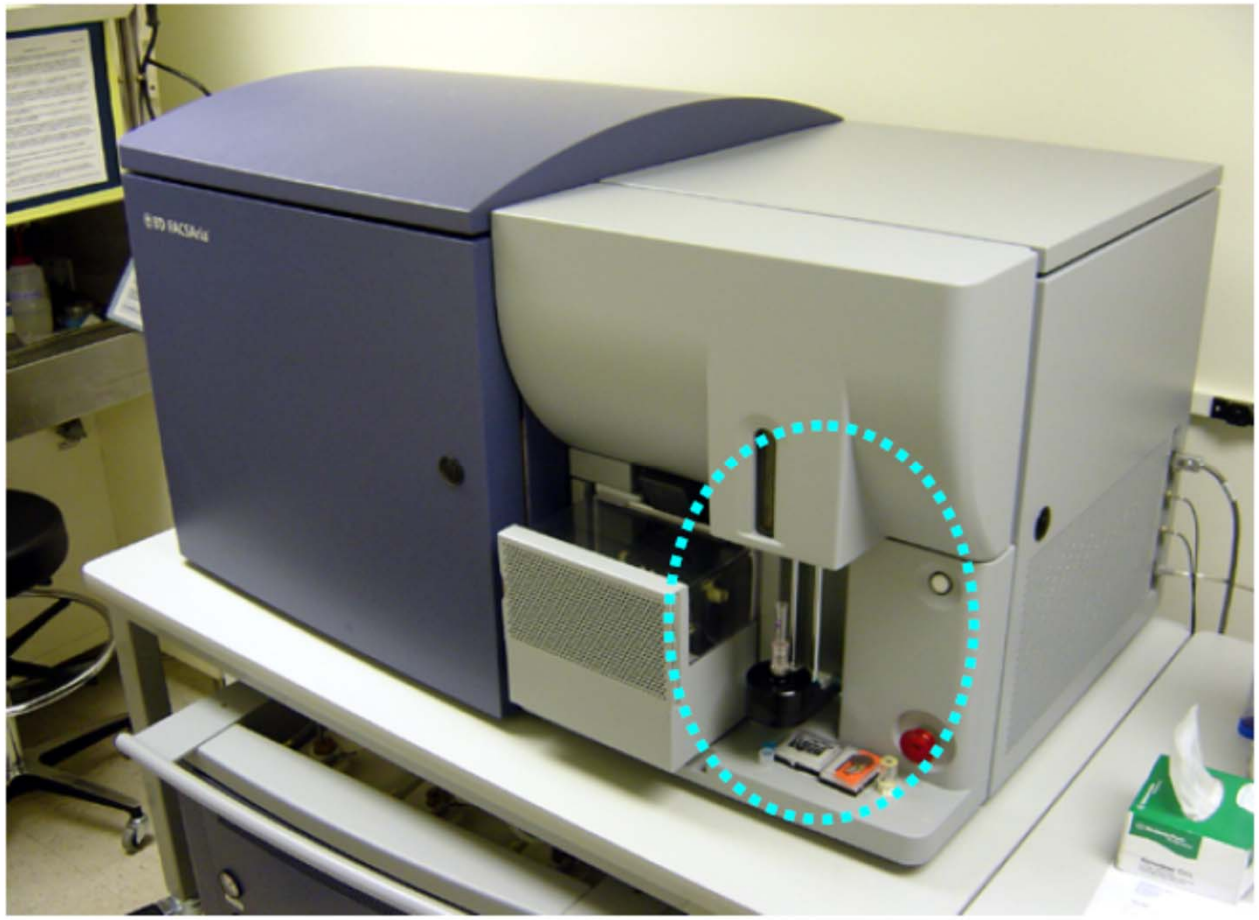
Clinical use of flow cytometry

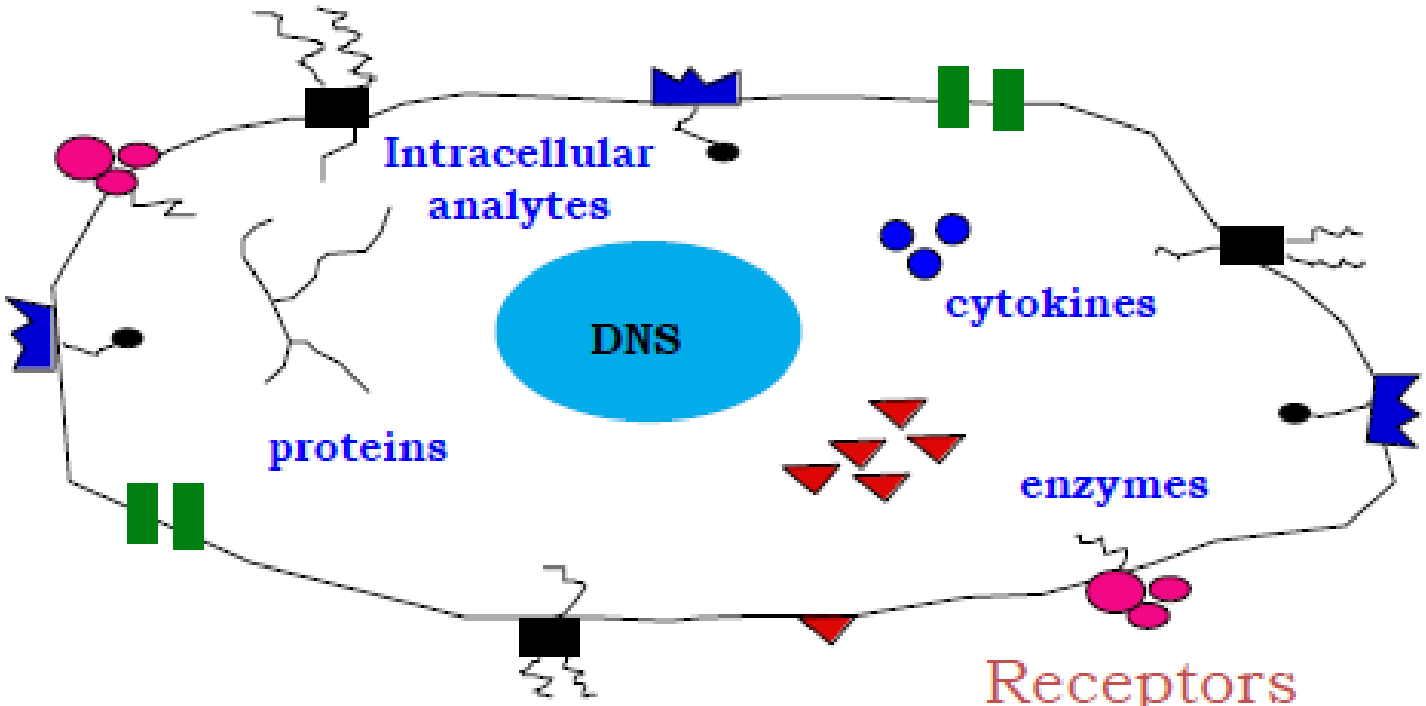
http://www.microbiol.unimelb.edu.au/images/facsca.jpg



http://cyto.mednet.ucla.edu/images/FACSAria.jpg

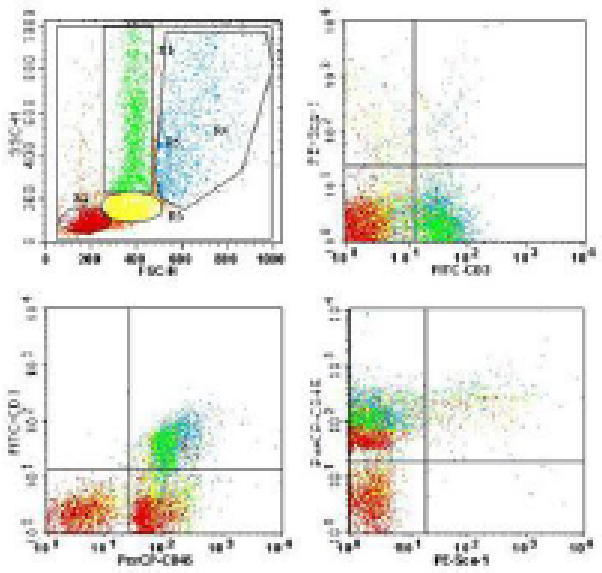
https://www.youtube.com/watch?v=UcjhdjsOG1E

Flow cytometer



Receptors
CD: cluster
of differentiation

Immune phenotyping



COMBINATION POPULATION IDENTIFIED

CD4+/CDw29+ Helper/effector, more mature memory cells

CD4+/CD45R+ Suppressor inducer, less mature non-memory cells

CD4+/Leu8+ Suppressor inducer, some helper function

CD4+/Class II MHC Activated cells, immature cells

CD4+/CD25+ Activated cells (IL2 receptor)

CD4+CD38+ Immature cells, activated cells

CD8+/CD11b+ Of the CD11b+ cells the suppressors are bright CD8+ and NK are dim

CD8+ CD8+/CD28+ Cytotoxic precursor/effector cells

CD8+/CD57+ Cytotoxic function

CD8+/Class II MHC+ Activated cells, immature cells

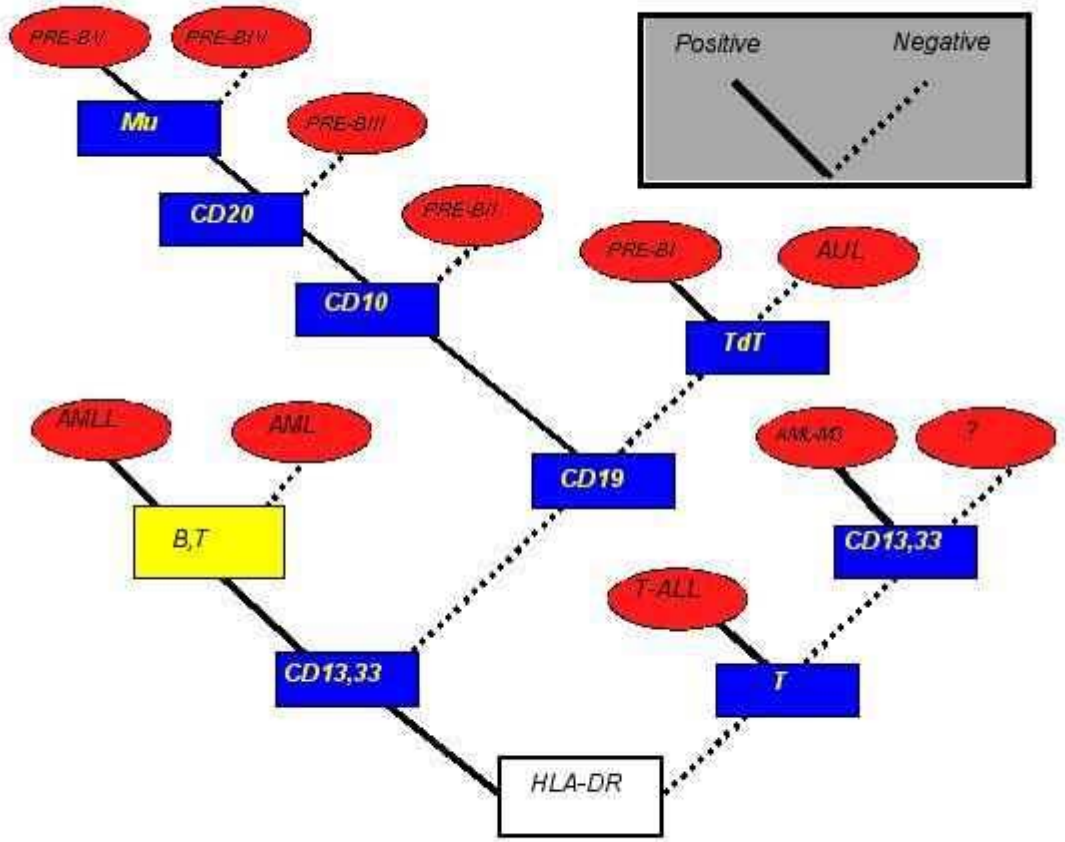
CD8+/CD25+ Activated cells (IL2 receptor)

CD8+/CD38+ Immature cells, activated cells

CD16+/CD57+ Low NK activity

CD16+/CD56+ Most potent NK activity

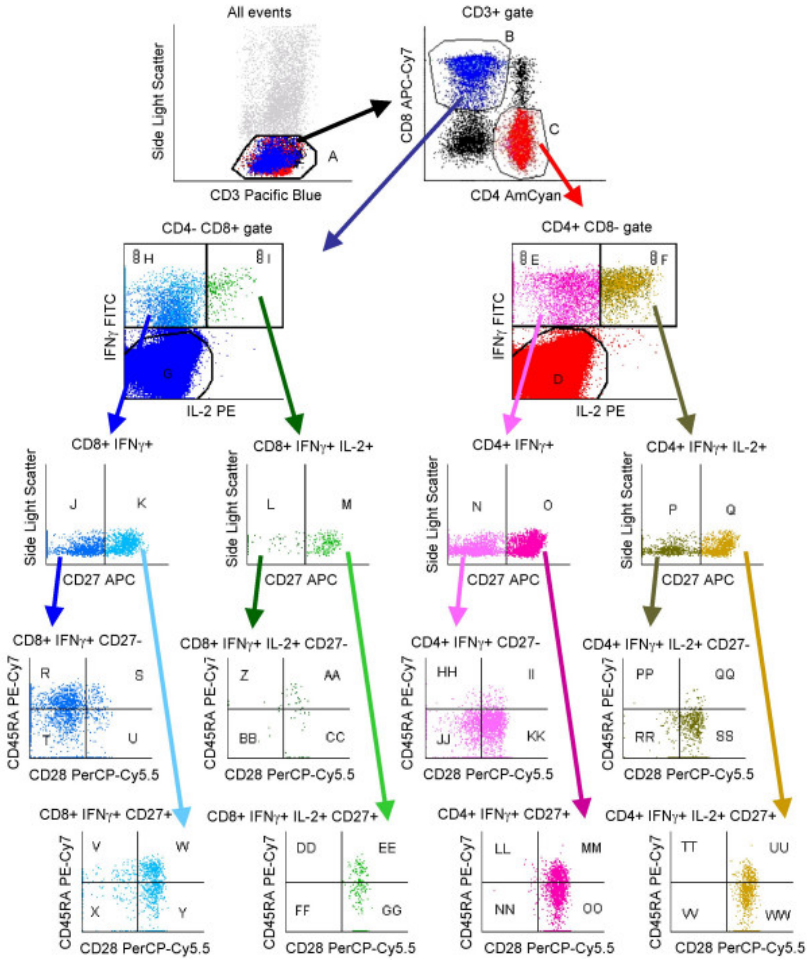
Diagnosis of leukemia



Decision Tree in Acute Leukemia

From Duque et al, Clin.Immunol.News.

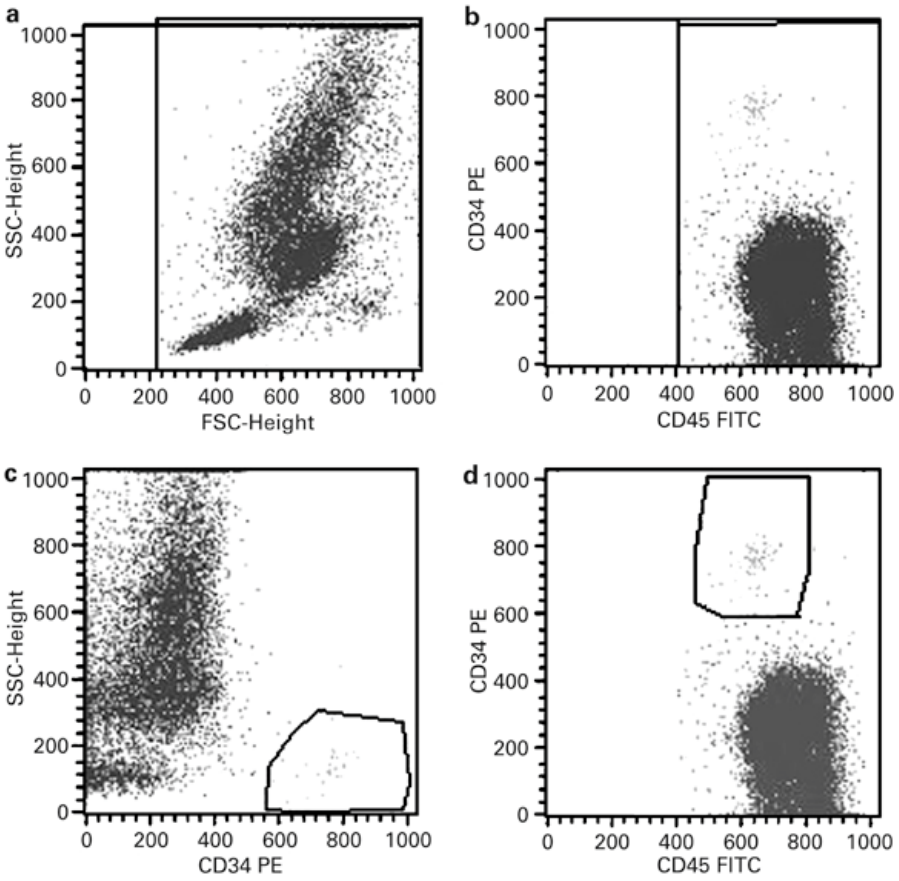
Combined use of intracellular staining and a cell surface marker



Detection of stem cells

- CD34+ stem cells

- Monitoring of stem cell count following irradiation therapy
- Success of stem cell mobilization



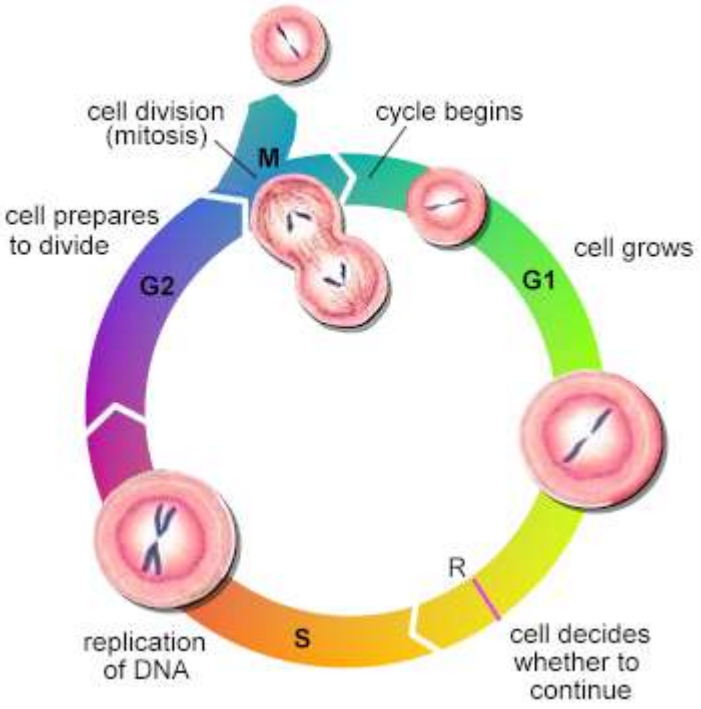
Analysis of cell life cycle

DNA probes

DAPI	}	
Hoechst	}	UV
Propidium iodide (PI)	}	
7-AAD	}	488
TOPRO-3	}	
DRAQ5	}	633

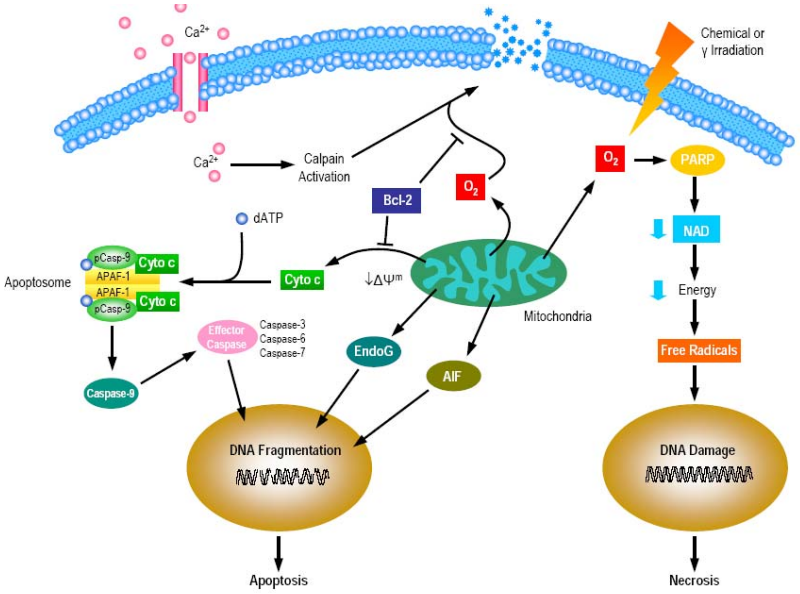
Amount of bound molecules is proportionate to DNA present in cells

Cell cycle



During cell cycle the size and DNA content of cells change

Apoptosis

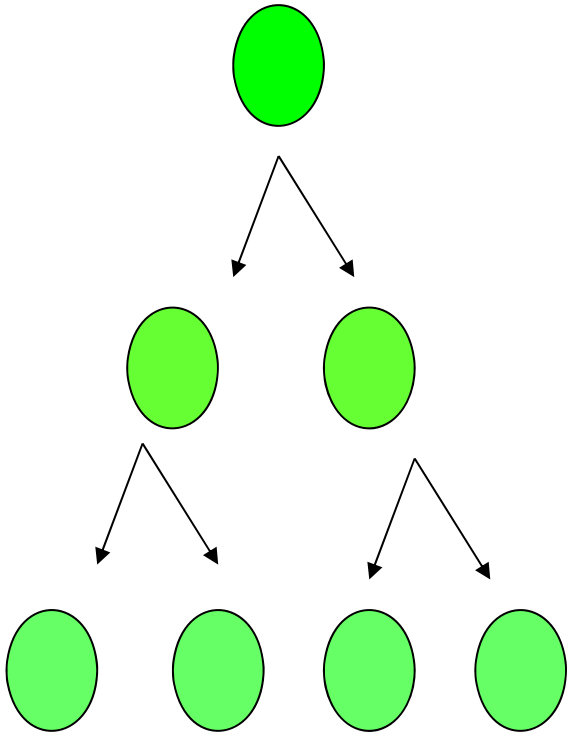


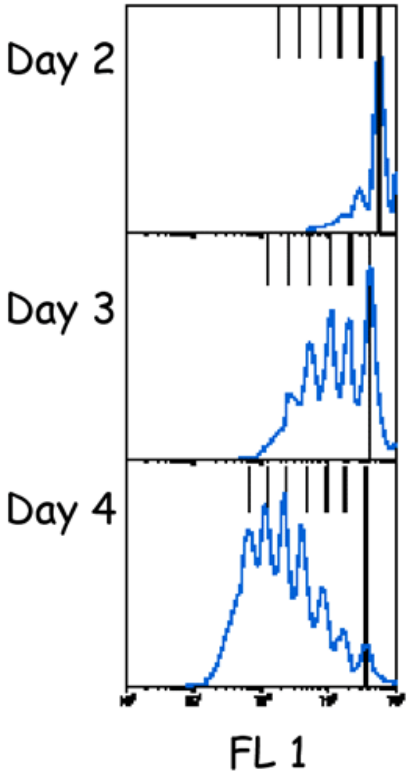
FACS measurement:

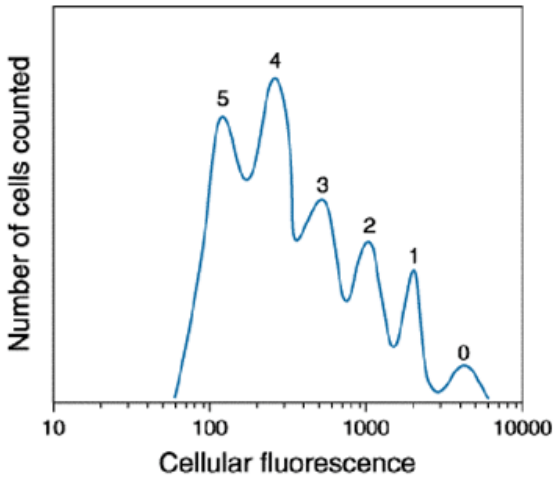
- DNA fragments
- Membrane structure and –integrity (Annexin-V, PI)
- Mitochondrion function (Mitotracker Red)
- Caspase activity (antibodies)

Cell proliferation

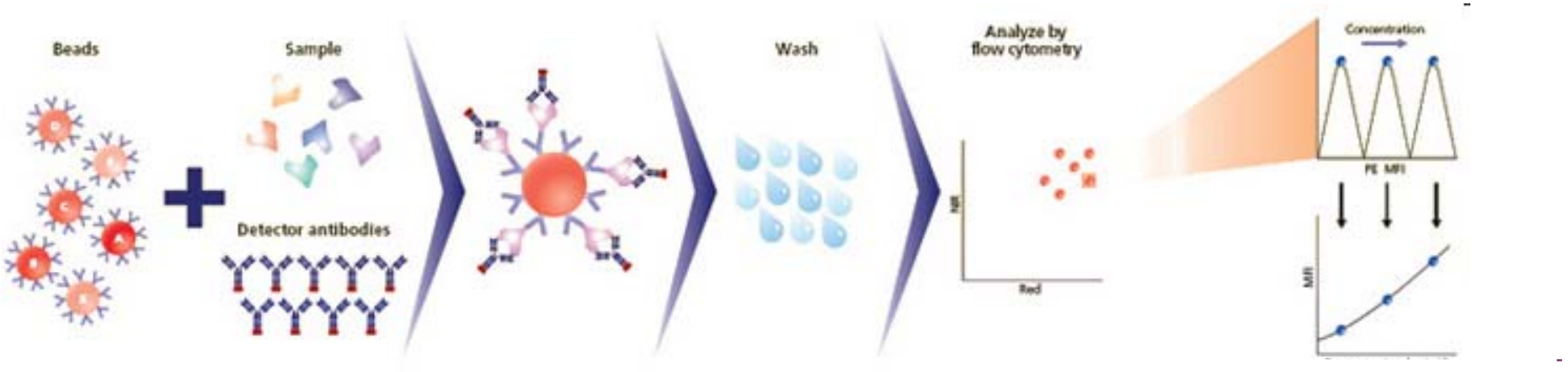
CFSE staining

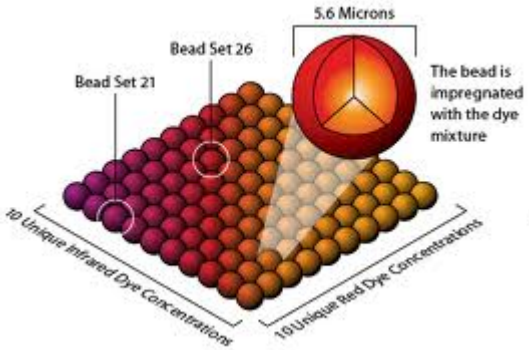




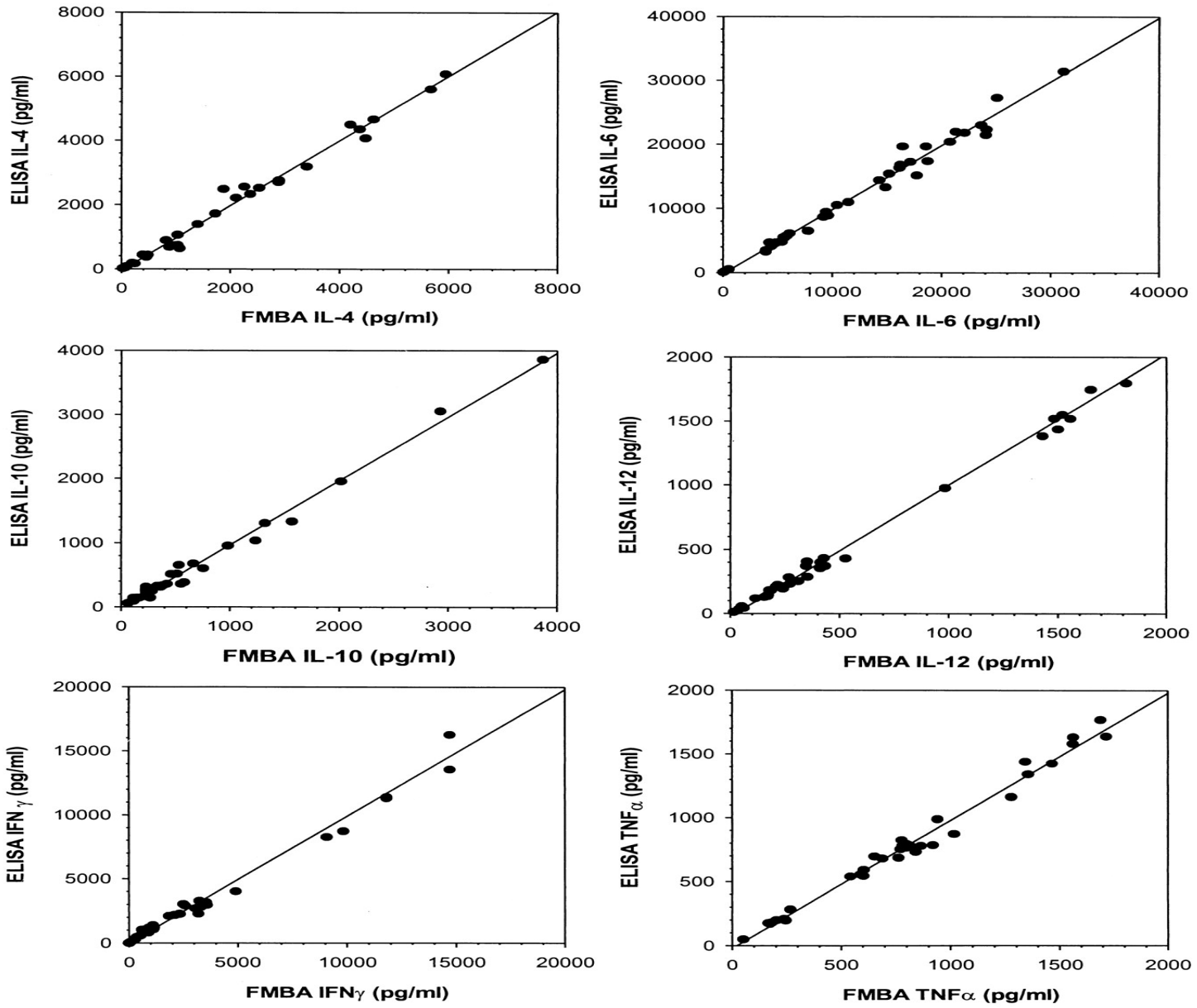


Determination of soluble parameters (cytokine levels)



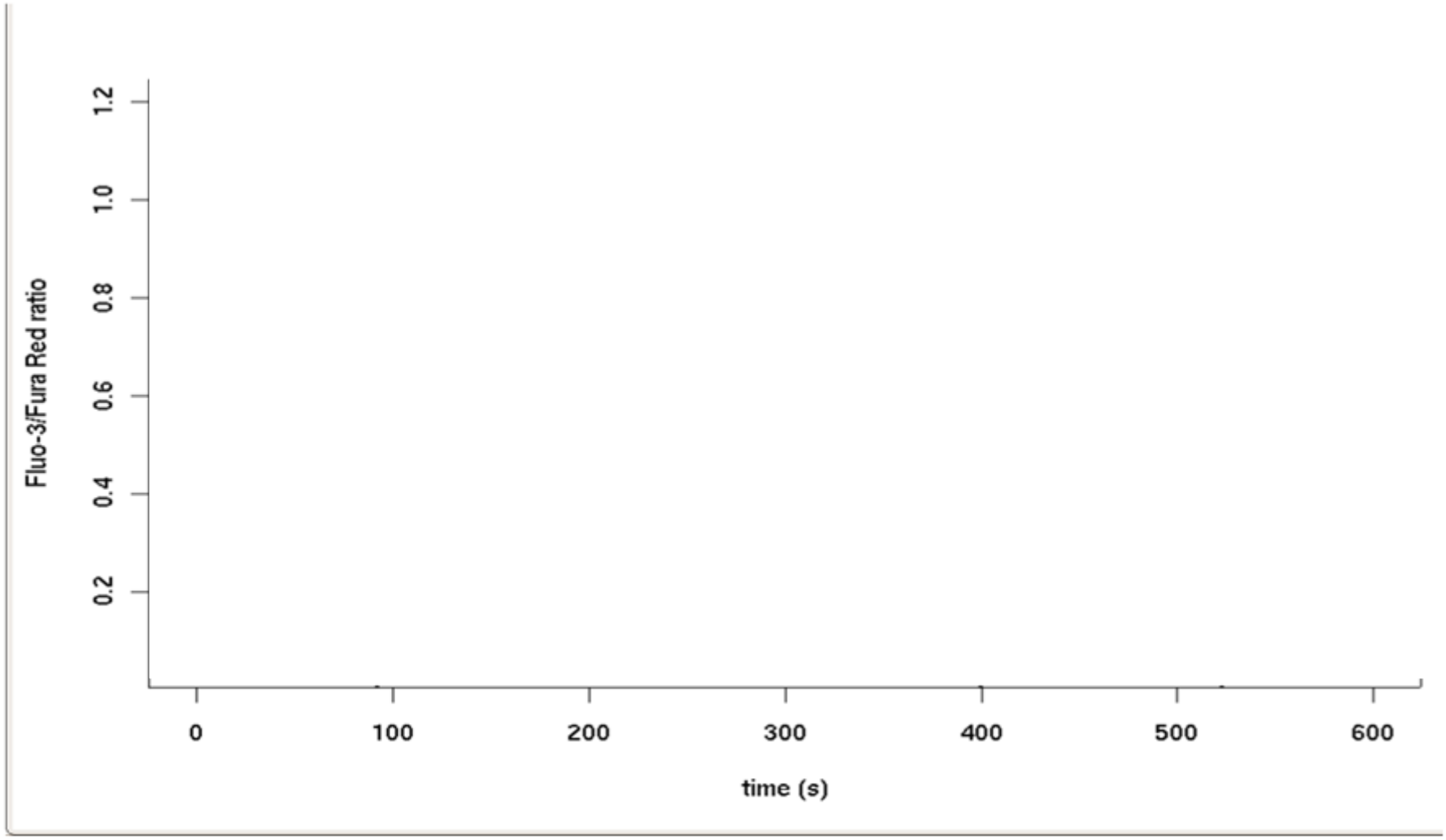


ELISA vs. FACS cytokine levels

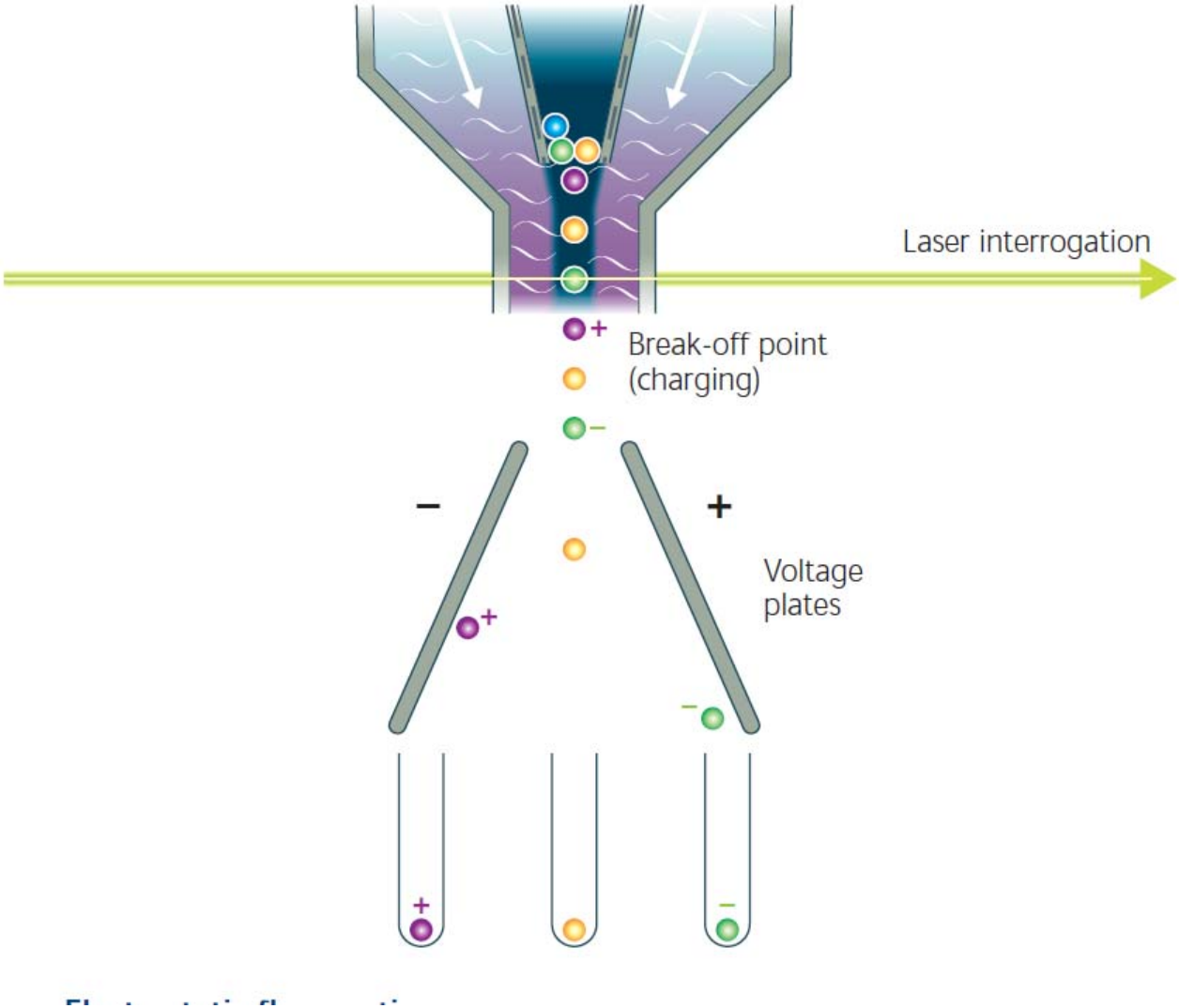


http://cvi.asm.org/cgi/content/full/8/4/776/F4

Intracellular parameters during cell activation



Cell isolation - sorting



Sample

Bone marrow: at least 3 ml, heparinic tube

ASAP send to the lab (20-25°C)

or Special storage for overnight (RPMI solution, 4- 8 °C)

Peripheral anticoagulated blood (20-25°C)

ACD (72 óra)

Na Heparin (48 óra)

K2-K3EDTA (30 óra)

Tissue (2–8°C)

cut for small pieces, RPMI solution (24 hours),

Saline should be avoided

Does not use fixation

Body fluid (RPMI solution, 4- 8 °C) 24 hours

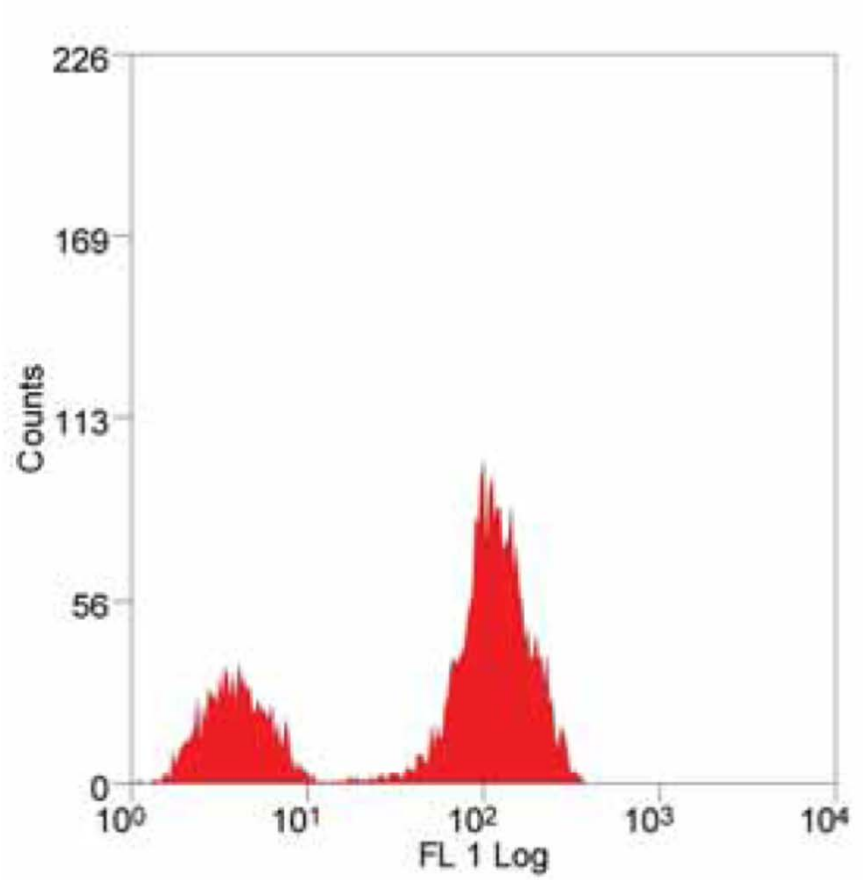
Liquor (CSF): send immediately to the lab

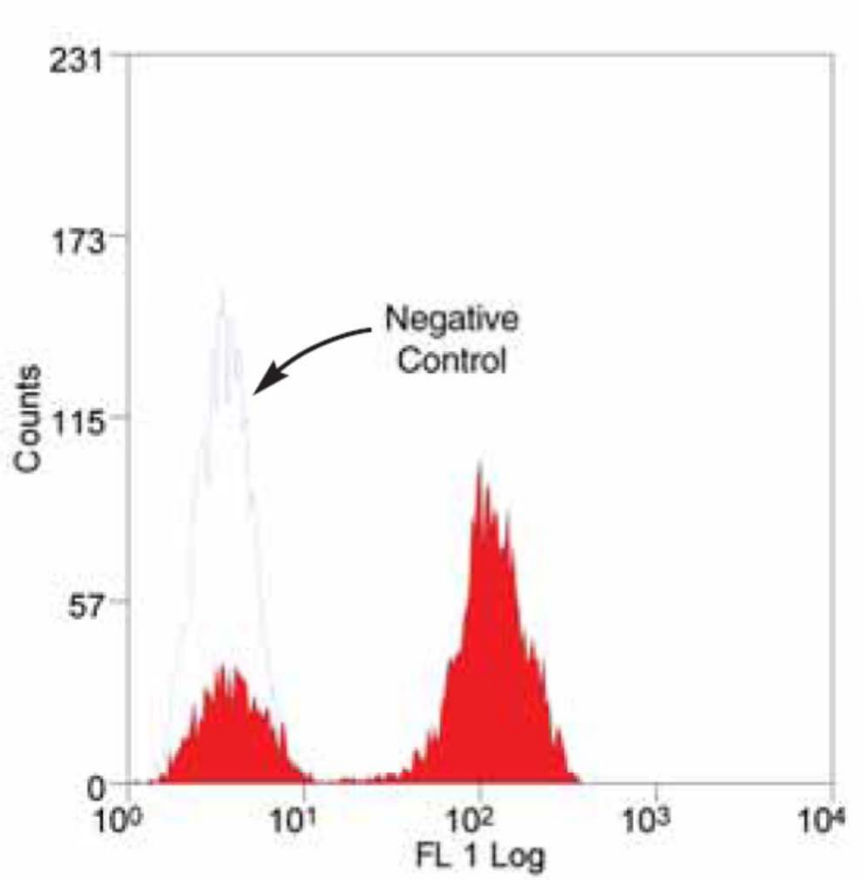
Sample processing

- Isolation

- Ficoll-gradient fuge (PBMC = peripheral blood mononuclear cells)
- Magnetic particle
- Digestion of tissues with proteases
- Specific device for chopping the tissues for one cell size

Sample processing





- formaldehyde

Benefits

- Speed
- Identification and characteriazion of individual cells
- Large number of data
- Simultaneous analysis of multiple parameters
- Detection of rare events
- Quantitative measurement based on fluorescence
- Sorting of specific cells

Drawbacks

- Expensive / complicated devices
- Tissue structure disappears
- Limited data on intracellular distribution

HEMATOLOGICAL ANALYZERS

Hematolological analyzers

Advia 2120 Siemens

Sysmex
XE

Indications for CBC

- General assessment of health status
- Inflammation & infection
- Endocrine and malignant disorders
- Blood loss
- Hematological malignancies
- Bleeding tendency
- Autoimmunity, allergies
- Monitoring of electrolyte and fluid homeostasis

Components of CBC report

CBC

- WBC 4-10 G/l
- RBC 4-5,5 T/l
- HGB 130-160 g/l
- HCT 0,4-0,52 l/l
- MCV 80-95 fl
- MCH 28-32 pg
- MCHC 330-370 g/l
- (CHCM) 330-370 g/l
- RDW 11,5-14,5%
- HDW 2,2-3,2 g/dL
- PLT 130-400 G/l
- MPV 7-11 fl
- PDW 25-65%
- PCT 0,12-0,36%
- FRAGM
- %MICRO
- %MACRO
- %HYPO
- %HYPER

Opportunities and limitations

- Measured directly and **completely reliable data**:
- WBC, RBC, hemoglobin levels, MCV

- **Less reliable**:
- Differential cell count, reticulocyte and platelet count (in case of low levels)

- **Depends on instrumentations (no standardisation)**
- RDW (RBC distribution width)
- MPV (mean platetet volume)
- PDW (platelet distribution width)

Calculated parameters

- MCV (mean corpuscular volume) = Htc/RBC
  Ref: 80-95 fl

- MCH (mean corpuscular hemoglobin) = Hb/RBC
  Ref: 28-32 pg

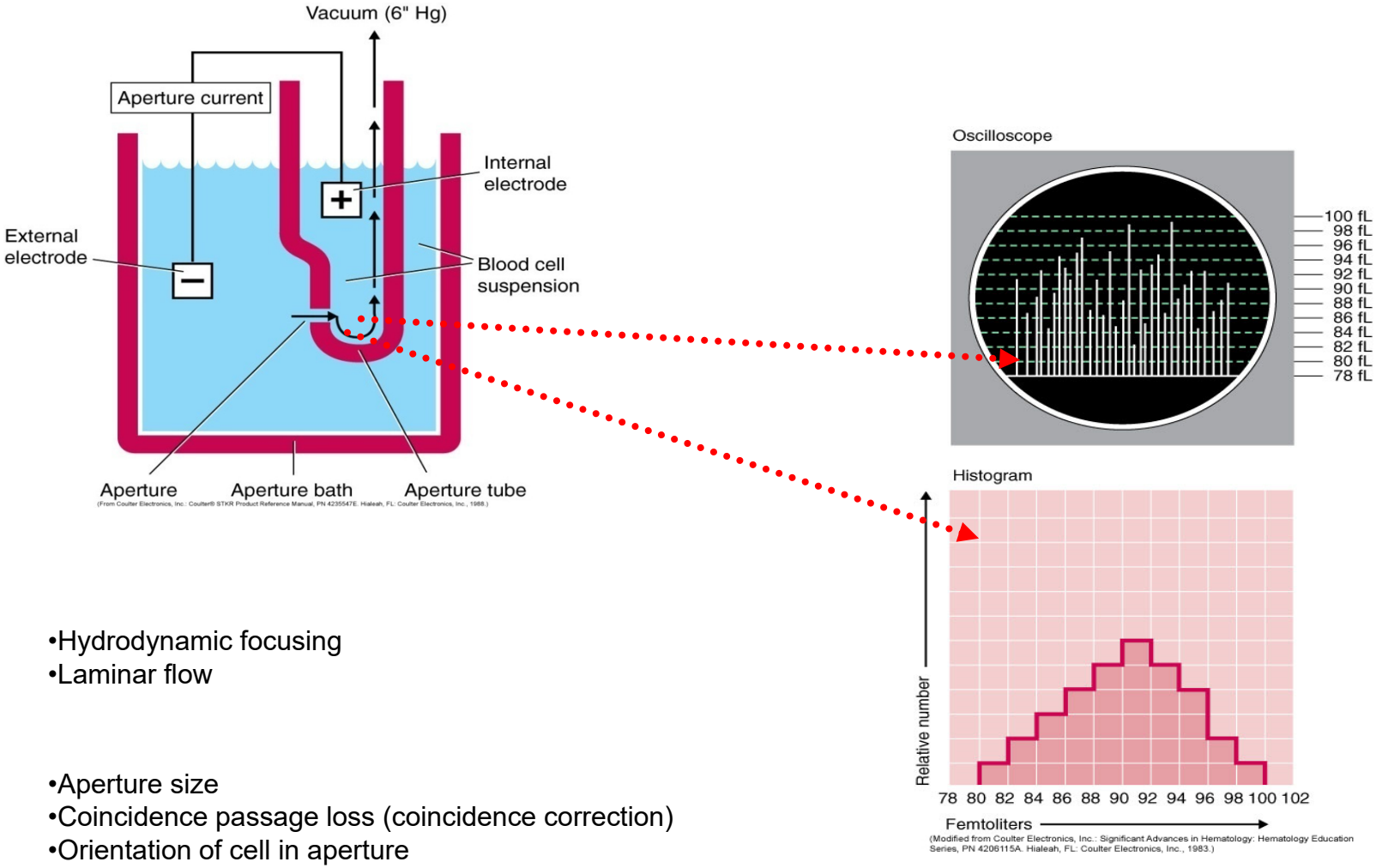
- MCHC (mean corp. hemoglobin contrentation) = Hb/Htc
  Ref: 330-370 g/l

Differential cell count

	Neonate	Infant	Adult
Lymphocyte	20-70%	25-50%	20-40%
Monocyte	1-10%	1-6%	3-8%
Neutrophyl granulocyte	15-60%	25-60%	40-70%
Stabs	1-8%	3-6%	1-2%
Eosinophyl granulocyte	1-5%	1-5%	1-5%
Basophyl granulocyte	0-1%	0-1%	0,5-1%

Principles of measurement

Electric impedance (DC) :

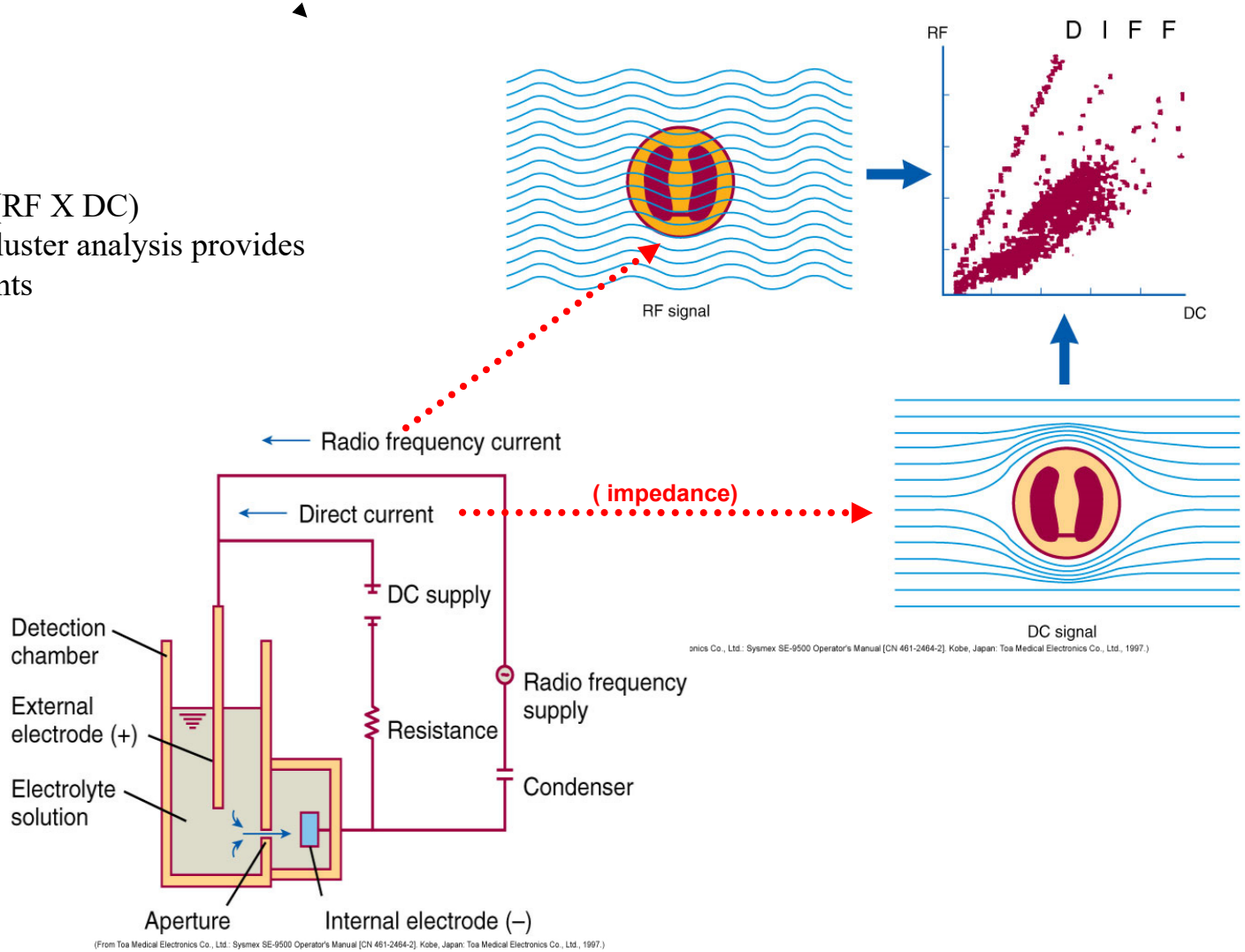


•Hydrodynamic focusing
•Laminar flow

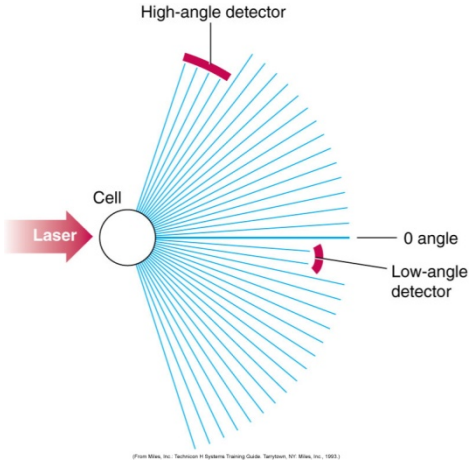
•Aperture size
•Coincidence passage loss (coincidence correction)
•Orientation of cell in aperture
•Low hemoglobin (RBC parameter)

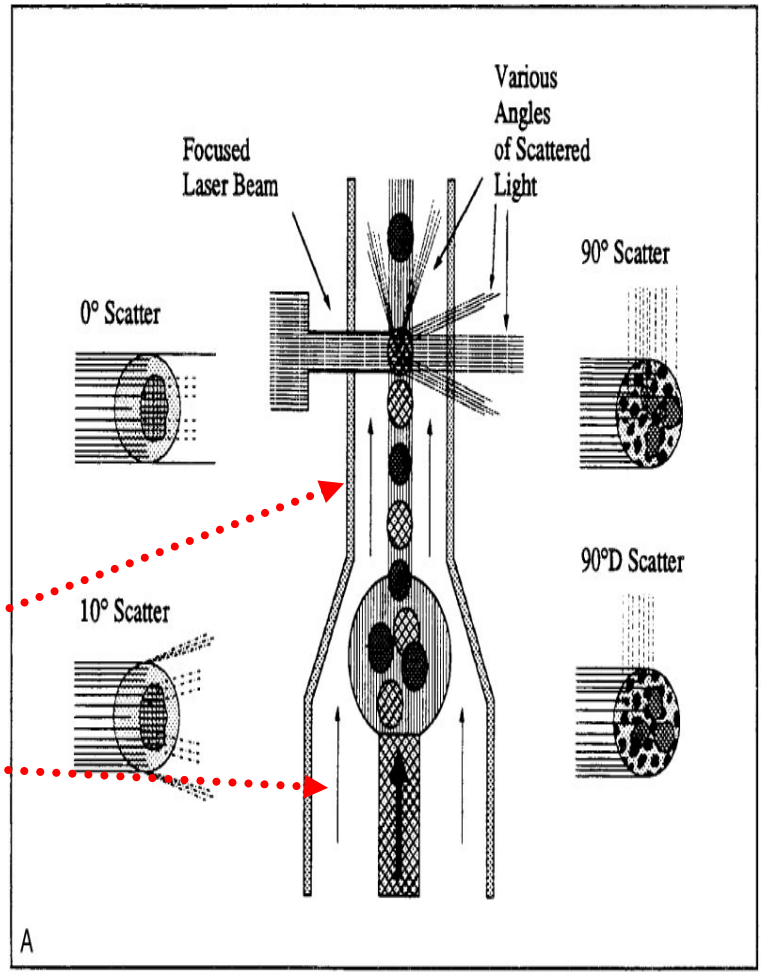
Radiofrequence resistence (RF):

- Scatterplot (RF X DC)
- Computer cluster analysis provides absolute counts



Optical based measurement:

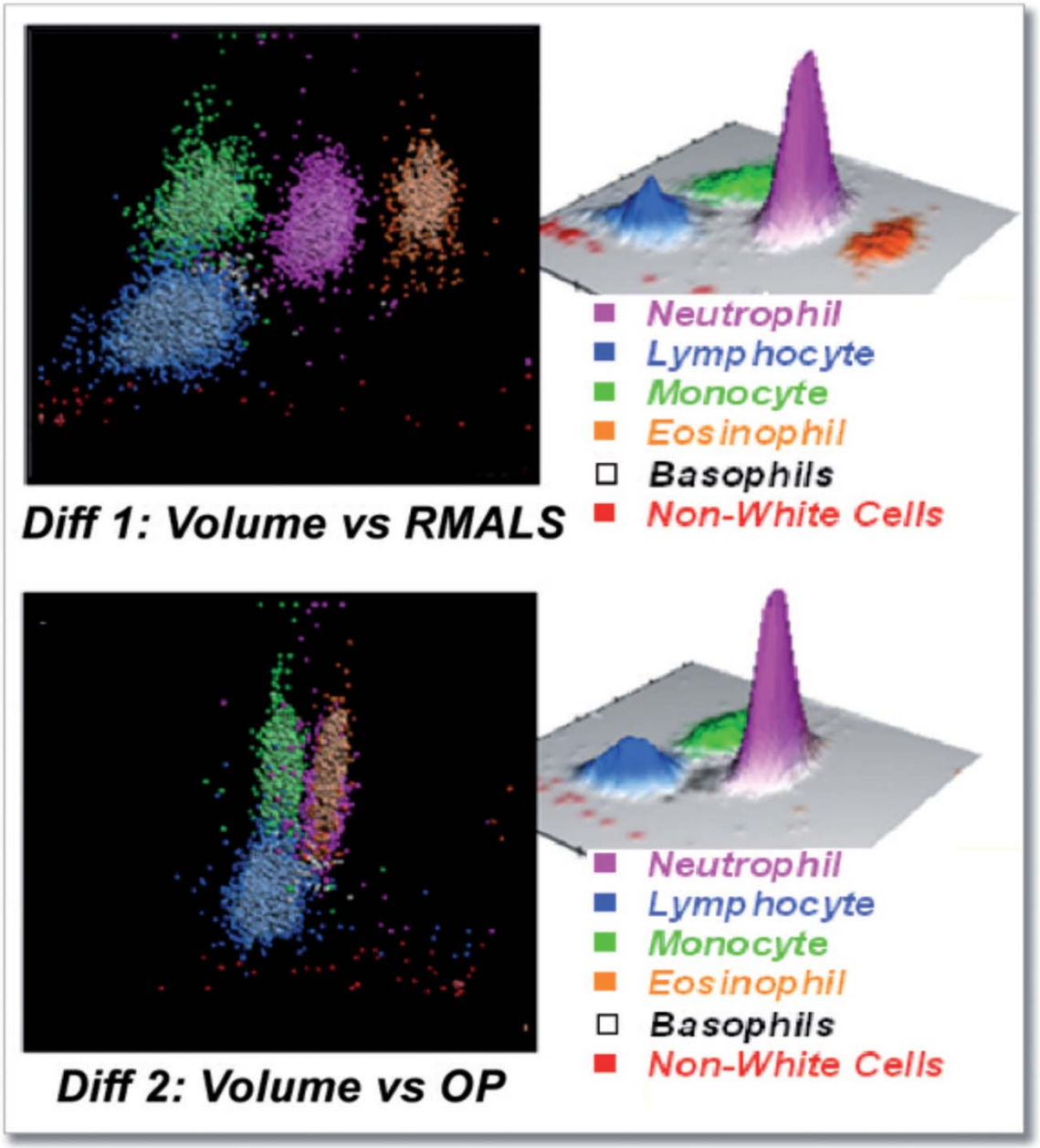




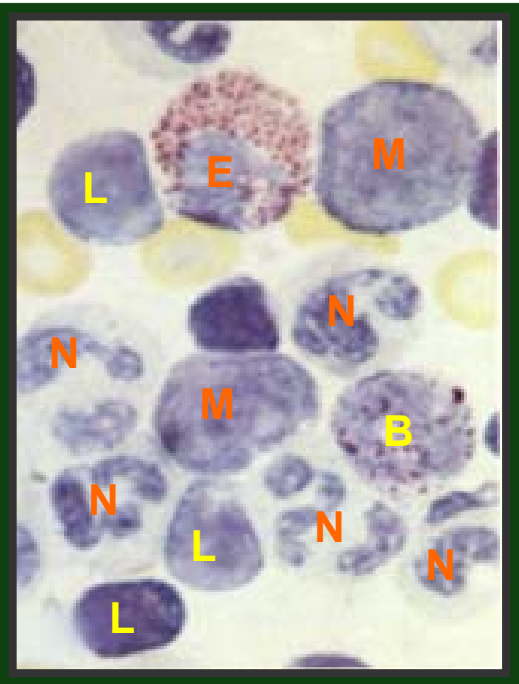
(From Abbott Laboratories: CELL-DYN® 3500 System Operator's Manual [LN 92722-05]. Abbott Park, Ill. Abbott Laboratories, 1996.)

- Cells in solution run through quartz flow cell
- Hydrodynamic focusing (uses sheath fluid) using principle of laminar flow
- Focused laser beam (coherent, monochromatic)
- Computer cluster analysis of cytograms provide quantitative and qualitative information

Differentiation of WBC without staining

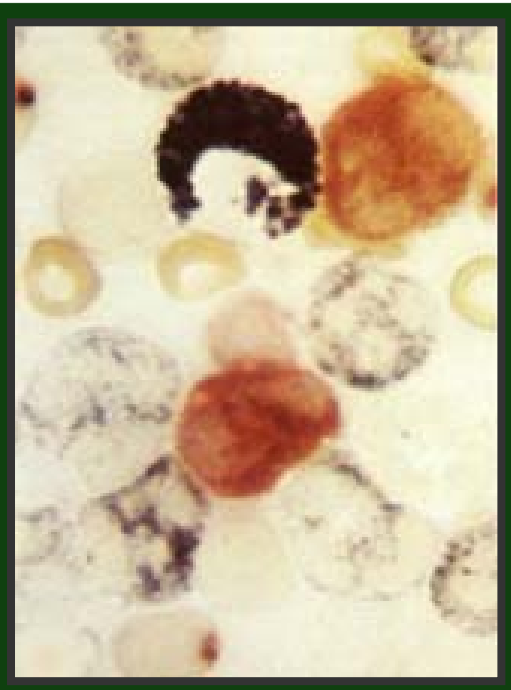


Peroxidase positive cells



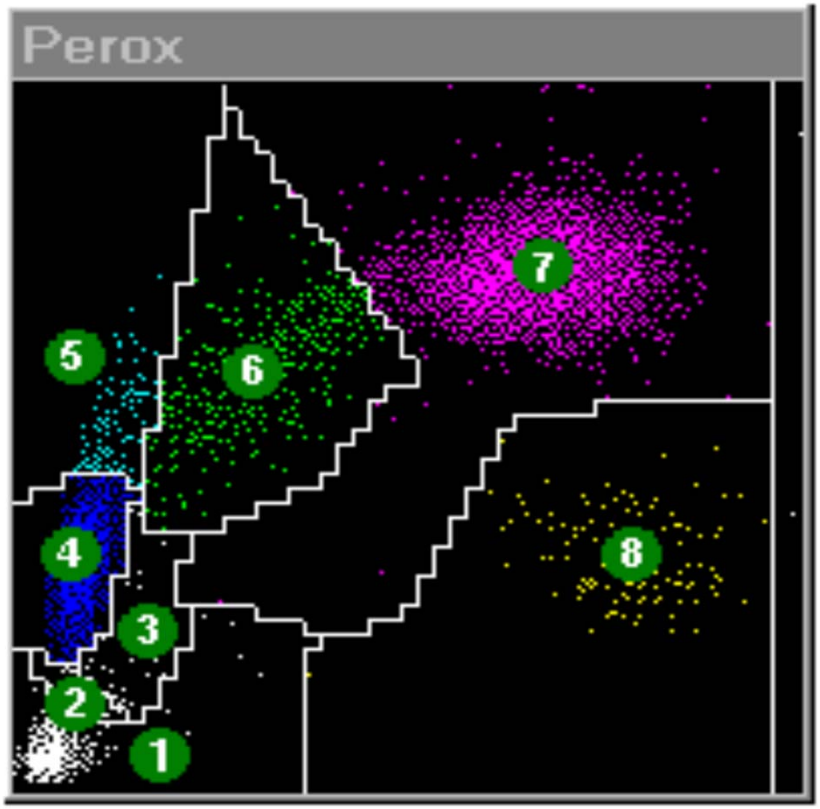
Wright staining

M = Monocyte
N = Neutrophil
E = Eosinophil
L = Lymphocyte
B = Basophil



Peroxidase staining

Cloud diagram with peroxidase staining

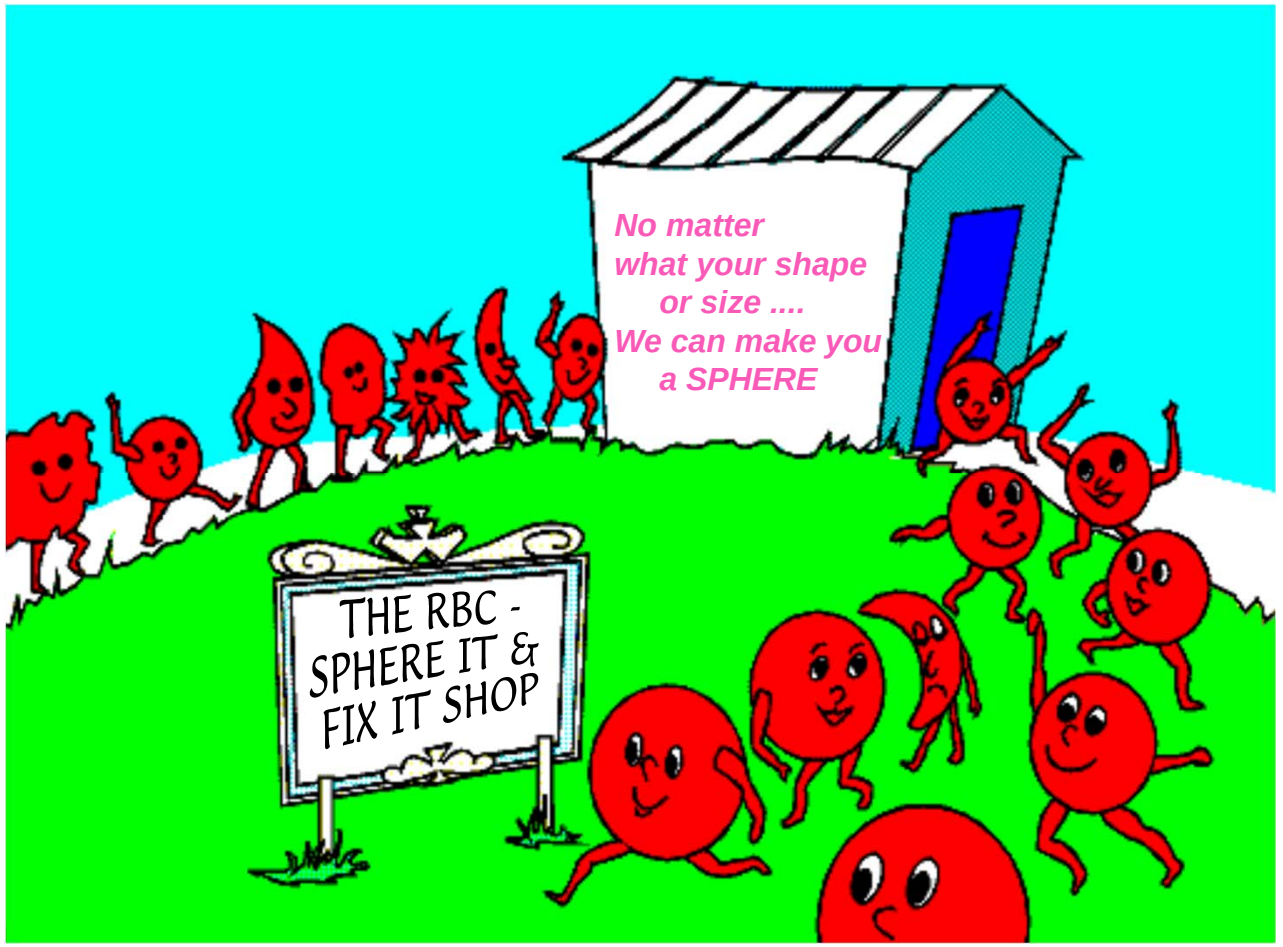


1 Noise
2 NRBC
3 aggregated platelets
4 Lymphocytes & basophyls
5 Large Unstained Cells (LUC)
6 Monocytes
7 Neutrophyls
8 Eosinophyls

Morphological flags

ATYP (Atypic lymphocytes)
IG (Immature granulocytes)
MPO (Myeloperoxidase deficiency)
NRBC (nucleated red cells)
PLT-CLM (clumped platelets)

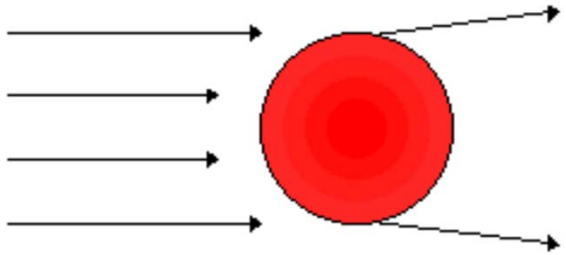
Red cell measurements



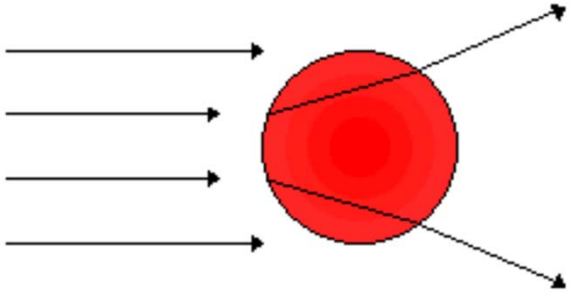
Transition to isovolumetric sphere

Optical detection of red cells

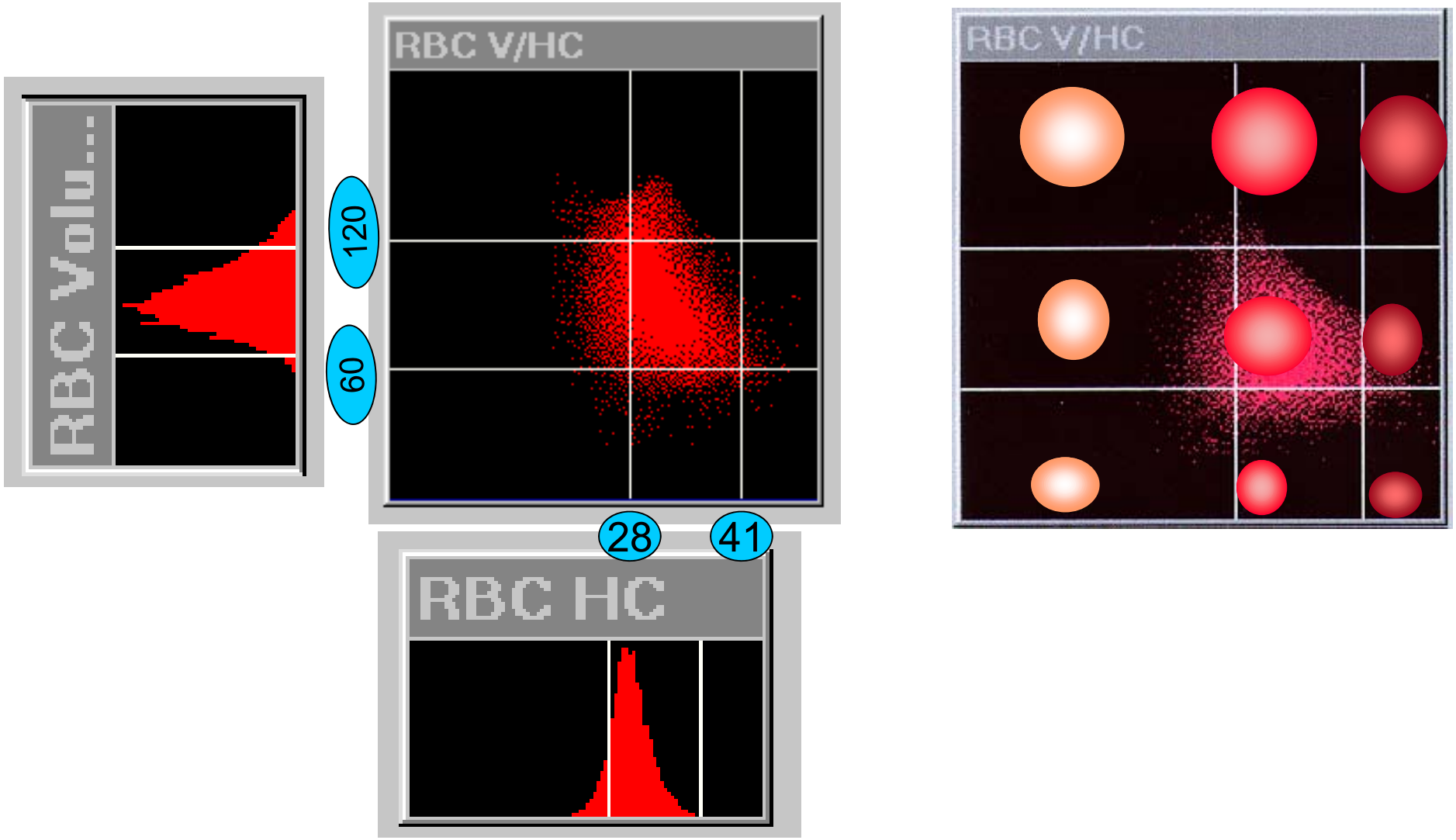
Low angle scatter 2^{o} - 3^{o}(Volume)



Large angle scatter 5^{o} - 15^{o} (HGB levels)

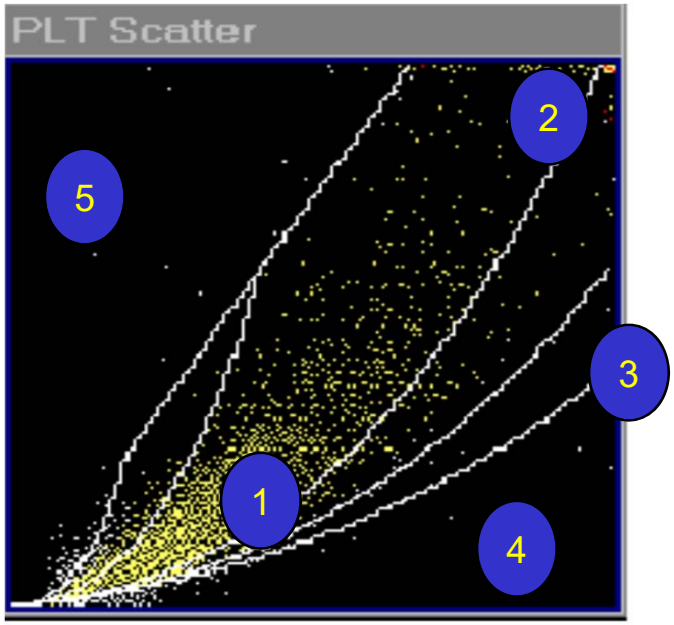


Volume / Hgb distribution



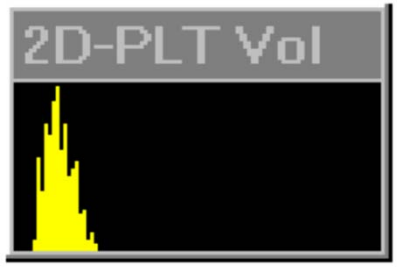
Platelet detection

Same as RBC detection.



Platelet cytogram

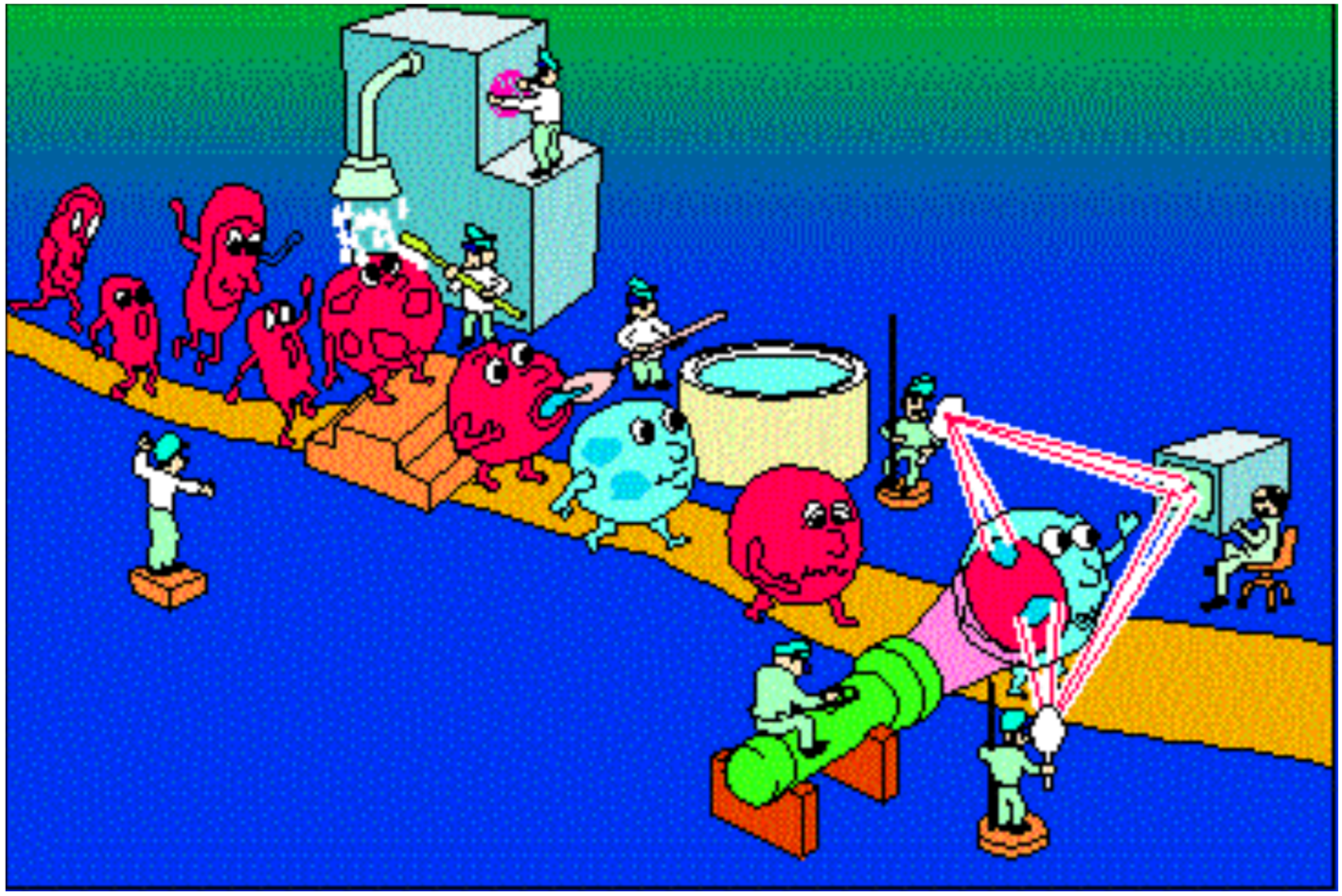
1 platelets
2 Giant thrombocytes
3 RBC
4 RBC fragments
5 RBC shade



Plt volume
0-60 fl

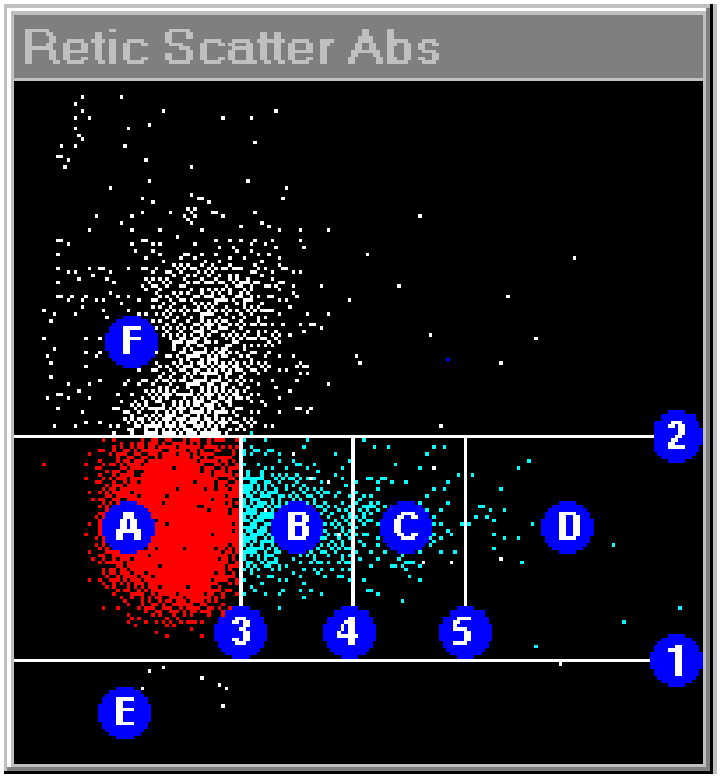
Parameters: PLT, MPV, L-PLT

Reticulocyte detection



Transition to sphere; then reticulocyte RNA detection with fluorescent dye

Reticulocyte detection



Reticulocyte citogram:
-absorption (maturity) on x axis
- Scatter (size) on y axis

Immature reticulocyte fraction (**IRF**): indicates the current erythropoetic activity (ESRD, bone marrow graft mobilization, anemia)

Mean reticulocyte content (**CHr vagy RetHe**): indicates the amount of functionally available iron (iron deficient anemia, assessment of response to iron therapy, monitoring EPO therapy

Interfering factors and problem solving

- Hemolysis: RBC ↓, Hct ↓
  - repeat using another sample
- Transfusion, iron and B12 therapy: RDW ↑, bimodal RBC histogram
- Lysis resistant red cells: WBC↑, lymphocytes ↑
  - Dilute samples, increase lysis time
- Fragmentocytes: RBC↓ PLT ↑
  - smear
- NRBC :WBC↑,lymphocytes ↑
  - NRBC program, smear

Interfering factors

- High WBC : RBC ↑, incorrect Hgb and calculated values
  - sample dilution
- Cryoglobulines: WBC ↑
  - 37ºincubation, repeated testing
- Cold agglutinins: RBC ↓,MCV ↑ ,MCHC ↑
  - 37ºincubation, repeated testing
- Lipemia: Hgb ↑, MCHC ↑, MCH↑
  - Repeated testing
- PLT aggregates: WBC ↑, PLT ↓
  - Smear, repeated testing of citrated blood
- Giant platelets: WBC ↑, MCV ↑, PLT ↓
  - smear

Preanalytical issues

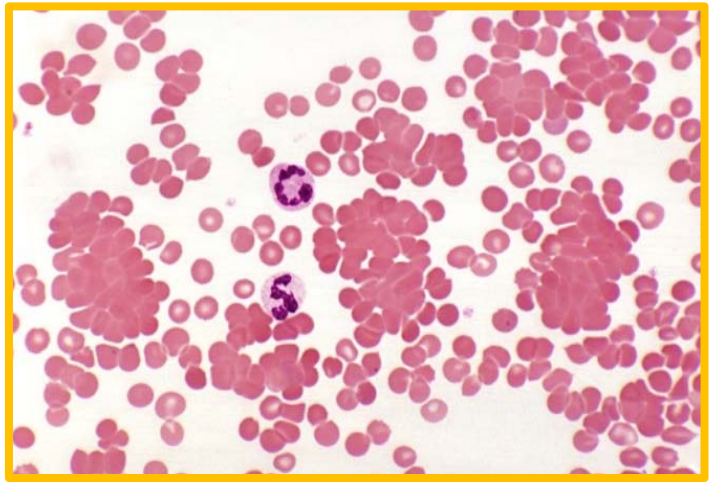
- K2-EDTA tubes, sufficient amount of blood
- In general app. 0.5 ml for manual measurements
- Should be measured within 8 hours
- Smear within 4 hours
- HEPARIN: not appropriate (background)
- Citrate: not appropriate (dilution)

CBC changes following 8 hours of storage at room temperature:

- RBC loose their biconcave shape
- MCV ↑, Ht↑
- WBC↓
- Absolute lymphocyte count↓
- Reticulocyte count after 6 hours↓
- NRBCs disappear after 24-36 hours
- MPV↑, IRF↓

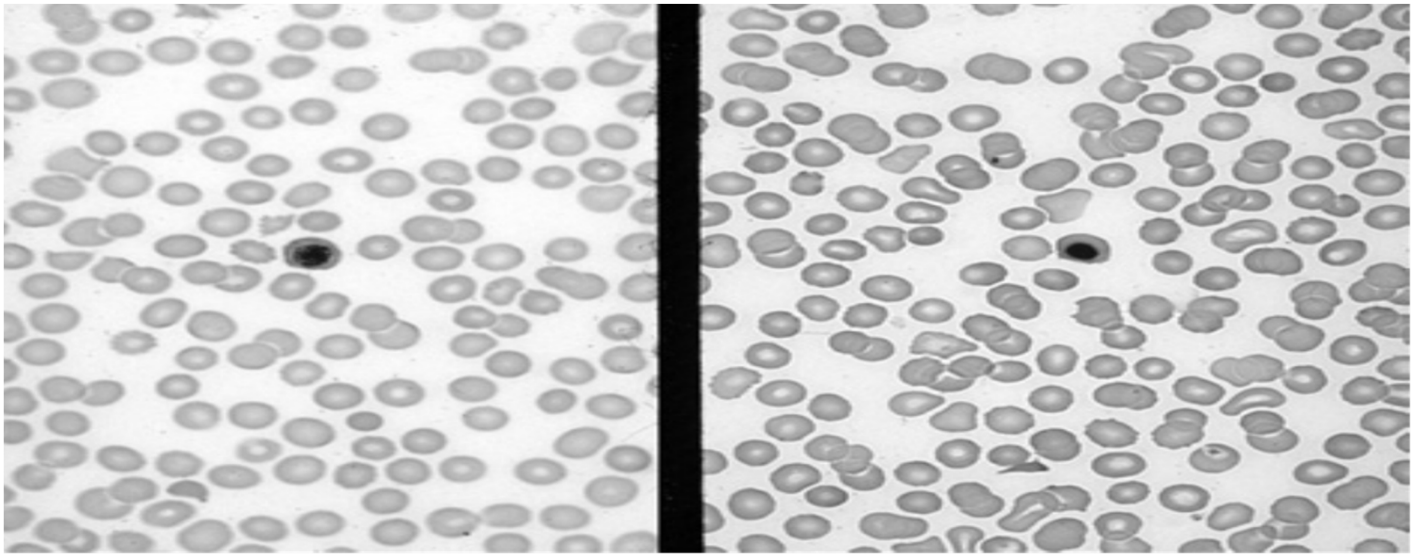
Analytical errors – some possibilities

- **Cold agglutinins**– indicative: low RBC, high MCV
- Uncorrect hematocrit and MCHC
- Alterations are present in cold sample
- Cold agglutinins may be present in autoimmunity, infectious mononucleosis and mycoplasma pneumonia infections
- **PROBLEM SOLVING:** warm the sample (or should sample be taken into prewarmed tube)



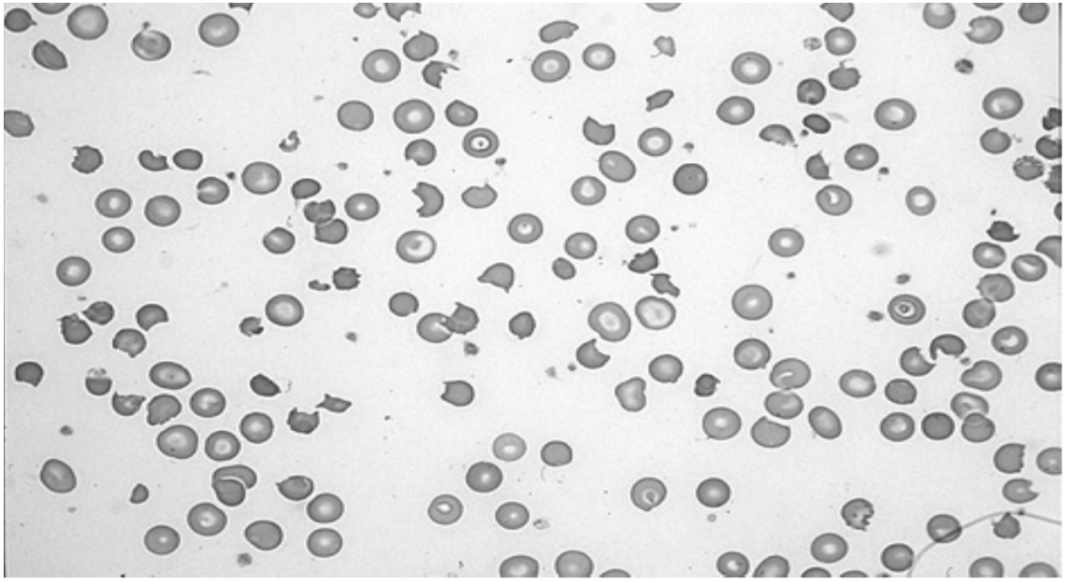
- **NRBCs (nucleated red blood cells):**

Same size as lymphocytes; device counts as WBC



Fragmented or very small RBCs

- Sometimes they are not differentiated from PLTs
- Histogram should be inspected



False hematocrit values

- Htc is a calculated parameter
- Presumption: RBC and volume should be measured correctly
- In case of false RBC or volume Htc is false

Possible cause of errors

- **Hgb**
  1. Very high WBC
  2. Severe lipemia
  3. Heparinated blood
  4. RBC resistant to lysis
  5. Jaundice

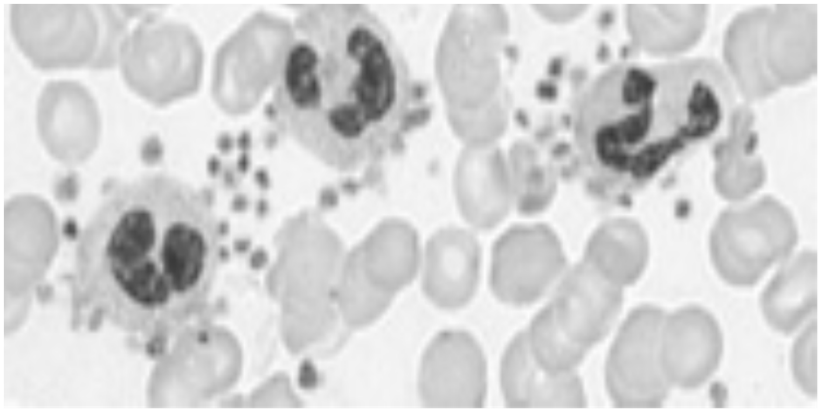
- **MCV**
  1. Very high WBC
  2. Giant PLTs
  3. Agglutinated RBCs
  4. Small (<36 fL) RBC fragments
  5. Rigid RBCs

- **Aggregated PLTs:**

PLT is falsely low

Increase of right side PLT histogram

Located at the end of smear

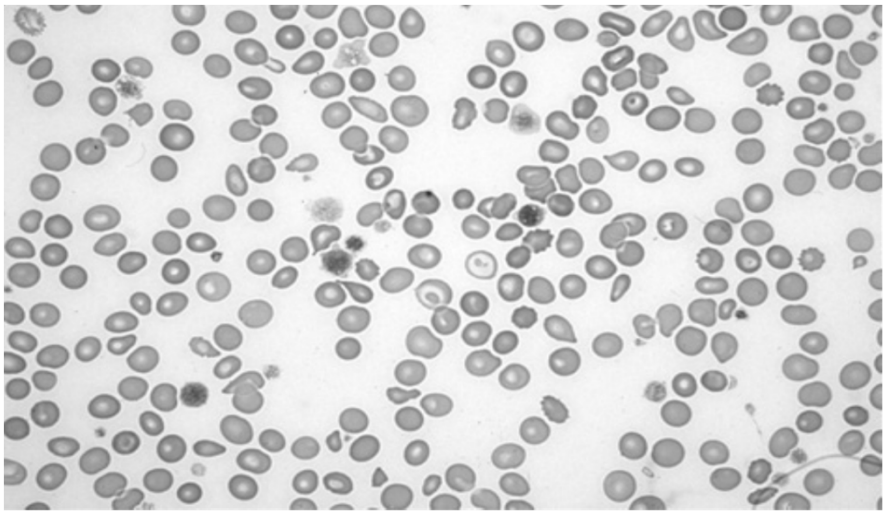


Giant PLTs:

Instrument considers them as RBC

RBC count is not affected

Elevation at right side of PLT and left side of RBC histogram



Problem solving

- **PLT < 40 G/L**
  1. Check the presence of microclots
  2. Smear, look at fragments, giant PLT, or very small RBC
- **WBC ++++**

  Dilute with 1:2 saline, multiply the result by 3

  Do not use: HGB, MCH, MCHC.

  PLT count is not affected by WBC
- **PLT++++**

  Smear (RBC-fragment, microcytes)

  If there is no abnormal RBC, should be diluted
- **RBC > 7.0 T/L**

  Sample should be diluted