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Flow Cytometry Core Facility

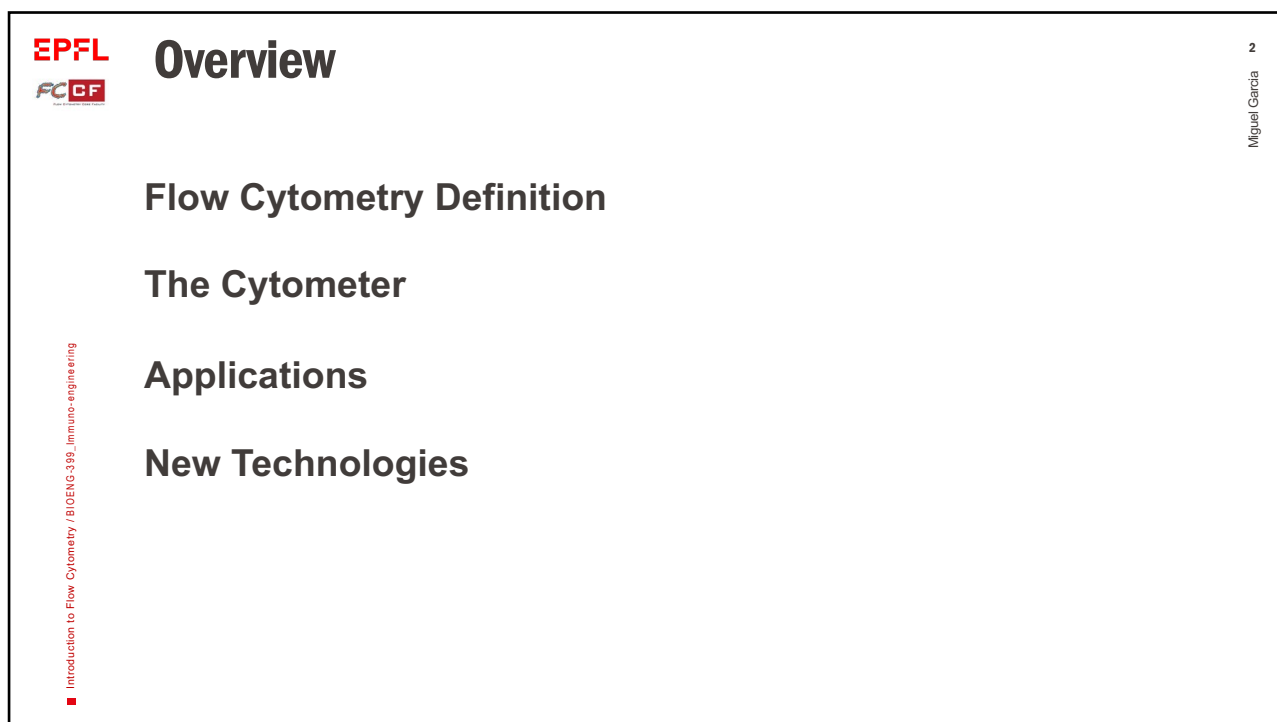
Introduction to Flow Cytometry
BIOENG-399_Immuno-engineering

Miguel Garcia
Head of Unit
PTCF

École polytechnique fédérale de Lausanne

14 March 2025

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Overview

- Flow Cytometry Definition
- The Cytometer
- Applications
- New Technologies

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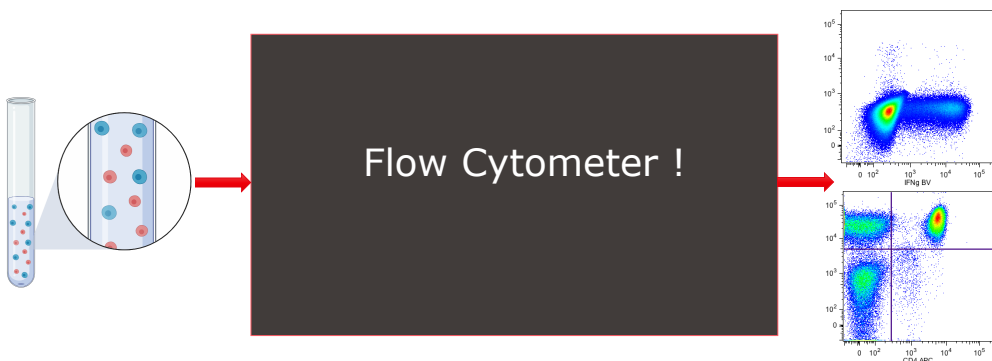
What is flow Cytometry ?

Flow Cytometry

Fluid Cell Measure

Flow Cytometer !

<http://mastery.expertcytometry.com>



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What are the advantages ?

- Analysis of thousands of cells per second detecting multiple parameters of individual cells within heterogeneous populations
- Fast sample processing (up to 35'000 evs/s)
- High statistical power
- Study of different cell (sub)populations
- Multi-parameter analysis - up to 20 parameters simultaneously in conventional, up to 50 on the latest instruments



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Technology - Analyzers

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Technology – High Speed Cell sorter

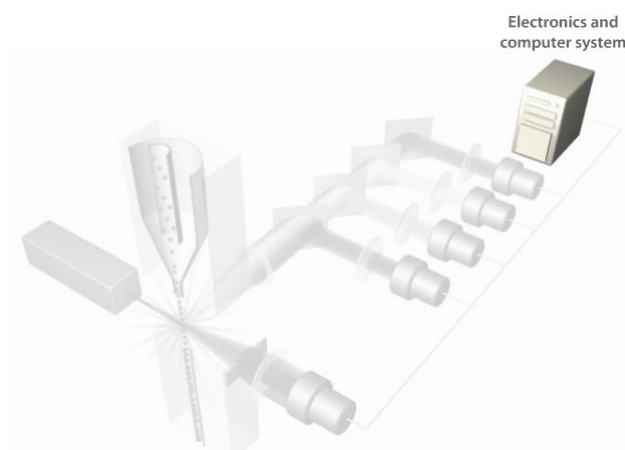
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Look *Inside* the Box

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<http://probes.invitrogen.com>

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3 major components of a Cytometer

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- **Fluidics**
 - Moving cells to the interrogation point
 - Fluidics dynamics
- **Optics**
 - Interrogation point
 - Measuring light
- **Electronics**
 - Signal conversion from light to electronics
 - Signal Quantification

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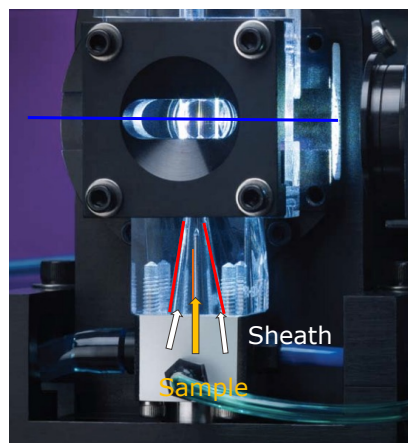
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Fluidics System

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- Cells in suspension flow in a single file through a laser intercept.
- Cells are hydrodynamically focused by injecting sample into a stream of sheath fluid as it passes through an orifice
- Sample fluid flows with the sheath fluid in laminar flow



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2 Fluidics principles

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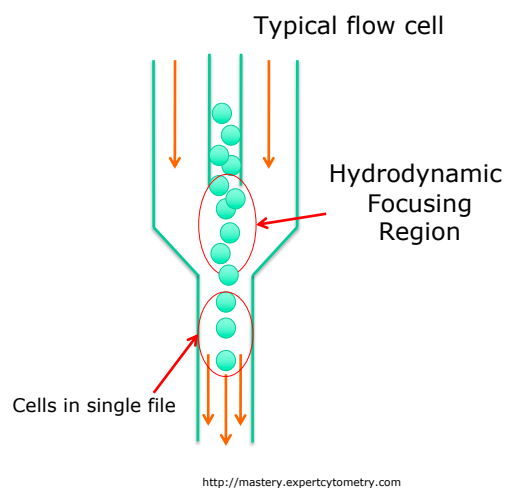
- **Laminar Flow**
 - Uniform fluid flow in parallel layers
 - The sample flows in the very center of the sheath
 - Sample and sheath fluids don't mix
- **Hydrodynamic focusing**
 - Differential pressure between Sample and Sheath
 - Sample enters the sheath stream where its diameter is constrained, and the cells are spread out along the flow velocity axis
 - This is how you get the cells to stay "in line" and pass through the laser in single file

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The Flow Cell

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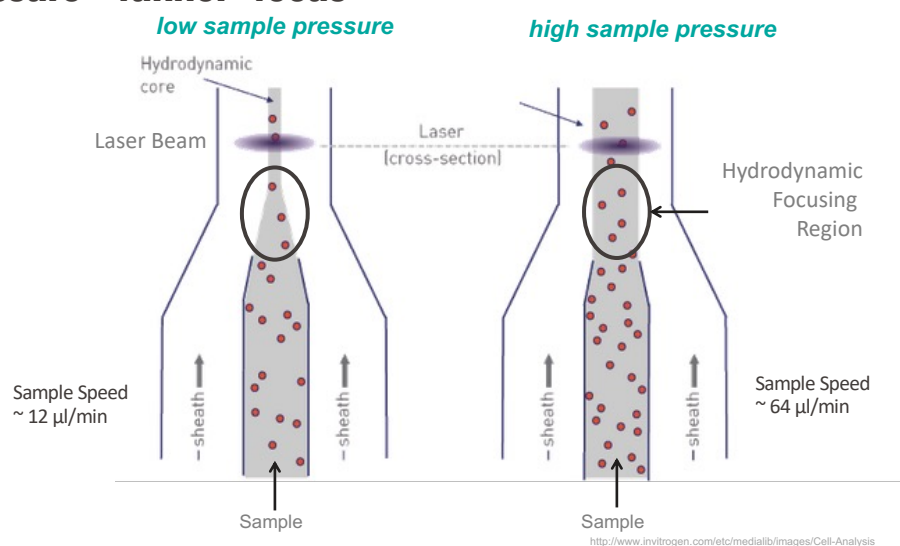
- The place where the sample is introduced to the sheath fluid
- Sample is hydrodynamically focused so the cells spread out.
- Sheath flow rate sets fluid flow rate
- Differential pressure sets core stream size



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Hydrodynamic Focusing Pressure – funnel - focus

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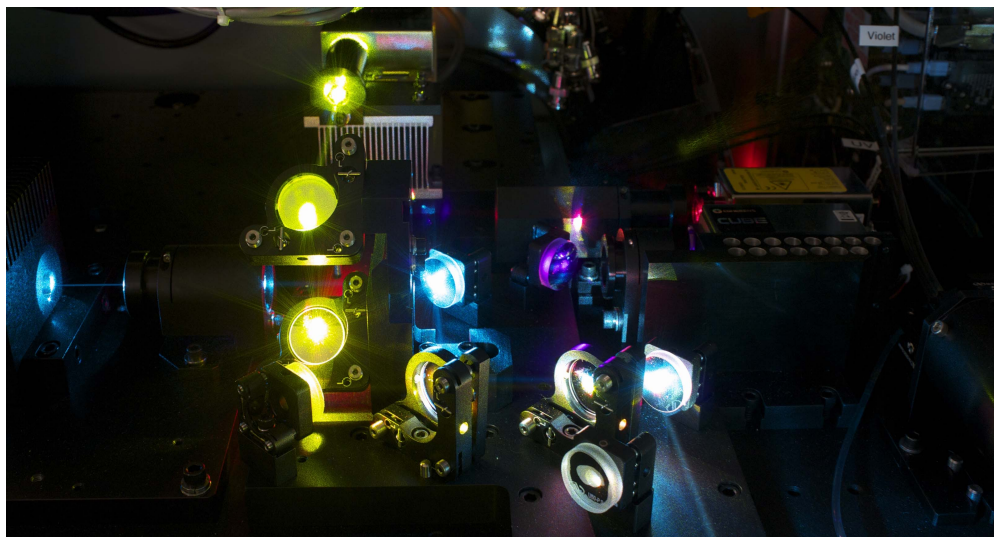
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Optics – Lasers/Filters/Detectors

Moving photons of light through the instrument

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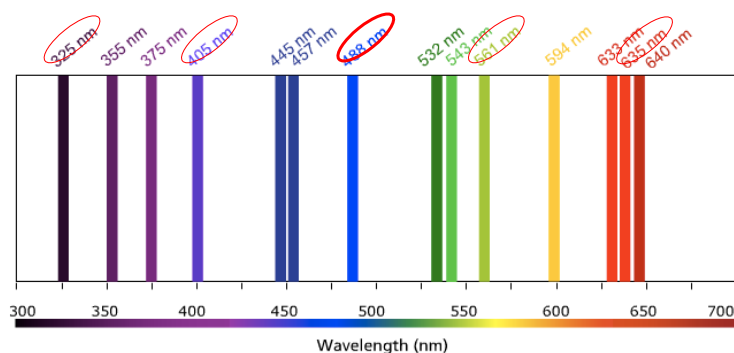
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Lasers

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Laser = Light Amplification by Stimulated Emission of Radiation

- **MONOCHROMATIC** - Lasers can provide a single wavelength of light
- **COHERENT** - The motion of all photons are coordinated

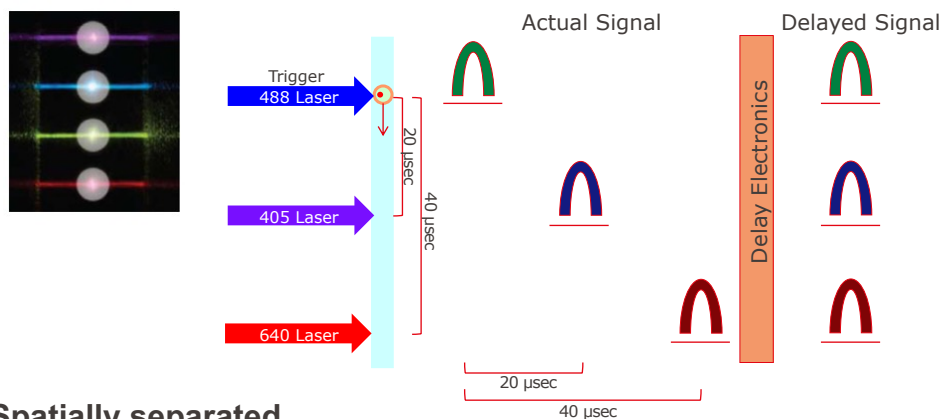


<http://www.bdbiosciences.com/>

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Light Source configuration

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- **Spatially separated**
 - Lasers separated
 - Multiple interrogation points
 - Must set time delay between lasers

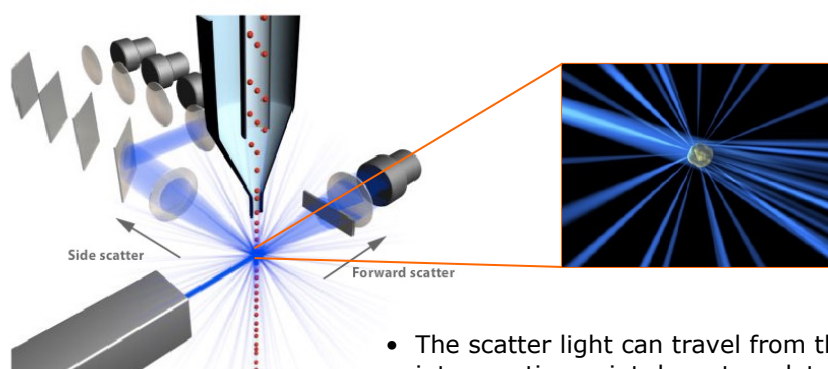
<http://mastery.expertcytometry.com>

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Interrogation Point

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Where the laser and the sample intersect → the optics collect the resulting scatter and fluorescence



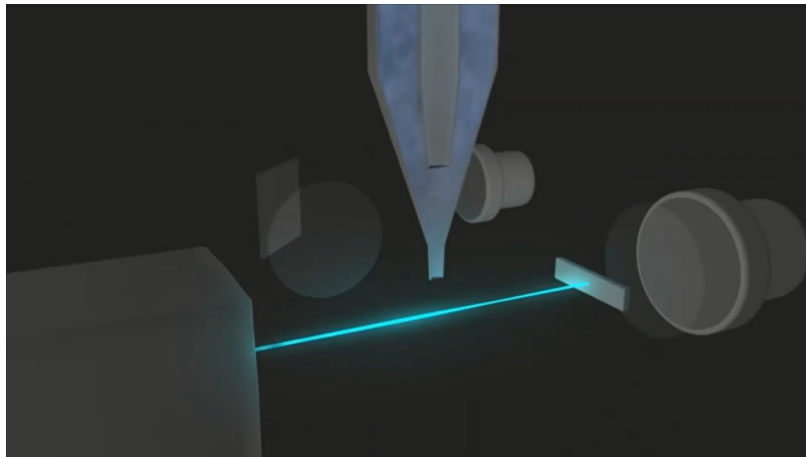
- The scatter light can travel from the interrogation point down to a detector

<http://probes.invitrogen.com>

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Interrogation Point

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<http://probes.invitrogen.com>

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Measure light

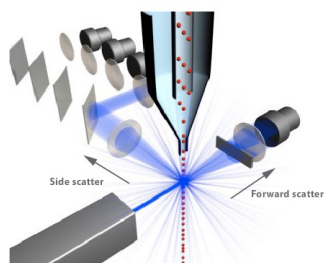
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- Two types of light are measured as particles pass through the illumination source
- **Laser light scatter:** refraction of illuminating beam by the particle
 - Forward scatter (FSC)
 - Side scatter (SSC)
- **Fluorescence:** emitted from fluorescent tags after being excited by the illumination source.

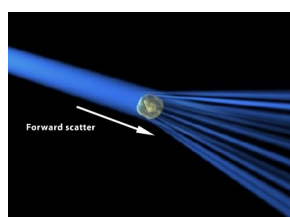
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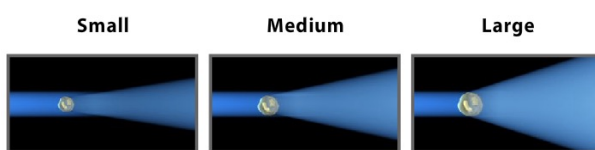
Forward Scatter (FSC)

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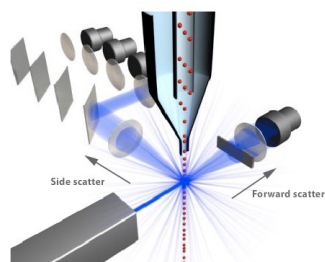
- Light that is scattered in the *forward* direction (along the same axis the laser is traveling) is detected in the Forward Scatter Channel
- The intensity of this signal is roughly proportional to cell/particle size and membrane integrity



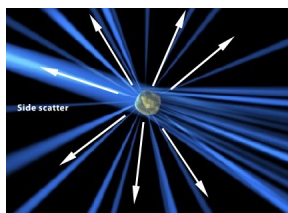
<http://probes.invitrogen.com>



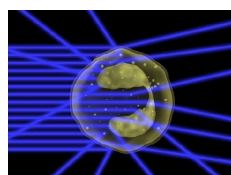
Side Scatter (SSC)

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- Light that is scattered at 90 degrees to the axis of the laser path is detected in the Side Scatter Channel
- Side scatter is caused by granularity and/or structural complexity inside the cell/particle (eg. Granulated nuclei, cell inclusions, etc.)



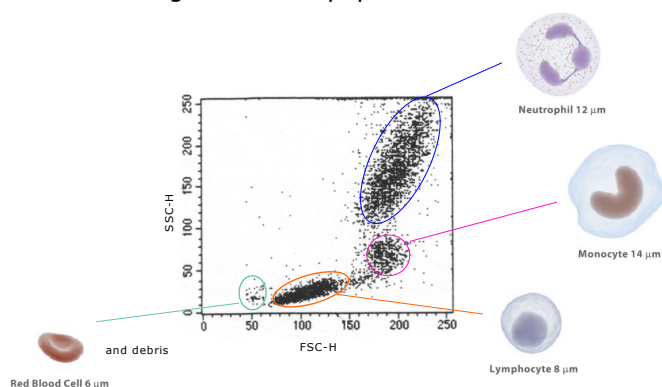
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FSC v. SSC

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- Since FSC \sim size and SSC \sim internal structure, a correlated measurement between them can allow for a differentiation of cell types in a heterogeneous cell population



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What is Fluorescence ?

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Emission of light by a compound that has absorbed a photon of light

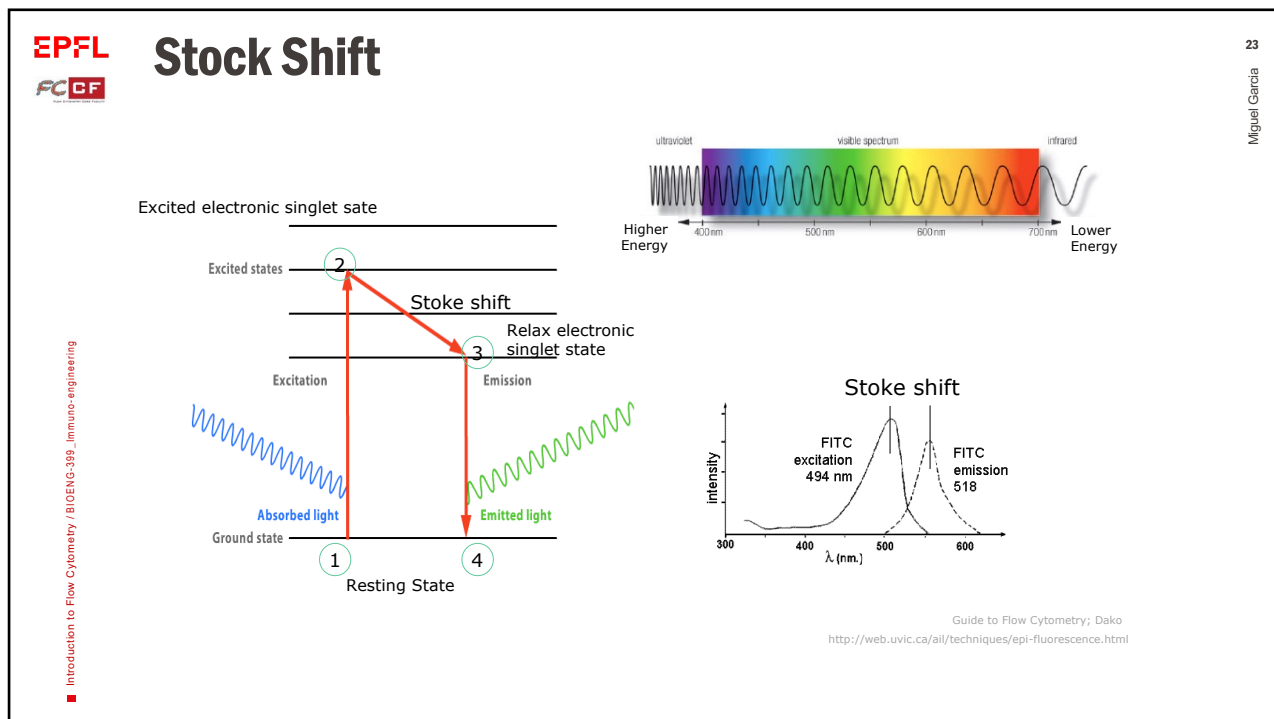
Excitation - Lasers

Absorbance of photon of light
Promotes electron to higher energy state

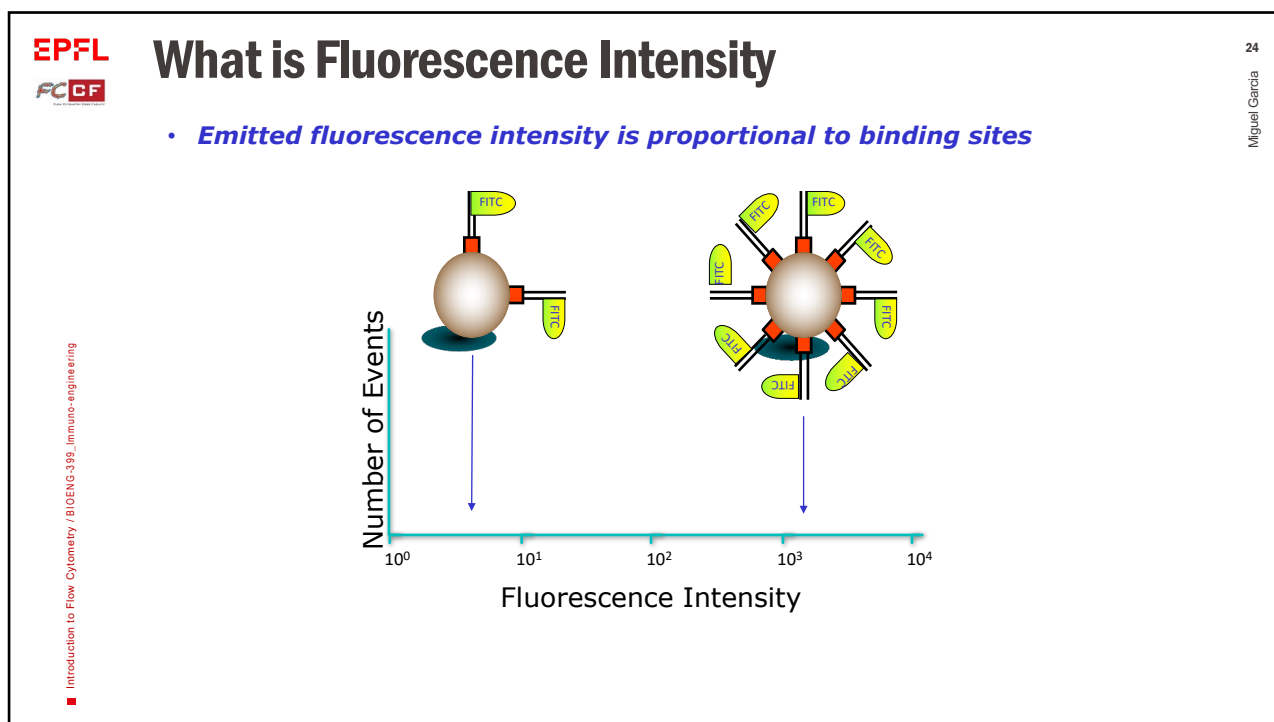
Emission – Detected by Optics

Return of excited electron to ground state
Emitted wavelength longer (less energy) than exciting wavelength named “**Stoke Shift**”

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Fluorophores

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Polymer dyes

Phycoerythrin: a naturally occurring fluorescent protein

Nanocrystal dyes

FITC: Fluorescein Isothiocyanate

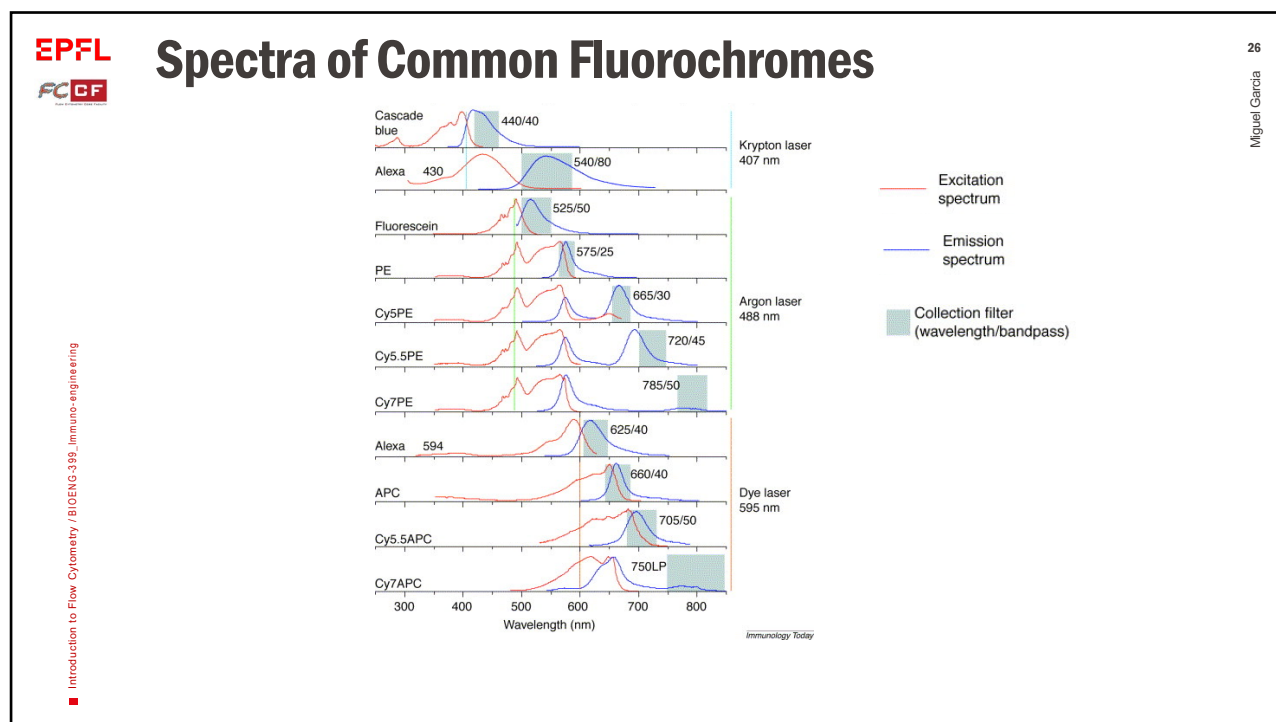
Alexa 488

Cyanine 3
Cyanine 5

source : Excyte Expert Cytometry

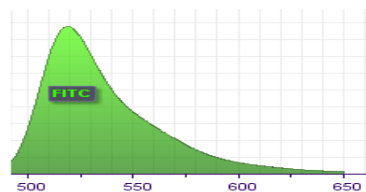
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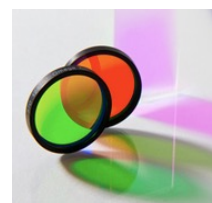
Filters



As many wavelengths of light will be scattered from a cell, we need a way to split the light into its specific wavelengths in order to detect them independently



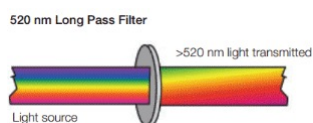
- Filters separate light based on photon wavelength
- Dichroic mirrors
 - Pass light of one signal, deflect the remainder
 - Most common filters used in current instruments
- Types of Filters
 - Longpass (e.g., LP560)
 - Shortpass (e.g., SP560)
 - Bandpass (e.g., BP 530/30)
 - Transmits light between a given range



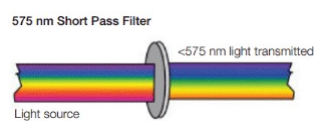
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Filters -Types

Longpass



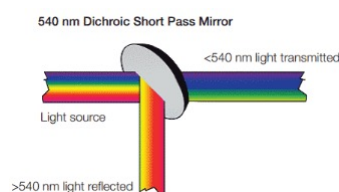
Shortpass



Bandpass

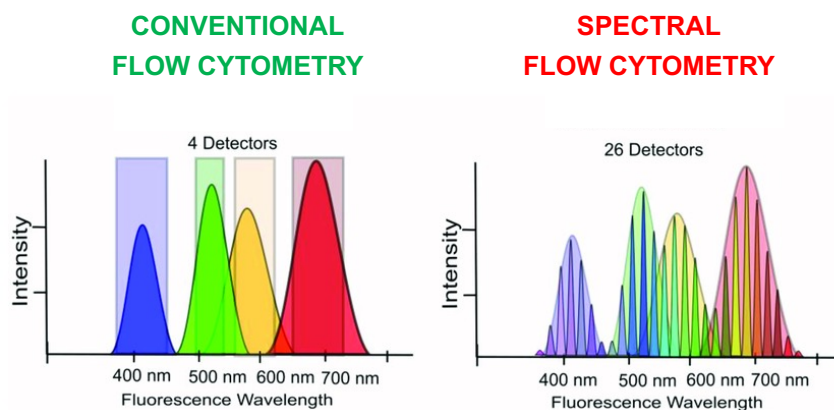


Dichroic mirror



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Different ways to collect fluorescence emission

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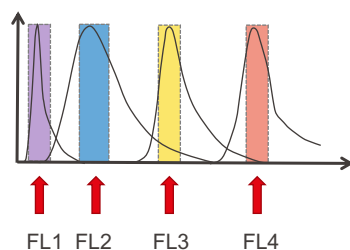
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Conventional Flow Cytometry

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Conventional

In conventional cytometry, one detector is assigned to one fluorophore



FL1 → PB
FL2 → FITC
FL2 → PE
FL3 → APC

Each fluorochrome is detected in
ONE channel

Limitations:

- Photons emitted outside of the filter will be lost
- # Fluors limited by # detectors
- Need to adapt the panel to the filter configuration
- Cannot combine fluorochromes with overlapping emission peaks

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PMTs

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The diagram illustrates the optical path of a flow cytometer. A sample is injected into a stream of fluid, which is then focused by a **Collection Lens**. The emitted light is collected and passes through a series of dichroic mirrors (**DM 510LP**, **DM 550LP**, **DM 640LP**) and filters (**488/10**, **530/30**, **585/42**, **660LP**) to be detected by three photomultiplier tubes (**FL 1 PMT**, **FL 2 PMT**, **FL 3 PMT**). A **SSC PMT** is also shown, and a **FSC Photodiode** is used for forward scatter detection. A photograph of a PMT wheel shows the arrangement of these tubes, with labels for **SSC**, **FL 1**, **FL 2**, **FL 3**, and various filters like **488/10**, **530/30**, **585/42**, and **660LP**.

<http://probes.invitrogen.com>
<http://www.labome.com/>

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Conventional Flow Cytometry

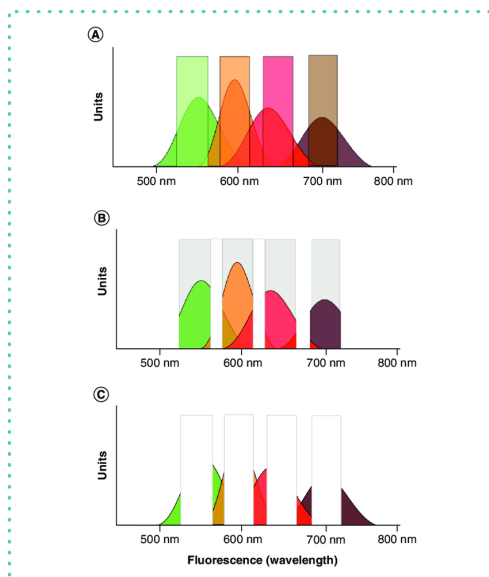
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The graph shows the **APC Emission Spectrum** (a broad peak) and the **670/30 Filter** (a narrow bandpass filter). The x-axis is **Wavelength** and the y-axis is **Emission Intensity**. A photograph of an iceberg is shown to the right, illustrating the concept of the 'iceberg' model of the cell surface, where only a small portion of the total surface area is visible.

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Conventional Flow Cytometry

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Bandpass filters create unique bands of light for each fluorochrome

Spectral overlap: multiple fluorescence emissions may be measured on any particular detector

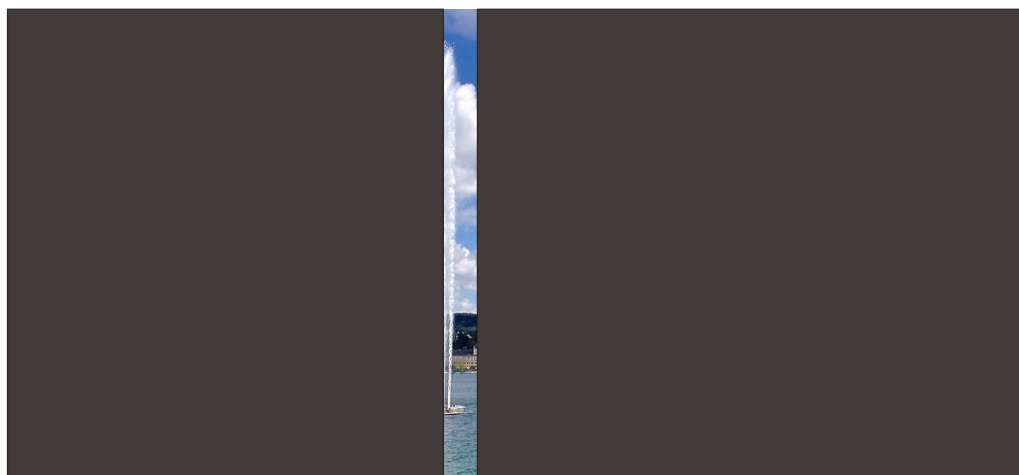
Only fluorescence signals within the bandpass filters are collected all the rest of the signal is lost

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Full Spectrum Flow Cytometry

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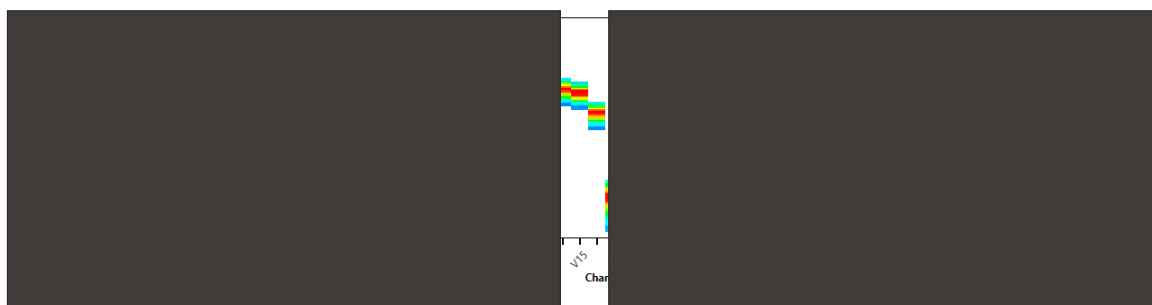
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Full Spectrum Flow Cytometry Allows you to see the full picture

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Is a fluorochrome only the section of the spectrum that we choose to view?



Fluorochromes can be excited by several lasers
 → We sample the signal generated by **every** laser

More photons sampled
 → Better identification of the signal

With spectral cytometry, all detectors are used for all fluorochromes
 Fluorophores are identified by their distinct spectra signature

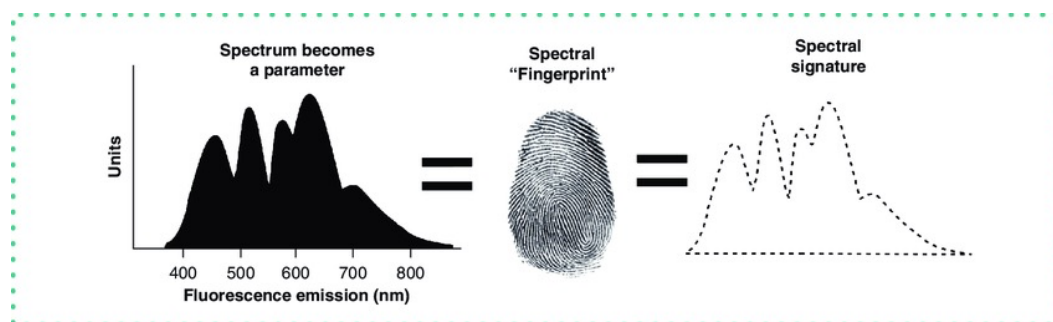
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Full Spectrum Flow Cytometry

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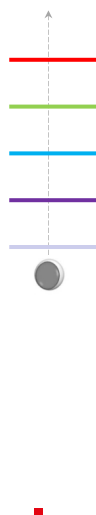


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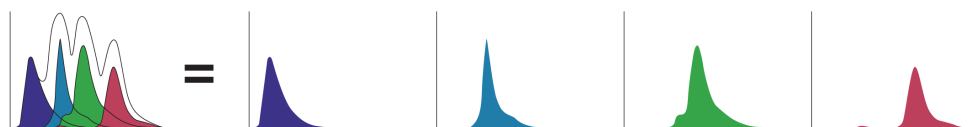
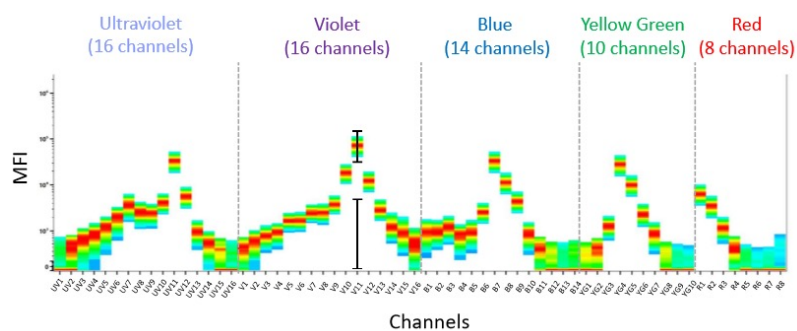
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Full Spectrum Flow Cytometry: How does it work?

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Full Spectral Flow Cytometry

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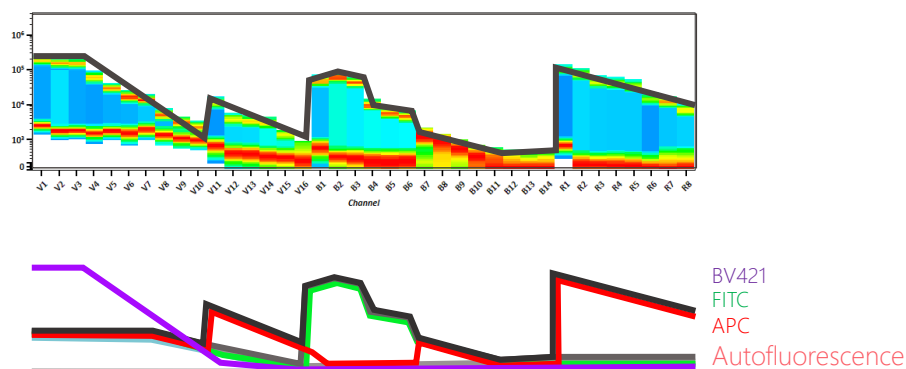
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Full Spectral Flow Cytometry Spectral Unmixing algorithm

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We use spectral unmixing to calculate the contribution of each fluorochrome to the total collected emission signal

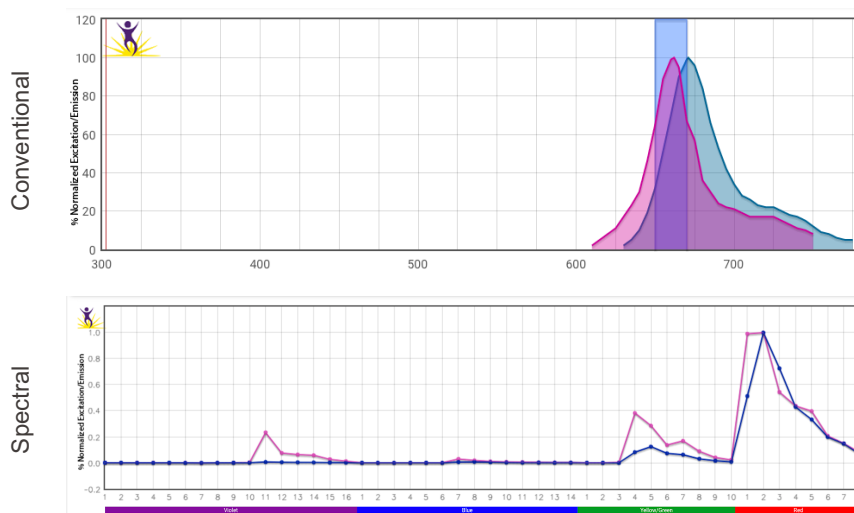


We can think of this as extracting or deconvoluting each component until we have nothing left.

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Full Spectral Flow Cytometry

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APC (purple) and AlexaFluor® 700 (blue) emission spectra

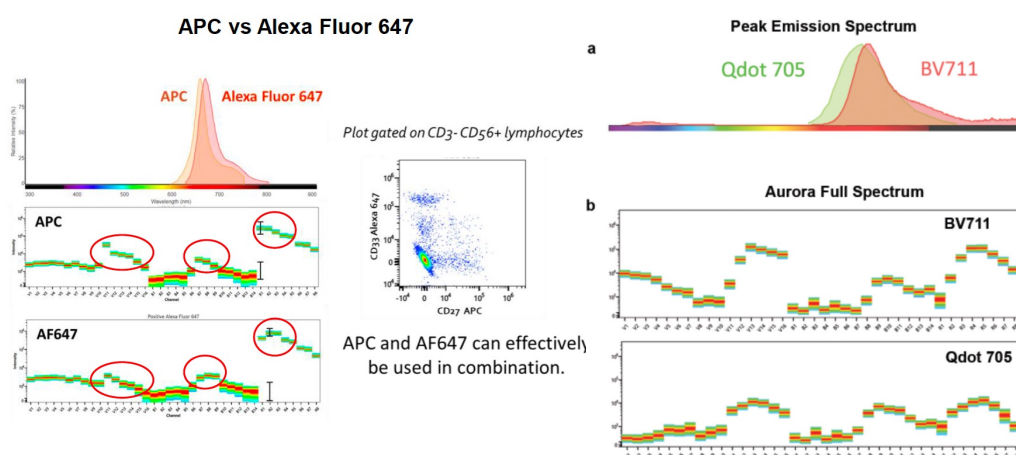
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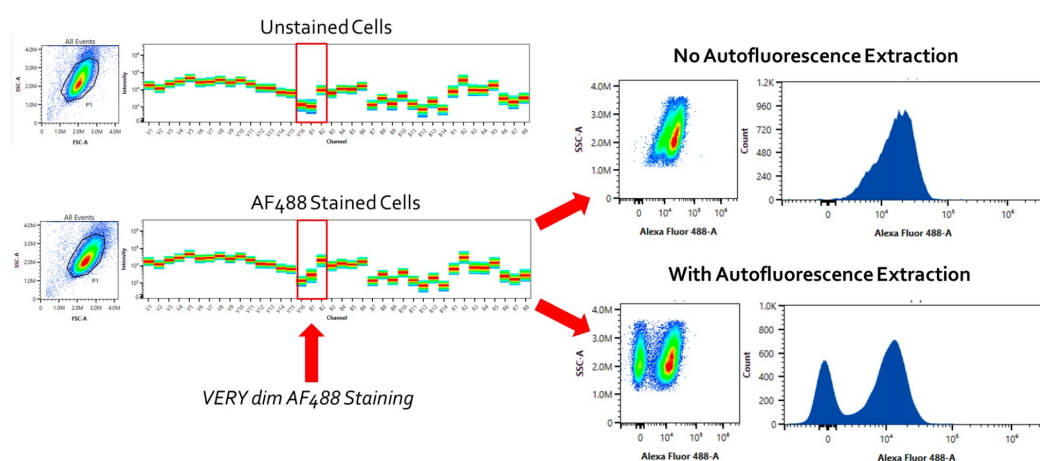


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Full Spectral Flow Cytometry

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Full Spectrum Flow Cytometry

Basic Optical Components

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Excitation Source (Laser)

Labeled Cell

focussing lens

Dispersing Element

Multichannel Light Detector

Eur J Immunol. (2017) 47:1584-1797. doi: 10.1002/eji.201546632.
Guidelines for the use of flow cytometry and cell sorting in immunological studies.

Light Dispersion Methods

Coarse Wavelength
Division Multiplexing
(CWDM)

https://www.pricom.com/education/cwdm_cascade.asp

Prism

<https://lightfermins.com/prism-grating/>

Light Detection Methods

Avalanche Photodiode
(APD) Arrays

Multichannel PMT

<https://www.sonybiotechnology.com/us/instruments/sp800system/>

<https://www.microscopyu.com/>

Adapted from Monica Delay (CytekBiosciences)

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Full Spectrum Flow Cytometry Commercial analyzers

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Support up to 7 Lasers excitation

188ch detectors
* the image is part of them

Micro lens array

Grating

7 Detection decks

Flow cell

Auto sampler

Optical fiber

SONY

Released in September, 2020

5 Laser System

375 nm Laser

405 nm Laser

473 nm Laser

532 nm Laser

633 nm Laser

660 nm Laser

690 nm Laser

730 nm Laser

780 nm Laser

830 nm Laser

850 nm Laser

880 nm Laser

910 nm Laser

940 nm Laser

970 nm Laser

1000 nm Laser

1064 nm Laser

1319 nm Laser

1550 nm Laser

1625 nm Laser

1650 nm Laser

1675 nm Laser

1700 nm Laser

1725 nm Laser

1750 nm Laser

1775 nm Laser

1800 nm Laser

1825 nm Laser

1850 nm Laser

1875 nm Laser

1900 nm Laser

1925 nm Laser

1950 nm Laser

1975 nm Laser

2000 nm Laser

2025 nm Laser

2050 nm Laser

2075 nm Laser

2100 nm Laser

2125 nm Laser

2150 nm Laser

2175 nm Laser

2200 nm Laser

2225 nm Laser

2250 nm Laser

2275 nm Laser

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4000 nm Laser

4025 nm Laser

4050 nm Laser

4075 nm Laser

4100 nm Laser

4125 nm Laser

4150 nm Laser

4175 nm Laser

4200 nm Laser

4225 nm Laser

4250 nm Laser

4275 nm Laser

4300 nm Laser

4325 nm Laser

4350 nm Laser

4375 nm Laser

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5000 nm Laser

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5075 nm Laser

5100 nm Laser

5125 nm Laser

5150 nm Laser

5175 nm Laser

5200 nm Laser

5225 nm Laser

5250 nm Laser

5275 nm Laser

5300 nm Laser

5325 nm Laser

5350 nm Laser

5375 nm Laser

5400 nm Laser

5425 nm Laser

5450 nm Laser

5475 nm Laser

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6000 nm Laser

6025 nm Laser

6050 nm Laser

6075 nm Laser

6100 nm Laser

6125 nm Laser

6150 nm Laser

6175 nm Laser

6200 nm Laser

6225 nm Laser

6250 nm Laser

6275 nm Laser

6300 nm Laser

6325 nm Laser

6350 nm Laser

6375 nm Laser

6400 nm Laser

6425 nm Laser

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6925 nm Laser

6950 nm Laser

6975 nm Laser

7000 nm Laser

7025 nm Laser

7050 nm Laser

7075 nm Laser

7100 nm Laser

7125 nm Laser

7150 nm Laser

7175 nm Laser

7200 nm Laser

7225 nm Laser

7250 nm Laser

7275 nm Laser

7300 nm Laser

7325 nm Laser

7350 nm Laser

7375 nm Laser

7400 nm Laser

7425 nm Laser

7450 nm Laser

7475 nm Laser

7500 nm Laser

7525 nm Laser

7550 nm Laser

7575 nm Laser

7600 nm Laser

7625 nm Laser

7650 nm Laser

7675 nm Laser

7700 nm Laser

7725 nm Laser

7750 nm Laser

7775 nm Laser

7800 nm Laser

7825 nm Laser

7850 nm Laser

7875 nm Laser

7900 nm Laser

7925 nm Laser

7950 nm Laser

7975 nm Laser

8000 nm Laser

8025 nm Laser

8050 nm Laser

8075 nm Laser

8100 nm Laser

8125 nm Laser

8150 nm Laser

8175 nm Laser

8200 nm Laser

8225 nm Laser

8250 nm Laser

8275 nm Laser

8300 nm Laser

8325 nm Laser

8350 nm Laser

8375 nm Laser

8400 nm Laser

8425 nm Laser

8450 nm Laser

8475 nm Laser

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8600 nm Laser

8625 nm Laser

8650 nm Laser

8675 nm Laser

8700 nm Laser

8725 nm Laser

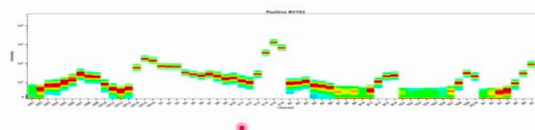
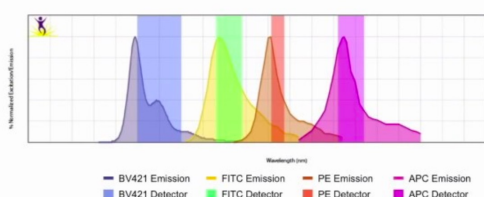
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Full Spectral Flow Cytometry

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Conventional vs Spectral Flow Cytometry

- In conventional cytometry, one detector is assigned to one fluorophore
- With spectral cytometry, all detectors are used for all fluorophores



Spectral Flow Cytometry uses dispersive optics, such as prisms or gratings, to disperse the collected light across a detector array, allowing the full spectra from each particle to be measured.

Nolan & Condello (2013) *Current Protocols in Cytometry*

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Detectors

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Light must be converted from photons into volts to be measured : **Detectors**

- Devices that sense the light, then convert it to an electronic signal
 - **Photodiode (PD)**
 - Light sensitive semiconductors
 - Not highly sensitive
 - Only used for strong signals (e.g., Forward scatter)
 - **Avalanche Photodiode (APD)**
 - Same as PD but with Higher sensitivity (High QE)
 - **Photomultiplier Tube (PMT)**
 - Much more sensitive than photodiode
 - Used for both fluorescence and side scatter (SSC)
 - Sensitivity adjusted using voltage



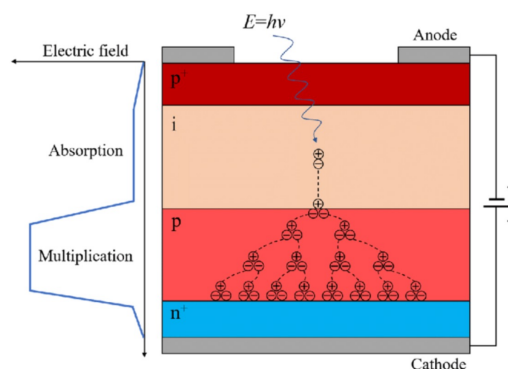
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APDs

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- Avalanche photodiodes (APDs) are silicon photodiodes with an internal gain mechanism.
- As with a conventional photodiode, absorption of incident photons creates electron-hole pairs.
- However, by placing a high reverse bias voltage a strong internal electric field is created, and this accelerates the electrons through the silicon crystal lattice to produce secondary electrons by impact ionization.
- The resulting electron avalanche can produce gain factors up to several hundred.



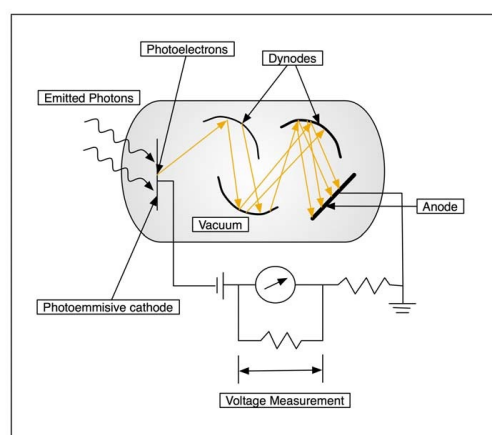
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PMTs

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- Produce current at their anodes when photons impinge upon their light-sensitive cathodes
Require external power source
- Their gain is as high as 10⁷ electrons out per photon in
- Noise can be generated from thermionic emission of electrons - this is called "dark current"
- If very low levels of signal are available, PMTs are often cooled to reduce heat effects
- Spectral response of PMTs is determined by the composition of the photocathode



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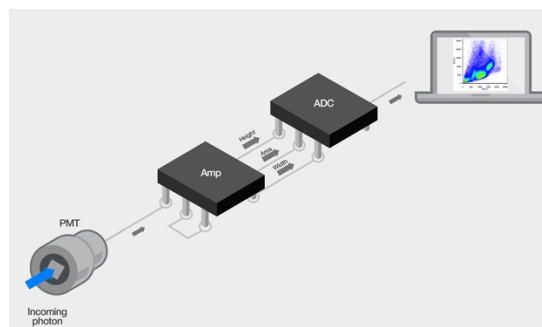
Signal Conversion

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Analog-to-Digital converters (ADC) translate analog signals such as voltages or light intensity into a digital representation of that signal.

This digital representation can then be processed, computed and stored.

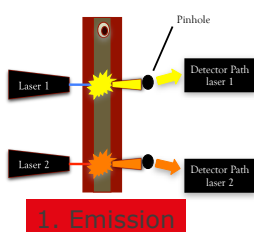
- Current is sent to amplifier(s)
- Signal pulse is sent to an analog to digital converter (ADC)
- The signal is converted to a digital value
- The values are entered into a spreadsheet and given a header
- A Flow Cytometry Standard (FCS) file is generated.



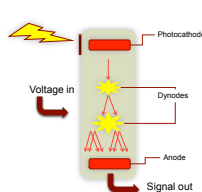
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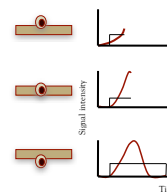
How flow data are generated

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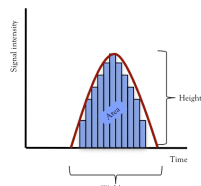
1. Emission



2. Detection



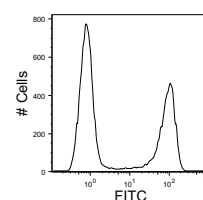
3. Converted to Voltage



4. Measured

Event	Time	FSC	SSC	FITC	PE	APC
1	0	100	500	10	650	4
2	0	110	505	700	700	6
3	0	90	480	720	670	10
4	0	95	490	700	720	15
5	0	12	76	15	15	13
6	0	120	600	14	810	785
7	0	108	530	16	595	18
8	0	117	654	12	720	12
9	1	54	276	378	576	18
10	1	193	803	690	912	790

5. File Generated



6. Plotted

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Storage Data – FCS files

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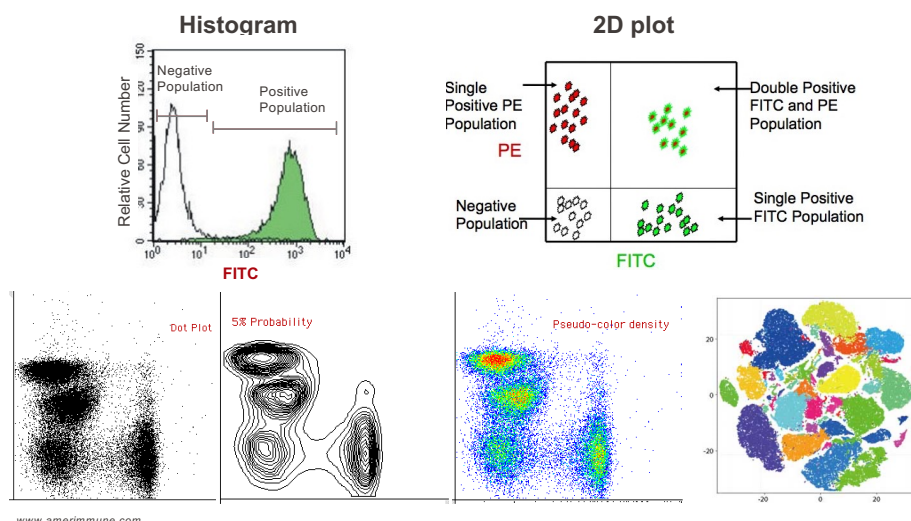
- **Flow Cytometry data are stored in a flow cytometry standard (FCS) file**
 - The standards for the file type are maintained by ISAC and contains:
 - All the discrete digital values in a “spreadsheet”
 - A header containing pertinent information about the file
 - Metadata (keywords)
 - values on Date run, PMT voltages, times, etc.
- **When the FCS file standard changes, the information required in the header changes, but the data values are still in a spreadsheet.**

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Data Visualisation

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Applications

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Extracellular and Intracellular Immunostaining
 Cell Cycle Analysis
 Fluorescent Proteins
 Cytotoxicity assays, Cell Death, Viability and Apoptosis
 Autophagy
 Cell Proliferation
 Calcium Flux
 ROS
 FRET
 CBA
 RNA analysis, Genomic cytometry
 Extracellular vesicles
 Microbiology / Marine biology
 Metabolism (NADH, GSH, Mitochondrial Activity...)

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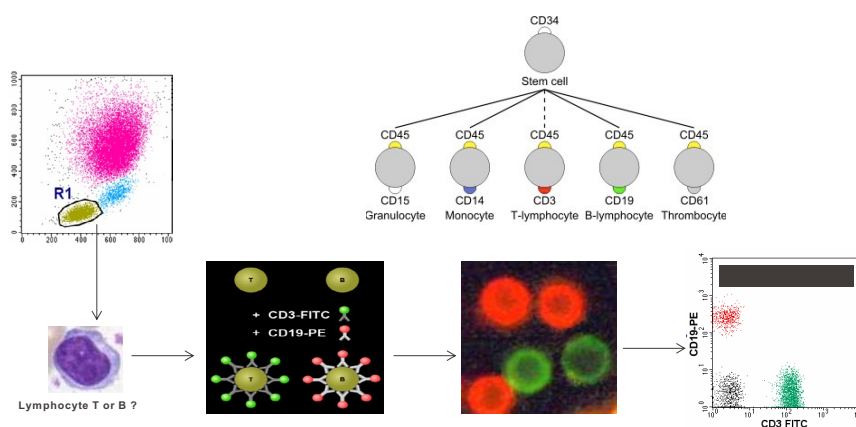
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Applications

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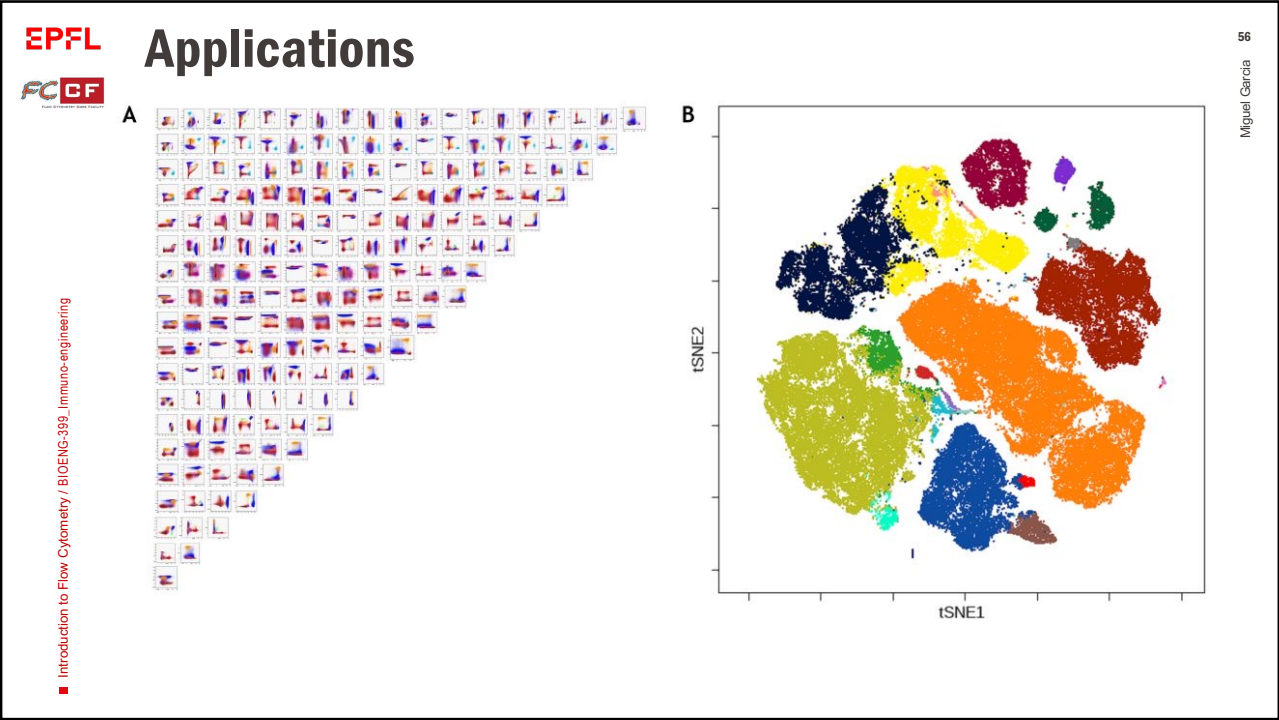
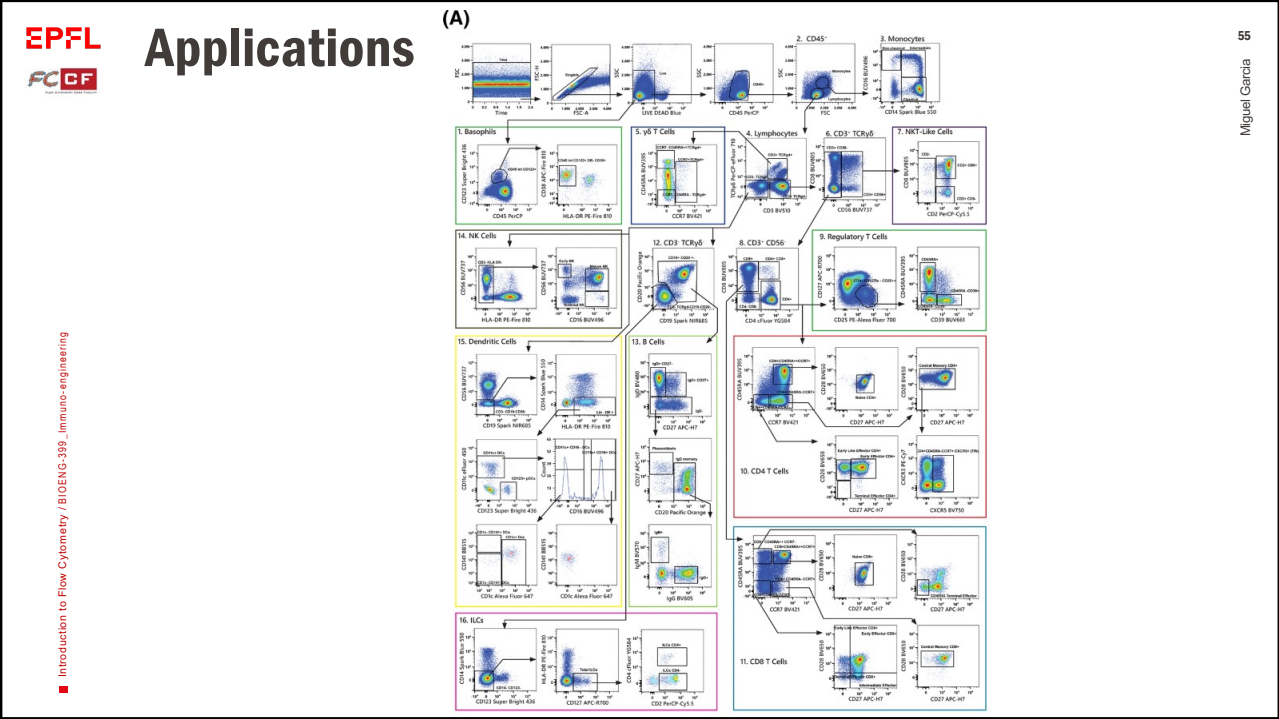
■ Immunophenotyping

- Detection of cell surface molecules as example cluster of differentiation



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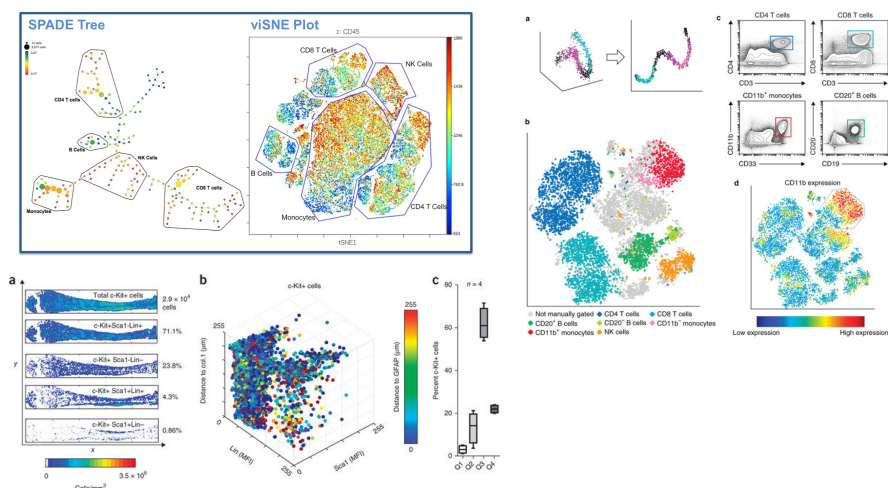
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Applications

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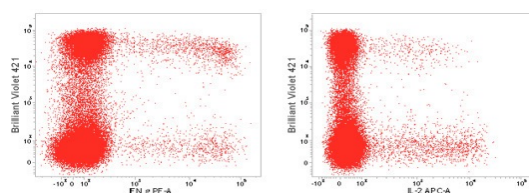
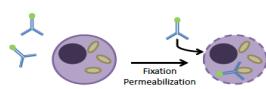
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Applications

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Cell function

- Intracellular staining of cytokines, cytoskeleton, enzymes, transcription factors, signaling molecules

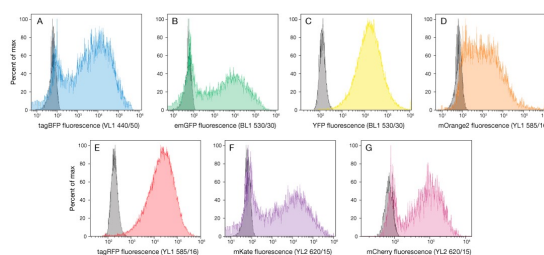
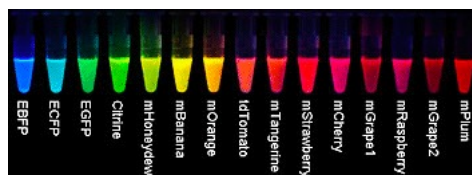


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Applications

■ Fluorescent Proteins



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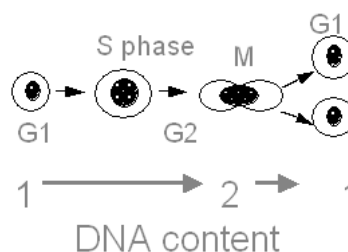
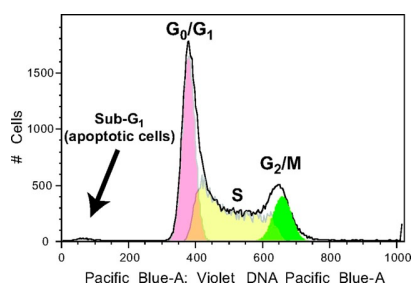
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Applications

■ Monodimensional DNA Analysis

- DNA content of individual cells gives information about their ploidy
- Suitable dyes: PI, DAPI, Hoechst, DRAQ5, DyeCycle...
- Combination with other parameter



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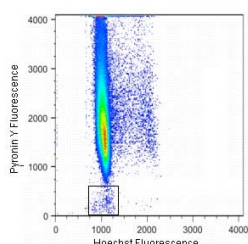
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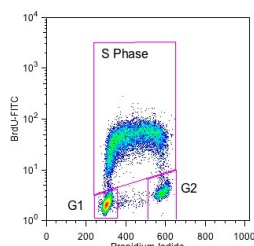
Applications

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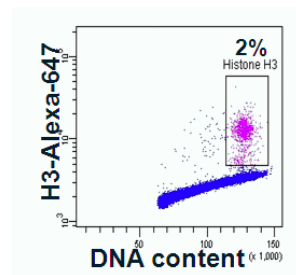
- **Bi-dimensional DNA Analysis**



G0-phase



S-phase



M-phase

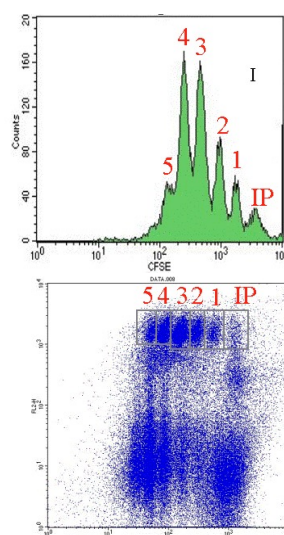
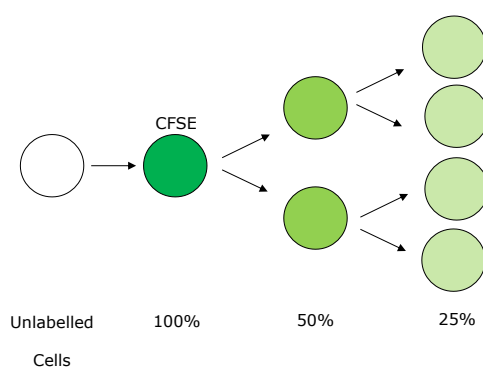
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Applications

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- **Cell Proliferation**



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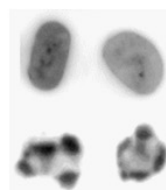
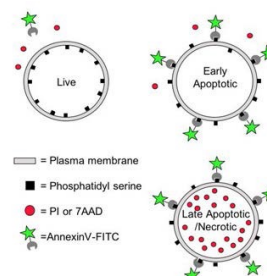
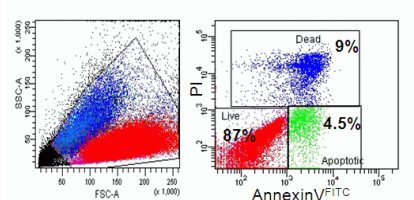
62

Applications

Cell death

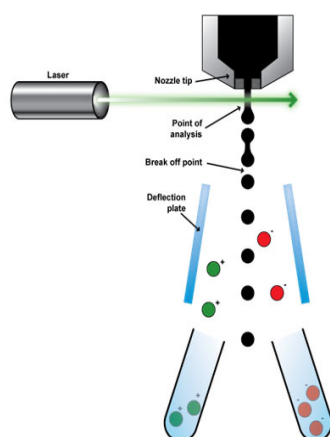
Measurements of cell death:

- Expression of proteins involved in apoptosis
- Activation of caspases
- Changes in the mitochondrial membrane potential
- Changes in the plasma membrane
- DNA degradation



Applications

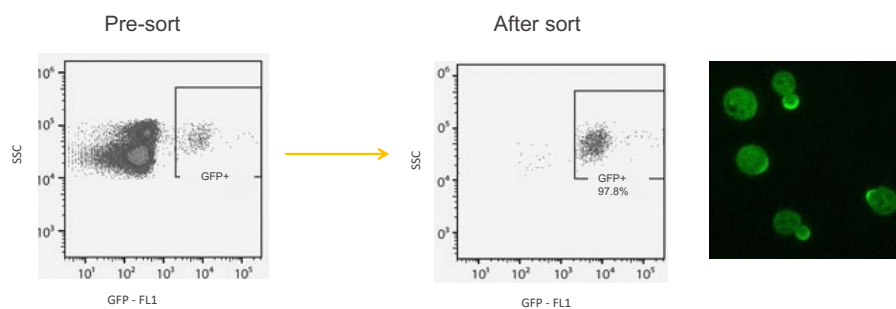
Sorting



- Same principle as analysers for detection of the fluorescence
- Physical separation of the cells of interest
- Possible to sort Single-Cell – Clones or single-cell gene expression analysis
- Possible to sort into tubes, plates or slides

Applications

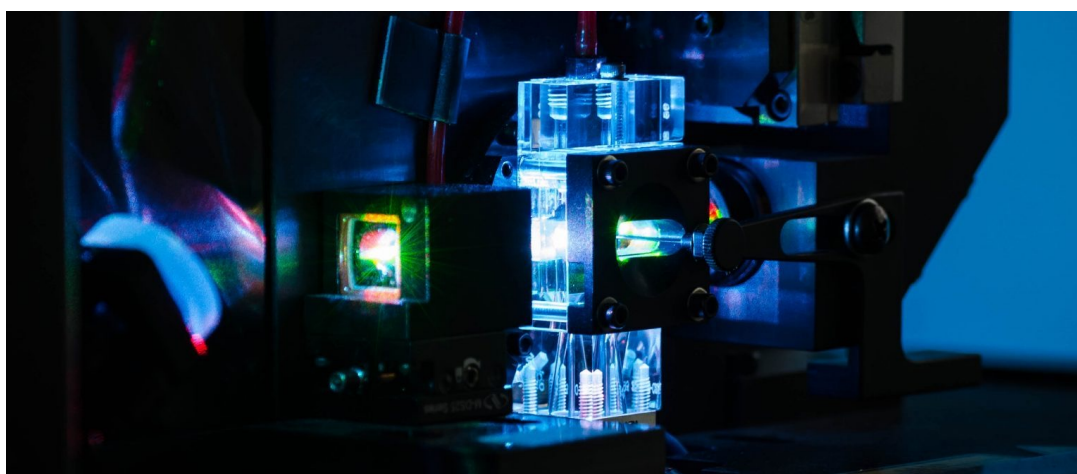
■ Sorting



Downstream applications : Cell culture, functional assays, clonal colony generation, omics technologies....

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Bridges with other Technologies



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New Technologies

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Imaging Flow Cytometry

Mass Cytometry (CyTOF)

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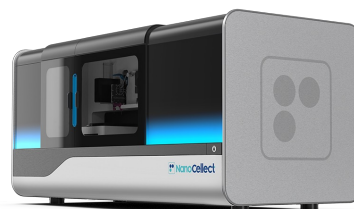
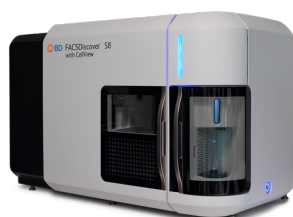
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Imaging Flow Cytometry

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- Powerful combination of quantitative images analysis and flow cytometry

For each dot in the plot, we can see the correspondent image



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Imaging Flow Cytometry Cytek Amnis

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- Conventional Flow Cytometer

- Up to 6 lasers
- 12 Fluorescent Channels
- 20x, 40x & 60x objective

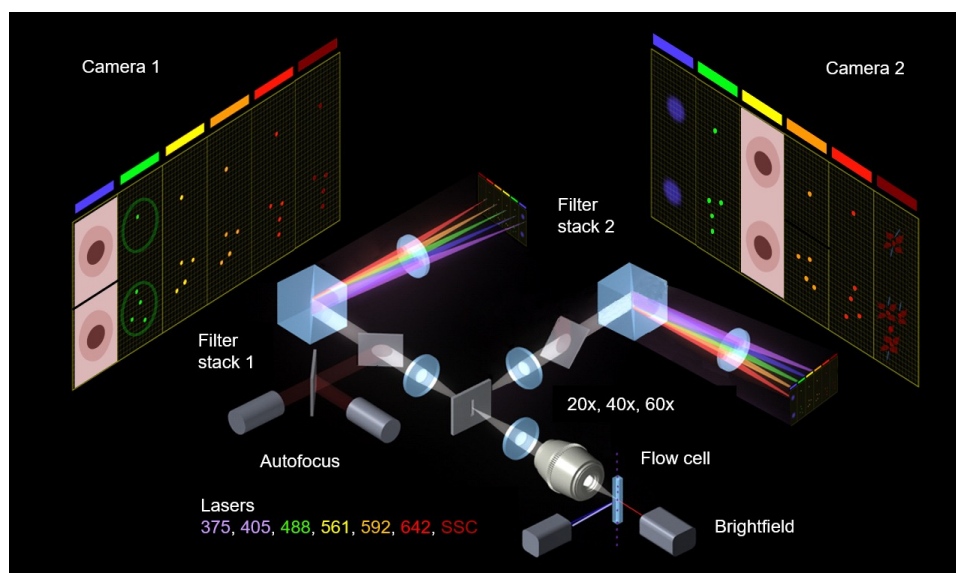
For each dot in the plot, we can see the correspondent image in all fluorescent channels



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Imaging Flow Cytometry – Cytek Amnis

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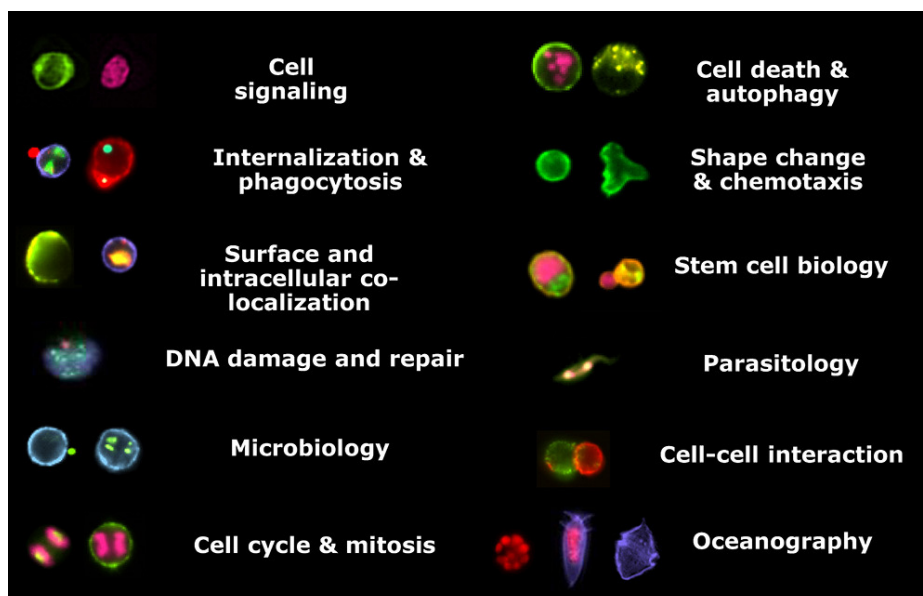
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Amnis – Applications

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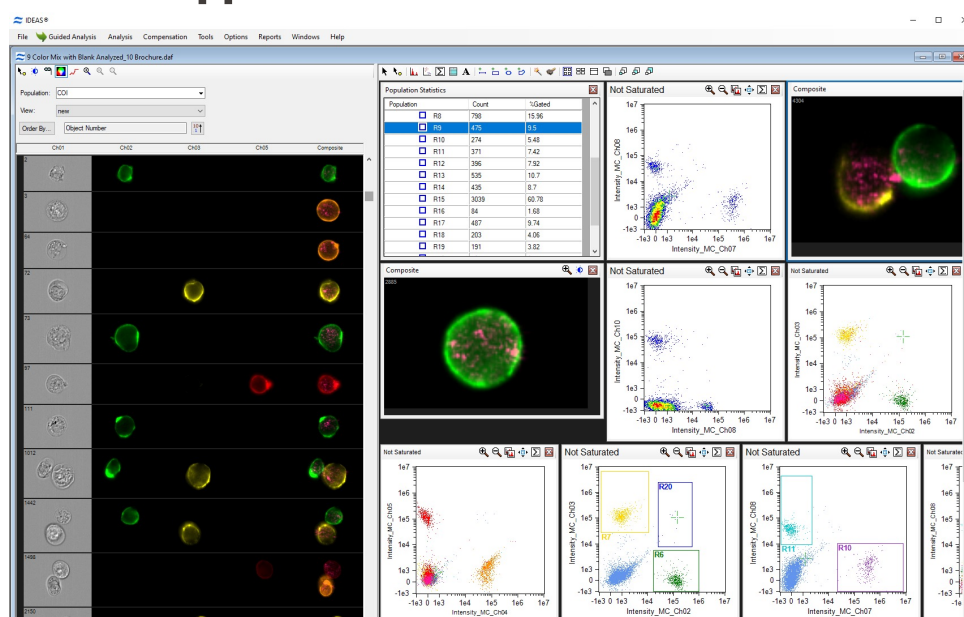


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Amnis – Applications

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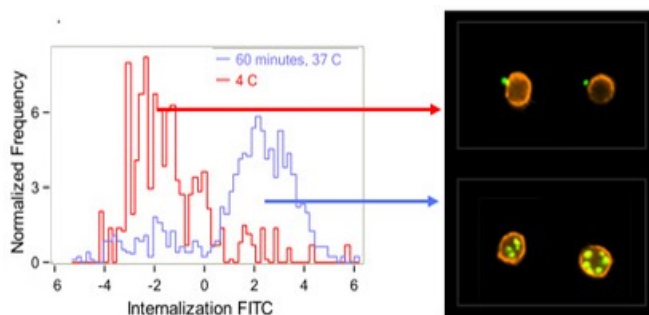


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Amnis – Applications

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Phagocytosis by macrophages



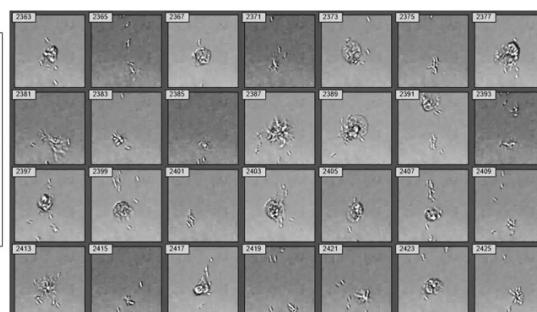
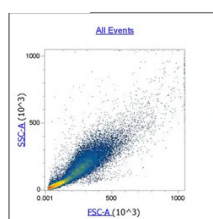
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Attune CytPix

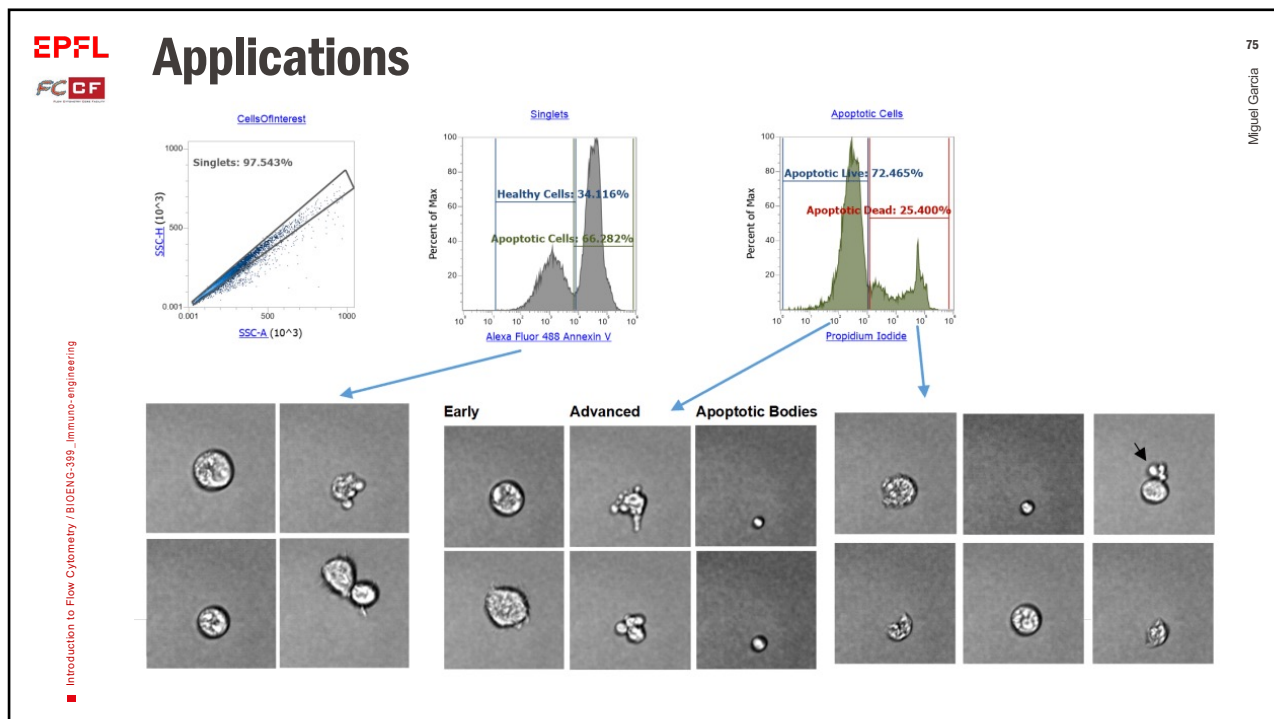
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Brightfield images
no fluorescence



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EPFL
FCBF

BD FACSDiscover™ A8 & S8

Combining Full Spectral Flow Cytometry with Real-Time Imaging

Integrates **real-time imaging (RTI)** with **full spectral flow cytometry**, providing both **morphological** and **spatial insights** alongside traditional cytometry data.

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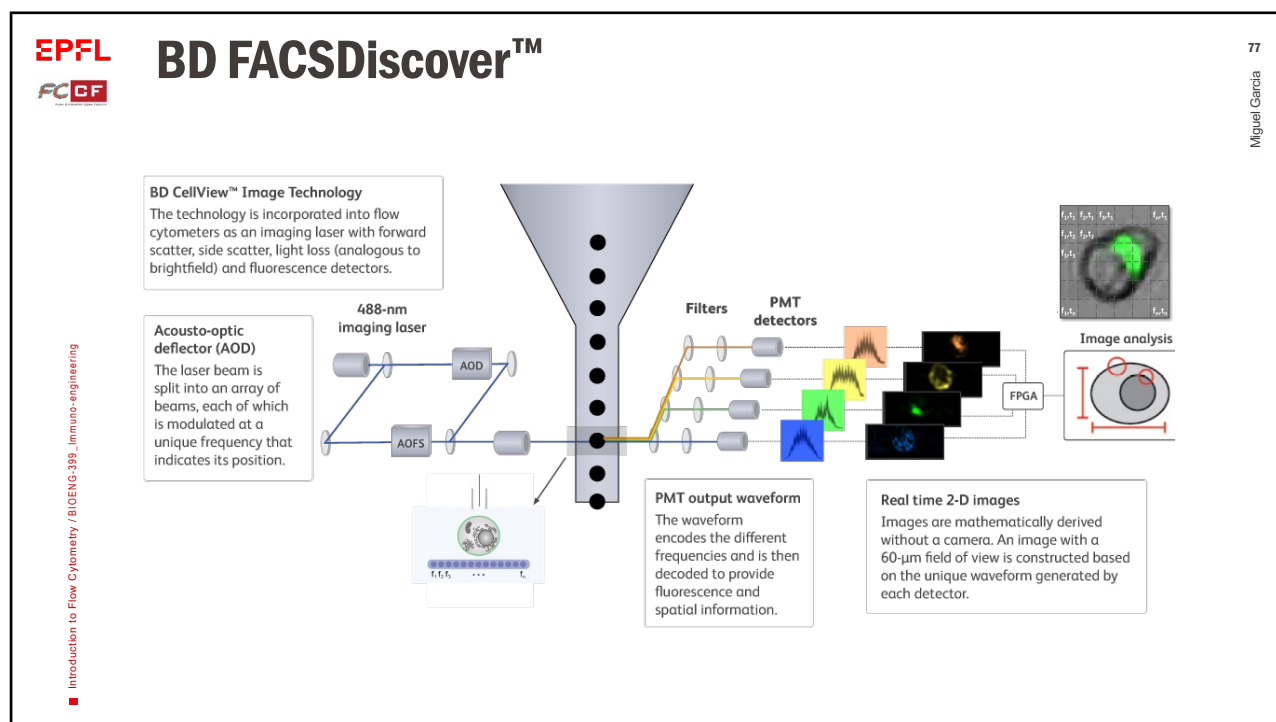
BD FACSDiscover S8 with CellView

S8 Cell Sort

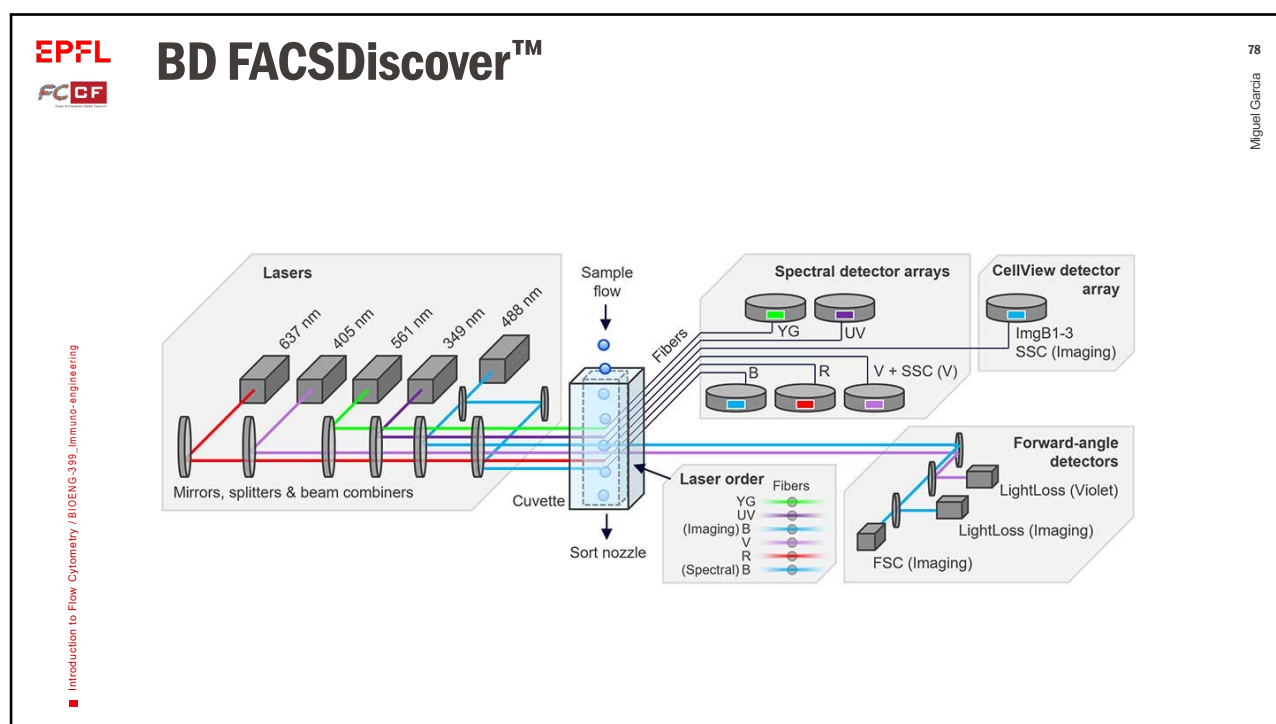
BD FACSDiscover A8 with CellView

A8 Analyzer

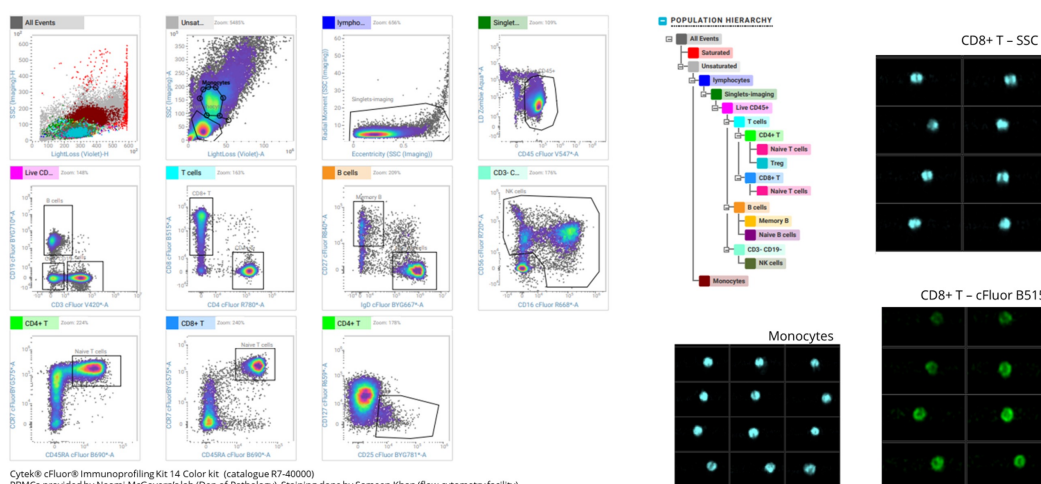
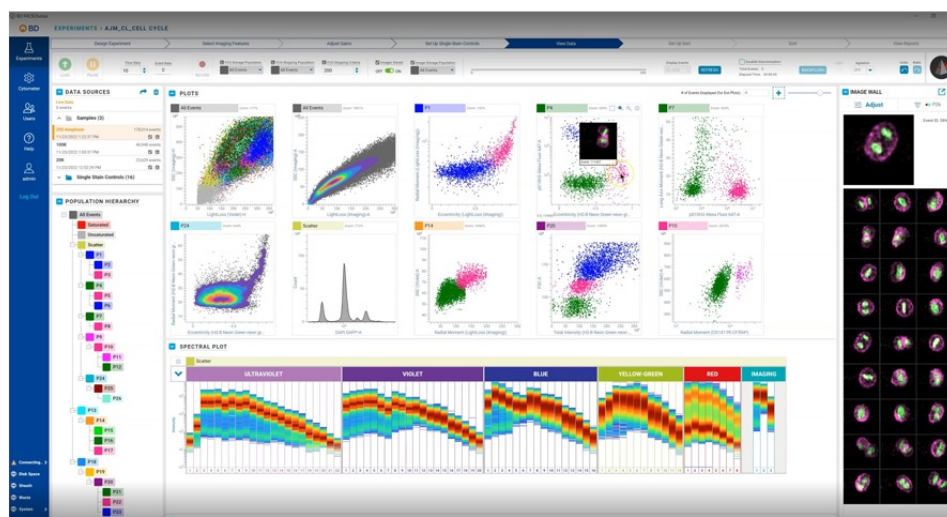
76

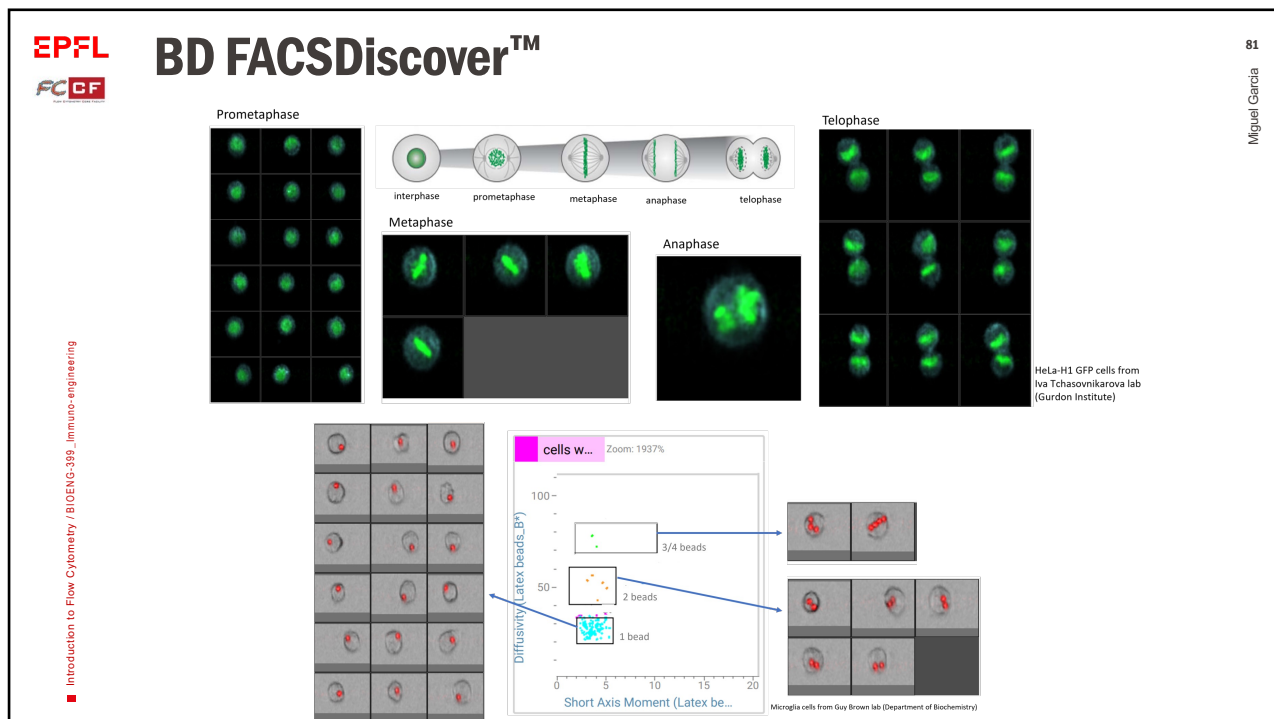


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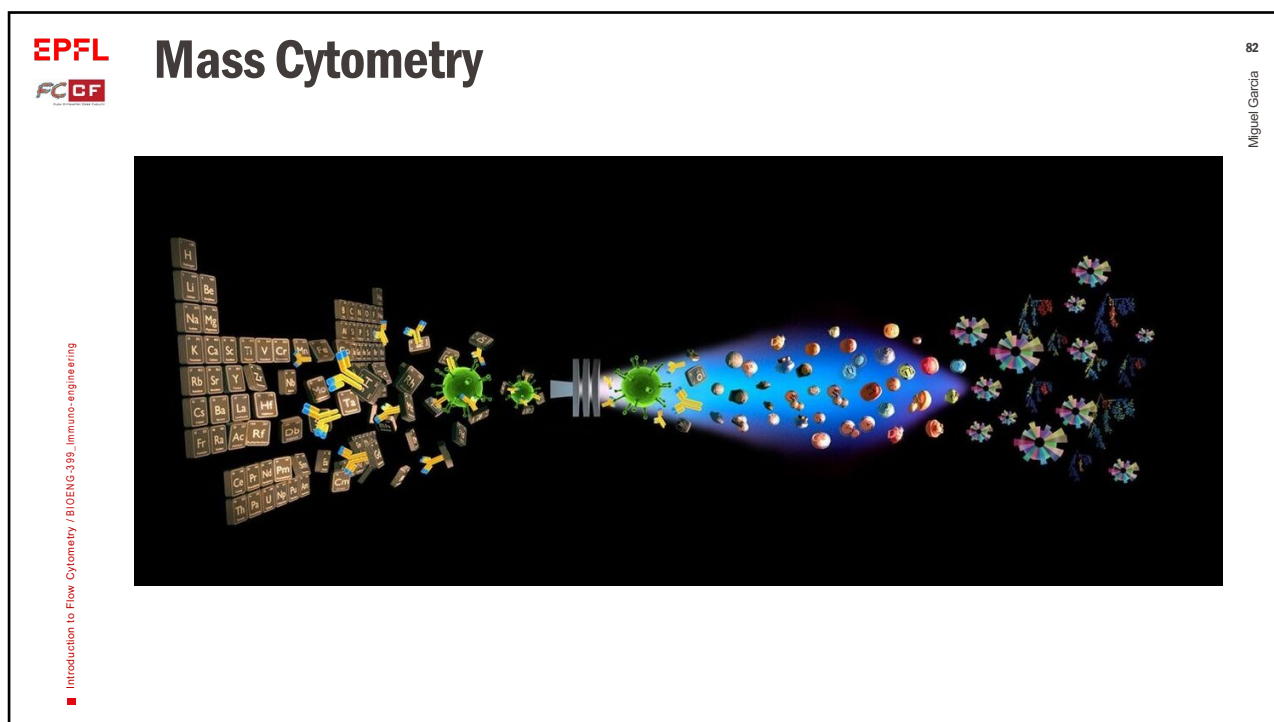


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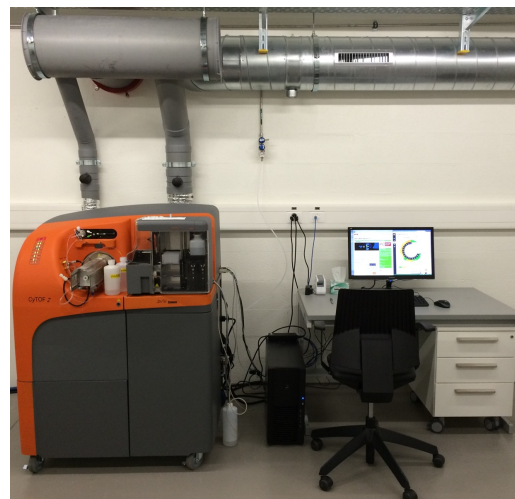


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Mass Cytometry

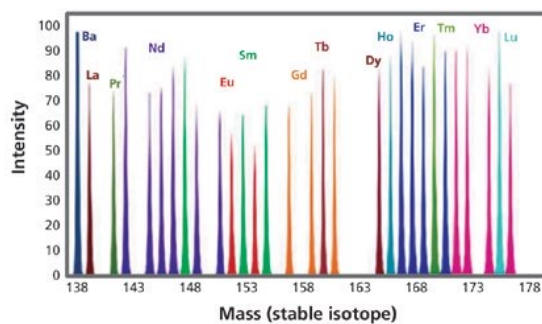
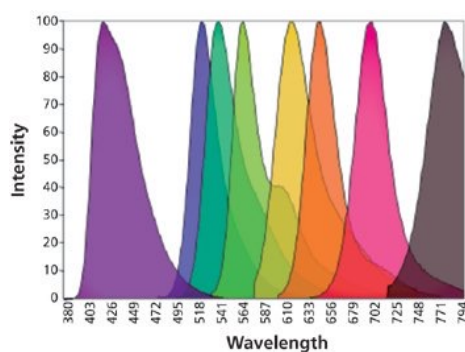
■ CyTOF : Mass Spec + Flow Cytometry

- Ability to resolve over 100 metal probes with minimal signal overlap common to atomic mass spectroscopy
- Cells are stained in suspension with a panel of metal-conjugated probes directed against targets of interest - antibodies
- The quantities of isotopes bound to each cell are then analysed by a time-of-flight **mass spectrometer**.
- The intensity of the signal detected in each channel is directly proportional to the number of specific probe-derived ions striking the detector and thus the number of antibodies originally bound per cell

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Mass Cytometry



Standard Biotools

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[illegible]

The diagram illustrates the Mass Cytometry workflow, which combines flow cytometry with mass spectrometry for high-dimensional cell analysis.

- Sample Preparation:** Cells are labeled with antibodies conjugated to elemental isotopes (e.g., barium, cerium, lanthanum).
- Ionization and Separation:** The sample is introduced into a **Nebulizer**, then passes through an **ICP** (Inductively Coupled Plasma) for ionization. The ions are then separated by a **Quadrupole** mass filter.
- Detection:** The ions are detected by a **Time-of-flight** mass analyzer, which measures the mass-to-charge ratio of the ions.
- Data Processing:** The raw data is processed to **Integrate-per-cell**, resulting in mass spectra for individual cells (e.g., Cell 1, Cell 2, Cell 3). These spectra are categorized into **Heavy (>100 Da) Reporter atomic ions** and **Light (<100 Da) Overly abundant ions**.
- Analysis:** The data is exported as a **.FCS file** and analyzed using software like **FlowJo**. The analysis shows **Element A** vs **Element B** scatter plots, identifying distinct cell populations (e.g., red and blue gates).

