Flow Cytometry

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Technology

- Tool
- Hypothesis

Definition

Flow cytometry is a method for quantitating components or structural features of cells, by optical means.

Requirments

Must be:

cells in suspension

well defined monoclonal antibodies

Can be:

alive or fixed

Ability

Density: 10^5-10^7 cells /ml
but upto 10^8

Speed: 50,000 events/sec
typically 1,000

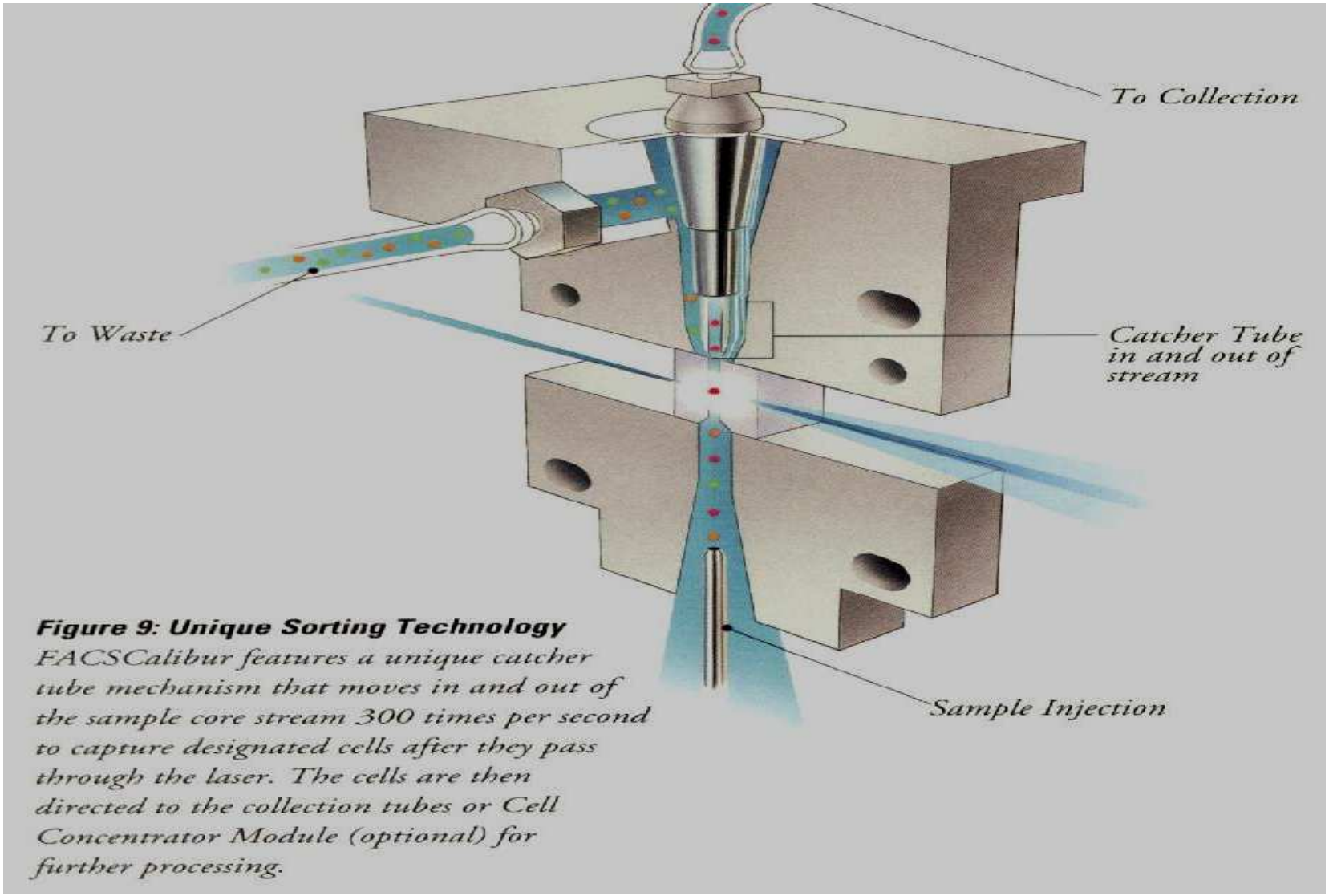
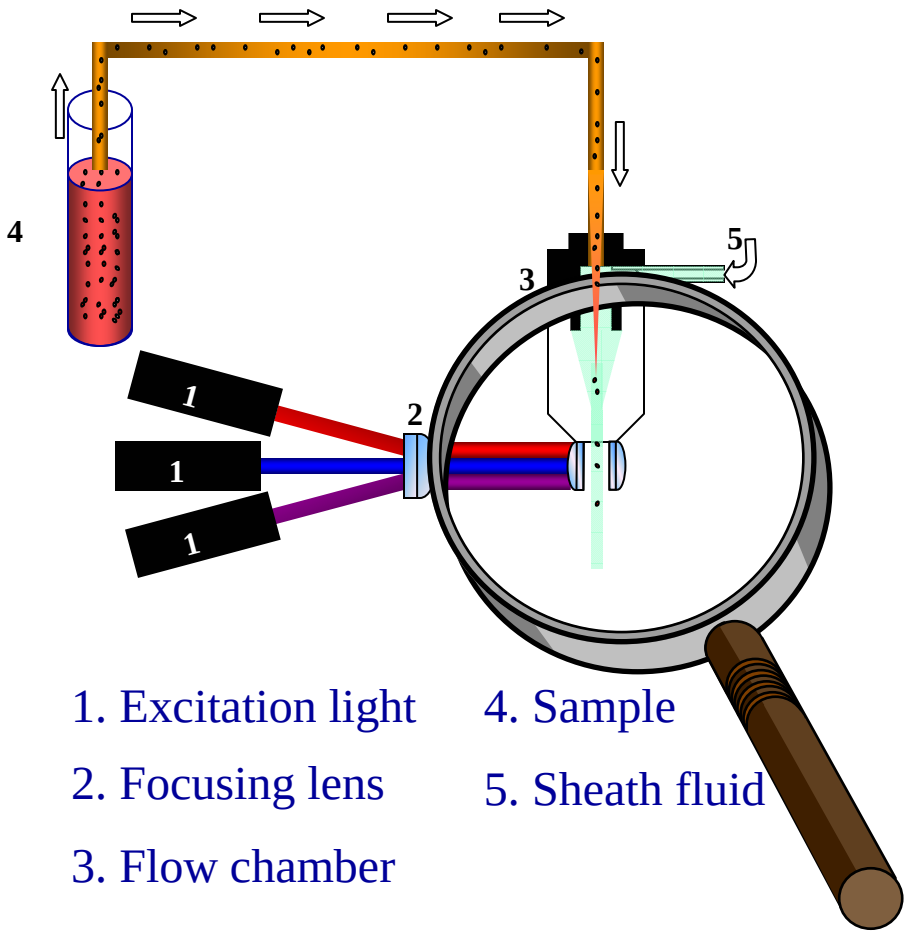


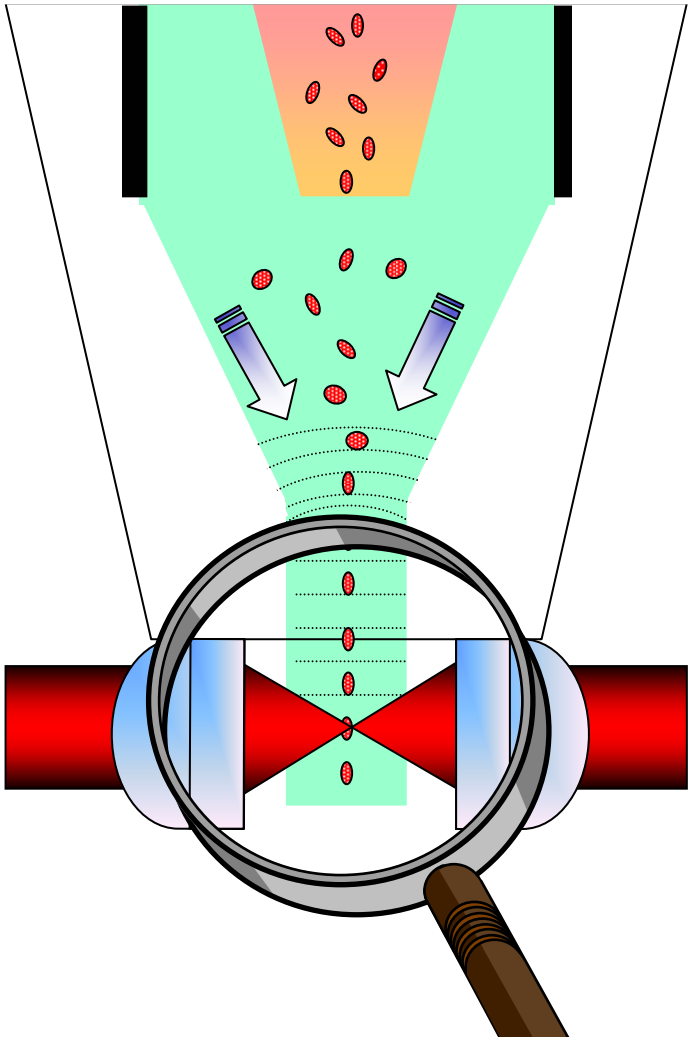
Figure 9: Unique Sorting Technology
FACSCalibur features a unique catcher tube mechanism that moves in and out of the sample core stream 300 times per second to capture designated cells after they pass through the laser. The cells are then directed to the collection tubes or Cell Concentrator Module (optional) for further processing.

Parameters

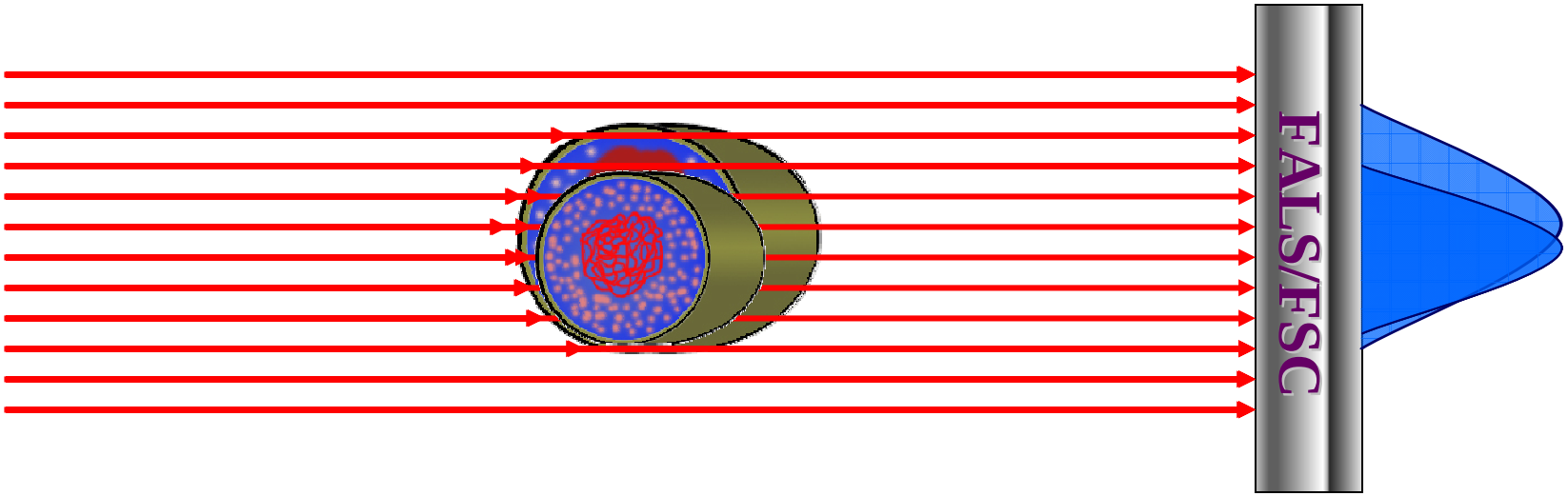
- Low angle forward scatter intensity
  - Cell diameter
- 90 degree scatter intensity
  - Granularity/density
- Fluoresence intensity
  - Several wavelenghts



1. Excitation light
2. Focusing lens
3. Flow chamber
4. Sample
5. Sheath fluid



Forward Angle Light Scatter
FALS/FSC

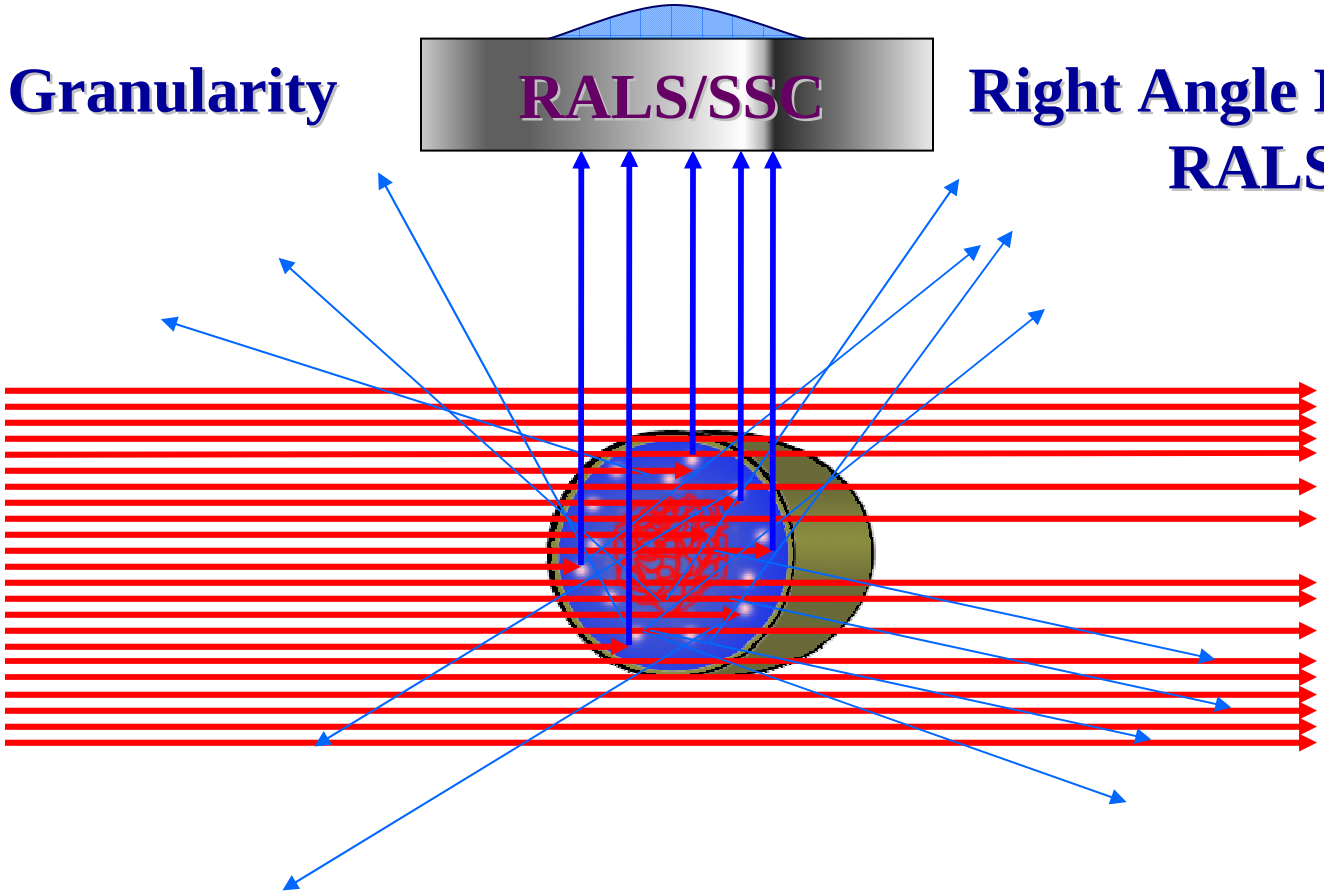


Size

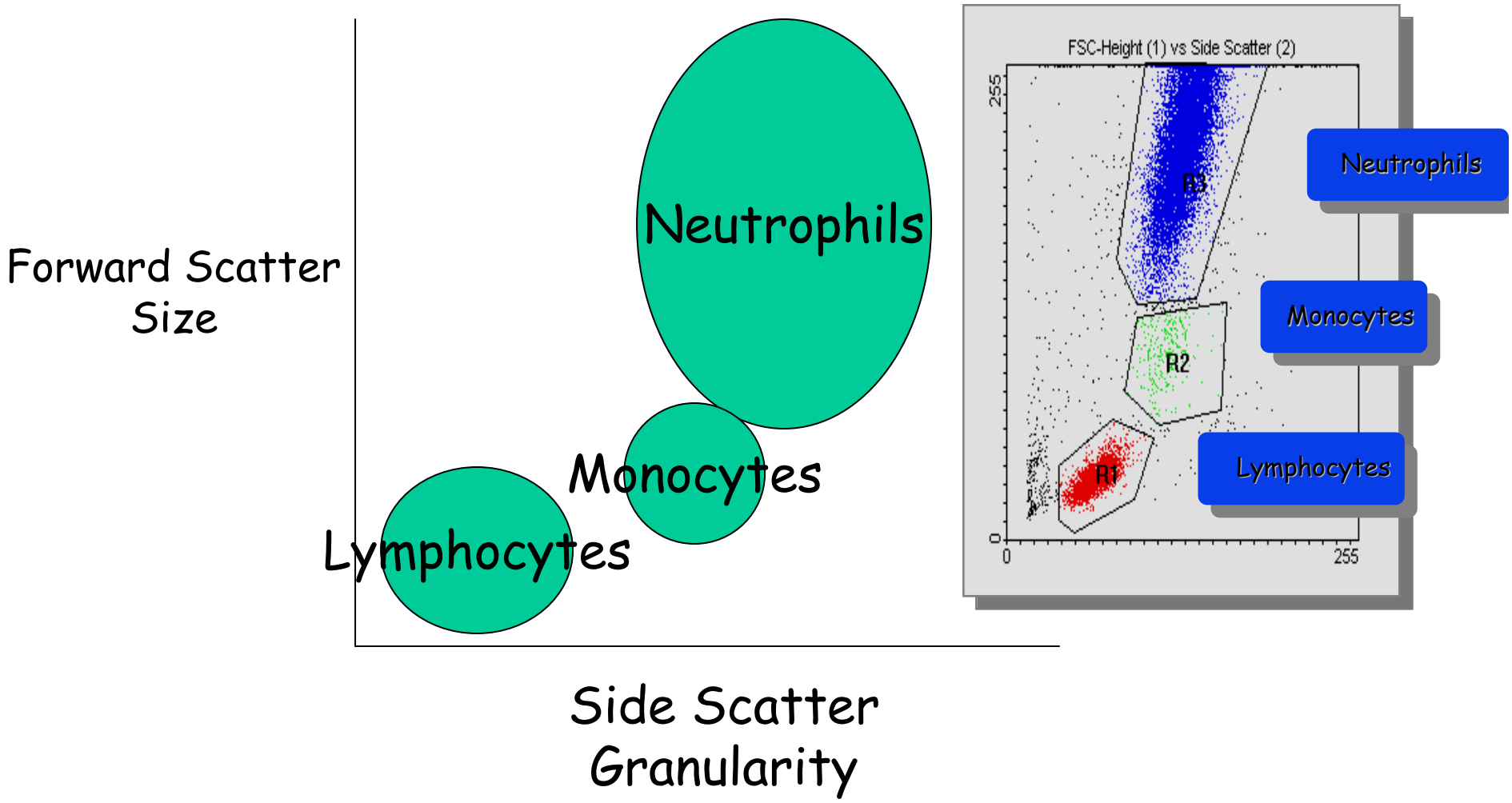
Granularity

RALS/SSC

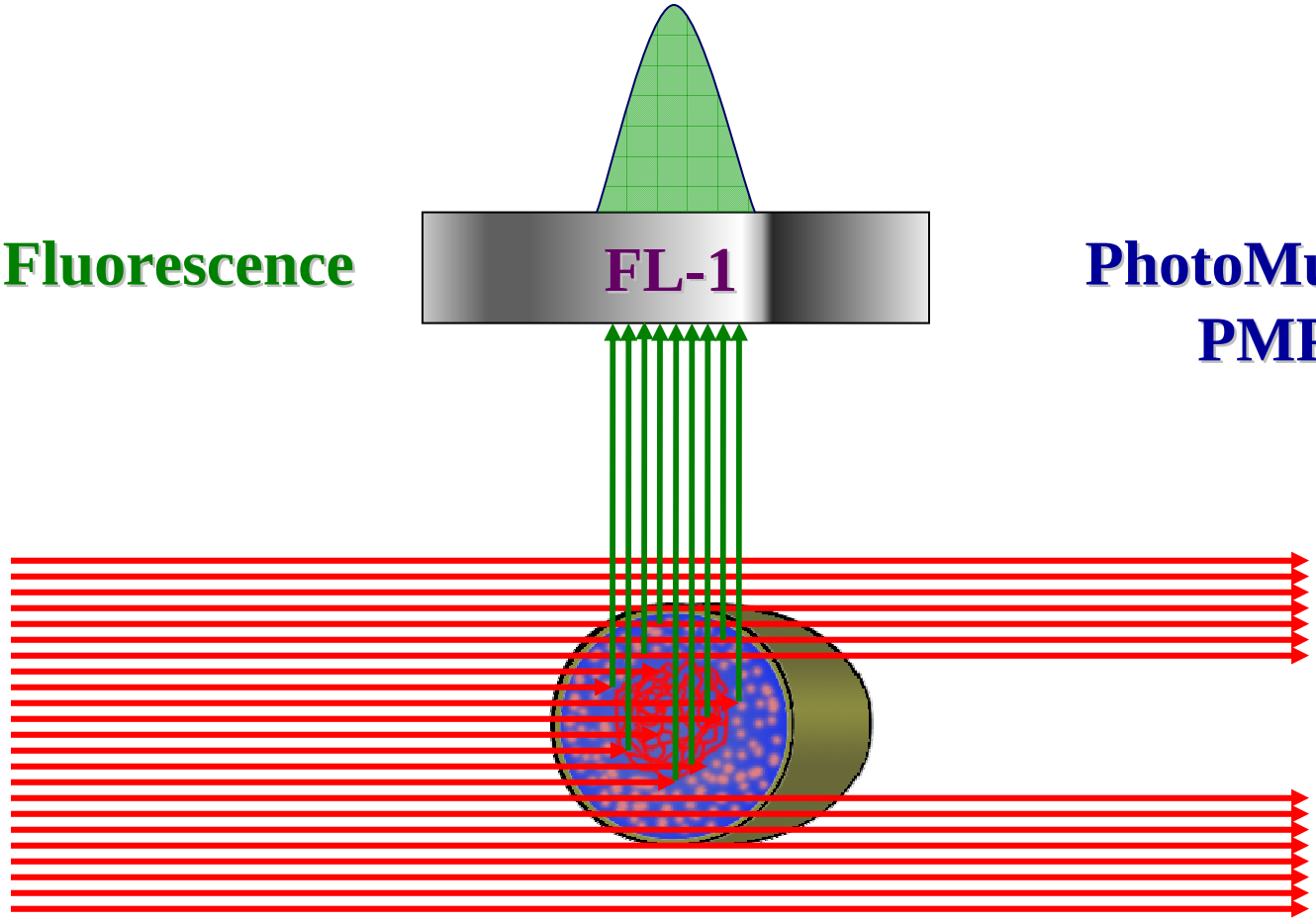
Right Angle Light Scatter
RALS/SSC



Size/Granularity

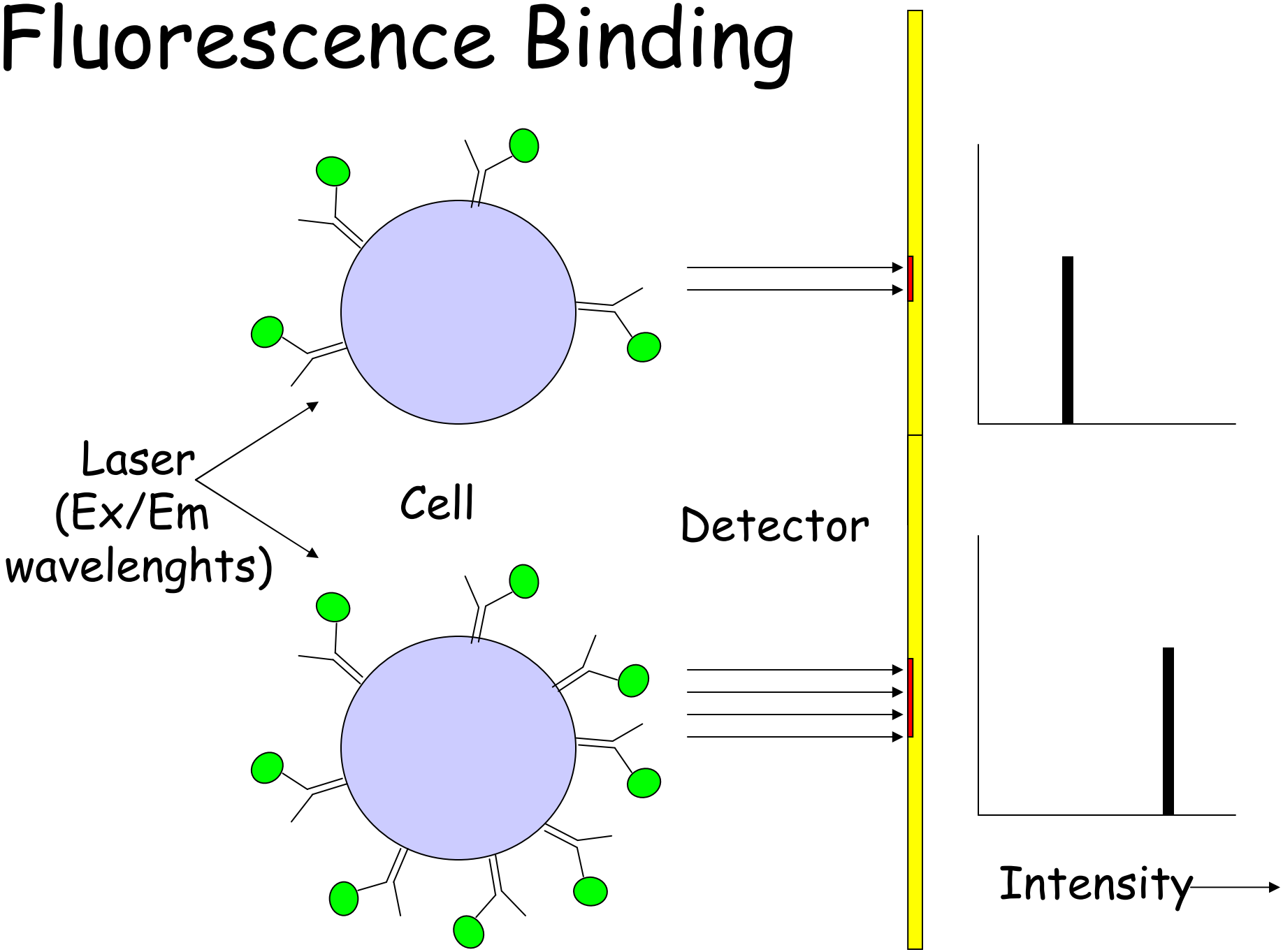


Fluorescence

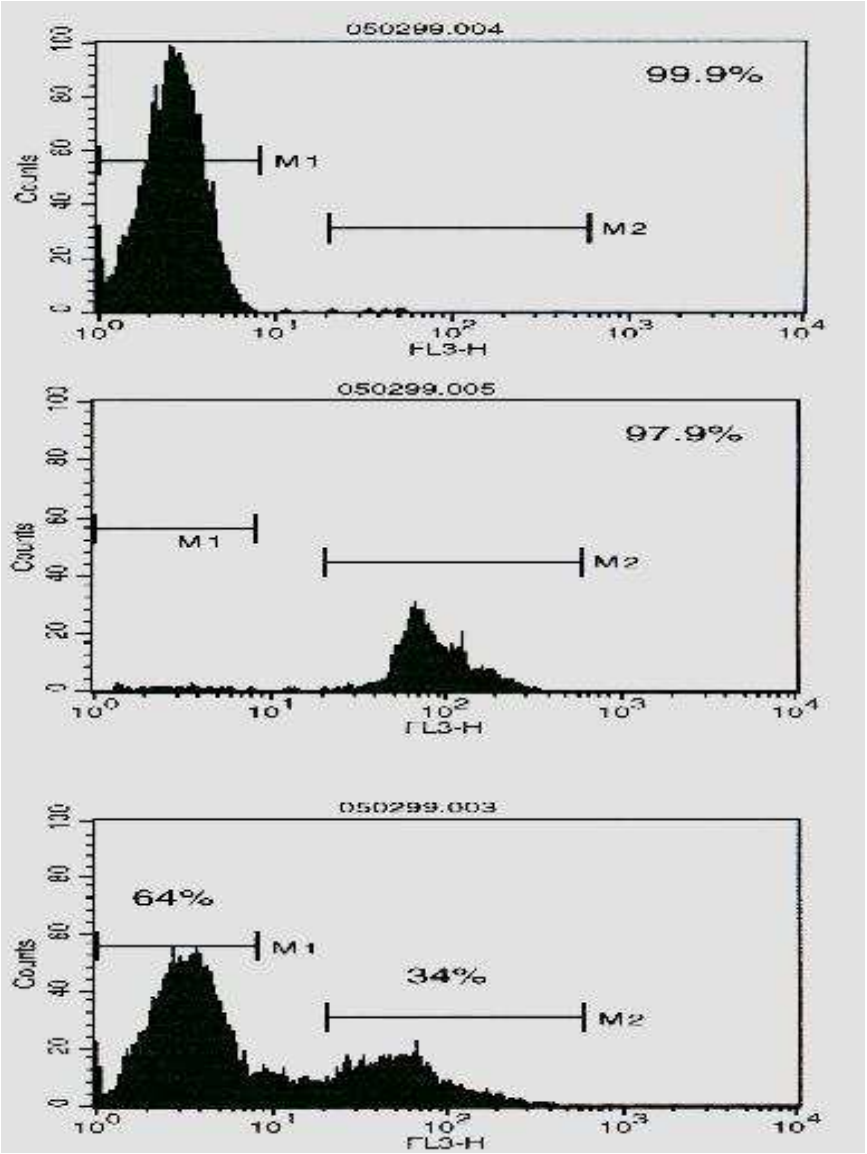


PhotoMultipliers
PMP/FL

Fluorescence Binding



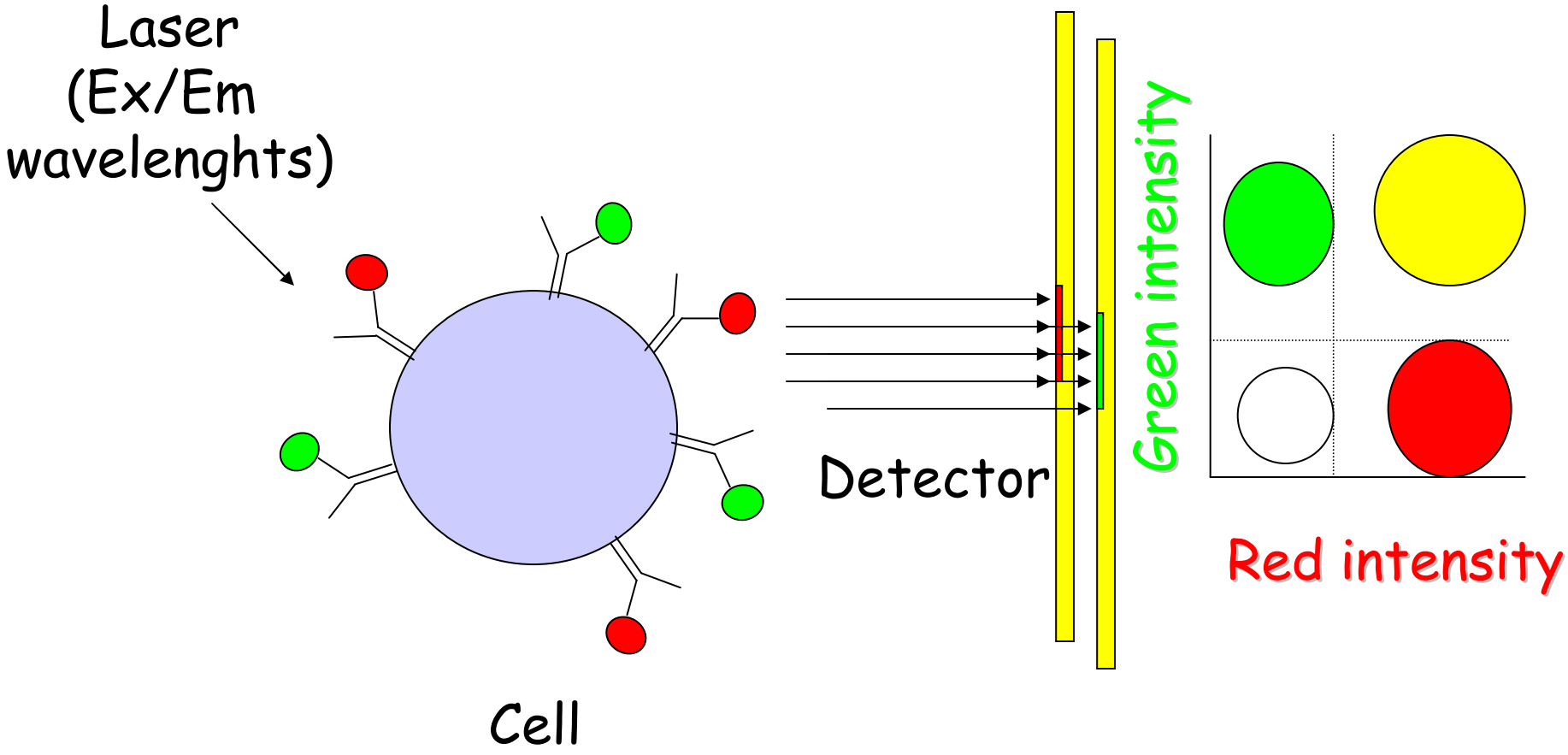
Cell Numbers



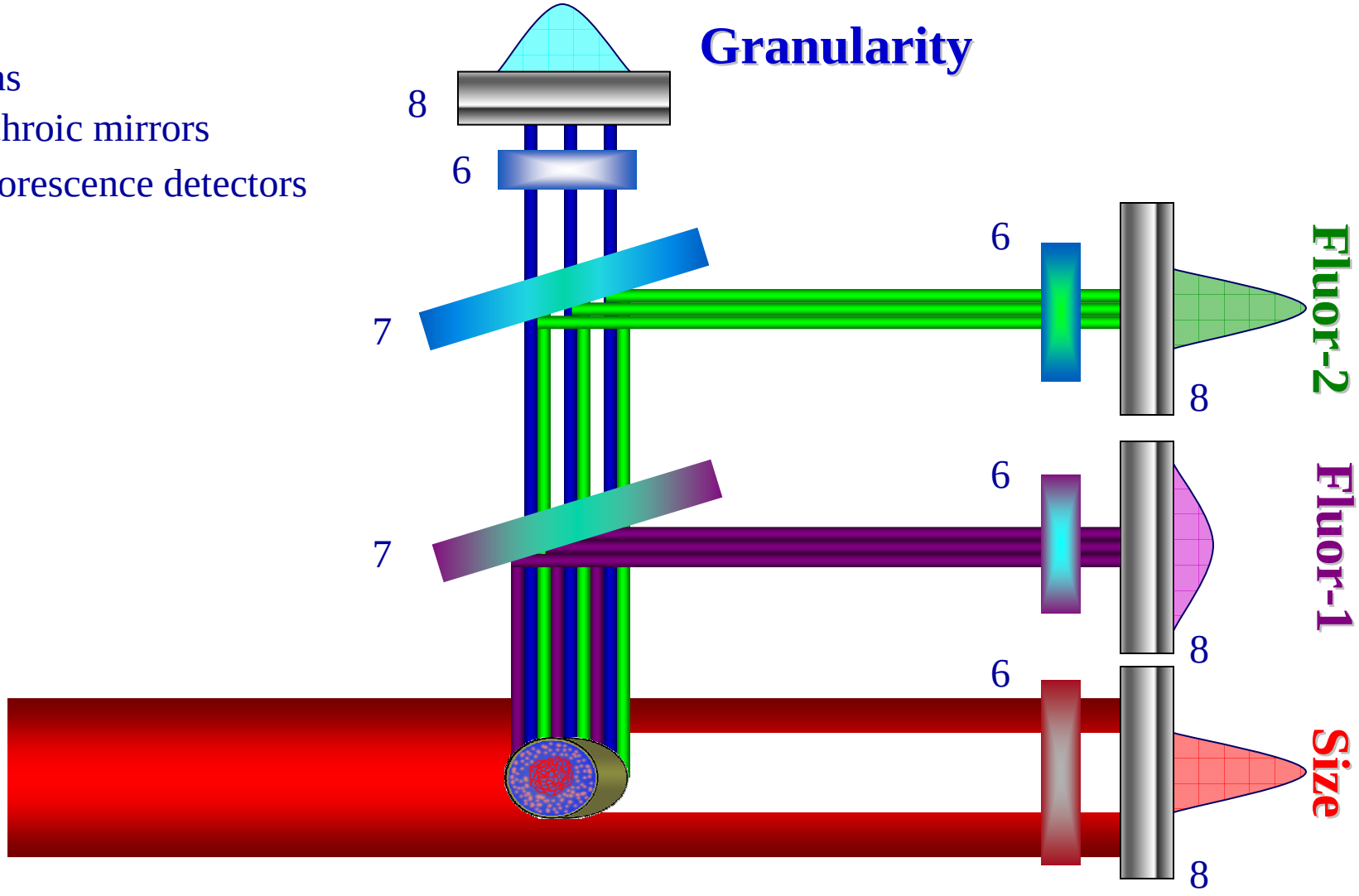
IgG Control

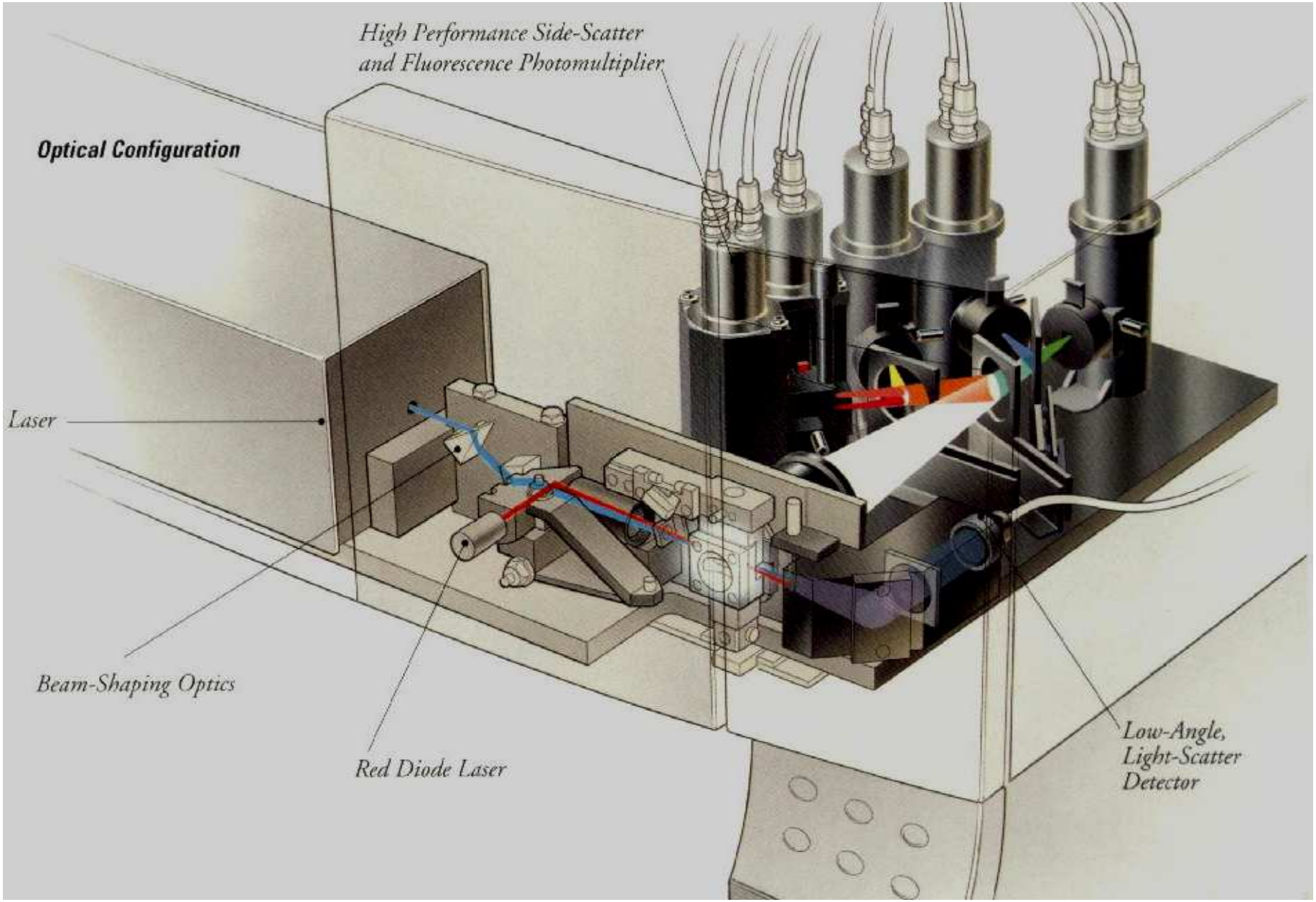
Intensity ⟶

Fluorescence Binding



6. Lens
7. Dichroic mirrors
8. Fluorescence detectors




Optical Configuration
High Performance Side-Scatter
and Fluorescence Photomultiplier
Laser
Beam-Shaping Optics
Red Diode Laser
Low-Angle,
Light-Scatter
Detector

Wavelenghts and Application

Several laser types

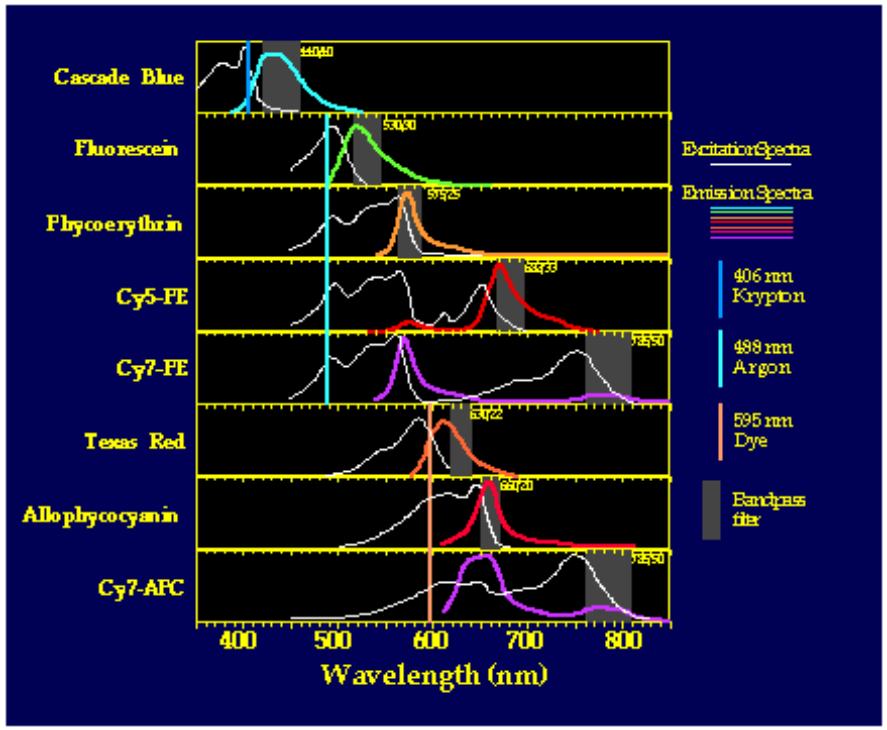
- "differ in gain medium that is used to amplify light"
- Gas (Helium-Neon, Argon, Helium-Cadmium)
- Solid state (NdYAG)

Fluorochrome used dependent on

- Application
- Excitiation
- Detector

Dyes

- Fluorescein (FITC)
- Phycoerythrin (PE)
- Cy5PE
- Cy7PE
- Texas Red (TR)
- Allophycocyanin (APC)
- Cy5
- Cy7APC
- Cascade Blue



FLUOROCHROME	EX.	EM.	APPLICATION
Indo-1 (unbound)	335	490	Calcium Flux
Indo-1 (Bound to Calcium)	335	405	Calcium Flux
Hoechst 33342	350	470	DNA analysis
DAPI	359	462	DNA analysis
Alexa350	350	442	Phenotyping
PerCP	470	670	Phenotyping
R-Phycoerythrin	480	578	Phenotyping
Green Fluorescent Protein (GFP)	488	510	Reporter molecule
YO-PRO-1	488	510	Apoptosis analysis
Fluorescein diacetate	488	530	Cell viability
Alexa488	488	530	Phenotyping
Sytox Green	488	530	DNA analysis
SNARF-1	488	530-640	pH measurement
Fluo-3	488	530	Calcium flux
dsRED	488	588	Reporter molecule
PE-Cy5 (TriColor, Cychrome)	488	670	Phenotyping
PE-Cy7	488	770	Phenotyping
Propidium Iodide	495	637	DNA analysis
Rhodamine 123	515	525	Membrane potential
Yellow Fluorescent Protein (YFP)	519	534	Reporter molecule
LDS-751	543	712	Nucleated cell detection
7-Aminoactinomycin D	546	655	DNA analysis
Alexa 546	546	573	Phenotyping
Cy3	550	565	Phenotyping
CMXRos (Mitotracker Red)	560	610	Mitochondrial membrane potential
Texas Red	596	615	Phenotyping
TO-PRO-3	643	661	DNA analysis
Alexa 647	647	667	Phenotyping
APC-Cy7	647	774	Phenotyping
Allophycocyanin (APC)	650	660	Phenotyping

Applications

- Analysis
- Sorting

Applications

- Intrinsic:
  - Size, shape, cytoplasmic granularity, Autofluorescence and pigmentation
- Extrinsic:
  - DNA content, composition, synthesis
  - Enzyme activity…
  - Membrane structure, cytoskeletal…
  - Internal and external receptors….
  - Charge, Calcium, pH…
  - Apoptosis, Necrosis..
  - Drug kinetics…

Applications

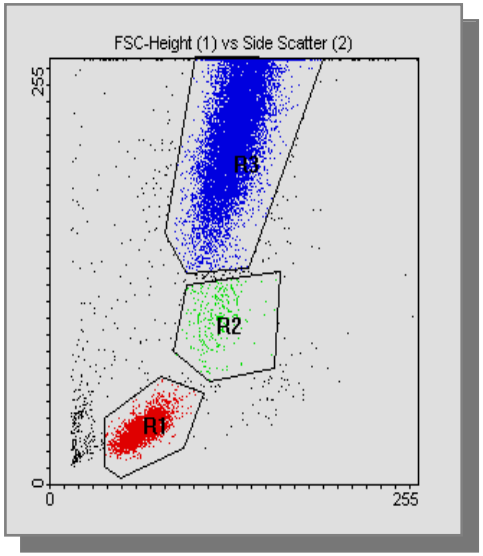
- Phenotyping – cell surface antigens
- Functional assays
  - Calcium flux (Indo-1, Fura, Fluo dyes)
  - pH (SNARF etc)
  - Apoptosis (Annexin V, Caspase 3, Propidium Iodide)
  - Cell cycle (PI, BrdU, Cyclins, CDKs, CDKIs)
  - Mitochondrial membrane potential (DiOC6)
- Cytokine production
  - Intracellular & secreted (multiplex CBA)
- Intracellular signalling
  - Phosphorylation status
  - Gene/Reporter molecule analysis
  - Living Color™ expression

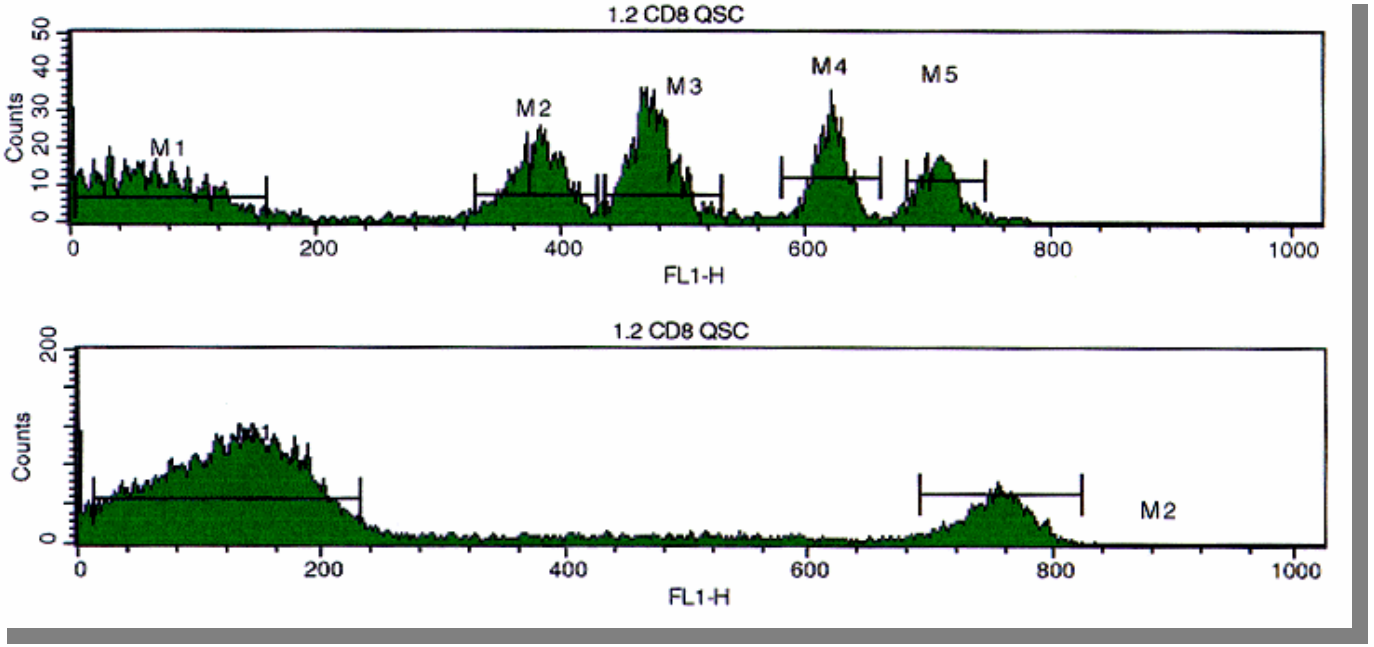
Multiple Colours

- Advantages:-
  - Save time, reagents and samples
  - Exponential increase in information
  - Identify new/rare populations (<0.05%)

- Problems:-
  - Care in the choice of fluorochrome combinations
  - Greater potential problems with compensation

Using Immunofluorescence

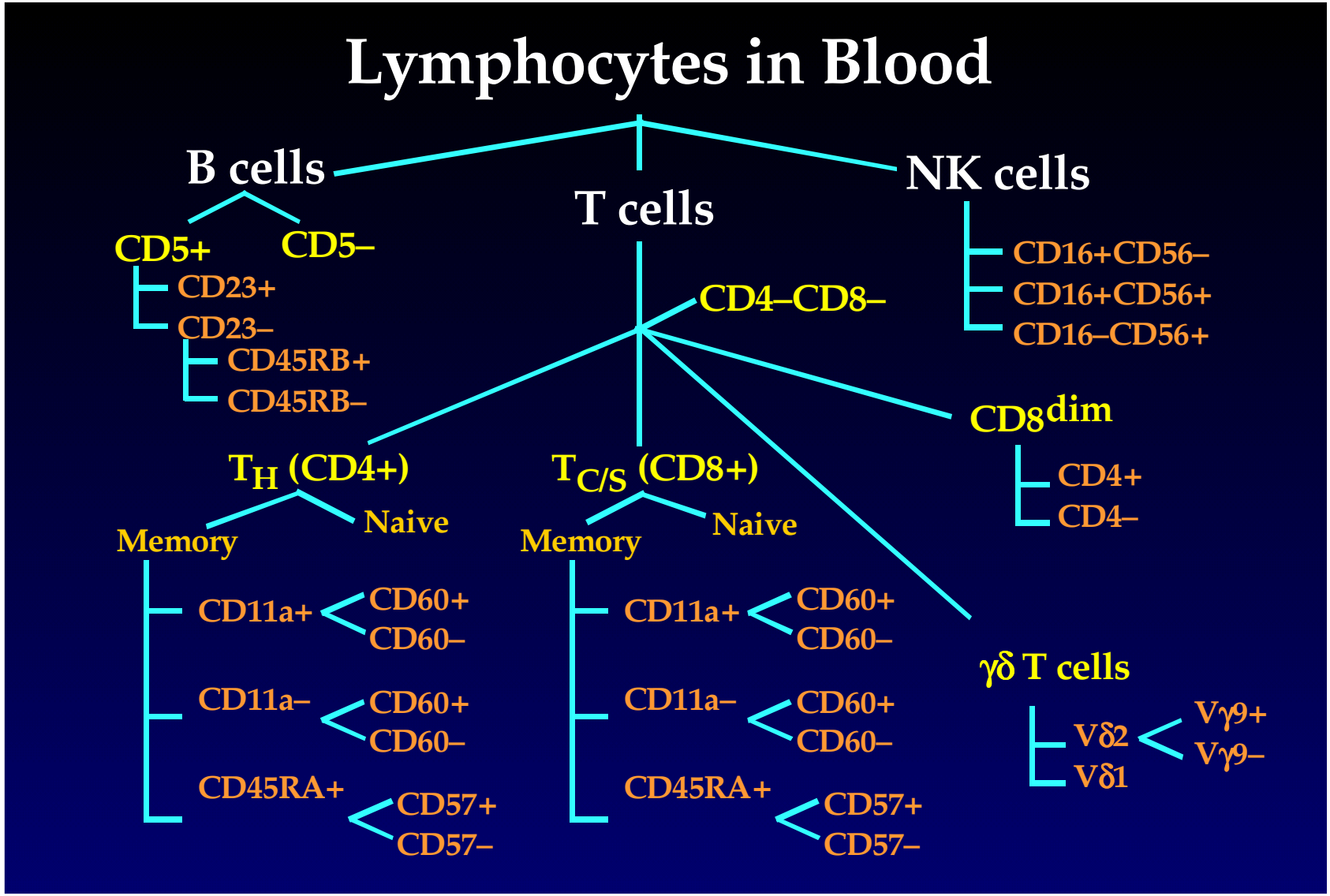




CD4+

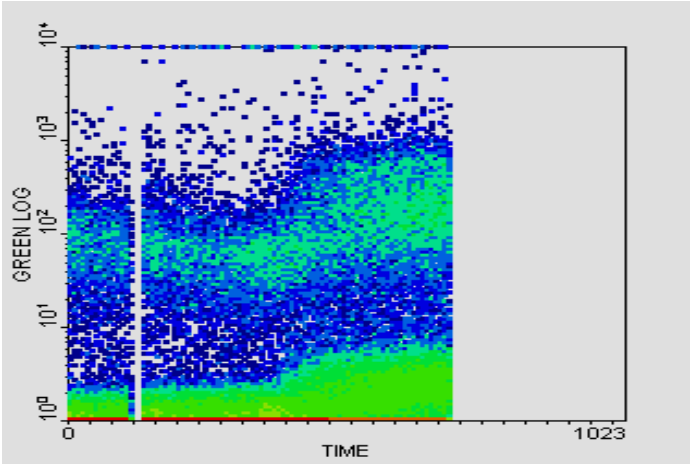
CD8+

T Cell Subsets

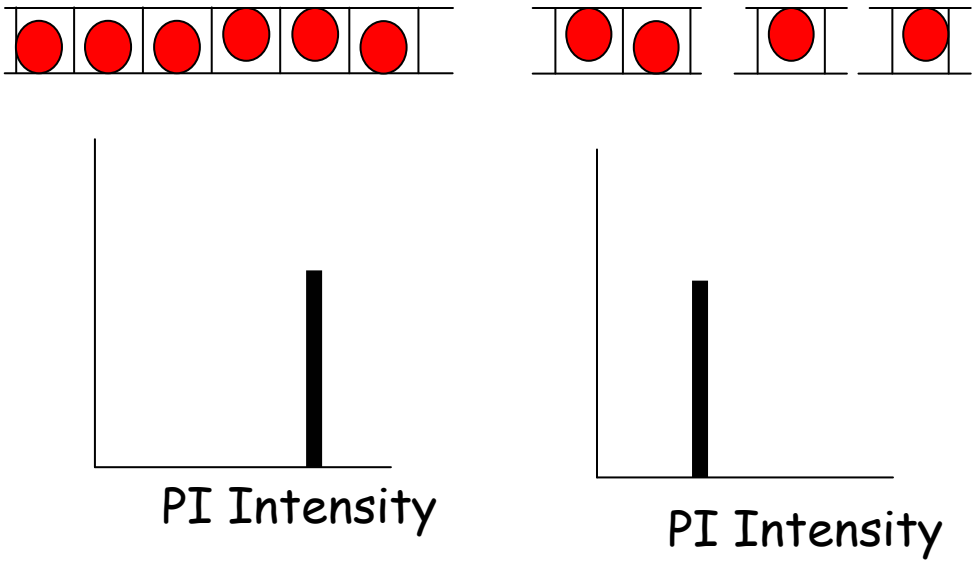


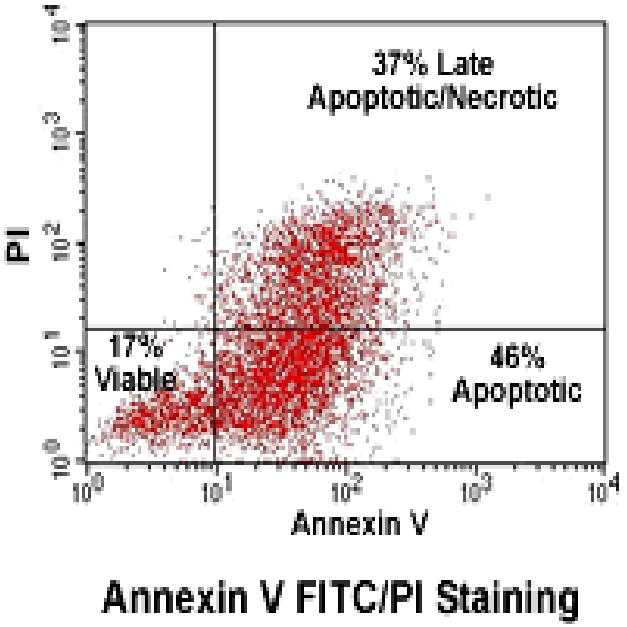
Calcium Flux

- Calcium measurements use Indo-1 (UV), Fura Red & Fluo-3 (488nm)

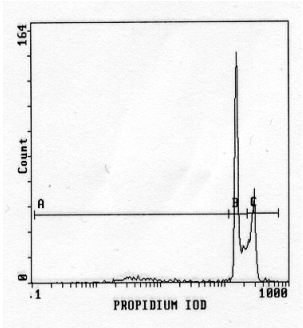


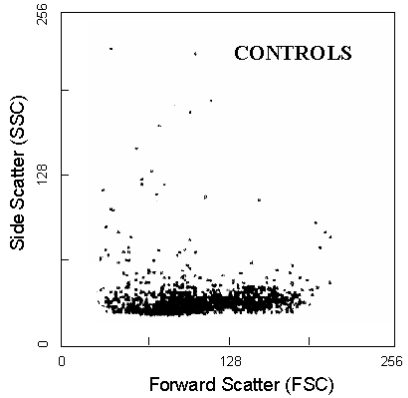
Apoptosis

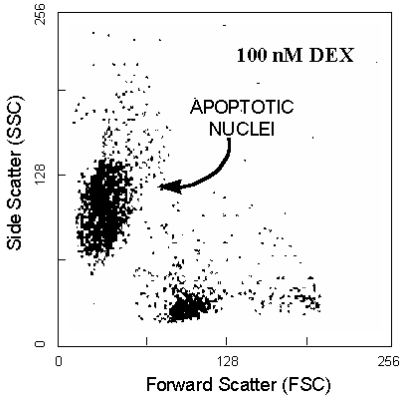




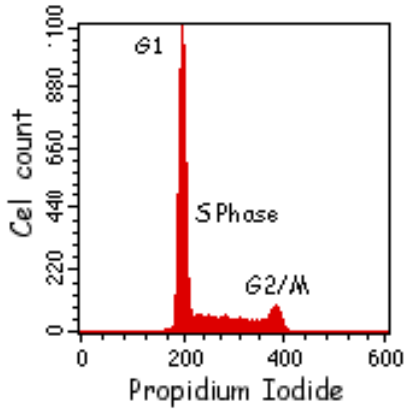
Annexin V FITC/PI Staining





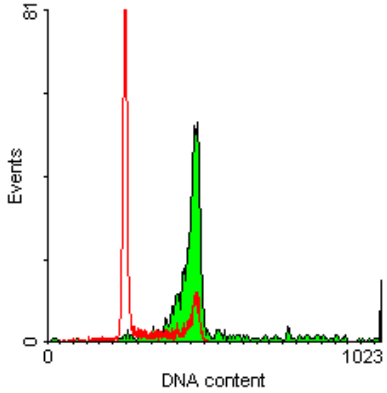


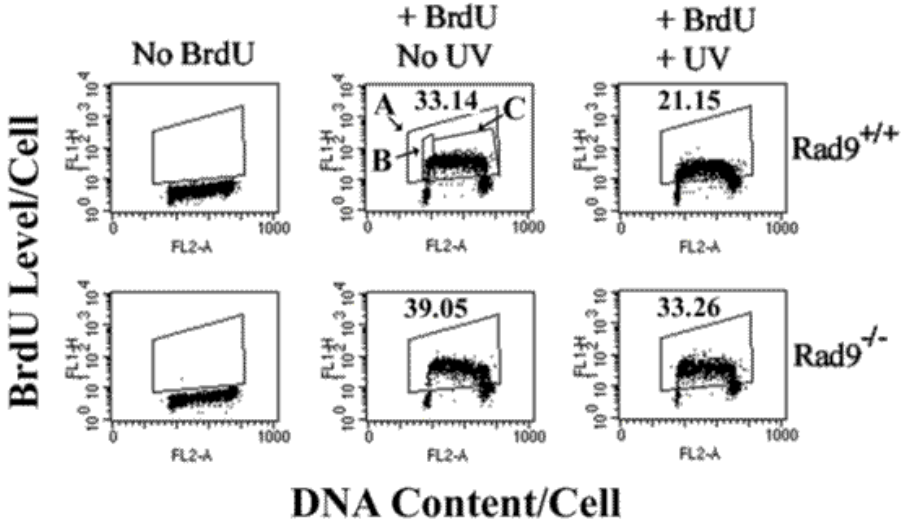
Cell Cycle



There are a number of dyes that can be used. In general, for quantitaive DNA analysis, cells are fixed in ethanol. After this we can use a number of DNA-binding dyes, e.g.:

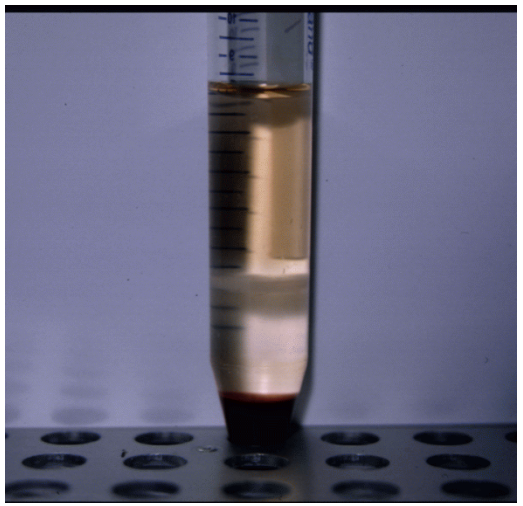
- Hoeschst 33342
- DAPI
- Mithramicin
- Propidium Iodide
- 7 Aminoactonomycin D
- DRAQ5
- TO-PRO-3

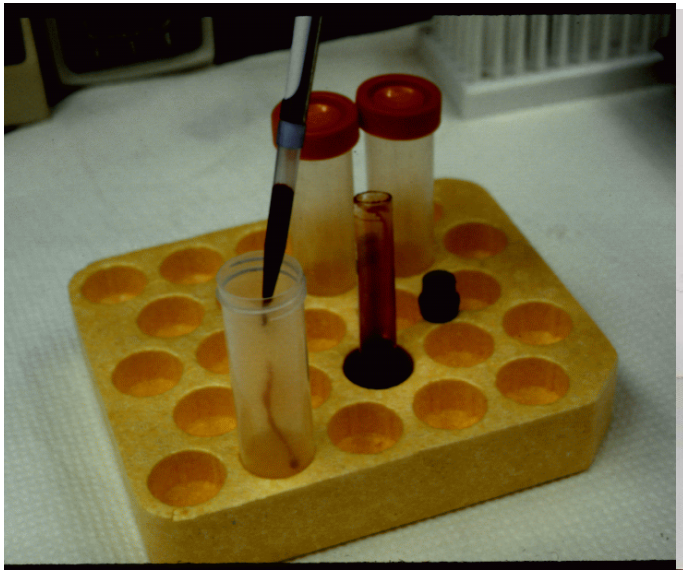


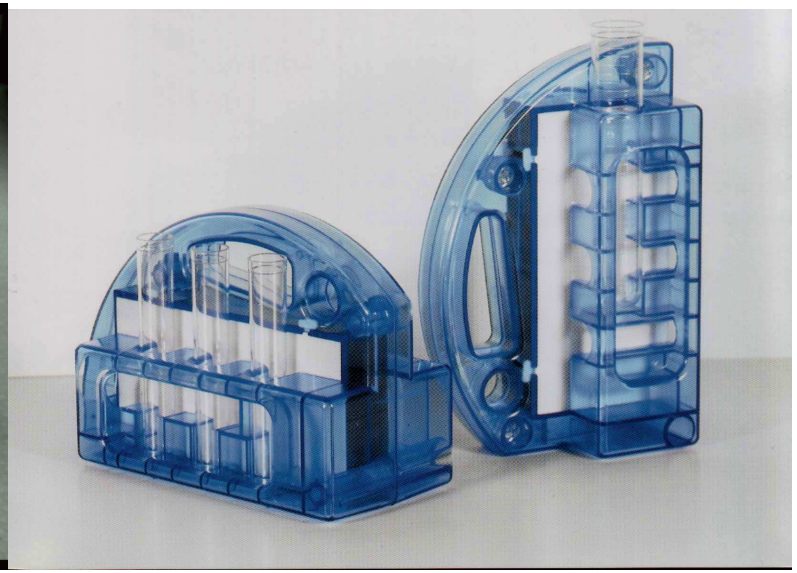


Cell Sorting

- Density gradient separation
- Magnetic bead separation
- Panning/solid phase separation
- Limiting dilution culture techniques
- Microscope based manipulations



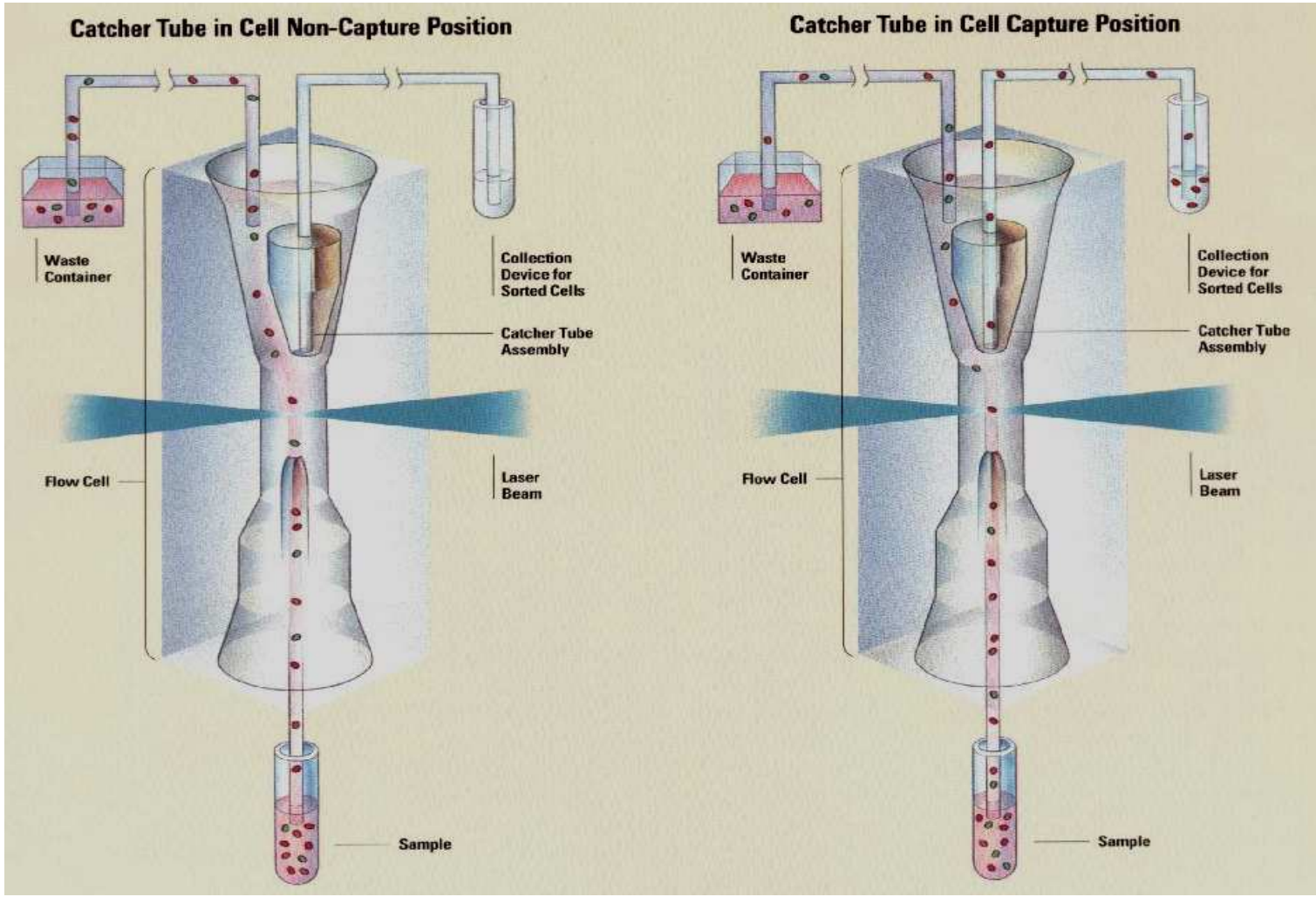


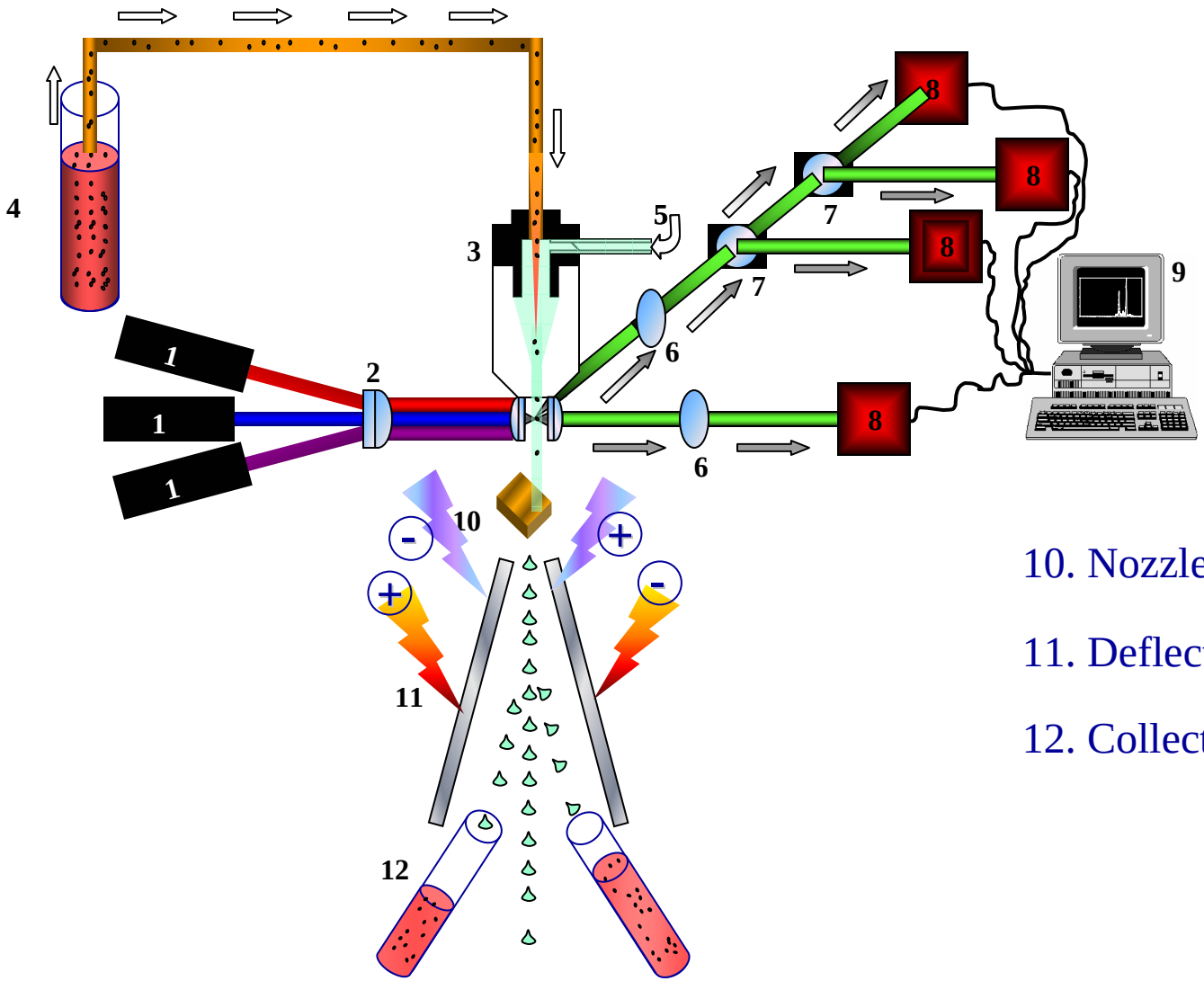


Sort Modes

- **enrichment** (a rapid sort to increase the relative frequency of the desired population, whilst not generating a high purity sort)
- **normal** (the mode used for most cell sorts, balancing speed with a good recovery and high purity)
- **counter/CloneCyte** (used to generate the highest purity possible but with greatly reduced yield as many sorted events will be aborted)

Catcher Principle

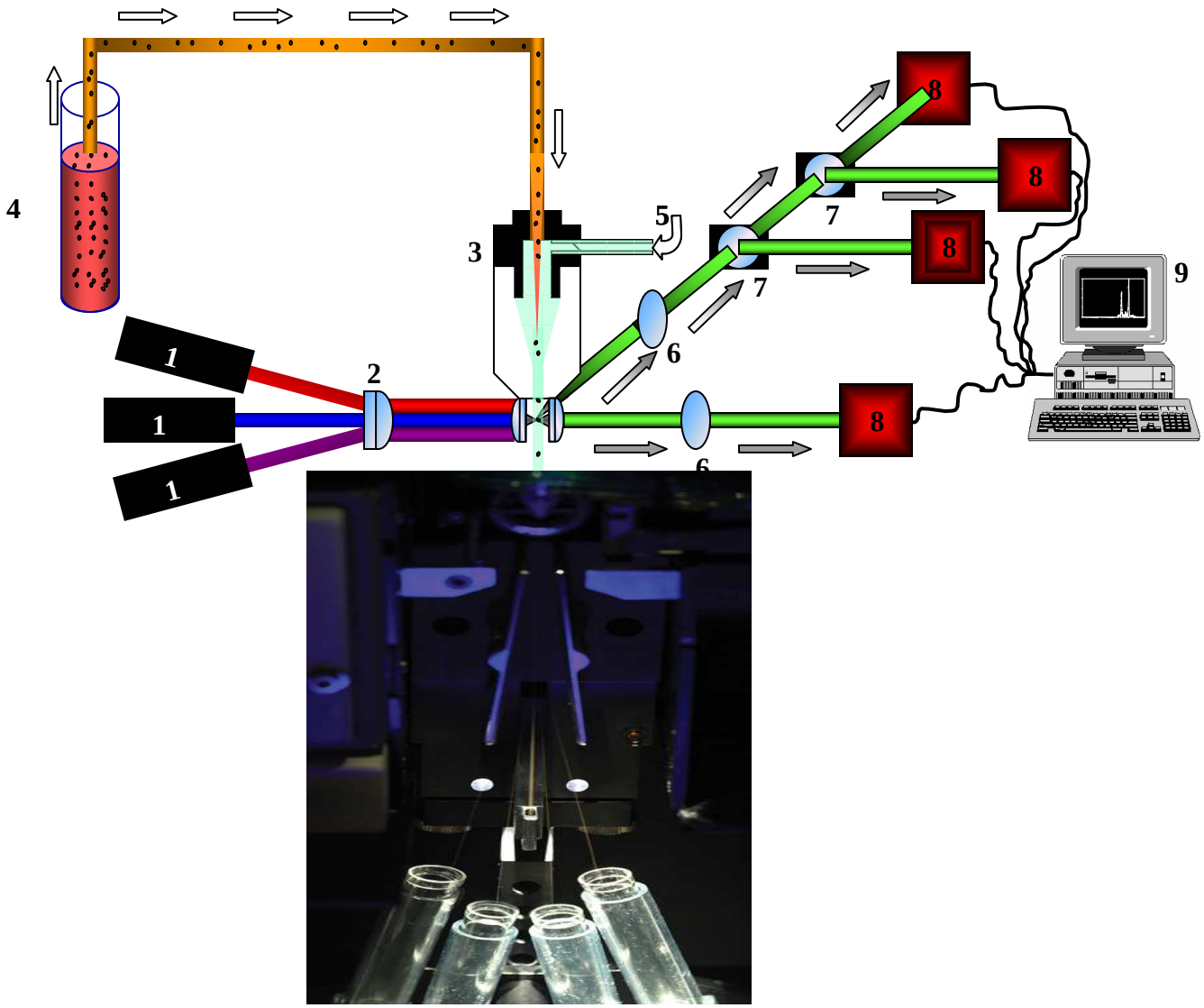




10. Nozzle

11. Deflection Plates

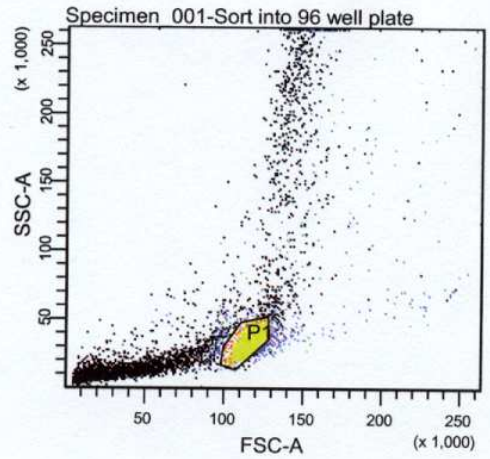
12. Collection Tubes

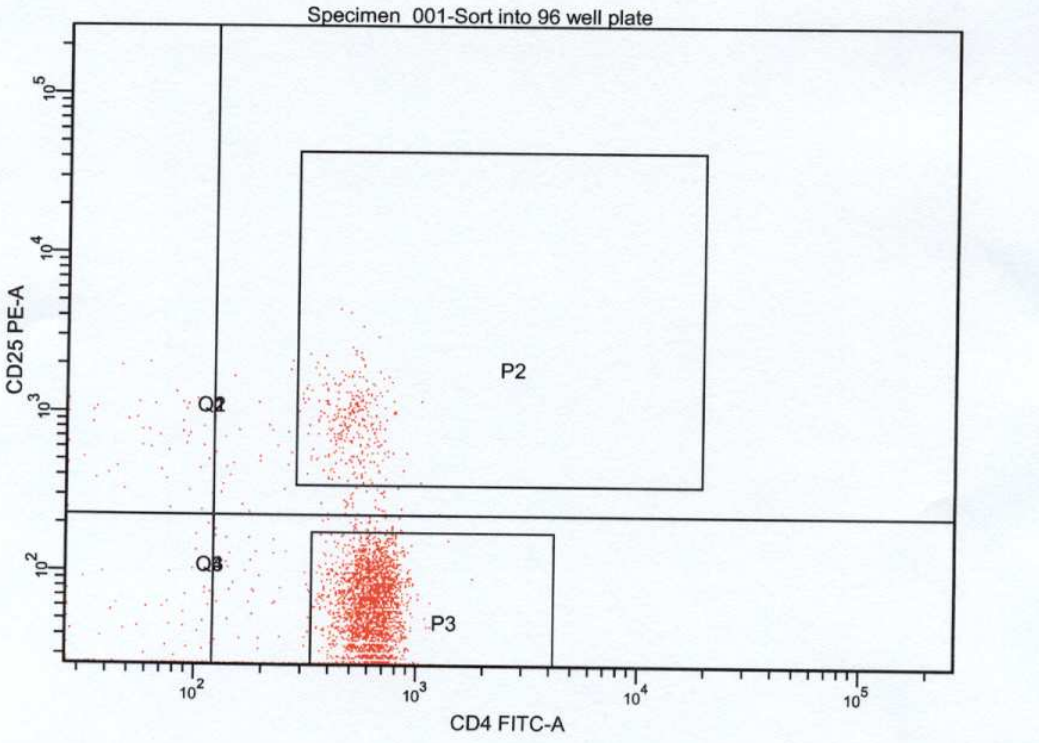

4
3
5
1
1
1
2
6
7
8
8
8
7
6
8
9

Electrostatic vs Mechanical

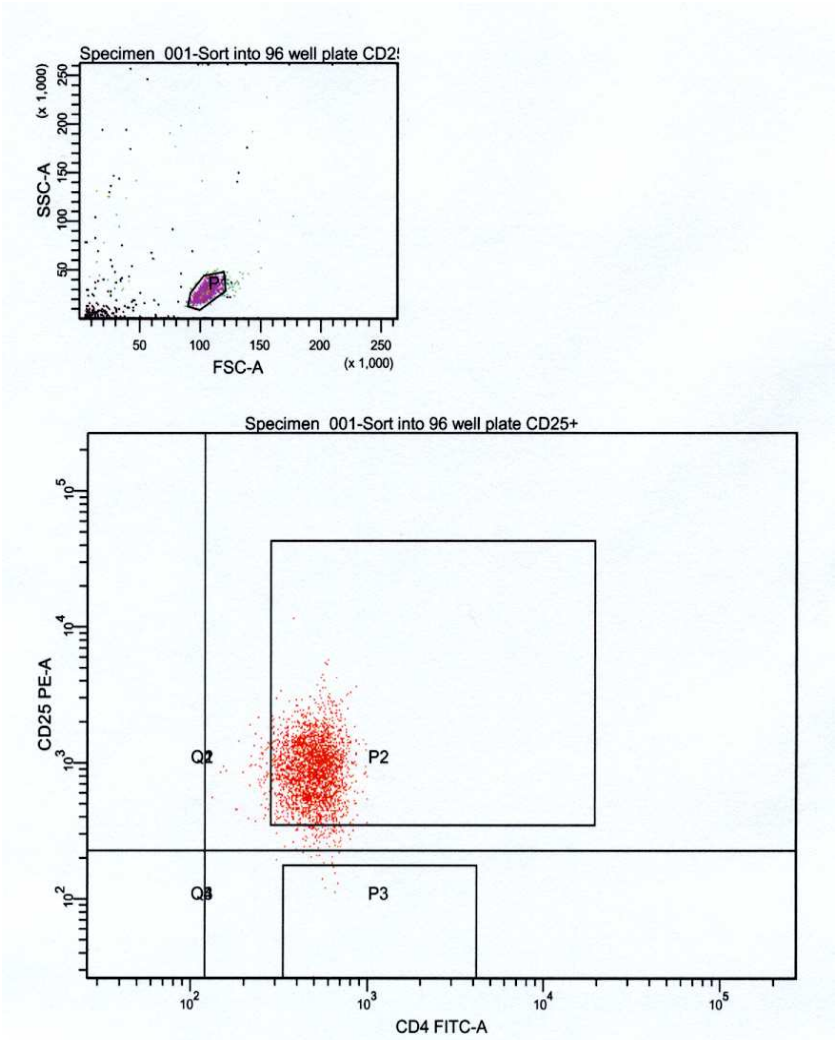
100,000/sec	300/sec
Aerosols	fully contained
Nozzle prone to blocking	no Nozzle
Two-four sorts	only one sort

Sort into tubes, slides,
multiple plates, cloning (single cell)

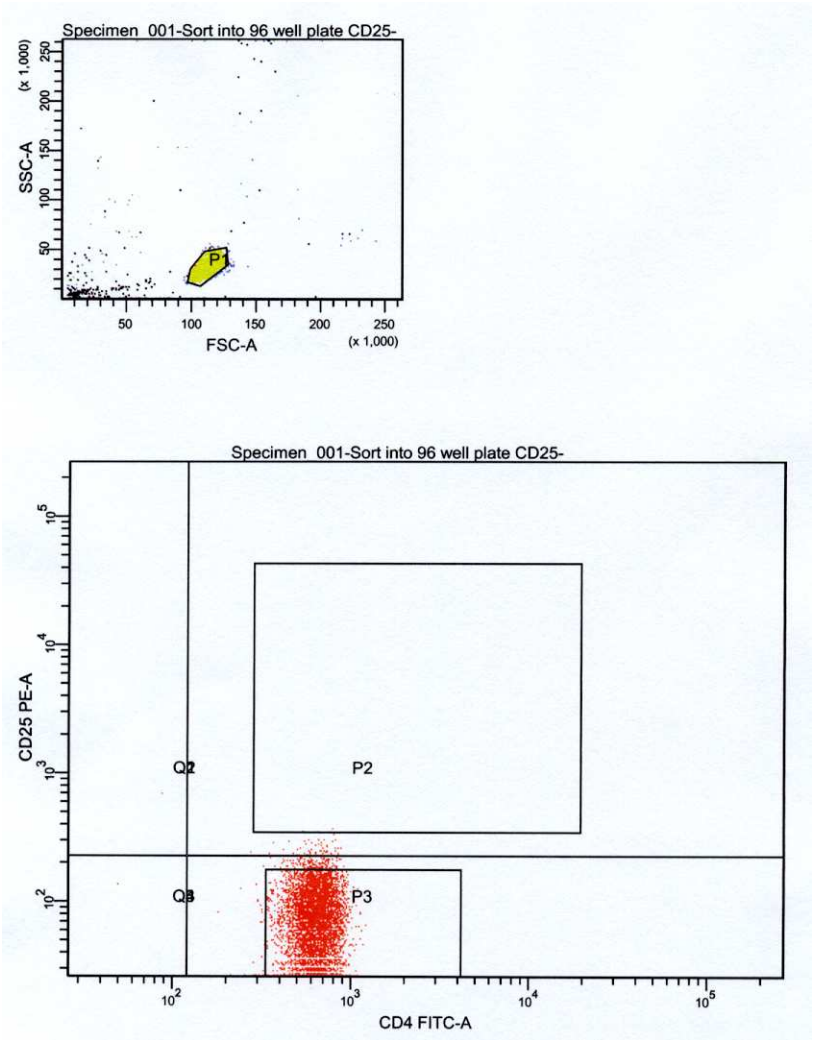

Specimen_001-Sort into 96 well plate
SSC-A
(x 1,000)
250
200
150
100
50
P1
50
100
150
200
250
FSC-A
(x 1,000)


Specimen_001-Sort into 96 well plate
CD25 PE-A
10^5
10^4
10^3
10^2
Q2
Q3
P2
P3
10^2
10^3
10^4
10^5
CD4 FITC-A

P2


Specimen 001-Sort into 96 well plate CD25+
SSC-A
(x 1,000)
FSC-A
(x 1,000)
P2
Specimen 001-Sort into 96 well plate CD25+
CD25 PE-A
CD4 FITC-A
Q2
Q3
P2
P3

P3


Specimen 001-Sort into 96 well plate CD25-
SSC-A
(x 1,000)
FSC-A
(x 1,000)
P1
Specimen 001-Sort into 96 well plate CD25-
CD25 PE-A
CD4 FITC-A
Q2
Q3
P2
P3

Sorting

Purity

Recovery/Yield

Viability

All dependent on speed

Flow Cytometry Core Facility
Conway Institute

BD FACSAria Flow Cytometer

Coulter Epics XL-MCL
Flow Cytometer

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