

## **INTRODUCTION**

Flow Cytometry involves the use of a beam of laser light projected through a liquid stream that contains cells, or other particles, which when struck by the focused light give out signals which are picked up by detectors. These signals are then converted for computer storage and data analysis, and can provide information about various cellular properties.

The term "flow cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. The fundamental concept is that cells flow one at a time through a region of interrogation where multiple biophysical properties of each cell can be measured at rates of over 1000 cells per second. These biophysical properties are then correlated with biological and biochemical properties of interest. The high through-put of cells allows for rare cells, which may have inherent or inducible differences, to be easily detected and identified from the remainder of the cell population.

In order to make the measurement of biological/biochemical properties of interest easier, the cells are usually stained with fluorescent dyes which bind specifically to cellular constituents. The dyes are excited by the laser beam, and emit light at longer wavelengths. This emitted light is picked up by detectors, and these analogue signals are converted to digital so that they may be stored, for later display and analysis.

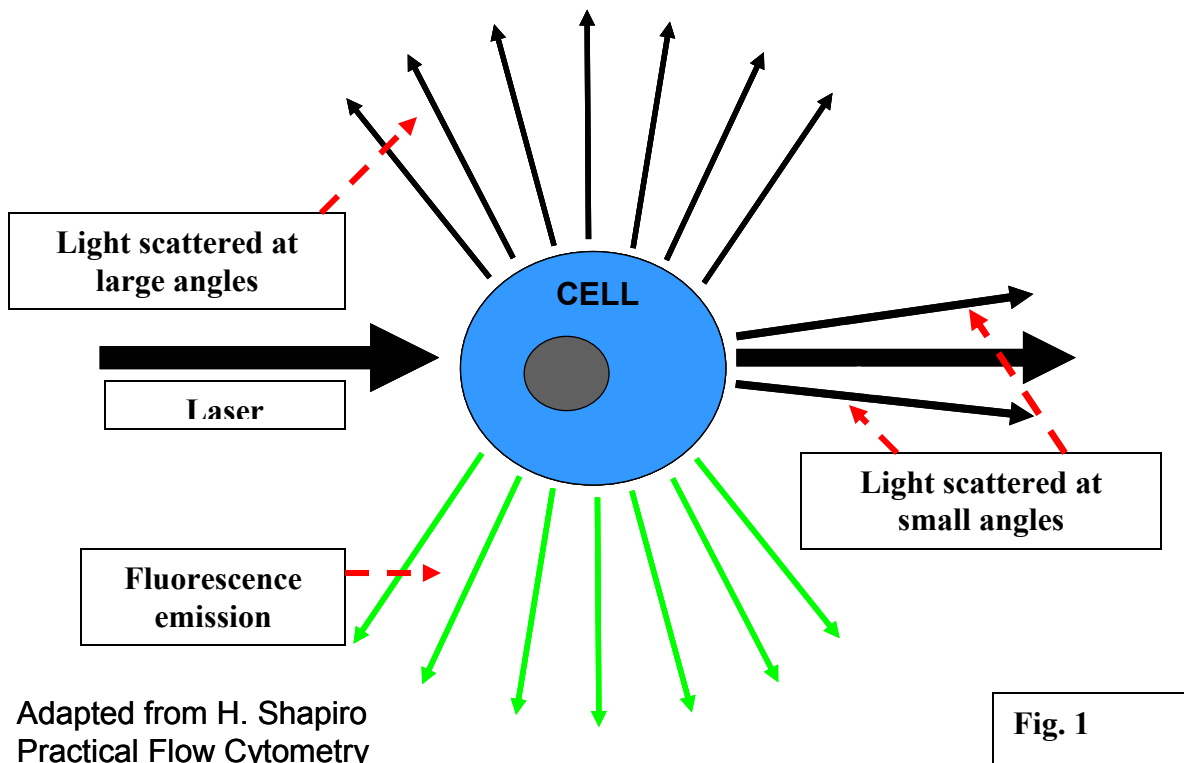
Computer Analysis: Following acquisition of data on the flow cytometer, analysis is undertaken to find out how many cells from the sampled population meet a criteria of interest. For example, does a population of cells express one molecule, but not another. The data can be displayed in a number of different formats, each having advantages and disadvantages. The common methods are histogram, or dot plots.

Many larger flow cytometers are also "cell sorters", instruments which have the ability to selectively deposit cells from particular populations into tubes, or other collection vessels. These selected cells can then be used for further experiments, cultured, or stained with another dye/antibody and reanalyzed. In order to sort cells, the instruments electronics interprets the signals collected for a each cell as it is interrogated by the laser beam, compares the signal with sorting criteria set on the computer. If the cell meets the required criteria, an electrical charge is applied to the liquid stream which is being accurately broken into droplets containing the cells. This charge is applied to the stream at the precise moment the cell of interest is about to break off from the stream, then removed when the charged droplet has broken from the stream. As the droplets fall, they pass between two metal plates, which are strongly positively or negatively charged. Charged droplets get drawn towards the metal plate of the opposite polarity, and deposited in the collection vessel, or onto a microscope slide, for further examination.

## MEASURABLE PARAMETERS:

Flow cytometers are capable of measuring a variety of cellular characteristics such as:

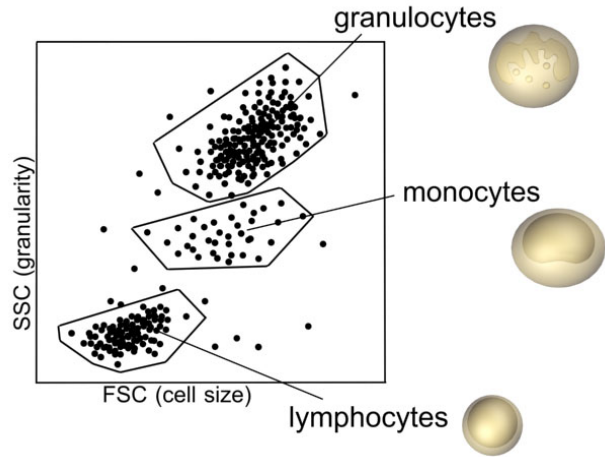
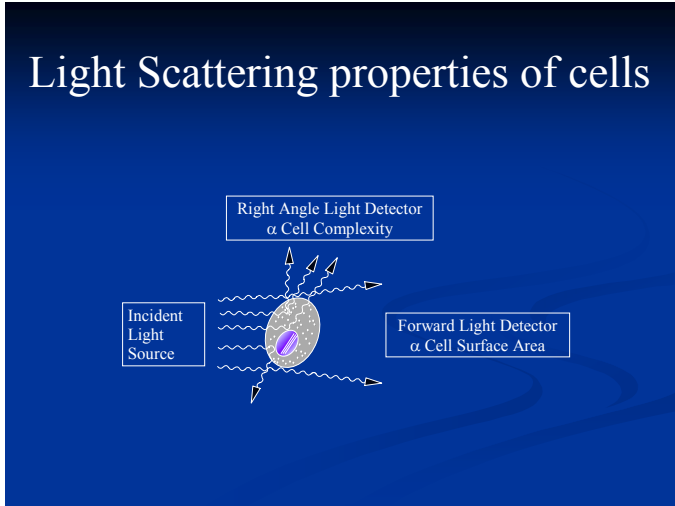
- Relative cell size
- Internal complexity/granularity
- Cell surface properties/refractive indices
- Levels of autofluorescence
- Presence or absence of an exogenous fluorescent probe
- Relative fluorescence intensity



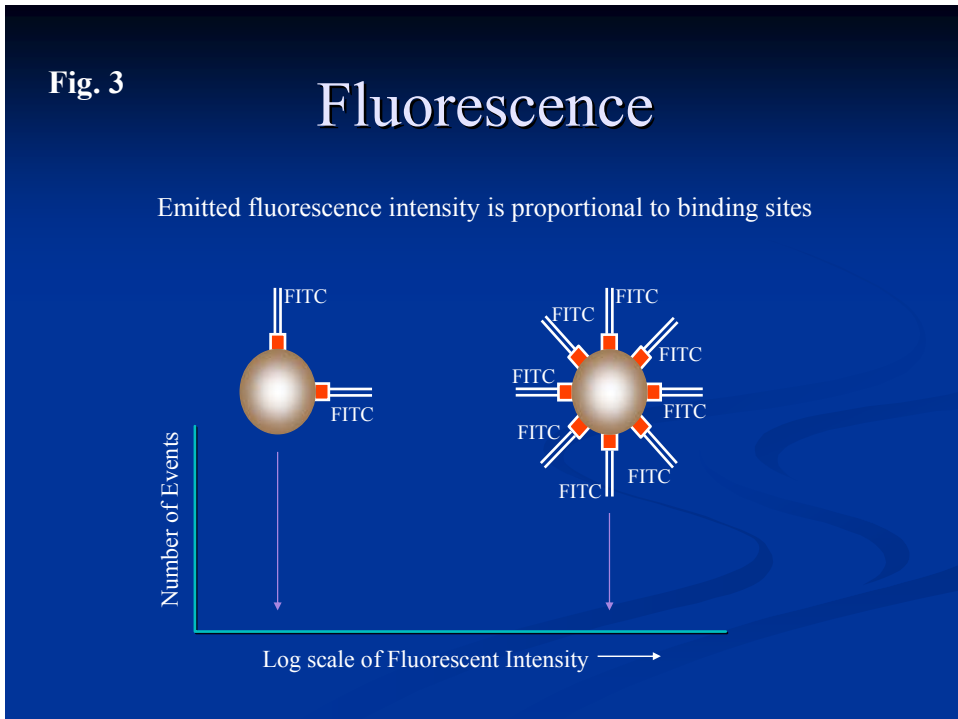
**Figure 1:** The amount of laser light scattered in a forward direction is an indicator of the “relative” size of a cell. We use the term “relative” here because other factors such as the refractive index of a particle will also contribute to the scattering of light in the forward direction. This is referred to as Forward Scatter or FSC. Likewise, laser light scattered at higher angles, i.e. perpendicular to the laser interrogation point ( $90^\circ$ ), gives rise to information relative to the internal complexity and granularity of a cell, such as cytoplasmic granules, multi-lobed nucleus, cytoplasmic vacuoles, etc. This is referred to as Side Scatter or SSC. Light scatter, both high and low angles, can also provide information regarding cell surface properties, i.e. smooth surface vs. dendritic projections. Fluorochromes, once excited by a laser beam, can emit fluorescence in a  $360^\circ$  pattern, however, in a flow cytometer are generally measured at  $90^\circ$  along with SSC.

**Figure 2:** Correlating FSC and SSC measurements provides the ability to discriminate different cell subpopulations in a heterogeneous population, even without the use of a fluorescent probe.

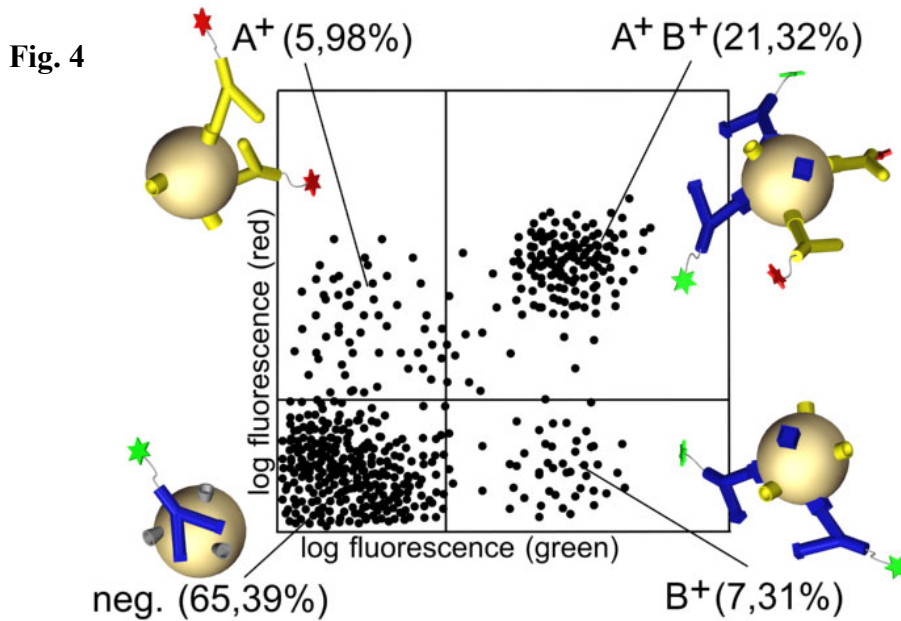
**Fig. 2**



**Figure 3:** With the use of electronic gating, specific subpopulations can then be interrogated with regard to the binding of specific fluorescent probes. The level of fluorescence intensity is proportional to the amount of the target to which the fluorophore is bound.



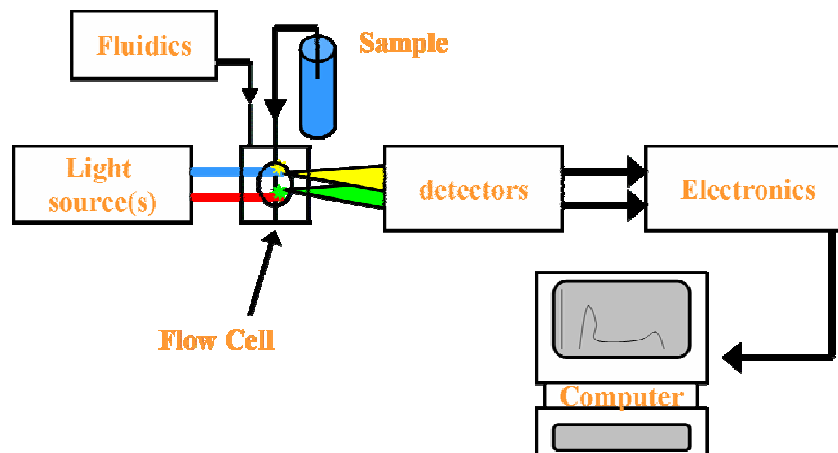
**Figure 4:** The use of multiple fluorescent probes provides information regarding the co-expression of multiple targets, as well as the relative density of each target. These targets can be proteins that are expressed on the surface or inside of the cell which have been identified by the use of a fluorescent antibody, a fluorescent probe that binds to some cellular constituent (DNA, mitochondria, vacuoles, etc) or a fluorescent probe which is an indicator of some cellular function (Ca<sup>++</sup>, Fluorescent protein for gene expression, etc).



## HOW A FLOW CYTOMETER WORKS

A flow cytometer is comprised of three sub systems:

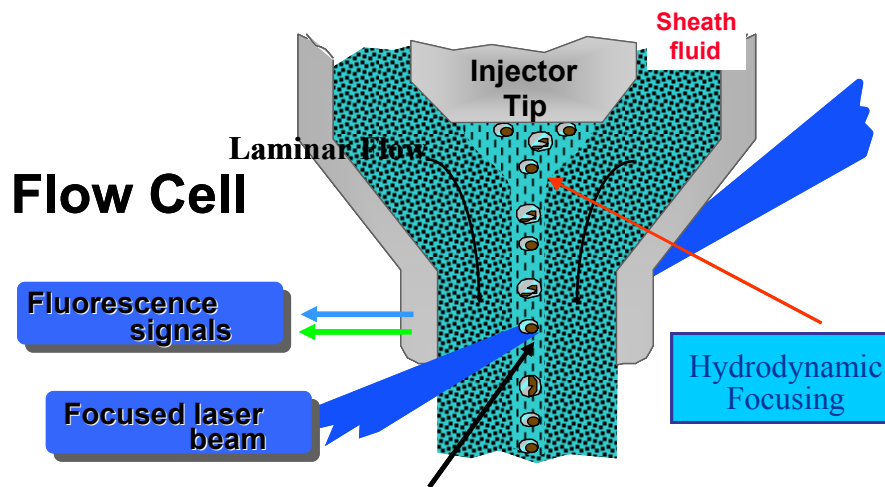
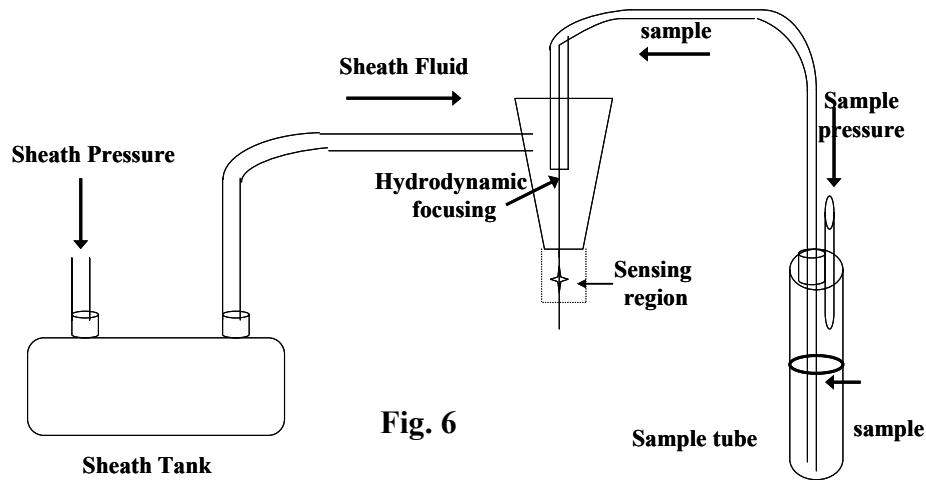
- **Fluidics System** – responsible for the delivery and focusing of cells at the point of laser intercept
- **Optical System** – responsible for the generation and collection of light signals
- **Electronics System** – responsible for the conversion of light signals to a measurable electronic signal, and then measuring, amplifying, and digitizing that signal to be communicated to the computer



### Fluidics System:

The fluidics system is comprised of the flow cell, a pressurized sample, and a pressurized sheath (PBS) tank (Figure 6). Two important processes are used to deliver cells in suspension to the point of laser interrogation so that only one cell is interrogated at a time:

- **Laminar Flow** – the process by which the sample fluid flows in the central core of the sheath fluid without mixing. This is accomplished by a difference in pressure between the sample tube and the sheath tank, with the sample tube being at a high pressure than the sheath pressure so when the sample is injected into the sheath fluid it creates a “core” or space for the sample without mixing with the sheath fluid
- **Hydrodynamic Focusing** – the process by which cells are focused in the center of the laser beam one cell at a time. This is accomplished by forcing a large volume of sample into a very small volume in the flow cell. (Figure 7)

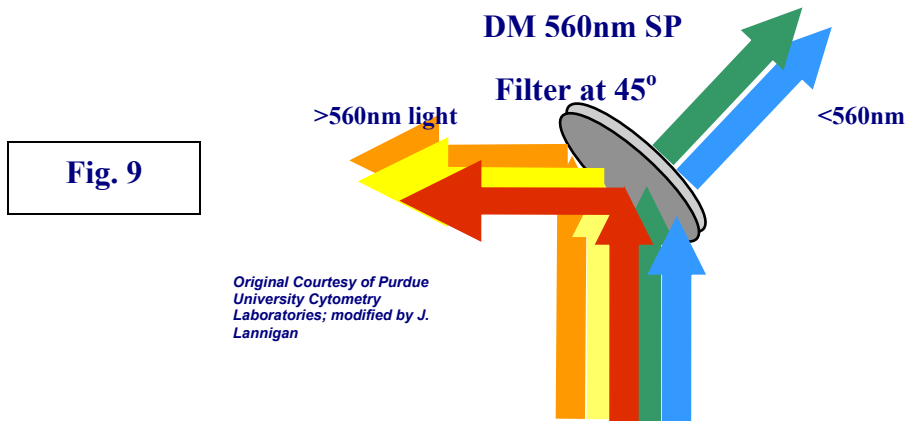
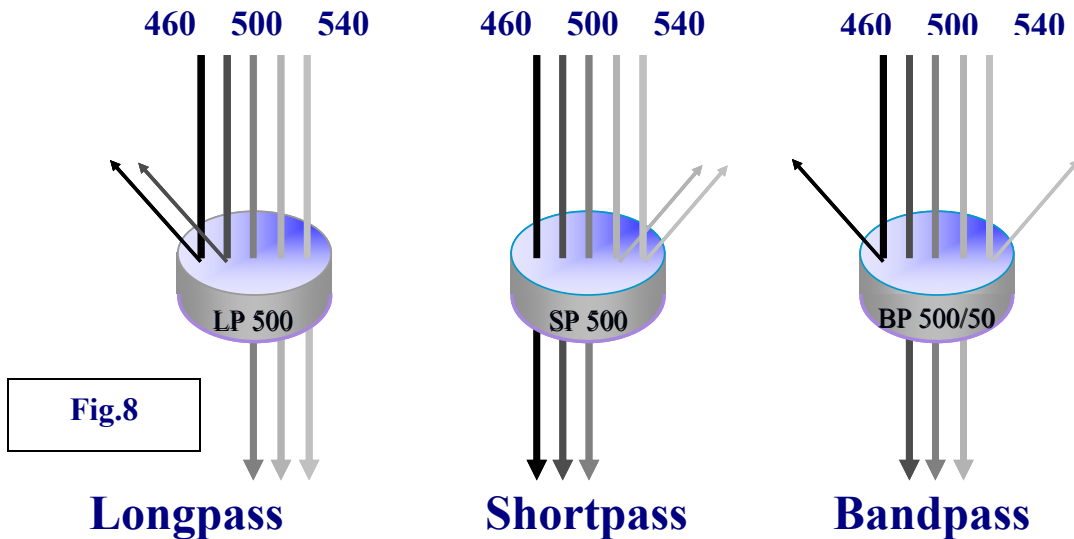


Courtesy of Purdue University Cytometry Laboratories

### Optical System:

The optical system is comprised of light excitation source(s), usually laser(s) of a specific wavelength, and a series of optical filters and mirrors which direct specific wavelengths of light to their appropriate detector.

- **Lasers** – the most commonly used wavelengths of lasers used in flow cytometers are 488nm (blue), 633-637nm (red), 405-407nm (violet), and 514nm (green). Fluorochromes have specific spectra of wavelengths with which they are maximally excited, so the more lasers an instrument has the more fluorescent colors one can measure.
- **Optical Filters/Mirrors** – a flow cytometer uses a combination of long pass, short pass, band pass, and dichroic mirrors to direct specific wavelengths of light to a particular detector
  - **Long Pass Filter** – transmits wavelengths of light equal to or greater than the spectral band of the filter. (Figure 8)
  - **Short Pass Filter** - transmits wavelengths of light equal to or shorter than the spectral band of the filter. (Figure 8)
  - **Band Pass Filter** – transmits wavelengths of light within a specific range of wavelengths. (Figure 8)
  - **Dichroic Mirror** – either a short pass or long pass filter placed at a 45° which splits wavelengths of light into two directions.(Figure 9)



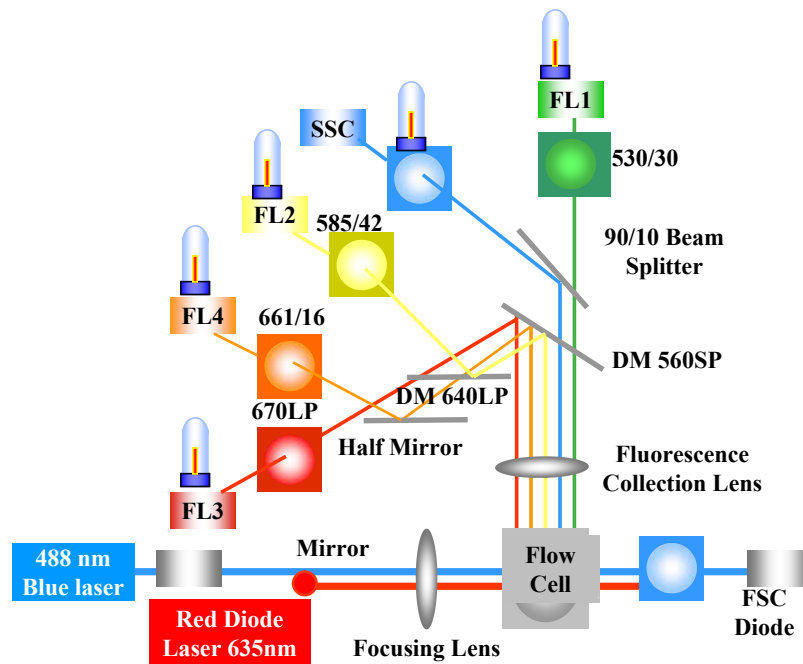


Fig. 10

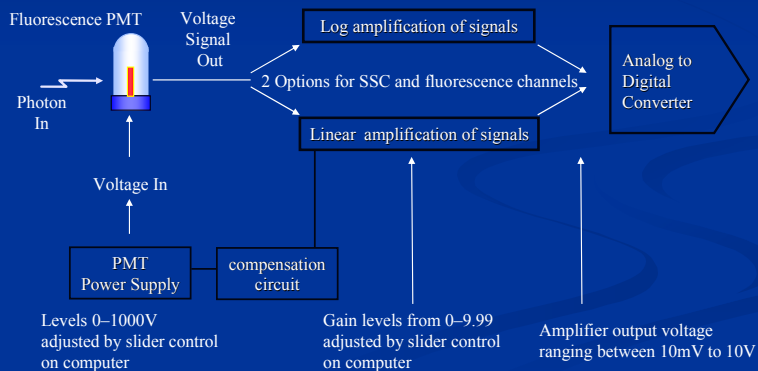
Figure 10: Optical Layout of BD FACSCalibur™ Dual Laser Flow Cytometer

### Electronics System:

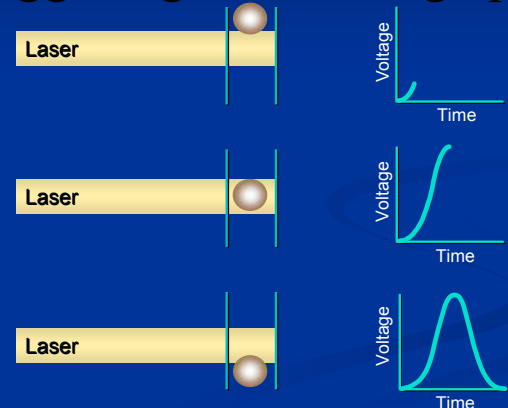
The electronics system is responsible for:

- Converting an optical signal (photons) to a proportional and measurable electronic signal (photoelectrons). This is accomplished with photomultiplier tubes which take in a photon and output several thousand photoelectrons.
- Measuring the amplitude, area and width of the photoelectron pulse
- Compensating for spectral overlap of fluorochromes
- Amplifying the pulse through the use of linear or log amplification pulse boards
- Converting the output from the amplification boards to a digitized value
- Communicating the digitized data to the computer

### Conversion of optical signals to proportional digital output



### Electronics: Triggering on a voltage pulse



### Fluorescence Measurements:

Fluorochromes have specific excitation and emission spectra which are uniquely characteristic to the molecule. When a fluorescent molecule is excited by its appropriate wavelength of light it absorbs that light energy and when the molecule returns to its unexcited state it releases the energy in the form of dissipated heat and photons. Since some of the energy is lost through heat the photons that are released are of a lower energy (longer wavelength) than the exciting wavelength. This is referred to as the “Stokes Shift”. (Figure 12) The fluorochrome molecule which can be excited by a range of wavelengths will therefore emit a range of wavelengths of light. This is referred to as the excitation and emission spectra. A fluorochrome molecule will have an excitation (or absorption) maximum, as well as an emission maximum. These are the wavelengths which optimally excite the fluorochrome as well the strongest emitted wavelengths. A fluorochrome may have several different ranges of excitation but it will always emit over the same range of wavelengths but to different extent (Figure 13).

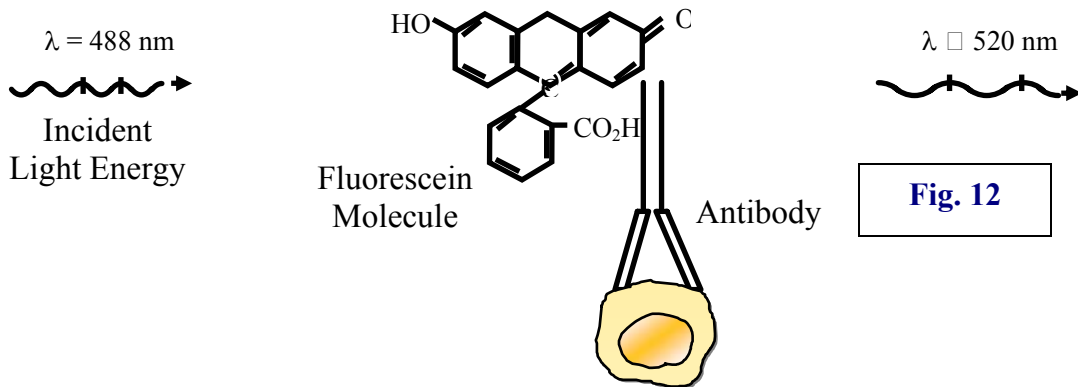


Fig. 12

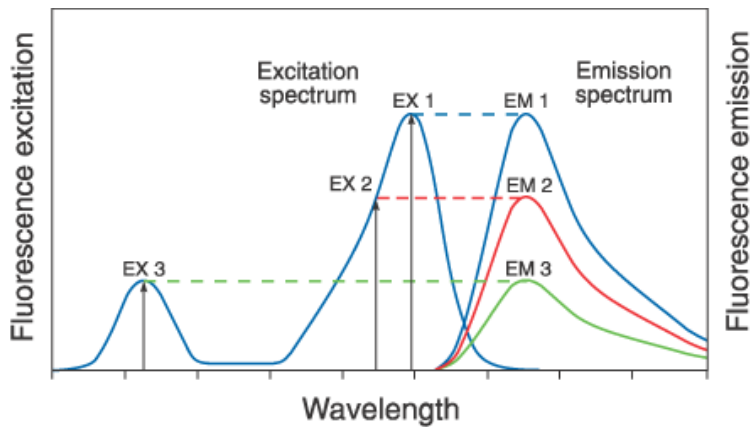


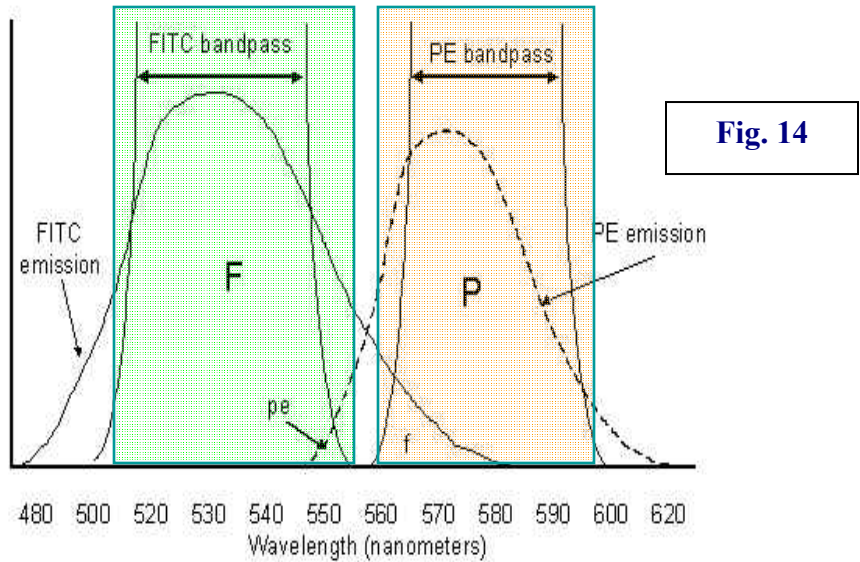
Fig. 13

### Spectral Overlap:

The optical filters in a flow cytometer are designed to capture a range of wavelengths which are centered around the maximum emission of specific fluorochromes. Because fluorochromes emit over a range of wavelengths there is the potential of some of the wavelengths to be detected in other detectors (Figure 14). In order to be sure your measurements in each of your fluorochrome specific detectors are coming only from that specific fluorochrome there must be some way to “compensate” for the spectral overlap



from the other fluorochromes. In order to do this one must have a set of “compensation” controls, each of which only contains one of your fluorochromes to be used in an experiment. In this way, one will be able to see to what extent each fluorochrome overlaps into the other fluorescent detectors. By doing so we are able to “subtract” the overlapping signal from the other detectors. This process is referred to as “Spectral Compensation” and can be accomplished either through the electronics or the software (if available).



**Fig. 14**

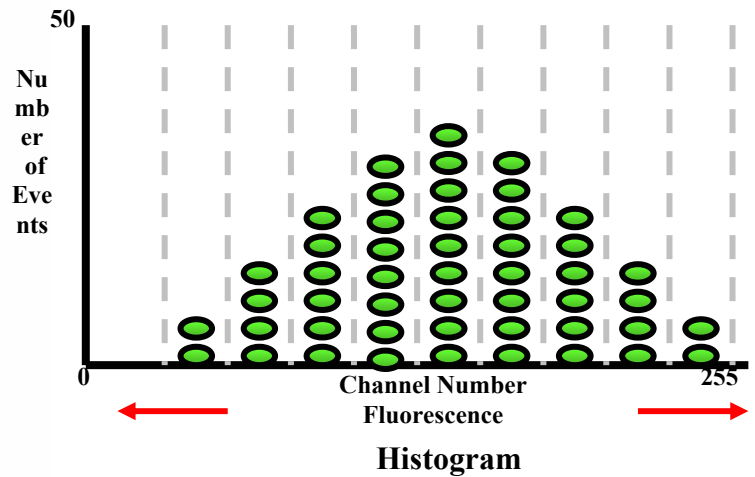
**Data Analysis**

Once a signal has been digitized, this digitized value is assigned a “channel” number to which it will be plotted on a graph. This is done for each cell for each parameter collected (i.e. FSC, SSC, FL1, FL2, etc). This data is stored in the form of a list for each cell as it came through the cytometer. **(Figure 15)** Once all the data is stored in this manner, which is referred to as “Listmode”, one can analyze any of the parameters as a single parameter or a combination of correlated parameters. **(Figure 16)** There are also multiple options for the way in which data can be displayed.

**FCS DATA FILE (TRANSLATED)**

CELLS IN SEQUENCE	FSC-H	SSC-H	FL1-H	FL2-H
1	120	28	152	24
2	190	169	42	60
3	175	149	56	52
4	107	25	149	0
5	97	22	151	26
6	174	136	47	36
7	190	127	42	47
8	106	14	148	0
9	66	17	165	23
10	90	16	149	31
11	184	163	58	50
12	191	160	39	48
13	101	24	152	19
14	126	36	153	0
15	126	28	157	0
16	96	17	155	0
17	215	224	61	59
18	165	95	55	46
19	173	73	49	43
20	91	27	158	0
21	210	180	59	52
22	179	161	60	52
23	165	93	54	35
24	187	45	67	52
25	192	184	46	50
26	111	25	149	17
27	207	206	58	40

**Fig. 15**



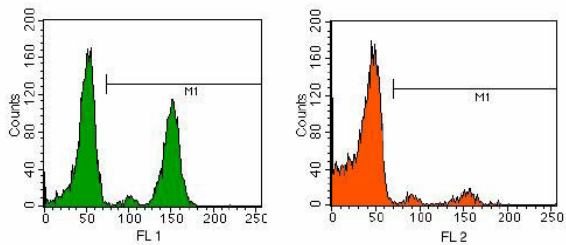
**Fig. 16**

## Data Display Options

- **Histograms** – used for displaying single parameter data where the “X” axis represents scatter or fluorescence intensity and the “Y” axis represents the number of events. Histograms represent a single dimension and as a result have limited usefulness. They are best suited for well resolved homogeneous populations or for comparing intensities of multiple samples for a single parameter. **(Figure 17A)**
- **Dot Plots** – plots data values for each cell for any 2 acquired parameters “X” and “Y” where one dot or point on the display is related to the amount of parameter “X” and “Y” for each cell which passed through the instrument These are good for detecting small numbers of events which are well separated from the main populations of the cells present but give little or no indication of the relative density of numbers of events in populations. **(Figure 17B)**
- **Density Plots** - simulates a three dimensional display of events with the " *third*" parameter being the number of events. These can dramatically emphasize or hide altogether, populations of cells. **(Figure 17C)**
- **Contour Plots** - another two dimensional display of relative X and Y amounts of two parameters, with contour lines being drawn to form x and y co-ordinates which have similar numbers of cells. **(Figure 17D)**

SINGLE COLOR HISTOGRAM WITH STATS

Fig. 17

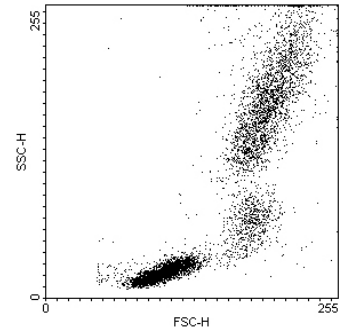


Total Events: 6000

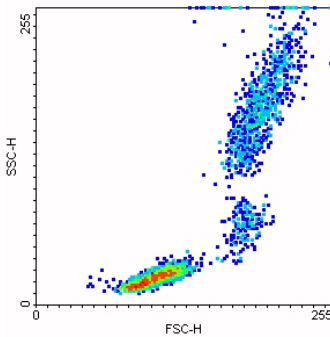
Marker	Mean	Median	Peak Ch	% pos
All	87.76	58.00	56	
M1	146.08	149.00	150	43

Total Events: 6000

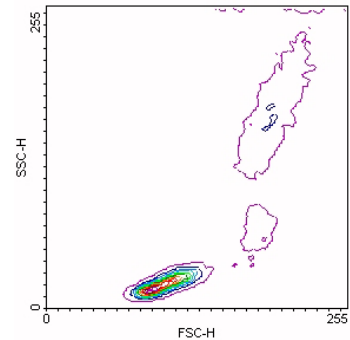
Marker	Mean	Median	Peak Ch	% pos
All	41.32	42.00	0	
M1	134.06	148.00	156	8



Dot Plot



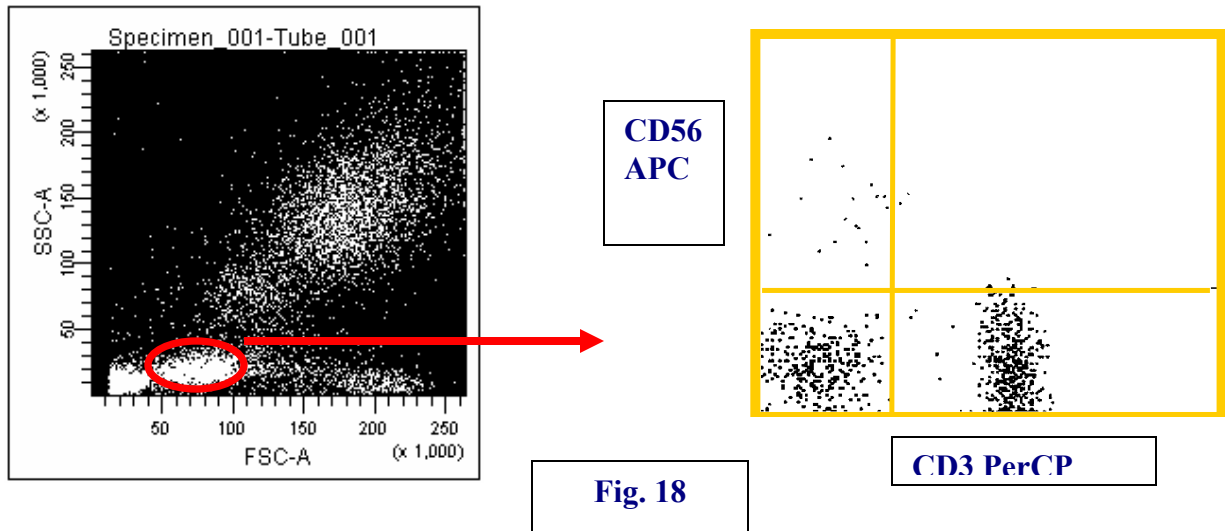
Density Plot



Contour Plot

- **Gating**

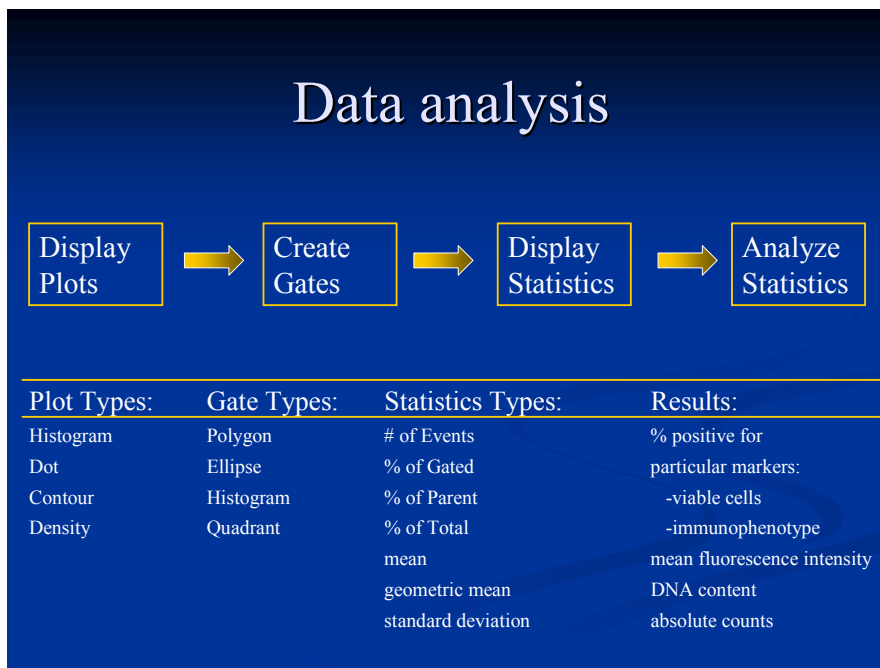
Gating is the process by which data is reduced by selecting populations with specific characteristics for display in one, two or three dimensions for further analysis. For example, if one has a heterogeneous population of cells which contain lymphocytes, monocytes, and granulocytes and is only interested in evaluating the fluorescence of the lymphocyte subpopulation one could define an analysis gate around the lymphocyte population and then display any other fluorescent parameter through that gate. **(Figure 18)**



### Data Analysis

Analyzing data is a process by which you:

- display data
- establish your regions of interest through gating
- calculate statistics for relevant parameters
- analyze the statistics and determine significance

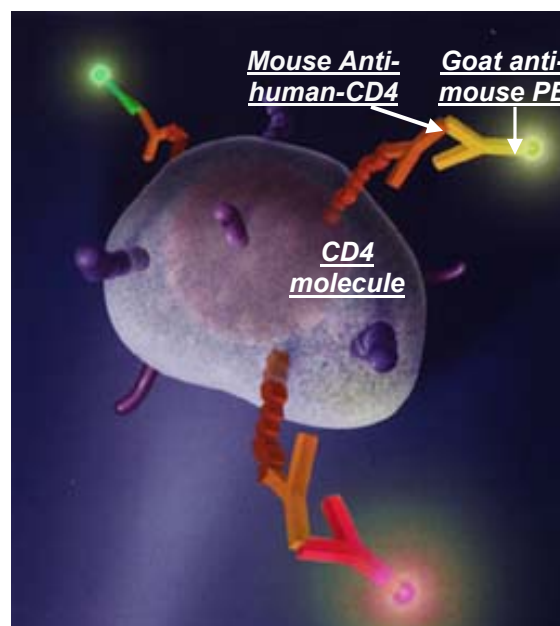
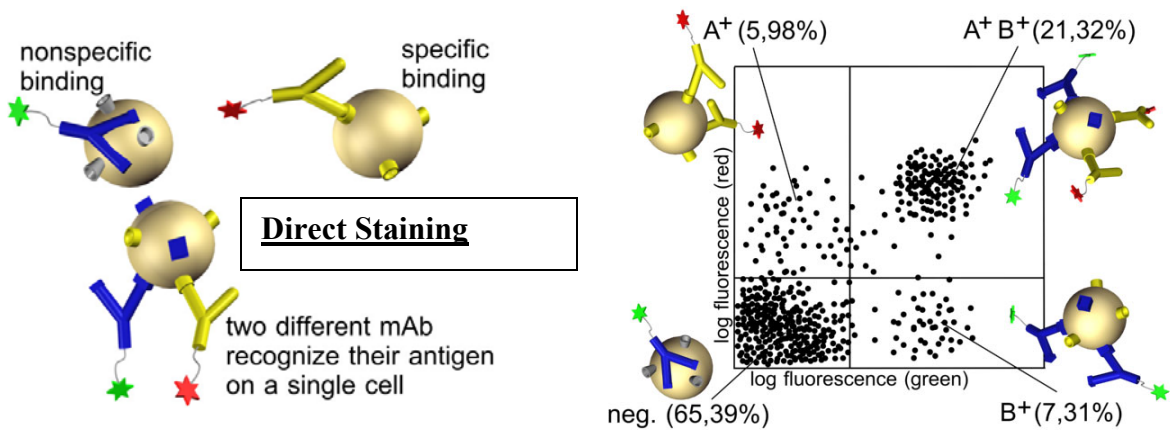


## Common Applications

Flow cytometry is adaptive to studying many types of biological questions. These can be broken up into several main categories:

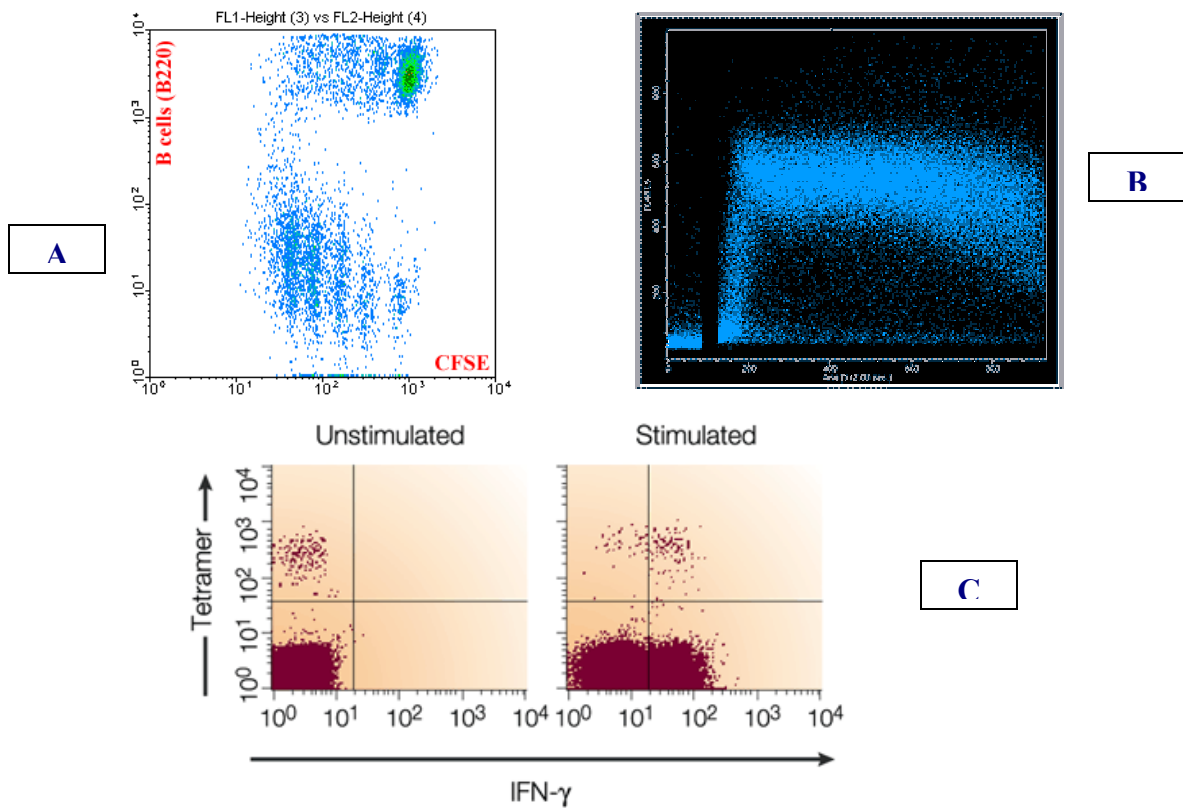
- **Immunophenotyping** – most commonly used application in which cells are labeled with a monoclonal or polyclonal antibody to some cell surface protein to identifying the presence, absence or levels of cell surface molecules to determine:
  - Cell lineage
  - Activation status
  - Adhesion, migration, and homing capacity
  - Ability to respond to stimuli
  - Ability to interact with other cells

This procedure can be performed with either an antibody that is directly conjugated with a fluorescent probe (direct) or with a second antibody conjugated with a fluorescent probe that is specific for the first antibody (indirect).



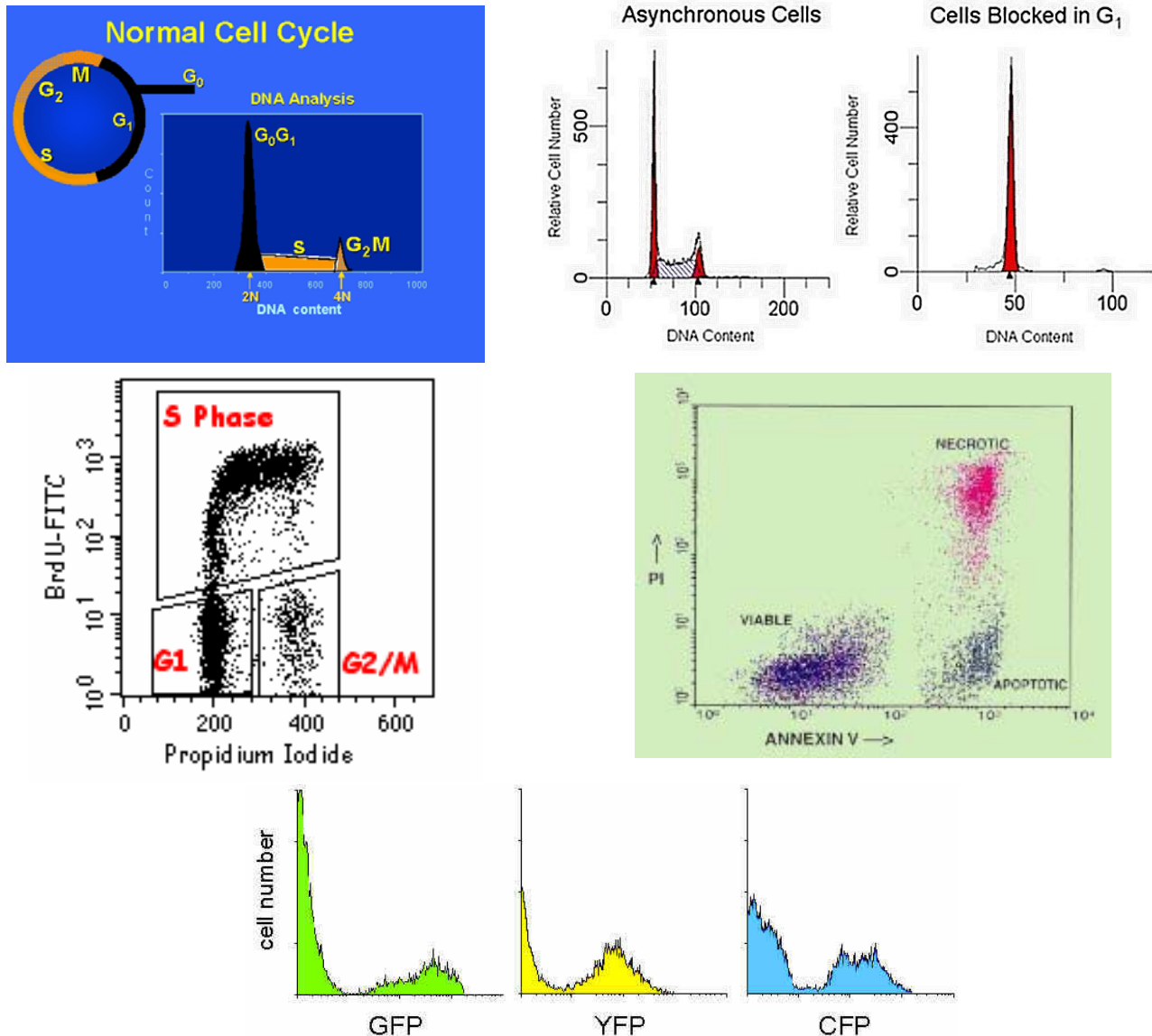
## Indirect Staining

- **Functional Assays** – these types of applications primarily involve the measurement of functional byproducts as an indicator of the cells capacity to respond to a specific set of conditions. Some examples of these types of experiments are:
  - Intracellular cytokines
  - Ca<sup>++</sup> flux
  - Intracellular pH
  - Phosphorylation of proteins
  - Reactive Oxygen Species
  - Cellular proliferation
  - Phagocytosis



**Figure 19 – (A) Cellular proliferation measured by the dilution of the fluorescent dye CFSE with each round of division. Each daughter cell contains half the amount of fluorescence as the parent. (B) Ca<sup>++</sup> Flux as measured by the dye Fluoro 4 which is non-fluorescent except when bound to Ca<sup>++</sup>. The area to the left of the blank spot is the baseline, the blank area is where the sample was stopped and the stimulus added, and the area to the right of the blank area is the increase in fluorescence intensity due to an influx of Ca<sup>++</sup>. (C) Intracellular measurement of IFN-γ on antigen specific T cells which have bound the antigen specific peptide.**

- **DNA Studies** – these assays involve the measurement of fluorescence associated with DNA to determine:
  - Stages of cell cycle
  - Apoptosis
  - Gene transfections
  - Chromosomal aberrations
  - Endoreduplication
  - Proliferation checkpoints (cyclins)
  - Doubling times



**Fig. 20 – (A) Fluorescent DNA dyes exhibit stoichiometric binding so that the fluorescence intensity of the dye correlates to the amount of DNA content (2N vs. N) from which cell cycle statistics can be calculated. (B) Synchronizing cells by blocking them in a particular phase of the cell cycle with drugs and then releasing them provides an estimate of the cell’s doubling time. (C) Culturing cells with BrdU and then labeling them with a fluorescent antibody to BrdU and a DNA dye is an accurate way to estimate the % of cells in S phase. (D) The binding of Annexin V to phosphatidyl serine on the outer membrane of a cell is a marker of apoptosis. (E) Transfecting a fluorescent protein gene along with a gene of interest is a way to determine which cells have been transfected with your gene of interest.**

## **Summary**

Flow Cytometry is a very powerful and valuable tool for evaluating many immunological processes. The technology incorporates the use of lasers, detectors, filters, fluidics, electronics, fluorescence and light scatter to provide great detailed data with regard to a cell's size, shape, health, cell surface and intracellular expression of proteins, functional byproducts, ability to proliferate, and gene expression. Each of the instrument's subsystems; fluidics, optics, and fluidics, plays a critical role in the accuracy of the data that is collected. Because of its ability to collect thousands of data points in short periods of time it provides great statistical power when analyzing data. Software programs allow the gating and manipulation of all acquired parameters (FSC, SSC and all FL channels) for large data sets to provide statistically relevant results.