Introduction into Flow Cytometry

Katharina Lückerath

(AG Dr. Martin Zörnig)

adapted from Dr. Jörg Hildmann

BD Biosciences,Customer Service

How does a FACS look like?

FACSCalibur

FACScan

What is Flow Cytometry?

- Technique to analyse multiple physical characterisitcs of single particles
- A single cell suspension is traveling through a laser light beam.

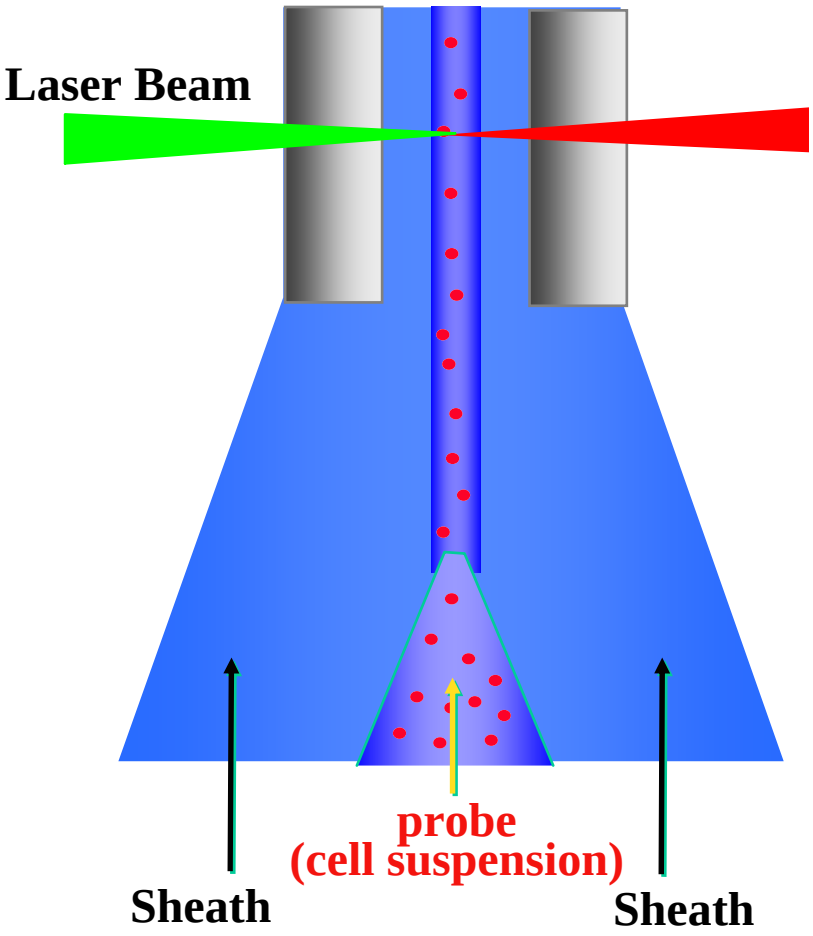
  Each cell will emit characteristic light signals which are captured by suitable detectors.

What can a Flow Cytometer do?

- A Flow Cytometer quantifies simultaneously different optical parameters obtained from whole cells in high throughput mode.

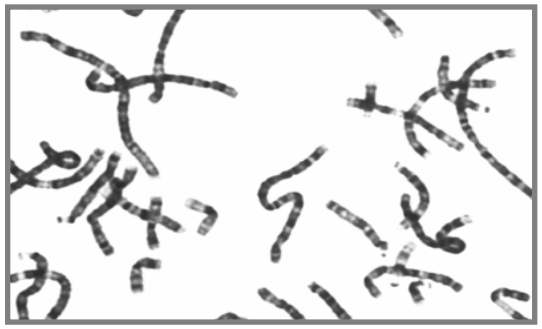
- Maximum speed of analysis is 4,000 cells per second (BD FACSCalibur™; in reality you should not go above 1,700 cells per second).

How does a Flow Cytometer function?

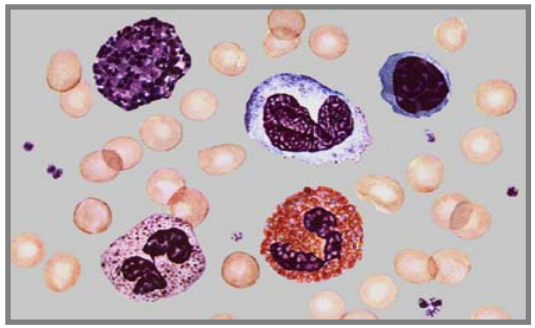


- Sheath liquid generates a laminar flow
- Reduction of the diameter within the flow cell leads to an acceleration and tapering of the sheath and the sample flow (hydrodynamic (horizontal) focussing). Both flows do not mix!
- The distance between two adjacent cells within the sample flow is increasing, so that each cell passes the laser beam individually.
- Laser light is scattered and fluorescence is emitted. These signals are detected by suitable detectors.

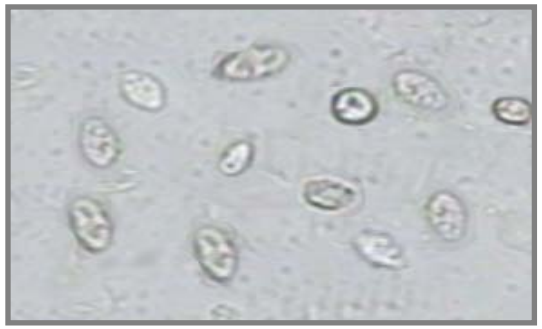
Which parameters of these particles can be measured by Flow Cytometry?



chromosomes



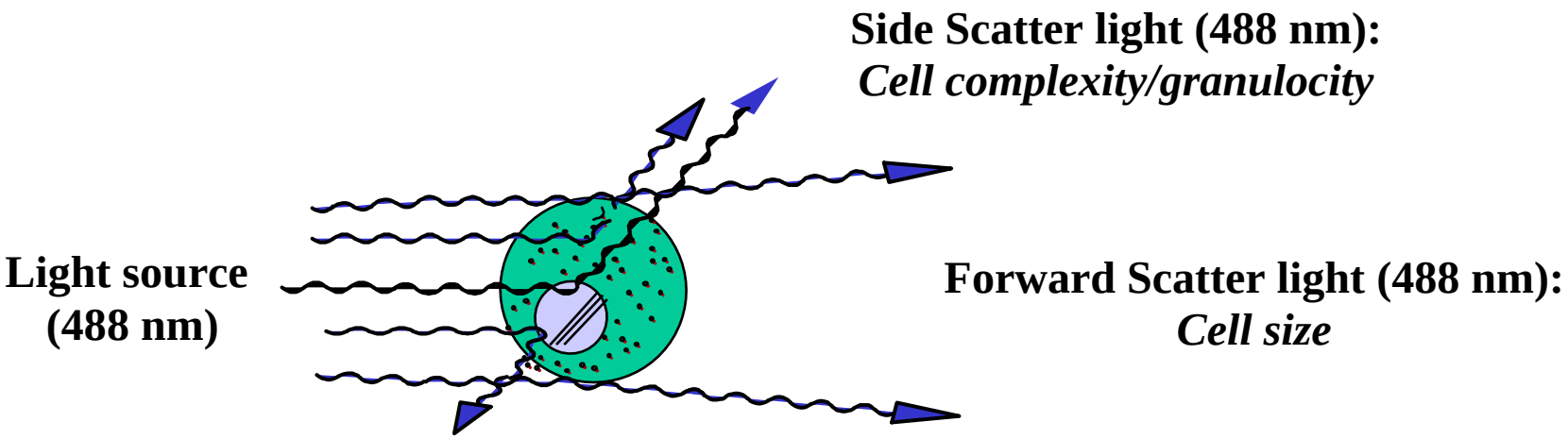
blood cells



protozoans

- their relative size (Forward Scatter - FSC)
- their relative granulocity/internal complexity (Side Scatter - SSC)
- their specific fluorescence (FL1, FL2, FL3, FL4…) and the respective relative fluorescence intensities

Forward and Side Scatter



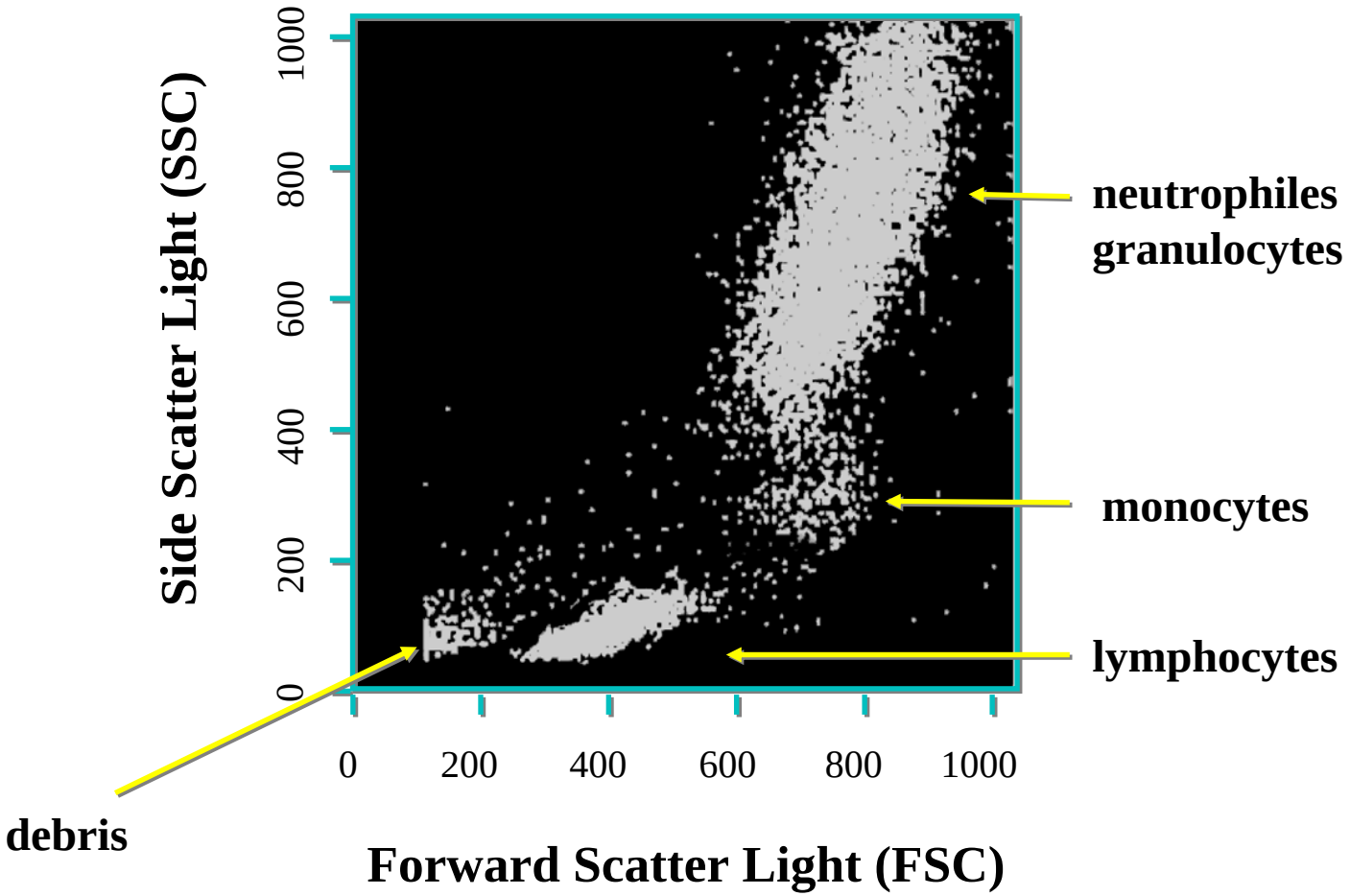
Forward Scatter light ***(FSC)*** -Diffraction

- proportional to cell surface (cell size)
- measured along the axis of the incoming light

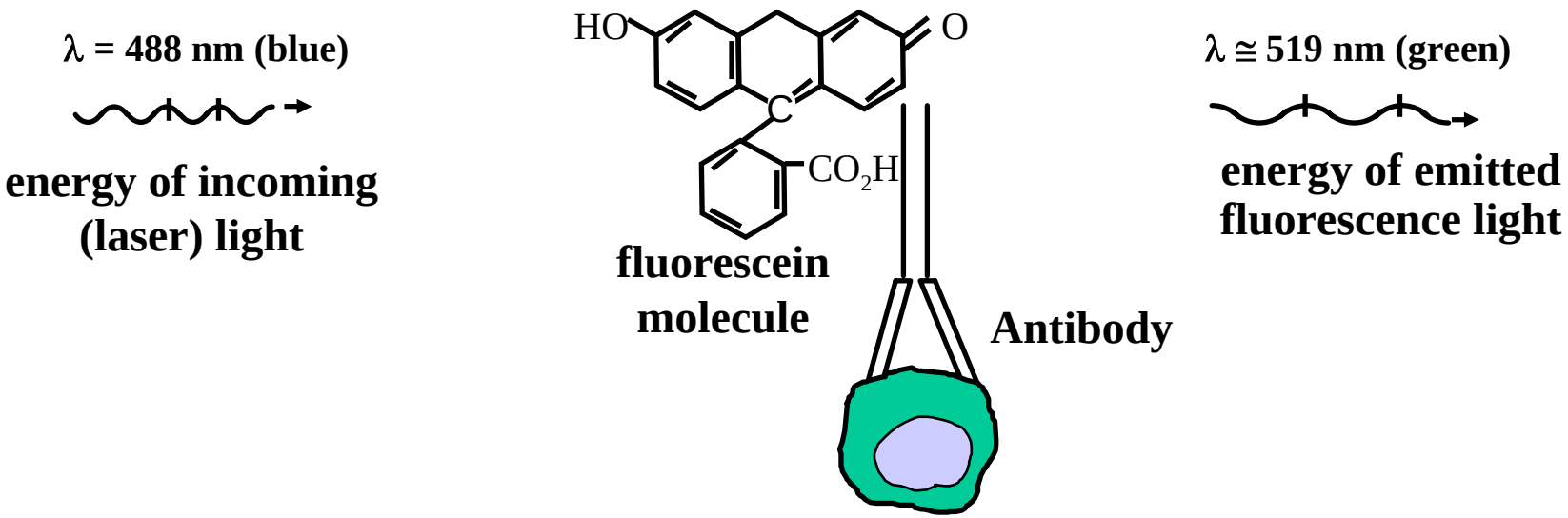
Side Scatter light ***(SSC)*** - Refraction and Reflection

- proportional to cell complexity or cell granulocity
- measured perpendicular to the axis of the incoming light

An example for FSC/SSC: Blood lysis

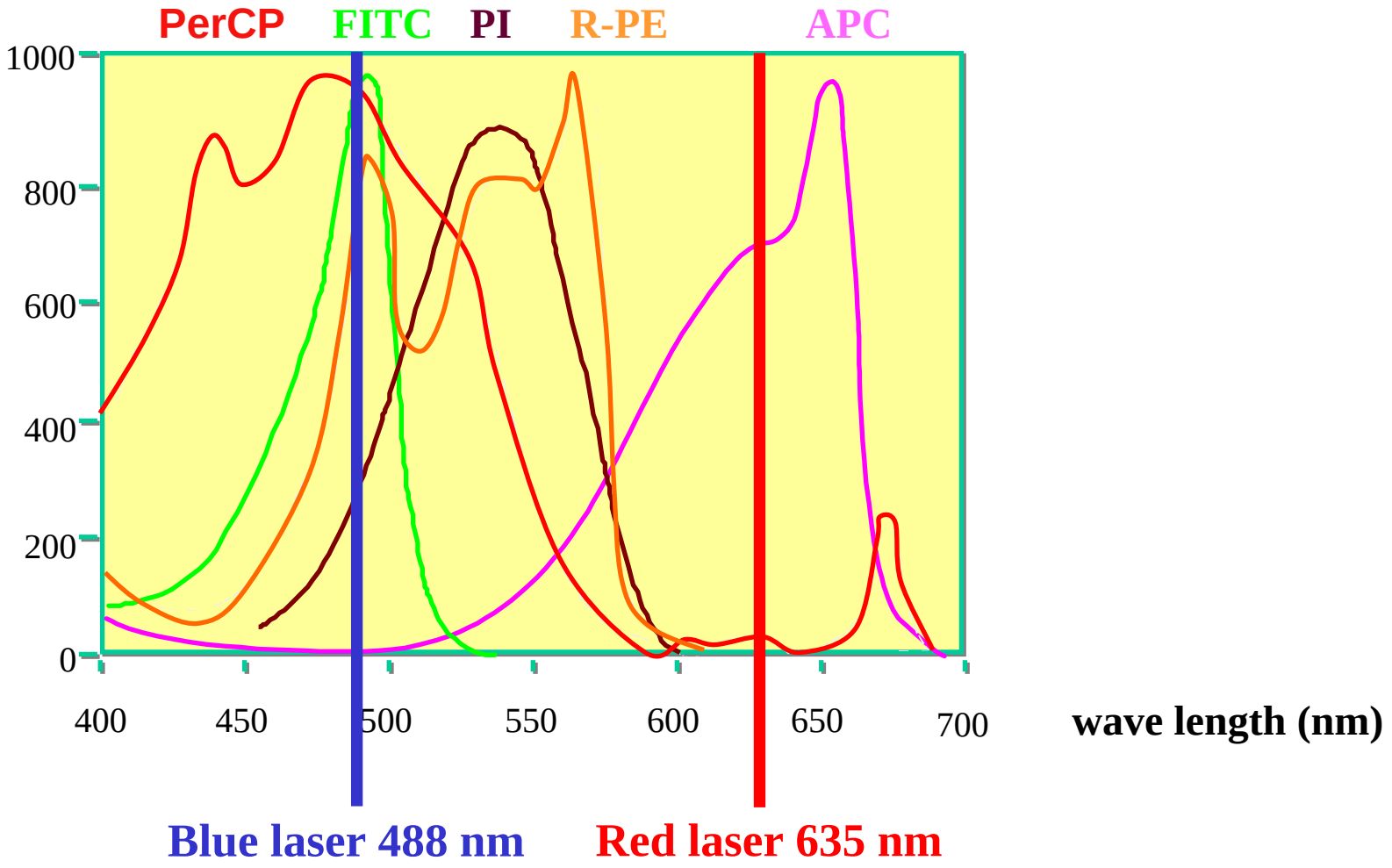


How is fluorescent light generated & measured?

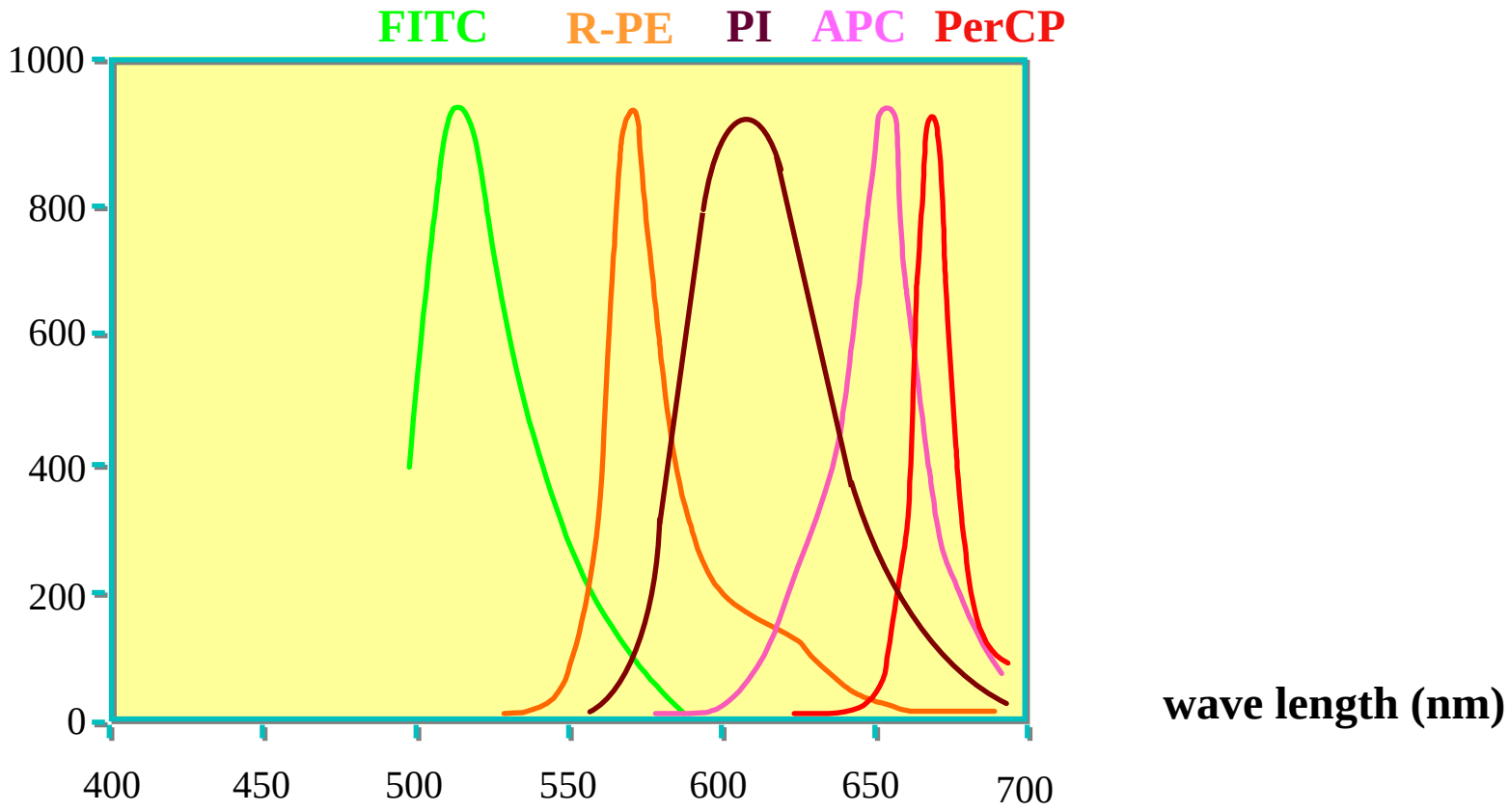


- The fluorochrome absorbs a specific amount of laser light energy
- The fluorochrome releases the absorbed energy:
  - The difference in wavelength between the absorbed and the emitted light is called Stoke´s shift
- Fluorescent signal is quantified by different detectors positioned 90° to the axis of incoming laser light

Absorption spectra of useful fluorochromes

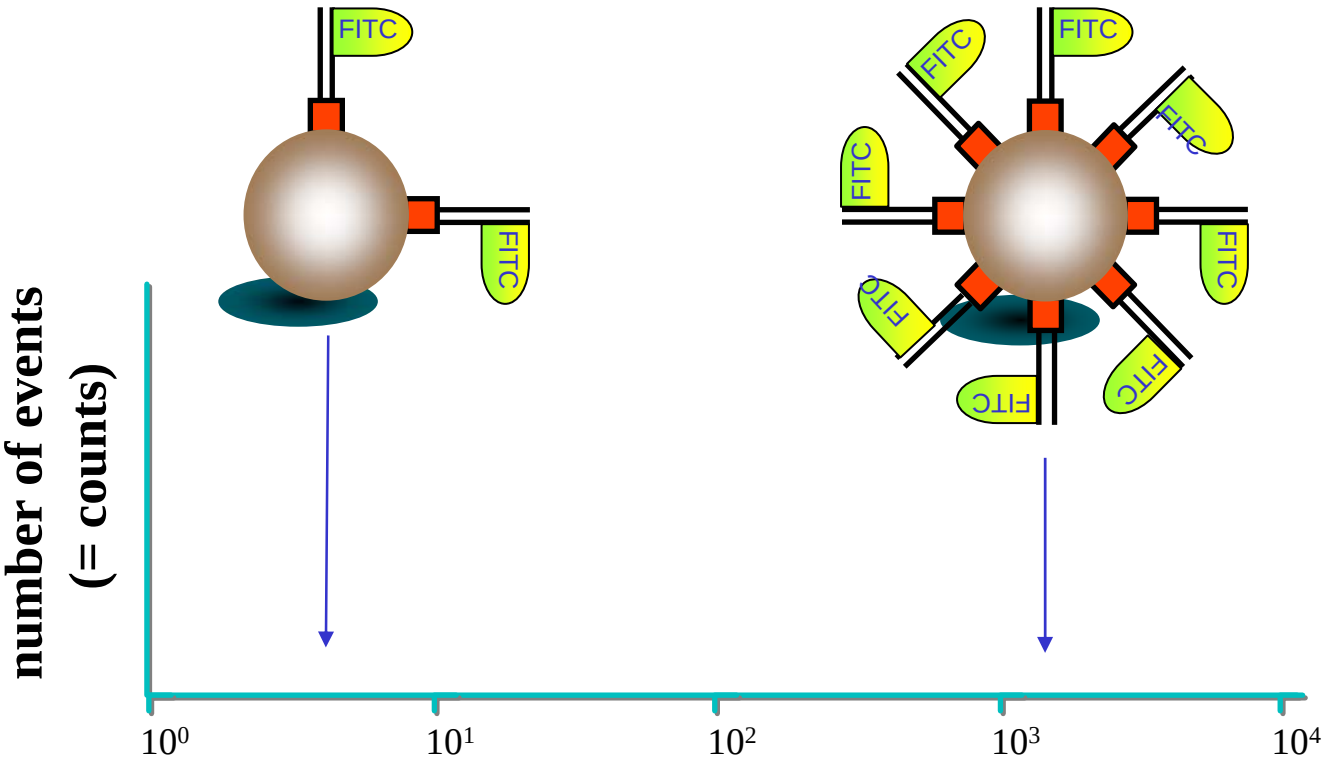


Emission spectra (= Fluorescence spectra) of useful fluorochromes



Fluorescence Intensity

The amount of emitted fluorescent light is proportional to the number of bound fluorochrome molecules



A Flow Cytometer consists of three components

☆ Liquid system

- Transport and focusing of cells on the point of measurement

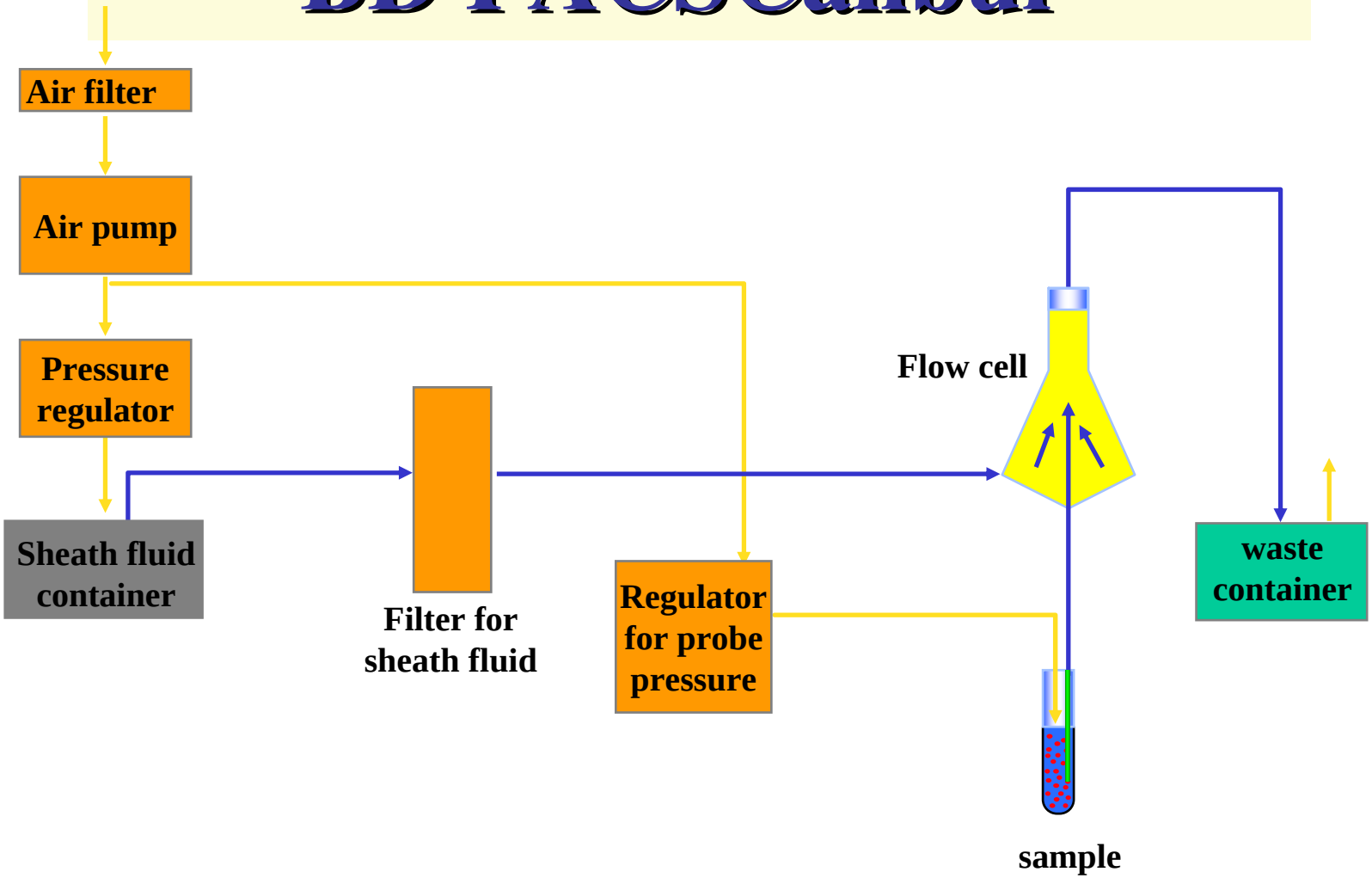
Optical system

- Excitement
- Detection

Electronic system

- Conversion of optical into electrical (digital) signals for subsequent computer analysis

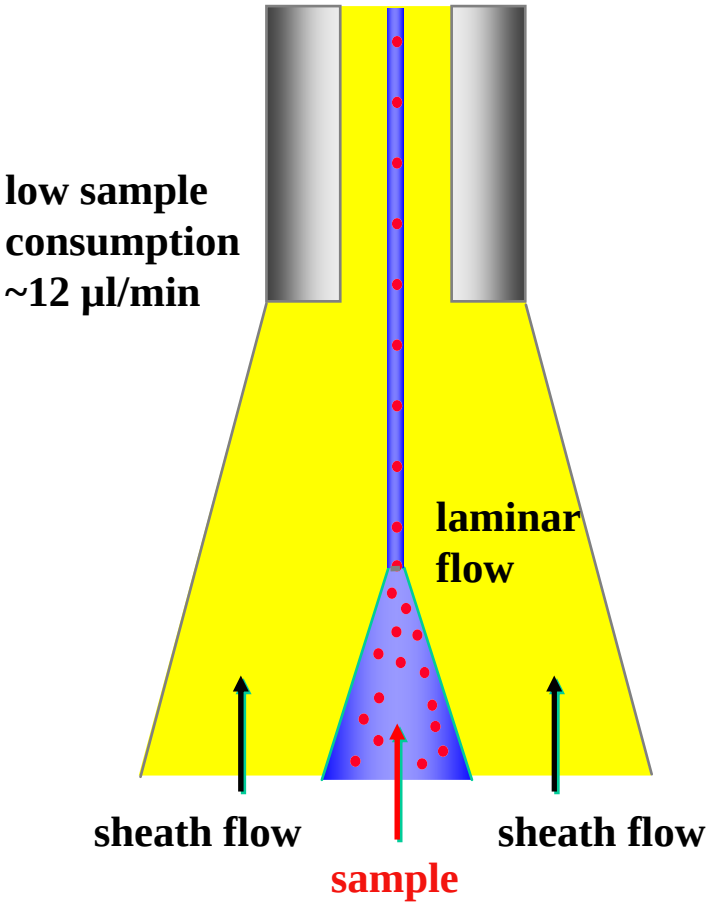
☆ Liquid system of the BD FACSCalibur™



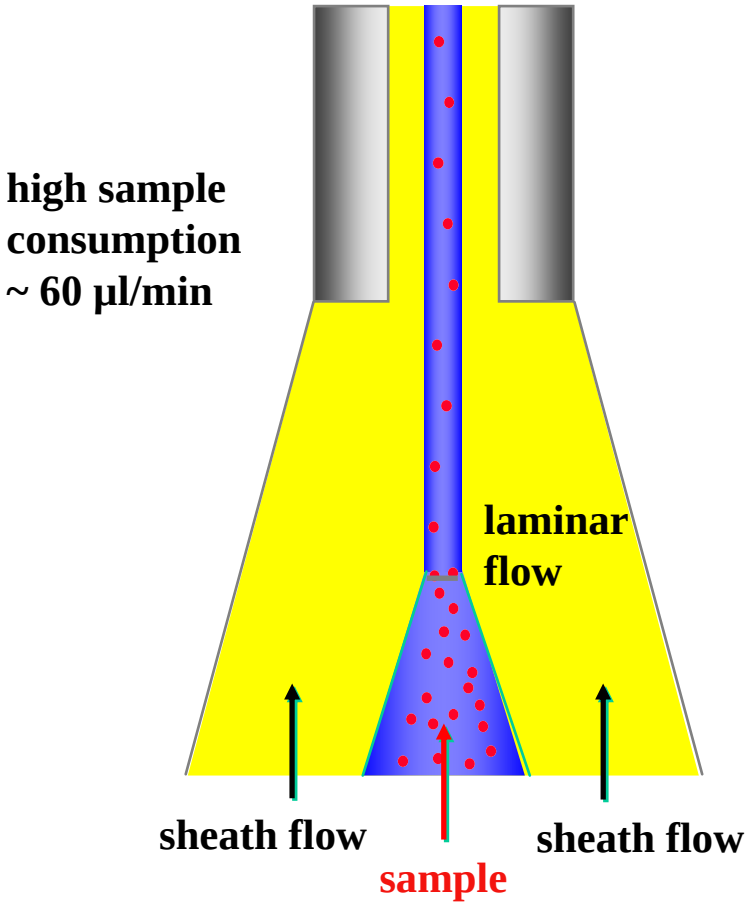
Sample Flow in the Flow cell

(BD FACSCalibur™, BD FACScan™, BD FACSort™)

low sample pressure (LOW)



high sample pressure (HIGH)



The optics I

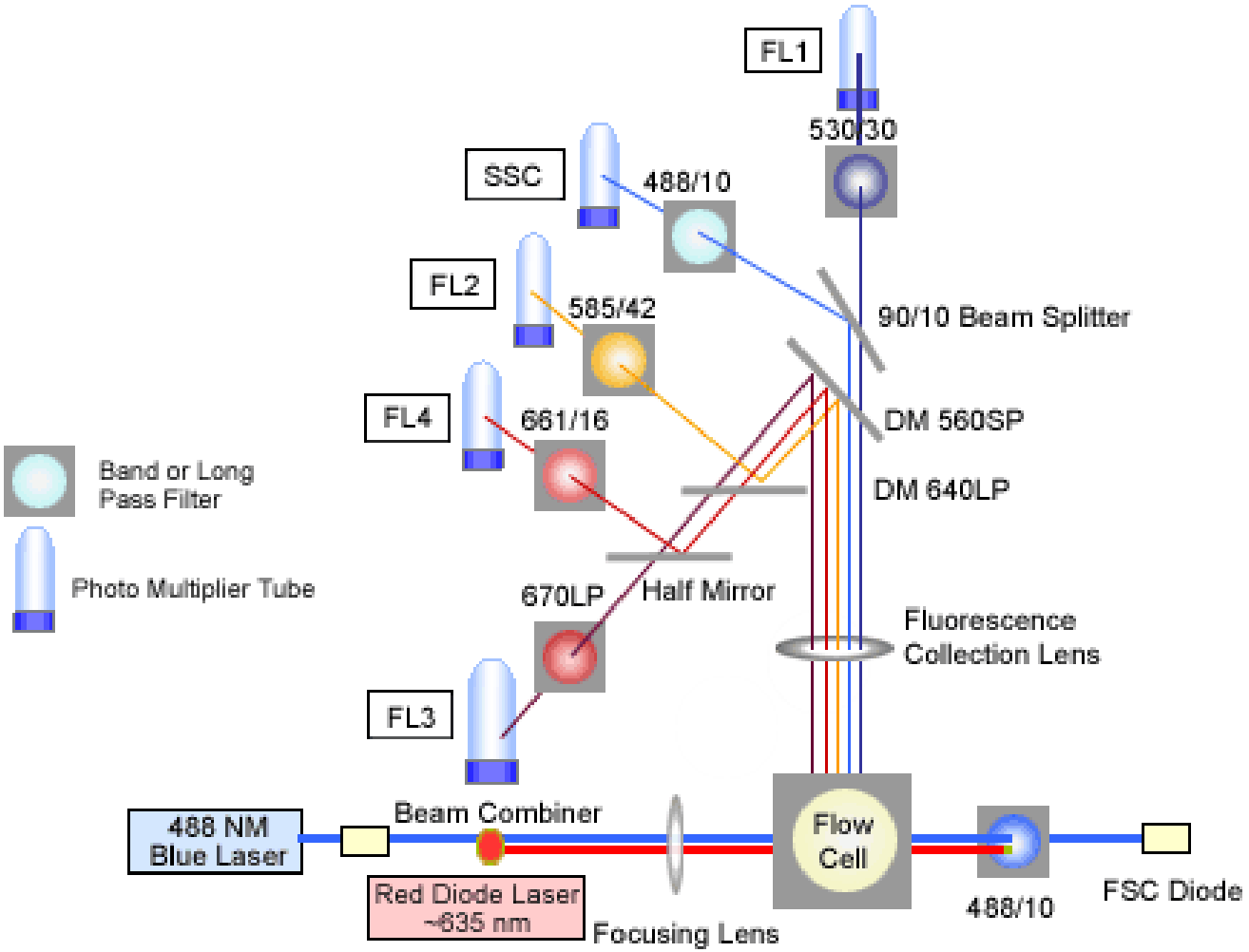
The excitation optics consists of:

- an air-cooled argon laser (488 nm), optional with a diode laser (635 nm)
- prisms (Beam Expander) to form and focus the laser beam (lenses)

The detection optics consists of:

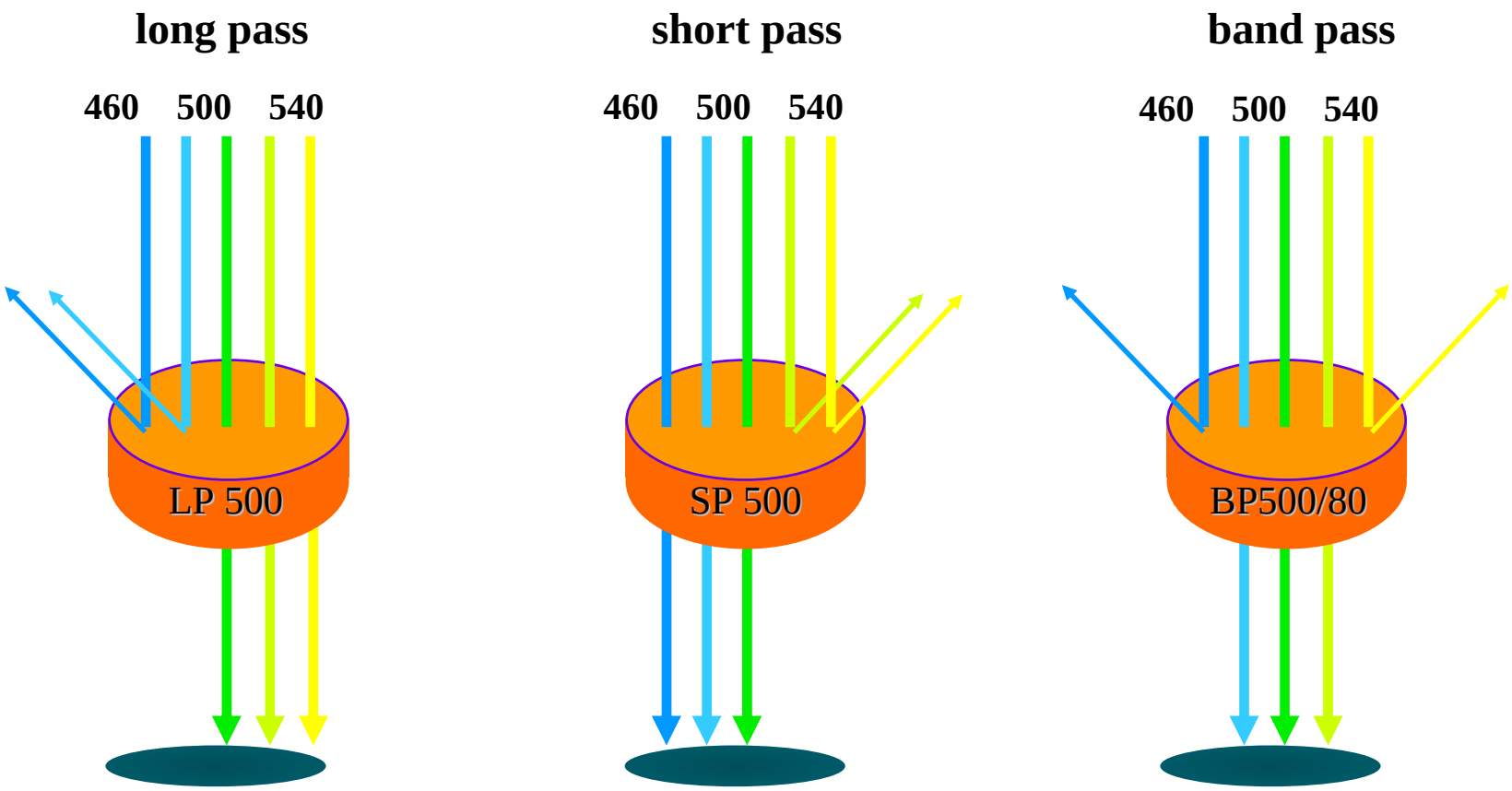
- a converging lense which collects the emitted light perpendicular to the axis of the incoming light
- a system of mirrors and filters (beam splitters) which divert the specific wave lengths from the emitted light to the corresponding detectors

The optics II



http://www.sickkids.ca/fcf/images/FACSlayout.gif

Optical filters

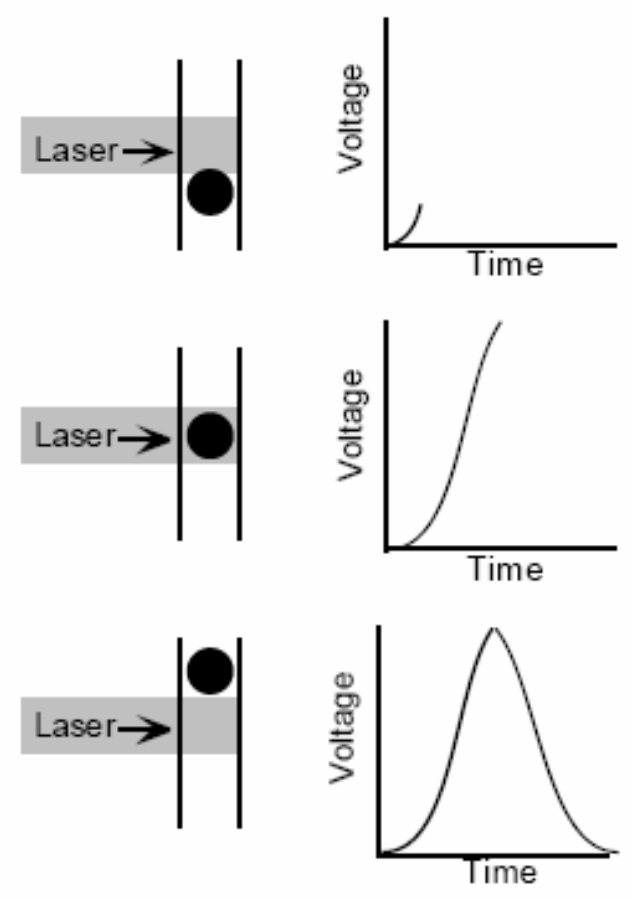


Electronic system I

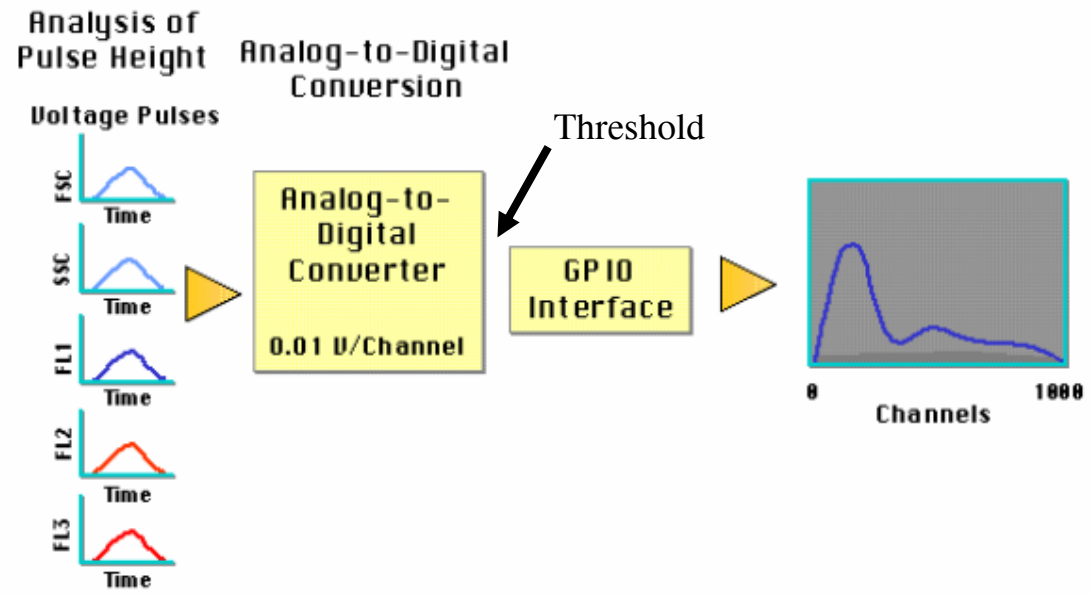
- Converts optical signals (photons) into electronic signals (voltage pulses)
- Voltage pulse is analysed:
  - Height
  - Area
  - Width
- Electronic signals are digitalised and send to the computer for analysis

Electronic system II

Optical → electronical signal

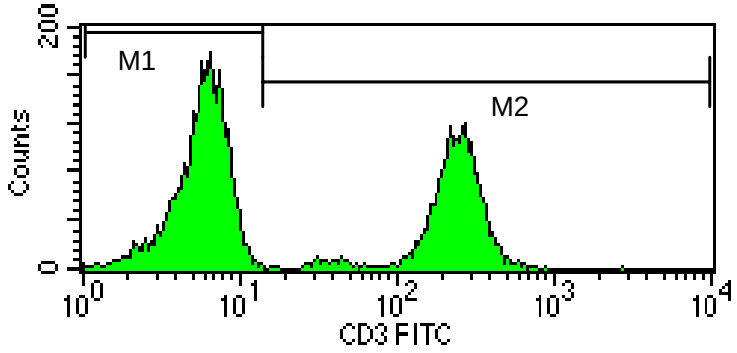


Electronical → digital signal

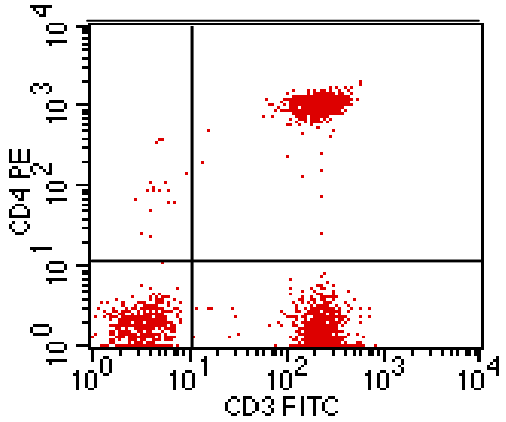


Different possibilities to present

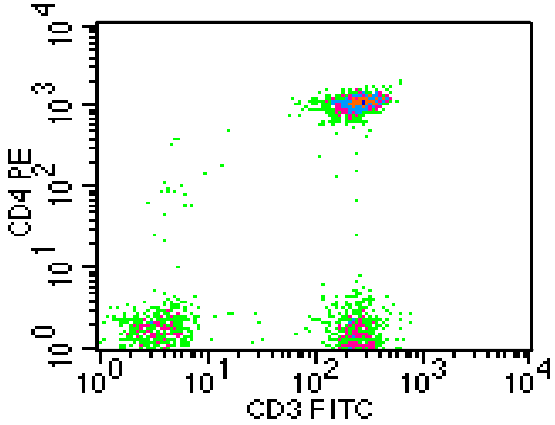
- Histogram



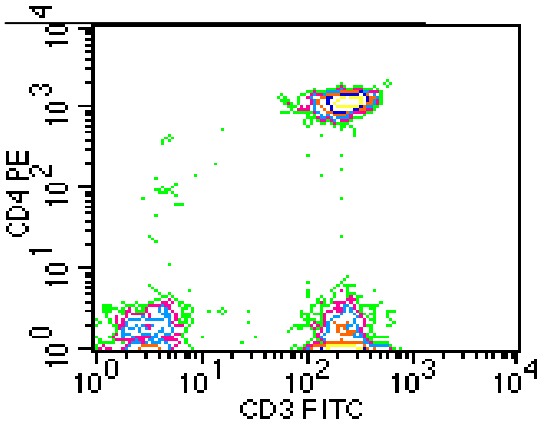
- Dot plot



- Density plot



- Contour plot



Instrument Settings

☆ Detectors/Amplifiers

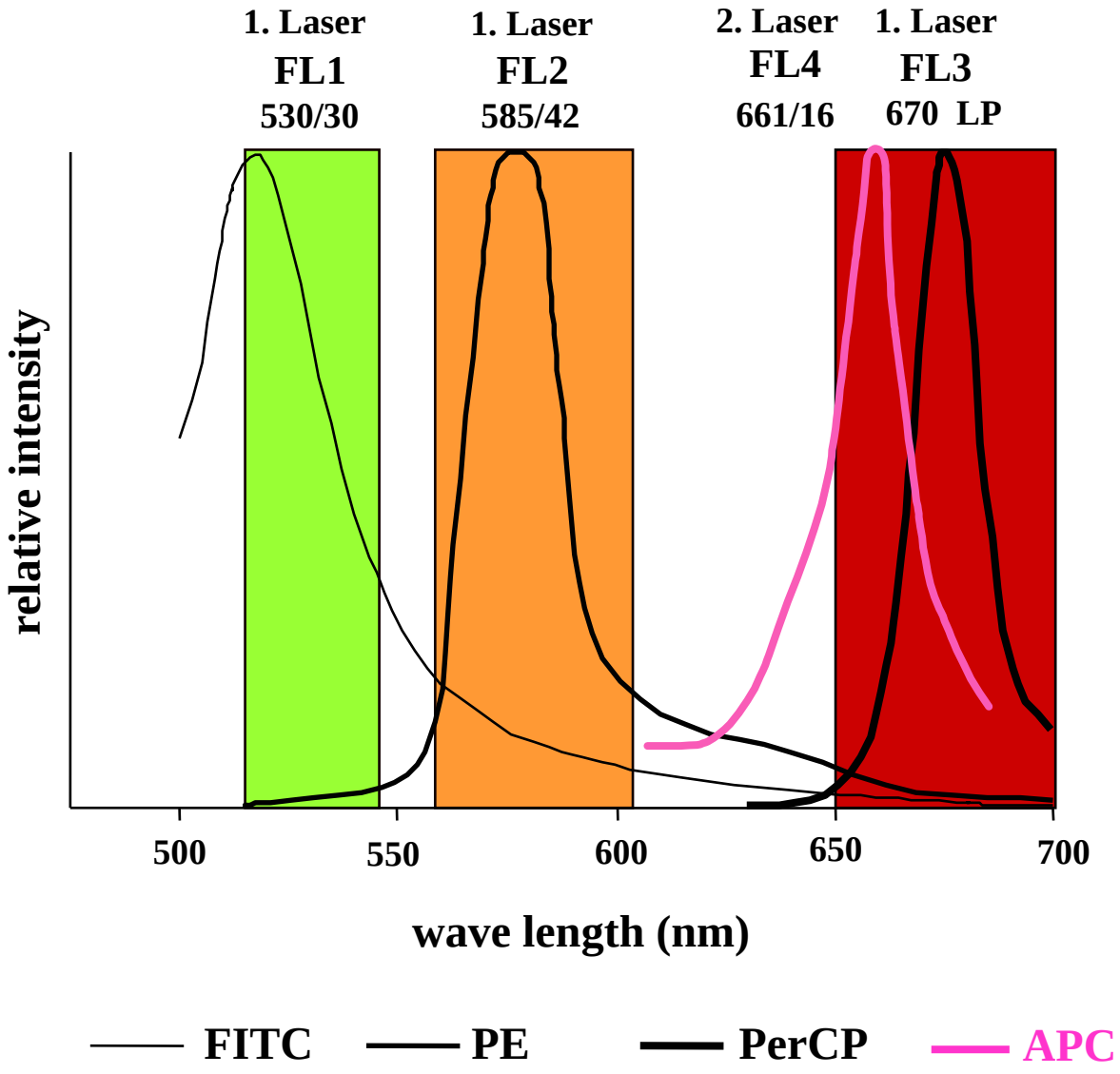
☆ Sensitivity: proportion optical : electronical signal

Threshold

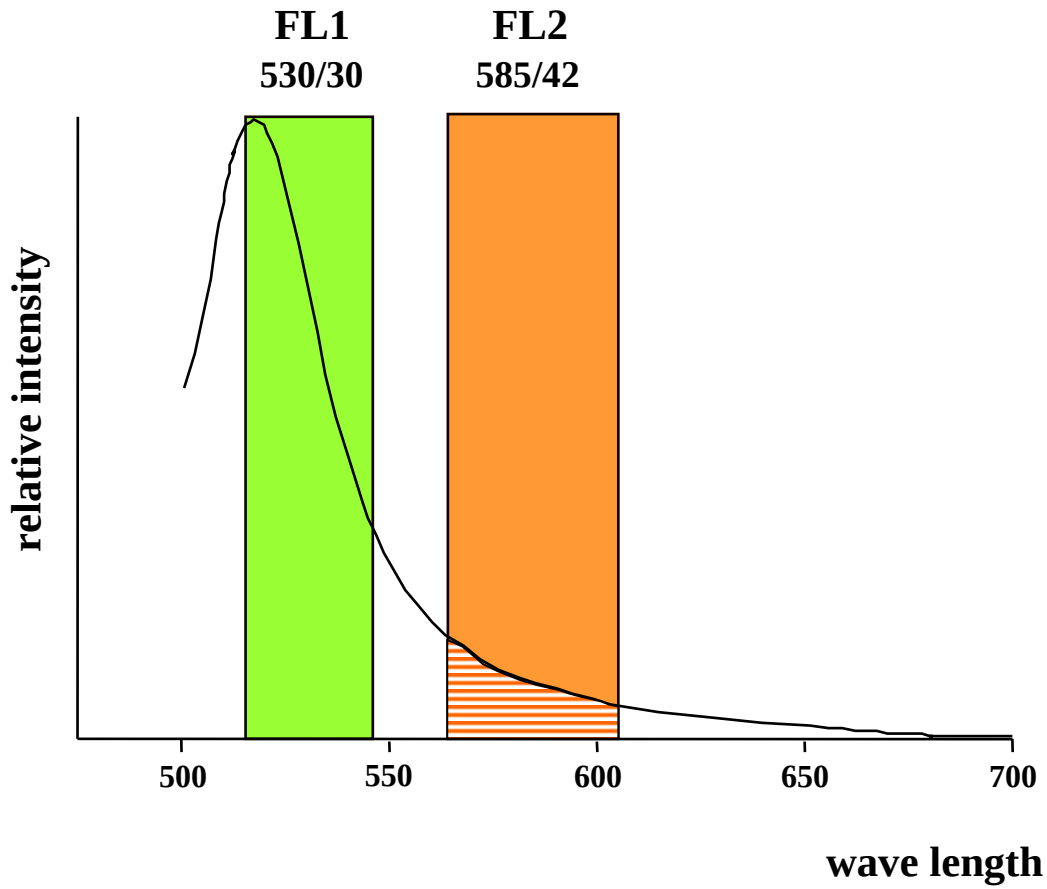
- only signals with an intensity greater or equal to the threshold value will be processed an sent to the computer

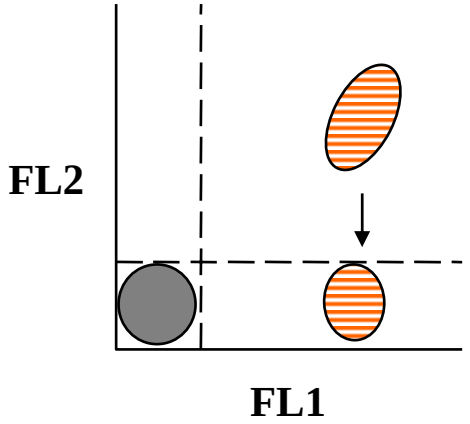
Compensation

Fluorescence emission spectra

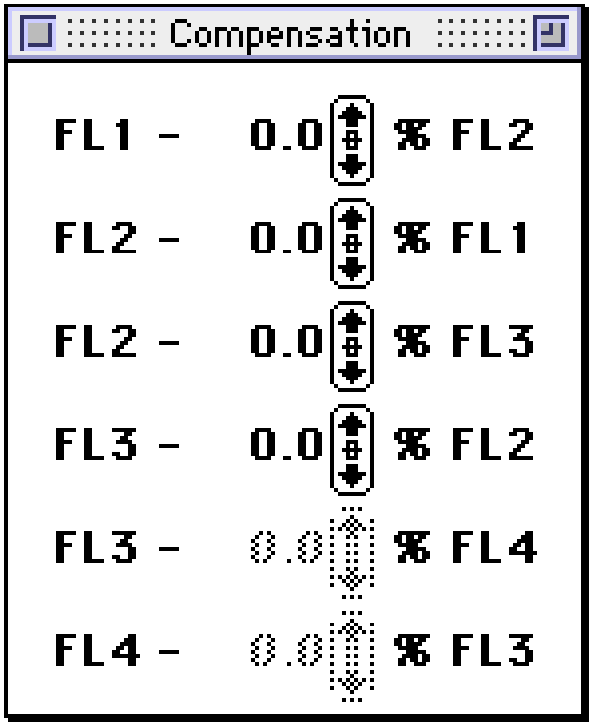


FITC fluorescence overlap





Compensation (*1*)



- Compensation allows to correct for spectral overlap.
- Compensation needs to be applied whenever more than one (adjacent) fluorescence is measured and analyzed.
- The settings for compensation have to be defined after the PMT voltage settings for each fluorescence were found and set fix (functional dependency of both).

Compensation (2)

- Compensation values depend on the fluorochromes used.
- Compensation values also depend on the individual Flow Cytometer with its specific optical properties (because of the functional dependency on the PMT voltage settings).
- Compensation is applied during acquisition and is not possible afterwards during software analysis (not true for newer software [Diva software]).

FACS analysis in research laboratories

- analysis of immune functions
- stem cell hematopoiesis
- multi drug resistance (cancer)
- kinetic studies (cell function)
- analysis of microorganisms (bacteria, protozoa, yeast)
- environmental analysis e.g. of water (giardia, cryptosporidium)
- FISH

Summary

- Basic principles
  - Laminar flow, hydrodynamic focussing, measured parameters
- Instrument components
  - Liquid -, Optical - , Electronic – system
- Instrument settings
  - Detectors, Threshold, Compensation