Basics of Flow Cytometry

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ISAC Live Education Task Force

What is flow cytometry?

A technology which allows us to measure:

Light scatter

fluorescence intensity

on cells or other particles

one by one (cells are in suspension)

.

What are the advantages of flow cytometry?

Speed

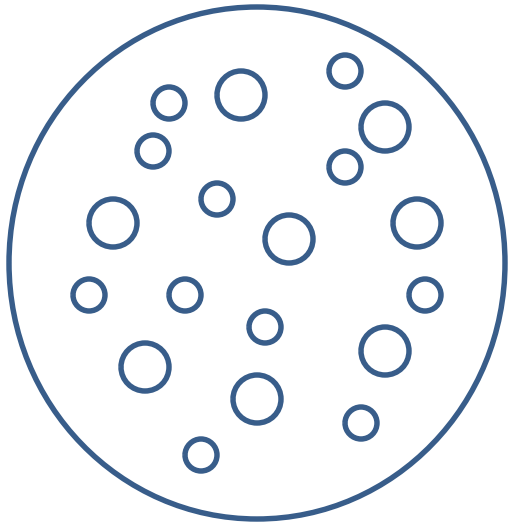
Millions of single cells

Increased statistical significance

Multi-parameter

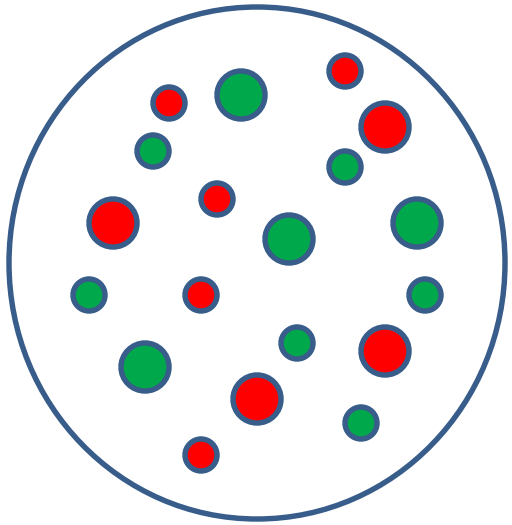
Cell sorting

When should we use a flow cytometer?



How many Small and/or Big Cells are there ?

Parameter: Size



How many Small cells are Green and/or Red?

How many Big cells are Green and/or Red?

Parameter: Color (Fluorescence)

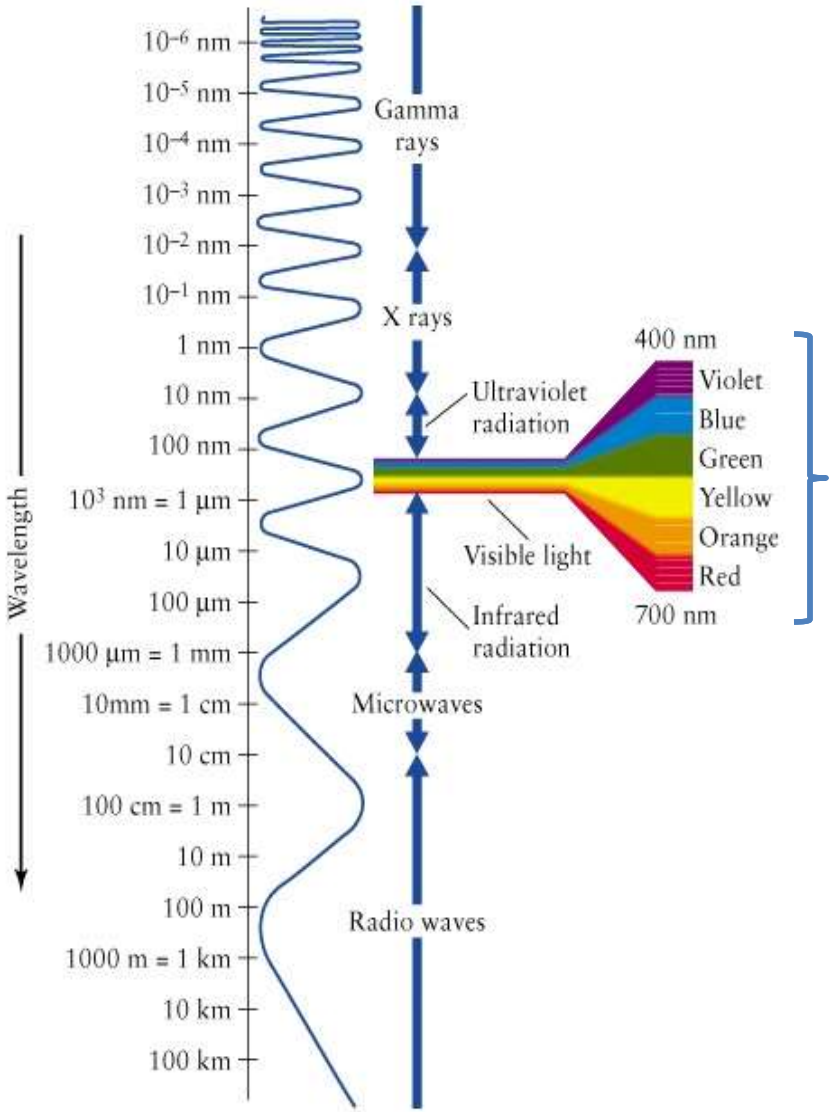
Courtesy of Dr Krishnamurthy

ncbs TIFR

Overview

- Light
- What we measure:
    - Fluorescence
    - Light scatter
- How a flow cytometer works
    - Fluidics
    - Optics
    - Electronics
    - Cell sorting

Light: the range of wavelengths used in cytometry



350-800 nm

Short wavelength
Higher energy

Long Wavelength
Lower energy

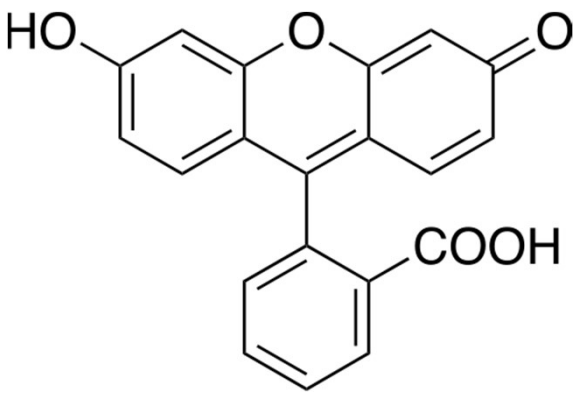
Fluorescence

Fluorochromes

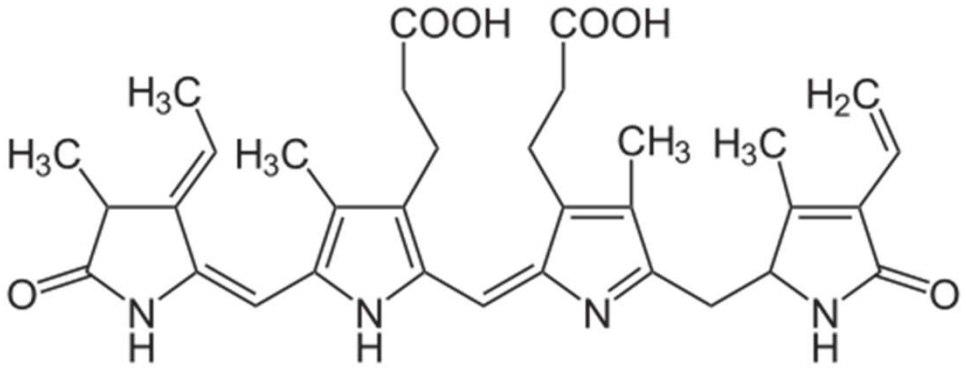
Fluorochromes are molecules
which absorb light at one wavelength
then re-emit the light energy at a longer wavelength

Structures are generally aromatic rings

Fluorescein (FITC)

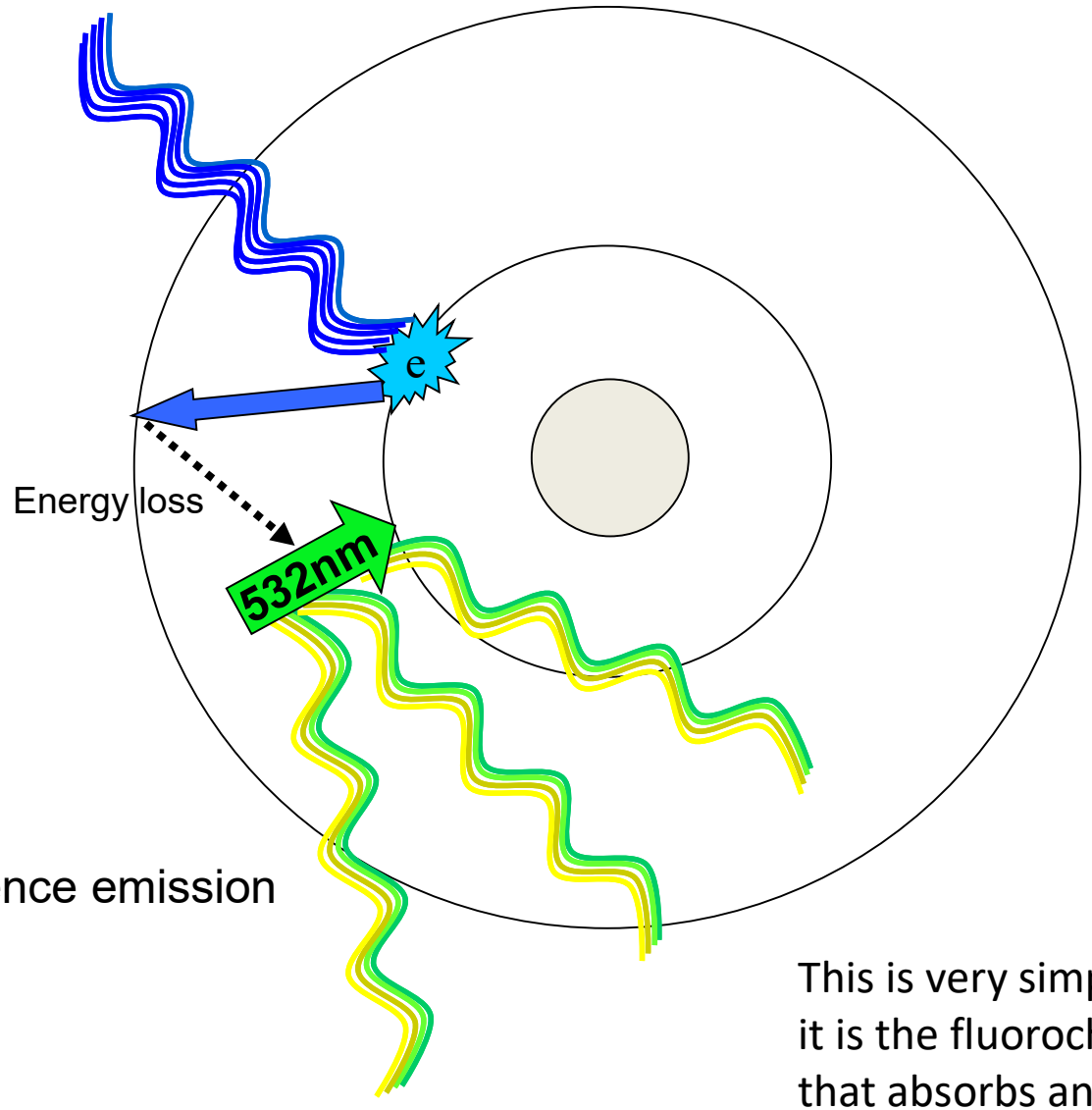


Phycoerytherin (PE)



Fluorescence

Blue 488 Laser excitation

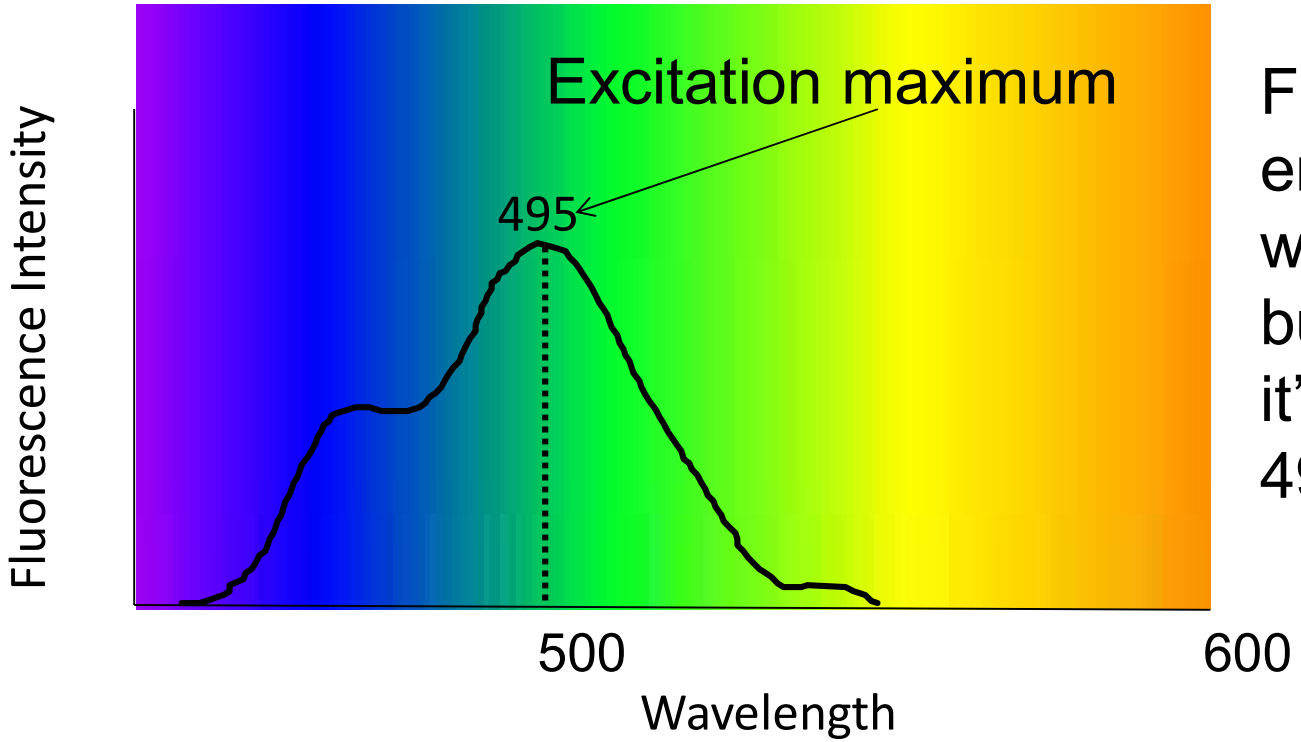


Green fluorescence emission

This is very simplified:
it is the fluorochrome's electron cloud
that absorbs and emits light energy

Excitation spectrum

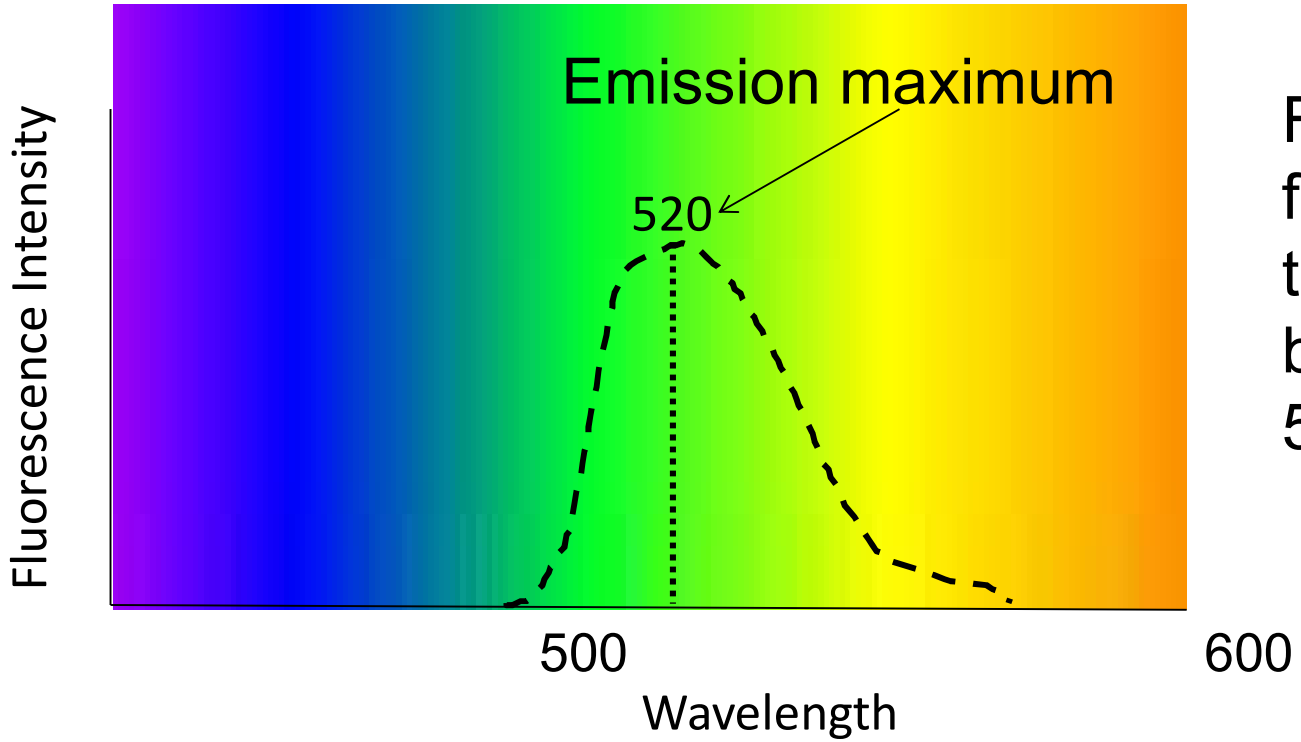
Each fluorochrome is capable of absorbing light energy over a specific range of wavelengths



FITC can absorb energy at all these wavelengths but absorbs best at it's excitation max: 495nm

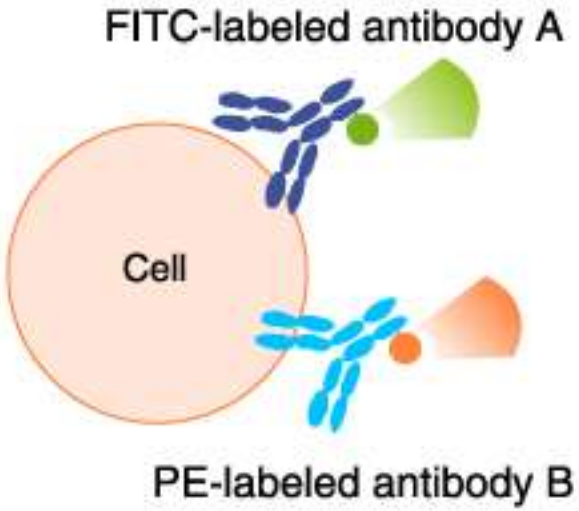
Emission spectra

Each fluorochrome is also capable of emitting light energy over a specific range of wavelengths

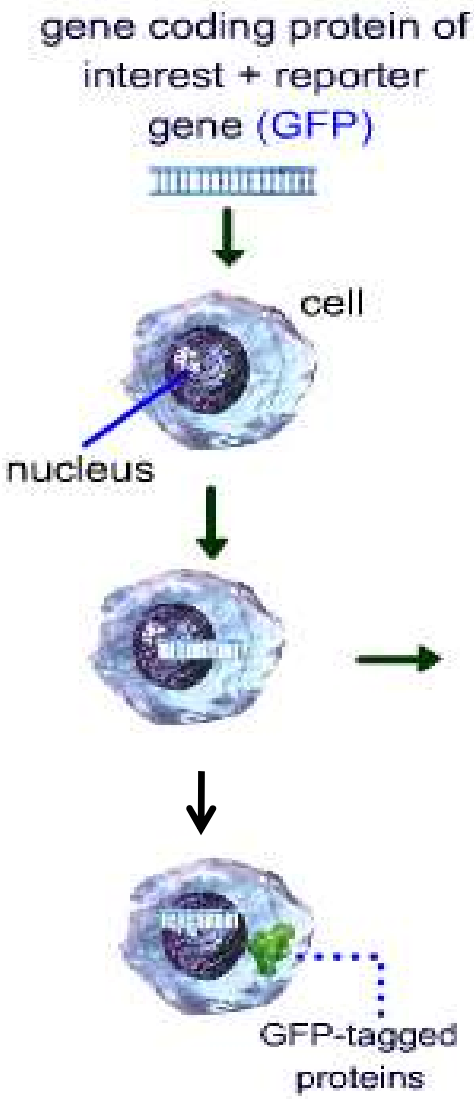


FITC will emit fluorescence at all these wavelengths but highest at 520nm

How do we use fluorochromes?



https://ruo.mbl.co.jp/bio/e/support/method/conjugated.html

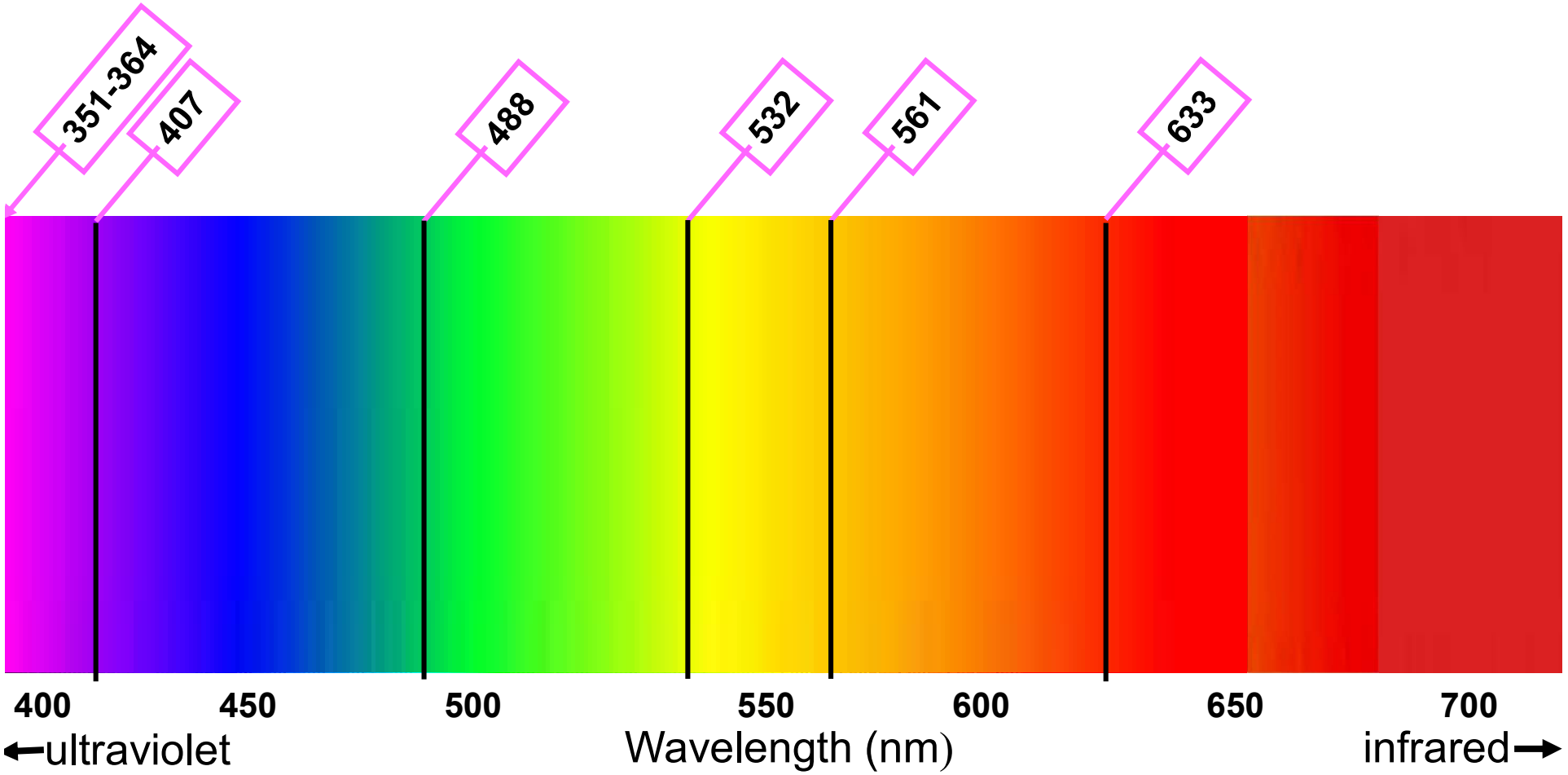


Many more specific for:

DNA
pH sensitive
Organelle specific
Calcium flux
Live/dead
Membrane potential
Oxidative states

Laser light is used to excite fluorochromes

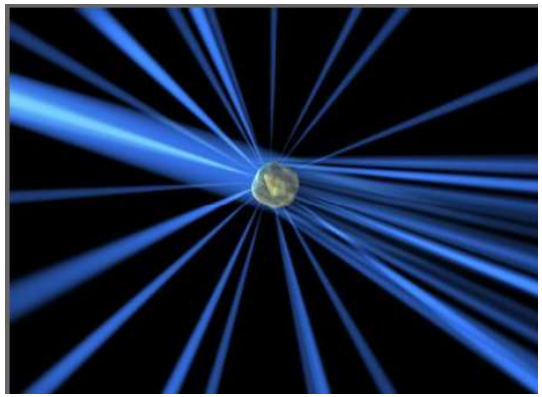
Lasers found on standard flow cytometers

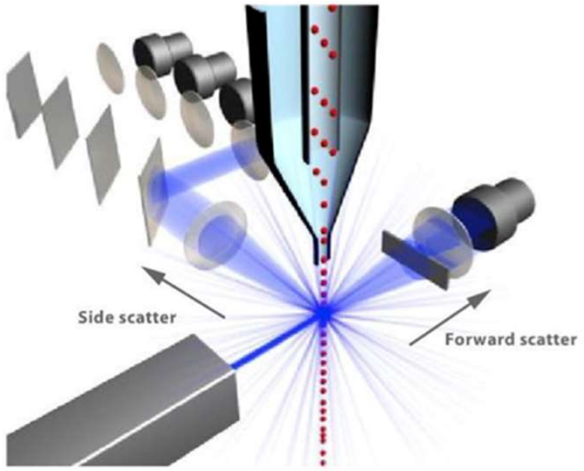


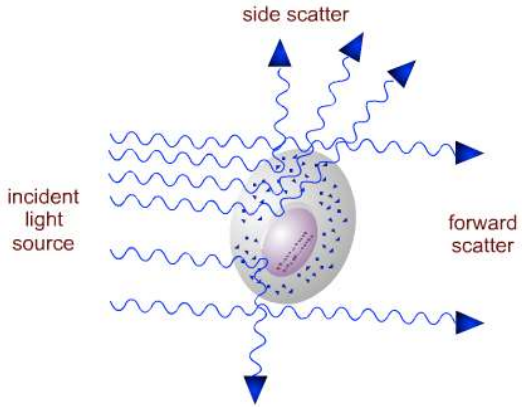
Light Scatter

Light scatter is also measured by flow cytometry

Light scatter is a physical property of the cell or particle which refracts or “scatters” light when it passes a laser beam







Light is scattered in all directions but we measure it at 2 angles:

Forward scatter (FSC): light scattered in the axis of the laser beam

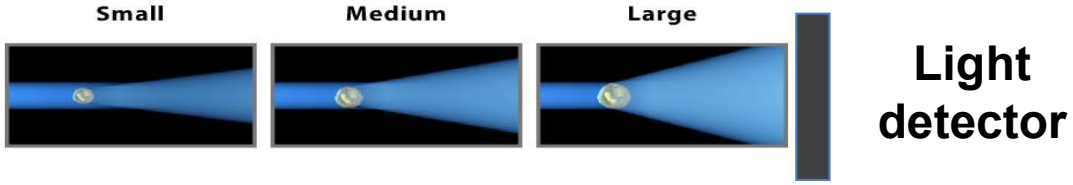
Side scatter (SSC): light scattered at a 90° angle to the laser beam.

Images from Life Technologies Flow Cytometry Tutorials

Courtesy of Kylie Price
Malaghan Institute

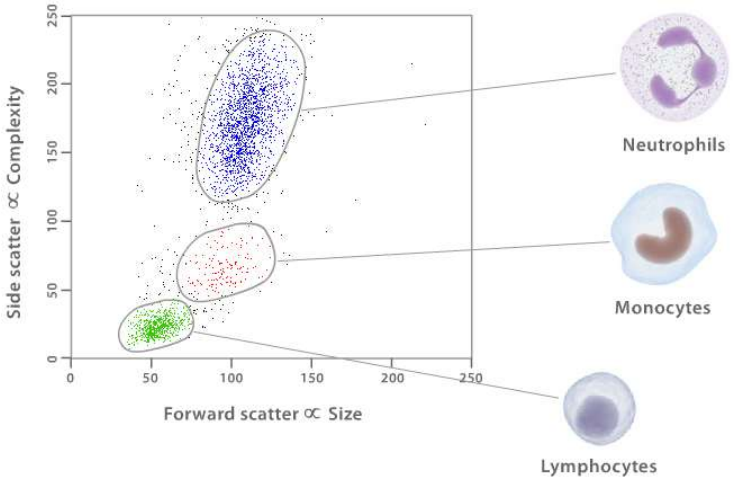
What does light scatter tell us?

Forward scatter is roughly proportional to cell surface properties and size



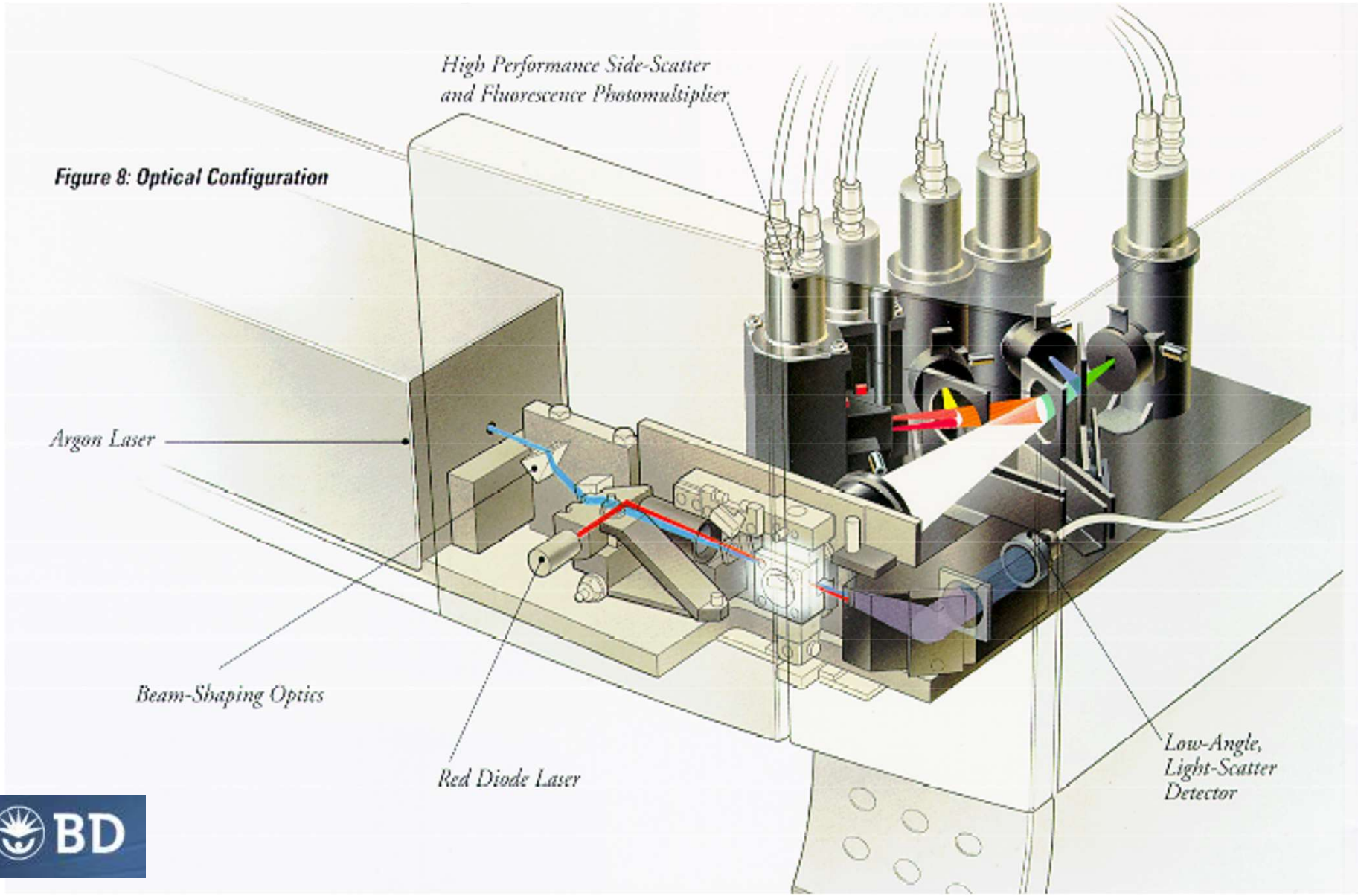
Side Scatter is affected by cell structural complexity and granularity

Neither of these are can be used to quantitate the size of cells, however they can be used to distinguish different types of cells



Courtesy of Kylie Price
Malaghan Institute

It’s not a black box!

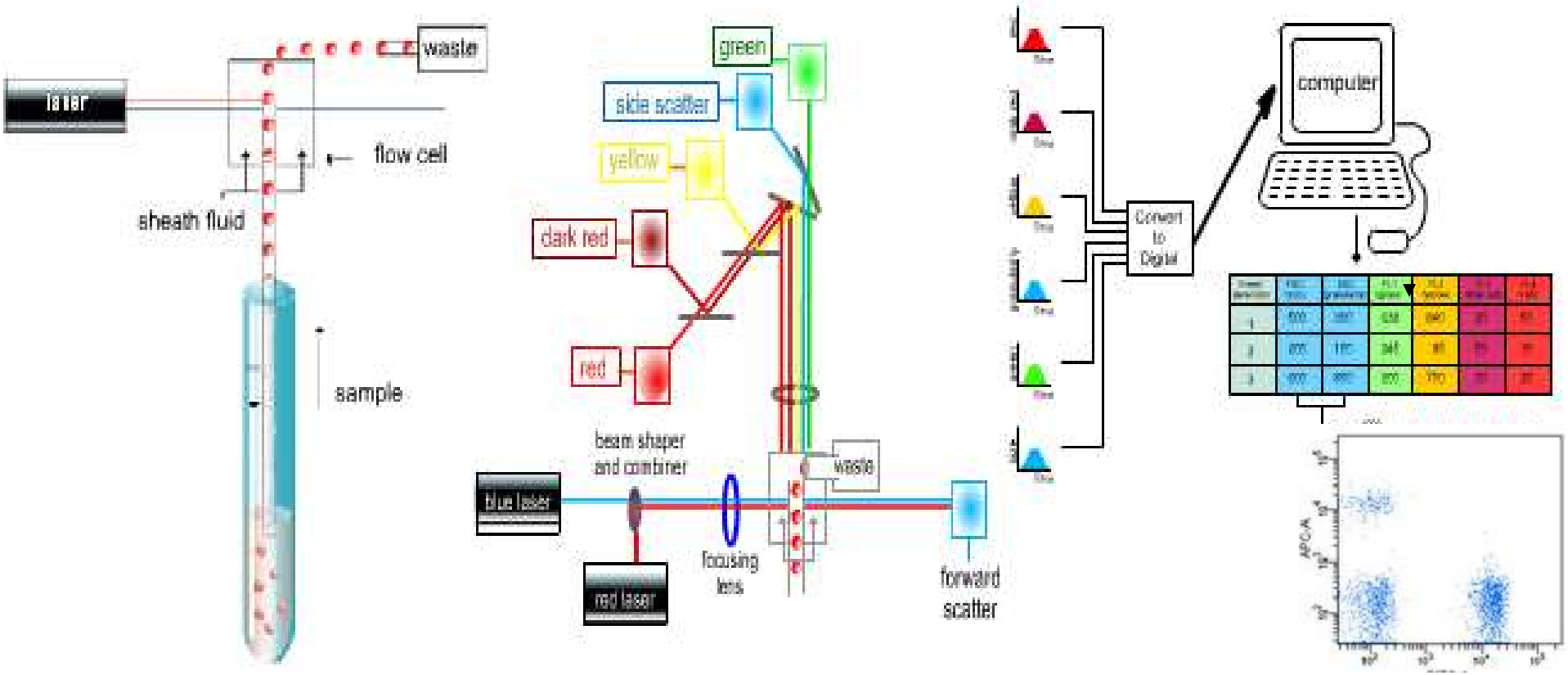


What do you find inside a Flow Cytometer?

Fluidics
Position cells
to flow one by one
past the laser beam

Optics
Separate the light emission
from different fluorochromes
and direct towards detectors

Electronics
Detectors convert light
emission to voltage pulses
which are digitalized



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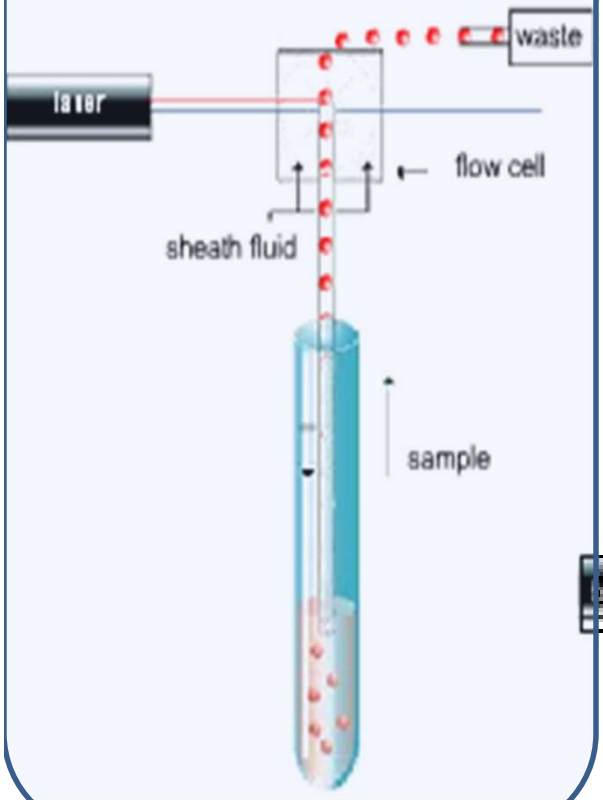
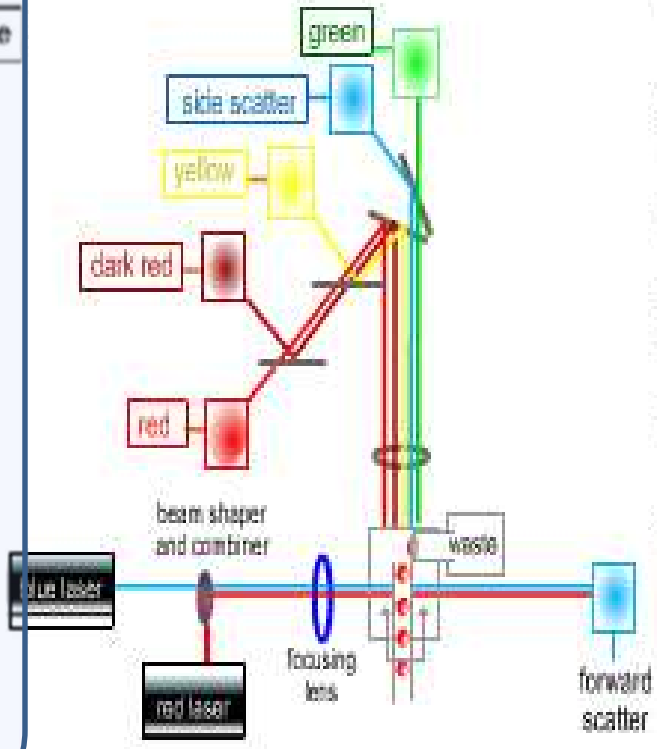
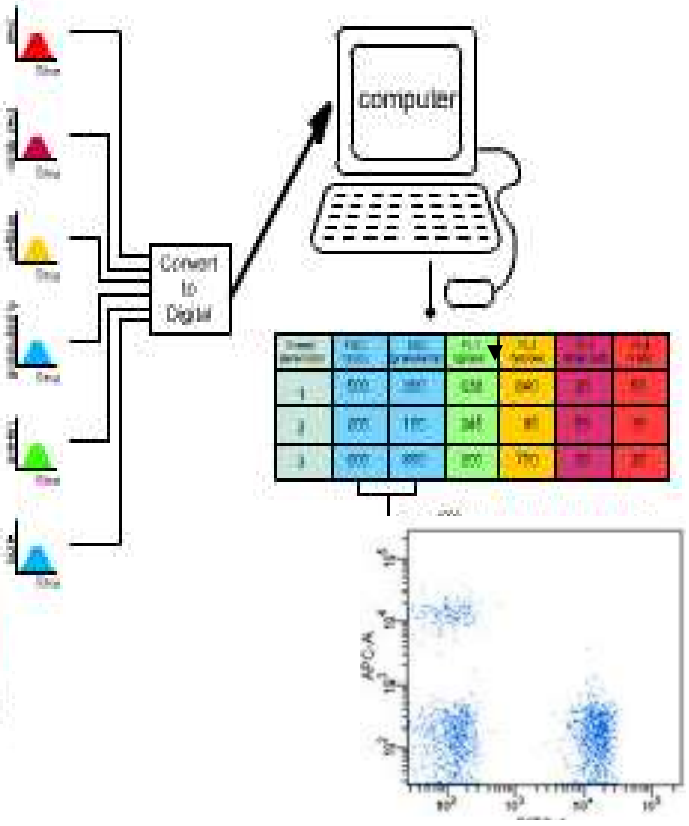
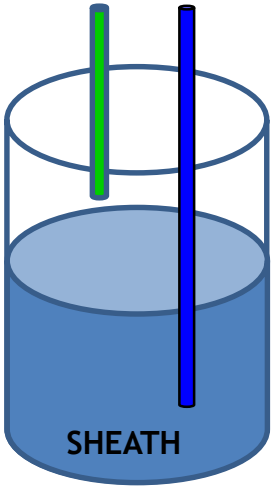
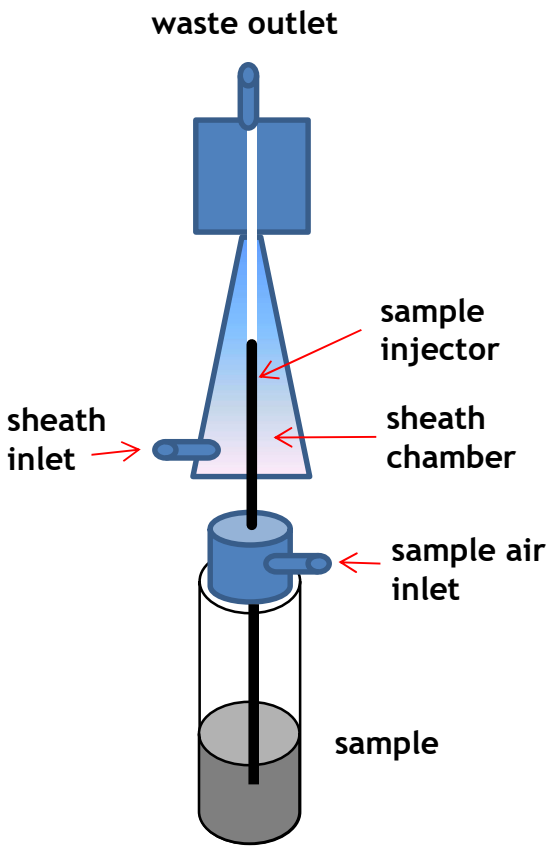


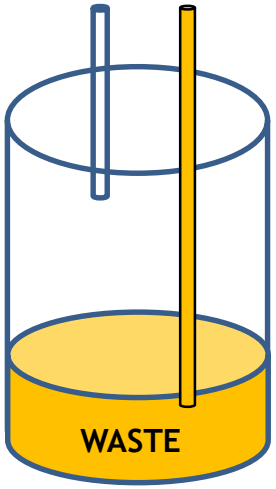




Instrument Fluidics:
positive air pressure system

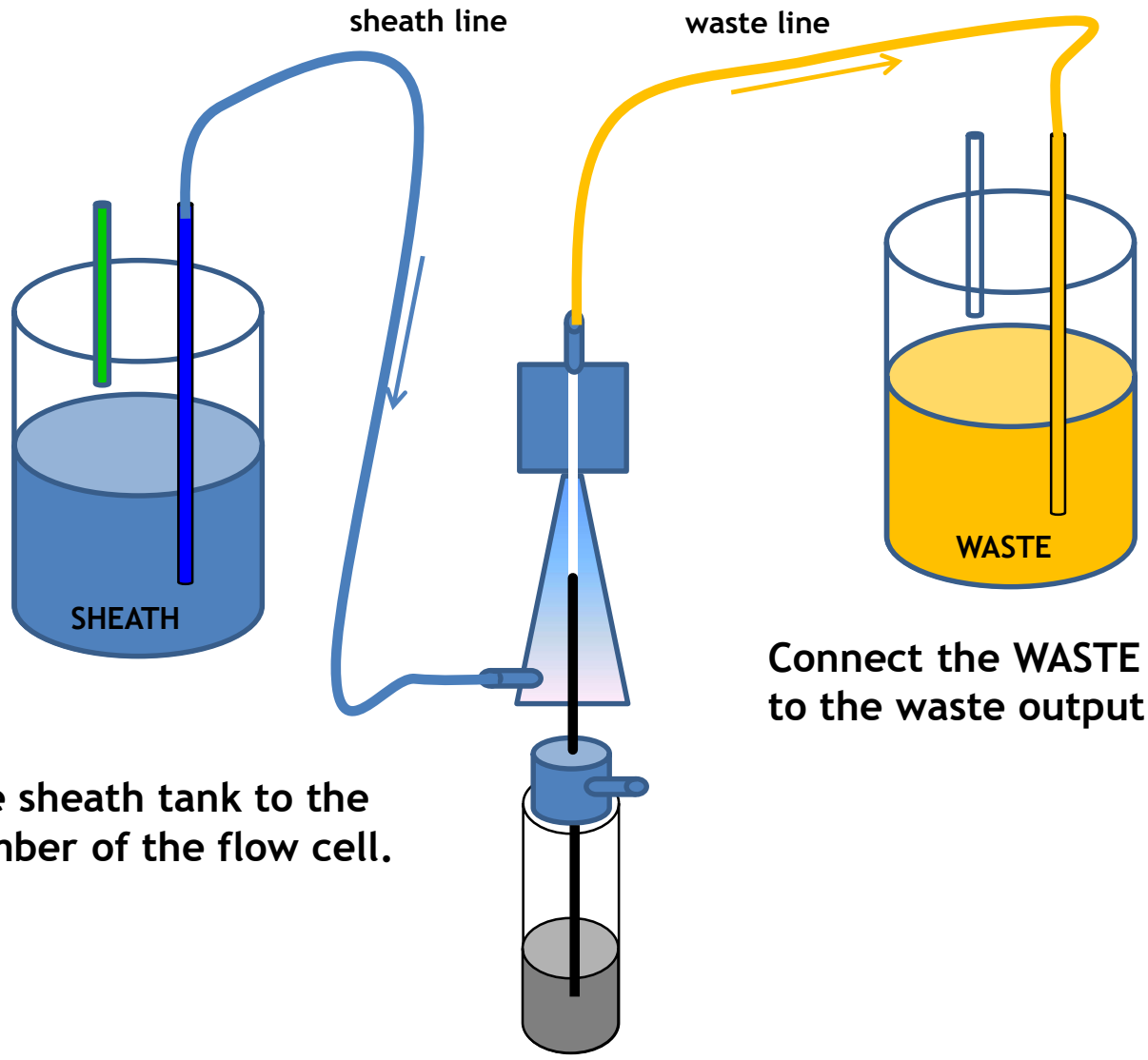






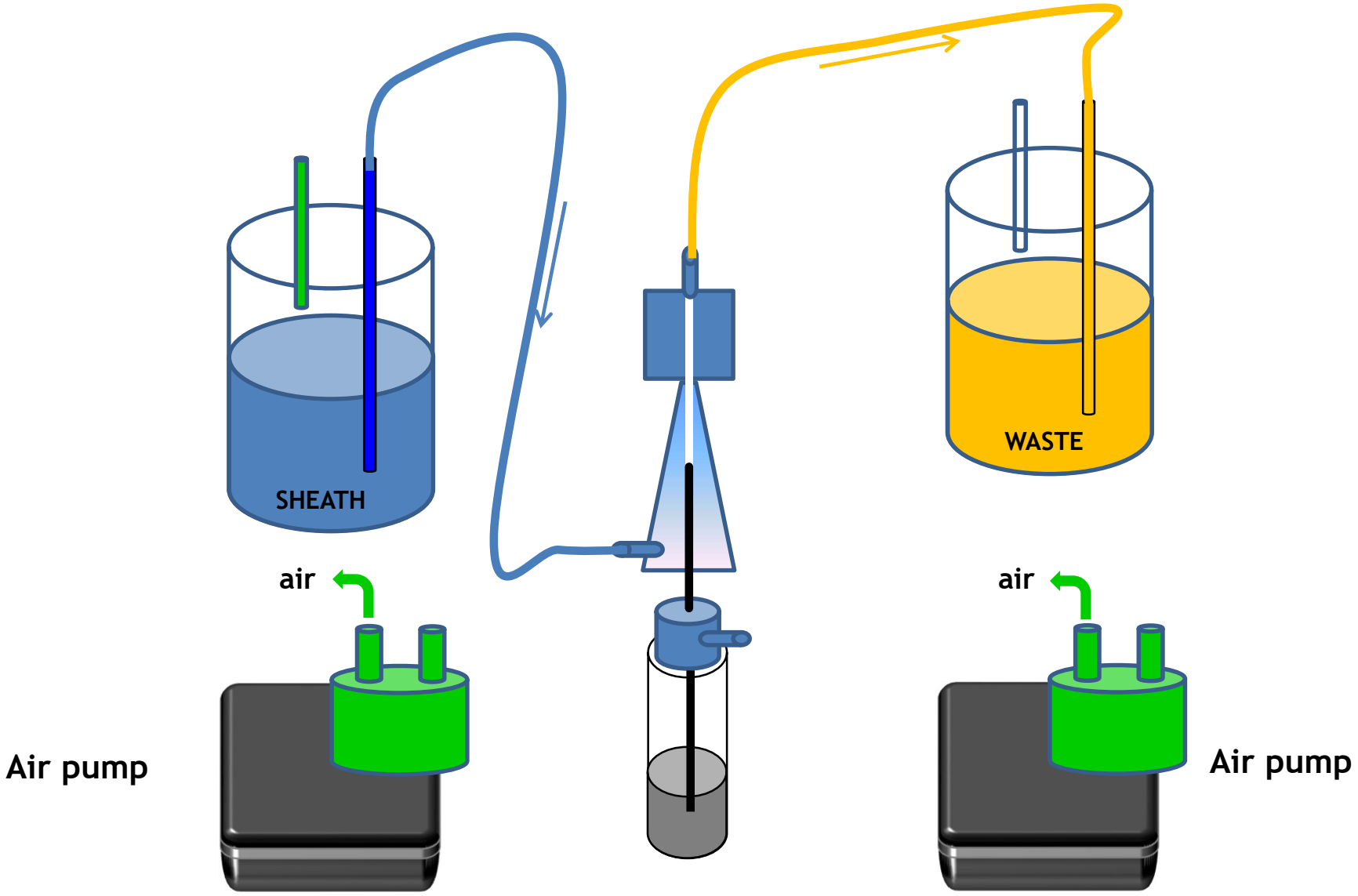
Sheath fluid can be
a saline solution, PBS, water

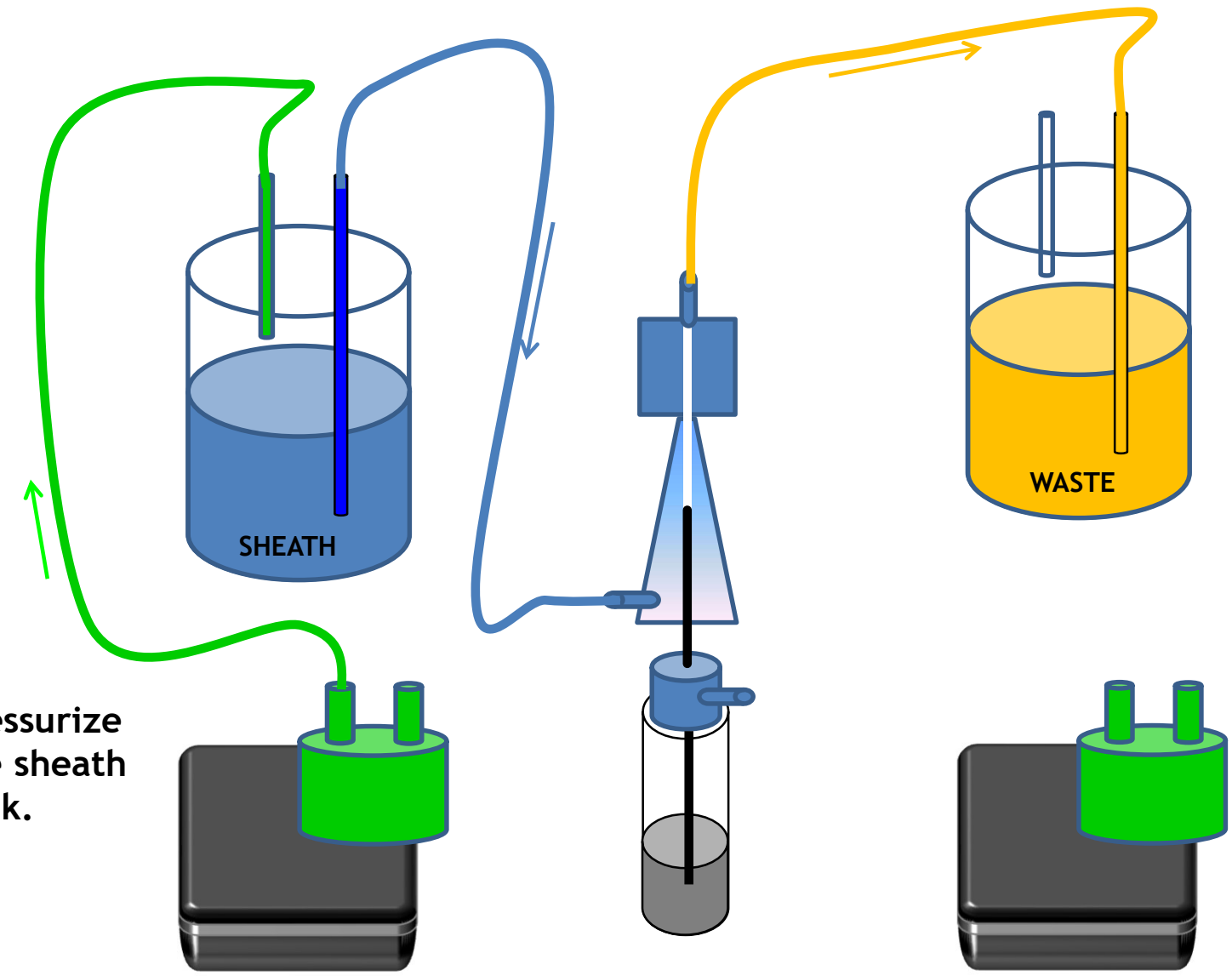
Slides courtesy of Bill Telford NIH



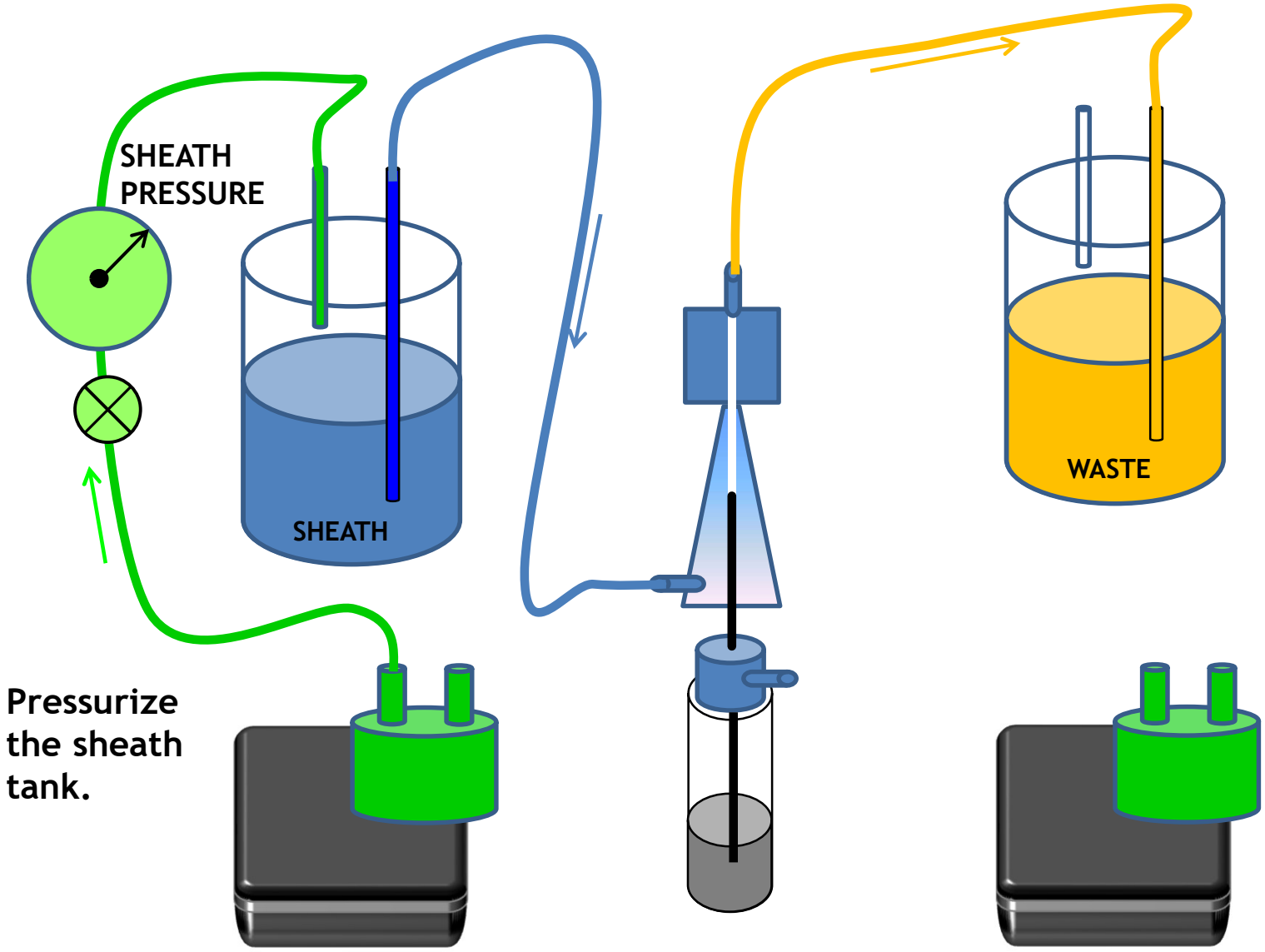
Connect the sheath tank to the sheath chamber of the flow cell.

Connect the WASTE tank to the waste output

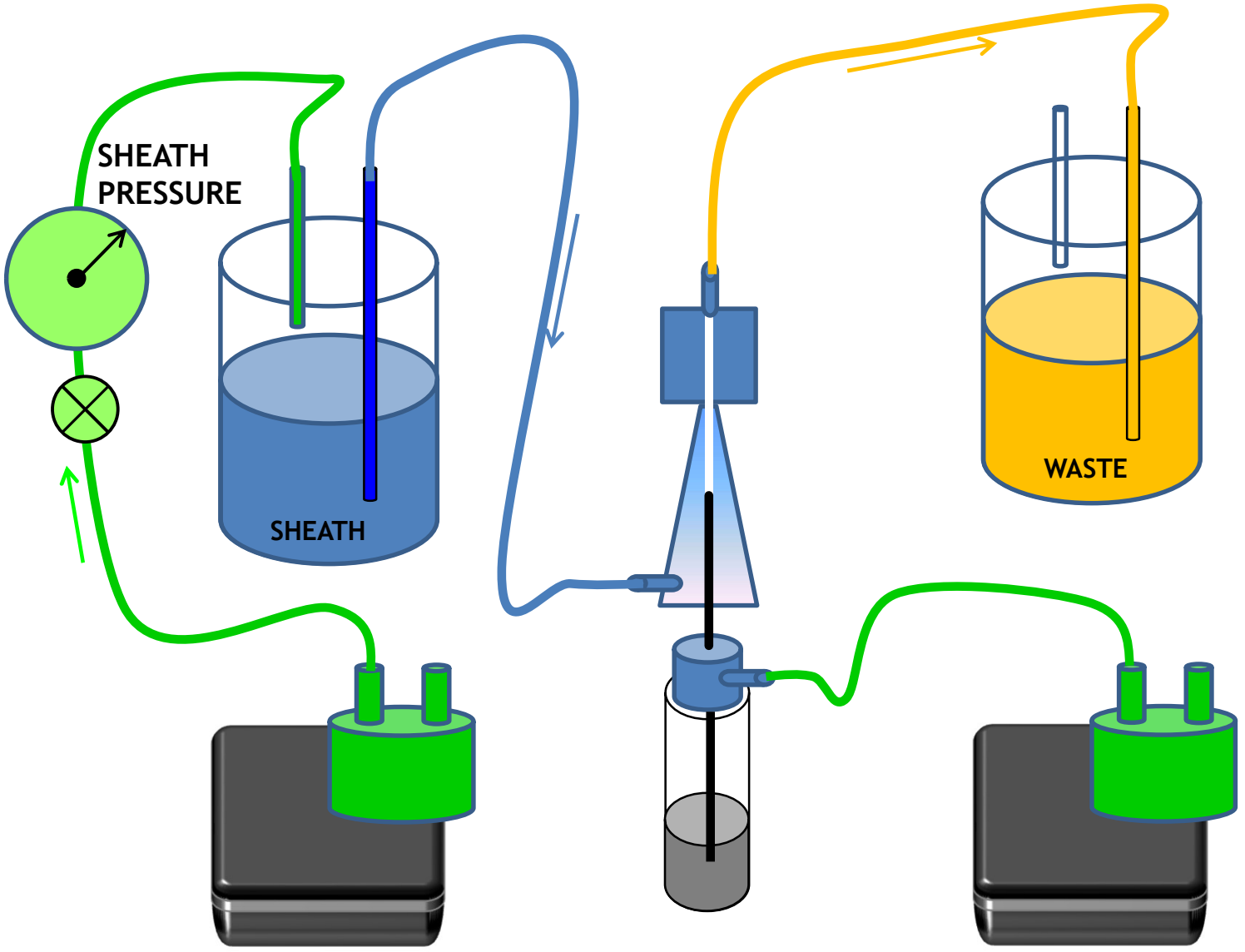

SHEATH
WASTE
air
air
Air pump
Air pump



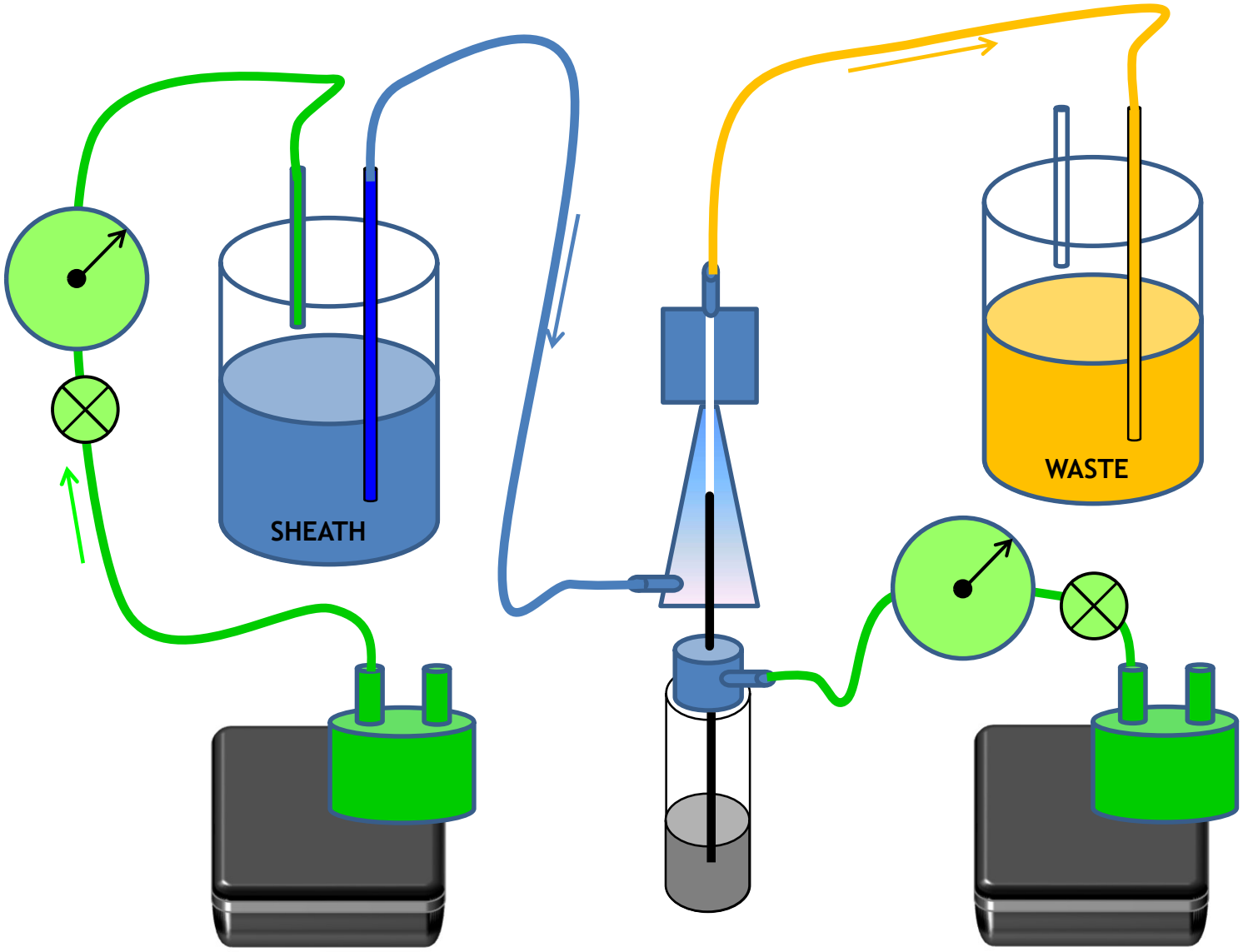
Pressurize the sheath tank.



Pressurize the sheath tank.



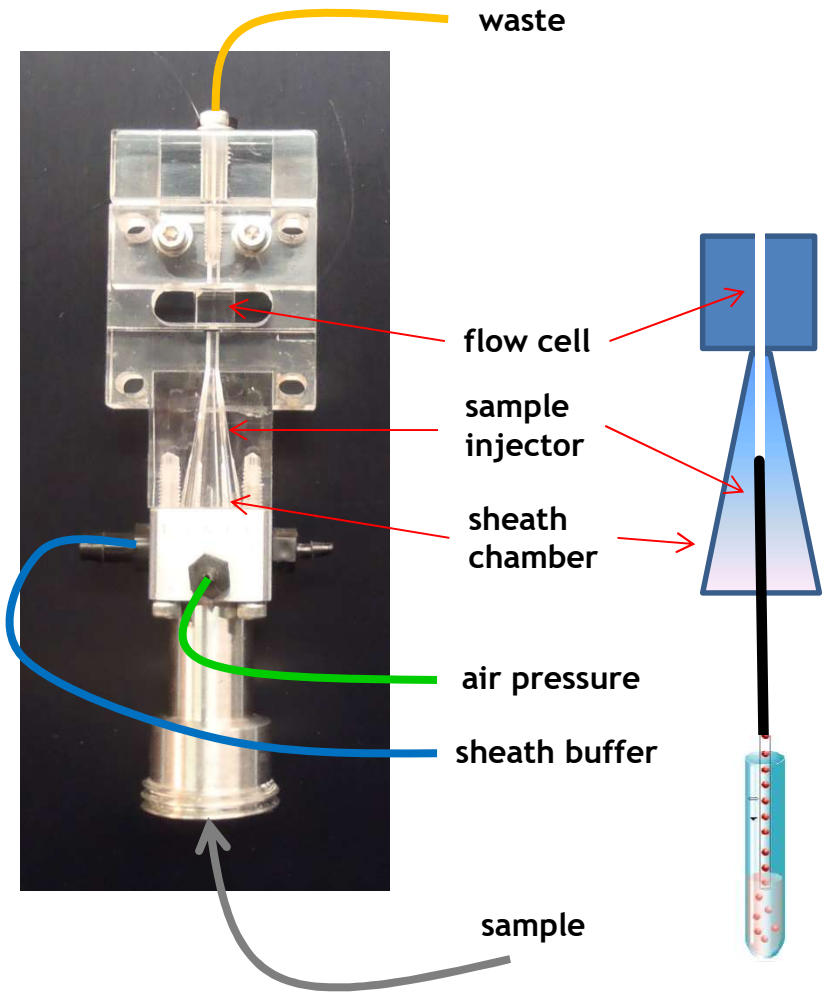
Pressurize the sample port.

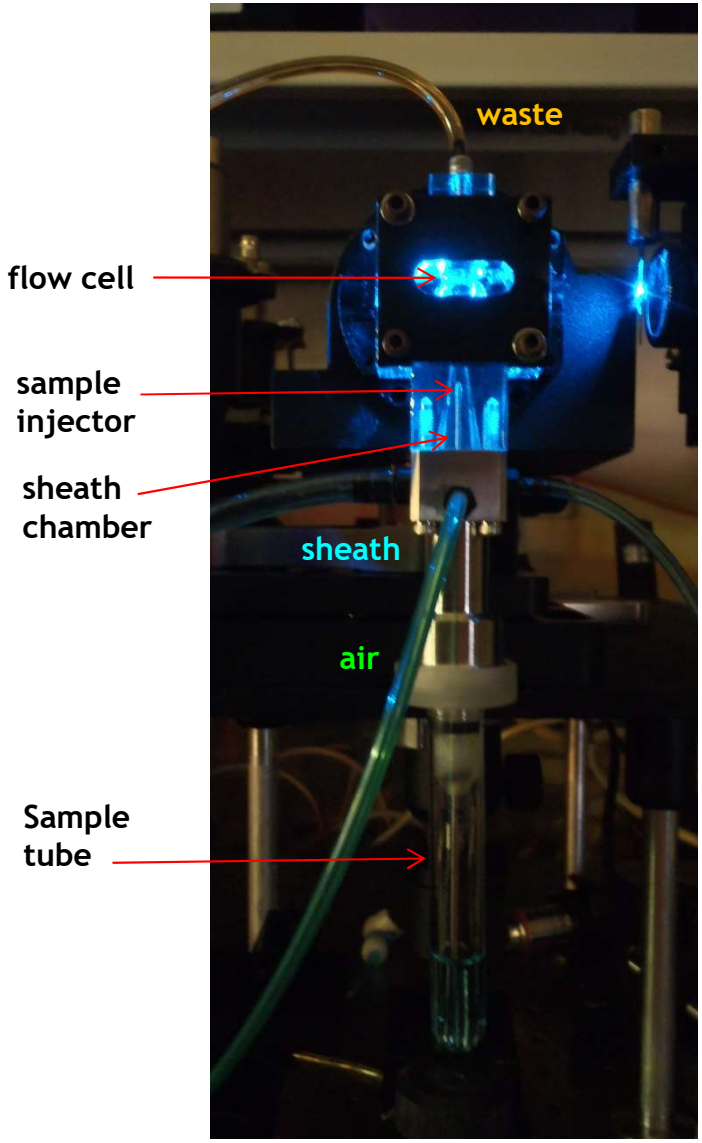


This is very simplified! Most commercial systems have complex pressure regulation mechanisms to carefully control sheath and sample delivery.

What a flow cell looks like

BD LSRII, Fortessa, FACSCalibur





Slide from Bill Telford NIH

Different ways to pump sheath and sample through the cytometer

1. Positive air pressure (which we've just seen)
   - LSRII, Fortessa, Calibur
   - Gallios
   - Sorters (Aria, Astrios, S3, etc)

2. Syringe pump
   - Guava
   - Attune
   - Novocyte (sample)

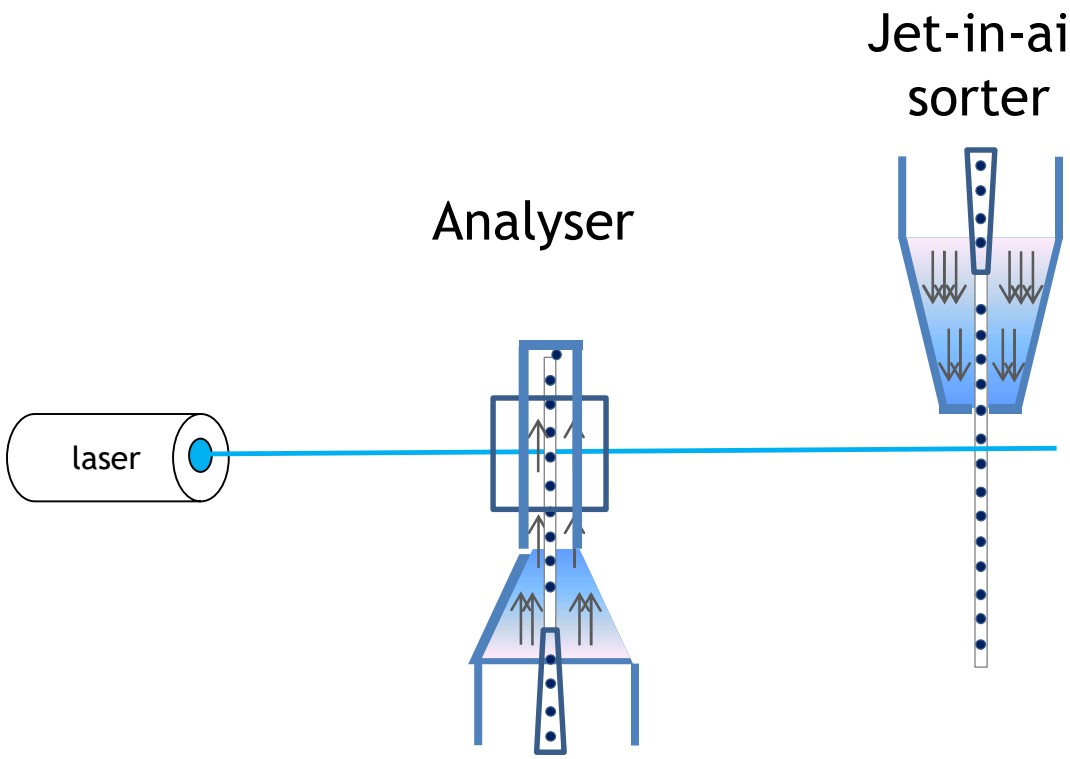
3. Peristaltic pump
   - Accuri
   - Cytoflex
   - Novocyte (sheath)
   - ZE5

Intercepting the sample stream with a laser

The laser beam is focused on the point in the sample stream where the cells will be analyzed.

On an analyser, this is inside the flow cell

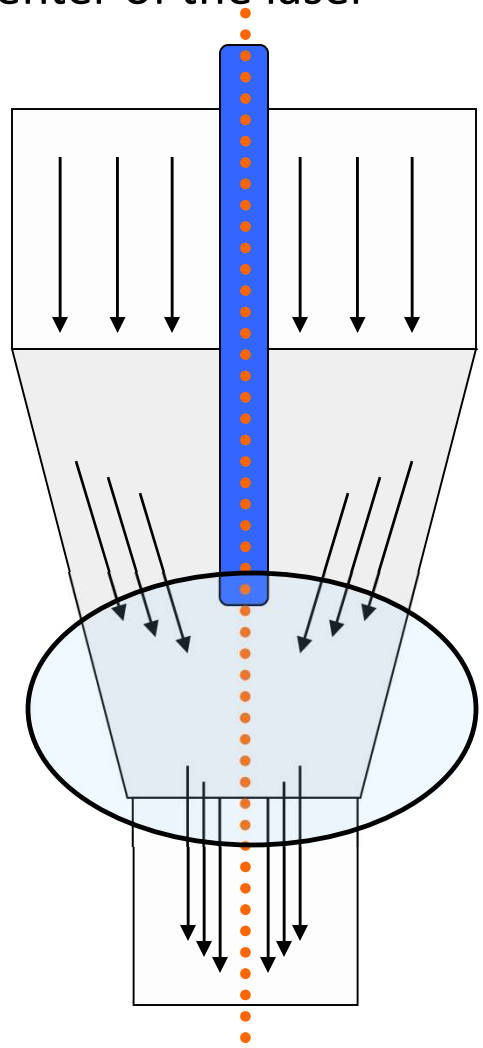
On a jet-in-air sorter, this is just below the nozzle

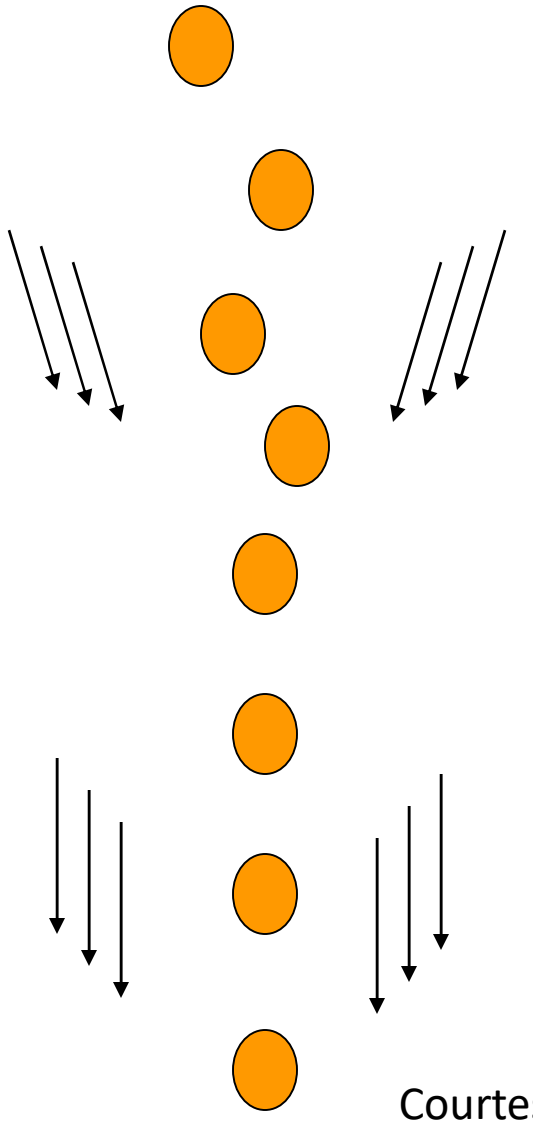


Slide courtesy of Bill Telford

Stream within a Stream: the role of hydrodynamic focusing

Cells are injected into the center of the sheath fluid so that they will be positioned in the center of the laser



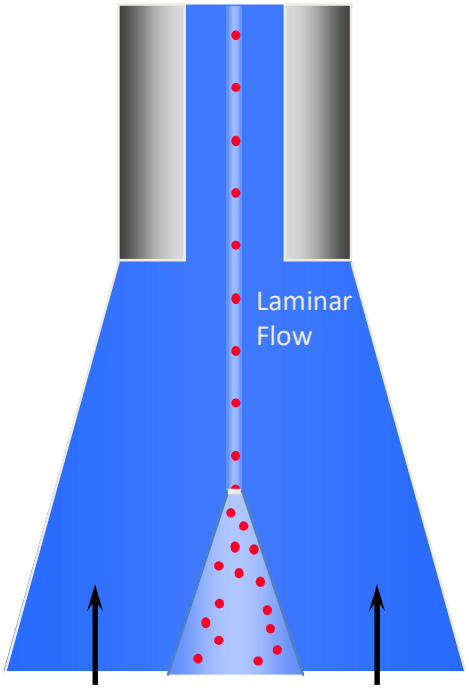


Courtesy of Alan Saluk

The effect of changing the sample pressure

Cytometer **sheath pressure** always remains **fixed**!

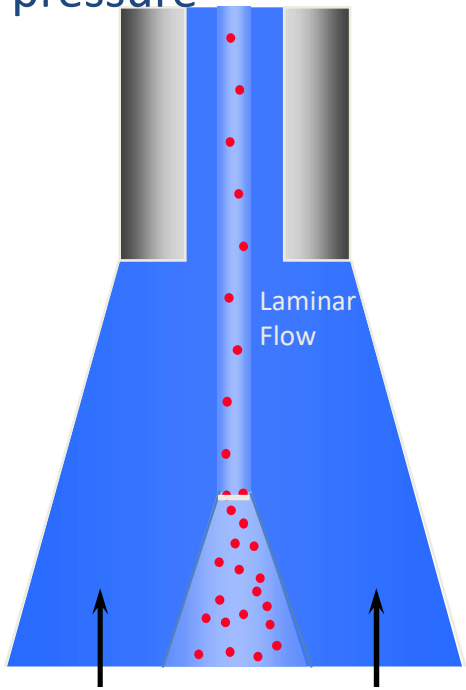
Low sample pressure



Narrow core

All cells pass through center of laser beam
Excitation and emission very uniform
Important to use low for DNA cell cycle analysis!

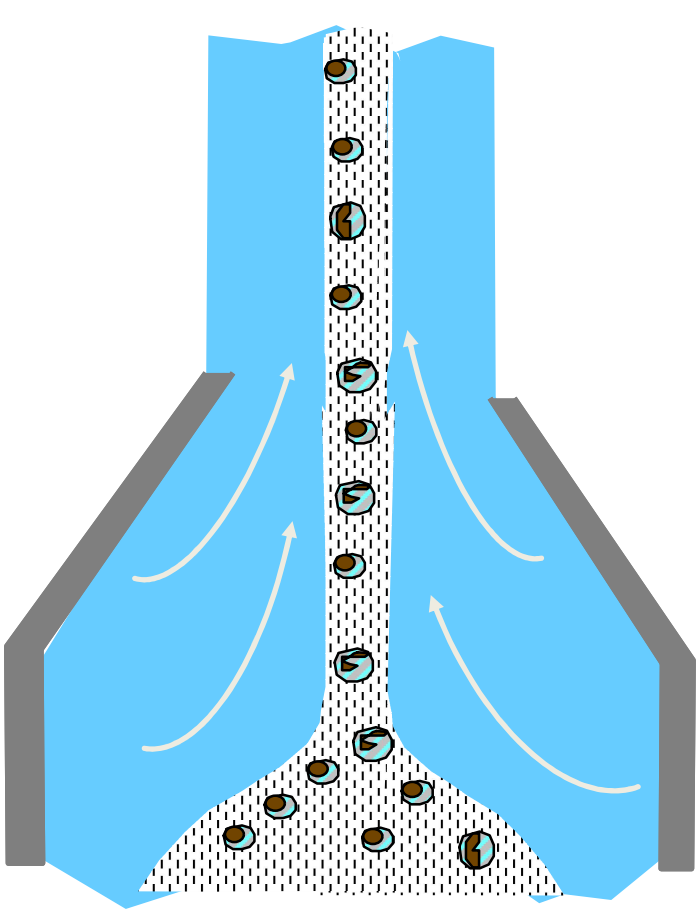
High sample pressure

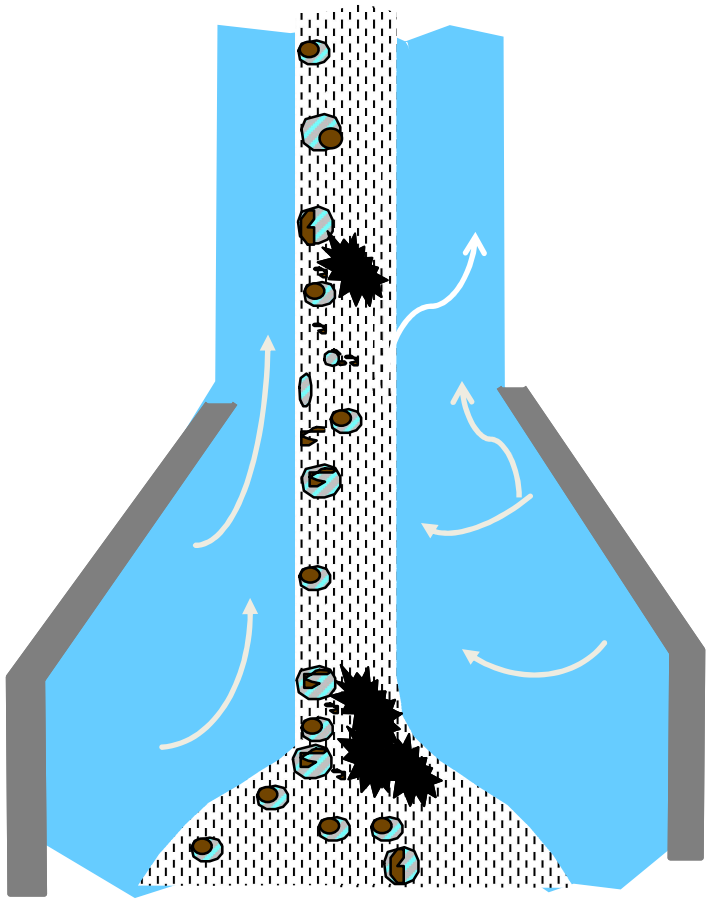


Wide core

Not all cells pass through center of laser beam
Excitation and emission not uniform

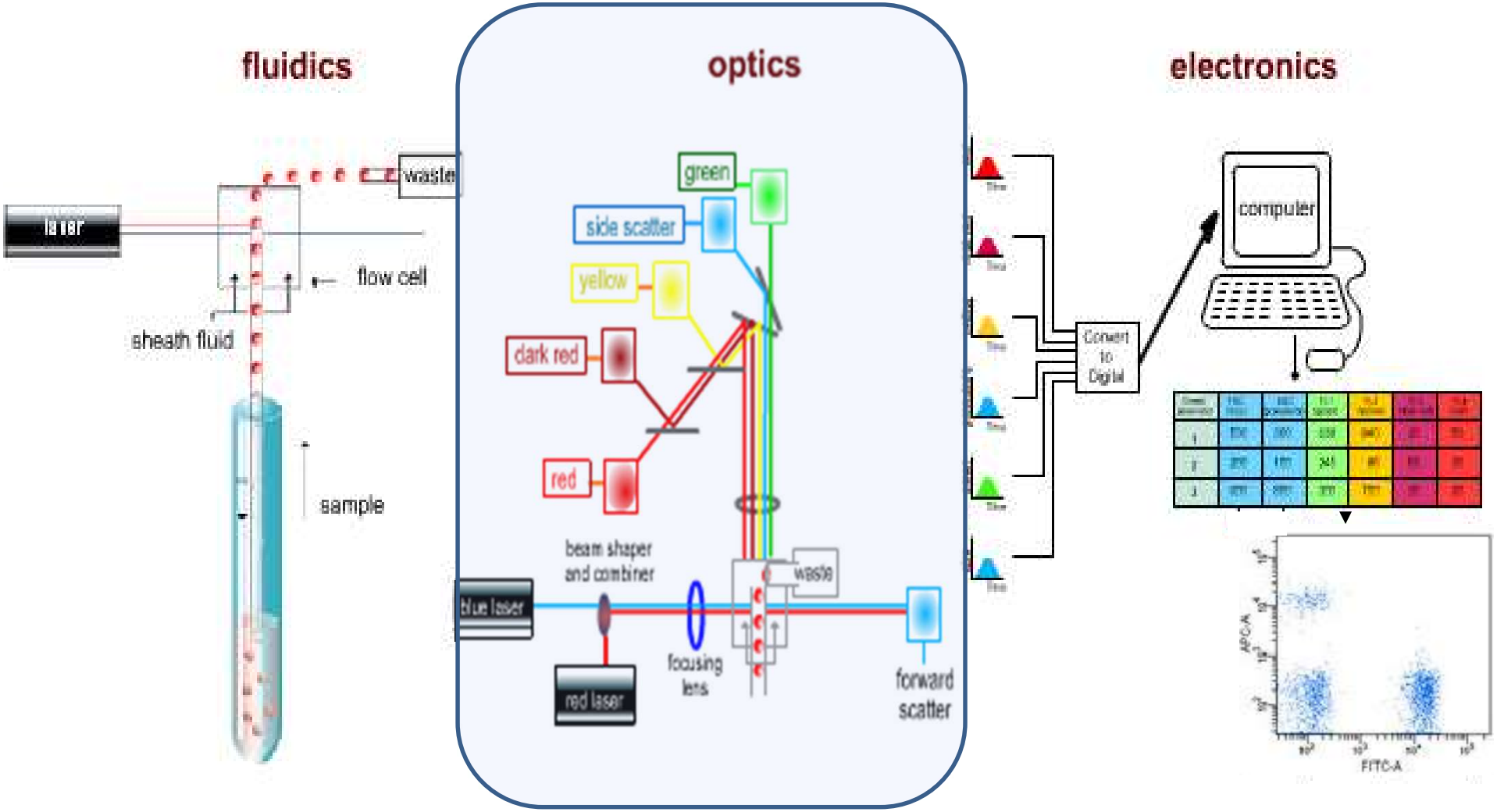
Air bubbles or dirt will decrease signal





Courtesy of Grace Chojnowski

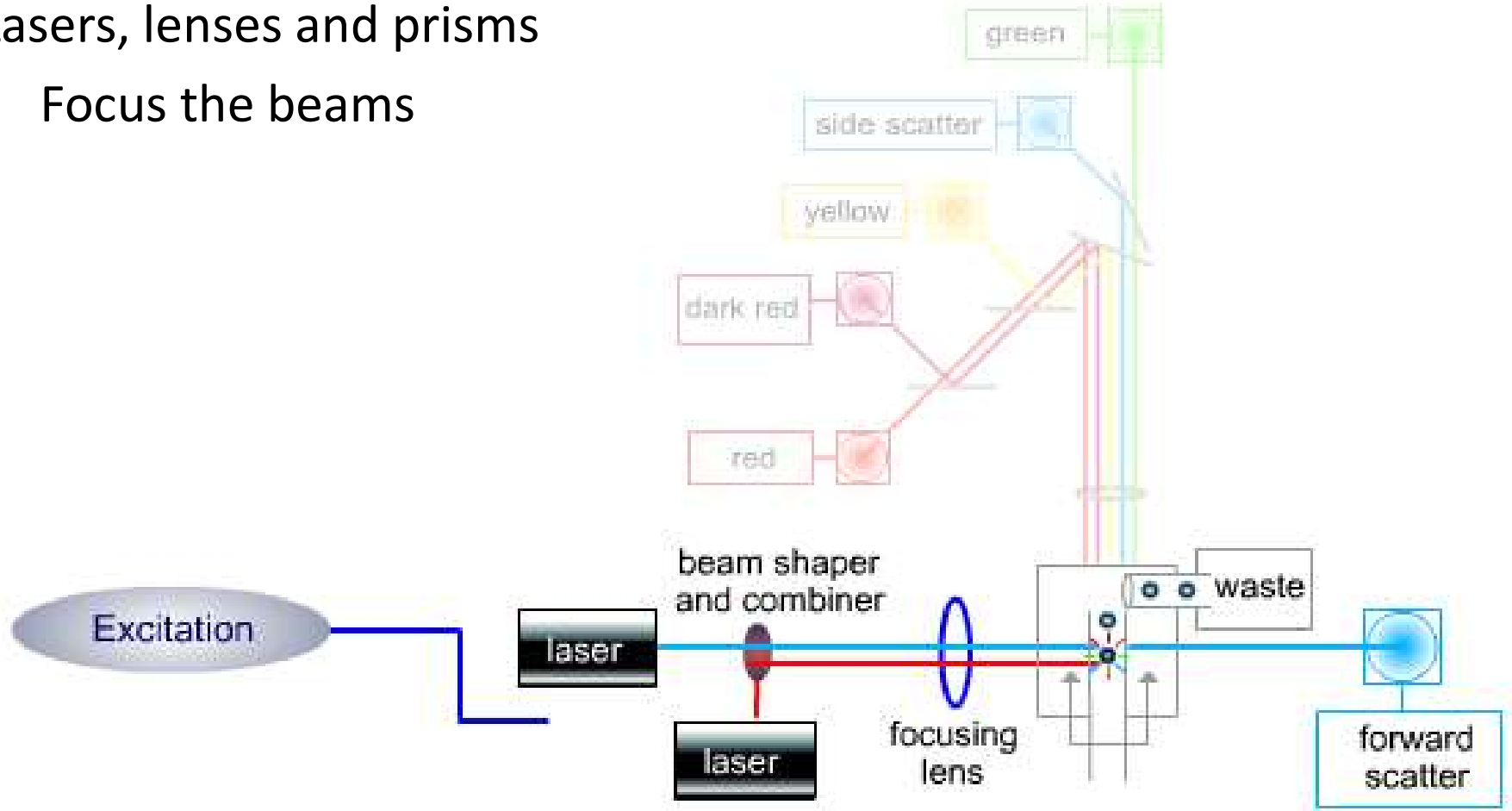
Flow Cytometer Elements



Excitation Optics

Lasers, lenses and prisms

Focus the beams



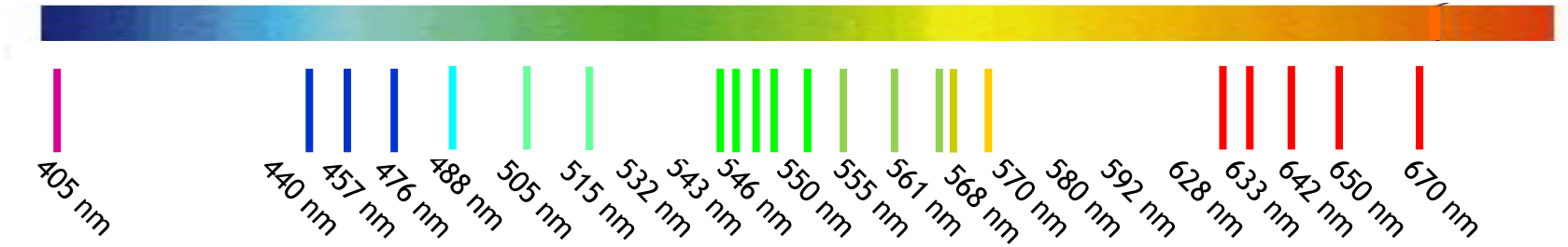
Let there be Light!

Laser characteristics

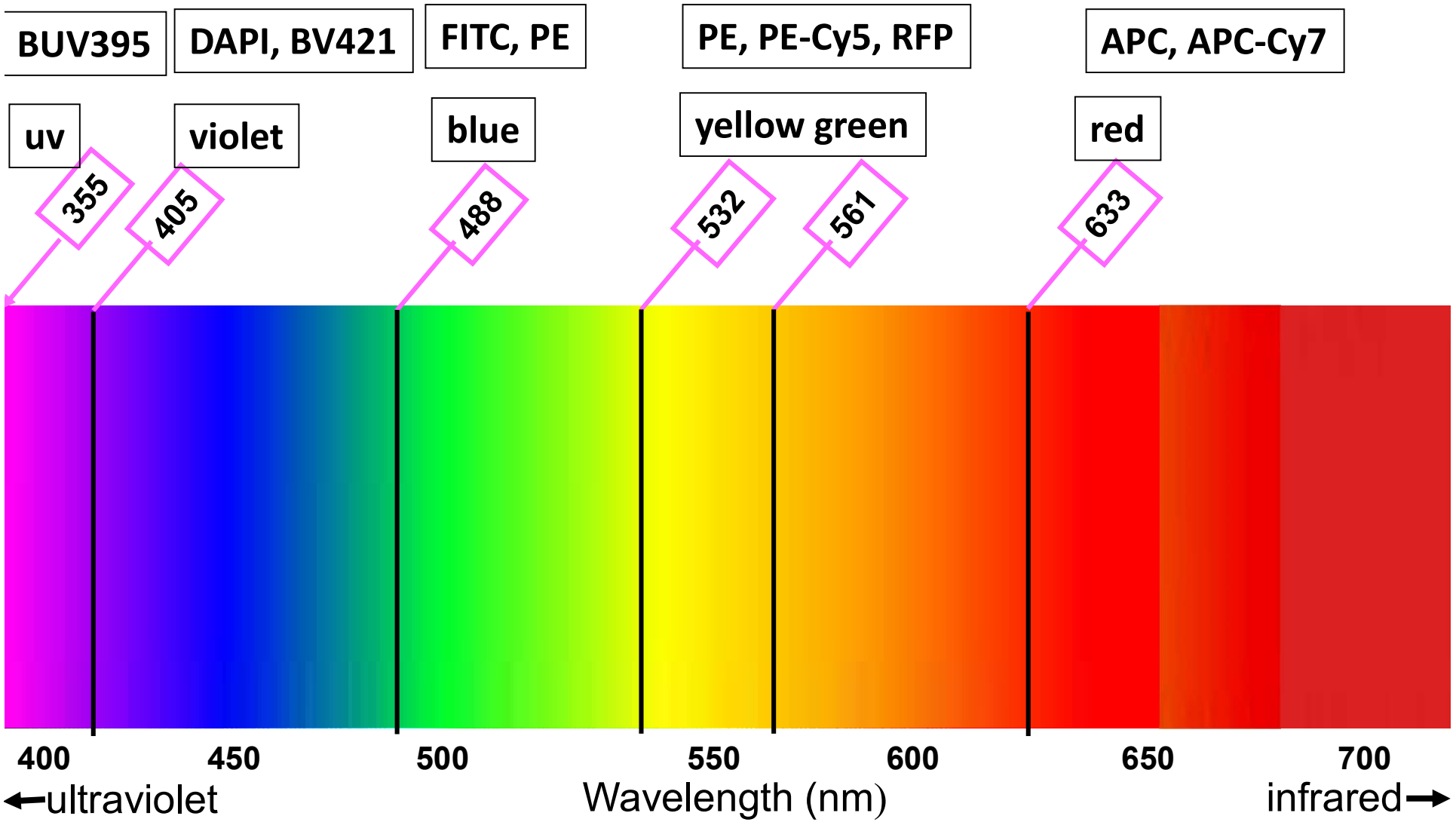
Bright
Coherent
Emit at a single wavelength
Stable
Focus to a tight spot on a tiny area
(like a sample stream)
getting smaller and cheaper!

New Generation Solid State Lasers

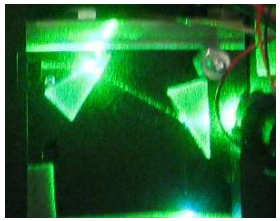
available in virtually **any color** allowing excitation of almost any fluorescent molecule



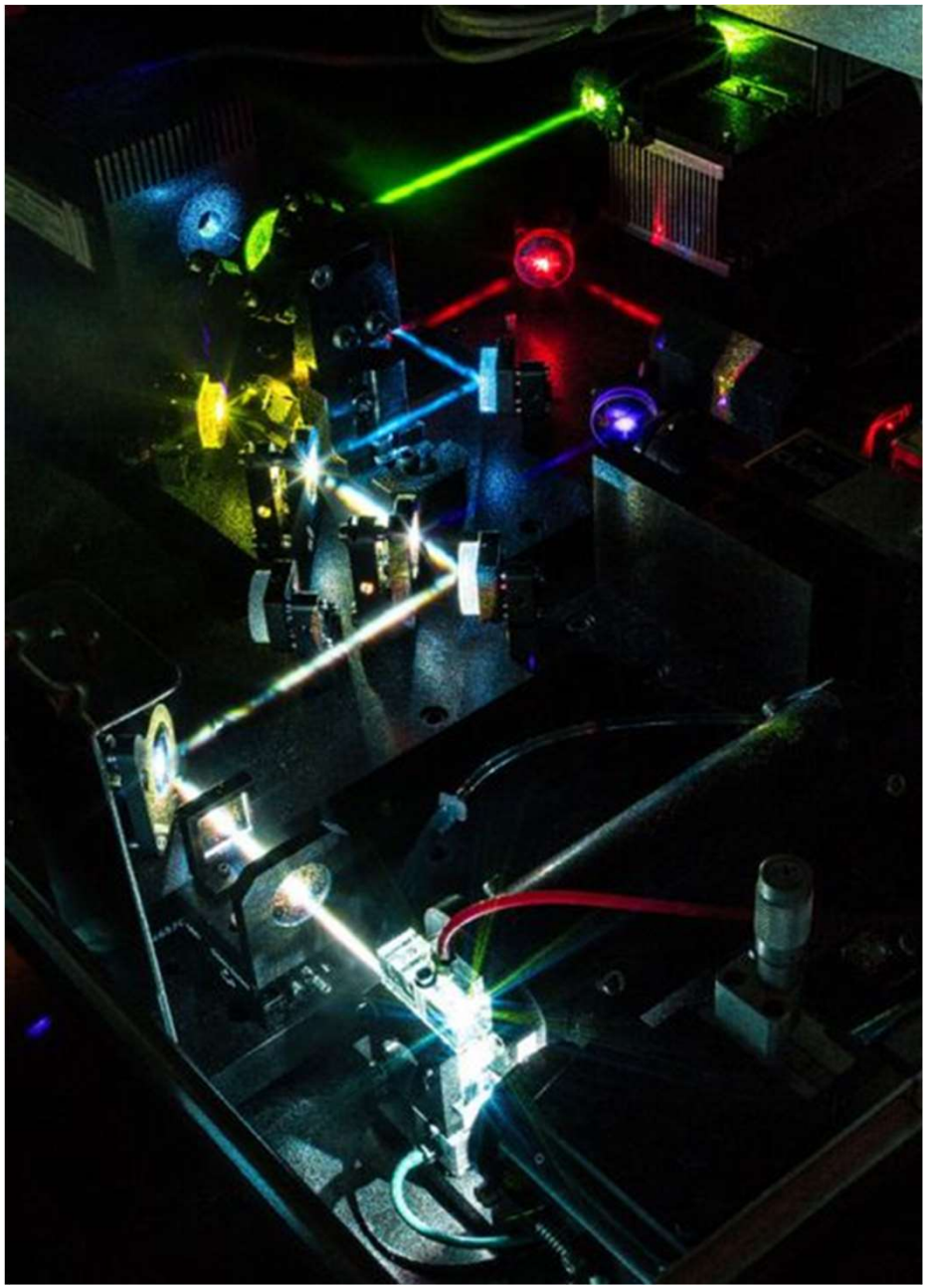
Laser wavelengths on typical cytometers



Lenses and prisms
direct and focus the laser
beams on the cells as they pass
through the flow cell



Here we can see a blue laser beam, a yellow-green, a red and a violet

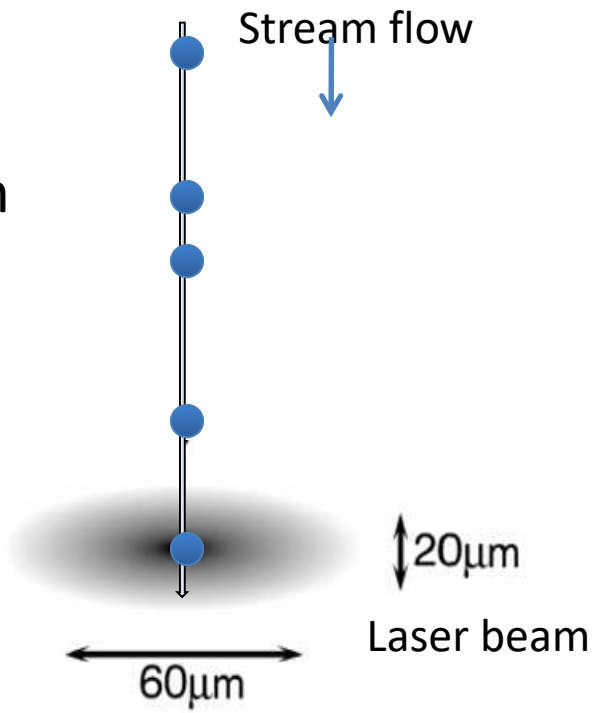


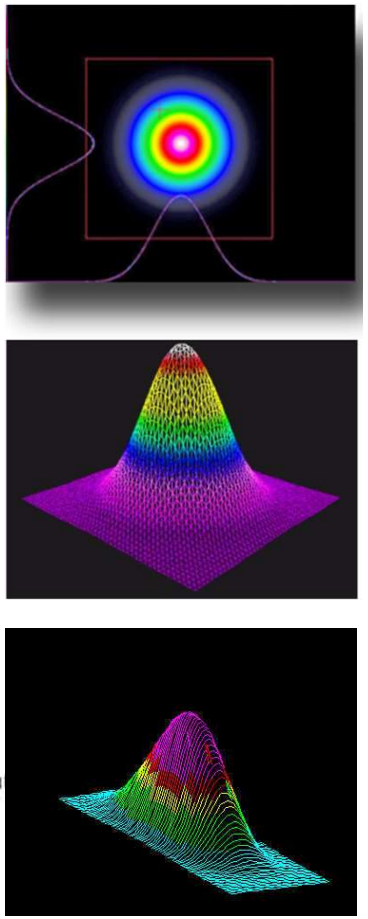
Laser beam geometry

Cells MUST pass through
- center of the laser beam
- for maximum uniform excitation

If they don't:
Decreased excitation means
Decreased fluorescence

Dirt or bubbles can cause this by deflection of the cell path

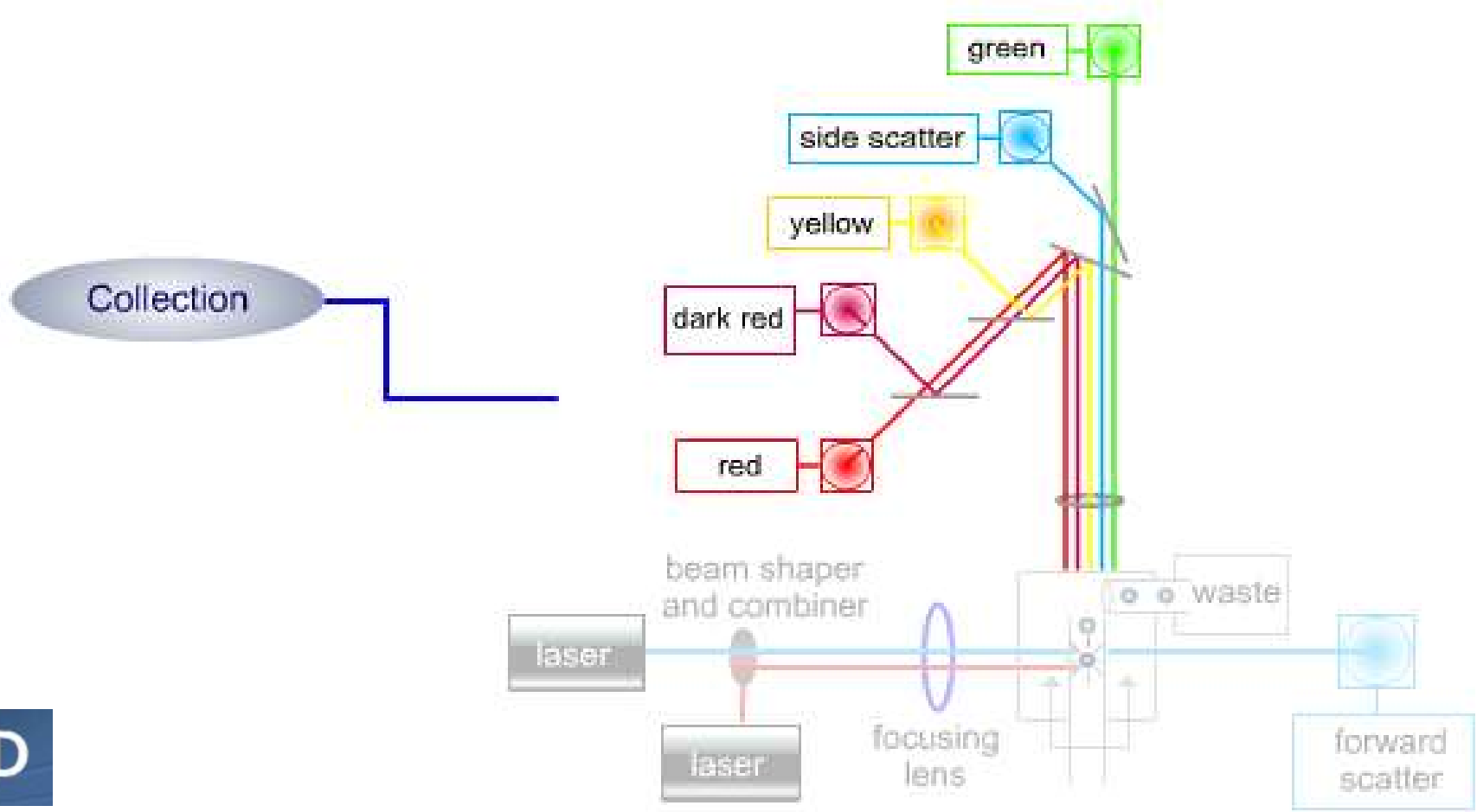




Collection Optics

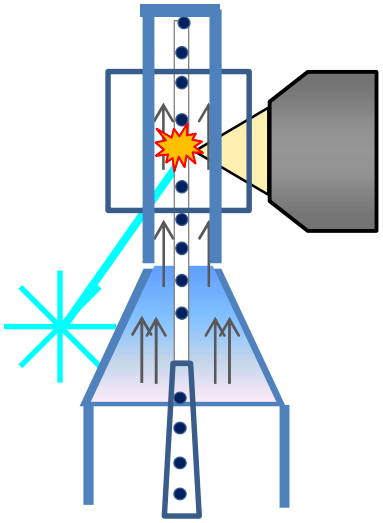
Lenses, mirrors and filters

separate wavelengths and direct to detectors

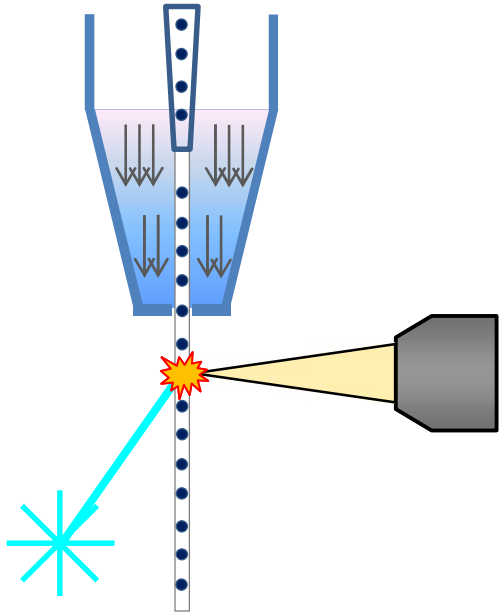


Fluorescent light emission is first collected through a lens

flow cell



Stream-in-air



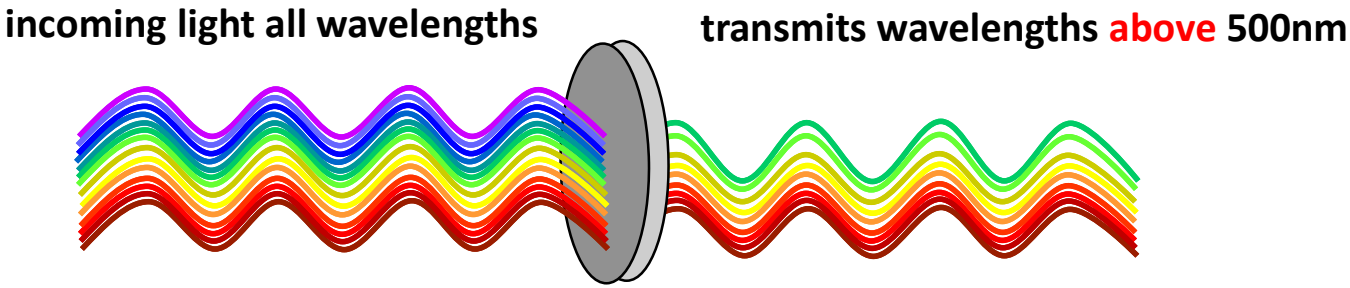
Here the lenses are shown at 90° to the axis of the lasers

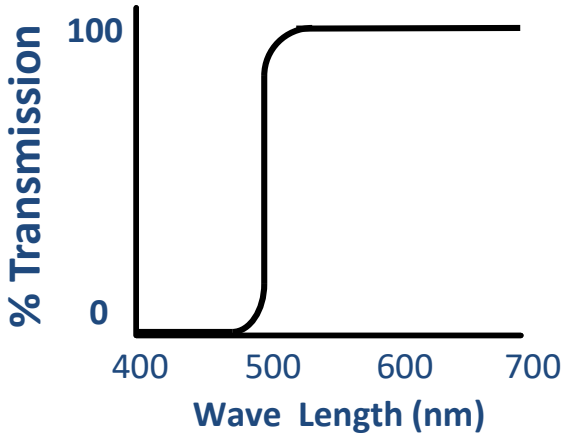
After collection by the lens, the emitted light then

- passes through optical mirrors and filters
- which separate the different wavelengths
- and direct them to the right detectors

Optical Filters: Long Pass

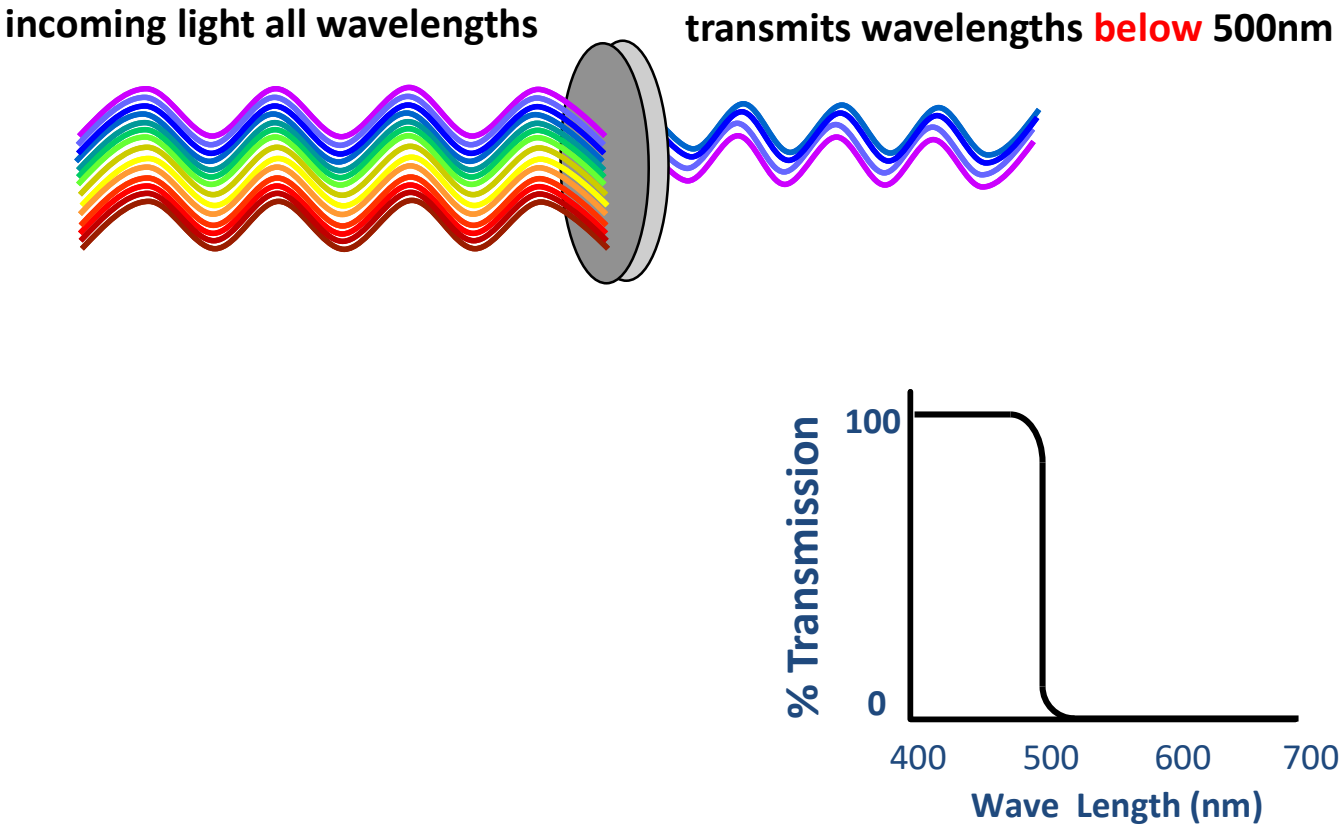
Long Pass LP500





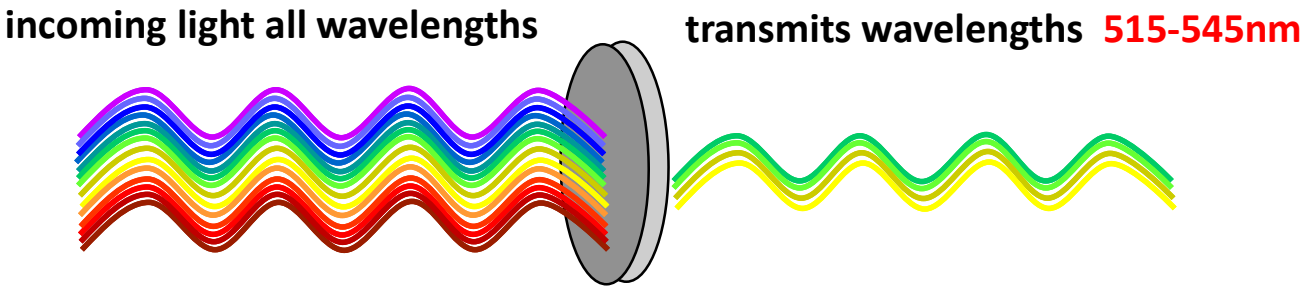
Optical Filters: Short Pass

Short Pass Filter SP500



Optical Filters: Band Pass

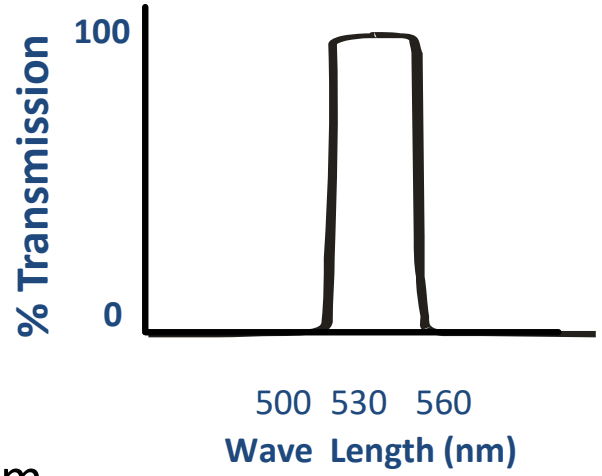
Band Pass Filter BP530/30



Bandpass → BP 530/30 nm

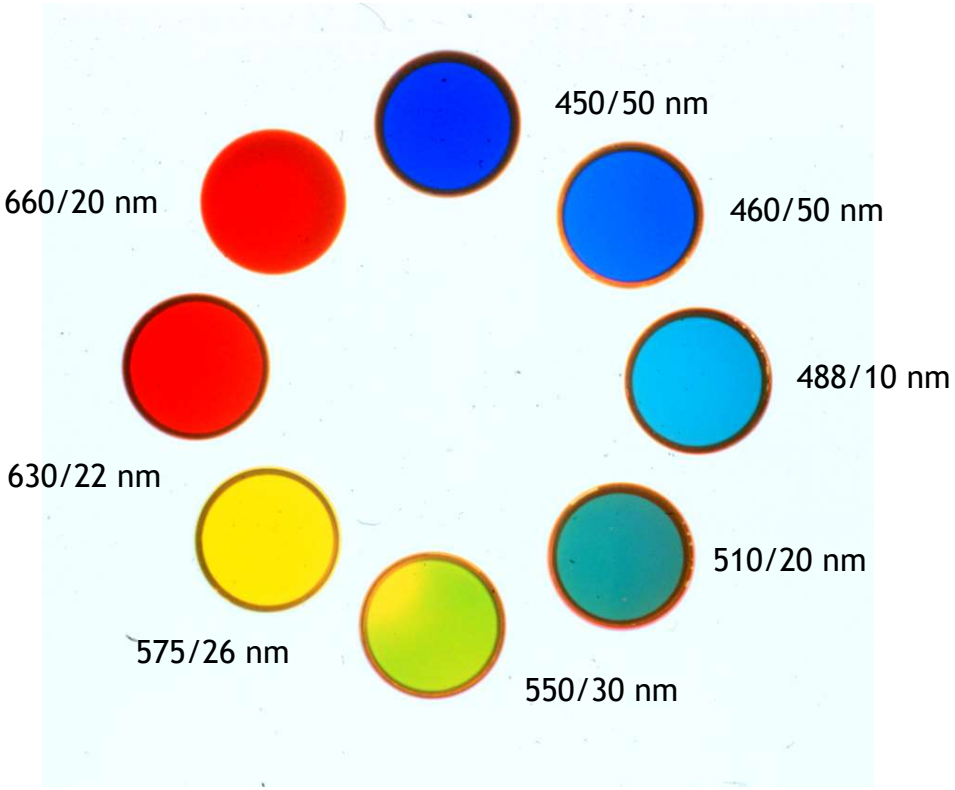
The first number refers to the center wavelength of the filter.

The second number refers to the size of the filter window.



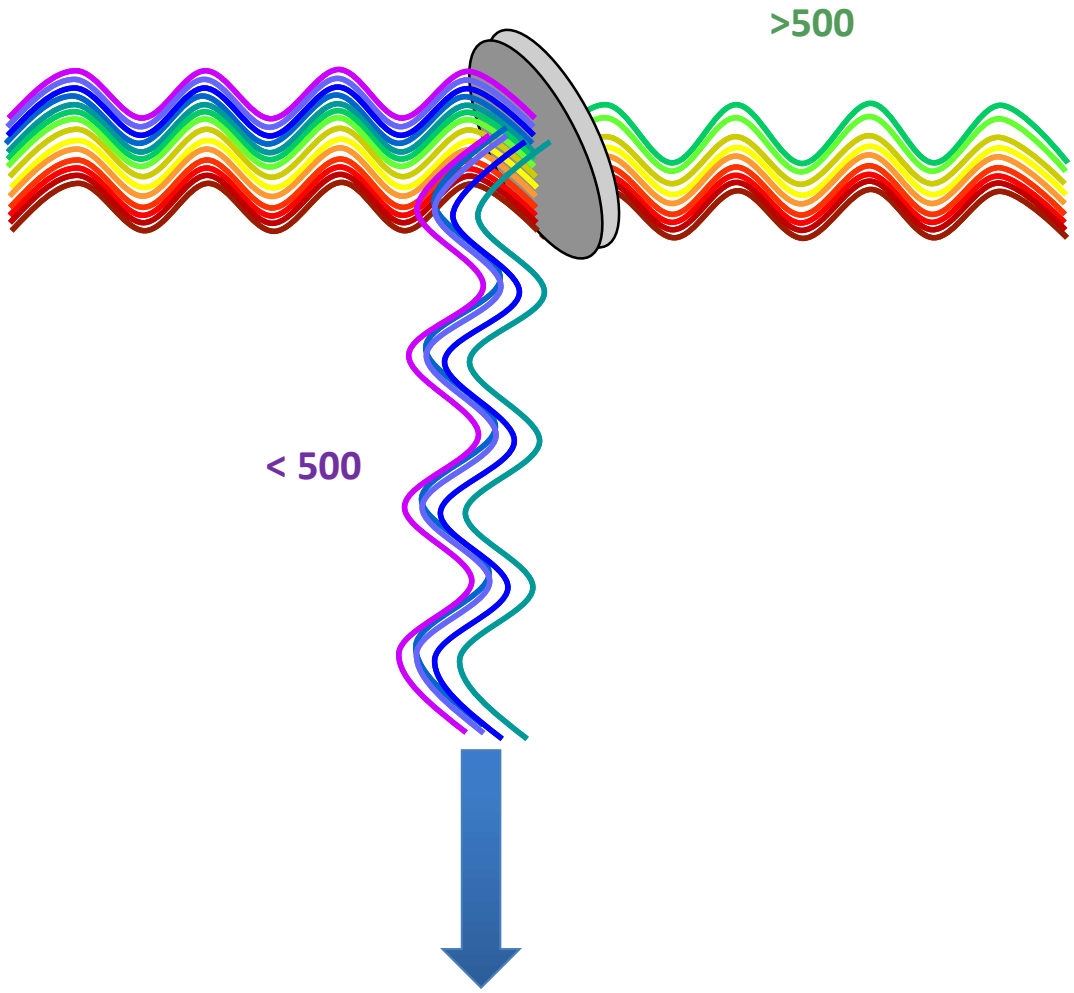
This means it transmits 530+/-15 or 515-545 nm

A rainbow of bandpass filters are available in a wide range of wavelengths

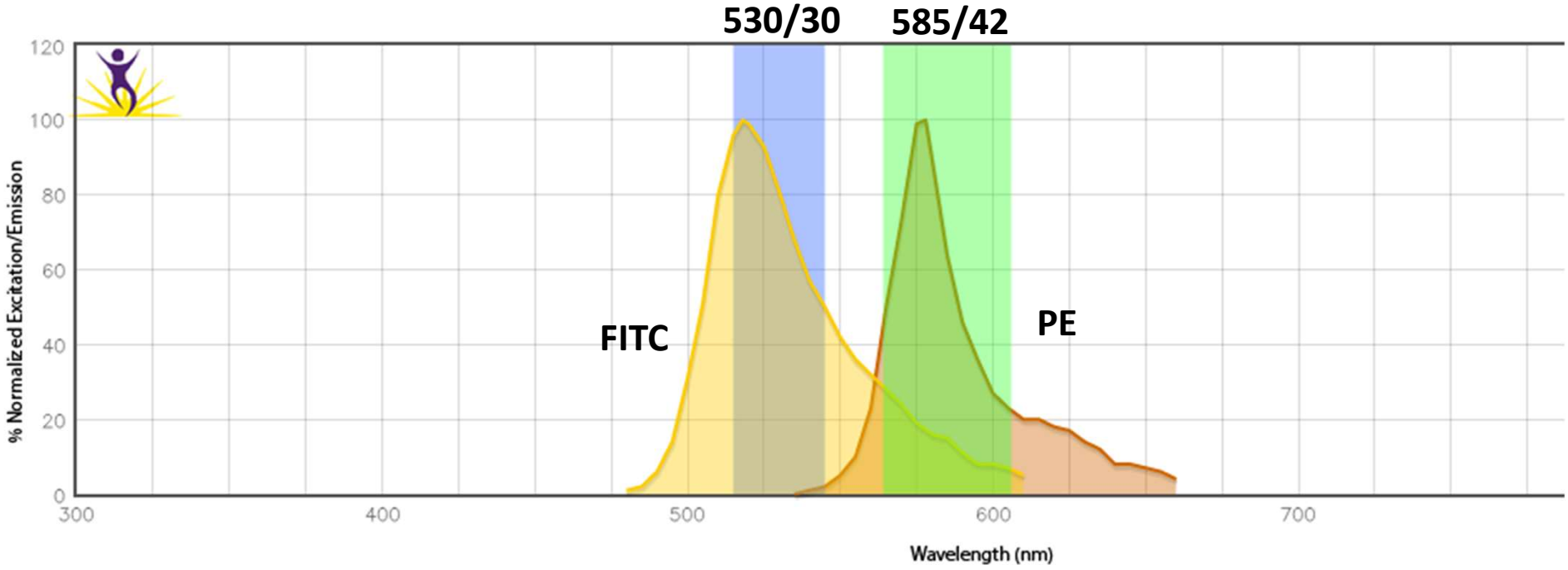


Dichroics: filters and mirrors

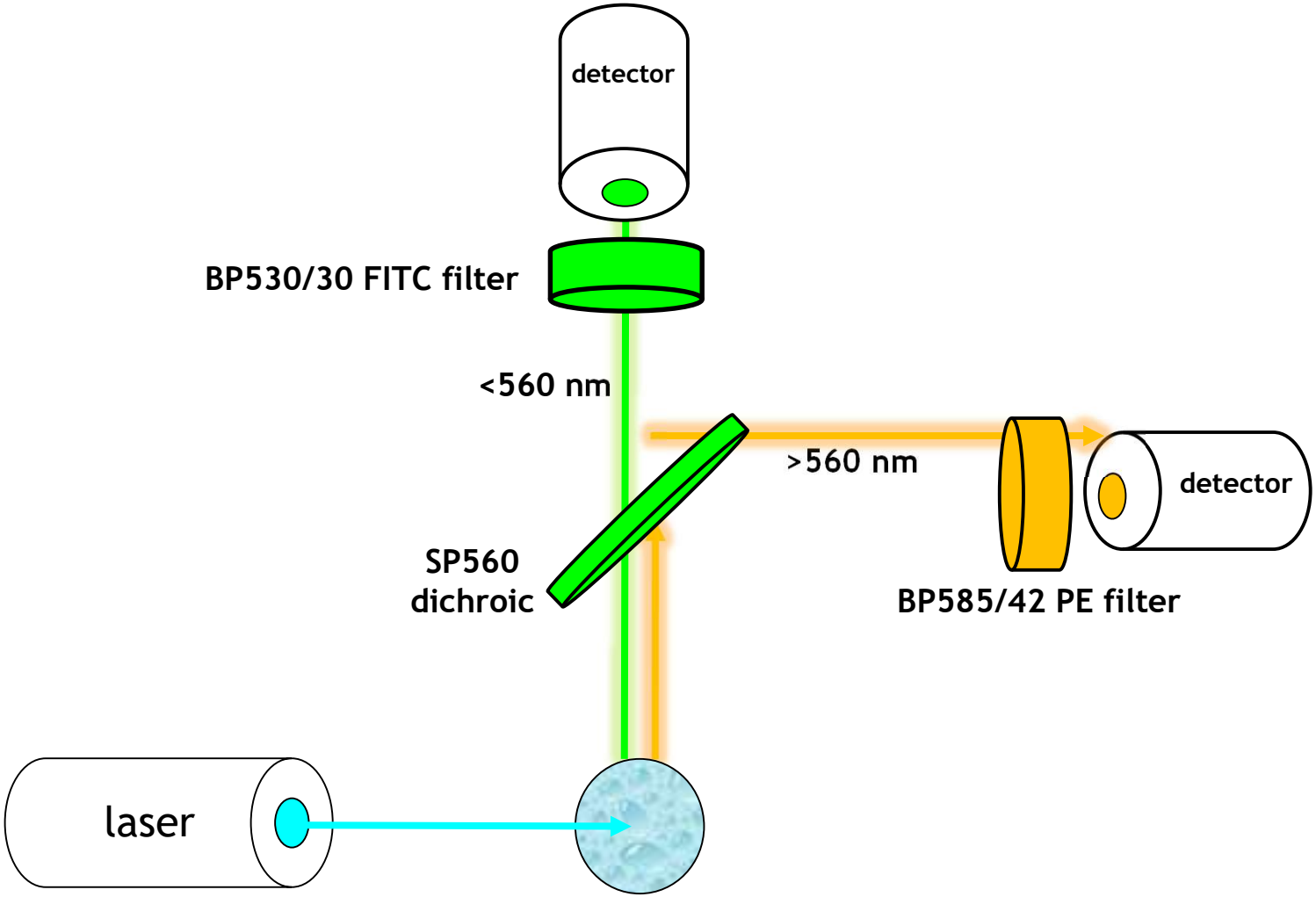
LP500 filter is angled to use as a dichroic mirror



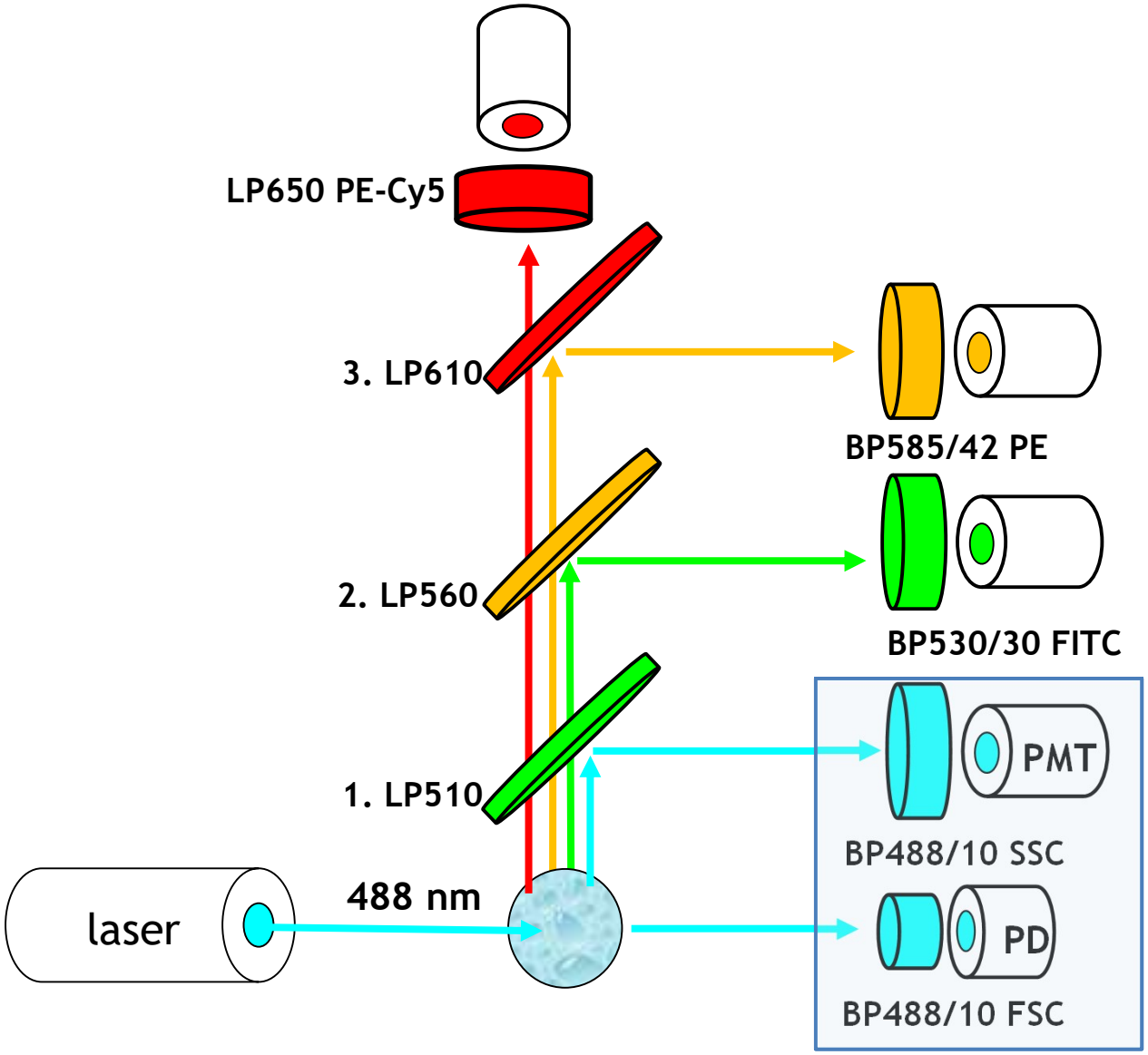
Know the emission spectra of your fluorochromes and which filters are best adapted

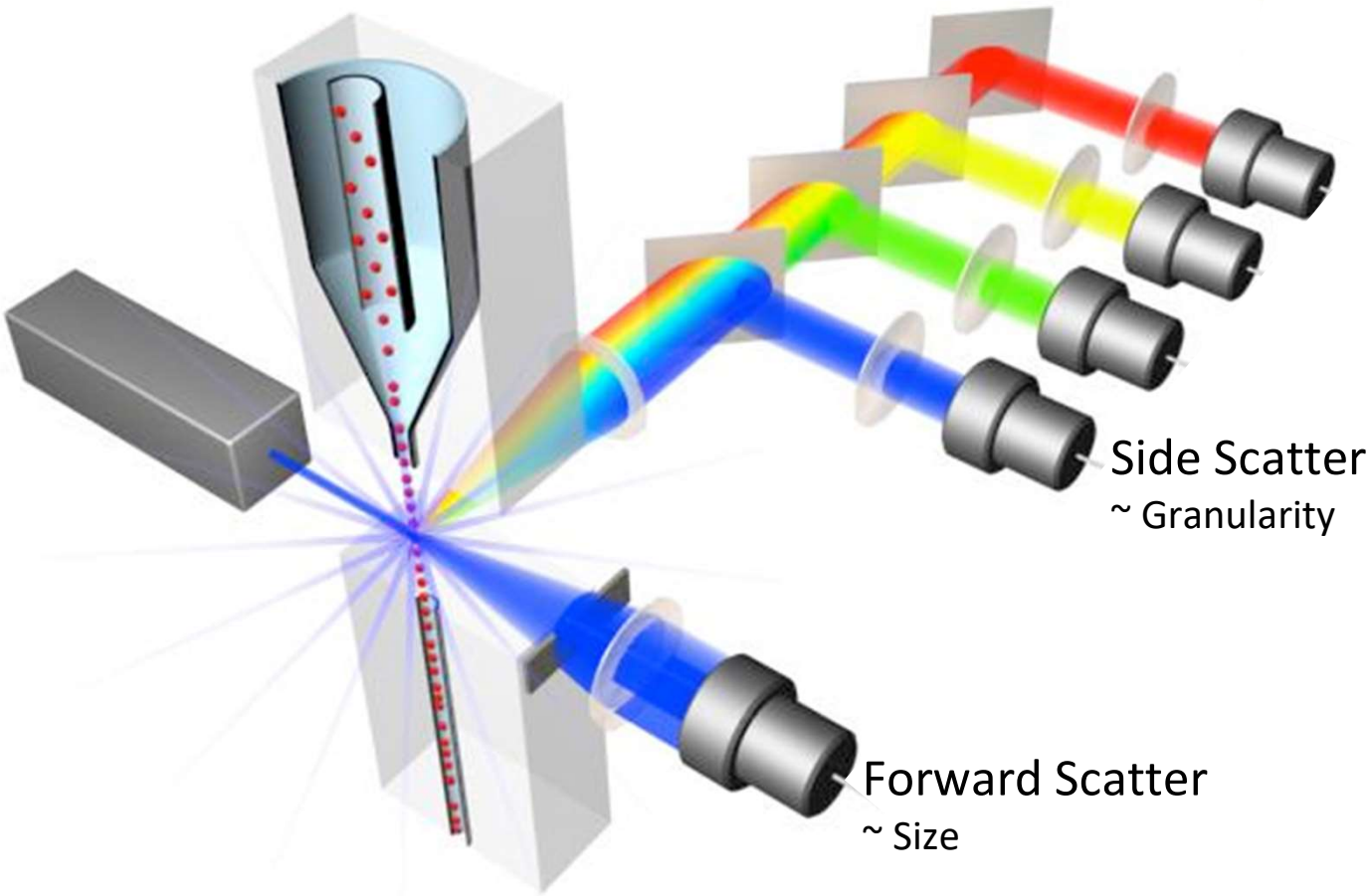


2 color fluorescence detection
FITC and PE



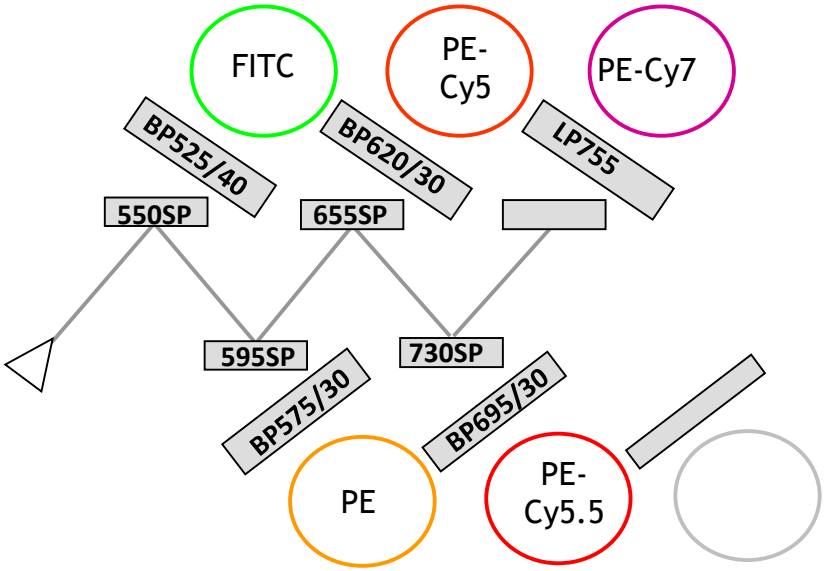
3 color fluorescence plus scatter detection



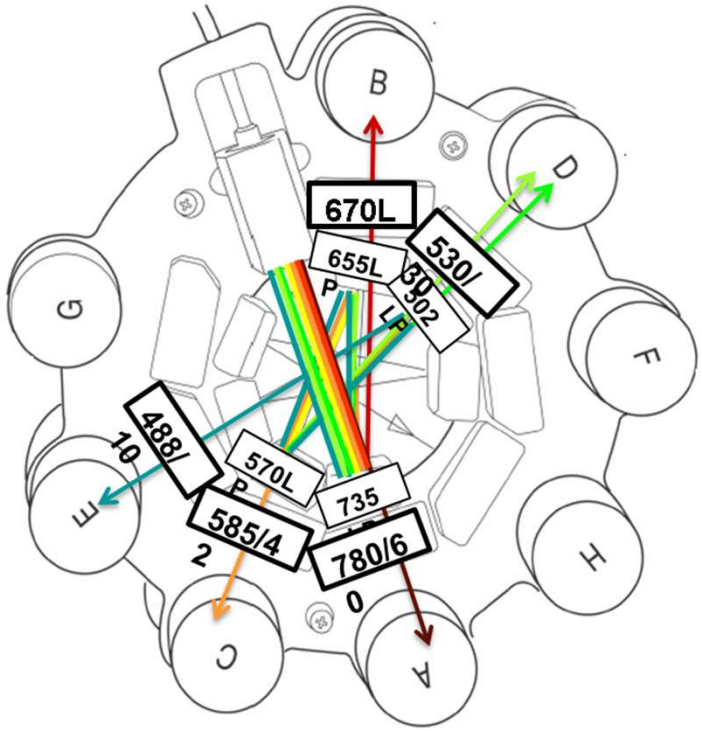

Side Scatter
~ Granularity
Forward Scatter
~ Size

Some Typical Optical Schemes

Linear array

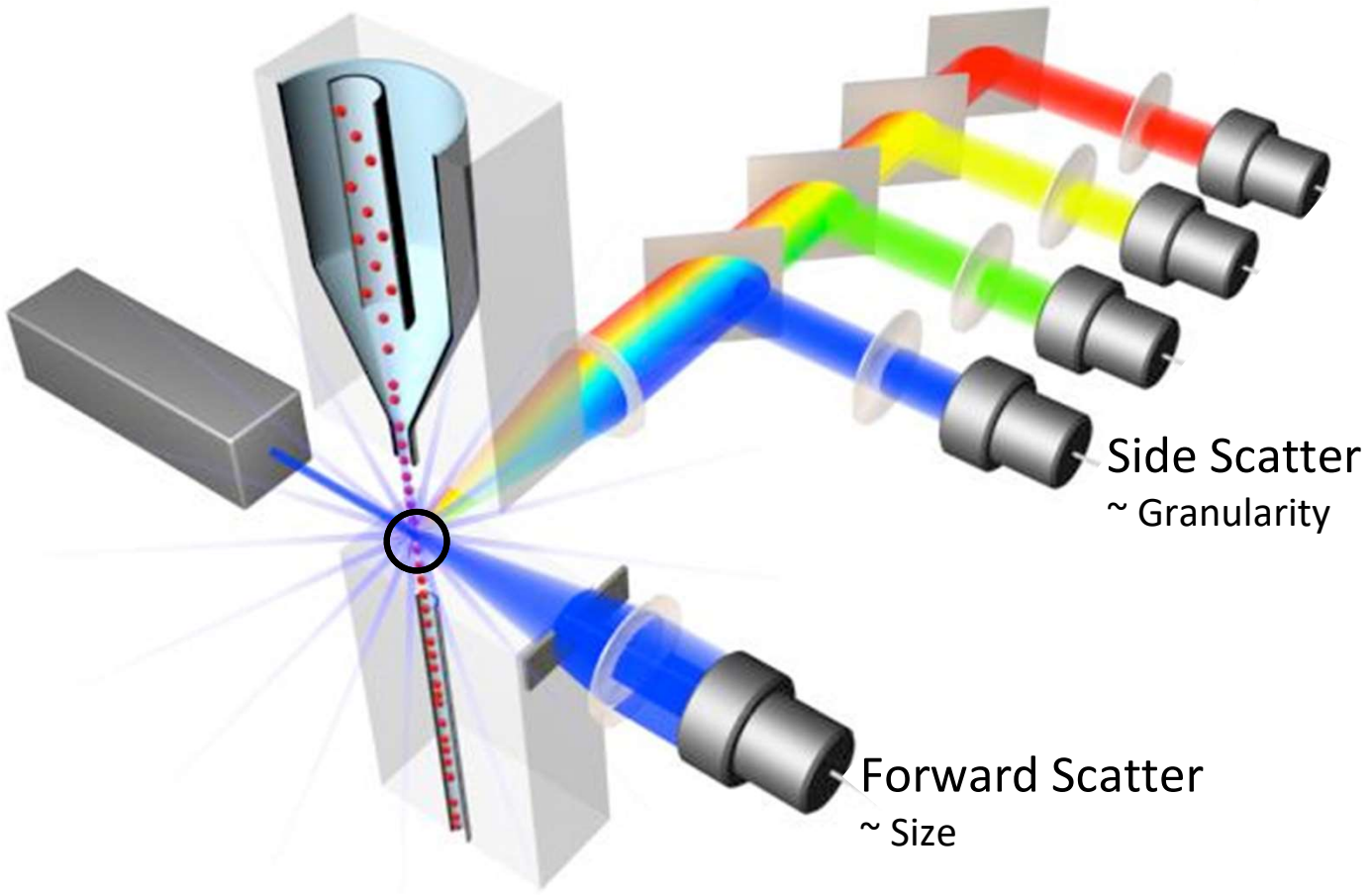


Octagon

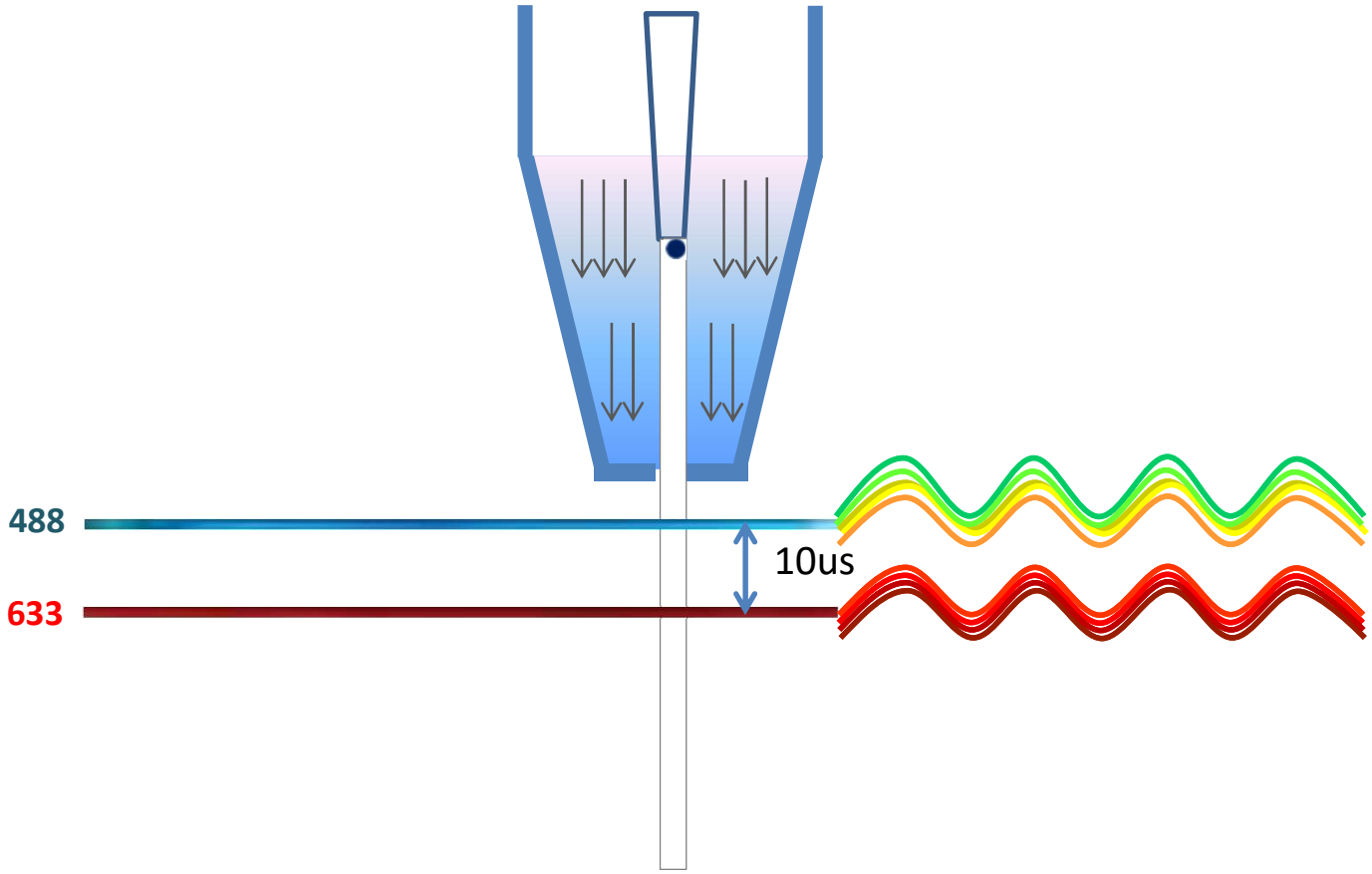


Single laser

So far, we have been looking at the excitation and emission from only one laser



What happens when there are 2 lasers? separation in space and time



Most cytometers have 3 to 5 lasers

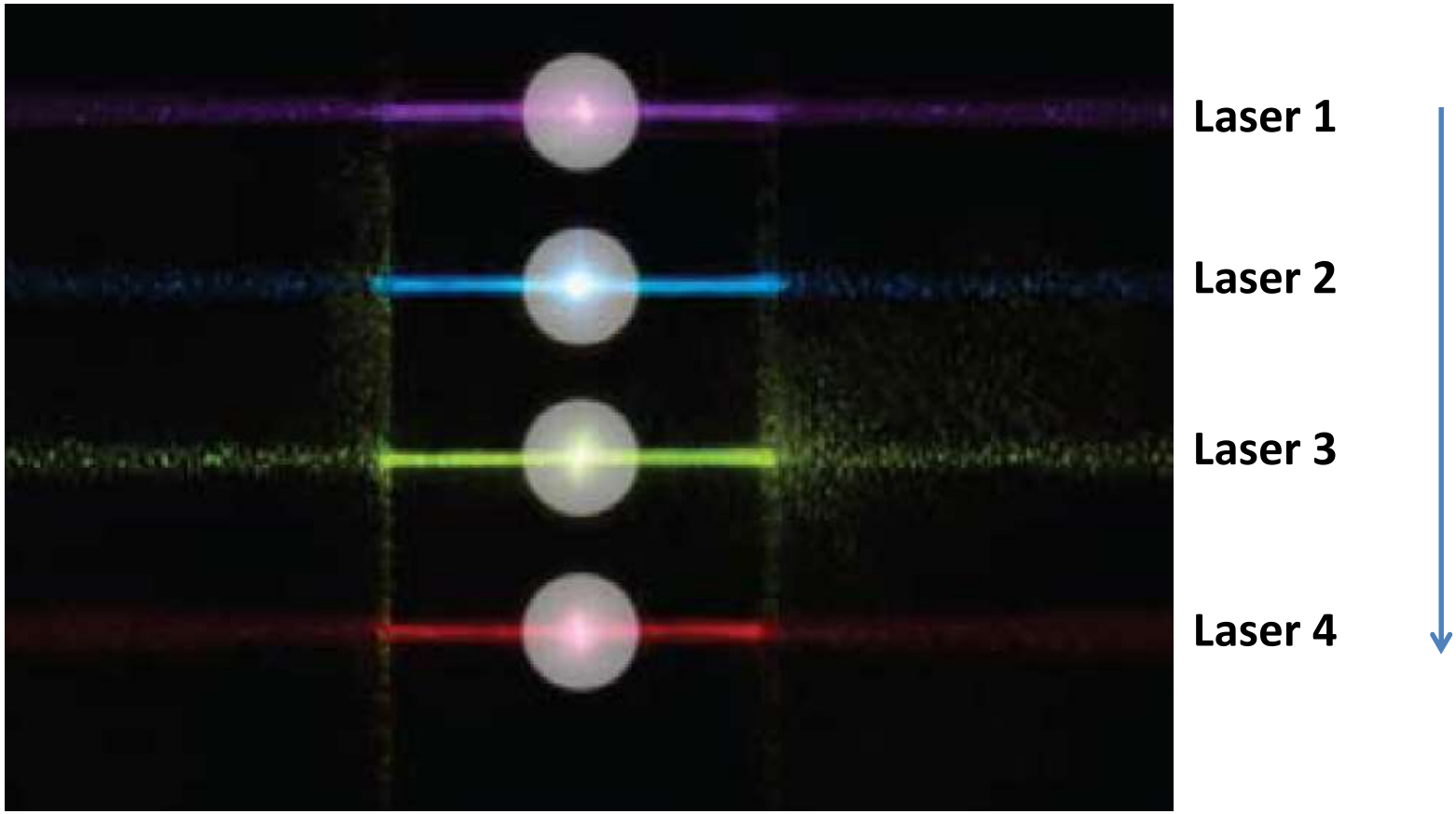
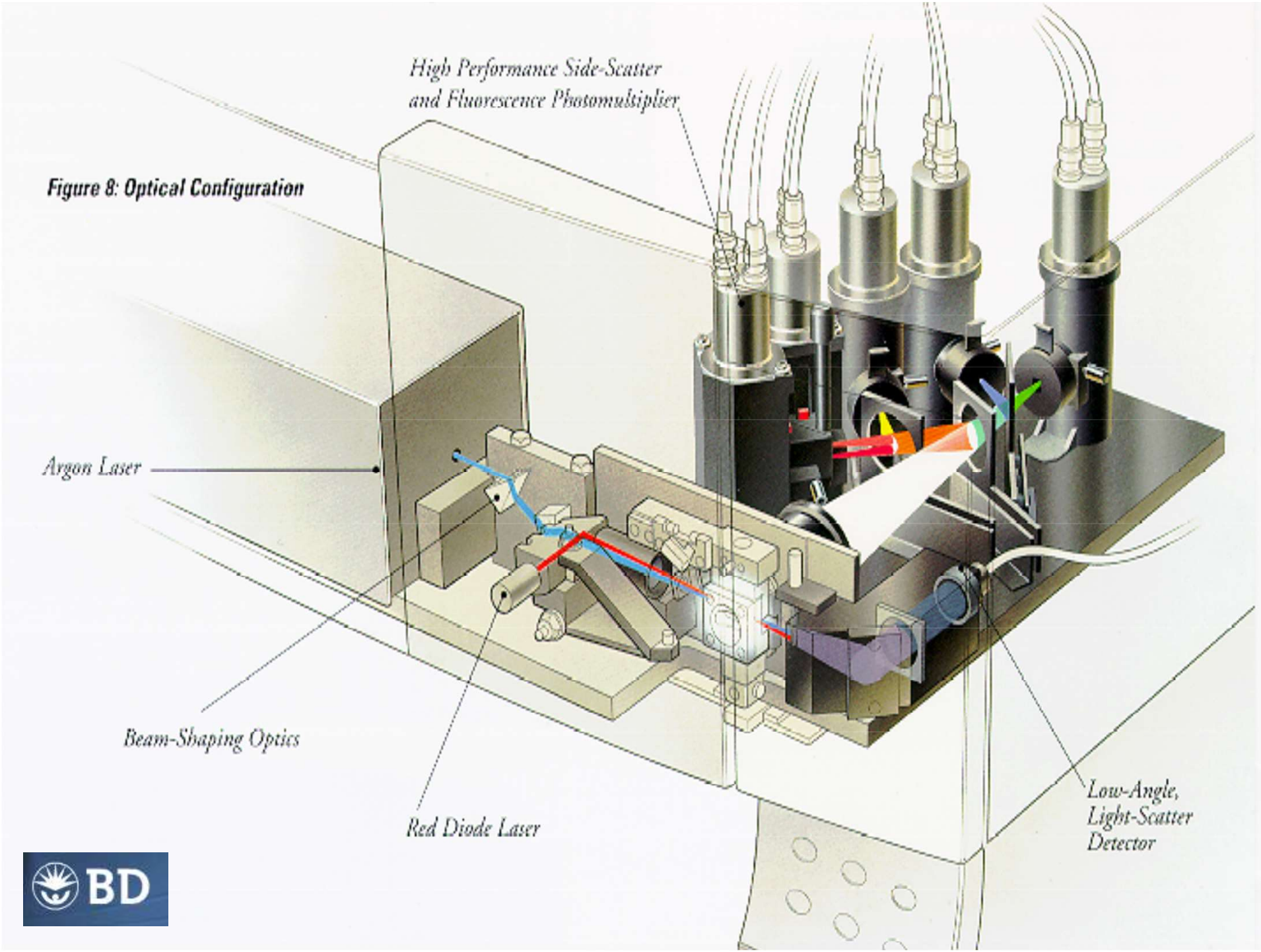
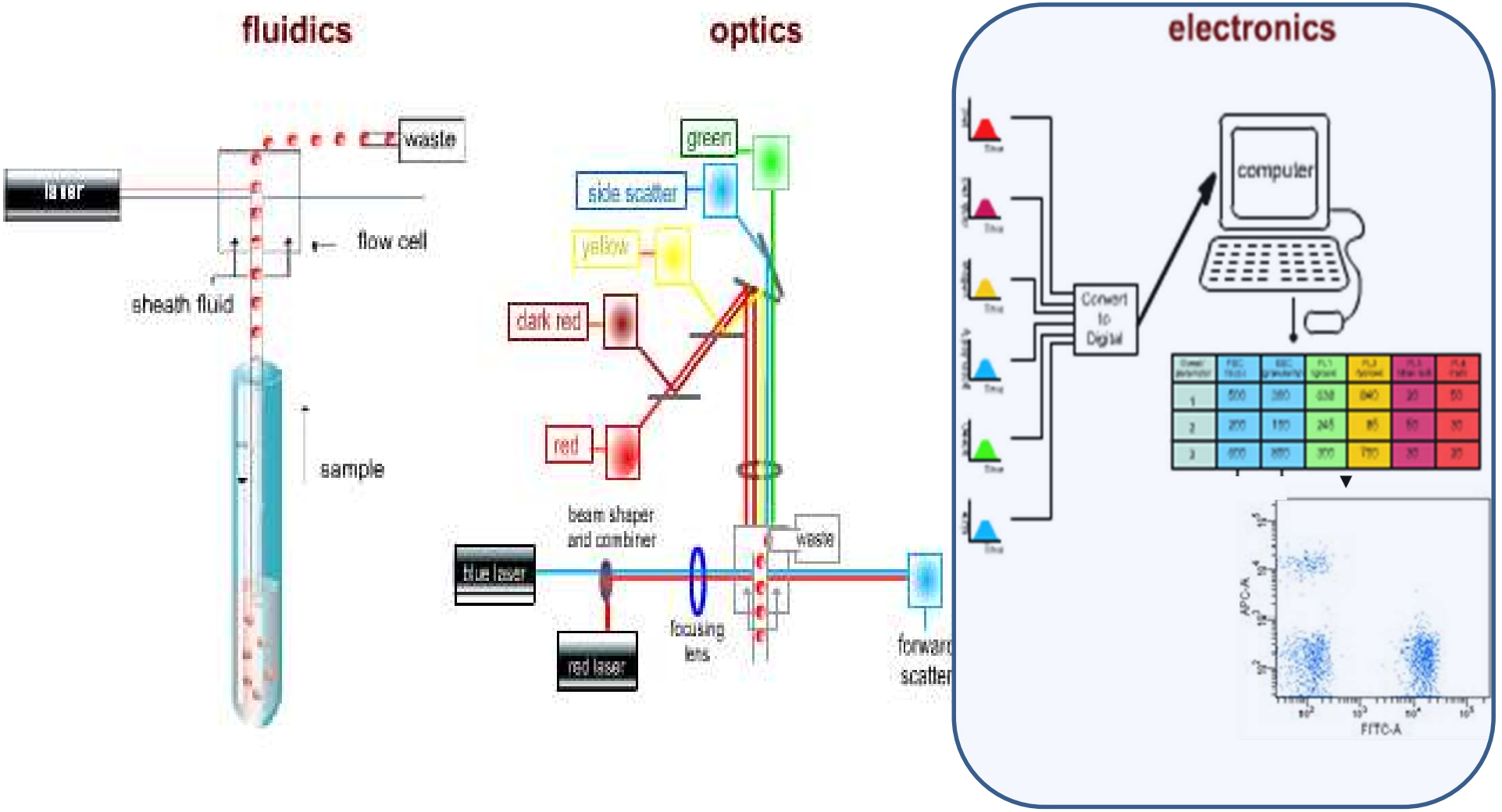


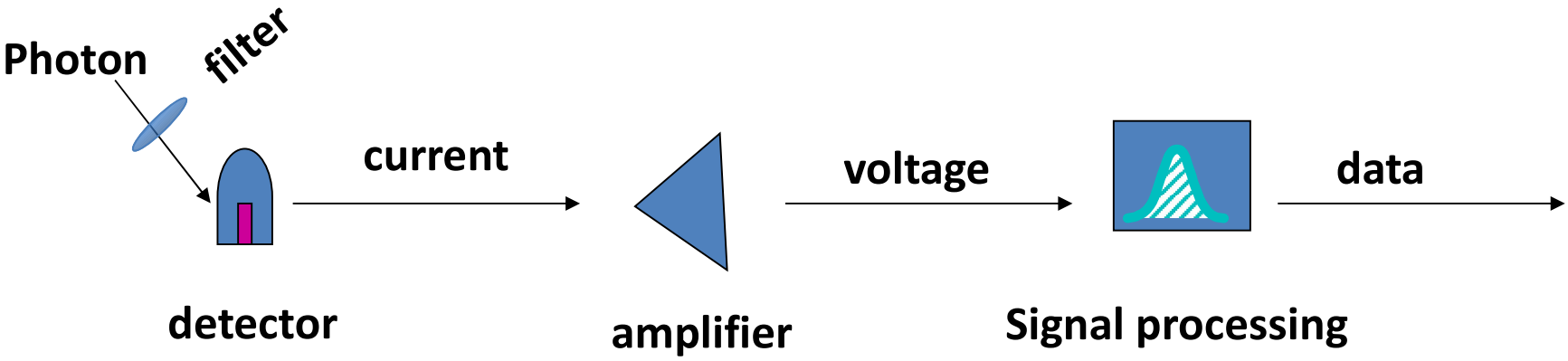
Figure 8: Optical Configuration



Flow Cytometer Elements



Electronics overview



Light detectors transform light photons into electrical current

voltage pulses proportional to each fluoresence emission are generated

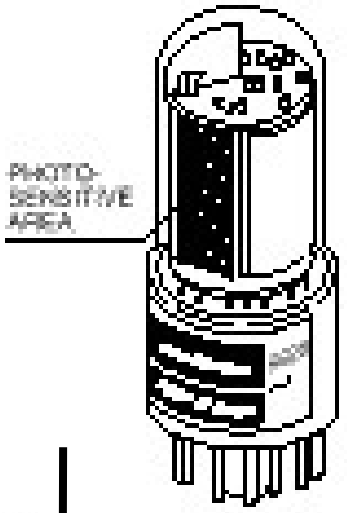
then digitalized To a numerical value

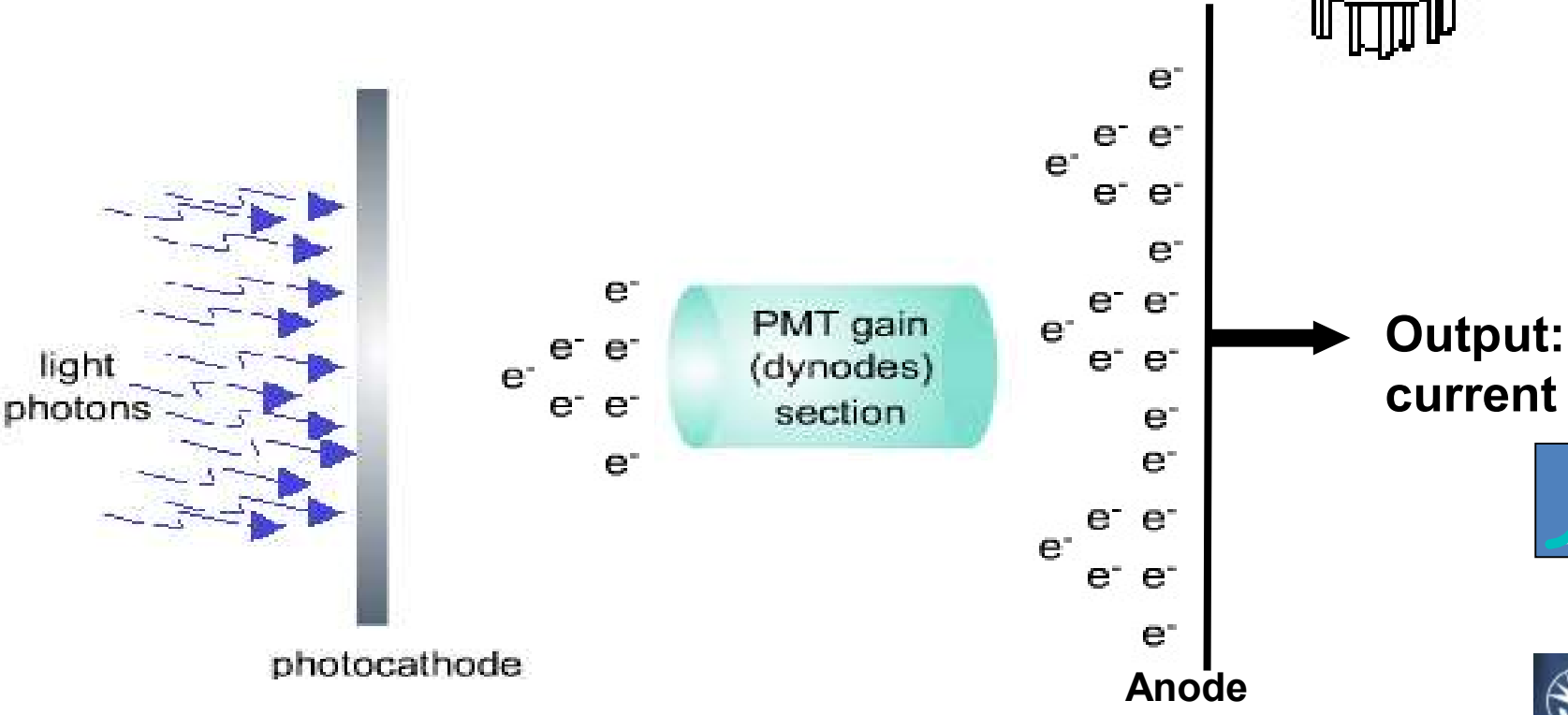
Photodetectors

- Photodetectors transform light into electrical current
- types of photodetectors used in cytometers
    - Photodiodes:
        - Forward scatter (used for strong light signals)
        - Avalanche photodiodes APD (Cytoflex)
    - Photomultiplier tubes (PMT): used for weak light signals
        - Side scatter and all fluorescence parameters

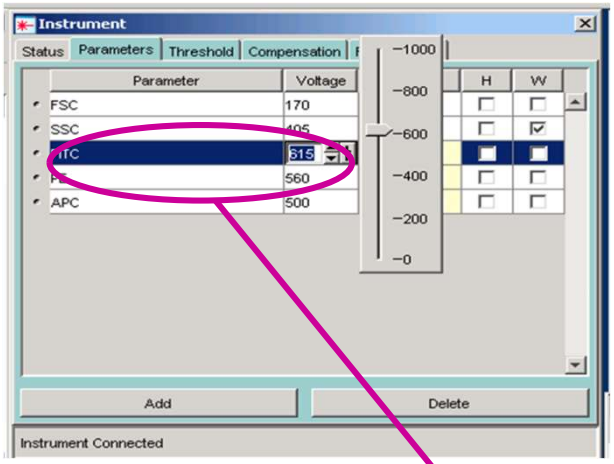
Light Detectors

Photomultiplier Tube (PMT)





Changing the PMT voltage



- Changing the voltage applied to the dynode chain increases or decreases output signal (current) from the PMT

- This is done using the PMT voltage control on the software

- 10^3 to 10^8 electrons may reach the anode for every electron that left the cathode, depending on the voltage applied

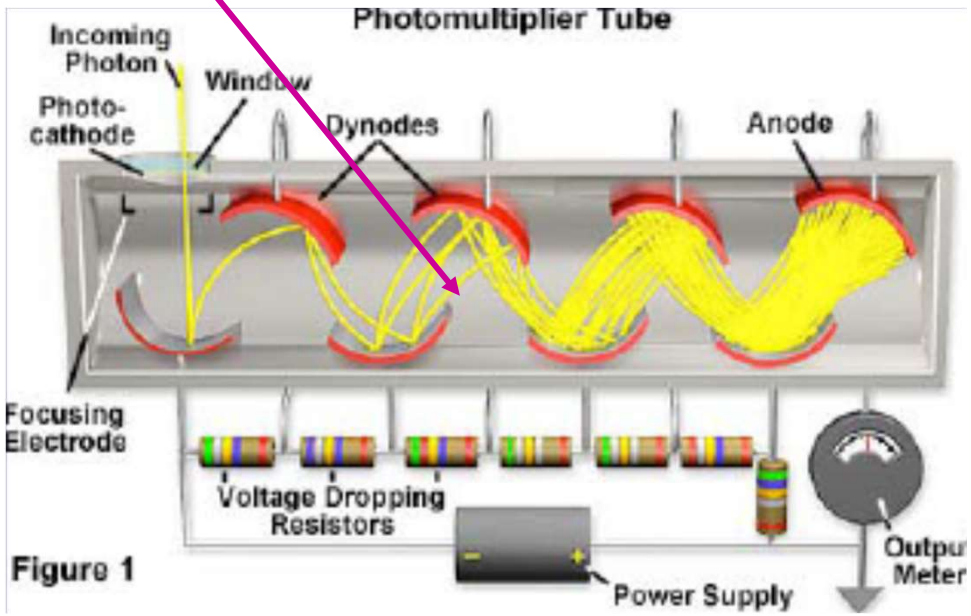
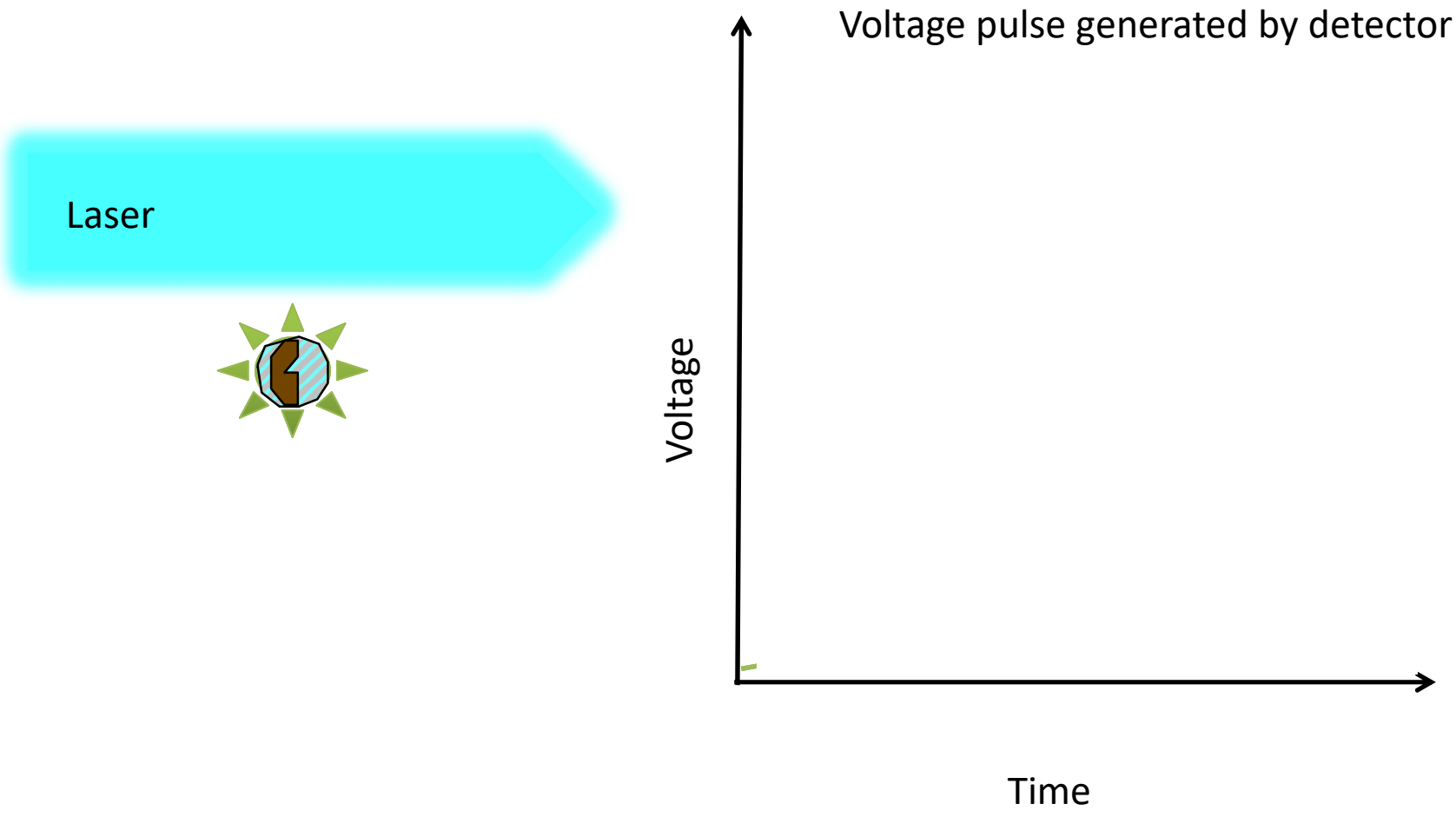


Diagram from Dakocytomation

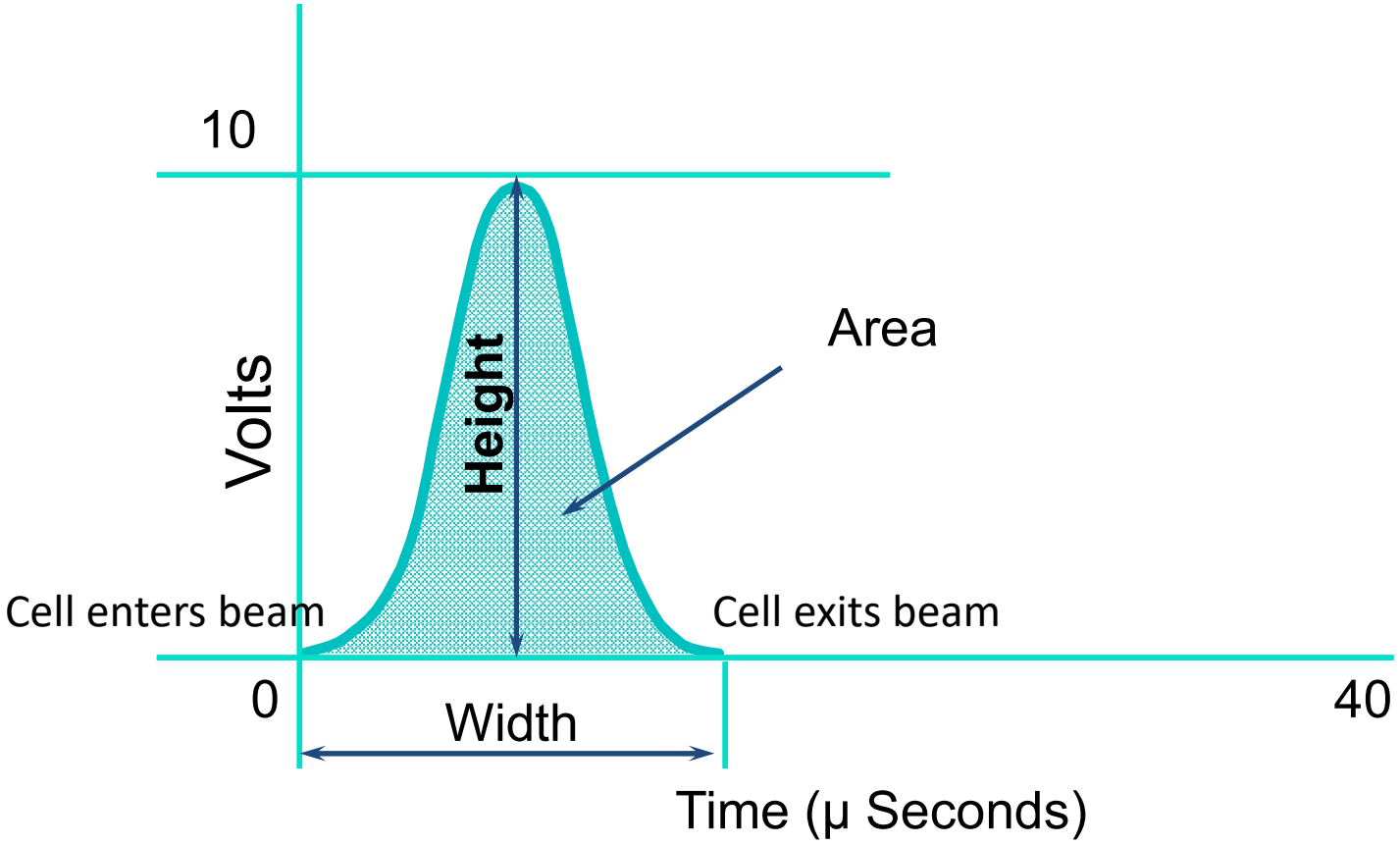
How is a pulse/signal created on a Flow Cytometer ?



Courtesy of Grace Chojnowski

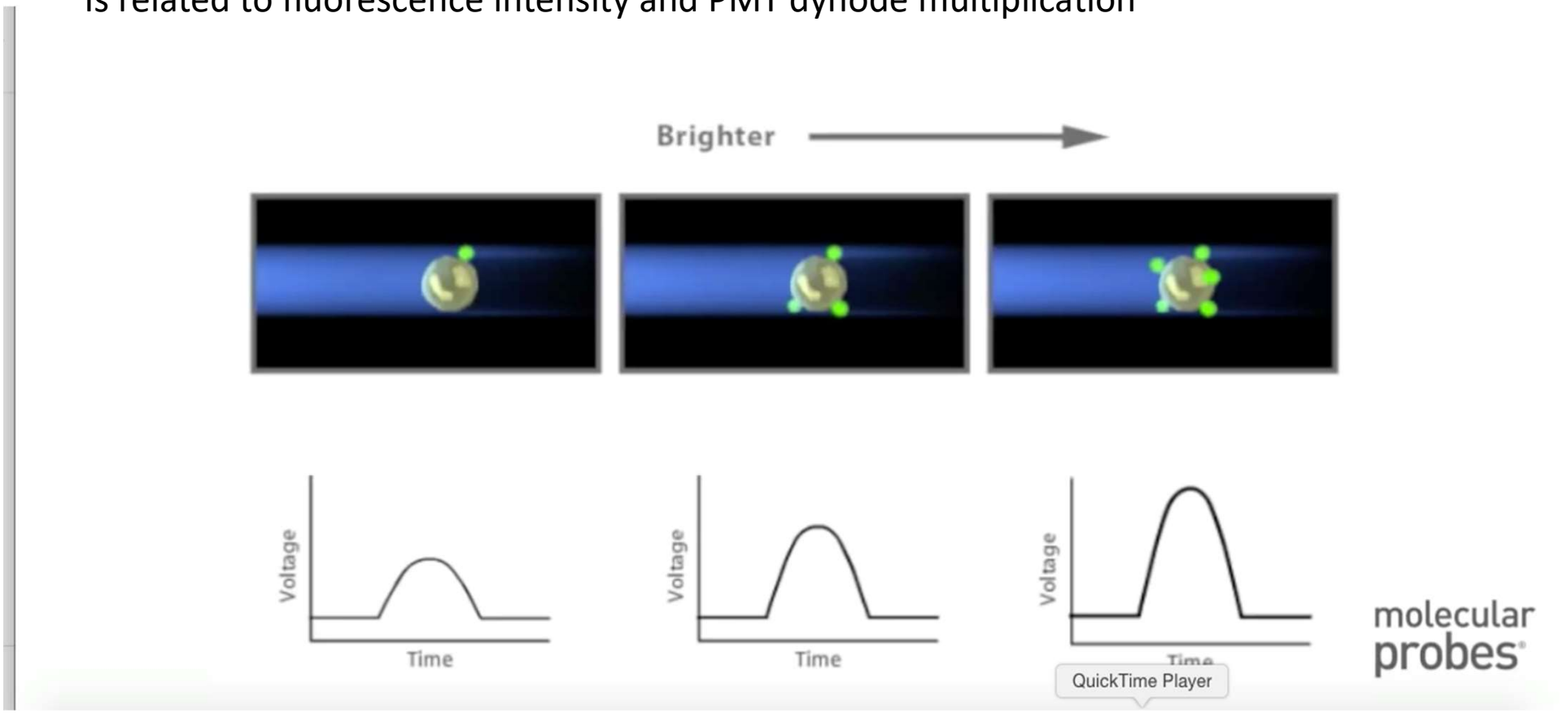
Signal Processing

- The signal processors quantify the voltage pulses
- They generate a numerical channel value for pulse height, area and width



Voltage pulse size

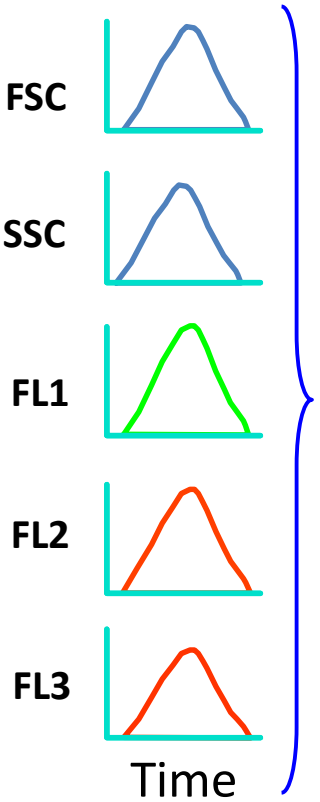
Is related to fluorescence intensity and PMT dynode multiplication



Digitalization

The pulse size numerical values are recorded as channel numbers
The data is saved as a list mode (.fcs) file which records all values for each event

Voltage Pulses from all detectors



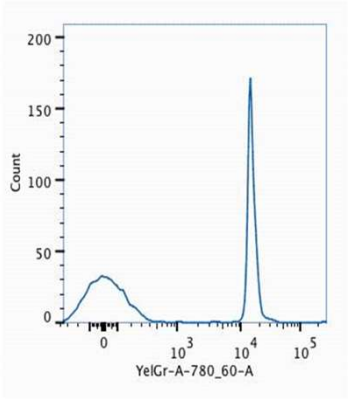
Pulse processing

Data Acquisition - Listmode

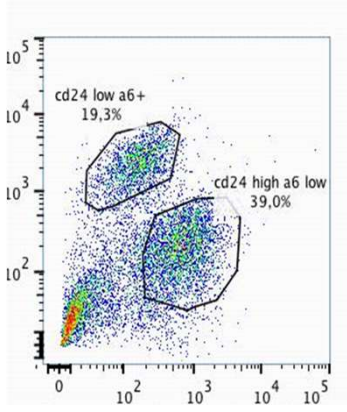
Event	Param1 FS	Param2 SS	Param3 FITC	Param4 PE
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

[RFM]

Single parameter frequency histogram



Dual parameter dotplot



List mode file

A list mode (.fcs) files contains scatter and fluorescence values for each event as well as instrument settings and cytometer information.

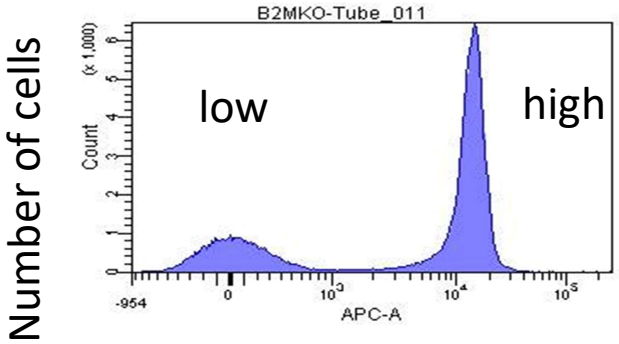
FCS DATA FILE (TRANSLATED)

CELLS IN SEQUENCE	FSC-H	SSC-H	FL1-H	FL2-H
1	120	28	152	24
2	190	169	42	60
3	175	149	56	52
4	107	25	149	0
5	97	22	151	26
6	174	136	47	36
7	190	127	42	47
8	106	14	148	0
9	86	17	165	23
10	90	16	149	31
11	184	163	58	50
12	191	160	39	40
13	101	24	152	19
14	126	36	153	0
15	126	28	157	0
16	96	17	155	0
17	215	224	61	59
18	165	95	55	46
19	173	73	49	43
20	91	27	158	0
21	210	180	59	52
22	179	161	60	52
23	165	93	54	35
24	187	45	67	52
25	192	184	48	50
26	111	25	149	17
27	207	206	58	40

From Data File to Data Display

CELLS IN SEQUENCE	FSC-H	SSC-H	FL1-H	FL2-H
1	120	28	152	24
2	190	169	42	60
3	175	149	56	52
4	107	25	149	0
5	97	22	151	26
6	174	136	47	36
7	190	127	42	47
8	106	14	148	0
9	86	17	165	23
10	90	16	149	31
11	184	163	58	50
12	191	100	39	40
13	101	24	152	19
14	126	36	153	0
15	126	28	157	0
16	96	17	155	0
17	215	224	61	59
18	165	95	55	46
19	173	73	49	43
20	91	27	158	0
21	210	180	59	52
22	179	161	60	52
23	165	93	54	35
24	187	45	67	52
25	192	184	48	50
26	111	25	149	17
27	207	206	58	40

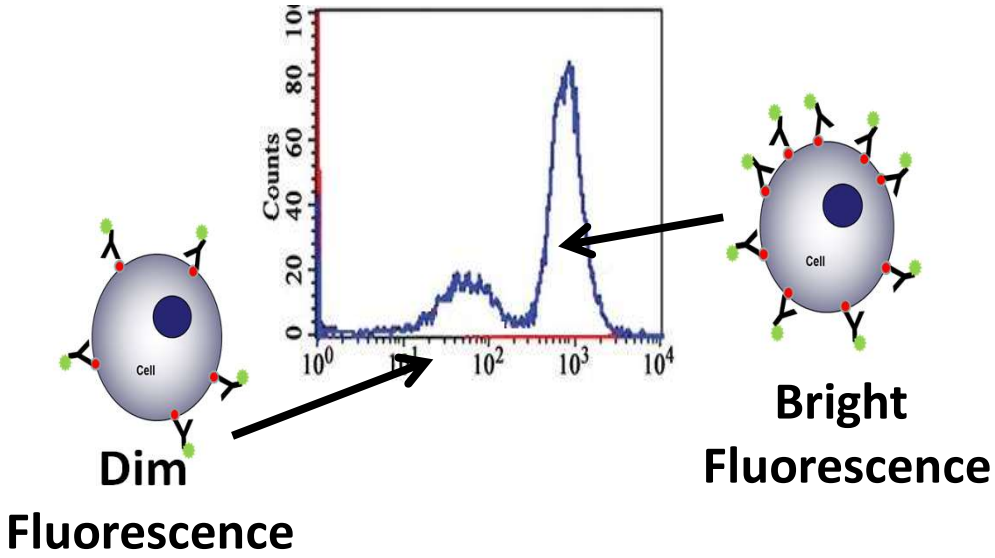
List Mode File



Fluorescence intensity

Fluorescence Intensity

Fluorescent dye or antigen abundance on the cell is proportional to the fluorescence level detected



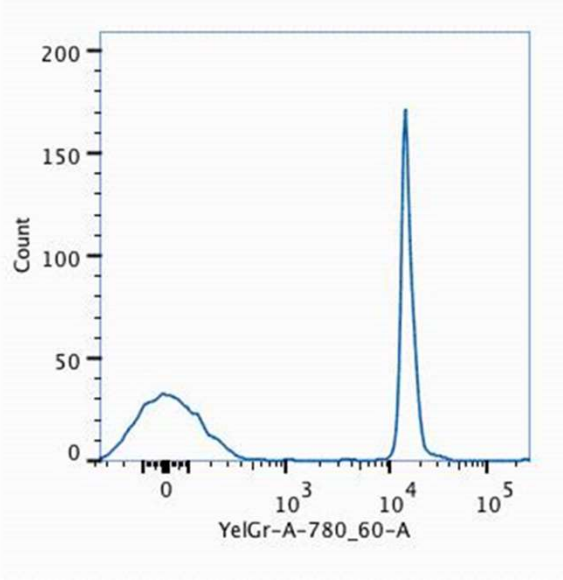
Slide courtesy of Celine Lages and Sherry Thornton

Data Acquisition - Listmode

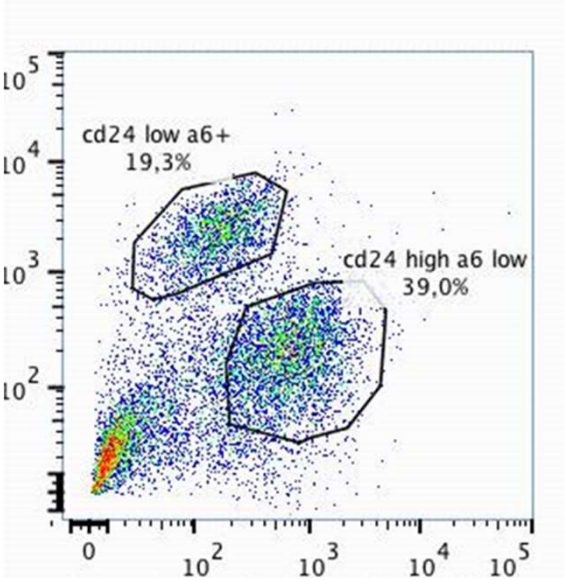
Event	*Param1 FS*	*Param2 SS*	*Param3 FITC*	*Param4 PE*
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

[RFM]

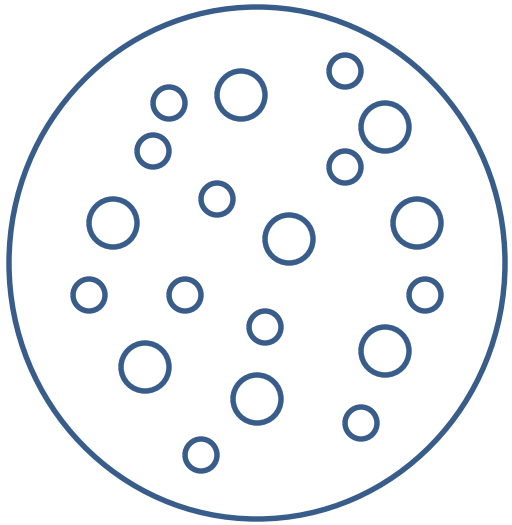
Single parameter frequency histogram

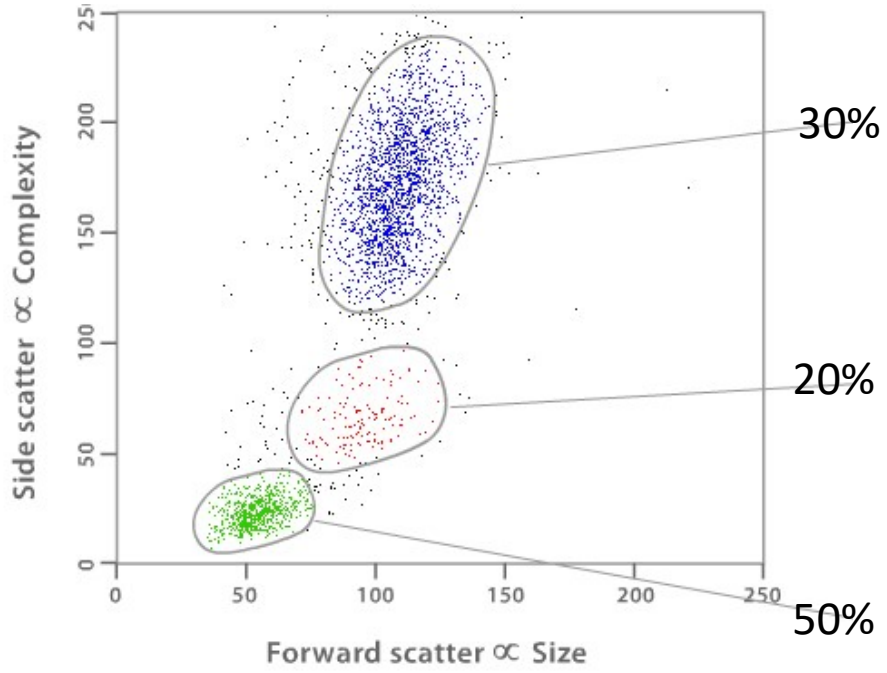


Dual parameter dotplot



So now we can answer the questions





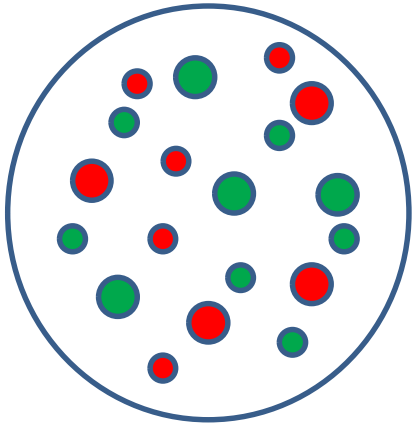
How many Small and/or Big Cells are there ?

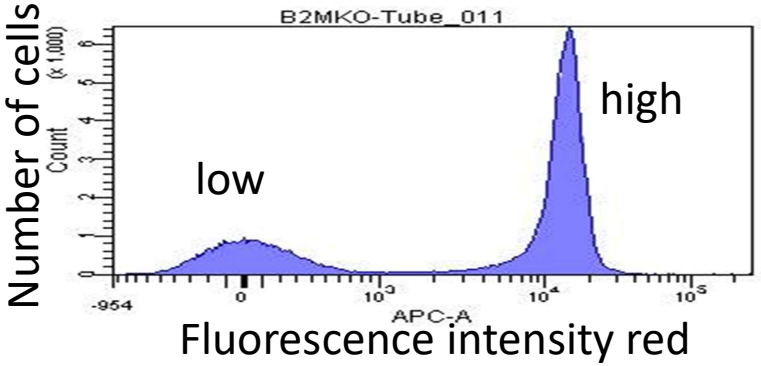
Parameter: Size

Courtesy of Dr Krishnamurthy

ncbs TIFR

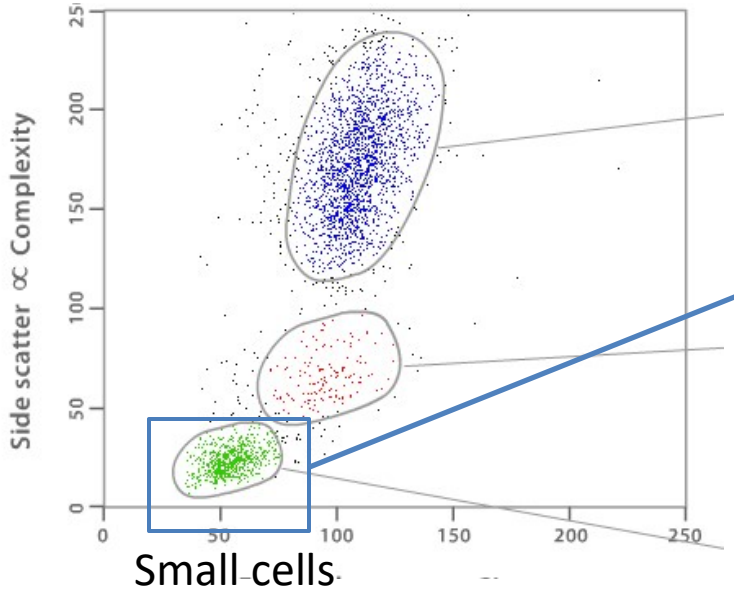
And the next questions:

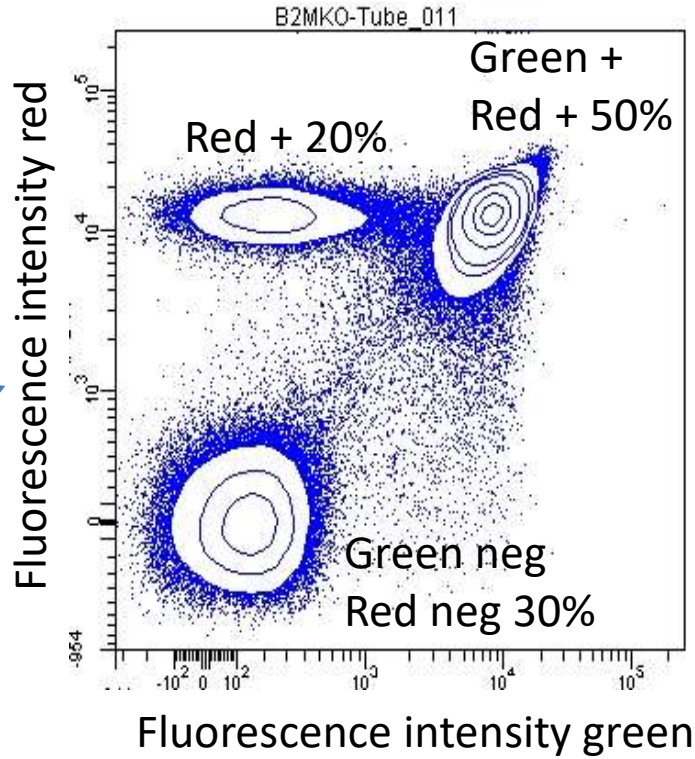




How many Small cells are Green and/or Red?

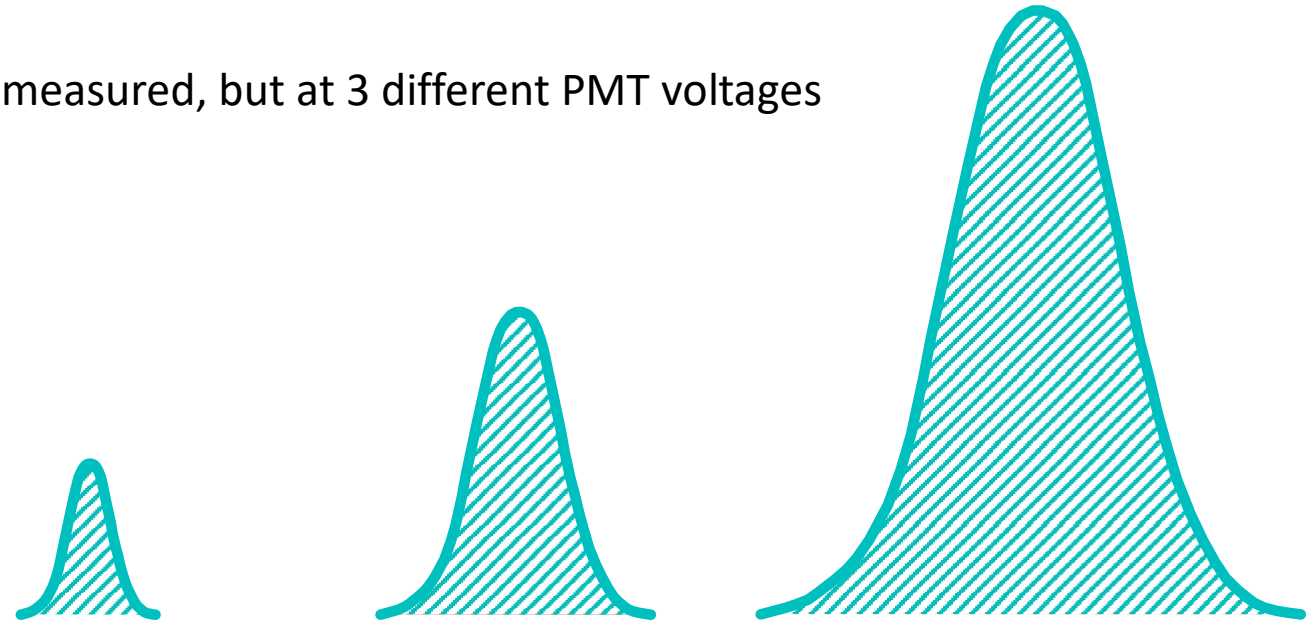
How many Big cells are Green and/or Red?



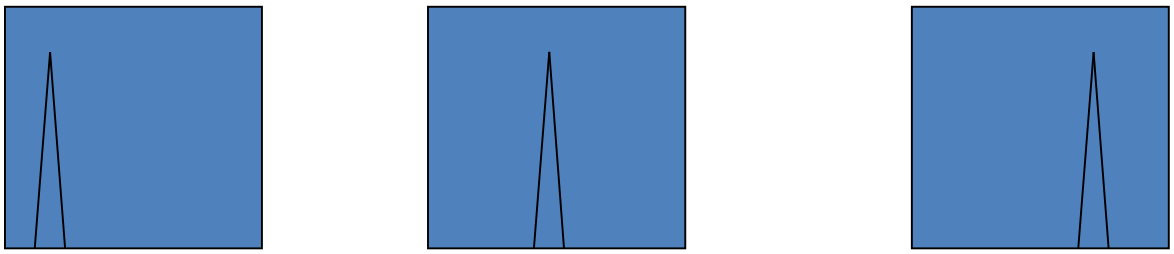


Changing the PMT voltage

The same cell is measured, but at 3 different PMT voltages



300v **400v** **500v**



Forward scatter

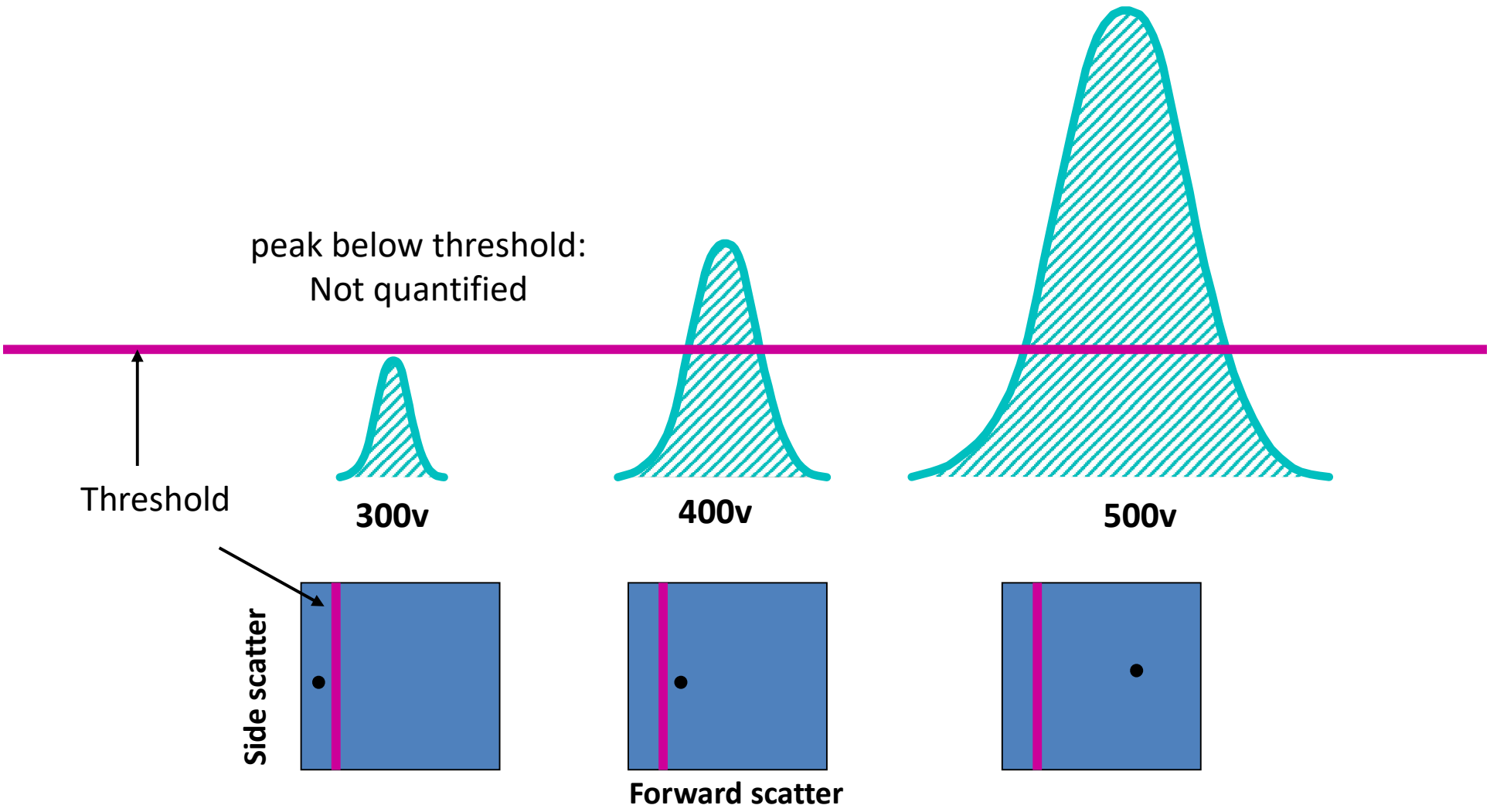
Threshold

The cytometer needs a threshold to determine what is considered an event (or cell or bead etc) and what is background or debris

Threshold: the level above which detected signals will be processed.

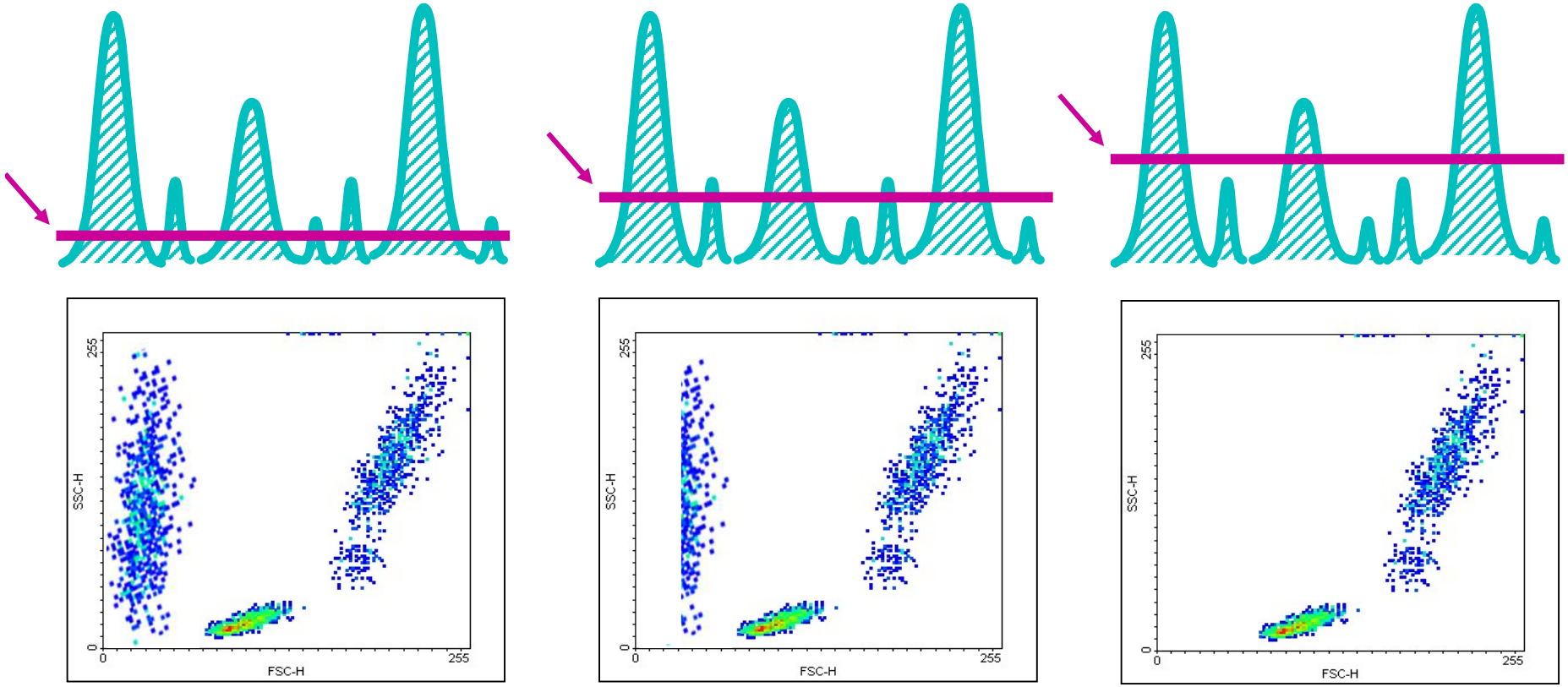
If a pulse is lower than the threshold, **it will not be seen.**
Anything below threshold is excluded from analysis.

Threshold



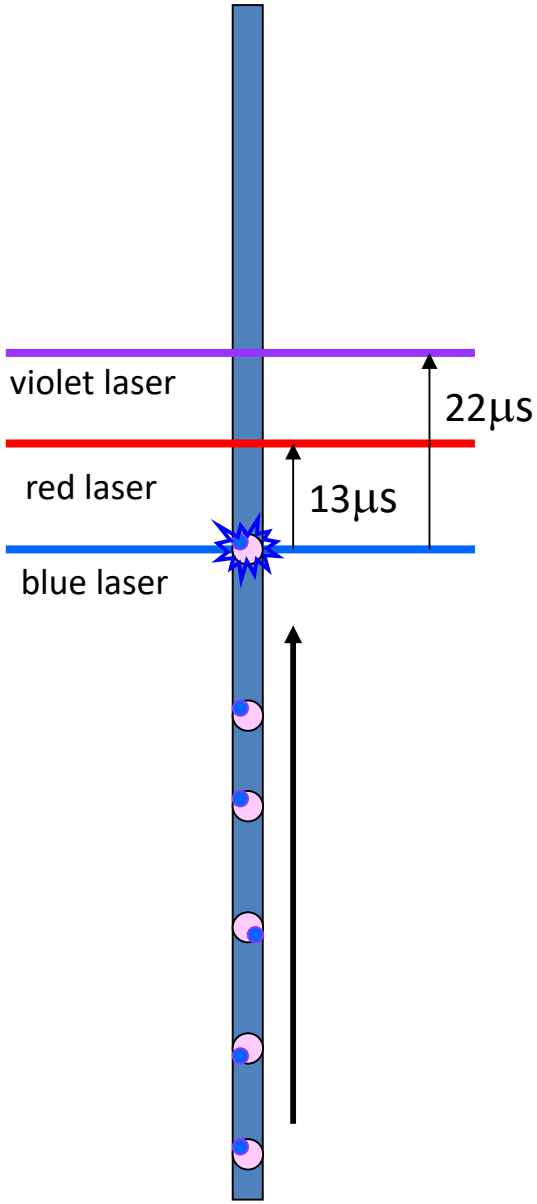
No events seen!

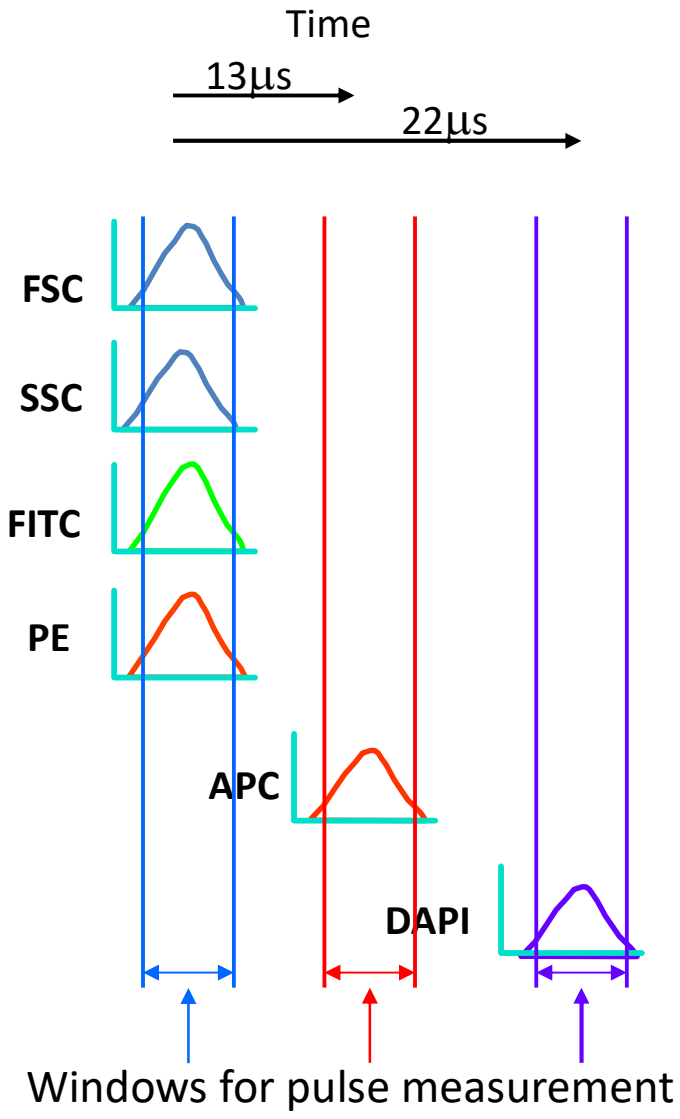
Threshold



- Increasing the threshold removes smaller pulses thus smaller events from analysis
- Events below threshold are not recorded, thus lost for good.

Laser time delay





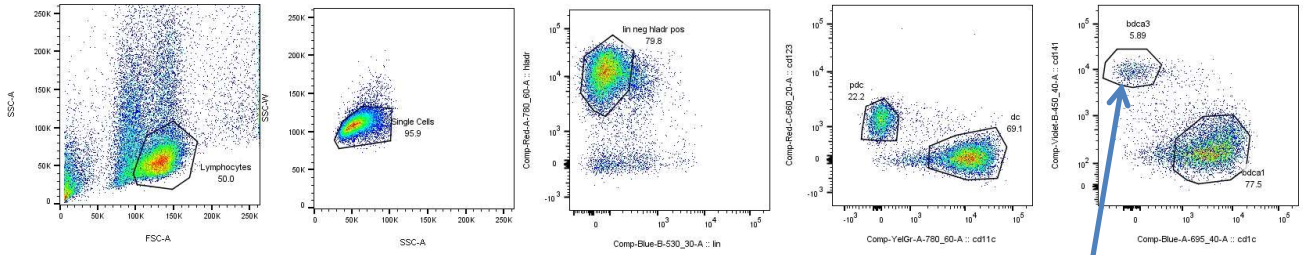
list mode (.fcs) file

	Cell # 1	Cell #2
FSC	360	450
SSC	345	375
FITC	35040	205
PE	125000	85000
APC	230	160000
DAPI	405	650

Cell Sorting

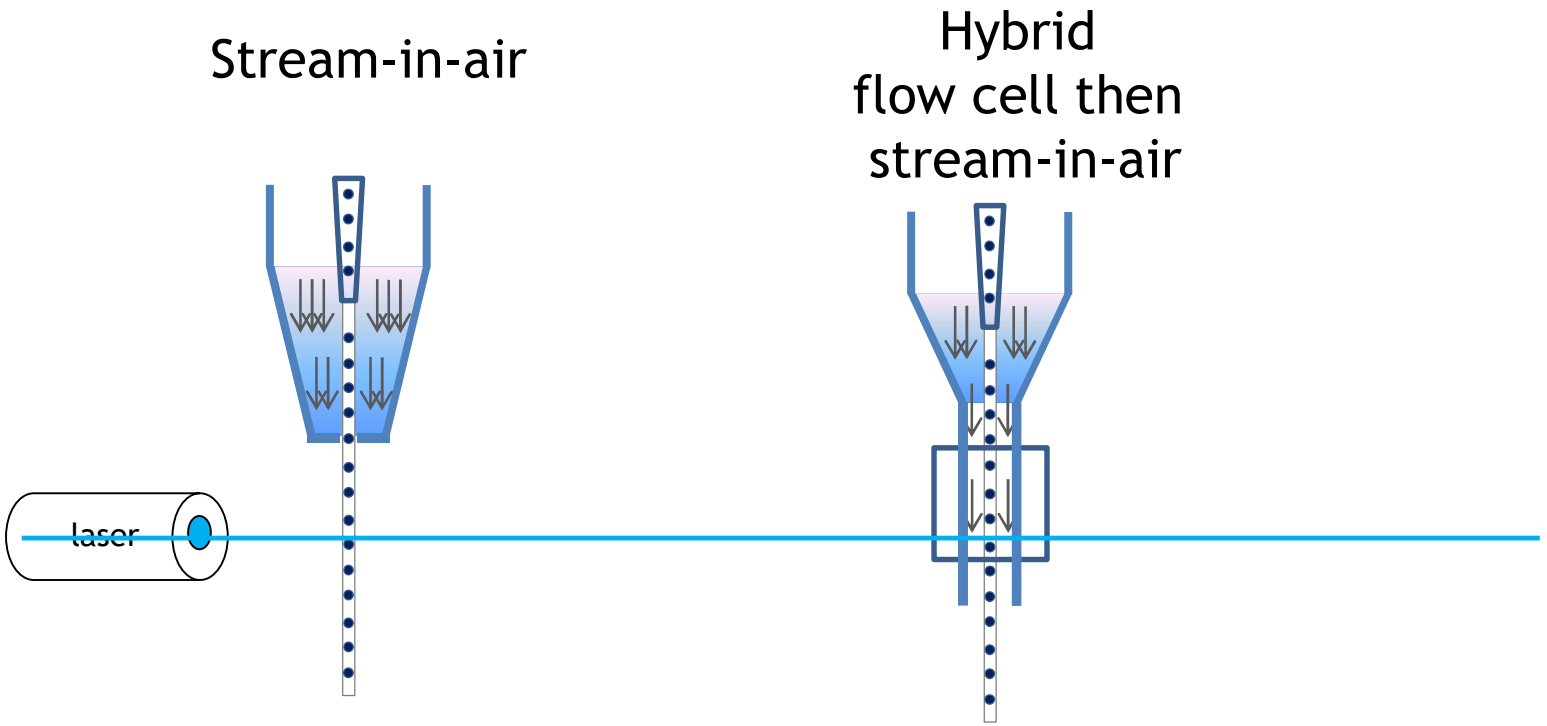
Why would we want to sort cells?

We have a very mixed population of cells



And we want to do experiments with a pure subset of these DC cells

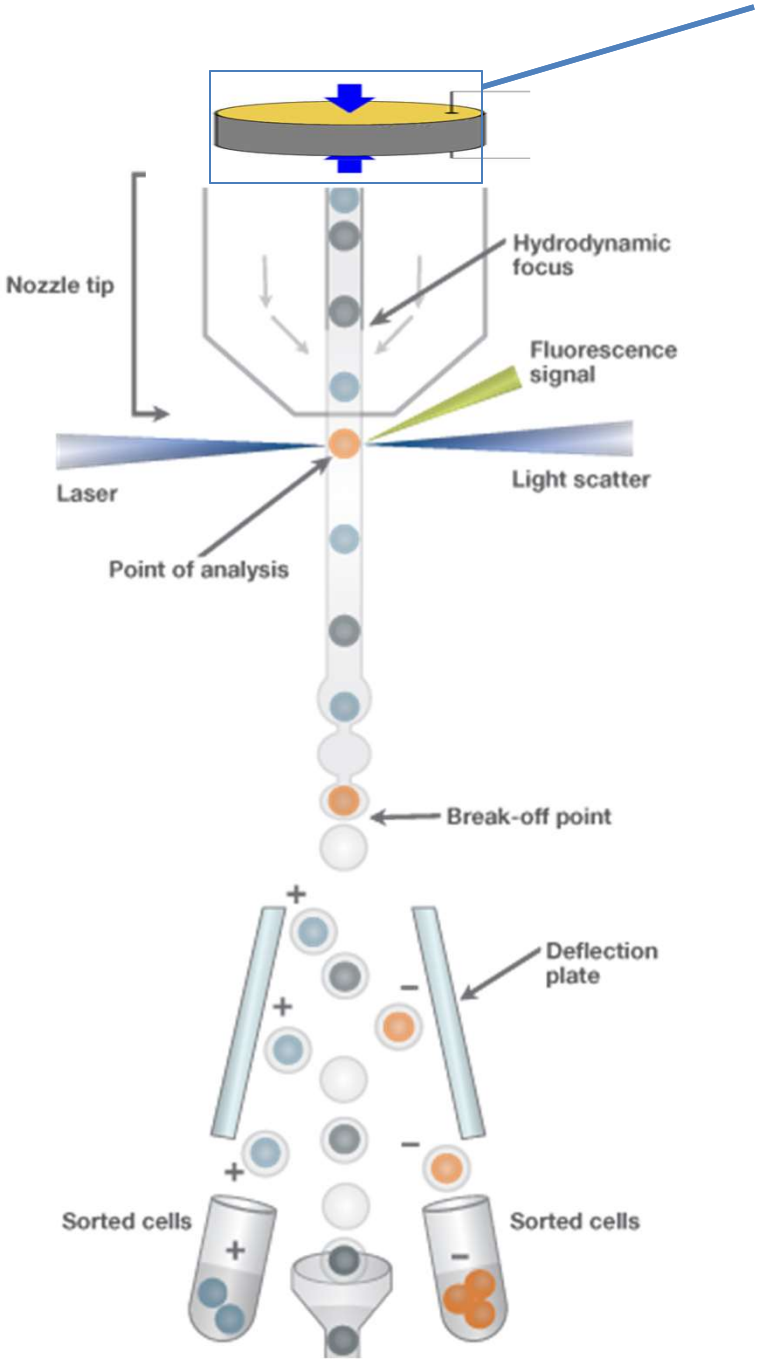
Most sorters are “stream in air”



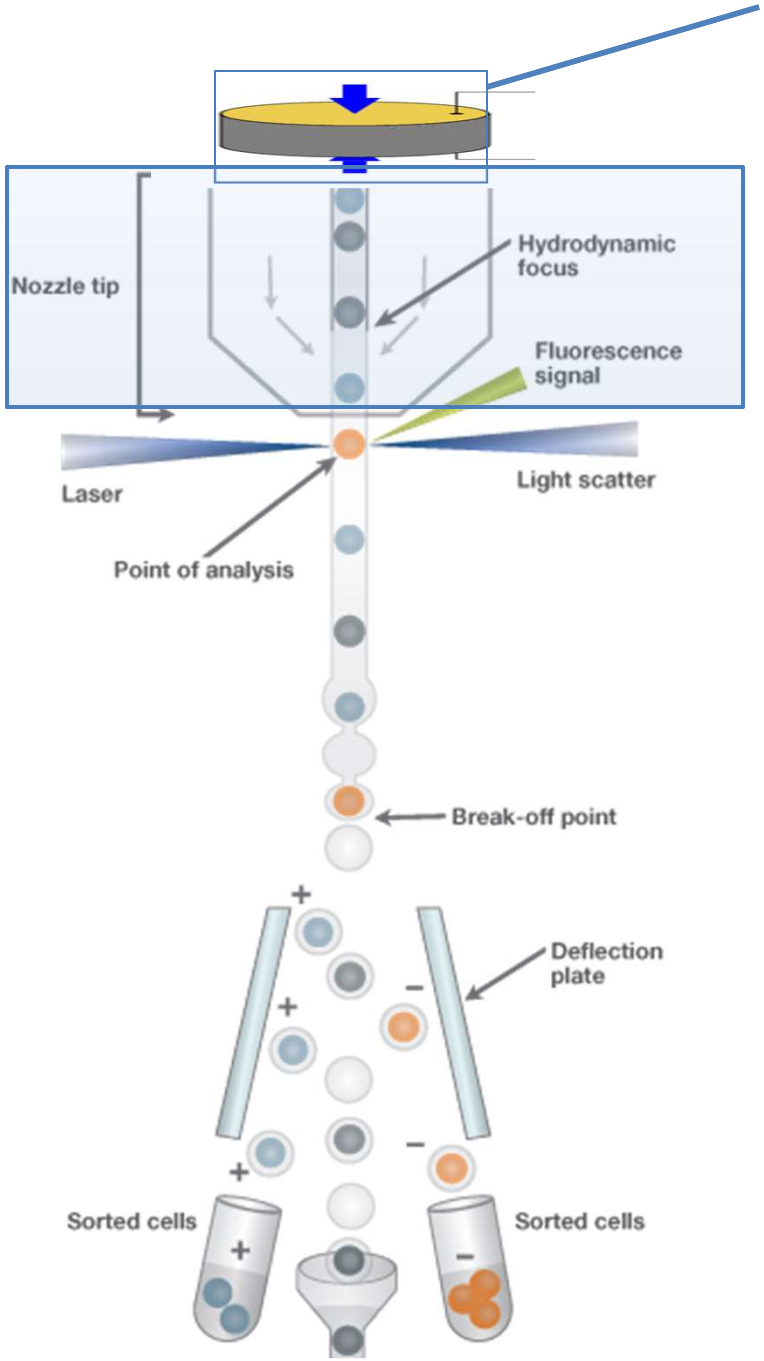
Astrios
S3
Moflo

Aria
Melody

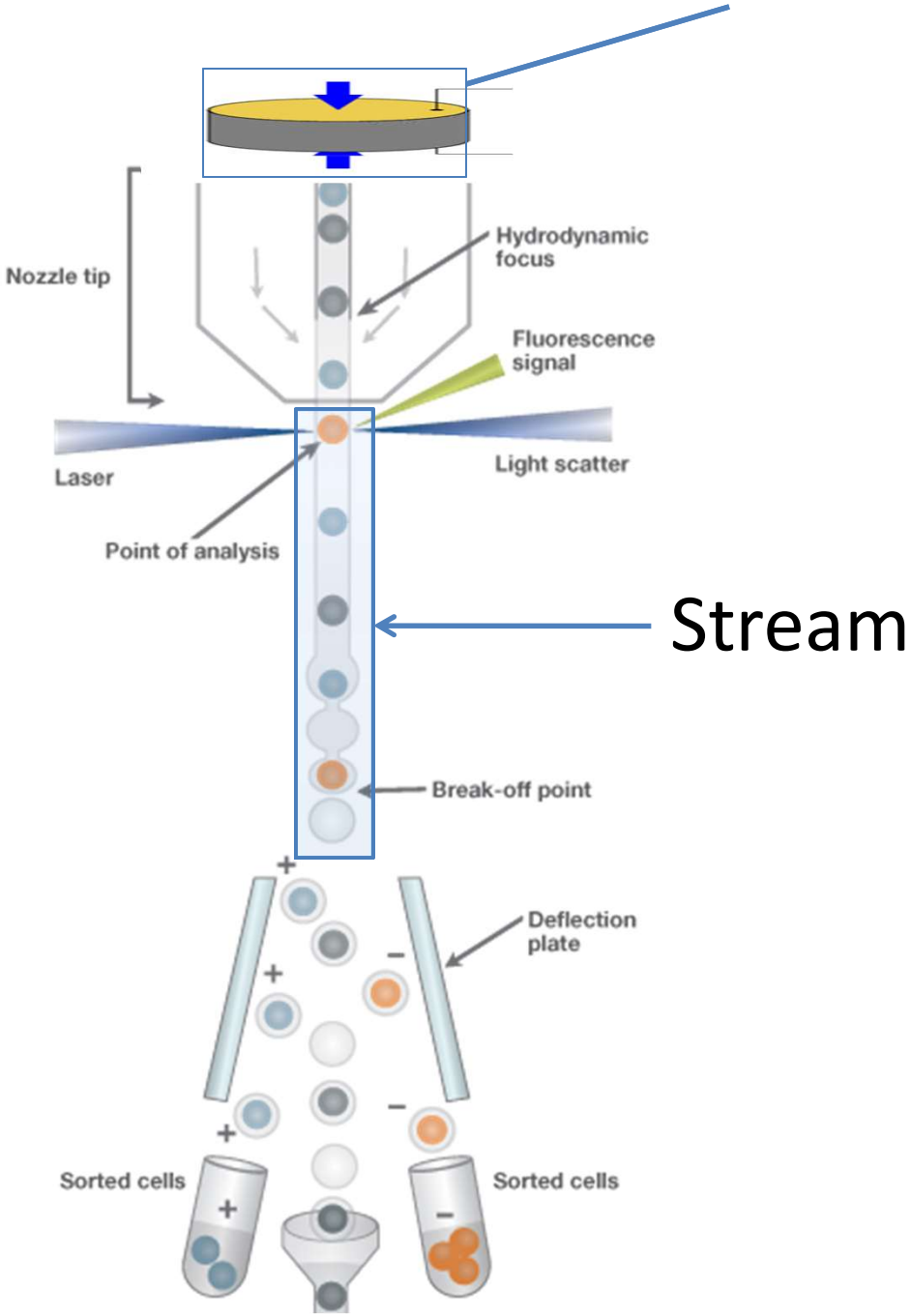
Slide from Bill Telford NIH

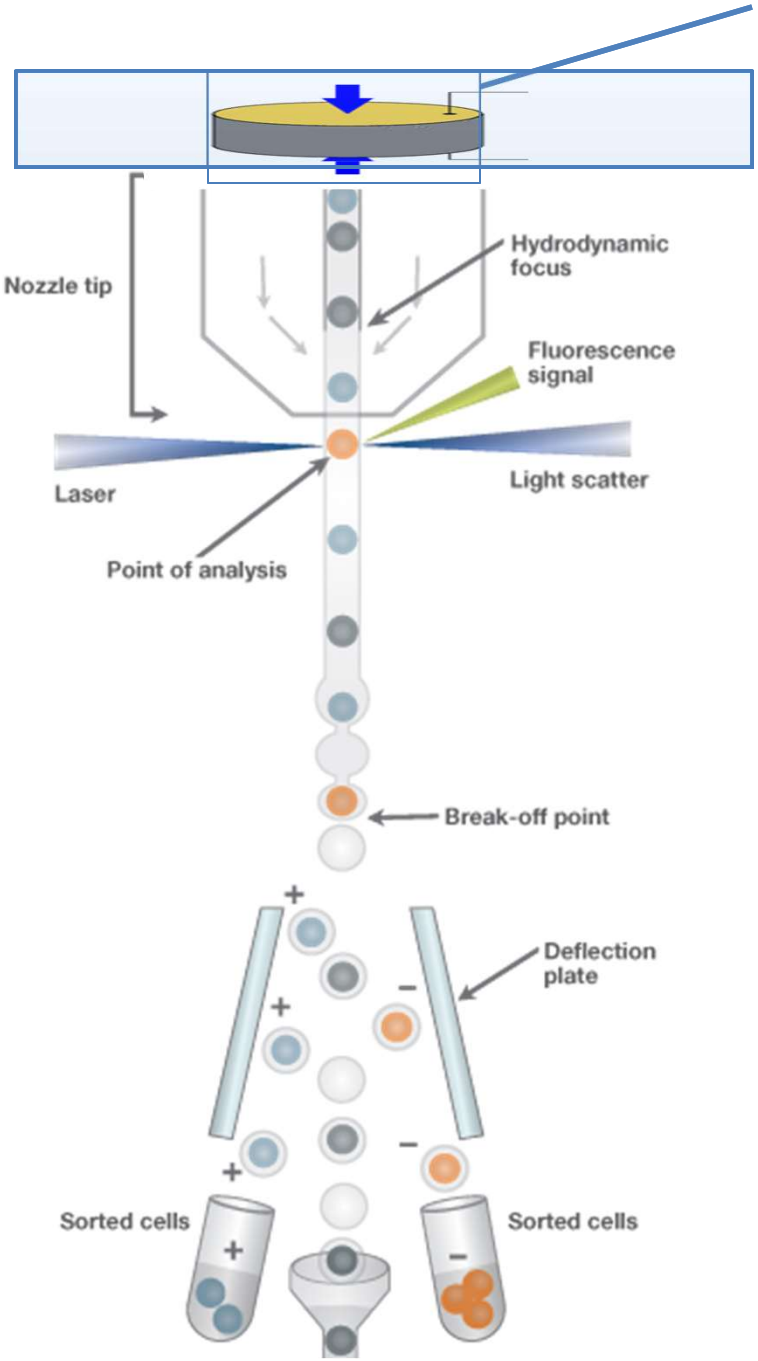


Elements of a Sorter


Nozzle tip
Hydrodynamic focus
Fluorescence signal
Laser
Light scatter
Point of analysis
Break-off point
Deflection plate
Sorted cells
Sorted cells

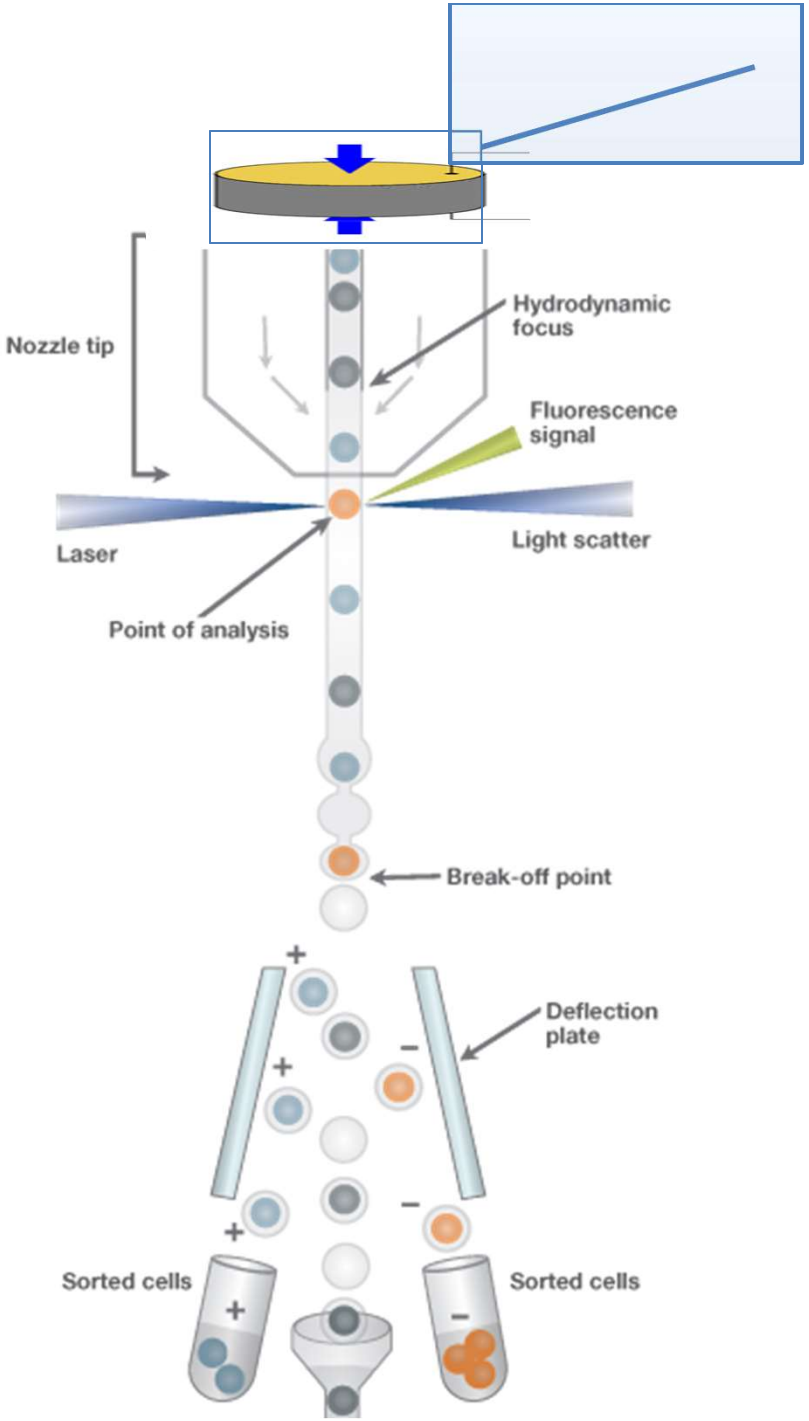
Nozzle

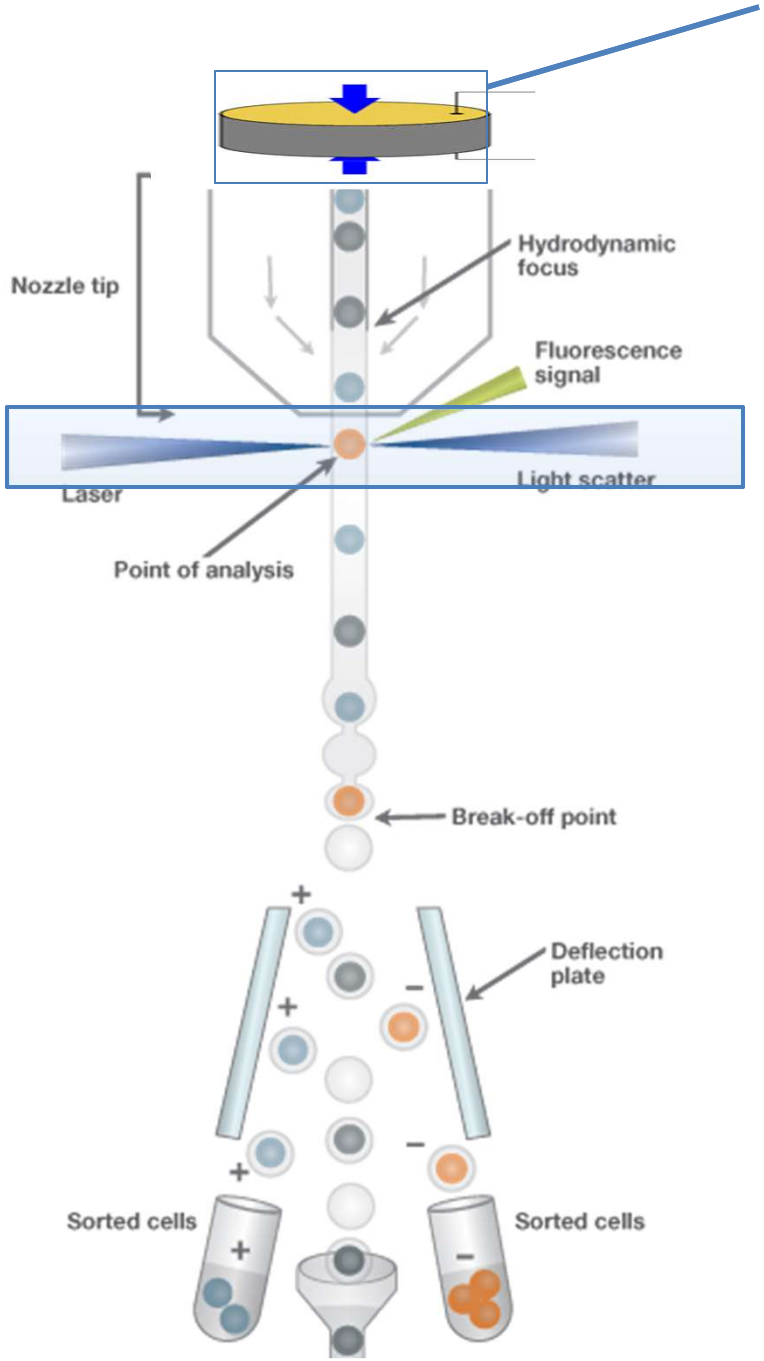

Nozzle tip
Hydrodynamic focus
Fluorescence signal
Laser
Light scatter
Point of analysis
Break-off point
Deflection plate
Sorted cells
Sorted cells
Stream



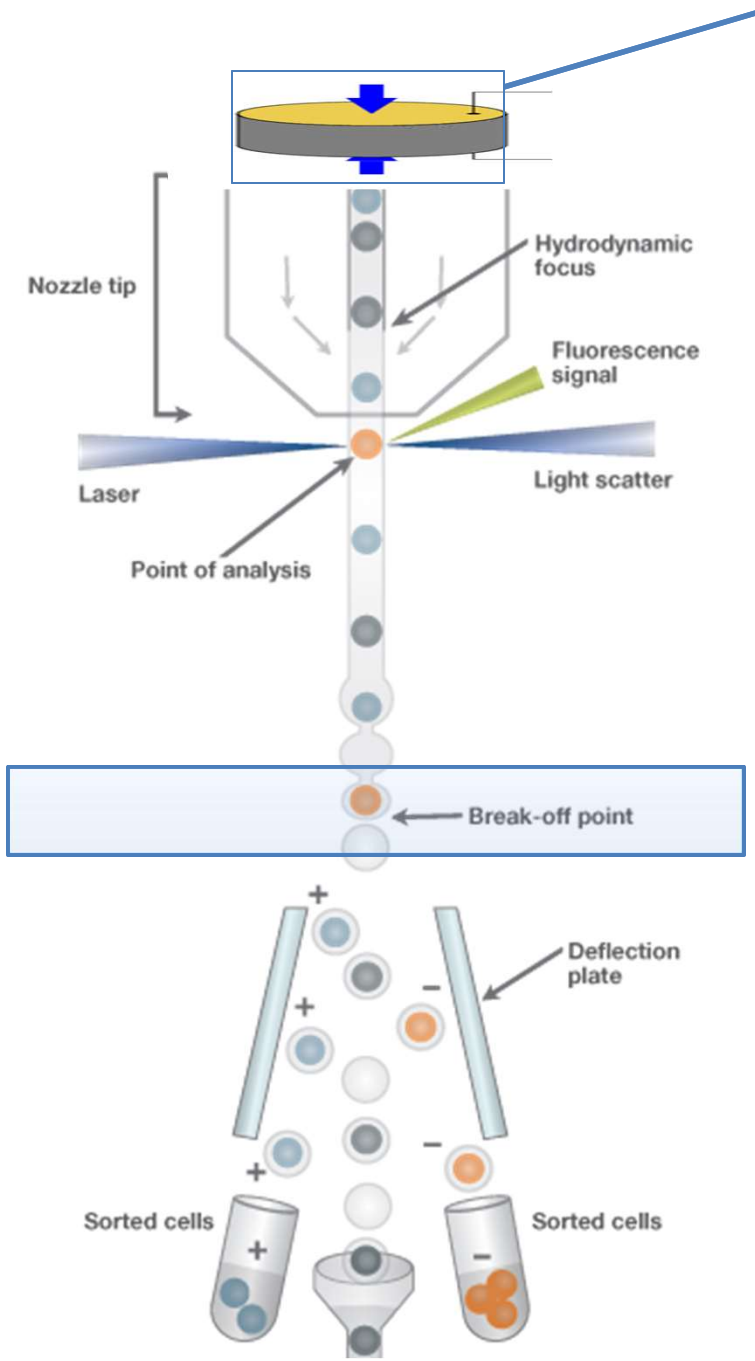
Piezoelectric crystal

Charging wire

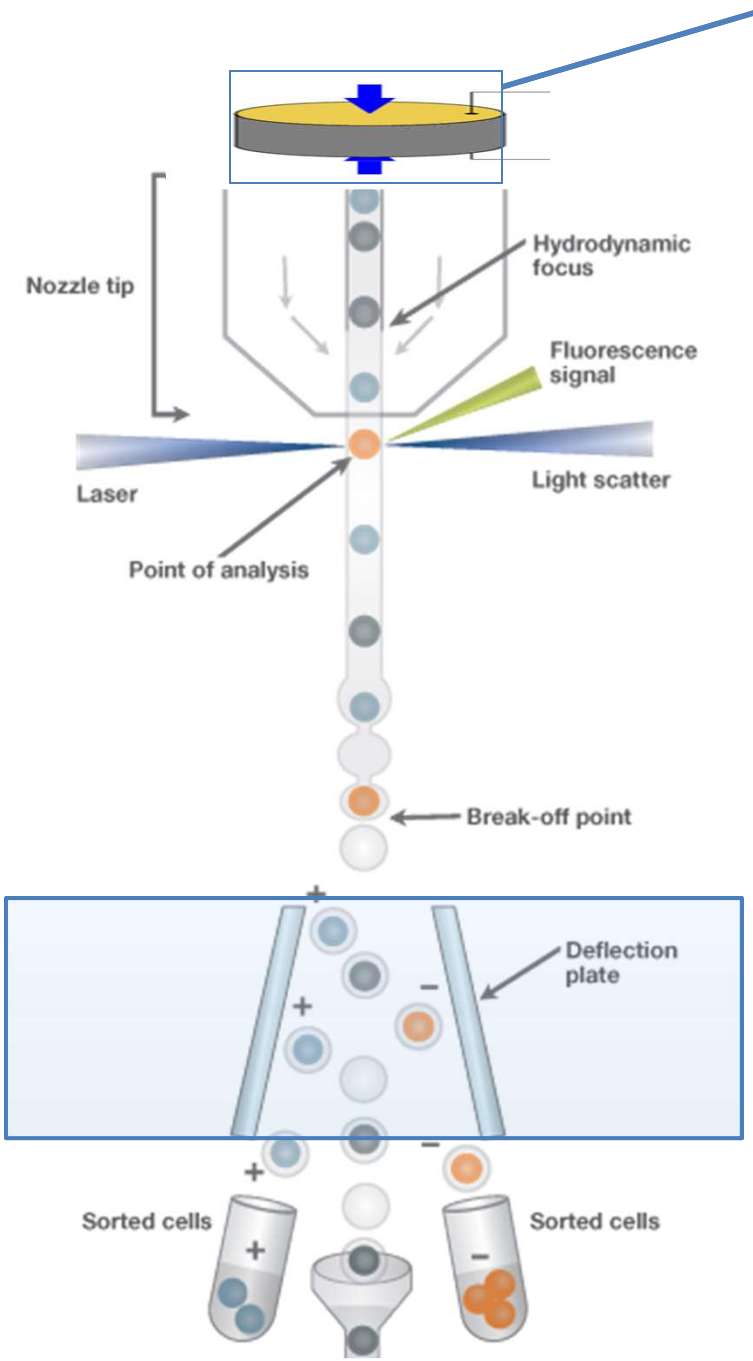




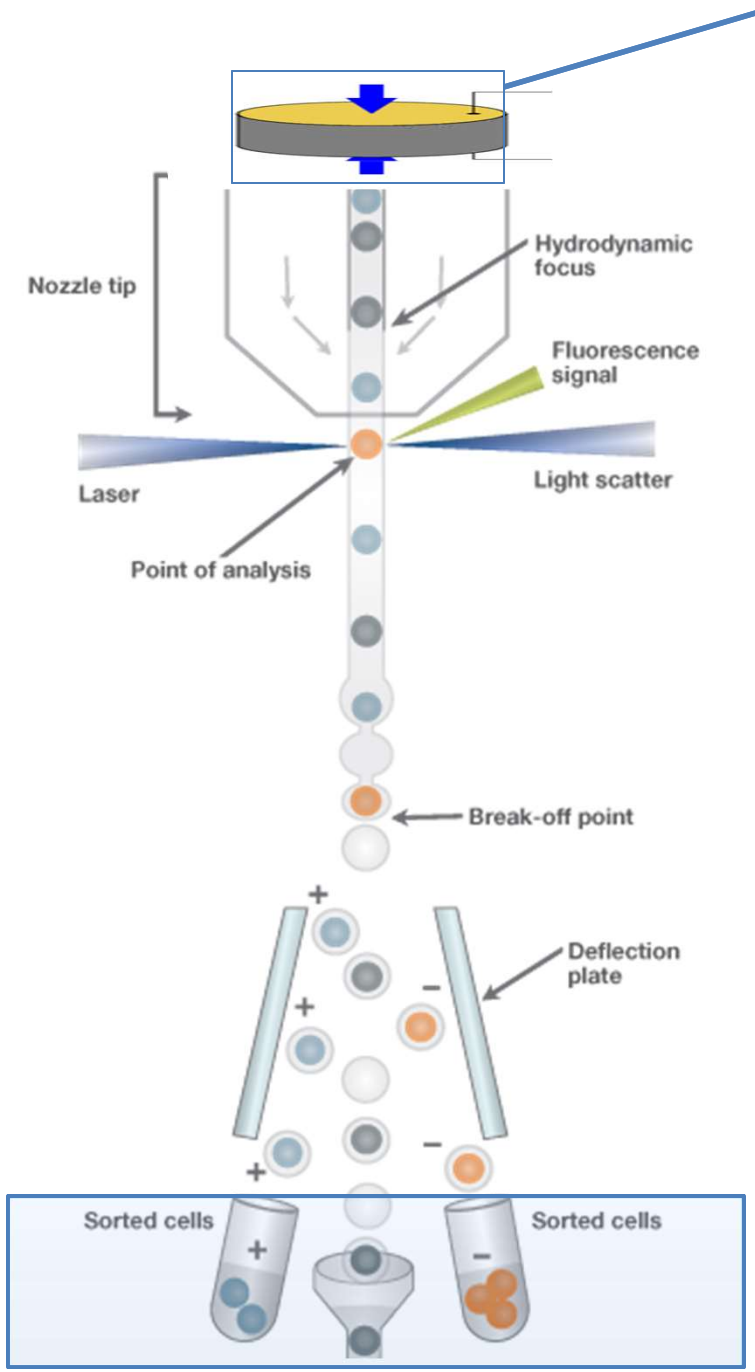
Laser Intercept



Droplet formation and Breakoff Point

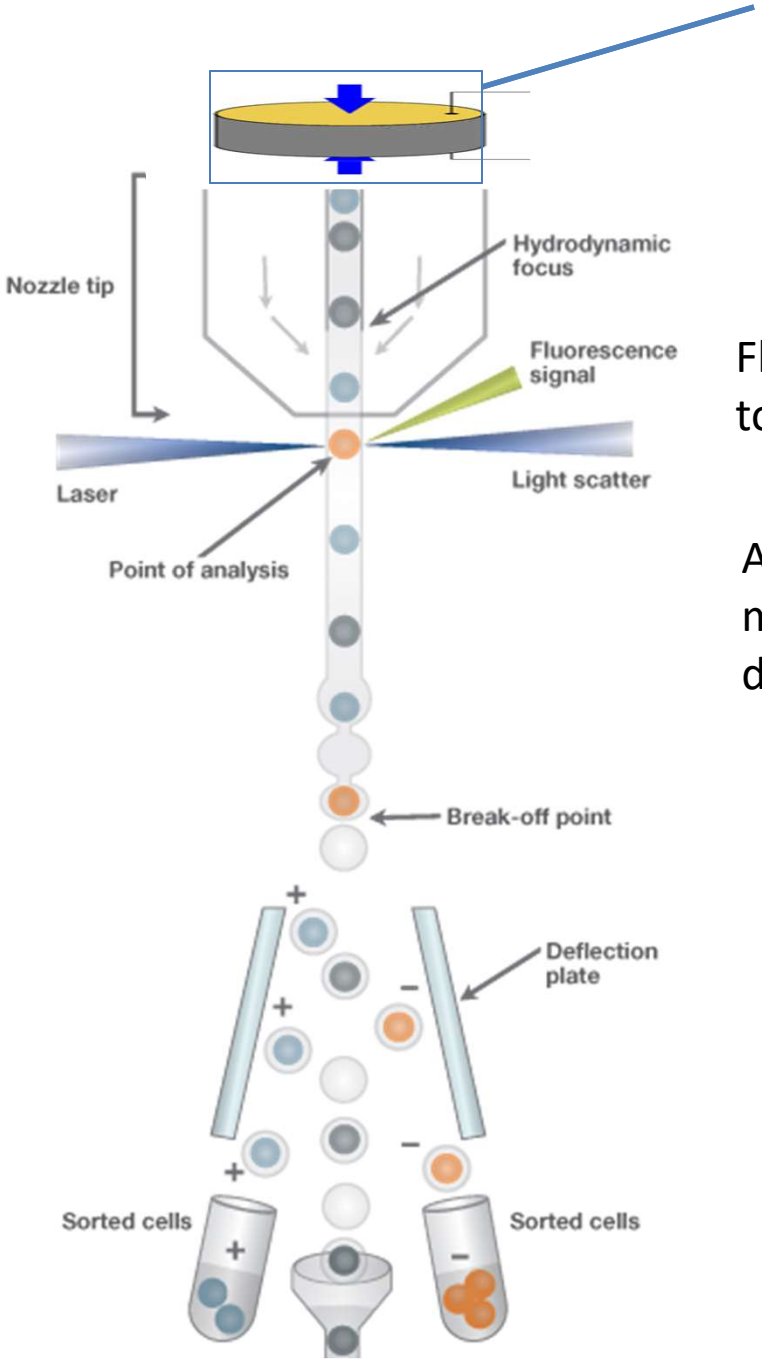


Deflection Plates



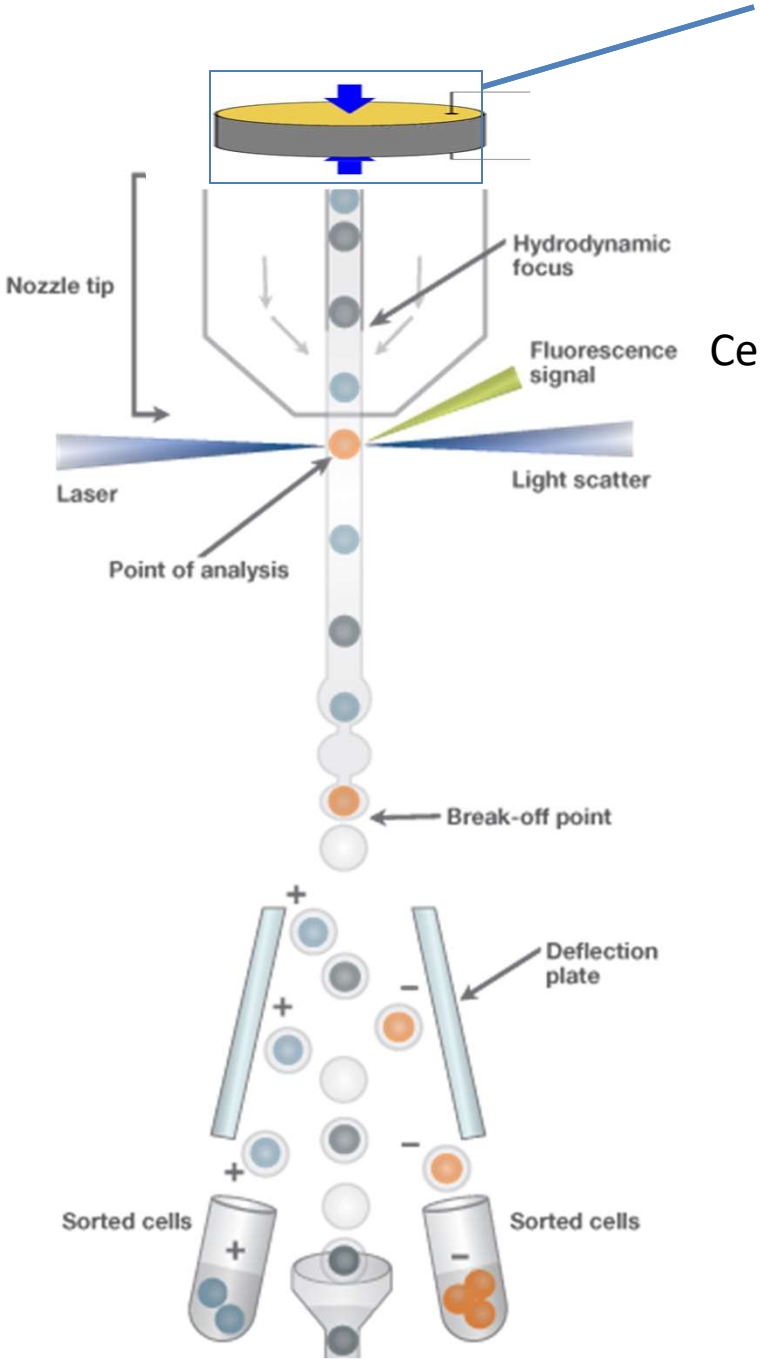
Collection Tubes

How does it work?



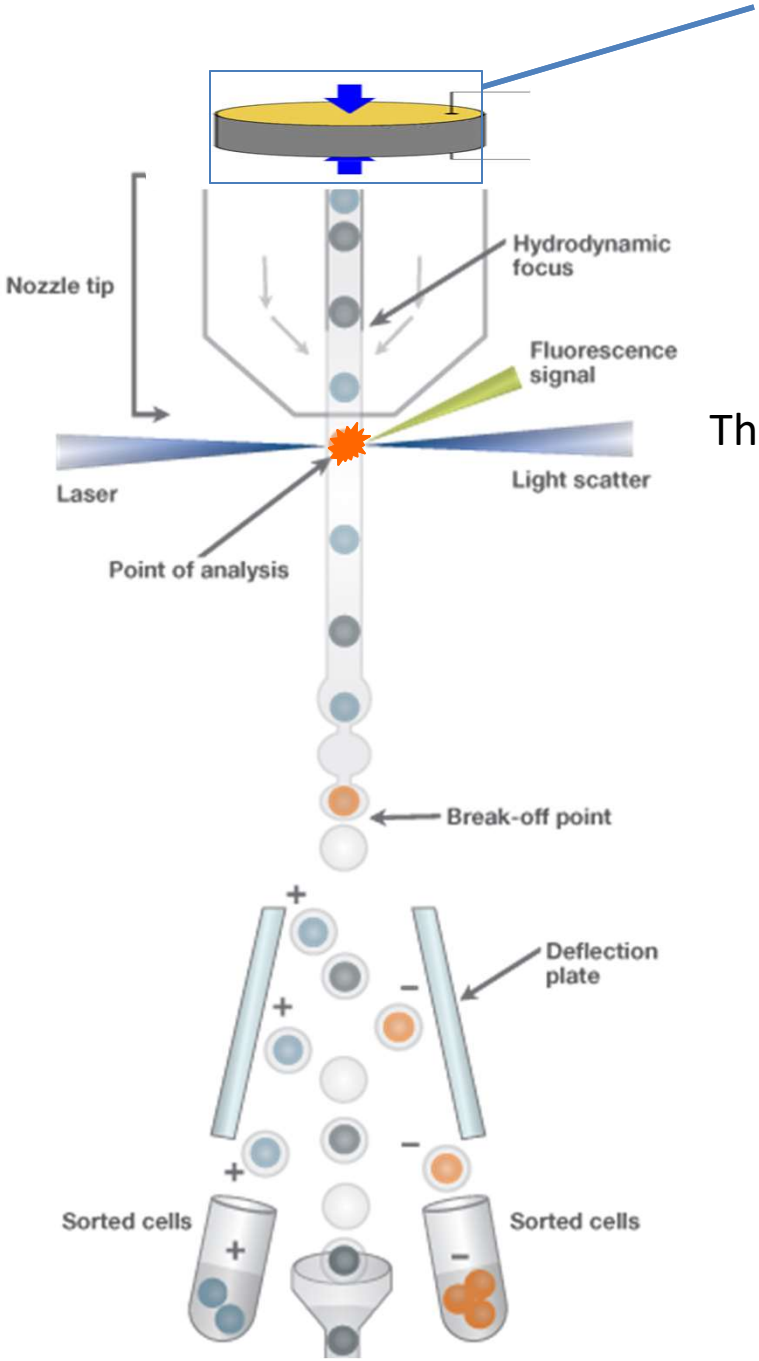
Fluid is pushed out the nozzle tip by pressure to form a stream

An oscillation is applied by the piezoeletric crystal to make waves in the stream so that it breaks into droplets

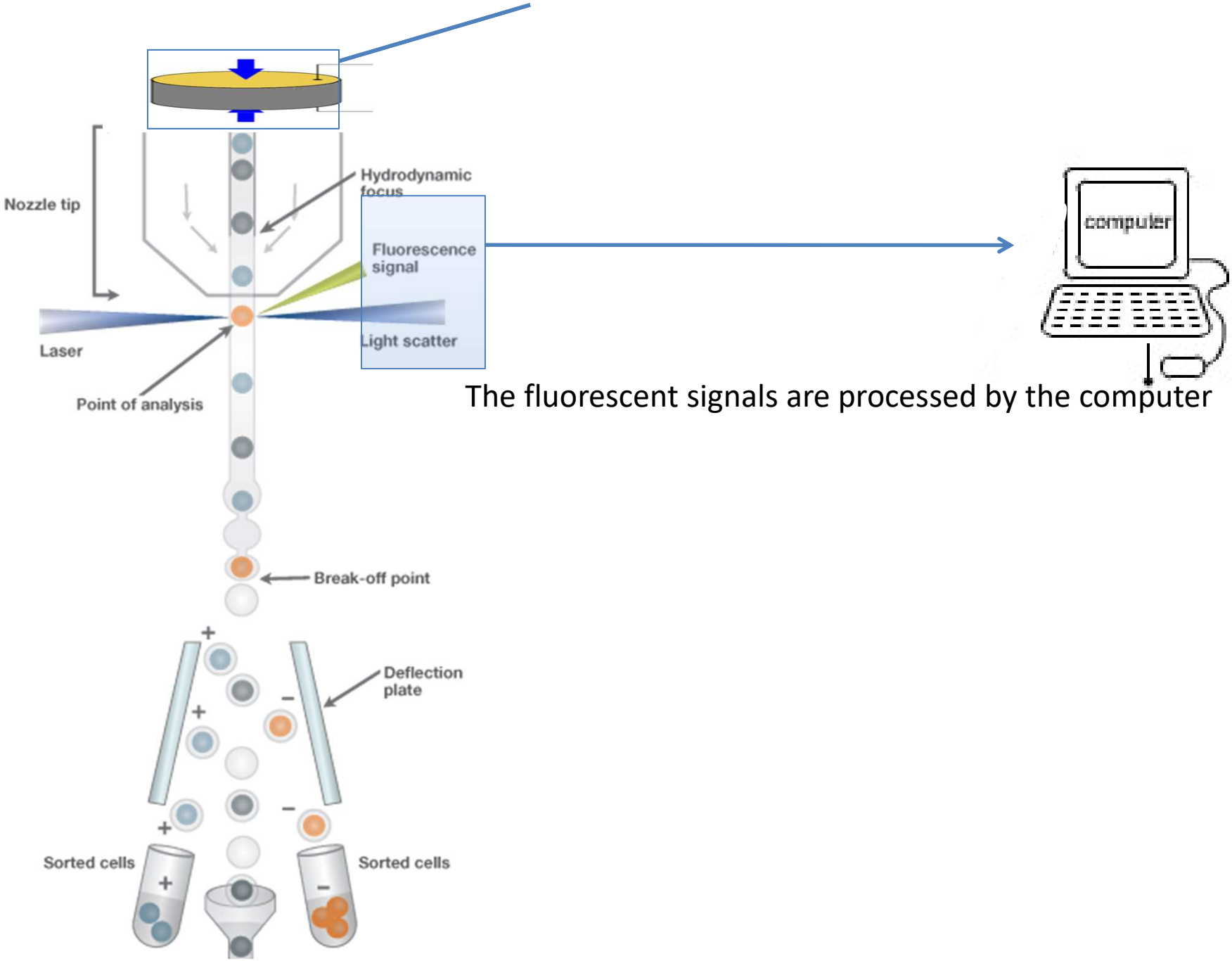


Cells pass one by one through the nozzle into the stream

Detection

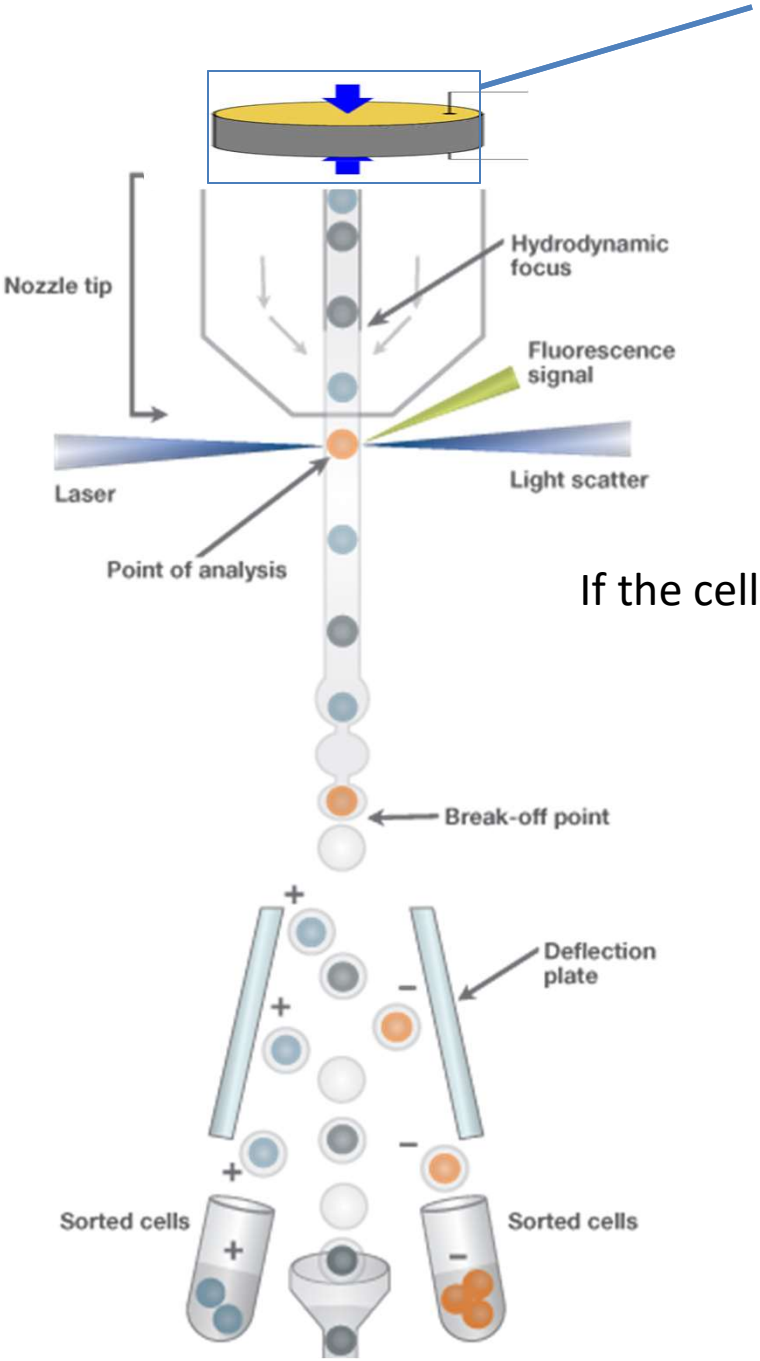


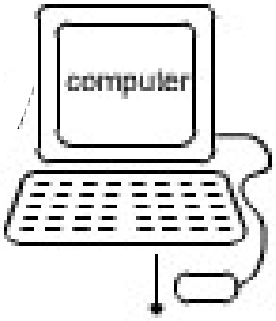
The cells pass through the laser beam and fluoresce



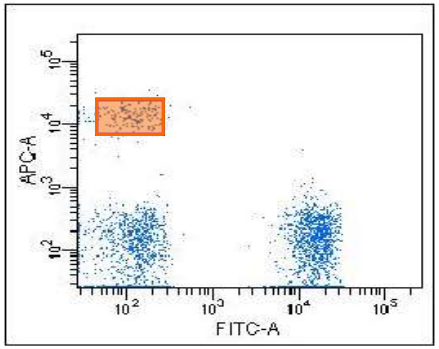
The fluorescent signals are processed by the computer

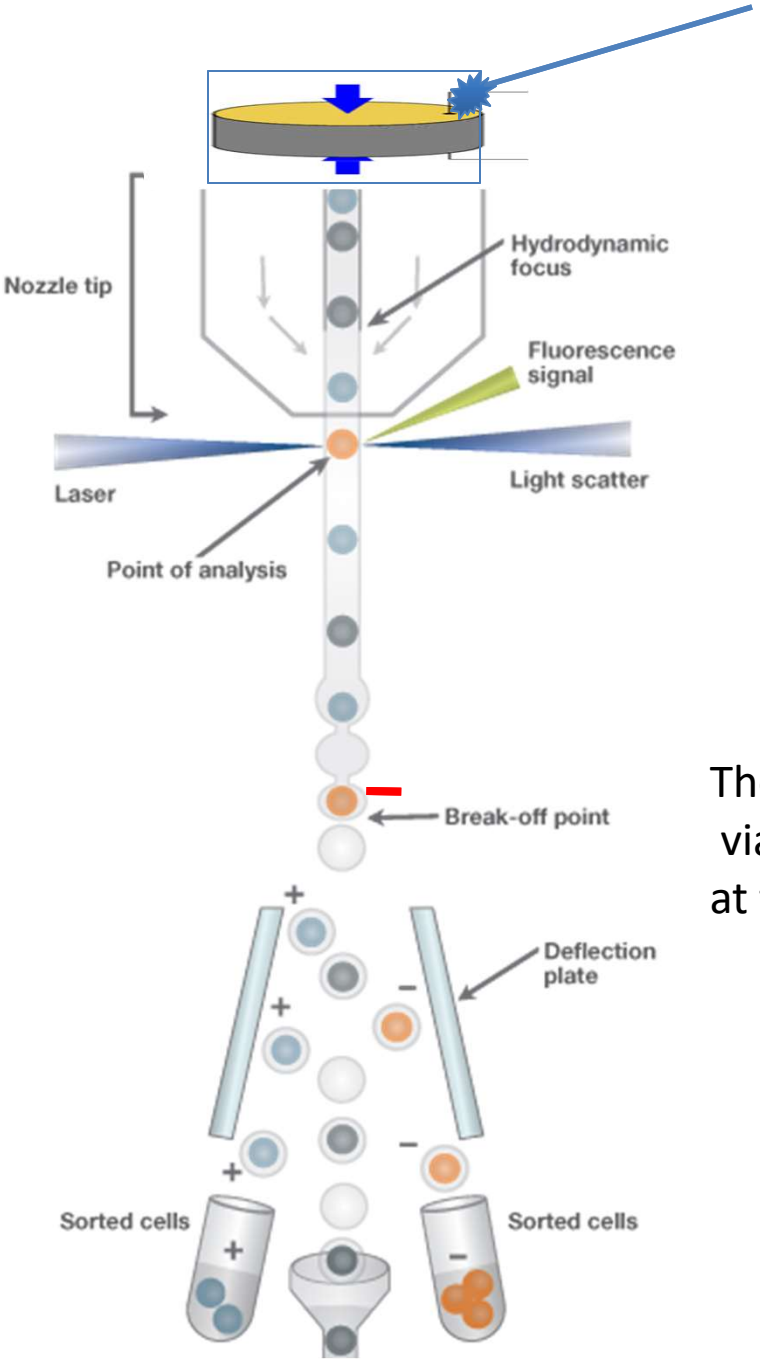
Decision

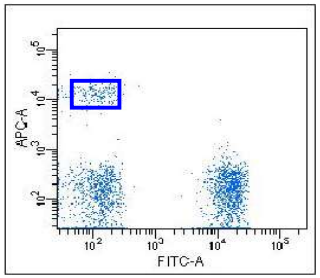




If the cell is within the defined sort gate

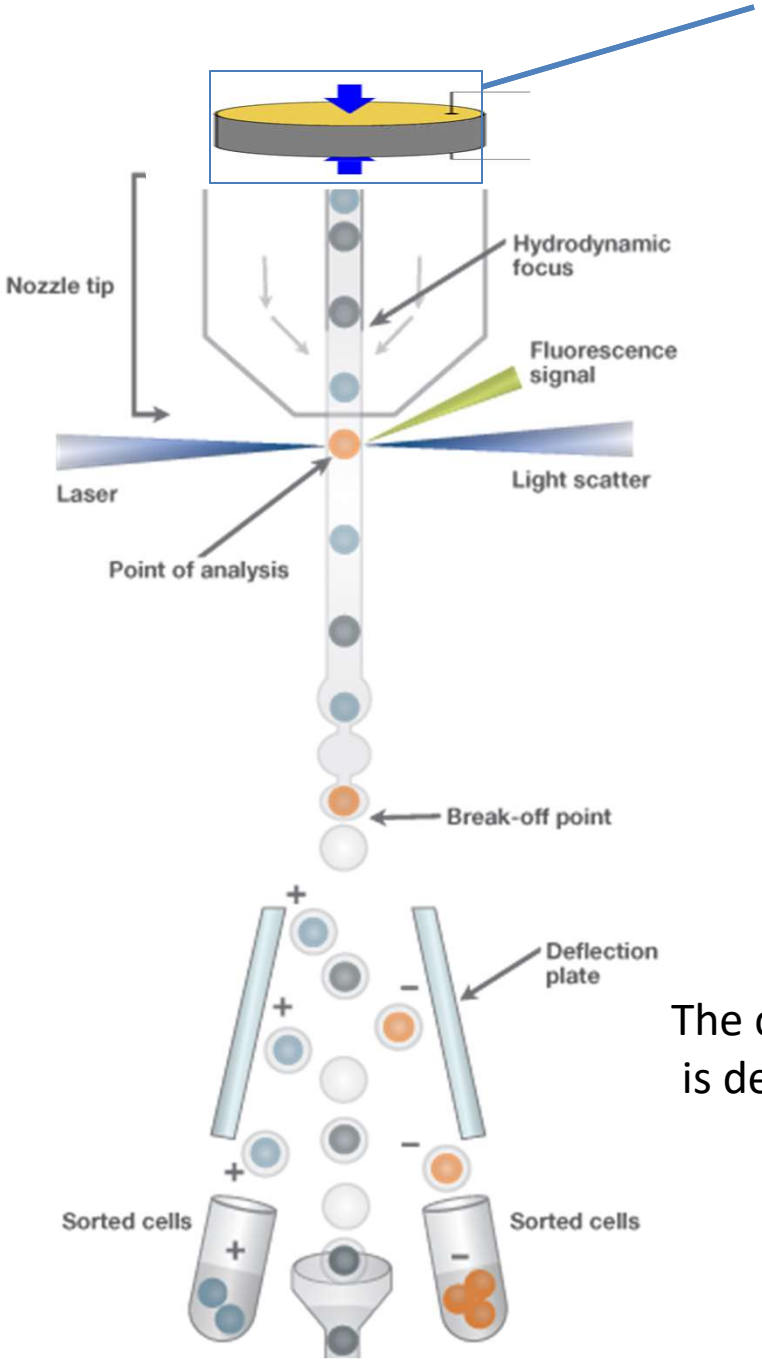






The cytometer sends a signal to charge the stream
via a charging wire in the nozzle
at the very moment that cell reaches the breakoff point

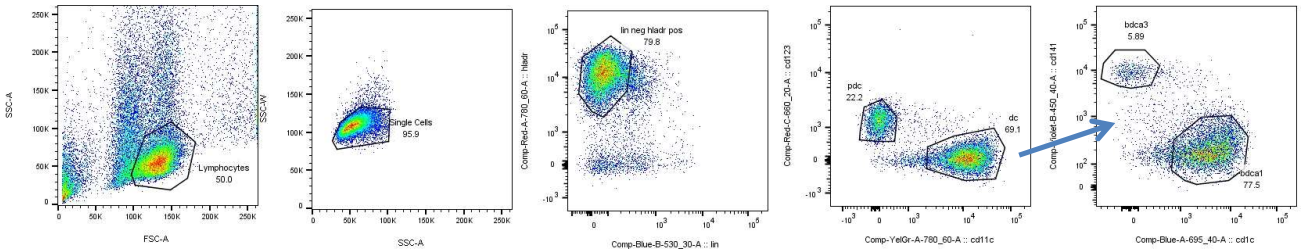
Deflection



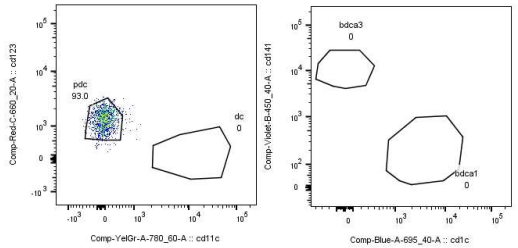
The charged droplet containing that cell
is deflected by charged plates into a collection tube

Sort results

Before sort

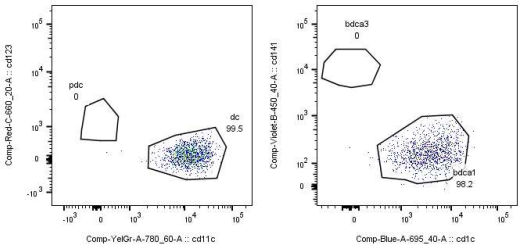


after sort purity checks pdcs

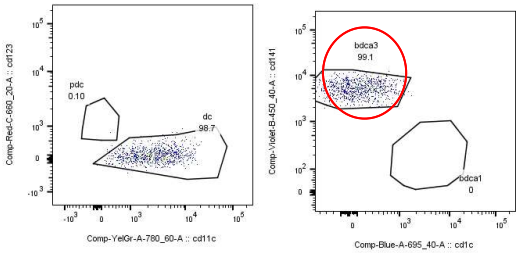


All this happens at
8,000-40,000 cells per second!

dcs bdca1



dcs bdca3



What can Flow Cytometry do?

The cells can be stained with multiple markers coupled to different fluorochromes, currently 28 different colors can be done

The data acquired allows rapid quantitation and complex analysis of all the different populations of cells in the sample.

Pure subpopulations of cells of interest can sorted at high speed into tubes or or cloned in 96 or 384 well plates for subsequent experimentation.

Applications include multicolor phenotyping, measurement of apoptosis, cell cycle, cell kinetics, minimum residual disease, stem cell analysis.

Applications

Research

- Cellular biology:
  - Phenotyping
  - Immunology
  - Membrane potential
  - pH
  - Calcium Flux
  - Proliferation
  - Apoptosis
  - Cell Cycle
  - Gene transfections: Fluorescent Proteins
  - Stem Cell Analysis
  - Cell Signaling
- Plant Biology
- Marine Biology
- Microbiology
- Extracellular vesicles

Clinical

- Pathology and Laboratory Medicine
  - Leukemia and Lymphona
  - Immunology
  - Minimal Residual Disease
  - Stem Cell Enumeration
  - Crossmatching
  - Autoantibodies
  - HIV/AIDS – CD4 enumeration
  - Fetal RBC
  - Immunodeficiencies
  - Paroxysmal nocturnal haemoglobinuria
  - Reticulocytes
  - Microbiology

References

Mike Ormerod's Basic Flow Cytometry book:
http://flowbook.denovosoftware.com/Flow_Book

Howard Shapiro's Flow Cytometry book:
http://www.beckmancoulterreagents.com/us/?page_id=1660

Good basic tutorials free on the web:
https://www.thermofisher.com/fr/fr/home/support/tutorials.html?cid=cid-mptutorials

https://www.bdbiosciences.com/us/support/training/s/itf_launch

Thank you!

Slide courtesy of Celine Lages and Sherry Thornton