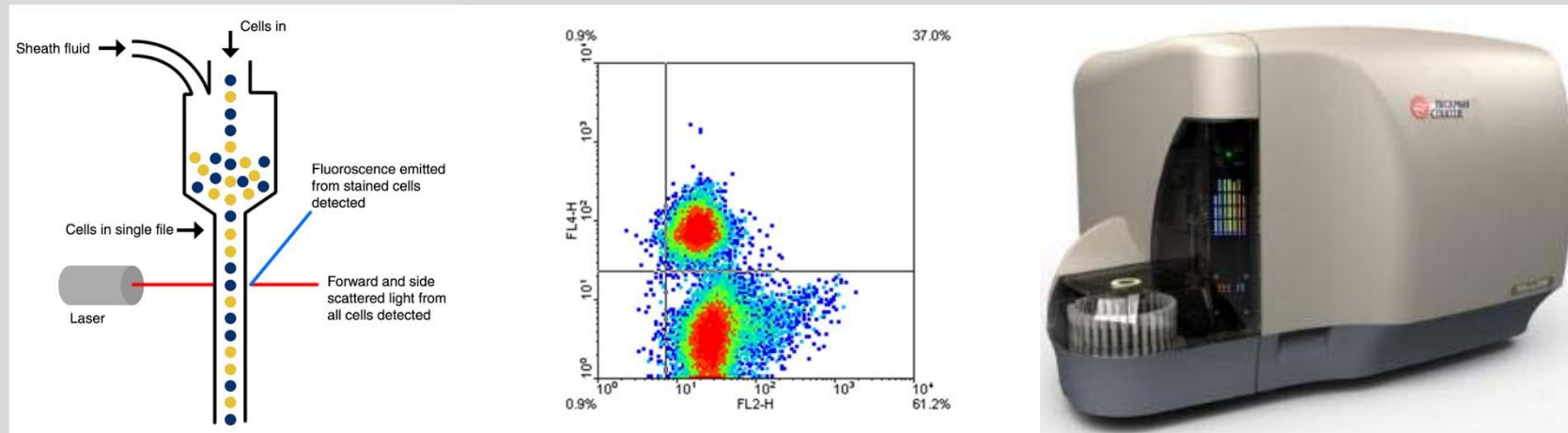


Principles and applications of flow cytometry

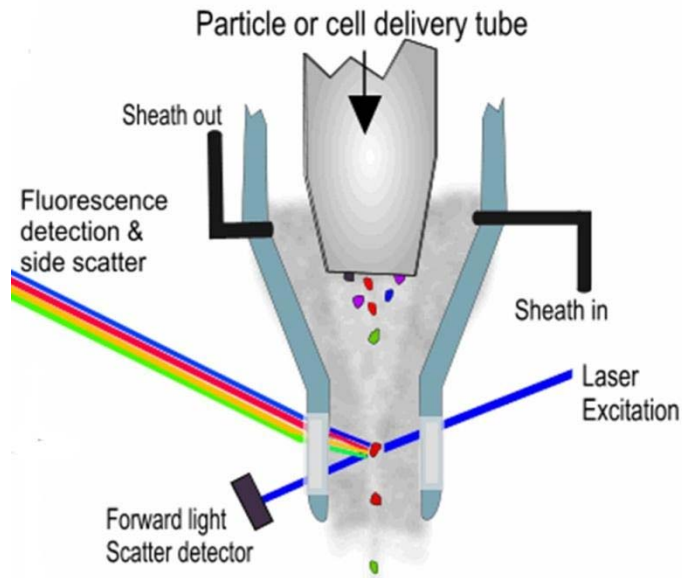
Peter Nagy

Department of Biophysics and Cell Biology, University of Debrecen



- Principles of the device
 - Flow system
 - Optical parts
 - Measurement, display and analysis of signals
- Representative applications
 - Immunophenotyping, DNA content, cell cycle, FRET, ion concentrations, multiplexing applications

Principles of flow cytometry

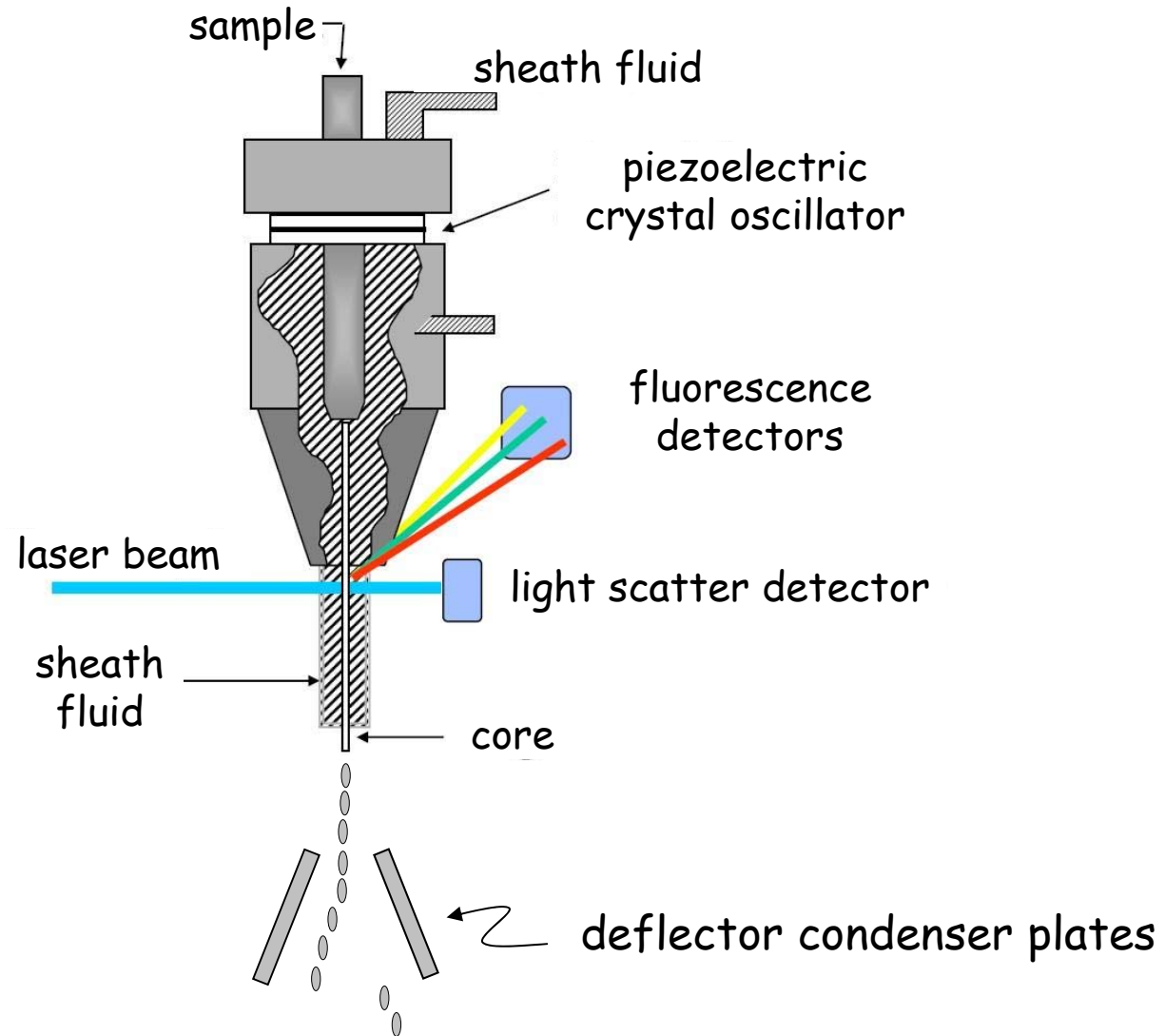


An instrument which measures

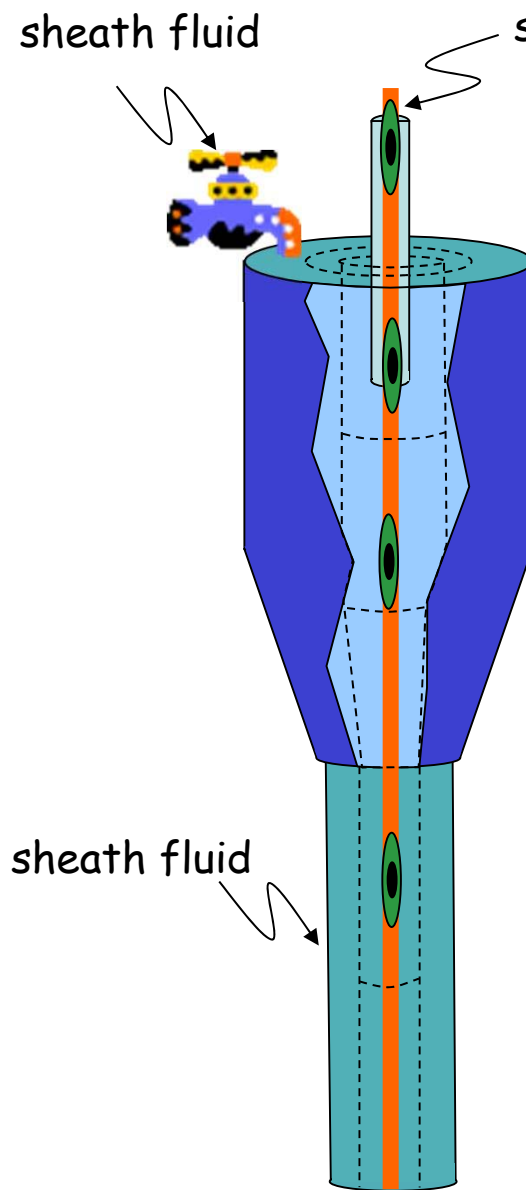
- the fluorescence and light scatter parameters of
- suspended
- single cells
- with high speed (as high as several thousand cells/sec)

	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



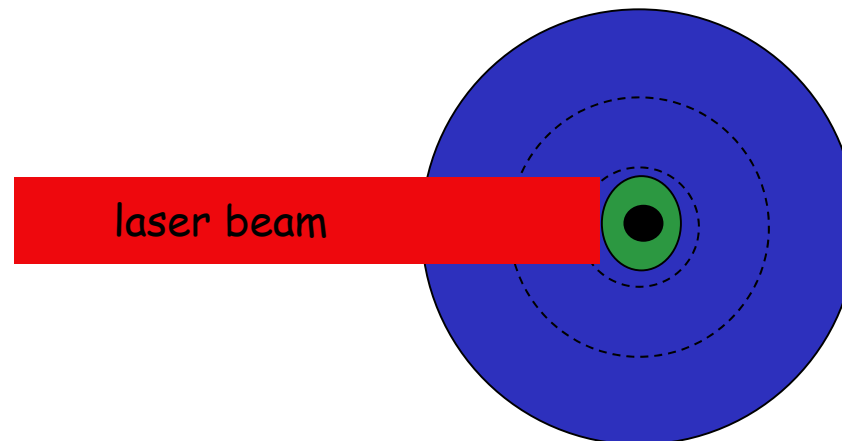
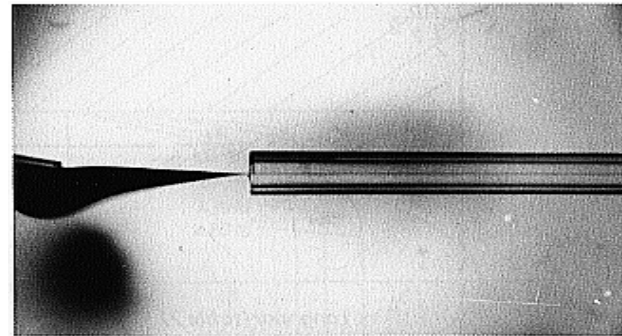
The sheath fluid surrounds the sample in concentric layers (laminar flow).

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

Purpose of hydrodynamic focusing:

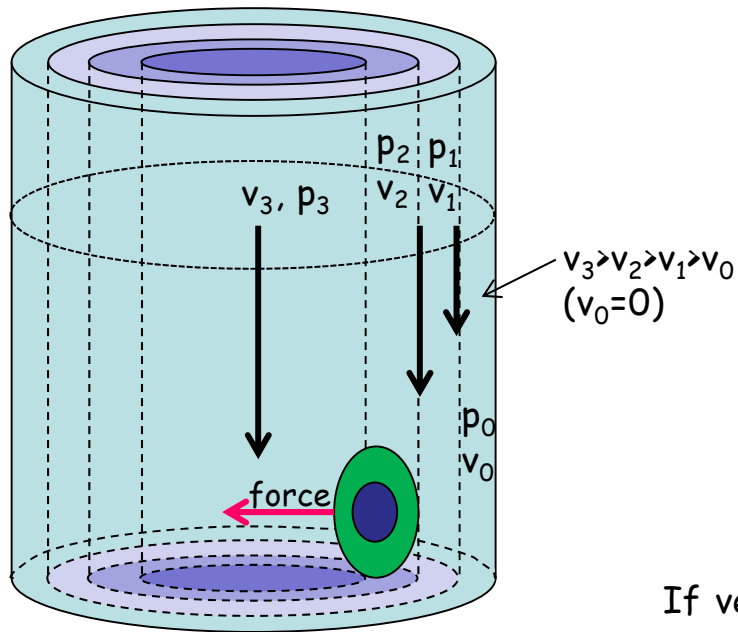
The cells shall be where the laser beam illuminates them.

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:

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

If investigated at a given height (h):

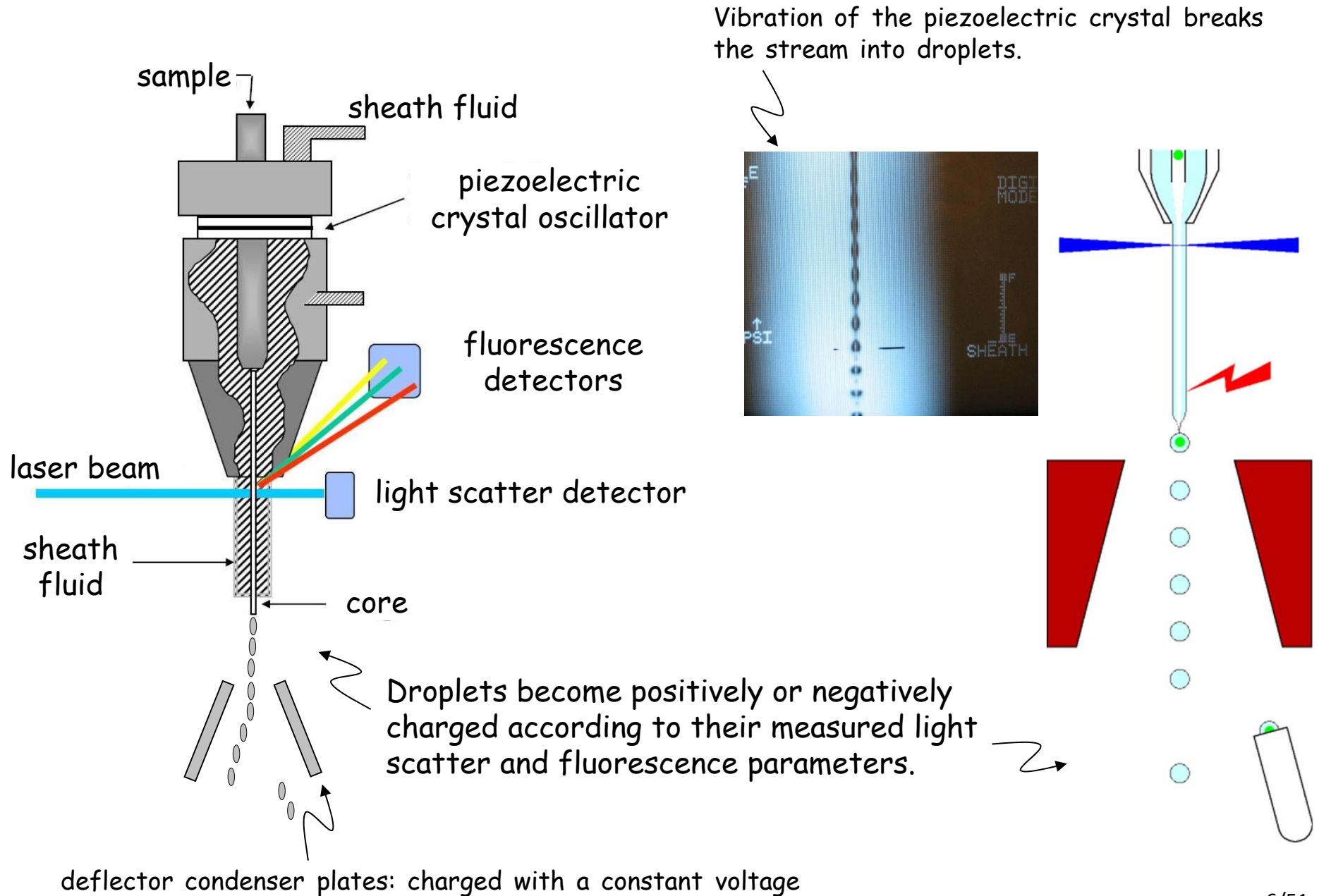
$$p + \frac{1}{2}\rho v^2 = \text{const.}$$

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.

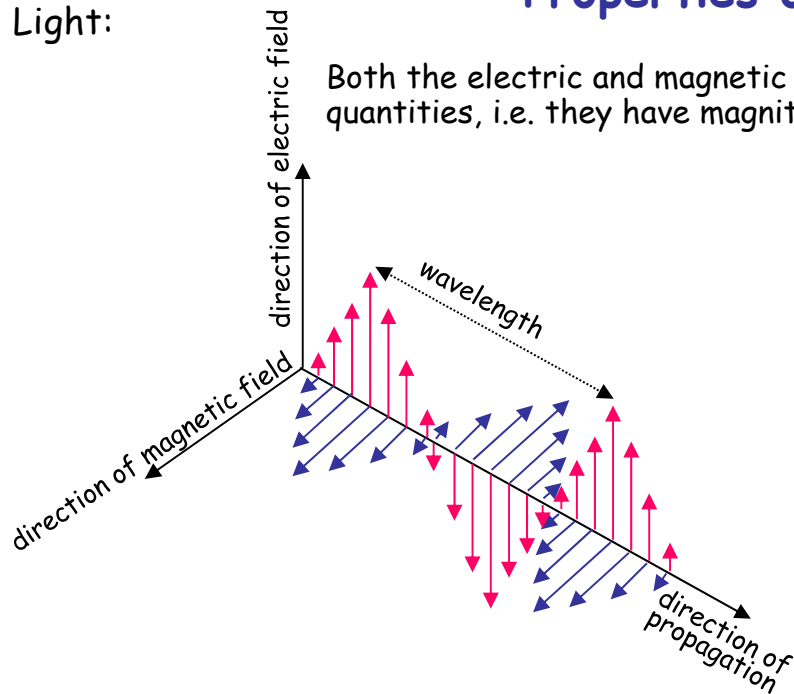
Cell sorting



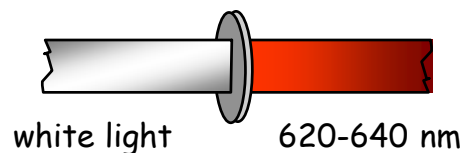
Properties of light and fluorescence

Light:

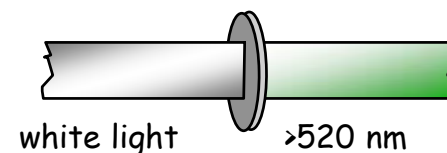
Both the electric and magnetic field are vector quantities, i.e. they have magnitude and direction.



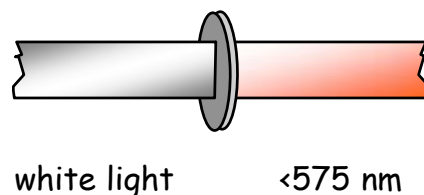
630 nm band-pass (BP)



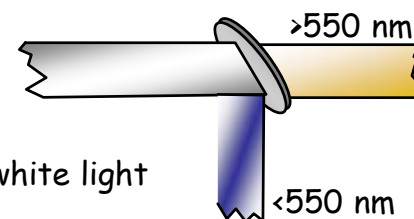
520 nm long-pass (LP):



575 nm short pass (SP):



550 nm dichroic mirror:



Fluorescence:

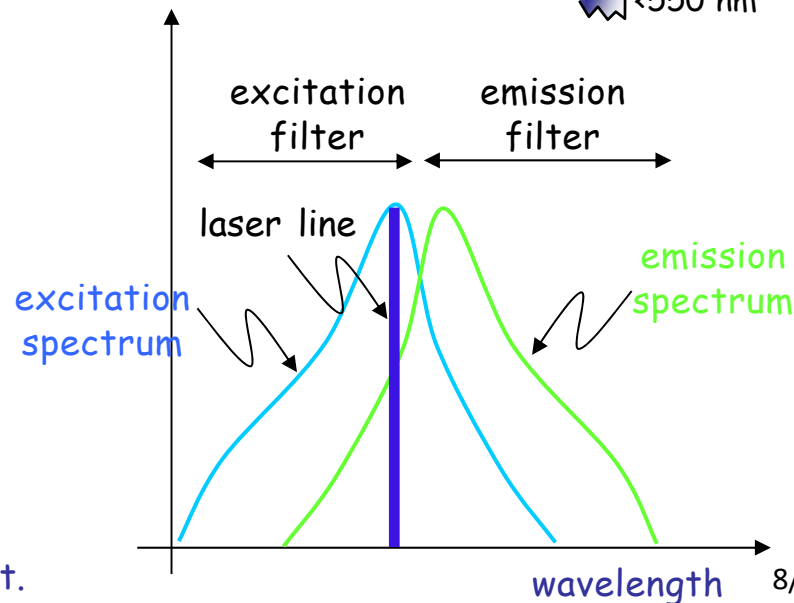
excited state



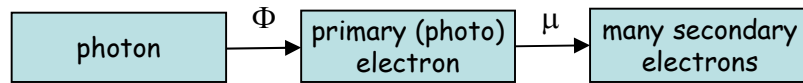
ground state



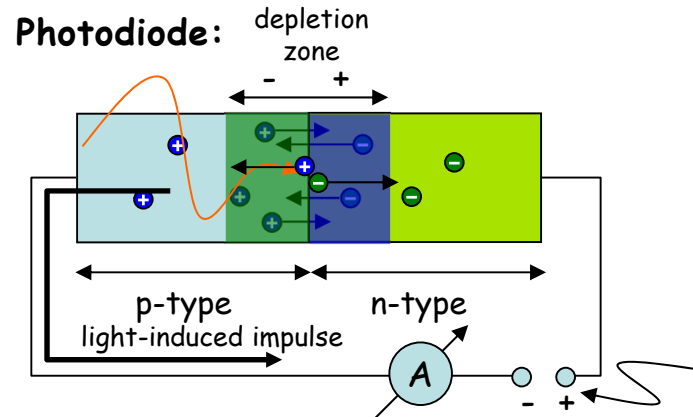
- After excitation the molecule gets back to the lowest (vibrational) level of the first excited state. Every subsequent process start from this level.
- Fluorescence has a longer wavelength than excitation light.



Detectors

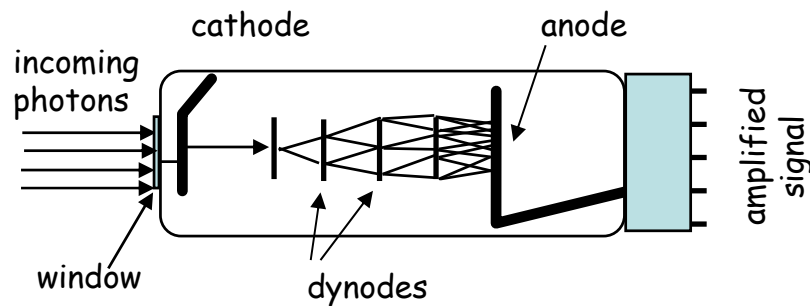


- quantum yield (Φ): $\Phi = \frac{\text{number of electrons}}{\text{number of photons}} \times 100$
 - amplification (μ): $\mu = \frac{\text{final number of electrons}}{\text{number of photoelectrons}} \times 100$
- e.g. every dynode emits 10 secondary electrons, and there are 8 dynodes:
- $$\mu = 10^8$$



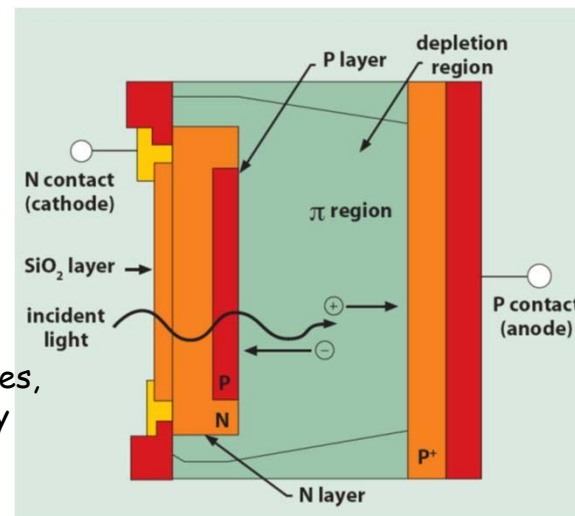
- high quantum yield
 - zero amplification
- two modes: 1. no voltage, 2. reverse bias

Photomultiplier (PMT):



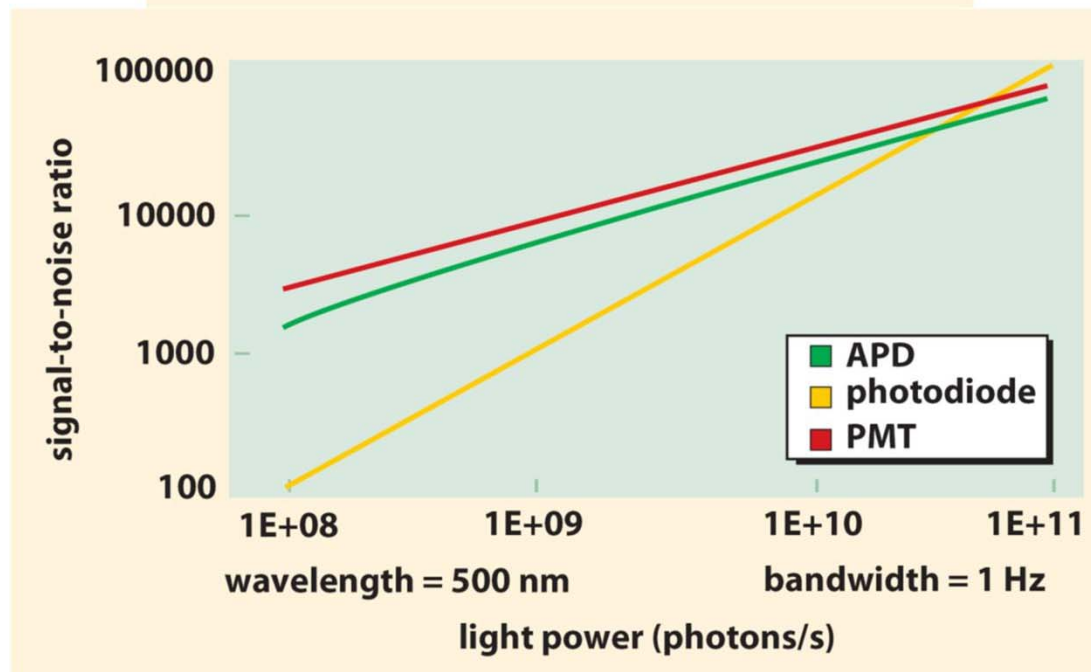
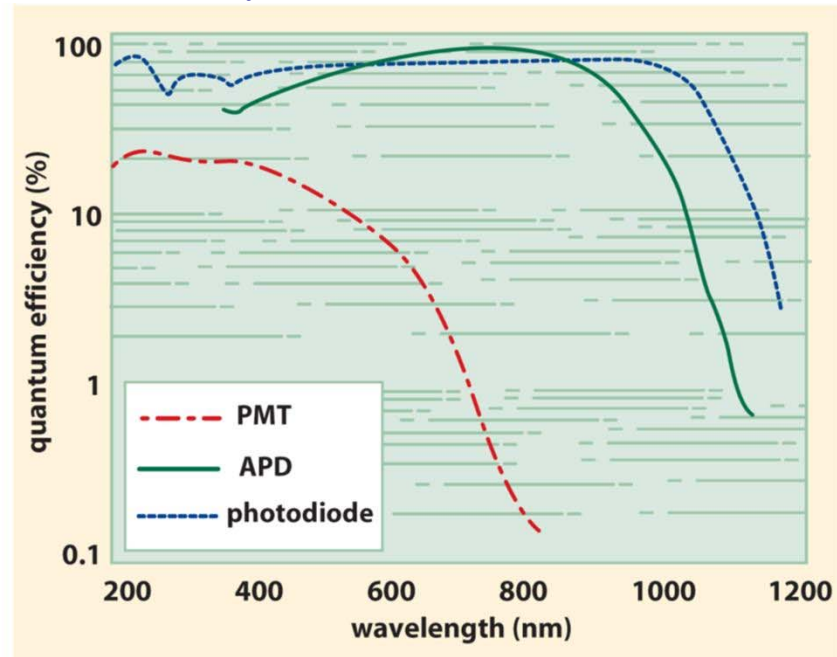
- an increasingly positive voltage is applied to the dynodes, and the accelerated electrons evoke several secondary electrons when they impinge into the dynodes
- low quantum yield
- high amplification

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 ($\mu \neq 0$)
- 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

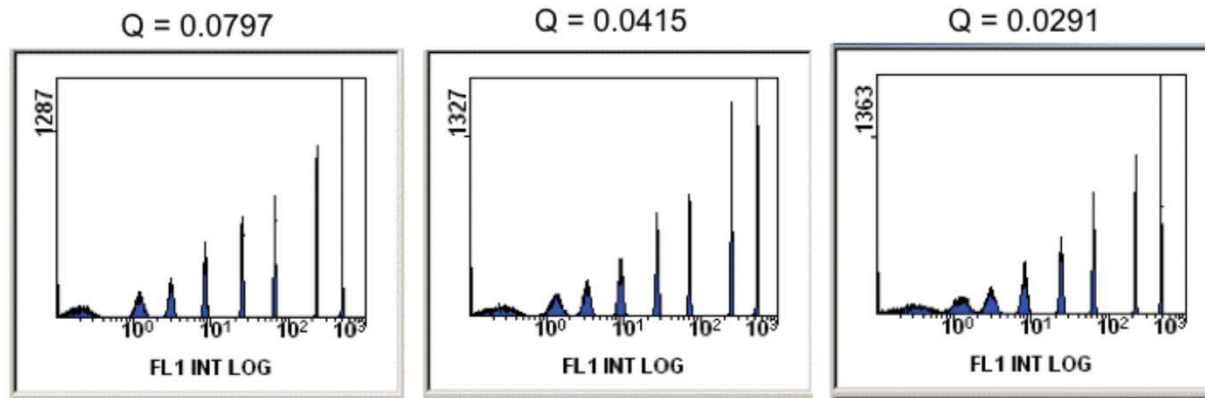
- The quality of flow cytometric data is determined by the signal to noise ratio.
- The signal to noise ratio can be characterized by two factors:
 - detection efficiency (Q), a.k.a. quantum yield (Φ): number of photoelectrons produced per molecule of fluorophore
 - background light level (B)

due to the Poisson nature
of photon counting

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

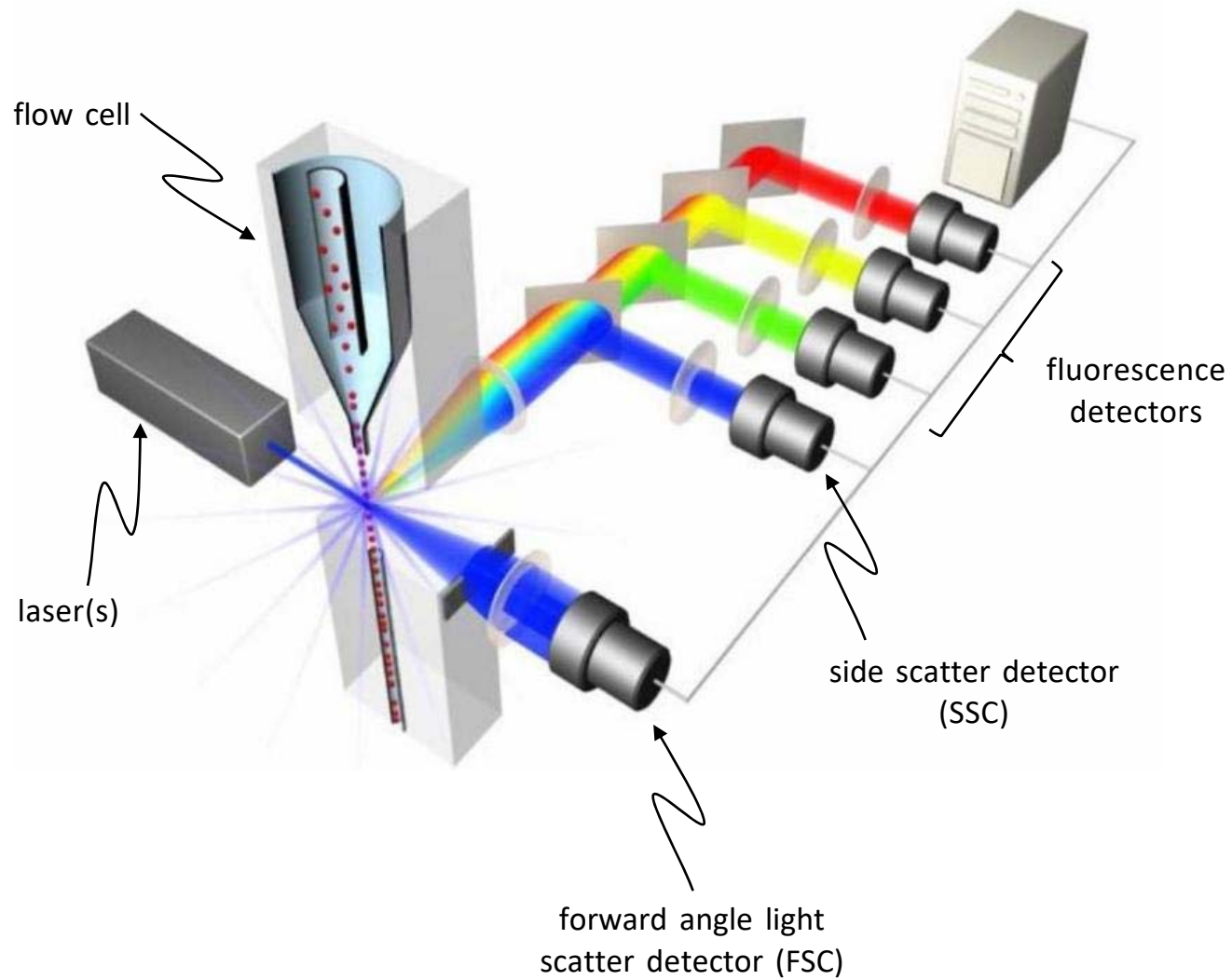
$$\text{relative noise} \sim \frac{\sqrt{n}}{n} = \frac{1}{\sqrt{n}}$$

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



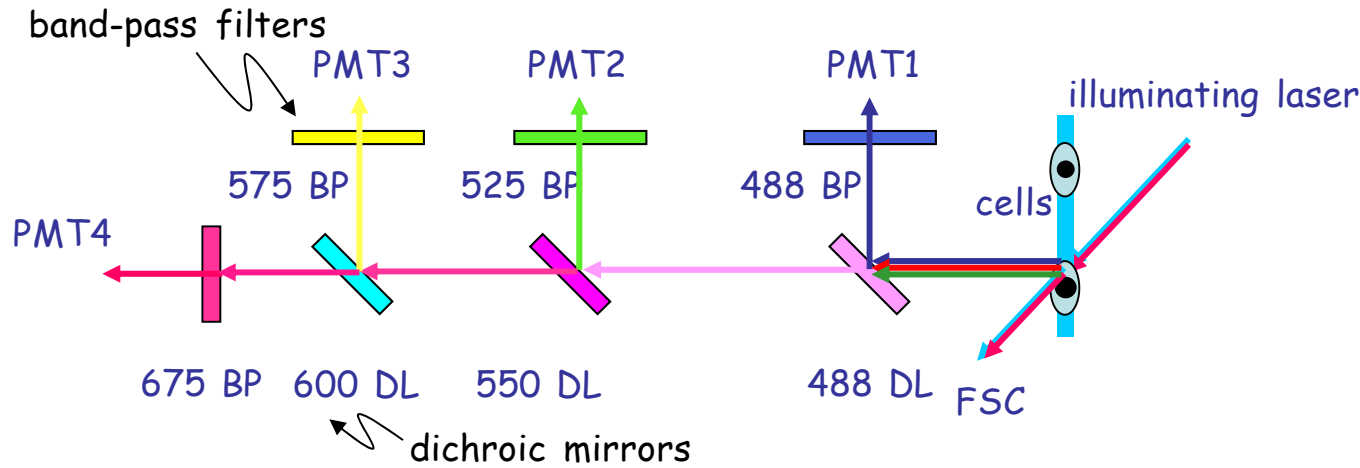
The Q factor was reduced by decreasing the laser intensity (while maintaining the brightest population in the same mean channel by increasing the detector voltage) resulting in the loss of resolution.

Arrangement of detectors I.



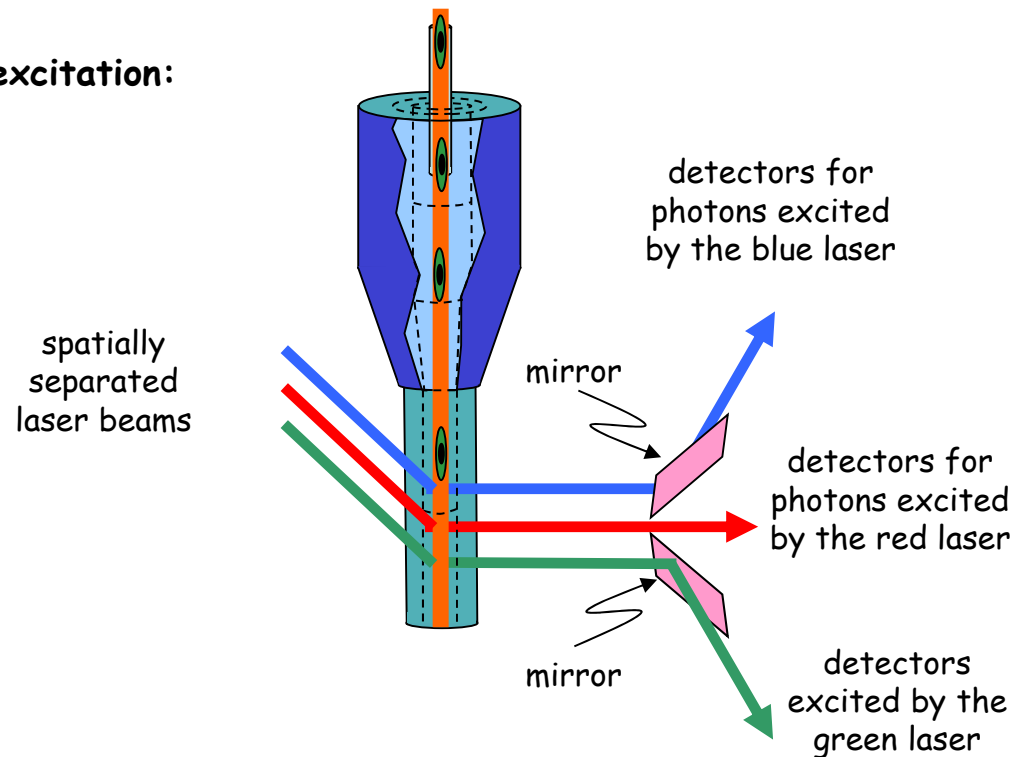
Arrangement of detectors II.

Excitation at the same place with different lasers:

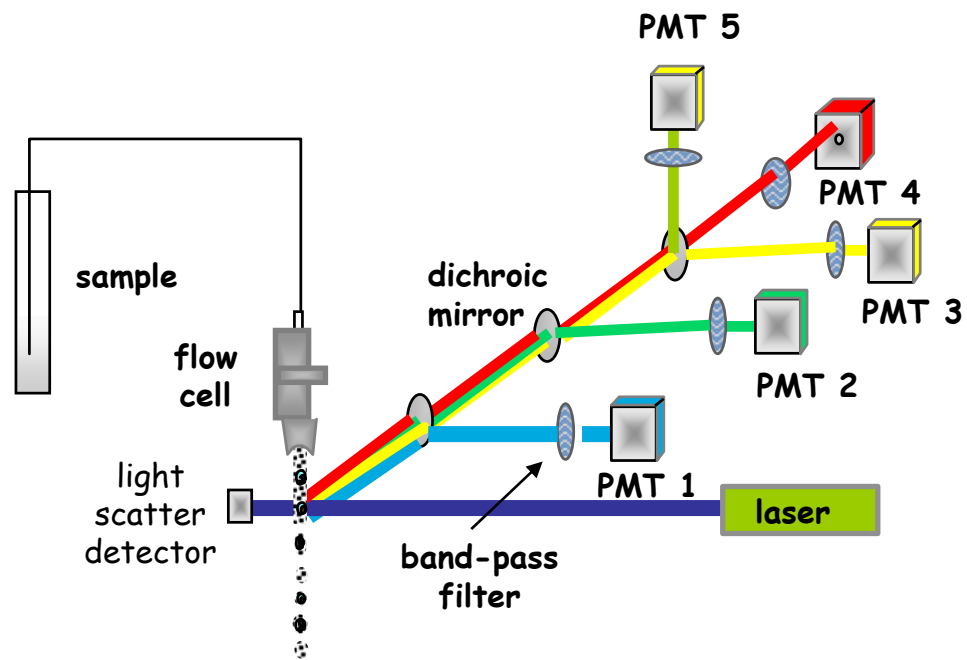


Beams separated from the common beam by dichroic mirrors are further filtered by band-pass filters.

Spatially separated excitation:



Arrangement of detectors III.

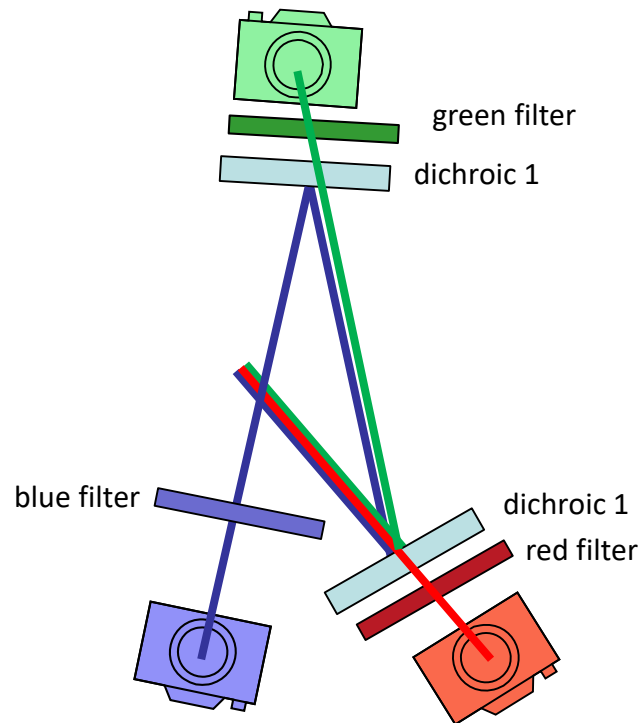


Arrangement of detectors IV.

New development: octagon or trigon arrangement of detectors

Advantage and principles:

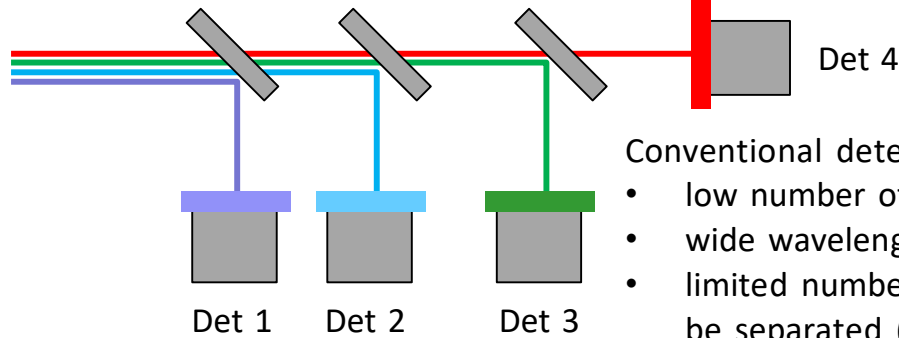
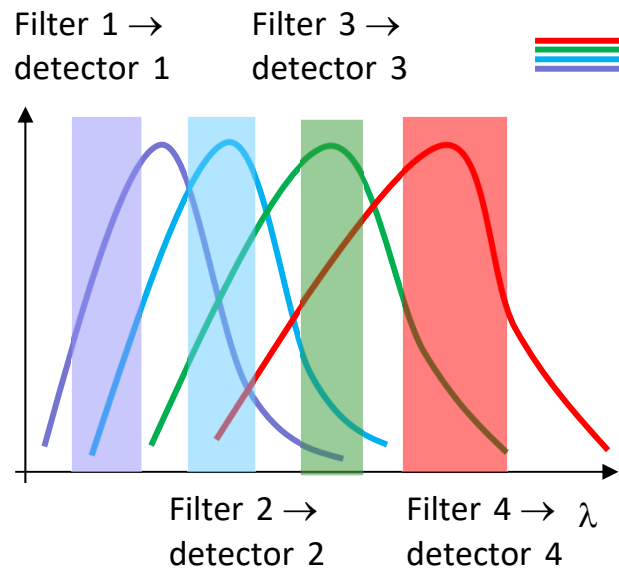
- The detector closest to the site of emission records the highest wavelength fluorescence usually having the fewest photons.
- Lower wavelength photons are reflected to the rest of the detectors by (high-pass) dichroic mirrors.
- Light reflection is usually more efficient than transmission.



Arrangement of detectors V.

New development: spectral flow cytometry

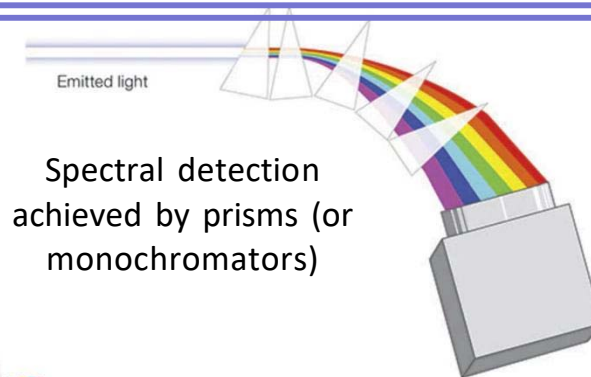
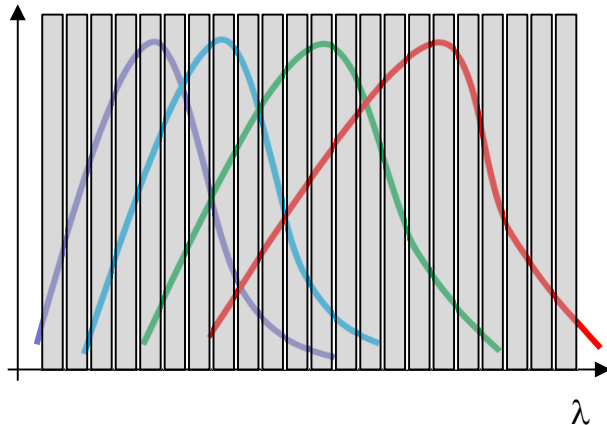
Conventional



Conventional detection:

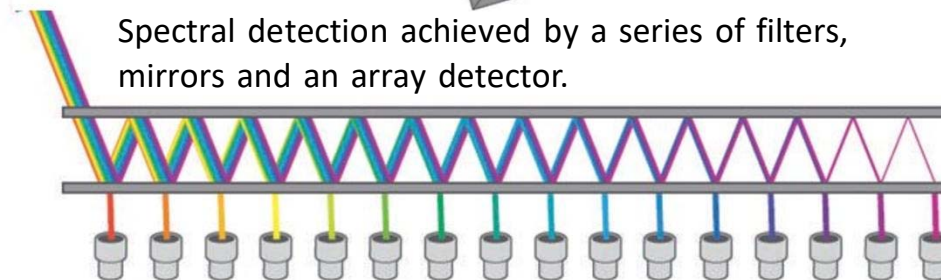
- low number of detectors
- wide wavelength range of detectors
- limited number of fluorophores can be separated (see compensation later)
- not all photons are detected

A large number of detectors (e.g. 16) each with a narrow wavelength range



Spectral detection:

- whole spectrum is recorded (with low resolution)
- with a large number of detectors
- all photons detected
- many fluorophores can be separated

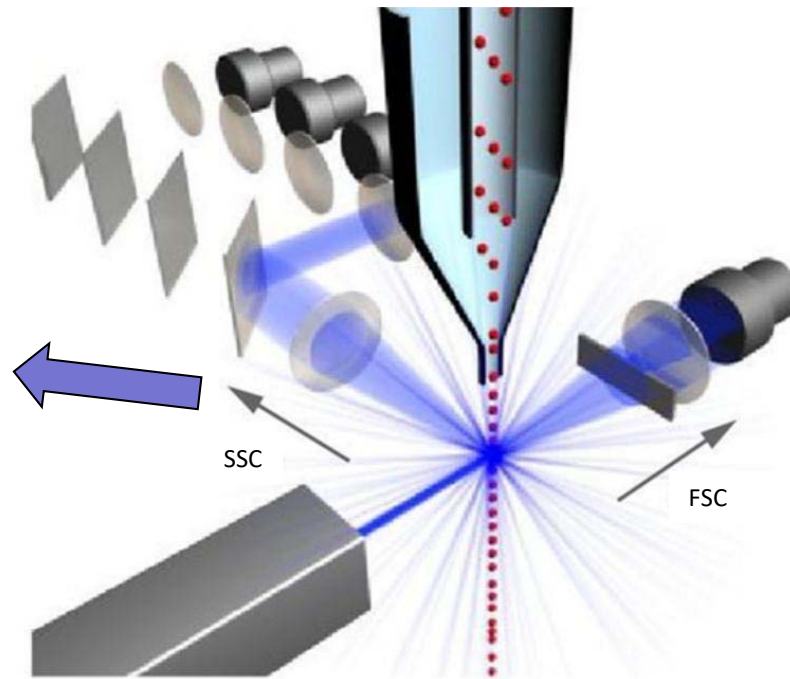
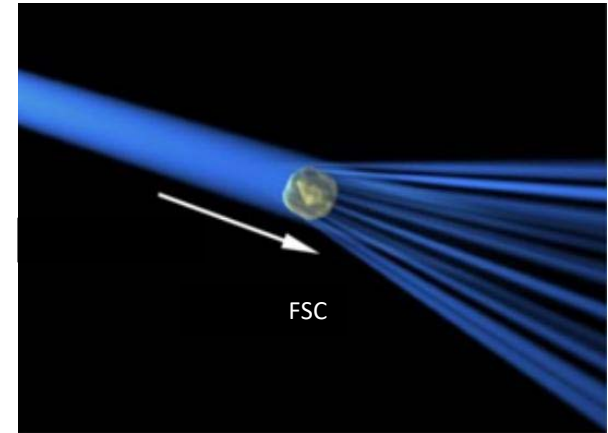
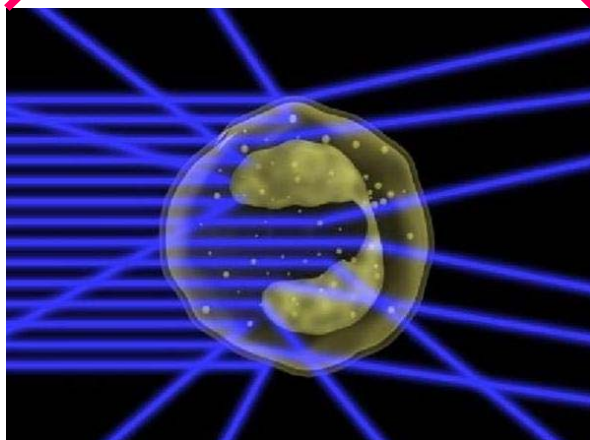
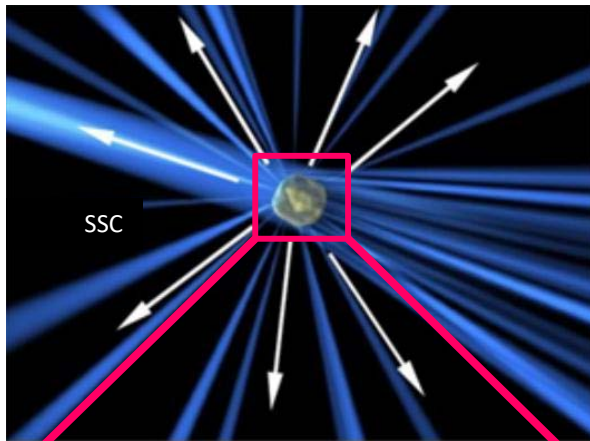


Light scatter signals

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

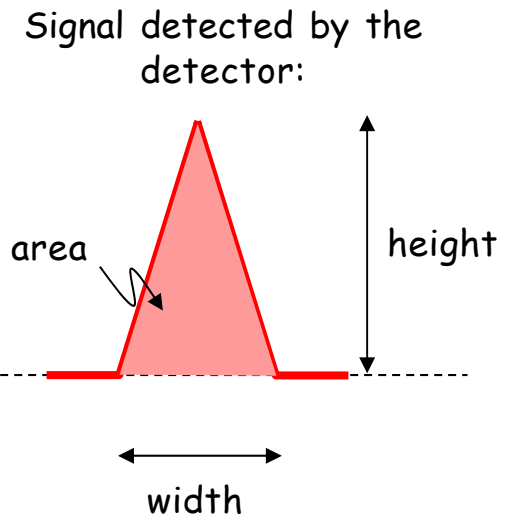
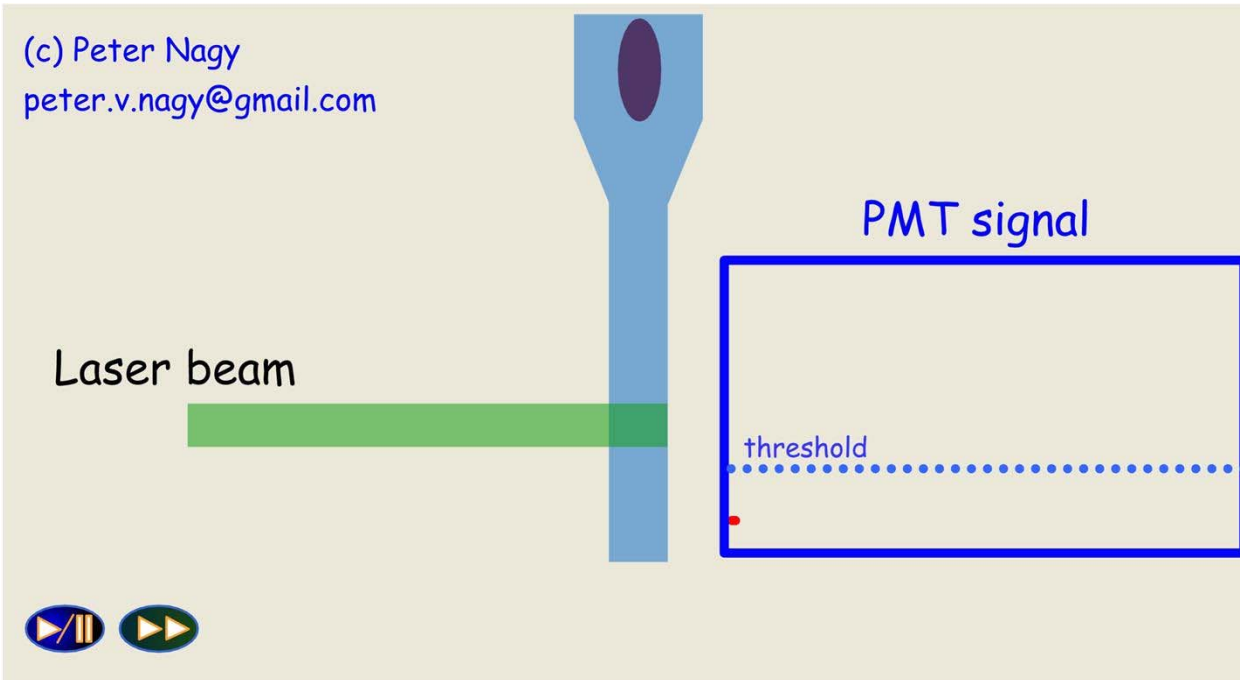
SSC signal: proportional to the internal granularity of cells



FSC signal: proportional to cell size

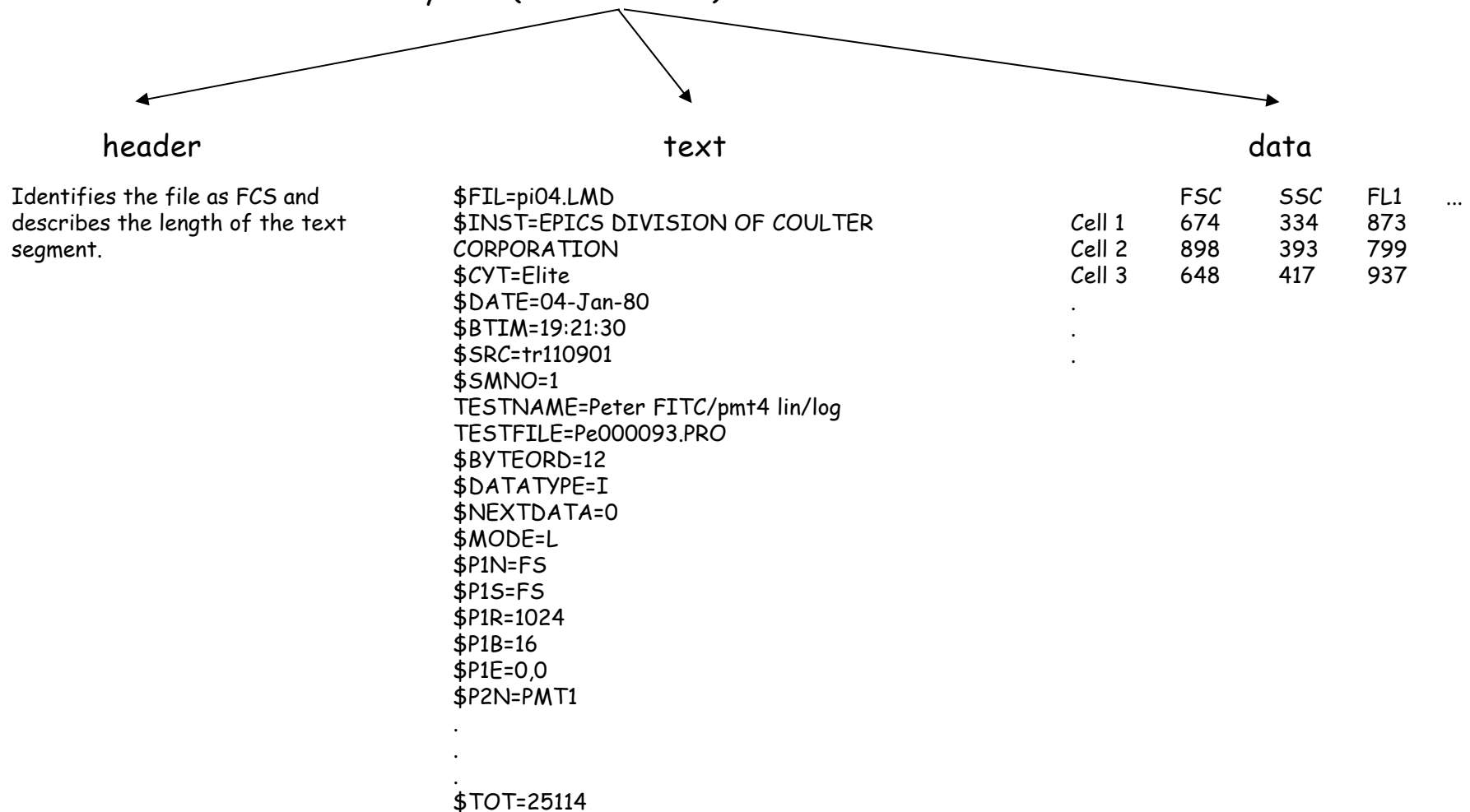
Detection of signals

(c) Peter Nagy
peter.v.nagy@gmail.com



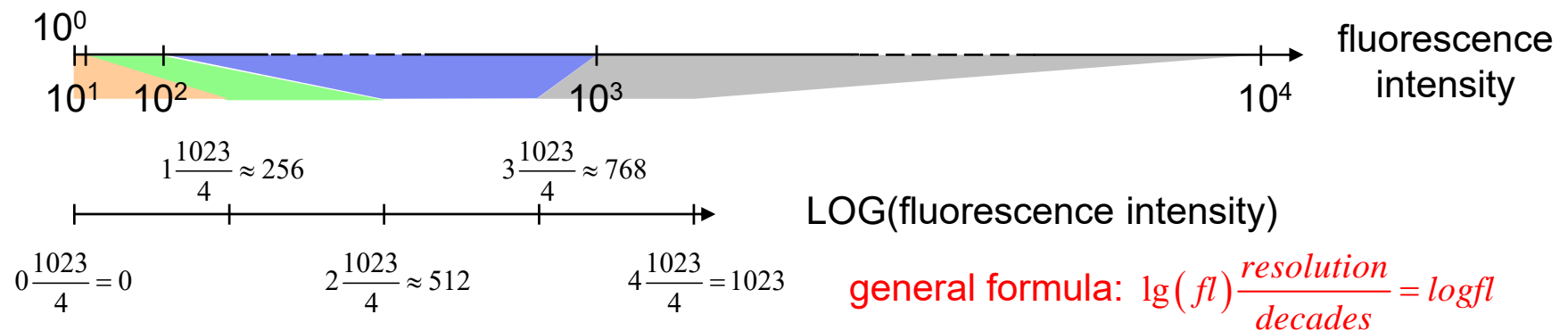
Data storage

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).



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



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

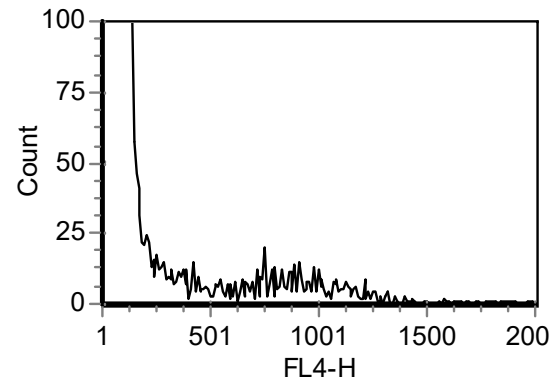
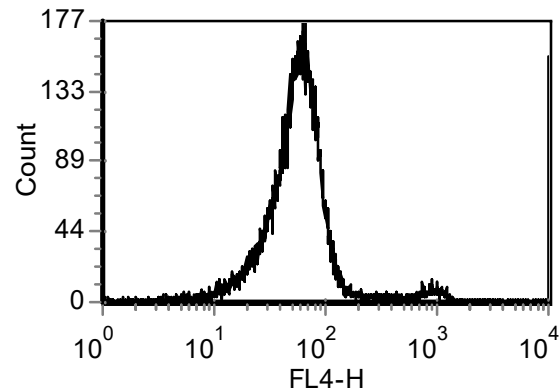
Display of data I.

One dimensional (one parameter) display: histogram

the same data on

logarithmic scale

linear scale

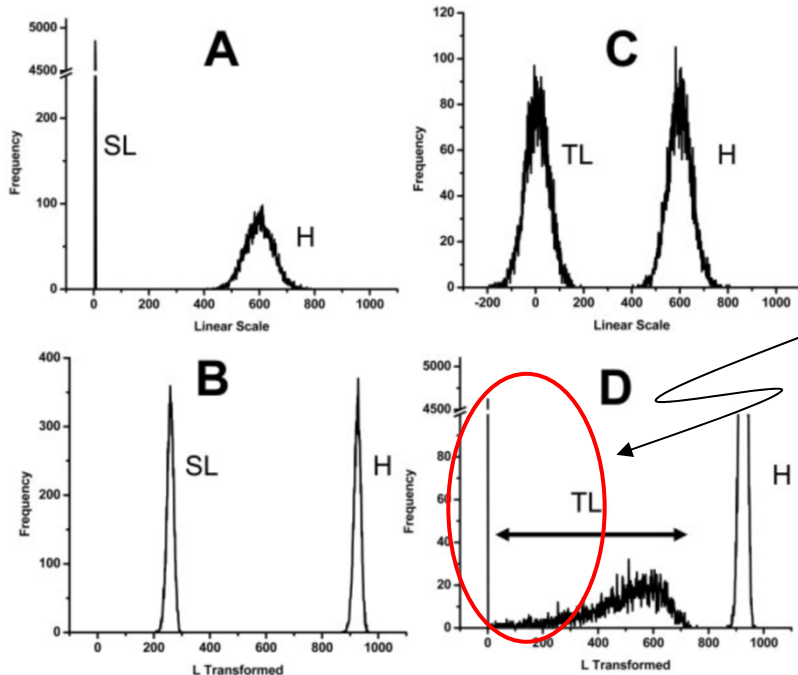


Advantages of logarithmic scale:

- 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.

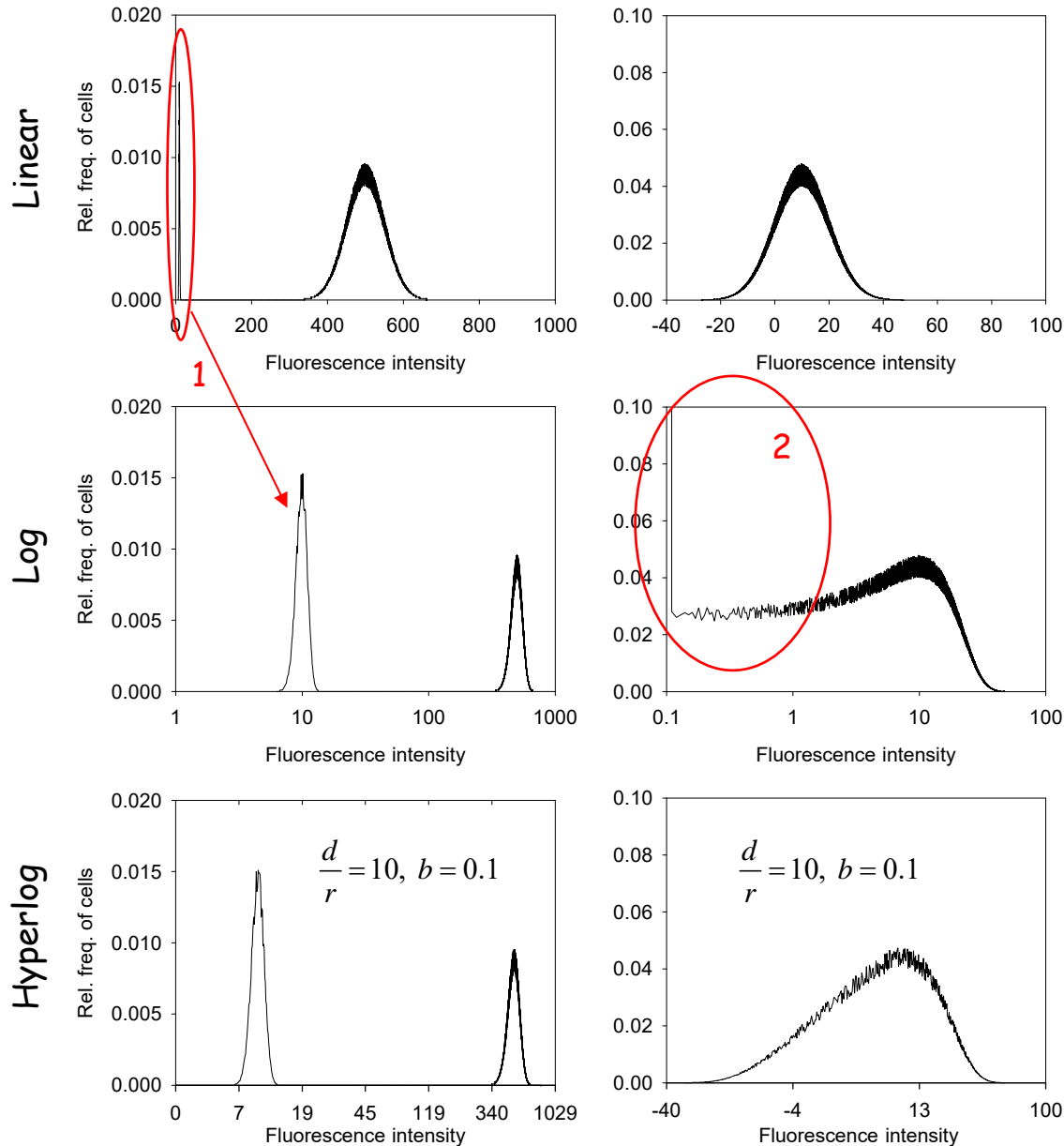
Disadvantages:

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



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

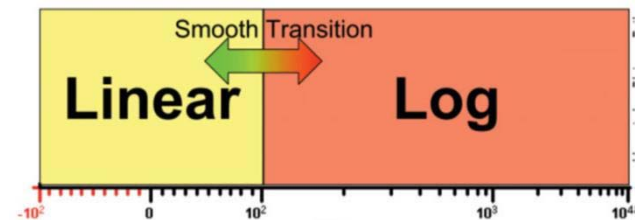
Scale types



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)



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

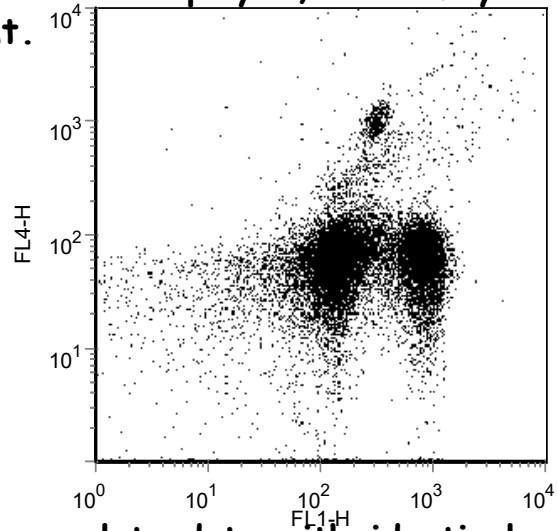
$$LIN = -10^{-\frac{d}{r}x_{HL}} + b \frac{d}{r} x_{HL} + 1, \text{ if } x_{HL} < 0$$

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

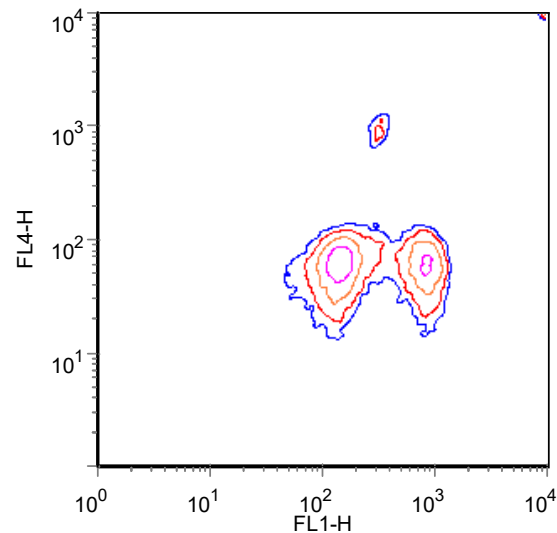
1-2. On the HL scale both populations are resolved, and there is no binning artifact.

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.

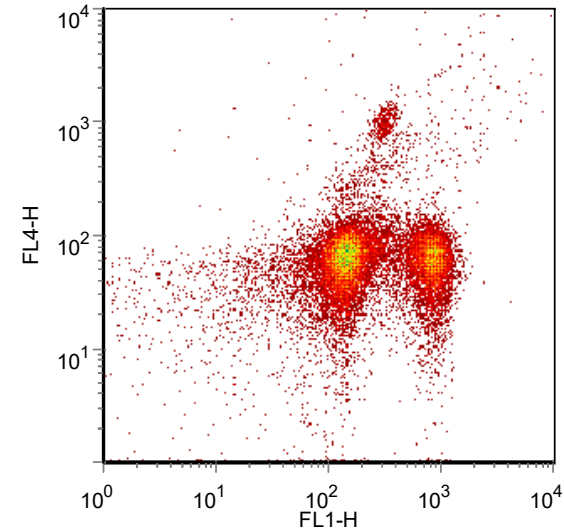


3. contour plot: dots with identical cell numbers are connected with lines

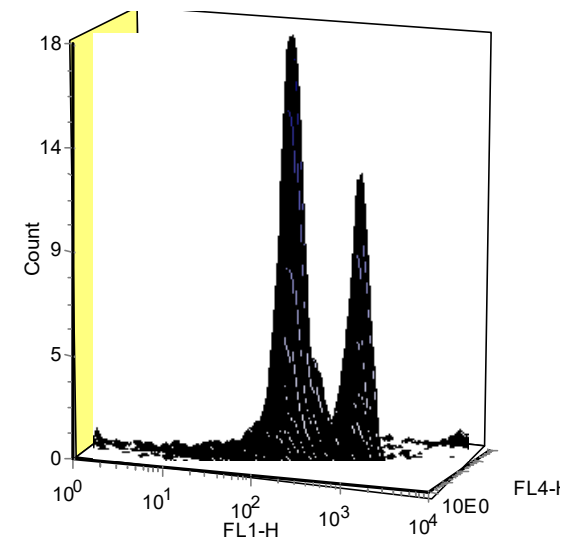


Display of data II.

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

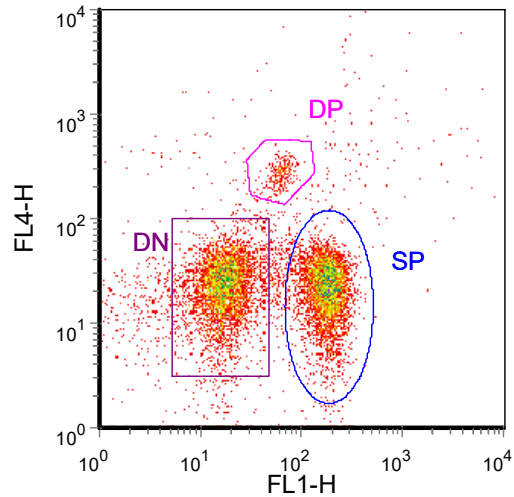


4. 3D (surface) plot: the number of cells is displayed on the z axis (rarely used)



Regions, quadrants and markers:

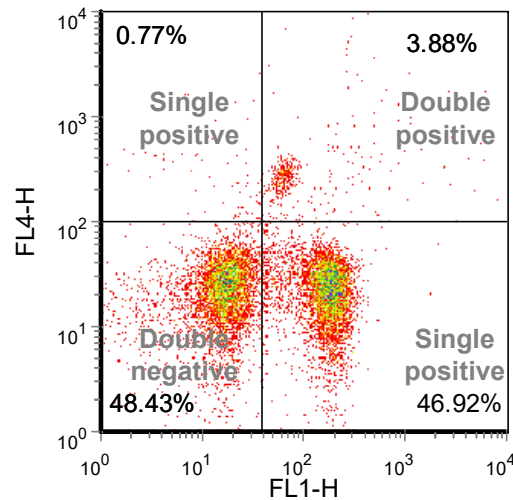
tools to identify subpopulations



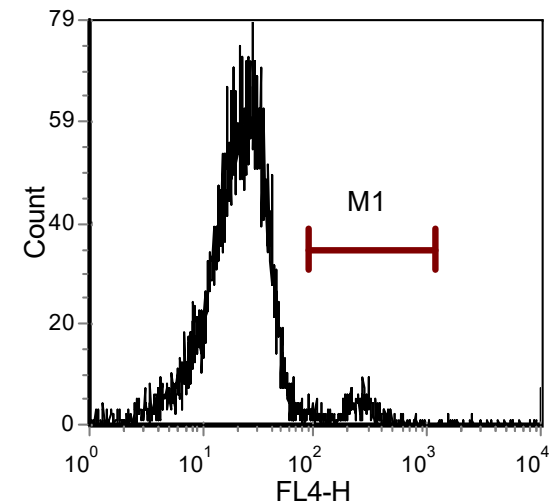
Region: a set of points selected by the user that specifies an area in a 2D graph.

Several regions can be defined in the same graph.

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

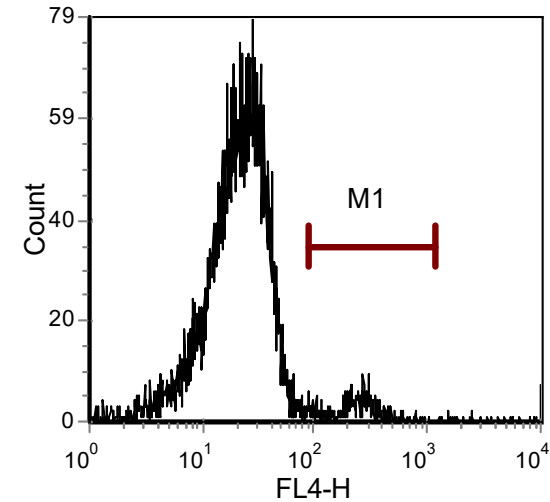
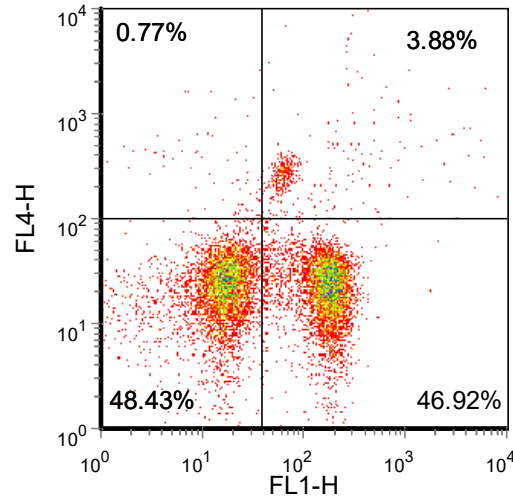
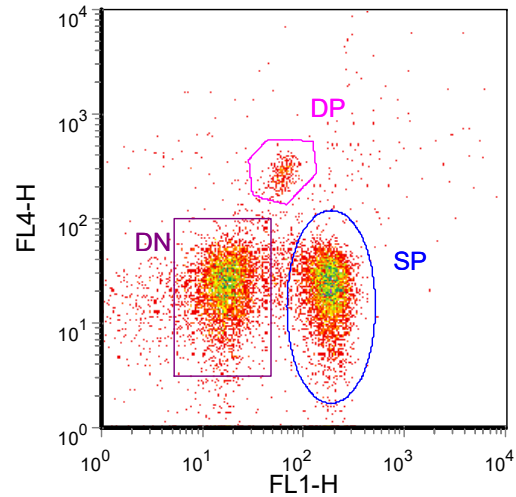


Quadrant: rectangular selection of 4 areas discriminating positive and negative cells on both axes of a 2D graph.



Marker: selection of cells in a histogram

What can we do with regions, quadrants and markers?



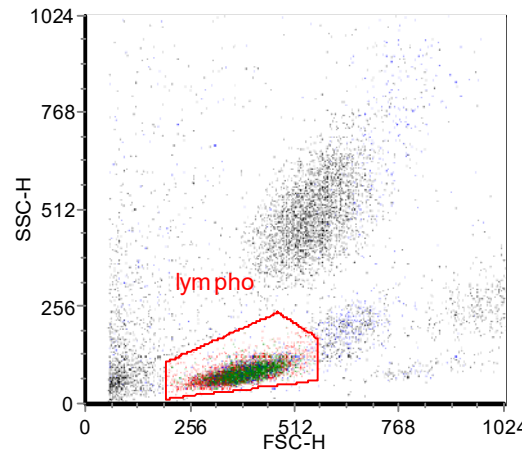
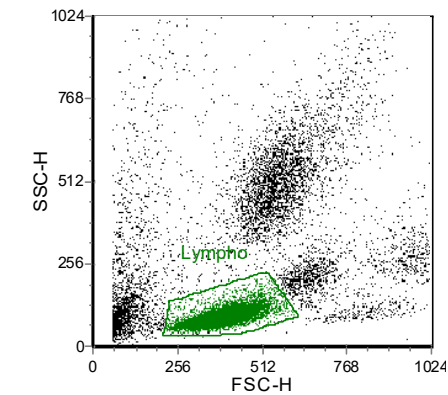
Gate	X Geometric Mean	Y Geometric Mean	% of All Cells
None	48.09	21.7	100.0
SP	171.29	19.51	42.91
DP	61.56	253.34	2.62
DN	16.86	21.08	45.19

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

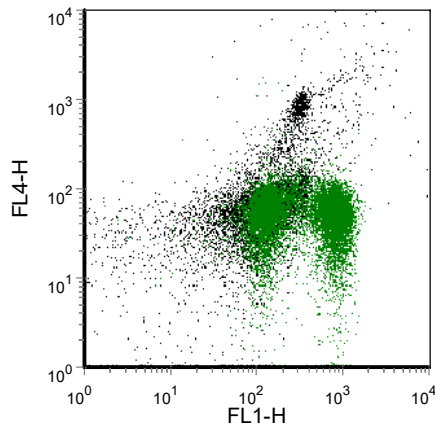
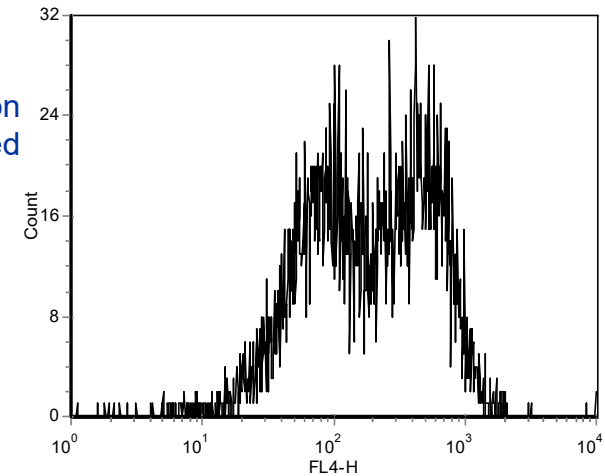
Marker	% of all cells	Geometric Mean	CV
None	100.0	21.7	677.57
M1	4.49	240.38	54.55

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

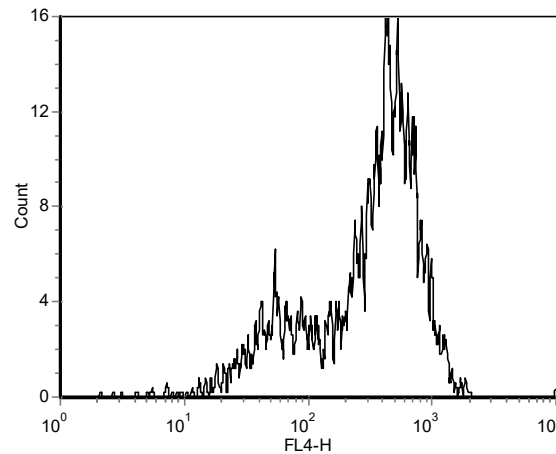
Gating



FL4 distribution of all measured cells



FL4 distribution of cells in the red (lymphocyte) gate



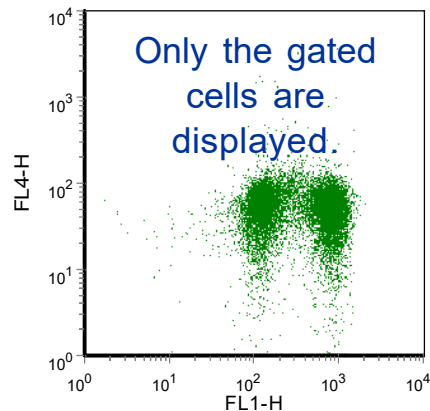
A **gate** can be defined as one or more regions/quadrants/markers combined using logical operators (AND, OR, NOT)



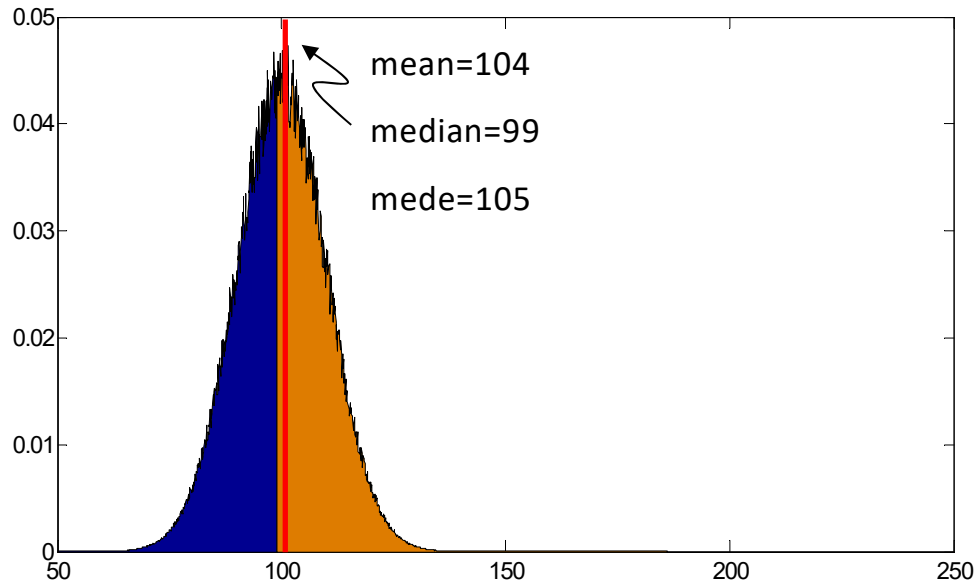
Defines a subset of the data to be displayed or analyzed.



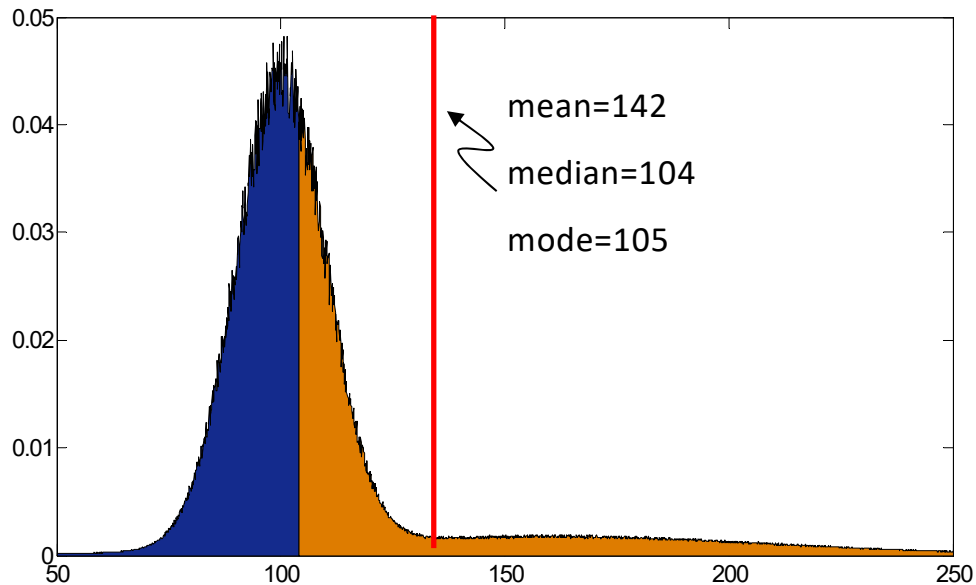
- Used to compute **statistics** and characterize the subset of events selected
- Get rid of unwanted events, e.g. cell debris



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

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

Coefficient of variation (CV):

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

describes the relative width of the distribution

Robust SD (rSD):

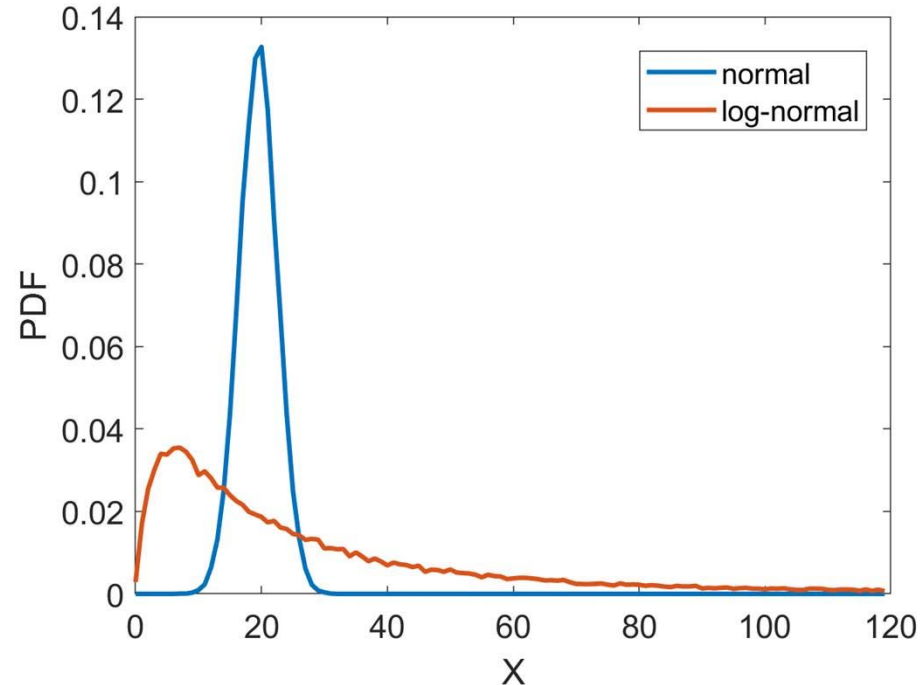
$$rSD = 1.4826 \left(median of \left\{ |X_i - median(X)| \right\} \right)$$

Robust CV (rCV):

$$rCV = 100 \cdot rSD / median(X)$$

Statistics

- The distribution of many biological parameters is log-normal
 - the logarithm of the parameter, and not the parameter itself, follows a normal distribution
 - distribution of the parameter is skewed toward large values



- | | |
|---|---|
| <ul style="list-style-type: none"> • Parameters of the normal distribution: <ul style="list-style-type: none"> • $\mu=20$ • $\sigma=3$ • Statistics of the normally-distributed, blue histogram <ul style="list-style-type: none"> • (arithmetic) mean: 19.99 • geometric mean: 19.76 | <ul style="list-style-type: none"> • Parameters of the log-normal distribution: <ul style="list-style-type: none"> • $\mu=20$ • $\sigma=3$ } Parameters of the normal distribution characterizing the distribution of the logarithm of X. • Statistics of the log-normally distributed, red histogram <ul style="list-style-type: none"> • (arithmetic) mean: 36.53 • geometric mean: 19.97 |
|---|---|

The geometric mean is often used due to the common occurrence of the log-normal distribution in biology.

Flow cytometers



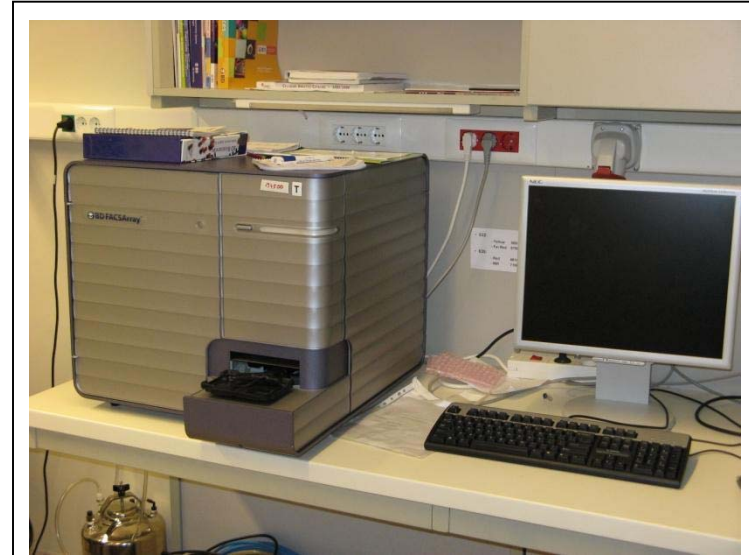
Coulter Epics Elite



Becton Dickinson FACS Vantage DiVa

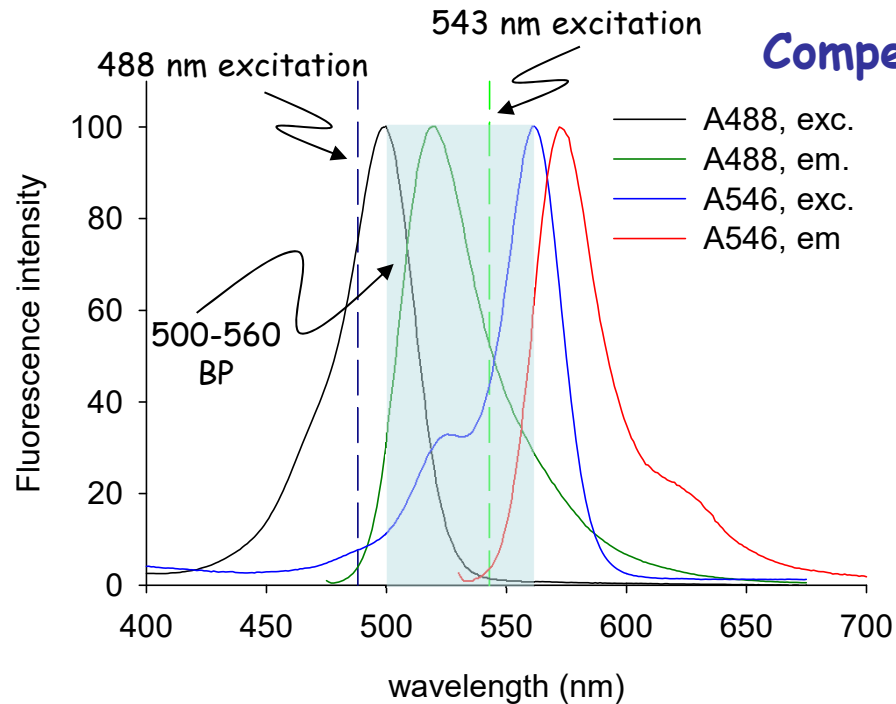


Becton Dickinson FACScan



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.

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

A488channel - measured fl. intensity in the A488 channel

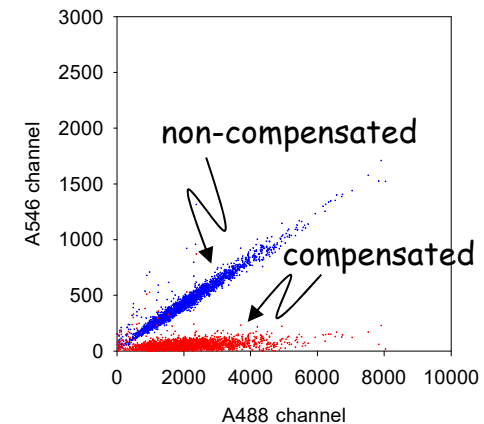
I_{A488}, I_{A546} - „pure“ fluorescence intensities of dyes A488 and A546

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

A similar equation holds for the A546 channel:

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

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.

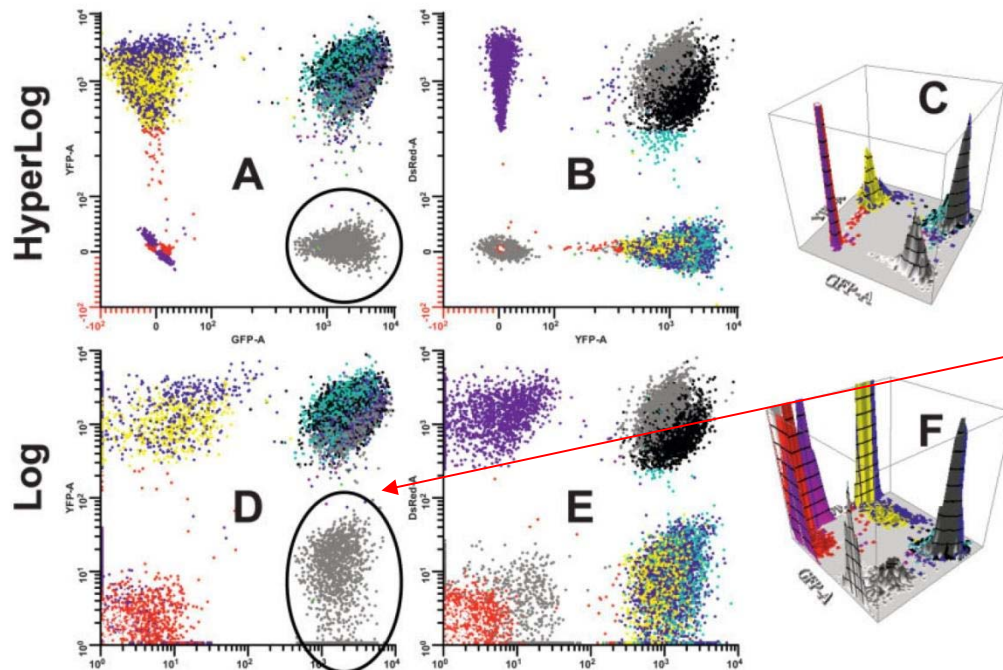
Simple case: one-way spillover, e.g. $S_{A546 \rightarrow A488} \neq 0$, $S_{A488 \rightarrow A546} = 0$

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

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

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

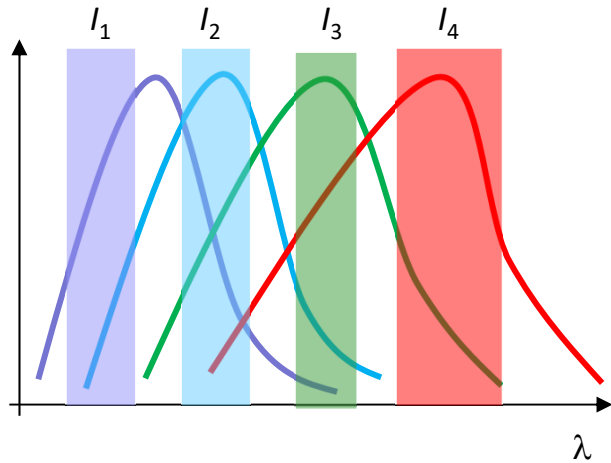
$$\left. \begin{aligned} A488channel &= I_{A488} + I_{A546} \times S_{A546 \rightarrow A488} \\ A546channel &= I_{A546} + I_{A488} \times S_{A488 \rightarrow A546} \end{aligned} \right\} \begin{aligned} I_{A488} &= \frac{A488channel - A546channel \times S_{A546 \rightarrow A488}}{1 - S_{A546 \rightarrow A488} \times S_{A488 \rightarrow A546}} \\ I_{A546} &= \frac{A546channel - A488channel \times S_{A488 \rightarrow A546}}{1 - S_{A546 \rightarrow A488} \times S_{A488 \rightarrow A546}} \end{aligned}$$



Logarithmic scale often shows a misleading picture after compensation:
it seems to be undercompensated.

Compensation vs. spectral unmixing

Conventional



F_1 – concentration of 1st fluorophore
 $S_{1,2}$ – spectral overspill of 1st fluorophore to the 2nd detector

$$I_1 = F_1 + F_2 S_{2,1} + F_3 S_{3,1} + F_4 S_{4,1}$$

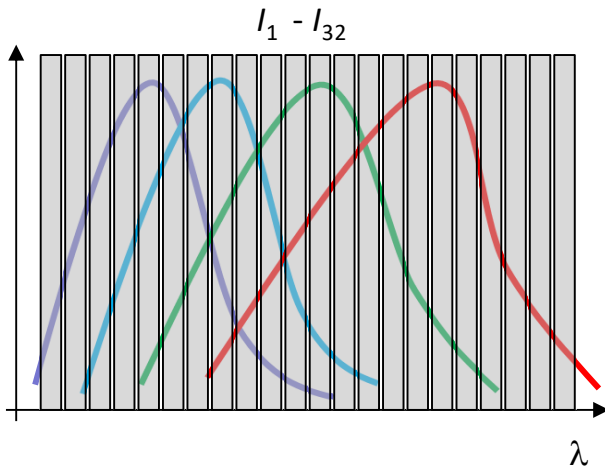
$$I_2 = F_1 S_{1,2} + F_2 + F_3 S_{3,2} + F_4 S_{4,2}$$

$$I_3 = F_1 S_{1,3} + F_2 S_{2,3} + F_3 + F_4 S_{4,3}$$

$$I_4 = F_1 S_{1,4} + F_2 S_{2,4} + F_3 S_{3,4} + F_4$$



- 4 unknowns (F_1 - F_4), 4 equations
- F_1 - F_4 can be calculated



$$I_1 = F_1 + F_2 S_{2,1} + \dots$$

$$I_2 = F_1 S_{1,2} + F_2 + \dots$$

...

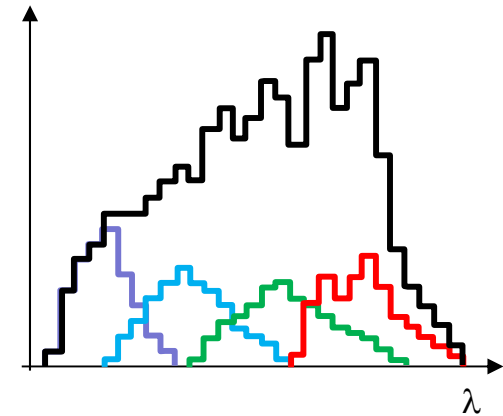
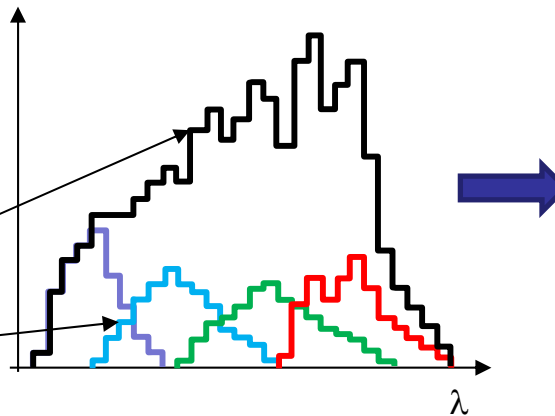
$$I_{32} = F_1 S_{1,32} + F_2 S_{2,32} + \dots$$



- 4 unknowns (F_1 - F_4), 32 equations
- system of equations is overdetermined
- but it can be „solved” by optimization

measured spectrum of sample

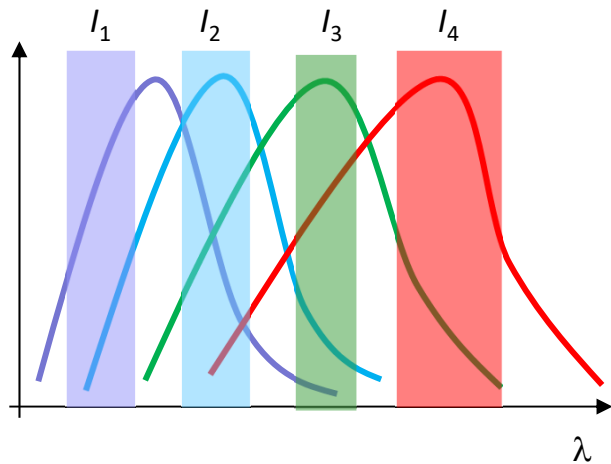
reference spectra of fluorophores



Spectral

Compensation vs. spectral unmixing

Conventional



F_1 – concentration of 1st fluorophore
 $S_{1,2}$ – spectral overspill of 1st fluorophore to the 2nd detector

$$I_1 = F_1 + F_2 S_{2,1} + F_3 S_{3,1} + F_4 S_{4,1}$$

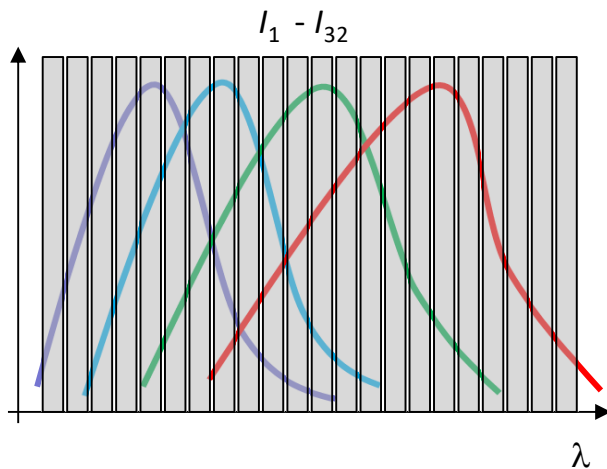
$$I_2 = F_1 S_{1,2} + F_2 + F_3 S_{3,2} + F_4 S_{4,2}$$

$$I_3 = F_1 S_{1,3} + F_2 S_{2,3} + F_3 + F_4 S_{4,3}$$

$$I_4 = F_1 S_{1,4} + F_2 S_{2,4} + F_3 S_{3,4} + F_4$$



- 4 unknowns (F_1 - F_4), 4 equations
- F_1 - F_4 can be calculated



$$I_1 = F_1 + F_2 S_{2,1} + \dots$$

$$I_2 = F_1 S_{1,2} + F_2 + \dots$$

...

$$I_{32} = F_1 S_{1,32} + F_2 S_{2,32} + \dots$$

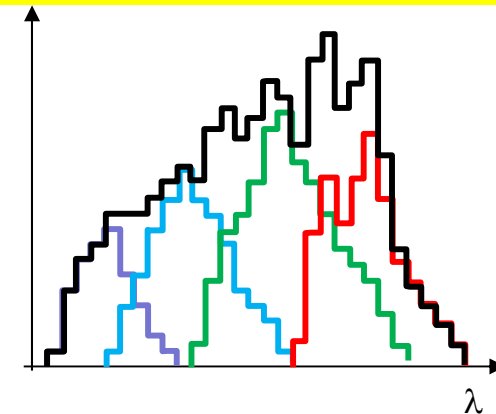
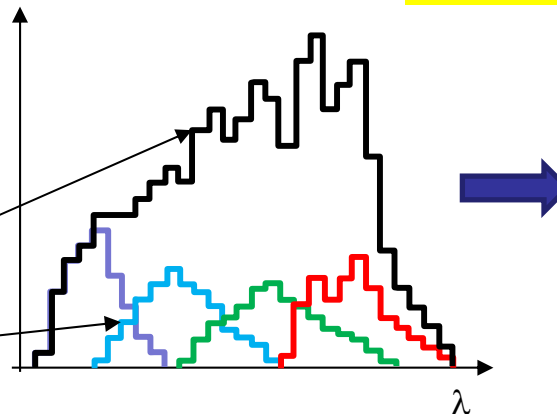


- 4 unknowns (F_1 - F_4), 32 equations
- system of equations is overdetermined
- but it can be „solved” by optimization

- Approximation of the measured spectrum with a combination of the reference spectra
- More photons detected → more accurate

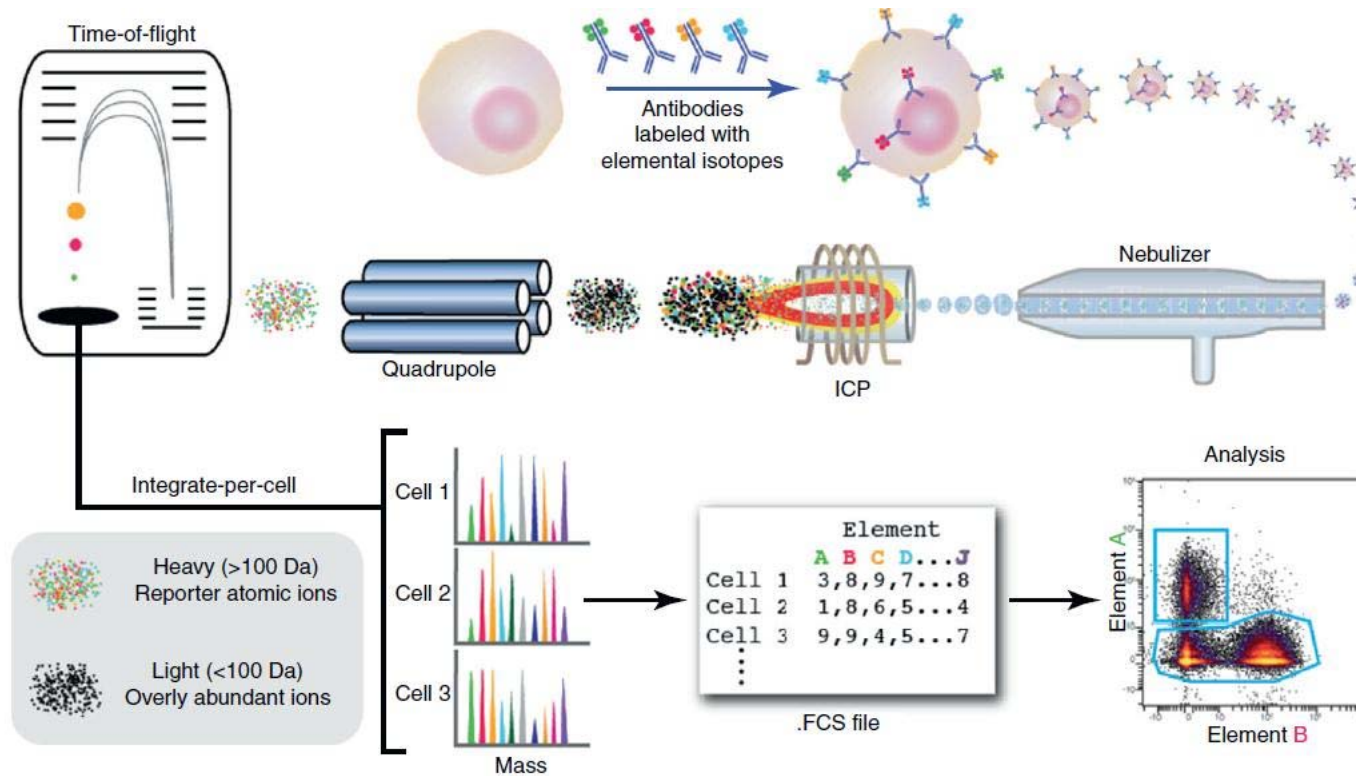
measured spectrum of sample

reference spectra of fluorophores



Spectral

Mass cytometry

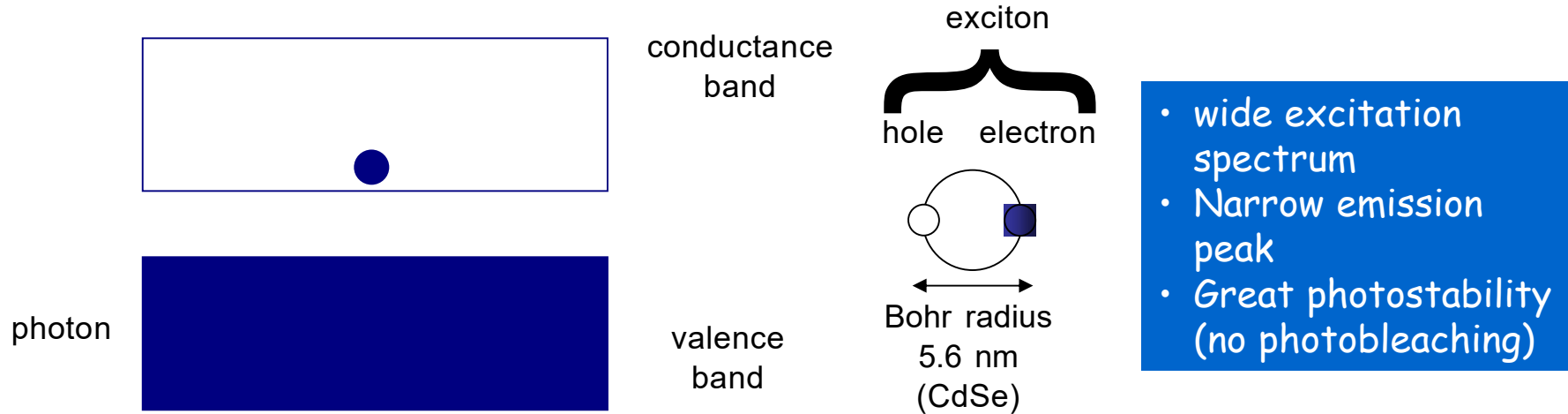


- The number of fluorophores selectively detectable at the same time is limited due to the problem of spectral overlap.
- Antibodies can be labeled with stable metal isotopes, typically lanthanides.
- Cells are ionized in an ICP (inductively coupled plasma) followed by TOF-MS (time-of-flight mass spectrometry)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

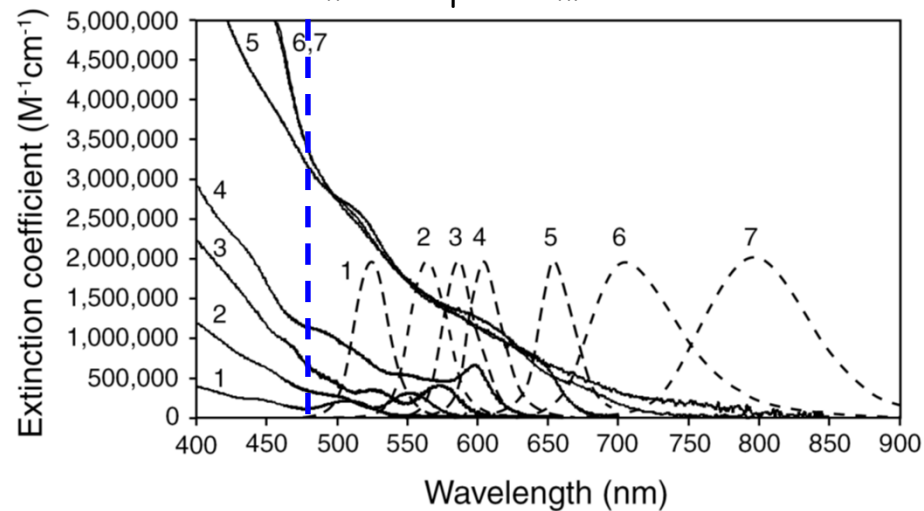
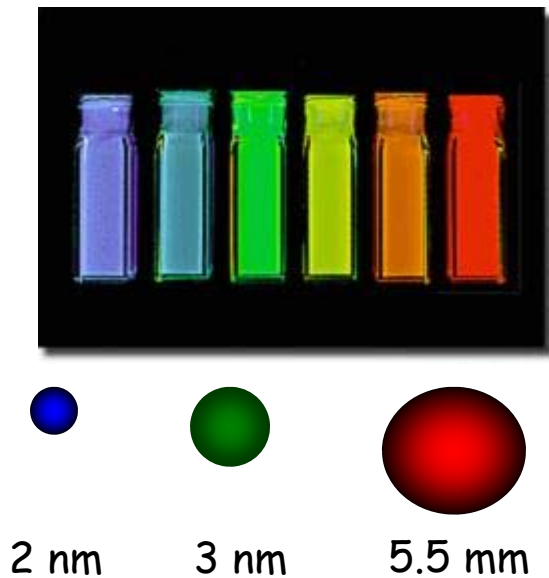
- Bendall, S. C., G. P. Nolan, M. Roederer, and P. K. Chattopadhyay. 2012. A deep profiler's guide to cytometry. *Trends Immunol* 33:323-332.
- Bandura, D. R., V. I. Baranov, O. I. Ornatsky, A. Antonov, R. Kinach, X. Lou, S. Pavlov, S. Vorobiev, J. E. Dick, and S. D. Tanner. 2009. Mass cytometry: technique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



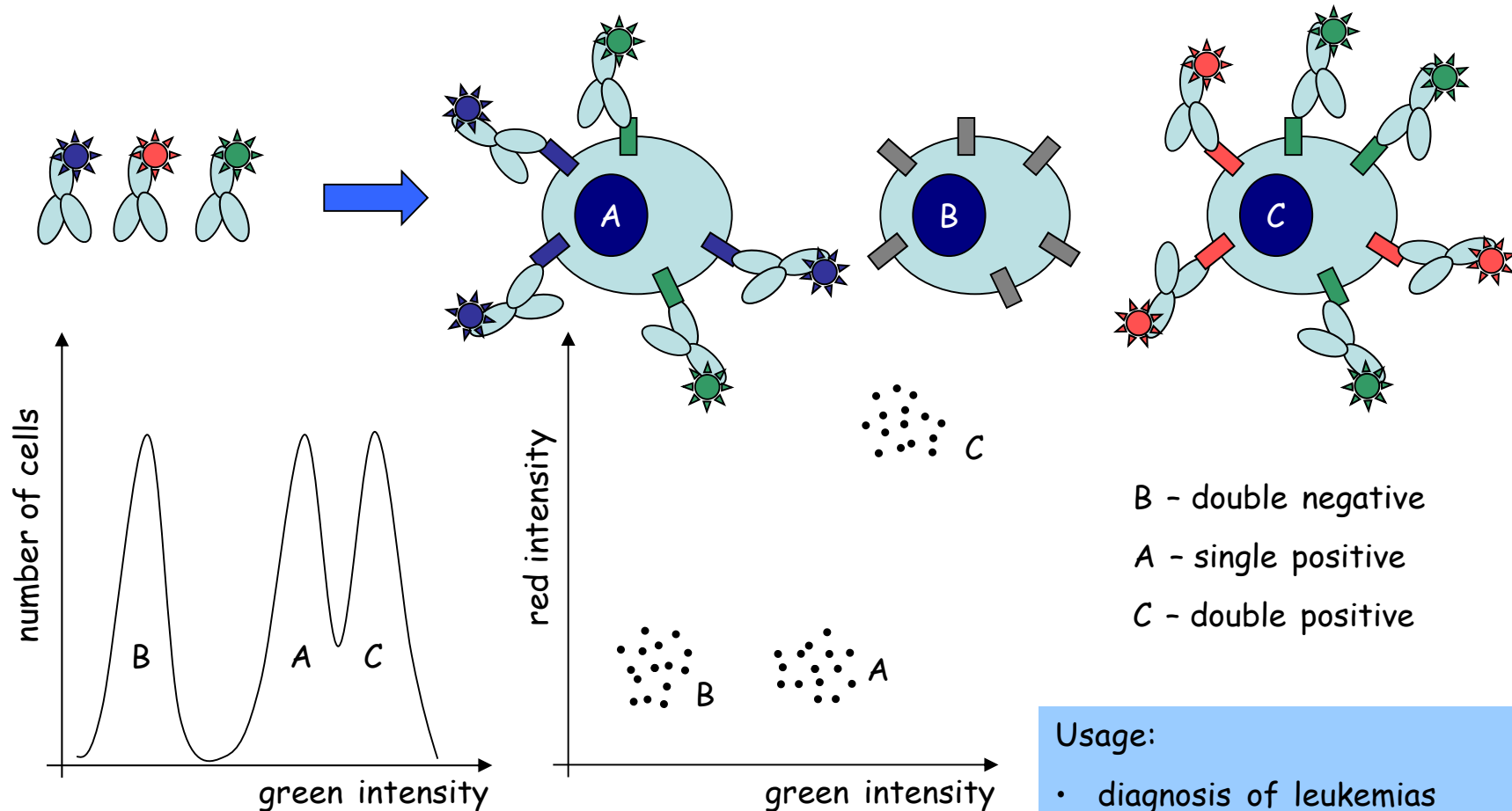
Due to the wide excitation spectrum a single laser (e.g. 488 nm) can excite several different quantum dots whose fluorescence can be detected without spectral overspill by different detectors as a result of the narrow emission spectrum.



1. Qdot[®] 525 conjugate
2. Qdot[®] 565 conjugate
3. Qdot[®] 585 conjugate
4. Qdot[®] 605 conjugate
5. Qdot[®] 655 conjugate
6. Qdot[®] 705 conjugate
7. Qdot[®] 800 conjugate

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.



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

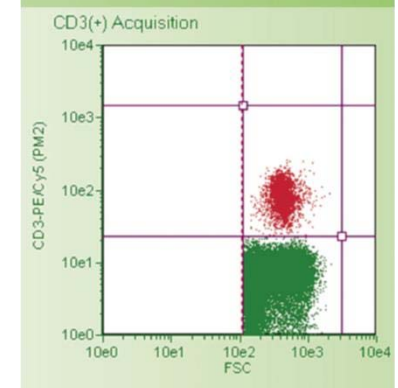
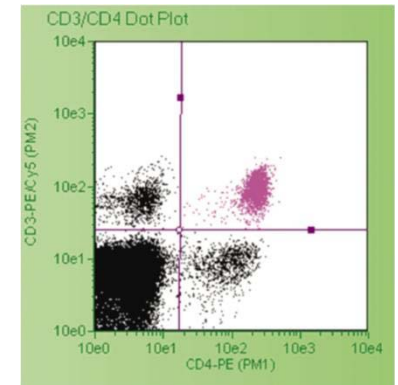
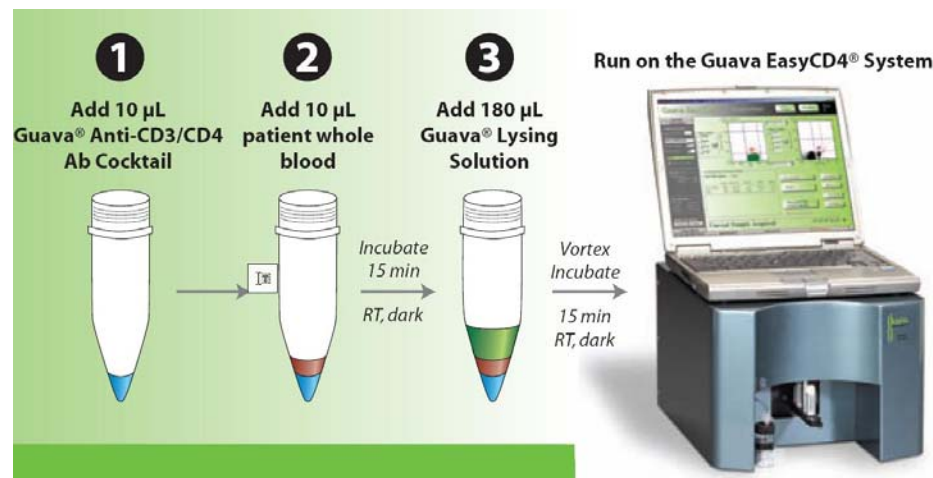
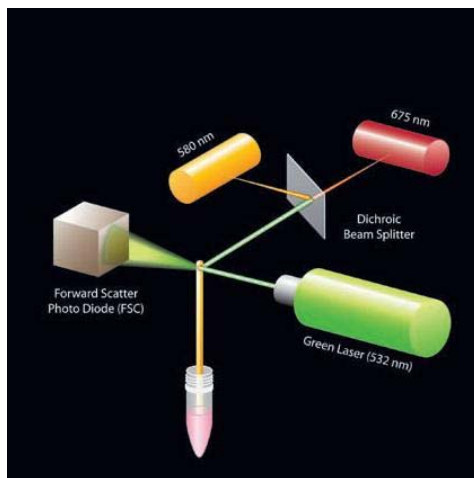
Solution:

- development of such a specialized, dedicated platform, which is simple, cheap and does not need service

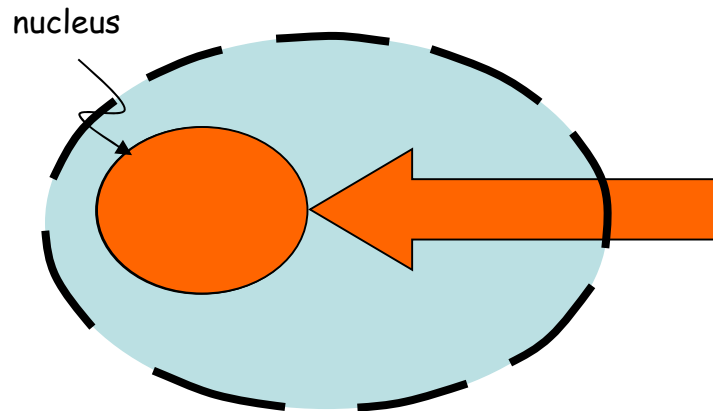
- such instruments can practically only determine the CD4 count, but cheaply

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



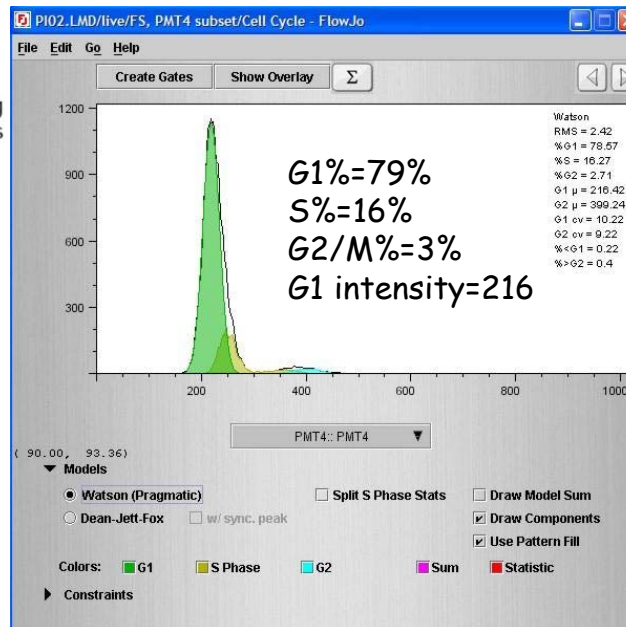
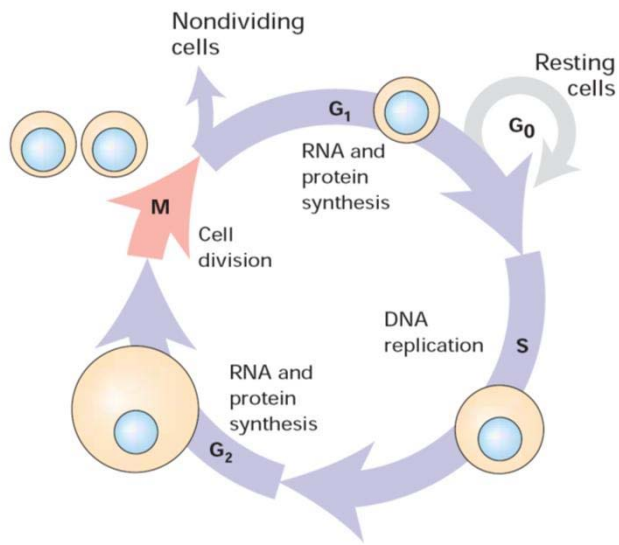
Cell cycle and DNA content analysis



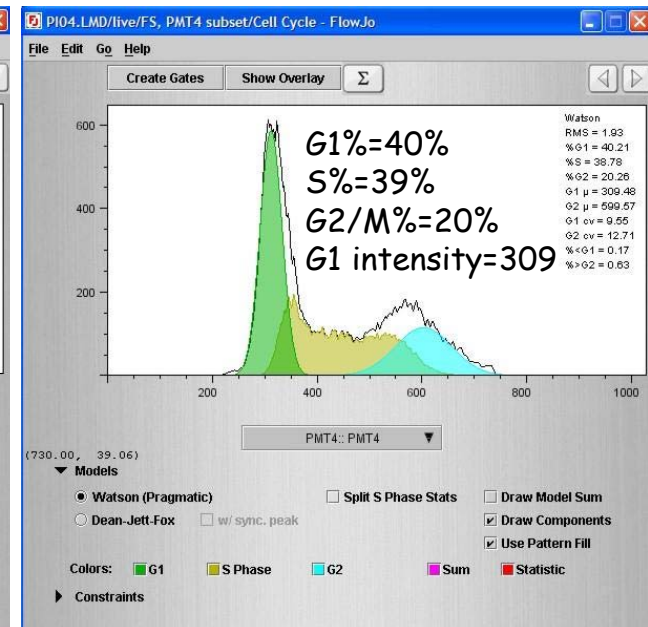
1. cells are fixed and permeabilized so that the DNA-specific dye can gain access to DNA
2. the DNA-specific dye (e.g. propidium iodide) gets to the nucleus and binds to DNA stoichiometrically
3. measured fluorescence intensity is proportional to the DNA content of the cells

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

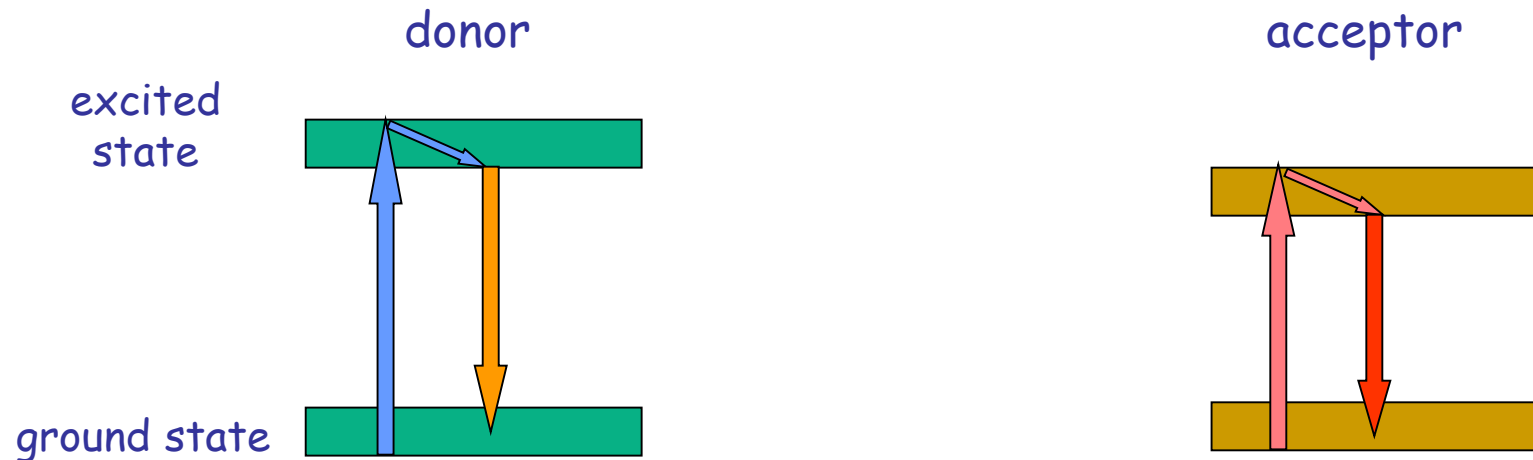


human diploid cell



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:

$$k_{FRET} = const \cdot J n^{-4} k_f R^{-6} \kappa^2$$

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

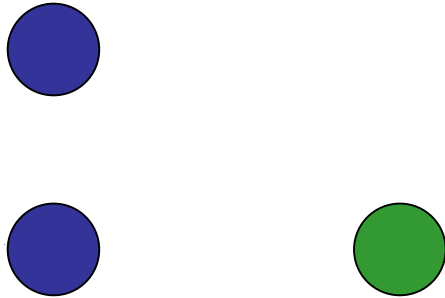
$$E = \frac{R_0^6}{R_0^6 + R^6}$$

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)

$$E = \left(\frac{F_{AD}}{F_A} - 1 \right) \frac{\epsilon_A C_A}{\epsilon_D C_D}$$

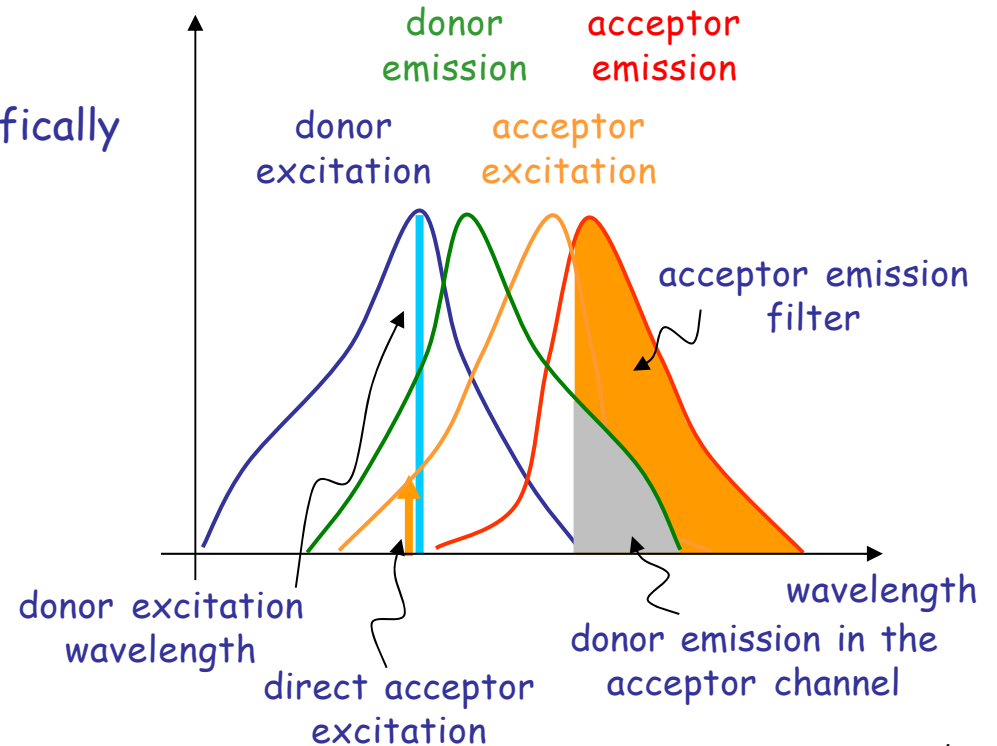


Problems:

- 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.

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

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

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

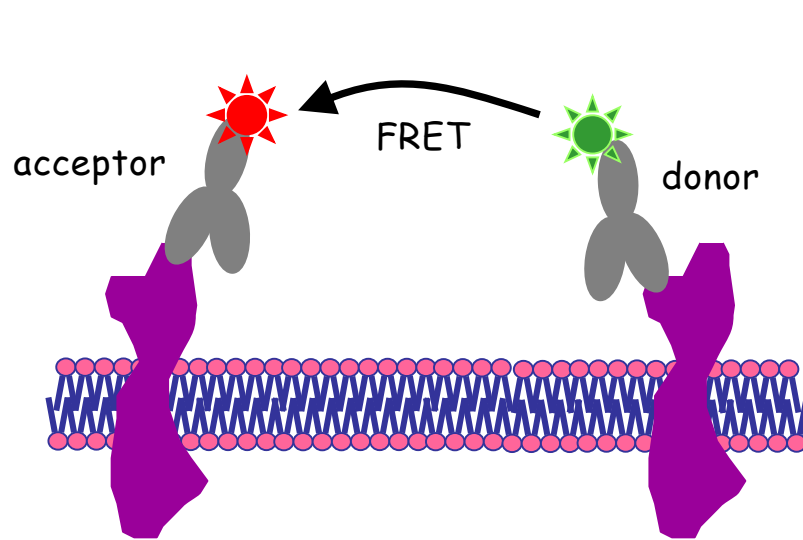
donor signal acceptor signal FRET signal

$$S_1 = \frac{I_{2,D}}{I_{1,D}} \quad S_3 = \frac{I_{3,D}}{I_{1,D}} \quad S_2 = \frac{I_{2,A}}{I_{3,A}} \quad S_4 = \frac{I_{1,A}}{I_{3,A}} \quad \alpha = \frac{I_2}{I_1} \frac{\epsilon_D L_D}{\epsilon_A L_A}$$

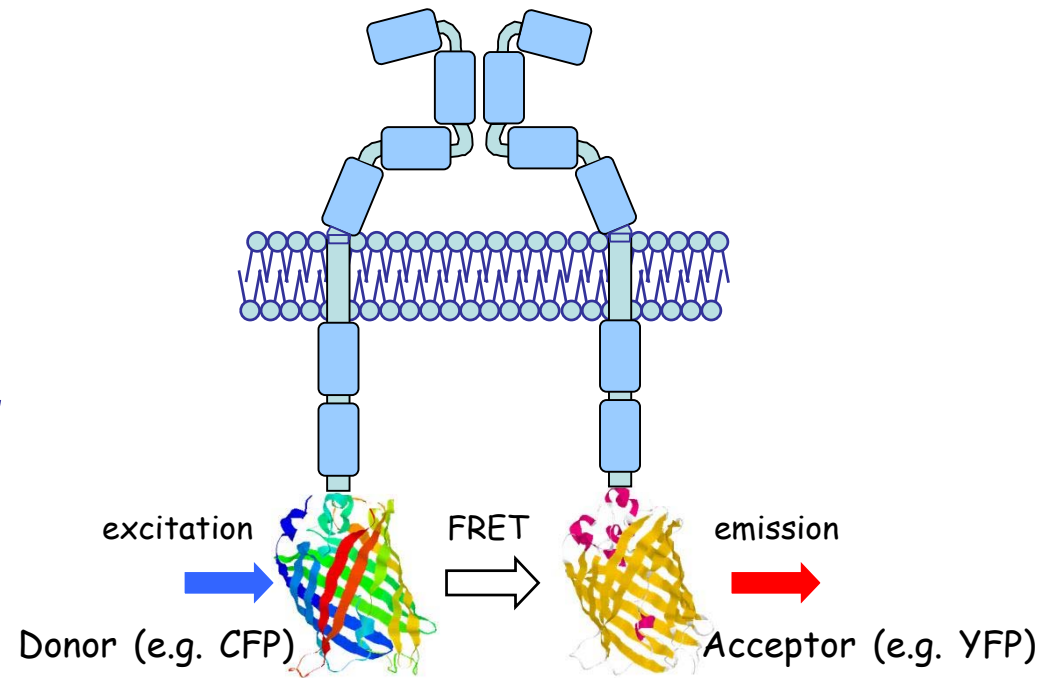
$$\frac{E}{1-E} = A = \frac{1}{\alpha} \left[\frac{I_1(S_1 - S_2 S_3) + I_2(S_3 S_4 - 1) + I_3(S_2 - S_1 S_4)}{I_1(S_2 S_3 / S_1 - 1) + I_2(S_4 / S_2 - S_3 S_4 / S_1)} \right]$$

Measurement of FRET by flow cytometry III.

Fluorescent labeling of cells



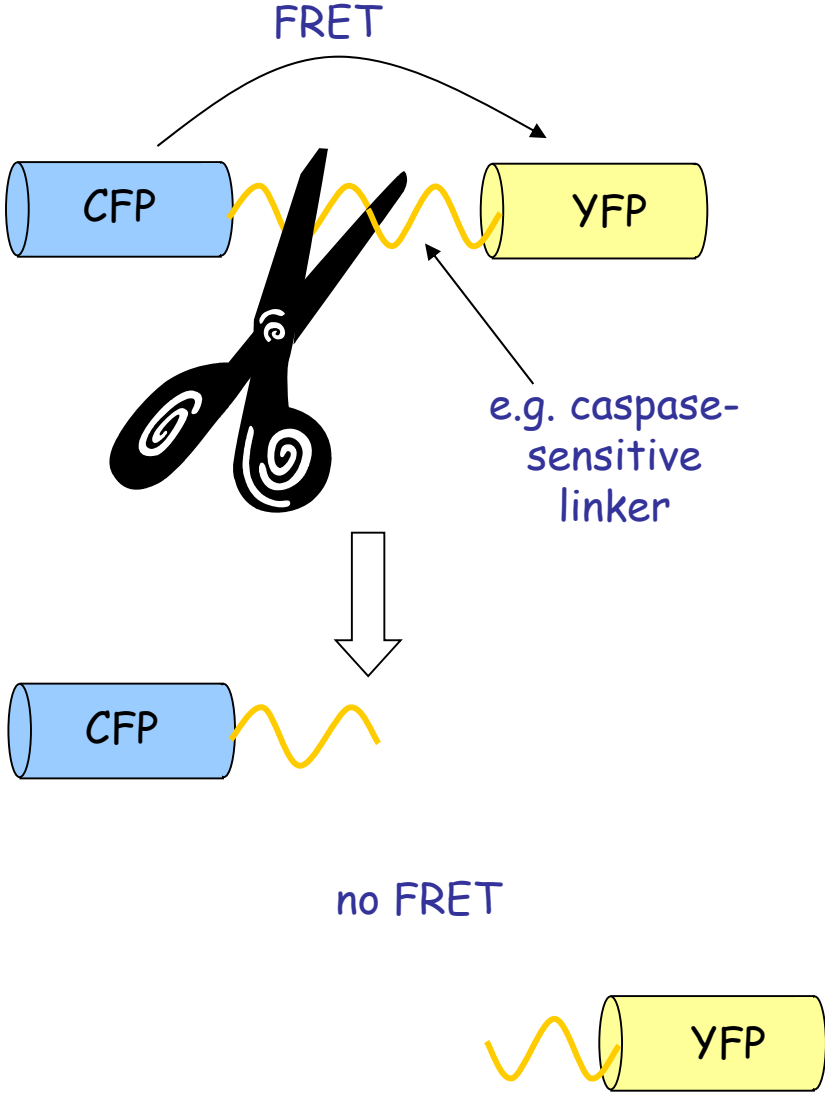
labeling by antibody or Fab



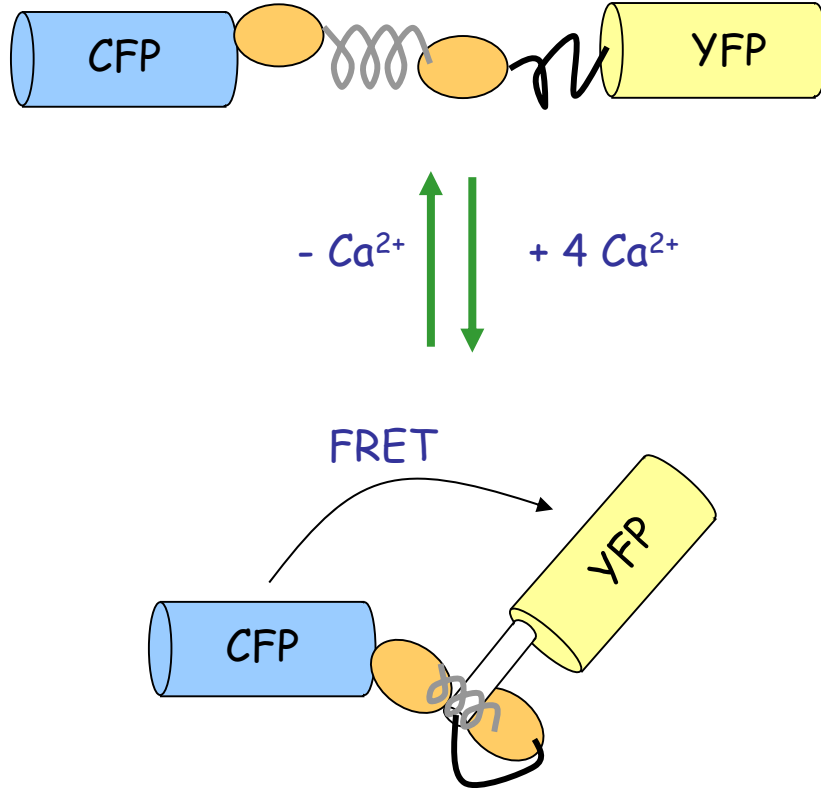
fusion protein labeled by
GFP or one of its spectral
variants

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.

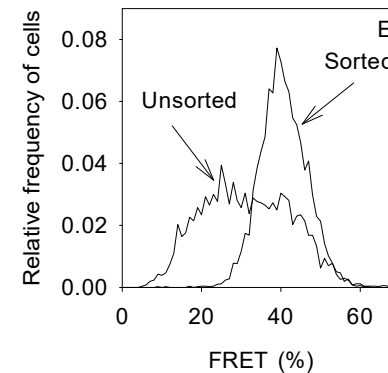
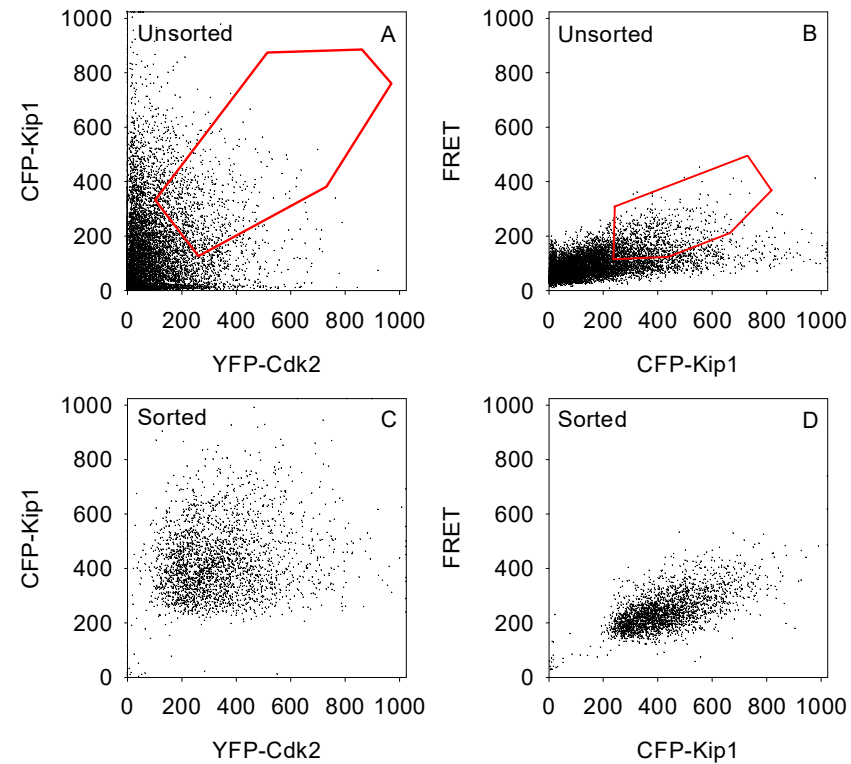
Yeast cells were transfected with YFP-CDK2-vel and CFP-Kip1.

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

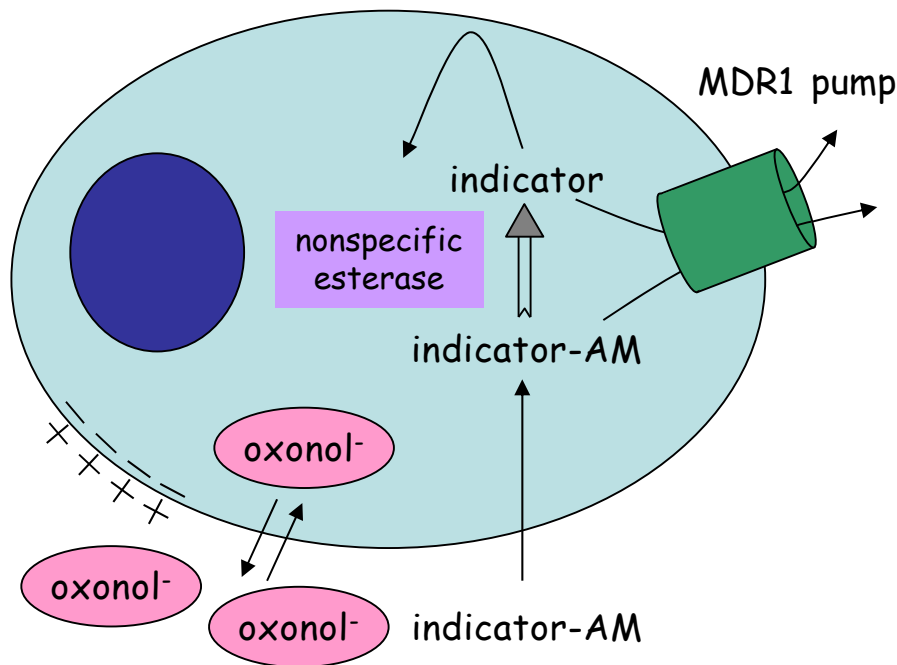
Cells were sorted according to gates displayed in A and B, and the sorting was checked. Those cell were sorted which showed

- 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

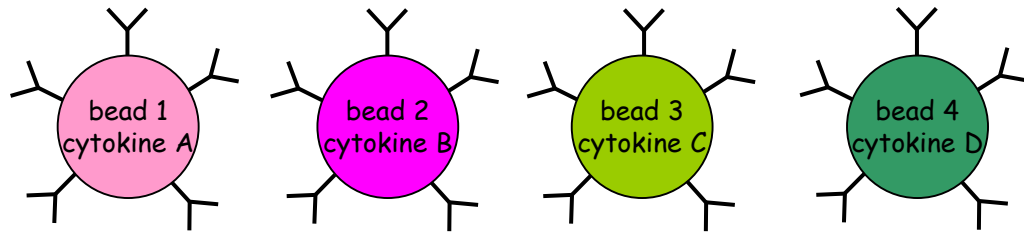


1. The hydrophobic, acetoxy methyl ester (AM) form of the indicator gets across the membrane.
2. It is hydrolyzed in the intracellular space by non-specific esterases.
3. The released hydrophilic indicator is not able to get across the membrane of living cells, so it is trapped intracellularly.
4. The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

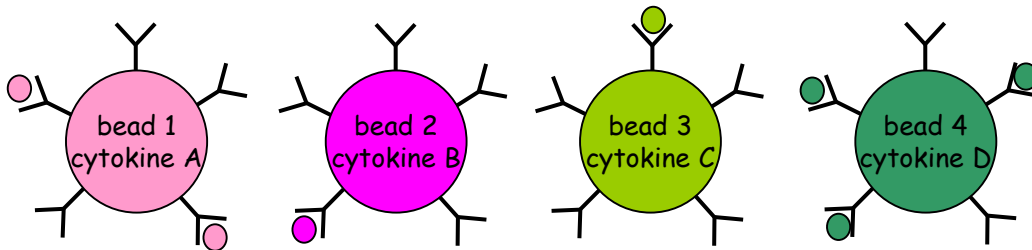
5. measurement of multidrug resistance: MDR proteins pump the indicator and/or its AM form from the intracellular space. Calcein is such in indicator, which does not indicate anything, i.e. its accumulation, or the lack thereof, is measured in cells showing MDR.
6. Negatively charged oxonol is distributed 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

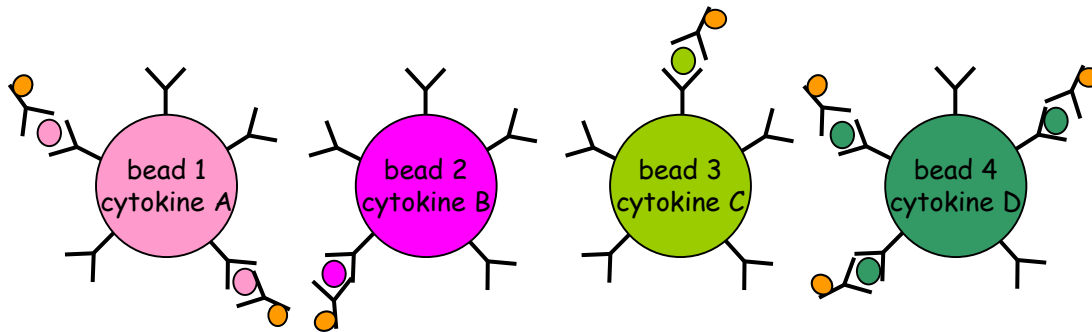
Multiplex bead analysis



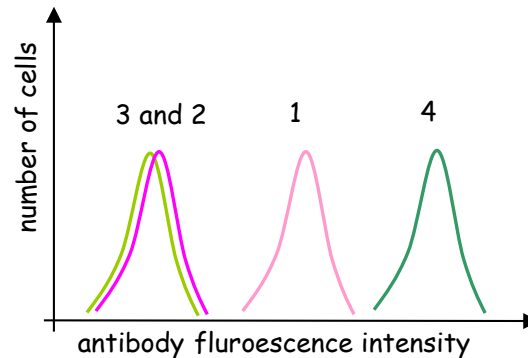
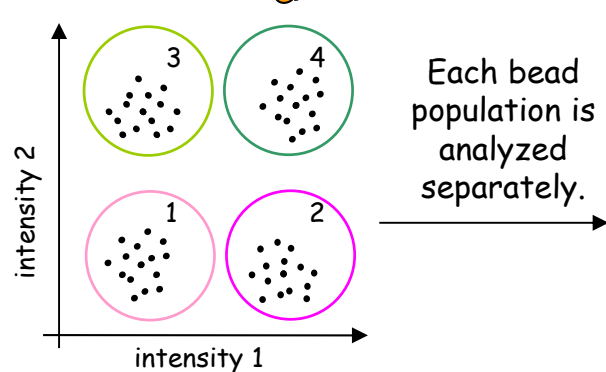
Beads with different colors. Different, cytokine-specific antibodies are bound to each of them



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



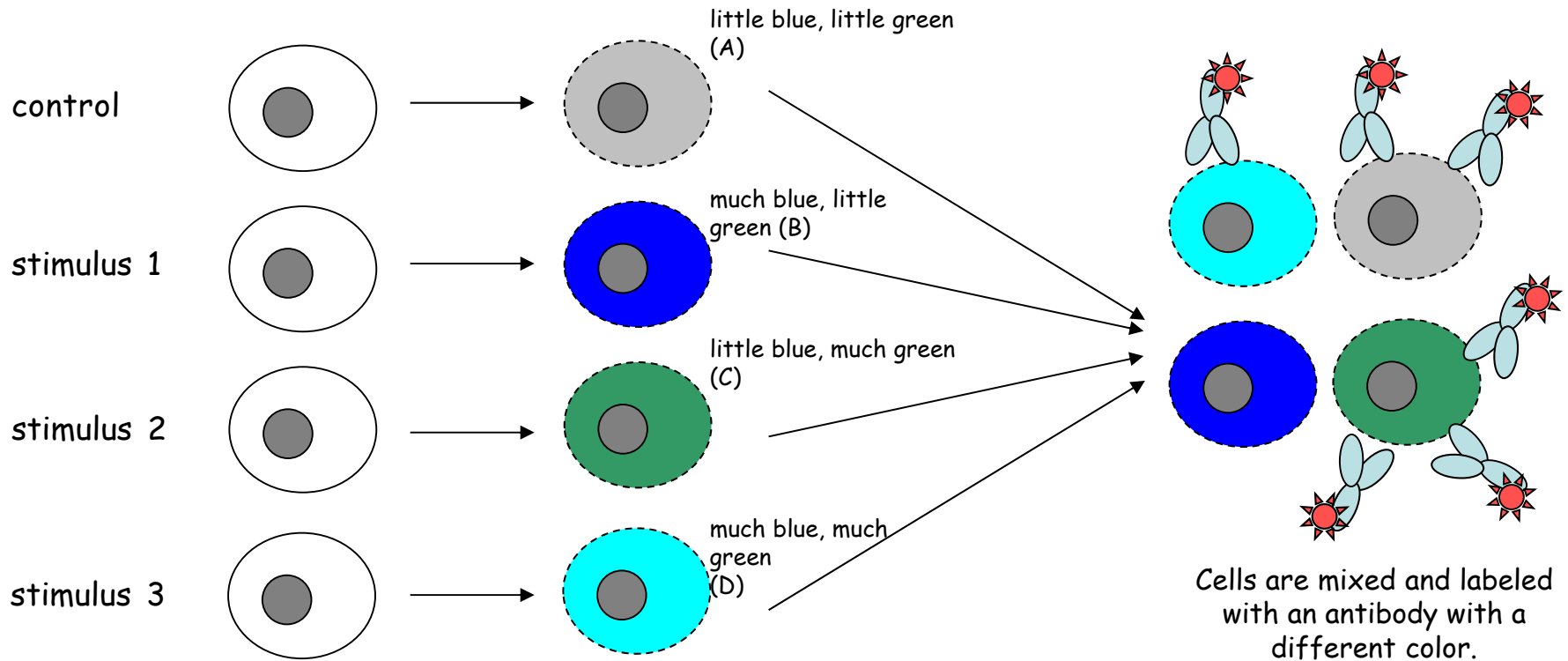
A cytokine specific, detecting antibodies labeled with a fluorescent dye with a color different from that of the bead is added to the beads. The antibody binds to and labels cytokines already bound to the beads.



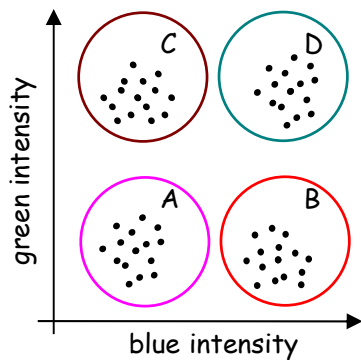
The beads can be run simultaneously on a flow cytometer.

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

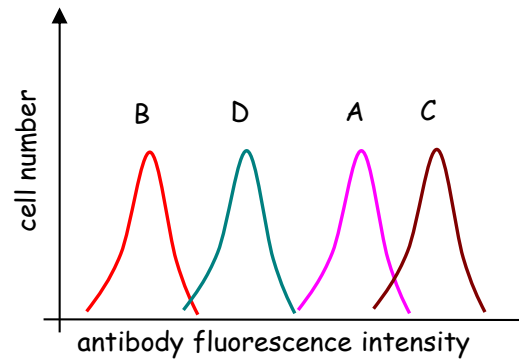
FCB: fluorescent cell barcoding *Nat. Methods, 3, 361*



Cells are fixed, permeabilized and labeled with fluorescent dyes with different color and concentration.



Each population is analyzed separately.

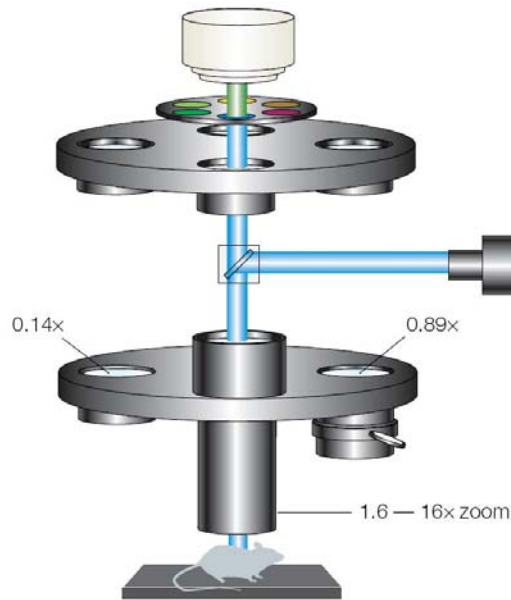


Advantage:

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

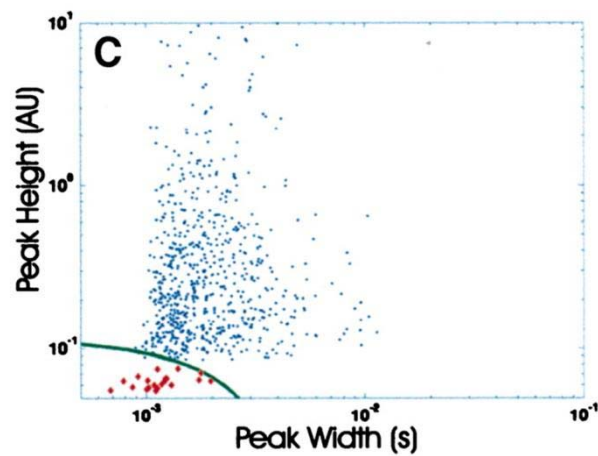
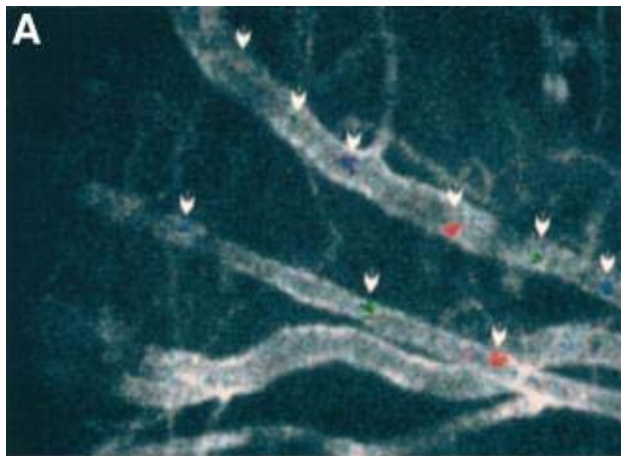
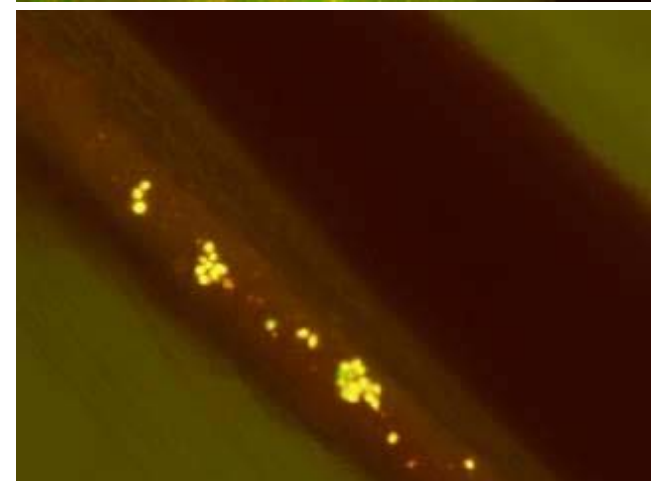
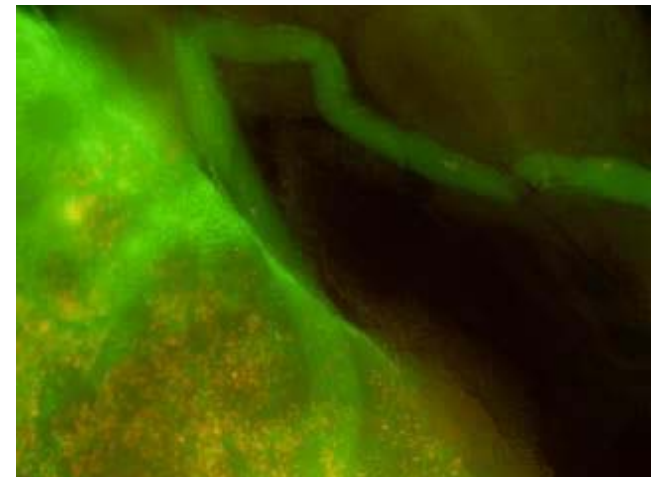
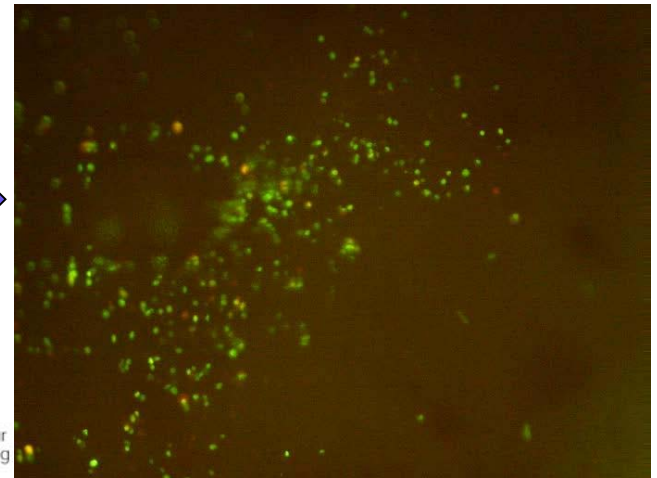
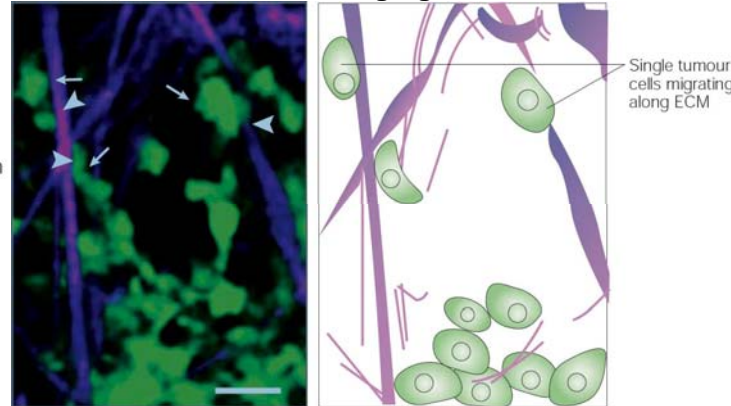
In vivo flow cytometry

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



Olympus OV100

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):

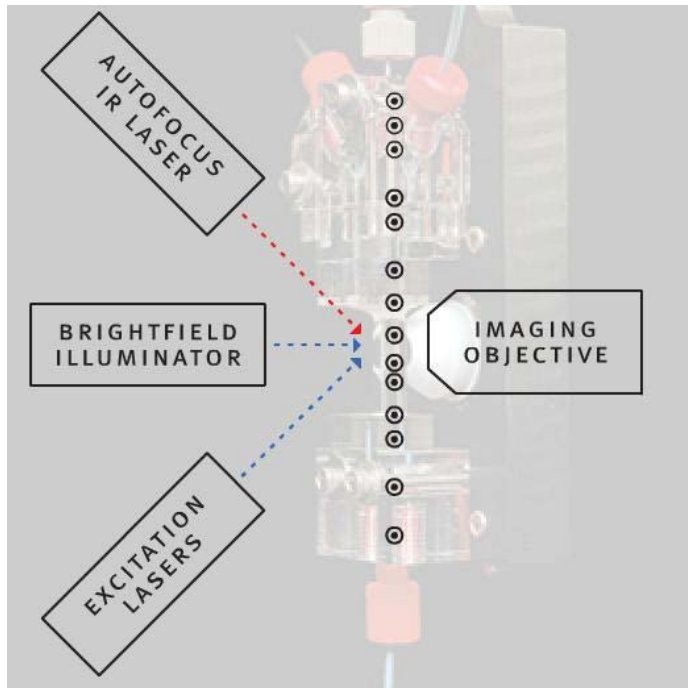
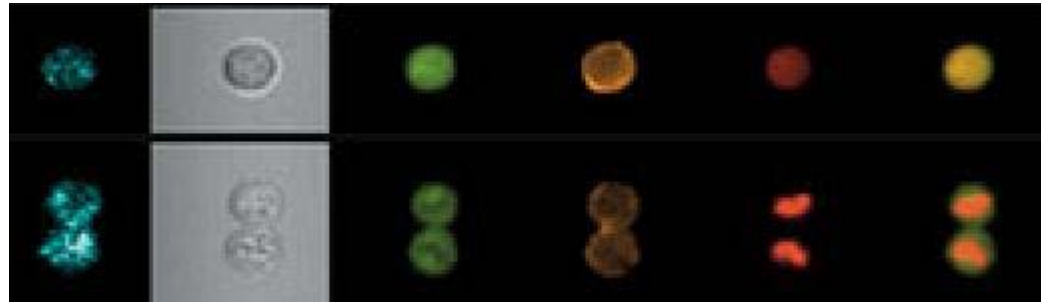
- investigates cells attached to a slide
- the whole slide (i.e. not only a microscopic field) is scanned with a low NA objective
- cells are identified automatically
- morphological and colocalization measurements can be done with the cells
- the fluorescence intensity of cells can be displayed in one-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



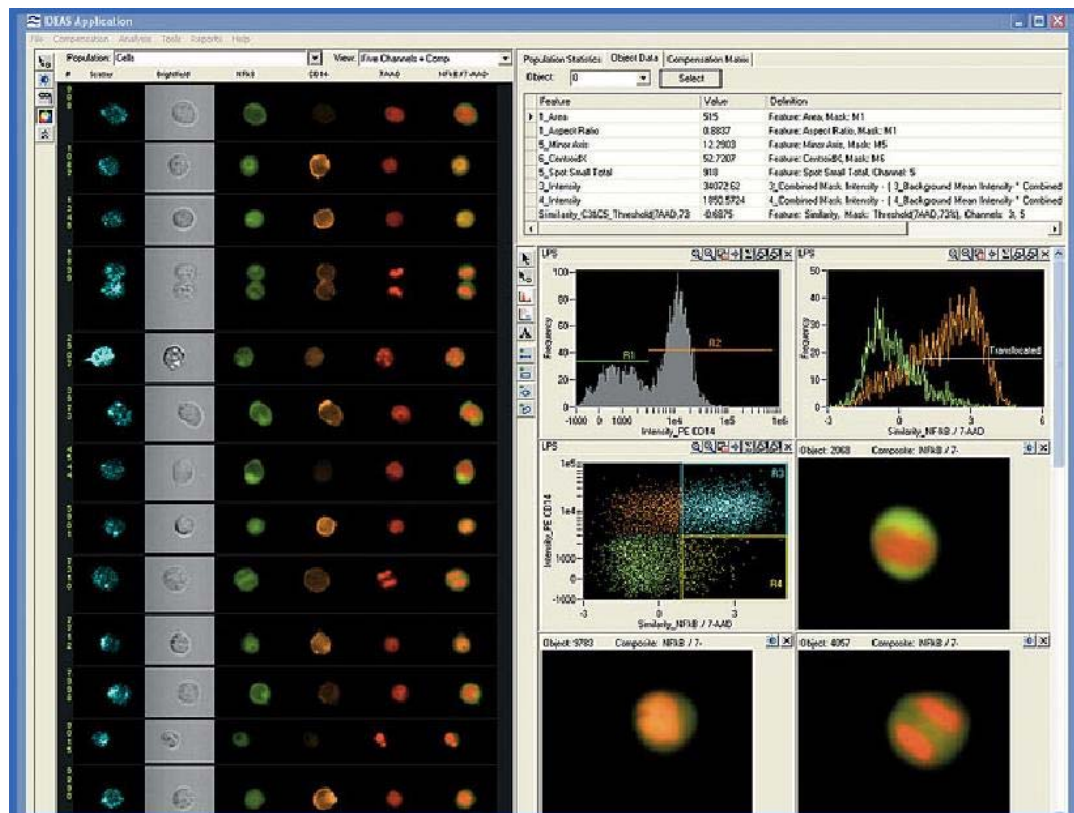
measurement of many cells automatically with subcellular resolution

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.

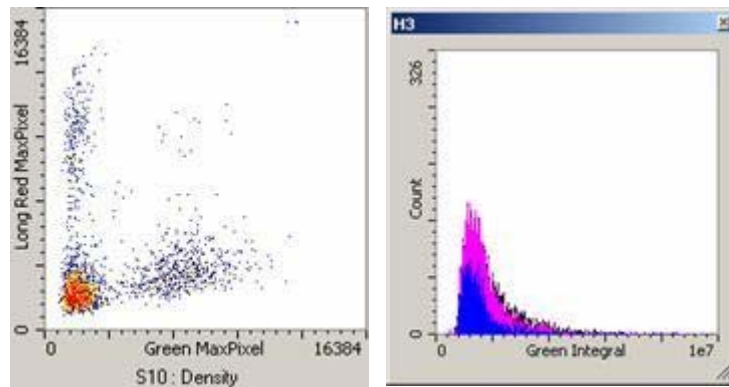
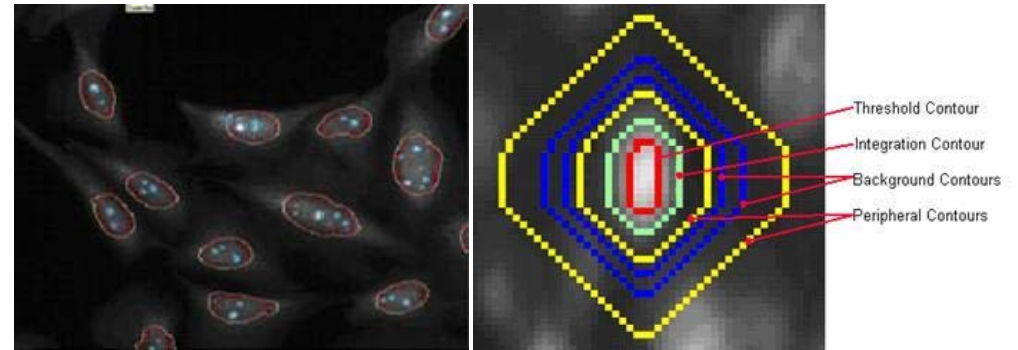


Laser scanning cytometry (LSC)

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.

