FLOW CYTOMETRY : PRINCIPLES

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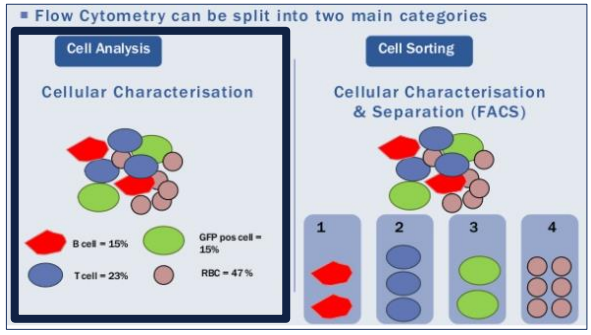
03-12-2019

FLOW CYTOMETRY

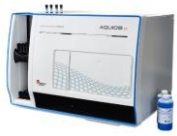
Cytometry : the counting of cells

Flow cytometry : cytometry performed by suspending cells in a liquid and passing them through a light beam, often after applying fluorescent stains



CLINICAL FLOW CYTOMETERS

Navios EX Flow Cytometer



AQUIOS CL Flow Cytometry System

Navios Flow Cytometer

- 3 lasers (blue, red and violet)
- fourth yellow laser (561 nm) is available as an upgrade.

Omnicyt

BD FACSCanto™

- up to 3 laser (blue, red and violet)
- 8-, 10- and 12-color configurations

BD FACSLyric™ Clinical System

WHAT IS INSIDE A FLOW CYTOMETER?

Flow cytometers have 3 key systems

WHAT IS INSIDE A FLOW CYTOMETER?

Flow cytometers have 3 key systems

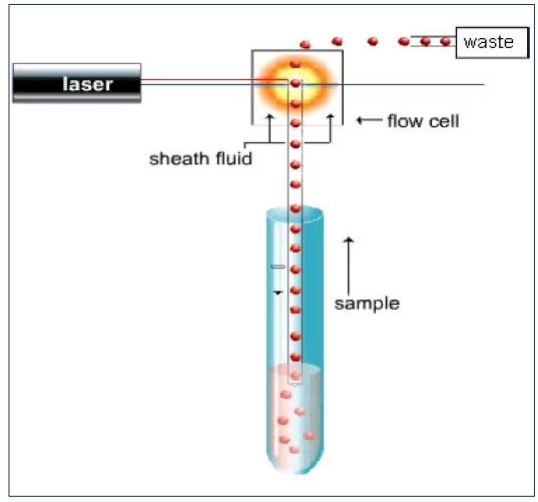
1) FLUIDICS

2) OPTICS

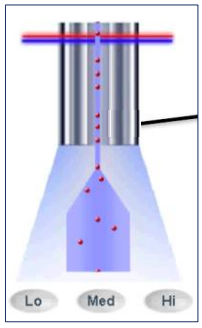
3) ELECTRONICS

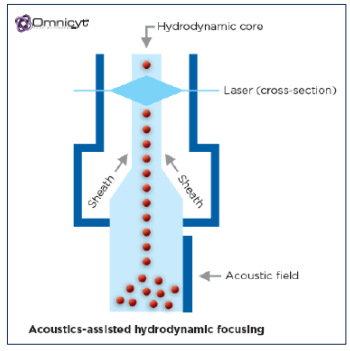
FLUIDIC SYSTEM

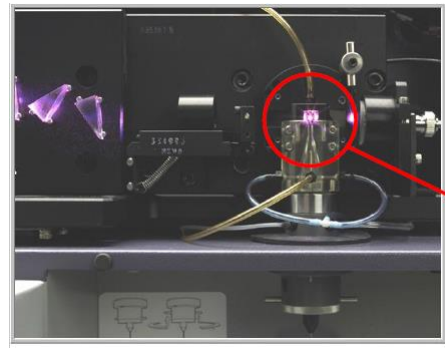
- Removal of air bubbles (filter)
- Alignment of particles before passing through the flow cell **"SINGLE CELL ANALYSIS"**
  - Hydrodynamic focusing
  - Acoustic-assisted hydrodynamic focusing
- Waste



FLUIDIC SYSTEM







WHAT IS INSIDE A FLOW CYTOMETER?

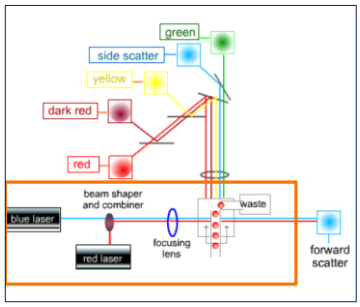
Flow cytometers have 3 key systems

1) FLUIDICS

2) OPTICS

OPTICS

- **Excitation** of light
  - Laser
  - Lenses



OPTICS

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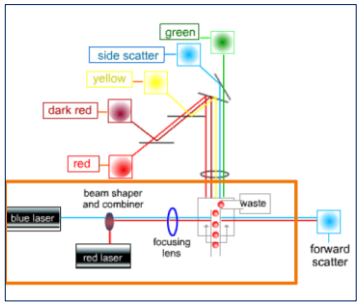
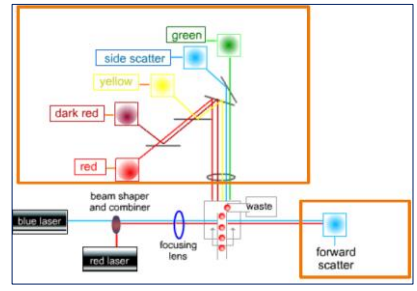
  - Lenses



- **Collection** of the emitted light
  - Filters
  - Scatter detectors
  - Fluorescence detectors



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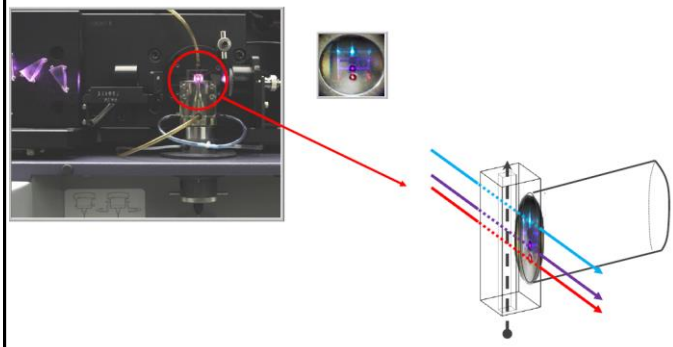

Overview of lasers and emission filters in Canto, Lyric and Navios.

Dynamic range (Number of channels)	BD FACS Canto II	BD FACS Lyric	Navios
	18-bit (262144)	18-bit (262144)	20-bit (1048576)
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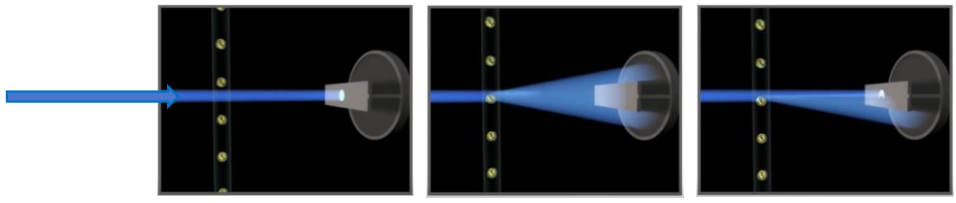
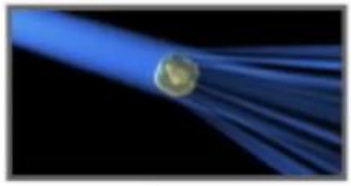
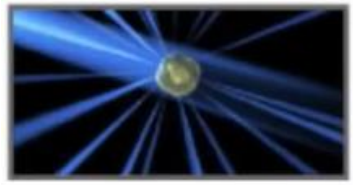
OPTICS

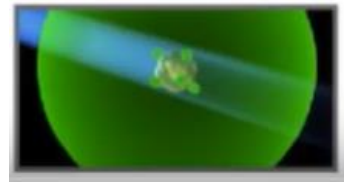




Forward scatter

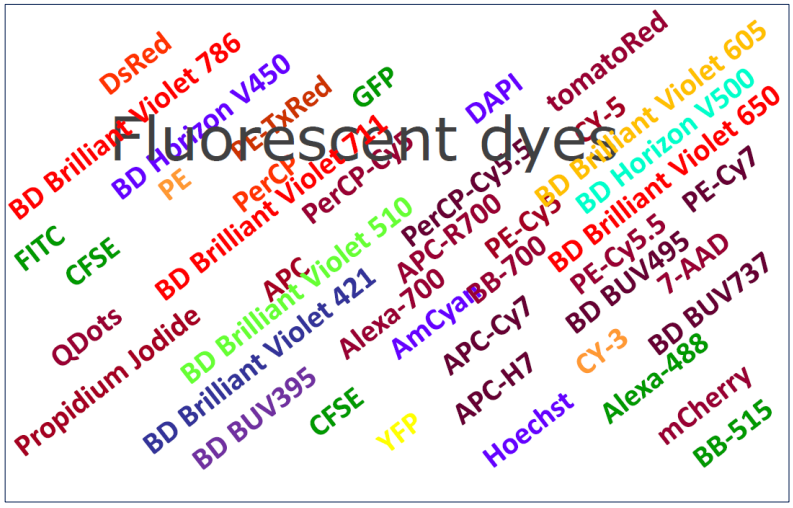


Sideward scatter



Fluorescence

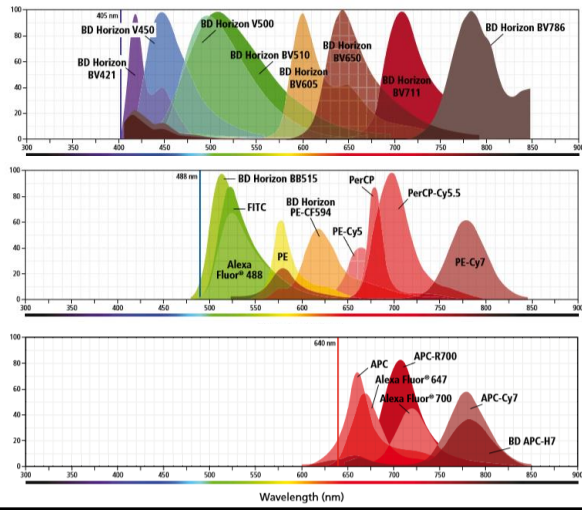
OPTICS



- Absorption of a photon at a specific wavelength
- Emission of photon at a longer wavelength

OPTICS

BD Facs Lyric



Violet (405 nm)

Bleu (488 nm)

Red (640 nm)

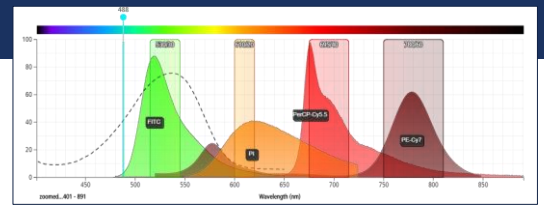
OPTICS

PI

7-AAD

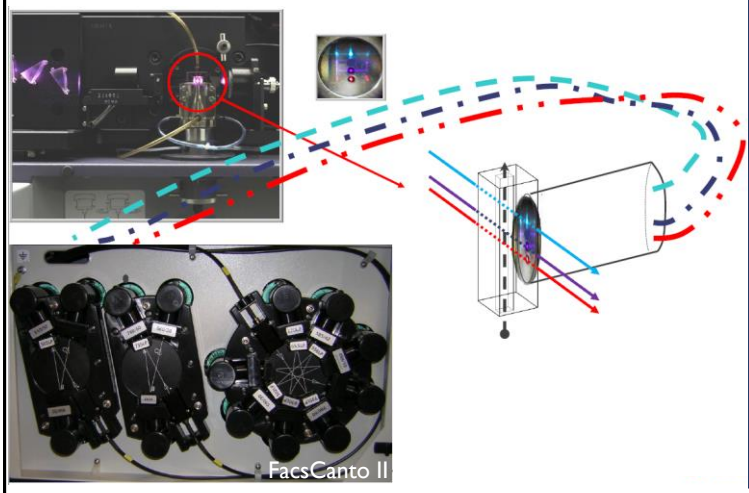
Fluorescent dyes

DAPI



- intercalate into intra-cellular nucleic acid structures (DNA or RNA)
- applications: viability, cell cycle analysis, proliferation assays

OPTICS



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Pacific Orange (OC515, V500)	510/50	528/45	550/40
FITC	530/30	527/32	525/40
PE	585/42	586/42	570/30
	–	–	620/30
PerCP-Cy5.5	670LP	700/54	695/30
PE-Cy7	780/60	783/56	755 LP
APC	660/20	660/10	660/20
	–	720/30	725/20
APC-C750 (APC-H7)	780/60	783/56	755 LP

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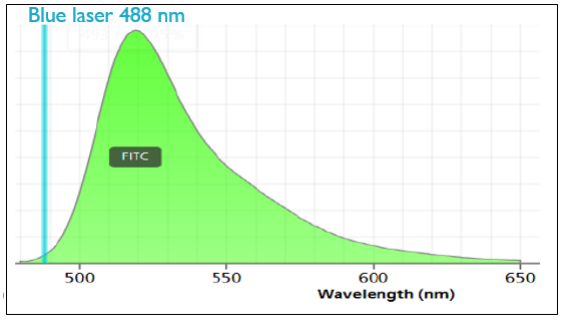

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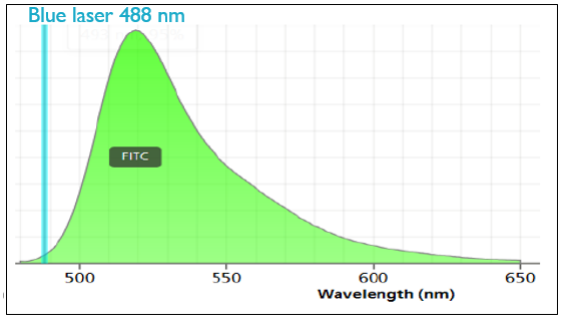
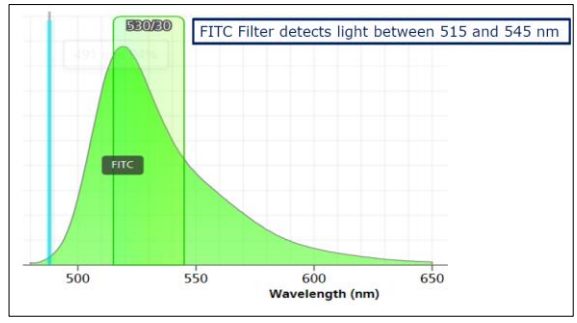
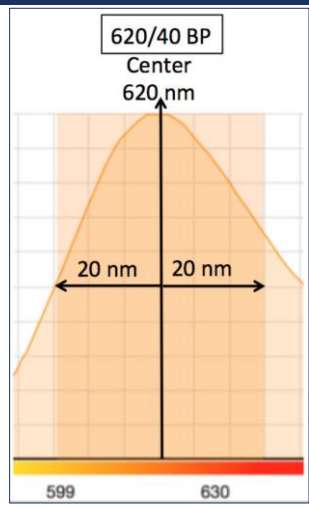




OPTICS

manufacturers label filters with the center of the range and the size of the window of light that can pass through the filter

example: a 620/40 band pass filter will allow light between 600 to 640 nm through



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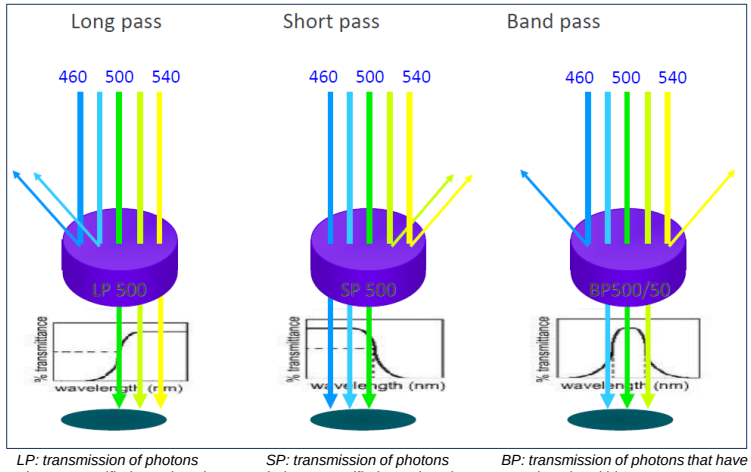
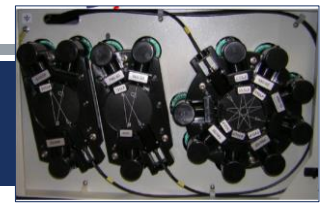


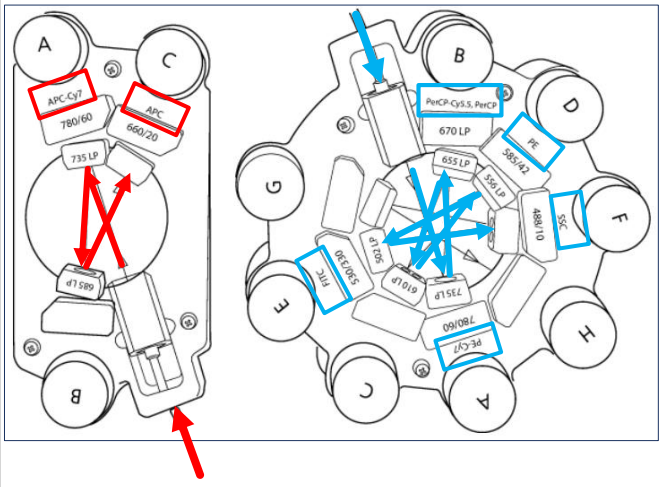
LP: transmission of photons above a specified wavelength

SP: transmission of photons below a specified wavelength

BP: transmission of photons that have wavelengths within a narrow range

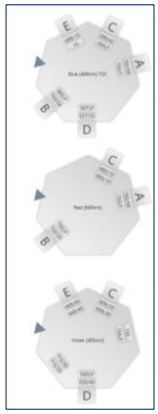
OPTICS

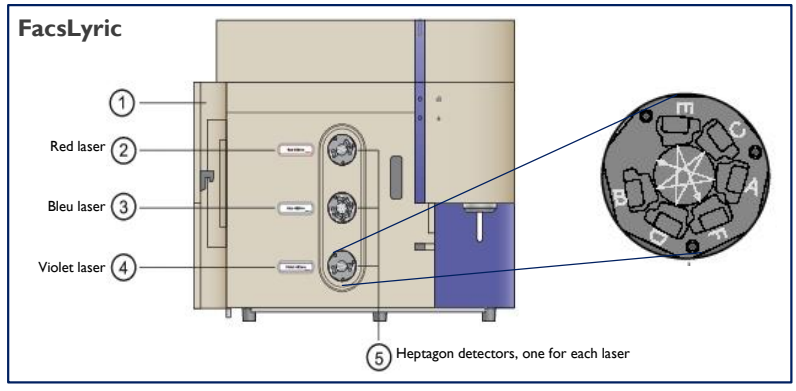




- BD FacsCanto detectors
  - an **octagon**: contains five PMTs and detects light from the 488-nm blue laser. A PMT in the octagon collects side scatter signals.
  - a **trigon**: contains two PMTs and detect light from the 633-nm (red) and the 405-nm (violet) lasers.
- use serial light reflections to guide signals to their target detectors
- collecting dimmest emission signals first, moving from longest wavelengths (typically PE-Cy™7) to shortest (FITC)

OPTICS





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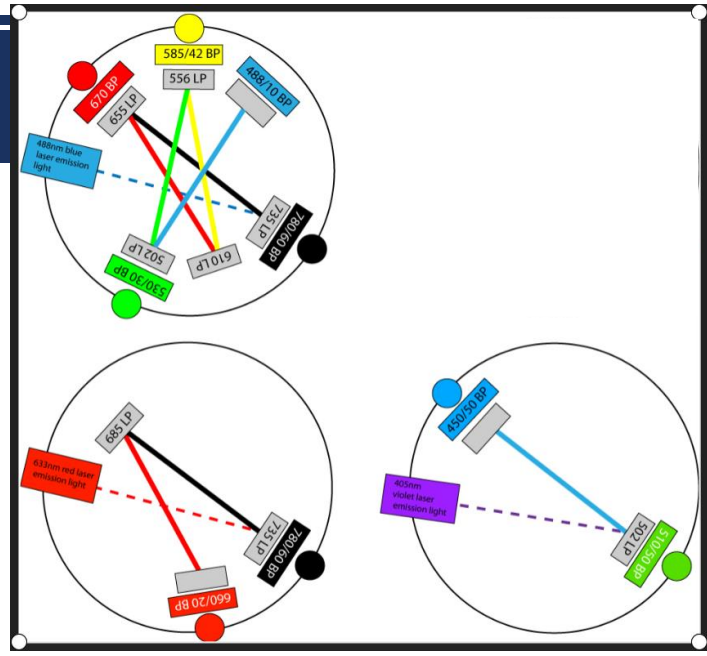
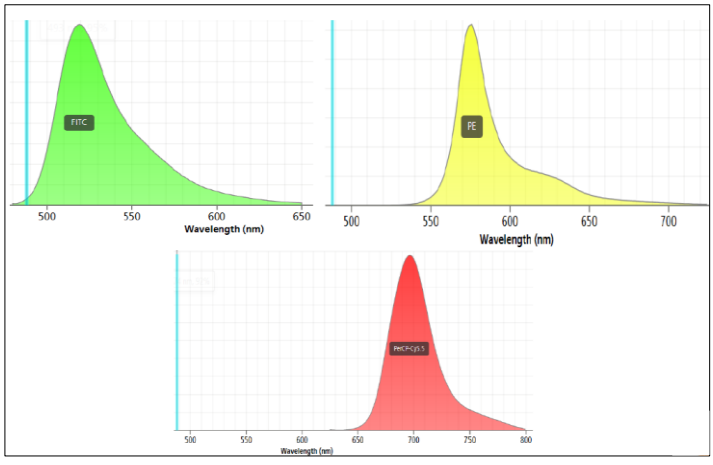
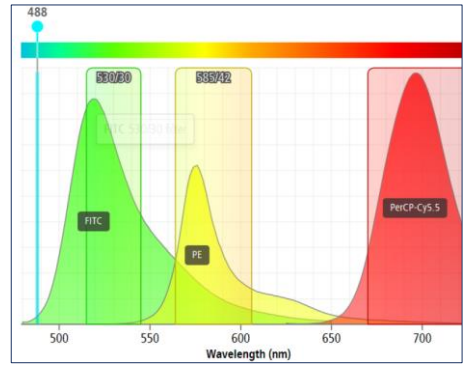


OPTICS

- different fluorochromes have a different emission spectrum => multiple filters to detect all fluorescent signals





WHAT IS INSIDE A FLOW CYTOMETER?

Flow cytometers have 3 key systems

1) FLUIDICS

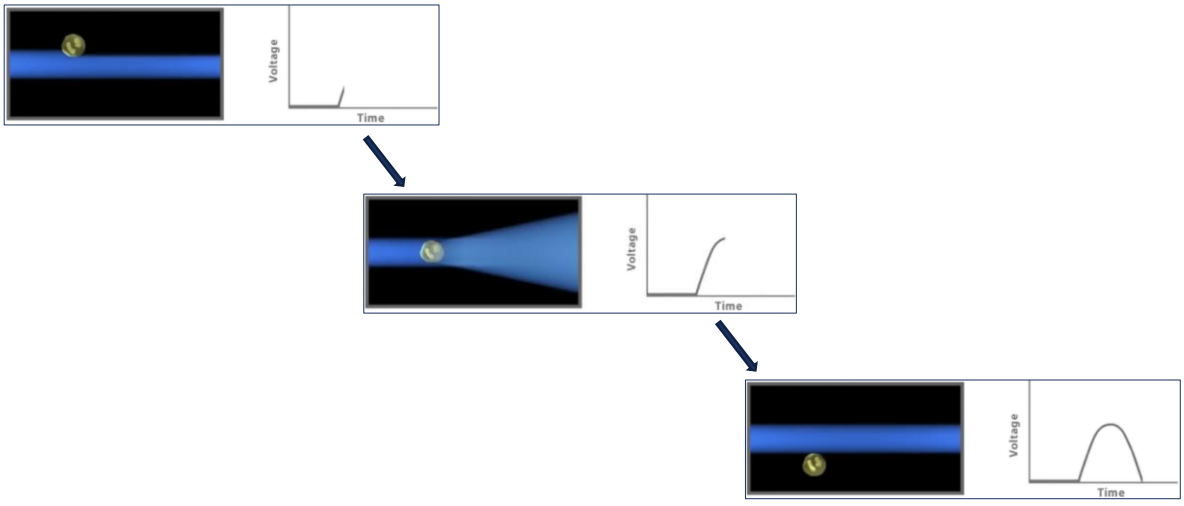
2) OPTICS

3) ELECTRONICS

ELECTRONICS

- photomultiplier tubes (**PMT**s) are **optical detectors** which detect fluorescence
- each sensed particle will generate a **signal** on the optical detectors
- PMT converts light signal **into electronic pulse**

ELECTRONICS



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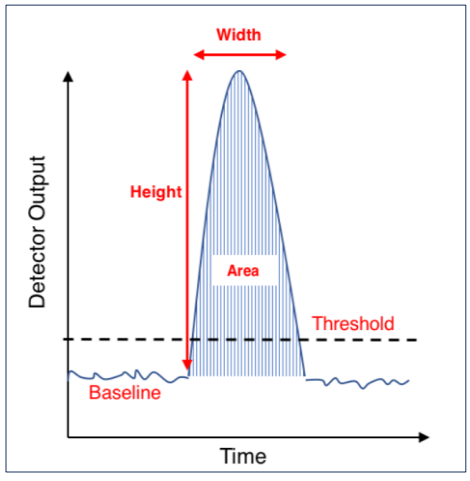


Cell passes through laser beam

⇒ signal from detectors

⇒ pulse

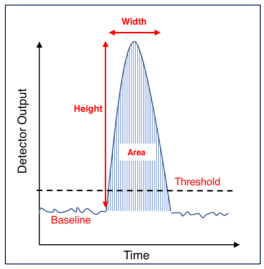
Pulse parameters

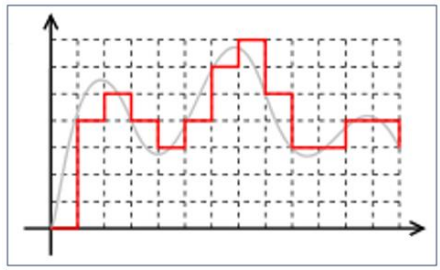
- Area (A)
- Height (H)
- Width (W)

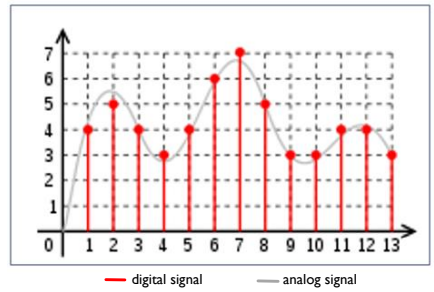
e.g. "SSC-A," "SSC-H," or "SSC-W"

ELECTRONICS

- signal will **be digitized** by an analog-to-digital converter (ADC)

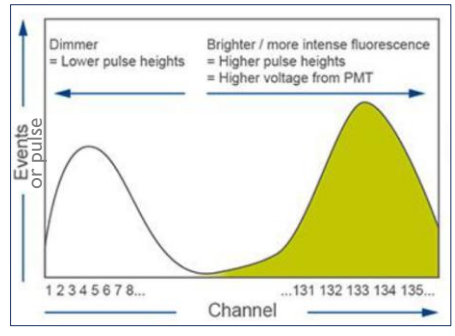






ELECTRONICS

- assignment in channels based on pulse intensity/area
  - eg. 8 bit ADC can divide the range of signals into 256 (2^8) discrete values (depending on its measured intensity); the more intense the fluorescence, the higher the channel number the event is assigned
    - channel 1 can contain up to the dimmest events
    - channel 256 can contain up to the brightest events



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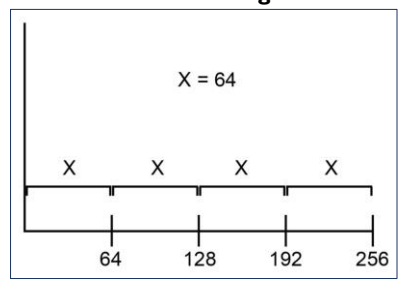
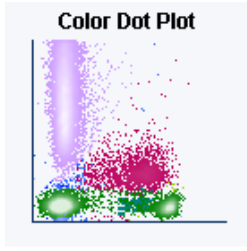
Linear scaling



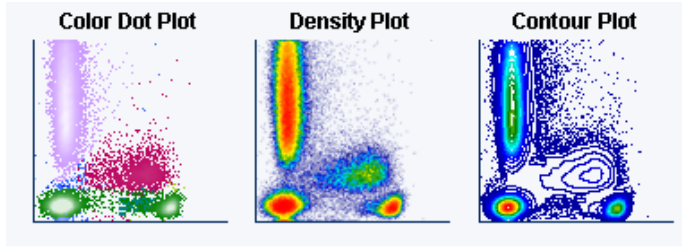
VISUALIZATION OF DATA

- Dot plots 2D



VISUALIZATION OF DATA

- Dot plots 2D
- Density plots
- Contour plots



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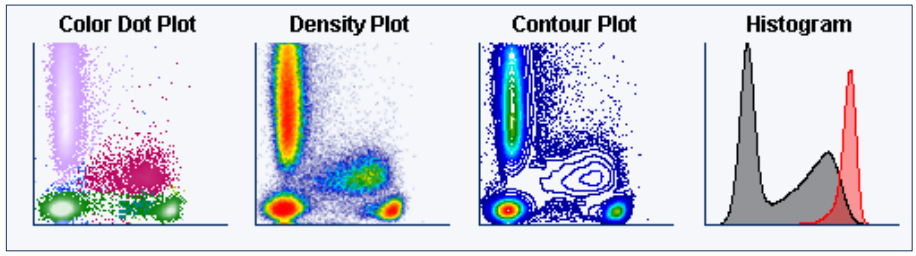
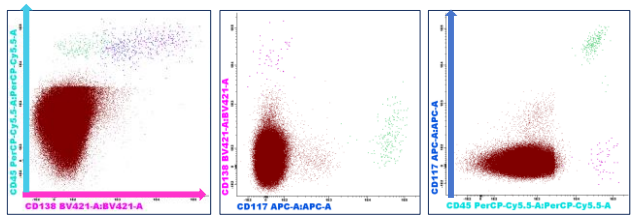
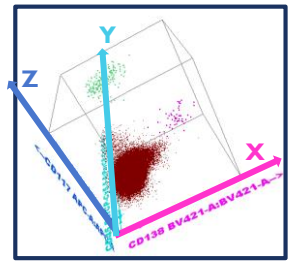


- Histograms
  - most useful when **only 1 parameter** (e.g. intensity from a single fluorescent channel) is important
  - multiple **overlaid** histograms can be used to compare a single parameter from 2 different populations (e.g. experimental vs. control)

VISUALIZATION OF DATA

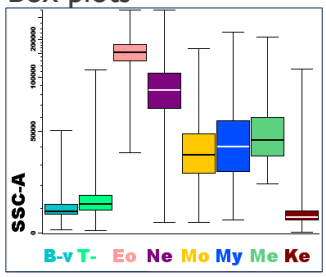
- 3D plots





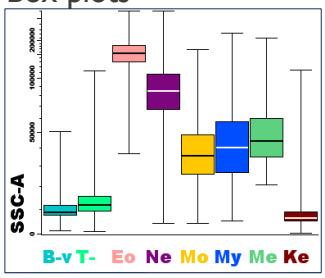
VISUALIZATION OF DATA

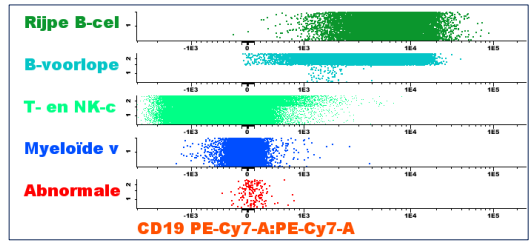
- Box plots



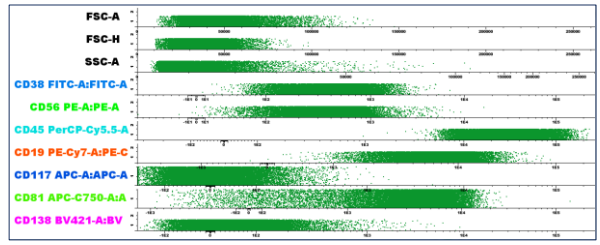
VISUALIZATION OF DATA

- Box plots



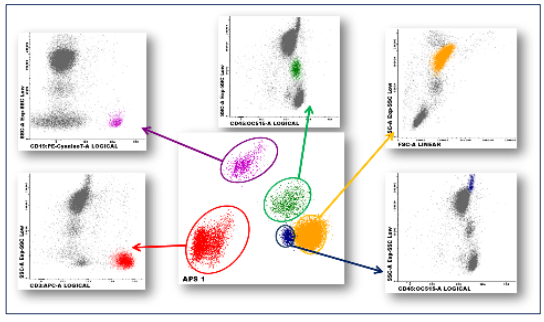


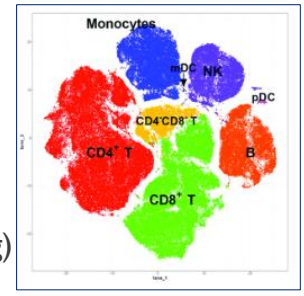
- Band plots (population, parameter)



VISUALIZATION OF DATA

- APS view (automatic population separarator)

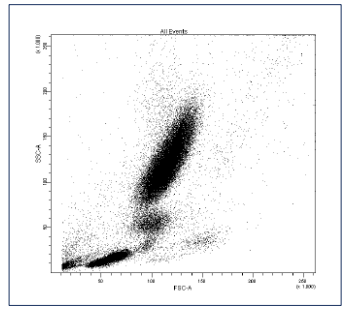




- t-SNE plots (T-distributed Stochastic Neighbor Embedding)

INSTRUMENT SETTINGS

- Adjustment of scatter voltages
  - adjust FSC and SSC voltages to put events on desired spot in scatter dot plot
  - PB sample



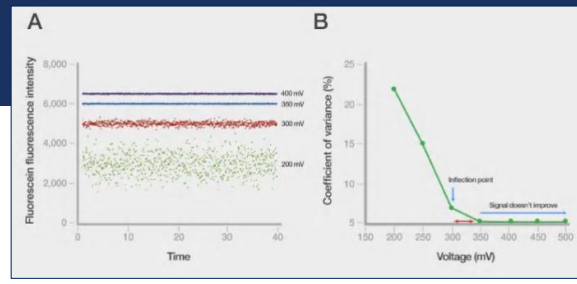
Parameters | Co

Parameter

Parameter	Voltage
FSC	267
SSC	387

INSTRUMENT SETTINGS

- Adjustment of scatter voltages
  - adjust FSC and SSC voltages to put events on desired spot in scatter dot plot
  - PB sample

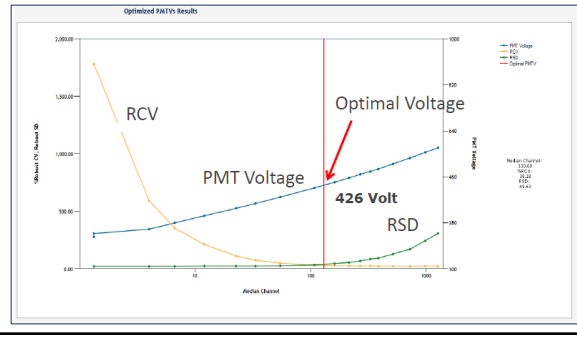


Parameters | Co

Parameter

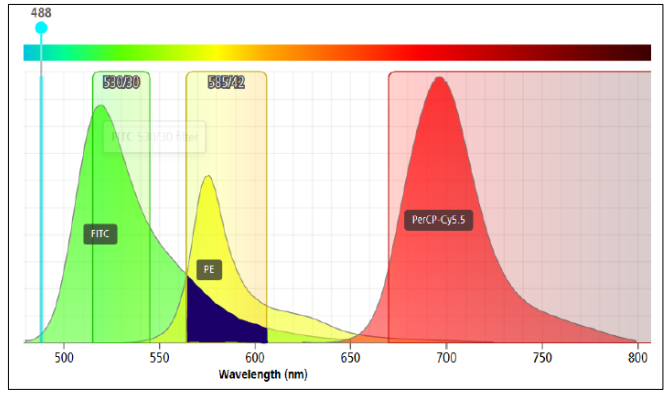
Parameter	Voltage
FSC	267
SSC	387
FITC	446
PE	369
PerCP-Cy5-5	522
PE-Cy7	561
APC	540
APC-H7	579
V450	416
V500	442

- Optimal voltages for each PMT
  - use voltage with lowest CV and maximum signal/background ratio
  - CS&T beads



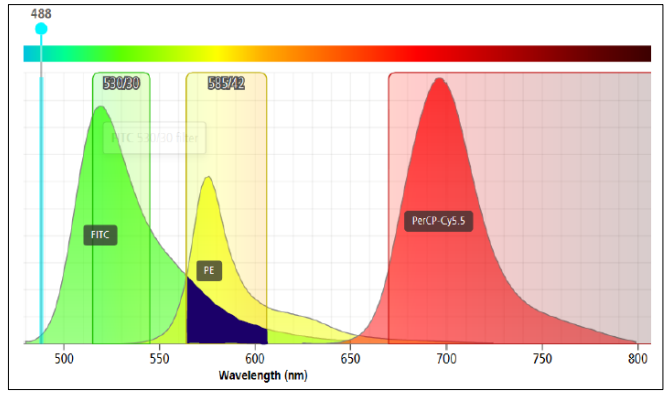
INSTRUMENT SETTINGS

- Adjustment of scatter voltages
  - adjust FSC and SSC voltages to put events on desired spot in scatter dot plot
  - PB sample
- Optimal voltages for each PMT
  - use voltage with lowest CV and maximum signal/background ratio
  - CS&T beads
- Calculation of compensation
  - dependant of used fluorochromes and voltages
  - compensation beads / fresh cells



COMPENSATION

- due to spectral overlap: false positive signals if **NOT** compensated
- compensation = mathematical correction of a signal overlap between channels of the emission spectra of different fluorochromes



INSTRUMENT SETTINGS

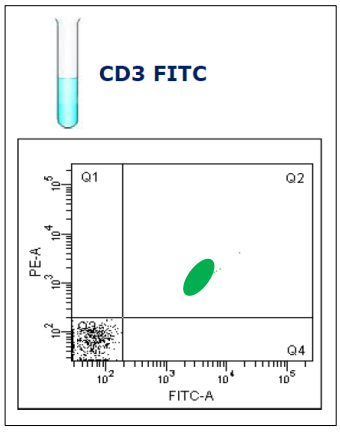
- Adjustment of scatter voltages
  - adjust FSC and SSC voltages to put events on desired spot in scatter dot plot
  - PB sample
- Optimal voltages for each PMT
  - use voltage with lowest CV and maximum signal/background ratio
  - CS&T beads
- Calculation of compensation
  - dependant of used fluorochromes and voltages
  - compensation beads / fresh cells

Parameters | Compensation

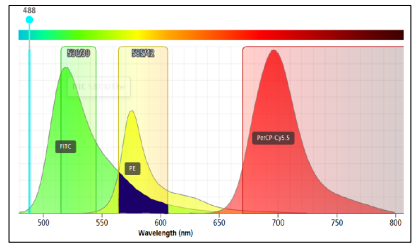
☑ Enable Compensation | Clear

Fluorochrome	- % Fluorochrome	Spectral Overlap
PE	FITC	9.89
PerCP-Cy5-5	FITC	3.34
PE-Cy7	FITC	0.31
APC	FITC	0.01
APC-H7	FITC	0.00
V450	FITC	0.18
V500	FITC	5.17
FITC	PE	1.40
PerCP-Cy5-5	PE	41.80
PE-Cy7	PE	3.17
APC	PE	0.05

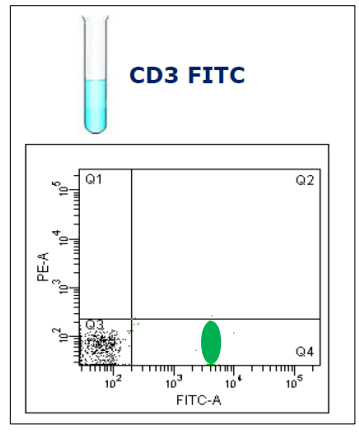
COMPENSATION



Not compensated

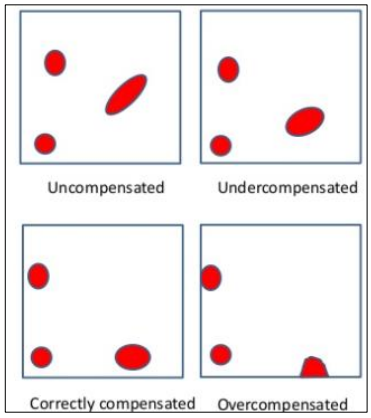


Fluorochrome	- % Fluorochrome	Spectral Overlap
PE	FITC	20.10
PerCP-Cy5-5	FITC	0.00



Compensated

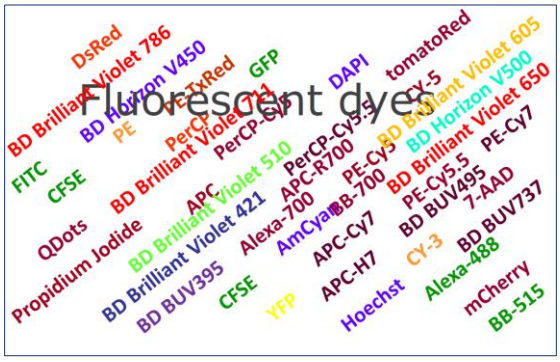
COMPENSATION



Fluorochrome	PMTV	Spillover Values											
		FITC	PE	PerCP-Cy5.5	PerCP	PE-Cy7	APC	APC-R700	APC-H7	APC-Cy7	V450	V500-C	BV605
		Generic	Generic	Generic	Generic	Generic	Generic	Generic	Generic	Generic	Generic	Generic	Generic
FITC	643.7	100.00	1.10	0.03	0.03	0.17	0.01	0.02	0.06	0.04	0.02	5.36	0.00
PE	468.6	14.58	100.00	0.03	0.08	1.23	0.01	0.01	0.04	0.02	0.02	1.08	2.13
PerCP-Cy5.5	581.0	2.77	22.95	100.00	100.00	1.05	1.62	5.26	0.09	0.11	0.02	0.45	4.53
PE-Cy7	603.5	0.29	1.76	16.29	6.58	100.00	0.21	1.31	1.64	1.49	0.01	0.05	0.56
APC	551.0	0.00	0.01	1.16	1.79	0.03	100.00	6.50	1.91	7.62	0.00	0.01	0.11
APC-R700	494.9	0.00	0.00	4.79	1.33	0.09	18.55	100.00	0.77	2.80	0.00	0.00	0.02
APC-H7	553.7	0.00	0.00	4.33	1.20	8.40	14.51	66.95	100.00	100.00	0.00	0.00	0.02
V450	537.6	0.17	0.03	0.08	0.08	0.03	0.03	0.00	0.19	0.13	100.00	6.25	13.49
V500-C	410.5	3.43	0.47	0.09	0.14	0.05	0.03	0.00	0.17	0.12	17.38	100.00	1.56
BV605	521.0	0.55	10.01	0.05	0.13	0.14	0.07	0.01	0.06	0.04	0.77	15.46	100.00

MULTICOLOR DESIGN

- How to choose the CD markers / panel?



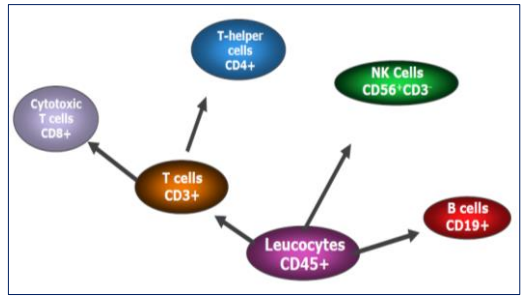
Online tools (panel builders, guided panel solutions, ...)

MULTICOLOR DESIGN

- Which populations do we want to target?

Identification human lymphocyte subsets (B, T, NK cells):

- CD3
- CD19
- CD45
- CD56
- CD4
- CD8



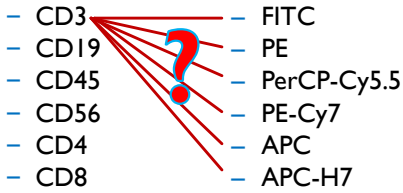
MULTICOLOR DESIGN

- Which populations do we want to target?
- Select fluorochromes according to instrument configuration

- FITC
- PE
- PerCP-Cy5.5
- PE-Cy7
- APC
- APC-H7

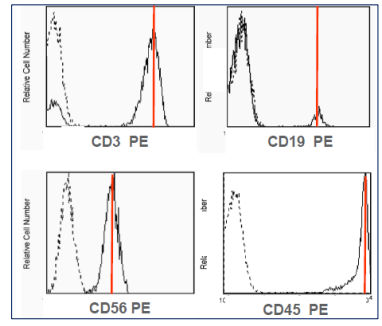
MULTICOLOR DESIGN

- Which populations do we want to target?
- Select fluorochromes according to instrument configuration
- Match the fluorochrome brightness with the antigen-expression levels



Antigen	Antigen Density	Expression Level
CD3	90.000	++
CD4	100.000	++
CD8	124.000	+++
CD19	18.000	+
CD45	200.000	+++
CD56	10.000	+

Antigen density expressed as molecules per cell



MULTICOLOR DESIGN

- Which populations do we want to target?
- Select fluorochromes according to instrument configuration
- Match the fluorochrome brightness with the antigen-expression levels
  - bright antibodies go on dim fluorochromes
  - low antigen density with (very) bright fluorochromes
  - intracellular antigens are usually dimmer and/or less discrete populations than surface antigens

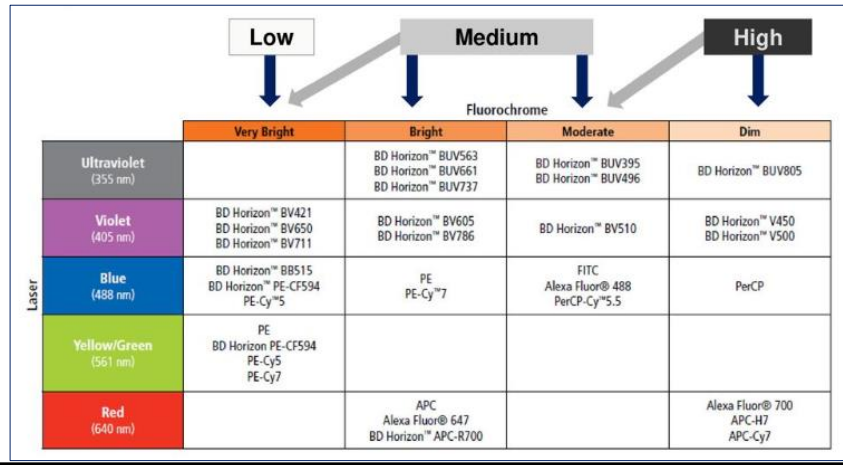
MULTICOLOR DESIGN

- Overview of brightness of fluorochromes

Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV563 BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	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-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	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

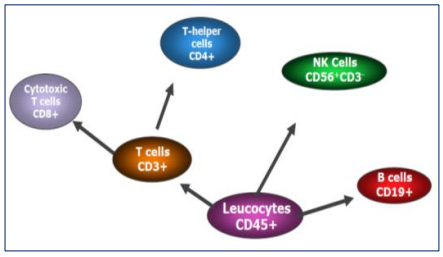
MULTICOLOR DESIGN

- Antigen / fluorochrome combinations

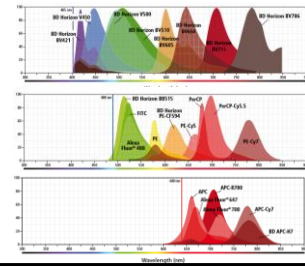


MULTICOLOR DESIGN

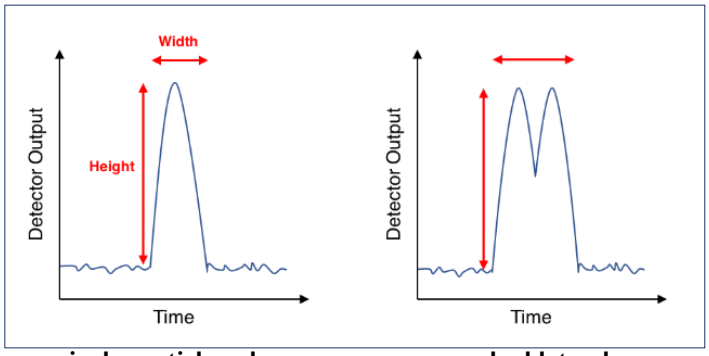
- Which populations do we want to target?
- Select fluorochromes according to instrument configuration
- Match the fluorochrome brightness with the antigen-expression levels
- Minimize the potential for spectral overlap on the same cell



CD3	FITC
CD19	PE
CD45	PerCP-Cy5.5
CD56	PE-Cy7
CD4	APC
CD8	APC-H7



SINGLETS - DOUBLETS



single particle pulse **doublet pulse**

single particle pulse *vs.* doublet pulse:

- area and width of doublet pulse is larger than the single cells (because 2 cells spend longer passing through a laser beam than one cell)
- heights of the 2 pulses are very close (may differ), if not identical

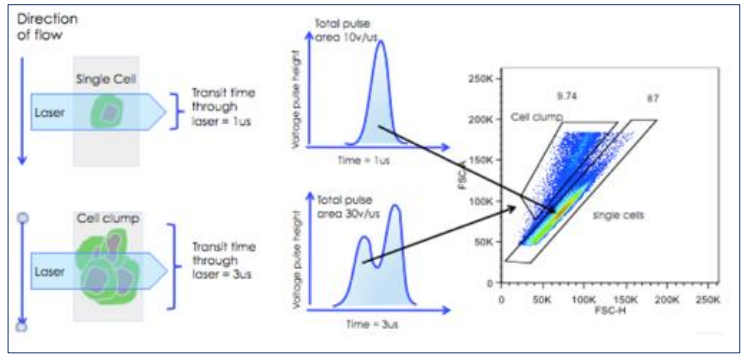
SINGLETS - DOUBLETS

- Single cells *versus* cell clumps passing through the laser intercept

  ⇒ differences in time

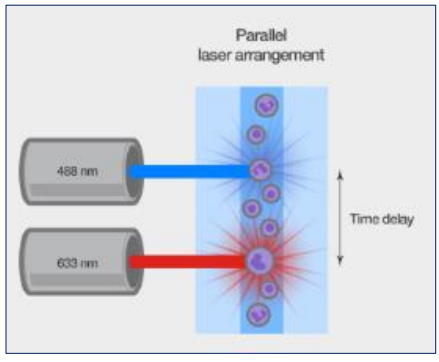
  ⇒ affects the area of the signal

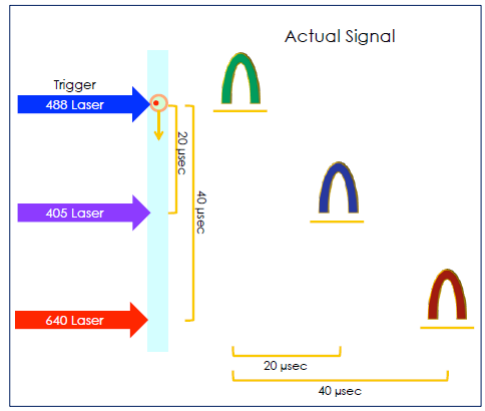
  ⇒ elimination of doublets/clumps



TIME DELAY

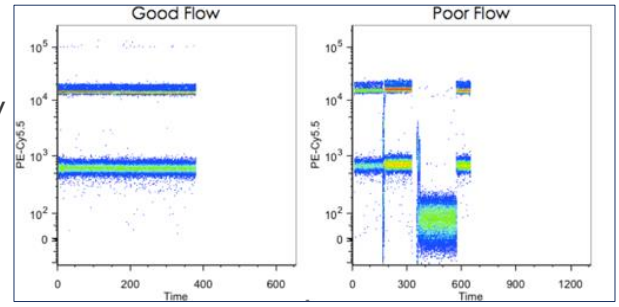
- a **time delay is** inherent in the system



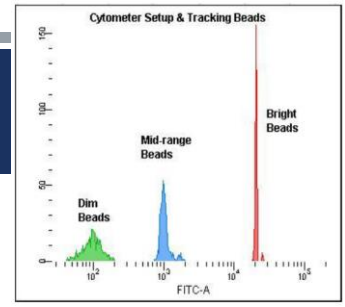


TIME AS PARAMETER

- To monitor and check the stability of your instruments during your measurements
- To see how even the flow rate was during the entire run (in-run QC parameter)
  - Time *versus* scatter
  - Elimination of artefacts caused by poor flow

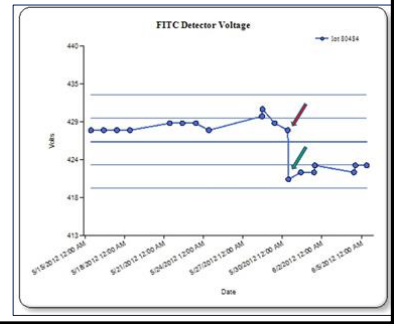


MONITORING



- CS&T beads: 3 different fluorescent intensities
- **Baseline performance:** setting of optimal voltages/target values
- **Daily monitoring:** PMTV are automatically adjusted by the software, so that MFI target values defined at baseline are achieved

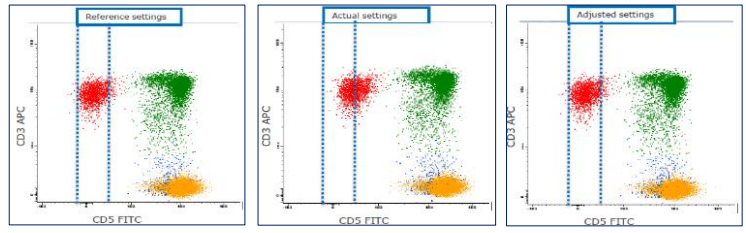
Example:
- red arrow data of maintenance service
- green arrow shows lower PMTV (to obtain the same MFI output signal) than voltage needed before service



MONITORING

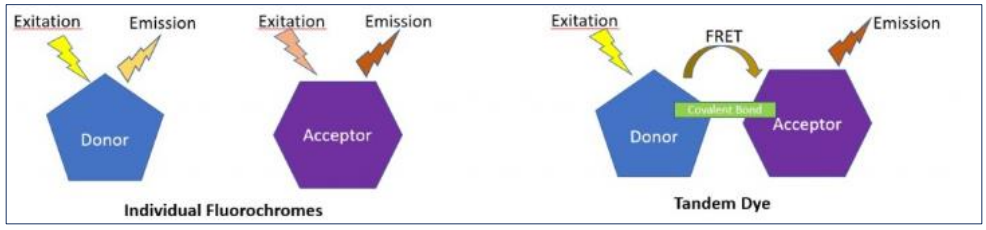
- CS&T beads: 3 different fluorescent intensities
- **Baseline performance:** setting of optimal voltages/target values
- **Daily monitoring:** PMTV are automatically adjusted by the software, so that MFI target values defined at baseline are achieved

Example:
- FITC PMT showed increased MFI on CS&T beads
- ensure consistent results over time, experiment to experiment



TANDEM CONJUGATES

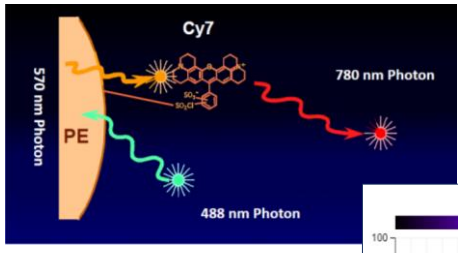
- a pair of covalently linked fluorescent molecules which contain a donor and an acceptor molecule
  - donor molecule is generally a protein-based dye (eg. PE)
  - acceptor molecule is a synthetic dye (eg. Cy7) which absorbs energy emitted by the donor molecule
  - examples: PE-Cy7, PE-CY5.5, APC-Cy7, …

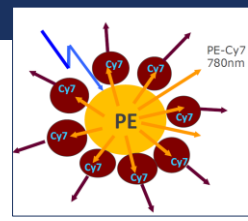


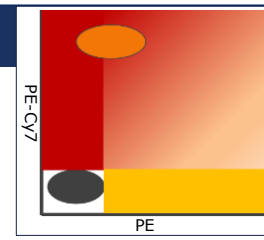
TANDEM CONJUGATES

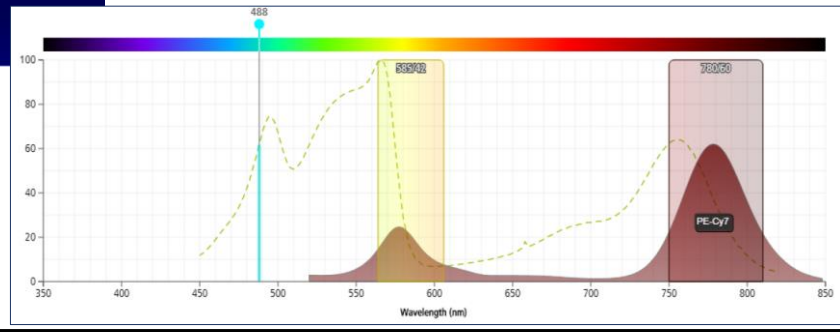
Considerations for tandem dyes

- Take into consideration residual donor emission.









TANDEM CONJUGATES

- Causes of degradation of covalent bonds :
  - light exposure (repeated illumination and/or exposure to direct light during storage)
  - temperature (NEVER be stored at -20°C or other freezing temperatures)
  - ….
- Impact of tandem dye breakdown
  - false positive signals in the donor channel