DNA / Cell cycle measurement

DNA/ RNA dyes

- Propidium Iodide
- Ethidium Bromide
- Hoechst dyes
- DRAQ5
- Cyanine dyes e.g. TO-PRO-3, SYTO/SYTOX dyes
- Acridine Orange (RNA/ DNA ratio)
- Pyronin Y
- Styryl Dyes e.g. LDS-751
- Mithramycin, Chromomycin
- 7 Aminoactinomycin D (7AAD)
- Diamino-2-phenylindole (DAPI)

Which dye to use?

Excitation wavelength available	UV: Hoechst, DAPI 488: PI, 7AAD 633: TO-PRO-3
Specificity (Sequence)	None: PI A-T: Hoechst, DAPI G-C: 7AAD, Chromo-Mithramycin
Viability	Hoechst 33342 DRAQ5

We can use the DNA dyes in 2 ways

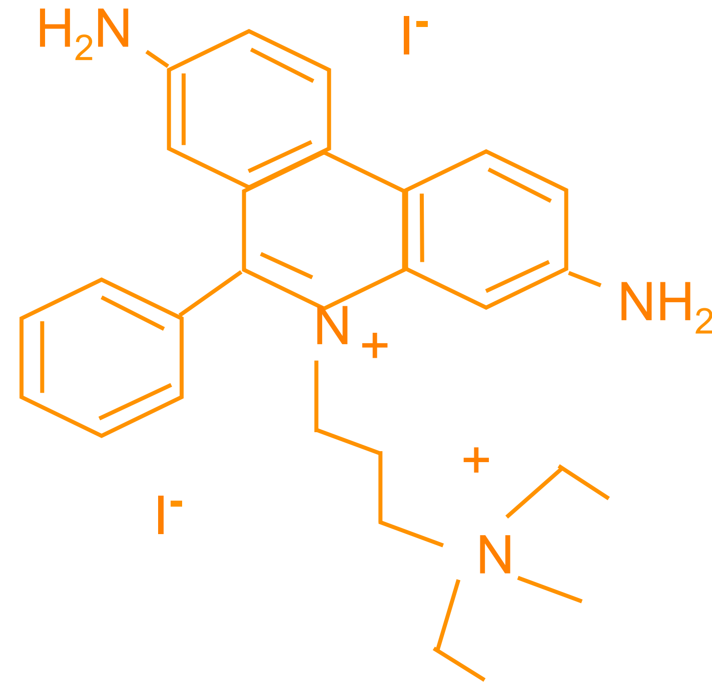
1. **To measure relative cellular DNA content**

2. For discrimination of live / dead cells

Propidium Iodide (PI)

Excitation: 488 nm

Emissionsmaxima: 575 nm; 620 nm



Intact cells exclude PI
(live/ dead cell discrimination)

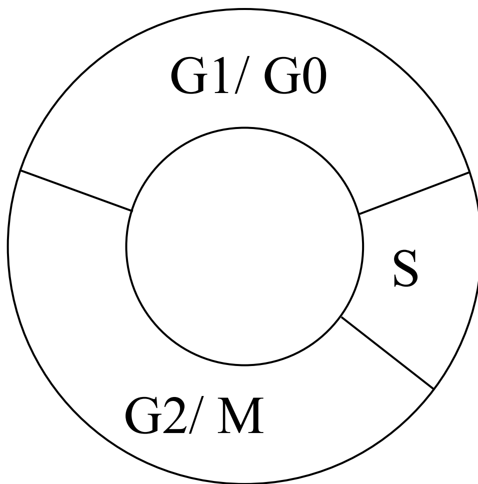
Fixated/ Permalized cells show PI staining
(DNA / cell cycle staining)

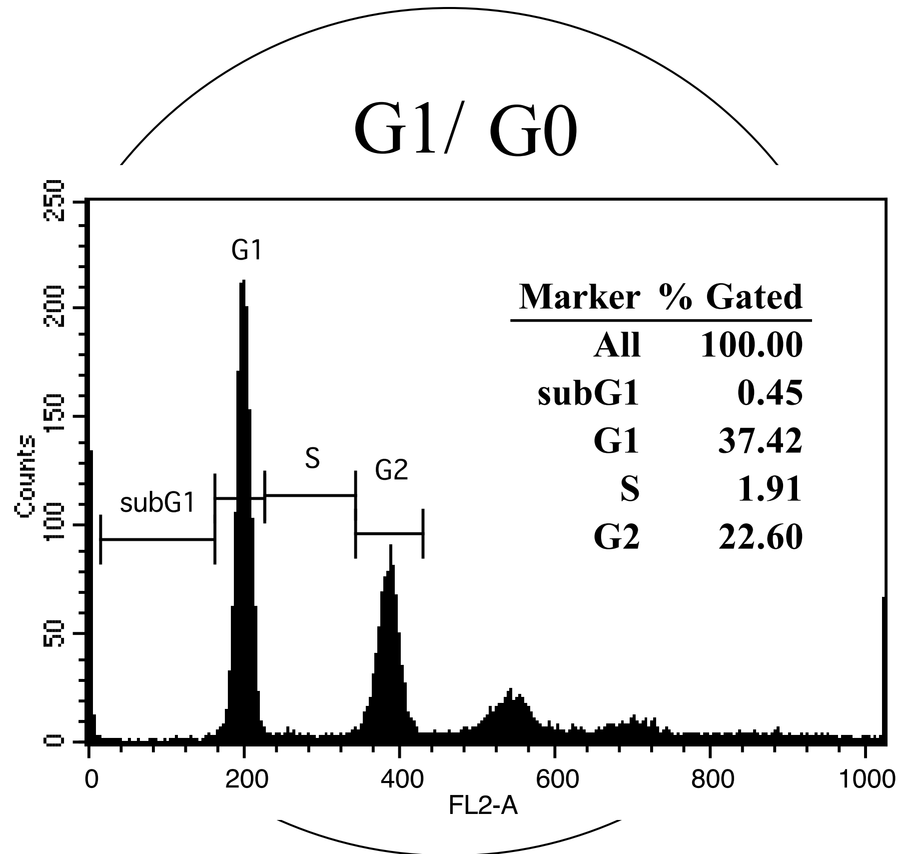
Staining concentrations:

1 µg/ ml for live / dead discrimination
50 µg/ ml for DNA / cell cycle analysis

For dead cell exclusion you can add PI shortly before your analysis, otherwise at least 10-15 min before your cell cycle measurements

Cell cycle



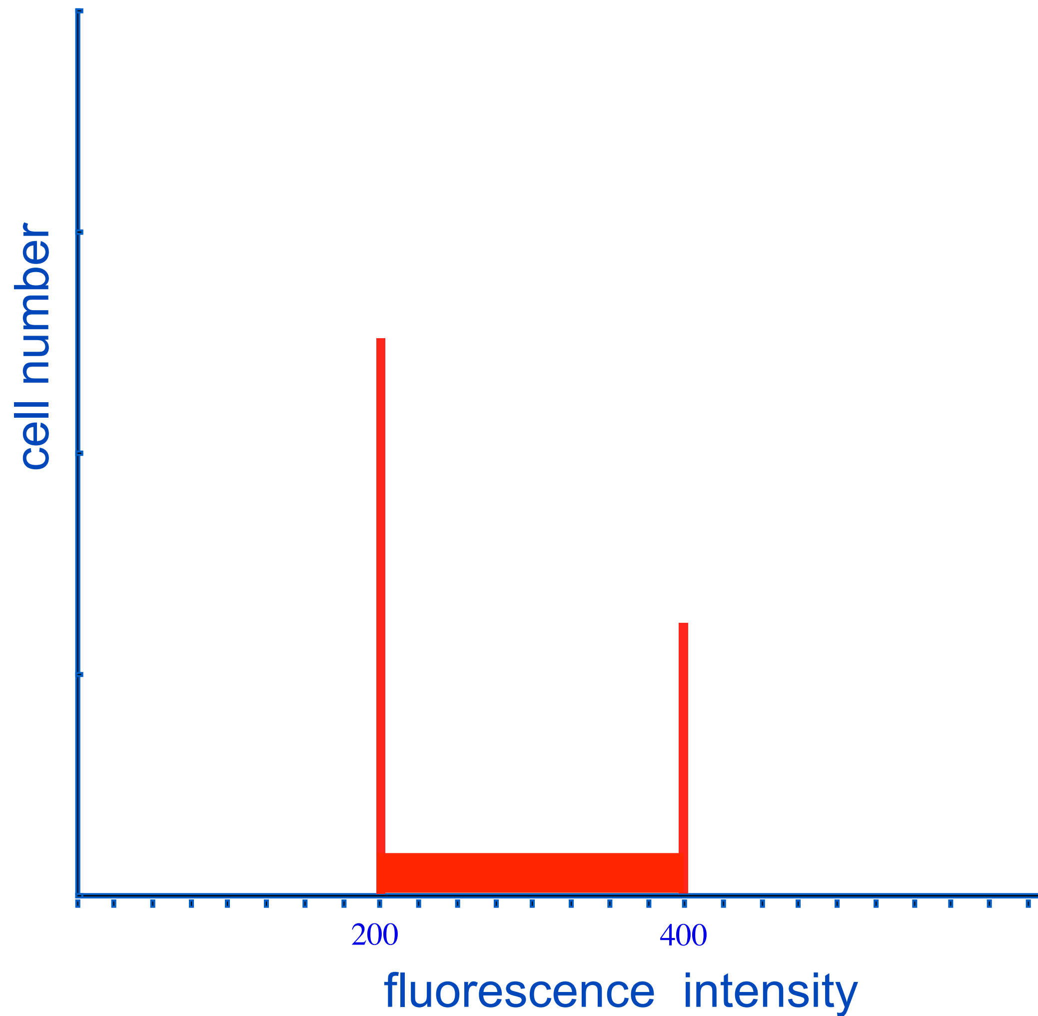


Marker	% Gated
All	100.00
subG1	0.45
G1	37.42
S	1.91
G2	22.60

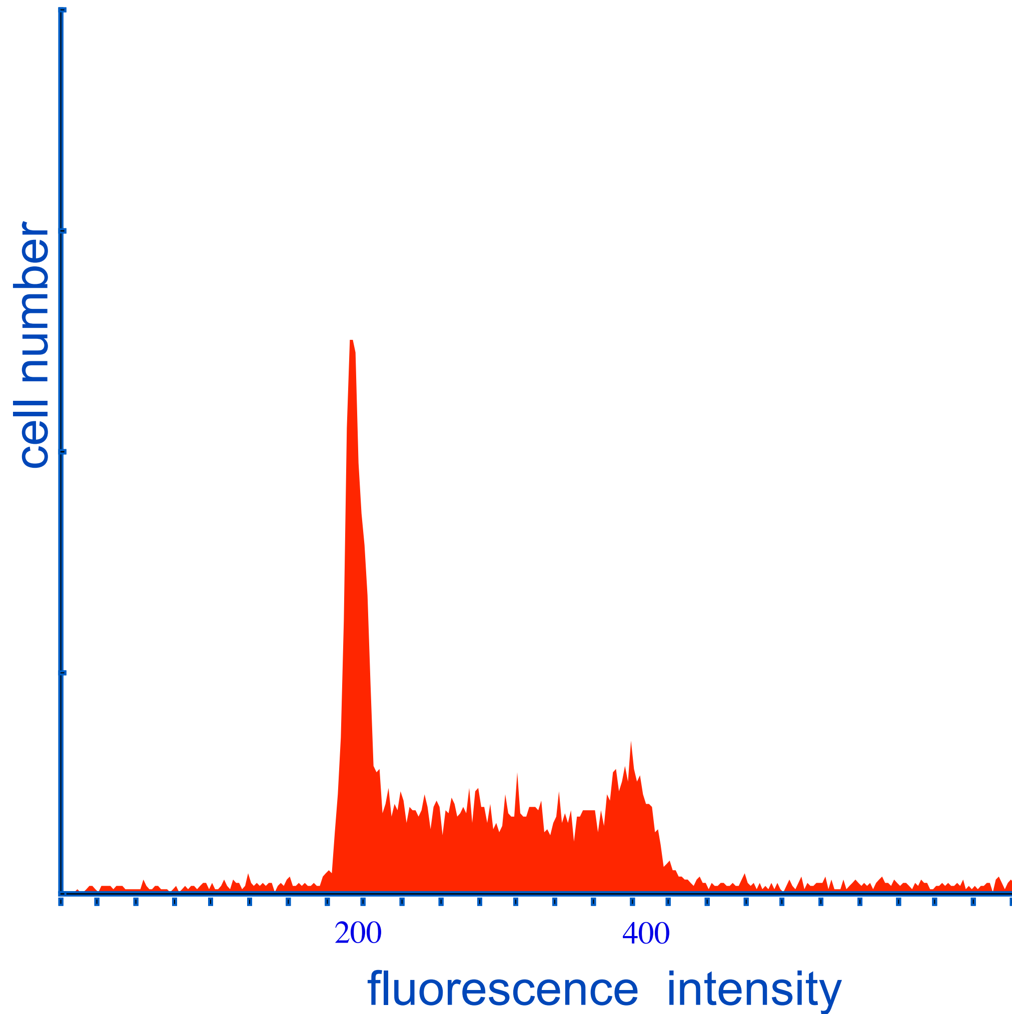
From 5 different cell cycle-phases only 3 can be distinguished by Flow Cytometry (due to their DNA-content)

n = chromosome-content

In an ideal world ...



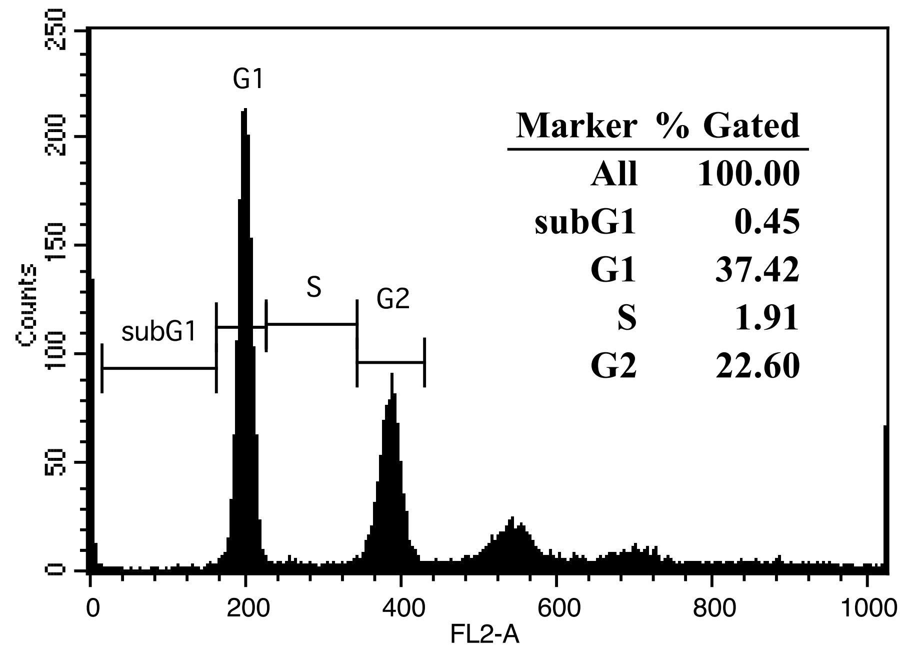
In the real world ...



Cell cycle-Analysis

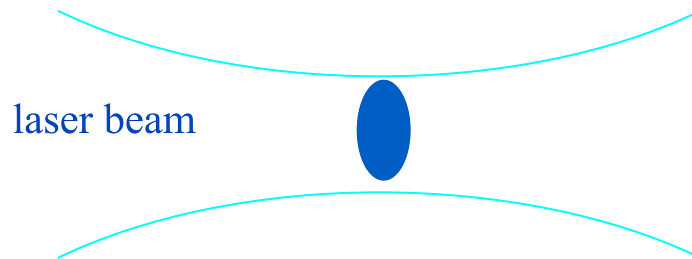
- A pre-requisite for flow cytometry is, that cells should be in a single cell suspension.
- How do cell clumps affect quantitation of DNA content?

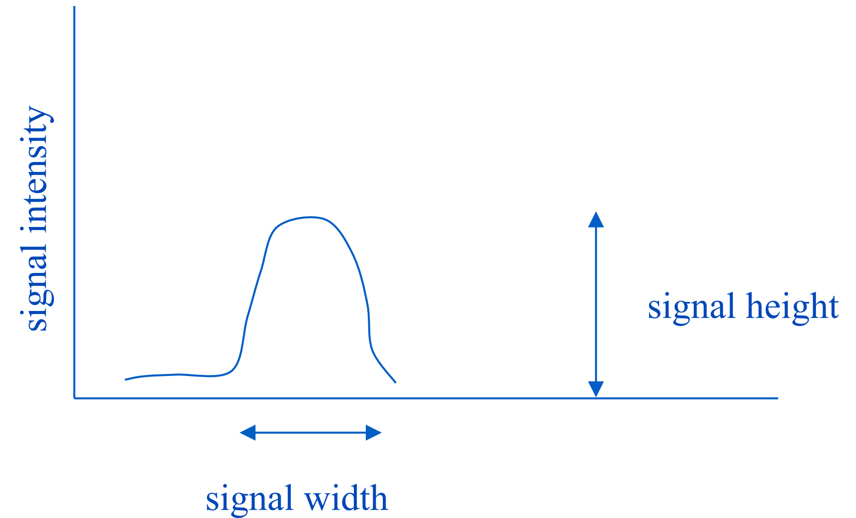
Cell cycle-Analysis

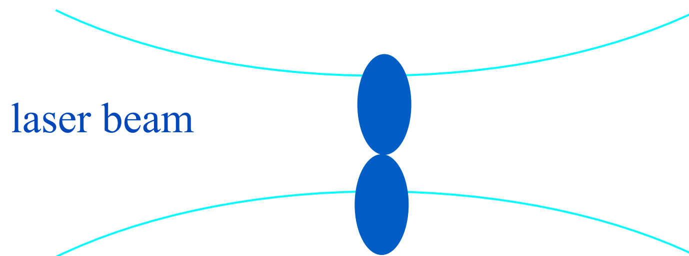


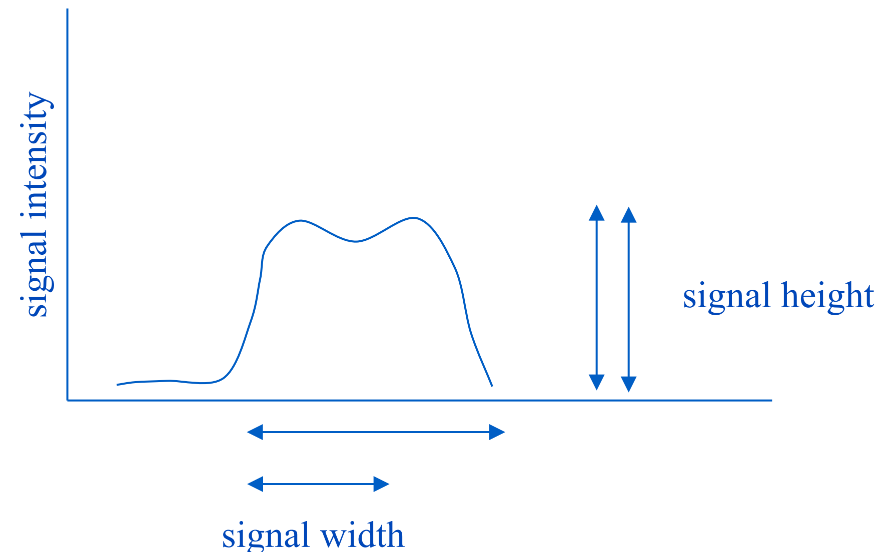
Note: linear scale !!

Doublet discrimination



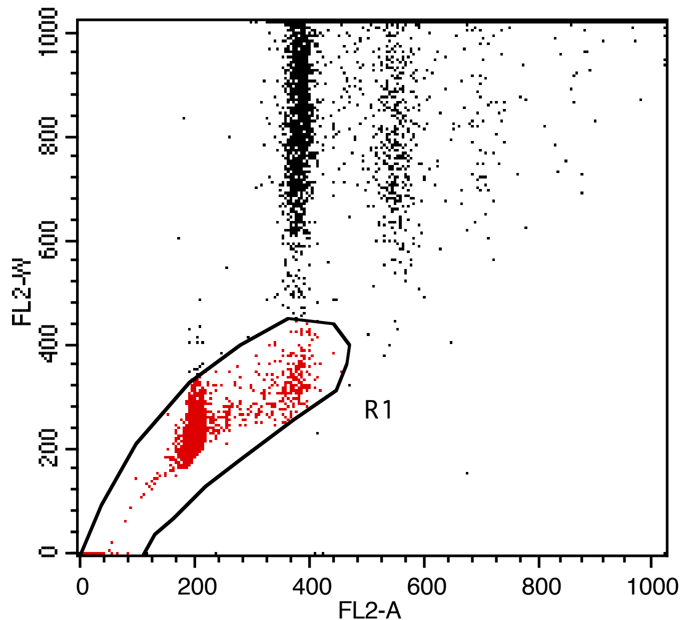




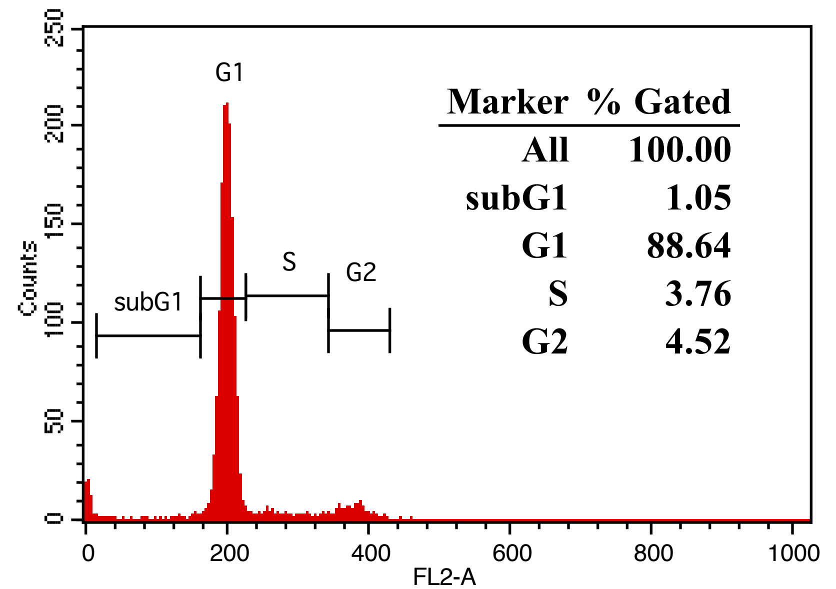


Cell cycle-Analysis

gating out the doublets



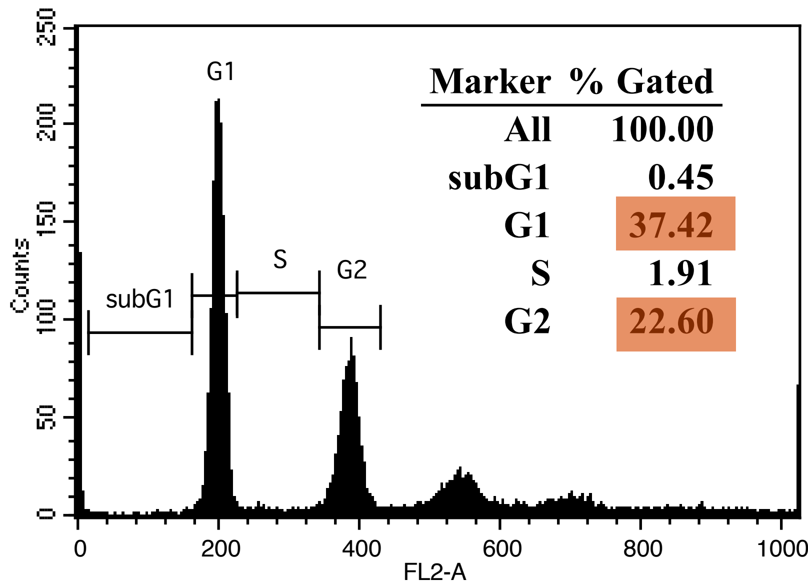
cell cycle-analysis



Marker	% Gated
All	100.00
subG1	1.05
G1	88.64
S	3.76
G2	4.52

Cell cycle-Analysis

gating out the doublets



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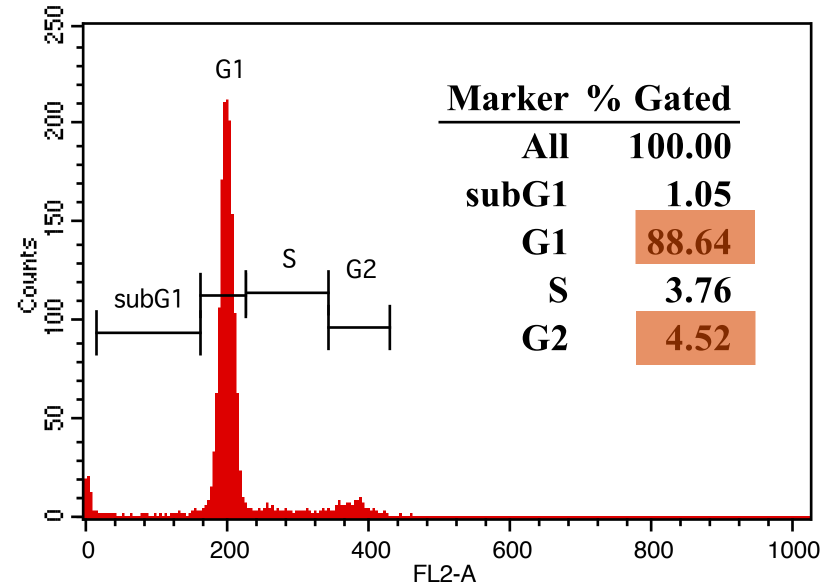
cell cycle-analysis



Marker	% Gated
All	100.00
subG1	1.05
G1	88.64
S	3.76
G2	4.52

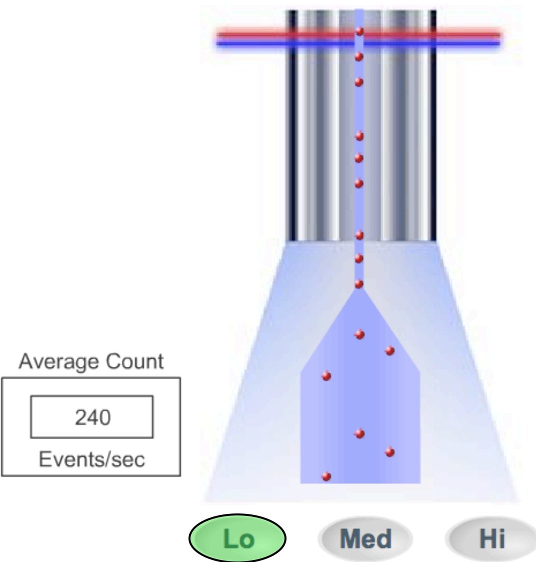
with doublets the cell cycle phase G2 is overestimated
other populations (e.g. G1) were underestimated

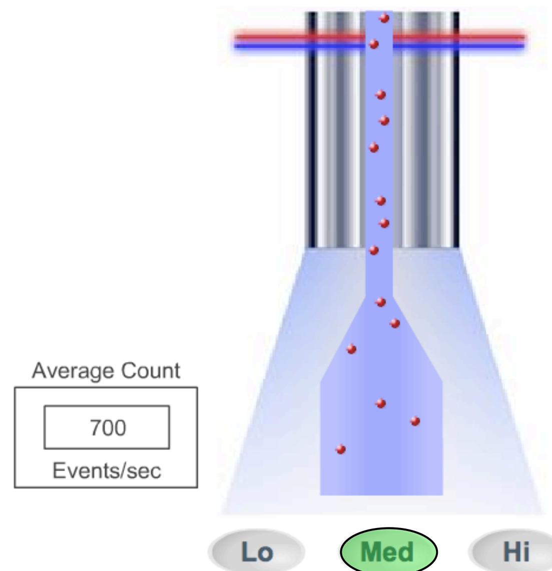
Flow rates have an impact on signal precision

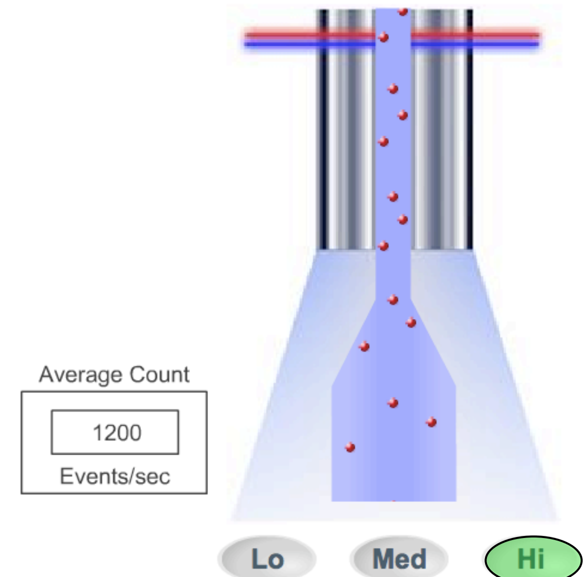
Low: ≈ 10 µl/ min

Medium: ≈ 60 µl/ min

High: ≈ 120 µl/ min

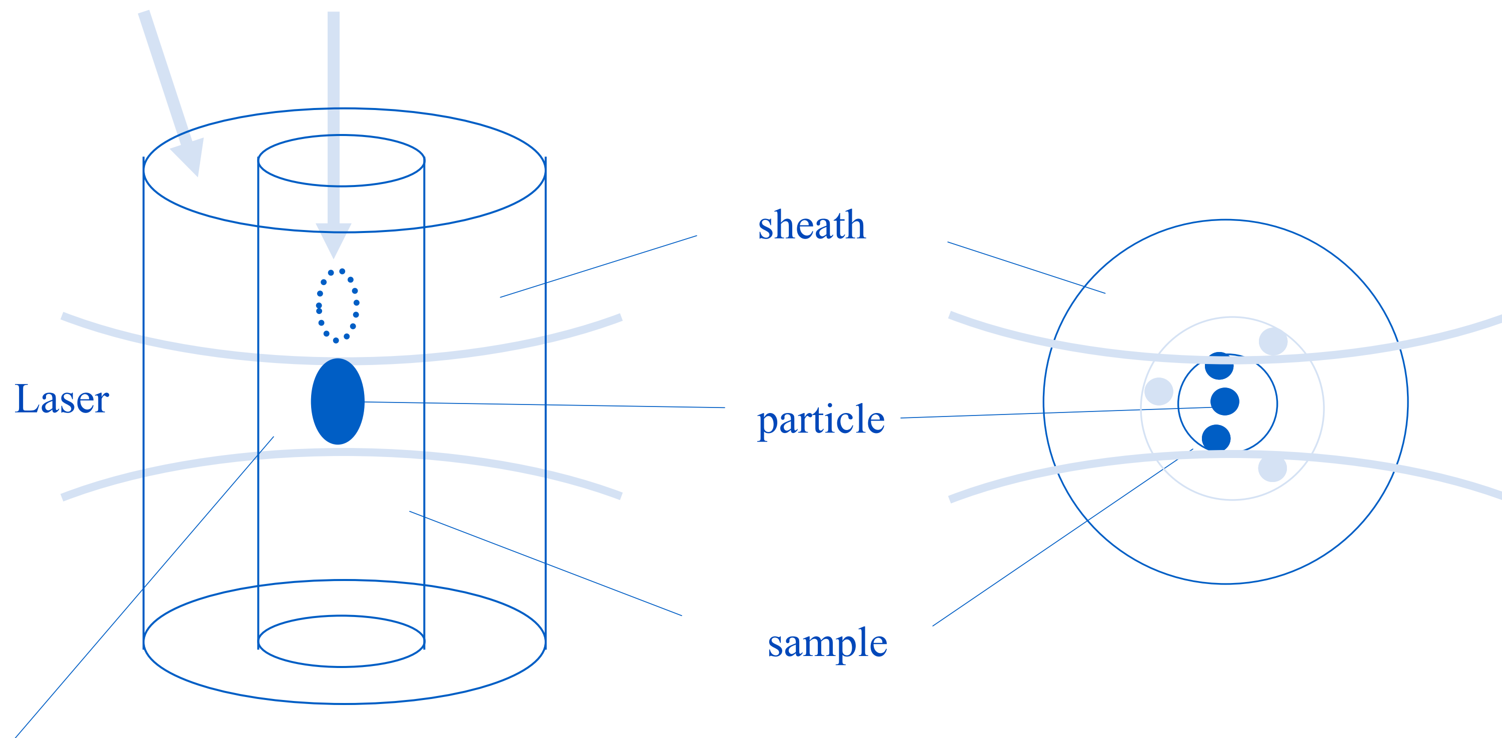






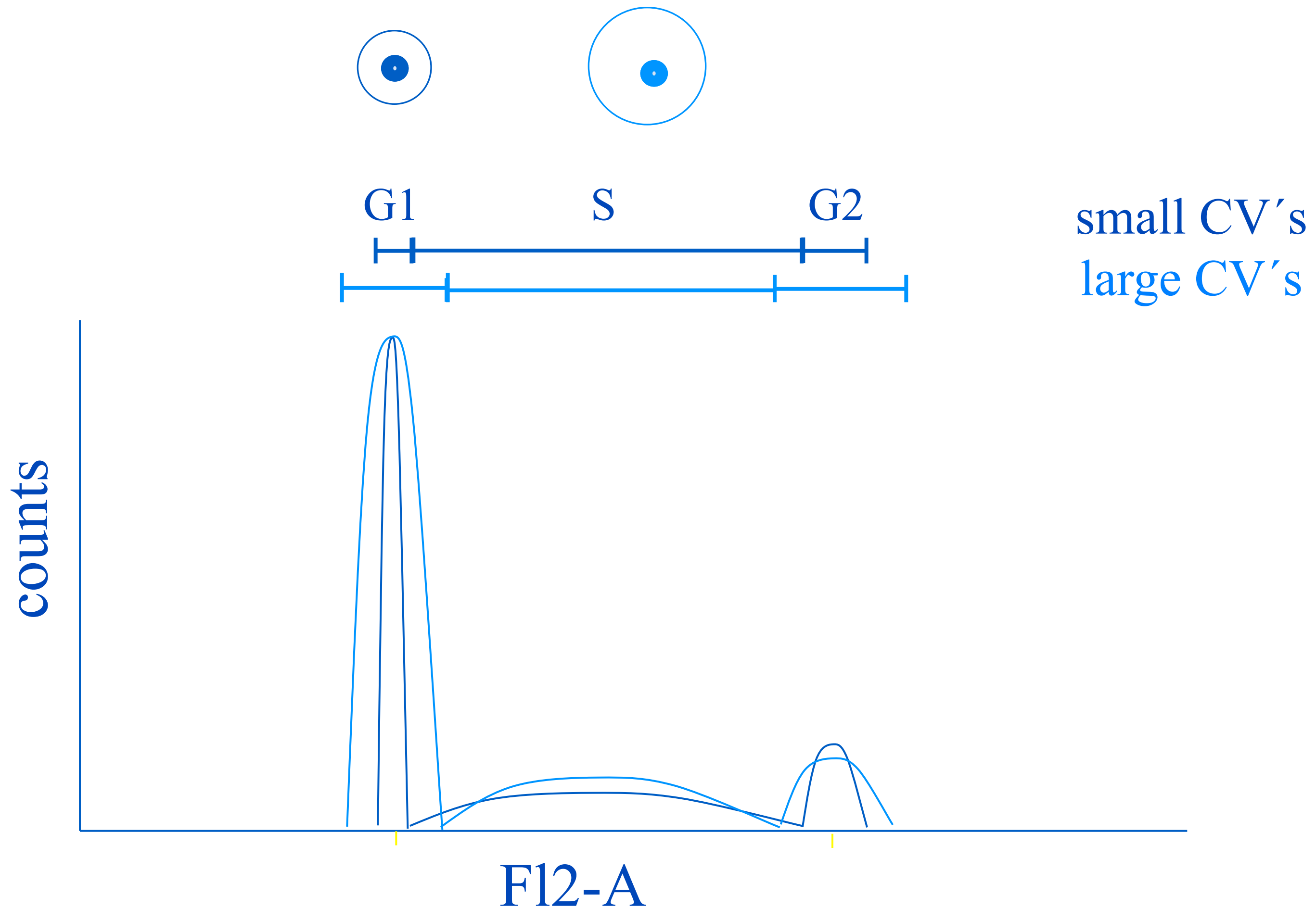
modified from BD online tutorial

Hydrodynamic Focus

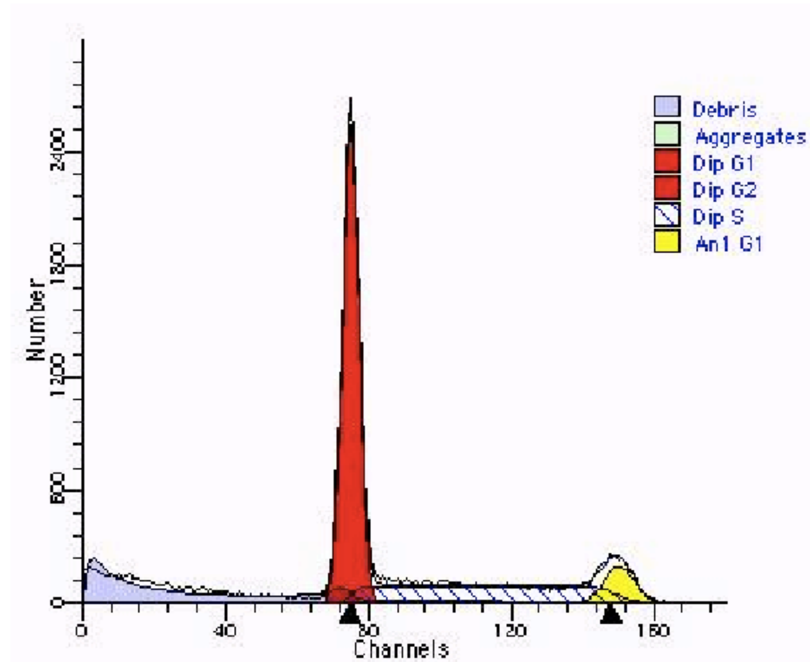


Focussing the cells in the stream

Flow rate and Quality of histograms



Analysis of DNA histograms ...



special software, e.g. ModFIT LT, FlowJo, ...
automate the process of analysis with mathematical modelling

Possible Problems

Problems	Likely reasons
Population shifts during measurment	To much PI in your stained sample; Or minimal residual ETOH in sample
Population shifts between samples	Cell numbers are different
Large CVs and weak resolution of histograms	To much free DNA/ RNA in the cell suspension
Large CVs	The flow rate is to high

Protocol for DNA-Analysis/ Cell cycle

- plate, cultivate and treat the cells
- harvest cells (1x 10^6 / ml)
- fixate cells for at least 30-60 min (cold Ethanol (-20°C))
  - be sure cells are well resuspended
  - add the cell suspension drop by drop to the alcohol while mixing suspension
  - (centrifugate cells and resuspend in cold PBS (for storage))
- treat cells (at least 30 min at RT) with RNase (50 µg/ ml)
- (count cells) and resuspend in PI (50 µg/ ml)
- FACS analysis

DNA + additional stainings

We can combine antigen staining or fluorescent protein expression with DNA staining and...

... see how many cells are expressing a particular antigen

or

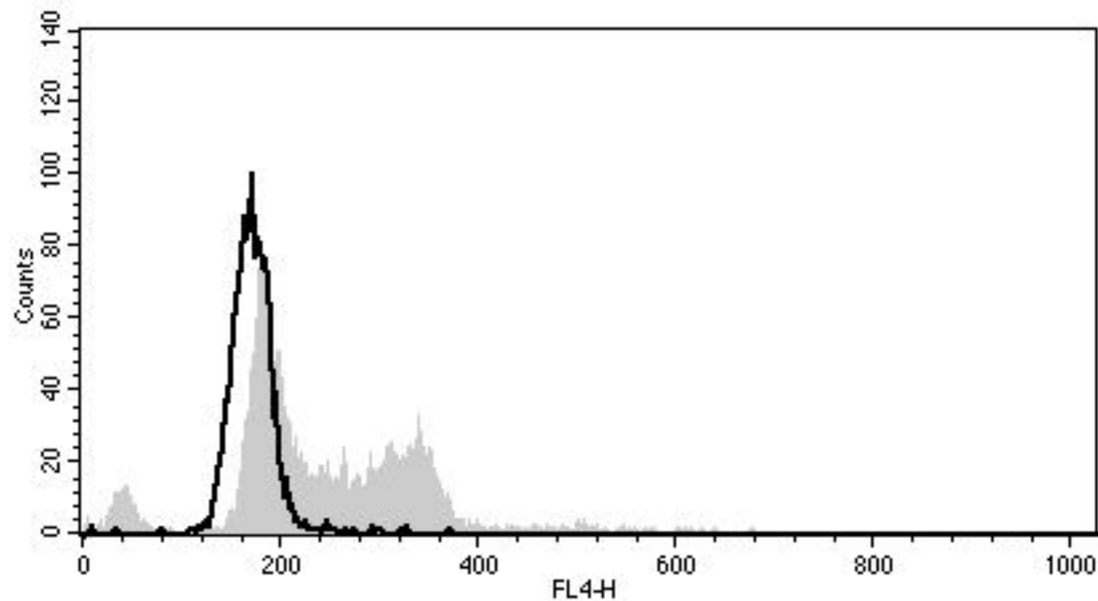
... see which phase an antigen is expressed

or

... look at the DNA profile of a selected subset of cells

Cell cycle-analysis with viable cells

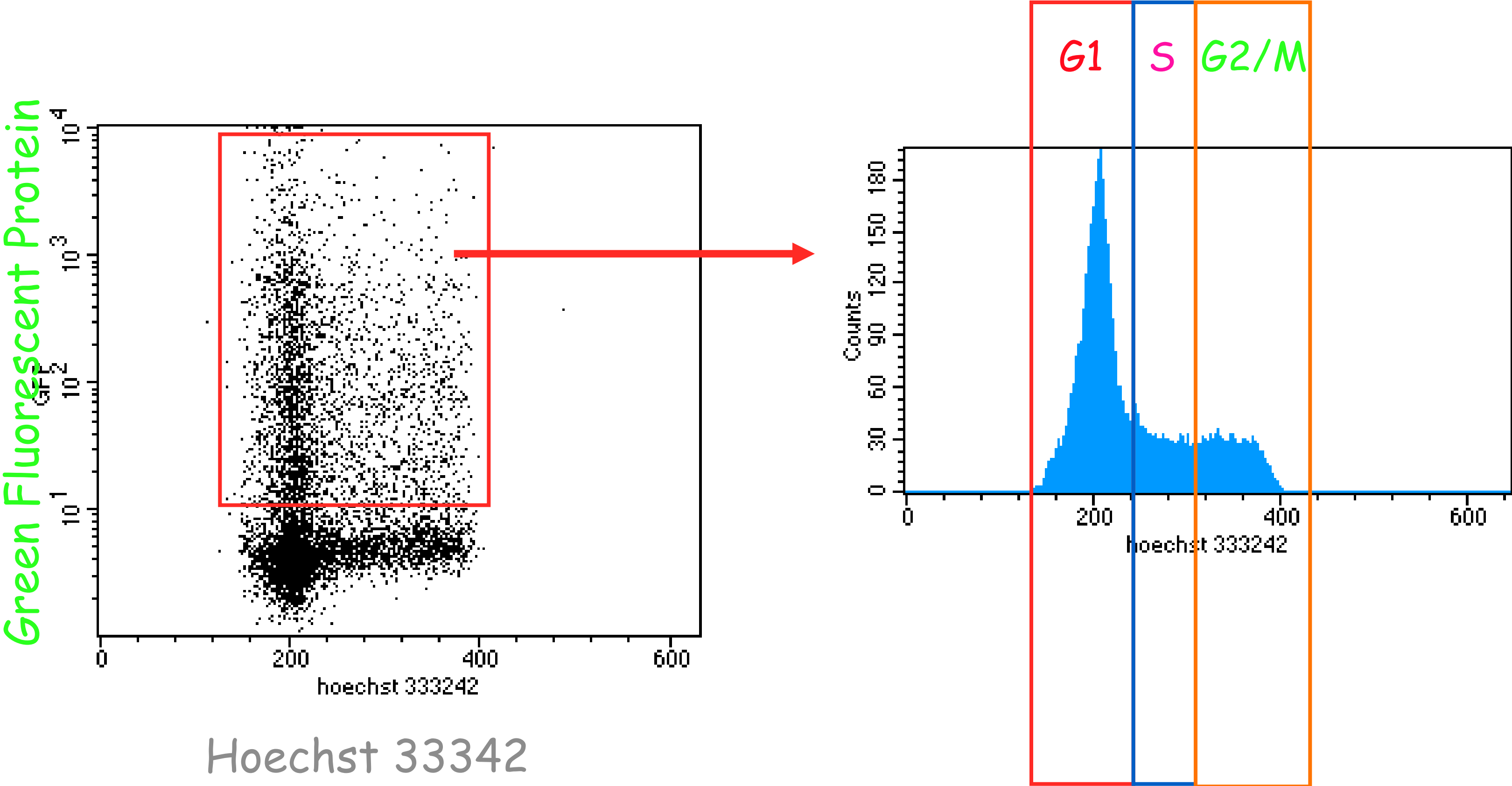
(e.g. Hoechst 33342)



Hoechst 33342

Cell cycle-analysis + fluorescent proteins

(e.g. EGFP)



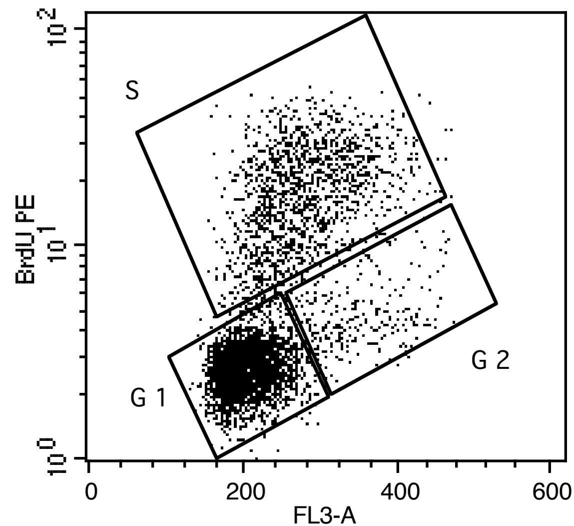
Specific S-Phase Analysis - BrdU labelling

- Thymidine analogue
- Taken up by cycling cells
- Use for comparative growth rates, length of cell cycle, pulse labelling
- Staining procedure involves unwinding DNA
- Combine with Propidium Iodide

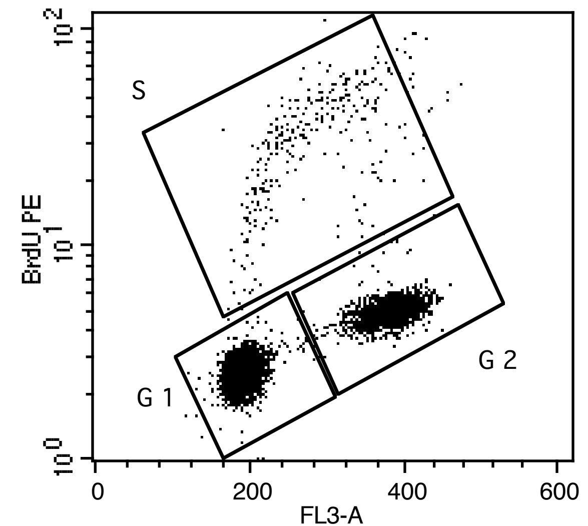
S-Phase analysis with BrdU

Bromo-deoxy Uridine is incorporated in DNA of cyclin cells during S phase and can be detected with specific antibodies.

untreated control

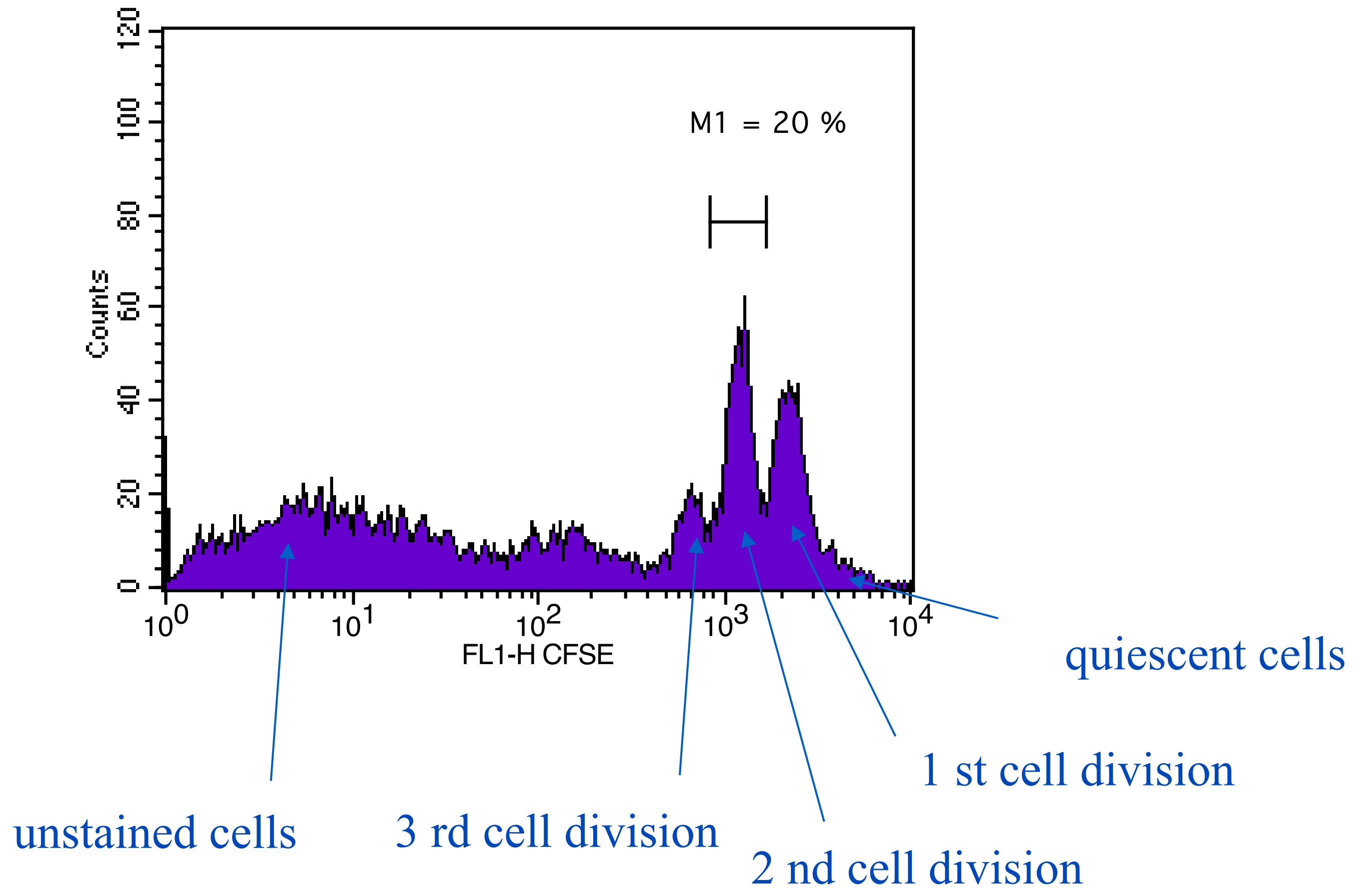


treated sample



Tatjana Trost

Following cell proliferation with CFSE



CFSE = carboxyfluorescein succinimidyl ester

ΑΠΟΠΤΩΣΗ

Different possibilities to die

- Nekrosis
- Apoptosis

Apoptosis = programmed cell death

regulated elimination of cells, e.g. for:

- Formation of parts of the body (during embryogenesis)
  (e.g. finger formation; death of interdigital mesenchymal tissues)

- Depletion of injured cells
  (e.g. infection, DNA-damage)

- Thymic selection
  (elimination of autoreactive and non reactive thymocytes)

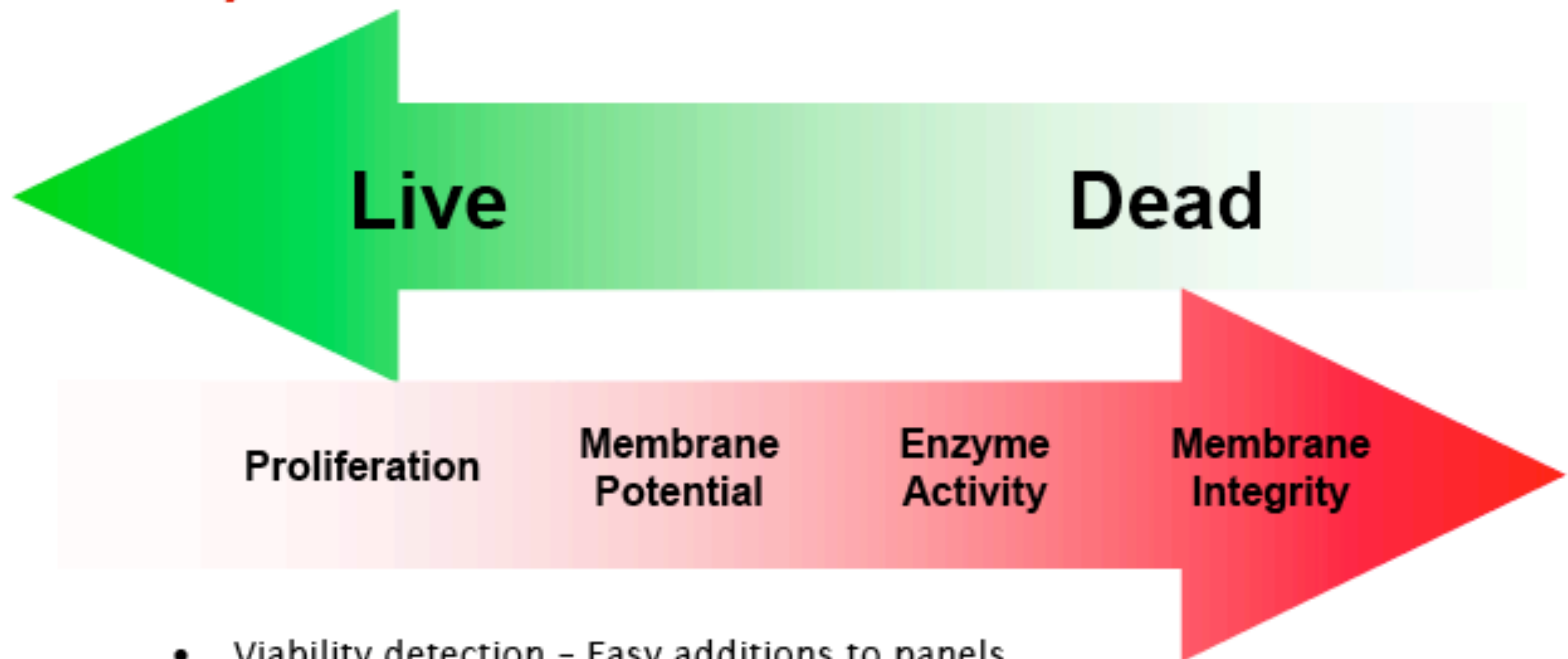
- Homöostasis of adult organs
  (turnover: 1/2 mio. cells/ min)

Apoptosis versus Nekrosis

- Mitochondria and Lysosoms stay intact (reduced $\Delta\psi$)
- No change in plasmamembrane-integrity and function (Phosphatidylserin-exposition)
- Mobilisation of intracellular Ca^{2+}- Ions
- Chromatin-condensation
- Activation of endonucleases (DNA-degradation)

- Mitochondria swell and break down
- Desintegration of plasmamembrane
- Release of proteolytic enzyms
- local Chromatin-condensation („patchy areas“)
- Karyolysis

Vitality Continuum



- Viability detection – Easy additions to panels
  - *Traditional* and *fixable* membrane integrity reagents
  - Metabolic activity
- Cell Cycle and Proliferation
- Apoptosis detection – Value of multiple parameters
  - Early events: Mitochondrial and membrane changes
  - Mid-game: caspase activity, metabolic changes
  - Late events: Nuclear changes, membrane integrity

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Why Use a Viability Indicator?

FSC-Area
SSC

Live Cells
Dead Cells
FSC-Area
Fixable Violet

Phenotyping based on the Live Cell Gate

CD4 Cy55PE
59%
0.6%
15%
24%
CD8 Q705

CD45RO TRPE
7%
32%
2.4%
59%
CD27 Cy5PE

CD4+ / Dead Cells Excluded
0.001%
CD107a
IFN-g

Phenotyping based on the Dead Cell Gate

CD4 Cy55PE
65%
26%
3%
6%
CD8 Q705

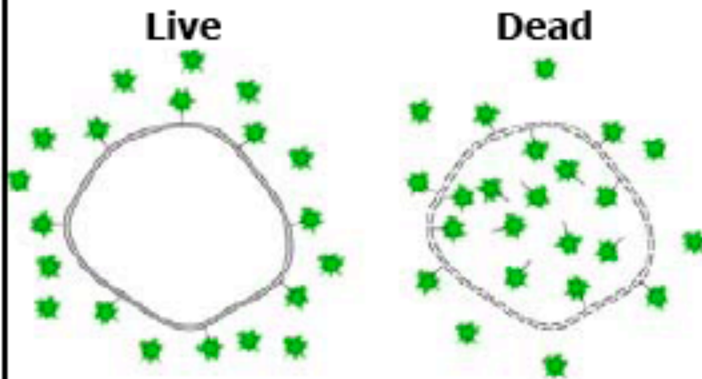
CD45RO TRPE
9.6%
10%
8%
72%
CD27 Cy5PE

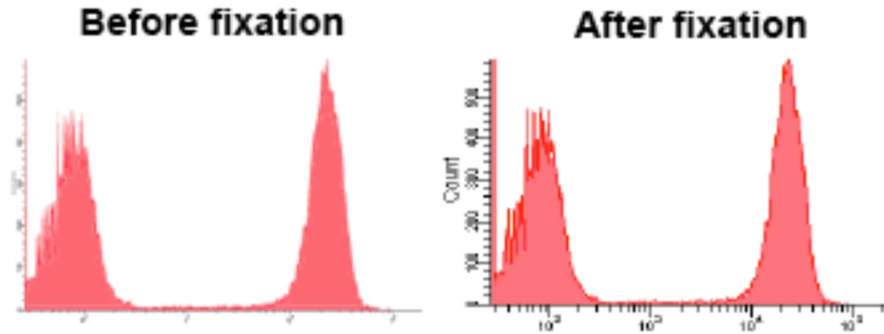
CD4+/ Dead Cells Included
0.55%
CD107a
IFN-g

Stimulated with SEB + Brefeldin A

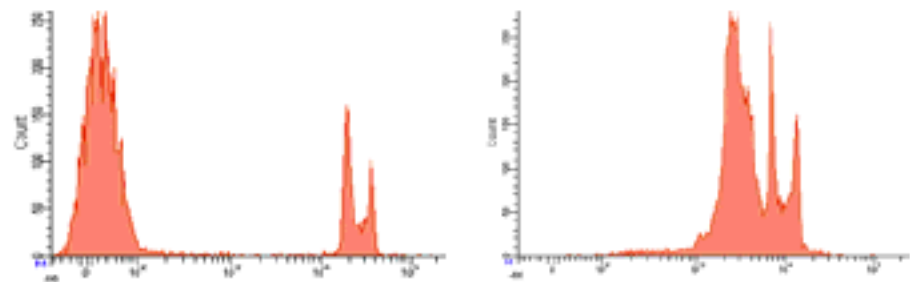
Perfetto et al. (2006) J Immunol Methods *313*:199

LIVE/DEAD® Fixable Dead Cell Stains





Fixable violet dead cell dye
(405 nm excitation, 440/40)



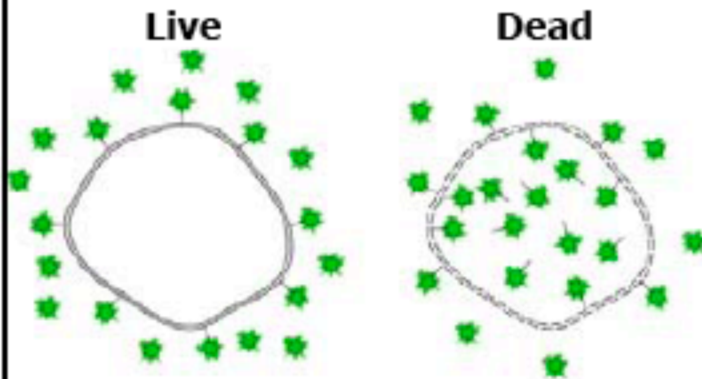
Propidium iodide
(488 nm excitation, 610/20)

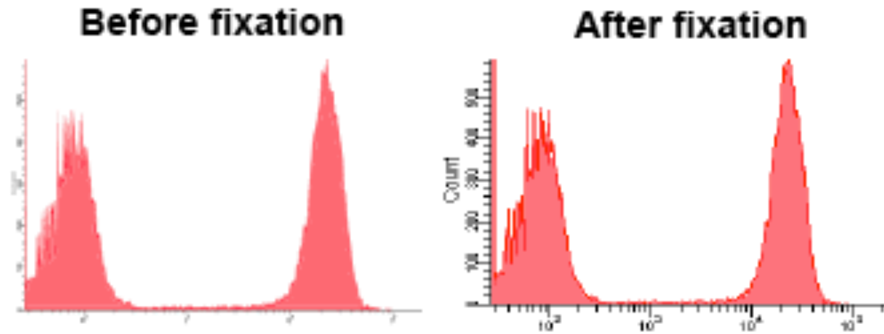
Live cells: react with the kit's fluorescent reactive dye only on their surface to yield weakly fluorescent cells.

Cells with compromised membranes: react with the dye throughout their volume, yielding brightly stained cells.

Jurkat cells: mixed live & heat-treated

LIVE/DEAD® Fixable Dead Cell Stains





Fixable violet dead cell dye
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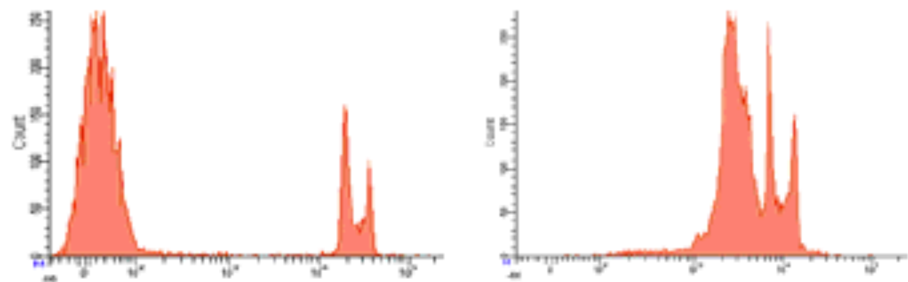

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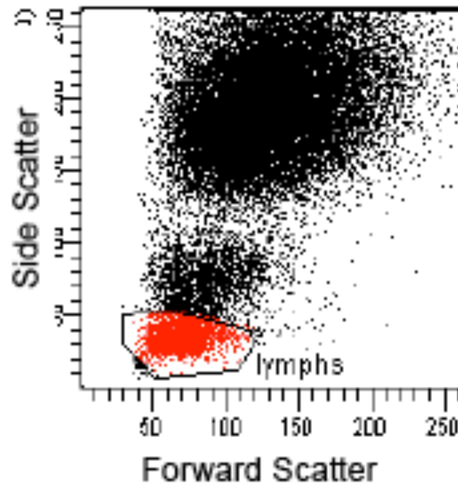
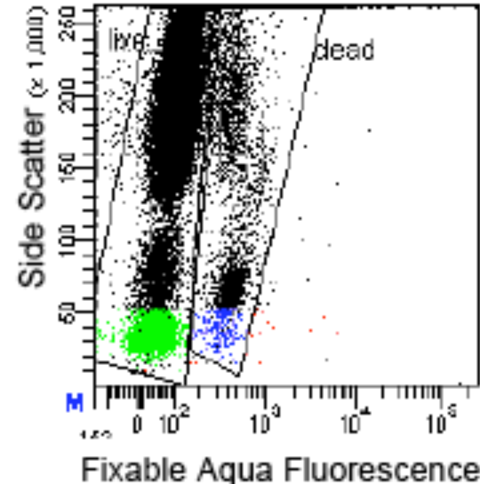
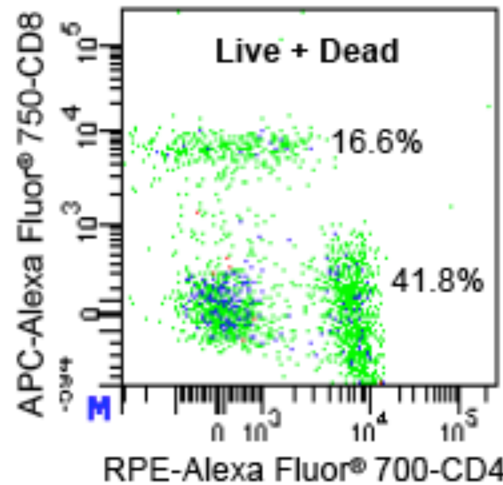
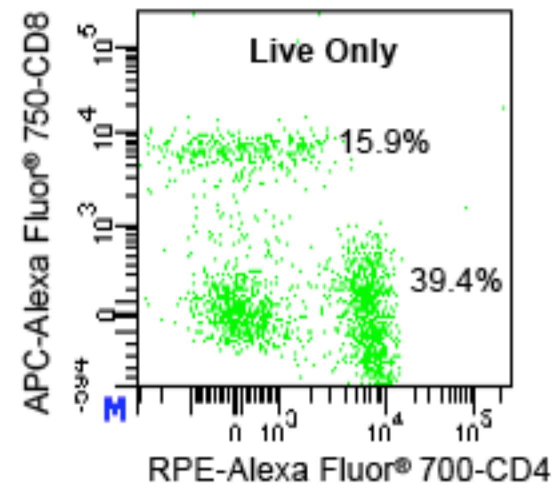


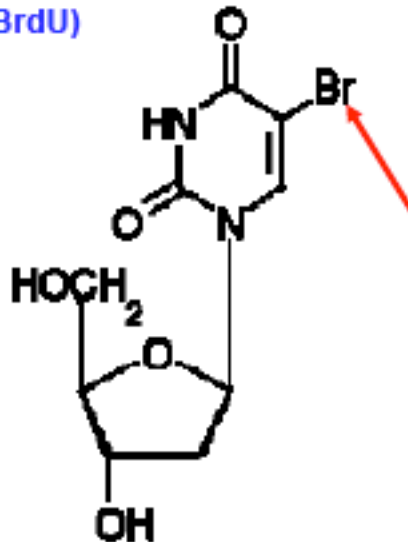


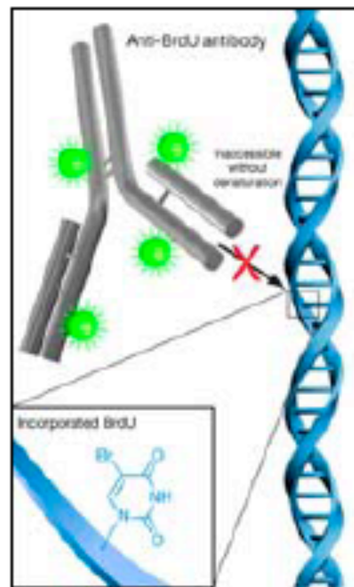


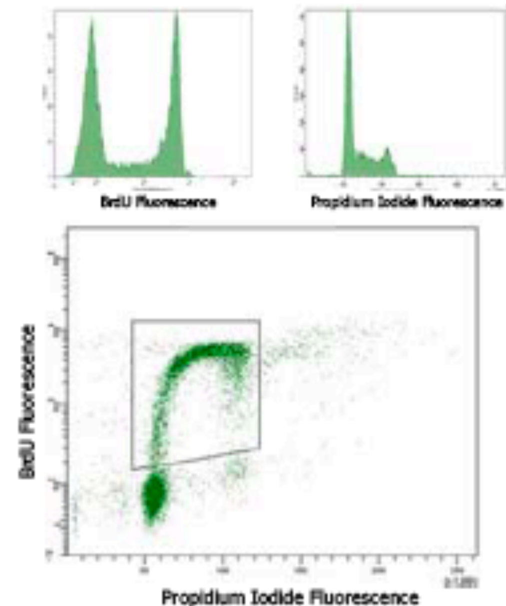


Proliferation: Measuring DNA Synthesis

- Detection and measurement of newly synthesized DNA in cells began in the 1960s with the incorporation of radioactive nucleotides (^{3}H-thymidine).
- This was replaced by antibody-based detection of the nucleoside analog bromo-deoxyuridine (BrdU).





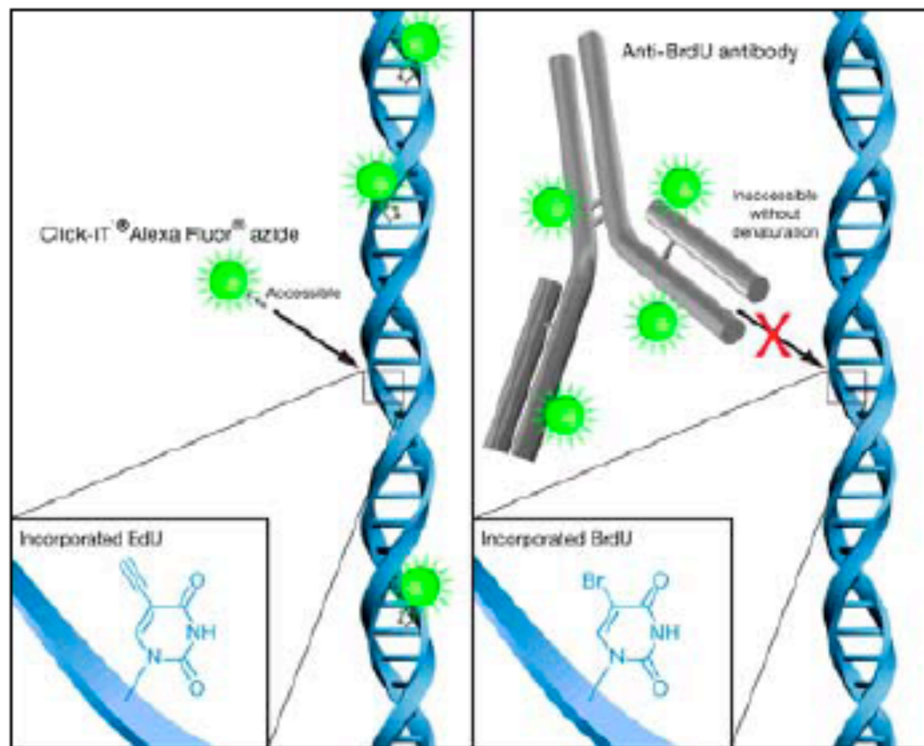


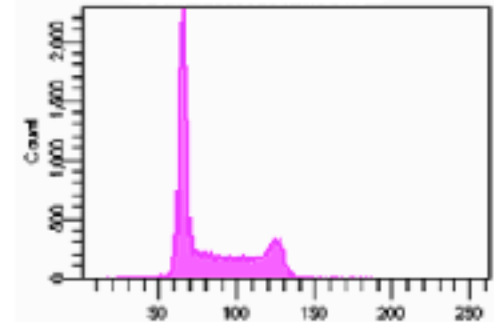
- Requires DNA denaturation for strand separation to make the incorporated BrdU accessible for antibody staining: acid, heat, or nucleases
- Difficult to optimize

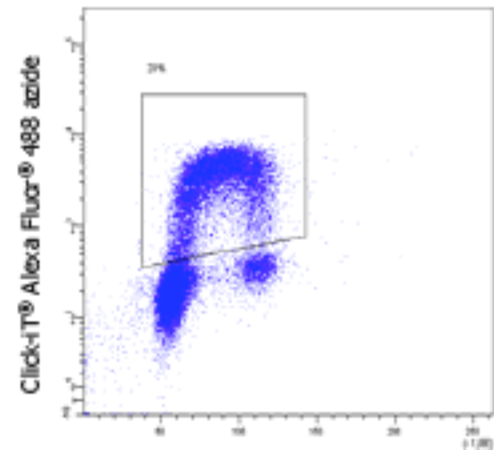
invitrogen

EdU Labeling

Click-iT® EdU Cell Proliferation Assay for Flow Cytometry







Flow cytometric methods for analysis of Apoptosis/ cell death

- Changes in cell morphology
- Changes in plasmamembrane-structure and in transport-functions
- Loss of function of cell organelles (e.g. Mitochondria)
- DNA-content (endonucleolytic DNA-degradation)
- Apoptosis associated proteins (e.g. Caspases)

Flow cytometric methods for analysis of Apoptosis/ cell death

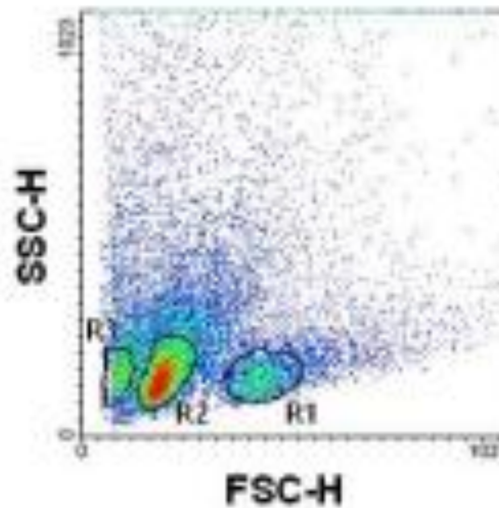
- Changes in cell morphology

  e.g. different FSC/ SSC signals

- Changes in plasmamembrane-structure and in transport-functions
- Loss of function of cell organelles (e.g. Mitochondria)
- DNA-content (endonucleolytic DNA-degradation)
- Apoptosis associated proteins (e.g. Caspases)

Reduced FSC / SSC signal

CD8 $^{+}$ Lymphocytes

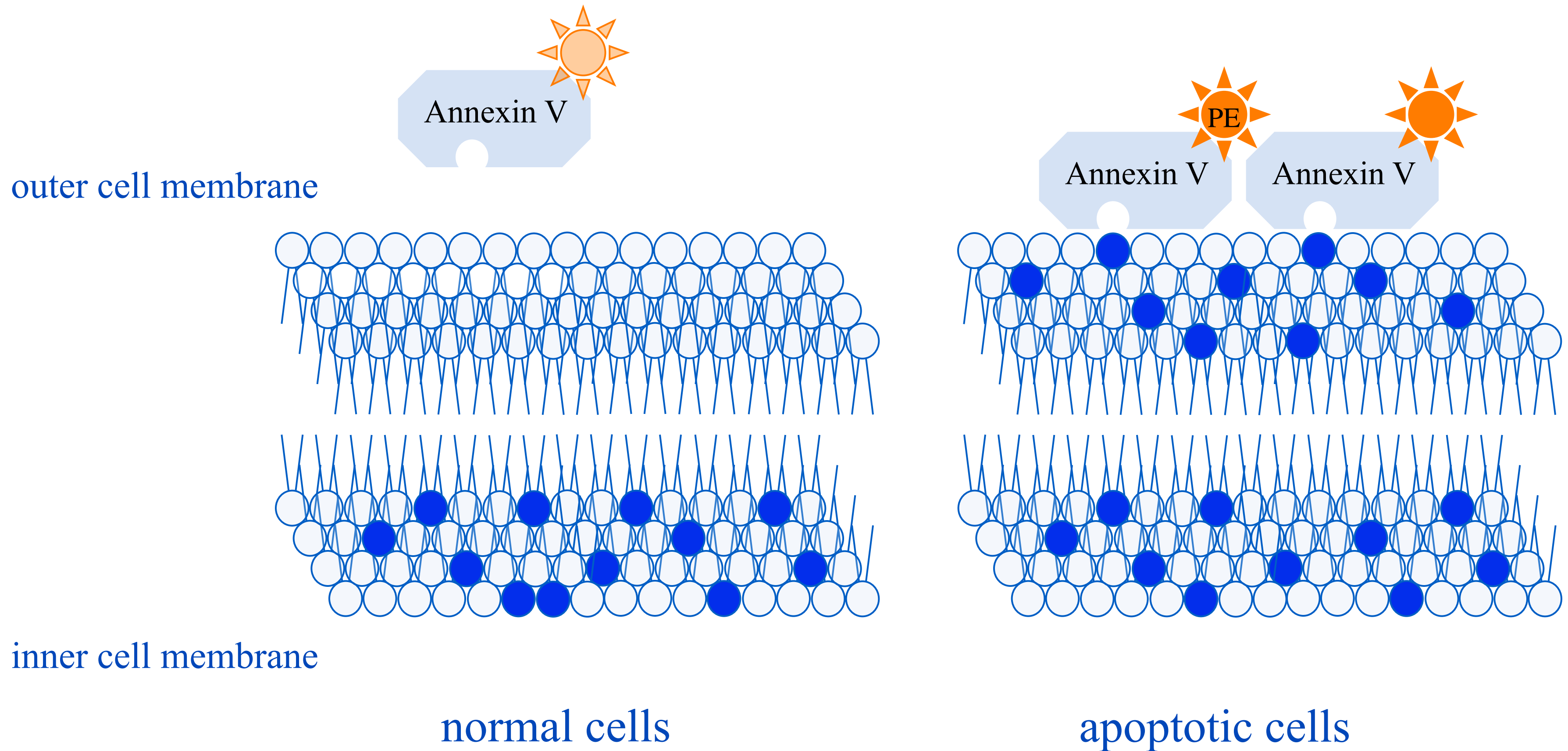


Flow cytometric methods for analysis of Apoptosis

- Changes in cell morphology
- Changes in plasmamembrane-structure and in transport-functions
  e.g. - membrane “flipping”
  - strong uptake of dyes
- Loss of function of cell organelles (e.g. Mitochondria)
- DNA-content (endonucleolytic DNA-degradation)
- Apoptosis associated proteins (e.g. Caspases)

Annexin V - Staining

Theory



Annexin V - Staining

Protocol

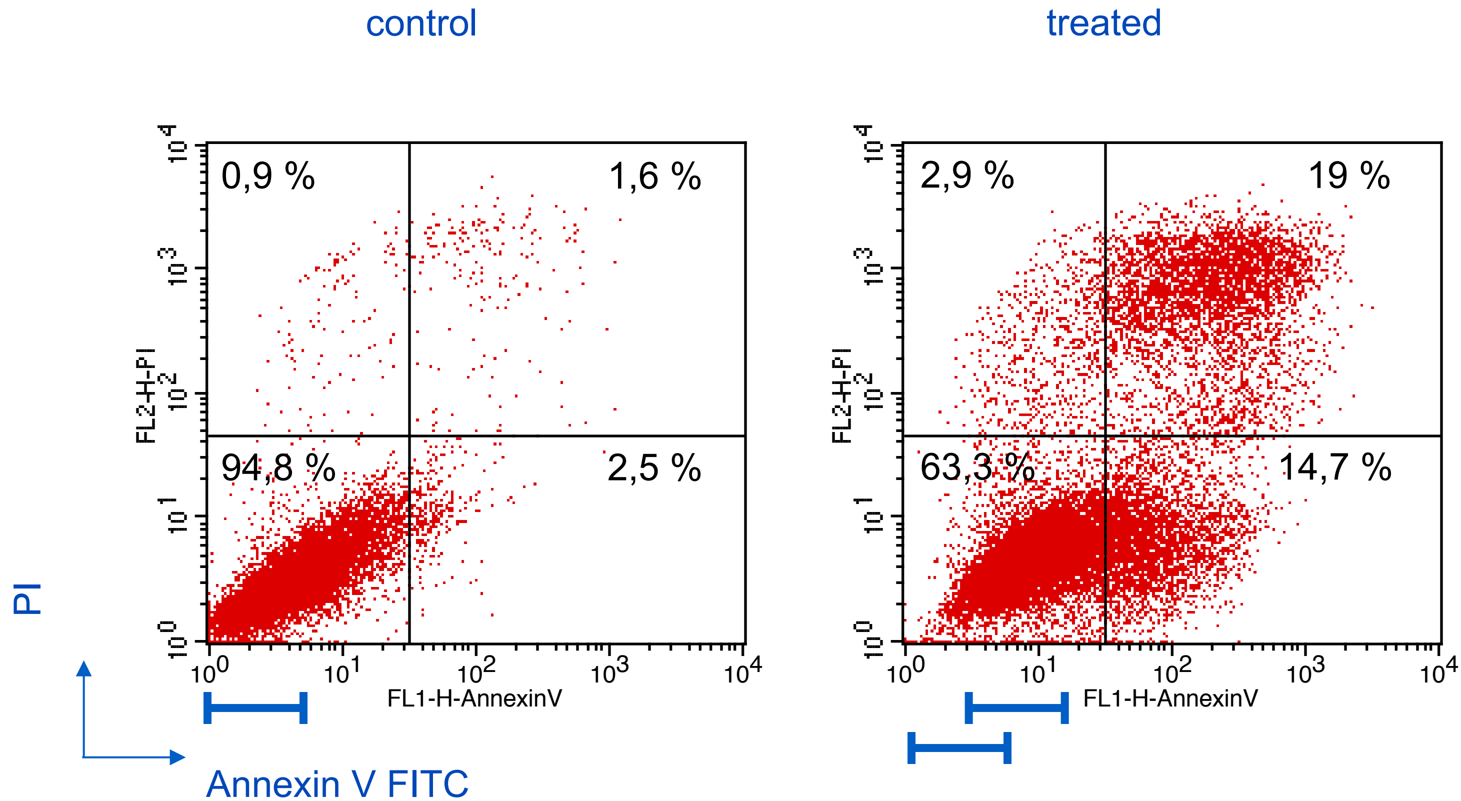
- Annexin V - binding requires **Ca^{2+}**-Ions

  Attention: be careful if you use EDTA to block Trypsin after harvesting your adherent cells (EDTA binds Calcium!)

- Use fresh buffers and reagents
- Typical concentration:
  - 0.25 µg/ml Annexin V, (1-) 5 µg/ml PI
- Incubation for 15 min at RT in the dark
- Add PI and analyse with FACS ≤ 1h

Annexin V - Staining

Data analysis



Tobias Nübel,
University Mainz

Uptake of dyes

- Sytox Green
  Excitation: 488 nm (blue); Emission: green Fluorescence

- Propidium Iodide
  Excitation: 488 nm (blue); Emission: orange/ red Fluorescence

- 7-Actinomycin D (7AAD)
  Excitation: 488 nm (blue); Emission: red Fluorescence

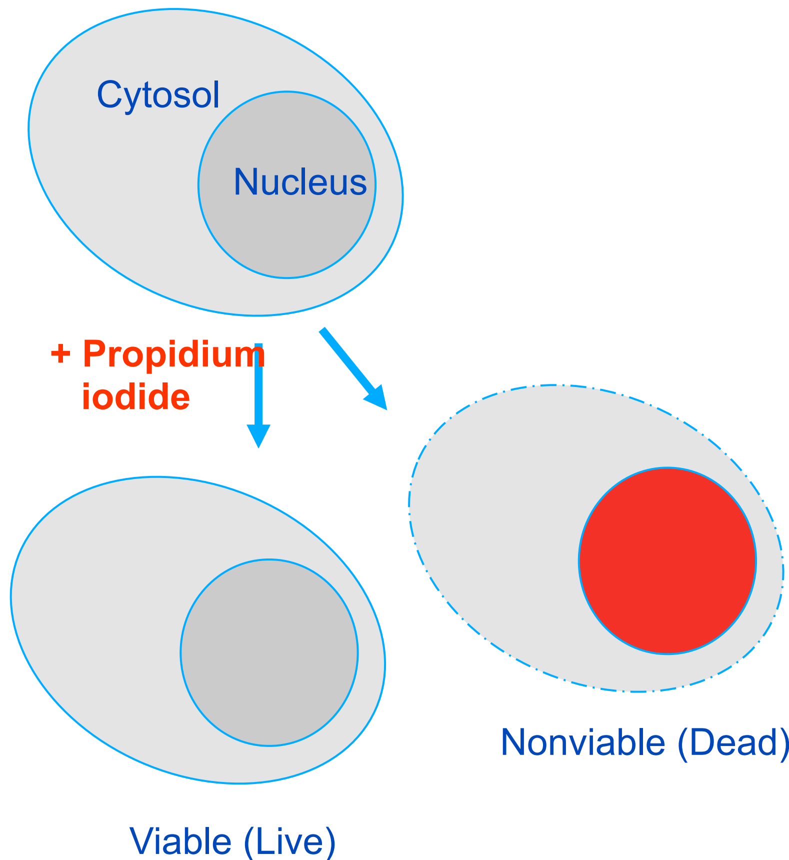
- To-Pro3
  Excitation: 633 nm (red); Emission: red Fluorescence

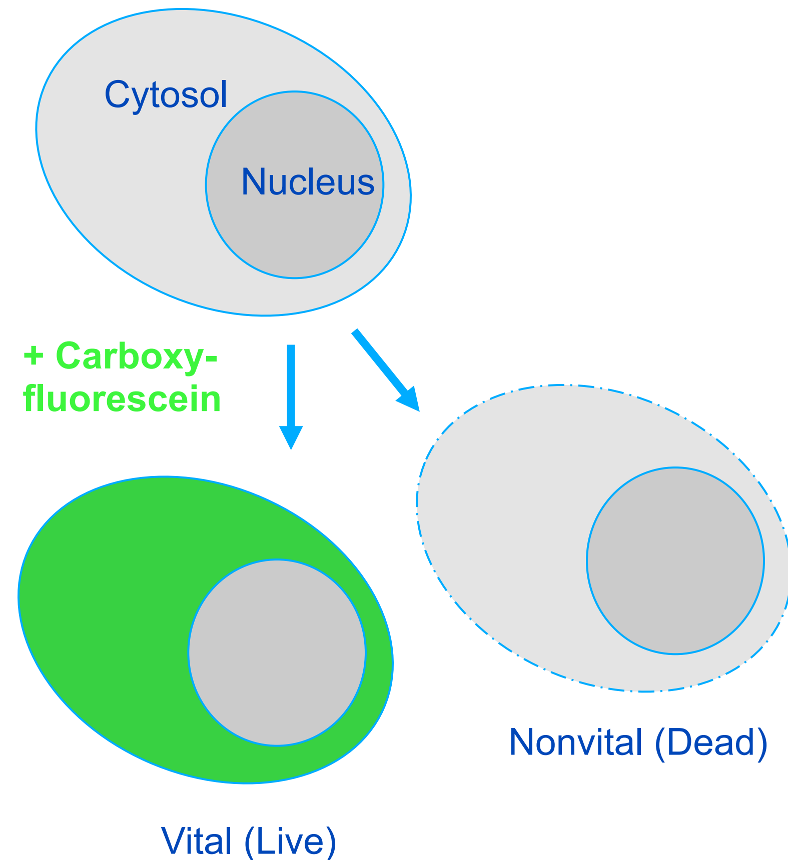
...

Viability & Vitality

Viability:
Cell membrane forms intact barrier

Vitality:
Cell mediates active processes

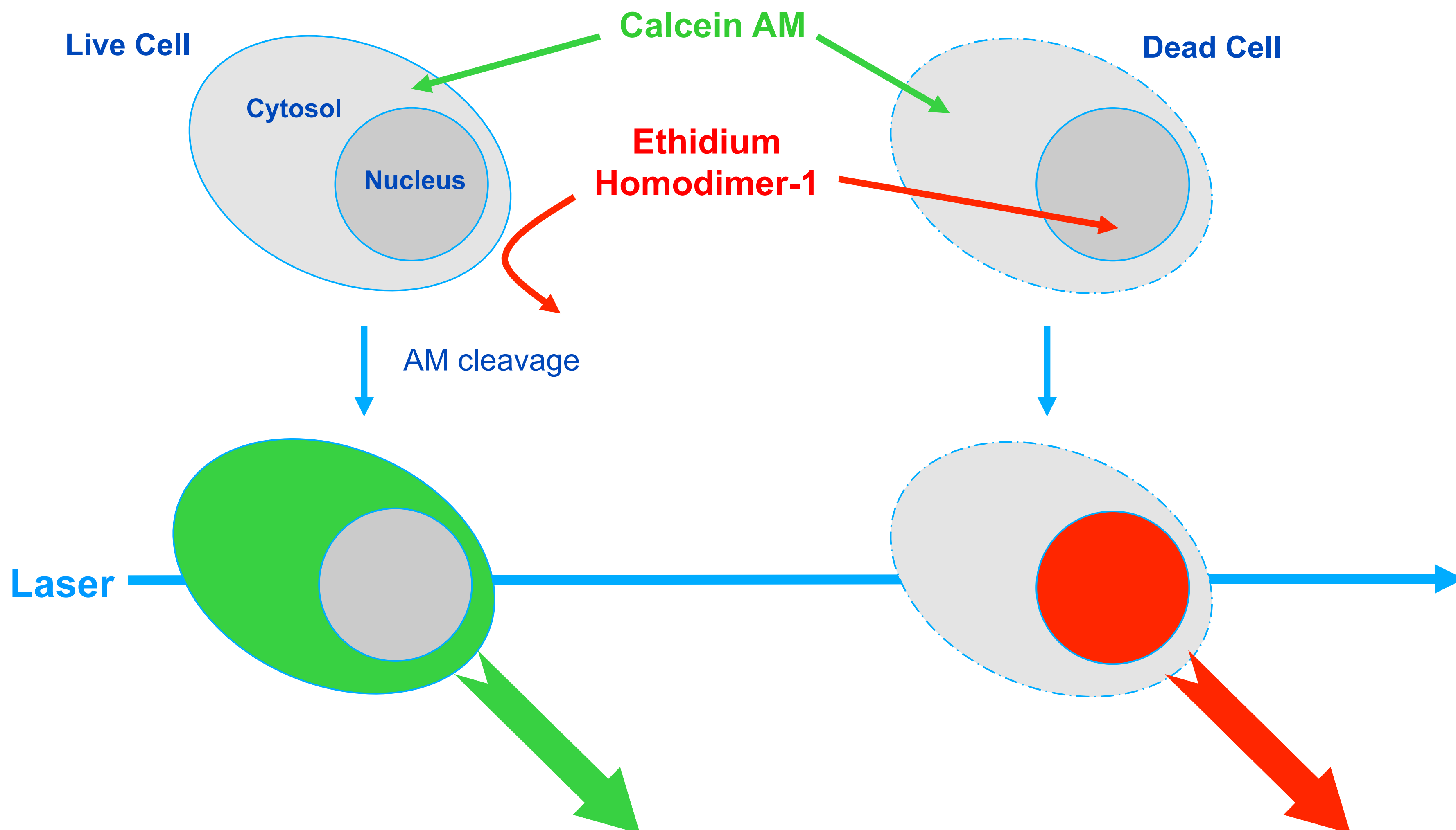




How it works

from Invitrogen

Vitality by Enzyme / Metabolic Function

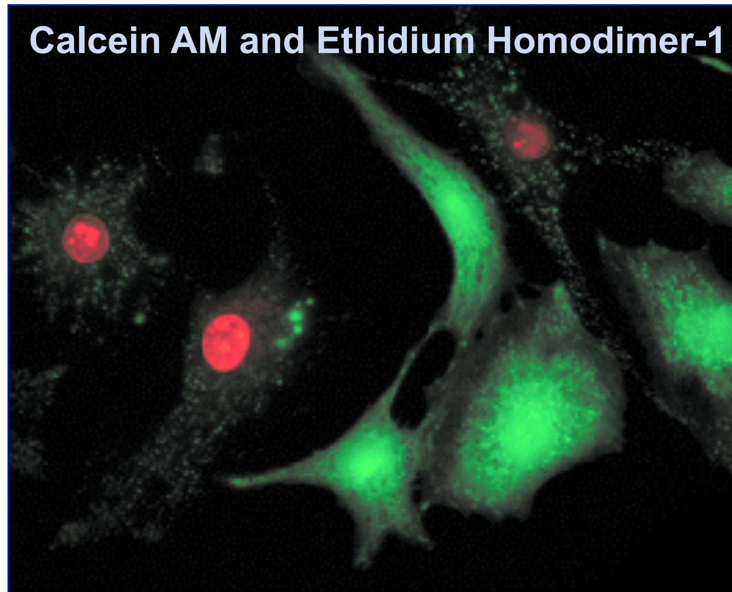


"It isn't easy being green"

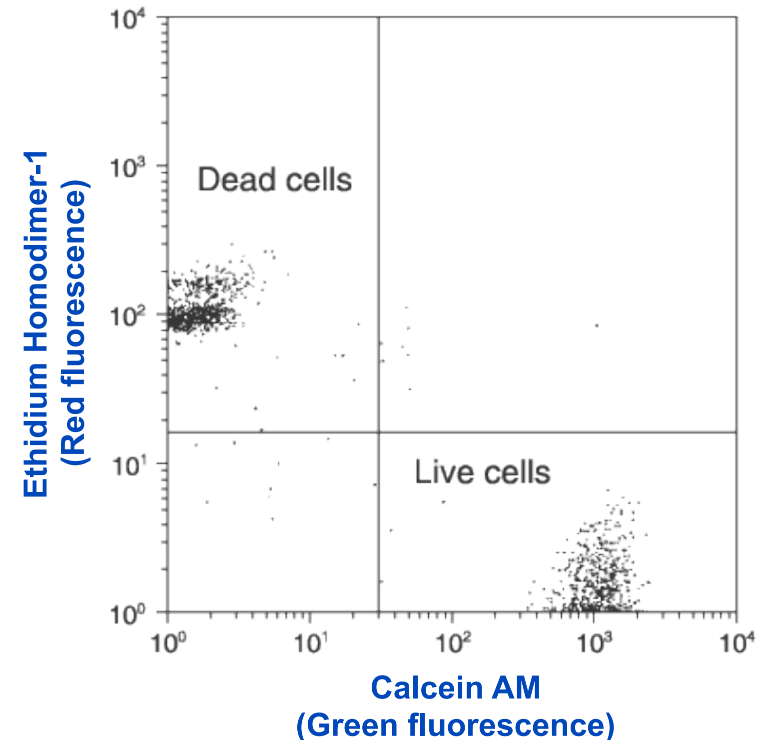
from Invitrogen

LIVE/DEAD® Viability / Cytotoxicity Kit

Measurement of intracellular esterase activity and membrane integrity



BPAE cells stained with the LIVE/DEAD Viability/Cytotoxicity Kit (L3224)



- Rapid assay
- Detects live and dead cells simultaneously
- The most popular viability assay kit for Microscopy, Flow Cytometry and Multiwell plate scanner

Vitality = metabolic activity

from Invitrogen

Flow cytometric methods for analysis of Apoptosis

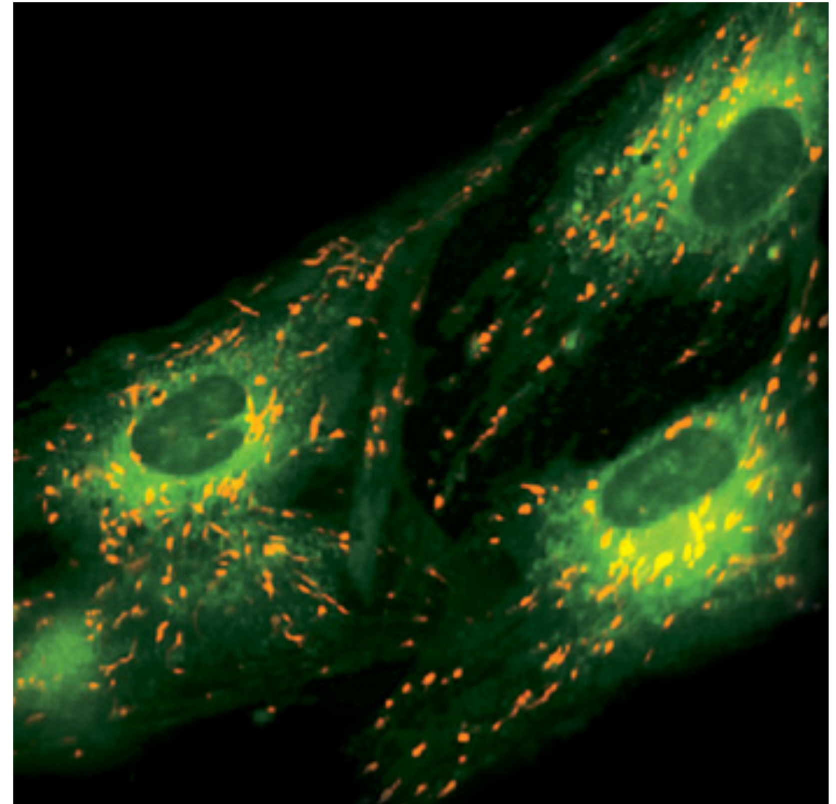
- Changes in cell morphology
- Changes in plasmamembrane-structure and in transport-functions
- Loss of function of cell organelles (e.g. Mitochondria)

  e.g. - JC-1 as indicator for mitochondrial membrane potential
  - generation of reactive oxygen species (ROS)
- DNA-content (endonucleolytic DNA-degradation)
- Apoptosis associated proteins (e.g. Caspases)

Mitochondrial Membrane potential

JC-1

- Combining signals from the green-fluorescent JC-1 monomer and the red-fluorescent J-aggregate
- For flow cytometry, JC-1 can be excited at 488 nm and detected using the green channel for the monomer and the red channel for the J-aggregate form



from Invitrogen

JC-1

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanin Iodide

- J-aggregates are formed at the mitochondrial membrane dependent on the membrane potential. This results in a shift of the Fluorescence emission (red fluorescence).
- With loss of membrane potential the J-aggregates disintegrate in monomers (green fluorescence).

Flow cytometric methods for analysis of Apoptosis/ cell death

- Changes in cell morphology
- Changes in plasmamembrane-structure and in transport-functions
- Loss of function of cell organelles (e.g. Mitochondria)
- DNA-content (endonucleolytic DNA-degradation)
  e.g. sub G1 quantification ("Nicoletti")
- Apoptosis associated proteins (e.g. Caspases)

We can use the DNA dyes in 2 ways

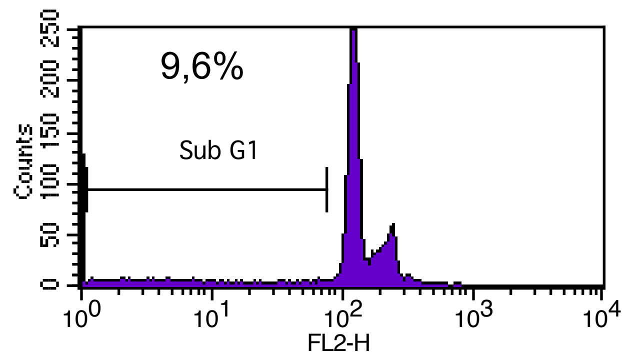
1. To quantify cellular DNA content

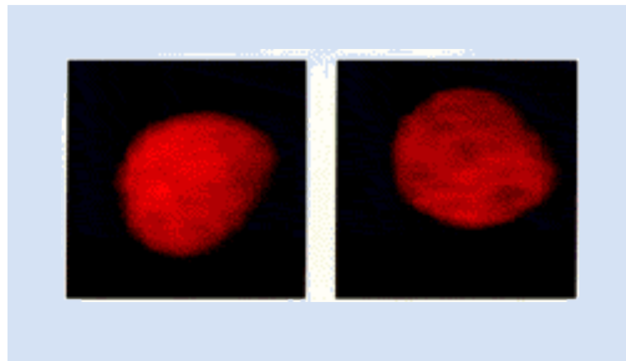
2. As a dead cell discriminator

DNA-Degradation

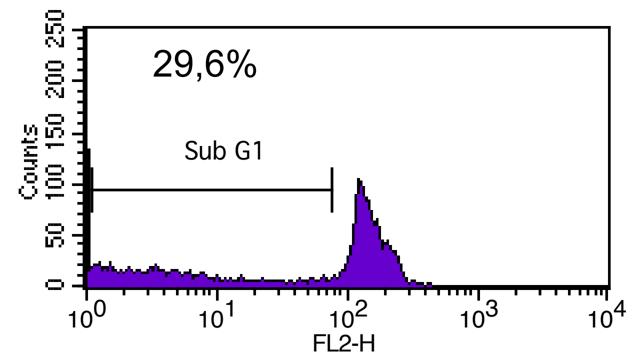
sub G1

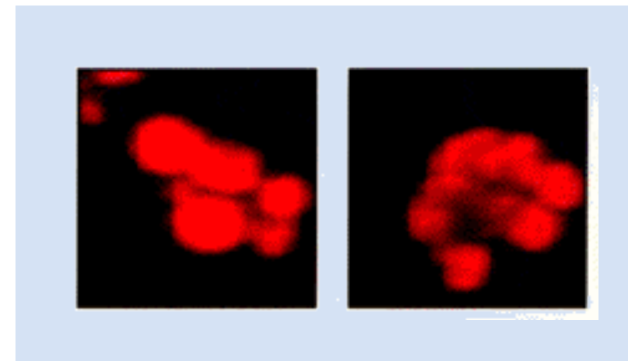
control





irradiated





Binje Fleischer,
University Mainz

Flow cytometric methods for analysis of Apoptosis/ cell death

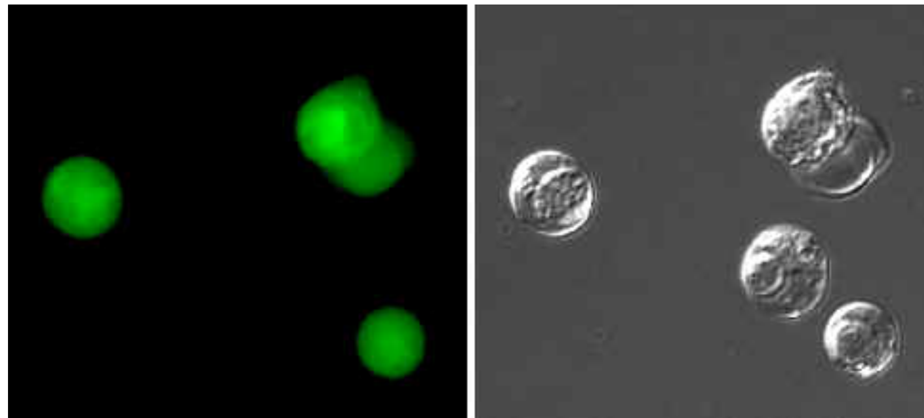
- Changes in cell morphology
- Changes in plasmamembrane-structure and in transport-functions
- Loss of function of cell organelles (e.g. Mitochondria)
- DNA-content (endonucleolytic DNA-degradation)
- **Apoptosis associated proteins (e.g. Caspases)**

  e.g. - PARP- cleavage

  - FLICA

FLICA Apoptosis Kits

- **Fluorescent-Labeled Inhibitor of Caspases**
- quantitate apoptosis via active caspases in whole, living cells
- Using inibitor proteins like VAD



4 out of 5 cells are apoptotic: Jurkat cells were labeled with ICT's Poly-Caspases FLICA™ kit . 4 cells fluoresce green (left), while the grey image (right) reveals 5 cells in the field. The 4 green cells are apoptotic = 80% of cells in this experiment had active caspases. The level of fluorescence can be quantified on a fluorescence plate reader or flow cytometer. Data courtesy of Dr. Brian W. Lee, ICT.

taken from immunochemistry Technologies

Summary Apoptosis

- Consider the cells/model used:
  - Include supernatant if working with adherent cells
  - What positive control to use
  - When to look for apoptosis (time point, kinetic)

- Complement flow studies with other methods:
  - Microscopy

  (- DNA laddering, TUNEL-Assay)

Acknowledgements

Some slides were generated through stimulation/ support of following companies:

BD Biosciences
Beckman Coulter, (Cytomation)
Invitrogen
Partec

Some other slides were adapted from slides you can find in the www or in the sources shown on slide 17.

Special thanks to Derek Davis (UK / cell cycle) and
Mario Roederer (USA / compensation, bi-exponential display)