Immunological Techniques

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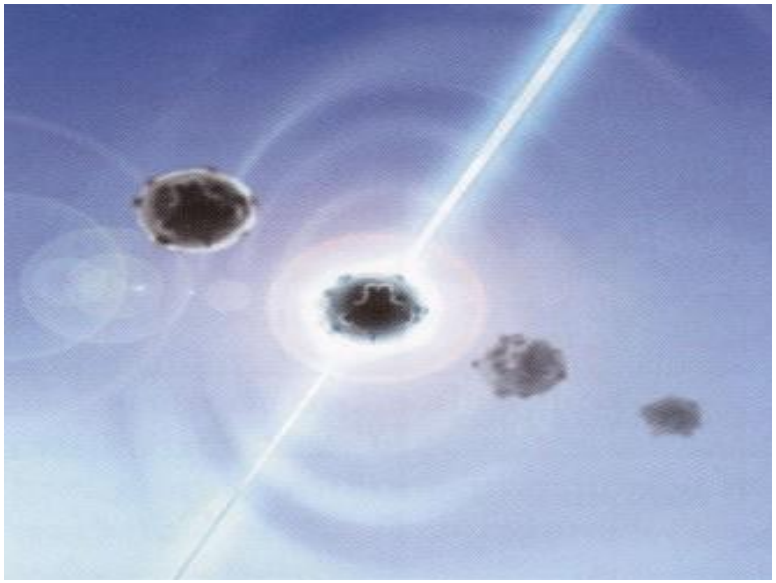
Objectives

- At the end of this lecture students should be able to understand the principles underlying common immunological techniques such as FACS and ELISA.

- The application of these techniques will be discussed



Flow cytometry is a technique for measuring physical and chemical properties of individual cells as they travel in suspension one by one past a sensing point.

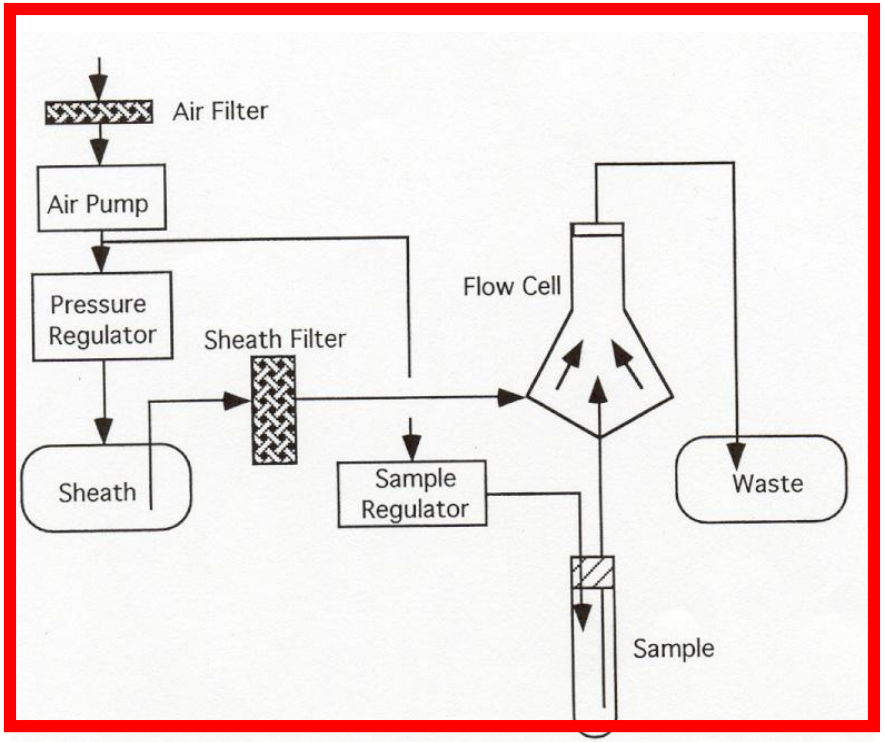
- The cells in suspension are forced to pass in a fluid stream through a flow cell.
- The fluid stream intersects the focus of a laser.
- The laser light is scattered and, if the cells are fluorescent, they produce fluorescent signals.
- These light signals are then converted to electronic signals (voltages).

INSTRUMENTATION

1. Fluidics
2. Optics
3. Electronics

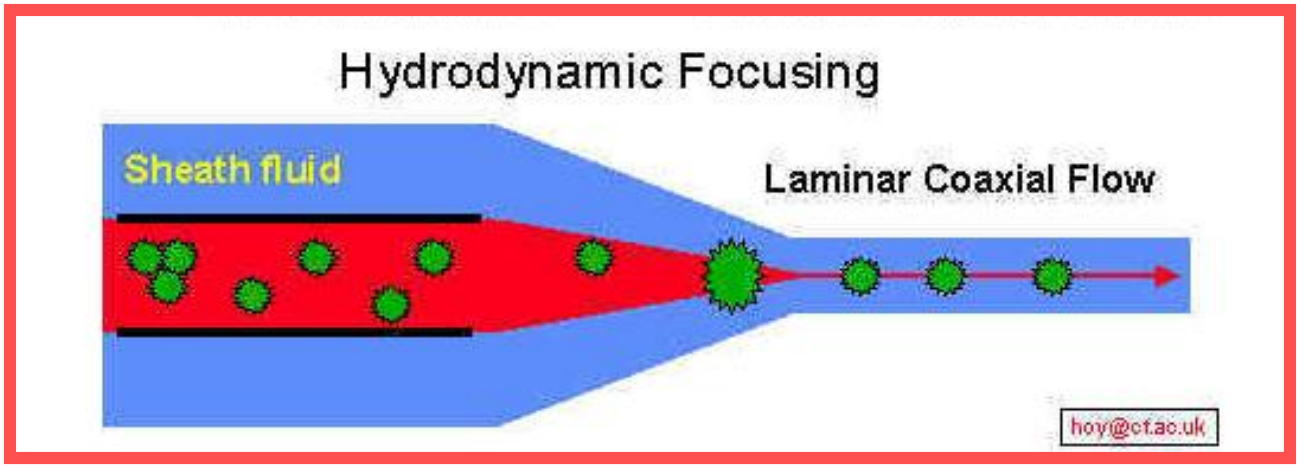
FLUIDICS

•The purpose of the fluidics system is to transport cells in a fluid stream to the laser beam for interrogation.



OPTICS

The optics system consist of a laser to illuminate the cells in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors (i.e . to resolve different colors).

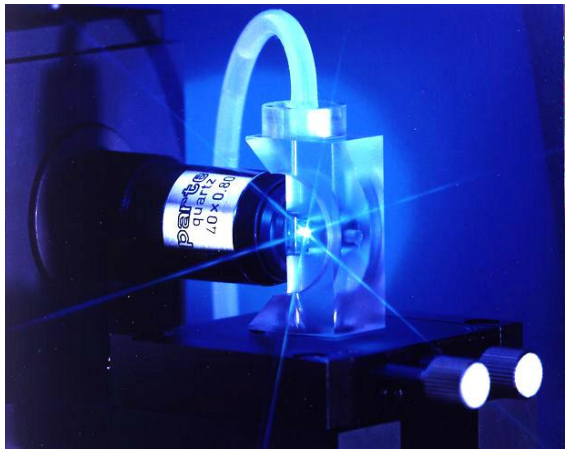


1. The flow of sheath fluid restricts the cells to the center of the sample core for optimal illumination (hydrodynamic focusing).

2. Only one cell should move through the laser beam at a given moment.

“THE LASER”

- Lasers emit coherent light, in a fine, straight beam at a specified wavelength.
- The use of a laser allows the beam of light to be focused on single cells so that basic measurements based on beam disturbance can be taken (FSC, SSC) .



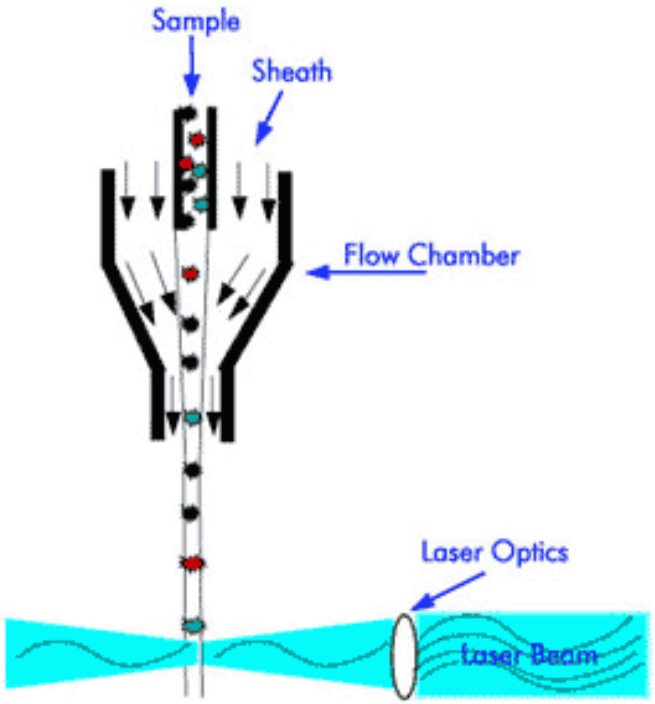
ELECTRONICS

- Processing of signals from detectors

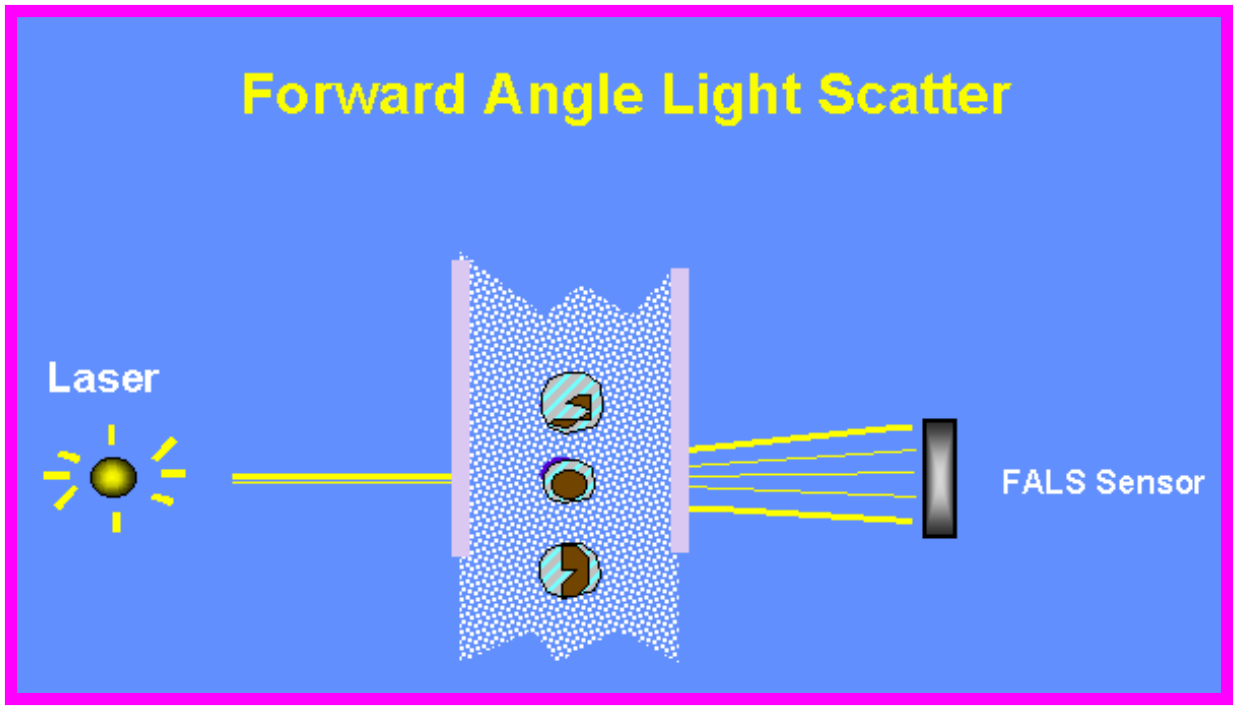
-Cells passing through the laser beam generate light signals. These light signals are then converted to electronic signals (voltages).

-The electrical voltage generated will be proportional to the number of photons (amount of light) emitted by the cell/particle.

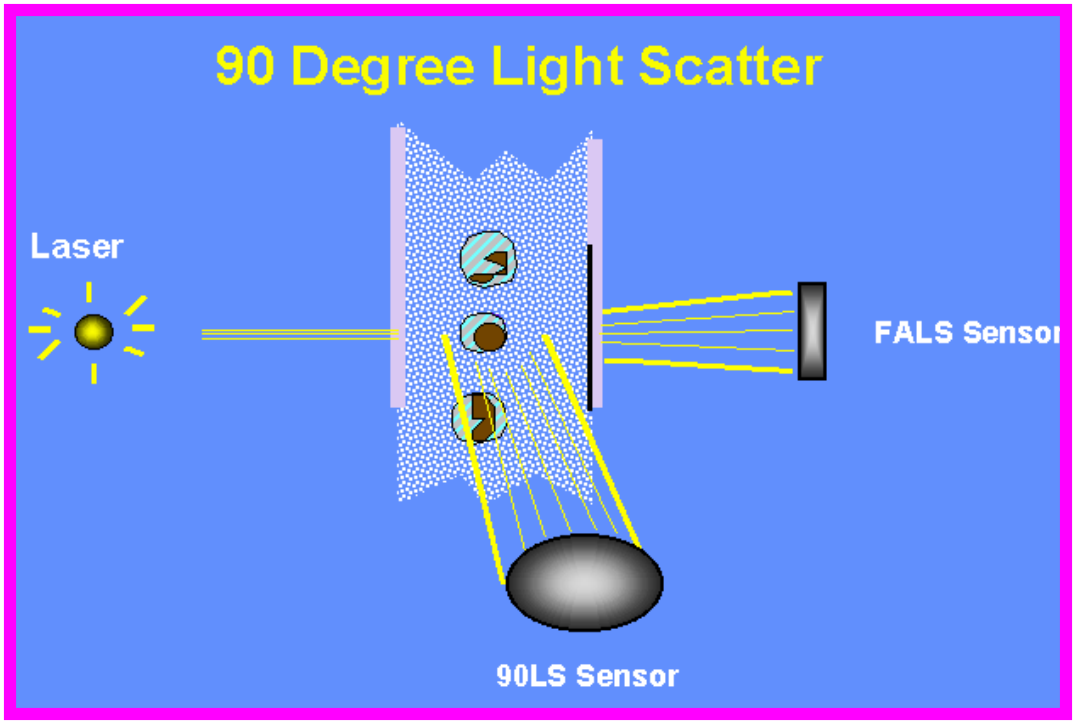
-The voltages are processed by the computer.



The sample is injected into a stream of sheath fluid within the flow chamber; the sample core remains separate but coaxial within the sheath fluid.

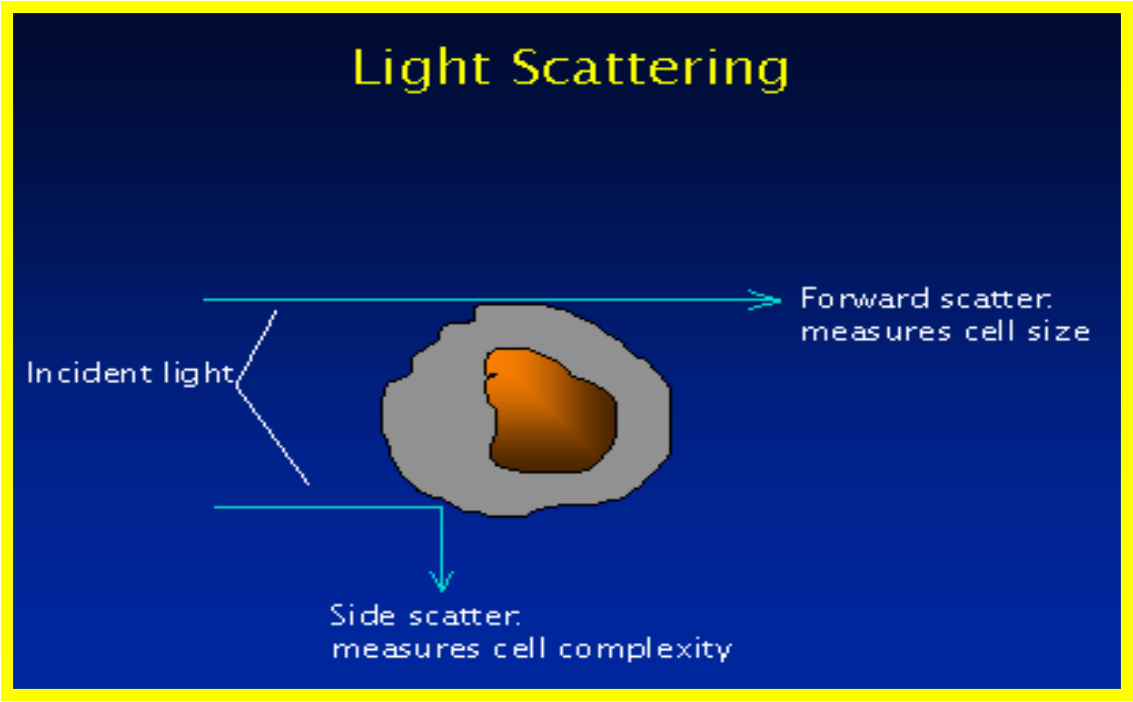


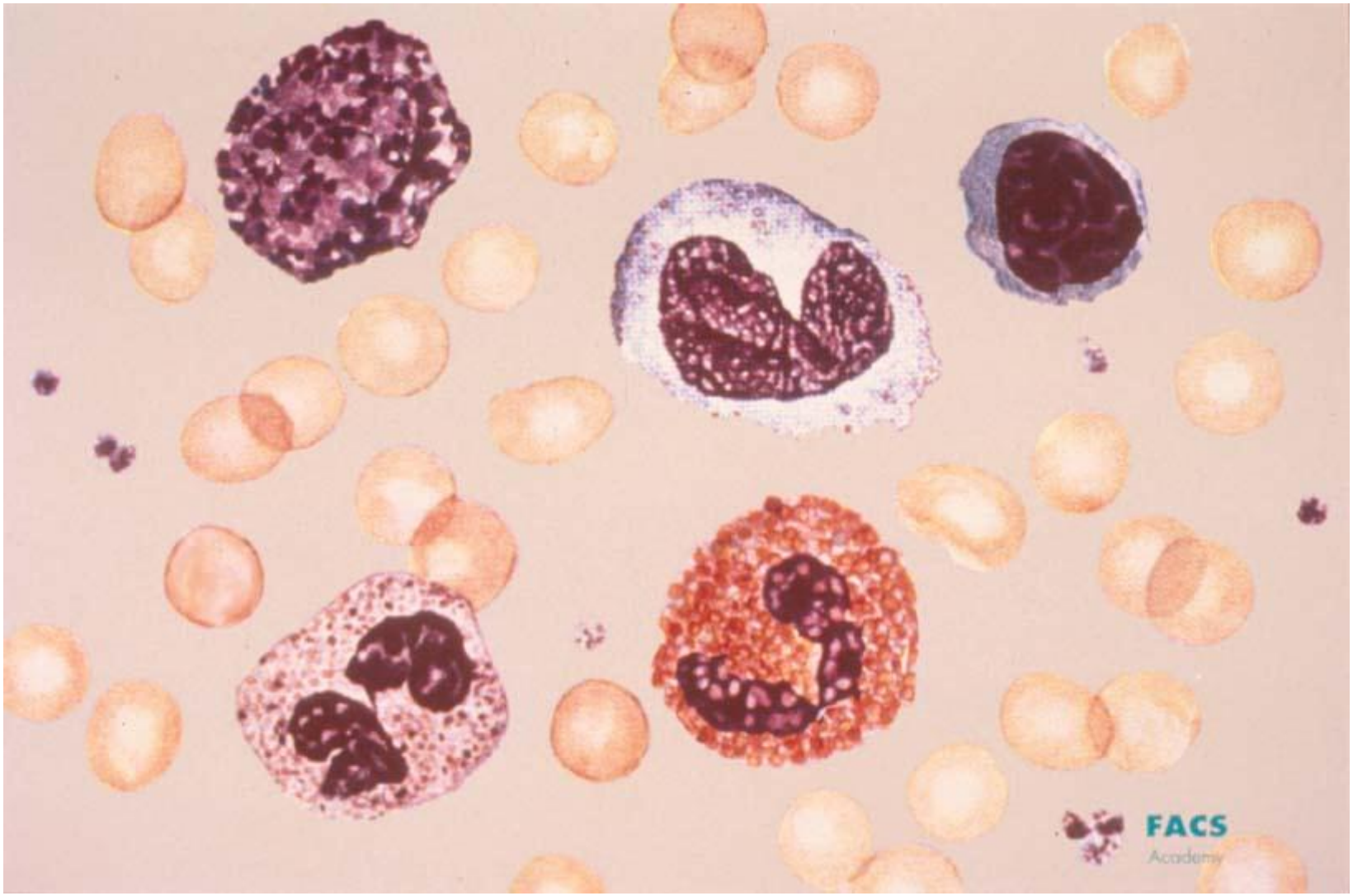
- Light is bent (diffracted) depending on the size and refractive index of the cell
- Detected along the axis of incident light ($0\text{-}10^0$)



- Light is reflected/bounced to the side
- Proportional to cell granularity
- Detected at 90º to incident light axis

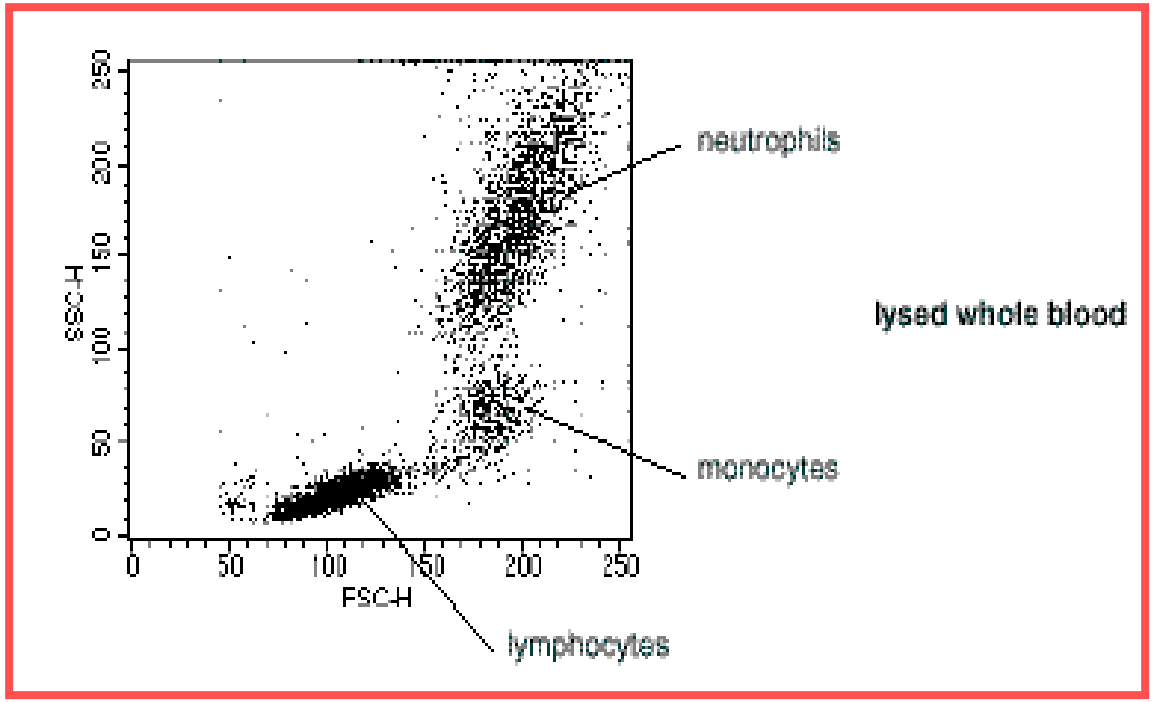
When cells pass through the laser intercept, they scatter laser light and the system measures the degree and direction of scattered light-indicators of the cell's size, shape and structure.




FACS
Academy

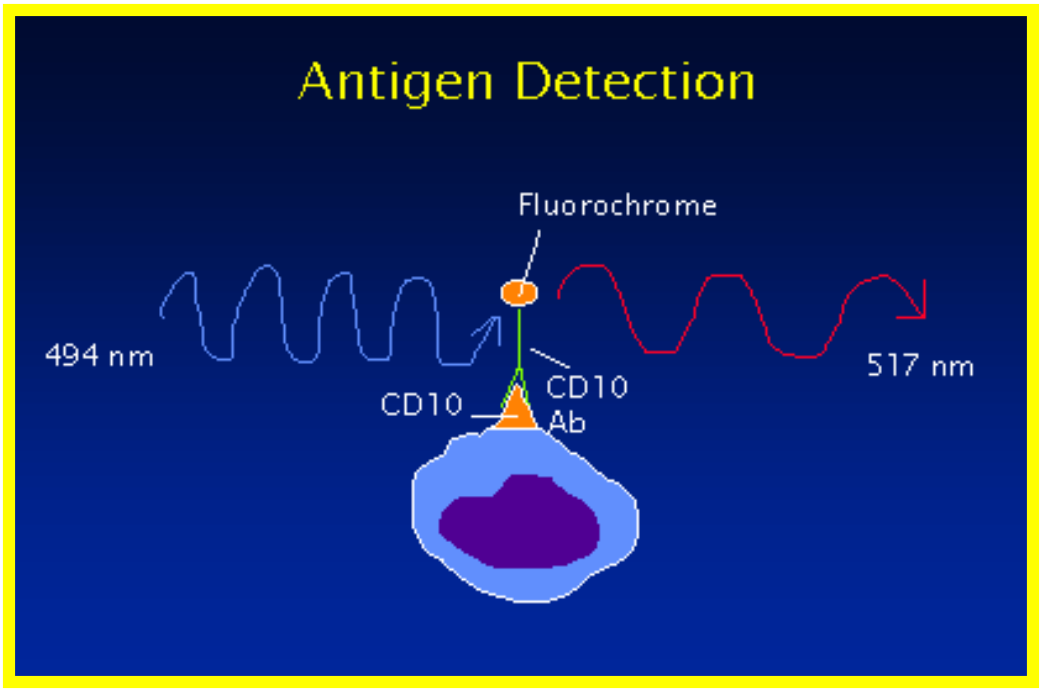
What do the scatter signals tell us?

- Together the forward and side scatter signals can provide useful ways to characterize different cell types.
  - Example: Leucocytes (white blood cells).

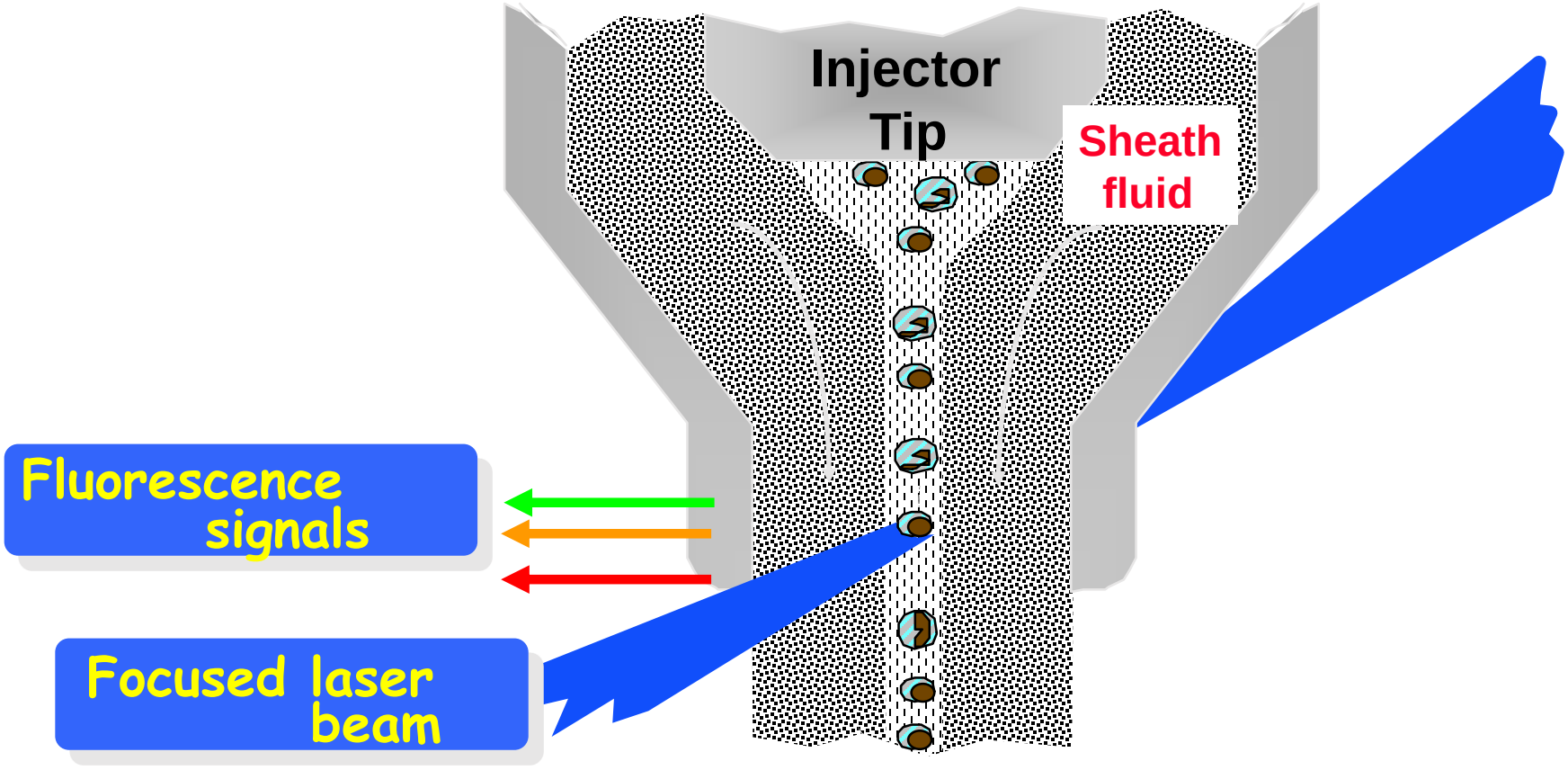

SSC-H
FSC-H
0
50
100
150
200
250
neutrophils
monocytes
lymphocytes
lysed whole blood

FLUOROSCENCE

- Fluorescent compounds absorb light energy over a range of wavelengths, which causes an electron in the compound to be raised to a higher energy level.
- The excited electron emits this excess energy as a photon of light.
- While each fluorochrome will have an optimal, or peak emission wavelength, the spectra will actually be distributed over a number of wavelengths.



Any fluorescent molecules present on the cell fluoresce.



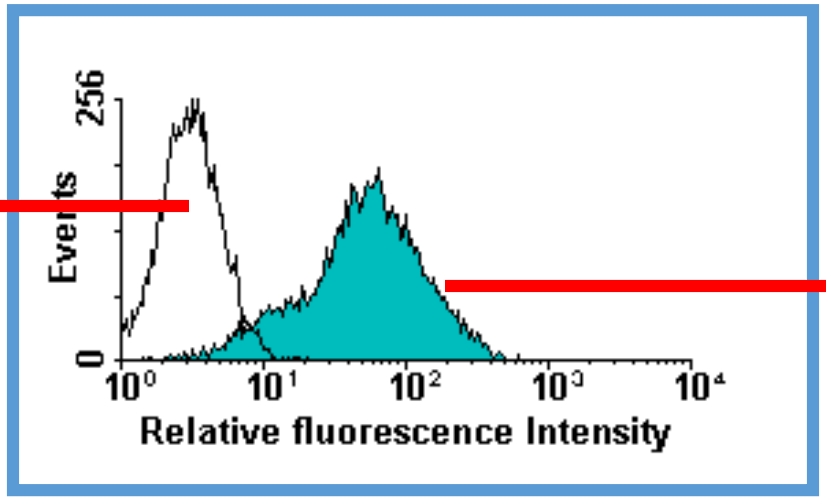
- **The cells are forced to pass in a fluid stream through a flow cell where the fluid stream intersects the focus of the laser.**

Detector	Fluorochrome	Emission
FL1	FITC, GFP, Alexa 488, CFSE, Fluo 3	525 nm
FL2	PE, PI	575 nm
FL3	PER-CP, Cychrome, 7-AAD	660-675 nm

More than one fluorochrome can be used simultaneously if each is excited at 488 nm and if the peak emission wavelengths are not extremely close to each other.

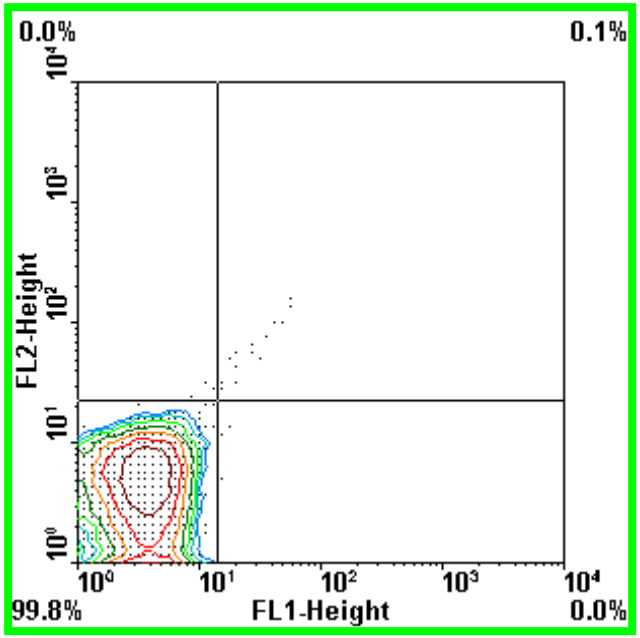
DATA DISPLAY

Negative Control



Sample

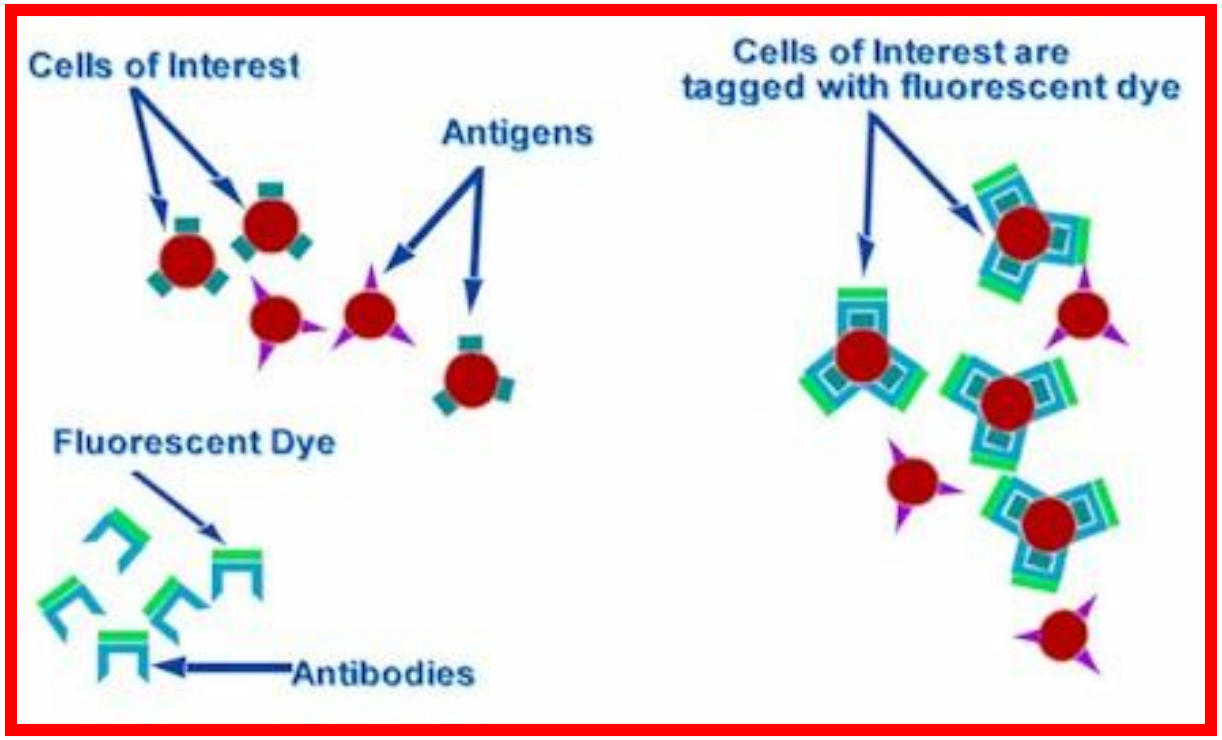
DATA DISPLAY



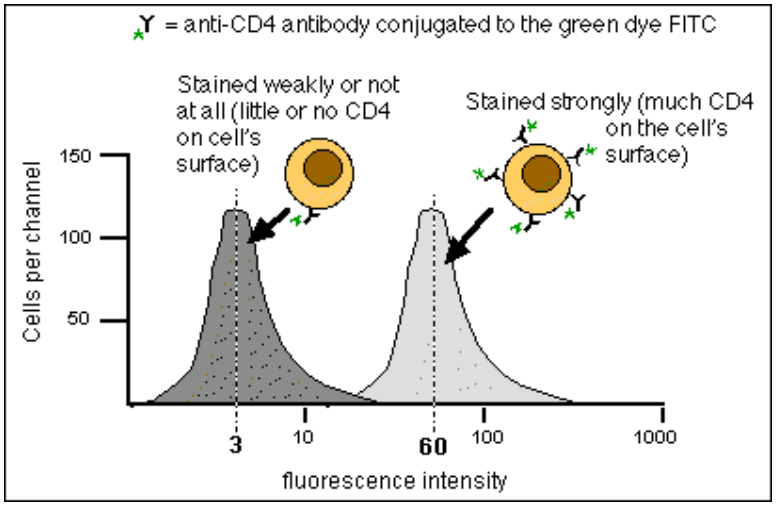
Quadrants can be applied to any 2 parameter display.

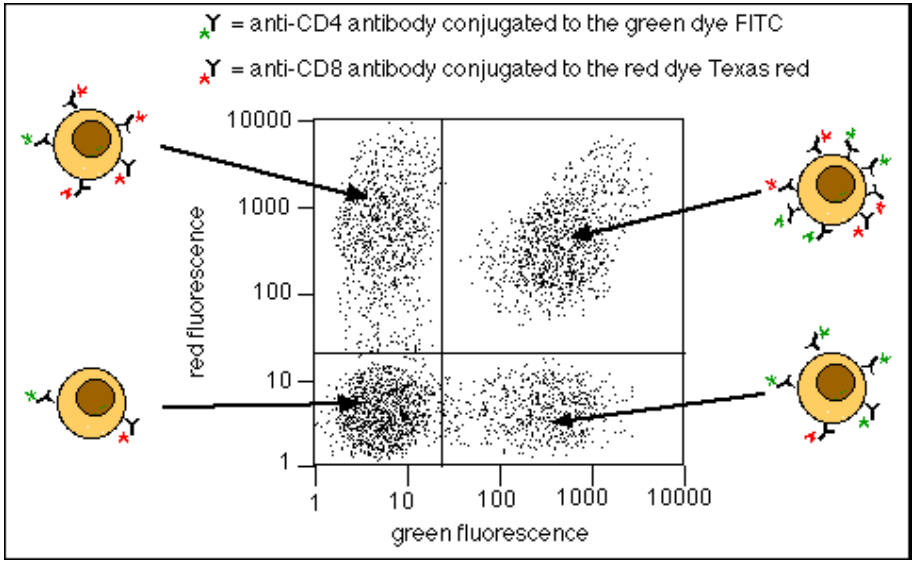
IMMUNOPHENOTYPING

Antibody-fluorescent dye conjugates bind to antigens and the quantity of the fluorescent light emitted is correlated with the cellular marker in question.



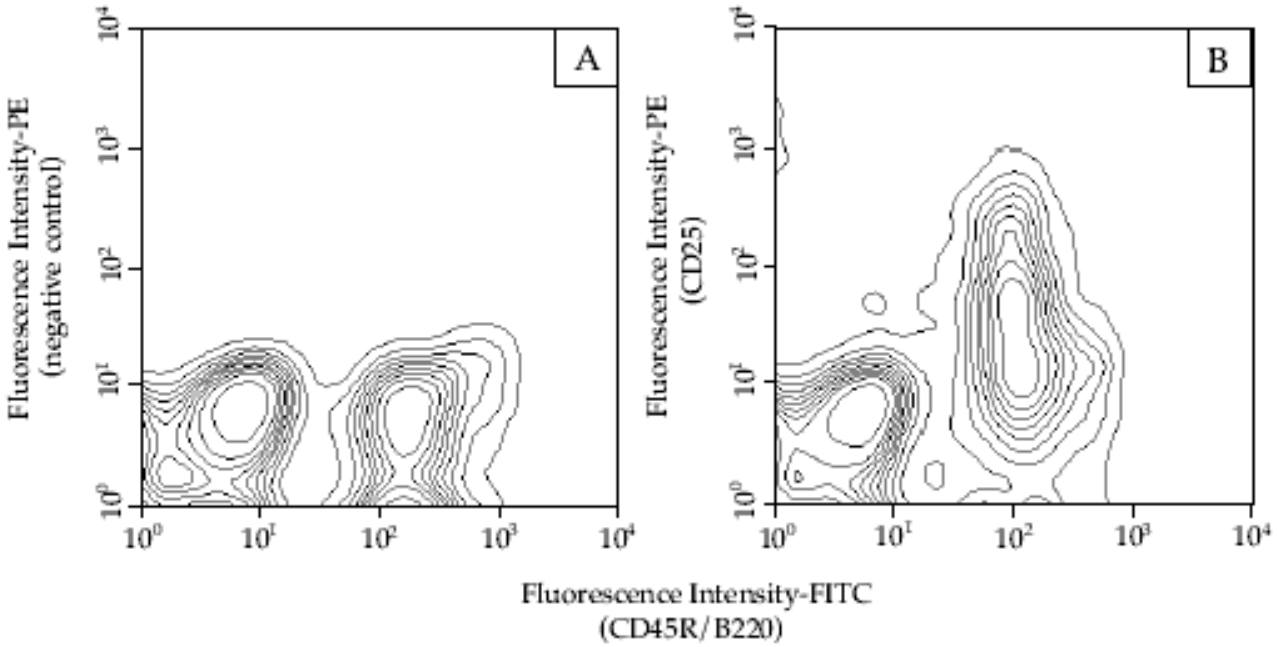
DATA DISPLAY



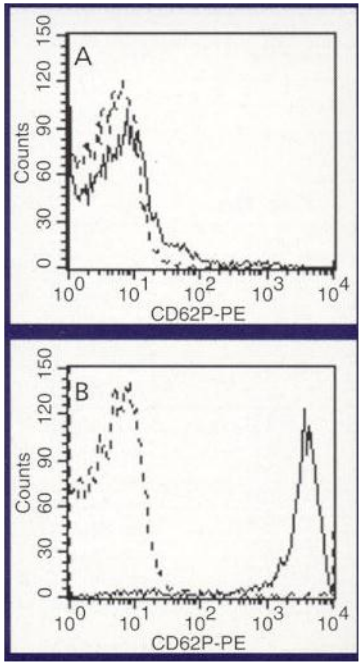


Activation: Surface Receptor Expression

Up Regulation of IL-2 Receptor on Mouse B Cells



CELL ADHESION MOLECULES



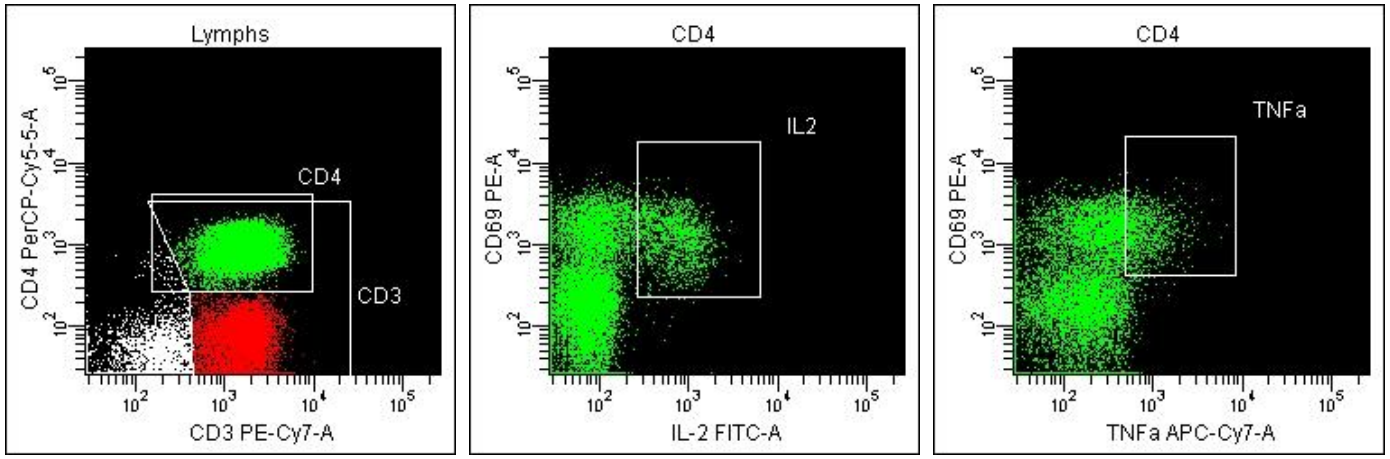
A. Unstimulated

--- Isotype
__ Anti-P-Selectin

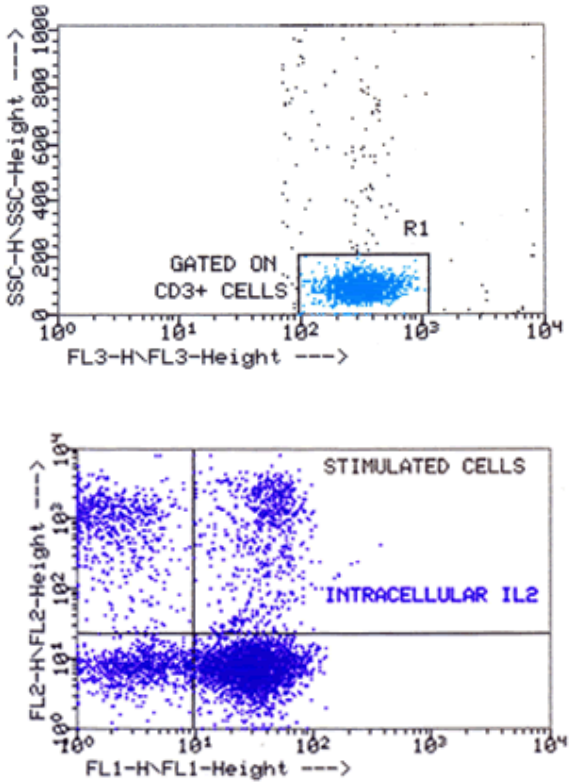
B. Activated

Expression of P-selectin is up-regulated on activated peripheral blood platelets.

Activation: Intracellular Cytokines



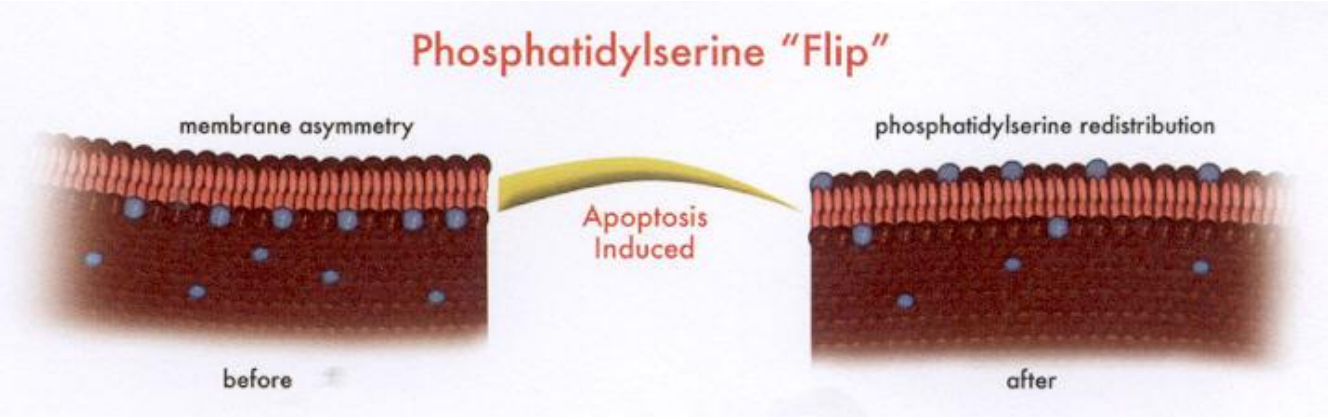
INTRACELLULAR CYTOKINE MEASUREMENT



APOPTOSIS

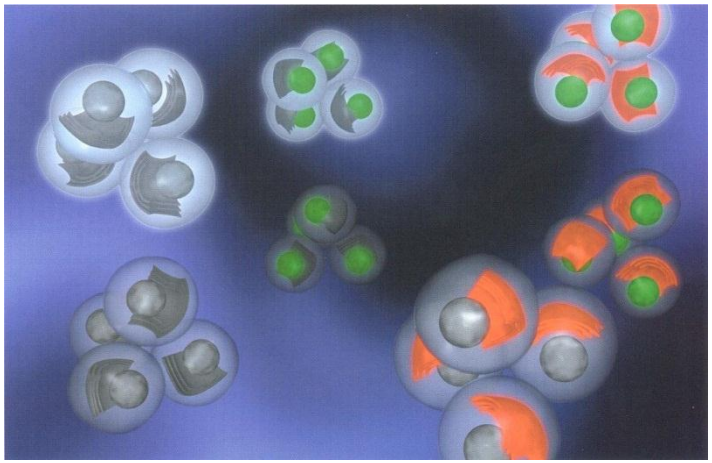

FasL
TNF-α
Fas
TNFR-1
RIP/RAIDD
FADD
Caspase-8
Bid
Cytochrome C
Caspase
Activation

METHODS FOR DETECTING APOPTOSIS: Annexin V assay



INTRACELLULAR CYTOKINE MEASUREMENT

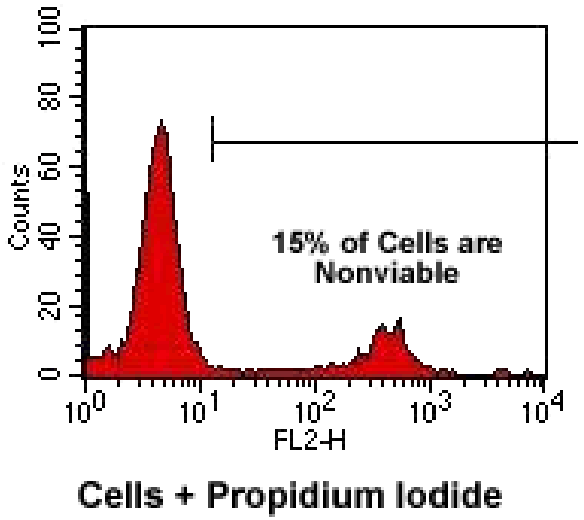
- The production of cytokines by specific cell types can be determined as opposed to measuring the amount of secreted cytokine present in the serum or supernatant.



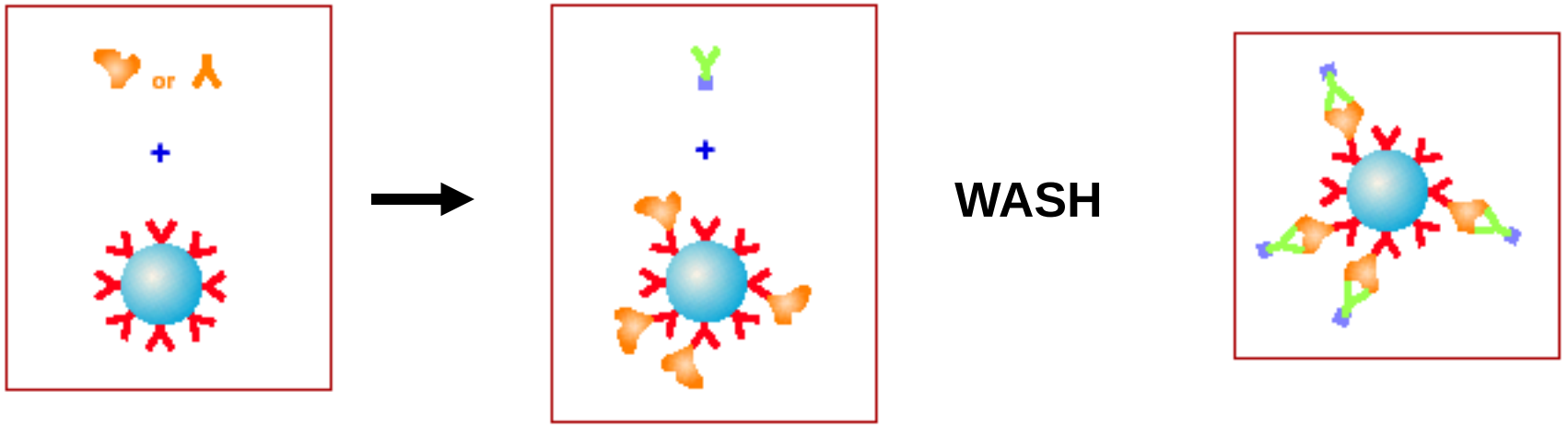
ESTIMATING CELL VIABILITY

PROPIDIUM IODIDE (PI)

-Excluded by viable cells and when taken up by dying cells, binds to nucleic acids and fluoresces orange.



BD CYTOMETRIC BEAD ARRAY SYSTEM



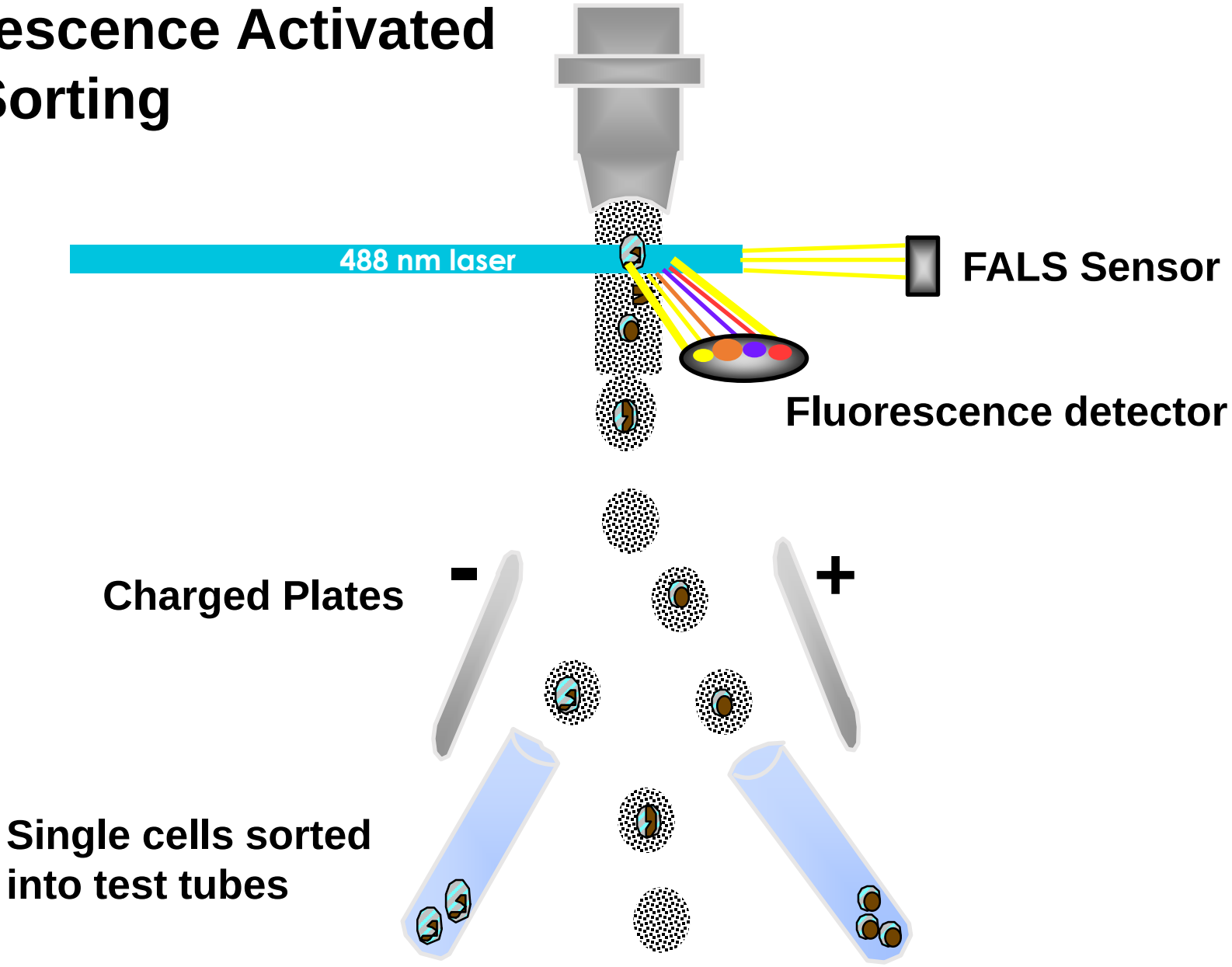
1. Add unknowns or standards to capture bead array
2. Add detection reagents and incubate
3.Acquire samples

Cell Sorting

- The population of interest can be separated during flow and deposited into a tube for later analysis (>95% purity).

- Any combination of analytical parameters can be used to set the criteria for sorting as opposed to single parameter methods (I.e. separating the negatives from the positives).

Fluorescence Activated Cell Sorting



ADVANTAGES

- ✓ Evaluating large number of cells quantitatively and reproducibly increases the statistical confidence and precision of data.

  -The ability to measure several parameters on thousands of single cells within minute (10,000 cells/second).

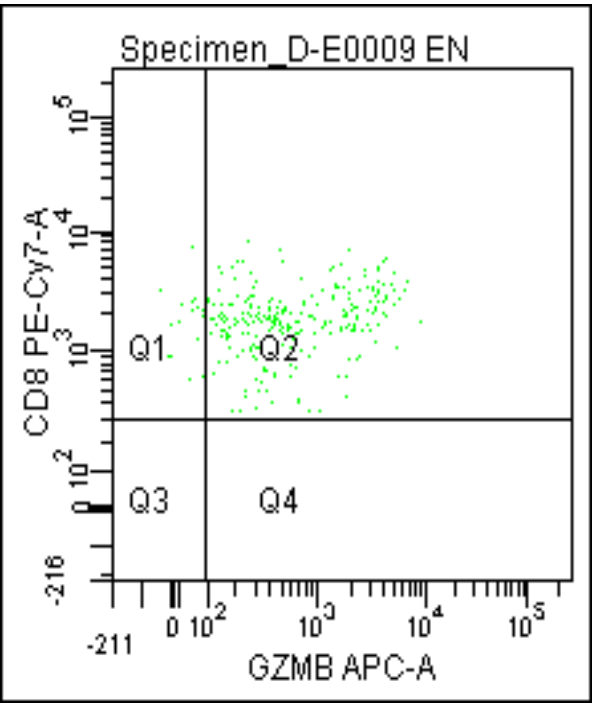
  -Measurements are made separately on each cell.

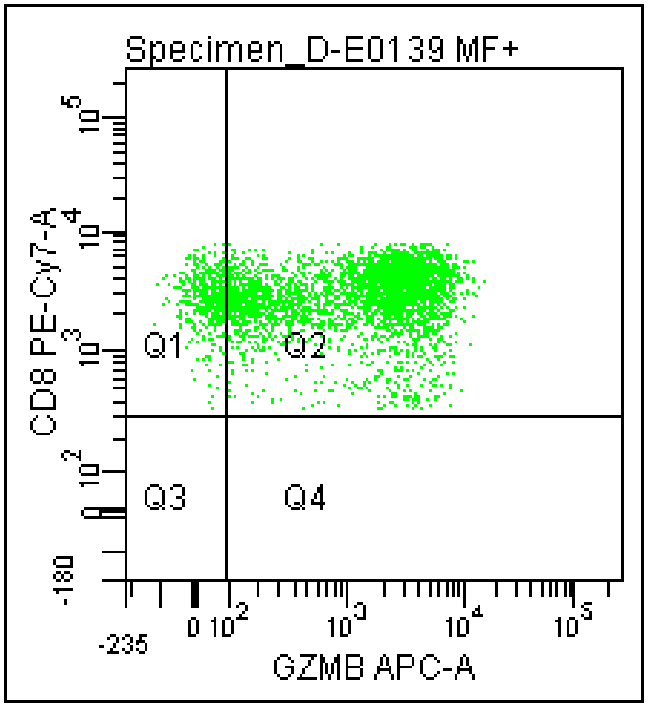
  -Simultaneous, multi-parameter analyses in complex cell populations.

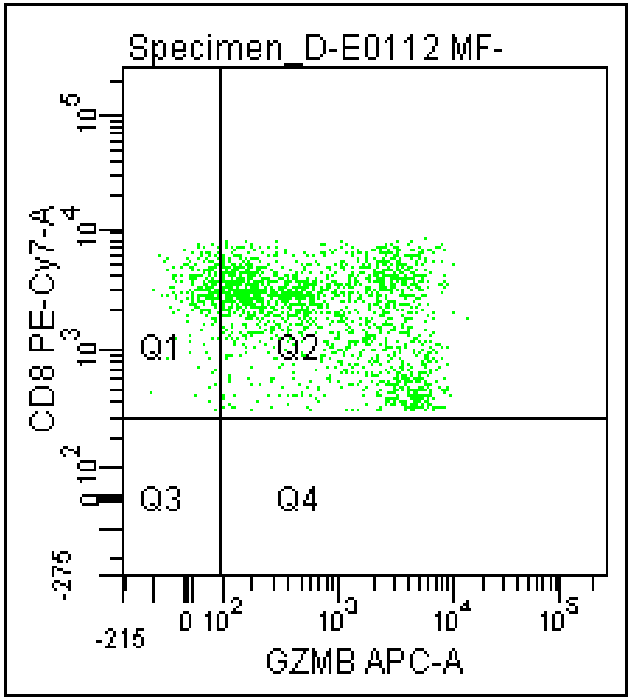
The power of flow cytometry lies in the ability to measure several parameters on thousands of single cells within minutes (1000 cells/second).

BD FACScan is a 3-colour, fixed alignment bench top analyzer, equipped with a 488 nm air-cooled argon laser. It is capable of simultaneously measuring and analyzing FSC, SSC, and 3 spectral regions of fluorescence.

Frequency of $CD8^{+}GZMB^{+}$ expressing T cells in persons with filarial infection







Peripheral Blood Mononuclear Cells (PBMCs)

- Consist of lymphocyte and monocytes

- PBMCs are obtained using the density ficol gradient method.

PBMC isolation

Whole Blood