Introduction to Flow Cytometry

BIOENG-399_Immuno-engineering

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1

Overview

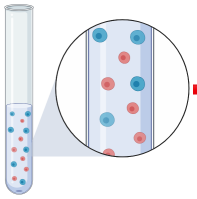
- Flow Cytometry Definition
- The Cytometer
- Applications
- New Technologies

2

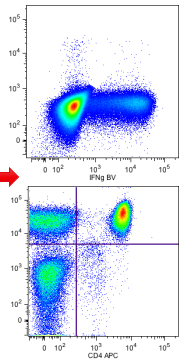
What is flow Cytometry ?

Flow Cytometry

Fluid Cell Measure



Flow Cytometer !



http://mastery.expertcytometry.com

3

What are the advantages ?

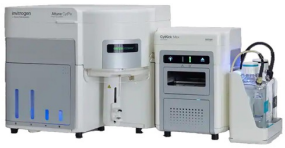
- Analysis of thousands of cells per second detecting multiple parameters of individual cells within heterogeneous populations

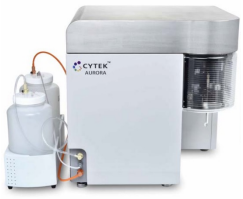
- Fast sample processing (up to 35'000 evs/s)
- High statistical power
- Study of different cell (sub)populations
- Multi-parameter analysis - up to 20 parameters simultaneously in conventional, up to 50 on the latest instruments

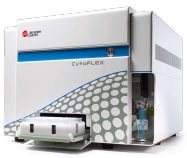
4

Technology - Analyzers









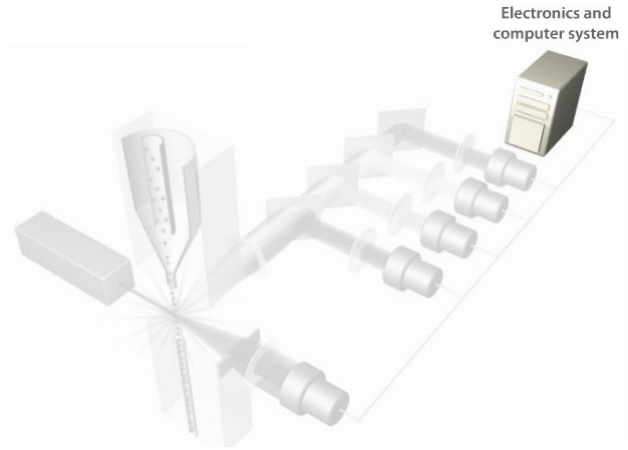
5

Technology – High Speed Cell sorter




6

Look *Inside* the Box



http://probes.invitrogen.com

7

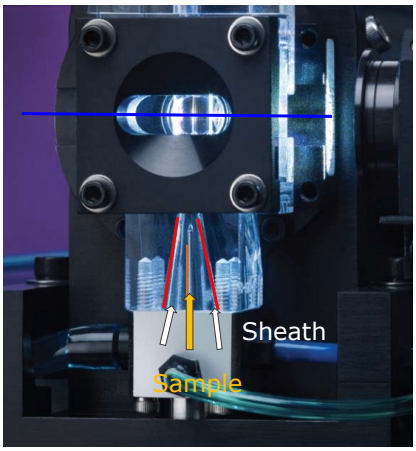
3 major components of a Cytometer

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

8

Fluidics System

- 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



9

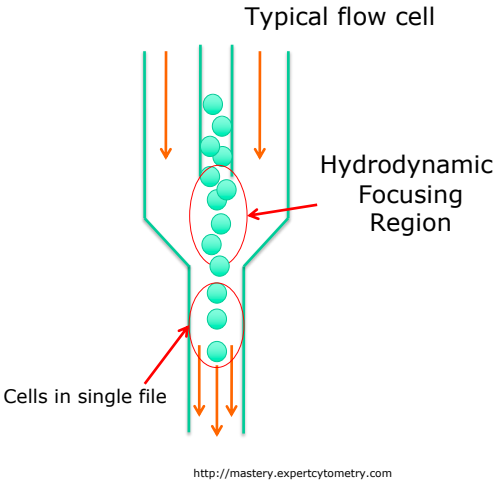
2 Fluidics principles

- ***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

10

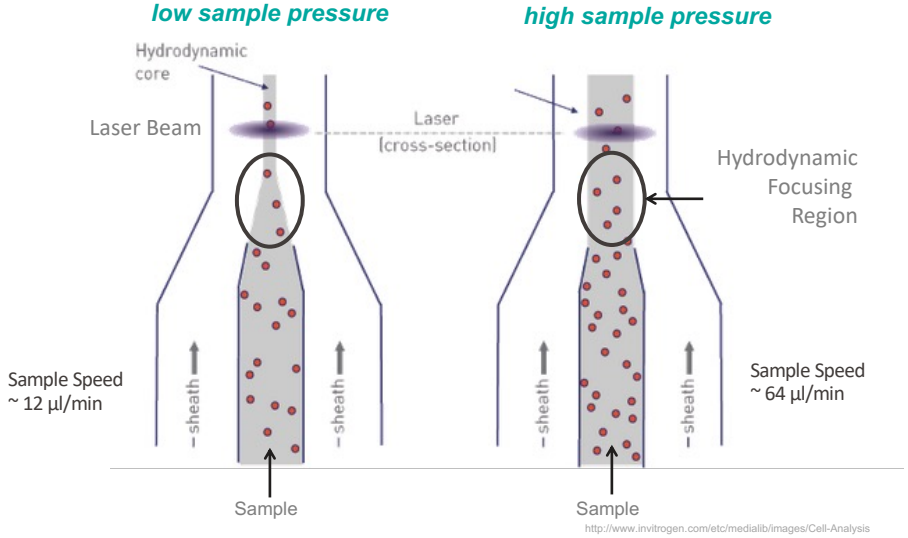
The Flow Cell

- 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



11

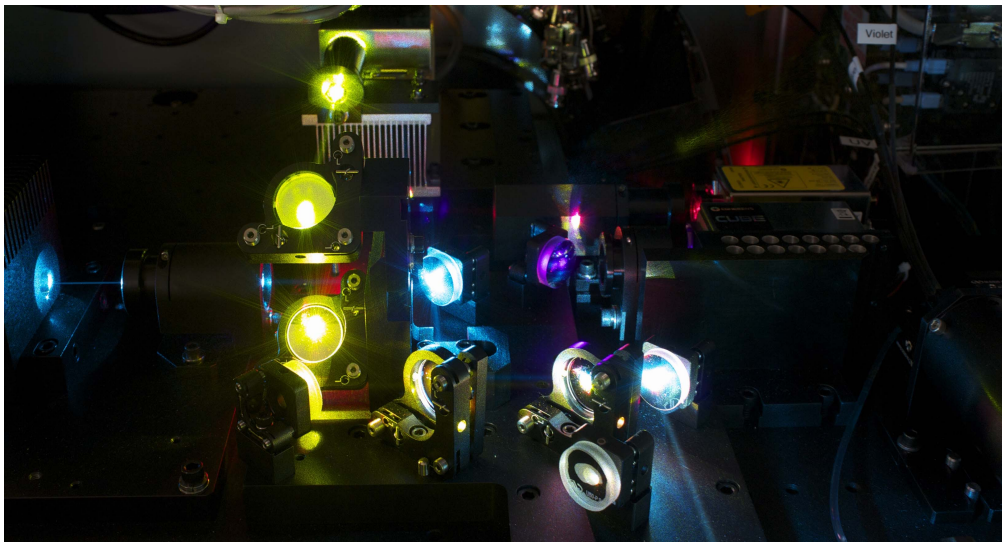
Hydrodynamic Focusing
Pressure – funnel - focus



12

Optics – Lasers/Filters/Detectors

Moving photons of light through the instrument

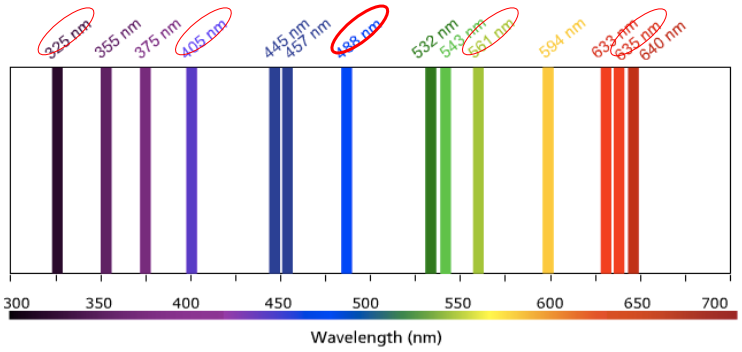


13

Lasers

Laser = **L**ight **A**mplification by **S**timulated **E**mission of **R**adiation

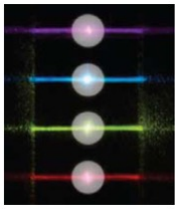
- **MONOCHROMATIC** - Lasers can provide a single wavelength of light
- **COHERENT** – The motion of all photos are coordinated

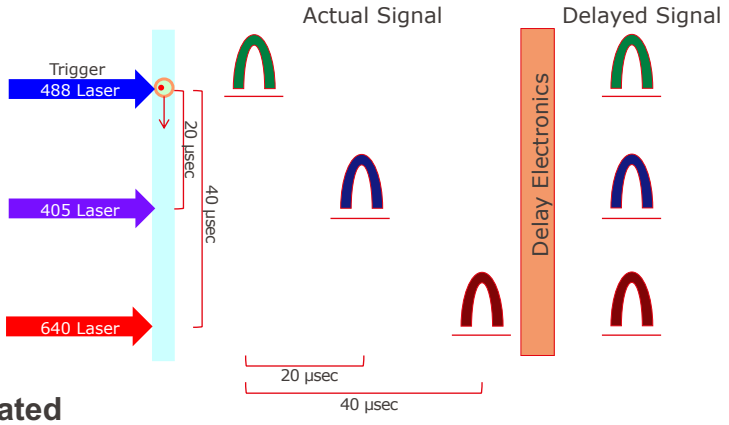


http://www.bdbiosciences.com/

14

Light Source configuration





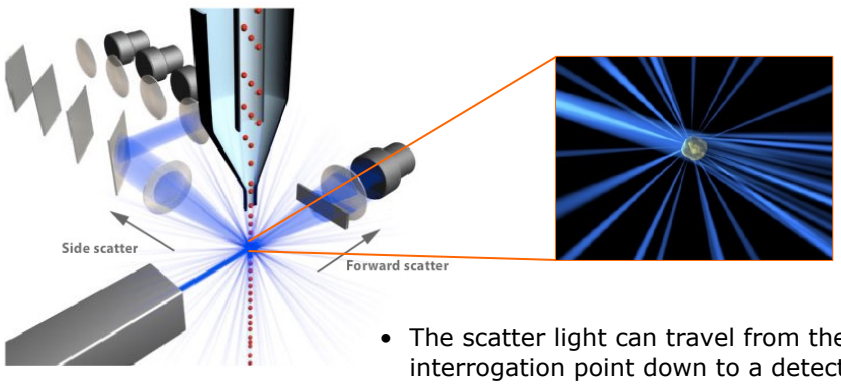
- **Spatially separated**
  - Lasers separated
  - Multiple interrogation points
  - Must set time delay between lasers

http://mastery.expertcytometry.com

15

Interrogation Point

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

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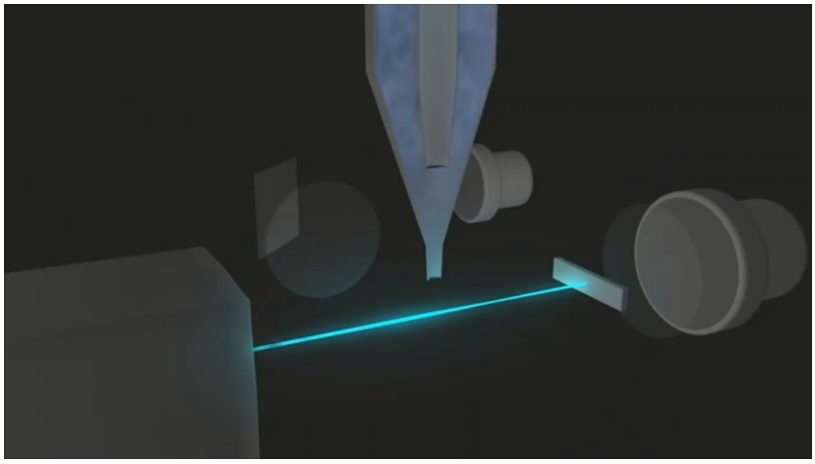
16

Interrogation Point



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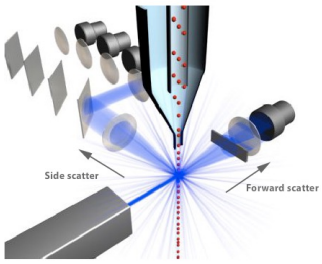
17

Measure light

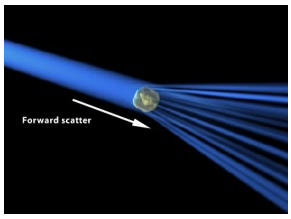
- **Two types of light are measured as particles pass through the illumination source**
- **Laser light scatter**: refraction of illuminating beam by the particle
  - Forward scatter (FSC)
  - Side scatter (SSC)
- **Fluorescence**: emitted from fluorescent tags after being excited by the illumination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

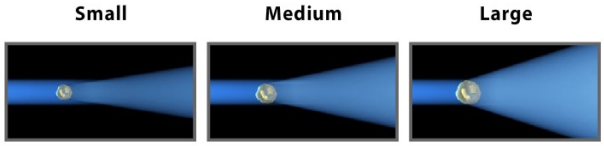
18

Forward Scatter (FSC)



- 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





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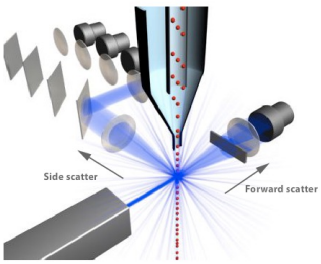
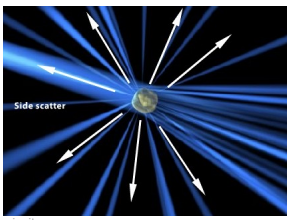
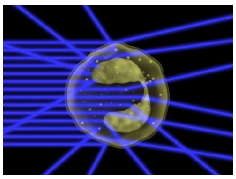
19

Side Scatter (SSC)



- 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 (eg. Granulated nuclei, cell inclusions, etc.)





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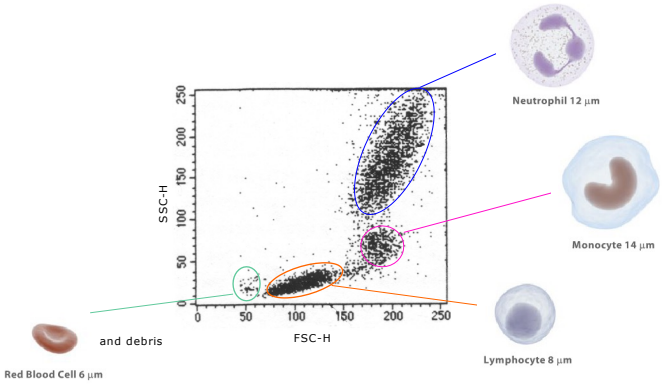
20

FSC v. SSC

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



21

What is Fluorescence ?

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

Excitation - Lasers

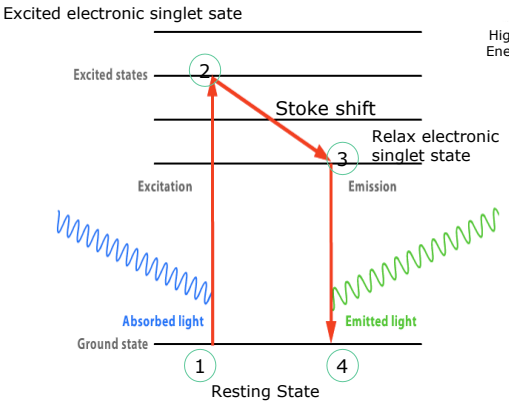
Absorbance of photon of light
Promotes electron to higher energy state

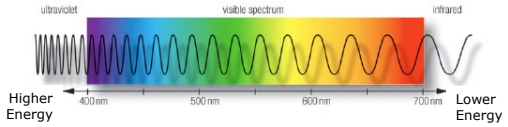
Emission – Detected by Optics

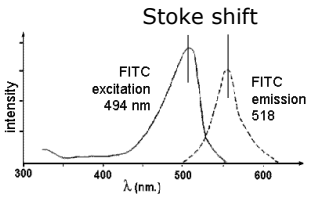
Return of excited electron to ground state
Emitted wavelength longer (less energy) than exciting wavelength named "***Stoke Shift***"

22

Stock Shift





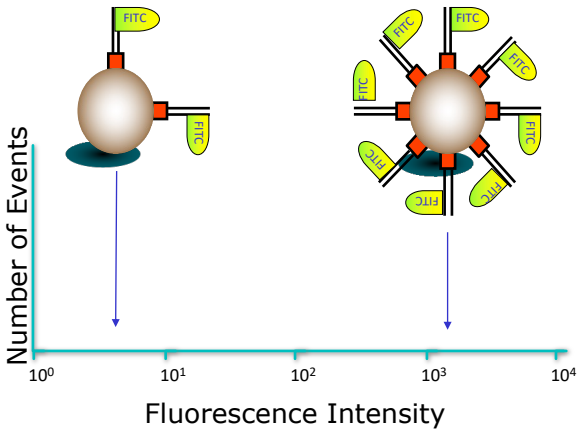


Guide to Flow Cytometry; Dako
http://web.uvic.ca/ail/techniques/epi-fluorescence.html

23

What is Fluorescence Intensity

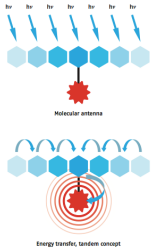
- ***Emitted fluorescence intensity is proportional to binding sites***



24

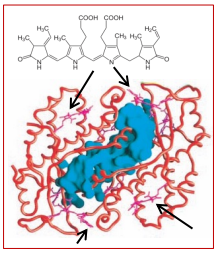
Fluorophores

Polymer dyes

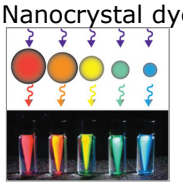


Polymer dyes

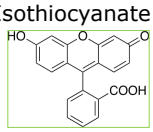
Phycoerythrin: a naturally occurring fluorescent protein



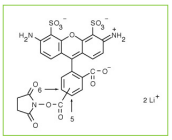
Nanocrystal dyes

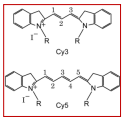


FITC: Fluorescein Isothiocyanate



Alexa 488



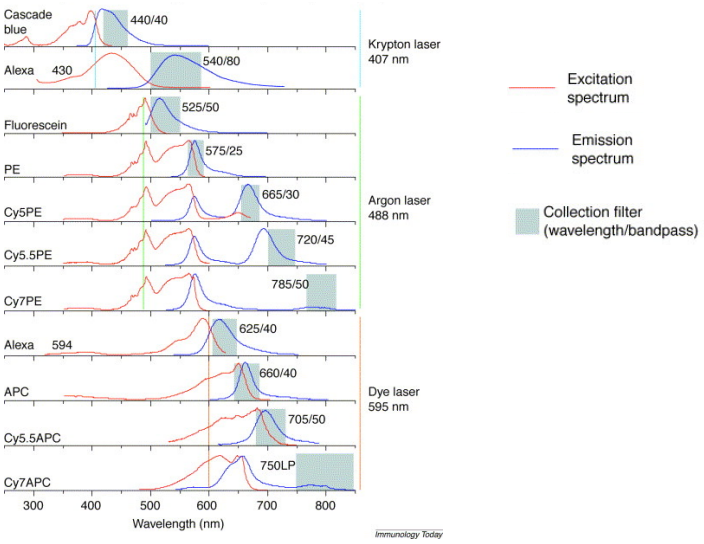


Cyanine 3
Cyanine 5

source : Excyte Expert Cytometry

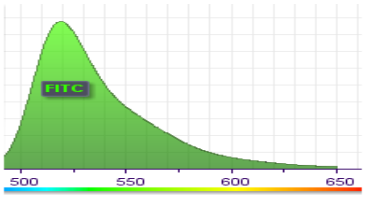
25

Spectra of Common Fluorochromes



26

Filters



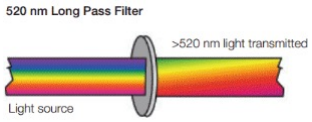
As many wavelengths of light will be scattered from a cell, we need a way to split the light into its specific wavelengths in order to detect them independently

- 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

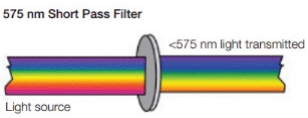
27

Filters -Types

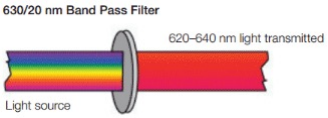
Longpass



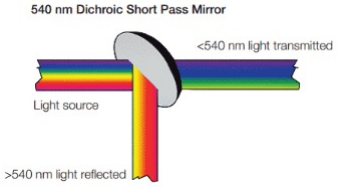
Shortpass



Bandpass



Dichroic mirror

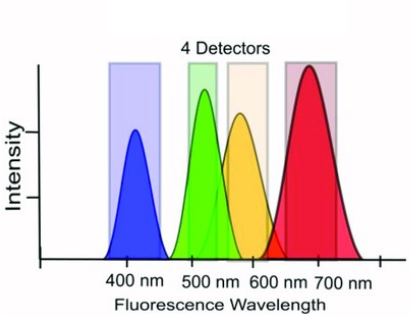


https://analyticalscience.wiley.com/do/10.1002/imaging.3828

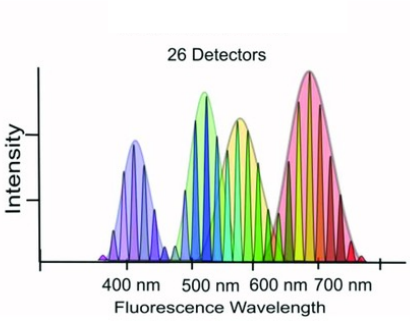
28

Different ways to collect fluorescence emission

CONVENTIONAL FLOW CYTOMETRY



SPECTRAL FLOW CYTOMETRY

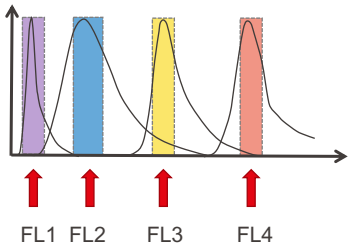


29

Conventional Flow Cytometry

Conventional

In conventional cytometry, one detector is assigned to one fluorophore



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

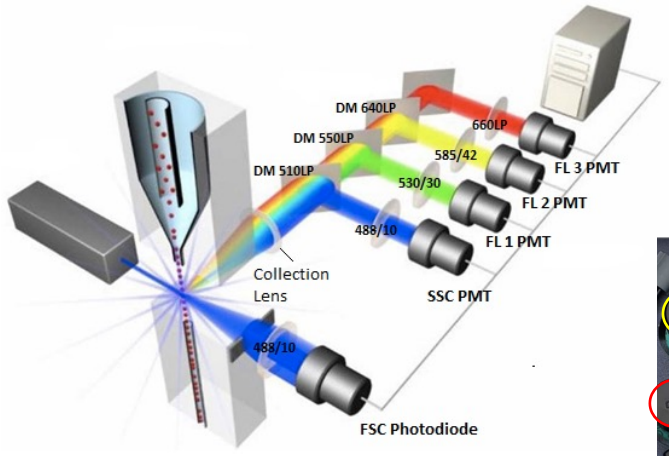
Each fluorochrome is detected in **ONE** channel

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

30

PMTs



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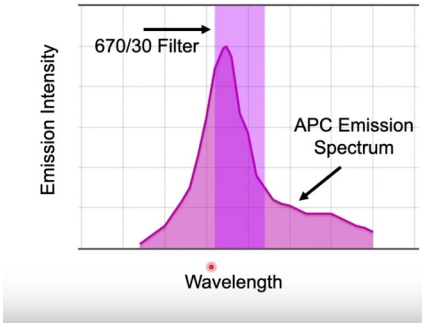
http://www.labome.com/

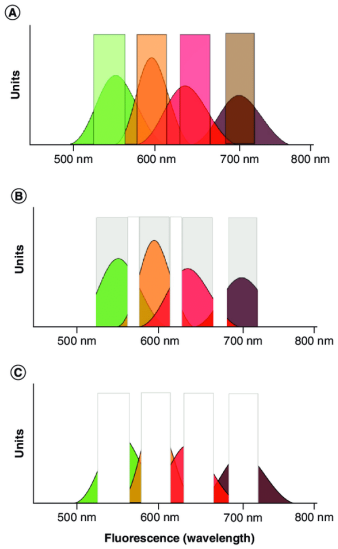
31

Conventional Flow Cytometry



32

Conventional Flow Cytometry



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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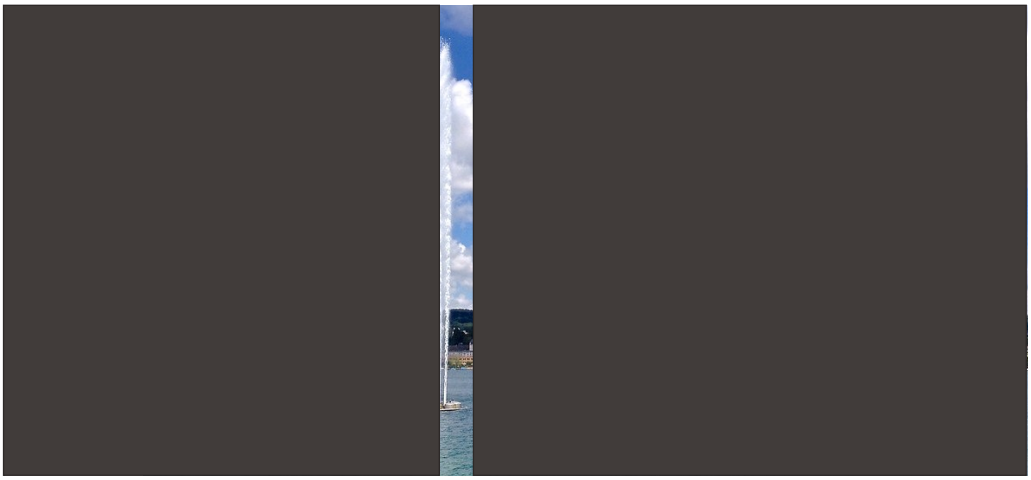
33

Full Spectrum Flow Cytometry

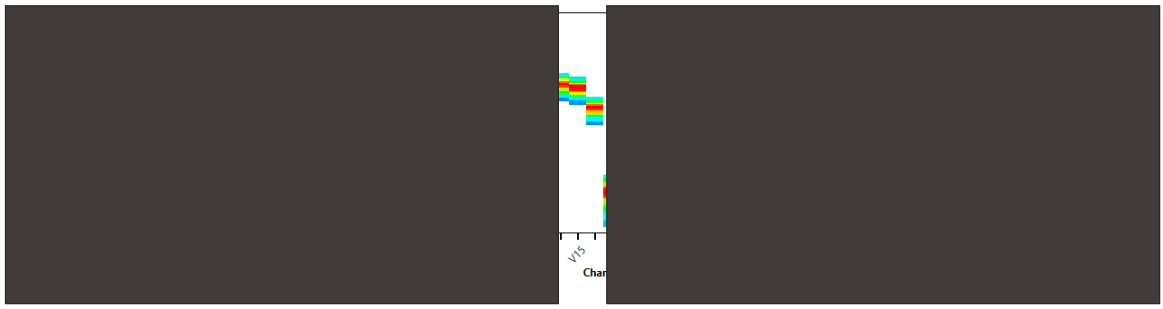


34

Full Spectrum Flow Cytometry
Allows you to see the full picture

35

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

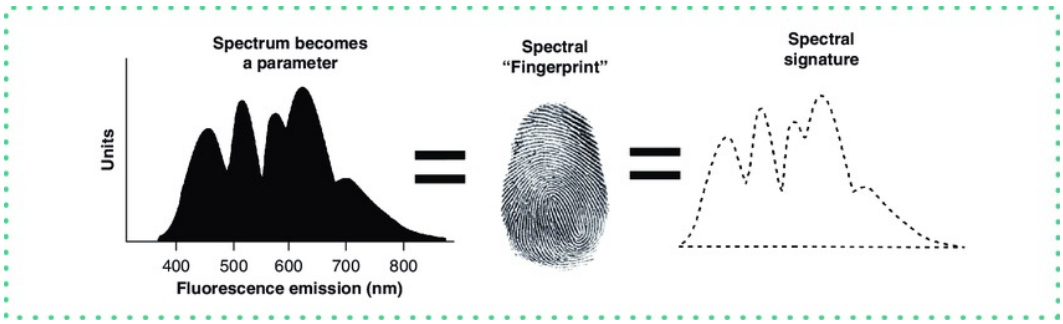
Fluorophores are identified by their distinct spectra signature

35

Full Spectrum Flow Cytometry

36

Miguel Garcia



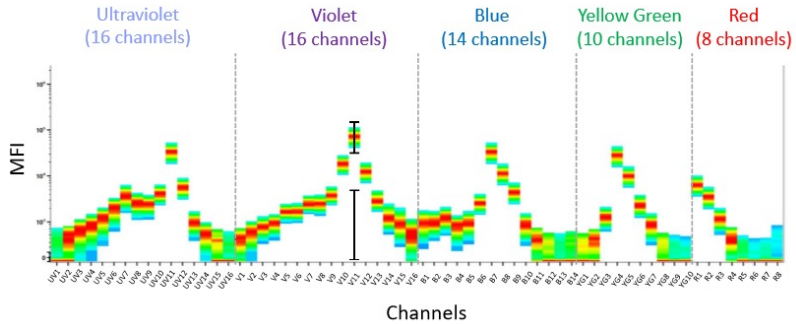
Reproduced with permission from cyto.purdue.edu.

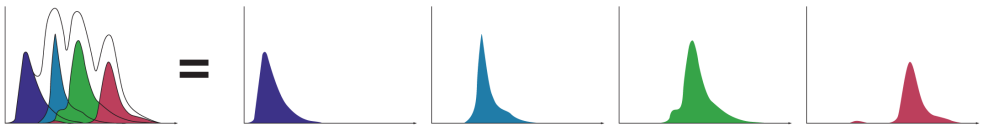
36

Full Spectrum Flow Cytometry: How does it work?

37

Full Spectral Flow Cytometry

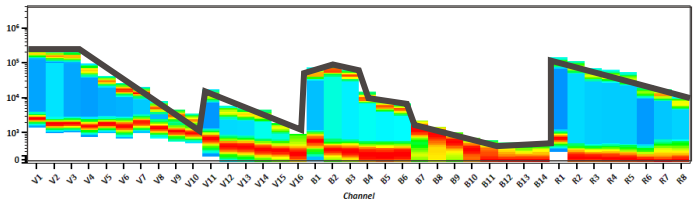


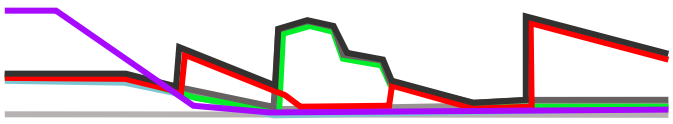


38

Full Spectral Flow Cytometry
Spectral Unmixing algorithm

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



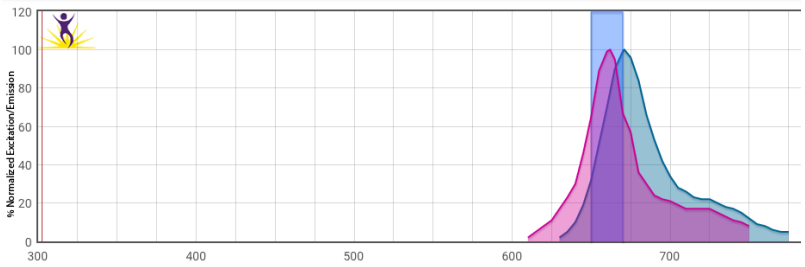


We can think of this as extracting or deconvoluting each component until we have nothing left.

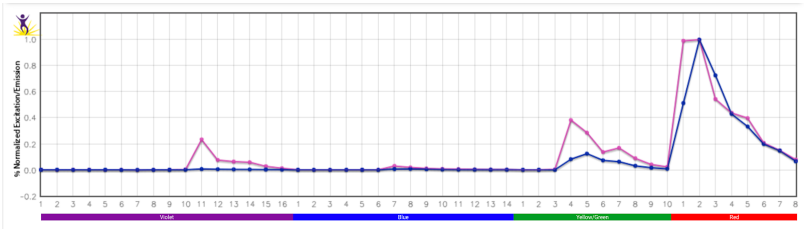
39

Full Spectral Flow Cytometry

Conventional



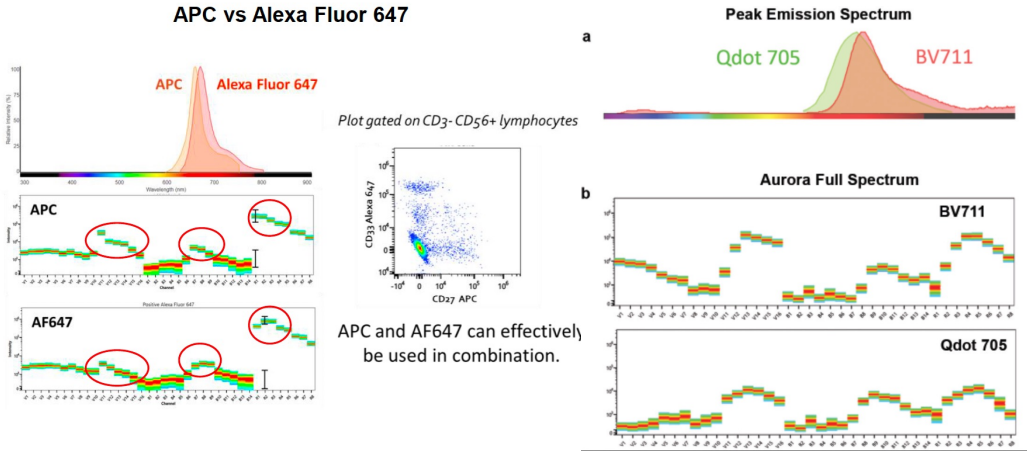
Spectral



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

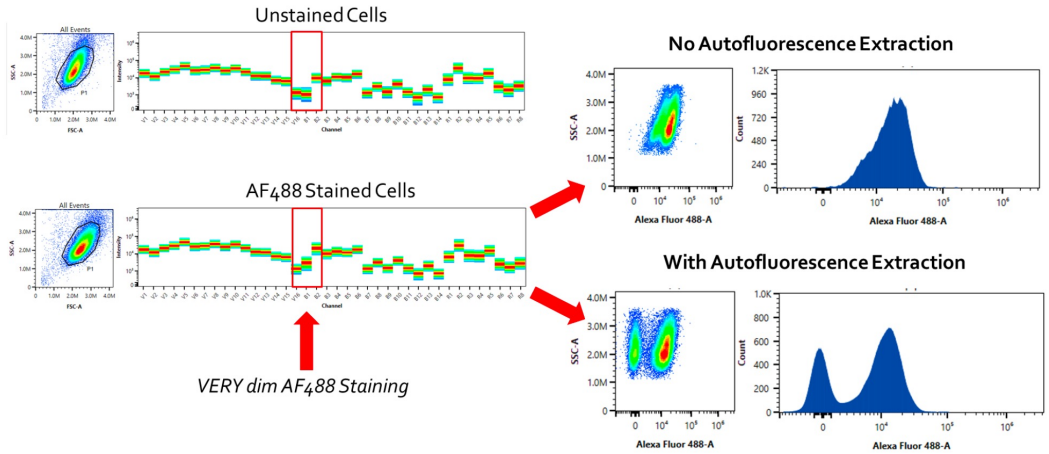
40

Full Spectral Flow Cytometry



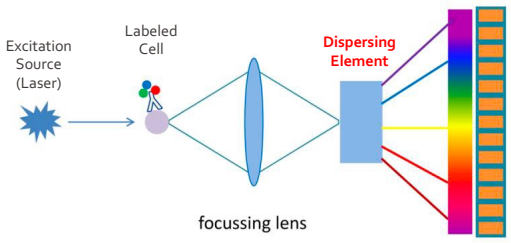
41

Full Spectral Flow Cytometry



42

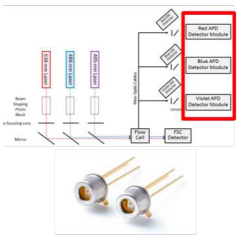
Full Spectrum Flow Cytometry
Basic Optical Components



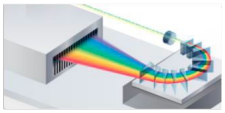
Eur J Immunol.(2017) 47:1584-1797. doi: 10.1002/eji.201646632.
Guidelines for the use of flow cytometry and cell sorting in immunological studies.

Light Detection Methods

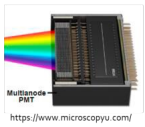
Avalanche Photodiode (APD) Arrays



Multichannel PMT



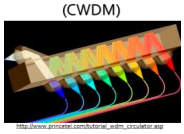
https://www.sonybiotechnology.com/us/instruments/sp680o/system/



https://www.microscopyu.com/

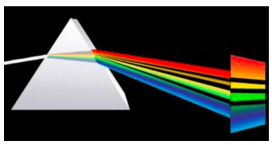
Light Dispersion Methods

Coarse Wavelength Division Multiplexing (CWDM)



http://www.princetel.com/tutorial_wdm_circulator.asp

Prism

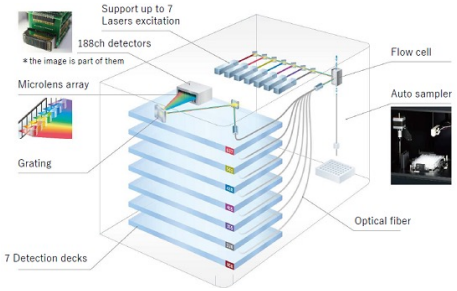


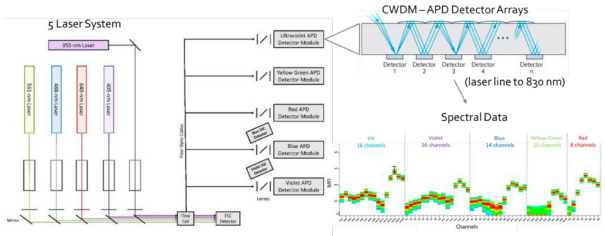
https://lightforminc.com/prism-grating/

Adapted from Monica Delay (CytekBiosciencies)

43

Full Spectrum Flow Cytometry
Commercial analyzers





SONY

Released in September, 2020

CYTEK

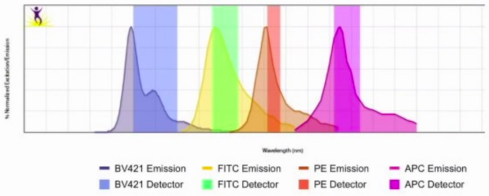
Released in June, 2017

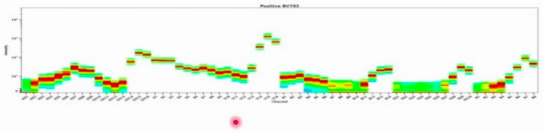
44

Full Spectral Flow Cytometry

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 prims or ratings, 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

45

Detectors

Light must be converted from photos 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

46

APDs

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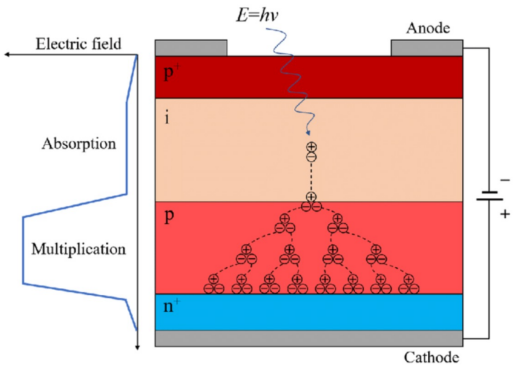
- Avalanche photodiodes (APDs) are silicon photodiodes with an internal gain mechanism.
- As with a conventional photodiode, absorption of incident photons creates electron-hole pairs.
- However, by placing a high reverse bias voltage a strong internal electric field is created, and this accelerates 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.



47

PMTs

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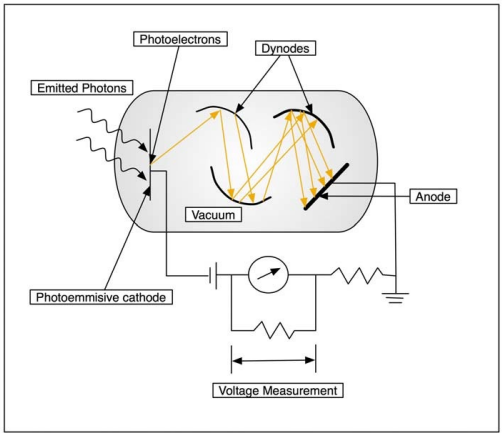
- Produce current at their anodes when photons impinge upon their light-sensitive cathodes Require external power source
- Their gain is as high as 107 electrons out per photon in
- Noise can be generated from thermionic emission of electrons - this is called “dark current”
- If very low levels of signal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



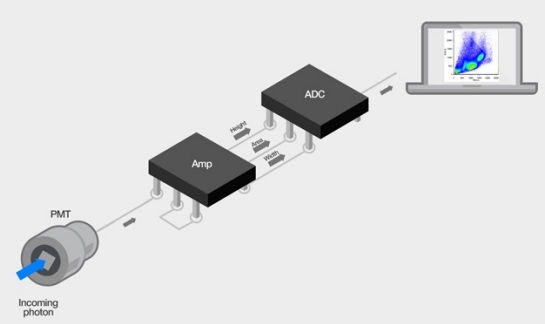
48

Signal Conversion

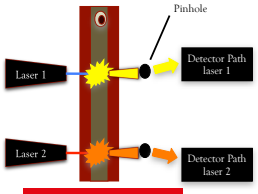
Analog-to-Digital converters (ADC) translate analog signals such voltagesor light intensity into a digital representation of that signal.

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

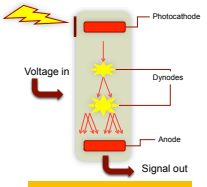
- Current is sent to amplifier(s)
- Signal pulse is sent to an analog to digital converter (ADC)
- The signal is converted to a digital value
- The values are entered into a spreadsheet and given a header
- A Flow Cytometry Standard **(FCS)** file is generated.



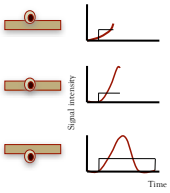
How flow data are generated



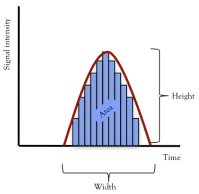
1. Emission



2. Detection



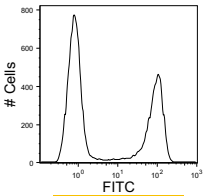
3. Converted to Voltage



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

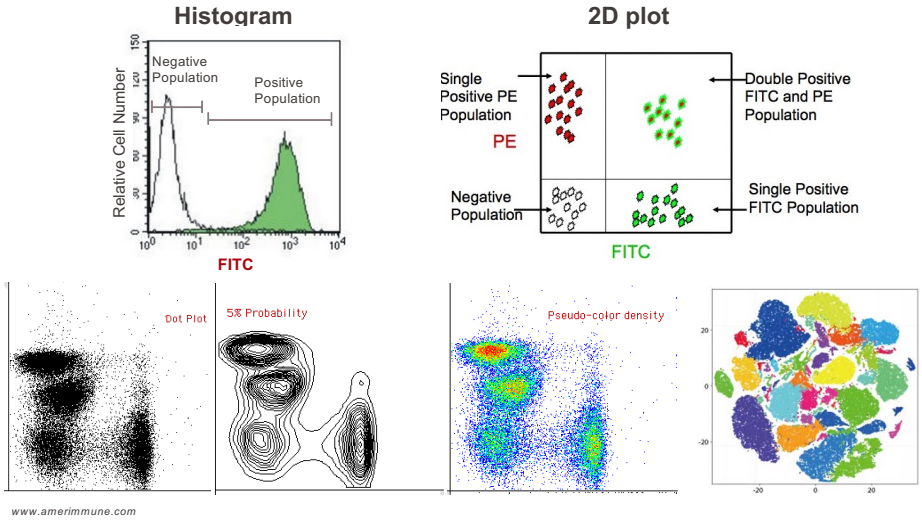


6. Plotted

Storage Data – FCS files

- **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.**

Data Visualisation

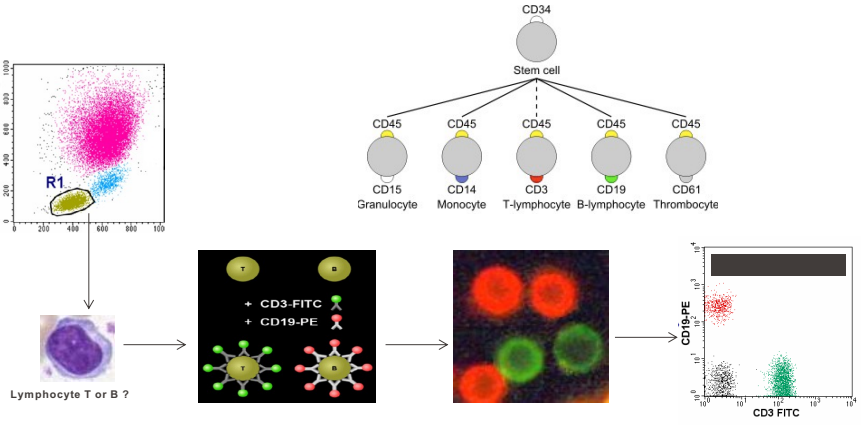


Applications

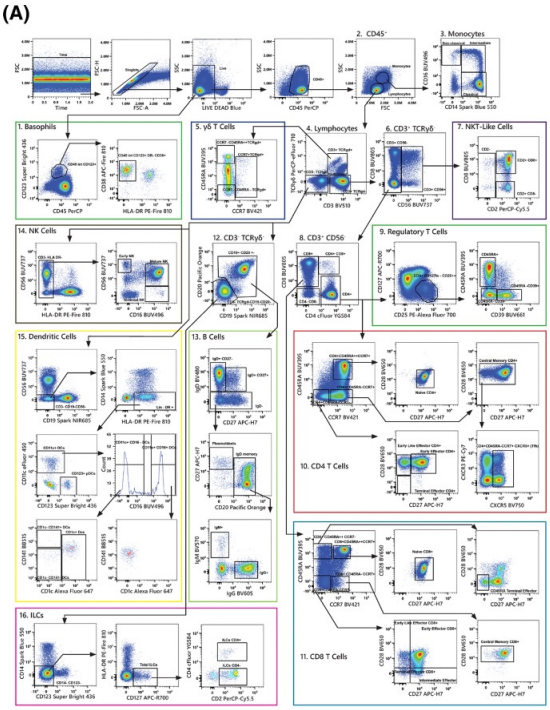
Extracellular and Intracellular Immunostaining
Cell Cycle Analysis
Fluorescent Proteins
Cytotoxicity assays, Cell Death, Viability and Apoptosis
Autophagy
Cell Proliferation
Calcium Flux
ROS
FRET
CBA
RNA analysis, Genomic cytometry
Extracellular vesicles
Microbiology / Marine biology
Metabolism (NADH,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

Applications

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

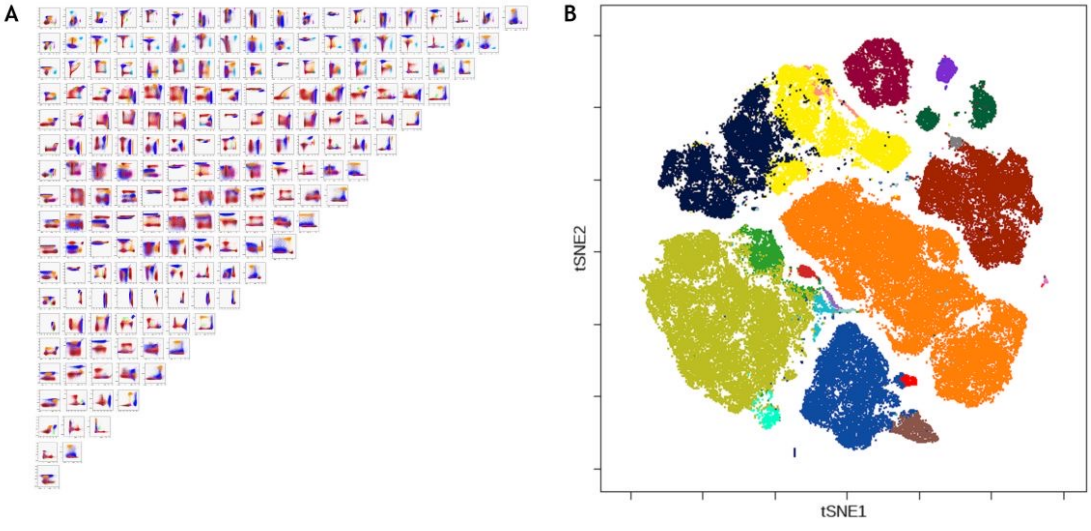


Applications



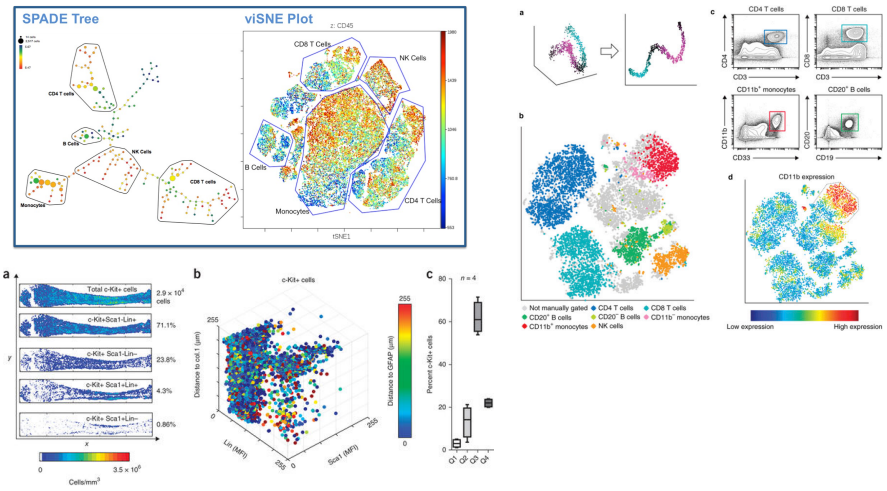
55

Applications



56

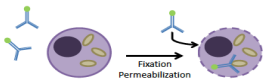
Applications

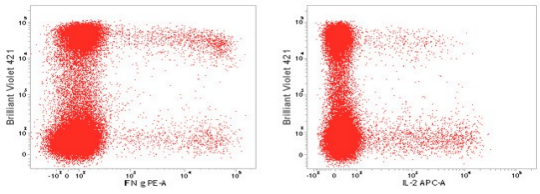


57

Applications

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

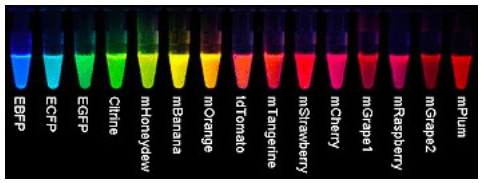


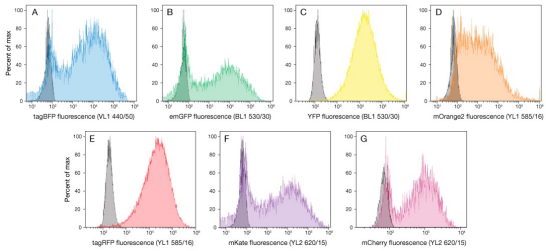


58

Applications

- **Fluorescent Proteins**

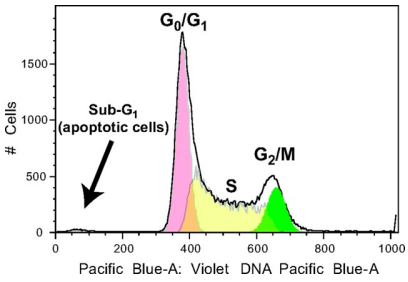


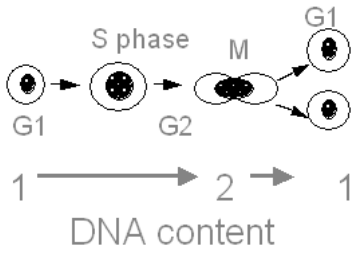


59

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





60

Applications

61

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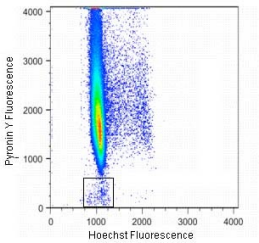
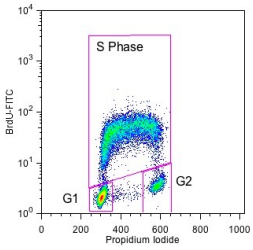
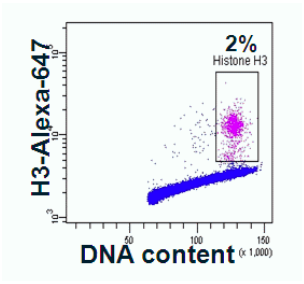
- **Bi-dimensional DNA Analysis**







G0-phase **S-phase** **M-phase**

61

Applications

62

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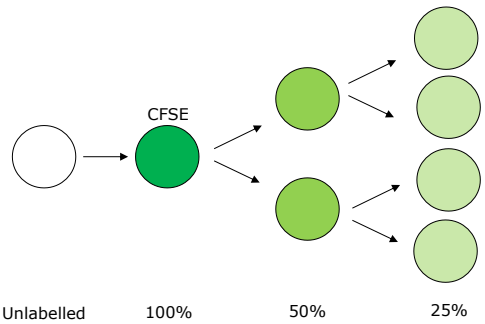
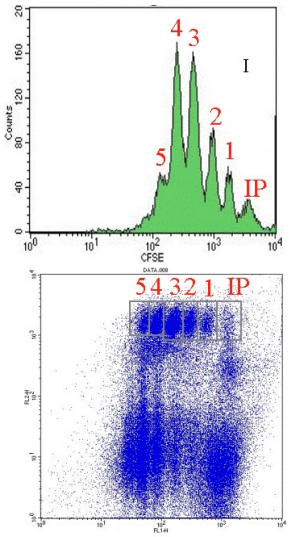
- **Cell Proliferation**

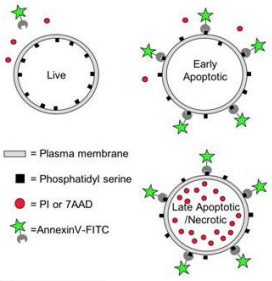


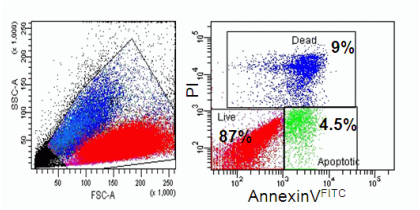


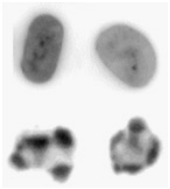
62

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



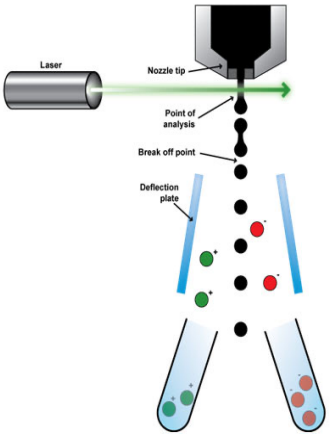




63

Applications

- **Sorting**

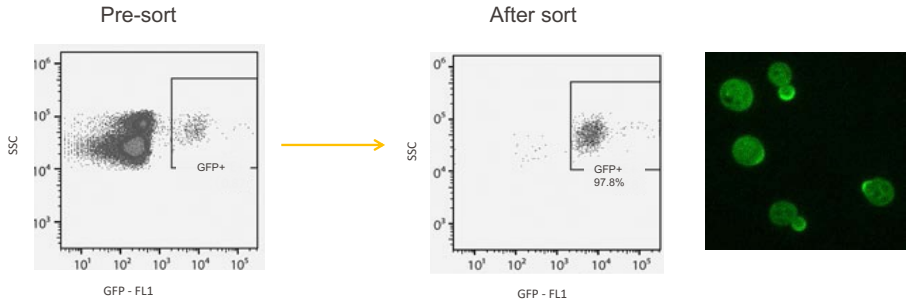


- Same principle as analysers for detection of the fluorescence
- Physical separation of the cells of interest
- Possible to sort Single-Cell – Clones or single-cell gene expression analysis
- Possible to sort into tubes, plates or slides

64

Applications

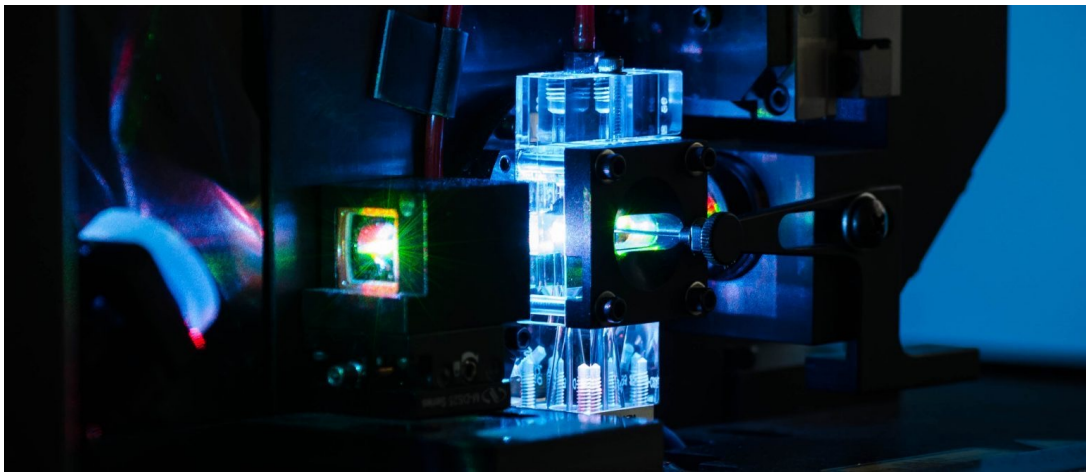
- **Sorting**



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

65

Bridges with other Technologies



66

New Technologies

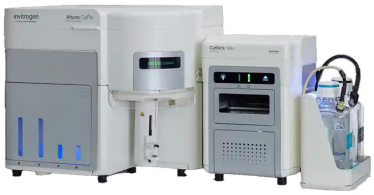
Imaging Flow Cytometry

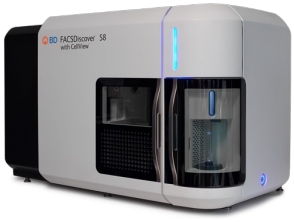
Mass Cytometry (CyTOF)

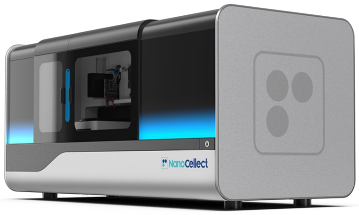
Imaging Flow Cytometry

- Powerful combination of quantitative images analysis and flow cytometry

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







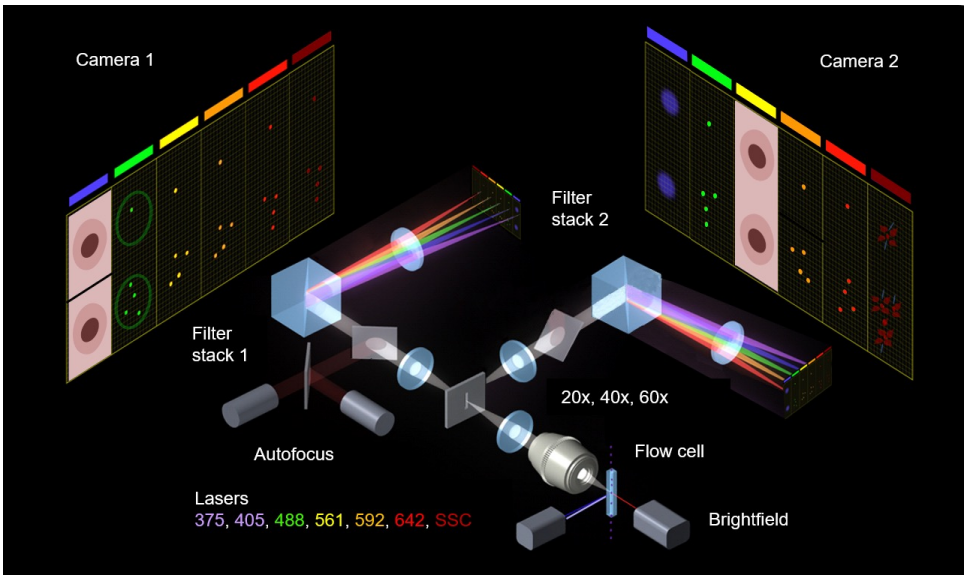
Imaging Flow Cytometry
Cytek Amnis

- 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

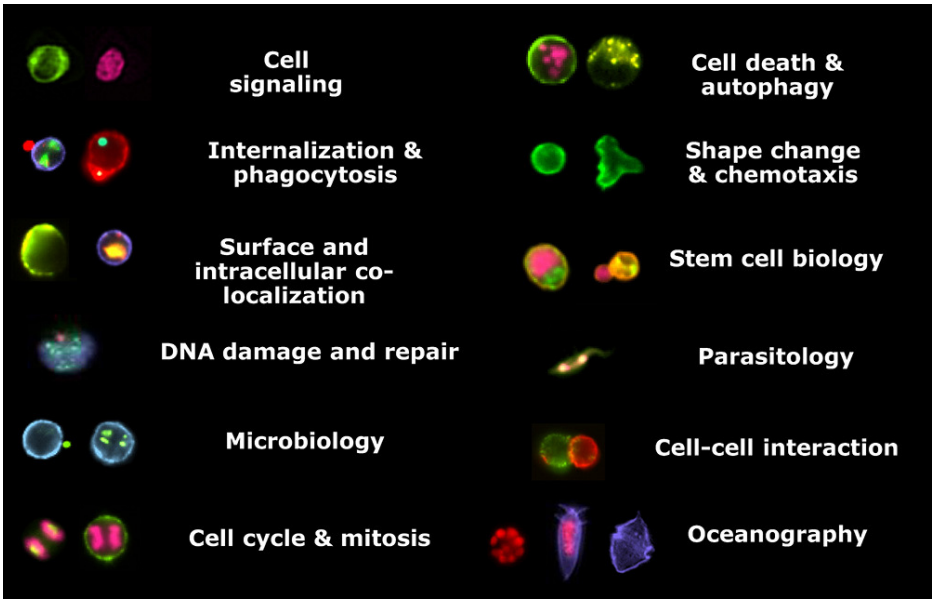
69

Imaging Flow Cytometry – Cytek Amnis



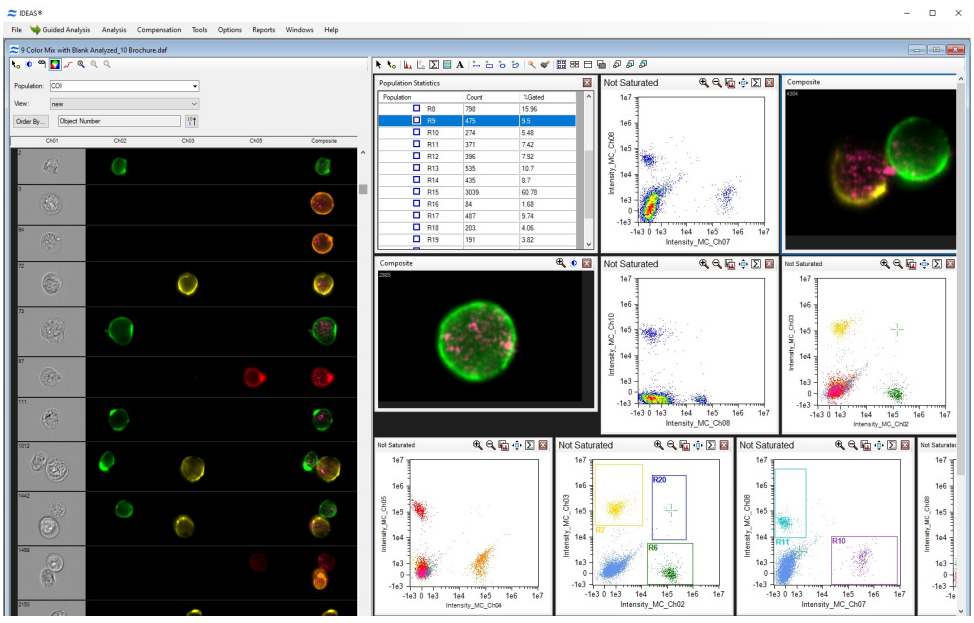
70

Amnis – Applications



71

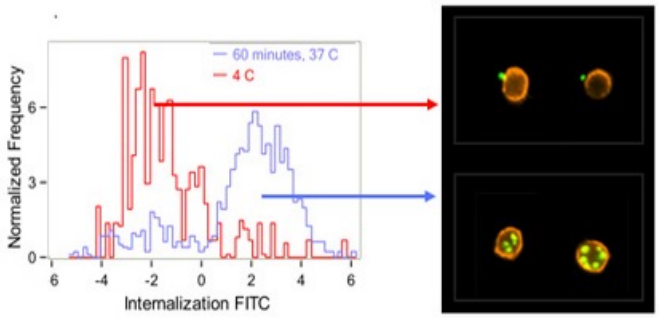
Amnis – Applications



72

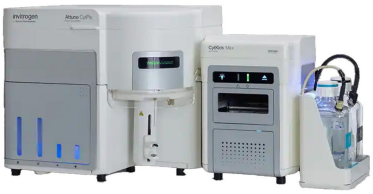
Amnis – Applications

Phagocytosis by macrophages

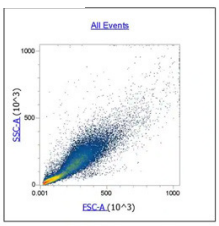


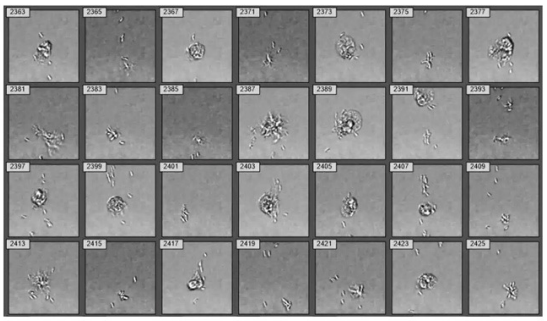
73

Attune CytPix



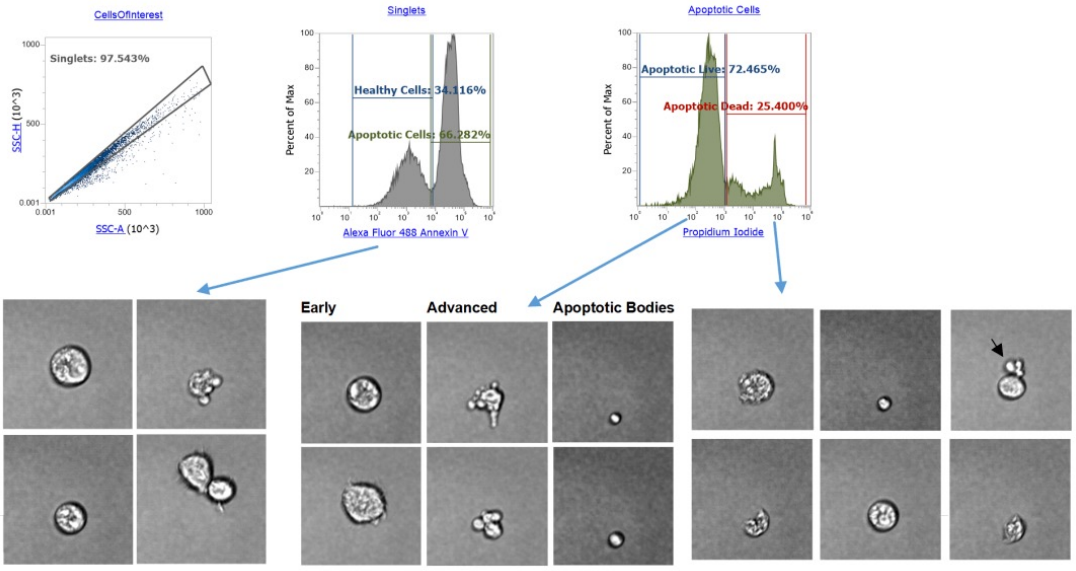
Brightfield images
no fluorescence





74

Applications

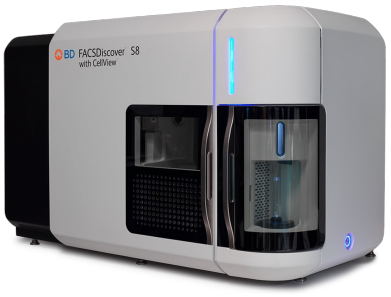


75

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.



S8 Cell Sort

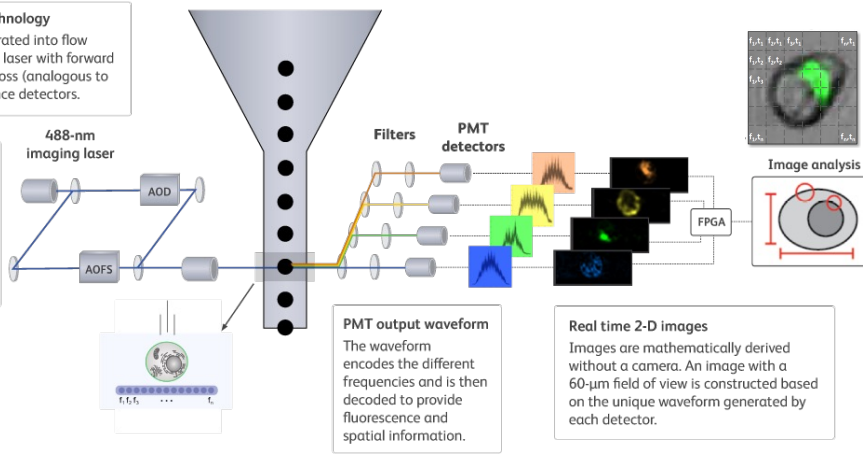
A8 Analyzer

76

BD FACSDiscover™

BD CellView™ Image Technology
The technology is incorporated into flow cytometers as an imaging laser with forward scatter, side scatter, light loss (analogous to brightfield) and fluorescence detectors.

Acousto-optic deflector (AOD)
The laser beam is split into an array of beams, each of which is modulated at a unique frequency that indicates its position.

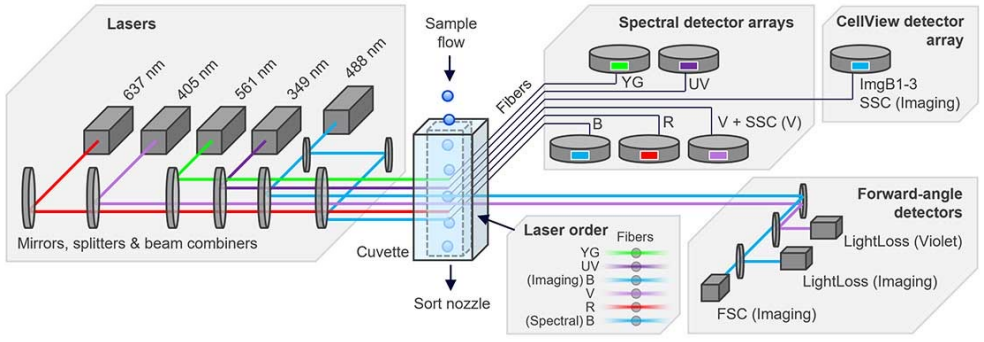


PMT output waveform
The waveform encodes the different frequencies and is then decoded to provide fluorescence and spatial information.

Real time 2-D images
Images are mathematically derived without a camera. An image with a 60-µm field of view is constructed based on the unique waveform generated by each detector.

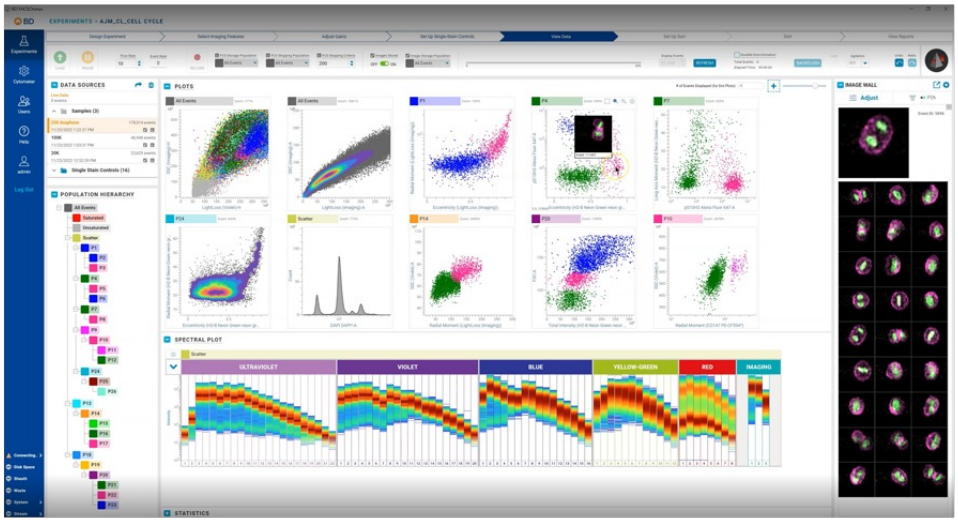
77

BD FACSDiscover™



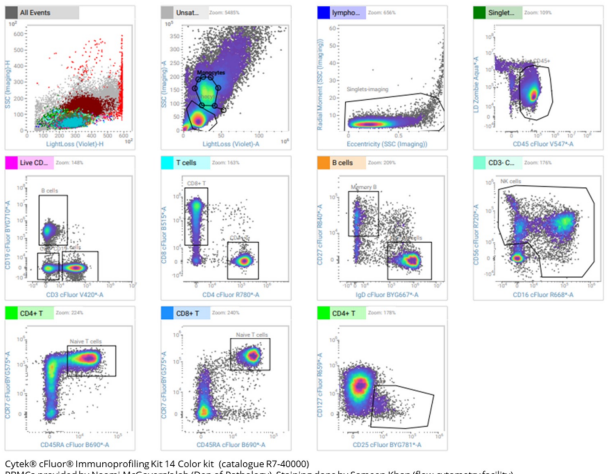
78

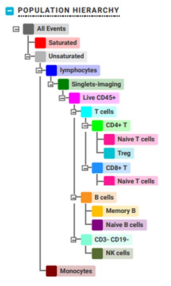
BD FACSDiscover™



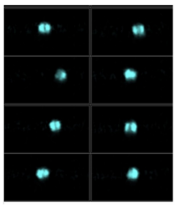
79

BD FACSDiscover™

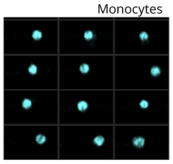




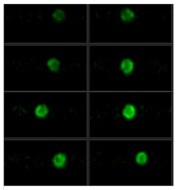
CD8+ T – SSC



Monocytes



CD8+ T – cFluor B515

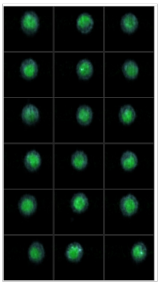


Cytek® cFluor® Immunoprofiling Kit 14 Color kit (catalogue R7-40000)
PBMCs provided by Naomi McGovern's lab (Dep of Pathology). Staining done by Sameen Khan (flow cytometry facility)

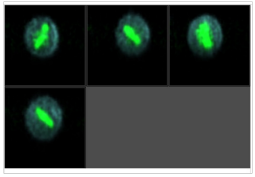
80

BD FACSDiscover™

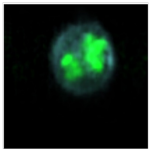
Prometaphase



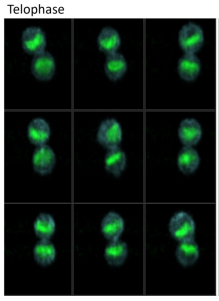
Metaphase



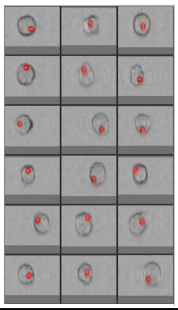
Anaphase

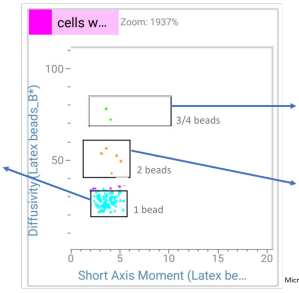


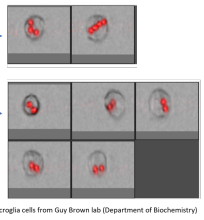
Telophase



HeLa-H1 GFP cells from Iva Tchasovnikarova lab (Gurdon Institute)



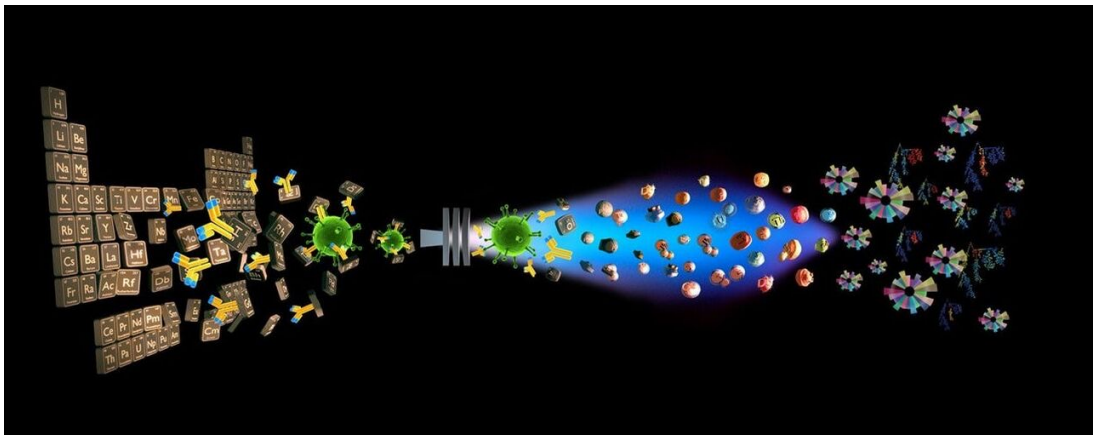




Microglia cells from Guy Brown lab (Department of Biochemistry)

81

Mass Cytometry



82

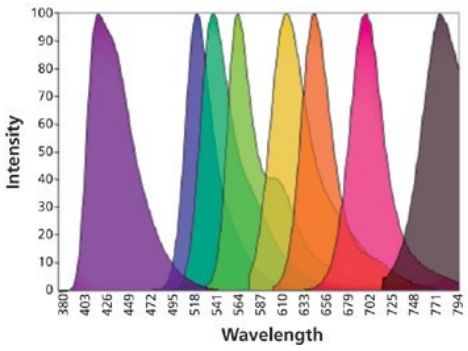
Mass Cytometry

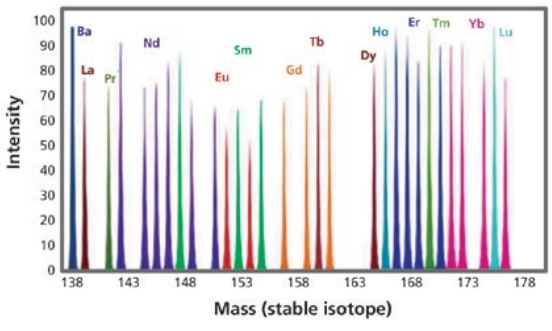
- **CyTOF : Mass Spec + Flow Cytometry**

- 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

83

Mass Cytometry

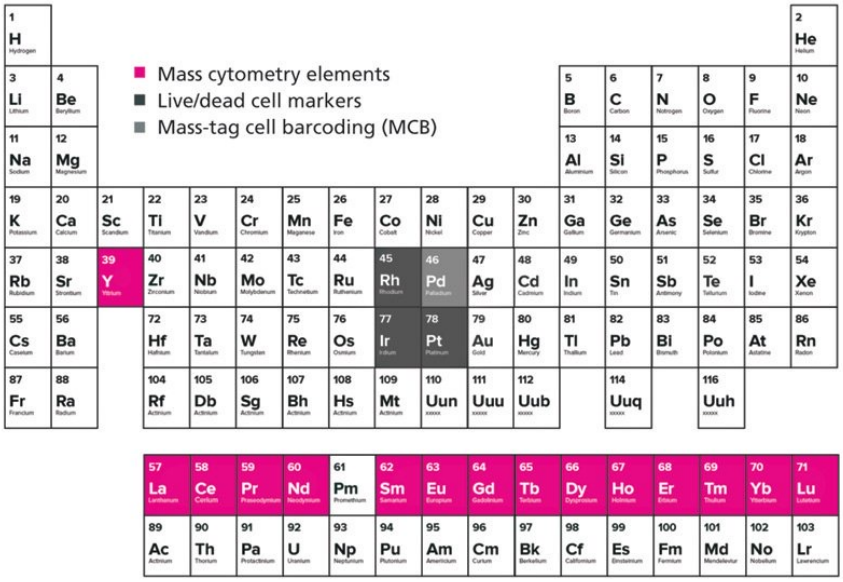




Standard Biotools

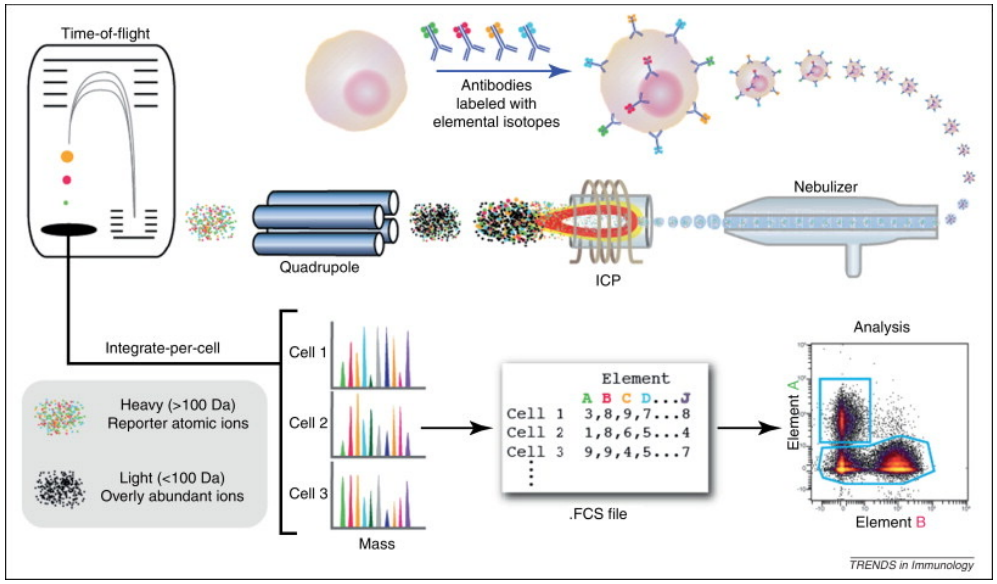
84

Mass Cytometry



85

Mass Cytometry



86

Mass Cytometry

Miguel Garcia