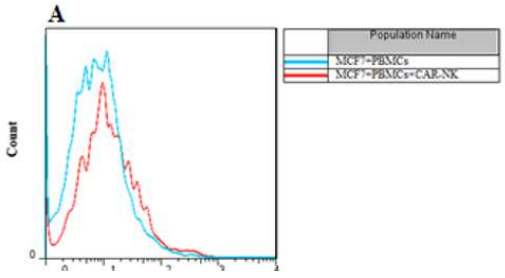
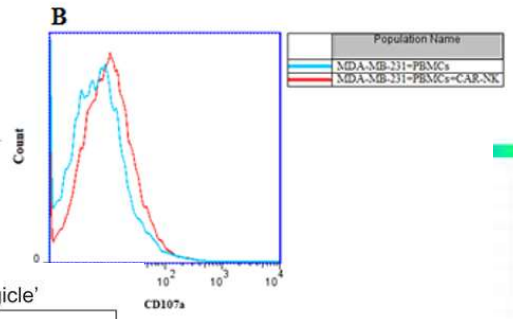
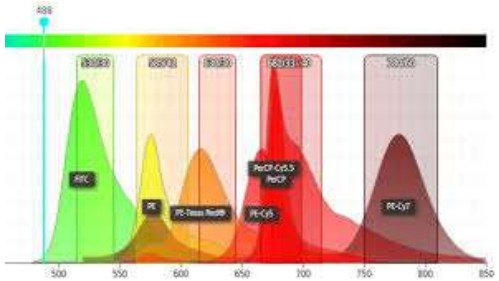
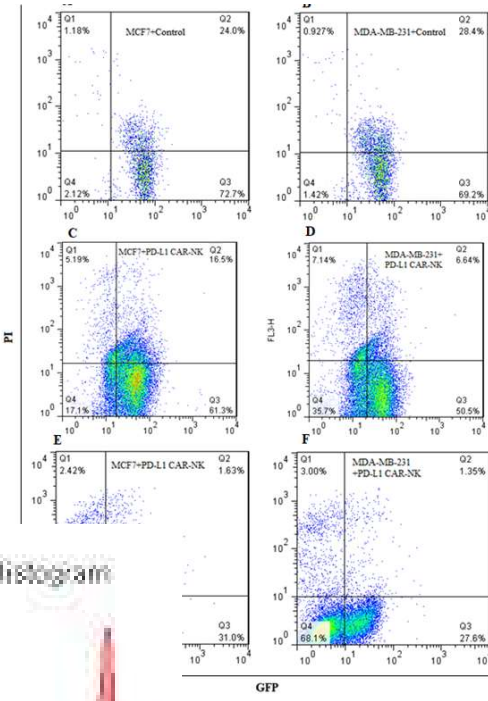
Flow Cytometry Workshop

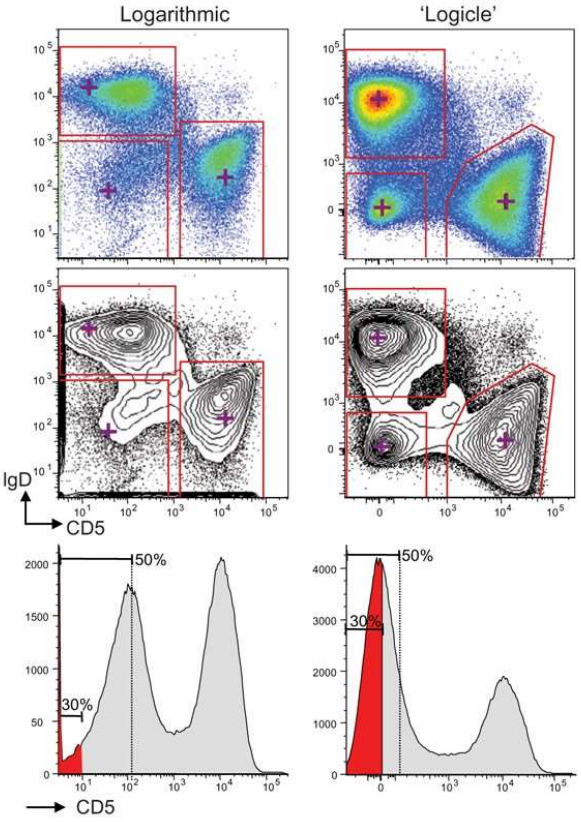
- Nafiseh Esmaeil
- Ph.D. of Immunology

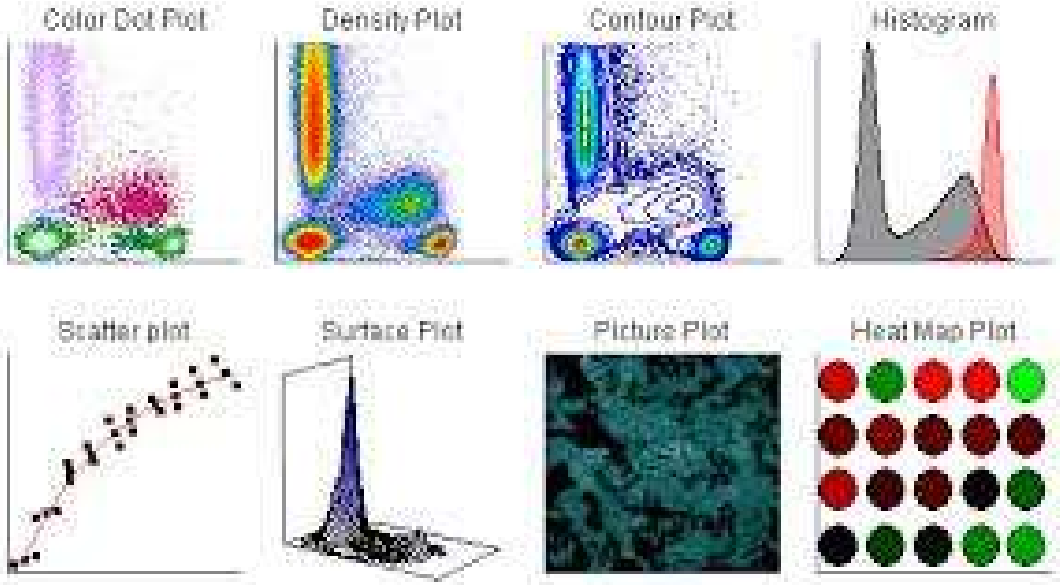

A
Population Name
MCF7-PBMCs
MCF7-PBMCs+CAR-NK
Count


B
Population Name
MDA-MB-231-PBMCs
MDA-MB-231-PBMCs+CAR-NK
Count
CD107a



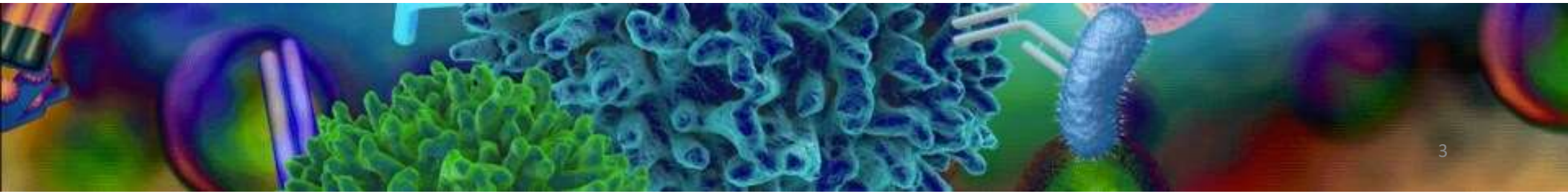

MCF7+Control
MDA-MB-231+Control
MCF7+PD-L1 CAR-NK
MDA-MB-231+PD-L1 CAR-NK
PI
GFP


Logarithmic
'Logicle'
IgD
CD5
50%
30%
CD5


Color Dot Plot
Density Plot
Contour Plot
Histogram
Scatter plot
Surface Plot
Picture Plot
Heat Map Plot

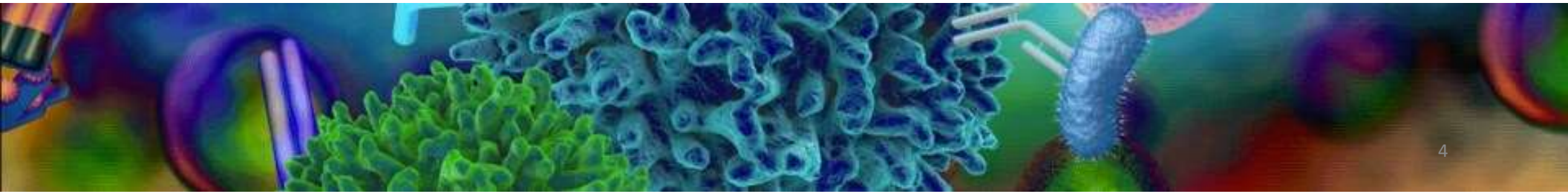
Section I

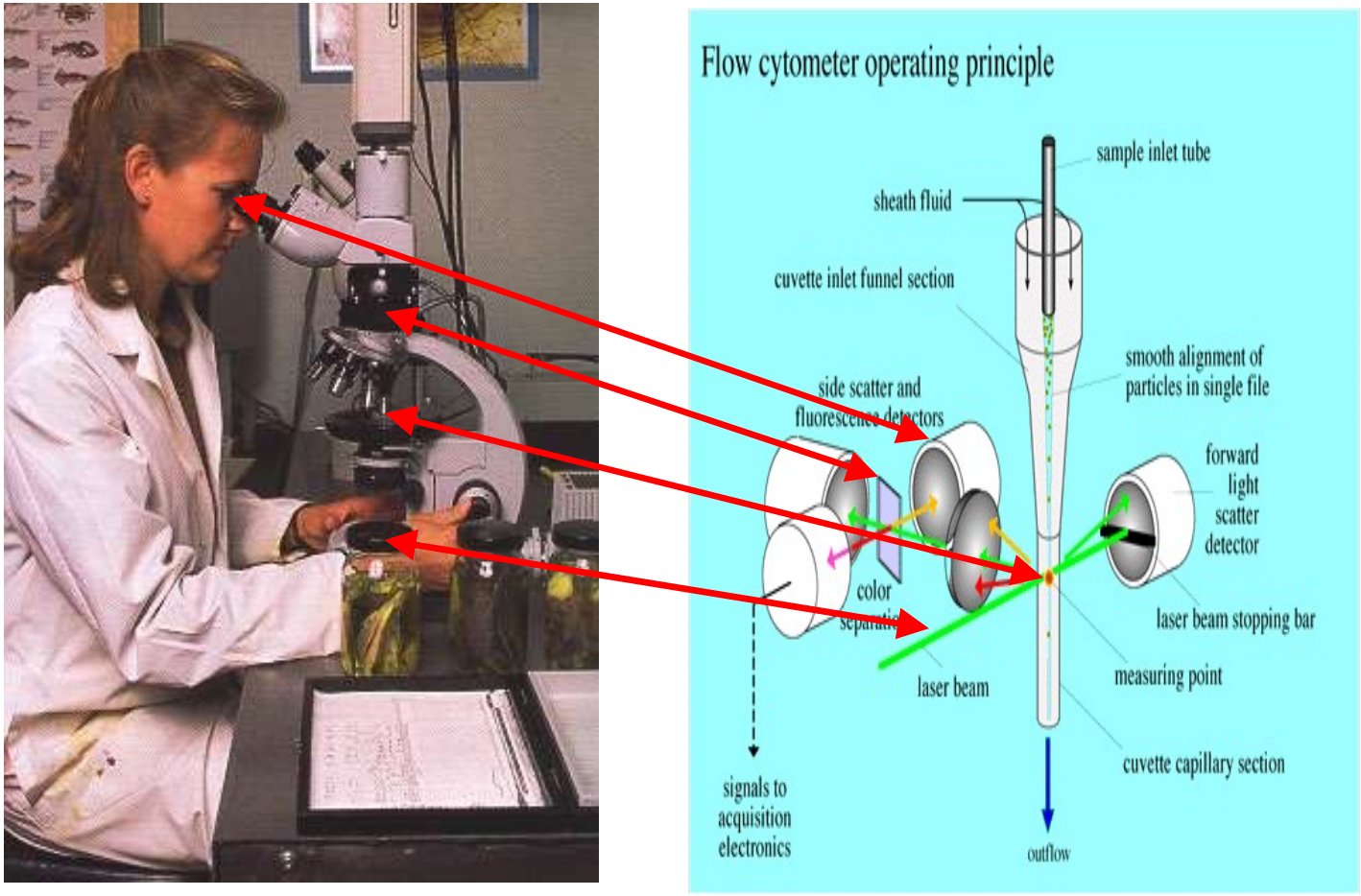
Background Information on Flow Cytometry



What Is Flow Cytometry?

- Flow ~ cells in motion
- Cyto ~ cell
- Metry ~ measure
- Measuring properties of cells while in a fluid stream




Flow cytometer operating principle
sample inlet tube
sheath fluid
cuvette inlet funnel section
smooth alignment of particles in single file
side scatter and fluorescence detectors
forward light scatter detector
color separation
laser beam stopping bar
laser beam
measuring point
cuvette capillary section
signals to acquisition electronics
outflow

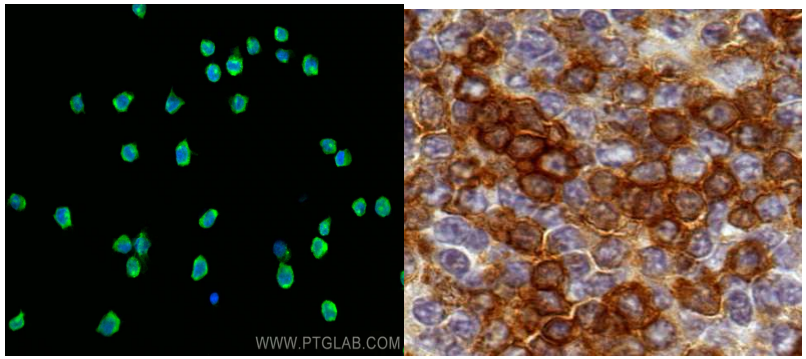
Cytometry vs. Flow Cytometry

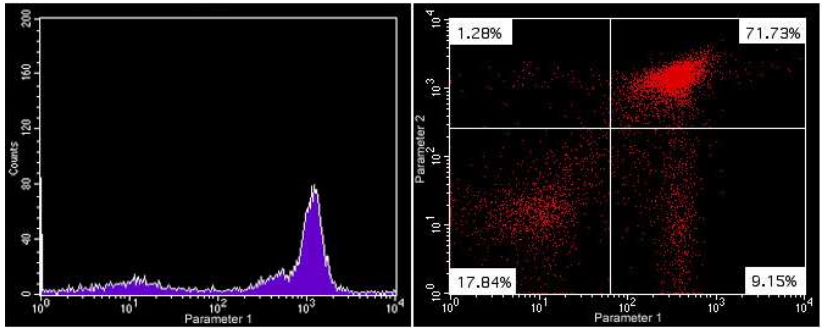
Cytometry

- Localization of antigen is possible
- Poor enumeration of cell subtypes
- Limiting number of simultaneous measurements

Flow Cytometry

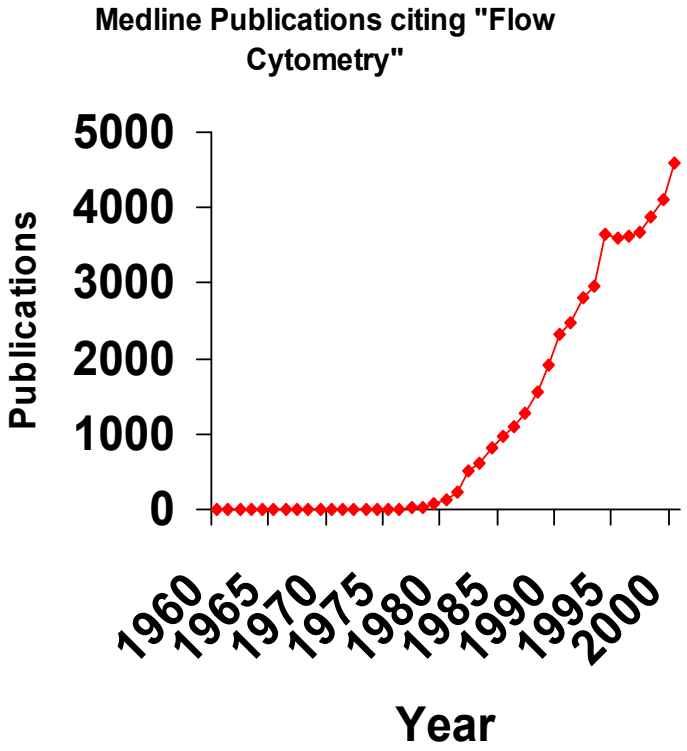
- Cannot tell you where antigen is.
- Can analyze many cells in a short time frame.
- Can look at numerous parameters at once.





Uses of Flow Cytometry

- It can be used for…
  - Immunophenotyping
  - DNA cell cycle/tumor ploidy
  - Membrane potential
  - Ion flux
  - Cell viability
  - Intracellular protein staining
  - pH changes
  - Cell tracking and proliferation
  - Sorting
  - Redox state
  - Chromatin structure
  - Total protein
  - Lipids
  - Surface charge
  - Membrane fusion/runover
  - Enzyme activity
  - Oxidative metabolism
  - Sulfhydryl groups/glutathione
  - DNA synthesis
  - DNA degradation
  - Gene expression
- The use of flow in research has boomed since the mid-1980s



The Many Parts of Flow

- Experimental design
- Sample preparation
- Choosing the proper instrument
- Setting up the instrument
- Collecting the proper data
- Interpreting the data
- Graphics presentation and publication
- Sorting

Specific Applications Courses

Flow Basics

Data Analysis

Basic mechanism

Biological sample

↓

Label it with a fluorescent marker

↓

Cells move in a linear stream through a focused light source (laser beam)

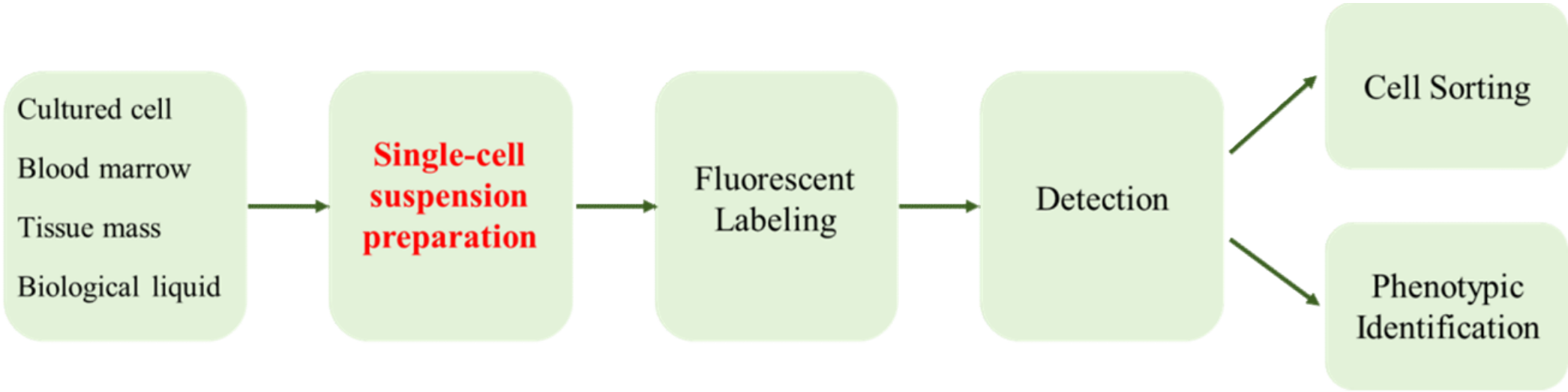
↓

Fluorescent molecule gets activated and emits light that is filtered and detected by sensitive light detectors (usually a photomultiplier tube)

↓

Conversion of analog fluorescent signals to digital signals

Experimental design and Sample preparation



Peripheral blood cells or cells growing in suspension are most suitable for flow cytometry analysis.

Peripheral whole blood

- Peripheral whole blood anticoagulated with EDTA or sodium heparin is often used in flow cytometry experiments. Approach:

- 1. Use hemolysin to lyse red blood cells

- 2. Use Ficoll or Percoll to isolate PBMCs from peripheral whole blood, and obtain the target cells from the corresponding density layer for cleaning and staining.

Common Categories	Anticoagulant Selection	Storage Time
Lymphocyte Phenotypic Analysis	Sodium Heparin or EDTA	Less than 72 hours
Bone Marrow Immunophenotypic Analysis	EDTA	Immediately
Neutrophil Function Analysis	Sodium Heparin or EDTA	Immediately
Platelet Activation	EDTA	Immediately
Platelet Marker Analysis	EDTA	Immediately
Erythrocyte Analysis	EDTA	Less than 72 hours (4°C)
Cell Cycle Analysis	Sodium Heparin or EDTA	Immediately
DNA Ploidy Analysis	Sodium Heparin or EDTA	Less than 72 hours

Methods	Cell Yield	Experiment Speed	Selection Basis	Advantages
Lysis of Red Blood Cells	High	Fast	Used for lymphocyte phenotypic identification or when the research subjects are mononuclear cells, granulocytes, and lymphocytes that do not require culture or cryopreservation.	① Simple experimental operation ② Cell morphology can be used for differentiation of granulocytes, mononuclear cells,and lymphocytes
Ficoll/Percoll Density Gradient Centrifugation	Moderately Low	Moderately Slow	Used for lymphocyte functional testing or when lymphocyte subpopulations, monocytes, and other cells need further culture, blocking, and long-term storage.	① Can remove most neutrophils ② PBMCs obtained can be cryopreserved for long-term storage ③ The cells obtained are more pure ④ Most dead cells are removed

Peripheral whole blood split red experimental protocol

1) Add an appropriate amount of surface antibody to anticoagulated whole blood and incubate at room temperature for 15 minutes

2) Add 1× red blood cell lysis solution, incubate at room temperature in the dark for 10 minutes, centrifuge at 300-400 g for 5 minutes, and remove the supernatant

3) Add PBS/flow cytometry staining buffer and mix;

4) Centrifuge again according to the above conditions and disperse the pellet in PBS/flow cytometry staining buffer

5) Test on the machine within 3 hours (if you cannot get on the machine immediately, resuspend the cells use 500 ul 4% paraformaldehyde, store in the dark at 4°C, and analyze on the machine within 24 hours).

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Peripheral whole blood split red experimental protocol

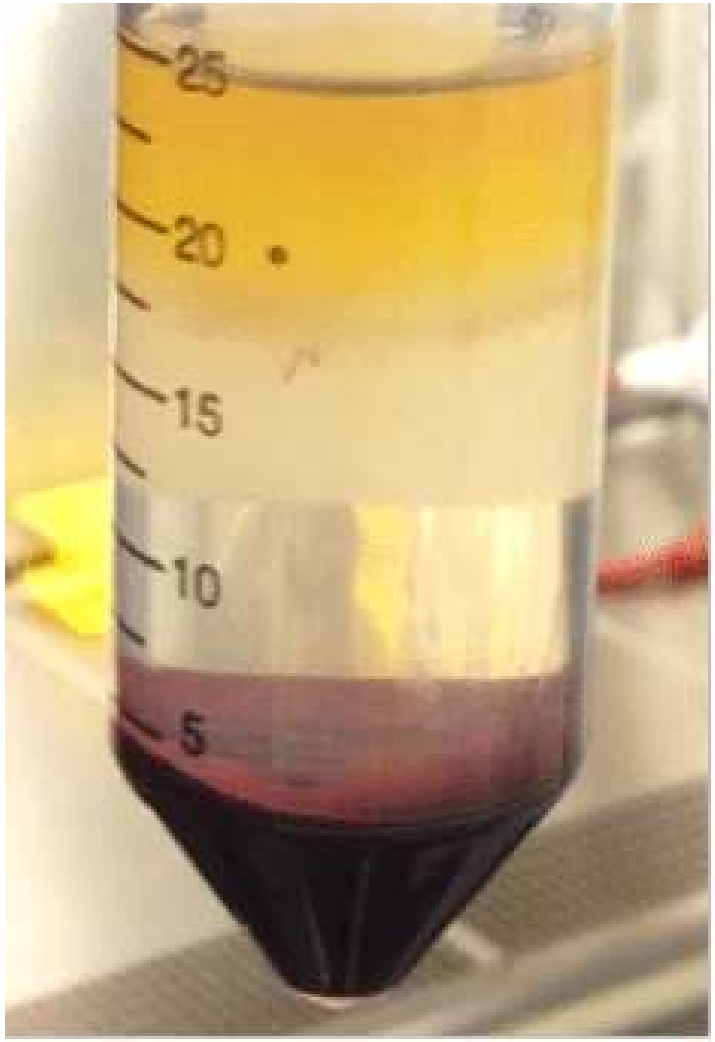
1) Add an appropriate amount of surface antibody to anticoagulated whole blood and incubate at room temperature for 15 minutes;

2) Add 1× red blood cell lysis solution and incubate at room temperature in the dark for 10 minutes;

3) Mix well and test on the machine.

Isolation of peripheral blood mononuclear cells

- 1) Dilute the blood sample 2-4 times with sterile diluent;
- 2) First add 15-20 mL of separation liquid into a 50mL conical bottom centrifuge tube, and then slowly add 20-30 mL of diluted whole blood or tissue cell suspension to the surface of the separation liquid
- 3) Centrifuge at 400 g for 15-30 minutes at room temperature to ensure that the rotor speed decreases smoothly
- 4) Carefully take out the centrifuge tube from the centrifuge and slowly suck off the top layer to avoid contact with the mononuclear cell layer
- 5) Slowly transfer the mononuclear cell layer to another 50 mL conical bottom centrifuge tube
- 6) Add sterile washing solution and mix well, centrifuge at 300 g for 10 minutes at room temperature, and carefully discard the supernatant
- 7) To remove residual platelets, resuspend the cells in 30-50 mL sterile washing solution, centrifuge at 200 g for 10-15 mins at room temperature, and discard the supernatant
- 8) You can optionally repeat step 7 to remove most of the platelets
- 9) Resuspend the cells in buffer or culture medium and perform subsequent operations such as detection and culture.
- Note: The experimental steps for bone marrow samples are the same as those for peripheral whole blood.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15
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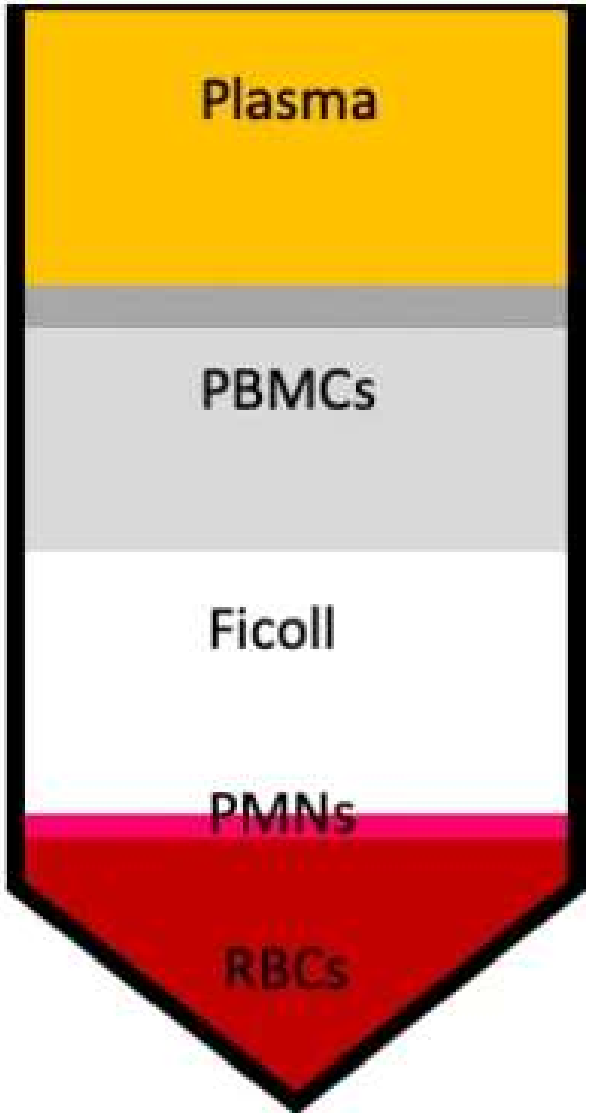

Plasma
PBMCs
Ficoll
PMNs
RBCs

Experimental design and Sample preparation

- Identification of cells through their marker expression is one of the key techniques in flow cytometry.
- Some cells are easy to identify using one or two markers, however some subsets require identification of multiple markers to accurately identify a cell subset.
- Moreover, if you want to identify memory status, activation state and cytokine profiles, the number of markers can significantly increase.
- Another important consideration is the level of marker expression or antigen density.
- This will influence your choice of fluorophore as dim fluorophores should be paired with highly expressed markers and vice versa.
- Use the antigen density function of the panel builder to help choose the best fluorophore for your experiment.

Experimental design and Sample preparation

- Cell frequency can influence both the choice of fluorophore and how many cells you need to collect to obtain meaningful data.
- If you are searching for rare cells such as stem cells, or have a complex gating strategy, you may have to collect many more cells compared to more common cell types such as T cells.

Experimental design and Sample preparation
Choosing a Fluorophore

- Choosing a fluorophore rapidly becomes complicated as you increase the number of fluorophores.
- As already noted, fluorophore excitation and brightness depends on your flow cytometer and your cell of interest's abundance.
- However, choosing a fluorophore can depend on many other factors, including emission wavelengths as overlapping emission spectra affect staining and require compensation.
- The recommended way to avoid compensation and obtain the best resolution is to separate out fluorophores as much as possible across lasers and filters.
- With pre-loaded cytometer settings and the ability to exclude incompatible fluorophores, our panel builder can help you choose the best options.

Experimental design and Sample preparation
Choosing the Right Antibody

- Using antibodies conjugated to fluorophores, cells from virtually any source can be identified.
- However, finding the right antibody can be challenging.
- Search companies' website to find antibodies by marker, clone, isotype and target species.
- Alternatively use panel builder to find antibodies with the right fluorophore.
- Once you have sourced your antibody, and before starting your experiment, we recommend titration of the antibody.
- This can improve your data, by reducing the levels of background staining whilst maintaining a bright, positive population, and save you money!
- If you can't find the antibody conjugated to the fluorophore you want, you could use a secondary antibody or a conjugation kit depending on availability and complexity of staining.

Experimental design and Sample preparation Controls

- Controls are essential in any experiment to confirm positive results from background.
- In flow cytometry these can be biological, positive, negative, viability, isotypes, Fc blocking and fluorescence minus one (FMO) controls depending upon your experiment.
- Dead cells bind antibodies non-specifically therefore it is essential to remove them from your analysis.
- The use of forward and side scatter may not always be sufficient. Viability dyes are available in a wide range of excitation and emission wavelengths; easily fit controls to your experiment using our multicolor panel builder.

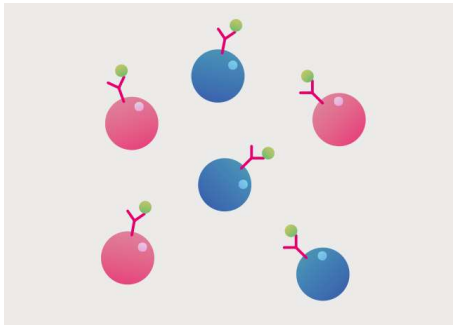
Experimental design and Sample preparation Staining Protocol

- Depending on the location of your antigen on your cell, the staining protocol may change.
- Surface staining with antibodies can be relatively straightforward but intracellular staining requires fixation and permeabilization, and a golgi inhibitor for cytokine staining.
- There are various methods and reagents for fixation and permeabilization.
- Depending on the antigen or technique being performed, these may also require optimization. Use the panel builder to create dump channels to improve resolution by excluding unwanted cells.

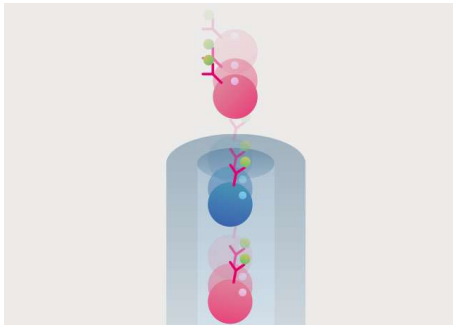
Experimental design and Sample preparation Staining Protocol

- Flow cytometry does not have to be difficult, and considering these critical steps before you start will improve your chances of obtaining great results.
- As mentioned, the multicolor panel builder is not only a useful tool to build large panels but also to:

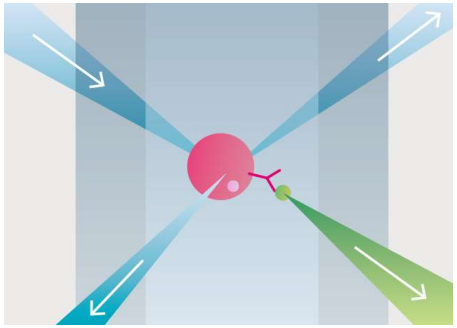
- Understand your cytometer
- Choose and separate out fluorophores by lasers and detectors
- Include compatible viability dyes
- Select flow validated antibodies
- Exclude unwanted cells using a dump channel



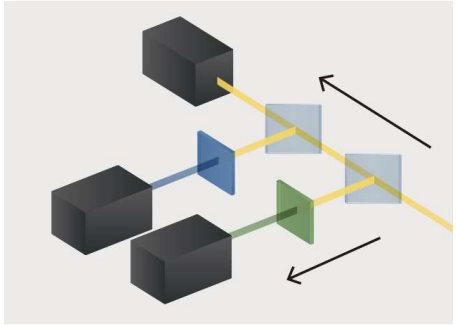
1. Target cells are fluorescently labeled.



2. Cells are poured into flow path system and move through tube at fixed intervals.



3. Cells are irradiated with laser beams.



4. Light is detected via the optical system.

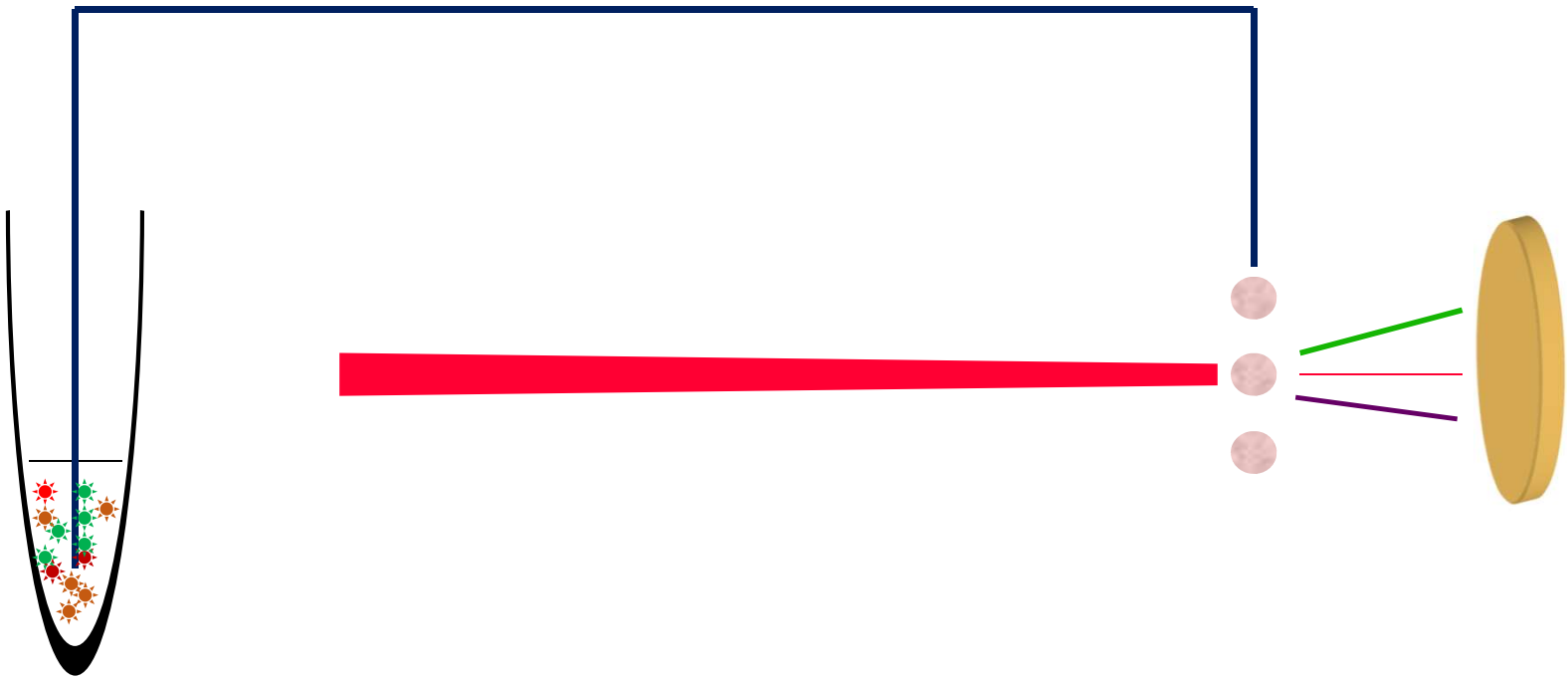
Section II

The 4 Main Components of a Flow Cytometer

What Happens in a Flow Cytometer?

- Cells in suspension flow single file past — Fluidics
- A focused laser where they scatter light and emit fluorescence that is filtered and collected — Interrogation
- Then converted to digitized values that are stored in a file — Electronics
- Which can then be read by specialized software. — Interpretation

What Happens in a Flow Cytometer (Simplified)

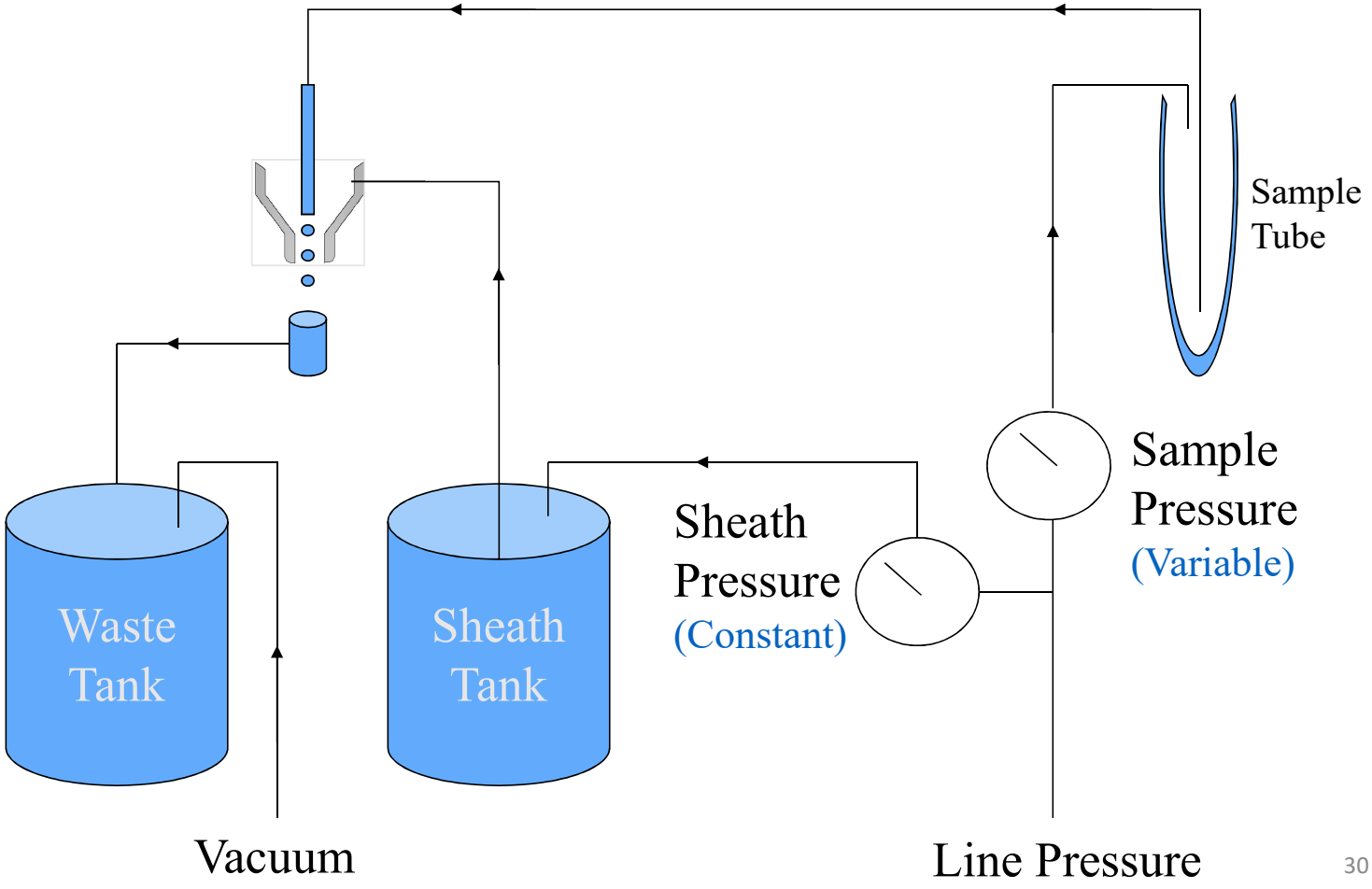


The Fluidics System

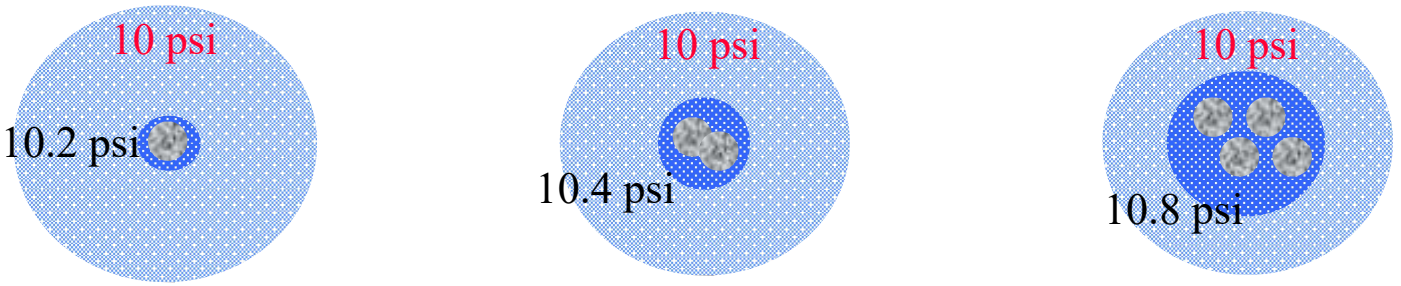
"Cells in suspension flow single file"

- You need to have the cells flow one-by-one into the cytometer to do single cell analysis
- Accomplished through a pressurized laminar flow system.
- The sample is injected into a sheath fluid as it passes through a small orifice (50um-300um)

Fluidics Schematic



Sample Differential



- Difference in pressure between sample and sheath
- This will control sample volume flow rate
- The greater the differential, the wider the sample core.
- If differential is too large, cells will no longer line up single file
- Results in wider CV's and increase in multiple cells passing through the laser at once. No more single cell analysis!

What Happens in a Flow Cytometer?

- Cells in suspension flow single file past — Fluidics
- a focused laser where they scatter light and emit fluorescence that is filtered, collected — Interrogation
- and converted to digitized values that are stored in a file — Electronics
- Which can then be read by specialized software. — Interpretation

Interrogation

- Light source needs to be focused on the same point where cells are focused.

- Light source
  - On all flow lab instruments-Lasers

Lasers

Light amplification by stimulated emission of radiation

- Lasers can provide a single wavelength of light (monochromatic)
- They can provide milli watts to watts of power
- Also provide coherent light
- All help to create a stable and reliable signal

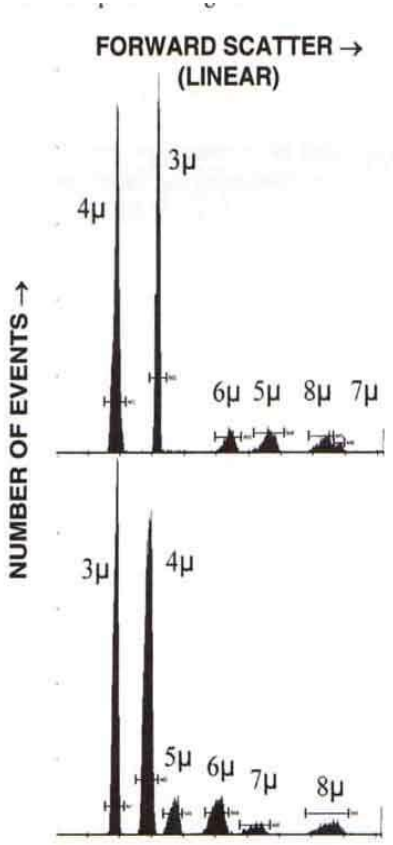
Coherent: all emitting photons have same wavelength, phase and direction as stimulation photons

Light Scatter

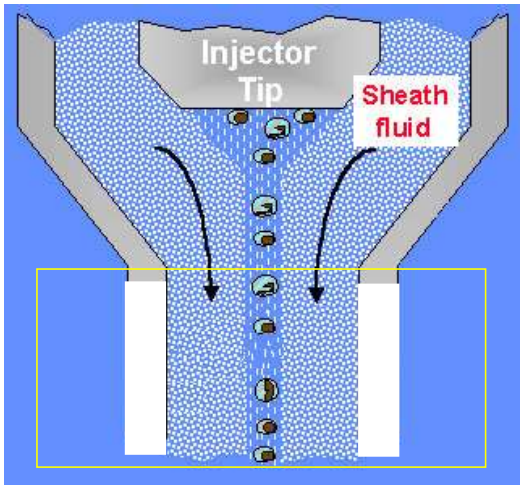
- When light from a laser interrogates a cell, that cell scatters light in all directions.
- The scattered light can travel from the interrogation point down a path to a detector.

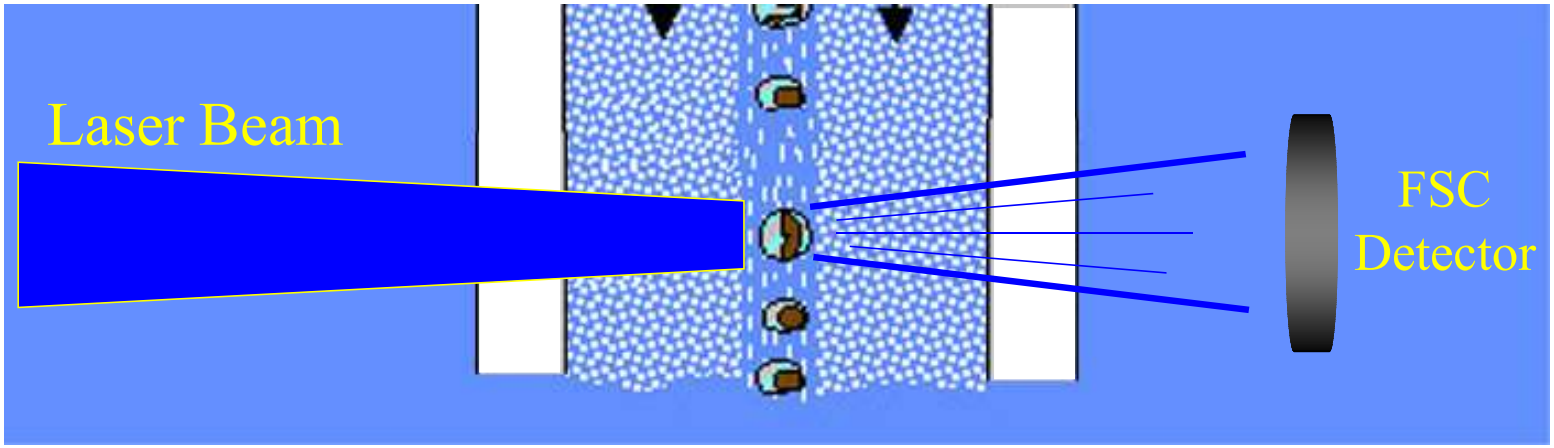
Forward Scatter

- Light that is scattered in the *forward* direction (along the same axis the laser is traveling) is detected in the Forward Scatter Channel.
- The intensity of this signal has been attributed to cell size, refractive index (membrane permeability)
- Forward Scatter=FSC=FALS=LALS



Forward Scatter

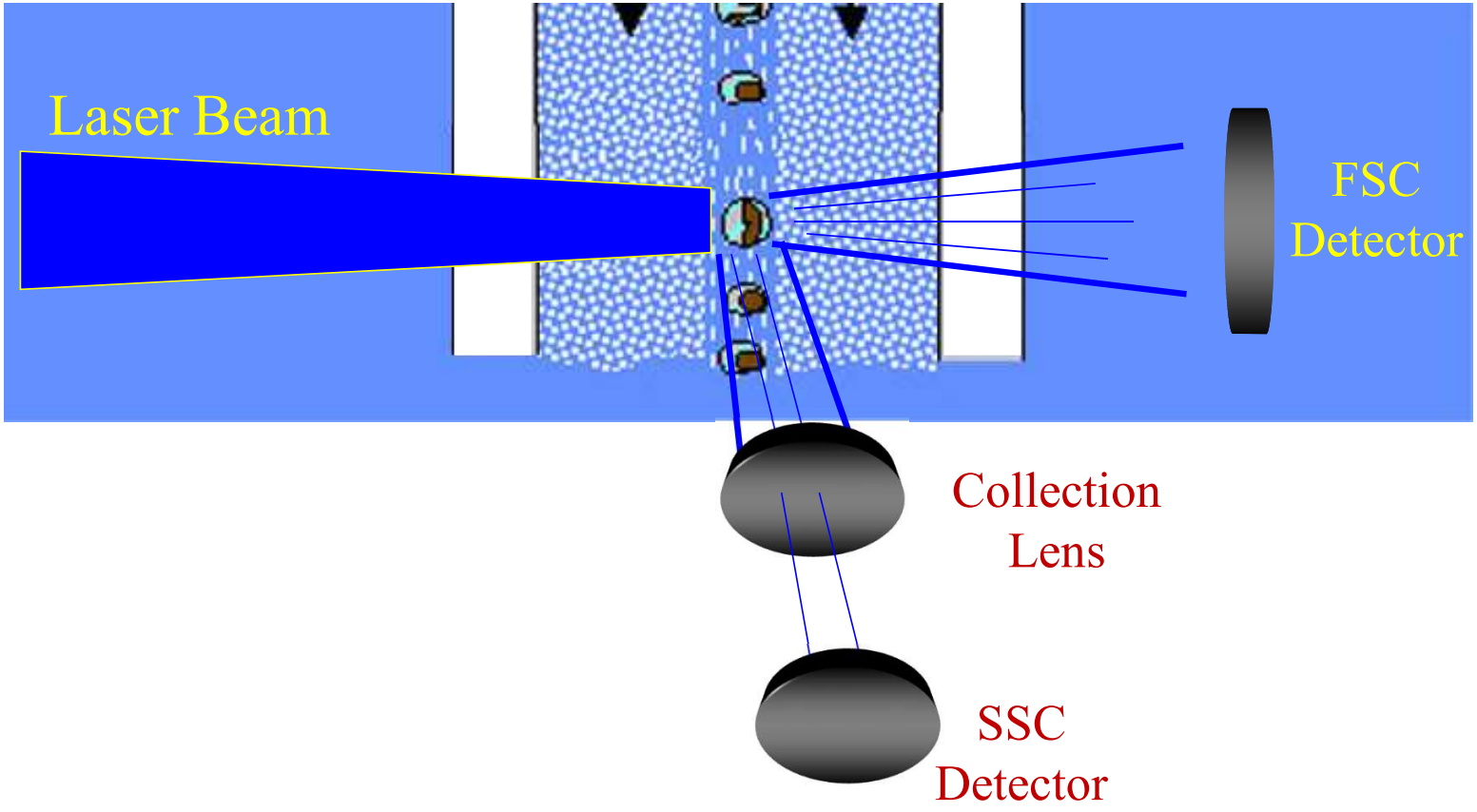




Side Scatter

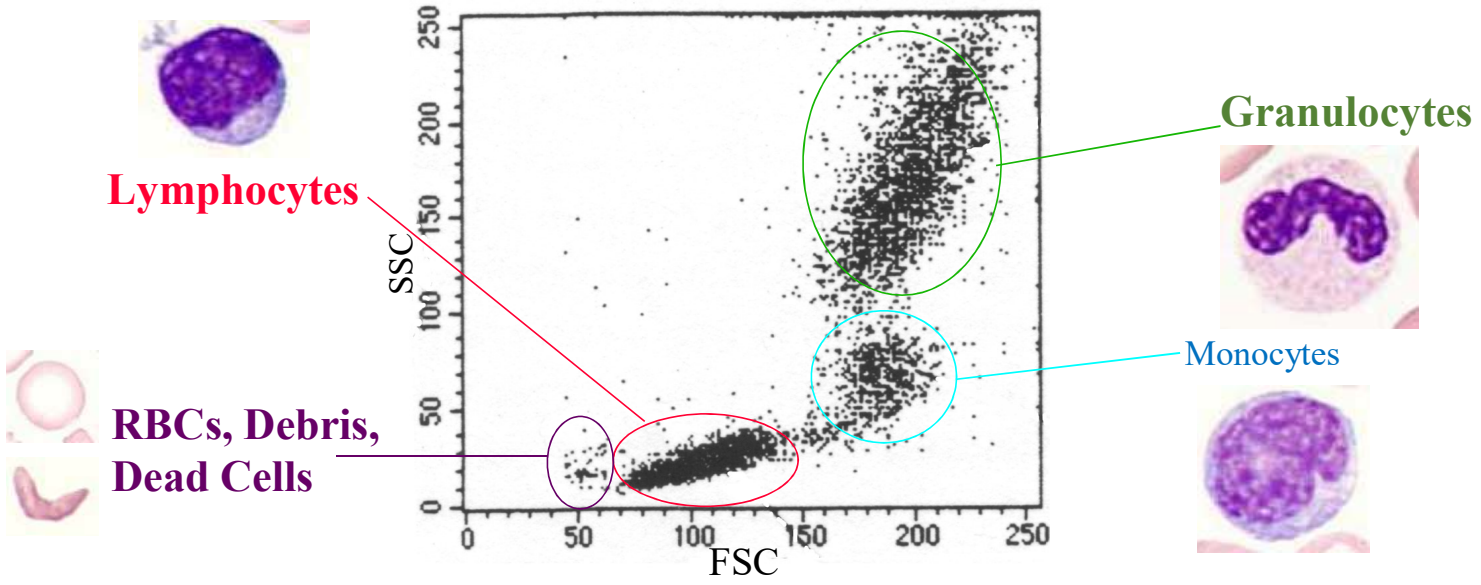
- Laser light that is scattered at 90 degrees to the axis of the laser path is detected in the Side Scatter Channel
- The intensity of this signal is proportional to the amount of cytosolic structure in the cell (eg. granules, cell inclusions, etc.)
- Side Scatter=SSC=RALS=90 degree Scatter

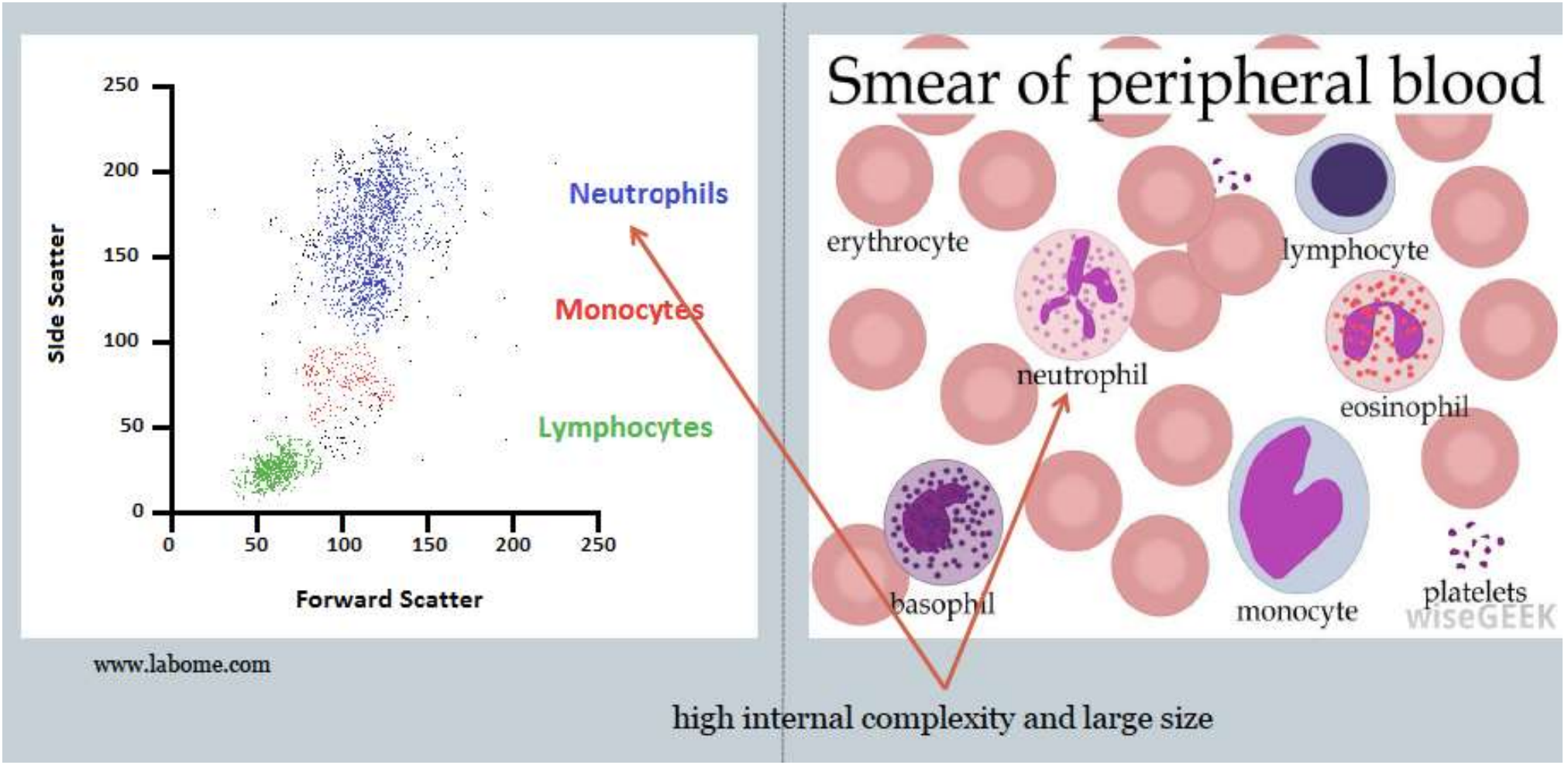
Side Scatter



Why Look at FSC v. SSC

- Since FSC ~ size and SSC ~ internal structure, a correlated measurement between them can allow for differentiation of cell types in a heterogenous.

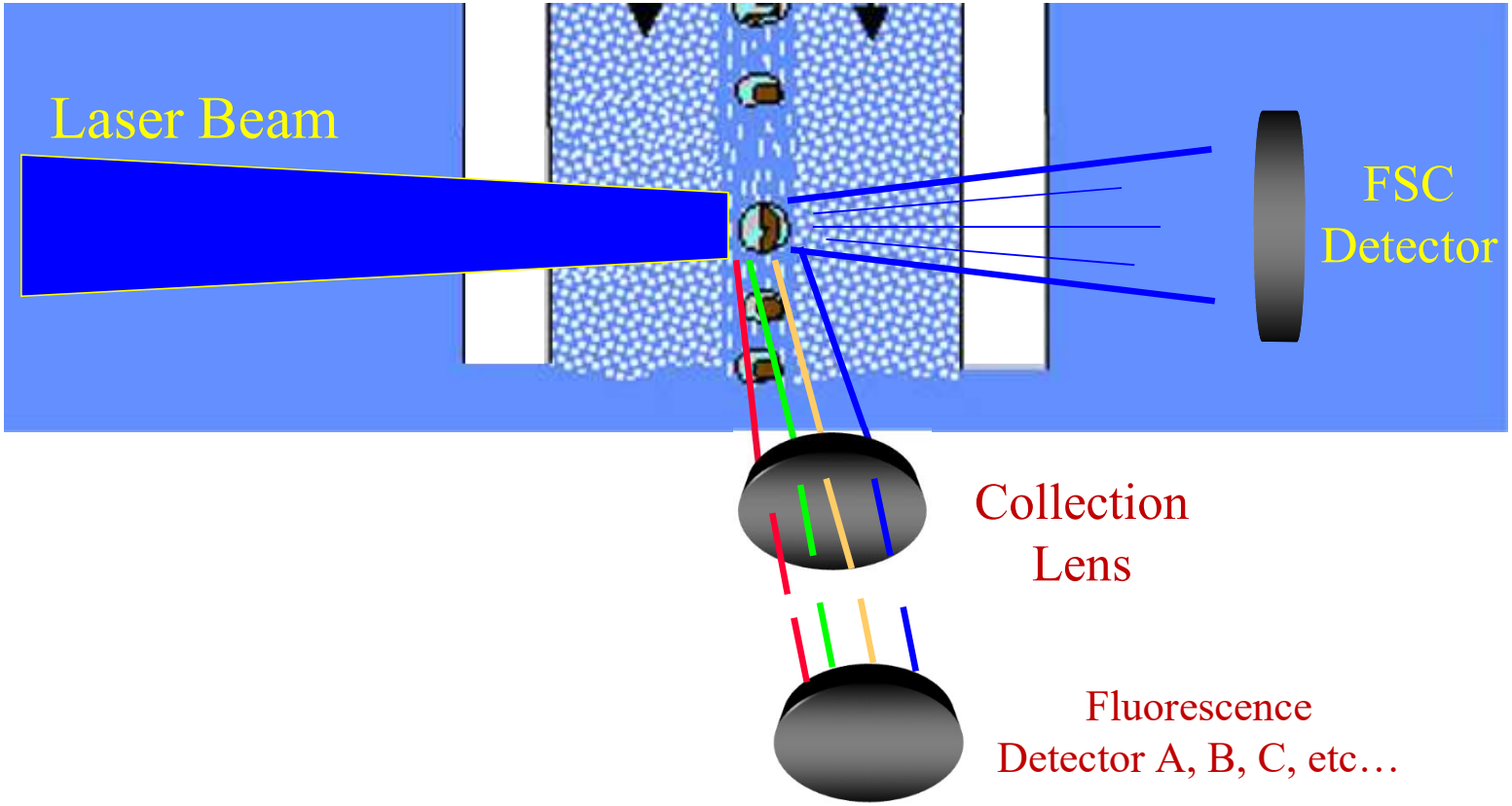



Side Scatter
250
200
150
100
50
0
0
50
100
150
200
250
Forward Scatter
Neutrophils
Monocytes
Lymphocytes
www.labome.com
Smear of peripheral blood
erythrocyte
lymphocyte
neutrophil
eosinophil
basophil
monocyte
platelets
wiseGEEK
high internal complexity and large size

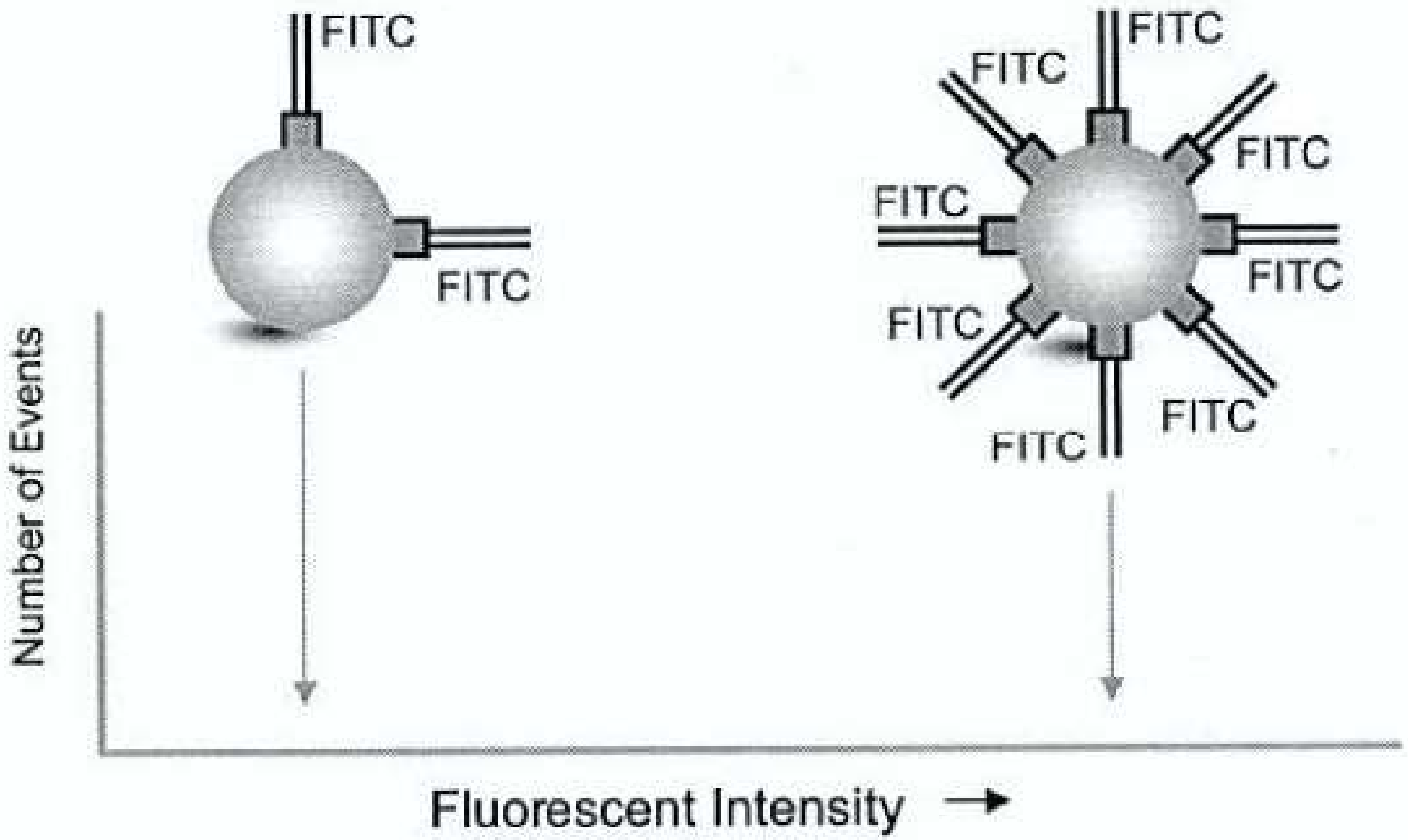
Fluorescence Channels

- As the laser interrogates the cell, fluorochromes on/in the cell (*intrinsic or extrinsic*) may absorb some of the light and become excited.
- As those fluorochromes leave their excited state, they release energy in the form of a photon with a specific wavelength, longer than the excitation wavelength.
- Those photons pass through the collection lens and are split and steered down specific channels with the use of filters.

Fluorescence Detectors



Emitted fluorescence intensity proportional to binding sites

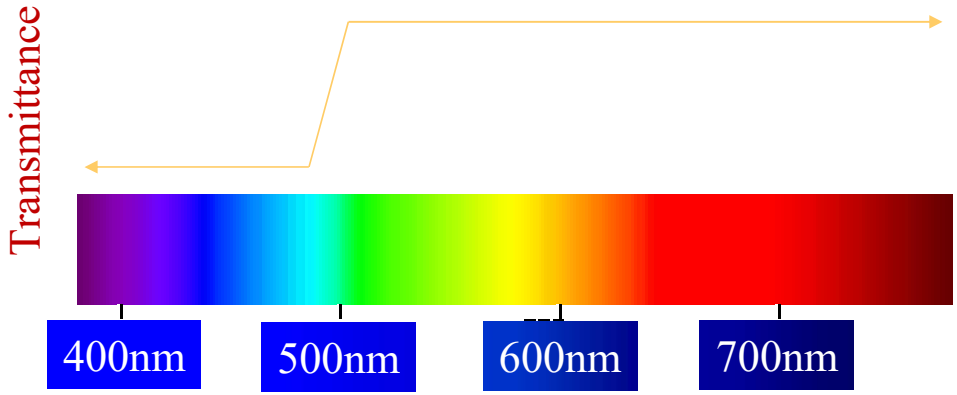


Filters

- Many wavelengths of light will be scattered from a cell, we need a way to split the light into its specific wavelengths in order to detect them independently. This is done with filters
- Optical filters are designed such that they absorb or reflect some wavelengths of light, while transmitting other.
- 3 types of filters
  - Long Pass filter
  - Short Pass filter
  - Band Pass filter

Long Pass Filters

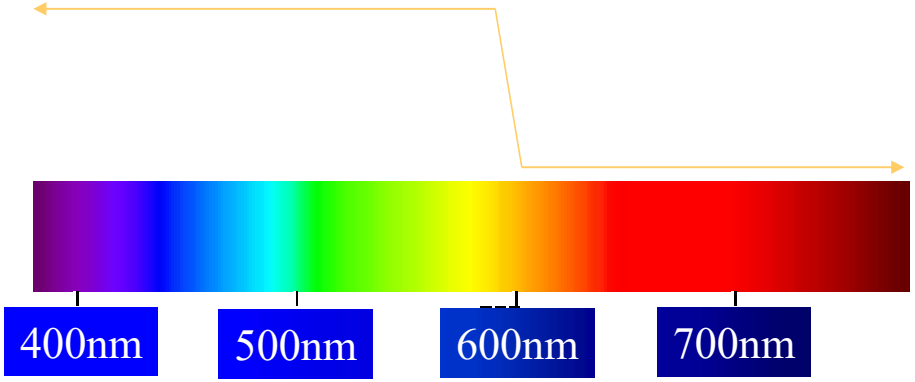
- Transmit all wavelengths greater than specified wavelength
  - Example: 500LP will transmit all wavelengths greater than 500nm



Short Pass Filter

- Transmits all wavelengths less than specified wavelength

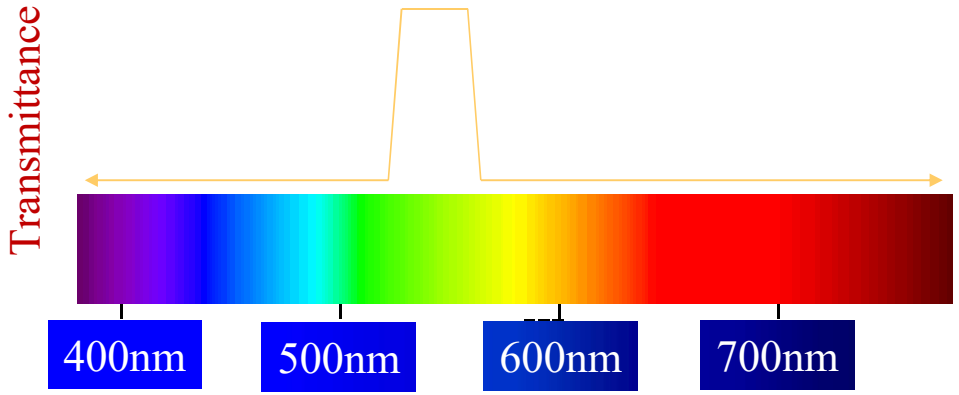
  Example: 600SP will transmit all wavelengths less than 600nm.



Band Pass Filter

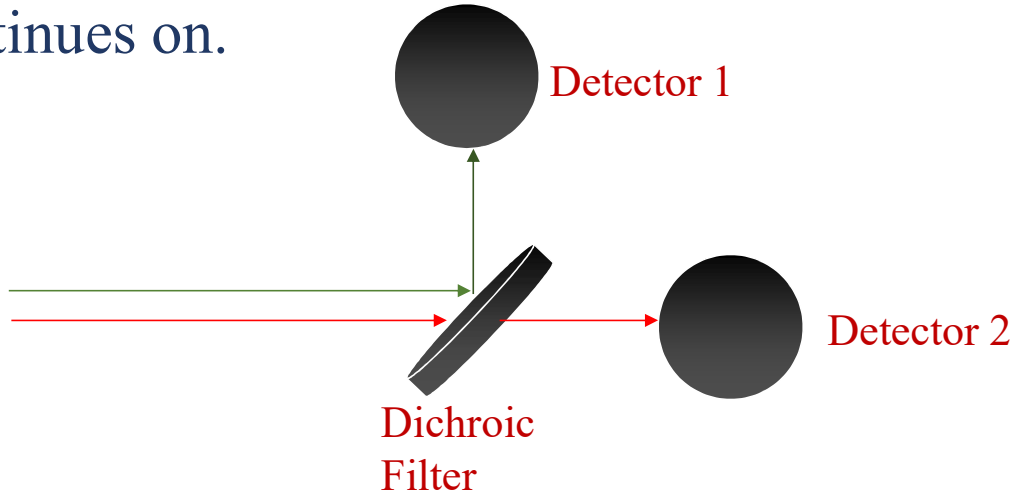
- Transmits a specific band of wavelengths

  Example: 550/20BP Filter will transmit wavelengths of light between 540nm and 560nm *(550/20 = 550+/-10, not 550+/-20)*



Dichroic Filters

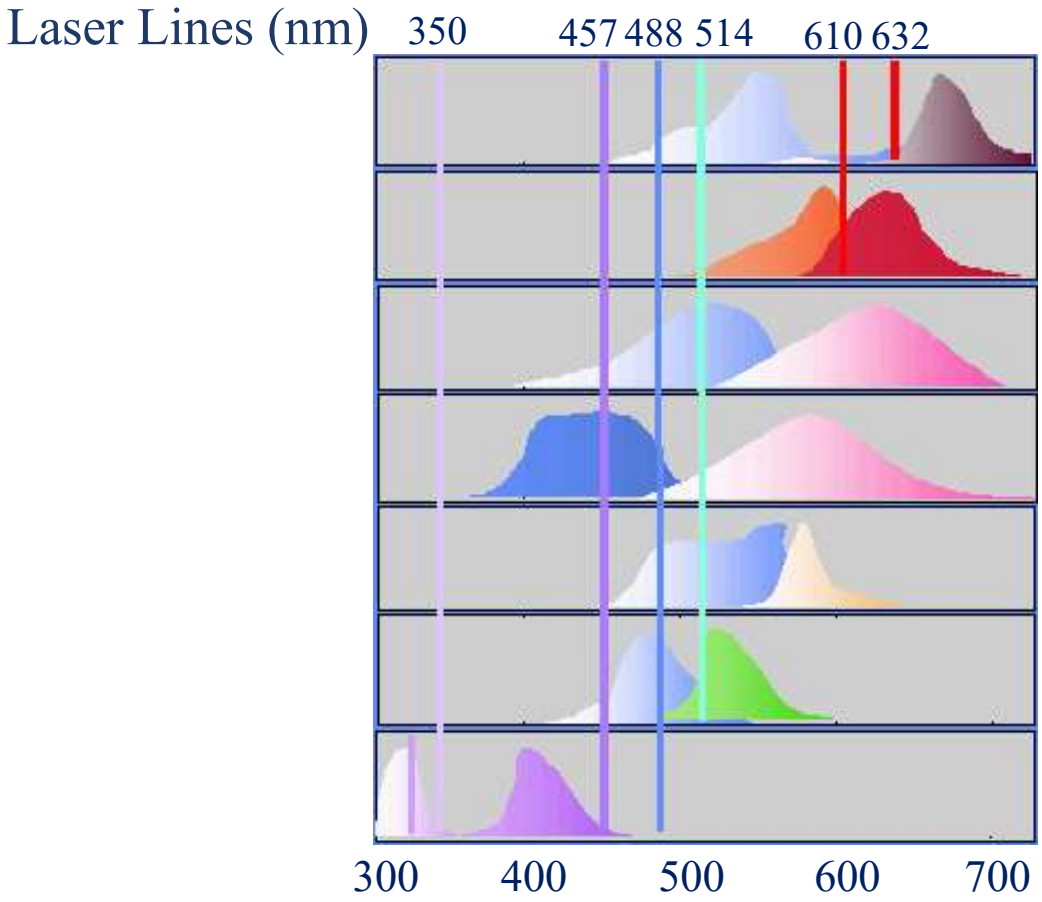
- Can be a long pass or short pass filter
- Filter is placed at a 45º angle to the incident light
- Part of the light is reflected at 90º to the incident light, and part of the light is transmitted and continues on.



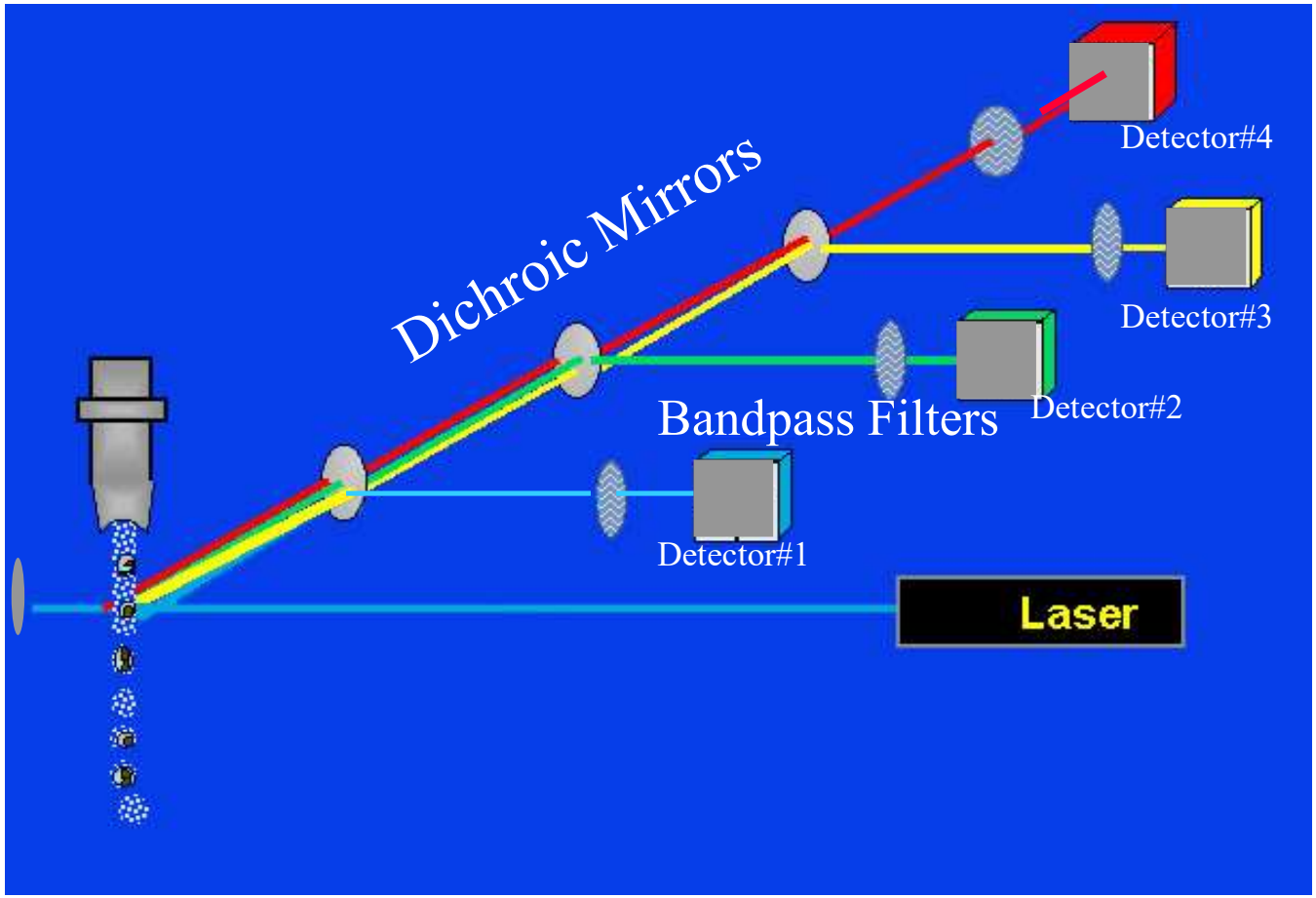
Optical Bench Layout

- To separate scatter and multiple fluorescence wavelengths simultaneously from each cell,
- The design of a multi-channel layout must consider
  - Spectral Properties of the fluorochromes used
  - The appropriate positioning of filters

Spectra of Common Fluorochromes



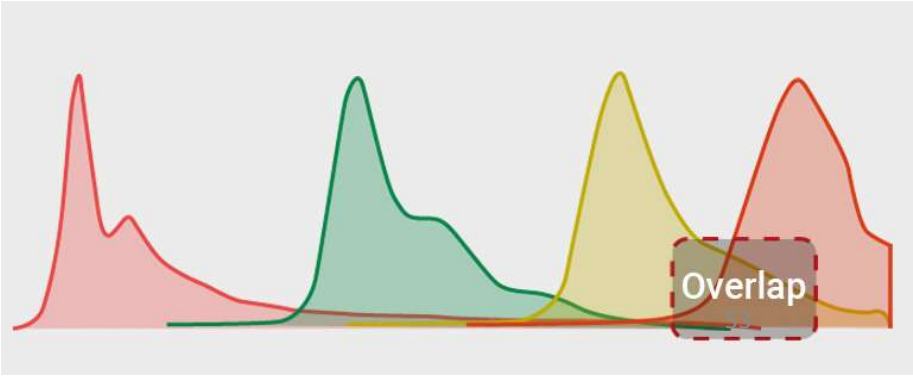
Example Channel Layout

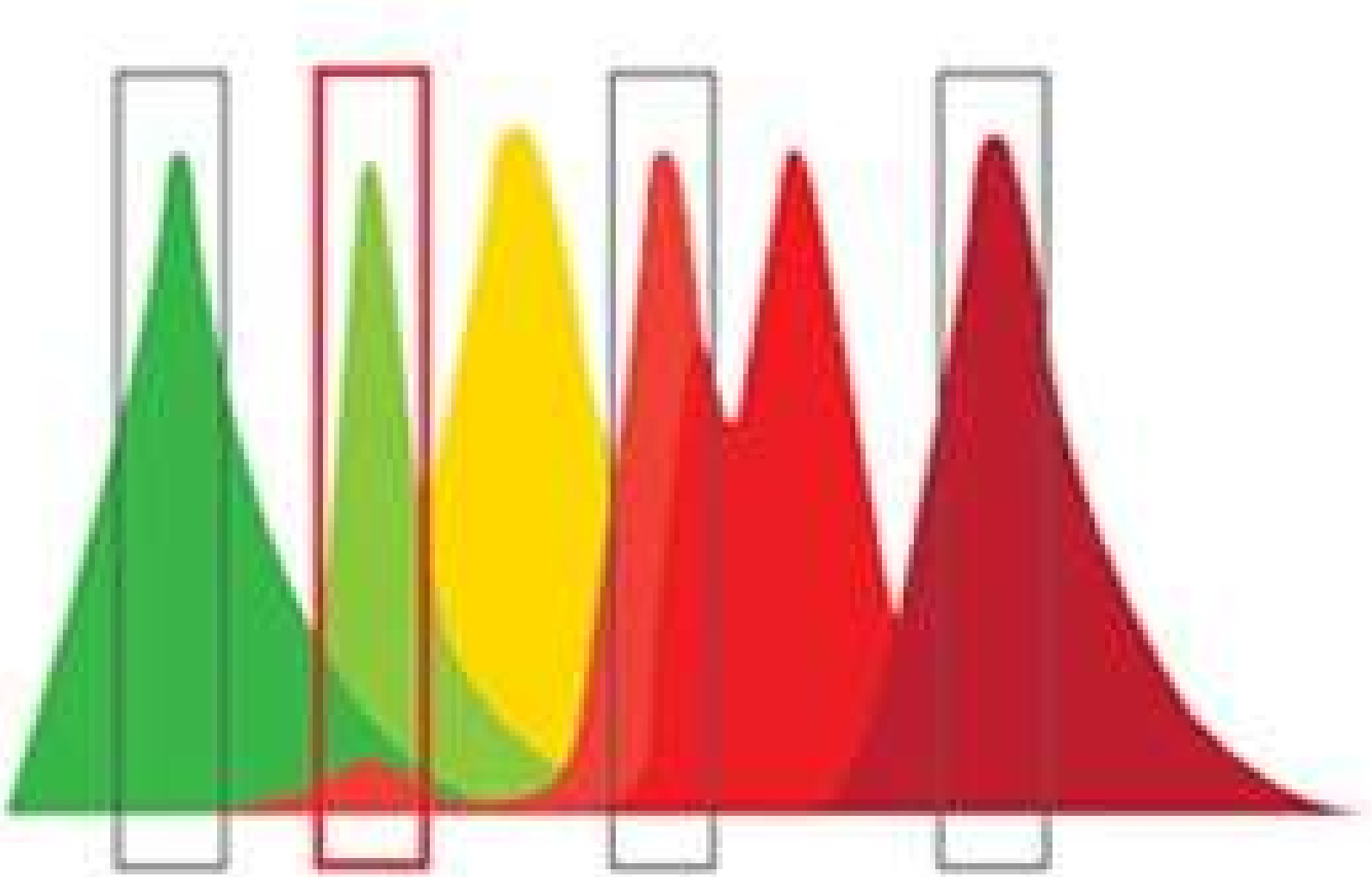


Original from Purdue University Cytometry Laboratories

Compensation

- Fluorochromes typically fluoresce over a large part of the spectrum (100nm or more).
- Depending on filter arrangement, a detector may see some fluorescence from more than 1 fluorochrome. (referred to as bleed over).
- You need to “compensate” for this bleed over so that 1 detector reports signal from only 1 fluorochrome.





Detectors

- There are two main types of photo detectors used in flow cytometry
  - Photodiodes
    - Used for strong signals, when saturation is a potential problem (eg. FSC detector)
  - Photomultiplier tubes (PMT)
    - More sensitive than a Photodiode, a PMT is used for detecting small amounts of fluorescence emitted from fluorochromes.

Photodiodes and PMTs

- Photo Detectors usually have a band pass filter in front of them to only allow a specific band width of light to reach it

- Therefore, each detector has a range of light it can detect, once a filter has been placed in front of it.

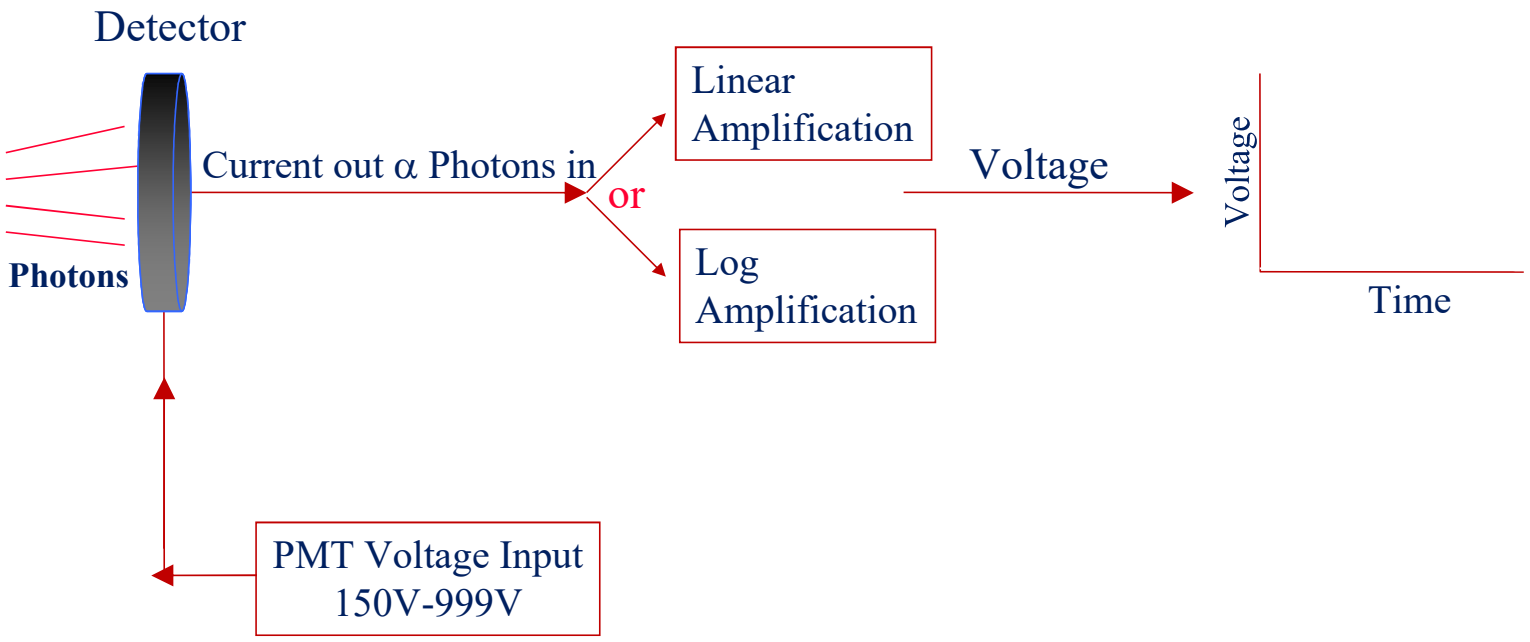
What Happens in a Flow Cytometer?

- Cells in suspension flow single file past — Fluidics
- a focused laser where they scatter light and emit fluorescence that is collected, filtered — Interrogation
- and converted to digitized values that are stored in a file — Electronics
- Which can then be read by specialized software. — Interpretation

Electronics

- Detectors basically collect photons of light and convert them to current
- The electronics must process that light signal and convert the current to a digitized value/# that the computer can graph

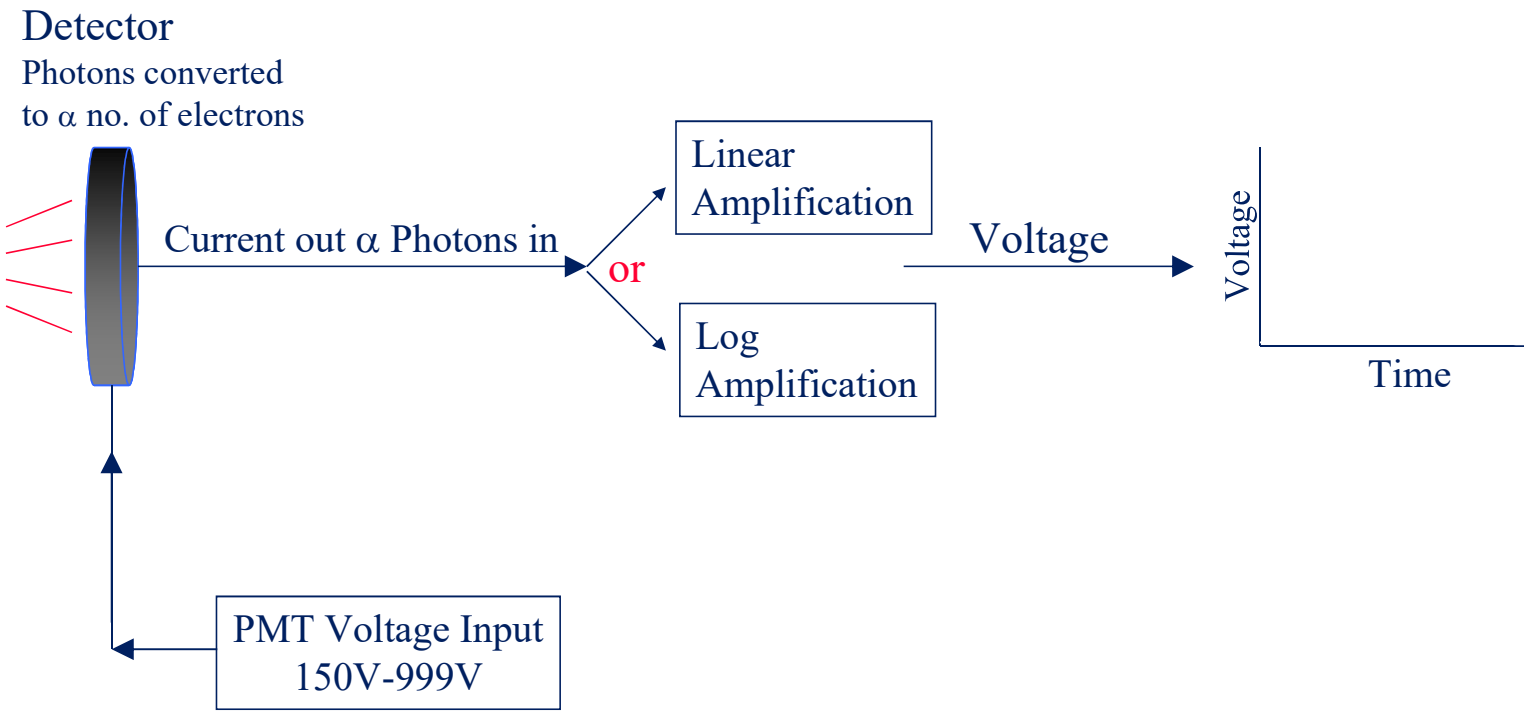
Electronics Schematic



Threshold

- When the laser interrogates an object, light is scattered.
- If the amount of light scattered surpasses a threshold, then the electronics opens a set *window* of time for signal detection
- The threshold can be set on any parameter, but is usually set on FSC

Photons In ~ Voltage Out



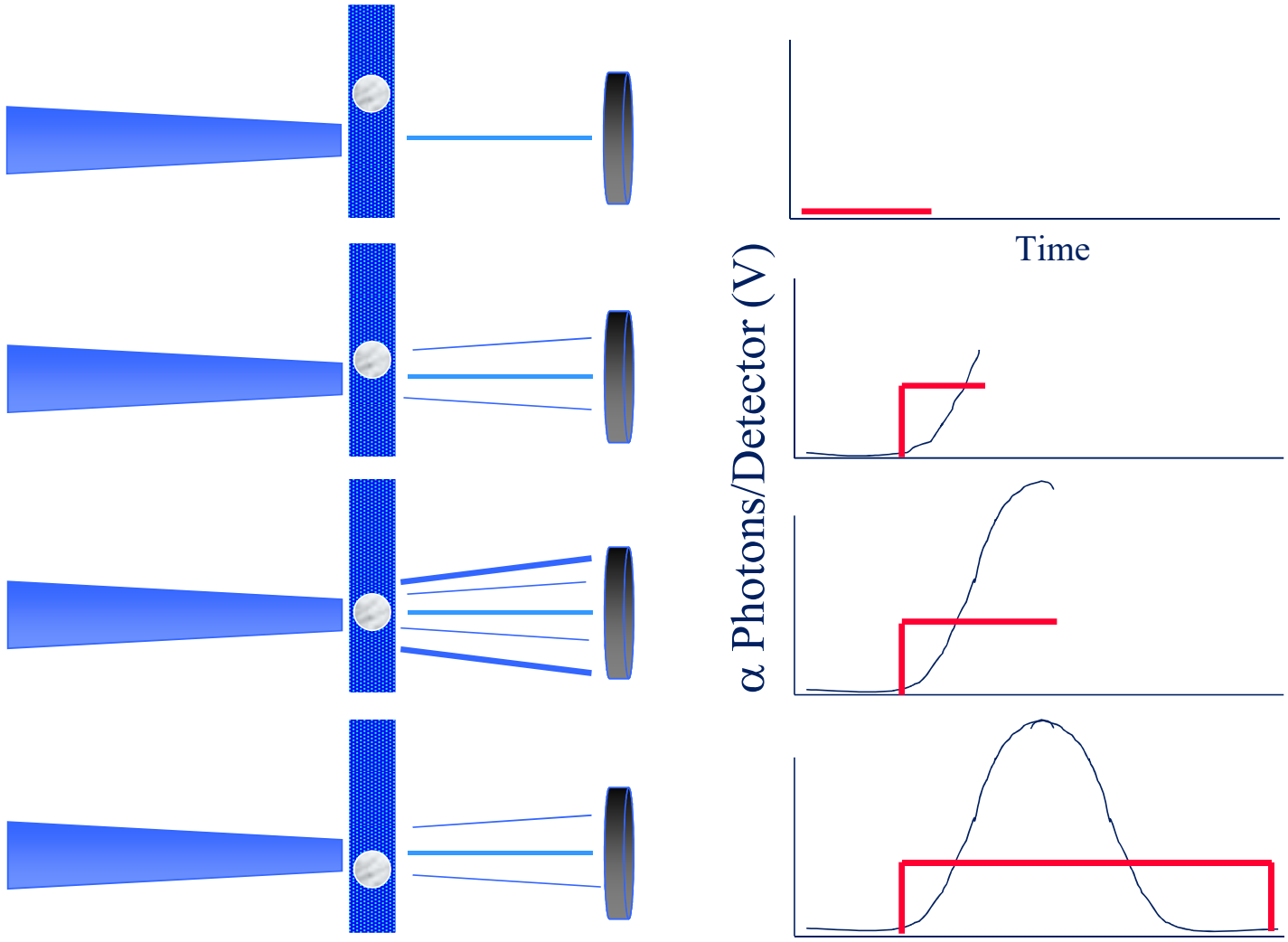
The Voltage Pulse

- As the cell passes through the laser, more and more light is scattered until the cell is in the center of the laser (maxima)

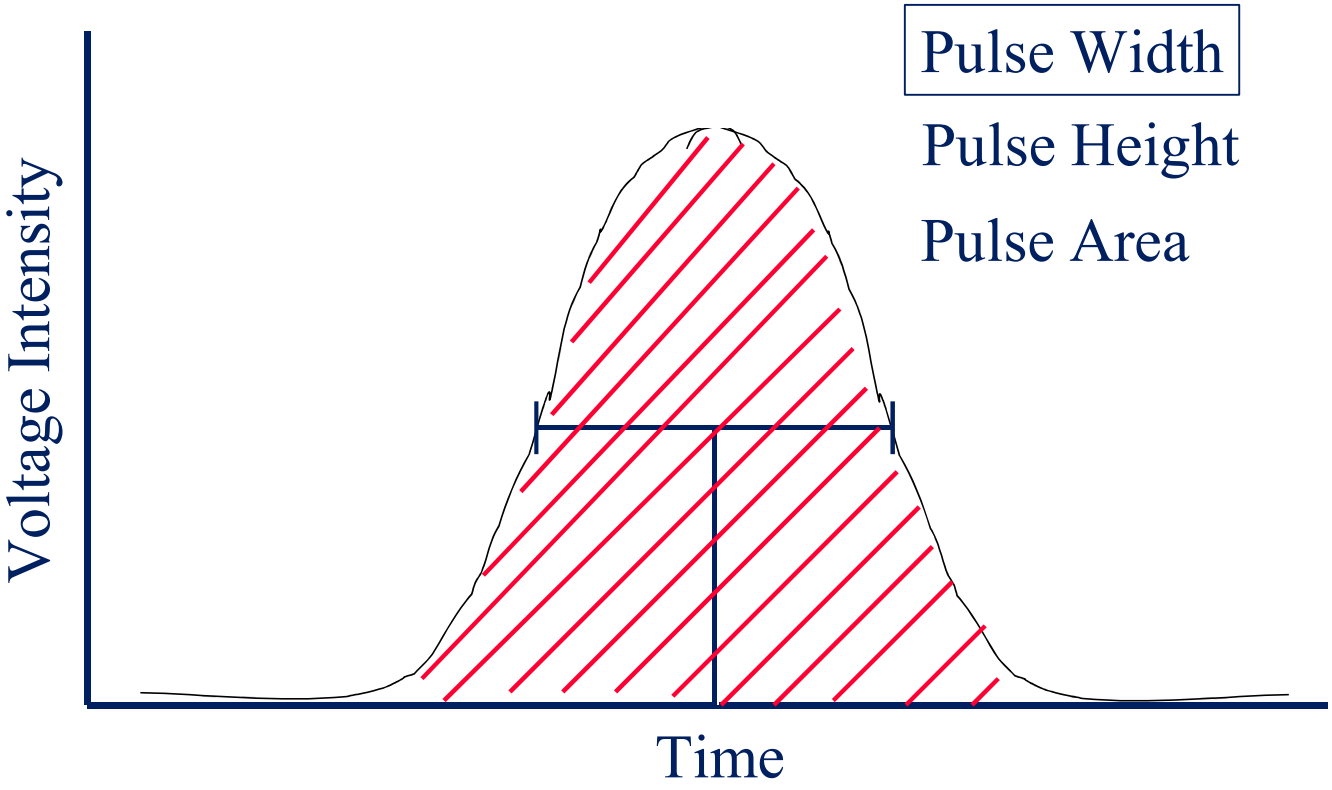
- As the cell leaves the laser, less and less light is scattered

- After a set amount of time, the window closes until another object scatters enough light to be triggered.

The Pulse



Measurements of the Pulse



Linear and Log Amplifiers

- The current exiting the detector passes through either a linear or log amplifier where it is converted into a voltage pulse.
- You can adjust the intensity of the voltage by amplifying it on a linear scale or converting it to a logarithmic scale
- The use of a log amp is beneficial when there is a broad range of fluorescence as this can then be compressed; this is generally true of most biological distributions.
- Linear amplification is used when there is not such a broad range of signals e.g. in DNA analysis and calcium flux measurement.

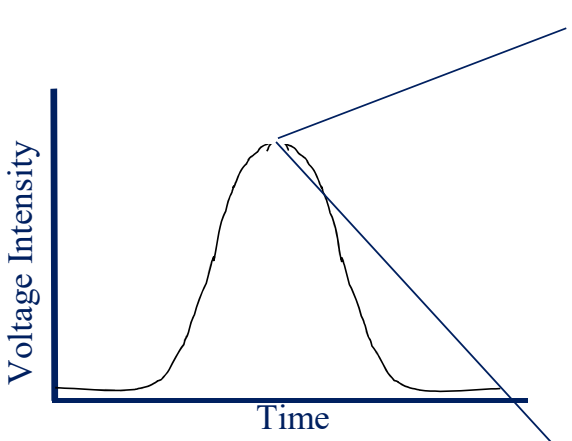
Analog to Digital Converters

- An ADCs takes the voltage pulse and converts it to discrete binary numbers depending on total resolution

- The binary signal generated is converted to a relative bin number

- Those relative bin numbers are acquired as a list of values from each detector for each event (cell) and are eventually plotted on a graph.

Analog to Digital Conversion



ADC — 11001100

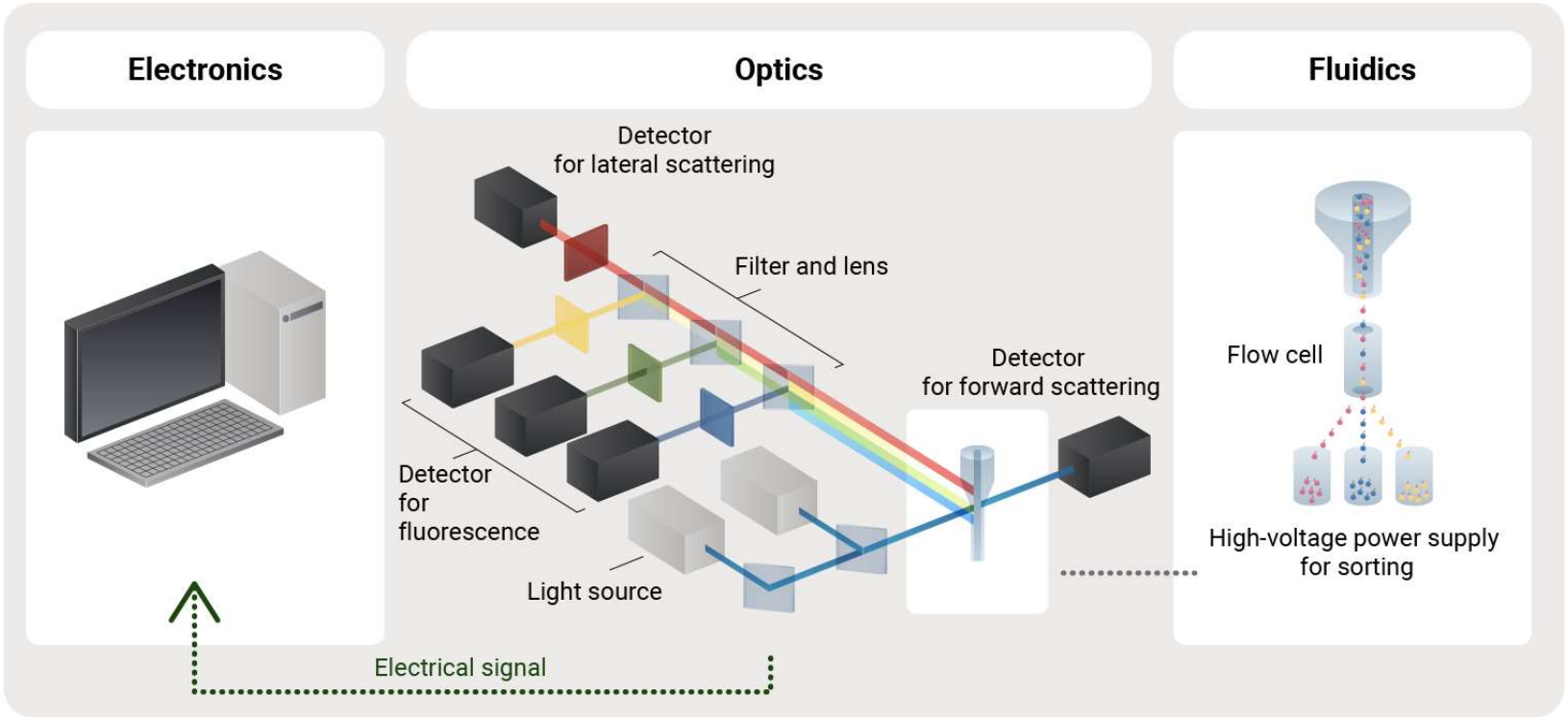
2^7 2^6 2^3 2^2

204 Relative bin number

ADC — 0011100100

2^7 2^6 2^5 2^2

228 Relative bin number


Electronics
Optics
Fluidics
Detector
for lateral scattering
Filter and lens
Detector
for forward scattering
Flow cell
Detector
for
fluorescence
Light source
High-voltage power supply
for sorting
Electrical signal

What Happens in a Flow Cytometer?

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Interpretation

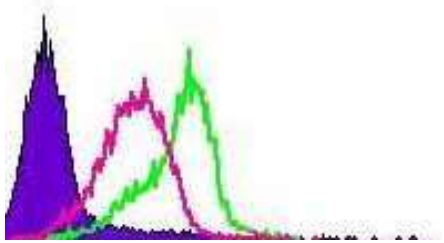
- Once the values for each parameter are in a list mode file, specialized software can graphically represent it.
- The data can be displayed in 1, 2, or 3 dimensional format
- Common programs include…
  - CellQuest
  - Flowjo
  - WinMDI
  - FCS Express

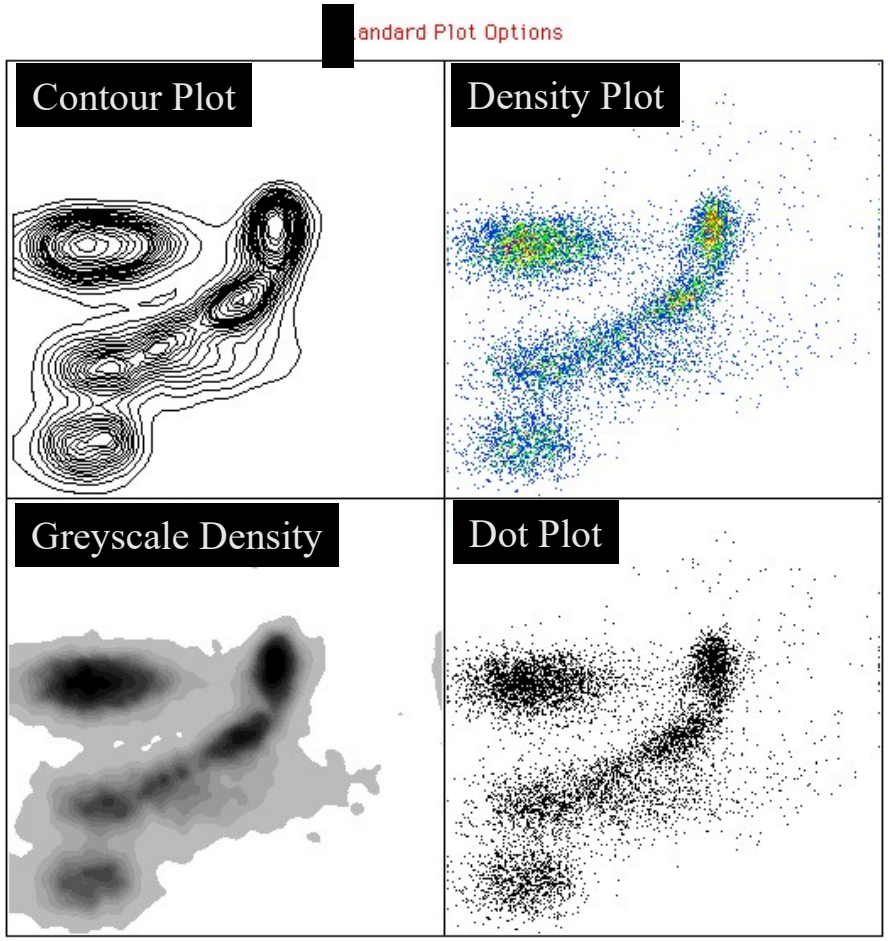
Types of Plots

- Single Color Histogram
  - Fluorescence intensity (FI) versus count
- Two Color Dot Plot
  - FI of parameter 1 versus FI of Parameter 2
- Two Color Contour Plot
  - FI of P1 versus FI of P2. Concentric rings form around populations. The more dense the population, the closer the rings are to each other
- Two Color Density Plot
  - FI of P1 versus FI of P2. Areas of higher density will have a different color than other areas

Plots

Histogram

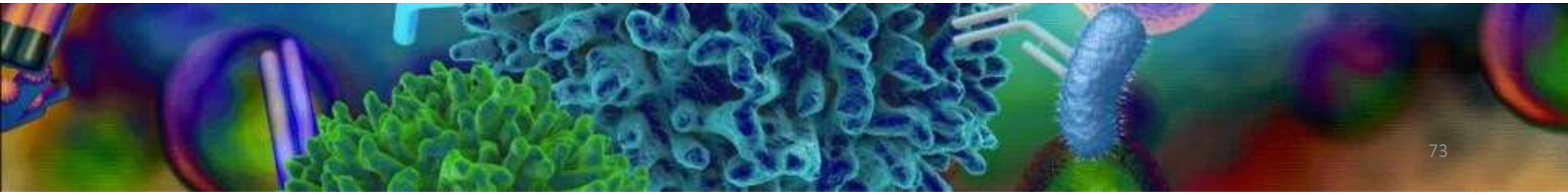


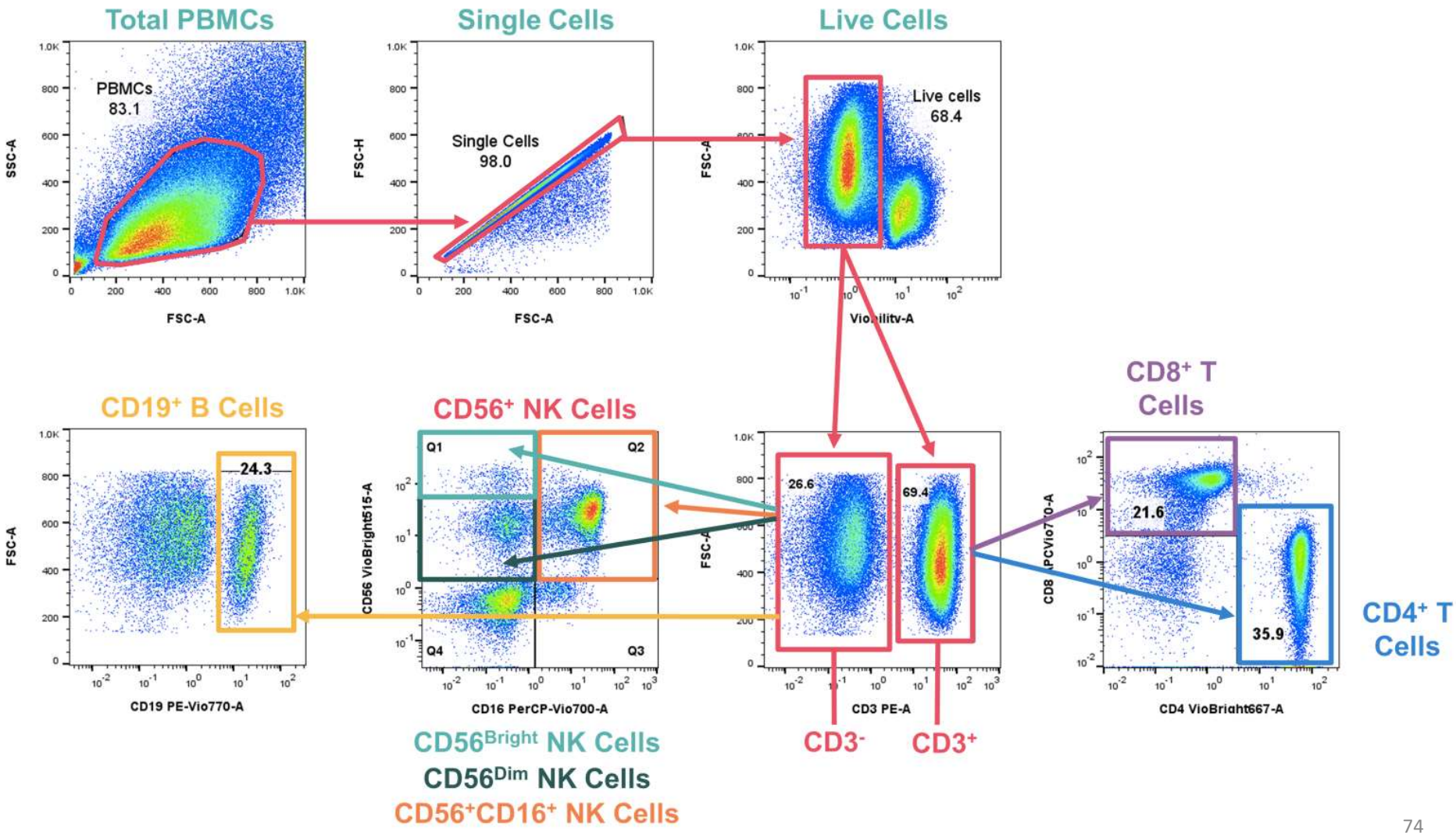


www.treestar.com

Gating

- Is used to isolate a subset of cells on a plot
- Allows the ability to look at parameters specific to only that subset



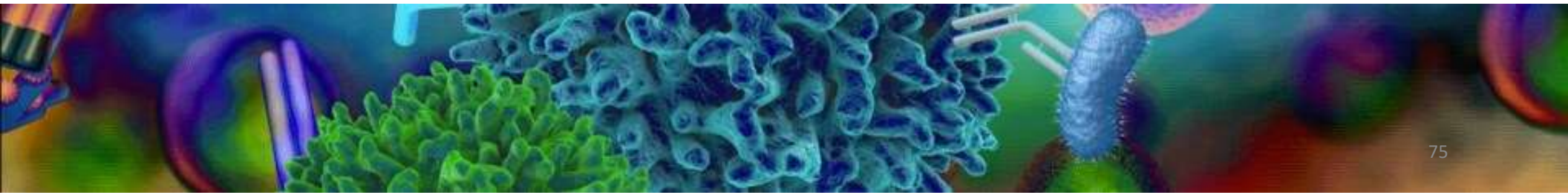

Total PBMCs
PBMCs
83.1
SSC-A
FSC-A
Single Cells
Single Cells
98.0
FSC-H
FSC-A
Live Cells
Live cells
68.4
FSC-A
Viability-A
CD19+ B Cells
24.3
FSC-A
CD19 PE-Vio770-A
CD56+ NK Cells
Q1
Q2
Q4
Q3
CD56 VioBright515-A
CD16 PerCP-Vio700-A
CD3 PE-A
26.6
69.4
CD8+ T Cells
21.6
35.9
CD8 PCVio770-A
CD4 VioBright667-A
CD4+ T Cells
CD56Bright NK Cells
CD56Dim NK Cells
CD56+CD16+ NK Cells
CD3-
CD3+

Three common modes for dot plots are:

- Forward scatter (FSC) vs. side scatter (SSC):
- To look at the distribution of cells based upon size & granularity
- Single color vs. side scatter
  To visualize the expression of the fluorescence of the cells
- Two-color fluorescence plot.

To differentiate between those cells that express only one of the particular fluorescent markers, those that express neither, and those that express both.

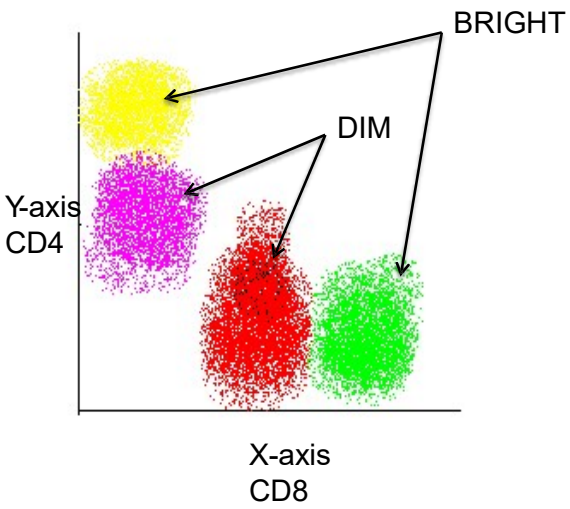
used to discriminate dead cells from the live ones that are expressing the desired fluorescence.

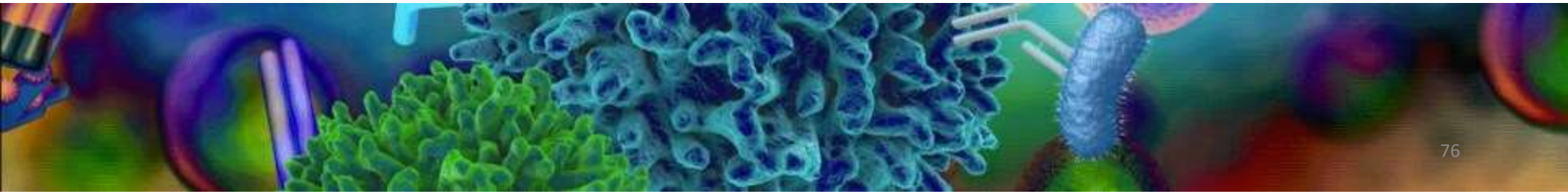


How to differentiate dim & bright expression of an antigen?

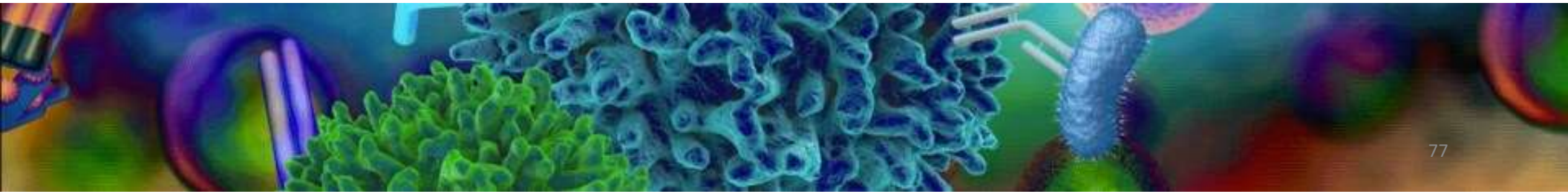
- Dim : cells are present more towards the origin(0) on x(red) - y axis (pink)

- Bright : cells are present away from the origin(0) on x(green) & y(yellow) axis.



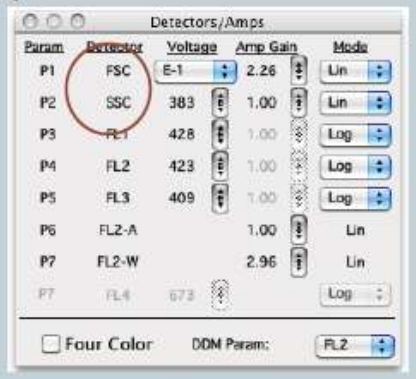


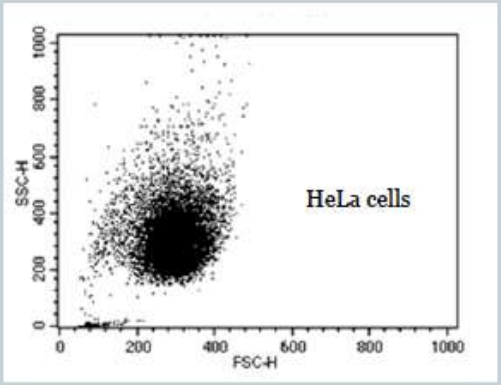
Flow Cytometer Set tup

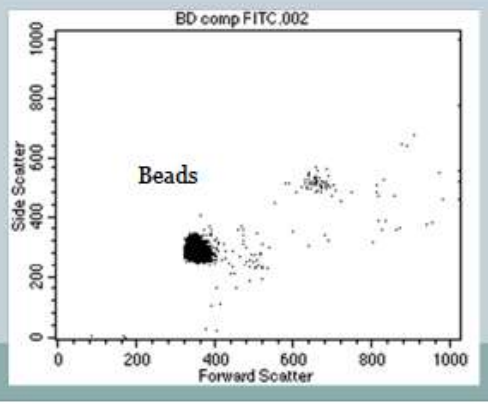


Setting FSC and SSC

- Set forward and side scatter detectors to untreated cells.

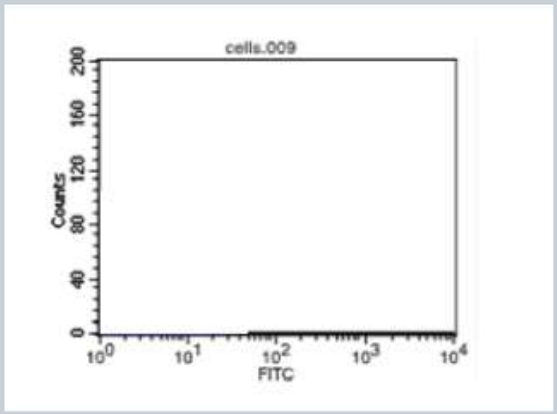


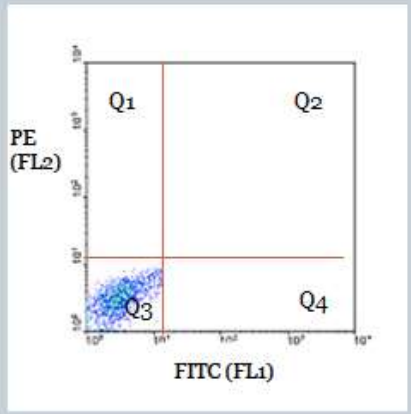




Setting Fluorescence Detectors

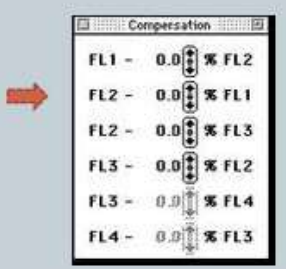
- Set FL1 and FL2 detectors so that the auto fluorescence from unstained cells are set within the first log decade (10^0 to 10^1)

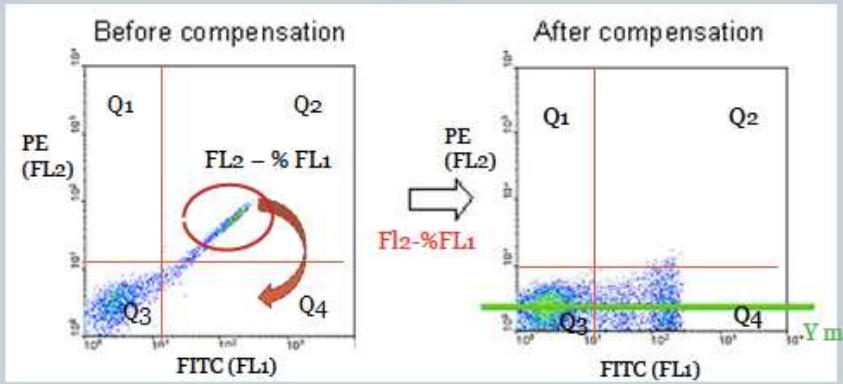




Compensation

Cells + FITC



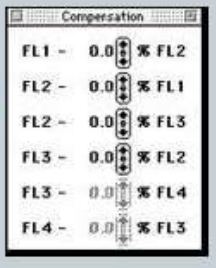


Quadrant Statistics

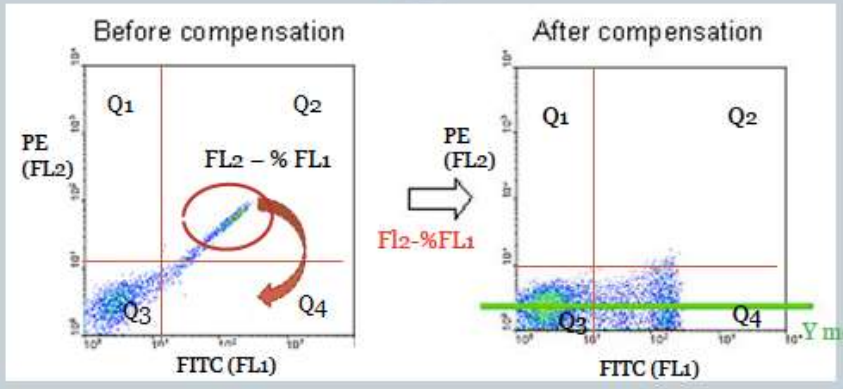
Quad	Events	X Mean	Y mean
Q1	0	***	***
Q2	0	***	***
Q3	7198	3.87	3.27
Q4	2786	336.3	3.32

Compensation

Cells + FITC



Cells + PE



Quadrant Statistics

Quad	Events	X Mean	Y mean
Q1	0	***	***
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Q3	7198	3.87	3.27
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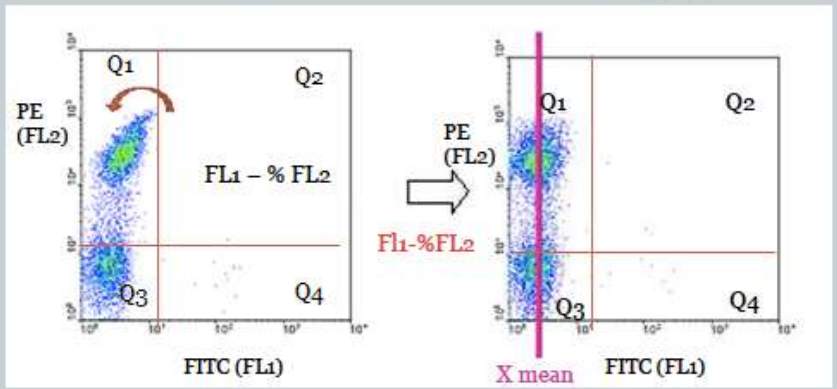
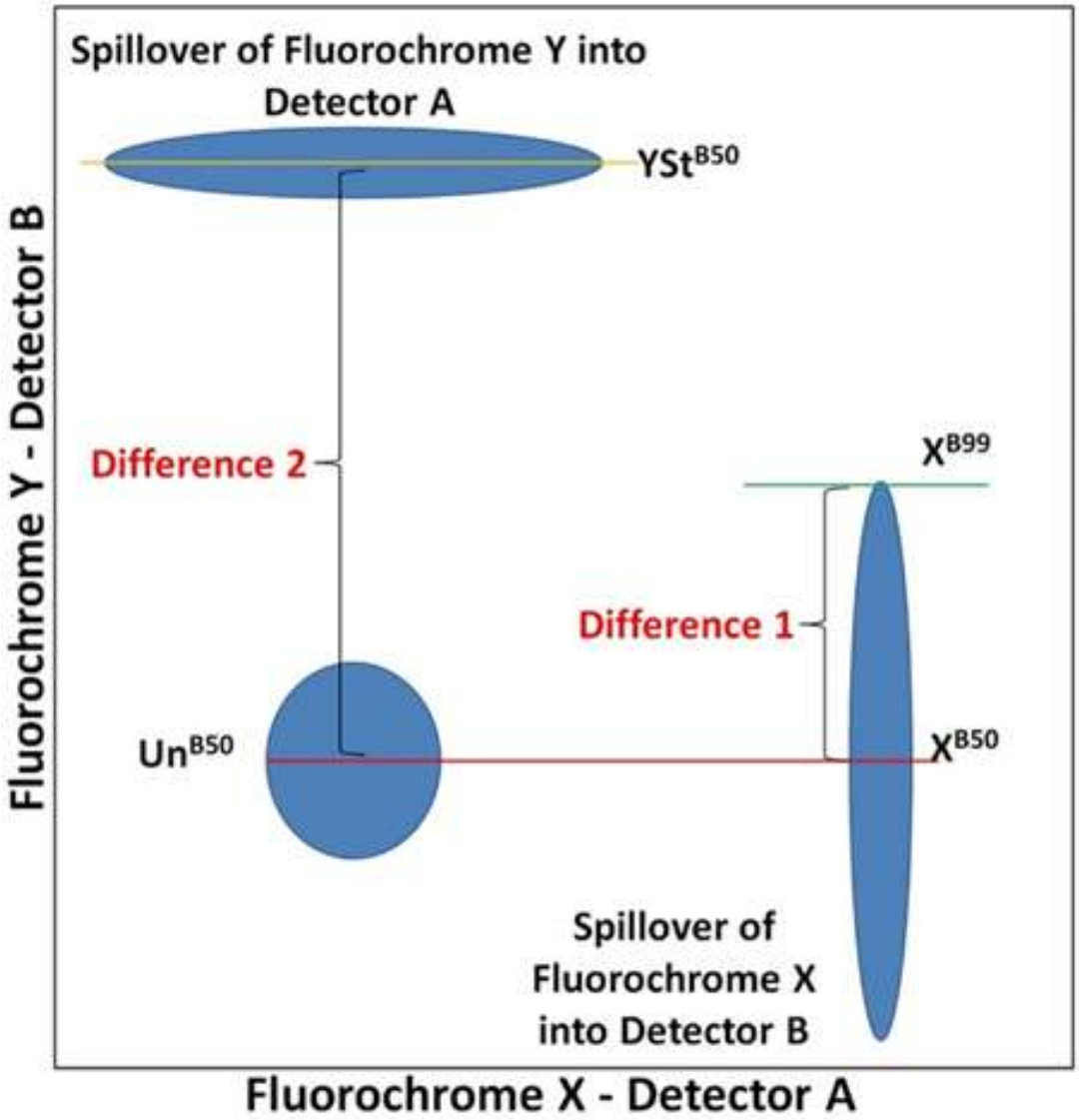


Quadrant Statistics

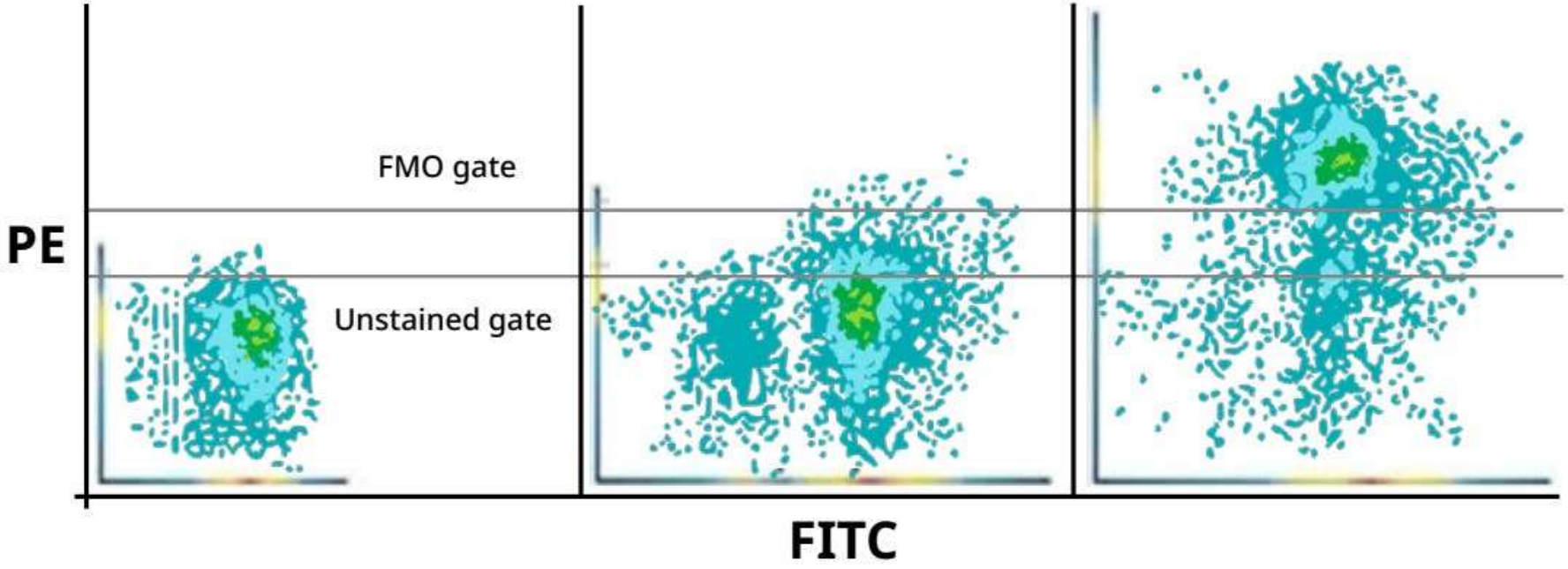
Quad	Events	X Mean	Y mean
Q1	318	3.51	600.71
Q2	0	***	***
Q3	1035	3.86	4.03
Q4	0	***	***


Spillover of Fluorochrome Y into Detector A
YSt^B50
Fluorochrome Y - Detector B
Difference 2
X^B99
Difference 1
Un^B50
X^B50
Spillover of Fluorochrome X into Detector B
Fluorochrome X - Detector A

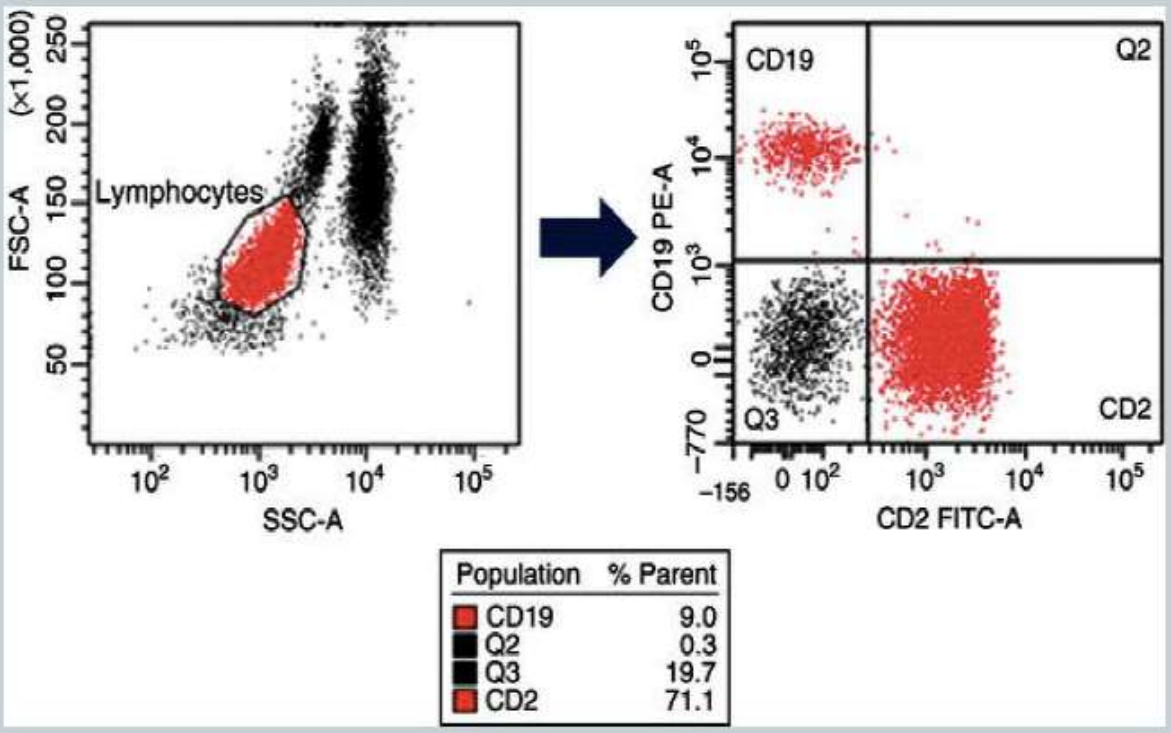
Fluorescence Minus One (FMO)

- Fluorescence Minus One (FMO) controls are samples stained with all the fluorophores in your panel, minus one of them.
- They are used to set the upper boundary for background signal on the omitted label, and thus to identify and gate positive populations in multicolor experiments.

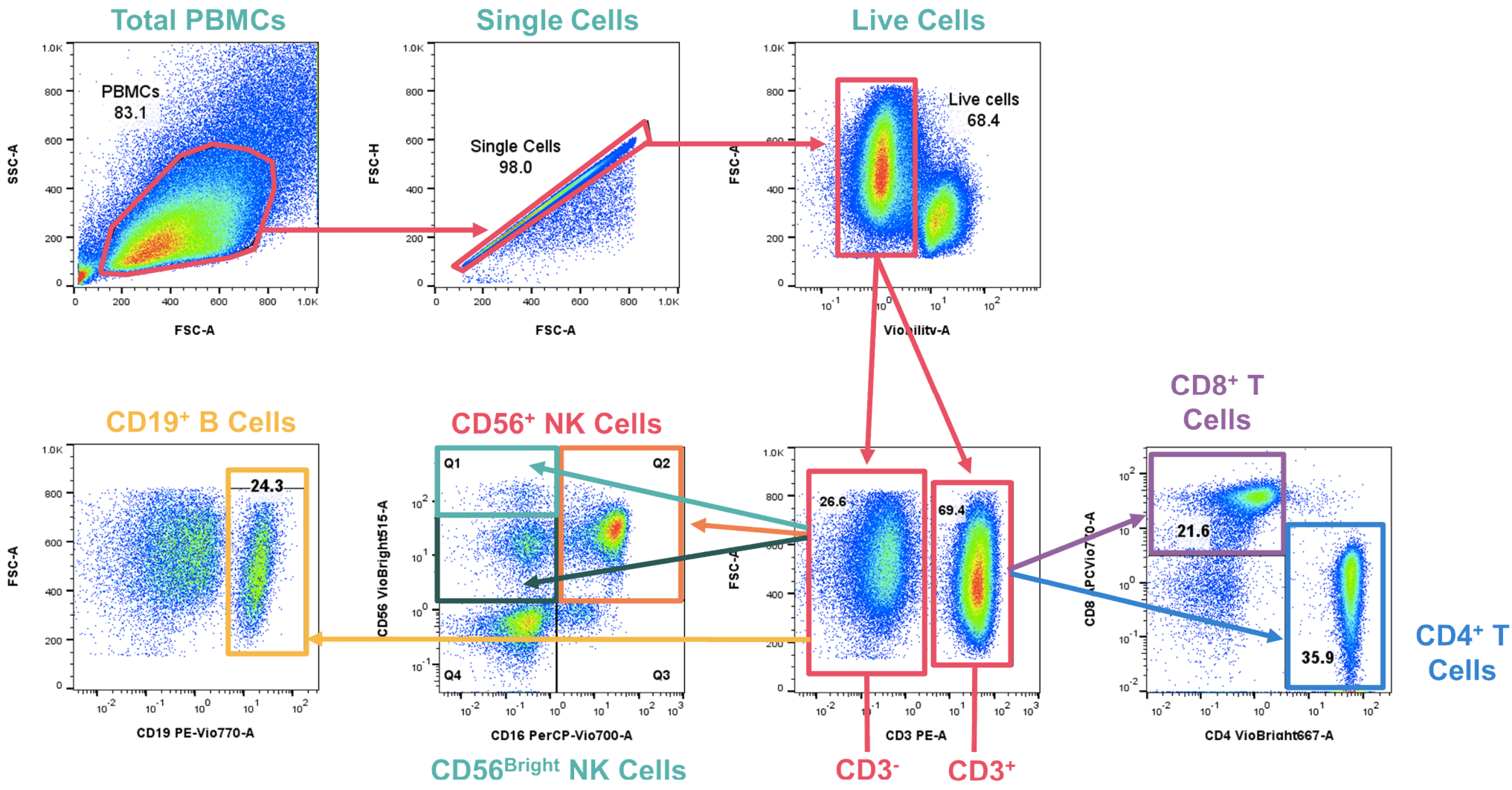
Dye	Unstained control	FMO control	Sample
FITC	-	CD3	CD3
CyPE	-	CD8	CD8
Cy7PE	-	CD45RO	CD45RO
PE	-	-	CD4



Gating Example

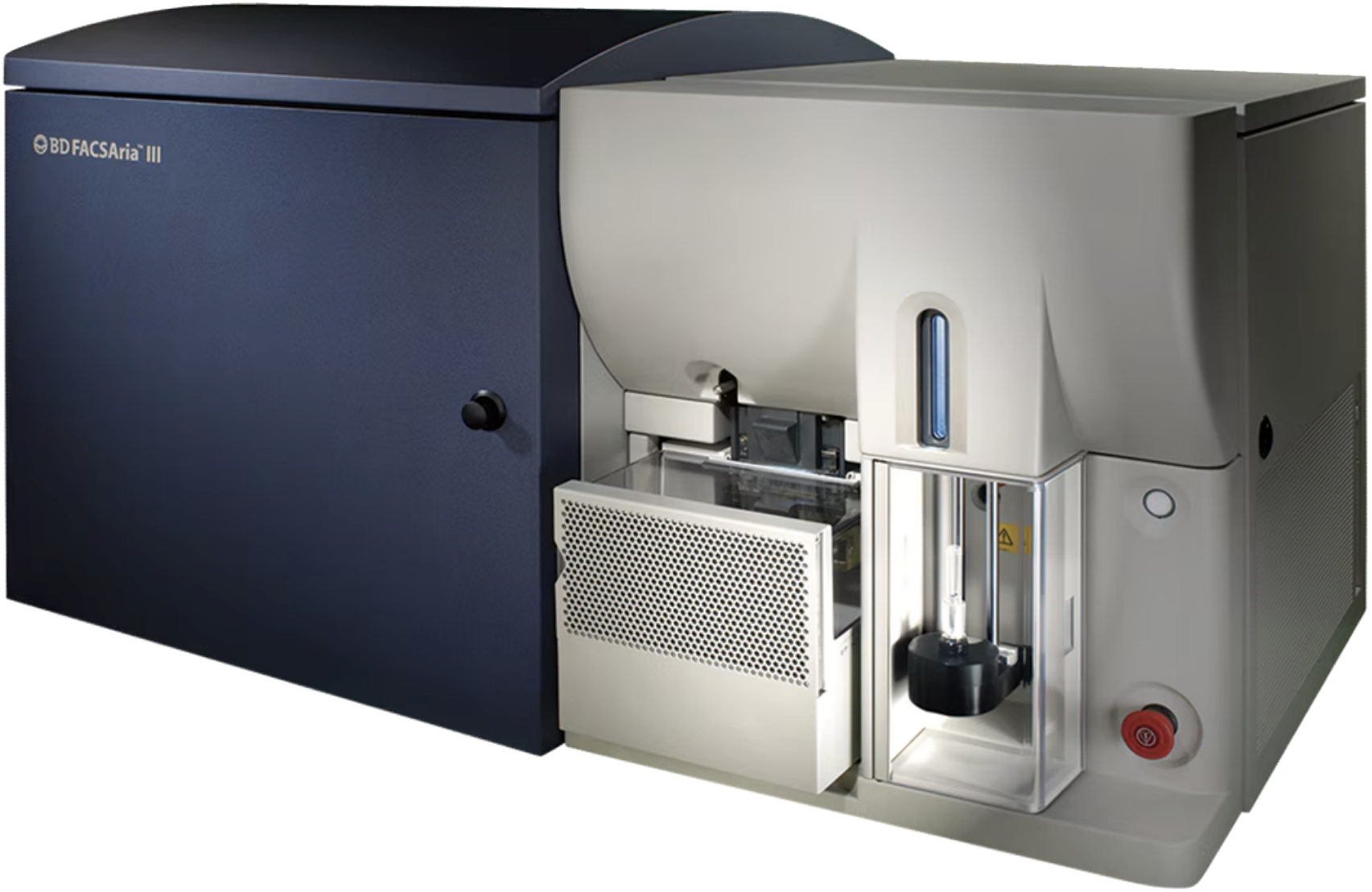


Cannot discriminate between cells with the same scattering properties


Total PBMCs
PBMCs
83.1
SSC-A
FSC-A
Single Cells
Single Cells
98.0
FSC-H
FSC-A
Live Cells
Live cells
68.4
FSC-A
Viability-A
CD19+ B Cells
24.3
FSC-A
CD19 PE-Vio770-A
CD56+ NK Cells
Q1
Q2
Q4
Q3
CD56 VioBright515-A
CD16 PerCP-Vio700-A
26.6
69.4
FSC-A
CD3 PE-A
CD8+ T Cells
21.6
35.9
CD8 APCVio770-A
CD4 VioBright667-A
CD4+ T Cells
CD56Bright NK Cells
CD56Dim NK Cells
CD56+CD16+ NK Cells
CD3-
CD3+

Clinical Flow Cytometry


BD FACSCalibur
BD


BD FACSAria™ III

BD Launches New Robotic System to Automate Clinical Flow Cytometry


SONY


mindray

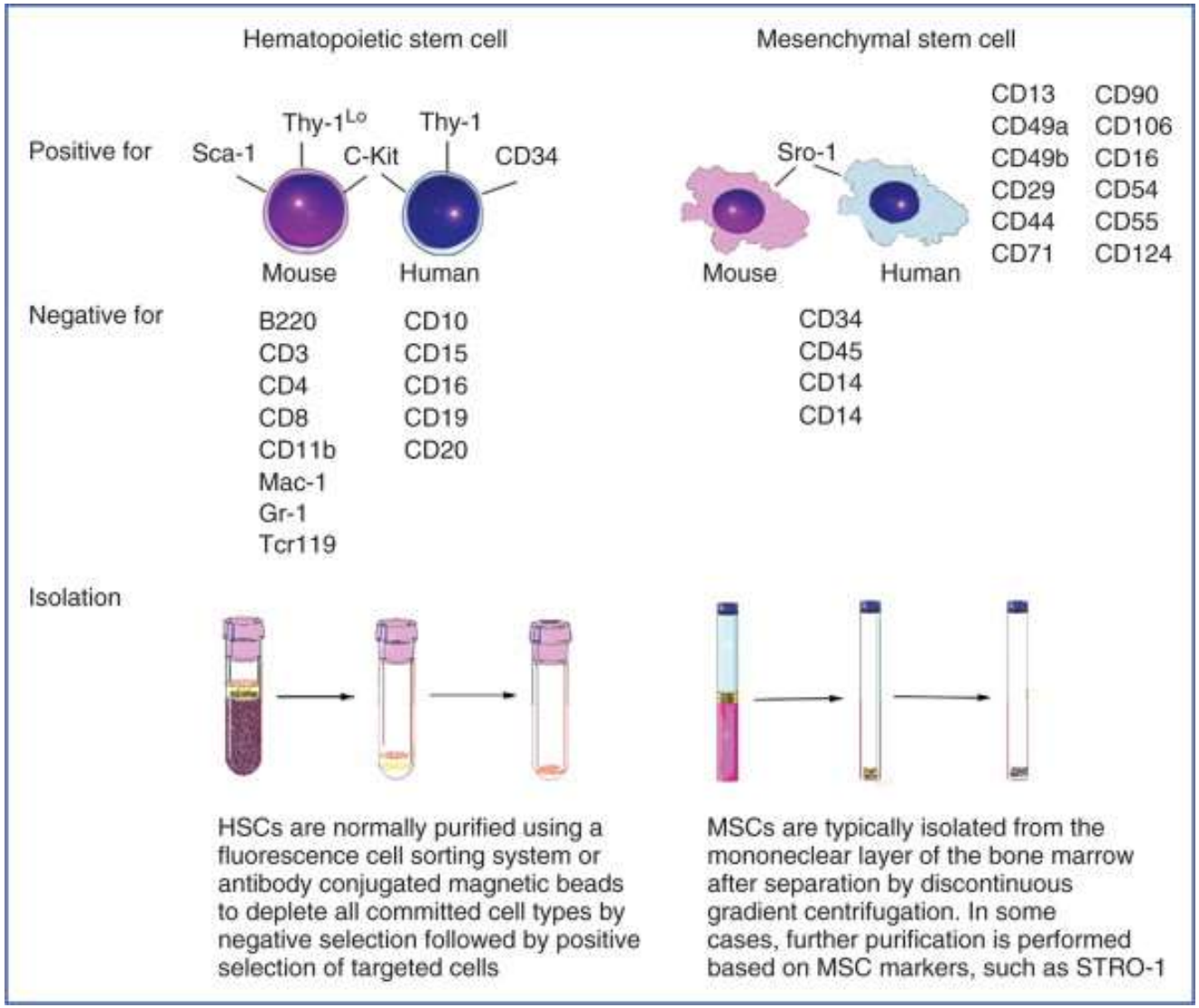
Beckman Coulter's CytoFLEX Mosaic (launched 2025)

Core Clinical Applications

- **Leukemias and Lymphomas (Hematopoietic Neoplasms)**: Immunophenotyping for diagnosis, subclassification (e.g., B- vs. T- vs. myeloid), and MRD monitoring. Includes acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), other chronic lymphoproliferative disorders, multiple myeloma, and non-Hodgkin lymphomas (B- and T-cell). It distinguishes lineages, detects aberrant antigen expression, and assesses prognosis.
- **Myeloid Neoplasms and Myelodysplastic Syndromes (MDS)**: Evaluation of blasts, aberrant myeloid/monocytic cells, and monitoring.
- **Paroxysmal Nocturnal Hemoglobinuria (PNH)**: Gold-standard test detecting GPI-anchor protein deficiencies (e.g., CD55, CD59) on red cells, granulocytes, and monocytes. High-sensitivity assays identify small clones in bone marrow failure syndromes.
- **Immune Deficiencies and Function**: HIV/AIDS: CD4+ T-cell enumeration and monitoring. Primary immunodeficiencies (e.g., severe combined immunodeficiency/SCID, chronic granulomatous disease/CGD, X-linked agammaglobulinemia).
- Lymphocyte subset analysis (T, B, NK cells) for inherited or acquired defects.
- **Other Hematologic/Immune Conditions**: Mast cell neoplasms, platelet disorders, autoimmune or inflammatory conditions (e.g., some allergy testing via basophil activation), and post-transplant monitoring (e.g., chimerism, graft-vs-host).
- **Emerging/Additional Uses**: Immuno-oncology (tumor-infiltrating lymphocytes, CAR-T monitoring), infectious disease immune responses, and rare event analysis. It is less routine for solid tumors but useful in some contexts (e.g., effusions, fine-needle aspirates).
- **Minimal Residual Disease (MRD) Tracking:** Hunting for highly specific, lingering abnormal cell populations (often down to 1 in 10,000 or 100,000 cells) after chemotherapy to detect early relapse.

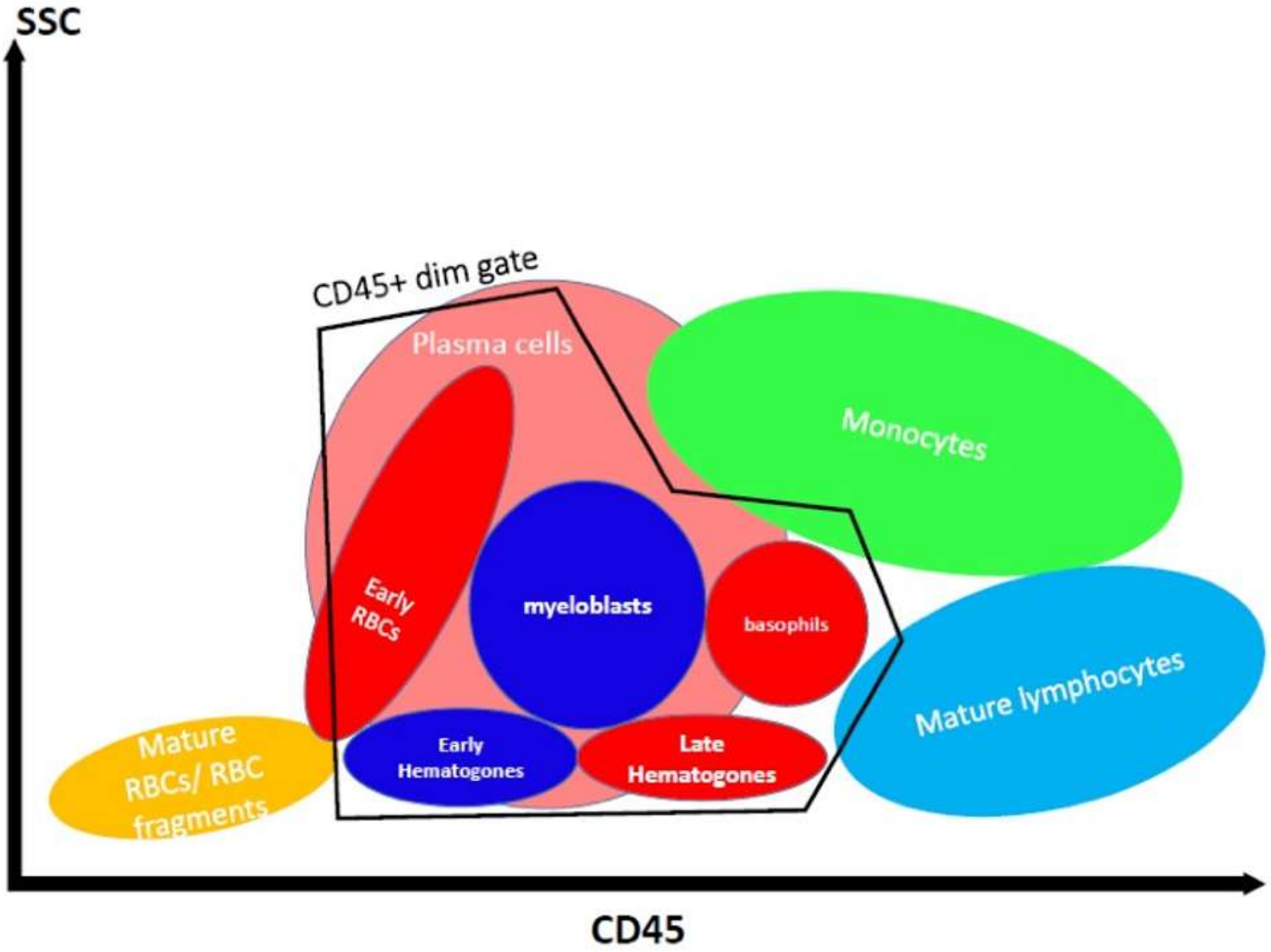
Leukemia Panels

- We look for **lineage assignment** (Is it myeloid, B-cell, or T-cell?) and **maturation abnormalities** (Are there blasts present?).

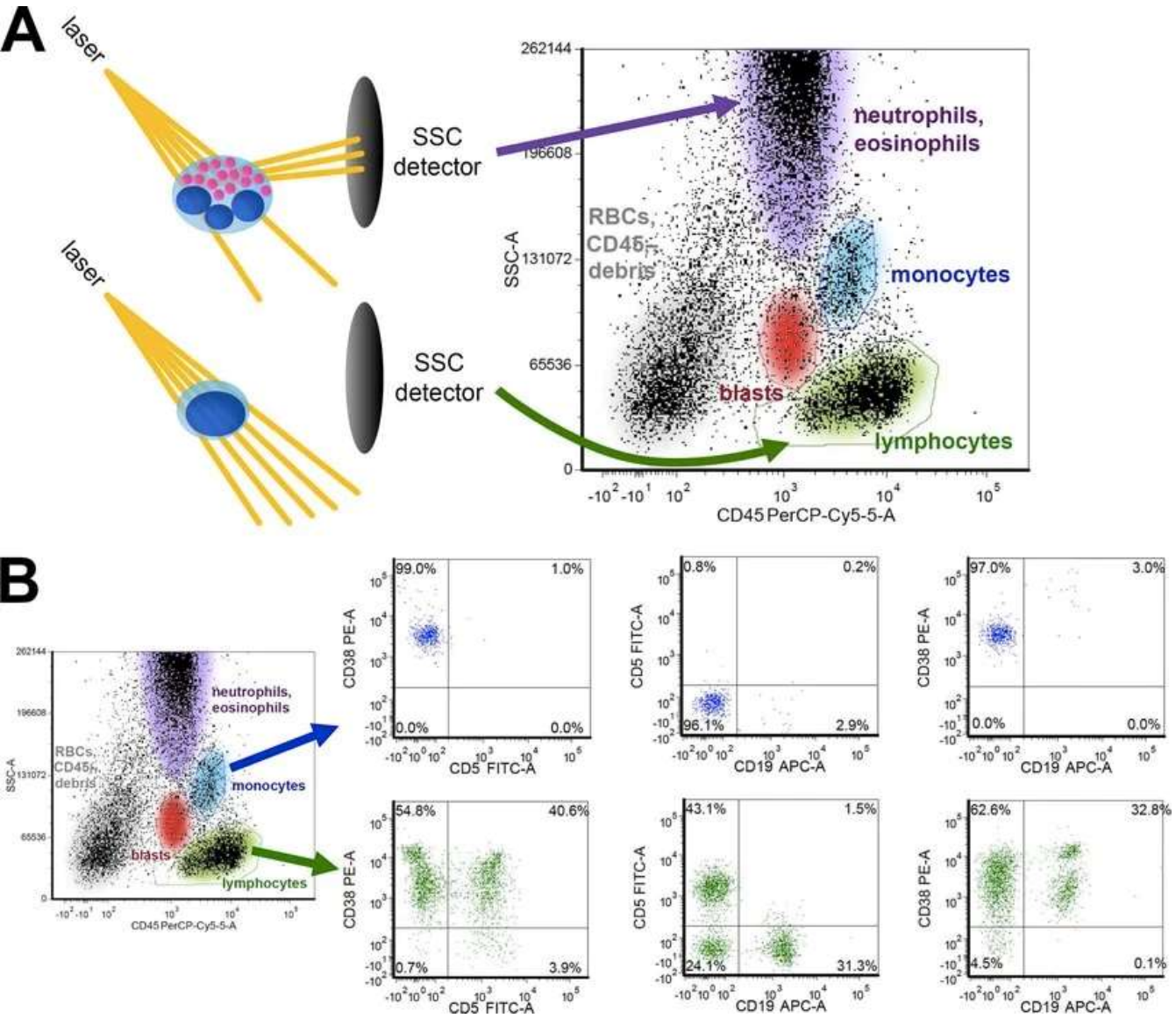

Hematopoietic stem cell
Mesenchymal stem cell
Positive for
Thy-1Lo
Thy-1
Sca-1
C-Kit
CD34
Mouse
Human
Sro-1
Mouse
Human
CD13
CD49a
CD49b
CD29
CD44
CD71
CD90
CD106
CD16
CD54
CD55
CD124
Negative for
B220
CD3
CD4
CD8
CD11b
Mac-1
Gr-1
Tcr119
CD10
CD15
CD16
CD19
CD20
CD34
CD45
CD14
CD14
Isolation
HSCs are normally purified using a fluorescence cell sorting system or antibody conjugated magnetic beads to deplete all committed cell types by negative selection followed by positive selection of targeted cells
MSCs are typically isolated from the mononeclear layer of the bone marrow after separation by discontinuous gradient centrifugation. In some cases, further purification is performed based on MSC markers, such as STRO-1

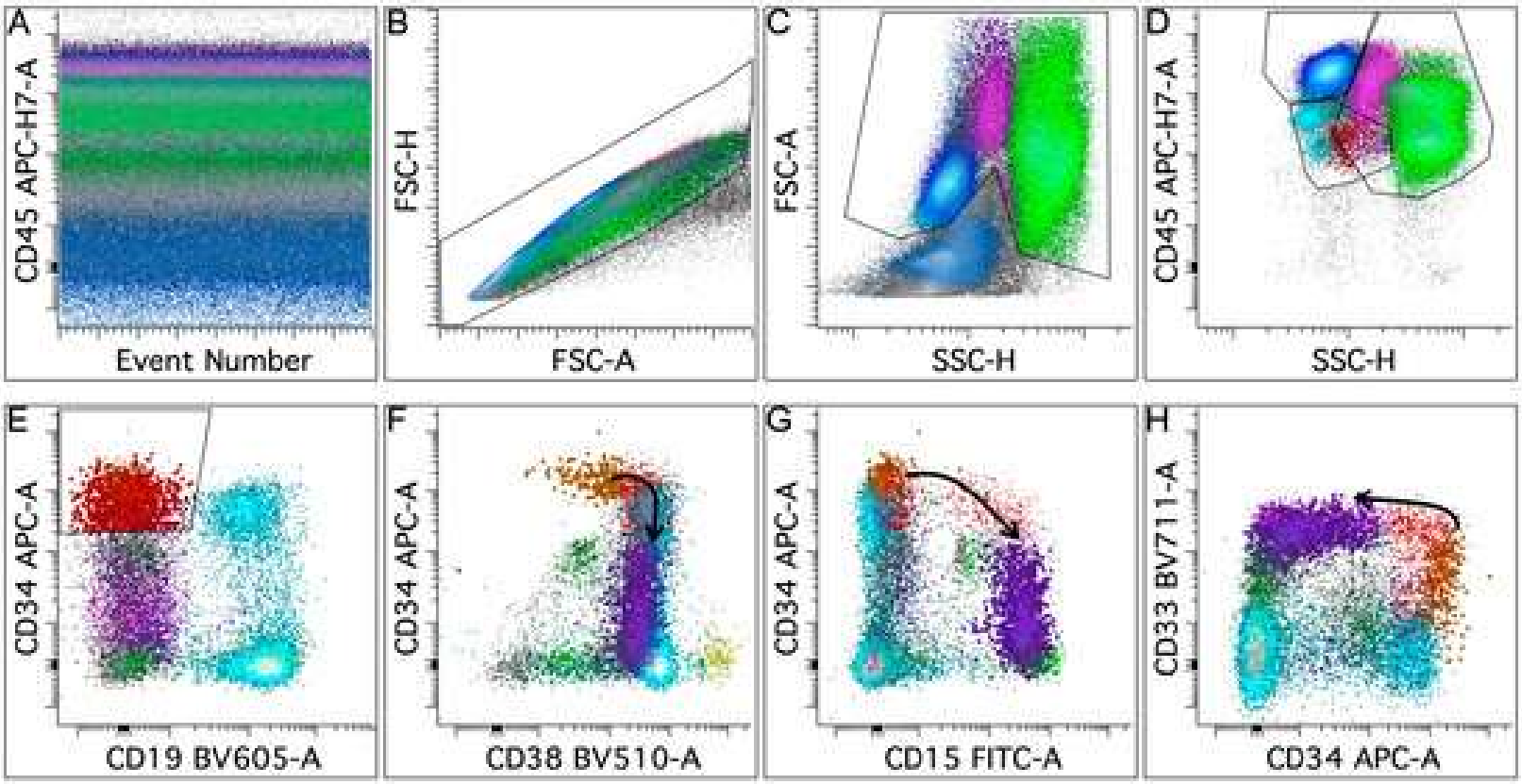
Leukemia Panels

- **Backbone Marker:** Always **CD45**.
- Paired with **Side Scatter (SSC)**, CD45 is the compass of flow cytometry, separating lymphocytes, monocytes, granulocytes, and debris.
- **Immature/Blast Markers: CD34**, **CD117**, and **HLA-DR**.
- **Lineage Specifics:**
- **CD19/CD20** (B-cell)
- **CD3/CD5/CD7** (T-cell)
- **CD13/CD33** (Myeloid).

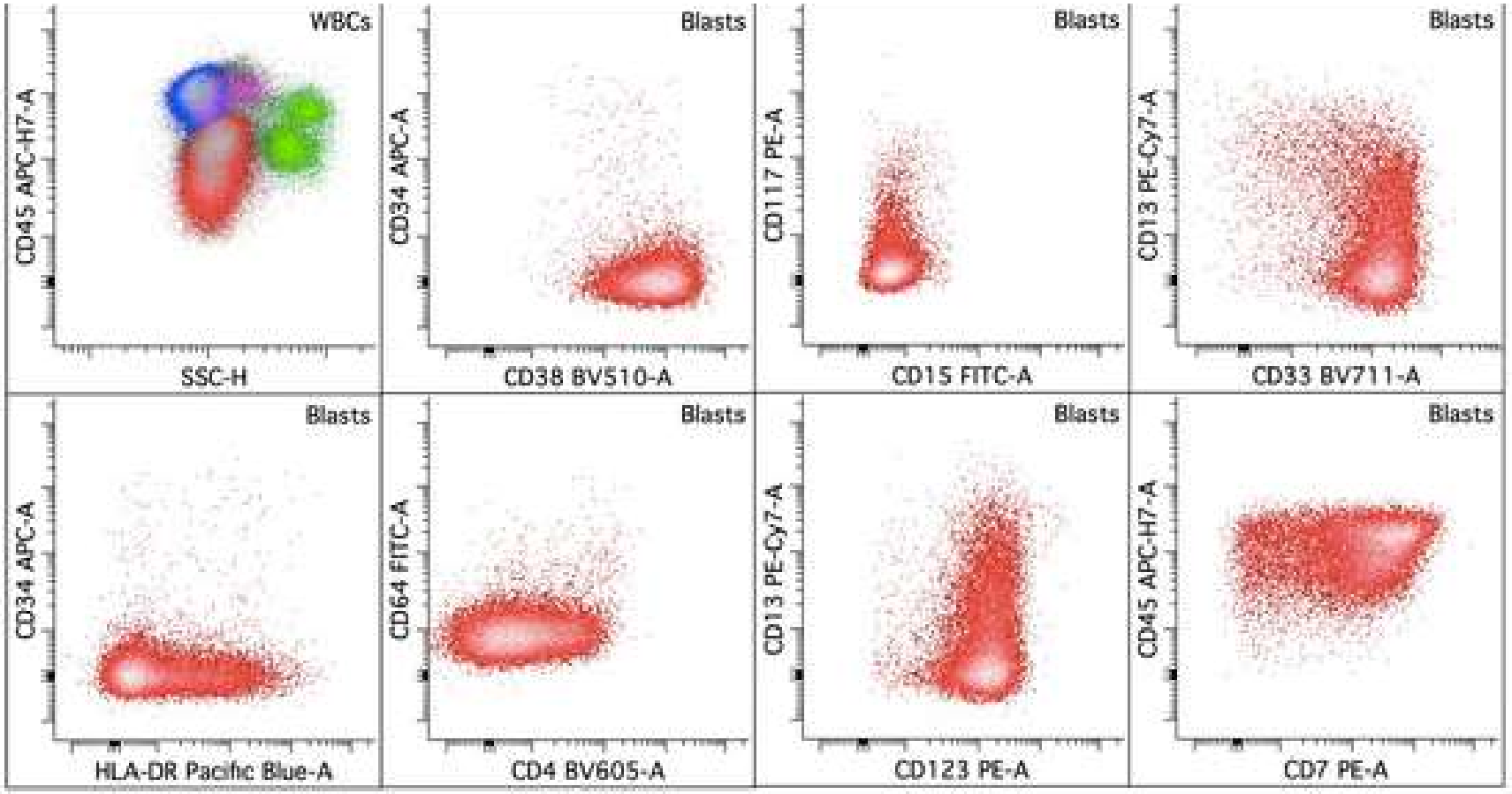

SSC
CD45+ dim gate
Plasma cells
Monocytes
Early RBCs
myeloblasts
basophils
Mature lymphocytes
Mature RBCs/ RBC fragments
Early Hematogones
Late Hematogones
CD45

Panel Type	Key Diagnostic Markers to Highlight	Clinical Significance
Acute Myeloid Leukemia (AML)	CD13, CD33, CD14, CD15, CD64, CD11b, MPO (intracellular)	Differentiates monocytic, granulocytic, or erythroid lineages; identifies blast percentages.
B-Cell Malignancies (ALL / CLL)	CD19, CD20, CD10, CD5, CD23, Kappa & Lambda Light Chains	Assesses clonality via light chain restriction (monoclonal vs. polyclonal) and identifies aberrant CD5 expression in CLL.
T-Cell Malignancies (ALL / T-NHL)	CD2, CD3 (surface & cytoplasmic), CD4, CD8, CD5, CD7	Look for the loss of normal pan-T cell markers (e.g., a CD3+ cell missing CD7 is a classic flag for T-cell lymphoma).

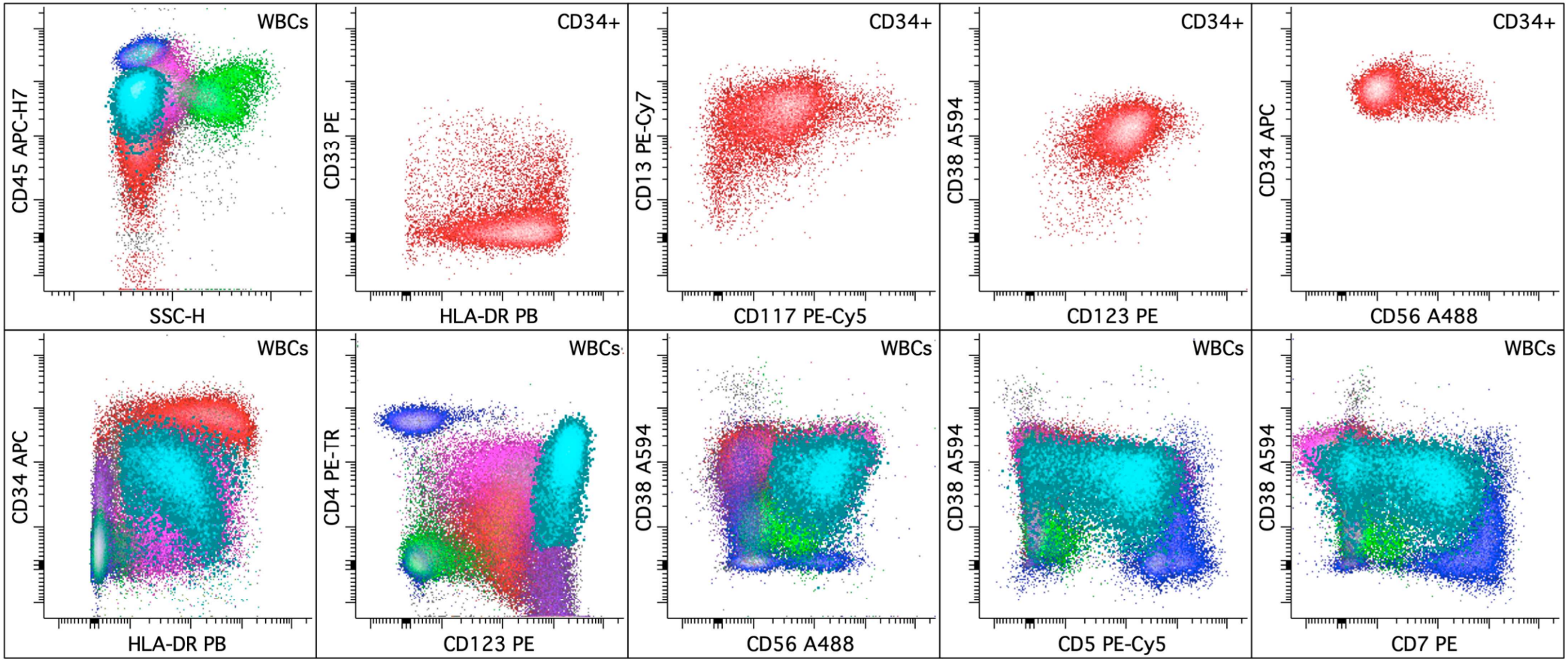

A
laser
SSC detector
laser
SSC detector
262144
196608
131072
65536
0
SSC-A
neutrophils, eosinophils
RBCs, CD45+, debris
monocytes
blasts
lymphocytes
CD45 PerCP-Cy5-5-A
B
neutrophils, eosinophils
RBCs, CD45+, debris
monocytes
blasts
lymphocytes
CD45 PerCP-Cy5-5-A
CD38 PE-A
CD5 FITC-A
99.0%
1.0%
0.0%
0.0%
0.8%
0.2%
96.1%
2.9%
CD19 APC-A
97.0%
3.0%
0.0%
0.0%
54.8%
40.6%
0.7%
3.9%
43.1%
1.5%
24.1%
31.3%
62.6%
32.8%
4.5%
0.1%



Gating strategy and normal myeloid maturation in normal bone marrow. (**A**) All the events are displayed on a CD45 versus event number plot to ensure stable data acquisition. (**B**) A singlet gate is applied to exclude doublets. (**C**) A viability gate is applied to exclude poorly viable cells and debris. (**D**) The CD45 versus side scatter (SSC) plot defines the blast gate (low CD45 and low SSC), lymphocyte gate (high CD45 and low SSC), and myelomonocytic gate (maturing myeloid cells colored green and monocytes in pink). (**E**) Events in the blast gate are displayed. A CD34-positive myeloid blast gate is generated to separate myeloid blasts from CD19-positive hematogones (colored aqua), as well as myeloid and monocytic cells. (**F**–**H**) Normal myeloid maturation from early progenitors to promyelocytes. The black line follows changes in the intensity of antigen expression. Early progenitors (highlighted in orange) express bright CD34, dim CD38, and dim CD33 without CD15. During differentiation, the progenitors gradually lose CD34 while acquiring CD15 and express higher levels of CD33 and CD38 as they reach the promyelocyte stage (highlighted in purple).

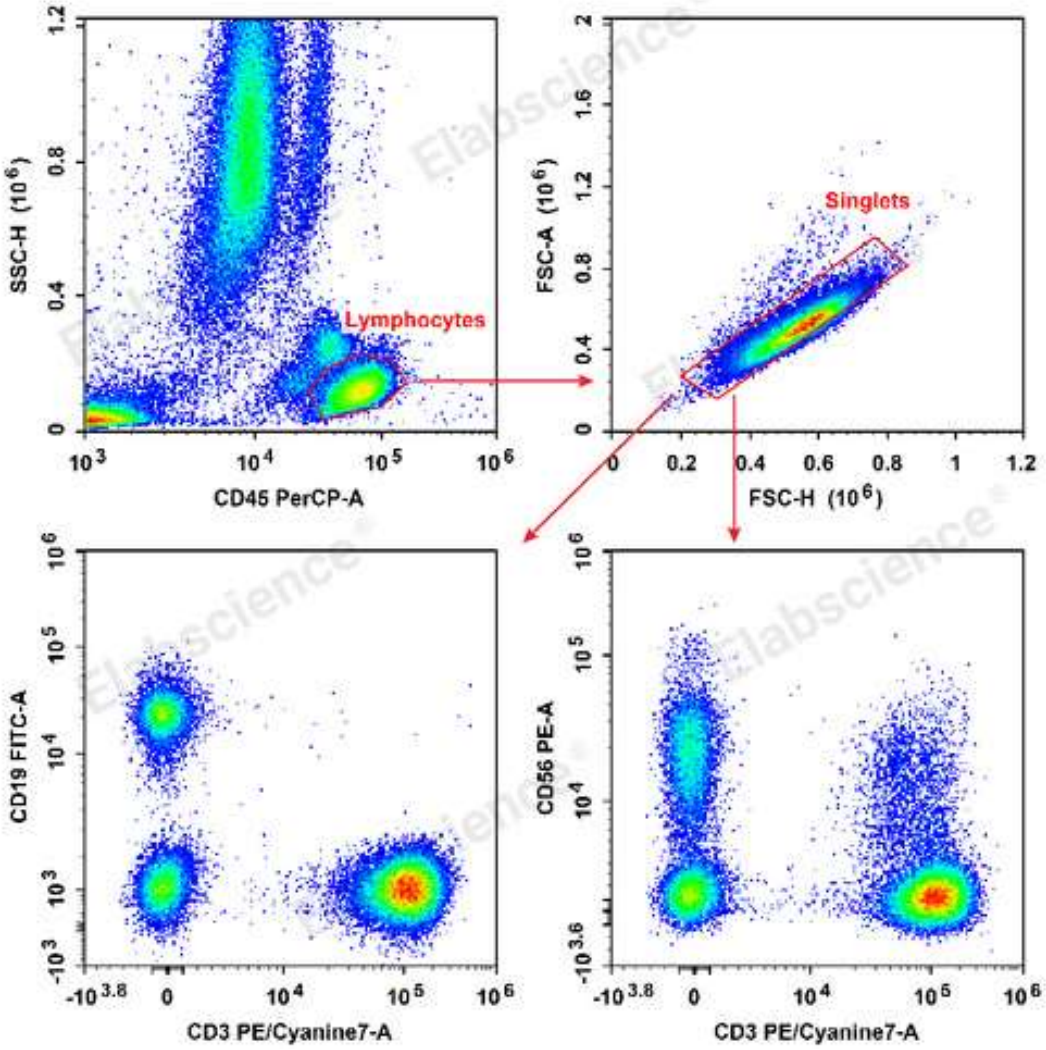


Acute myeloid leukemia with *FLT3 ITD* and *NPM1* mutations. The upper left dot plot displays the total viable white blood cells with blasts colored in red, lymphocytes in blue, monocytes in pink, and granulocytic cells in green. The rest of the dot plots selectively display leukemic blasts. The leukemic blasts (colored red; ~63% of the white blood cells) in the bone marrow express CD7, CD13 (variably decreased to absent), CD33 (uniform), CD38 (bright), CD117 (dim to mostly absent), CD123 (uniform), and HLA-DR (variably decreased to absent) without CD34 or CD64.



Acute myeloid leukemia with plasmacytoid dendritic cell (pDC) expansion and a *RUNX1* mutation. The upper left dot plot displays the total viable white blood cells with blasts colored in red, pDCs in aqua (highlighted), lymphocytes in blue, monocytes in pink, and granulocytic cells in green. The rest of the dot plots in the upper panel selectively display CD34+ leukemic blasts. The leukemic blasts (colored red; ~20% of the white blood cells) in the peripheral blood express CD13 (bright), CD33 (dim to mostly absent), CD34 (bright), CD38, CD56 (small subset), CD117 (decreased), CD123, and HLA-DR (variably decreased). In the lower panel, all the dot plots display the total white cells. The pDCs (highlighted in aqua; ~15% of the white blood cells) express CD4, CD5 (major subset), CD7 (major subset), CD34 (dim, variable), CD38, CD45, CD56, CD123, and HLA-DR (dim, variable) without CD2, CD13, CD14, CD33, CD64, and CD117.

NK cells Evaluation



Minimal Residual Disease

- Minimal Residual Disease (MRD), also called Measurable Residual Disease, refers to the detection of very small numbers of residual cancer cells (typically at levels of 10^{-4} to 10^{-6} or lower) that persist after treatment, even when a patient is in complete remission by conventional criteria.
- Flow-based MRD is widely used because it is relatively fast, widely available, applicable to most patients (>95% in many diseases), and does not require patient-specific primers (unlike some molecular methods).
- It complements molecular techniques like PCR or NGS. Sensitivity depends on the number of cells acquired (often $\geq 10^6$–10^7 events), panel design, sample quality (fresh bone marrow preferred), and analysis standardization.

Flow cytometry-based diagnosis of primary immunodeficiency diseases

Application of flow cytometry in the diagnosis of primary immunodeficiency diseases.

Disease	Test
Evaluate for specific cell population and subpopulation	
XLA	Absent B cells
SCID	Absent T cells and variable number of B and NK cells (depending on defect)
XLP1	Markedly reduced iNKT cells
ALPS	Increased TCR-$\alpha/\beta+$ double-negative ($CD4^-CD8^-$) T cells
HIES and CMCD	Decreased Th17 cells
Evaluate of specific cell surface protein	
CVID	CD19 on B cells
	BAFF-R on B cells
	ICOS on activated T cells
X-linked HIGM	CD40L on activated T cells
Autosomal recessive HIGM	CD40 on B cells
MSMD	IFN-γR1 on monocytes
	IL-12Rβ1 on activated T cells
LAD1	CD18 on granulocytes
X-SCID	CD132 on lymphocytes
gp91-phox and p22-phox deficient CGD	Cytochrome b558 on granulocytes and B cells
IL-17RA deficiency	IL-17RA on lymphocytes and monocytes

Flow cytometry-based diagnosis of primary immunodeficiency diseases

Evaluate of specific intracellular protein	
XLA	BTK in monocytes and platelets
Wiskott-Aldrich syndrome and X-linked thrombocytopenia	WASp in lymphocytes and myeloid cells
XLP1	SAP in $CD8^+$ T cells and NK cells
XLP2	XIAP in lymphocytes
FHL2	Perforin in $CD8^+$ T cells and NK cells
FHL3	Munc13-4 in platelets
ZAP70 deficiency	ZAP70 in T cells
p47-phox and p67-phox deficient CGD	p47-phox and p67-phox protein in granulocytes
DOCK8 deficiency	DOCK8 in lymphocytes
CTLA4 haploinsufficiency and LRBA deficiency	CTLA4 in $CD4^+FOXP3^+$ T cells
Evaluate of specific nuclear protein	
IPEX	FOXP3 in $CD4^+CD25^+$ T cells
Evaluate biologic effects	
CVID	Decreased switched memory B cells
Omenn syndrome and hypomorphic SCID	Oligoclonal TCR/diversity
X-linked HIGM	Decreased memory B cells and memory $CD4^+$ T cells

Flow cytometry-based diagnosis of primary immunodeficiency diseases

Evaluate function	
MSMD	STAT1 phosphorylation in monocytes in response to IFN-γ
CMCD (STAT1 gain-of-function)	STAT1 phosphorylation in monocytes in response to IFN-γ
FHL3/4/5, Chédiak-Higashi syndrome and Griscelli syndrome	CD107a expression in NK cells and CTLs
XLP2	TNF-α in monocytes in response to muramyl dipeptides
CGD	DHR123 assay in granulocytes, monocytes and B cells
X-SCID and JAK3-deficient SCID	STAT phosphorylation in lymphocytes in response to cytokine stimulation
X-linked HIGM	No binding of CD40L and CD40-Ig
IRAK4 and MyD88 deficiency	TNF-α in monocytes in response to LPS
IL-10R deficiency	STAT3 phosphorylation in lymphocytes in response to IL-10
Infantile-onset multisystem autoimmune disease 1 (heterozygous GOF mutation in STAT 3)	Increased STAT3 phosphorylation

Flow cytometry-based diagnosis of primary immunodeficiency diseases

Evaluate function	
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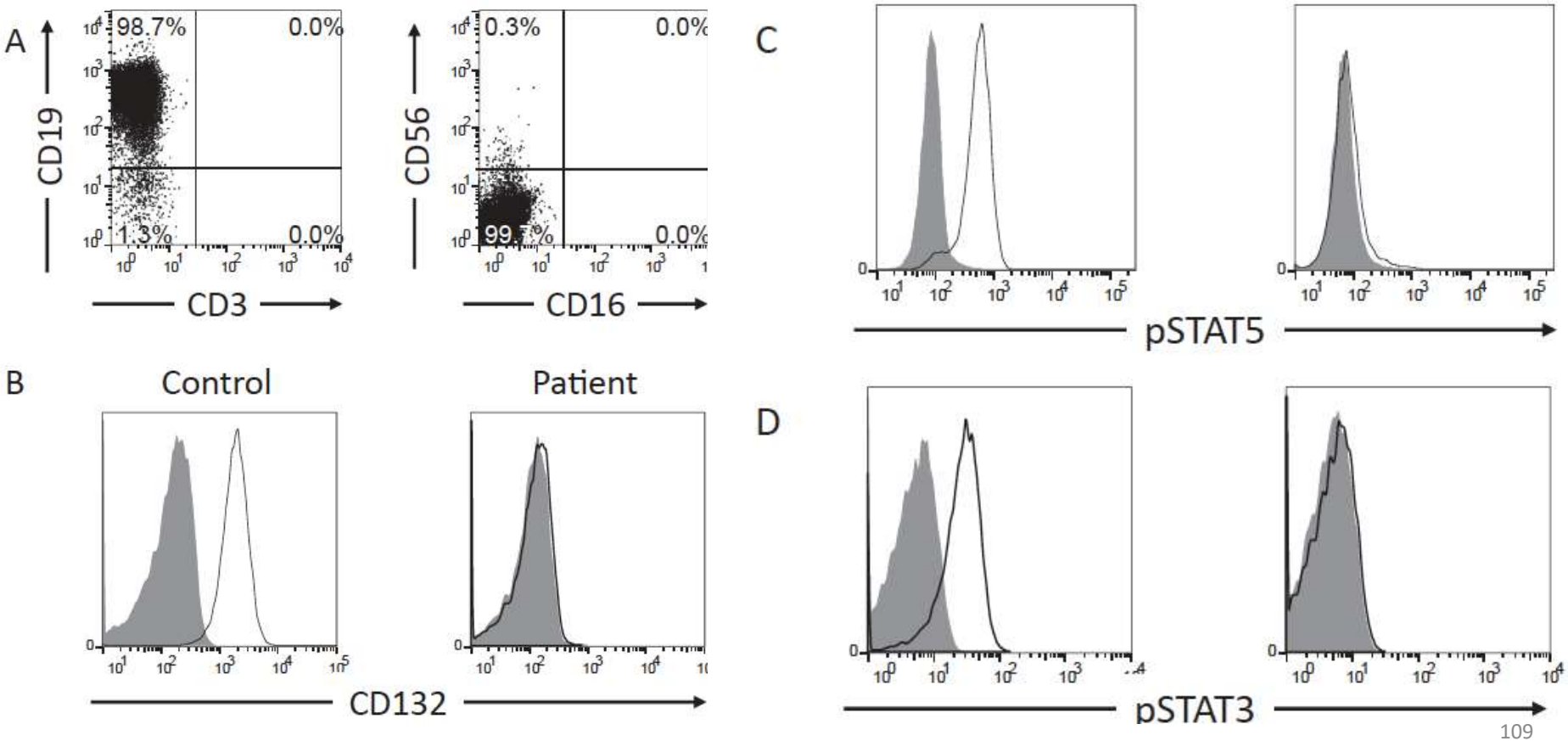
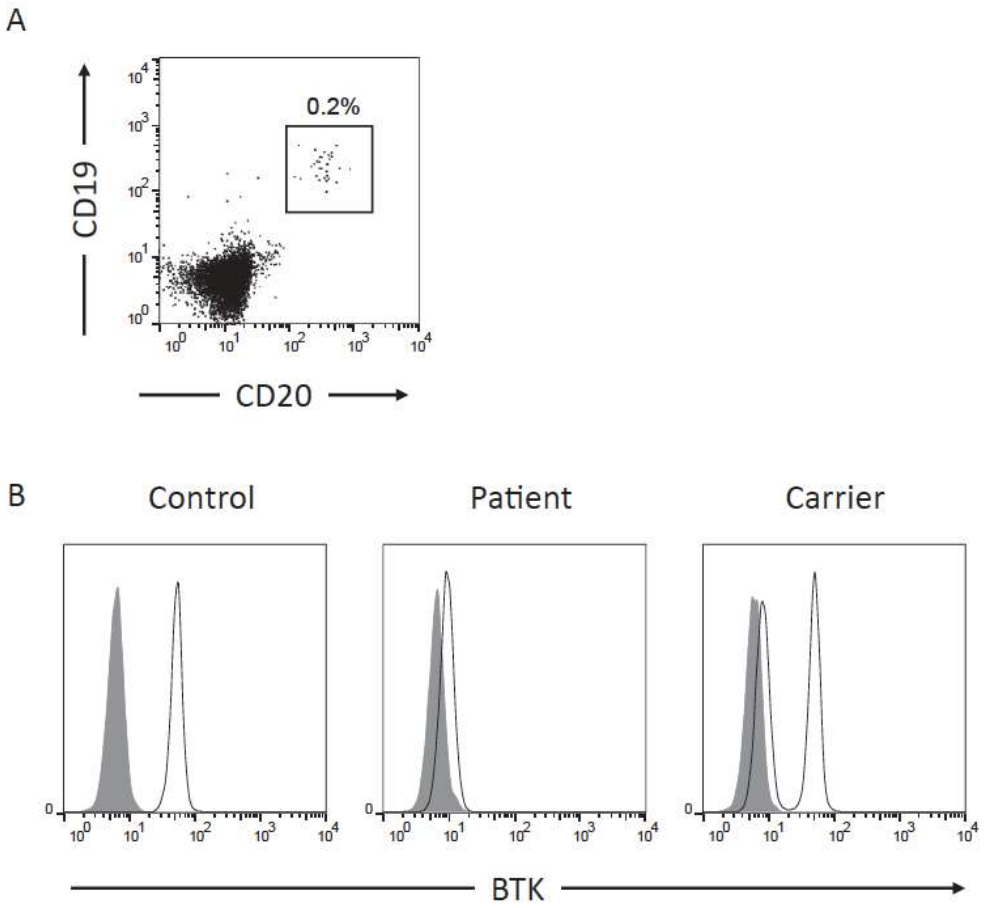
Infantile-onset multisystem autoimmune disease 1 (heterozygous GOF mutation in STAT 3)	Increased STAT3 phosphorylation

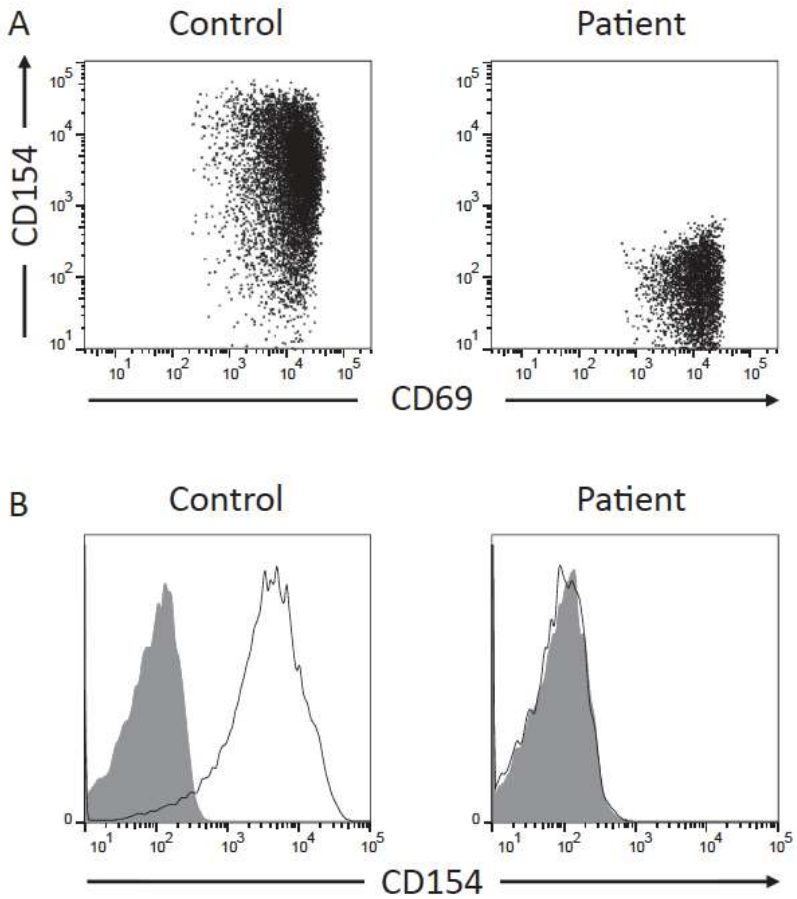
X-linked severe combined immunodeficiency



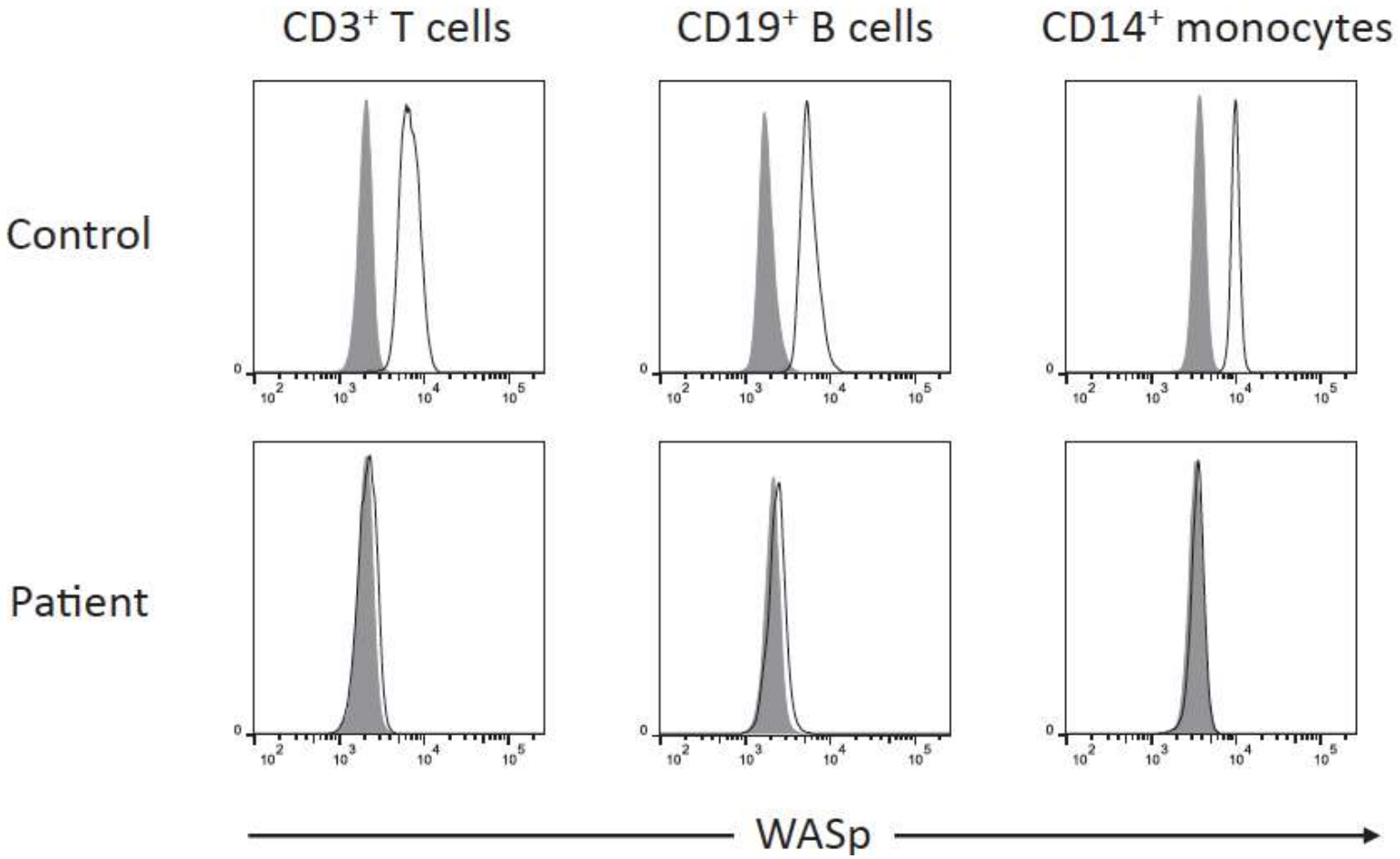
X-linked agammaglobulinemia (XLA)



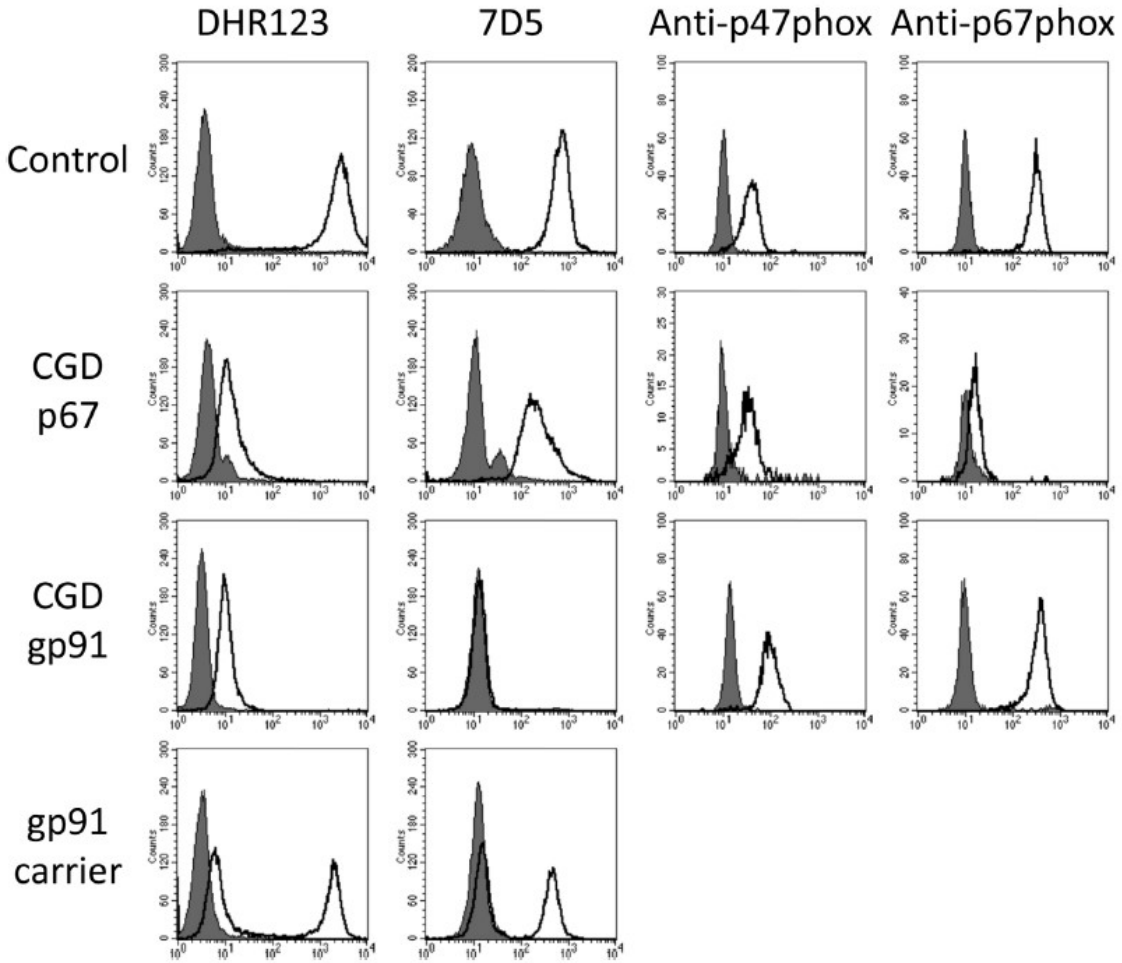
Flow cytometric identification of CD154 (CD40L) on activated T cells in a patient with X-linked hyper IgM syndrome



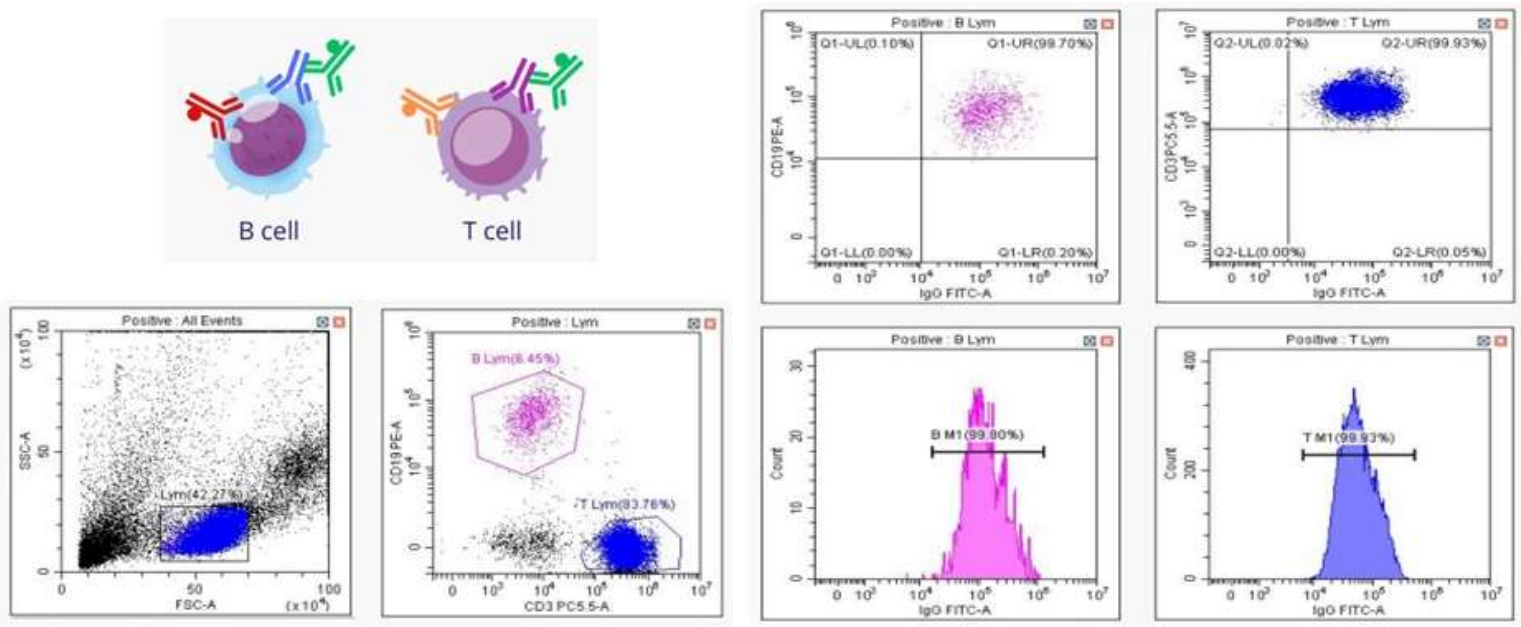
Flow cytometric detection of WASp in a patient with Wiskott-Aldrich syndrome



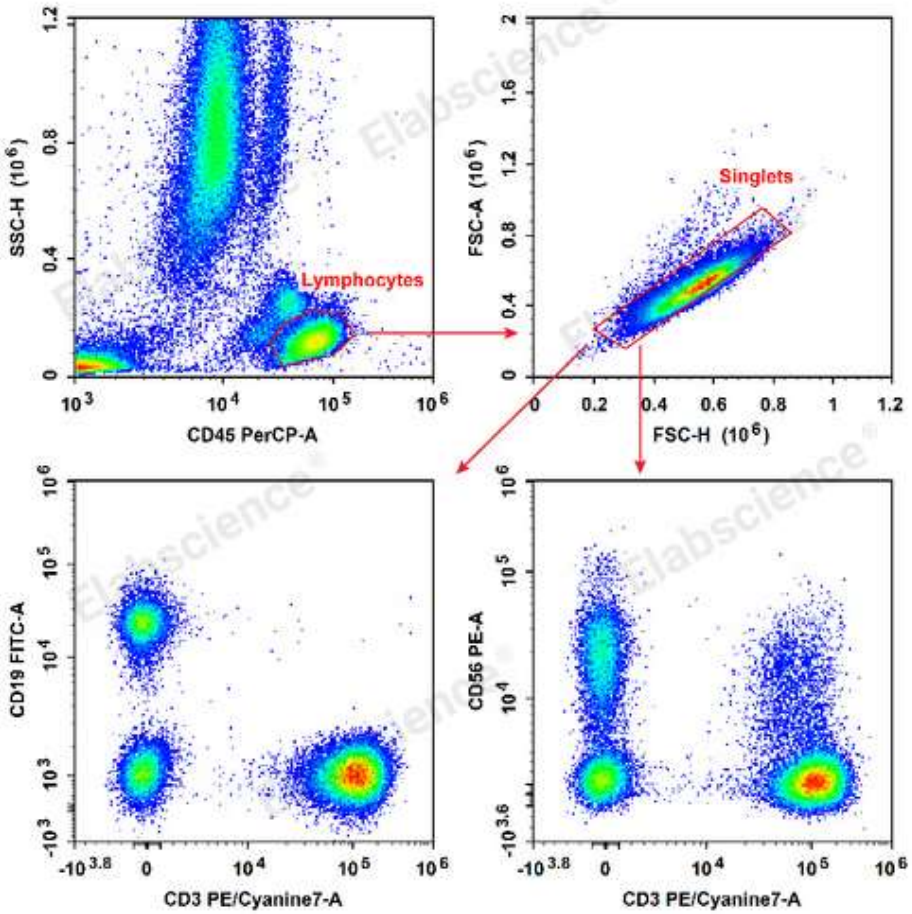
Flow cytometric detection of CGD



Flow Cytometry Crossmatch



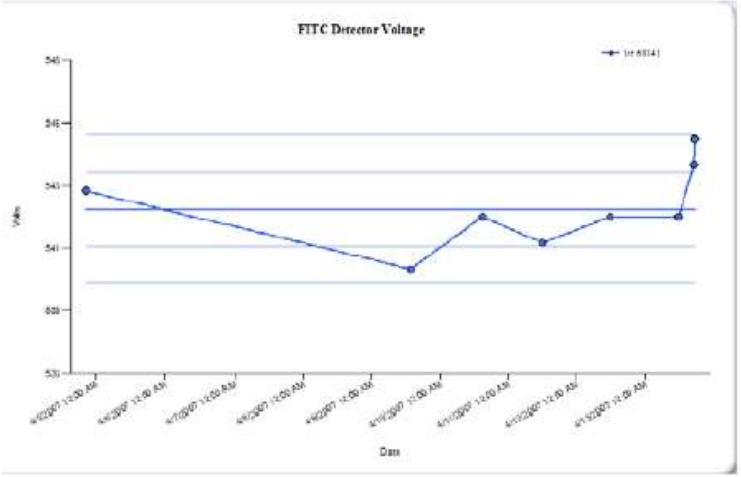
NK Cells Evaluation



Daily Instrument QC & Laser Alignment

- **Fluidics Integrity:** Startup cleaning, bubble checks, and pressure stability.
- **Hard QC (Beads):** Tracking Levey-Jennings (L-J) charts for Target Channels.
- **Laser Health:** Monitoring **rCV** (robust Coefficient of Variation) for alignment.
- **PMT Voltage Baseline:** Ensuring consistent signal amplification.

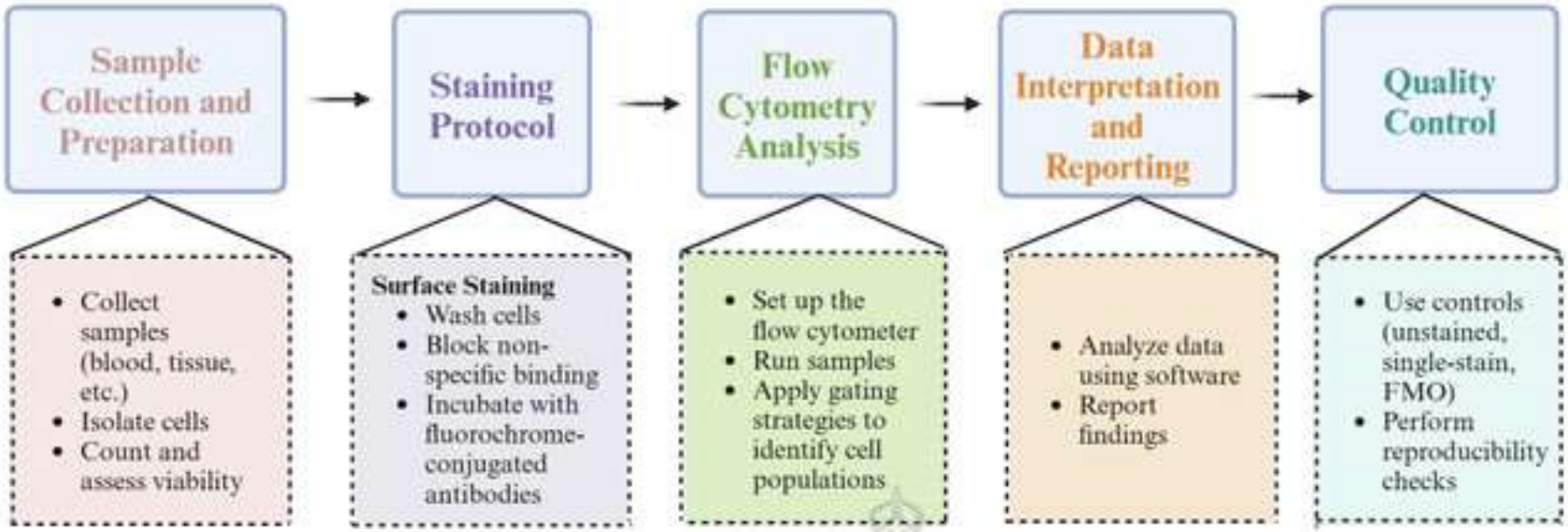
Levey-Jennings



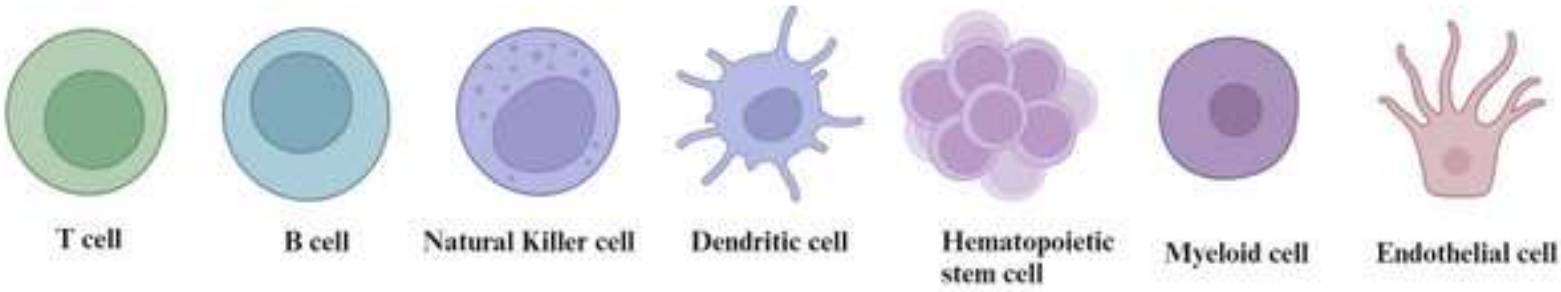
Bench Troubleshooting Guide

Problem Observed	Potential Root Cause	Immediate Action / Troubleshooting Step
High Background / Poor Resolution	Incomplete RBC lysis or inadequate washing leaving plasma proteins.	Re-wash the sample with PBS. Increase lysis incubation time by 5 minutes (do not exceed 15 mins total).
Sudden Drop in Event Rate	Micro-clog or air bubble in the sample line / flow cell.	Pause the run. Perform a 'Prime' or 'Flush' cycle. Check sample tube for fibrin strands or microclots.
Fluorescence Leaking into Wrong Channel	Compensation matrix is incorrect or tandem dyes have degraded.	Re-run single-stain compensation controls. Ensure tandem dye conjugates (like PE-Cy7) were protected from light.
Unexpected Broad or Shifted Peaks	High number of dead/apoptotic cells or incorrect sample-to-antibody ratio.	Run a viability dye (e.g., 7-AAD) to gate out dead cells. Ensure bone marrow samples are diluted to standard white blood cell counts before staining.

Immunophenotyping by Flow Cytometry



Cell type markers



References

- Numerous References available in the Flow Lab
  - Cytometry
  - Current Protocols in Flow Cytometry
  - Many more reference books available
- Purdue University Cytometry Laboratories website: http://www.cyto.purdue.edu/
  - Dr. Robert Murphy, Carnegie Mellon University- Basic Theory 1 and 2 PowerPoint slides
- The Scripps Research Institute Flow Cytometry Core Facility: http://facs.scripps.edu/