Flow Cytometry

PRINCIPLES AND APPLICATIONS

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What is Flow Cytometry?

• Cytometry refers to the measurement of **physical/chemical characteristics of cells** or other **biological particles.**

•Flow Cytometry is the process whereby such measurements are made upon **cells/particles** as they pass through a **single file** suspended **in a fluid stream.**

•It is very important to note that all the measurements are performed on **a cell by cell basis.**

•An additional function that flow cytometers can perform apart from cellular analysis is their **physical sorting.** This sorting takes just few minutes and gives purity of any cellular subtype in excess of 95%.

What are the various parameters assayed by flow cytometry?

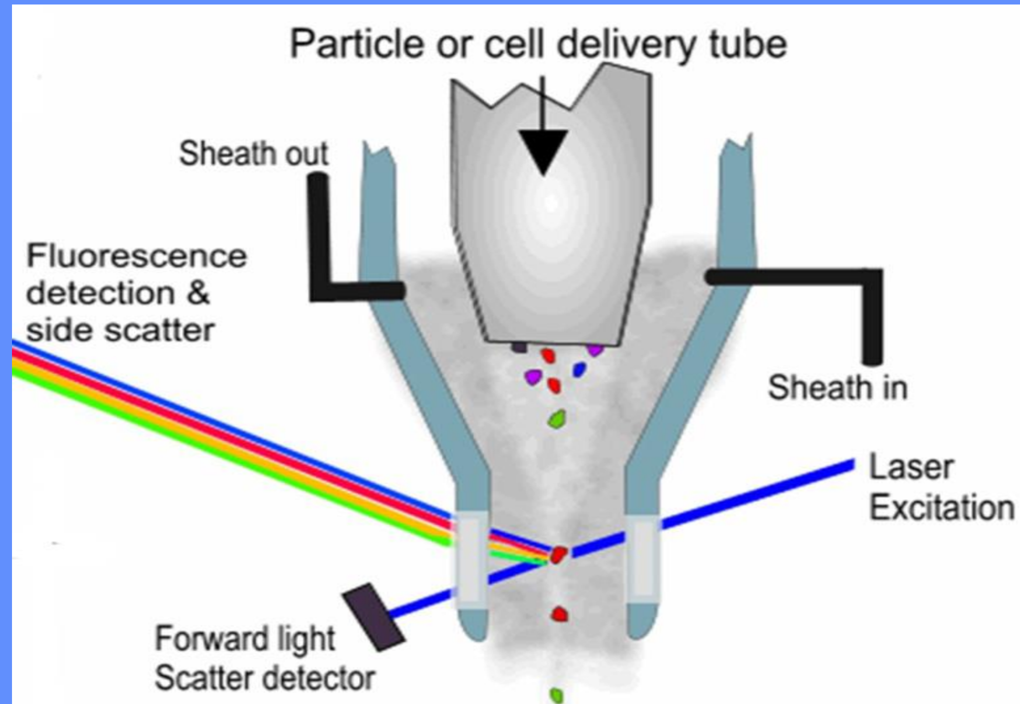
Physical parameters	Chemical parameters
Cell size Cell shape Cytoplasmic granularity Cytoskeletal organization Redox state Membrane integrity Endocytosis Surface charge Membrane fluidity Structuredness of cytoplasmic matrix Membrane potential of biological membranes	Animal and plant Pigment content Total protein Basic protein Sulfhydryl groups DNA content DNA base ratio DNA synthesis RNA content Antigens Surface sugars Enzyme activity Membrane permeability Intracellular receptors Surface receptors Membrane bound Ca^{2+} Cytosolic Ca^{2+} Intracellular pH

Working principle

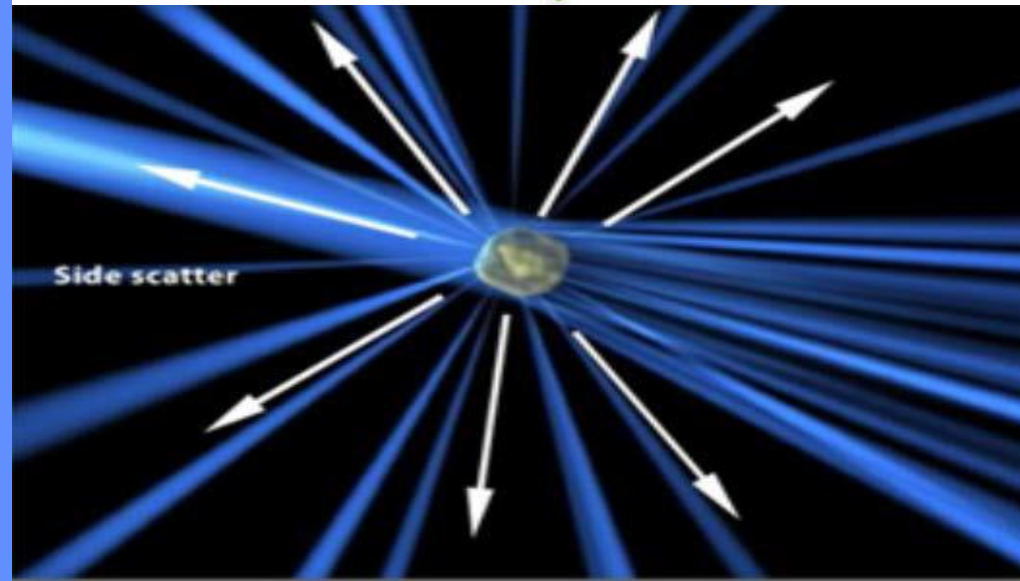
- The flow cytometer analysis and sorting instruments combine electrical and optical sensing techniques thereby permitting several measurements to be performed simultaneously on the same cell.
- Typical measurements include electronic cell volume, fluorescence of individual cell constituents, light scatter due to extrinsic or intrinsic cellular features, absorption or loss of extinction of light due to cellular components and fluorescence polarization.
- If a heterogeneous cell population is being assayed, the simultaneous measurements allow a biochemical, functional, cytological relationship to be established between different cell types.

Working principle contd…

- Cells incubated with **fluorescent or absorption dyes** are suspended in **physiological saline.** This suspension is allowed to pass through the flow chamber @ of about **1000 cells/sec.**



- As the cells pass, they either **scatter, absorb or fluoresce** the light impinging upon them. This scattered, transmitted or fluoresced light is then measured by appropriate **optical sensors.** Additionally electronic sensors detect the particle volume and other related parameters.



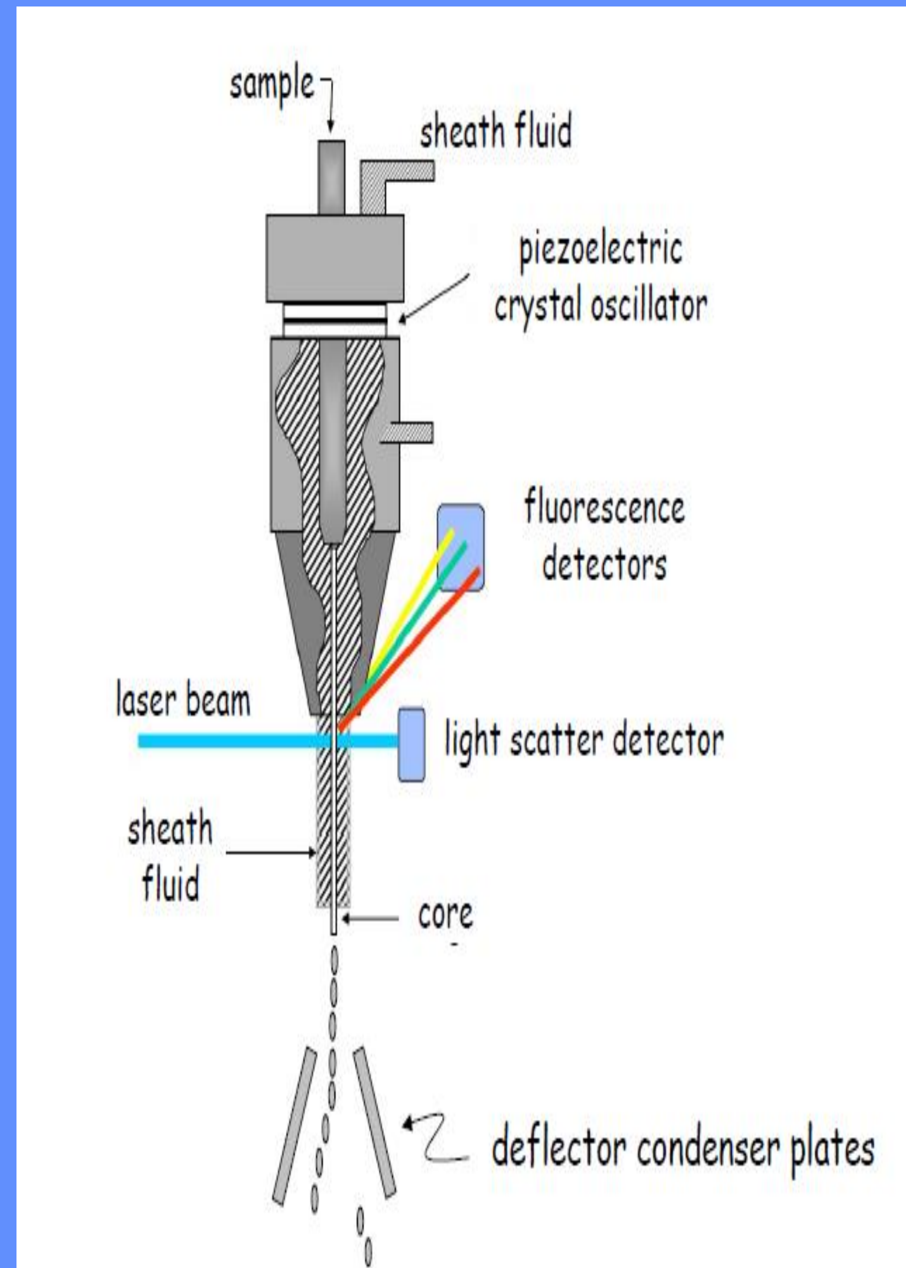
Working principle contd…

- Signals from each of the sensors are processed on a **cell by cell basis** and the resulting data are displayed as **frequency distribution histograms.**

- The stream which exits from the flow chamber is disturbed by a **piezoelectric transducer** to give **uniform droplets.**

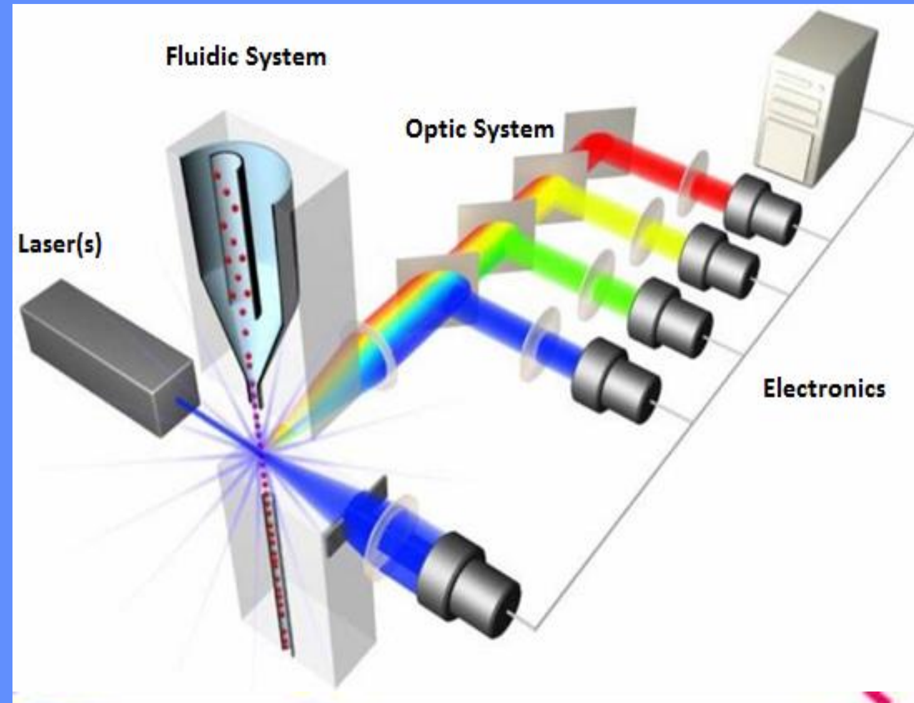
- Processed signals from cell sensors now activate the cell sorting device. If the **amplitude of the signals fall within a preset range** a **droplet charging device** is activated.

- The droplets, which are so charged, are deflected by a **static electric field** and are collected in a container. Other droplets not containing the desired cells are not charged and these are therefore not deflected.



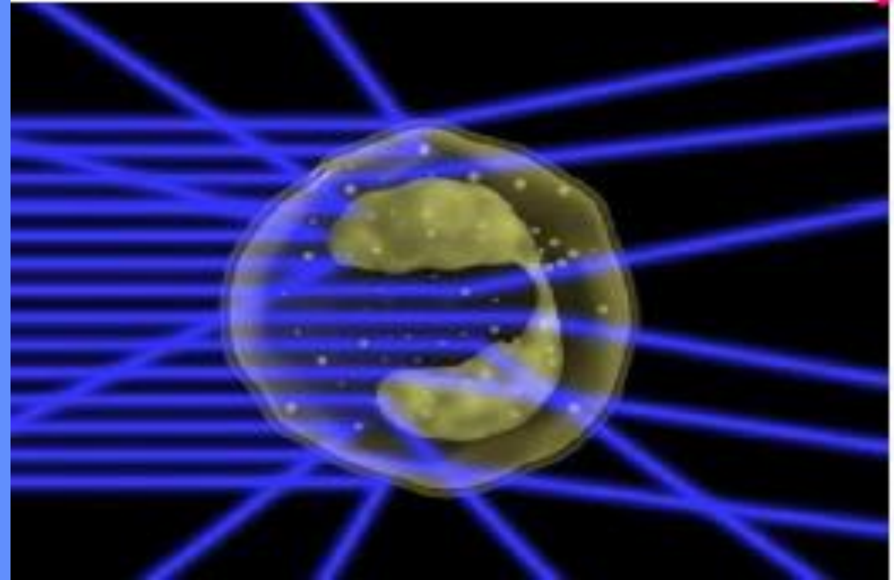
Instrumentation

- Composed of basic units like fluid transport system, flow chamber, excitation source and optics, optical and electronic sensors, collection optics, signals processing electronics and data display, storage and analysis system.



- The sample in the sample chamber, is continuously stirred to mix the heterogeneous cell population.

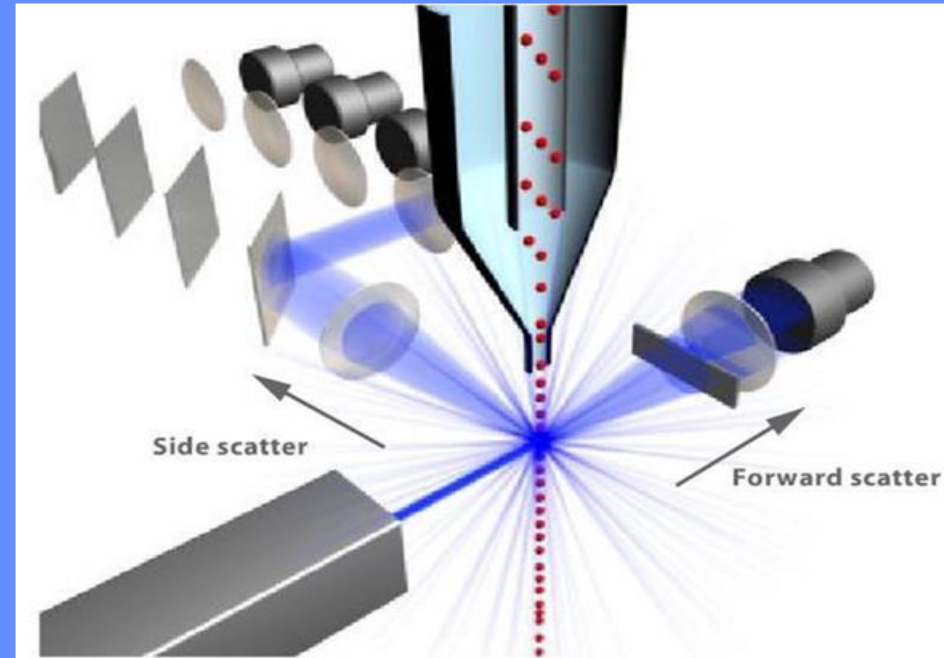
- In flow chamber the sample stream is injected into the centre of a cell free stream of sheath fluid. The sample along with the sheath fluid now enters a constriction region, which increases the flow velocity.

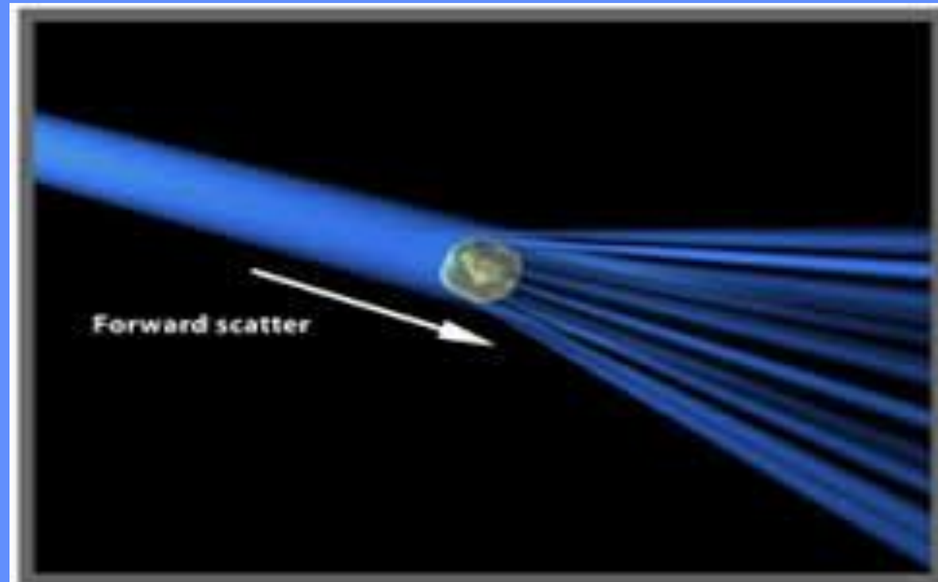


Instrumentation contd…

- The source of light is the **argon-ion laser.** A series of **detectors** and **filters** are positioned appropriately to measure fluorescence at a number of wavelengths.

- The cells passing through the flow chamber **scatter** the incident light. **Forward light scatter** gives an idea **about cell size and shape** whereas **side scatter** gives important information about the **granularity and fine structure** of the cell.



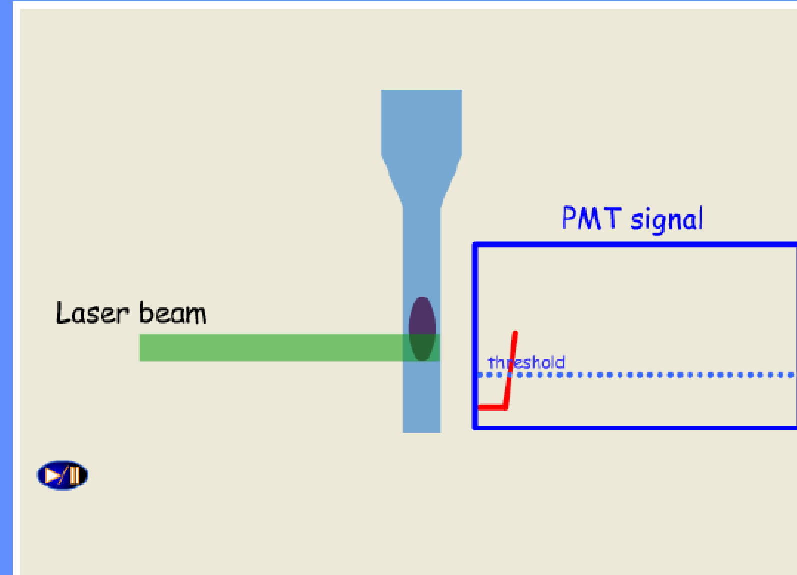


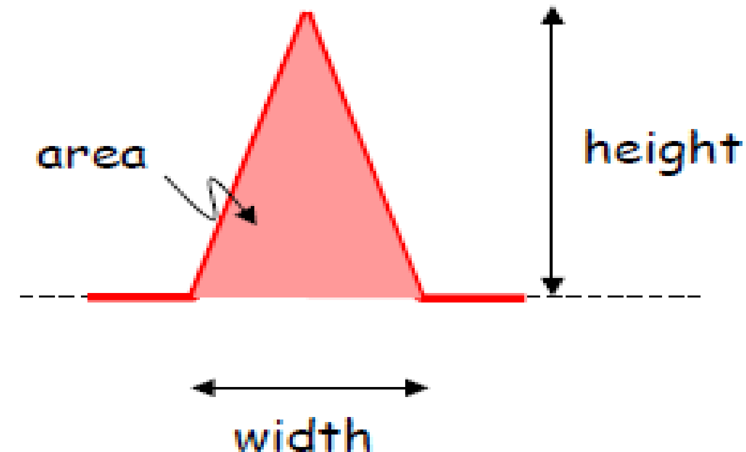
Instrumentation contd…

- Each detector in the flow cytometer gives the signal in the form of an **electrical pulse** that is proportional to the **concentration of a cellular substance** or to a **cellular feature.**

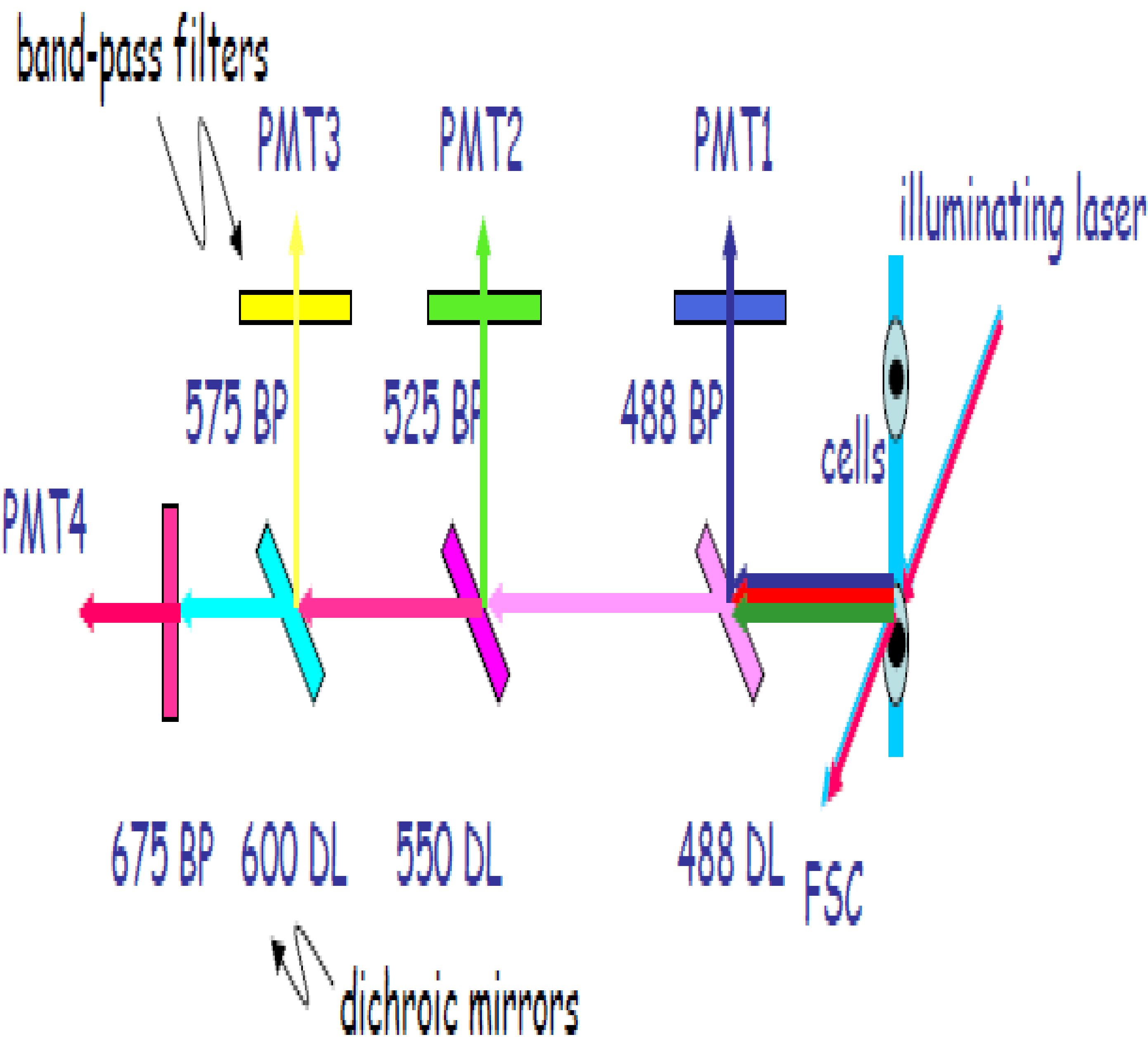
- This electrical pulse is **amplified** and converted to a **digital value** before being sent to a **digital computer** which **analyses, store or displays the signal.**

- For every pulse, its peak amplitude, width and area are recorded. The **peak** gives us the idea about the **maximum fluorescent intensity of the cell**; the **width** tells us about the **width of the fluorescing part of the cell** and the **area of the pulse** informs us about the **total fluorescence of the cell.**





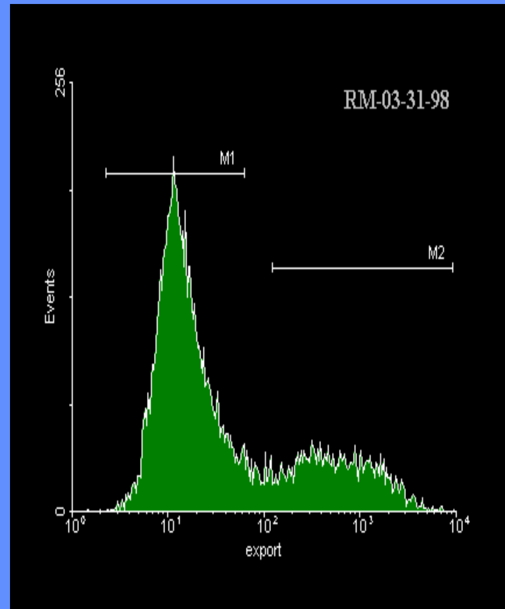
Arrangement of detectors

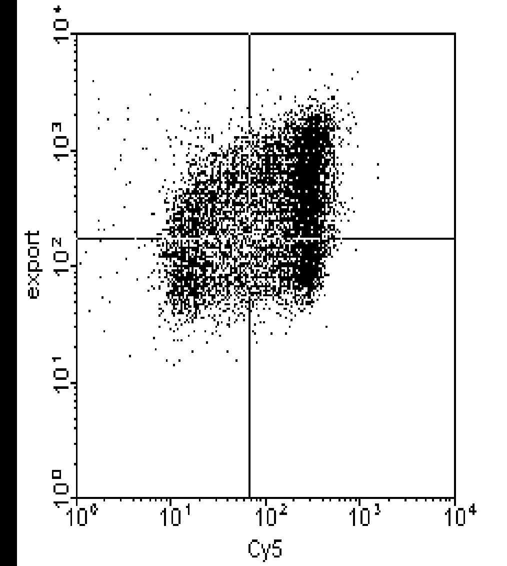


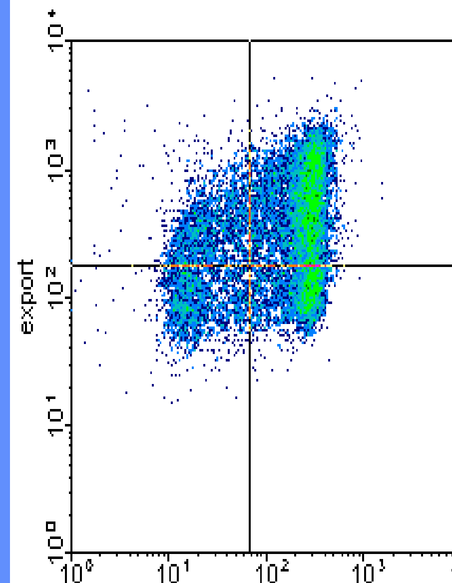
Beams separated from the common beam by dichroic mirrors are further filtered by band-pass filters.

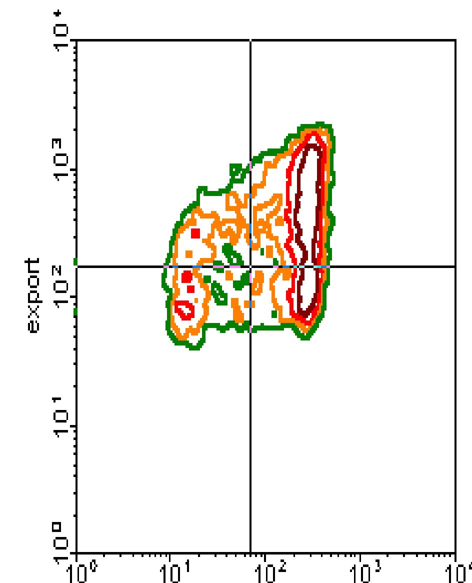
Common display formats

- **Histogram-**only single parameter assessed.
- **Dot plot-**two measured parameters are displayed on the x and y axes, every dot in the plot corresponds to a single cell. **Drawback:** if many cells are displayed, dots may become confluent.
- **Density plot-** the color of dots corresponds to the number of cells.
- **Contour plot-**dots with identical cell numbers are connected with lines.



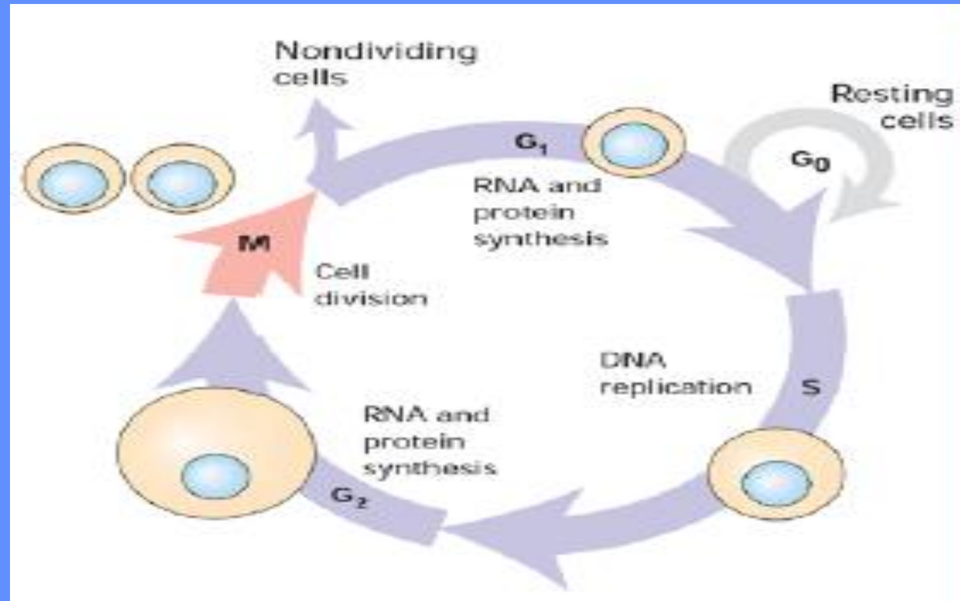


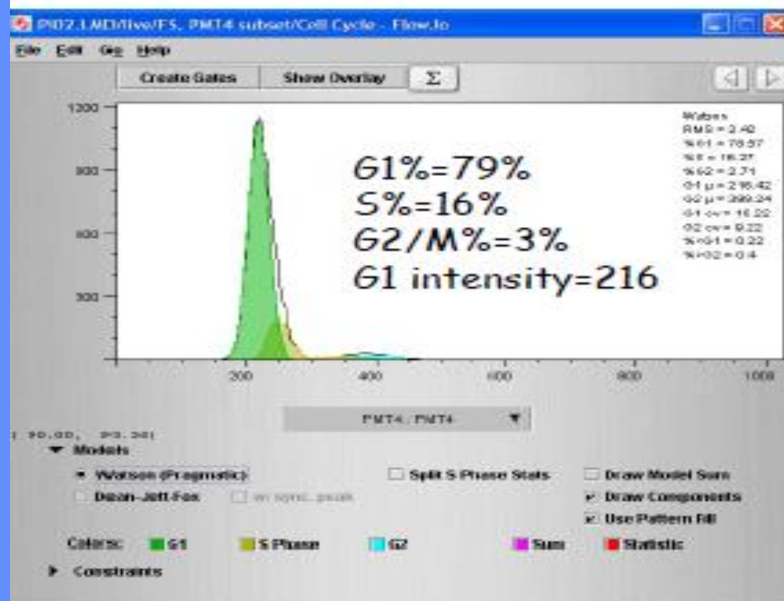




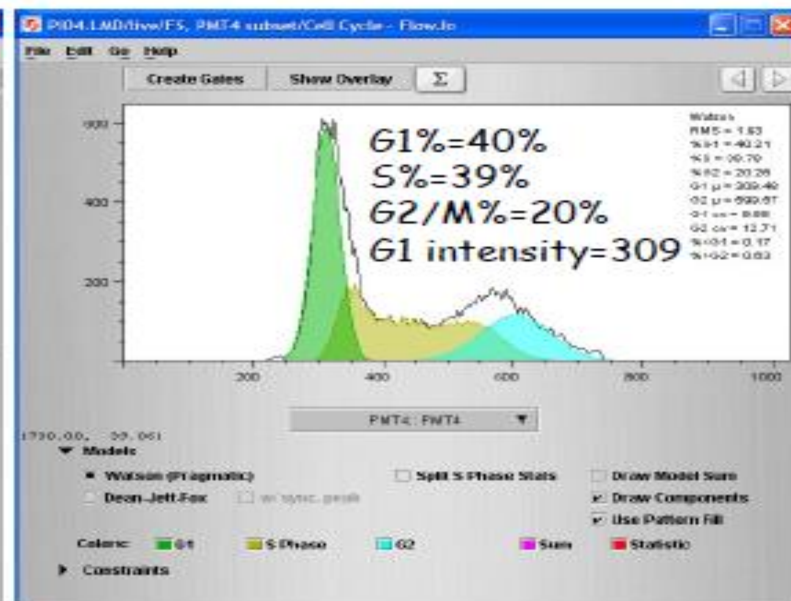
Applications

- **Cell cycle analysis:** By simultaneous measurements of DNA, RNA, cell size and protein, it becomes possible to define **cells' position in the cell cycle.** It also becomes possible to **sort** cell populations in **different cell cycle phases** and to subject each population to biochemical analysis.





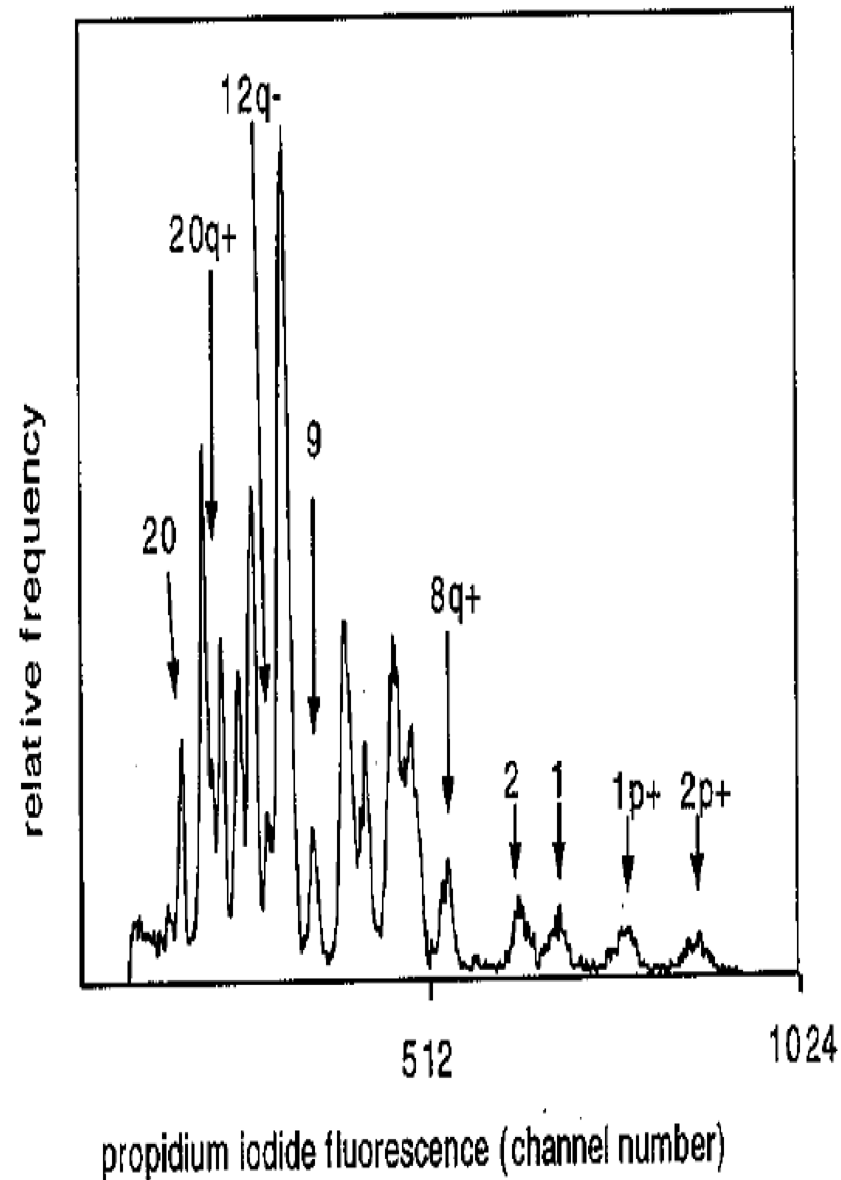
human diploid cell



JIMT-1 (human breast tumor cell)

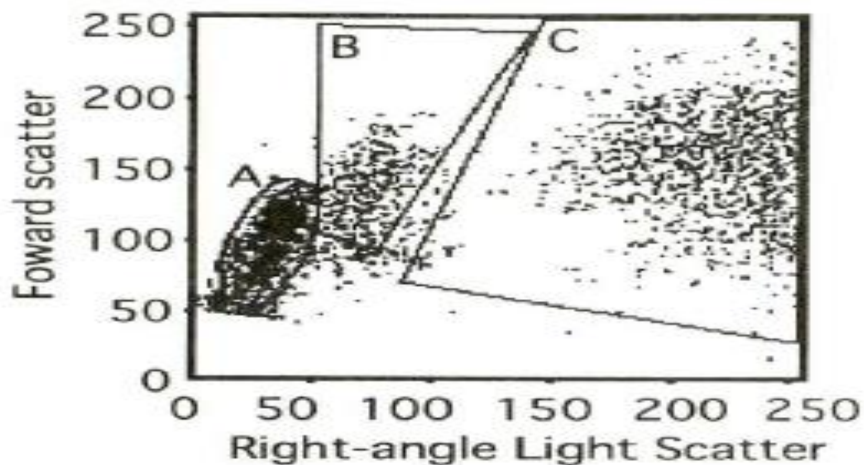
Applications contd…

- **Studies on chromosomes:** It is possible to determine karyotype of a cell using flow cytometry. For example, **Brown Norway myelocytic leukemia cell line** were treated with the fluorochrome **propidium iodide.** The chromosomes were then analysed by exciting at a particular wavelength and studying the fluorescence at another wavelength. **All** the **chromosomes** gave a **different peak.** By extension it can be said that **altered karyotype** would give an **altered fluorescence** pattern. Such studies therefore achieve great significance in **studying malignant cells** that often demonstrate an altered karyotype.

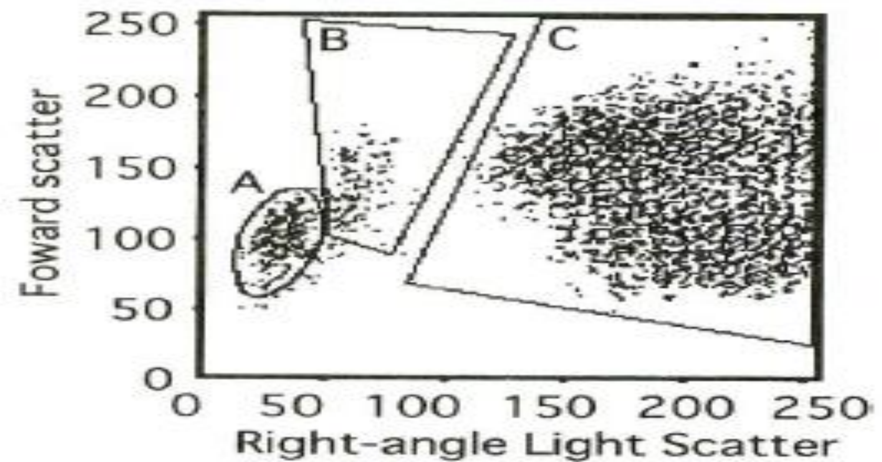


Applications contd…

- **Leucocyte characterization:** Two colour fluorescence characterization of acridine orange treated leucocytes makes it possible to **distinguish between lymphocytes, monocytes and granulocytes** and to sort them out. Acridine orange is also utilized to characterize leucocytes from patients with various kinds of neoplasm such as **leukemias, lymphomas** etc. In addition flow cytometry is also utilized for **differential leucocyte counting** where it gives very precise values.



Cytoron (%)		NE-8000 (%)	
A	46.2	Lym	44.0
B	7.8	Mon	5.6
C	38.8	Gran	50.4

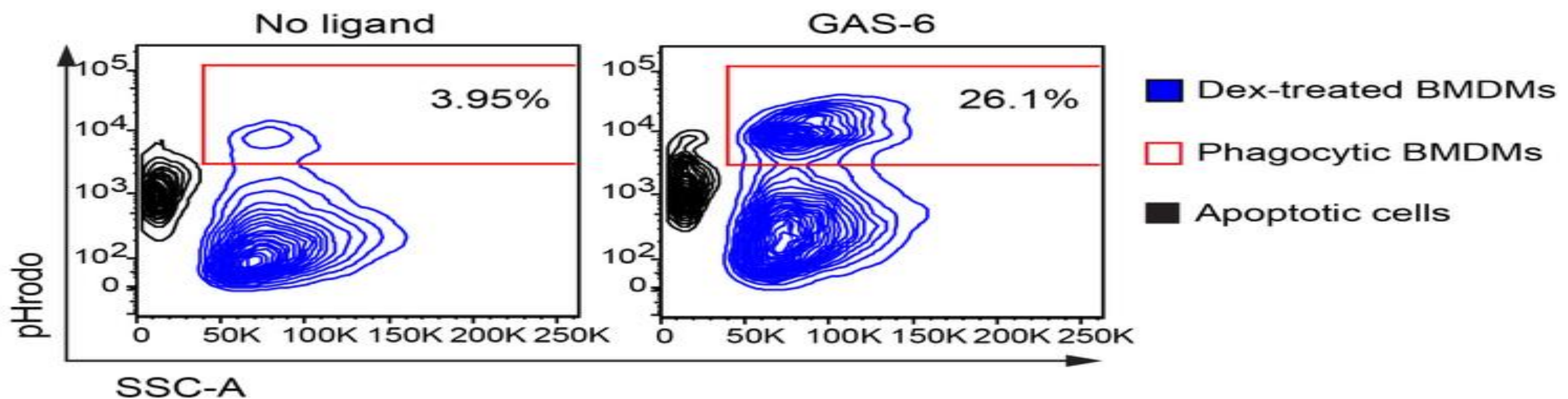


Cytoron (%)		NE-8000 (%)	
A	9.5	Lym	9.0
B	6.0	Mon	2.0
C	79.9	Gran	78.0

Fig. 2. Cytograms of specimens with a high percent lymphocytes (left) and high percent granulocytes (right).

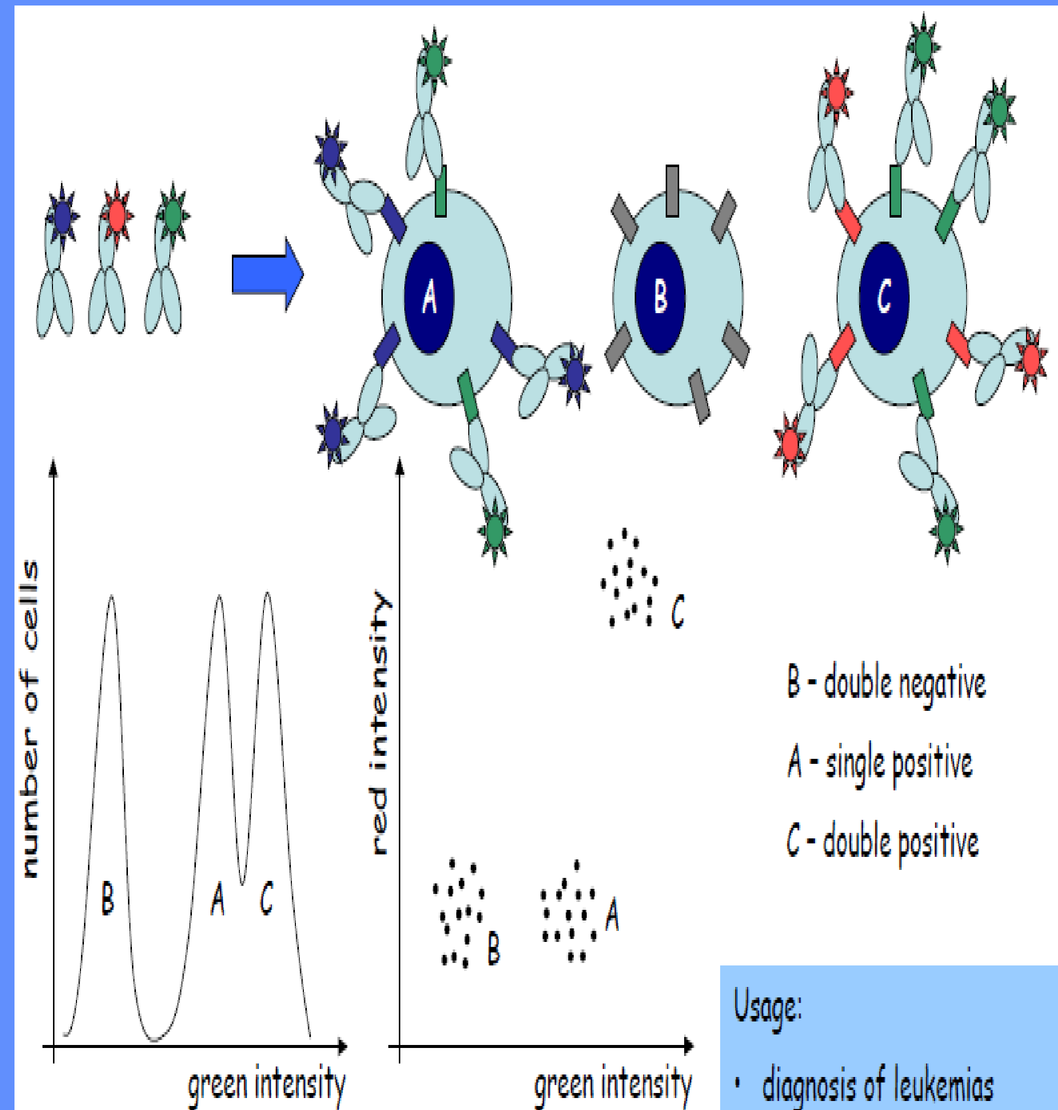
Applications contd…

- **Tests of granulocyte and platelet function:** Tests for granulocyte function such as phagocytic assays are possible using flow cytometry. Platelet viability can be assayed easily by either a dye exclusion test or by measuring the cytoplasmic Ca^{2+} concentration. Apoptotic cells are labeled with pH-sensitive dye, pHrodo. Once engulfed into the acidic environment of phagosomes, pHrodo fluorescence is enhanced and phagocytic macrophages are distinguished based on their side scatter (SSC-A) and pHrodo fluorescence intensity using flow cytometry. In this experiment, the percent cells undergoing phagocytosis is quantified in an 1-hour assay, in absence or presence of ligand GAS-6.



Applications contd…

- Immunophenotyping is the identification of antigens using detection antibodies.
- The manufactured antibody (CD marker) is attached to a fluorochrome like FITC and then added to the sample.
- If the cell has the Ag that the Ab specifically binds, the antibody and fluorochrome attach to the cell.

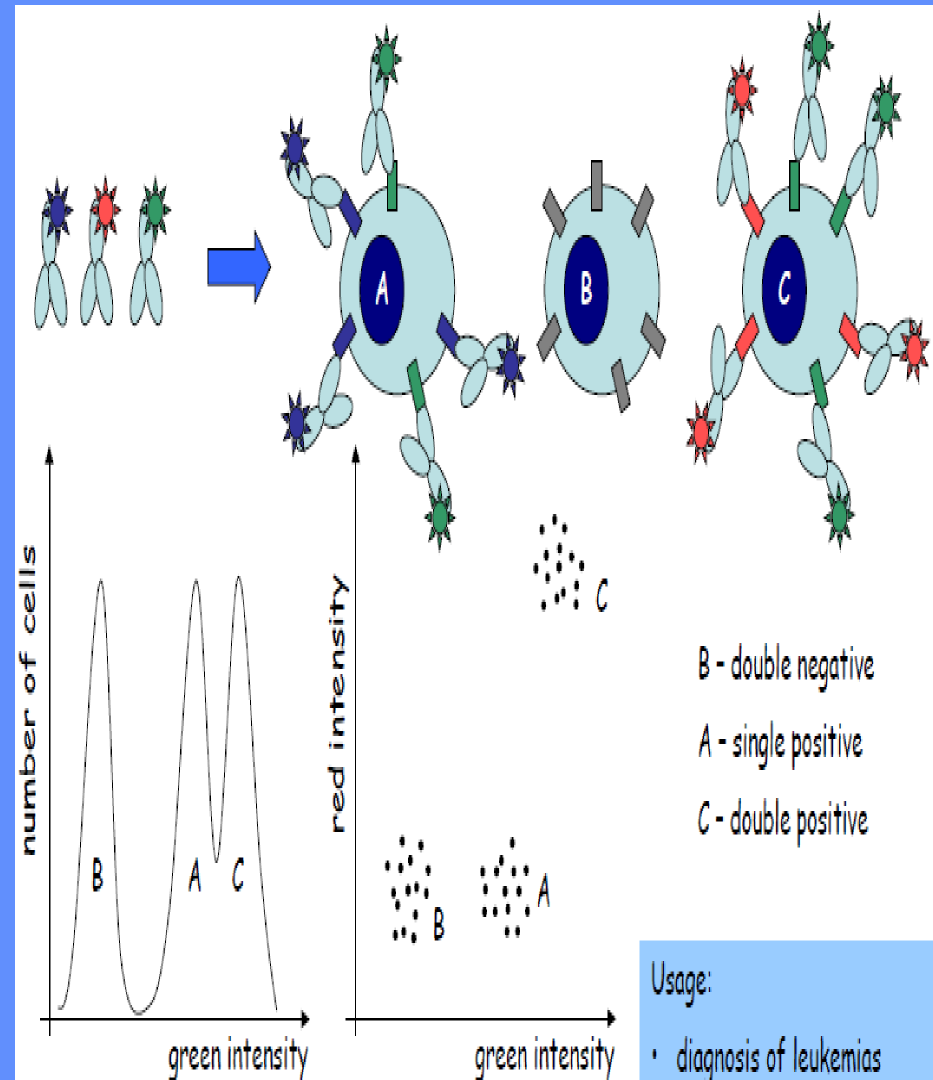


Applications contd…

- When the cell/antibody/fluorochrome complex passes through the laser beam, the fluorochrome is excited and fluoresces at a measurable wavelength detected by the photomultiplier tube in the flow cytometer.

- Computer software connected with the flow cytometer generate a histogram that visually represent the cells present.

- No target molecule ⟶ no fluorescence.



Applications contd…

- **Quantification of cell-to-cell communication:** Flow cytometry makes it easy to quantify cell-to-cell communication. Cells are loaded with **lucifer yellow** with or without **rhodamine labeled dextran.** The **transfer** of the two dyes **between donor and recipient cells** can be studied easily using 2-colour fluorescence flow cytometry.

- It is also used in **cell differentiation, immunology, parasitology, sperm analysis, food science, pharmacology and toxicology, cancer biology and carcinogenesis, bone marrow analysis, tissue typing and lymphocyte applications, T-cell subset analysis etc.** It is increasingly being used for sorting different cellular populations. Thus, leucocytes, macrophage and many other types of cells are routinely sorted using flow cytometry.