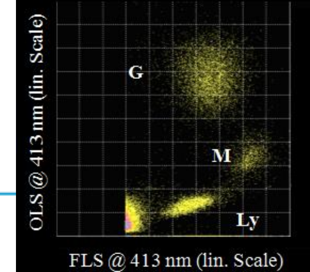
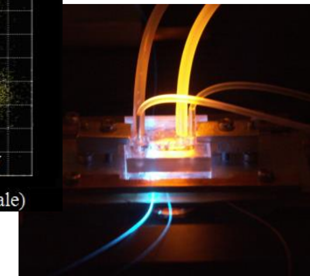
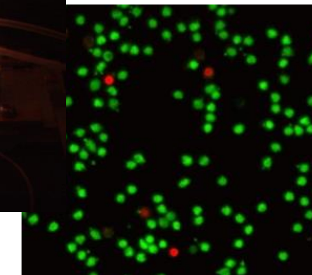
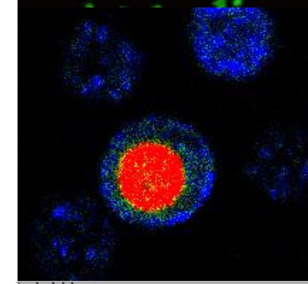
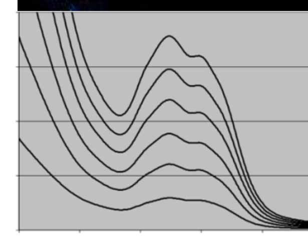
Physikalisch-Technische Bundesanstalt
Braunschweig und Berlin
Nationales Metrologieinstitut











Metrological traceability in cell concentration determination

Jörg Neukammer
*Working Group **"Flow Cytometry and Microscopy"***

Reliable support of diagnosis in Medicine,
e.g. Haematology, Oncology, Virology

New challenges
for traceability in laboratory medicine ?

Z. ges. exp. Med. 151, 331—349 (1969)

Freie Universität Berlin

Die elektronische Volumenbestimmung von Blutkörperchen und ihre Fehlerquellen

R. Thom, A. Hampe und G. Sauerbrey

Medizinische Klinik und Poliklinik der Freien Universität Berlin (Klinikum Westend) und Physikalisch-Technische Bundesanstalt, Institut Berlin

Eingegangen am 23. September 1969

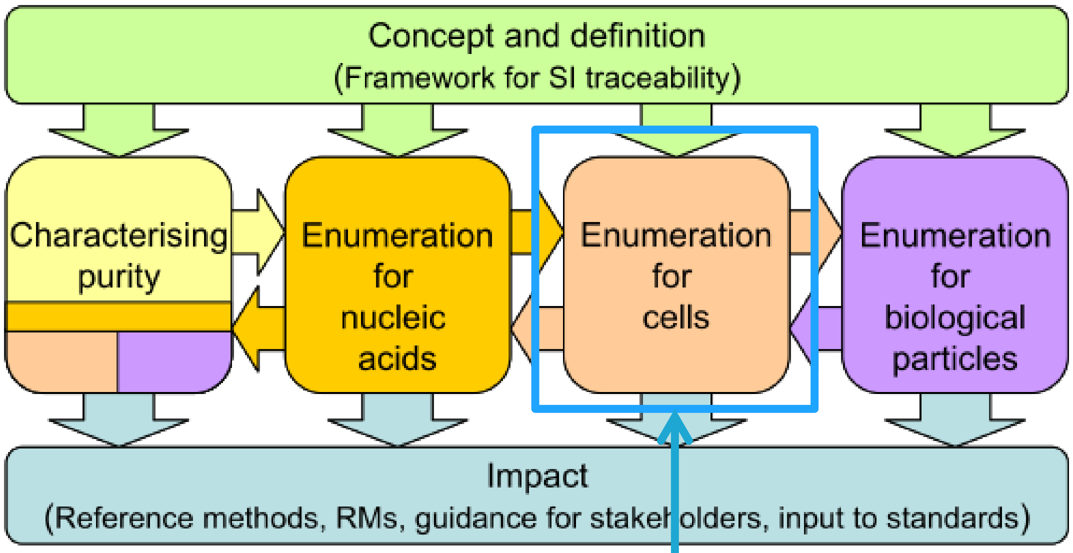
- ➢ Inter-laboratory (about 75 laboratories) comparisons started 1985 on a voluntary basis
- ➢ Evaluation based on instrument specific (consensus) target values

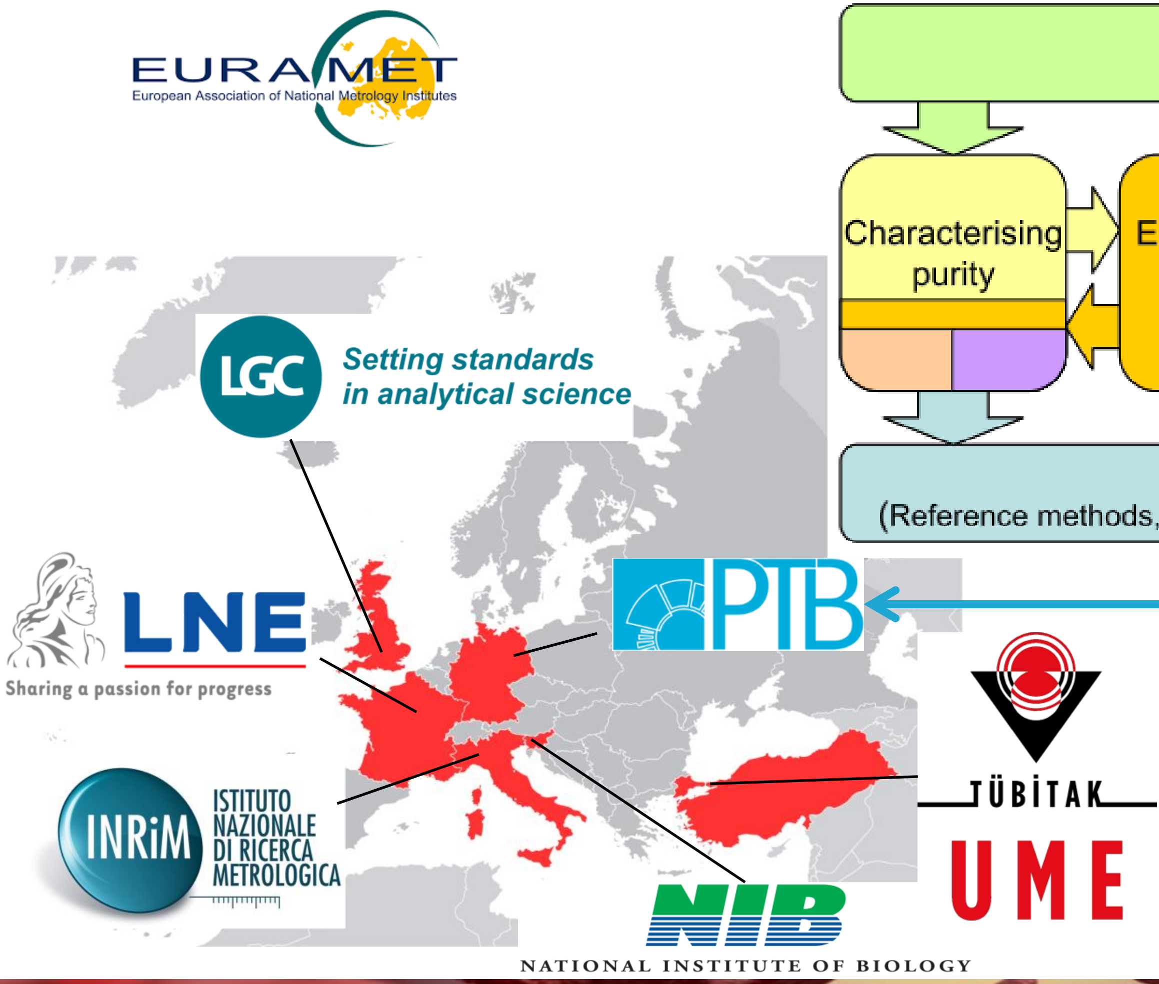
⇒ Request for reference measurement procedures

Cell counting in flow: Dilution series as “gold standard”

- Moldavan A. Photo-electric technique for the counting of microscopical cells. Science 1934;80:188 – 9.
- Lagercrantz C. Photo-electric counting of individual microscopic plant and animal cells. Nature 1948;161:25 – 6.
- Crossland-Taylor PJ. A device for counting small particles suspended in a fluid through a tube. Nature 1953;171:37 – 8.
- Coulter WH. Means for counting particles suspended in a fluid. United States Patent. October 20, 1953; 2,656,508.
- Wales M, Wilson JN. Theory of coincidence in Coulter particle counter. Rev Sci Instrum 1961;32:1132 – 6.
- Strackee J. Coincidence loss in bloodcounters. Med Biol Eng Comput 1966;4:97 – 9.
- Dittrich W, Göhde W. Impulsfluorometrie bei Einzelzellen in Suspensionen. Zeitschr Naturforsch 1969;24b:360 – 1.
- Bader H, Gordon HR, Brown OB. Theory of coincidence counts and simple practical methods of coincidence count correction for optical and resistive pulse particle counters. Rev Sci Instrum 1972;43:1407 – 12.
- Helleman PW. Chemical and physical aspects of electronic cell counting. In: Izyk G, Lewis SM, Path MR, editors. Modern concepts in hematology. New York: Academic Press Inc, 1972: 164–90.
- Lewis SM, England JM, Kubota F. Coincidence correction in red blood cell counting. Phys Med Biol 1989;34:1239 – 46.
- Helleman PW. Letter to the Editor: More about coincidence loss and reference methods. Phys Med Biol 1990;35:1159 – 62.

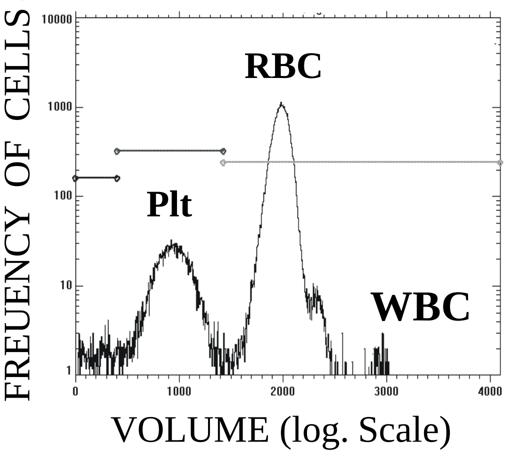
Traceability (to SI units) for biologically relevant molecules and entities

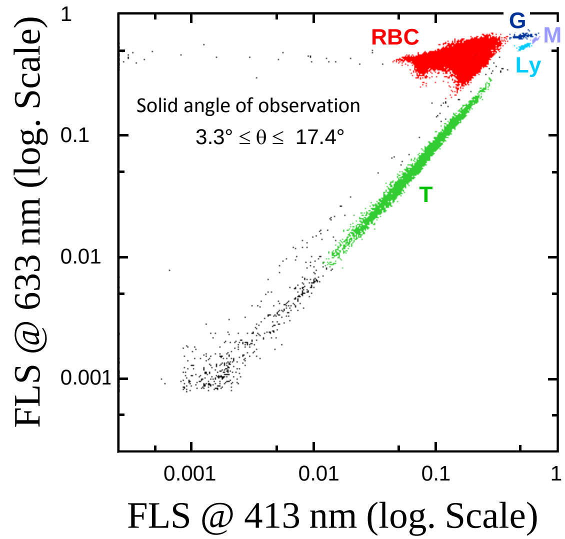


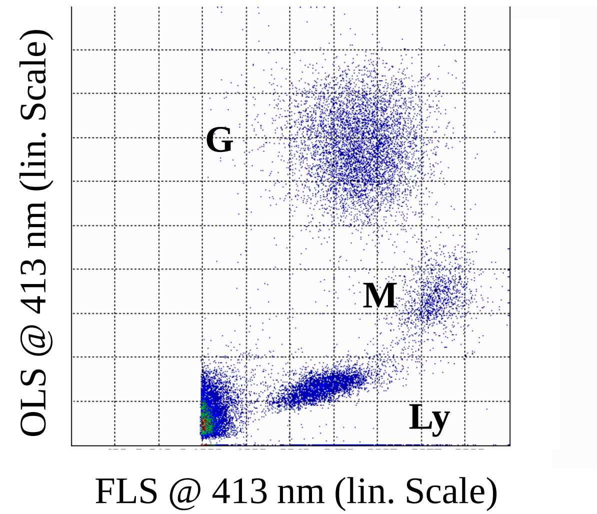


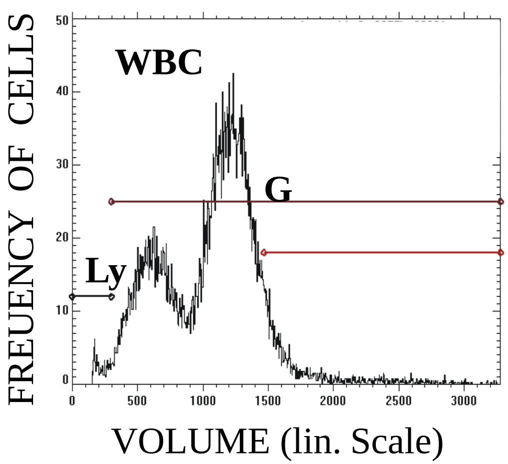
http://biositrace.lgcgroup.com

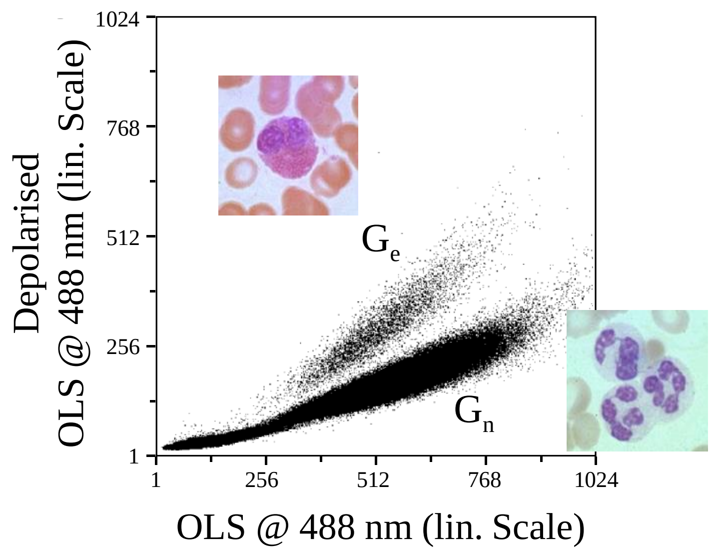
Flow Cytometric Differentiation of Blood Cells











RBC: Red Blood Cells
Plt: Platelets
WBC: White Blood Cells
G: Granulocytes
Ly: Lymphocytes
M: Monocytes

FLS: Forward Light Scatter
OLS: Orthogonal Light Scatter

Cell counting in the Working Group “Flow Cytometry and Microscopy”

PTB

Determination of reference values

for quality assurance of haematological laboratories in Germany

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

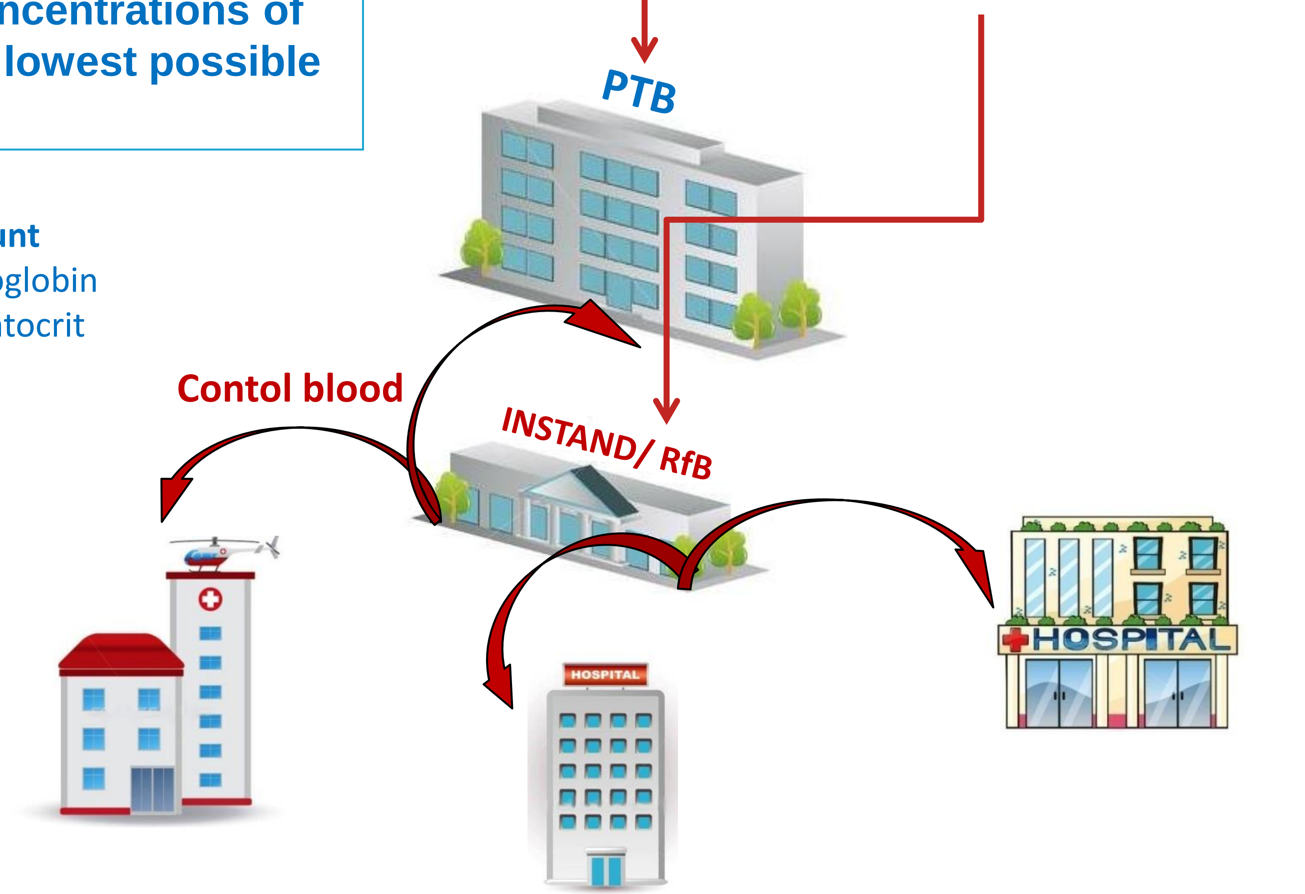
IVD-Directive 98/79/EC

MPG (2.8.1994 / 7.8.2013)

RiliBÄK (2014)

CBC: Complete Blood Count

Erythrocytes	Haemoglobin
Thrombocytes	Haematocrit
Leucocytes	



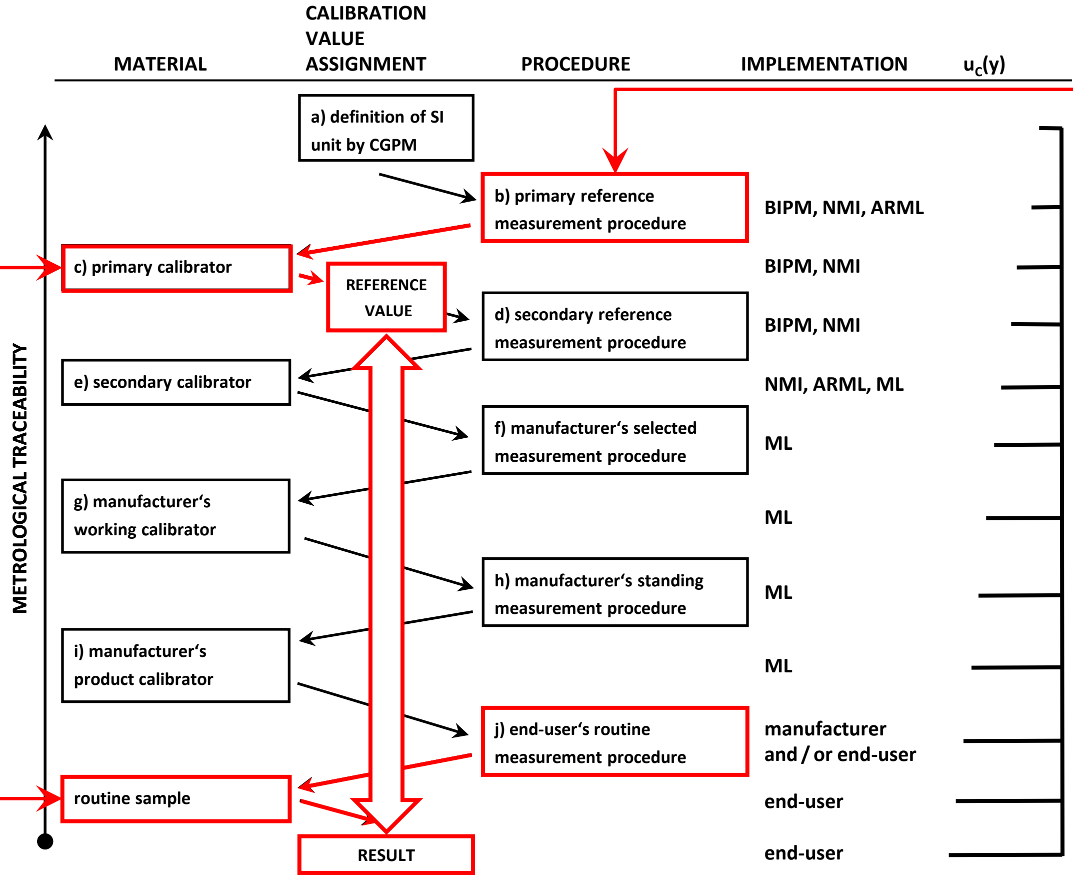
German Medical Scientific Associations:

RfB Referenzinstitut für Bioanalytik

INSTAND

Complete Blood Count: Validation of traceability of results by round robin tests

PTB



development of reference instruments & reference measurement procedures @ **PTB**

round robin tests using control blood

RfB INSTAND

disadvantage:

routine instruments are calibrated to provide reliable results for fresh blood,

control blood does not mimic properties of fresh blood sufficiently

EQUALIS

uses fresh samples !

Extensive calibration hierarchy and metrological traceability to SI

EN ISO 17511 (2003): In vitro diagnostic medical devices

Measurement of quantities in biological samples – Metrological traceability of values assigned to calibrators and control materials

Requirements for internal and external quality assurance

Guideline of the German Medical Association on
Quality Assurance in Medical Laboratory Examinations – Rili-BAEK
(J Lab Med 2015; 39(1): 26–69, Table B1a)

no.	Analyte	Quantity	Acceptable relative deviation of a single result or of the relative root mean square	Acceptable relative deviation in interlaboratory tests	range of measurement			Type of target value
					from	to	unit	
20	erythrocytes	cell concentration	4%	8%	1,5	7	1/pL	RMV
27	haematocrit	volume ratio	5%	9%	10	60	%	STV
28	haemoglobin	mass ratio	4%	6%	20	200	g/L	RMV
40	leucocytes	cell concentration	6.5%	18%	2	30	1/nL	RMV
56	thrombocytes	cell concentration	7.5% 8.5% 13.5%	13% 15% 18%	>300 >150 40	700 300 150	1/nL 1/nL 1/nL	STV

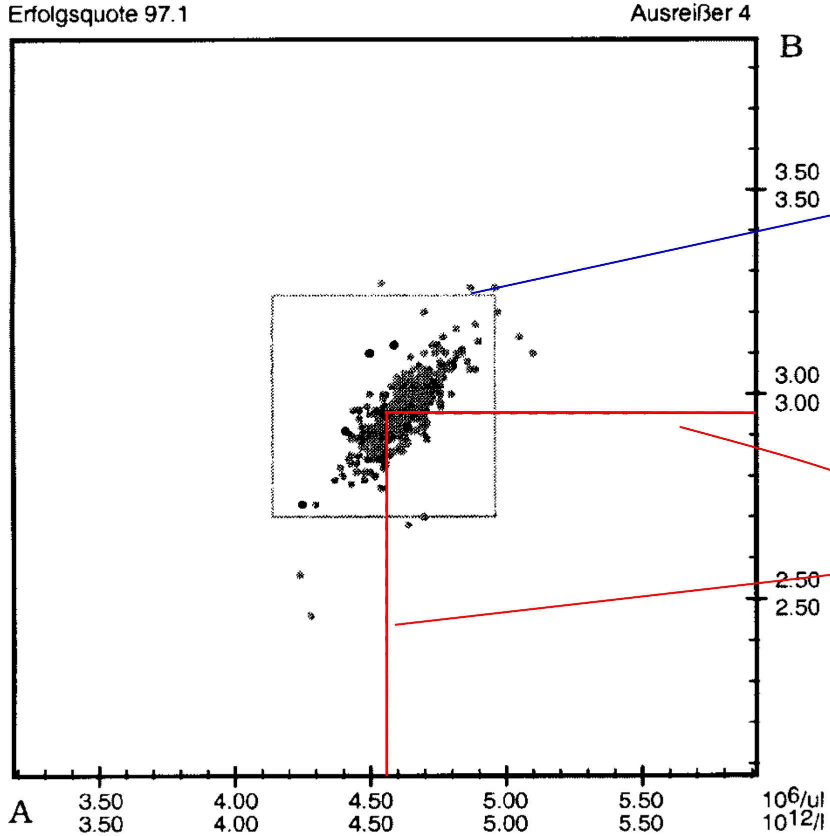
external quality assurance

Participation mandatory since 2002

RMV = reference method value, STV = target value specific for the particular test method

Erythrocyte concentration of all participants

Analyt	Methode
Erythrozyten	**Alle Methoden**



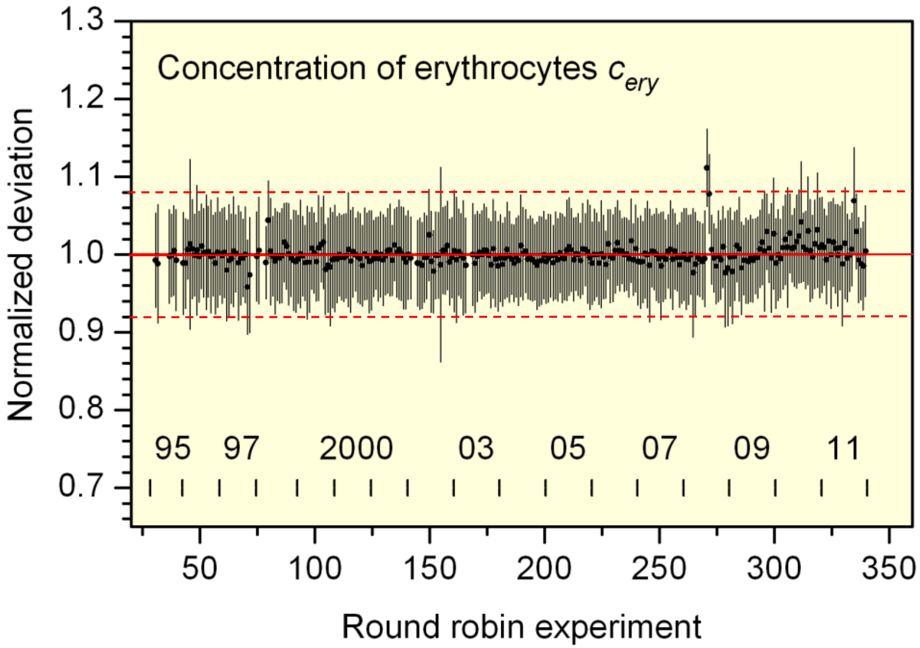
Teilnehmerzahl	448			
Probe/Einheit	A 10^6/ul	B	A 10^{12}/l	B
Mittelwert	4.62	2.96	4.62	2.96
Standardabweichung	0.107	0.085	0.107	0.085
Variationskoeffizient	2.33	2.89	2.33	2.89

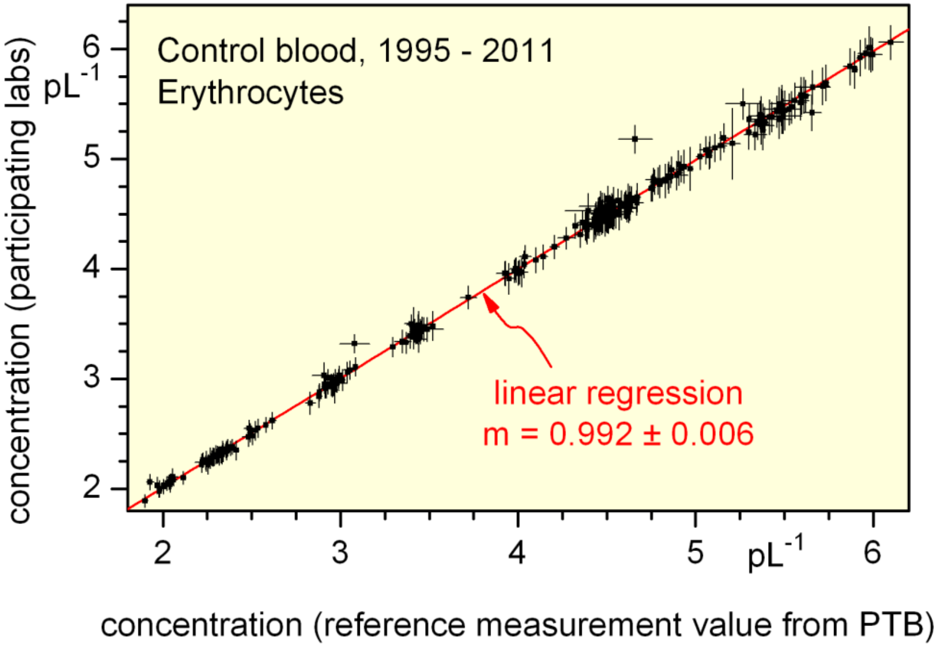
Youden – Diagram

Evaluation limits according to RiLiBÄK
(± 8%)

PTB reference values
$U(C_{ery})$ = (± 0,8%)

External quality assurance since 1995: Erythrocyte concentrations





normalized reference measurement values (PTB)

acceptable deviations in interlaboratory tests (RiLiBÄK)

mean of collective and standard deviation

Primary reference measurement procedure applied at PTB

Concentration C of primary sample

$$C = \frac{N}{V}$$

Recorded concentration C of analytical suspension

$$C_{ri,j} = \frac{N_{ri,j}}{V_{i,j} \cdot \phi_i}$$

Coincidence correction by dilution series $\Rightarrow$ *N*

$$\ln \frac{\overline{N}_{ri}}{\phi_i} = \ln N - \phi_i \cdot N \cdot p$$

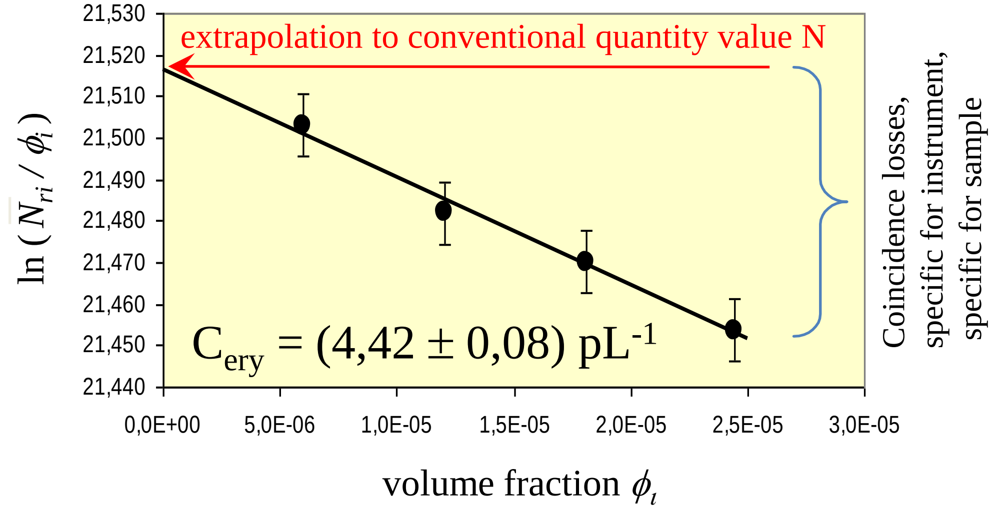
Determination of volume and density

V, ϕ_i gravimetrical measurement of volume V and volume fraction ϕ_i

ρ density measurement using the mechanical oscillator method

Definition of symbols

- N conventional quantity value of the number of particles
- V volume of primary sample derived from $V_{i,j}$
- $N_{ri,j}$ recorded number of events
- $V_{i,j}$ volume of analytical solution i, repeat measurement j
- ϕ_i volume fraction of primary sample in the analytical solution i
- p coincidence parameter

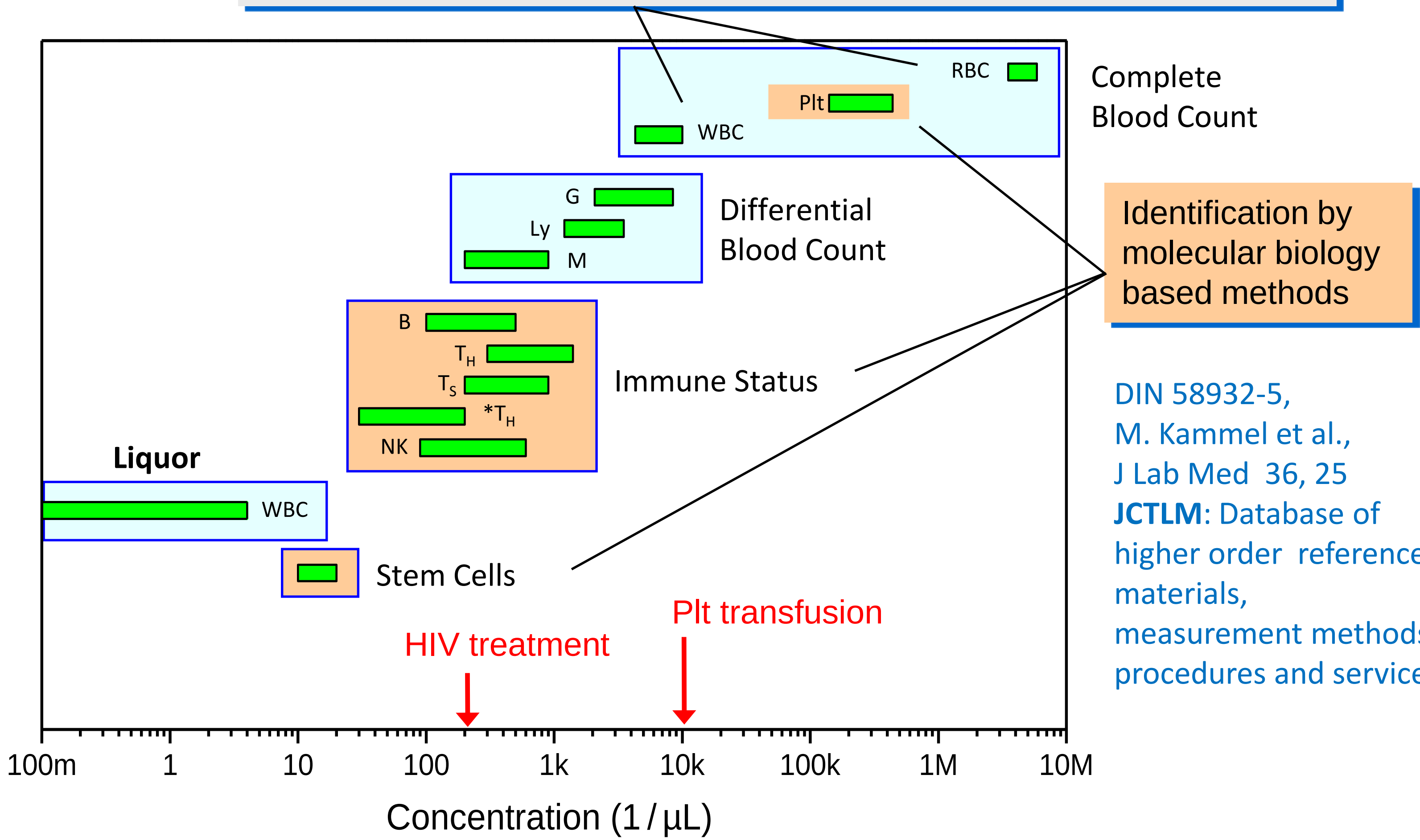


Control of influence quantities

adhesion:	determination of concentration immediately and 30 min after preparation
agglomeration:	analysis of pulse height distributions, scatter plots, integrated dead time
sedimentation:	stirring during measurement, time dependence of $N_{ri,j}$
carry over:	background determination between different series of measurements
RBC ghosts:	different methods (impedance change, FLS, mAb-staining)
lysis:	comparison experiments using various reagents

Development of reference measurement procedures

Reference Procedures (DIN 58932-3, DIN 58932-4, **DIN 58932-5**)

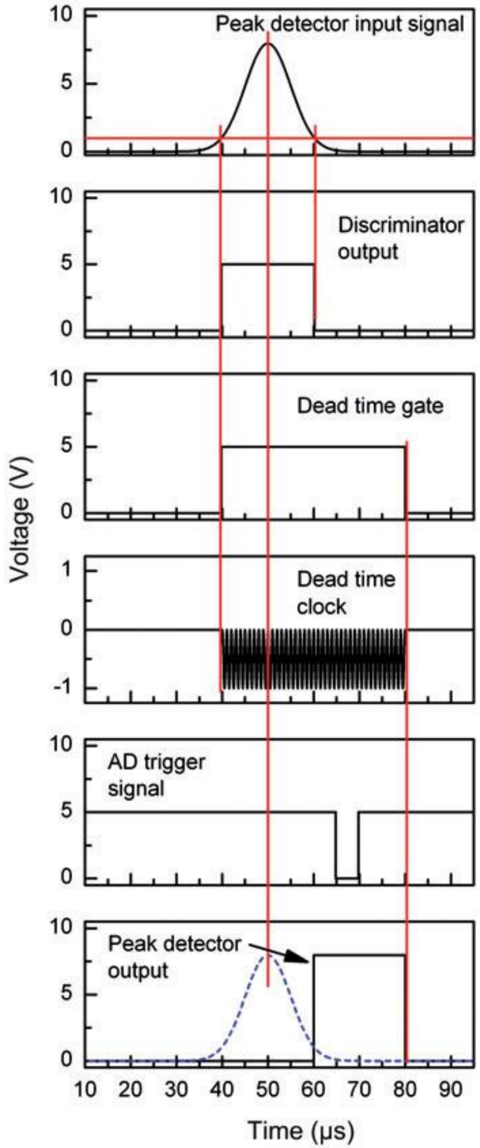


DIN 58932-5,
M. Kammel et al.,
J Lab Med 36, 25
JCTLM: Database of higher order reference materials, measurement methods / procedures and services

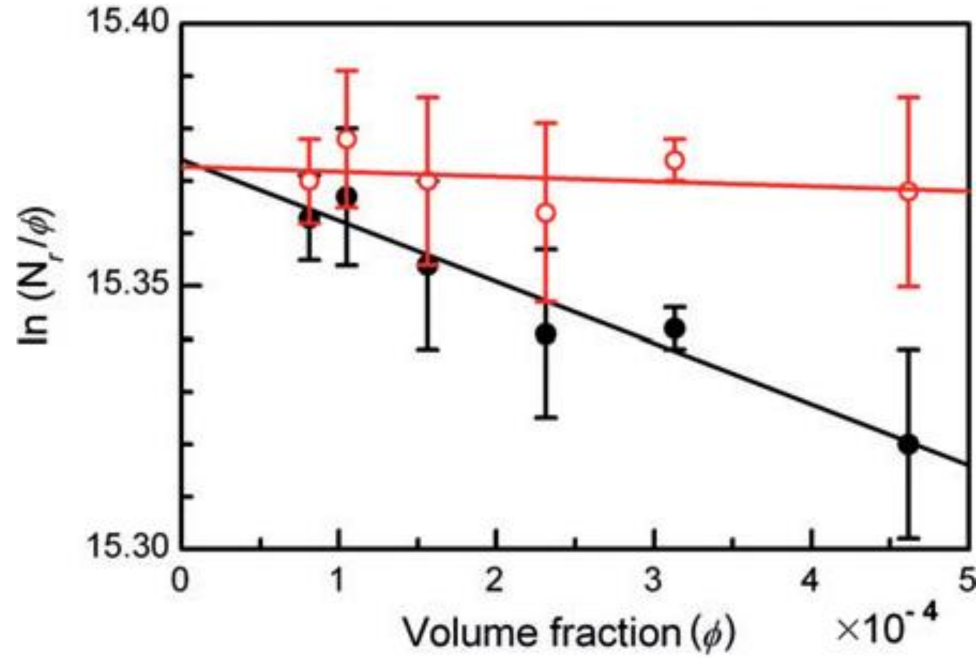
Flow cytometry: Identification - Counting – Sorting

Validation of target cells: Microscopy (Morphology, Localization of mAb,…)
Nucleic acid amplification (NAAT), e.g. qPCR, isothermal NAAT

Pulse widths measurements to quantify coincidence loss

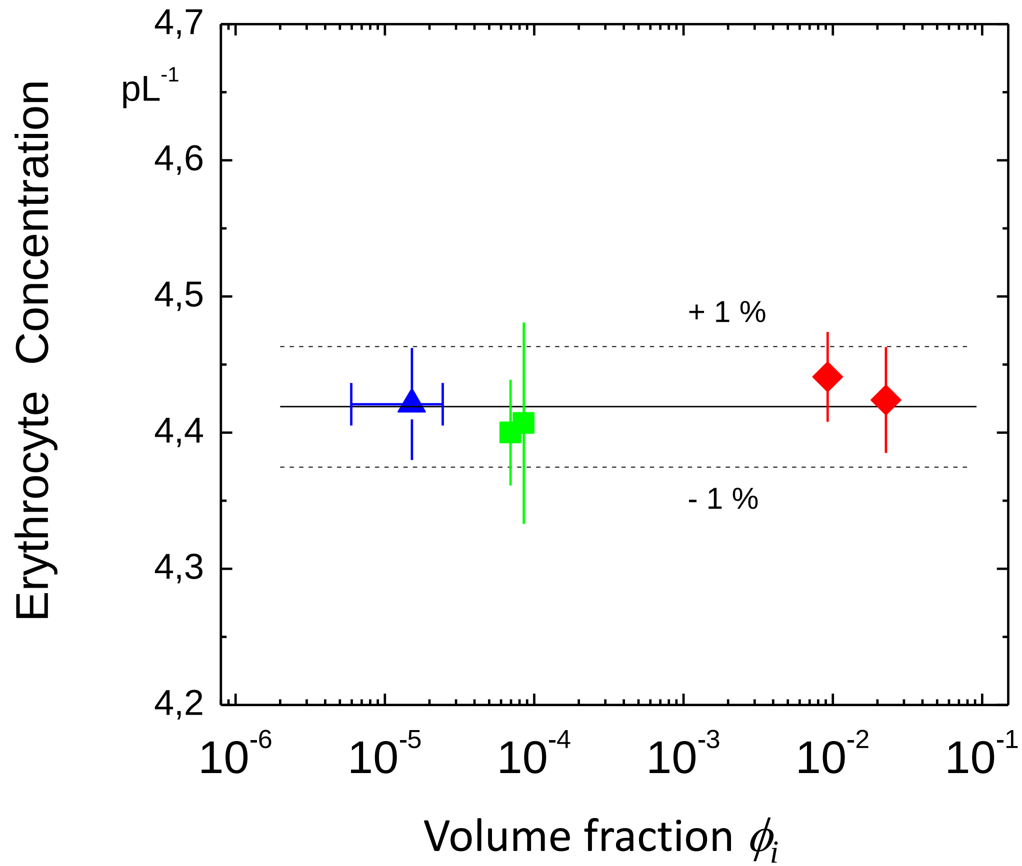


$$\left(N\ \ \right)\approx\frac{N_{ri}}{\phi_i}\frac{1}{1-\left(N_{ri}\,\tau/t\right)}$$



M. Kammel et al., J Lab Med 36, 25 (2012)

Comparison of Different Reference Instruments Using Integrated Pulse Width Measurement



Laser flow cytometer

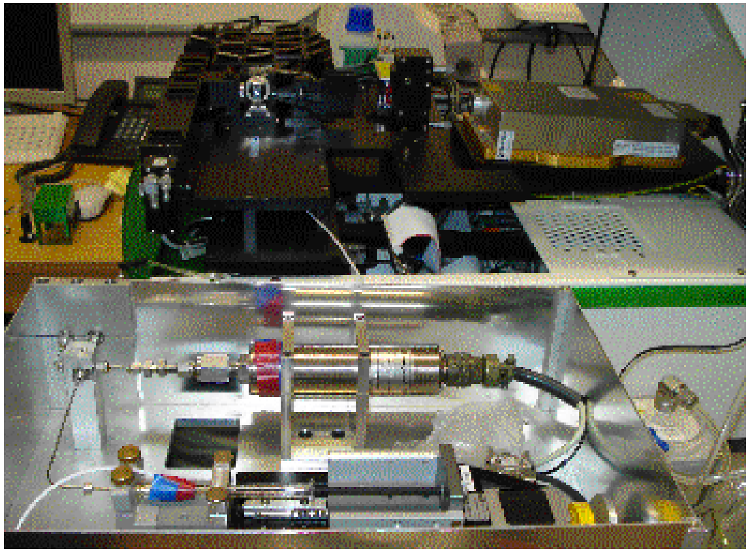
Impedance based flow cytometer, hydrodynamic focussing

Impedance based flow cytometer, <u>no</u> hydrodynamic focussing (Coulter ZM, Bonn)

Result: Good agreement between different techniques for cell detection

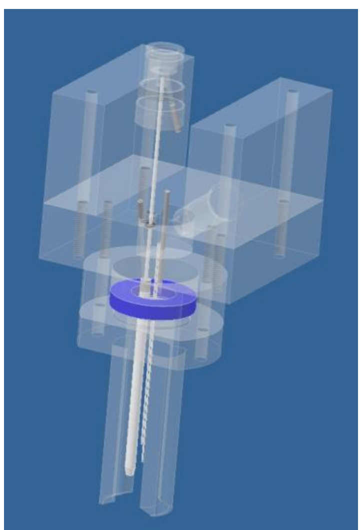
Conclusion: Concentration is obtained from a single measurement,
Dilution series serves as independent control,
Method is suited as primary reference procedure

Modification of commercial instruments to allow application of primary procedure



- direct volume measurement by motor driven, gravimetrically calibrated syringe & high accuracy pressure determination

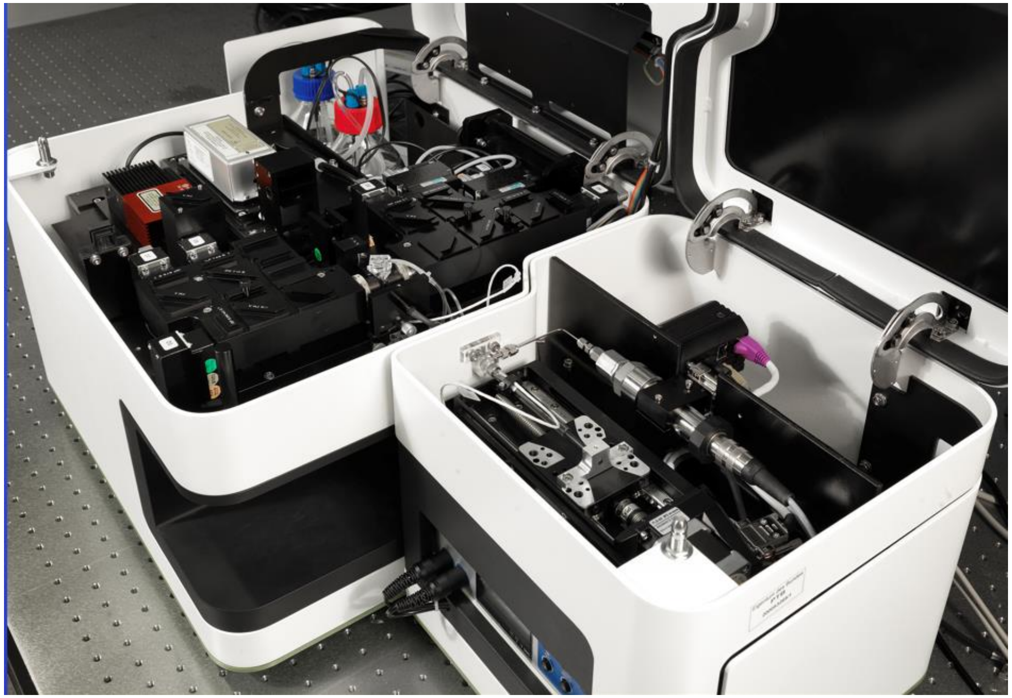
- new hard & software for control of measurement and data acquisition



- direct injection of sample in flow cell

Modification of commercial instruments to allow application of primary procedure

PTB



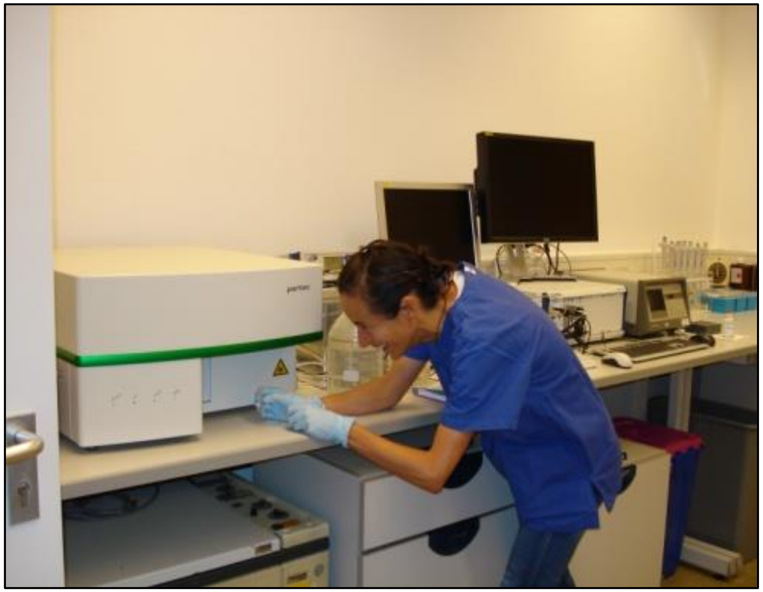
TÜBİTAK

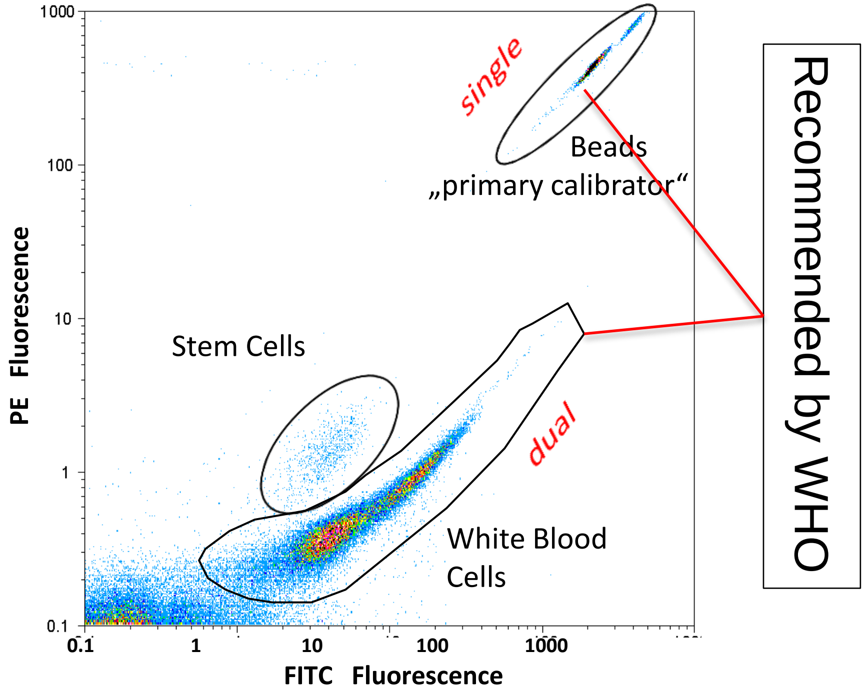
RfB
Referenzinstitut
für Bioanalytik

INSTAND

Determination of reference values for stem cell concentrations

Partec / PTB-reference instrument
@ Klinikum Karlsruhe*





Martin Kammel

Andreas Ruf

*Department of Transfusion Medicine and Haemostasis

Secondary reference measurement procedure: Relative enumeration of lyophilised CD4$^+$ cells

CCQM Pilot Study 102:

Surface (pre-) labelled lyophilised cells for inter-laboratory comparison

Stebbings R.[1], *Sutherland J.*[1], *Wang L.*[2], & *Neukammer J.*[3]

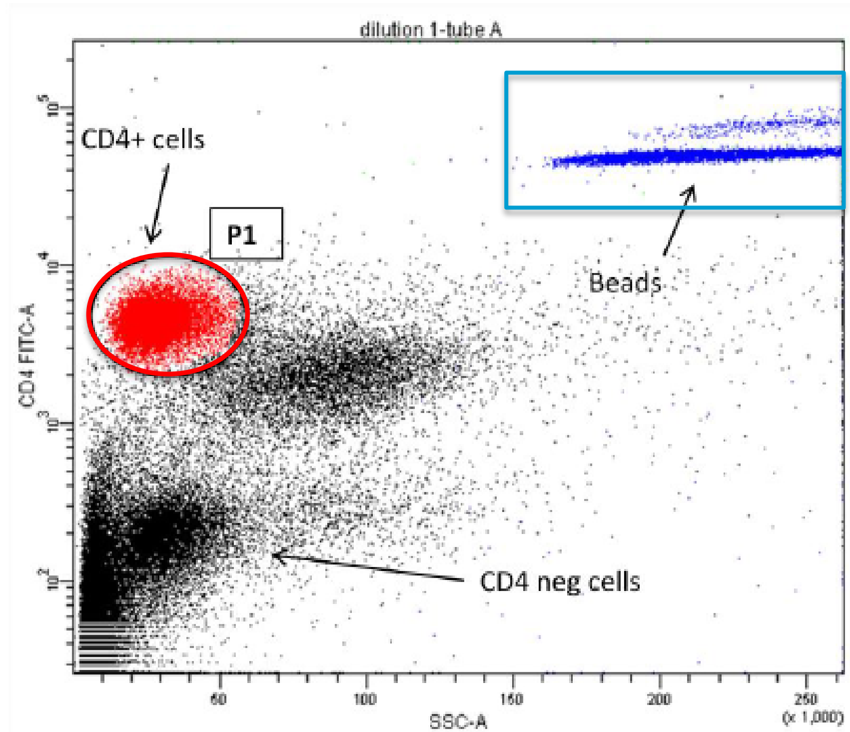
[1] NIBSC (United Kingdom), [2] NIST (U.S.A.), [3] PTB (Germany)

sLL

+ 1mL Reinstwasser

1) Entnahme 200µL
in TruCount Röhrchen
+ 800µL Isoton
⇒ 1: 5

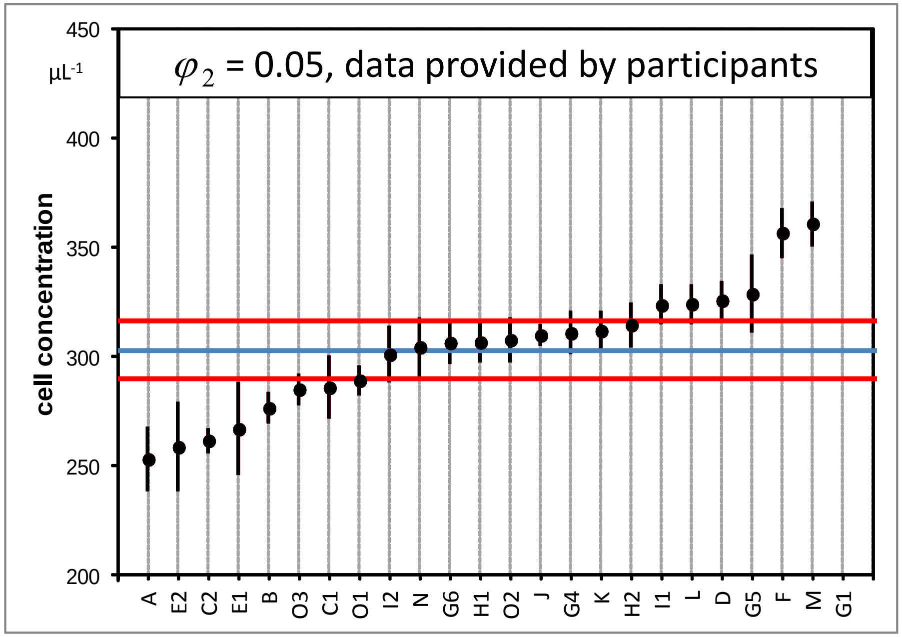
2) Entnahme 50µL
in TruCount Röhrchen
+ 950µL Isoton
⇒ 1: 20



Reference value for bead number assigned by PTB

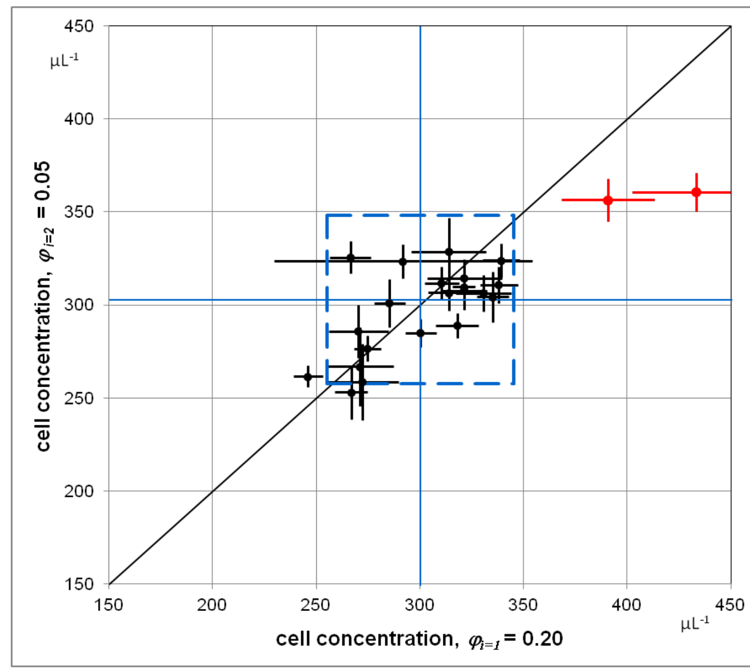
$u(N) \approx 7\%$, vial to vial variation

Results of CCQM Pilot study 102: Relative enumeration of lyophilised CD4$^+$ cells



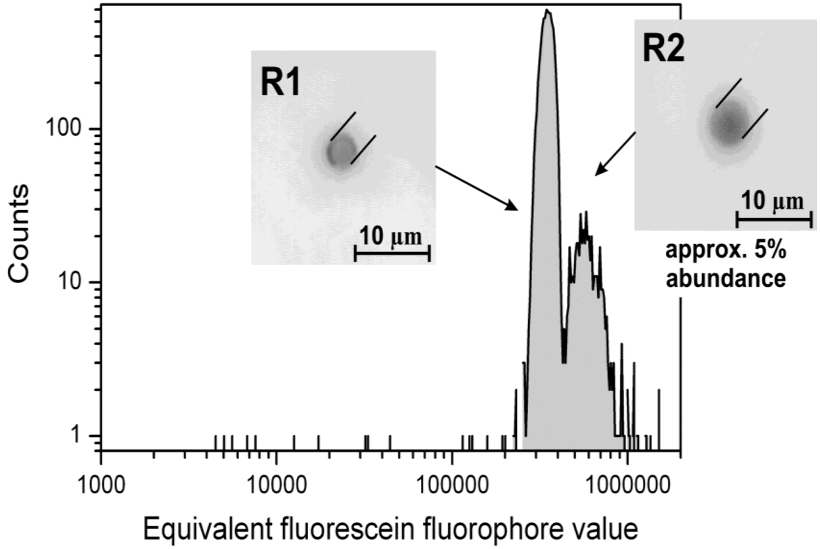
- Participants average for
  - dilution 1: (300 ± 44) μL^{-1} (level of
  - dilution 2: (303 ± 48) μL^{-1} confidence ≈ 95%)
- Target value (NIBSC preparation) ≈ 300 μL^{-1}
- Reference and routine protocols were used
  - no instrumental / procedure dependent clusters
  - no effect of dilution factor observed

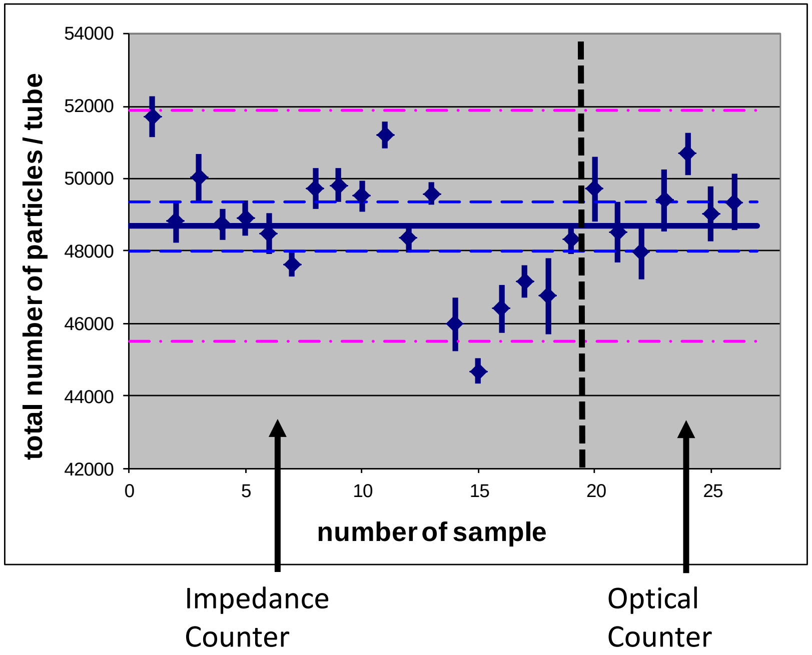
⇒ Acceptable deviation in ring trials: 15% - 20%



blue rectangle: ± 15%

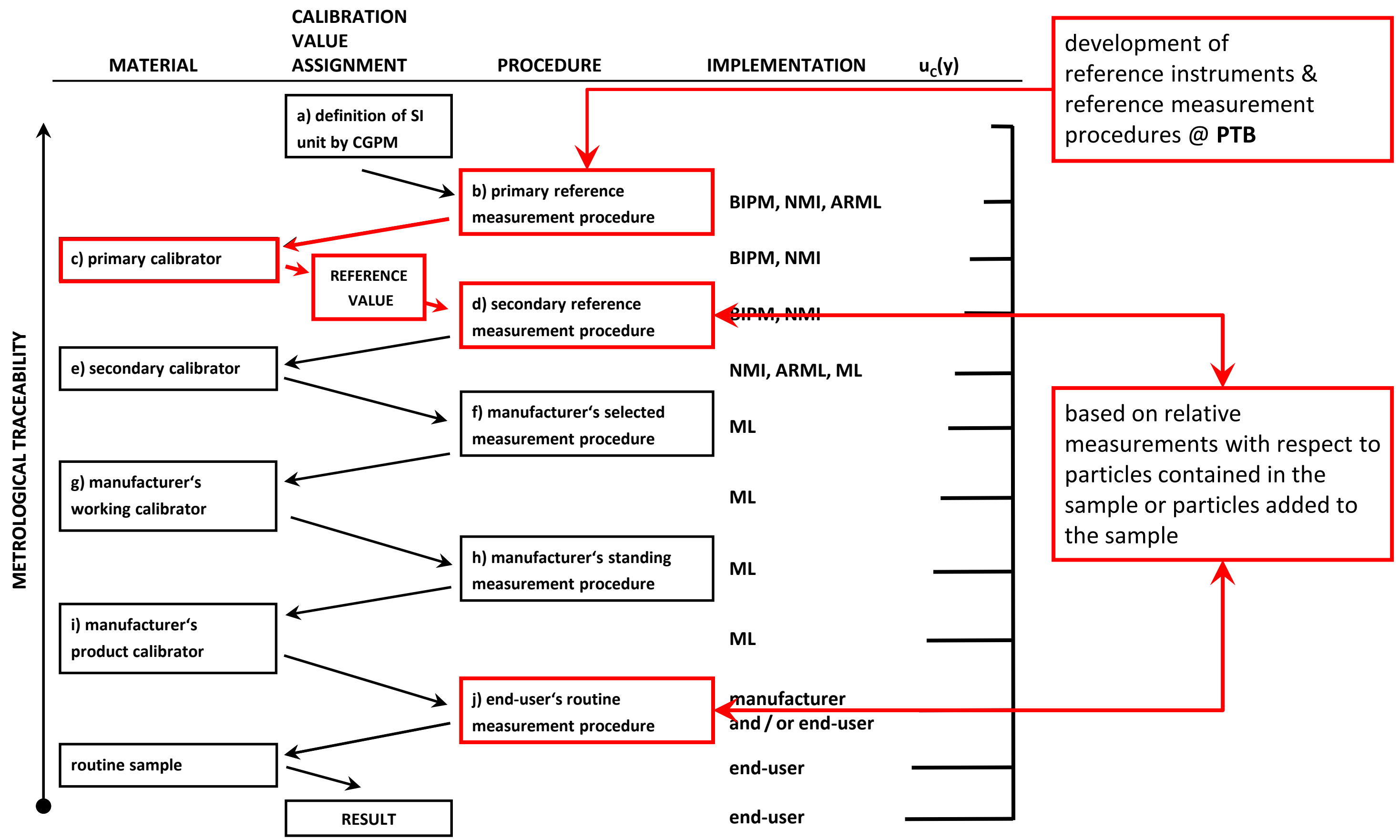
Characterisation of calibrators: TruCount, Flow Count, Flow Check, ...





instruments	value specified by manufacturer	weighted mean value	expansion factor	uncertainty of mean value	
		$<N_n>$	k	$k\,u(<N_n>)$	$k\,u_{rel}(<N_n>)$
optical and impedance reference counters	51511	48660	2.04	627	1.3 %
				uncertainty for single vial measurement	
				$\sqrt{32}\;k\,u(<N_n>)$	$\sqrt{32}\;k\,u_{rel}(<N_n>)$
				3544	7.3 %

Assignment of reference values to primary calibrators



Extensive calibration hierarchy and metrological traceability to SI

EN ISO 17511 (2003): In vitro diagnostic medical devices
Measurement of quantities in biological samples – Metrological traceability of values assigned to calibrators and control materials

Complete Blood Count: DIN Standards for reference procedures

DIN 58931	Determination of **haemoglobin concentration** in blood - Reference method, August 2010 $\Rightarrow$ PNWI CEN TC 140 (77% agreed, but experts are needed!)

Haematology — Determination of the concentration of blood corpuscles in blood

DIN 58932-1	Blood collection, sample preparation, biological influence quantities, interference factors, April 2012
DIN 58932-2	Characteristic quantities for erythrocytes (erythrocyte indices), June 1998
DIN 58932-3	Determination of the concentration of **erythrocytes**, Reference method (in German), Comments by February 2016 http://www.din.de/de/mitwirken/normenausschuesse/named
DIN 58932-4	Reference procedure for the determination of the concentration of **leukocytes**, July 2003
DIN 58932-5	5: Reference method for the determination of the concentration of **platelets**, May 2007
DIN 58932-6	Reference method for the determination of the concentrations **CD4 positive cells**
DIN 58932-7	Determination of blood cell concentrations by **relative enumeration**
DIN 58933-1	Procedure for determining the volume fraction of erythrocytes (**packed cell volume**) in blood - Part 1: Reference method based on centrifugation (in German), January 1995

DIN Committee NAMed "Haematology"

INSTAND
Society for Promoting
Quality Assurance
in Medical Laboratories e.V.

DGKL
Deutsche Vereinte Gesellschaft
für Klinische Chemie und Laboratoriumsmedizin e.V.

Abbott

Axonlab
connecting ideas

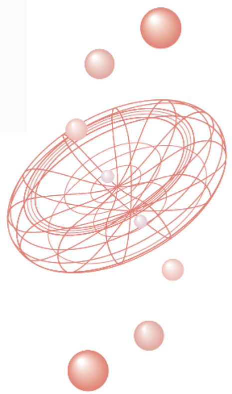
BECKMAN COULTER Life Sciences

BD

PARTEC
a Sysmex Company

sysmex

REVIEW
ARTICLE



The Reference Measurement Procedure for Erythrocyte Enumeration

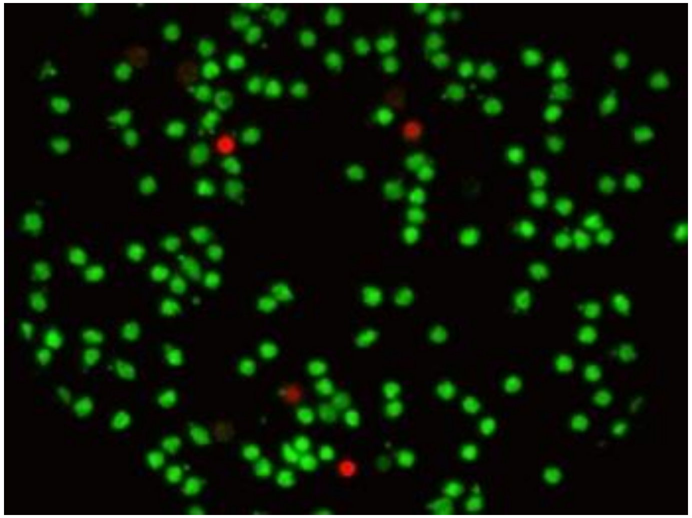
Etsuro Shinkai, Atsushi Shirakami and Keiji Fujimoto

Scientific Affairs, Sysmex Corporation, 1-3-2 Murotani, Nishi-ku, Kobe, 651-2241, Japan

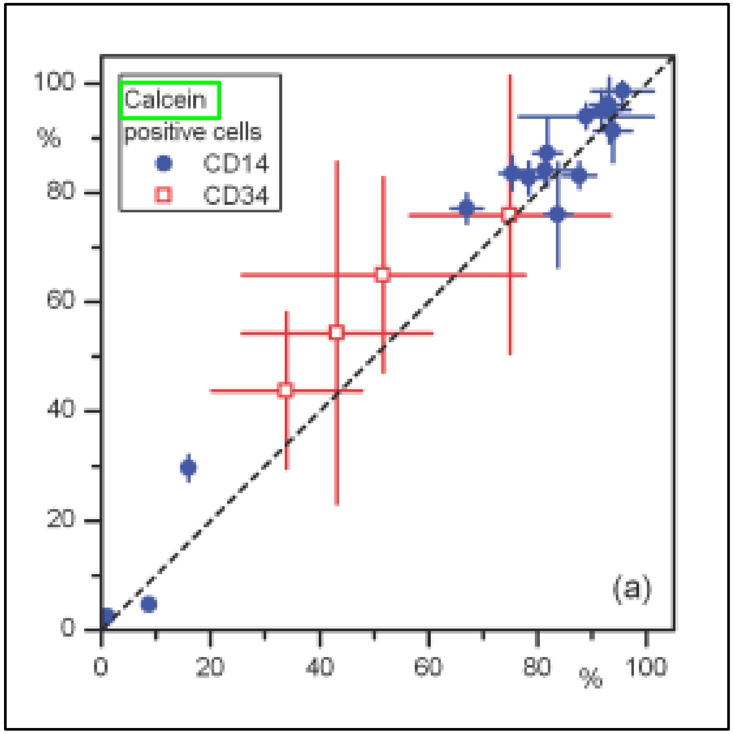
Annex A. ISO/TC276/WG3 Work Programme Status

Project #	Project Title	Stage Date	Target IS Date	Stage	Reference	Comments	WG	Lead Author(s)	Secretary
ISO/NP 20391-1	*Biotechnology - Cell Counting – Part 1. General Guidance on Cell Counting Methods*	*7-Aug-2015*	*7-Aug-2018*	10.99	WG3/N6,N20,N22, N32, N58 TC276/N81, N123	WG3/N29, N36, N47, N70	3	Lin-Gibson, Sarkar (US)	Allocca
ISO/NP 20391-2	*Biotechnology - Cell Counting – Part 2. Experimental Design and Statistical Analysis to Quantify Counting Method Performance*	*7-Aug-2015*	*7-Aug-2018*	10.99	TC276/N82, N122 WG3/N21, N22, N32, N33, N34, N35, N45, N46,	WG3/N30, , N37, N38, N48	3	Lin-Gibson, Sarkar (US)	Allocca
	Cell Characterization Strategy: *Characterization of Cells – Guide for Cell Measurement Methods*			------	WG3/N41, N60		3	Heki-san (Japan)	Allocca
	Characterization of Cells – Best Practice to Design Cell Measurement Methods			------			3	Lin-Gibson (US)	Allocca
	Characterization of Cells – Cell Measurement Process			------	WG3/N59		3	Heki-san (Japan)	Allocca
	Analytical Methods for Mesenchymal Stem Cells			------			3		Allocca

REGENMED*: Microscopic cell counting to determine cell viability



live (calcein)



cell fraction by microscopy →

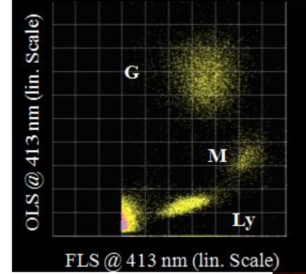
cell fraction by FCM →

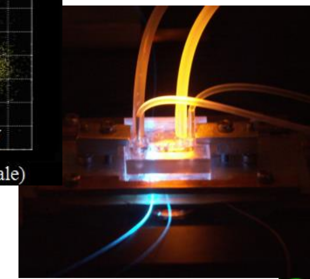
Kummrow et al., Cytometry 83A (2013)

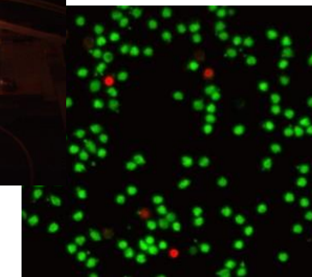
***REGENMED:** Metrology on a cellular scale for regenerative medicine

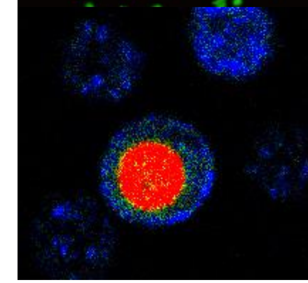
Coordination: Paul Tomlins

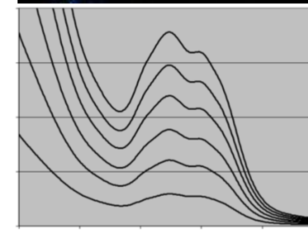
Thank you for your attention











Working Group „Flow Cytometry and Microscopy“

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