Introduction to Flow Cytometry

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BD

Outlines

- Definition
- Principle and Flow Cytometer Components
- Interpretation of Flow Cytometry
- Multicolor and Compensation

Definition

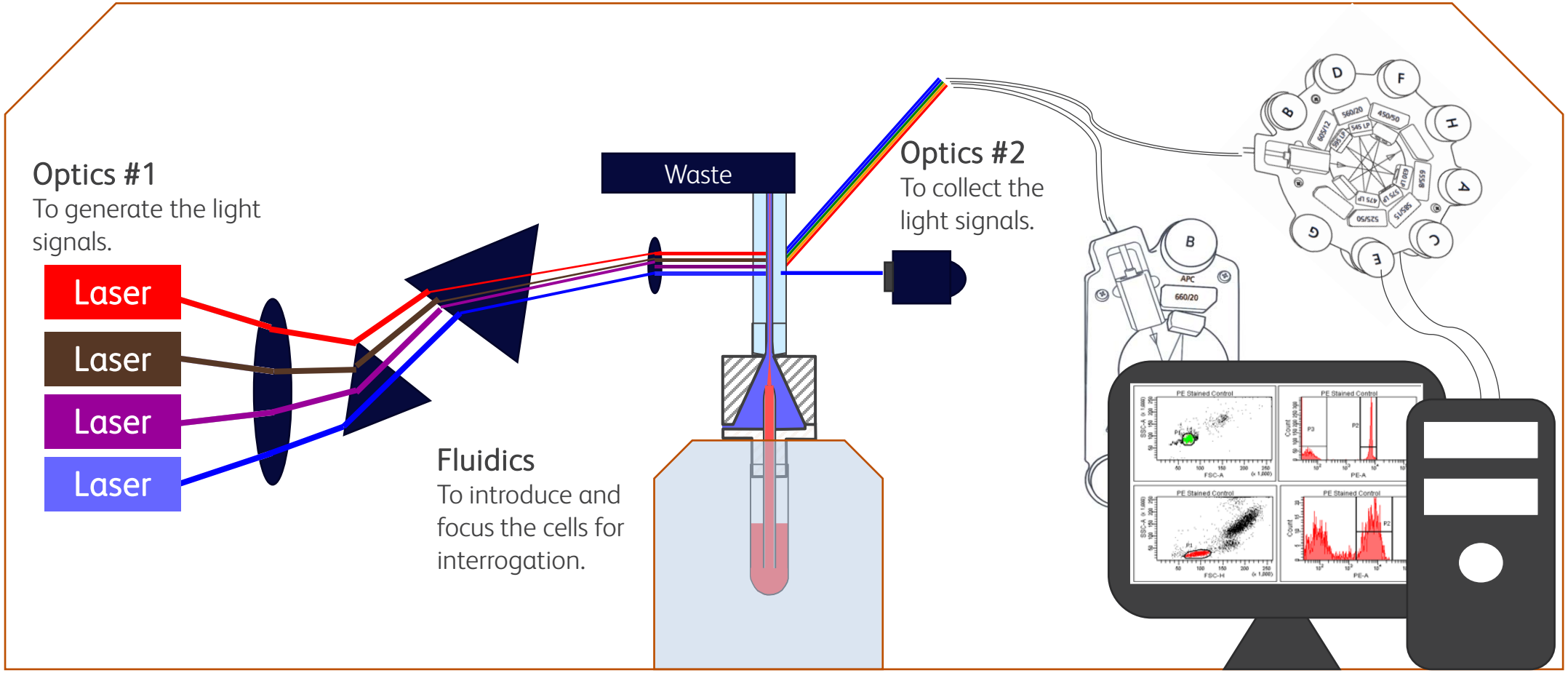
- **Flow** = in Fluid **Cyto** = Cell **Metry** = Measurement

- Measurements are made on a per-cell basis
  - *Not an average*

- Routine rates of thousands of cells per second
  - *Quick*

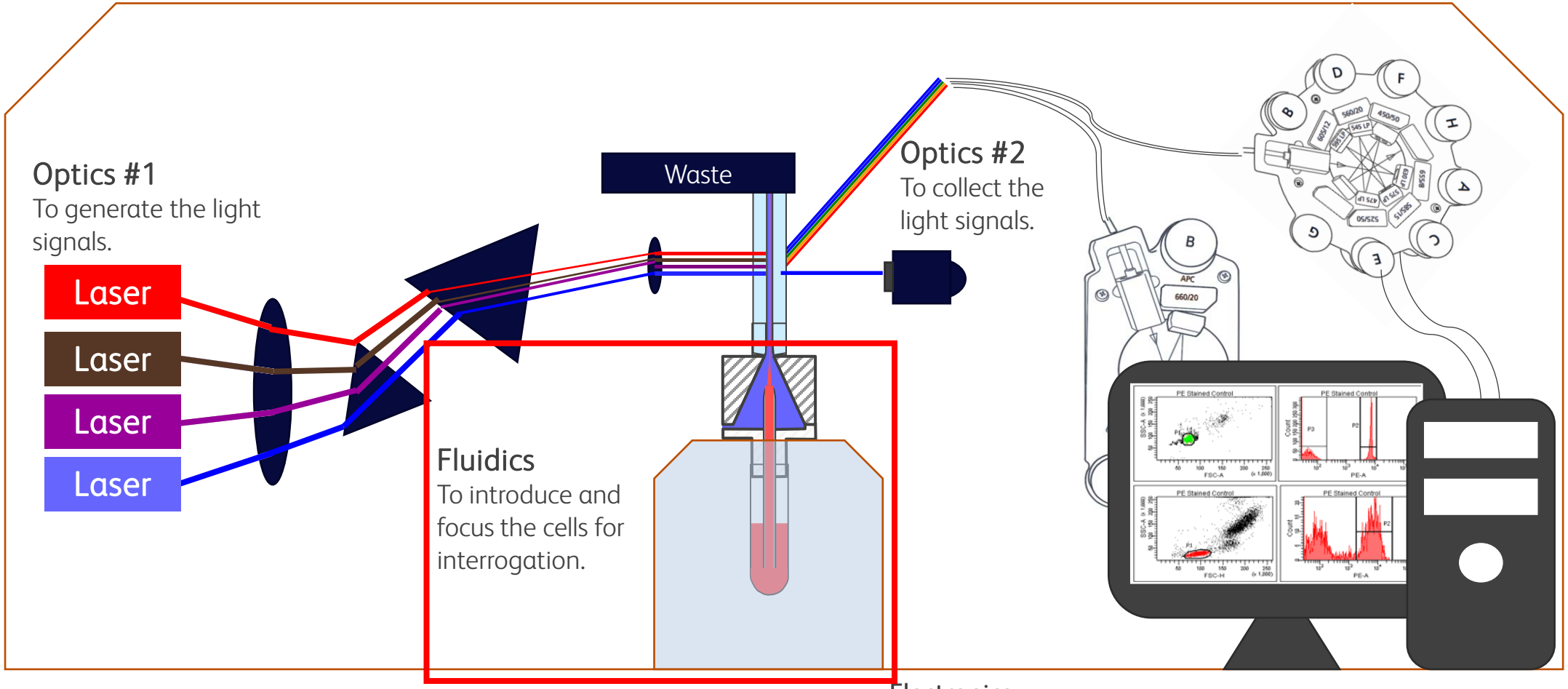
- Simultaneous measurements of multiple characteristics of a single cell through its light scatter
  - *Multi-parametric*

Subsystems of Flow Cytometer

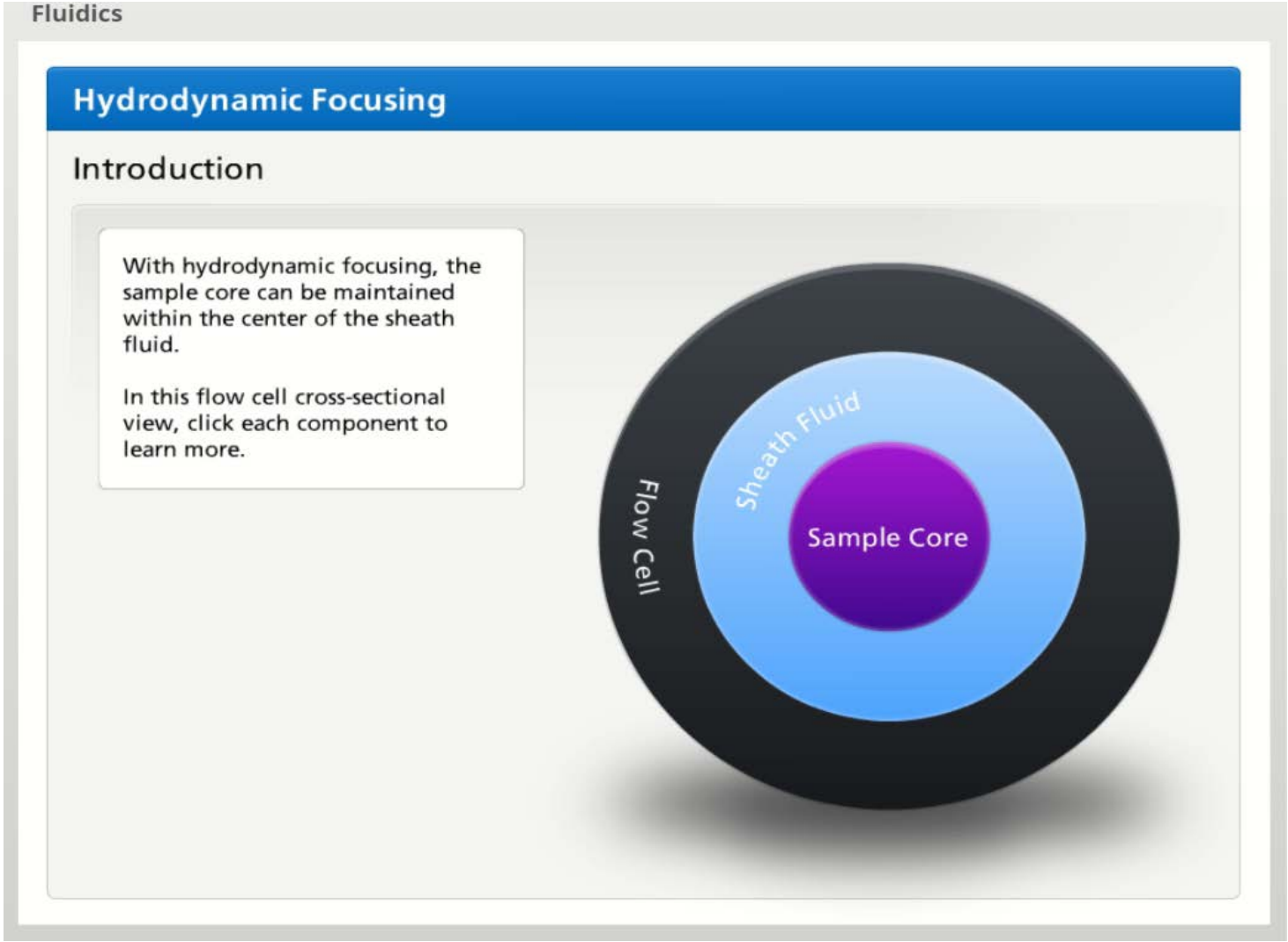


Electronics
To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer.

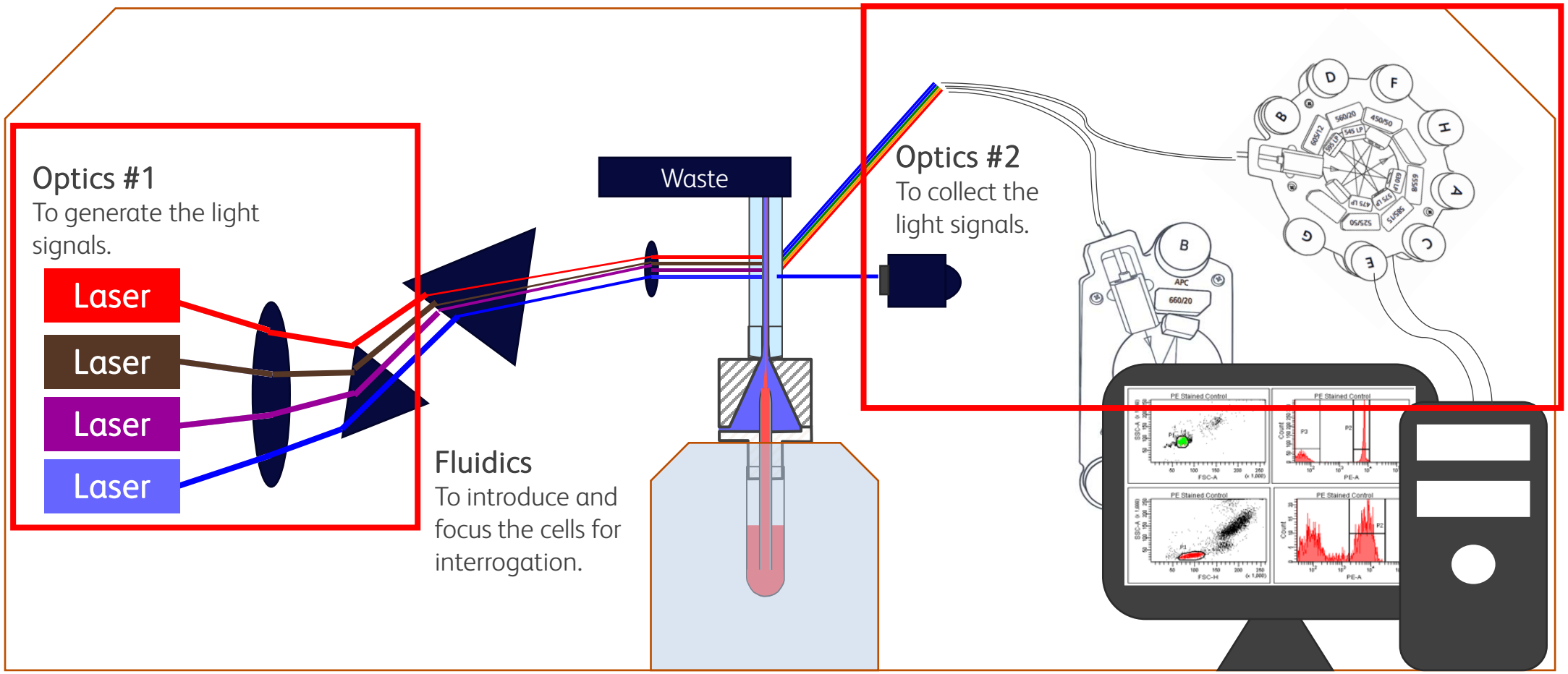
Fluidic System



Fluidic System

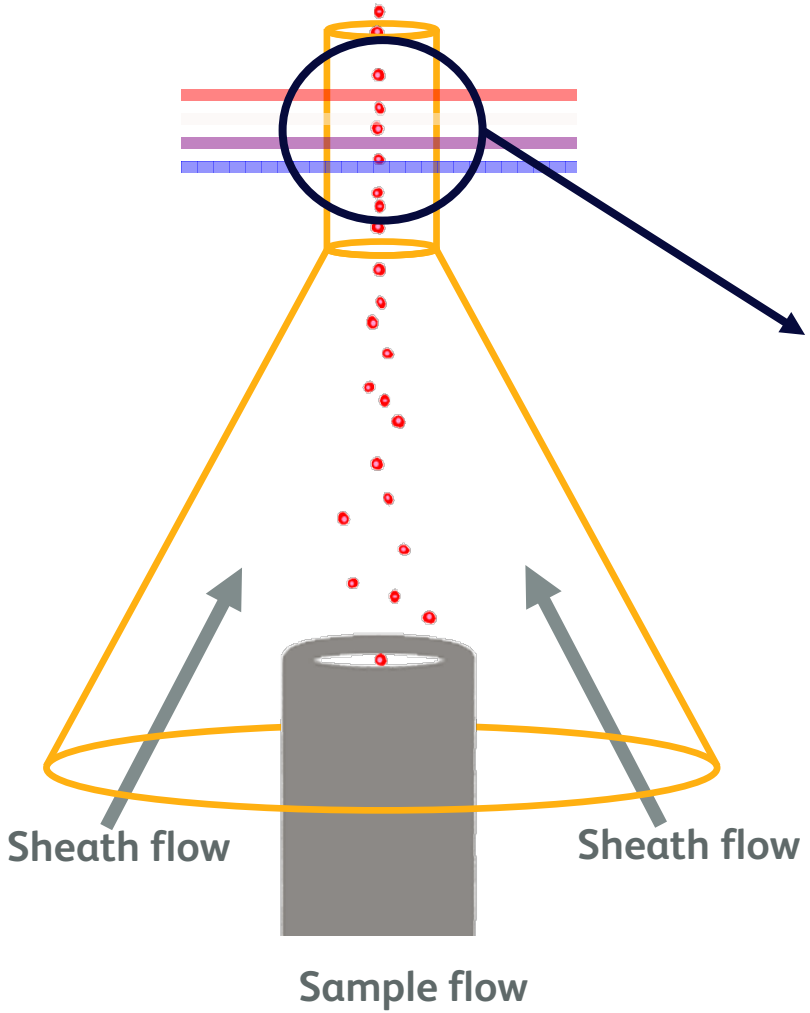


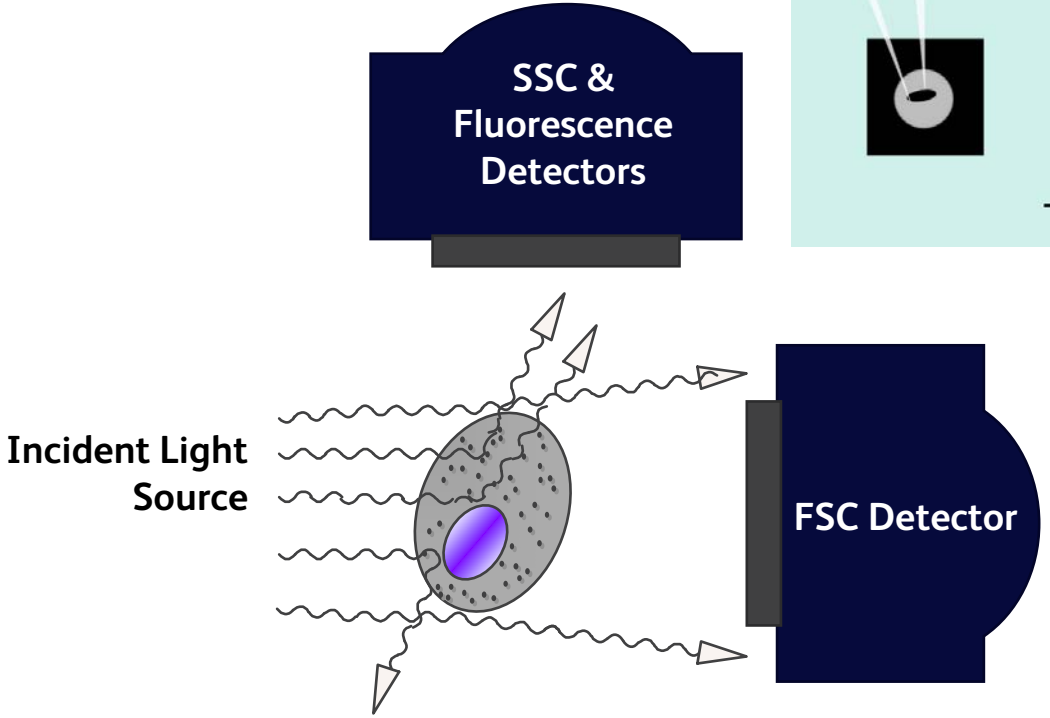
Optic System

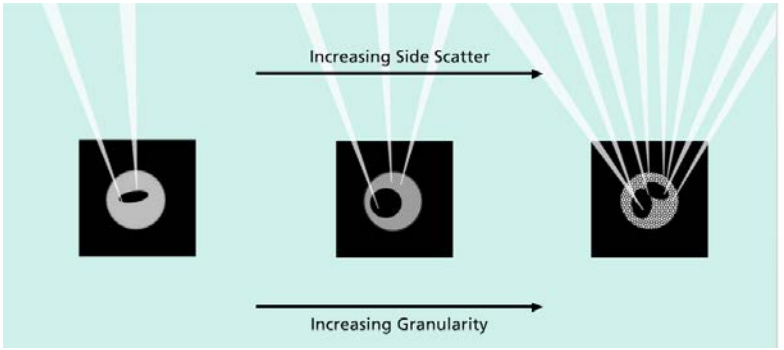


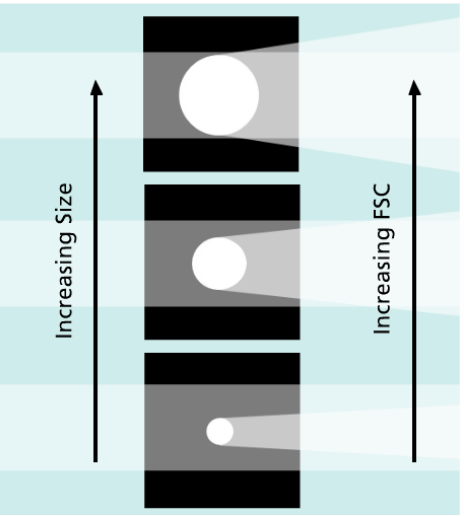
Electronics
To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer.

Flow Cell Dynamic



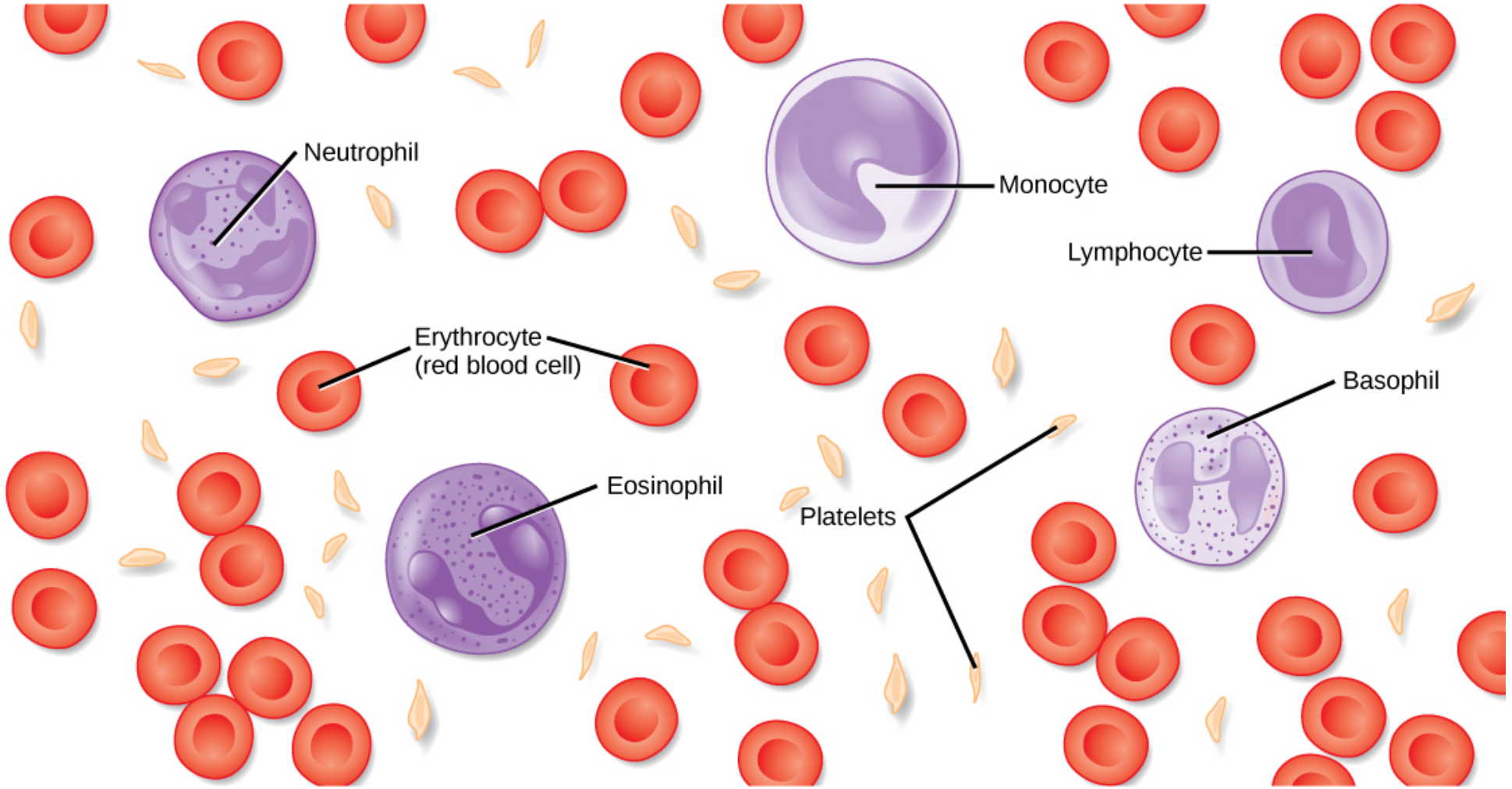




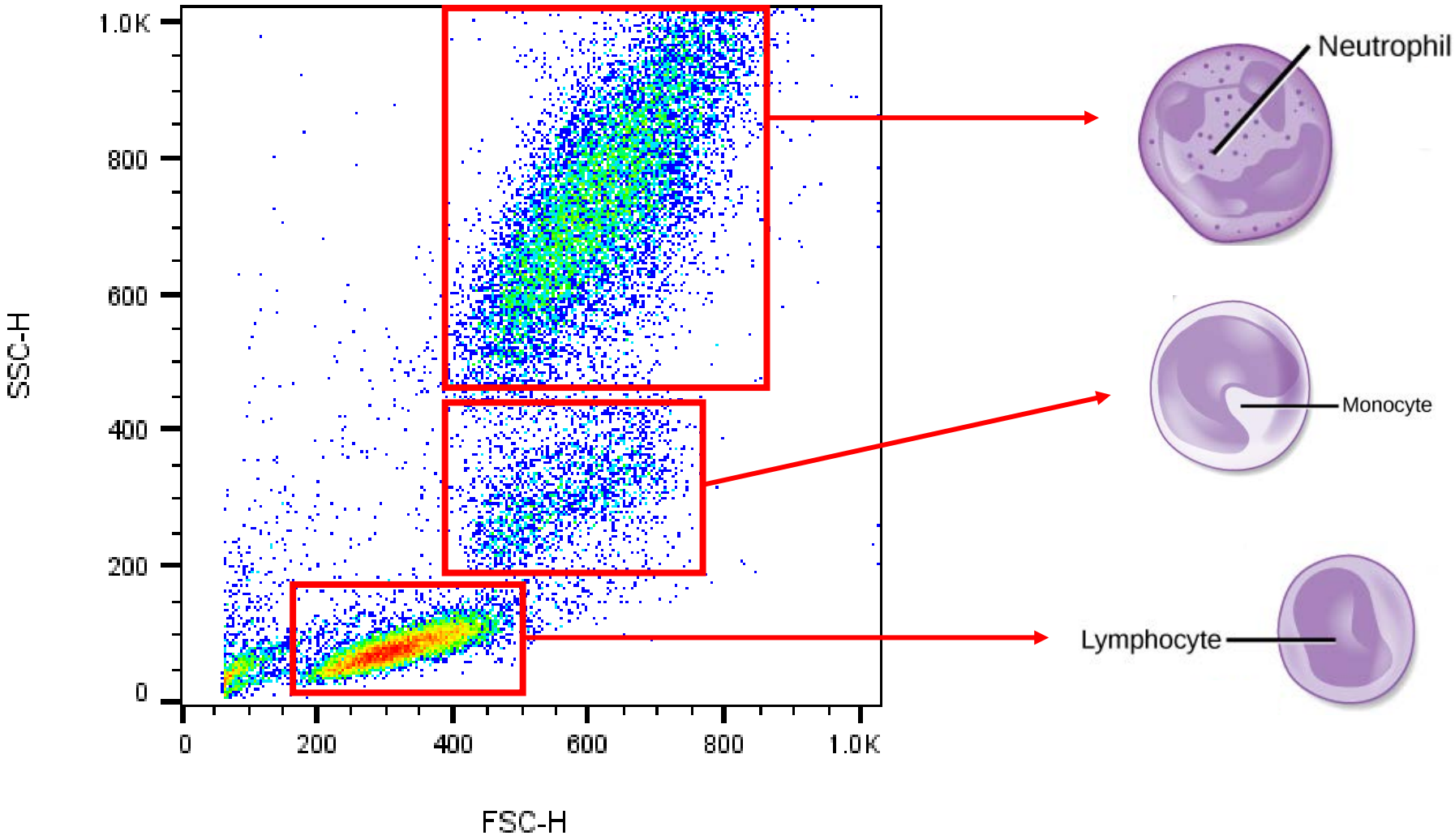


- Relative size: Forward Scatter (FSC)
- Relative granularity or internal complexity: Side Scatter (SSC)
- Relative fluorescence intensity

Example: Cellular distribution of Lysed Whole Blood

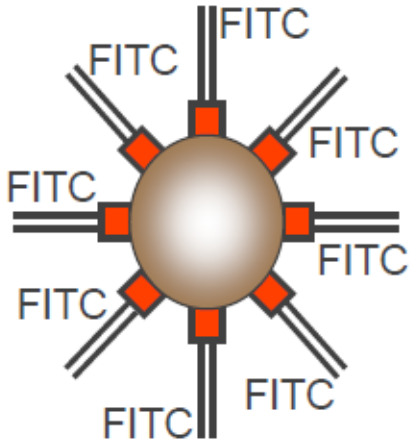


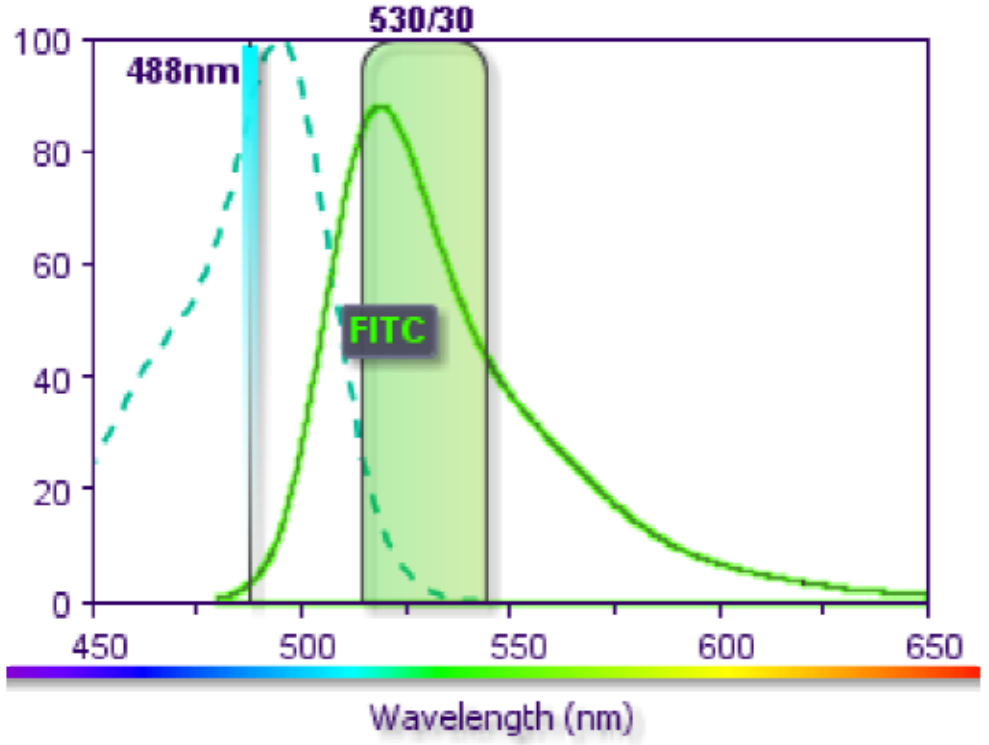
Example: Cellular distribution of Lysed Whole Blood



Fluorescence

- The **excitation wavelengths** of a fluorochrome direct the choice of laser used to excite it.
- The **emission wavelengths** of a fluorochrome direct the choice of filters and PMTs used to measure the emission signal.





Fluorescence

Fluorochrome	Fluorescence Emission Color	Ex Max (nm)	Excitation Laser Line (nm)	Em Max (nm)
BD Horizon™ BV421	Blue	405	405, 407	421
BD Horizon™ V450	Blue	404	405, 407	448
BD Horizon™ V500-C	Green	415	405, 407	500
BD Horizon™ BV510	Green	405	405, 407	510
AmCyan	Green	457	405, 407	491
FITC	Green	495	488	520
PE	Yellow	496, 694	488	578
BD Horizon™ BV605	Orange	407	405, 407	602
APC	Red	650	633, 638, 640	659
PerCP	Red	482	488	675
PerCP-Cy™5.5	Far Red	482	488	693
BD Horizon™ APC-R700	Far Red	652	633, 638, 640	704
PE-Cy™7	Infrared	496, 694	488	777
APC-Cy™7	Infrared	650	633, 638, 640	777
APC-H7	Infrared	650	633, 638, 640	777

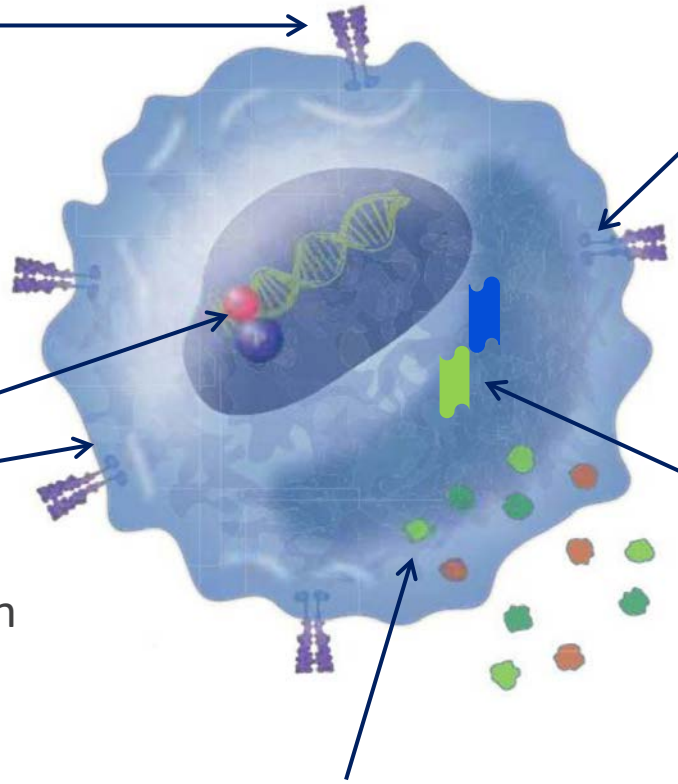
Application for Flow Cytometry

Cell surface

Lineage marker, Activation marker,
Cytokine and chemokine receptors

Intracytoplasmatic or intranuclear

Cell signaling proteins



Cell processes

Apoptosis, Cell viability, Proliferation

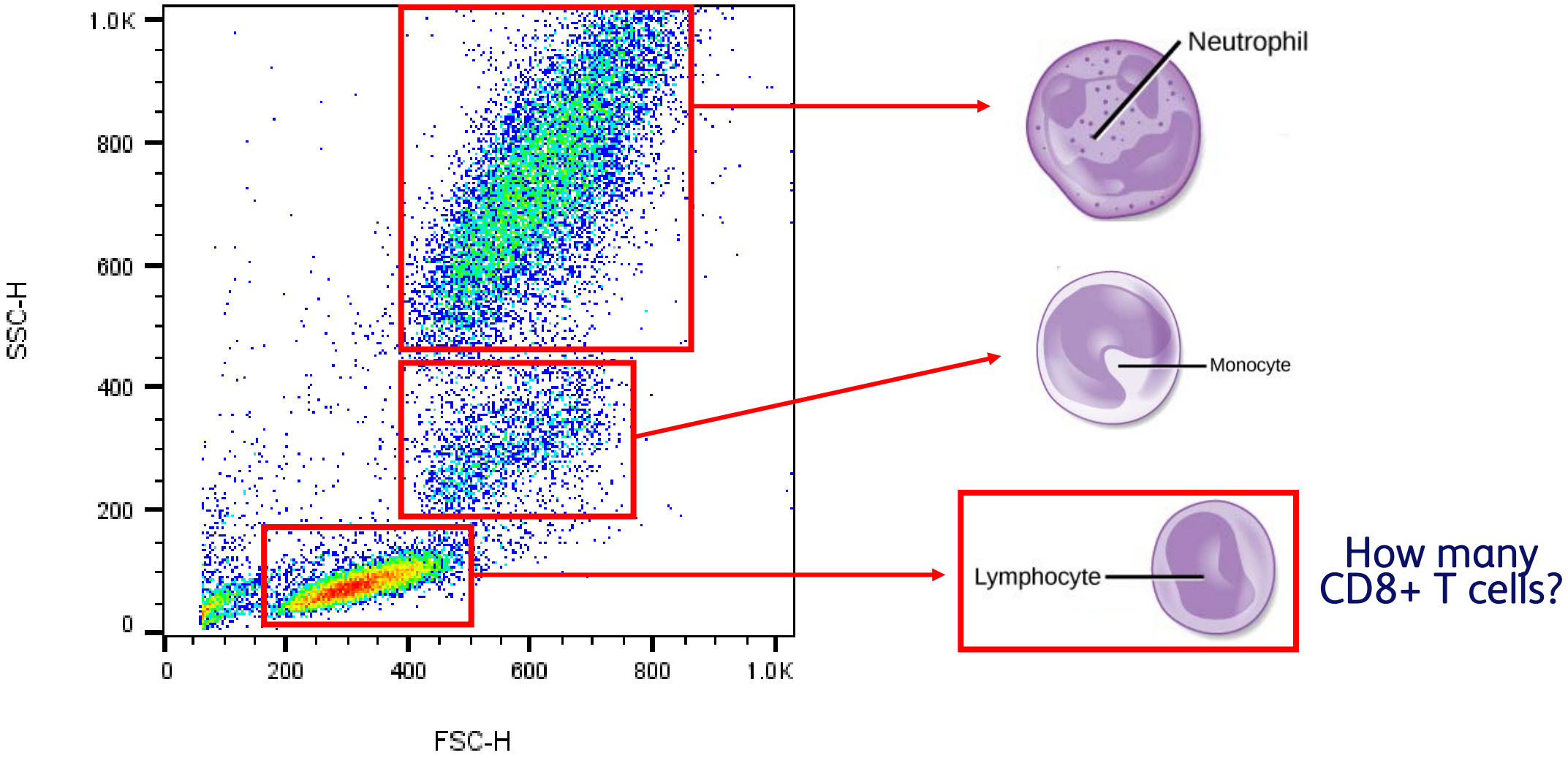
Fluorescent proteins

GFP, YFP, mCherry etc.

Intracytoplasmatic

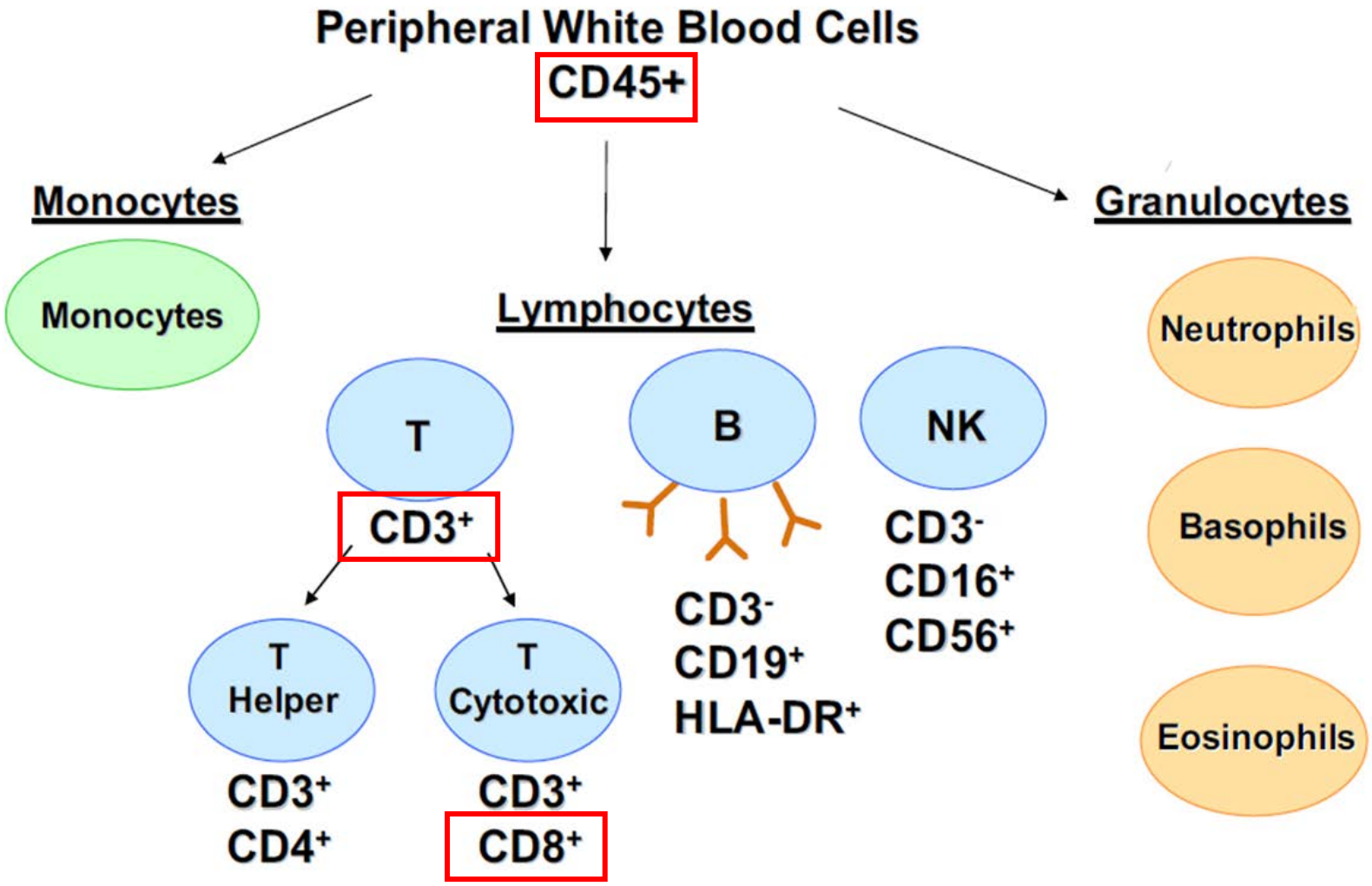
Cytokine, chemokines, various proteins

Example: Cellular distribution of CD8+ in Lymphocytes



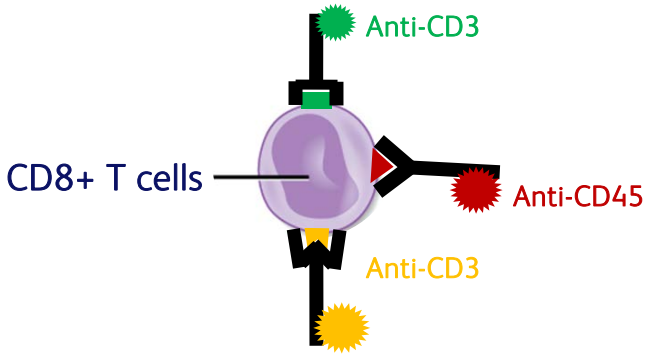
How many CD8+ T cells?

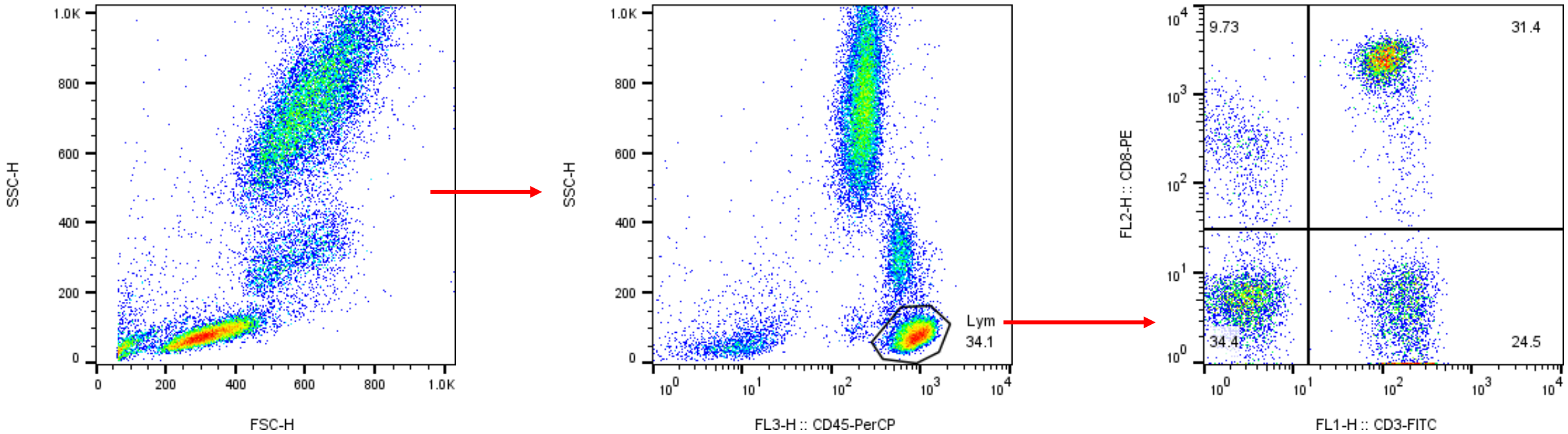
Lymphocyte subsets



Example: Cellular distribution of CD8 in Lymphocytes

- Adding
  - Anti-CD45-PerCP: WBCs
  - Anti-CD3-FITC: T cells
  - Anti-CD8-PE: CD8+ T cells (Cytotoxic T cells)

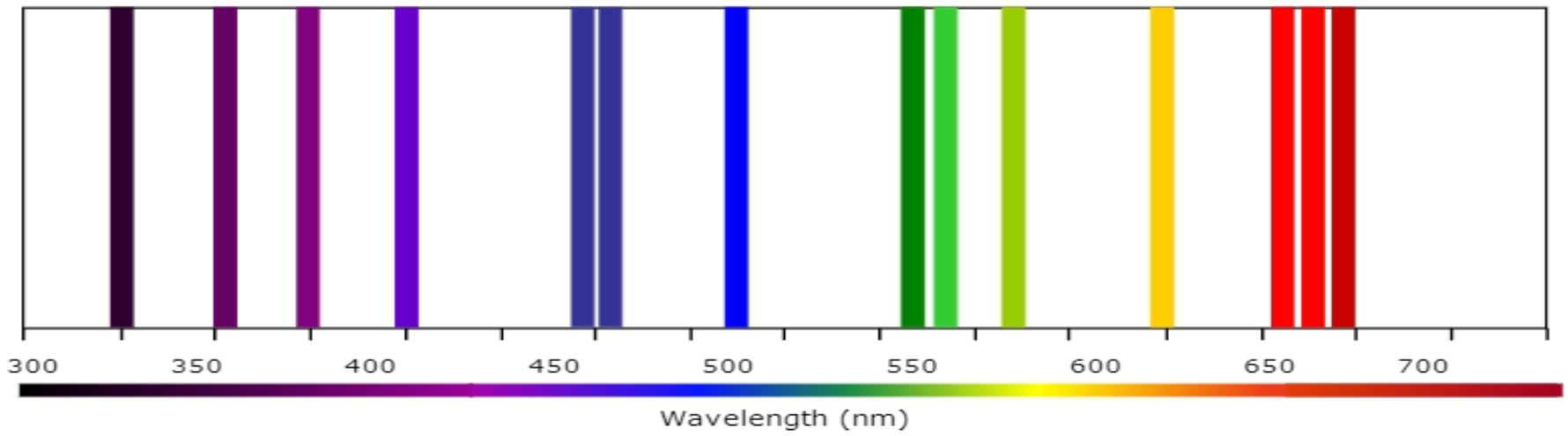




Optical Filters

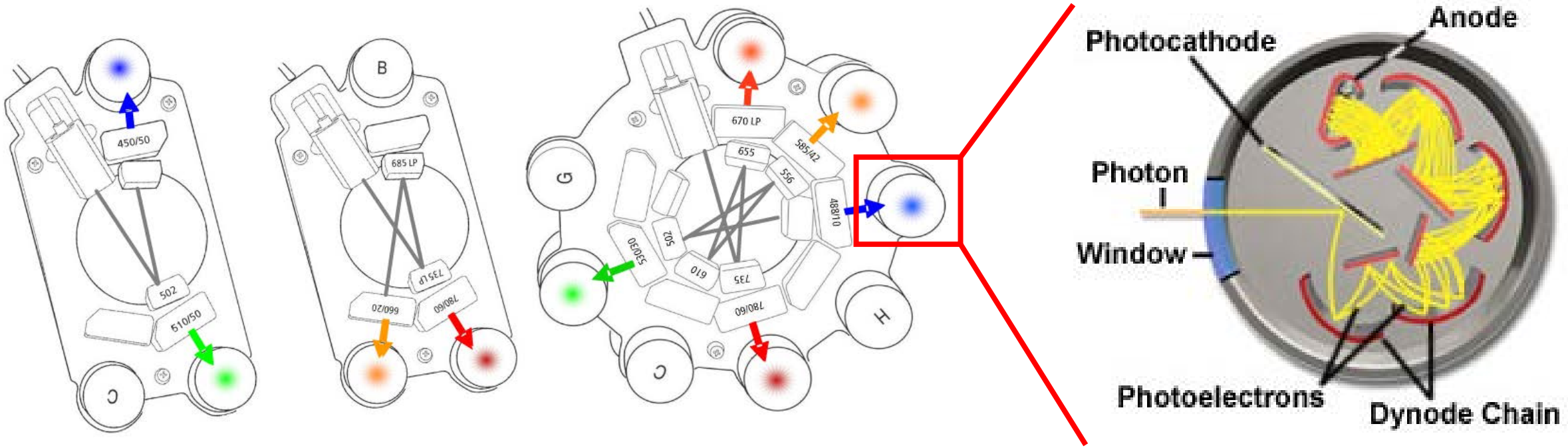
Lasers in BD Cytometers

Light Amplification by Stimulated Emission of Radiation



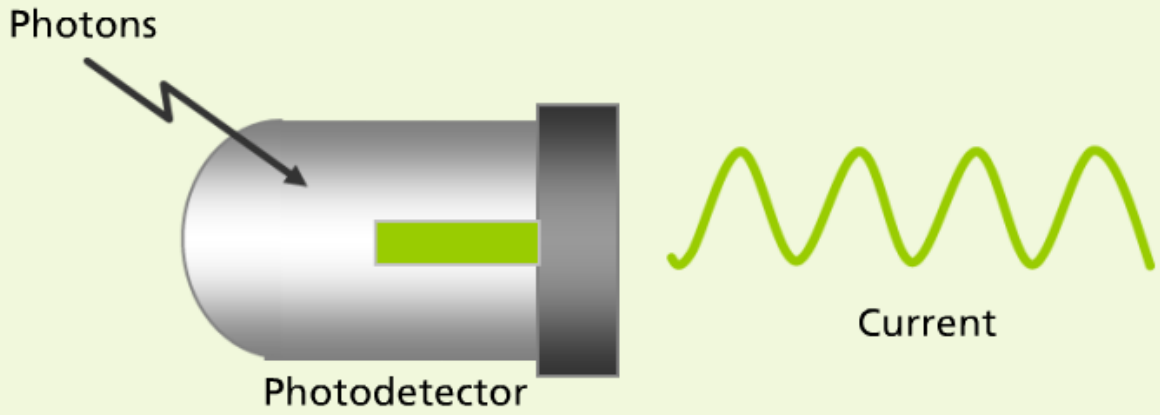
Detectors

- PMTs: Photomultiplier tubes



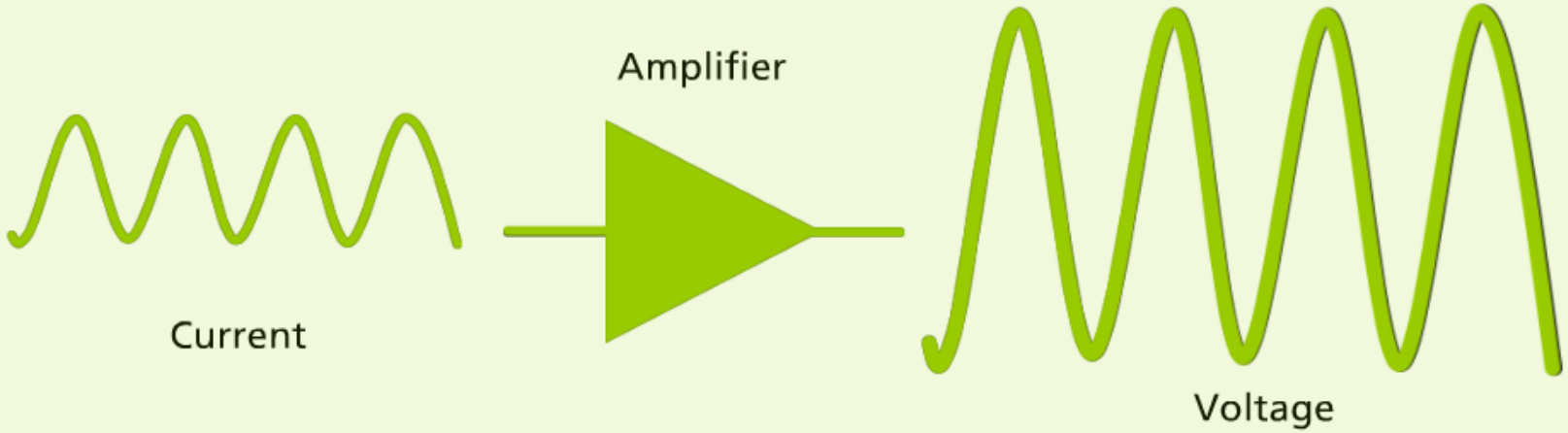
Detectors

- Photodetectors are light sensors that can detect photons of light.
- Incoming photons cause photodetectors to produce electrical current.

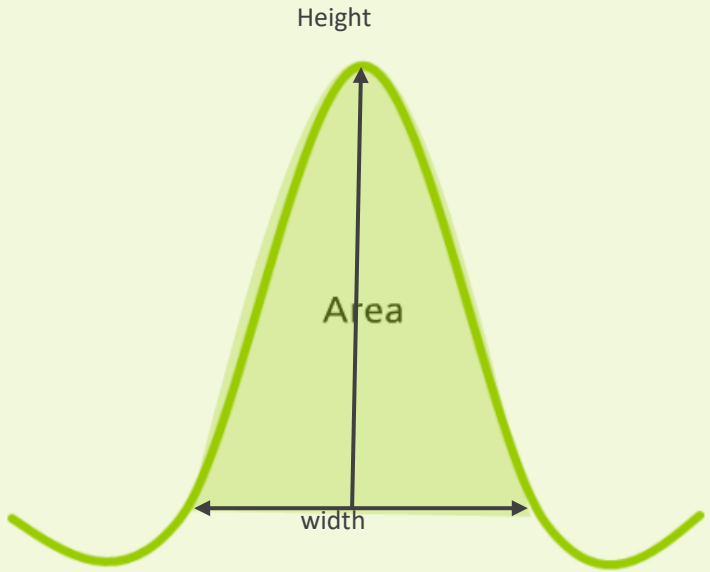


Detectors

- Amplifiers convert electrical current from photodetectors into a voltage.
- The resulting voltages are larger in magnitude than the incoming currents.

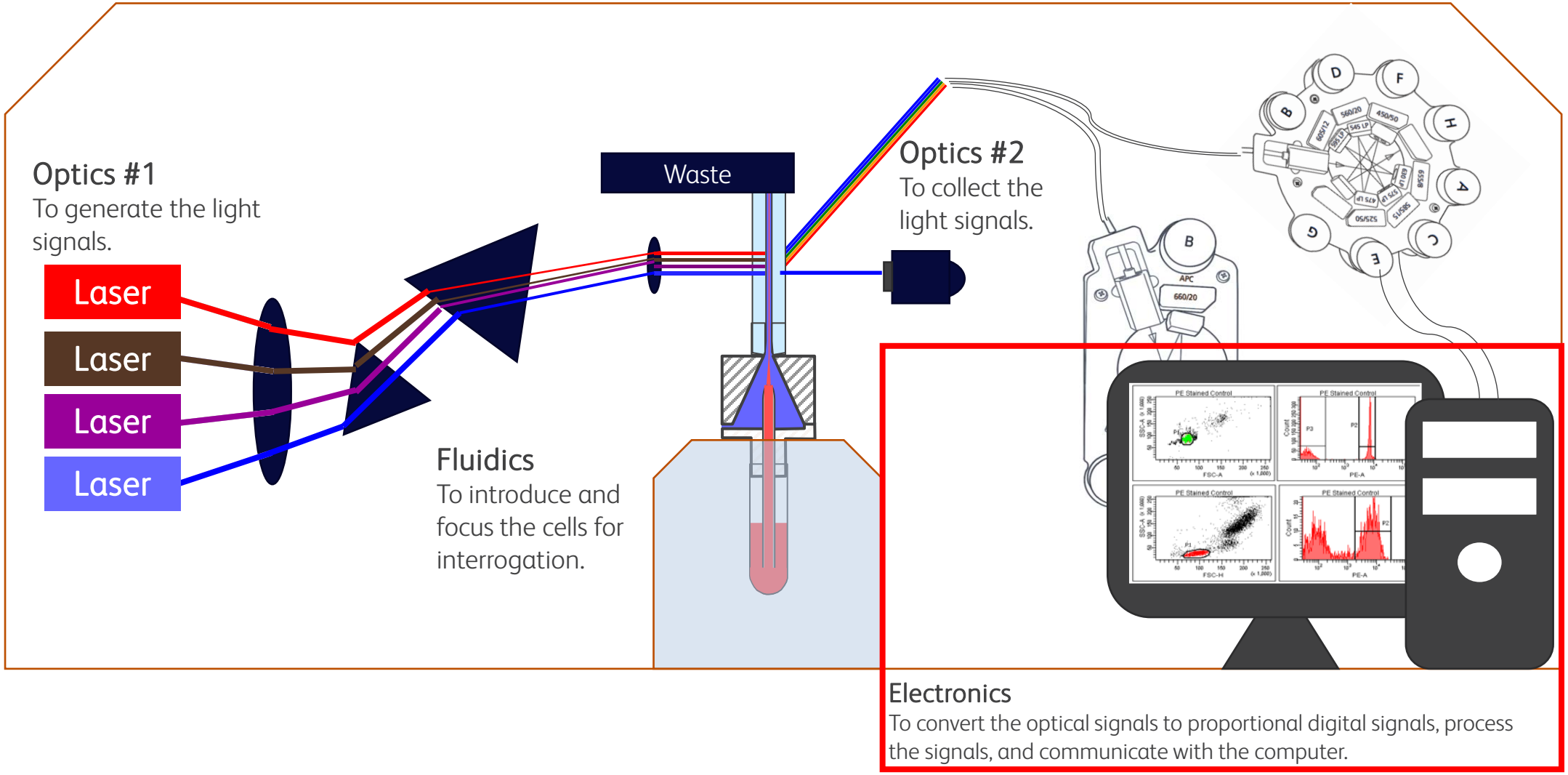


Detectors

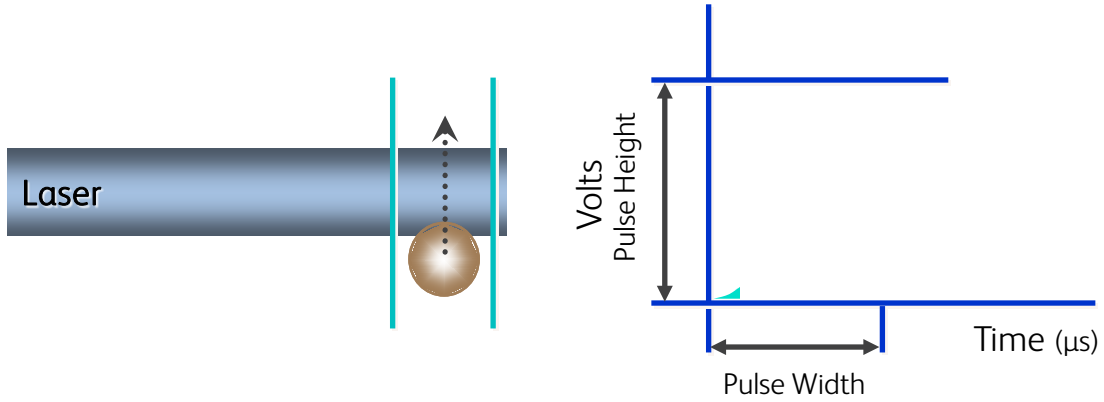


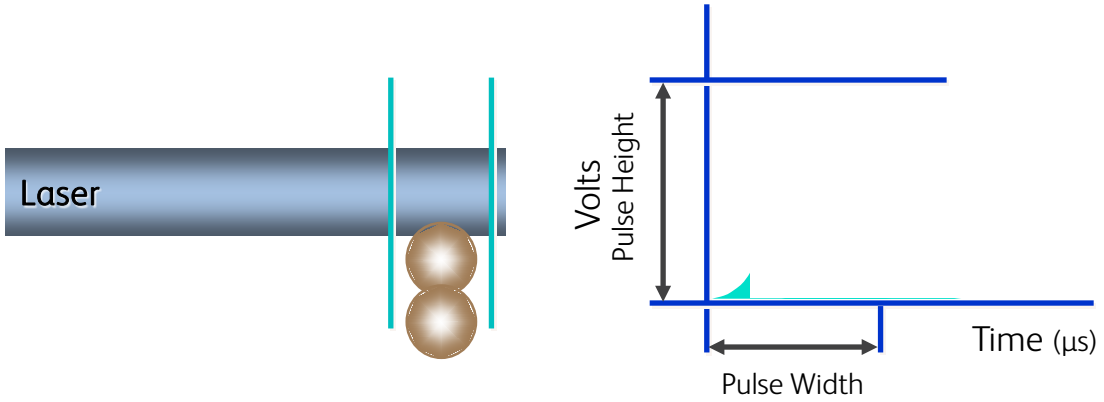
Signal processors quantify voltage pulses, providing numerical values for pulse height, width, and area.

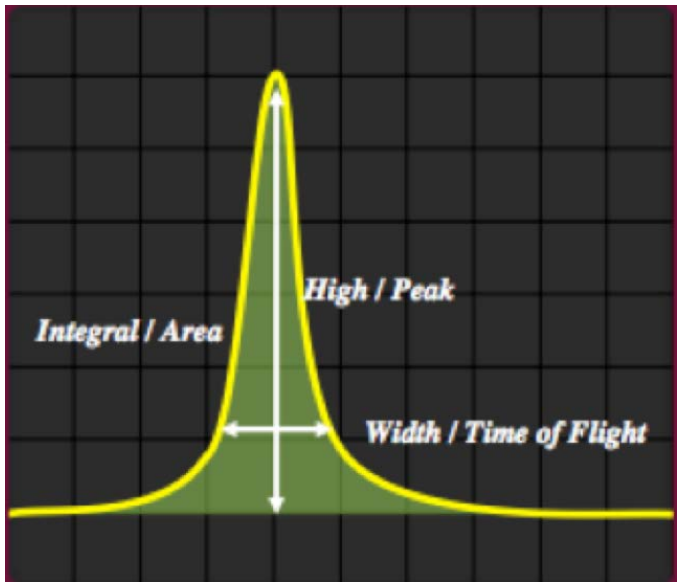
Electronic System

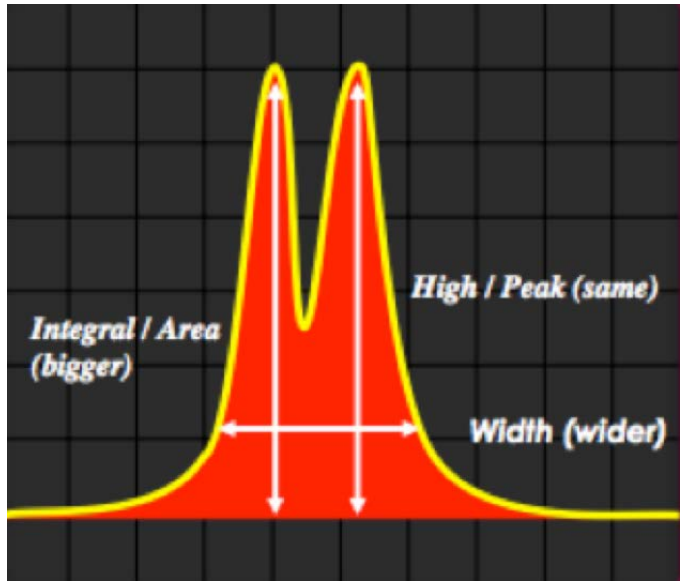


Signal Processing

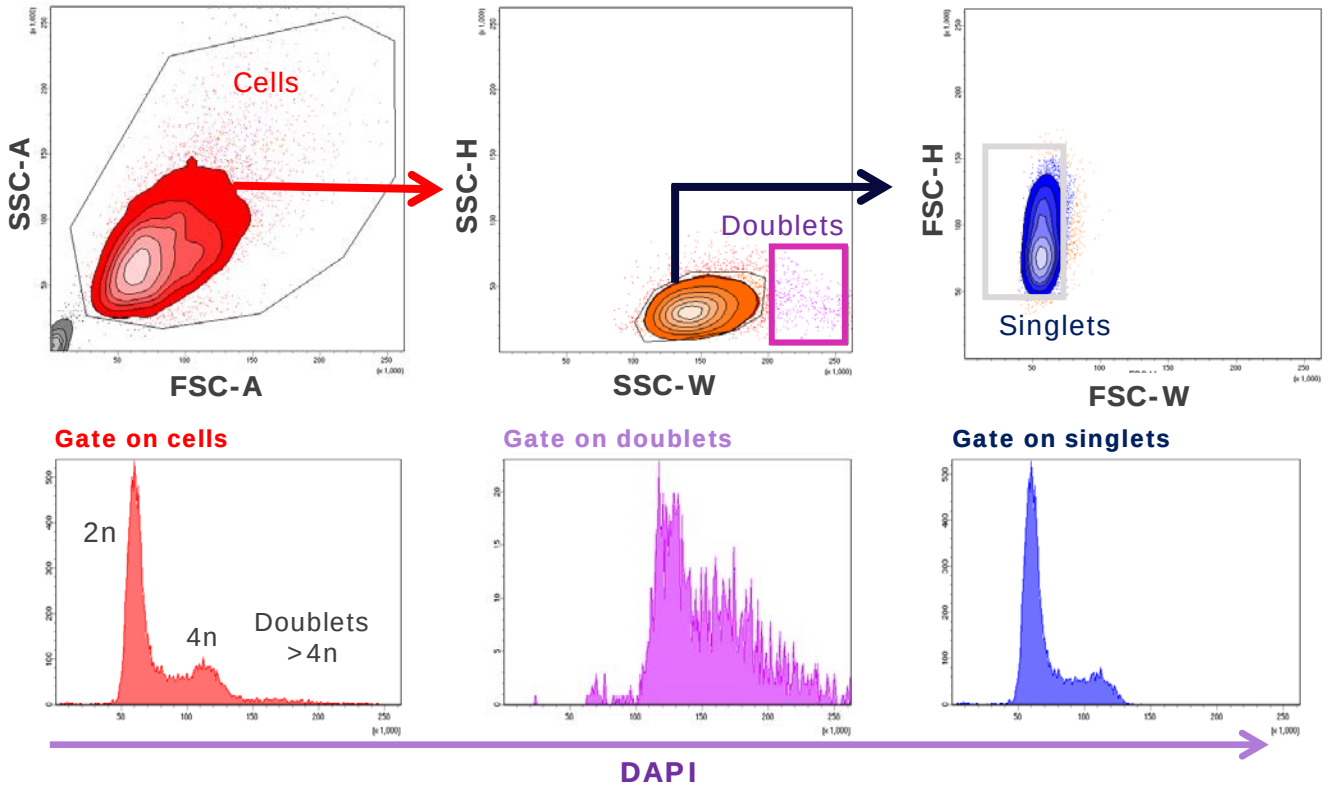


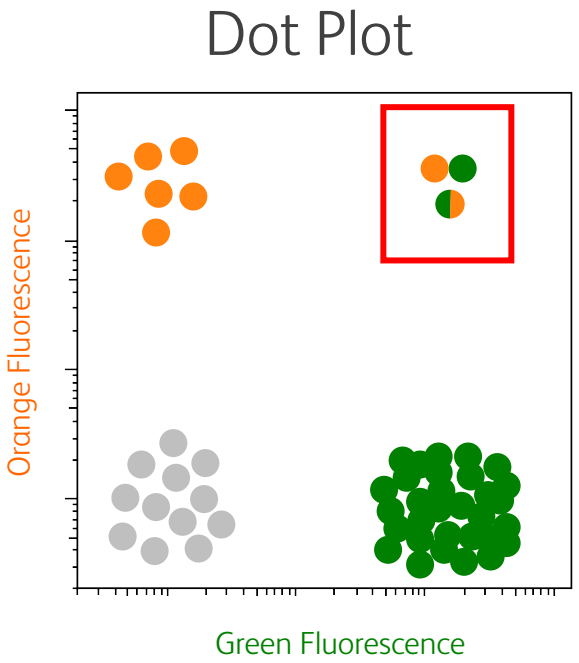






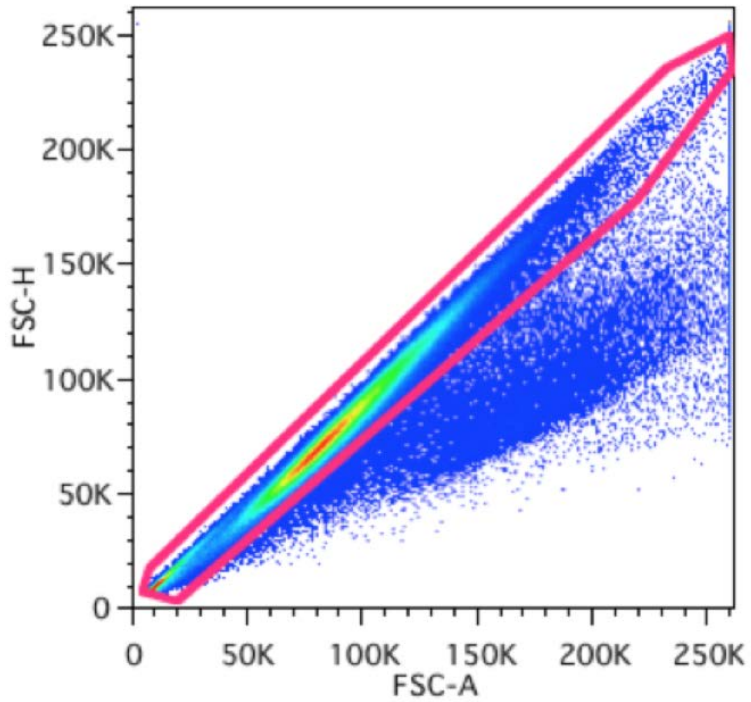
Doublet discrimination



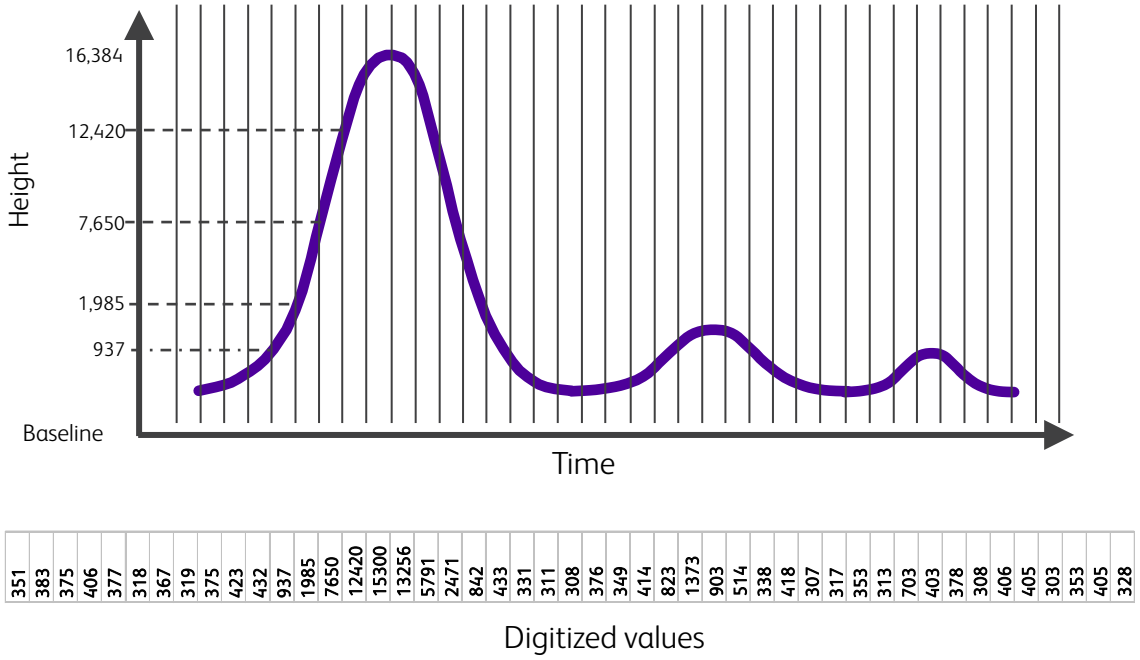


Doublet discrimination

- As shown in the graph below, cells along the diagonal are the single cells to be gated on. The cells off this diagonal should be excluded from the data. For this gate, use FSC-Height (FSC-H) by FSC-Area (FSC-A). SSC-H by SSC-A can also be used.

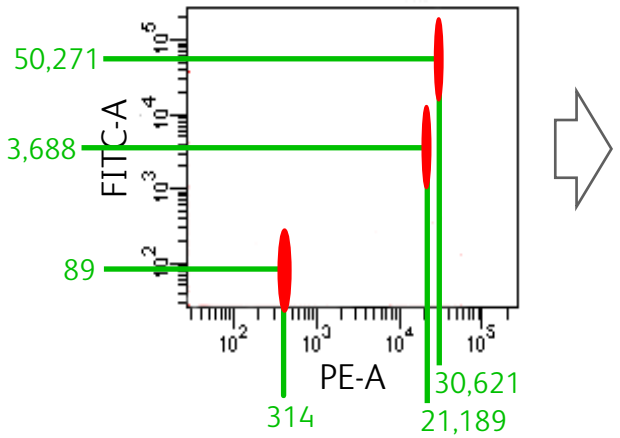


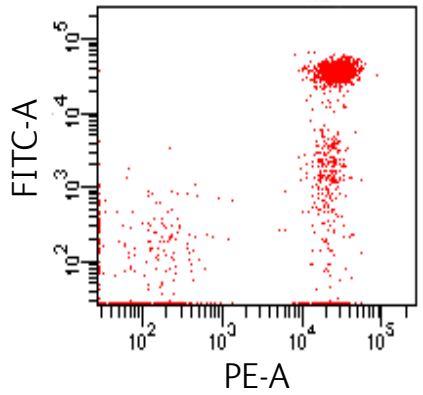
Data Generation



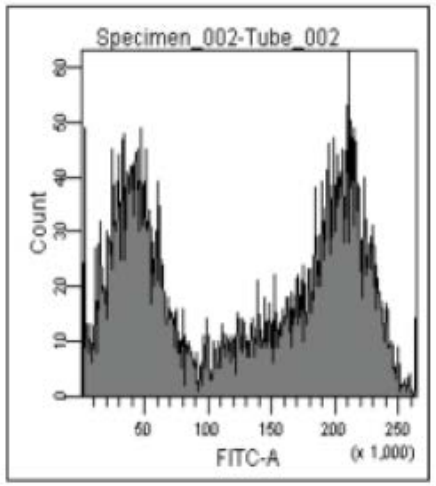
List-Mode Data

	Time	FSC	SSC	FITC	PE
Event 1	0	60	120	89	314
Event 2	10	160	65	50,271	30,621
Event 3	25	650	160	3,688	21,189

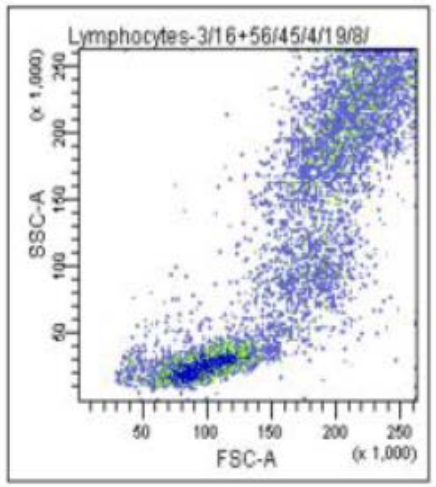




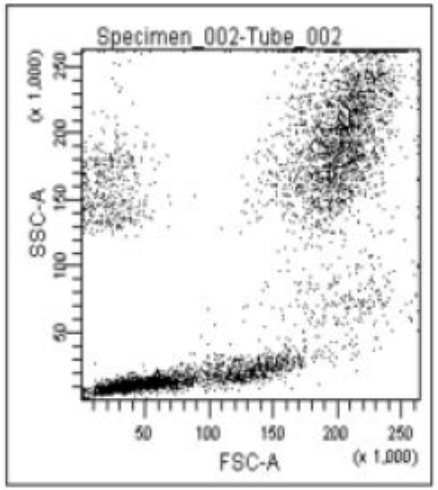
Plot types



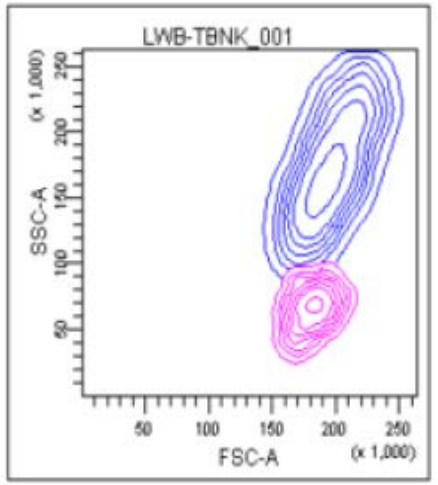
Histogram



Density Plot

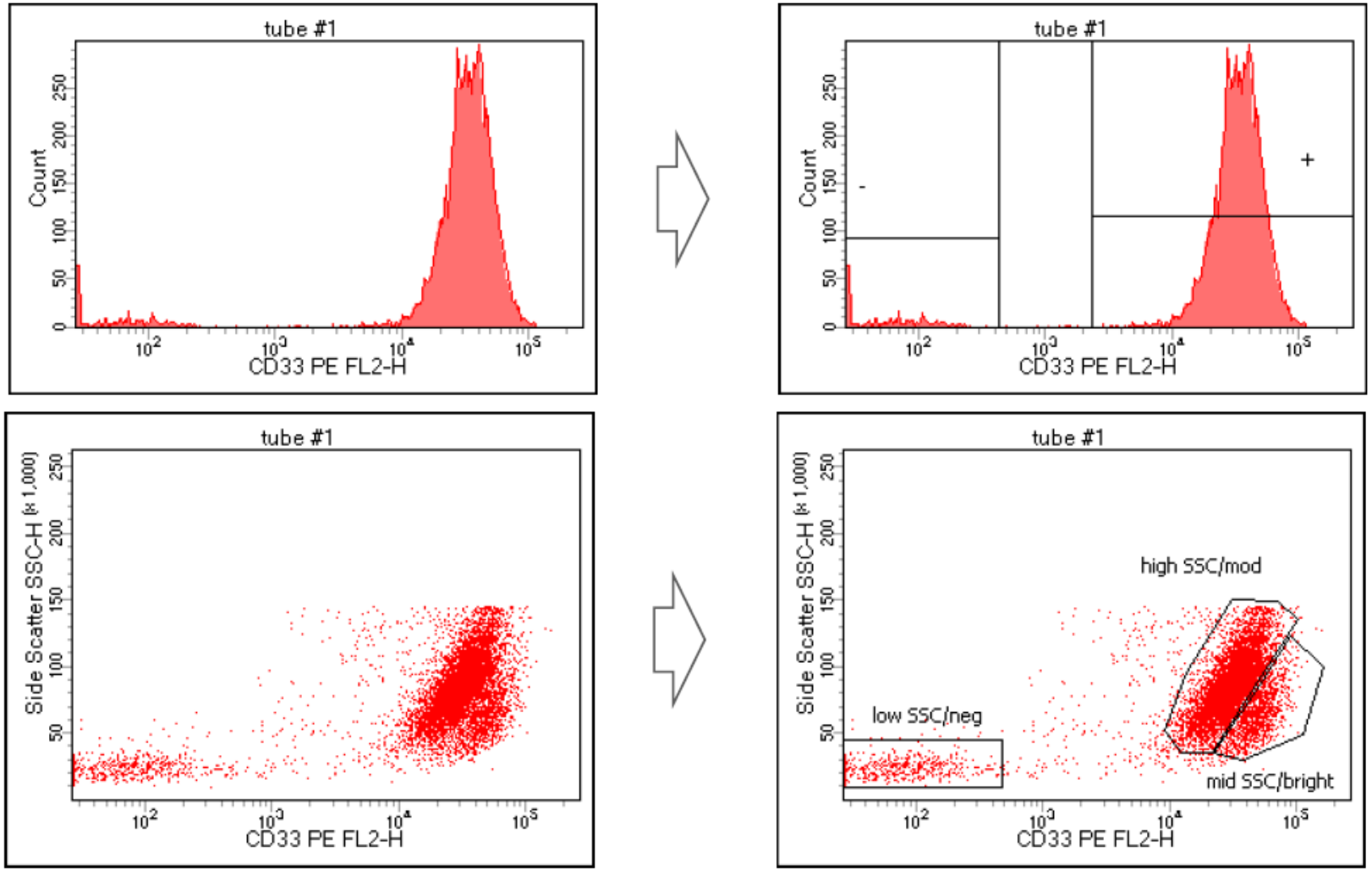


Dot Plot

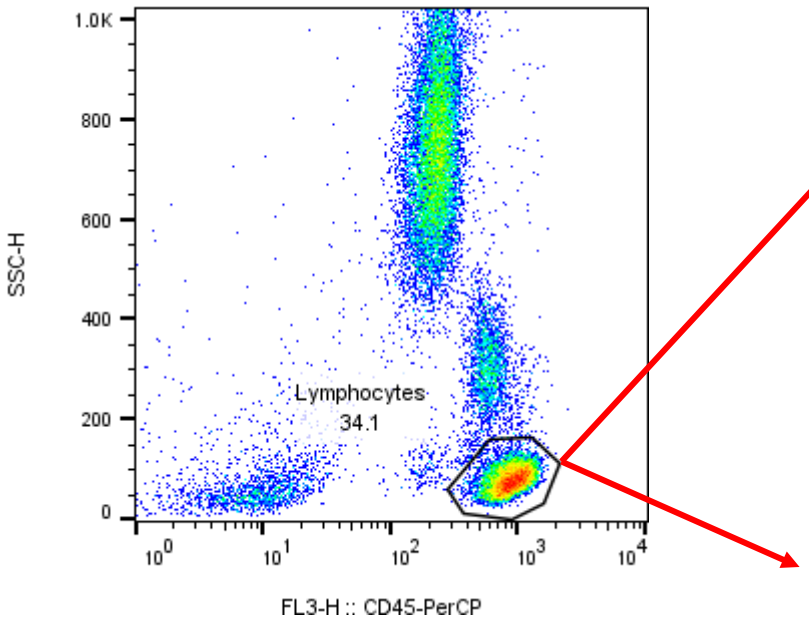


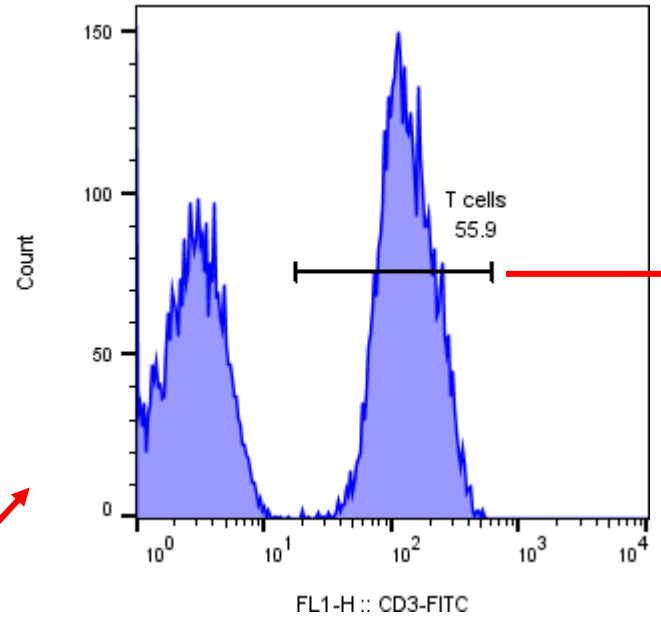
Contour Plot

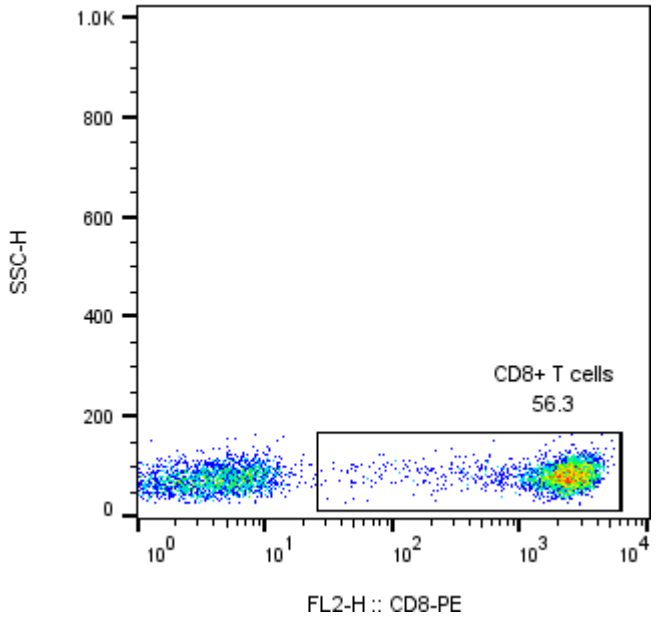
Dot Plot vs Histogram

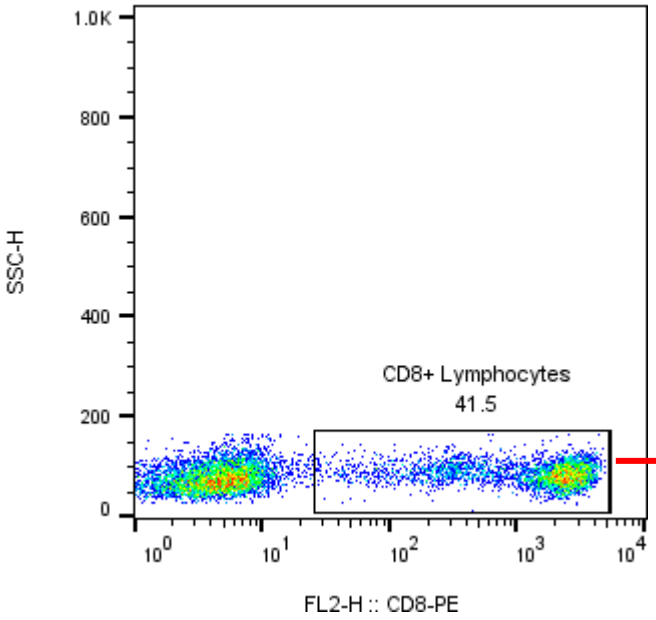


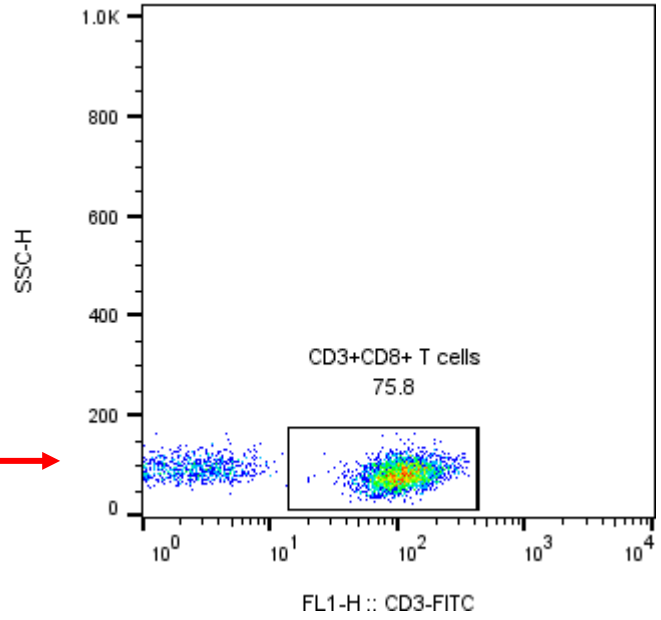
Gating


SSC-H
1.0K
800
600
400
200
0
Lymphocytes
34.1
10^0 10^1 10^2 10^3 10^4
FL3-H :: CD45-PerCP

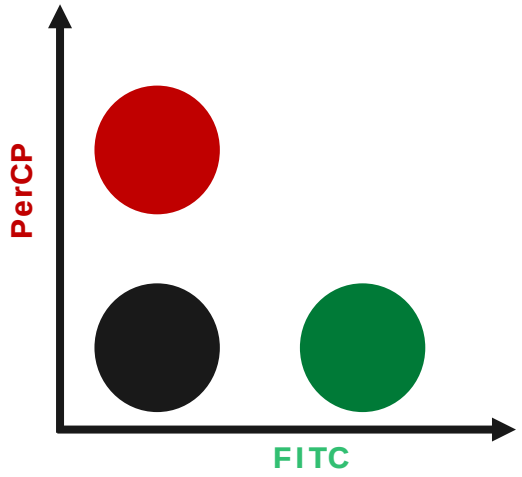

Count
150
100
50
0
T cells
55.9
10^0 10^1 10^2 10^3 10^4
FL1-H :: CD3-FITC

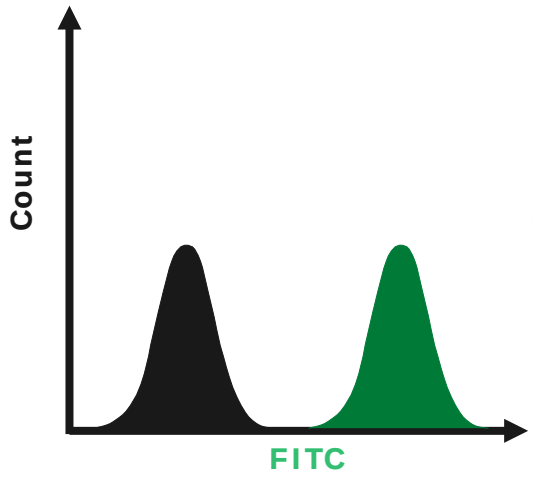

SSC-H
1.0K
800
600
400
200
0
CD8+ T cells
56.3
10^0 10^1 10^2 10^3 10^4
FL2-H :: CD8-PE

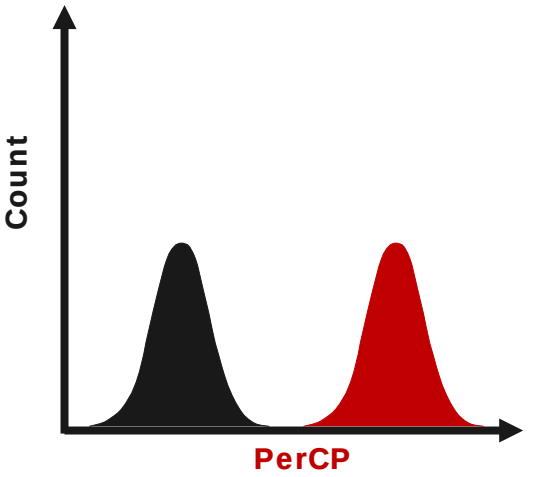

SSC-H
1.0K
800
600
400
200
0
CD8+ Lymphocytes
41.5
10^0 10^1 10^2 10^3 10^4
FL2-H :: CD8-PE


SSC-H
1.0K
800
600
400
200
0
CD3+CD8+ T cells
75.8
10^0 10^1 10^2 10^3 10^4
FL1-H :: CD3-FITC

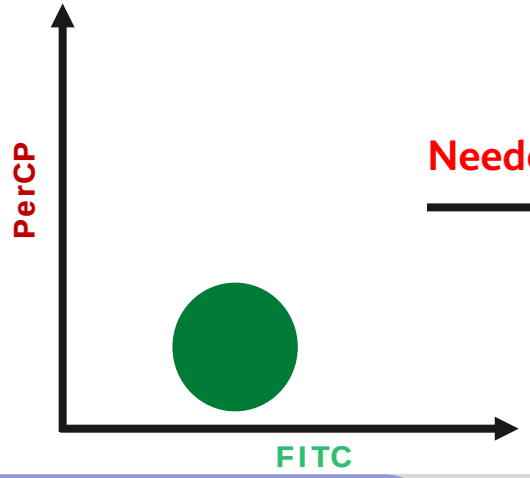
Interpretation in Flow Cytometry





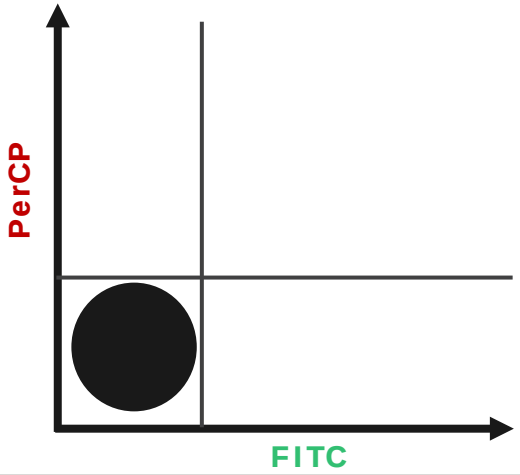


Cells + Ab-FITC

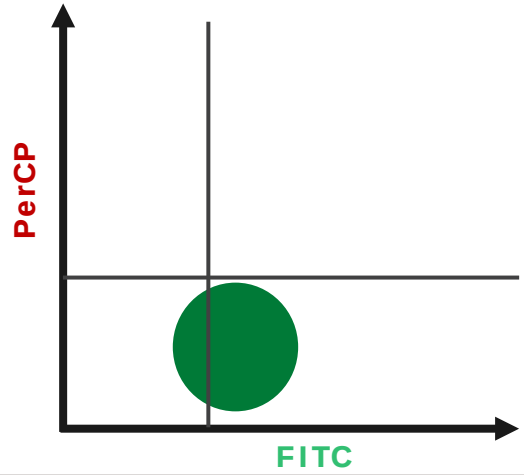


Needed control

Cells only or
Cells + Isotype-FITC



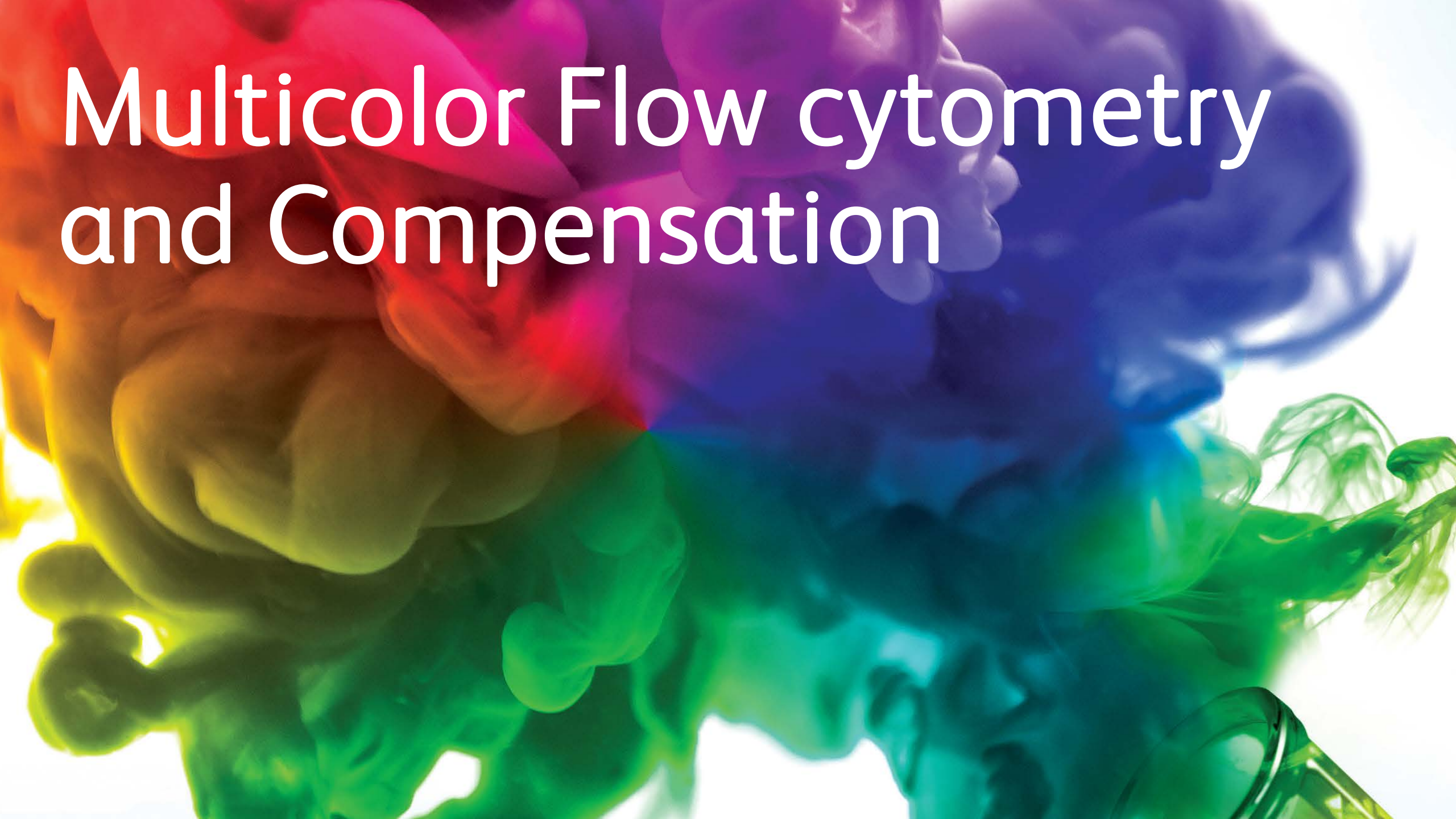
Cells + Ab-FITC



Q&A

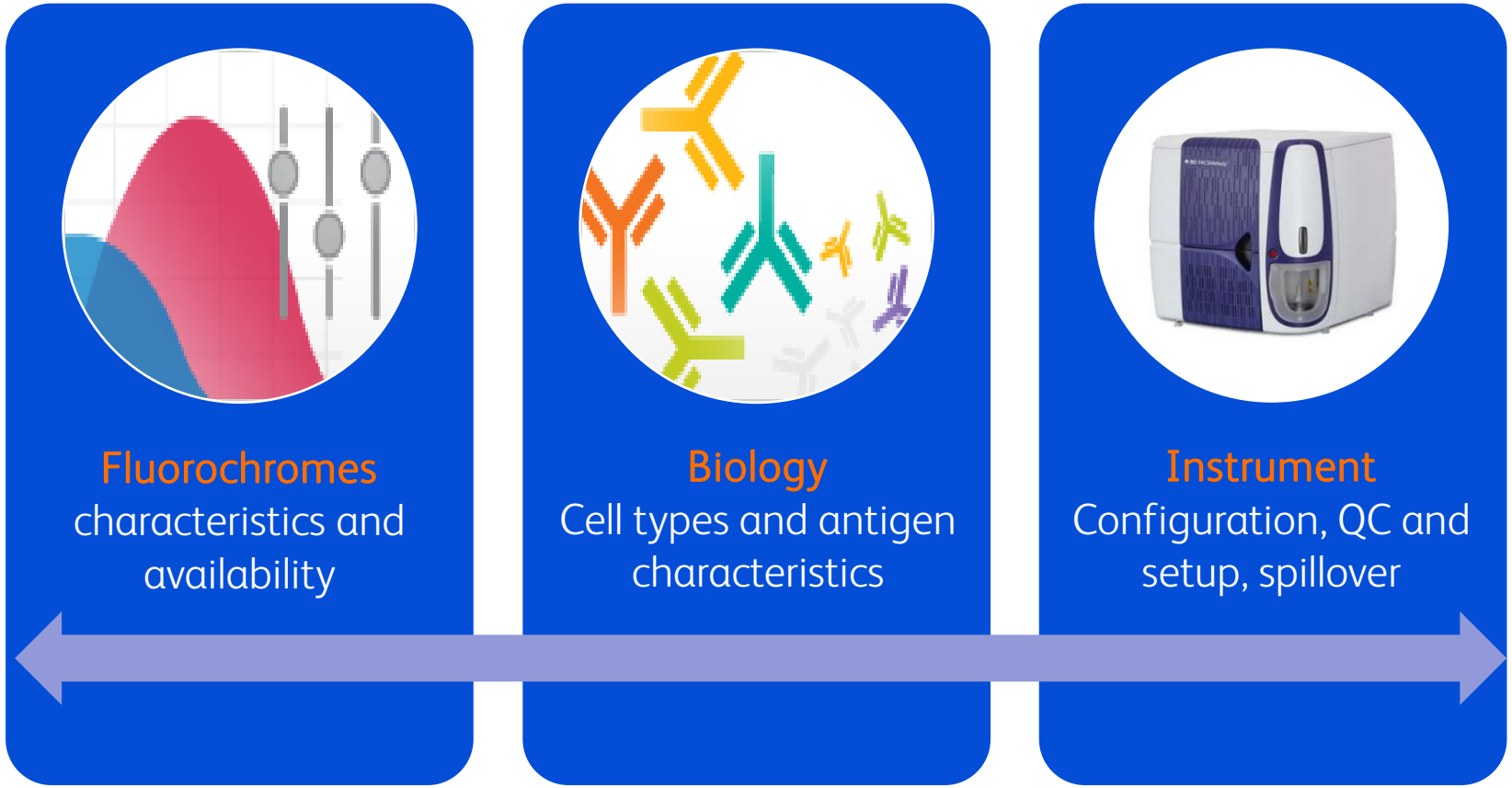
Break Time

Multicolor Flow cytometry and Compensation



Elements of Multicolor Flow Cytometry

- Considerations in designing panels



Fluorochromes

Emission

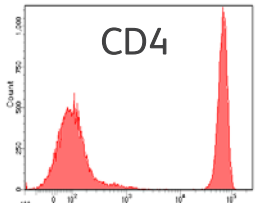
Laser								
Ultraviolet (355 nm)	BUV395		BUV496	BUV563		BUV661	BUV737	BUV805
Violet (405 nm)		BV421 V450	BV480 BV510 V500		BV605	BV650	BV711	BV786
Blue (488 nm)			BB515 FITC Alexa Fluor® 488	PE	PE-CF594	PE-Cy™5	PerCP PerCP-Cy5.5	PE-Cy™7
Yellow/Green (561 nm)				PE	PE-CF594	PE-Cy5	PE-Cy5.5	PE-Cy7
Red (640 nm)						APC Alexa Fluor® 647	APC-R700 Alexa Fluor® 700	APC-H7 APC-Cy7

Choice of fluorochromes depends on the available instrument configuration and the total number of markers being used in an experiment.

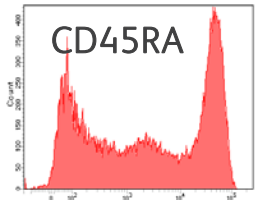
Antigen Characteristics

Leucocyte antigens can be categorized based upon their patterns of expression:

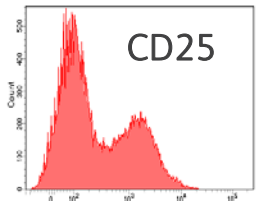
- Primary: Well characterized, easily classified as positive or negative, typically define broad subsets or lineages
  - Examples: CD3, CD4, CD19



- Secondary: Well characterized, typically expressed at a higher density, often over a continuum
  - Examples: CD27, CD28, CD45RA, CD45RO

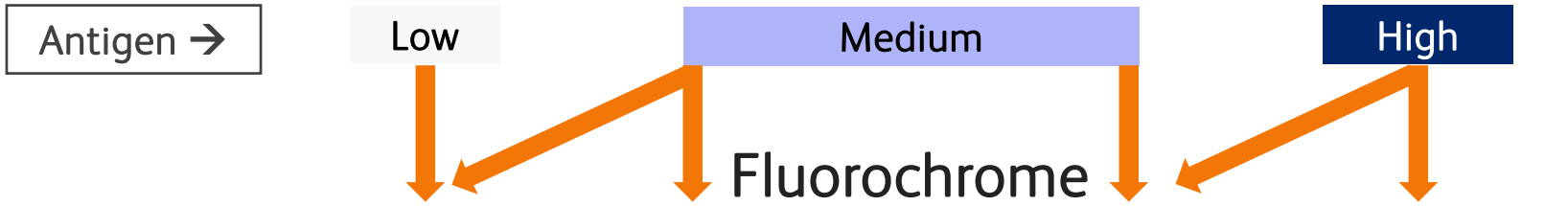


- Tertiary: Expressed at low levels, variable upon activation unknown, critical
  - Examples: CD25, STAT5, FoxP3



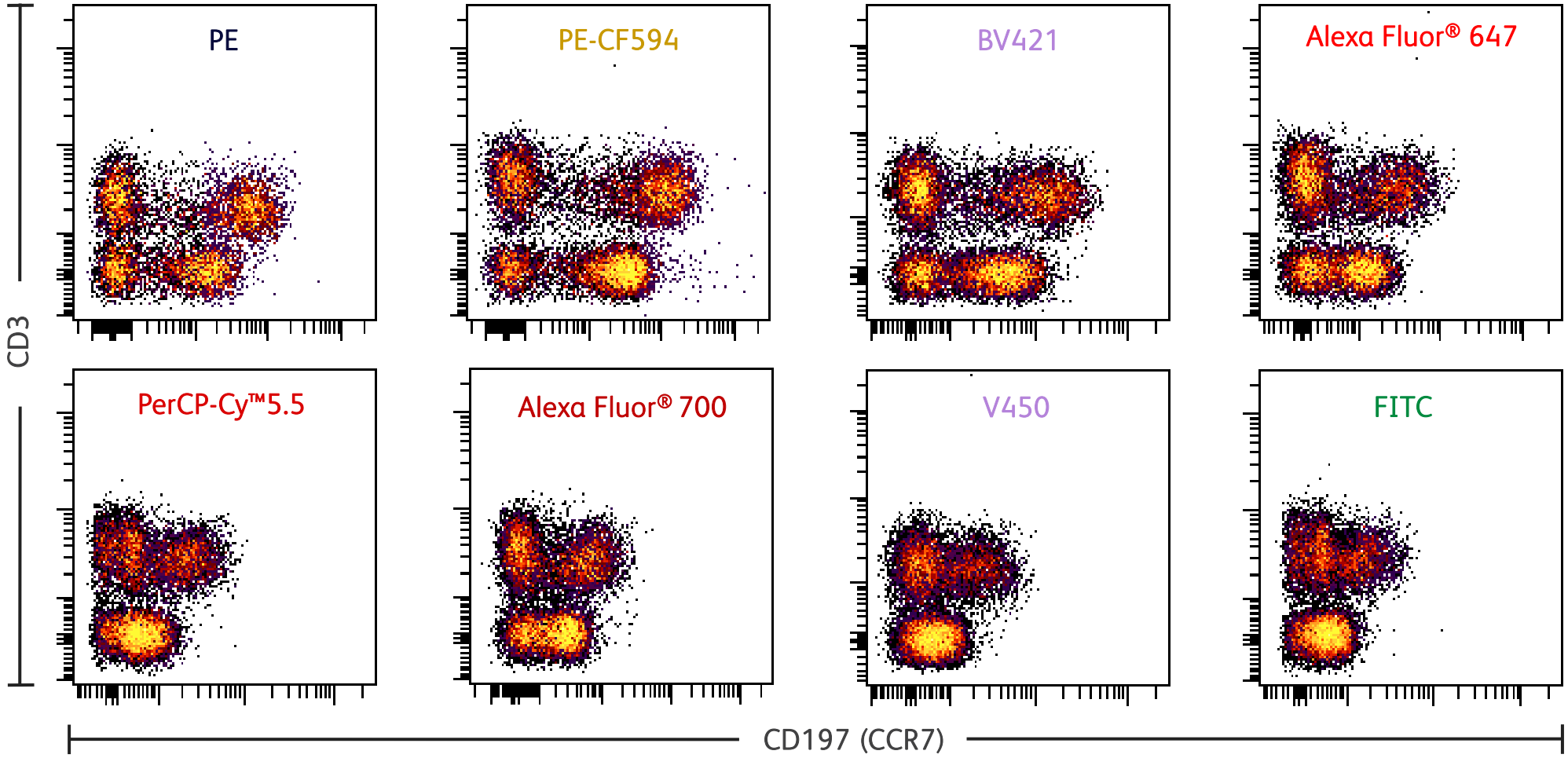
Mahnke YD, Roederer M. Optimizing a multicolor immunophenotyping assay. *Clin Lab Med*. 2007;27:469-485.

Antigen/Fluorochrome Combinations



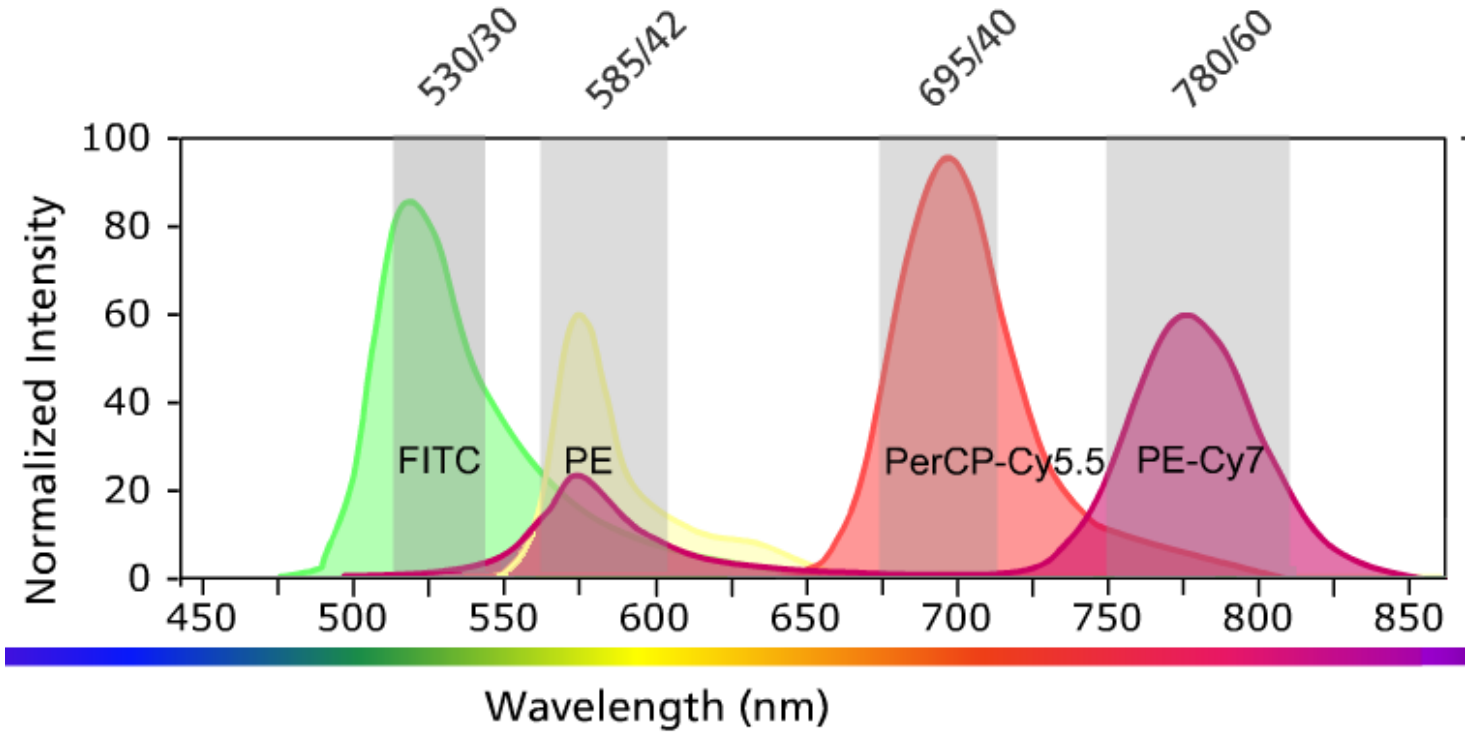
Laser	Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon BUV661 BD Horizon BUV737 BD Horizon BUV563	BD Horizon BUV395 BD Horizon BUV496	BD Horizon BUV805
Violet (405 nm)	BD Horizon BV421 BD Horizon BV650 BD Horizon BV711	BD Horizon BV480 BD Horizon BV605 BD Horizon BV786	BD Horizon BV510	BD Horizon V450 BD Horizon V500
Blue (488 nm)	BD Horizon BB515 BD Horizon PE-CF594 PE-Cy5	PE PE-Cy7	FITC Alexa Fluor® 488 PerCP-Cy5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

Resolution Effect of Fluorochrome



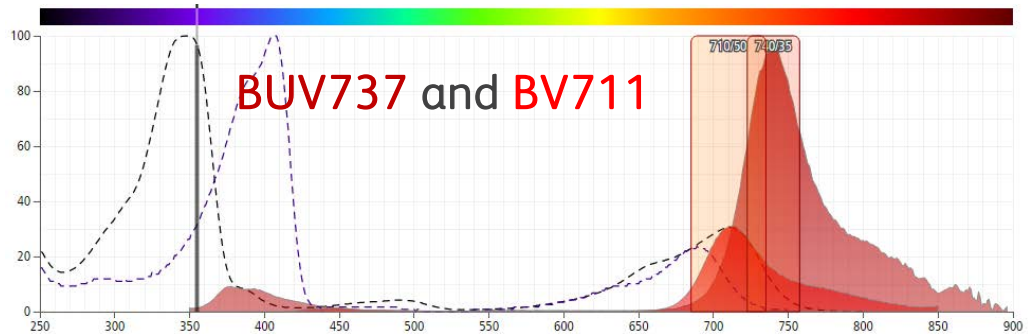
Spectral Overlapping

- Emission from multiple fluorochromes results in spectral overlap
- Detectors are selected to minimize fluorescence spillover
- BD Fluorescence Spectrum Viewer: bdbiosciences.com/spectra

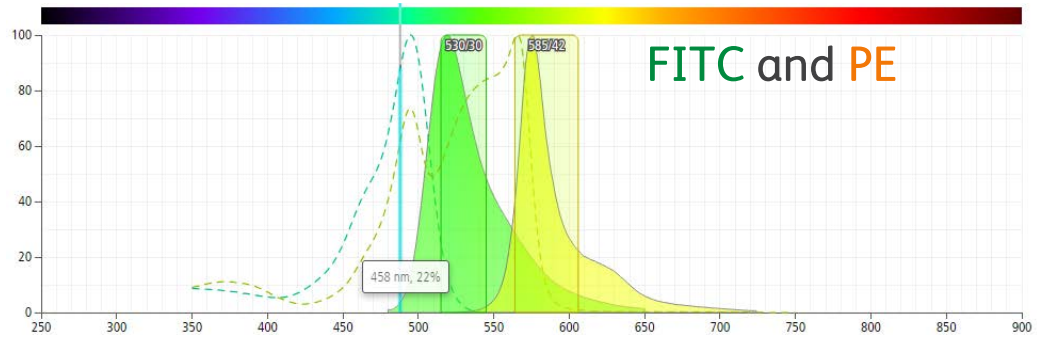


Spectral Overlapping

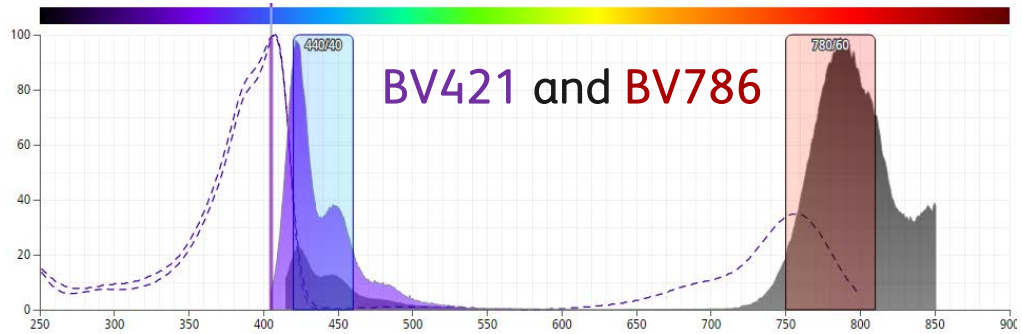
Similar emission spectra (cross-laser)



Adjacent detectors



Residual base fluorescence



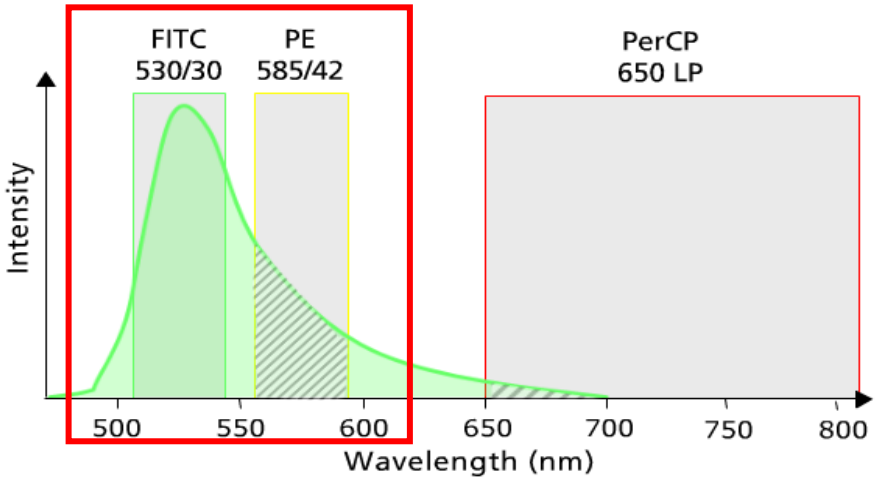
Spillover

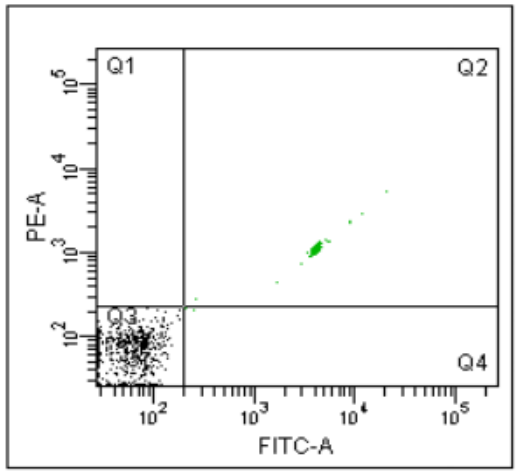
	BD Biosciences Fluorochromes									
	~380	~480	~530	~575	~610	~660	~685	~710	~740	~780
Ultraviolet (355 nm)	BUV395	BUV496				BUV661			BUV737	BUV805
Violet (405 nm)		BV421 V450	BV510 V500		BV605	BV650		BV711		BV786
Blue (488 nm)			FITC BB515	PE	PE-CF594	PE-Cy5	PerCP PerCP-Cy5.5			PE-Cy7
Yellow/Green (561 nm)				PE	PE-CF594	PE-Cy5	PE-Cy5.5			PE-Cy7
Red (640 nm)						APC		APC-R700		APC-H7 APC-Cy7

- Fluorochromes with similar emission spectra will have the greatest potential for cross-laser spillovers
- Spillover into adjacent detectors
- Residual spillover between tandems and their base

Compensation

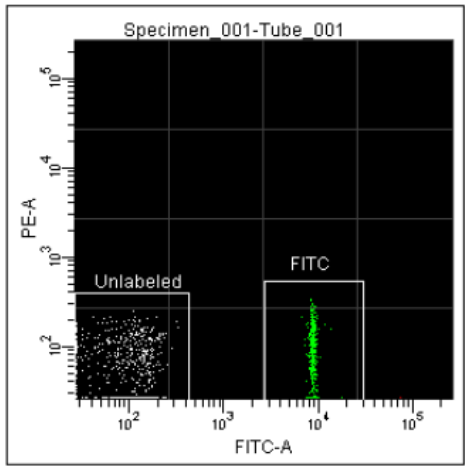
- Remove spillover signals so that Median fluorescence intensity (MFI) of populations concerned agree





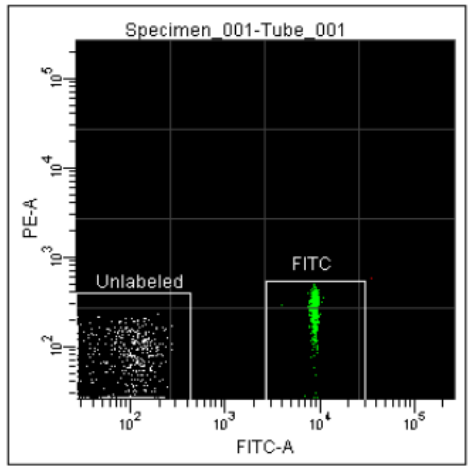
Incorrect Compensation

Correct Compensation



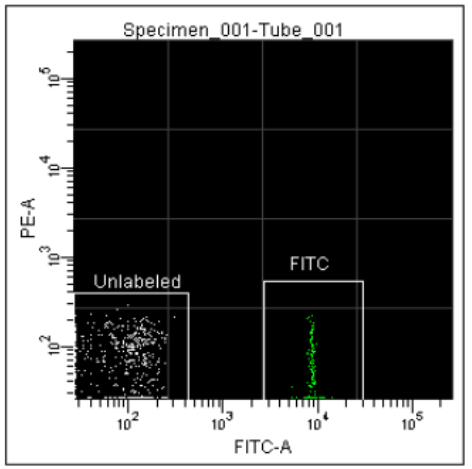
Population	PE-A Median
Unlabeled	77
FITC	73

Undercompensation



Population	PE-A Median
Unlabeled	72
FITC	245

Overcompensation



Population	PE-A Median
Unlabeled	70
FITC	-54

Thank You

BD

Q&A