The dos and don’ts of single extracellular vesicle detection

Edwin van der Pol

August 26th, 2021

Conflict of interest

- Cofounder and shareholder of EXOMETRY

Goal: 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

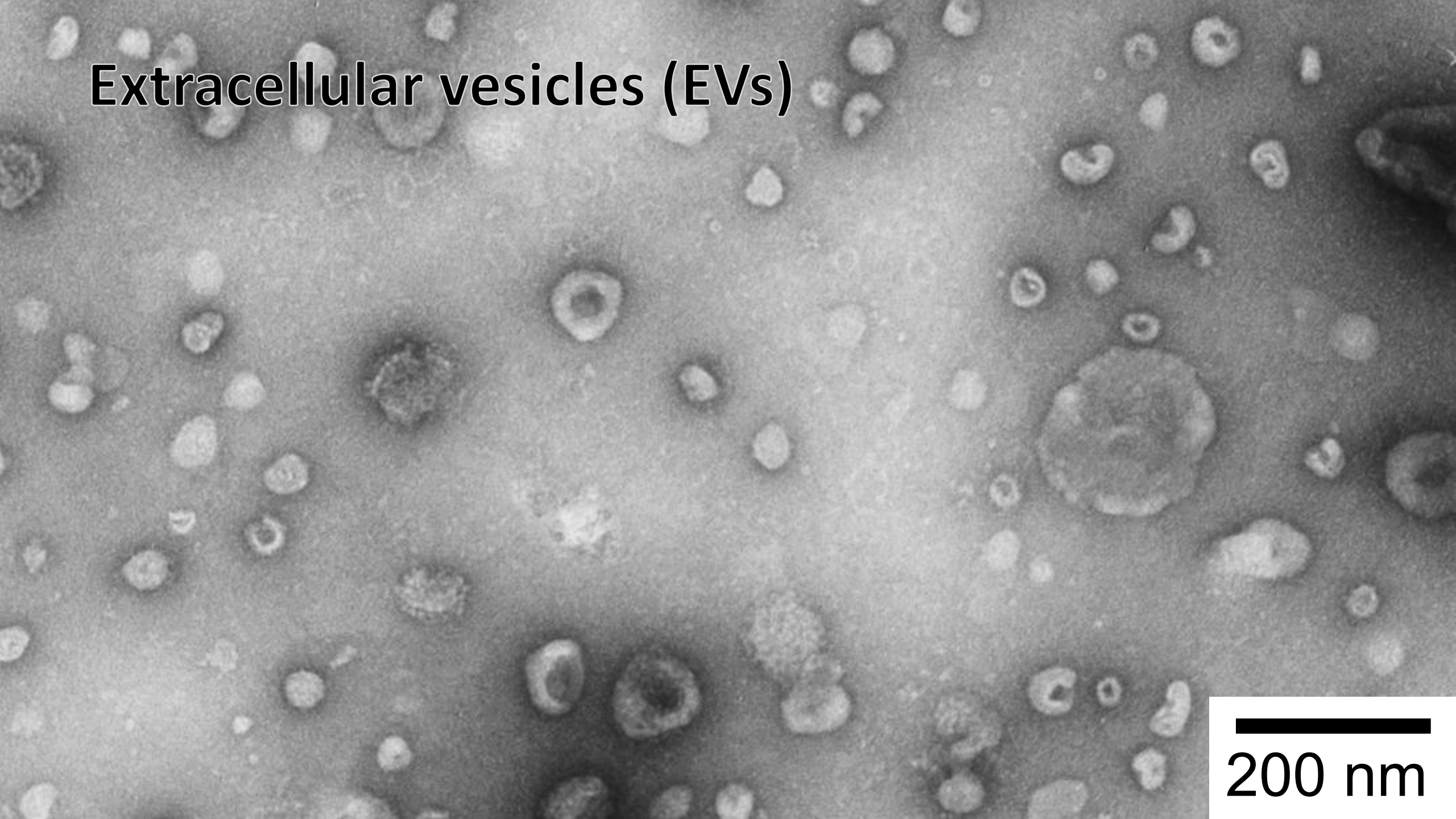
EV-based liquid biopsy: difficulties

- Single EV characterization
- Data interpretation
- Data comparison

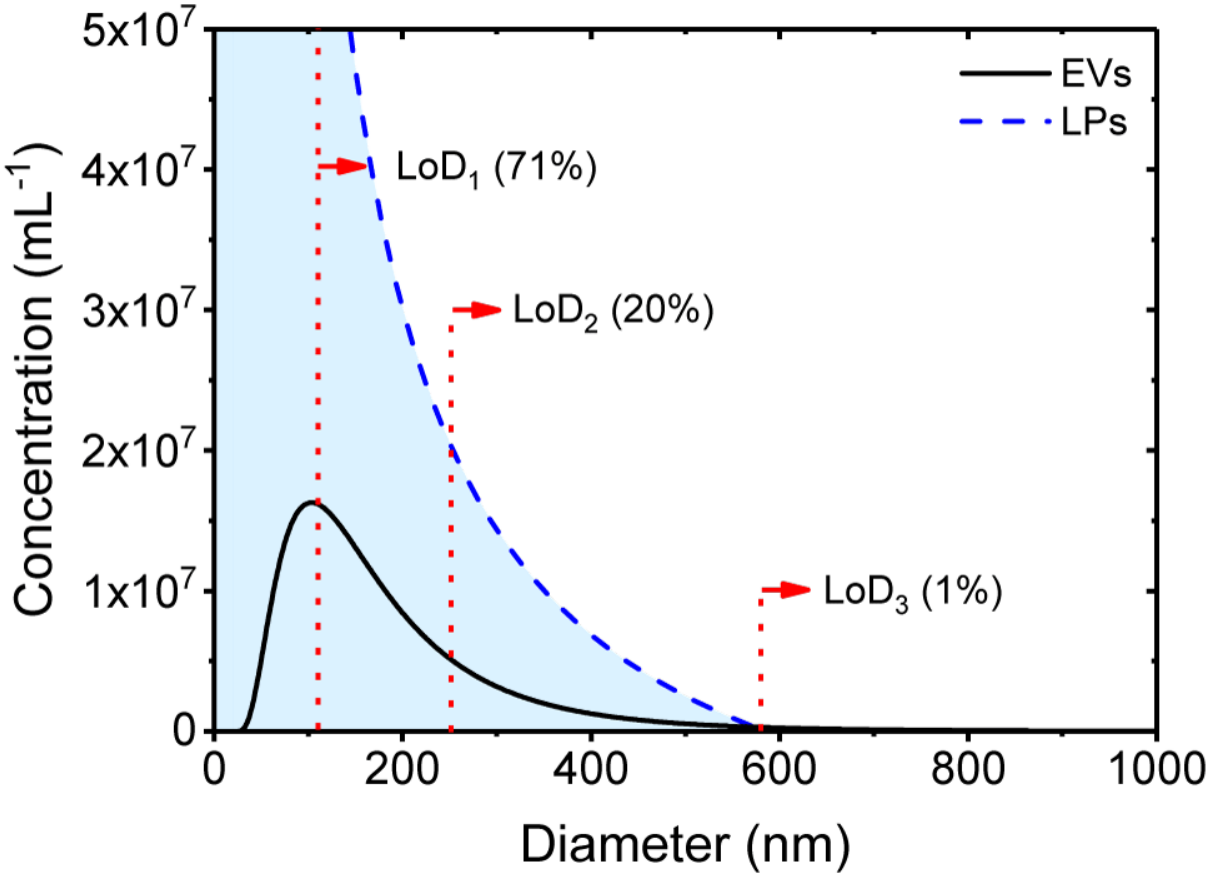
Outline

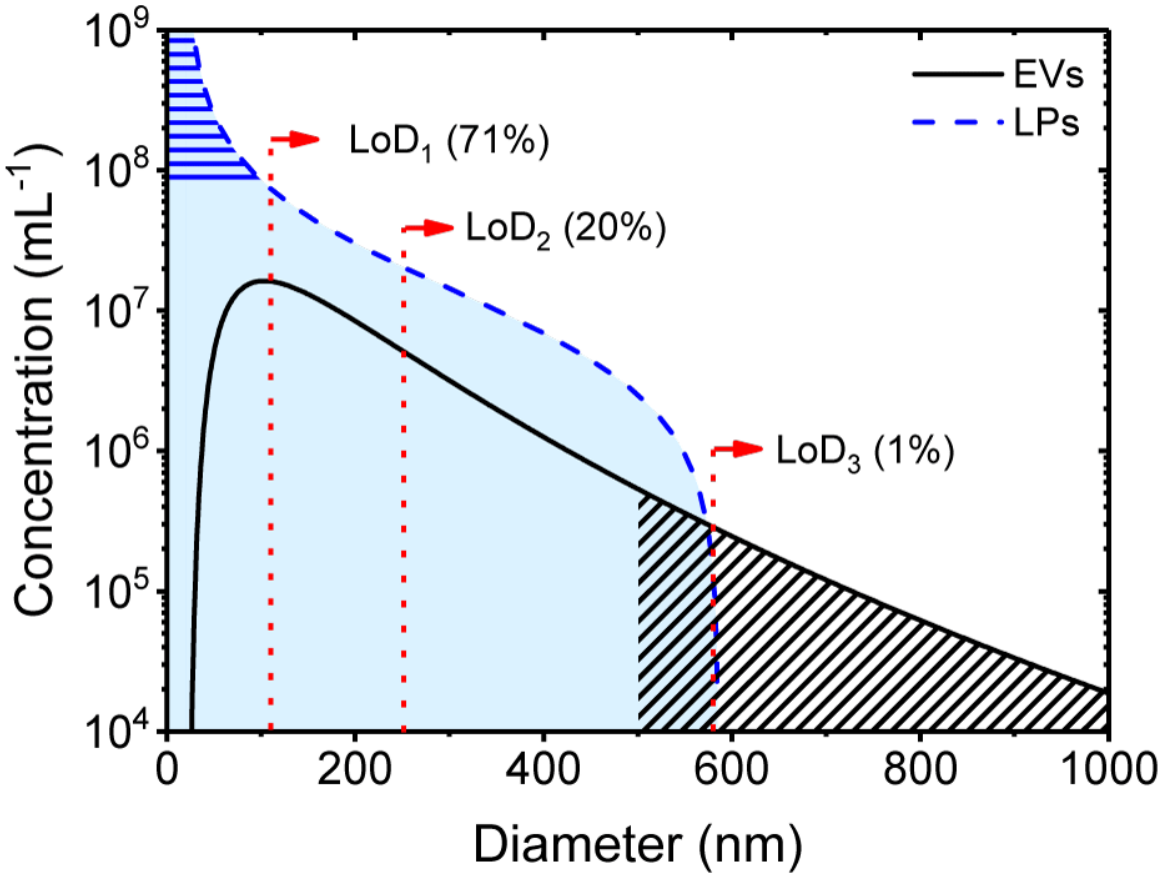
- EV detection: size does matter
- Flow cytometry
  - Magic of calibration: scatter-based sizing
  - Relevance of calibration: 2 examples
  - Data reporting
- Outlook
- Dos and don’ts

Extracellular vesicles (EVs)



Extracellular vesicle samples and size distribution

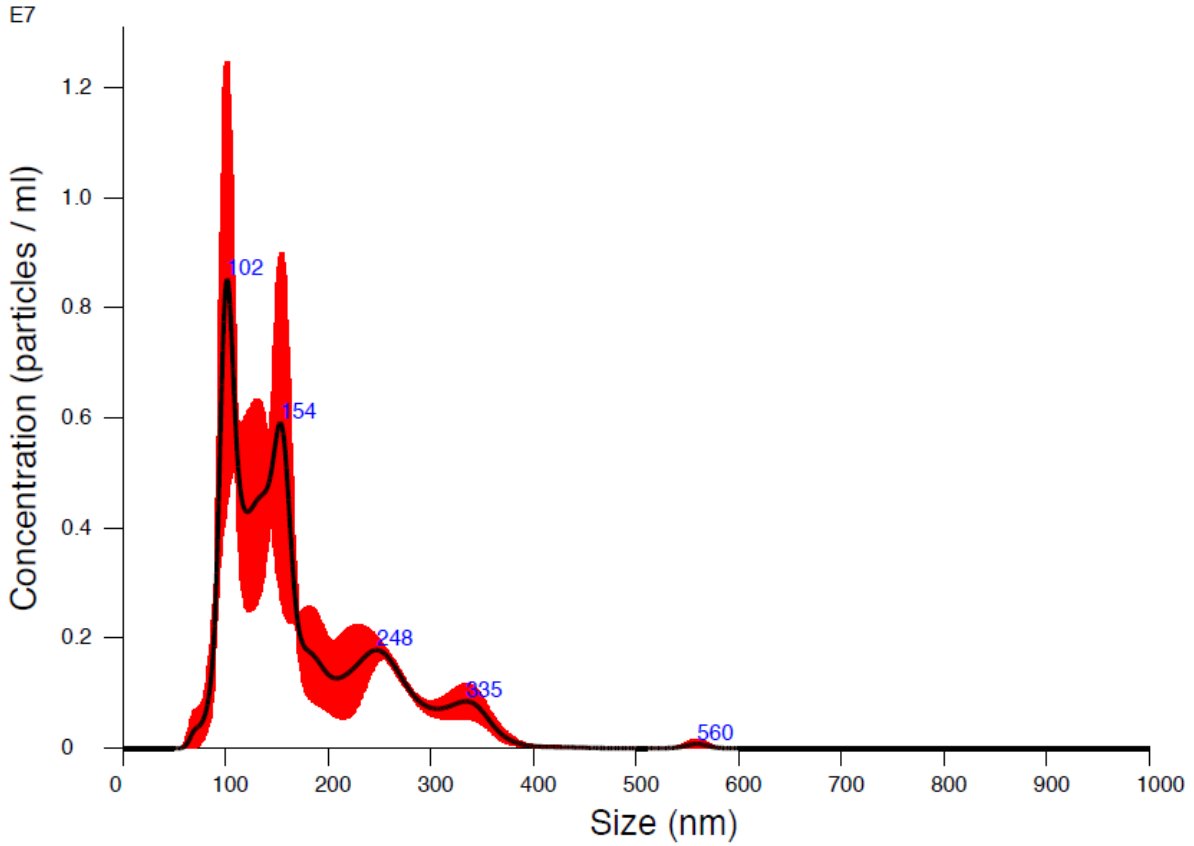




LoD: limit of detection, LPs: lipoprotein particles

~~Hurray: we measured EVs!~~

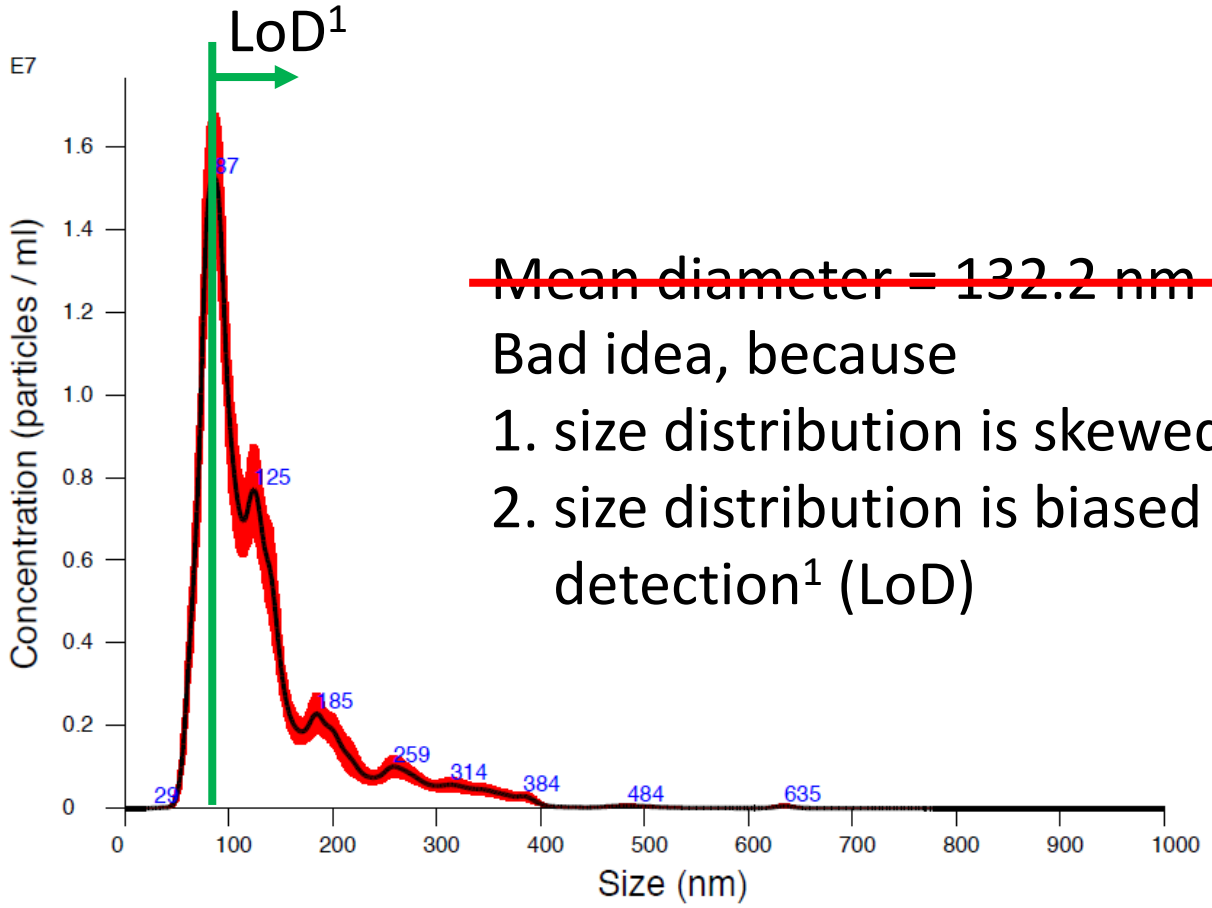
No, you measured too little particles



Example: MCF7 EVs by nanoparticle tracking analysis

~~Hurray: we measured EVs?~~

Almost. You measured sub-µm particles. And now?



~~Mean diameter = 132.2 nm~~

Bad idea, because

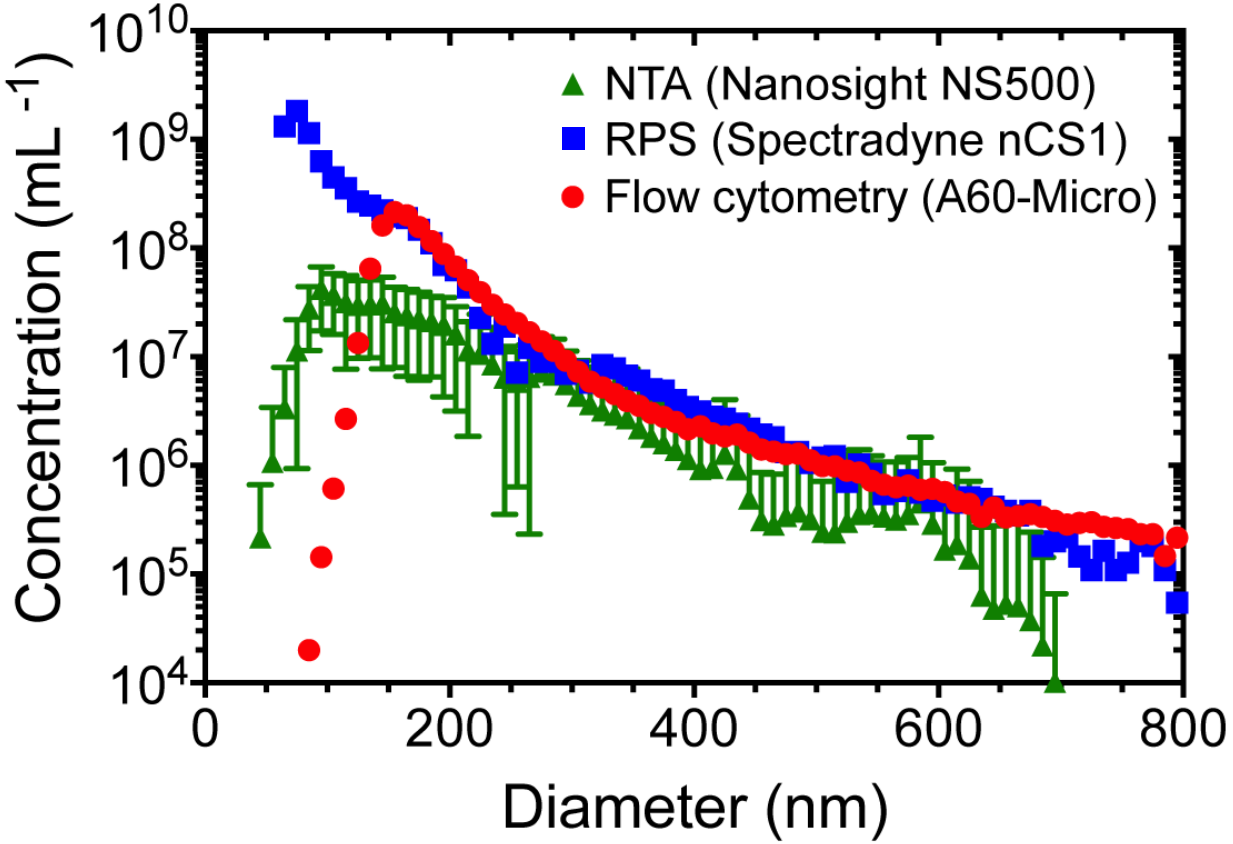
1. size distribution is skewed
2. size distribution is biased by lower limit of detection[1] (LoD)

Example: Urine EVs by nanoparticle tracking analysis

1. van der Pol et al. *J Thromb Haemost* 2014

Reporting mean diameters: example

Particles in human urine



- Bad practice for biofluids:
  - report mean diameters
  - report concentration without size range

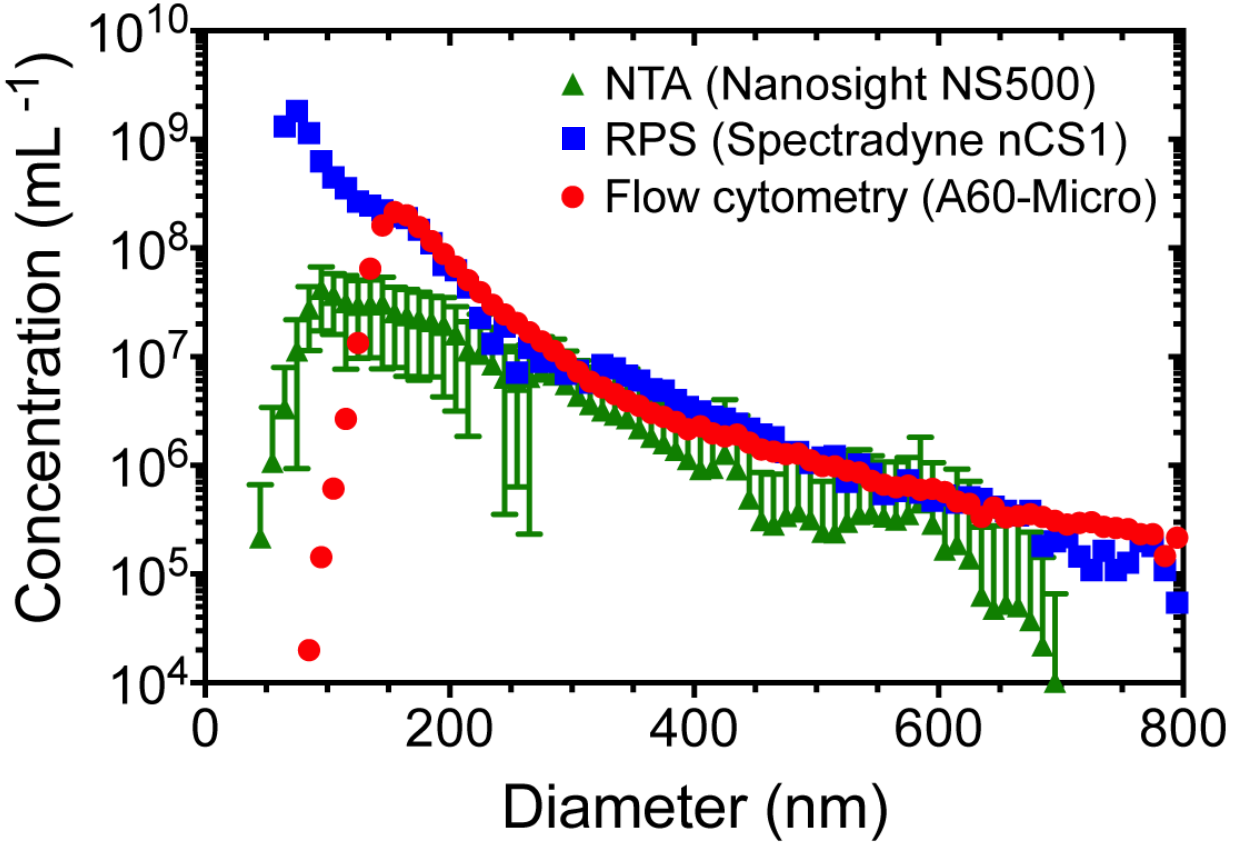
	Mean diameter (nm)	Concentration (mL^{-1})
NTA	169	$4.8 \cdot 10^{8}$
RPS	104	$7.5 \cdot 10^{9}$
Flow cytometry	191	$1.4 \cdot 10^{9}$

Data and figure by Michael Cimorelli, manuscript in prep.

Filipe et al. *Pharm Res* 2010 | Gross et al. *Eur J Pharm Biopharm* 2016

Analyzing size distributions and concentrations

Particles in human urine

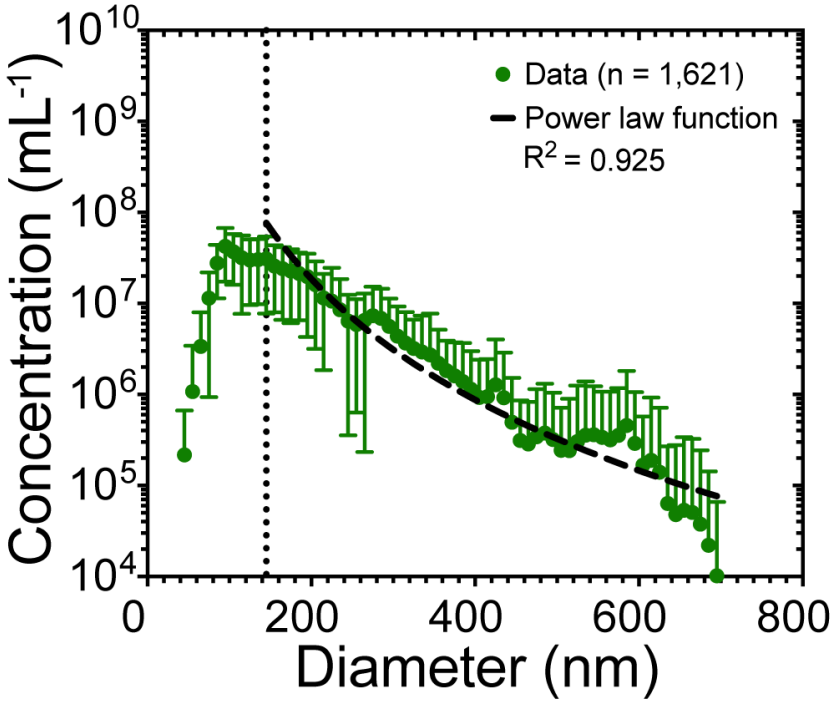


- Better practice:
  - describe size distribution with mathematical function
  - quantify detection limits
  - report concentration within size detection range

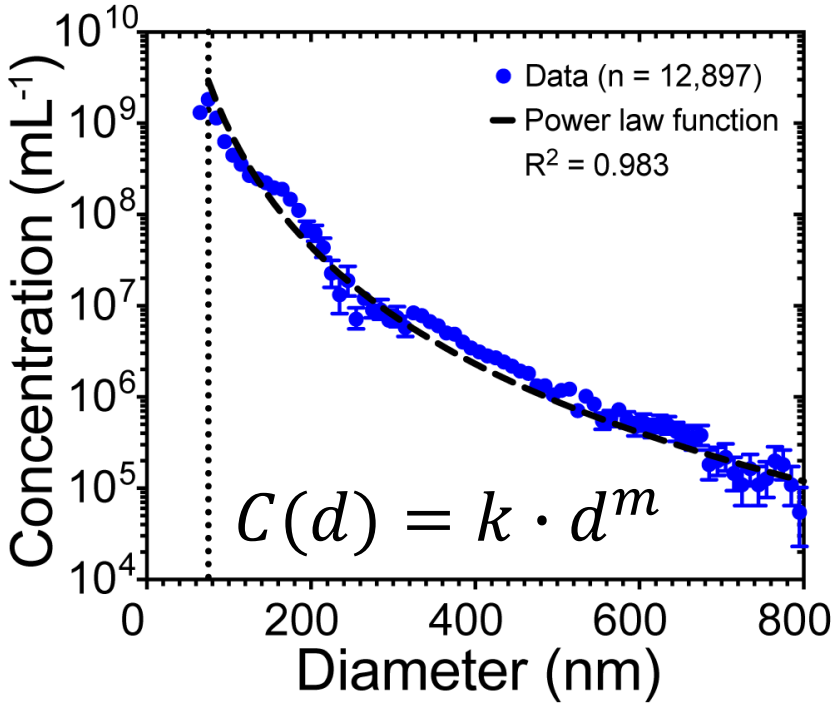
Data and figure by Michael Cimorelli, manuscript in prep.

Analyzing size distributions and concentrations

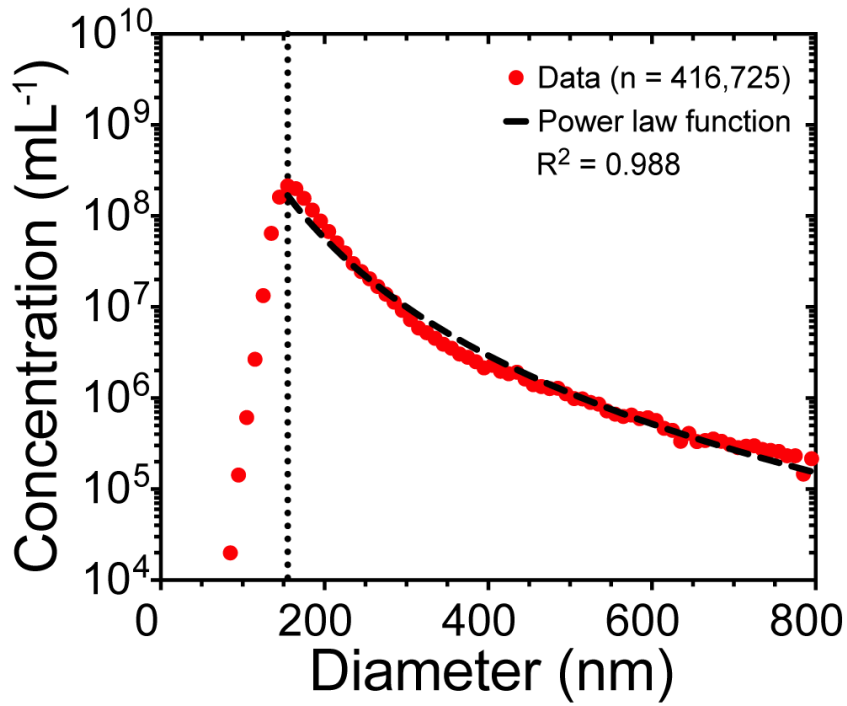
NTA
(Nanosight NS500)



RPS
(Spectradyne nCS1)



Flow cytometry
(Apogee A60-Micro)



Data and figure by Michael Cimorelli, manuscript in prep.

Analyzing size distributions and concentrations

$$C(d) = k \cdot d^m$$

Method	# particles measured	Power-law *m*	Power-law *k* (mL^{-1})	Fit quality R^2	Measured concentration (mL^{-1})	Size range (nm)
NTA	1,621	-4.41	$2.6 \cdot 10^{17}$	0.925	$2.7 \cdot 10^{8}$	140 - 1,000
RPS	12,897	-4.15	$2.1 \cdot 10^{17}$	0.983	$6.1 \cdot 10^{9}$	70 - 810
Flow cytometry	416,725	-4.27	$3.8 \cdot 10^{17}$	0.988	$1.1 \cdot 10^{9}$	150 - 810

More on reporting: MISEV by Théry et al. *JEV* 2018
MIFlowCyt-EV by Welsh et al. JEV 2020

Reporting size distributions and concentrations

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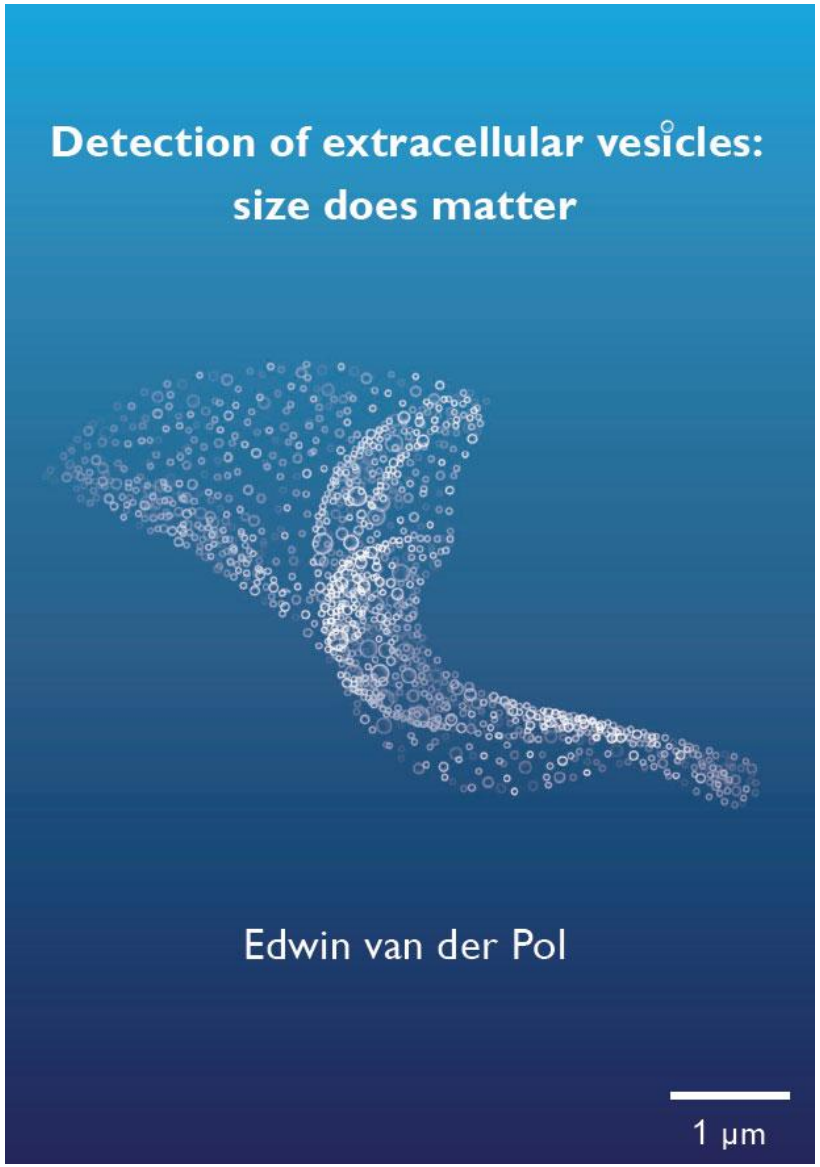
MIFlowCyt-EV by Welsh et al. JEV 2020

EV detection: size does matter

- Report EV concentration within size detection range



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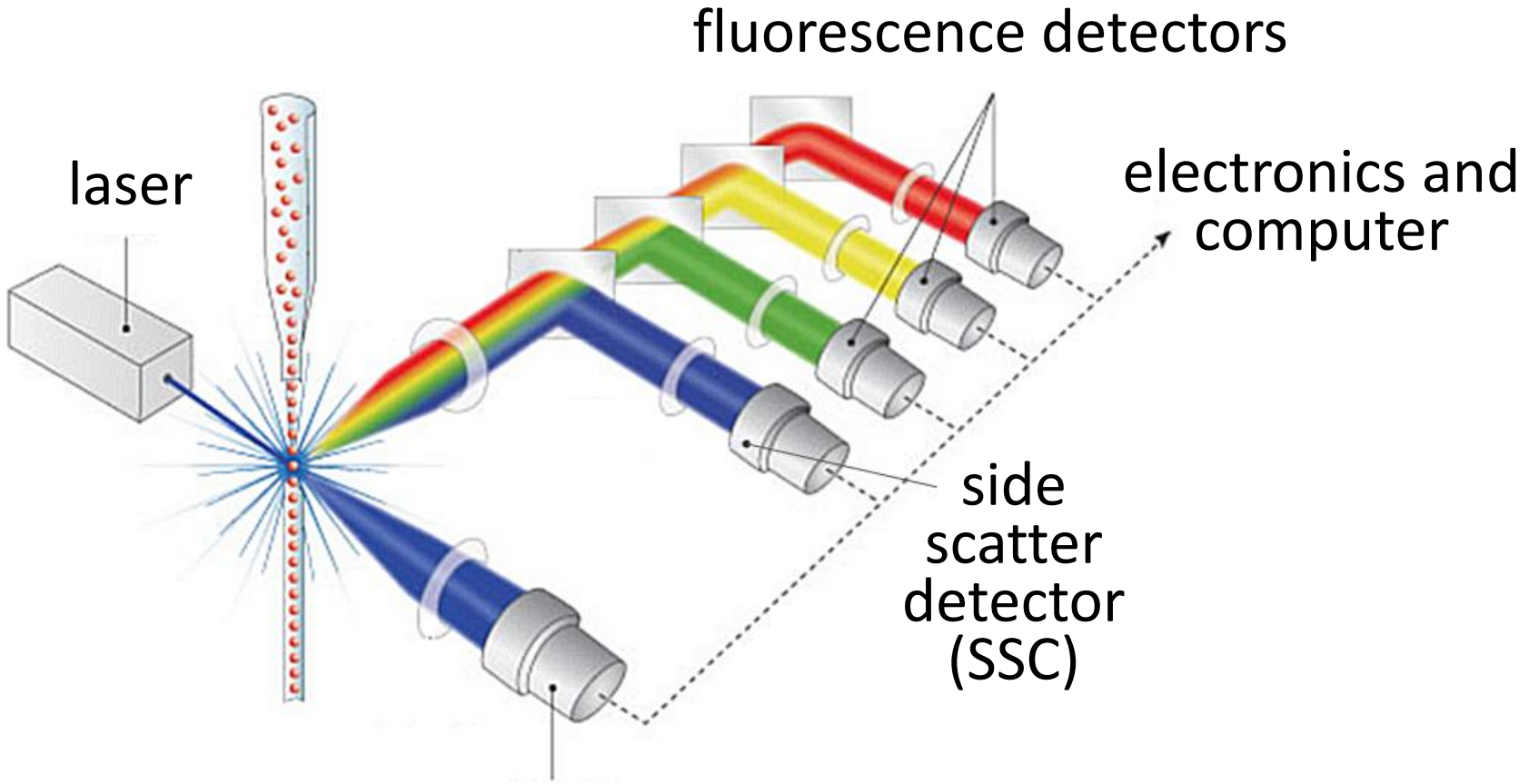
Flow cytometry does not measure size



>10,000 events/s

Image: semrock.com

Other techniques also do not measure “size”

- Nanoparticle tracking analysis
  - measures mean square displacement
- Resistive pulse sensing
  - measures electrical resistance
- Flow cytometry
  - measures light scattering…

EV size gate based on polystyrene beads

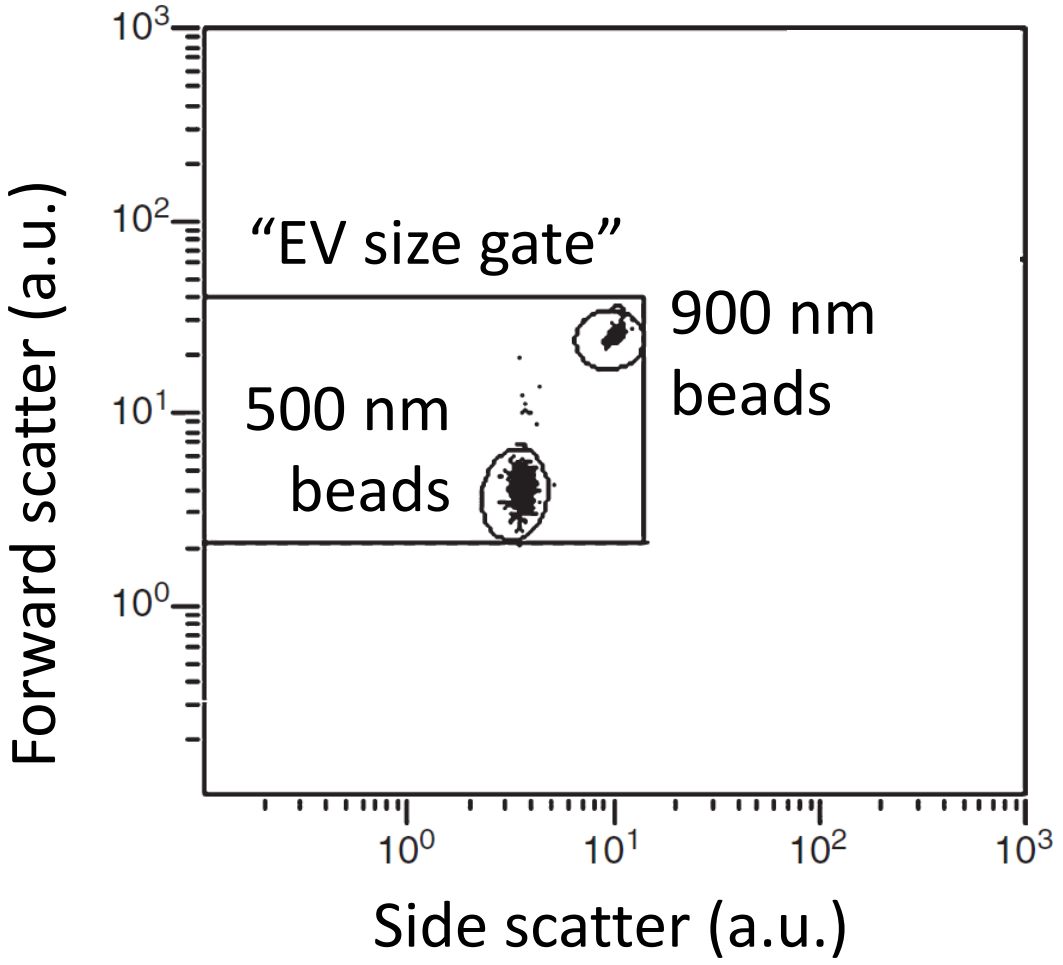
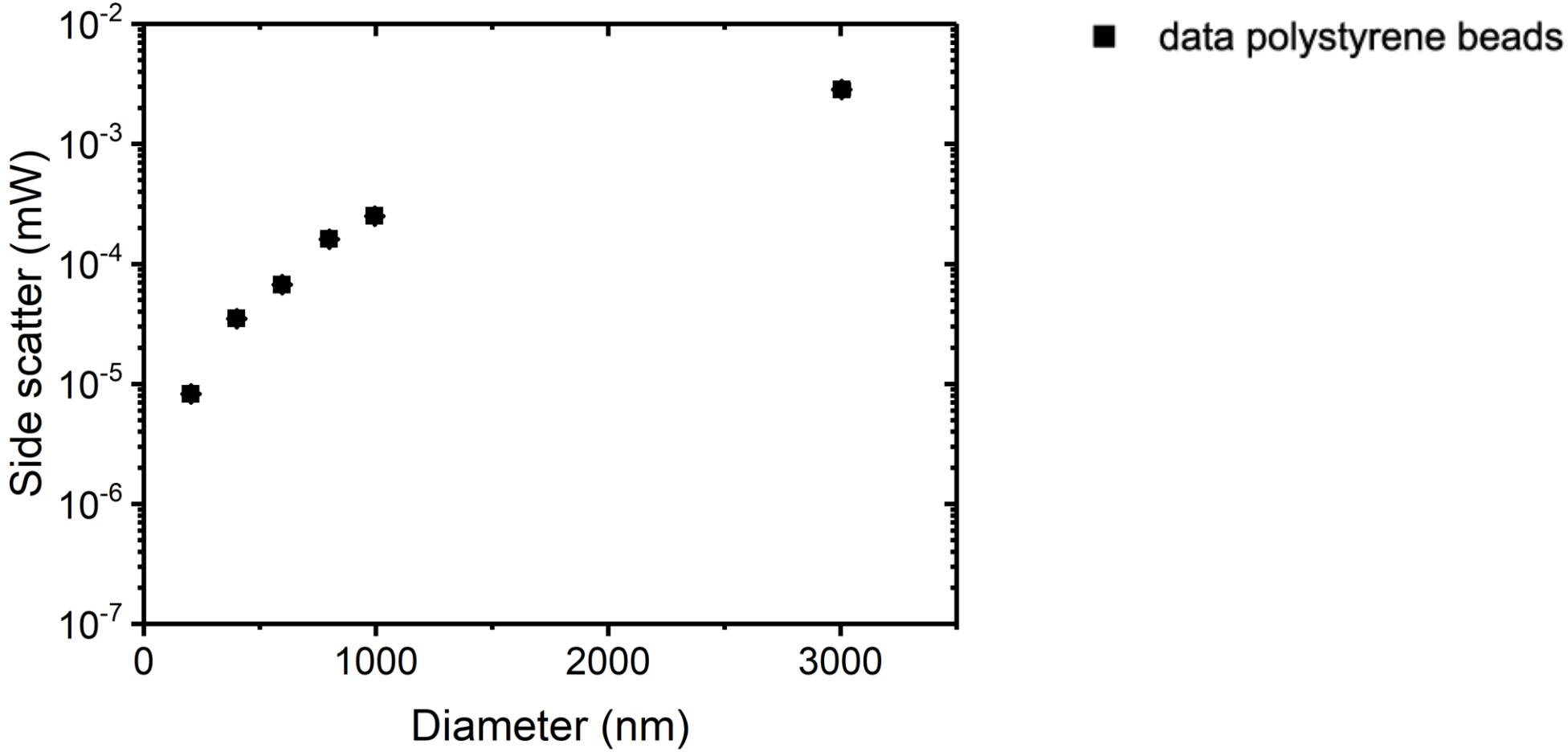
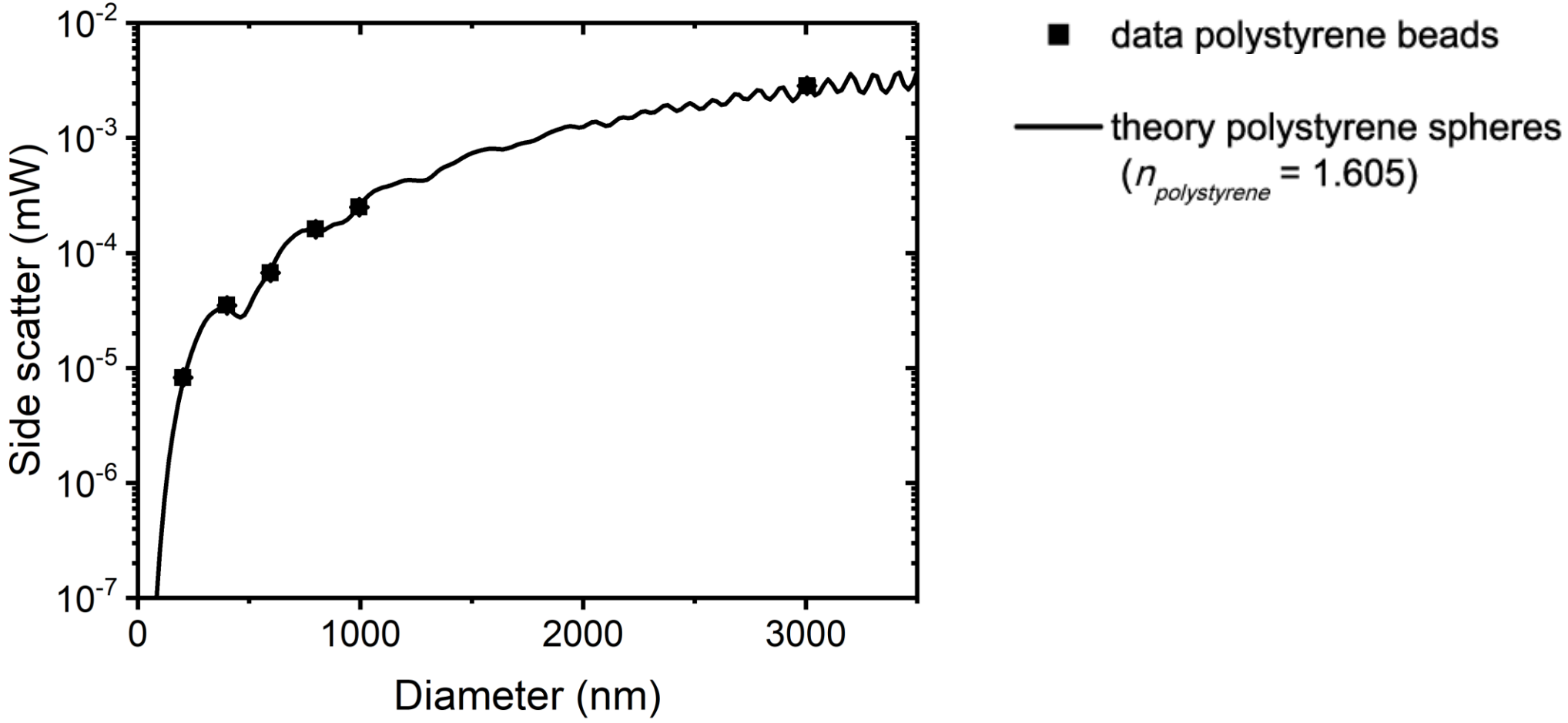


image adopted: Robert et al. *J Thromb Haemost* 2008

Relate scatter to diameter of beads

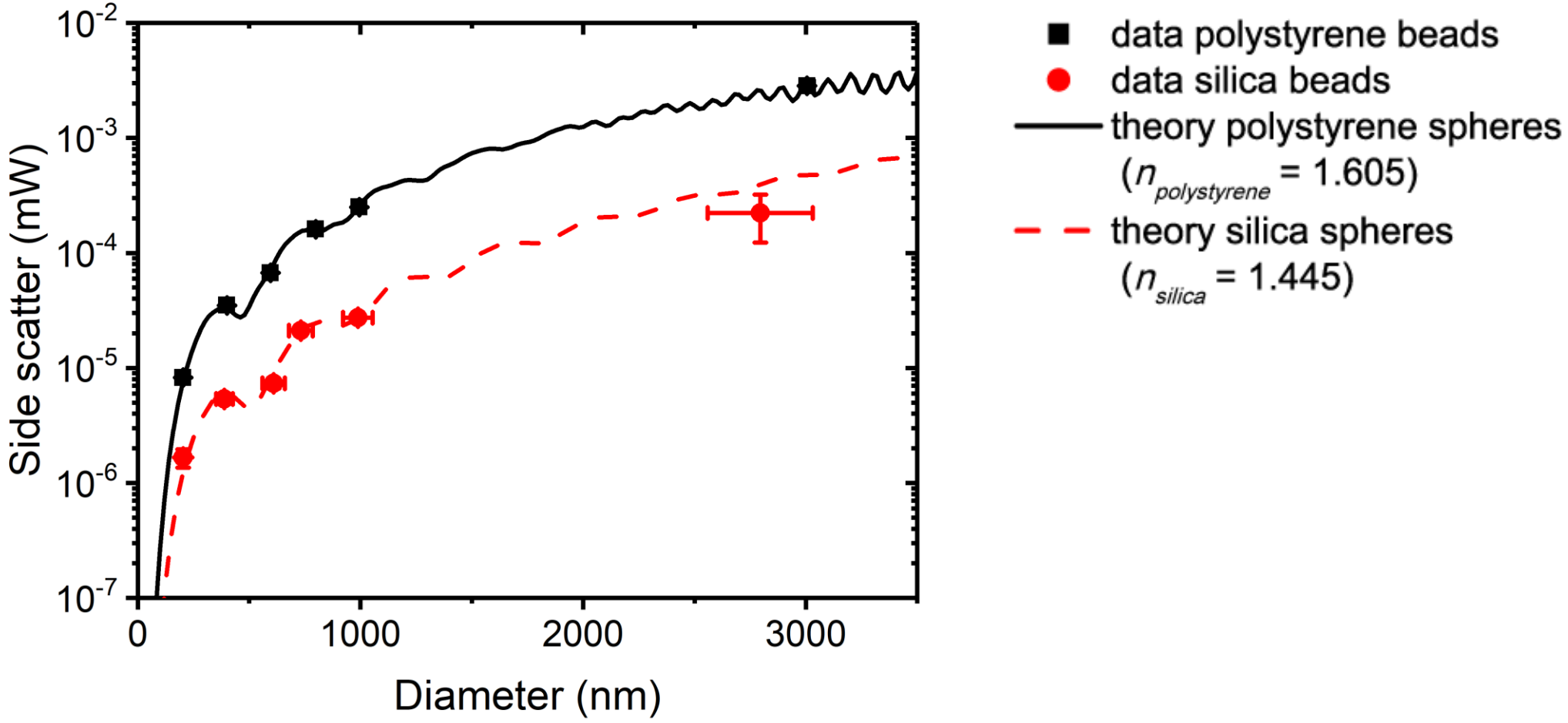


Relate scatter to diameter of beads



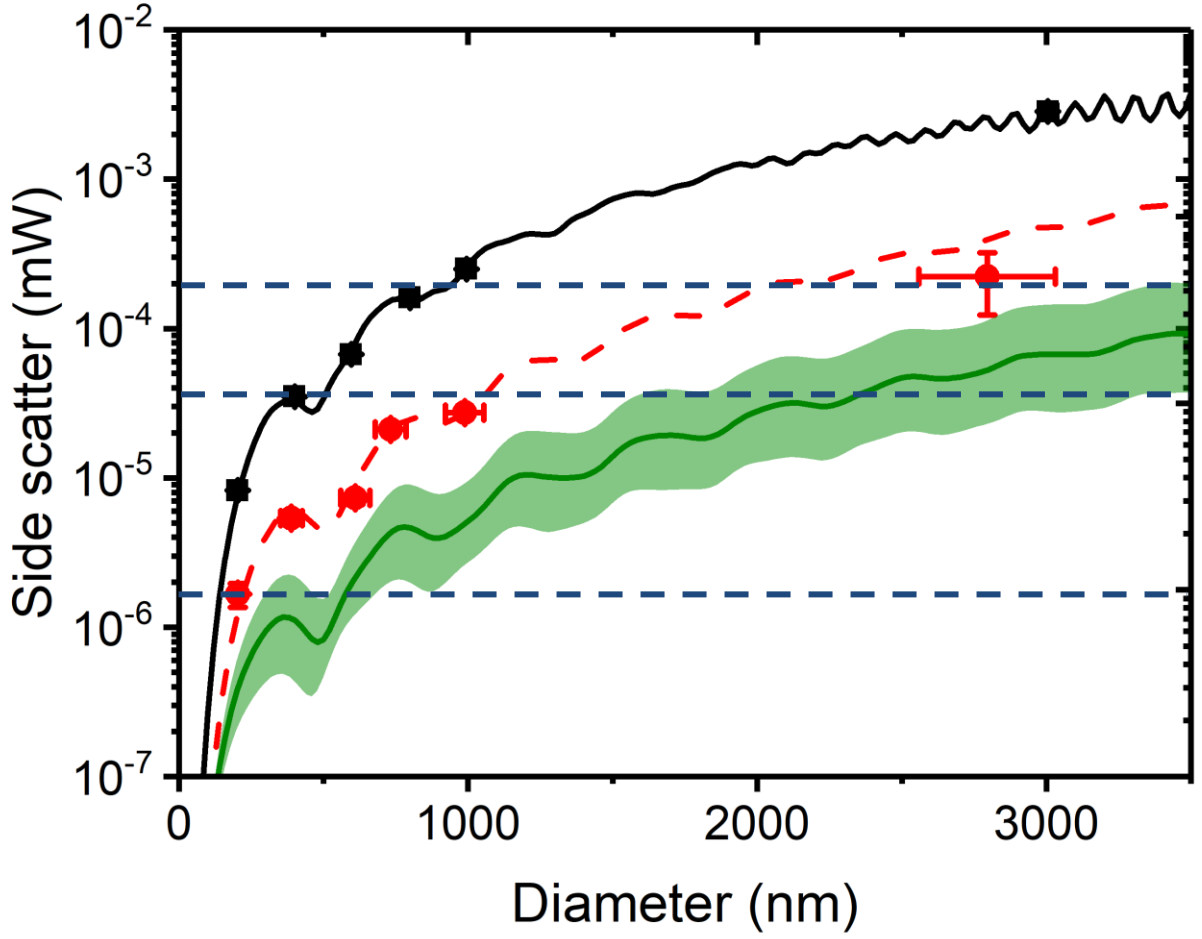
Model: de Rond et al. *Curr Protoc Cytom* 2018

Relate scatter to diameter of beads

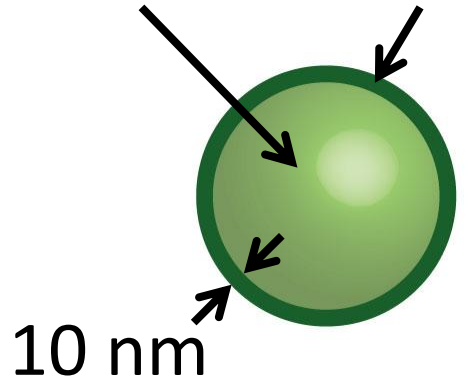


Model: de Rond et al. *Curr Protoc Cytom* 2018

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)



Calibrate light scattering of a flow cytometer

- Measuring your daily bead mix is not a calibration

Heterogeneity of the nucleic acid repertoire of plasma extracellular vesicles demonstrated using high-sensitivity fluorescence-activated sorting

K Kondratov, Y Nikitin, A Fedorov… - Journal of …, 2020 - Taylor & Francis

… Events corresponding to **calibration** beads are gated within the "**Megamix** FS Beads" region. Beads of specific diameter are highlighted in different colours … VioletSSC-A dot plot. Events corresponding to **calibration** beads are gated within the "**Megamix** FS Beads" region …

[HTML] Circulating endothelial cells and microparticles as prognostic markers in advanced non-small cell lung cancer

T Fleitas, V Martínez-Sales, V Vila, E Reganon… - PLoS One, 2012 - ncbi.nlm.nih.gov

… Standard fluorescent beads of different diameters were used for size **calibration** (0.5–3.0 microns, **Megamix**, BioCytex) and to set the gate of MP detection at a diameter from 0.5 to 1 μm, following a consensus guideline on MP measurement [29] …

ction and sorting of microbial cells and sub-micron particles
Biomedical - nanocellect.com
n size **calibration bead mix** from **Apogee** Flow Systems (CAT #1493) containing
tios of … to most currently available commercial flow cytometers, where the 0.5 µm
ds to … The 450 nm **beads** (“0.45” red gate) can be efficiently sorted from 880 nm …
endothelial cells
Sirois, C Bell, LA Hanafi, K Hamelin… - …, 2013 - Wiley Online Library
dary and primary necrotic cells revealed PI positivity. 2.6 Flow cytometry. All
eriments were performed on a Fortessa flow cytometer (BD Biosciences),
gamix calibration before every acquisition. Fluorescent …
microns. **Megamix**, BioCytex) and to set the gate of MP detection at a …
outine detection of extracellular vesicles in clinical samples
eel, M Krawczyk-Durka… - … journal of laboratory …, 2014 - Wiley Online Library
ple, the ISTH has recommended the use of 0.5-µm and 0.9-µm **Megamix**
beads (Biocytex, Marseille, France) for **calibration** of platelet-derived
ments with wide angle (1–19°) flow cytometers 27 …
manner in human endotoxemia
f a flow cytometric approach
012 - Wiley Online Library
R Bradford, EL Hobl… - … of Ton a fluorescence versus forward light-scatter dot plot to determine microparti
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·6 µm) and **Megamix**™ **calibration**
cles (MPs). Scatter plot (A) and histogram (B) of **Gigamix**® beads used for
g. The MP gate was set between 0.2 and 1 µm, as shown. With new … with
ted automated thrombogram (CAT). The contribution
salem treated platelets over 7 days of storage with an
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automated cytometry assay pa
High correlation between 2 flow cytometry platforms in the microparticles
andinieres
M Diquattro, G De Francisci… - … journ
analysis using a new calibrated beads strategy
ere selecte
… respectively). To standardize the PMI
V Sánchez-López, V Vila-Liante, E Arellano-Orden… - Translational …, 2015 - Elsevier
calibration beads (**Megamix**; Biocytex,
… (A–E) Beckman Coulter Navios **calibration** using **Megamix**-Plus FSC beads, (A) density plot of
ment of platelet microparticles
The good correlations between the 2 cytometers observed in our study are in agreement with
Lacroix, F Dignat-George… - Platelets and …, 201
a recent research comparing PMPs numbers using the **Megamix**-Plus **calibration** beads …
n beads are utilized to validate microparticle measurements … 3.3.1 Standardization
ytometry Part …, 2016 - Wiley Online Library
rescent polystyrene beads (Megamix FSC & SSC
es … To demonstrate that the cytometer is clean
uring the **Gigamix** solution, beads were …
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ds are gated within the “**Megamix** FS Beads” region.
markers in
ours … VioletSSC-A dot plot. Events
Red cell microparticle enumeration: validation of a flow cyt
Z Xiong, TB Oriss, JP Cavaretta, MR Rosengart… - Vox …, 2012 - Wiley On
… Defining the parameters using a mixture of **Megamix** and absolute count
beads. A mixture of Absolute Counting Standard microbead (7·6 µm) and **Me**
·9 and 3 µm) of differing ratios was generated …
cell–derived microparticles loaded with iron oxid
MR imaging monitoring in mice
zeau, C Wilhelm, C Devue, CL Guérin… - Radiology, 2012 - p
gate was defined, by using **calibration** beads (**Megamix**, Bioc
0.1-µm beads (Invitrogen, Eugene, Ore), as events with a 0.1- to 1-µm diamet
M Levy, CL Guerin… - Pe
, 15 First, MVs were sele
calibration beads (**Meg**
recommendations for the cytometer s

Turn your flow cytometer into a particle sizer!

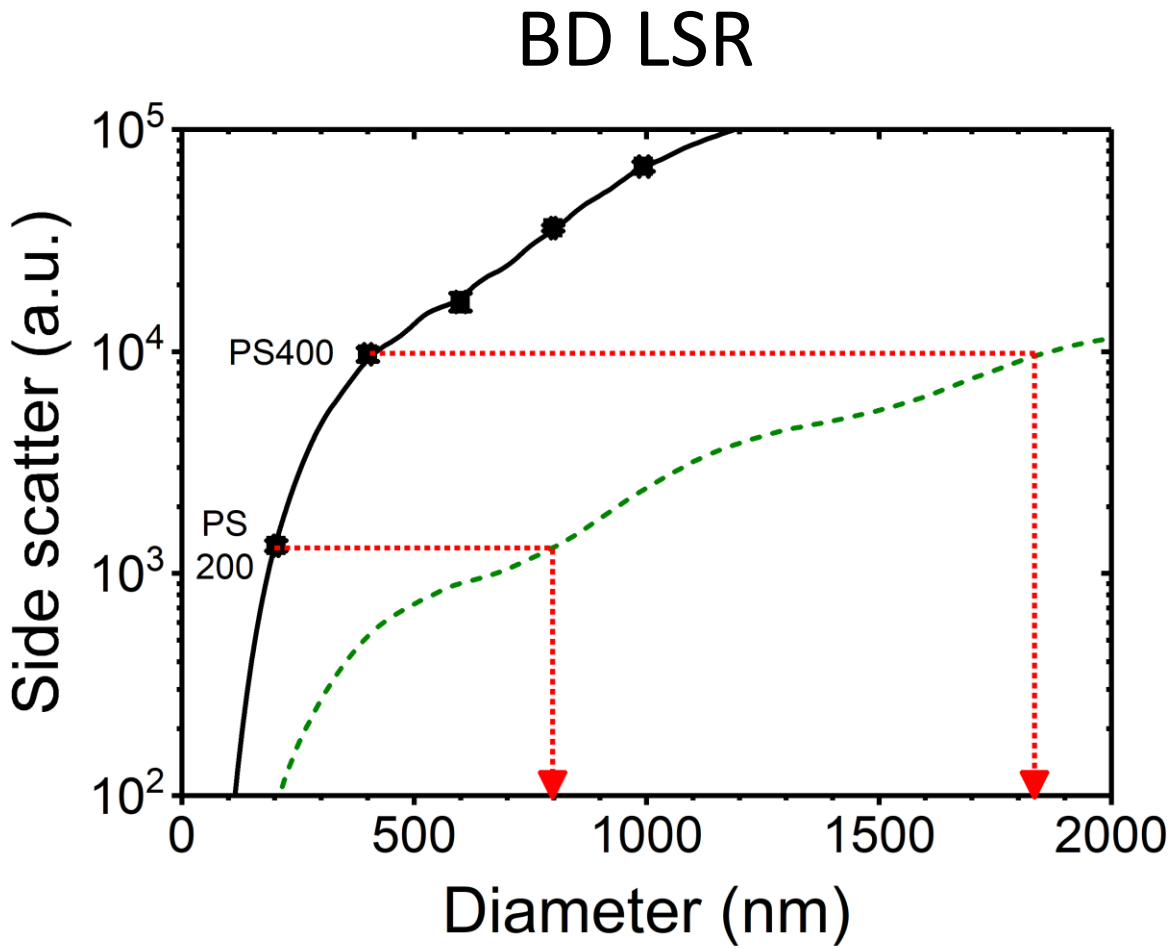
Deriving Extracellular Vesicle Size From Scatter Intensities Measured by Flow Cytometry

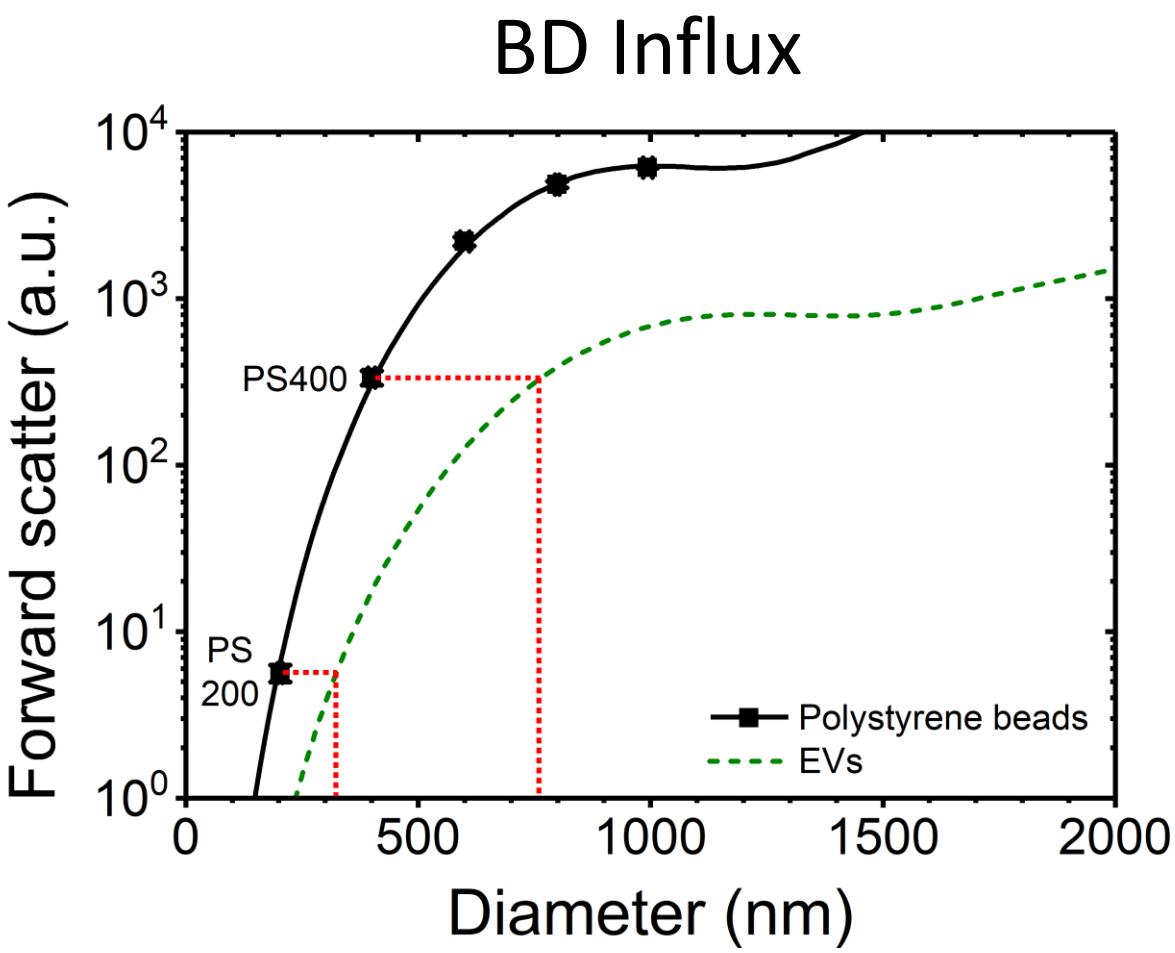
Leonie de Rond,[1,2,3] Frank A. W. Coumans,[1,2,3] Rienk Nieuwland,[2,3] Ton G. van Leeuwen,[1,3] and Edwin van der Pol[1,2,3,4]

Current Protocols in Cytometry, e43 (2018)

- Easy
- Cheaper than daily quality control with polystyrene beads
- More accurate than NTA (flow cytometer dependent)

Scatter to diameter relationship differs between instruments





Scatter calibration: de Rond et al. *Curr Protoc Cytom* 2018

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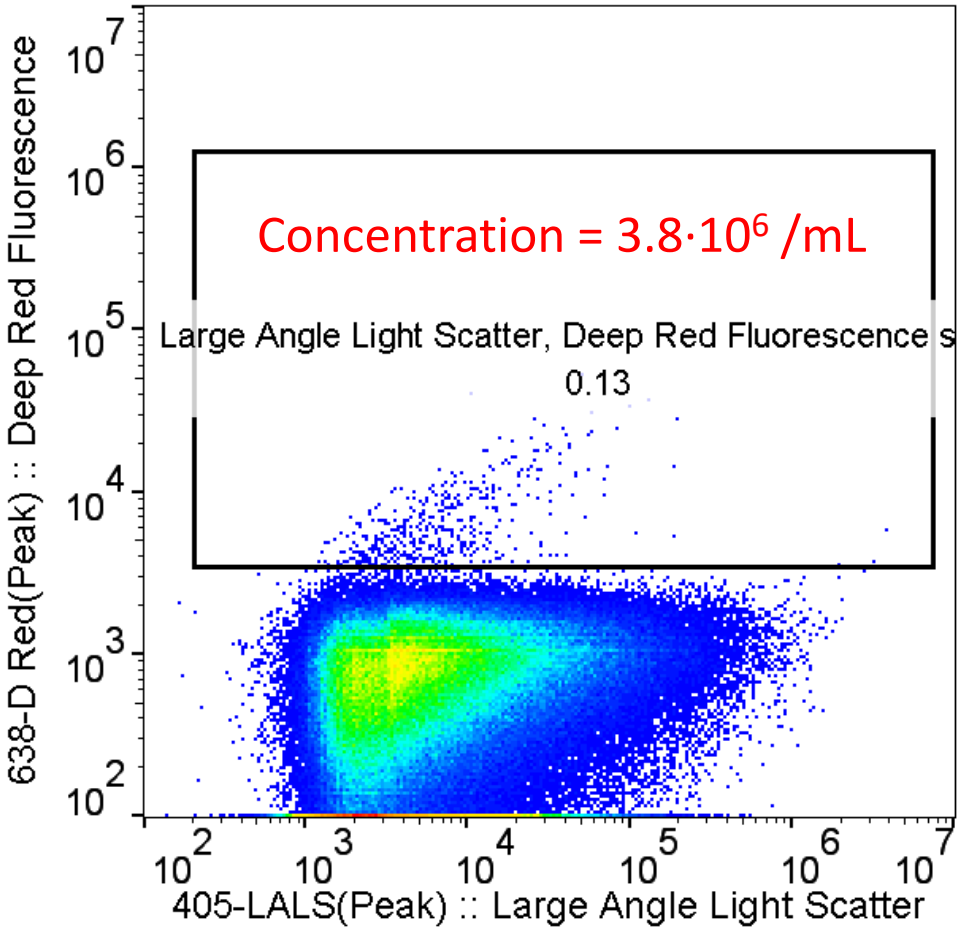
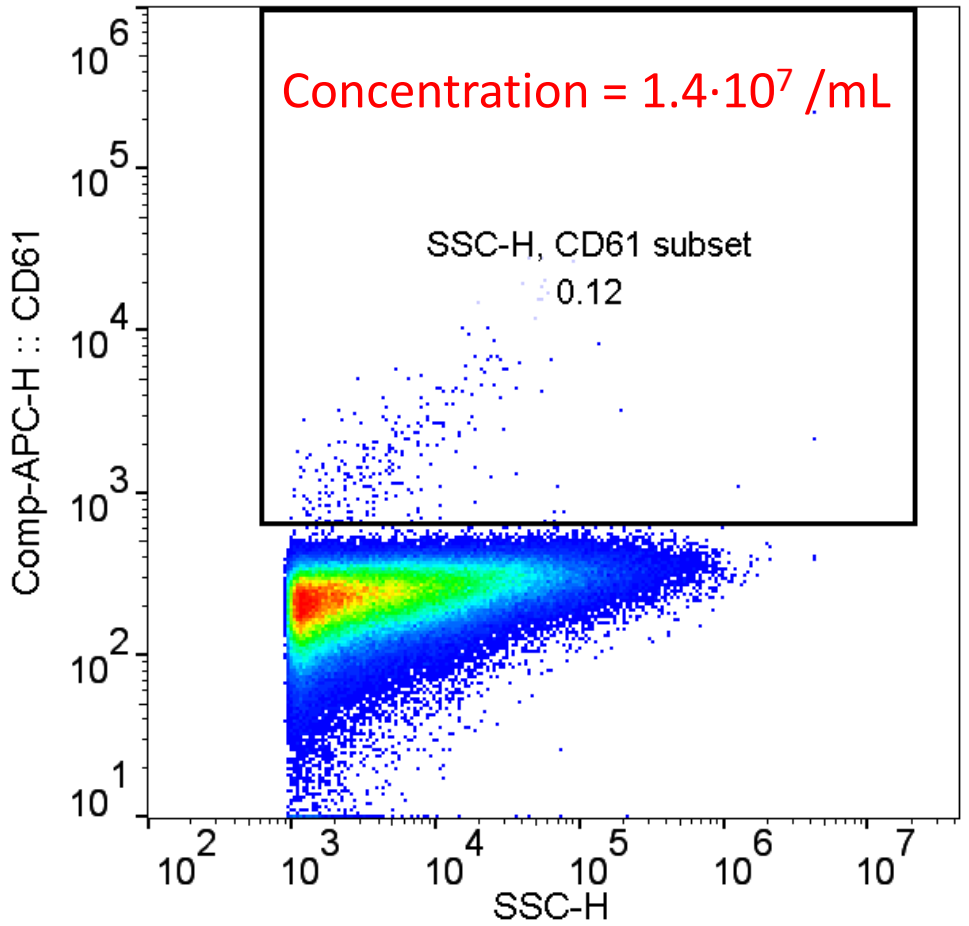
Usefulness of size-calibration to data comparison

Apogee A60-Micro



Cytek Aurora

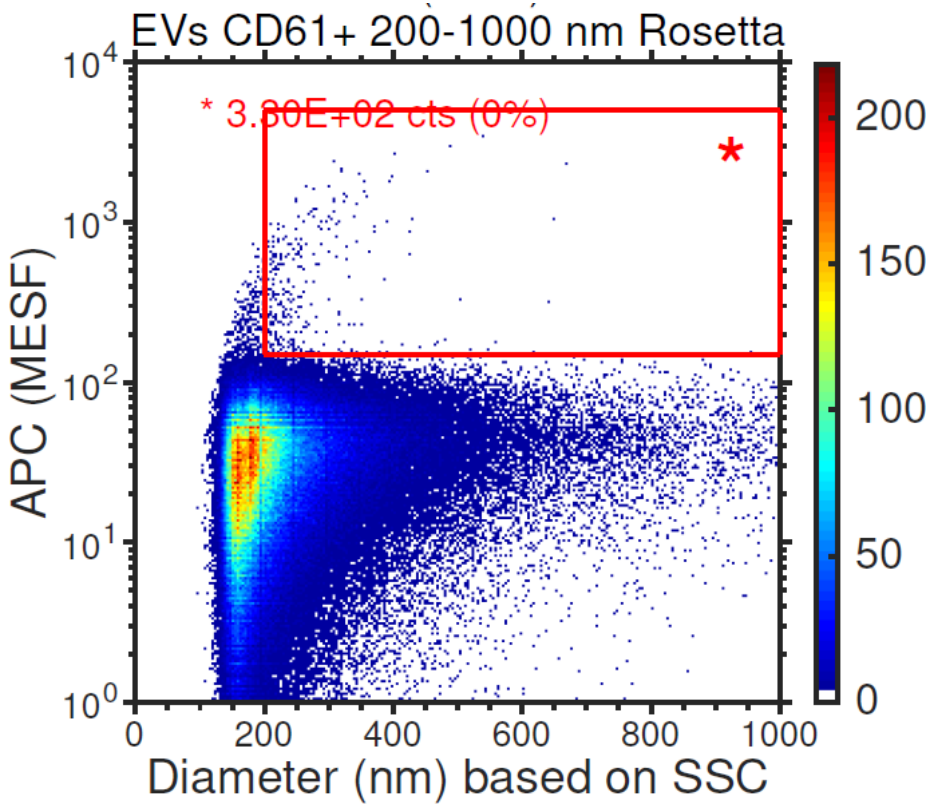


EV sample prepared by Britta Bettin, AMC

Usefulness of size-calibration to data comparison

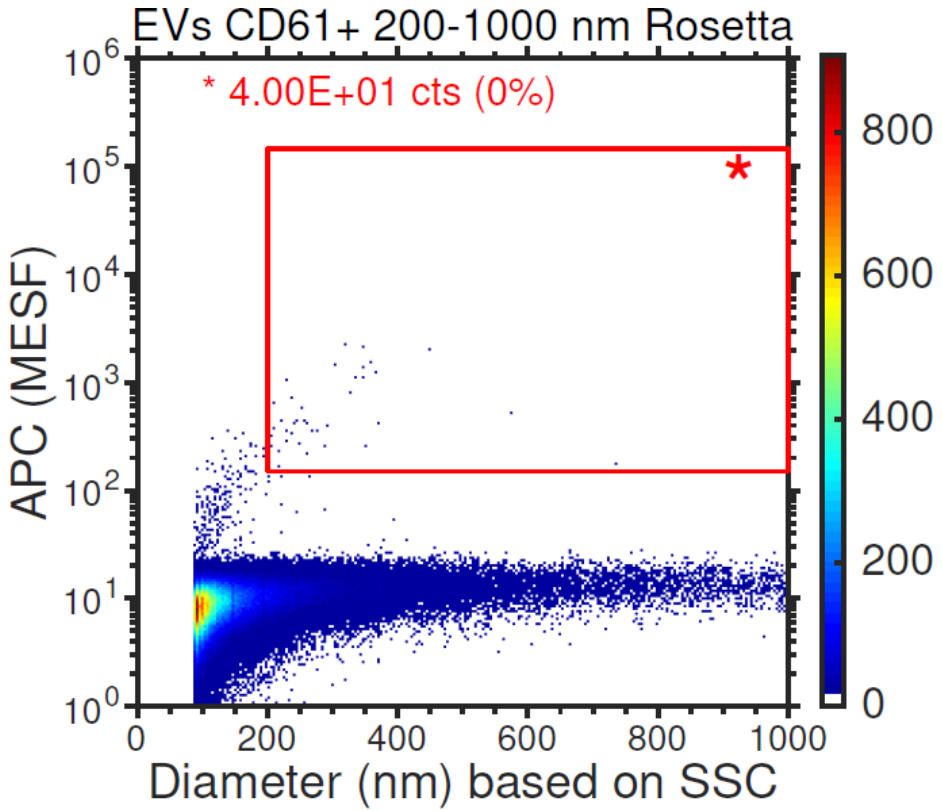
Apogee A60-Micro

Concentration = $2.7 \cdot 10^6$ /mL



Cytek Aurora

Concentration = $2.2 \cdot 10^6$ /mL



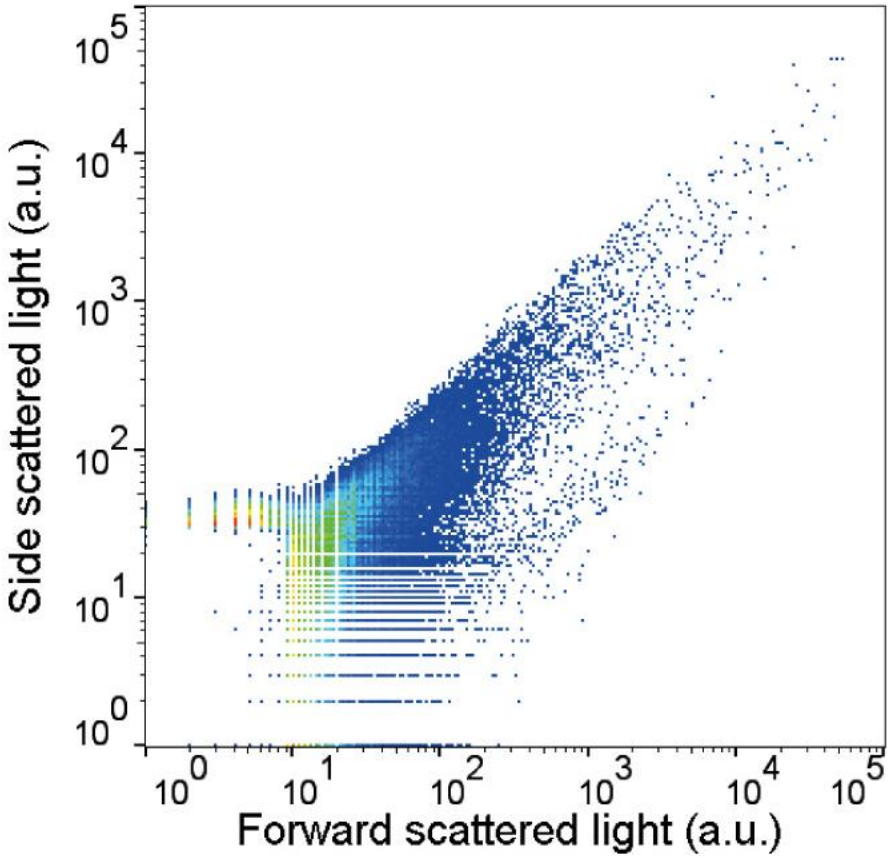
EV sample prepared by Britta Bettin, AMC

MISEV 2018

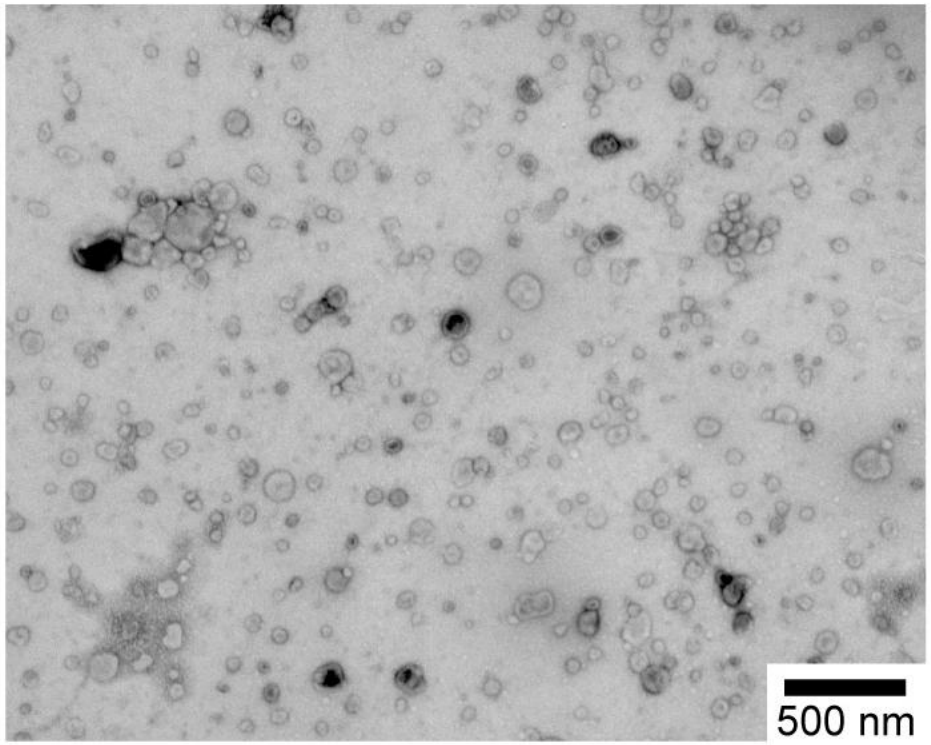
"**EV characterization by multiple, complementary techniques is important** to assess the results of separation methods and to establish the likelihood that biomarkers or functions are associated with EVs"

EVs measured by FCM and TEM

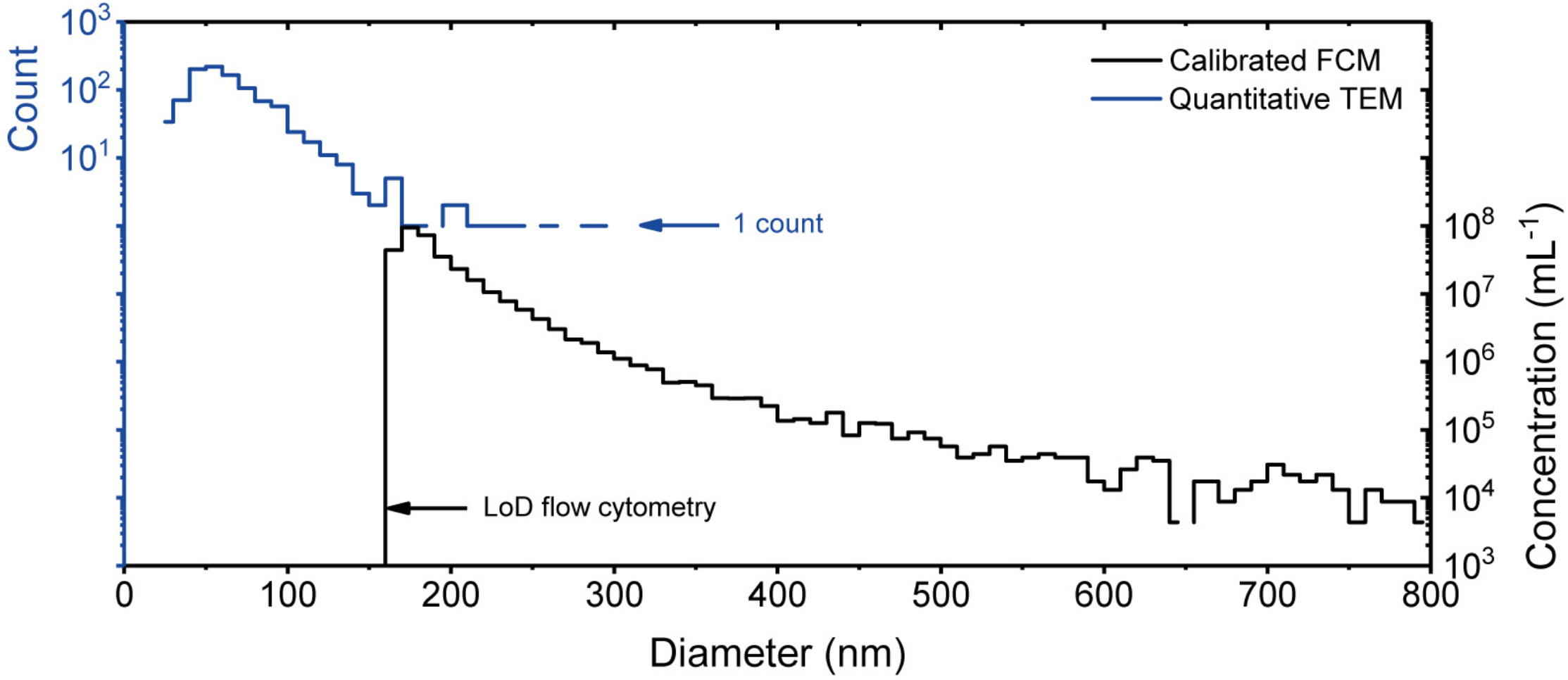
Flow cytometry (FCM)



Transmission electron microscopy (TEM)



Usefulness of calibration to data interpretation



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Reporting

- Calibrate measured signals[1]
  - Fluorescence to MESF
  - Scatter to diameter (nm)
- Report EV concentration within calibrated fluorescence and size ranges[1]
- Use assay controls to verify that events are EVs[1]

[1]Welsh et al. *JEV* 2020

MESF: molecules of equivalent soluble fluorophores

Reporting

JOURNAL OF EXTRACELLULAR VESICLES
2020, VOL. 9, 1713526
https://doi.org/10.1080/20013078.2020.1713526

Taylor & Francis
Taylor & Francis Group

OPEN ACCESS

MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments

Joshua A. Welsh [a], Edwin Van Der Pol [b,c,d], Ger J.A. Arkesteijn [e], Michel Bremer [f], Alain Brisson [g], Frank Coumans [b,c,d], Françoise Dignat-George [h,i], Erika Duggan [j], Ionita Ghiran [k], Bernd Giebel [f], André Görgens [f,l,m], An Hendrix [n], Romaric Lacroix [h,i], Joanne Lannigan [o], Sten F.W.M. Libregts [e,p], Estefanía Lozano-Andrés [e], Aizea Morales-Kastresana [a], Stephane Robert [m], Leonie De Rond [b,c,d], Tobias Tertel [f], John Tigges [k,q], Olivier De Wever [n], Xiaomei Yan [r], Rienk Nieuwland [c,d], Marca H.M. Wauben [e], John P. Nolan [j] and Jennifer C. Jones [a]

Minimum information about a flow cytometry experiment on extracellular vesicles (MIFlowCyt-EV)

Outline

- EV detection: size does matter
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Outlook

- Improving
  - Knowledge (evflowcytometry.org)
  - Procedures (Current Protocols in Cytometry)
  - Reference materials (metves.eu)
  - Reporting (MIFlowCyt-EV)
- Comparable data on concentrations of EVs with specific surface markers: within known fluorescence intensity and size ranges
- Reference value study (224 healthy volunteers)

Don’ts of single EV detection

- Do not make size distributions based on “insufficient” numbers of EVs
- Be careful describing size distributions of EVs with statistical parameters *mean*, *median*, *mode*
- Do not standardize flow cytometry experiments using bead gates

Dos of single EV detection

- Describe size distributions of EVs with a mathematical function
- Calibrate: relate arbitrary units to standard units
- Report EV concentration within size detection range
- Report all experimental details (MIFlowCyt-EV)

Acknowledgements

- Amsterdam University Medical Centers
  - Vesicle Observation Center
- EV flow cytometry working group

- More information
  - edwinvanderpol.com
  - evflowcytometry.org
  - metves.eu

- Funding
  - EURAMET project 18HLT01 (METVES II)