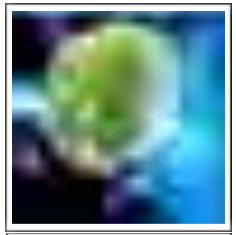
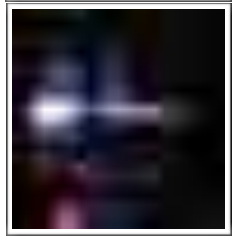
The Heidelberg Flow Cytometry Workshop

18-21 April 2016

Flow Cytometry Principles Overview
Assays and Tools
Practical Sessions (Limited places)
Data Analysis





Free Registration for EMBL and DKFZ Scientific Communities

Theoretical Sessions at EMBL and Practical Sessions at BD Headquarters in Heidelberg.

For more information and registration contact:

Malte Paulsen and Diana Ordonez (fccf-team@embl.de) or

Steffen Schmitt (steffen.schmitt@dkfz-heidelberg.de).

Partners:

EMBL European Molecular Biology Laboratory

Monday 18 April 2016

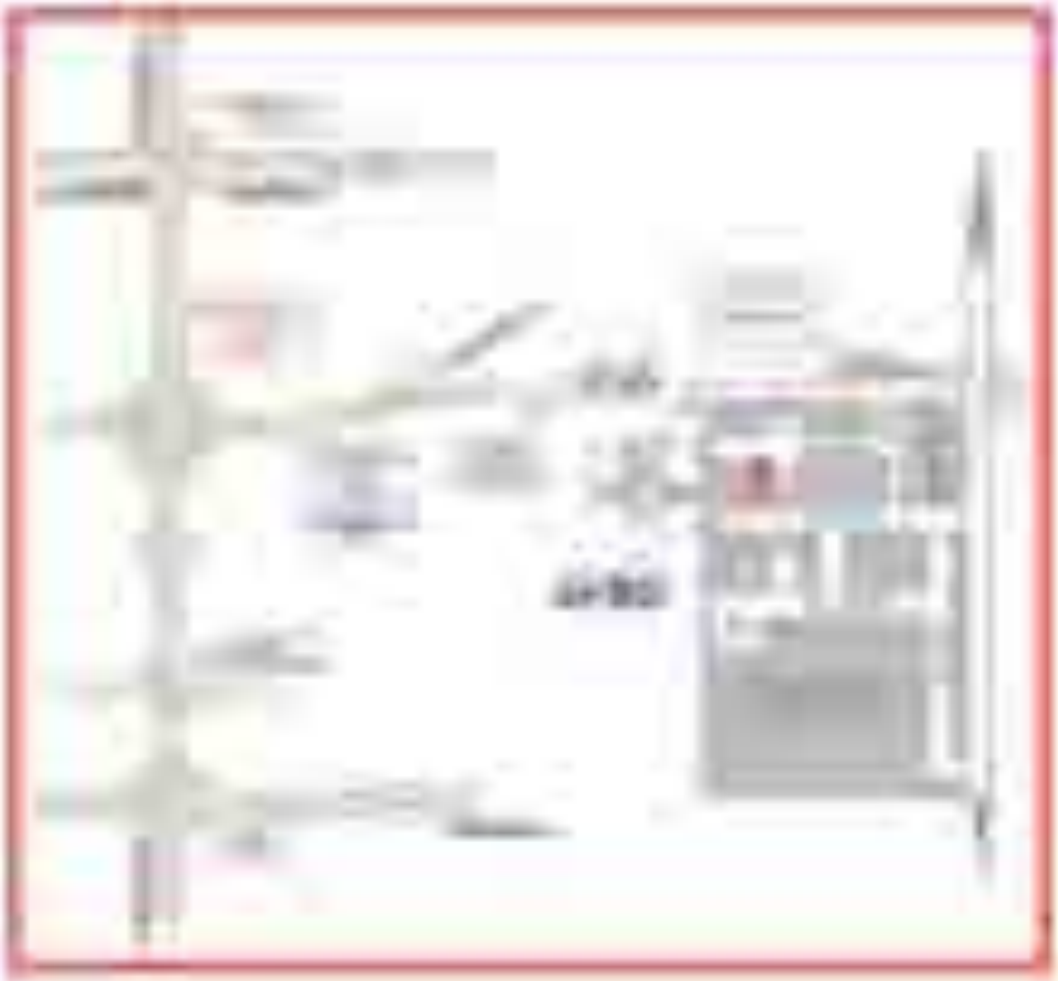
Session I 9.00 – 14.00 General knowlegde

Lecture	Topic	Location	Speaker
1	Flow Cytometry, History and current state of technology		Schmitt
2	Fluorochromes and Fluorescence		Paulsen
3	Generating Signals		Schmitt
	Coffee Break	EMBL small Operon	
4	Compensation		Chadick
5	Panel design and sample preparation		Ordonez
	Lunch Break		
6	Workshop: Panel design		all

Thursday 21 April 2016

Session IV 9.00 – 14.00 Data Analysis - FlowJo

Lecture	Topic	Location	Speaker
18	Data from practical parts will be analysed	EMBL	Ordonez



Local Transportation:

Rohrbach-Einkaufszentrum
- RN Bus 757

Rohrbach Süd
- Tram 24

Warm up:

History and current state of technology

History of Flow Cytometry

The beginnings

1940s - 1965s

Lou Kamentsky develops spectrometer based flow cytometer

Mack Fulwyler designs and built first cell sorter based on electrostatic principle

Wallace Coulter invents Coulter Counter

Wallace Coulter first patent issued

Lou Kamentsky publishes paper on cell spectrometry

1947 1949 1953 1961 1963 1964 1965

Gucker develops air sheath flow system

Crossland-Tayler develops sheath flow system

Boris Rotman develops methods for cellular fluorescence

Mack Fulwyler hears about Richard Sweet´s electrostatic printer (Standford)

1963: The dawn of Flow Cytometry

© by Cytomation (from L. Kamentsky)

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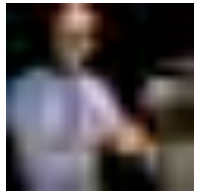
The beginnings

1940s - 1965s



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History of Flow Cytometry

The first description of sorting

dates back to 1812

First sorting reference:
Grimm, J. et al. (eds.):
"Cinderella" pp. 88 -101
Reimer Verlag (1812)

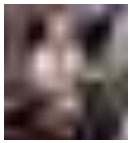
First biological sort: „the good into the pot, the bad into the crop"

History of Flow Cytometry

The roaring 60ties and 70ties

1965 - 1975

1966 — Marvin van Dilla publishes first paper on fluorescence flow cytometry



1966 — Mack Fulwyler contacted by Len Herzenberg asking about sorter

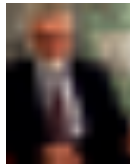
1967 — Mack Fulwyler builds a copy of original sorter for Boris Rotman

1969 — Wolfgang Göhde submits patent on fluorescence cytometer and produce commercial cytometer

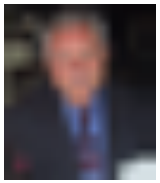
1969 — Dittrich and Göhde publish second paper on FACS

1972 — Herzenberg coins term „FACS“

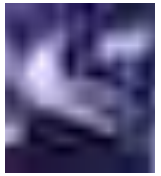
1972 — BD builds first FACS instrument for NIH



1974 — Crissman shows cell cycle in 20 minutes



1974 — Phil Dean and Jim Jett develop models of cell cycle



1975 — Milstein and Köhler publishes paper on monoclonal antibodies

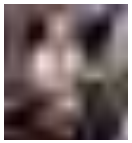
1968 - ImpulsCytoPhotometrie

History of Flow Cytometry

The roaring 60ties and 70ties

1965 - 1975

Marvin van Dilla publishes first paper on fluorescence flow cytometry



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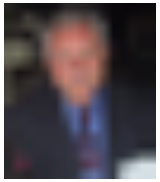

Crissman shows cell cycle in 20 minutes

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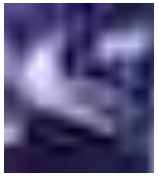
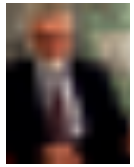


1966 1967 1969 1972 1974 1975

Mack Fulwyler contacted by Len Herzenberg asking about sorter

Herzenberg coins term „FACS“

BD builds first FACS instrument for NIH



Milstein and Köhler publishes paper on monoclonal antibodies

1972: The Early Days

Len Herzenberg a „Life for FACS“

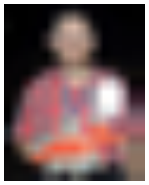
As the father of modern flow cytometry and with numerous fundamental breakthroughs in cellular immunology, Len was recognized with numerous awards and accolades. He was a member of the National Academy of Sciences and was awarded a Special Albert Lasker Prize in Immunology 2004 and the Kyoto Prize 2006.

1931–2013): The Life of FACS

History of Flow Cytometry

Make it colorful and speed it up

1975 - 1995



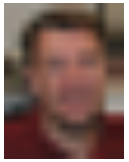
Howard Shapiro publishes „Practical Flow Cytometry“ beginning of documentation

Len Herzenberg sabbatical with Cesar Milstein in Cambridge coins the term „hybridoma“

Cesar Milstein publishes first paper using monoclonal antibodies and FACS

Leon Wheeless „Slit scanning“ Flow cytometer

Parks, Hardy, Herzenberg develop 3 color analysis; beginning of multicolor FC



Mario Roederer breaks the color barrier again and again and again -

1995 1997 2001 2004

Robert Murphy develops FCS 1.0 file standard

1975 1976 1977 1982 1984 1986 1994 1995

EPICS II laser based 2 color fluorescence detection + scatter + Coulter volume

Loken, Parks and Herzenberg 2 color immuno-fluorescence

Bob Auer develops device for rapid immunophenotyping Q-prep

Cytomation built the first „high“ speed cell sorter

1985 - FACS®

Int. Cl.: 9

Prior U.S. Cl.: 26

United States Patent and Trademark Office

Reg. No. 1,367,436

Registered Oct. 29, 1985

TRADEMARK

PRINCIPAL REGISTER

FACS

[illegible] FIRST USE [illegible] IN COMMERCE [illegible]

SER. NO. [illegible], FILED 1-4-1985.

FOR: LABORATORY FLOW CYTOMETER, BLOOD ANALYZER, CELL SEPARATOR, CELL SORTER, IN CLASS 9 (U.S. CL. 26).

G. T. [illegible], EXAMINING ATTORNEY

History of Flow Cytometry

Digitalization and Specialization

1996 - 2016

Noble Prize Chemistry for
Conductive Organic Polymers

1997 Digital FCS 3
Standardformat;
Moore, Parks et al.

2000

2001 BD FACSAria
Sorting with Cuvette

Patent on
„Image Cytometry“
(Amnis Inc.)

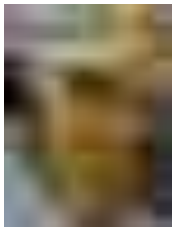
2005 „Biexp. Display“
Parks, et al.,

Large Particle Sorter
(Union Biometra)

2006

Ward M., Patent on
„Accoustic Focusing“

2008 Dep Array: Chip-based
Sorting and Imaging
(Silicon Biosystems)



2009 Mass Cytometry
Nolan G et al.,
DVS Toronto

2011 Brilliant
(Violet) Dyes
(Sirigen)

2012 Spectral Analyser
(Sony Biotec)

2013 „MoFlo Astrios“;
6-way sorting
(Beckman Coulter)

2016 FACSymphony
theoretical
50 Parameter

Is there a perfect solution?

… which is optimized

for **all** expectations
for **everybody**
and **always available**?

Specialization in (Flow) Cytometry

Label-free cell analysis

Slide-based Cytometer

Chip-based sorting

Spectral Cytometer

Cell Imaging

Multi-Parameter Analysis

Multiplex Bead Arrays

“Portable” kit based

Cell sorting

Mass Cytometry

High Throughput

Modern Flow Instruments

Actual available Cell sorters

What a Flow Cytometer measure

from 0.2µm

to 50µm

Typically particles or cells from 0.2-50 micrometers in size are suitable for flow cytometric analysis. On some cytometer larger particles can be analyzed

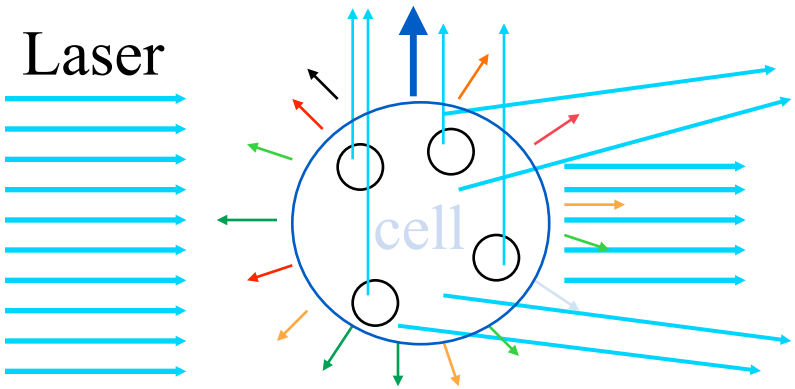
modified from BD Biosciences

What a Flow Cytometer can do

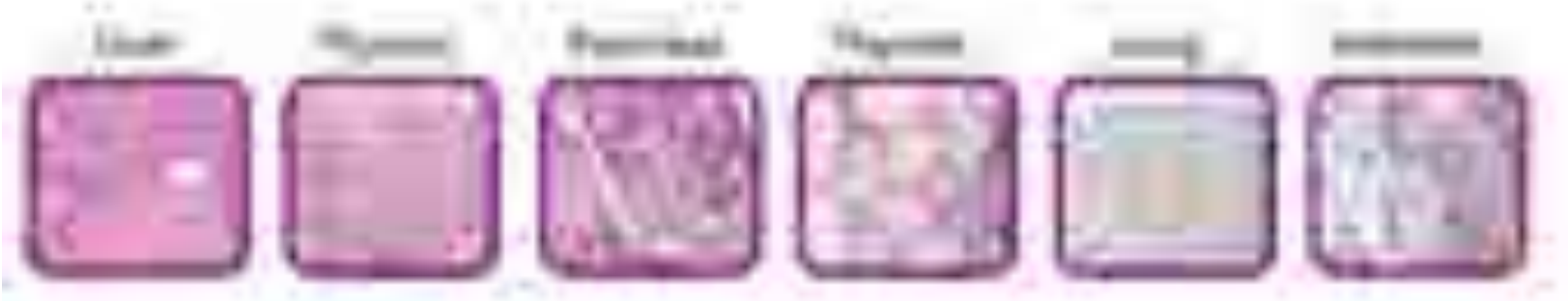
- Measure particles with following sizes

  > 1/2 wavelength of excitation source

  < 1/3 of diameter of the fluidic stream



What a Flow Cytometer can´t do...

1) 

Cells from solid tissue must be disaggregated before analysis.

2) Intracellular location of molecules (e.g. membrane vs. nucleus)

3) Translocation of proteins (e.g. plasma into nucleus)

4) Colocalization of molecules (exception: FRET)

5) Cellular structure or morphology

modified from BD Biosciences

What a user should know

Why we are here?

- Is the analyser in a good technical condition?
- Know your cells!!!!

- Optimize/ adjust the settings, depending on your preparation and question.

- Be familiar with the theoretical background
- Know how to operate the instrument and software

Advantages of FACS-Analysis

- Quick sample processing
- Quantitative analysis of single cells
- Multi-parameter analysis

Typical FACS-Measurements

- Absolut-cell-count analysis
- Lymphocyte phenotyping
- Cell cycle analysis (PI) / DNA-content of tissues
- Apoptosis / Necrosis / Viability
- Phagozytosis
- Functional tests (e.g. metabolism; Ionflux [Ca^{2+}, pH])
- Transfection efficiacy / reporter gene expression (e.g. GFP)

- Cytometric Bead Arrays (CBA) / Flex Sets
- Phospho-Profiling / Cytokine production
- ...

How does flow cytometry work?

Single particles in a fluidic stream pass a focused laser beam

↓

Particles emit characteristic light signals

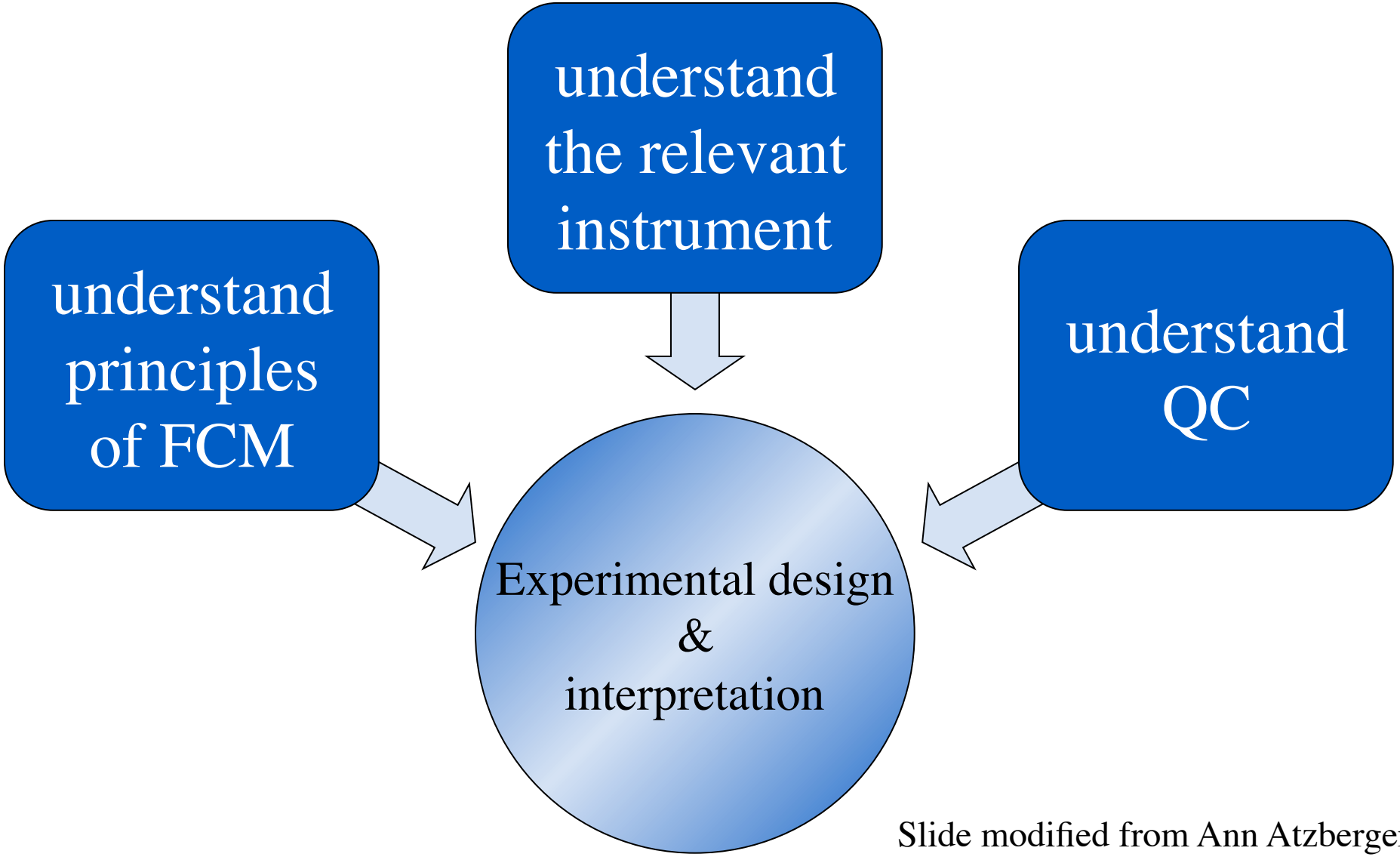
↓

Detection and amplification of light signals with photodetectors

↓

The measured, relative amount of light will be plotted via a computer

What is required?



Slide modified from Ann Atzberger

…

"Unfortunately, there is no RIGHT way to do a FACS experiment - but there are a whole bunch of wrong ways."

…

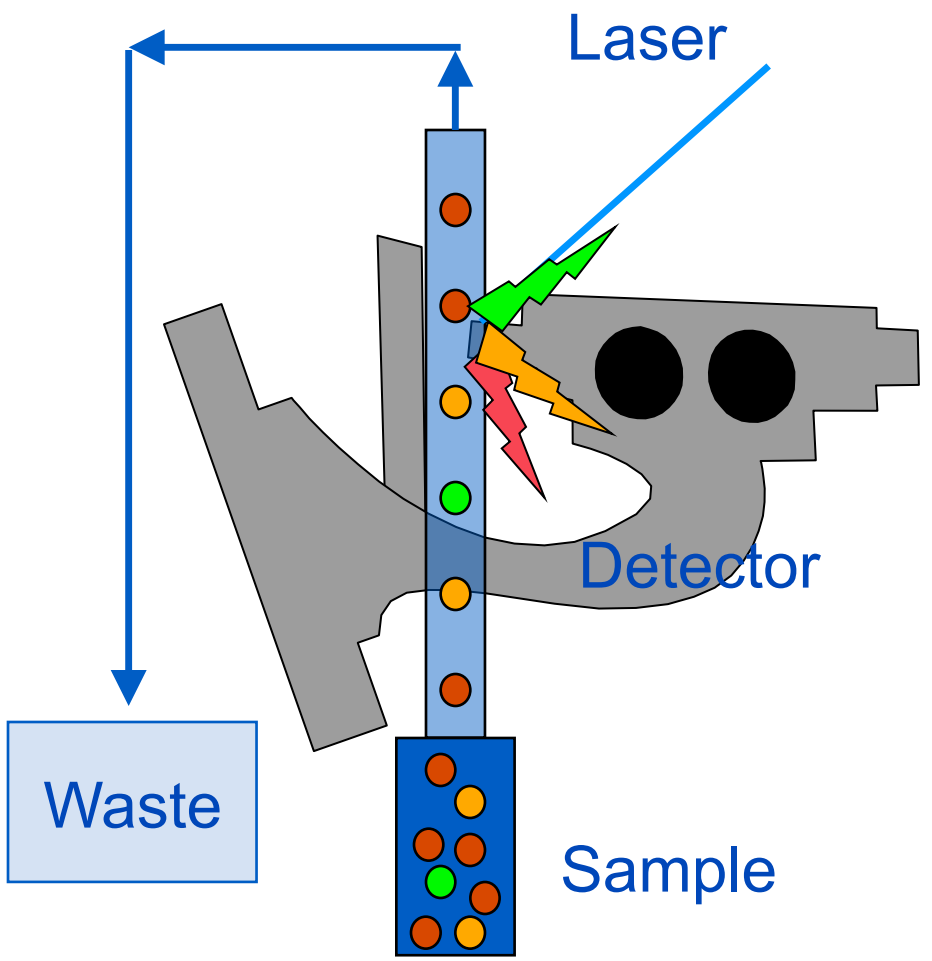
Ultimately, flow cytometry is a very complex technology. The shear number of variables that can directly impact the output measurement, sometimes in extremely subtle ways -- makes it daunting. There is no substitute for experience -- and that's the other thing I tell people:

Don't be afraid to get help! Even when you "know" the answer! …

M. Roederer (comment from 08.05.2012 on cytometry perdue list about teaching flow)

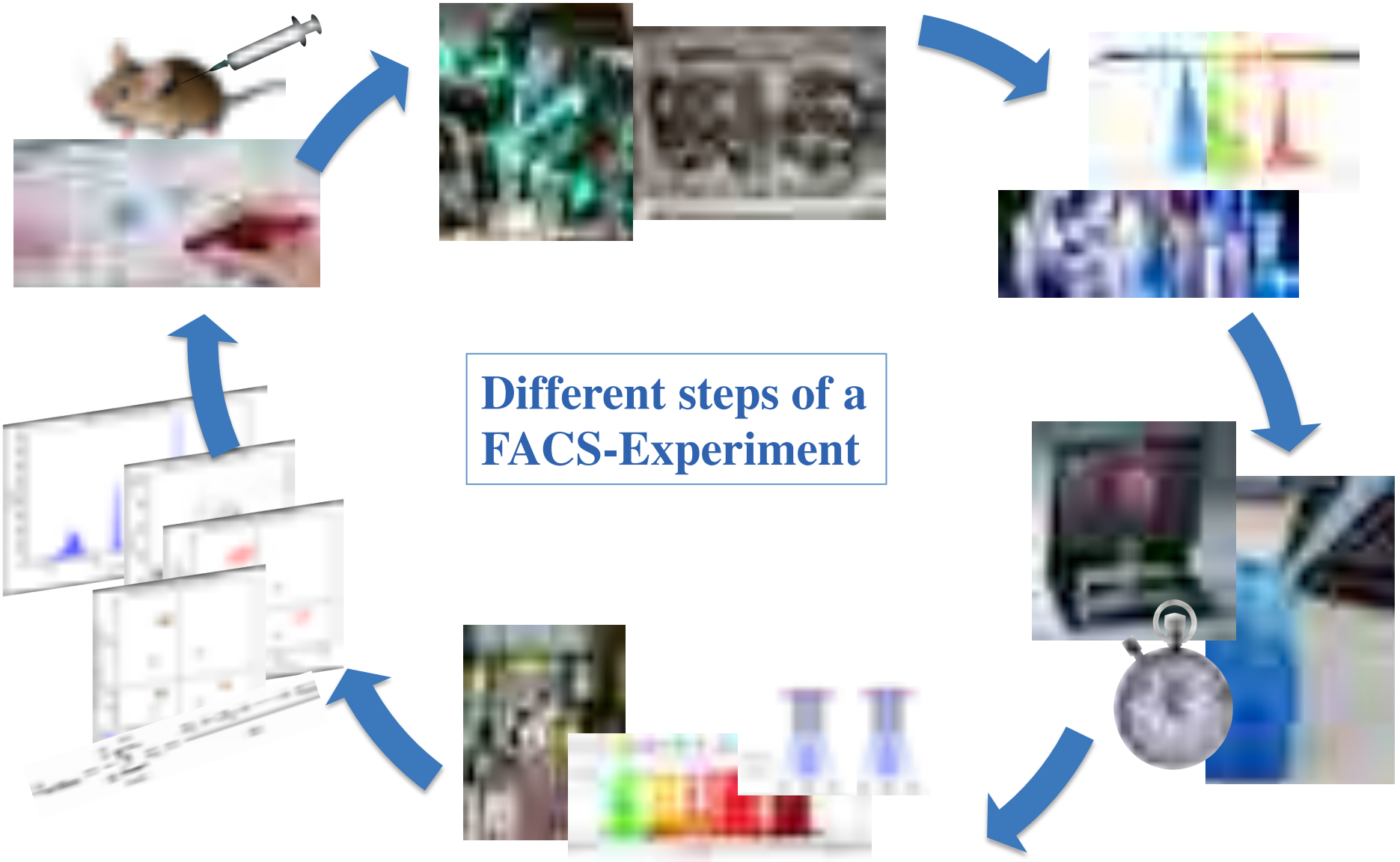
FACS®

- F luorescence
- A ctivated
- C ell
- S orting/ caning

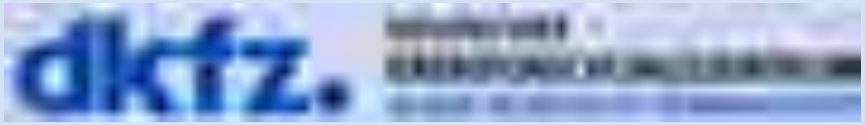


Flow Cytometry translates cellular structures and properties into light!

Where to optimize your results



Major Components of a Flow Cytometer

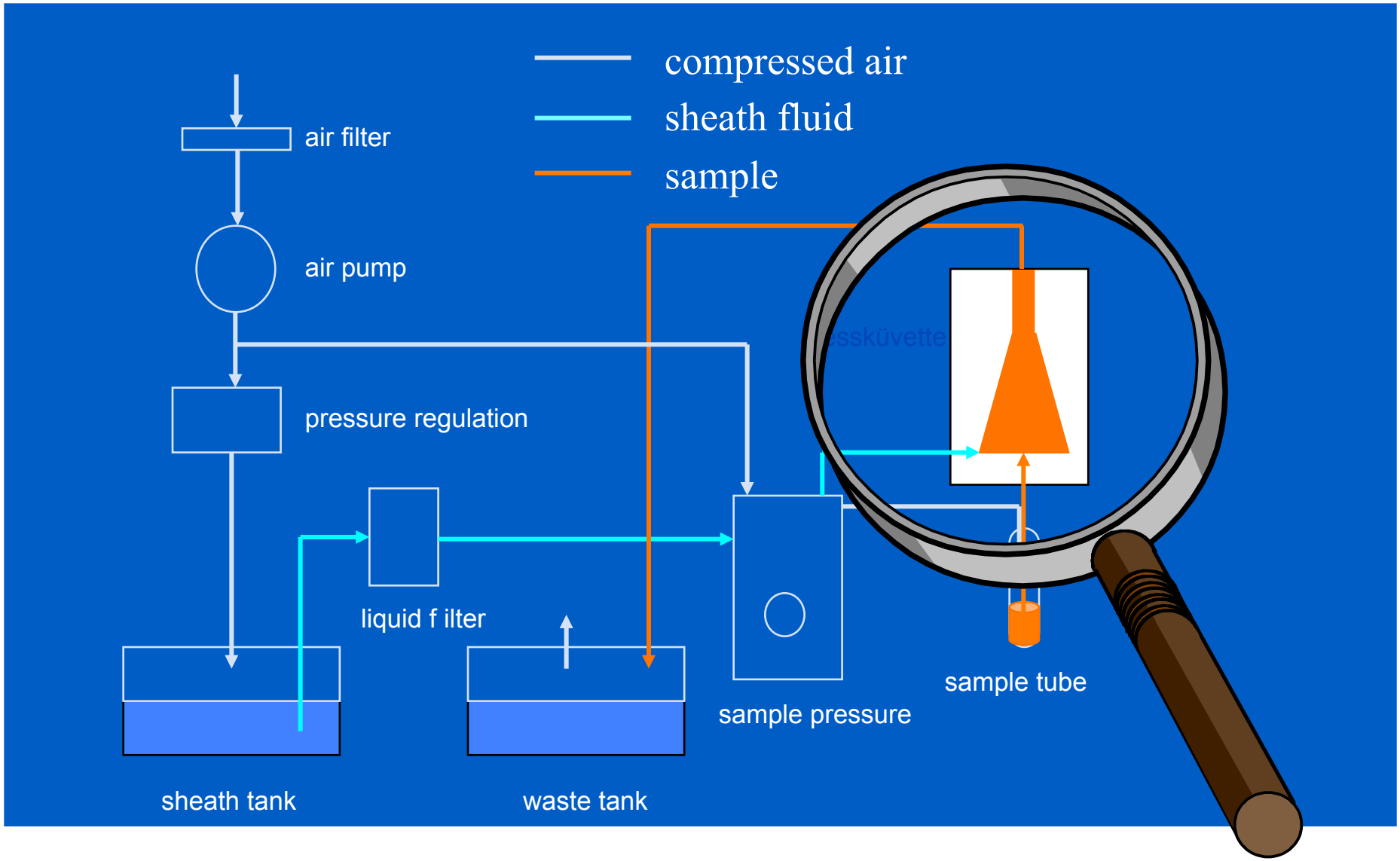


What do we need for that?

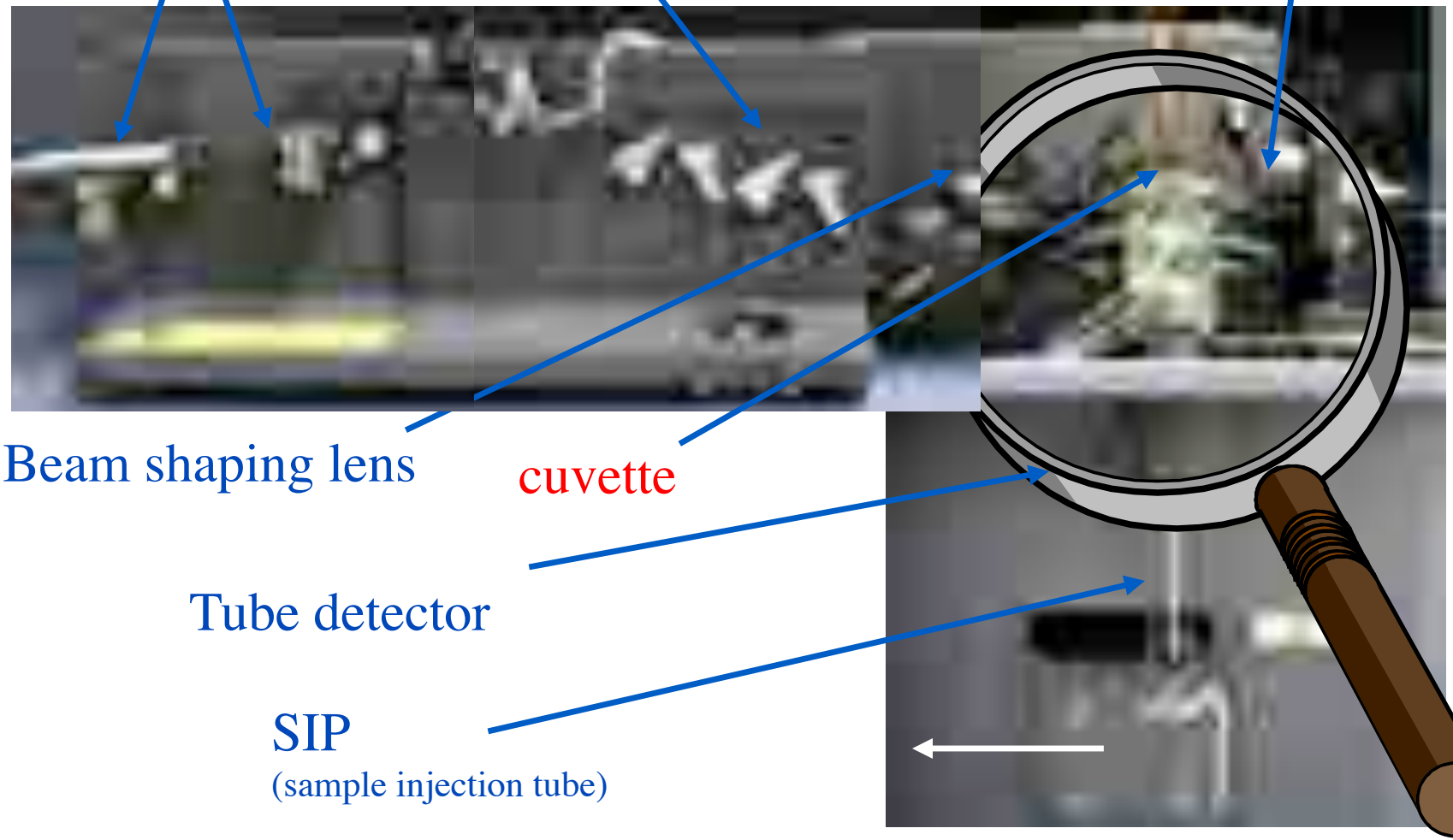
„Anatomy“ of a flow cytometer

- Liquid reservoir with pressure regulation
- Optical system (detection of fluorescence)
- Electronic compounds (signal processing)

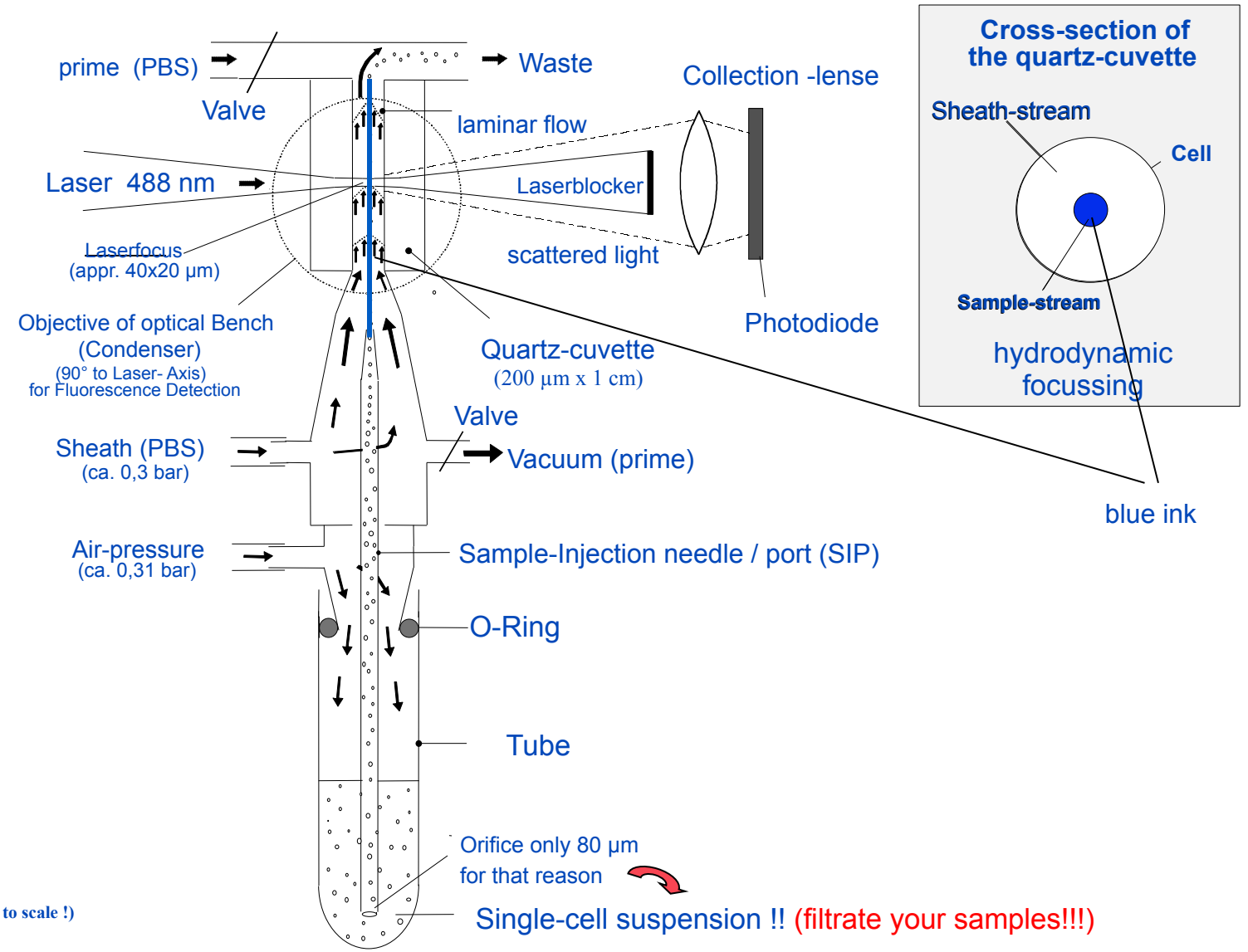
Liquid handling



FACSCanto II



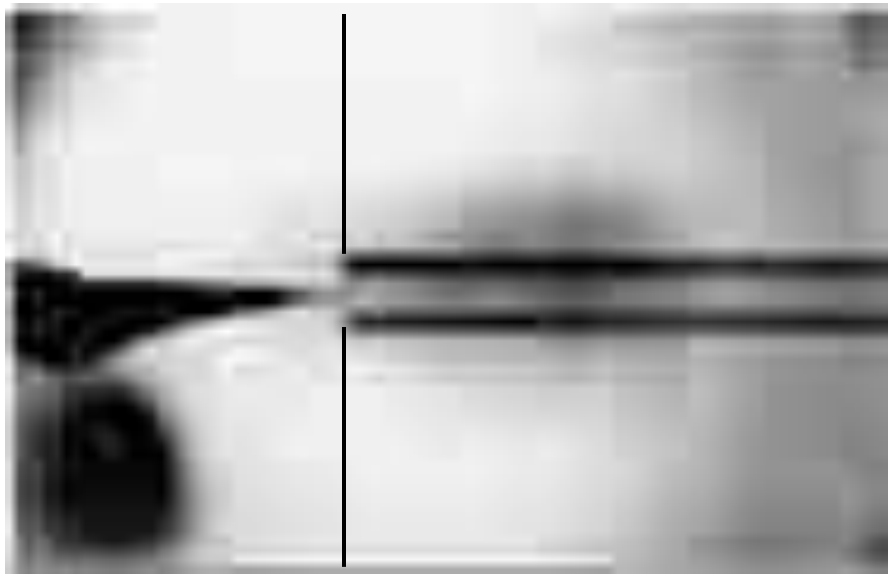
Quartz cuvette



(Picture is not true to scale !)

Laminar Flow of liquids

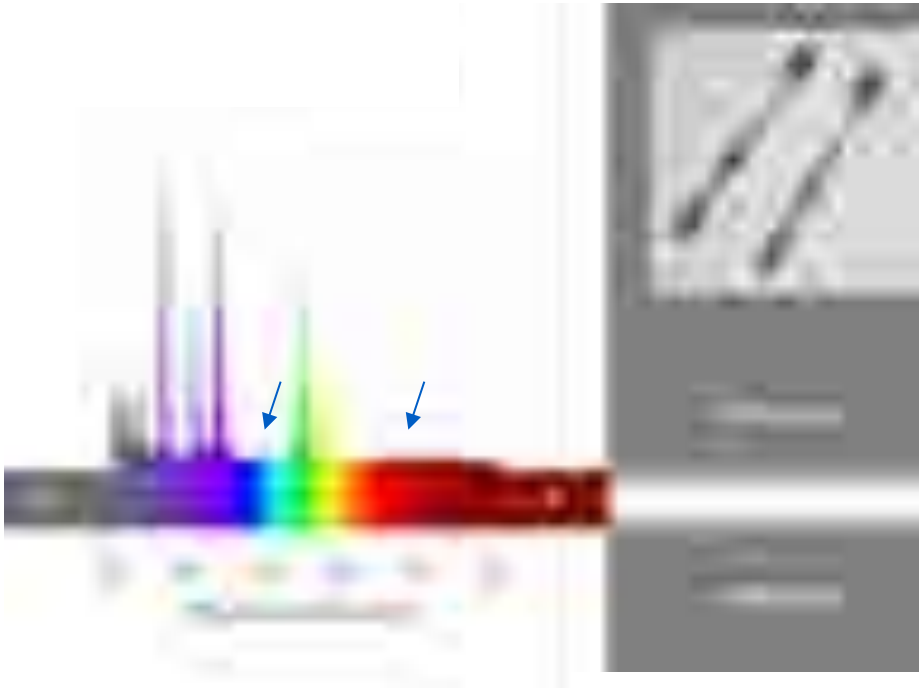
Notice how the ink is focused into a tight stream as it is drawn into the tube under laminar flow conditions.



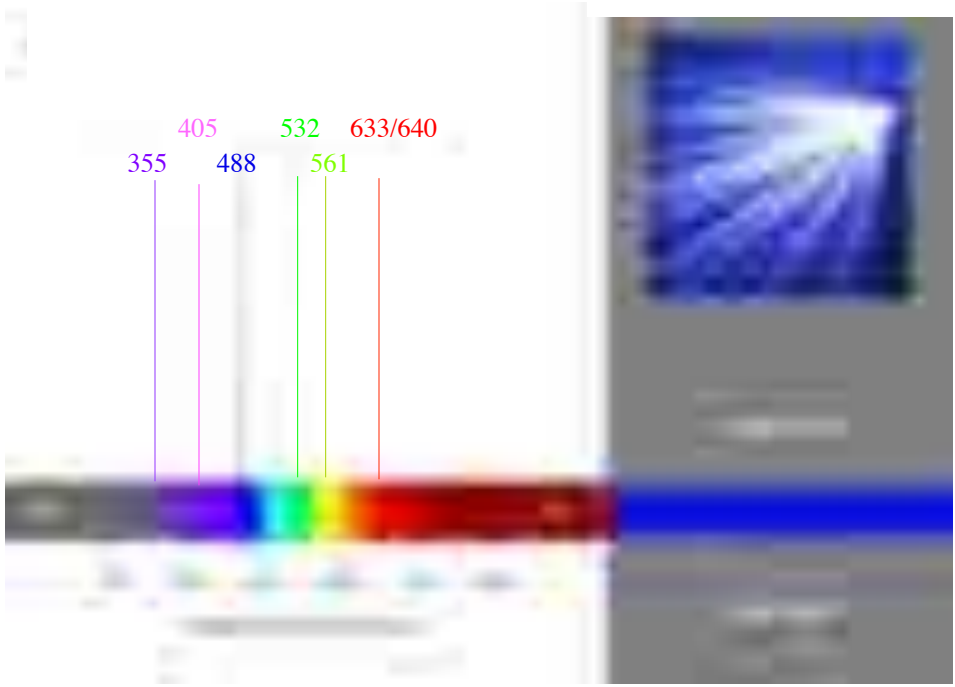
V. Kachel, H. Fellner-Feldegg & E. Menke - MLM Chapt. 3

Compare different Excitation sources

Excitation with multiple wavelengths
(e.g. Microscope)

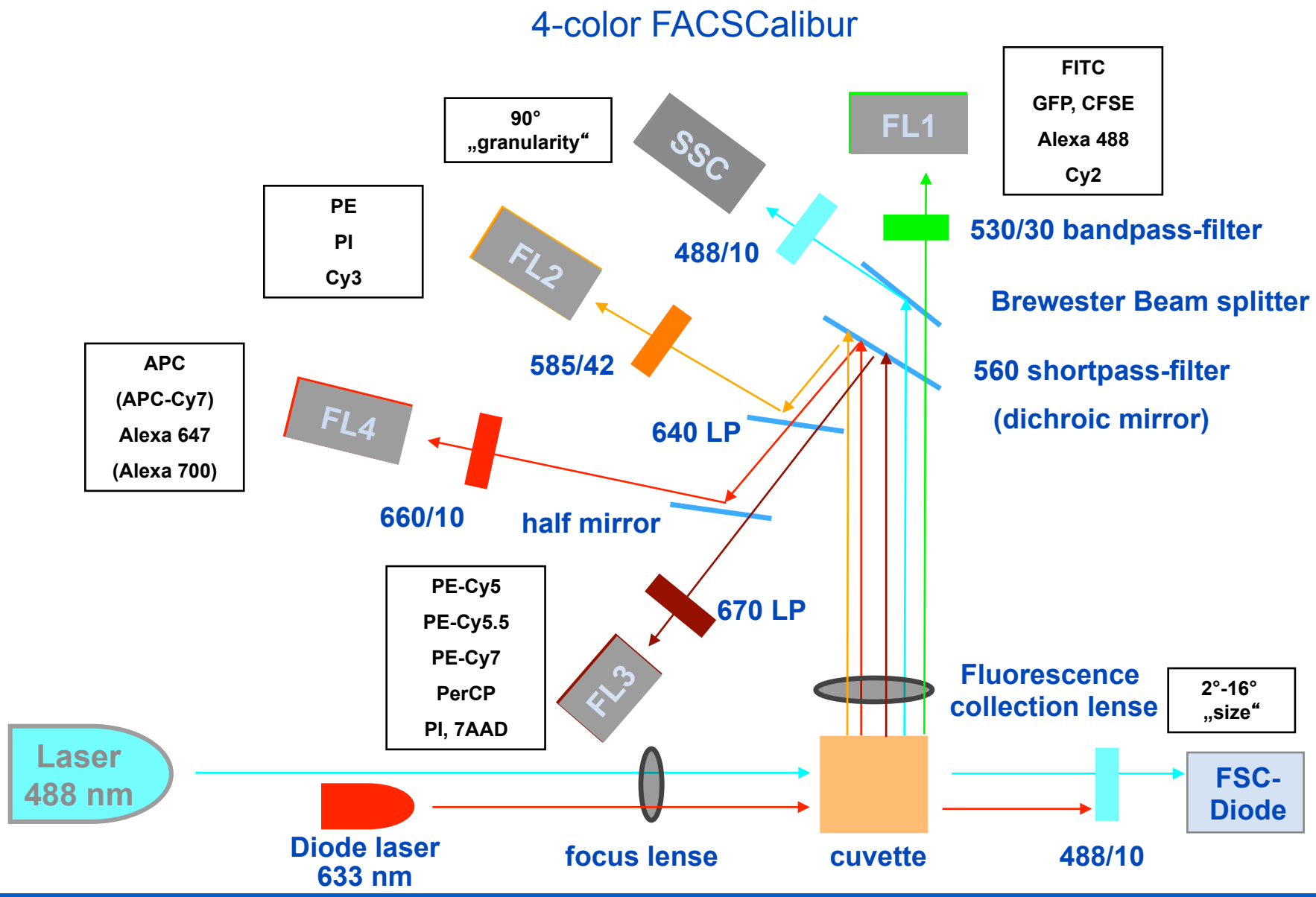


Monochromatic-excitation
(e.g. LSM, FACS)

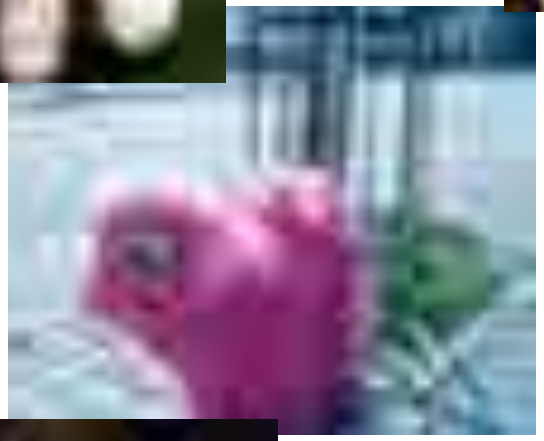


modified from Invitrogen

The optical system



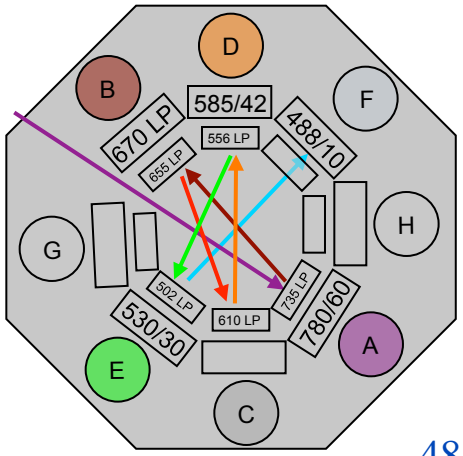


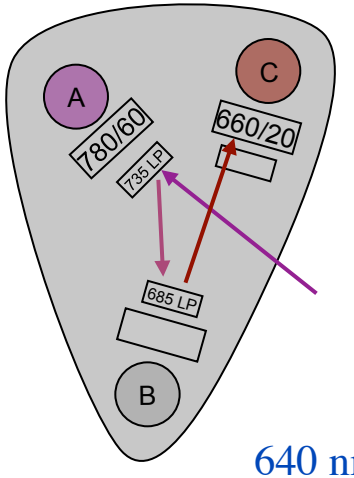



Optical Benches of the FACSCanto II

Configuration: 4-2 = 6 colors (2 laser)

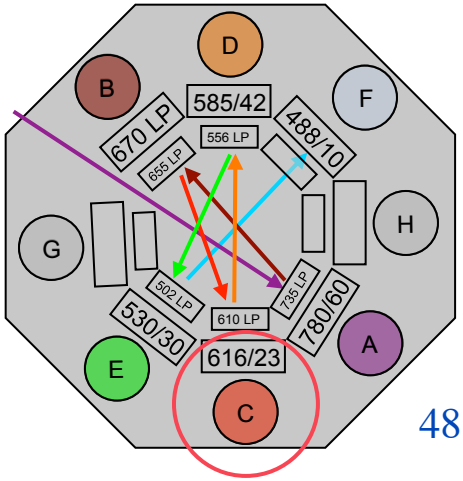


488 nm

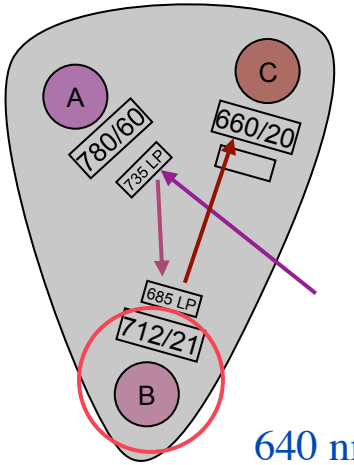


405 nm 640 nm

Configuration: 5-3 = 8 colors (2 laser)

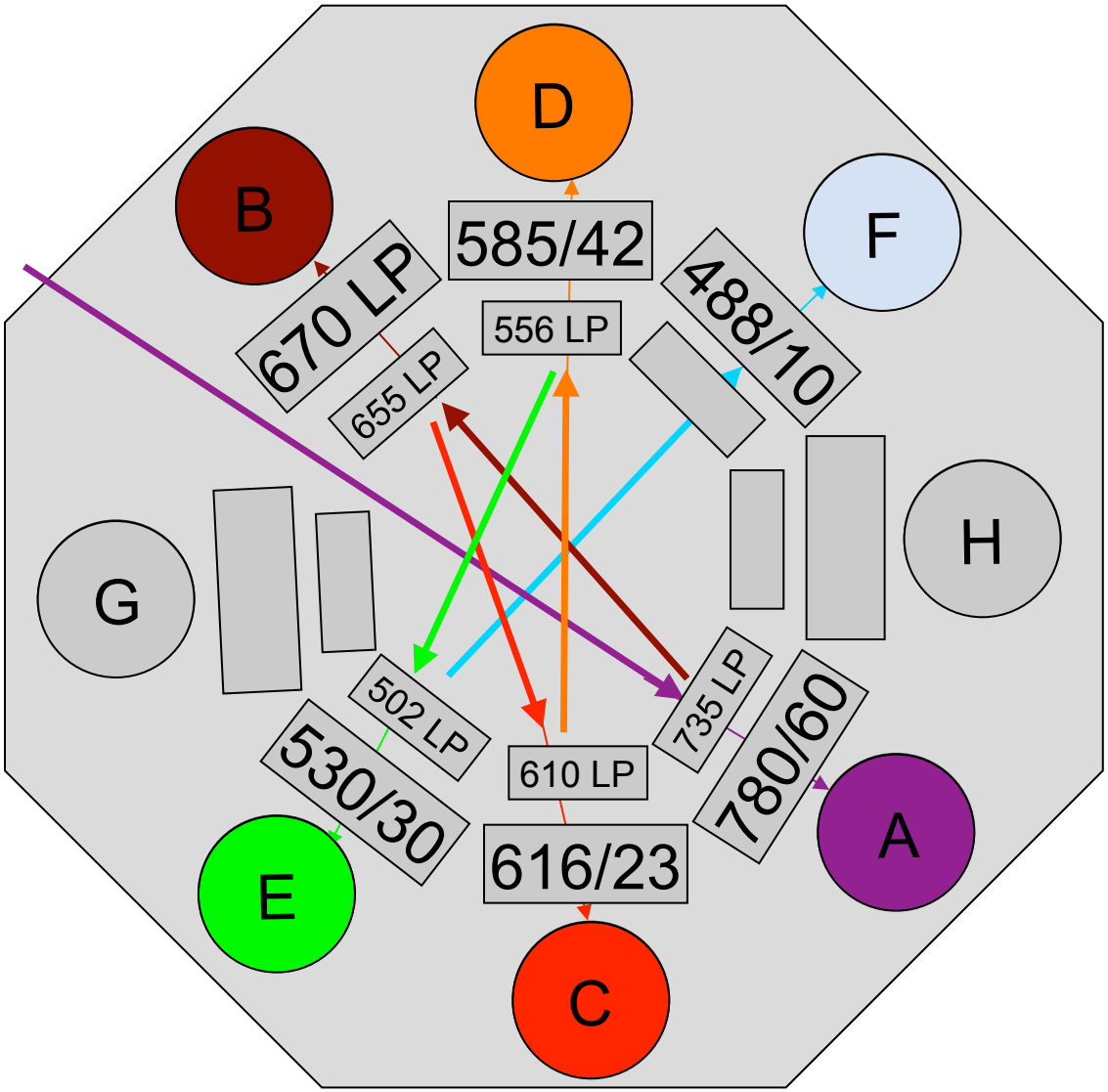


488 nm



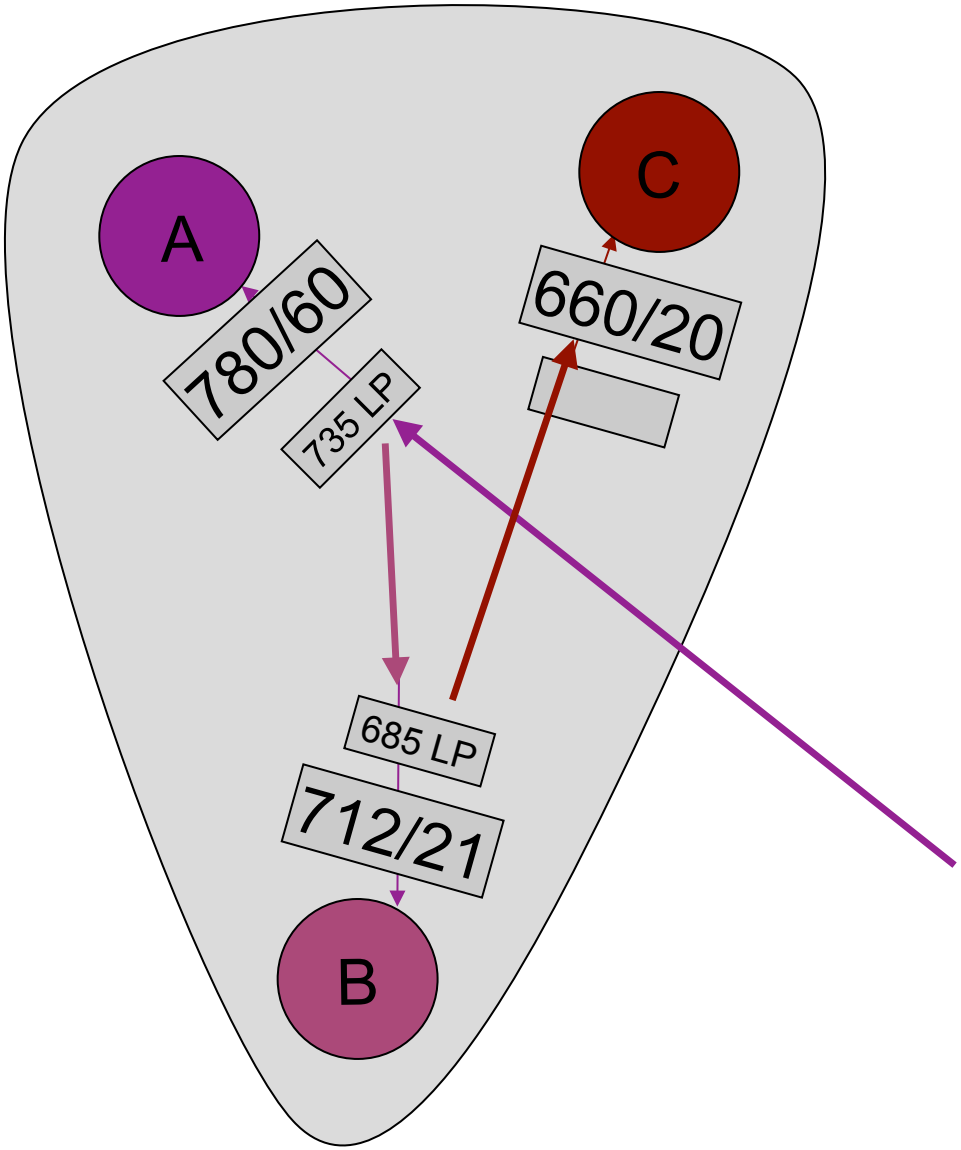
640 nm

FACSCanto II (488nm)



Detector	Dye
A	PE=Cy7, PE=Alexa750
B	PerCP, PE=Alexa647 PE=Cy5, PE=Cy5.5 PerCP=Cy5.5 7AAD, PI, ECD. AO (red)
C	PI, PE=TexasRed
D	PE, PI (orange), Cy3 DsRed, EYFP, DHE JC-1 (high), t-dimer2 (12)
E	FITC, Alexa 488, Cy2 EGFP, GFP, CFSE Fluo-3 und -4, JC-1 (low) Calcein, DCFH, DCFDA BODIBY, Rhodamine 123 NAO, TOTO-1 TO-PRO-1, AO (green)
F	Scatter

FACSCanto II (635 nm)

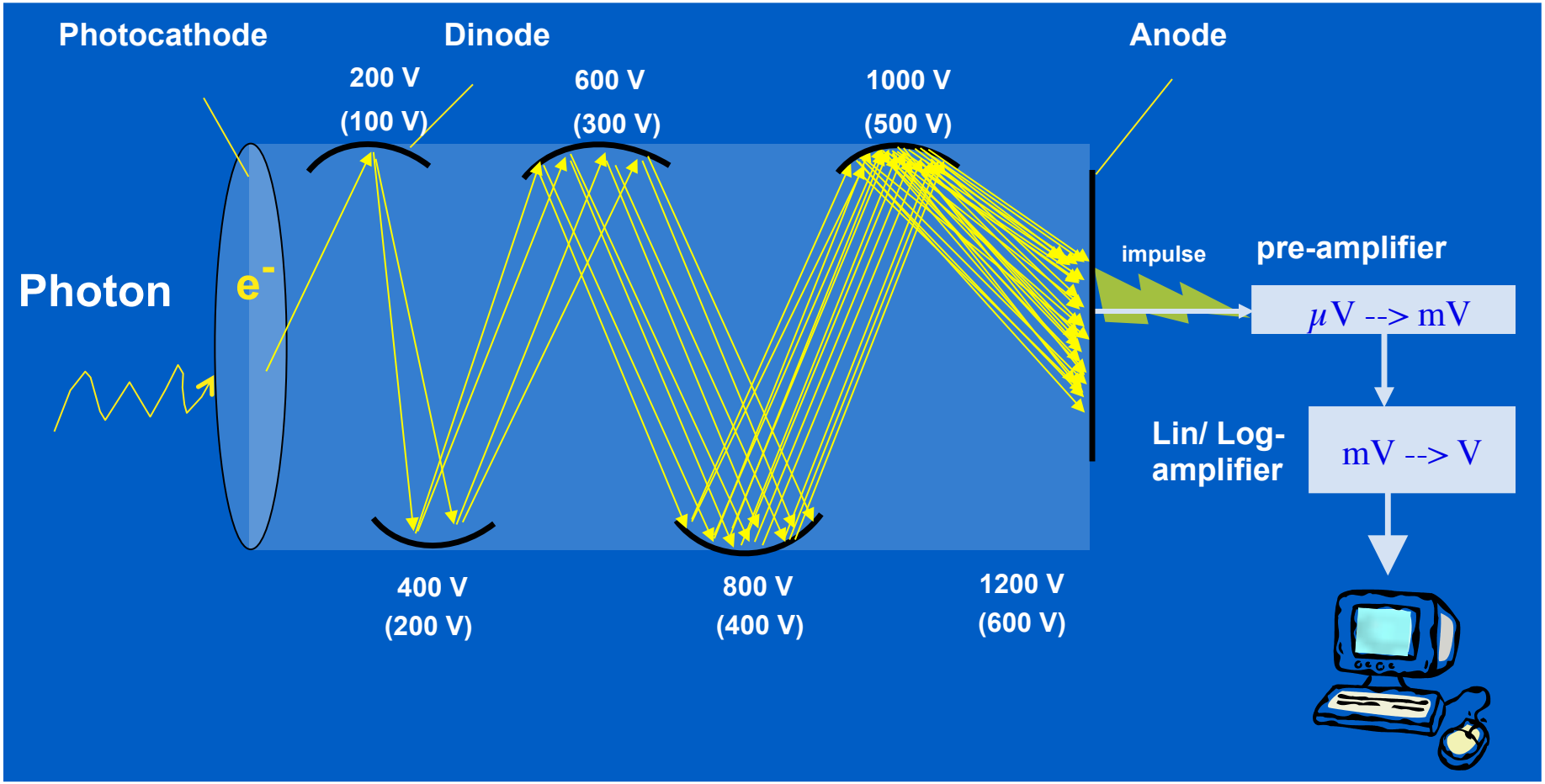


Detector	Dye
A	APC=Cy7 APC=Alexa750
B	Alexa 680 **Alexa 700**
C	APC Alexa 647 Cy5 TO-PRO 3 TOTO 3

Generation of a (FACS-) signal

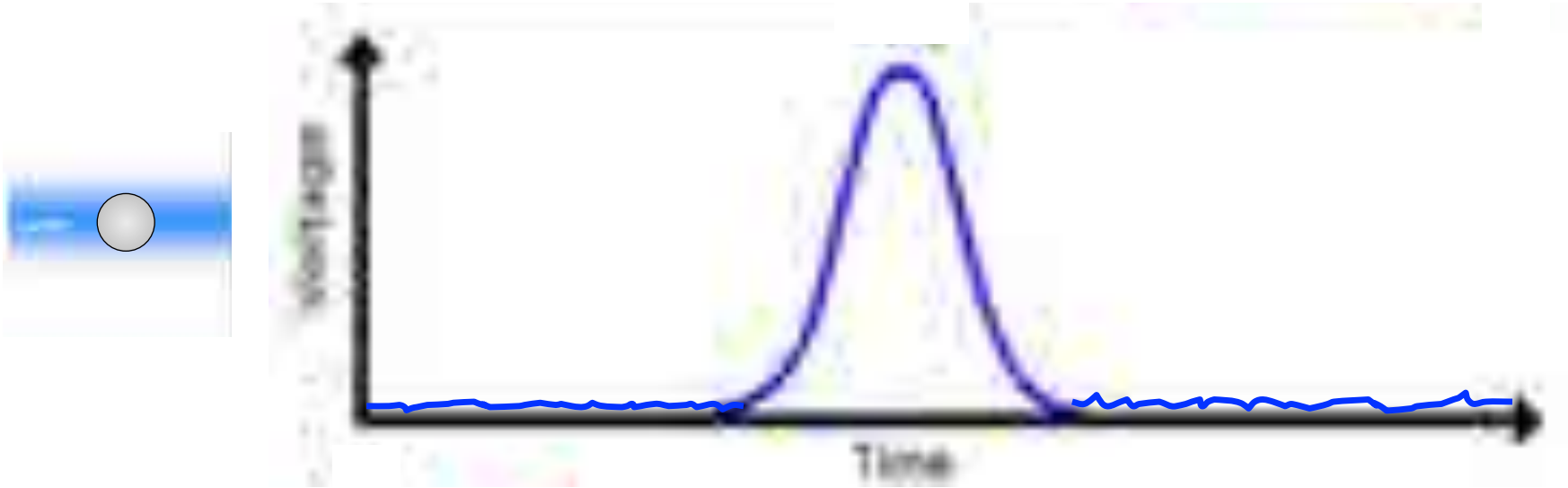
Photomultipliertube (PMT)

A photomultiplier converts incoming light into electrons. Out of one photon up to 10^8 (photon)electrons can be generated („electron amplification“).



Generation of a Pulse

A PMT converts the emitted light into an electrical signal. This signal is called a pulse. The resulting signal intensity is proportional to the light intensity.

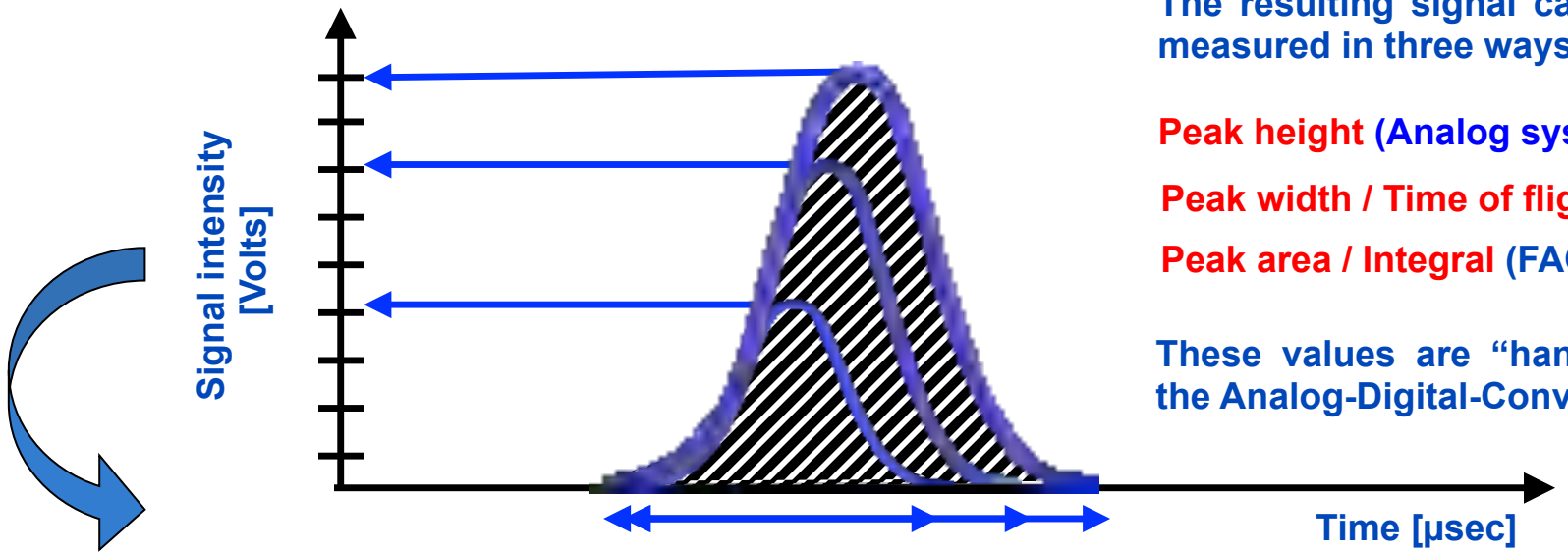


The pulse starts, when a particle enters the laser beam. At this point are both intensities (laser and signal) low.

A pulse reaches its maximal intensity (signal), when the cell is in the middle of the laserfocus.

The particle leaves the laser beam and the signal gradually returns down to zero.

Presentation of the Data



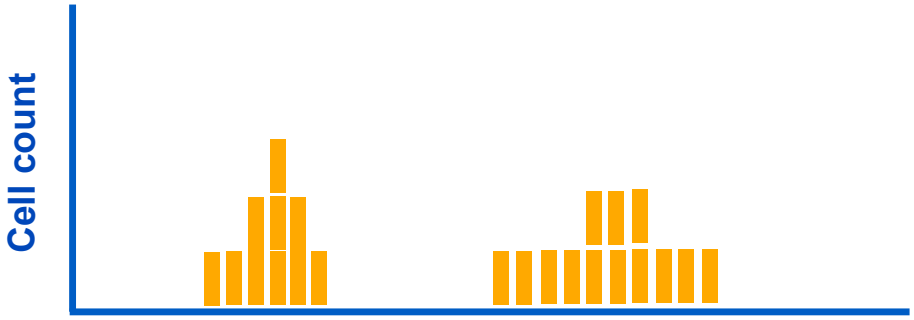
The resulting signal can be measured in three ways:

Peak height (Analog systems)

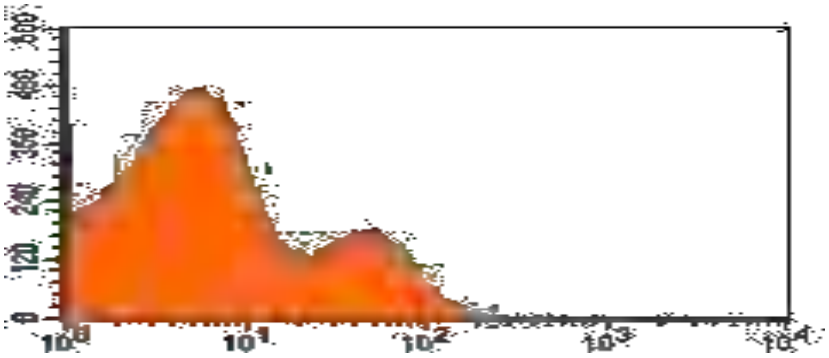
Peak width / Time of flight

Peak area / Integral (FACSCanto)

These values are “handed over” to the Analog-Digital-Converter (ADC)

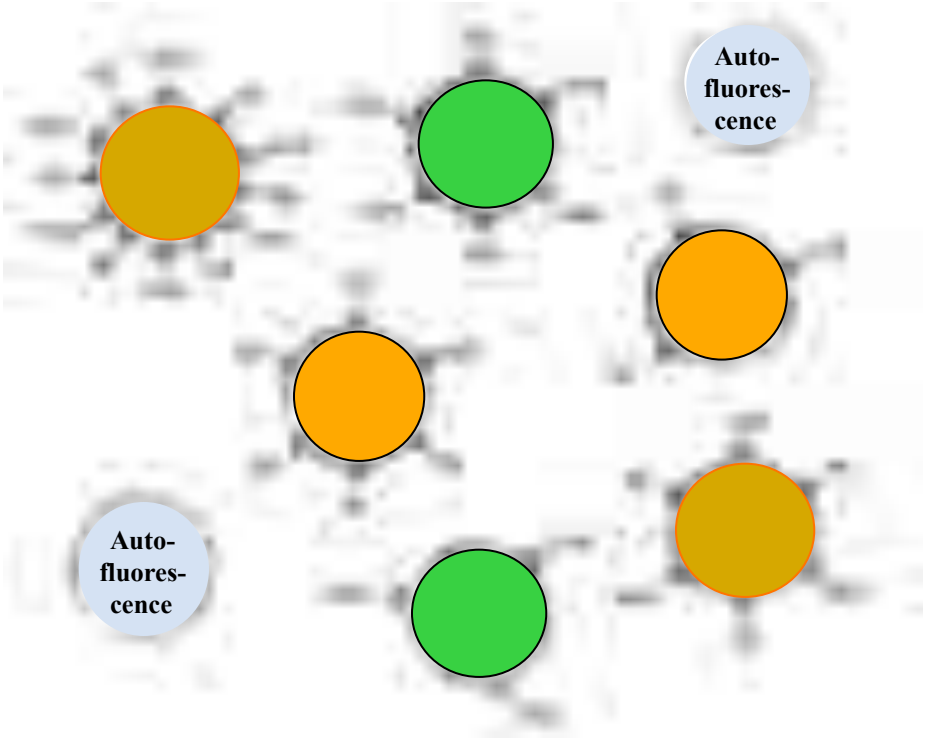


Signal intensity (area / height / width)



Example: surface antigenes

The classical method to count fluorescent cells is under the fluorescence-microscope (e. g. standard microscope with high-pressure mercury-arc lamp and lightfilter-block). Determination of CD4/CD8 T-cell ratio of peripheral blood-lymphocytes (after Erylyse) or thymocytes labeled with specific fluorescent antibodies against their respective surface-antigenes .



Antigene A e.g. CD4 => T-helper-cell

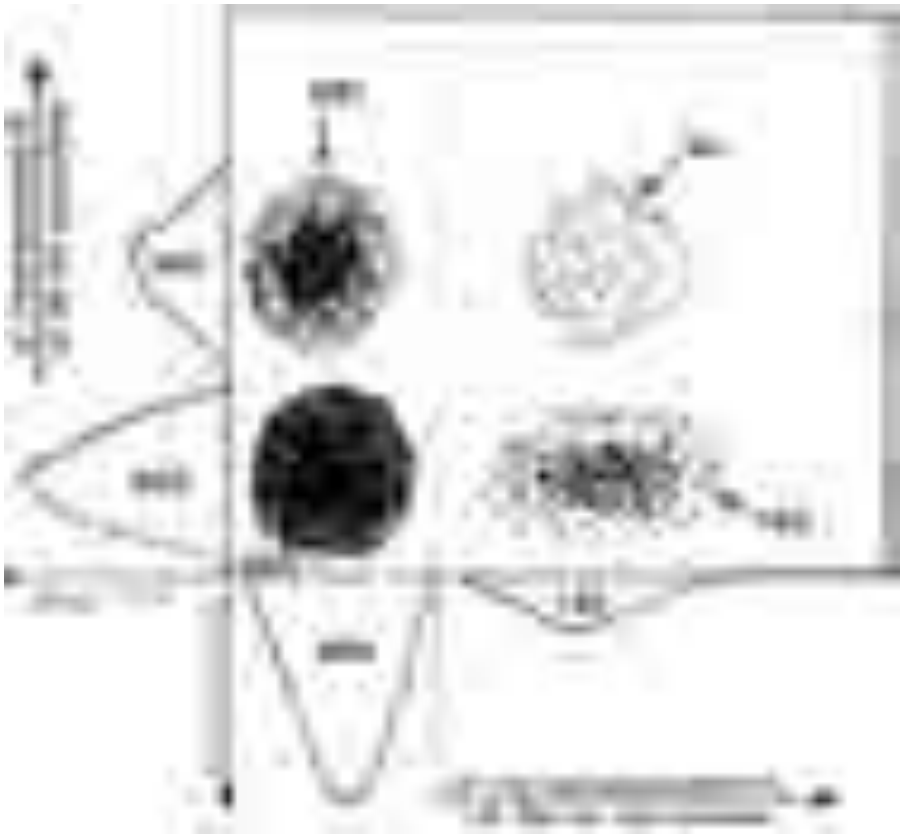
Antibodies versus antigene A, conjugated with Fluorescein-Iso-Thio-Cyanat (FITC)

Antigen B e.g. CD8 => cytotoxic T-Cell

Antibodies versus antigene B, conjugated with Phycoerythrin (PE)

Why Dot - Plot?

Example : Double - Fluorescence with FITC / PE (example surface antigens)



4 possible subpopulations :

1. auto-fluorescent cells
2. only FITC labelled cells (Antigen A)
3. only PE labelled cells (Antigen B)
4. FITC and PE labelled cells (Antigen A and B)

Conclusion : the double-fluorescent cells can not be statistically captured through the respective single-histograms → only possible through Dotplot and „Quadrant“-statistic !

Data Storage as List Mode Data

. . . 19 parameter

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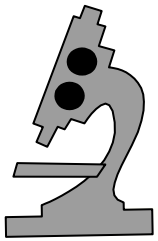
.

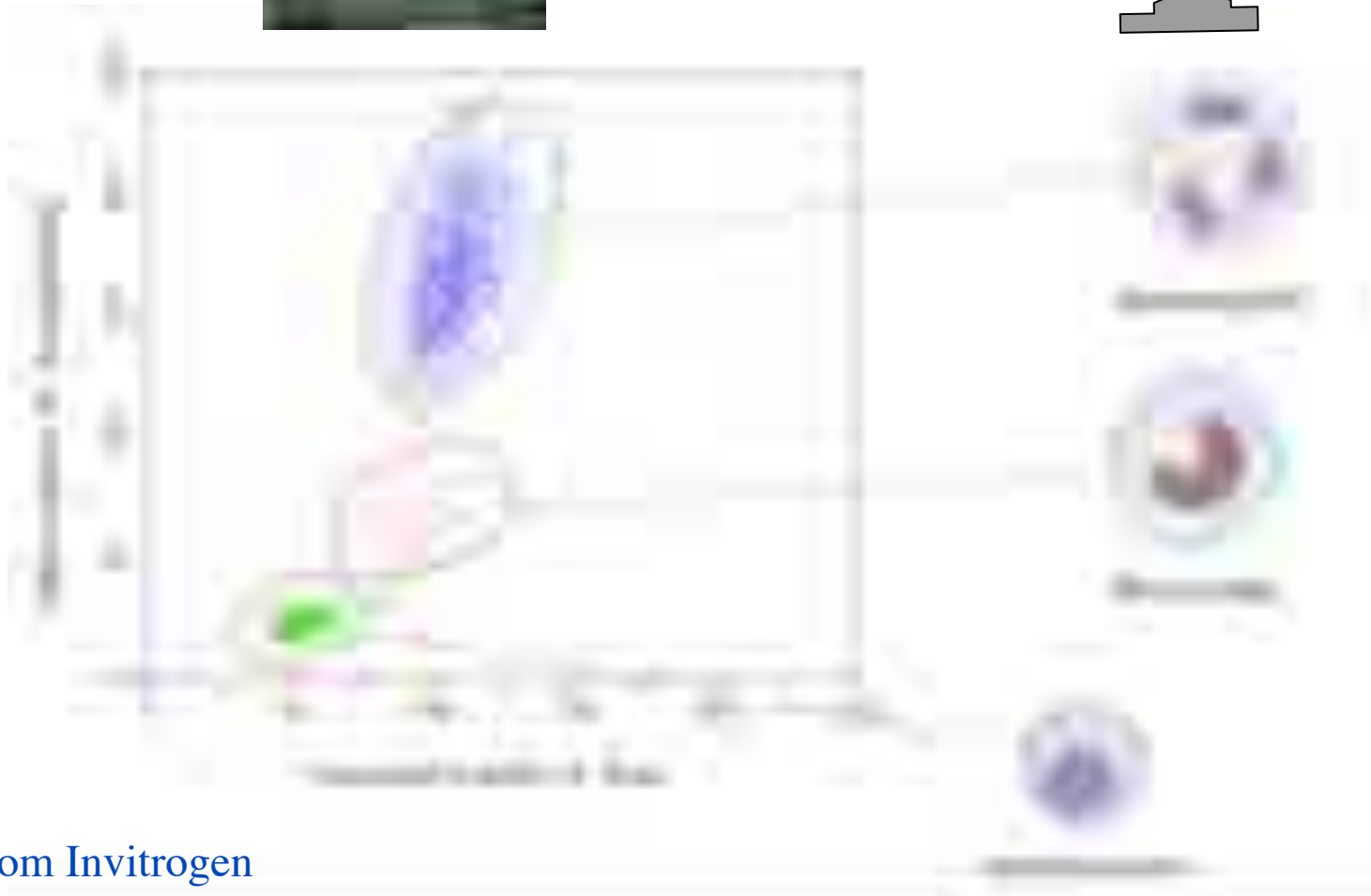
event n (often 10.000)

When a computer saves data from the cytometer, it is saved as list-mode data. This is simply a listing of cell (or particle) parameters and their measurements on a cell by cell basis. Data can be displayed in different plot types. For example, a dot plot take two parameter and plot them against each other.

modified from BD Biosciences

Visualizing Blood cells





adapted from Invitrogen

Acknowledgements

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BD Biosciences
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Invitrogen
Partec

Some other slides were adapted from slides you can find in the www or in the sources shown on slide 17.

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