Introduction to Flow Cytometry

BIOENG-519
October 2025

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1

Overview

- What is Flow Cytometry
- Flow cytometer components:
  - Fluidics (hydrodynamic focusing)
  - Optics (FSC, SSC, fluorescence)
  - Electronics (detectors, signal)
- Data presentation
- Overview of applications
- Instruments
  - Flow cytometers, cell sorters, mass cytometers, full spectral cytometers

2

What is Flow Cytometry

Flow Cytometry ≈ FACS: **F**luorescence **A**ctivated **C**ell **S**orter

Fluid Cell Measure

Definition :

Single cells in suspension that pass a laser bean produce characteristic light signals which are analyzed by different detectors.

Flow cytometry is a technique used to measure the physical and chemical properties of cells or cellular components.

Cells are measured individually, but in large numbers.

3

What is the advantages

Analysis of thousands of cells per second detecting multiple parameters of individual cells within heterogeneous populations.

- Quick sample processing (up to 35'000 evs/s)
- High statistical power
- Study of (sub)populations of cells
- Multi-parametric analysis – up to 20 parameters simultaneously in conventional, up to 50 on most recent instruments

4

Flow cytometer components

Understand what is happening inside the “black box” (flow cytometer) is critical to the design and execution of flow cytometry experiments.

5

Flow cytometer components

Fluidics
- Cells in suspension
- flow in single file line through

Optics Detectors
- an illuminated volume where they
- scatter light and emit fluorescence
- that is filtered, collected and

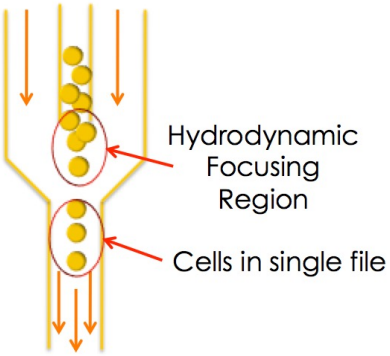
Electronics
- converted to digital values
- that are stored on a computer

6

Flow Cytometer Fluidics

Fluidics

- Cells in suspension
- flow in single-file through



2 Fluidics principles

Hydrodynamic focusing

Laminar flow

7

Flow Cytometer Fluidics

2 Fluidics principles

Hydrodynamic focusing

- Sample is injected into the center of sheath fluid
- Difference of pressure,
  $P_{sheath} > P_{sample}$
  (different velocities)
- Design of the Flow cell

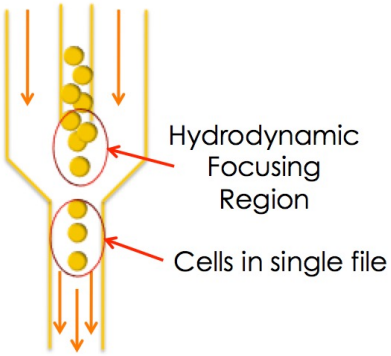
Laminar flow

If two fluids differ enough in density and/or velocity, so they do not mix ! They form a two layer stable flow

8

Flow Cytometer Fluidics

- The cell sample is injected into a stream of sheath fluid.
- By the laminar flow principle, the sample remains in the center of the sheath fluid.
- The cells in the sample are accelerated and individually pass through a laser beam for interrogation.

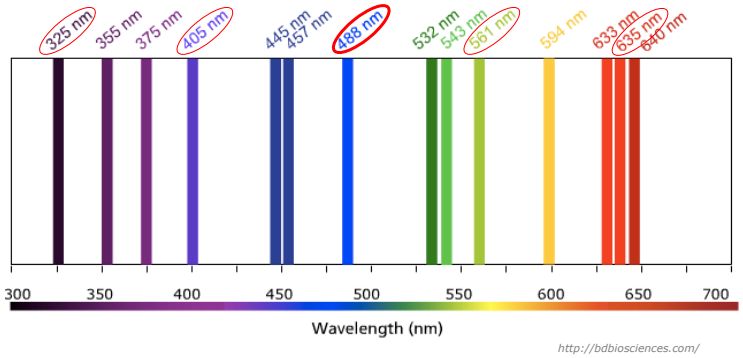


9

Flow Cytometer Optics

Illumination Source - Lasers

Laser light is coherent and monochromatic
(synchronized, identical wave frequency and single wavelength)

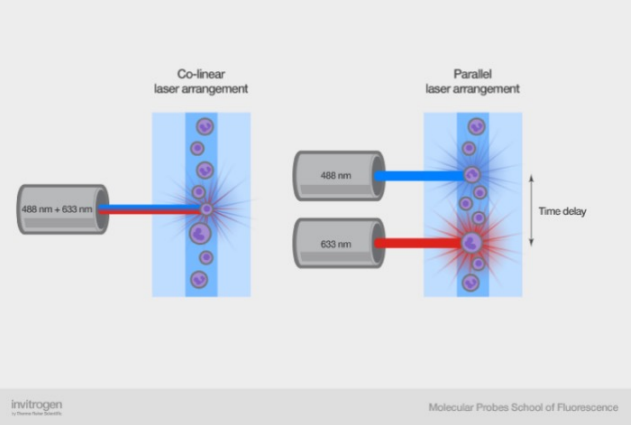


http://bdbiosciences.com/

10

Flow Cytometer Optics

Lasers configuration

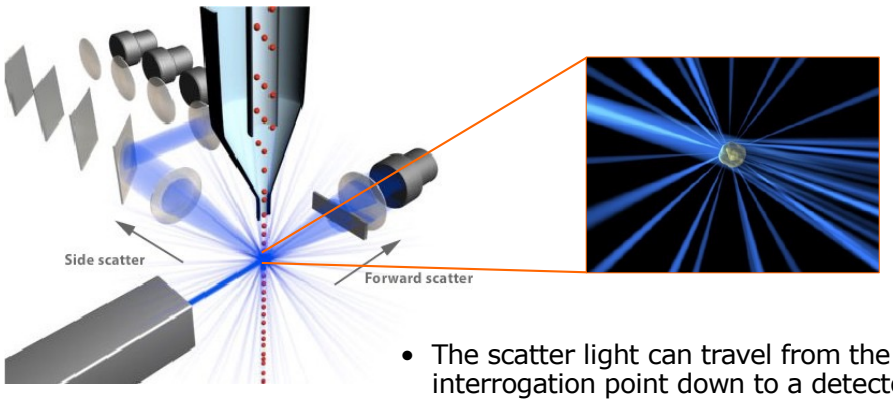


- 1 to 7 lasers
- Co-linear
- Parallel or Spatially separated

11

Flow Cytometer Optics

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

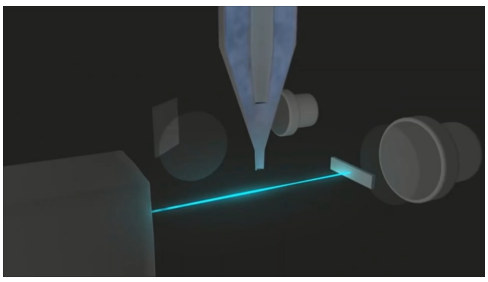


- The scatter light can travel from the interrogation point down to a detector

12

Flow Cytometer Optics

Lights measured by flow cytometry



Laser light scatter: Refracted light when the laser hits the particle

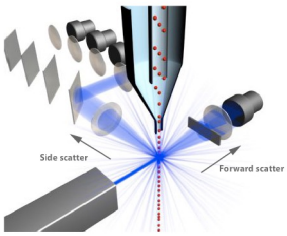
- Forward scatter (FSC)
- Side scatter (SSC)

Fluorescence: emitted light from fluorescent tag(s) added to the sample, when excited by the laser

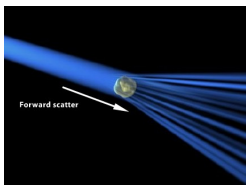
13

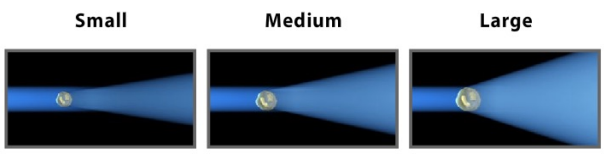
Flow Cytometer Optics

Forward Scatter



- 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

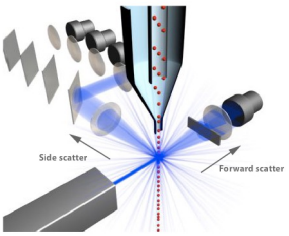


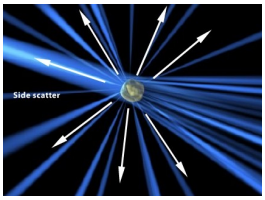


14

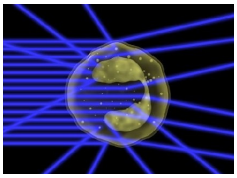
Flow Cytometer Optics

Side Scatter





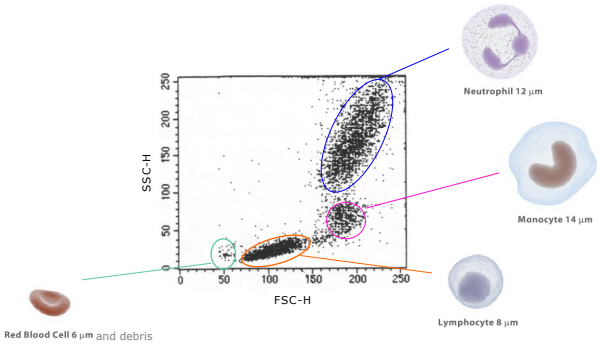
- Light that is scatter at 90 degrees to the axis of the laser path is detected in the Side Scatter Channel
- Side scatter is caused by granularity and/or structural complexity inside the cell/particle (e.g. Granulated nuclei, cell inclusions, etc.)



15

Flow Cytometer Optics

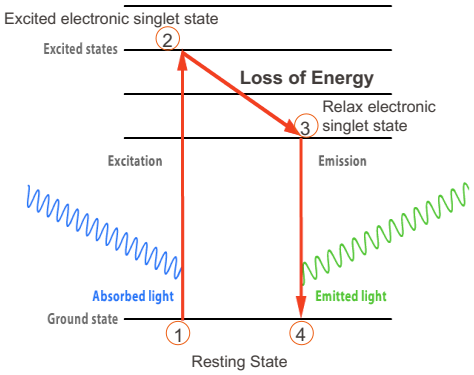
- Since FSC ~size and SSC ~internal structure, a correlated measurement between them may allow the differentiation of different cell types in a heterogeneous cell population



16

Flow Cytometer Optics

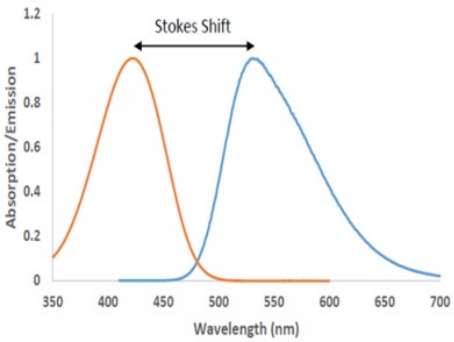
Fluorescence : Emission of light by a compound that has absorbed a photon of light



1. Excitation : Energy Intake
   Absorbing a photon raises an electron up to a higher energy level
2. Excited state lifetime
   Loss of energy by vibration, rotation
3. Emission : Energy release
   The electron falls back to the ground state and emits a photon with less energy than the absorbed one

17

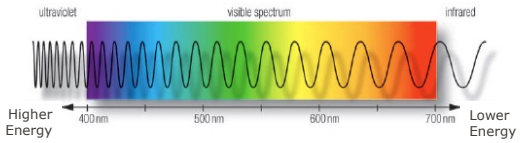
Flow Cytometer Optics



The energy difference between an absorption and emitted photon is called :

Stokes Shift

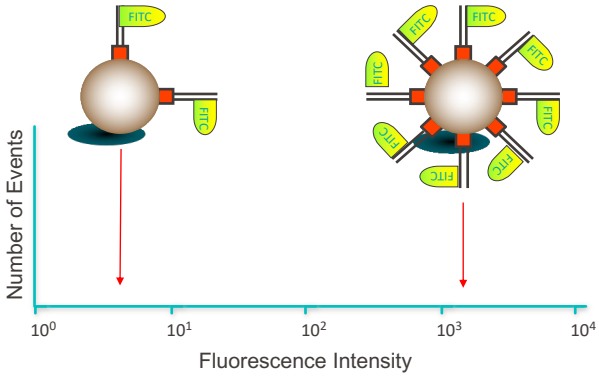
Each type of fluorochrome exhibits its own Stokes shift in this regard and emits light of a specific wavelength.



18

Flow Cytometer Optics

Emitted fluorescence intensity is proportional to the amount of bound fluorescence molecules

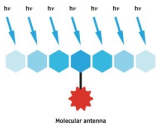


19

Flow Cytometer Optics

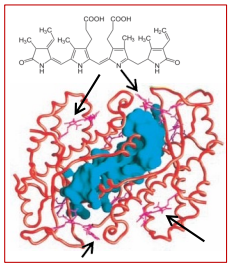
Fluorophores

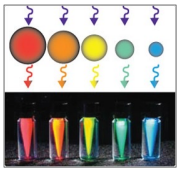
Polymer dyes



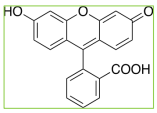
Polymer dyes

Phycoerythrin: a naturally occurring fluorescent protein

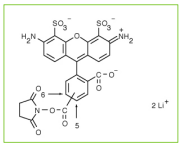


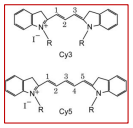


FITC: Fluorescein Isothiocyanate



Alexa 488





Cyanine 3
Cyanine 5

source : Excyte Expert Cytometry

20

Flow Cytometer Optics

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 and Dichroic mirrors

- Used to guide and split the light accordingly to the its wavelength in order to be collected independently
- **Types of Filters**
  - Longpass (e.g., LP 560)
  - Shortpass (e.g., SP 560)
  - Bandpass (e.g., BP 530/30)
- **Dichroic mirrors**
  - Longpass or shortpass filters that contain a mirror coating
  - Allow some light to pass and reflect the remainder
  - Most common filters used in current instruments

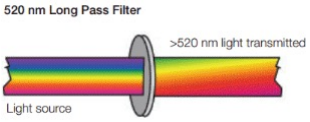
https://analyticalscience.wiley.com/do/10.1002/imaging.3828

21

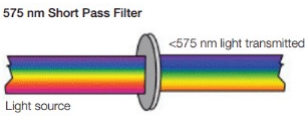
Flow Cytometer Optics

Filters and Dichroic mirrors

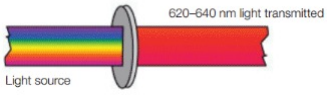
Longpass



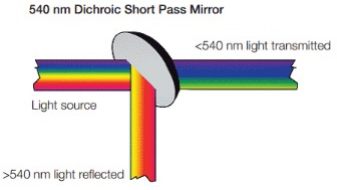
Shortpass



Bandpass



Dichroic mirror

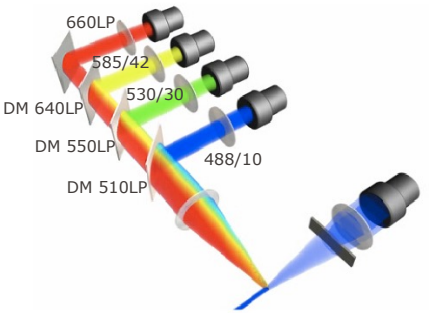


22

Flow Cytometer Optics

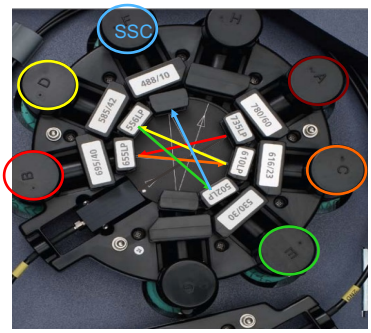
Different optical configurations

Transmission principle



http://probes.invitrogen.com

Reflection principle (BD)



http://bdbiosciences.com/

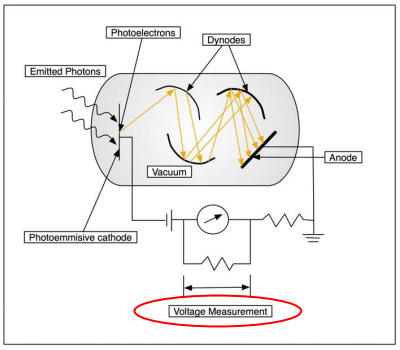
23

Flow Cytometer Electronics

Detectors

- Light must be converted from photons into volts (current) to be measured
  - Photodiodes
  - APDs

  - **PMTs**
    - Conversion and signal amplification
    - Provides current output proportional to light intensity

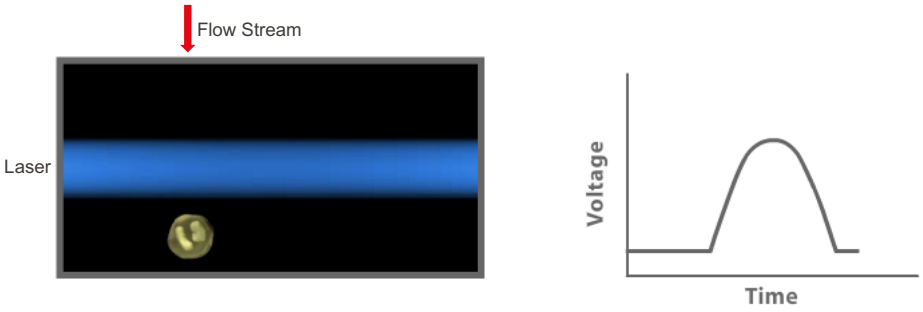


Quinn, John & Hrebien, L. & Leonid,. (2020). Development of a pattern recognition approach for analyzing flow cytometric data

24

Flow Cytometer Electronics

Signal Pulse

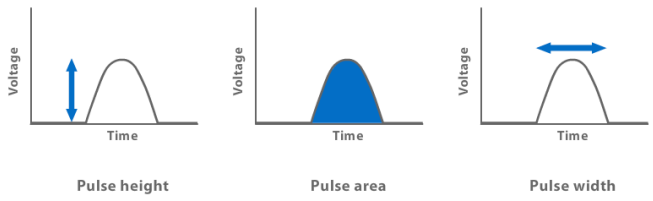


- A voltage pulse is generated each time a cell or particle passes through the laser beam
- Each pulse for each cell/particle is known as an **event**

25

Flow Cytometer Electronics

- From each pulse we can obtain:
  - The peak of the pulse – **Height, H**
  - The time that it takes to pass through the laser – **Width, W**
  - The total area of the pulse – **Area, A**



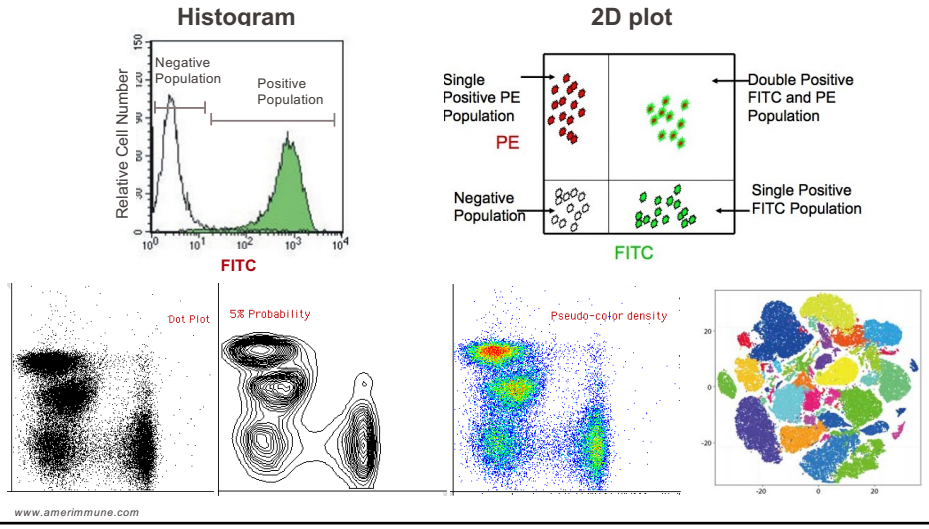
The voltage pulse height, width, and area are determined by the particle's size, speed, and fluorescence intensity.

26

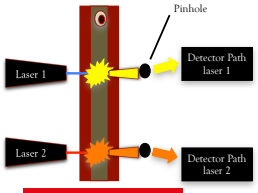
Flow Cytometer Electronics

- **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.**

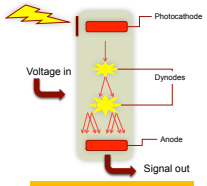
Flow Cytometer Data presentation



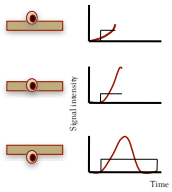
Flow Cytometer Overview



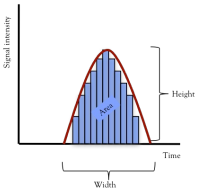
1. Emission



2. Detection



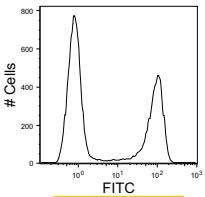
3. Converted to Voltage



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

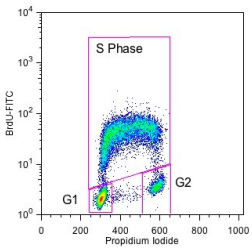


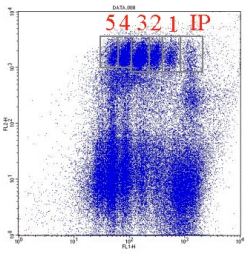
6. Plotted

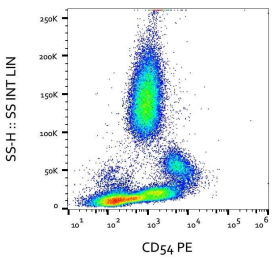
29

Flow Cytometer Applications

- Extracellular and Intracellular Immunostaining
- Cell Cycle Analysis
- Fluorescent Proteins
- Cell Death, Viability and Apoptosis
- Autophagy
- Cell Proliferation
- Calcium Flux
- ROS
- FRET
- CBA
- RNA analysis
- Extracellular vesicles
- Microbiology
- Metabolism (NADH, GSH, Mitochondrial Activity.



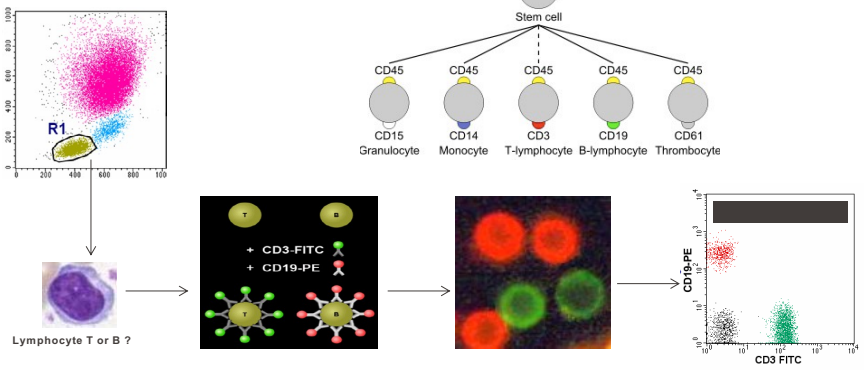




30

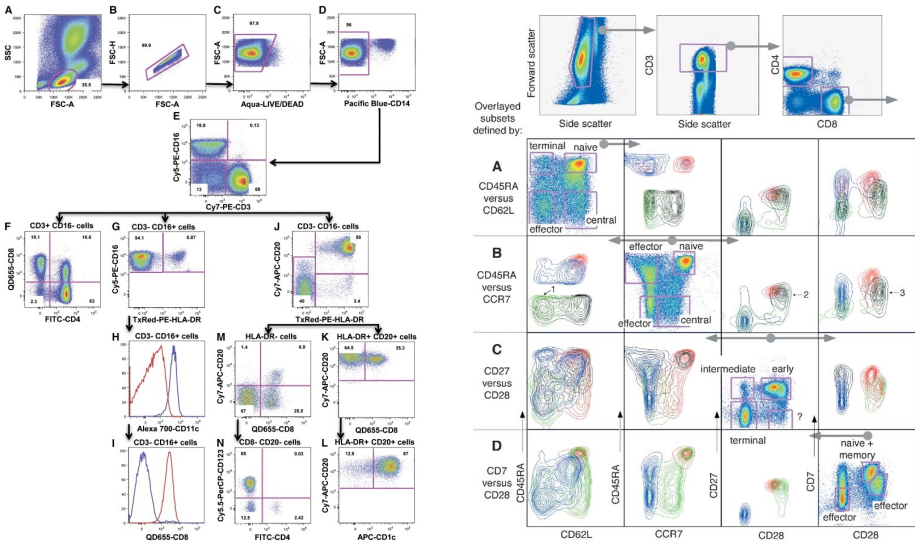
Flow Cytometer Applications

- **Immunophenotyping**
  - Detection of cell surface molecules as example cluster of differentiation



31

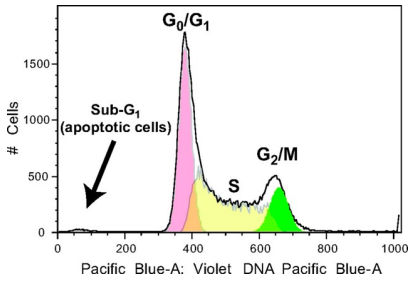
Flow Cytometer Applications

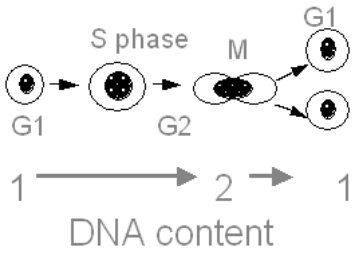


32

Flow Cytometer Applications

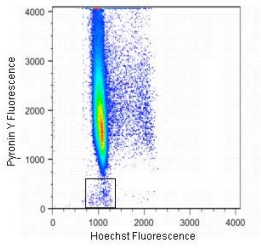
- **DNA Analysis**
  - DNA content of individual cells gives information about their ploidy
  - Suitable dyes: PI, DAPI, Hoechst, DRAQ5, DyeCycle…
  - Combination with other parameter



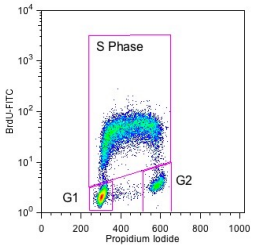


33

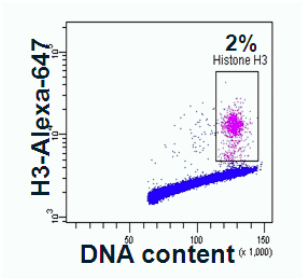
Flow Cytometer Applications



G0-phase



S-phase

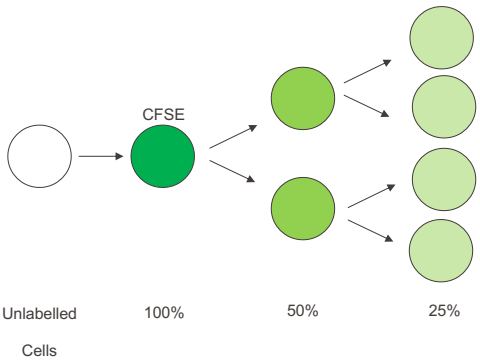


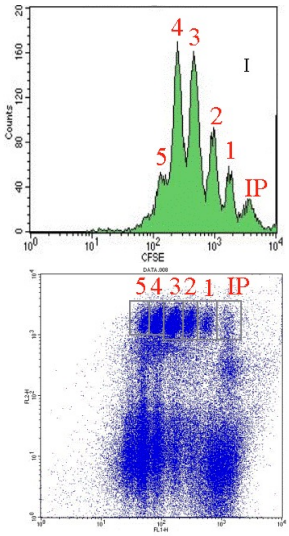
M-phase

34

Flow Cytometer Applications

- **Cell Proliferation**

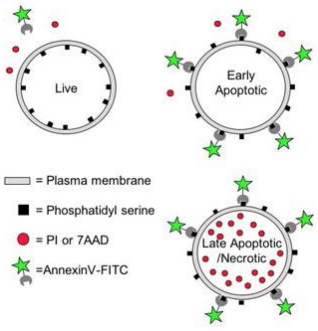


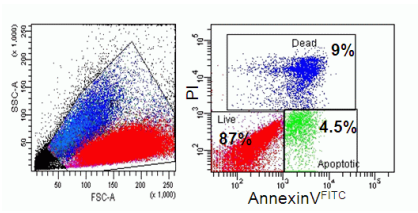


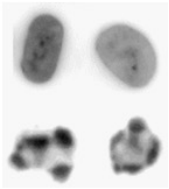
35

Flow Cytometer 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







36

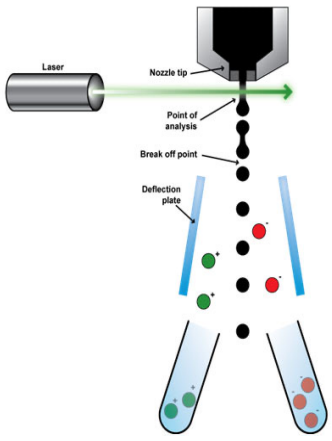
Flow Cytometer Applications

- Bone marrow cells are evaluated based on SSC and CD45 expression to diagnose acute lymphoblastic leukemia.
- $CD4^+$ T cell counts are used to monitor the progression of AIDS in HIV-infected patients
- Erythropoietin and blood doping
- Monitoring oenological fermentation in wine
- ……..

37

Flow Cytometer Applications

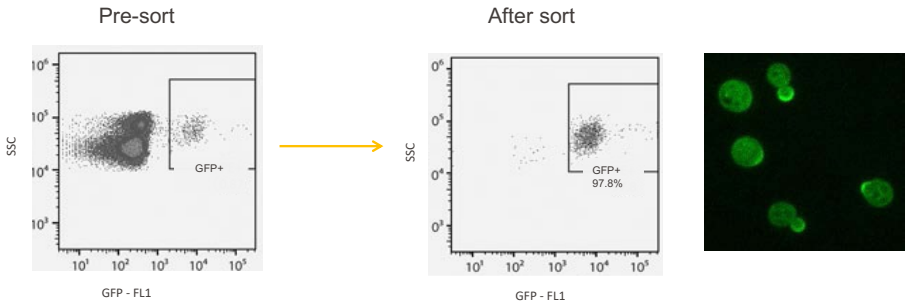
Sorting



- 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

38

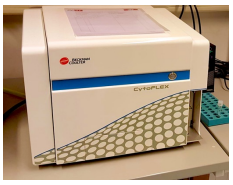
Flow Cytometer Applications

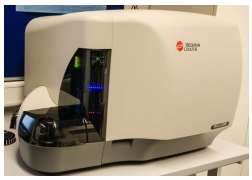


39

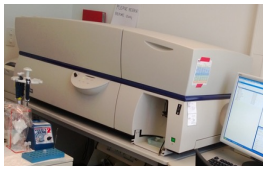
Flow Cytometer Instruments

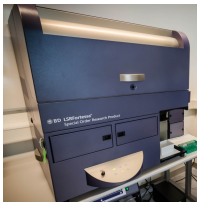

Aurora


CytoFLEX


Gallios (x2)


Attune NxT


LSR II (x2)

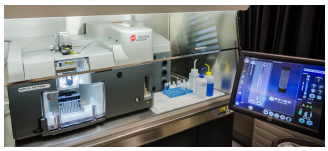

LSRFortessa

40

Flow Cytometer Instruments

Sorters
Droplet based

Jet-in-air

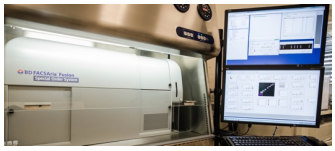


MoFlo ASTRIOS

SONY SH800

Cuvette

FACSARIA II



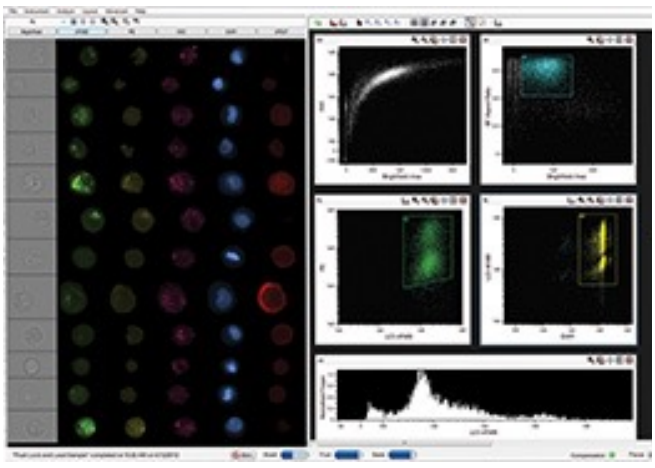
FACSARIA FUSION

Flow Cytometer Instruments

ImageStream

Powerful combination of quantitative images analysis and flow cytometry

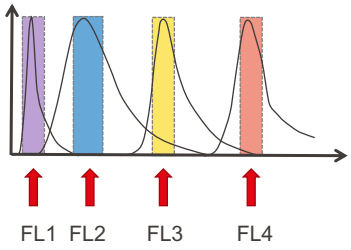
These instruments produce multiple high-resolution images of every cell directly in flow, including brightfield and darkfield (SSC), and up to 10 fluorescent markers



Conventional Flow Cytometry

Conventional

In conventional cytometry, one detector is assigned to one fluorophore



Each fluorochrome is detected in **ONE** channel

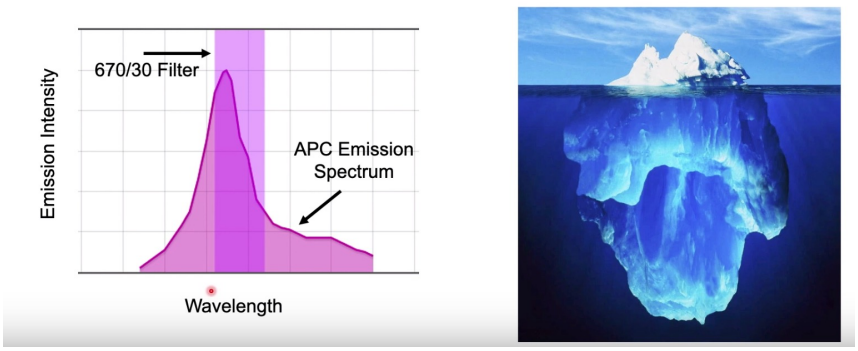
FL1 → PB
FL2 → FITC
FL2 → PE
FL3 → APC

Limitations:

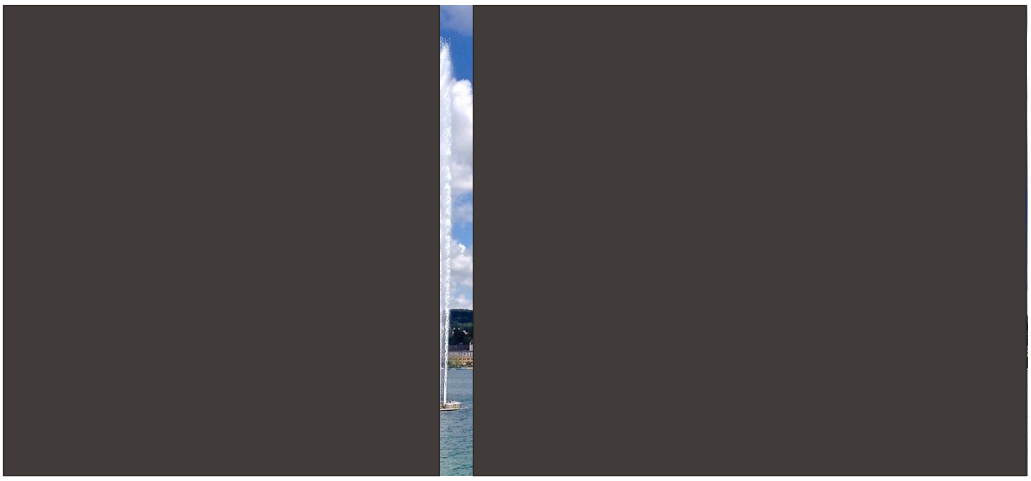
- Photons emitted outside of the filter will be lost
- # Fluors limited by # detectors
- Need to adapt the panel to the filter configuration ☹
- Cannot combine fluorochromes with overlapping emission peaks ☹

Full Spectral Flow Cytometry

In conventional cytometry, one detector is assigned to one fluorophore



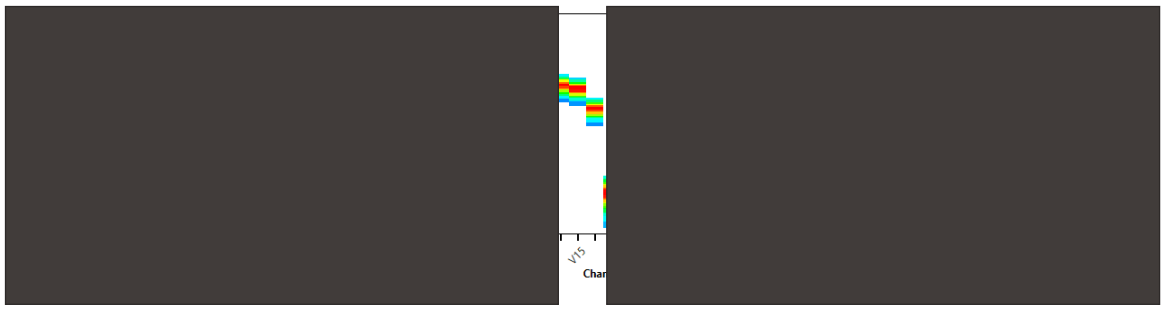
Full Spectrum Flow Cytometry



45

Full Spectrum Flow Cytometry
Allows you to see the full picture

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

46

SPECTRAL

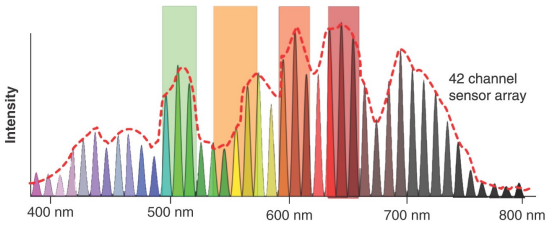
47

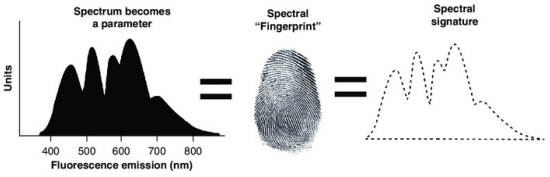
Full Spectral Flow Cytometry

M.Garcia

THE SIGNAL COLLECTED BY EACH DETECTOR CONTRIBUTES TO THE SPECTRAL SIGNATURE OF EACH FLUOROPHORE

BY RELATING THE SIGNATURE TO THE FLUORESCENCE OF A PARTICULAR FLUOROPHORE, THE SPECIFIC SIGNATURE OF A FLUOROPHORE CAN BE IDENTIFIED FROM THE MIXTURE (UNMIXING).

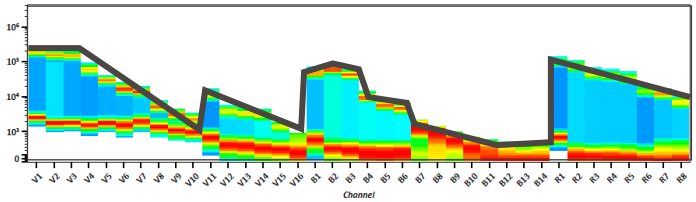


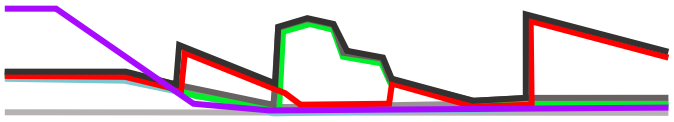


48

SPECTRAL CYTOMTRY: UNMIXING

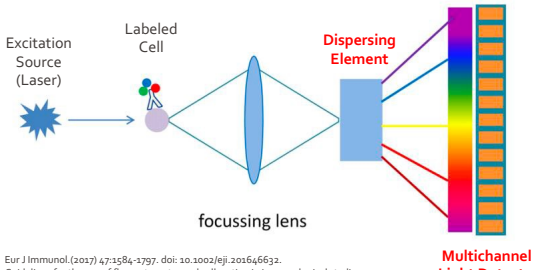
We use spectral unmixing to calculate the contribution of each fluorochrome to the total collected emission signal





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

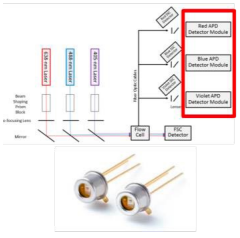
Full Spectrum Flow Cytometry Basic Optical Components



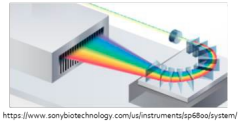
Eur J Immunol.(2017) 47:1584-1797. doi: 10.1002/eji.201646632.
Guidelines for the use of flow cytometry and cell sorting in immunological studies.

Light Detection Methods

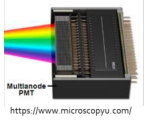
Avalanche Photodiode (APD) Arrays



Multichannel PMT



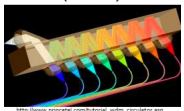
https://www.sonybiotechnology.com/us/instruments/sp680a/system/



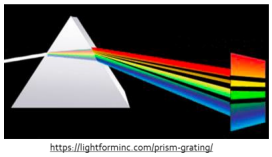
https://www.microscopyu.com/

Light DispersionMethods

Coarse Wavelength Division Multiplexing (CWDM)



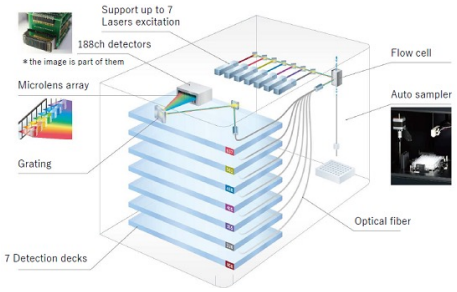
Prism

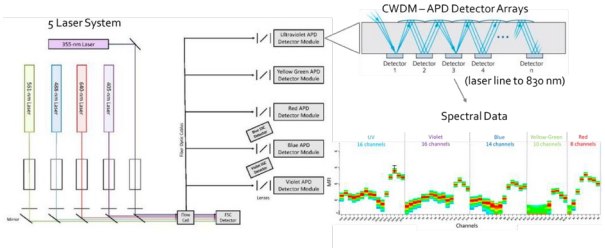


https://lightforminc.com/prism-grating/

Adapted from Monica Delay (CytekBiosciencies)

Full Spectrum Flow Cytometry
Commercial analyzers





SONY

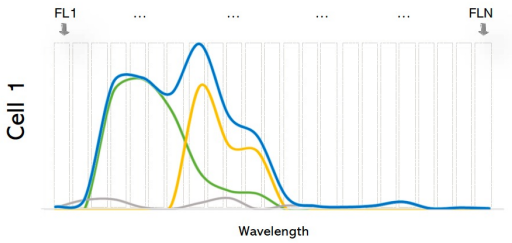
Released in September, 2020

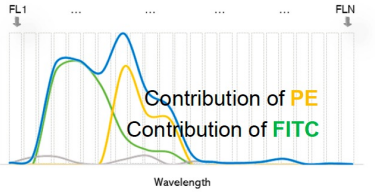
CYTEK

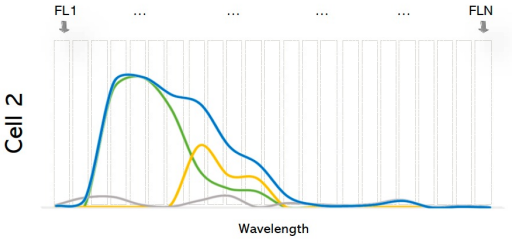
Released in June, 2017

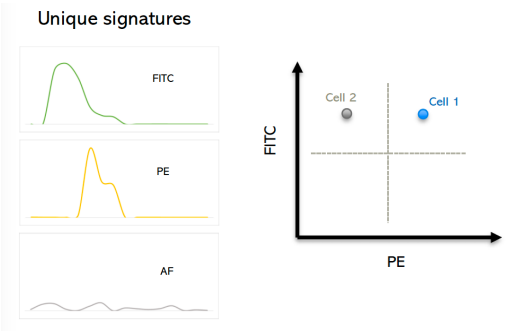
51

Full Spectral Flow Cytometry
Aurora



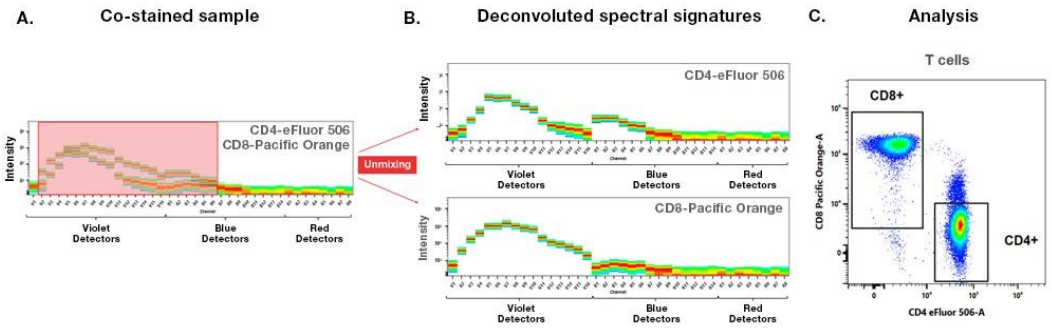






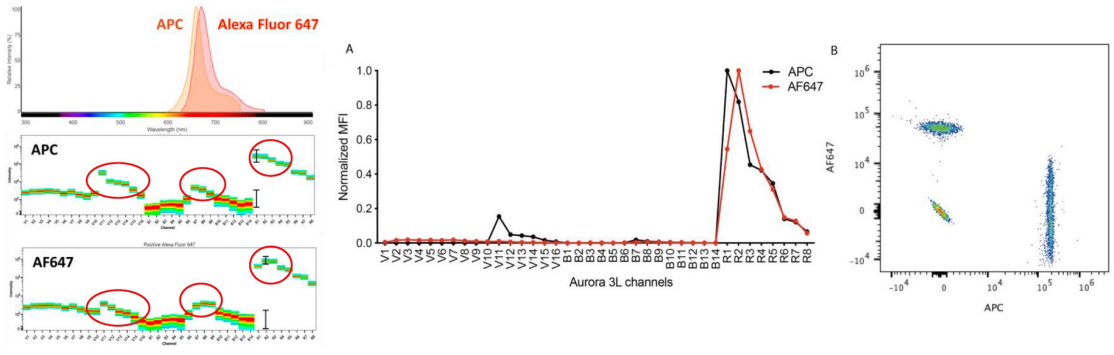
52

Full Spectral Flow Cytometry
Aurora

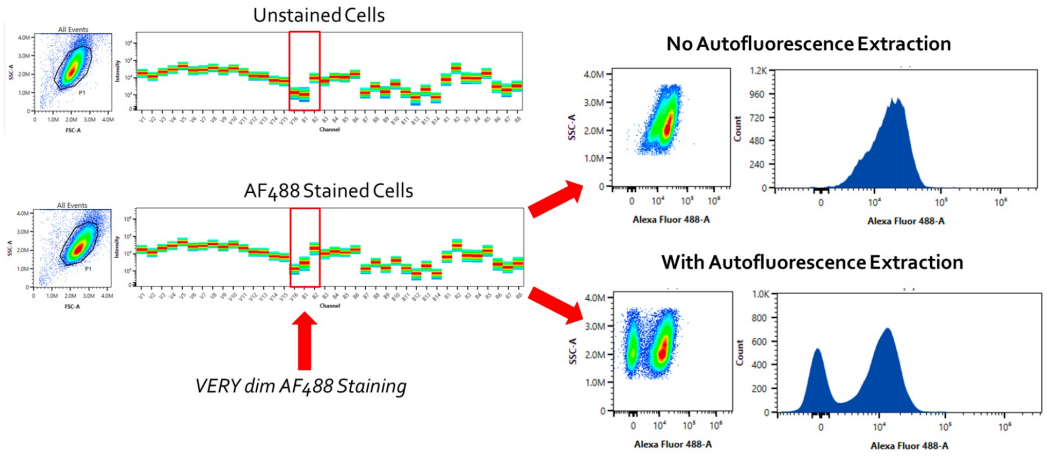


Full Spectral Flow Cytometry
Aurora

APC vs Alexa Fluor 647



Full Spectral Flow Cytometry Aurora



55

Flow Cytometer Instruments

Take Home message

- Parameters measured in conventional flow cytometers are relative size (forward scatter), granularity (side scatter) and several fluorescence parameters (from 1 to 30 simultaneously)
- In the flow cell, cells are aligned in a liquid stream by hydrodynamic focussing and then pass one by one through the laser beam
- Flow cytometry results are produced at high speed : analysis of several thousands of cells per second with statistical output.
- Option to isolate cell population of interest on cell sorters
- New technologies are now on the market allowing to increase the number of parameters up to 60.

56