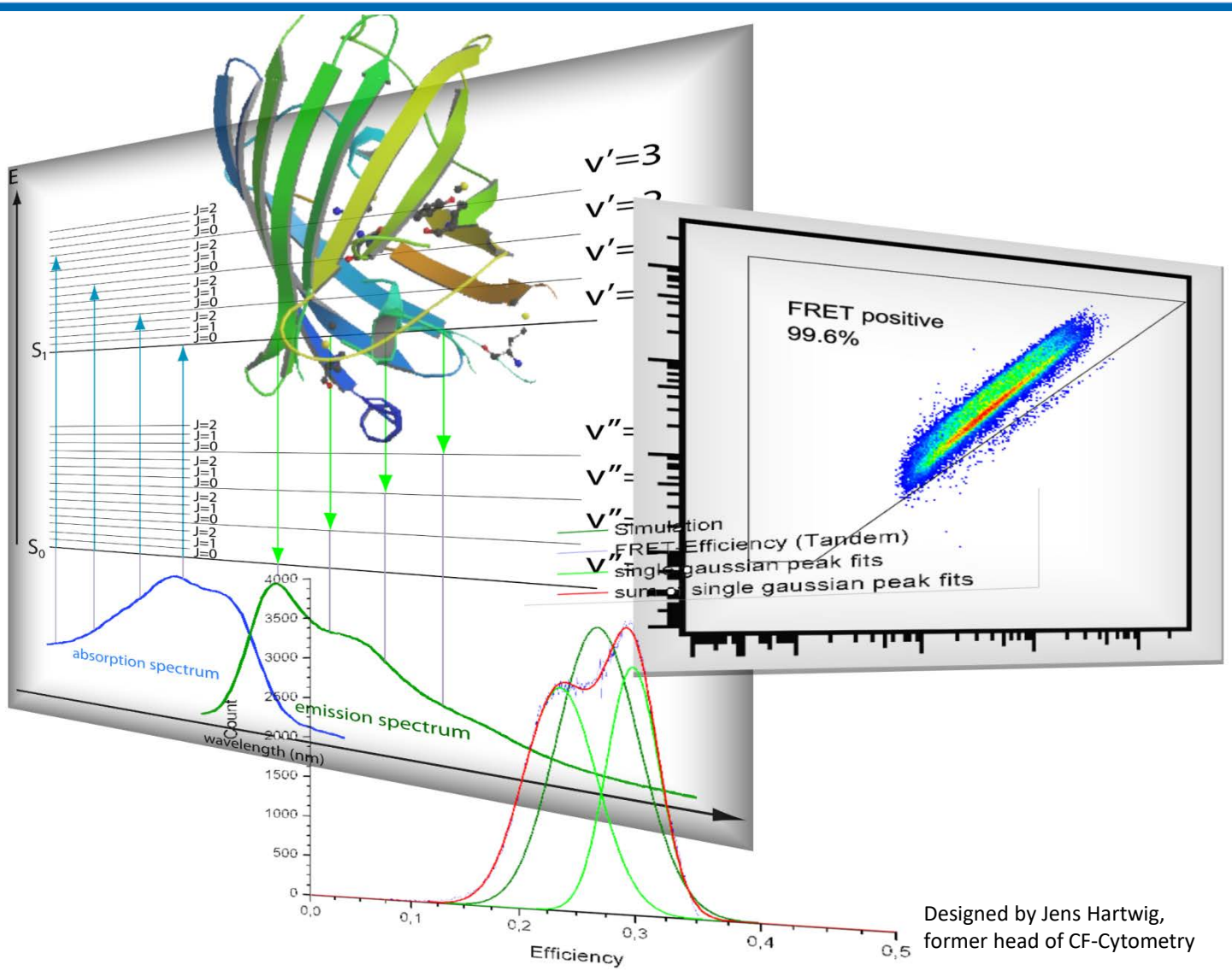
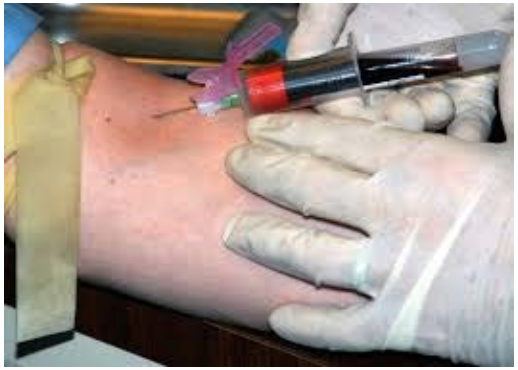
Introduction to Flow Cytometry



Designed by Jens Hartwig,
former head of CF-Cytometry

Flow Cytometry Core Facility
Dr. Stefanie Bürger
s.buerger@imb-mainz.de

Flow cytometry in daily life


- Routine blood diagnostics and blood count is still done by Coulter Counters, which are forerunners of today‘s cytometers and sorters
- Especially leukemia diagnostics is done by flow cytometry (surface marker staining of blood cells with fluorescently labelled antibodies)

Overview

- History of Flow Cytometry
- What is Flow Cytometry
- Flow cytometry parameters: FSC, SSC, fluorescence
- Flow Cytometer Components:
  - Fluidics,Optics, Electronics
- Data presentation
- Cell Sorting
- Overview of applications
- Instruments
  - Flow cytometers, cell sorters, special instrumentation

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History of Flow cytometry and cell sorting

1930s: Andrew Moldavan designed a photoelectric apparatus to count individual cells flowing through a capillary tube that was mounted on a microscope stage

1950s: Wallace Coulter began the development of the first instrument that could electronically calculate cell volume: The Coulter Counter. This is still used in todays haematological cytometers

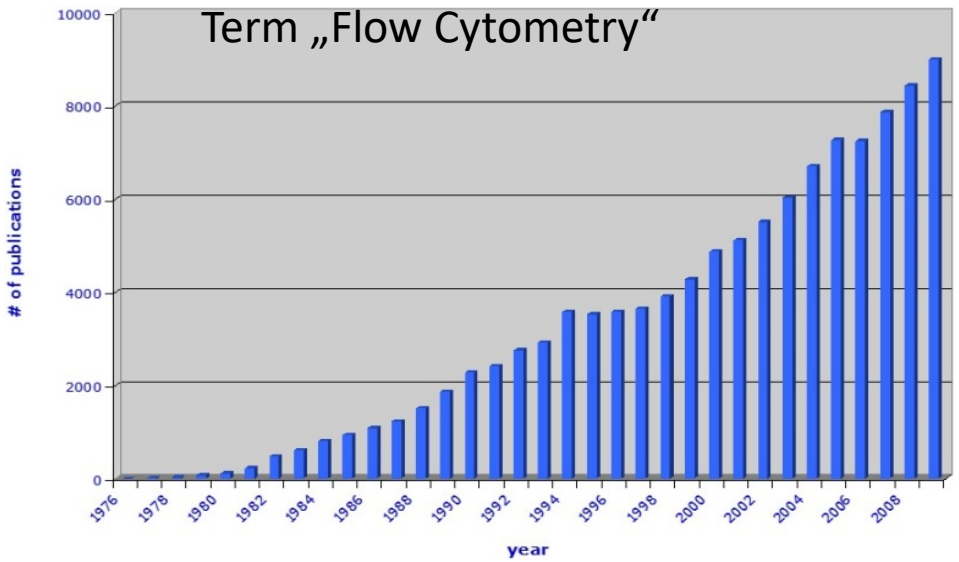
1965: Mack Fulwyler invented the forerunner to today's type of flow cytometers, particularly the cell sorter as he included techniques described by Richard Sweet concerning charging and deflection of droplets (originally used in printers)

1969: Len Herzenberg (Stanford) combined the invention with simultaneous detection of fluorescence parameters

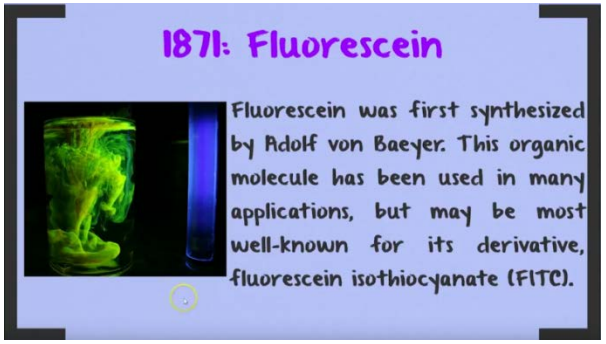
1972: Invention of the term „FACS" by Herzenberg

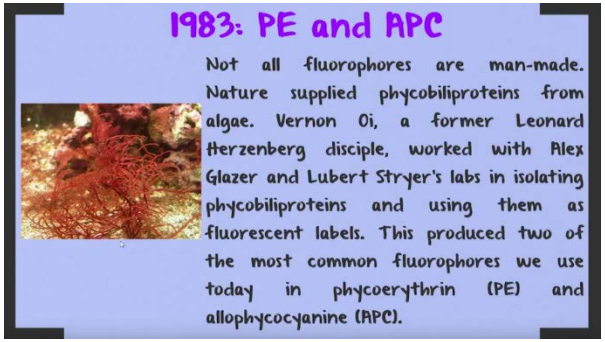
Herzenberg teamed up with Becton-Dickinson

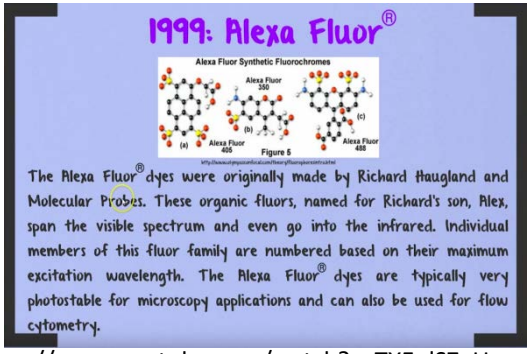
History of Flow cytometry and cell sorting

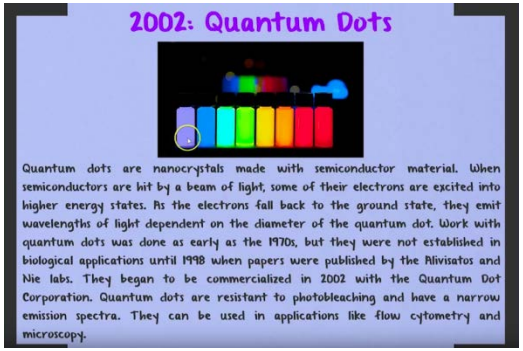


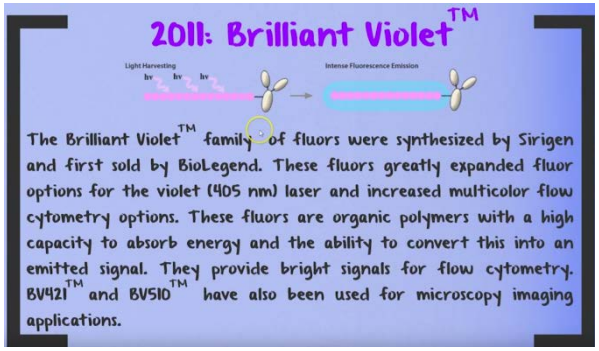
Year 2016 - 11.000 of publications











https://www.youtube.com/watch?v=TXEnlSZeU-c

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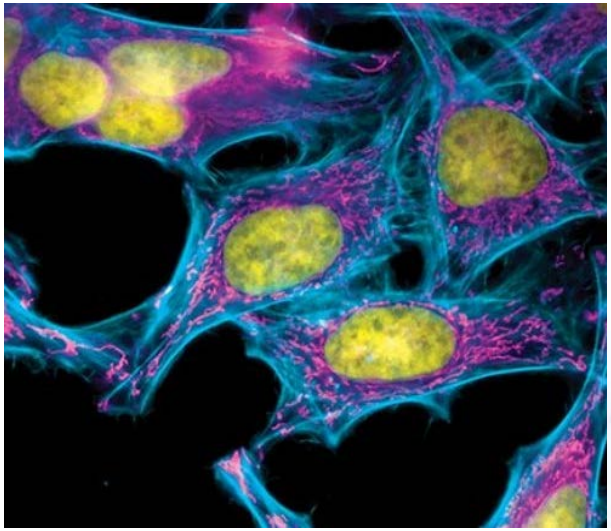
What is Flow Cytometry?

Flow - Fluid

pbase.com

Cyto - Cell



smithsonianmag.cm

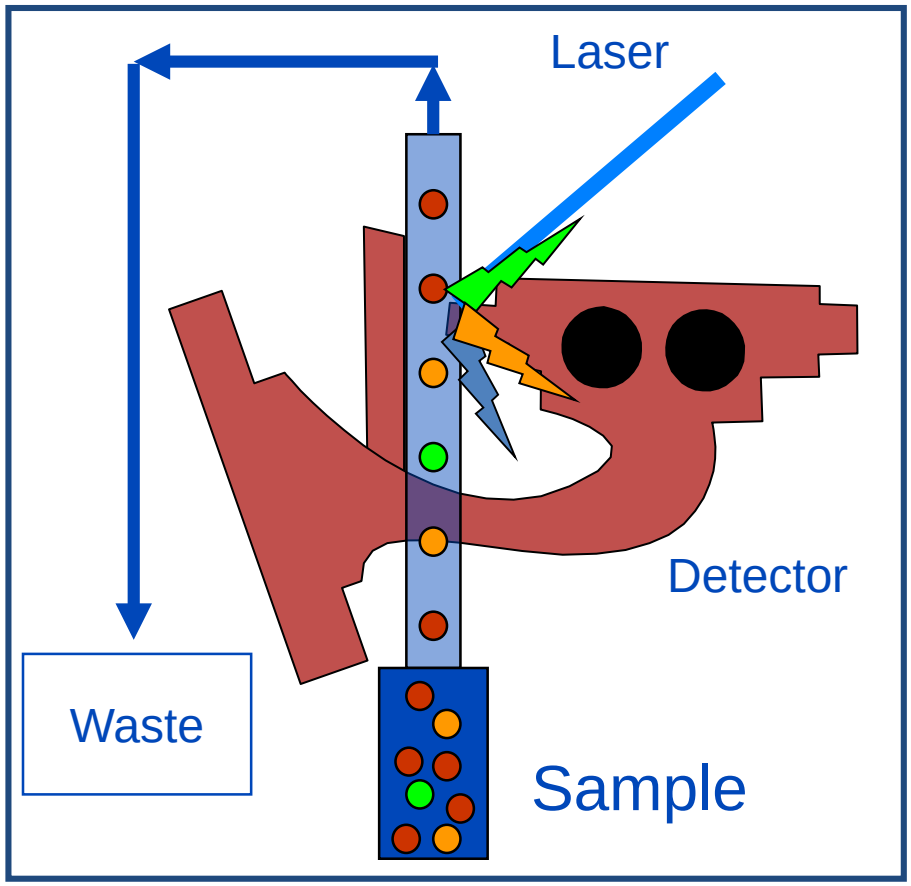
Metry - Measurement

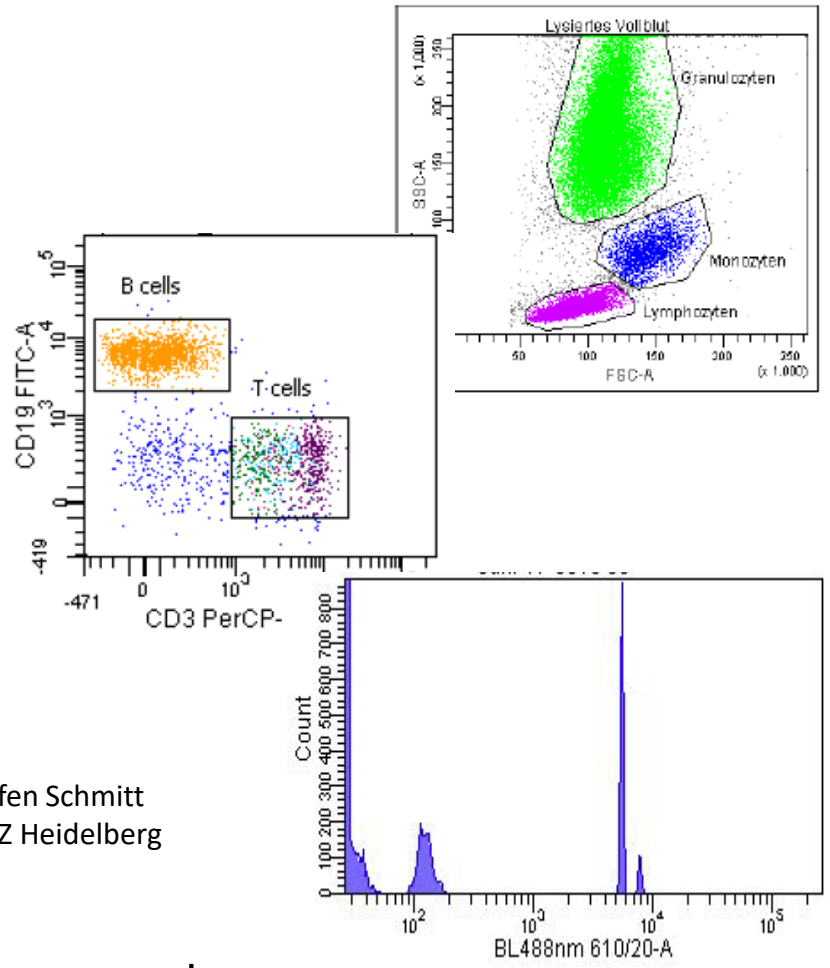
clipartkid.com

Definition of flow cytometry:

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

What is Flow Cytometry?





Steffen Schmitt
DKFZ Heidelberg

Definition of flow cytometry:

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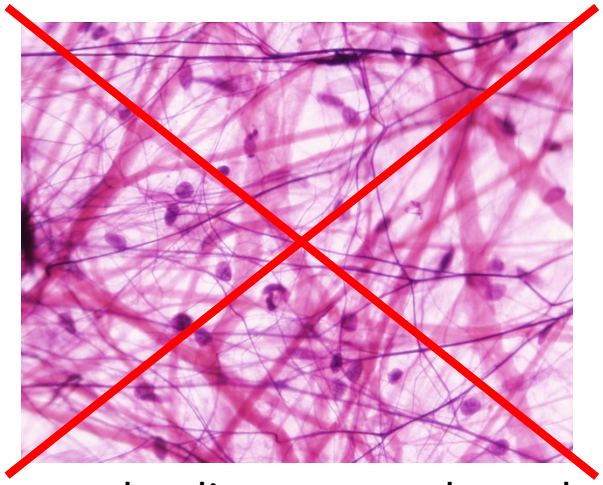
What a flow cytometer cannot measure

No solid tissue .... only cells in suspension



Tissue must be disaggregated, e.g. by using enzymes (e.g. Dispase or Collagenase)

- Cannot ordinarily locate a component within the cell
- Cannot see how the fluorescent component is uniformly distributed in the cell
- No detailed intracellular morphology

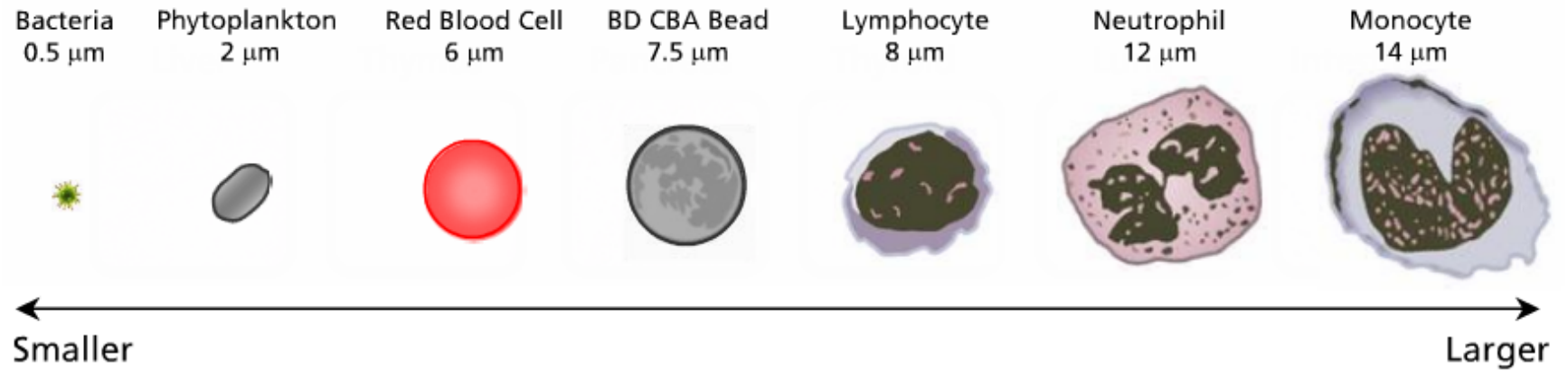
Flow cytometry sample preparation

- Preparation of single cells in suspension
  - Suspension cell lines
  - Detachment of adherent cells with trypsin or EDTA, etc.
  - Tissue digest with collagenase, dispase, liberase, DNase, etc.
  - Filtration to remove cell clumps

- Enrichment of rare cell populations
  - Magnetic sorting
  - Density gradient centrifugation (e.g. Percoll or Ficoll)

Size range of suitable particles

Coventional flow cytometer



Modified from BD Biosciences

From 0.5 µm

Up to 50 µm

Special instruments

Smaller particles: Extracellular vesicles (0.03 - 1 µm)

Larger particles: Whole organisms or cell clusters like *C.elegans, Drosophila* larvae, pancreatic islets (100 µm – 1800µm)

Examples of flow cytometrical measurements

Analysis parameters	Examples – Details later
Surface antigens Intracellular antigens	Phenotyping, cell type identification by surface marker analysis, protein expression (e.g. GFP fusion proteins), developmental stages, activation markers
DNA/RNA content	Cell cycle analysis
Cell activation	cell death, apoptosis, proliferation,membrane potential, Ca^{2+} release, phosphorylation
Intracellular distances protein-protein-interaction	FRET, YFP complementation
Cell or particle number	Counting of cells, quantification

Advantages of flow cytometrical measurements

- Quick sample processing
  In most instruments up to 20.000 events per second
- High statistical power
- Study of (sub)populations of cells
- Rare cell detection, e.g.: CTCs (circulating tumor cells)
- Sort option (e.g. clonal cell lines)
- Single cell sorting
- Multi-parametric analysis – up to 20 parameters simultaneously at IMB; up to 50 on most recent instruments (more to come)

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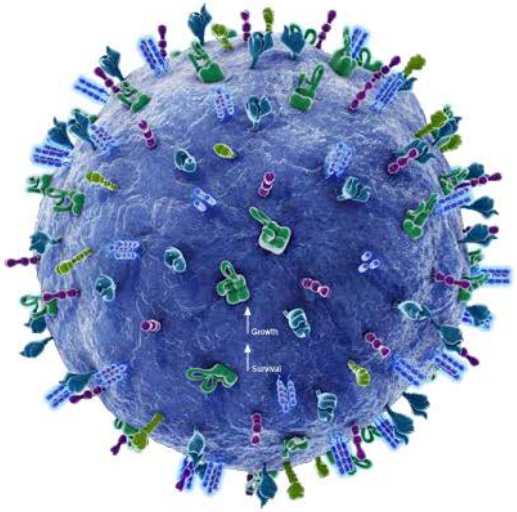
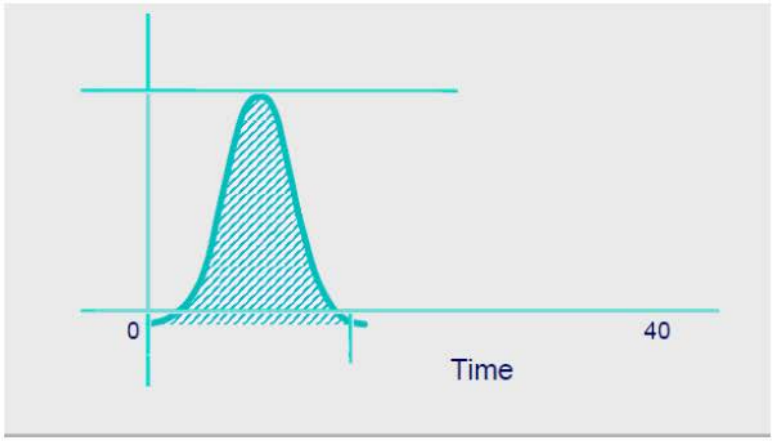
What is happens in a flow cytometer?

Properties of a cell

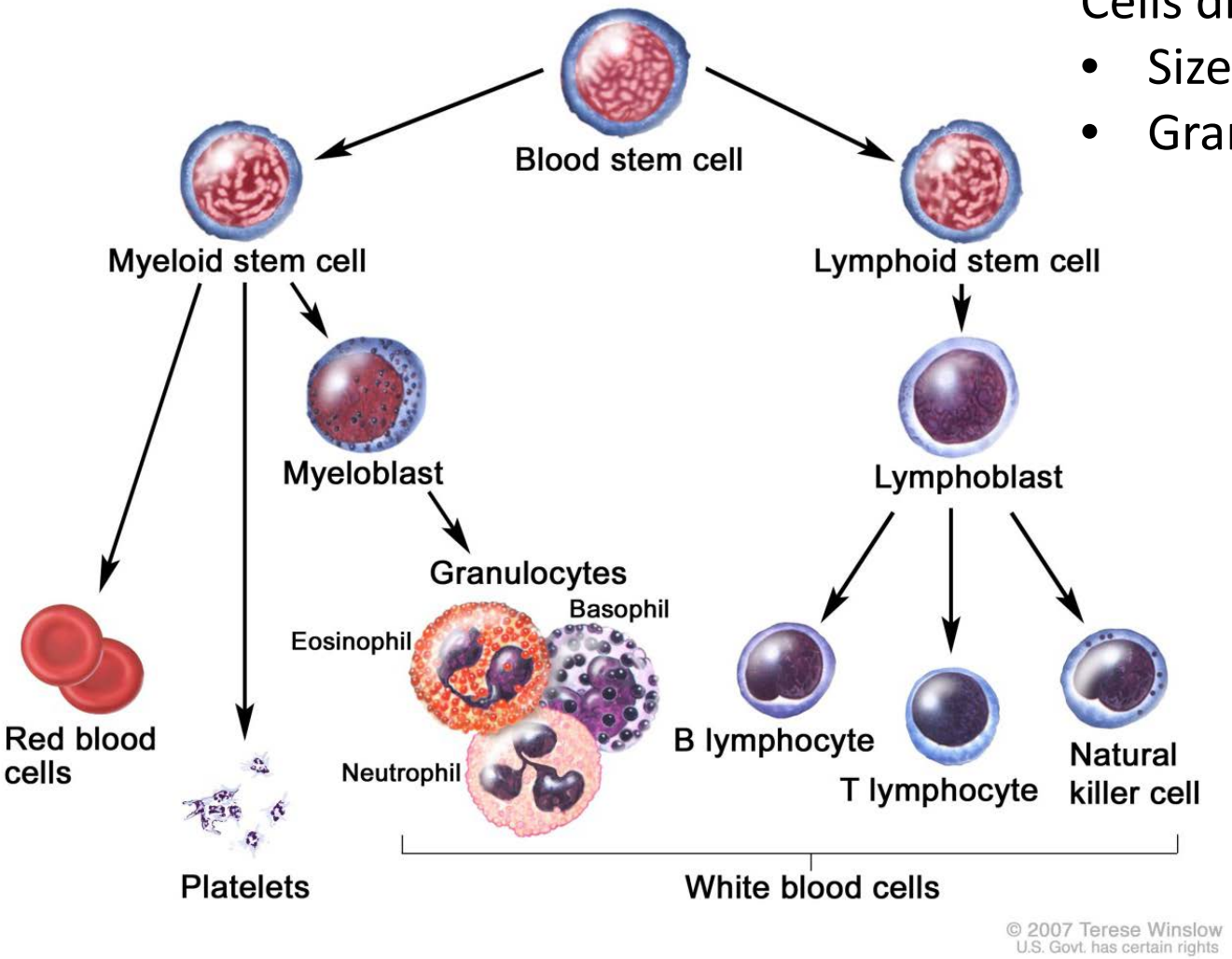


Detectable light signal



Size
Granularity/complexity/
Fluorescence (antibody staining or intrinsic like GFP)

Analysis based on cell morphology



Cells differ in

- Size
- Granularity/Complexity

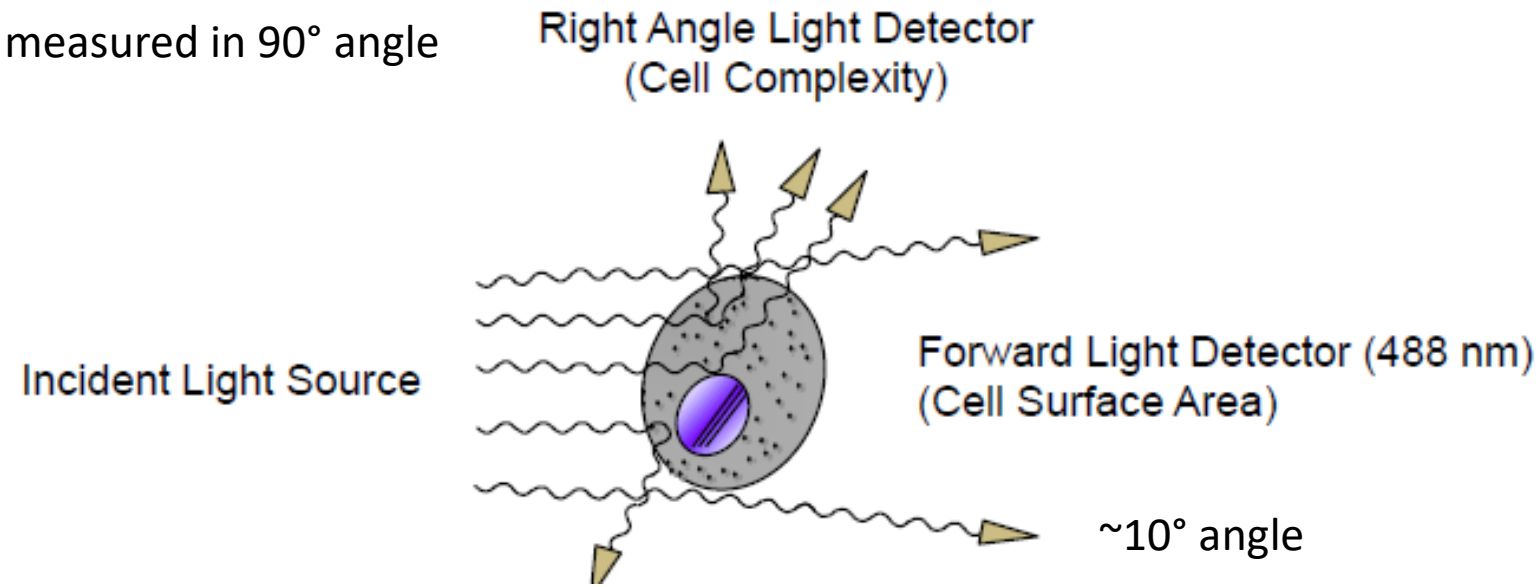
Forward and Side Scatter

Forward Scatter (FSC)

- Measured along the axis of the incoming light (diffracted light)
- **Roughly proportional to cell size or cell surface area**
  (only true for perfectly round cells, not applicable for small particles)

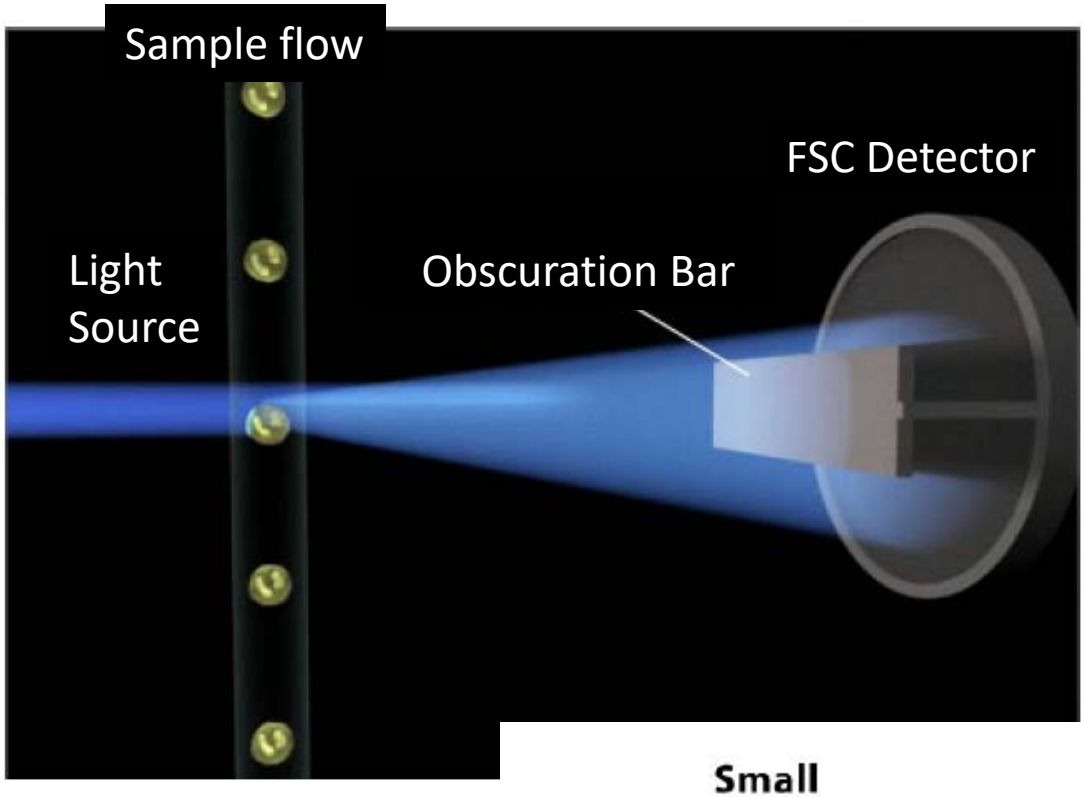
Side Scatter (SSC)

- Measured in 90° direction to the incoming light (reflected or refracted light)
- **∝ cell "complexity" or granularity**

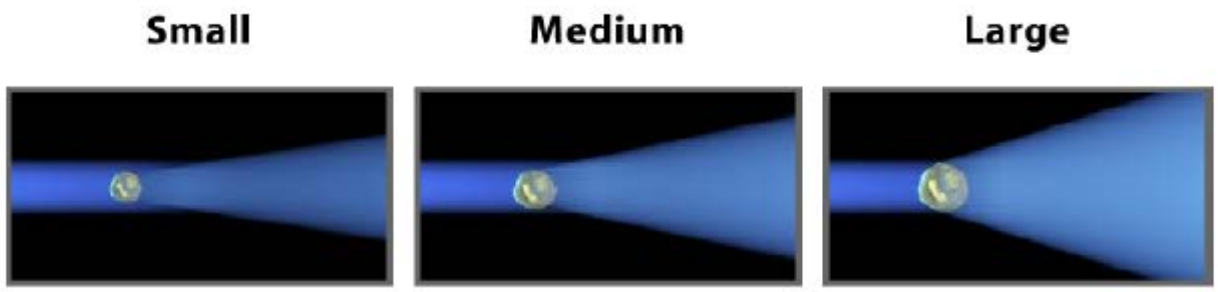


BD Biosciences
Flow cytometry tutorials

Forward Scatter

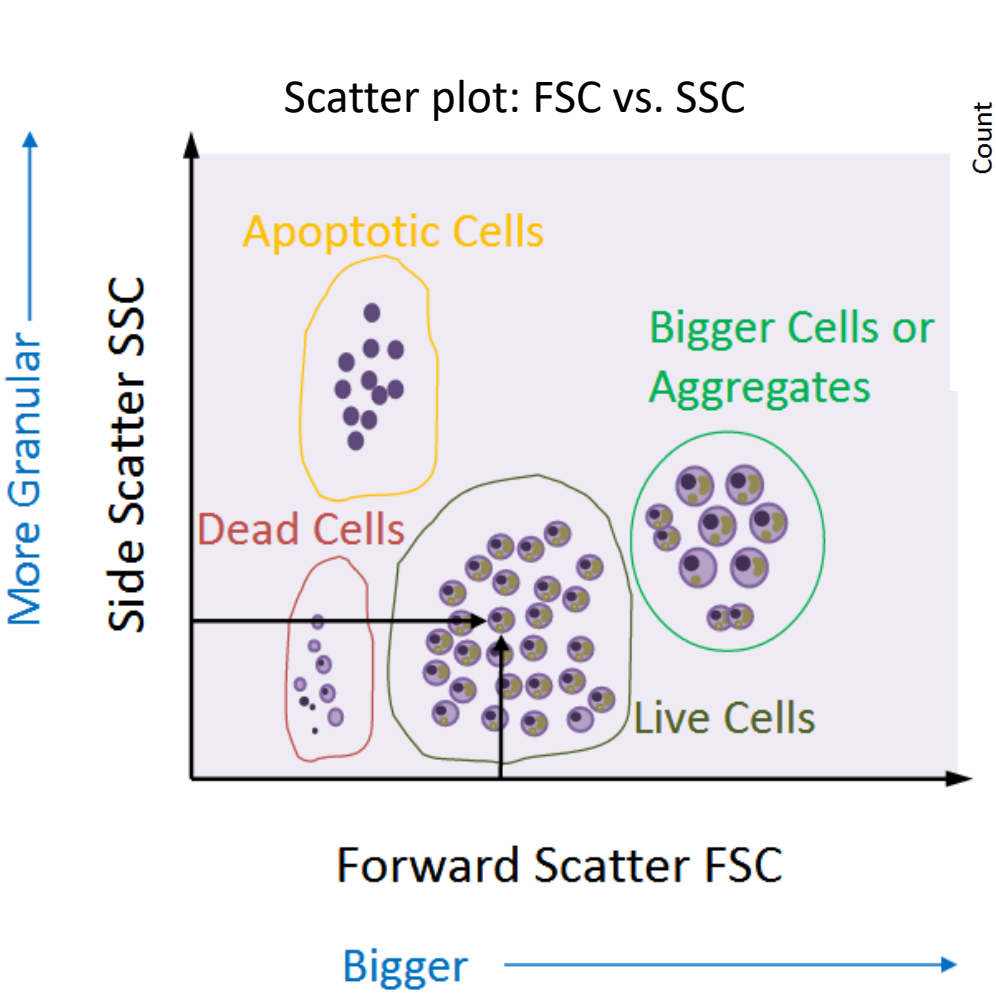


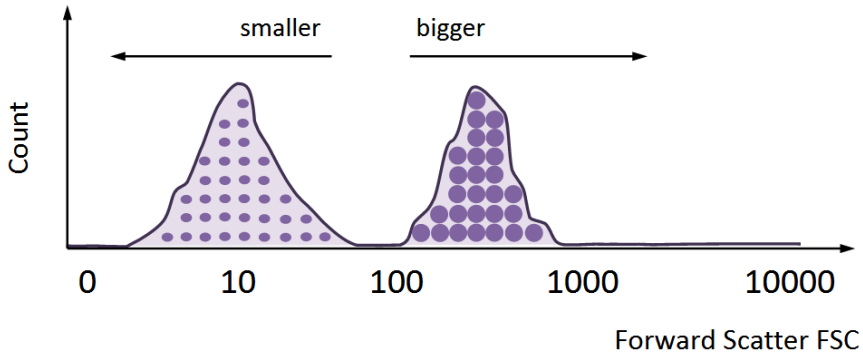
The obscuration bar blocks the intense laser light from reaching the FSC detector



Attune Acoustic Focusing Training Guide, Thermo Scientific

Cell analysis based on morphology

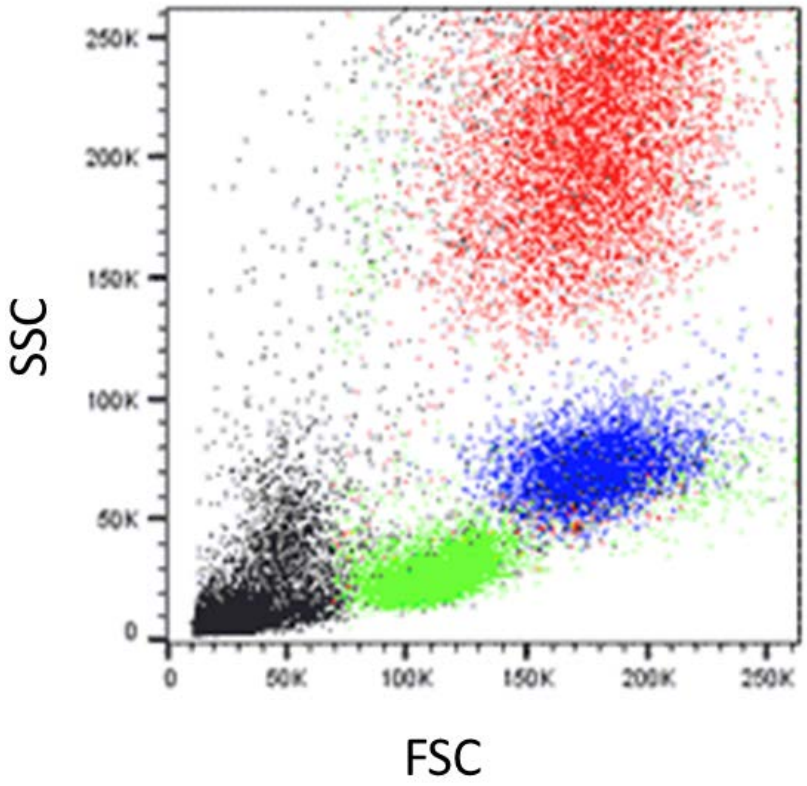


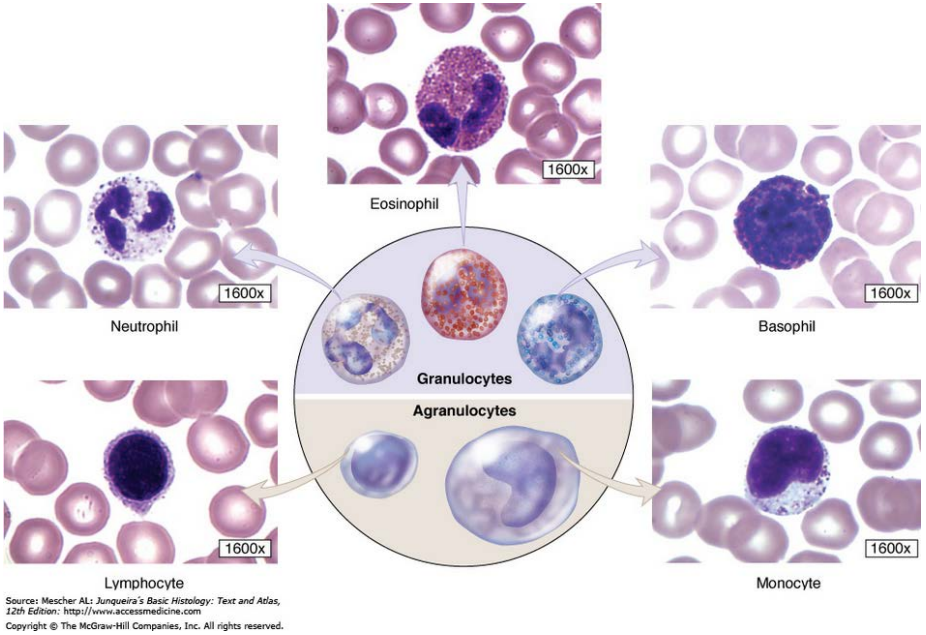


BD Biosciences

Cell analysis based on morphology

Scatter plot
Lysed whole blood





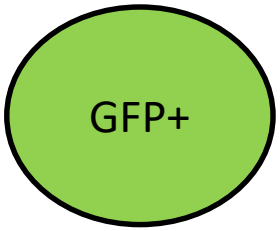
Lymphocytes: small, low granularity

Monocytes: big, low granularity

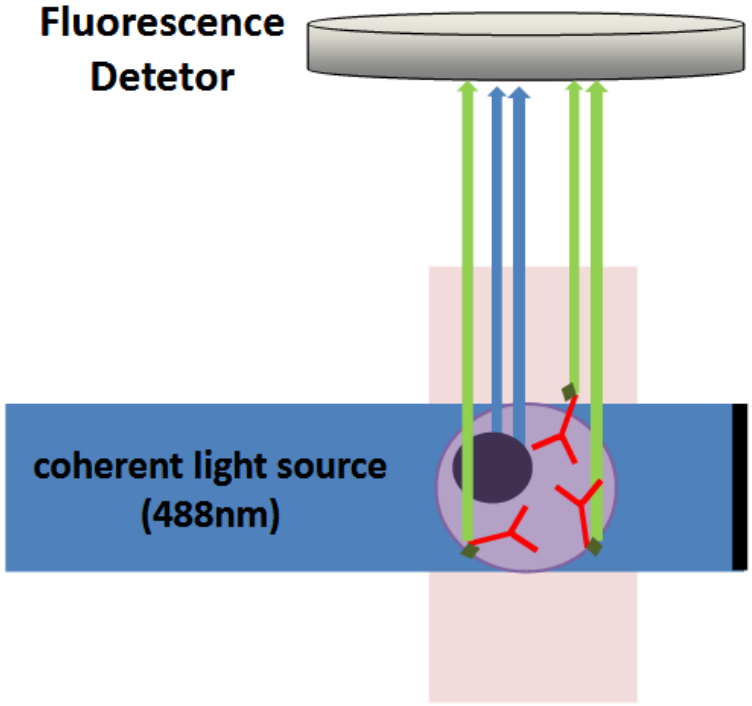
Granulocytes: big, high granularity

Which population show lymphocytes, monocytes and granulocytes?

Analysis of fluorescence by flow cytometry

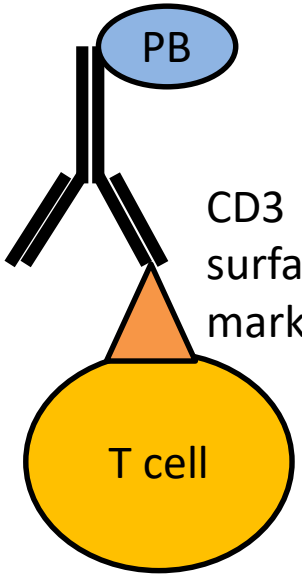


Emitted fluorescence is measured in a 90° angle, just as the side scatter



Fluorescence emission
lower energy than source light
(green)

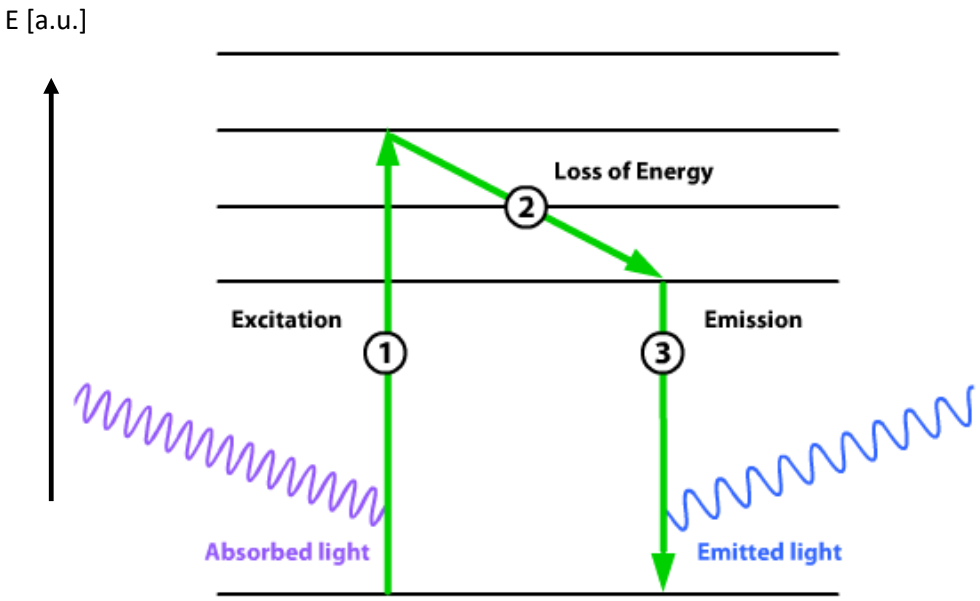
Anti CD3 Antibody with fluorescent label



CD3 surface marker

BD Biosciences

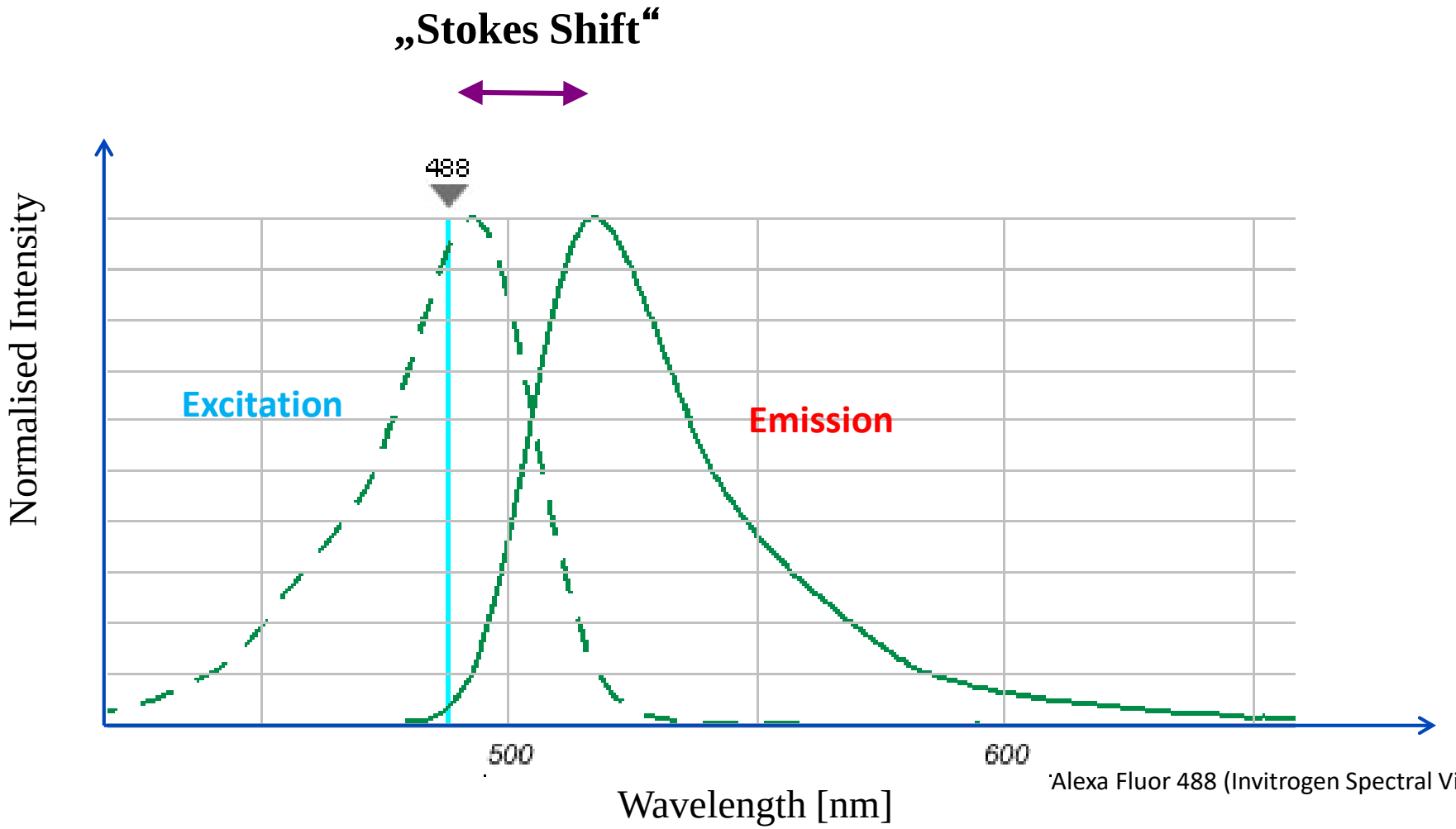
Fluorescence electronic state diagram (Jablonski diagram)



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

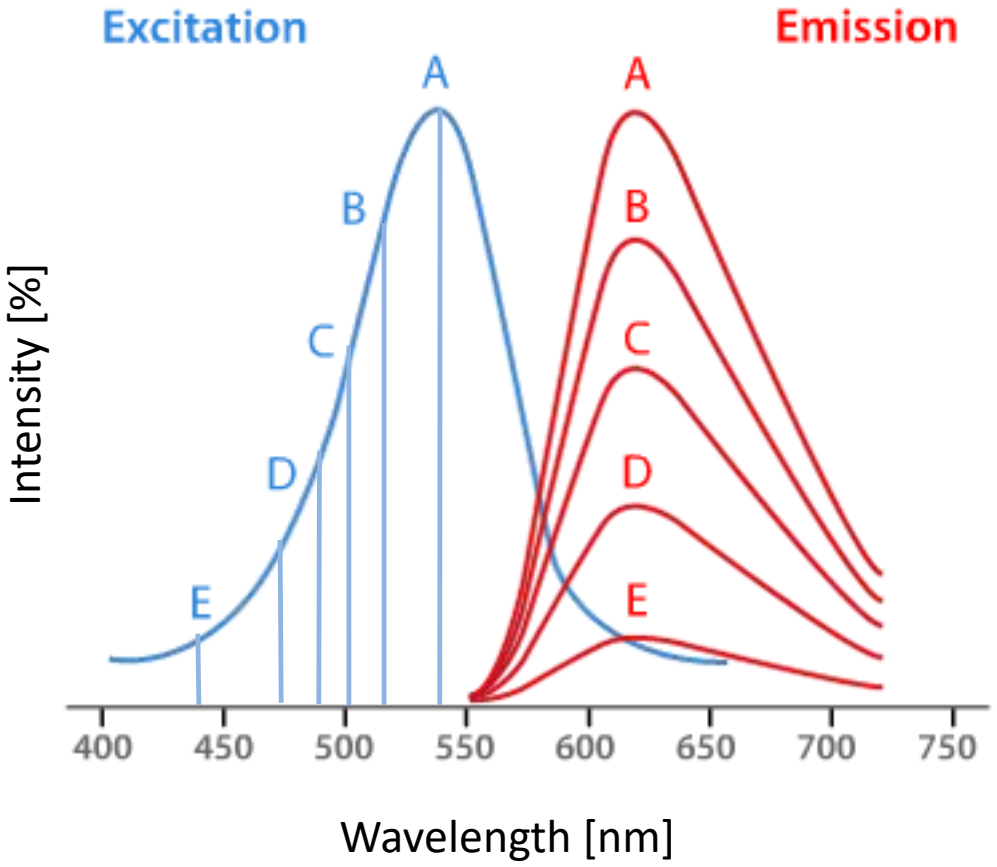
Fluorescence

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



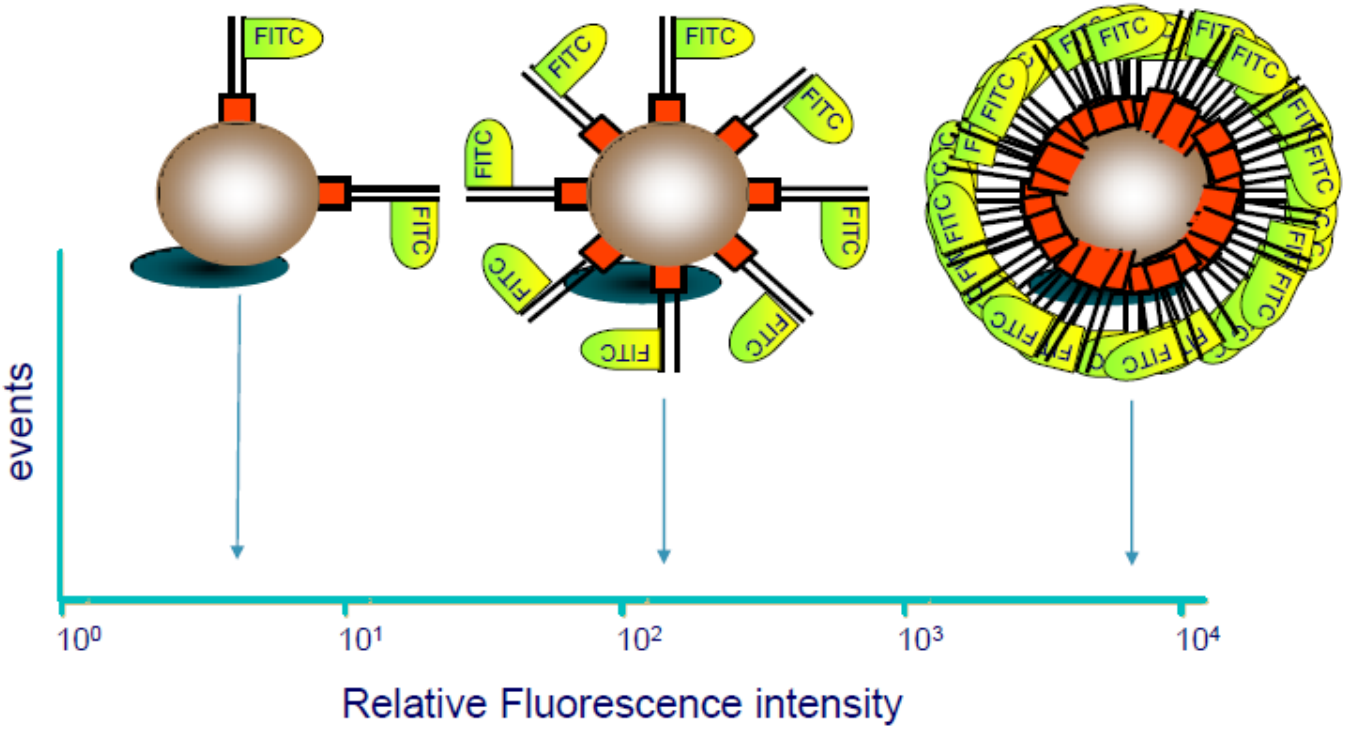
Alexa Fluor 488 (Invitrogen Spectral Viewer)

Fluorescence



- The illumination at lower or higher wavelength affects only the intensity of the emitted light (area below the curve)
- the spectrum of the emitted light is not affected (shifted)

Fluorescence intensity



The emitted fluorescence light is proportional to the amount of bound fluorescence molecules

BD FACSAria Operator Manual

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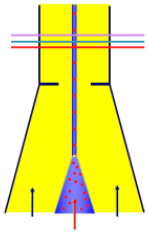
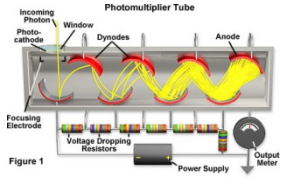
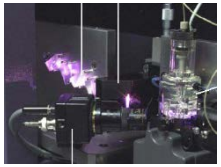
Flow cytometer components

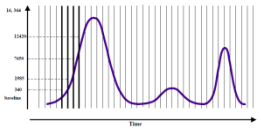


fluidics

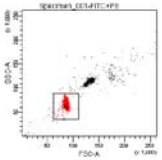
Flow Cytometer

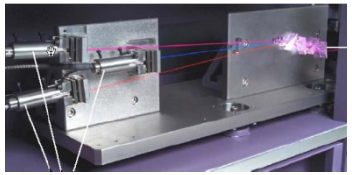






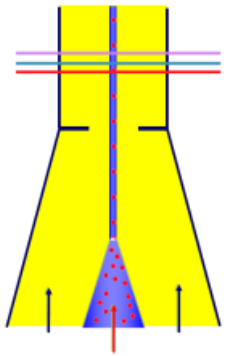
electronics



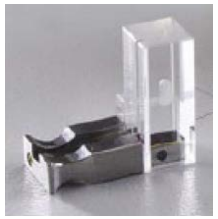


optics

Fluidics



fluidics



Flow Cytometer

Fluidics: Fluidic cart or reagent bottles

Sheath Waste

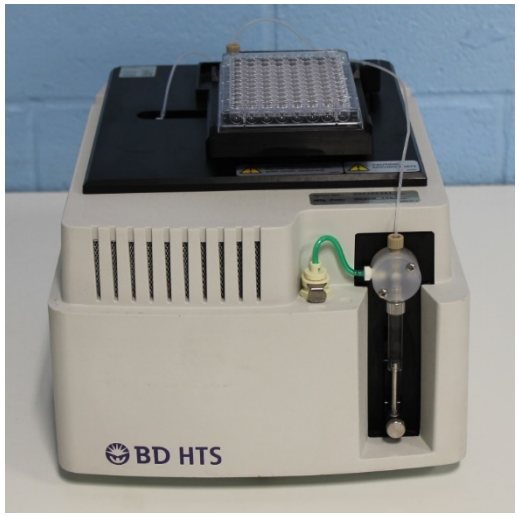
„Carrier liquid“
Normally PBS with stabilizers

Waste Sheath (PBS)

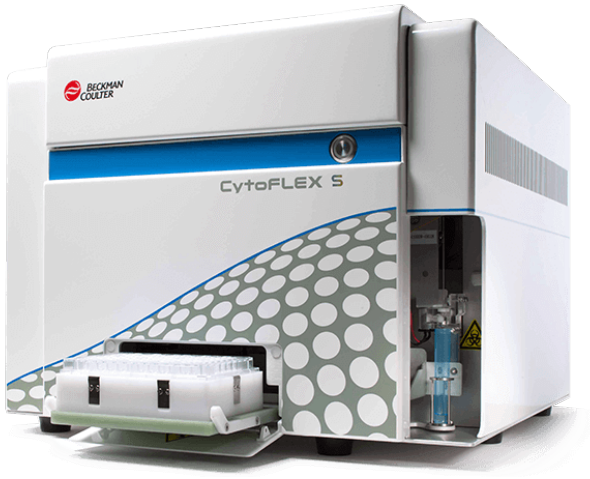
Sheath Waste

Fluidics: Sample injection

Sample Injection Port (SIP)
Sample Injection Tube (SIT)

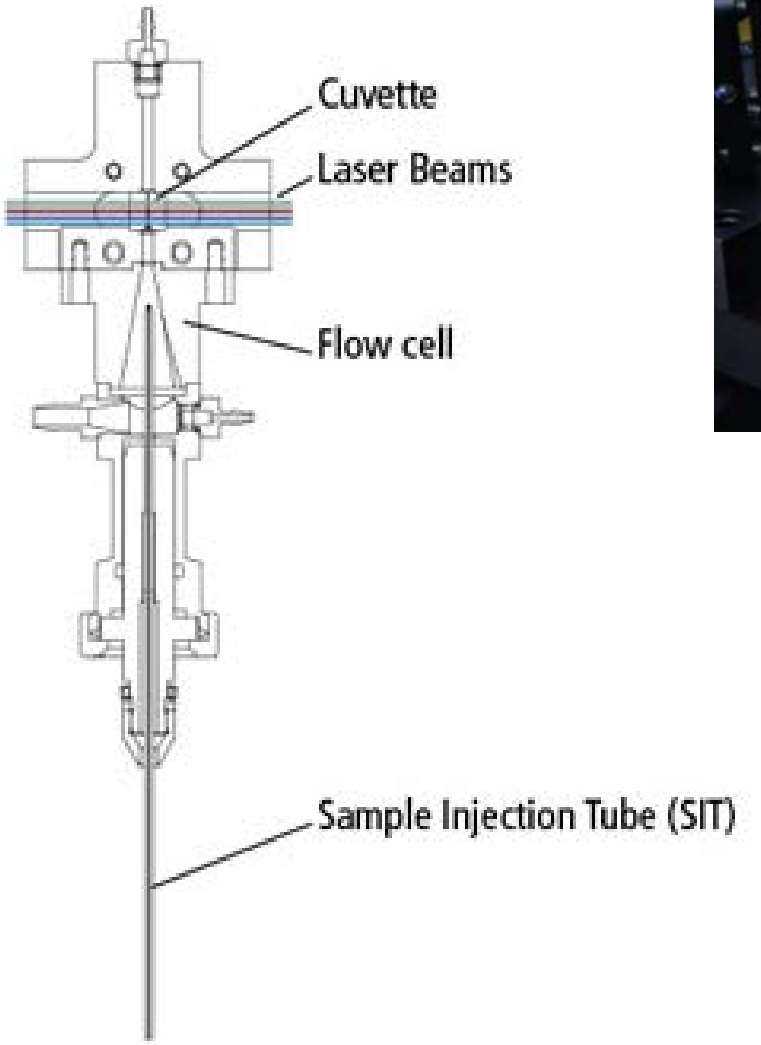


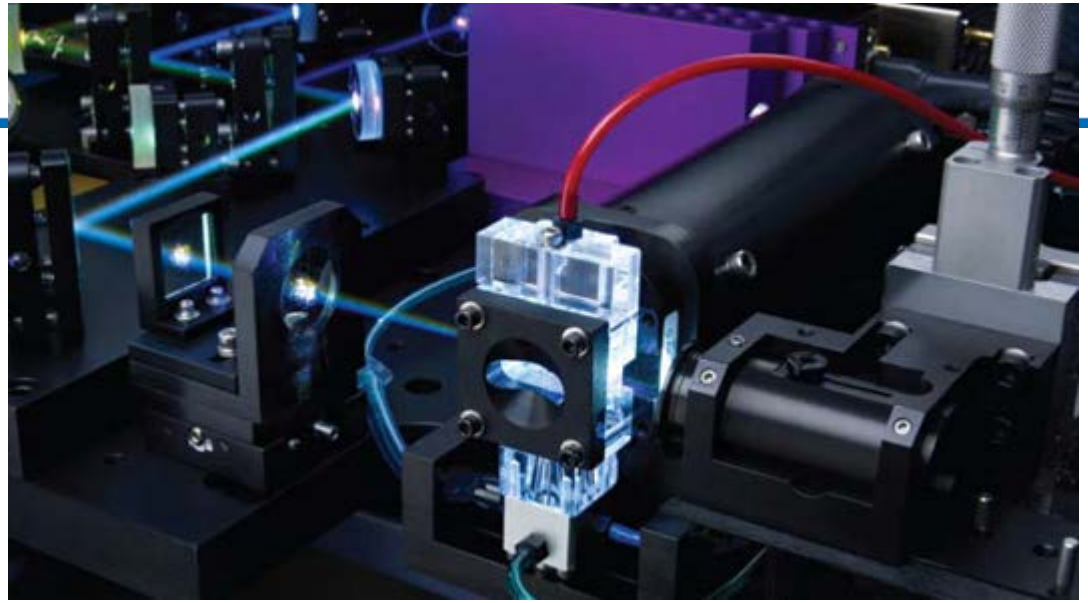
High Throughput Sampler (HTS)
for 96 or 384 well plates

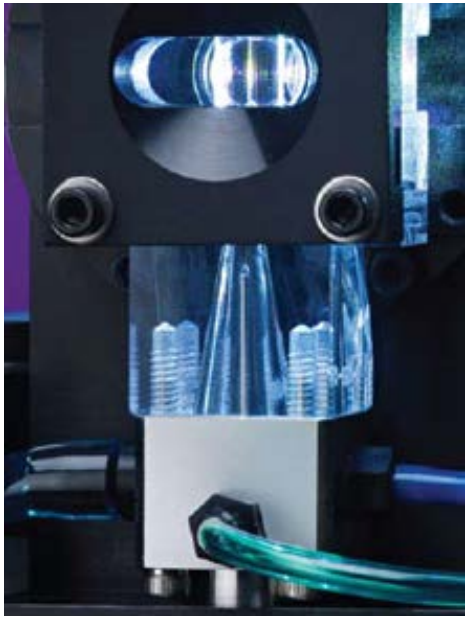


Adapter for tube racks

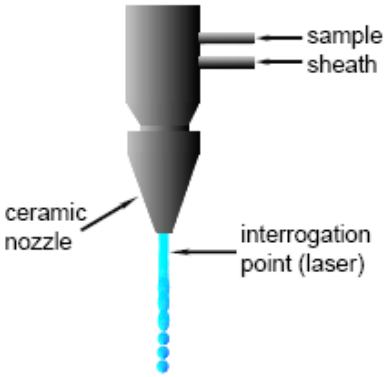
Fluidics: Flow cell



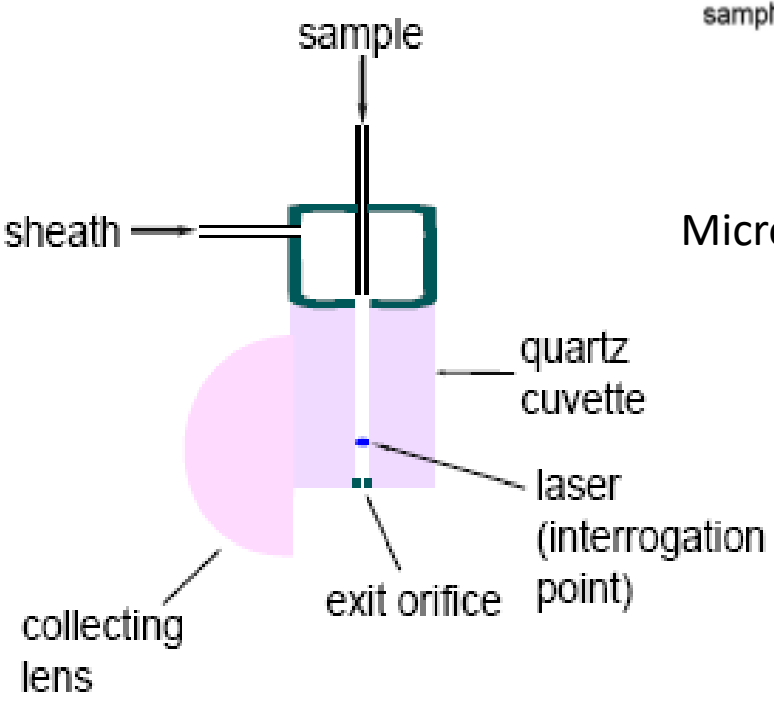




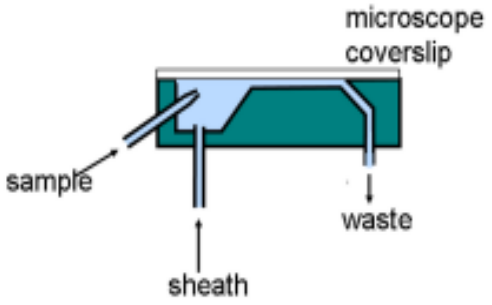
Fluidics: Flow cell types



„Stream-in-air“ system
(Cell sorters only)



Cuvette based system



Microscope based flow chamber

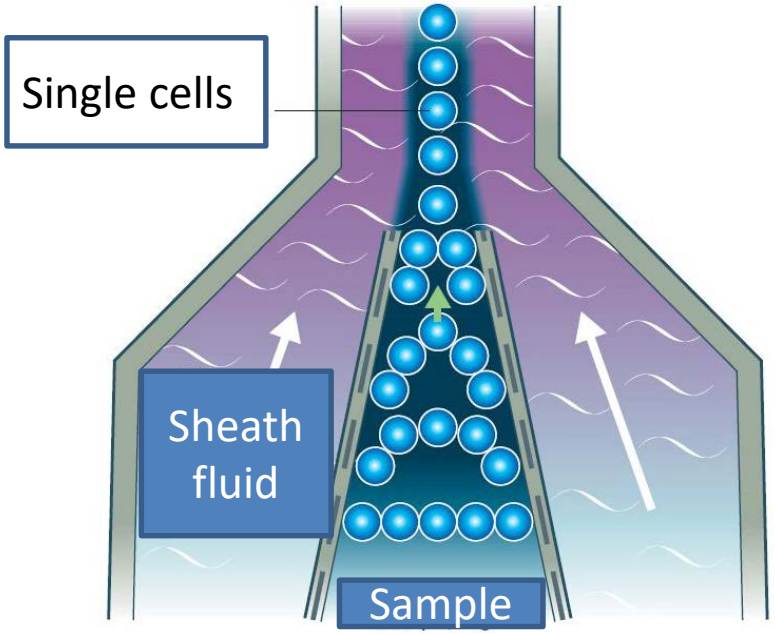
http://flowbook.denovosoftware.com/chapter-2-flow-cytometer

Fluidics: Hydrodynamic focussing

Principle of fluid dynamics

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

Rio Negro & Amazon river Avesci.com



Only one cell or particle can pass through the laser beam at a given moment

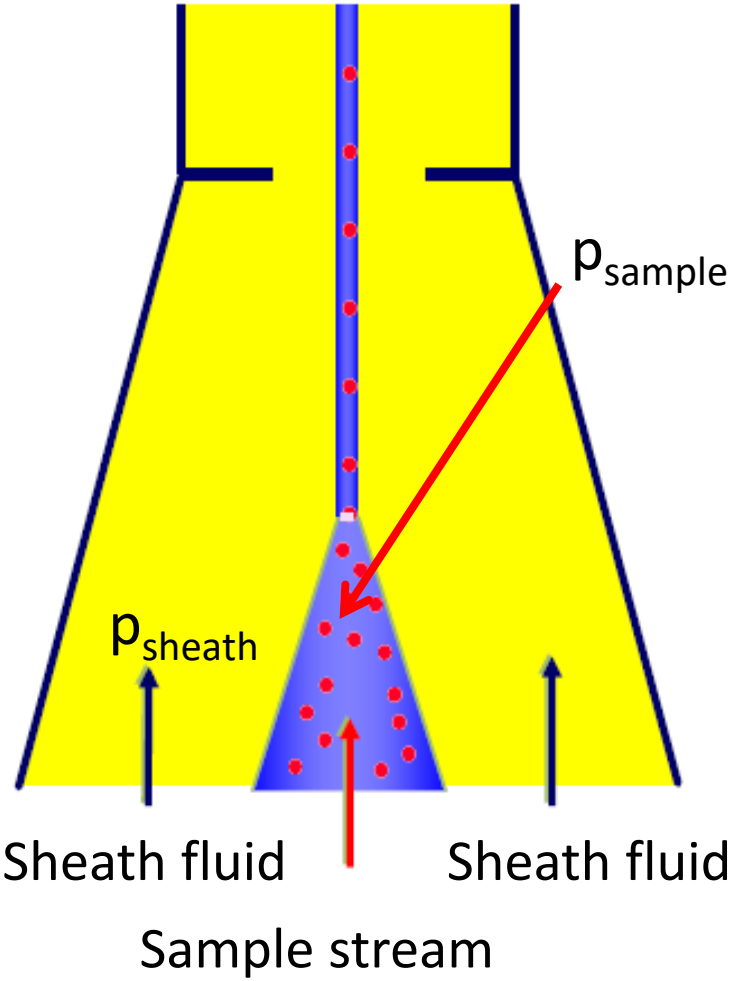
http://www.biologydiscussion.com/biochemistry/flowcytometry/principles-of-flowcytometry-with-diagram-2/12973

Fluidics- Hydrodynamic focussing in flow cell

Laminar flow (stream) by

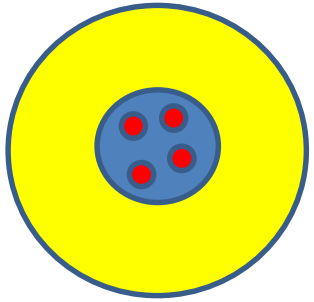
„hydrodynamic focussing“

- Sample is injected into the centre of sheath fluid
- Difference of pressure, $p_{sample} > p_{sheath}$ (different velocities)
- Design of flow cell

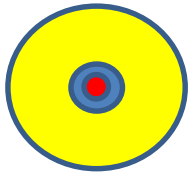


BD Biosciences, modified

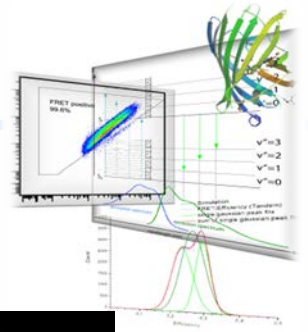
View from above
Core diameter and cells at time of sample injection



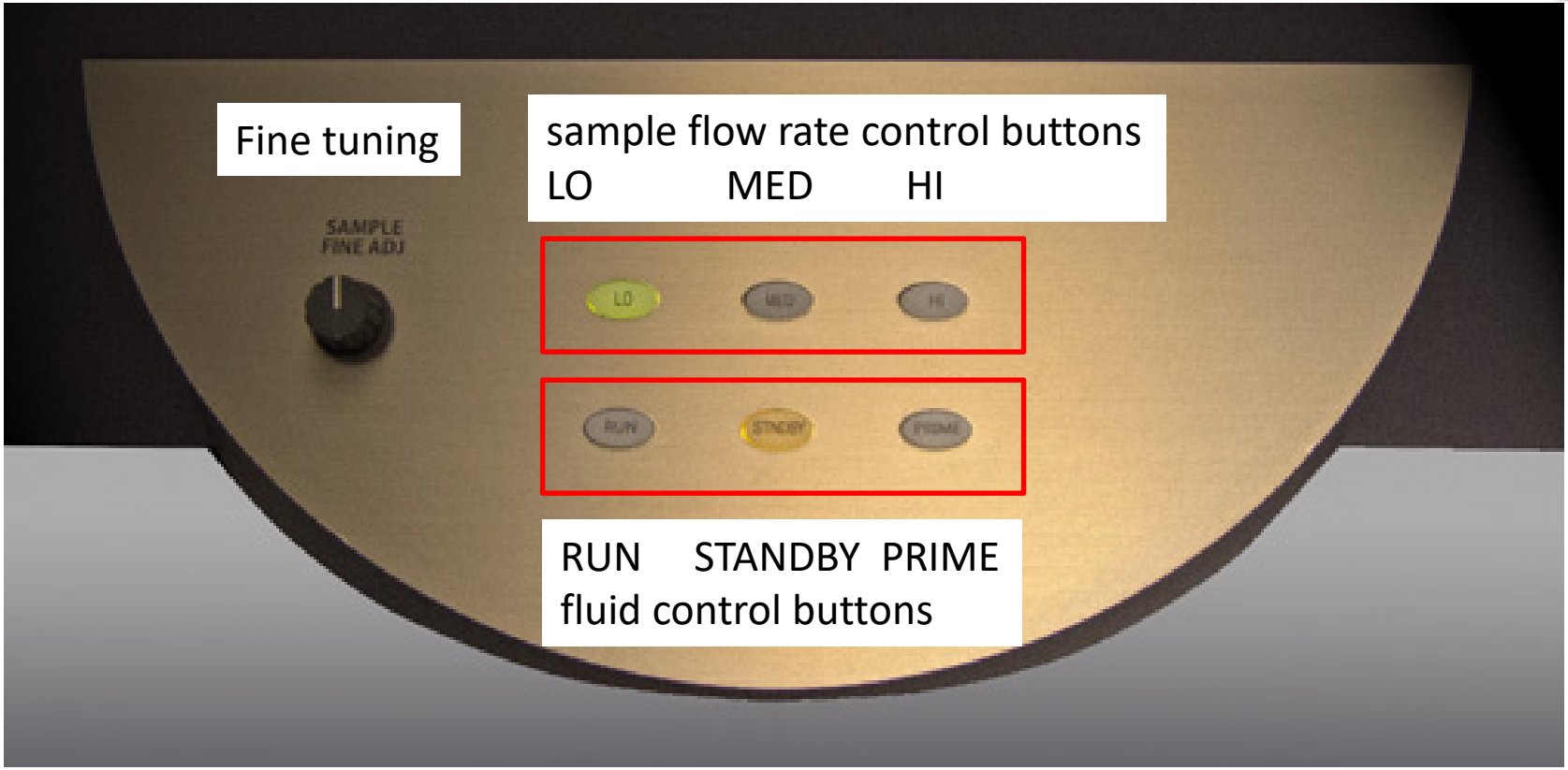
Core diameter and cells at time of measurement



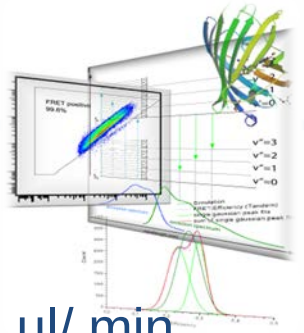
Fluidics – Flow rate



Front panel of the LSRFortessa



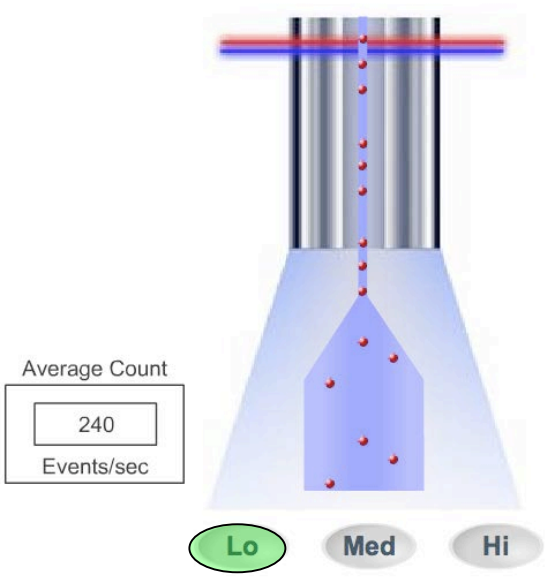
Fluidics – Flow rate

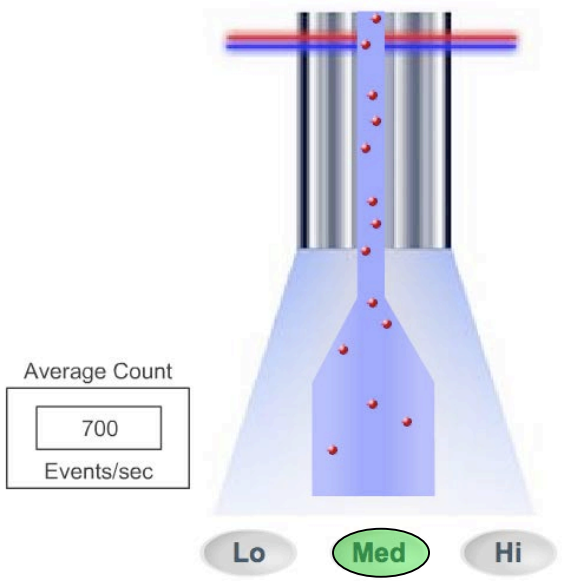


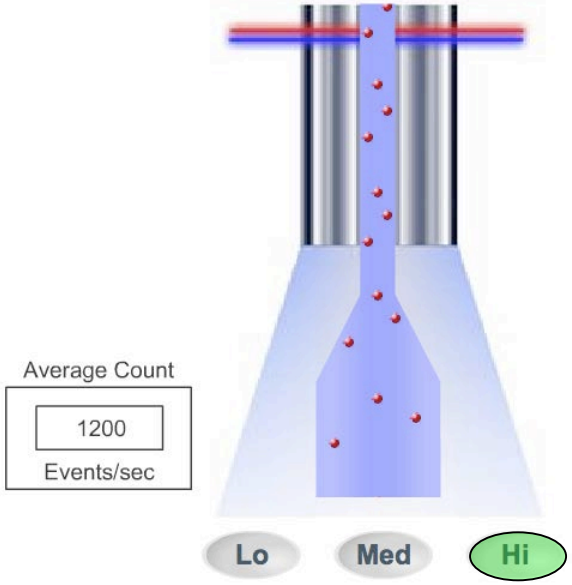
Low: ≈ 12 µl/ min

Medium: ≈ 35 µl/ min

High: ≈ 60 µl/ min





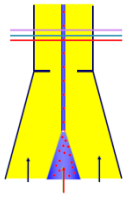


BD Biosciences (modified)

Higher flow rate → higher sample pressure → higher core stream diameter
(lower signal resolution)

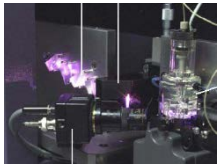
Flow Cytometry Core Facility
Dr. Stefanie Bürger
s.buerger@imb-mainz.de

Optics

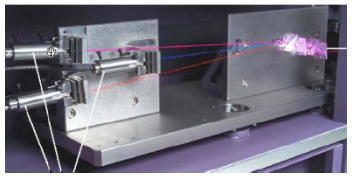


fluidics

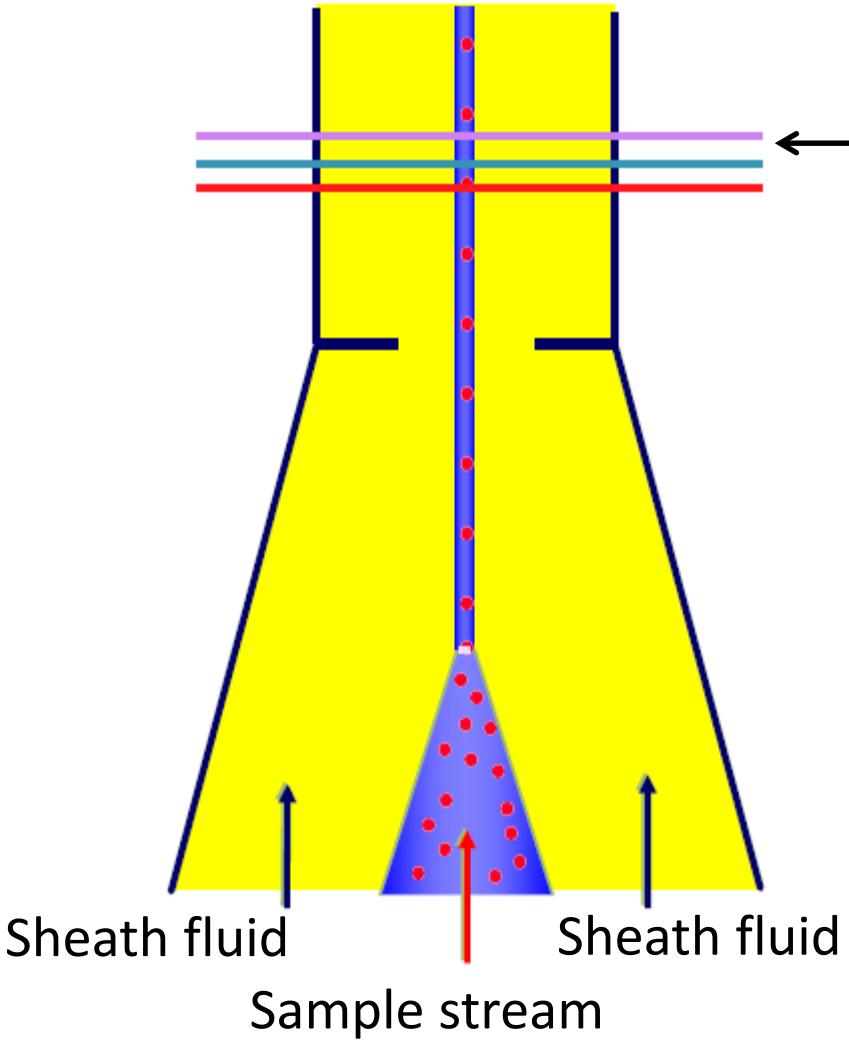
Flow Cytometer



optics



Optics - Excitation



- Excitation by multiple lasers when cells are aligned in the liquid stream and pass one by one through the laser beam
  → **Interrogation point**
- @IMB: 355nm (UV), 405nm, 488nm, 561nm, 640nm
- Horizontally aligned with separate pinholes for the lasers

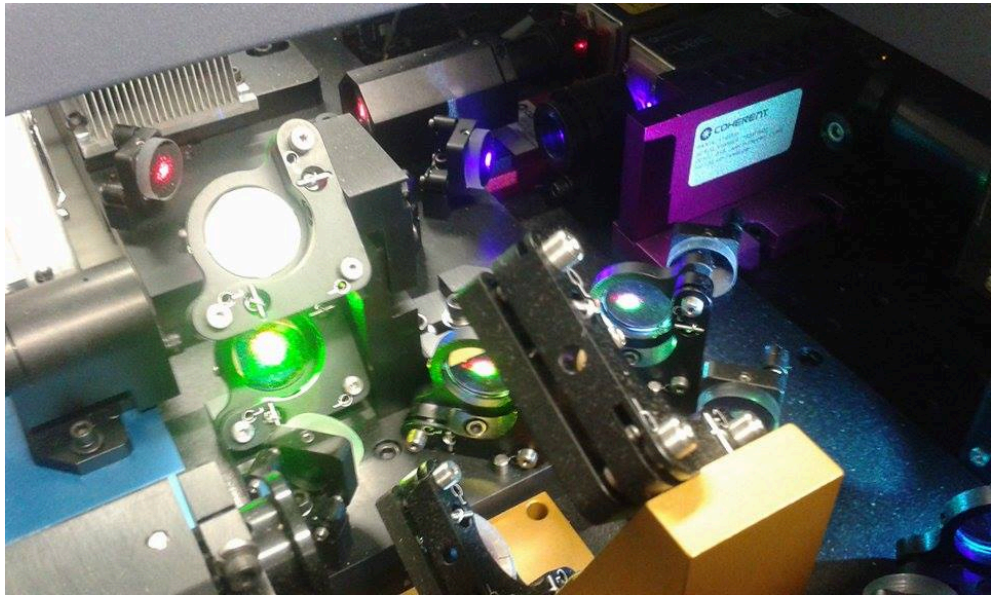
BD Biosciences, modified

Excitation optics

BD FACSAria Operator Course Manual

BD FACSAria

- Laser light comes from fiberoptic cables
- Steering optics with prisms and lenses direct the laser light to the cuvette

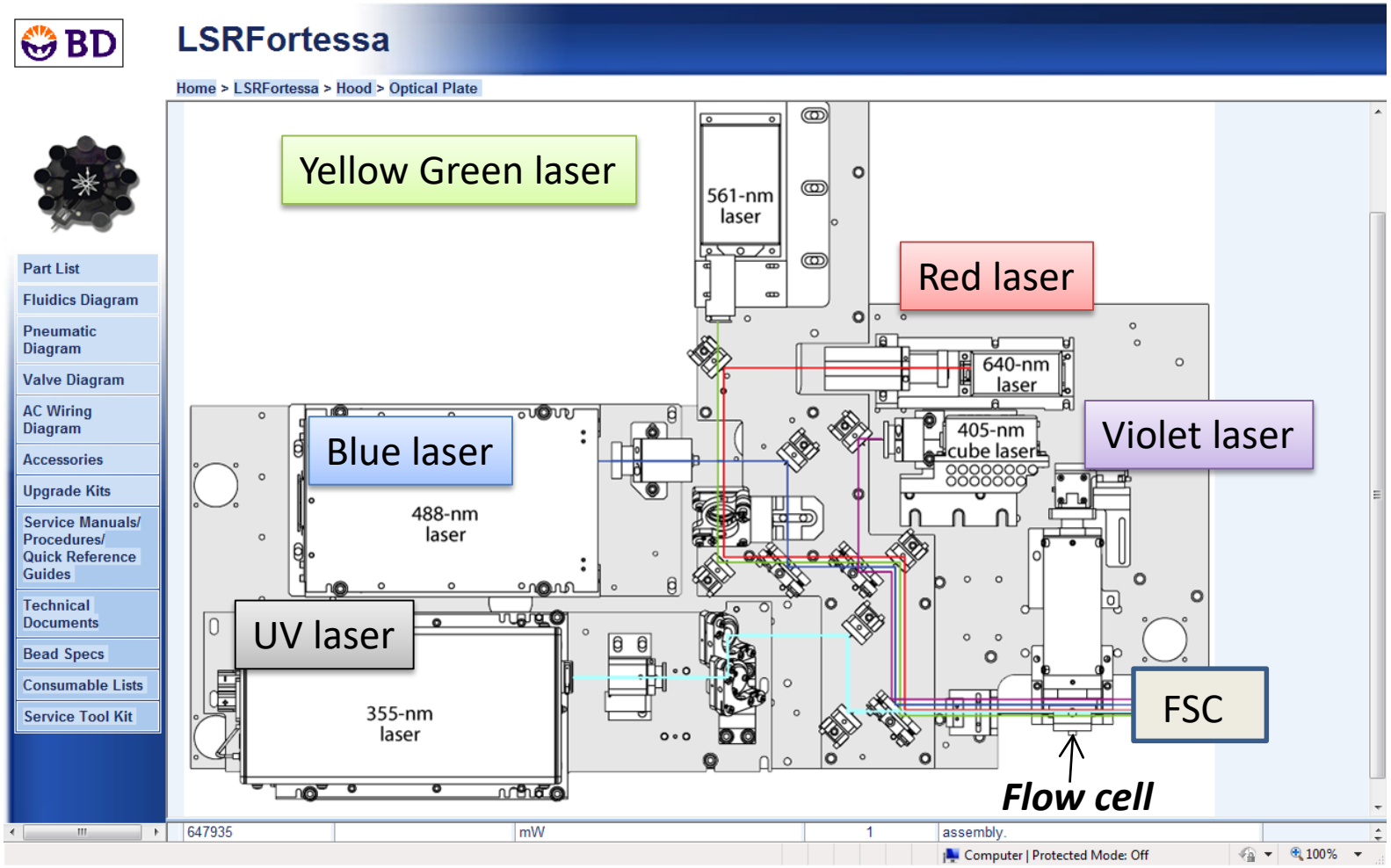


BD LSR Fortessa

- Laser light is directed via mirrors and focussing lenses towards the cuvette

Optics - Excitation

- LASER array of the analyzer LSRFortessa

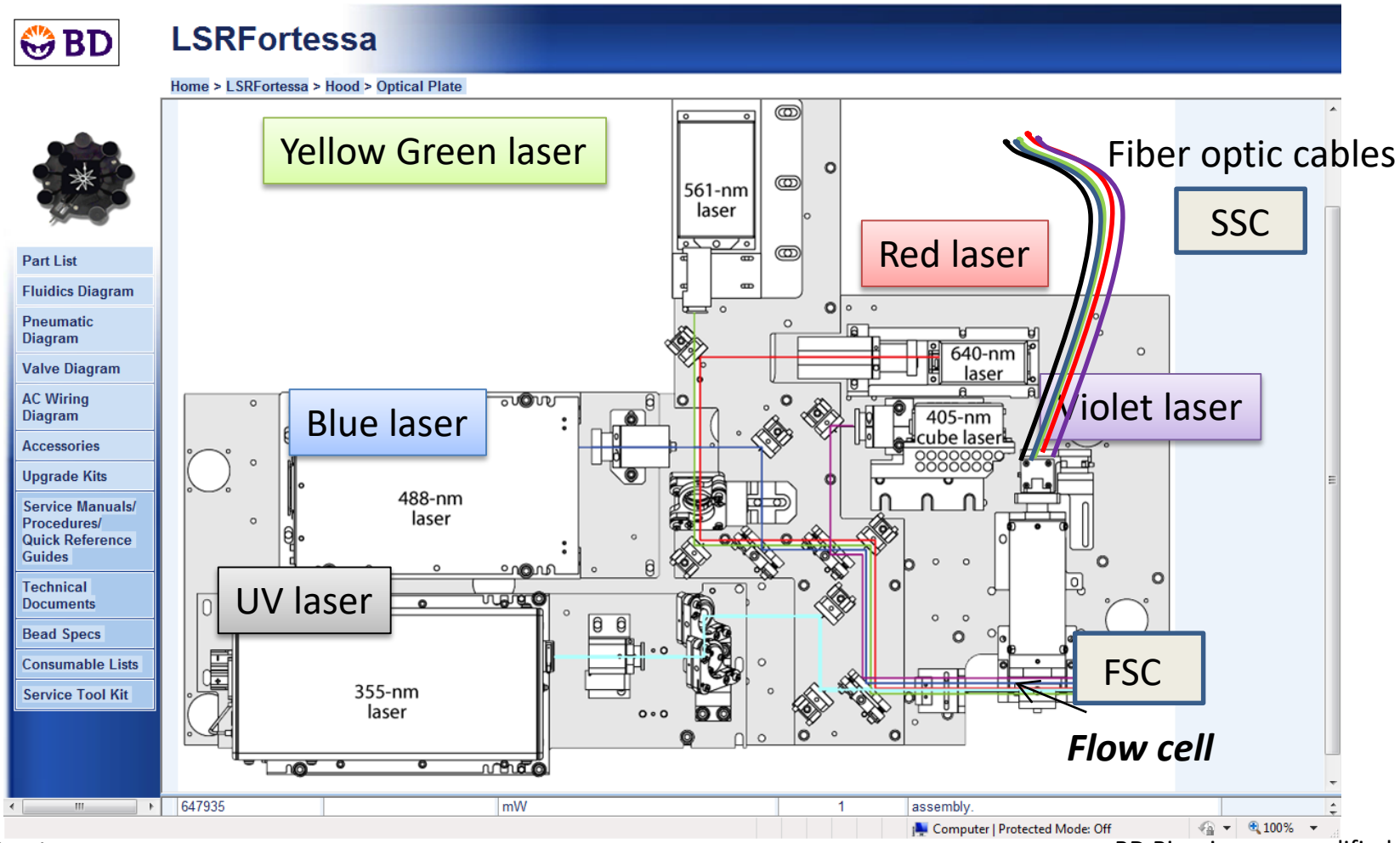


BD Biosciences

BD Biosciences, modified

Optics – Excitation and collection of emitted light

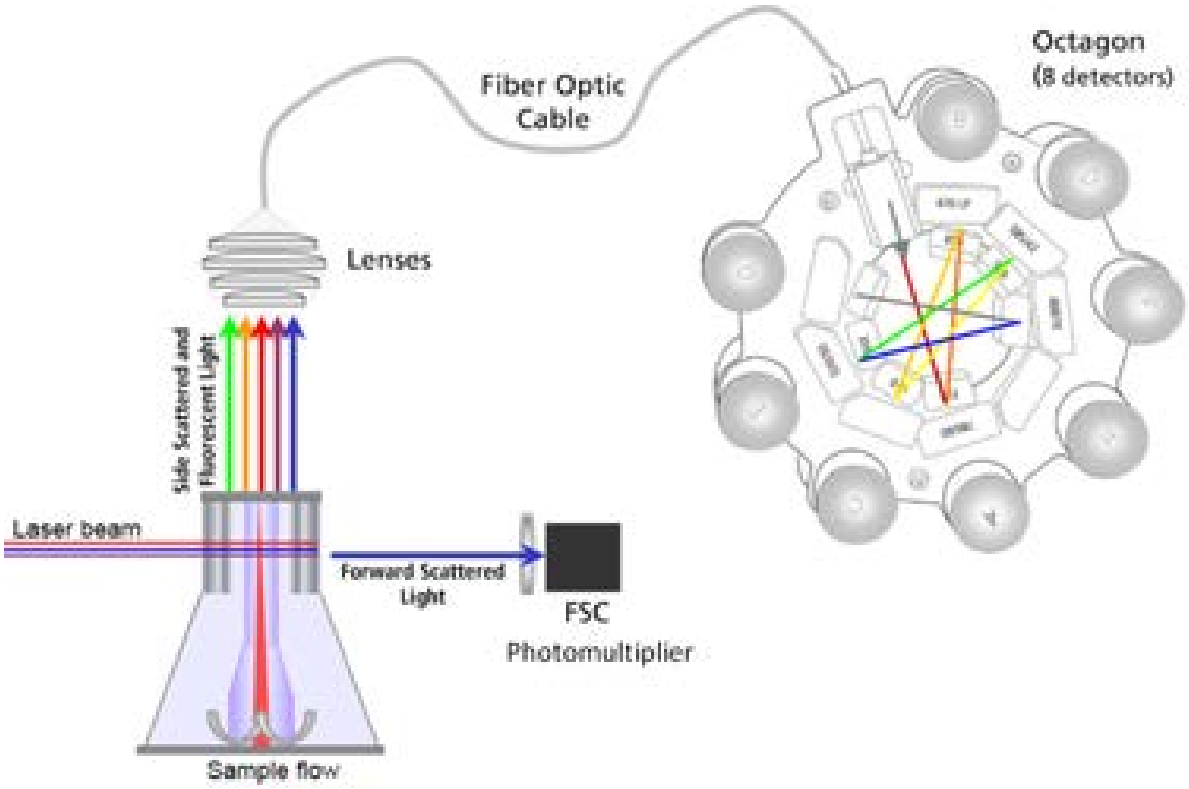
- LASER array of the analyzer LSRFortessa



BD Biosciences

BD Biosciences, modified

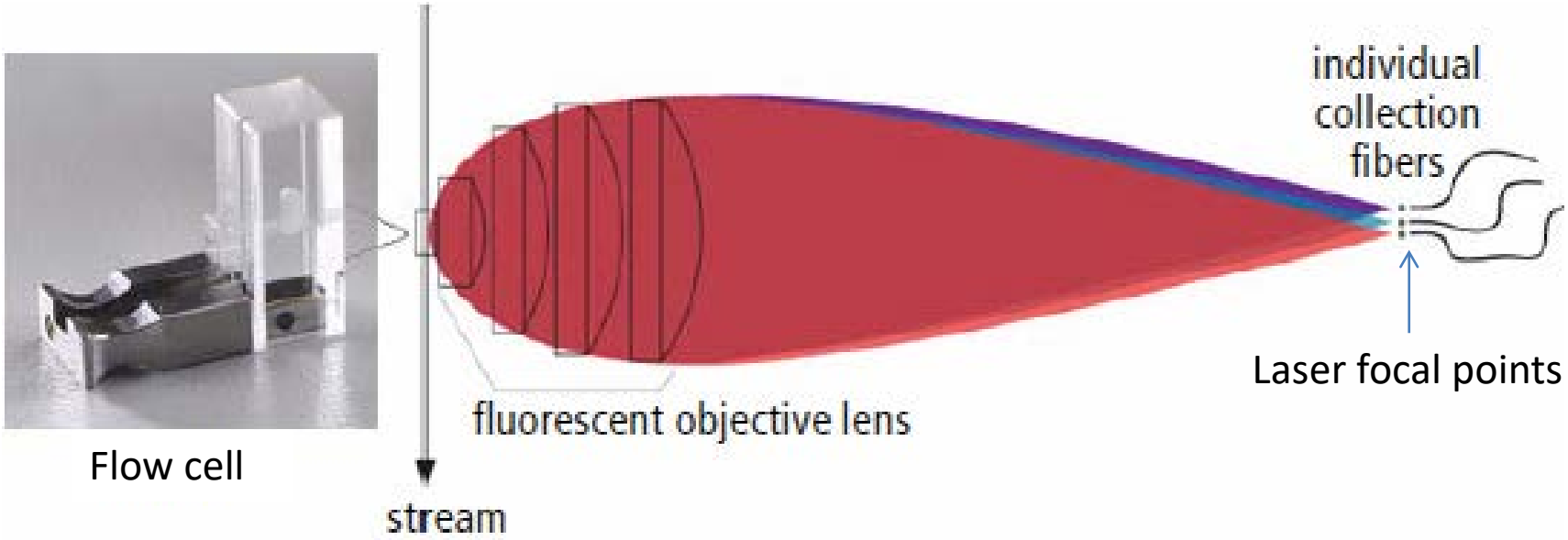
Optics - Excitation and detection of emitted light



Lenses collect and focus fluorescent light into individual collection fibres which transfer the emitted light to detector arrays.

https://www.google.de/search?q=LSRFortessa+Flow+cuvette&client=firefox-b&source=lnms&tbm=isch&sa=X&ved=0ahUKEwiRi-b88enTAhVJIcAKHdqcApsQ_AUICigB&biw=1920&bih=1089#imgrc=FNP6rEj3zy9opM:

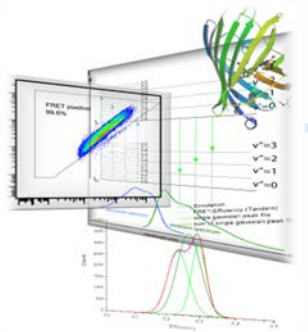
Optics - Emission



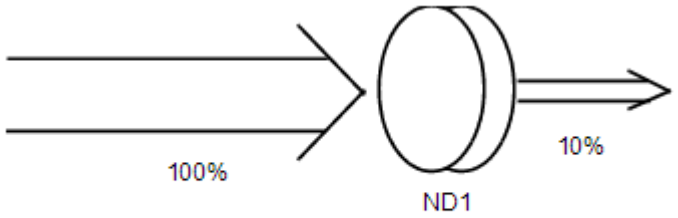
Lenses collect and focus fluorescent light into individual collection fibres which transfer the emitted light to the optic photomultiplier tubes (PMTs).

BD Biosciences

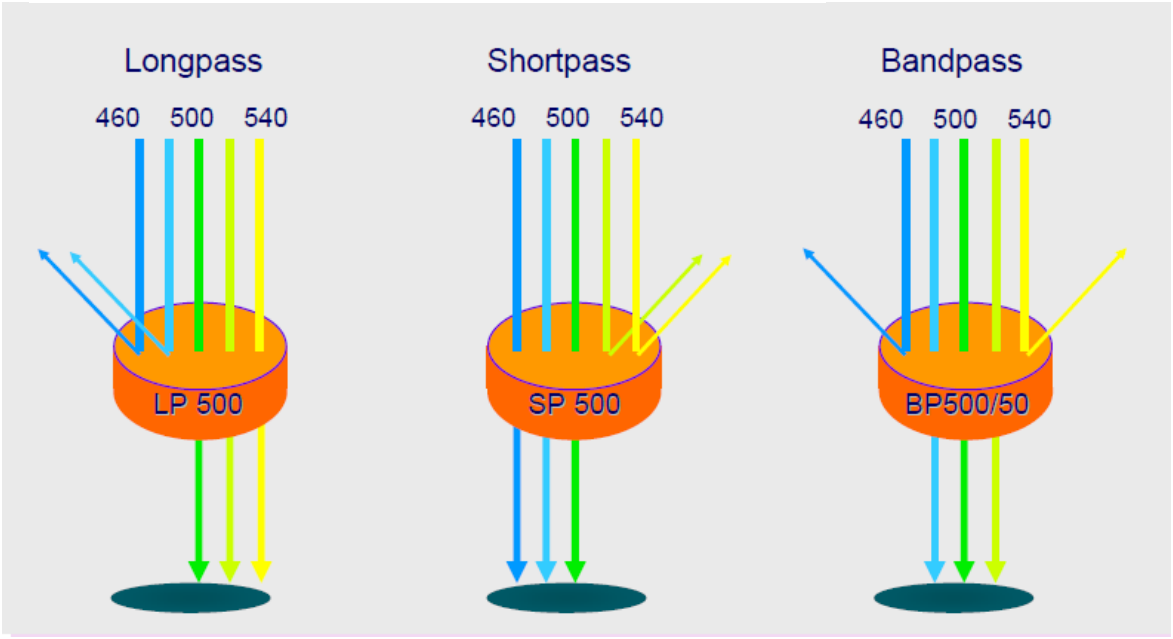
Optics – Filters



Neutral density filter (ND) – in front of FSC detector



10 % of light transmitted
FSC is a very strong signal!



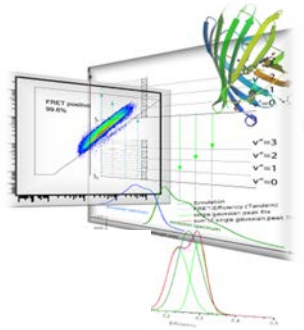
BP500/50 = BP500+/-25nm

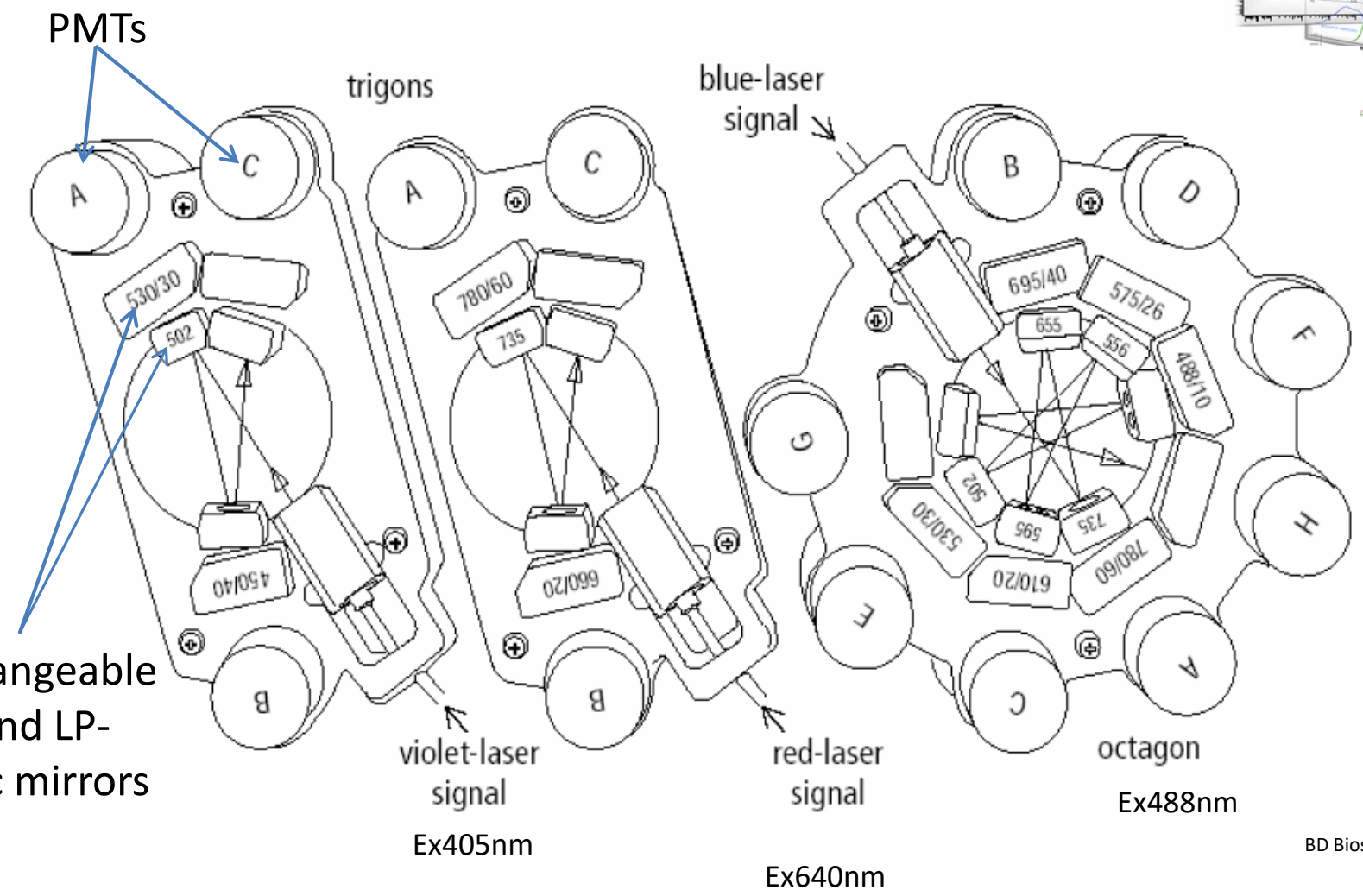
LP and SP filters are normally coated with a reflective surface, so unwanted light is not only blocked, but reflected in a certain angle

→ Dichroic mirror

https://www.bdbiosciences.com/documents/BD_FACSAria_III_User_Guide.pdf

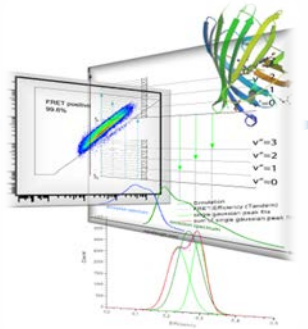
Optics – Detector arrays: trigons and octagons





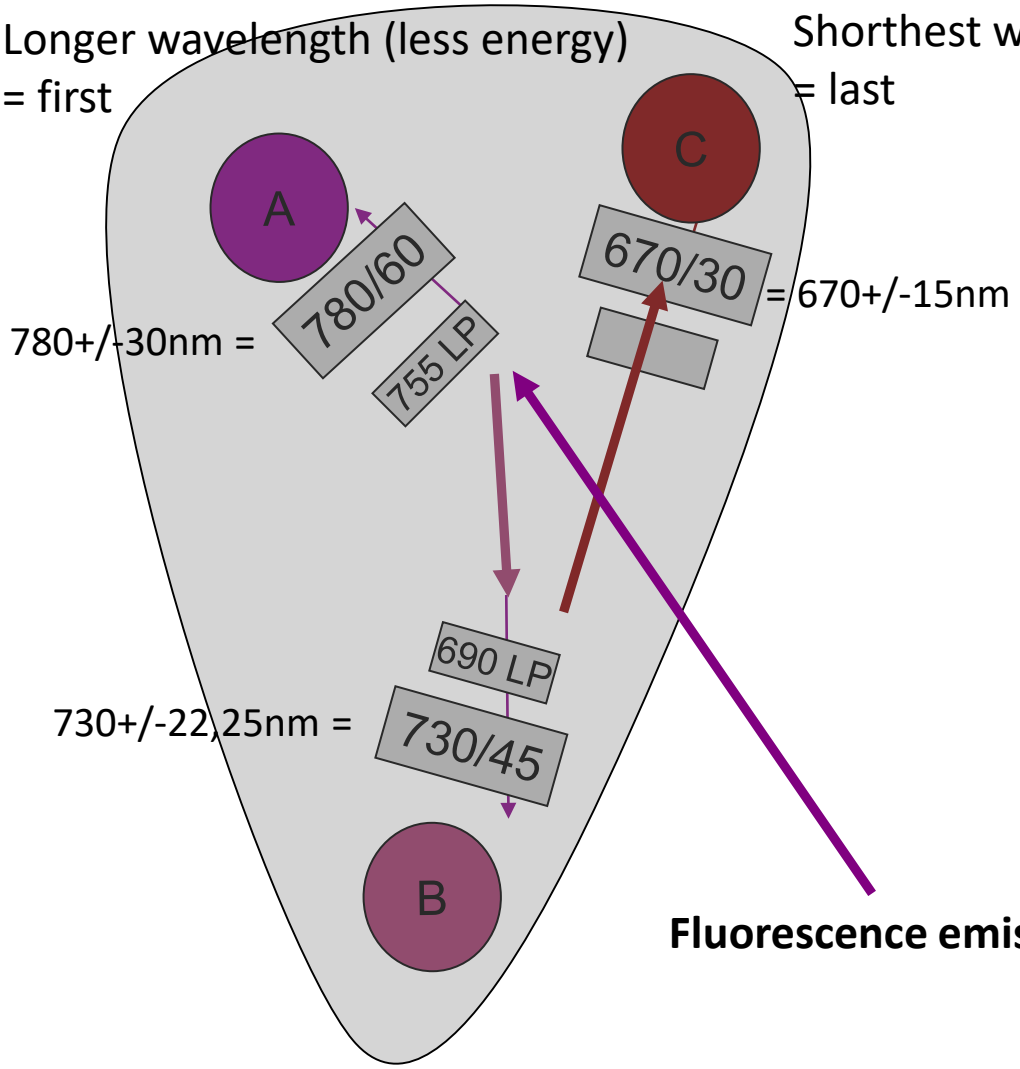
BD Biosciences

Optics – Trigon technology from BD



Longer wavelength (less energy) = first

Shorthest wavelength (high energy) = last

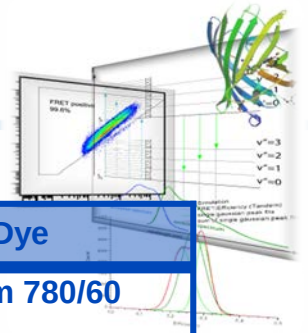


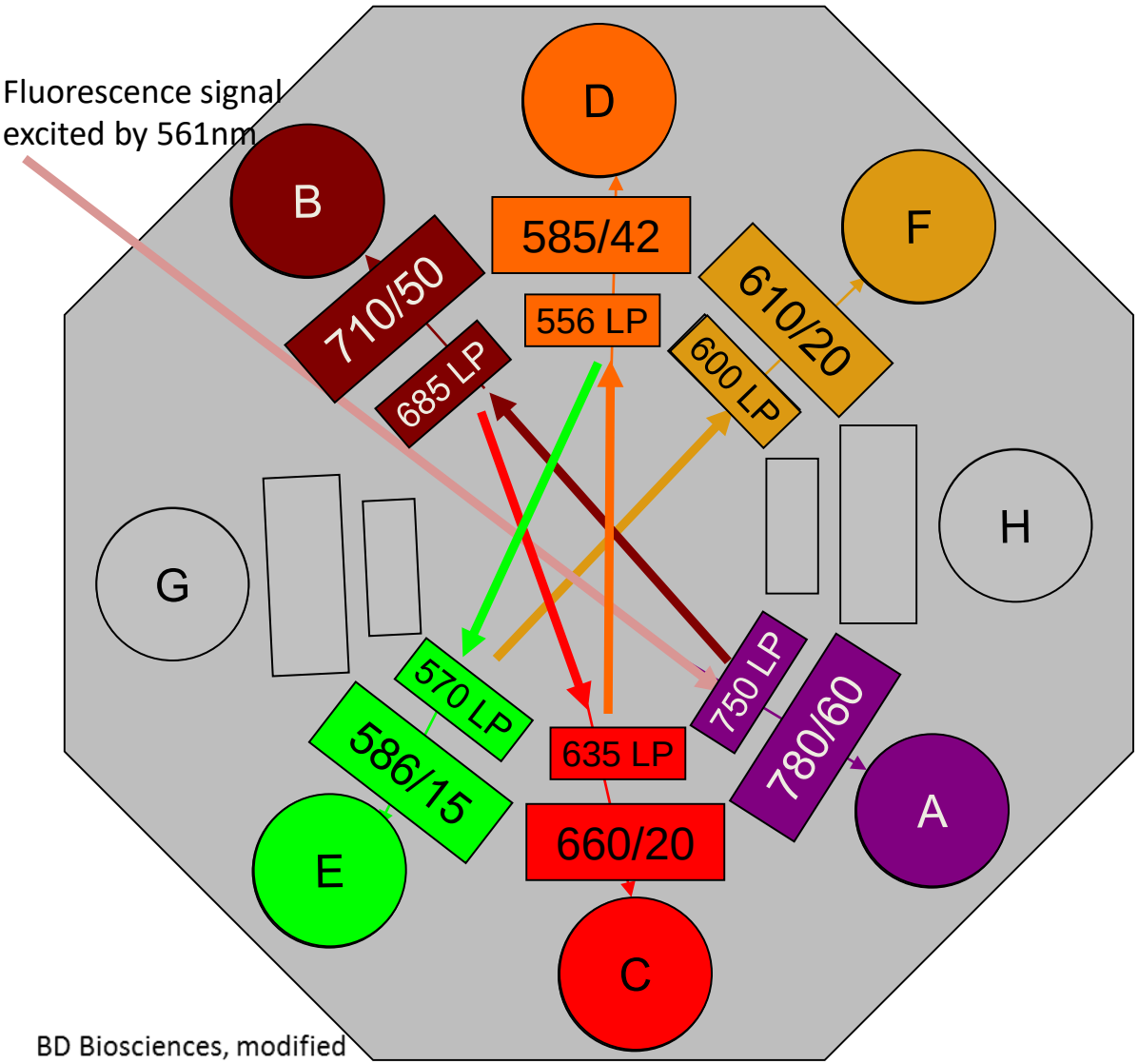
Detector	Dye
A	**RL640nm 780/60** APC-Cy7
B	**RL640nm 730/45** Alexa 700
C	**RL640nm 670/30** APC Alexa 647 Cy5 TO-PRO 3 TOTO 3

Fluorescence emission of dyes excited by 640nm

BD Biosciences, modified

Optics – Octagon technology from BD

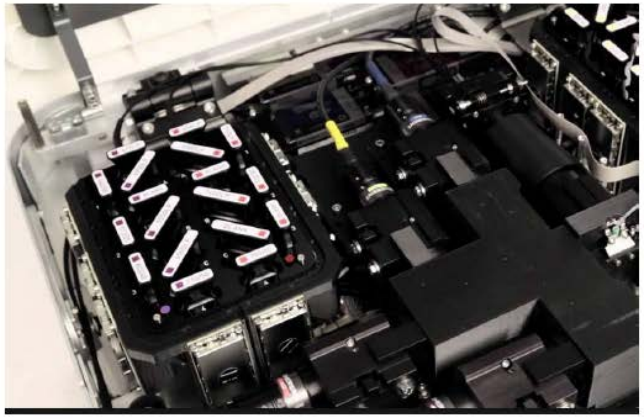




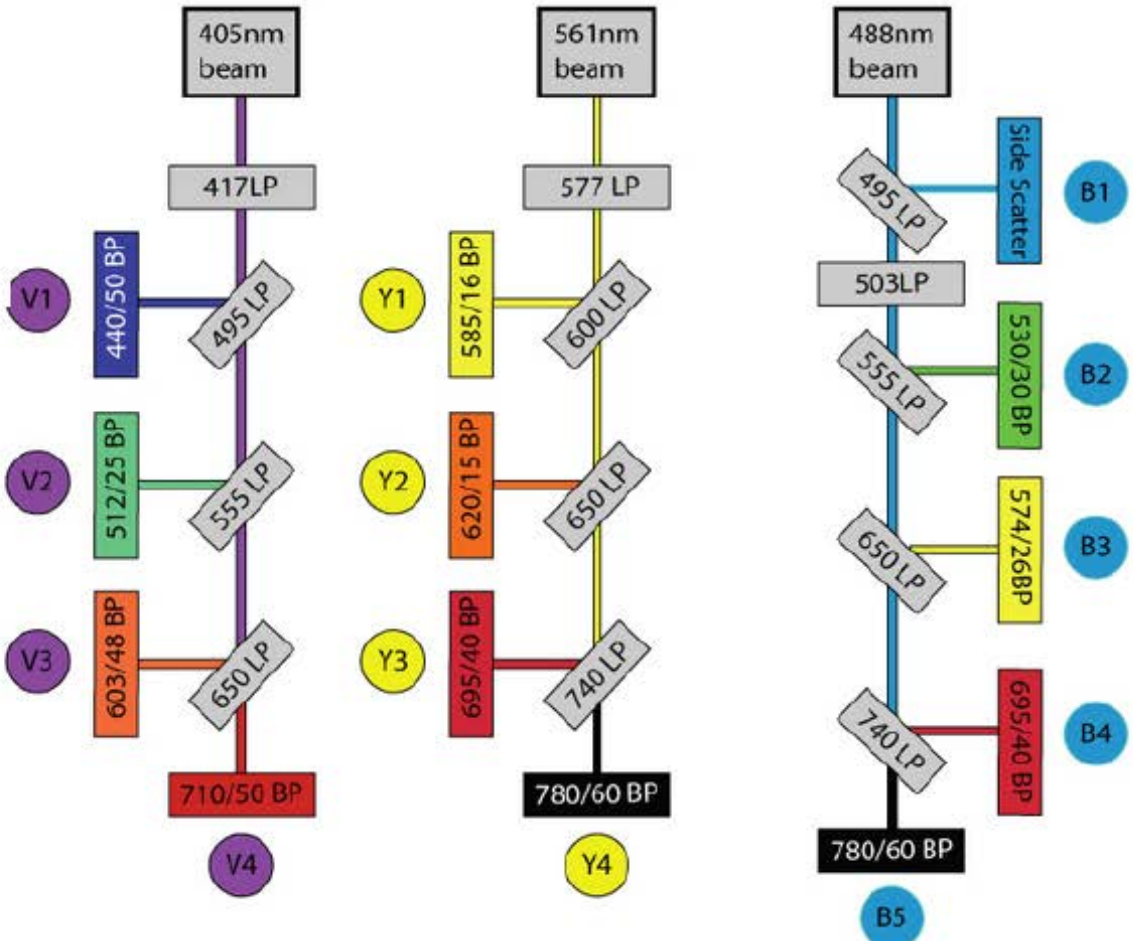
BD Biosciences, modified

Detector	Dye
A	**YG561nm 780/60** PE-Cy7 PE-AlexaFluor750
B	**YG561nm 710/50** AlexaFluor700 PE-Cy5.5
C	**YG561nm 660/20** PE-Cy5 mPlum
D	**YG561nm 610/20** PI mCherry
E	**YG561nm 586/15** PE Cy3 DsRed RFP tdTomato

Optics – Detector Array from Attune Nxt (Thermo Fisher)

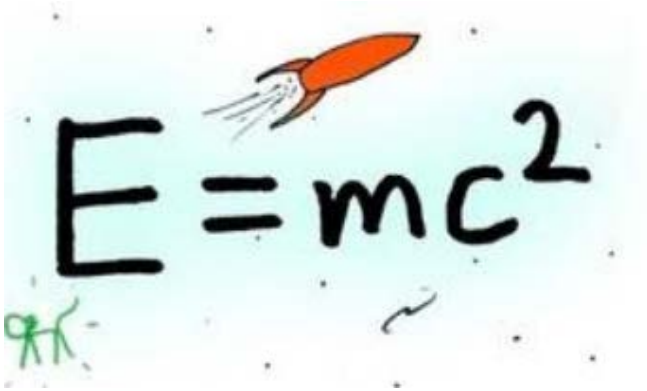


Thermo Fisher Scientific



Optics – Signal detectors

- :



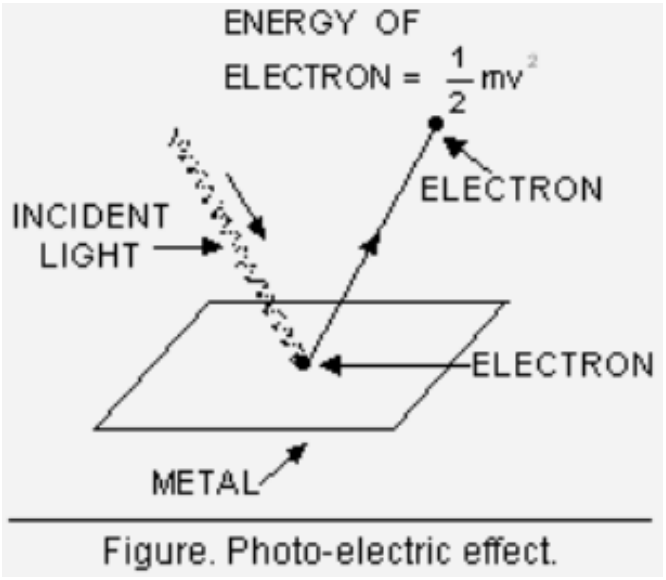


Figure. Photo-electric effect.

Photodiodes

- Less sensitive to light signal than the PMTs
- Is used to detect the stronger **FSC signal**
- **No amplification inside the photodiodes**

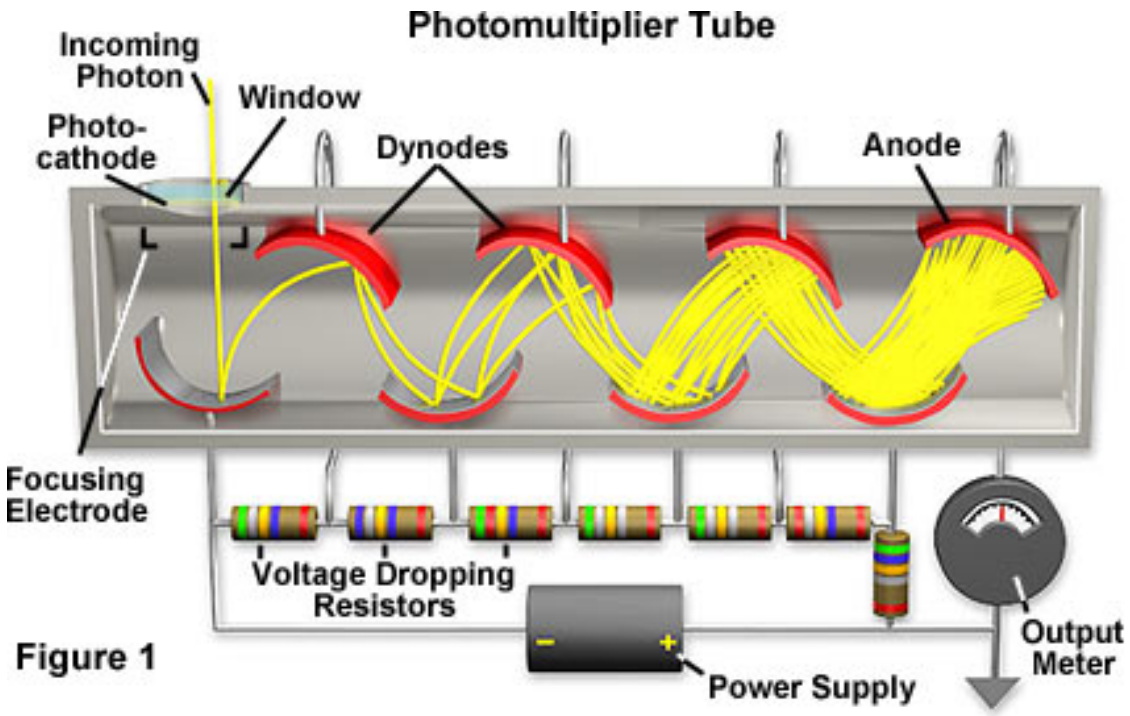
Photomultiplier tubes (PMTs)

- Detection of weaker signals by **Side Scatte**r and **all fluorescence channels**
- Signal amplification inside the PMT via Dynodes
- Applying voltage to the PMT is changing sensitivity

https://www.google.de/search?q=photons+into+electrons&source=lnms&tbm=isch&sa=X&ved=0ahUKEwj3rPPj7pHUAhWCNxQKHRamBxkQ_AUIBigB&biw=992&bih=531#tbm=isch&q=photoelectric+effect+comic&imgrc=1ElZauVyWxcyRM

Optics - Photomultiplier tube (PMT)

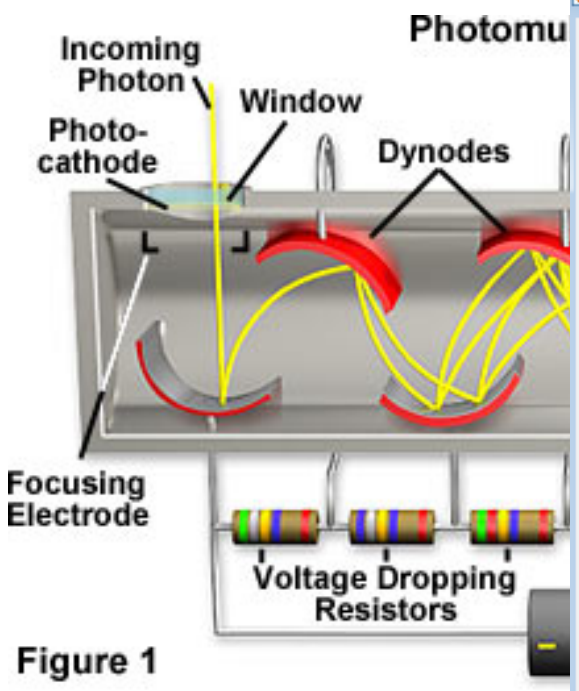
www.olympusmicro.com

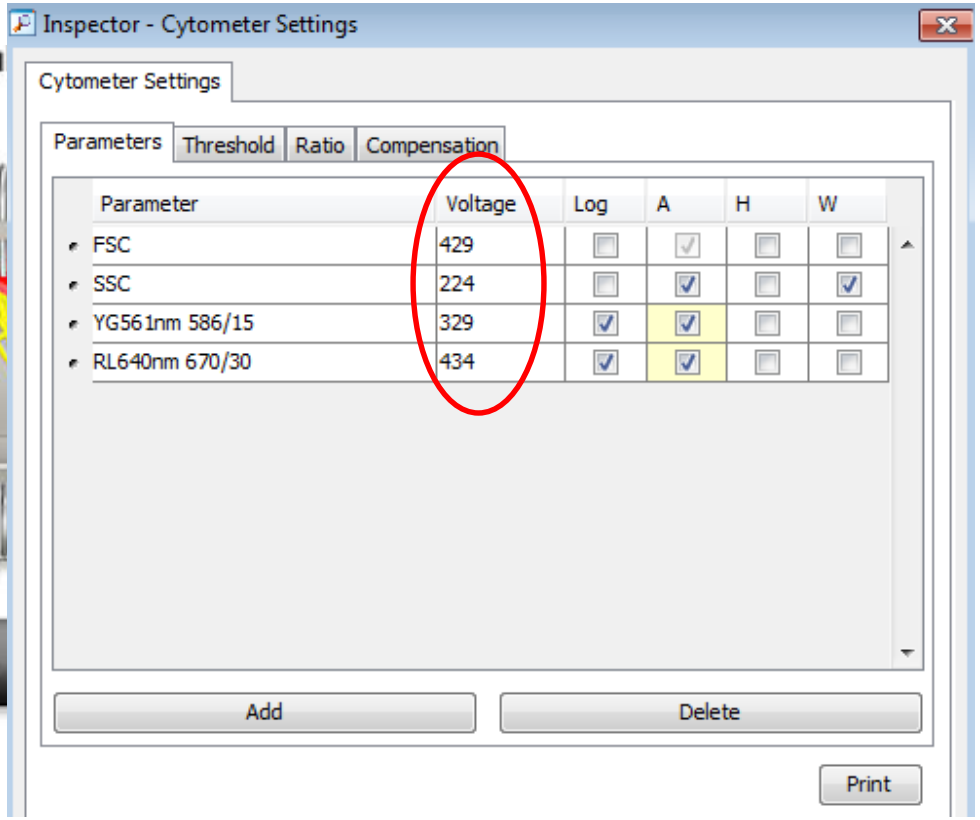


- Amplification: up to 10^8 (voltage dependent)
- Supply voltage: up to 1000 V
- PMT provides current output proportional to light intensity
- Bright dyes have an intrinsically high photon to electron conversion rate

Optics - Photomultiplier tube (PMT)

www.olympusmicro.com

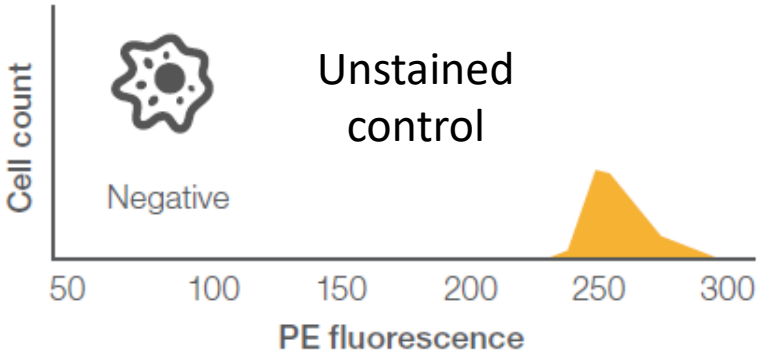


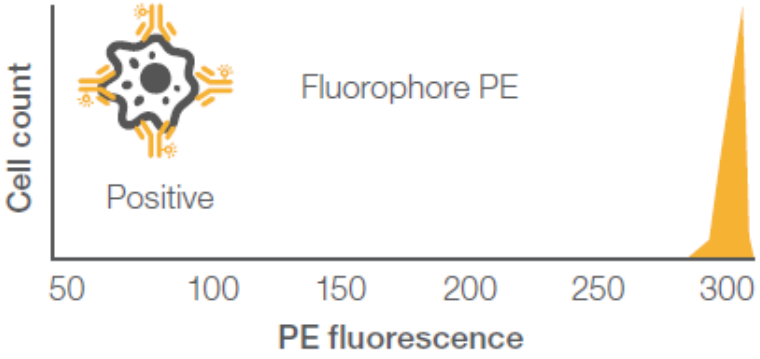


- Amplification: up to 10^8 (voltage dependent)
- Supply voltage: up to 1000 V
- PMT provides current output proportional to light intensity
- Bright dyes have an intrinsically high photon to electron conversion rate

Optics – Examples of PMT settings

PMT voltage too high: Positive signal off scale

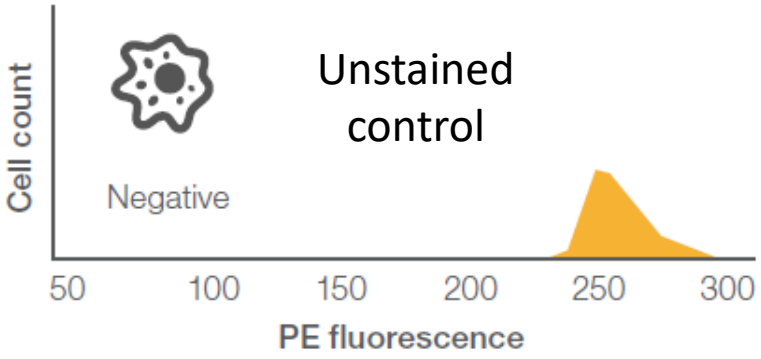


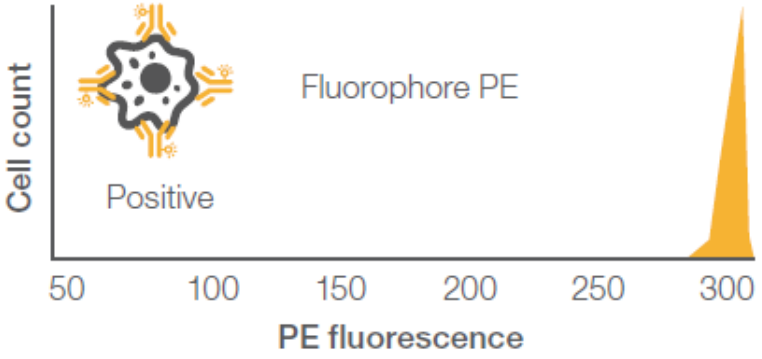


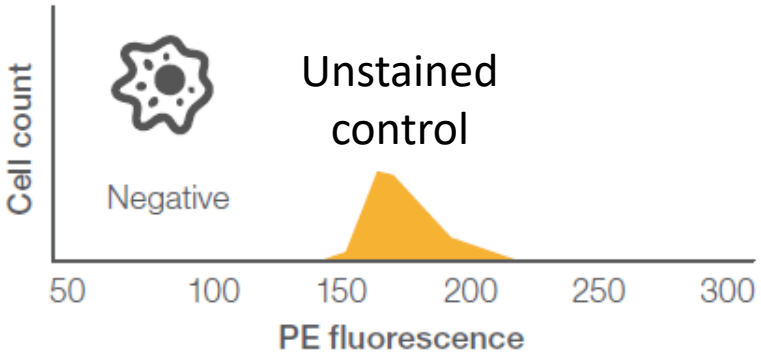
Thermo Fisher Scientific: Flow Cytometer Evaluation Guide

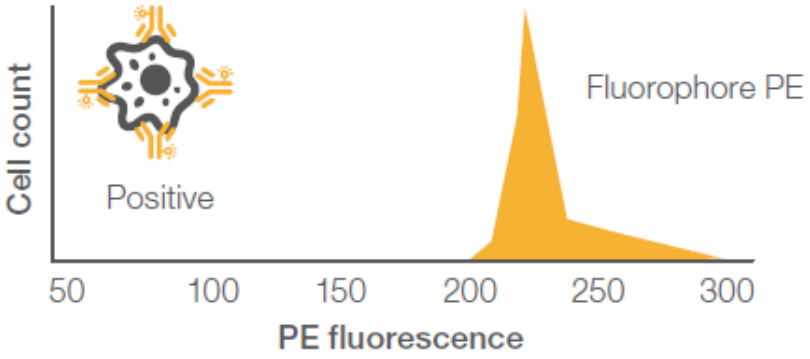
Optics – Examples of PMT settings

PMT voltage too high: Positive signal off scale





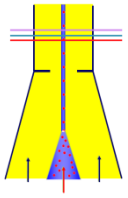




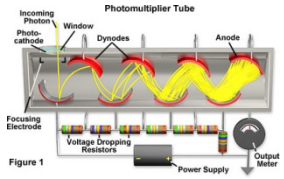
PMT voltage lowered: Positive signal fully resolved

Thermo Fisher Scientific: Flow Cytometer Evaluation Guide

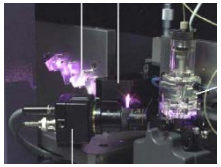
Electronics

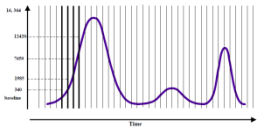


fluidics

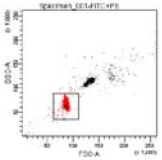


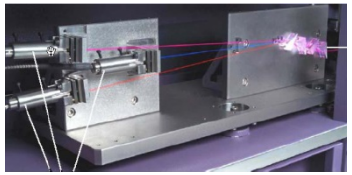
Flow Cytometer





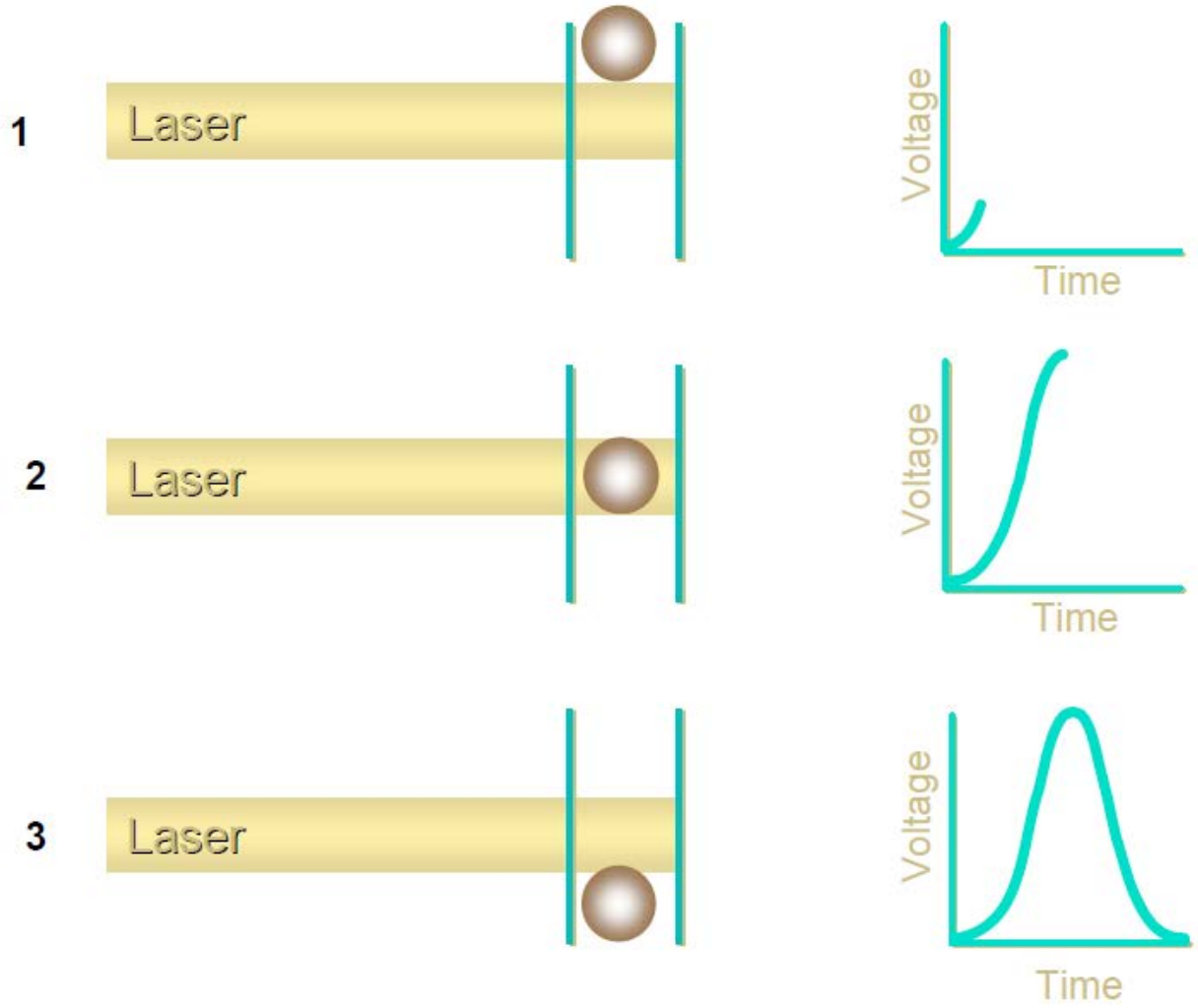
electronics





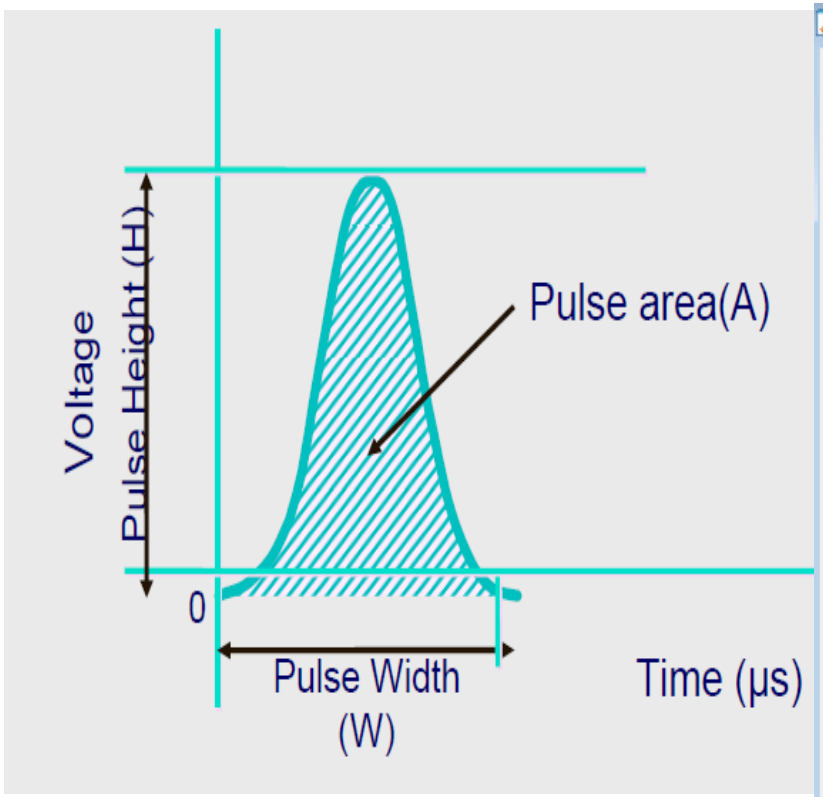
optics

Electronics – Generation of a signal (voltage pulse)

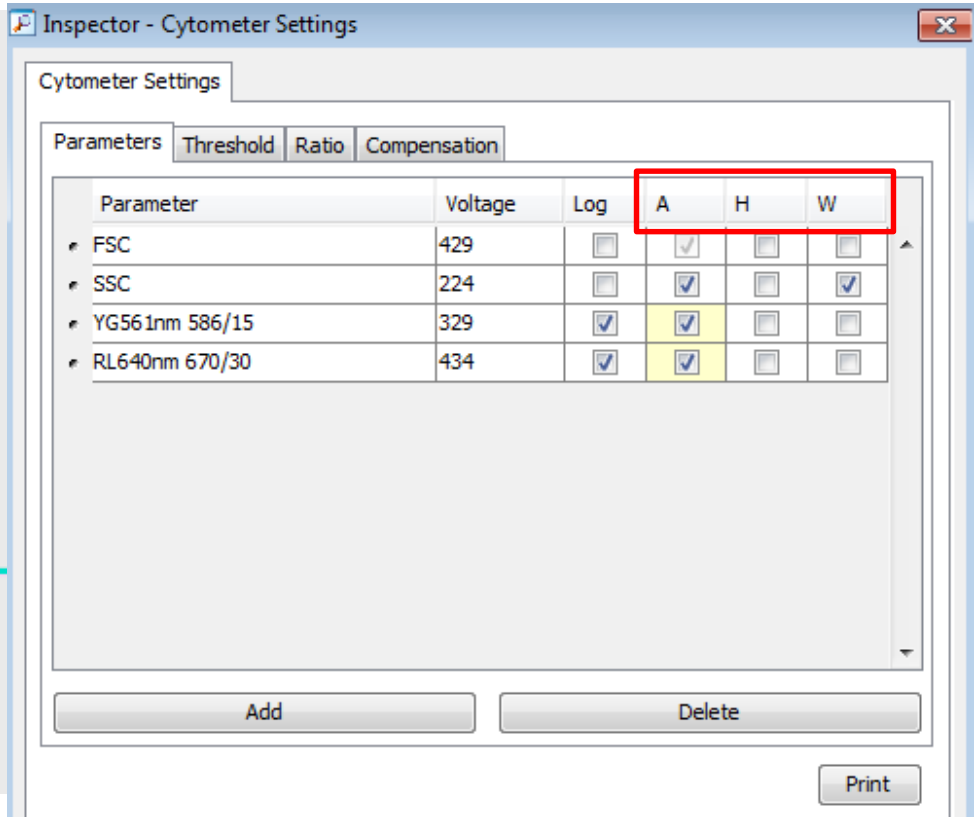


BD Biosciences

Electronics – Generation of a signal -single cells-



BD Biosciences

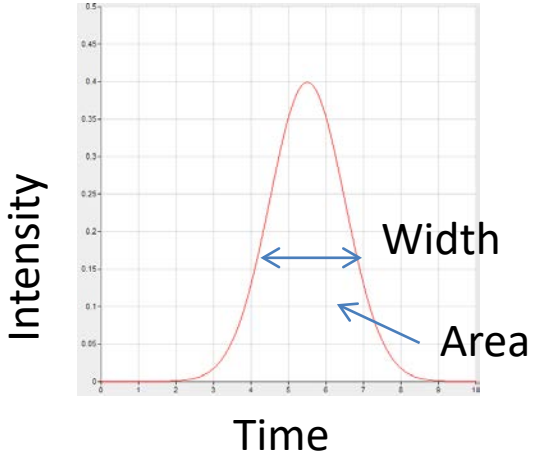


Example:
FSC-H, FSC-W, FSC-A

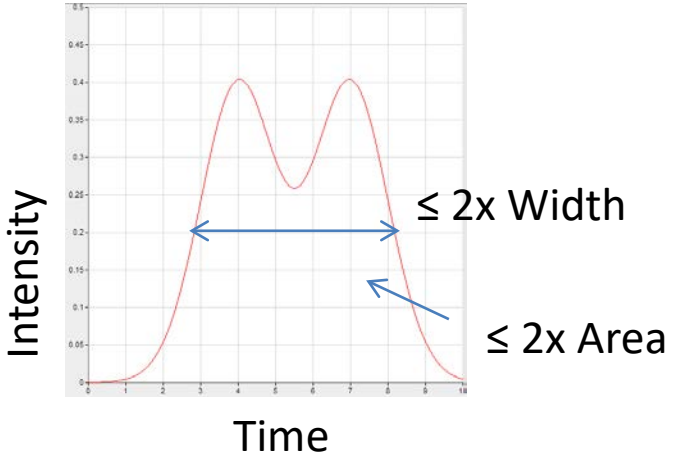
Electronics – Doublet discrimination

Exclusion of aggregates to avoid false (double-) positive cells

Single cell

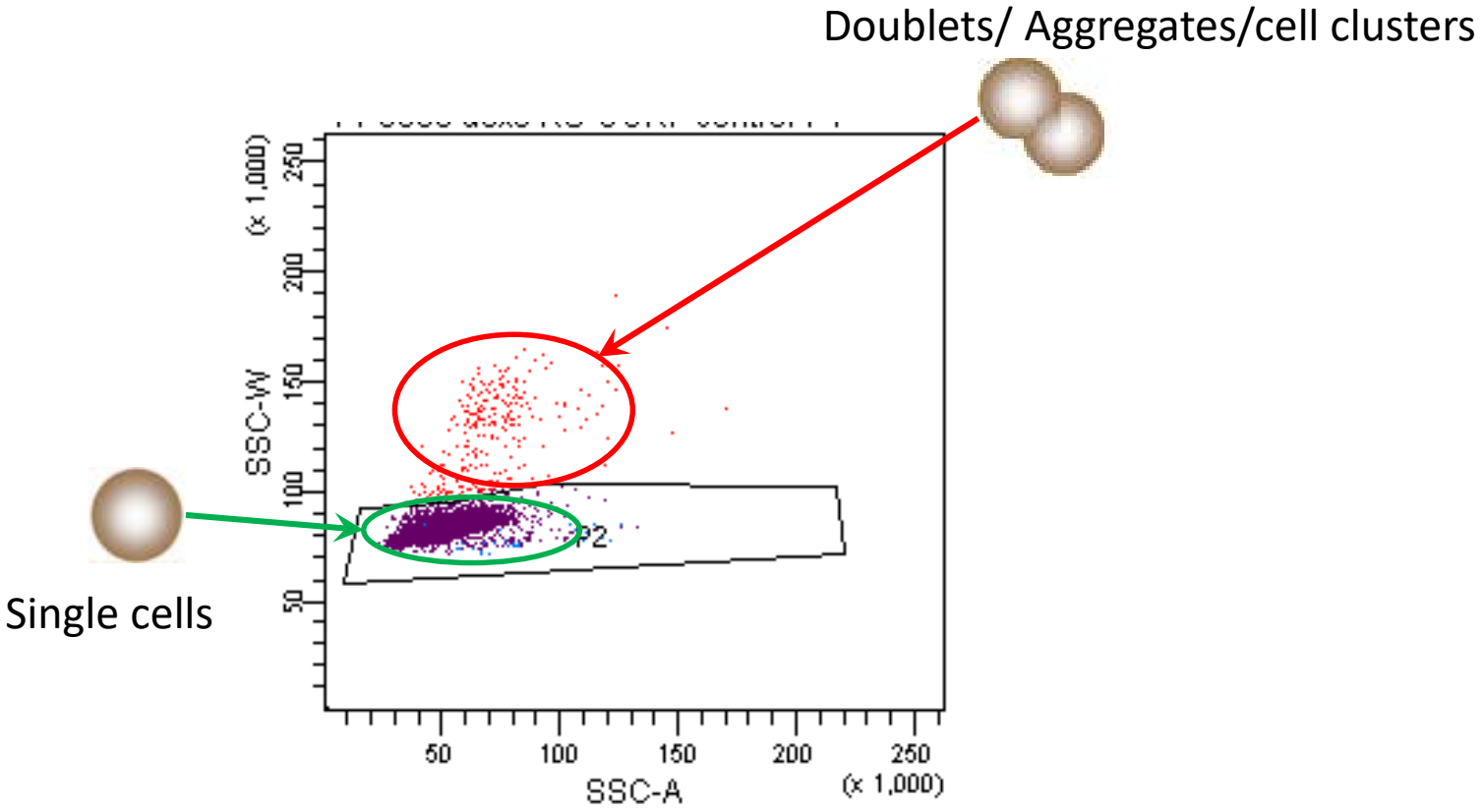


Doublets/Aggregate/cell cluster



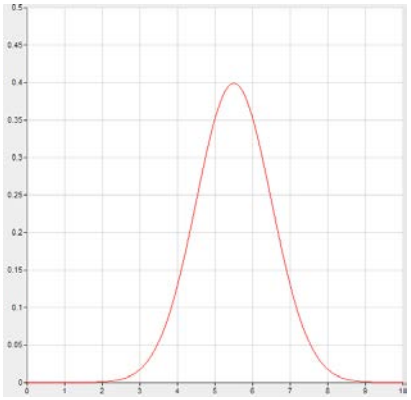
Electronics – Doublet discrimination

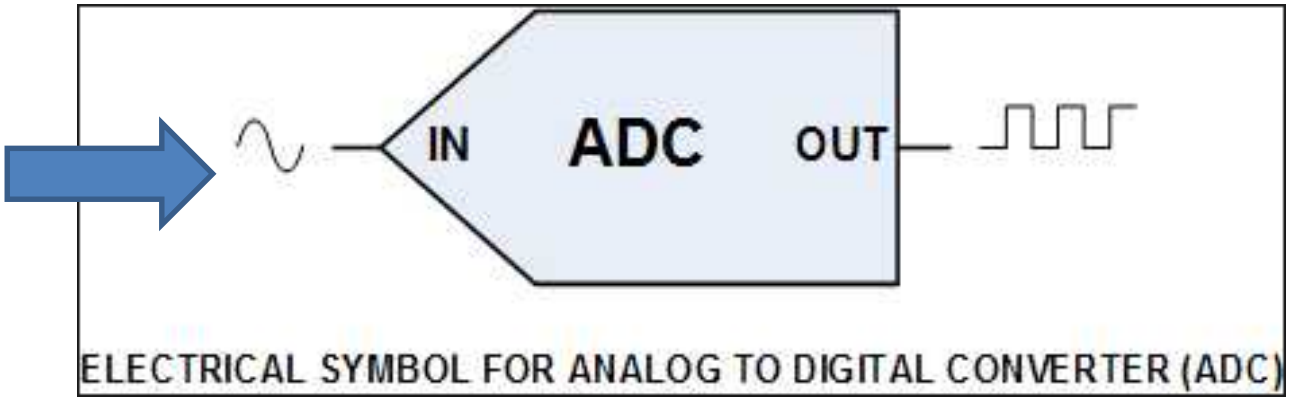
Best with SSC parameter (sensitivity & stability)

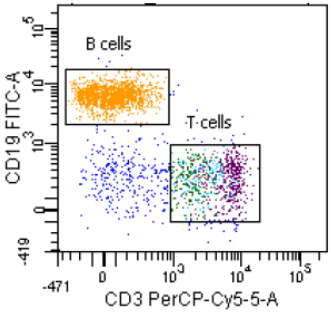


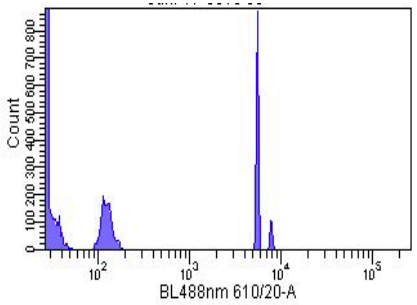
Electronics

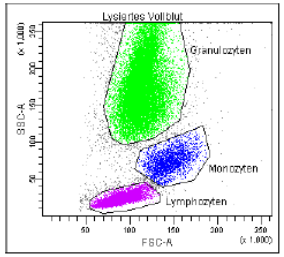
Aquisition board: Analog-to-digital converter(ADC)











http://regmed.musc.edu

Overview

- History of Flow Cytometry
- What is Flow Cytometry
- Flow cytometry parameters: FSC, SSC, fluorescence
- Flow Cytometer Components:
  - Fluidics,Optics, Electronics
- **Data presentation**
- Cell Sorting
- Overview of applications
- Instruments
  - Flow cytometers, cell sorters, special instrumentation

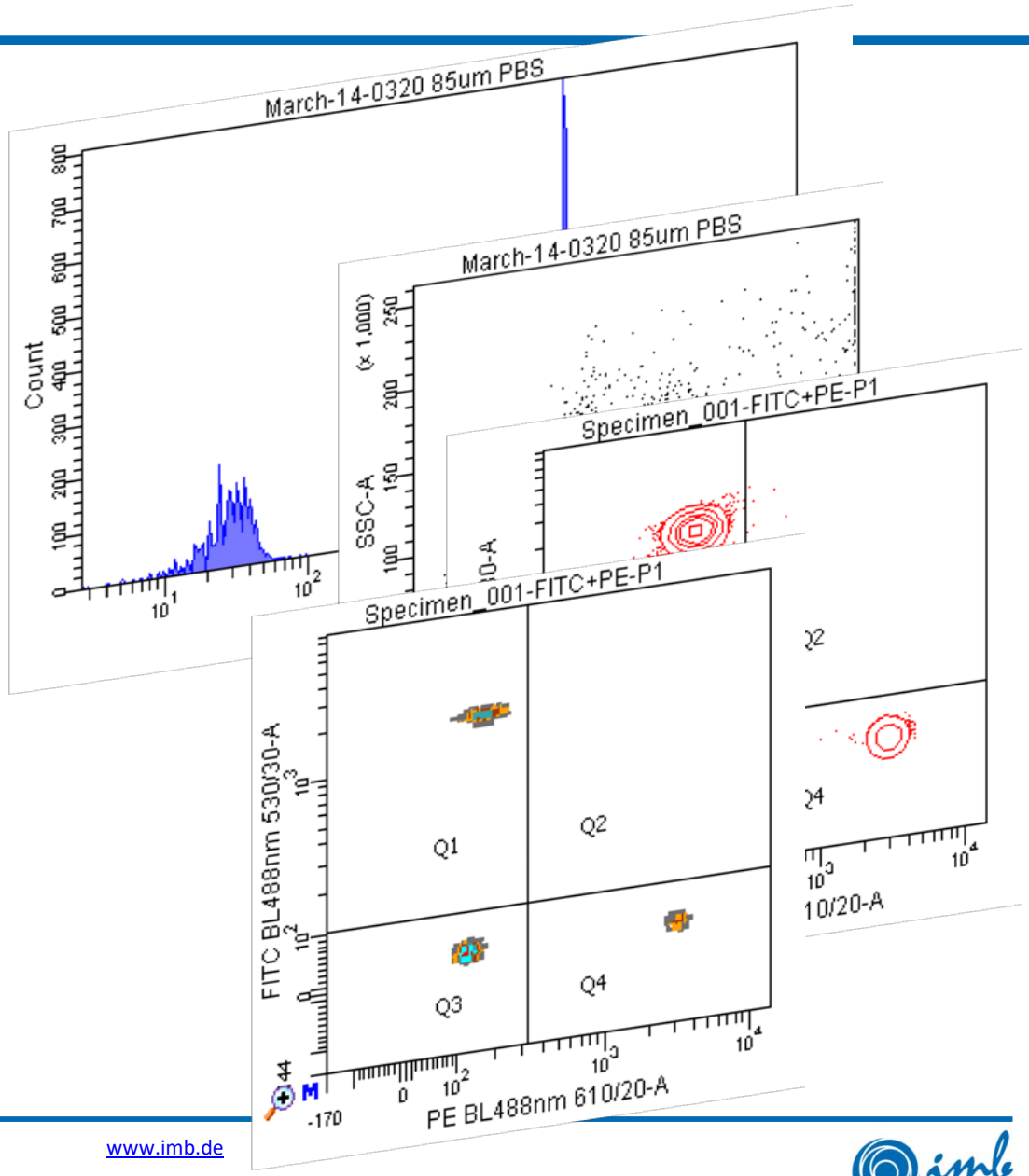
Data presentation

Plot types

- Histograms
- Dot plots
- Contour plots
- Density plots

Data display

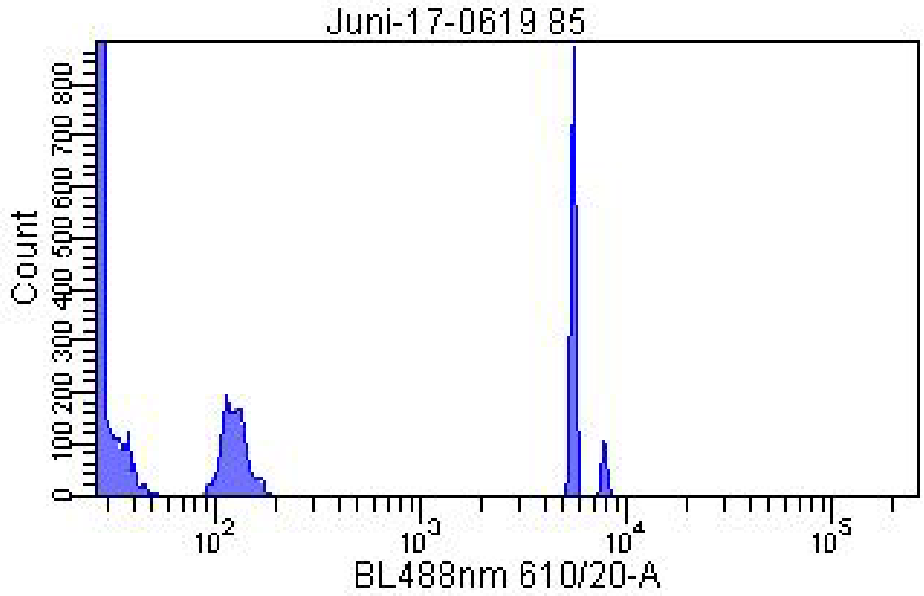
- Linear scale
- Logarithmic scale
- Biexponential scale



Data presentation

Plot types

- **Histograms**
- Dot plots
- Contour plots
- Density plots



Data display

- Linear scale
- **Logarithmic scale**
- Biexponential scale

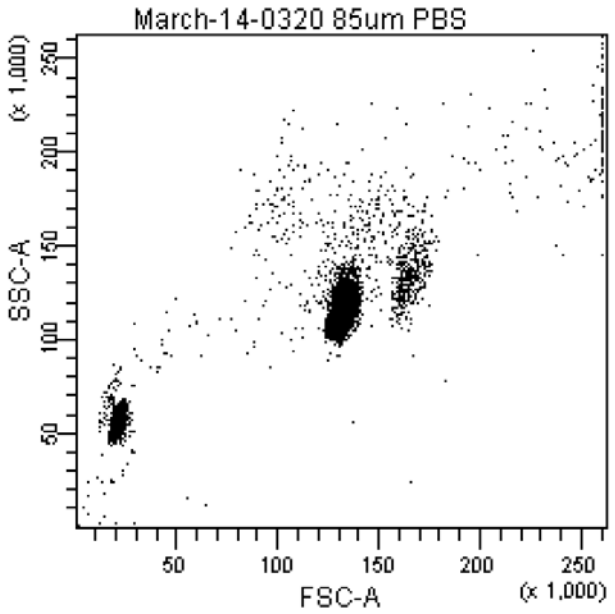
Histogram

- Binning of data
- Display of one parameter vs. counts per bin

Data presentation

Plot types

- Histograms
- **Dot plots**
- Contour plots
- Density plots



Data display

- **Linear scale**
- Logarithmic scale
- Biexponential scale

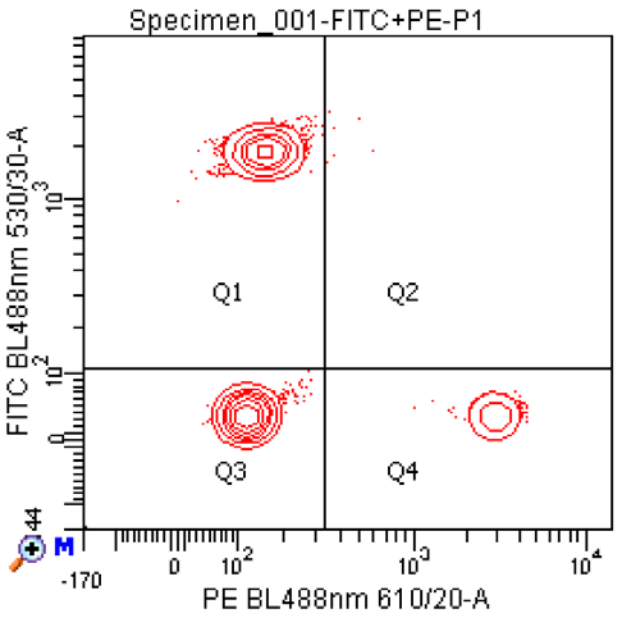
Dot plot

- Display of two parameters
- Each dot represents one cell

Data presentation

Plot types

- Histograms
- Dot plots
- **Contour plots**
- Density plots



Data display

- Linear scale
- **Logarithmic scale**
- Biexponential scale

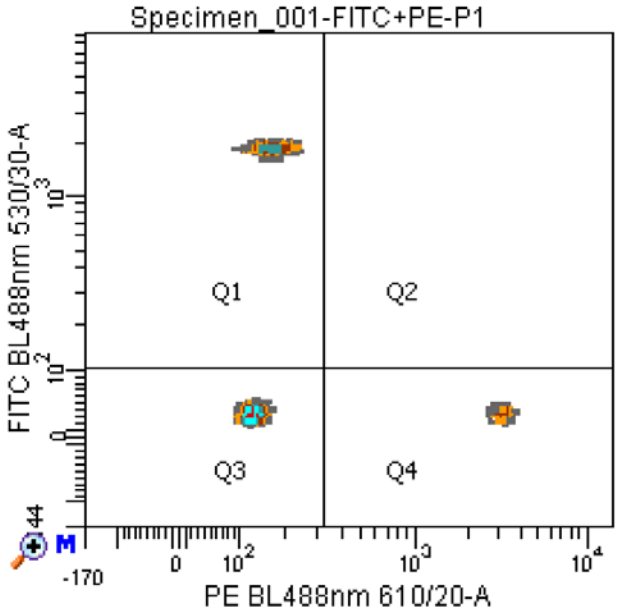
Contour plot

- Display of two parameters
- Contour line connects values of the same particular value (frequency as a third dimension)

Data presentation

Plot types

- Histograms
- Dot plots
- Contour plots
- **Density plots**



Data display

- Linear scale
- **Logarithmic scale**
- Biexponential scale

Density plot

- Color coded density
  (A relative number of events in a given region)

Data presentation

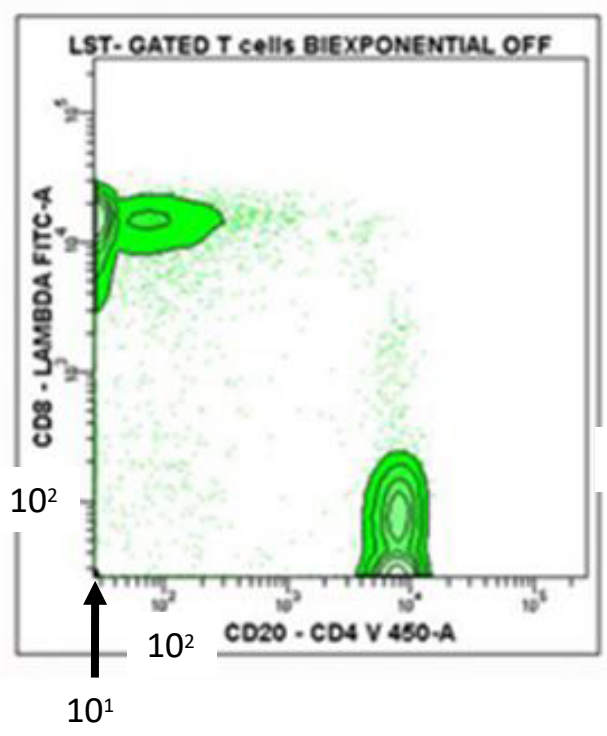
Plot types

- Histograms
- Dot plots
- **Contour plots**
- Density plots

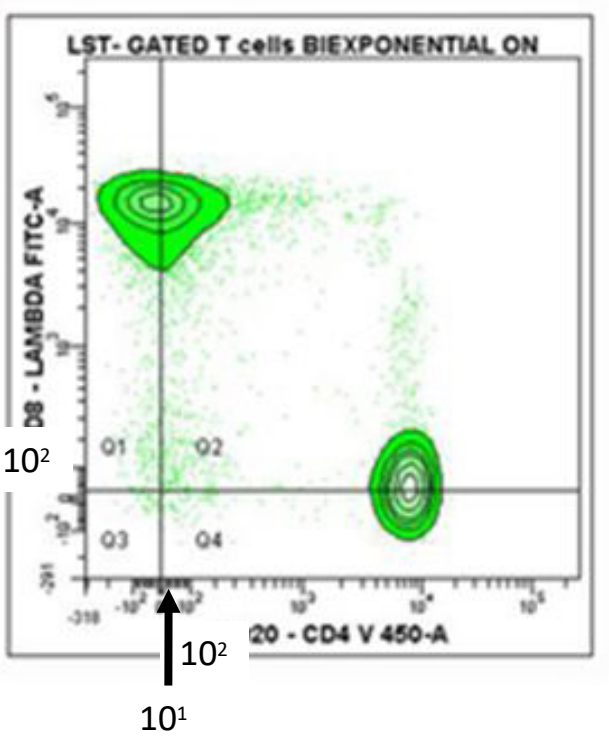
Data display

- Linear scale
- Logarithmic scale
- **Biexponential scale**

Biexponential off



Biexponential on

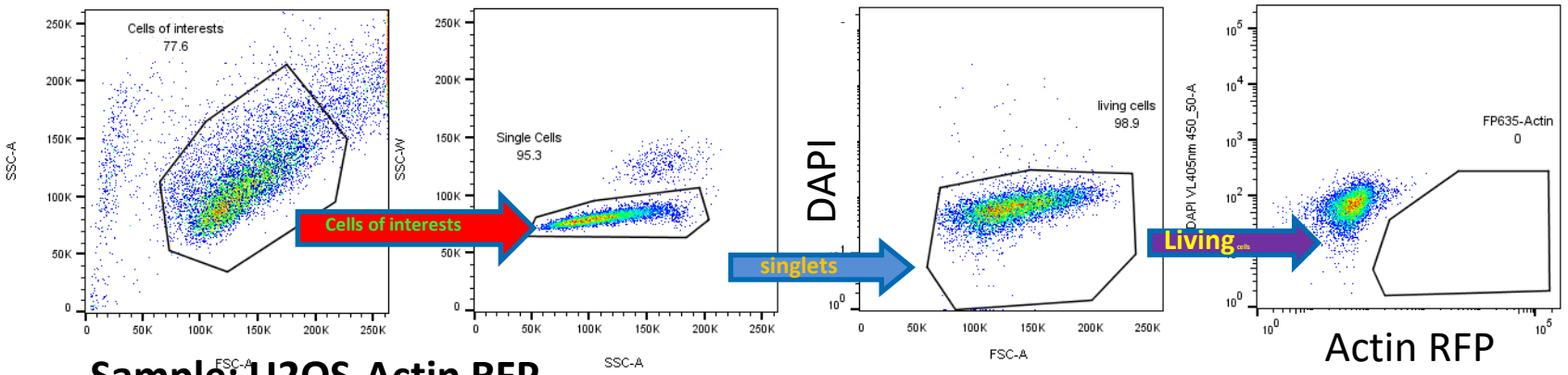


- Biexponential display enables signal resolution in negative range (unstained population)
- May be helpful in case of high fluorescence intensity, e.g. highly expressed marker stained with bright dye

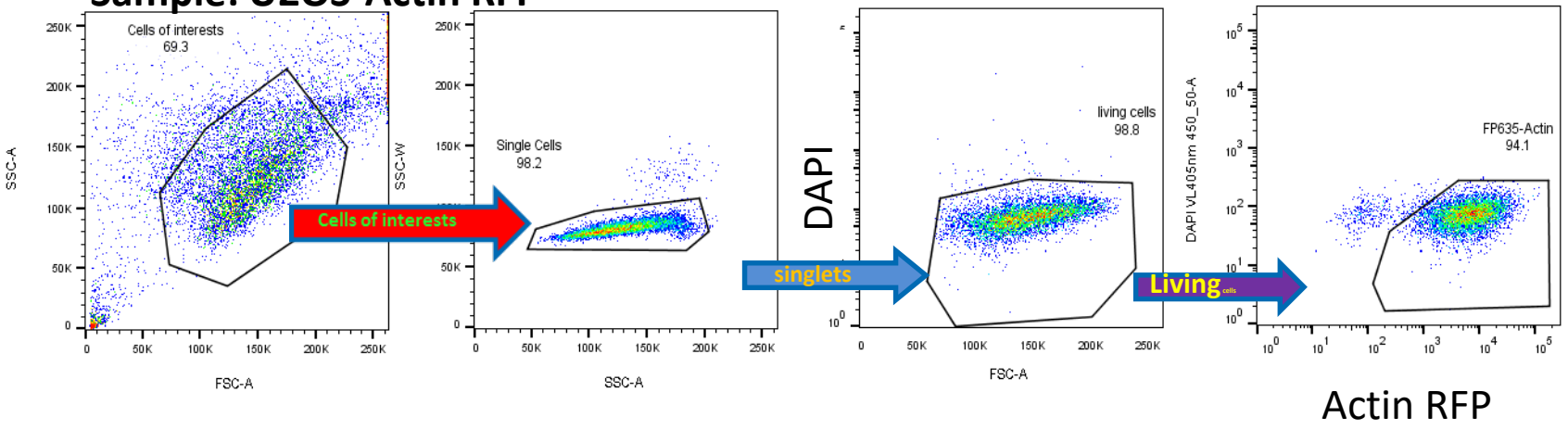
Data presentation – Gating

Cell line to analyze: U2OS-Actin RFP

U2OS cells, wt

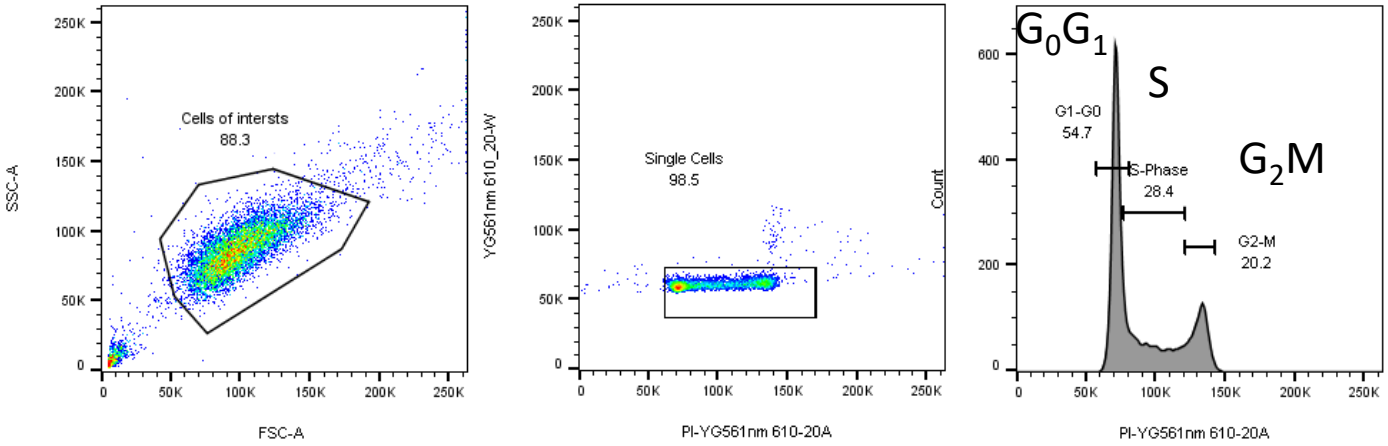


Sample: U2OS-Actin RFP



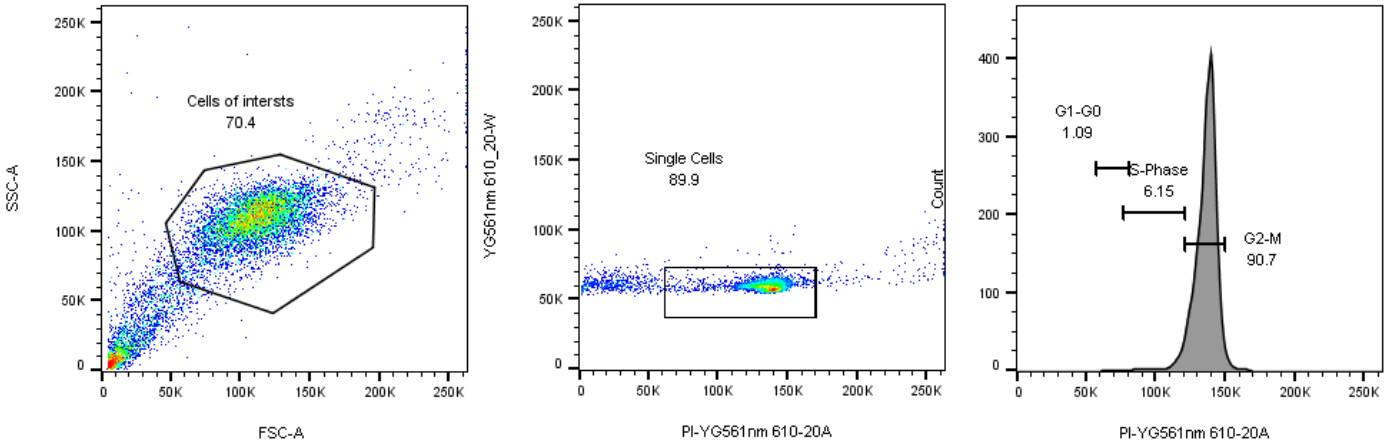
Data presentation: Gating

Negative control



HeLa cells synchronized with Nocodazole 50ng/mL

Nocodazole 50ng/mL

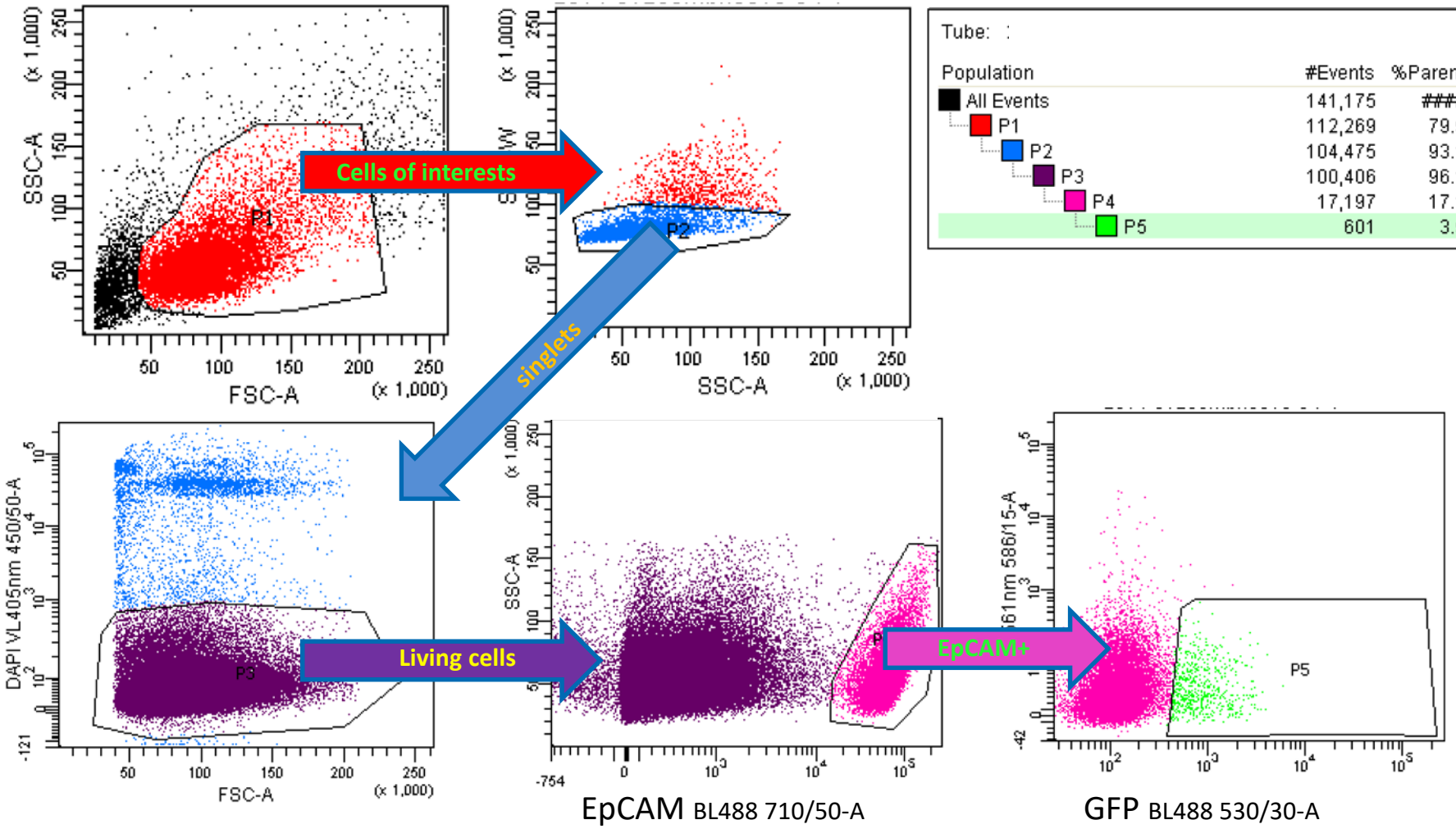


Nocodazole inhibits microtubule polymerization - arrest in G2/M phase

Data presentation – Gating

Analysis of subpopulations

AG Soshnikova – Lira Nigmatullina



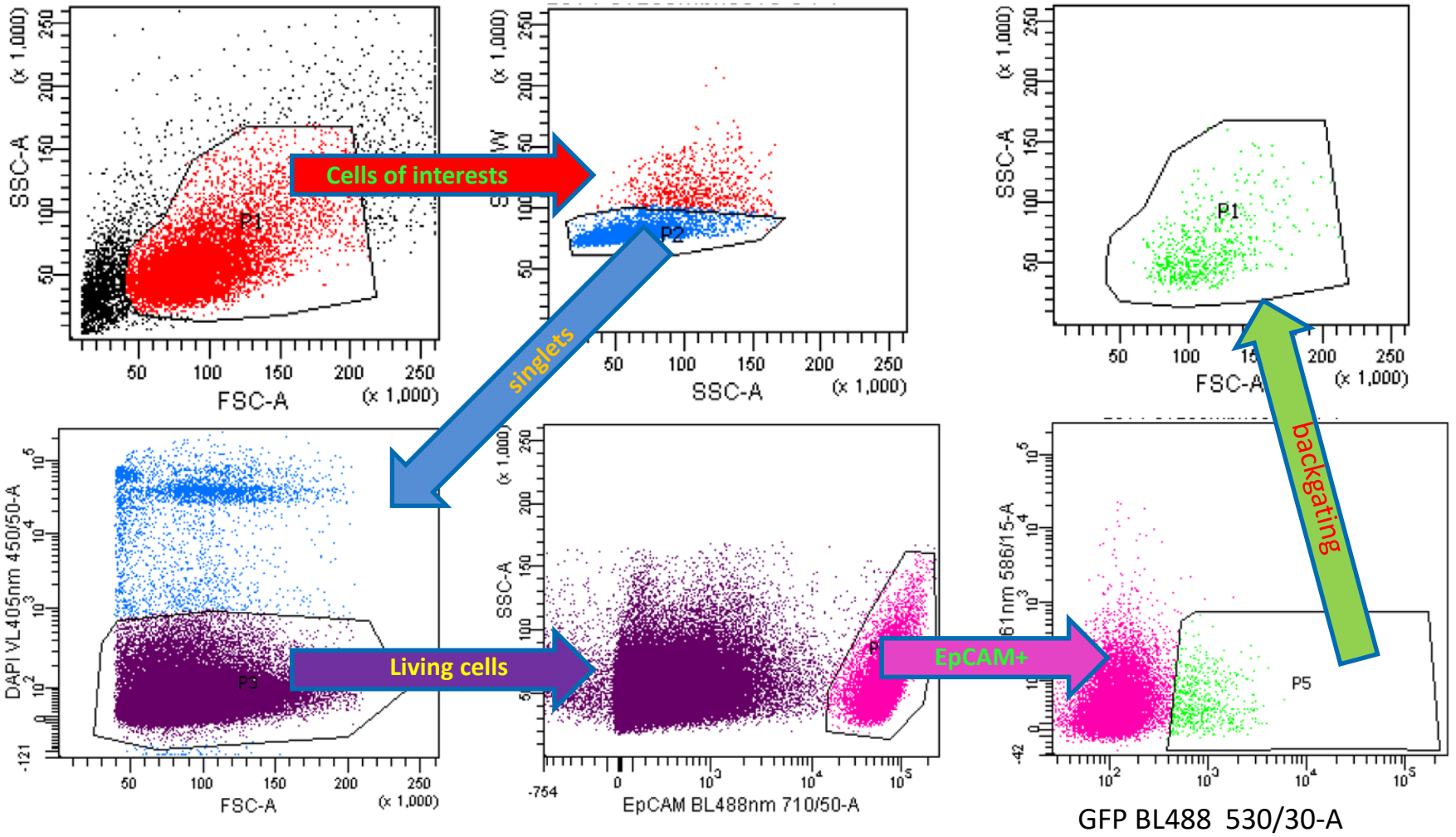
Tube: :

Population	#Events	%Parent	%Total
All Events	141,175	####	100.0
P1	112,269	79.5	79.5
P2	104,475	93.1	74.0
P3	100,406	96.1	71.1
P4	17,197	17.1	12.2
P5	601	3.5	0.4

Data presentation: Gating

Backgating!

AG Soshnikova – Lira Nigmatulli



Overview

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- Flow cytometry parameters: FSC, SSC, fluorescence
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Terminology

FACS: Fluorescence-activated cell sorting

- Registered trademark of Becton Dickinson

Flow cytometry or FACS?

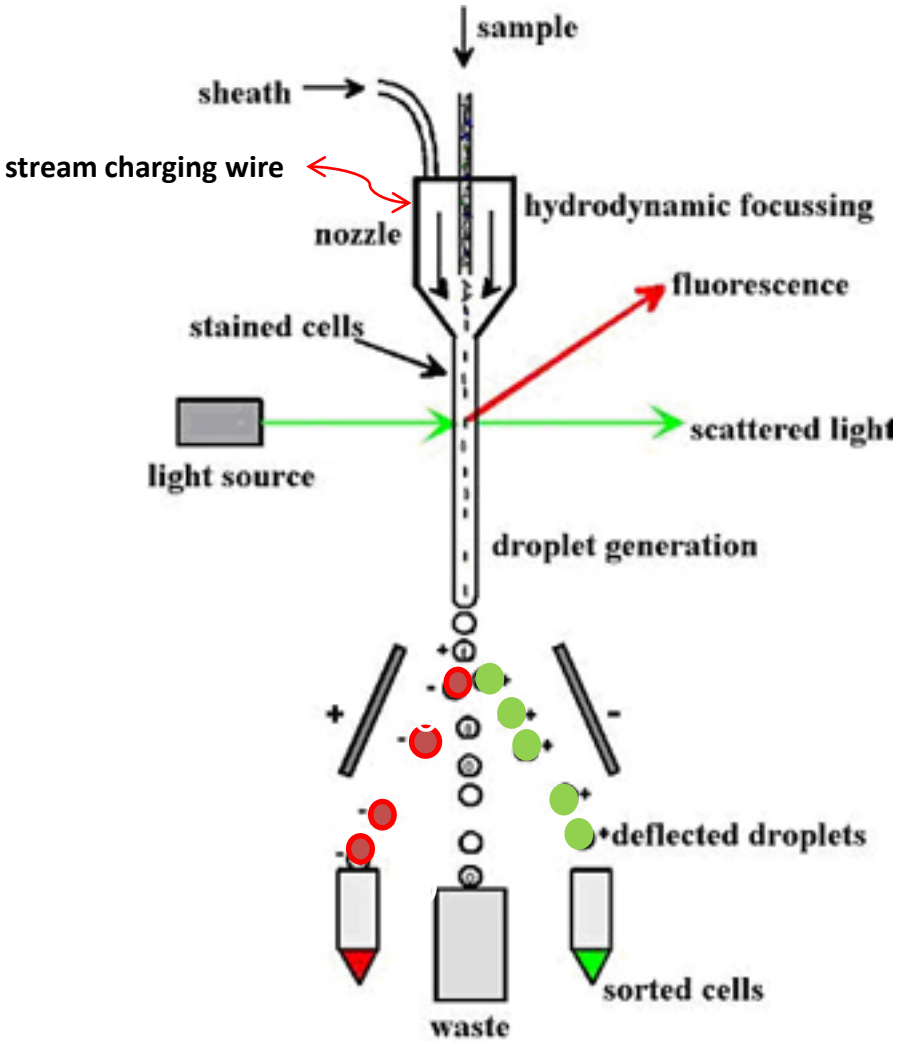
- Analysis principle is similar
- Analysis only versus analysis & cell sorting

Recommendation for publications or theses

- Use the general terms **flow cytometry** or **cell sorting** in your material & methods section
- Especially don’t use the term FACS if you sort on a Beckman Coulter instrument
- Very bad example: “Cells were measured by FACS”

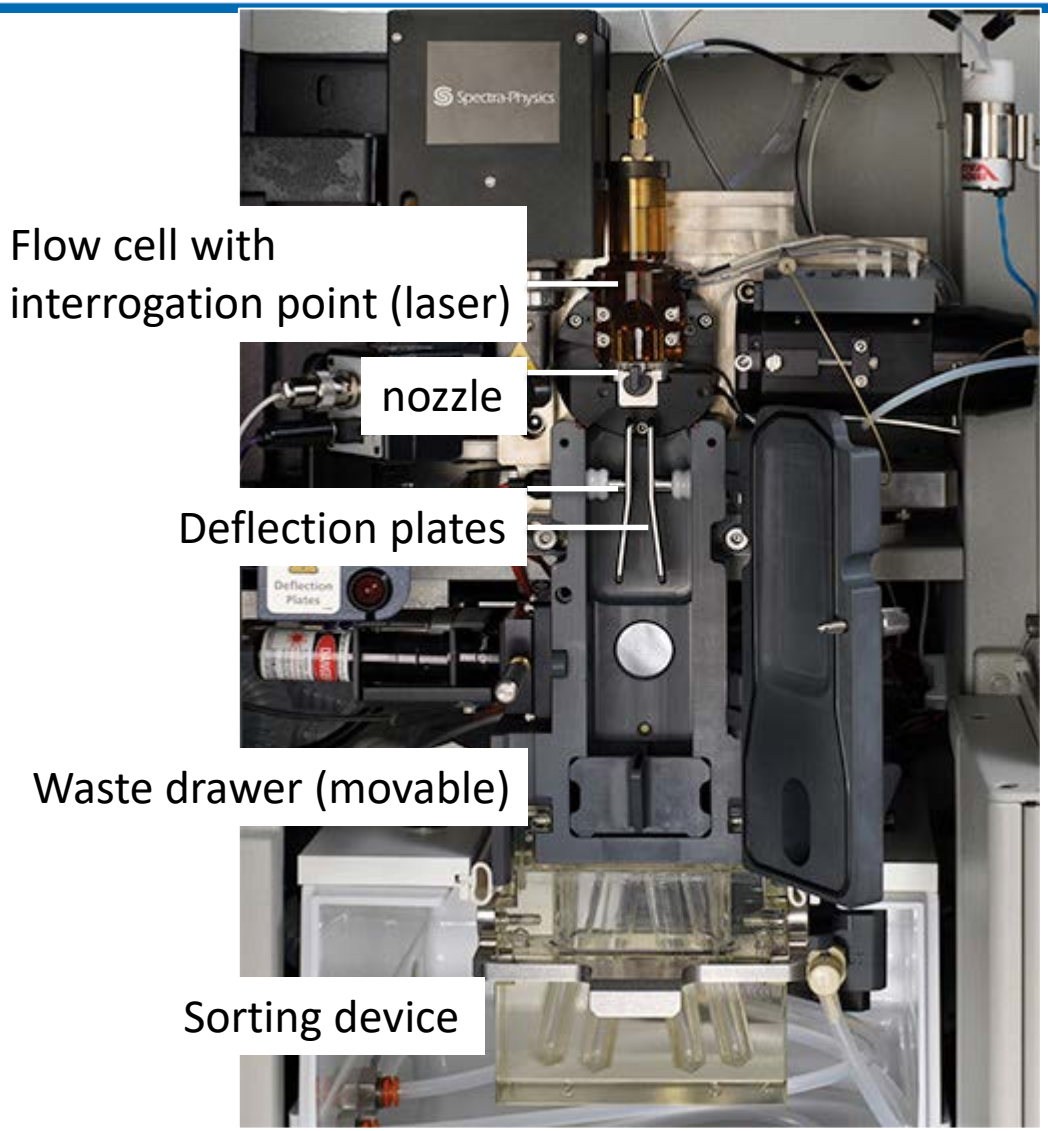
Cell sorting – the principle

- A vibrating mechanism („transducer“) causes a liquid stream to break into single droplets
- One drop contains one cell (in theory)
- Cells are interrogated before the droplet breaks off
- At the stream break-off point, the droplet is charged, if it contains a cell of interest
- Charged droplets are deflected in an electric field and collected in the respective sorting device

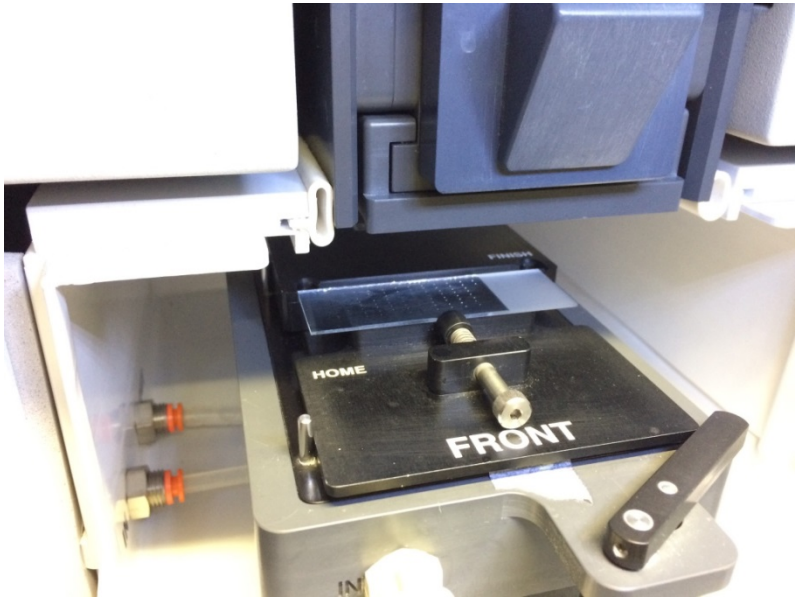


http://www.appliedcytometry.com/flow_cytometry.php

Cell sorting – the principle



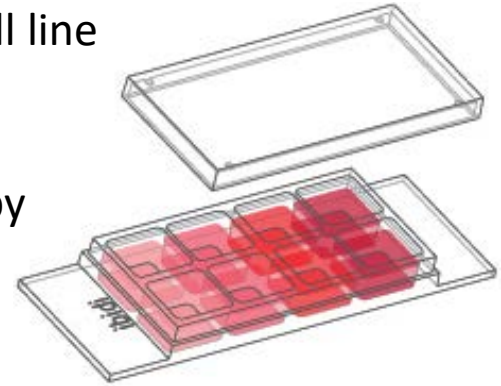
Possible collection devices for cell sorting



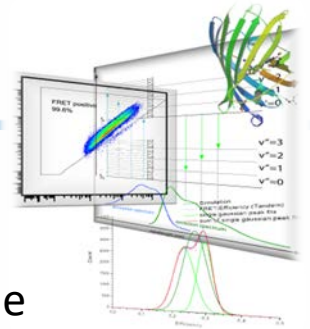
Sorting of (sub)populations into

- **Eppendorf tubes** to isolate e.g. RNA, for RNA sequencing or qPCR
- **Microtiter plates 96w, 384w** e.g. in order to create a stable cell line

  or to do single cell sorting for sequencing
- **Microscopy slides** to analyze certain populations by microscopy
- **Ibidi chamber** for live cell imaging



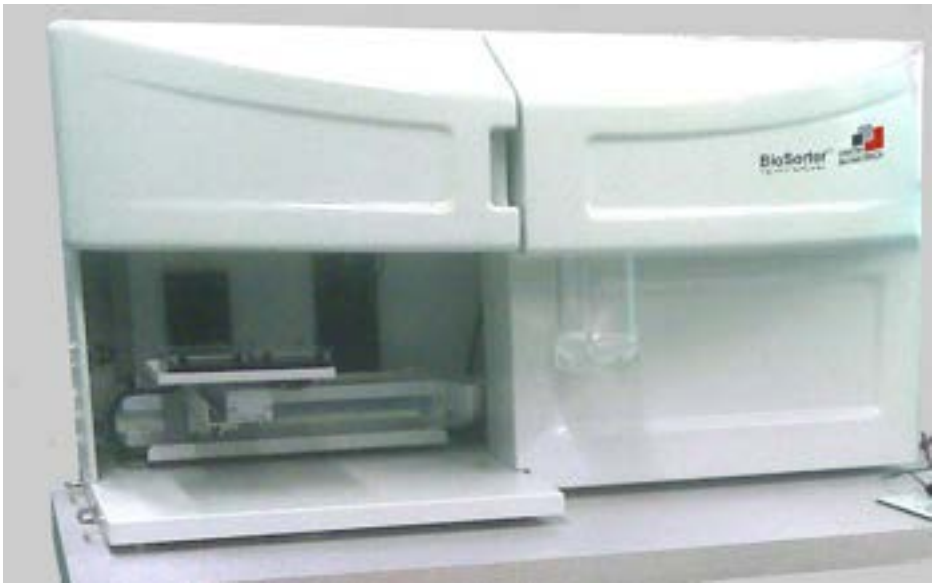
Large Particle Sorter/BioSorter



Union Biometrica BioSorter

Particles from 20-1500µm, like *C.elegans*, Zebra fish embryos, *Drosophila* larvae

2 excitation lasers: 488nm, 561nm

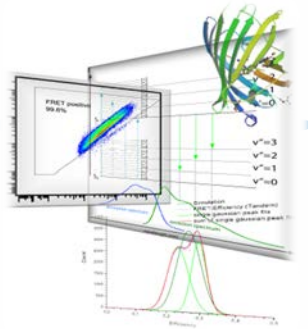


Sorting parameters:

- Axial length /Size (time of flight-TOF)
- Optical density (extinction)
- 3 fluorescence channels to detect, e.g. GFP, YFP or DsRed

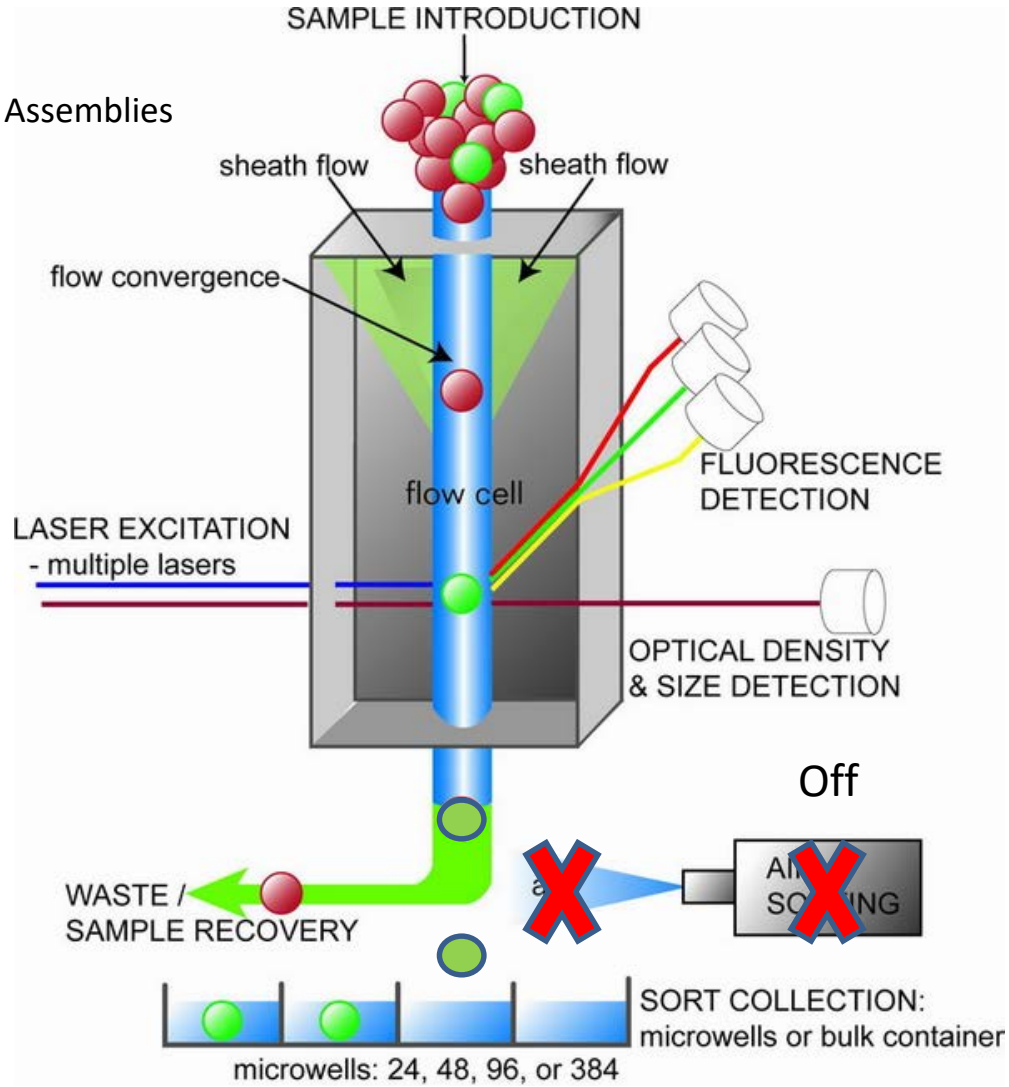
Applications:
Small multi cellular animals/ large cells/ small plant models/ beads and particles or encapsulation

Large Particle Sorter: Air sorting



FOCA

Fluidics and Optics Core Assemblies

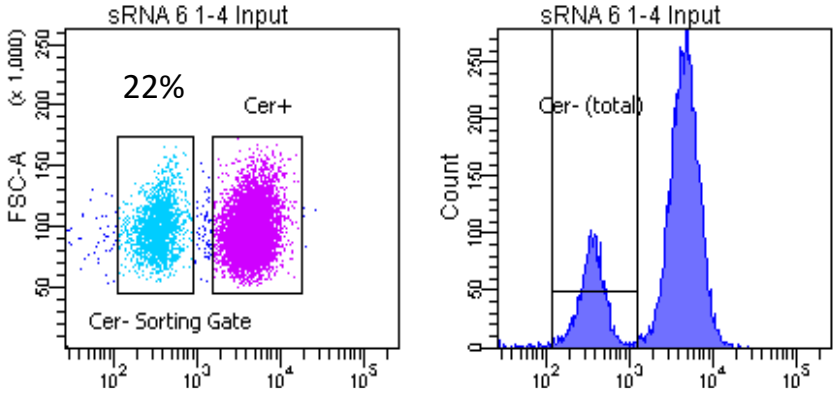


http://www.unionbio.com

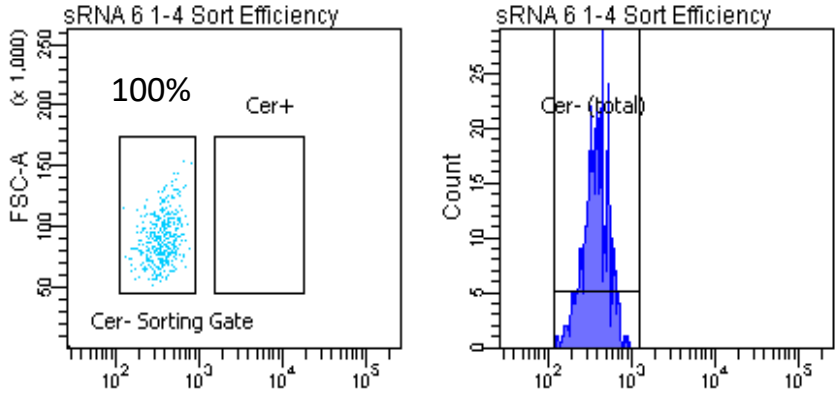
Sort example: Creating a knockout cell line

- Targeted knockout using CRISPR/Cas9 with fluorescent H2B reporter (H2B Cerulean)
  - Guide RNA against gene of interest and H2B-Cerulean
  - If the Cerulean fluorescence is gone, the gene of interest should also be knocked out

Input



Reanalysis after sorting



Cerulean fluorescence intensity

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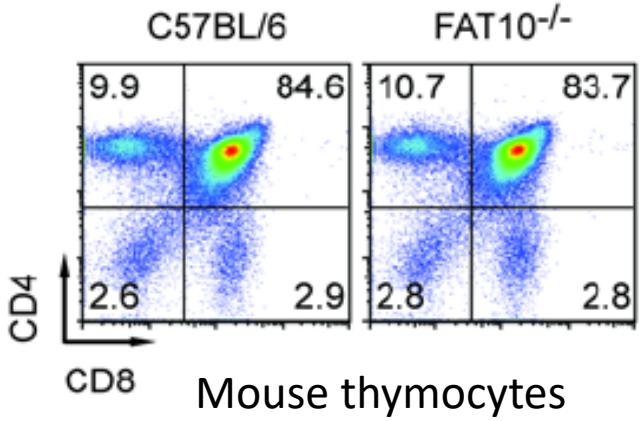
Phenotyping

- Lineage-& Subsetmarker
  - Analysis of the expression of proteins on the cell surface or inside a cell

- Cytokines & Chemokines and their Receptors
  - Detection of various cytokines or chemokines in different cell types
  - Detection of their receptors on the cell surface

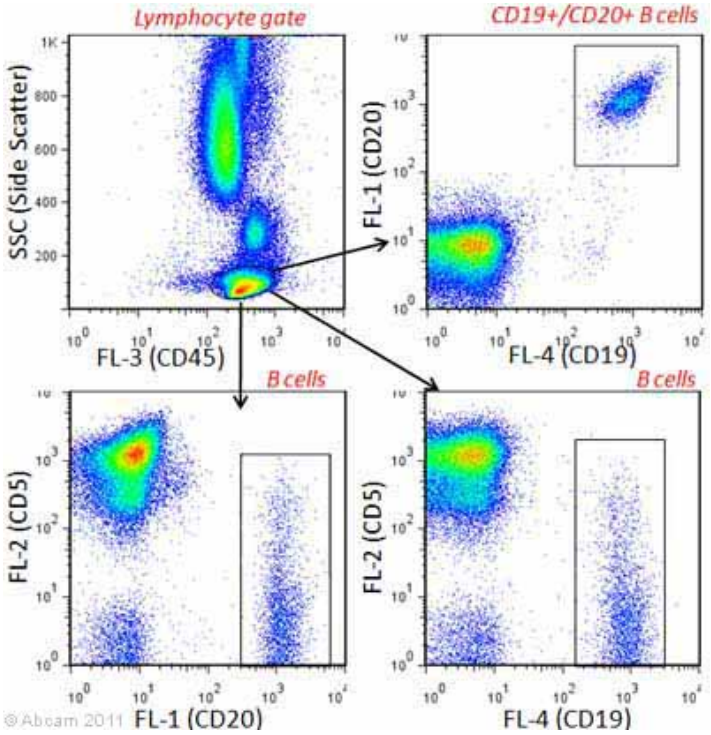
  - The expression pattern defines:
    - a certain cell type
    - the differentiation state
    - the grade of activation
    - a certain cell line and allows
    - monitoring of differentiation and progression

Phenotyping: Lineage marker



Mouse thymocytes

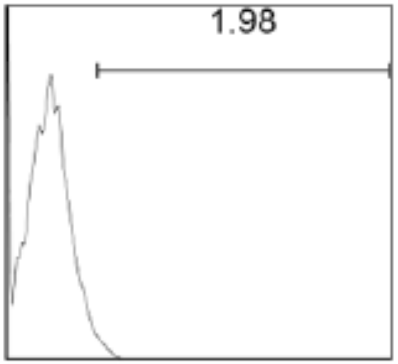
Mouse splenocytes



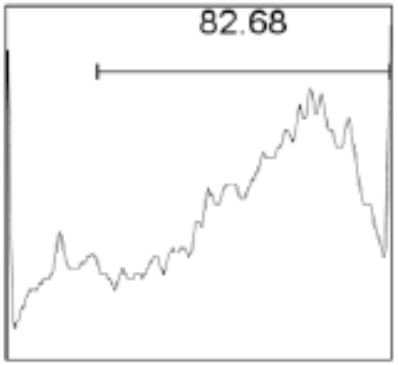
Human B cell marker panel
BD Biosciences

Analysis of transfection efficiency

Electroporation of MoDCs



untreated

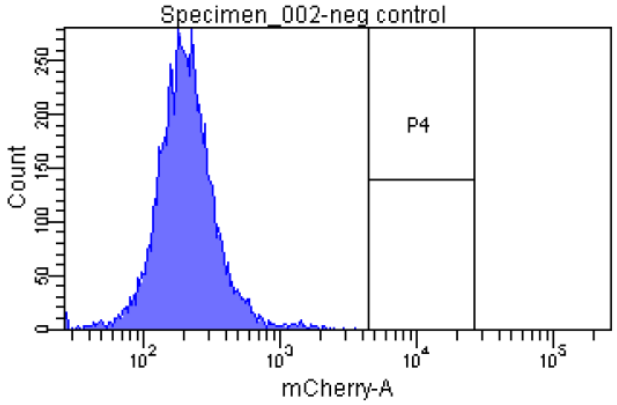


transfection
pEGFPN1

Cell count

GFP fluorescence intensity

HeLa transfection with a mCherry fusion construct



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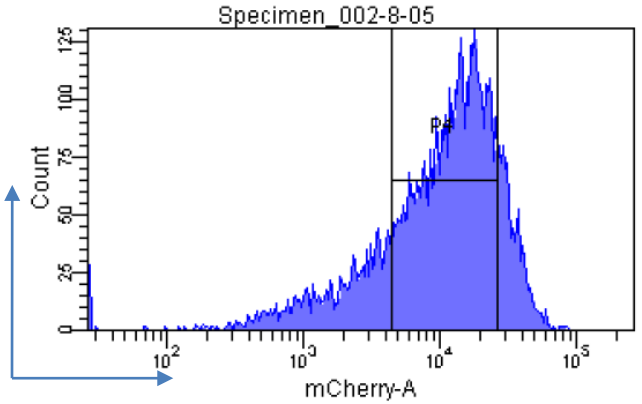


transfection
mCherry

Cell count

mCherry fluorescence intensity

Phospho-Protein Profiling

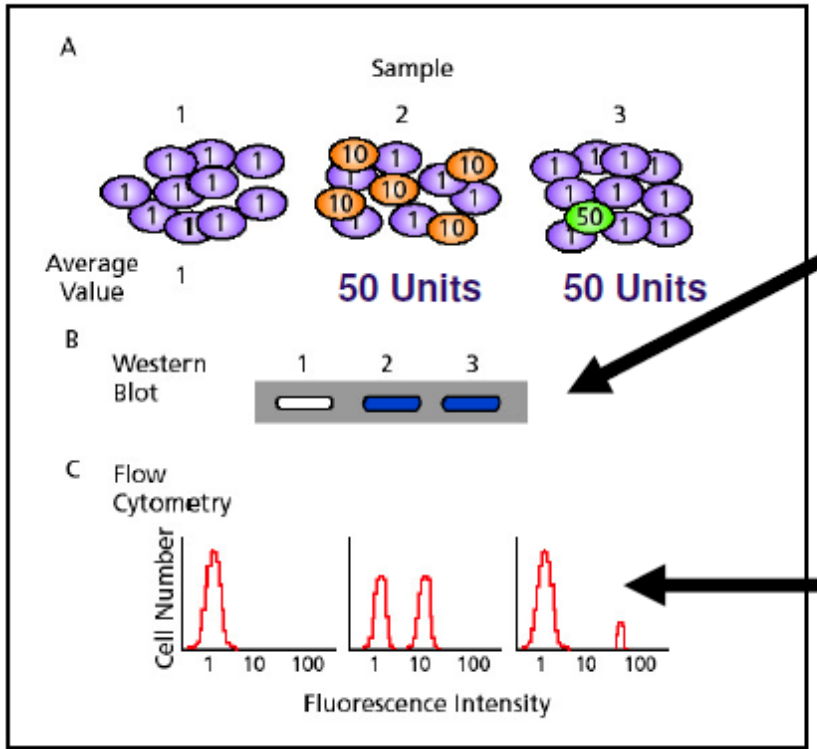
- Distinguish and analyze phospho-protein signaling in single cells
  - Nonphosphorylation “specific” antibodies

    Detect the total protein: unphosphorylated + phosphorylated isoforms

    Antigen: recombinant protein fragments
  - Phospho-specific antibodies

    Detect defined phosphoepitopes of the phosphorylated isoform

    Antigen: Synthetic 4-10mer phospho-peptides



http://www.bdbiosciences.com/research/phosflow/

Western Blot

- No opportunity to view variability
- Requires sorting of subsets to gain access to intracellular antigens
- Requires larger numbers of cells 10^6

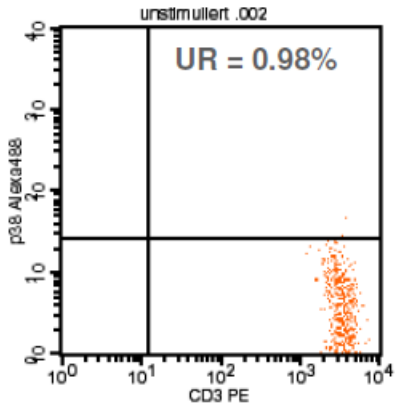
Flow Cytometry

- Differentiation possible
- Subset Typing via surface markers possible
- Possibility to observe heterogeneity in the population
- Requires fewer cells 10^3-10^4

Phospho-Protein Profiling

Kinetics of MAPK p38 on CD4+ T cells out of a mixture of cells

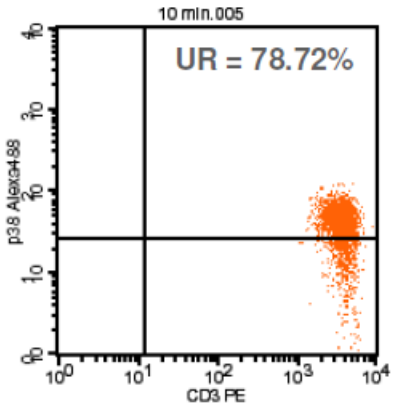
unstimulated



File: unstimulert .002
Tube: 20µl p38
Gate: CD4 pos T-Lymphozyten

Quad	% Gated	Y Geo Mean
UL	0.00	***
UR	0.98	29.12
LL	0.00	***
LR	99.02	2.54

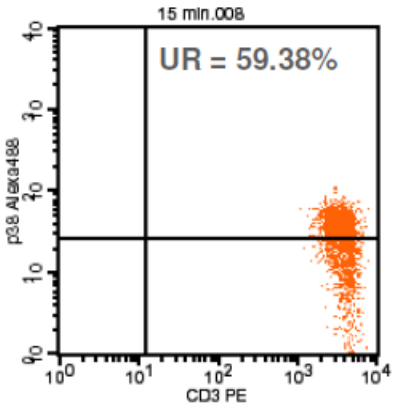
10 min. PMA



File: 10 min.005
Tube: 20µl p38
Gate: CD4 pos T-Lymphozyten

Quad	% Gated	Y Geo Mean
UL	0.00	***
UR	78.72	46.45
LL	0.00	***
LR	21.28	12.00

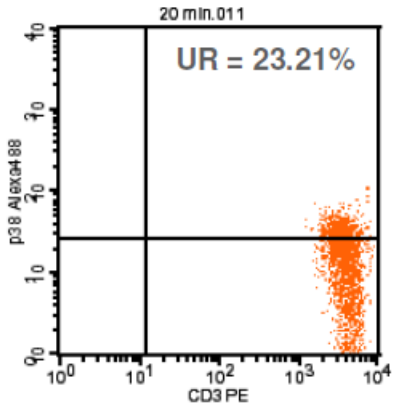
15 min. PMA



File: 15 min.008
Tube: 20µl p38
Gate: CD4 pos T-Lymphozyten

Quad	% Gated	Y Geo Mean
UL	0.00	***
UR	59.38	39.25
LL	0.00	***
LR	40.62	10.10

20 min. PMA



File: 20 min.011
Tube: 20µl p38
Gate: CD4 pos T-Lymphozyten

Quad	% Gated	Y Geo Mean
UL	0.00	***
UR	23.21	33.83
LL	0.00	***
LR	76.79	6.66

BD Biosciences PhosFlow Tutorial

Cell activation: Calcium flux

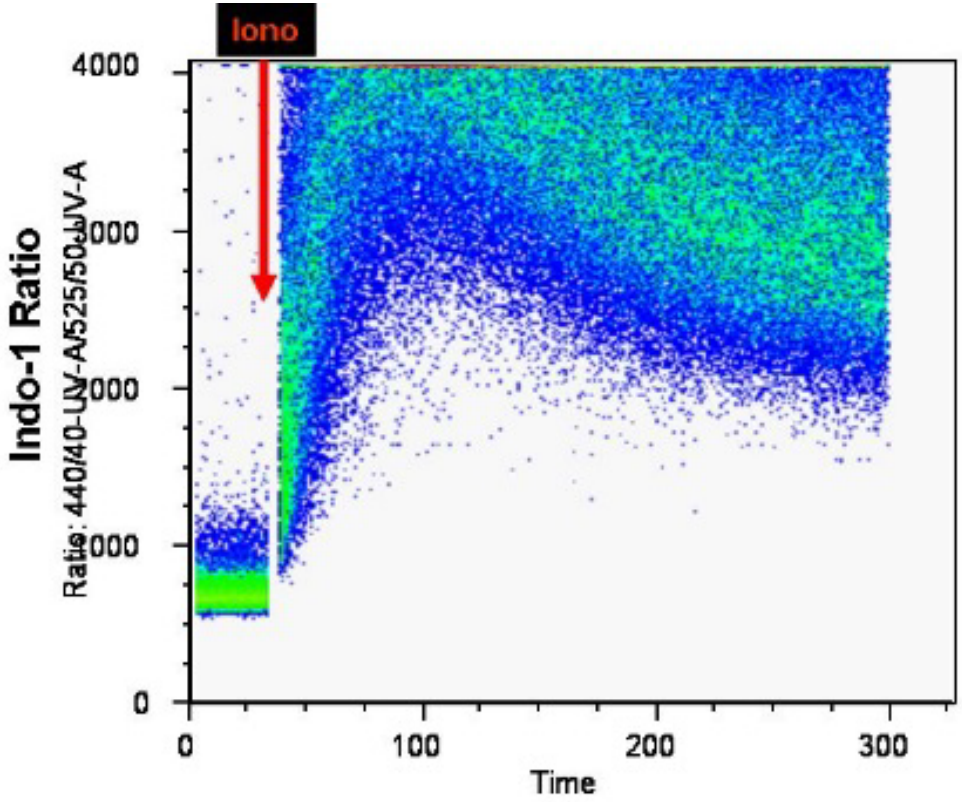
- Calcium is a second messenger after cell stimulation
  - After receptor-ligand interaction
  - In response to hormones or drugs
  - During cell injury

Multiparameter staining with parallel analysis of intercellular Ca^{2+} and cell surface markers

- **Indo-1**
  - Emission maximum after Ca^{2+} binding changes from 475nm to 400 nm
  - Excitation maximum at 346 nm (UV laser required!)
- **Fluo-4**
  - Emission maximum at 506 nm. Increases intensity after Ca^{2+} binding > 100 fold
  - Excitation maximum at 494 nm
- **FuraRed**
  - Excitation maximum at 657 nm without Ca^{2+}, intensity is reduced after binding
  - Excitation maximum at 472 nm

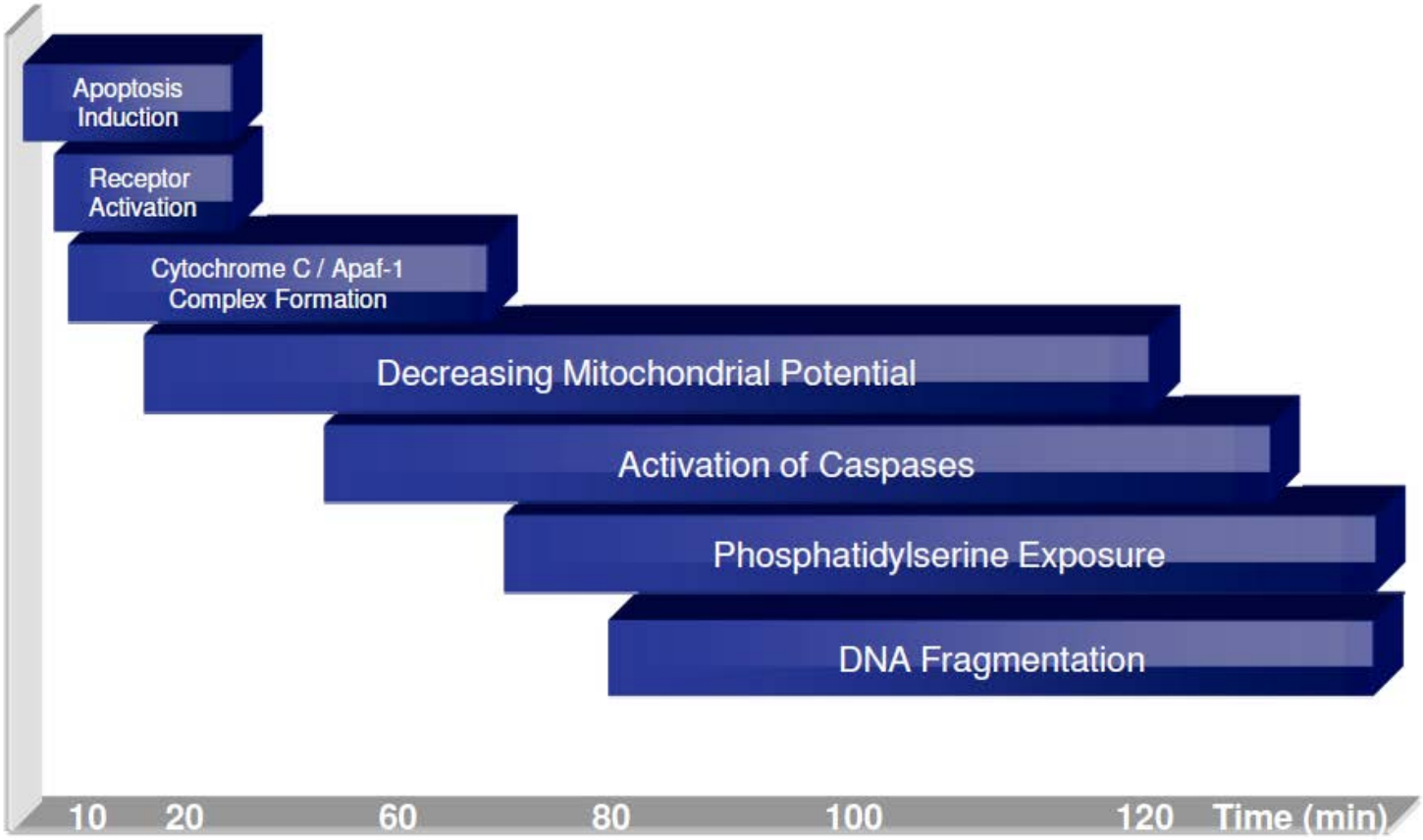
Cell activation: Calcium flux

- Stimulation of Jurkat T cells, monitoring of cell activation over time



http://www.icms.qmul.ac.uk/flowcytometry/uses/functionalanalysis/diagrams/Indo1RatioLegend.jpg

Analysis of apoptosis: Time scale



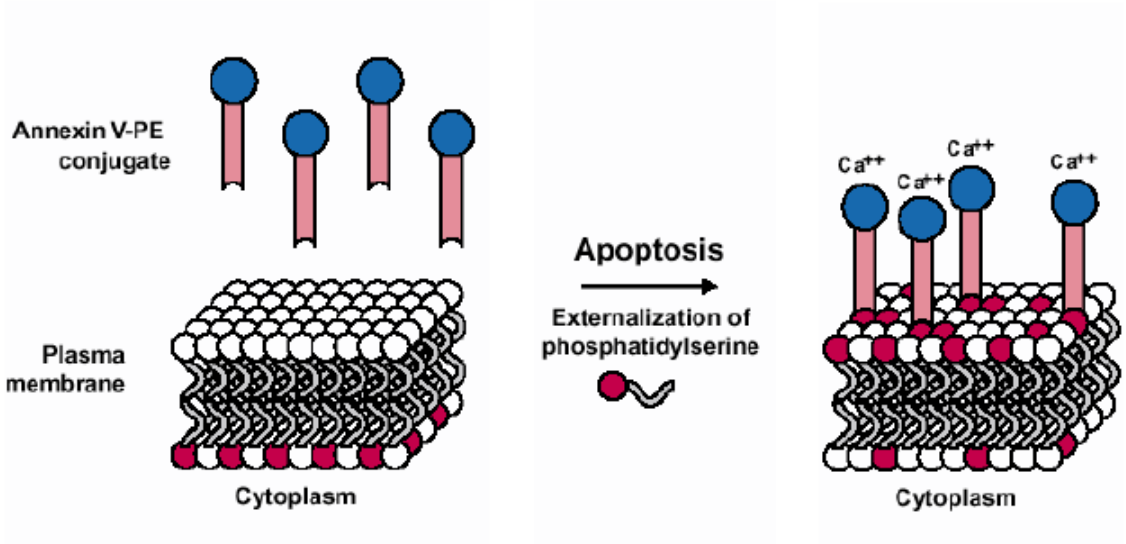
Applications in flow cytomety:
Tim Schenkel
BD Biosciences Training Center Heidelberg

Analysis of apoptosis: Possible parameters

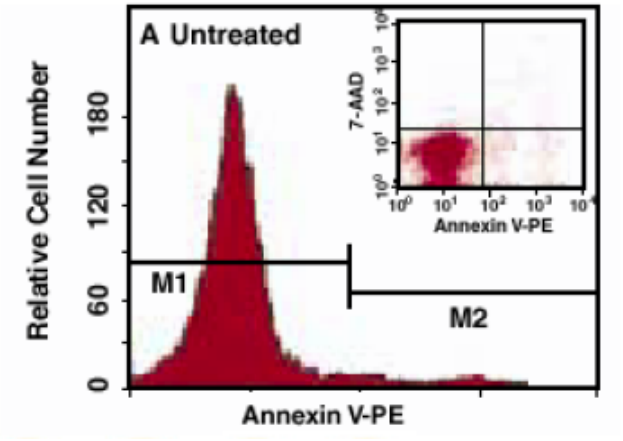
Change	Assays
Translocation of phophatidylserine from the inner to the outer leaflet of the plasma membrane	Annexin V
Changes of mitochondria membrane potential	BD MitoScreen test
Activations of casapses	e.g. activation of caspase-3
DNA fragmentation	APO BrdU Kit (TUNEL)

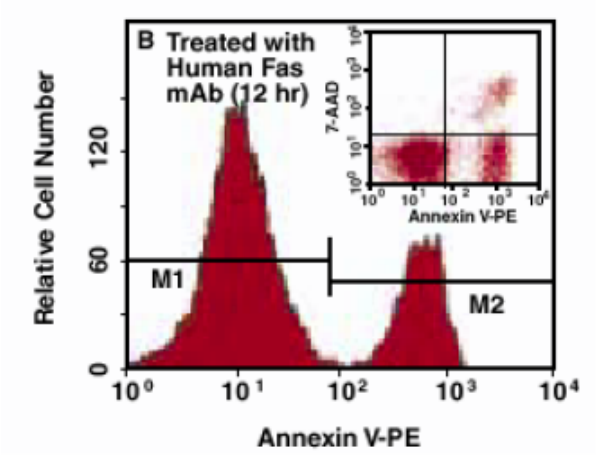
Applications in flow cytomety:
Tim Schenkel
BD Biosciences Training Center Heidelberg

Analysis of apoptosis: Annexin V staining



- Calcium essential!
- Assay does not work in PBS!

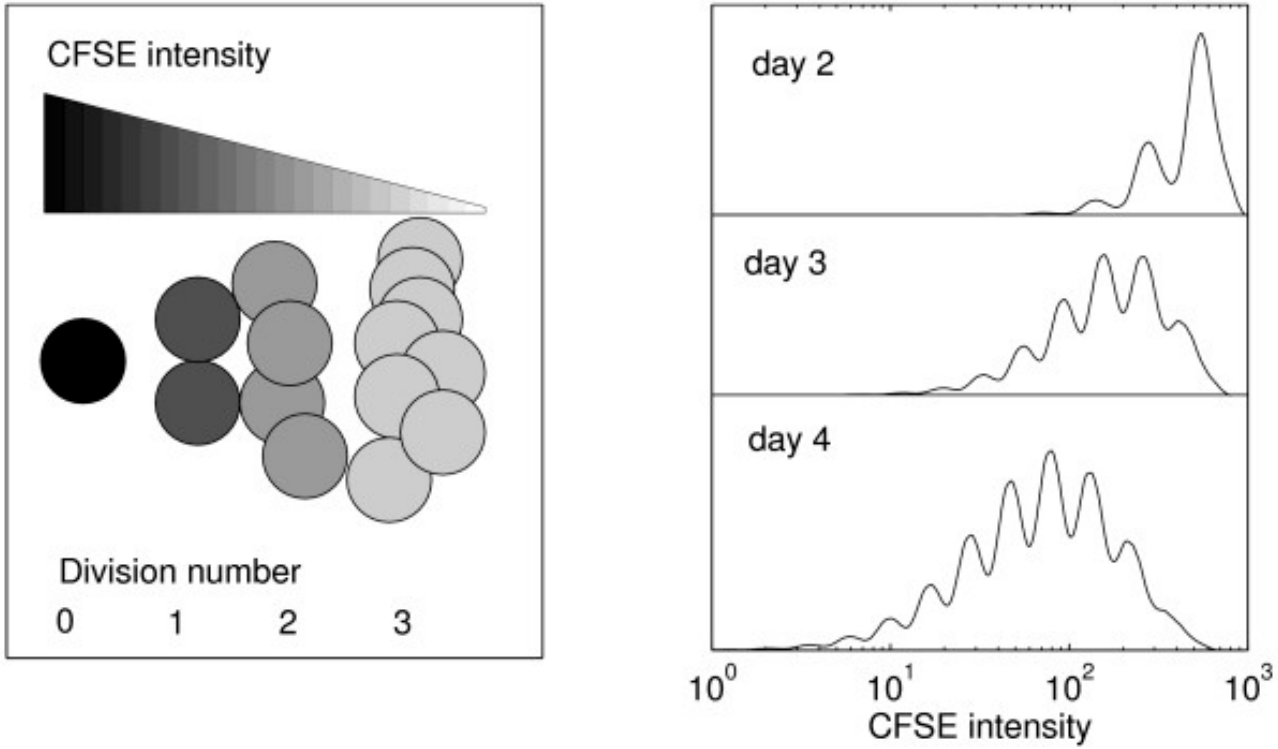




Applications in flow cytomety:
Tim Schenkel
BD Biosciences Training Center Heidelberg

Cell proliferation: CFSE staining

- Carboxyfluorescein Succinimidylester
  - Covalent binding to intracellular free amine residues (lysine)
  - Equal distribution in daughter cells upon cell division
  - Enables monitoring of distinct generations of proliferating cells

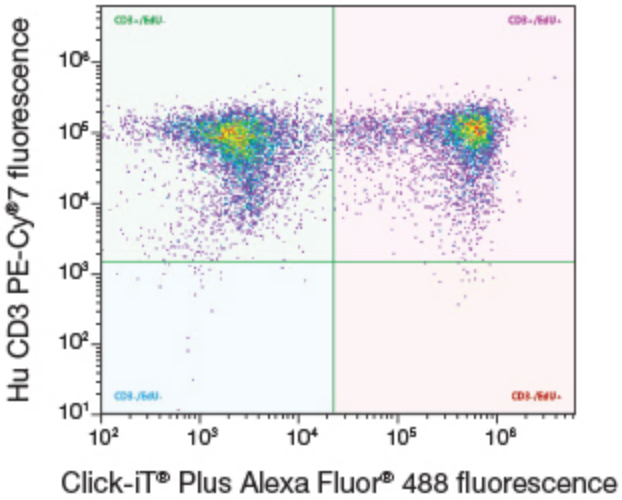


Luzyanina *et al. Theoretical Biology and Medical Modelling* 2007 **4**:26

Cell proliferation: Thymidine analogues

- DNA-synthesis-based cell proliferation
  - BrdU = thymidine analogue 5-Brom-2‘-deoxyuridine
  - Incorporated into newly synthesized DNA
  - Subsequent detection with a BrdU-specific antibody upon DNA denaturation

  - EdU, Click iT Technology
  - Click iT technology for fluorescence labeling does not require harsh fixation protocols (faster)



Click-iT Plus Alexa Fluor 488
Kit Manual, ThermoScientific

Overview

- History of Flow Cytometry
- What is Flow Cytometry
- Flow cytometry parameters: FSC, SSC, fluorescence
- Flow Cytometer Components:
  - Fluidics,Optics, Electronics
- Data presentation
- Cell Sorting
- Overview of applications
- **Instruments**
  - Flow cytometers, cell sorters, special instrumentation

Research flow cytometers from Becton-Dickinson

BD FACSCalibur™

4 colors

BD FACSCanto II™

8 colors

BD FACSVerse™

8 colors

6 colors at IMB

BD Accuri™ C6

4 colors

BD FACSymphony

Up to 50 colors

BD LSRFortessa™

> 8 colors

18 colors at IMB

BD LSRFortessa™ X-20

< 20 colors

www.bdbiosciences.com

Research flow cytometers from Beckman Coulter

CytoFLEX

Now up to 21 colors

Gallios

Up to 12 colors

www.beckman.com

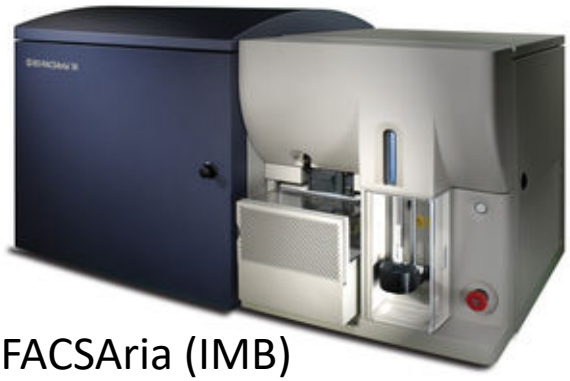
Flow cytometers from other companies

MACSQuant
www.miltenyibiotec.com

CyFlow Cube
www.sysmex.com

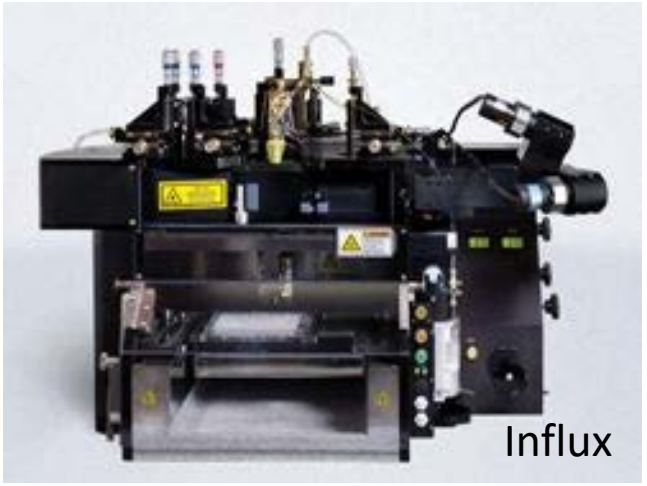
Attune Nxt
www.thermofisher.com

Cell sorters from Becton-Dickinson



FACSAria (IMB)

FACSMelody



Influx

FACSAriaFusion

www.bdbiosciences.com

Cell sorters from Beckman Coulter

MoFlo Astrios

www.beckman.com

MoFlo

Cell sorters from other companies

MACSQuant Tyto
www.miltenyibiotec.com

S3e Cell Sorter
www.bio-rad.com

BioSorter (Large Particle Sorter) IMB
www.unionbio.com

Sony SH800Z
www.sony.net

Special instrumentation: Mass cytometer (CyTOF)

- Mass cytometry combines time-of-flight mass spectrometry with Maxpar metal-labeling technology
- Cellular targets are labeled with metal-tagged antibodies and detected and quantified by time-of-flight mass spectrometry
- The high purity and choice of metal isotopes provide minimal background noise from signal overlap or endogenous cellular components

FLUIDIGM®

www.fluidigm.com

Mass cytometry

- Mass Cytometry elements
- Live/Dead cell markers
- Mass-tag cell barcoding (MCB)

1 H Hydrogen																	2 He Helium
3 Li Lithium	4 Be Beryllium											5 B Boron	6 C Carbon	7 N Nitrogen	8 O Oxygen	9 F Fluorine	10 Ne Neon
11 Na Sodium	12 Mg Magnesium											13 Al Aluminium	14 Si Silicon	15 P Phosphorus	16 S Sulfur	17 Cl Chlorine	18 Ar Argon
19 K Potassium	20 Ca Calcium	21 Sc Scandium	22 Ti Titanium	23 V Vandium	24 Cr Chromium	25 Mn Magnese	26 Fe Iron	27 Co Cobalt	28 Ni Nickel	29 Cu Copper	30 Zn Zinc	31 Ga Gallium	32 Ge Germanium	33 As Arsenic	34 Se Selenium	35 Br Bromine	36 Kr Krypton
37 Rb Rubidium	38 Sr Strontium	39 Y Yttrium	40 Zr Zirconium	41 Nb Niobium	42 Mo Molybdenum	43 Tc Technetium	44 Ru Ruthenium	45 Rh Rhodium	46 Pd Palladium	47 Ag Silver	48 Cd Cadmium	49 In Indium	50 Sn Tin	51 Sb Antimony	52 Te Tellurium	53 I Iodine	54 Xe Xenon
55 Cs Caselum	56 Ba Barium		72 Hf Hafnium	73 Ta Tantalum	74 W Tungsten	75 Re Rhenium	76 Os Osmium	77 Ir Iridium	78 Pt Platinum	79 Au Gold	80 Hg Mercury	81 Tl Thallium	82 Pb Lead	83 Bl Bismuth	84 Po Polonium	85 At Astatine	86 Rn Radon
87 Fr Francium	88 Ra Radium		104 Rf Rutherfordium	105 Db Dubnium	106 Sg Seaborgium	107 Bh Bohrium	108 Hs Hassium	109 Mt Meitnerium	110 Ds Darmstadtium	111 Rg Roentgenium	112 Cn Copernicium		114 Fl Flerovium		116 Lv Livermorium		

57 La Lanthanum	58 Ce Cerium	59 Pr Praseodymium	60 Nd Neodymium	61 Pm Promethium	62 Sm Samarium	63 Eu Europium	64 Gd Gadolinium	65 Tb Terbium	66 Dy Dysprosium	67 Ho Holmium	68 Er Erbium	69 Tm Thulium	70 Yb Ytterbium	71 Lu Lutetium
89 Ac Actinium	90 Th Thorium	91 Pa Protactinium	92 U Uranium	93 Np Neptunium	94 Pu Plutonium	95 Am Americium	96 Cm Curium	97 Bk Berkelium	98 Cf Californium	99 Es Einsteinium	100 Fm Fermium	101 Md Mendelevium	102 No Nobelium	103 Lr Lawrencium

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www.fluidigm.com

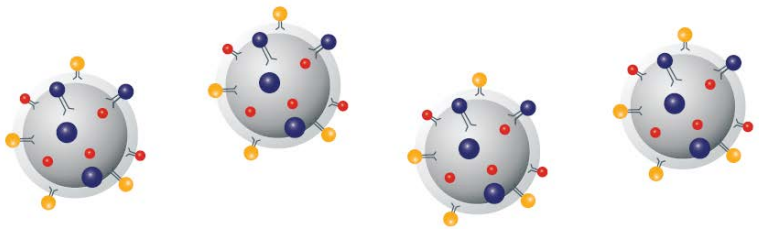
Flow Cytometry Core Facility
Dr. Stefanie Bürger
s.buerger@imb-mainz.de

www.imb.de

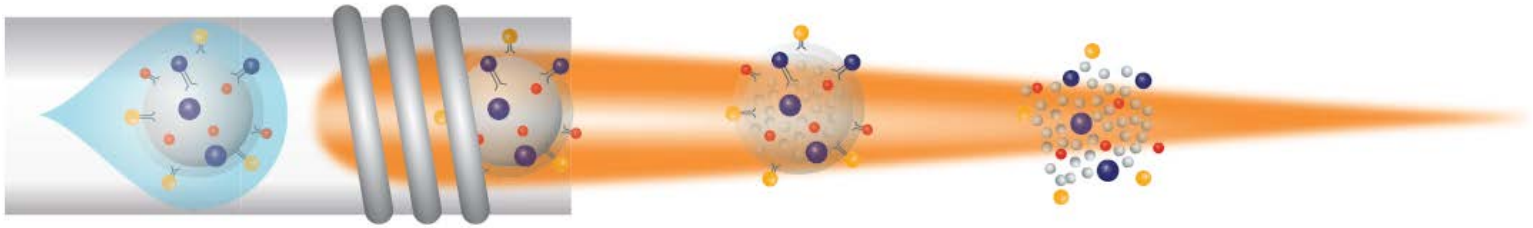
2018-06-25

Institute of Molecular Biology

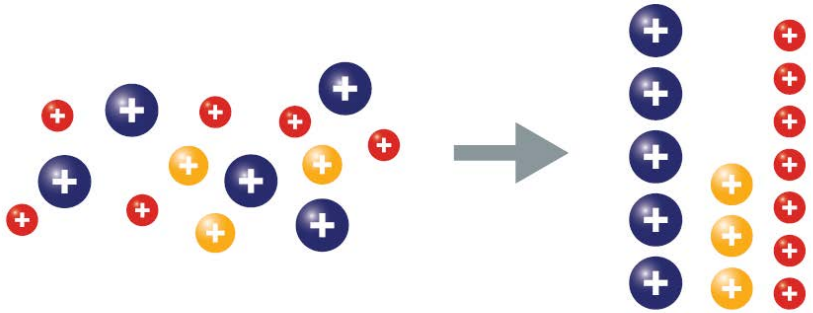
Mass cytometry



Inside the instrument, cells are individually atomized to release metal ions. Ions derived from each stained cell are maintained in discrete clouds



Metal ions of interest are resolved by mass in the time of flight (TOF) chamber



FLUIDIGM®

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Mass cytometry

Major advantage

- In multicolor flow cytometry, overlapping emission spectra call for mathemathical algorithms that need to be applied (“compensation”). Compensation may lead to a decrease in signal resolution, so it may be more difficult to identify rare cell populations

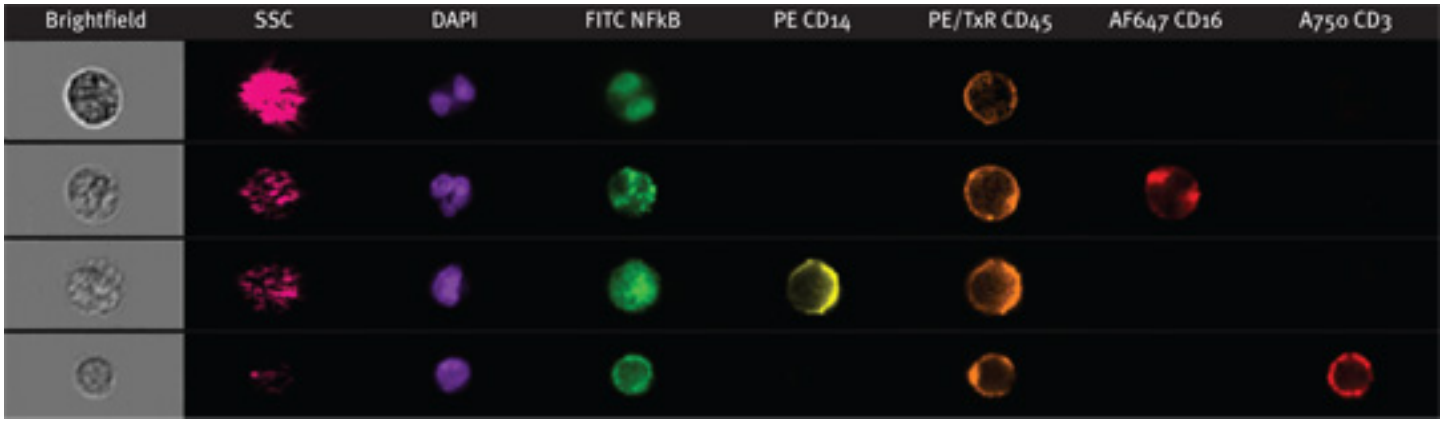
Disadvantage

- Low data acquisition rate: around 500 cells per second versus several thousand in flow cytometry
- Current chemical methods limits cytometer use to around 40 parameters per cell
- CyTOF is expensive (> 1 Mio EUR) and requires one dedicated operator and special lab conditions

www.fluidigm.com

Imaging Flow Cytometry: ImageStream

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



MERCK

Flow Cytometry Core Facility
Dr. Stefanie Bürger
s.buerger@imb-mainz.de

Imaging Flow Cytometry: ImageStream (Merck)

Advantage

- Information about signal distribution within the cell (spatial distribution) and about cell morphology of a high number of cells

Disadvantage

- Low data acquisition rate (5000 evts/sec)
- Only up to 10 colors
- Huge data amount per measurement
- Image quality

Next step in Imaging Flow Cytometry:
Image based sorting is already in development

Flow Cytometry: Take home message

- Parameters measured in standard flow cytometers are **relative size** (**forward scatter**), **granularity/complexity** (**side scatter**) and several **fluorescence parameters** (from 1 up to 50 colors 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 populations of interest on cell sorters