FLOWCYTOMETRY

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Basics of Flow Cytometry

Fluidics

Cells in suspension

flow in single-file

Optics

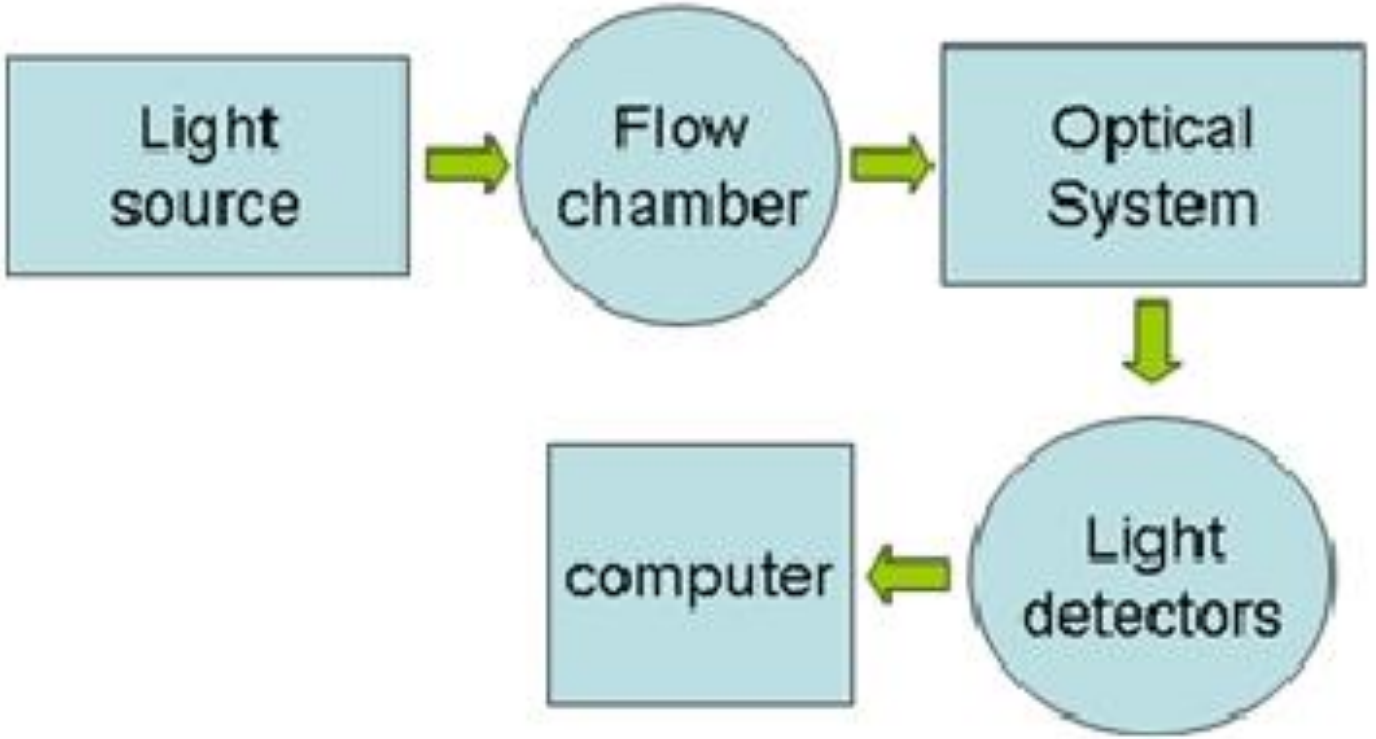
through an illuminated volume where they scatter light and emit fluorescence

that is collected, filtered and

Electronics

converted to digital values

that are stored on a computer

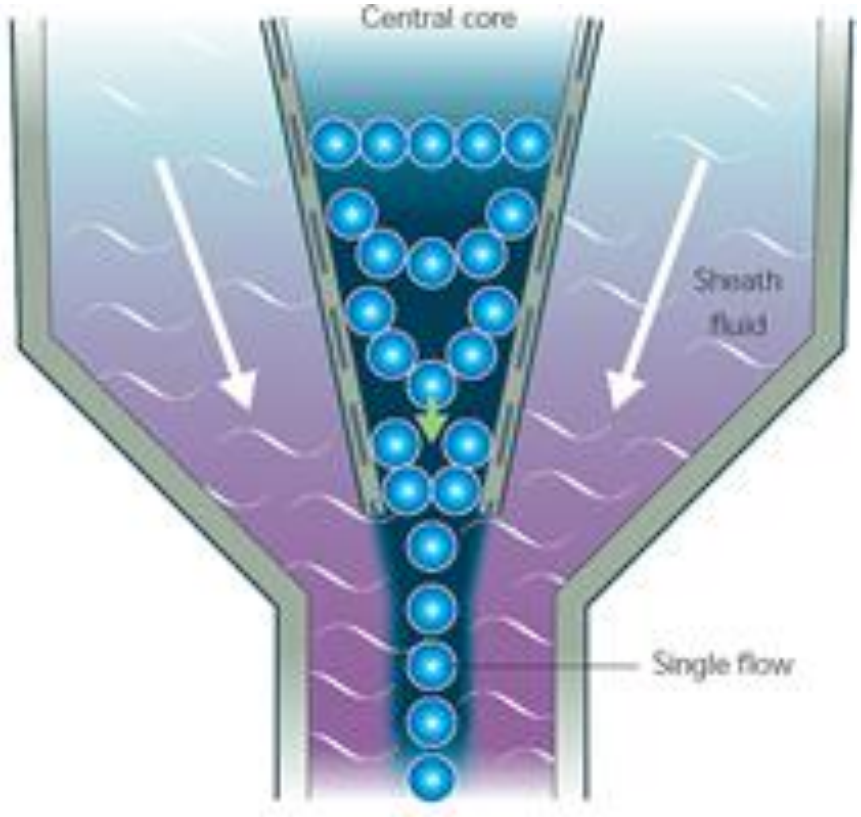

Light source
Flow chamber
Optical System
Light detectors
computer

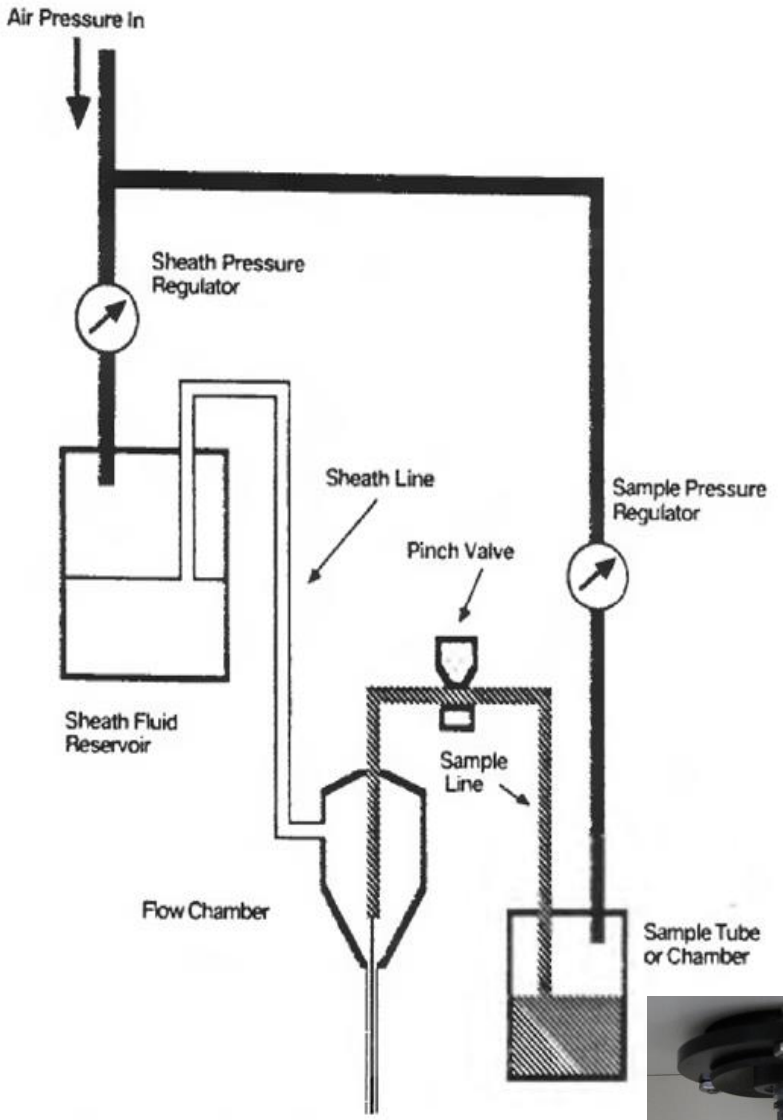
Fluidics

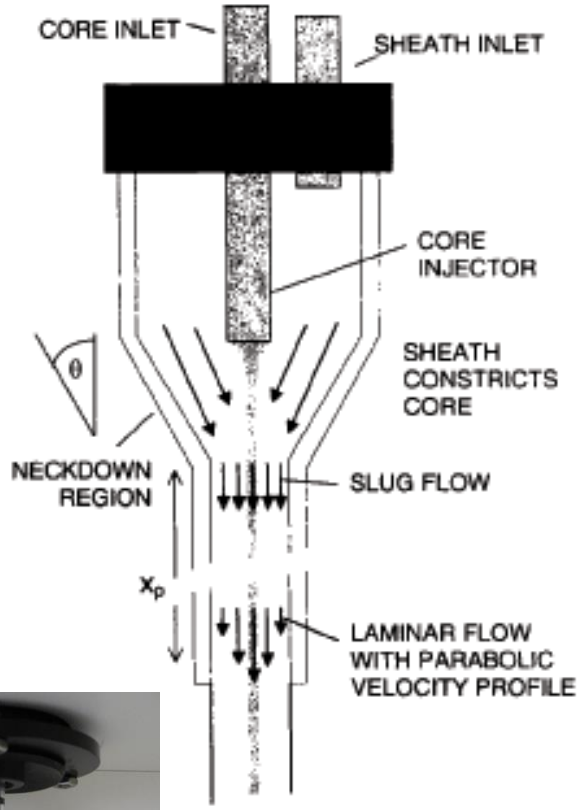
The fluidics system consists of sheath fluid (usually a buffered saline solution) that is pressurized to deliver and focus the sample to the laser intercept or interrogation point where the sample is analyzed.

Fluidics

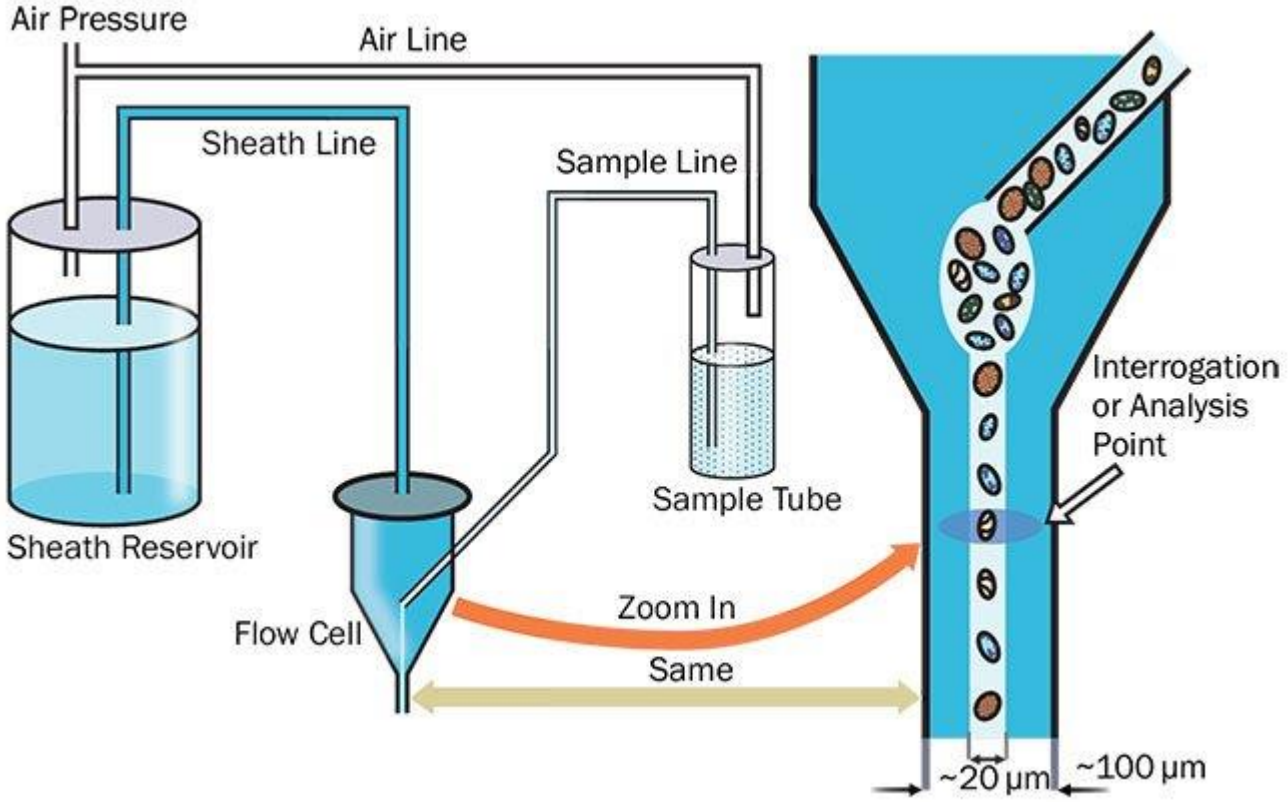
Flow cell







Fluidics


Air Pressure
Air Line
Sheath Line
Sample Line
Sheath Reservoir
Sample Tube
Flow Cell
Zoom In
Same
Interrogation or Analysis Point
~20 µm
~100 µm

Optics - Filter Layout

To simultaneously measure more than one scatter or fluorescence from each cell, we typically use multiple channels (multiple detectors)

Design of multiple channel layout must consider

- spectral properties of fluorochromes being used
- proper order of filters and mirrors

Multiple laser systems are common with instruments often having 20 parameters (FSC, SSC and 18 fluorescent detectors).

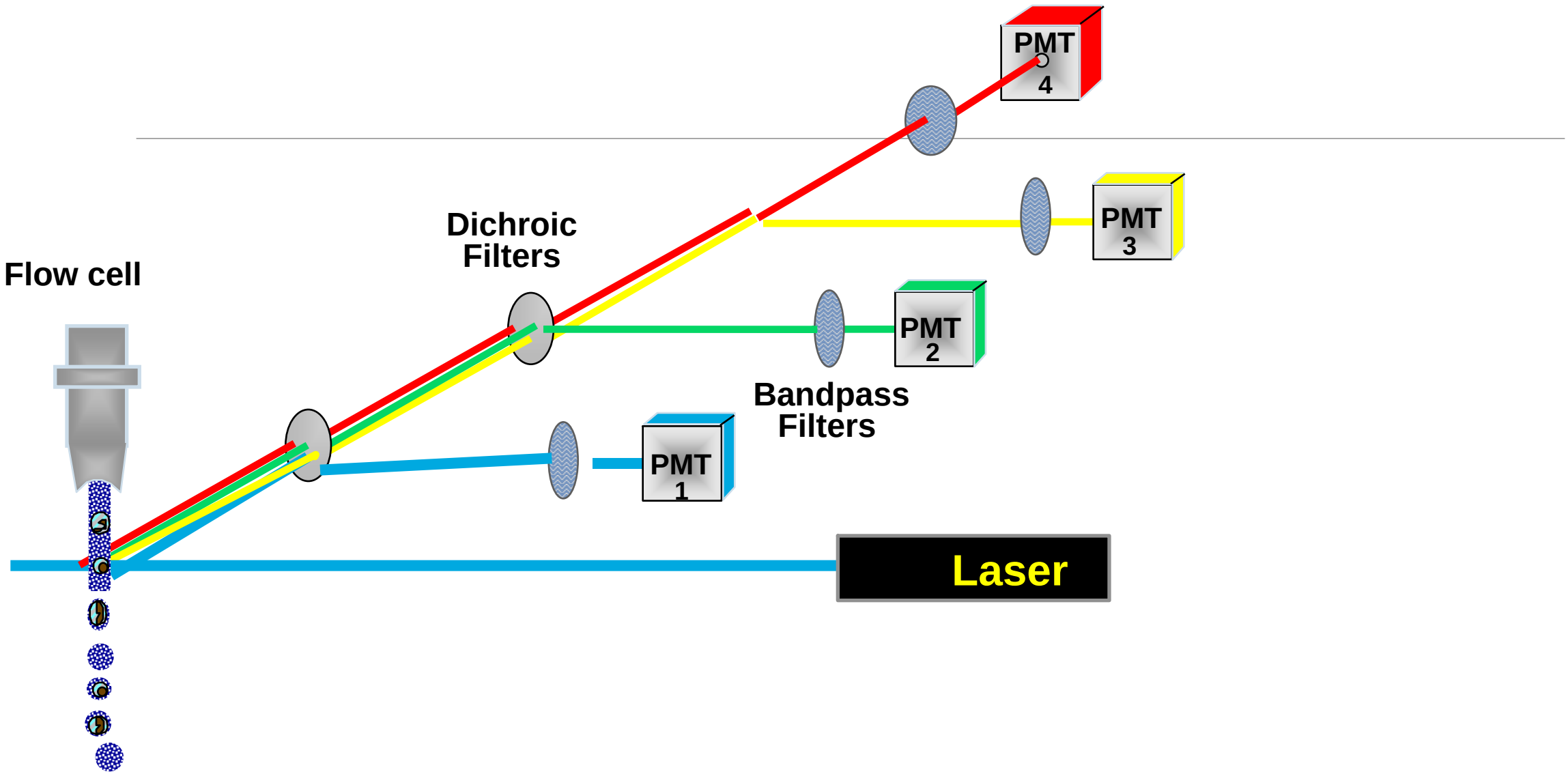
There are new instrument platforms being introduced with five or more lasers and 30–50 parameters.

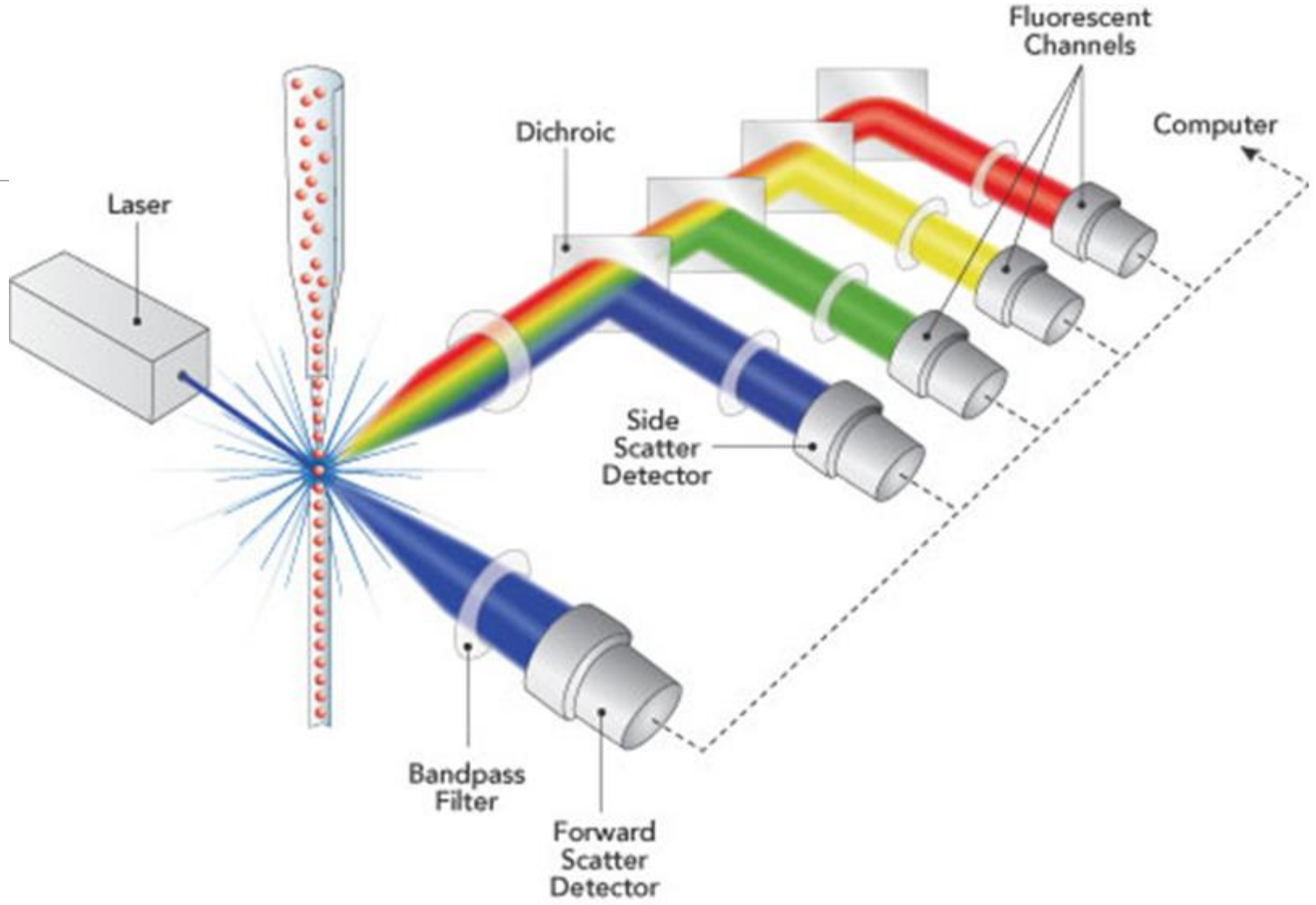
The most common lasers used are 355 nm (ultraviolet), 405nm (violet), 488 nm (blue), 532nm (green), 552nm (green), 561 nm (green-yellow) and 640 nm (red).

Why Laser?

Laser is coherent light , so :

- Can be focused in a very small point , nominally 10x100 um in flow cytometry.
- High energy in very small area.
- No shadow.
- Absolutely Mono color excitation .


PMT
4
PMT
3
Dichroic
Filters
Flow cell
PMT
2
Bandpass
Filters
PMT
1
Laser

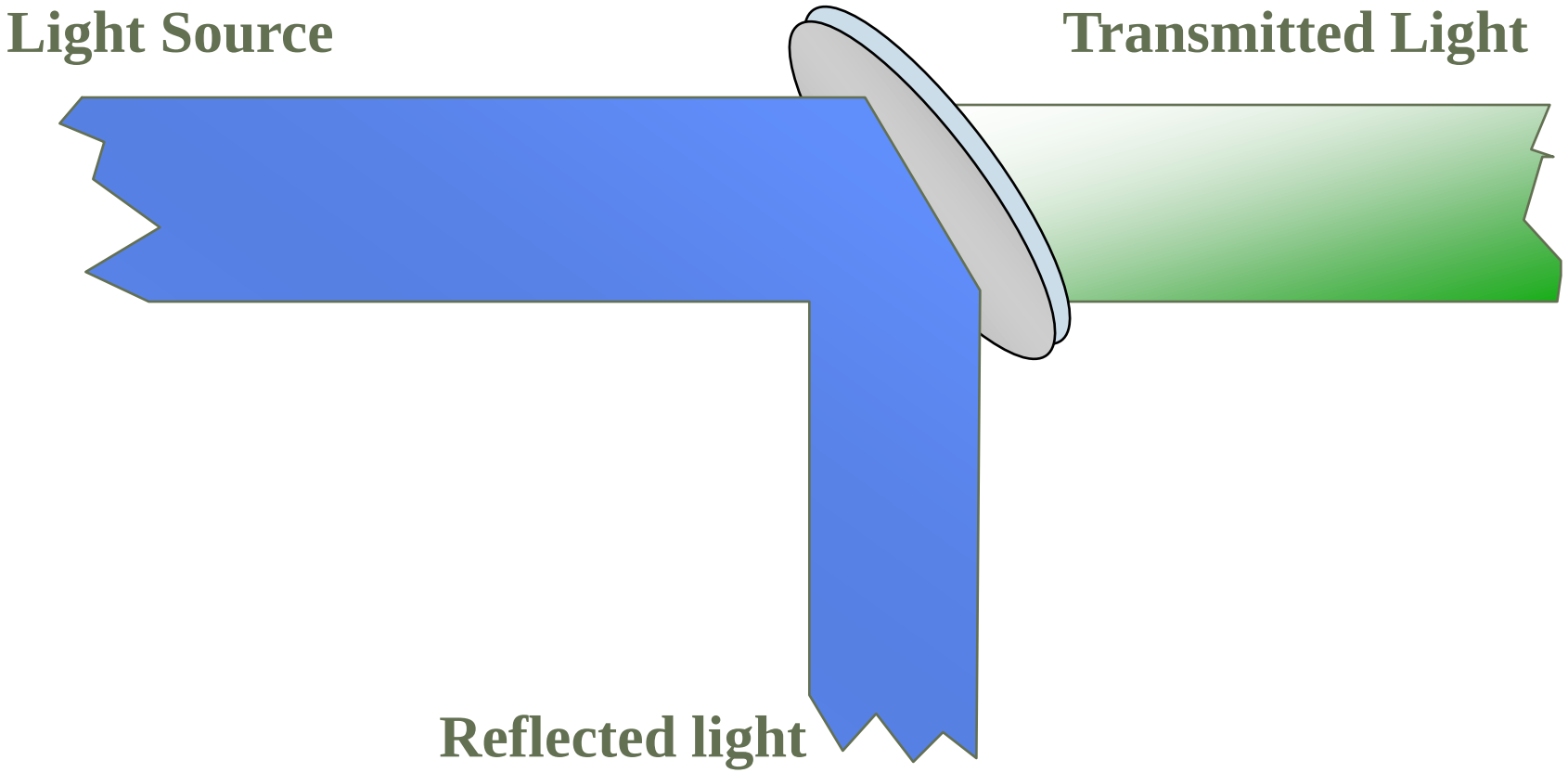

Fluorescent
Channels
Computer
Dichroic
Laser
Side
Scatter
Detector
Bandpass
Filter
Forward
Scatter
Detector

Optics

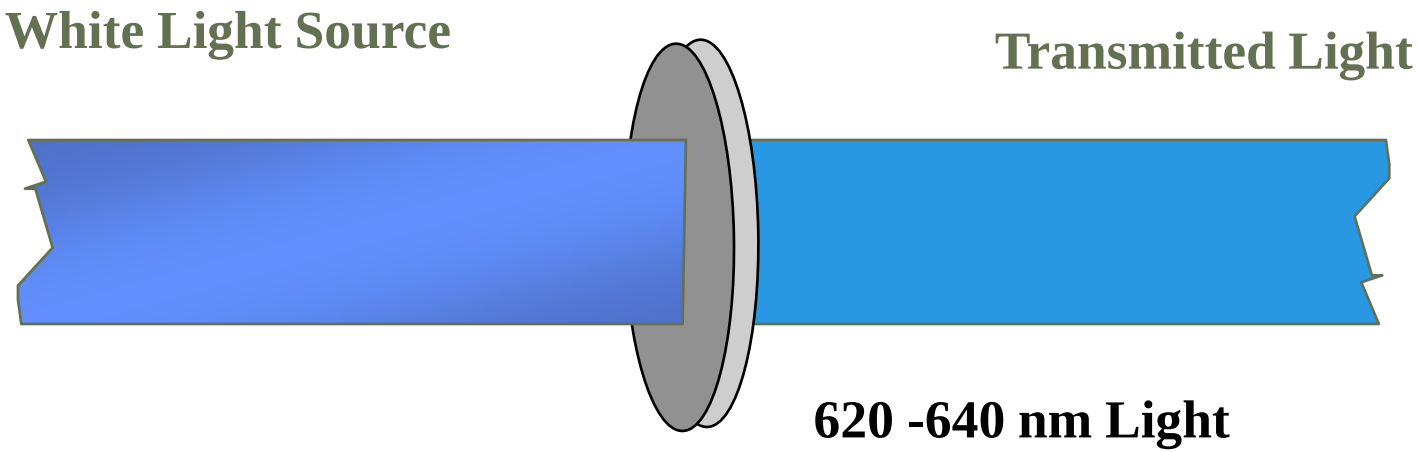
The optical system consists of excitation optics (lasers) and collection optics (photomultiplier tubes or PMTs) that generate the visible and fluorescent light signals used to analyze the sample.

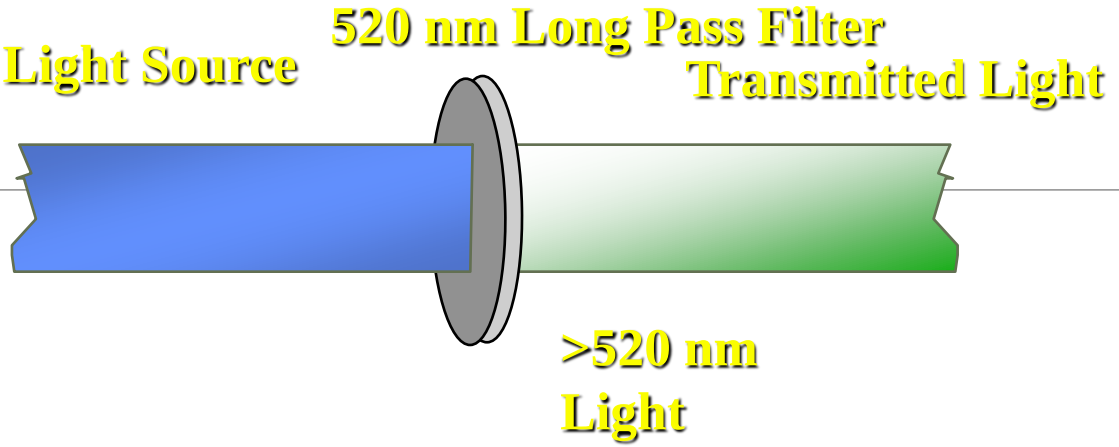
Dichroic mirror/filters, Long Pass, Short Pass, Band Pass filters.

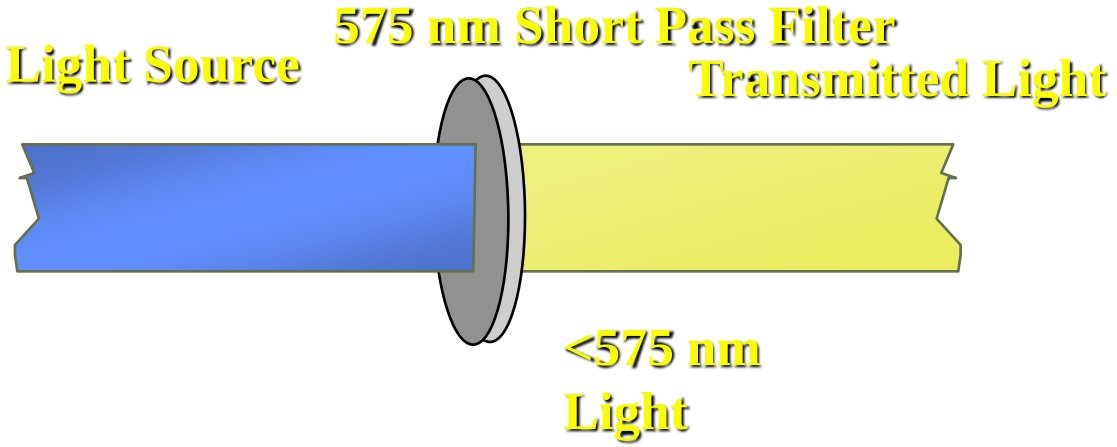
Dichroic Filter/Mirror at 45 deg



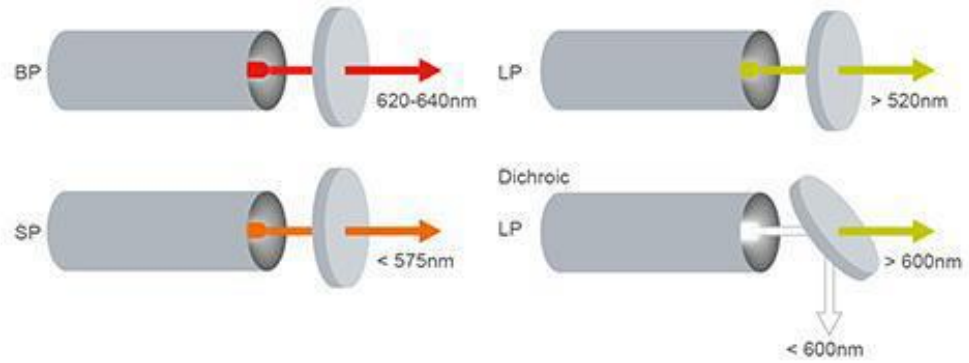
630/10 nm BandPass Filter

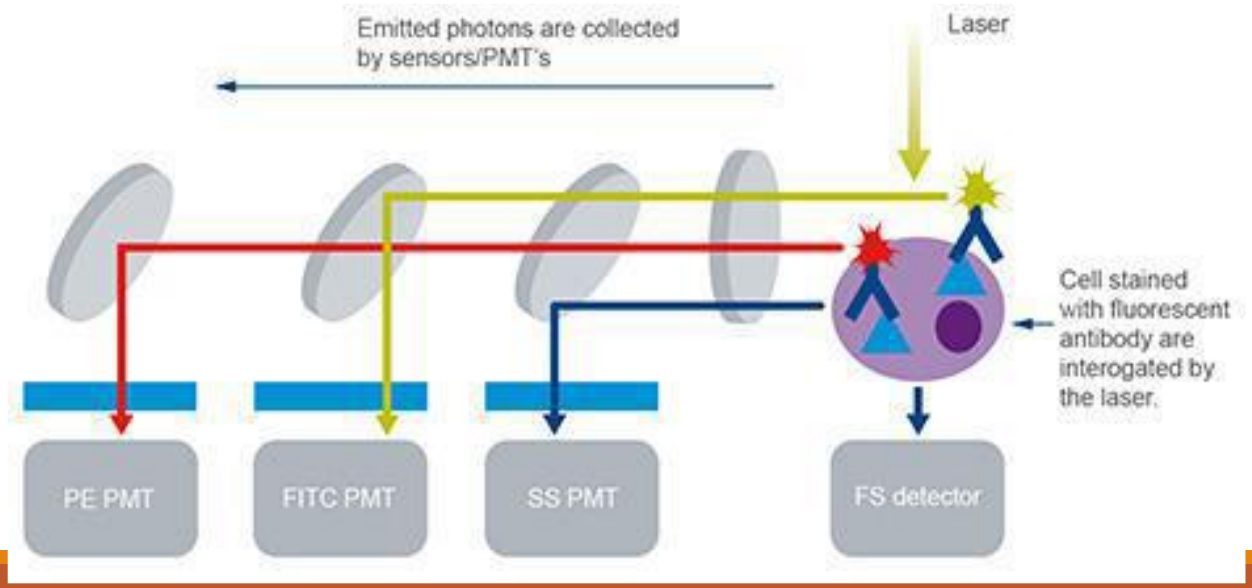


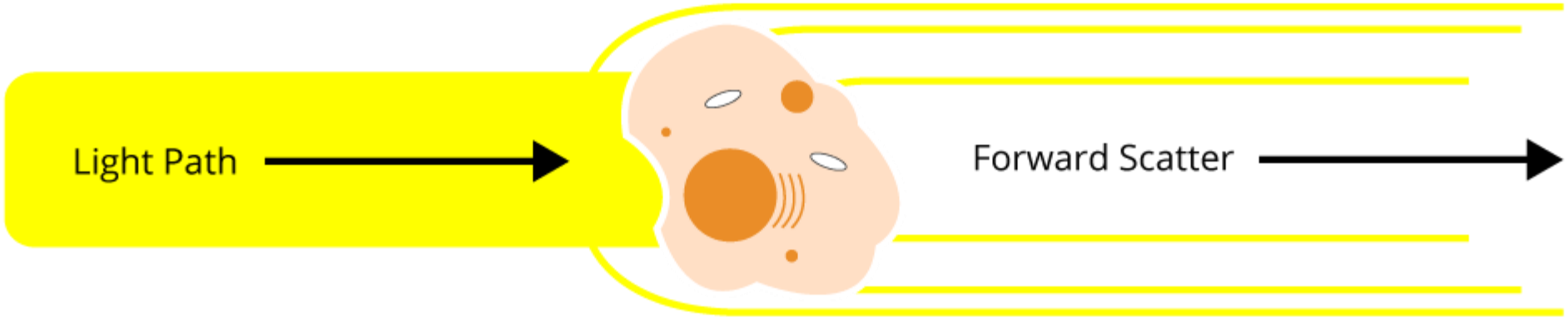

520 nm Long Pass Filter
Light Source
Transmitted Light
>520 nm
Light

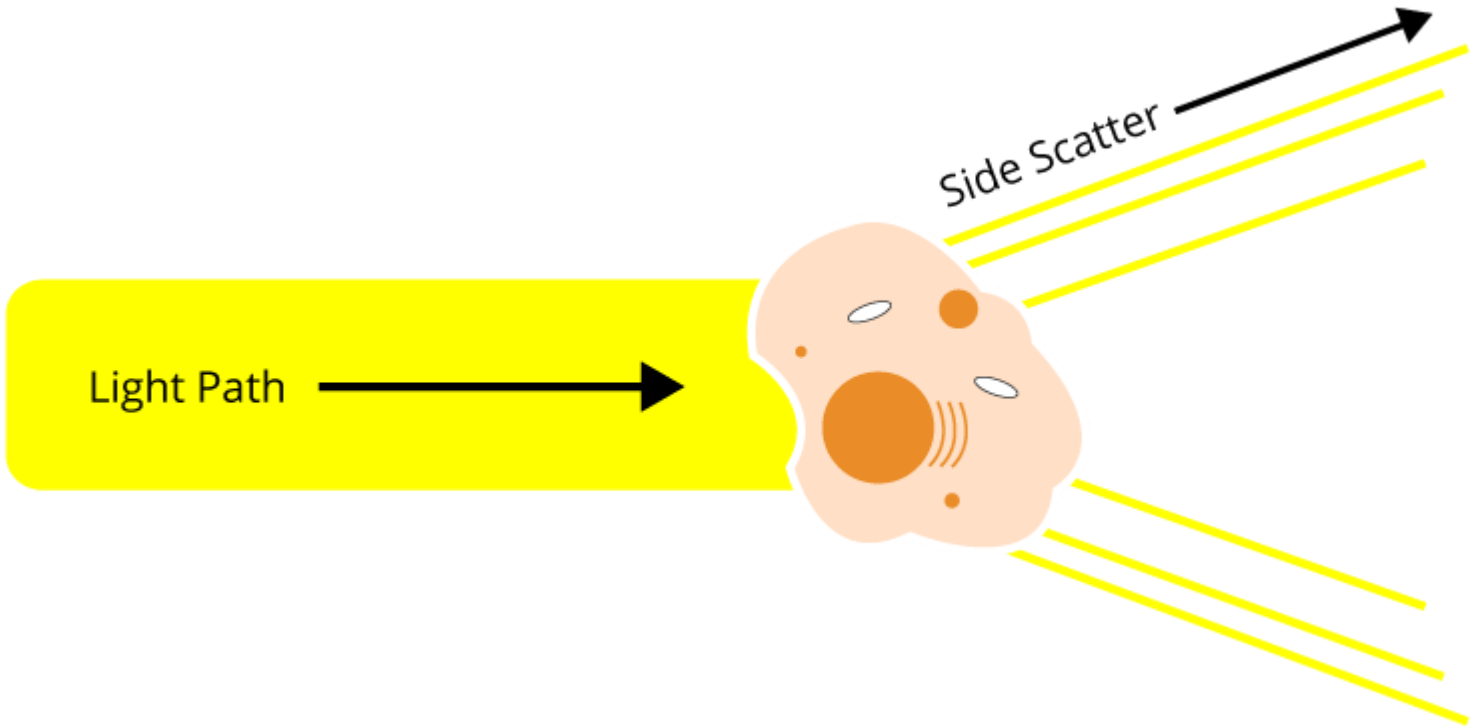

575 nm Short Pass Filter
Light Source
Transmitted Light
<575 nm
Light

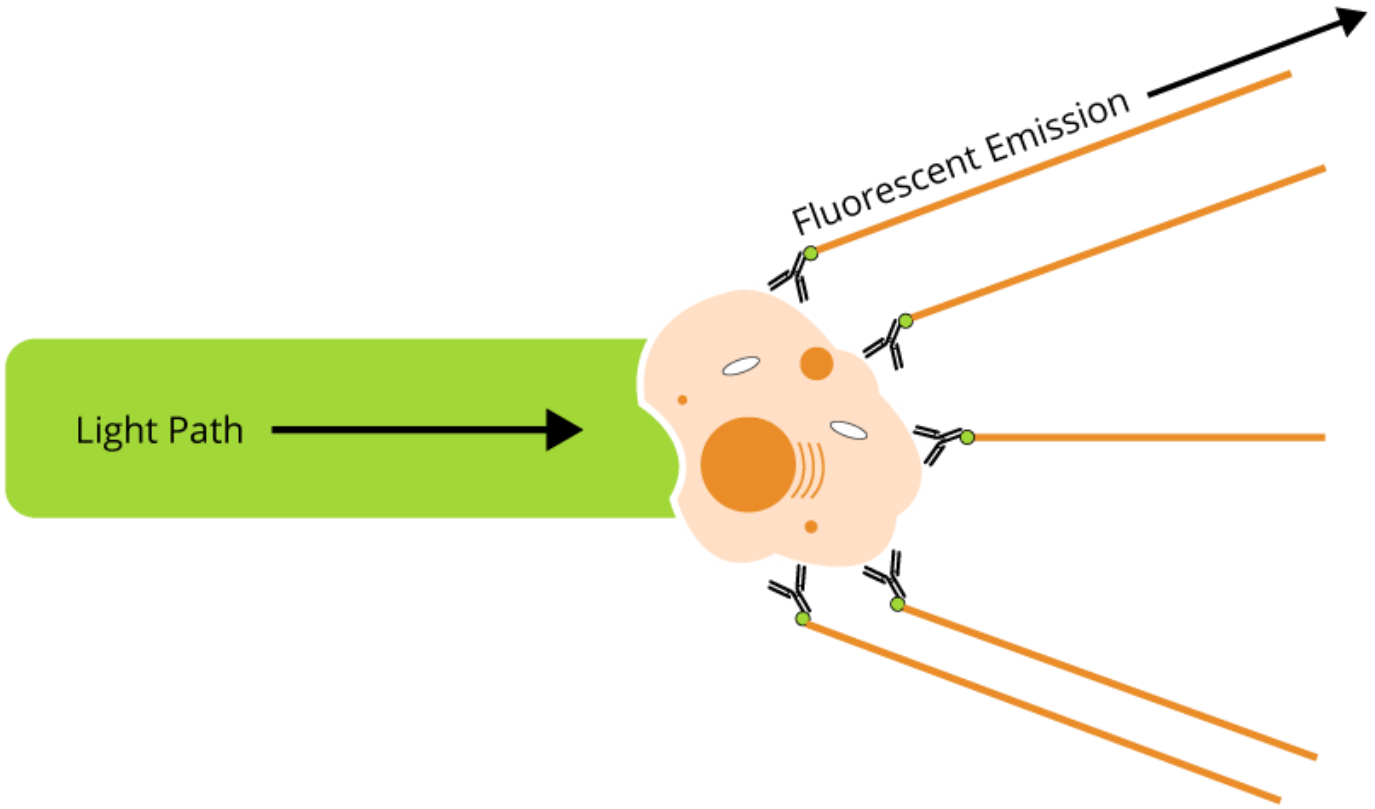
Forward and side scattered light and fluorescence from stained cells are split into defined wavelengths and channeled by a set of filters and mirrors within the flow cytometer. The fluorescent light is filtered so that each sensor will detect fluorescence only at a specified wavelength. These sensors are called photomultiplying tubes (PMTs).
(Various filters are used in the flow cytometer to direct photons of the correct wavelength to each PMT)

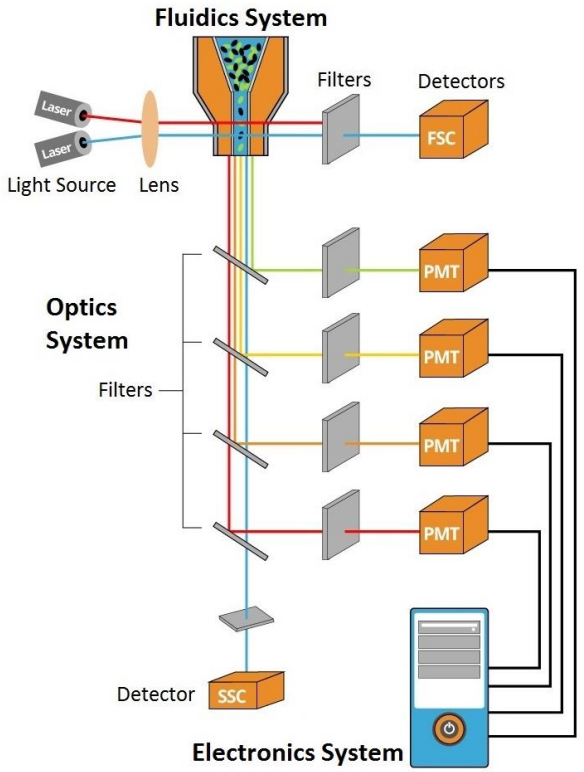


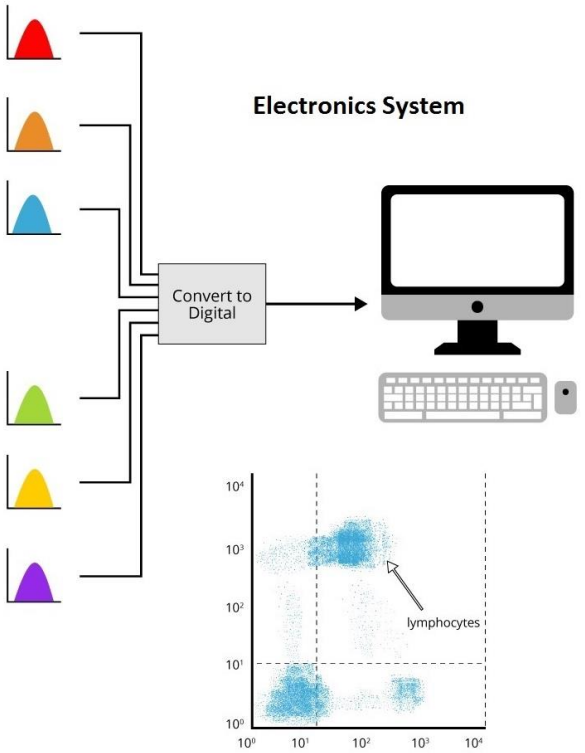



Light Path
Forward Scatter


Side Scatter
Light Path

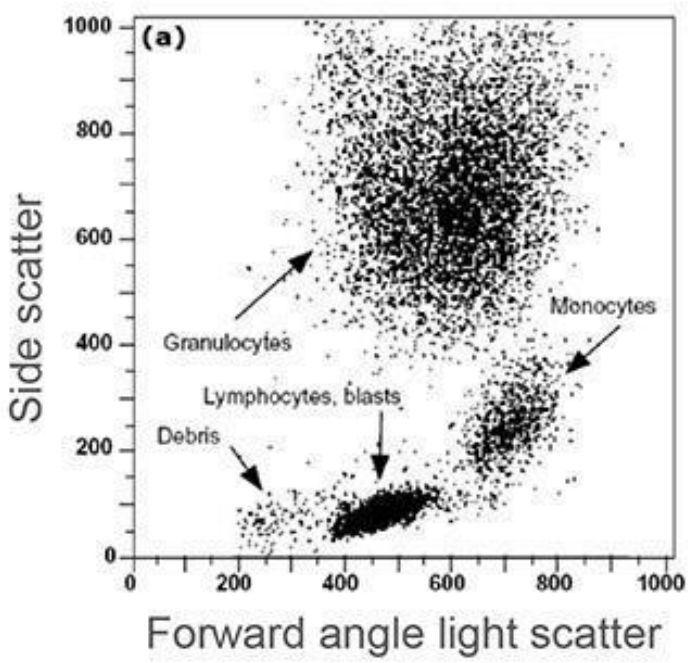

Fluorescent Emission
Light Path

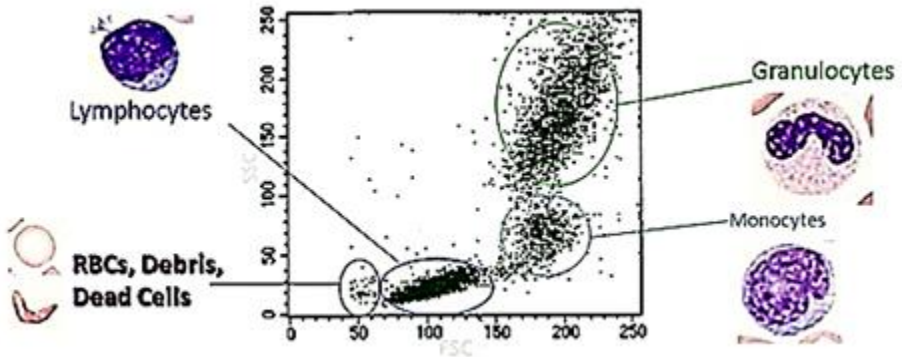

Fluidics System
Filters
Detectors
Laser
Laser
FSC
Light Source
Lens
PMT
PMT
PMT
PMT
Optics System
Filters
Detector
SSC
Electronics System

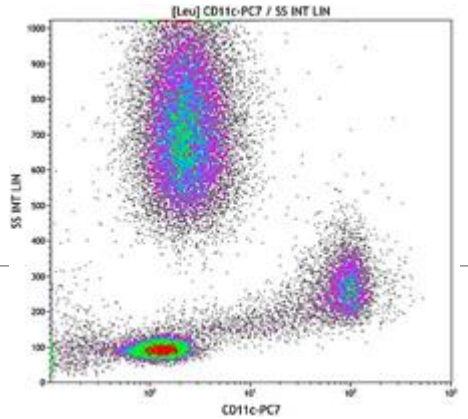

Electronics System
Convert to Digital
lymphocytes
10^4
10^3
10^2
10^1
10^0
10^0
10^1
10^2
10^3
10^4

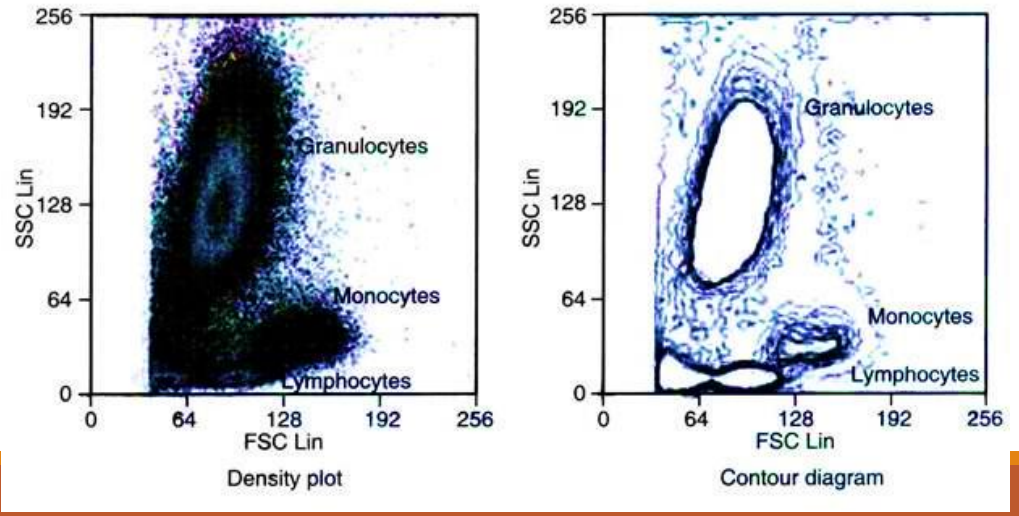
when running blood samples on the flow cytometer.
 Larger and more granular granulocyte cells produce a large population with high SS and FS.
 Monocytes are large cells, but not so granular, so these produce a separate population with high FS but lower SS.
 Smaller lymphocytes and lymphoblasts produce a separate population with less FS. They are not granular cells, so also have low SS.

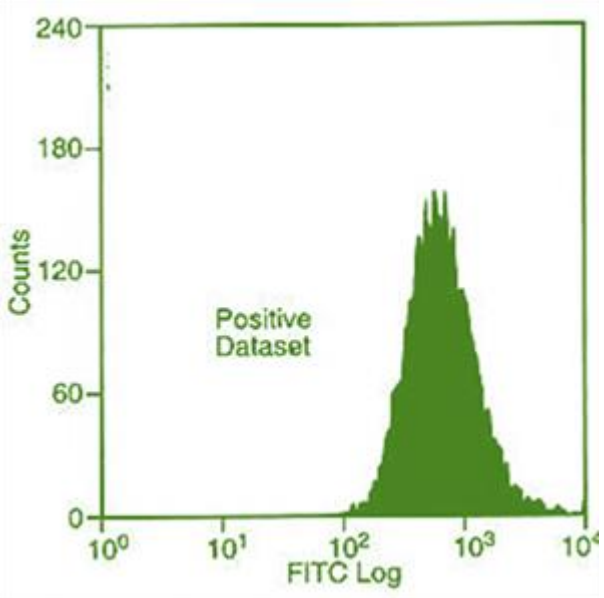
Therefore, these cells can be separated into different populations based on their FS and SS alone.




Lymphocytes
RBCs, Debris,
Dead Cells
Granulocytes
Monocytes
SSC
FSC
0
50
100
150
200
250


[Leu] CD11c-PC7 / SS INT LIN
SS INT LIN
CD11c-PC7


256
192
128
64
0
SSC Lin
Granulocytes
Monocytes
Lymphocytes
0
64
128
192
256
FSC Lin
Density plot
Contour diagram


240
180
120
60
0
Counts
Positive
Dataset
10^0
10^1
10^2
10^3
FITC Log

Stokes Shift

is the difference between the spectral position of the maximum of the first absorption band and the maximum of the fluorescence emission

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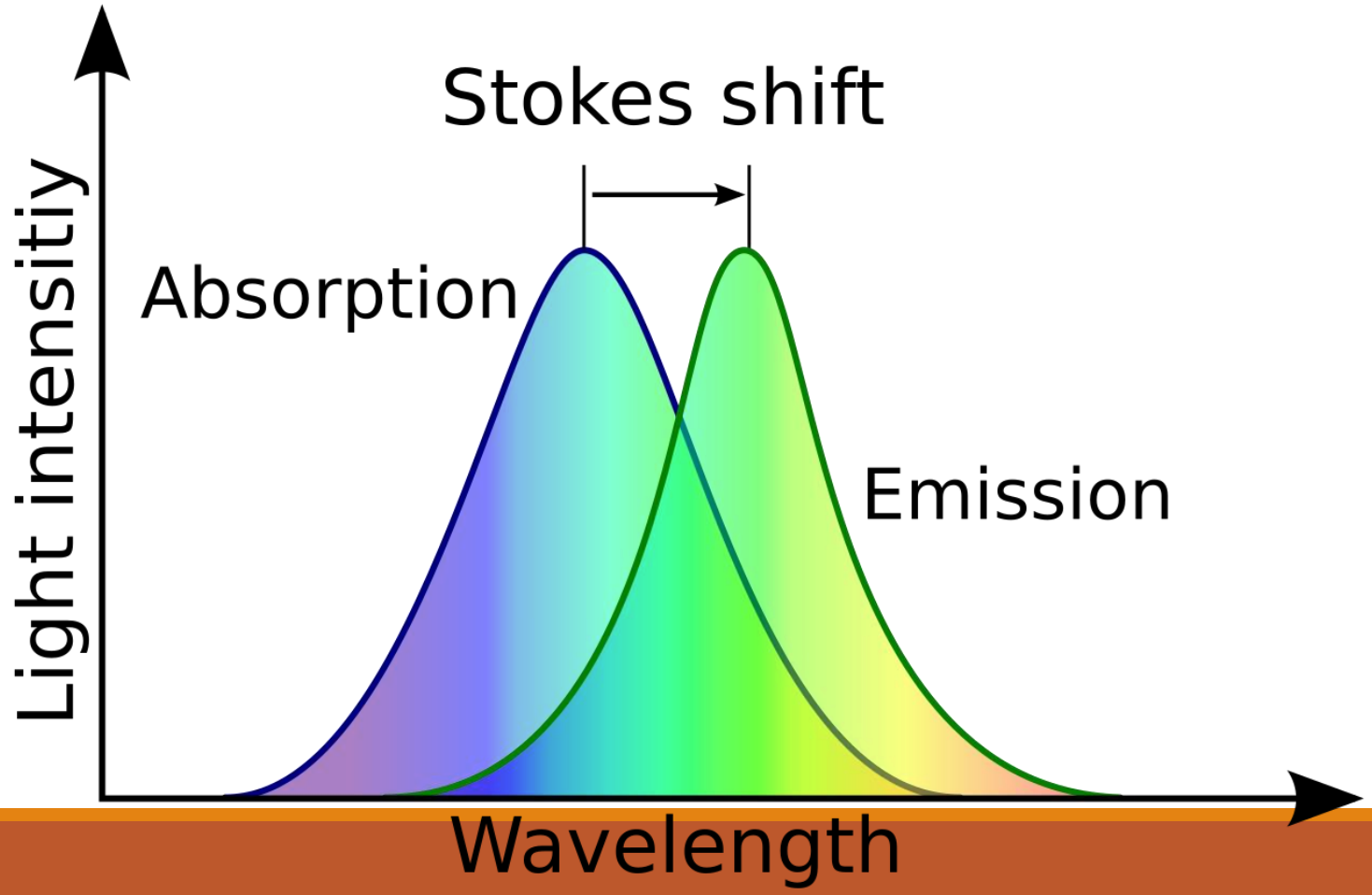


Reagents (Probes)

Small Organic Molecules—Small organic molecules such as fluoroscein (MW=389 D), Alexa Fluor 488 (fluorescein analog), Texas Red (325 D), Alexa Fluor 647 (1464 D), Pacific Blue and Cy5 (762 D) are commonly used for antibody conjugation. They have consistent emission spectra but a small Stokes shift (the difference between excitation wavelength and emission wavelength, approximately 50–100 nm). They are also stable and reasonably easy to conjugate to antibodies.

Phycobiliproteins—Phycobiliproteins are large protein molecules derived from cyanobacteria, dinoflagellates, and algae. They are large molecules, for example phycoerythrin (PE) has a molecular weight of 240,000 D. These proteins have large Stokes shifts (75–200 nm) are very stable with consistent emission spectra. Because of their large size, phycobiliproteins are excellent for quantitative flow cytometry since they usually have a 1:1 protein to fluorochrome ratio during conjugation. APC, PerCP

Quantum Dots—Quantum Dots (Qdots) are semiconductor nanocrystals that have tight fluorescence emission spectra associated with the size of the nanocrystal. They are optimally excited with UV or violet lasers but can be minimally excited by multiple lasers. This minimal excitation complicates fluorescence compensation when Qdots are used in multi-parameter experiments. Because of the compensation issues and difficulty in conjugating Qdots to antibodies, these reagents have largely been replaced with the polymer dyes in multi-parameter staining panels.

Reagents (Probes)

Polymer Dyes— Polymer dyes consist of polymer chains that collect light signals and can be “tuned” to absorb and emit light at specific wavelengths based on the length of the polymer chain and the attached molecular subunits. These dyes are very stable have similar quantum efficiency to phycobiliproteins with greatly increased photostability. Since polymer dyes can be made to absorb light only at specific wavelengths, they avoid the issues with multiple laser excitation that make Qdot reagents difficult to use in multi-parameter experiments. Examples of these reagents are the Brilliant Violet (BV), Brilliant Ultraviolet (BUV) and Brilliant Blue (BB) reagents.

Tandem Dyes—Tandem dyes chemically couple either phycobiliproteins (PE, APC, PerCP) or polymers dyes (BV421, BUV395) with small organic fluorochromes (Cy3, Cy5, Cy7) to create a dye that uses fluorescence energy transfer (FRET) to increase the available fluorochromes that can be excited with a single laser source.

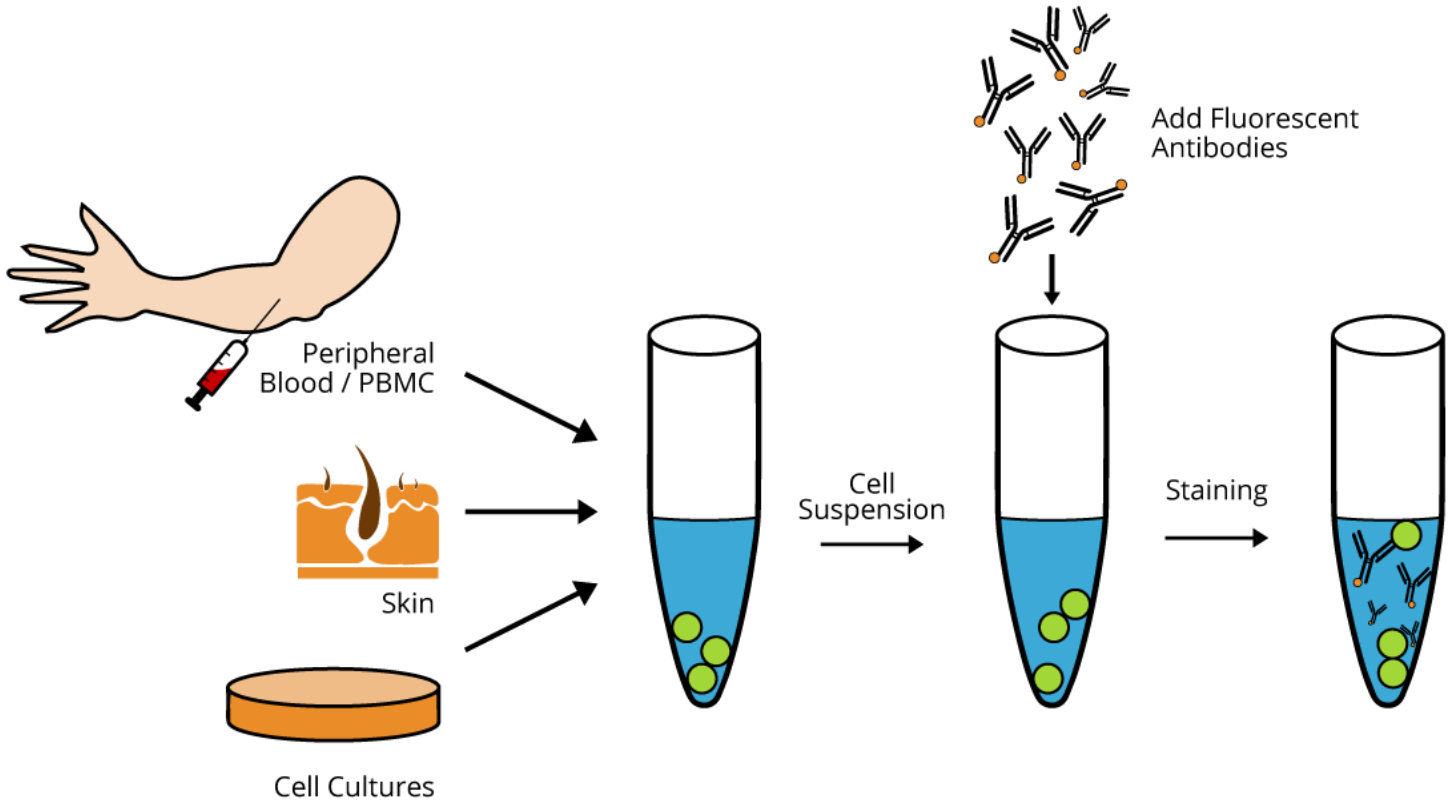
Fluorescent Proteins—Fluorescent proteins are frequently used as reporter systems for gene expression. The most commonly used is green fluorescent protein (GFP) derived from the jellyfish Aequorea victoria (Tsien, 1998).

Reagents (Probes)

Nucleic Acid Dyes—Nucleic acid dyes bind DNA, RNA or both. They are used to quantitate DNA for cell cycle analysis (Propidium Iodide, 7AAD, DyeCycle Violet, DAPI).

Proliferation Dyes—Carboxyfluoroscein succinimidly ester (CFSE) and other similar dyes can be used to follow multiple divisions of proliferating cells. Red and violet excited variants of these dyes are also now available. Each cell is permanently labeled with the dye and the subsequent generations of cells inherit lower amounts of the dye due to the dilution of the dye. These dyes do not affect cell growth or morphology and are suitable for long term proliferation studies.

Ab Staining


Add Fluorescent
Antibodies
Peripheral
Blood / PBMC
Skin
Cell Cultures
Cell
Suspension
Staining

Ab Staining

1. **Direct staining**

- In direct immunofluorescence staining, cells are incubated with an antibody directly conjugated to a fluorochrome (e.g. FITC). This has the advantage of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody. This approach is particularly useful for intracellular staining, where large antibody-fluorochrome complexes including secondary antibodies can become trapped causing non-specific binding, or fail to enter the cell preventing primary antibody detection.

2. **Indirect staining**

- In indirect staining, the primary antibody is not fluorochrome-labeled, but is detected by a fluorochrome-labeled secondary antibody. This second reagent may be an antibody with specificity for the first antibody. Alternatively, the avidin-biotin system can be used, whereby an antibody is conjugated to biotin and detected with fluorochrome-labeled avidin. With the wide range of conjugated antibodies now available, this method means that unconjugated primary antibodies raised against many different targets can be used in conjunction with a labeled secondary antibody for FACS analysis. This widens the choice of target proteins for the researcher.

Ab Staining

3. **Intracellular staining**

- Staining of intracellular antigens for flow cytometry protocols depends on various fixation and permeabilization methods to allow access of antibodies to internal cellular proteins. A successful staining procedure in all cases is dependent on optimization of experimental conditions through titering of antibodies, use of appropriate controls to set up the flow cytometer correctly and optimized fixation and permeabilization procedures.

4. **Detection of secreted proteins**

- Detection of secreted proteins is difficult as the protein will be released from the cell before detection, or may degrade rapidly. A Golgi block such as Brefeldin A can be used to inhibit secretion of expressed proteins, retaining them in the Golgi apparatus. The intracellular staining method can then be used for detection of the target protein.

Common Lasers & related probes

Violet Laser (405nm):

- A: BV786, Qdot800
- B: BV711 (CD3), Qdot705, eFluor700NC
- C: BV650, Qdot655, eFluor650NC
- D: BV605, Qdot605, eFluor605NC
- E: AmCyan, BV510 (CD56), Pacific Orange
- F: Alexa Fluor430, BV421 (PBSE), DAPI

Blue Laser (488nm):

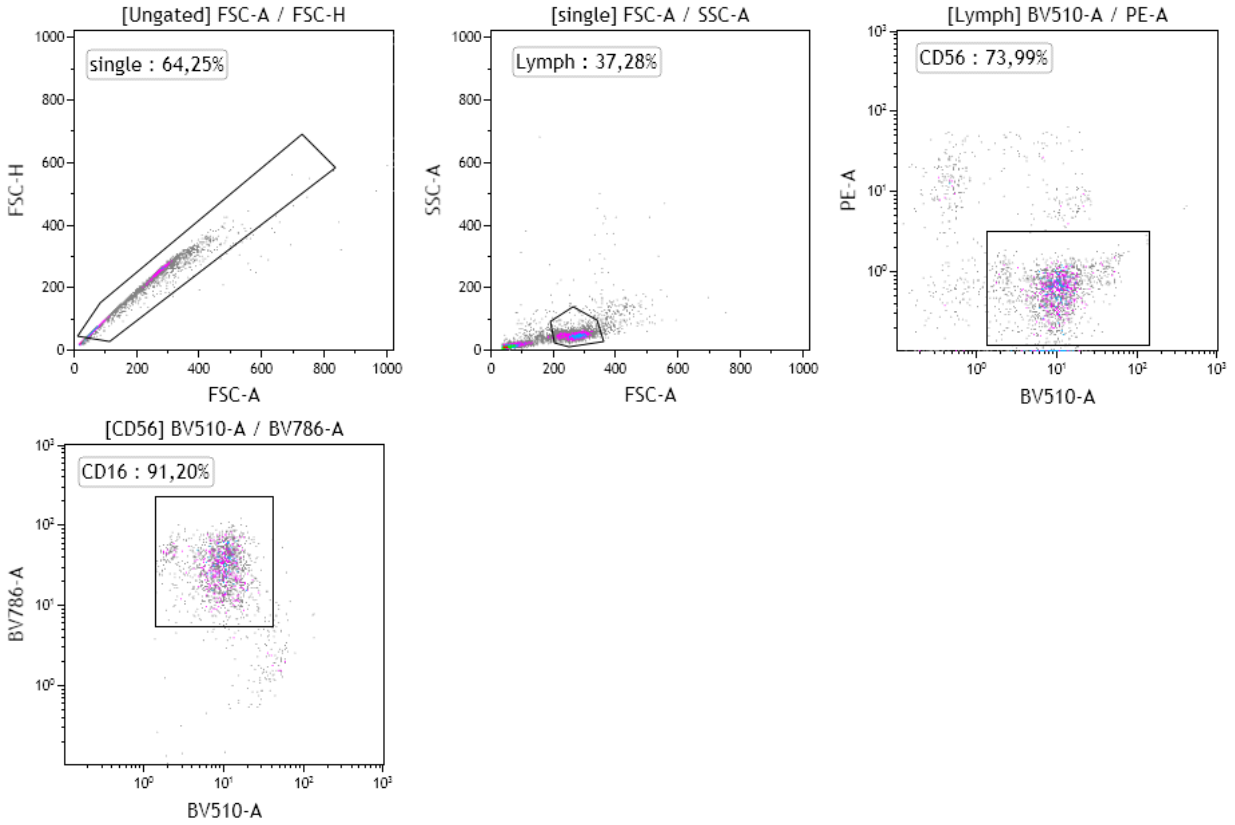
- A: PE-Cy7, PE-Vio700
- B: 7AAD, PE-Cy5, PE-Cy5.5
- C: PE-CF594, PE-Texas Red, PI
- D: PE (CD3, CD107a), PKH26
- E: Alexa Fluor 468, FITC (Llama), GFP
- F: SSC

Red Laser (640nm):

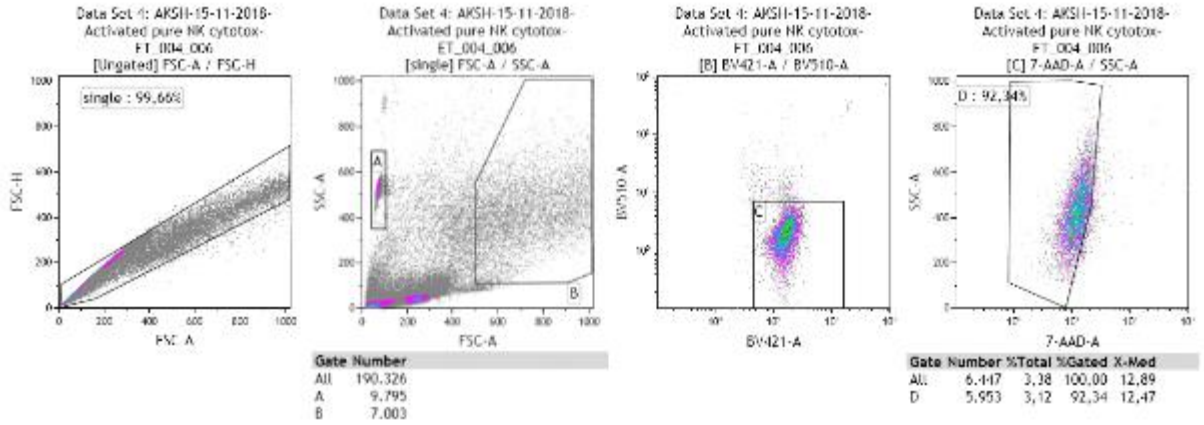
- A: APC-Cy7, APC-H7, Alexa Fluor 750
- B: Alexa Fluor 700, APC-Alexa 700, eFluor 710
- C: Alexa Fluor 647, APC, eFluor 660

Gating

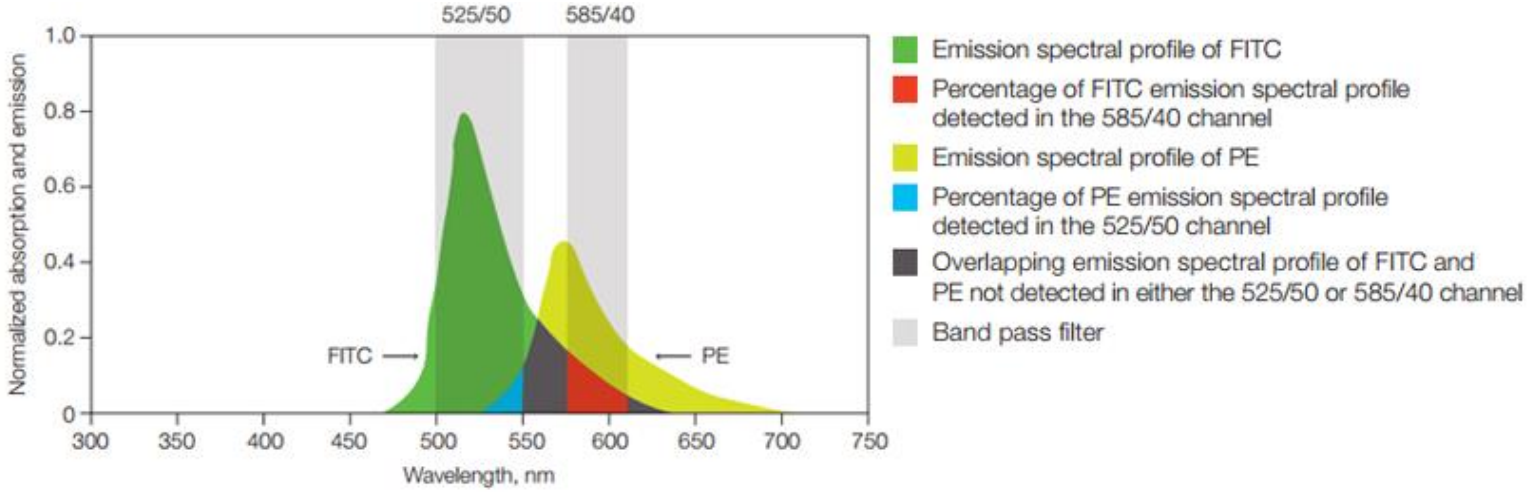
NK purity
Check

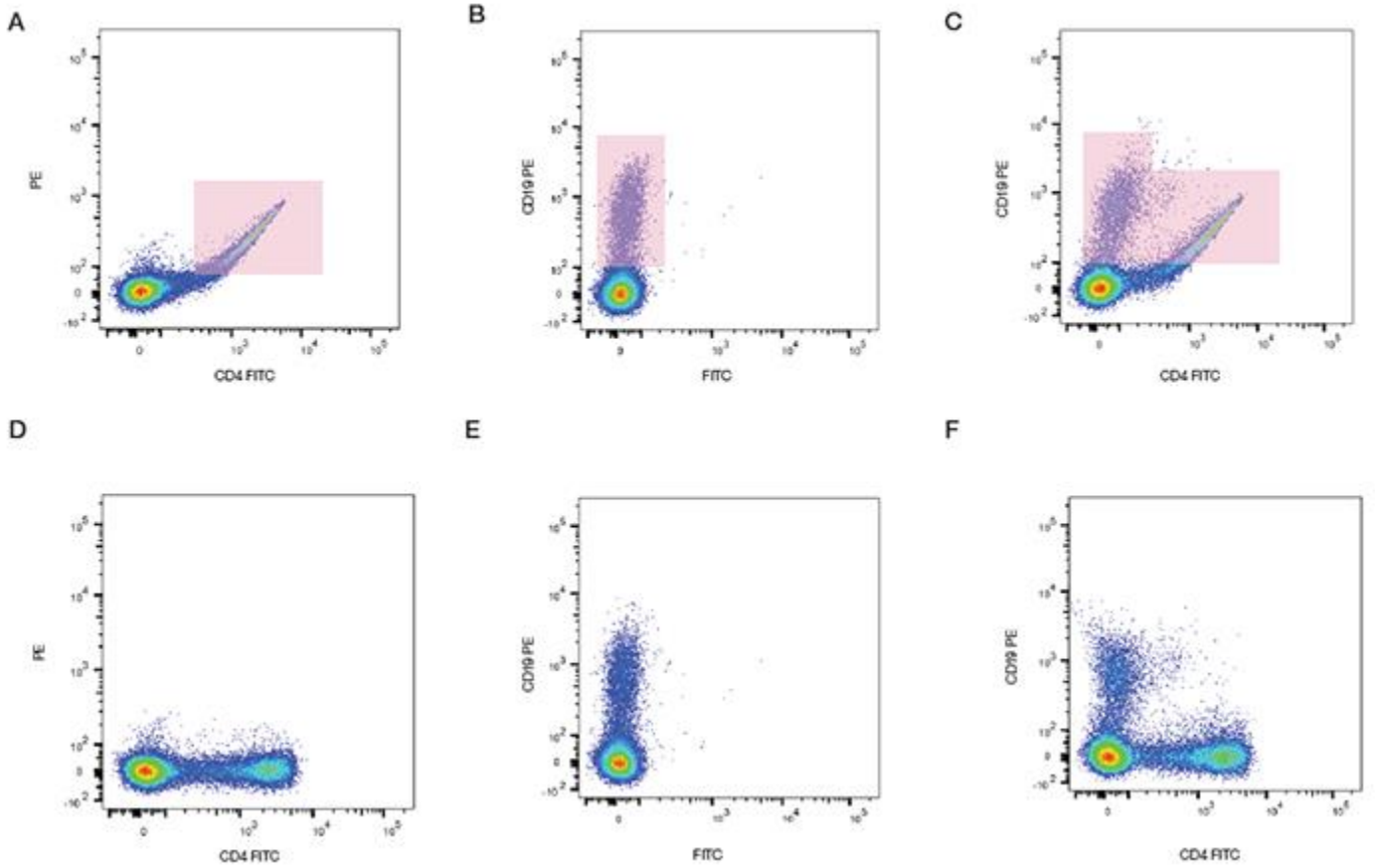


Gating


Data Set 4: AKSH-15-11-2018-
Activated pure NK cytotox-
ET_004_006
[Ungated] FSC-A / FSC-H
single : 99,66%
FSC-H
FSC-A
Data Set 4: AKSH-15-11-2018-
Activated pure NK cytotox-
ET_004_006
[single] FSC-A / SSC-A
A
B
SSC-A
FSC-A
Gate Number
All 190.326
A 9.795
B 7.003
Data Set 4: AKSH-15-11-2018-
Activated pure NK cytotox-
ET_004_006
[B] BV421-A / BV510-A
BV510-A
BV421-A
Data Set 4: AKSH-15-11-2018-
Activated pure NK cytotox-
ET_004_006
[C] 7-AAD-A / SSC-A
D : 92,34%
SSC-A
7-AAD-A
Gate Number %Total %Gated X-Med
All 6.417 3,38 100,00 12,89
D 5.953 3,12 92,34 12,47

Overlap/Spillover And Compensation





Applications

Immunophenotyping

Antigen Specific Responses

Binding Assay

Cytotoxicity Assay

Degranulation Assay

Intracellular Cytokine Analysis

Apoptosis Analysis

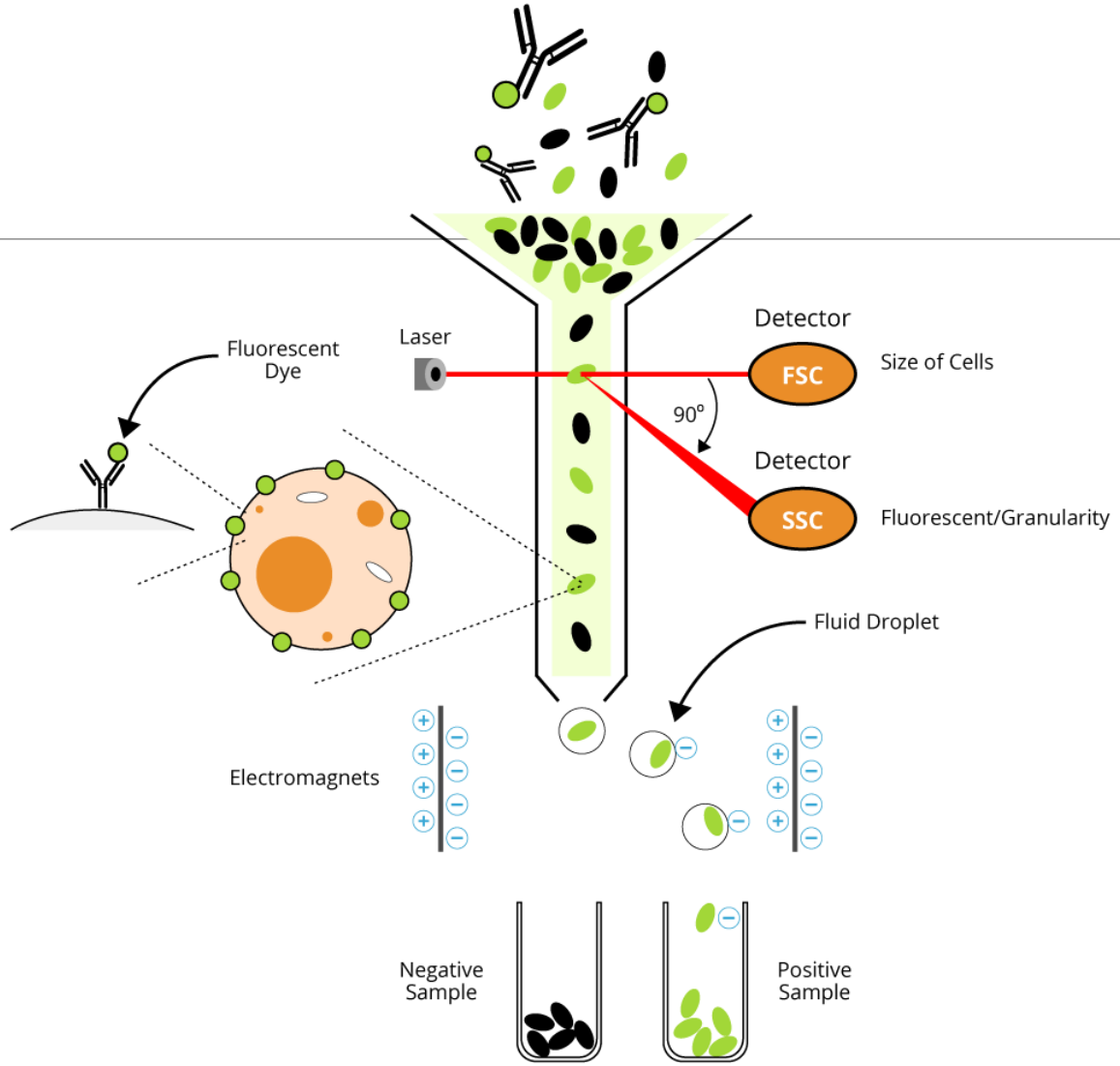
Proliferation Analysis

Fluorescent Protein Analysis

Cell Sorting

Phagocytosis Assays

ETC


Fluorescent
Dye
Laser
Detector
FSC
Size of Cells
90°
Detector
SSC
Fluorescent/Granularity
Fluid Droplet
Electromagnets
Negative
Sample
Positive
Sample

Thanks