Extracellular vesicle flow cytometry: what's possible and what's next

Edwin van der Pol

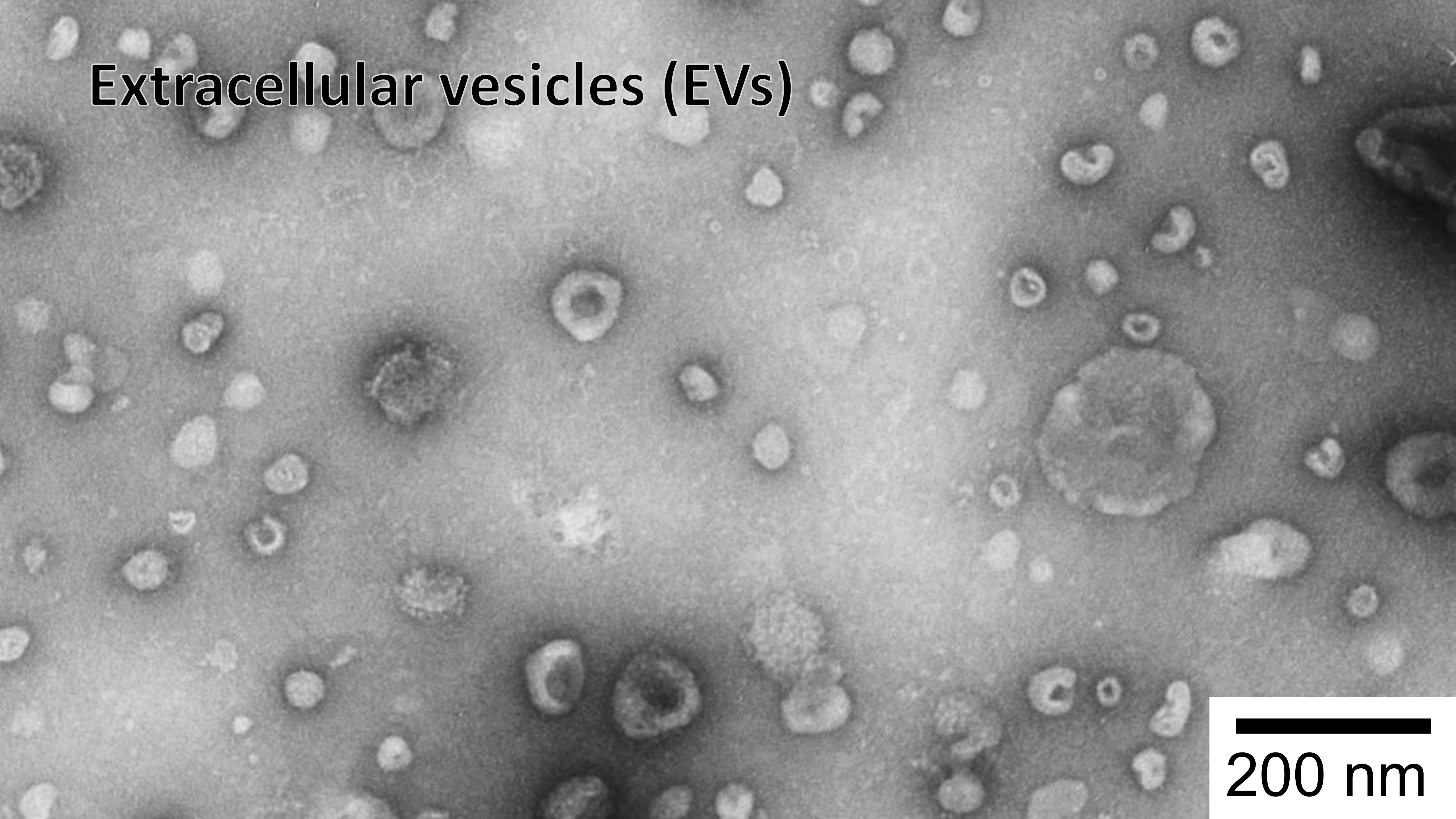
August 21st, 2025

Amsterdam UMC

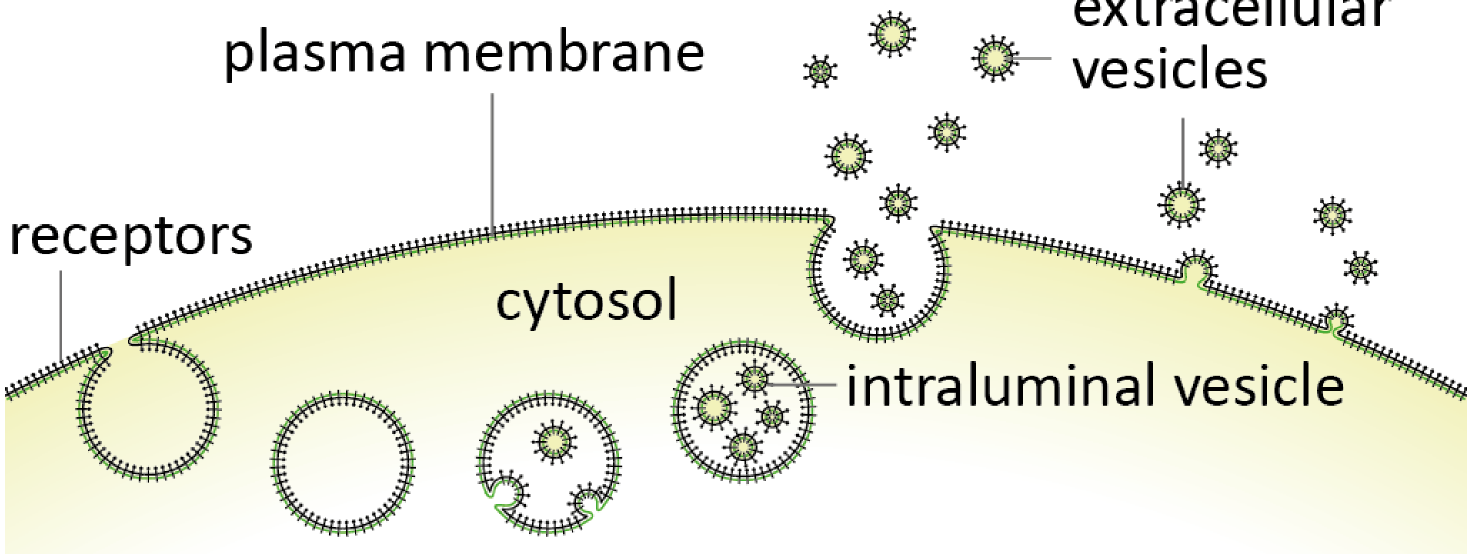
Amsterdam Vesicle Center

Conflicts of interest

- Cofounder and shareholder of EXOMETRY
- Cofounder of EVcount


Extracellular vesicles (EVs)
200 nm

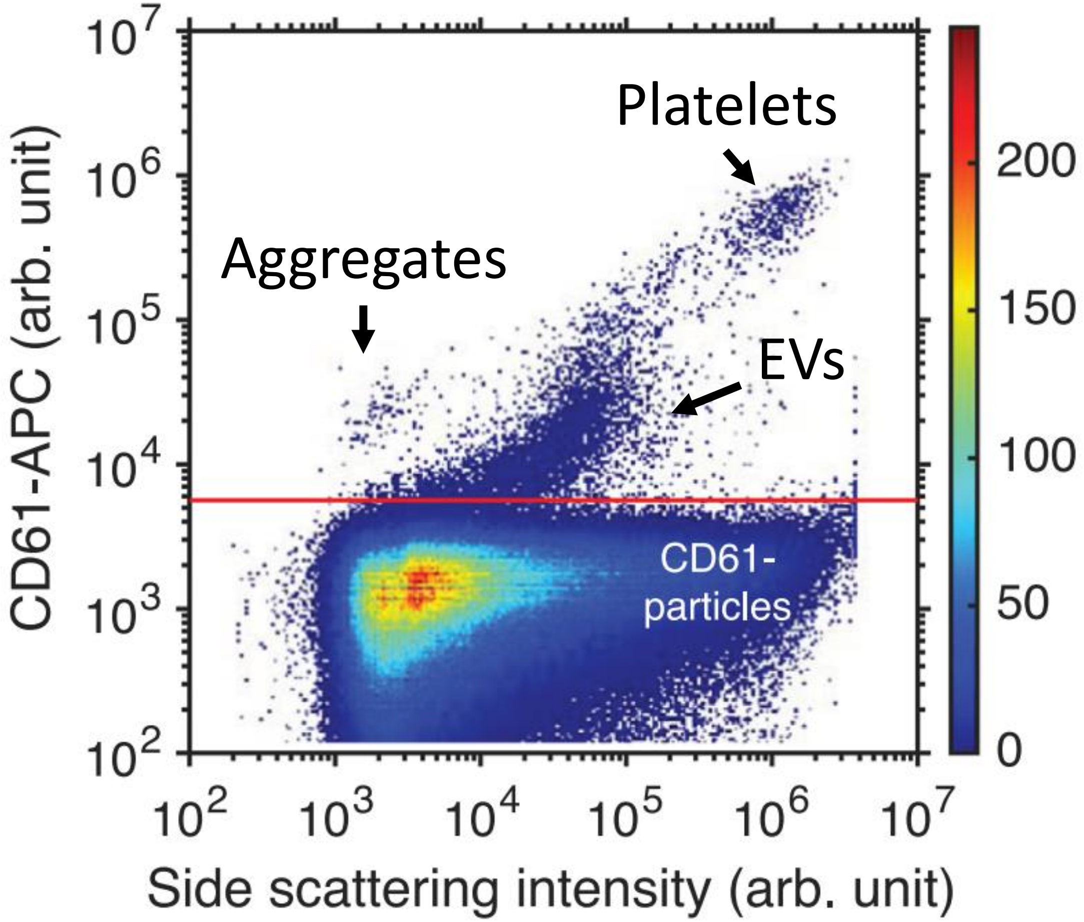
Extracellular vesicles (EVs)



- cells release vesicles:
  biological nanoparticles with receptors, DNA, RNA
- specialized functions
- potential biomarkers for disease

EV vs. cell detection by flow cytometry

Plasma labeled with anti-CD61



- EVs:
  - dim signals
  - signals overlap with
    - background noise
    - non-EV particles
  - heterogeneous populations

Question 1

- Who has experience with extracellular vesicle detection with flow cytometry?

Question 2

- Who is considering performing flow cytometry experiments on extracellular vesicles?

Question 3

- Who has calibrated a flow cytometry experiment on extracellular vesicles?

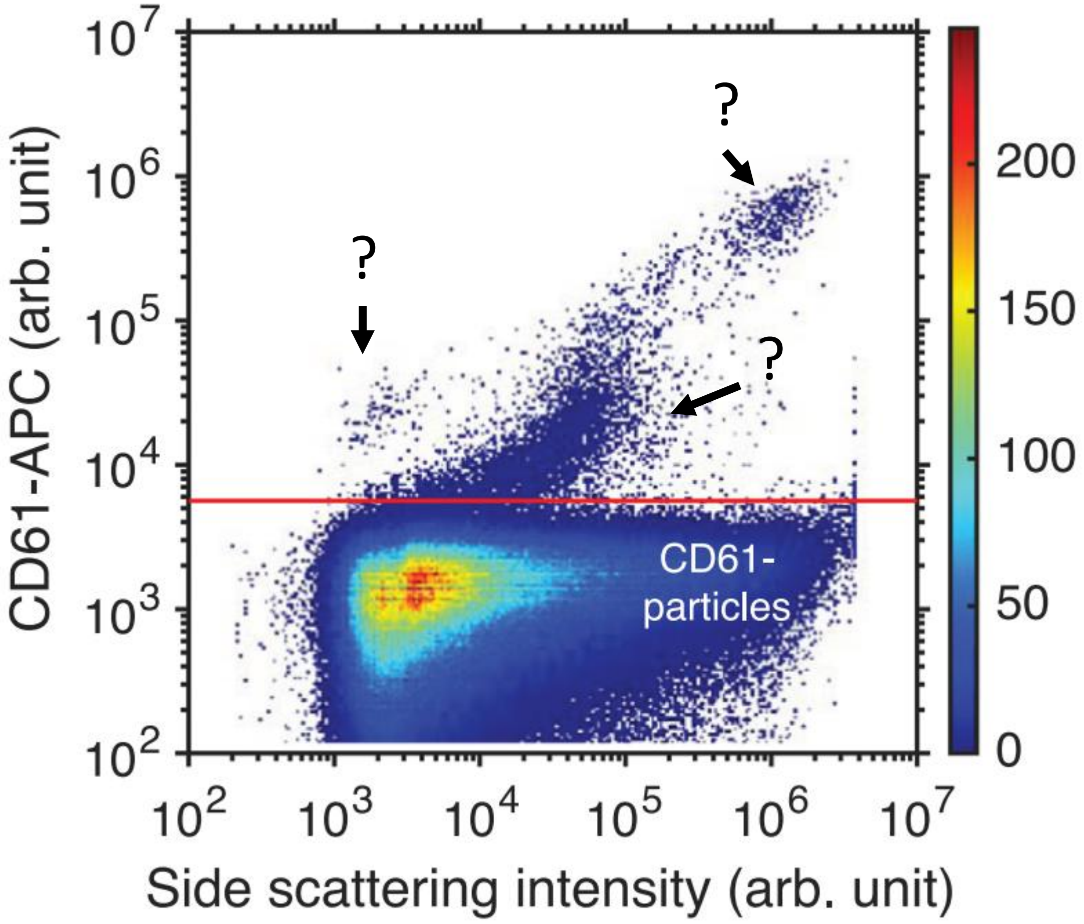
Outline

- Calibrate!
  - improve data interpretation
  - achieve data reproducibility
- What's possible
- What's challenging
  - Get scientists to calibrate and report
  - Dilution paradox
- What's next

Need for calibration

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

Plasma labeled with anti-CD61

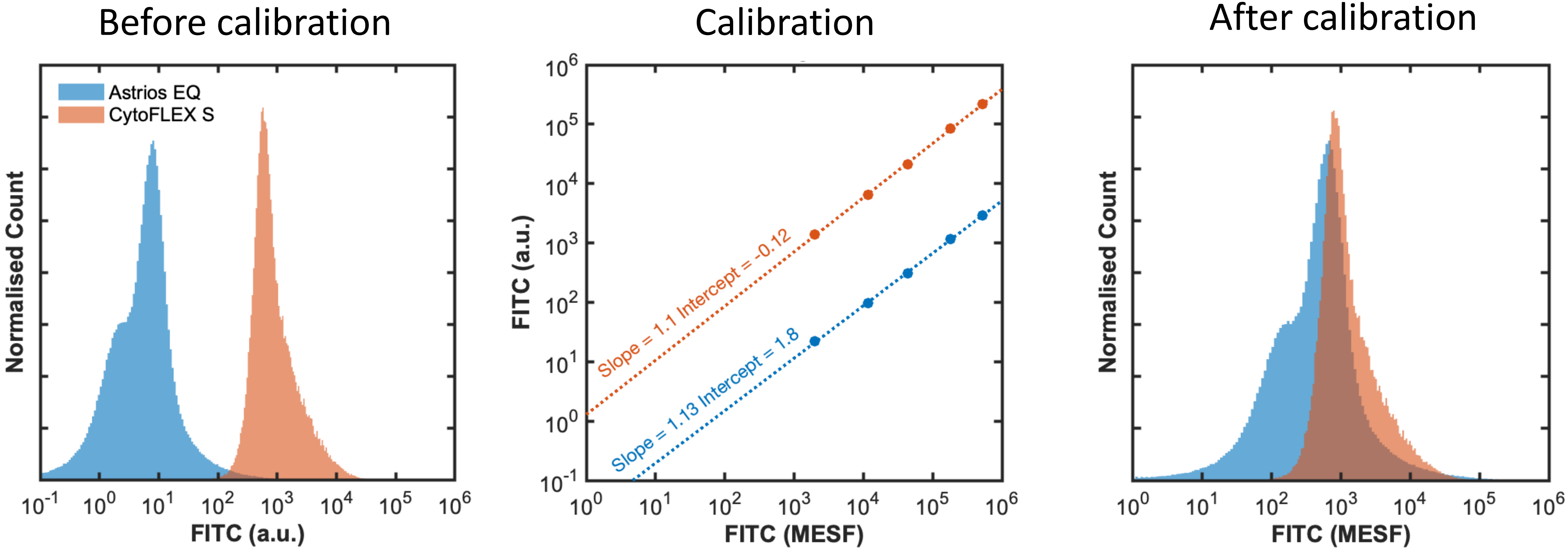


What is calibration?

- Relate arbitrary units to standard units*
  - Fluorescence intensity (a.u.) → (MESF)
  - Light scattering intensity (a.u.) → Diameter (nm)
  - Counts or percentages → Concentration (EVs mL^{-1})
    - Verify: flow rate ($\mu L\ min^{-1}$)

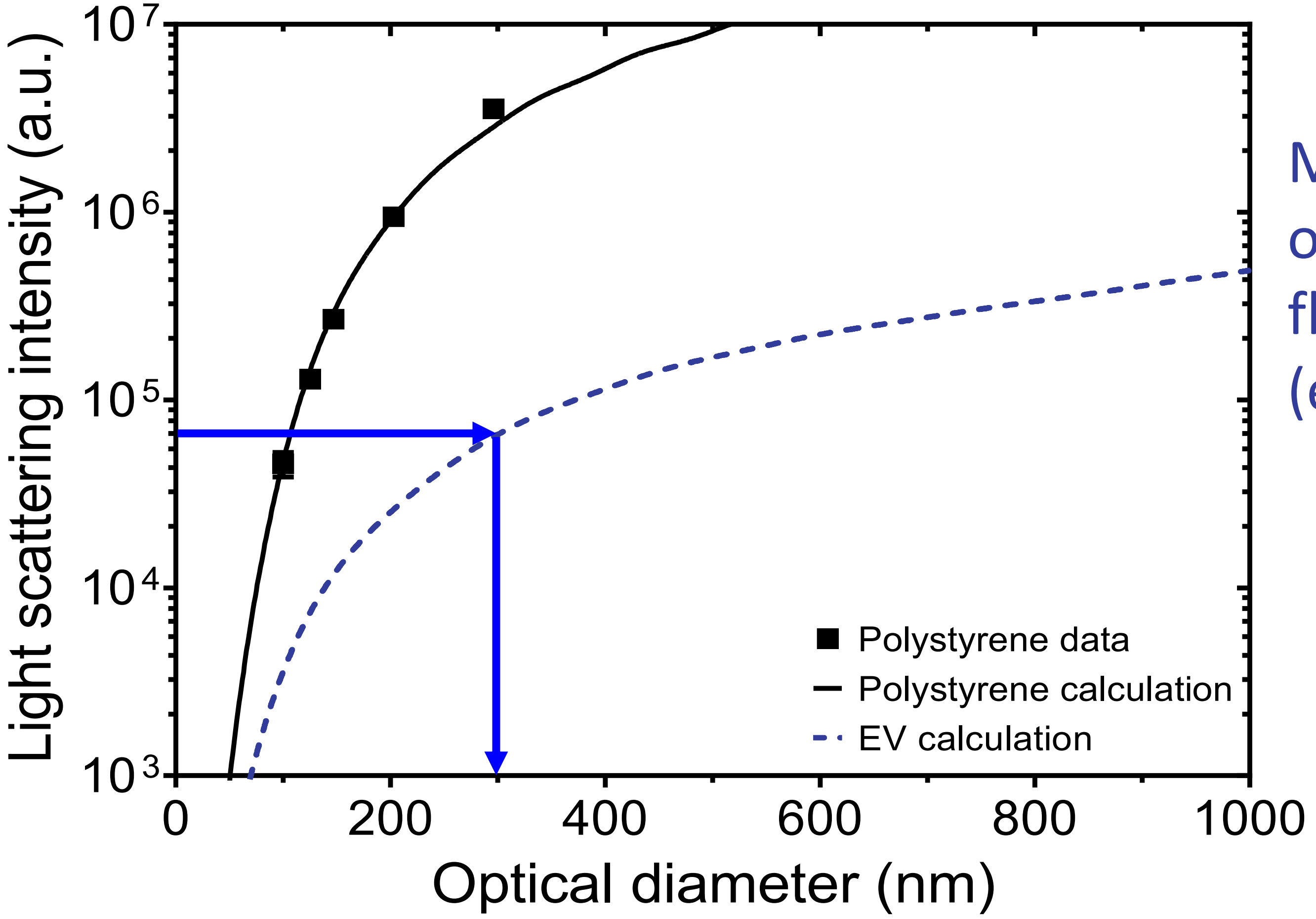
*Welsh et al. "EV FCM compendium" *J Extracell Vesicles* 2023
MESF: molecules of equivalent soluble fluorophore

Example of fluorescence calibration



Welsh et al. *Curr Protoc Cytom* 2020
Welsh et al. *Cytom Part A* 2020

Example of light scattering calibration

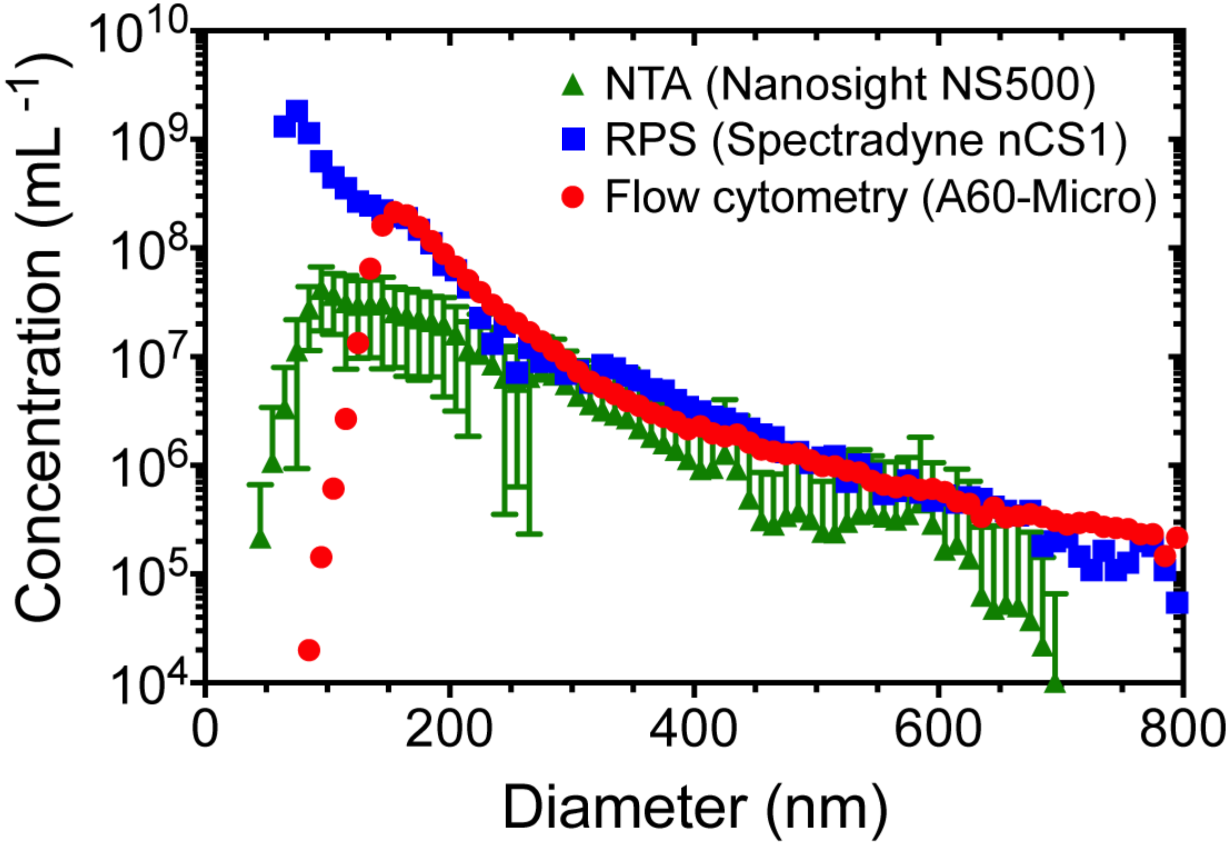


Mie theory takes into account optical properties of flow cytometer and particles (e.g. polystyrene beads or EVs)

Side scattering detector of flow cytometer with NA=1.2 lens and 405 nm wavelength illumination

Turn your flow cytometer into a particle size analyzer!

EVs in human urine



Data courtesy of Michael Cimorelli

RPS: resistive pulse sensing; NTA: nanoparticle tracking analysis

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]

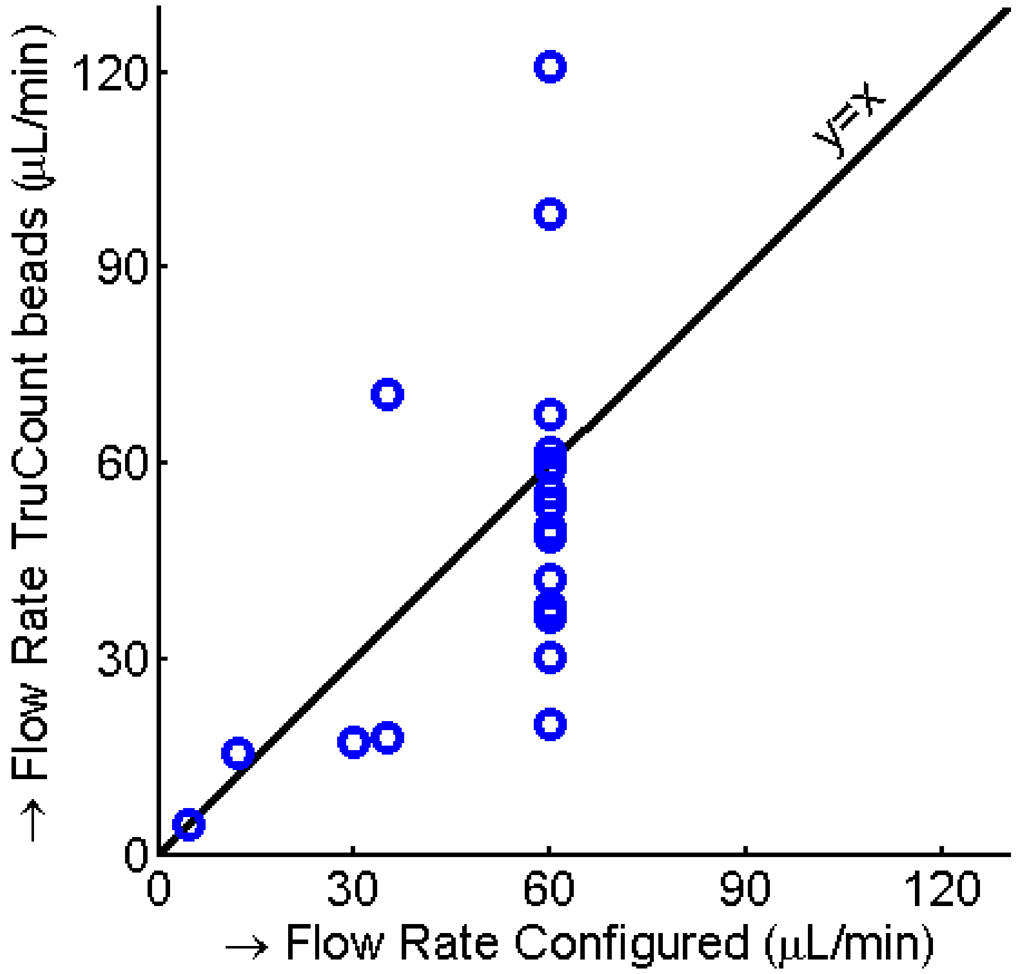
Current Protocols in Cytometry, e43 (2018)

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

NTA: nanoparticle tracking analysis

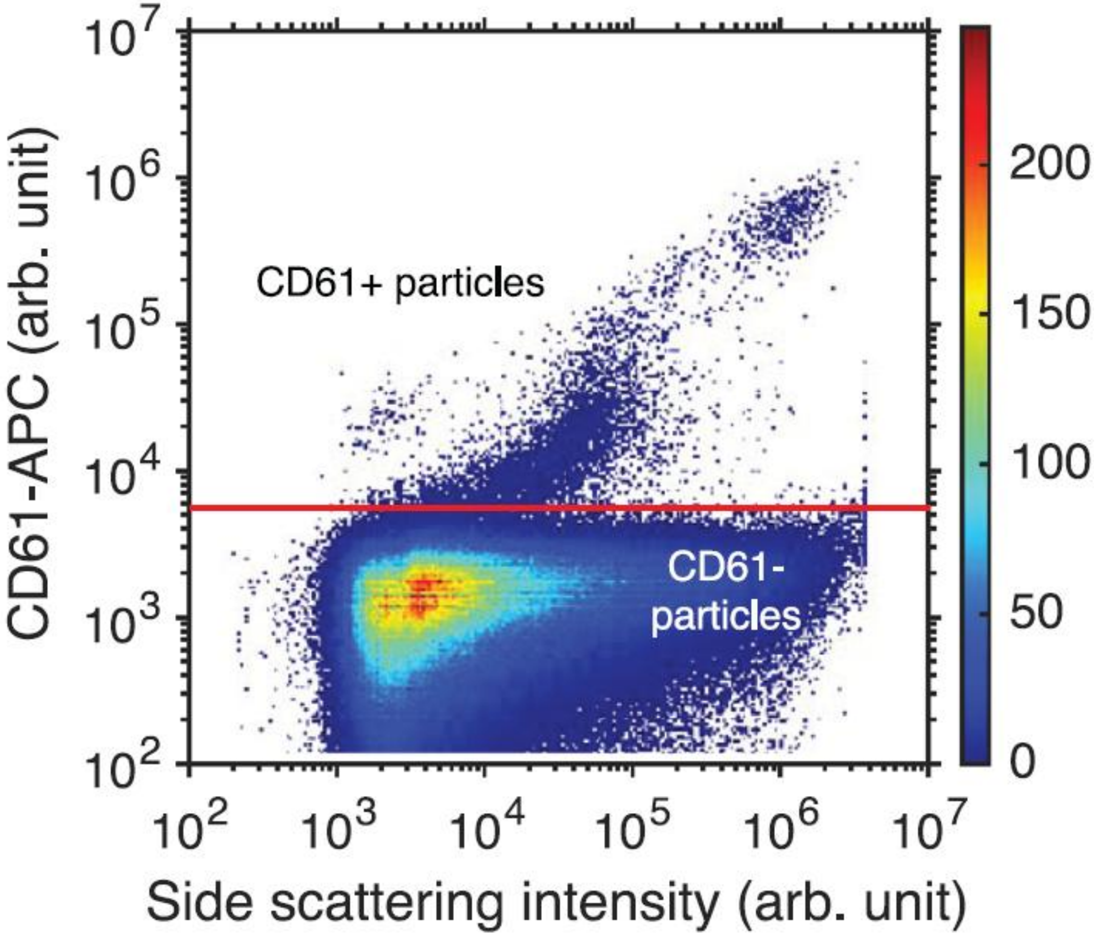
Importance of flow rate calibration

$$\text{concentration} = \frac{\#\text{ of EV}}{\text{flow rate} \times \text{measurement time}}$$

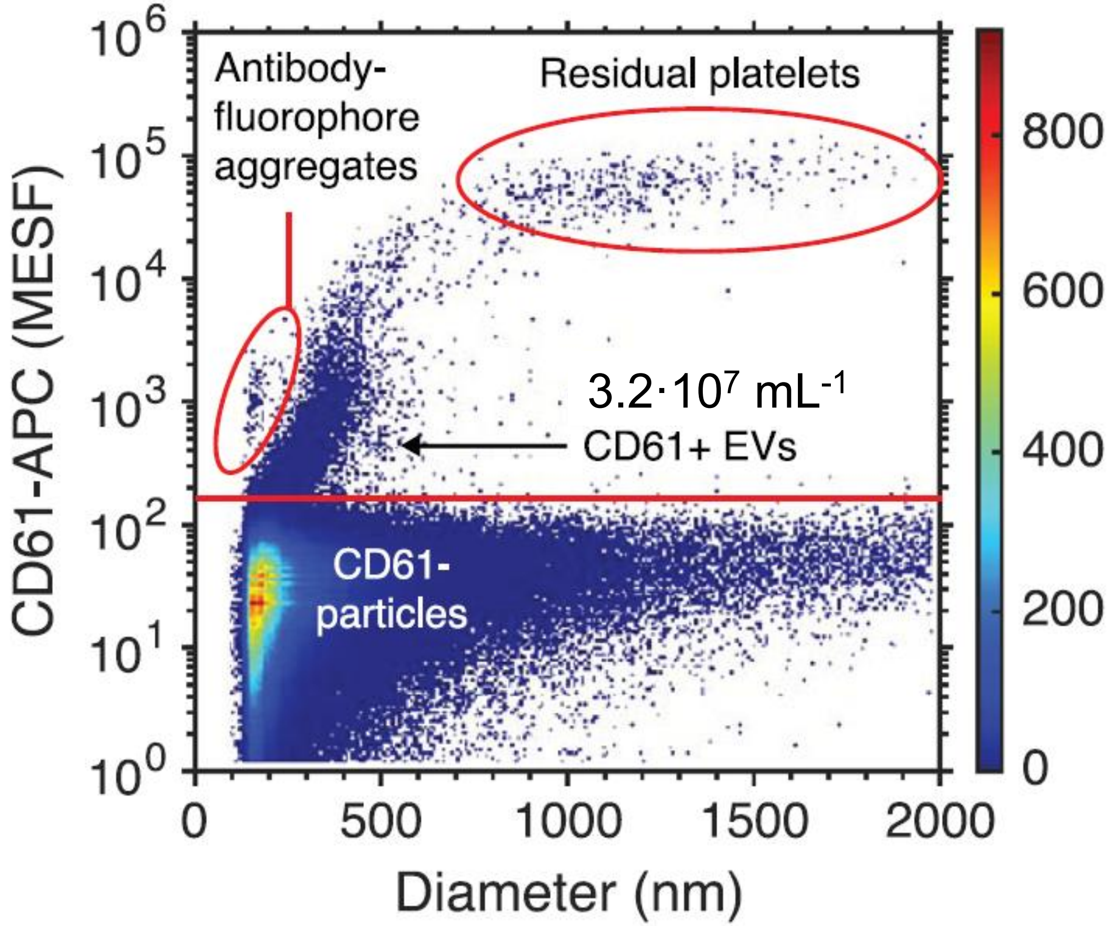


Calibrations enable enhanced data interpretation

Uncalibrated



Calibrated



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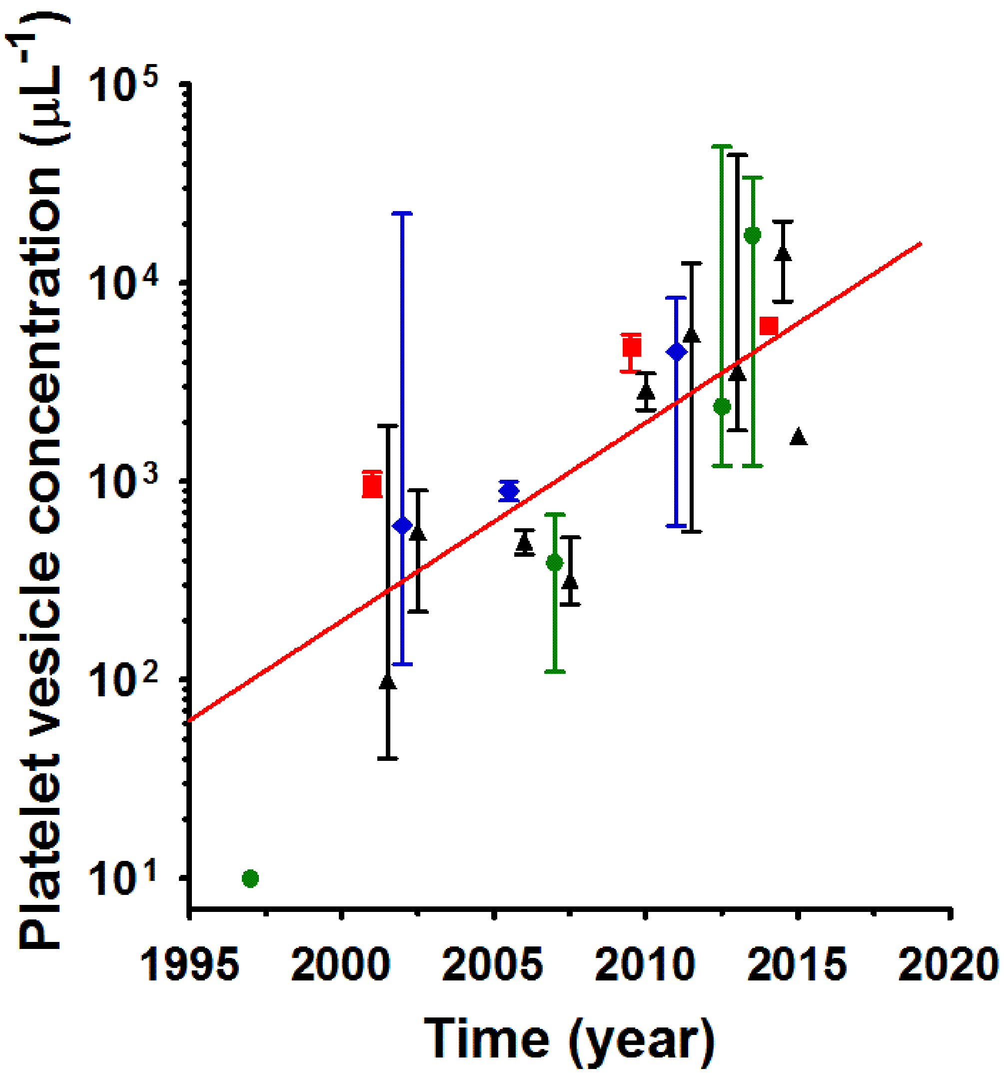
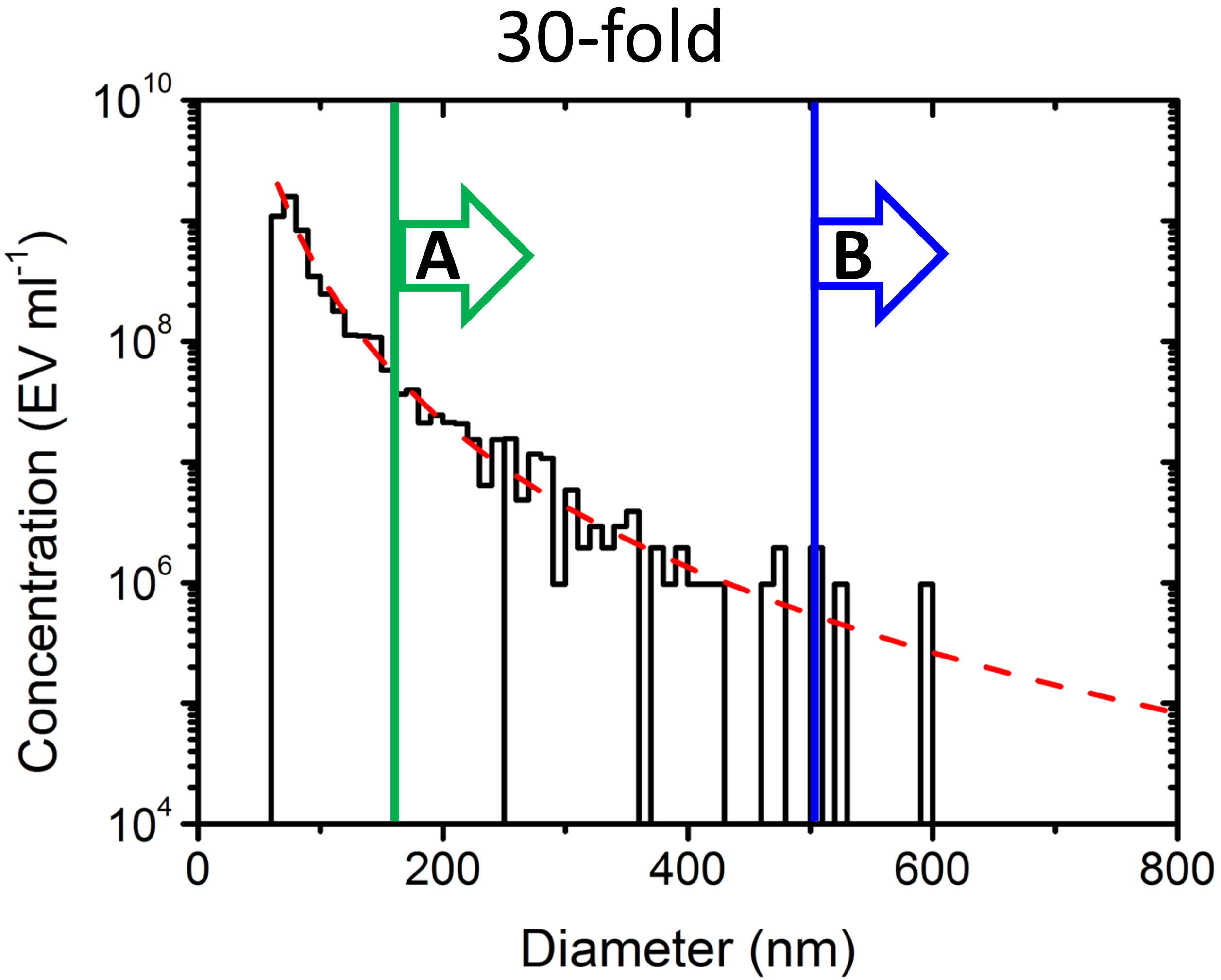
Problem

- Reported concentrations of blood plasma EV differ 10^4-fold
- Clinical data cannot be compared
- Standardization required

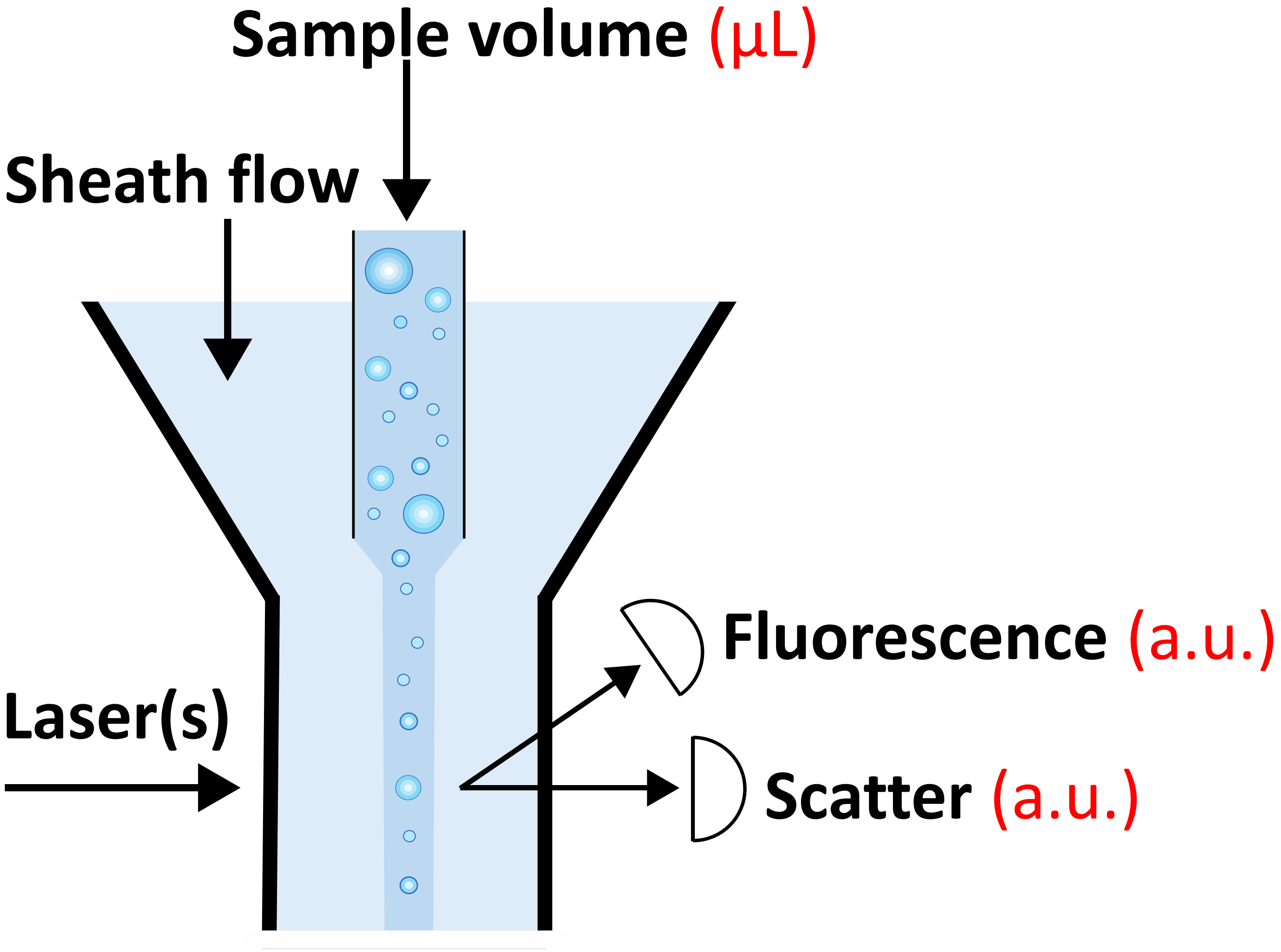


Cause 1: Flow cytometers differ in sensitivity



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

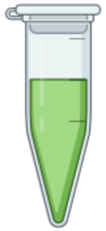
Cause 2: Data have arbitrary units



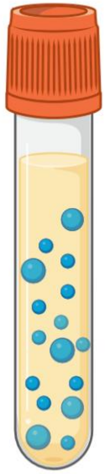
a.u.: arbitrary units
Slide courtesy of Britta Bettin

Does calibration allow comparison of EV data?

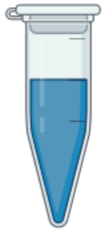
Assay controls

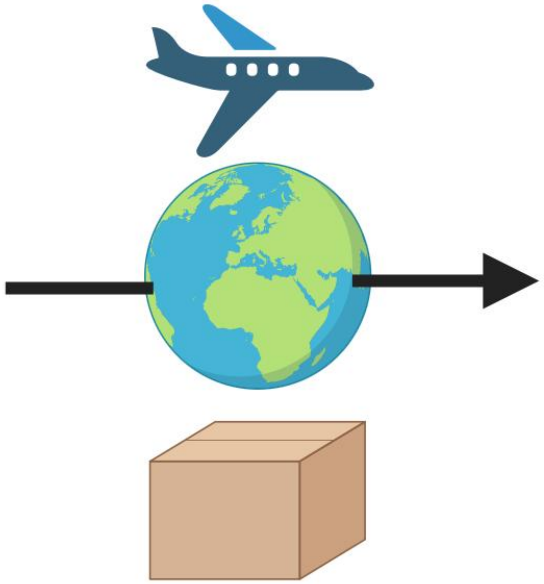


Plasma EV test sample



Calibration materials

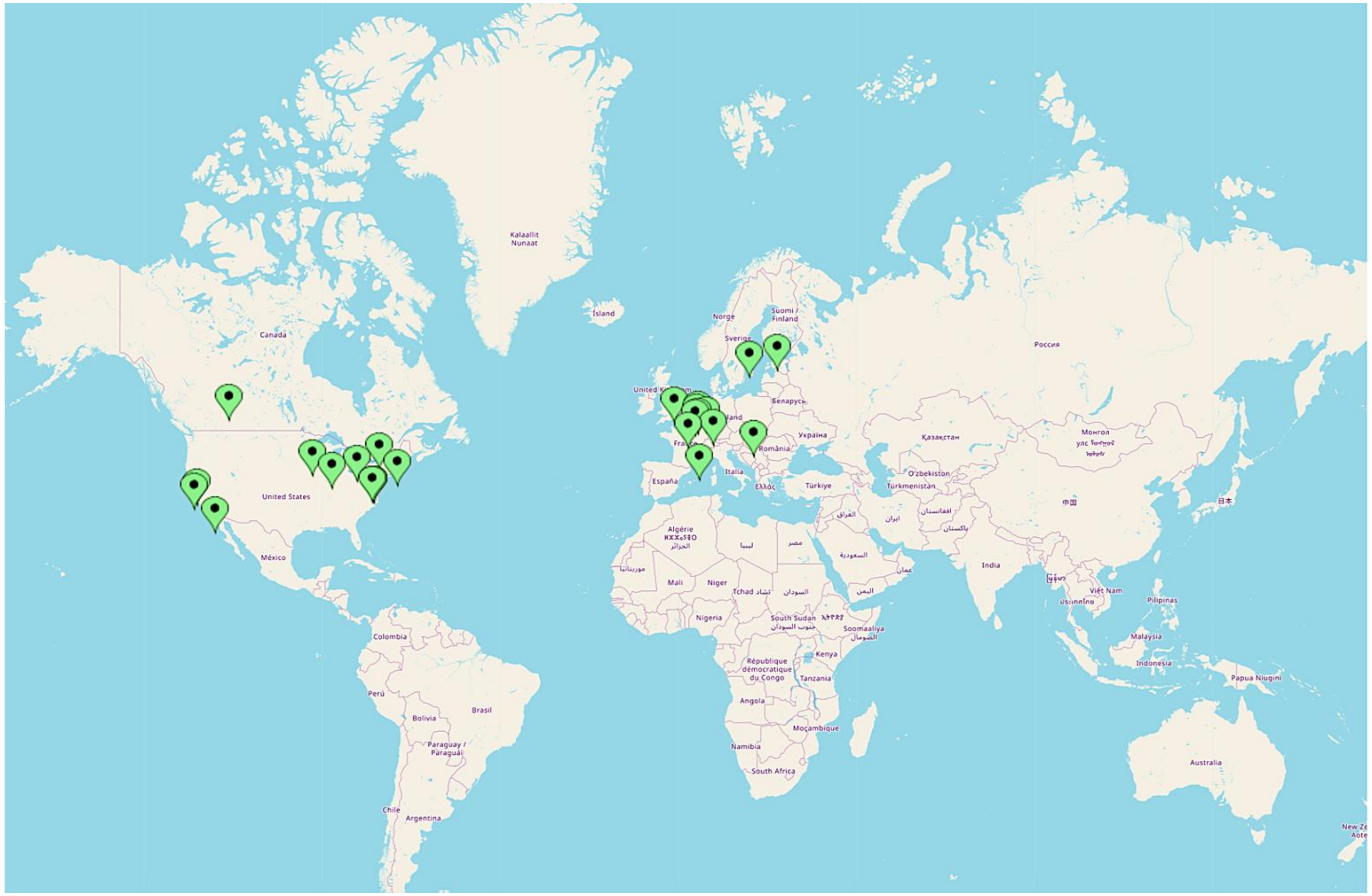




25 Flow cytometers

Slide courtesy of Britta Bettin
Submitted to *J Extracell Vesicles*

Interlaboratory comparison study

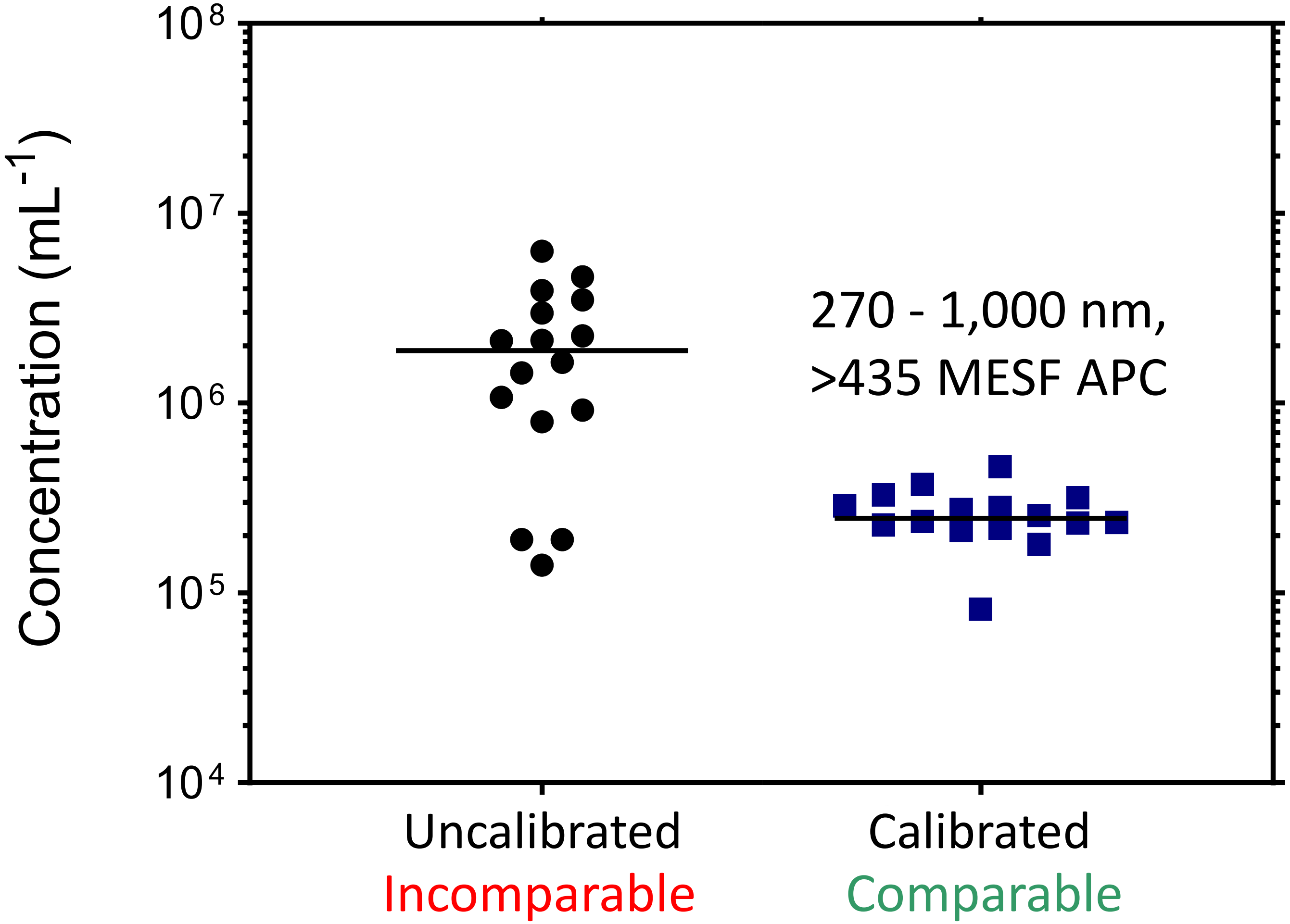


Slide courtesy of Britta Bettin

Submitted to *J Extracell Vesicles*

Britta Bettin shipping the samples

Platelet EV concentrations are comparable



$$MAD = \frac{median(\forall c_i \in \vec{c}, |c_i - median(\vec{c})|)}{median(\vec{c})} \cdot 100\%$$

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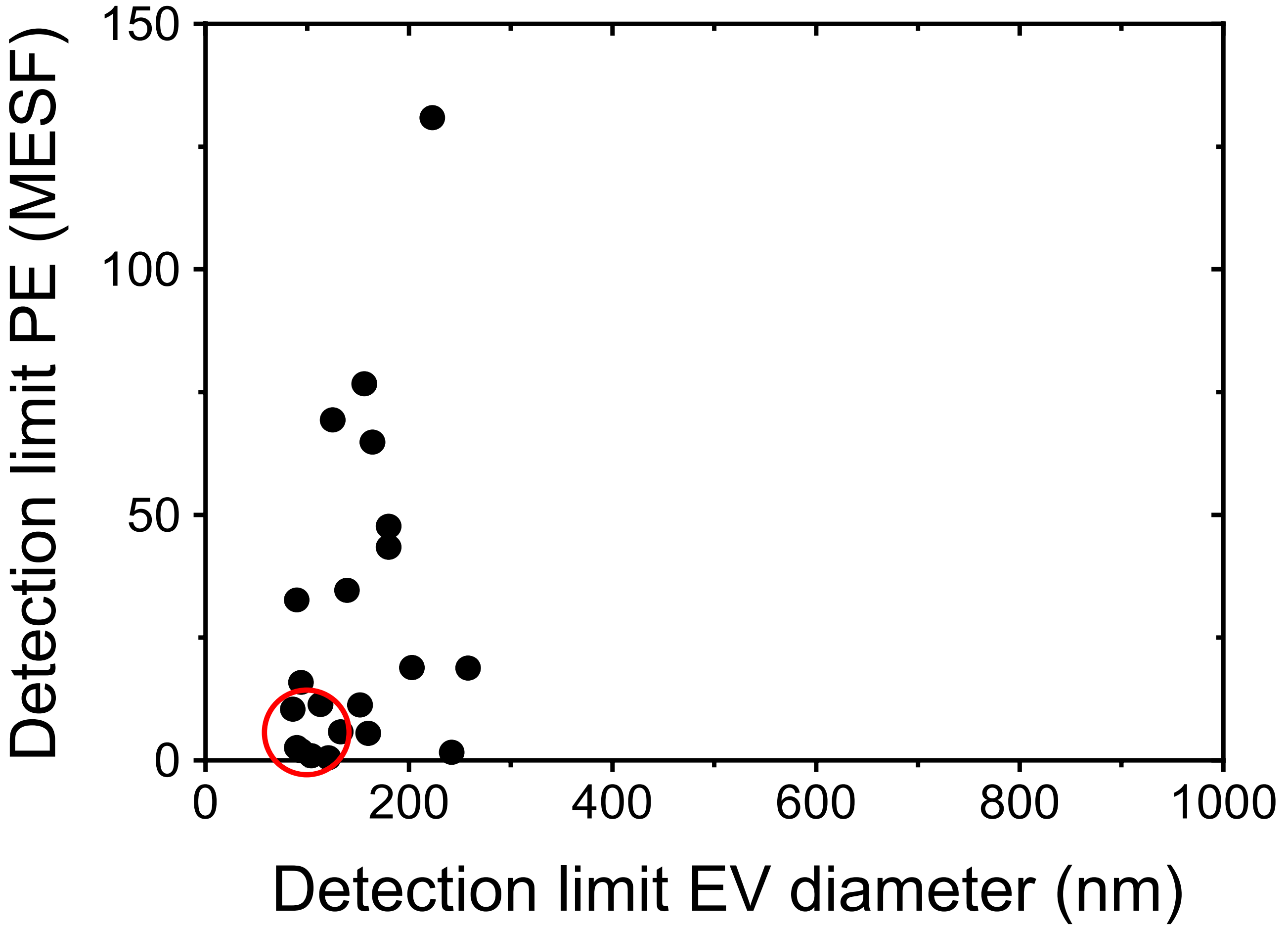
What’s currently possible with flow cytometry

Interlaboratory comparison study

- Apogee A50-Micro
- Apogee A60-Micro
- BC Astrios
- BC Cytoflex LX
- BC Cytoflex S
- BD FACSCanto
- BD FACSLyric
- BD FACSymphony A1 + SPD
- BD FACSymphony A5
- Cytek Aurora + ESP
- Cytek Northern Lights +ESP



Submitted to *J Extracell Vesicles*

MESF: molecules of equivalent soluble fluorophores

What's currently possible with flow cytometry

- Detect 100 nm EVs by light scattering
- Detect <10 MESF PE by fluorescence
- Detect EV concentrations down to 10^5 mL^{-1} *
- Characterize 1–2·10^4 particles s^{-1} with multiple labels
- Automatically measure 96 samples
- Generate calibrated, reproducible data

* Assay dependent

Submitted to *J Extracell Vesicles*

Is the technology ready for the clinic?


Is the technology ready for the clinic?
Is the clinic ready for the technology?

Is the technology ready for the clinic?

Flow cytometry enables concentration measurements of EVs ≥100 nm in a standardized, reproducible (16% MAD) and scalable way

EV flow cytometry is state-of-the-art nanoparticle characterization and thereby more demanding than routine (cellular) flow cytometry

Is the clinic ready for the technology?

Outline

- ✔ Calibrate!
  - ✔ improve data interpretation
  - ✔ achieve data reproducibility
- ✔ What's possible
- What's challenging
  - ➢ Get scientists to calibrate and report
  - ➢ Dilution paradox
- What's next

Question 4

- Who has heard about MIFlowCyt-EV?

Question 5

- Who has completed a MIFlowCyt-EV template?

Reporting

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

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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]

	Category	Components	
1	Preanalytical variables & experimental design	1.1. Report preanalytical variables conforming to MISEV guidelines* 1.2. Report experimental design according to MIFlowCyt guidelines*	Allow others to repeat your assay
2	Sample preparation	2.1. Sample staining* 2.2. Sample washing steps* 2.3. Sample dilution*	
3	Assay controls	3.1. Buffer-only* 3.2. Buffer with reagents* 3.3. Unstained controls* 3.4. Isotype controls** 3.5. Single-stained controls* 3.6. Procedural controls** 3.7. Serial dilution* 3.8. Detergent-treated EV samples	Provide evidence you are looking at single EVs
4	Instrument calibration & data acquisition	4.1. Trigger channel(s) and threshold(s)* 4.2. Flow rate & volumetric quantification (μL min^{-1} / μL)* 4.3. Fluorescence Calibration (MESF/ERF units)* 4.4. Light Scatter Calibration (nm^2)	Allow others to validate data across instruments and settings
5	EV characterization	5.1. EV diameter/surface area/volume approximation 5.2. EV refractive index approximation 5.3. Epitope number approximation	
6	FC data reporting	6.1. Complete MIFlowCyt checklist* 6.2. Calibrated channel detection range 6.3. EV number concentration 6.4. EV brightness	Provide transparency on the quality of data and how it was analyzed
7	FC data sharing	7.1. Share data to public repository	

Welsh et al. *J Extracell Vesicles* 2020

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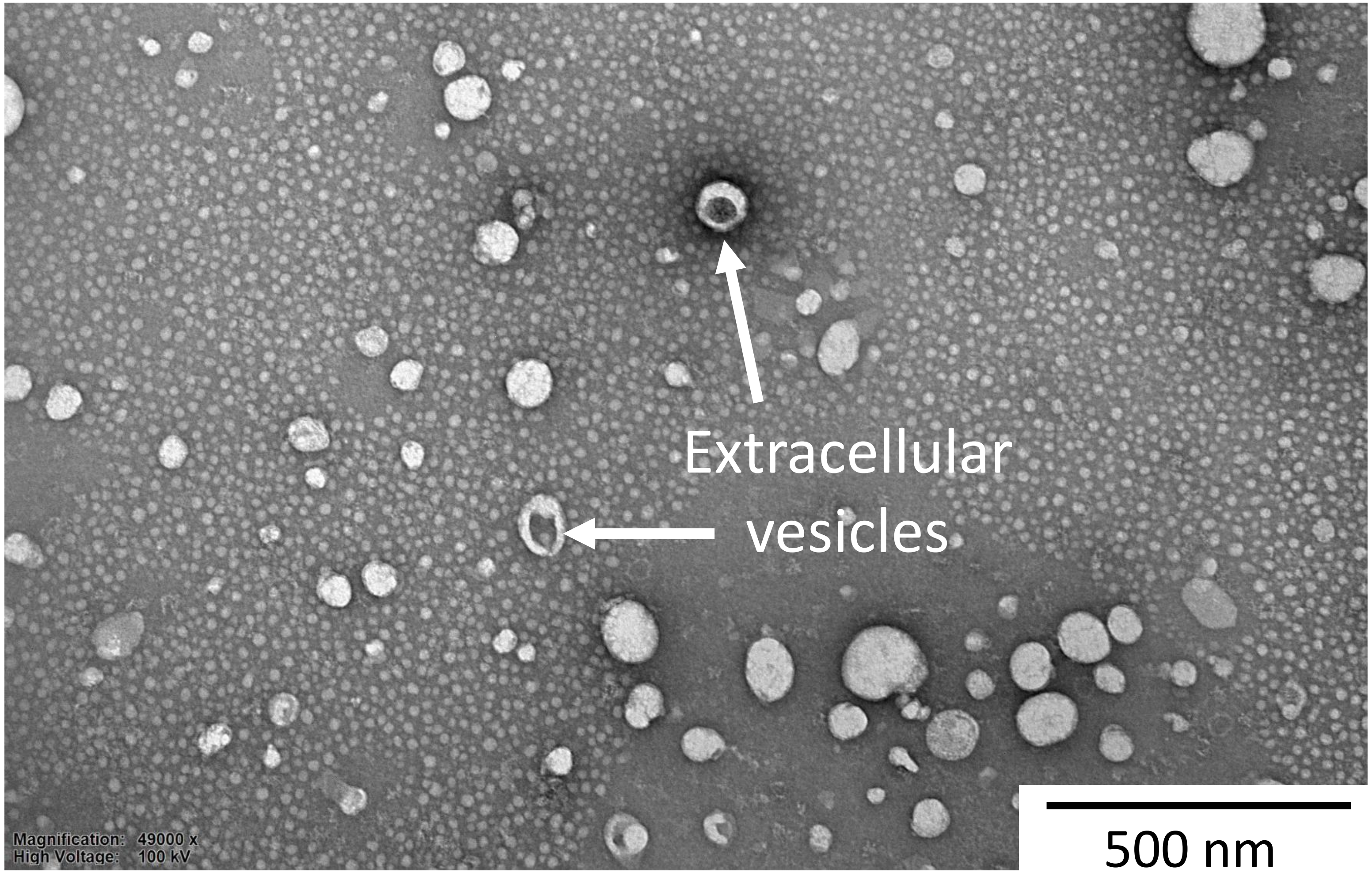
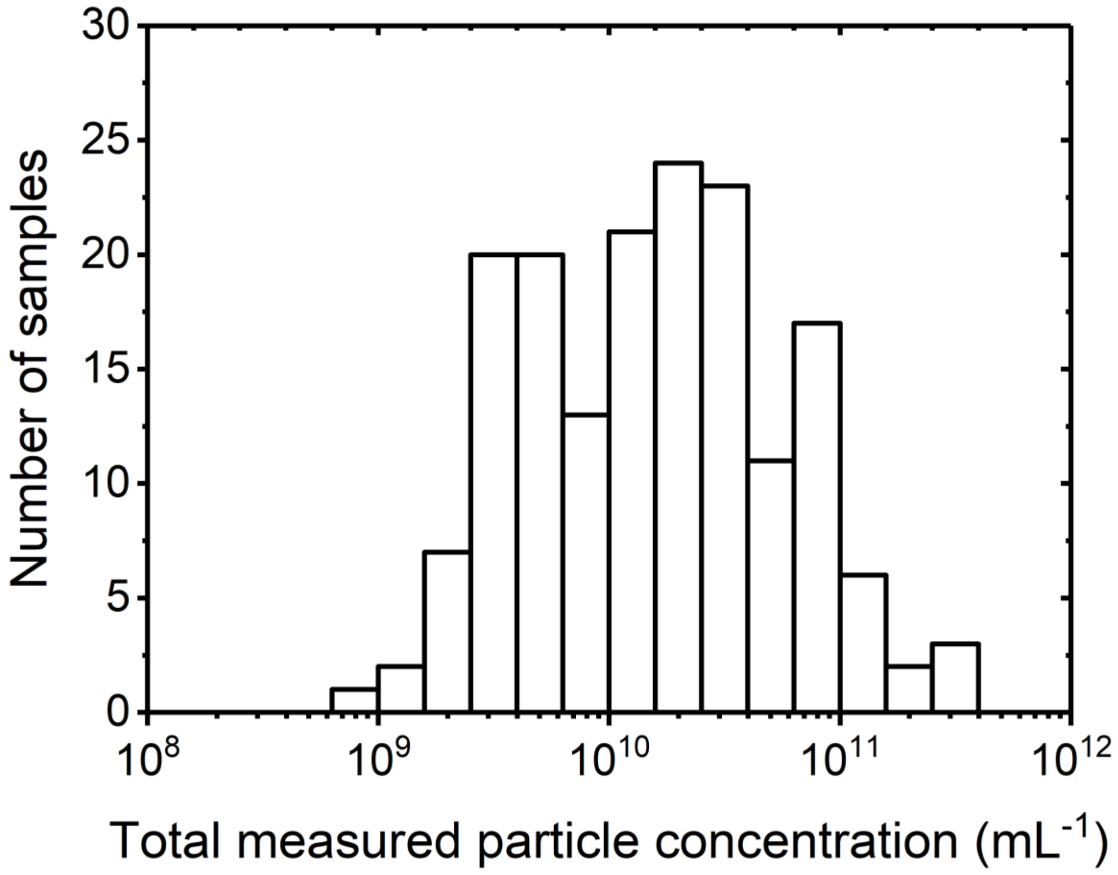
The dilution paradox for EVs in blood plasma

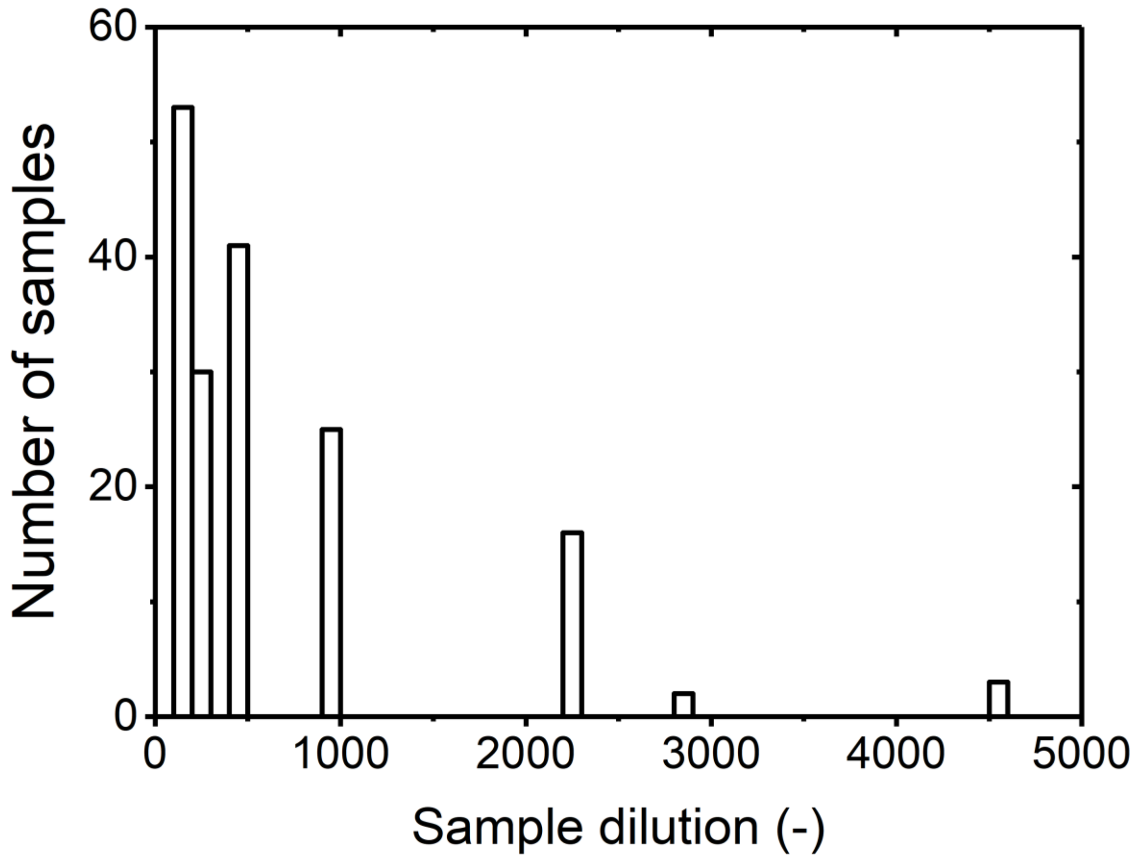


Sub-µm particle concentrations differ 100-fold in plasma from different donors in studies



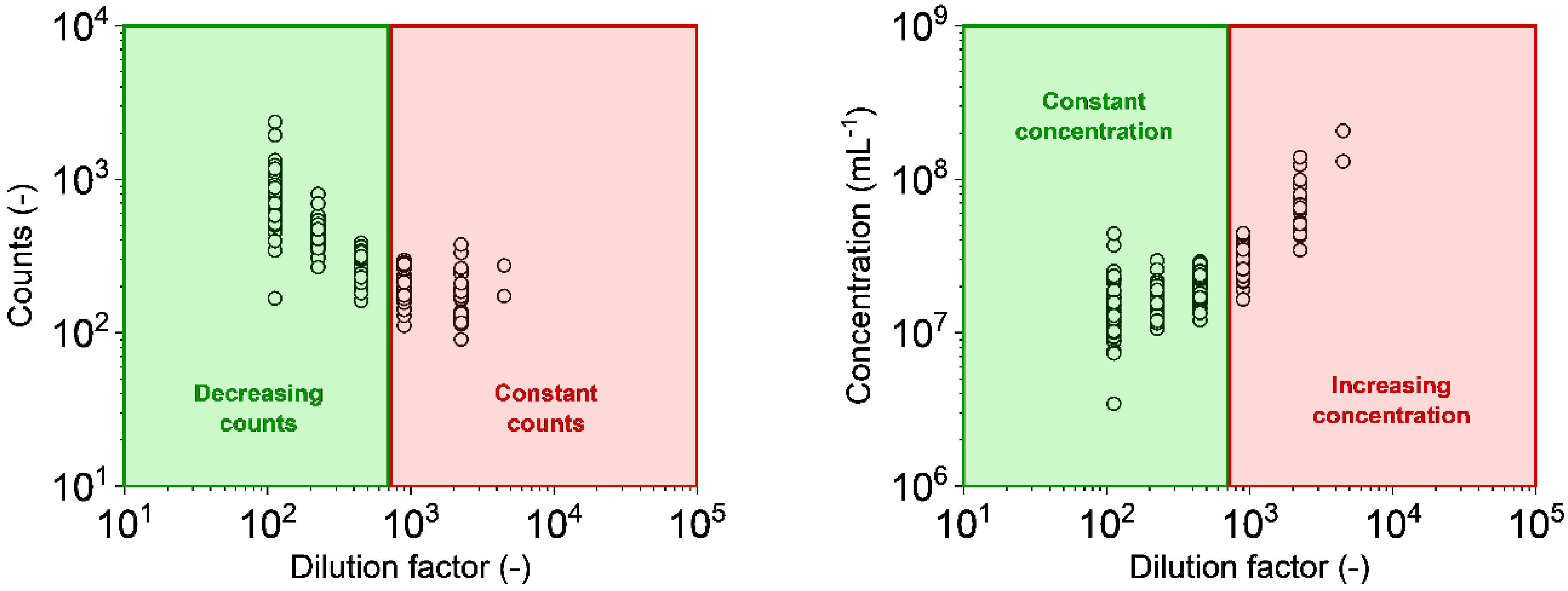
Data from Gasecka et al. *J Thromb Haemost* 2020
Measured with Apogee A60-Micro using scatter triggering

How we dealt with 100-fold difference in particle concentrations between donors



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

Custom dilutions affect measured EV concentrations



CD45+ EVs
Image courtesy of Joyce Rops (unsubmitted)

Custom dilutions lead to unreliable EV concentrations for part of data in our studies

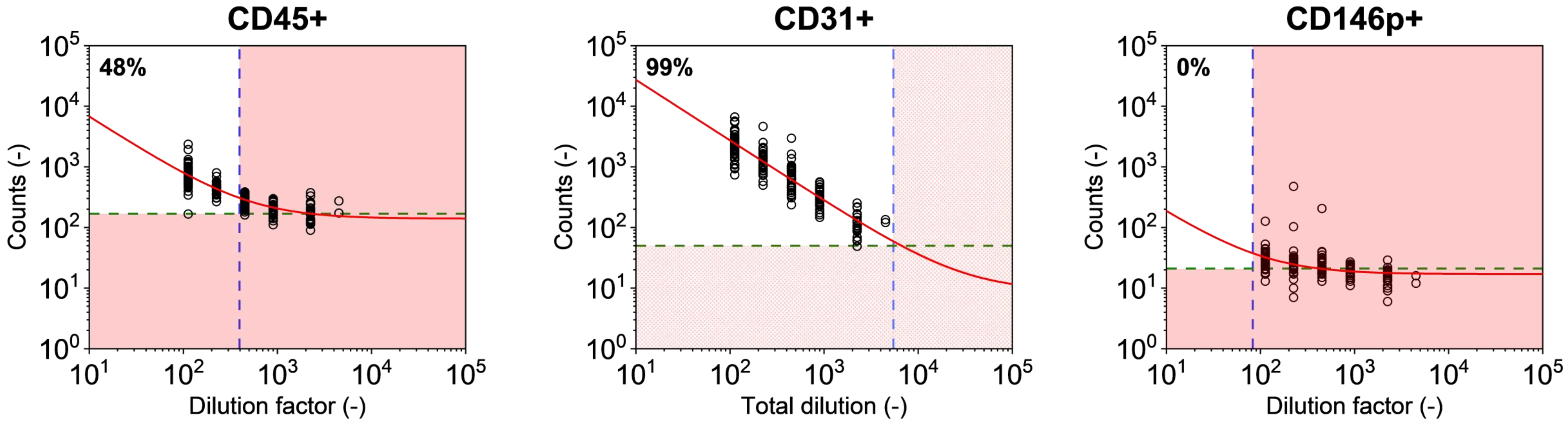


Image courtesy of Joyce Rops (unsubmitted)

The dilution paradox for EVs in blood plasma

- Reliable scatter detection of EVs in plasma requires sample dilutions that are often too high to detect sufficient EVs by fluorescence

Thus…

- After 15 years of optimizing EV flow cytometry
  - Light scattering calibration
  - Swarm detection
  - Refractive index determination with Flow-SR
  - MIFlowCyt-EV
  - EV flow cytometry compendium
  - Clinical research studies

  we partly measured background fluorescence ☹
- Hence, we will refine our assay ☺

Outline

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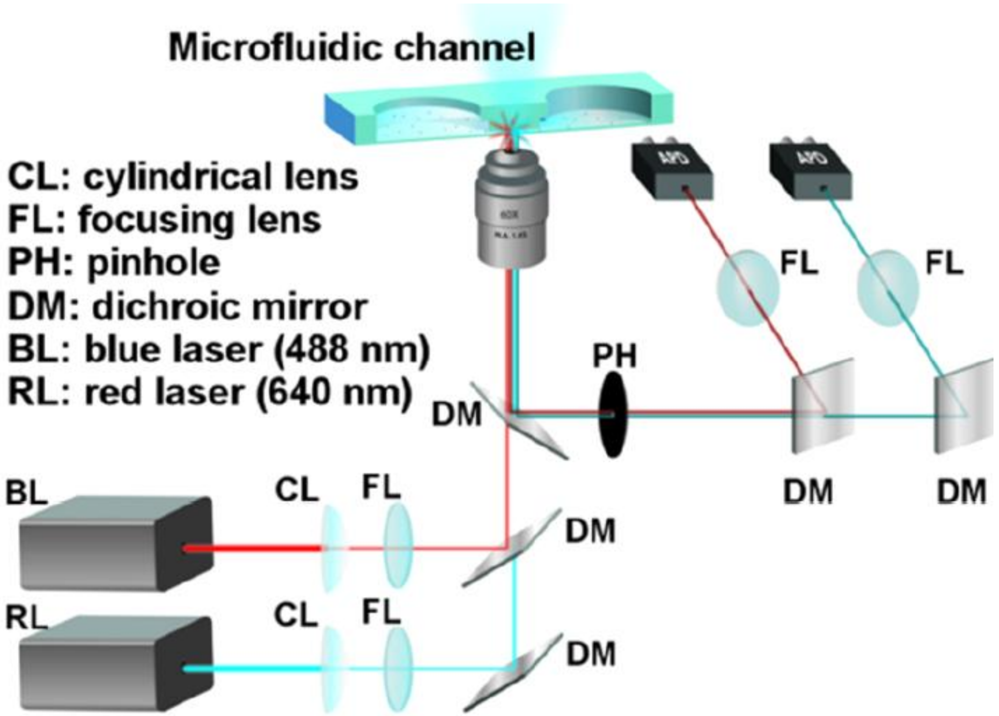
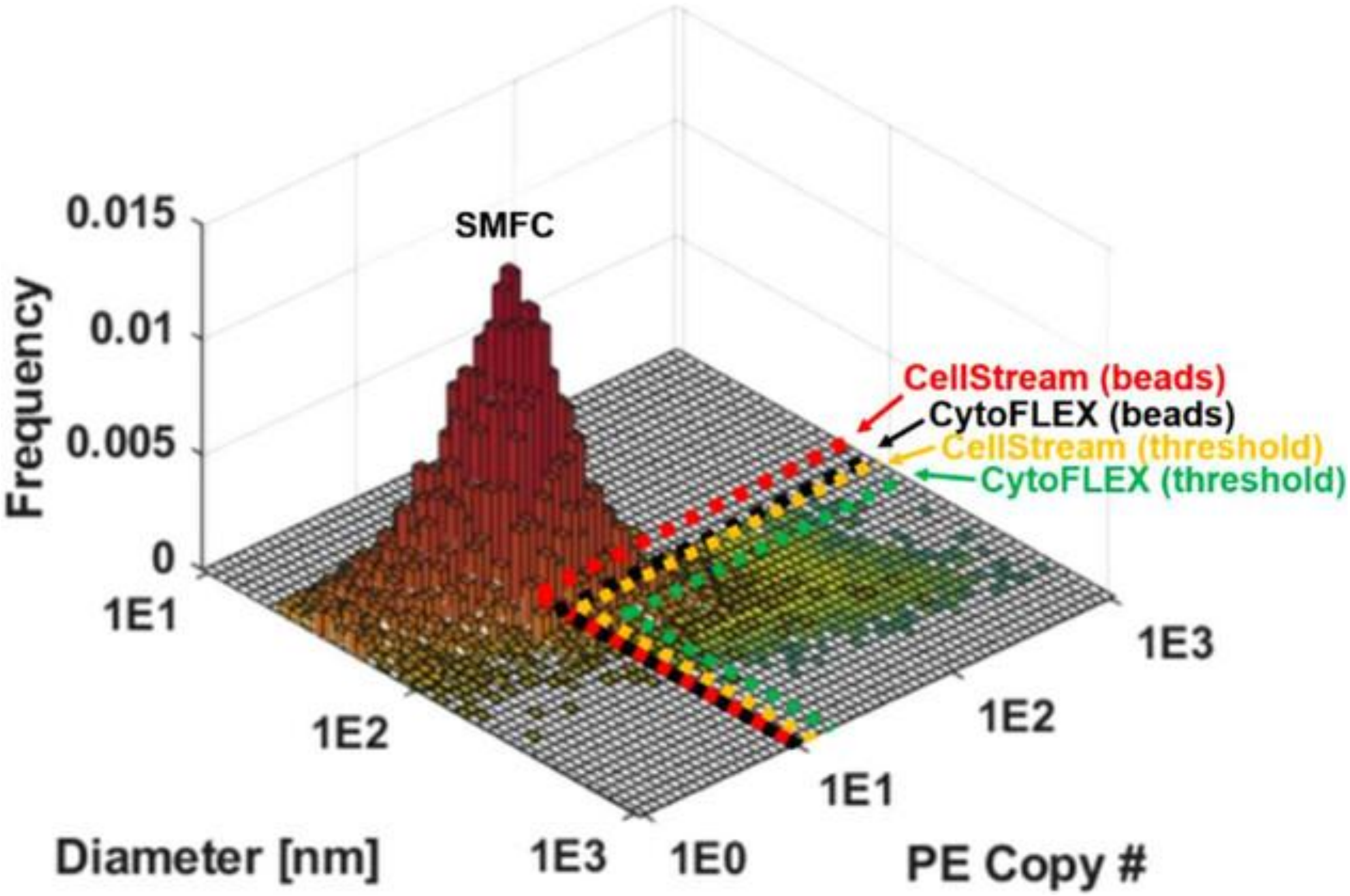
What Swedish churches and the field of EV flow cytometry have in common?

Source: nos.nl

What’s next





Andronico et al. *Anal Chem* 2021
Kim et al. *J Extracell Vesicles* 2024

Summary

- Calibrate!
  - Improve data interpretation
  - Achieve data reproducibility
- What’s possible: reliable 100 nm EV detection
- What’s challenging
  - Report details: MIFlowCyt-EV
  - Use fixed sample dilution
- What’s next: specialized flow-based EV devices

Question 2.2

- Who is considering performing flow cytometry experiments on extracellular vesicles?

DOI: 10.1002/jev2.12299

REVIEW ARTICLE

A compendium of single extracellular vesicle flow cytometry

Joshua A. Welsh[1] | Ger J. A. Arkesteijn[2] | Michel Bremer[3] | Michael Cimorelli[4,5,6] | Françoise Dignat-George[7,8] | Bernd Giebel[3] | André Görgens[3,9,10] | An Hendrix[11] | Martine Kuiper[4,5,12,13] | Romaric Lacroix[7,8] | Joanne Lannigan[14] | Ton G. van Leeuwen[4,12,15,16] | Estefanía Lozano-Andrés[2] | Shoaib Rao[3] | Stéphane Robert[7,8] | Leonie de Rond[4,5,12] | Vera A. Tang[17] | Tobias Tertel[3] | Xiaomei Yan[18] | Marca H. M. Wauben[2] | John Nolan[19,20] | Jennifer C. Jones[1] | Rienk Nieuwland[4,5,15] | Edwin van der Pol[4,5,12,15,16]

This summer school is endorsed by
INTERNATIONAL SOCIETY *for* EXTRACELLULAR VESICLES

Amsterdam
Vesicle Center

EV Flow Cytometry Summer School

July 6th to July 10th, 2026

Learn (almost) everything we know about EV flow cytometry,
by Jillian Bracht, Rienk Nieuwland and Edwin van der Pol

More information: c.m.hau@amsterdamumc.nl
Limited places

EVcount

INPUT

Extracellular vesicle samples

SERVICE

Labelling

Flow cytometry

Data analysis

OUTPUT

Cellular origin, concentration and diameter of extracellular vesicles

- Calibrated flow cytometry data files
- Standardized measurement report
- Completed MIFlowCyt-EV template

CONTACT INFORMATION

info@evcount.com www.evcount.com

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- Amsterdam University Medical Center
  - Biomedical Engineering & Physics
  - Laboratory of Experimental Clinical Chemistry

Amsterdam UMC

EMPIR EURAMET

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Amsterdam Vesicle Center

NWO Netherlands Organisation for Scientific Research

Hartstichting

METVES II

- More information
  - edwinvanderpol.com
  - evcount.com
  - evflowcytometry.org
  - exometry.com
  - isev.org
  - metves.eu
  - vesiclecenter.com