Standardization of extracellular vesicle measurements by flow cytometry

Edwin van der Pol

November 19th, 2019

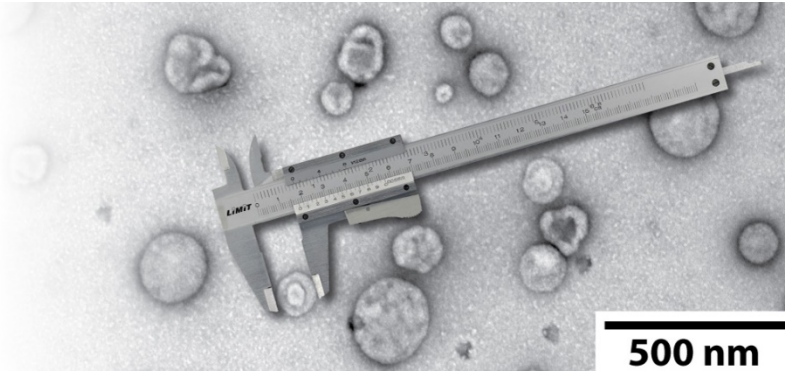
Vesicle Observation Center

METVES II

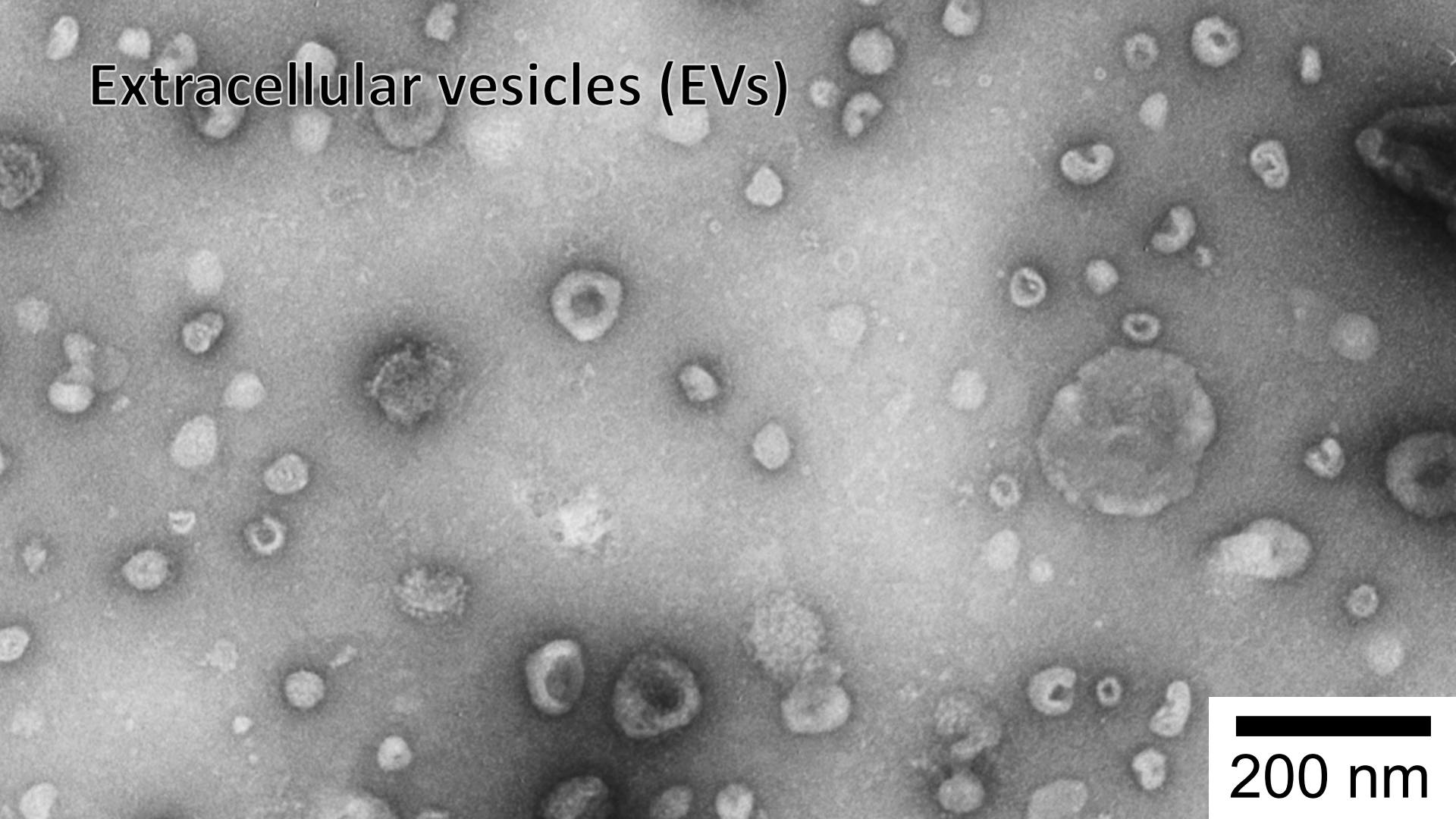
Amsterdam UMC
University Medical Centers

Outline

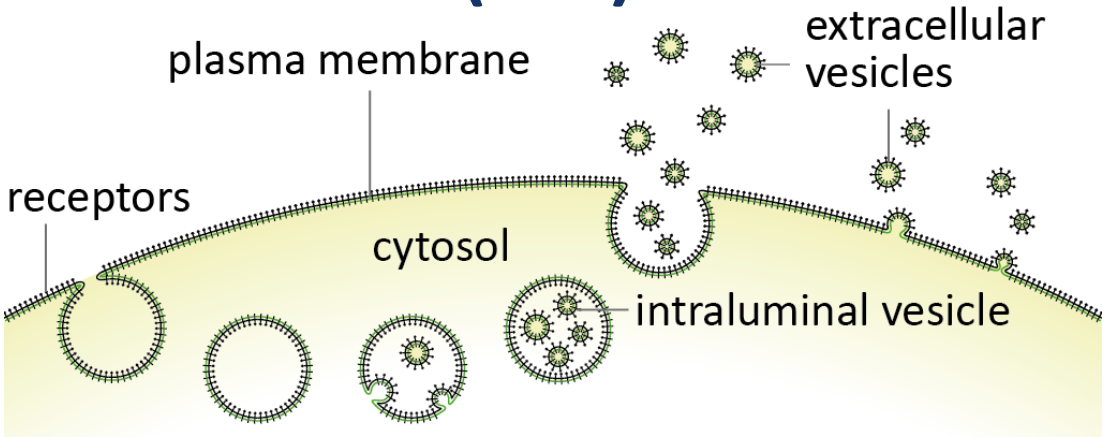
- Small particles: extracellular vesicles (EVs)
- Flow cytometry limitations
- Calibration
- Solid beads are misleading
- Swarm detection
- Standardisation of EV concentration measurements



Extracellular vesicles (EVs)



Extracellular vesicles (EVs)



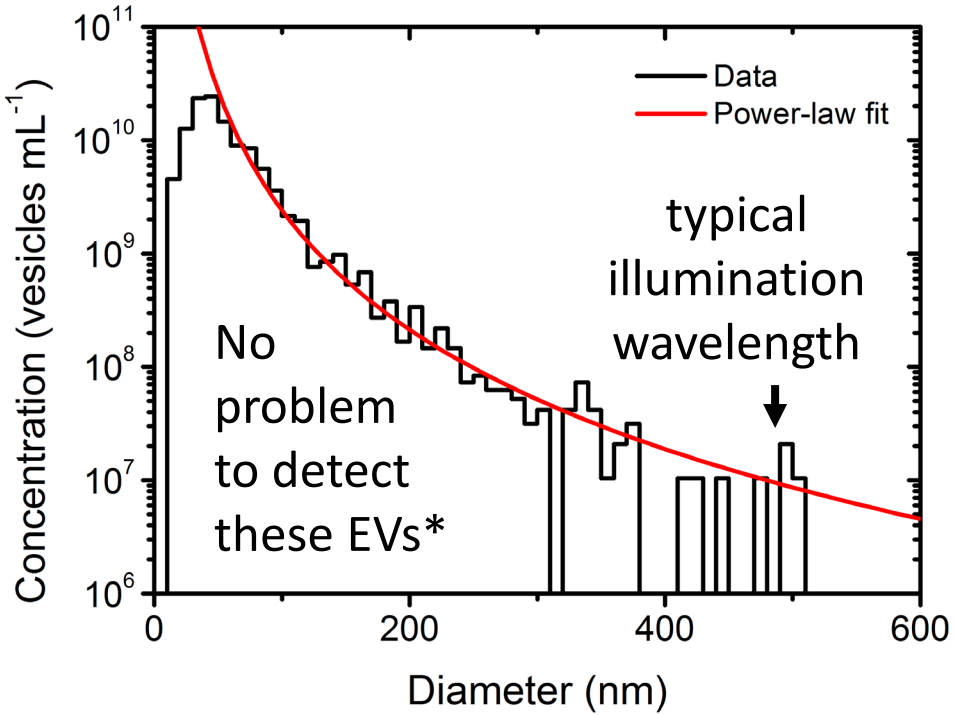
- cells release vesicles:
  biological nanoparticles with receptors, DNA, RNA
- specialized functions
- clinically relevant

EV-based liquid biopsy

Hematology parameter	Concentration (vesicles mL^{-1})
Platelet vesicle count	$2.3 - 6.2 \cdot 10^{9}$
Erythrocyte vesicle count	$7.0 - 8.6 \cdot 10^{10}$
Reticulocyte vesicle count	$3.9 - 15.6 \cdot 10^{8}$
Leukocyte vesicle count	$6.2 - 16.4 \cdot 10^{7}$
Total vesicle count	$7.3 - 9.4 \cdot 10^{10}$

Fictive values, PhD thesis van der Pol *UvA* 2015

Problem: EVs are small and heterogeneous



van der Pol et al. *JTH* 2014
*Zhu et al. ACS Nano 2014

Flow cytometry

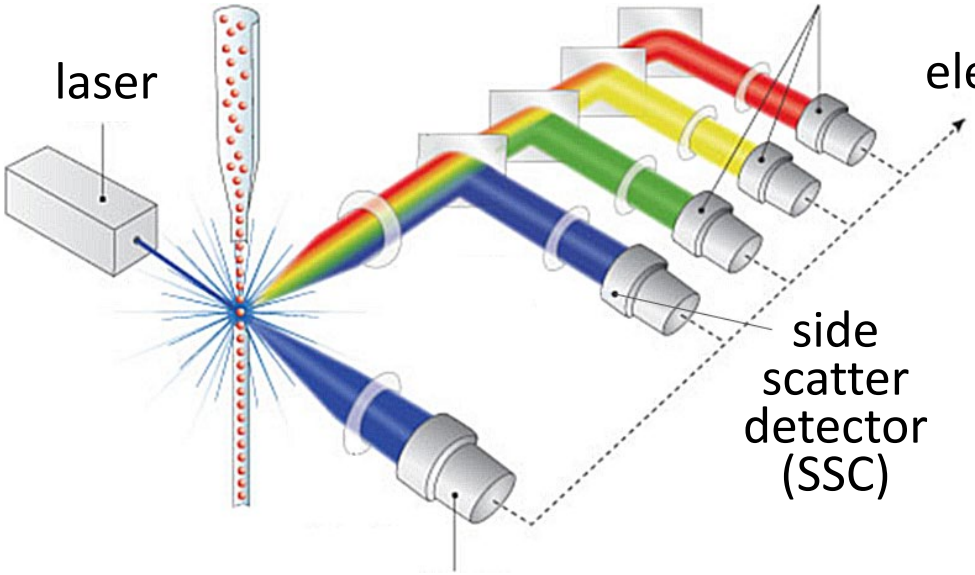
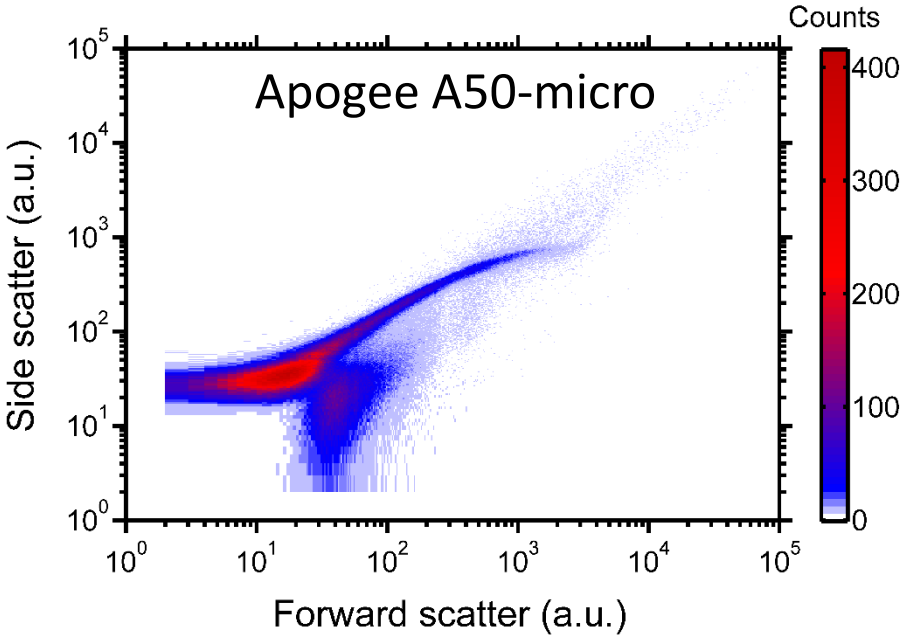
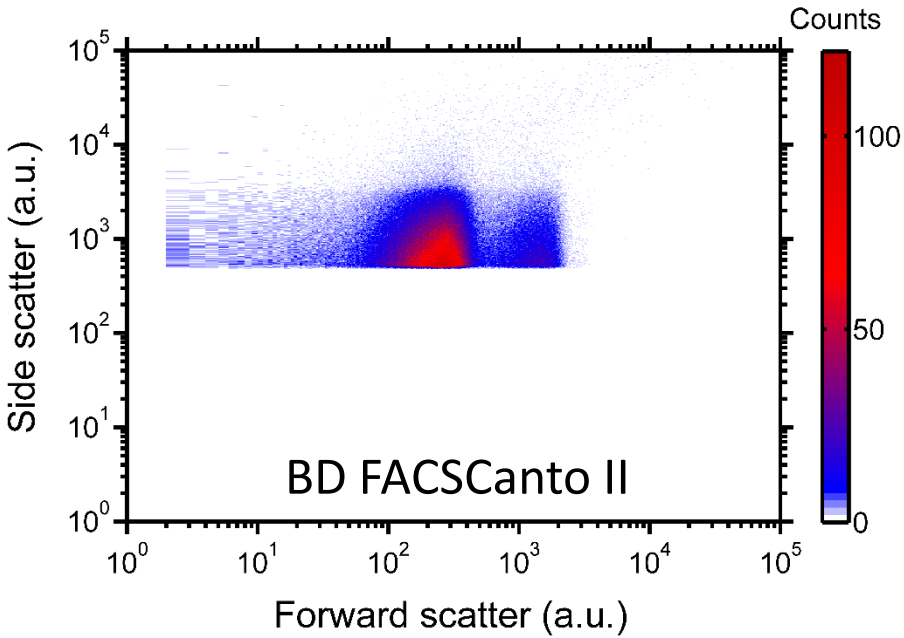


Image: semrock.com

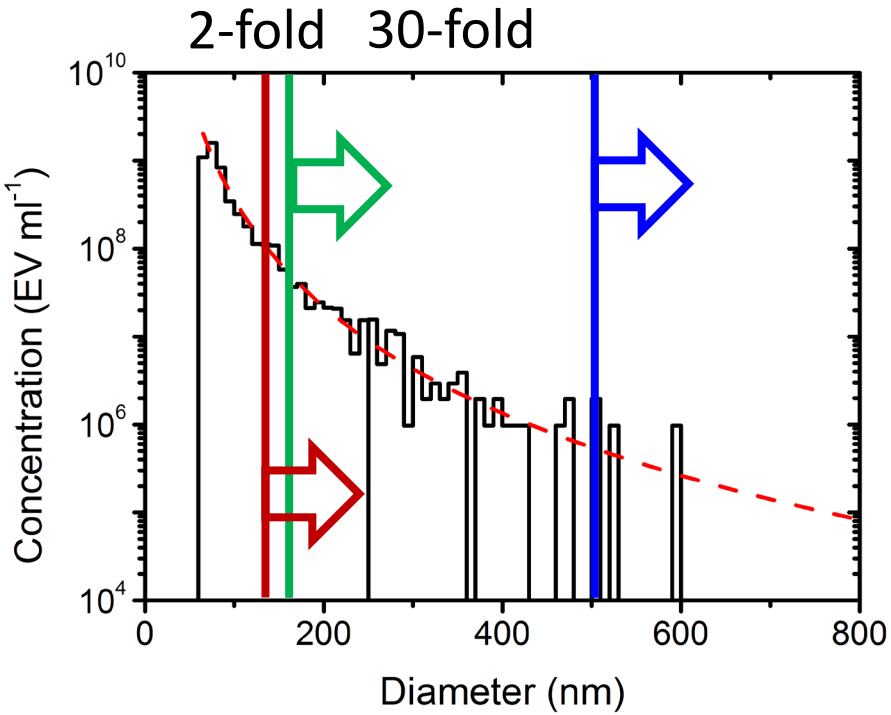
Problem 1: arbitrary units

same population of erythrocyte EVs



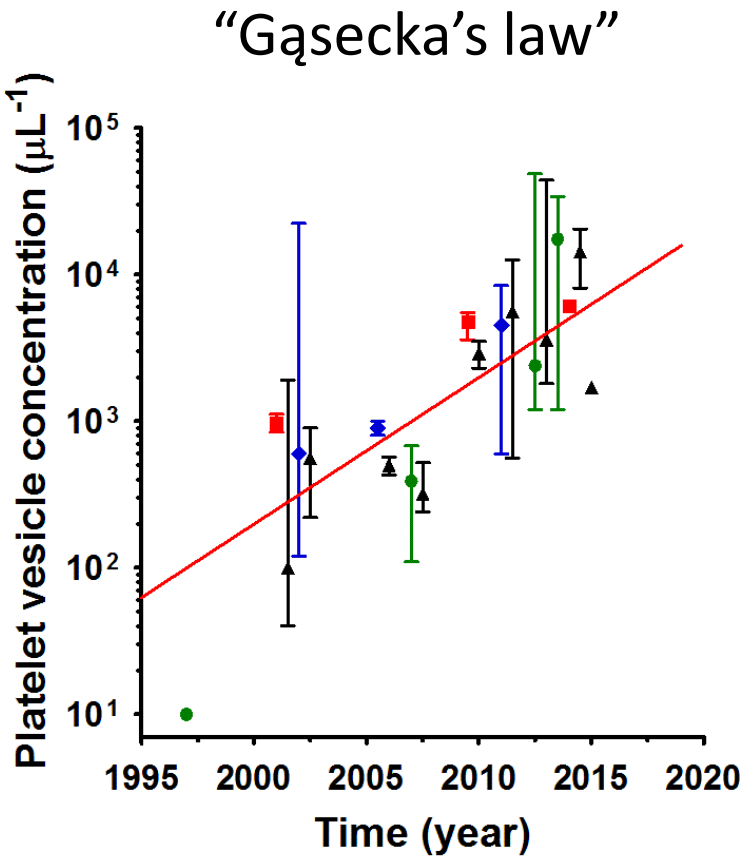


Problem 2: instruments differ in sensitivity



Clinical reality

- reported concentrations of blood plasma EV differ >10^6-fold
- clinical data cannot be compared
- standardization required

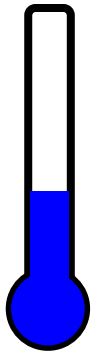


Solution

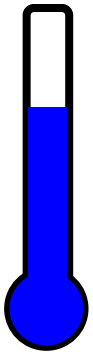
- Calibrate!

Thermometer: no calibration

Lab 1

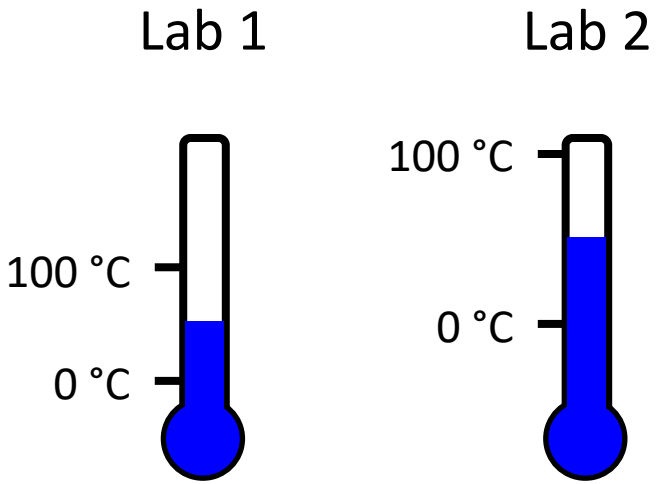


Lab 2



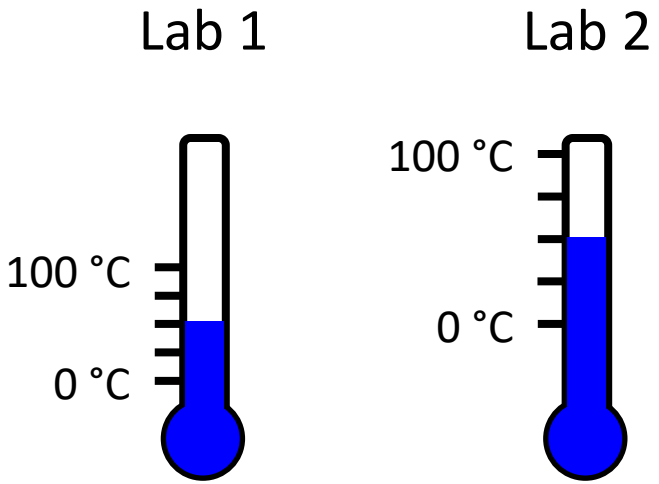
- Data interpretation
  - What is the temperature?
- Data comparison
  - Is the temperature equal?

Thermometer: measuring reference values



- Data interpretation
  - What is the temperature?
- Data comparison
  - Is the temperature equal?

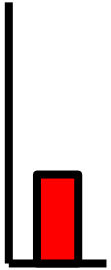
Thermometer: calibration



- Data interpretation
  - What is the temperature? 50 °C
- Data comparison
  - Is the temperature equal? Yes!

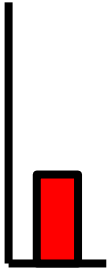
Flow cytometer: no calibration

BD LSR



Side scatter

BD Influx



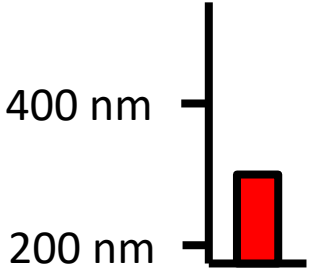
Forward scatter

- Data interpretation
  - What is the EV size?
- Data comparison
  - Do we study equal EV sizes?

Flow cytometer: measuring reference materials

BD LSR

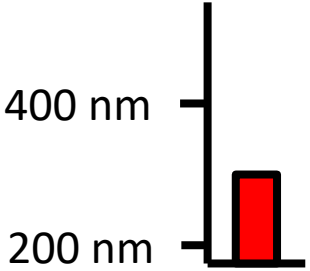
PS beads:



Side scatter

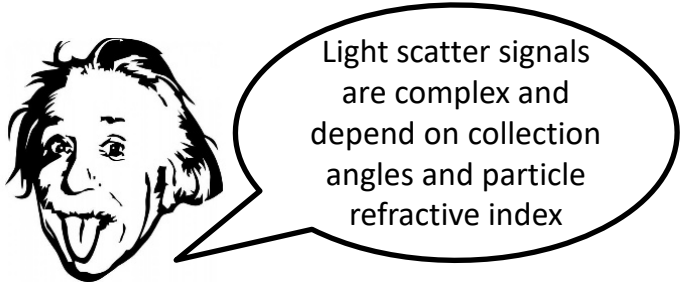
BD Influx

PS beads:

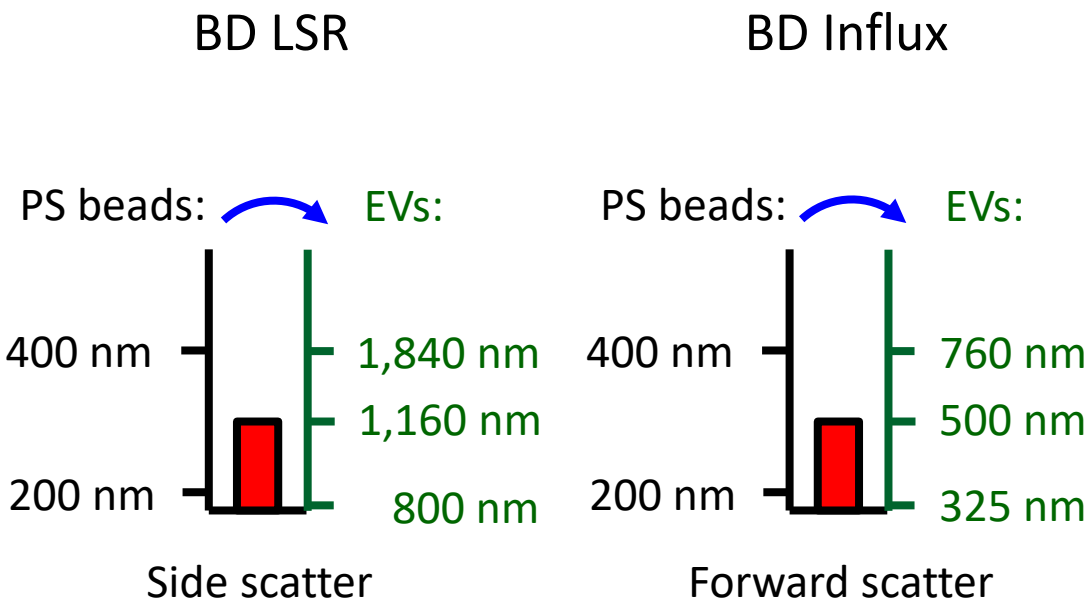


Forward scatter

- Data interpretation
  - What is the EV size? ~~300 nm?~~
- Data comparison
  - Do we study equal EV sizes? ~~Yes?~~

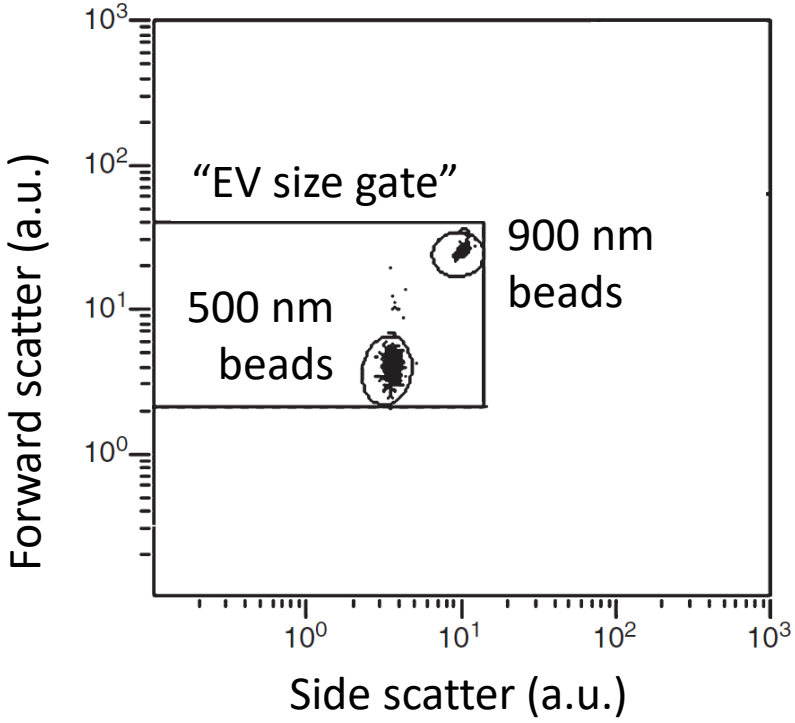


Flow cytometer: calibration



- Data interpretation
  - What is the EV size? 1,160 nm & 500 nm
- Data comparison
  - Do we study equal EV sizes? No!

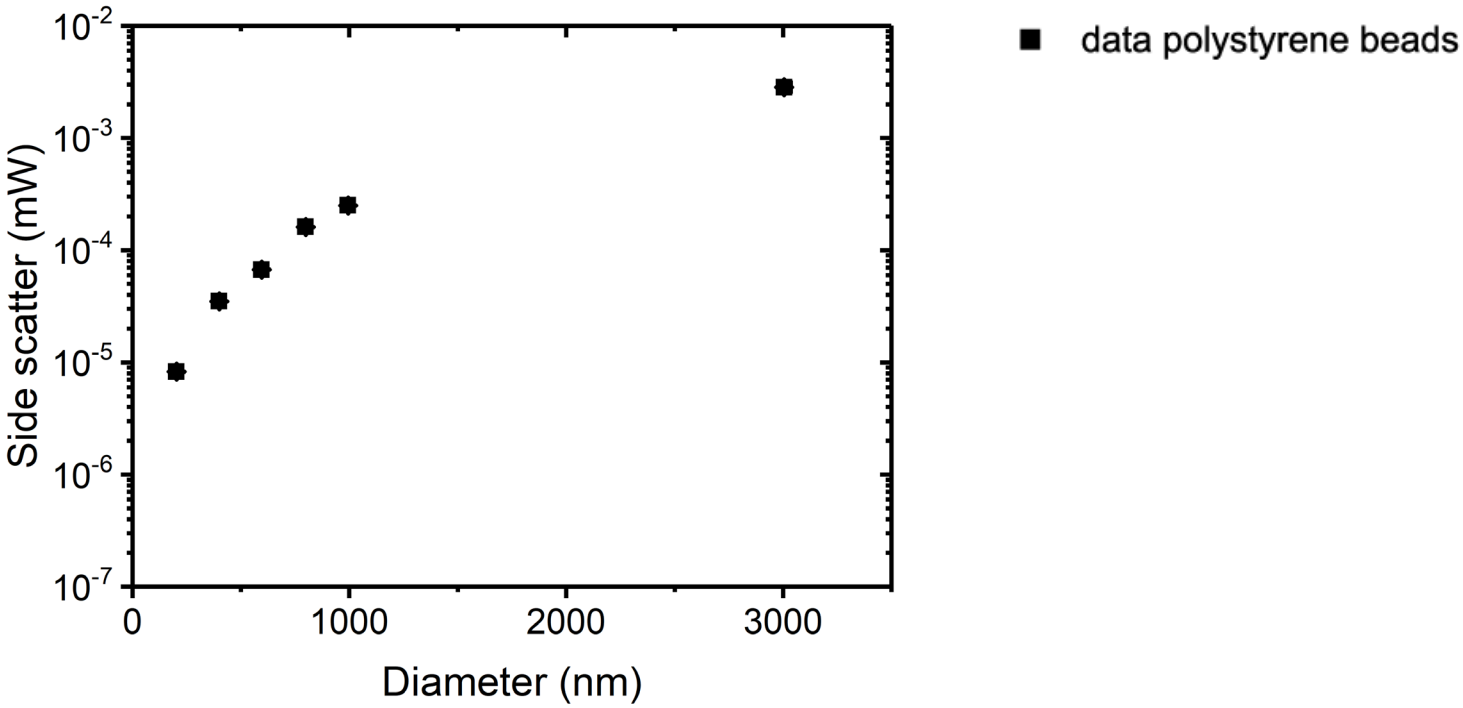
EV size gate based on polystyrene beads



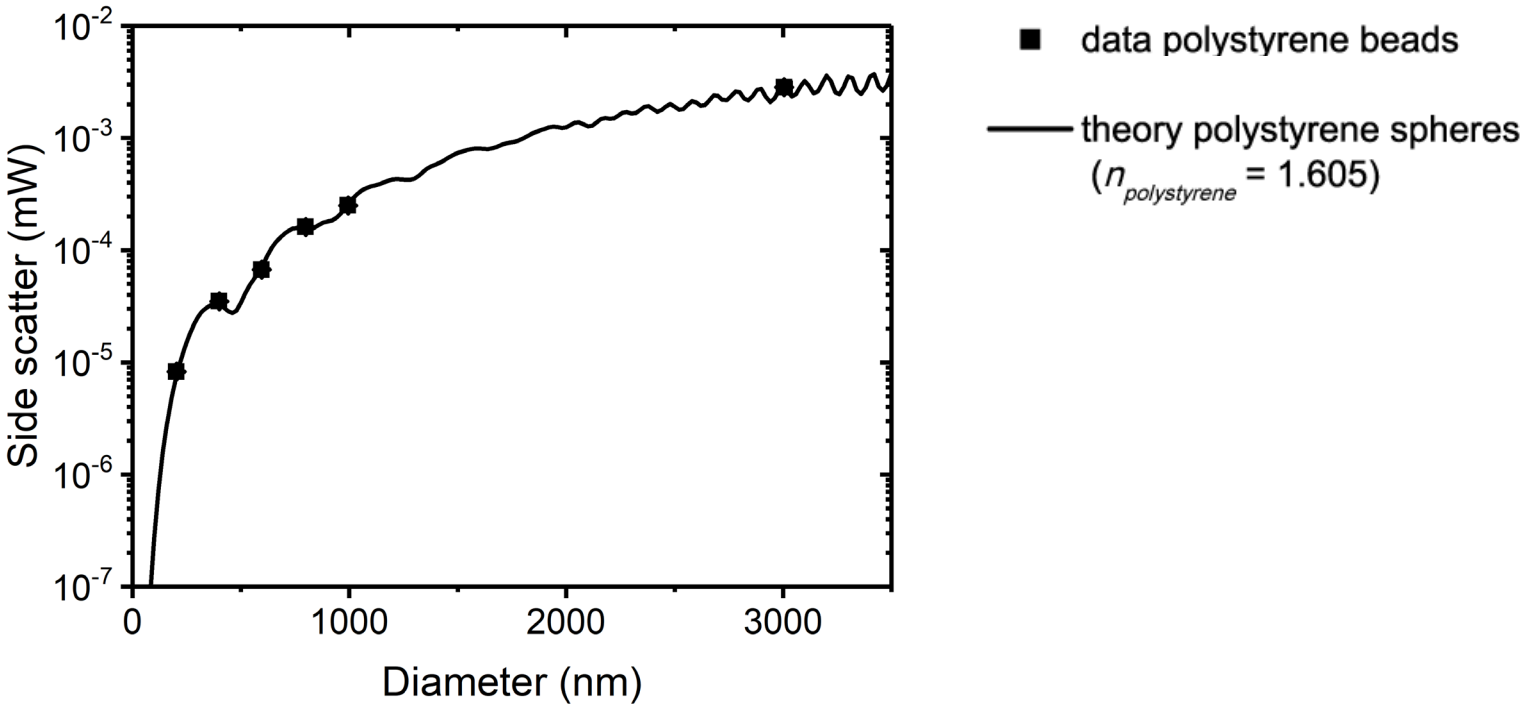
- Introduced in 2008
- Common practice
- Bad practice

image adopted: Robert et al. *J Thromb Haemost* 2008

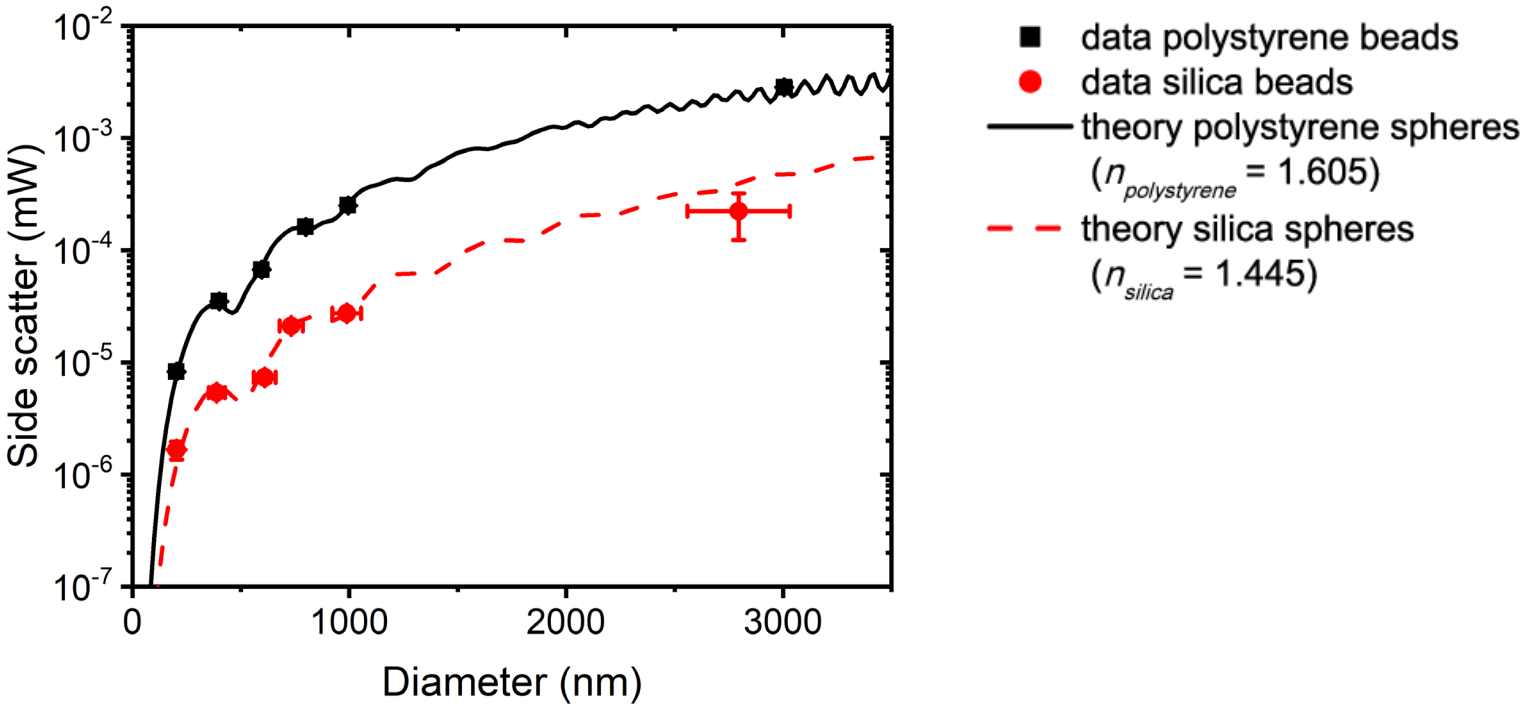
Relate scatter to diameter of beads



Relate scatter to diameter of beads

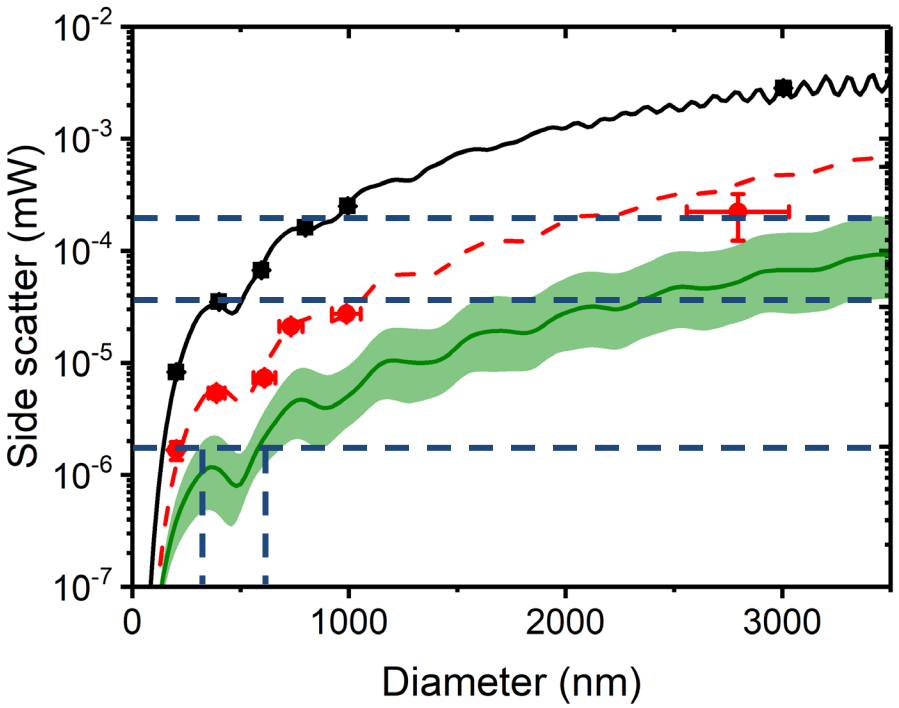


Relate scatter to diameter of beads

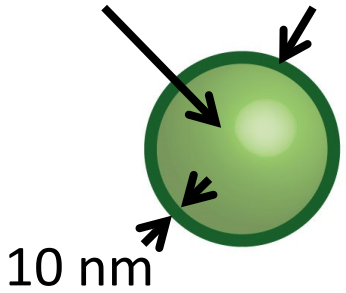


Model: de Rond et al. *Curr Protoc Cytom* 2018

Relate scatter to diameter of EVs

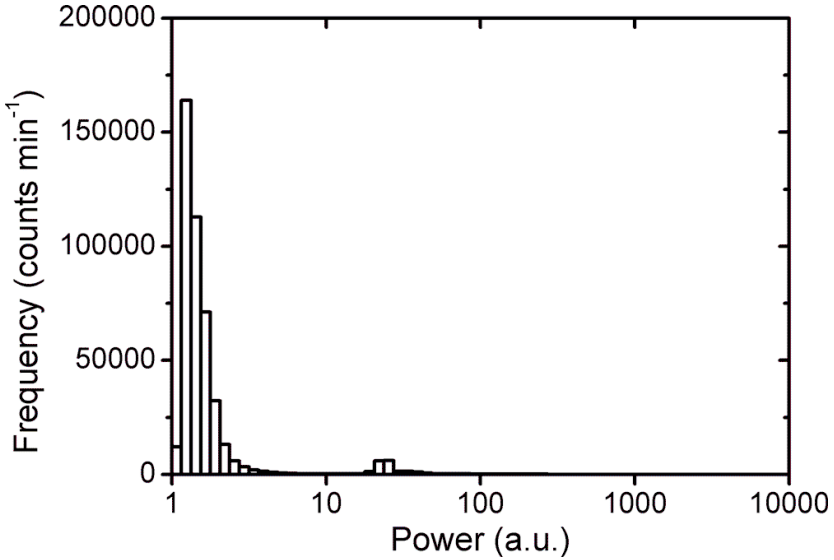


- data polystyrene beads
- data silica beads
- theory polystyrene spheres ($n_{polystyrene}$ = 1.605)
- theory silica spheres (n_{silica} = 1.445)
- theory vesicles (n_{core} = 1.38±0.02, n_{shell} = 1.48)

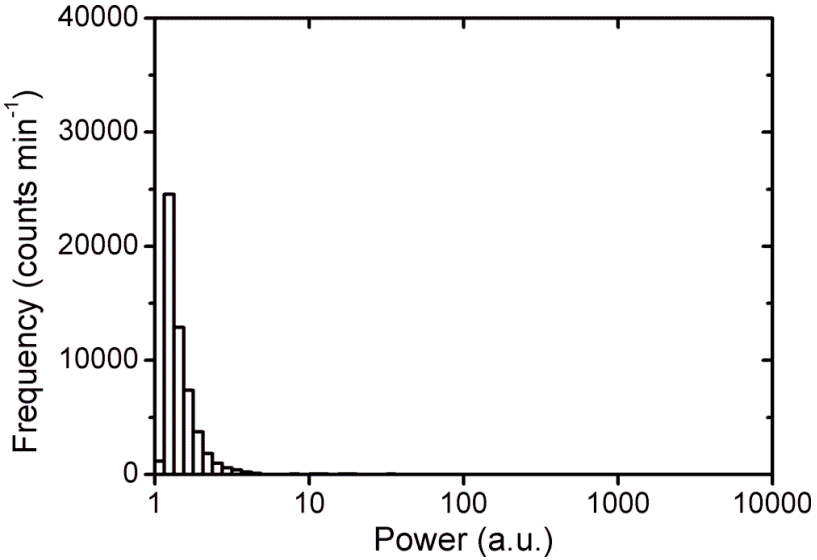


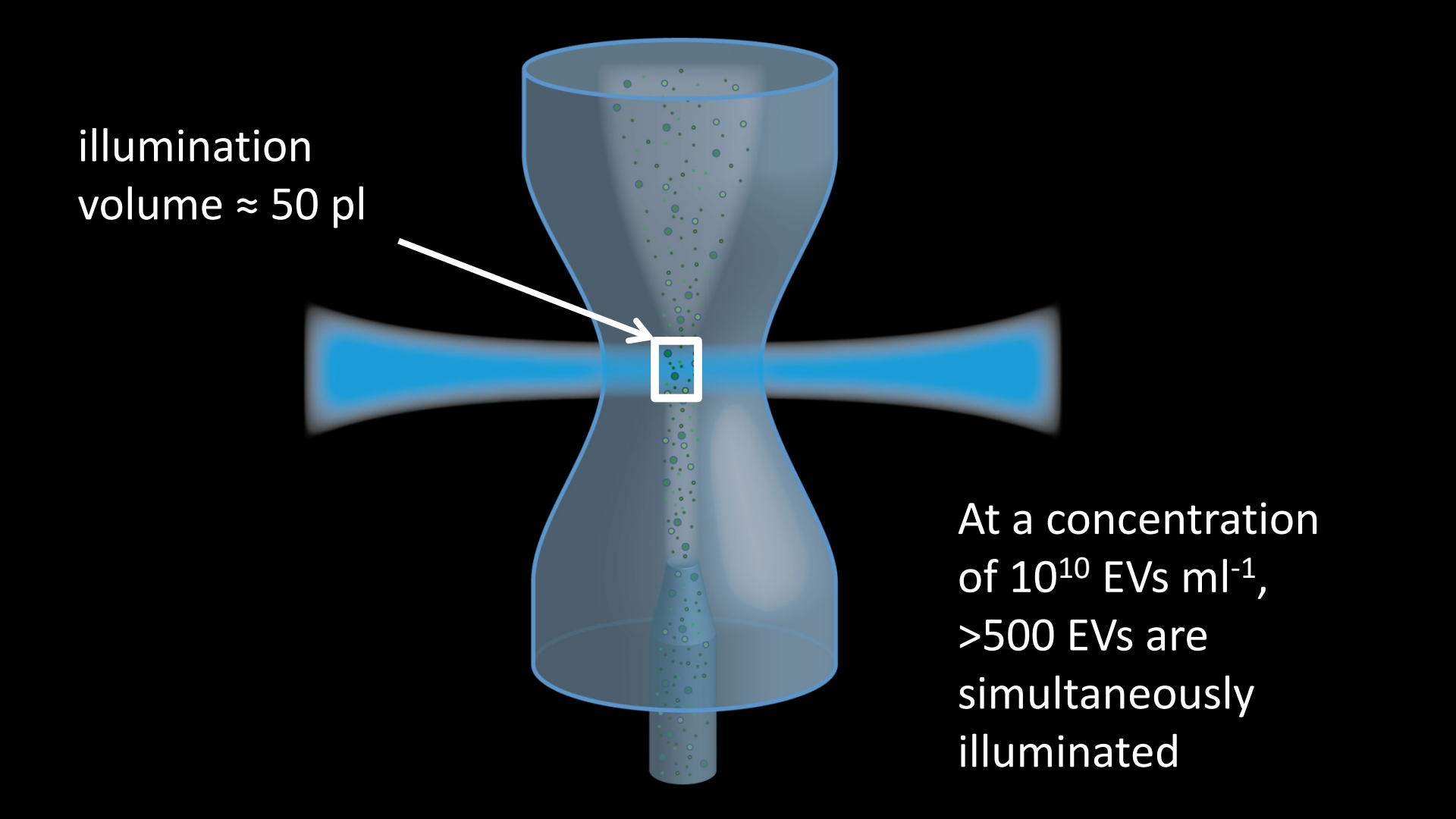
Particles below detection limit are detected

89 nm silica beads
(10^{10} ml^{-1})



220 nm filtered urine
(10^{10} EVs ml^{-1})




illumination
volume ≈ 50 pl
At a concentration
of 10^{10} EVs ml^{-1},
>500 EVs are
simultaneously
illuminated

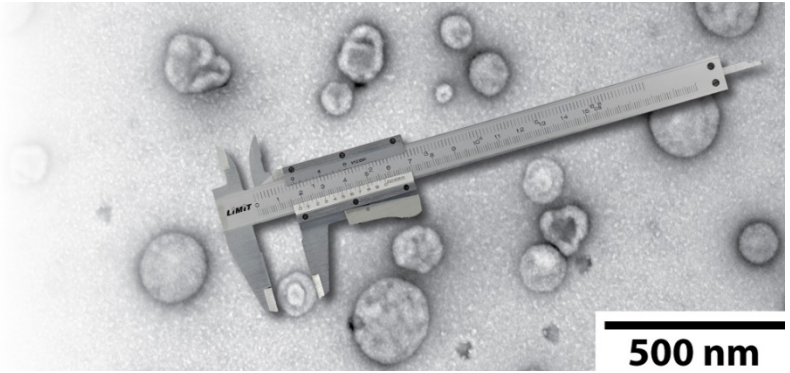
Swarm detection

Invisible vesicles swarm within the iceberg

Harrison & Gardiner *JTH* (2012)

Outline

- Small particles: extracellular vesicles (EVs)
- Flow cytometry limitations
- Calibration
- Solid beads are misleading
- Swarm detection
- Standardisation of EV concentration measurements



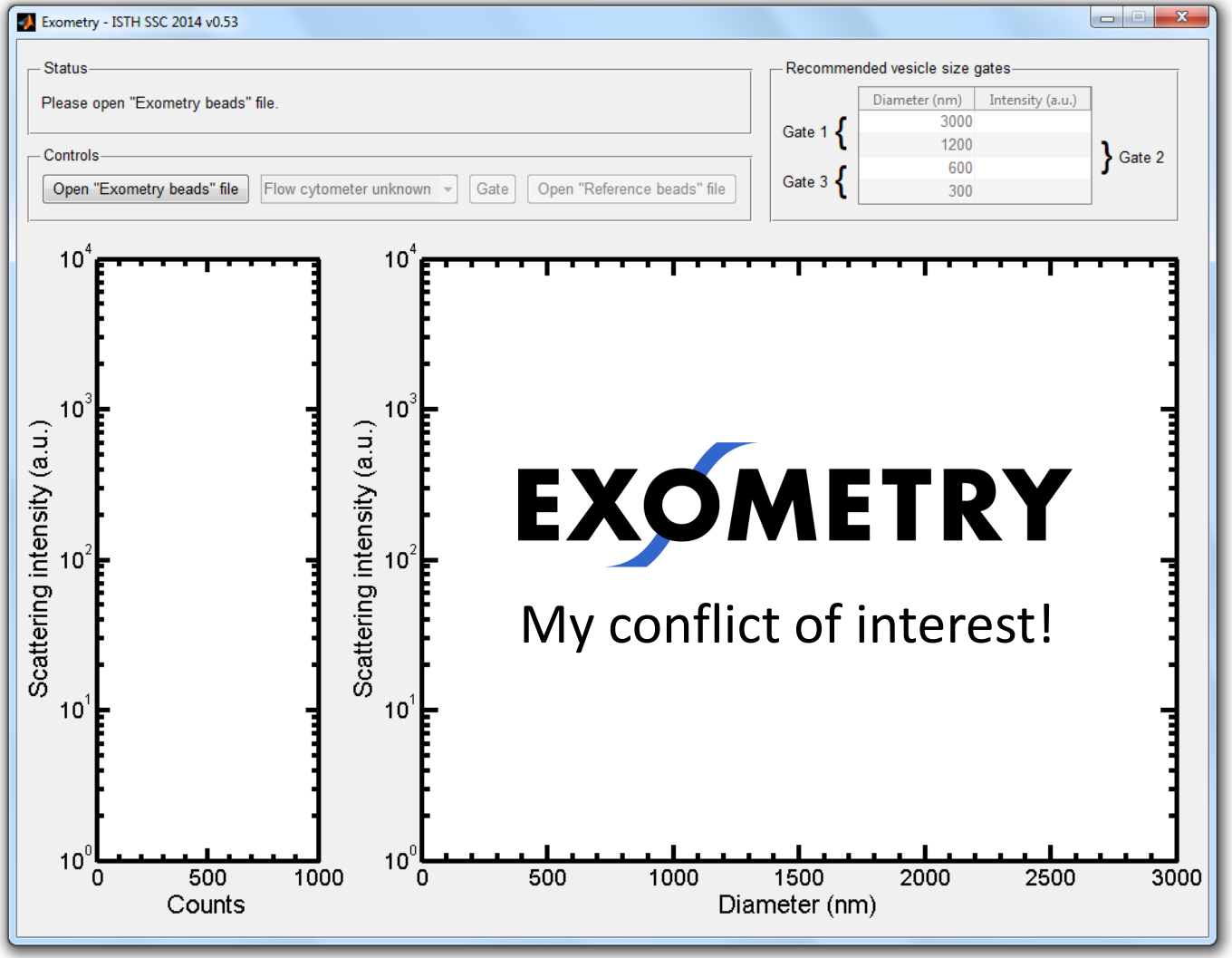
Study comprises 33 sites (64 instruments) worldwide

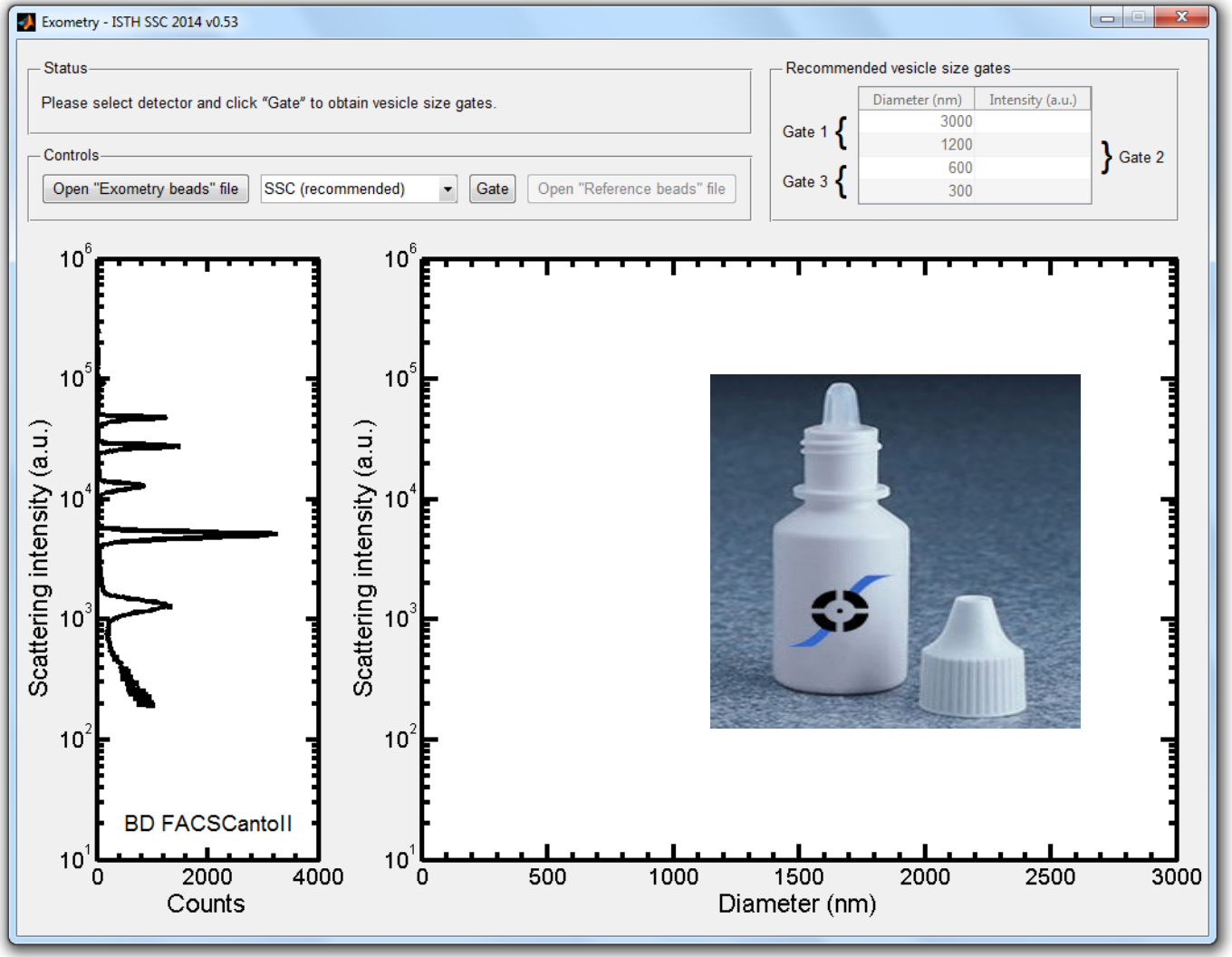
METVES

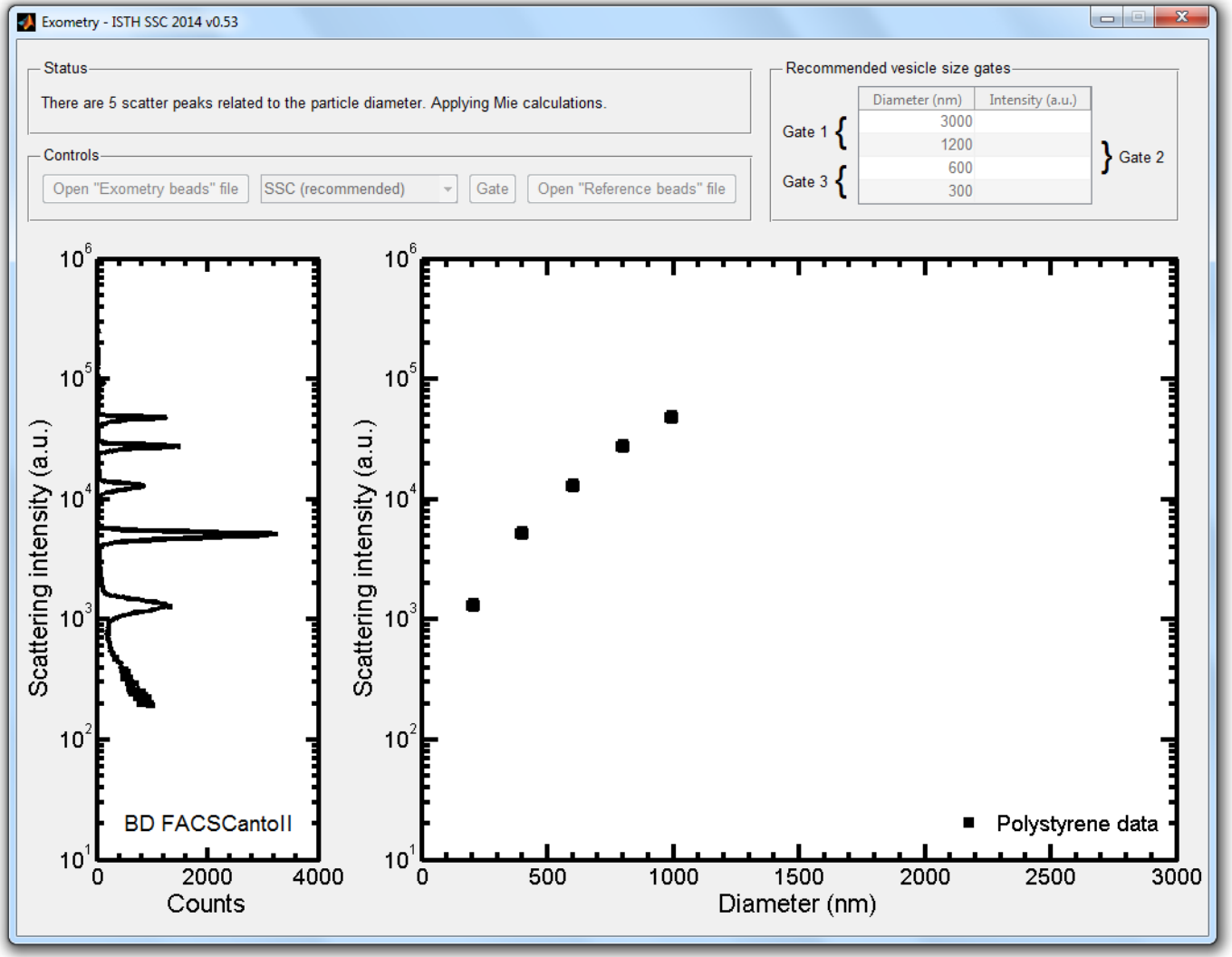
International Society on Thrombosis and Haemostasis

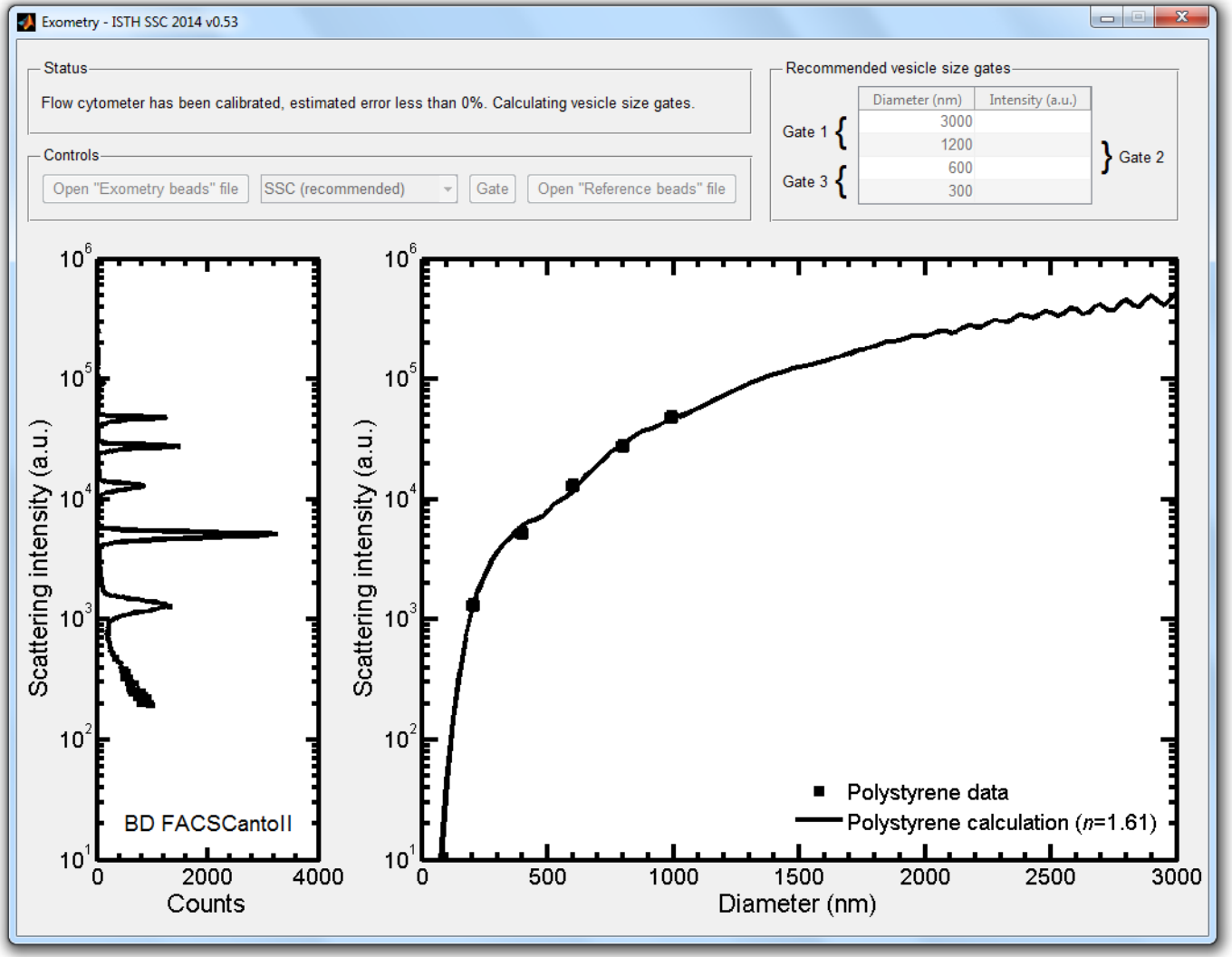
Vesicle Observation Center

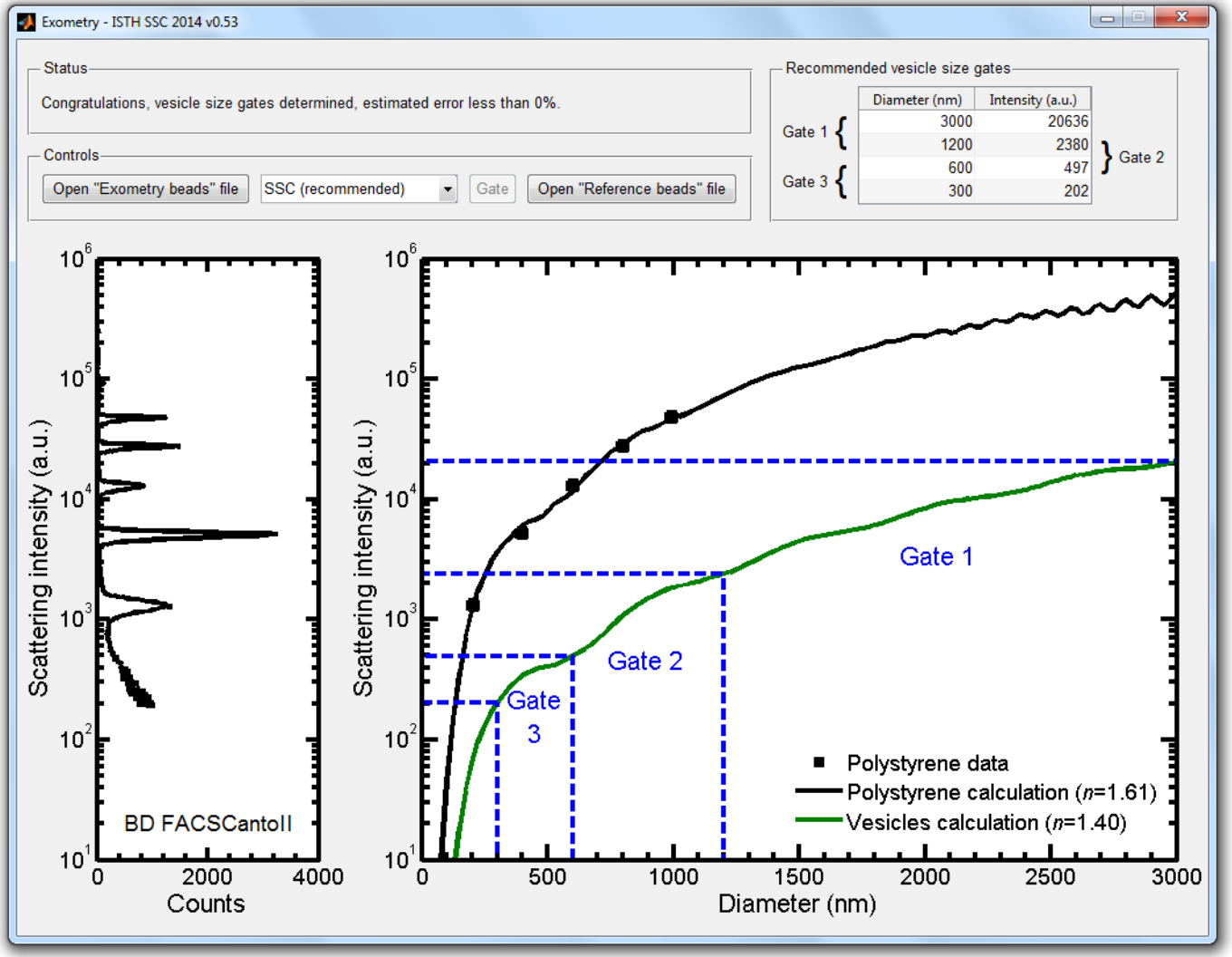
EXOMETRY

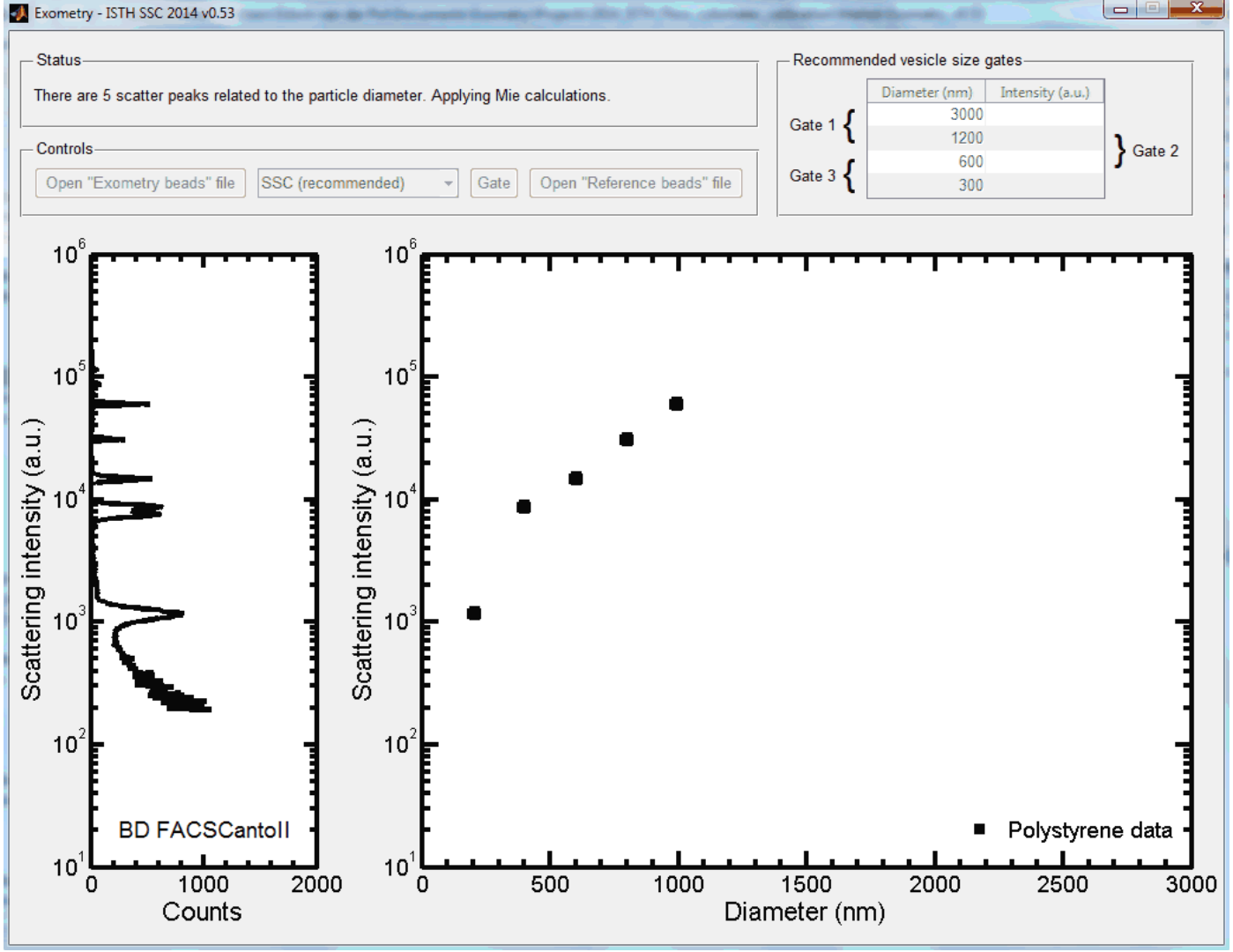

Exometry - ISTH SSC 2014 v0.53
Status
Please open "Exometry beads" file.
Controls
Open "Exometry beads" file
Flow cytometer unknown
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
Gate 3
Gate 2
3000
1200
600
300
Scattering intensity (a.u.)
Counts
Diameter (nm)
EXOMETRY
My conflict of interest!


Exometry - ISTH SSC 2014 v0.53
Status
Please select detector and click "Gate" to obtain vesicle size gates.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
BD FACSCantoII
Counts
Diameter (nm)

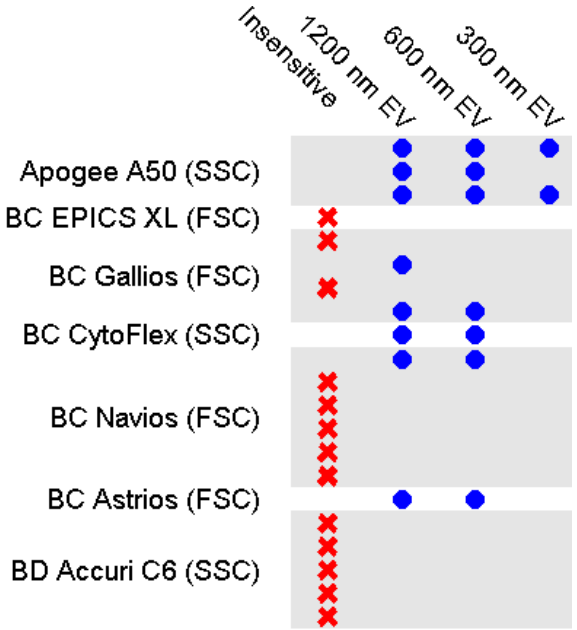

Exometry - ISTH SSC 2014 v0.53
Status
There are 5 scatter peaks related to the particle diameter. Applying Mie calculations.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
BD FACSCantoII
Counts
Polystyrene data
Diameter (nm)

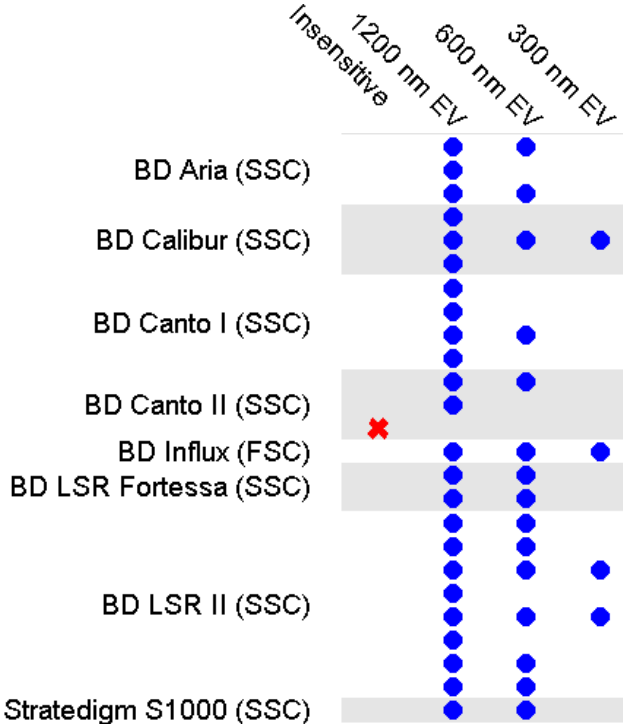

Exometry - ISTH SSC 2014 v0.53
Status
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
BD FACSCantoII
Counts
Diameter (nm)
Polystyrene data
Polystyrene calculation (n=1.61)


Exometry - ISTH SSC 2014 v0.53
Status
Congratulations, vesicle size gates determined, estimated error less than 0%.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
20636
1200
2380
Gate 2
Gate 3
600
497
300
202
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)
Gate 1
Gate 2
Gate 3
Polystyrene data
Polystyrene calculation (n=1.61)
Vesicles calculation (n=1.40)


Exometry - ISTH SSC 2014 v0.53
Status
There are 5 scatter peaks related to the particle diameter. Applying Mie calculations.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
BD FACSCantoII
Counts
Polystyrene data
Diameter (nm)

Sensitivity of 46 flow cytometers in the field





✖ = unable to detect 400 nm fluorescent polystyrene beads

Reproducibility of 1200-3000 nm EVs, 31 FCMs

	CV(%)
Gate on beads	139%
Gate on EV size with light scatter theory	81%

Requires improvement!

%CV = standard deviation / mean * 100%

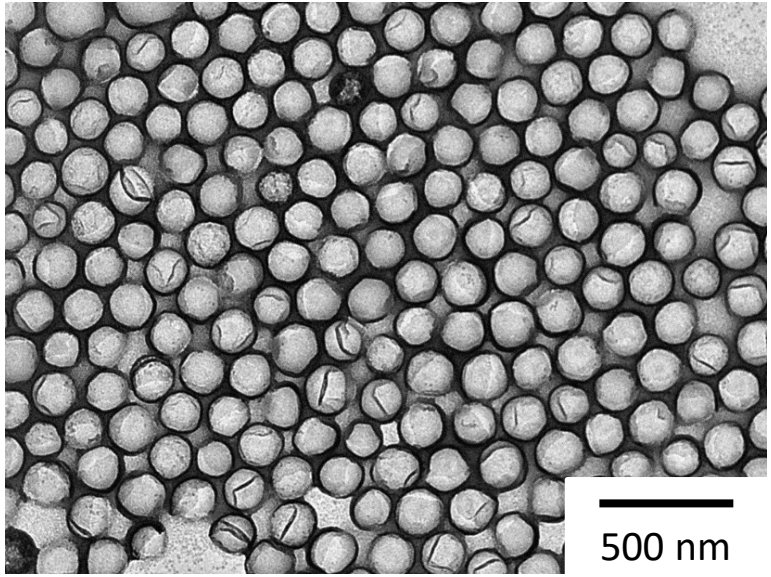
Outlook: METVES II

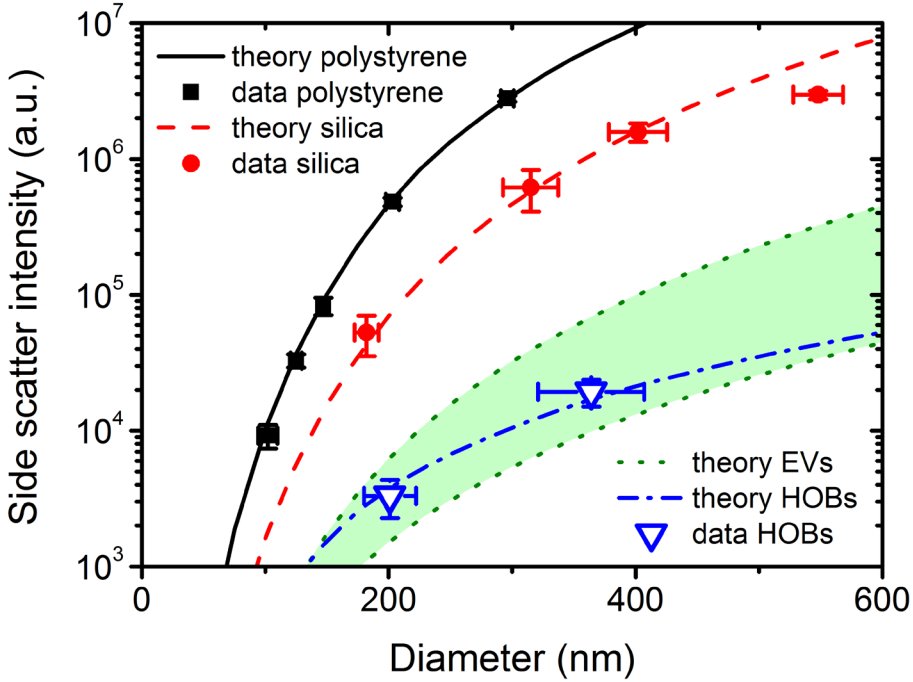
- one bead to calibrate them all
  - fluorescence
    - 100 – 100,000 fluorescent molecules
  - number concentration
    - 10^9 – 10^{12} particles mL^{-1}
  - scatter
    - discrete diameters between 50 nm – 1,000 nm
    - refractive index between 1.37 – 1.42

METVES II consortium

- National metrology institutes
  - BAM, LGC, LNE, PTB, VSL, VTT
- Academic partners
  - AMC, UH, MTA TTK
- Industry
  - BD, Exometry, PolyAn

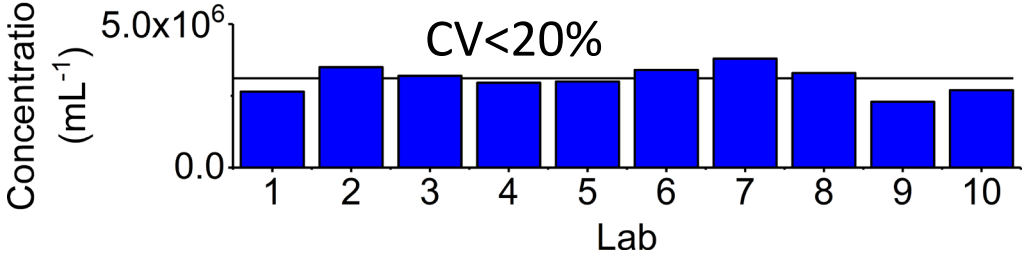
Example: hollow organosilica beads (HOBs)

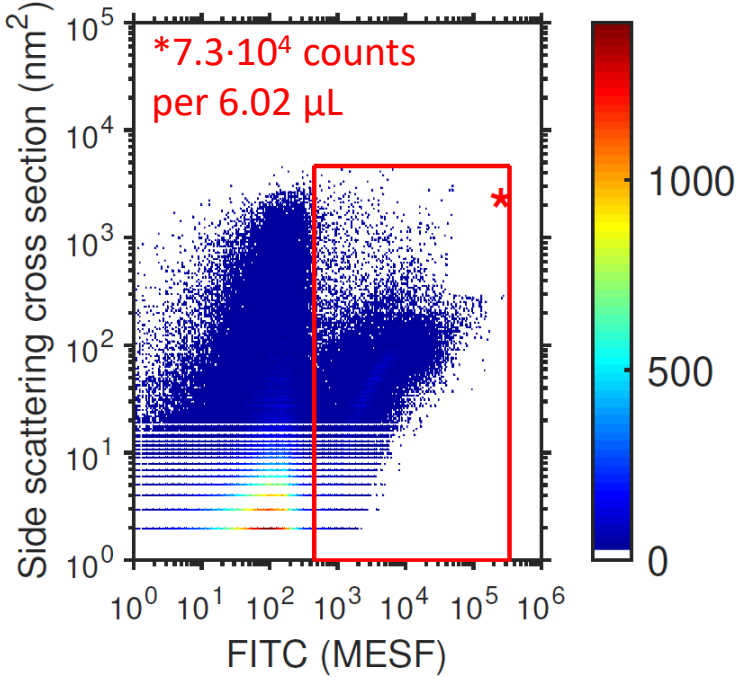




Anticipated outcome comparison study

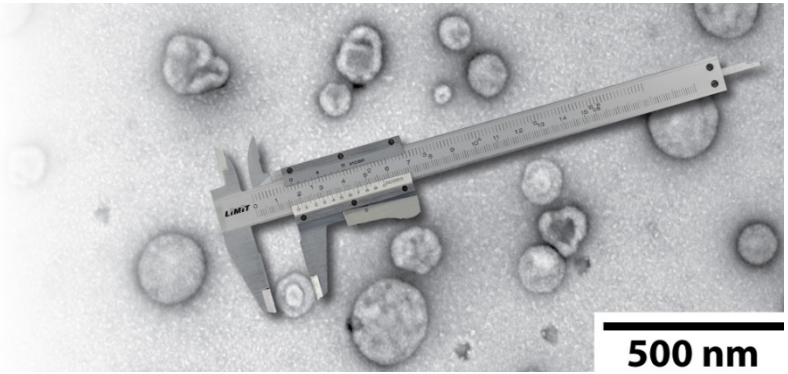
- Per lab:
  - flow cytometry
    - reference materials
    - biological test samples
  - fully automated calibration & data analysis





Summary

- Extracellular vesicles (EVs): small and heterogeneous
- Flow cytometry limitation: arbitrary units
- Calibrate flow, fluorescence & scatter!
- Solid bead gates are misleading
- Avoid swarm detection
- Standardize



Acknowledgements

- Amsterdam University Medical Centers
  - Vesicle Observation Center
  - Biomedical Engineering & Physics
  - Laboratory Experimental Clinical Chemistry
- Hungarian Academy of Sciences
  - Zoltan Varga
- Funding
  - EURAMET
  - ISTH
  - NWO-TTW

Relevant websites

- edwinvanderpol.com

- evflowcytometry.org

INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

isth International Society on Thrombosis and Haemostasis

ISAC INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

- exometry.com

EXOMETRY

- metves.eu

METVES II

- nlsev.nl

NLSEV