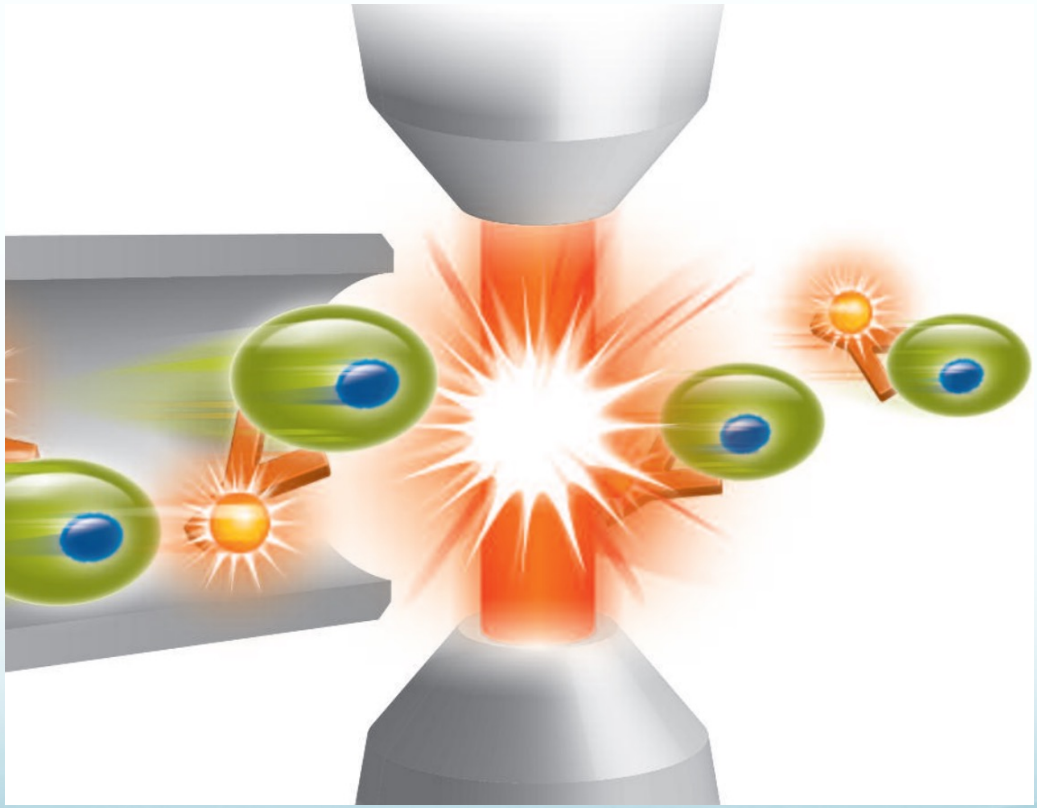
UBC Flow Cytometry Facility



Cytometry is changing

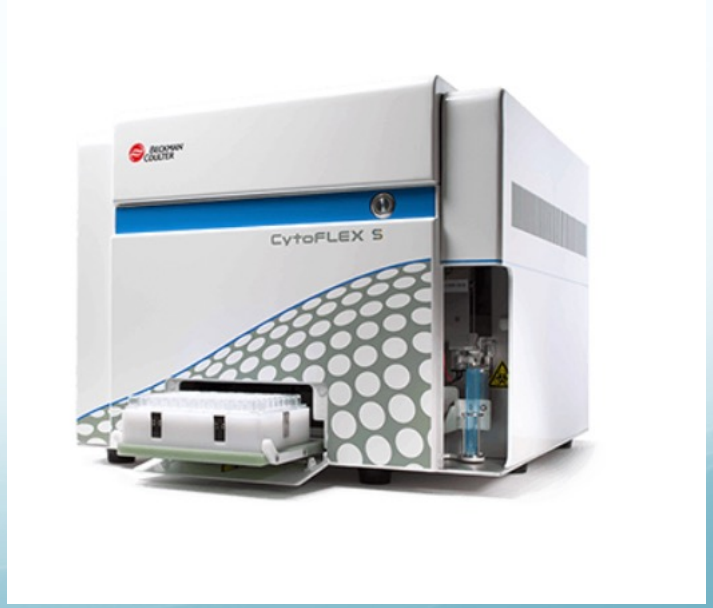
- Conventional flow cytometry
- Spectral flow cytometry
- Image flow cytometry
- Mass Cytometry
- Imaging Mass Cytometry

Quick Bio

- Graduated from Liverpool University with a degree in Medical Cell Biology.
- Trained as a Clinical Scientist for the Blood Transfusion Service, UK.
- MSc in Transfusion Medicine.
- Worked at Cambridge Antibody Technology.
- Flow Core, MRC Addenbrookes, Cambridge, UK
- Moved to UBC in 2001

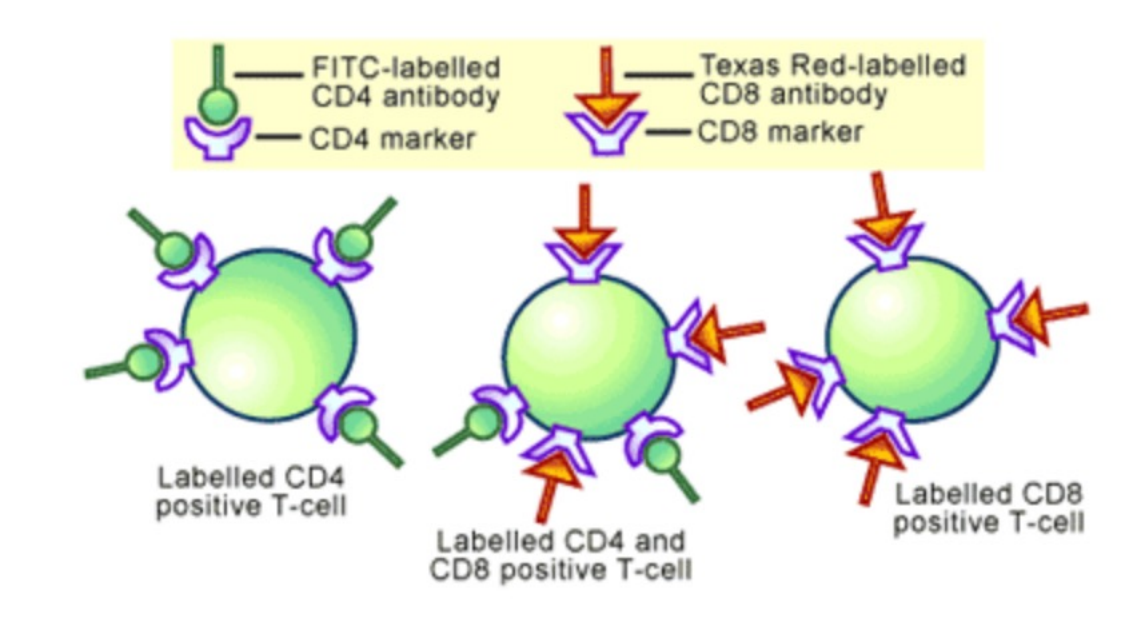
What is flow cytometry

- Flow Cytometry is a technique used to detect and measure all the physical characteristics of a population of cells or particles.
- In this process, a sample containing cells or particles is suspended in a fluid and injected into the **flow cytometer** instrument.

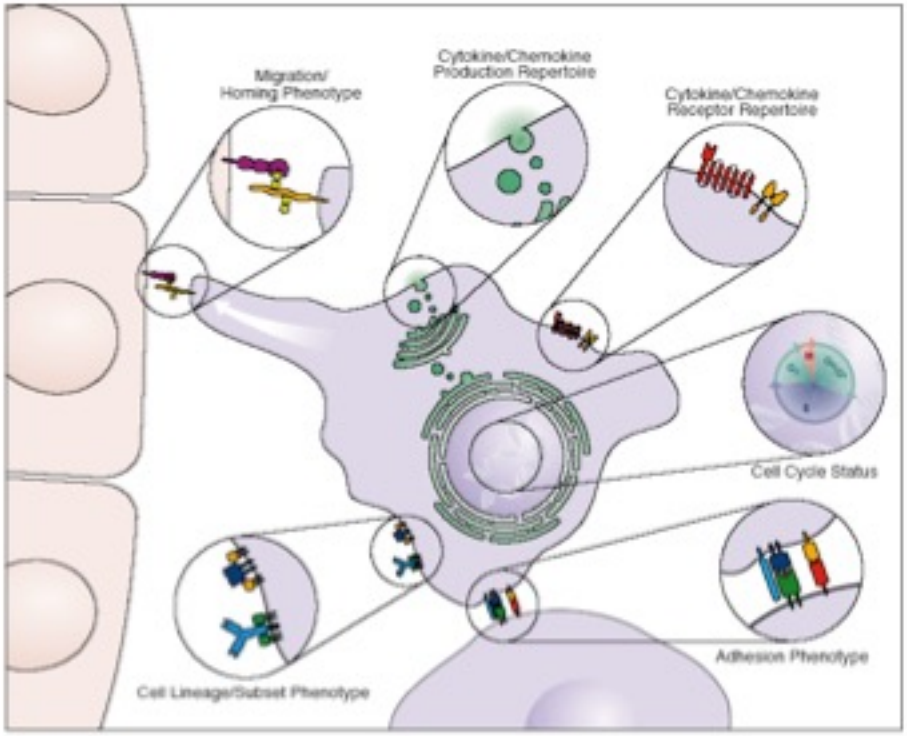


The reason we use flow

- Unlike microscopy we can look at millions of cells.
- Flow cytometry provides a well-established method to identify cells in solution and is most commonly used for evaluating peripheral blood, bone marrow, and other body fluids.
- Flow cytmetry studies are used to identify and quantify immune cells and characterize hematological malignancies.[1] They can measure:

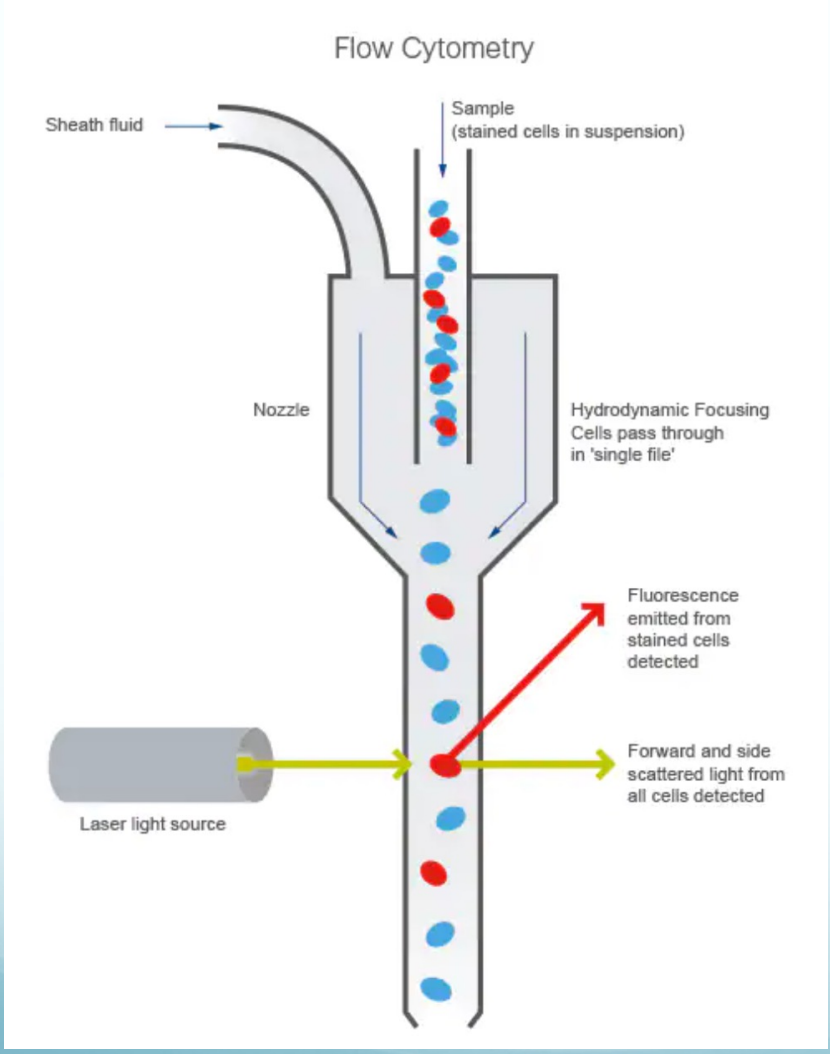


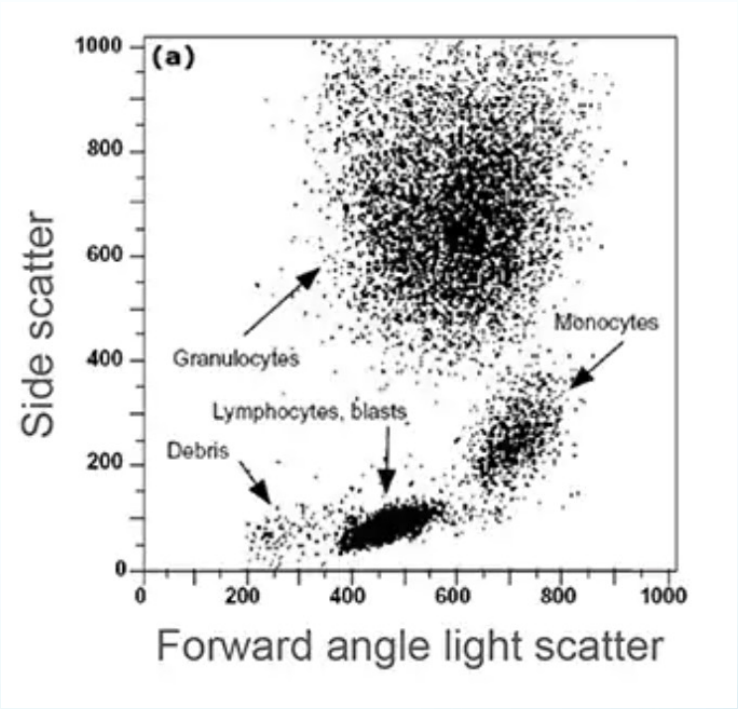
What can we detect



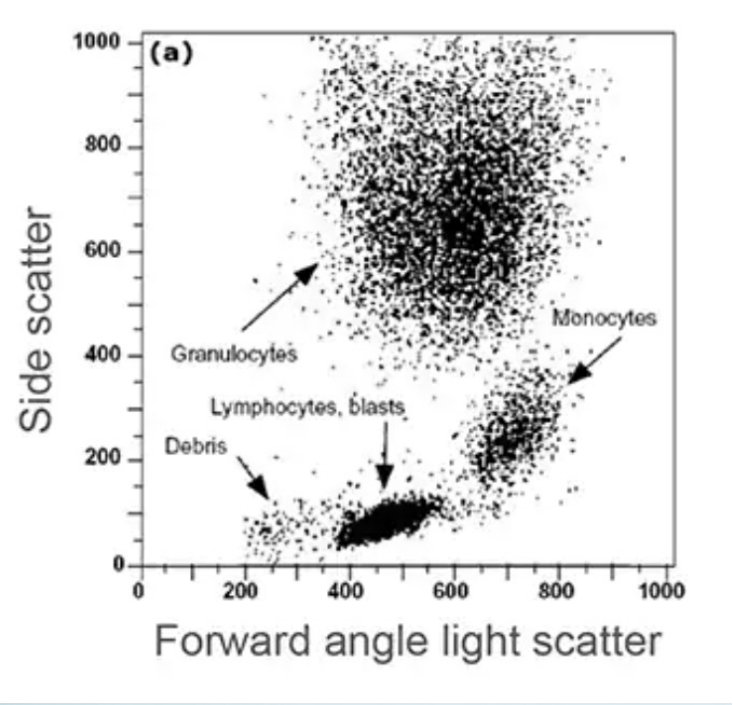
- cell size
- cell granularity
- total DNA
- new synthesized DNA
- gene expression
- surface receptors
- intracellular proteins

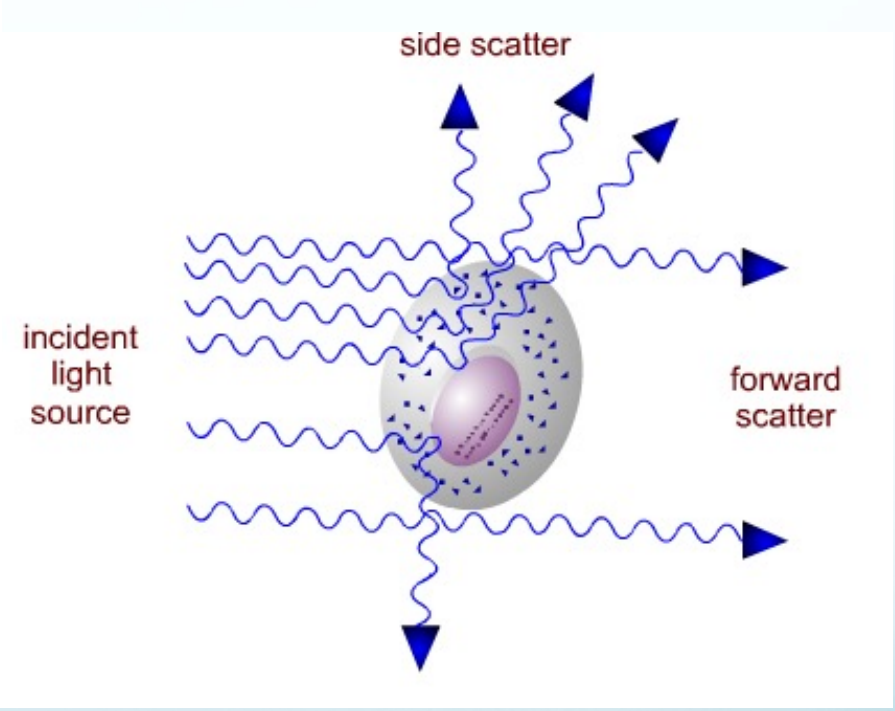
Fluidics: How they work





FSc and SSc: How they work

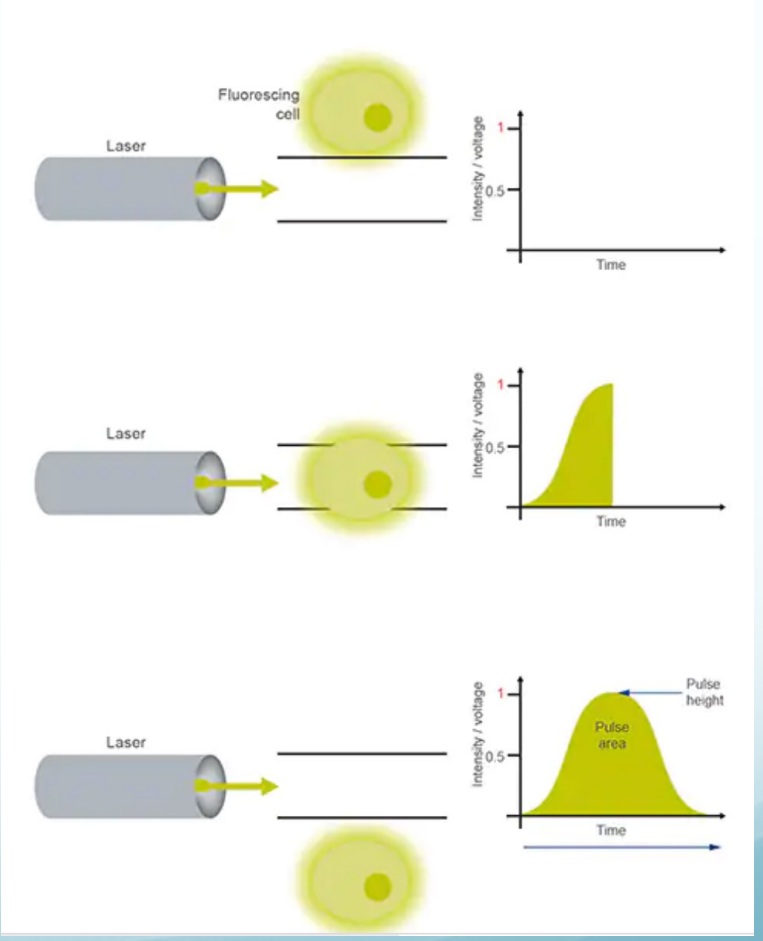




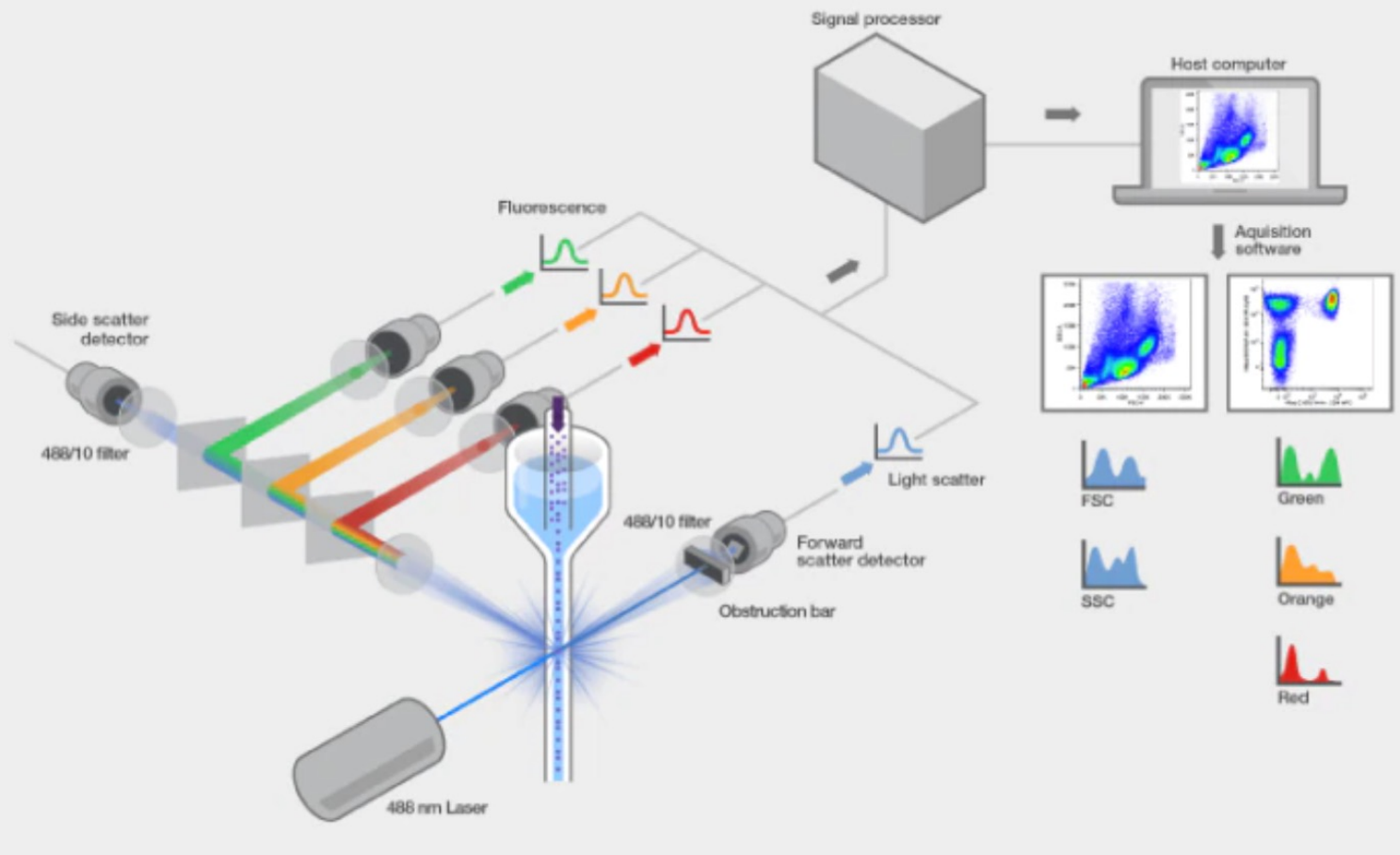
Detectors: How they work

As the fluorescing cell passes through the laser beam, it creates a peak or pulse of photon emission over time. These are detected by the PMT and converted to a voltage pulse, known as an event.

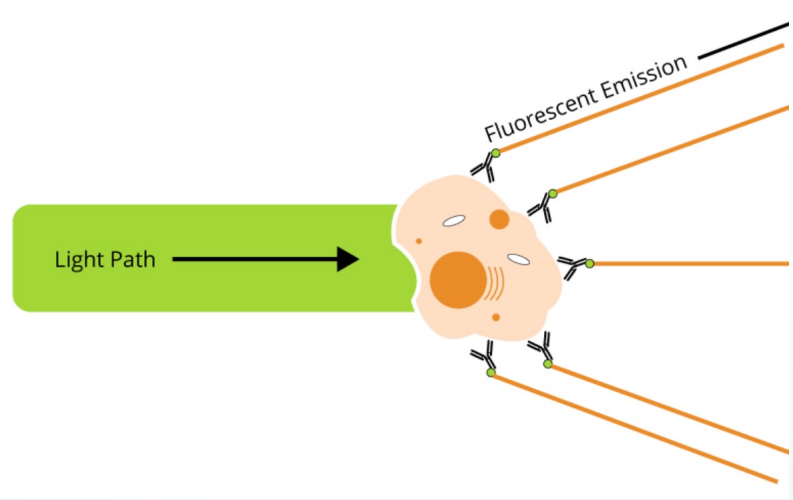
The total pulse height and area is measured by the flow cytometer. The measured voltage pulse area will correlate directly to the intensity of fluorescence for that event.



Instrument: How it comes together

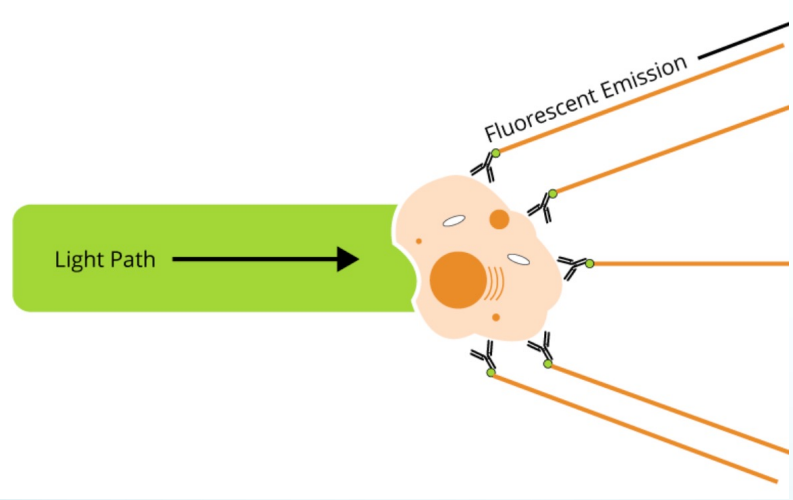


Fluorphores



- Cell populations can sometimes be separated based on FSC and SSC, but cells can also be separated by whether they express a specific protein.
- In this case, a fluorophore is usually used to stain the protein of interest.
- Fluorophores used for the detection of target proteins emit light after excitation by a laser of compatible wavelength.

Fluorphores



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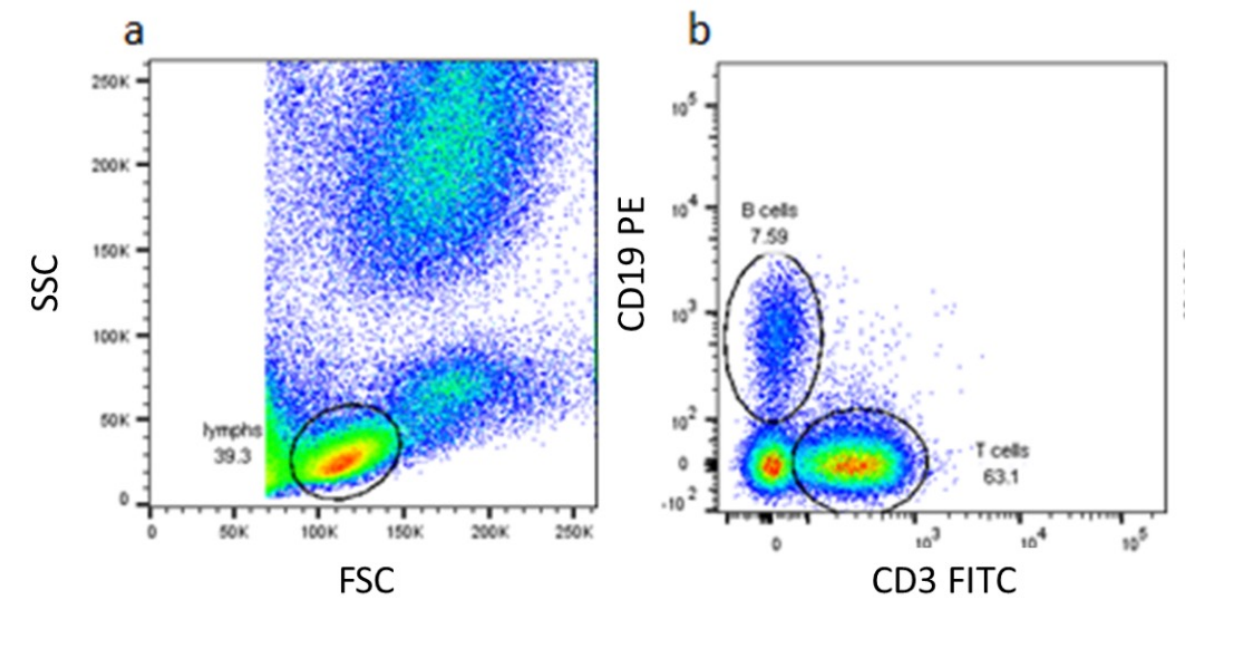
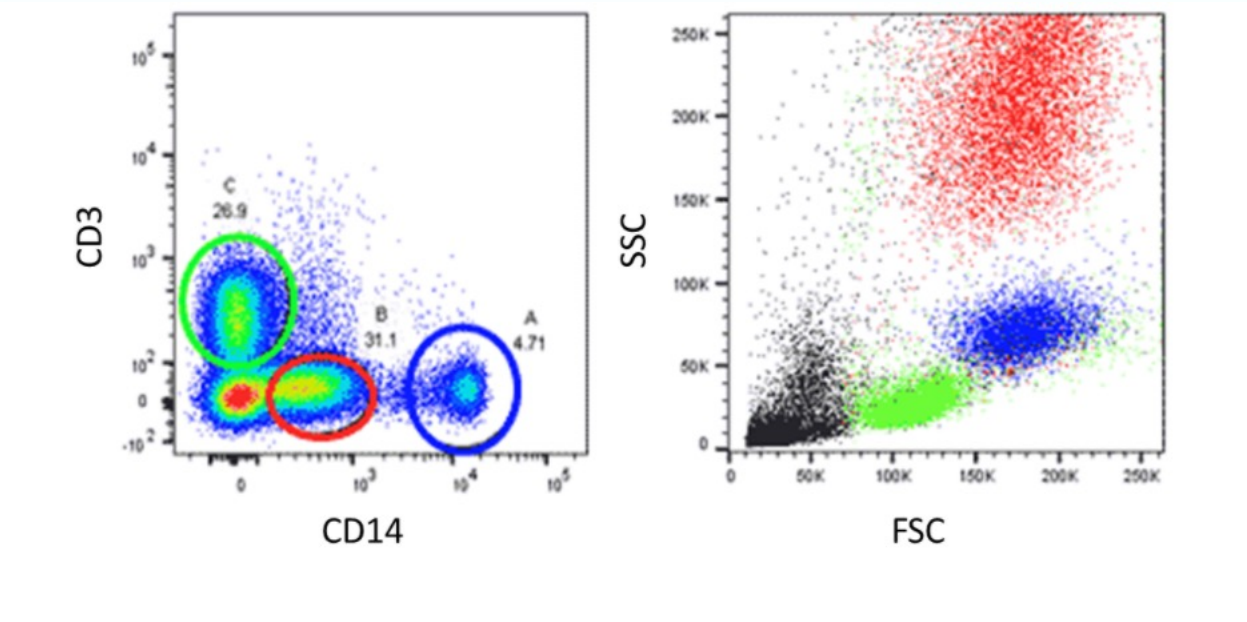
Antibody Gating



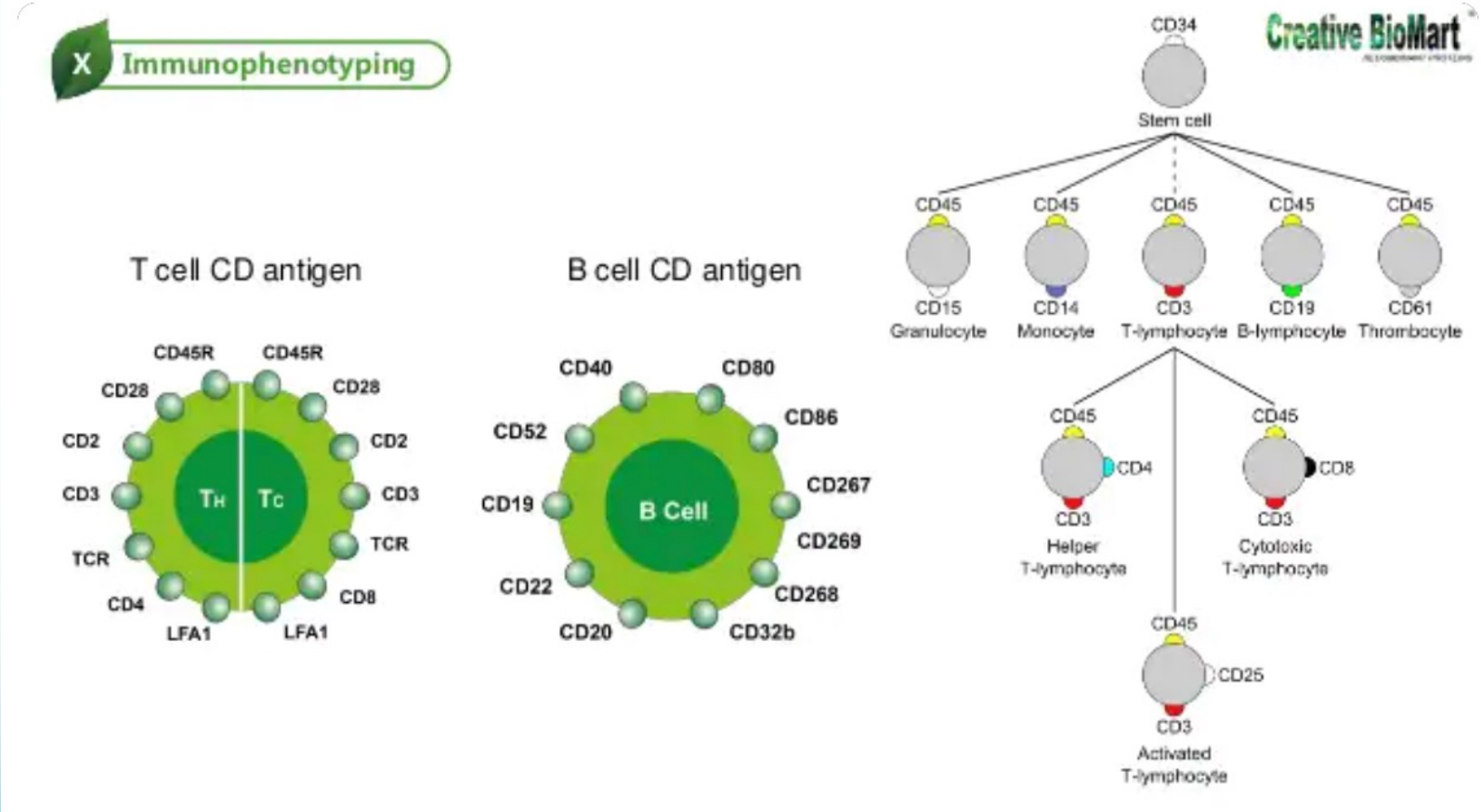
- Once a certain population has been gated out on the basis of scatter properties, the next step is to further divide it into sub-populations based on surface (or intracellular) markers. Lymphocytes are first gated on the basis of FSC and SSC and then divided into T cells and B cells on the basis of surface expression of CD3 and CD19 respectively..

Back Gating



- Back-gating is a method to confirm a gating pattern. It is usually done when one is trying out a new gating strategy or there is a concern of non-specific staining and false positives.

CD Markers

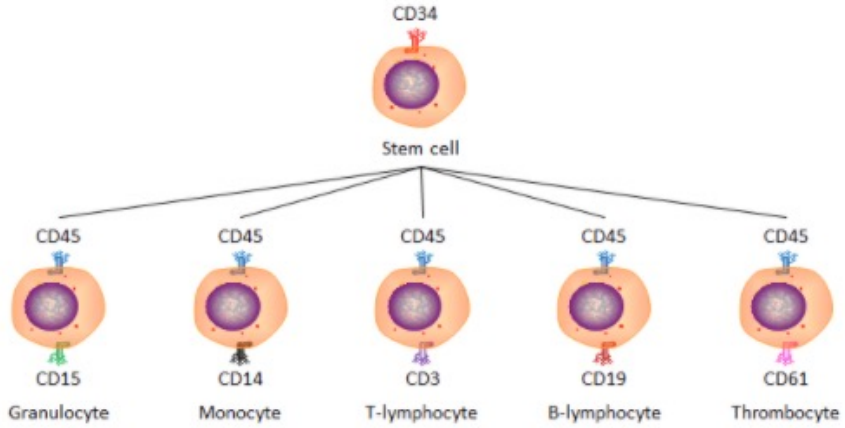


https://www.slideshare.net/ShangeryFang/cluster-of-differentiation

CD Markers

	Key Markers - Human
T Cell	CD3 CD4 CD8
B Cell	CD19 CD20
Dendritic Cell	CD11c CD123
NK Cell	CD56
Stem Cell/ Precursor	CD34 *hematopoetic stem cell only*
Macrophage/ Monocyte	CD14 CD33
Granulocyte	CD66b
Platelet	CD41 CD61 CD62
Erythrocyte	CD235a
Endothelial Cell	CD146
Epithelial Cell	CD236

2



Setting up the cytometer

- Quality control: is the flow cytometer working correctly?
- Have we prepared the best sample possible?
- Detecting ‘good’ cells FSc vs SSc: cells vs debri vs clumps.
- Training the instrument: what does an unstained cell look like?
- Compensating the different fluorochromes
- Gating order: removing autofluorescent/dump channel
- Gating the target populations: using FMOs

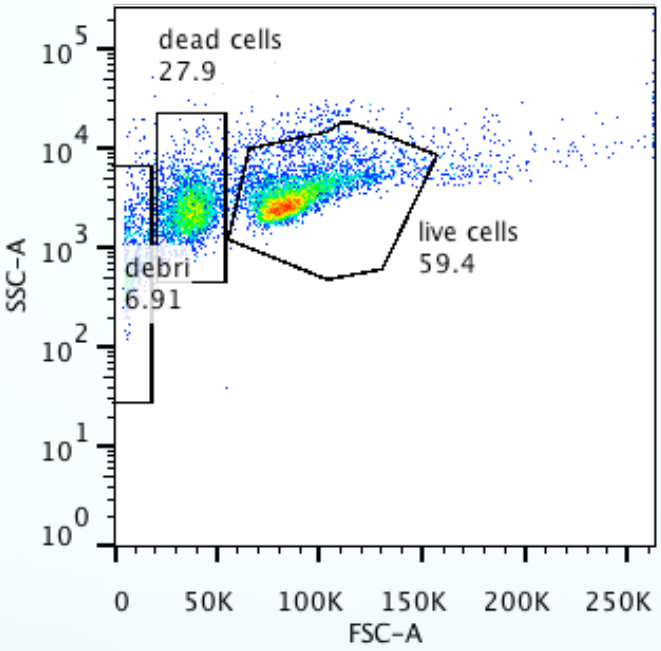
Four stages for setting up

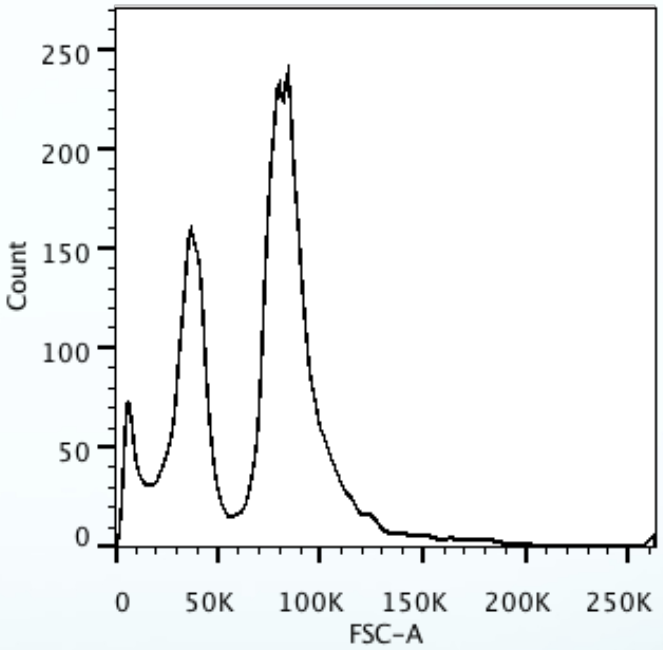
- identify the cells of interest, excluding the dead cells.
- set-up the instrument by defining a positive cell vs a negative cell.
- use controls to compensate the fluorochromes to identify cell labeled with two colours.
- use isotype/FMOs to correctly gate populations of interest.

Detecting ‘good’ cells FSc vs SSc: cells vs debri vs clumps.

Dead cells stick antibodies rather than live cells binding to antibodies.
Clumps of cells can be a mixture of positive and negative cells.

Identify Cell Population

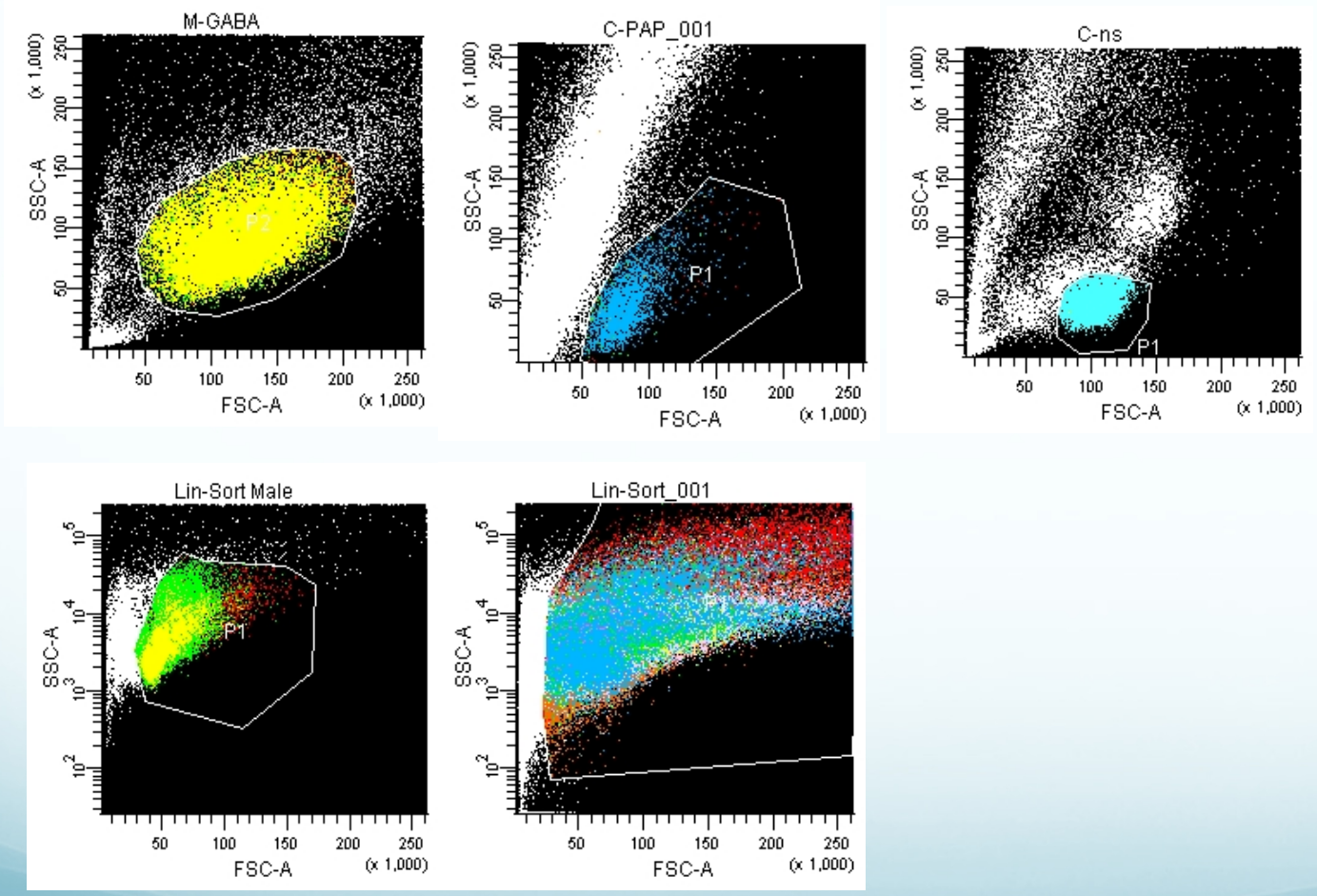




Sea water sample contains too many events to collect, so we need need to use the threshold and exclude some of the
events.

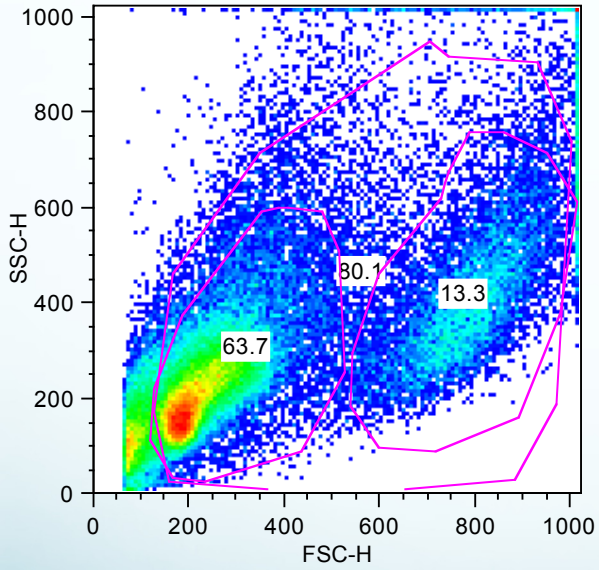
The population of interest now becomes visible and we are able to work with it and analyse the profile.

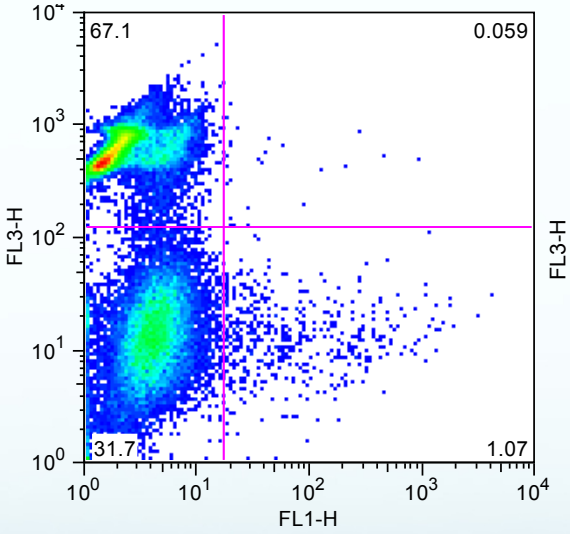
Finding our cells

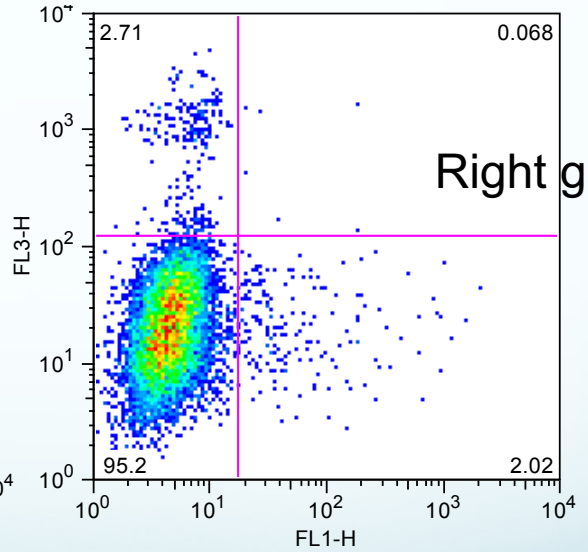


Viability dye (PI or 7AAD)

vs fixable dyes





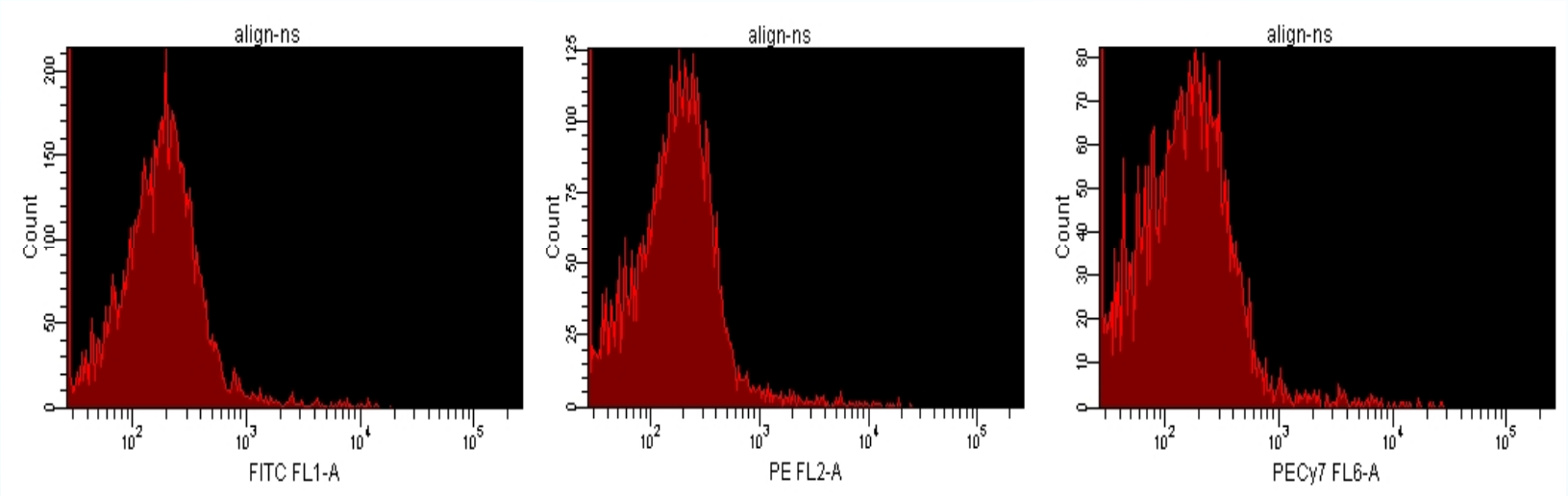


Right gate

Using a viability dye allows you to exclude dead cells which may appear viable. PI works on fixed cells, but you can get fix-perm viability dyes

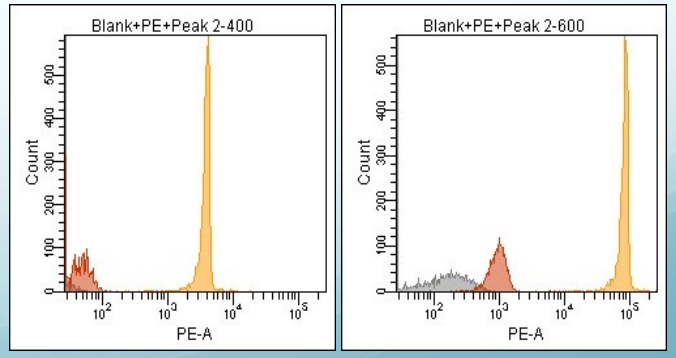
- Training the instrument: what does an unstained cell look like?

Setting the correct voltage (or gain)



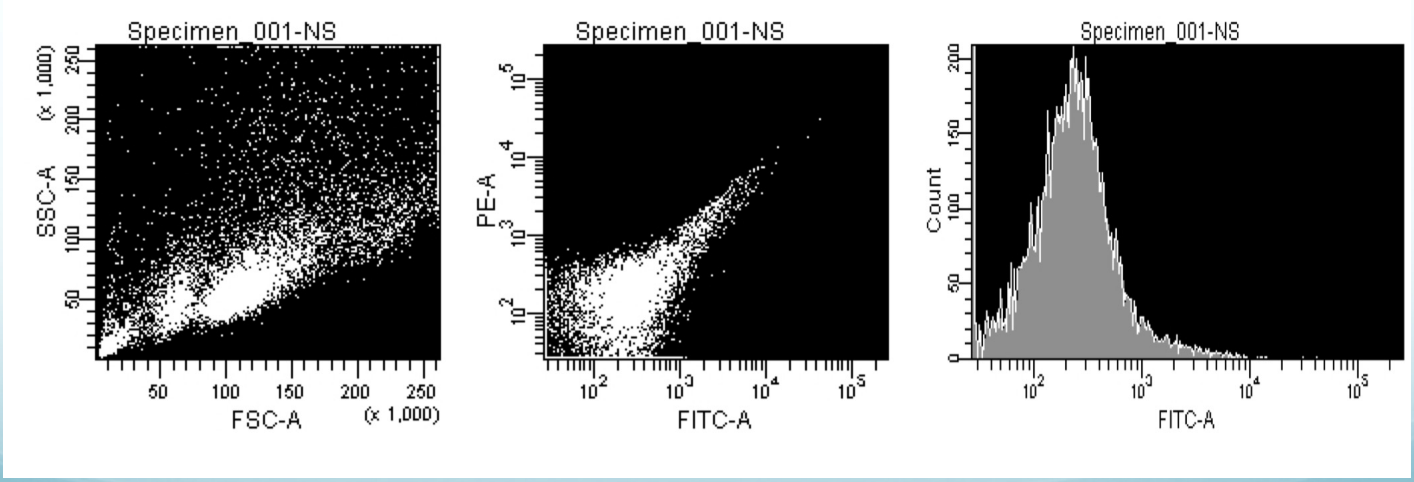
The negative cells are used to set the voltage for each PMT, as it is tissue specific. CS&T beads could be used to give you starting voltages if your sample was limited.

400 V **600 V**

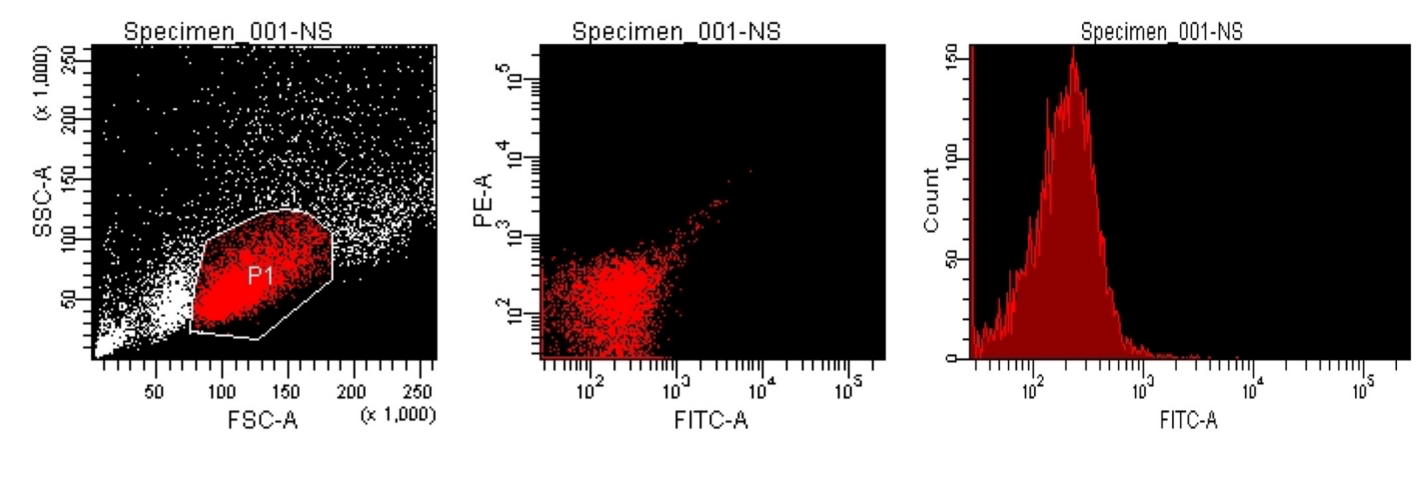


- Work Flow

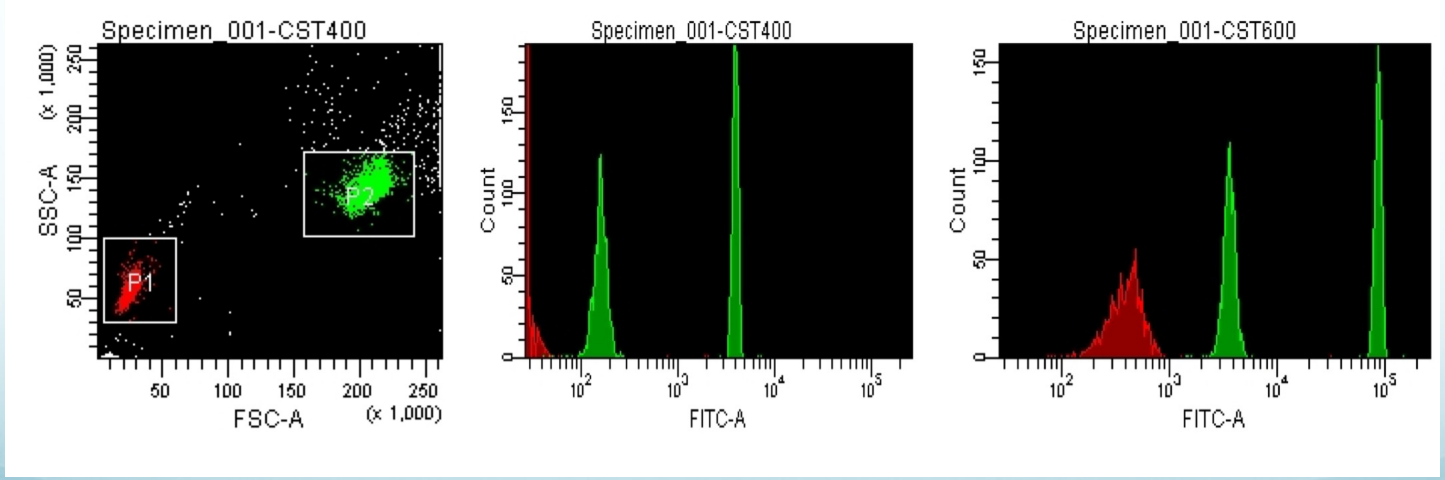
Run the no stain control to set the correct voltage for the FSc and SSc parameters. Position the cells population in the middle of the plot, so that we can see debri and doublets.



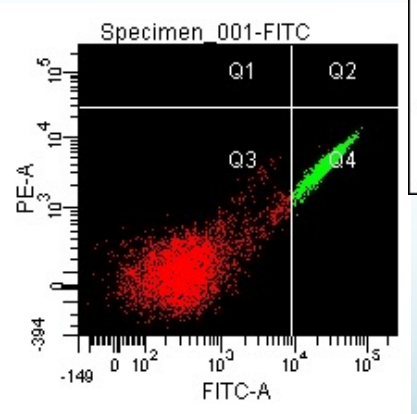
Gate around the cell of interest, or gate out what you know to be doublets and debri; this reduces the background noise from all the other cells. Correct the voltage for the fluorescent parameter so the peak is positioned just before 10^3.



The optimal voltage can be determined by using CS&T beads, but as these are optimized for human lymphocytes, you need a system that would work for any cell type. Increase the voltage until you can resolve all three populations.



Compensation is much easier than you think and the trouble comes from poor quality samples and mixed populations of cells which make interpreting the data much harder.
Most instruments come with an Auto-compensation option in the software, but compensation should be fully understood before using this.
Once the no stain has been run and the voltages corrected, there should be no further need to change the voltages, so all that is left to do is compensate.



Tube Name: FITC

Population	FITC-A Mean	PE-A Mean
☒ Q3	580	163
☒ Q4	29,690	4,363

Tube Name: FITC

Population	FITC-A Mean	PE-A Mean
☒ Q3	580	79
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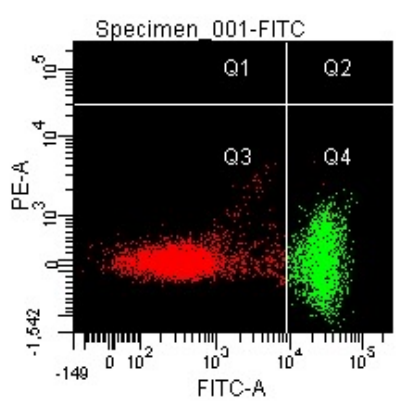


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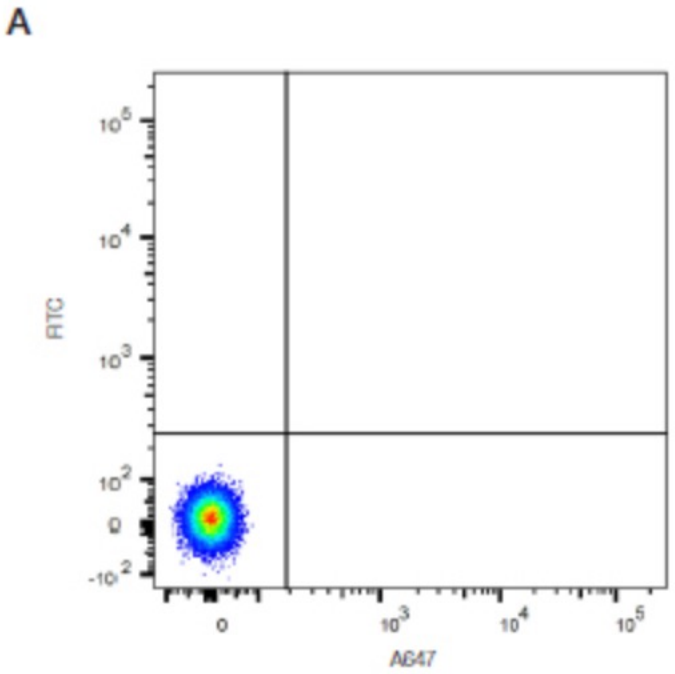
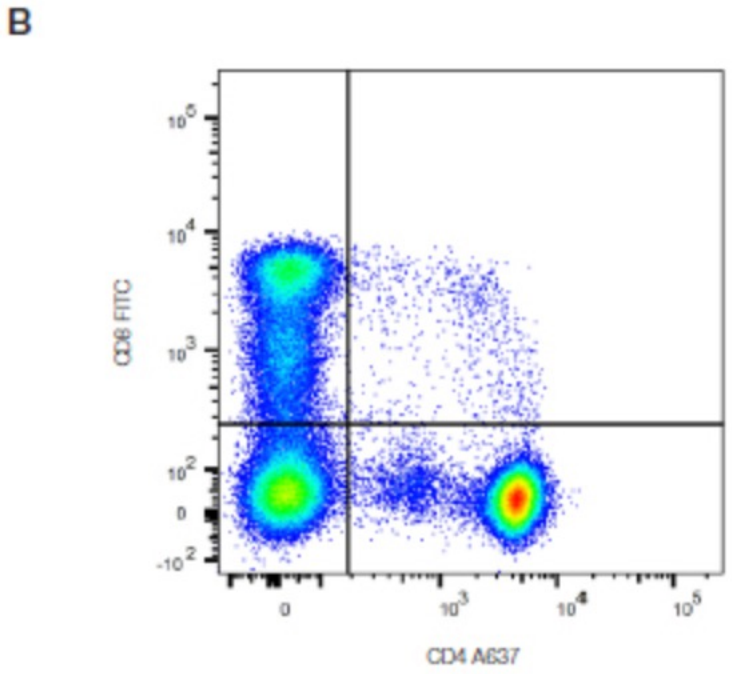
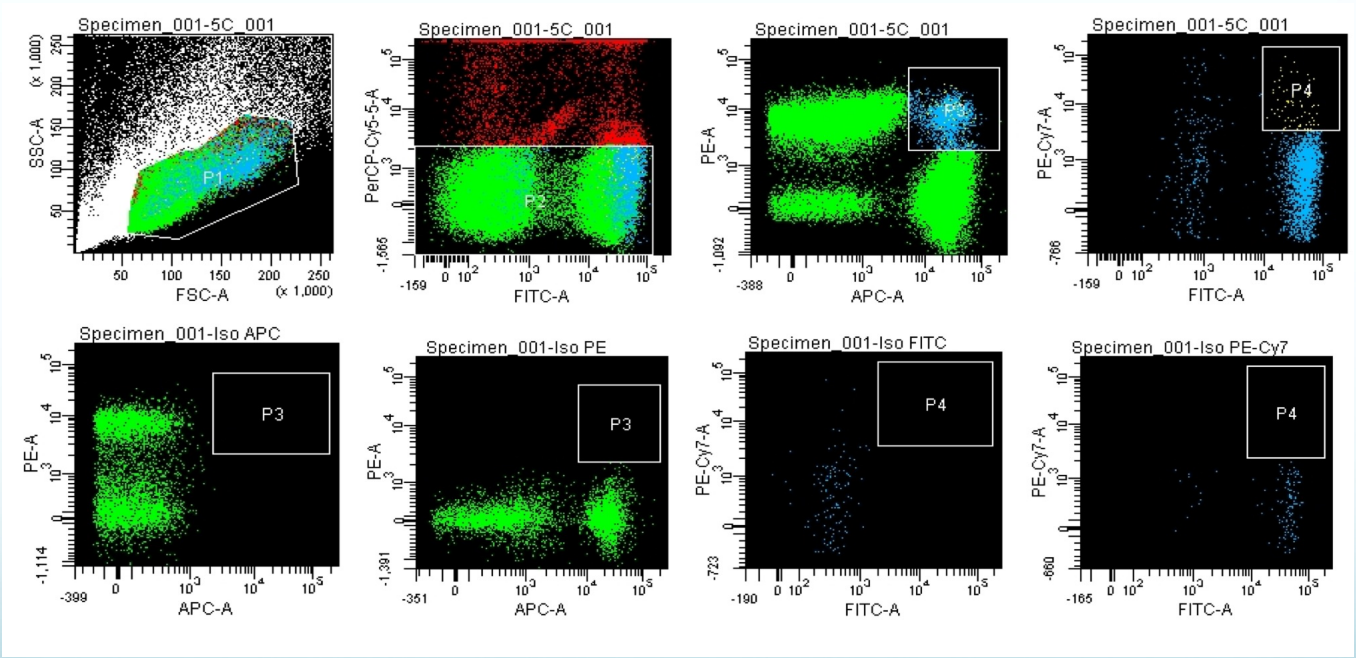

A
FITC
A647
0
10^3
10^4
10^5
10^2
-10^2


B
CD8 FITC
CD4 A637
0
10^3
10^4
10^5
10^2
-10^2

Once the sample is run and a large file is collected, we need to run the FMOs to determine the correct gating.



Compensating the different fluorochromes.

- Antibody capture beads vs flourescent beads vs stained sample
- Auto-compensation vs manual

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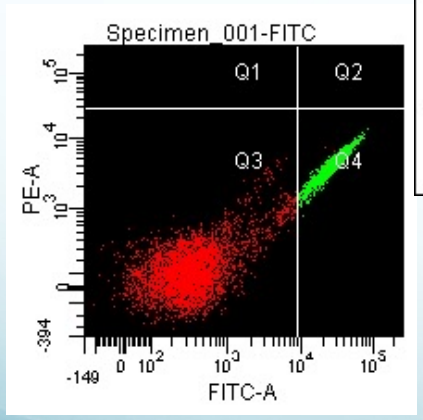

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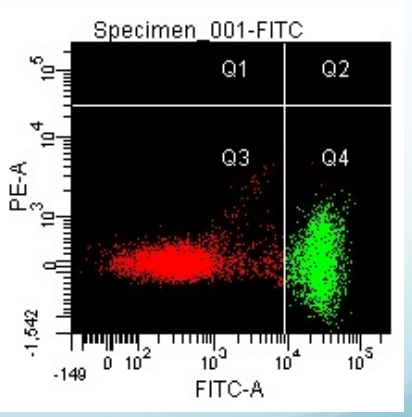
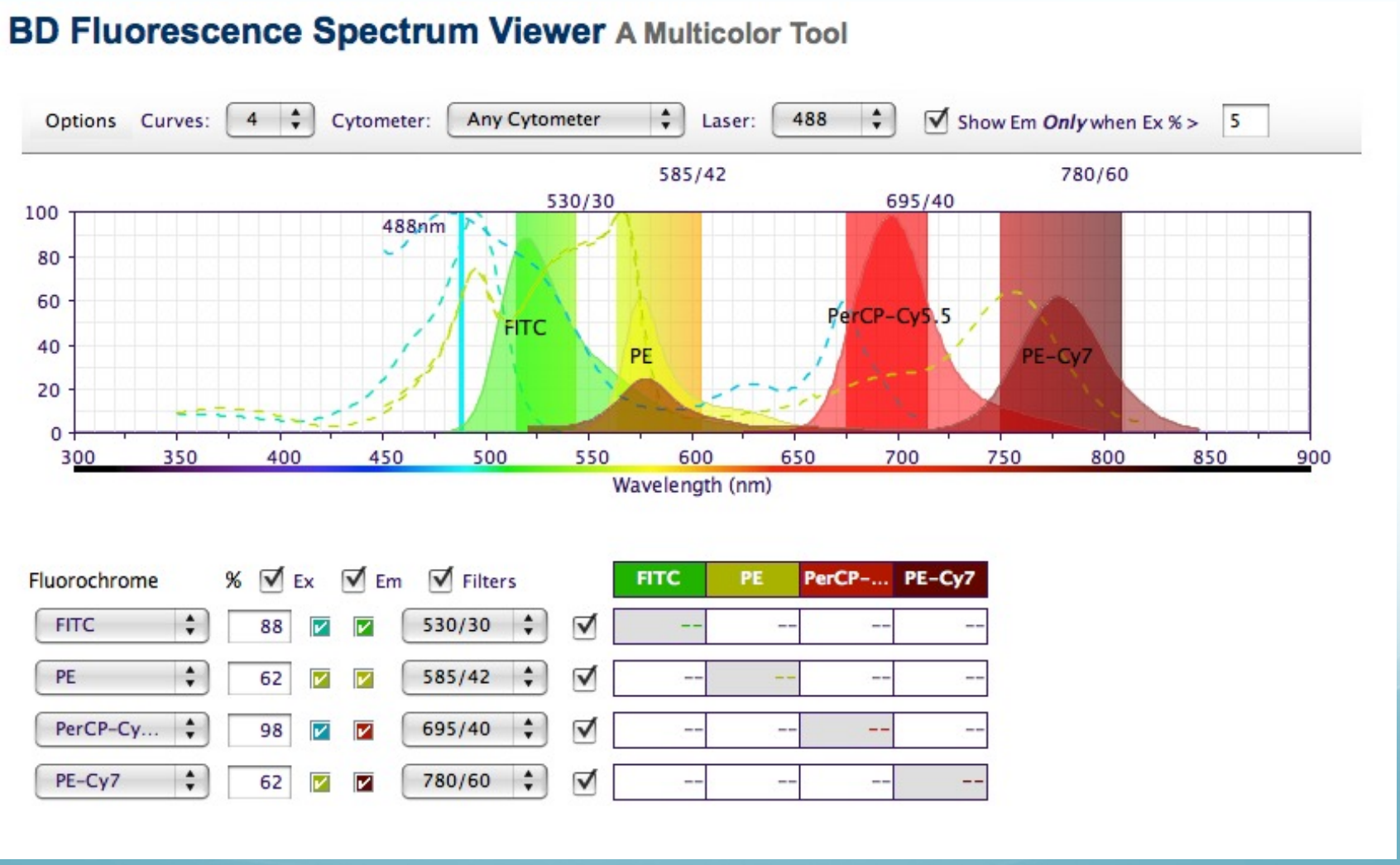


Compensation



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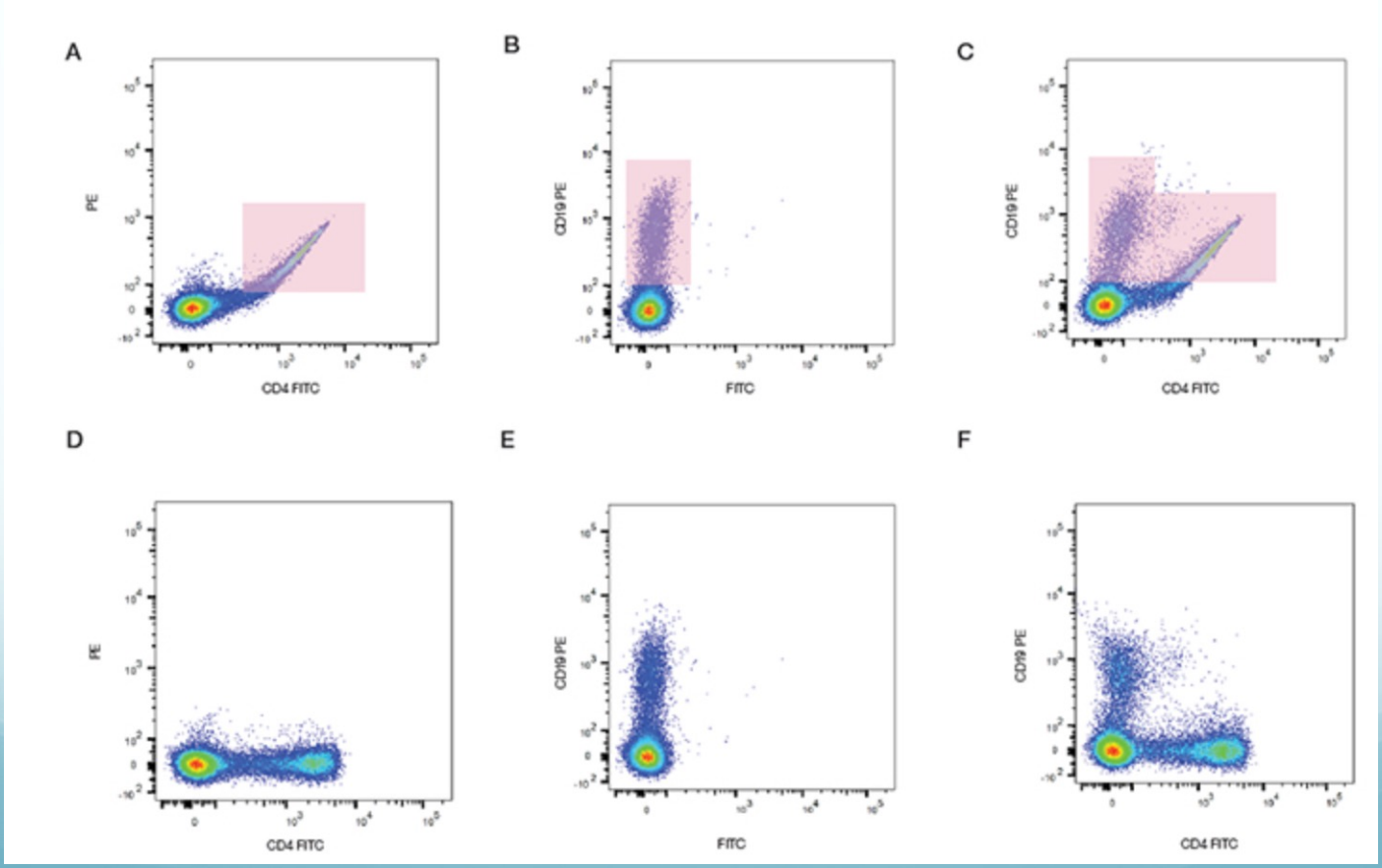
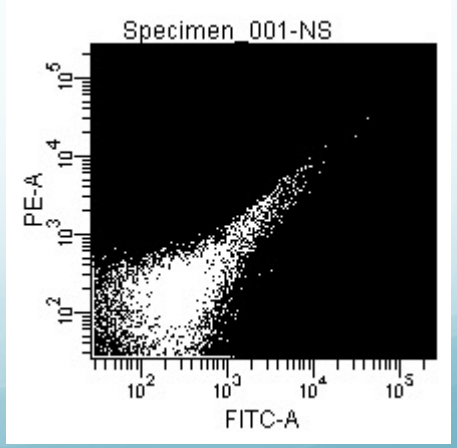


Gating order: removing autofluorescent/dump channel

- Gate the live/viable cells, then remove the cells with autofluoresence, leaving the ‘good’cells.



Resolving positive from negative

- The main objective is to define multiple populations of cells that are identified by different combinations of markers.
  - Positive for some and negative for others
- A positive cell can only be defined as positive if we can identify it over the background noise.
  - Dim positive cells can be hidden by poor compensation
- Once we can see a population we can ask more questions.
  - Is the population dim positive, or bright positive?
  - Does the population have a tight CV, uniformed expression?

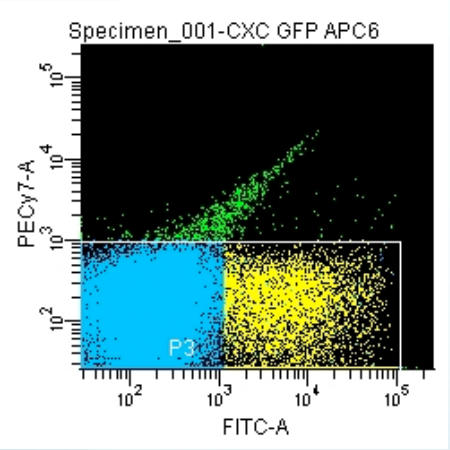
Influencing factors

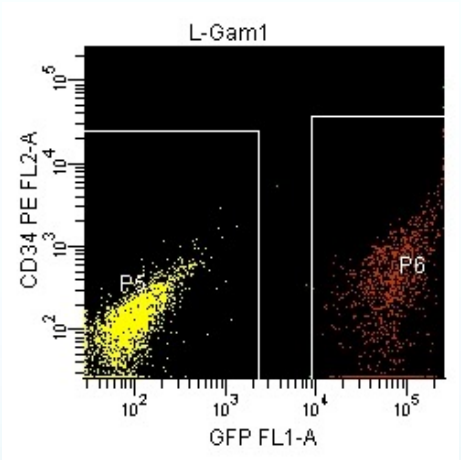
Background fluorescence

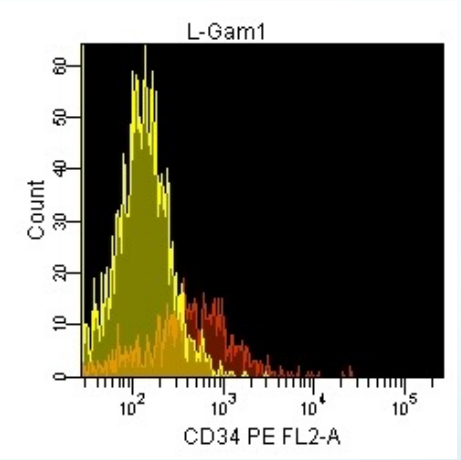
- non-specific binding by antibodies
- auto fluoresence due to a mixed population

Fluorescence spread

- spill over
- incorrect PMT settings

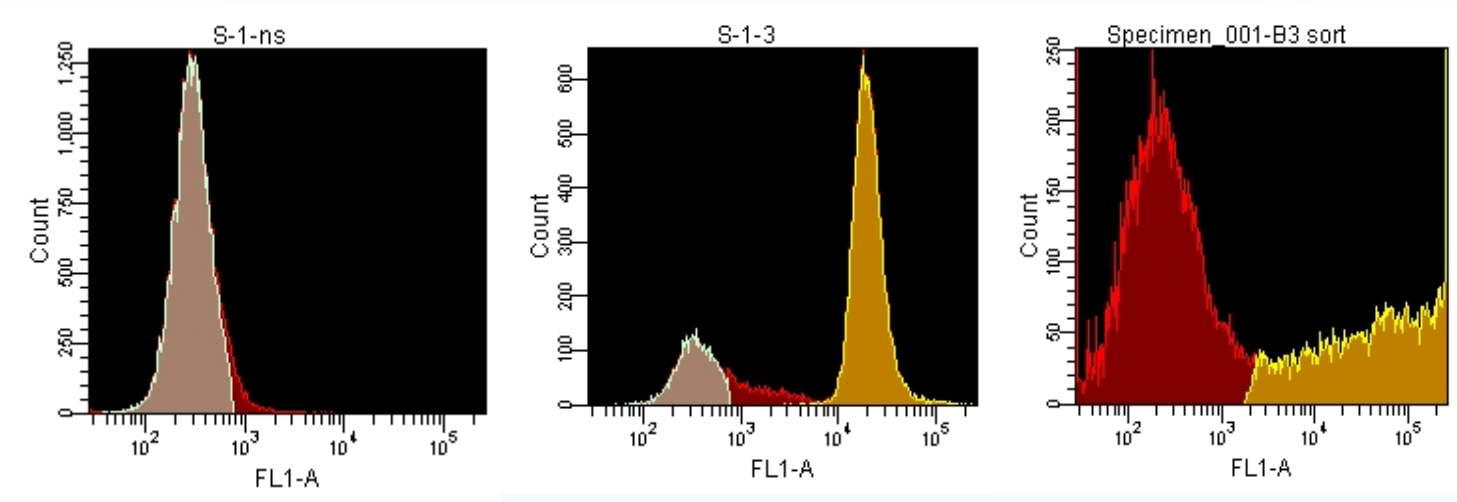






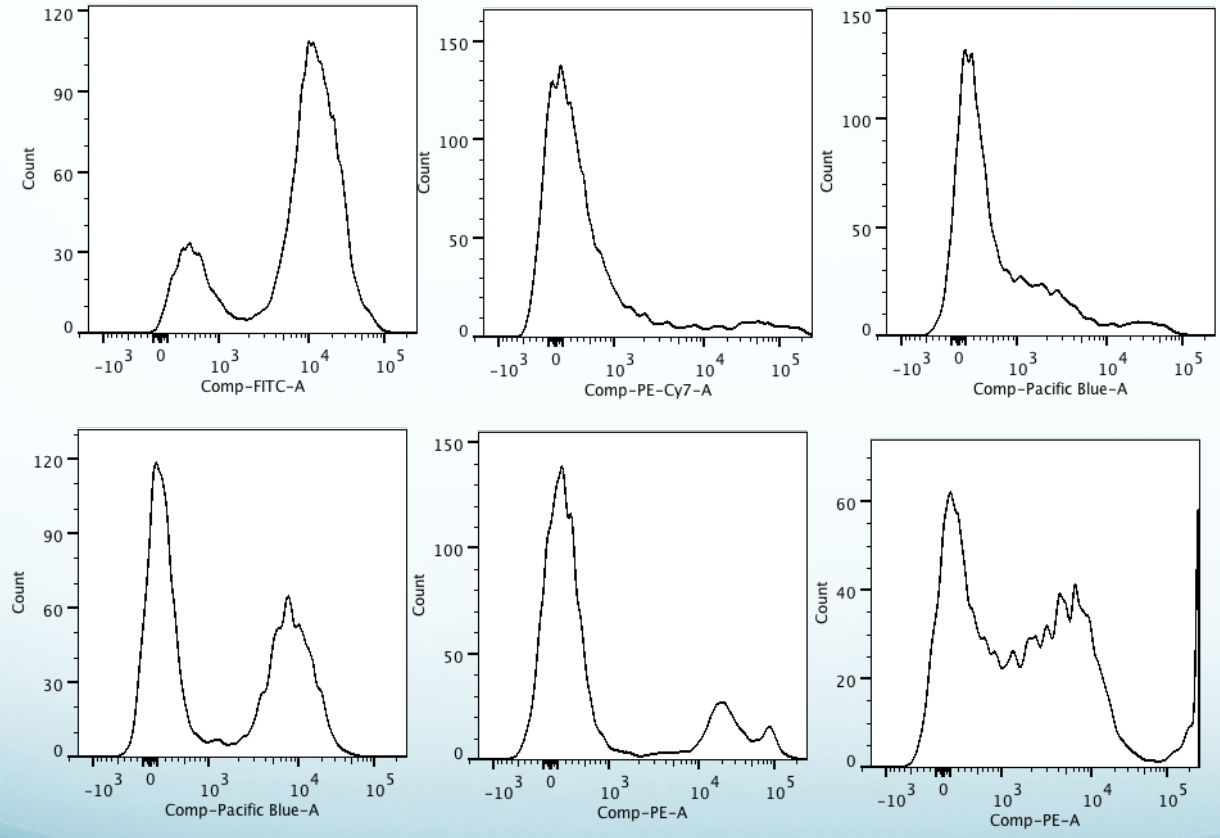
Some of these factors we can control, others are down to sample preparation and correct instrument set-up.

Profile of antibody binding

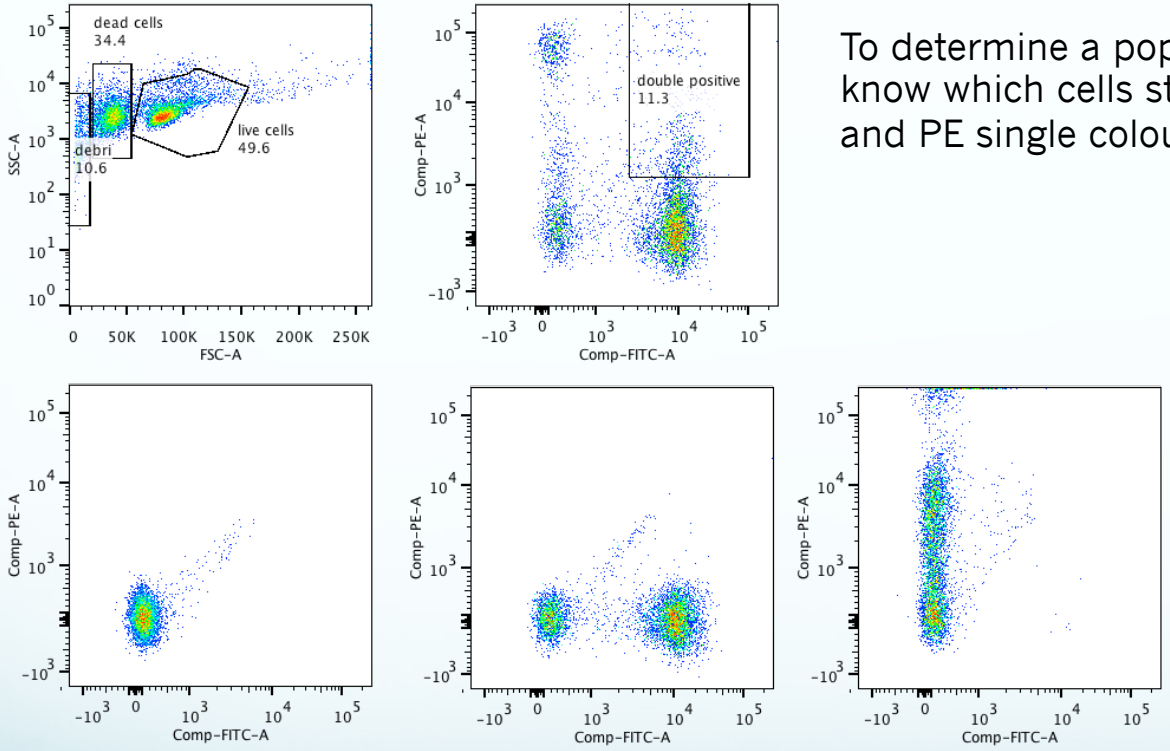


The shape of the peaks gives a lot of information about the expression of the surface receptors. A tight peak indicates that all the cells have the same expression; a broad peak indicates a range of expression levels.

Ab Binding Profiles

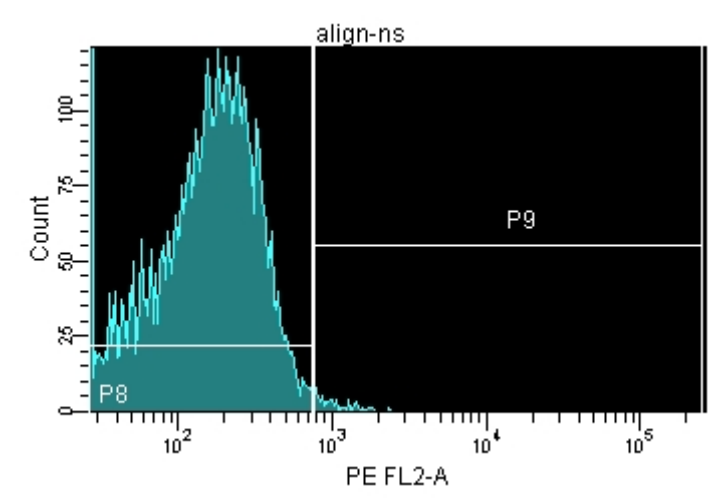


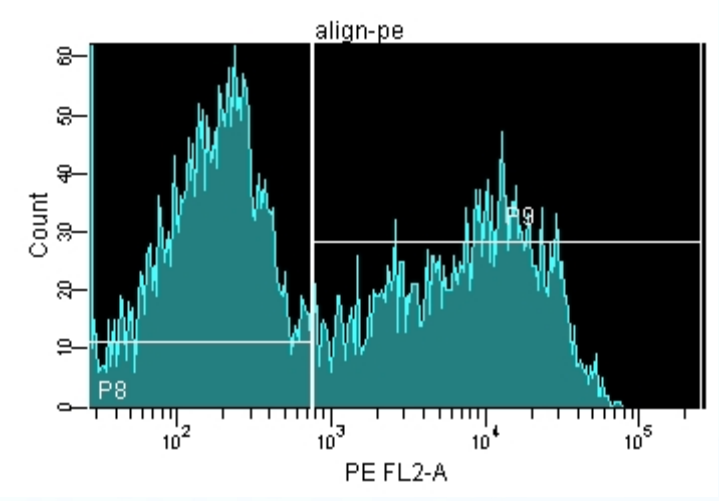
Population Gating Strategy



To determine a population we first need to know which cells stain. We use a blank, FITC and PE single colour controls

Report values





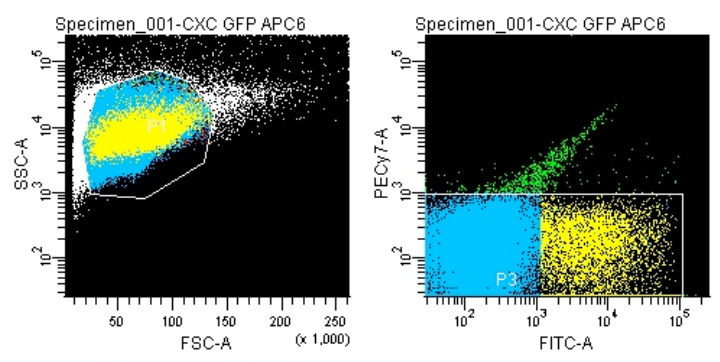
% Negative = 98%
% Positive = 2%

Mean Negative = 97

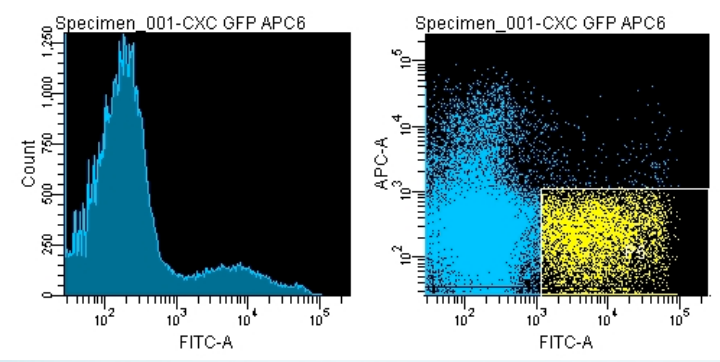
% Negative = 67%
% Positive = 33%

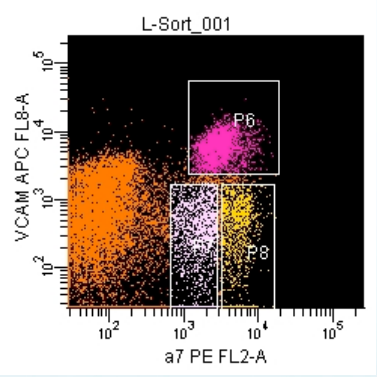
Mean Negative = 110
Mean Positive = 11,180

Examples of analysis

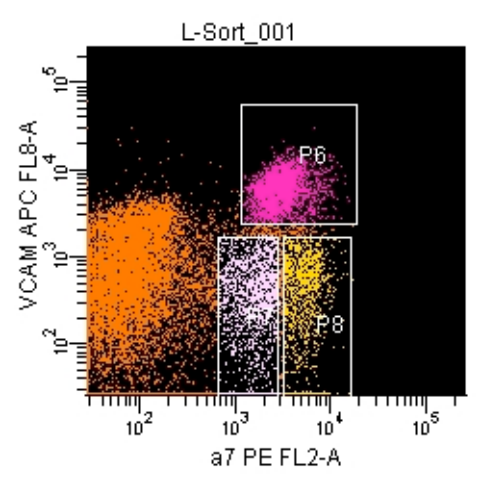


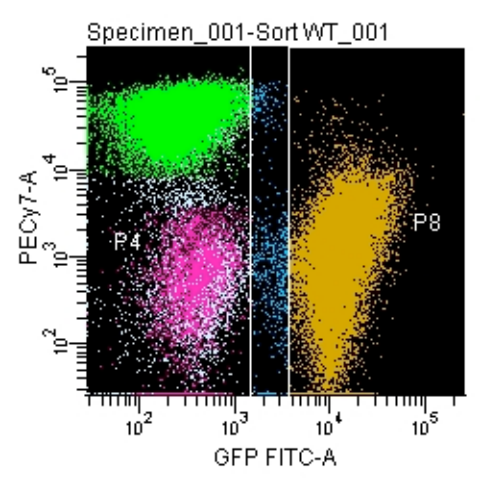
- Gate the live cells
- Exclude the autofluorescence
- Gate the positive population
- Calculate subpopulations

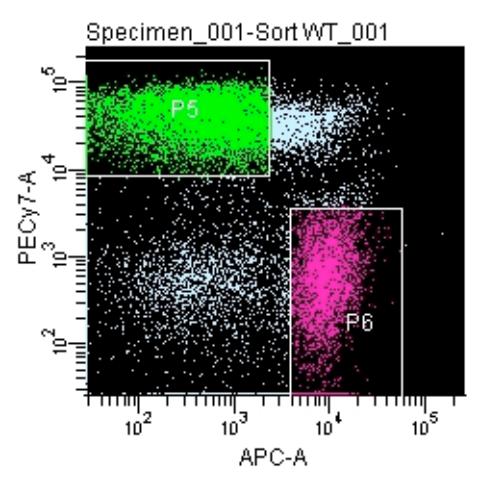




Examples of analysis

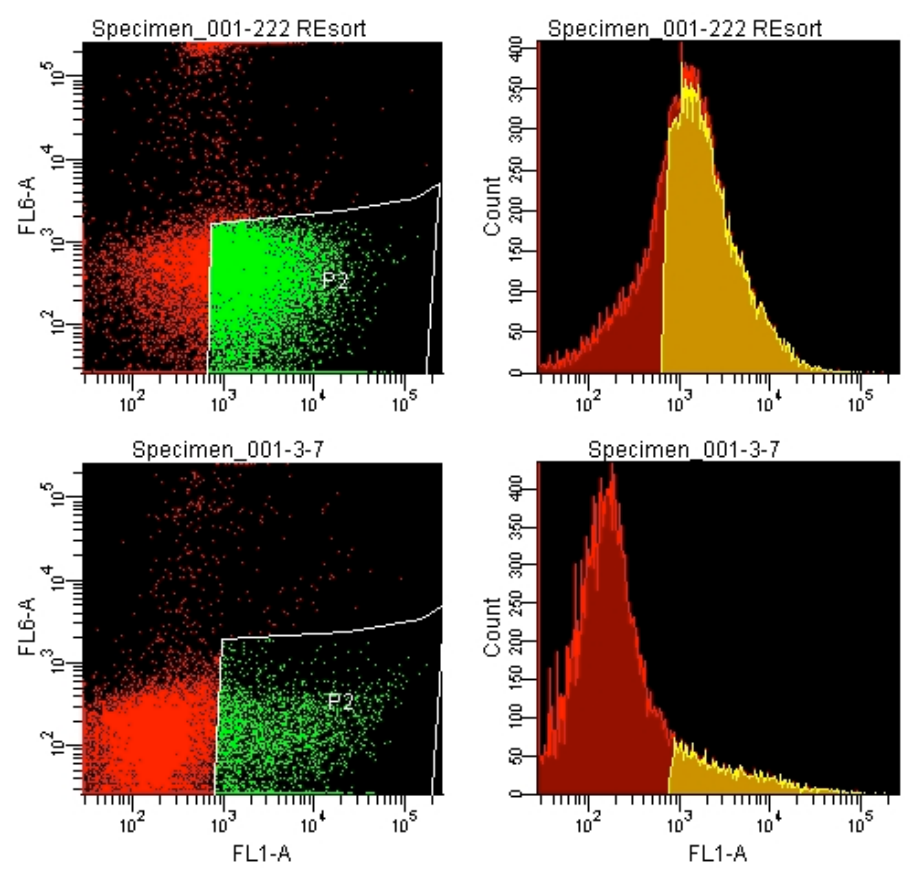






- Gate the live cells
- Exclude the autofluorescence
- Gate the positive population
- Calculate subpopulations

Examples of analysis

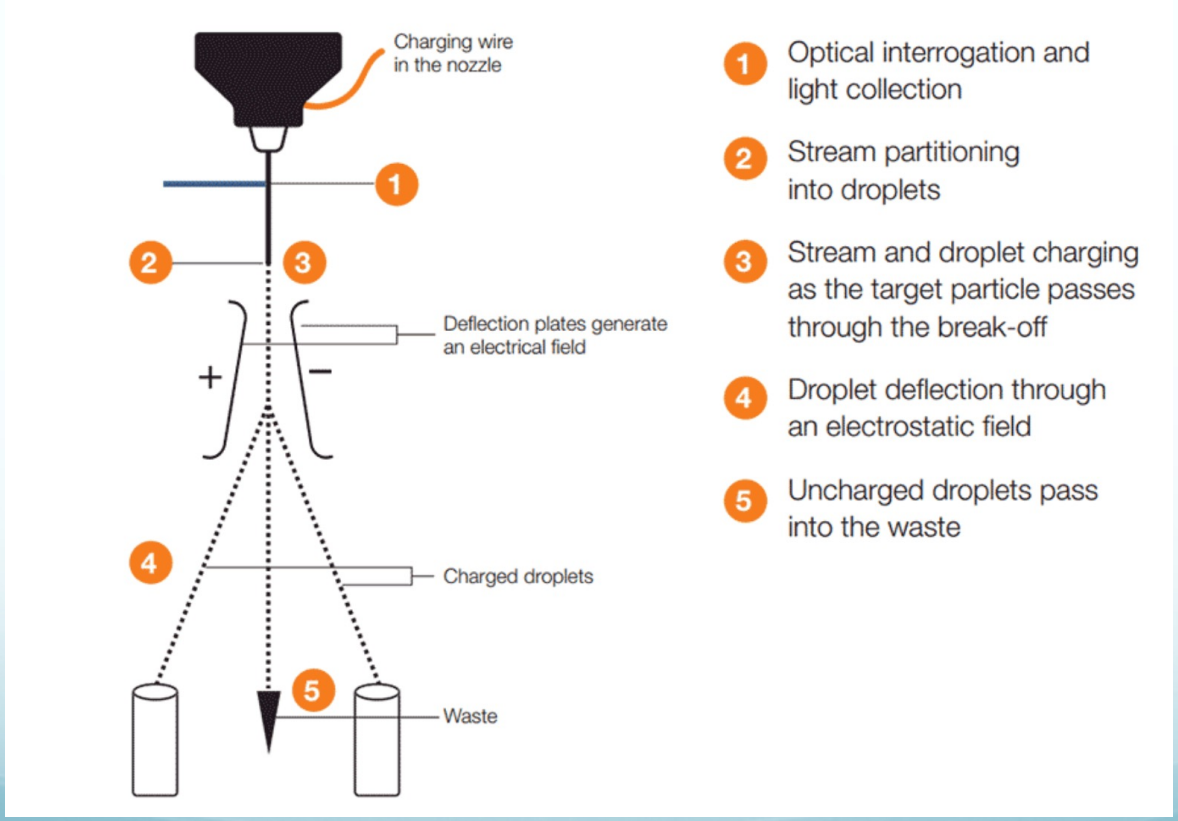


Your population might be 80% positive or 5% positive.

You need to know what to expect.

Is the staining real?

Cell Sorting: isolating the cells we want back



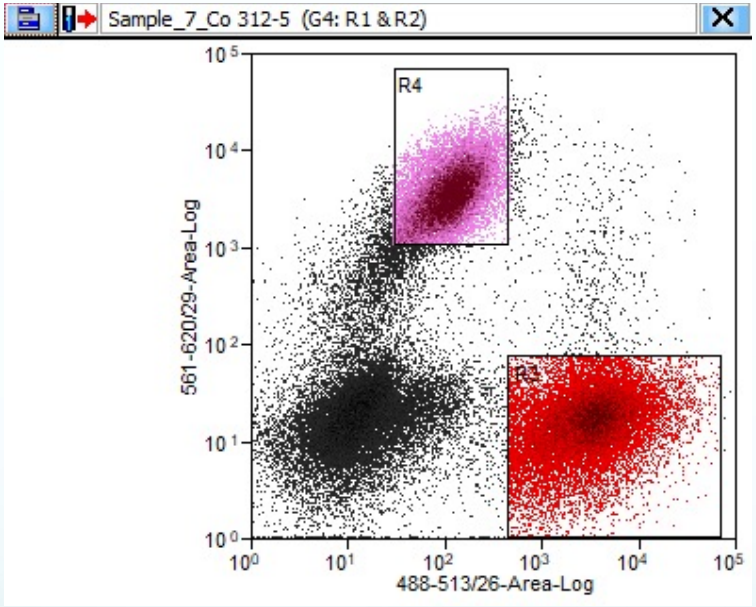
Core Facilities

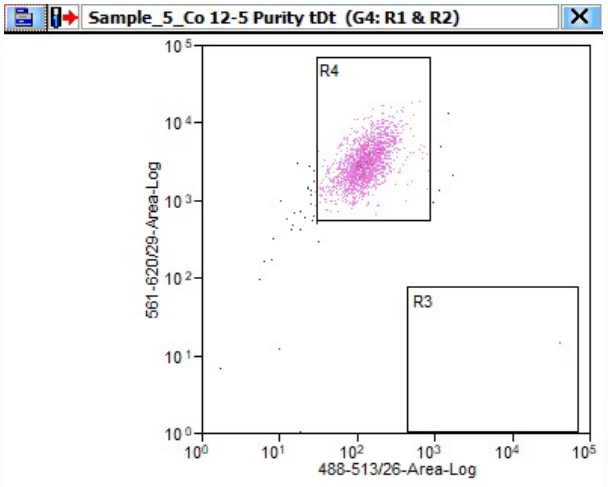
THE UNIVERSITY OF BRITISH COLUMBIA

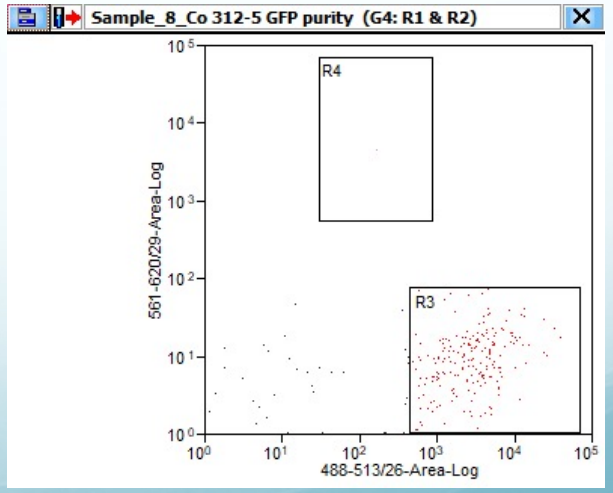
Life Science Institute

UBC Flow Cytometry Facility

Flow







Panel Design

Multicolor flow cytometry panel design

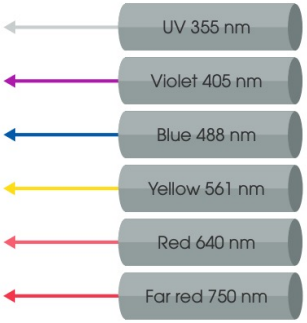
abcam

Our guide to help you build successful multi-color flow cytometry panels

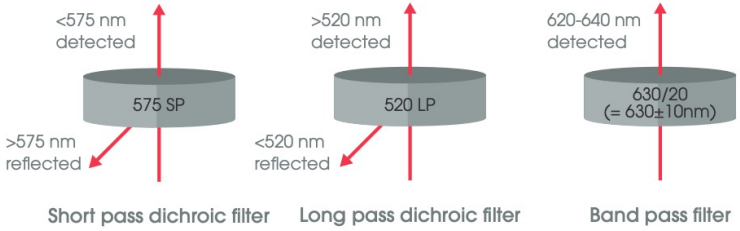
1. Know your flow cytometer

Lasers: only fluorochromes excited by the corresponding wavelength of light from the laser can be used

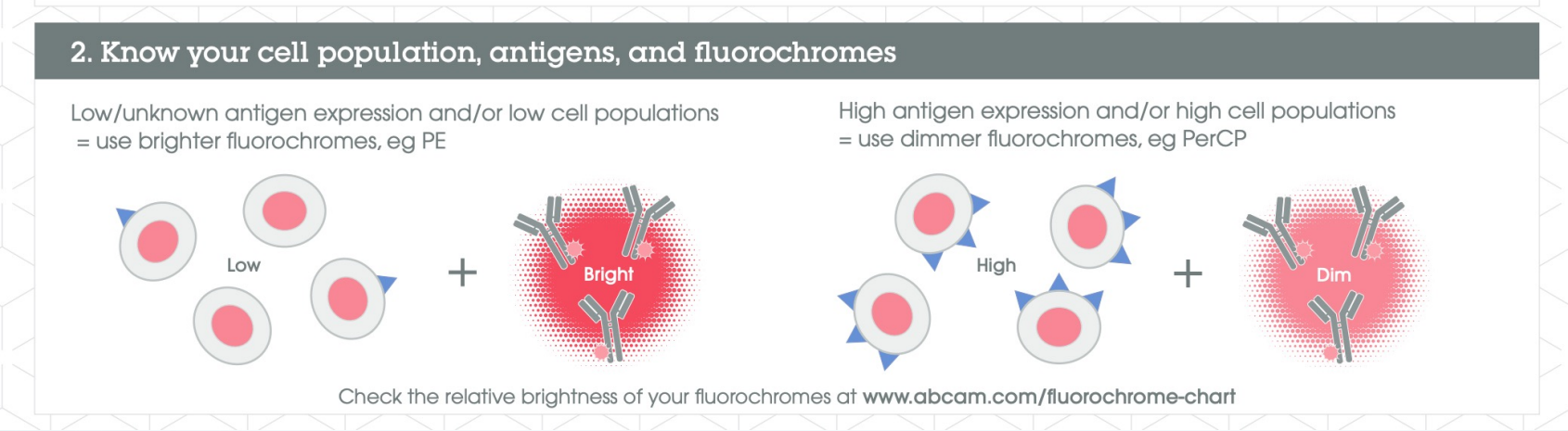
To ensure optimal detection, make sure you understand the combination of lasers /filters on your machine. Refer to your instrument's manual or speak to your core facility



Filters: detection of light emitted from fluorochromes is controlled by filters



Panel Design

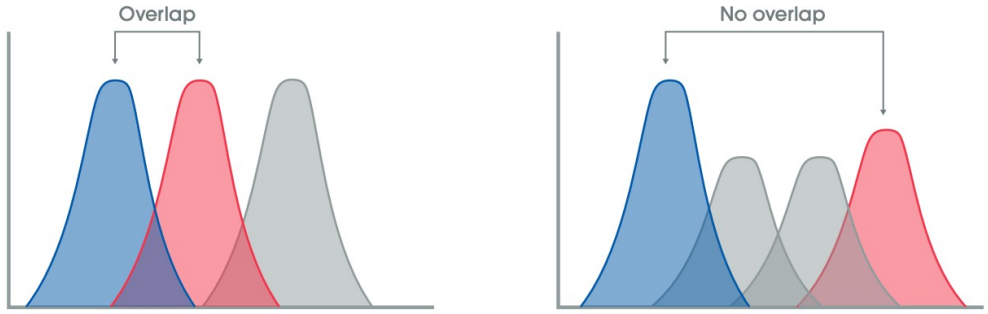


Panel Design

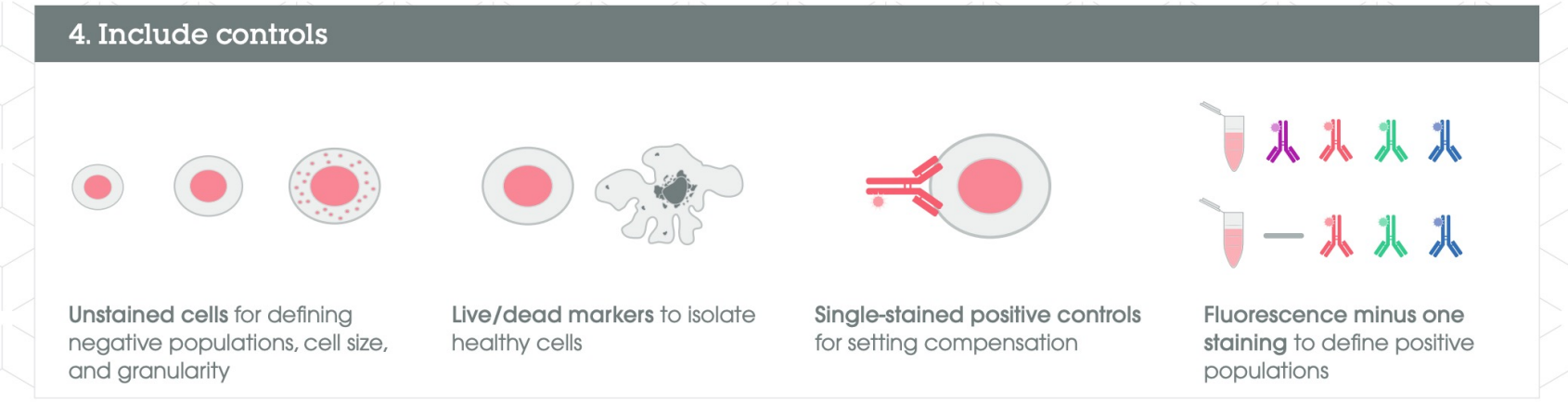
3. Minimize spectral overlap

Minimize emission spectra overlap

- Sacrifice bright fluorochromes to avoid overlap
- Compensation can be used to control the effects of spectral overlap

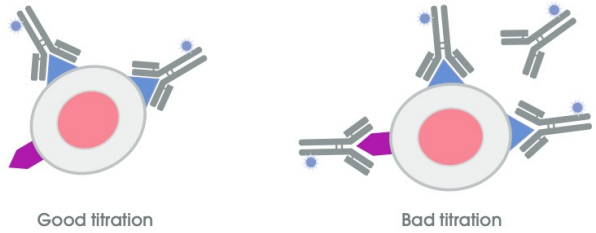


Panel Design

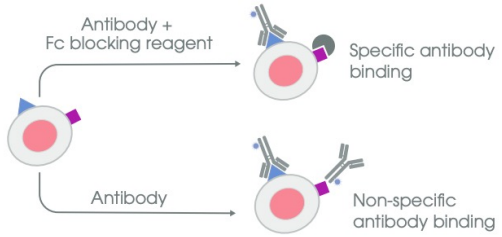


Panel Design

5. Optimize your staining protocol



Antibody concentration: titrate your antibodies to avoid non-specific binding or reduced sensitivity



Fc blocking reagents:
Human IgG for human
Anti-CD16+ CD32 for mouse

Fc blocking: use Fc blocking reagents in cells with high content of Fc receptors (eg phagocytic cells) to avoid non-specific binding

Spectral Flow Cytometry

Spectral Flow Cytometry

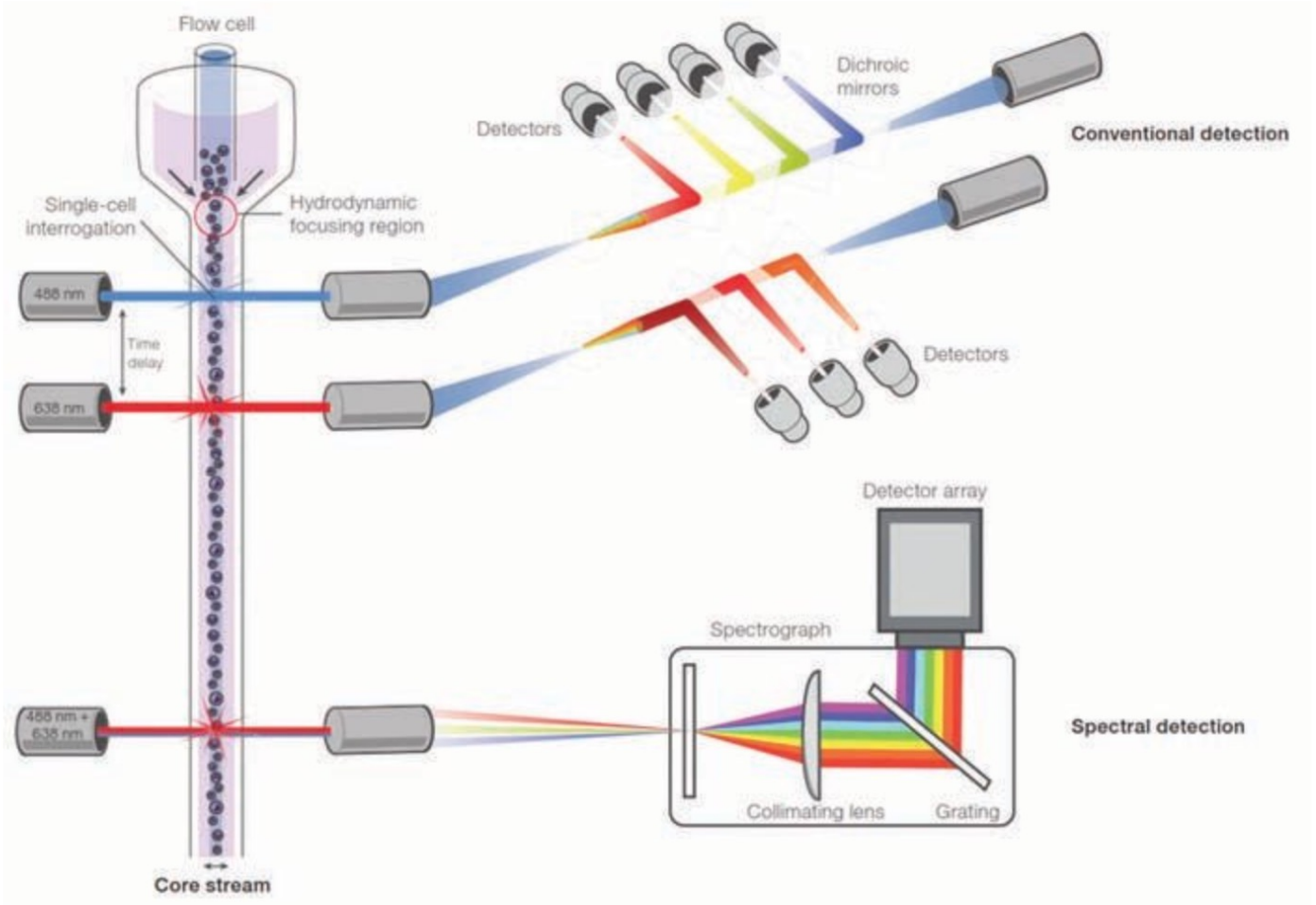
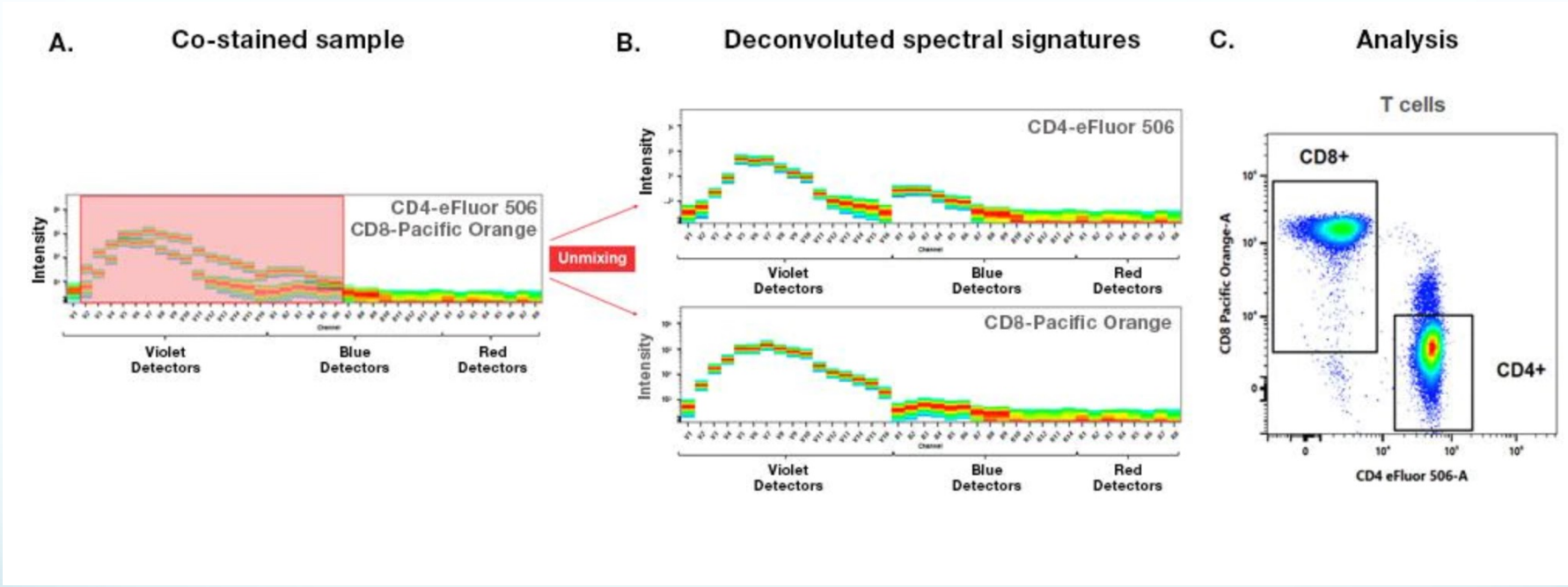
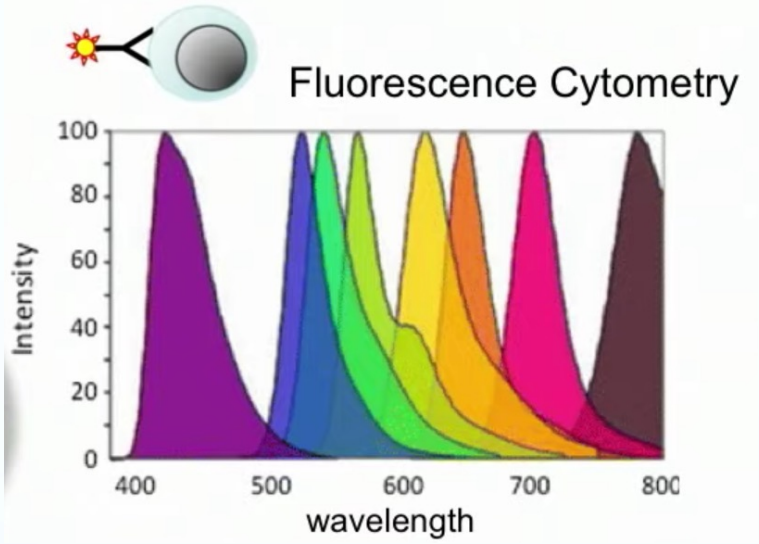


Figure 1. Comparison of a conventional and spectral flow cytometer system. (Top) A conventional flow cytometer relies on a series of band

Spectral Flow Cytometry



Move to Spectral Flow Cytometry



Alexa 488, GFP and FITC all emit at 530nm but have different spectral profiles.

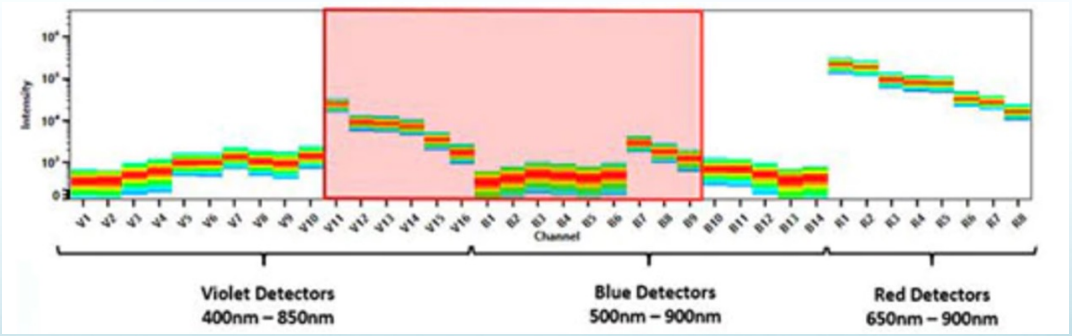


Image Cytometry

Image Cytometry

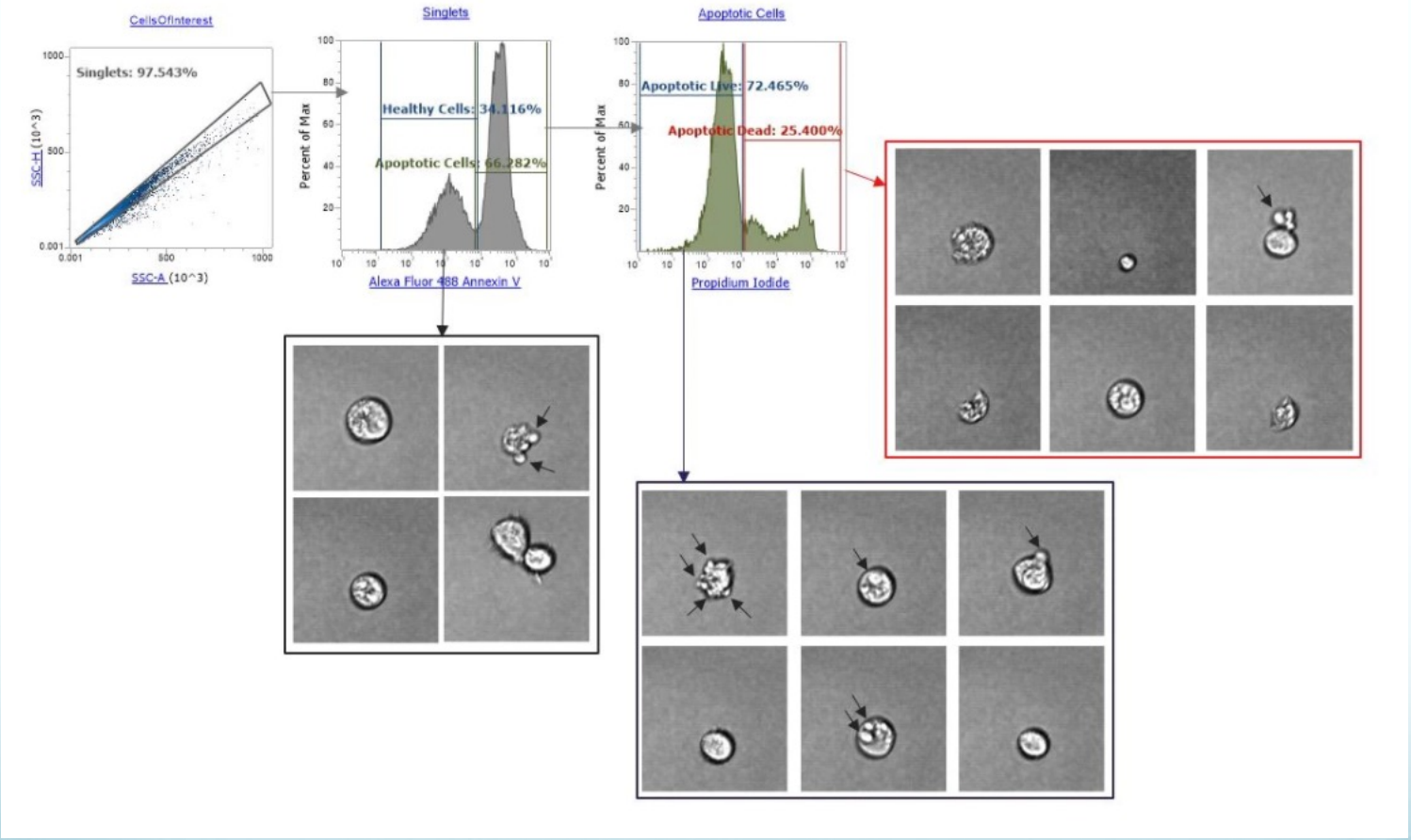
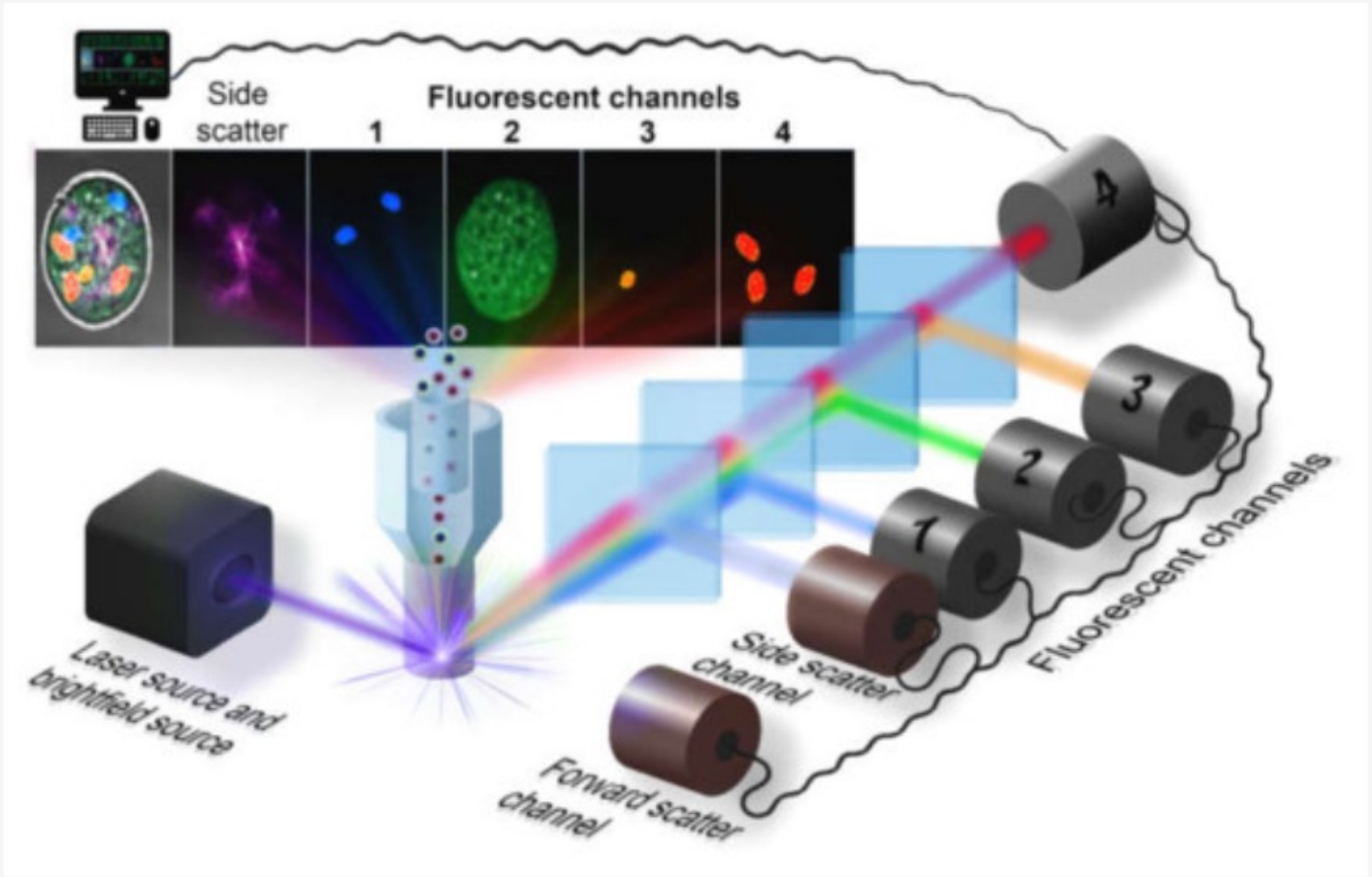
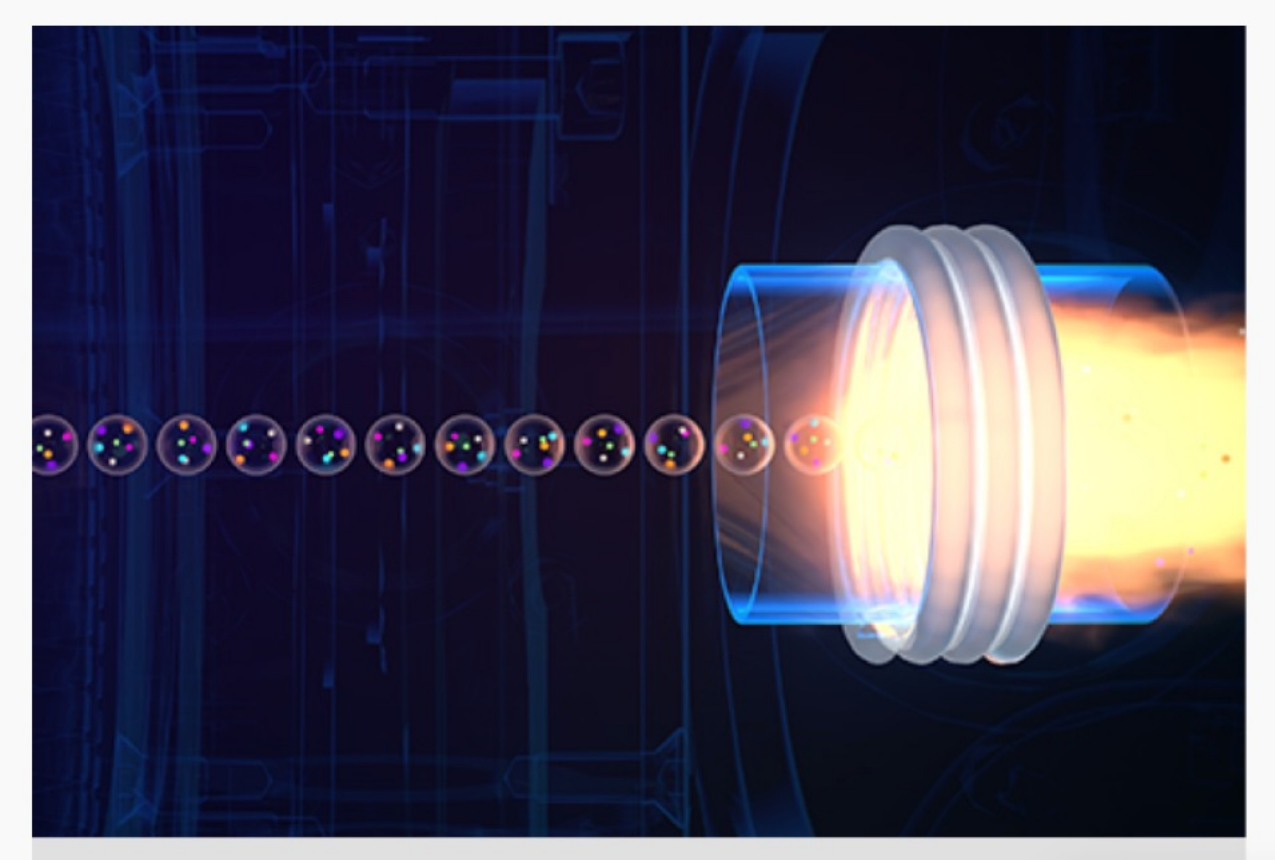


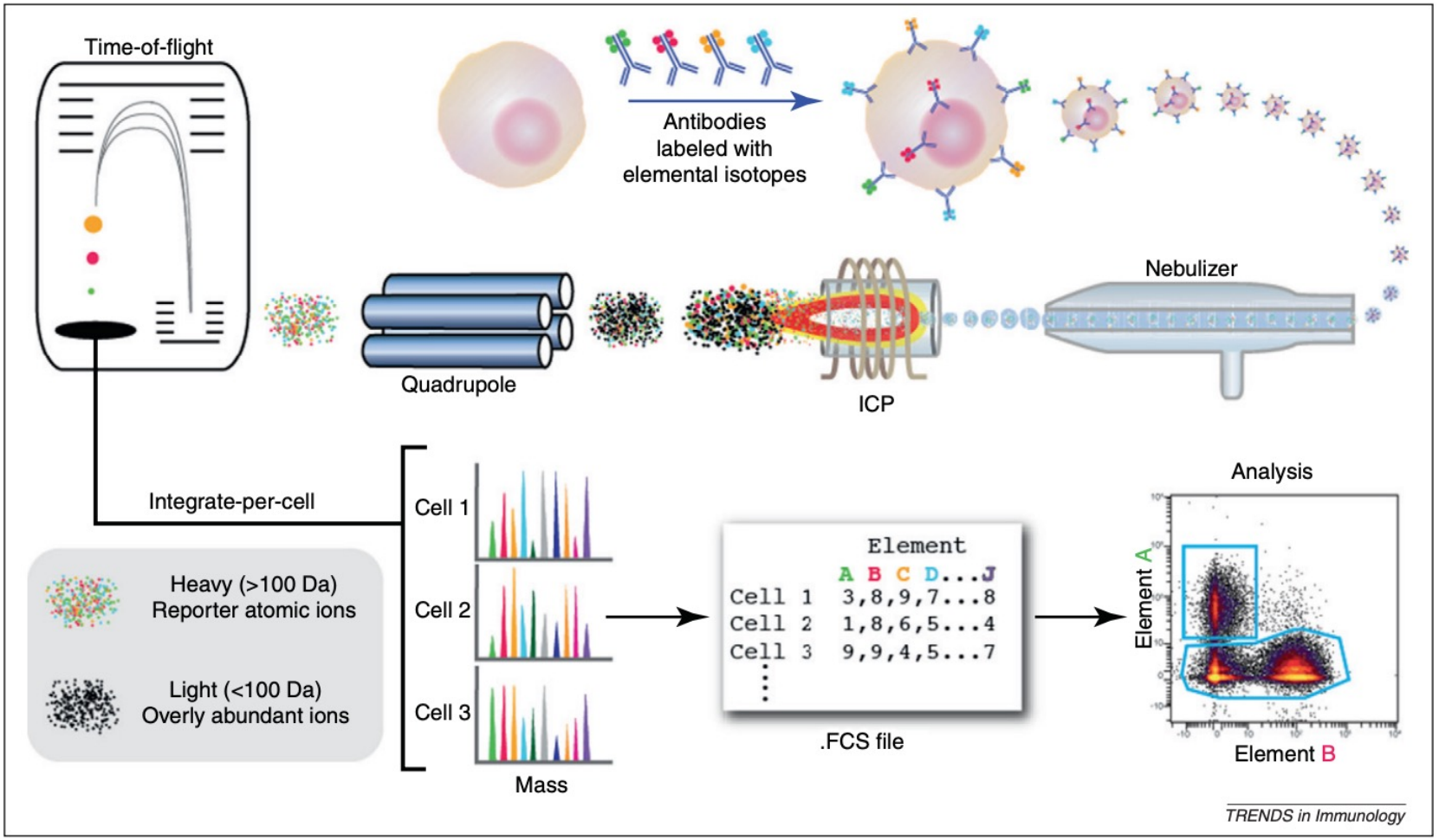
Image Cytometry



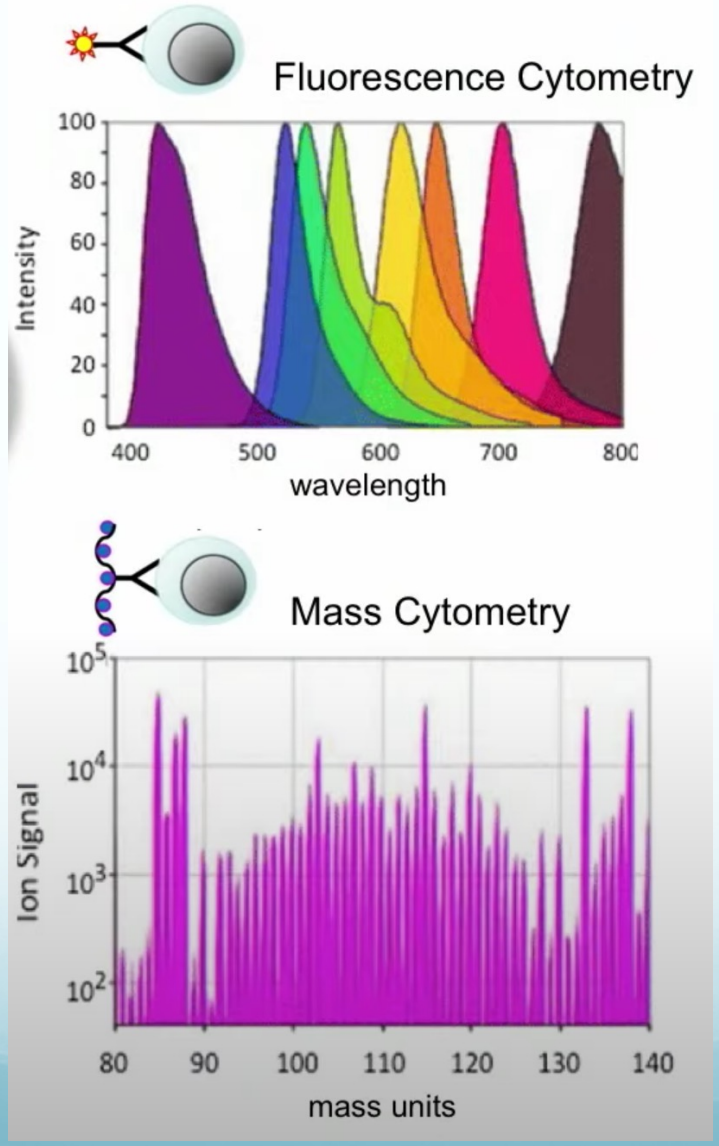
Mass Cytometry



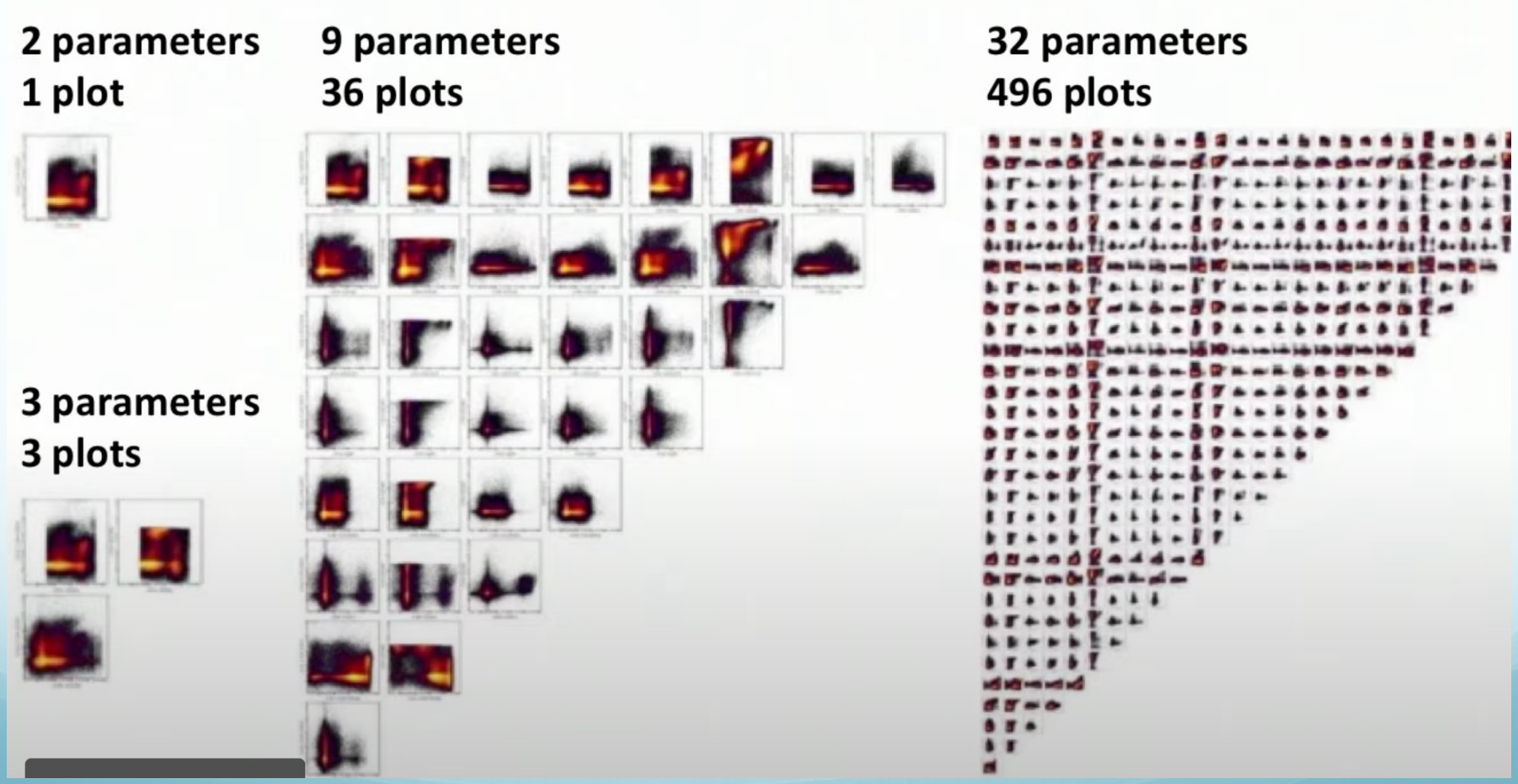
Mass Cytometry



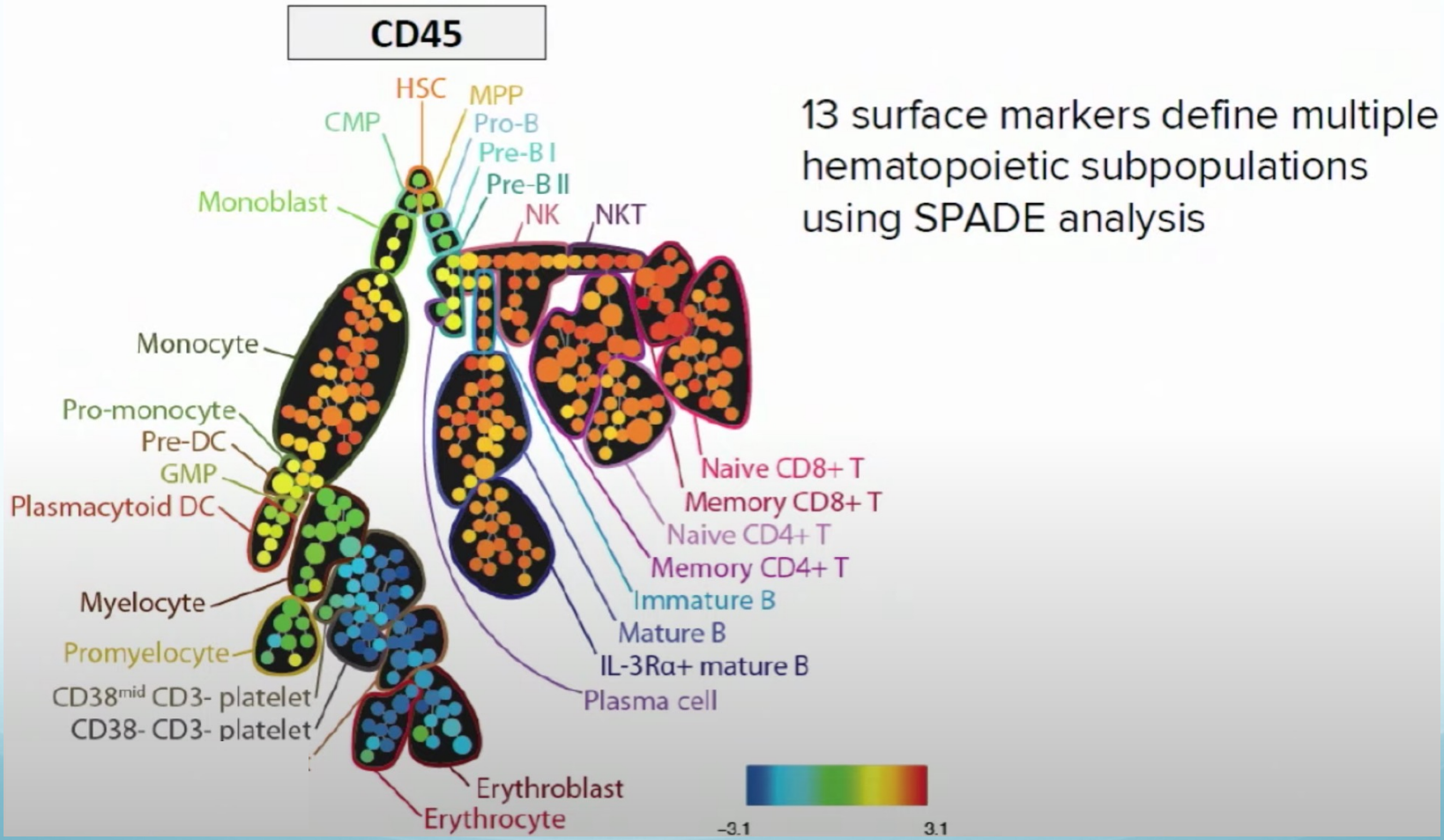
Mass Cytometry



Mass Cytometry



Mass Cytometry



13 surface markers define multiple hematopoietic subpopulations using SPADE analysis

Image Mass Cytometry

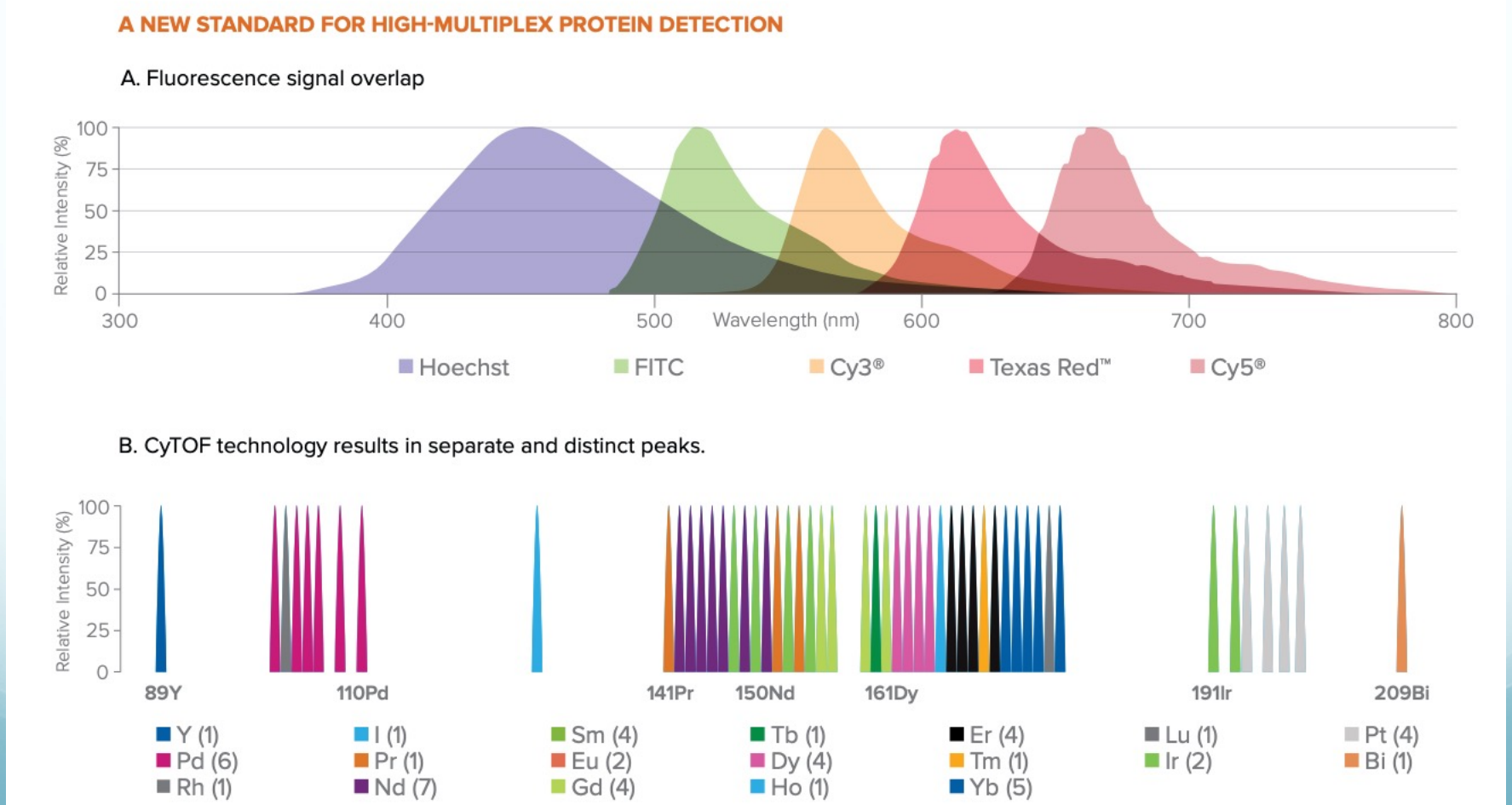
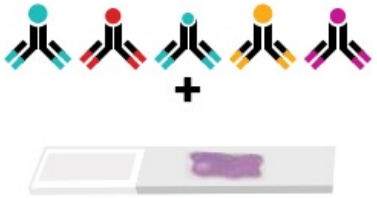
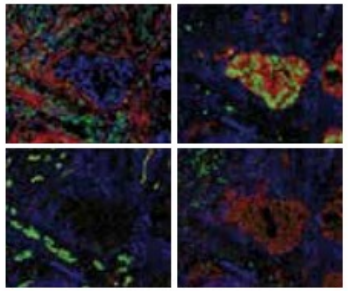


Image Mass Cytometry

IMAGING MASS CYTOMETRY WORKFLOW





1. DESIGN

Design panels using pathologist-verified Maxpar® antibodies conjugated to metal tags.

2. STAIN

Stain tissues (FFPE or frozen) or fixed cells using familiar IHC protocols.

3. IMAGE

Image protein markers at subcellular resolution using the Hyperion Imaging System.

4. ANALYZE

Analyze images in minutes using MCD Viewer and easily export for secondary analysis.

Image Mass Cytometry

HOW IMAGING MASS CYTOMETRY WORKS

Load sample into the Hyperion Imaging System.

FIGURE 3A

Load the slide stained with a panel of Maxpar antibodies into the Hyperion Imaging System. Select the region of interest to be imaged.

Precise laser imaging of the region of interest

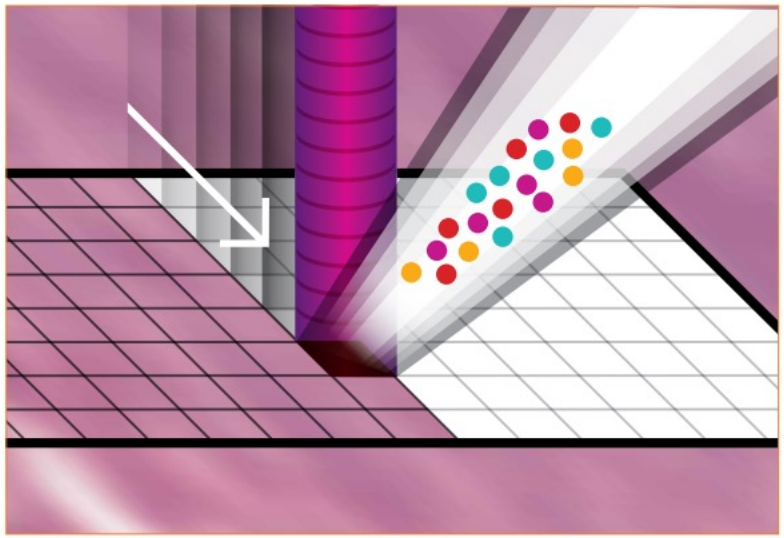


FIGURE 3B

A laser beam focused at 1 μm^2 spots samples proteins stained with metal-tagged antibodies and directs these tags to analysis by inductively coupled time-of-flight (TOF) technology, the basis for CyTOF mass cytometry. This occurs in a single scan as the laser samples each 1 μm^2 pixel in the selected region.