



1

EPFL

FC CF
Flow Cytometry Core Facility

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

M.Garcia 2

▪ Introduction to Flow Cytometry – BIOENG-519

2

EPFL
FCBF

What is Flow Cytometry

M.Garcia

Flow Cytometry ≈ FACS: Fluorescence Activated Cell Sorter

↓ ↓ ↓
Fluid Cell Measure

Definition :

Single cells in suspension that pass a laser beam 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.

■ Introduction to Flow Cytometry – BIOENG-519

3

EPFL
FCBF

What is the advantages

M.Garcia

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

■ Introduction to Flow Cytometry – BIOENG-519

4

EPFL
FCBF

Flow cytometer components

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

■ Introduction to Flow Cytometry – BIOENG-519

M.Garcia

5

EPFL
FCBF

Flow cytometer components

Fluidics

- Cells in suspension
- flow in single file line through
- an illuminated volume where they

Optics Detectors

- scatter light and emit fluorescence
- that is filtered, collected and

Electronics

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

■ Introduction to Flow Cytometry – BIOENG-519

M.Garcia

6

EPFL
FCBF

Flow Cytometer Fluidics

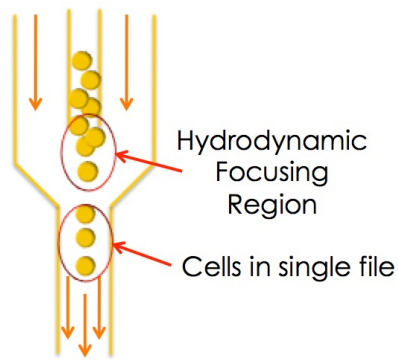
7
M. Garcia

Fluidics

- Cells in suspension
- flow in single-file through

2 Fluidics principles

Hydrodynamic focusing Laminar flow



Introduction to Flow Cytometry – BIOENG-519

7

EPFL
FCBF

Flow Cytometer Fluidics

8
M. Garcia

2 Fluidics principles

Hydrodynamic focusing Laminar flow

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

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

Introduction to Flow Cytometry – BIOENG-519

8

EPFL
FC/CF

Flow Cytometer Fluidics

M. Garcia

- 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.

Introduction to Flow Cytometry - BIOENG-519

9

EPFL
FC/CF

Flow Cytometer Optics

M. Garcia

Illumination Source - Lasers

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

Wavelength (nm)

<http://bdbiosciences.com/>

Introduction to Flow Cytometry - BIOENG-519

10

EPFL
FCBF

Flow Cytometer Optics

11
M. Garcia

Lasers configuration

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

introduction to Flow Cytometry - BIOENG-519

invitrogen
Molecular Probes School of Fluorescence

11

EPFL
FCBF

Flow Cytometer Optics

12
M. Garcia

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

introduction to Flow Cytometry - BIOENG-519

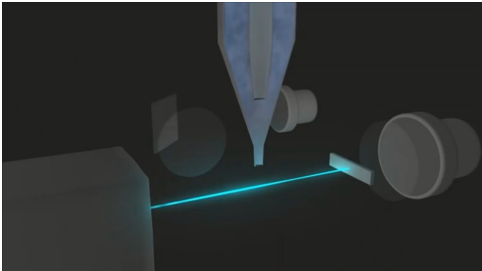
12

EPFL
FCBF

Flow Cytometer Optics

13
M. Garcia

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

■ Introduction to Flow Cytometry – BIOENG-519

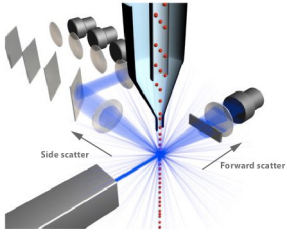
13

EPFL
FCBF

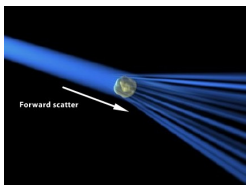
Flow Cytometer Optics

14
M. Garcia

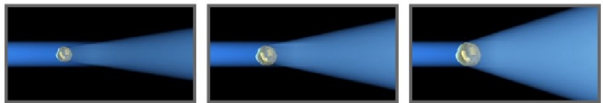
Forward Scatter



- 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



Small Medium Large



■ Introduction to Flow Cytometry – BIOENG-519

14

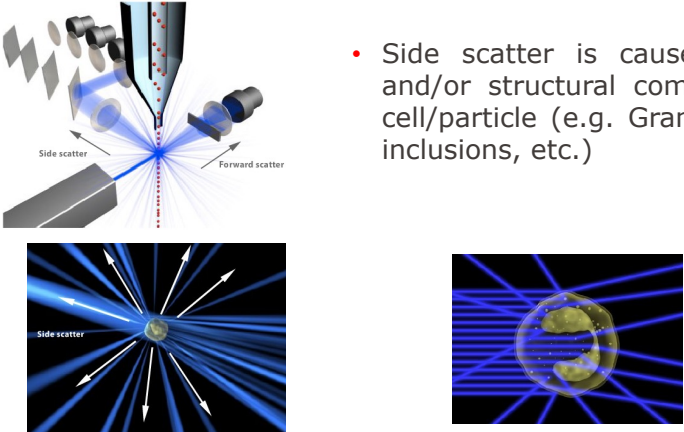
EPFL
FC/CF

Flow Cytometer Optics

15
M. Garcia

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.)



■ Introduction to Flow Cytometry – BIOENG-519

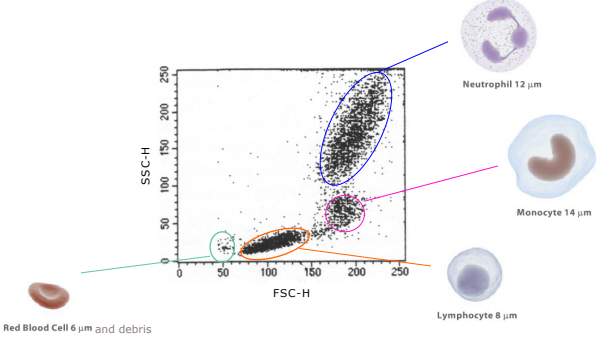
15

EPFL
FC/CF

Flow Cytometer Optics


16
M. Garcia

- 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



■ Introduction to Flow Cytometry – BIOENG-519

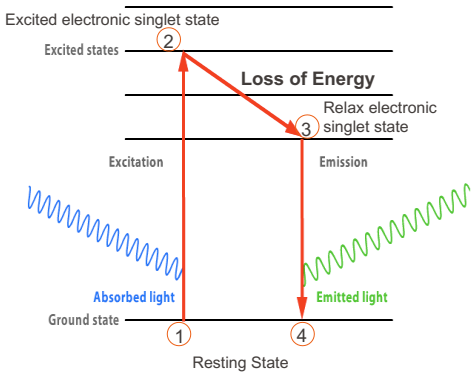
16



Flow Cytometer Optics

17
M. Garcia


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

■ Introduction to Flow Cytometry – BIOENG-519

17



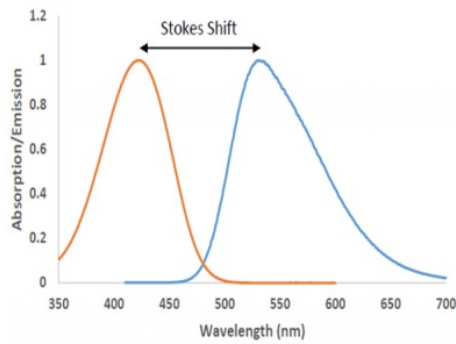
Flow Cytometer Optics

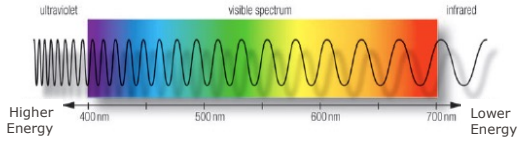
18
M. Garcia

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.





■ Introduction to Flow Cytometry – BIOENG-519

18

EPFL
FCBF

Flow Cytometer Optics

19 M. Garcia

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

Number of Events

Fluorescence Intensity

10⁰ 10¹ 10² 10³ 10⁴

■ Introduction to Flow Cytometry – BIOENG-519

19

EPFL
FCBF

Flow Cytometer Optics

20 M. Garcia

Fluorophores

Polymer dyes

Molecular antenna

Energy transfer, tandem concept

Polymer dyes

BV421™ BV525™ BV570™ BV612™ BV660™

Phycoerythrin: a naturally occurring fluorescent protein

FITC: Fluorescein Isothiocyanate


Alexa 488

Cyanine 3
Cyanine 5

source : Excyte Expert Cytometry

■ Introduction to Flow Cytometry – BIOENG-519

20




Flow Cytometer Optics

21
M. Garcia

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



■ Introduction to Flow Cytometry – BIOENG-519

<https://analyticalscience.wiley.com/doi/10.1002/imaging.3828>

21



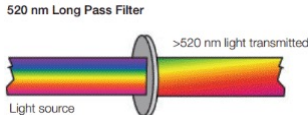
Flow Cytometer Optics

22
M. Garcia

Filters and Dichroic mirrors

Longpass

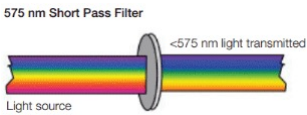
520 nm Long Pass Filter



Light source

Shortpass

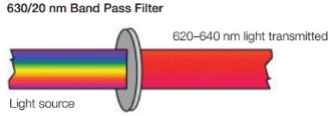
575 nm Short Pass Filter



Light source

Bandpass

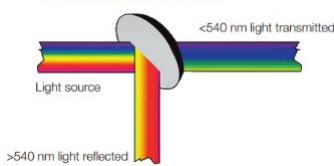
630/20 nm Band Pass Filter



Light source

Dichroic mirror

540 nm Dichroic Short Pass Mirror




Light source

>540 nm light reflected

■ Introduction to Flow Cytometry – BIOENG-519

22

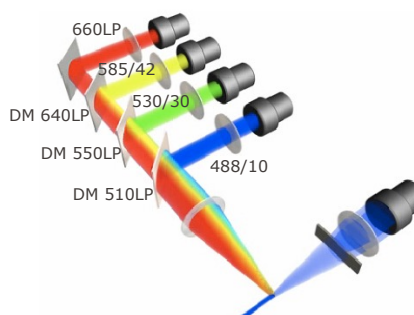


Flow Cytometer Optics

23
M.Garcia


Different optical configurations

Transmission principle



http://probes.invitrogen.com


Reflection principle (BD)



http://bdbiosciences.com/

■ Introduction to Flow Cytometry – BIOENG-519

23




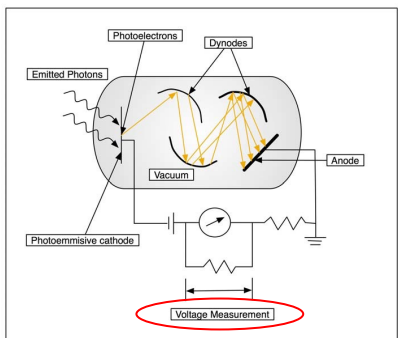
Flow Cytometer Electronics

24
M.Garcia

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

■ Introduction to Flow Cytometry – BIOENG-519

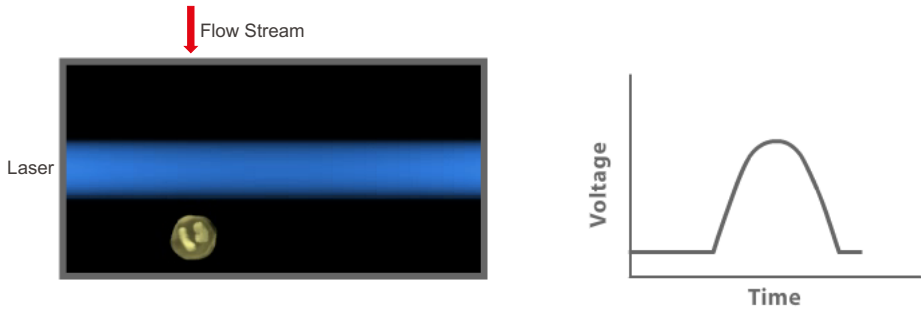
24

EPFL
FCBF

Flow Cytometer Electronics

25
M.Garcia

Signal Pulse



The diagram shows a laser beam (blue) passing through a flow stream (indicated by a red arrow) containing a cell (yellow). A graph to the right shows a voltage pulse (a bell-shaped curve) over time.

- 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**

■ Introduction to Flow Cytometry – BIOENG-519

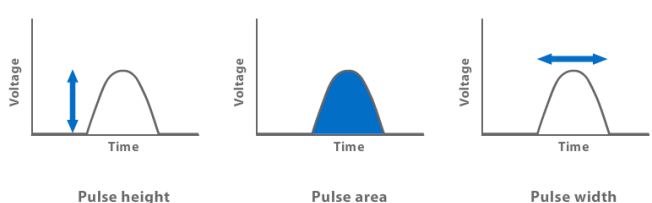
25

EPFL
FCBF

Flow Cytometer Electronics

26
M.Garcia

- 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 three graphs show: 1) Pulse height: a vertical double-headed arrow indicating the peak voltage. 2) Pulse area: the area under the pulse curve shaded in blue. 3) Pulse width: a horizontal double-headed arrow indicating the duration of the pulse.

Pulse height Pulse area Pulse width

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

■ Introduction to Flow Cytometry – BIOENG-519

26

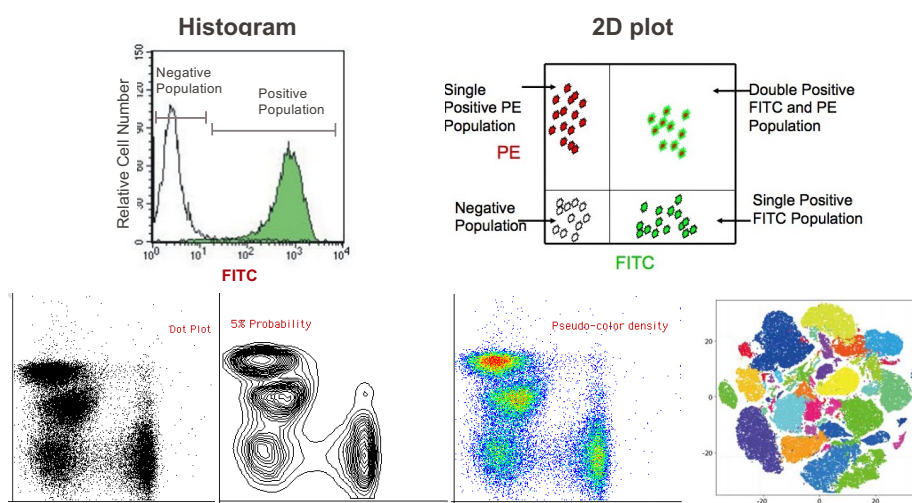
Flow Cytometer Electronics

27
M. Garcia

- 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.

27

Flow Cytometer Data presentation

28
M. Garcia

28

EPFL
FC/CF

Flow Cytometer Overview

29
M. Garcia

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

■ Introduction to Flow Cytometry – BIOENG-519

EPFL
FC/CF

Flow Cytometer Applications

30
M. Garcia

- 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).

■ Introduction to Flow Cytometry – BIOENG-519

EPFL
FC/CF

Flow Cytometer Applications

Immunophenotyping

- Detection of cell surface molecules as example cluster of differentiation

Introduction to Flow Cytometry – BIOENG-519

31 M. Garcia

31


EPFL
FC/CF

Flow Cytometer Applications

Introduction to Flow Cytometry – BIOENG-519

32 M. Garcia

32

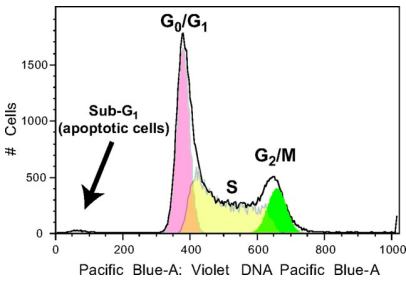


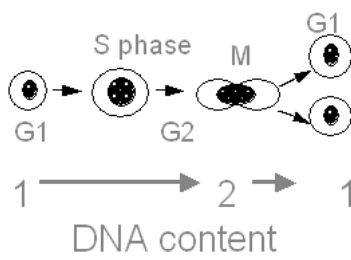
Flow Cytometer Applications

33

M. Garcia


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





■ Introduction to Flow Cytometry – BIOENG-519

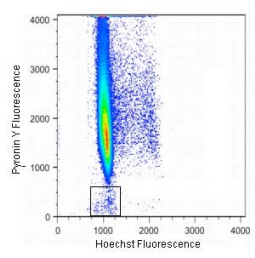
33



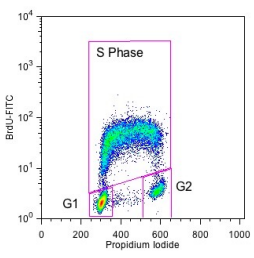
Flow Cytometer Applications

34

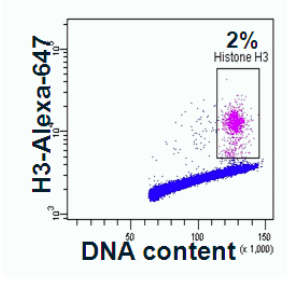
M. Garcia



G0-phase



S-phase



M-phase

■ Introduction to Flow Cytometry – BIOENG-519

34

EPFL
FC/CF

Introduction to Flow Cytometry – BIOENG-519

Flow Cytometer Applications

Cell Proliferation

Unlabelled
Cells

100%
50%
25%

Counts

CFSE

5 4 3 2 1 IP

35

M. Garcia

35

EPFL
FC/CF

Introduction to Flow Cytometry – BIOENG-519

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

○ = Plasma membrane
■ = Phosphatidyl serine
● = PI or 7AAD
★ = AnnexinV-FITC

SSC-A (x 1,000)

FSC-A (x 1,000)

PI (x 10³)

AnnexinV-FITC

Live 87%
Apoptotic 4.5%
Dead 9%

36

M. Garcia

36

Flow Cytometer Applications

37
M. Garcia

- 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
-

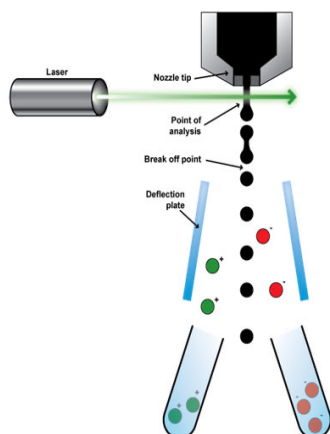
■ Introduction to Flow Cytometry – BIOENG-519

37

Flow Cytometer Applications

38
M. Garcia

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

■ Introduction to Flow Cytometry – BIOENG-519

38

EPFL
FCBF

Flow Cytometer Applications

Introduction to Flow Cytometry – BIOENG-519

Pre-sort

After sort

39
M. Garcia

39

EPFL
FCBF

Flow Cytometer Instruments

Introduction to Flow Cytometry – BIOENG-519

Aurora

CytoFLEX

Gallios (x2)

Attune NxT

LSR II (x2)

LSRFortessa

40
M. Garcia

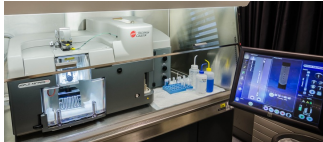
40

EPFL
FC/CF

Flow Cytometer Instruments


Sorters
Droplet based

Jet-in-air




MoFlo ASTRIOS

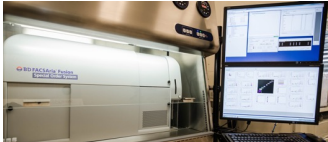
Cuvette



FACSARIA II



SONY SH800



FACSARIA FUSION

Introduction to Flow Cytometry – BIOENG-519

41
M.Garcia

41

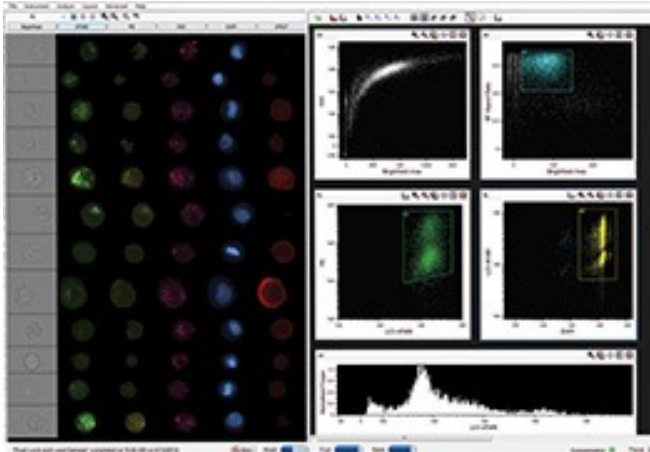
EPFL
FC/CF

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



Introduction to Flow Cytometry – BIOENG-519

42
M.Garcia

42

EPFL
FCBF

Conventional Flow Cytometry

43
M. Garcia

Conventional

In conventional cytometry, one detector is assigned to one fluorophore

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 ☹

Introduction to Flow Cytometry – BIOENG-519

43

EPFL
FCBF

Full Spectral Flow Cytometry

44
M. Garcia

In conventional cytometry, one detector is assigned to one fluorophore

Emission Intensity

Wavelength

670/30 Filter

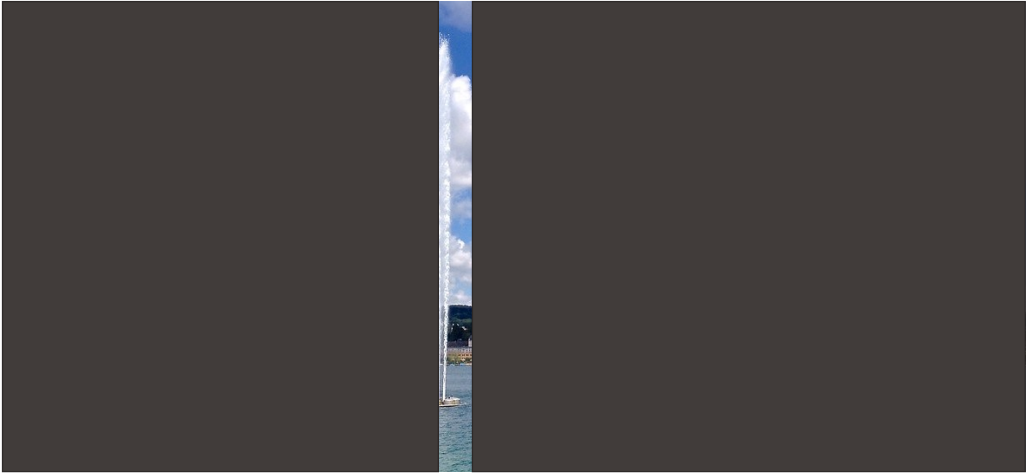
APC Emission Spectrum

Introduction to Flow Cytometry – BIOENG-519

44

EPFL
FCBF

Full Spectrum Flow Cytometry



■ Introduction to Flow Cytometry – BIOENG-519

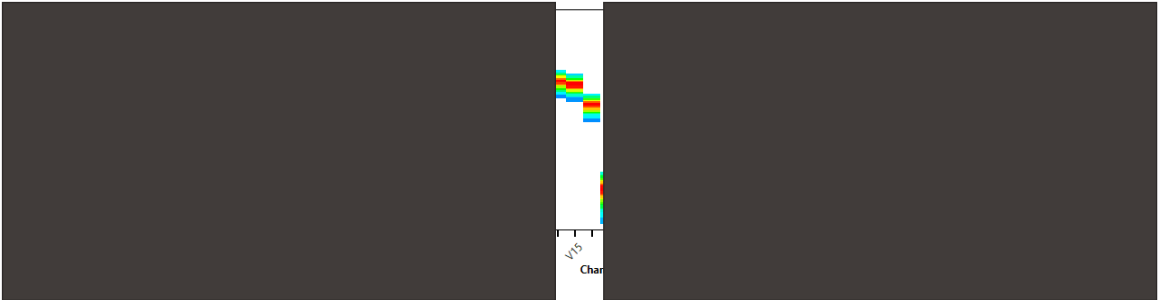
45

EPFL
FCBF

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?




■ Introduction to Flow Cytometry – BIOENG-519

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





Introduction to Flow Cytometry - BIOENG-519

47

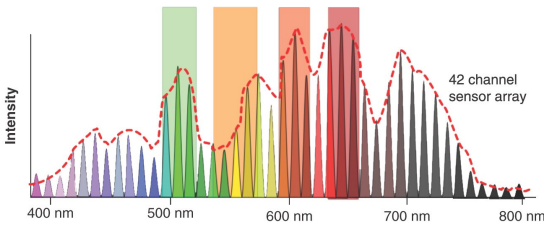


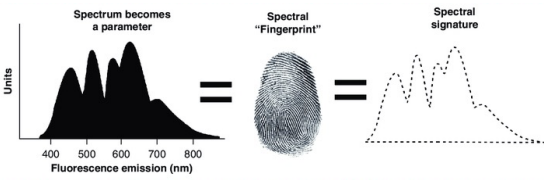
Full Spectral Flow Cytometry

48
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).





Introduction to Flow Cytometry - BIOENG-519

48

EPFL **SPECTRAL CYTOMETRY: UNMIXING** **EPFL FCGF** 49
M. Garcia

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

■ Introduction to Flow Cytometry – BIODENG-519

BV421
FITC
APC
Autofluorescence

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

49

EPFL **Full Spectrum Flow Cytometry** **EPFL FCGF** 50
M. Garcia

Basic Optical Components

Excitation Source (Laser) → Labeled Cell → focussing lens → Dispensing Element → Multichannel Light Detector

Light Dispersion Methods

- Coarse Wavelength Division Multiplexing (CWDM)
- Prism

Light Detection Methods

- Avalanche Photodiode (APD) Arrays
- Multichannel PMT

Eur J Immunol. (2017) 47:1584–1597. doi: 10.1002/eji.201646632. Guidelines for the use of flow cytometry and cell sorting in immunological studies. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5404640/>

<https://www.zanyibotechnology.com/us/instruments/p6600/system/>

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

Adapted from Monica Delay (CytekBiosciences)

50

EPFL
FCBF

Full Spectrum Flow Cytometry Commercial analyzers

51
M.Garcia

Support up to 7 Lasers excitation
188ch detectors
* the image is part of them
Microlens array
Grating
7 Detection decks
Flow cell
Auto sampler
Optical fiber

5 Laser System
CWDM-APD Detector Arrays
Detector 1, Detector 2, Detector 3, Detector 4, Detector 5, Detector 6, Detector 7, Detector 8, Detector 9, Detector 10
(laser line to 830 nm)
Spectral Data
101 16 channels, 104 16 channels, 106 14 channels, 108 16 channels, 110 16 channels, 112 16 channels, 114 16 channels, 116 16 channels, 118 16 channels, 120 16 channels

SONY
Released in September, 2020

CYTEK
Released in June, 2017

■ Introduction to Flow Cytometry – BIOENG-519

51

EPFL
FCBF

Full Spectral Flow Cytometry Aurora

52
M.Garcia

FL1 ... FLN
Cell 1
Wavelength
Contribution of PE
Contribution of FITC

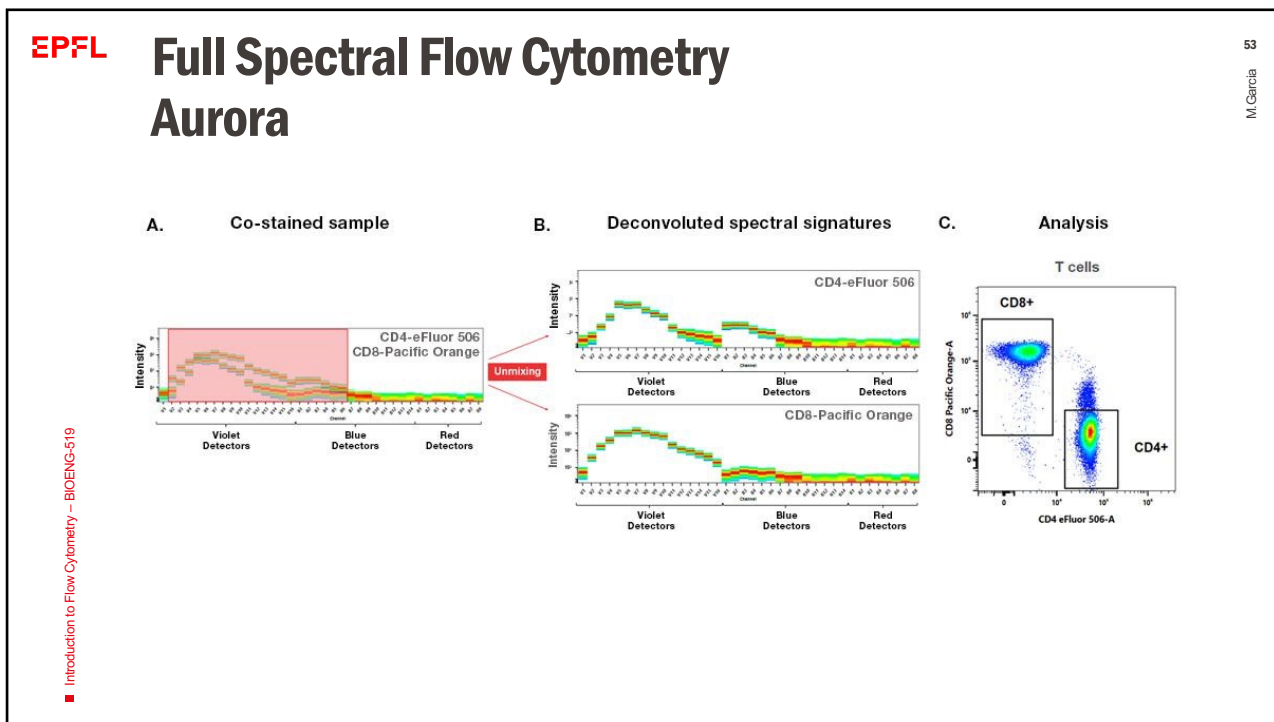
FL1 ... FLN
Cell 2
Wavelength

Unique signatures
FITC
PE
AF

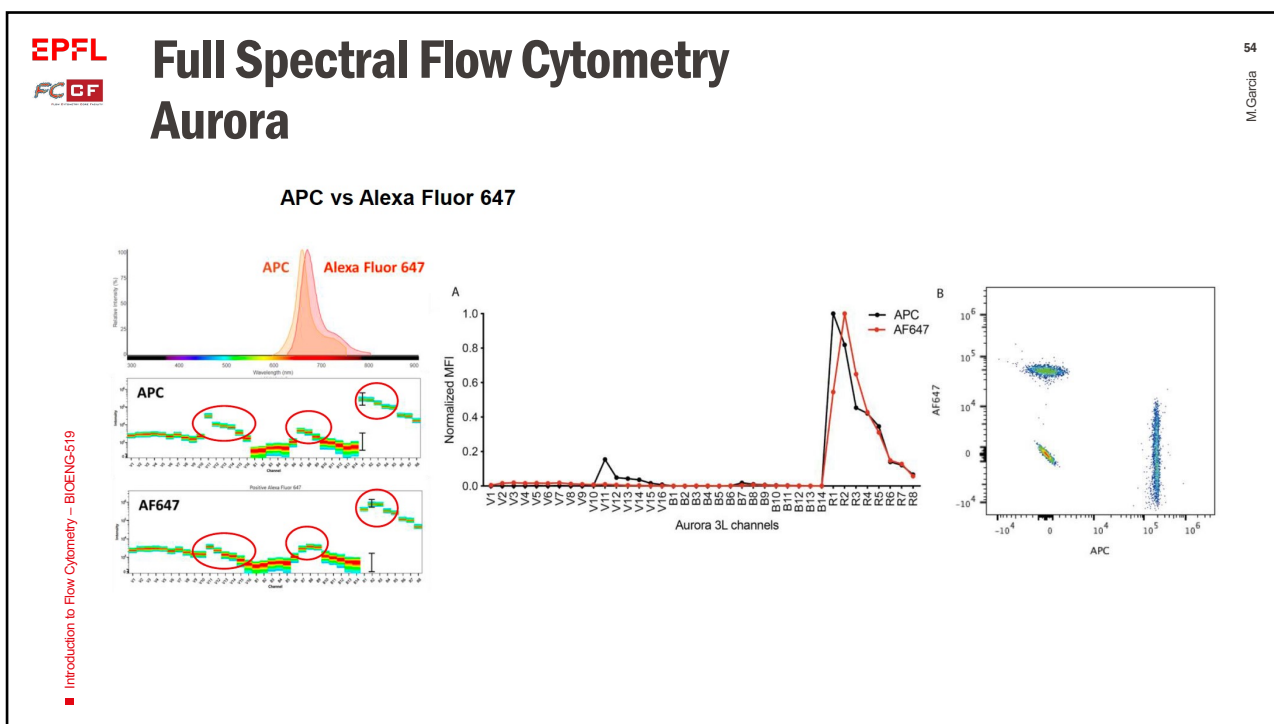
FITC
PE
Cell 2
Cell 1

■ Introduction to Flow Cytometry – BIOENG-519

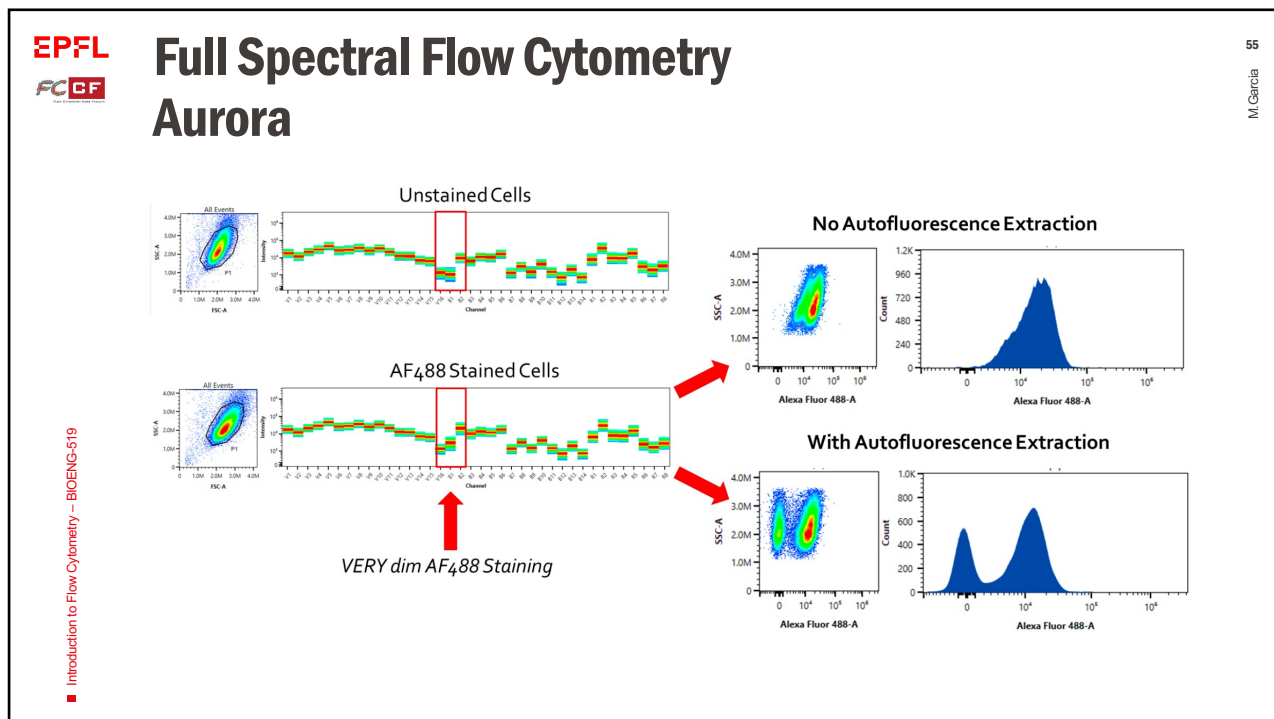
52



53



54



55

EPFL
FCBF

Flow Cytometer Instruments

56
M.Garcia

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.

■ Introduction to Flow Cytometry – BIOENG-519

56