Biochemical techniques for detection of cell death

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Features of Apoptosis and Necrosis

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological or Pathological	Pathological (injury)
Occurrence	Single cells	Groups of cells
Reversibility	Limited	Limited
Cellular level		
Cell shape	Shrinkage and formation of apoptotic bodies	Swelling and later disintegration
Adhesion between cells	Lost (early)	Lost (late)
Phagocytosis by other cells	Present	Absent
Exudative inflammation	Absent	Present
Cellular organelles		
Membranes	Blebbing	Blebbing prior to lysis
Cytoplasm	Late-stage swelling	Very early swelling
Mitochondrial permeability transition	Present	Present
Nucleus	Convolution of nuclear outline and breakdown (karyorrhexis)	Disappearance (karyolysis)

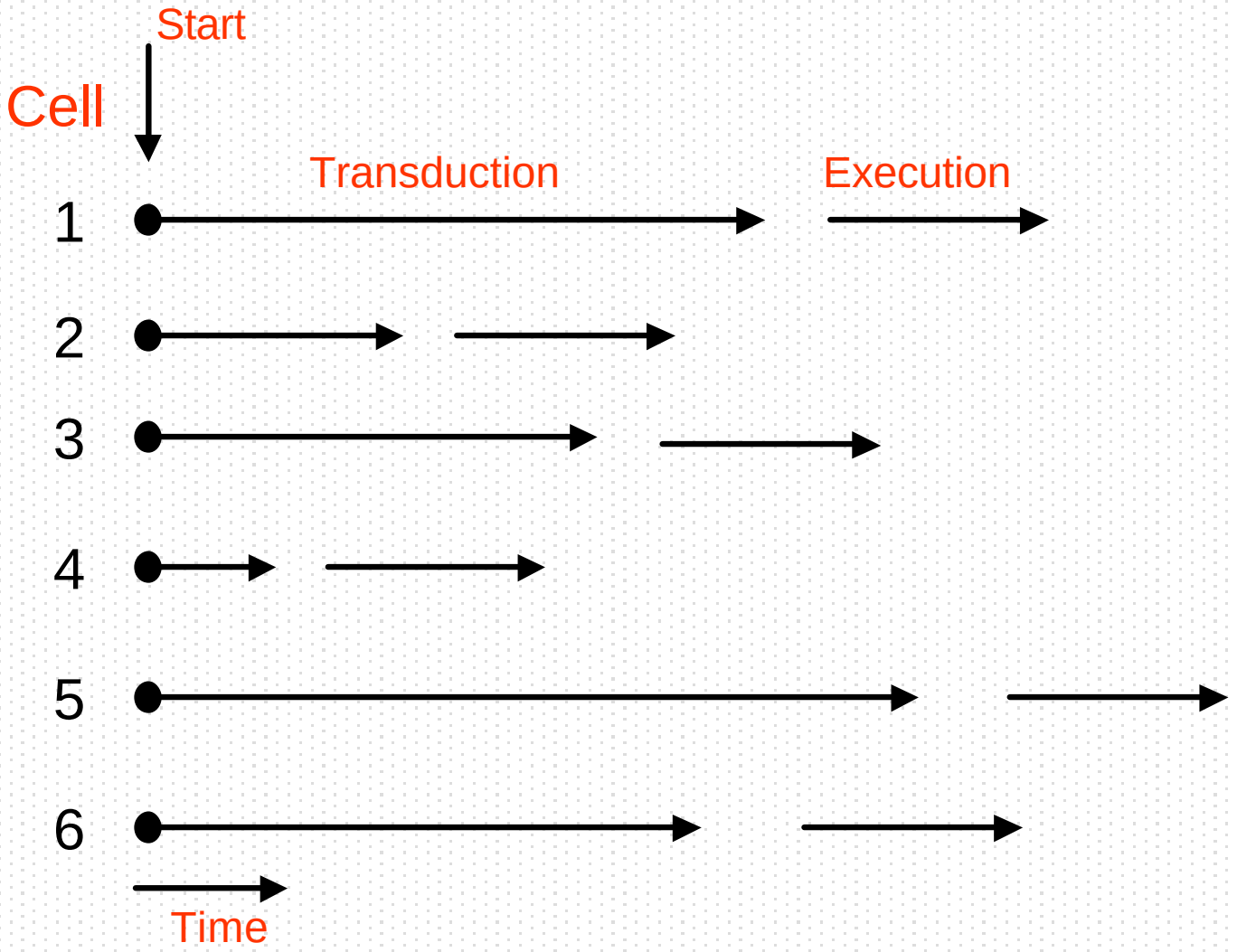
Features of Apoptosis and Necrosis (cont.)

Biochemical level

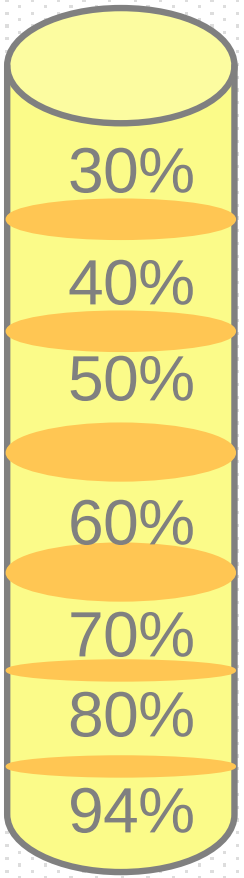
Gene activation	Present (?)	Absent (?)
Requirement for protein synthesis	Present (?)	Absent (?)
Lysosomal enzyme release	Present	Present
Activation of non-lysosomal enzymes	Present	Present
Activation of caspases	Present	Absent
Cleavage of specific proteins	Present	?
Changes in cytoskeleton	Present	Present
Level of ATP required	High	Low
Bcl-2 protection	Present	Present
Nuclear chromatin	Compactization in uniformly dense masses	Clumping not sharply defined
DNA breakdown	HMW and internucleosomal	Randomized
RNA degradation	Present	?
Phosphatidylserine exposure	Present	Present

(?)- This feature is not a universal event or there are conflicting reports.

Measuring Apoptosis in Cell Culture: the inherent problem of asynchrony



Percoll fractionation of apoptotic lymphocytes



	Viability (% of total)	% Nuclear fragmentation
Necrotic	3.4	6.4
Necrotic	4.9	14.9
Viable	92.0	1.9
Viable	91.6	1.6
Pre-apoptotic	89.4	22.6
Apoptotic	65.2	78.5

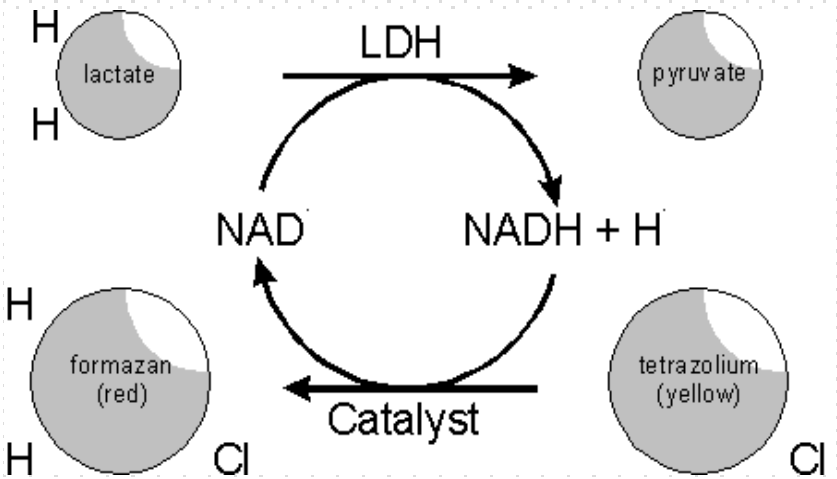
- **Are you studying apoptosis or necrosis?**
  - test for general cytotoxicity (LDH, MTT, etc)

- **Are you studying cell population, individual cells or clinical material (sections, biopsies, etc)?**

Analysis of plasma membrane changes

1. Lactate dehydrogenase (LDH) activity (transformation of tetrazolium, yellow, to formazan, red)

- LDH, a stable cytosolic enzyme, is released upon cell lysis and can therefore be used as a marker for cell death
- Release can be measured spectrophotometrically (max abs. at about 500 nm)
- The amount of enzyme activity correlates to the number of damaged cells (both apoptotic and necrotic)



Analysis of plasma membrane changes

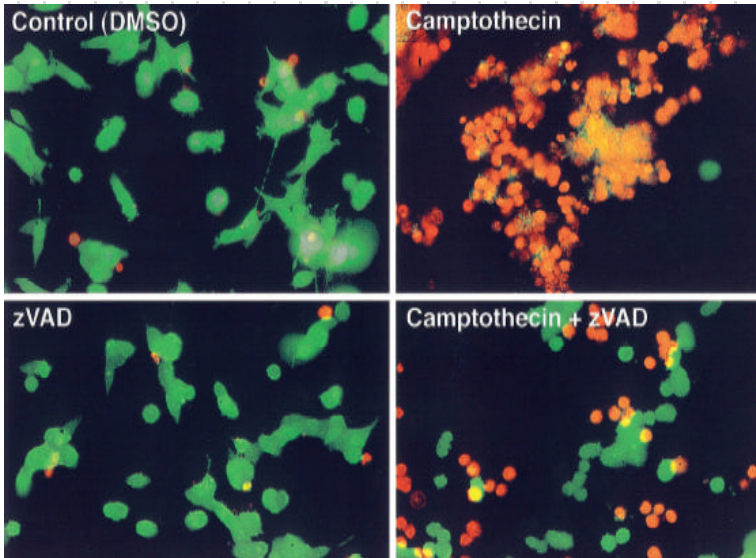
2. Live-dead cell assay (staining with calcein AM and ethidium homodimer-1)

- Calcein acetoxymethyl ester is a membrane-permeant esterase substrate, which easily stains living cells. Dead cells cannot convert Calcein-AM to its fluorescent substrate

- Ethidium homodimer-1 (EH-1) enters dead cells through deteriorating cellular membranes and binds DNA and RNA

Human neuroblastoma cells treated with camptothecin.
Cells were viewed with fluorescein (emission at 530nm) and rhodamine (emission at 590nm) optics.

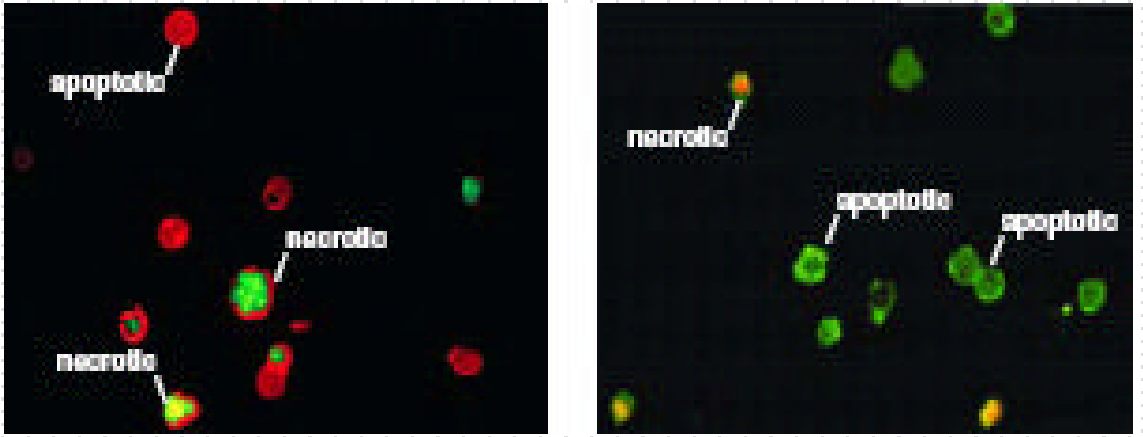
Calcein-positive cells (green fluorescence) indicate healthy cells with an intact membrane, whereas ethidium homodimer-1-positive cells (orange fluorescence) represent dead or severely damaged cells



Analysis of plasma membrane changes

3. Staining with Annexin V, followed by (A) fluorescence or confocal microscopy, or (B) FACS (co-staining with propidium iodide)

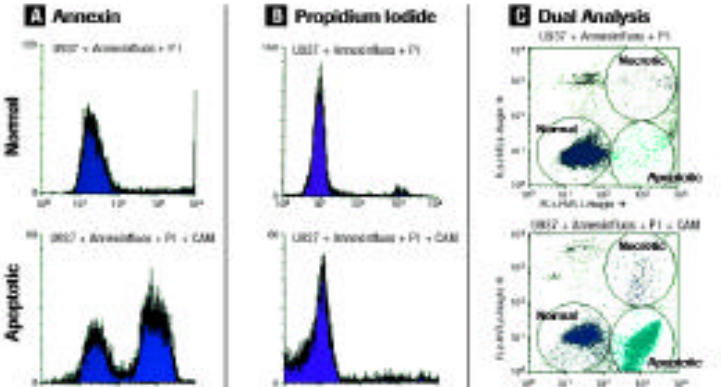
A



Annexin V-Alexa 568 (red)
BOBO-1 (green)

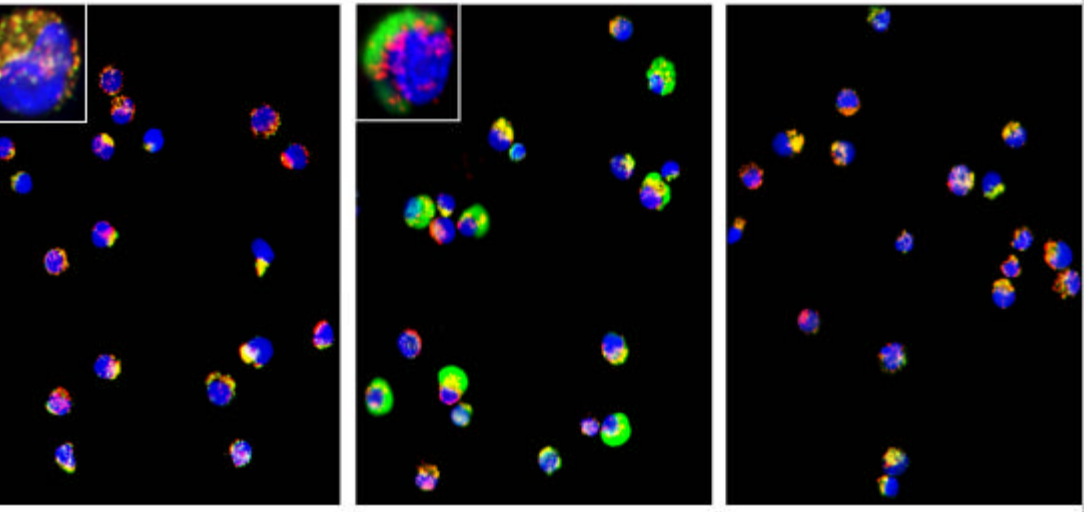
Annexin V-Fluo (green)
PI (red)

B



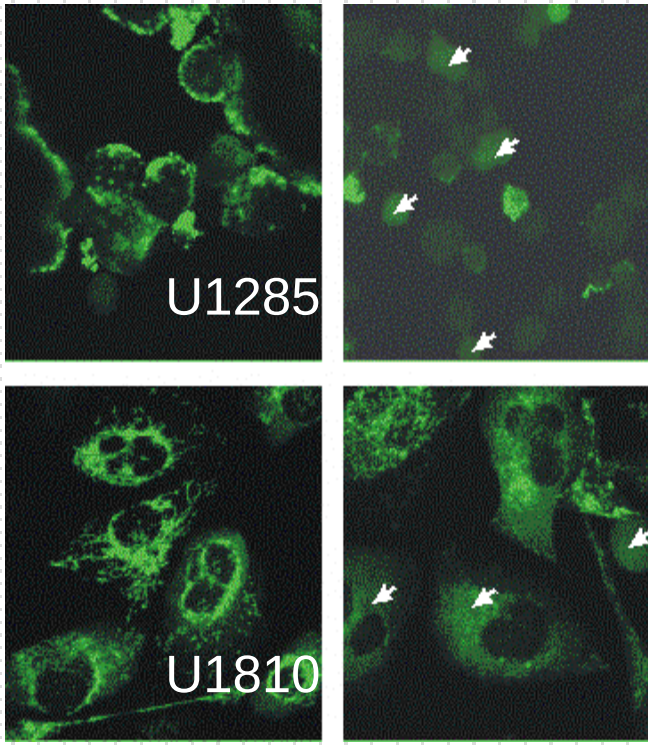
Analysis of mitochondrial integrity

1. Staining with MitoTrackerRed and anti-cytochrome *c* Abs



NBM RARS RARS+G-CSF

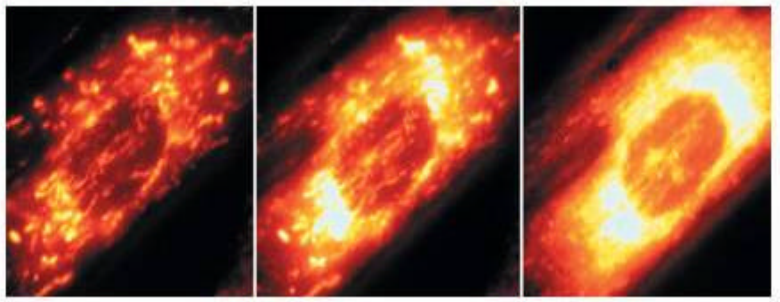
Control γ-irradiation



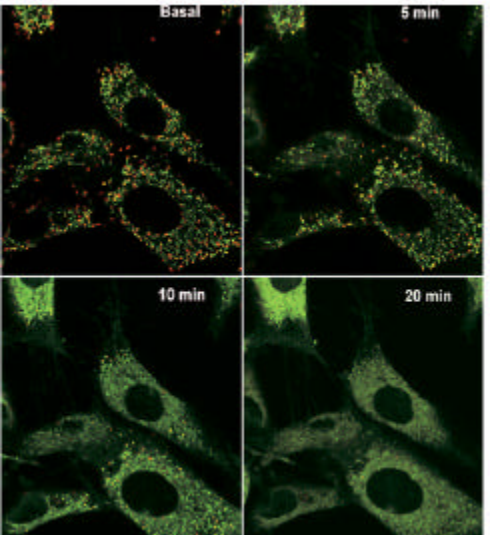
Analysis of mitochondrial integrity

2. Staining with Rhodamine 123 or JC-1 or TMRE, followed by confocal microscopy analysis

Staining of rat cortical astrocytes by rhodamine 123



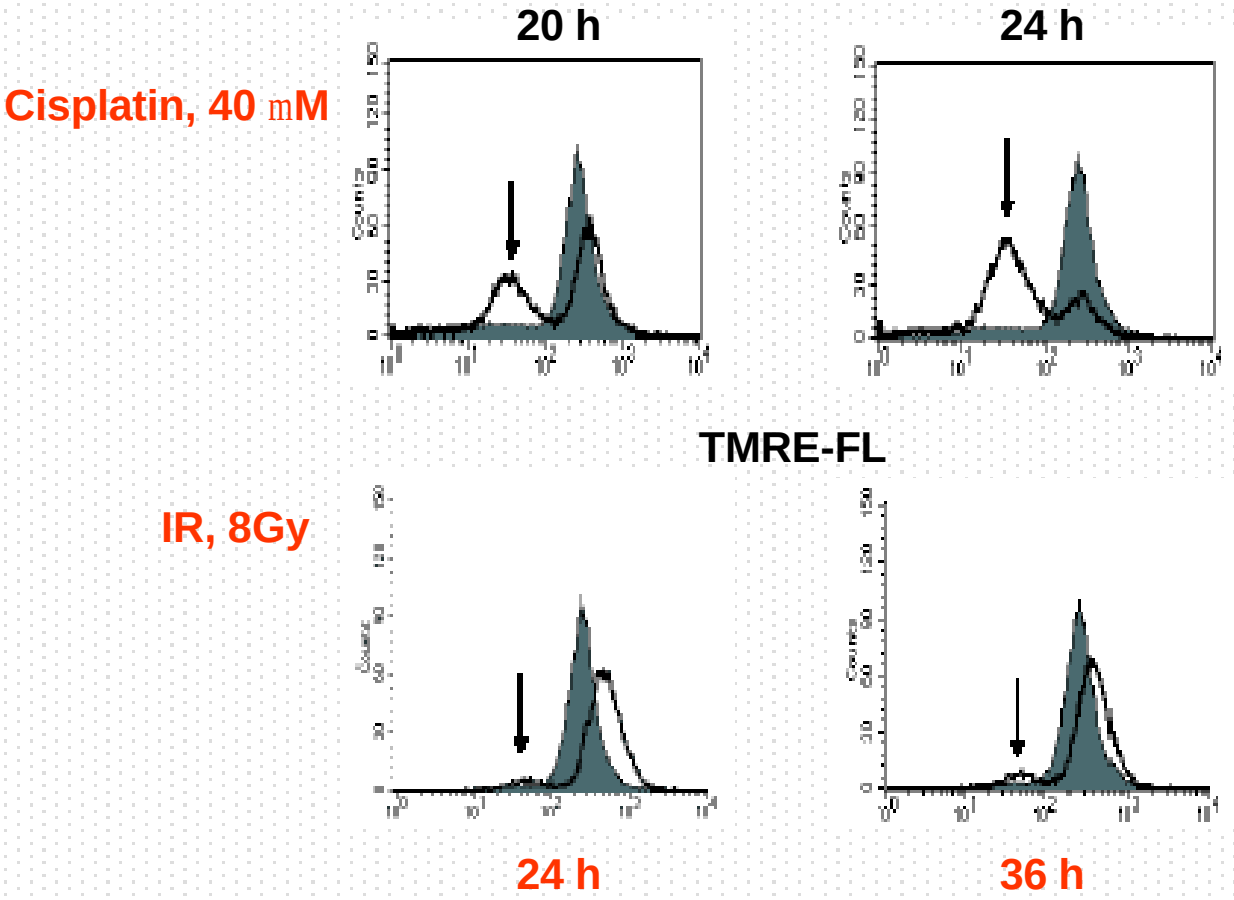
Potential-dependent accumulation of the cationic dye in mitochondria results in a relatively weak fluorescence signal due to self-quenching (left panel).
Dissipation of the mitochondrial membrane potential by the uncoupler FCCP is marked by increasing fluorescence (middle panel) and subsequent redistribution of the dye throughout the cell (right panel)



NIH 3T3 fibroblasts stained with JC-1, showing the progressive loss of red J-aggregate fluorescence and cytoplasmic diffusion of green monomer fluorescence following exposure to hydrogen peroxide

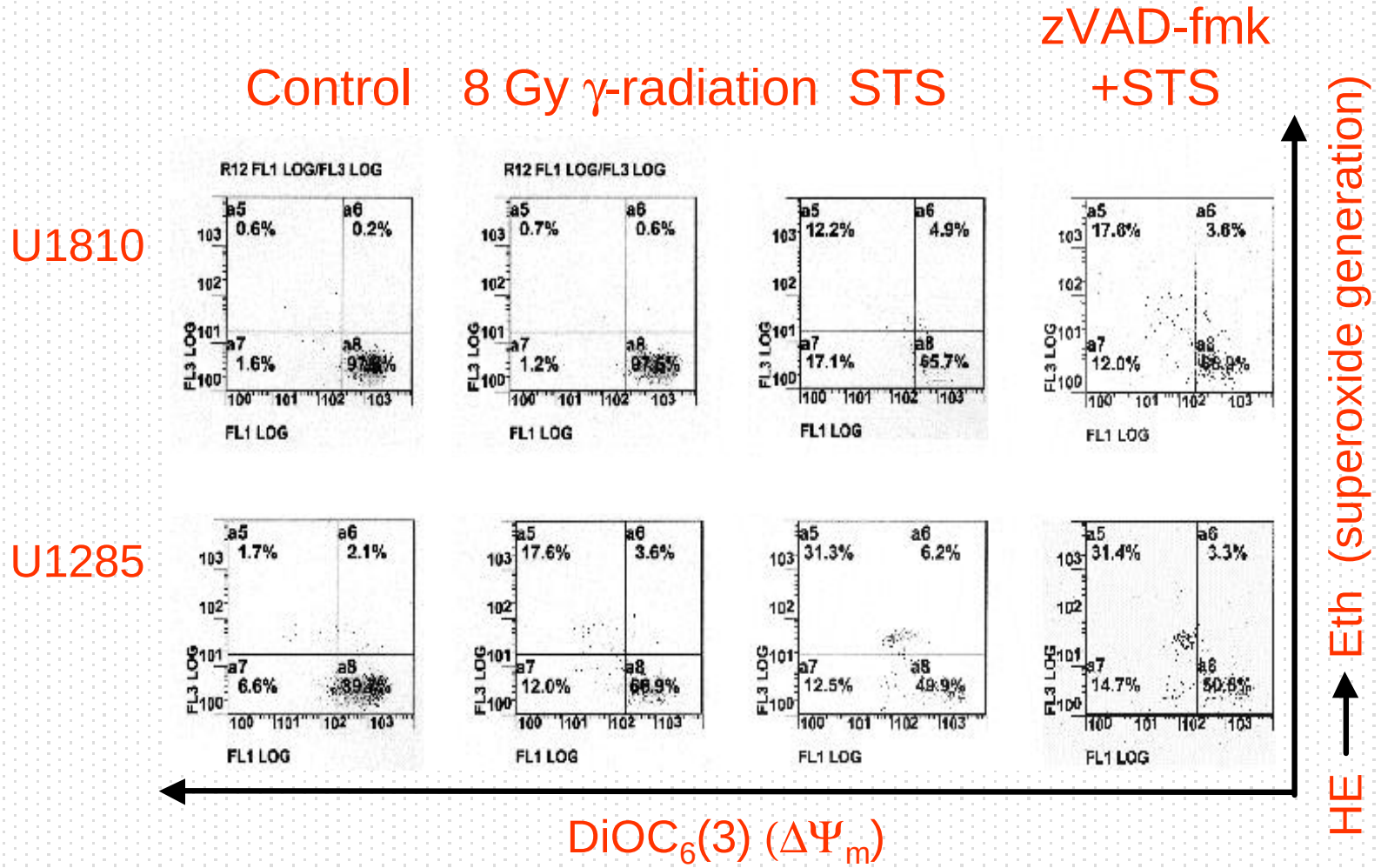
Analysis of mitochondrial integrity

3. Staining with Rhodamine 123 or JC-1 or TMRE, followed by FACS analysis



Analysis of mitochondrial integrity

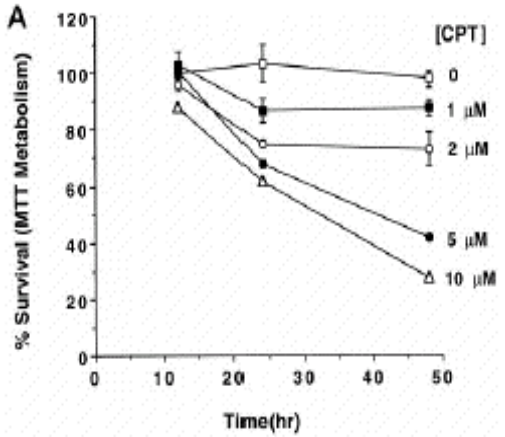
4. Measurement of the disruption of mitochondrial membrane potential and superoxide generation



Analysis of mitochondrial integrity

5. **MTT assay** to measure changes in mitochondrial membrane potential (conversion of the **yellow**, water-soluble, tetrazolium MTT to the **blue**, water insoluble formazan) (measured with an optical density reader)

- **Conversion is catalyzed by cellular mitochondrial dehydrogenases, which is proportional to the number of surviving cells**



6. **Staining with anti-Apo 2.7 Abs followed by FACS**

Analysis of metabolic changes

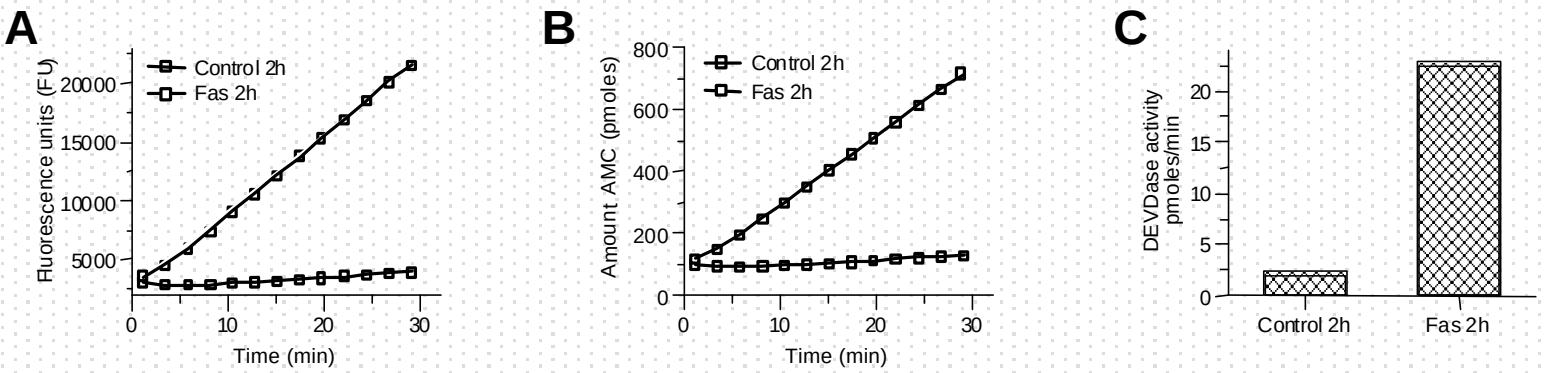
1. Intracellular acidification (SNARF-1, acetoxymethyl ester, shifts the color from **red** to **yellow**)

2. Changes in intracellular Ca^{2+} concentration (fura-2, fluo-3 or fluo-4)

3. Changes in cellular oxidative activity:
   a) Dihydroethidine **(mitochondria)**
   b) 5-(and-6-)-carboxy-2' 7'-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA) or RedoxSensorRed **(cytosol)**
   c) cis-parinaric acid **(lipids)**
   d) Monochlorobimane, or FluoReporter **(glutation depletion)**

Analysis of protease activation

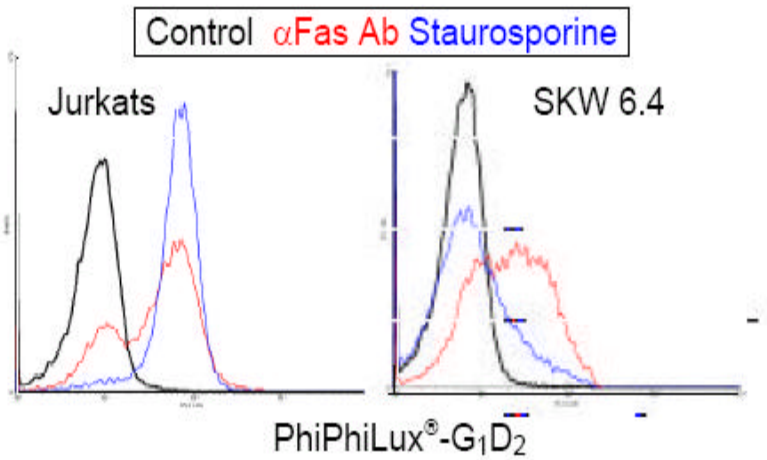
1. Cleavage of fluorogenic or chromogenic substrates, which are specific for different proteases

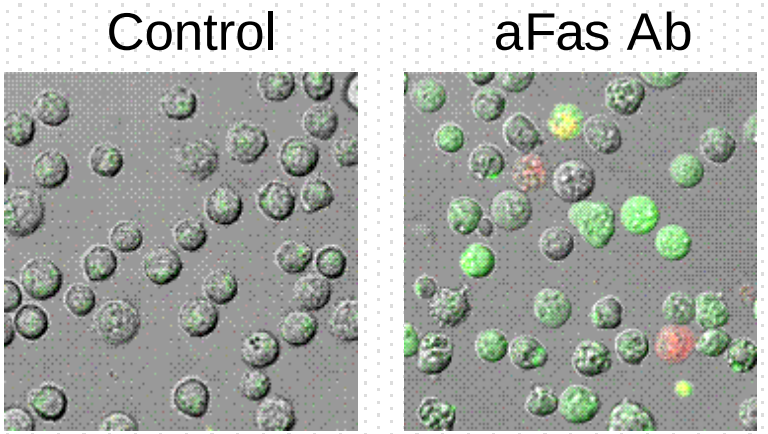


Fluoroscan or spectrophotometer

Analysis of protease activation

2. FACS or fluorescent microscopy analysis of cleavage of fluorogenic substrates (PhiPhiLux)

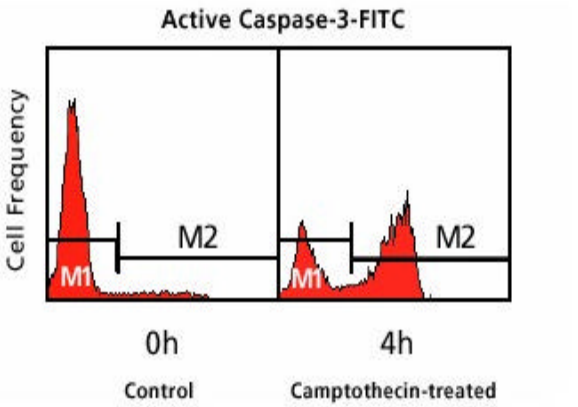




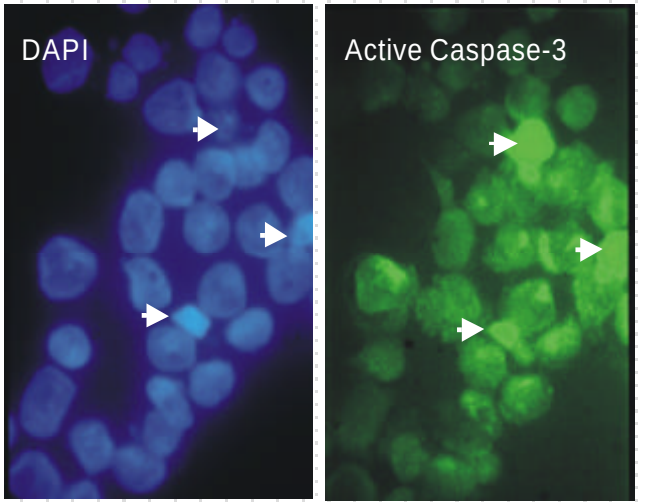
Analysis of protease activation

3. FACS (A) or fluorescent (B) or confocal microscopy analysis of active caspases using mAbs

A



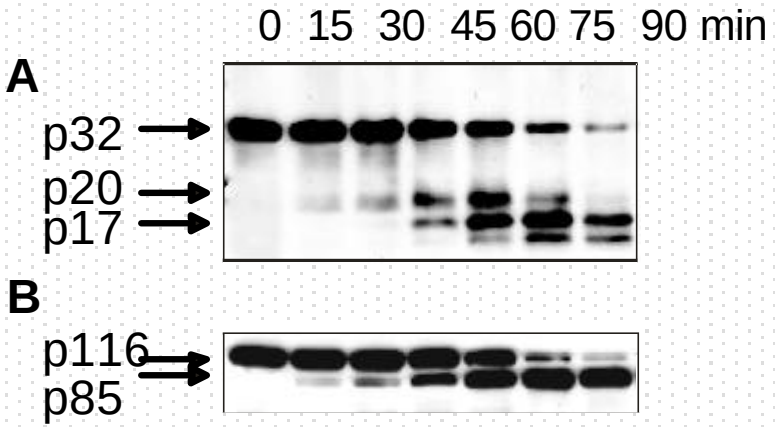
B



Analysis of protease activation

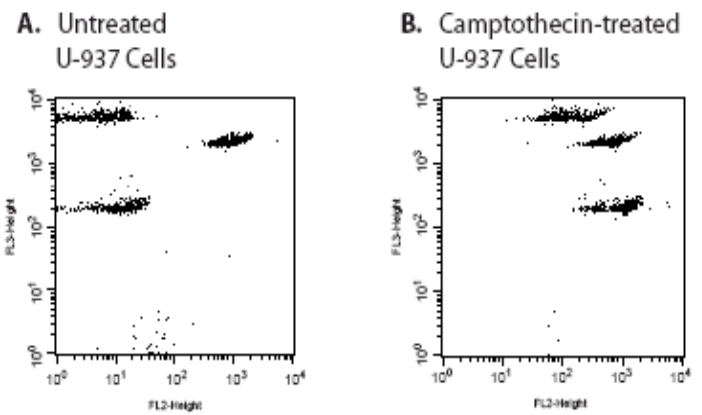
4. Western-blot or FACS analysis of targeted proteins

Caspase-3 processing (A) and PARP cleavage (B) analysis by immunoblotting



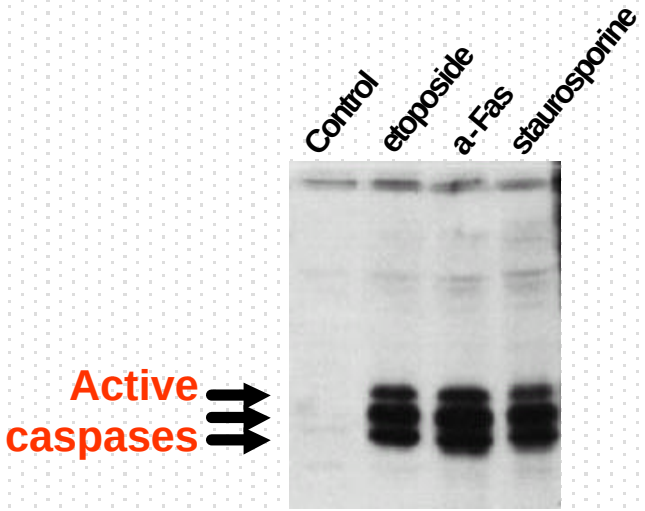
Cytometric Bead Array

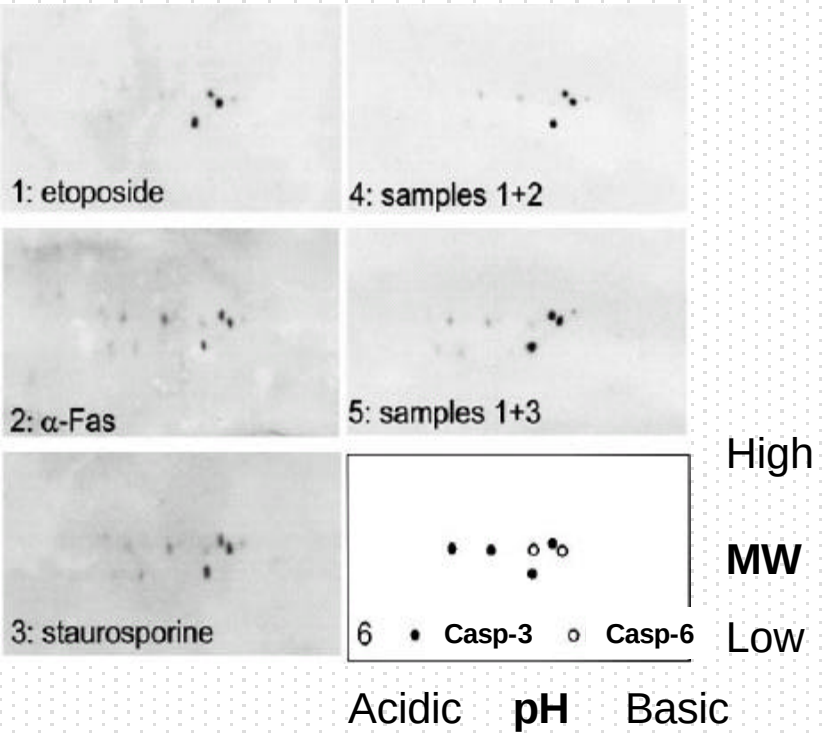
Human apoptosis kit for detection
of cleaved PARP, active caspase-3 and Bcl-2



Analysis of protease activation

5. Affinity labeling of active caspases with biotin-labeled tetrapeptides

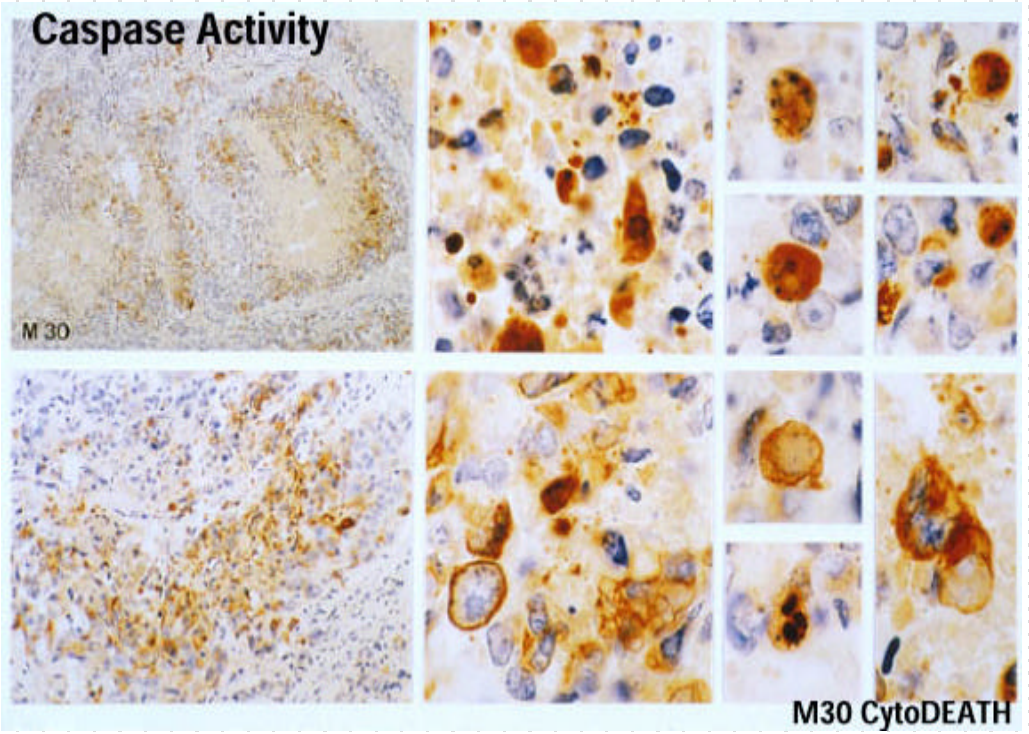




Caspases were labeled with biotin-YVAD-amk and visualized by 1D or 2D affinity blots

Analysis of protease activation

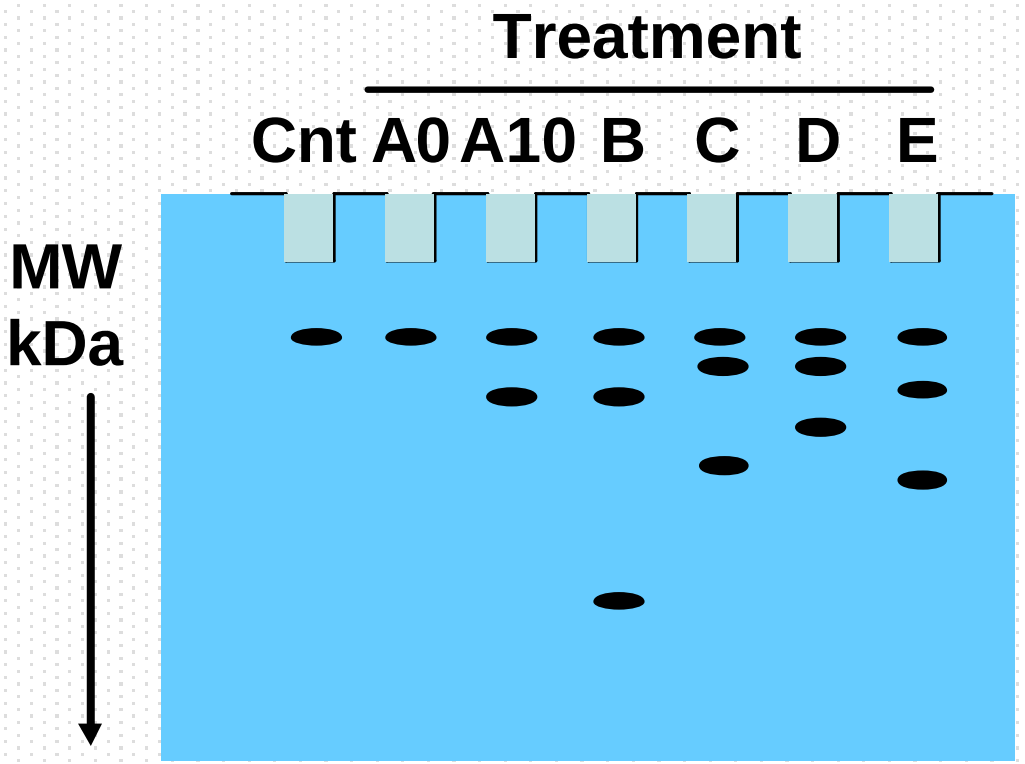
6. Caspase activity measured by staining with Abs against cleavage product of keratin



Spontaneous apoptosis in tissues from mouse and rat.
Staining with M30-biotin Abs, counterstaining with Hematoxilin

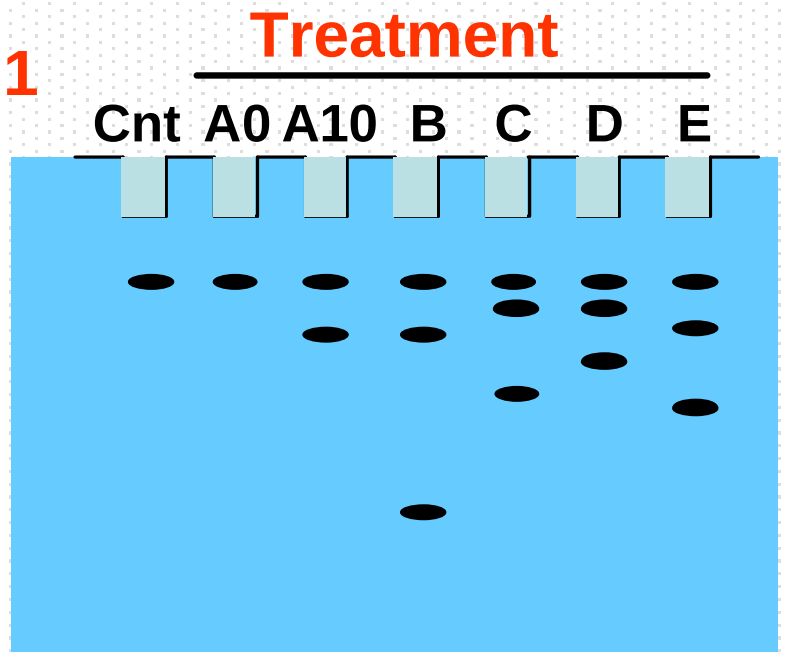
Analysis of protease activation

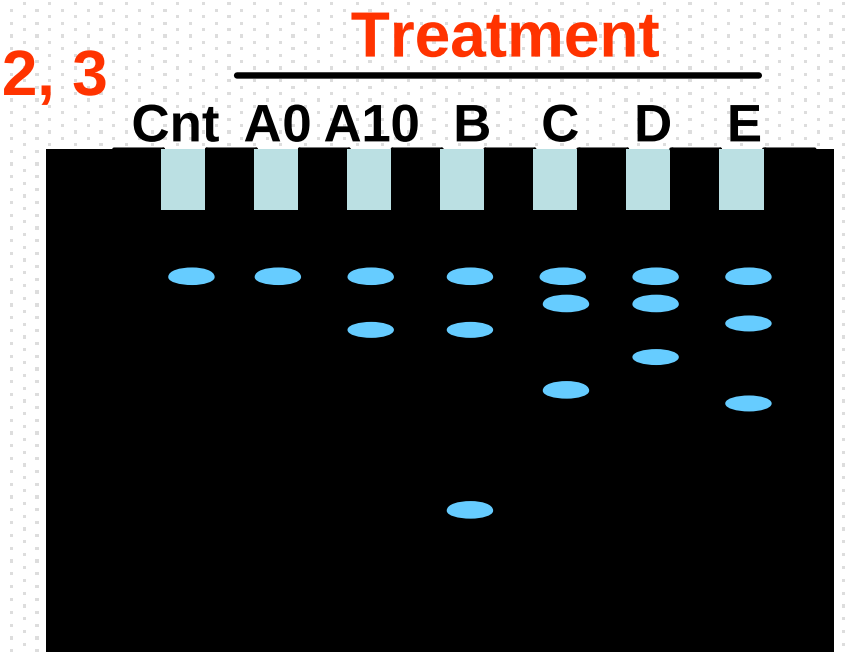
7. Cleavage of gelatin or other substrates in the gel followed by gel staining



Analysis of endonucleases

1. Direct DNA-degrading activity in SDS-PAGE
2. Incubation of isolated nuclei with nuclear protein extracts followed by electrophoresis
3. Cleavage of plasmid DNA with nuclear protein extracts





Analysis of DNA fragmentation

1. Diphenylamine reaction (quantitative assay) (Burton, 1956)

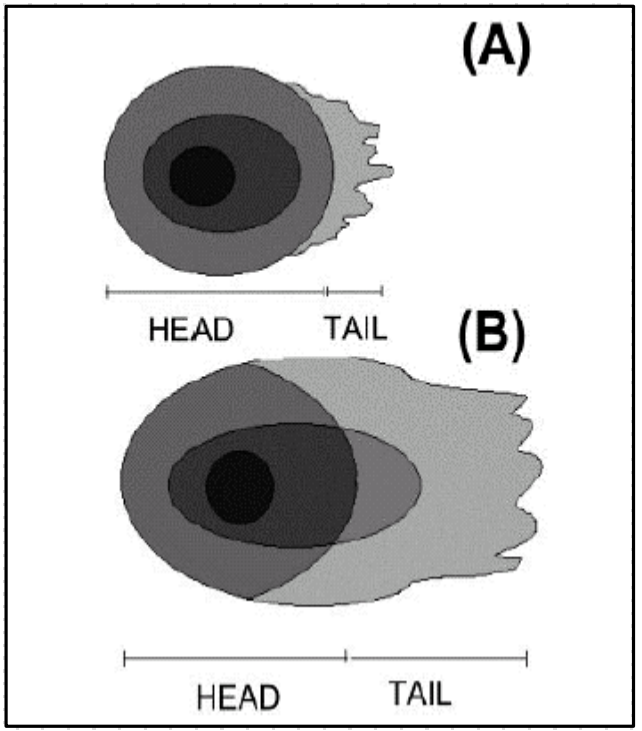
Recipe:
100 ml glacial acetic acid
1.5 g diphenylamine
1.5 ml concentrated sulfuric acid
0.5 ml 16 mg/ml acetaldehyde stock
Prepare just before use

Read absorbance at 600nm. Express results as the percentage of DNA fragmented

$$\% \text{ fragmented DNA} = \frac{\text{Absorb. supernatant}}{\text{Absorb. Supernatant + pellet}} \times 100$$

Analysis of DNA fragmentation

2. Comet assay



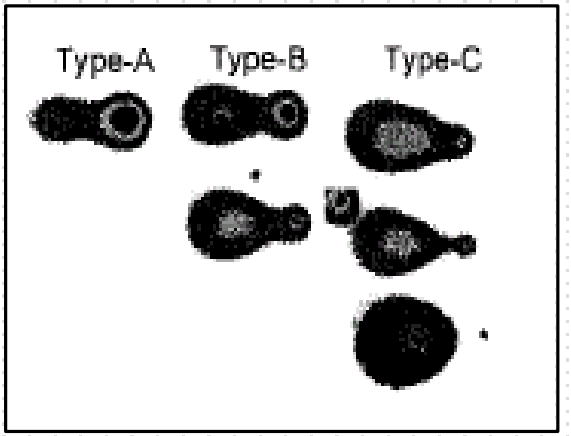
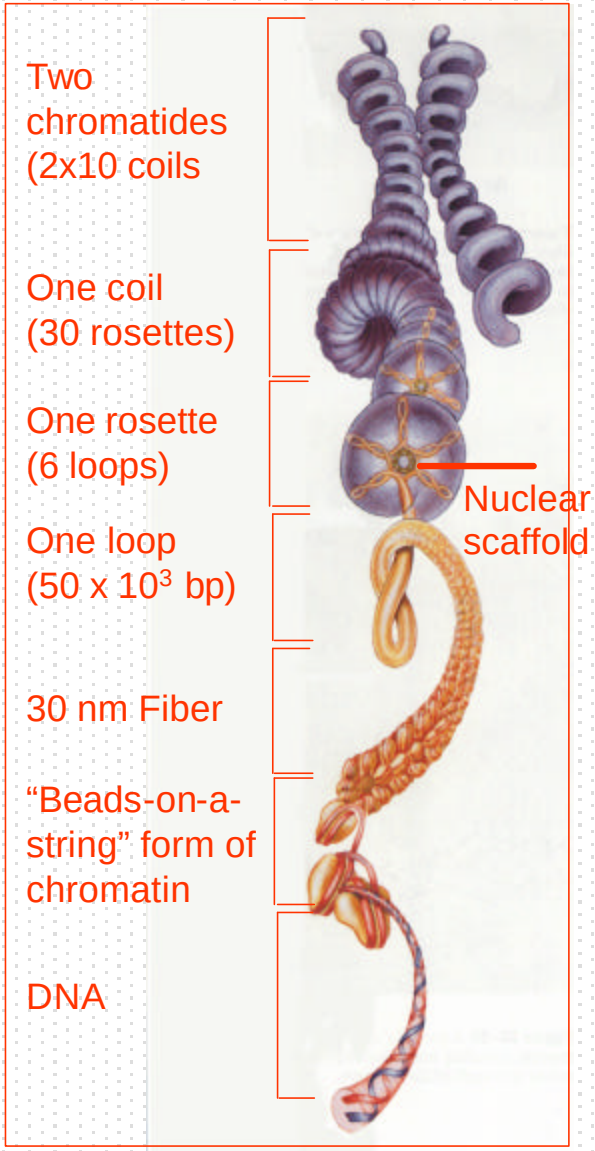


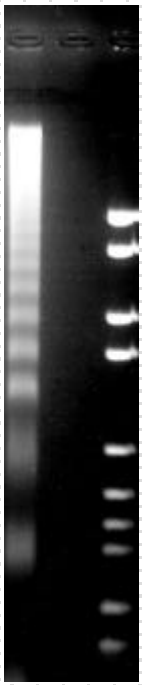
Illustration showing comets obtained from an untreated cell (A) and a cell with damaged DNA (B) with neutral comet assay

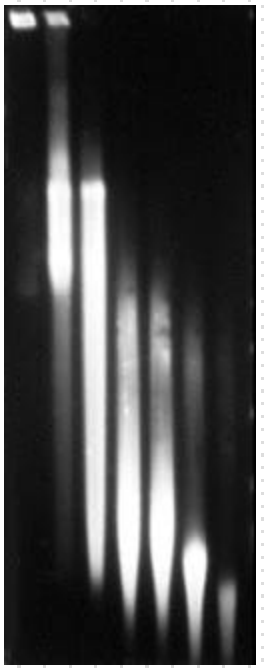
Analysis of DNA fragmentation



Modified from Leninger *et al.*, 1993 Principles of Biochemistry

3. Conventional gel electrophoresis

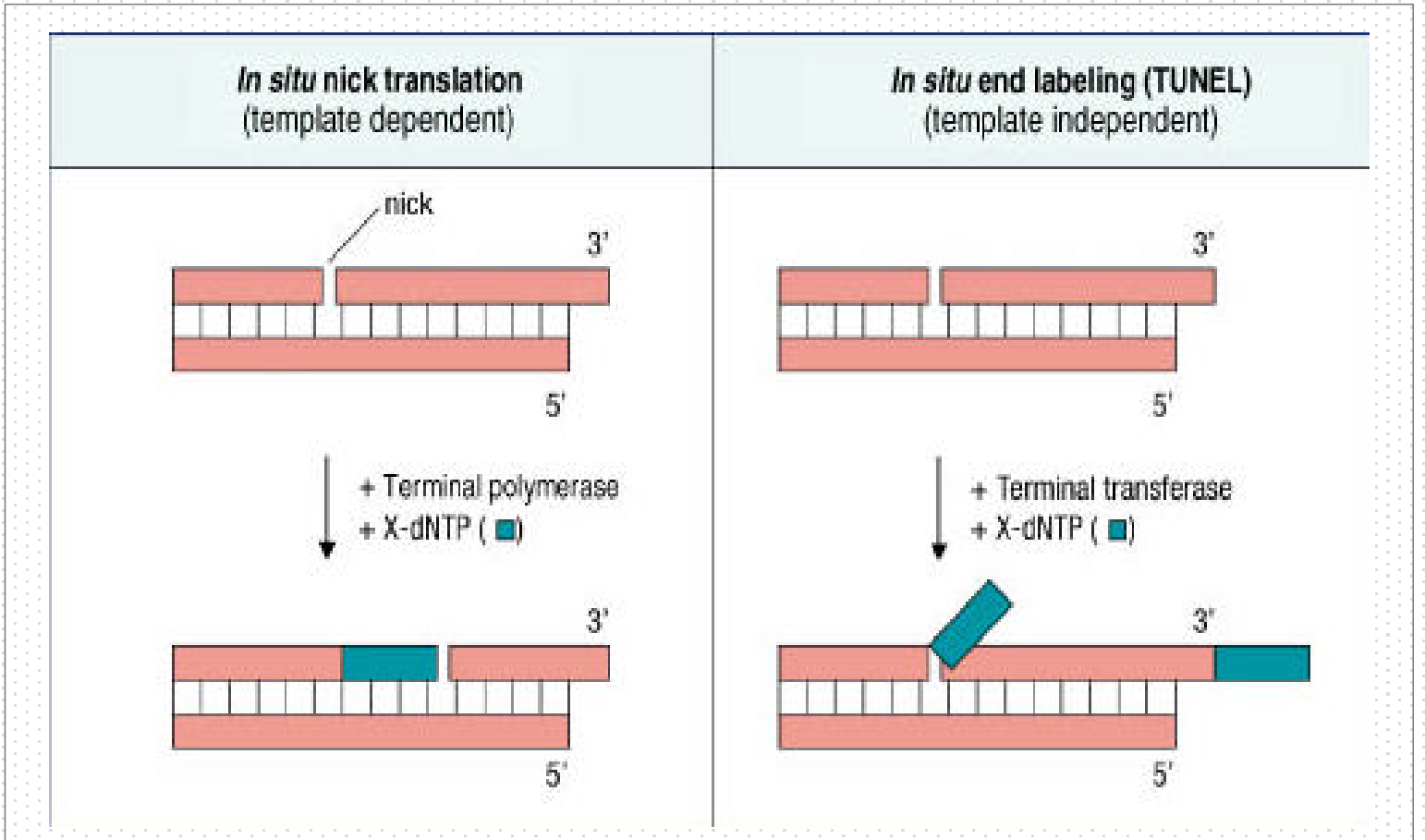




4. Pulse-field gel electrophoresis

Analysis of DNA fragmentation

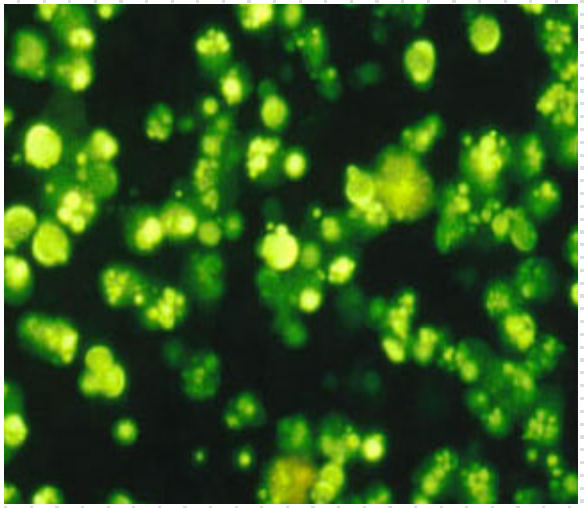
5. 3´-OH-end labeling (Klenow polymerase, followed by gel electrophoresis and autoradiography)
6. ISNT (*In situ* nick translation mediated by DNA polymerase I)



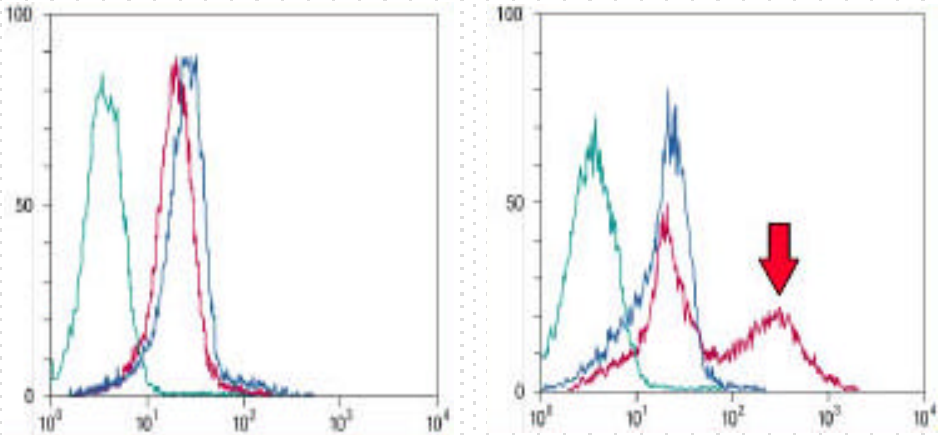
Analysis of DNA fragmentation

7. TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling)
(biotin-dUTP, digoxigenin-dUTP, or FITC-dUTP)
a) Single cells - analysis with microscopy (A) or by FACS (B)

A



B



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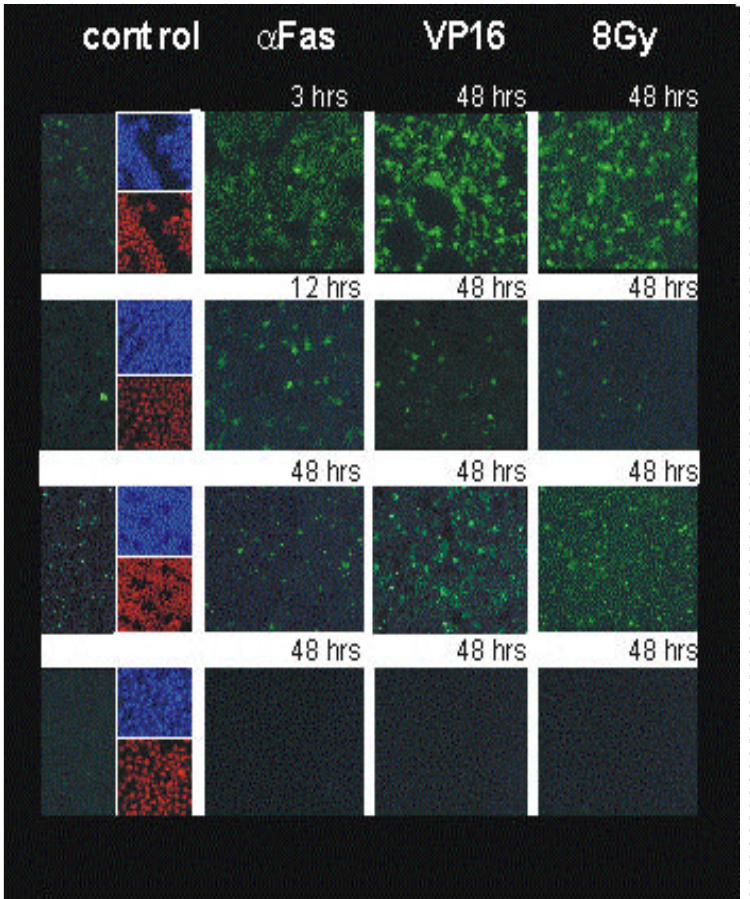

Analysis of DNA fragmentation

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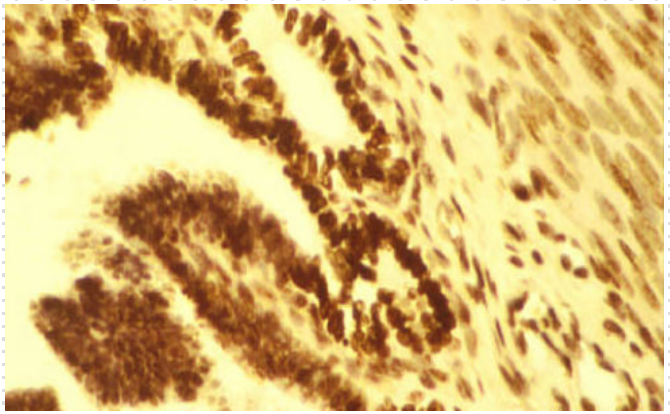
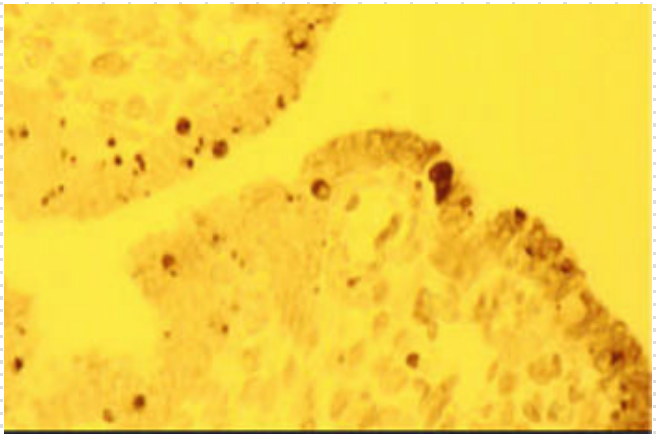
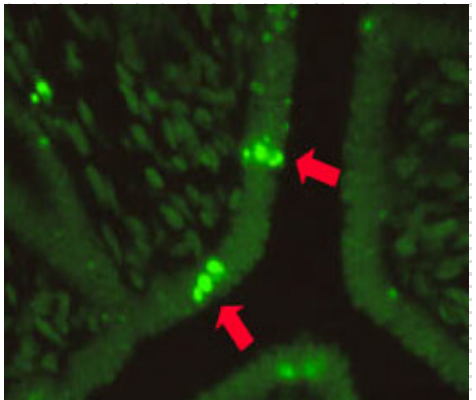
(biotin-dUTP, digoxigenin-dUTP, or FITC-dUTP)
b) Tissue sections - analysis with microscopy







Identification of apoptosis-related genes by techniques that analyze gene expression

1. Comparative approaches, including gene microarray analysis, serial analysis of gene expression, and differential display provide global information about expression levels.

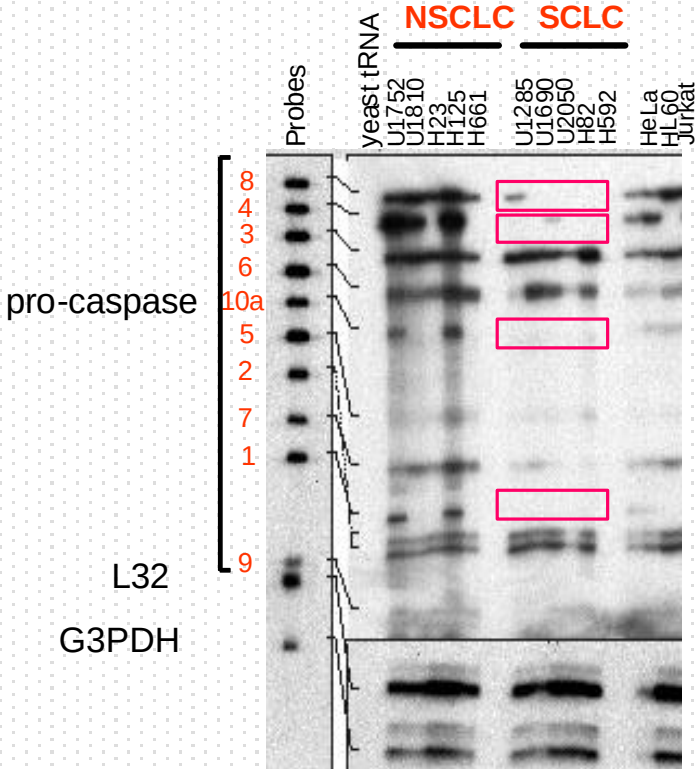
2. Subtractive approaches like complementary DNA representational difference analysis (cDNA RDA) and suppression subtractive polymerase chain reaction identify a focused set of differentially expressed genes.

3. A retroviral insertion mutagenesis approach identifies apoptosis regulatory genes.

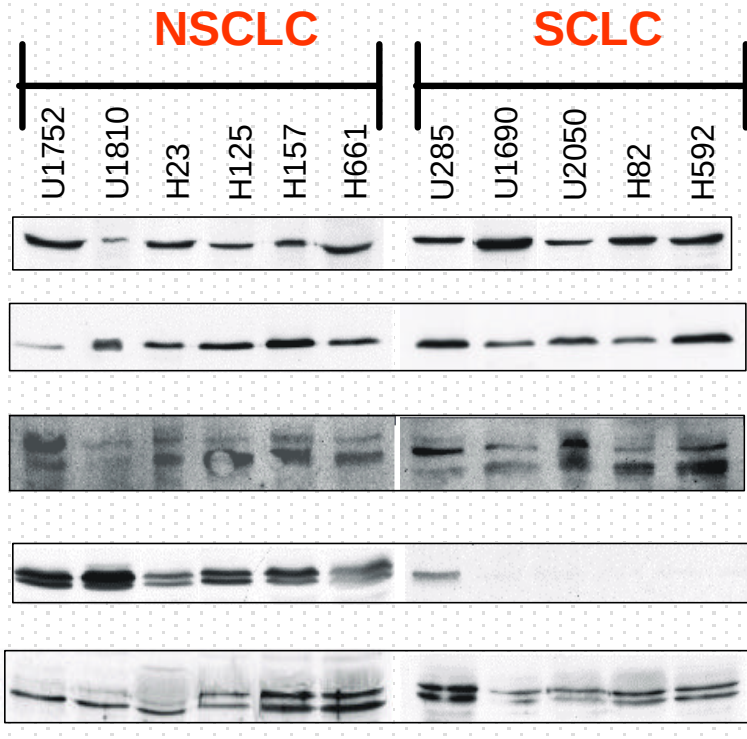
Methods for the Detection of Apoptosis

RNase protection assay for the detection of the expression of mRNA species for caspases, Bcl-2-related proteins, Death receptors, Death ligands, Signal proteins and Inhibitor proteins (IAPs)

RNase protection assay



Verification



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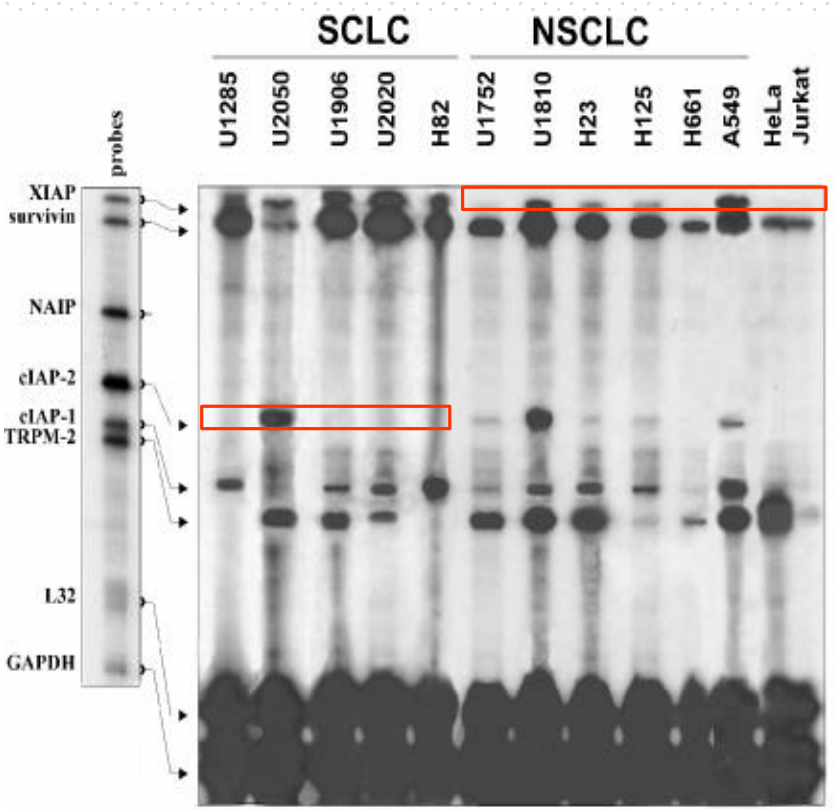
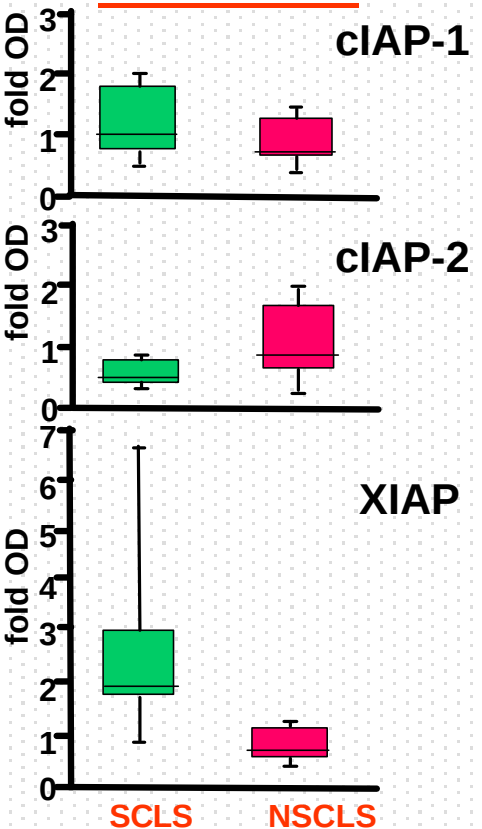
RNase protection assay for the detection of the expression of mRNA species for caspases, Bcl-2-related proteins, Death receptors, Death ligands, Signal proteins and Inhibitor proteins (IAPs)

RNase protection assay



Verification



Assays to examine the requirement of putative cell death genes

1. Targeting gene deletions by means of homologous recombination (time consuming, technically demanding, and expensive)

2. Transfection of antisense oligonucleotides (ineffective if the targeted gene is expressed at high levels)

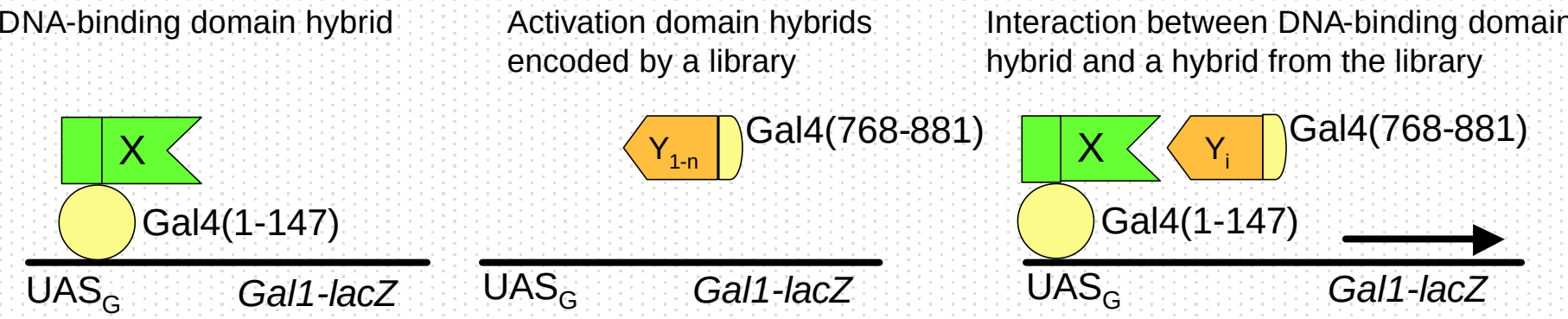
3. siRNA approach

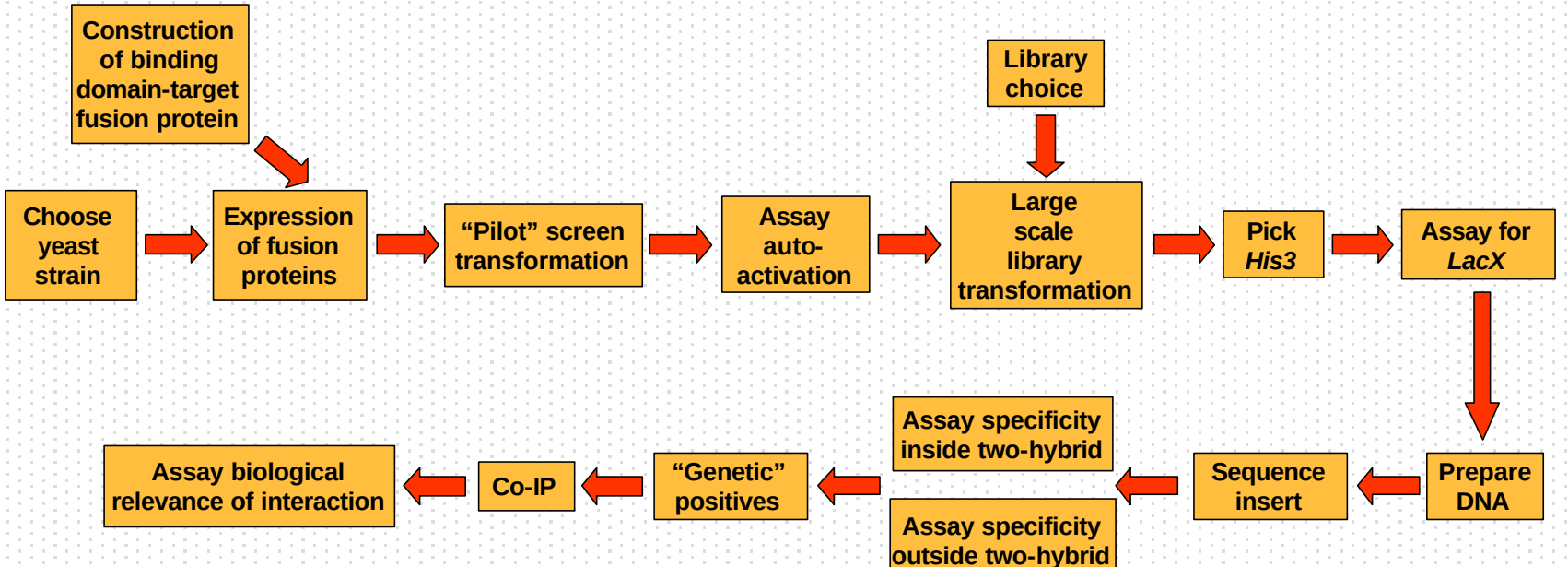
4. A retroviral insertion mutagenesis approach identifies apoptosis regulatory genes

5. Overexpression of genes

Identification of protein interactions by yeast two-hybrid screening and coimmunoprecipitation

Method is based on the Gal4 or LexA systems





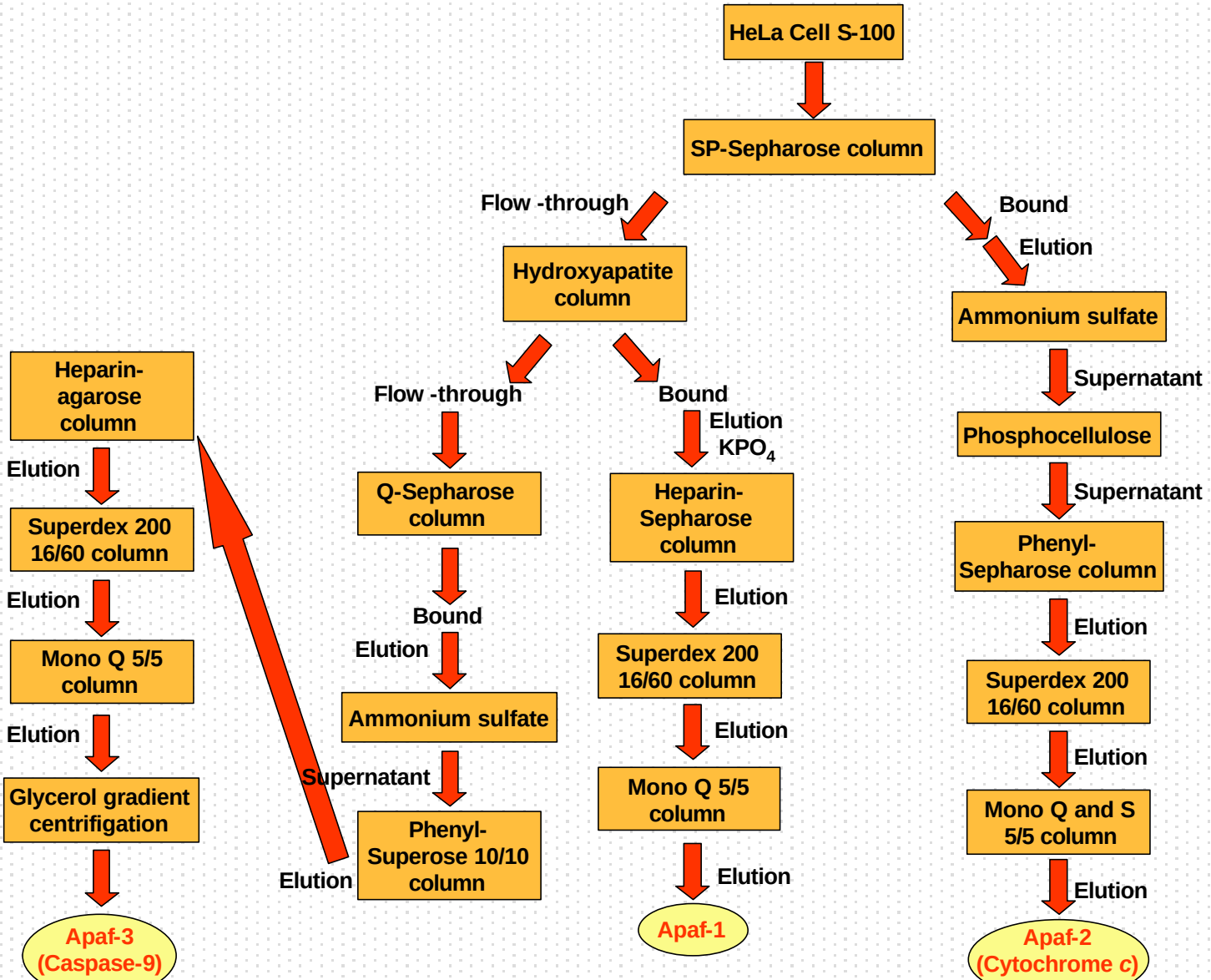
1. **Identification of protein interactions by yeast two-hybrid screening**
   (Field, S. and Song, O. Nature, 1989: 340, 245-246).

2. **Identification of protein interactions by bacterial two-hybrid screening**
   (Joung, JK, Ramm, EI, and Pabo, CO. Proc. Natl. Acad. Sci, USA 2000; 97, 7382-7387).

3. **A combined yeast/bacteria two-hybrid system**
   (Serebriiskii IG, et al., Mol. Cell Proteomics, 2005: 4.6, 819-826).

4. **Mammalian two-hybrid assay for detecting protein-protein interactions**
   (Lee, JW, and Lee, S.-K. Meth. Mol. Biol. 2004, 261: 327-336).

Identification of proteins involving in cell death by fractionation



Identification of proteins involving in cell death by fractionation

1. Protein fractionation, following western blot analysis and/or Mass spectroscopy, Matrix-assisted laser desorption ionization post-source decay (MALDI-PSD), etc.

2. Proteomic approach

Cell-free systems to investigate cell death

1. **Cytoplasmic extracts from mitotic chicken hepatoma cells were found to induce chromatin condensation and DNA cleavage reminiscent of apoptosis in exogenously added nuclei**
   (Lazebnik, Y. *et al*., J. Cell Biol., 1993; 123, 7-22).

2. **These extracts were used to identify a protease activity capable to cleave protein substrates during apoptosis**
   (Lazebnik, Y. *et al*., Nature, 1994; 371, 346-347).

3. **Cytoplasmic extracts of "aged" *Xenopus* eggs were found to induce apoptotic changes in nuclei added to these extracts**
   (Newmeyer, DD. *et al.,* Cell, 1994; 79, 353-364).

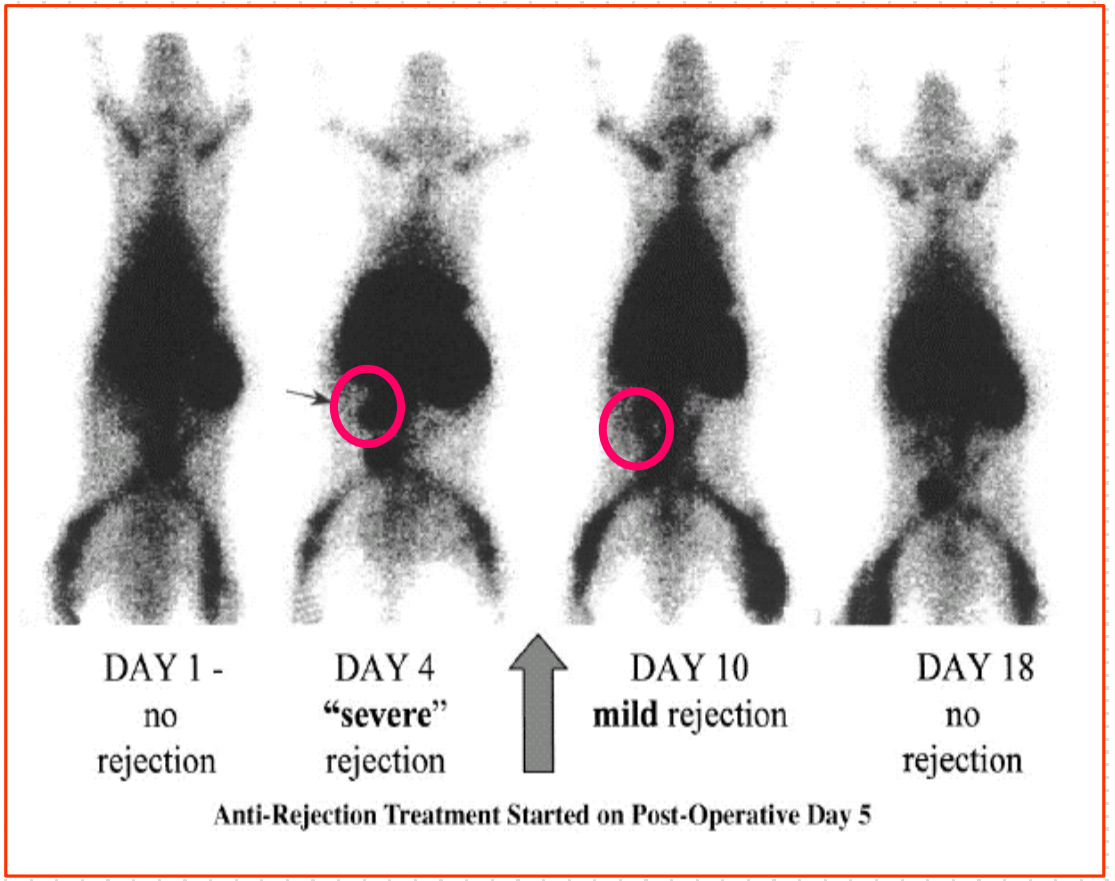
4. **Cytoplasmic S-100 extracts were used to reproduce aspects of the apoptotic program *in vitro.* This program is initiated by addition of dATP**
   (Liu *et al.,* Cell, 1996; 86, 147-157).

Methods for the Detection of Apoptosis

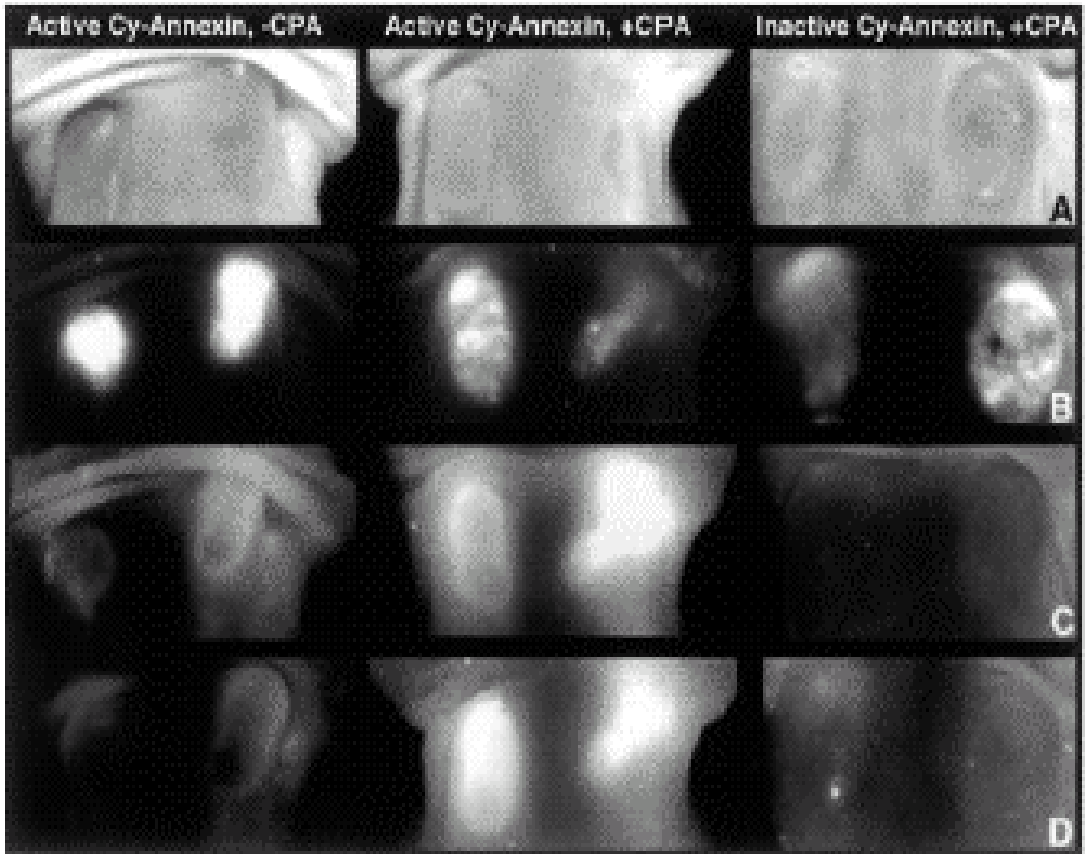
In vivo measurements

Methods for the Detection of Apoptosis

Staining with Annexin V labeled with 99mTc, followed by radionuclide imaging



In vivo imaging using a near infrared fluorescent-labelled (NIRF) annexin V (complex of annexin V with Cy5.5)



Visible light image of implanted tumors

Expression of DsRed2 in tumor (red fluorescence channel)

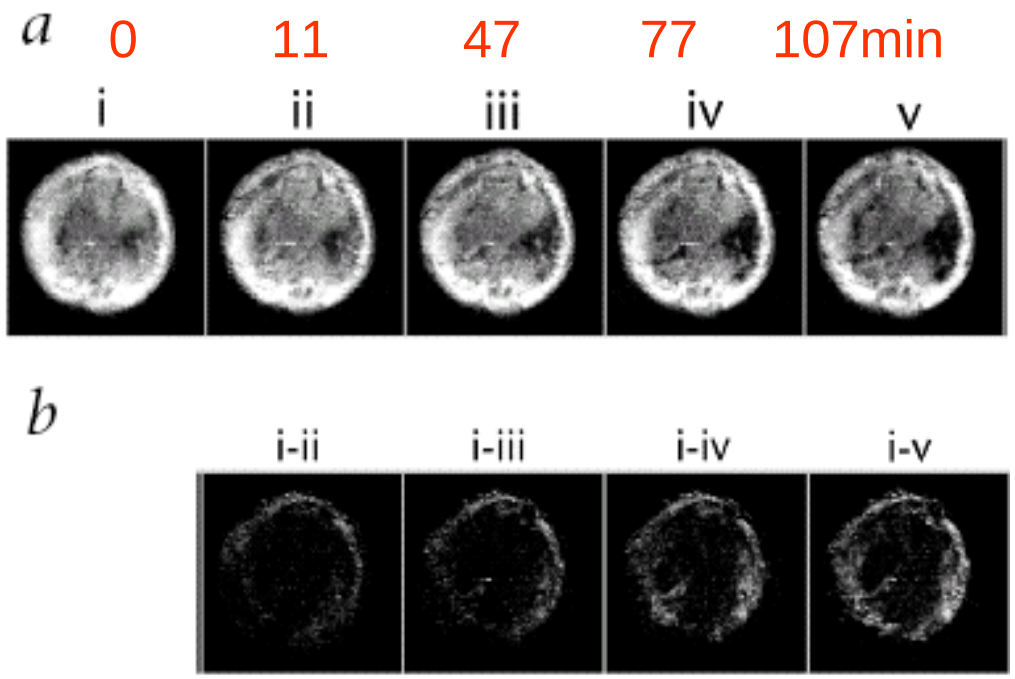
Near-infrared signal measured in tumors after injection of Cy-annexin (75 min)

Near-infrared signal measured in tumors after injection of Cy-annexin (20 h)

This method requires fluorescent endoscope an a minimally invasive fashion

Non-invasive detection of apoptosis using magnetic resonance imaging and a targeted contrast agent

C2 domain of synaptotagmin I is conjugated with superparamagnetic iron oxide (SPIO) nanoparticles. This complex binds to anionic phospholipids in the plasma membranes

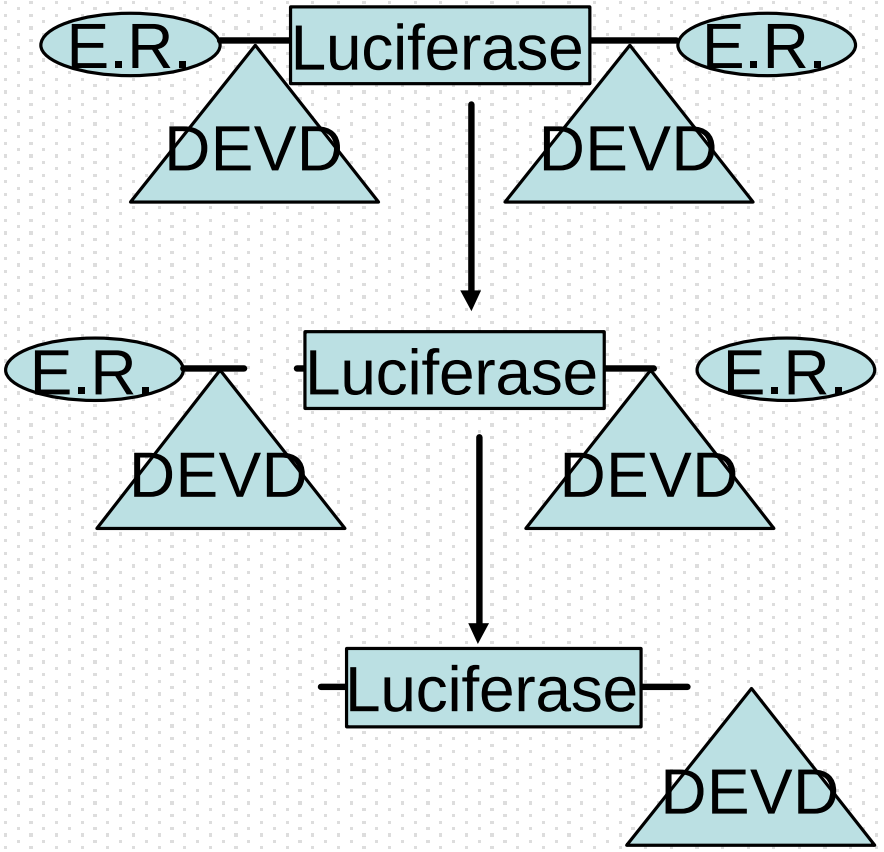


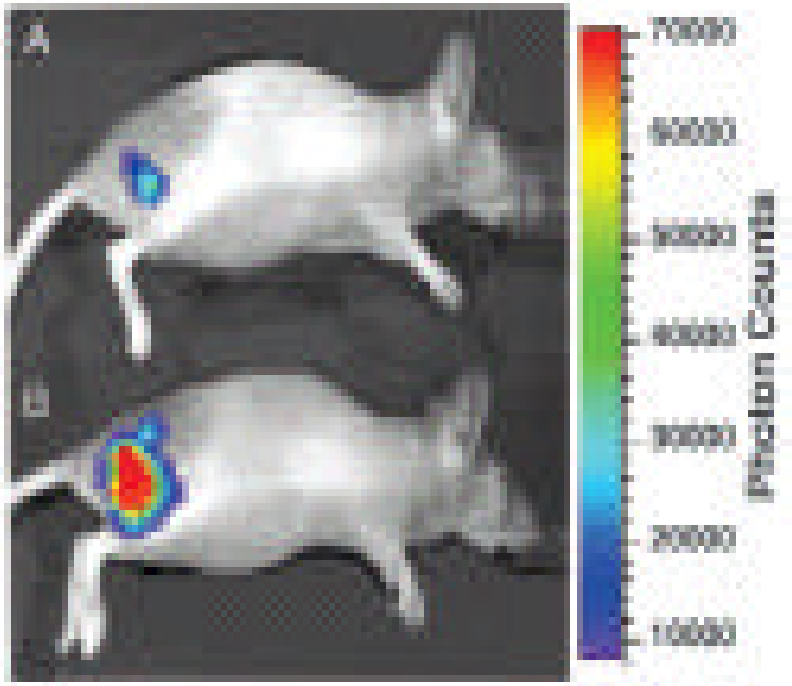
MR images of a tumor in a drug-treated mouse following injection of C_2-SPIO

Images obtained by subtracting the post-contrast images from the image acquired before injection of C_2-SPIO

Non-invasive detection of apoptosis using magnetic resonance imaging and a targeted contrast agent

A recombinant luciferase reporter molecule, E.R.–DEVD–Luc–DEVD–E.R., is cleaved and restore the luciferase activity, that can be detected in living animals with bioluminescence imaging





Cautions when assessing and characterizing cell death

- Irrespective of the insult, the time-course of cell death might be very fast
- Clearance of apoptotic cells (phagocytosis) is also might be fast, especially *in vivo*
- In any static analysis only a small fraction of apoptotic cells might be detected
- Some techniques are not selective for apoptosis and should be use in combination, and other may not be as sensitive

Cautions when assessing and characterizing cell death

- Damaged vs. dead cells. Which damage is irreversible?
- Dose matters! High dose = necrosis, Mild injury = apoptosis
- Apoptosis and necrosis may simply represent two extremes of biochemically overlapping cell death pathways
- Some forms of cell death contain features of both, e.g. apoptosis is sometimes accompanied by secondary necrosis
- Paraptosis, Anoikis, Autophagy- all have some features of apoptosis
- If you prevent apoptosis you may not prevent cell death, but simply shift the mode of death to necrosis

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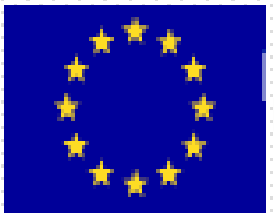
Work in our group is supported by the grants from:



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