

Label-free Cell Differentiation In Microfluidic Flow Cytometers by Light Scattering and HF-Impedance Measurements

Jörg Neukammer
Cytometry and Particle Analysis

Disposable devices

- to support rapid diagnosis in medicine
- for process control in bioreactors



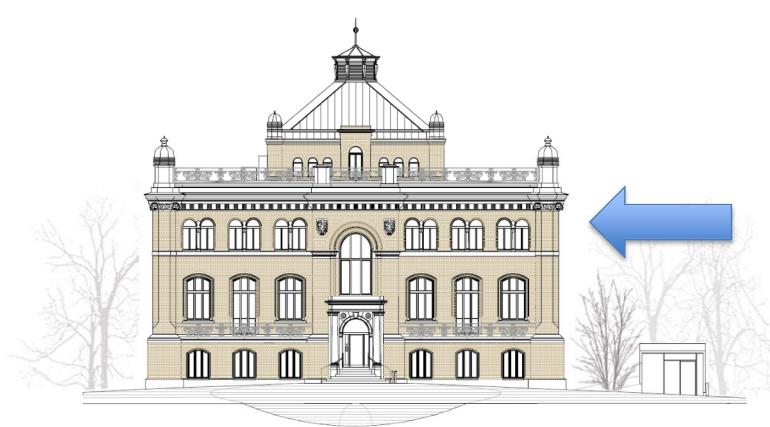
Counting and Measuring since > 125 Years



Founded in 1887 as the Physikalisch-Technische Reichsanstalt (PTR) on the basis of the ideas of Werner von Siemens and Hermann von Helmholtz and on their joint initiative



Hermann von Helmholtz (1887):
"Zählen und Messen erkenntnis-theoretisch betrachtet"
"Epistemological considerations on Counting and Measuring"



About PTB

PTB – Germany's national metrology institute
is a higher federal institute providing
scientific and technical services

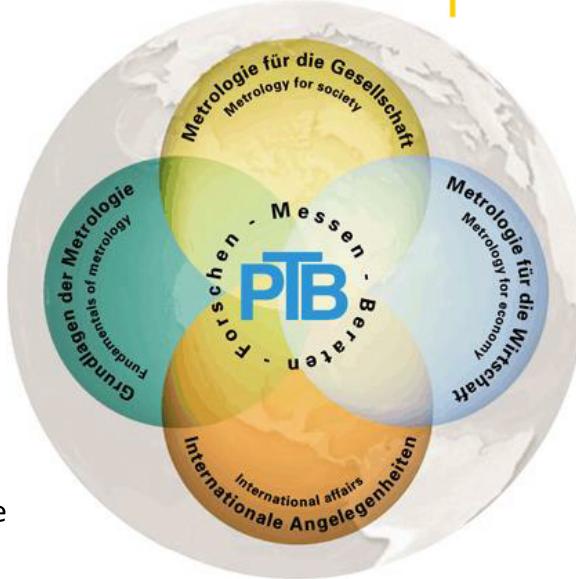
What are PTB's capabilities?

PTB measures with the highest
accuracy and reliability
– metrology as the core competence

For whom does PTB work?

Progress and reliability in metrology for the
benefit of society, trade and industry, and science

e.g. determination of fundamental and natural constants;
the realization, maintenance and dissemination of the legal units of the SI;
development of reference procedures & instruments; standardization; ...



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Metrology – The Science of Measurement

The Second:

- PTB uses atomic clocks of the latest generation
- Measurement uncertainty: $<10^{-15}$ ($0.03 \mu\text{s/year}$)
- Development of “optical clocks” for the future



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The Kilogram:

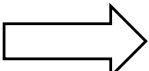
- PTB keeps and uses the national copies of the kilogram prototype

• Avogadro - Project:

Redefinition of the kilogram on an atomic basis

- Reduction of uncertainty from 0.1 ppm to 0.01 ppm

- Redefinition of the kilogram using a 1kg ^{28}Si silicon sphere



External Quality Assurance in Laboratory Medicine

- In vitro diagnostic medical devices (Directive 98/79/EC)

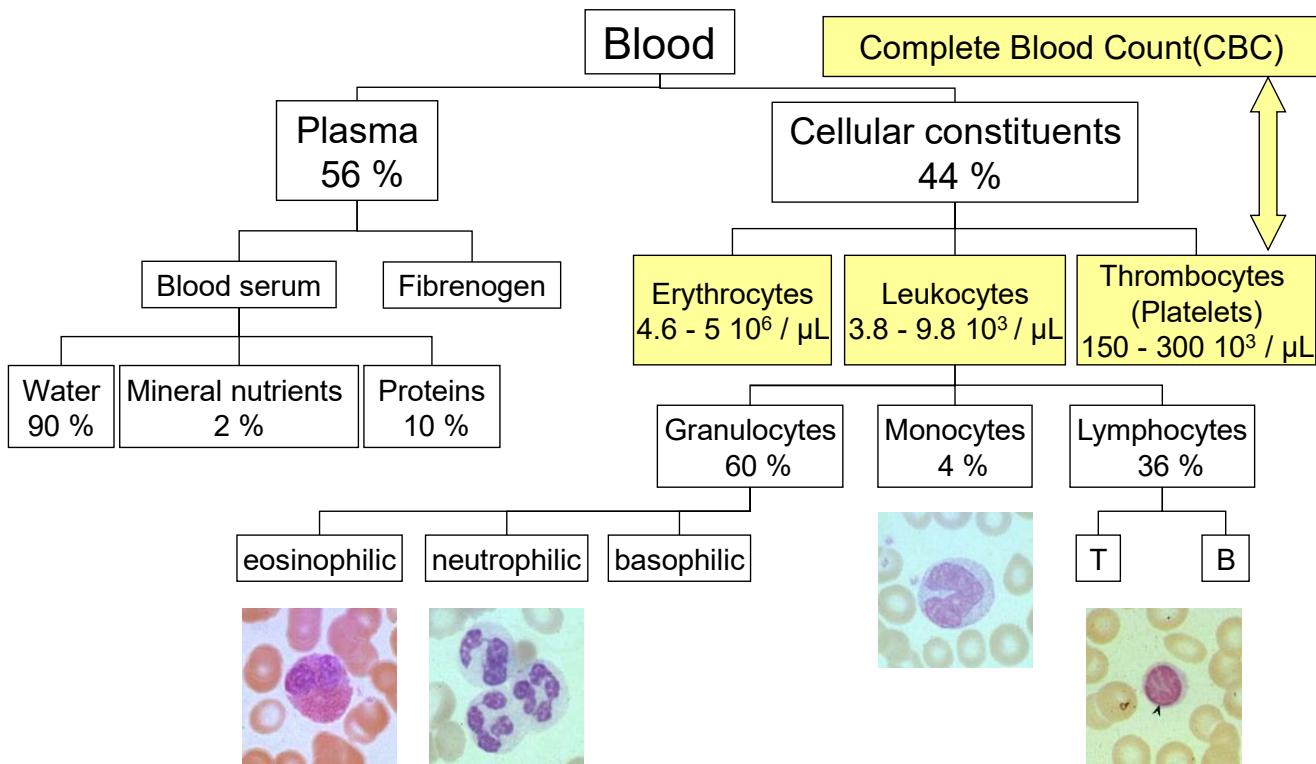
- Medical Devices Act, § 32 (3):

The Physikalisch-Technische Bundesanstalt is responsible for fostering and safeguarding metrology in medicine. In particular, the PTB is required

1. *to provide expert opinion on medical measuring devices and - if notified according to § 15 - to perform type approvals*
2. *to develop reference measuring techniques, reference instruments and test equipment, and to validate such techniques and instrumentation on request*
3. *to consult the competent authorities and notified bodies on scientific matters.*

- Guideline of the German federal medical association (RiLiBÄK) on Quality assurance of quantitative measurements in laboratory medicine

- Authorisation of medical scientific associations to carry out ring trials



Cell counting in the Working Group "Flow Cytometry and Microscopy"

Determination of reference values

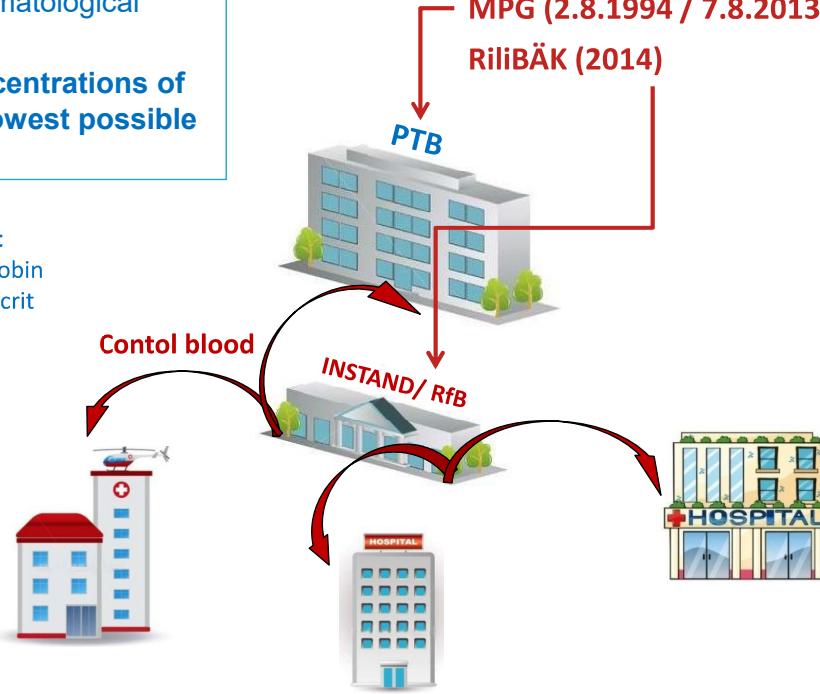
for quality assurance of haematological laboratories in Germany

Aim: Measurement of concentrations of blood cells with the lowest possible uncertainties

IVD-Directive 98/79/EC

MPG (2.8.1994 / 7.8.2013)

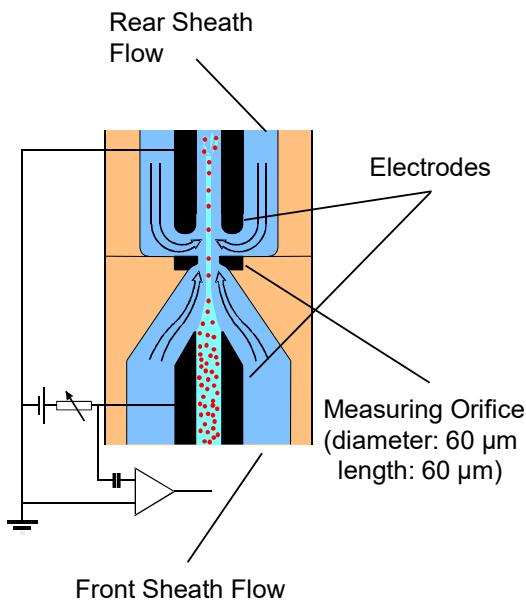
RiliBÄK (2014)



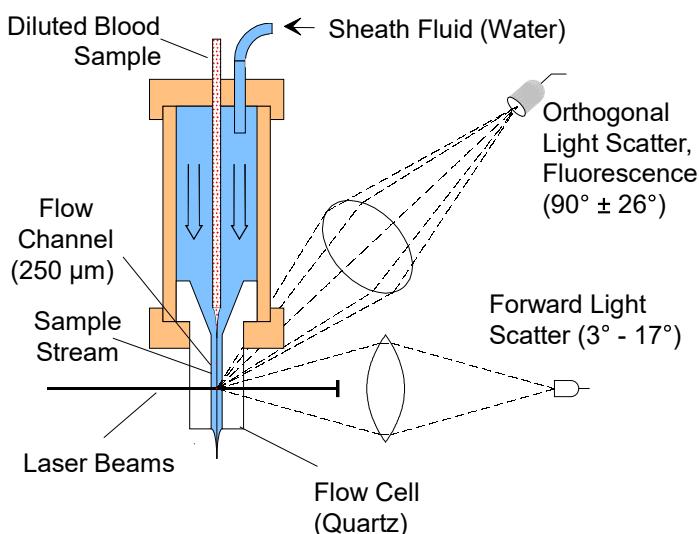
German Medical Scientific Associations:



Impedance counting

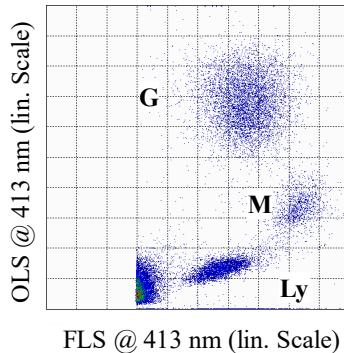
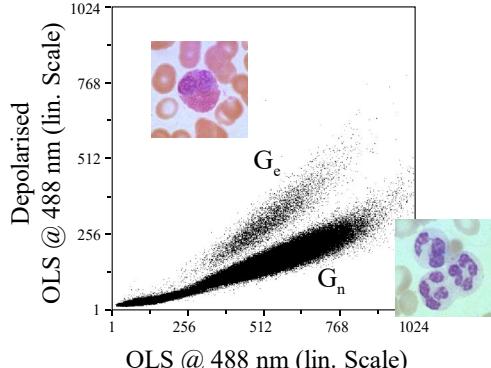
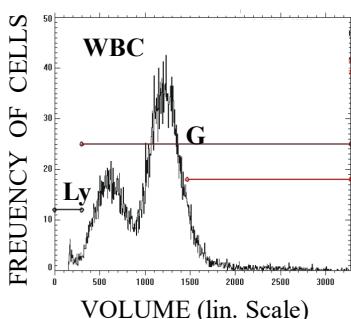
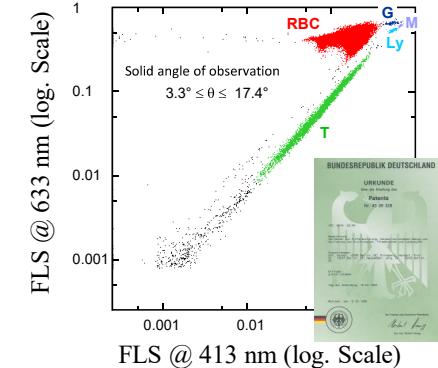
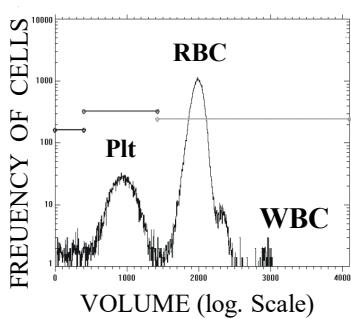


Optical cell counting



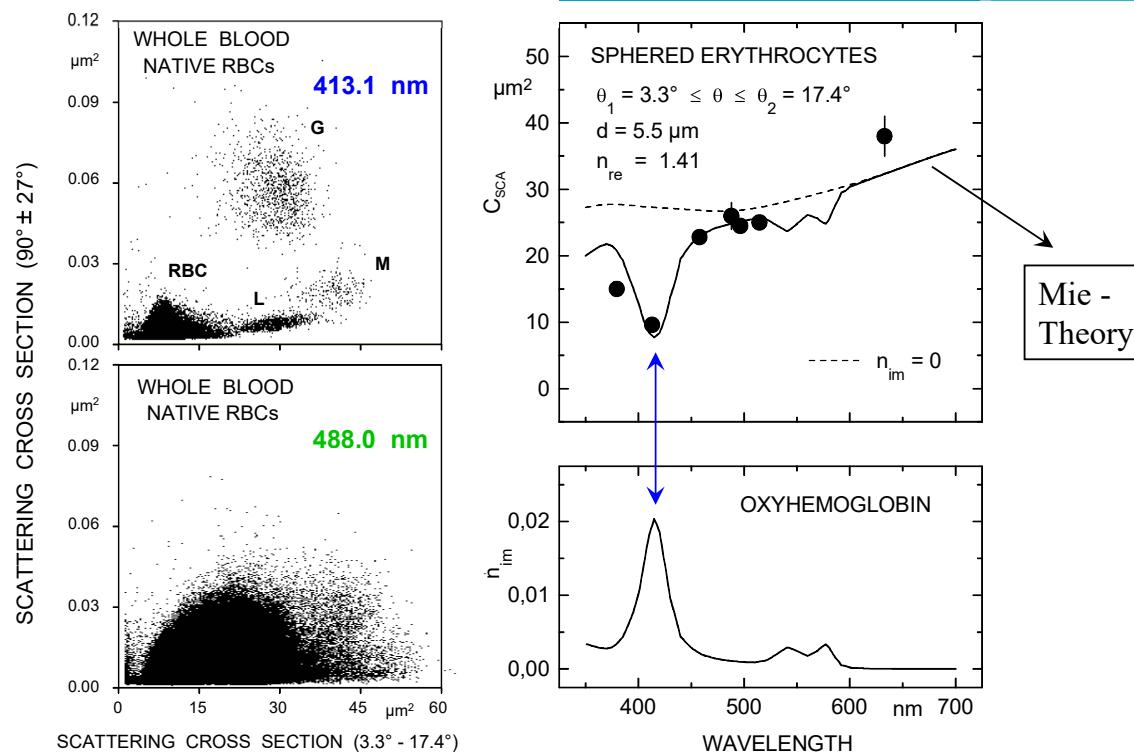
- Simultaneous detection of erythrocytes (RBC), leucocytes (WBC), and thrombocytes (Plt)
- Interaction time 2 μ s
- Analysis of typical 5000 cells / s
- Fluorescence sensitivity \approx 500 fluorochromes

Flow Cytometric Differentiation of Blood Cells



RBC: Red Blood Cells
Plt: Platelets
WBC: White Blood Cells
G: Granulocytes
Ly: Lymphocytes
M: Monocytes
FLS: Forward Light Scatter
OLS: Orthogonal Light Scatter

Differentiation of RBC / WBC by Light Scatter



V. Ost, J. Neukammer, H. Rinneberg, *Cytometry* 1998, 32, 191-197

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Label-free Blood Cell Differentiation in Disposable Micro Flow Cytometers

Point of care tests (POCT)

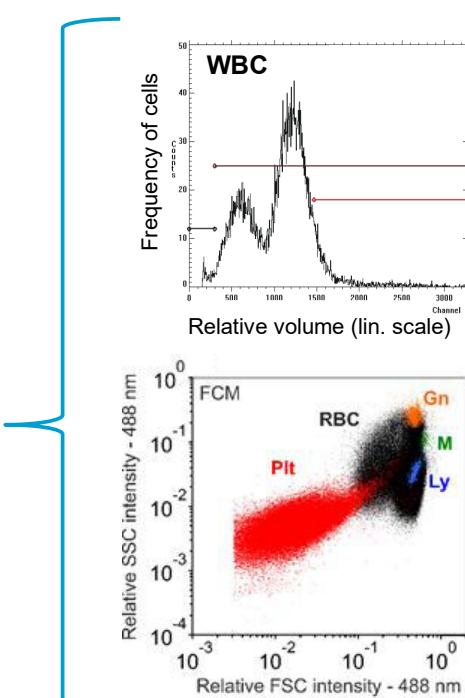
- to initiate transfusion during surgery
- for emergency medicine
- combined with telemedicine for remote monitoring (ships, airplanes)
- applicable in resource poor countries

Present status:

- DC-impedance requires lysis of RBC
- light scattering @ 488nm does not allow to identify WBC subpopulations
- staining with antibodies is time consuming

Control of bioreactors

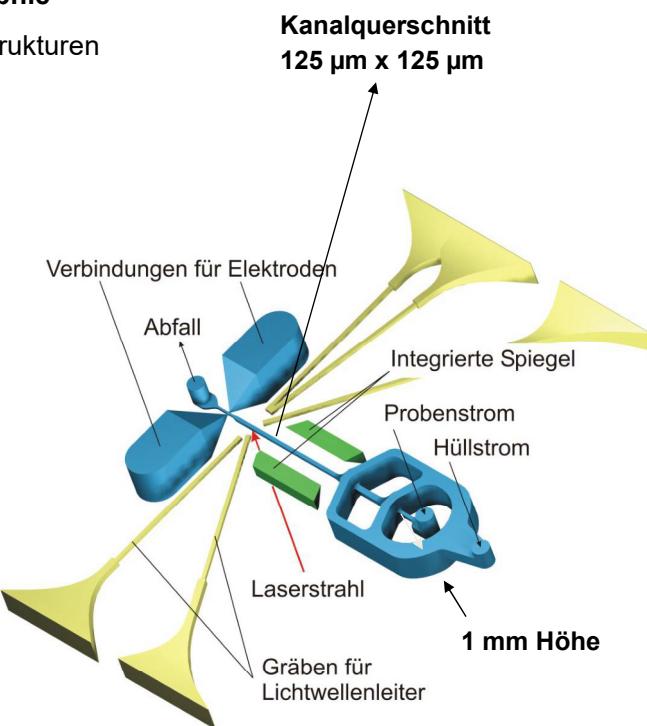
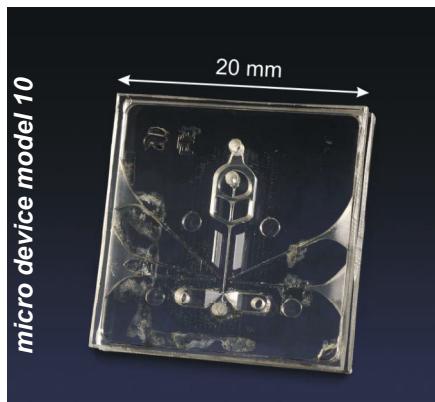
- H_2 producing algae



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Ultra-Präzisionsfrästechnik statt Lithographie

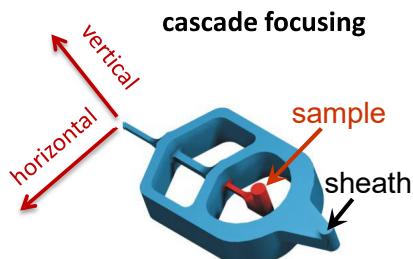
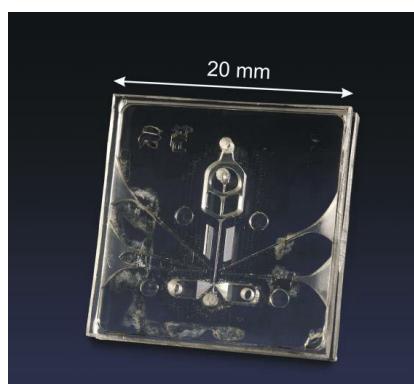
- komplexe 3D (statt 2,5 - dimensionale) Strukturen
- kürzere Entwicklungszeiten
- kostengünstigere Fertigung
- höhere Variabilität, z.B. 45° Spiegel
- Geschwindigkeit ≈ 3 m/s



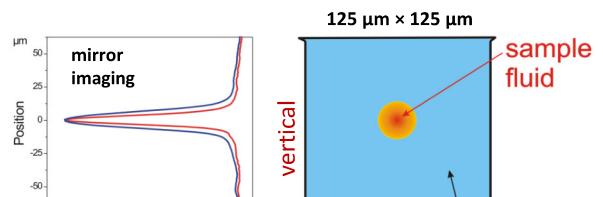
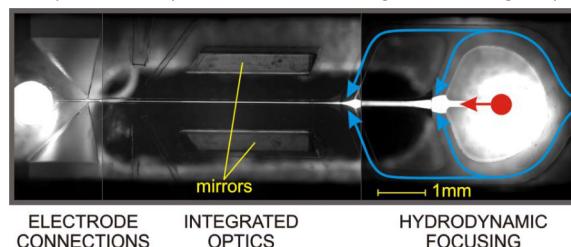
- 13 -

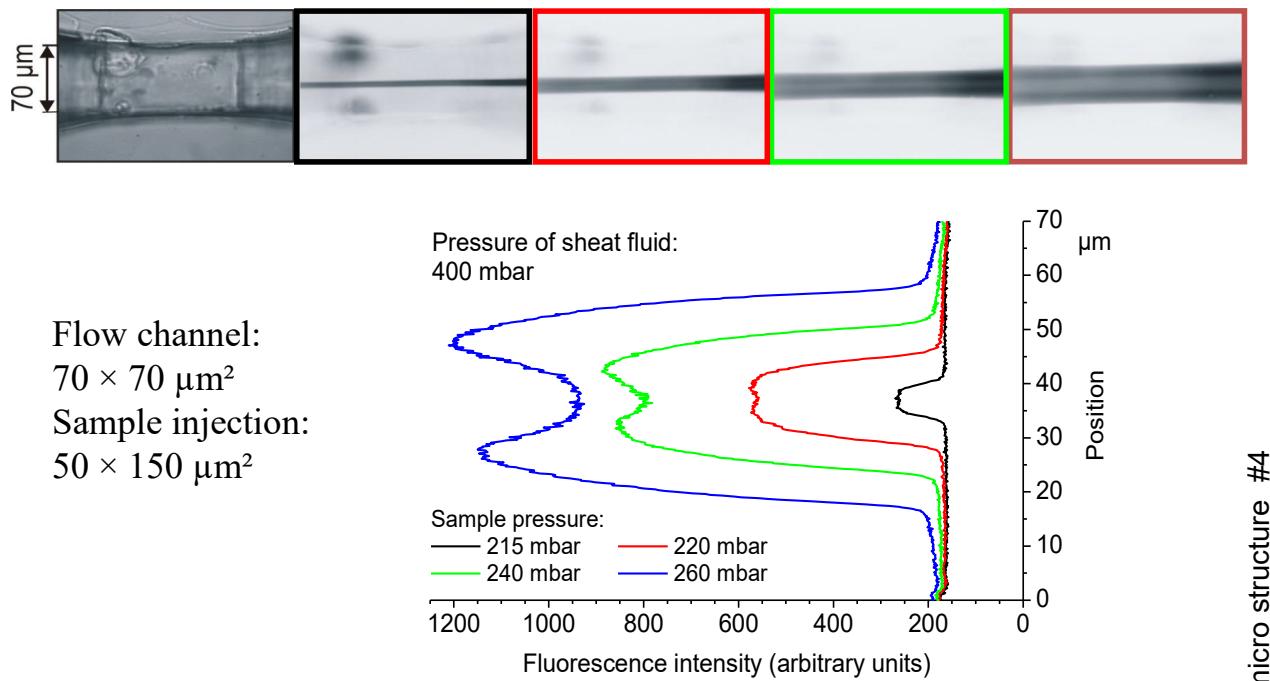
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Development of POCT: Hydrodynamic Focusing in Micro Fluidic Structures

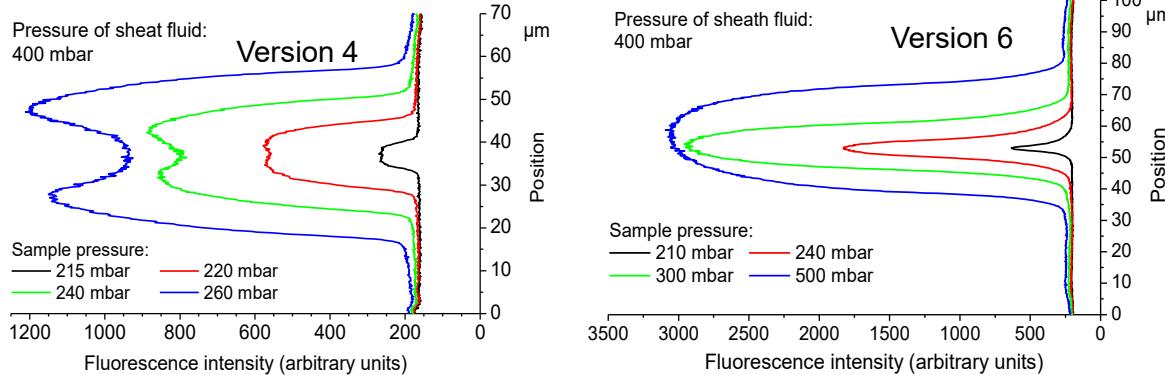


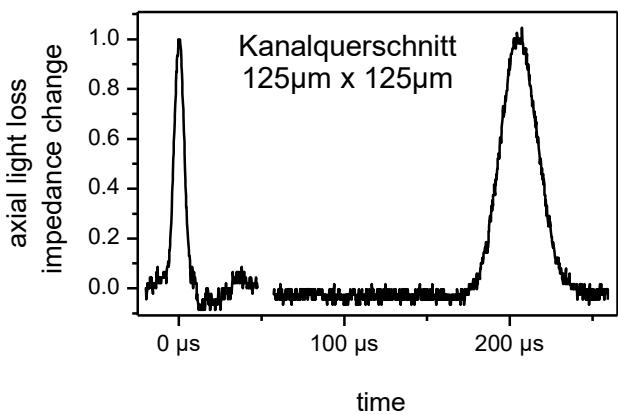
microscopic fluorescence image:
sample = Rh6G dye, sheath = water , light source : Hg lamp



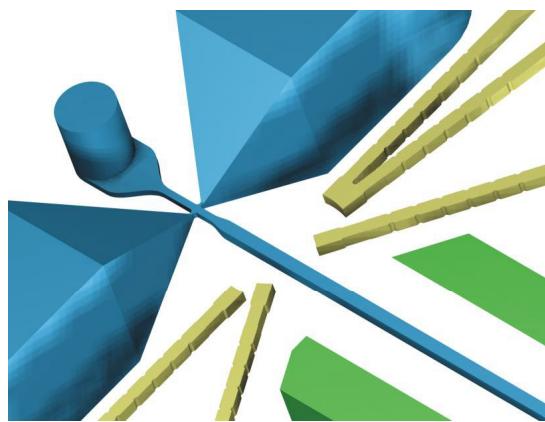


Comparison of FEM Calculations and Experimental Observation





Steigerung der Empfindlichkeit:
Reduktion des Kanalquerschnittes
auf 70μm x 70μm

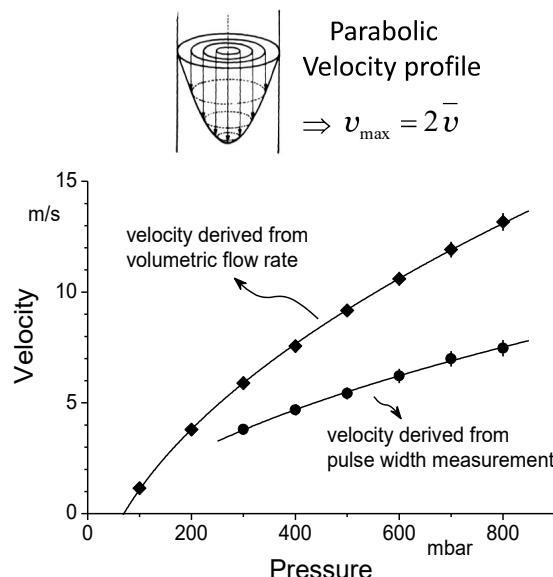
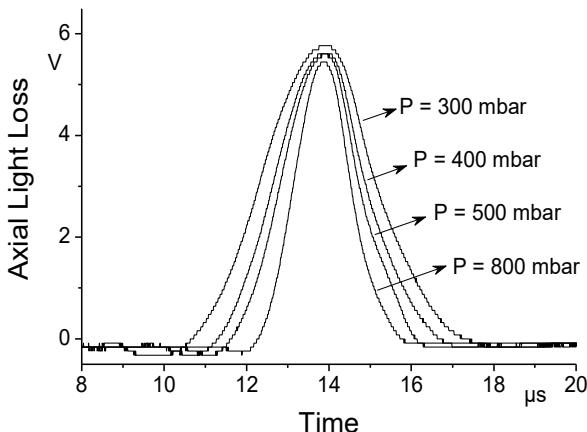


Ergebnisse:

- Externe Pt-Elektroden im Anschlusskopf
- Reduktion des Kanalquerschnittes erfordert integrierte Kammfilter

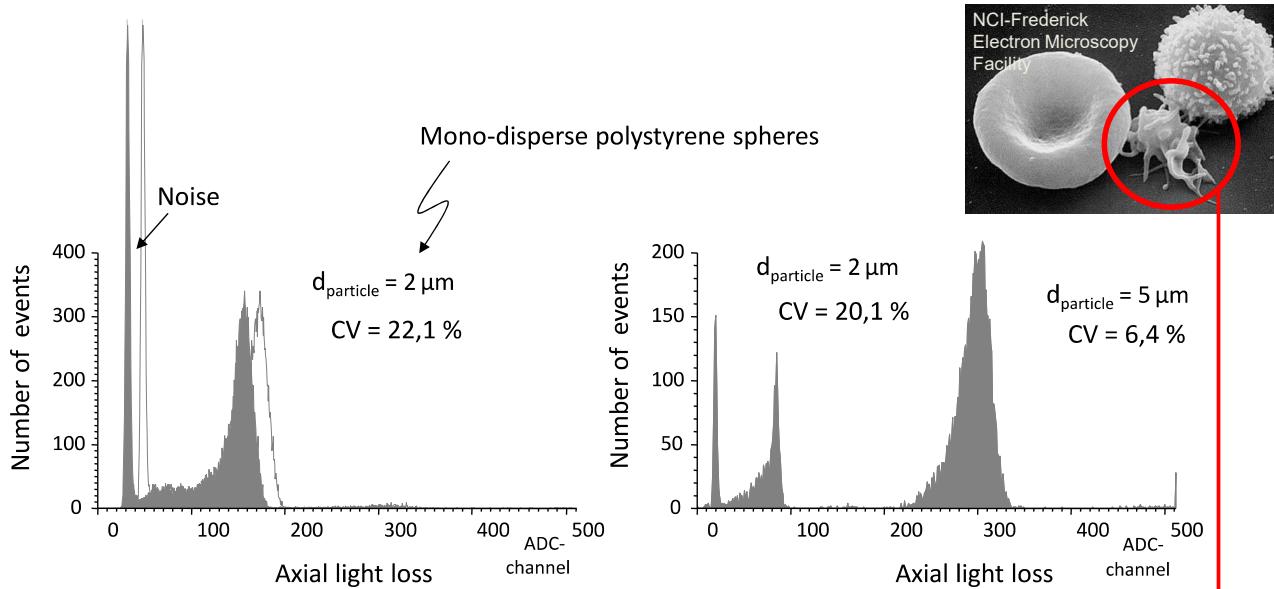
Velocity of Flow Derived from Pulse Width Measurements of ALL

Pulse width dependence
on sheath fluid pressure
ALL: Axial Light Loss



- ⇒ no parabolic velocity profile in structure #4,
- ⇒ to small distance between sample injection and interaction region

Axial Light Loss Measurements: Sensitivity & Pulse Height Variation (Chip Version #4)



$d_{particle} = 2 \mu\text{m}$ (633 nm) \Rightarrow not sufficient to detect **platelets**
(average diameter 2.3 μm , but significantly smaller refractive index)

CV typically 25 – 30 % for microstructures, 6.4 % should be further reduced to 2% for 5 μm particles

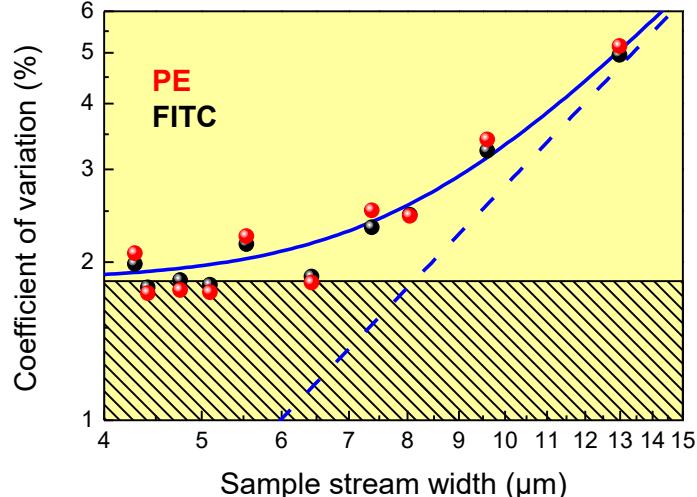


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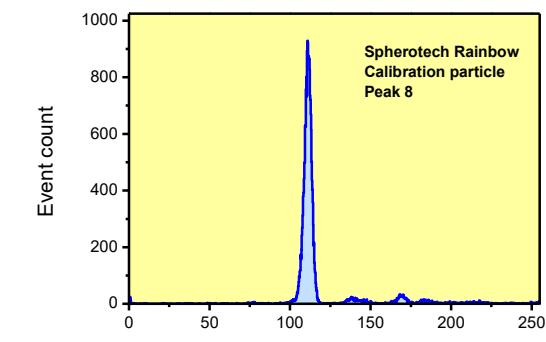
Performance of Micro Flow Cytometers: Signal Stability Determined by CV Measurements



- Typical for 1D-focusing: CV 20%
- Routine laser flow cytometer: CV 2-3%
- Microfabricated flow cytometer: CV<2%



Monodisperse calibration particles
(fluorescence, peak 8)



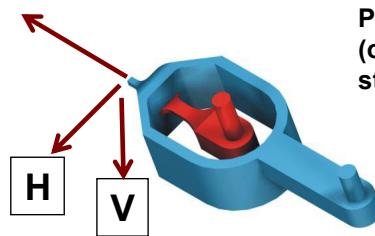
CV limited by
calibration
material used

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Development of POCT: Hydrodynamic Focusing in Micro Fluidic Structures



- Single step focusing

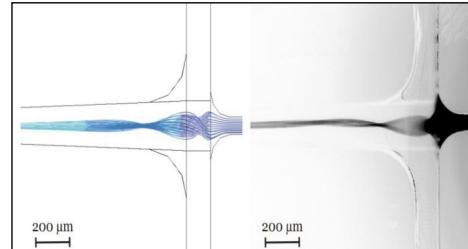


Patent US 2004 0043506
(one dimensional single step focussing)

- Cascade focusing



- Spin-focusing

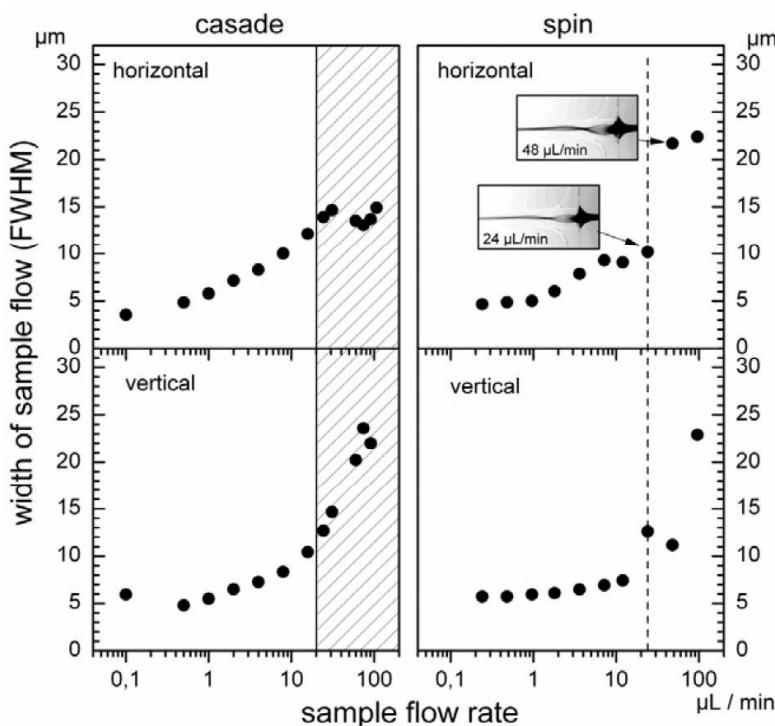


J. Theisen et al., Patent DE102007017318B4 2007

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Development of POCT: Stability of Hydrodynamic Focusing in Micro Fluidic Structures



⇒ easy adjustment
of count rate by
variation of flow
rate

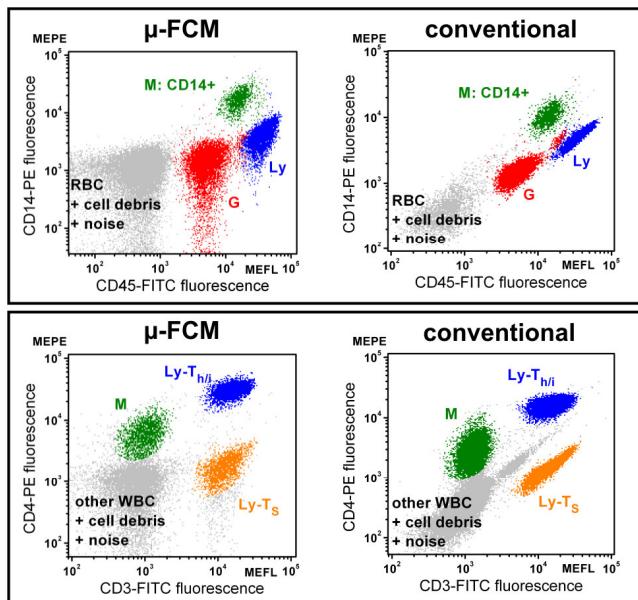
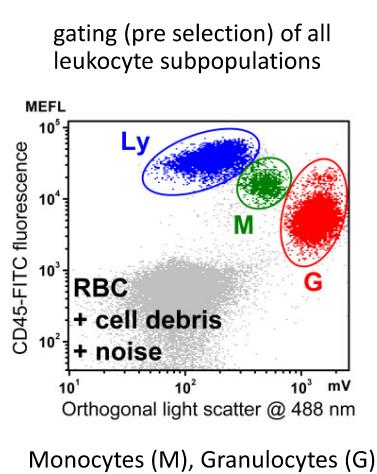
Microflow Cytometer for the Determination of the Immune Status



Lysis of RBCs,

Differentiation of **lymphocytes** by antibody staining (CD: cluster of differentiation)

T-Helper-Cells (Ly-T_h) , T-Suppressor-Cells (Ly-T_s) , B-Cells



M. Frankowski et al., Cytometry Part A, 2011, **79**, 613–624



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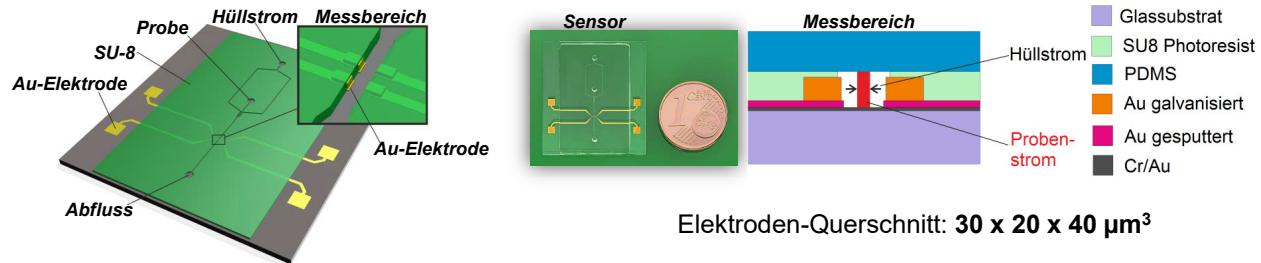
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Mikrofluidische Zytometer: μFCM

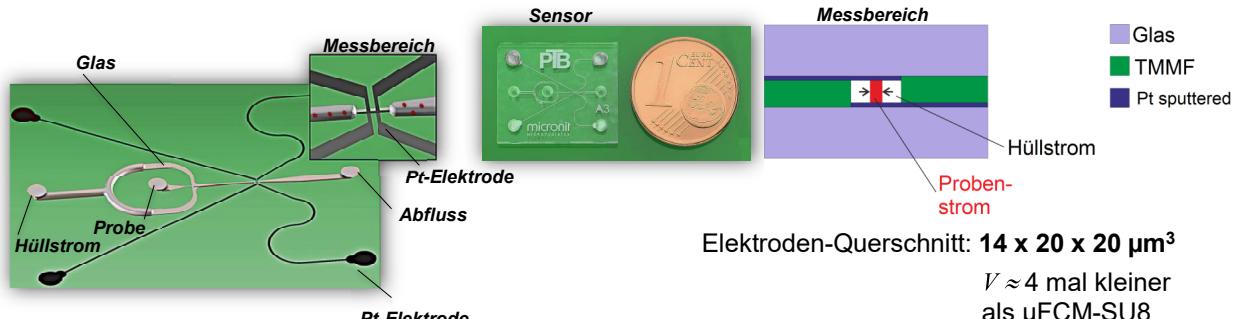


RWTH AACHEN
UNIVERSITY

μFCM-SU8 Herstellung: Lithographie

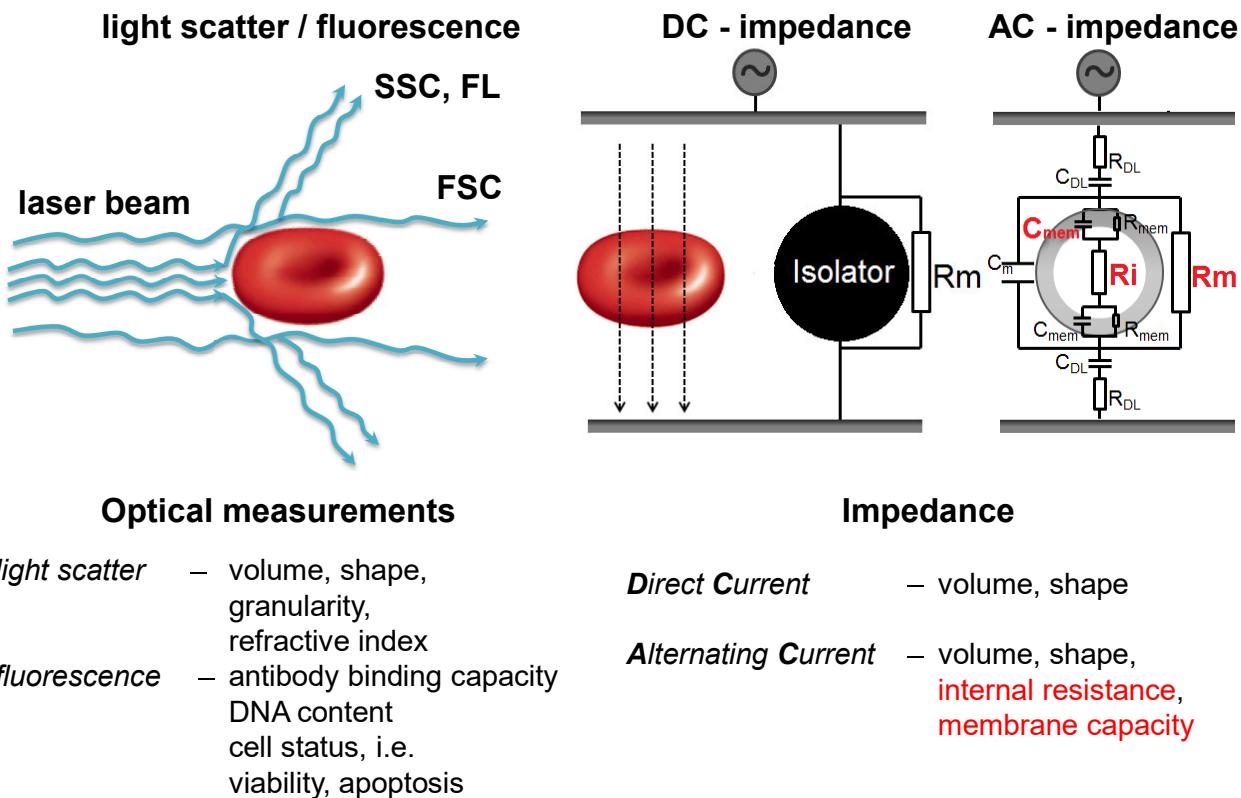


μFCM-Glas Herstellung: Nasschemische Ätzverfahren

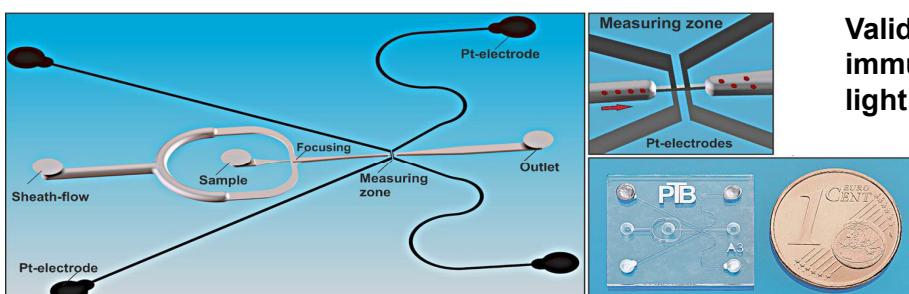
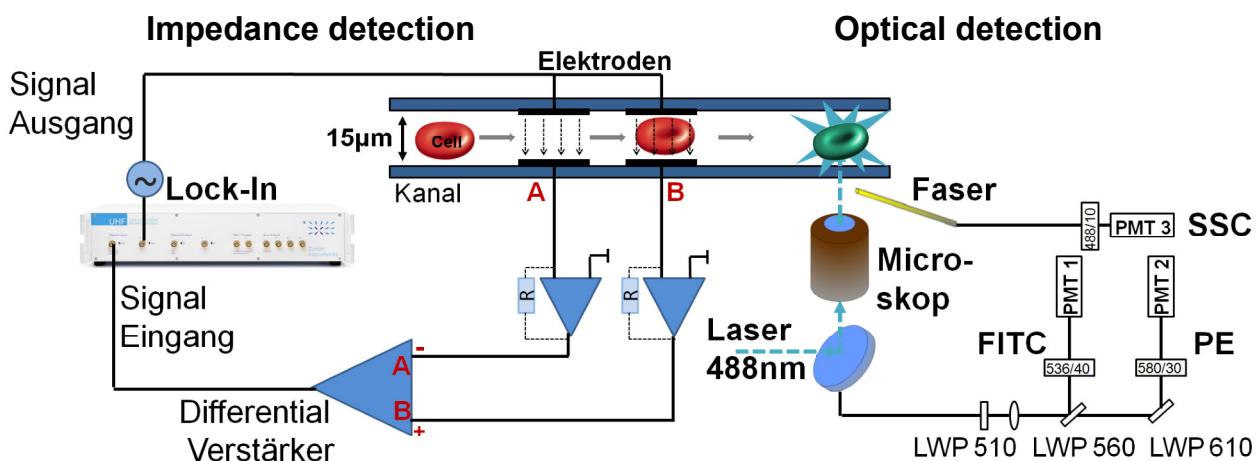


M. Frankowski et al., Eng. Life Sci. 2015, **15**, 286–296

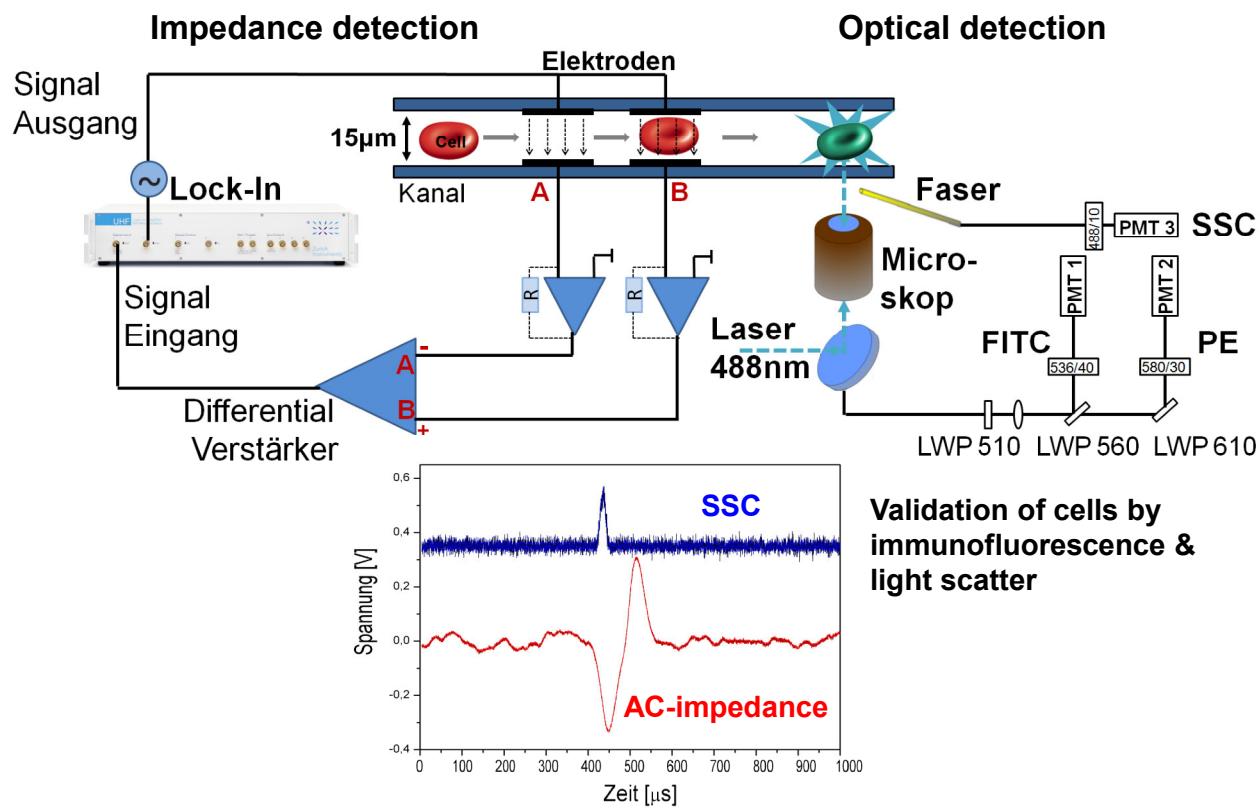
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Cell Differentiation by Combined Optical and AC– Impedance Measurements



Validation of cells by immunofluorescence & light scatter

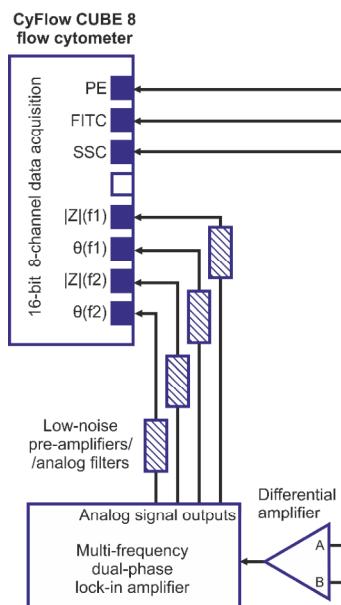


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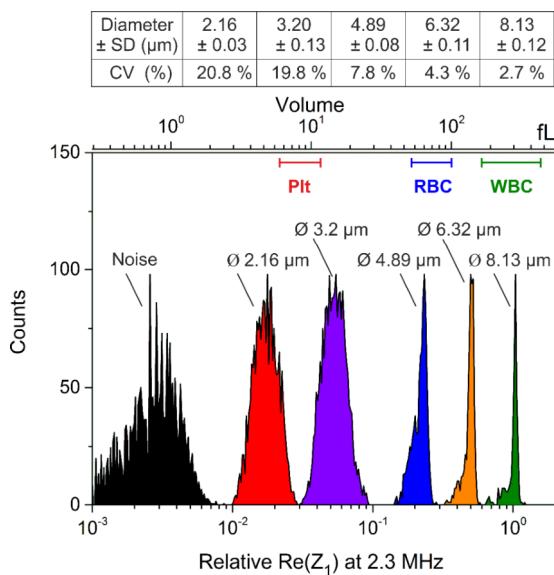
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Cell Differentiation by Combined Optical and AC– Impedance Measurements

Data acquisition for pulse height analysis



Sensitivity of AC- impedance measurements

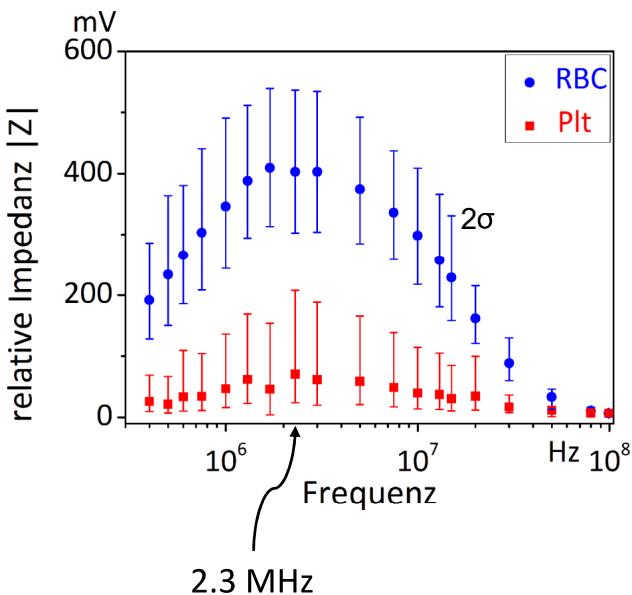


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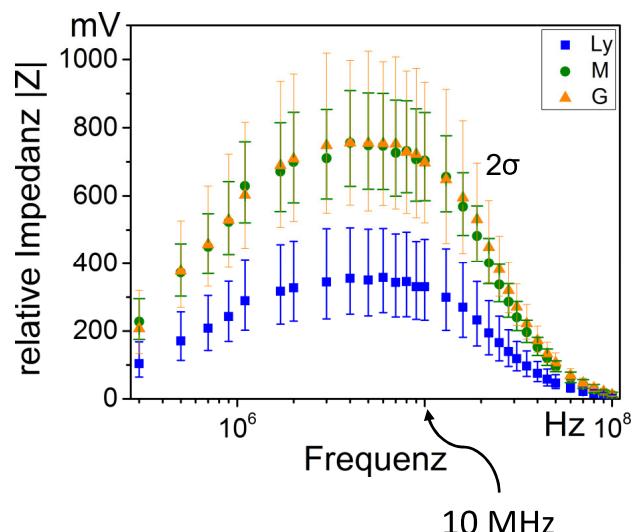
Whole blood sample:

Thrombocytes (Plt), erythrocytes (RBC)



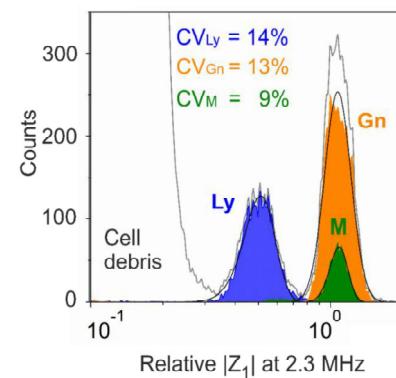
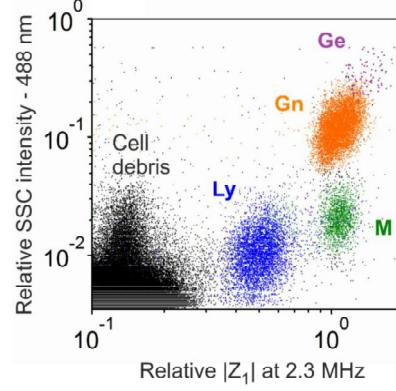
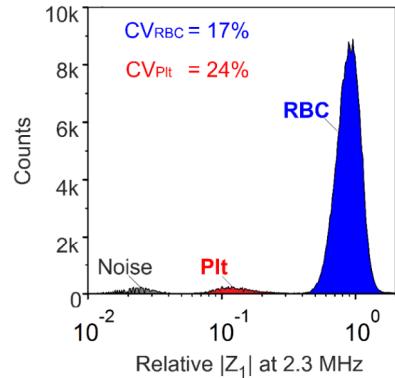
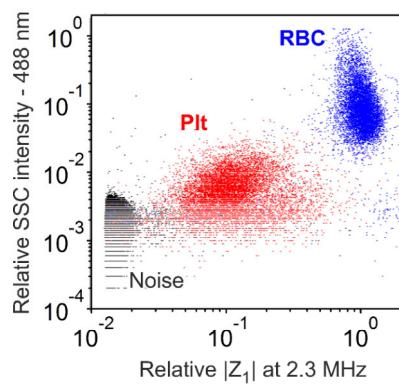
Lysis of RBC:

Granulocytes (G), monocytes (M), lymphocytes (Ly)

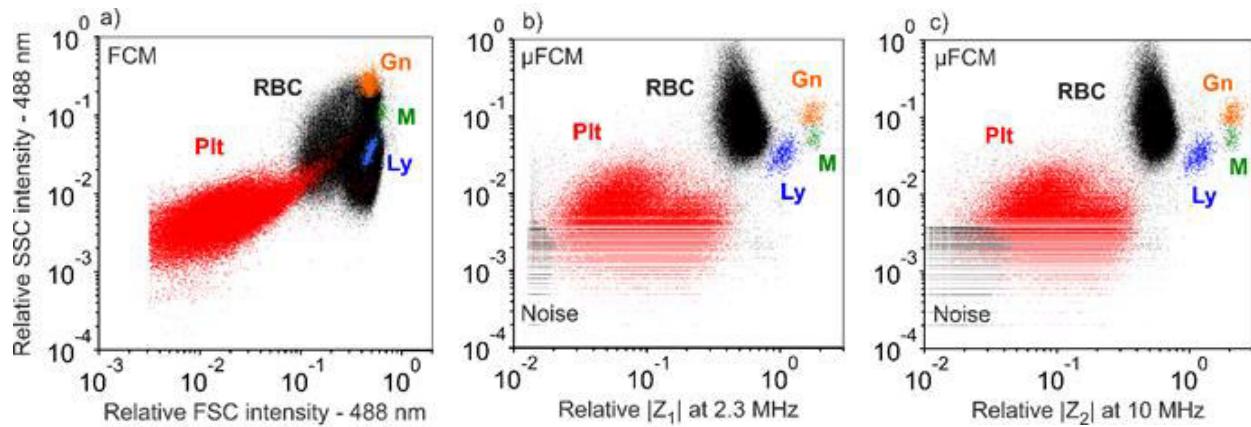


Cell Differentiation in Whole Blood and Lysed Blood Samples

Whole blood sample: Thrombocytes (Plt), erythrocytes (RBC)



Lysis of RBC: Granulocytes (G), monocytes (M), lymphocytes (Ly)



Whole blood sample - Plt, RBC, Ly, M, Gn

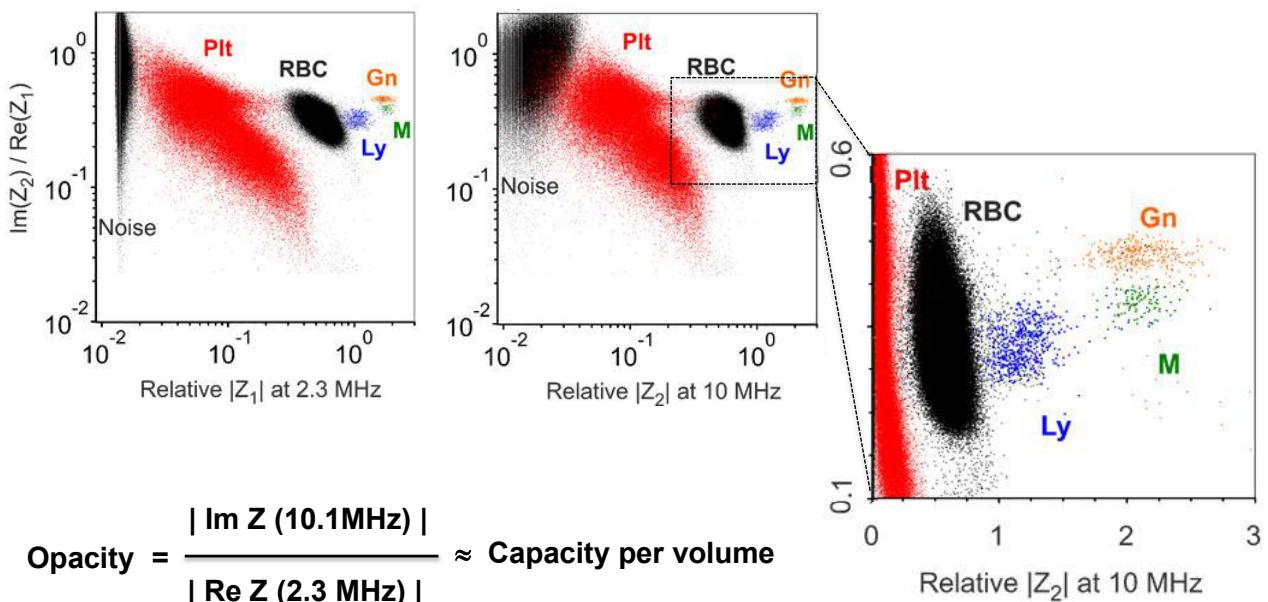
Ratio of concentrations:

$$C_{\text{WBC}} / C_{\text{RBC}} = 1 / 1000$$

Total number of events:

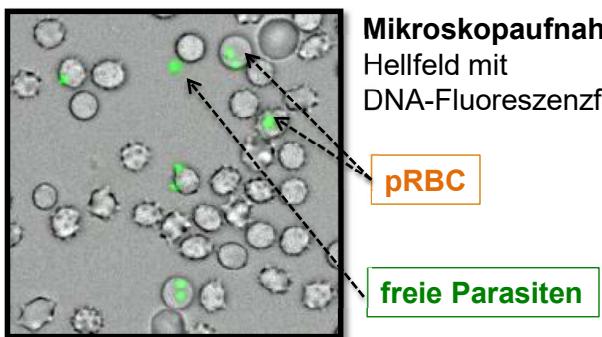
500 000 cells

*AC-Impedance Measurements
for Label-free Differentiation of Cells in Whole Blood*



$$\text{Opacity} = \frac{|\text{Im } Z(10.1\text{MHz})|}{|\text{Re } Z(2.3\text{ MHz})|} \approx \text{Capacity per volume}$$

Detection of Malaria Parasites in Red Blood Cells by AC-Impedance Measurements ?



Mikroskopaufnahme x20:
Hellfeld mit
DNA-Fluoreszenzfärbung

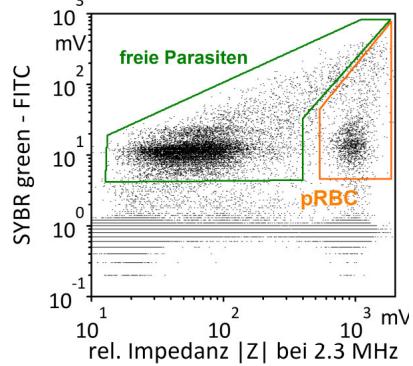
Klinische Proben:



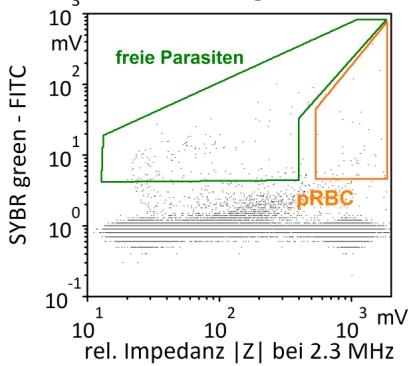
Proben in Kultur:



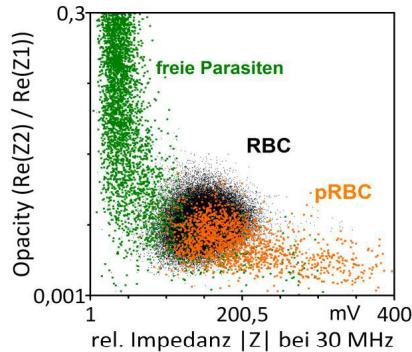
Malaria positiv:



Malaria negativ:



Label - frei:



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Development of Microflow Cytometers

Marcin Frankowski
Peter Simon
Nicole Bock
Andreas Kummrow
Kerstin Brattke (TFH Berlin)
Hülya Yıldırım / Ragusch (TFH Berlin)
Nuran Özdemir (FU Berlin)
Mesrure Baydaroglu (TFH Brandenburg)
Amin Chebbo (TFH Berlin)
Jörg Neukammer



Janko Theisen
Andrej Tuchscheerer
Markus Malcher
Katja Rehbach
Thilo Gutschauski
Christopher Sprenger
Robert Wahle
Cristian König
Roman Mästle
Martin Schmidt†



Fachgebiet

Mikro- und Feingeräte



Peter Simon
Akram El-Hasni
Uwe Schnakenberg

RWTH AACHEN
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