Flow Cytometry & Mass Cytometry

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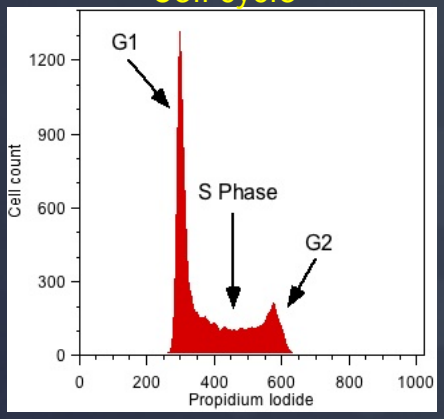
Applications of Flow Cytometry

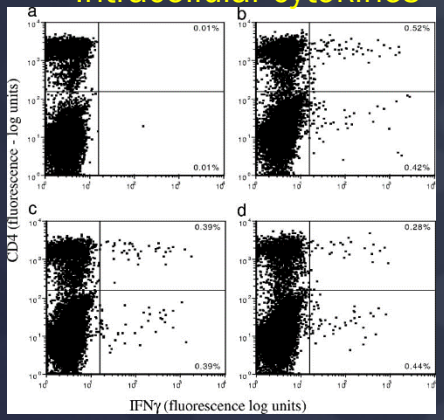
- MULTI-COLOUR IMMUNOPHENOTYPING Diagnosis of Hematologic Malignancies Detection of Minimal Residual Disease Intracellular Cytokines and phosphor-proteins Phagocyctosis Efficacy of Cancer Chemotherapy Platelet Function Analysis Mitochondrial activity Membrane Fluidity Fluorescent Protein expression Reticulocyte Enumeration Applications in Organ Transplantation Cell function Analysis Apoptosis Applications of Transfusion Medicine Analysis of DNA Ploidy and Cell Cycle Cell Viability Cell proliferation CELL SORTING

2 main instrument types – Analysers & Sorters

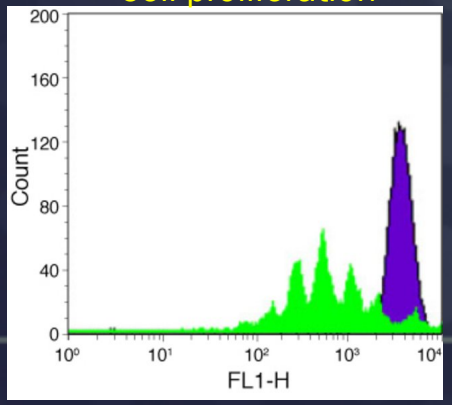
Cell cycle



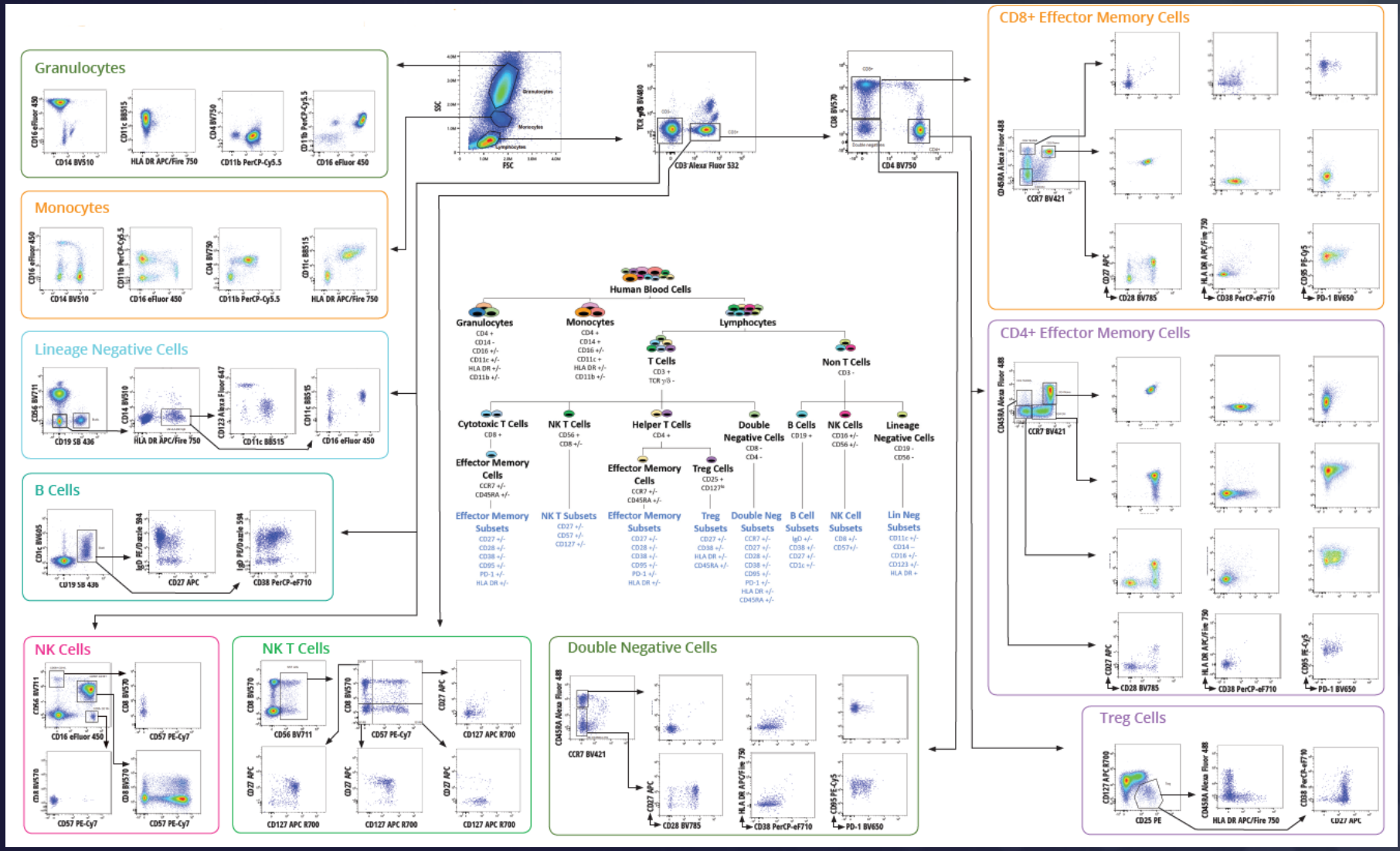
Intracellular cytokines



Cell proliferation



Data – 2D Hierarchal Gating

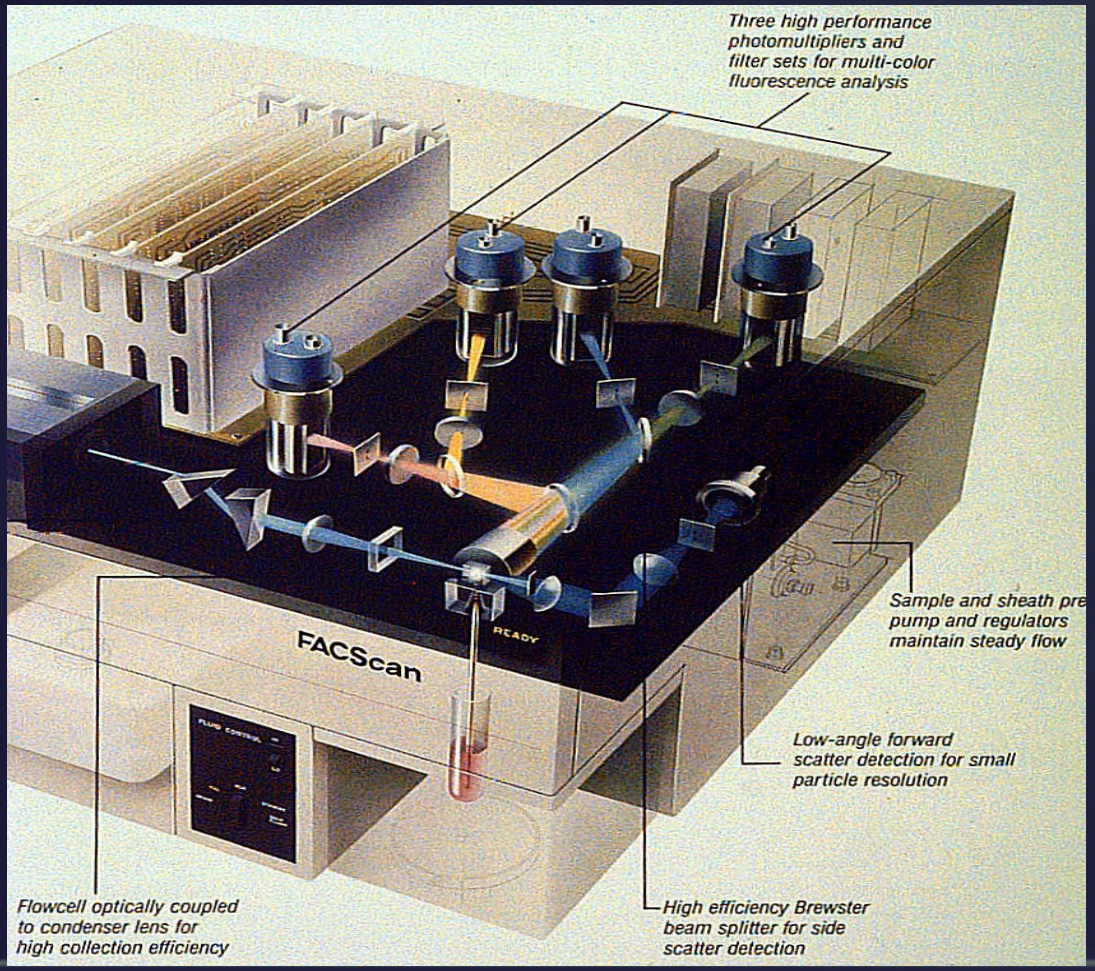


What is flow cytometry?

- Technology to measure one cell/particle at a time in a moving fluid stream
- Ability to measure and correlate many parameters from each cell
- It can rapidly record huge numbers of cells/events, good for rare event detection
- It is a mature technology, used worldwide for research and diagnostics
  - an expert user base
  - Large numbers of reagents and applications
- Specialised flow cytometers can sort populations of cells to high purity and also isolate single cells

What is Flow Cytometry?

Basic design

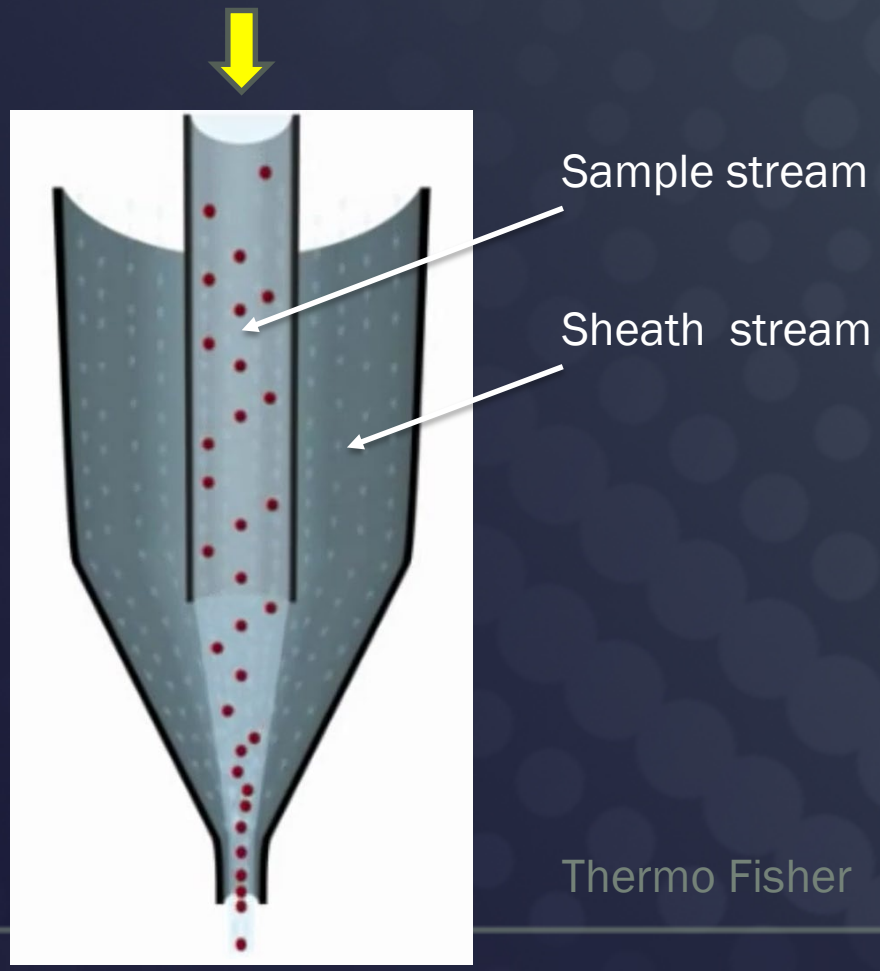


- Cells are passed from a sample tube one at a time into the flow cell
- Laser light interacts with the cells and causes light scatter and fluorescence
- Emitted light is collected and directed to detectors
- The photons are converted into digital values for analysis using specialised cytometry software

What is Flow Cytometry?

- The analysis of cells/particles one at a time in a moving fluid stream

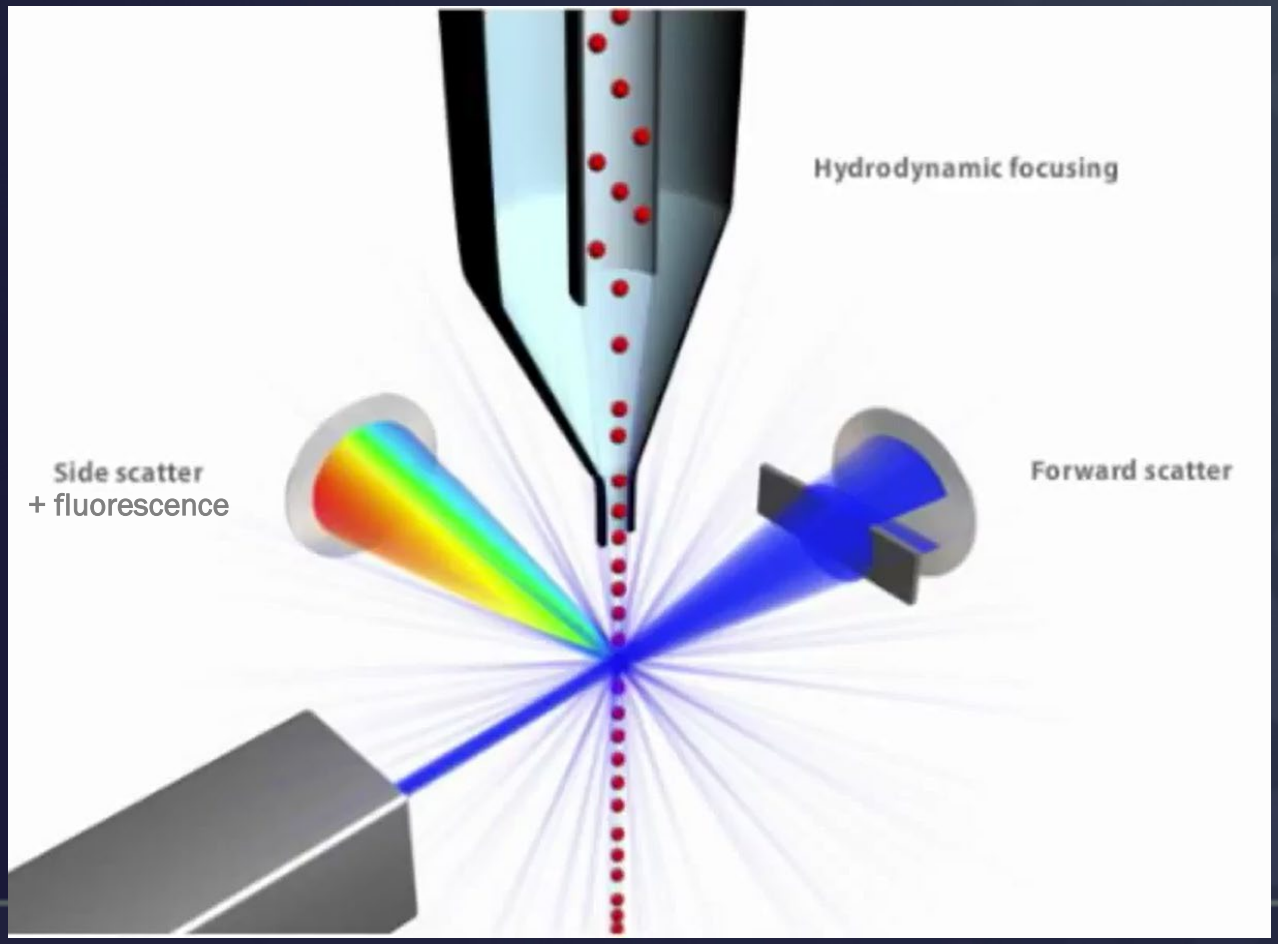
Hydrodynamic focussing - the sample is injected into an outer sheath stream. The pressure differential and design of the flow cell creates laminar flow where the sample core is focussed into a stream of single particles within the outer sheath layer **Acoustic** waves can also be used to improve focusing



Thermo Fisher

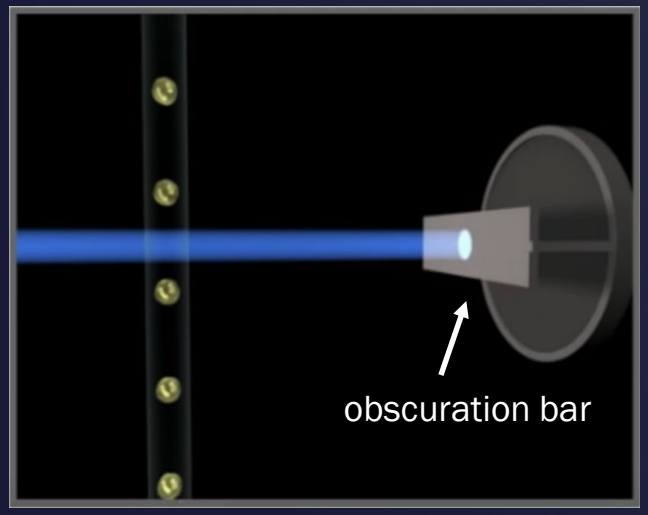
What do flow cytometers measure?

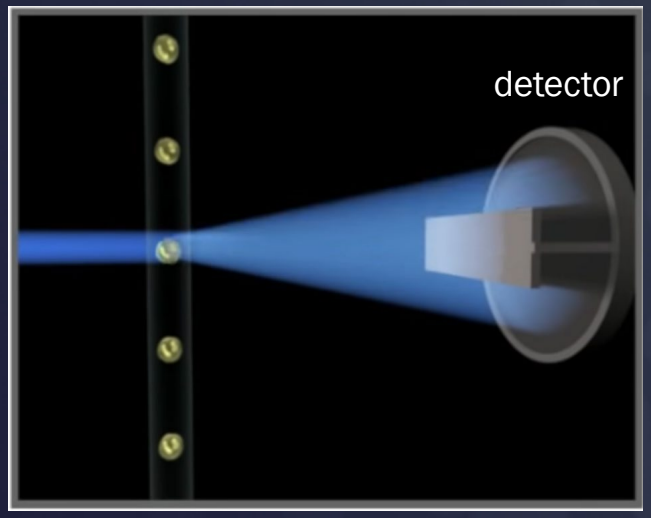
- Light scatter
- Fluorescence
- Time

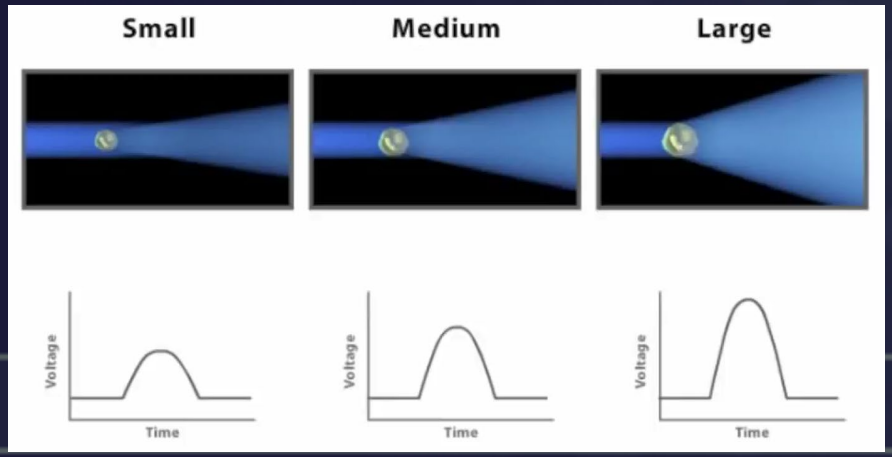


Forward Light Scatter

- Approximately proportional to particle size

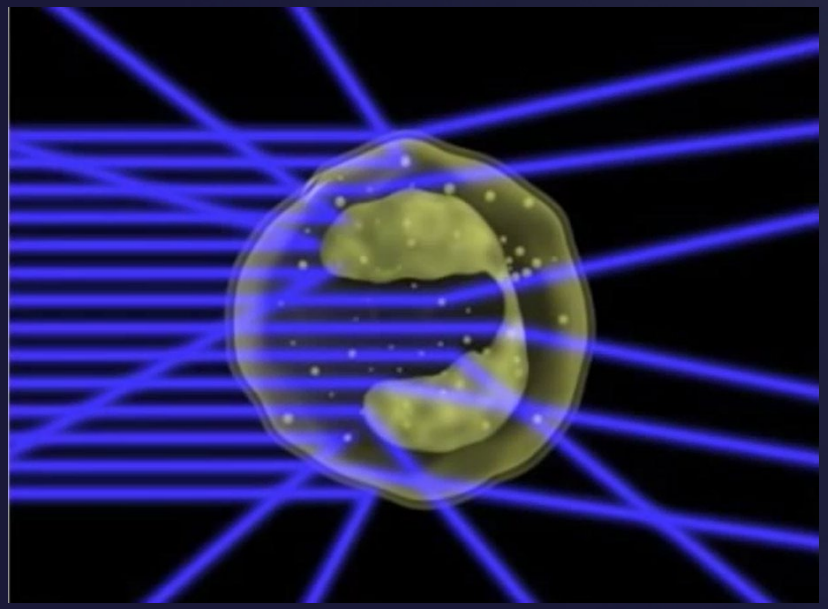


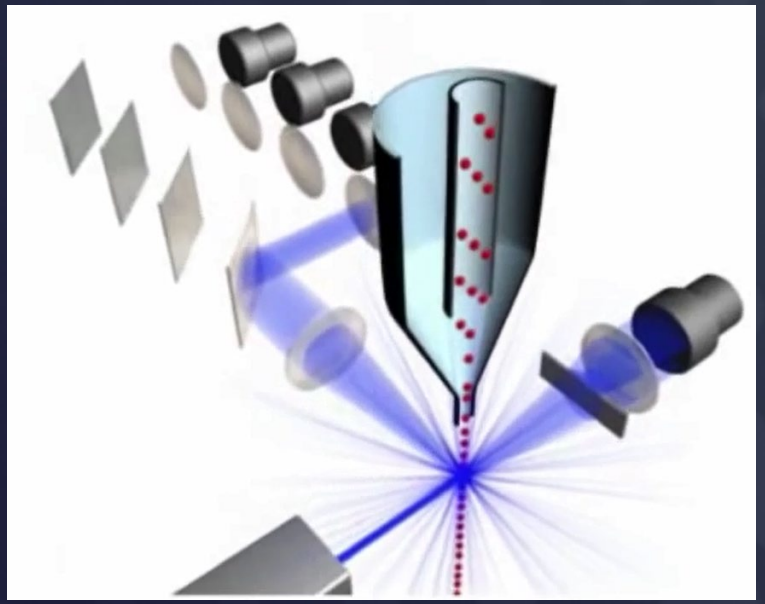




Side Scatter

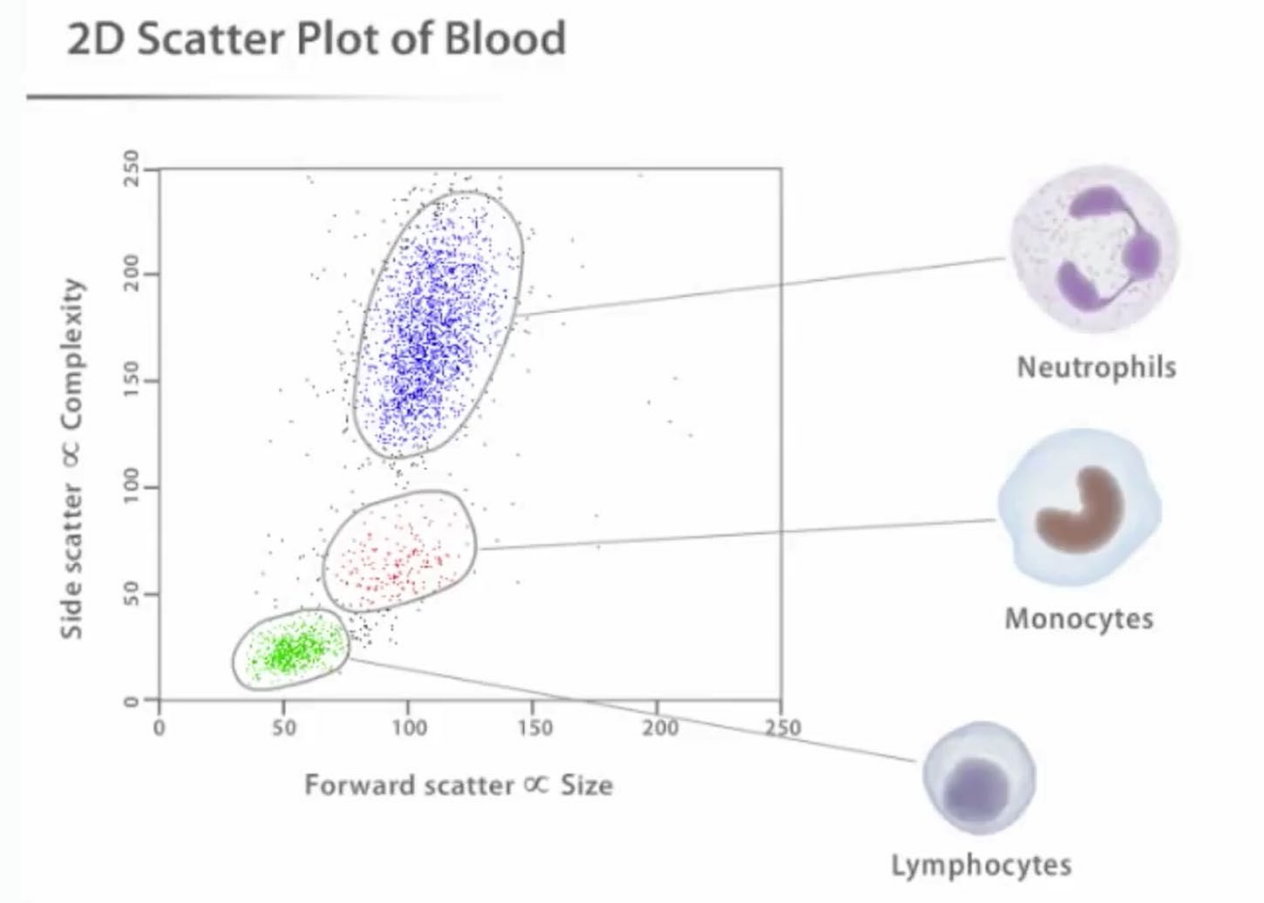
- Side scatter is linked to granularity and internal complexity
- Light is scattered in all directions, side scatter is a sample of this, measured at about 90º to the light source





Forward & Side Scatter

- When FSC and SSC signals are correlated it allows distinct clusters of cells to be identified



Thermo Fisher

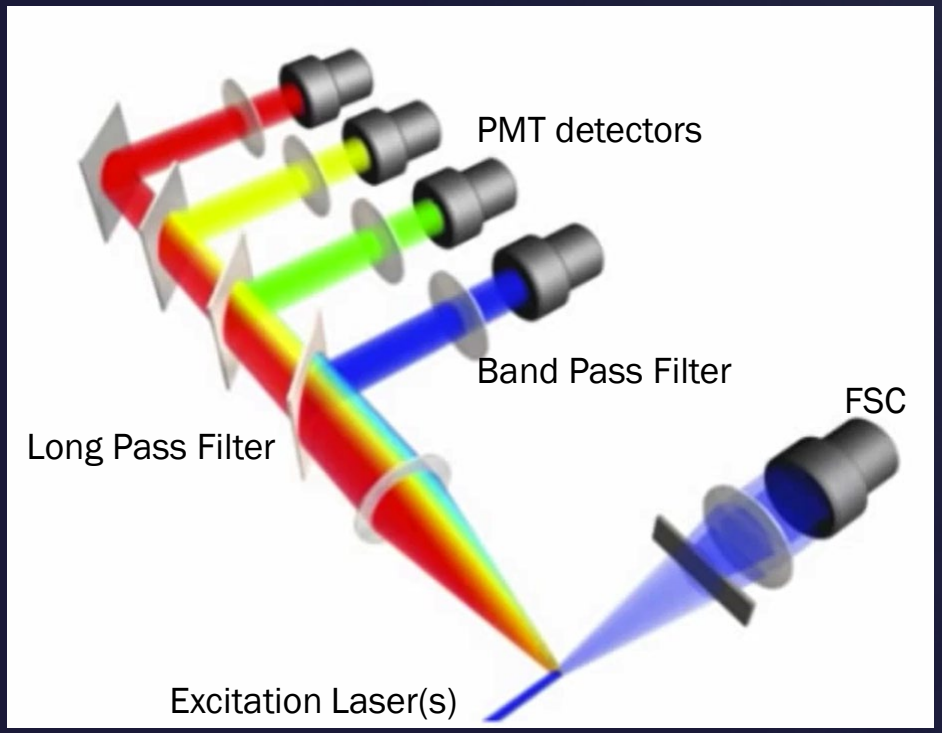
Fluorescence

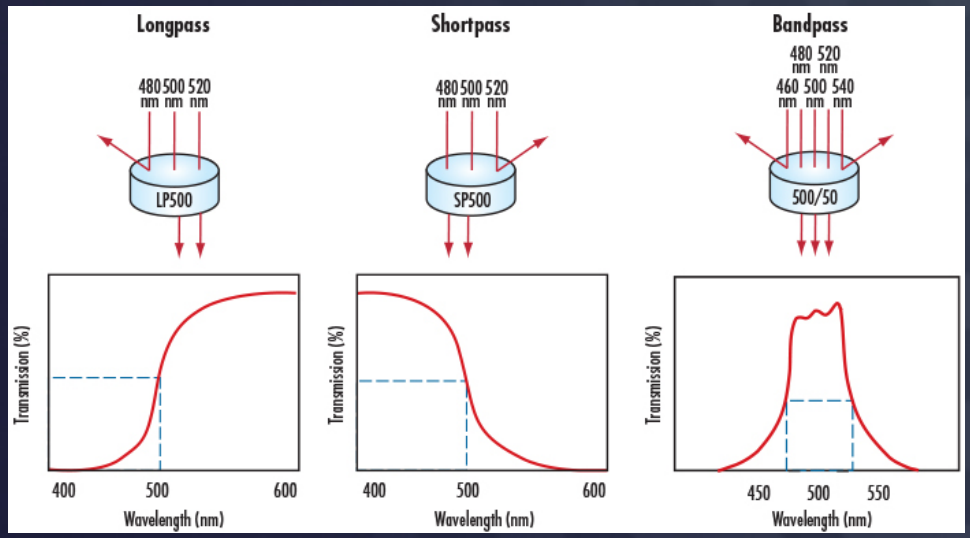
- Many fluorescent substances are used in FCM
  - Fluorochrome antibody conjugates
  - Nucleic acid binding dyes
  - Metabolic indicators
  - Fluorescent proteins
- All cells have some auto fluorescence and in general this is also linked to size
- There is normally some background non-specific binding of conjugates to cells – this should be minimised by blocking ie. FcBlock
- measured fluorescence =

  Specific binding + autofluorescence + non-specific binding

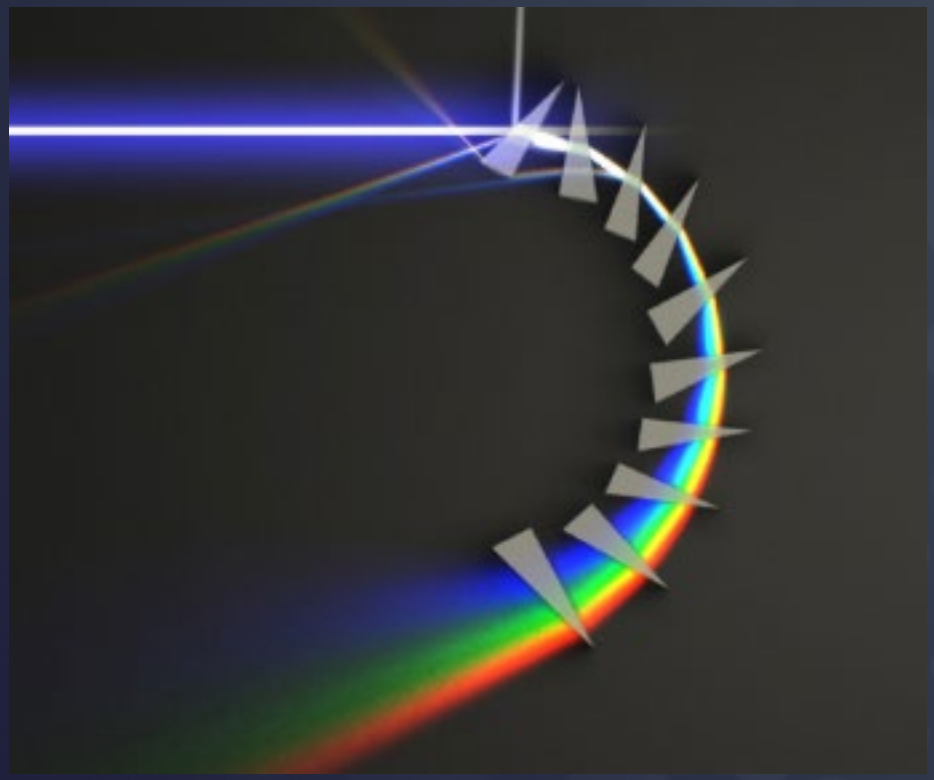
How is Fluorescence Measured?

- Most flow cytometers use filters and detectors to measure ranges of light that are associated with specific fluorochromes
- Some cytometers use spectral arrays / spectral unmixing



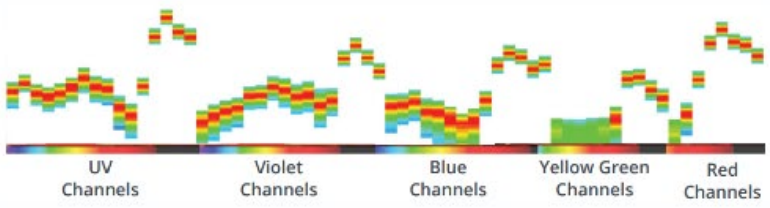


Spectral Analysers

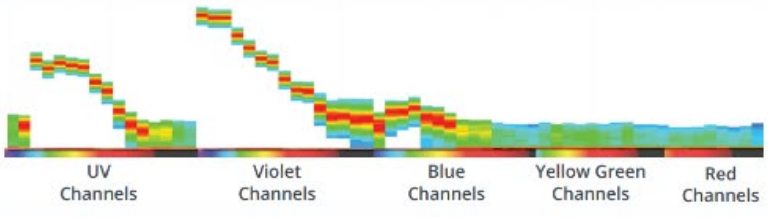


Cytek
Sony Biotechnology

Example 1: BUV737



Example 2: BV421



Spectral Analysers

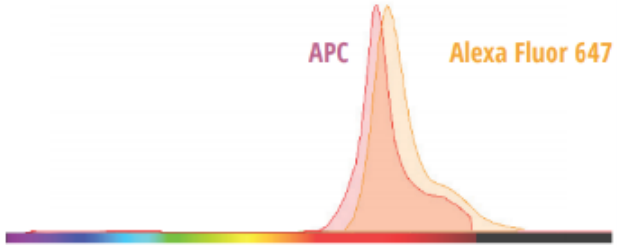


Figure 1: Spectrum plots from a conventional spectrum viewer shows heavy overlap between APC and Alexa Fluor 647.

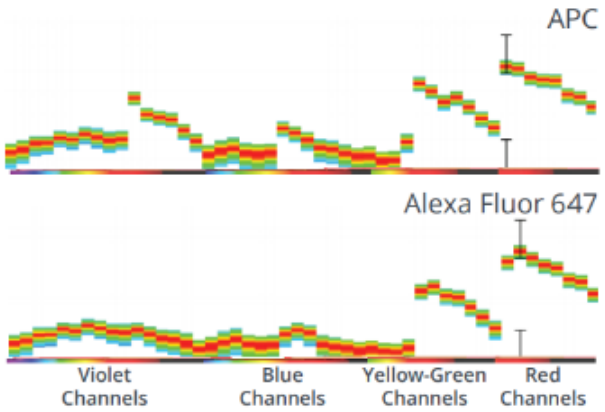


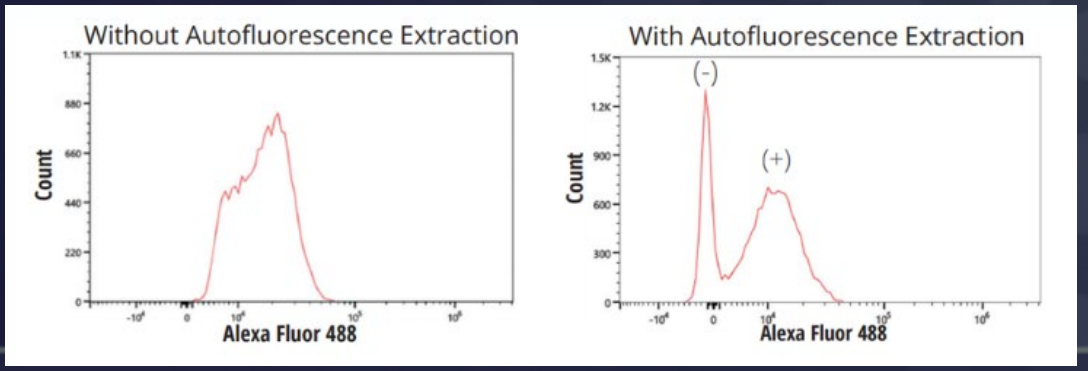
Figure 2: Spectrum plots from a four laser Aurora show distinct signatures for APC and Alexa Fluor 647.

Advantages

(1) Ability to identify dyes in the presence of very similar dyes

(2) Ability to subtract cellular auto-fluorescence

Cytek



How fast, How many?

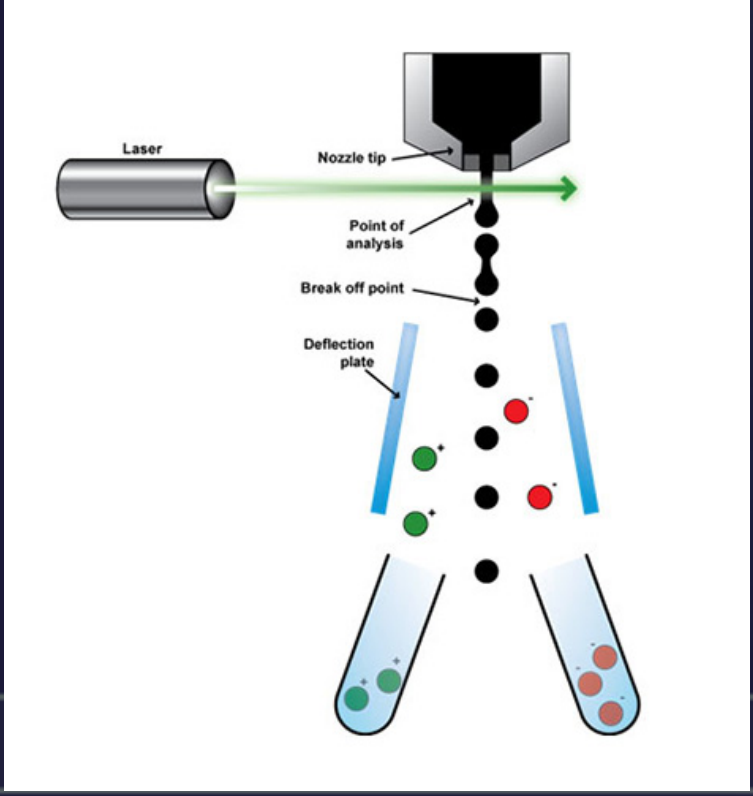
- Cells can be assayed one at a time at speeds of up to 100,000 cells per second
  - Typically 20-35,000 events per second (up to 126 million per hour)
  - High-end cytometers can measure up to 30 parameters (28 colours + FSC/SSC)
  - At high speed this is 3.7 billion separate measurements per hour
  - Pulse shape analysis (height, width and area) increases number to over 11 billion / hour
- Cell sorting speeds up to 25,000 per second
  - Approximately 6min 40 seconds to sort a million cells from a 10% target population
- Also possible to sort single cells into wells and record precise measurements from the cell (indexed sorting)

Limitations of flow cytometry

- Not ideal for analysis of solid tissues
  - Cells must be disrupted to achieve a single cell suspension
  - Mechanical and chemical methods of disruption can damage or change cells
  - Loss of proximity information
- Cannot easily determine if signals are localised in cells or provide morphology information (unlike microscopy)
- Uses of different fluorescence probes in multi-colour cytometry requires careful consideration to minimize signal overlap
- However…speed, accuracy and precision make FCM an excellent diagnostic tool as well as one of the most widely used research tools in the WIMM

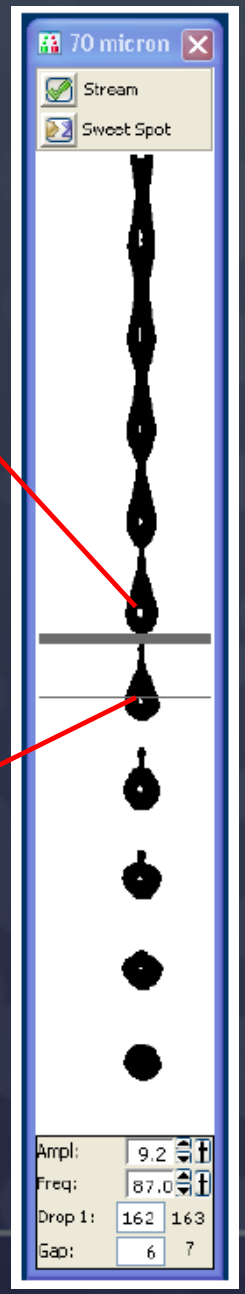
Cell sorting

- One of the main advantages of flow cytometry over mass cytometry is that cells are not destroyed during the measurement process – this allows cell sorters, to physically separate specific cells from mixtures of cells



When the target cell reaches this position the whole stream is charged

When the target cell reaches this position the whole stream is grounded (neutralised) The droplet containing the target cell – remains charged



Cell sorting

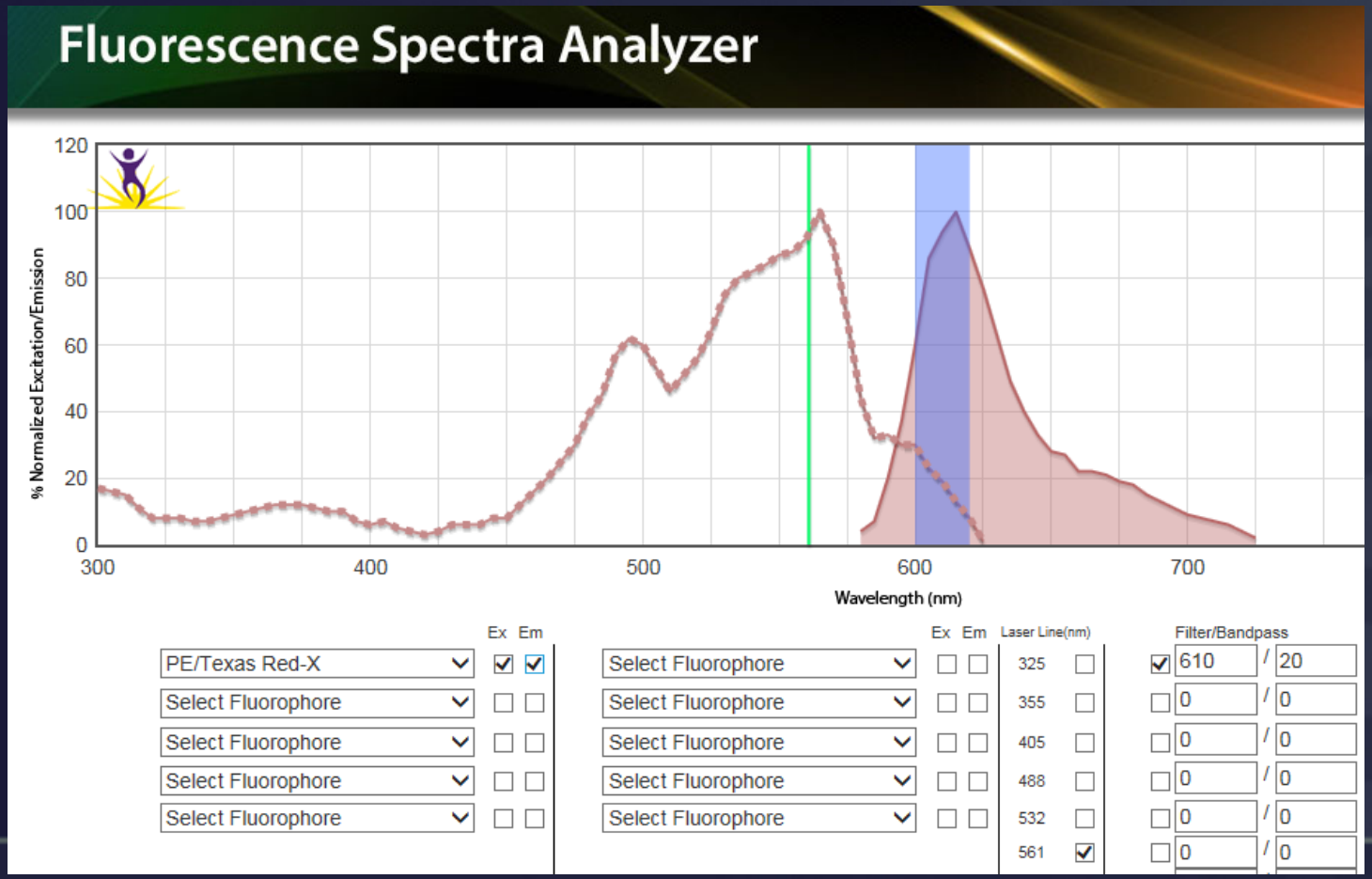
- Possible to sort 6 ways on some instruments – 6 different populations of cells
- Ability to sort into / onto a wide range of collection devices
  - 0.5ml, 1.5ml, 5ml, 15ml, 50ml tubes
  - 6, 12,24,48, 96 & 384 well plates
  - 60/72 well Terasaki plates, microscope slides
  - Other custom devices

Sorting applications in the WIMM

- Single cell sorting for cloning
- Single cell sorting for down-stream genomics (with and without phenotype indexing)
- Sorting of transformed cells using GFP and other fluorescent markers
- Bulk sorting cells based on single parameter or multi-colour staining

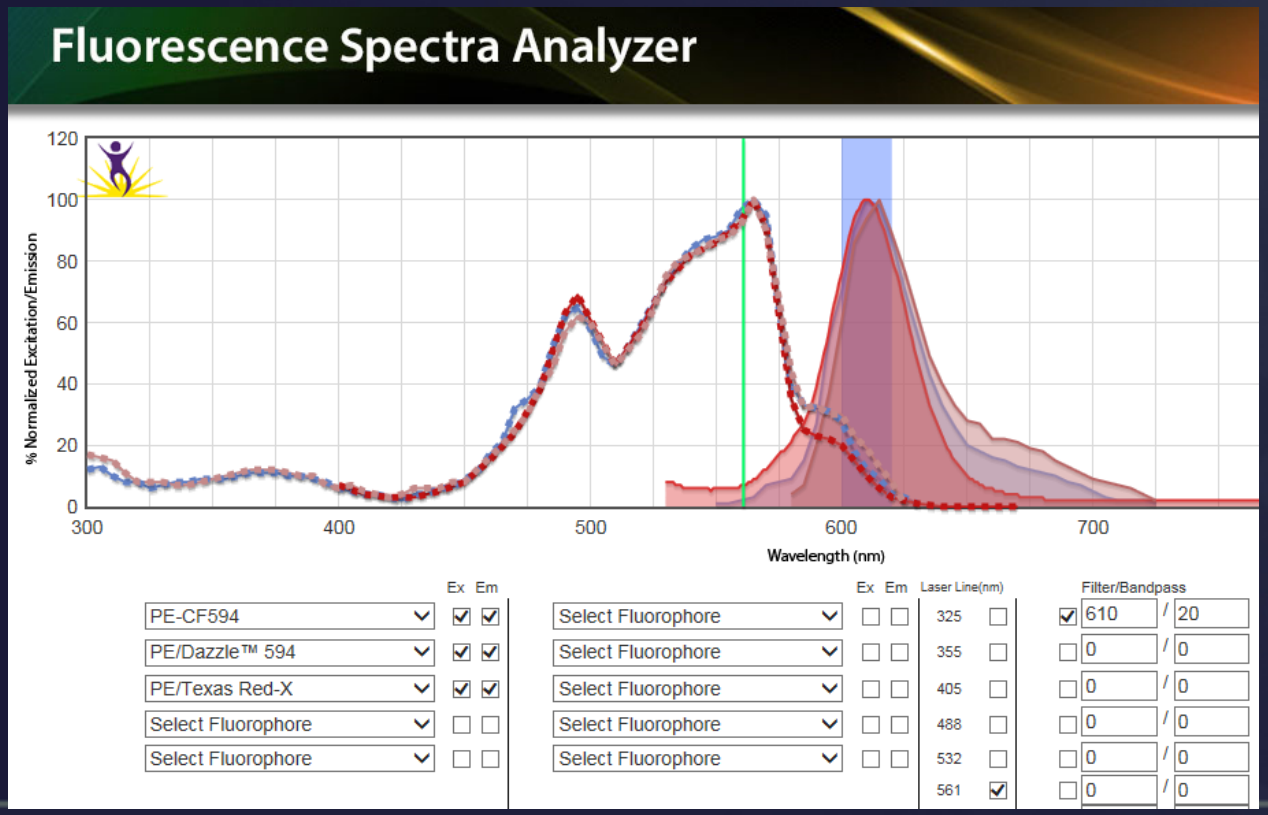
Understanding the properties of fluorochromes

- Spectrum viewers



Understanding the properties of fluorochromes

- Be aware that some fluorochromes with different names are very similar



561-610/20 channel

PE-Texas Red

ECD

PE/Dazzle 594

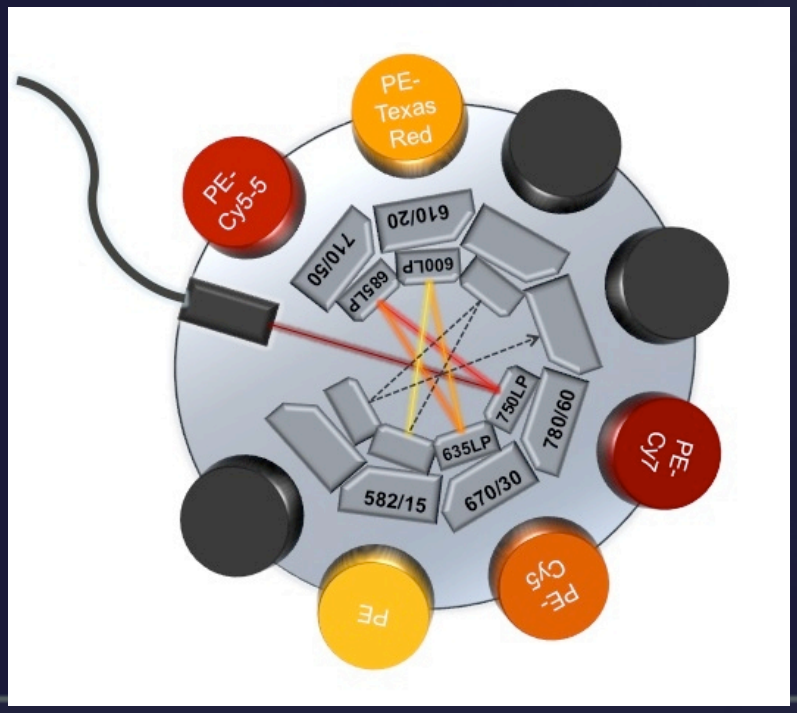
PE-CF594

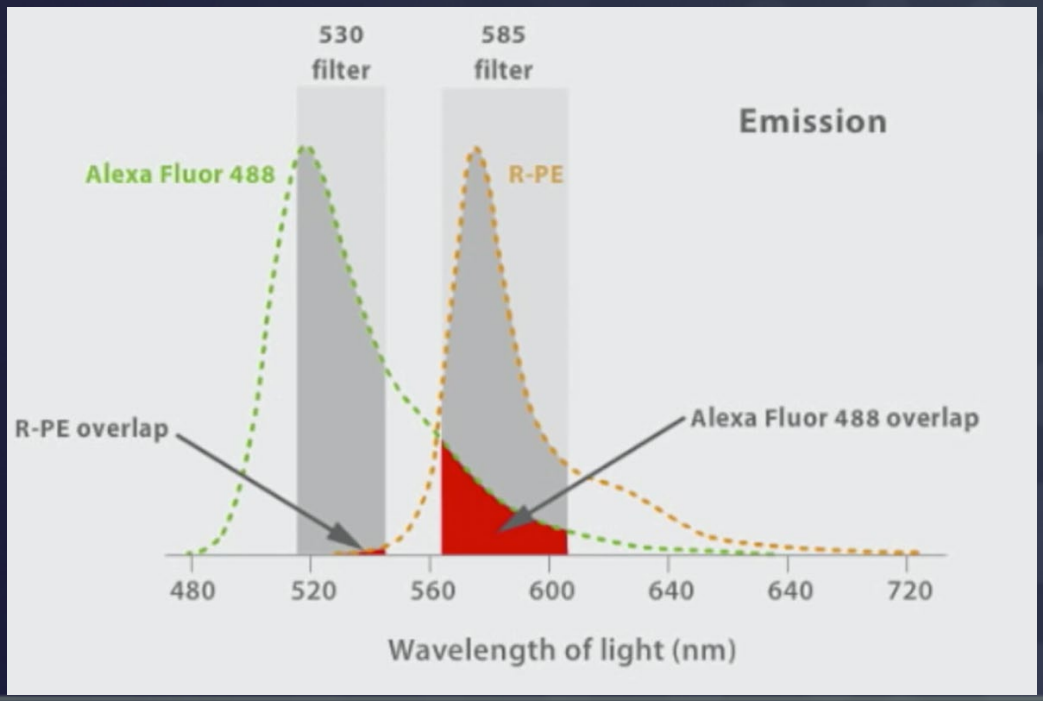
PE-eFluor 610

PE-Alexa Fluor 610

Multi-colour Flow

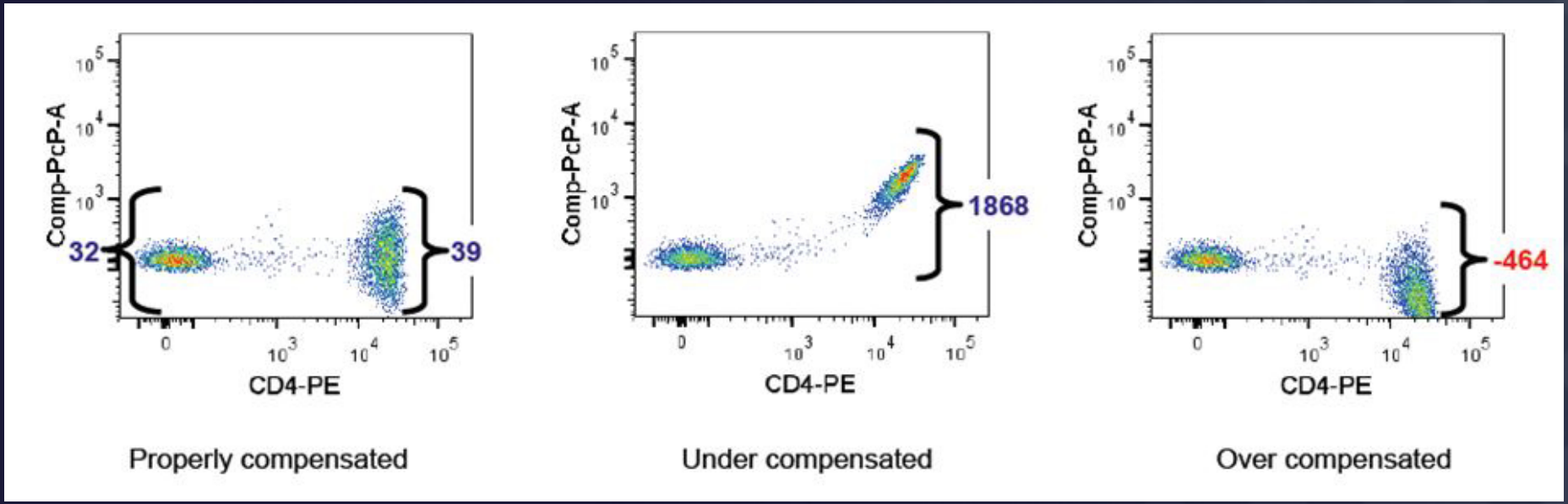
- It is difficult to select band-pass filters that only capture light from a single fluorochrome
- Fluorescence often overlaps or spills into adjacent detectors
- By selecting fluorochromes with very different excitation and emission spectra, spectral overlap will be minimised – this is not always possible



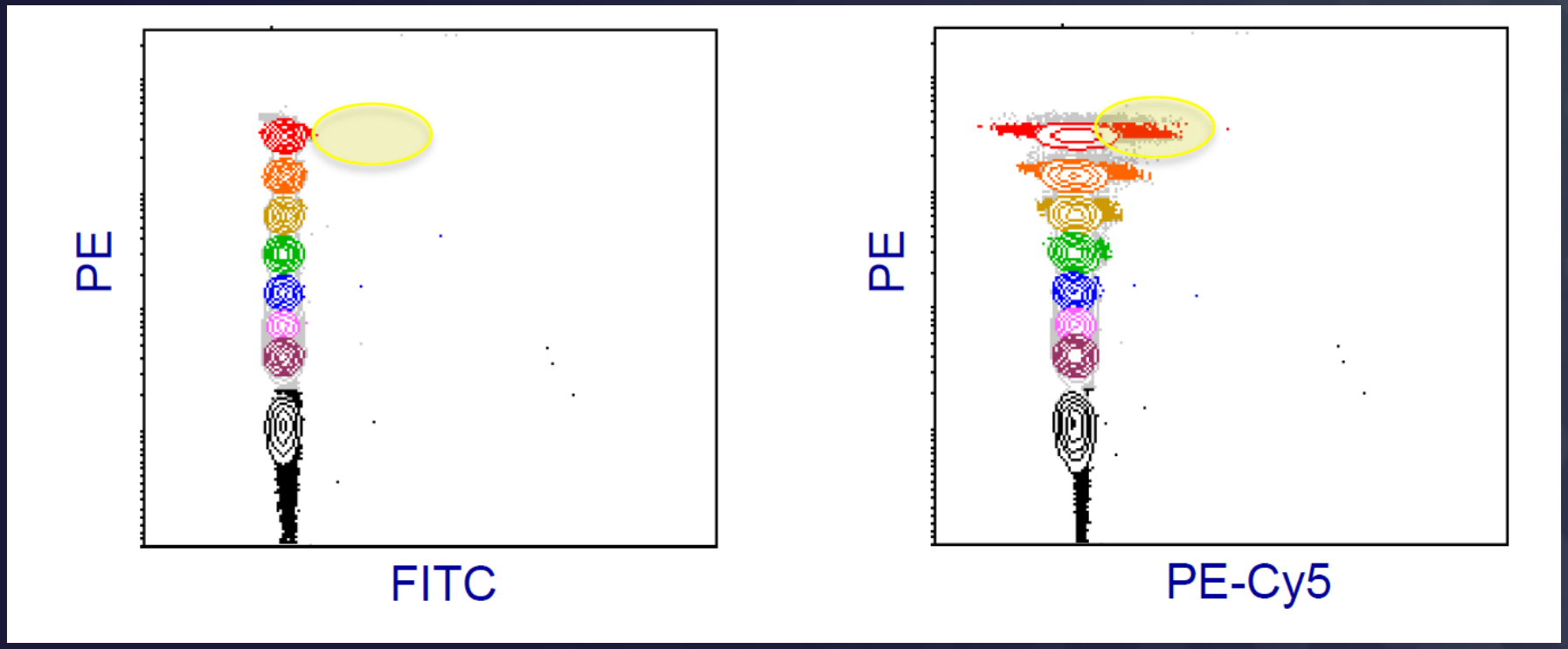


Fluorescence spill-over compensation

- Signal spill-over can be corrected using signal compensation
- The amount of signal spill-over is calculated using single stained controls

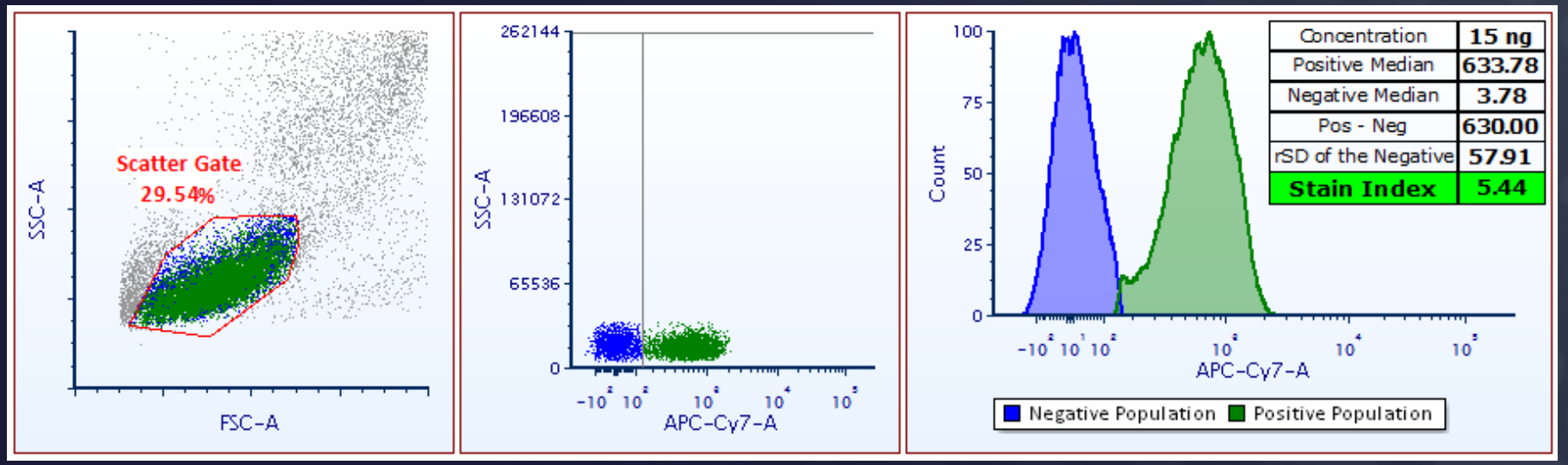


Spillover spread



Assay optimization – calculating stain index

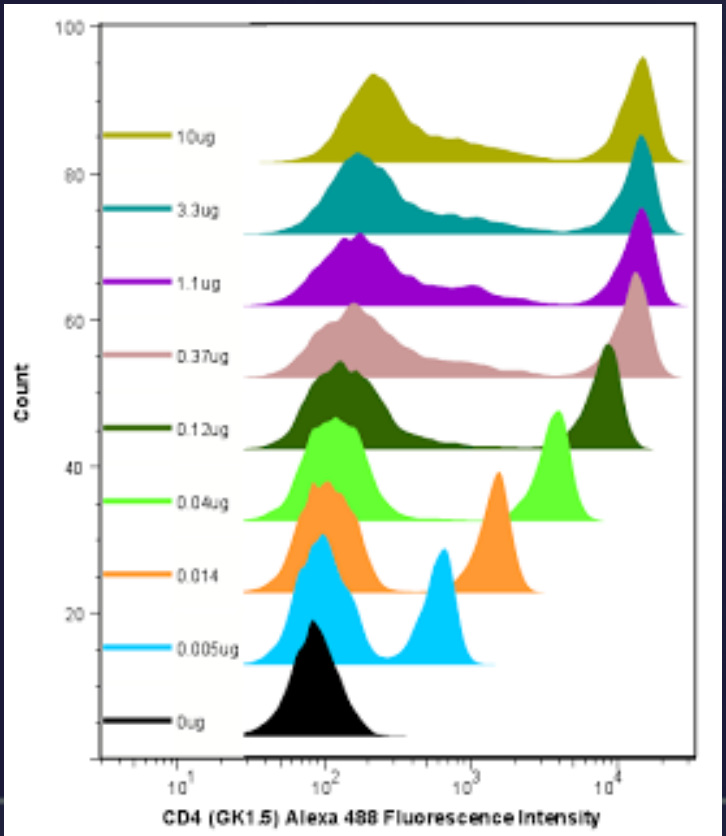
- Stain Index = (Median of Positive - Median of Negative) / (SD of Negative * 2)



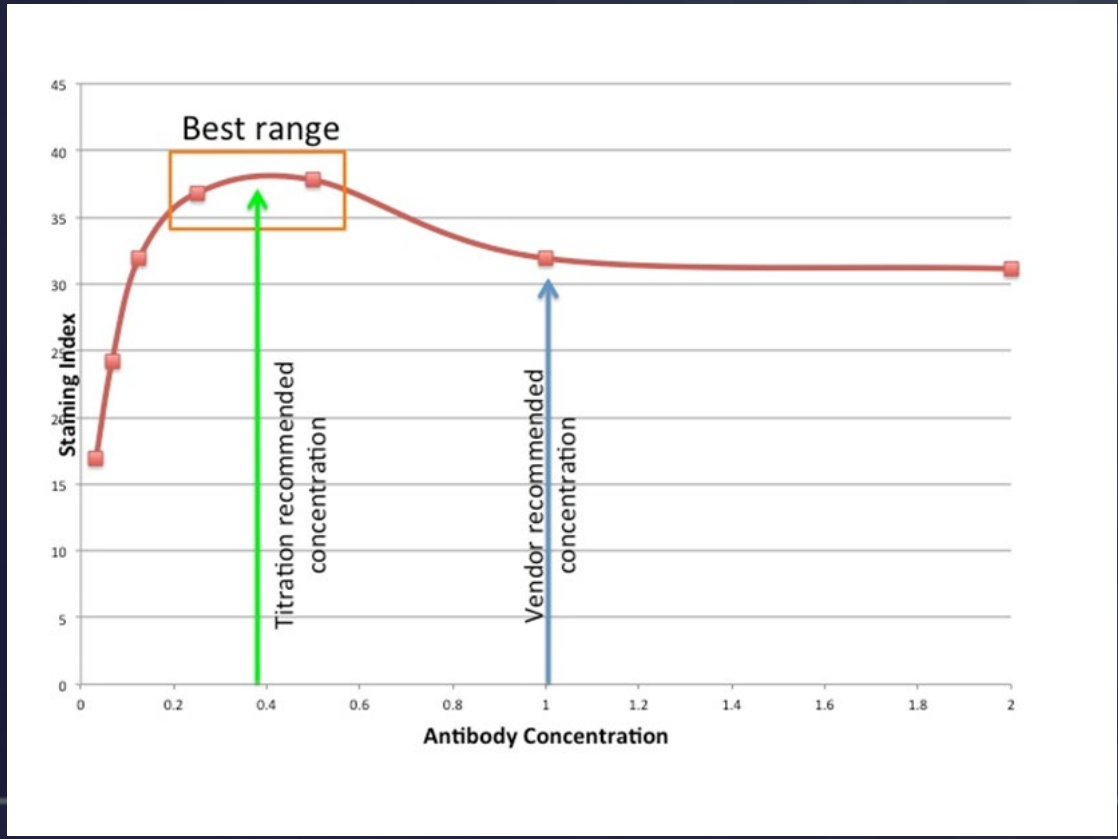
FCS Express

Assay optimization – antibody titration

- All antibodies must be titrated – do not rely on manufacturer's recommendations
- Using too little or too much antibody can have a detrimental effect on data quality



Ryan Duggan



Tim Bushnell

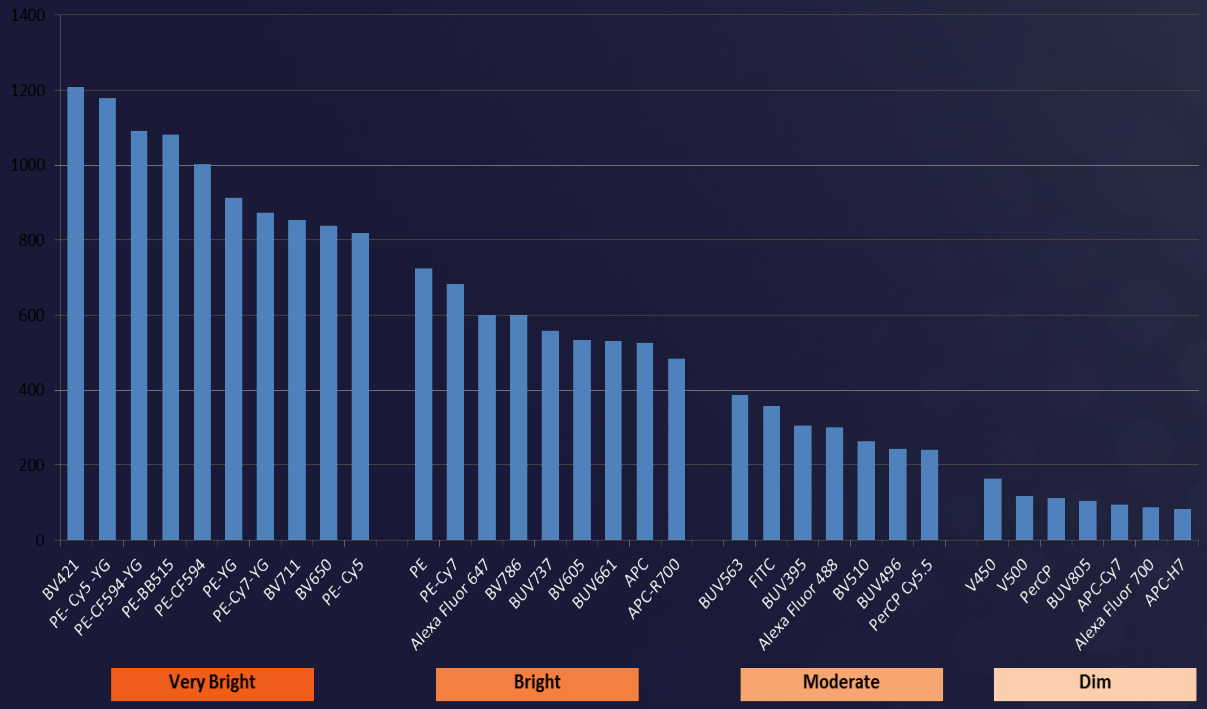
Panel design

- Best scenario is a panel with no or little compensation
  - This is achievable for up to 5 colours but becomes more difficult as more colours are added

		Fluor (Spillover into)				
		BUV395	BV421	FITC	PE-CF594	APC
Channel	BUV395		0.16	0.01	0.00	0.00
	BV421	0.29		0.00	0.03	0.00
	FITC	0.00	0.11		0.23	0.00
	PE-CF594	0.00	0.04	0.00		0.16
	APC	0.57	0.00	0.00	0.32	

Panel design rules

- Choose bright fluorochromes to stain weakly expressed antigens



Relative Brightness	Reagent	Filter
BRIGHTEST	Brilliant Violet™ 421	450/50
	PE	575/26
	Brilliant Violet 605	610/20
	BD Horizon PE-CF594	610/20
	PE-Cy5	670/14
	APC	660/20
BRIGHT	PE-Cy7	780/60
	Alexa Fluor® 647	660/20
	PerCP-Cy5.5	695/40
MODERATE	Alexa Fluor® 488	530/30
	FITC	530/30
	BD Horizon V450	450/50
	Pacific Blue™	450/50
DIM	Alexa Fluor® 700	730/45
	PerCP	695/40
	APC-Cy7	780/60
	AmCyan	525/20
	BD Horizon V500	525/20
	BD APC-H7	780/60

Panel design rules

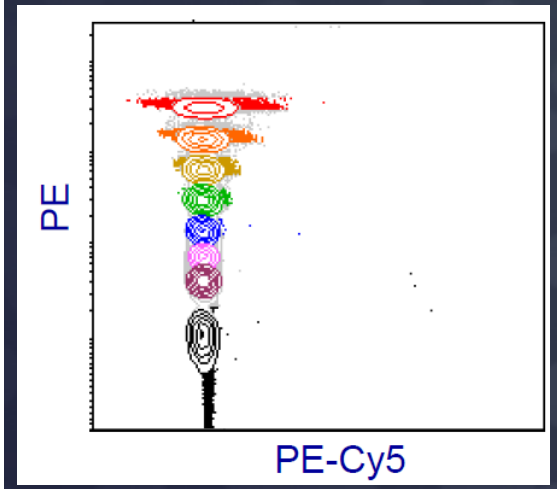
- Be aware of fluorochromes combinations that have high spectral overlap– adverse effects can be measured by making a Resolution Impact Matrix (RIM). This is particularly significant for fluorochromes that are used to identify markers that are co-expressed by cells

Resolution Impact Matrix (Color Coded)

Primary Fluorochrome \ Secondary Fluorochrome	FITC	PerCP-Cy5.5	BV421	BV510	BV605	BV650	BV711	BV786	BUV395	BUV496	BUV661	BUV737	APC	Alexa Fluor® 700	APC-H7	PE	PE-CF594	PE-Cy7
FITC	100																	
PerCP-Cy5.5		112																
BV421			457															
BV510				105														
BV605					77													
BV650						219												
BV711							129											
BV786								212										
BUV395									99									
BUV496										89								
BUV661											383							
BUV737												241						
APC													171					
Alexa Fluor ® 700														65				
APC-H7															48			
PE																487		
PE-CF594																	712	
PE-Cy7																		845

% loss of SP-SI				
<20%	20-40%	40-60%	60-80%	>80%

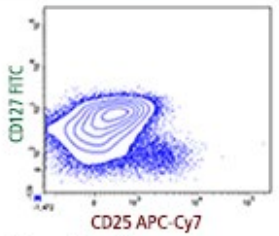
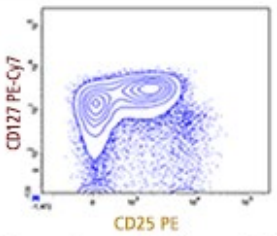
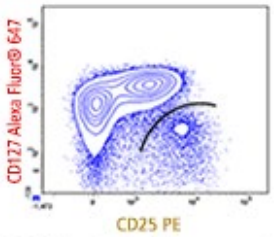
SI (Primary)



Panel design tools

- BD GPS

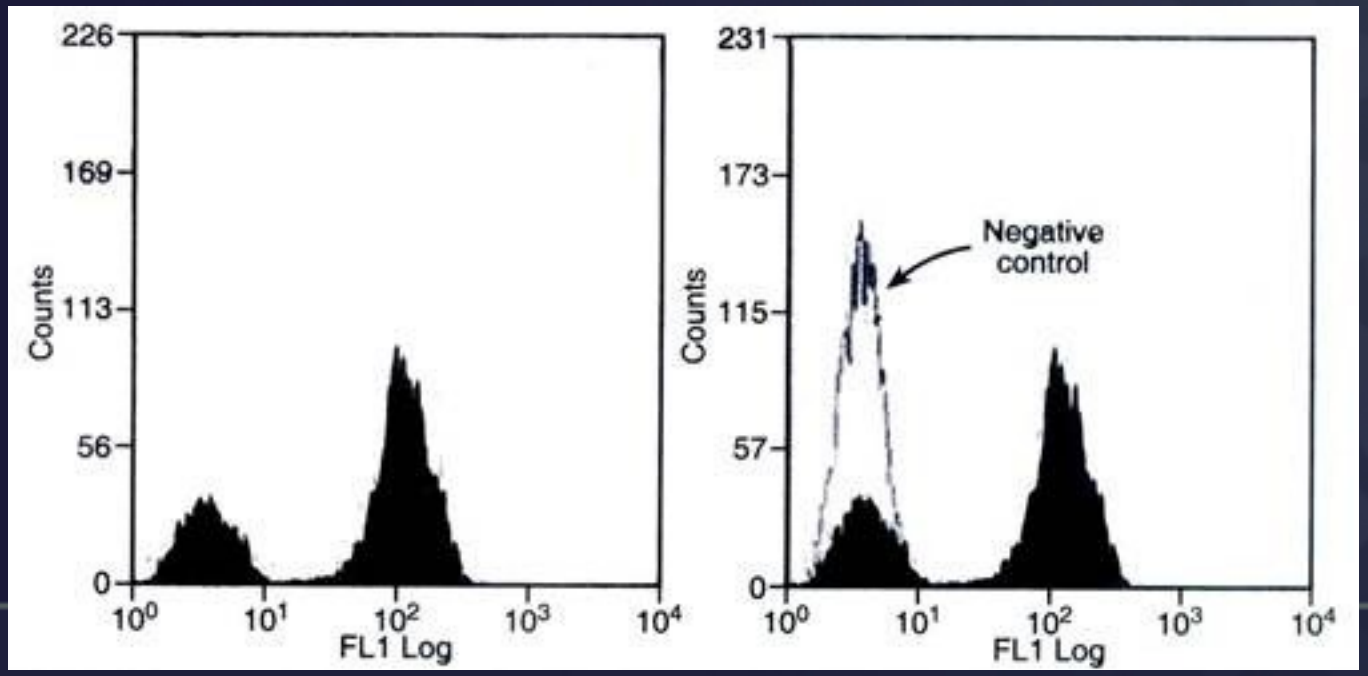
https://www.bdbiosciences.com/us/tools/s/gps

	Panel Iteration 1	Panel Iteration 2	Panel Iteration 3
Panel	CD127 FITC CD4 PE CD3 PerCP-Cy5.5 CD8 PE-Cy7 CD45RA APC CD25 APC-Cy7	CD127 PE-Cy7 CD4 PerCP-Cy5.5 CD3 FITC CD8 APC-Cy7 CD45RA APC CD25 PE	CD127 Alexa Fluor® 647 CD4 APC-Cy7 CD3 FITC CD8 PerCP-Cy5.5 CD45RA PE-Cy7 CD25 PE
	BD Horizon GPS notifications of reagent selections that may affect population resolution		
			
Data	 Mismatch of fluorochrome brightness and antigen resolution affects population resolution	 Fluorochrome brightness/antigen resolution rules applied, but spectral overlap on co-expressed markers prevents optimal resolution	 Principles taken into account and population is well resolved

CD25 and CD127 expression are low so in panel 1 APC-Cy7 and FITC are too dim to give good resolution. In panel 2 spectral overlap between PE and PE-Cy7 (both bright) prevent optimal separation. In panel 3 both AF647 and PE are very bright but with little spectral overlap

Interpretation of data

- Measurements are relative, but cytometers can be calibrated to accurately quantify parameters
- Some cytometers can provide absolute counts using volumetric sampling
- Use of controls is important



Controls

- Use as many controls as necessary for an experiment
  - Control cells
    - unstained
    - untreated
    - mock transfected
  - Reagent controls
    - Fluorescence minus one (FMO)
    - Isotype controls
  - Positive controls
  - Single stained (compensation controls)

Cell Analysers

In the WIMM:

LSR II – 5 laser
LSR Fortessa 4 laser
2 x LSR Fortessa X20 4 laser
LSR Fortessa X50 – 5 laser
3 x Attune NxT – 4 laser

Can be fitted with auto-samplers

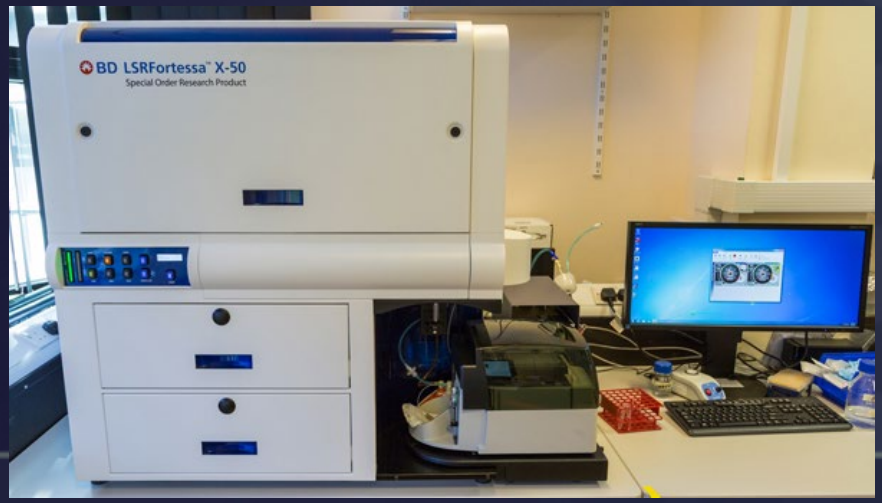
LSR II

Attune NxT

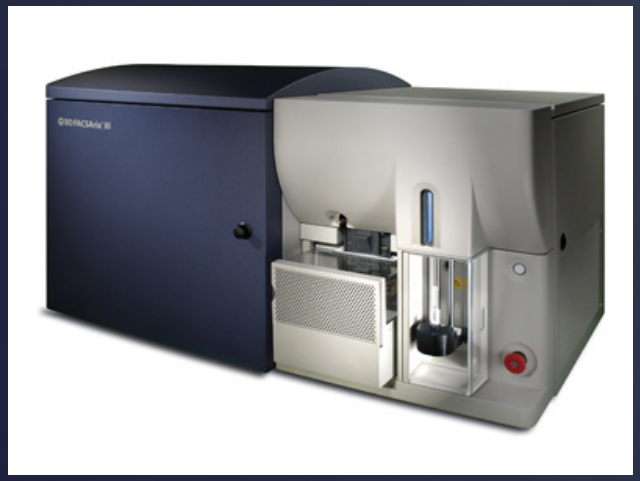
LSR Fortessa

LSR Fortessa X20

LSR Fortessa X50



Cell Sorters



In the WIMM:

FACSAria II
FACSAria III
2 x Aria Fusion
Sony MA900

Flow/Mass Cytometry common elements

- Assay one cell at a time
- Ability to measure many parameters on/in each cell
- Data format
- Some data analysis techniques
- Similar sample preparation and staining techniques