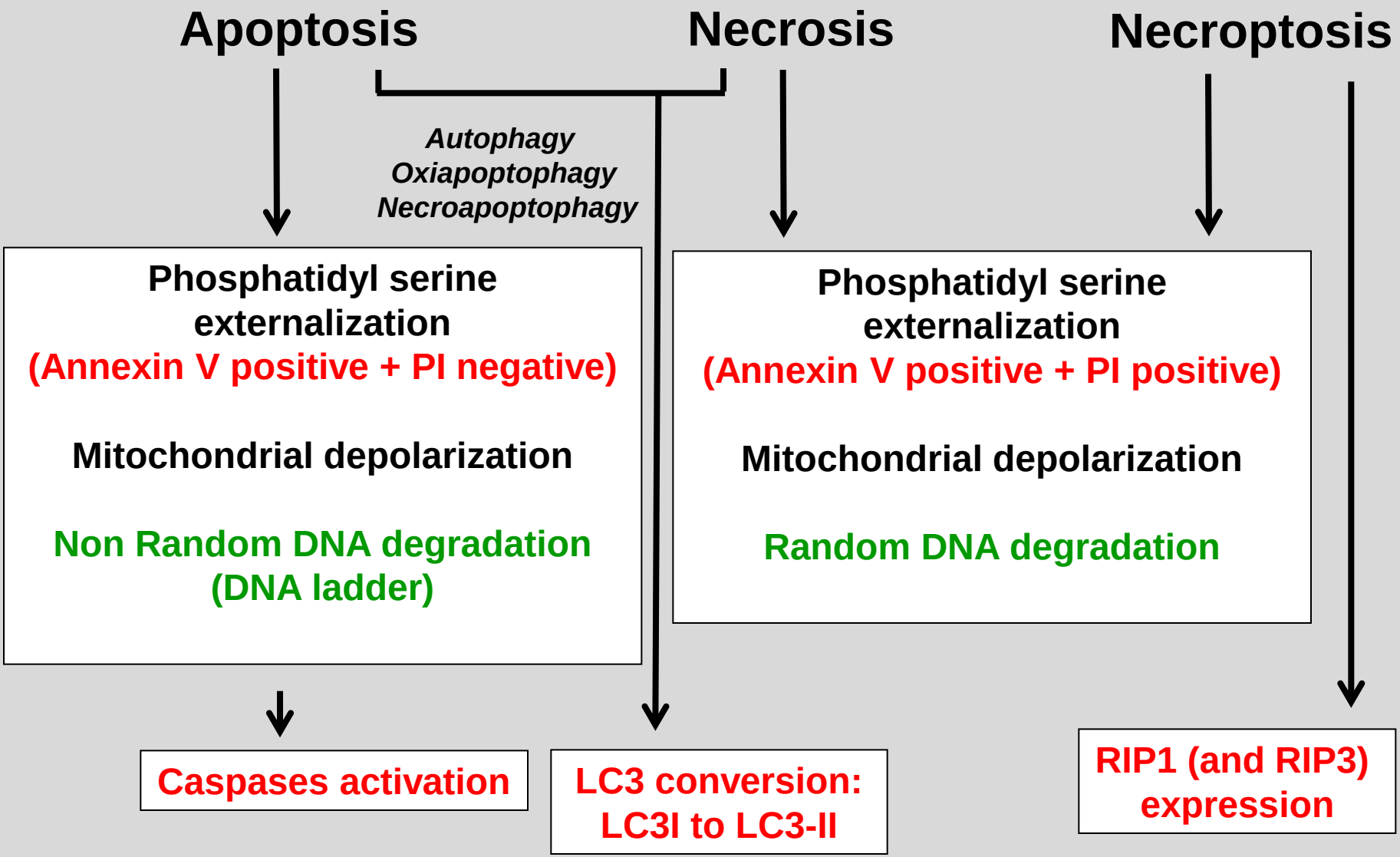

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