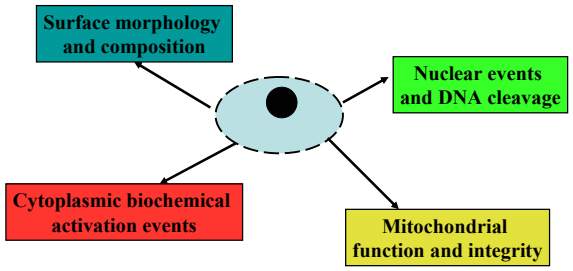
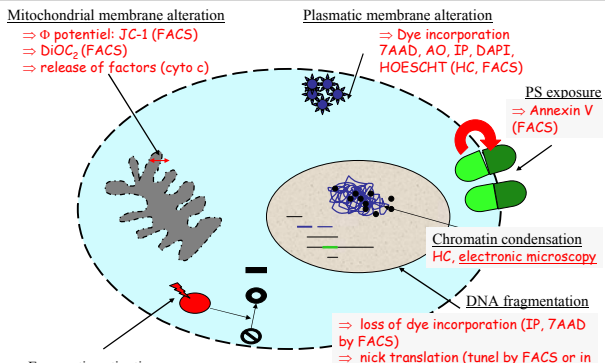
METHODS FOR THE DETECTION OF APOPTOSIS

Sylvie GARCIA

INSTITUT PASTEUR

Different levels of detection of apoptotic cells





Surface morphology and composition

Detection of cell shrinkage → EM, HF, Flow cytometry
Apoptotic bodies
Loss of impermeability → HF, Flow cytometry
Exposure of PS → Flow cytometry

Nuclear events and DNA cleavage

Chromatin condensation → EM, HF, Flow cytometry
DNA fragmentation → Electrophoresis, Flow cytometry

Morphological analyses of apoptotic cells

Electronic microscopy

Acridine orange staining under fluorescence microscopy

Zheng et al, Acta Pharmacol Sin, 2004, 25:1090.

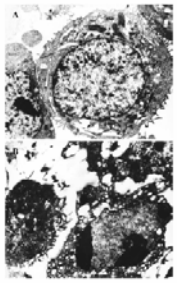


Fig 4. Characteristic morphology of AA-induced apoptosis of COLO 205 cells under electron microscope. A) control (×5000); B) Cells treated with AA 64 μmol/L for 48 h (×6000).

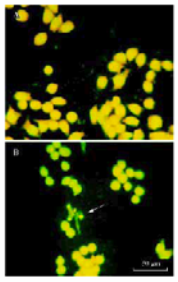


Fig 3. Morphological changes of COLO 205 cells stained with AO under fluorescence microscope. A) control; B) Cells treated with AA 64 μmol/L for 48 h. ×200.

In situ analysis of DNA fragmentation

ISNT DAPI Phase-contrast

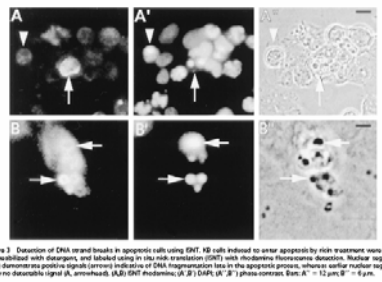
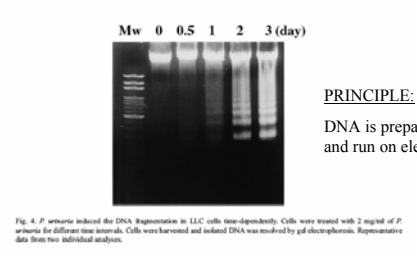


Figure 3 Detection of DNA strand-breaks in apoptotic cells using ISNT. KB cells induced to enter apoptosis by ricin treatment were fixed, permeabilized with detergent, and labeled using in situ nick-translation (ISNT) with rhodamine fluorescence detection. Nuclear segments (A,B) demonstrate positive signals (arrows) indicative of DNA fragmentation late in the apoptotic process, whereas earlier nuclear segments show no detectable signal (A, arrowhead). (A,B) ISNT rhodamine; (A',B') DAPI; (A'',B'') phase-contrast. Bars: A'' = 12 μm; B'' = 6 μm.

Willingham MC, 1999, J Histo Cyto, 1999, 47:1101

Internucleosomal DNA degradation



PRINCIPLE:

DNA is prepared from cells and run on electrophoresis gel

Huang ST, Life Science, 2003, 72:1705.

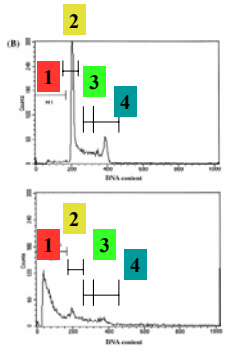
Hypodiploid DNA content measured by PI incorporation

1 Hypodiploid cells

2 Cells in G0-G1

3 Cells in S

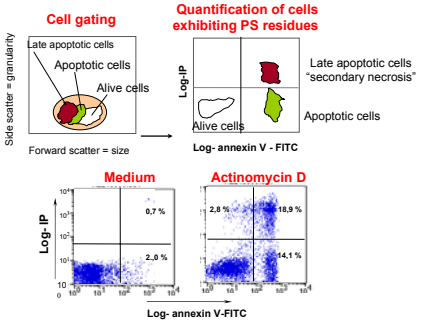
4 Cells in G2/M



PRINCIPLE:

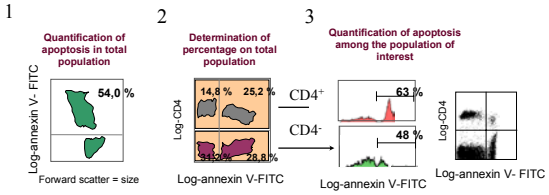
Incorporation of propidium iodide after plasma and nuclear membrane permeabilization - Stain DNA

Quantification of PS residue exposure on apoptotic cells



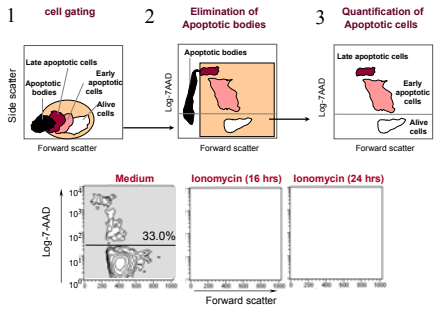
From H. Lecoeur, Institut Pasteur

Quantification of PS residue exposure on defined subsets of apoptotic cells



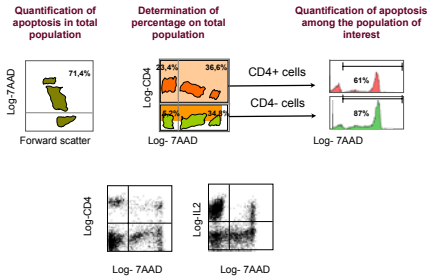
From H. Lecoeur, Institut Pasteur

Apoptosis quantification by membrane permeability using 7-amino actinomycin D (7-AAD) and size



From H. Lecoeur, Institut Pasteur

Apoptosis quantification in defined population using 7-amino actinomycin D (7-AAD) and surface/intracellular marker



From H. Lecoeur, Institut Pasteur

Quantification of DNA fragmentation by ISNT or TUNEL assay using flow cytometry

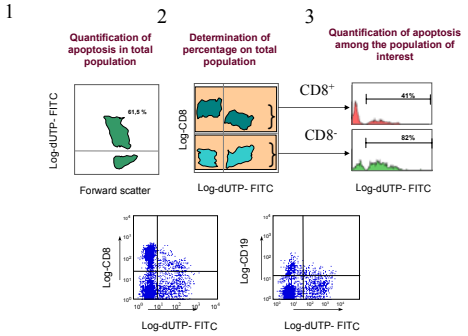
PRINCIPLE:

Detection of DNA fragmentation by addition of labeled nucleotides to 3'-ends of single-stranded DNA generated using in situ Nick-translation (ISNT) or terminal transferase (TUNEL)

ISNT

TUNEL

Quantification of DNA fragmentation by ISNT assay using flow cytometry



From H. Lecoeur, Institut Pasteur

Mitochondrial function and integrity

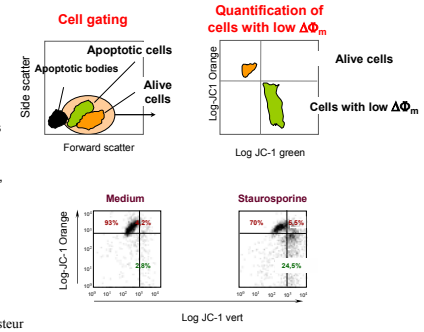
Φ potential	→	Flow cytometry
Cytochrome c release	→	HF, Flow cytometry
Bcl-2 family protein	→	Flow cytometry, WB, RT-PCR

Cytoplasmic biochemical activation events

Caspases	→	WB, Flow cytometry
PARP	→	WB, Flow cytometry

Measurement of mitochondria membrane potential loss

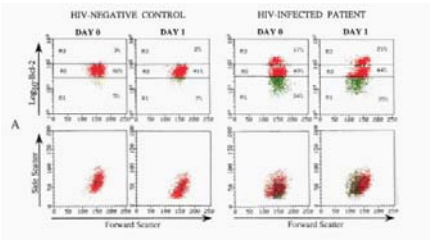
PRINCIPLE:
In non-apoptotic cells, JC-1 accumulates as aggregates in the mitochondria, which stain in red; whereas in apoptotic and necrotic cells, monomeric JC-1 stains the cytosol in green.



From H. Lecoeur, Institut Pasteur

Bcl-2 family protein analysis

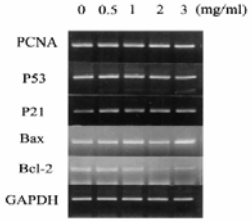
Flow cytometry



Boudet et al, JI, 1996, 156:2822

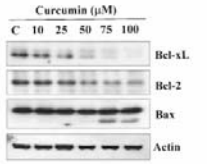
Bcl-2 family protein analysis

RT-PCR



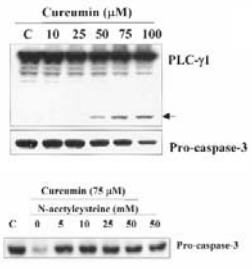
Huang ST, Life Science, 2003, 72:1705.

WB



Woo JH, Carcinogenesis, 2003, 24:1199.

Caspase activity detection by Western blot



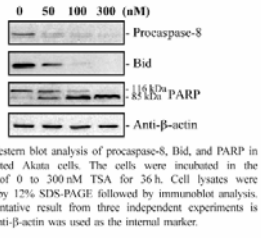


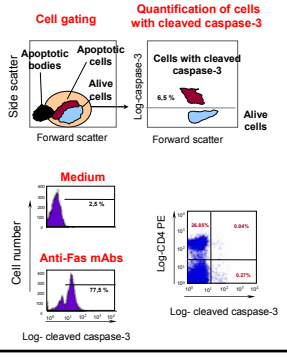
Fig. 2. Western blot analysis of procaspase-8, Bid, and PARP in EBV-infected Akata cells. The cells were incubated in the presence of 0 to 300 nM TSA for 36 h. Cell lysates were analyzed by 12% SDS-PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown. Anti-β-actin was used as the internal marker.

Woo JH, Carcinogenesis, 2003, 24:1199.

Kook SH, J Biochem & Mol Bio, 2005, 38:755

Caspase activity detection by FACS

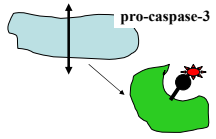
Flow cytometry

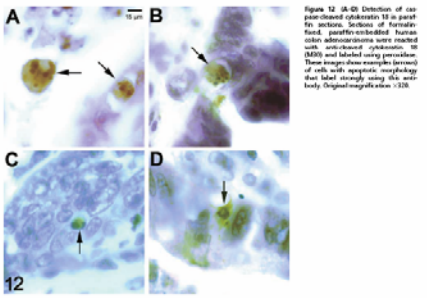


PRINCIPLE:

Use of caspase substrate decoy labeled with a dye and which covalently bind to cleaved caspases.

Specific substrates are available for each (almost) caspases.





Barret KL et al, J Histo & Cyto, 2001, 49:821

ELISA: cytochrome C release (1)

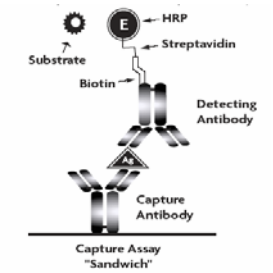


Figure 1: Sandwich ELISA schematic.

1) FunctionELISA Cytochrome c measures the amount of Cytochrome c present in cellular lysates or fluid samples. It can detect nanogram quantities of Cytochrome c and rapidly provides quantifiable results using a standard curve that is easily generated with the included Cytochrome c control.

Active motif company

ELISA: cytochrome C release (1)

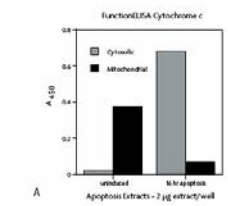
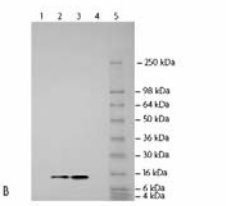


Figure 2: Location of cytochrome c in HeLa cells. HeLa cells were grown to 90-95% confluence and treated with 10 μM Actinomycin D. Cells were harvested after 16 hours of induction and mitochondrial and cytosolic extracts were isolated using the Mitochondrial Fractionation Kit (Active Motif Cat. No. 40015). Two μg of each lysate was tested using the FunctionELISA Cytochrome c Kit (Fig. 2A) and Western blot analysis using a 4-20% Tris-Glycine gel (Fig. 2B).



Lane 1: Cytosolic extract, no induction
Lane 2: Mitochondrial extract, no induction
Lane 3: Cytoplasolic extract, 16 hr post-induction
Lane 4: Mitochondrial extract, 16 hr post-induction
Lane 5: Protein standard

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Apoptosis and cell cycle by flow cytometry

- **Coupling CFSE and 7-AAD**
- **Coupling CFSE and Annexin V**

CFSE: carboxy fluorescein succimyl esterate
Binds to all cell proteins
Half dilution of CFSE intensity for each division

Conclusions (1)

- Viability alone (Trypan blue, MTT), and other techniques may not be specific for apoptosis => need to couple several techniques
- Because of engulfment by phagocytic cells, apoptotic cells may not "live" long in vivo => difficulty to detect ex-vivo apoptosis and need to "reveal" in vitro susceptibility
- Panel of techniques allowing to respond to "Who? How? Why?

Conclusions (2)

methods	Advantages	Limits
Flow cytometry	Single cell analysis Multiparametric analysis	Lack of mAbs
In situ analysis	Single cell analysis Some parameters at the same time Accurate to measure apoptosis	Lack of mAbs
WB	Biochemical modifications	Not single cell analysis
RT-PCR	No limits of tools	Pb with post-transcriptional modifications

Remarks:

- All apoptotic pathway do not lead to "scale" or fragmented DNA
- Some apoptotic pathways are caspase-independent
- Some are mitochondria-independent…..

References

- J.E. Ploski and PD Aplan, **Characterization of DNA fragmentation events caused by genotoxic and non-genotoxic agents**, *Mutation Research* (2001), 473:169-180.
- Katherine L. Barrett et al, **Advances in Cytochemical Methods for Detection of Apoptosis,** *The Journal of Histochemistry & Cytochemistry* (2001), 49:821-832.
- Lecoeur H et al, **Strategies to phenotype apoptotic peripheral lymphocytes comparing 7-AAD, AnnexinV and ISNT assays**. *J Immunol methods* (1997), 209:111-123.
- Lecoeur H et al, **Multiparametric flow cytometric analysis of biochemical and functional events associated with apoptosis and oncosis using 7-aminoactinomycin D assay**. *J Immunol methods* (2002), 265:81-86.