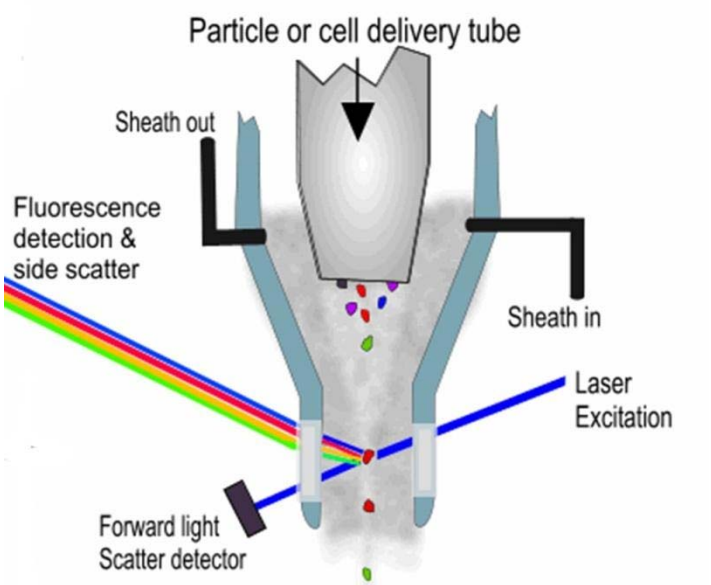
Principles of flow cytometry

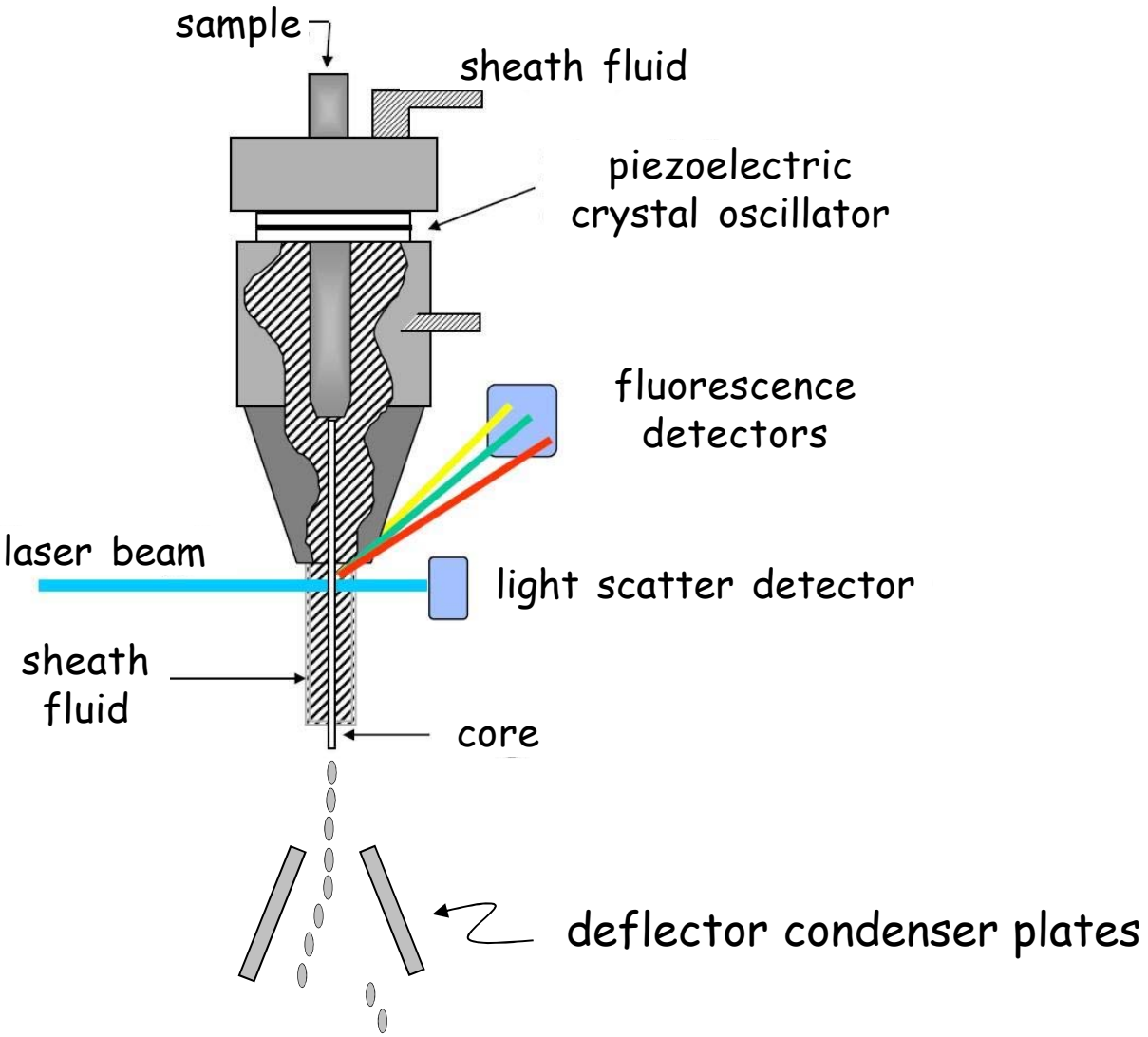


An instrument which measures

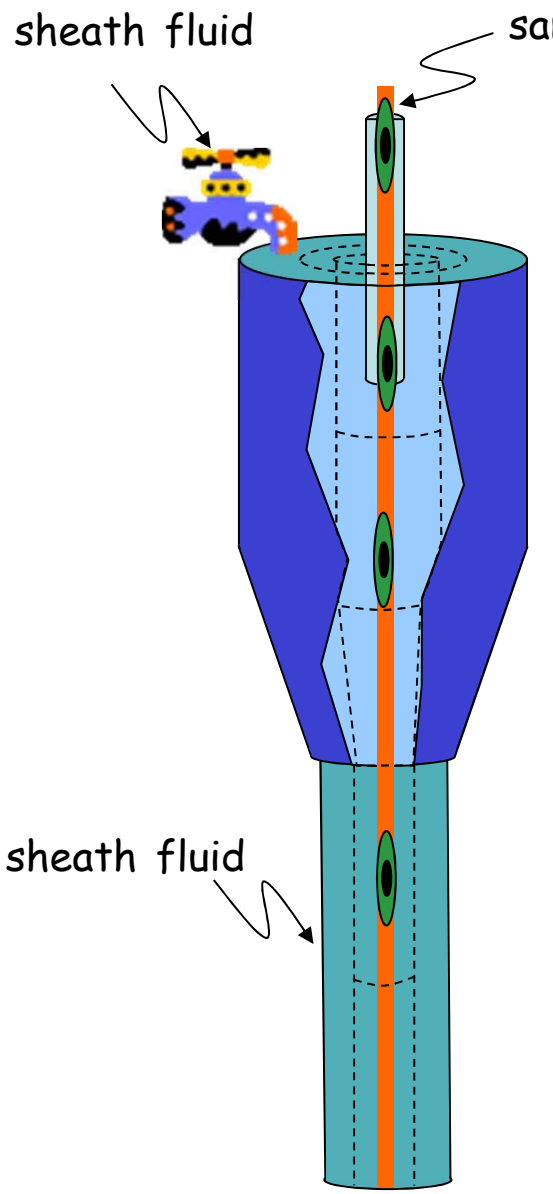
- the fluorescence and light scatter parameters of
- suspended
- single cells
- with high speed (as high as several thousand cells/sec)

	Flow cytometry	Fluorescence microscopy	Fluorometry
Types of cells	suspended	suspended and attached	suspended (or attached)
Single cell resolution	single cells (no subcell. resolution)	single cells with subcell. resolution	population
Measured parameter	fluorescent and light scatter	mainly fluorescent	mainly fluorescent
Speed	several thousand cells/sec	a couple of cells/sec	N.A.
Manipulation	sorting	manipulation of single cells	-

The working principle of flow cytometry



Fluidics



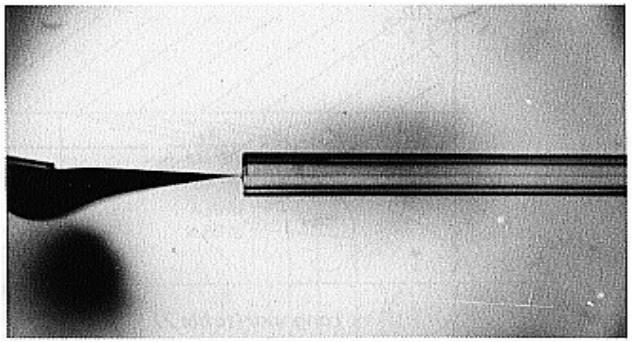
The sheath fluid surrounds the sample in concentric layers (laminar flow).

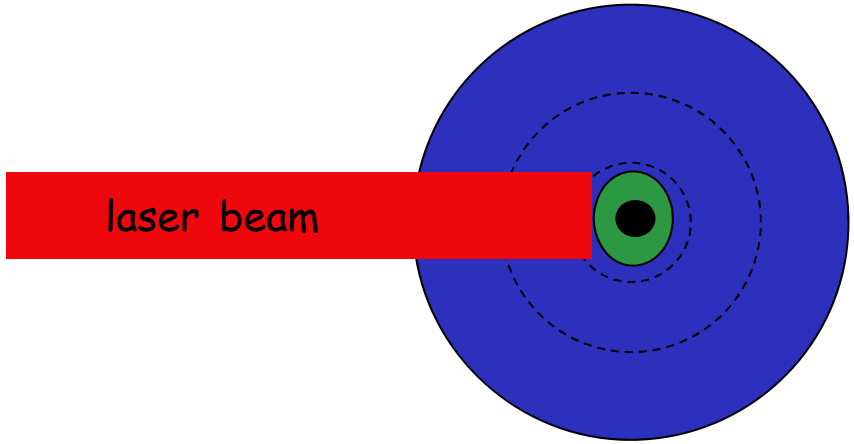
In this way the sample fluid is centered (focused) into the middle of the stream (hydrodynamic focusing).

Purpose of hydrodynamic focusing:

The cells shall be where the laser beam illuminates them.

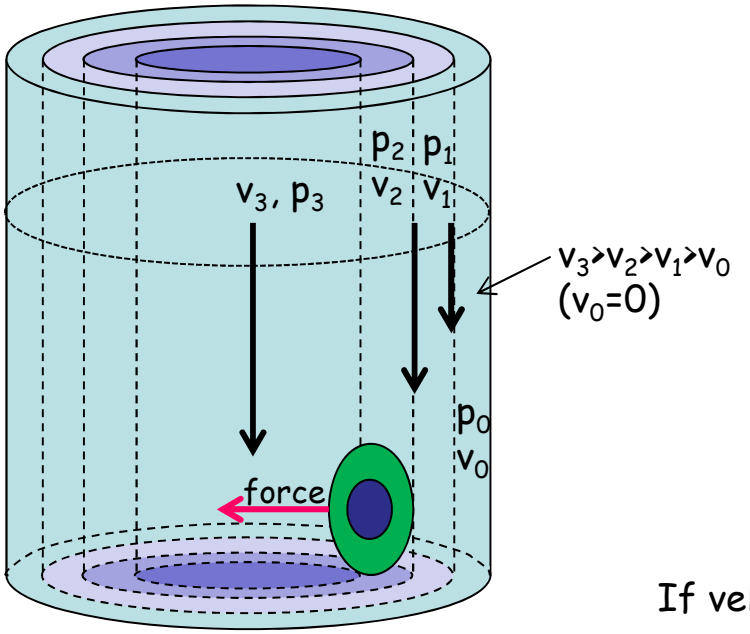
Hydrodynamic focusing of ink





Top view of a flow cell (nozzle)

Hydrodynamic focusing



Laminar flow: concentric layers of fluid flowing with increasing speed towards the center.

Bernoulli equation:

$$p + \frac{1}{2}\rho v^2 + \rho g h = const.$$

If investigated at a given height (h):

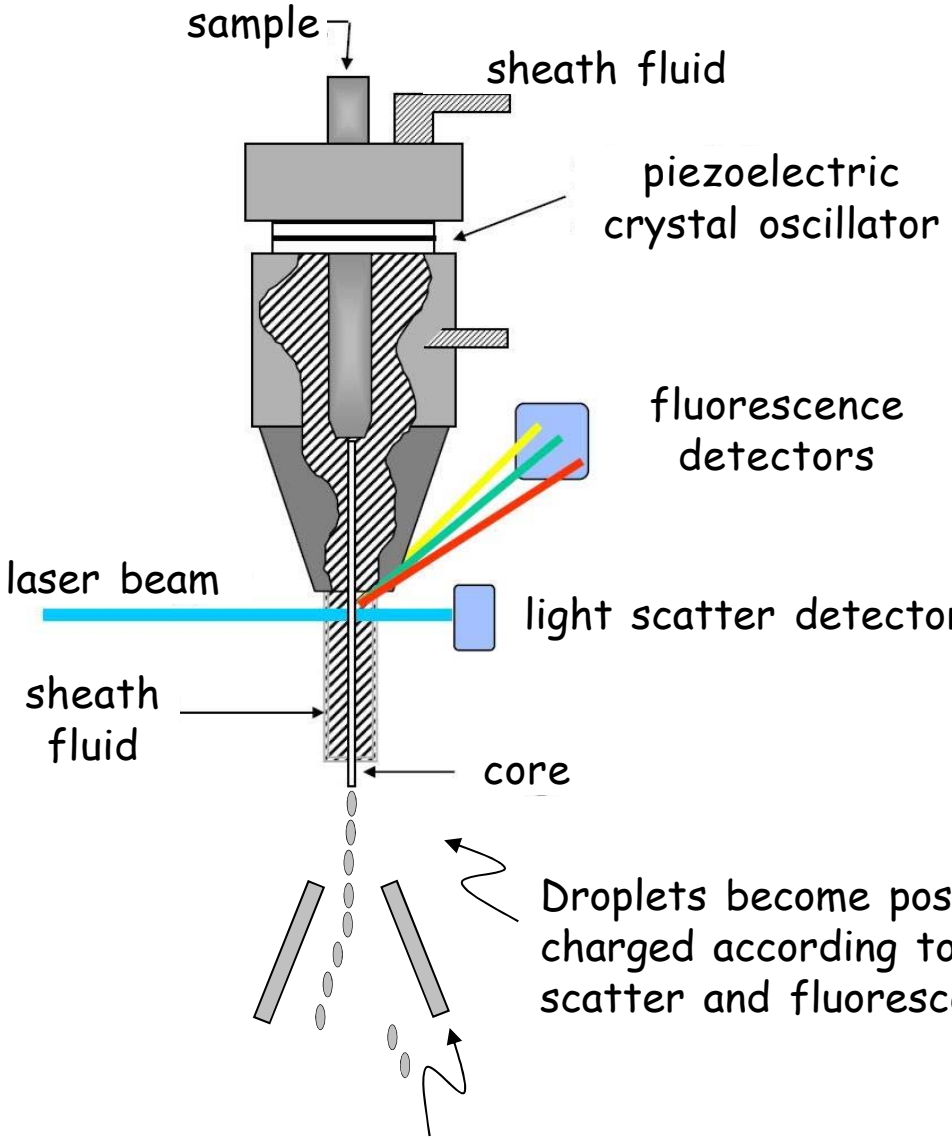
$$p + \frac{1}{2}\rho v^2 = const.$$

If velocity (v) increases, pressure (p) must decrease.

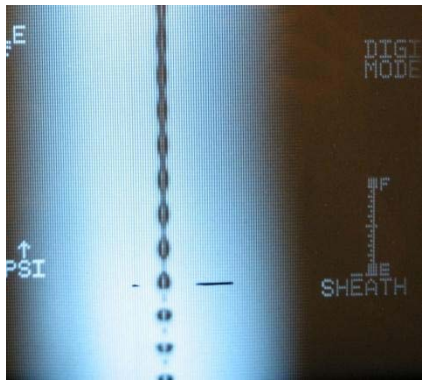
$p_0 > p_1 > p_2 > p_3$

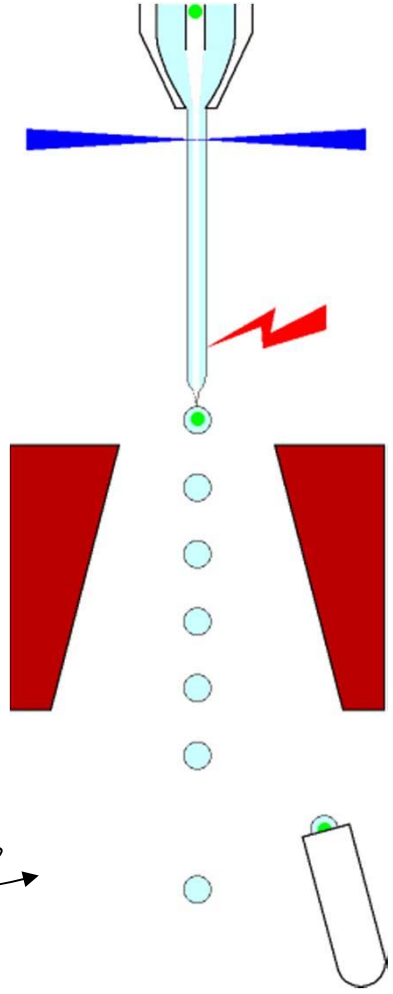
A cell is forced to move towards the center of the stream due to the pressure gradient.

Cell sorting

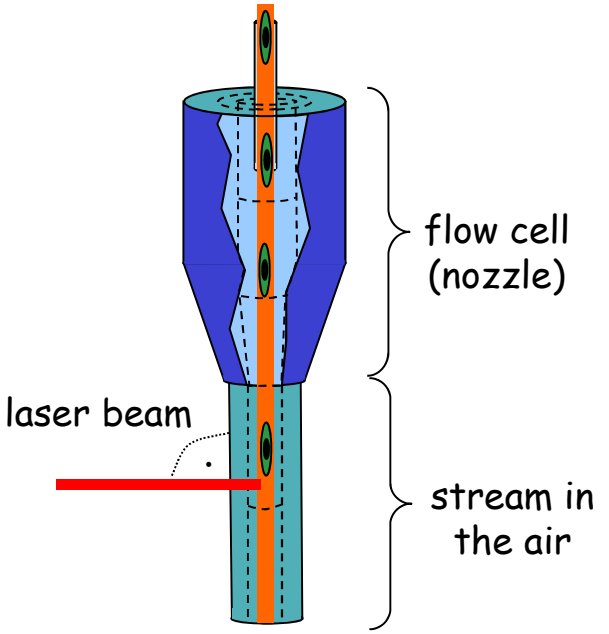


Vibration of the piezoelectric crystal breaks the stream into droplets.

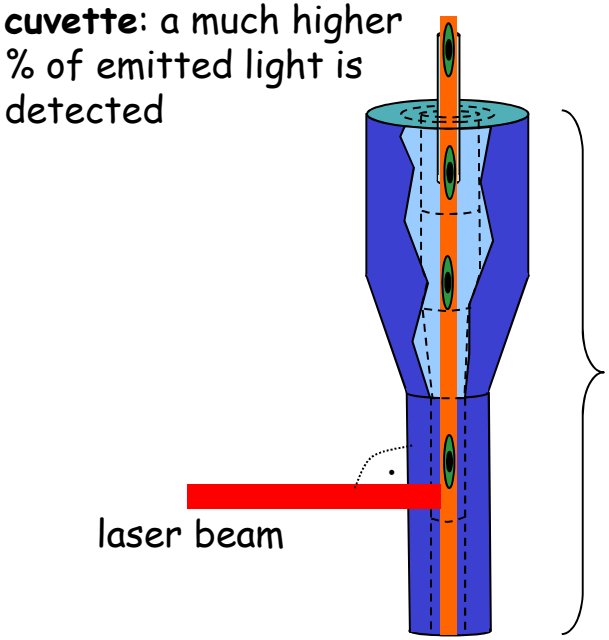




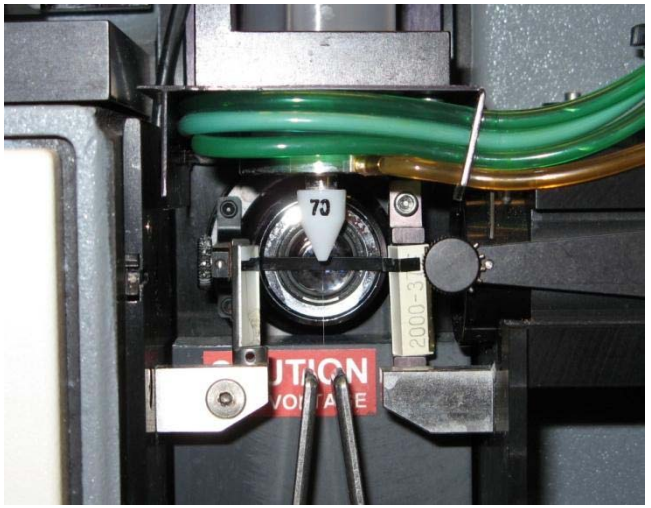
jet-in-air:

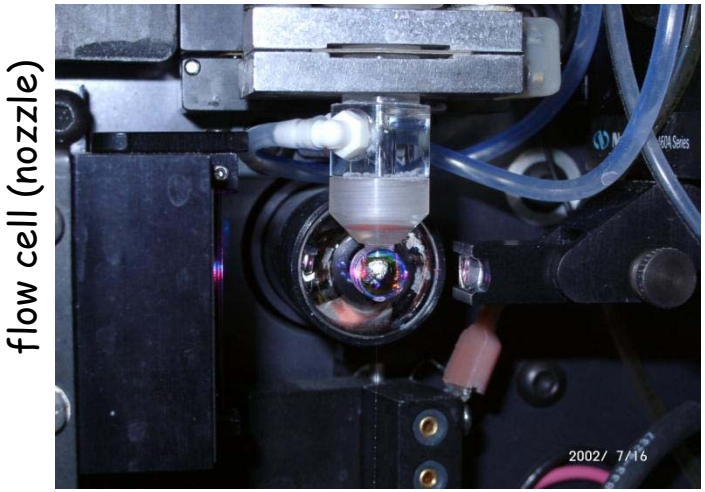


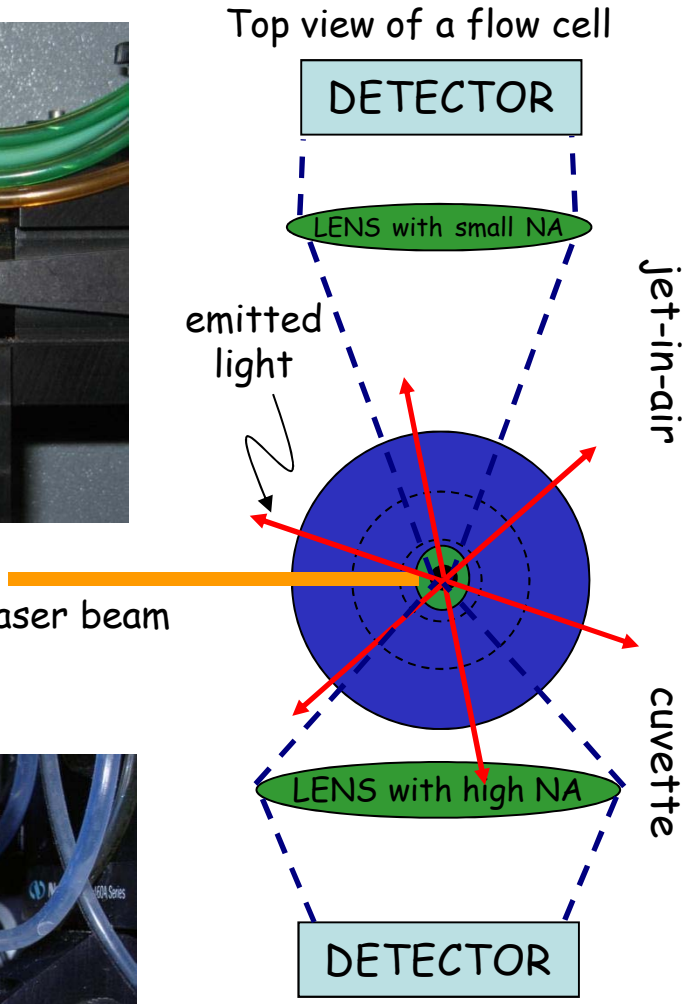
cuvette: a much higher % of emitted light is detected



Illumination

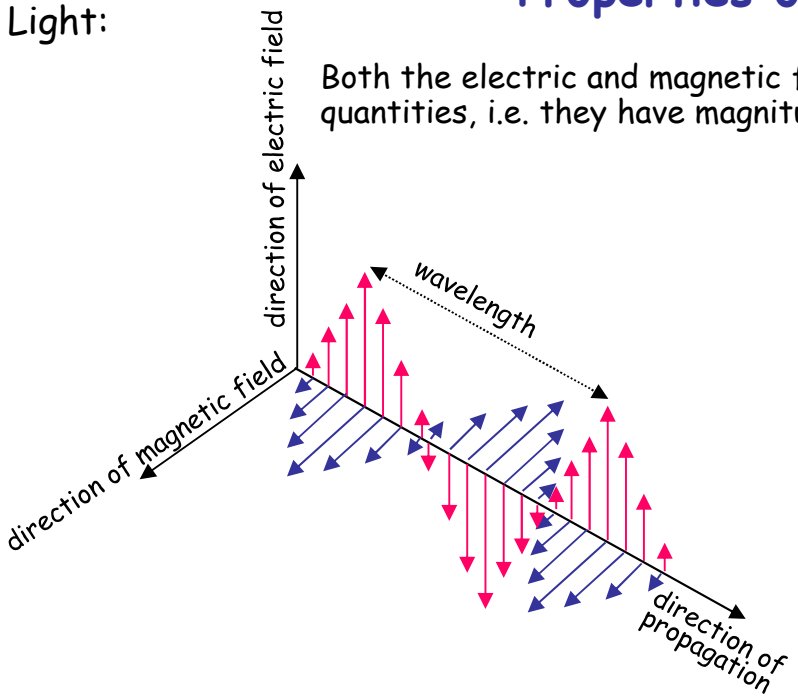






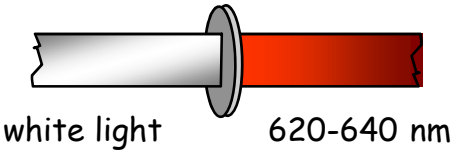
Properties of light and fluorescence

Light:

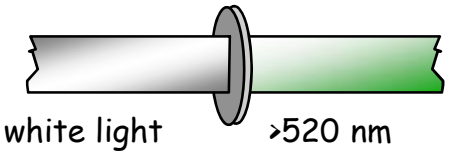


Both the electric and magnetic field are vector quantities, i.e. they have magnitude and direction.

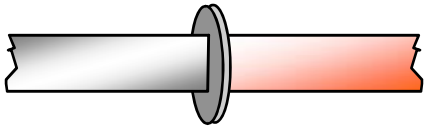
630 nm band-pass (BP)



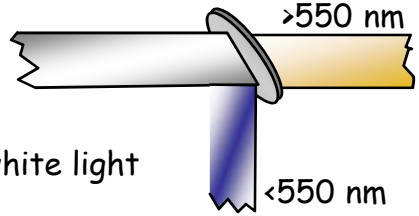
520 nm long-pass (LP):



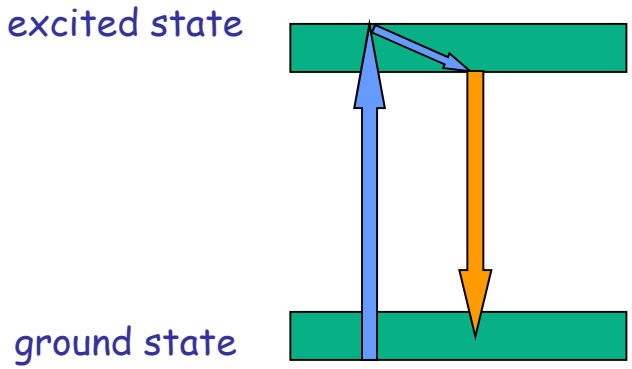
575 nm short pass (SP):

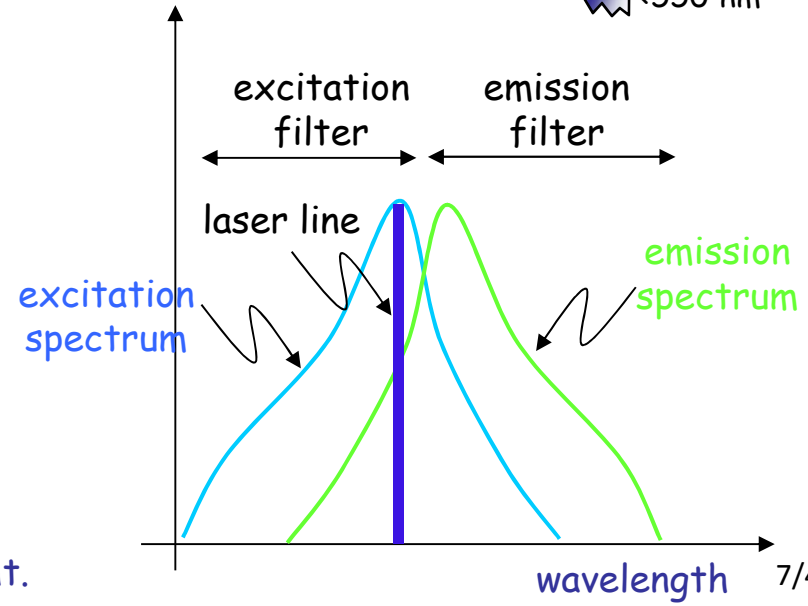


550 nm dichroic mirror:



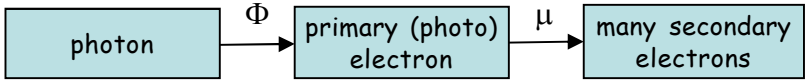
Fluorescence:





- After excitation the molecule gets back to the lowest (vibrational) level of the first excited state. Every subsequent process start from this level.
- Fluorescence has a longer wavelength than excitation light.

Detectors

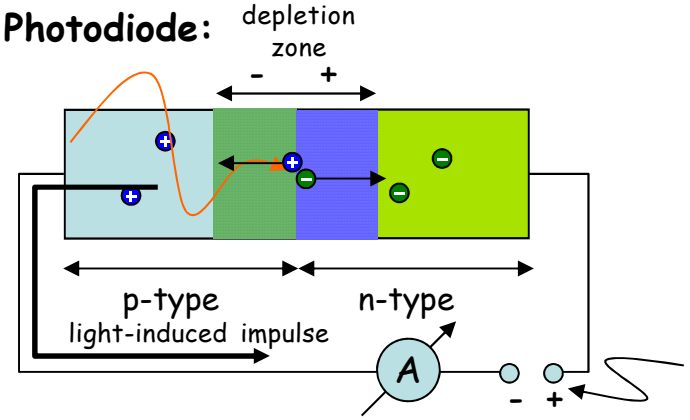


- quantum yield (Φ): $\Phi = \frac{number\ of\ electrons}{number\ of\ photons} \times 100$
- amplification (μ): $\mu = \frac{final\ number\ of\ electrons}{number\ of\ photoelectrons} \times 100$

e.g. every dynode emits 10 secondary electrons, and there are 8 dynodes:

$$\mu = 10^8$$

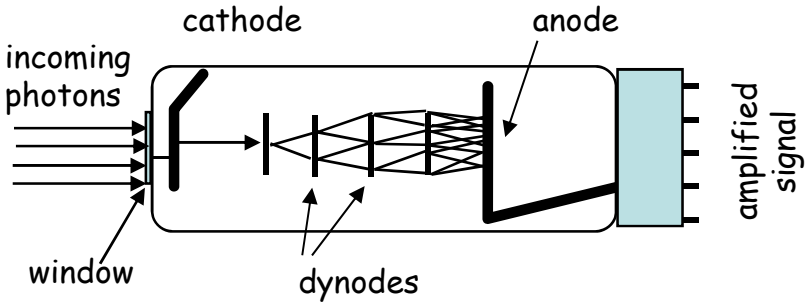
Photodiode:



- high quantum yield
- zero amplification

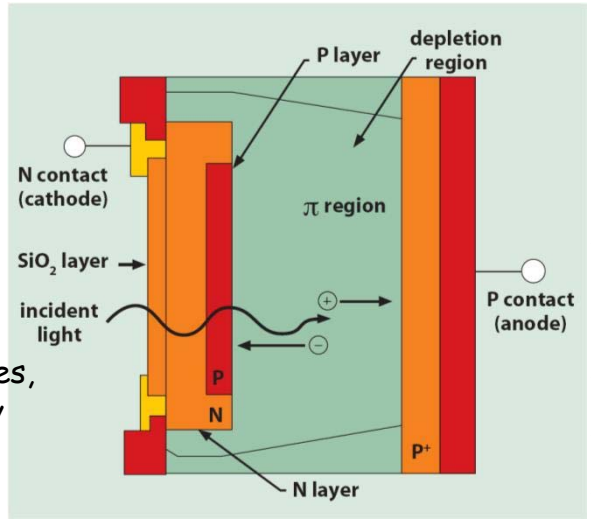
two modes: 1. no voltage, 2. reverse bias

Photomultiplier (PMT):



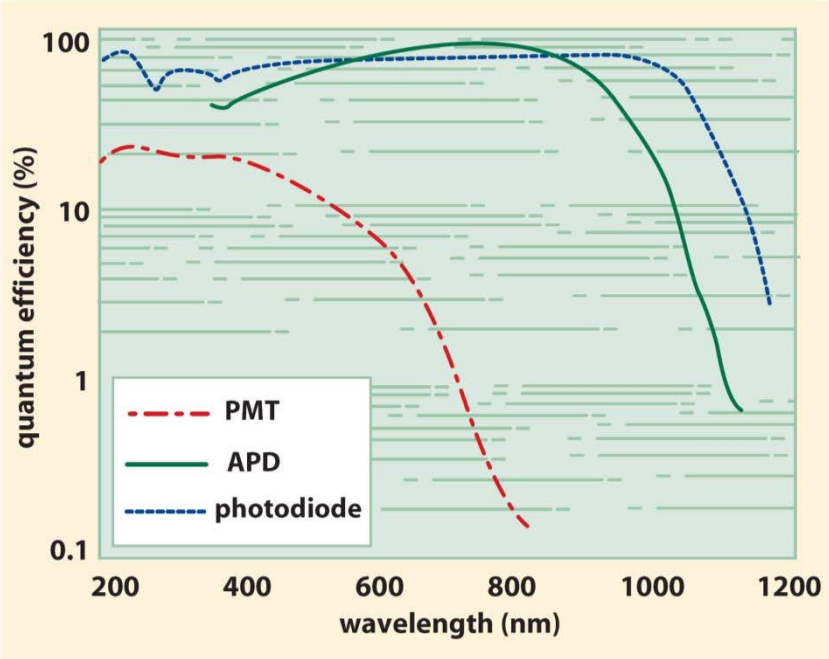
- an increasingly positive voltage is applied to the dynodes, and the accelerated electrons evoke several secondary electrons when they impinge into the dynodes
- low quantum yield
- high amplification

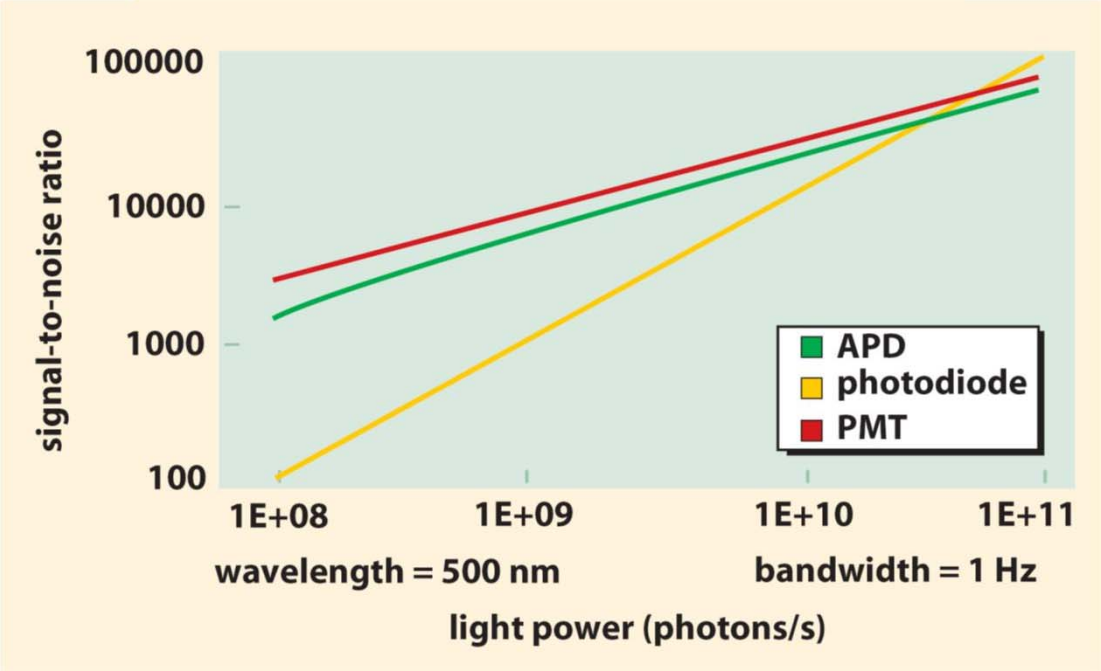
Avalanche photodiode:



- a high reverse bias is applied to the dynode, and the photoelectrons are accelerated to such an extent that they induce secondary electrons (μ≠0)
- combines the good properties of photodiodes and PMTs
- high quantum yield
- high amplification
- drawback: high dark current

Comparison of detectors





Signal to noise ratio in flow cytometry

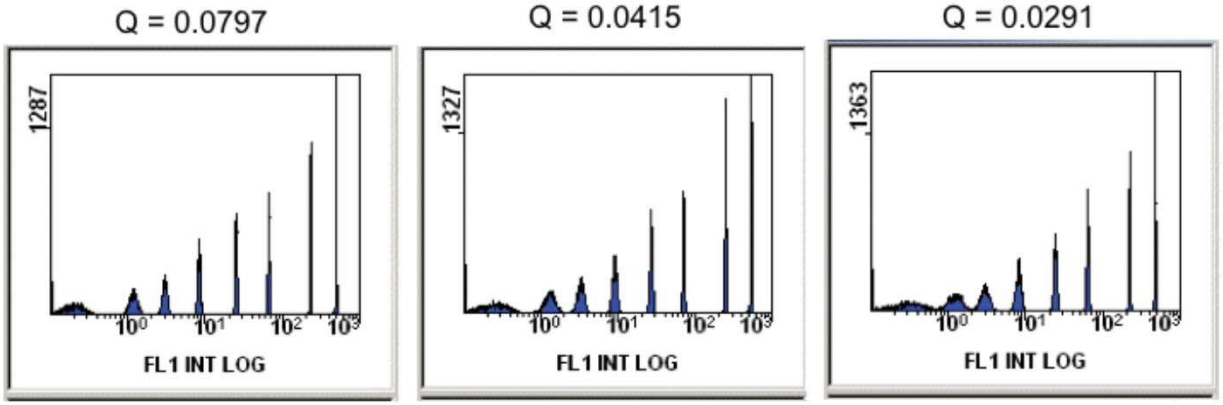
- The quality of flow cytometric data is determined by the signal to noise ratio.
- The signal to noise ratio can be characterized by two factors:
  - detection efficiency (Q), a.k.a. quantum yield (Φ): number of photoelectrons produced per molecule of fluorophore
  - background light level (B)

due to the Poisson nature of photon counting $\longrightarrow$

$$noise \sim \sqrt{n}$$

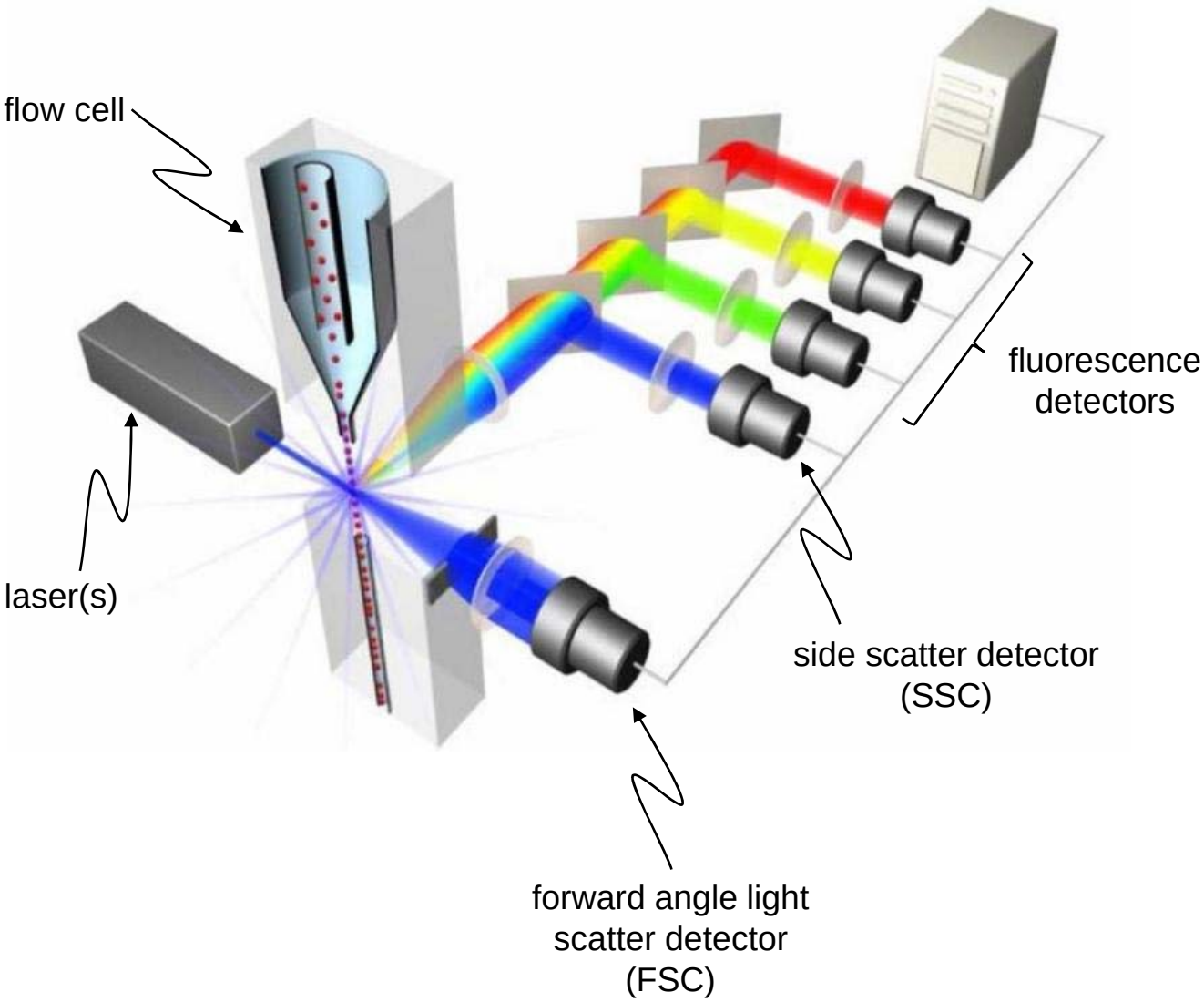
$$relative\ noise \sim \frac{\sqrt{n}}{n} = \frac{1}{\sqrt{n}}$$

The relative noise is inversely related to the square root of the number of photons.



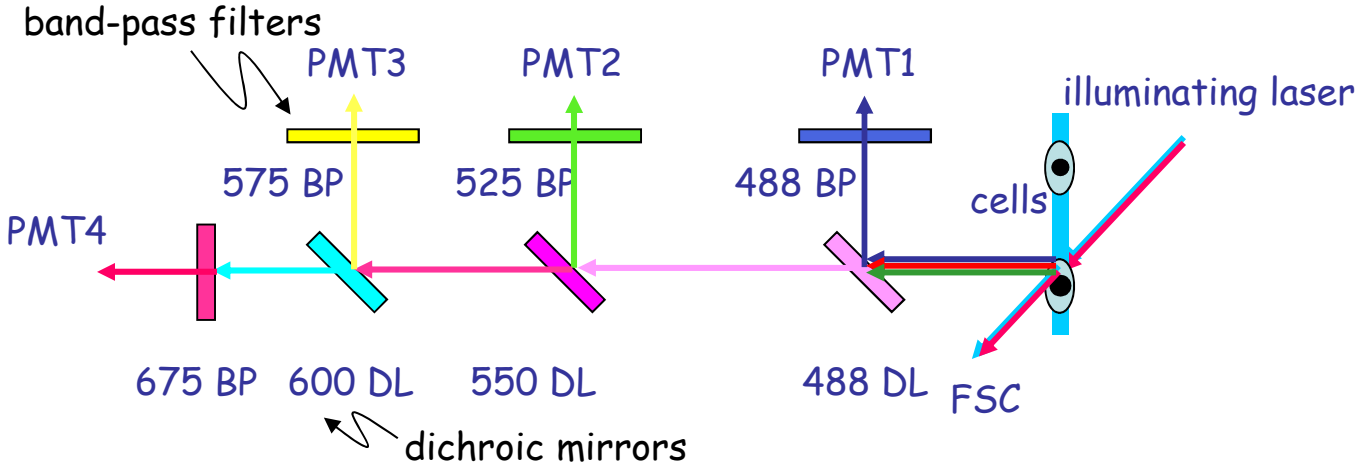
The Q factor was reduced by decreasing the laser intensity (while maintaining the brightest population in the same mean channel by increasing the detector voltage) resulting in the loss of resolution.

Arrangement of detectors I.



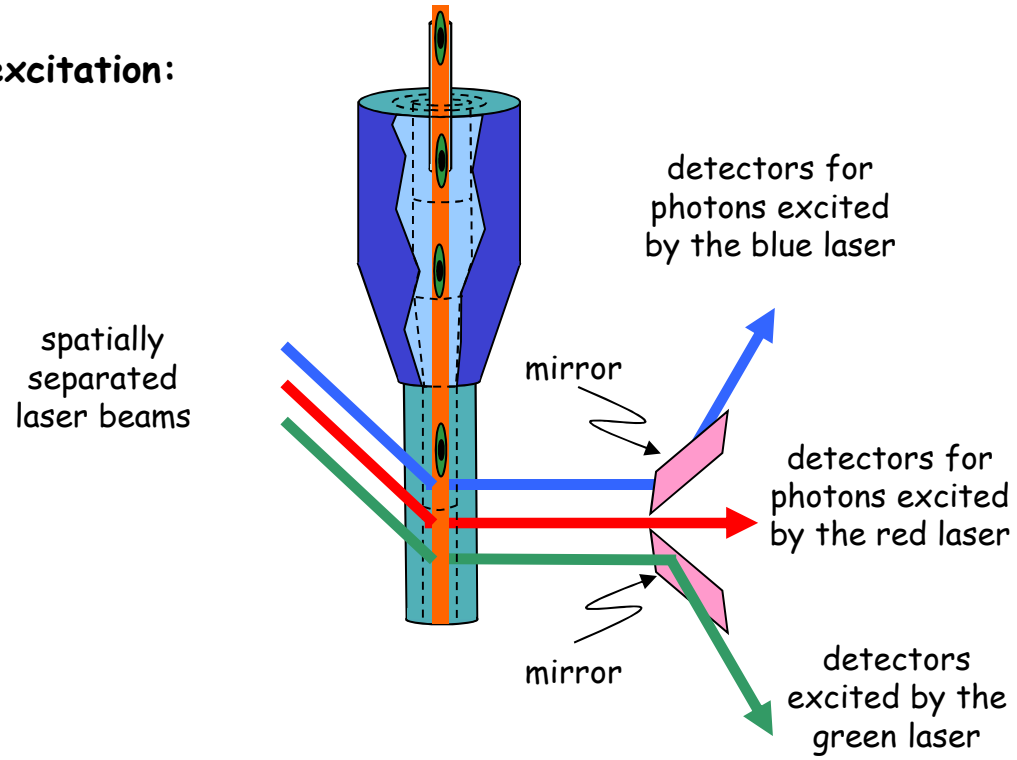
Arrangement of detectors II.

Excitation at the same place with different lasers:

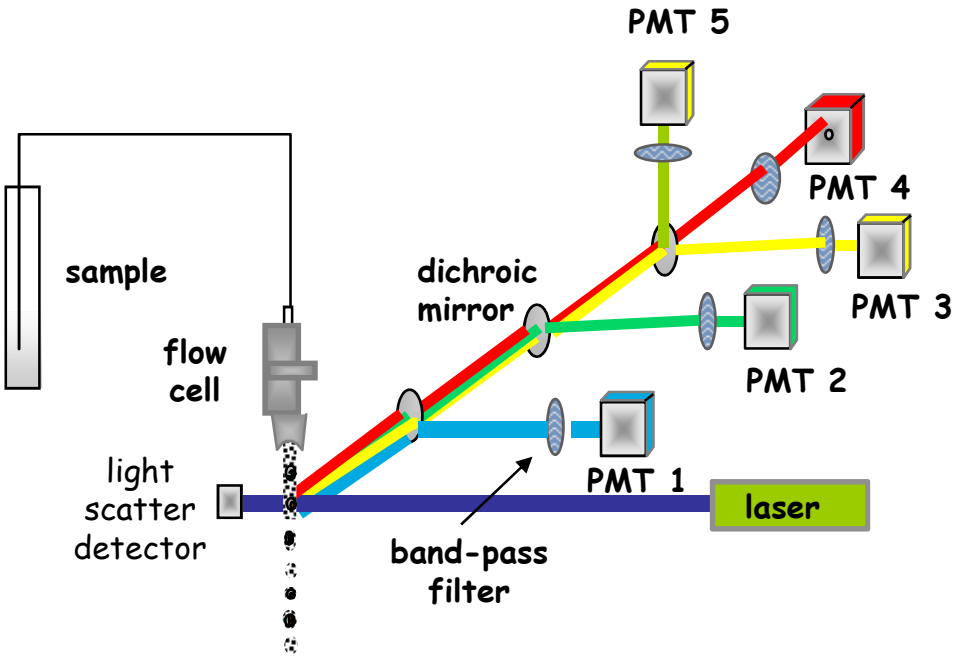


Beams separated from the common beam by dichroic mirrors are further filtered by band-pass filters.

Spatially separated excitation:



Arrangement of detectors III.

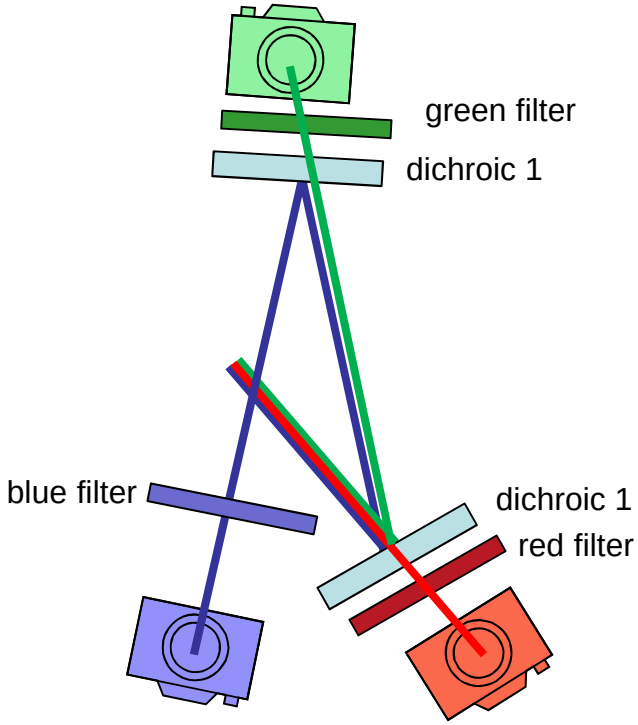


Arrangement of detectors IV.

New development: octagon or trigon arrangement of detectors

Advantage and principles:

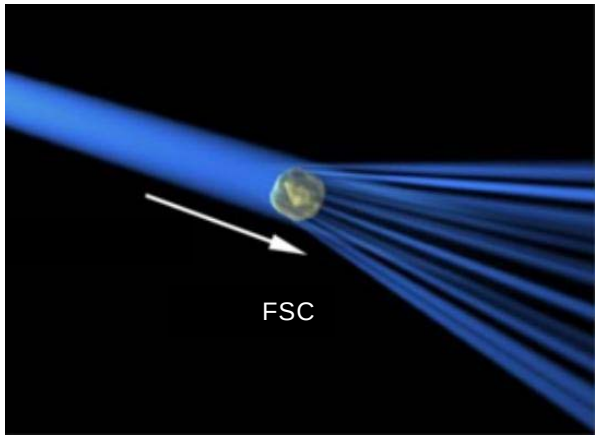
- The detector closest to the site of emission records the highest wavelength fluorescence usually having the fewest photons.
- Lower wavelength photons are reflected to the rest of the detectors by (high-pass) dichroic mirrors.
- Light reflection is usually more efficient than transmission.



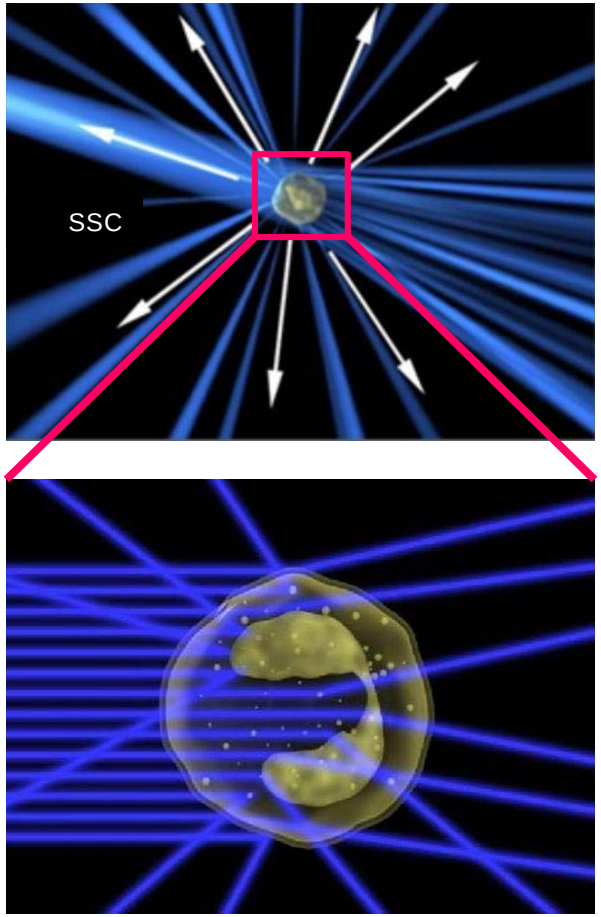
Light scatter signals

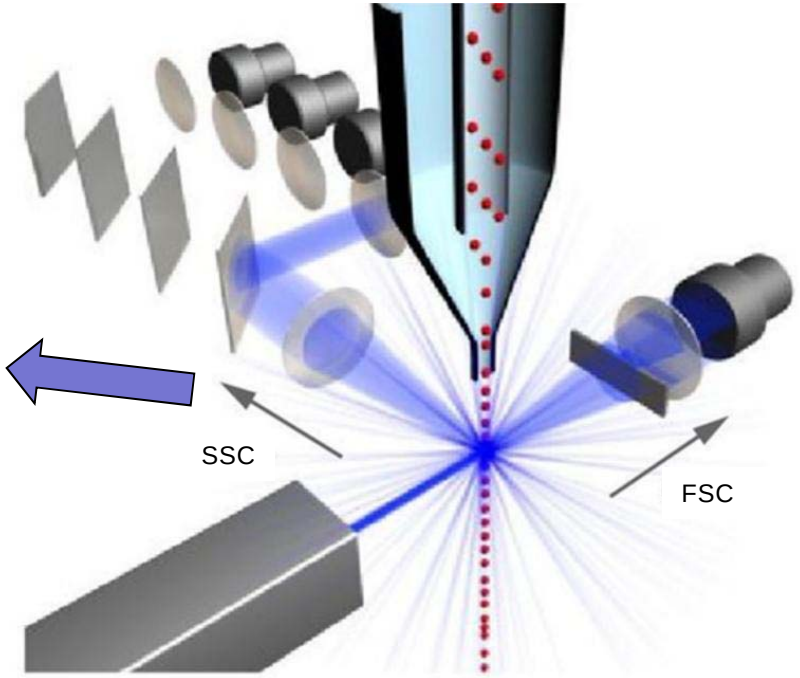
The intensity of light scatter signals (both FSC and SSC) depends on

- the index of refraction of cells (how different it is from the index of refraction of the surrounding buffer)
- the orientation of cells relative to the laser beam
- factors specific to FSC and SSC



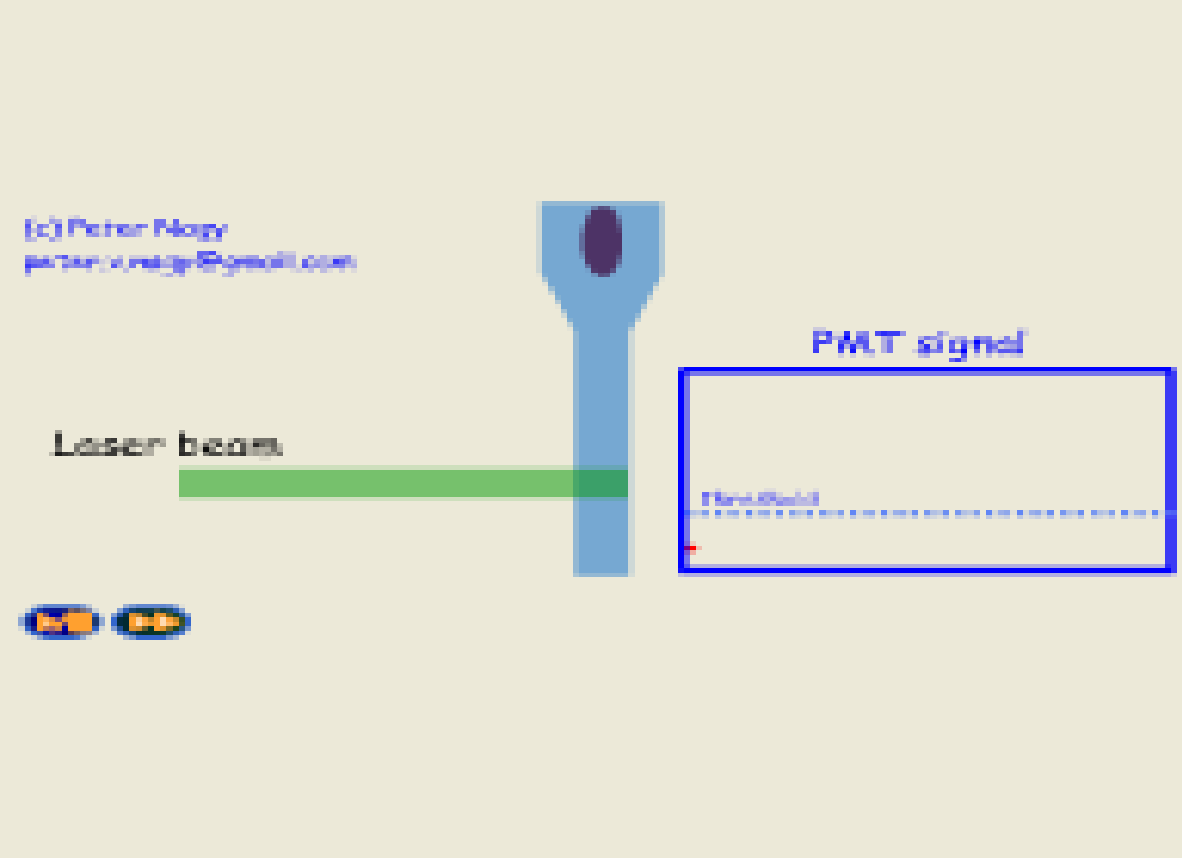
SSC signal: proportional to the internal granularity of cells



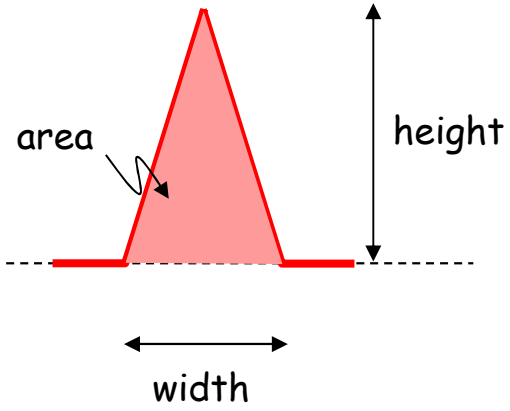


FSC signal: proportional to cell size

Detection of signals

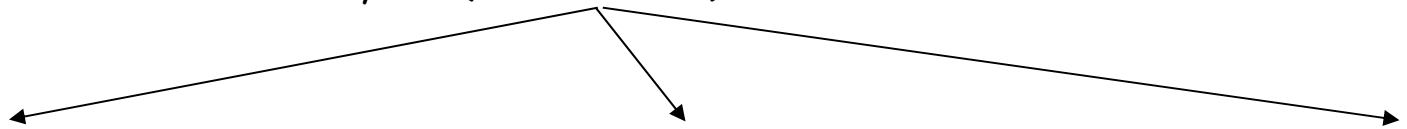


Signal detected by the detector:



Data storage

Data are usually saved in a so-called FCS (flow cytometry standard) file in which every measured piece of data is recorded for every cell (list-mode file).



header

Identifies the file as FCS and describes the length of the text segment.

text

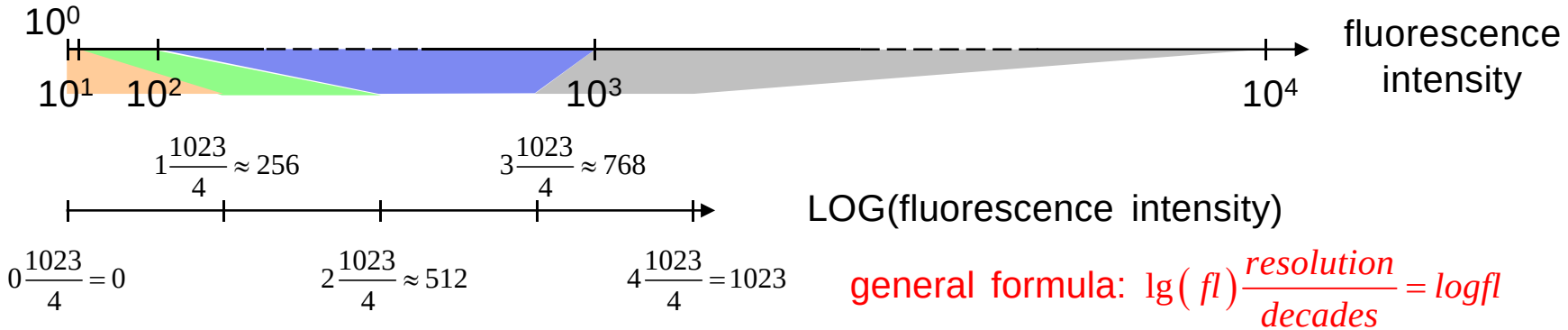
```
$FIL=pi04.LMD
$INST=EPICS DIVISION OF COULTER
CORPORATION
$CYT=Elite
$DATE=04-Jan-80
$BTIM=19:21:30
$SRC=tr110901
$SMNO=1
TESTNAME=Peter FITC/pmt4 lin/log
TESTFILE=Pe000093.PRO
$BYTEORD=12
$DATATYPE=I
$NEXTDATA=0
$MODE=L
$P1N=FS
$P1S=FS
$P1R=1024
$P1B=16
$P1E=0,0
$P2N=PMT1
.
.
.
$TOT=25114
```

data

	FSC	SSC	FL1	...
Cell 1	674	334	873	
Cell 2	898	393	799	
Cell 3	648	417	937	
.				
.				
.				

Data resolution

- Most biological parameters span several (3-4) orders of magnitude.
- Most flow cytometers used to have 10-bit resolution (because detectors with a higher resolution were prohibitively expensive), i.e. fluorescence intensities were recorded with a resolution of 10 bits (2^{10}=1024).
- The capability to measure 3 orders of magnitude difference in fluorescence intensity is not sufficient, therefore logarithmic amplifiers were used which compressed the high intensity part of the scale



general formula: $\lg(fl)\frac{resolution}{decades} = logfl$

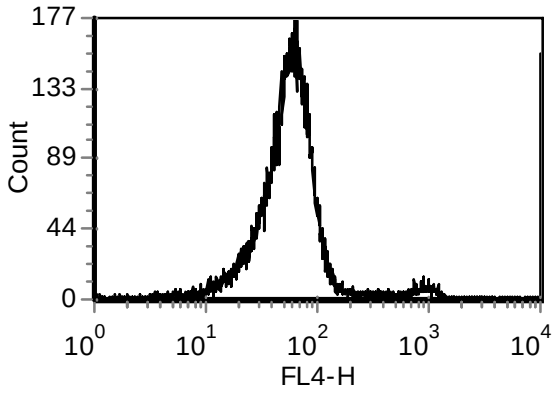
- Modern detectors record fluorescence data with 16-bit (or similar) resolution (2^{16}=65536) → no need for log amplifiers.

Display of data I.

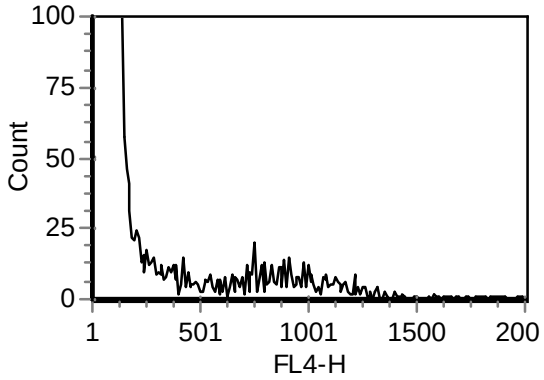
One dimensional (one parameter) display: histogram

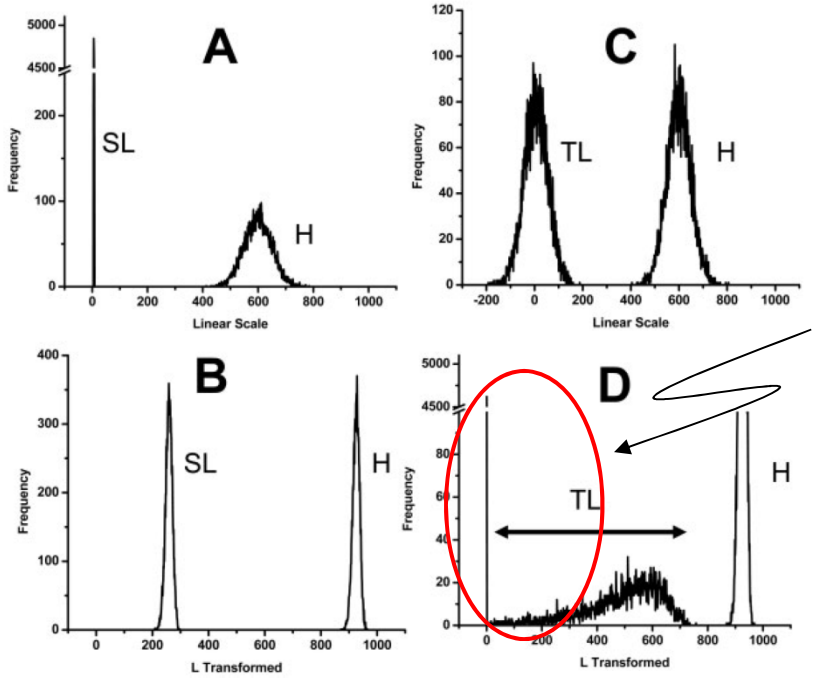
the same data on

logarithmic scale



linear scale





Binning artifact: it seems that the low intensity population is stretched to the left and a lot of cells accumulate in the first channel.

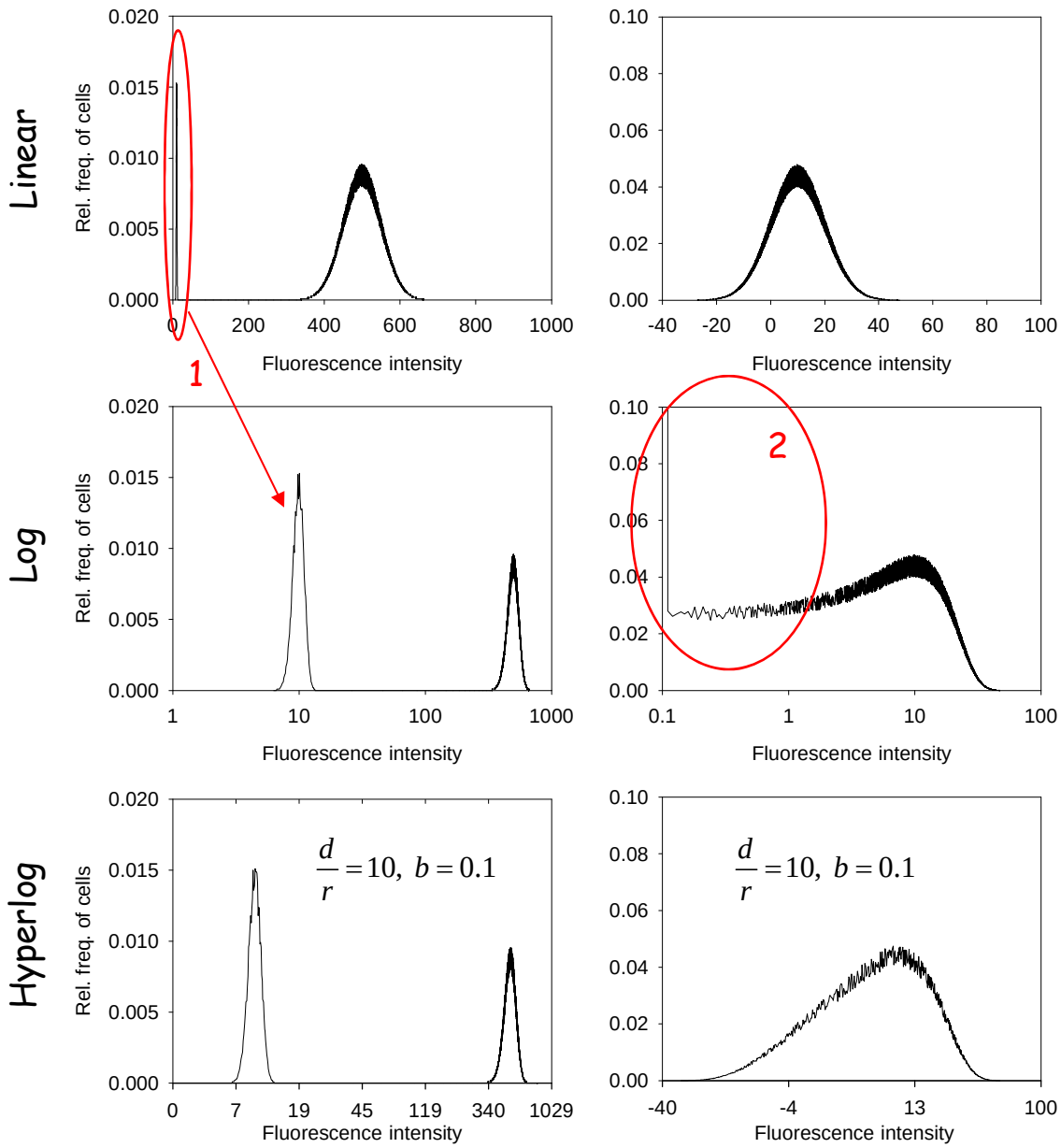
Advantages of logarithmic scale:

- contracts the scale in the high intensity range
- many biological parameters show log-normal distribution which seem to be a bell-shaped curve on a log scale.

Disadvantages:

- cannot display zero and negative values
- so-called binning artifact

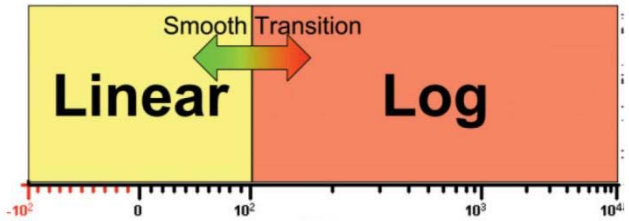
Scale types



1. Low intensity population is unresolved on linear scale, and it is resolved on log scale, BUT
2. Binning artifact on log scale.

Solution:

such a scale which is linear at low intensities and logarithmic at high intensities: hyperlog (HL) scale (Cytometry, 64A, 34)



$$LIN = 10^{\frac{d}{r}x_{HL}} + b\frac{d}{r}x_{HL} - 1,\ \text{if } x_{HL} \geq 0$$

$$LIN = -10^{-\frac{d}{r}x_{HL}} + b\frac{d}{r}x_{HL} + 1,\ \text{if } x_{HL} < 0$$

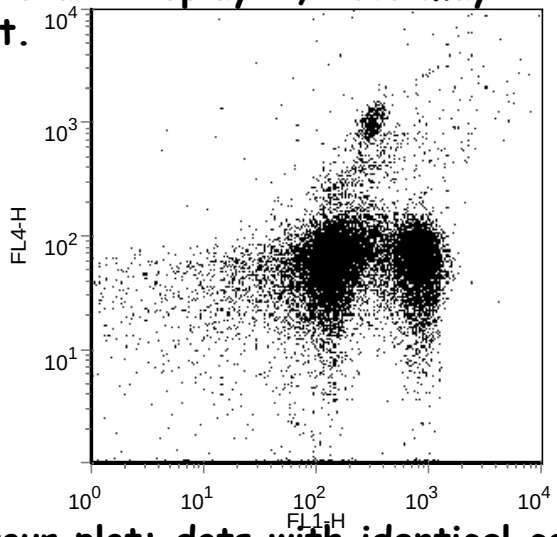
Find x_{HL}, so that the equations is satisfied.

1-2. On the HL scale both populations are resolved, and there is no binning artifact.

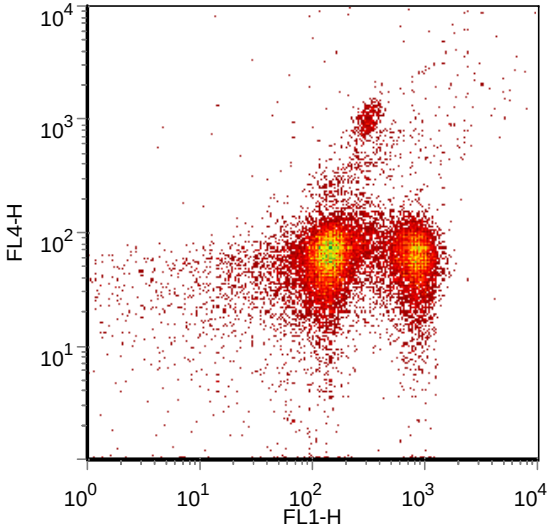
Display of data II.

Two dimensional (two parameter) display:

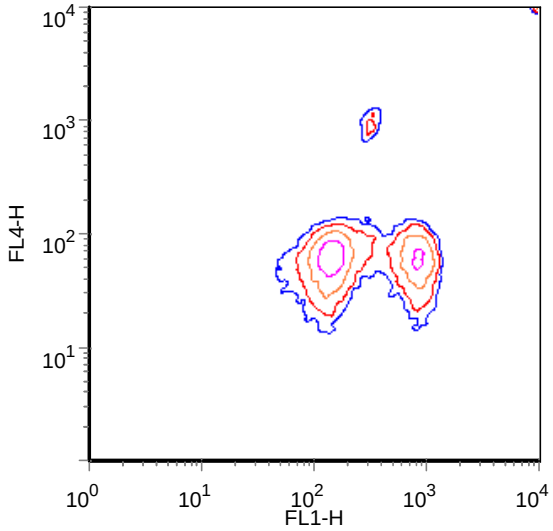
1. dot plot: two measured parameters are displayed on the x and y axes, every dot in the plot corresponds to a single cell. Drawback: if many cells are displayed, dots may become confluent.



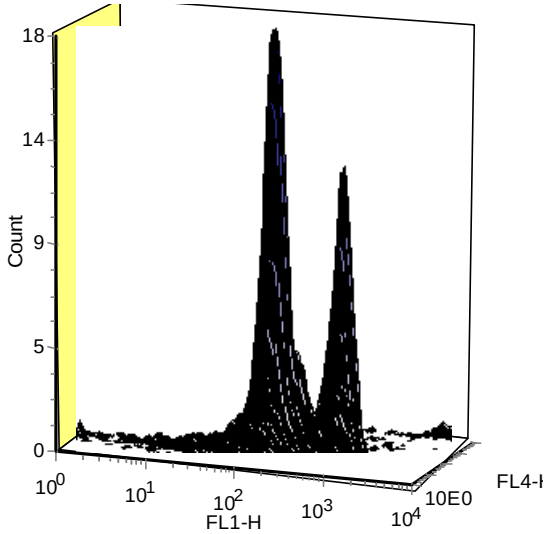
2. density plot: the color of dots corresponds to the number of cells



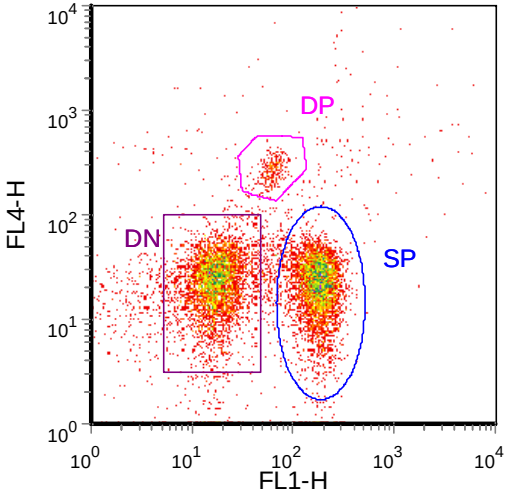
3. contour plot: dots with identical cell numbers are connected with lines

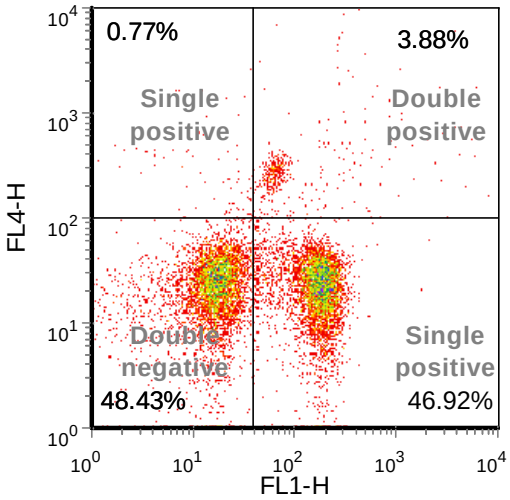


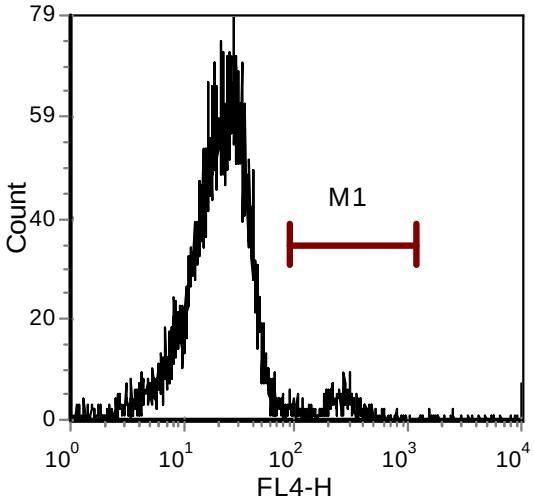
4. 3D (surface) plot: the number of cells is displayed on the z axis (rarely used)



Regions, quadrants and markers:
tools to identify subpopulations







Region: a set of points selected by the user that specifies an area in a 2D graph.

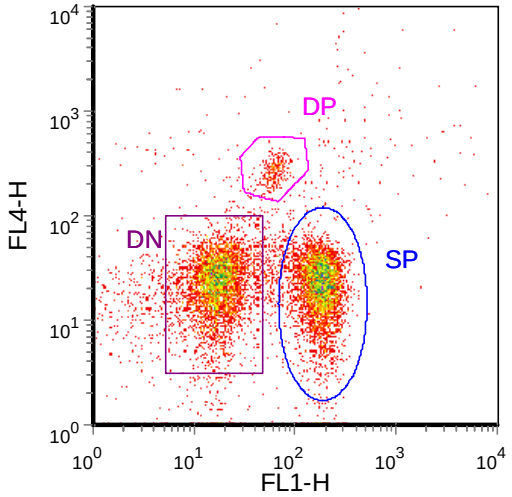
Several regions can be defined in the same graph.

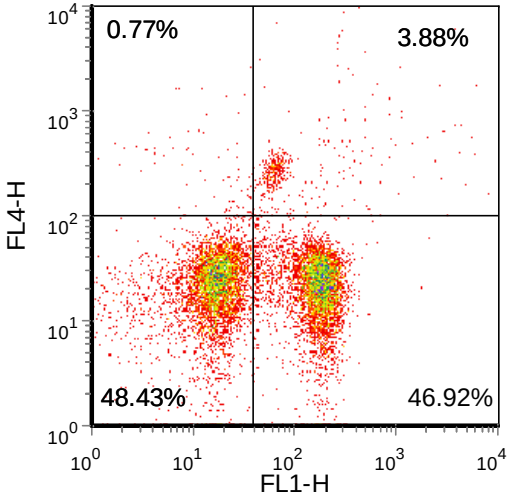
Regions can have different shapes (rectangular (DP), polygon (DP) and oval (SP)).

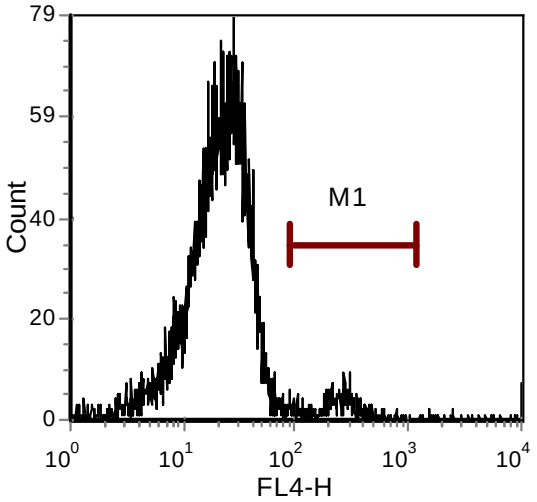
Quadrant: rectangular selection of 4 areas discriminating positive and negative cells on both axes of a 2D graph.

Marker: selection of cells in a histogram

What can we do with regions, quadrants and markers?







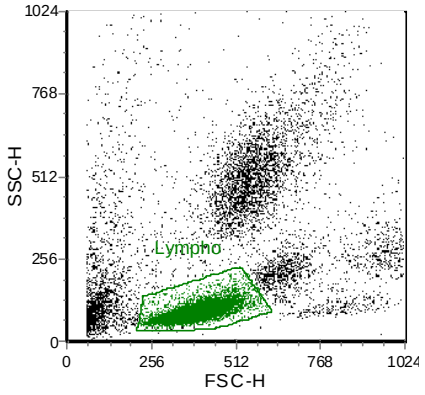
Gate	X Geometric Mean	Y Geometric Mean	% of All Cells
None	48.09	21.7	100.0
SP	171.29	19.51	42.91
DP	61.56	253.34	2.62
DN	16.86	21.08	45.19

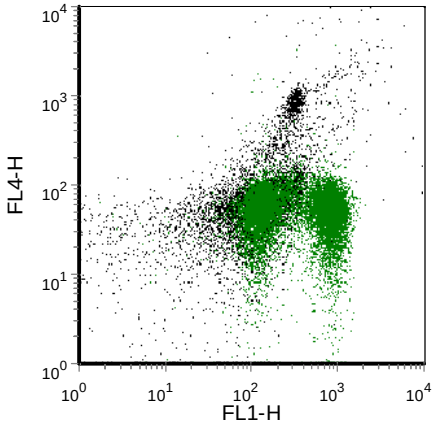
Gate	X Geometric Mean	Y Geometric Mean	% of All Cells
UL	13.55	286.44	0.77
UR	108.74	317.44	3.88
LL	14.45	18.95	48.43
LR	158.77	19.17	46.92

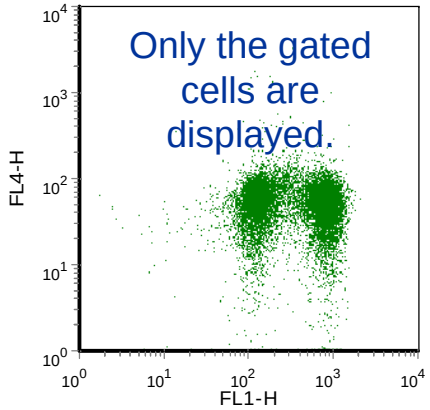
Marker	% of all cells	Geometric Mean	CV
None	100.0	21.7	677.57
M1	4.49	240.38	54.55

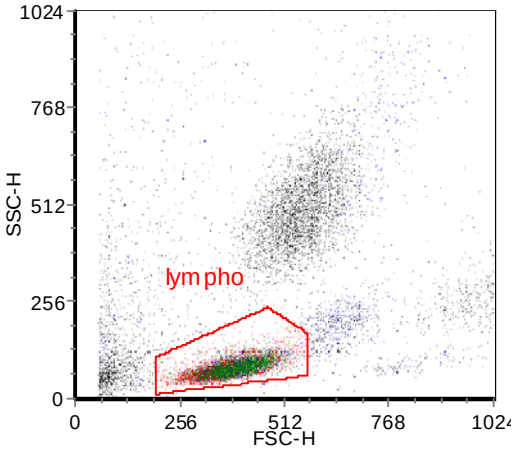
- the identified clusters can be analyzed separately
- statistics can be calculated on the whole population and on the regions, quadrants or markers

Gating

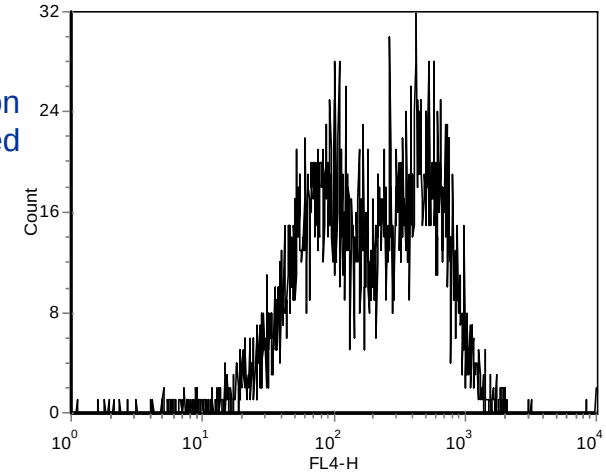




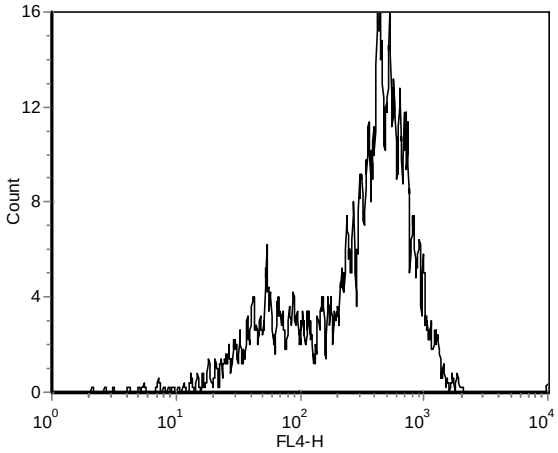




FL4 distribution of all measured cells



FL4 distribution of cells in the red (lymphocyte) gate

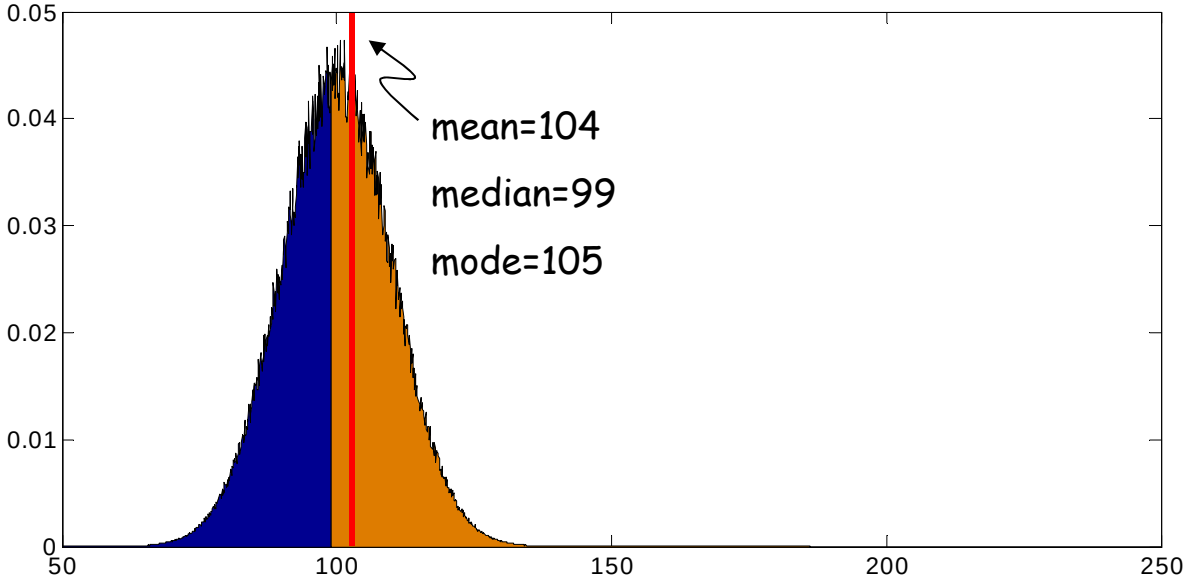


A **gate** can be defined as one or more regions/quadrants/markers combined using logical operators (AND, OR, NOT)

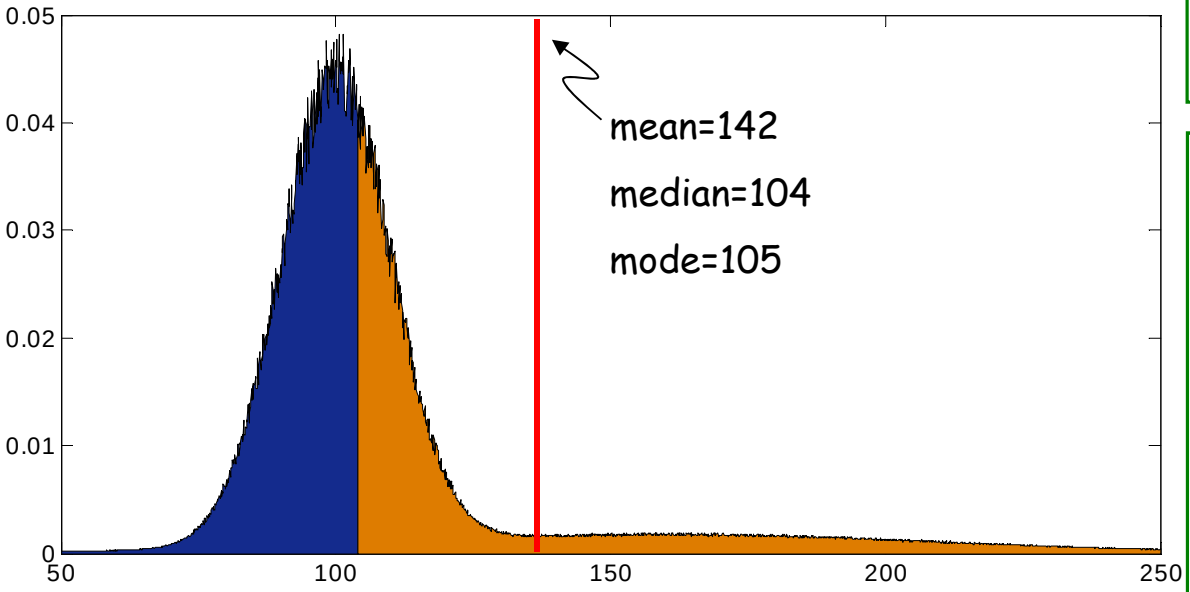
Defines a subset of the data to be displayed or analyzed.

- Used to compute **statistics** and characterize the subset of events selected
- Get rid of unwanted events, e.g. cell debris

Statistics



The distribution was contaminated by a high intensity subpopulation:



Measures of central tendency:

Mean:

$$mean = \frac{\sum_{i=1}^{n} x_i}{n}$$

Median:

the central value which divides the distribution into two equal parts (boundary between the blue and orange areas)

Mode:

the value with the highest frequency

The mean is shifted substantially by the presence of the contamination, whereas the median is a more robust estimate of central tendency.

Measures of dispersion:

SD: the dispersion of values around the mean

$$SD = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i - \bar{x}\right)^2}{n-1}}$$

Coefficient of variation (CV):

$$CV = \frac{SD}{mean} 100$$

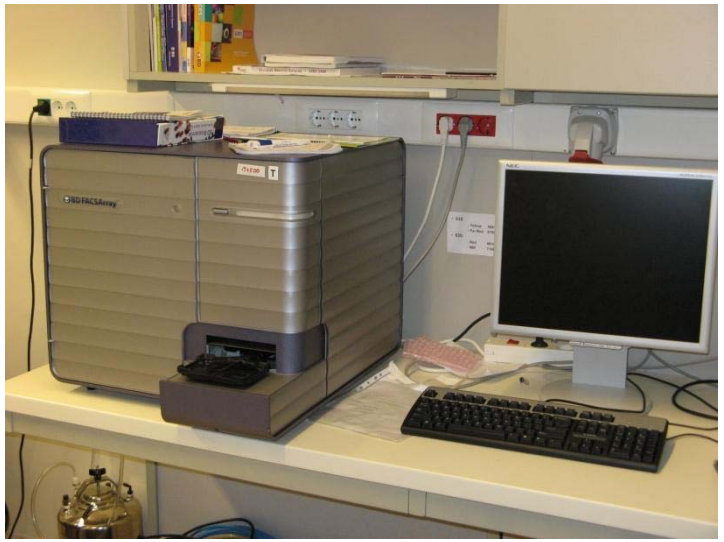
characterizes the relative width of the distribution

Flow cytometers

Coulter Epics Elite

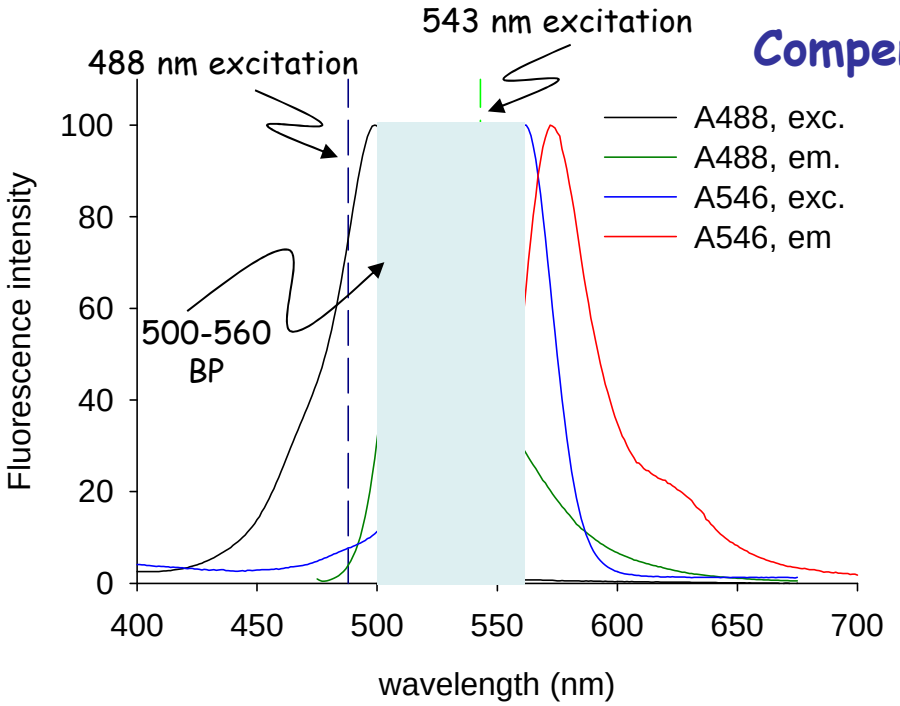
Becton Dickinson FacsVantage DiVa

Becton Dickinson FACScan



Becton Dickinson FacsArray

Compensation I.



If two fluorescent dyes (Alexa488, Alexa546) are examined, every measured parameter is a mixture of the contribution of both dyes.

$$A488channel = I_{A488} + I_{A546} \times S_{A546 \to A488}$$

A488channel – measured fl. intensity in the A488 channel

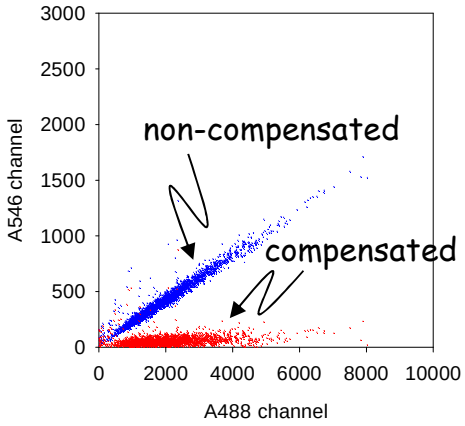
I_{A488}, I_{A546} – „pure" fluorescence intensiteis of dyes A488 and A546

$S_{A546 \to A488}$ – spectroscopic constant characterizing the overspill of A546 emission to the A488 channel

A similar equation holds for the A546 channel:

$$A546channel = I_{A546} + I_{A488} \times S_{A488 \to A546}$$

S factors are determined with samples labeled only with one dye (e.g. with A488), which should appear in horizontal or vertical position after compensation.



Compensation II.

In compensation pure intensities devoid of spectral overspill are calculated.
Simple case: one-way spillover, e.g. $S_{A546\rightarrow A488}\neq 0$, $S_{A488\rightarrow A546}=0$

$$\left.\begin{aligned} A488channel &= I_{A488} + I_{A546}\times S_{A546\rightarrow A488} \\ A546channel &= I_{A546} \end{aligned}\right\} \; I_{A488} = A488channel - A546channel \times S_{A546\rightarrow A488}$$

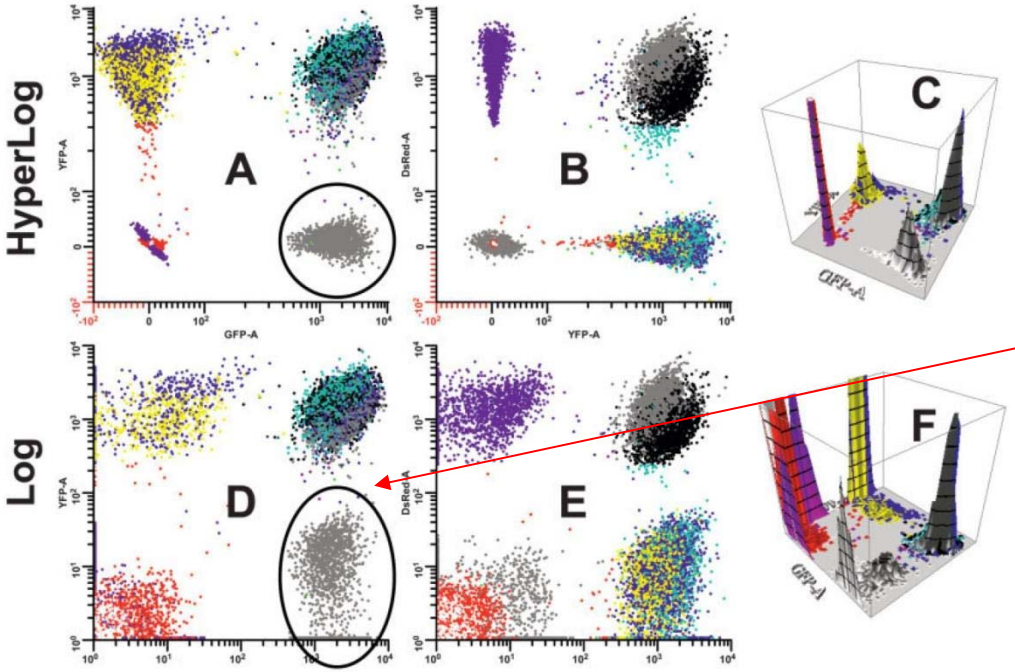
More complex case: two-way spillover, i.e. $S_{A546\rightarrow A488}\neq 0$, $S_{A488\rightarrow A546}\neq 0$

$$\left.\begin{aligned} A488channel &= I_{A488} + I_{A546}\times S_{A546\rightarrow A488} \\ A546channel &= I_{A546} + I_{A488}\times S_{A488\rightarrow A546} \end{aligned}\right\}$$

A system of equations with two unknowns has to be solved.

$$I_{A488} = \frac{A488channel - A546channel \times S_{A546\rightarrow A488}}{1 - S_{A546\rightarrow A488}\times S_{A488\rightarrow A546}}$$

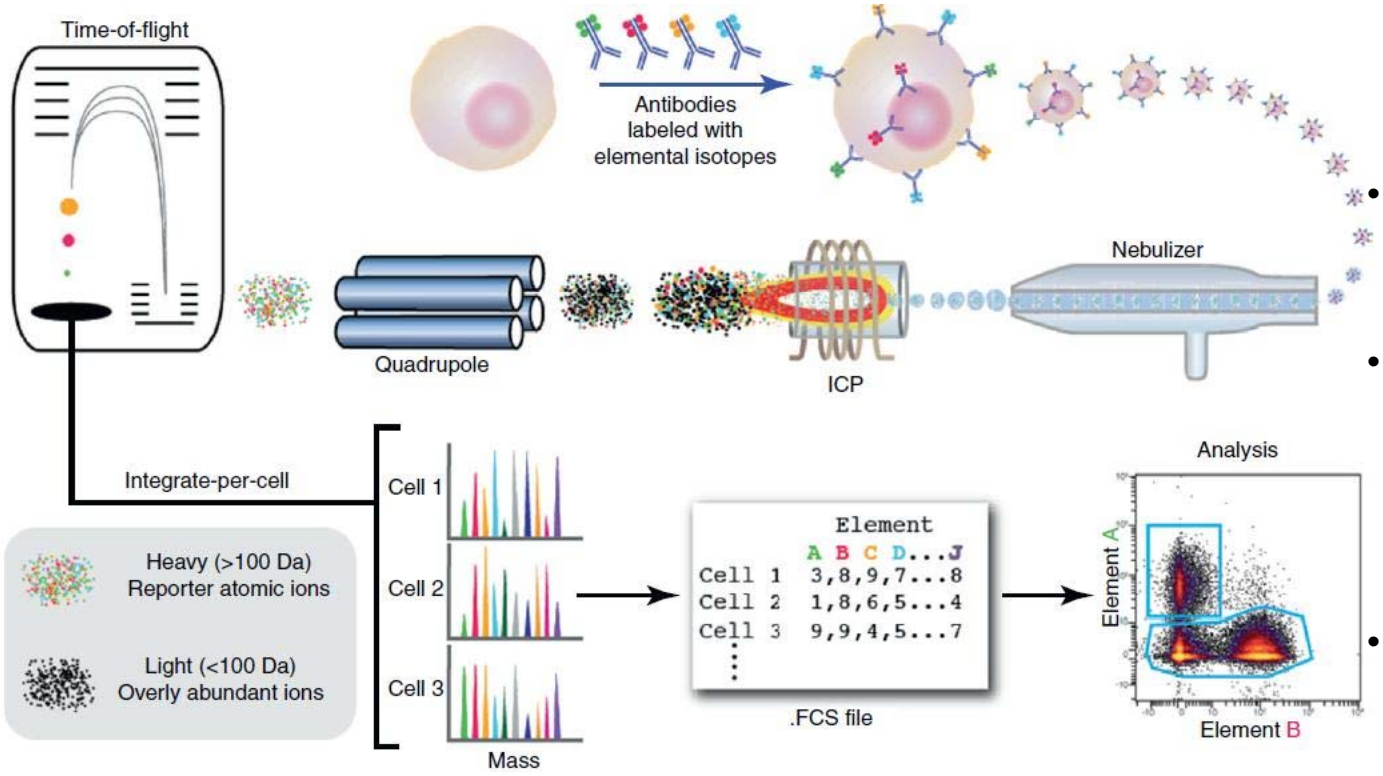
$$I_{A546} = \frac{A546channel - A488channel \times S_{A488\rightarrow A546}}{1 - S_{A546\rightarrow A488}\times S_{A488\rightarrow A546}}$$



Logarithmic scale often shows a misleading picture after compensation:

it seems to be undercompensated.

Mass cytometry

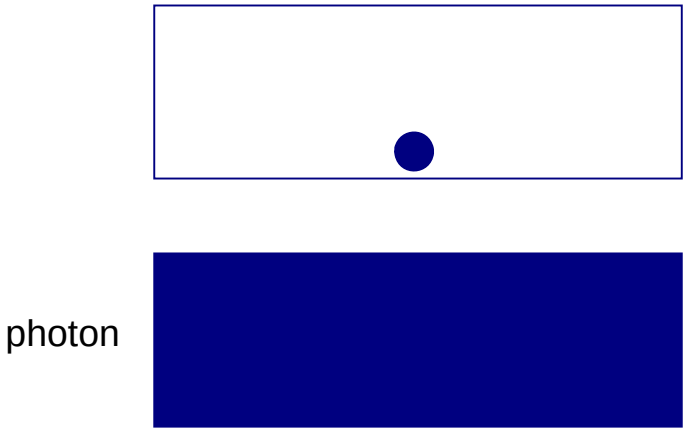


- The number of fluorophores selectively detectable at the same time is limited due to the problem of spectral overlap.
- Antibodies can be labeled with stable metal isotopes, typically lanthanides.
- Cells are ionized in an ICP (inductively coupled plasma) followed by TOF-MS (time-of-flight mass spectrometry)
- The amount of each isotope is quantified in a cell-by-cell basis.
- Data is presented and analyzed similar to flow cytometry.

- Bendall, S. C., G. P. Nolan, M. Roederer, and P. K. Chattopadhyay. 2012. A deep profiler's guide to cytometry. Trends Immunol 33:323-332.
- Bandura, D. R., V. I. Baranov, O. I. Ornatsky, A. Antonov, R. Kinach, X. Lou, S. Pavlov, S. Vorobiev, J. E. Dick, and S. D. Tanner. 2009. Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. Anal Chem 81:6813-6822.
- www.dvssciences.com/

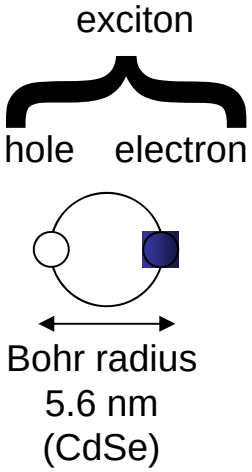
Optical properties of quantum dots (qdot, QD)

Compensation is not necessary, if such fluorescent dyes are used which have narrow emission spectrum, so only one fluorescence detector detects them. Quantum dots meet these criteria.

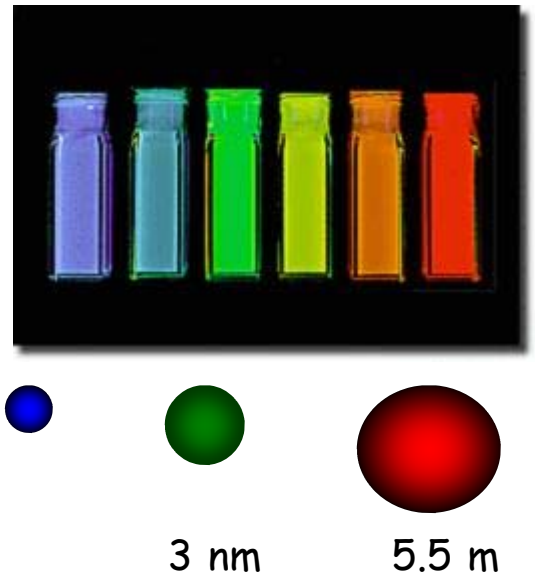


conductance band

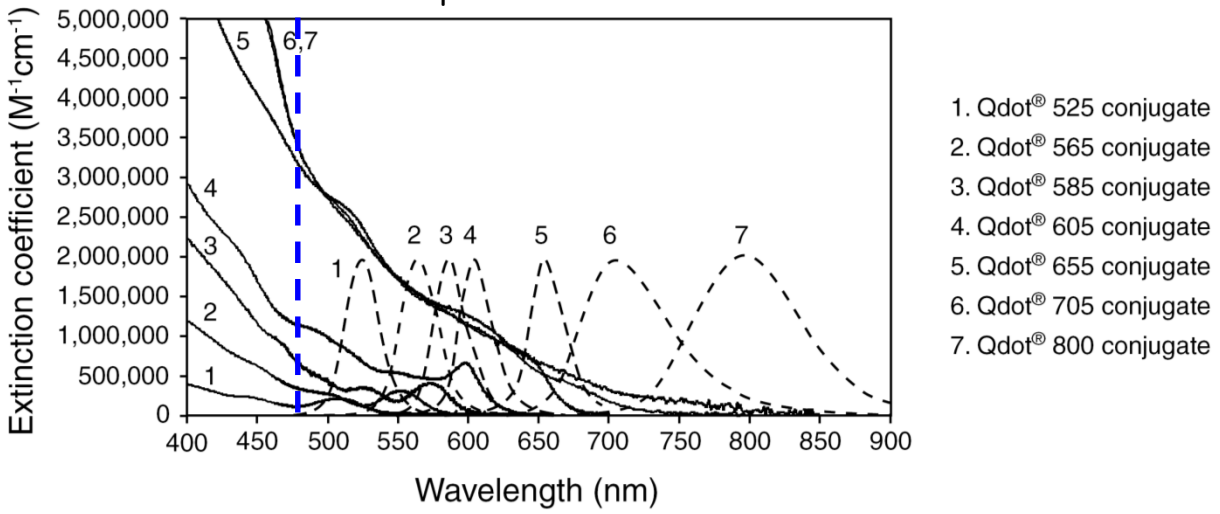
valence band



- wide excitation spectrum
- Narrow emission peak
- Great photostability (no photobleaching)



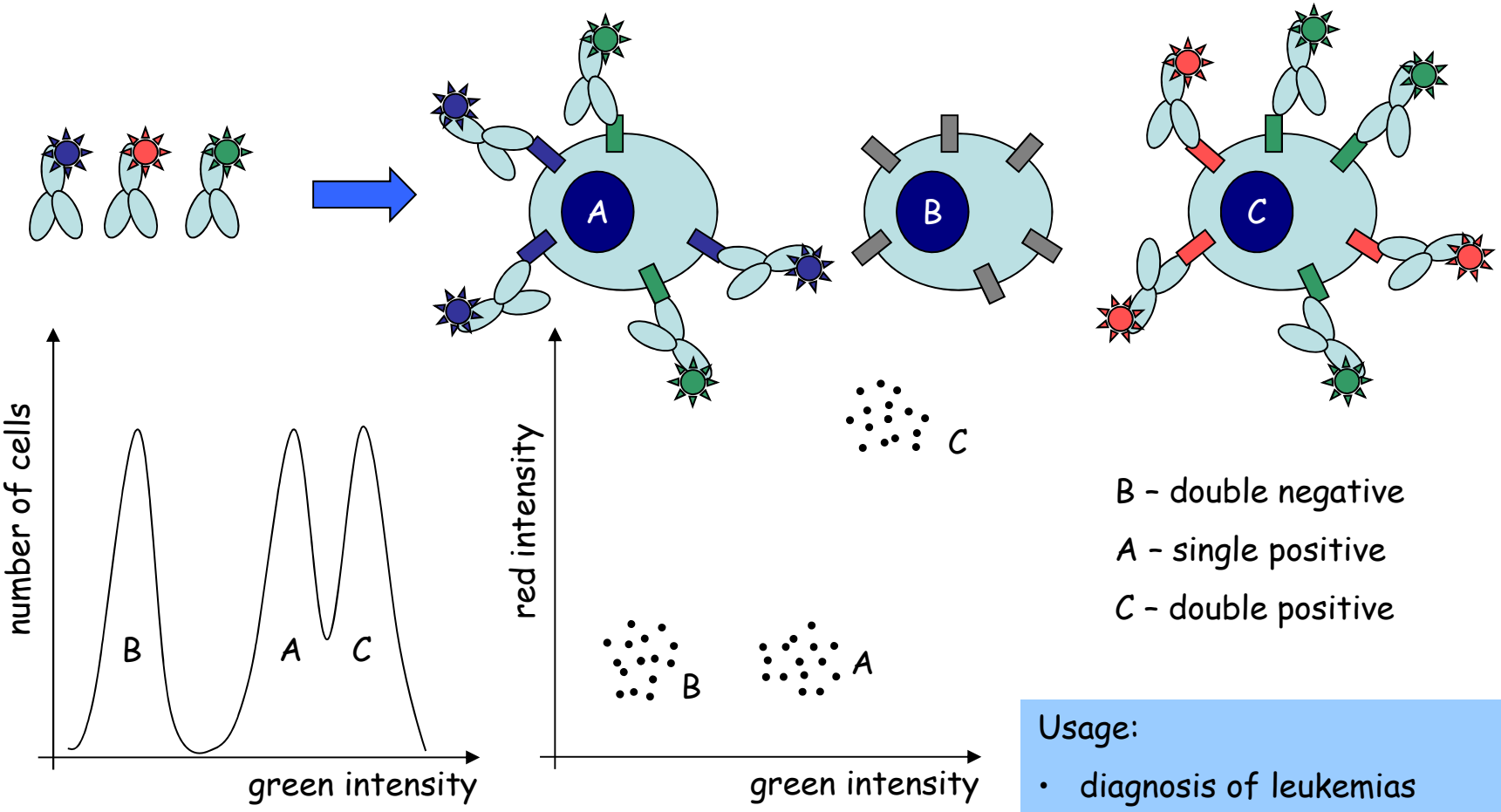
Due to the wide excitation spectrum a single laser (e.g. 488 nm) can excite several different quantum dots whose fluorescence can be detected without spectral overspill by different detectors as a result of the narrow emission spectrum.



Immunophenotyping with flow cytometry

- suitable for the investigation of suspended or easily suspendable cells

- cell surface antigens (or intracellular antigens of fixed and permeabilized cells) are labeled with monoclonal antibodies. In clinical practice primarily labeled antibodies are usually used.



B – double negative

A – single positive

C – double positive

Usage:

- diagnosis of leukemias
- AIDS diagnostics ($CD4^+$ lymphocyte count)

Counting $CD4^+$ lymphocytes in AIDS patients in Africa (www.cytometryforlife.org)

Problem:

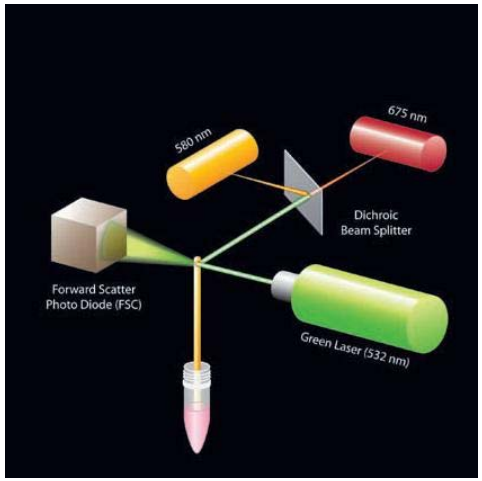
- 65% of new HIV infections occur in 3rd world, resource-poor countries (e.g. sub-Saharan Africa)
- progression of AIDS can be monitored by counting the number of $CD4^+$ lymphocytes (CD4 count)
- CD4 counts are usually determined by flow cytometry in developed countries
- flow cytometers are too expensive for 3rd world countries

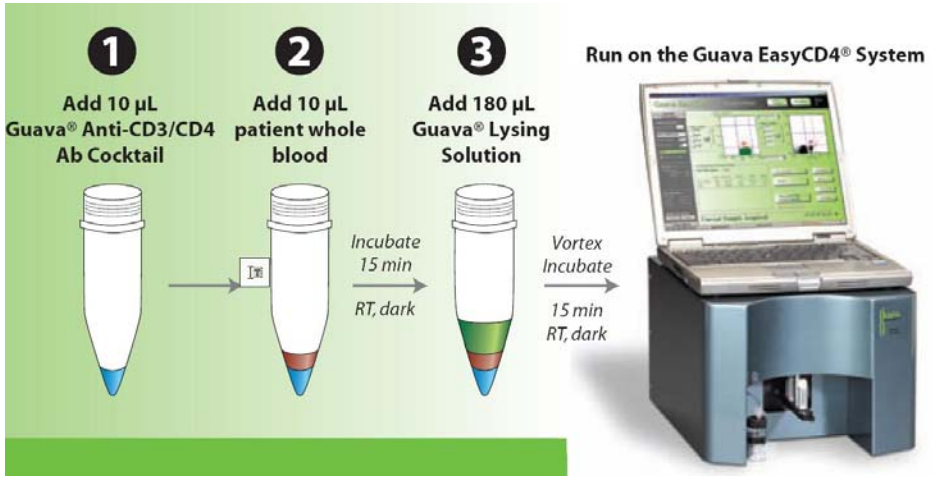
Solution:

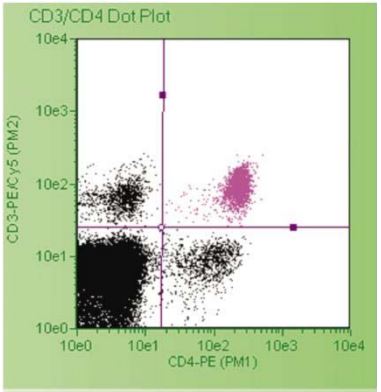
- development of such a specialized, dedicated platform, which is simple, cheap and does not need service
- such instruments can practically only determine the CD4 count, but cheaply

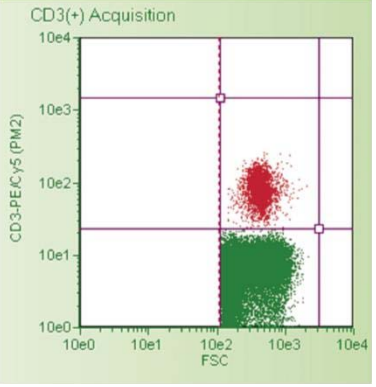
E.g. Guava EasyCD4 (www.guavatechnologies.com)

- microcapillary-based flow cytometer
- it does not need day-to-day adjustment, it can be used in harsh environments

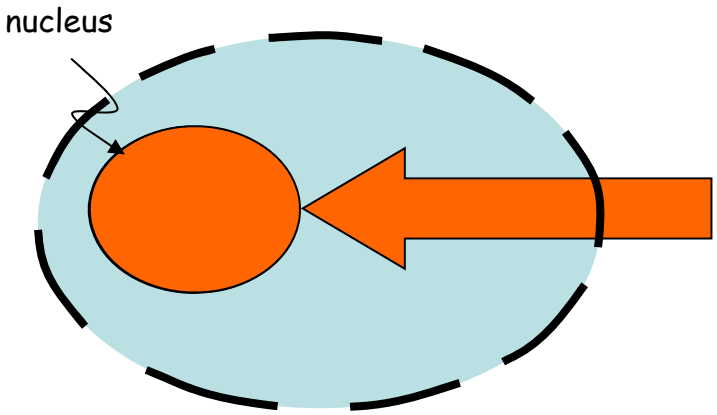








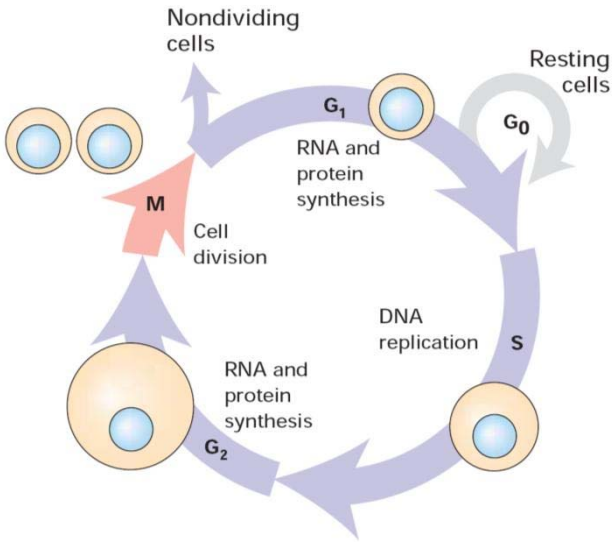
Cell cycle and DNA content analysis

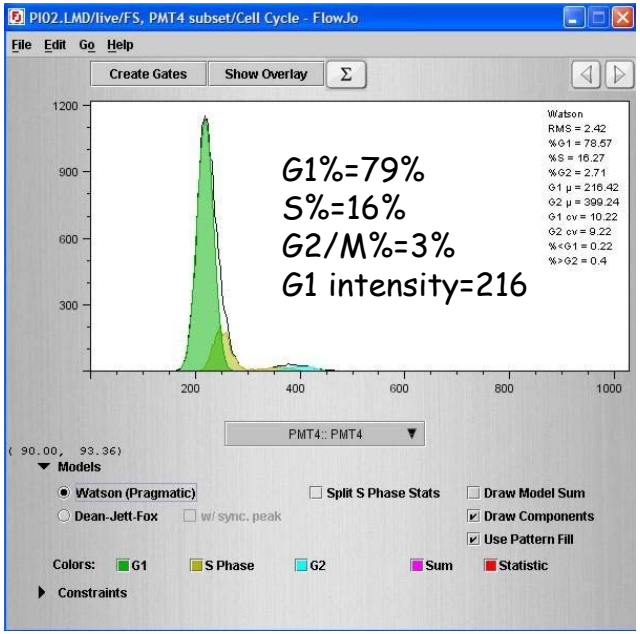


1. cells are fixed and permeabilized so that the DNA-specific dye can gain access to DNA
2. the DNA-specific dye (e.g. propidium iodide) gets to the nucleus and binds to DNA stoichiometrically
3. measured fluorescence intensity is proportional to the DNA content of the cells

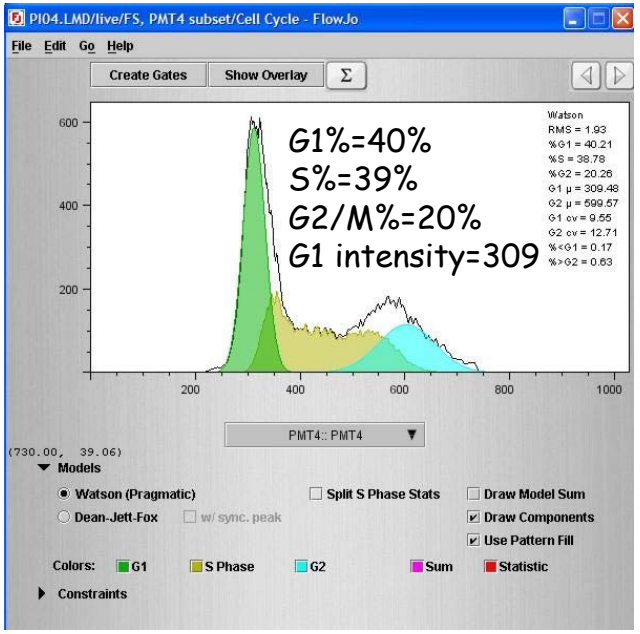
Application areas:

- cancer cells have
  - higher than normal DNA content
  - higher S and G2/M fraction
- apoptotic cells are characterized by sub-G1 DNA peak



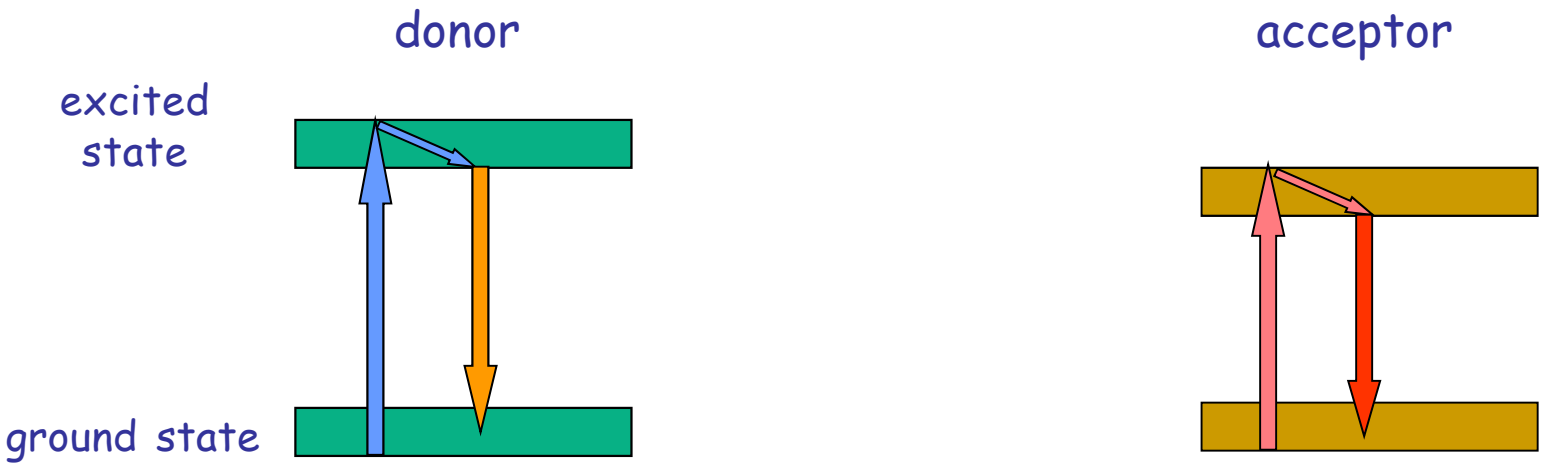


human diploid cell



JIMT-1 (human breast tumor cell)

FRET (Förster-type resonance energy transfer or fluorescence resonance energy transfer)



- After excitation the molecule gets back to the lowest (vibrational) level of the first excited state. Every subsequent process starts from this level.
- The acceptor molecule is in the close vicinity of the donor in FRET. The acceptor molecule receives the energy of the donor in a radiationless transition.
- FRET is manifested in emission of a photon by the acceptor after exciting the donor

The rate constant of FRET is described by the following equation:

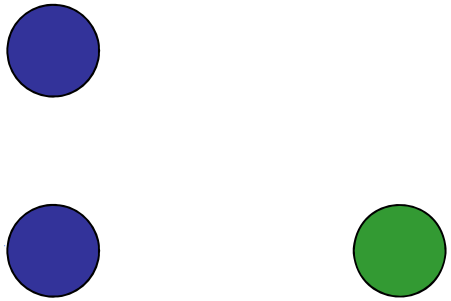
$$k_{FRET} = const \cdot Jn^{-4} k_f \kappa^2$$

FRET efficiency (E) depends steeply on the donor-acceptor distance (R):

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

R_0 - the distance at which E=50%
E - the fraction of excited donors relaxing by FRET

Measurement of FRET by flow cytometry I.



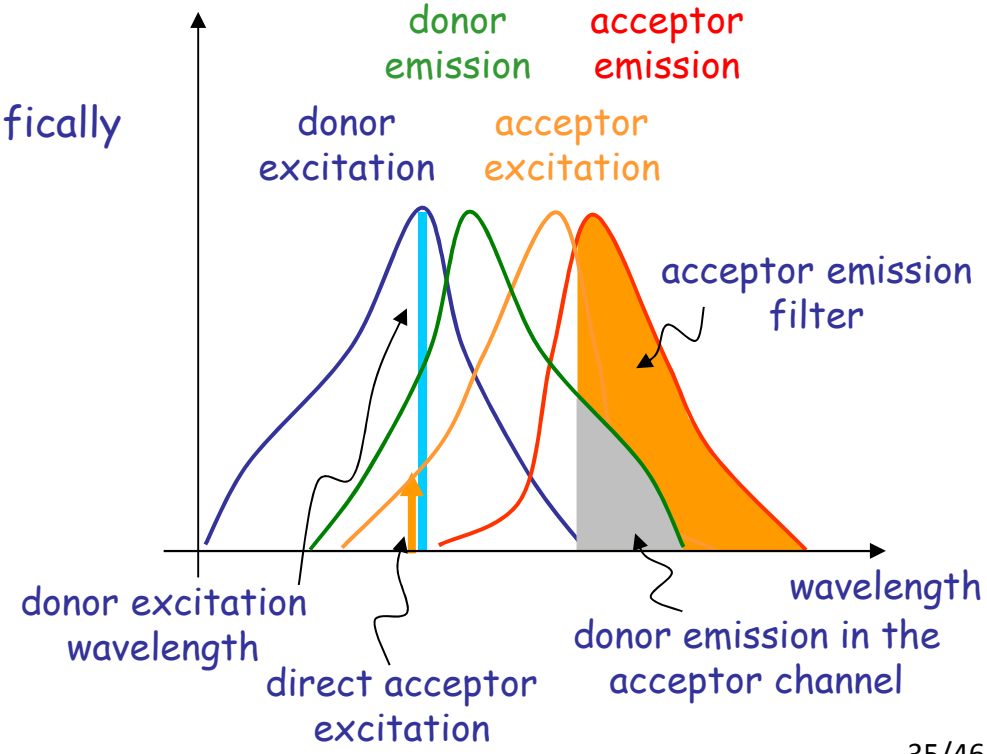
Sensitized emission: excitation of the acceptor through the donor (the acceptor fluoresces after donor excitation, F_{AD} - acceptor fluorescence intensity in the presence of donor; F_A - acceptor fluorescence intensity in the absence of donor)

$$E = \left(\frac{F_{AD}}{F_A} - 1 \right) \frac{\varepsilon_A c_A}{\varepsilon_D c_D}$$

Problems:

- it is impossible to excite the donor specifically
- it is impossible to detect the acceptor specifically

Spillover between the fluorescence channels has to be compensated.



Measurement of FRET by flow cytometry II.

$$I_1(\lambda_{ex,D},\lambda_{em,D}) = I_D(1-E) + I_A S_4 + I_D E\alpha \frac{S_4}{S_2}$$ donor channel

$$I_2(\lambda_{ex,D},\lambda_{em,A}) = I_D(1-E)S_1 + I_A S_2 + I_D E\alpha$$ FRET channel

$$I_3(\lambda_{ex,A},\lambda_{em,A}) = I_D(1-E)S_3 + I_A + I_D E\alpha \frac{S_3}{S_1}$$ acceptor channel

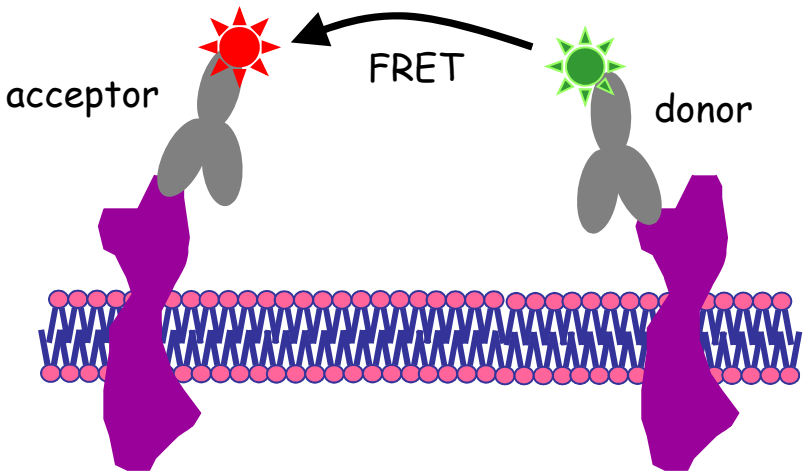
donor signal — acceptor signal — FRET signal

$$S_1 = \frac{I_{2,D}}{I_{1,D}} \qquad S_3 = \frac{I_{3,D}}{I_{1,D}} \qquad S_2 = \frac{I_{2,A}}{I_{3,A}} \qquad S_4 = \frac{I_{1,A}}{I_{3,A}} \qquad \alpha = \frac{I_2}{I_1}\frac{\varepsilon_D L_D}{\varepsilon_A L_A}$$

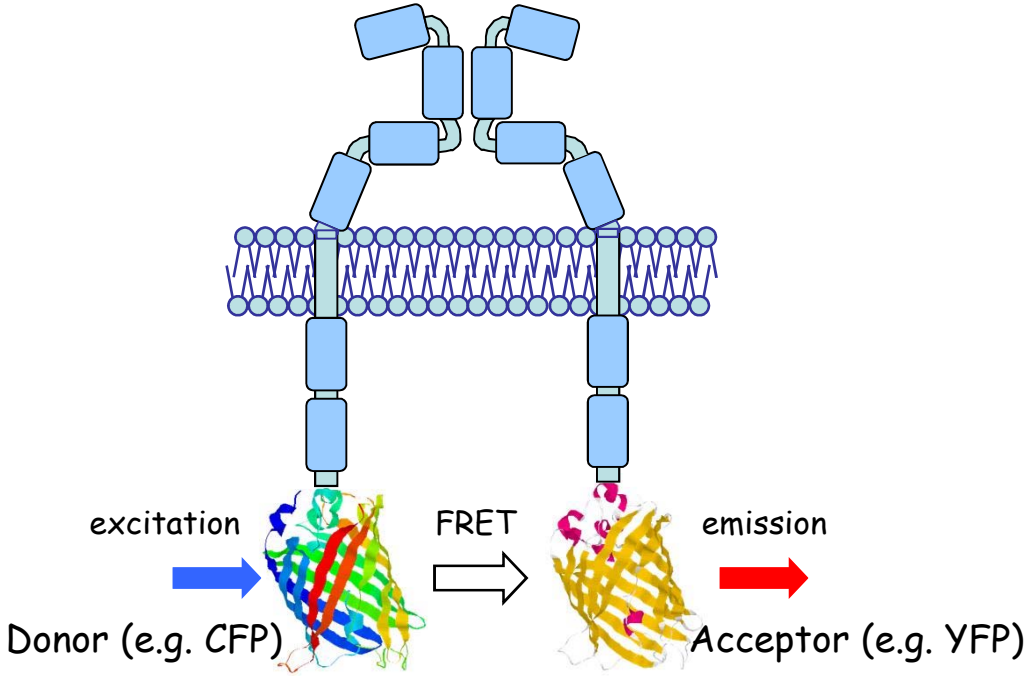
$$\frac{E}{1-E} = A = \frac{1}{\alpha}\left[\frac{I_1(S_1 - S_2S_3) + I_2(S_3S_4 - 1) + I_3(S_2 - S_1S_4)}{I_1(S_2S_3/S_1 - 1) + I_2(S_4/S_2 - S_3S_4/S_1)}\right]$$

Measurement of FRET by flow cytometry III.

Fluorescent labeling of cells



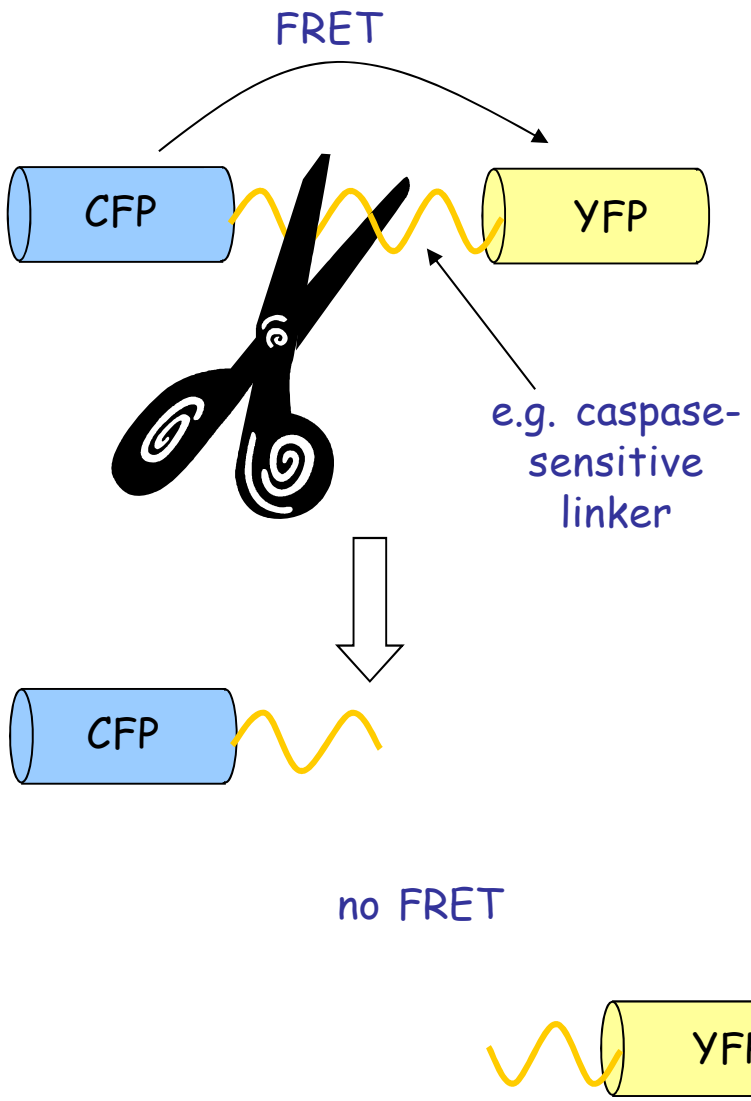
labeling by antibody or Fab



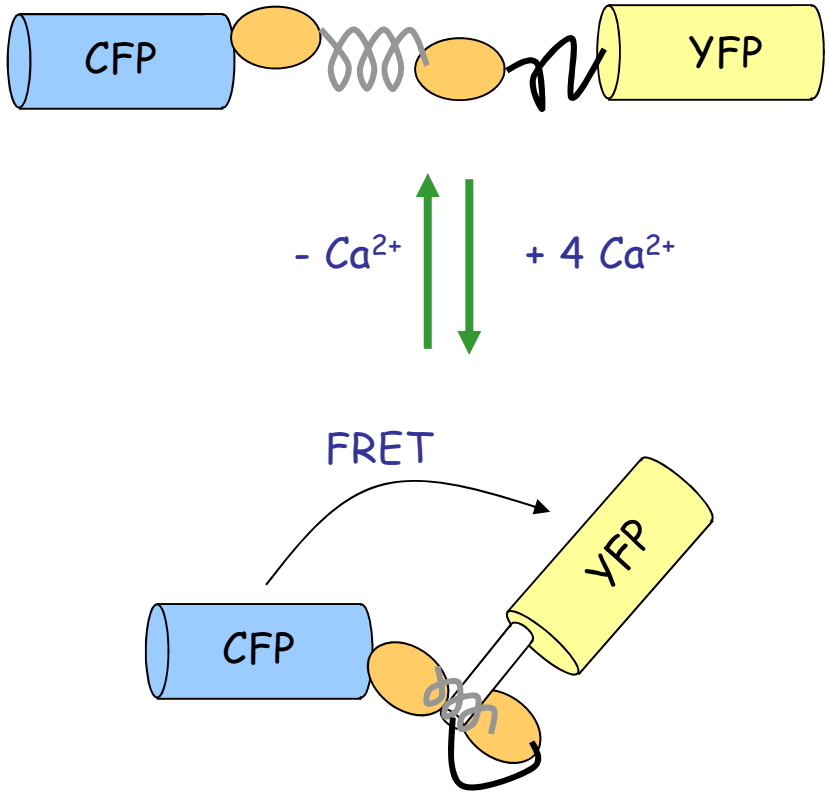
fusion protein labeled by GFP or one of its spectral variants

The application of FRET for the measurement of intracellular enzyme activity and ion concentration

Protease sensor:



Calcium sensor:



Nature, 388, 882.

FRET-based sorting

Cytometry, 67A, 86.

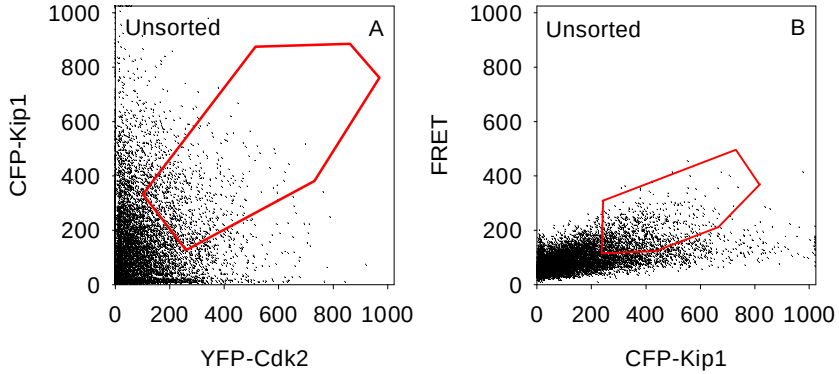
Yeast cells were transfected with YFP-CDK2-vel and CFP-Kip1.

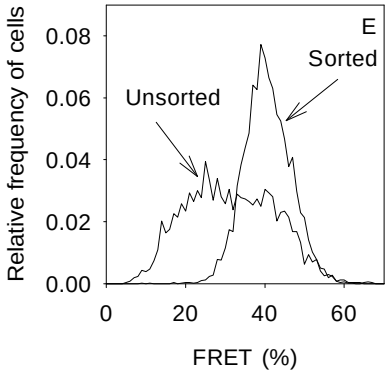
Non-sorted yeast cells, in which cells showing and do not showing FRET are mixed.

Cells were sorted according to gates displayed in A and B, and the sorting was checked. Those cell were sorted which showed

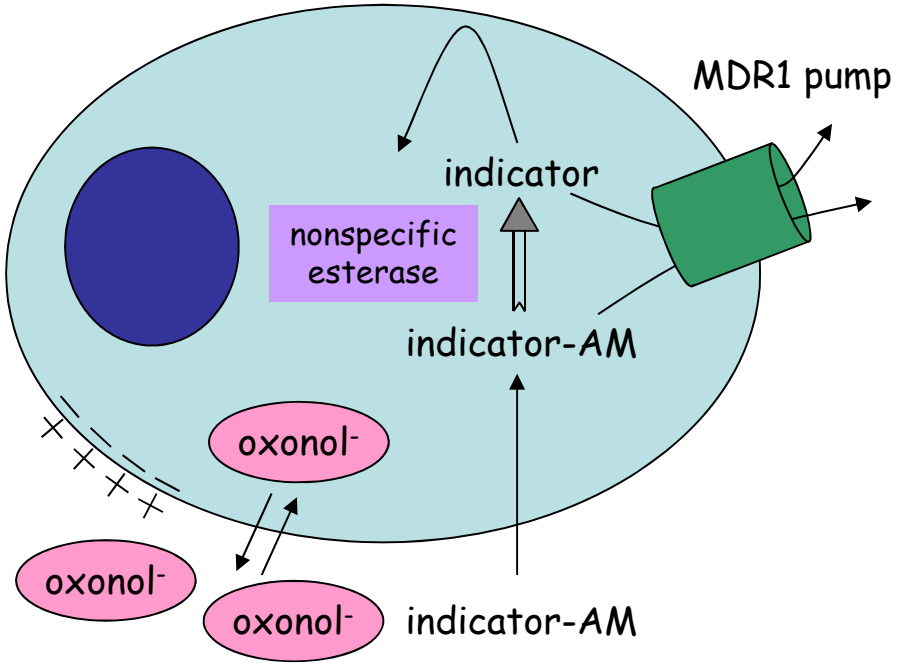
- both donor and acceptor fluorescence
- high intensity in the FRET channel

In the sorted population only cells showing FRET were present.





Measurement of ion concentrations, membrane potential and intracellular enzyme activity

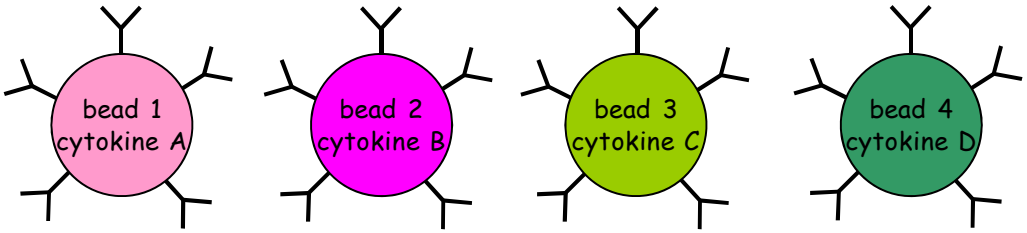


1. The hydrophobic, acetoxy methyl ester (AM) form of the indicator gets across the membrane.
2. It is hydrolyzed in the intracellular space by non-specific esterases.
3. The released hydrophilic indicator is not able to get across the membrane of living cells, so it is trapped intracellularly.
4. The fluorescence of some indicators depends on the ion concentration of the surrounding solution.

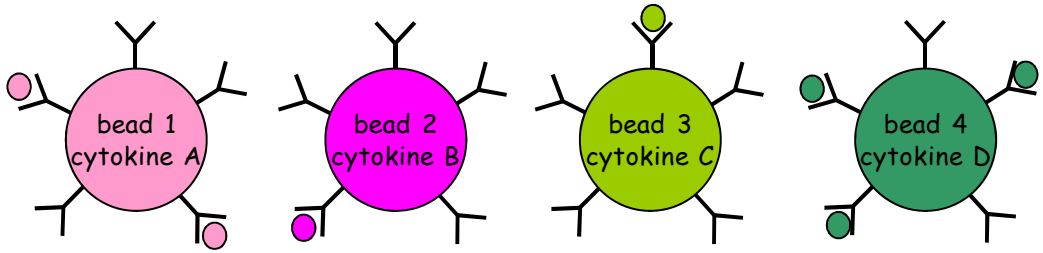
5. measurement of multidrug resistance: MDR proteins pump the indicator and/or its AM form from the intracellular space. Calcein is such in indicator, which does not indicate anything, i.e. its accumulation, or the lack thereof, is measured in cells showing MDR.
6. Negatively charged oxonol is distributed in the intra- and extracellular spaces according to the membrane potential (Nernst equation). After depolarization the amount of oxonol in the intracellular space, hence the fluorescence intensity of the cell, increases.

Name of indicator	Measured parameter
BCECF	pH
FURA-2, INDO-1, Fluo-3	Ca^{2+}
SBFI	Na^{+}
PBFI	K^{+}
fluorescein-diacetate (FDA)	viability, since the dye is not (very much) sensitive to anything, only its accumulation is measured in the intracellular space
calcein	multidrug resistance
oxonol	membrane potential

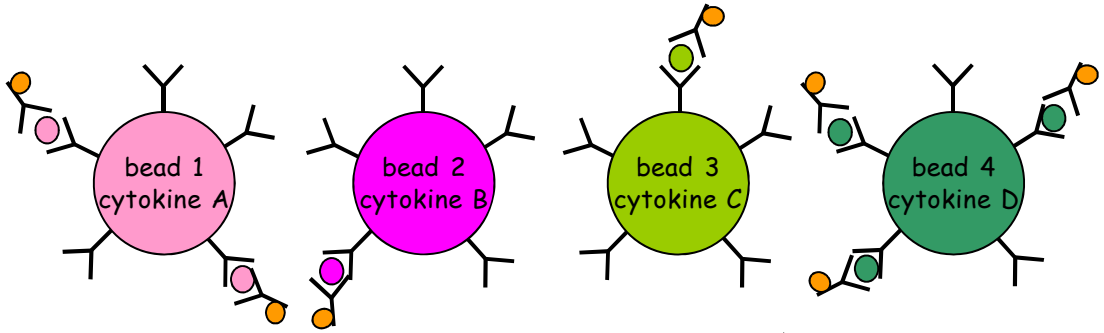
Multiplex bead analysis



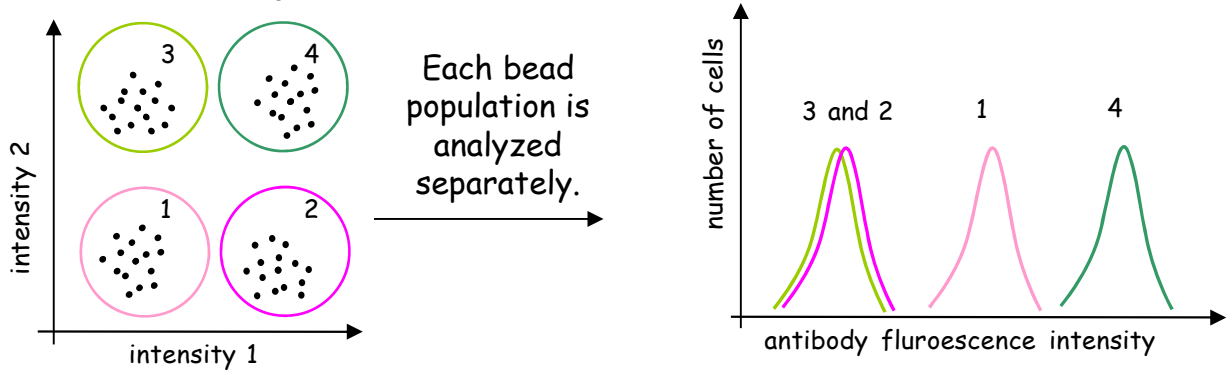
Beads with different colors. Different, cytokine-specific antibodies are bound to each of them



A cell lysate is added to the mixture of these beads. An amount of cytokine proportional to the concentration of the cytokine in the lysate binds to the bead.



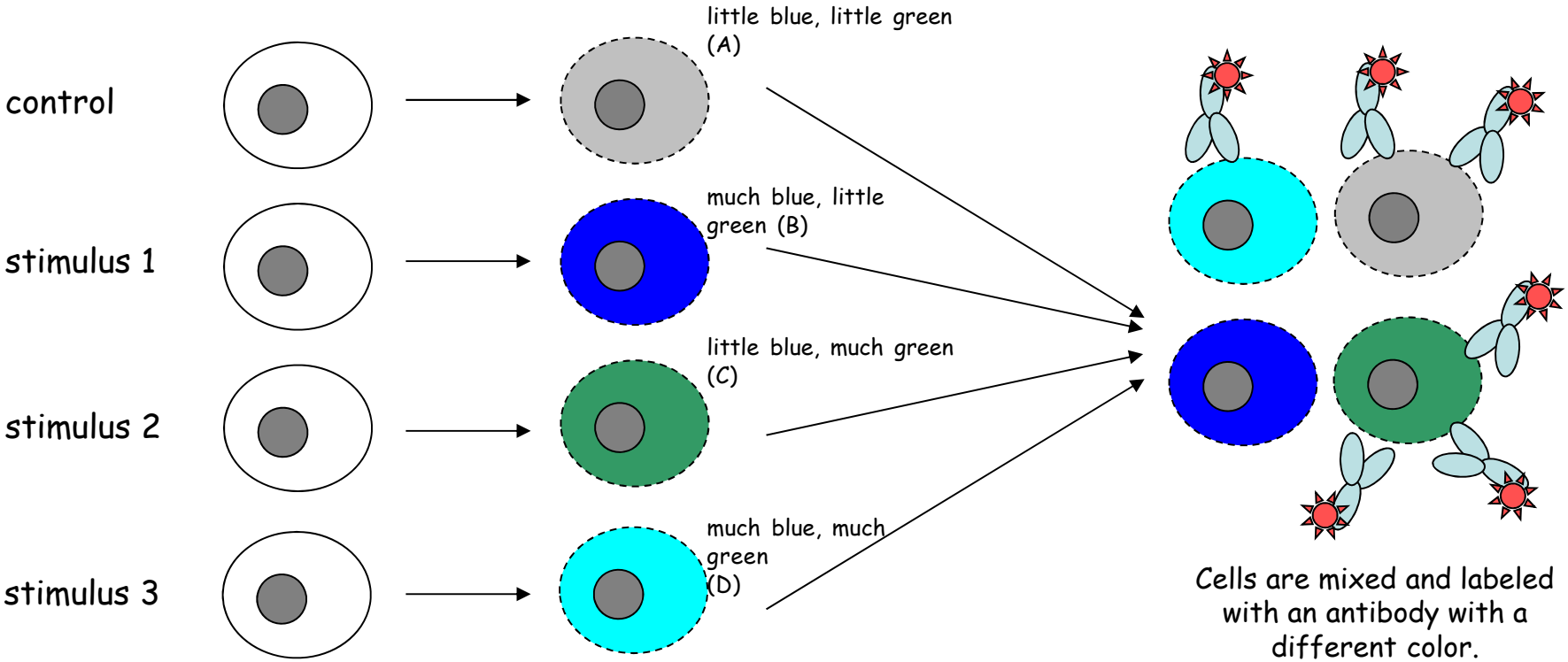
A cytokine specific, detecting antibodies labeled with a fluorescent dye with a color different from that of the bead is added to the beads. The antibody binds to and labels cytokines already bound to the beads.

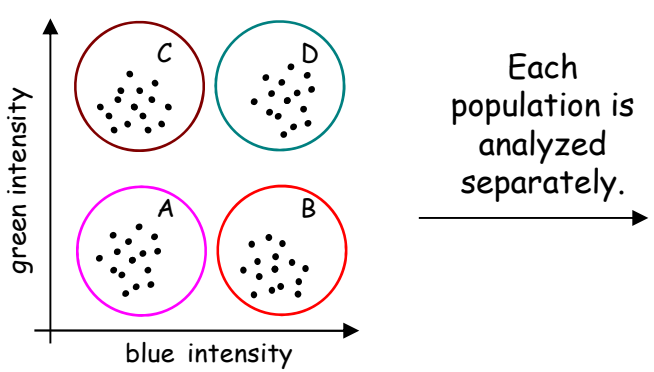


The beads can be run simultaneously on a flow cytometer.

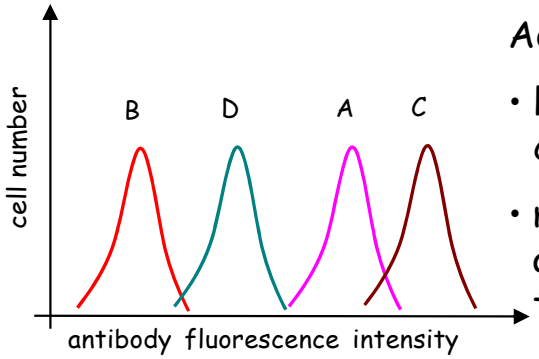
Advantage: the concentration of many citokines (or other proteins) can be measured at the same time.

FCB: fluorescent cell barcoding Nat. Methods, 3, 361





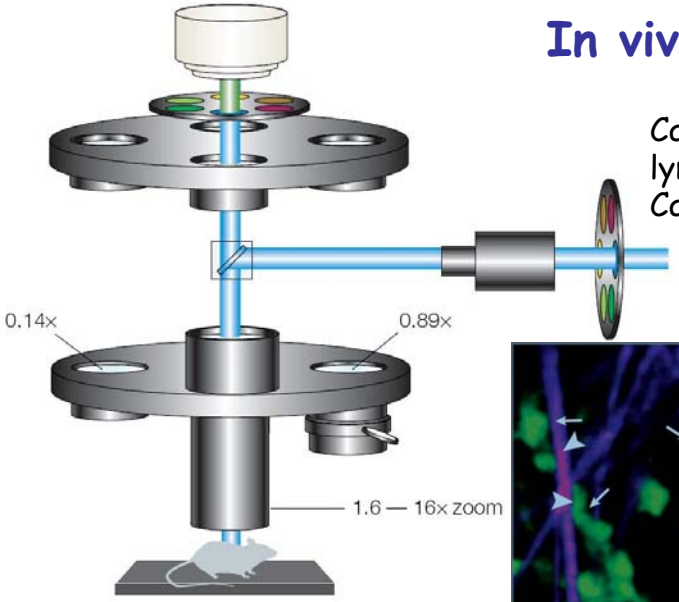
Each population is analyzed separately.



Advantage:

- low antibody consumption
- many samples can be analyzed at the same time

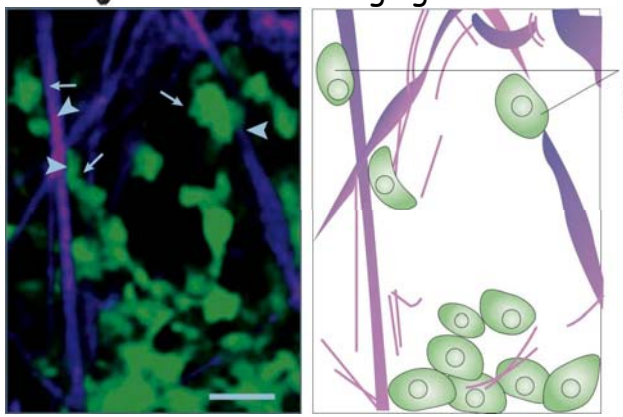
In vivo flow cytometry

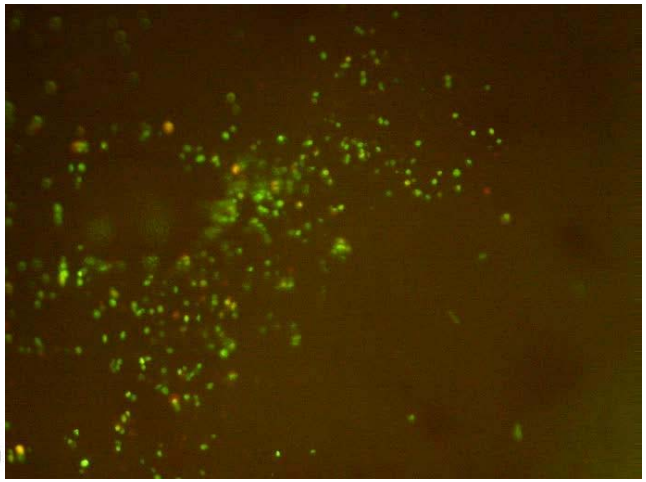


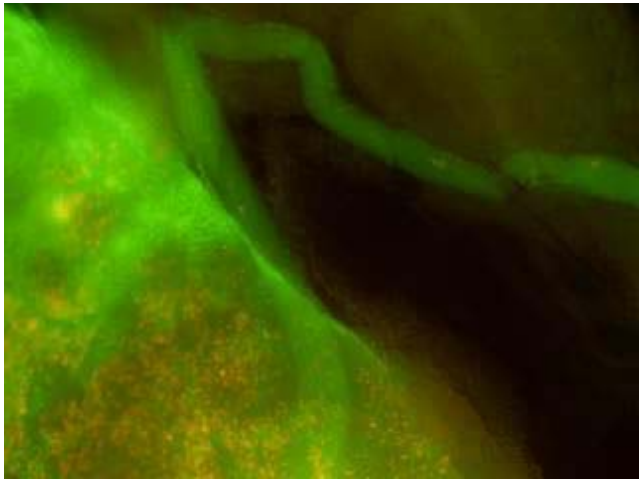
Olympus OV100

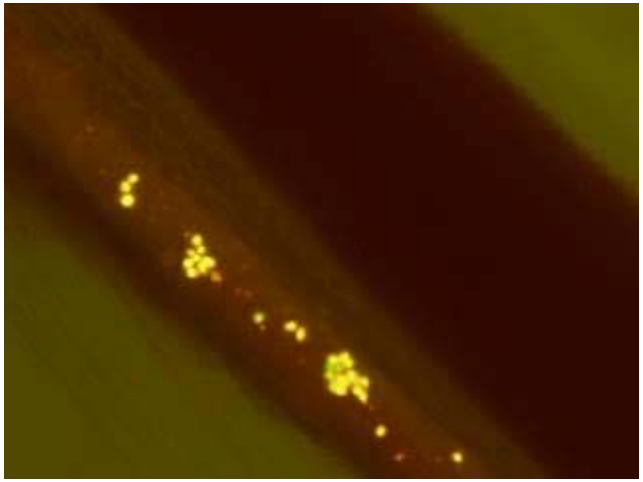
Cancer cells can be tracked in lymph vessels *in vivo*. Cancer Res 67:8223 (2007)

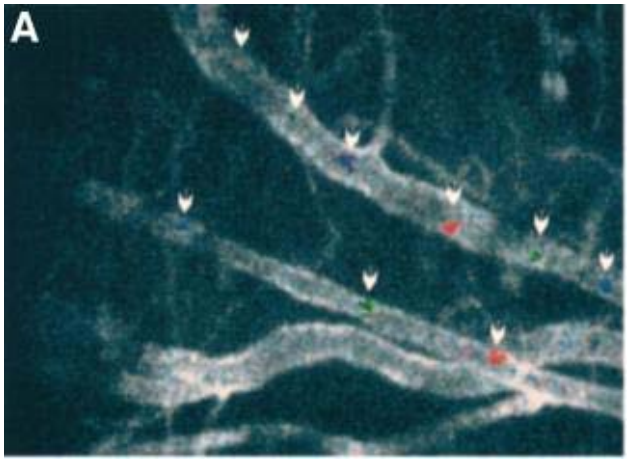
In vivo imaging

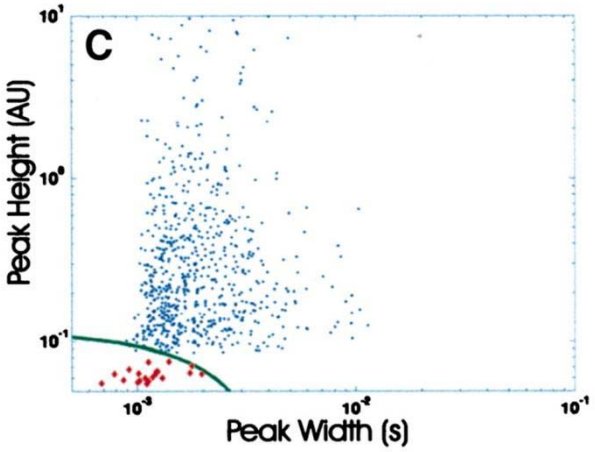












Fluorescently labeled cells can be investigated in living animals and analyzed in a similar way to conventional flow cytometric analysis. Cancer Res 64:5044 (2004), Nat. Rev. Canc. 3: 921 (2003)

Transition between flow cytometry and microscopy

Flow cytometry

- analysis of many suspended cells automatically
- without subcellular resolution

Microscopy:

- analysis of few attached cells with a huge workload
- subcellular resolution

Imaging flow cytometry:

- imaging of cells moving in the fluid stream
- the fluorescence intensity of cells is measured similarly to a flow cytometer, histograms and dot plots can be created similarly to a flow cytometer
- cells selected in one- and two-dimensional histograms can be traced back
- colocalization and morphological analysis can be carried out on the cells

Laser scanning cytometry (LSC):

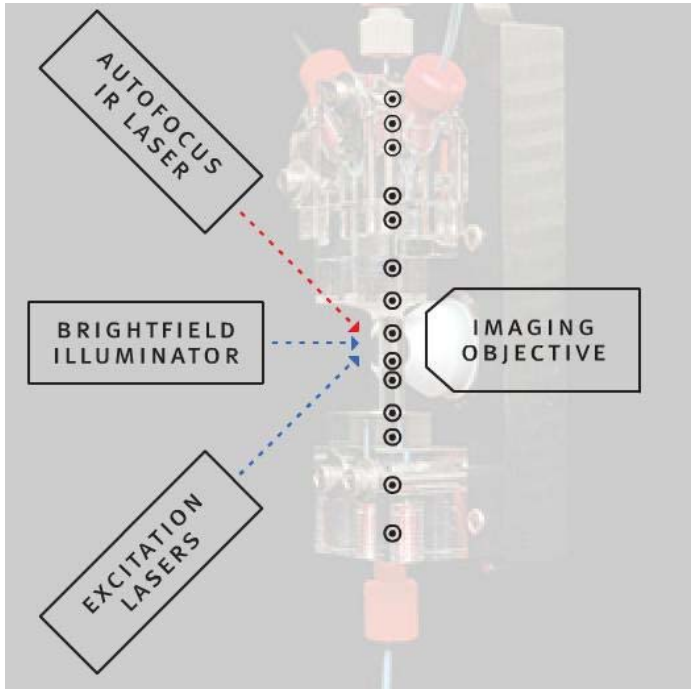
- investigates cells attached to a slide
- the whole slide (i.e. not only a microscopic field) is scanned with a low NA objective
- cells are identified automatically
- morphological and colocalization measurements can be done with the cells
- the fluorescence intensity of cells can be displayed in one- and two-dimensional histograms similarly to flow cytometry

measurement of many cells automatically with subcellular resolution

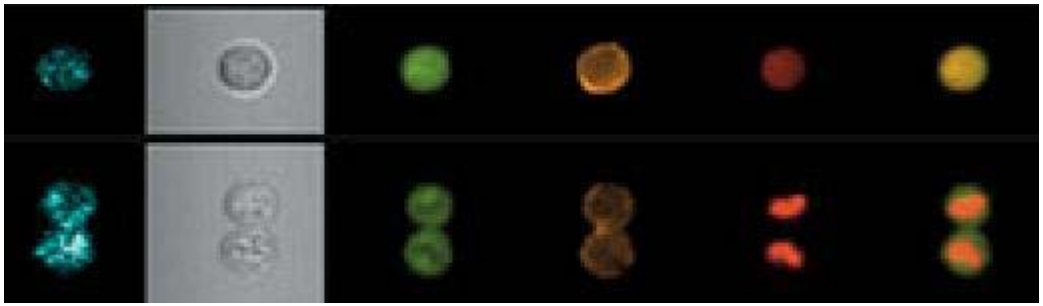
Imaging flow cytometry

1. Cells flow in a flow cell similarly to flow cytometry.

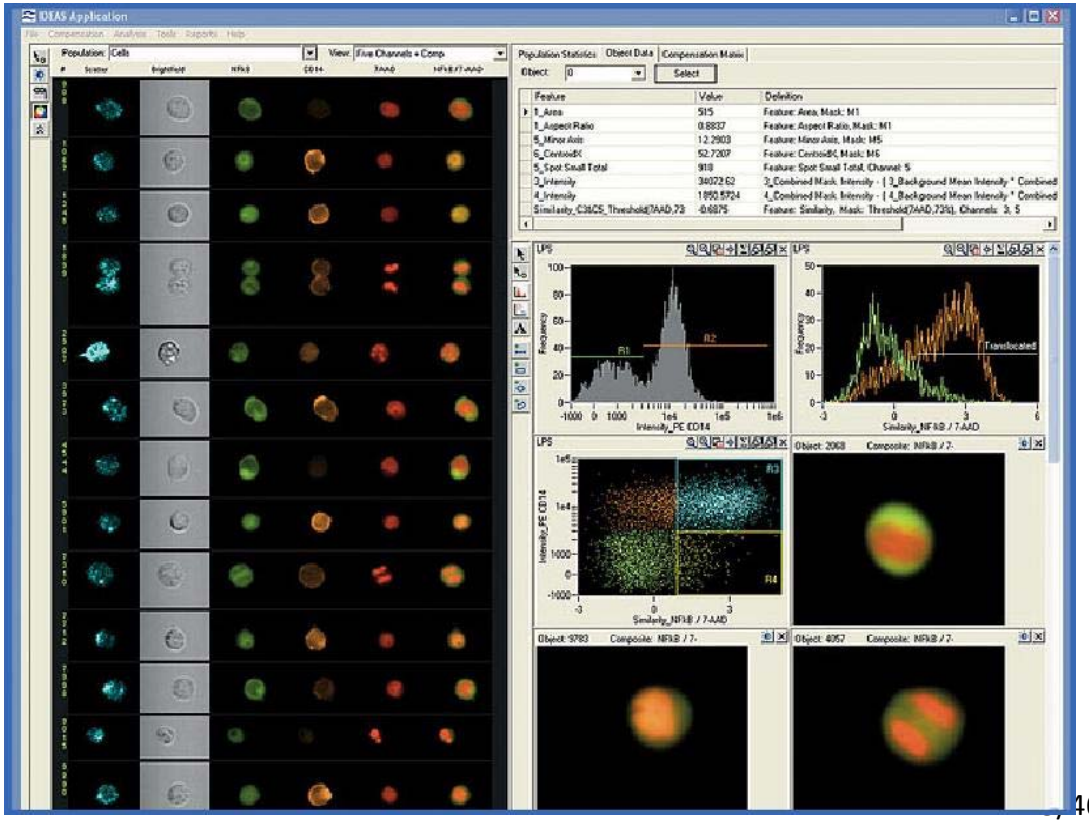
2. Not only the total (or maximal) fluorescence intensity of cells is measured, but images are also taken.



3. Images are saved by the device ...



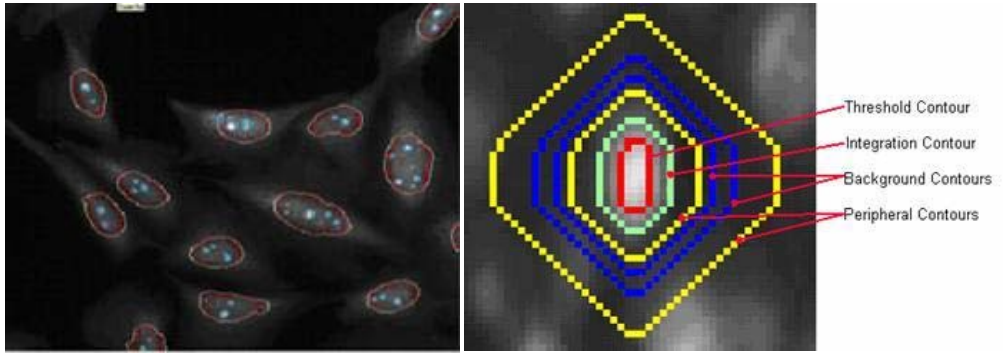
4. ... and the fluorescence intensity data of cells can be analyzed in a similar way to flow cytometry.

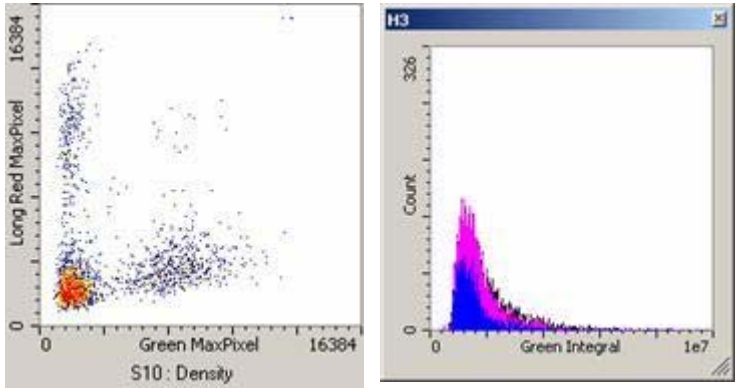


Laser scanning cytometry (LSC)

1. The microscope slide is scanned by a laser beam.

2. The software identifies cells based on nuclear staining, and the nucleus and the cytoplasm surrounding it is circumscribed (segmentation).





3. The fluorescence intensity of cells is calculated and displayed by the device, and it can be analyzed similarly to flow cytometry.