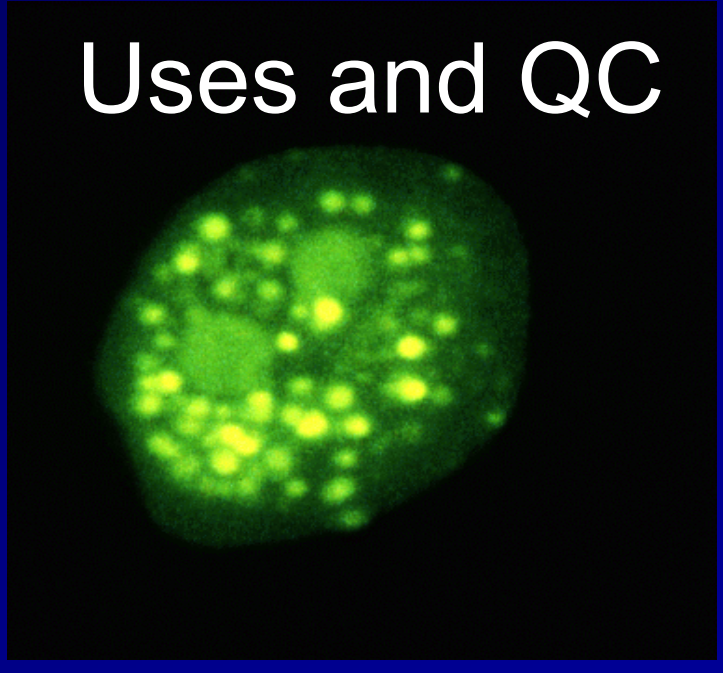
Immunophenotyping

(Flow Cytometry)



Flow Cytometry

" A system of sensing cells or particles in solution as they pass a laser light source one at a time"

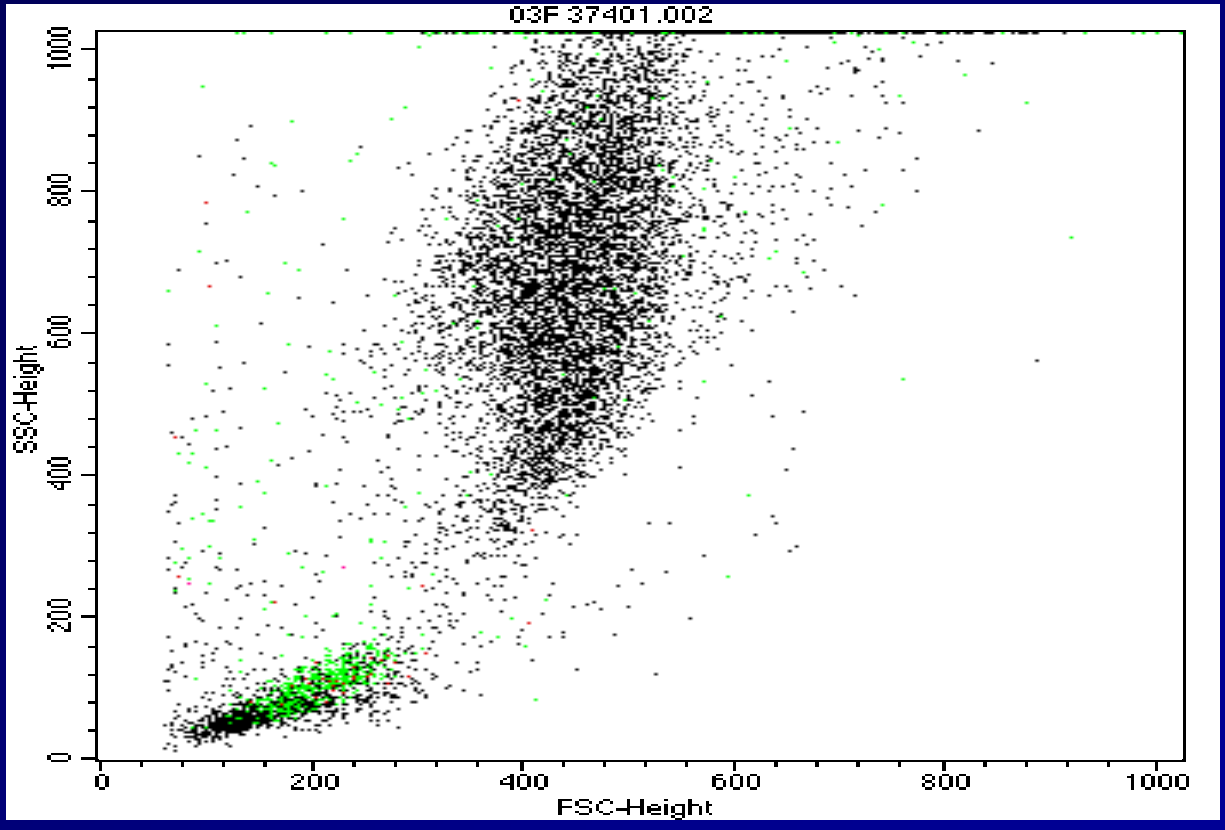
Technology available since late 1970's

Early instruments problematic – laser alignment daily

Terminology:

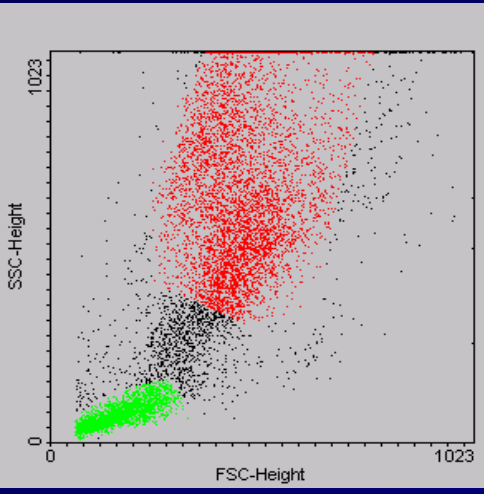
FACS Fluorescence Activated Cell Sorter

EPICS Electronic Programmable Individual Cell Sorter



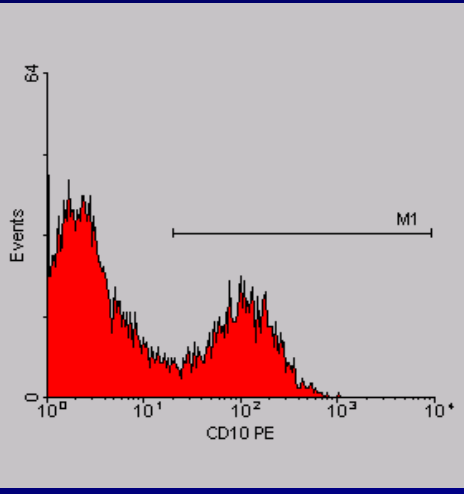
Human White cells separated based on Size (Forward Scatter) and complexity (Side Scatter)

Data Display:

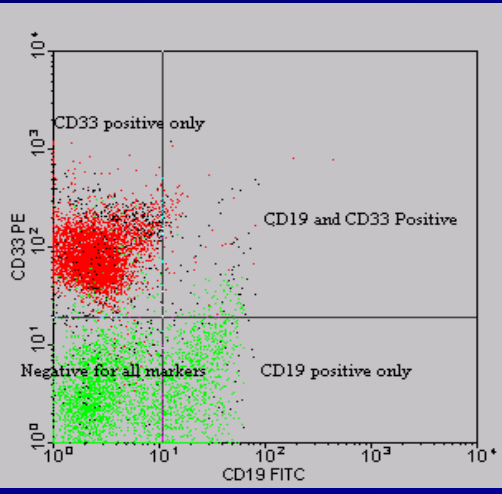


'Dot Plot' display of FSC versus SSC

i.e. Size versus complexity

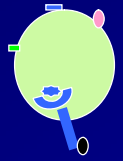


Histogram Plot display of FL2



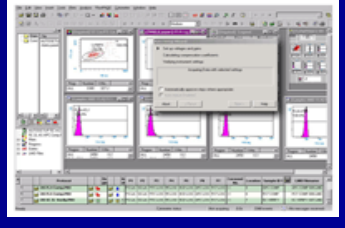
Dual Plot of FL1 versus FL2

- Flow Cytometry – Currently

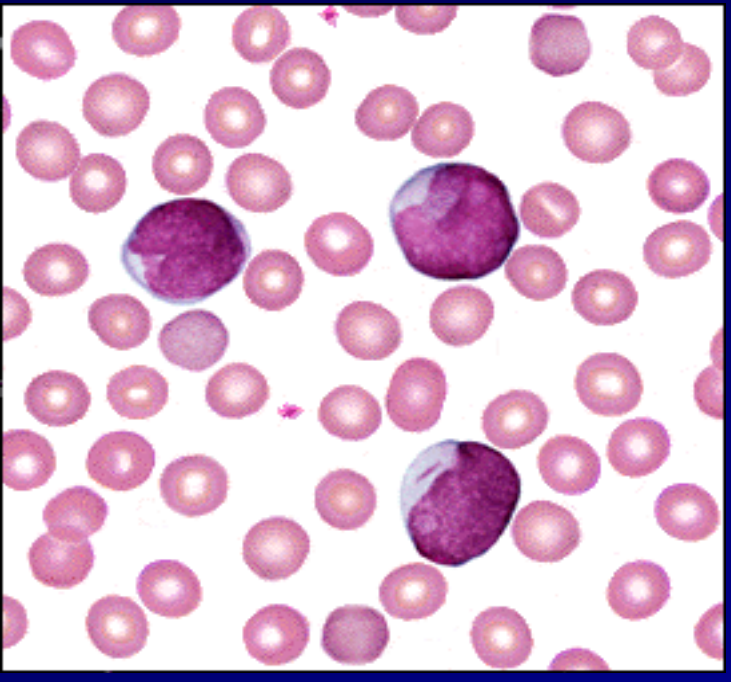


- Flow Cytometry – Future ?

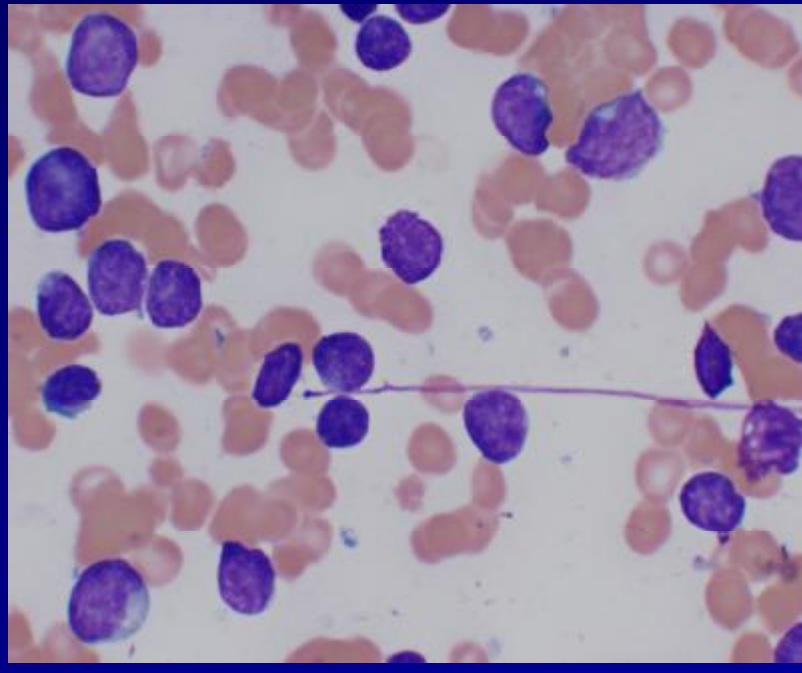
- Quality Assurance



Currently:

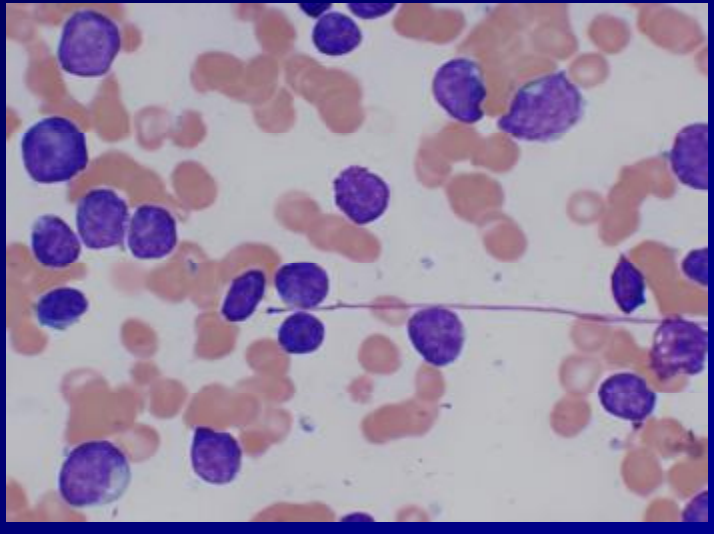


AML

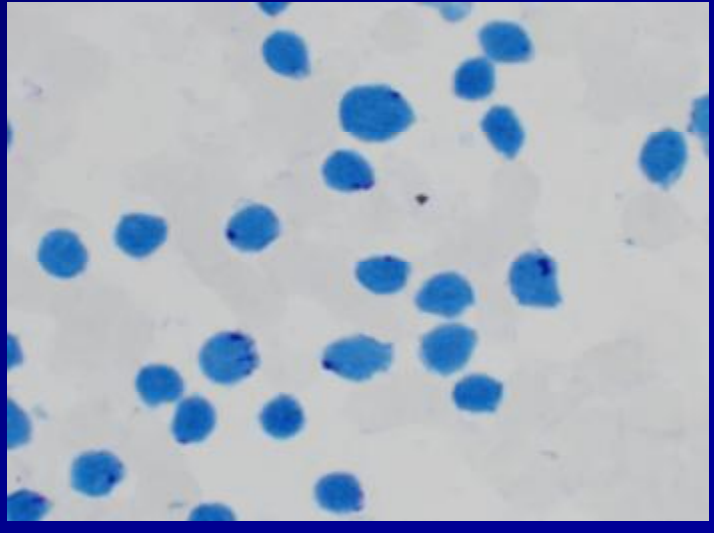


Early Pre-B-ALL

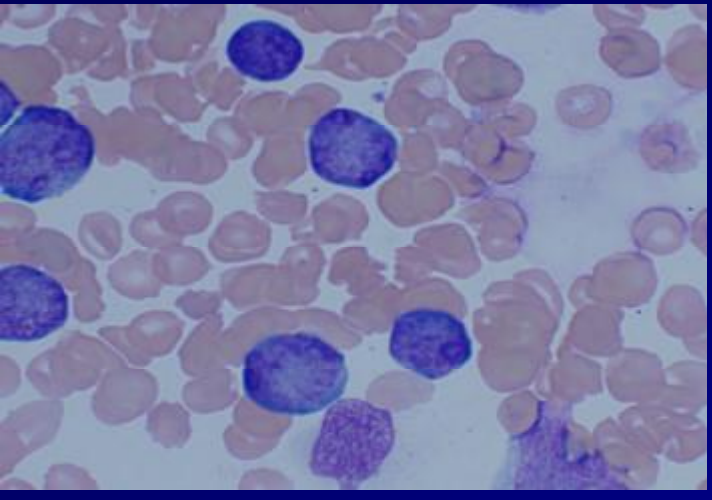
B-ALL



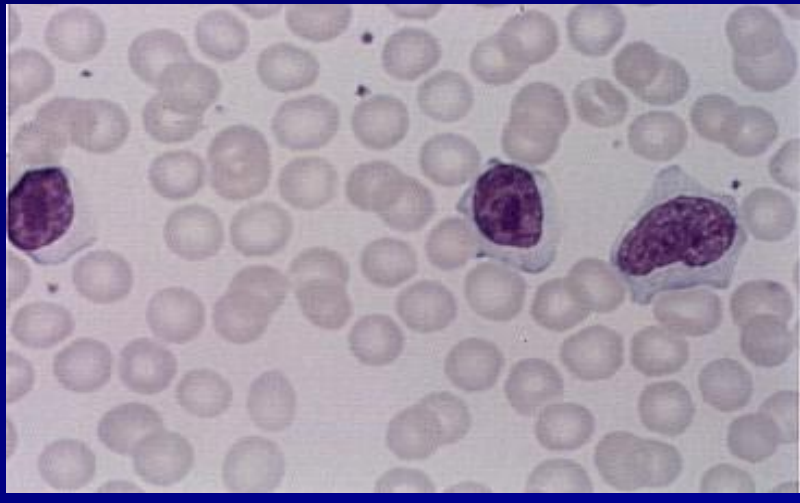
Early Pre B-ALL



PAS



T-ALL



CLL

Flow cytometry:

- Type antigens on surface and cytoplasm of white cells – Leukaemia
  Lymphoma
  Solid tumours e.g Neuroblastoma

$CD81^{+}$ / $GD2^{+}$ / 56^{++} / 45^{-}

Applications of Flow Cytometry

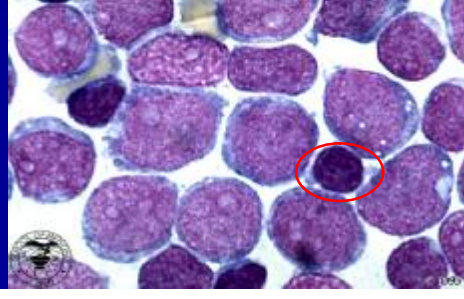
Rare event analysis:

Very powerful tool -In humans Residual disease in Acute Lymphoblastic Leukaemia can pick up 1 cell in a million

Instrument must be perfect!

PCR more powerful but not looking at every cell individually (extrapolating up)

Methodology can be adapted to almost any cellular model



MRD detection in Acute Leukaemia

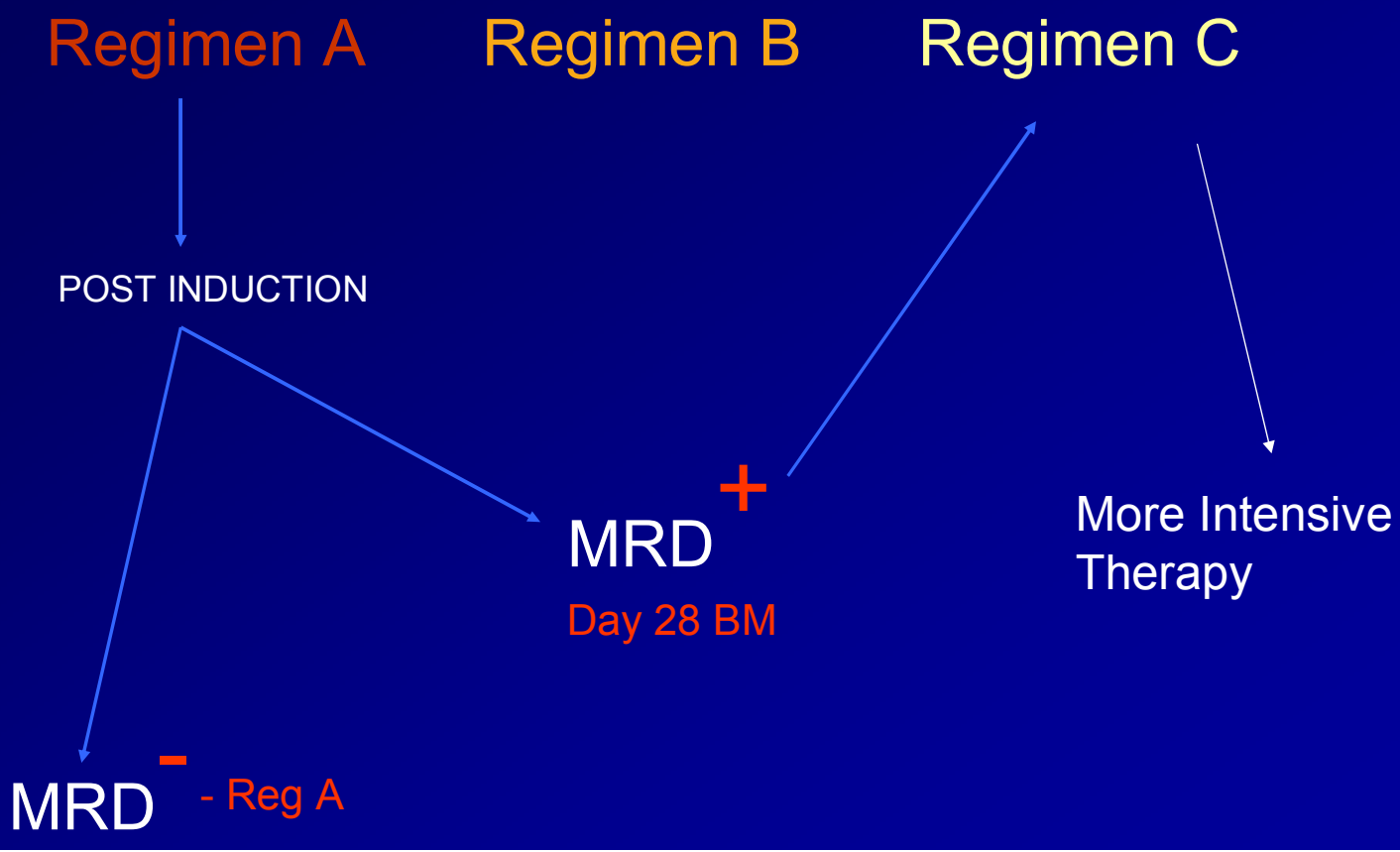
(Minimum Residual Disease)

- Evidence that MRD will detect relapse with a much greater sensitivity and specificity than clinical criteria/morphology
- MRD^- at Day 28 ? Reduce treatment !

==========

- Also MRD assays useful for assessing 'autologous' PB or BM harvests for presence of residual disease

UK-ALL 2003 Trial: ALL

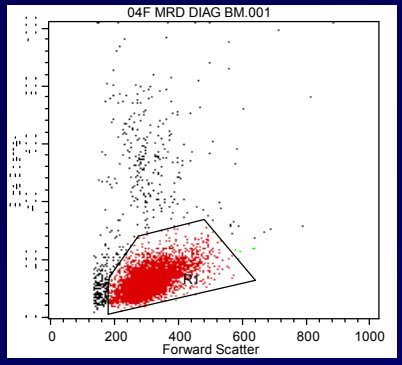


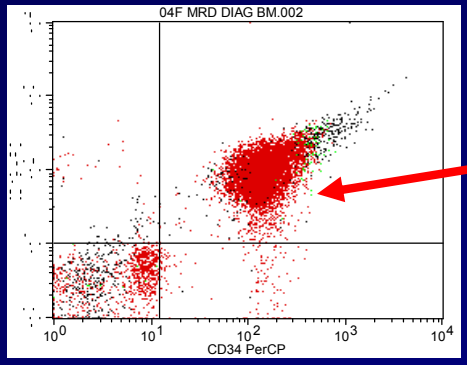
Treatment : B-ALL

75% Survival based on existing ‘risk stratification criteria’

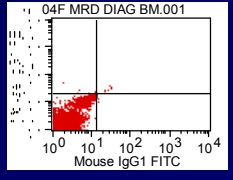
With MRD - Survival up to 91% in common types.

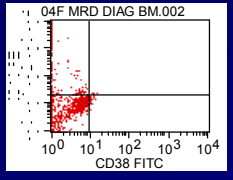
- Flow
- PCR

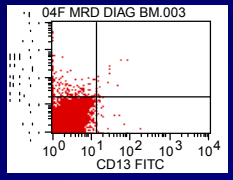


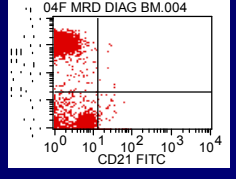


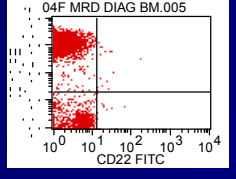
‘Gating’ Antibody

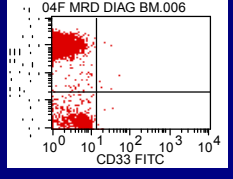


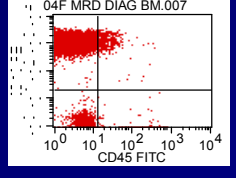


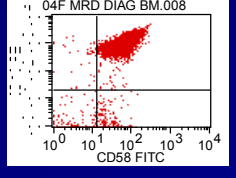


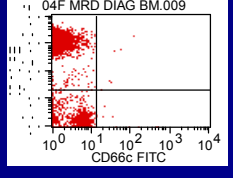










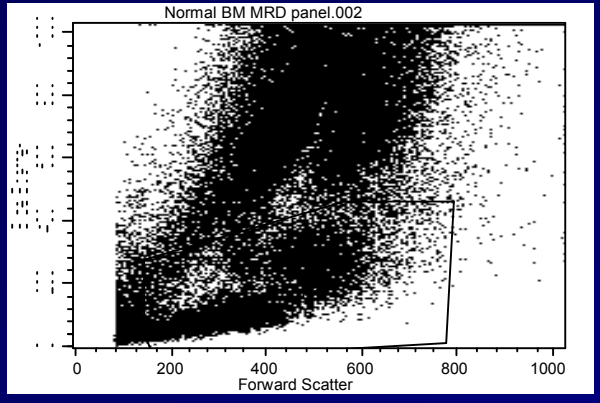


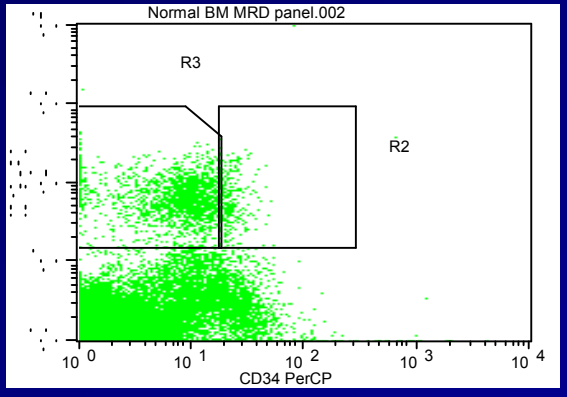
‘Normals’

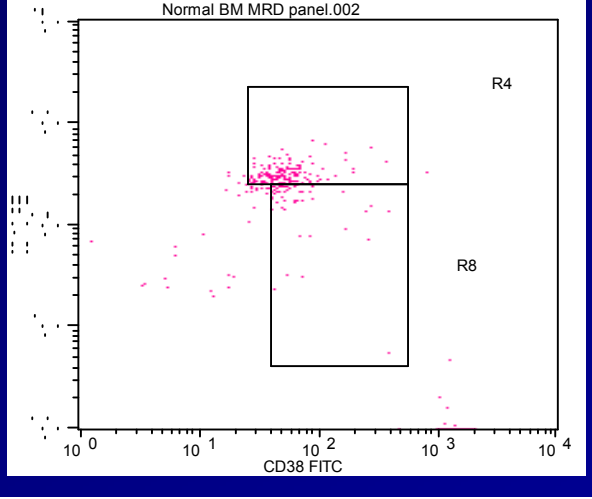
- Full panel run to develop a series of templates

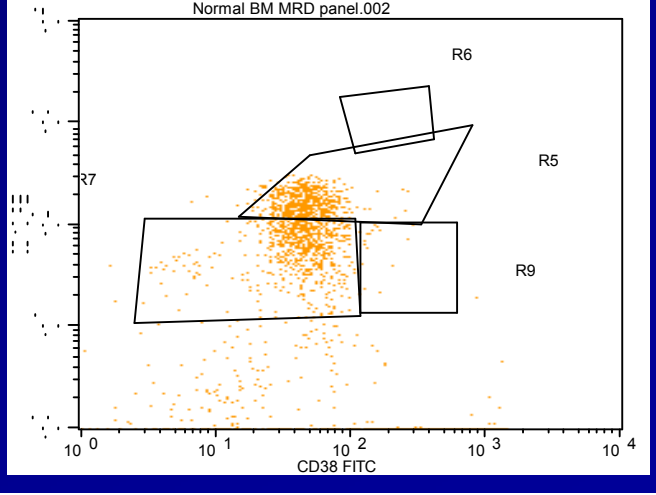
-each template specific for that antibody combination

Templates: Example - CD38 NORMAL

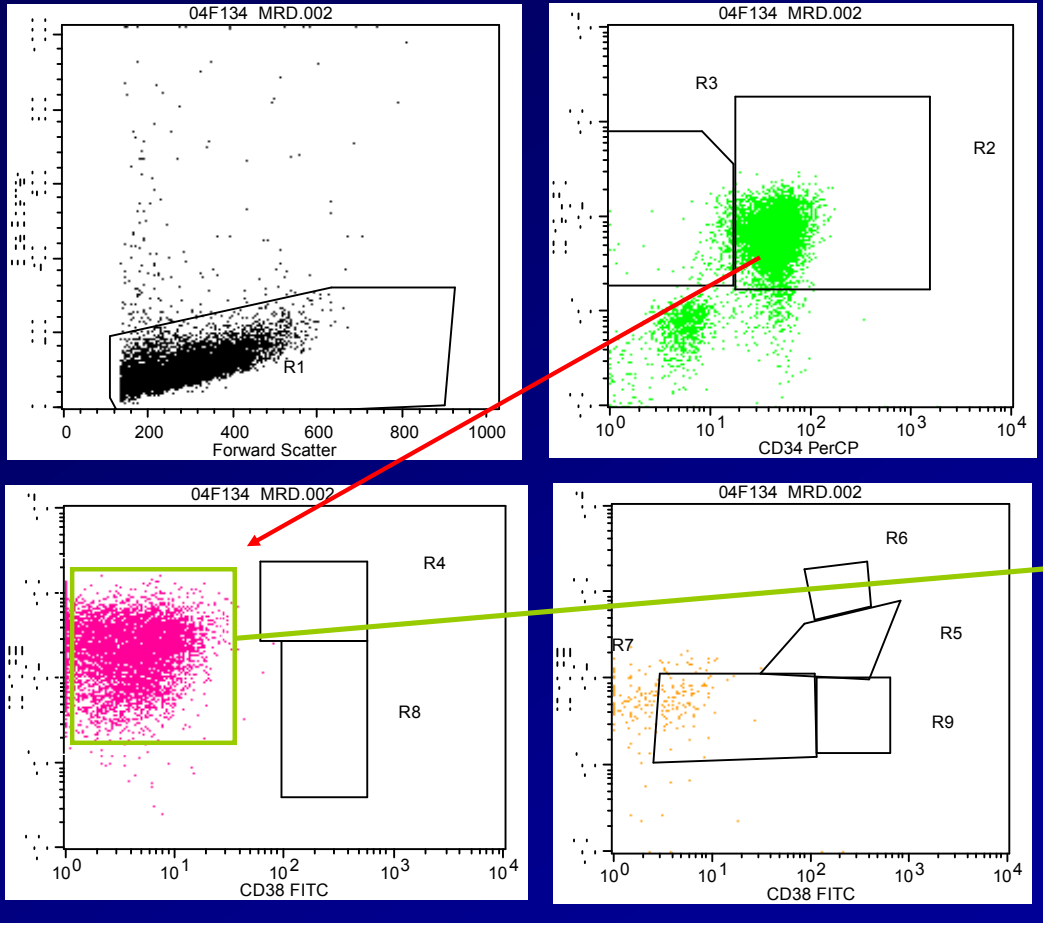




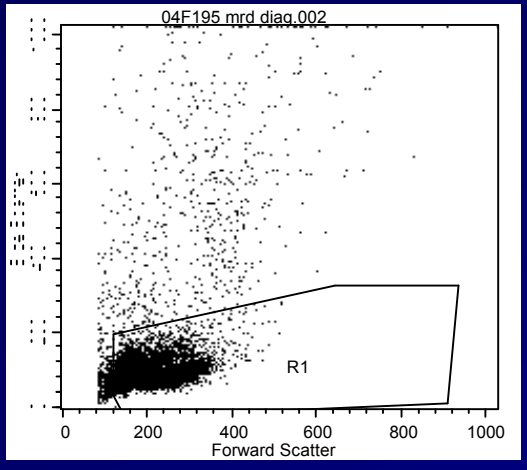


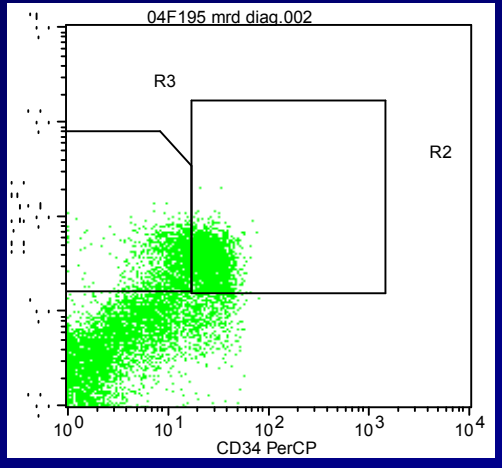


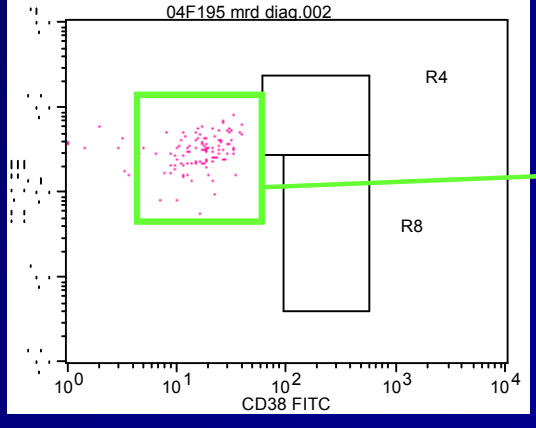
MRD → DIAGNOSIS (CD38) B-ALL

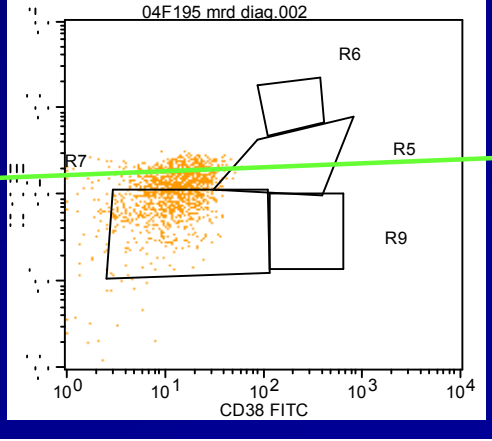


MRD – Follow up ? MRD at day 28







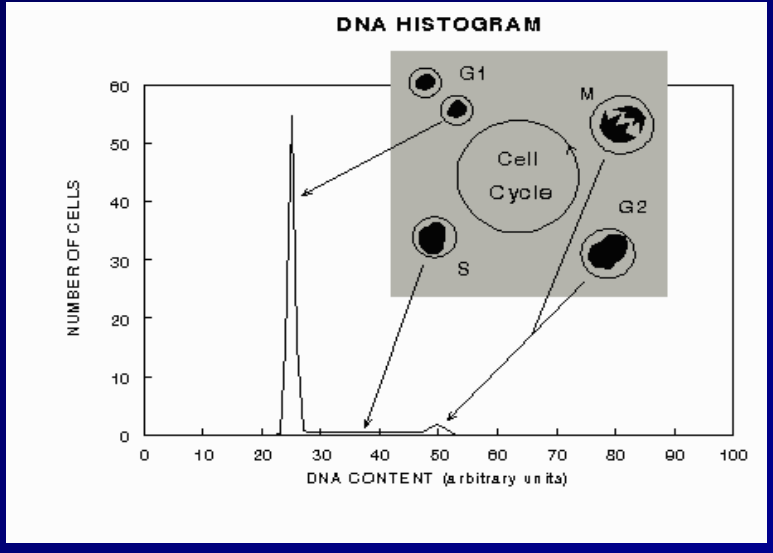


MRD +

0.66%

Applications of Flow Cytometry

DNA cell cycle analysis:



Cell membrane is removed and dye binds to DNA.

Level of fluorescence is directly proportional to each cell's DNA content (2N=G0/G1

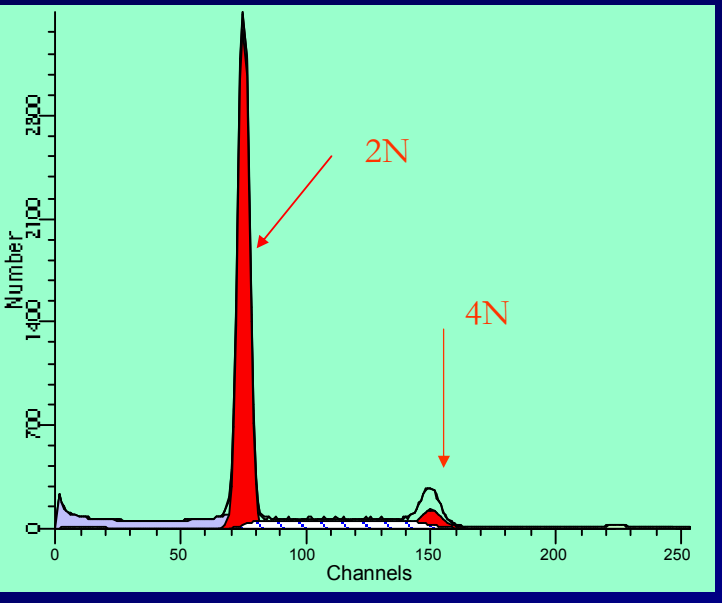
4N=G2/M

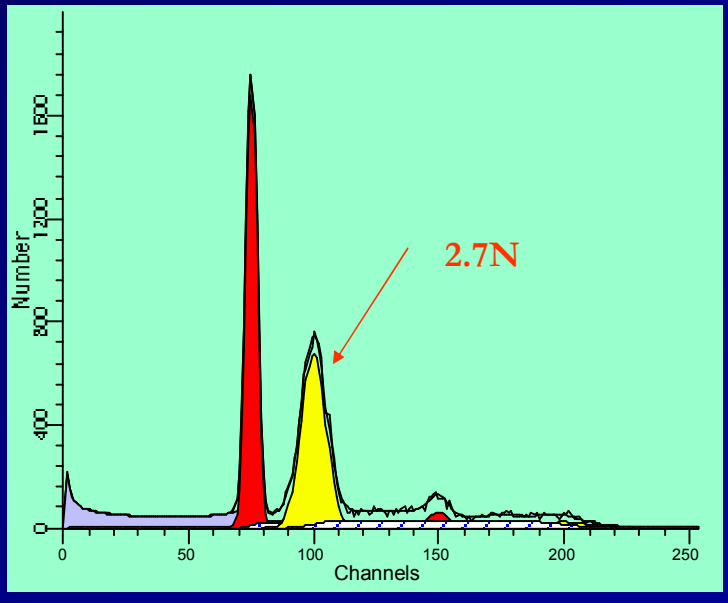
Ploidy analysis

Using a DNA fluorescent stain (Propidium Iodide) (after RNAase) – can get % of cells in G0/G1, G2/M and S phase

Very useful for indication of cell division/ tumour cells etc..

DNA cell cycle analysis: 2 yr old Acute Leukaemia





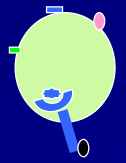
Diploid (2N)

Aneuploid (2.7N)

Knowledge of normal : e.g. Lymphocytes normally Diploid

Hyperdiploidy in Leukaemia – good prognosis

Flow Cytometry -applications



Cytoplasmic/Nuclear markers:

Using a combination of surface fixation, followed by permeabilisation of the cell membrane with a weak detergent- markers to intracellular elements can be monitored.

e.g. Nuclear marker TdT – Leukaemia

Cytoplasmic myeloid CD117 – Myeloid Leukaemia

Cytokines – IL1 – 6

-- TNFα /β etc..

Oxidative Burst in neutrophils

Thus cells can be classified or gated based on their surface markers and then results obtained from their cytoplasmic markers.

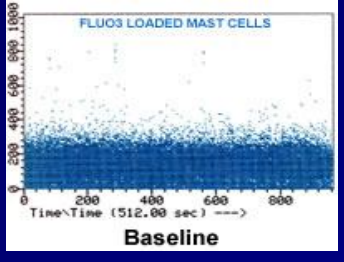
Flow Cytometry -applications

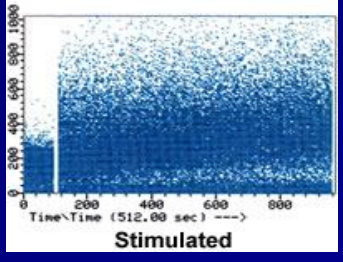
'Time 'as a parameter in Flow Cytometry

Induce a reaction 'in vitro' and monitor result or marker 'real time' on the cytometer

e.g. Calcium Flux FLUO 3 Calcium probe

A practically non-fluorescent material without CA^{++}





Calcium efflux as a measure of cell signalling

Flow Cytometry -applications

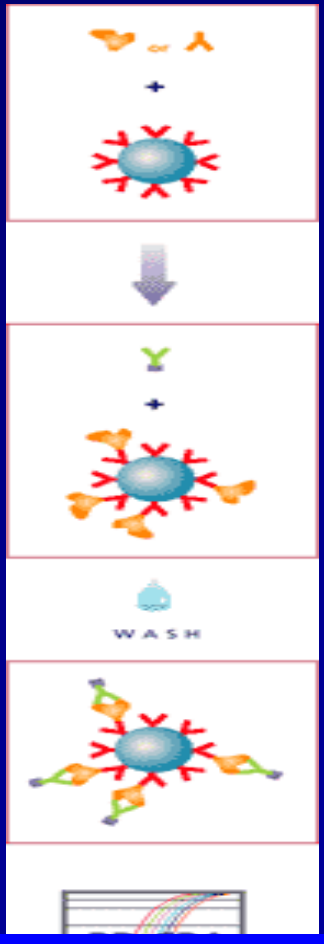
Multiplexing assays

A Cytometric Bead Array (CBA), commonly referred to as a multiplexed bead assay, is a series of spectrally discrete particles that can be used to capture and quantitate soluble analytes.

(up to 15 analytes per tube)

Unknowns calculated with use of standards and standard curve

Replaces ELISA etc. plus gating capabilities etc..



Flow Cytometry -applications

Use of FLUORESCENT DYES

Example: EMA Eosin-5-Maleimide

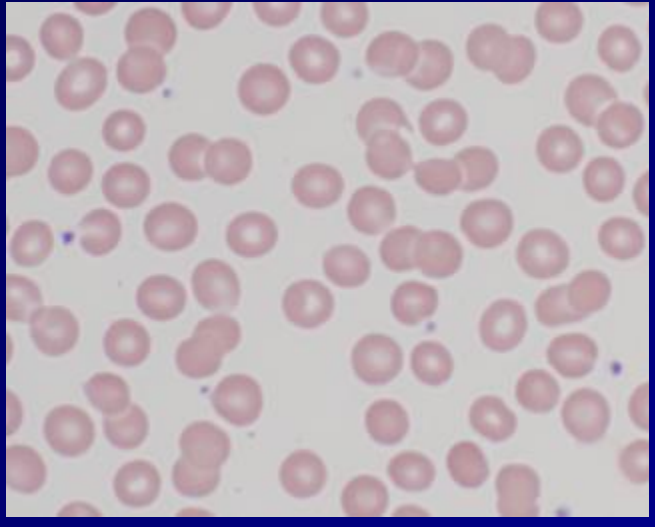
Binds/enters Red cells via Band 3 protein in membrane

In hereditary Spherocytosis this dye uptake is reduced – can be monitored 'real time'.

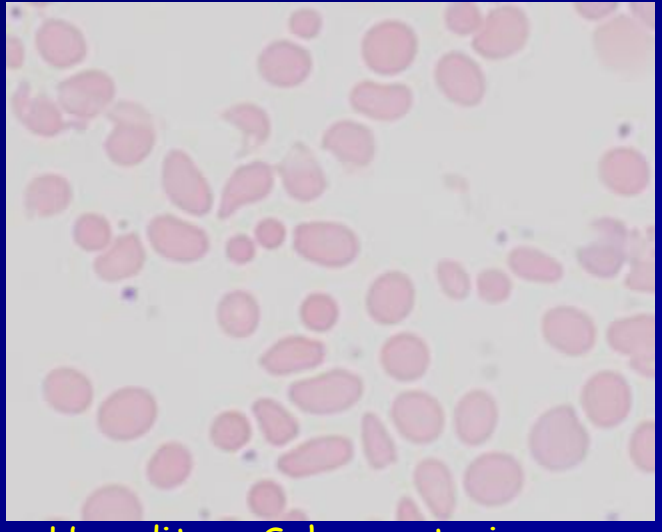
Many other fluorescent dyes available for

?? applications

Blood film - HS

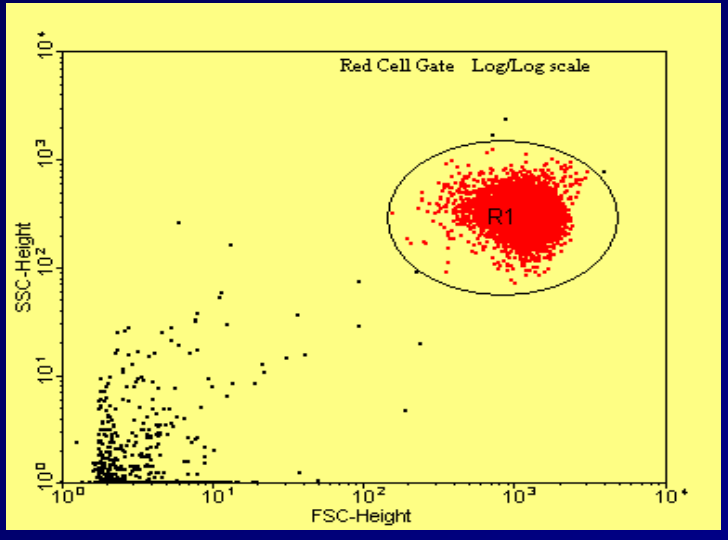


Normal RBC Morphology

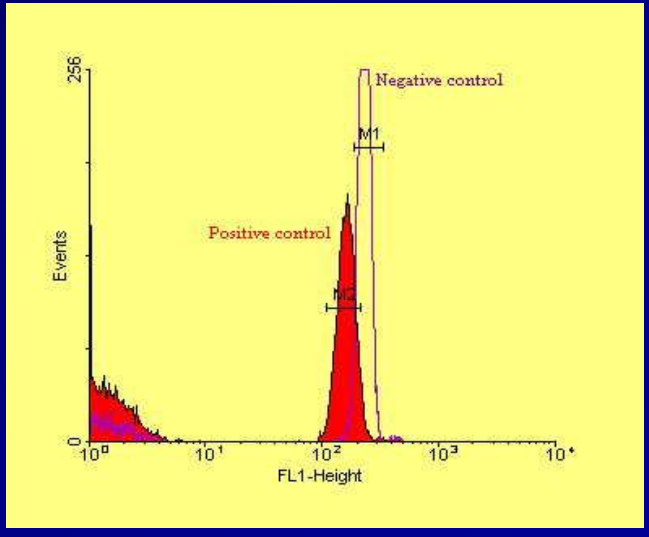


Hereditary Spherocytosis

No more osmotic fragility !



Plot of FSC v SSC of red blood cells on a log/log scale. 15000 red cells are counted within this gate.



Plot of positive HS sample overlayed with a normal sample

Flow Cytometry -applications

Apoptosis

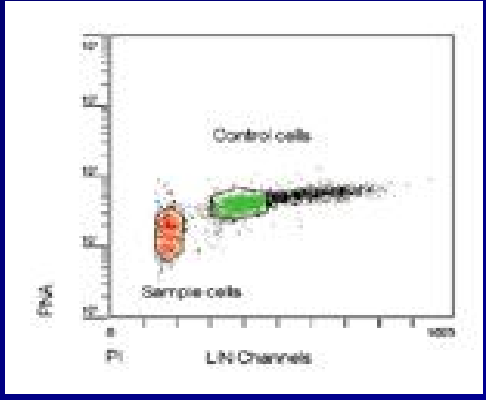
Fluorescein-conjugated annexin V, propidium iodide and binding buffer. Cells suspended in binding buffer are mixed with the fluorescein-conjugated annexin V and propidium iodide. After incubation for 10 minutes, cells are ready for analysis.

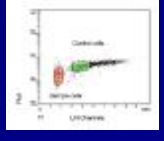
During apoptosis, cells expose phosphatidylserine at the cell surface. Annexin V is a phospholipid-binding protein, which in the presence of calcium ions binds selectively and with high affinity to phosphatidylserine

Flow Cytometry -applications

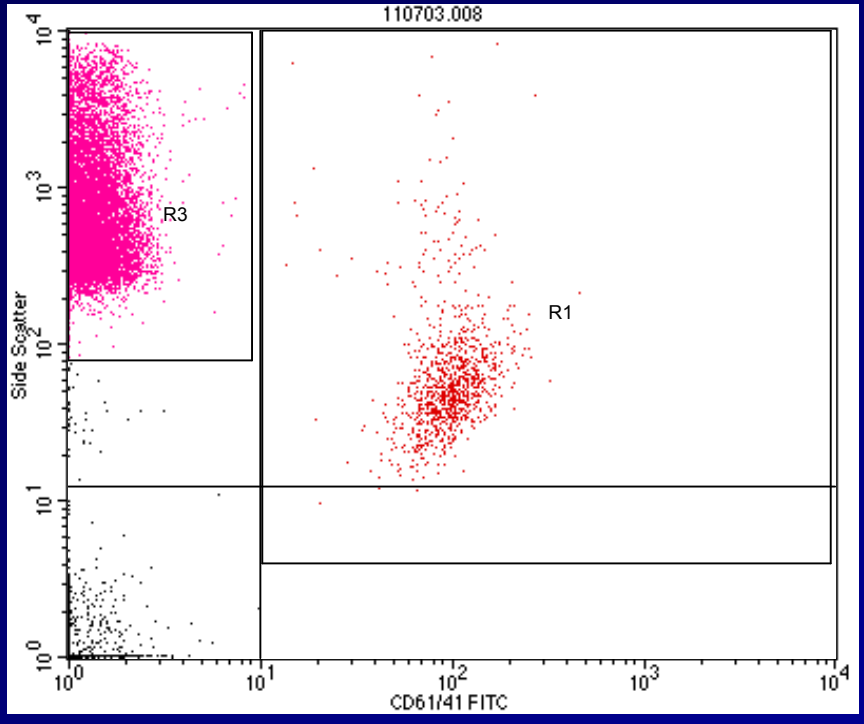
Molecular techniques

Telomere PNA-FITC for Flow Cytometry provides a convenient method for measuring telomeric sequences in vertebrate interphase haematopoietic cells



DNA → Denatured → Incubated with Hybridisation solution → PNA telomere probe added-FITC → 

FLOW F.I.S.H



Platelet counting by Flow cytometry -Gold Std.

PLT/RBC ratio Harrison et al

Other applications include

- PNH screening- CD55/59
- PLT phenotyping e.g Glanzman's
- Oxidative Burst test for CGD
- CD 34 stem cell counting
- TB subsets
- Perforin analysis - HLH disease

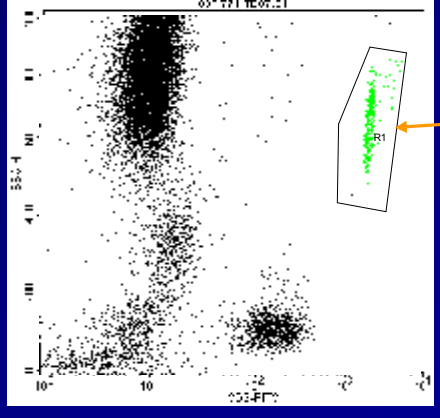
Flow Cytometry -applications

Quantitation

Use of exact concentration of beads added to each tube

Ratio of known bead concentration to unknown sample concentration yields value of unknown.

Can gate on populations of cells and count these.

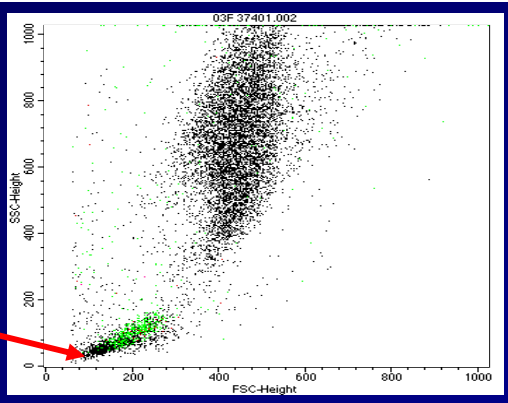


Beads

Can be used for almost any cell type

Other applications

- Microbiological



Use of flow cytometry to investigate morphological and physiological properties of individual organisms within bacterial populations is becoming increasingly popular.For example: The technique is an excellent tool for analyzing microbial responses to antibiotics

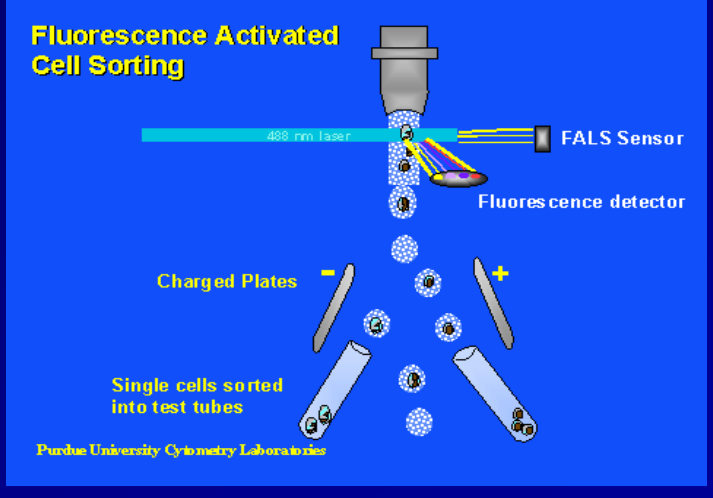
- Microparticle phenotyping

  1µm in size -Coagulation/ SCD etc.

Flow Cytometry -applications

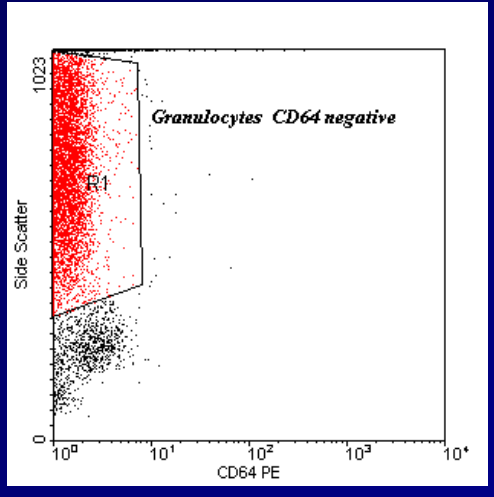
Cell Sorting – not on standard bench top cytometer

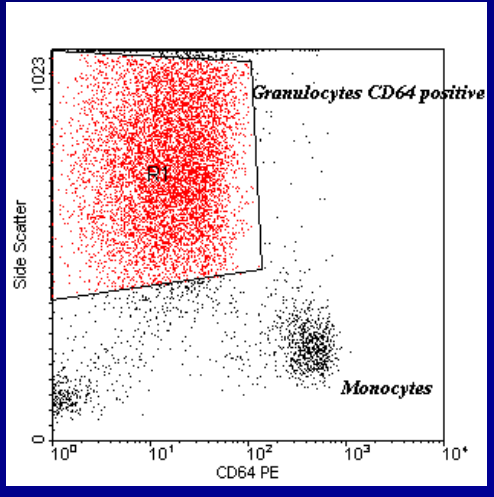
The ability to select any population defined by a logical combination of regions (a 'gate') and ***isolate*** *this population from the sample into a separate tube.*



CD64 levels in infection

- superior to CRP , PV , ESR

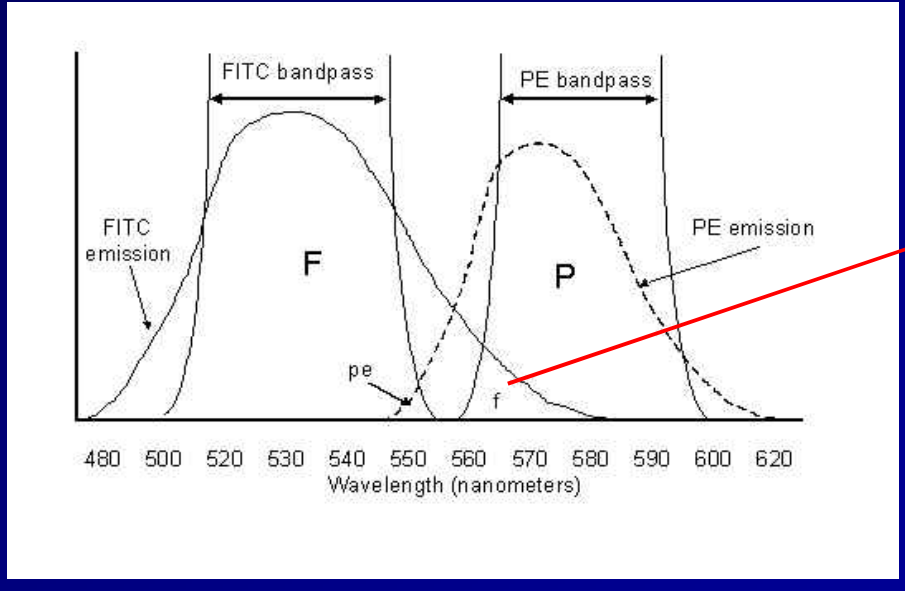




However required 24 hours a day for casualty etc..!

Compensation

Having amplified and transformed signals from the various detectors a correction can be applied for any residual overlap of fluorescent spectra through the optical filters This process is referred to as compensation. If two fluorochromes, FITC and PE are being measured with spectral characteristics as shown below



False positive PE result

Consider a cell labelled with only FITC, a large fraction of the emission (F) will be detected through the FITC bandpass filter and a small fraction f=(kF) through the PE bandpass filter. A plot of detected F versus apparent PE would appear as shown in this dot plot:

Quality Assurance

Monoclonal antibodies -Isotype matched controls

-Commercial controls – known values

-Known normal's/quantities

Molecular controls – Known normal and abnormals

DNA analysis - Inherent normal cells (also in malignancies)

Cytokine analysis – range of expected values

Other – Unexpected cells/particles – No controls !

Quality Assurance

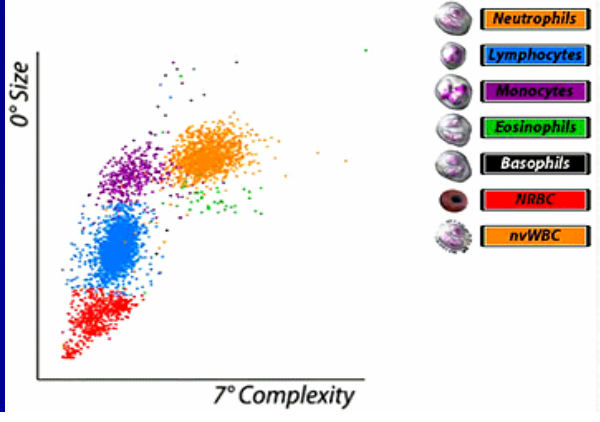
Most FBC analysers have laser technology

and newer analysers ‘ open’ antibody systems

Cell-Dyn Sapphire

So you can put on your own monoclonal antibodies for ‘fully automated analysis’ !!

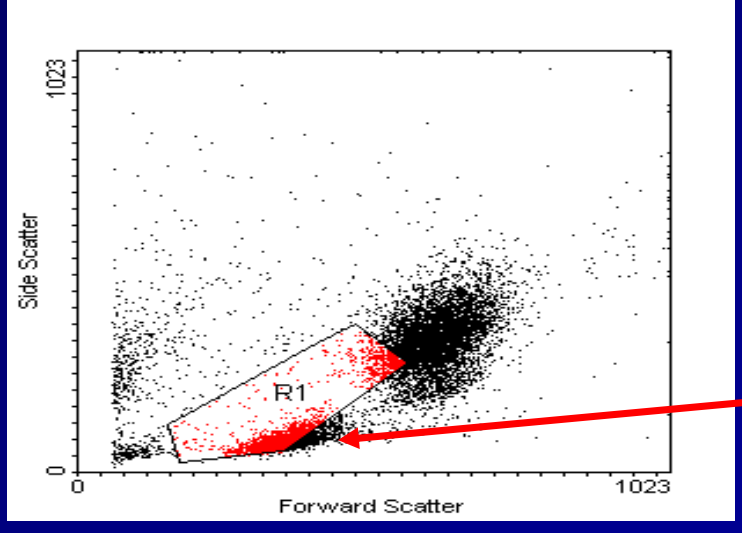
CD64 !



So new instruments:

word of caution - compensation

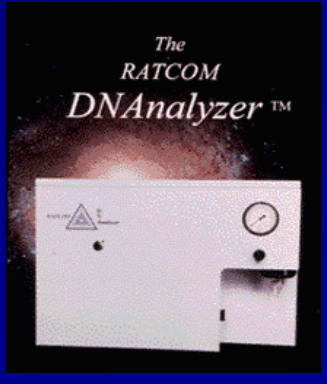
- failure of automated gating
- choice of antibodies etc..



Automated software choice of gate for Lymphocytes

And Finally

Flow Cytometers in space



Flow cytometry without gravity !