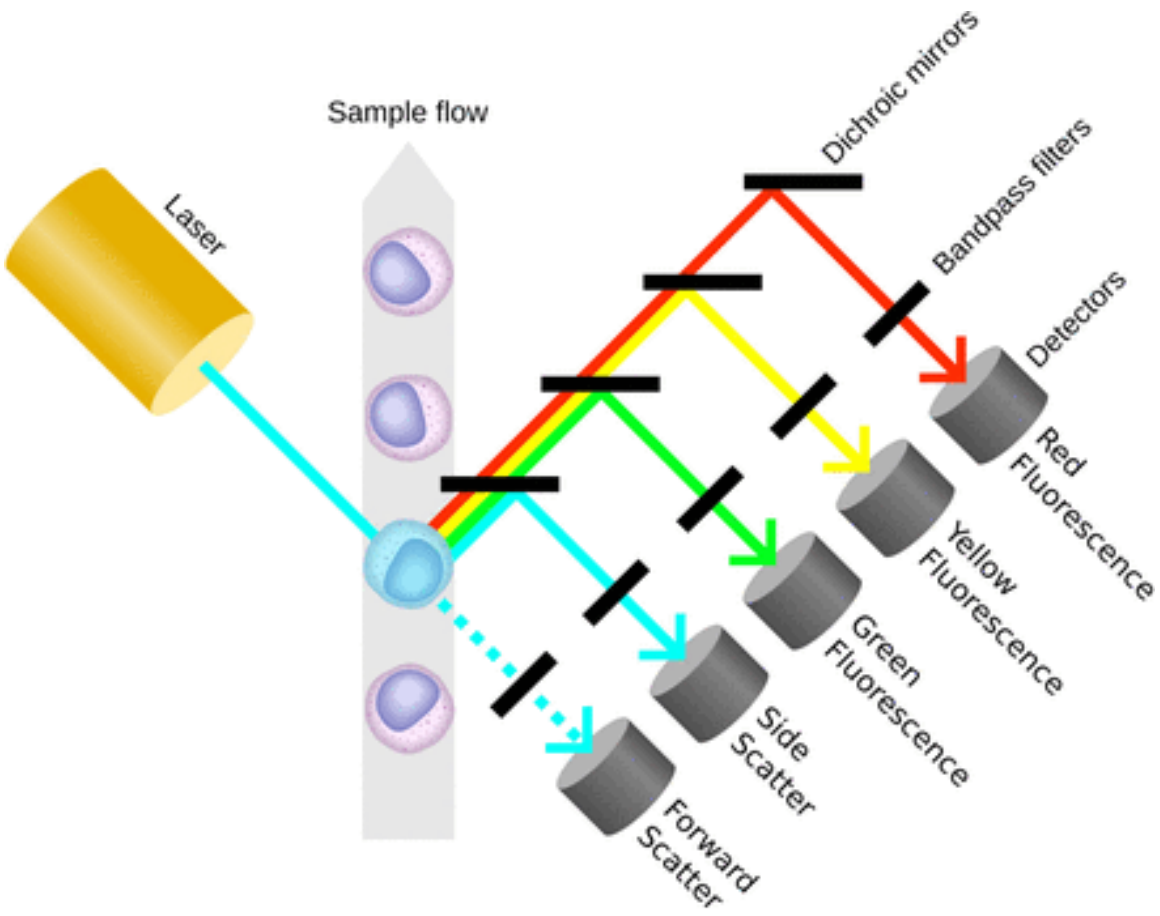
Flow Cytometry



Maike de la Roche

Overview

I: What is Flow cytometry?

- Principle
- Measurable parameters FSC, SSC, fluorescence
- Sample preparation

II: Applications of Flow cytometry

- Membrane antibody staining
- Intracellular staining
- DNA staining
- Apoptosis
- Ca flux analysis
- Cell/Molecule counts
- Cell Conjugation assay

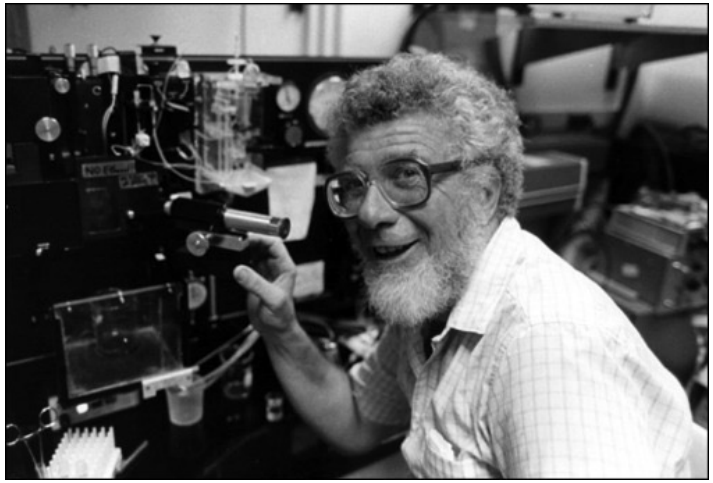
III: 'New' FACS techniques

Flow cytometry

Method of measuring cells (or other particles) in a high-speed fluid stream.

- Measuring parameter: fluorescence and light scattering
- Measurement rates: *thousands of cells per second*

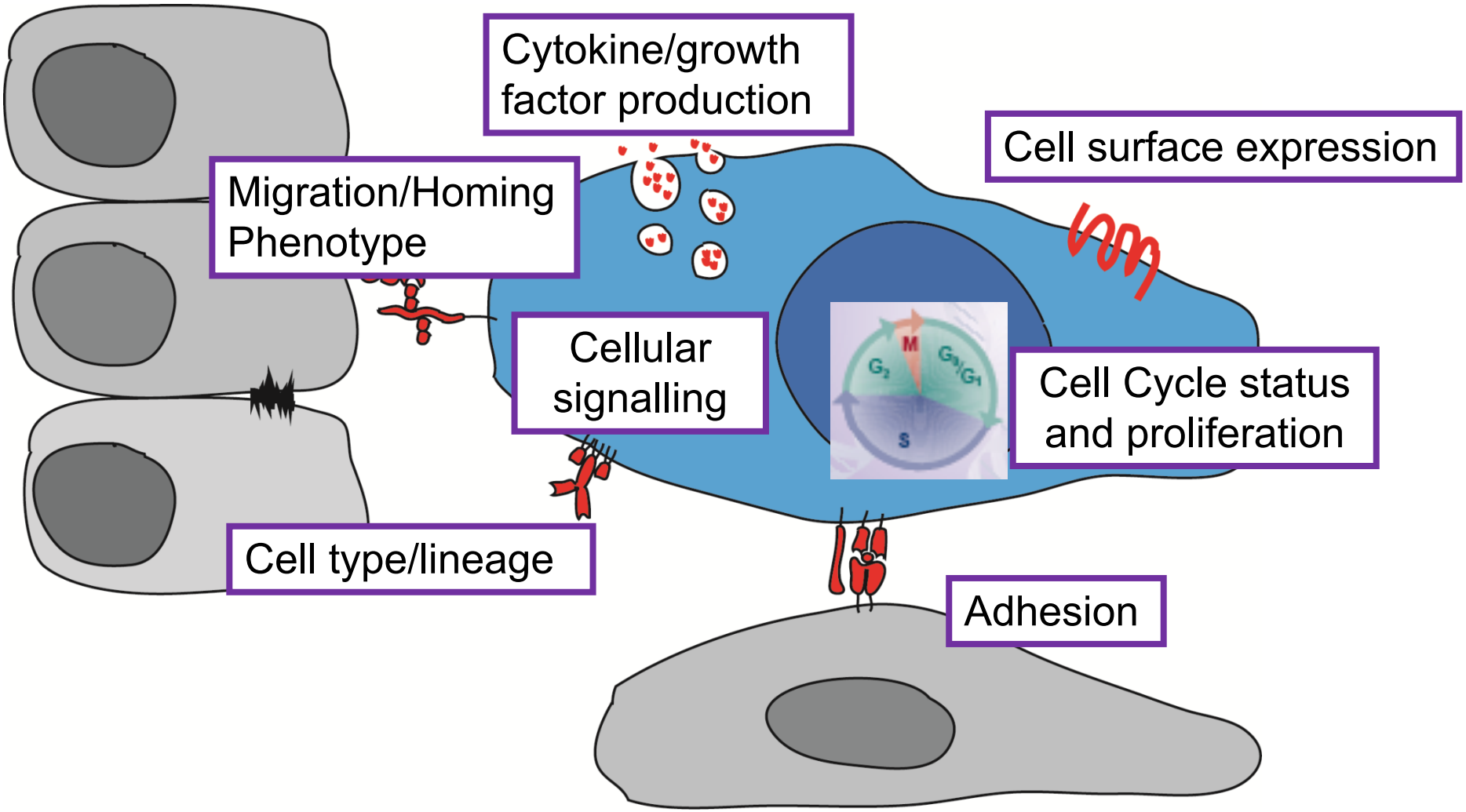
- The primary purpose is to discriminate cell populations based on phenotype >the information can be used to sort subpopulations of cells.



In 1972 Leonard Herzenberg (Stanford Univ.), developed a cell sorter that separated cells stained with fluorescent antibodies.

> ***Fluorescence Activated Cell Sorter (FACS)***

Cytometry - measuring cellular phenotypes



Flow Cytometry versus microscopy

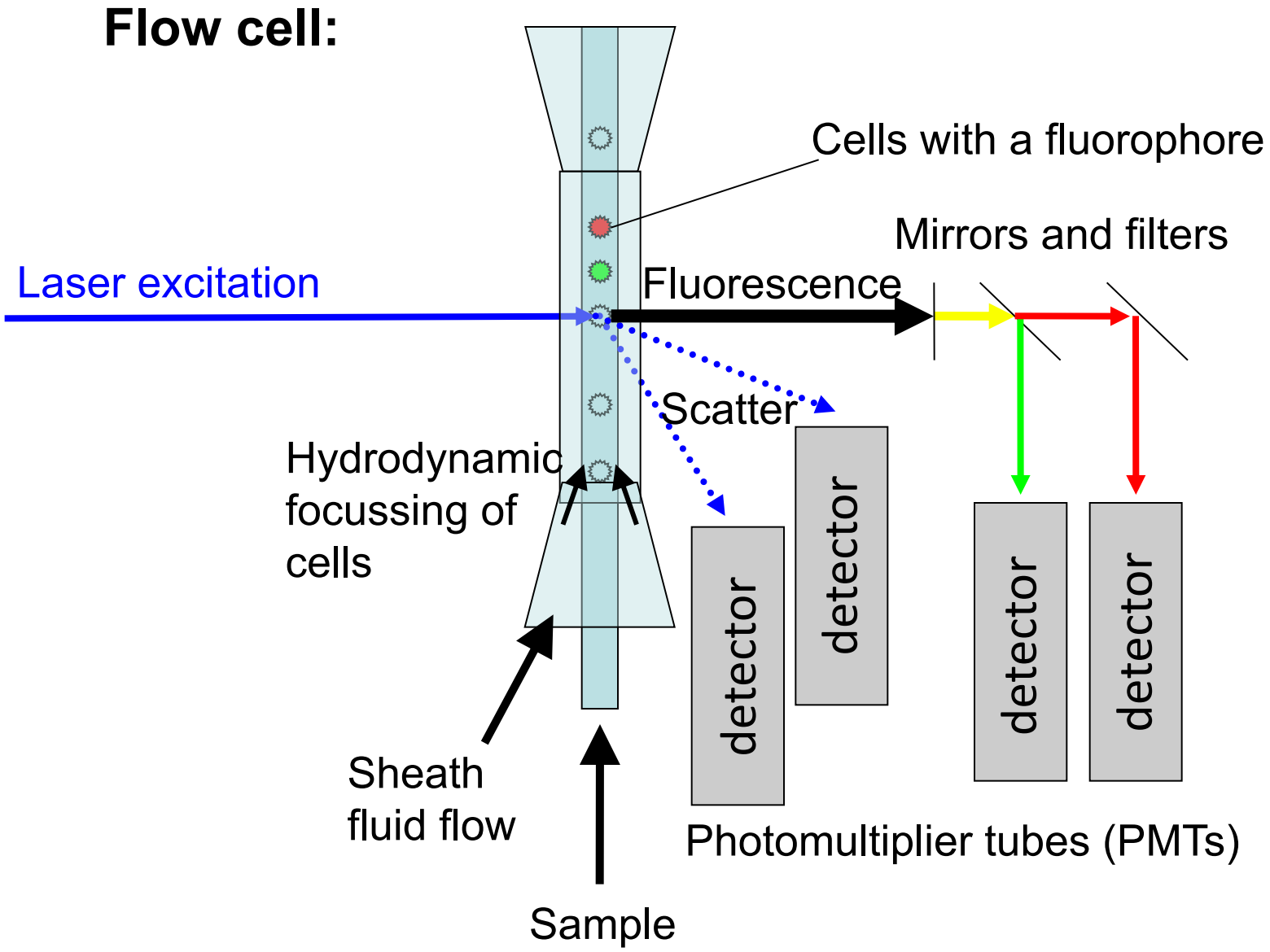
Measuring properties of cells in a fluid stream

Measuring properties of cells in a dish/slide/…

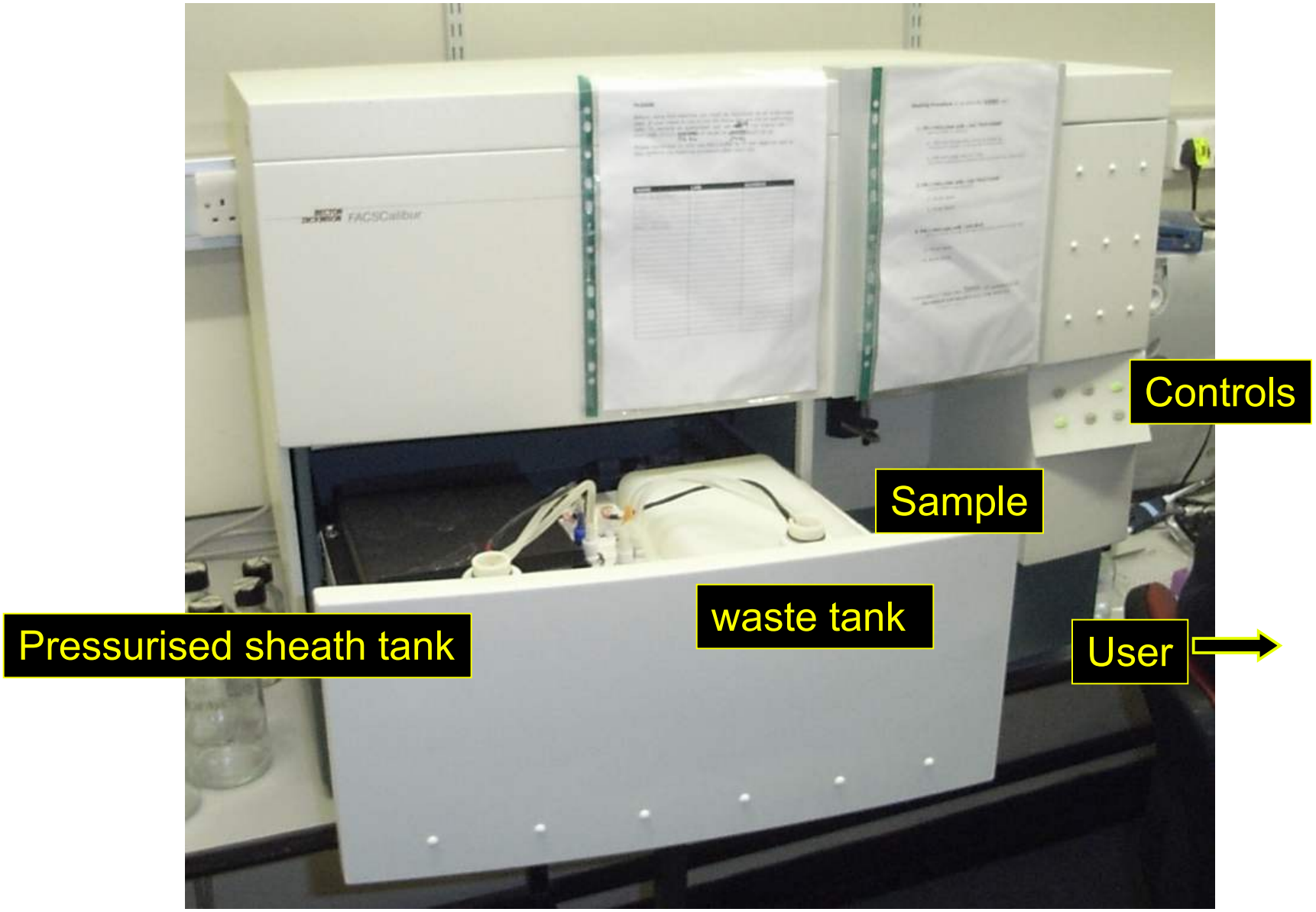
For Flow cytometry, large amounts of information can be gathered about a lot of cells:

- Much larger number of cells can be analysed very quickly
- Ability to look at more than 20 parameters at the same time

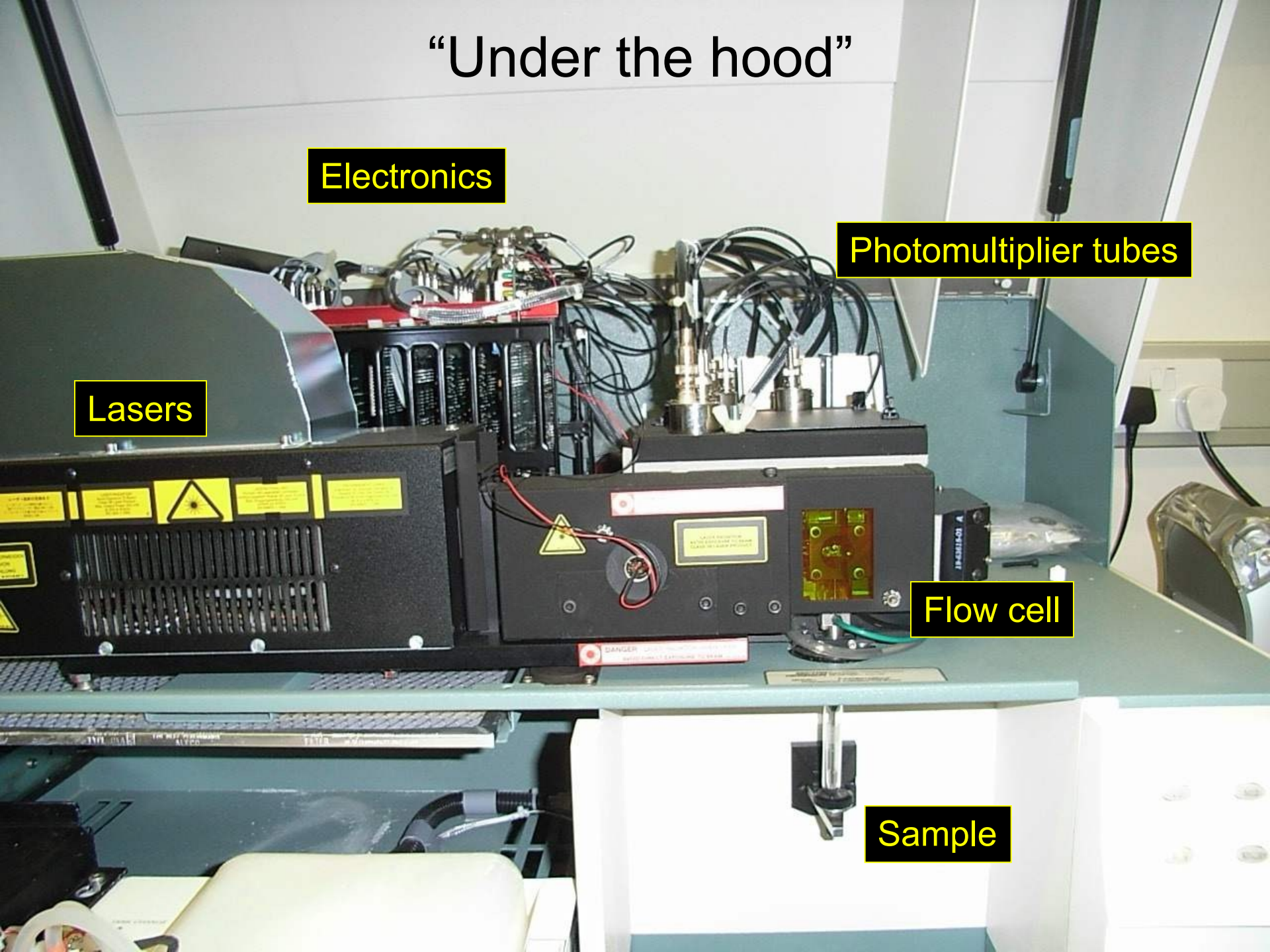
Technical configuration of a flow cytometer



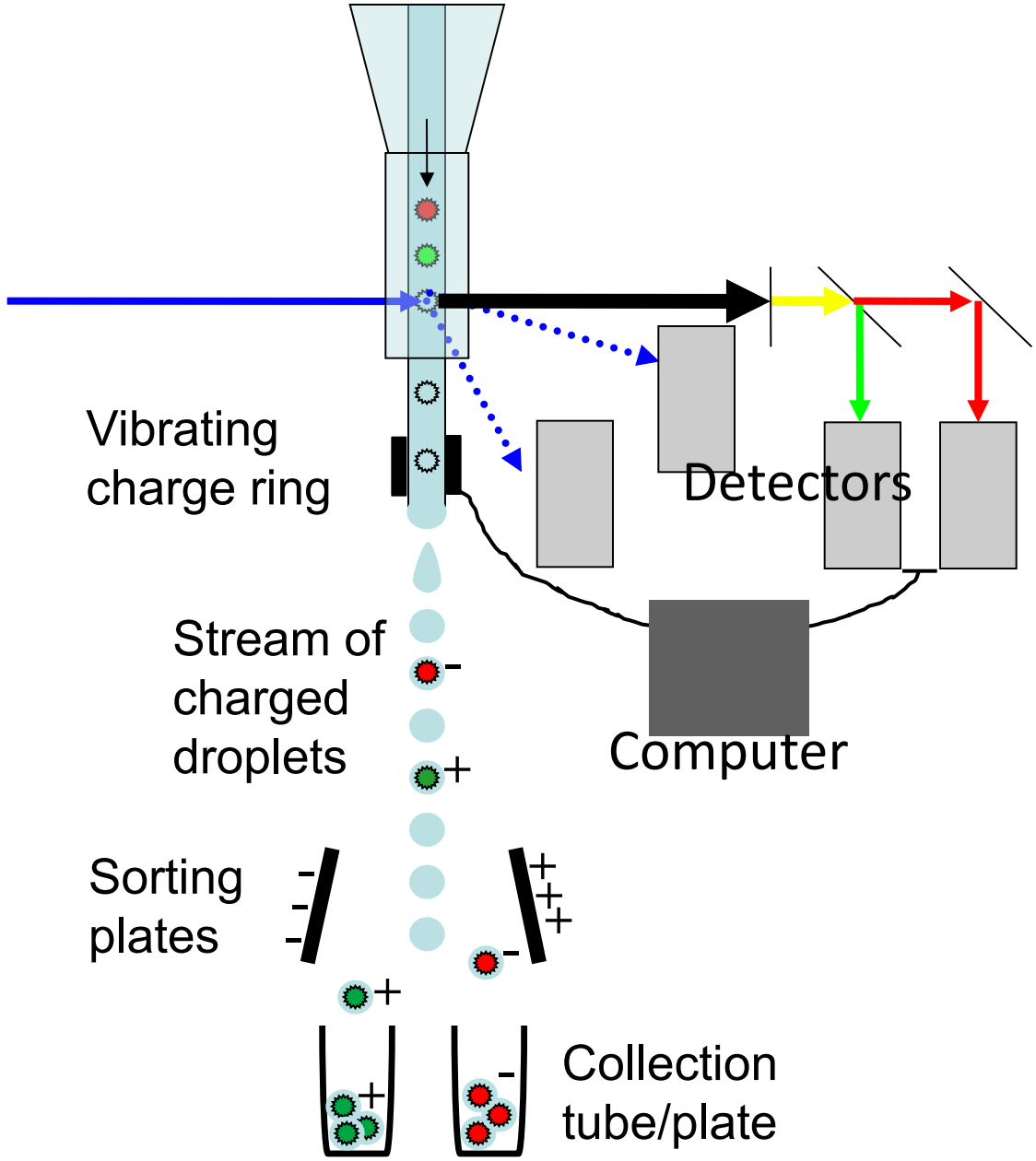
‘Classic’ flow cytometer – the FACSCalibur



“Under the hood”



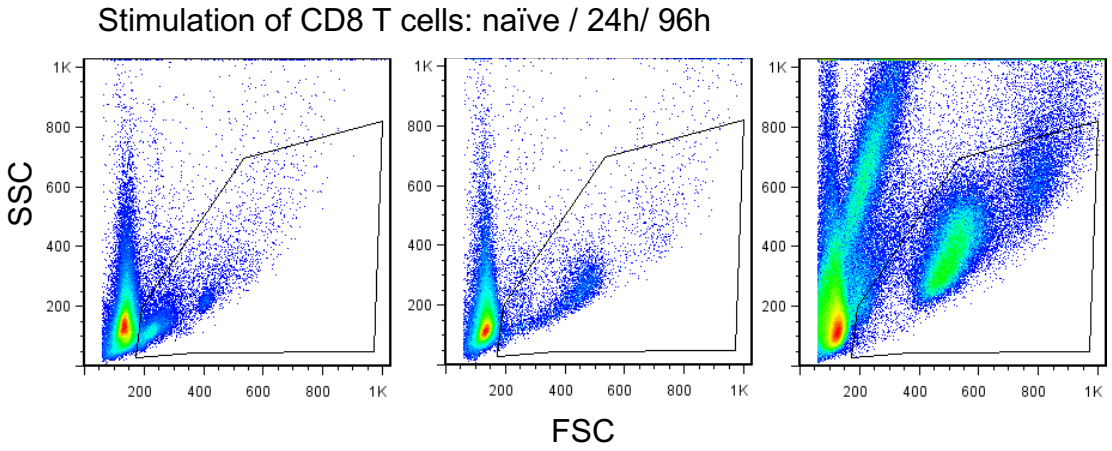
Fluorescence-activated cell sorting

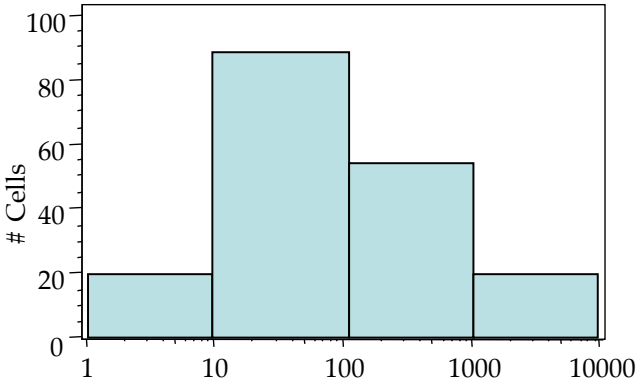


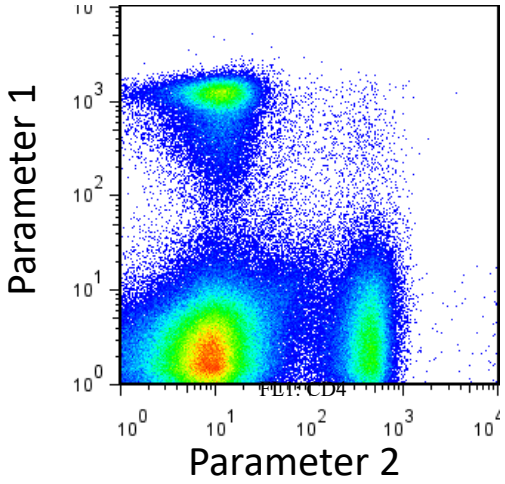
Representing cytometry data

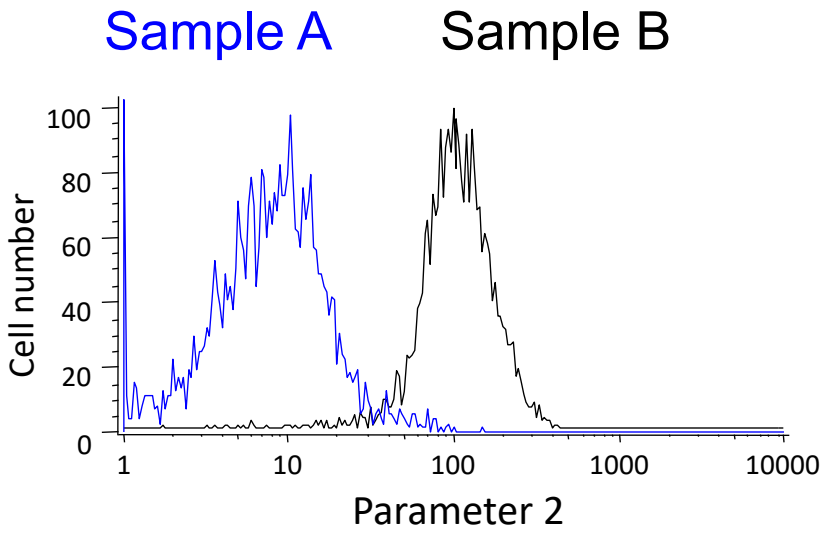
Histograms and dot plots

Linear and logarithmic scale



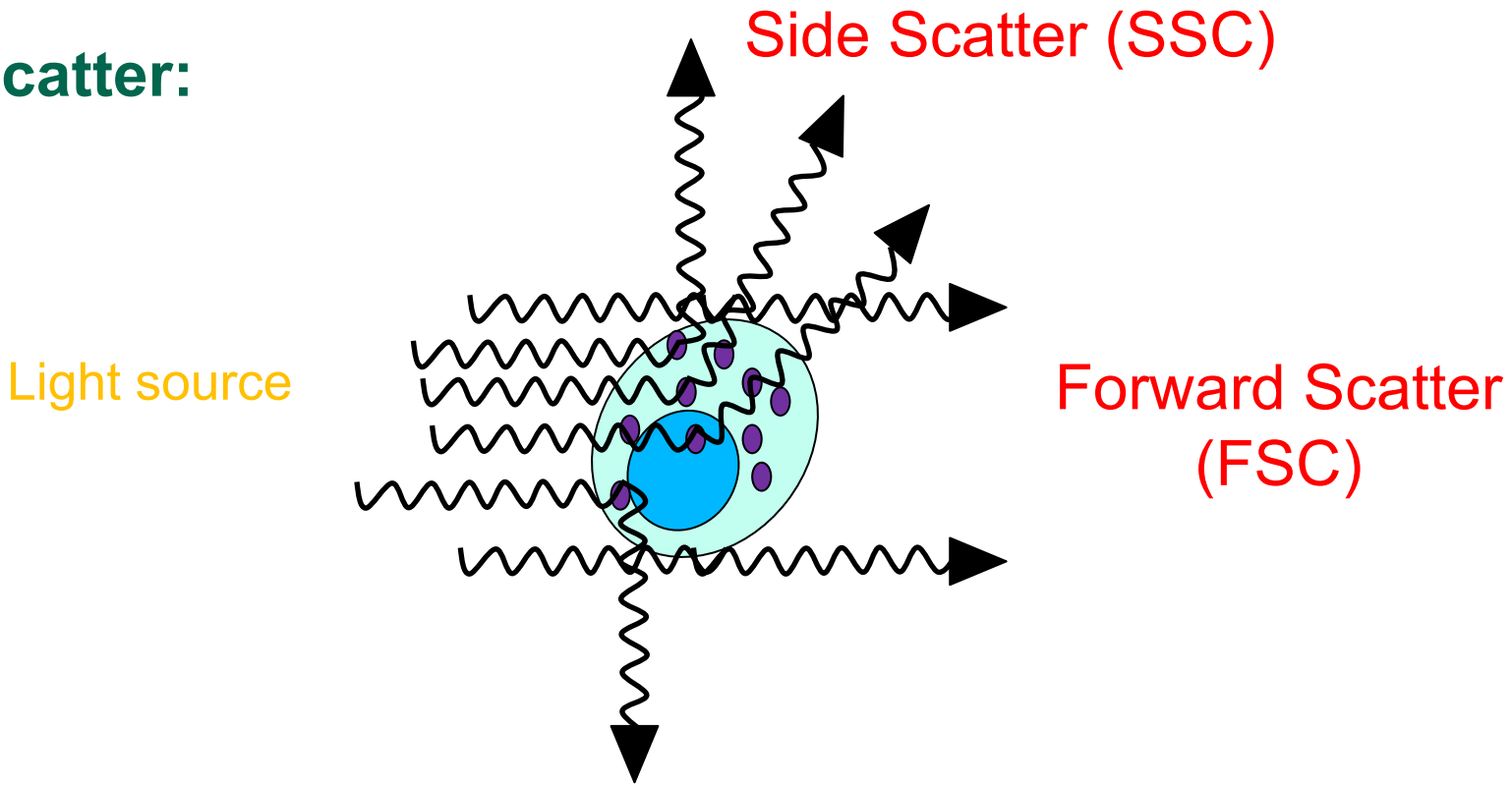






Parameters measured in Flow Analysis:

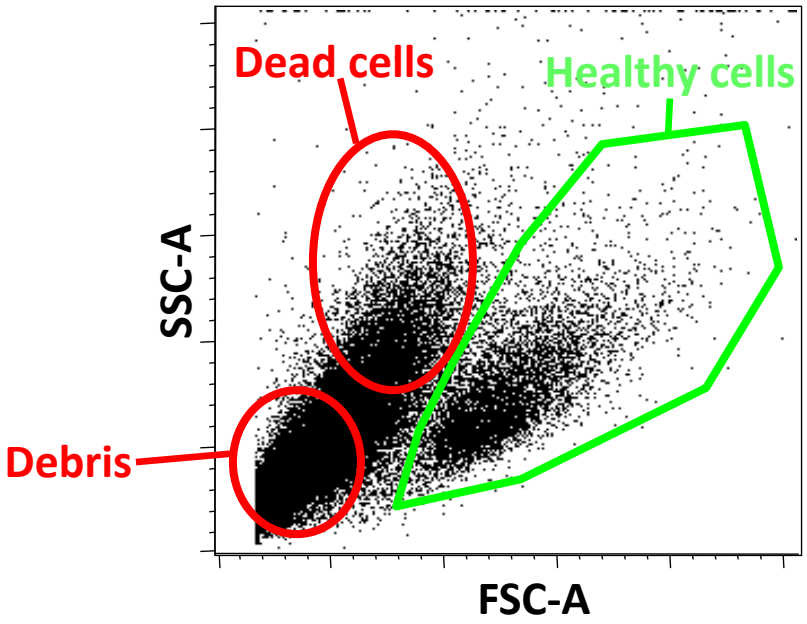
1) Scatter:



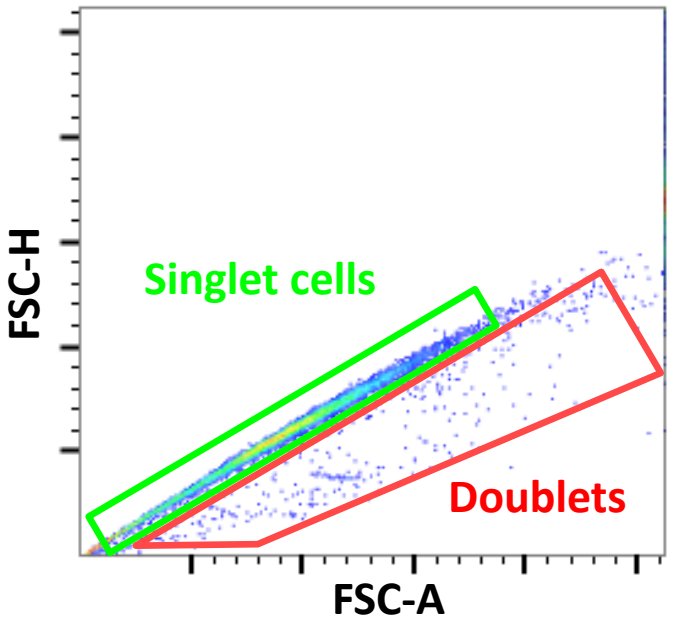
FSC: laser light that is scattered when cells pass through the laser. Correlates with cell size - the larger the cell the greater the FSC.

SSC: refracted laser light captured at a 90 degree angle. Correlates with complexity of the cell membrane, granularity or physical condition of the cell.

Forward Scatter (FSC) & Side Scatter (SSC)

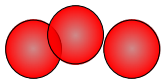


Correlates with live/dead/cellular debris. Viability dyes are required

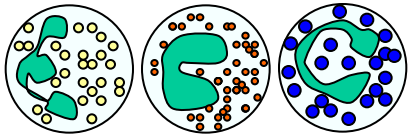


Discriminates single cells from doublets and cell clumps

FSC and SSC to distinguish cell subsets in blood



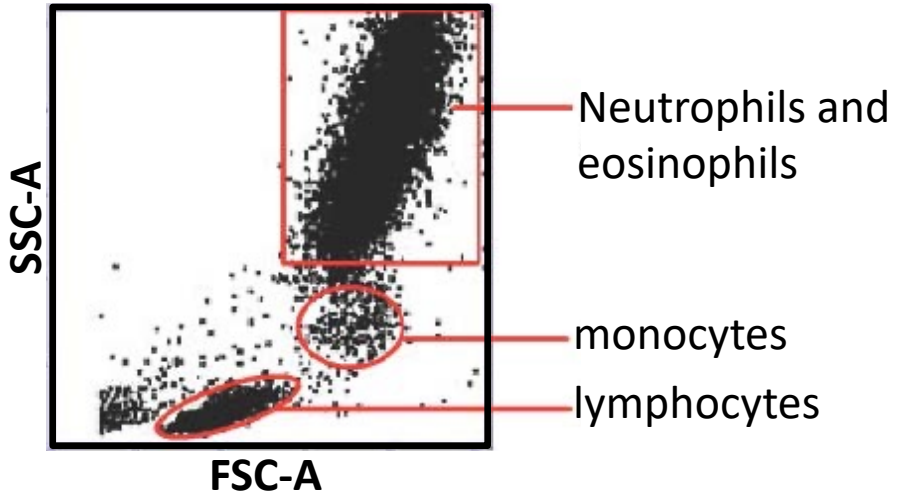
Erythrocyte



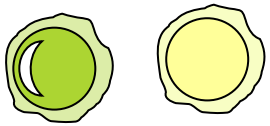
Neutrophil Eosinophil Basophil

Mature Monocyte

Whole blood cells



lymphocytes



B cell T cell

The use of cell type-specific fluorescently labelled antibodies add a further level of complexity for discriminating cell subsets.

Fluorochromes in flow cytometry

Consideration for the use of fluorophores:

- Fluorochromes with *large Stokes Shift* (energy of emitted fluorescence is less than that of absorbed light) are better for discriminating cell populations

- Fluorochromes with *excitation maximum close to the one of the laser lines* of the instrument should be selected

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Fluorescence on standard 4 colour instrument

488nm laser: 

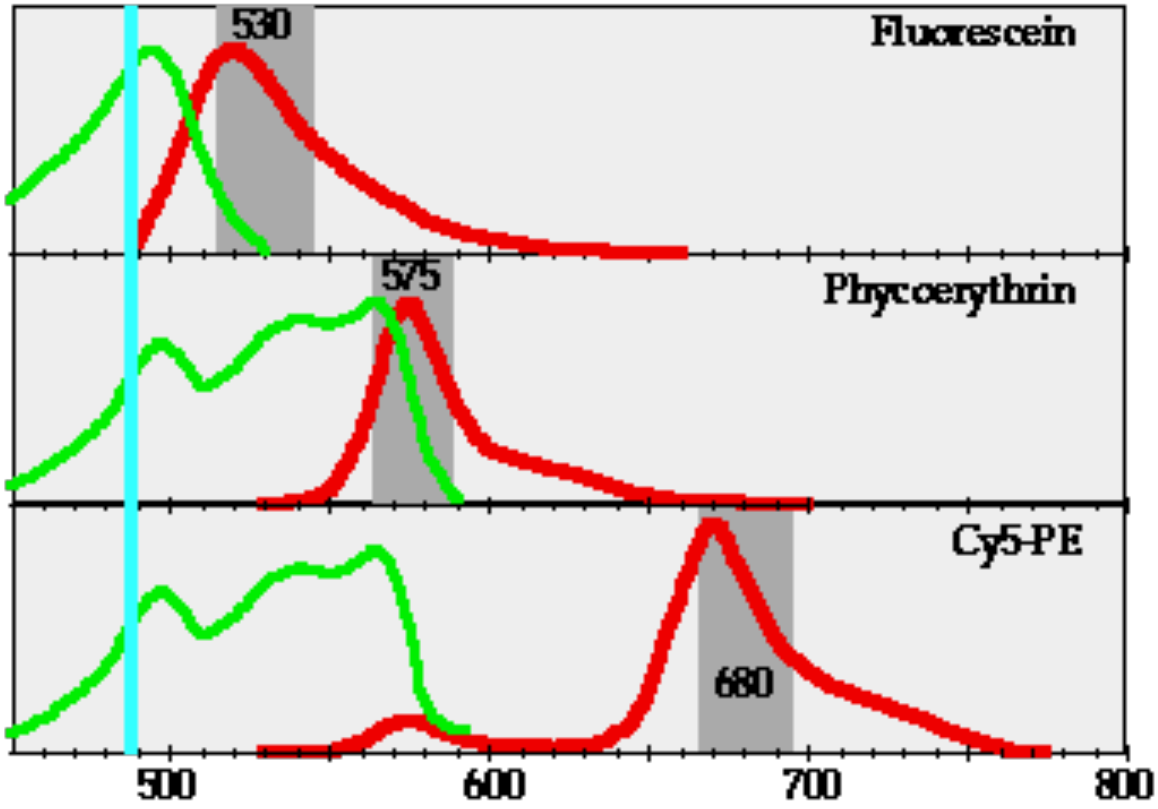
FL1 (FITC: ExMax 494, EmMax 520)
FL2 (PE: ExMax 496, EmMax 578)
FL3 (PE-Cy5:ExMax 496, EmMax 667)

633nm laser: 

FL4 (APC: ExMax 650nm, EmMax 660)

Fluorochromes in flow cytometry

Every fluorescent molecule emits light with a particular spectrum unique to that molecule.



Excitation spectra

Emission spectra

optical filters

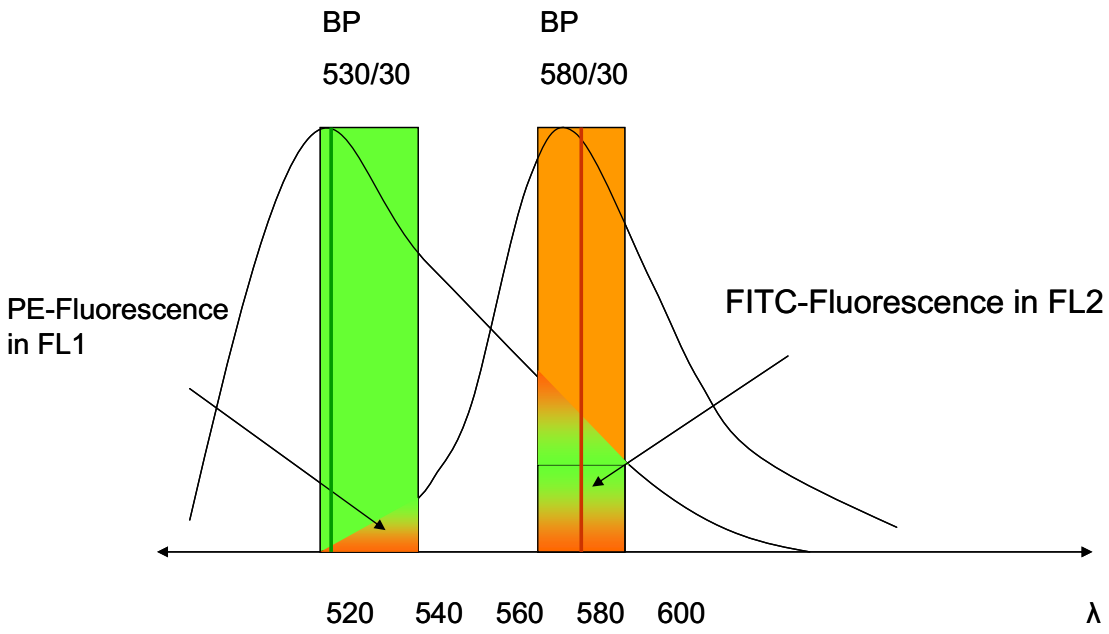
"bandpass filters"

To simultaneously measure these emissions we choose optical filters which only transmit specific wavelengths of light.

http://www.drmr.com/compensation/indexDetail.htm

Compensation

Fluorescence emission “spillover” originating from one channel into another.



FL2-%FL1= corrected FL2

FL1-%FL2= corrected FL1

Compensation
= correction of the ‘true’ fluorescent signal is through subtraction of the spillover

Fluorescent molecules used in flow cytometry

A: *Covalently labelled chemical probes*

Antibodies, lectins, hormones, avidin or streptavidin, or even cDNA

Fluorochrome synthesized in form that can be covalently linked to a protein: isothiocyanate >>> fluorescein isothiocyanate (FITC)

B: Fluorochromes used to label directly cell components

B.1: probes for nucleic acid (e.g. propidium iodide)
B.2: probes that reflect membrane potential (e.g. JC-1)
B.3: probes for lipids (e.g. PKH26)
B.4: probes sensitive to calcium (e.g. Fura Red)
B.5: probes that bind to cytoplasmic proteins (e.g. CFSE)
B.6: pH sensitive probes (SNARF)
B.7: probes identifying reporter genes (β-gal substrate, GFP)

Sample preparation

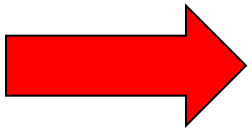
Aim: generation of a suspension of single particles, stained in a specific way which will be will pass through the system without disrupting the smooth flow of fluid or blocking tubes or orifices

Particles: whole cells, cell organelles, specific clumps of tissue
(e.g Islets of Langerhans)

Body fluids (e.g. blood): straightforward

Solid tissues: more difficult

Organelles (e.g.nuclei or chromosomes)



Be careful that your preparative method does not bias your result!!!

Sample staining

Usually cells are stained by incubation, under appropriate conditions, with a fluorescent dye or fluorescent-conjugated antibody or ligand

Problems:

-non specific binding
-cross-reactions
-spectral overlap of fluorochromes
-binding of antibodies by Fc-receptors on cells
-autofluorescence
-accessibility of antigens: fix and/or permeabilize

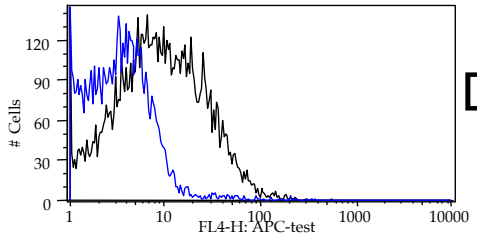
Problems in sample staining are easily assessed by the use of appropriate controls to ensure specificity and accuracy of measurements

Controls

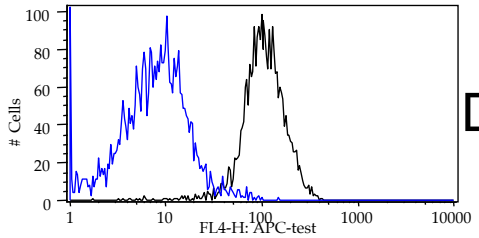
Controls:

-staining control
(isotype control,
FMO: fluorescence minus one)

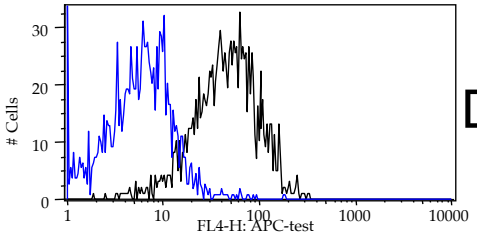
-transfection control
-stimulation control



DC type A



DC type B



DC type C

Isotype control antibody

Anti-CD1d

Applications of flow cytometry

-Membrane antibody staining

-Intracellular staining

-DNA staining

-Apoptosis

-Ca flux analysis

-Cell/ Molecule counts

-Conjugation assay

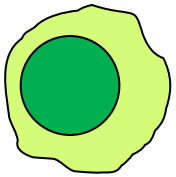
Membrane antibody staining

Key: discovery of monoclonal antibodies by *Köhler and Milstein* in 1975

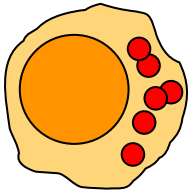
Detect cell populations, subsets, differentiation phenotypes

CD8 differention phenotypes in murine herpesvirus infection:

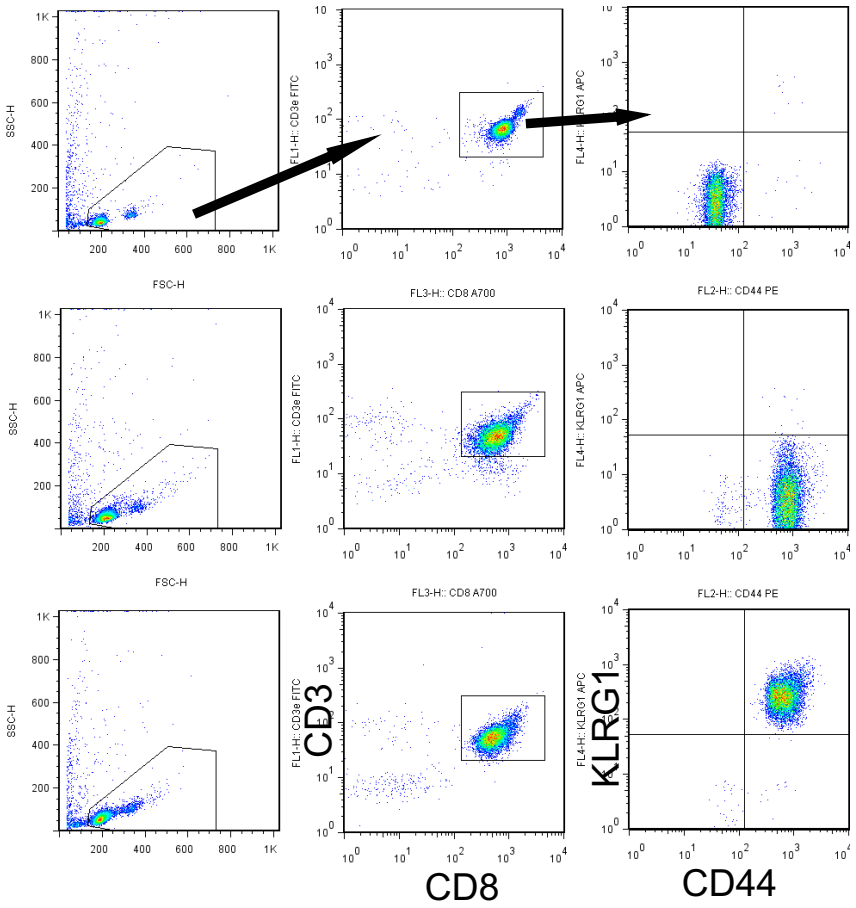
Naive



Memory



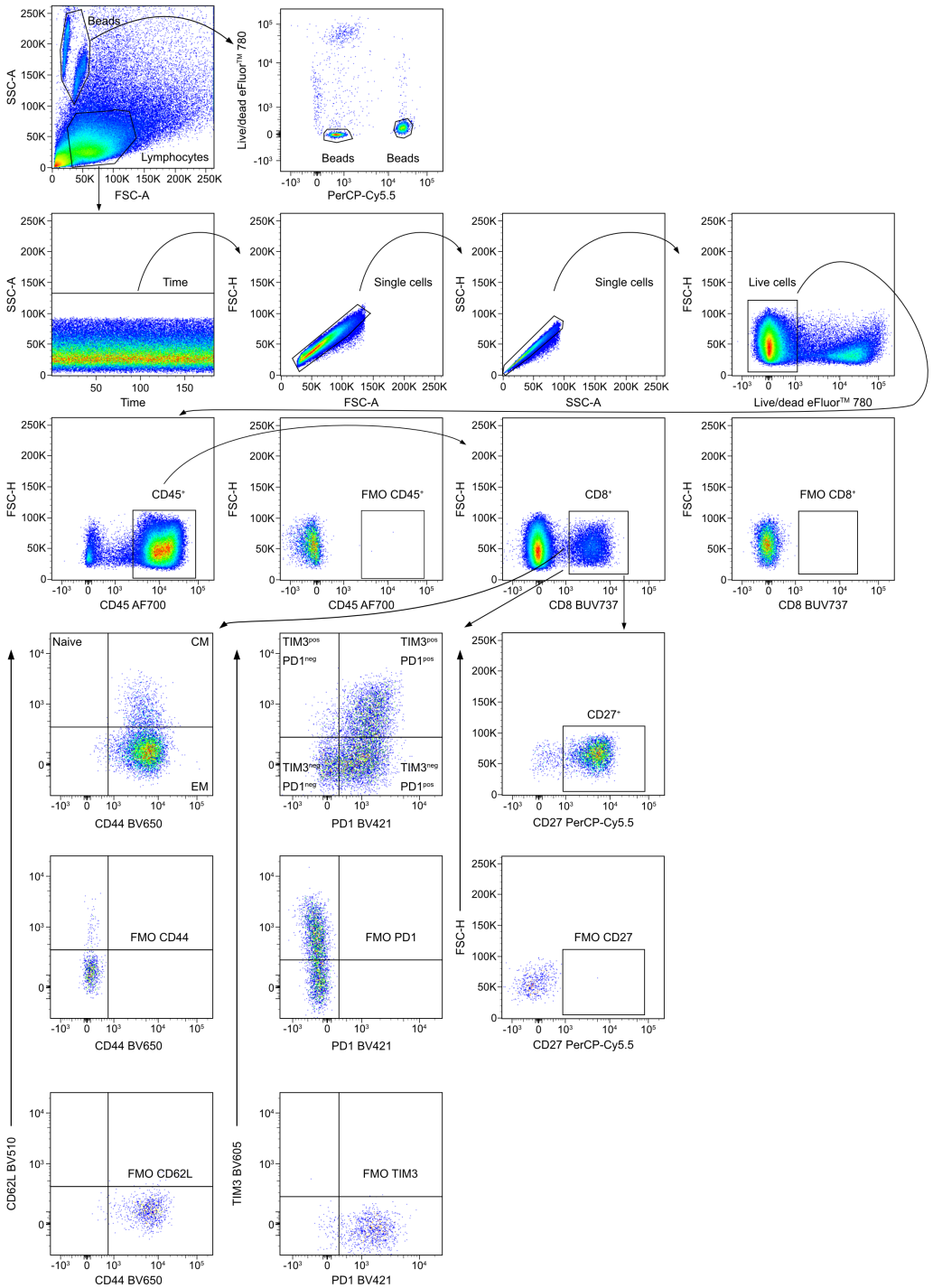
Effector



No replicative potential

replicative potential

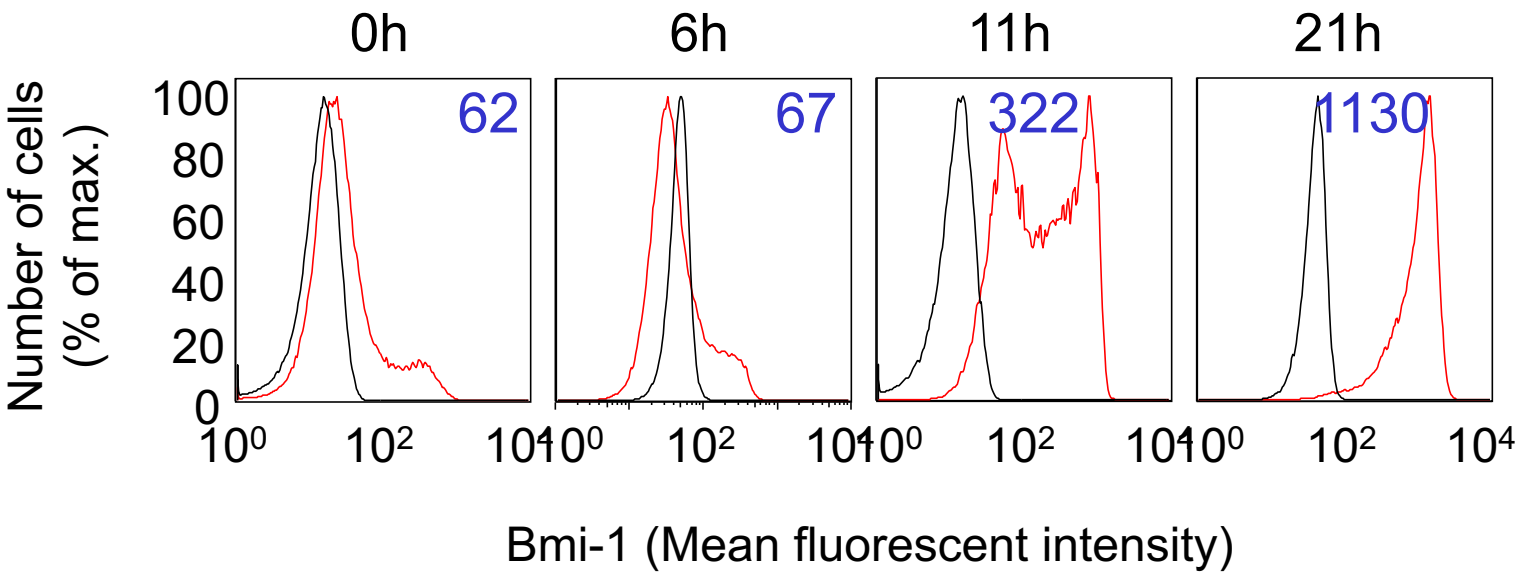
Senescent: no replicative potential



Example of gating strategy

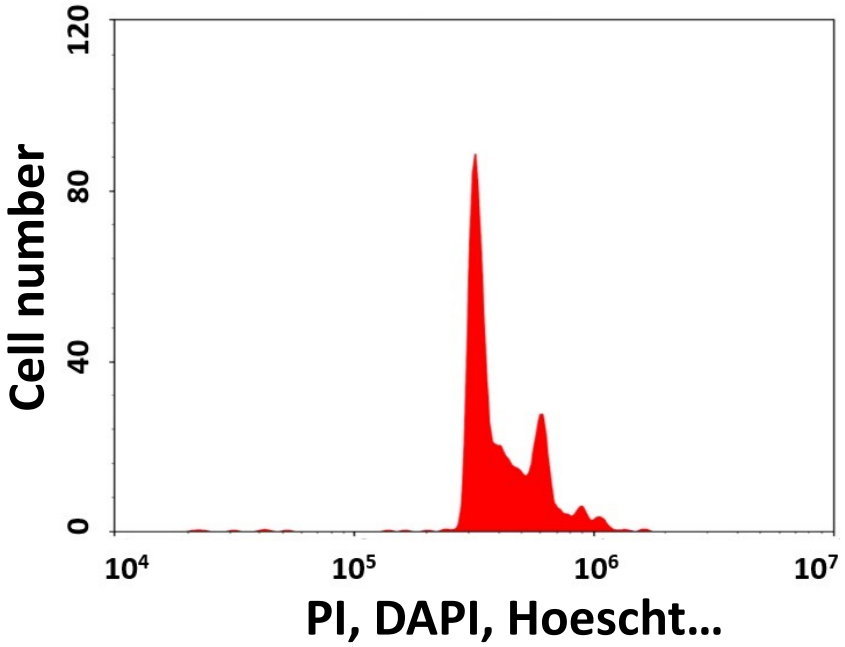
Intracellular staining

suitable antibodies, fixation and permeabilisation

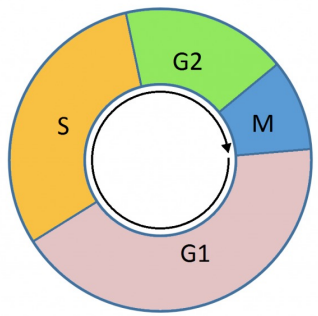


DNA staining to determine cell cycle

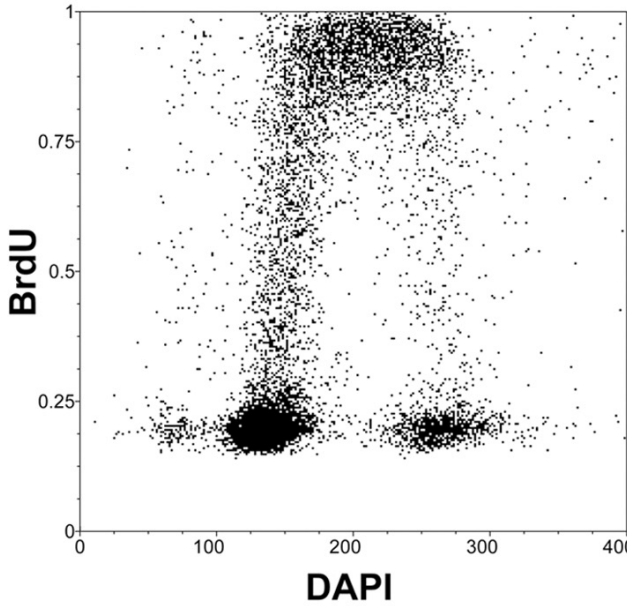
Information about:
Ploidy: malignant cells frequently aneuploid
(prognostic value in human tumors)



Cell cycle:
G0 = quiescent cell
G1 = RNA increases/
proteins ess. for DNA replication
are made
S = DNA synthesis
G2 = DNA duplicated
M = division and return to G0 or G1



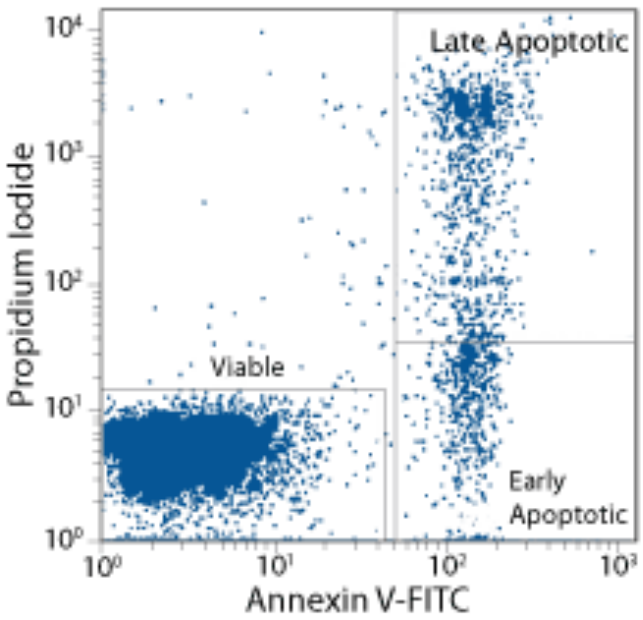
Pulse (chase) with BrdU

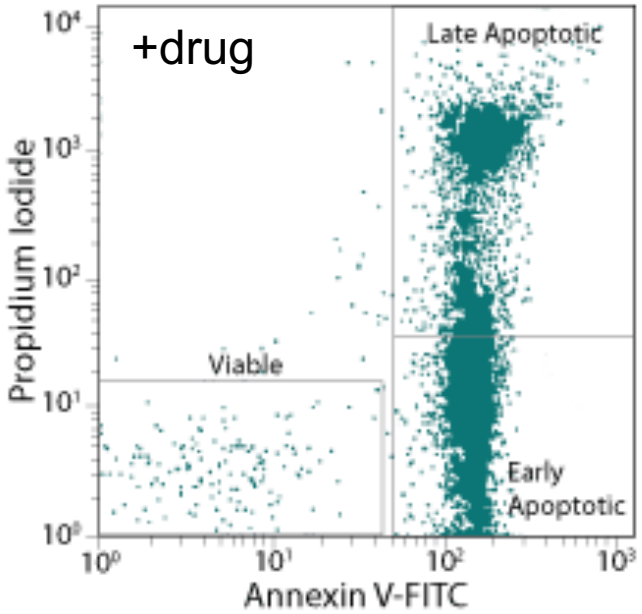


Apoptosis

Apoptosis (programmed cell death): condensation of nuclear chromatin, compaction of cytoplasmic organelles, cell shrinkage, collapse of mitochondrial membrane potential, changes on cell surface, no rupture of plasma membrane, DNA fragmentation

Necrosis: uncontrolled swelling, rupture of cell membrane, non-specific DNA degradation

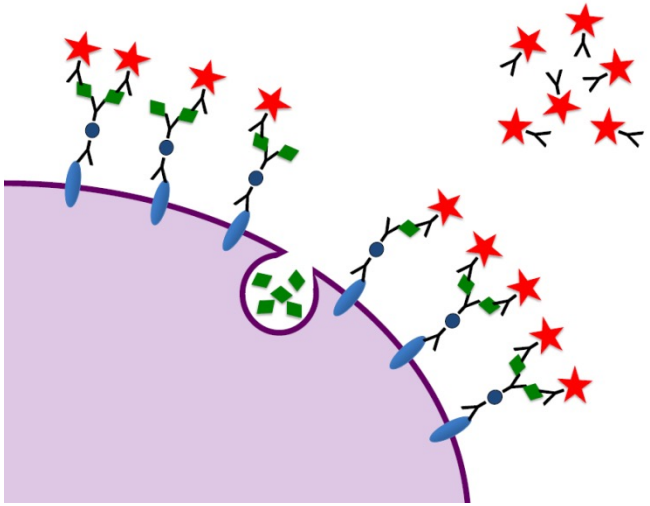




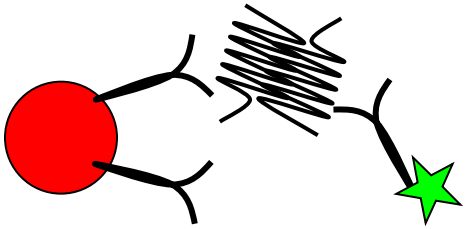
Annexin V: during apoptosis phosphatidyl serine "flips" from internal to external membrane and binds Annexin V (unfixed cells)

Cytokine measurements

Cytokine secretion assay



Molecular interactions on beads

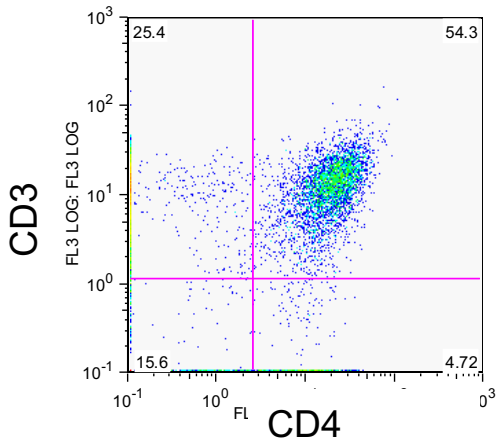


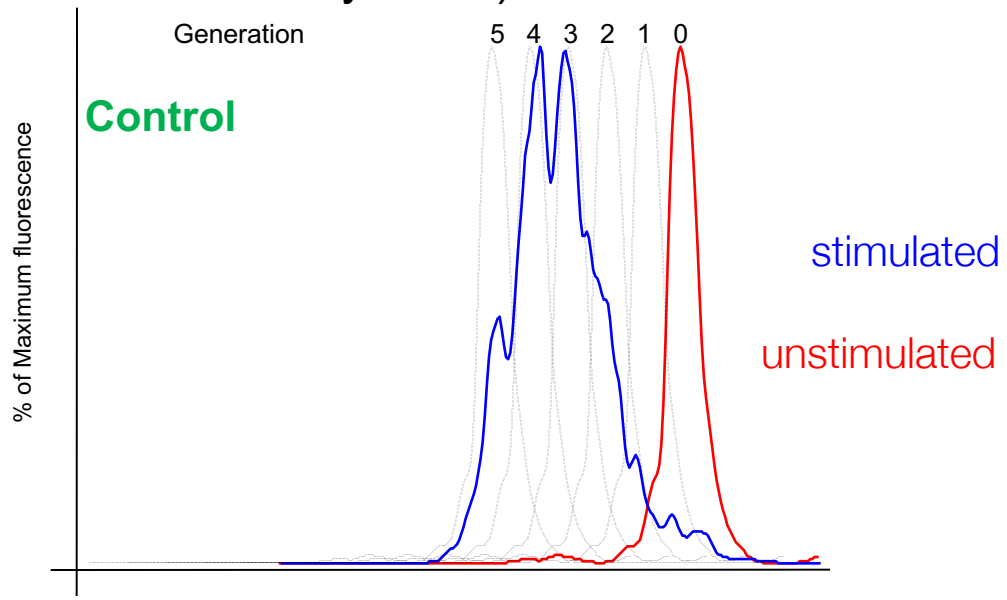
Fluorescent antibody
detects cytokine (epitope B)

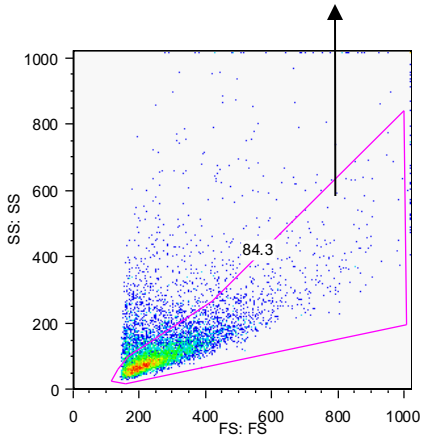
Antibody on beads
captures cytokine (epitope A)

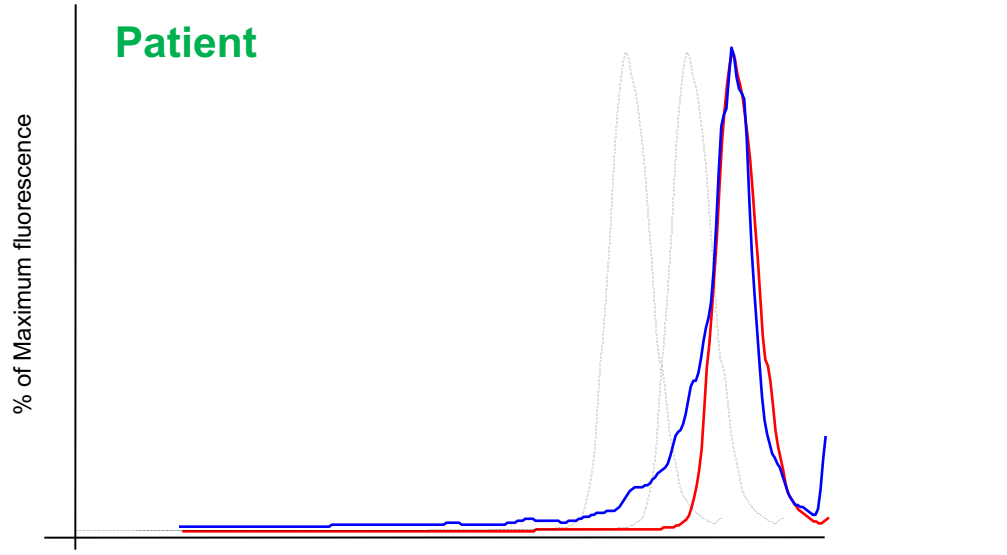
Cell division analysis with CFSE

(carboxyfluorescein succinyl ester)



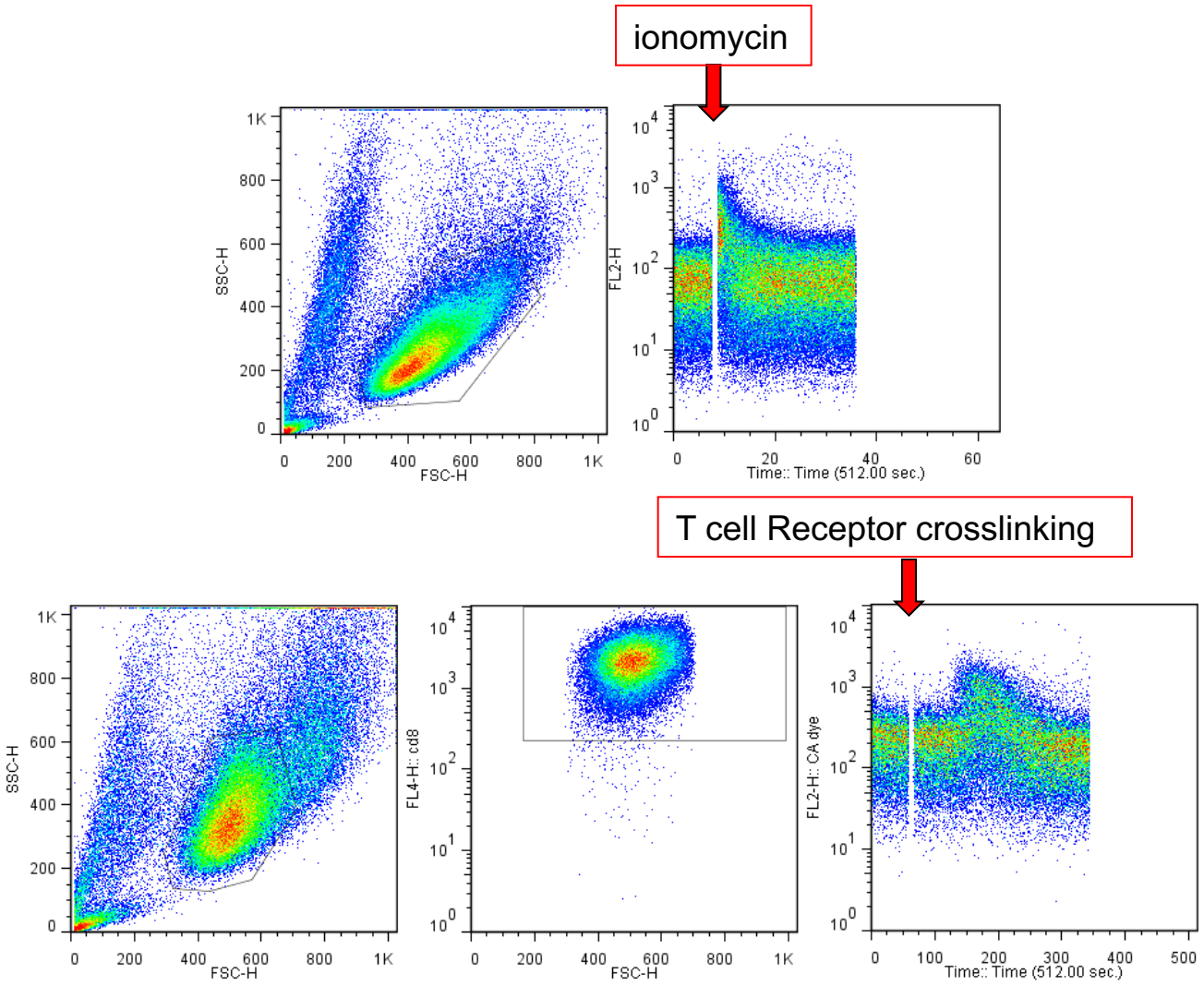






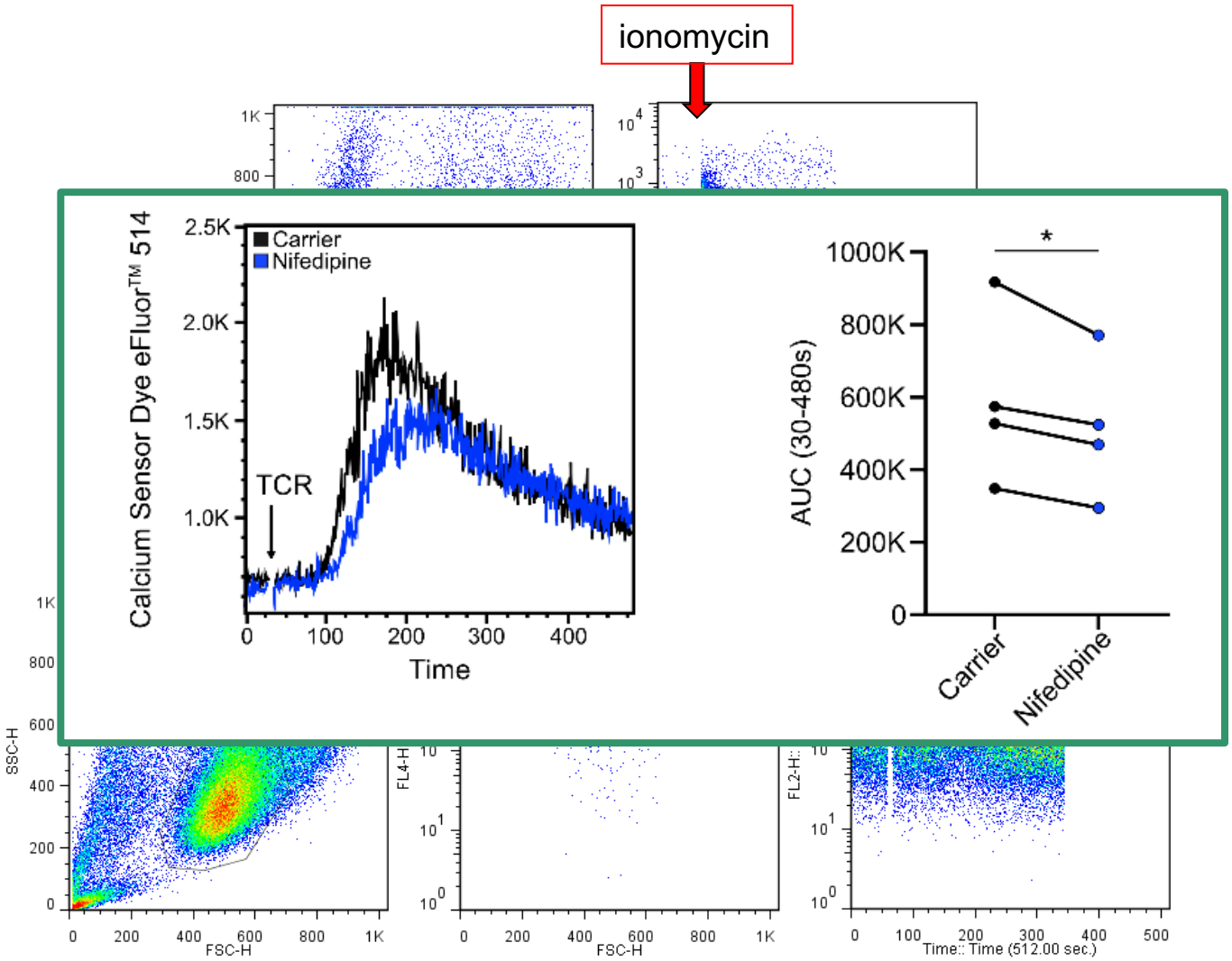
Thanks to Dr James Thaventhiran

Calcium Flux analysis



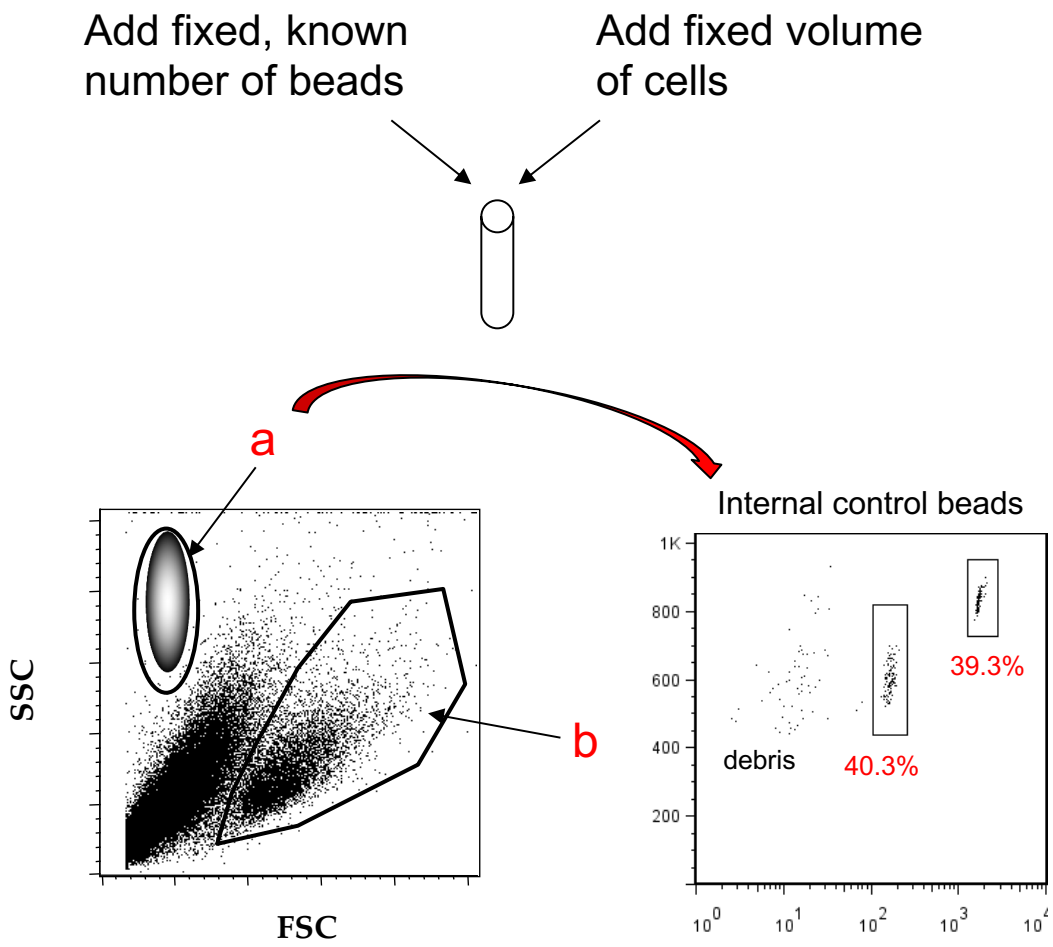
e.g. Calcium Sensor Dye eFluor514

Calcium Flux analysis



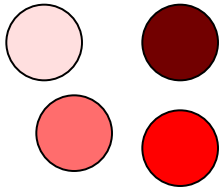
e.g. Calcium Sensor Dye eFluor514

Absolute number of cells using beads

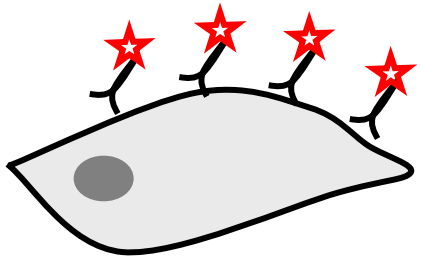


$$\text{Absolute count (cell/µl)} = \frac{\text{Number of cells counted (b)}}{\text{Total number of beads counted (a)}} \times \text{Number of beads/µl}$$

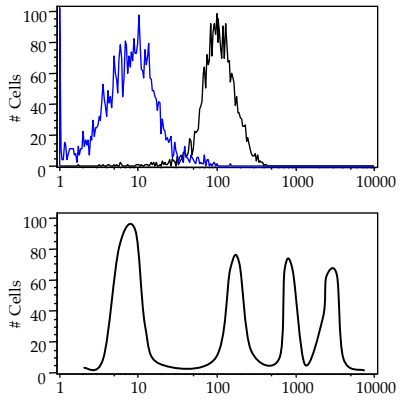
Absolute number of molecules using beads

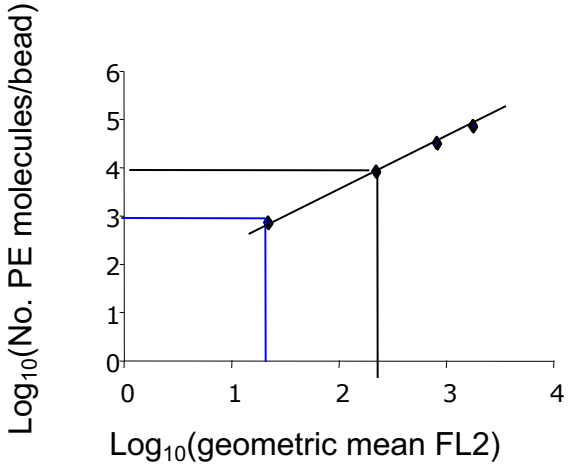


Pre calibrated beads with defined numbers of fluorescent molecules



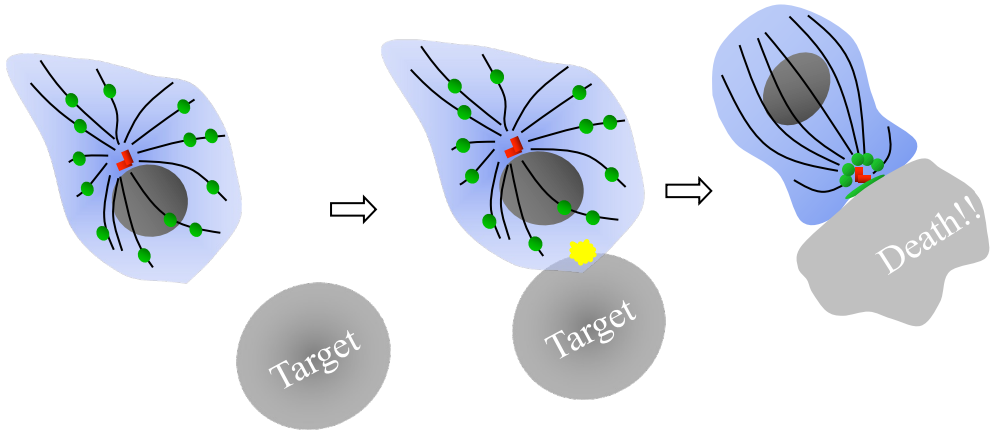
Stain molecule with excess of fluorescent antibody (same flurochrome)



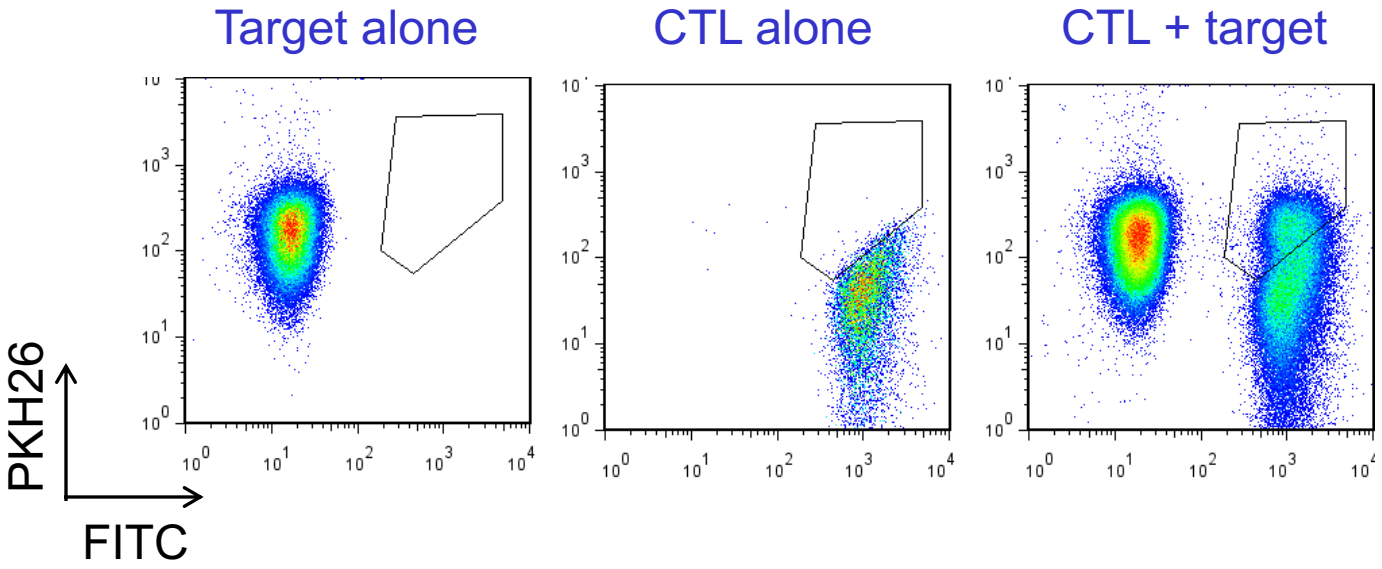


Cell Conjugation assay

Cytotoxic T lymphocyte (CTL):
kills infected and tumorigenic cells



label targets red (PKH26)
label CTLs green (CFSE)



Newer technologies

Multi parameters:

e.g. surface phenotyping, phosphorylation, cell cycle analysis, and cytokine production

- *new fluorophores, more laser lines*
- *Faster acquisition (e.g. Attune)*

- ***Spectral analysers (e.g. Cytek Aurora)***

- ***Imaging Flow cytometry***

- *Post-fluorescence era:* ***Mass cytometry***

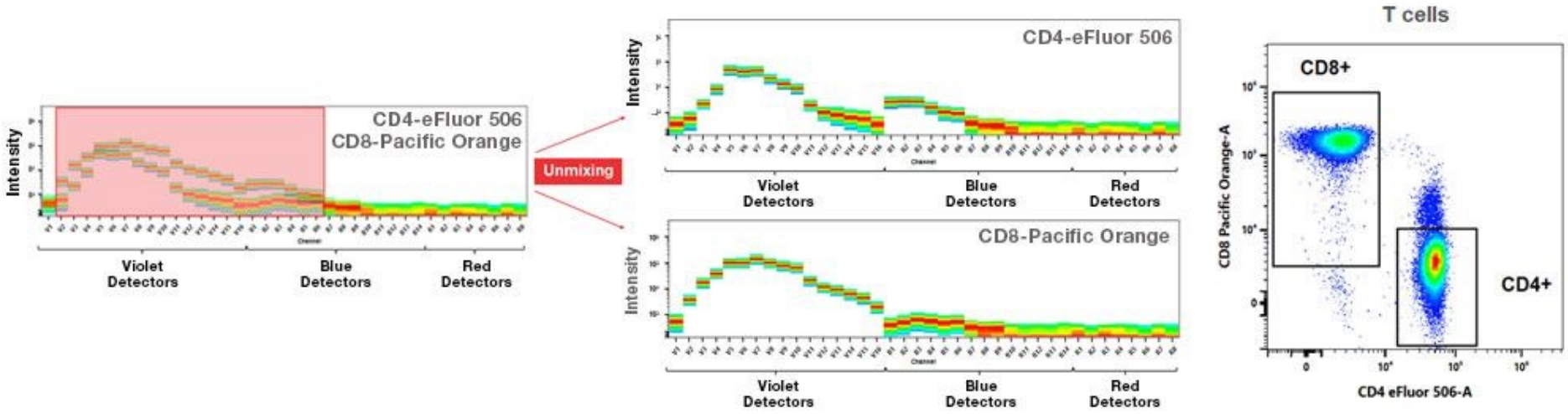
"Special application" cell sorting:

stem cells/sperm/multi colour/single cell PCR

Chromosome sorting

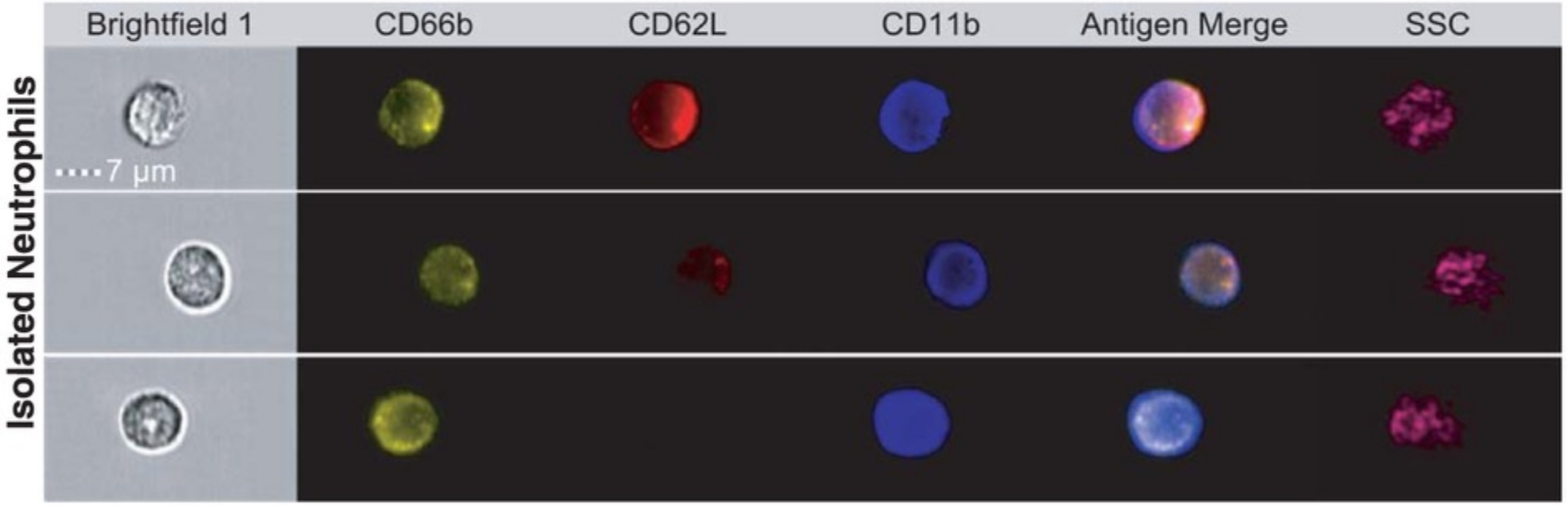
Spectral analysers and sorters

Co-stained sample > Deconvoluted spectral signatures > Analysis



Imaging flow cytometry

couples flow cytometry with microscopy

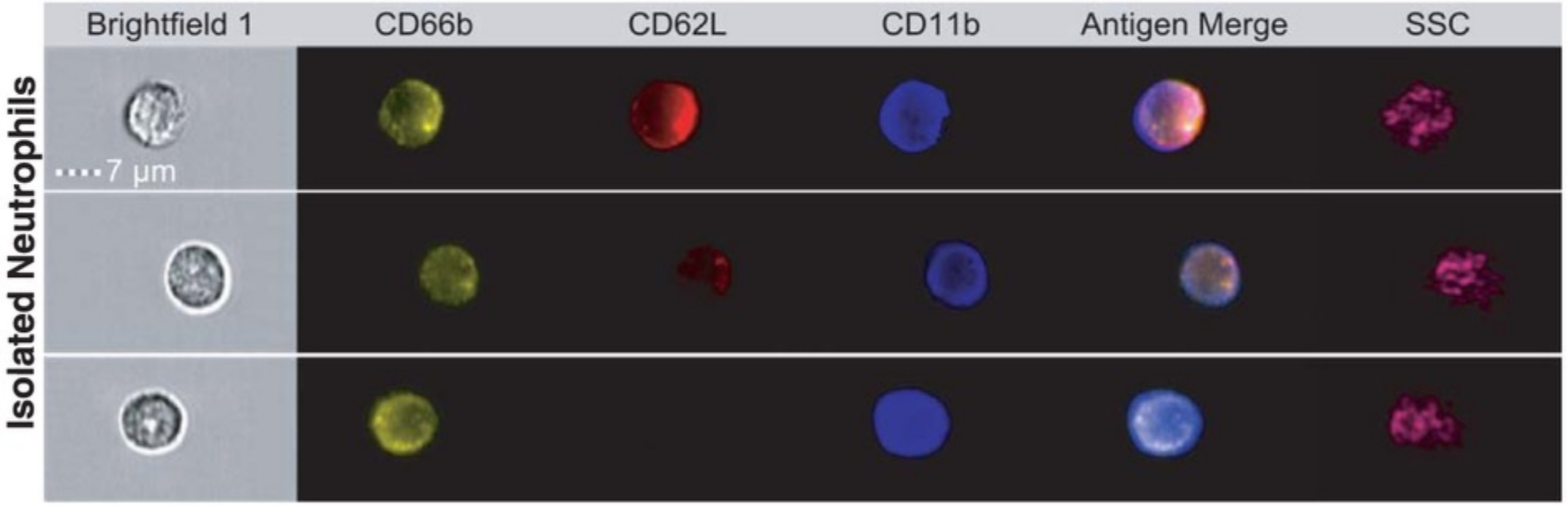


Headland *et al , Scientific reports, 2014*

- *Cell shape*
- *Cell conjugates (e.g. immune synapses)*
- *Distribution of antigen*
- …

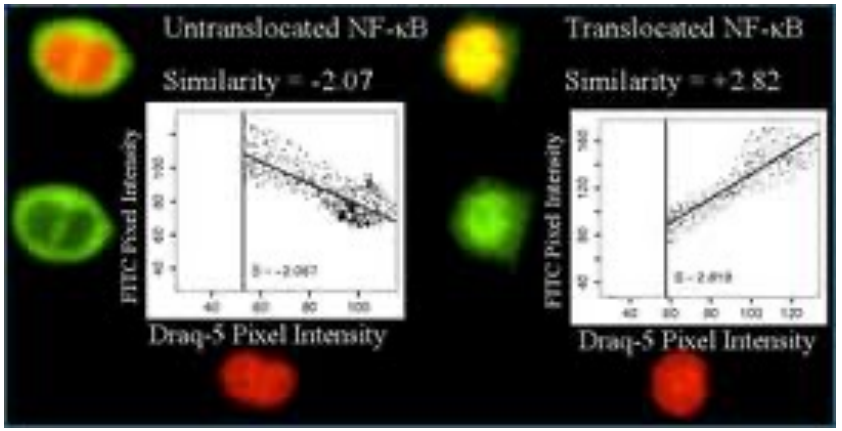
Imaging flow cytometry

couples flow cytometry with microscopy



Headland *et al , Scientific reports, 2014*

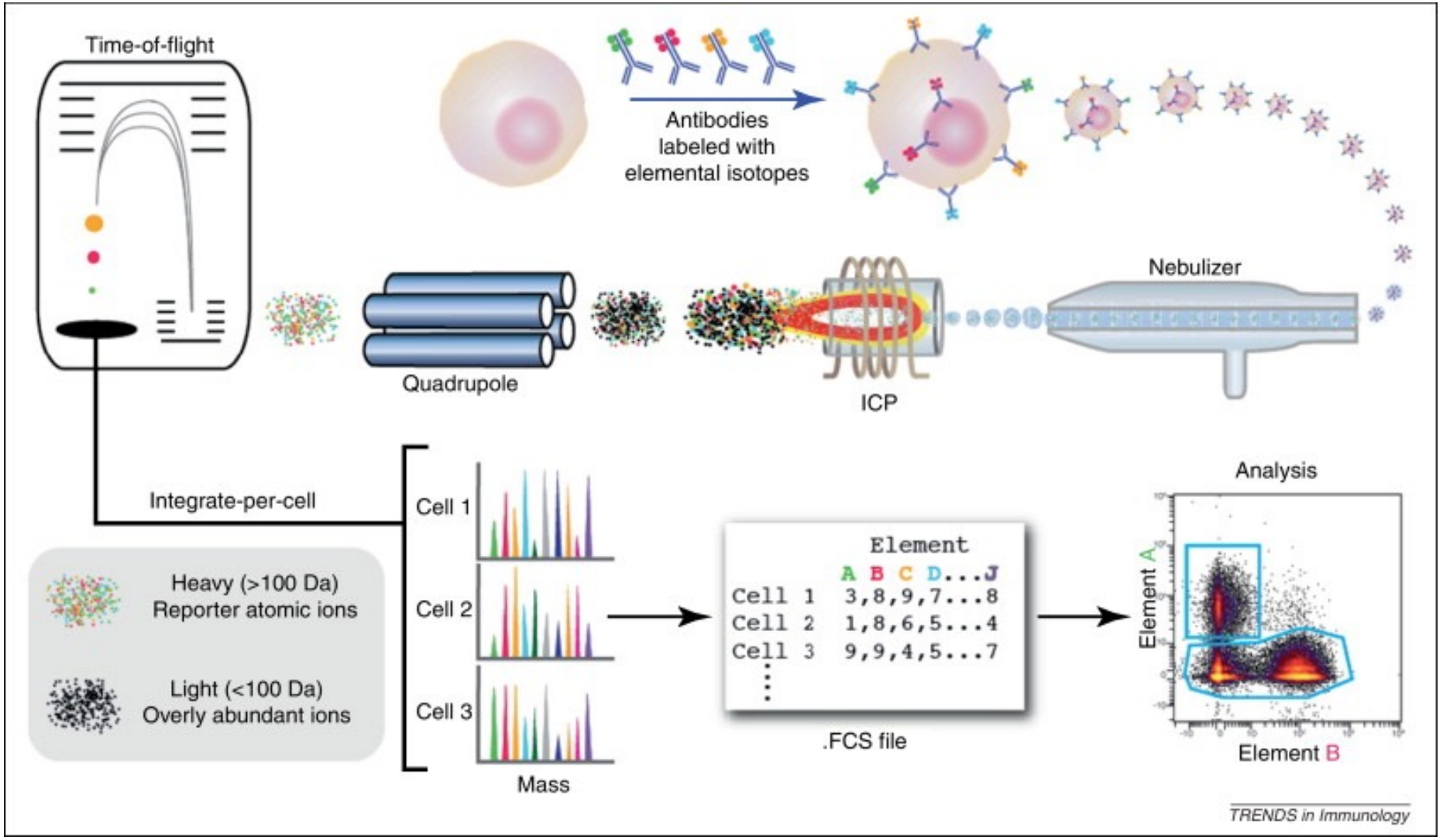
Famous assay
for the imaging cytometer:
NFκB translocation



Zuba-Surma *et al , Folia Histochemica et cytobiologica, 2007*

Mass cytometry

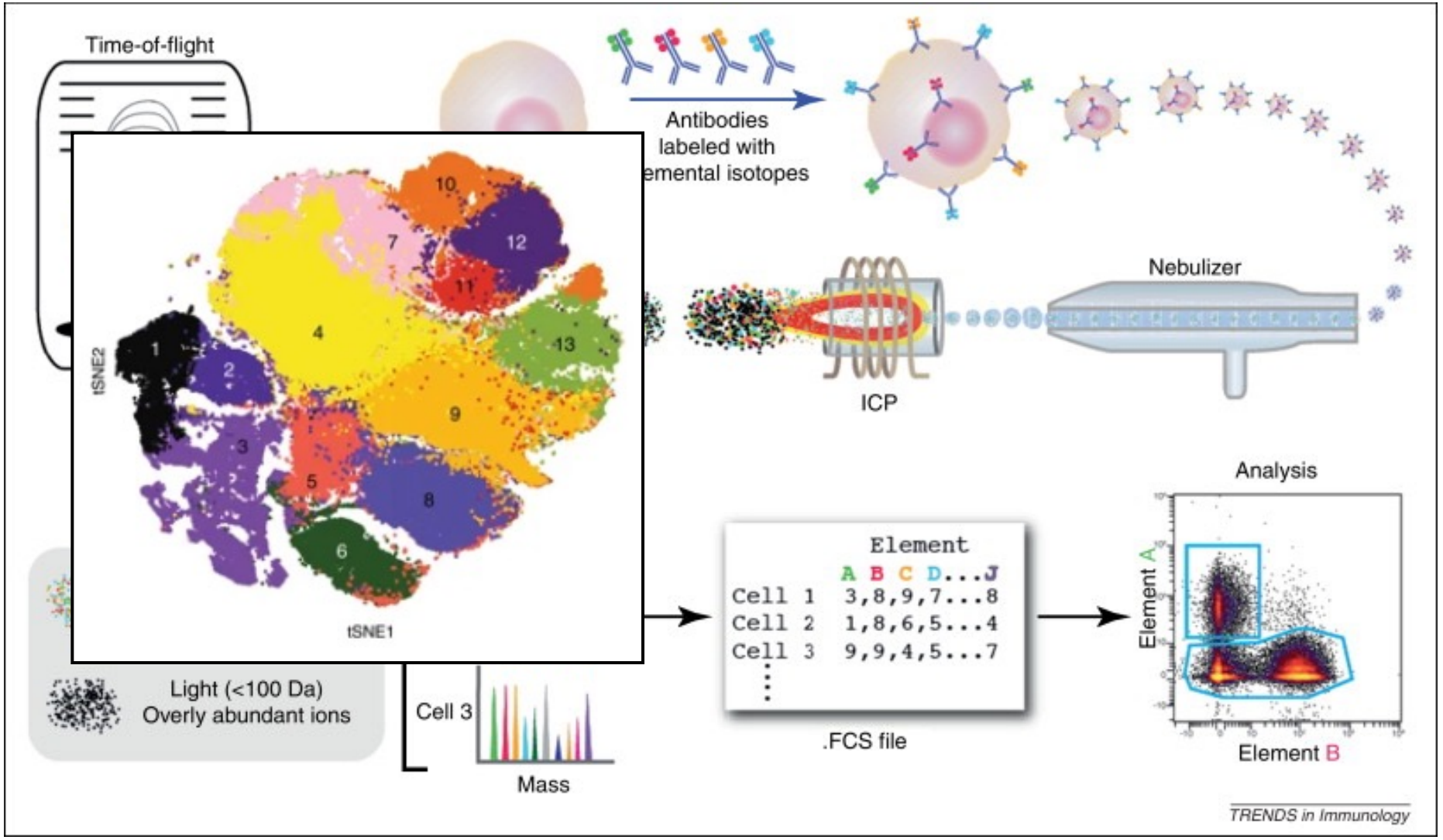
couples flow cytometry with mass spectrometry



Bendall *et al* , *Trends in Immunology, 2012*

Mass cytometry

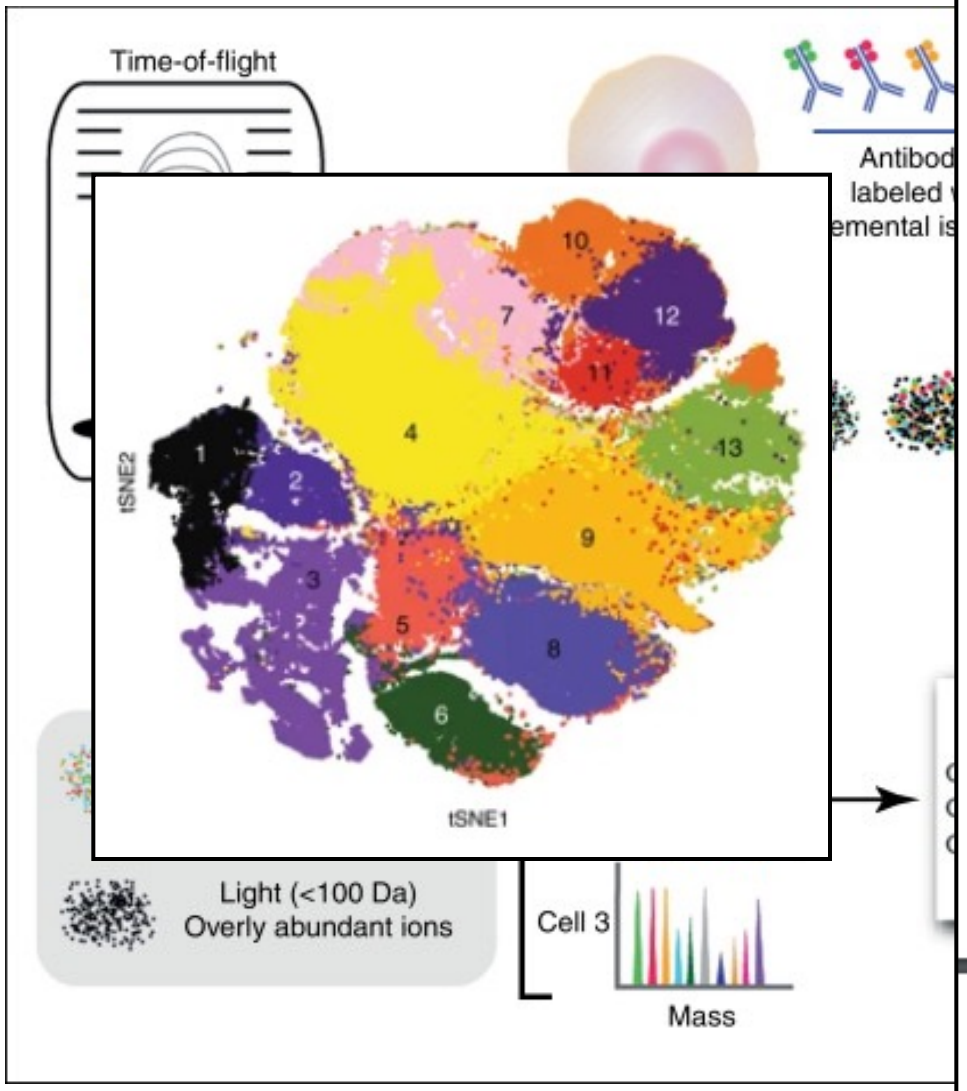
couples flow cytometry with mass spectrometry



Bendall *et al* , *Trends in Immunology, 2012*

Mass cytometry

couples flow cytometry with mass spectrometry



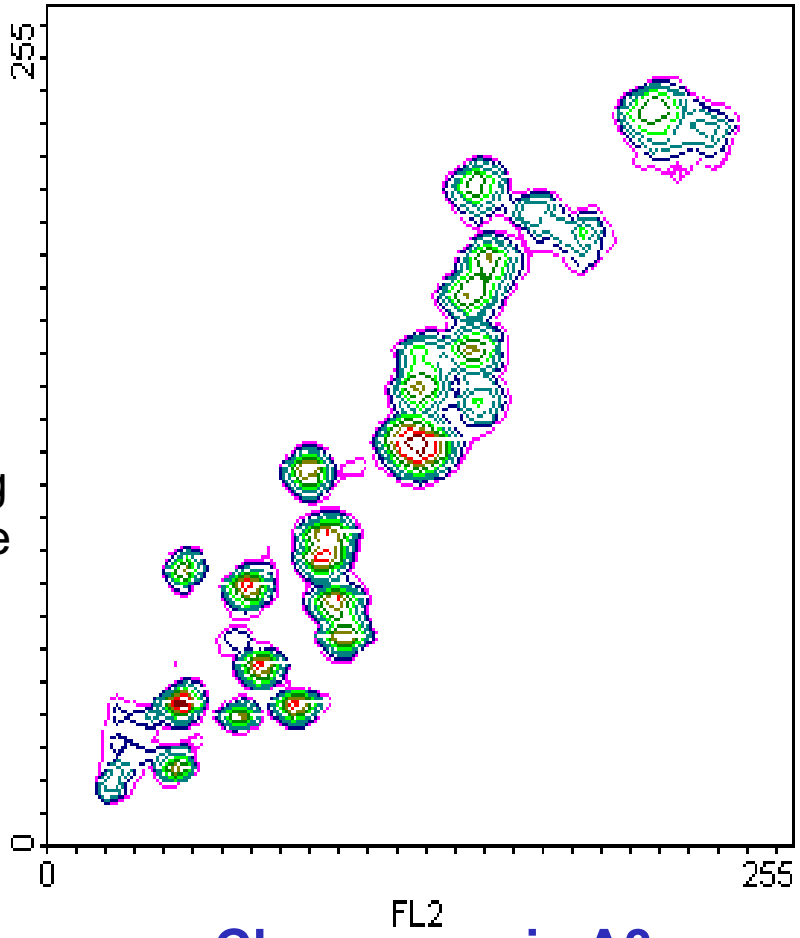
Isotope	Isotope Source	Marker	Antibody Clone	Antibody Source	viSNE Generation*
89Y	Fluidigm Sciences	CD45	HI30	Fluidigm Sciences	
112Cd		CD3 Qdot605	UCHT1	Thermofisher	
112Cd		CD14 Qdot605	Tük4	Thermofisher	
112Cd		CD19 Qdot605	SJ25-C1	Thermofisher	
112Cd		HLA-DR Qdot605	Tü36	Thermofisher	
115In	Sigma	CD57	HCD57	BioLegend	x
141Pr	Fluidigm Sciences	KIR2DS4	FES172	Beckman Coulter	x
142Nd	Fluidigm Sciences	CD103	Ber-ACT8	BioLegend	x
143Nd	Fluidigm Sciences	CD117	104D2	Fluidigm Sciences	x
144Nd	Fluidigm Sciences	CD69	FN50	Fluidigm Sciences	x
146Nd	Fluidigm Sciences	Granzyme B	CLB-GB11	Novus	x
147Sm	Fluidigm Sciences	MIP1β	D21-1351	BioLegend	
148Nd	Fluidigm Sciences	NKp30	P30-15	BioLegend	x
149Sm	Fluidigm Sciences	KIR2DL2/L3/S2	GL183	Beckman Coulter	x
150Nd	Fluidigm Sciences	IL-22	22URTI	Fluidigm Sciences	
151Eu	Fluidigm Sciences	CD107a	H4A3	Fluidigm Sciences	
152Sm	Fluidigm Sciences	Eomes	WD1928	eBioscience	x
153Eu	Fluidigm Sciences	MIP1α	1.2_3E8-2H6-2B6	Peprotech	
154Sm	Fluidigm Sciences	CD96	NK92.39	BioLegend	x
155Gd	Fluidigm Sciences	CD56	B159	Fluidigm Sciences	x
156Gd	Fluidigm Sciences	LILRB1	GHI/75	Fluidigm Sciences	x
157Gd	Trace Sciences	NKG2C	134591	R&D Systems	x
158Gd	Fluidigm Sciences	IFN-γ	B27	Fluidigm Sciences	
159Tb	Fluidigm Sciences	GM-CSF	BVD2-21C11	Fluidigm Sciences	
160Gd	Fluidigm Sciences	NKp44	P44-8	BioLegend	x
161Dy	Fluidigm Sciences	Tbet	4B10	Fluidigm Sciences	x
162Dy	Fluidigm Sciences	NKp46	BAB281	Fluidigm Sciences	x
163Dy	Fluidigm Sciences	CD49a	TS2/7	Fluidigm Sciences	x
164Dy	Fluidigm Sciences	CD161	HP-3G10	Fluidigm Sciences	x
165Ho	Fluidigm Sciences	CD127	A019D5	Fluidigm Sciences	x
166Er	Fluidigm Sciences	NKG2D	ON72	Fluidigm Sciences	x
167Er	Fluidigm Sciences	KIR3DL1	DX9	Fluidigm Sciences	x
169Tm	Fluidigm Sciences	NKG2A	Z199	Fluidigm Sciences	x
170Er	Fluidigm Sciences	XCL1	109001	R&D Systems	
171Yb	Fluidigm Sciences	DNAM-1	DX11	Fluidigm Sciences	x
172Yb	Fluidigm Sciences	Ki-67	B56	Fluidigm Sciences	x
173Yb	Fluidigm Sciences	KIR2DL1	143211	R&D Systems	x
174Yb	Fluidigm Sciences	CD94	HP-3D9	Fluidigm Sciences	x
175Lu	Fluidigm Sciences	AhR	FF3399	eBioscience	x
176Yb	Fluidigm Sciences	KIR2DL3	180701	R&D Systems	x
209Bi	Fluidigm Sciences	CD16	3G8	Fluidigm Sciences	x

2012

Chromosome analysis/sorting

Chromosomal abnormalities are endemic to some cancers and other genetic diseases

Conventional cytogenetics is time consuming and can be difficult



Hoechst 33258
AT-binding preference

Chromomycin A3
GC-binding preference

<u>Flow karyotyping</u>
Staining intensity depends on DNA content and base composition of each chromosome

Resolves all human chromosomes as separate peaks (exception chromosome 9,10,11,12)

<u>Chromosome sorting possible</u>
Study the human genome in health and disease

Considerations when planning your experiment

- ***Sample preparation*** (e.g. enzymatic digest, fixation, permeabilisation, labelling procedure, choice of fluorophores, future use)
- ***Instrument*** (e.g. sorter or analyser?, instrument/laser setup, containment level)
- ***Data collection*** (e.g. settings/compensation, timing)
- ***Data analysis and presentation*** (e.g. analysis software, graphics)

Most FACS units have dedicated operators who will help you to plan an experiment and use the instruments!

Abbreviations

APC:	Allophycocyanin
BrdU:	5’-bromodeoxyuridine
CFSE:	Carboxyfluorescein succinimidyl ester
FITC:	fluorescein isothiocyanate
PE:	Phycoerythrin
PE-Cy5:	Phycoerythrin-cyanine5
PI:	Propidium iodide