Methods of Biomaterials Testing
Lesson 7 - 8

Life and Death

Aspects of Cell Death/ Vitality

- Stop of cell metabolism ("functional" viability)
  - Good, but relatively late marker
  - Many enzymes are active for much longer time
  - No applicable at histological sections or at preserved cells
- Loss of cell integrity
  - Late marker
  - Not necessary required for cell death
  - Formalin- or ethanol-fixed cells will appear as vital
- Loss/ destruction of important components
  - Degradation of DNA and proteins is frequent, **but not required** for cell death
  - Red blood cells and blood platelets are vital cells without cell nucleus (RBC even without mitochondria)
- Irreversible process of dying
  - What is irreversible?
  - Still metabolically active
  - Maybe important communication/ signaling function
- Loss of reproductive capacity ("reproductive" viability)
  - Normal state for many cells in the body
  - Cells are definitely still vital, but will go to the process of dying
  - Can be a good criterion for anti-tumor drugs

Aspects of Cell Death/ Vitality

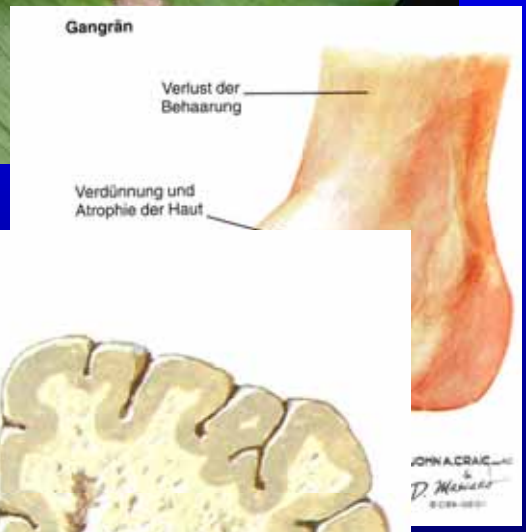
Vitality/ death have many different meanings for different applications/ situations

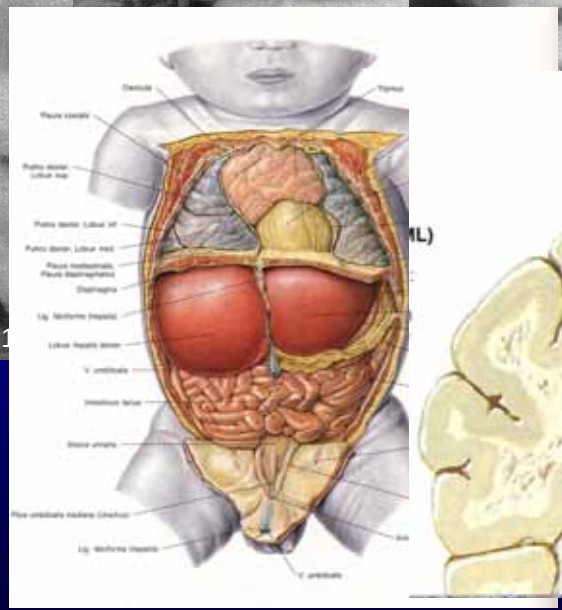
- Can the cell still reproduce?
- Is the cell still metabolizing?
- Are only some metabolic actions active, others not?
- (vita minima of some bacteria without metabolism but possibility to recover)

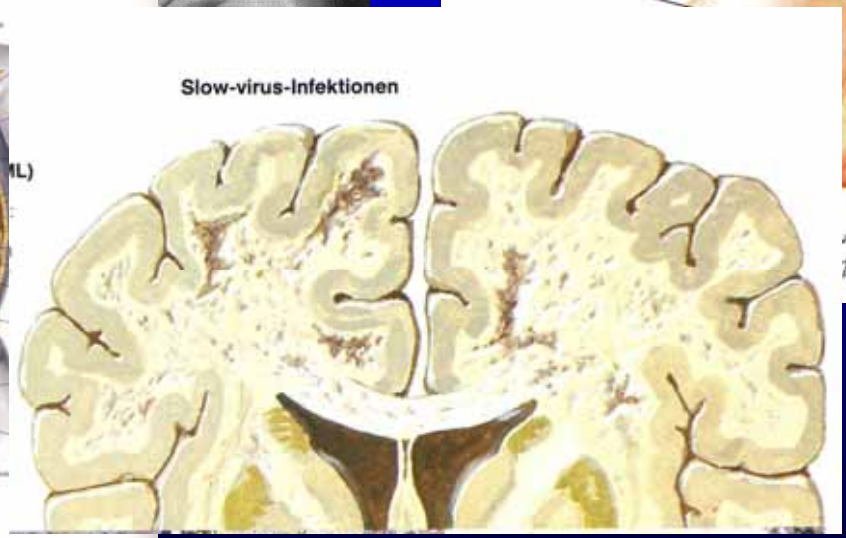
Consequence

- All vitality/ death tests are only tests for certain functions of the cells

Apoptosis vs. Necrosis







Two Ways of Cell Death

- Necrosis
  - "cruel" way of cell death
  - it is always harmful for the organism/ tissue.
  - Reasons:
    - Mechanically: disruption of the cell membrane
    - Toxic
    - Heat
    - Sudden lack of oxygen or nutrients
  - Consequence is immigration of inflammatory cells, inflammation, usually replacement by non-functional connective tissue (scar)
- Apoptosis
  - Important and necessary in the development of the body
  - Principal mechanism in some diseases
  - Highly organized and controlled cell death, energy consuming process
  - No (or very late) damage of the cell membrane
  - Organized degradation of proteins and DNA
  - Expression of "eat-me flags" on the cell surface leads to early and innocent removal of the cell

Quantification of Necrosis

Quantification of Necrosis

All tests on necrosis base on the lack of membrane integrity of necrotic cells. In this state important ion gradients can not be maintained $\Rightarrow$ Immediate cell death

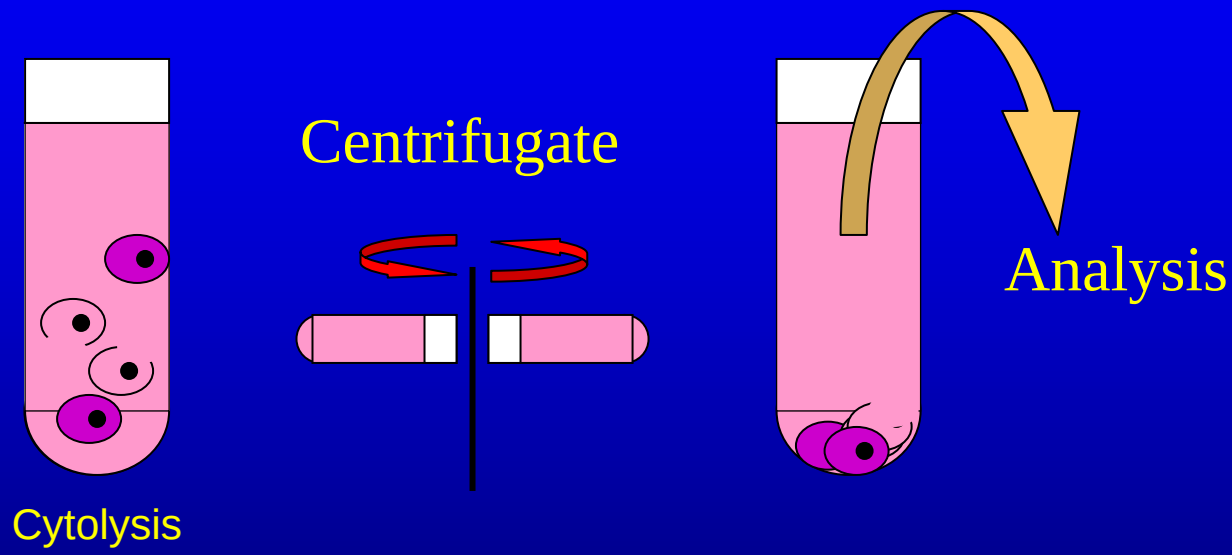
- Outflow of cell content
  - Substance, which is present only inside the cell
  - Substance, which is present at very constant concentration

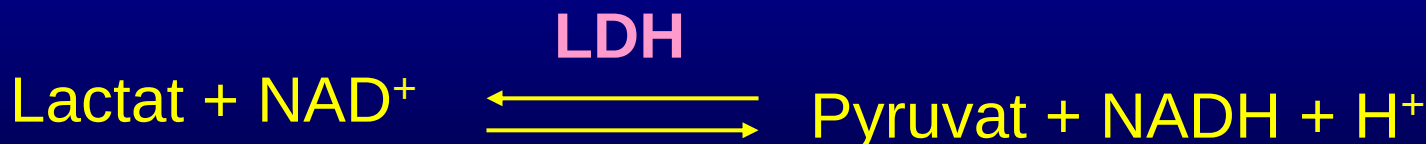
  $\rightarrow$ Lactate dehydrogenase (LDH)
- Inflow of substances (dyes) into the cell:
  charged and/or big molecules, which usually can not pass the cell membrane
  - Trypan Blue: stains intracellular proteins blue (standard method in cell culture)
  - (Fluorescent) DNA dyes: Ethidium Bromide, Propidium Iodide, 7-AAD, TOPRO-3, DAPI, Hoechst stains, SYTOX stains, …

Quantification of Necrosis (II)

- Conversion and Outflow of Substances
  - Substance passes the cell membrane freely in an inactive state
  - Substance is “activated” by enzymes (mainly esterases) of the cell:
    - Stained product
    - Charged/ non cell permeable product
  - Fluorescein diacetate (FDA) (first substance of the class, product shows relatively high leakage rate)
  - Calcein-AM (Calcein-Acetoxymethyl ester)
  - Many other products, which are derived from the first ones

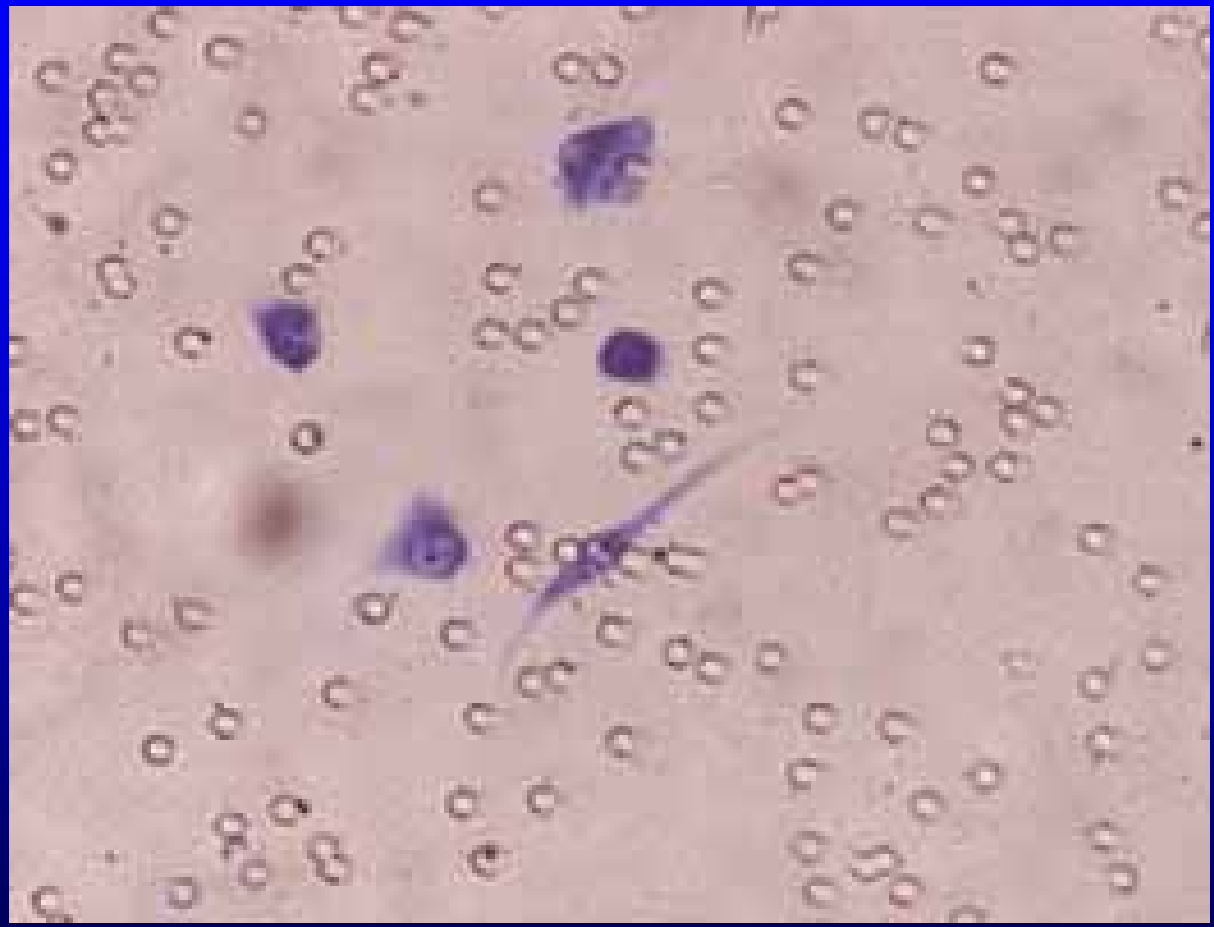
LDH Test





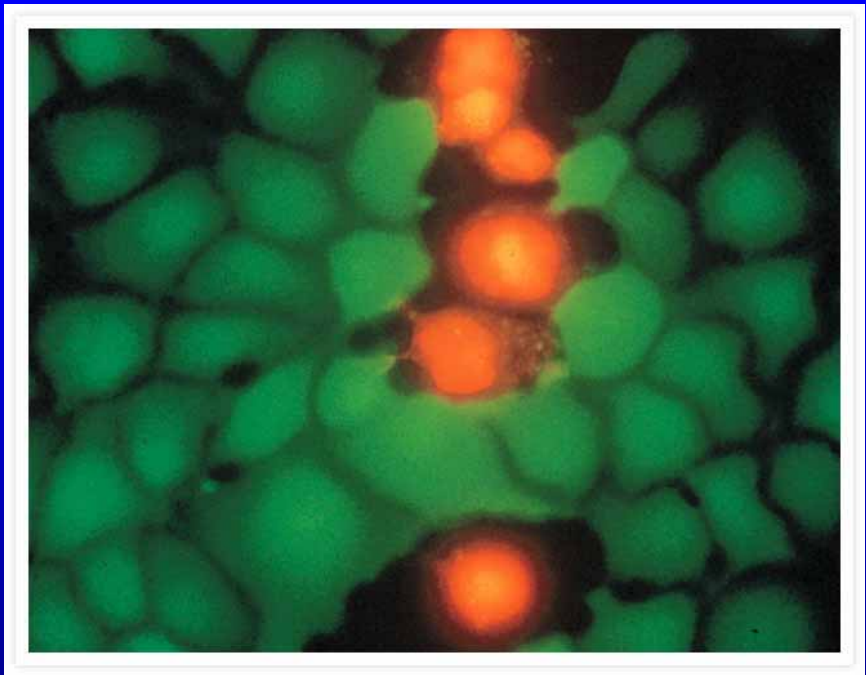
The reaction can be followed by the absorption of NADH at λ=340nm

Trypan Blue Stain

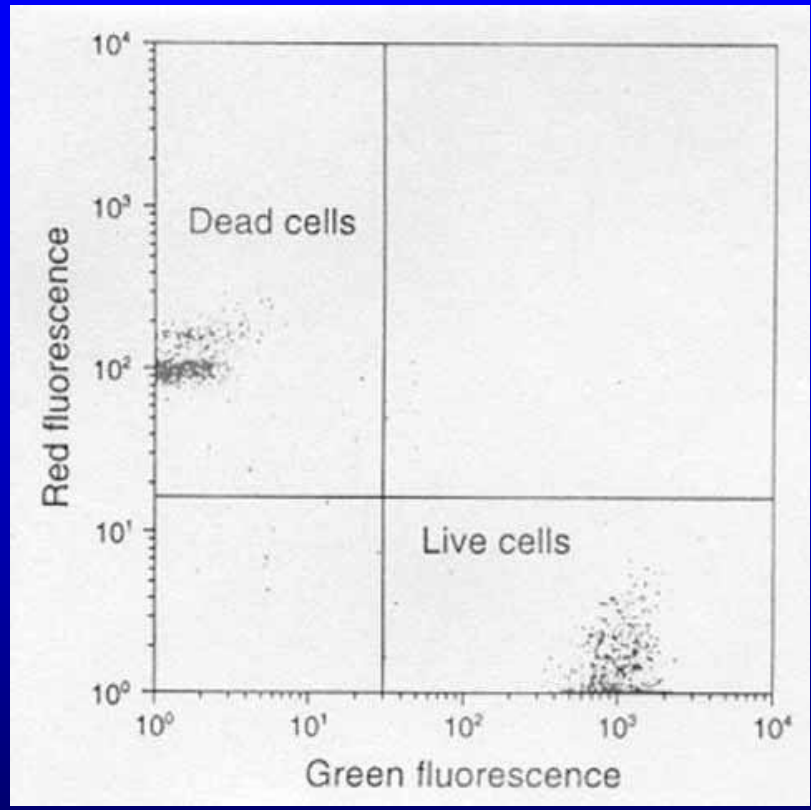


Trypan Blue stained, permeabilized cells

Fluorescent Double Staining



Live and dead kangaroo rat (PtK2) cells stained with ethidium homodimer-1 and the esterase substrate calcein AM. Live cells fluoresce a bright green, whereas dead cells with compromised membranes fluoresce red-orange.



Ideal Flow cytometry of such a double stained population allows direct quantification

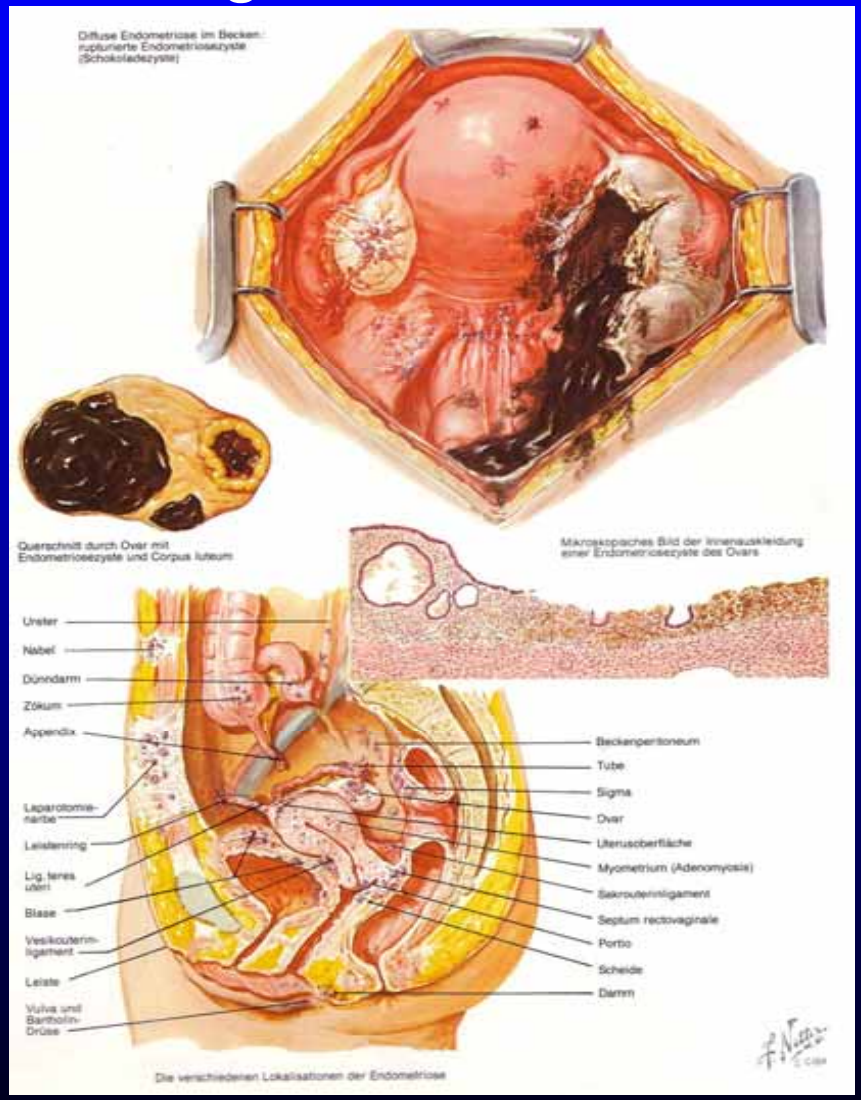
Apoptosis

Induction of Apoptosis

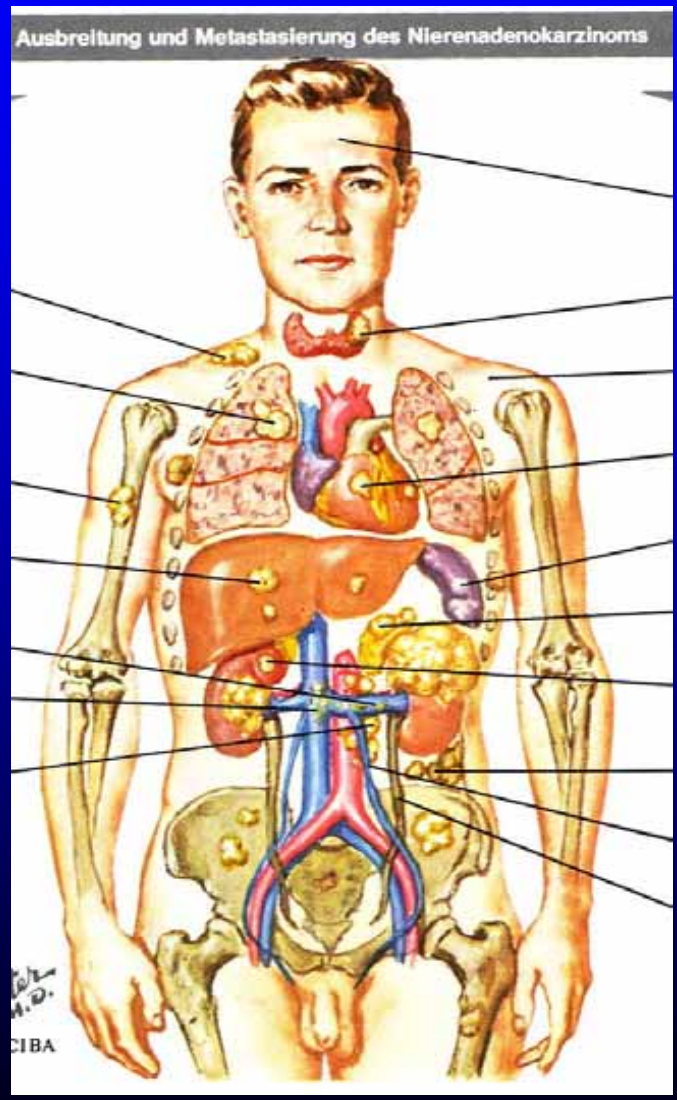
- Internal
  - Genetic program (?)
  - Severe DNA damage
  - Damage of mitochondria (ultra short high voltage pulses)
  - Virus infection
  - Toxic, many cytostatics
  - Free radicals
- External (receptor mediated)
  - Genetic program of development
  - Genetic program of development in the development of the immune system
  - Virus infection (detected from outside)
- ??
  - Cell displacement
  - (Many more)

Displaced Adherent Cells

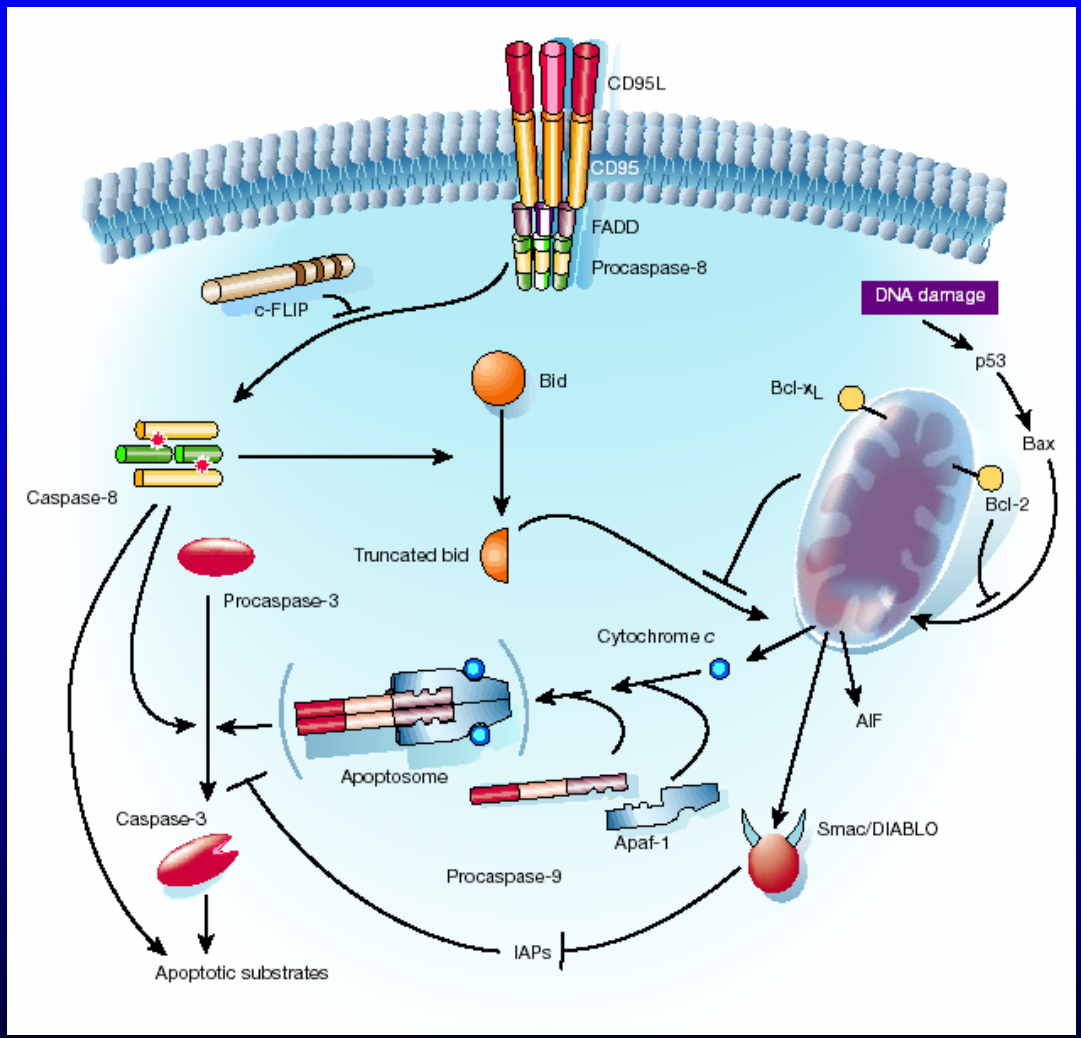
Benign: Endometriosis



Malign: Tumor Metastasis



Regulation of Apoptosis



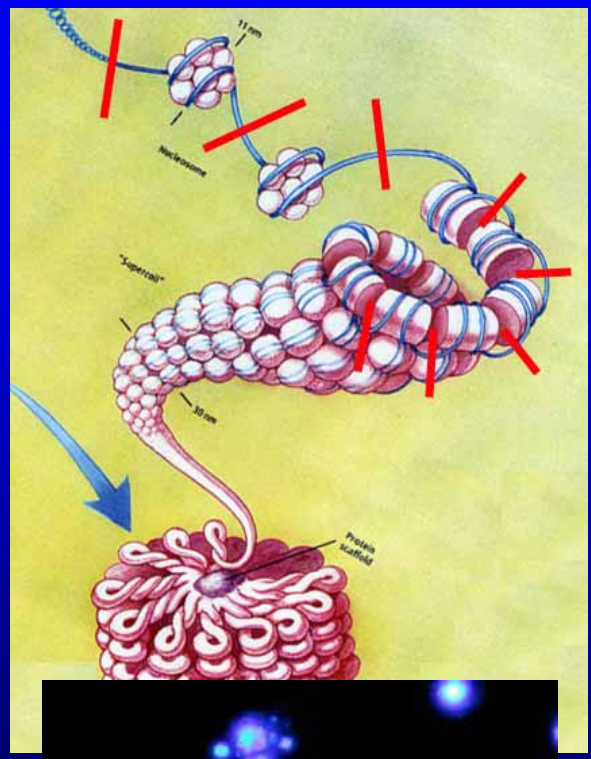
Nature 407 (2000): 773

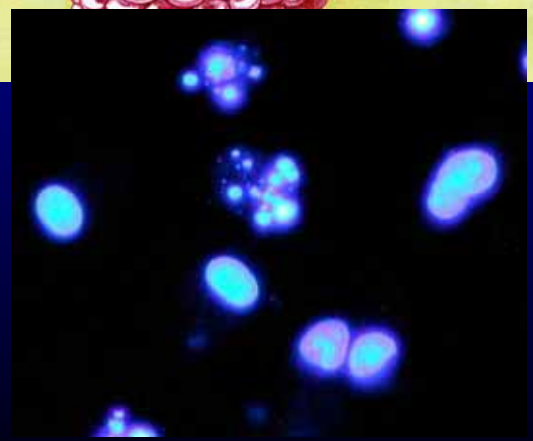
There are two major pathways for apoptotic cell death:

- One depends on cell death receptors (left). The key enzyme is caspase-8.
- Internal sensing of cell death signs is on a mitochondria centred pathway (right). Important further steps are cytochrom C assembly with Apaf-1 and caspase-9 activation.
- The common further pathway is caspase-3 activation.

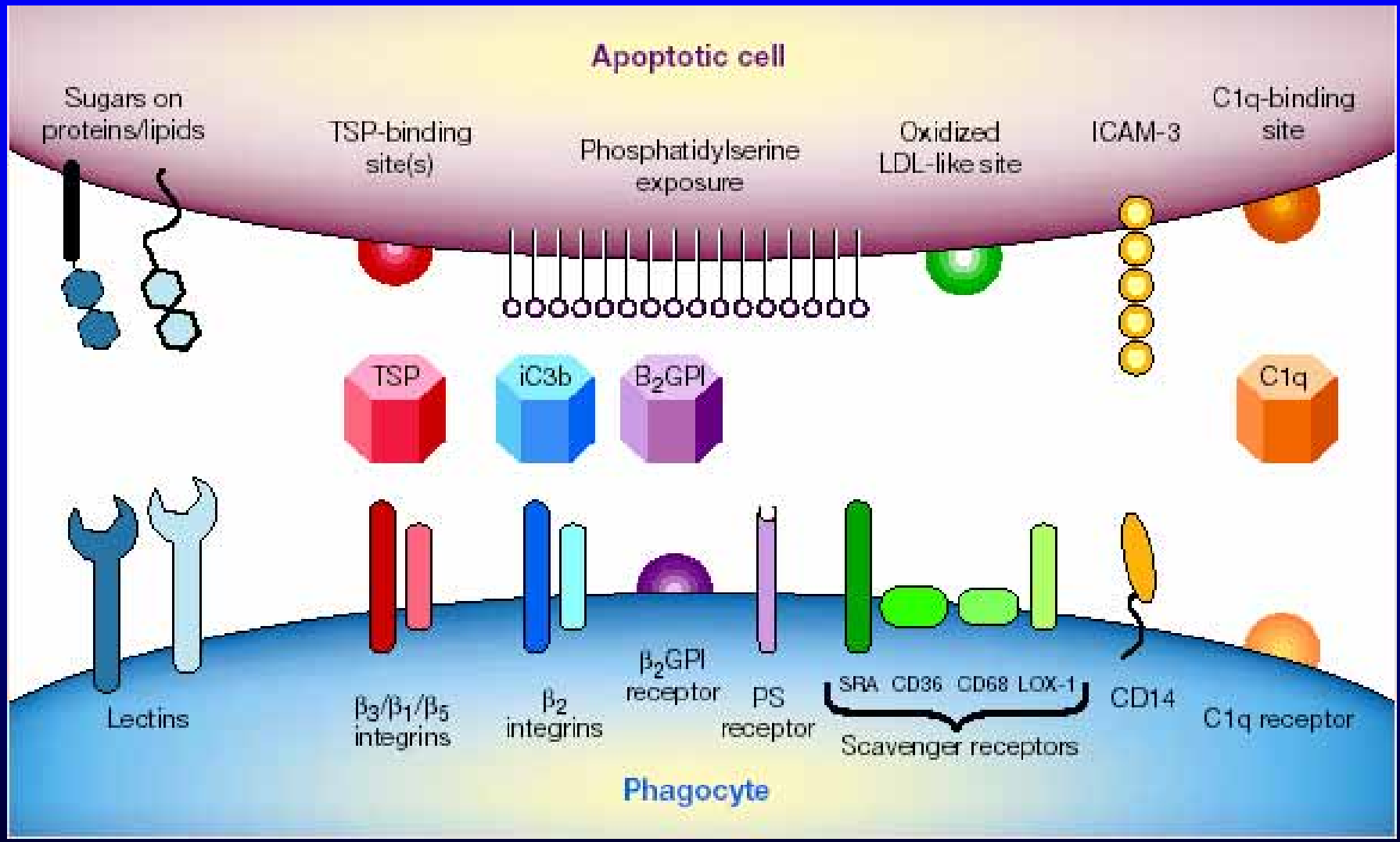
Consequences/ Steps of Apoptosis

- Activation of apoptosis by one of the two pathways: external or mitochondrial
- Assembly and activation of Caspases: Enzymes, which degrade proteins of the cell
- Degradation of structure proteins of the cell
- Expression of characteristic surface markers (“eat-me flags”)
- Caspase-dependent activation of a nuclease: Degradation of the DNA between the histones ⇒ Fragments integer multiples of 180 base pairs.
- Condensation and fragmentation of the cell nucleus
- Pore formation in the mitochondria





“Eat-me Flags” of Apoptotic Cells



Nature 407 (2000): 787

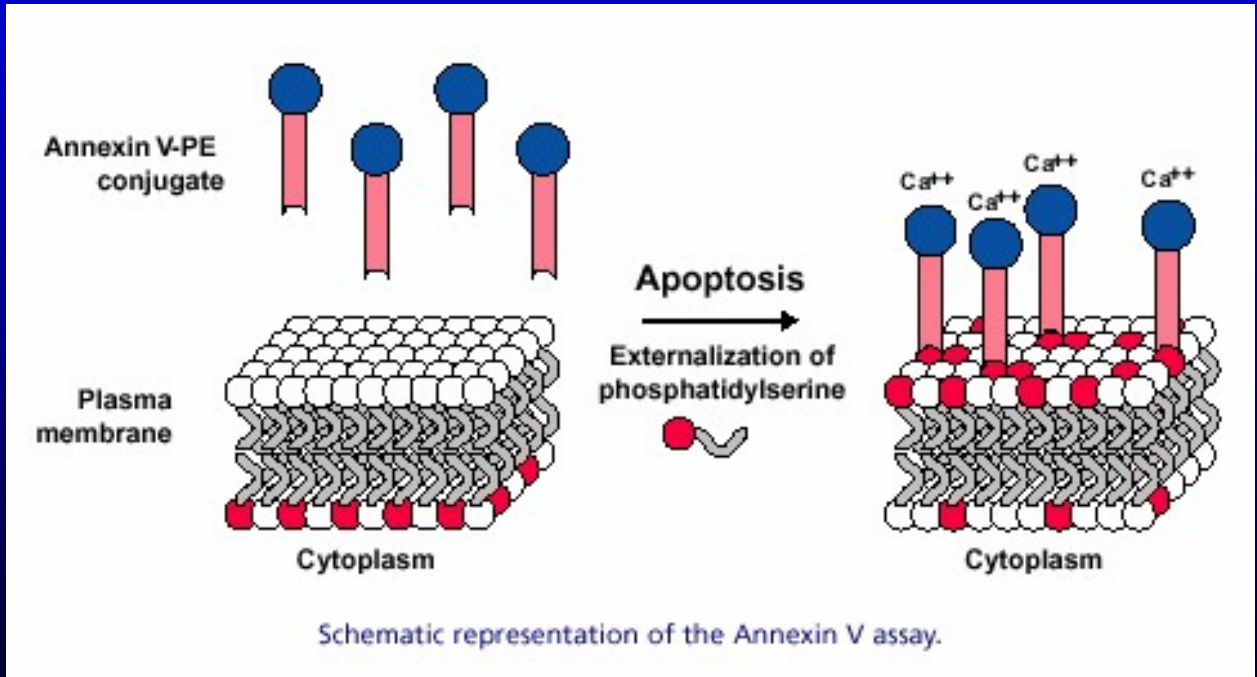
Analysis of Apoptosis

Most of the steps in the highly controlled pathways of apoptosis can be used to prove an apoptotic way of cell death

- Eat-me signals
  - Annexin V/ Propidium iodide stain
- Caspase activation
  - (Immunochemistry)
  - Or: Fluorescent or chromogenic substrates
- DNA fragmentation
  - TUNEL test
  - Electrophoresis: Proof of DNA-laddering
- Cell nucleus condensation and fragmentation
  - Light microscopy with DNA sensitive dyes
- Pore formation in mitochondria
  - Membrane potential sensitive dyes

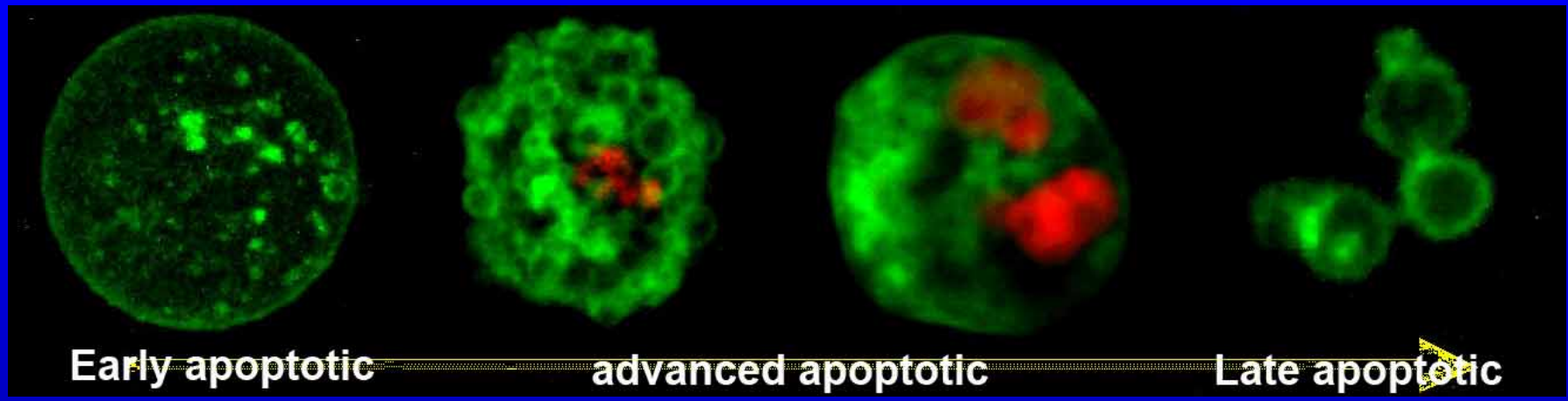
Annexin V Stain

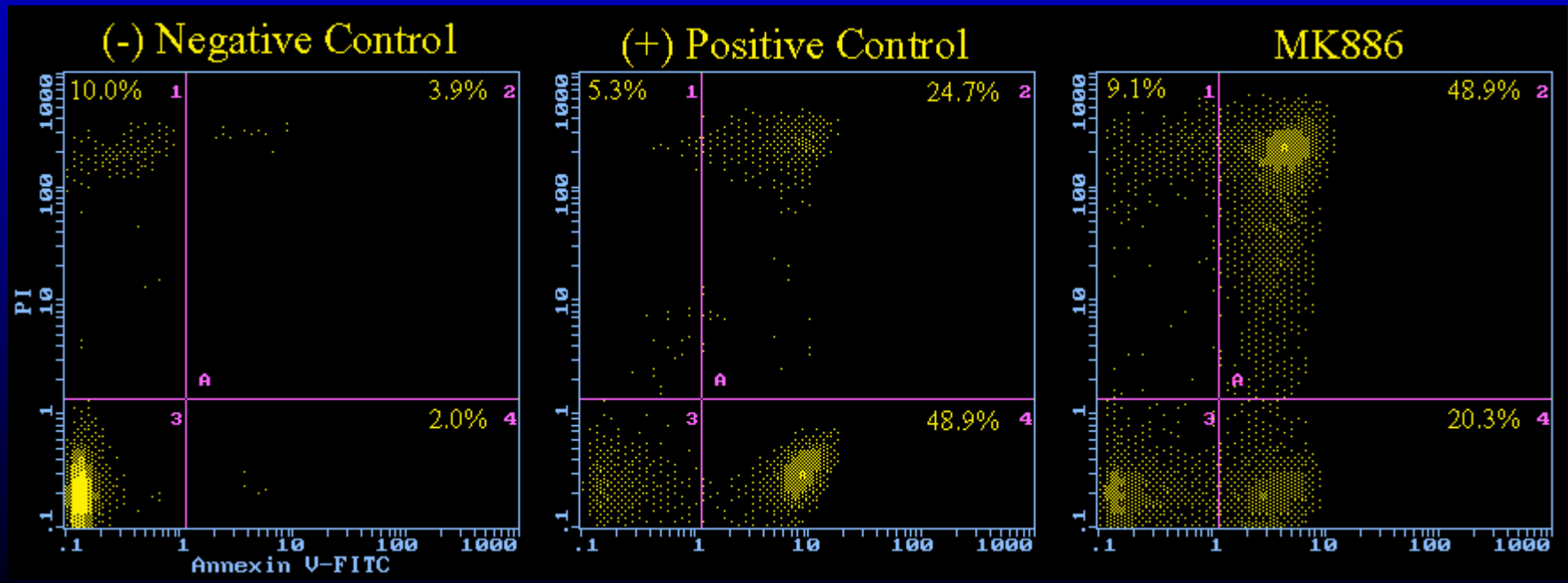
- Phosphatidyl serine usually is on the inner surface of the cell membrane. At apoptosis is switches to the outer surface (early marker)
- Annexin V is a protein, which binds specifically to phosphatidyl serine in a Ca^{2+} dependent reaction.
- Staining with (green) fluorescent labeled annexin V and counterstain of the cell nuclei with propidium iodide
  - No fluorescence: vital cell
  - Green fluorescence: apoptotic cell
  - Red+green fluorescence: necrotic or late apoptotic cell



Schematic representation of the Annexin V assay.

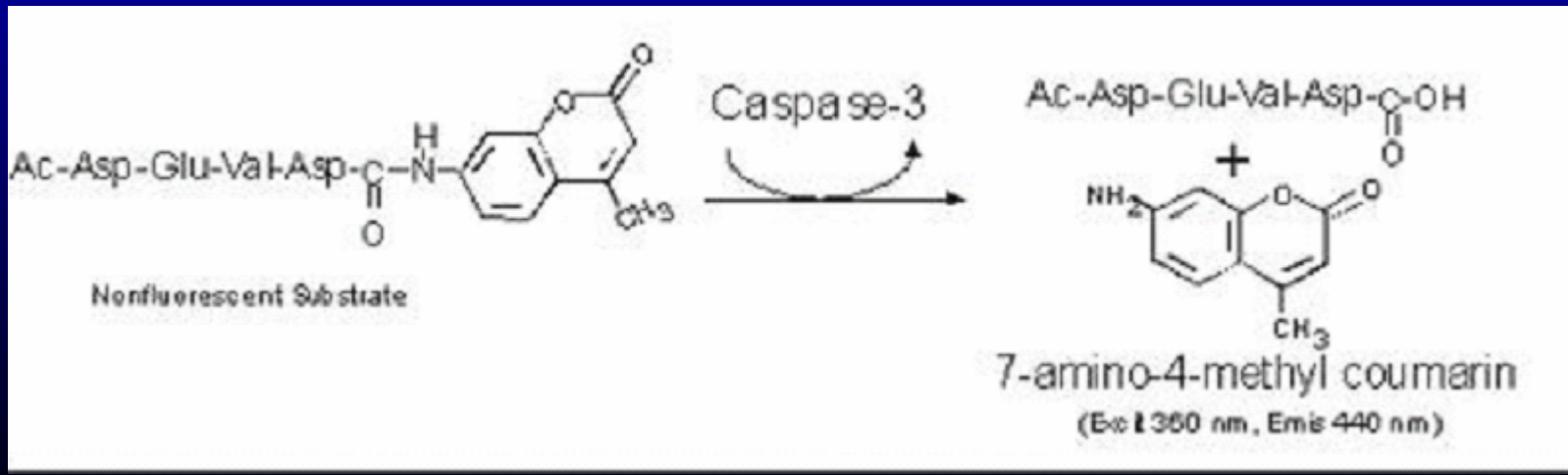
Annexin V / PI Stain



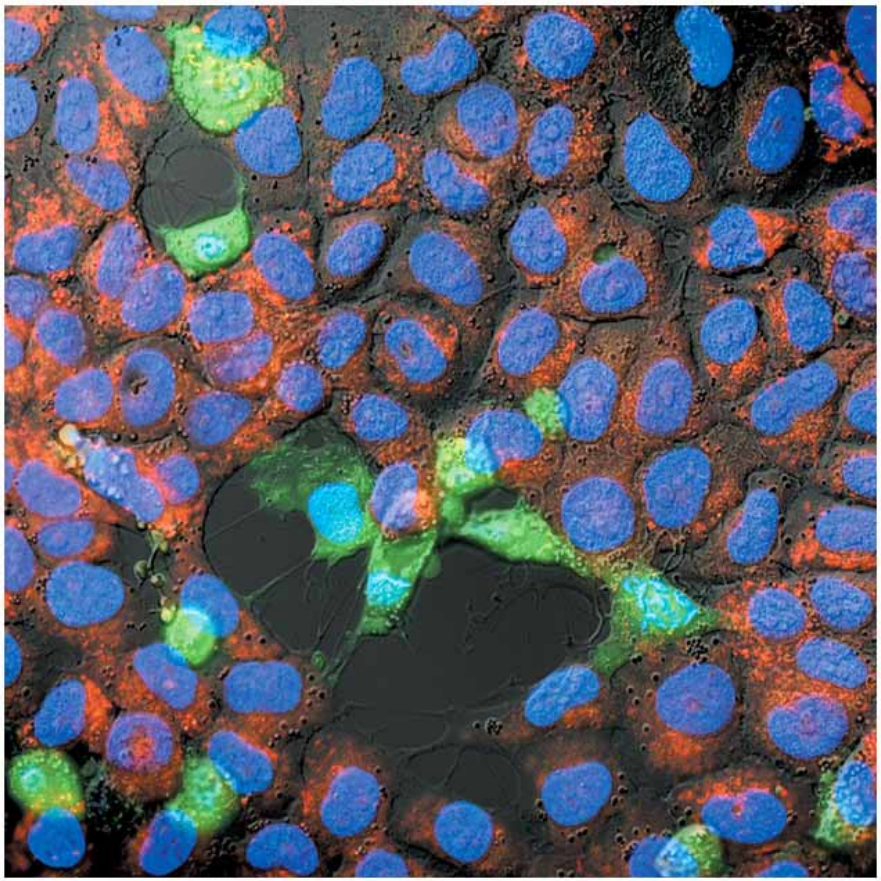


Caspases

- Caspases are Serin-Proteases.
- They are stored in the cell as inactive precursor molecules (Zymogenes) and get activated by assembly or limited proteolysis.
- They cleave proteins after very defined sequences
- Substrates for caspases are synthetically made with the same sequence and a dye molecule, which only has its color in the free form

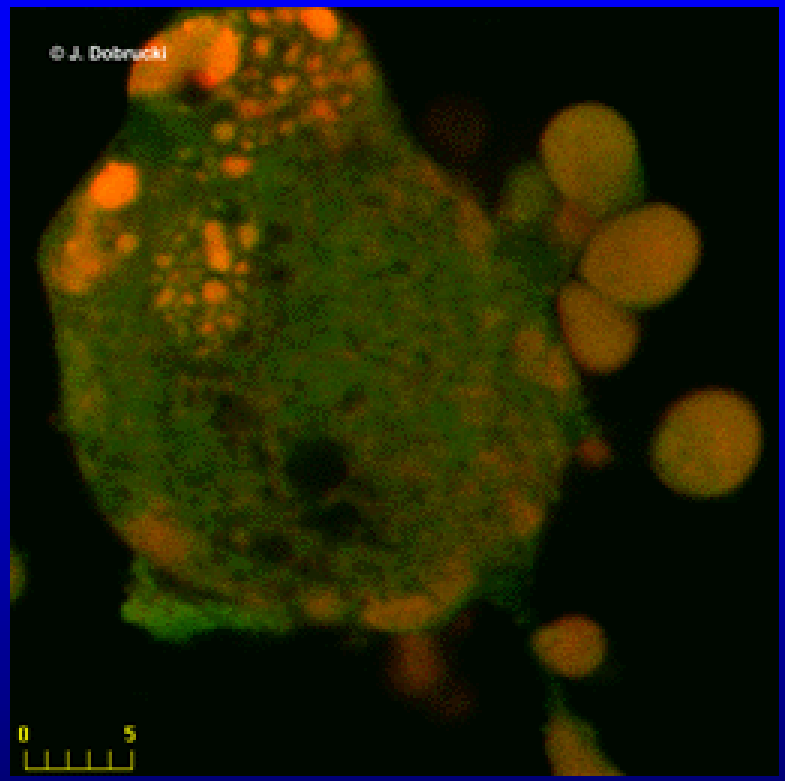


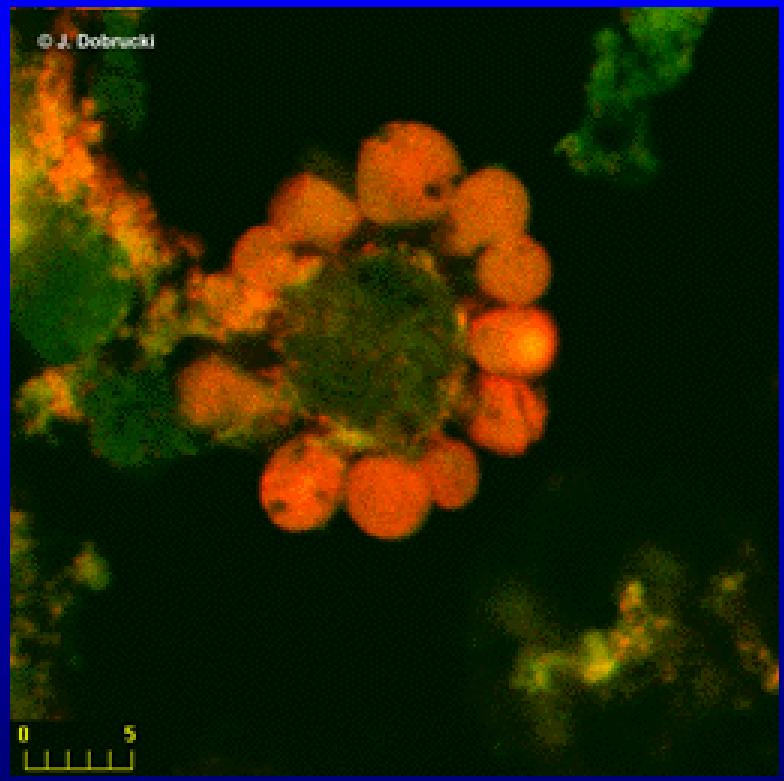
Caspase Substrates (Exemples)



Detection of apoptosis in SK-N-MC neuroblastoma cells. Following a six-hour exposure to hydrogen peroxide, cells were labeled with Hoechst 33342, tetramethylrhodamine ethyl ester (TMRE) and rhodamine 110, bis-L-aspartic acid amide for 15 minutes. Apoptotic cells show green cytosolic fluorescence resulting from cleavage of the rhodamine 110, bis-L-aspartic acid amide substrate by active caspase-3. The staining pattern of the Hoechst 33342 dye reveals that the majority of the rhodamine 110–positive cells also contain condensed or fragmented nuclei characteristic of apoptosis. Furthermore, the rhodamine 110–positive cells are also characterized by an absence of polarized mitochondria, as indicated by their failure to load the positively charged mitochondrial indicator TMRE. The image was contributed by A.K. Stout and J.T. Greenamyre, Emory University.

Nucleus Fragmentation





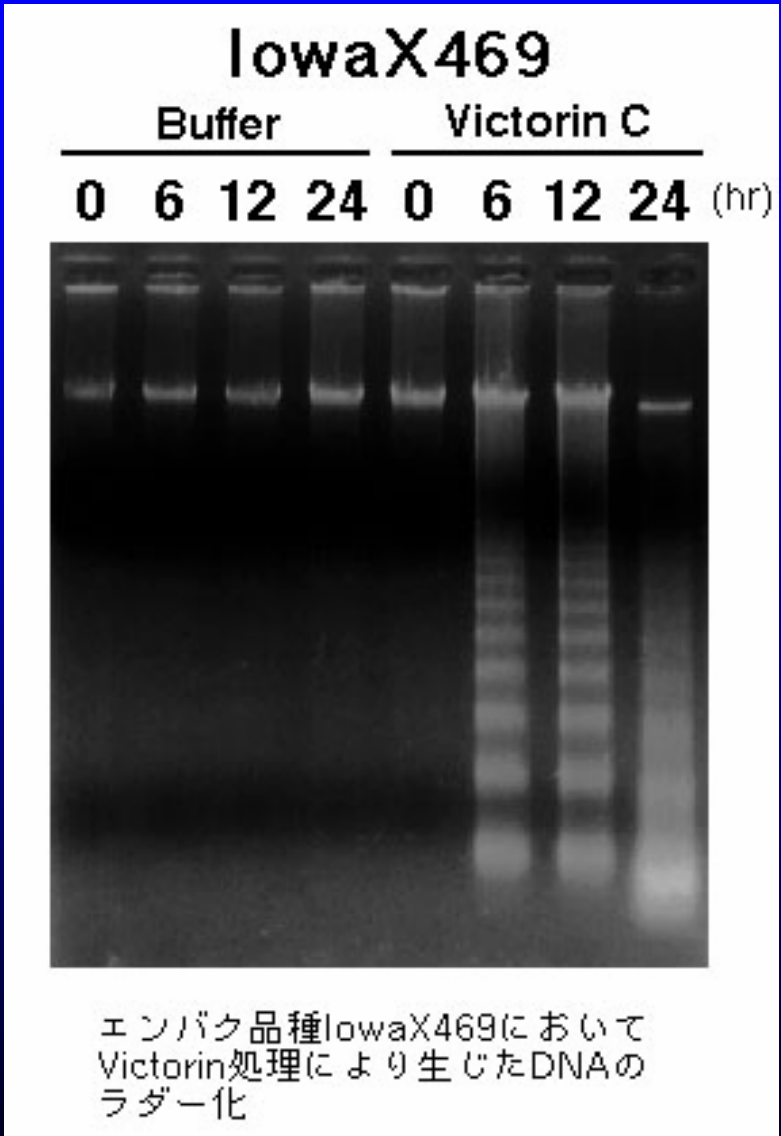
Fibroblast in early apoptosis

Condensation of the chromatin in the cell nucleus

See also the vacuoles in the cytoplasm

Fibroblast in advanced apoptosis

DNA Degradation

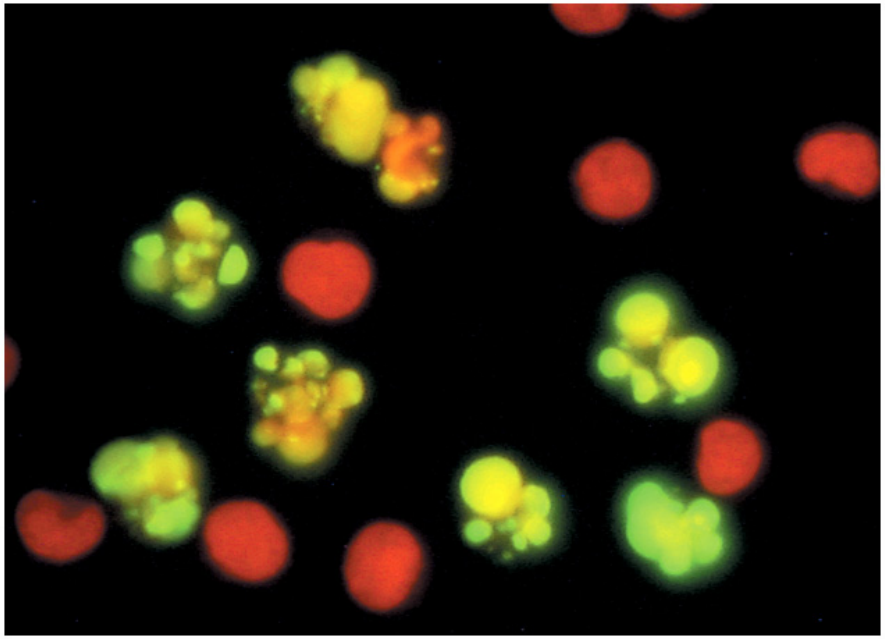


エンバク品種IowaX469において
Victorin処理により生じたDNAの
ラダー化

DNA Laddering

- At apoptotic DNA degradation the DNA is cleaved in integer multiples of 180 basepairs.
- DNA electrophoresis shows these fragments as a ladder. (degradation by other reasons would be only a homogeneous smear)

TUNEL Test



Human lymphoma cells treated with camptothecin for four hours and stained using the APO-BrdU TUNEL Assay Kit. Cells containing DNA strand nicks characteristic of apoptosis are detected by TUNEL and fluoresce green, while necrotic cells are stained with red-fluorescent propidium iodide.

- The endings of the apoptotic DNA fragments can be labeled specifically (TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling)

⇒ Wide range of fluorescence and color reactions and fluorescence, indirect immunochemistry… possible

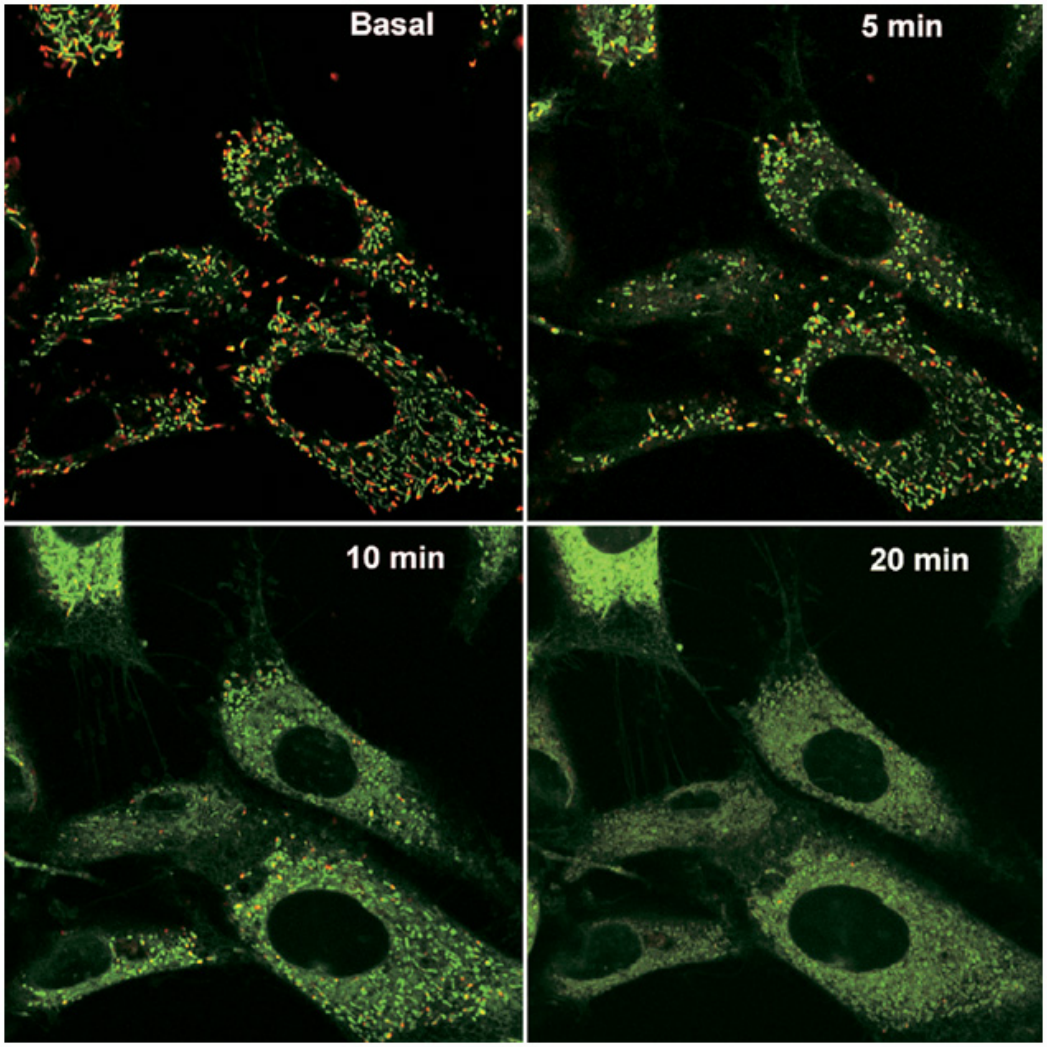
- Test can be performed at fixed material

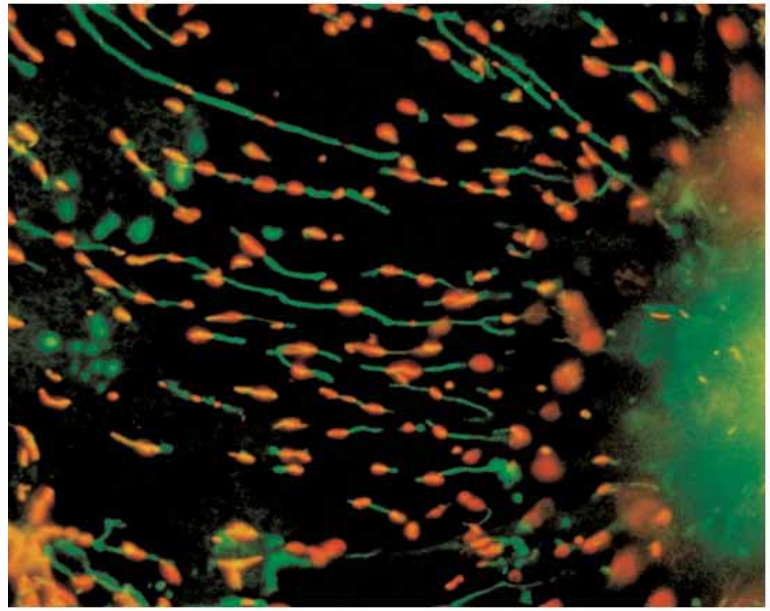
Mitochondrial Potential

Mitochondria generally build up an ion and electrical gradient over their membrane

- Due to pore formation in the mitochondrial membrane during apoptosis this potential breaks down.
- Fluorescent dyes JC-1, Rhodamine 123, MitoTracker Red CMXRos show fluorescence dependent on the mitochondrial potential
- Staining must be done at fresh material, but fixation of the MitoTracker Red stain is possible.

Examples JC-1





(Left) 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. The images were contributed by Ildo Nicoletti, Perugia University Medical School.

Toxicity Tests

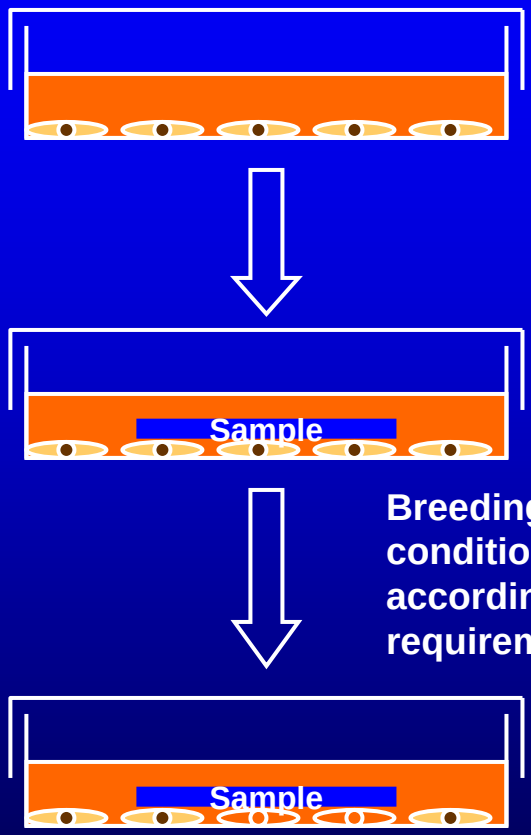
Toxicity Tests

A number of tests is established and standardized for toxicity testing (EN ISO 10993-5)

- Use of established cell lines (frequently L929)
- Defined sample preparation and handling
- Selection of appropriate controls

- Tests:
  - Direct Contact Test
  - Agar Overlay Test
  - Elution Test

Direct Contact Test (ISO ISO 10993-5 8.3)

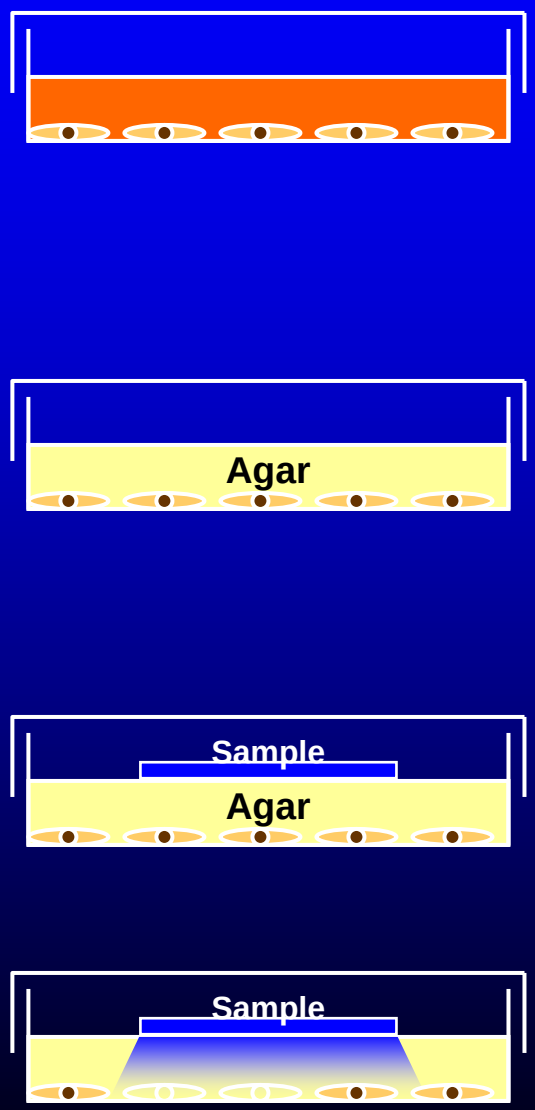


- Cells grow to ~80% confluency on the in the cell culture plate (exponential growth rate)
- Place test samples + controls **on** the cell layer, should cover ~10% of the cell layer (alternatively: seeding cells on the sample is acceptable)
- Do any appropriate toxicity test
  - Morphology
  - Uptake or outflow of a dye
  - LDH release
  - MTT test

Problems of the Test:

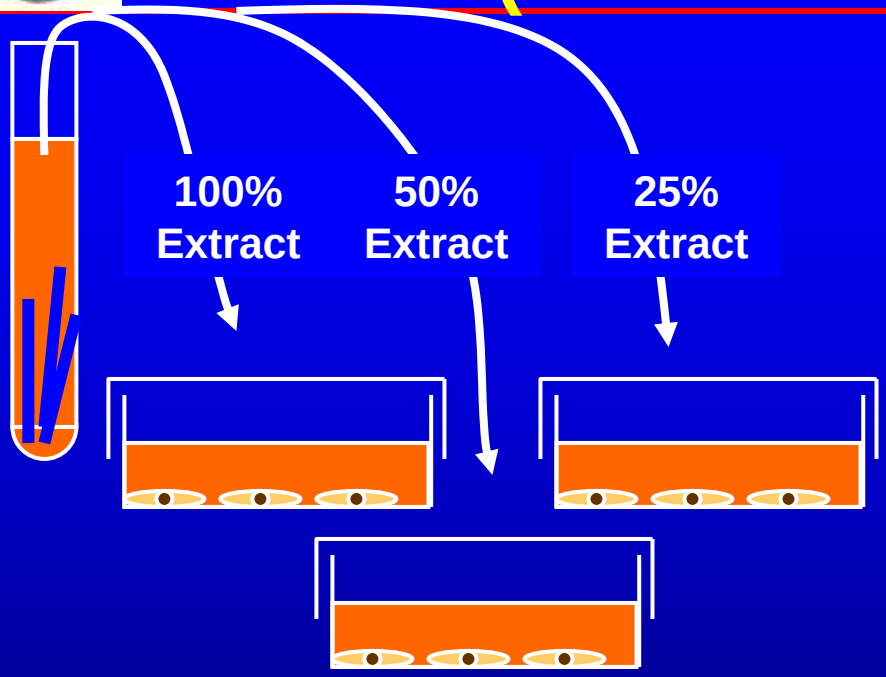
- Mechanical damage of the cells
- Reduced oxygen supply

Agar Overlay Test (ISO 10993-5 8.41)

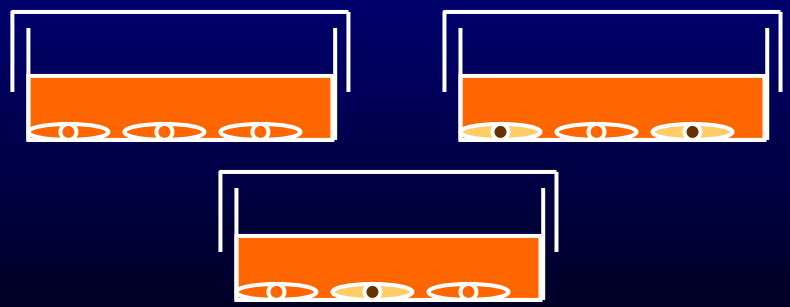


- Cells at the end of the exponential growth phase
- Overlay with medium with 0.5 – 2% agarose, which is suitable for cell culture
- Place samples + controls on the agar layer (~10% of the cell area)
- Any toxicity test

Elution Test (ISO ISO 10993-5 8.3)



Cell culture at appropriate conditions. 24-72 hours



- Prepare an extract of the samples. Conditions to be defined individually. Typically:
  - 3-5 cm^2/mL
  - > 24h at 37°C
  - 72h at 50°C or
  - 24h at 70°C or
  - 1h at 121°C
- Give extract at different dilutions to the cell cultures

- Any vitality/toxicity test

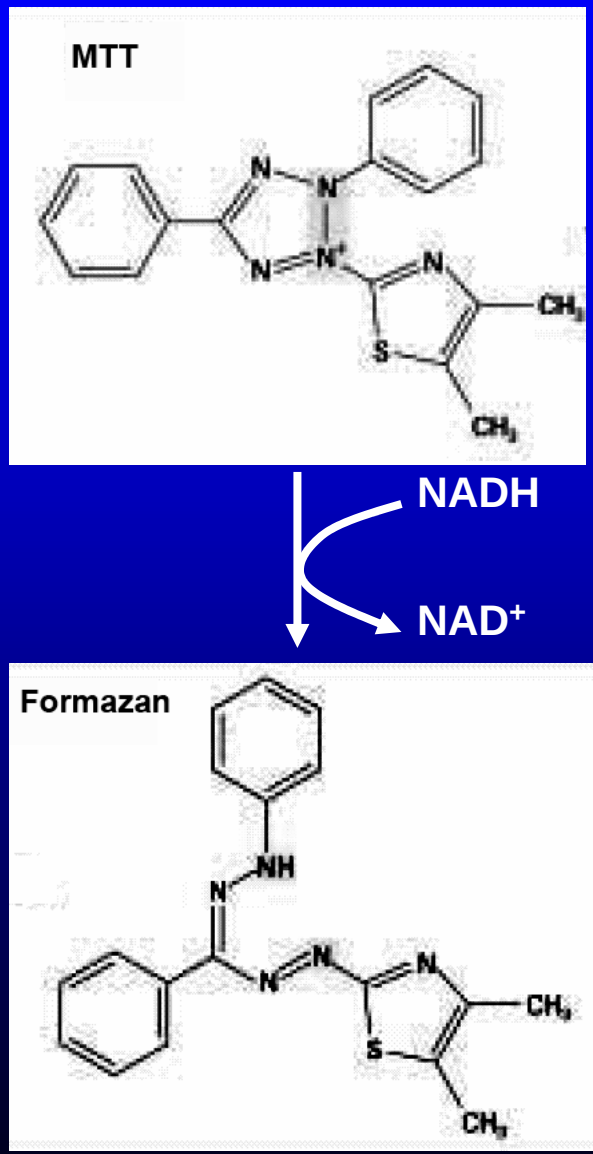
Vitality and Proliferation Tests

Vitality and Proliferation Tests

Check of cell functions, which relate to cell activity

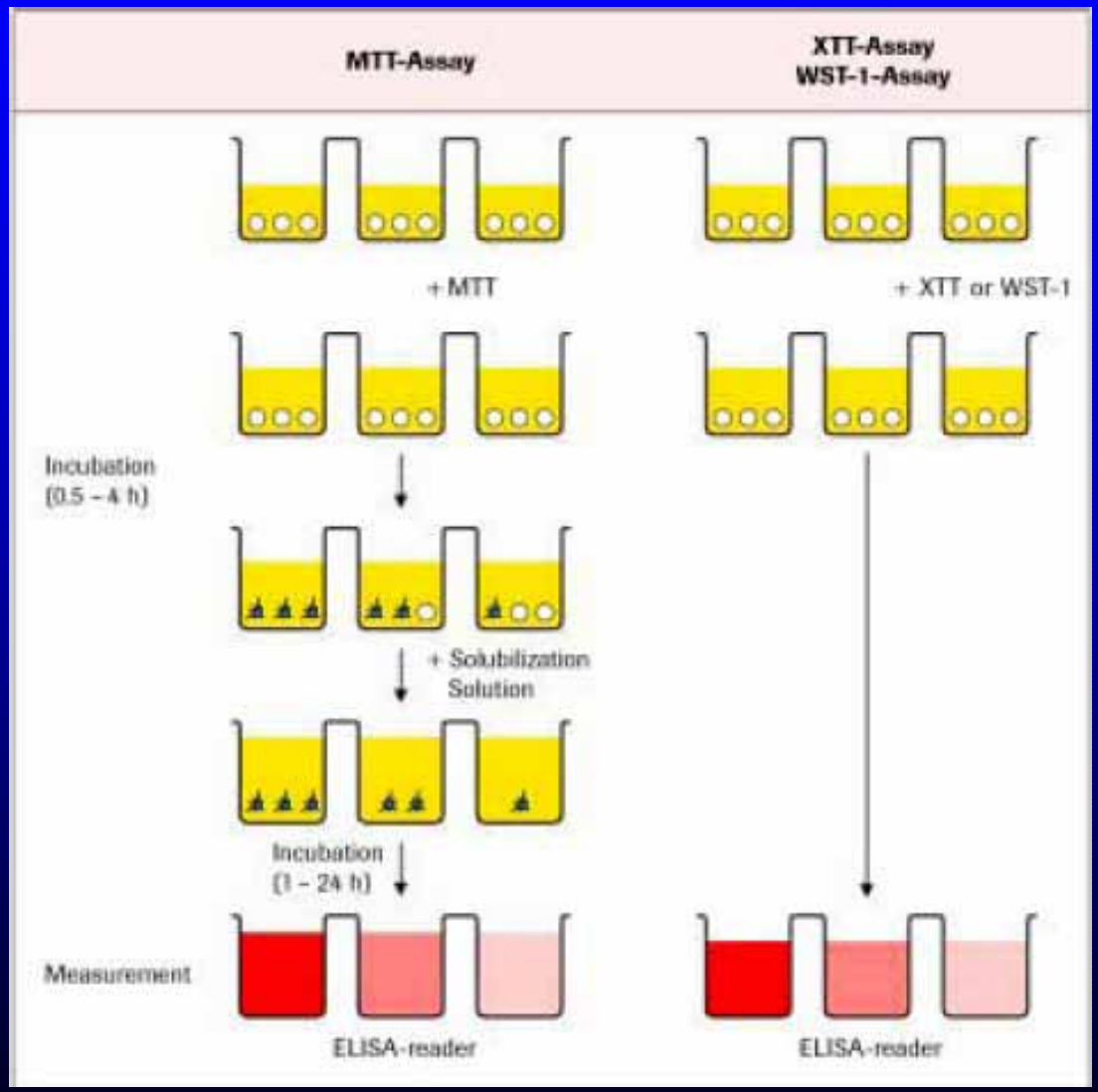
- Cell (energy) metabolism
  - Marker for cell activity
  - Mainly integral tests: total activity is proportional to the number of cells and the individual activity
  - Increased activity usually is a good sign but may also indicate activation and adverse signaling
- Cell proliferation
  - In general a good sign
  - Be aware that proliferation in cell culture is higher than in the body

MTT Test



- MTT is a (mainly colorless, slightly yellow) substance
- Vital cells reduce it in their mitochondria and in the cytoplasm to the blue colored, on water solvable formazan
- The formazan can be dissolved in acidic organic solvents and absorption measured at λ= 570nm.
- Pro:
  - The test is very simple
  - Good linearity with cell activity
- Contra
  - Cells/ samples are destroyed by the organic solvent
- Alternatives
  - XTT, WST-1 or Alamar Blue™ have solvable products

MTT Test



- In principle all steps of the test can be performed in the same well of the cell culture plate
- XTT, WST-1 and Alamar Blue™ test do not require dissolution of the product ⇒ No destruction of the cells, experiment can be repeated.

Analysis of Cell Proliferation

Cumulative (integral) tests

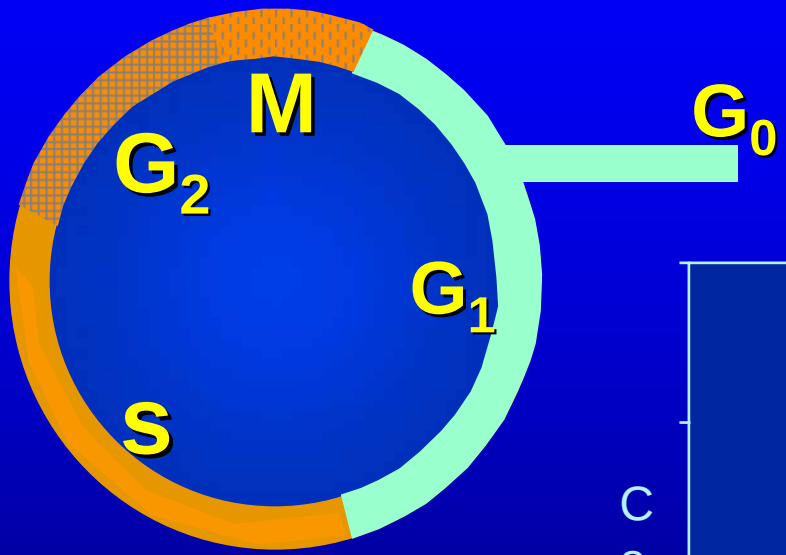
- Counting cells
  - Remove cells with trypsin from a sample and count
  - Fixate, count in SEM
  - Make fluorescent stain, count in fluorescent microscope
  - (Dry, count in dark field microscope)
- Measure a cell product
  destroy the cells and measure a product, which is proportional to the cell number
  - Total protein (Bradford, BCA, Coomassie Blue…)
  - Total DNA
  - LDH

Analysis of Cell Proliferation

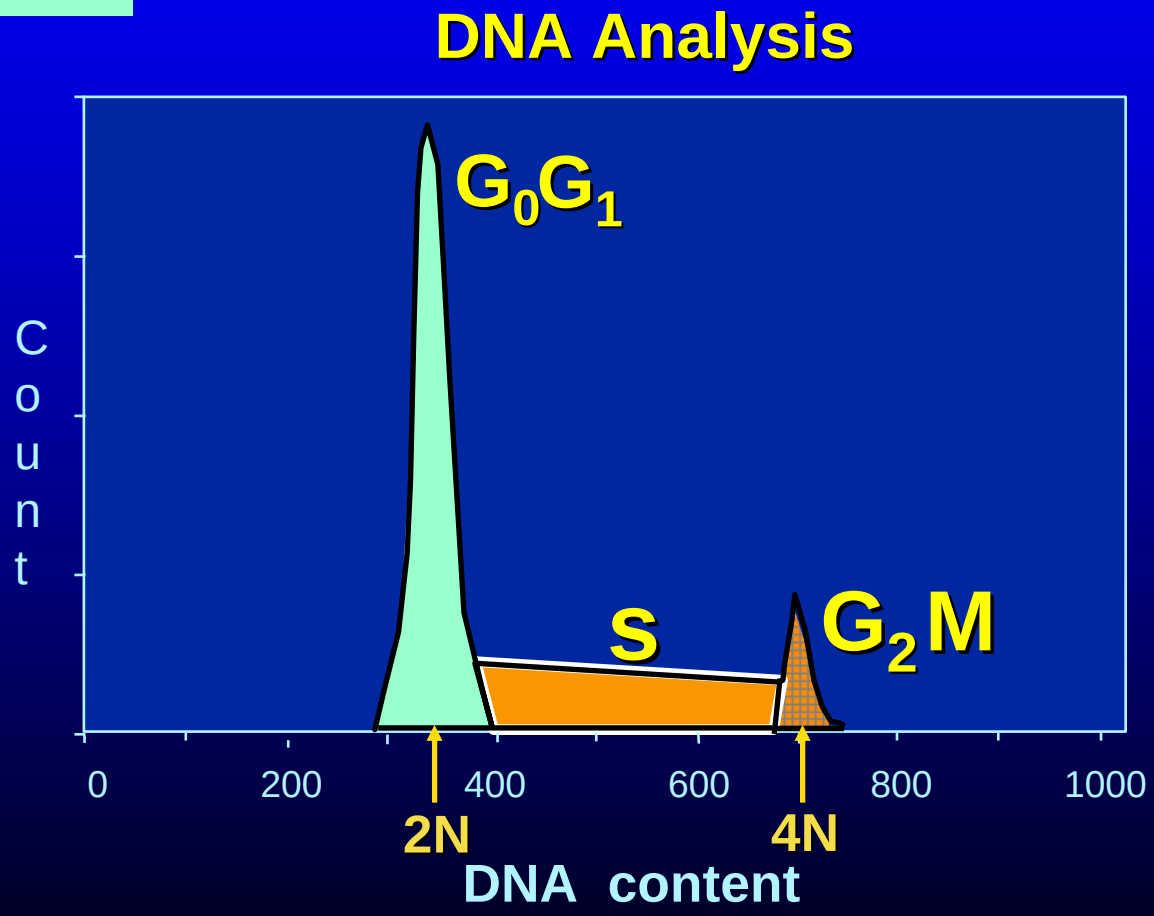
Punctual (differential) tests

- Proliferation markers
  dividing cells express specific proteins on their surface. Detection by immunochemistry
  - Ki67
  - Proliferating cell nuclear antigen (PCNA)
  - Transferrin receptor (CD71)
- Detection of DNA amplification
  before cell division the cells must duplicate their genetic information for the two daughter cells
  - The amount of DNA per cell can be measured with fluorescent DNA stains (propidium iodide) by FACS (Cell cycle analysis)
  - Measurement of the incorporation of thymidine or analogues
    - H^3 Thymidine $\Rightarrow$ detection of radioactivity
    - Bromodeoxy Uridine (BrdU) $\Rightarrow$ detection by immunochemistry

The Normal Cell Cycle



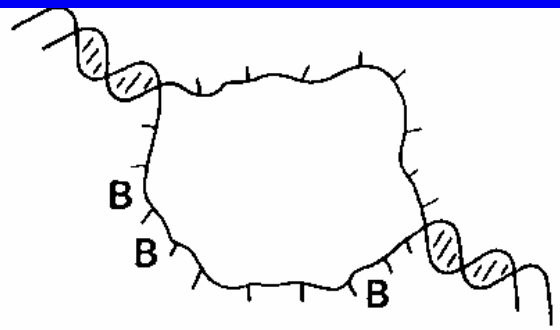
Cells most of the time are in a resting phase (G_0) with the standard amount of DNA. For proliferation they enter the cell cycle, duplicating the genetic information (S[ynthesis]) and performing the actual cell division (M[itosis])



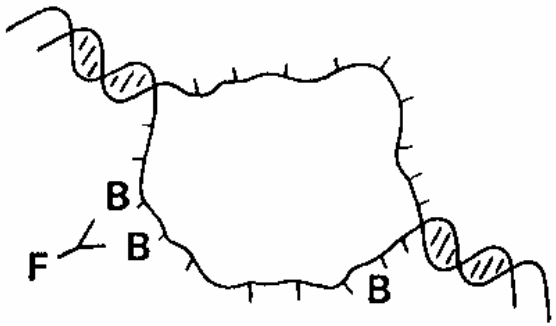
Purdue University Cytometry Laboratories

BrdU Detection

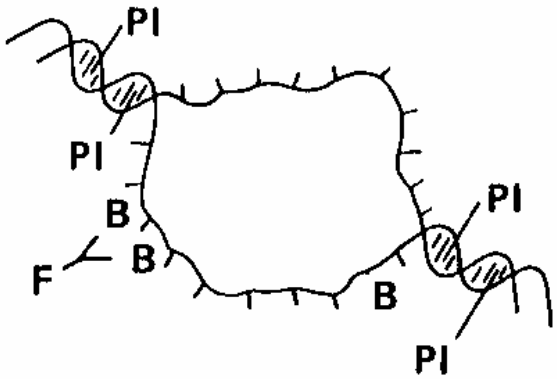
Partially denature cellular DNA



Immunochemically detect the incorporated BrdUrd and suppress nonspecific staining and/or autofluorescence



Fluorescently stain the remaining dsDNA



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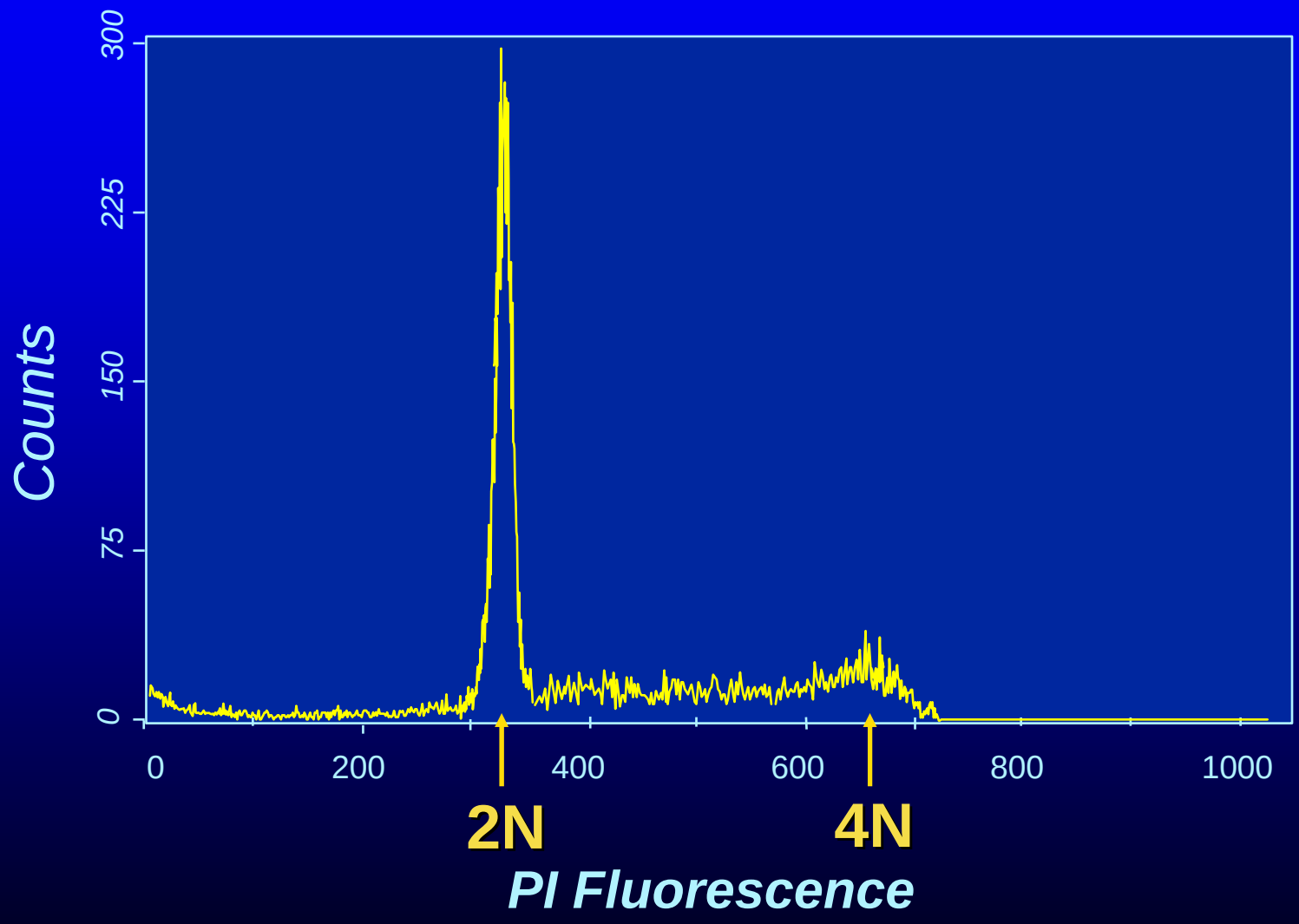
Cell Cycle Analysis in FACS



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