

## Introduction

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5µm to 40µm diameter.

Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source (See Figure 1.1). Lasers are most often used as a light source in flow cytometry.

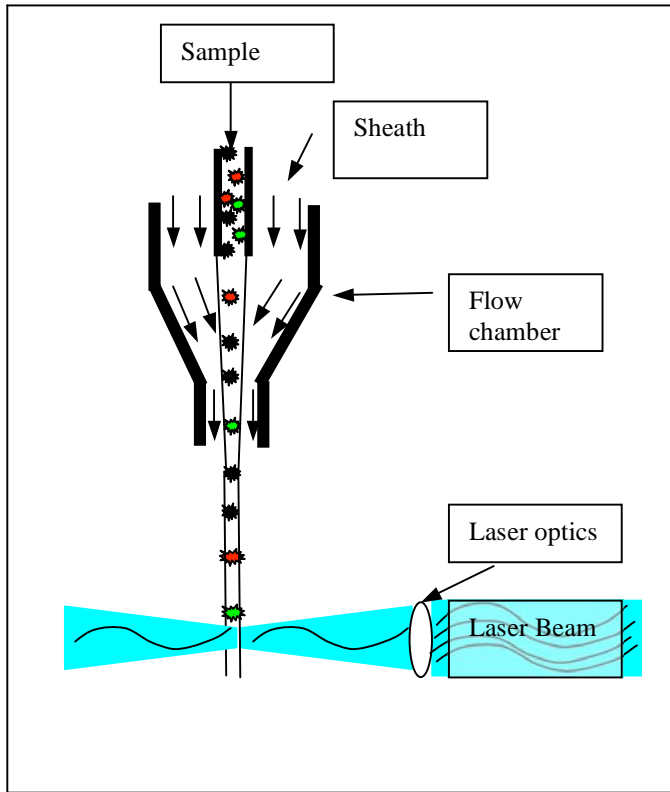


Figure 1.1 Flow cytometers use the principle of hydrodynamic focusing for presenting cells to the laser (or any other light excitation source). The sample is injected into the center of a sheath flow. The flow chamber is conical in shape, the reduced diameter forces the cell into the center of the stream. This way the cell passes through the laser one cell at a time. This schematic of the flow chamber in relation to the laser beam in the sensing area. (From Current Protocols In Cytometry, Unit 1.2, p 1.2.2)

As your cells or particles of interest intercept the light source they scatter light and fluorochromes are excited to a higher energy state. As the molecules relax to a lower state, energy is released as a photon of light with specific spectral properties unique to different fluorochromes (see Table 1.1 for a listing of commonly used fluorescent dyes and their excitation and emission spectra. This table also includes the most common laser light sources with their multiple lines of emission).

One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This contrasts with spectrophotometry in which the percent absorption and transmission of specific wavelengths of light is measured for a bulk volume of sample.



**Table 1.1(Previous page)** Fluorescence spectra of commonly used fluorochromes. Excitation spectra are represented by the gray lines while emission spectra is in black. The bottom part of the table summarizes the emission wavelengths of various light sources used in flow cytometry. The 488nm line of the argon ion laser is extended over the spectra. (From Practical Flow Cytometry, Third Edition, Howard M. Shapiro. P. 245)

Scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors. Collimated (parallel light waveforms) light quanta is picked up by confocal lenses focused at the intersection point of cells and the light source. Light is send to different detectors by using optical filters. For example , a 525 nm band pass filter placed in the light path prior to the detector will only allow “green” light into the detector. The most common type of detector used in flow cytometry is the photomultiplier tube (PMT). (See Figure 1.2 for a basic layout of the optical components in flow cytometry). PMT,s then convert light into electrical pulses.

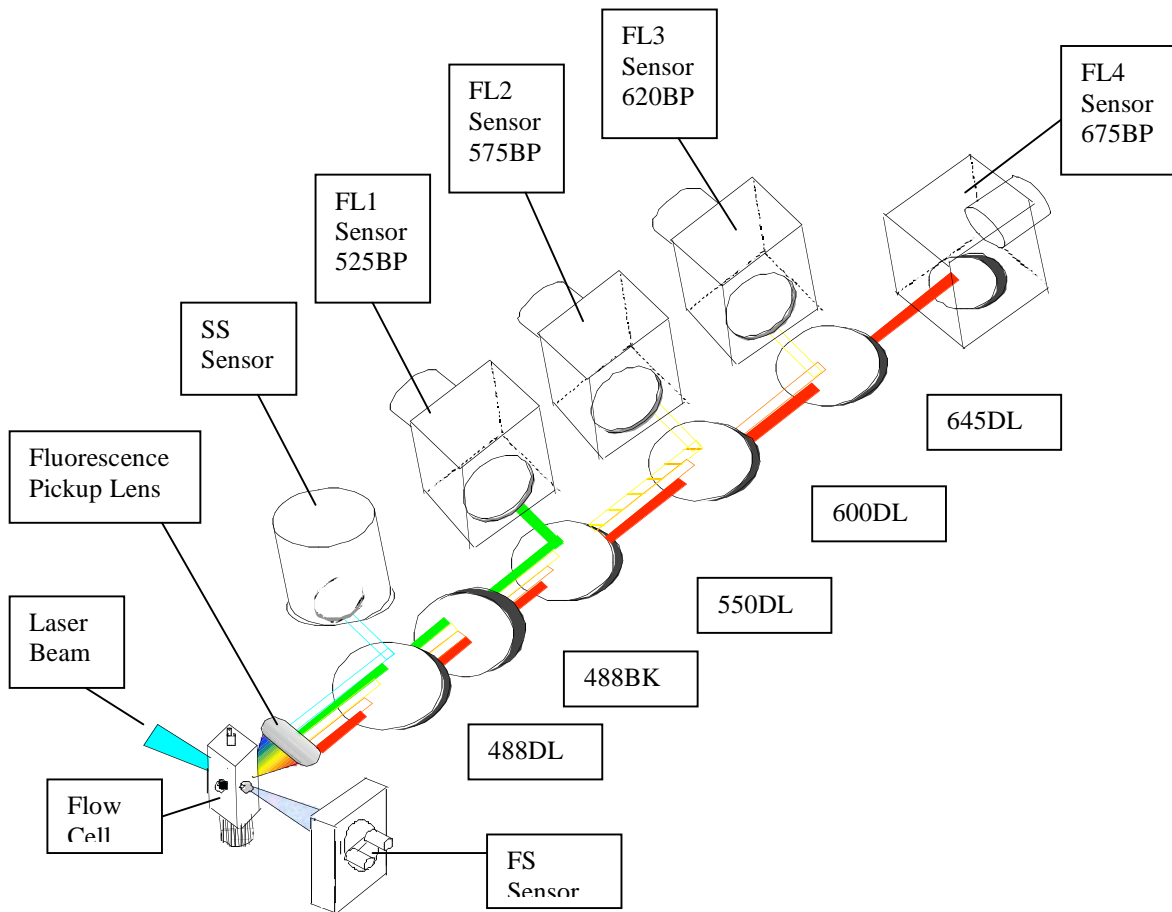


Figure 1.2 shows the optical system schematic for the XL analyser as well as the optical configuration for the four FL PMT sensors.

The electrical pulses originating from light detected by the PMTs are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for “strong” or specific fluorescence signals.

After the different signals or pulses are amplified they are processed by an Analog to Digital Converter (ADC) which in turn allows for events to be plotted on a graphical scale(One Parameter, Two parameter Histograms).

Flow cytometry data outputs are stored in the form of computer files using the FCS 2.0 or 3.0 standard. Data corresponding to one sample can be stored as a listmode file and/or histogram file.

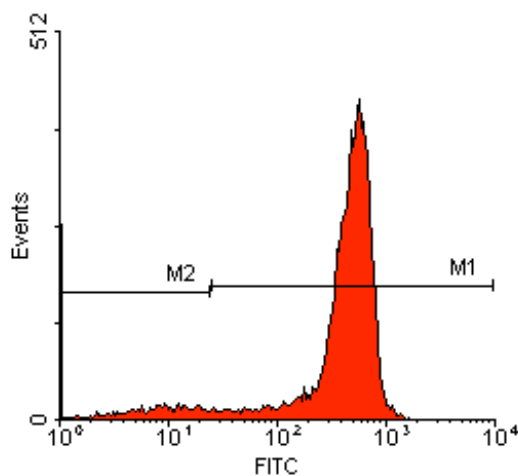
### **Histogram Files**

Histogram files can be in the form of one-parameter or two-parameter files.

Histogram files consist of a list of events for a 1 Parameter or to parameter histogram.

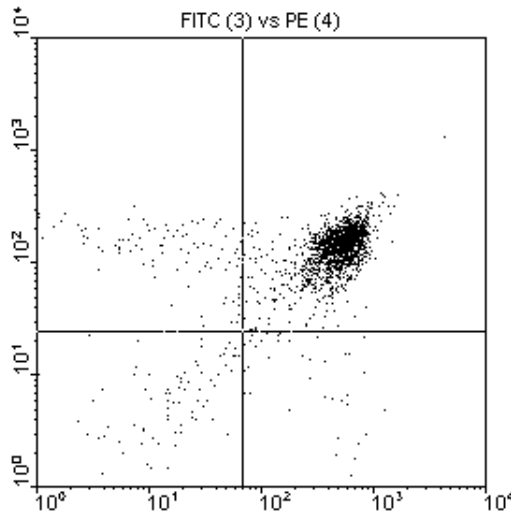
#### **One-Parameter Histograms**

A one-parameter histogram is a graph of cell count on the y-axis and the measurement parameter on x-axis. All one-parameter histograms have 1,024 channels. These channels correspond to the original voltage generated by a specific "light" event detected by the PMT detector. In other words, the ADC assigns a channel number based on the pulse height for individual events. Therefore, brighter specific fluorescence events will yield a higher pulse height and thus a higher channel number when displayed as a histogram.



## Two-Parameter Histograms

A graph representing two measurement parameters, on the x- and y-axes, and cell count height on a density gradient. This is similar to a topographical map. You can select 64 or 256 channels on each axis of two-parameter histograms. Particle counts are shown by dot density or by contour plots.



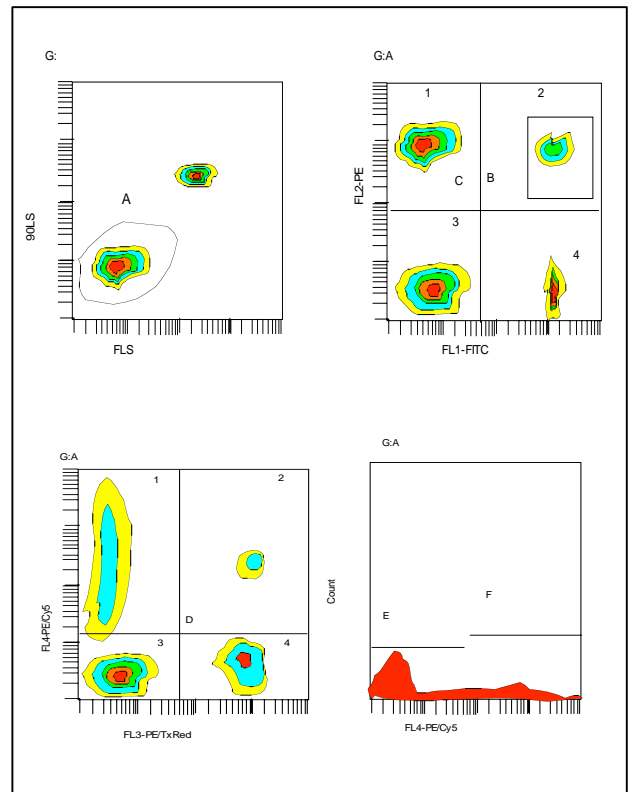
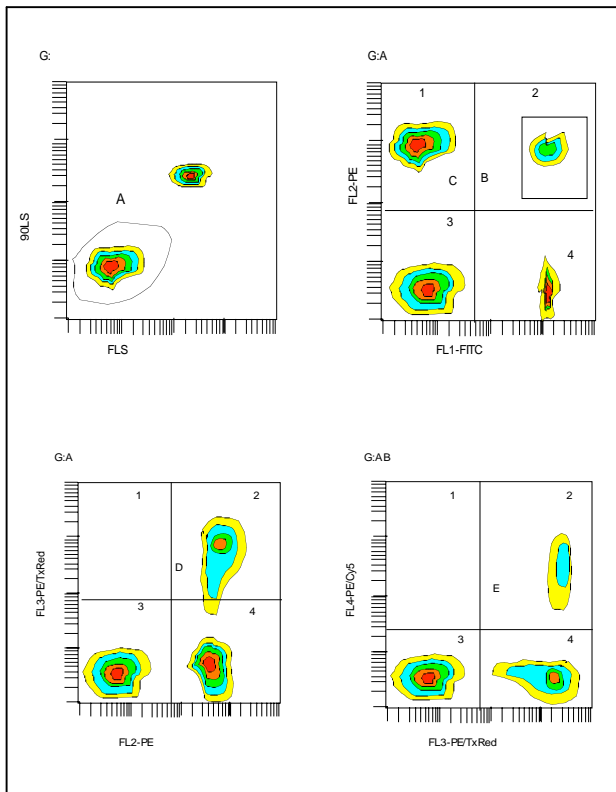
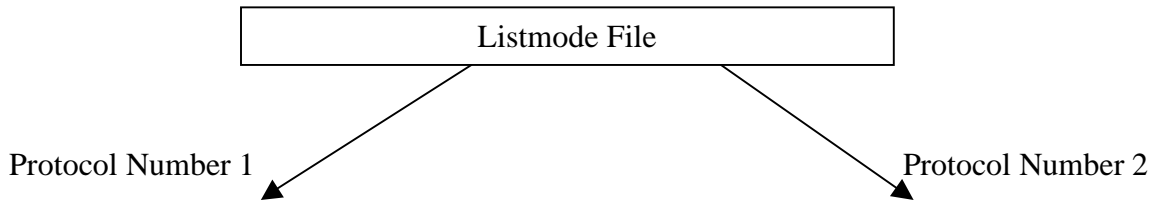
Two parameter histogram Dot Plot displaying FL1-FITC on the x axis and FL2-PE on the y axis.

## Listmode Data Files

Listmode files consist of a complete listing of all events corresponding to all the parameters collected, as specified by your acquisition *Protocol*. This file follows a format specified by the FCS 3.0 standard. Raw listmode data files can be opened or replayed using any program designed for analysis of flow cytometry data. You should keep in mind that a *Protocol* or *Layout* serves as a *template*. It allows you to specify *Parameters* of interest (i.e. FLS, FL1, FL2, etc.), and how these parameters are displayed. *Protocols* and *Layouts* also serve to determine how the data is *Gated*, and contains all the *Regions* from which your statistics will be generated. In addition, *Protocols* contain other specific information that serves as direct interface between the computer workstation and the cytometer. These pertain to high voltage settings for the PMT detectors, gains for amplification of linear parameters, sample flow rates, fluorescence compensation, discrimination settings, etc..

Once your data has been collected and written into a listmode file you can replay the file either using the specific *Protocol* used for collection or any other program specifically designed for analysis of flow cytometry data. However, you should keep in mind that you

can only adjust *Regions*, *Gating*, and *Parameters* to be displayed. Settings such as amplification voltages, and thresholds can not be modified. Depending on the software platform, older data may not be suitable for offline fluorescence compensation. Newer acquisition platforms offer the convenience of offline fluorescence compensation. Therefore, when collecting data make sure that your instrument settings are correct. Finally, if you open your listmode files using a programs such as FlowJo, WINMIDI, FCSExpress, etc..

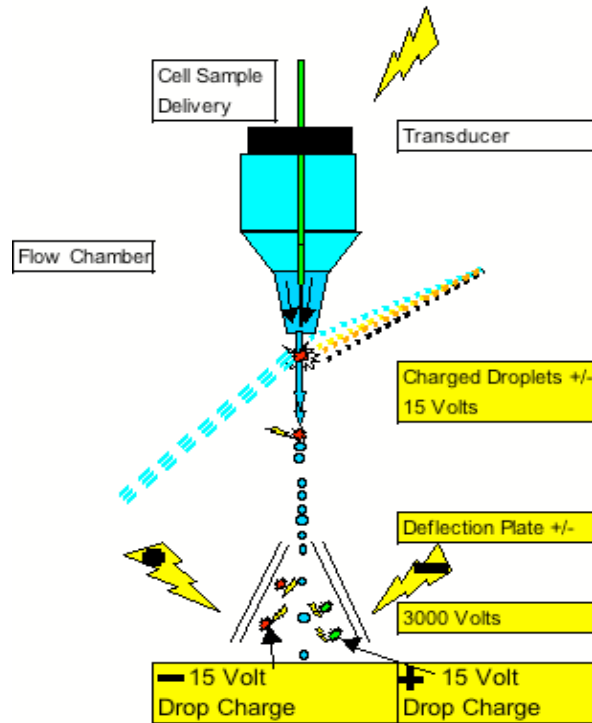


### Flow Cytometry Analysis and Sorting.

Flow cytometry analysis of a single cell suspension yields multiparameter data corresponding to Forward Light Scatter (FLS), 90° Light Scatter (90LS), and FL1-FLn. This information allows researchers to identify and characterize various subpopulations of cells. The process of separating cells using flow cytometry multiparameter data, is referred to as sorting.

The Beckman-Coulter XL instruments are bench-top, flow cytometer, analyzers. They are capable of acquiring multiparameter flow cytometry data but they can not separate or purify cells. **XL analyzers have self enclosed fluidics and thus do not generate aerosols.** Sorting is a specialized process that requires sophisticated electronic components not incorporated into most bench-top instruments.

Schematic of Sorting Components



The Beckman-Coulter ELITE-ESP is representative of a research grade cell sorter. Sorters include the following components:

A tunable transducer which permits the breaking of the fluid sheath into individual droplets. These individual droplets will encapsulate single cells.

Electric charge delays for charging individual droplets.

Deflection plates for deflecting individually charged droplets into collection tubes. Software settings for defining sorting criteria, these include regions defining populations to be sorted.

### Facility description

Our facility consists of 3 Beckman-Coulter EPICS XL flow cytometers. They use an argon ion air cooled laser (emission at 488nm/15mW power), both have 5 PMT detectors for 90LS and 4 color detection and 1 diode detector for FLS.

### Color Detectors

<u>Channel</u>	<u>Filter</u>	<u>Color</u>	<u>Fluorochrome</u>
FS	Forward Light Scatter(Size)	488nm	None
SS	Side Scatter(Granularity)	488nm	None
FL1	525nm Band Pass Filter	Green	FITC
FL2	575nm Band Pass Filter	Orange-Red	PE, PI
FL3	620nm Band Pass Filter	Red	613, (PE-Texas red)
FL4	675nm Band Pass Filter	Deep Red	Tricolor, Cychrome (PE-Cy5 Tandem)

Each instrument has its own computer workstation and laser printer. We currently use System II Data Acquisition and Display software. This program works in a DOS/Win 95-98 operating system environment.

In addition, each cytometer workstation has an Iomega ZIP 250mb disk drives for data downloading and CD burners for data archival.

We also have a Pentium II ,DELL computer with a HP LaserJet 2200 printer. This PC has WinCycle(Phoenix Flow Systems) for cell cycle analysis , WinMIDI for analysis of listmode flow cytometry data, and Summit (Dako-Cytomation).

### Cell Sorting

**Beckman-Coulter EPICS Elite cell sorter** equipped with an argon ion 15mW air cooled laser, a He-Ne 15mW air cooled laser, and a water cooled 5W argon ion laser.

This instrument is capable of sorting at data rates approaching 20 million events per hour. Sorting time has to be arranged with the facility's operator. It is not unusual to schedule sort time 2 weeks in advance.

**Dako-Cytomation MoFlo High Speed sorter** is equipped with 2 water cooled I-90 argon ion lasers tuned for 488nm and UV excitation and a Spectrum I-70, argon-krypton mixed gas laser tuned for 647nm excitation. This instrument can process upwards of 60 million cells per hour.

In addition, as with all flow cytometry samples, you have to **filter your sample through a 40um nylon mesh to remove large clumps of cells and debris.**

Recovered cells are generally sterile and can be subsequently cultured.

### Fees

<u>Instrument</u>	<u>UC Users Fee/Hour</u>	<u>Non-UC Users Fee/Hour</u>
XL Analysers	\$20.00	\$40.00
Elite Cell Sorter	\$40.00	\$80.00
MoFlo Sorter	\$60.00	\$120.00

