Approaching clinical feasibility with extracellular vesicle flow cytometry

Edwin van der Pol

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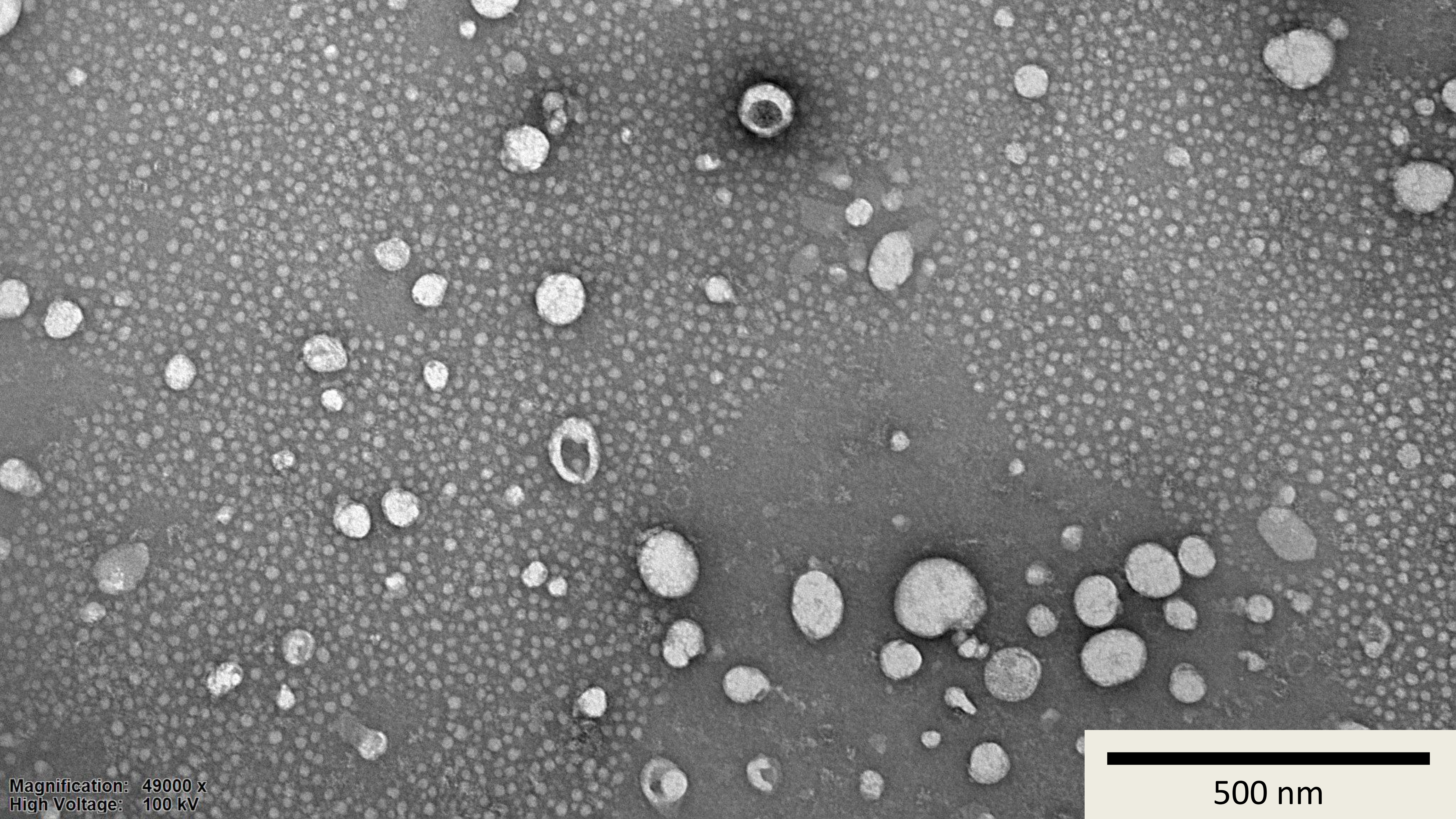
Amsterdam UMC
University Medical Centers

Vesicle
Observation Center

Outline

- Extracellular vesicles (EVs)
- Blood
- Flow cytometry characterization
  - EVs
  - Other particles
- Reproducibility
- Reference ranges

- Calibration
- Assay controls


Magnification: 49000 x
High Voltage: 100 kV
500 nm

Motive: EV-based biomarker

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 *University of Amsterdam* 2015

What are extracellular vesicles?

"Particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus."

ISEV

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Particles >30 nm in human blood

- EVs
- Other particles
  - Cells
  - Exomers
  - High molecular mass proteins (>100 kDa)
  - Lipoproteins
  - Plastics*

*Leslie et al. *Environ Int* 2022

Particles >30 nm in human blood plasma

- EVs
- Other particles
  - ~~Cells~~ Erythrocyte ghosts[1] & platelets[2]
  - Exomers
  - High molecular mass proteins (>100 kDa)
  - Lipoproteins
  - Plastics

[1]Arraud et al. *J Thromb Haemost* 2014
[2]Bettin et al. *J Thromb Haemost* 2022

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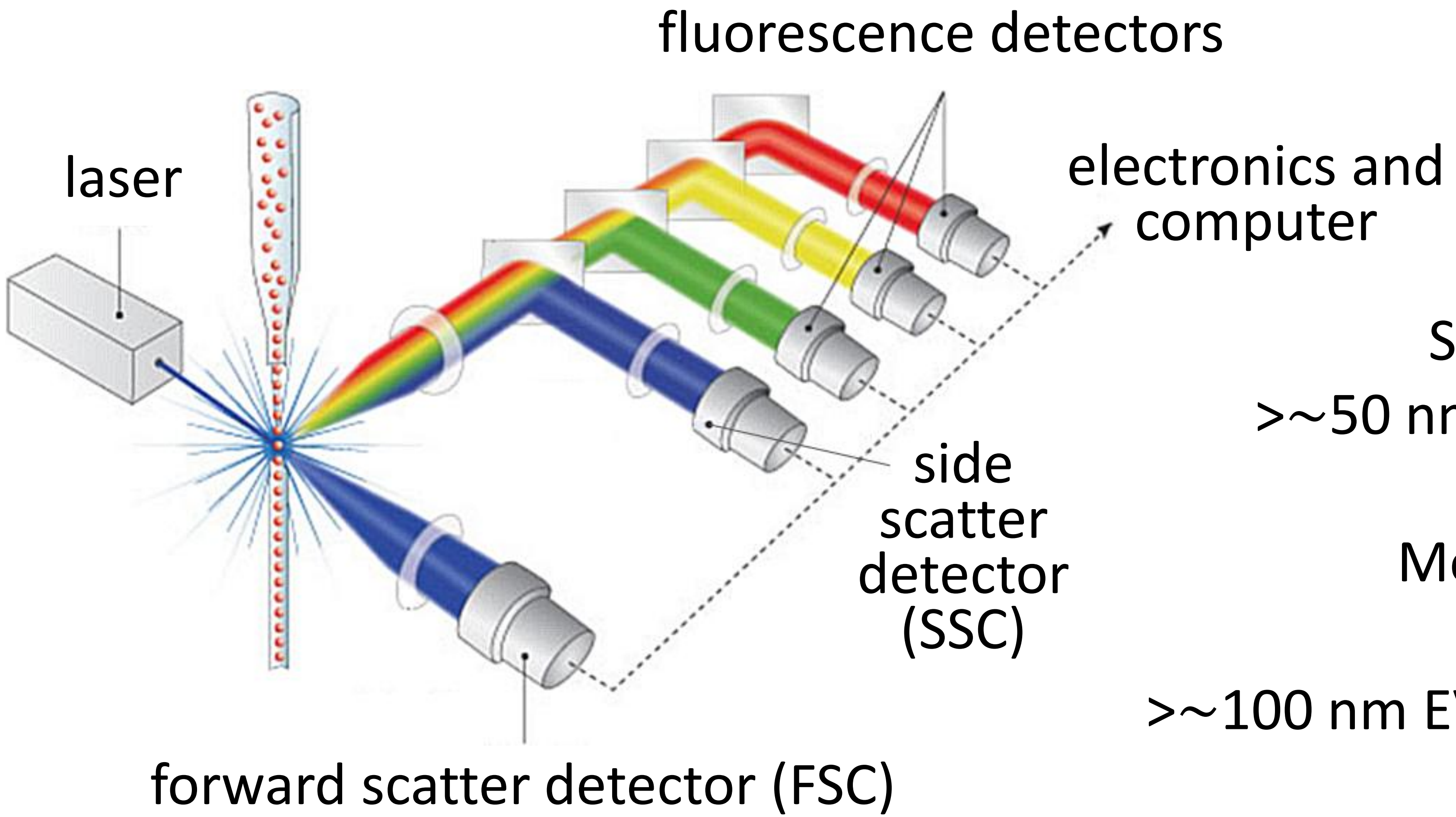
Flow cytometry

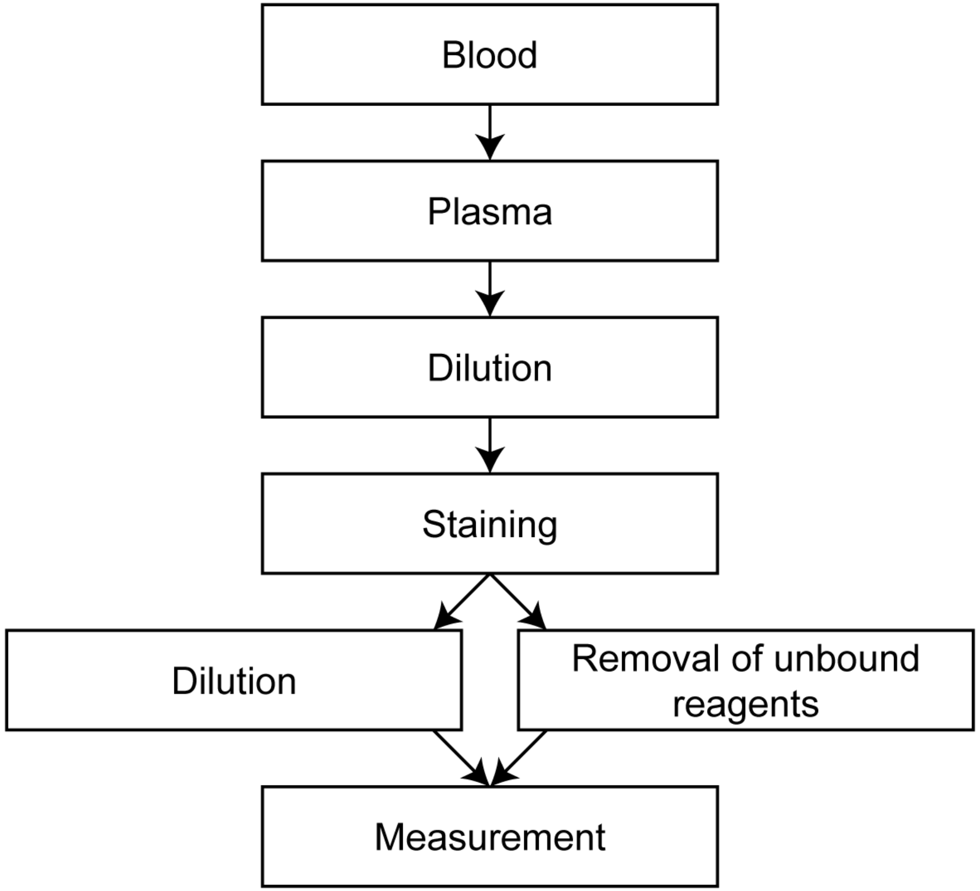


Specialized instrument:
>~50 nm EVs | 200 particles/s

Modern flow cytometer:
(also suitable for cells)
>~100 nm EVs | 10.000 particles/s
~100 samples per day

Image: semrock.com

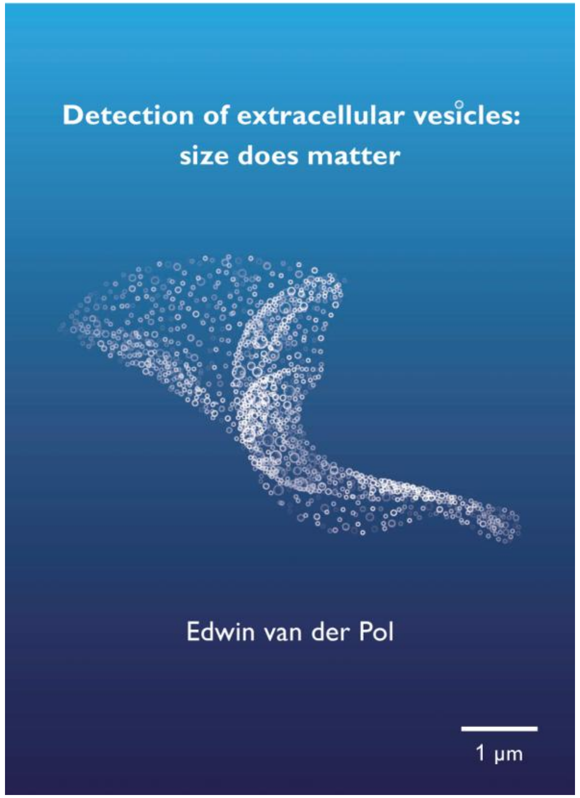
Flow cytometry sample work flow



Report number of EVs
per mL of plasma
(within detection ranges)

Flow cytometry characterization of EVs

- EV definitions:
  - "naturally released from the cell"
    - smaller than cells: sizing
  - "delimited by a lipid bilayer"
    1. transmembrane proteins: immunostaining
    2. lyse membrane: detergent
    3. membrane staining (difficult)
    4. low refractive index (specialistic)
  - "no functional nucleus"
    - smaller than nucleus: sizing



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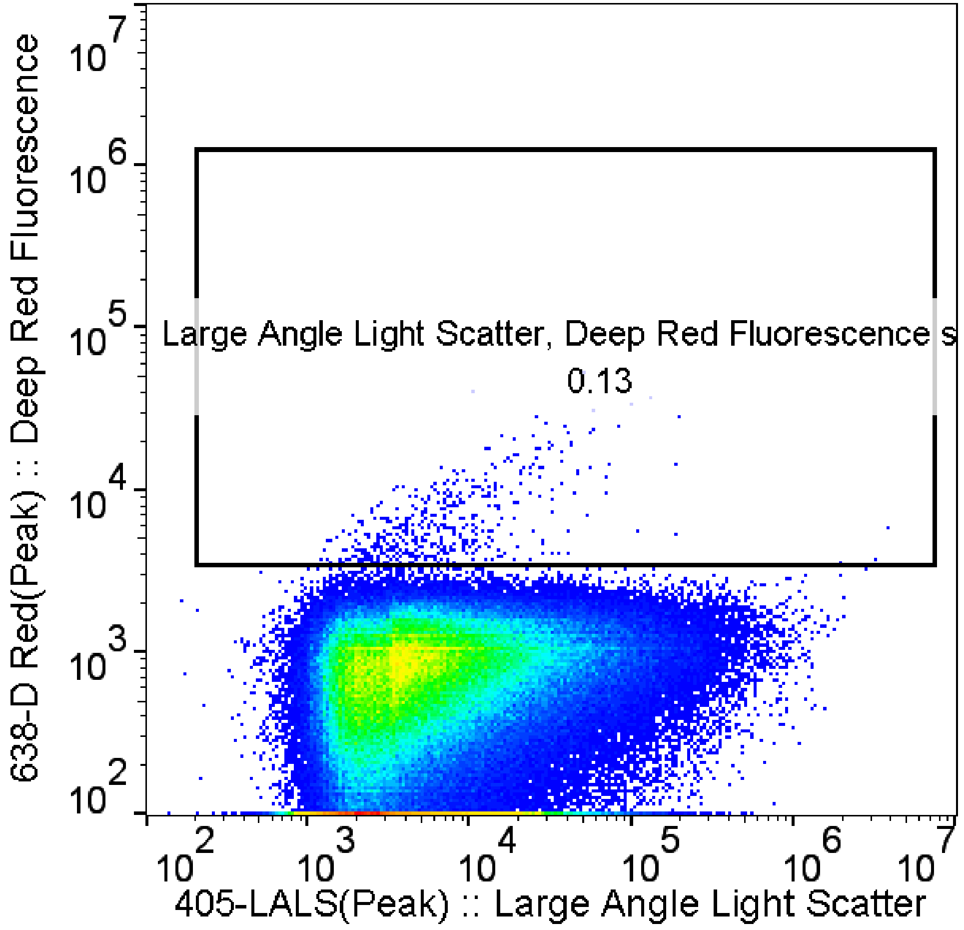
Need for calibration

- Flow cytometry data have arbitrary units
- It remains unclear whether EVs were measured



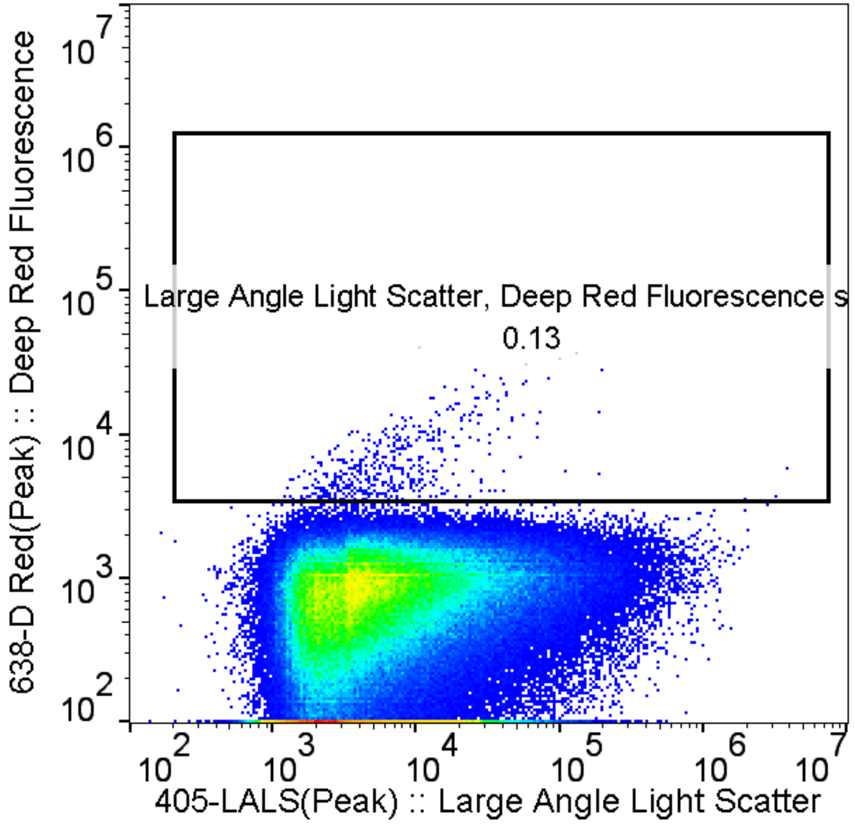
Calibration

- Relate arbitrary units to standard units*
  - Light scattering intensity (a.u.) → Diameter (nm)
  - Fluorescence intensity (a.u. → MESF)
  - Sample volume (unknown → µL)

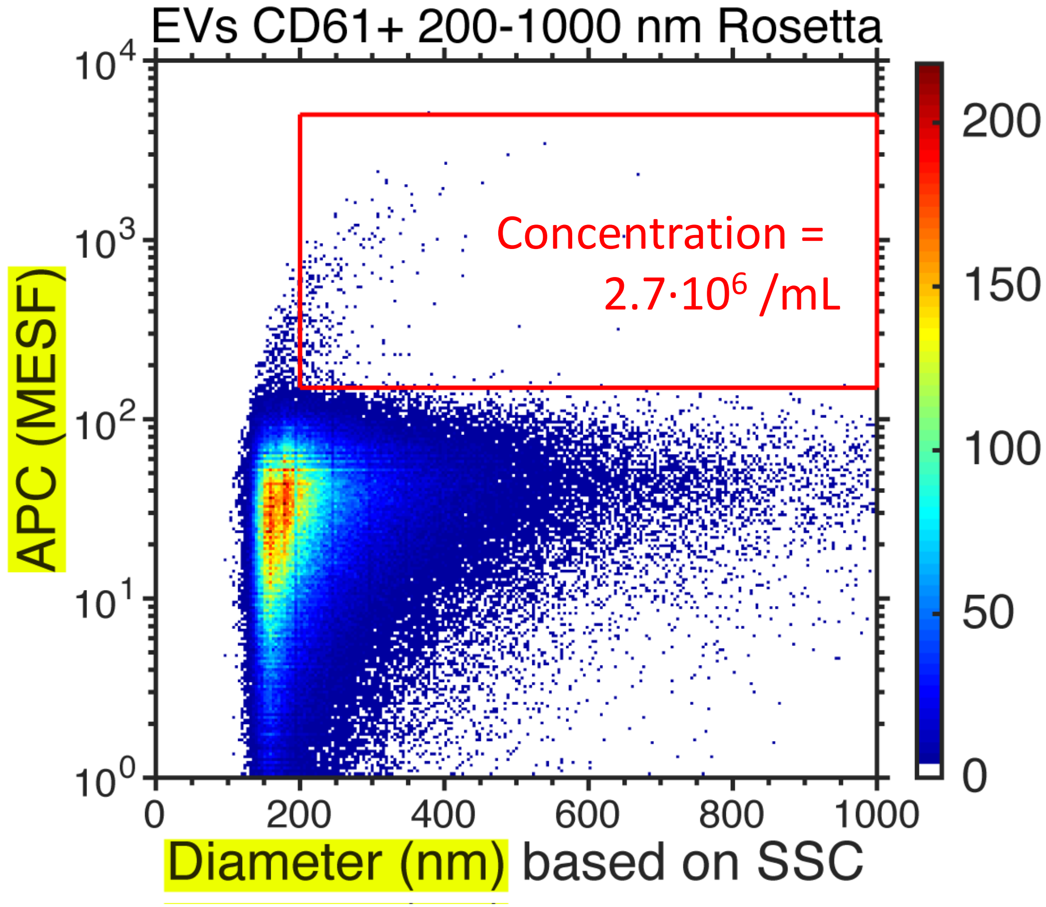
*EV flow cytometry compendium. Submitted to *J Extracell Vesicles*
MESF: molecules of equivalent soluble fluorophore

Example of calibration

Uncalibrated



Calibrated



Assay controls

- Confirm that signals originate from EVs*
- Examples
  - Buffer only controls (clean buffer and flow cytometer?)
  - Buffer with reagents controls (antibody aggregates?)
  - Isotype controls (Fc receptor binding?)
  - Detergent treatment controls (EVs?)
  - Serial dilutions (swarm detection?)

*MIFlowCyt-EV. Welsh et al. *J Extracell Vesicles* 2020

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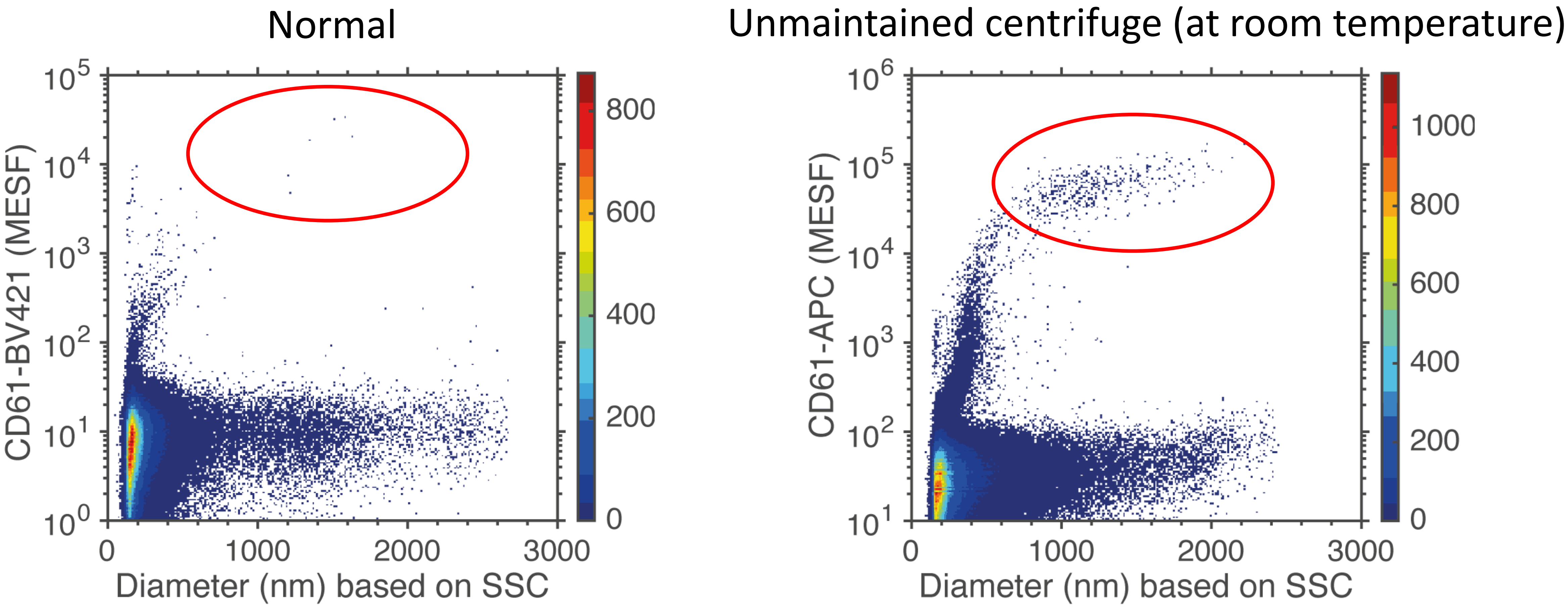
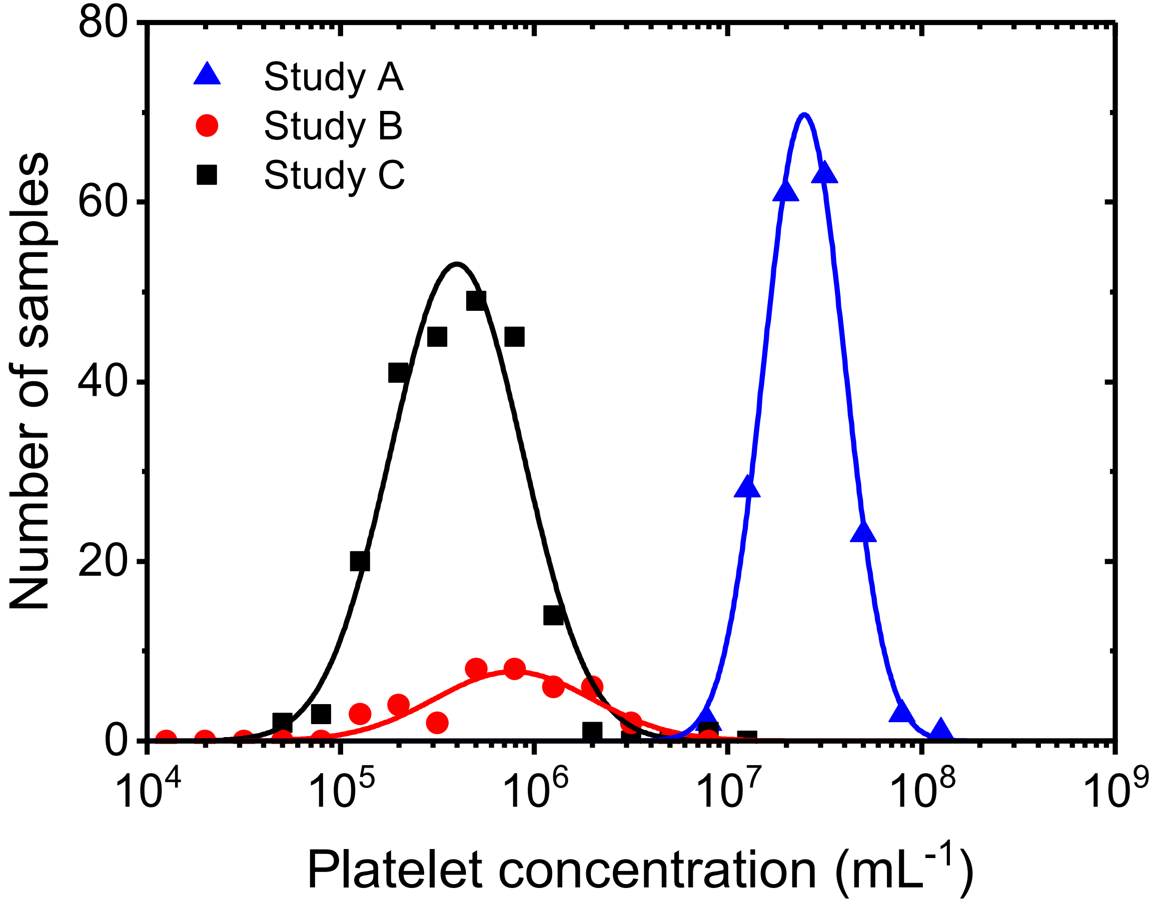
“Platelet free plasma” contains platelets



Right data: Gasecka et al. *J Thromb Haemost* 2020

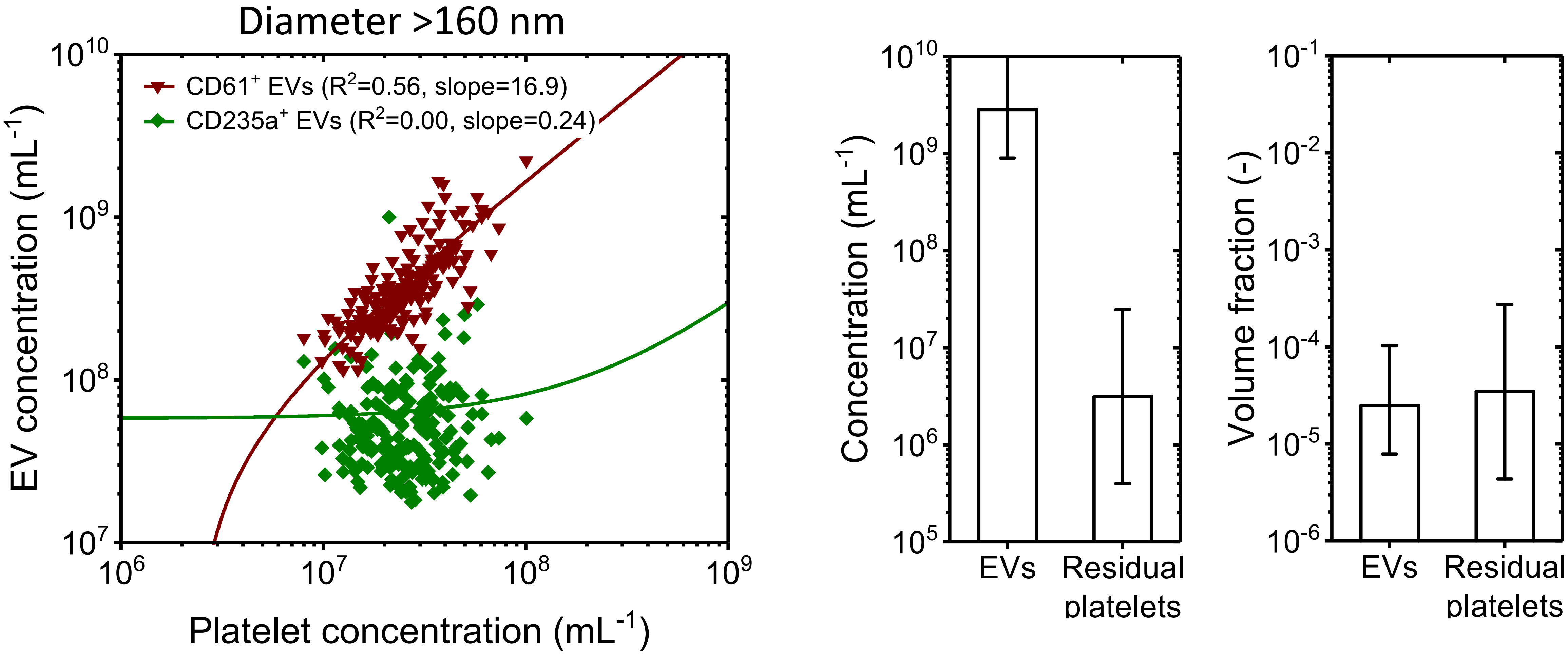
"Platelet free plasma" contains platelets

- Plasma prepared according to ISTH*/AHA** protocol
- Double centrifugation
  - 15 minutes
  - 2,500 *g*
  - 20 °C



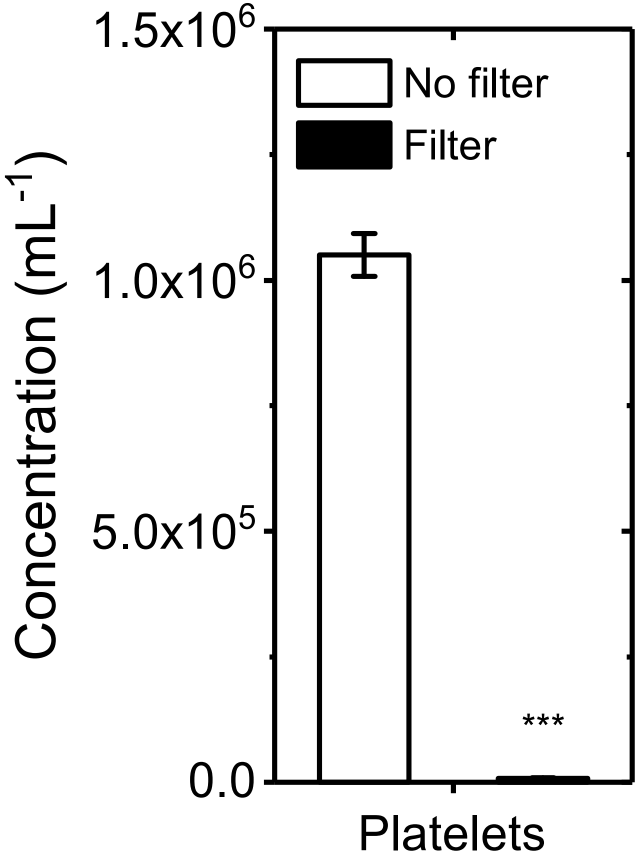
*Lacroix et al. *J Thromb Haemost* 2012
**Coumans et al. *Circ Res* 2017

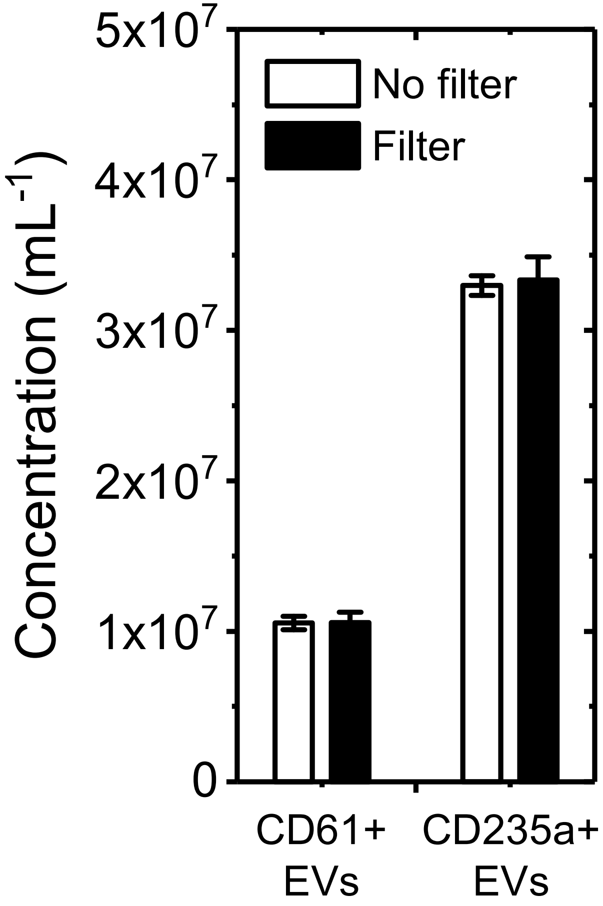
Platelets in plasma affect downstream analyses



Bettin et al. *J Thromb Haemost* 2022

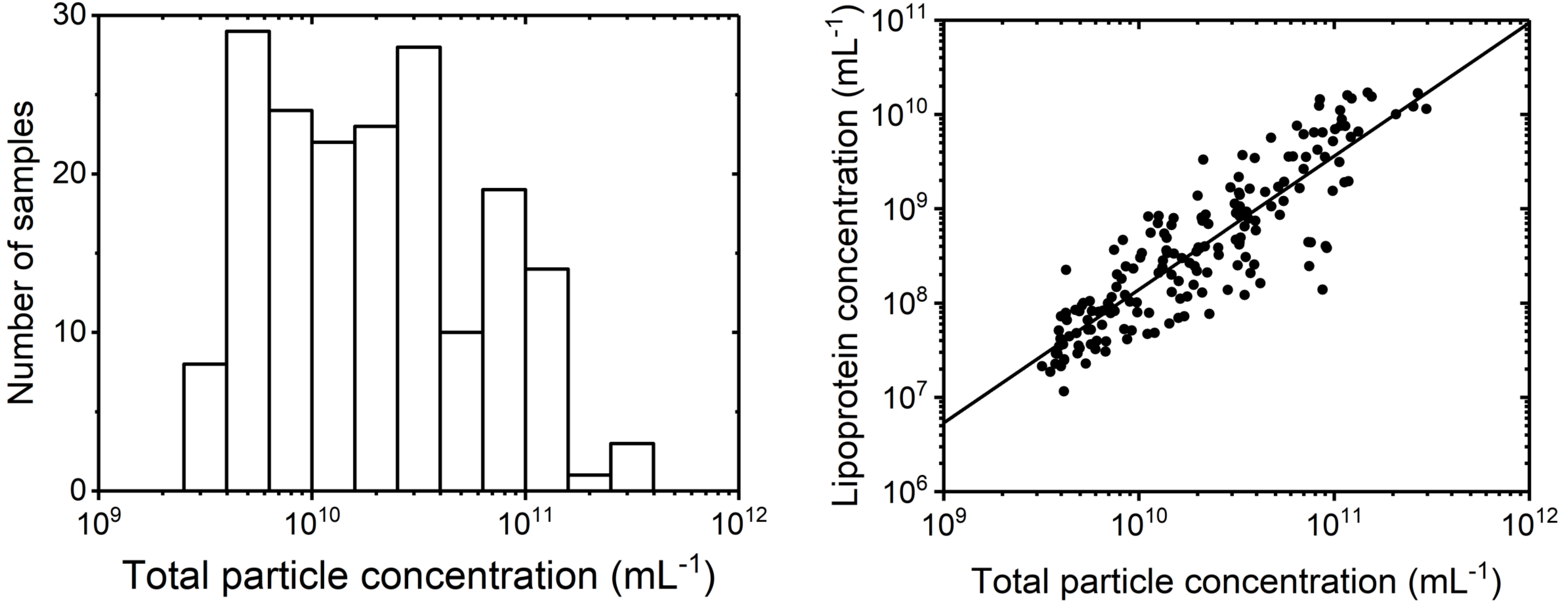
Platelets can be removed by filtration





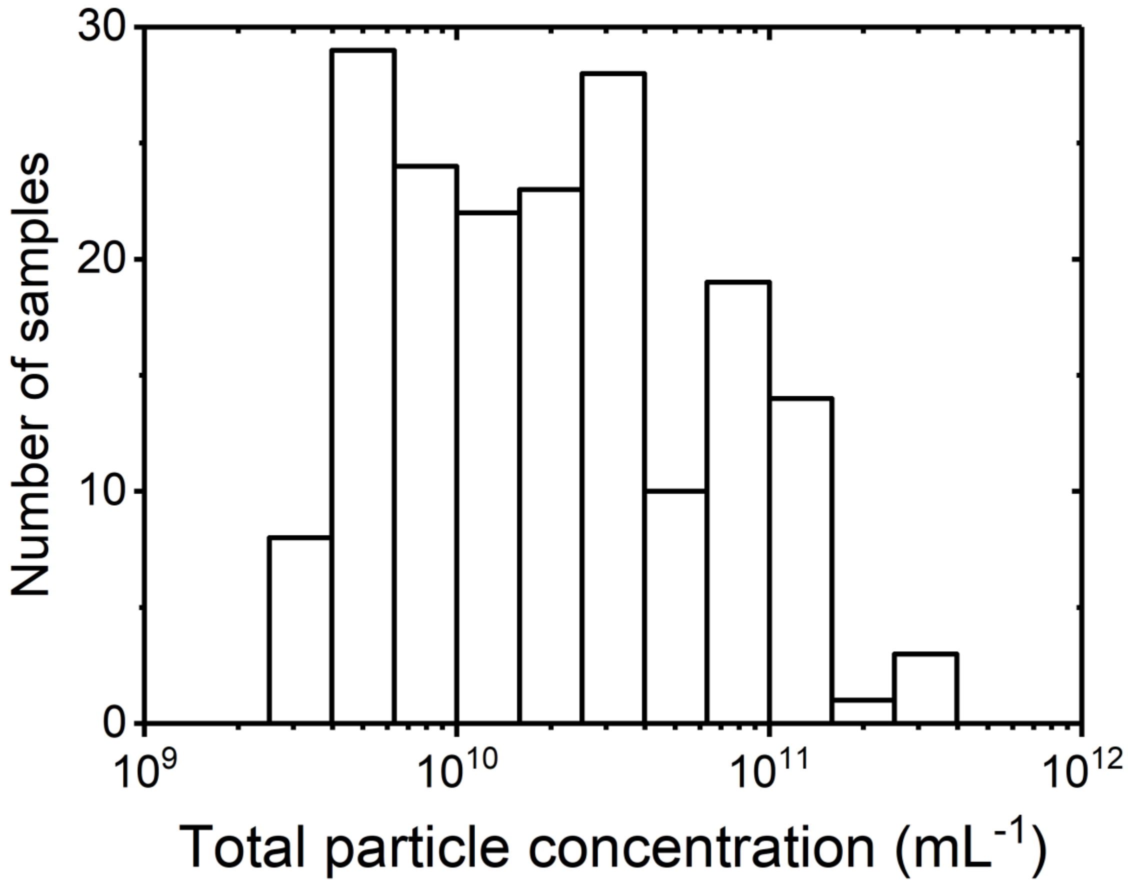
Polycarbonate track-etched membrane filter with 800-nm pores

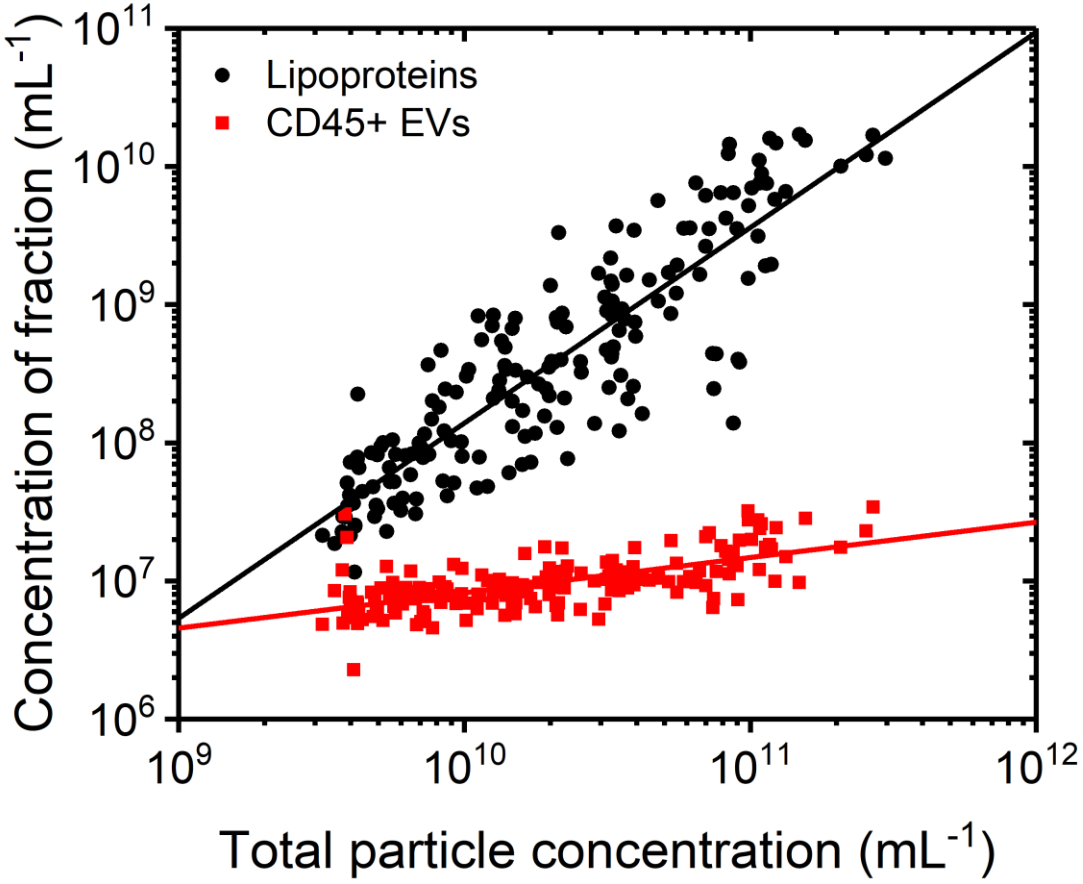
Lipoproteins dominate total particle count



Data from: Gasecka et al. *J Thromb Haemost* 2020

Lipoproteins may lead to false positive EV counts





Data from: Gasecka et al. *J Thromb Haemost* 2020

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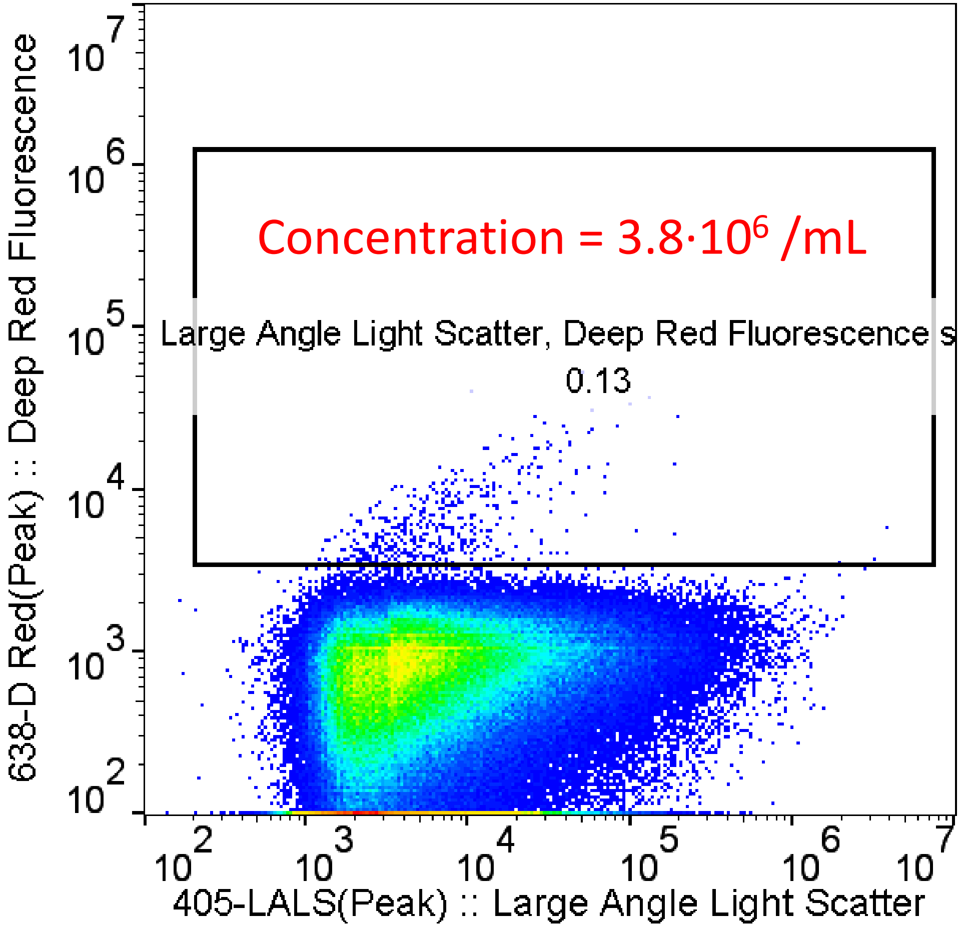
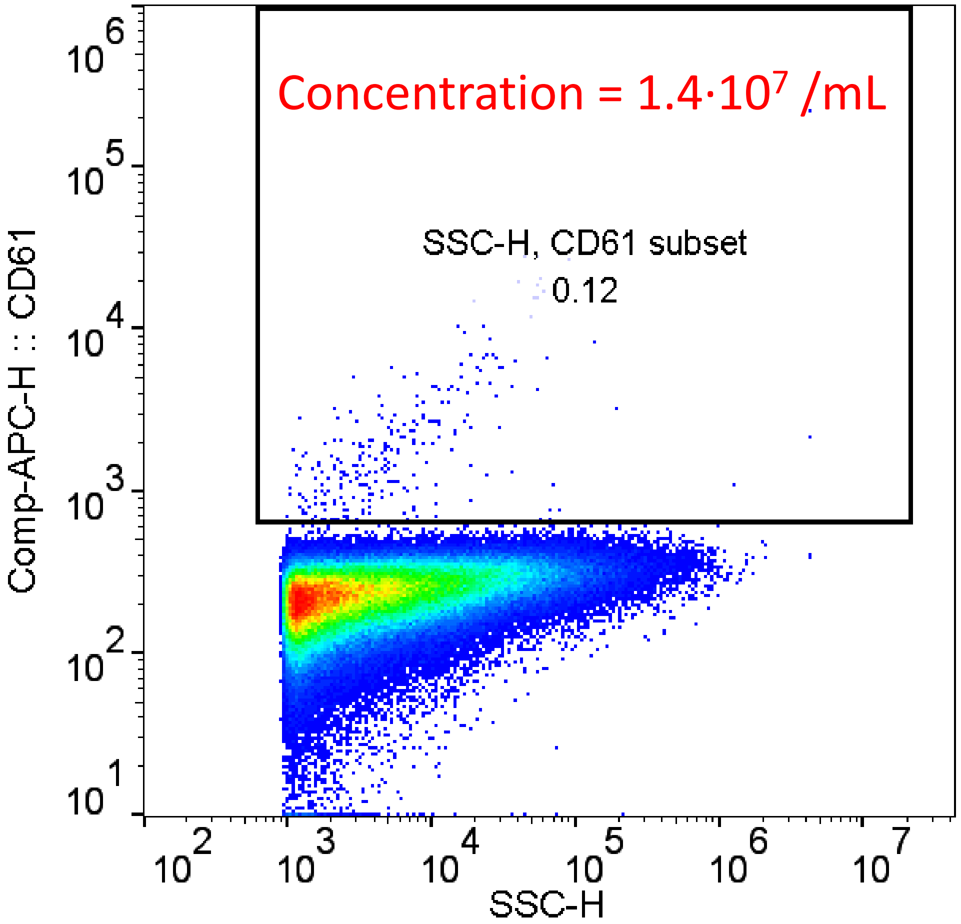
Without calibration, data do not reproduce

Apogee A60-Micro



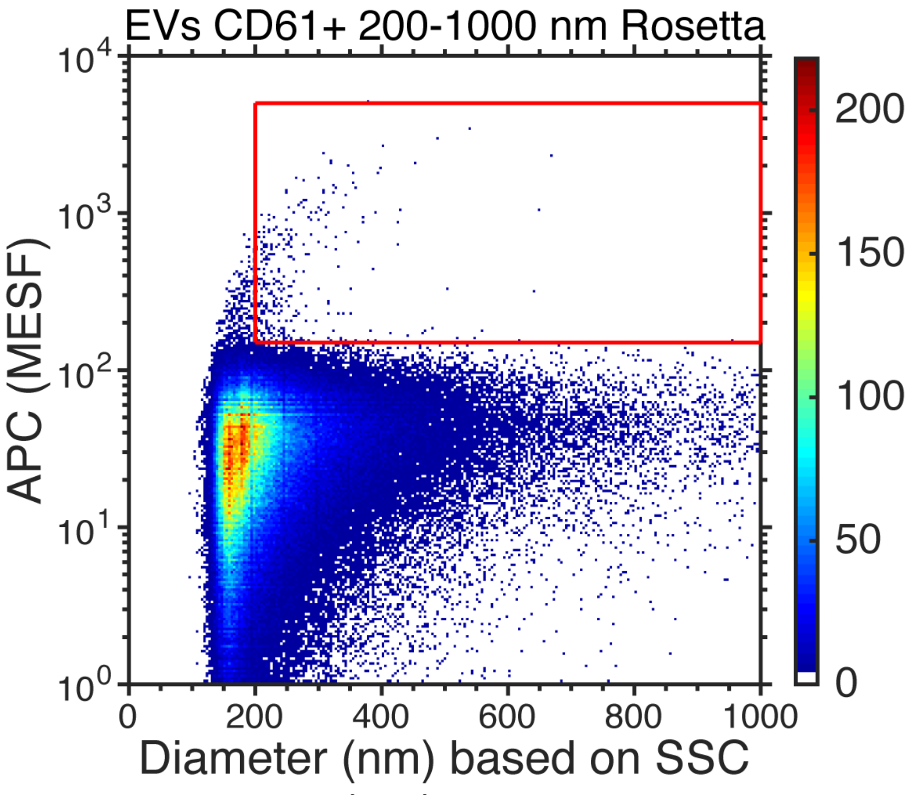
Cytek Aurora



Calibration improves data reproducibility

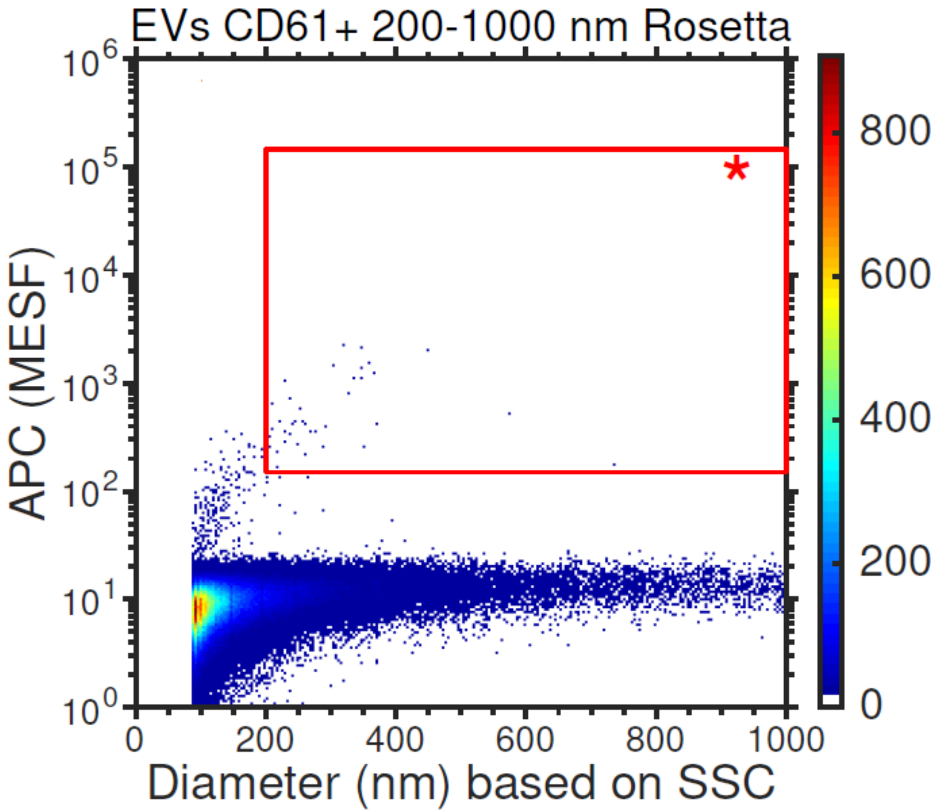
Apogee A60-Micro

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

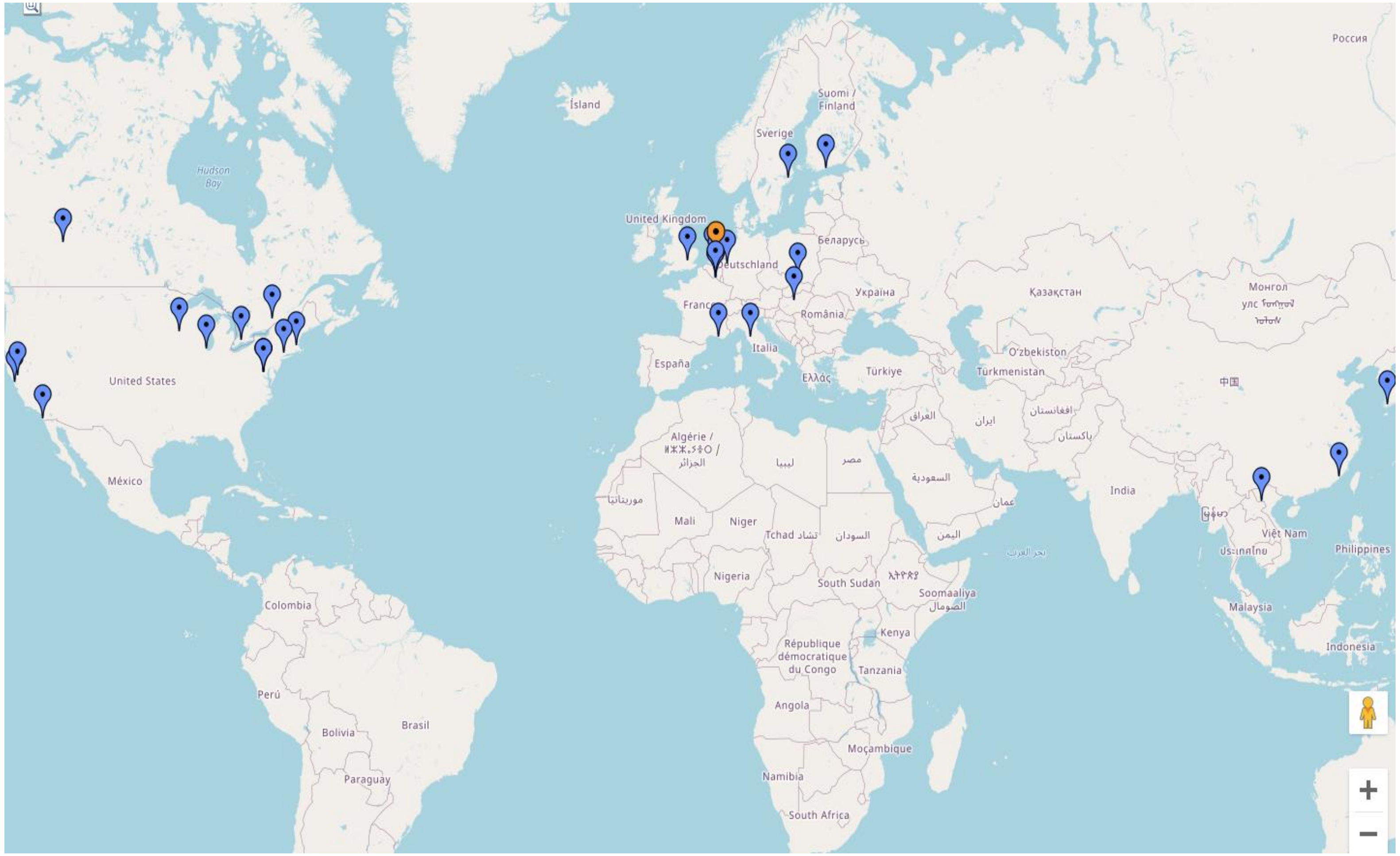


Cytek Aurora

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



METVES II: 23 participants and 35 flow cytometers



Interlaboratory comparison studies

J Thromb Haemost publication	Standardization	>1 FCM	Traceable reference materials	Calibration			Stable test sample
				Flow rate	Fluorescence	Light scattering	
Robert et al. 2008	✓			✓			
Lacroix et al. 2010	✓	✓		✓			
Cointe et al. 2018	✓	✓		✓			
van der Pol et al. 2018	✓	✓	~	✓		✓	
METVES II (upcoming)	✓	✓	~	✓	✓	✓	✓

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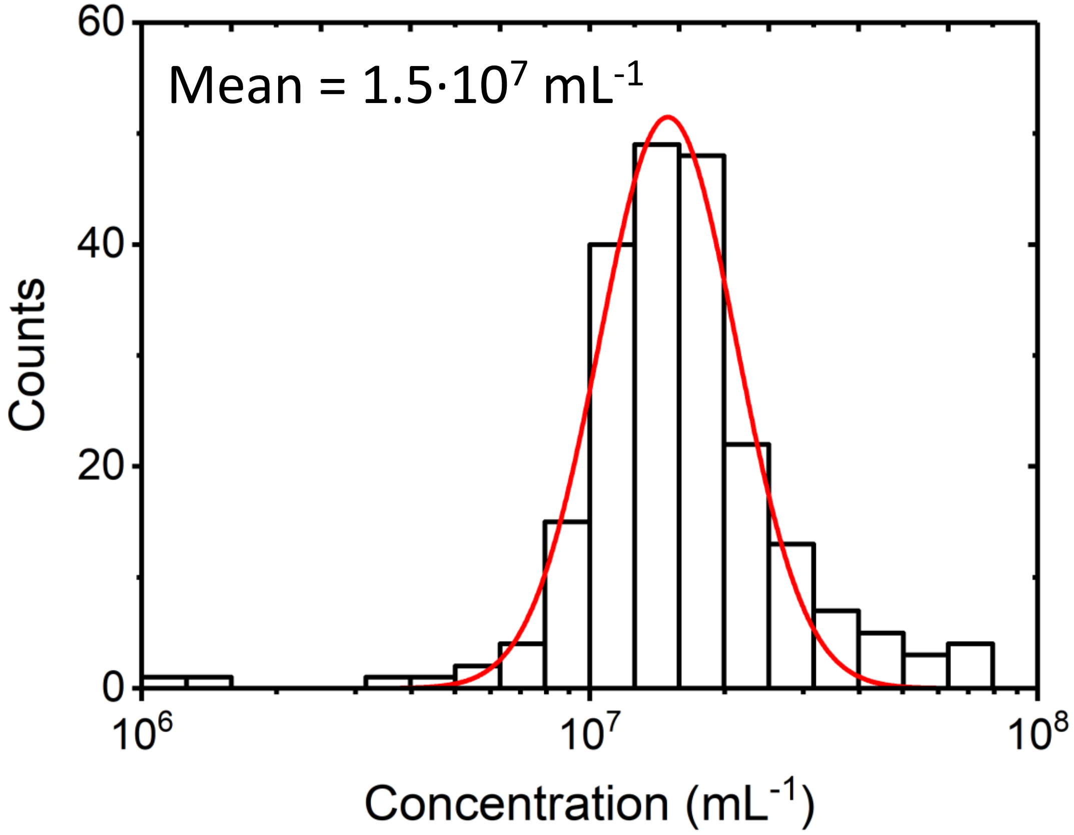
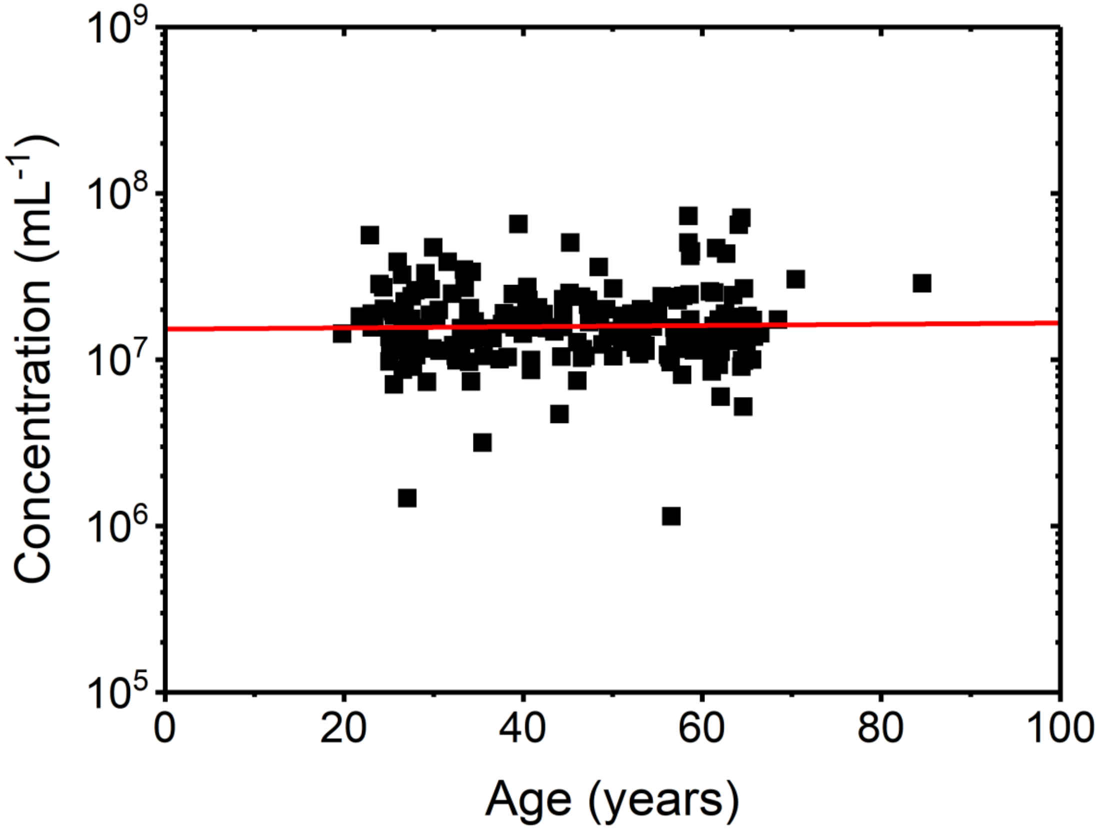
Reference values of EVs in human plasma

- 224 healthy volunteers
- Quality controls
- Assay controls
- Calibration
  - known size & fluorescence ranges

Reference values of EVs in human plasma example

CD61+ EVs, Diameter = 230 nm – 1,000 nm, >50 MESF BV-421





Data by Bo Li

Extracellular vesicle flow cytometry summary

- Clinical flow cytometers detect immunolabelled EVs >~100 nm in diluted plasma within minutes
- Calibration
  - data interpretation: characterize and identify EVs
  - data comparison and reproducibility
- Assay controls: confirm that particles are EVs
- Establishment of reference ranges confirms clinical feasibility

MIFlowCyt-EV. Welsh et al. *J Extracell Vesicles* 2020
EV flow cytometry compendium. Submitted to *J Extracell Vesicles*

Acknowledgements

- Amsterdam University Medical Centers
  - Biomedical Engineering & Physics
  - Laboratory of Experimental Clinical Chemistry
  - Vesicle Observation Center
- METVES II participants
  - Beckton & Dickinson
  - Cellarcus Biosciences

Amsterdam UMC
University Medical Centers

Vesicle
Observation Center

- More information
  - edwinvanderpol.com
  - evflowcytometry.org
  - metves.eu
- Funding
  - EURAMET project 18HLT01 (METVES II)