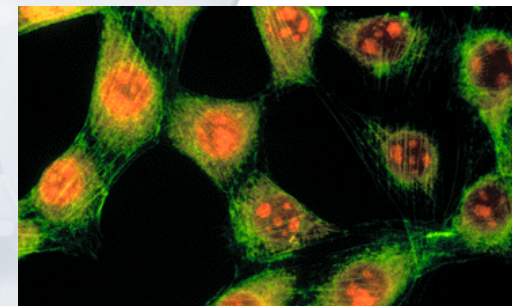




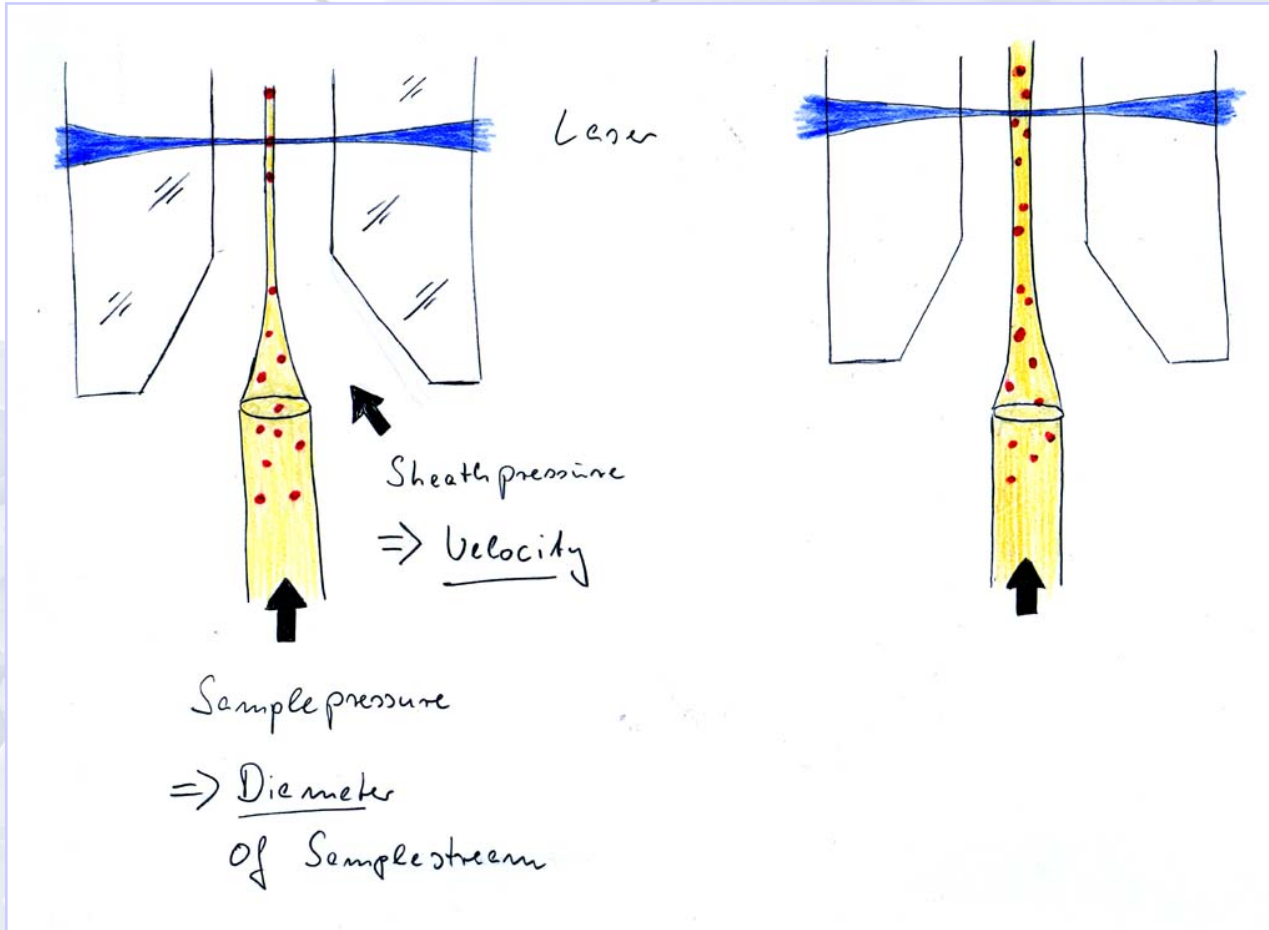
DNA and Cell Cycle Analysis by Flow Cytometry



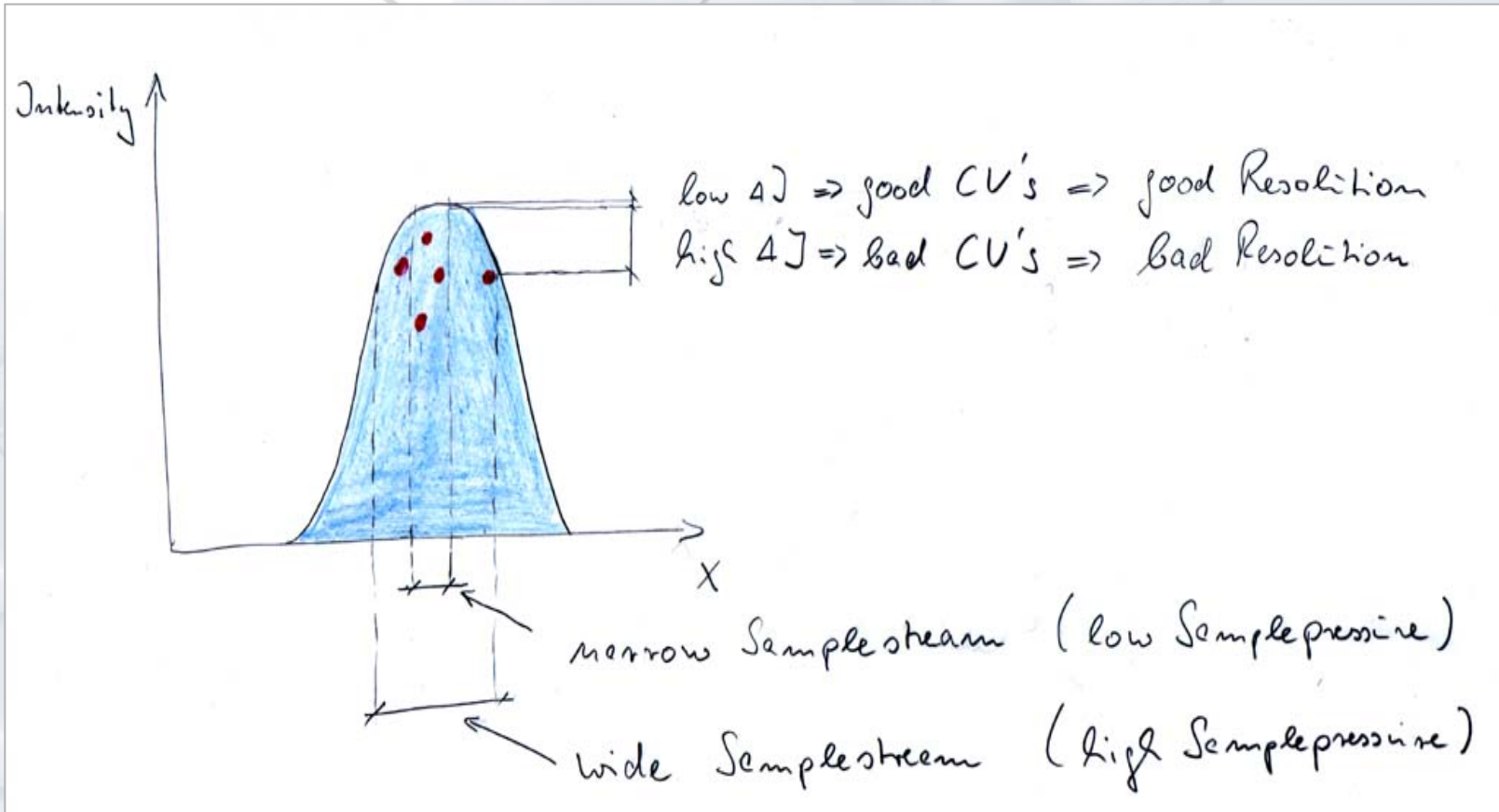
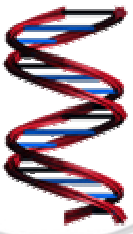
Dynamic Solutions in Immunology



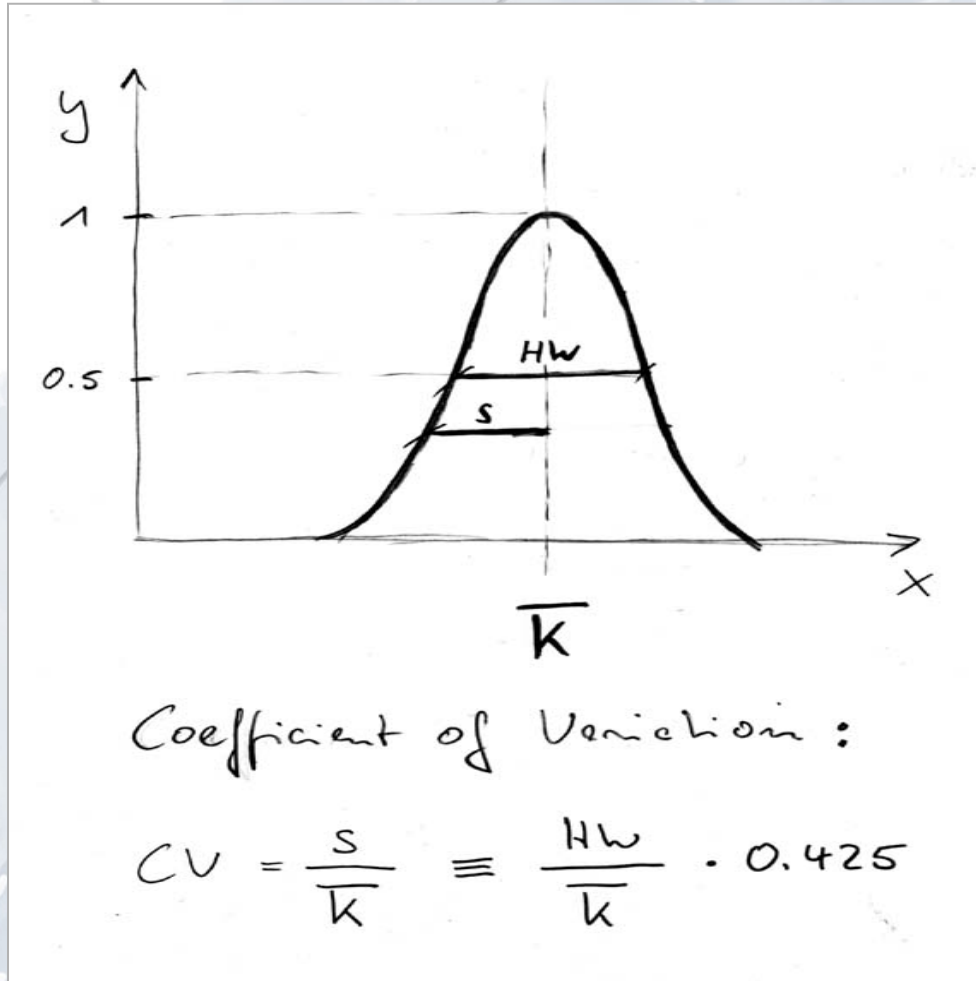
Sample Pressure



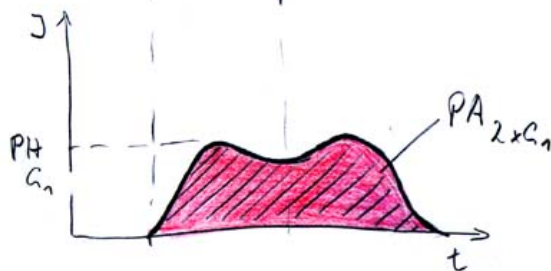
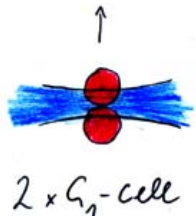
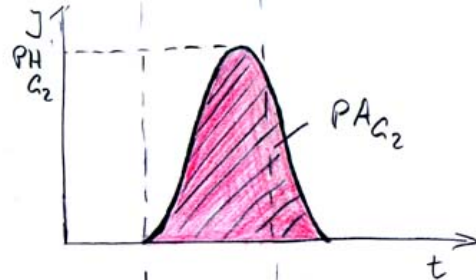
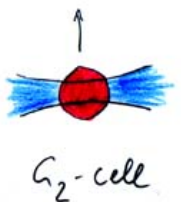
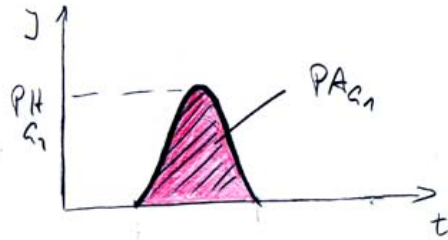
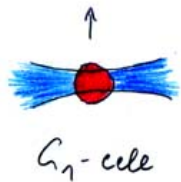
CV vs. Core diameter



Coefficient of Variation

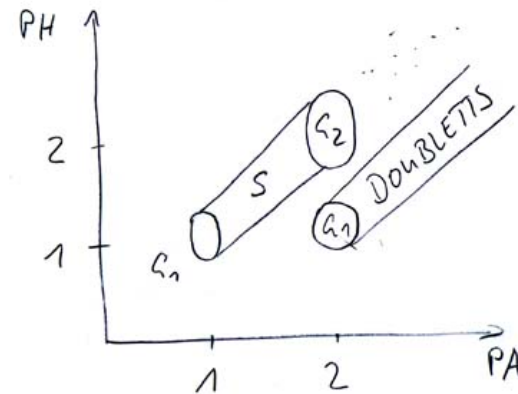


Doublet Discrimination

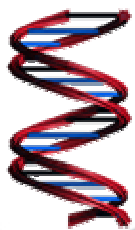


Fokus $\emptyset < \text{Cell } \emptyset$
 \Rightarrow PH not \propto DNA-content
 only PA \propto DNA-content

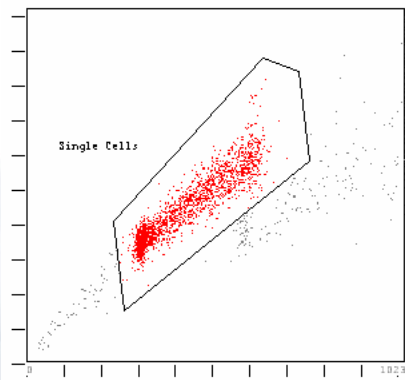
Doublet-Discrimination
 Only with small Fokus \emptyset



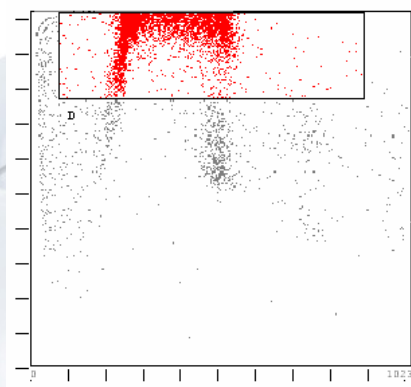
Methods for Doublet Discrimination



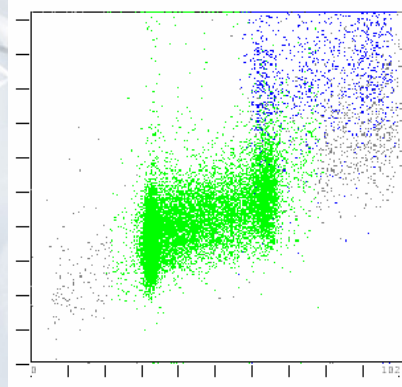
Peak vs. Area



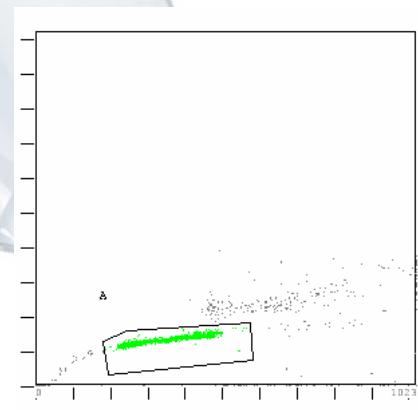
Ratio vs. Area



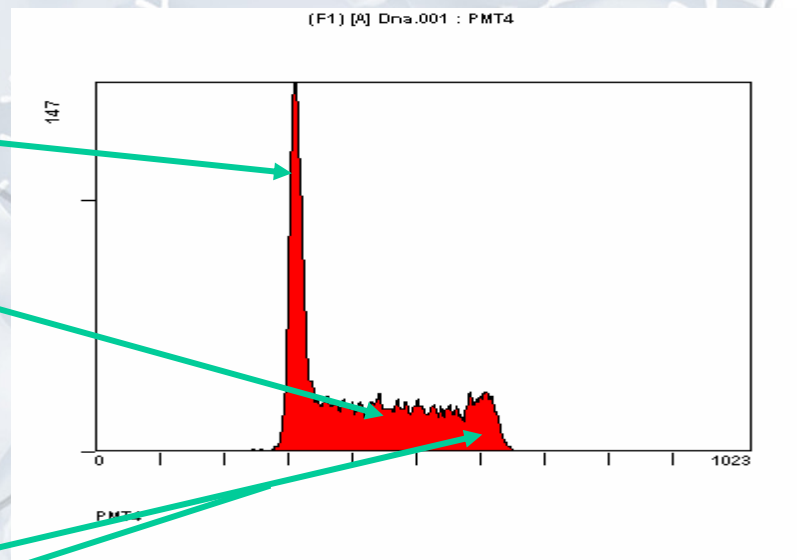
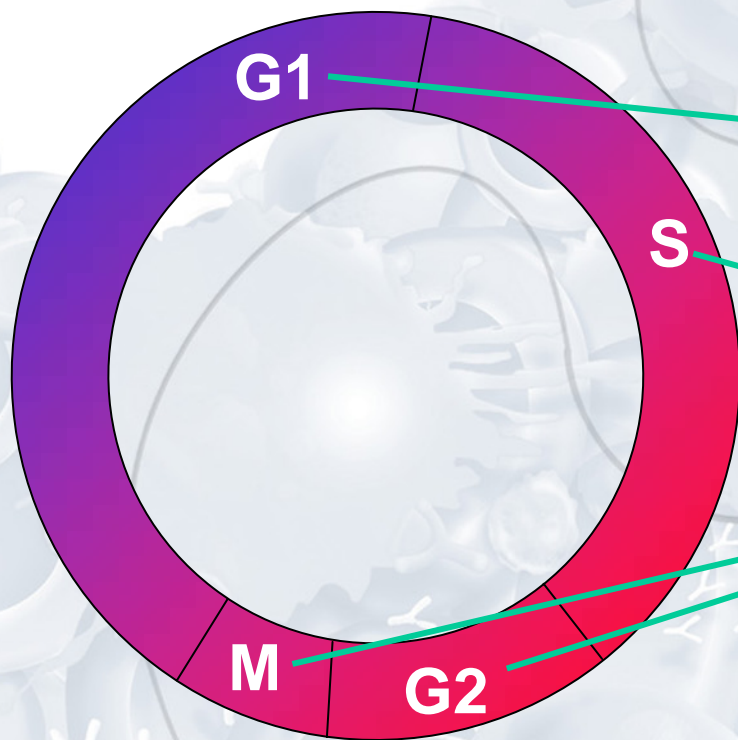
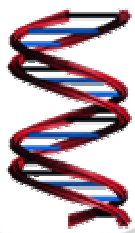
TOF vs. Area



Width vs. Area

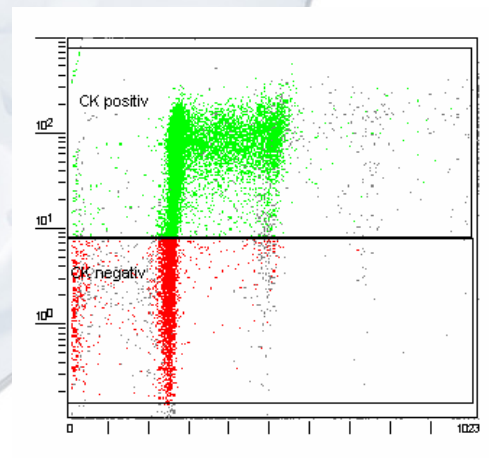
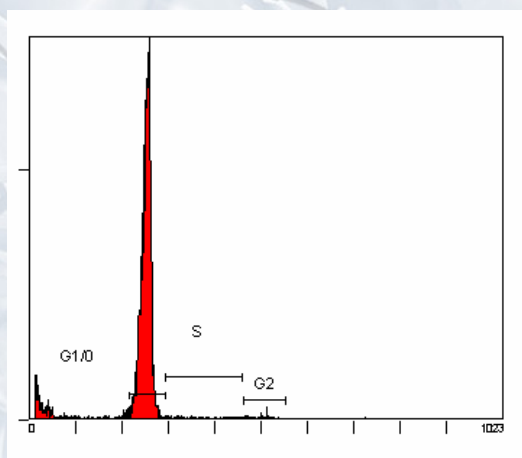
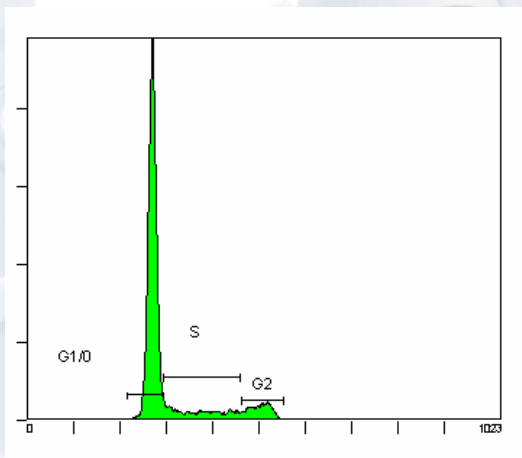


Cell cycle and DNA Staining



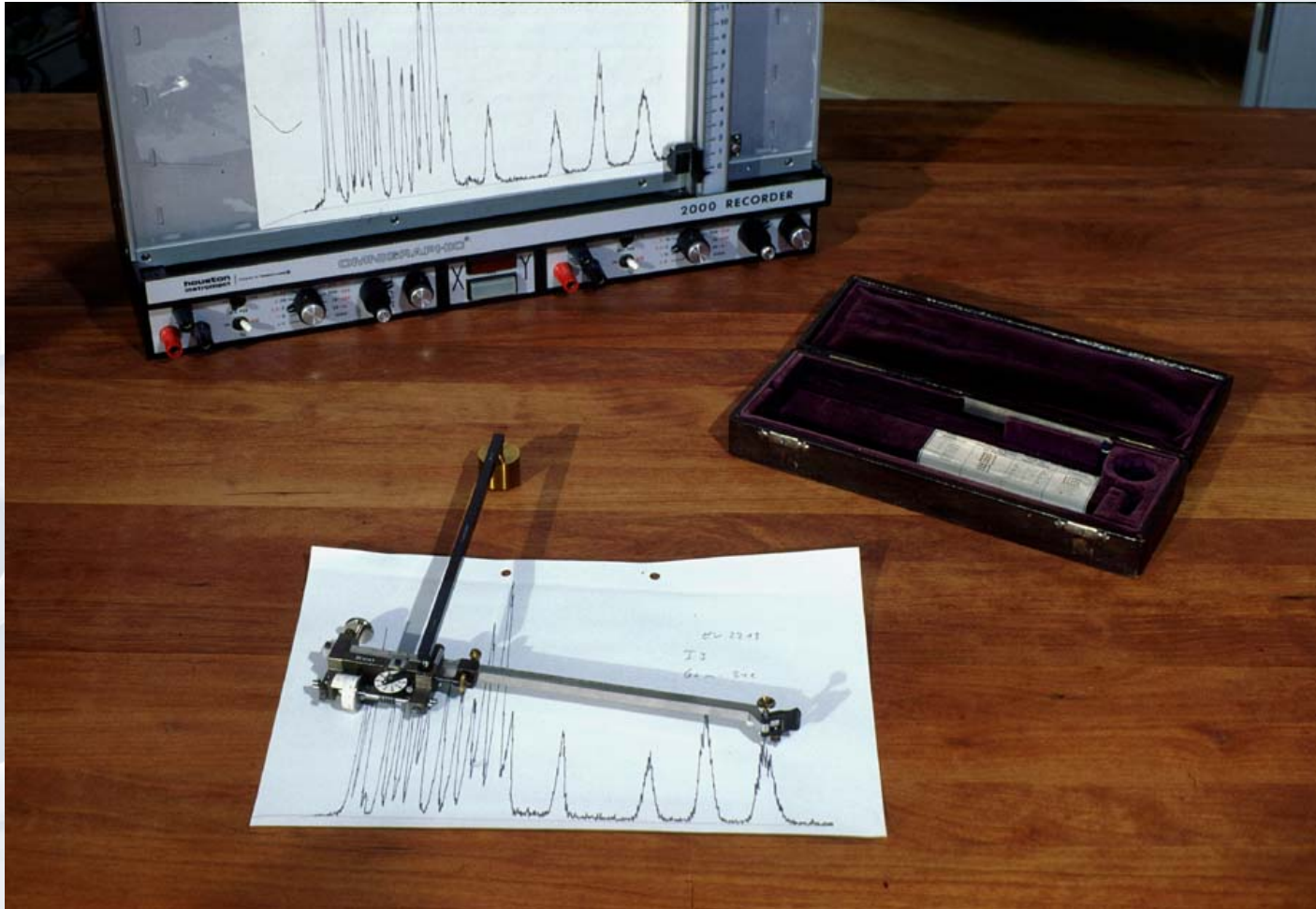
DNA and Cytokeratin Staining

Human bladder cancer cells, stained with PI and Cytokeratin





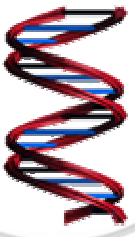
Plotter



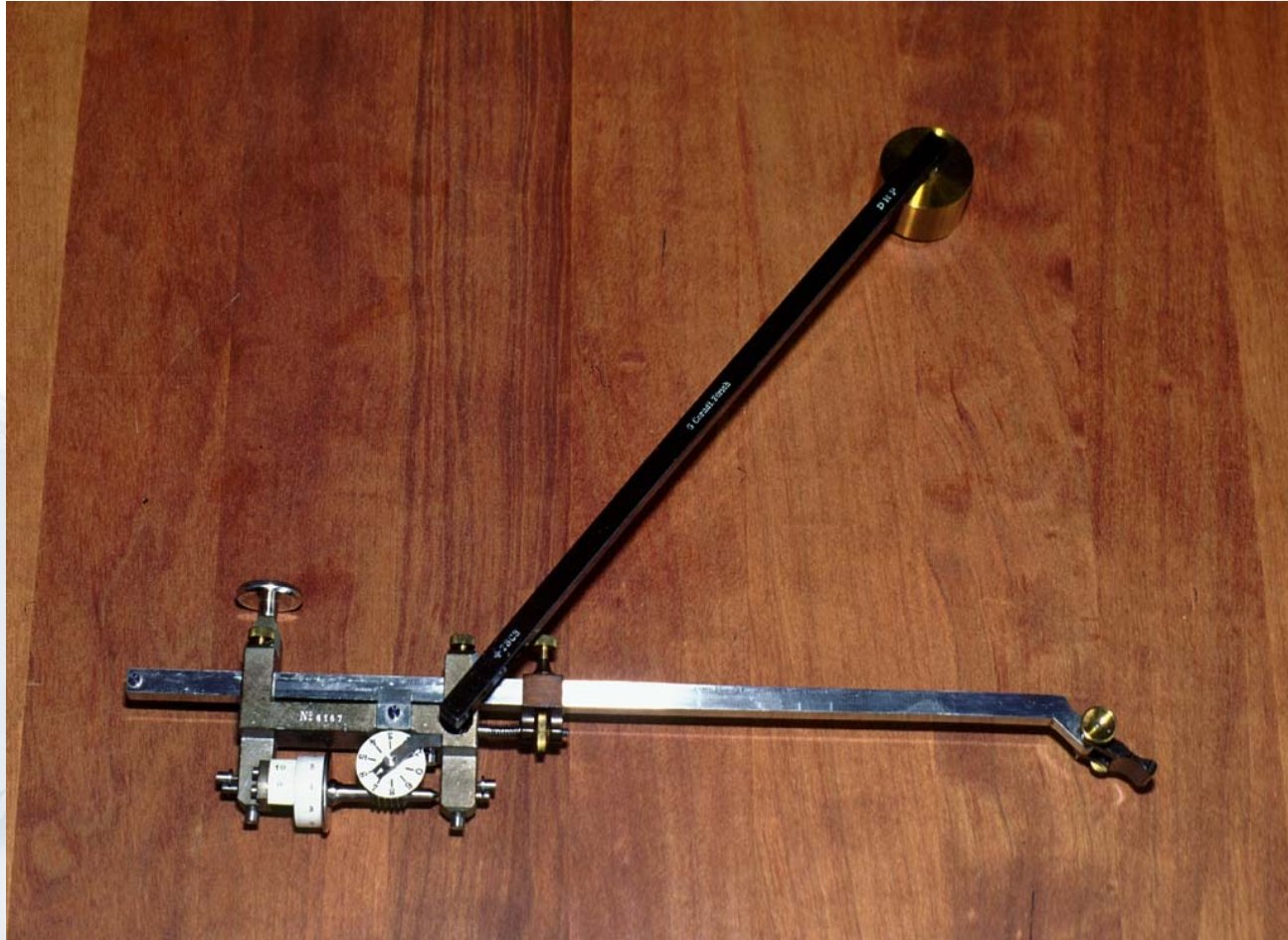


Ancient tools





Planimeter





DNA Analysis, Background

- **DNA Analysis and Cell Cycle Analysis is studied in:**
 - Routine Labs in the assessment of the malignancy and prognosis of cancers
 - Clinical research in the development of new treatment for malignant diseases
 - Pharmaceutical industry in drug development
 - Breweries for monitoring the fermentation process





Staining Principle

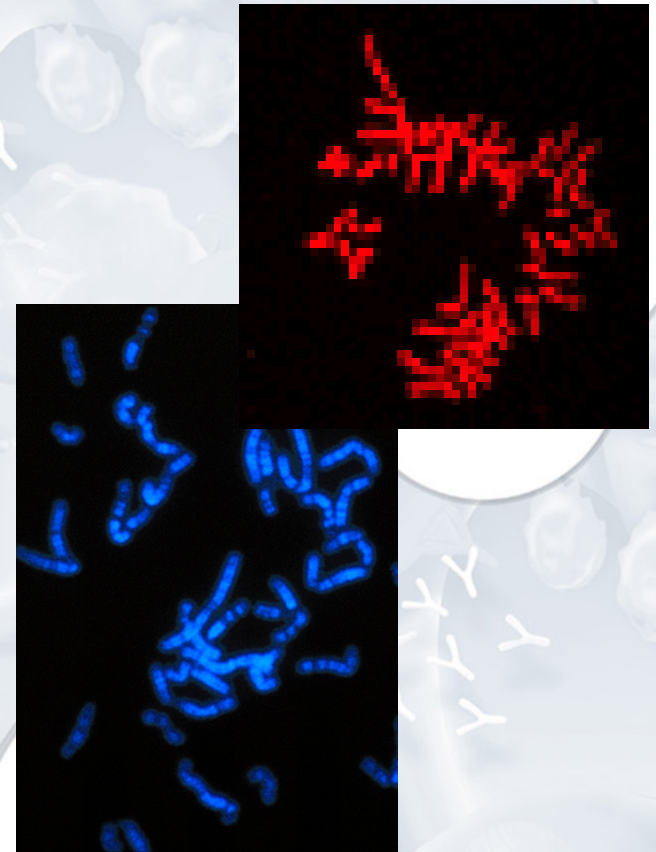
- DNA can be stained by different dyes that intercalate into the double strand
- The staining is stoichiometric, i.e. the amount of dye bound is directly proportional to the amount of DNA present in the cell
- The dye has to enter the nucleus to stain the DNA strands





DNA dyes

- **DNA dyes fall into two main categories:**
- **Excitable by 488nm laser light:**
 - Propidium iodide
 - Acridine Orange
 - Coriphosphine O
- **Excitable by UV light:**
 - DAPI
 - Hoechst





Dyes Useful for Cell Cycle Analysis

	Excitation (nm)	Emission (nm)	Fixation
Propidium Iodide	488	620	yes
7 AAD	488	675	yes
Ethidium Bromide	488	590	yes
TO-Pro 3	633	673	yes
Hoechst 33342 and 33358	U.V.	410 up	yes/no





The Cell Cycle

- G_0 - the cell is resting, the DNA content is $2N$
- G_1 - the cell enters the cycle, the DNA content is still $2N$
- S - the cell starts replicating its DNA strands, the DNA content varies between 2 and $4N$
- M - the cell is in Mitosis, the two daughter cells are still attached, the DNA content for each cell is again $2N$





Signal Shape Analysis

Since two cells attached to each other have the same DNA content as one cell with two times the DNA, the pulse shape has to be used to separate clumps from single cells. Several methods may be used that lead to the same result.

- DNA area vs. DNA width
- Time of Flight vs. DNA area
- DNA area vs. DNA peak
- Ratio (Integral/peak) vs. Integral





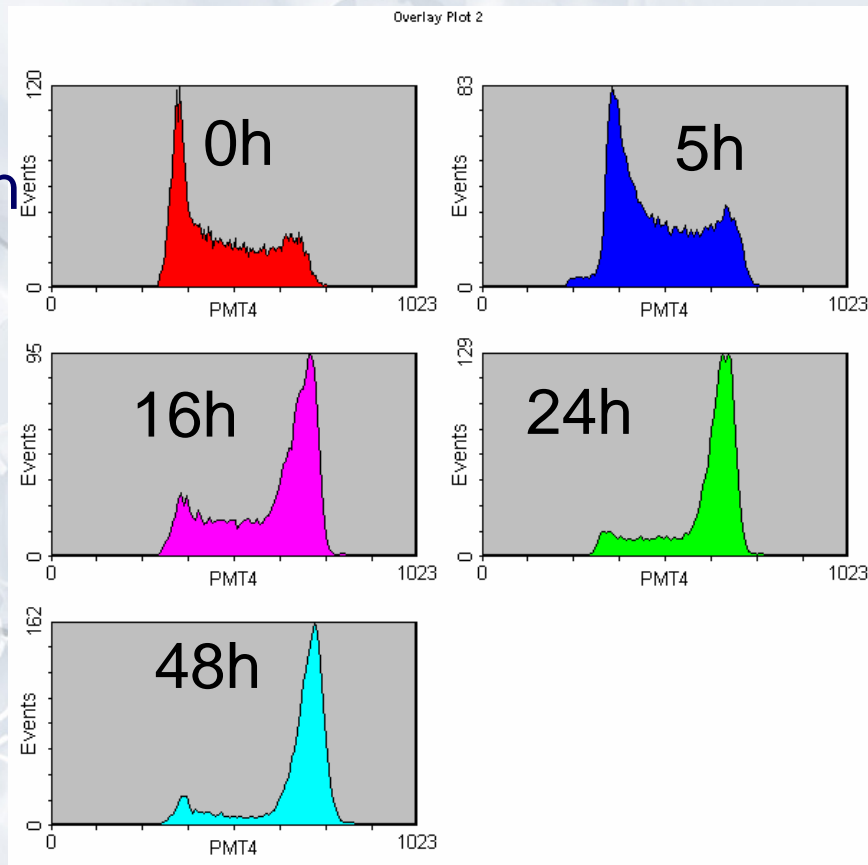
Information from Cell Cycle Analysis

- **People are mainly interested in two pieces of information:**
 - Size of the S-phase, i.e. how many cells are proliferating?
 - How many G_0 peaks are present, i.e. is more than one type of DNA content present in the cells? (malignant cells often display different DNA content, compared to healthy cells)



Drug Treatment and Cell Cycle

Cells were incubated with a cytostatic reagent, then grown in medium. Samples were taken at the timepoints shown.





Combined Immuno-phenotyping and DNA staining

Method:

- **For a surface antigen**
→ Stain and then fix
- **For an intracellular antigen**
→ Fix and then stain





DNA staining and BrdU Incorporation

- In order to study DNA synthesis, the base analog on BrdU (Bromodeoxyuridine), is introduced during cell proliferation and stained later.
- The cells are then permeabilised and stained with PI.



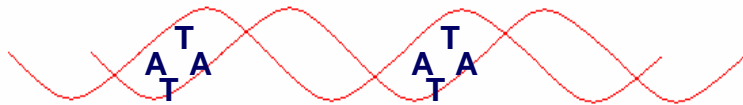


Proliferation Studies Using Cell Cycle Analysis

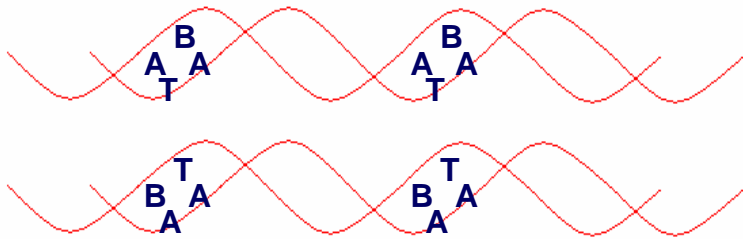
- **Pulse Labeling with Bromodeoxyuridine (BrdU)**
 - Analogous to Tritiated Thymidine Assay
- **Calculation of the Doubling Time of Cell Populations**
 - *In Vivo* and *In Vitro* labeling
- **Detection of Nuclear Antigens Associated with Proliferation**
 - Ki67, PCNA, p105



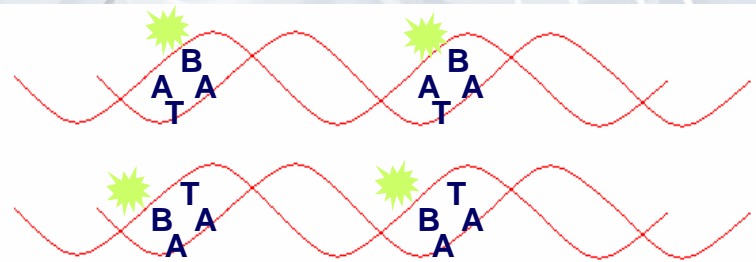
BrdU Labeling



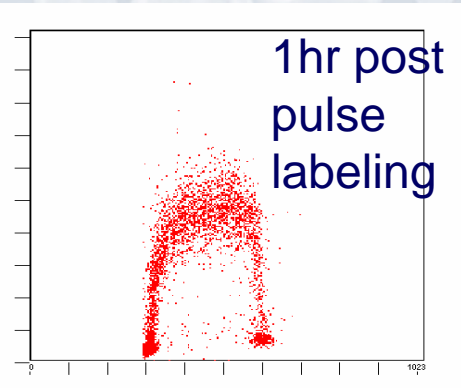
+BrdU



+ Anti BrdU FITC



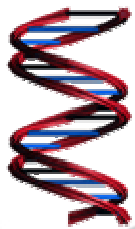
BrdU Green FL



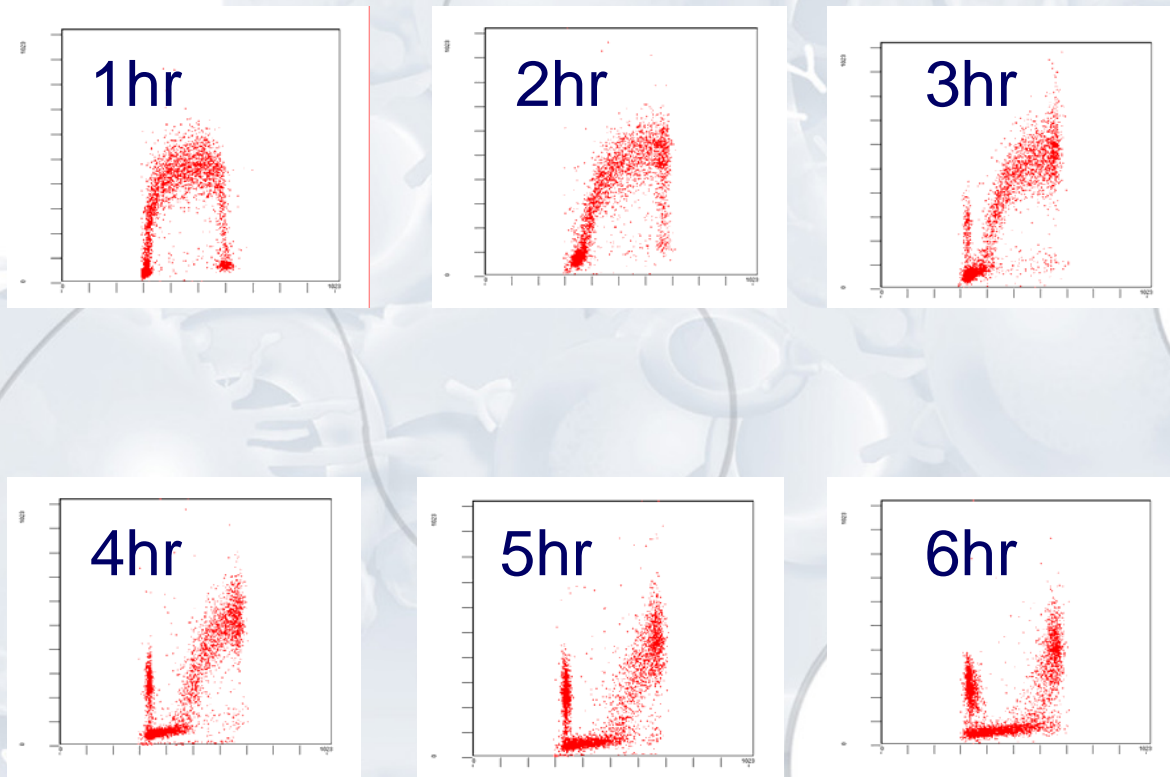
DNA Red FL



Cells Labeled with 5' Bromodeoxyuridine transiting the Cell Cycle



Green BrdU Staining



Red DNA Content





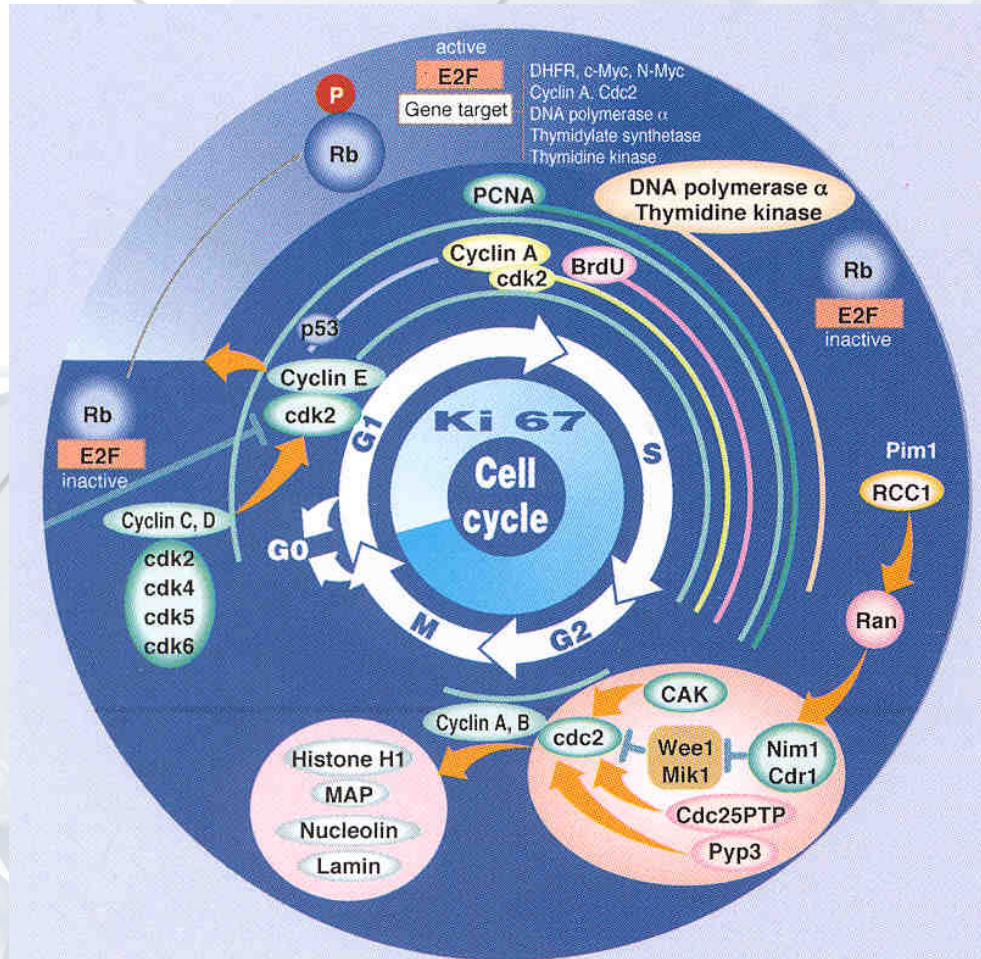
Proliferation Associated Antigens

- **Advantages of flow cytometry**
 - can relate directly to cell cycle phase
 - can measure cellular subsets
- **Disadvantages of flow cytometry**
 - sometimes difficult to confirm antibody specificity





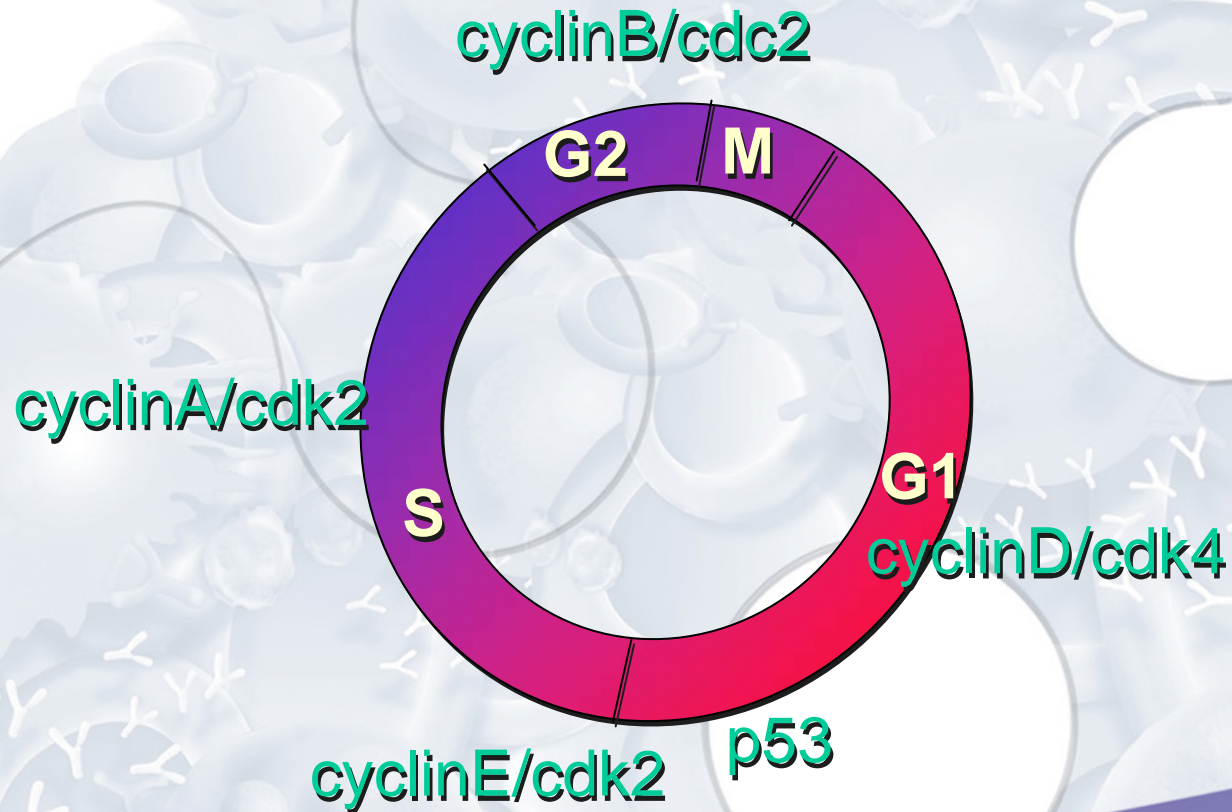
Cell Cycle Associated Antigens





Nuclear Antigens Associated With Proliferation

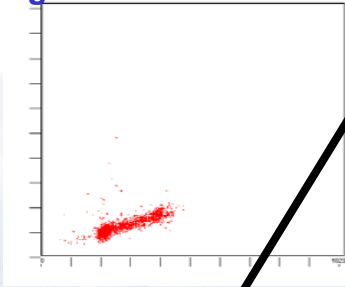
- Ki67
- PCNA
- Cyclins



Cyclin Expression

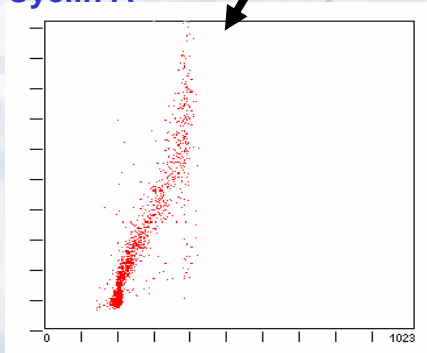


Control
IgG

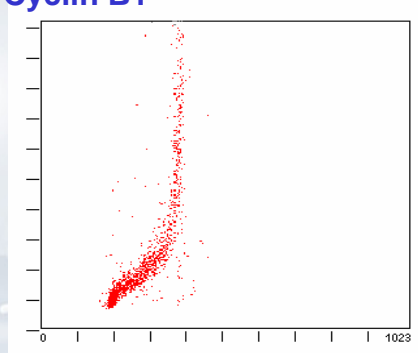


- Cyclin A reaches its maximum in G1, but is absent on mitotic cells
- Cyclin B1 reaches its maximum in G2 and mitotic cells
- Cyclin E reaches the maximum in early S-phase

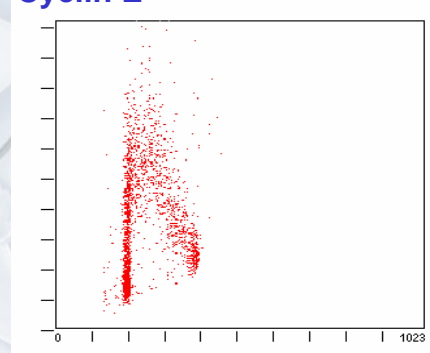
Cyclin A



Cyclin B1



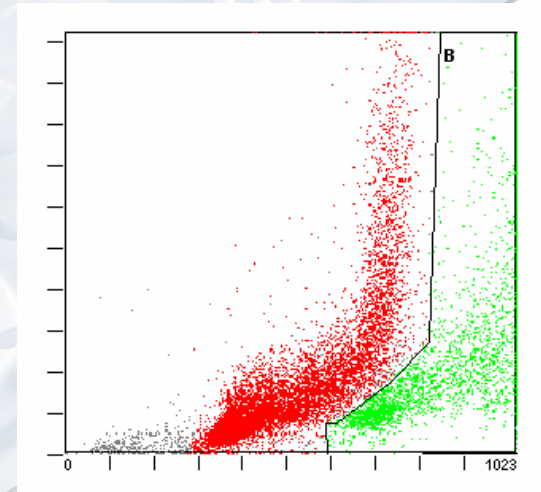
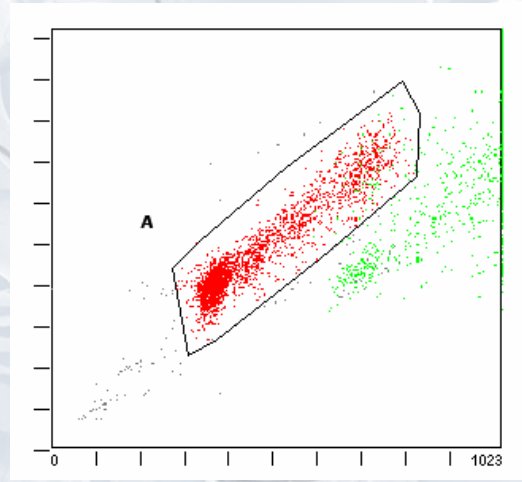
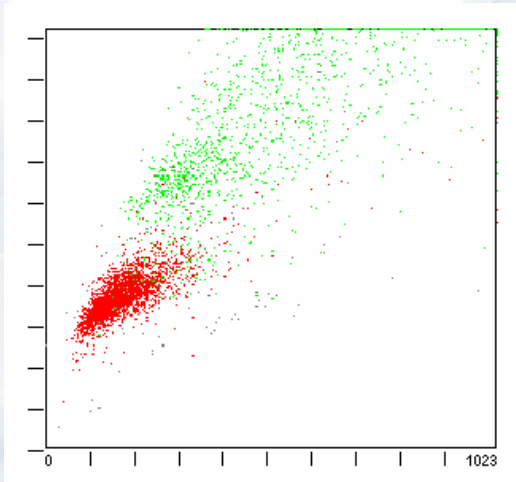
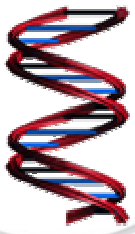
Cyclin E



Human Molt-4 Cell line



Cyclin Expression in Human Lymphoblastoid Cells



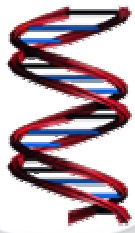


Ki67 and PCNA

- Ki67 is a nuclear antigen of 345 and 395 kDa
- Ki67 is expressed during all phases of the cell cycle except G_0
- Ki67 is a specific marker for proliferating cells, i.e. entering the cell cycle from G_1 to Mitosis
- PCNA expression increases during cell cycle, but is also present in G_0 phase

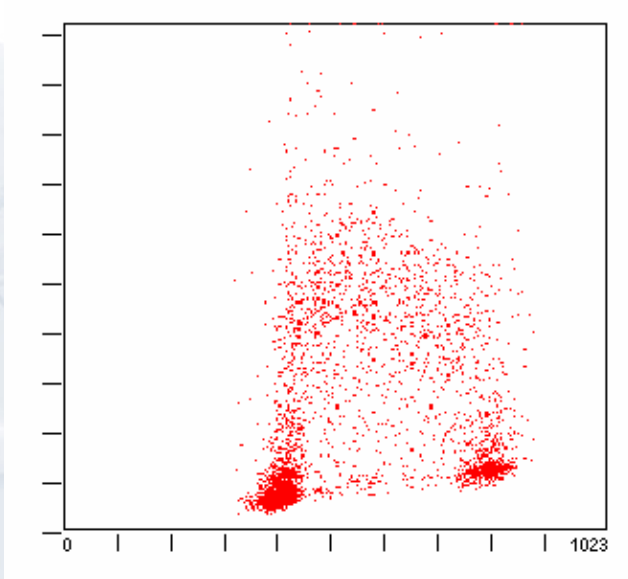


PCNA vs. PI



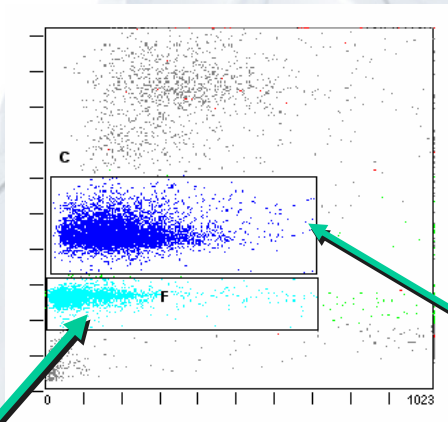
HELA cells stained simultaneously with PCNA and PI

The PCNA staining is directly related to the S-phase

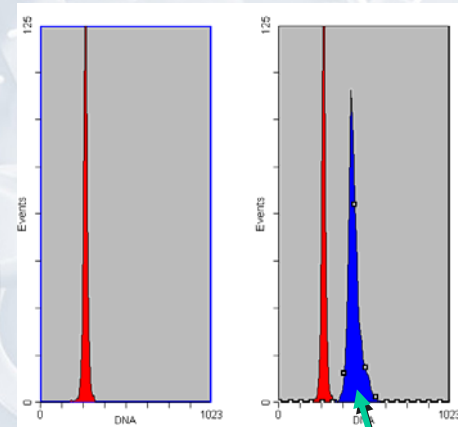


Ki67 and PI staining

Fine needle aspirate from a human breast cancer



Normal cells



Aneuploid
cancer cells





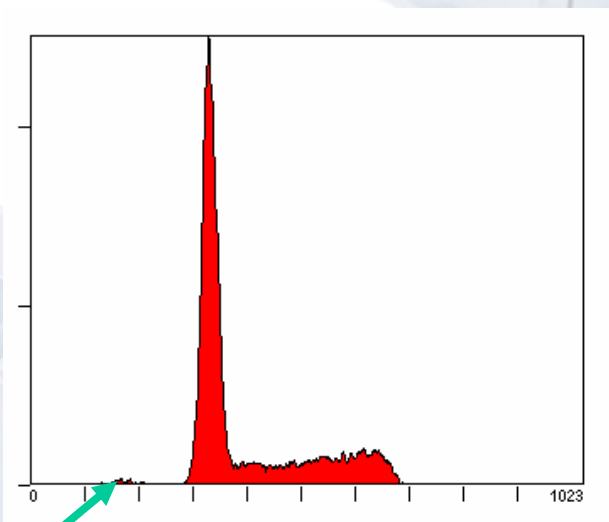
DNA staining and apoptosis

One of the simplest methods, but also one of the least reliable methods, is the staining of DNA of apoptotic cells with PI. The fragmented DNA strands will result in a typical sub- G_0 peak. The size of this peak can be used as a rough indicator of the number of apoptotic cells.



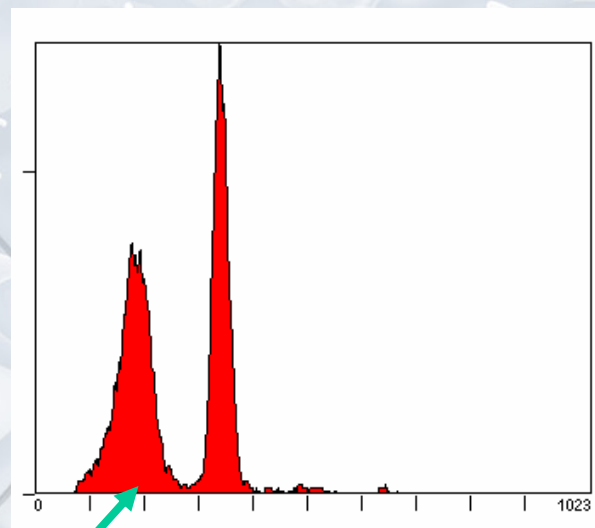
Sub-G₁ Peak

Before Induction



3% Apoptosis

After Induction



47% Apoptosis





DNA staining and Apoptosis

- In order to get a clearer picture of apoptotic cells, the cells may be stained simultaneously with PI and Annexin V.
- This assay allows a clear distinction of the various stages of apoptosis.

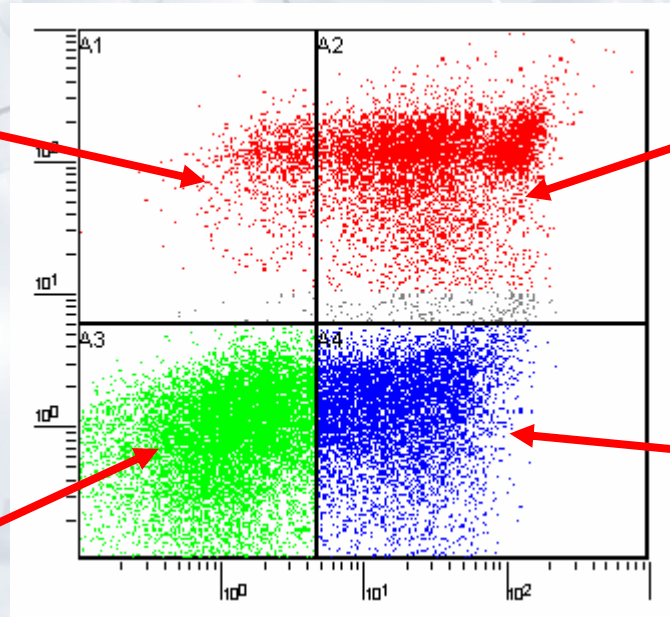


Annexin V and PI Staining



Dead Cells

**Secondary necrotic
Cells**



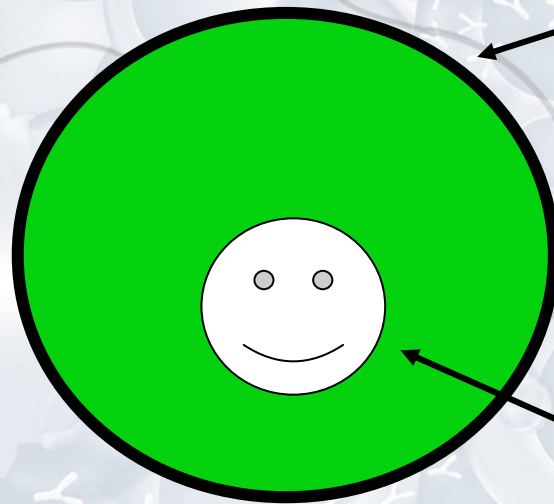
Live Cells

**Early apoptotic
Cells**



Proliferation Assays

On Day 0, CFSE (Carboxyfluorescein Succinyl Ester) used to stain cells

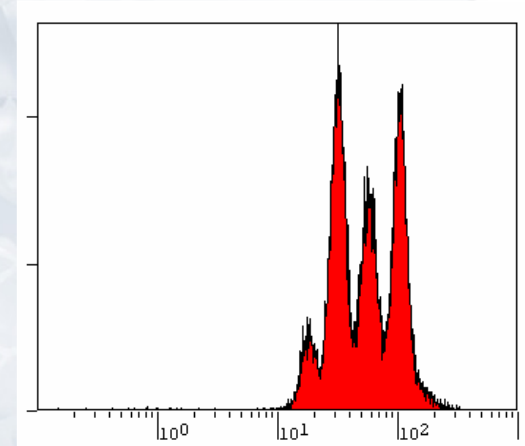
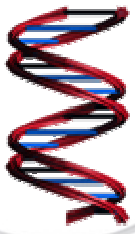


Dye binds irreversibly to cell constituents

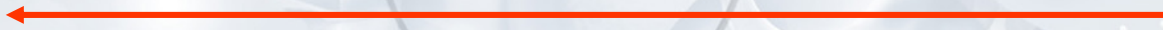
Cell Viability remains high (>99%)



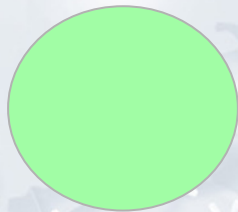
Proliferation Assays (cont.)



With Each Division Cell Intensity Halves



Day 4



Day 3



Day 2



Day 1



Day 0



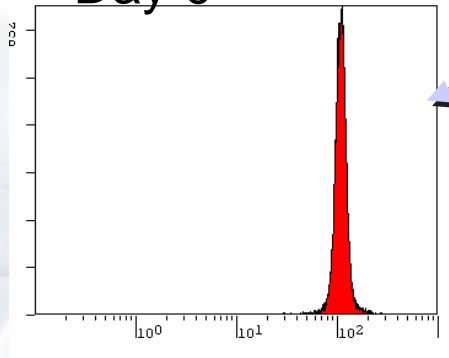
Proliferating T Cells



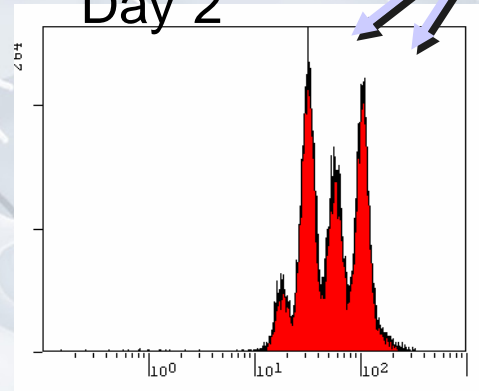
No proliferation, all cells same intensity

Some cells have gone through to 4th generation

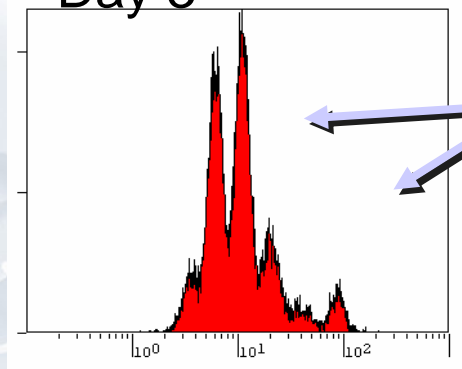
Day 0



Day 2



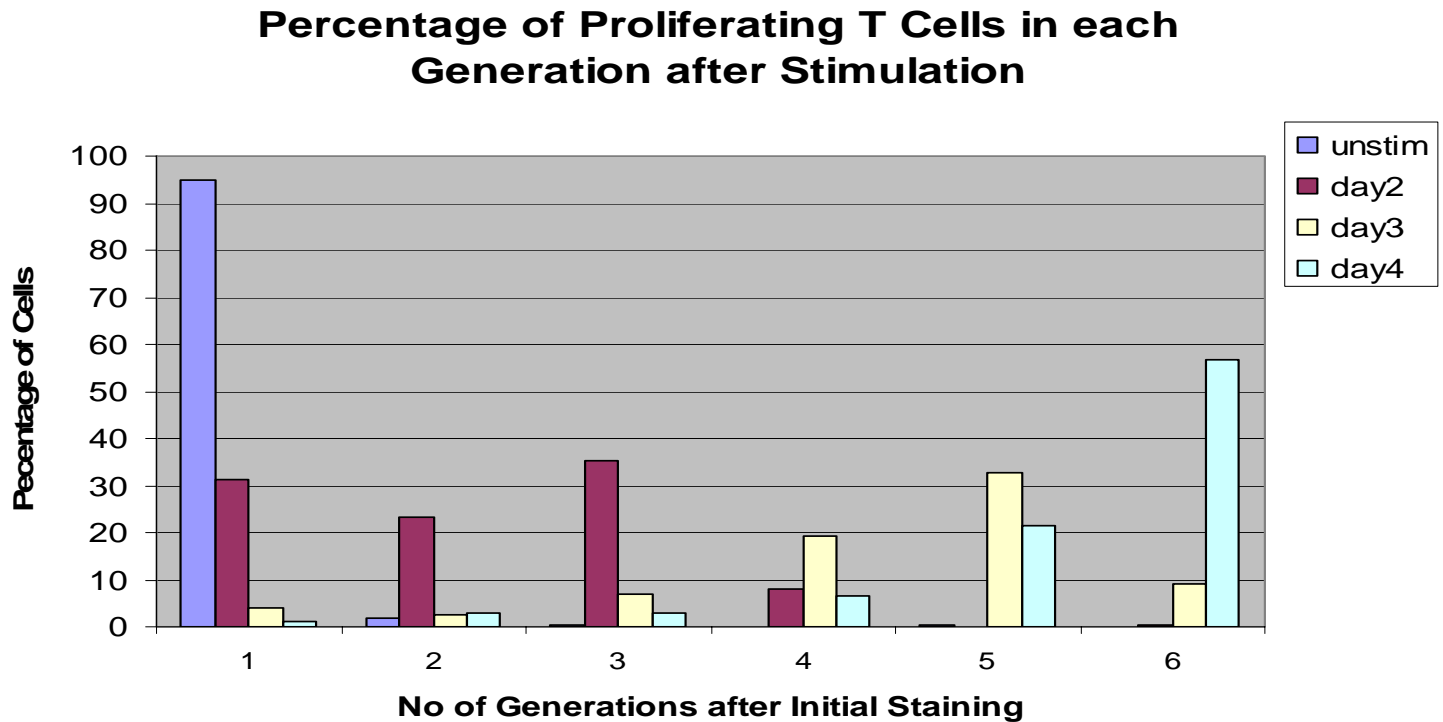
Day 3



Most cells have gone through 4 generations, some 5



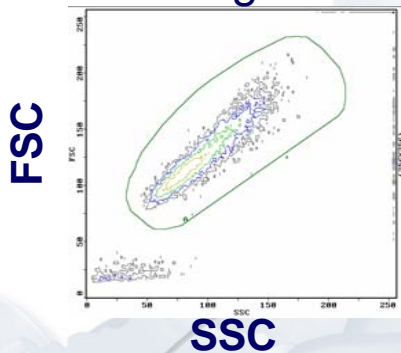
Proliferation Kinetics



Functionality Test (viability)



Scatterlight-Gate

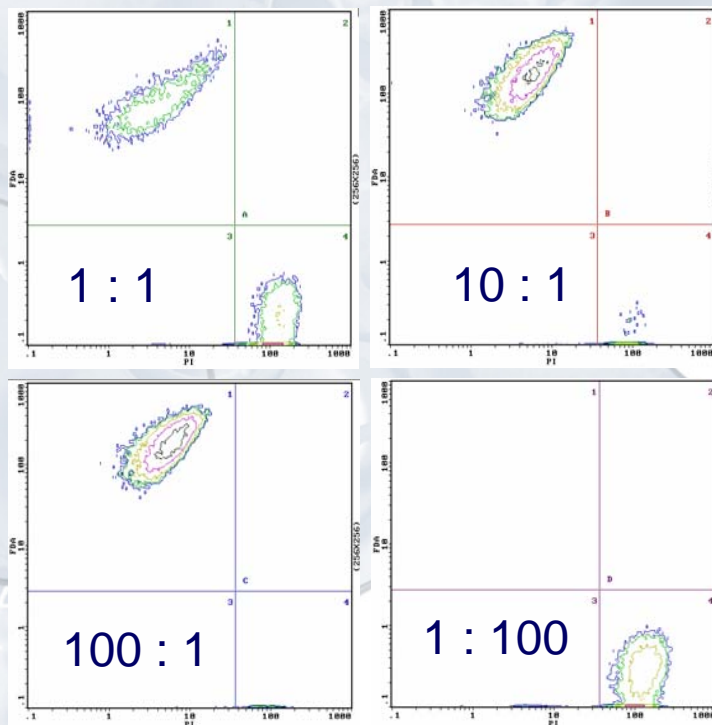


Statistics

ID	%	Count	ID	%	Count
A1	44.3	8862	B1	90.5	18091
A2	0.1	17	B2	0.2	31
A3	1.3	268	B3	1.2	233
A4	54.3	10853	B4	8.2	1645

ID	%	Count	ID	%	Count
C1	96.3	19255	D1	0.1	12
C2	0.1	14	D2	0.1	11
C3	0.8	156	D3	2.5	501
C4	2.9	575	D4	97.4	19476

FITC Fluorescence



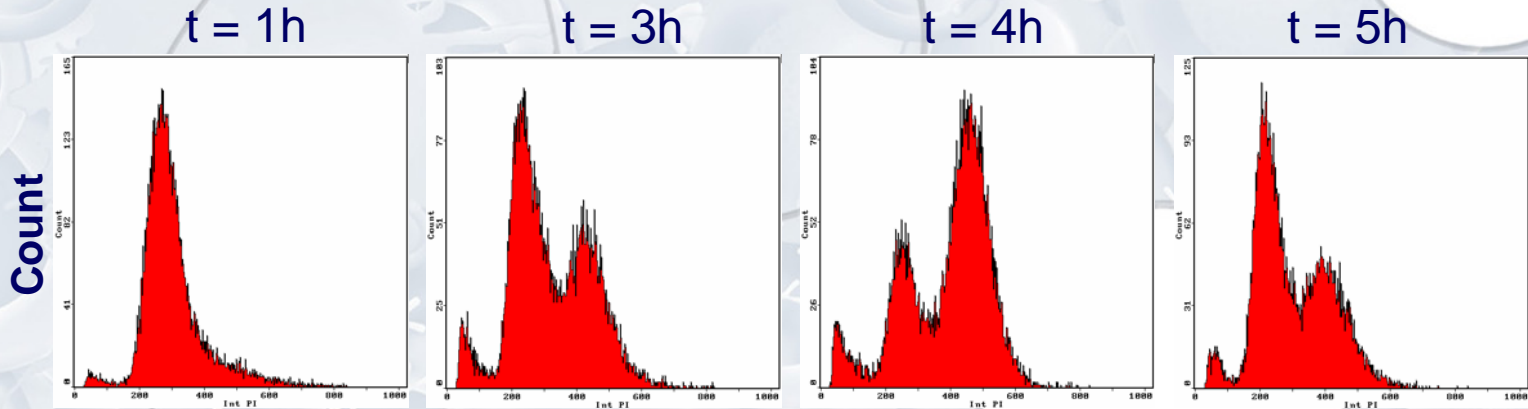
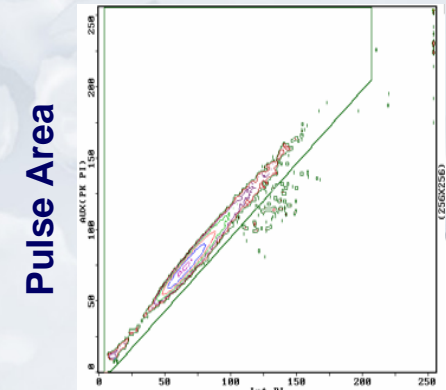
PI -Fluorescence



Cell Cycle Analysis DNA - Content/cell

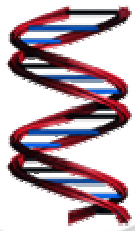


Discrimination of Doublets



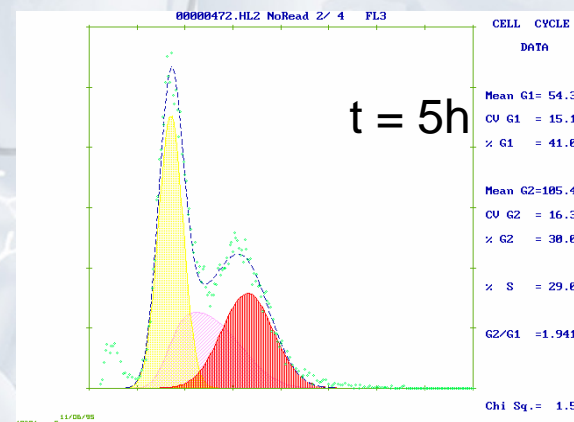
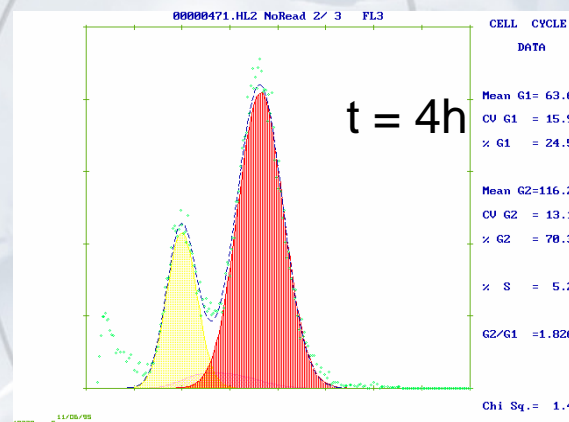
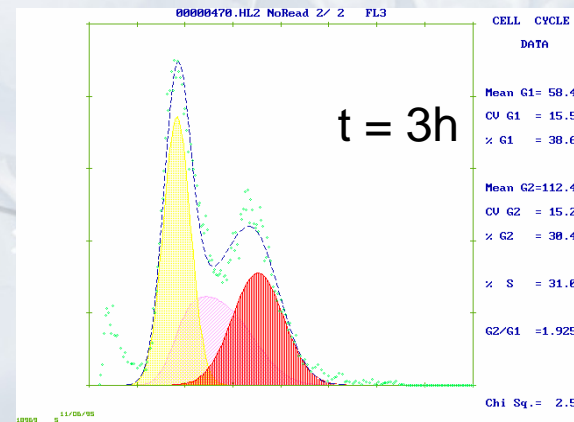
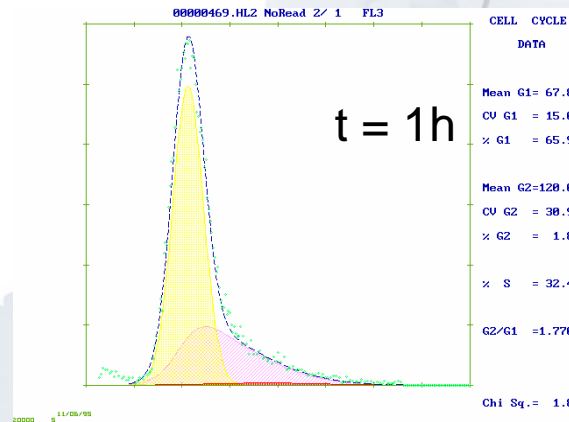
PI - Fluorescence (DNA - Content)



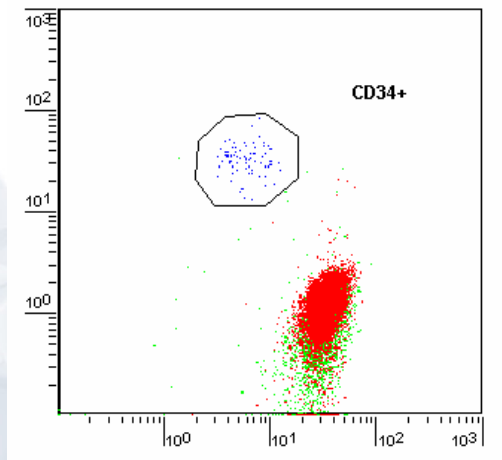
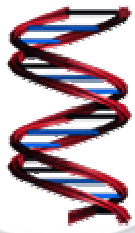


Cell cycle analysis

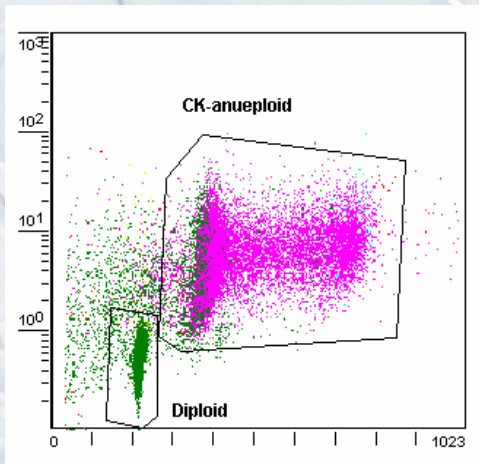
Calculation of the relative proportions in each cycle phase



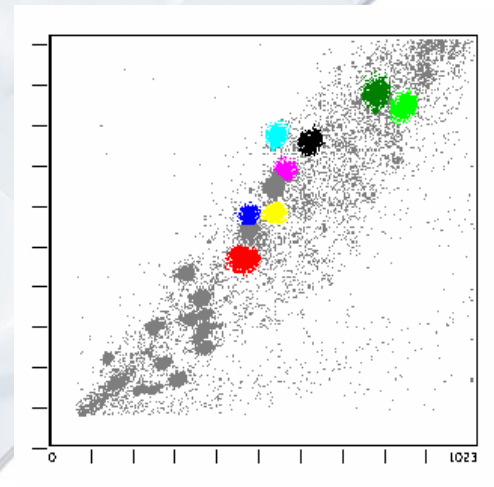
Applications



**Stem Cell
Enumeration &
Sorting**



DNA + Cytokeratin

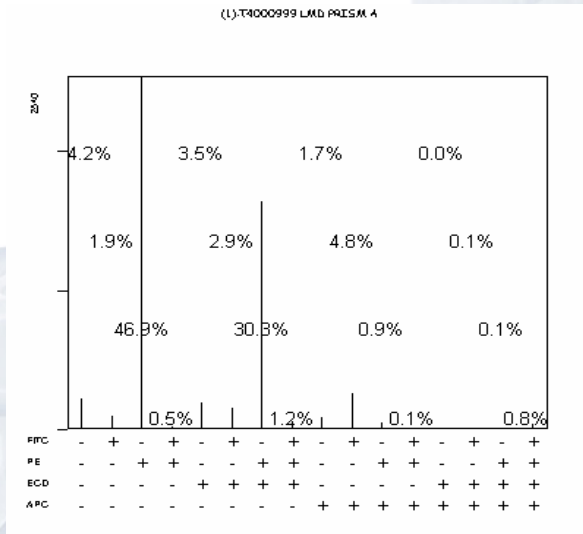


**Chromosome
Sorting**

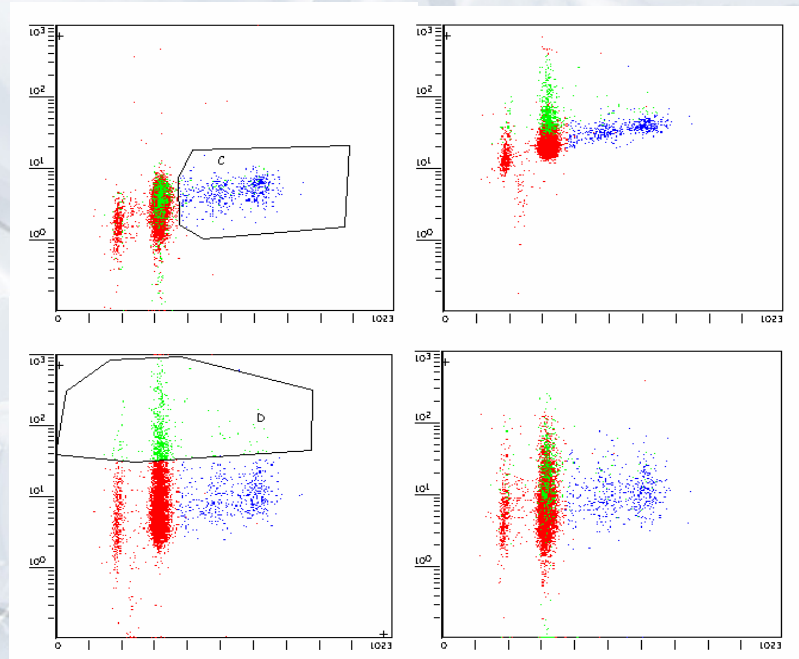




Applications II



Four Color Immunophenotyping
FITC, PE, ECD, APC

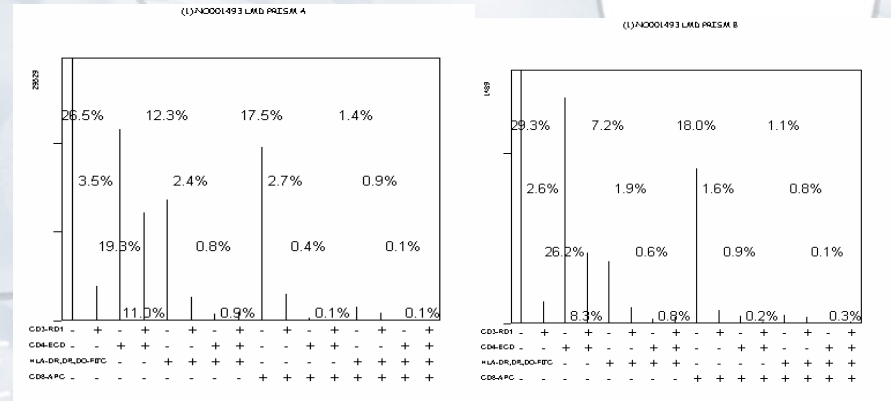
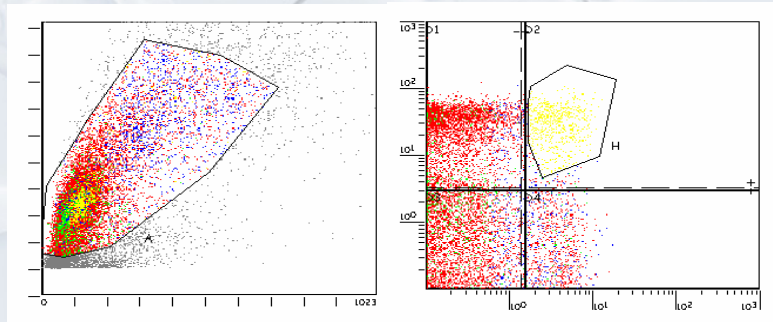
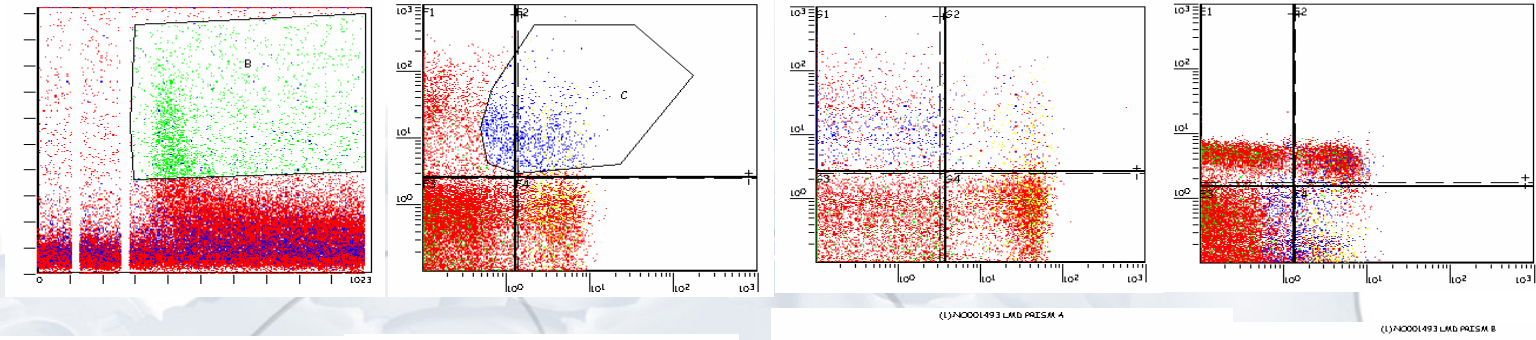


5 Color DNA Analysis:
DAPI, FITC, 7-AAD, PE, ECD





Applications III



6 Color: INDO-1 + 4 Color Immunophenotyping
Short, Long, FITC, PE, ECD, APC

