Flow cytometry – a step by step technical approach

Based on:

Introduction to Flow Cytometry:
A Learning Guide
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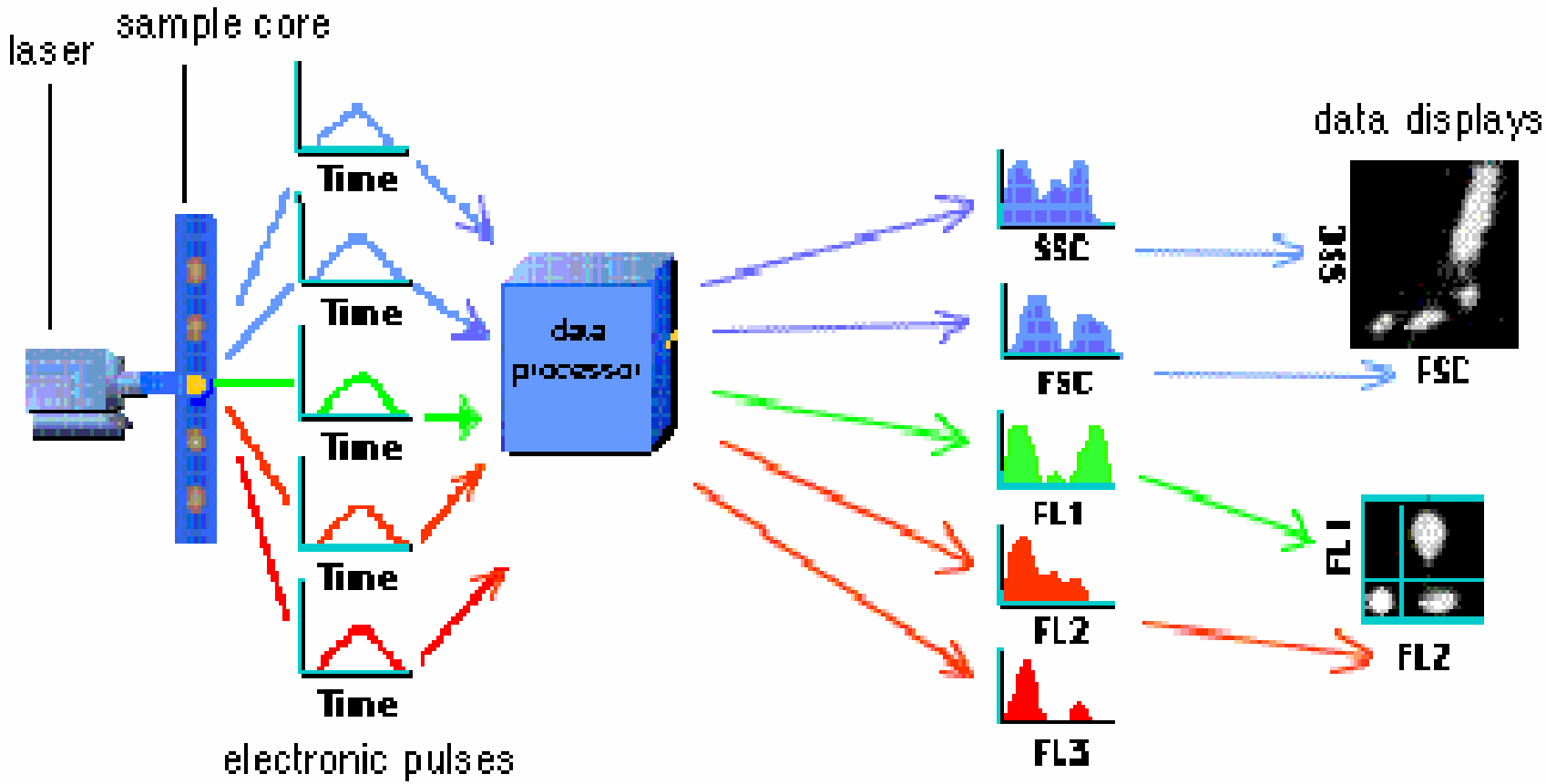
Overview

- Flow Cytometry measures and analyzes characteristics of cells:
  - relative size
  - relative granularity
  - relative fluorescence intensity
- A flow cytometer is made up of:
  - Fluidics system transporting particles to the laser beam
  - Optics system consisting of lasers to illuminate particles in the sample stream
  - Electronics system which converts the detected light signal into electric signals, which are processed by a computer

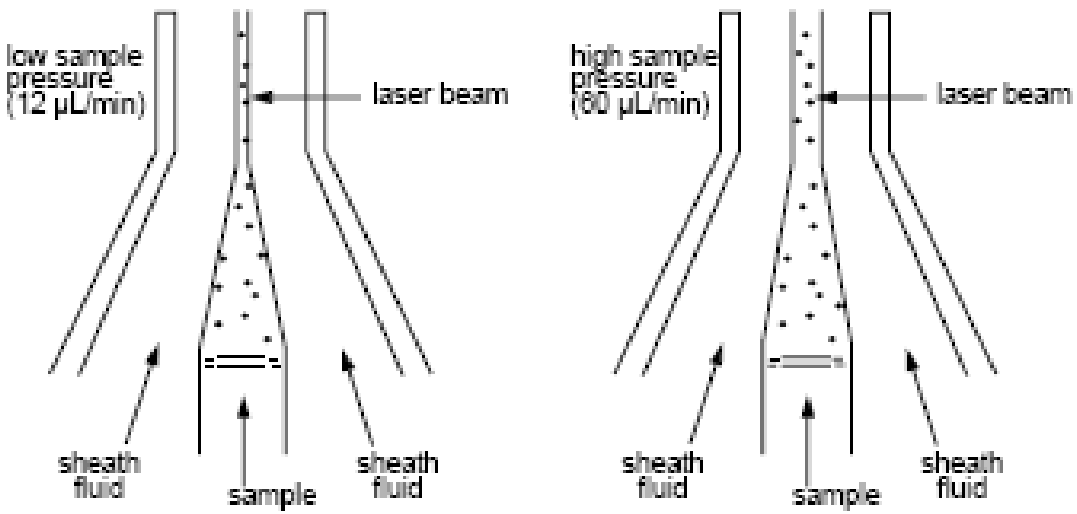
Overview



Scattered and emitted light signals are converted to electronic pulses that can be processed by the computer

Fluidics

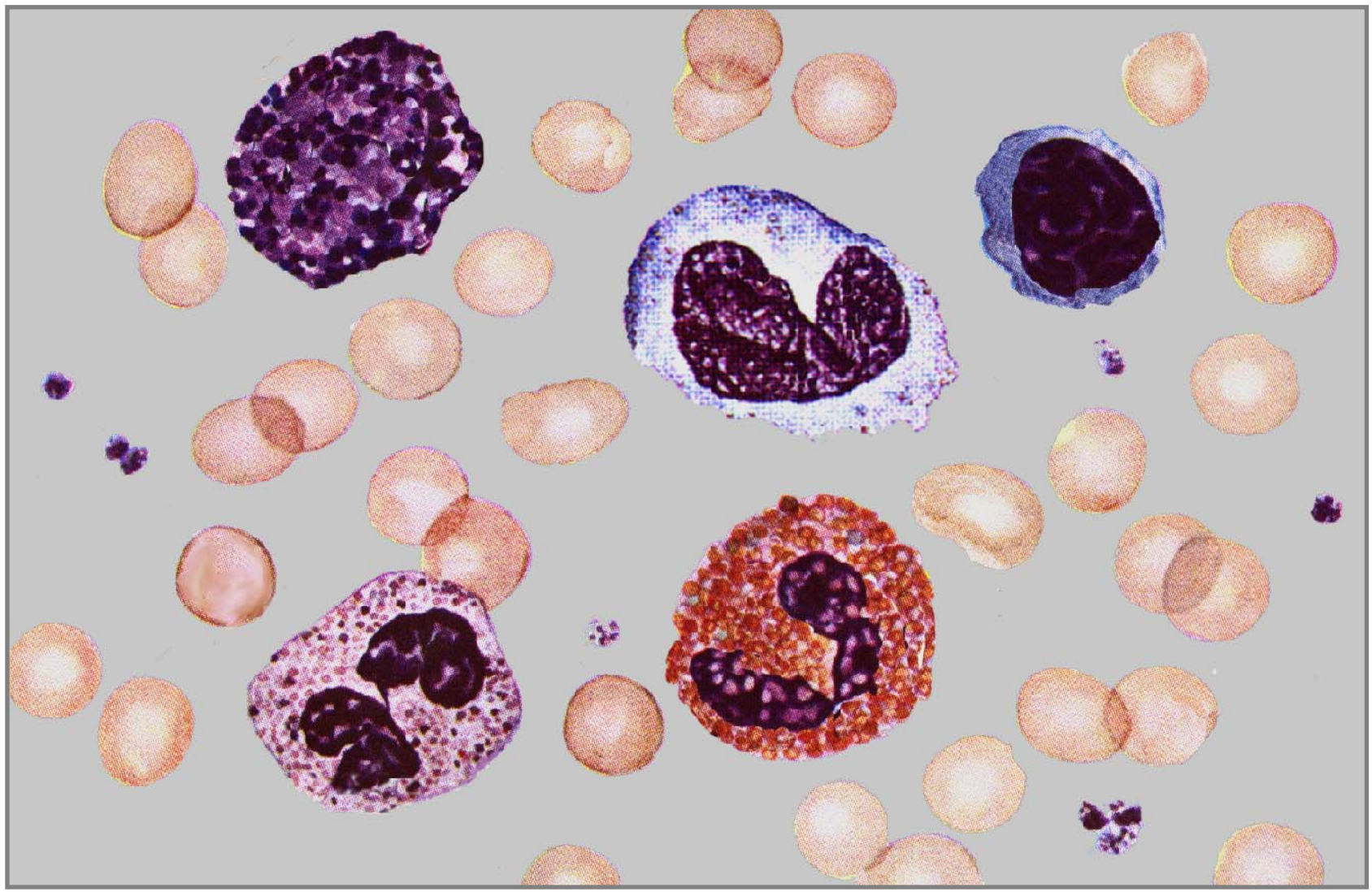
- Blood or bone marrow, or other cells in liquid suspension, are injected into a stream of sheath fluid within the flow chamber:
  - One cell at the time moves through the laser beam at any time
  - The laser beam will then interact with the cell



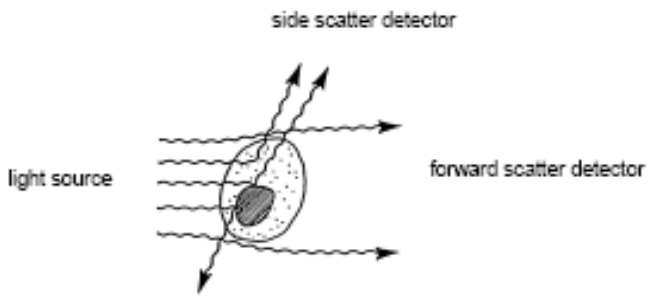
Generation of Scatter

- Light scattering occurs when a cell deflects laser light. The extent to which this occurs depends on:
  - Size of the cell
  - Internal complexity (granularity)
- Forward Scatter (FSC) is proportional to cell size
- It consists of diffracted light, and is just detected off the axis of the laser beam
- Side scattered light (SSC) is proportional to cell granularity of internal complexity
- It consists of refracted and reflected light, and is collected at approx. 90 degrees to the laser beam

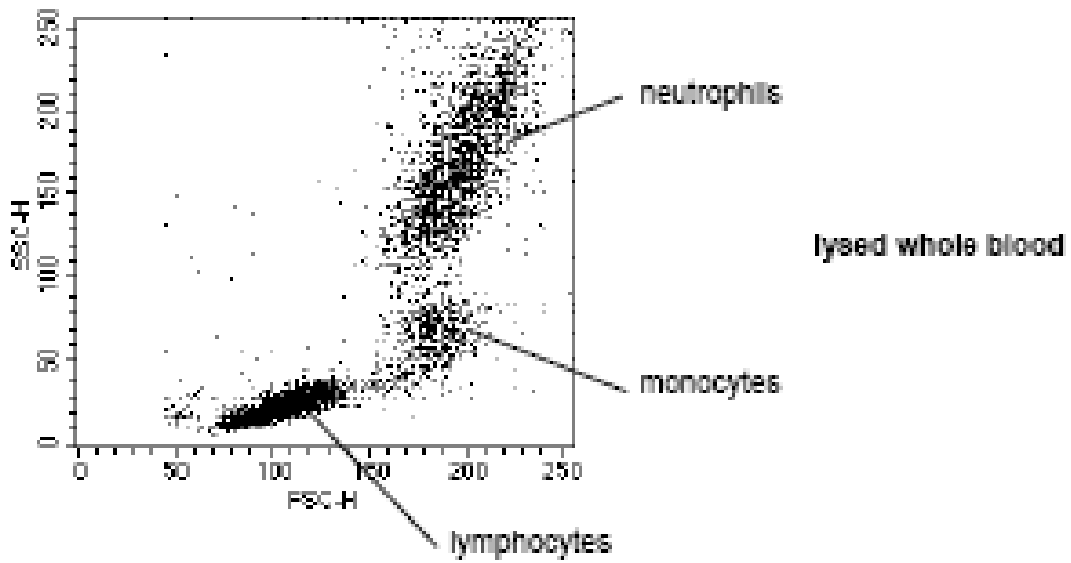
Generation of Scatter



Generation of Scatter



- Measuring the FSC and SSC of cells helps to differentiate different cell types in a given population, such as major leucocyte subpopulations in blood:

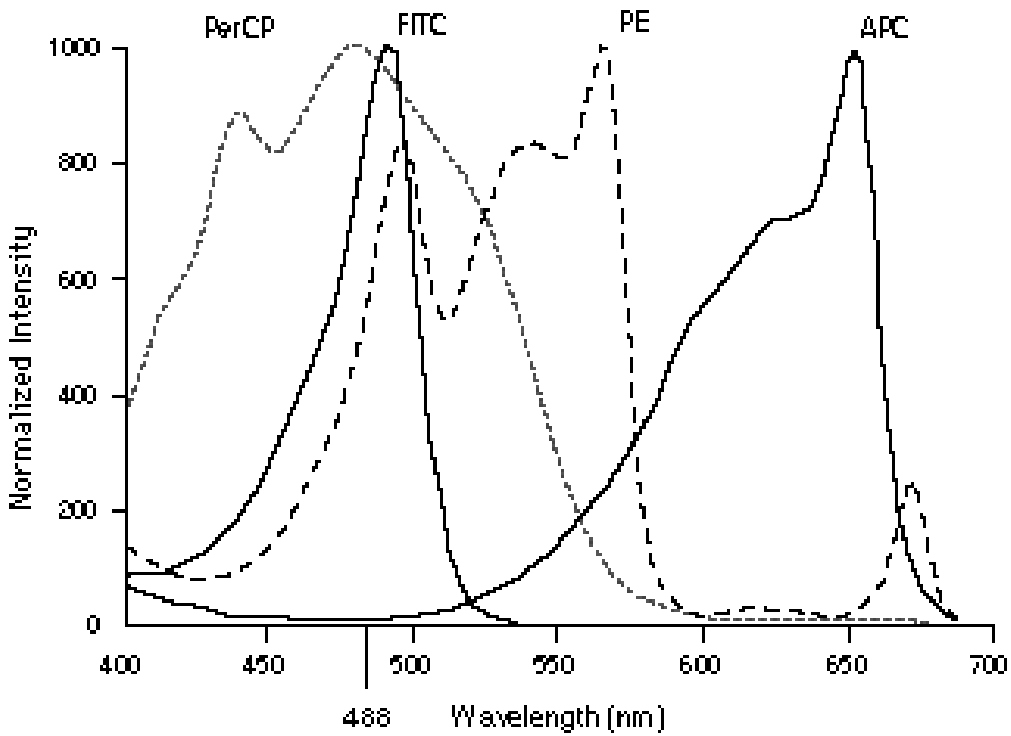


Fluorescence

- A fluorescent compound absorbs and emits light energy over a characteristic range of wavelength (absorption and emission spectrum)
- Fluorescent compounds or fluorochromes used in flow cytometry are:
  - Fluorescein isothiocyanate (FITC)
  - Phycoerythrin (PE)
  - Peridinin chlorophyll protein (PerCP)
  - Allophycocyanin (APC)

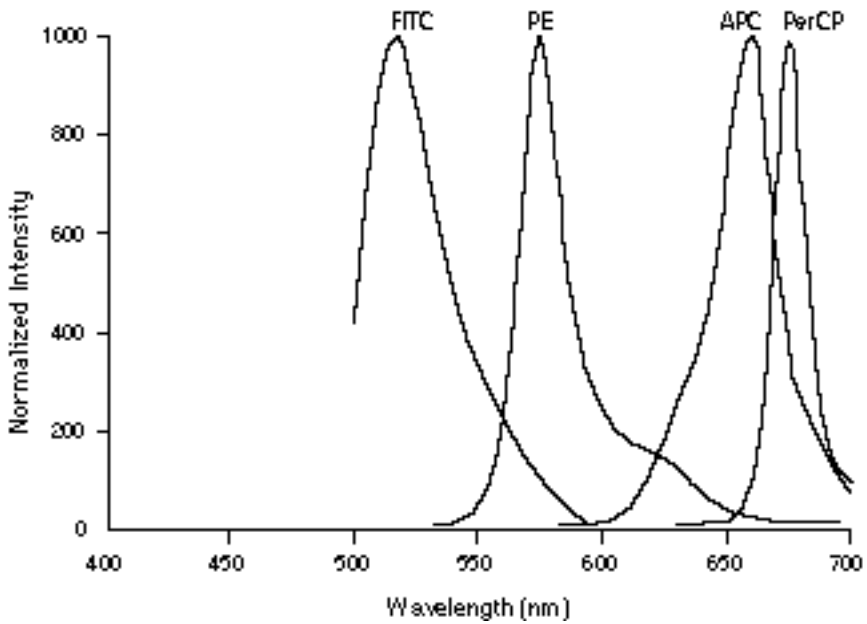
Fluorescence

- A laser used in flow cytometry, must produce light at a wavelength which excites one or more fluorochromes (commonly argon ion laser at 488nm)



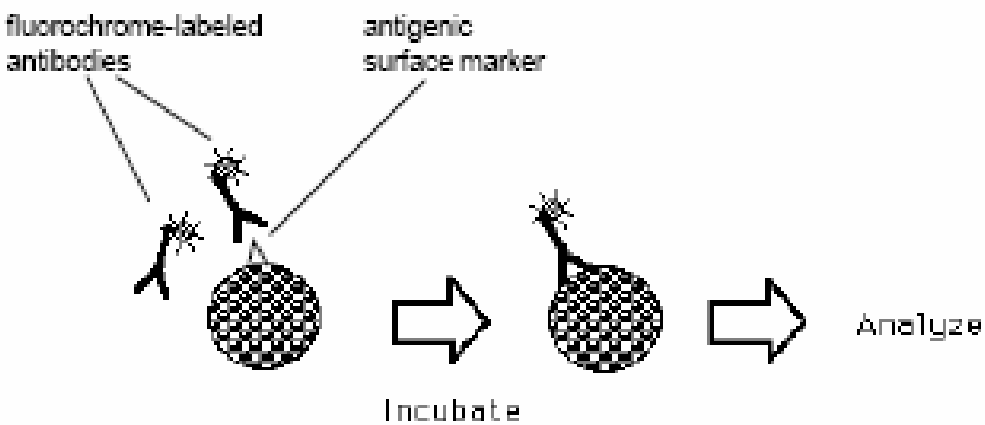
Fluorescence

- When more than one fluorochrome is excited, their emission spectrum must be sufficiently apart to enable separate detection
- The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the cell

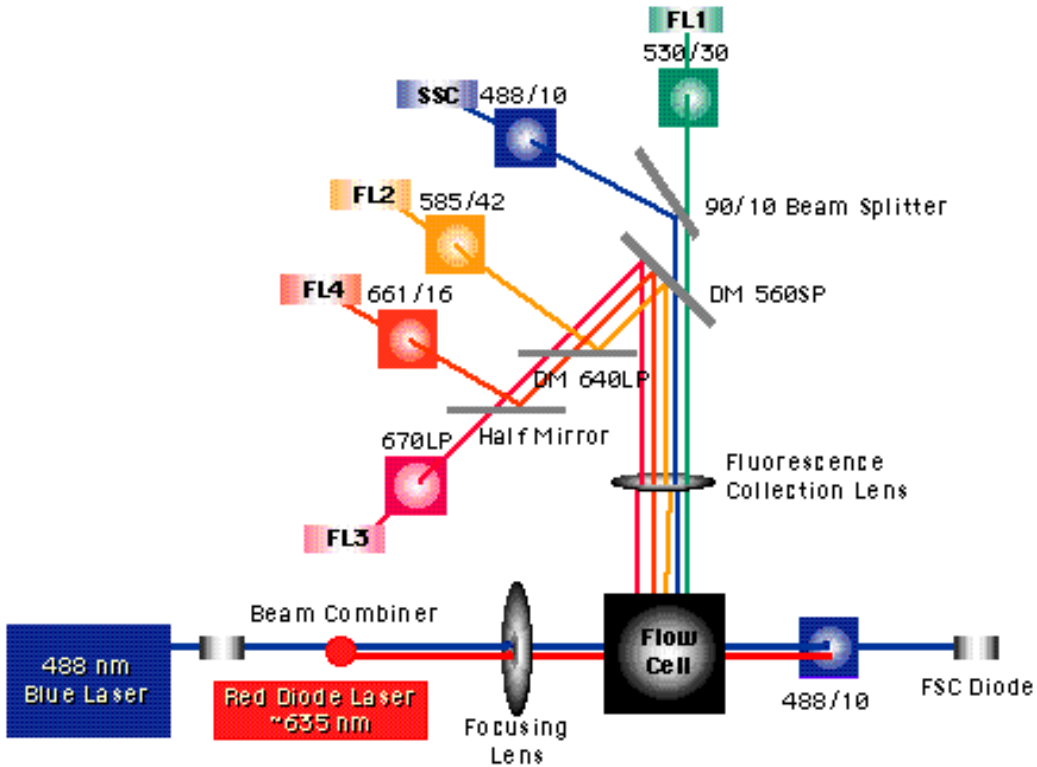


Fluorescence

- Fluorescent dyes used in flow cytometry are conjugated to monoclonal antibodies, so that a particular antigen on a cell can be identified
- In a mixed population of cells, different fluorochromes can be used to distinguish separate subpopulations

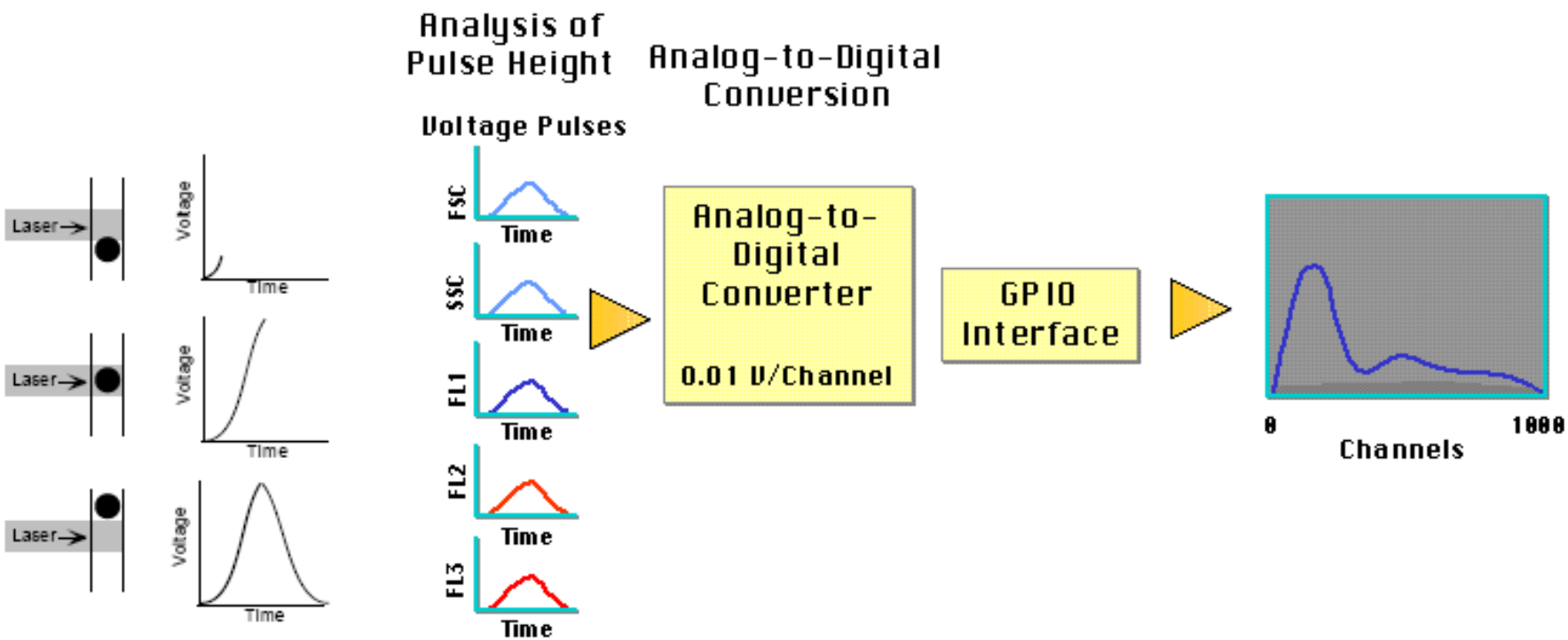


Synthesis


FL1
530/30
SSC
488/10
FL2
585/42
90/10 Beam Splitter
FL4
661/16
DM 560SP
DM 640LP
670LP
Half Mirror
Fluorescence
Collection Lens
FL3
Beam Combiner
488 nm
Blue Laser
Red Diode Laser
~635 nm
Focusing
Lens
Flow
Cell
488/10
FSC Diode

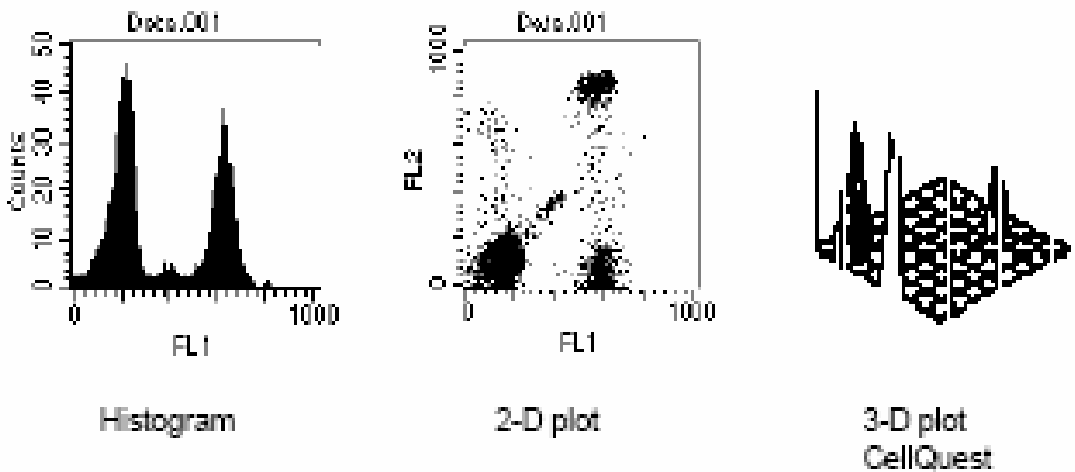
Data collection

- Light signals are converted to electronic pulses, and their intensity recorded (conversion to channel numbers by analog-digital converter)
- The light signal is then displayed in an appropriate position on the data plot



Data collection

- Data collected on cell populations can be displayed in several formats:
  - Histogram
    - Horizontal axis represents the parameters intensity, and the vertical axis represents the number of events at that intensity
  - 2-D plot, 3-D plot
    - 2 parameters are plotted against each other
    - Signals with identical intensities accumulate in the same channel
    - In the 3-D plot, the z-axis displays the number of events

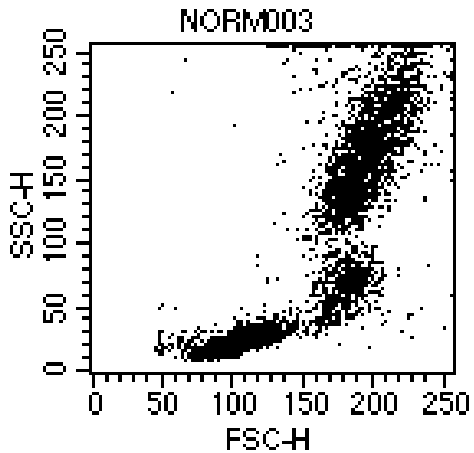


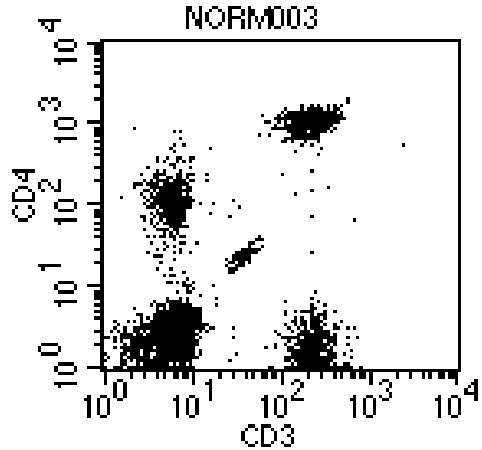
Histogram 2-D plot 3-D plot CellQuest

Gating

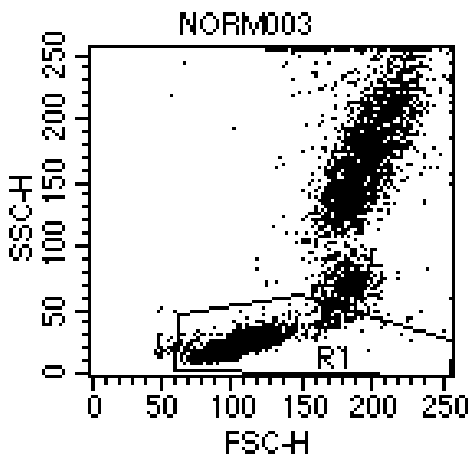
- Flow cytometry has the disadvantage that the FSC, SSC and fluorescence data obtained from a cell population are not related to a morphological picture of the cells examined
- Gating techniques are used in order to select the correct subgroup of cells for analysis
- Gating can be based on the light scattering characteristics indicating the size and granularity of a cell:
  - For example, in a blood sample one wants to restrict analysis to only the lymphocytes: A gate can be set on the FSC vs SSC plot around the cells of the size and granularity of the lymphocytes

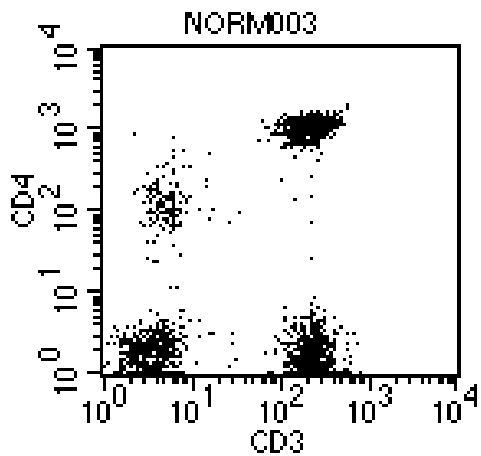
Gating





Fluorescence on ungated data





Data analysis for gated populations

- Several types of plots can be used to represent the data

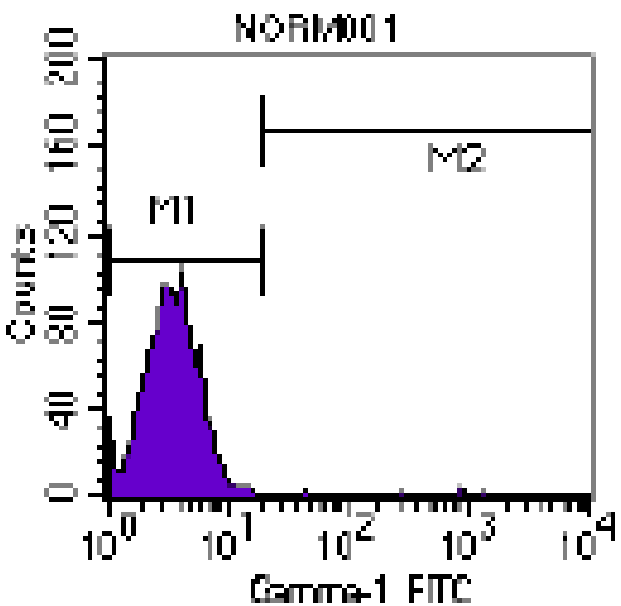
1) Histogramme

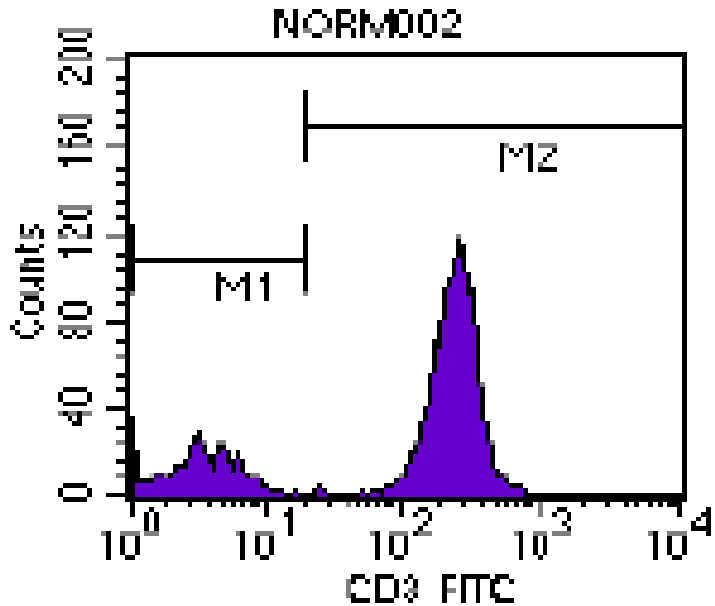
– In a histogram, a single parameter is plotted against the number of events

– A control marker is used to determine where a marker is to be placed to distinguish positive from negative events

Data analysis for gated populations

– On the first histogramme, marker M1 is placed around the negative peak of the control, marker M2 is placed to the right of M1 to designate positive events on subsequent analysis with CD3 (2nd histogramme)





Data analysis for gated populations

- Histogramme statistics give percentages of the negative and positive events, comparing the counted total events against the events found inside the lymphocyte gate
- 6000 events are on the data file, but 2891 events are within the lymphocyte gate
- The percentage of lymphocytes which are CD3 positive is indicated by M2

Histogram Statistics

File: NORM002 Sample ID: 481|A|
Tube: CD3/CD19 Gate: G1
Gated Events: 2891 Total Events: 6000
X Parameter: FL1-H CD3 (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	2891	100.00	48.18	176.92	86.78	62.94	191.10	220
M1	1, 18	619	21.41	10.32	3.75	3.26	51.90	3.40	1
M2	18, 9647	2272	78.59	37.87	224.10	212.20	32.71	220.67	220

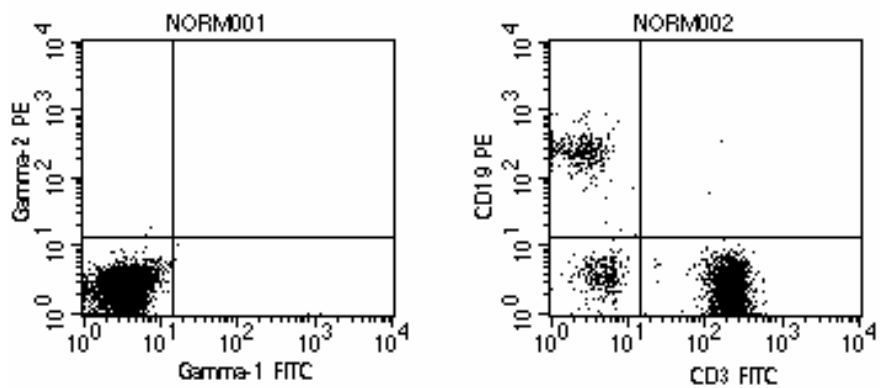
Data analysis for gated populations

- Several types of plots can be used to represent the data

2) Dot plot and dot plot statistics

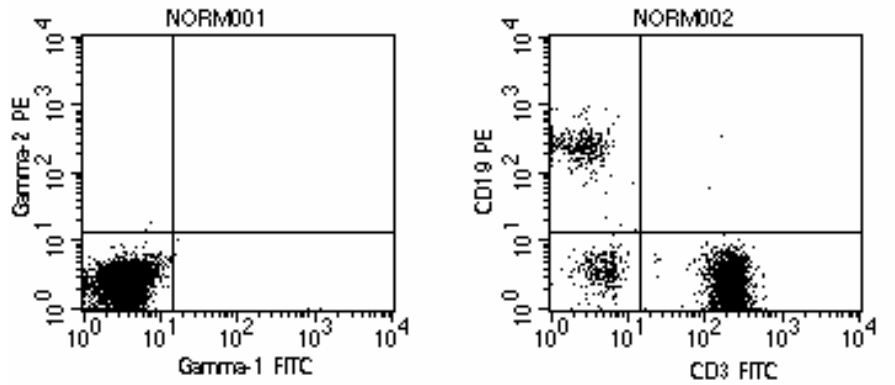
– In a dot plot, two parameters are plotted against each other, where each dot represents one or more events

– A control is used to determine where the quadrant markers are to be placed, which divide dot plots into 4 sections distinguishing populations which are negative, single positive, or double positive for each of the 2 parameters



Data analysis for gated populations

- The lower left quadrant (LL) displays events, which are negative for both parameters used, the upper left (UL) and lower right quadrant (LR) displays events which are positive for one parameter each, and the upper right (UR) quadrant displays events which are positive for both parameters (in this case CD3 and CD19)



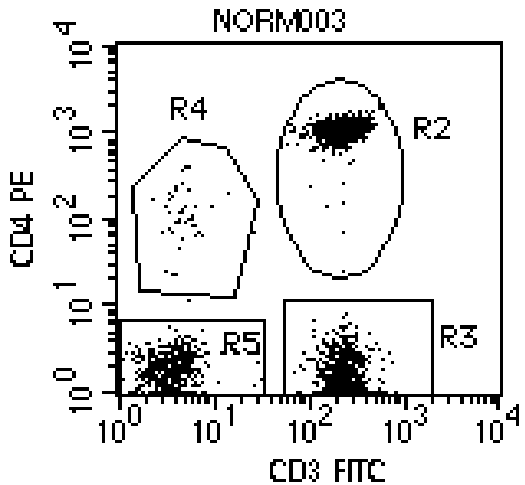
Quadrant Statistics

File: NORM002
Sample ID: 481|A|
Acquisition Date: 24-Sep-93
Gated Events: 2839
Log Data Units: Linear Values
Tube: CD3/CD19
Gate: G1
Total Events: 6000

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	296	10.43	4.93	2.74	2.39	270.11	239.43
UR	5	0.18	0.08	140.12	136.26	130.53	59.57
LL	279	9.83	4.65	4.66	4.34	3.87	3.57
LR	2259	79.57	37.65	224.31	212.28	2.14	1.78

Data analysis for gated populations

- Alternatively, regions can be created around populations, and regions statistics used



Region Statistics

File: NORM003 Sample ID: 481|A|
Tube: CD3/CD4 Gate: G1
Gated Events: 2866 Total Events: 6000
X Parameter: FL1-H CD3 Leu4 (Log) Y Parameter: FL2-H CD4 Leu3 (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	2866	100.00	47.77	196.91	102.19	484.05	29.25
R2	1271	44.35	21.18	246.06	234.08	1084.74	1061.58
R3	1035	36.11	17.25	240.91	227.57	1.53	1.37
R4	40	1.40	0.67	4.86	4.57	135.29	109.51
R5	517	18.04	8.62	3.93	3.45	1.92	1.77