The history of extracellular vesicle detection by flow cytometry

Edwin van der Pol

October 19th, 2022

Conflict of interest

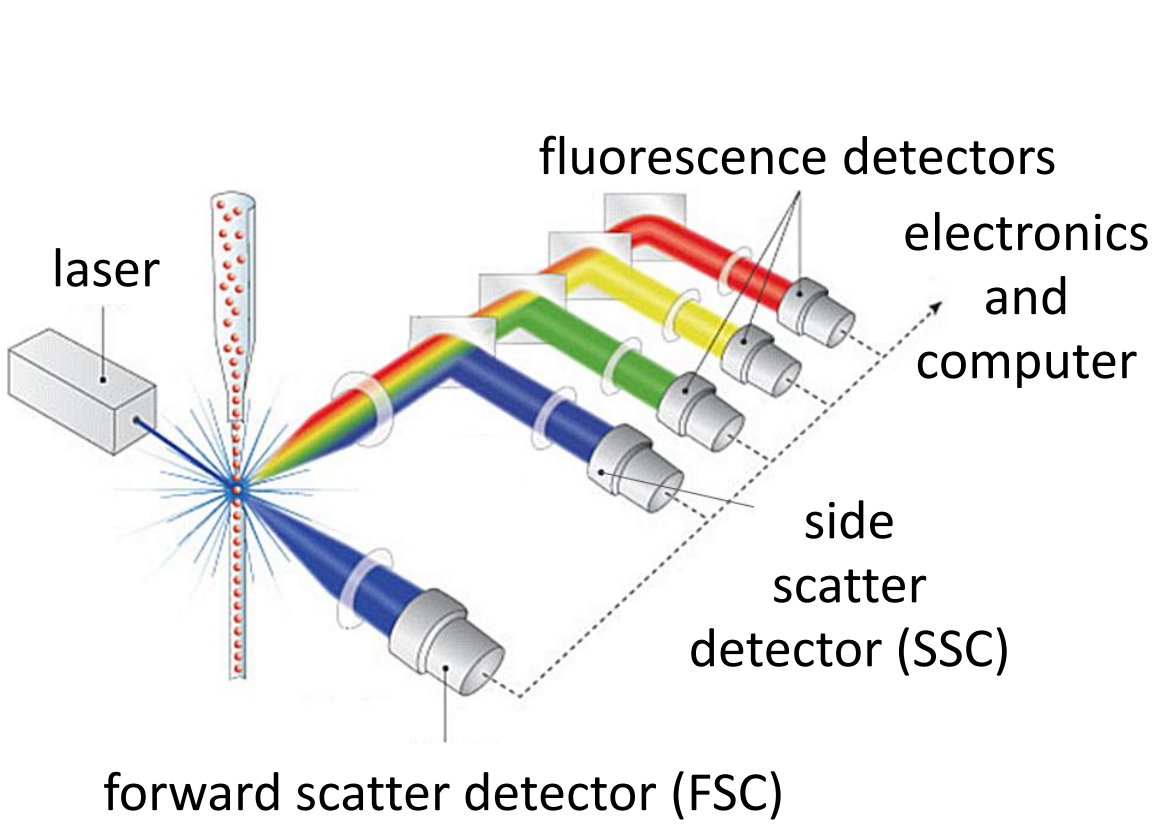
- Cofounder and shareholder of EXOMETRY

Outline

- Flow cytometry basics: parler la même langue

- A history of… *extracellular vesicle detection*
- The future of… *by flow cytometry*

Flow cytometer trigger detector(s) and threshold(s)



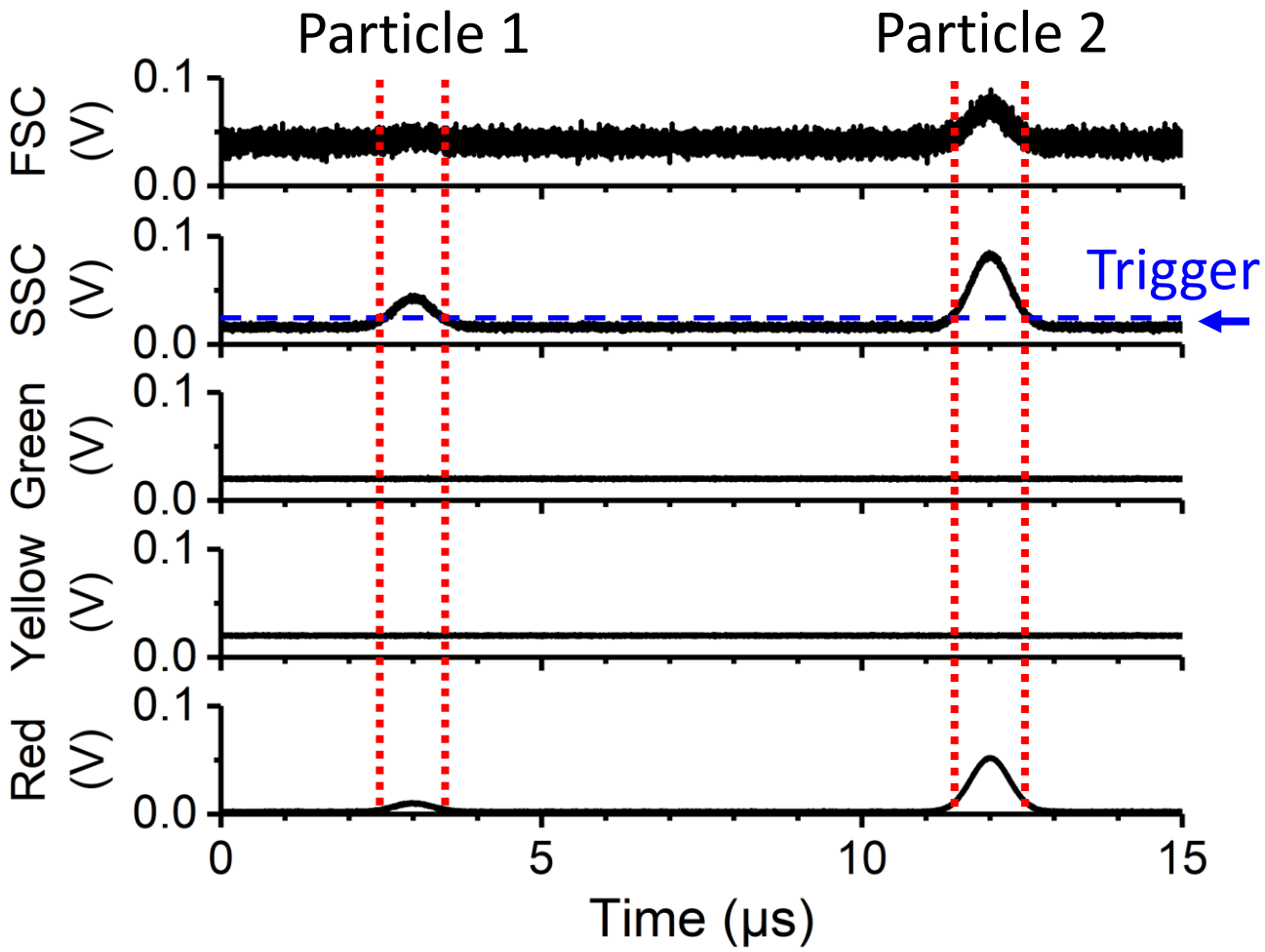
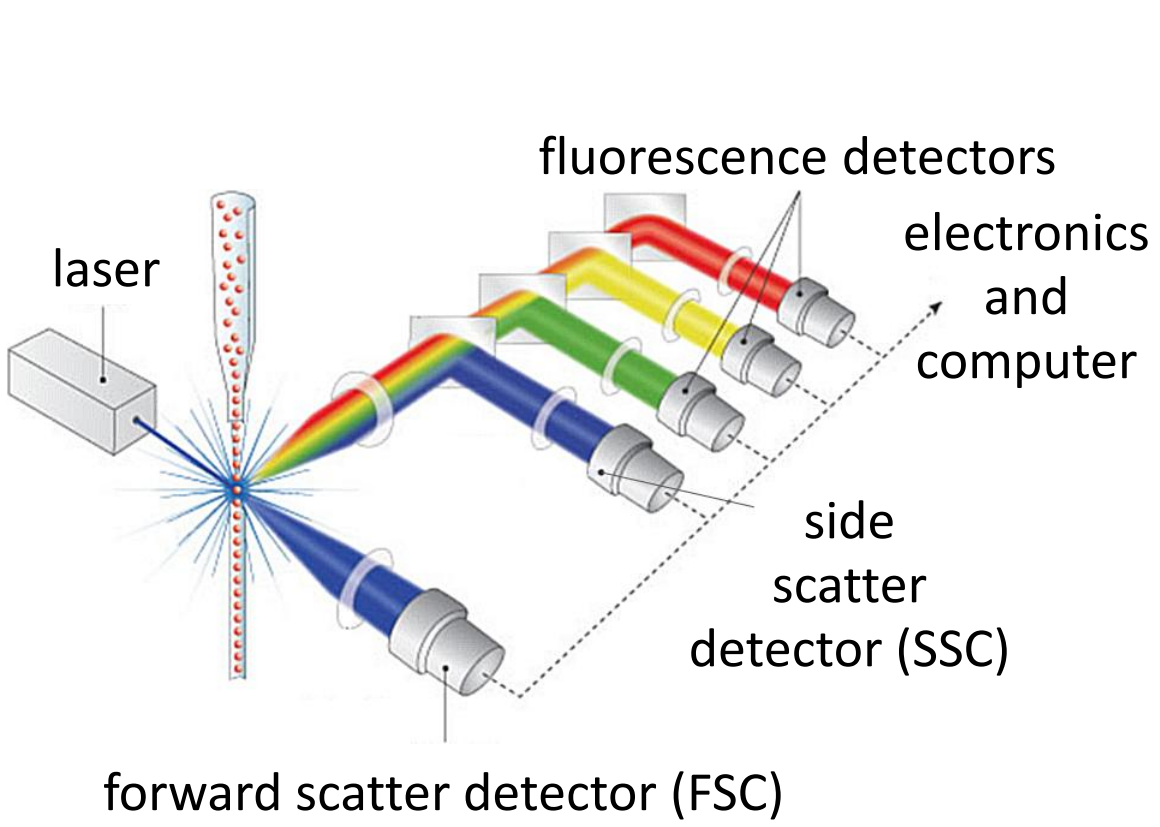


Image: semrock.com

Flow cytometer trigger detector(s) and threshold(s)



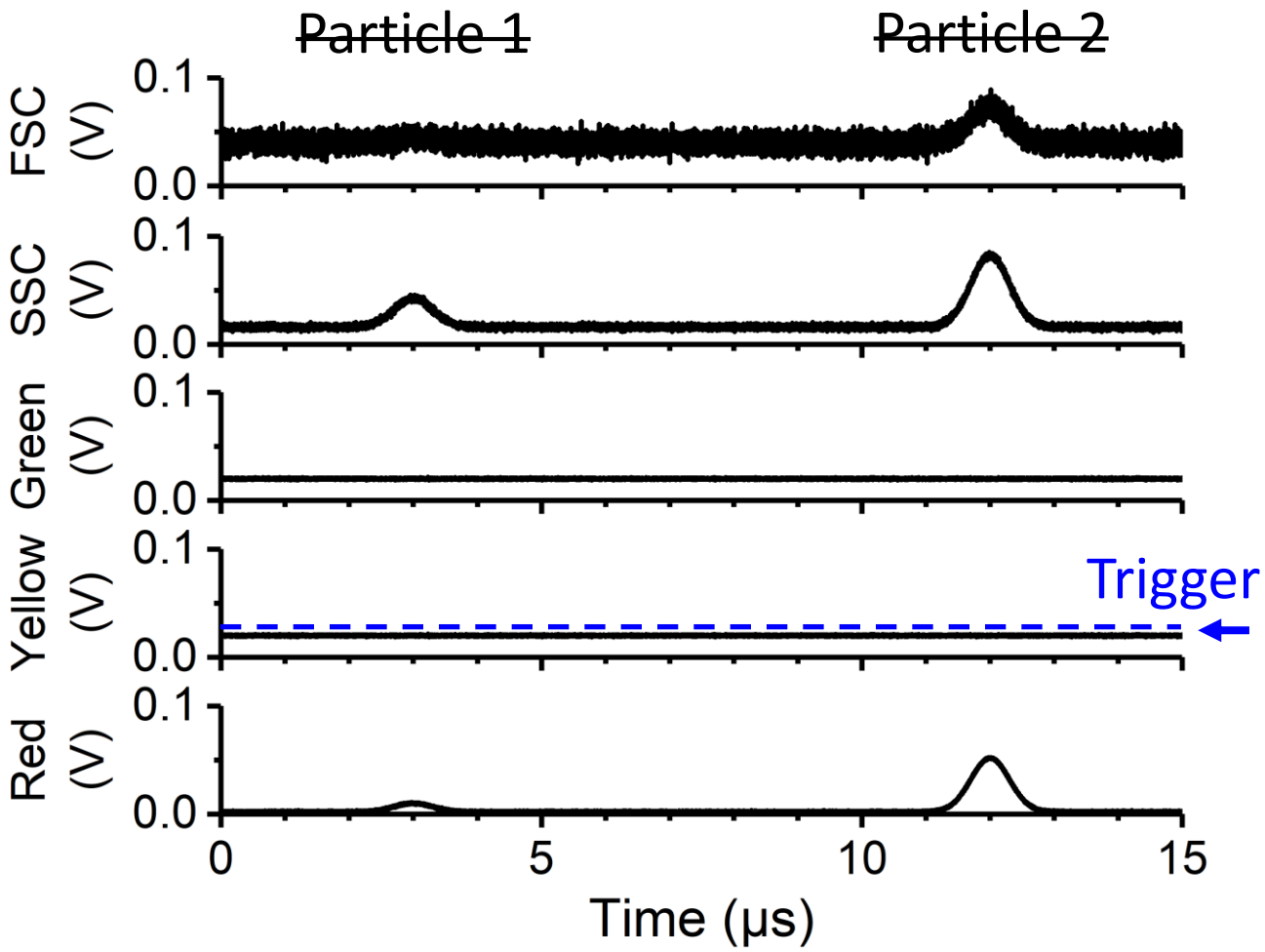
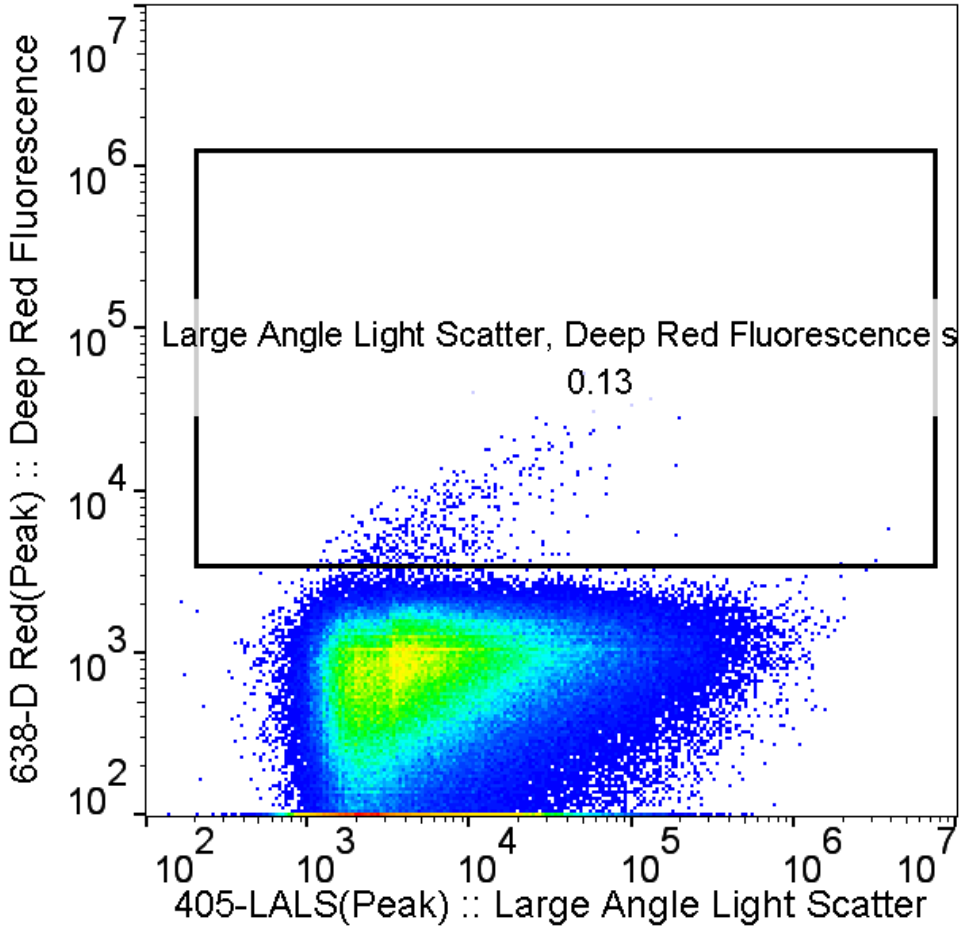


Image: semrock.com

Example of raw flow cytometry data

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



Outline

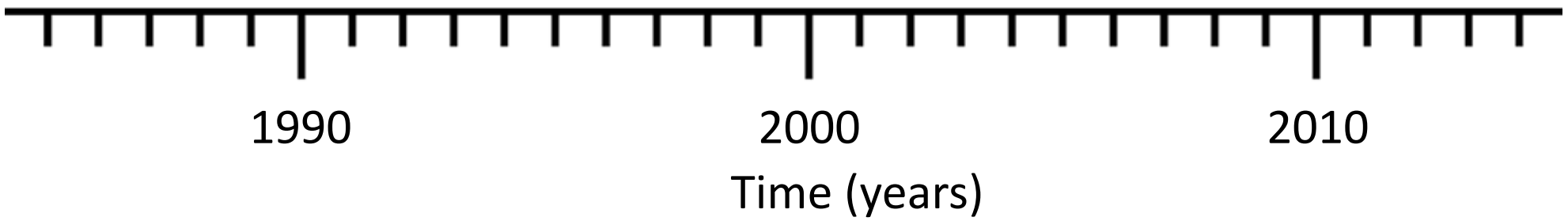
✅ Flow cytometry basics: parler la même langue

- A history of… *extracellular vesicle detection*
- The future of… *by flow cytometry*

A history of extracellular vesicle detection by flow cytometry

Abrams et al.
Forward scatter (a.u.)
∝ particle size

Sims et al.
Detection of “100-nm platelet-derived microparticles”



Disclosure: this story is incomplete

EVs: extracellular vesicles; MESF: molecules of equivalent soluble fluorophore

Activation of cellular responses

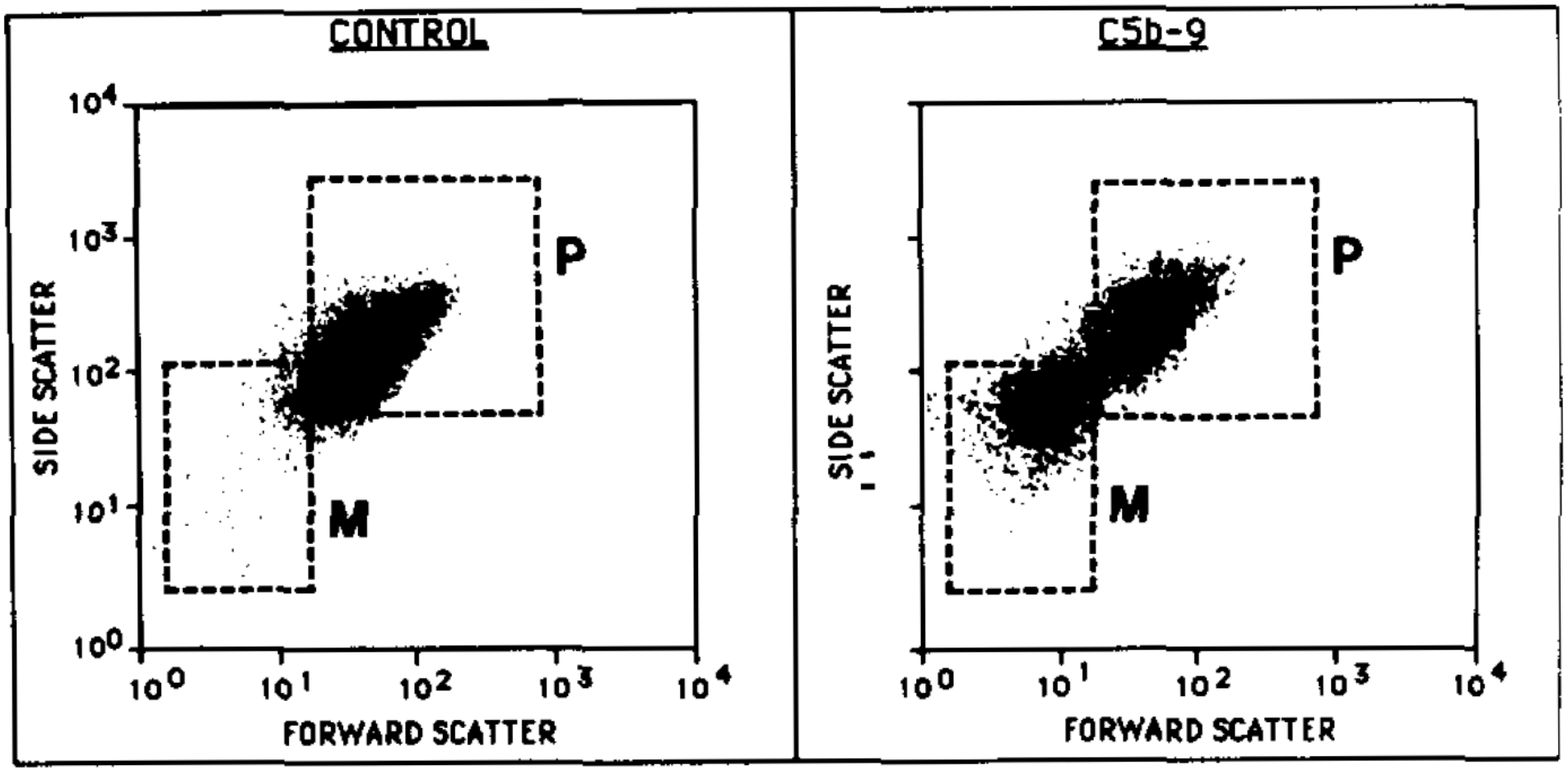


Figure adopted from Sims et al. *J Biol Chem* 1988
Glycoprotein Ib-positive events

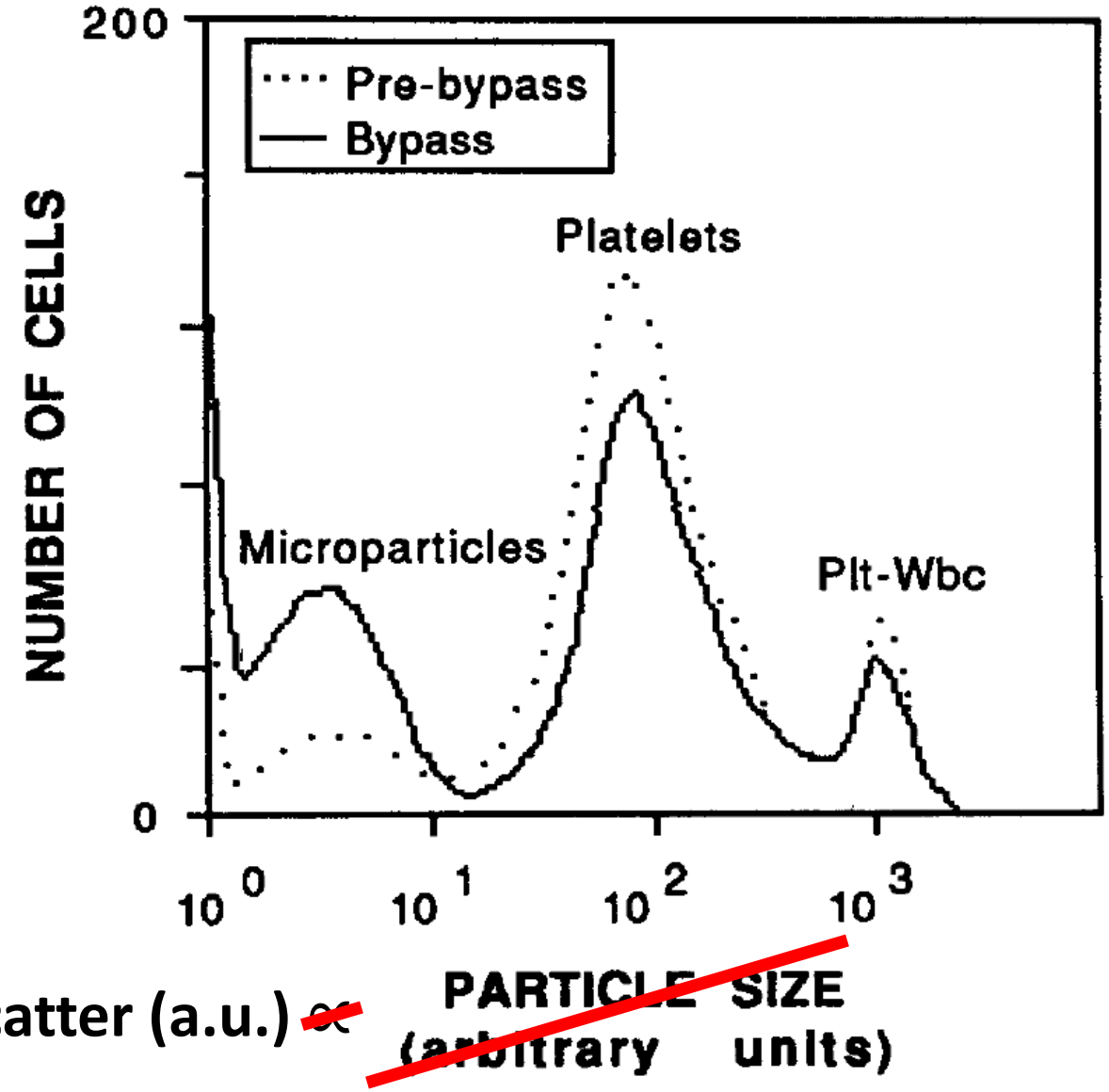


Figure from Abrams et al *Blood* 1990

Detection of “100-nm platelet-derived microparticles”

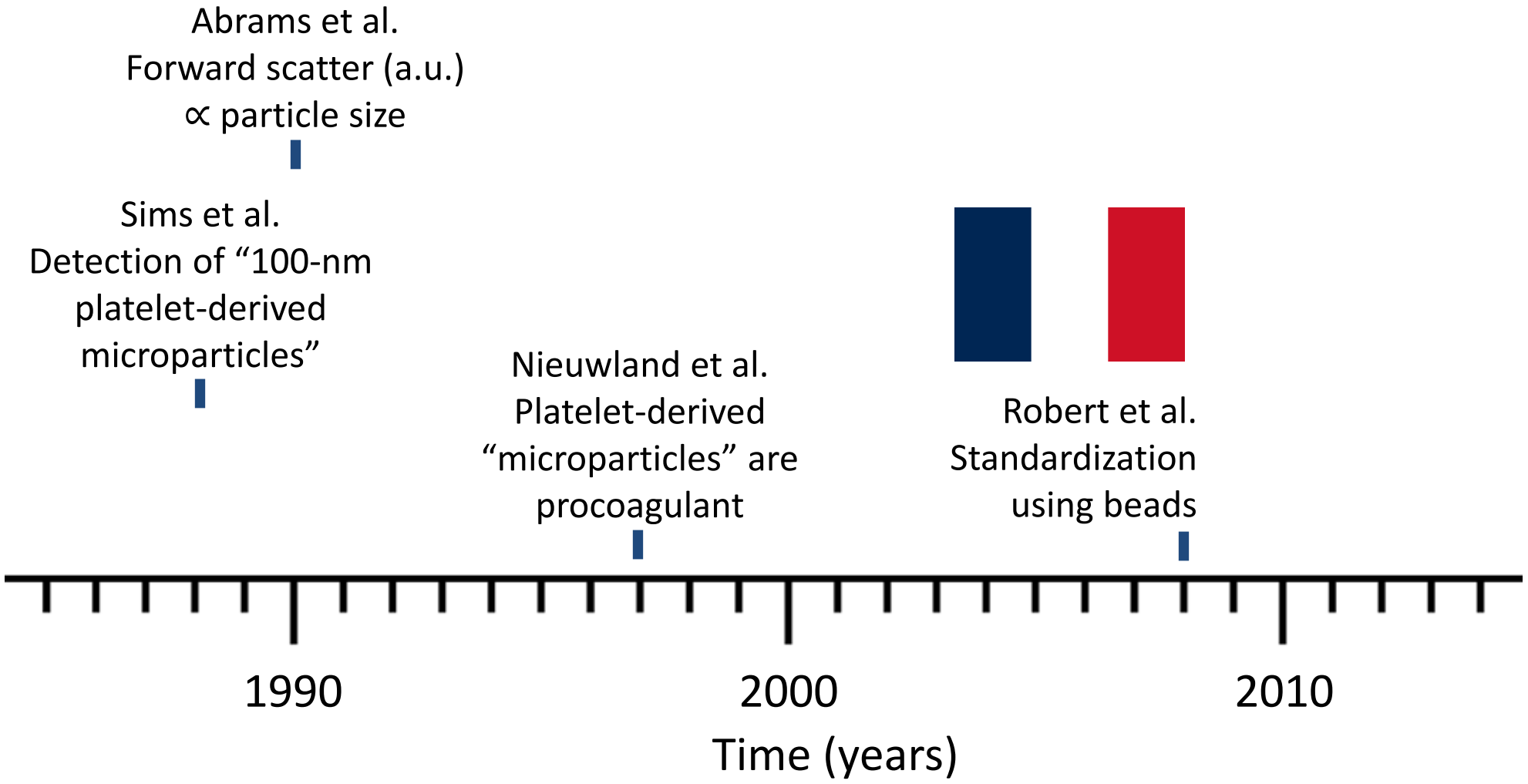
- Similar forward scatter for “microparticles” and 100-nm fluorescence beads

Based on this calibration, the average size of the platelet-derived microparticles was approximately 0.1 μm in diameter, similar to the 0.05 to 0.1-μm vesicles we have previously detected in electron micrographs of C5b-9-treated platelets (7).

Flow cytometer: BD FACStar

Image from spwindustrial.com

A history of extracellular vesicle detection by flow cytometry



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Standardization with polystyrene bead - gates: Megamix

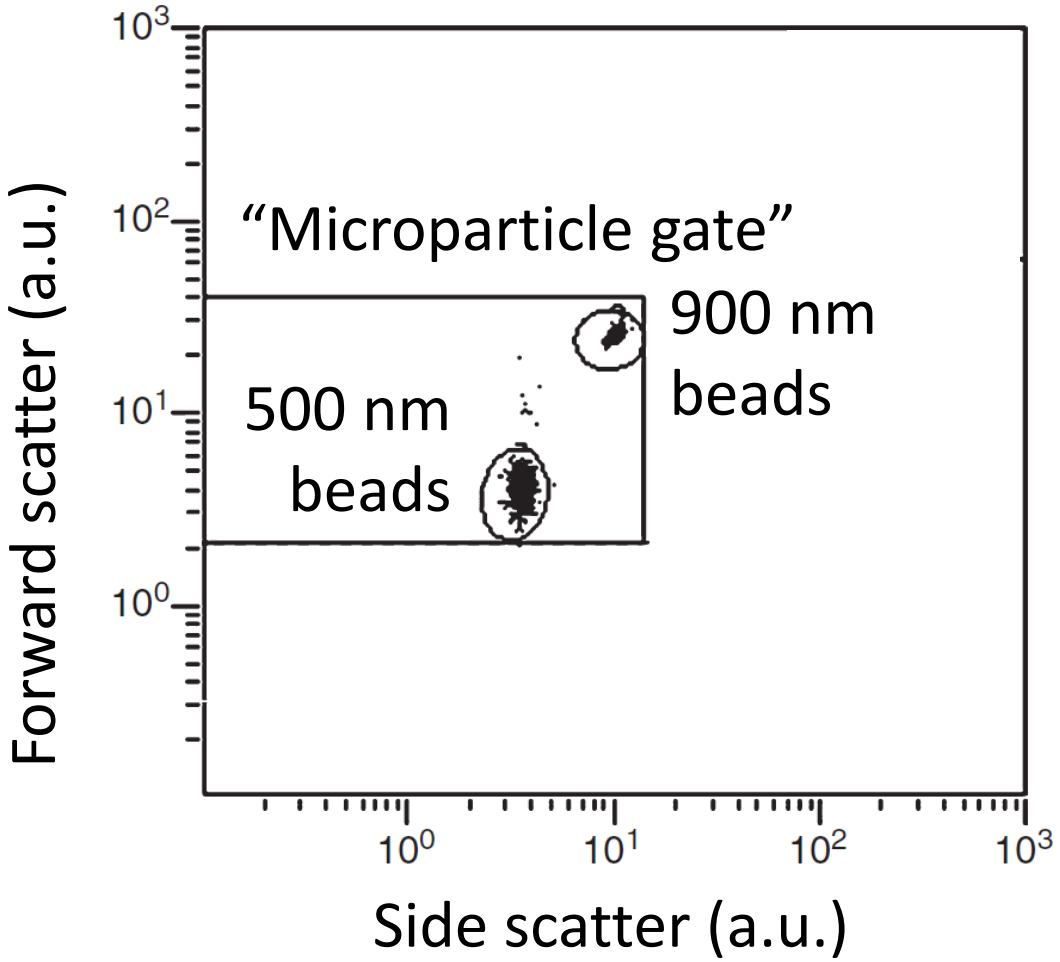
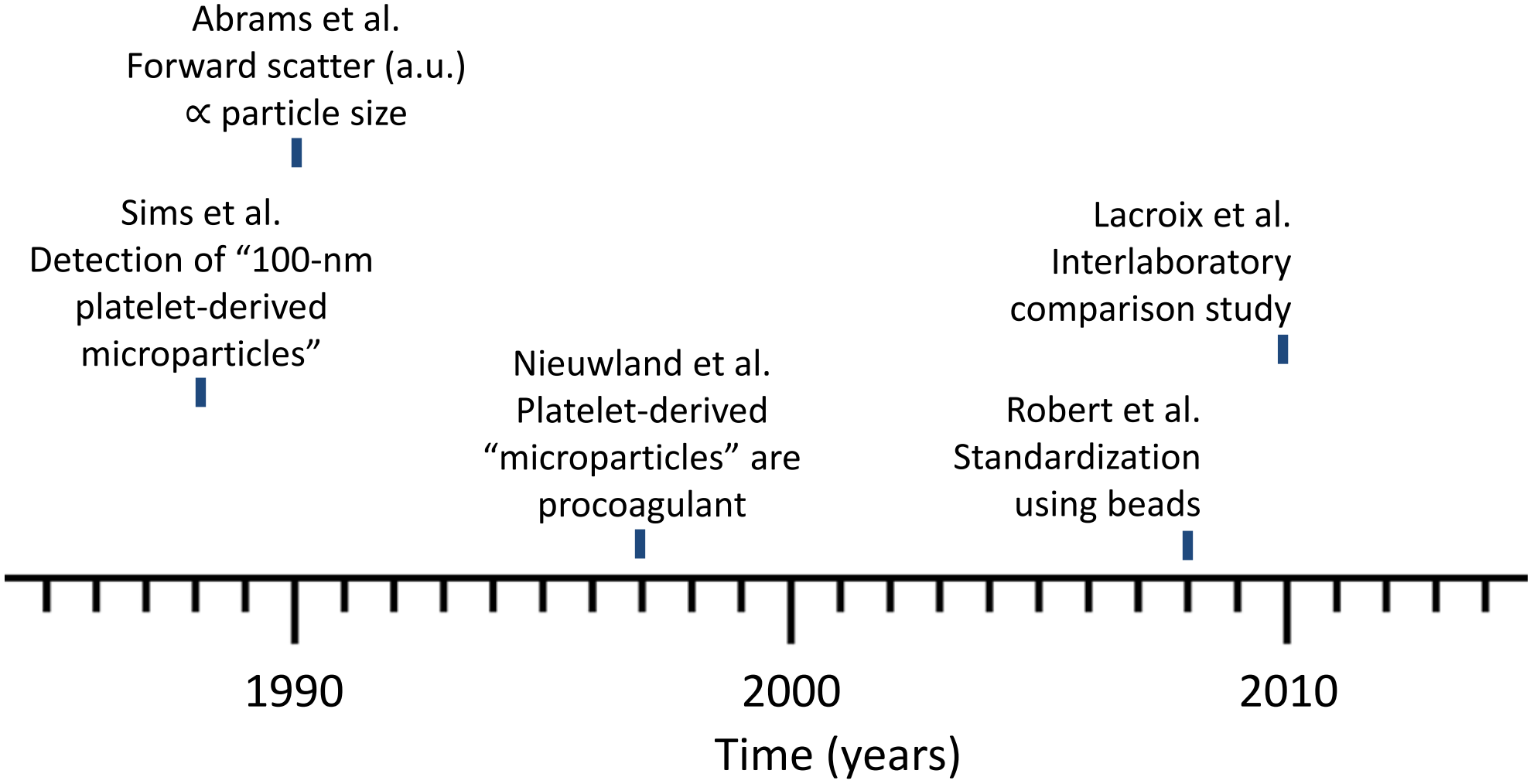


Figure adopted from Robert et al. *J Thromb Haemost* 2008

A history of extracellular vesicle detection by flow cytometry



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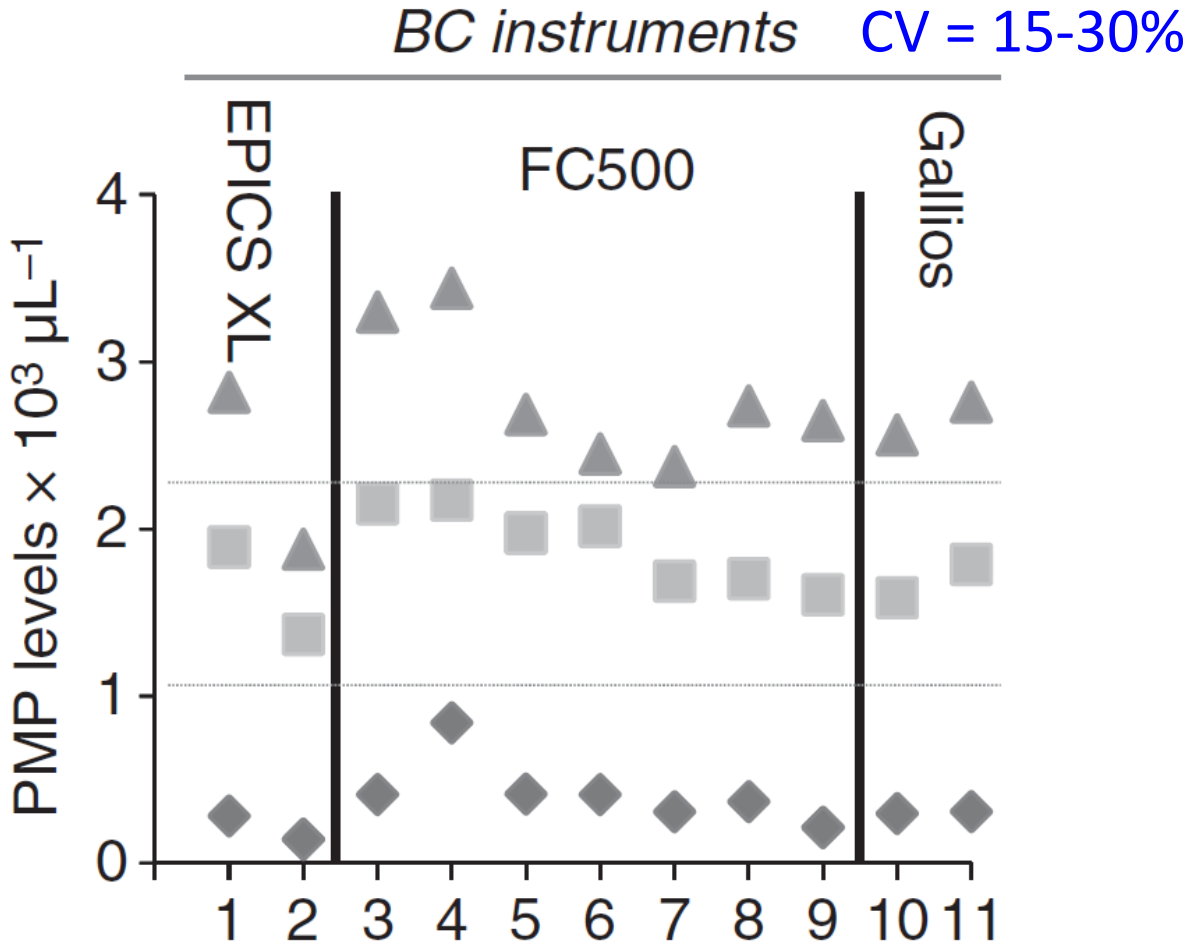
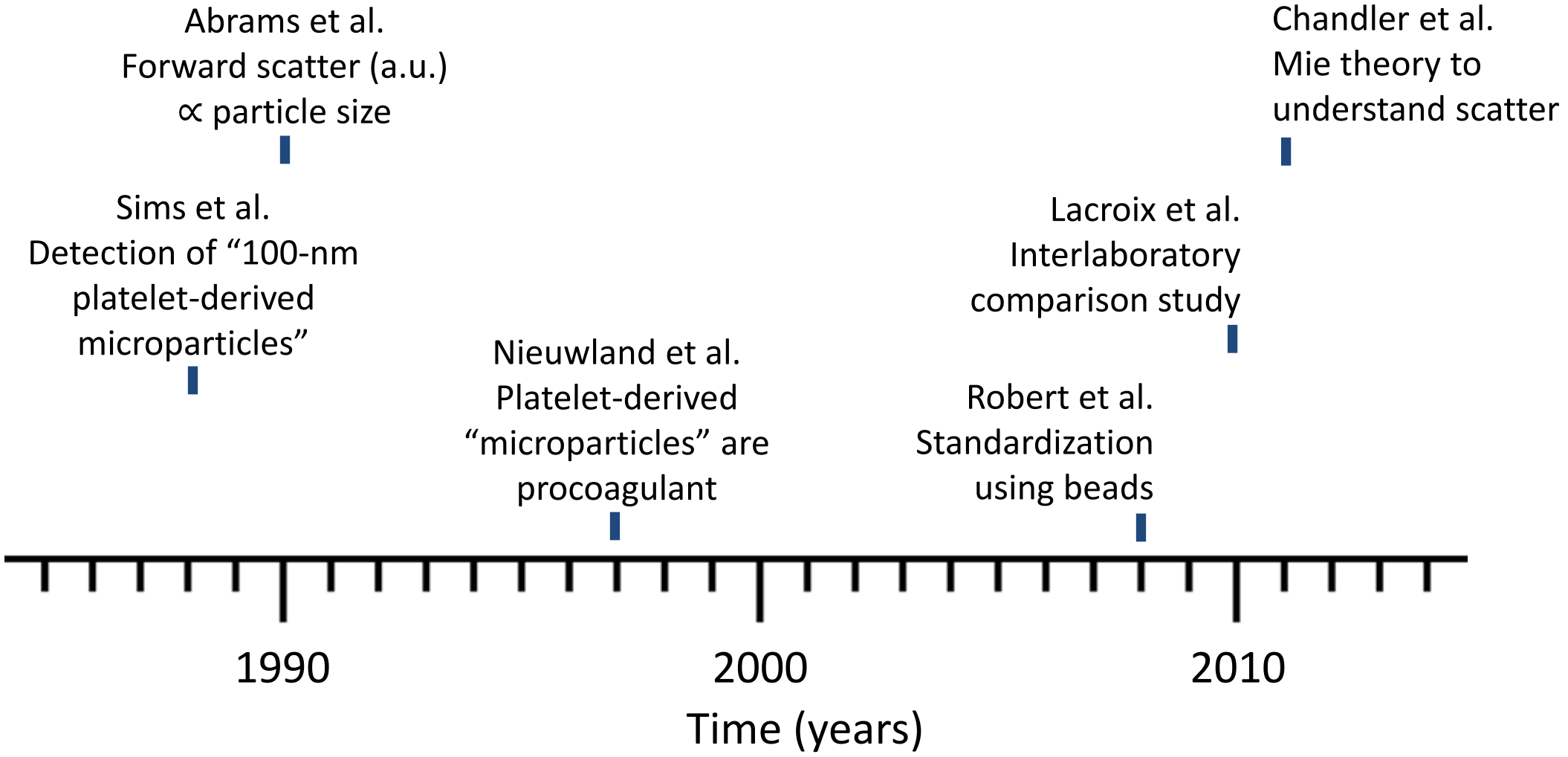
Interlaboratory comparison study: 49 tested FCMs



Figures from Lacroix et al. *J Thromb Haemost* 2010
FCMs: flow cytometers; PMP: platelet microparticles

A history of extracellular vesicle detection by flow cytometry



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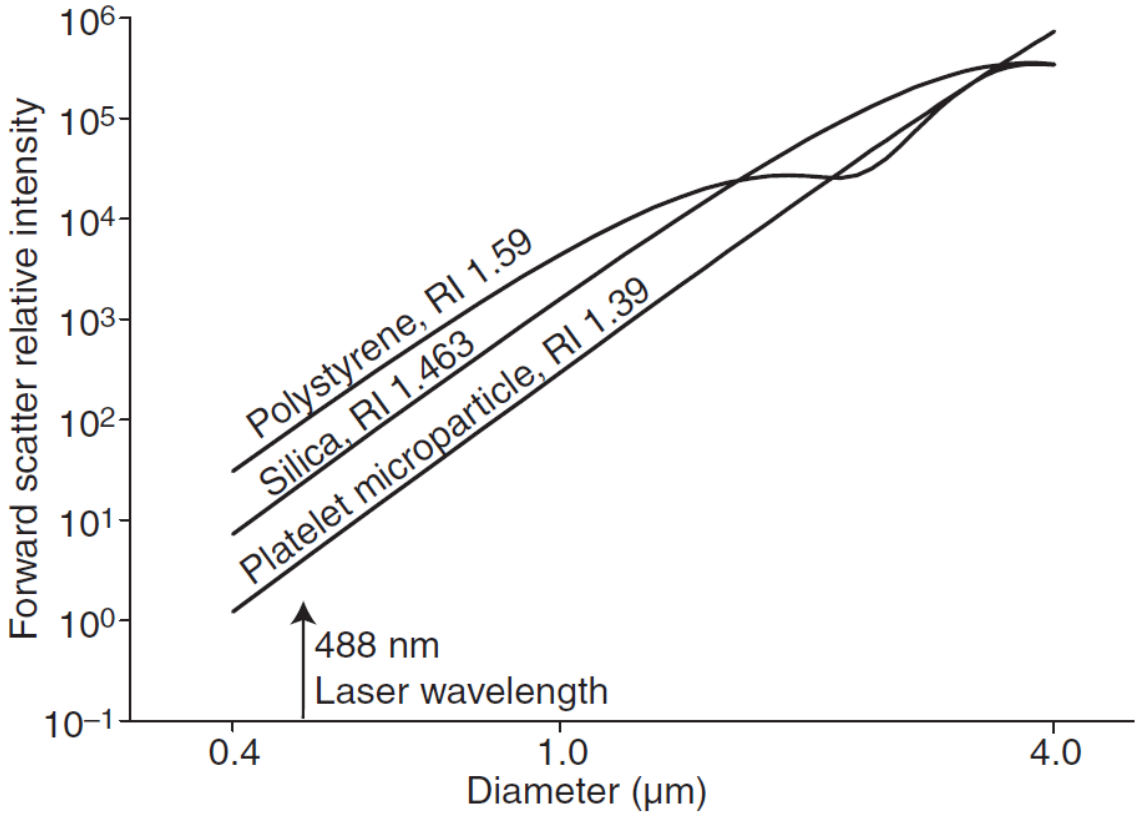
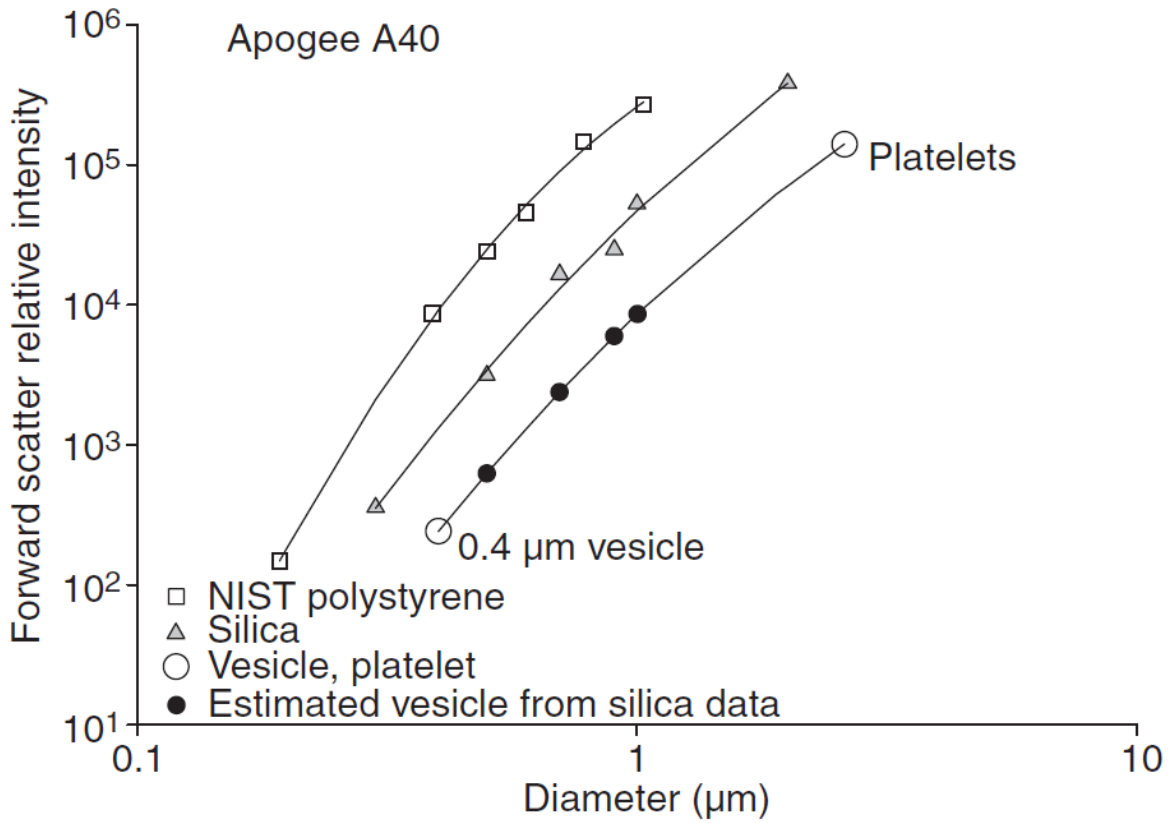
Mie theory to understand EV light scattering

Mie calculation



Measurements + polynomial fit



Figures from Chandler et al. *J Thromb Haemost* 2011

RI: refractive index

Mie theory to understand EV light scattering

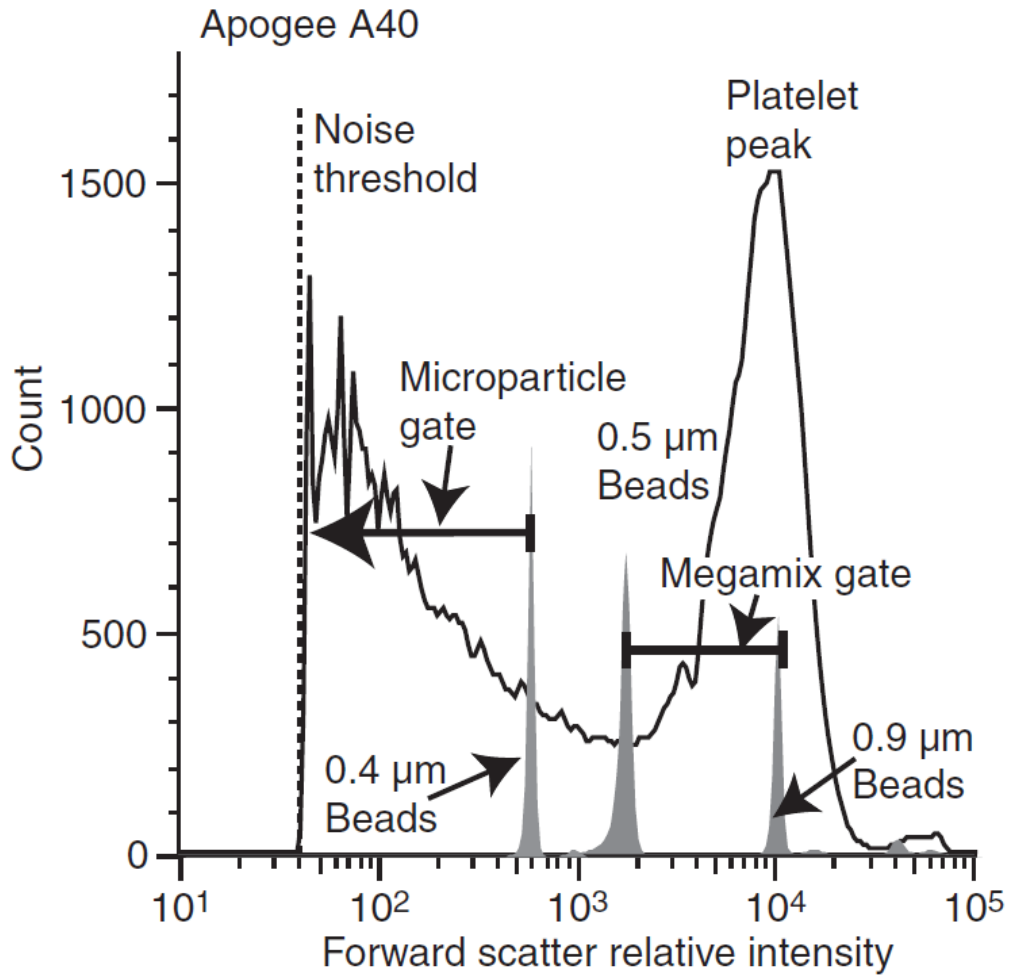


Figure from Chandler et al. *J Thromb Haemost* 2011

A history of extracellular vesicle detection by flow cytometry

Abrams et al.
Forward scatter (a.u.)
∝ particle size

Sims et al.
Detection of “100-nm platelet-derived microparticles”

Nieuwland et al.
Platelet-derived “microparticles” are procoagulant

Robert et al.
Standardization using beads

Lacroix et al.
Interlaboratory comparison study

Chandler et al.
Mie theory to calibrate scatter

van der Pol et al.
Mie theory & swarm detection

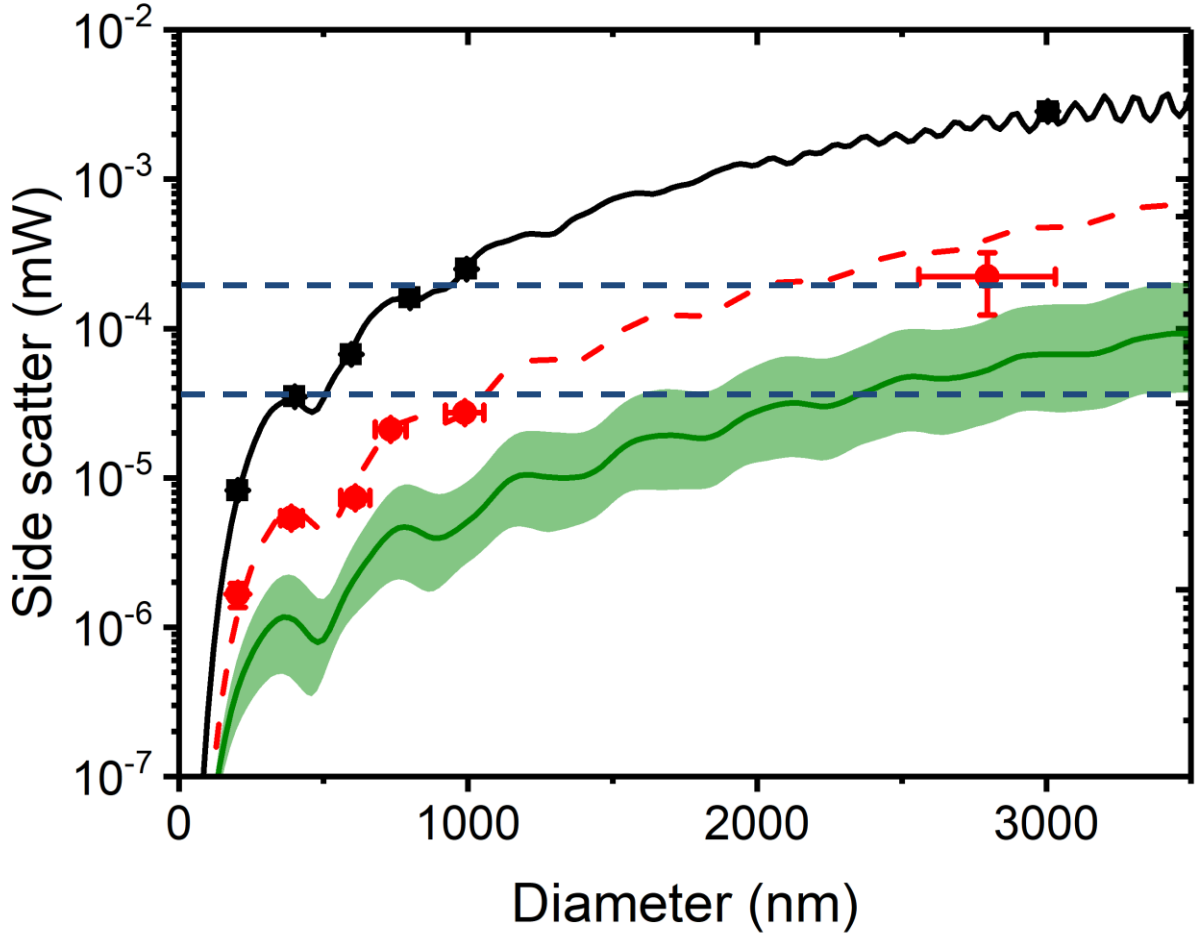
1990 2000 2010

Time (years)

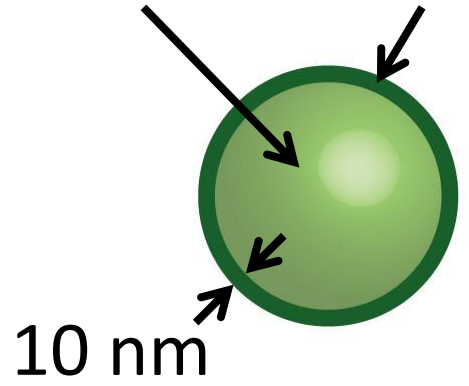
Disclosure: this story is incomplete

EVs: extracellular vesicles; MESF: molecules of equivalent soluble fluorophore

Relate scatter to diameter of EVs by Mie theory



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)



The heart of the flow cytometer

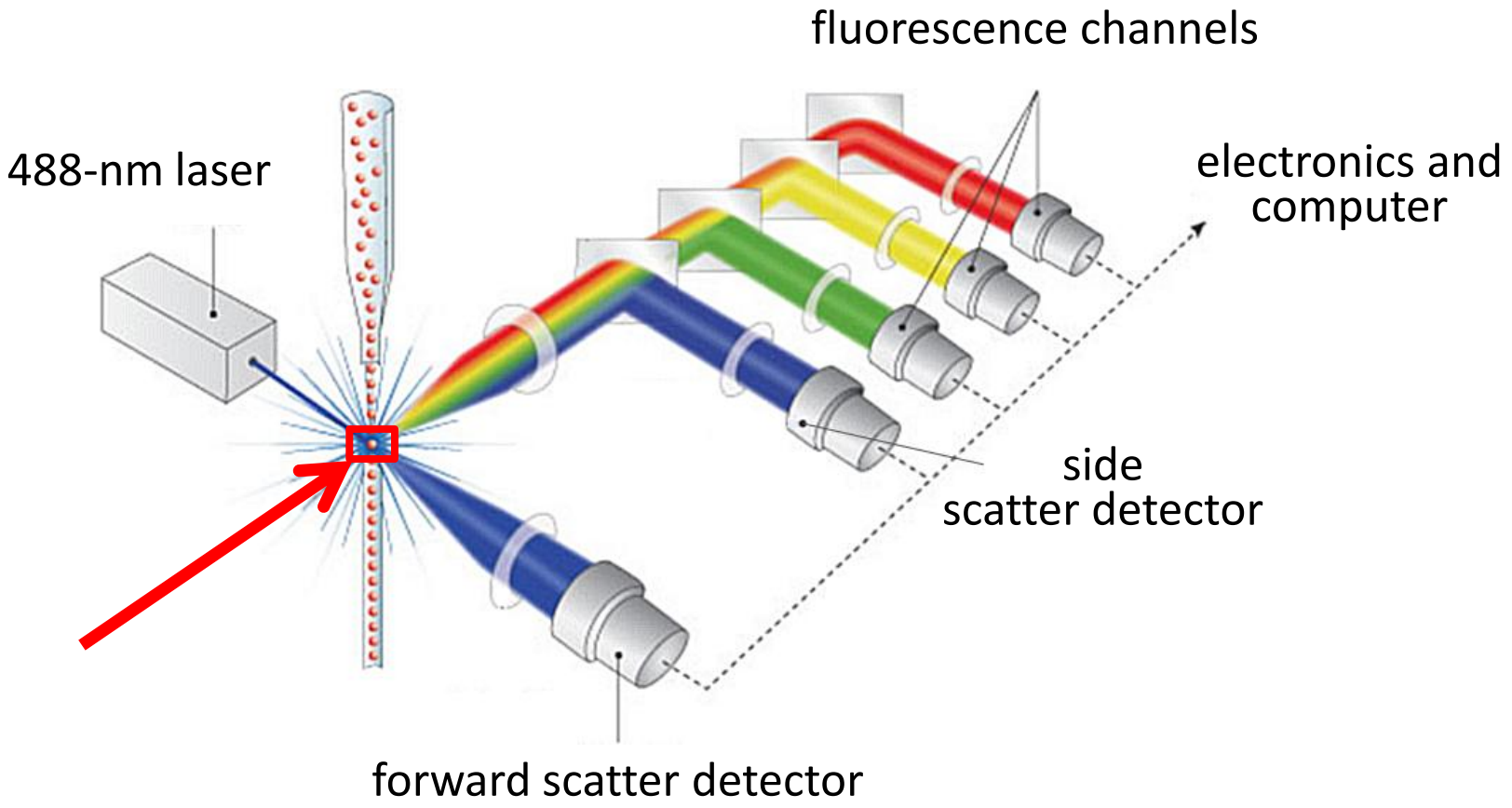
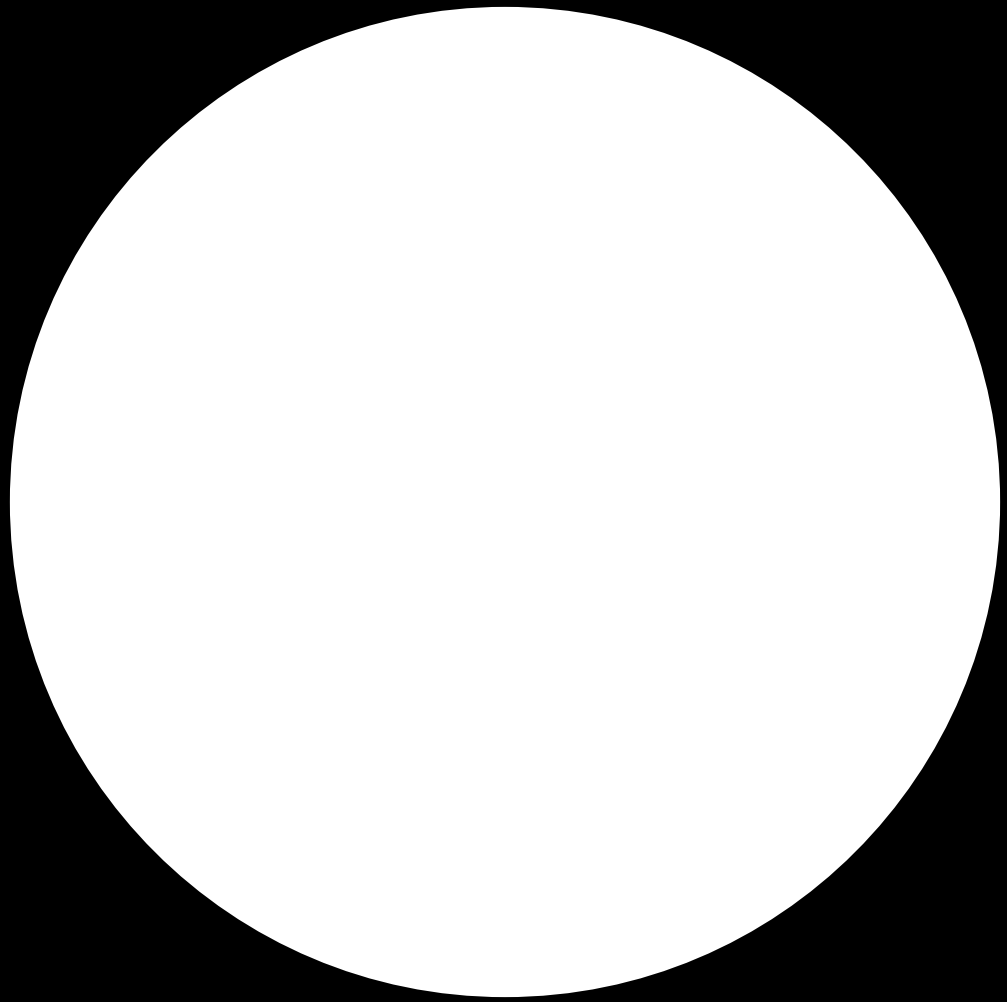
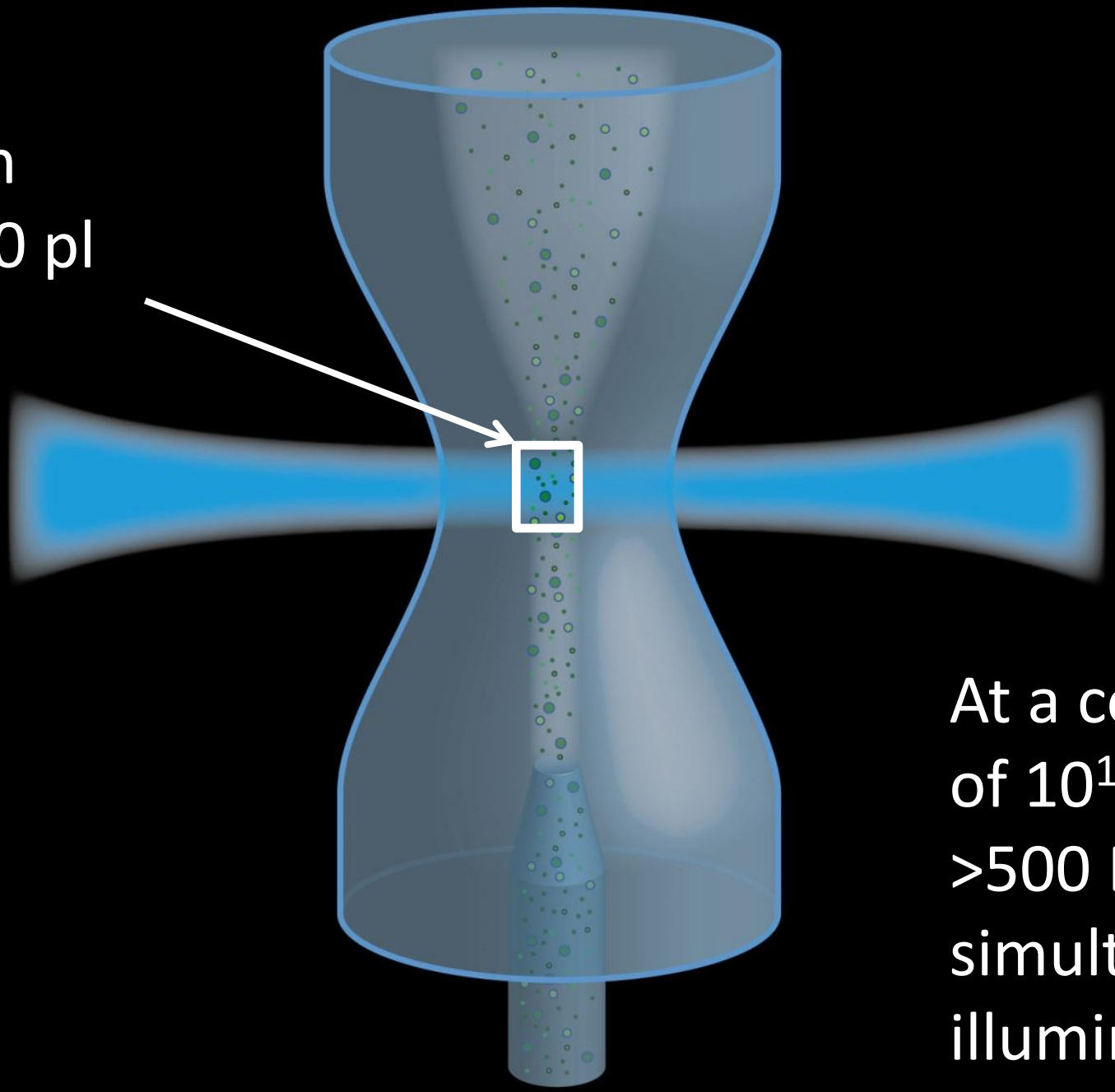


image: semrock.com

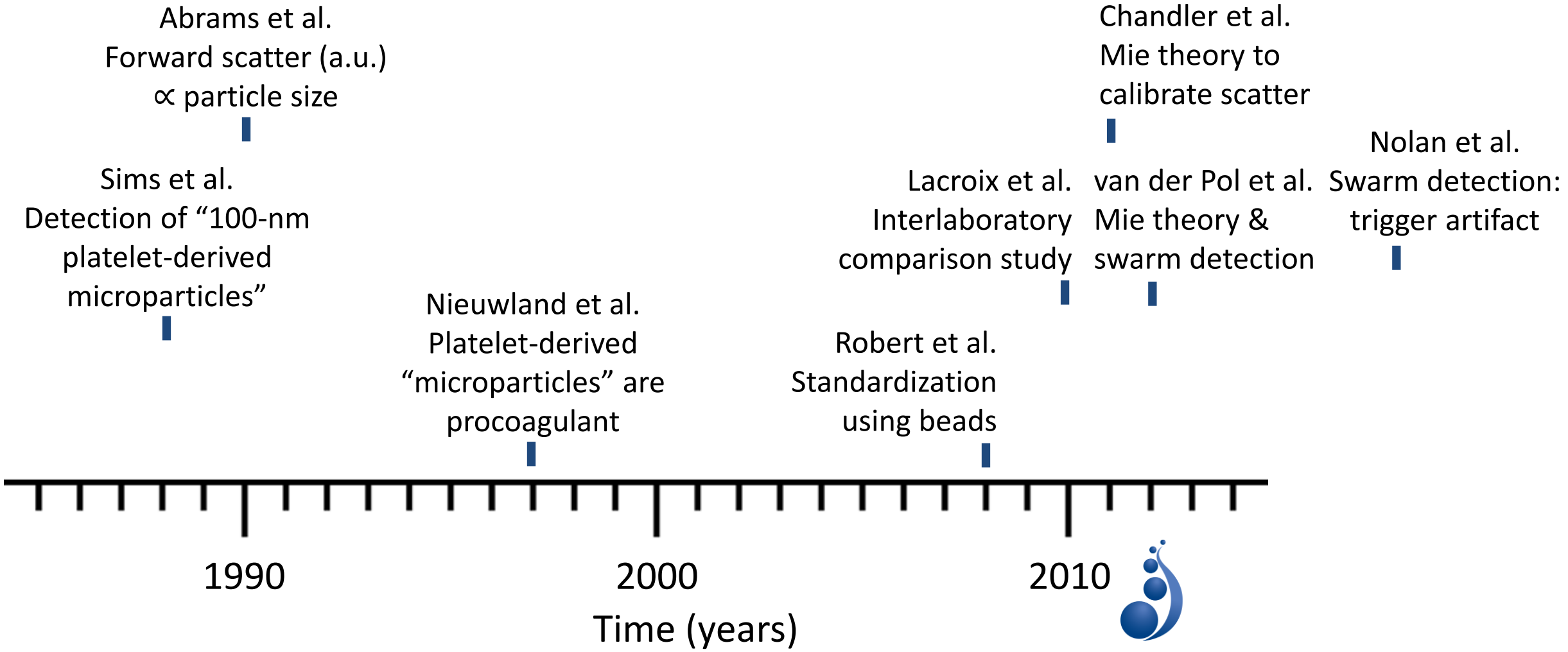


illumination
volume ≈ 50 pl



At a concentration of 10^{10} EVs ml^{-1}, >500 EVs are simultaneously illuminated

A history of extracellular vesicle detection by flow cytometry



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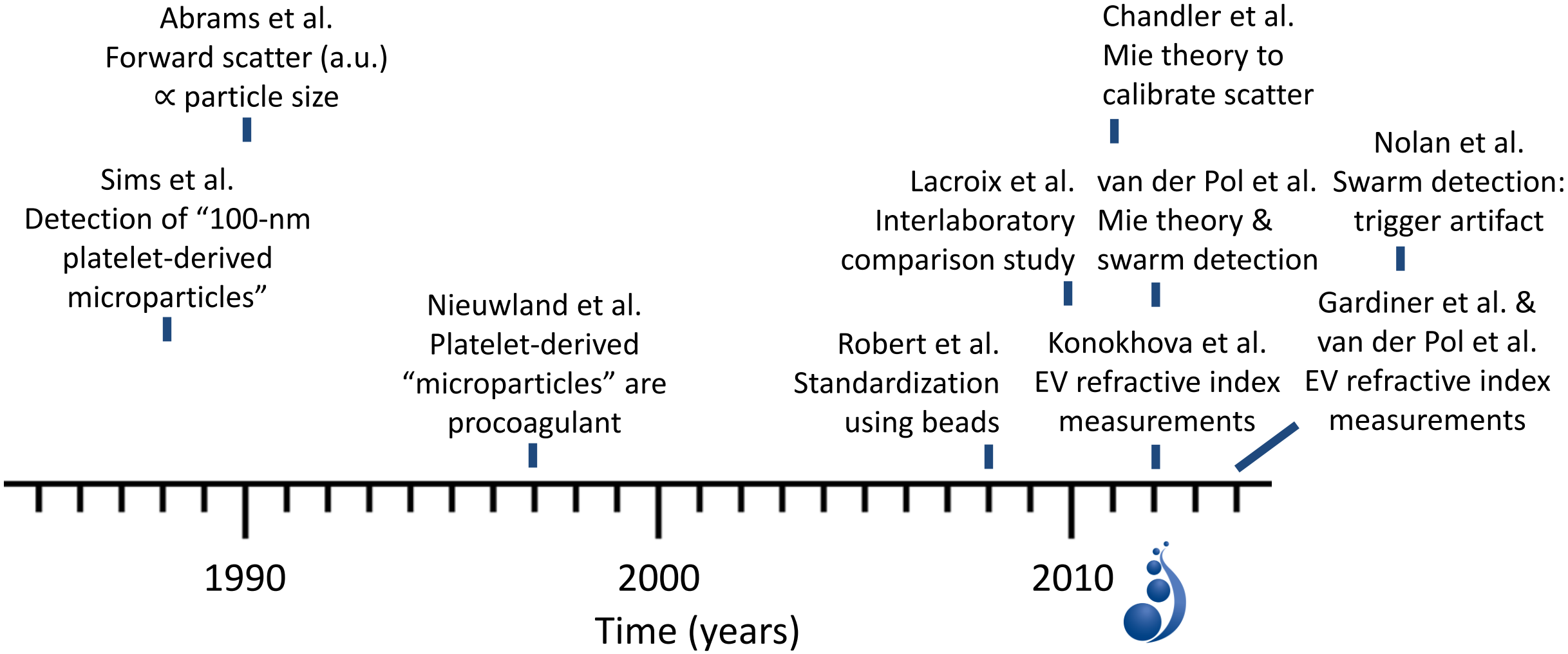
EVs: extracellular vesicles; MESF: molecules of equivalent soluble fluorophore

Coincidence vs. swarm detection

- Coincidence detection
  - simultaneous detection of two or a few particles
- Swarm detection
  - special case of coincidence detection
  - multiple particles at or below the detection limit
  - simultaneously and continuously in laser beam
  - erroneously registered as a single event

A history of extracellular vesicle detection by flow cytometry



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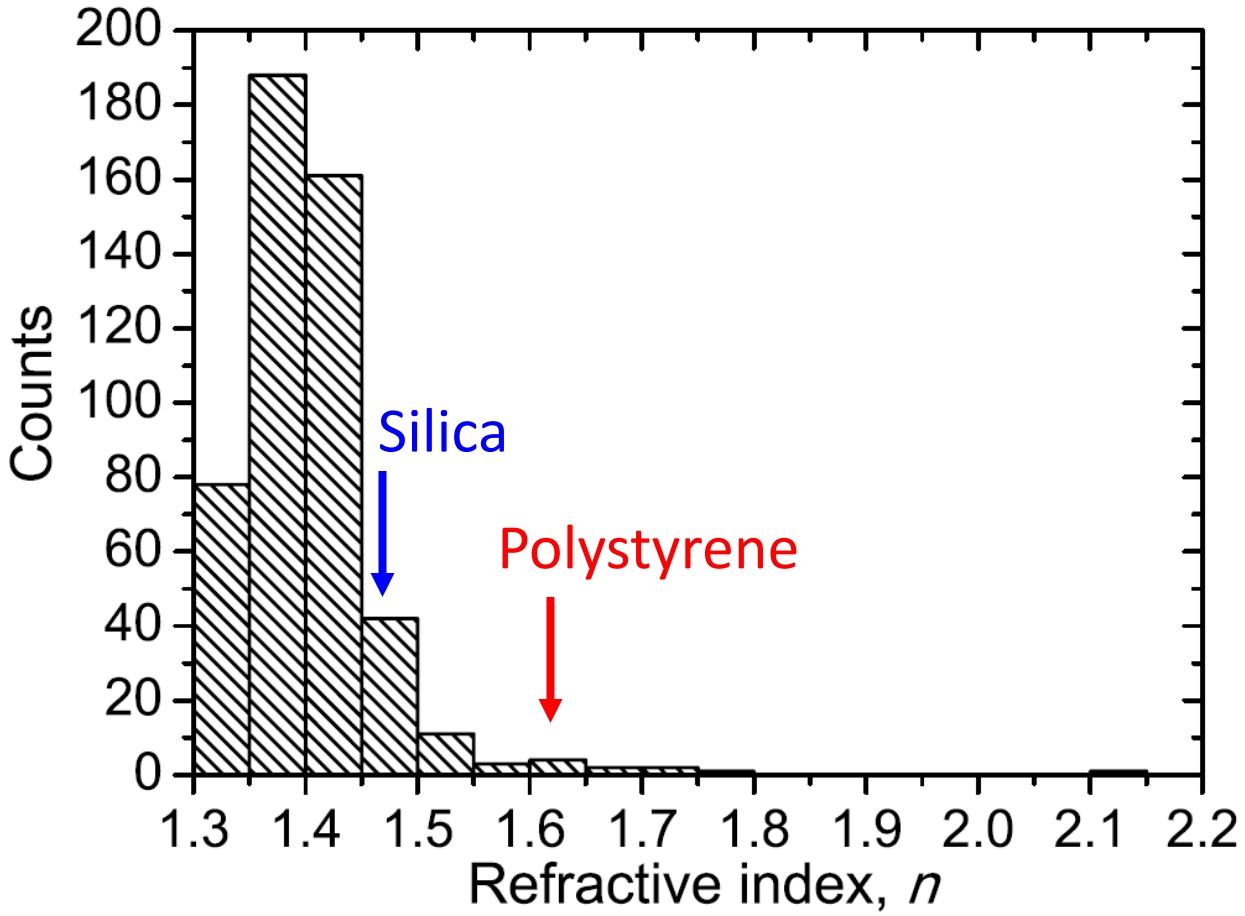
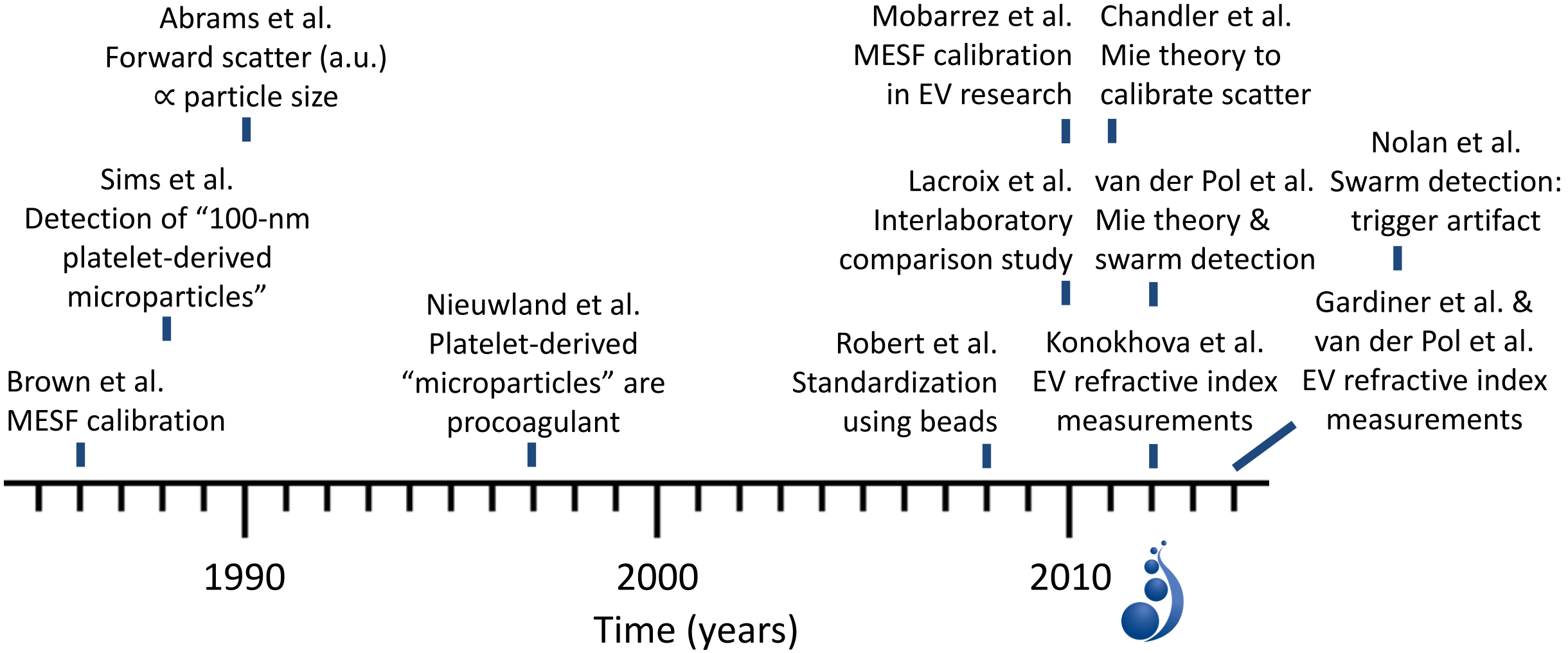
First refractive index measurements of EVs



Konokhova et al. *J Biomed Optics* 2012

A history of extracellular vesicle detection by flow cytometry



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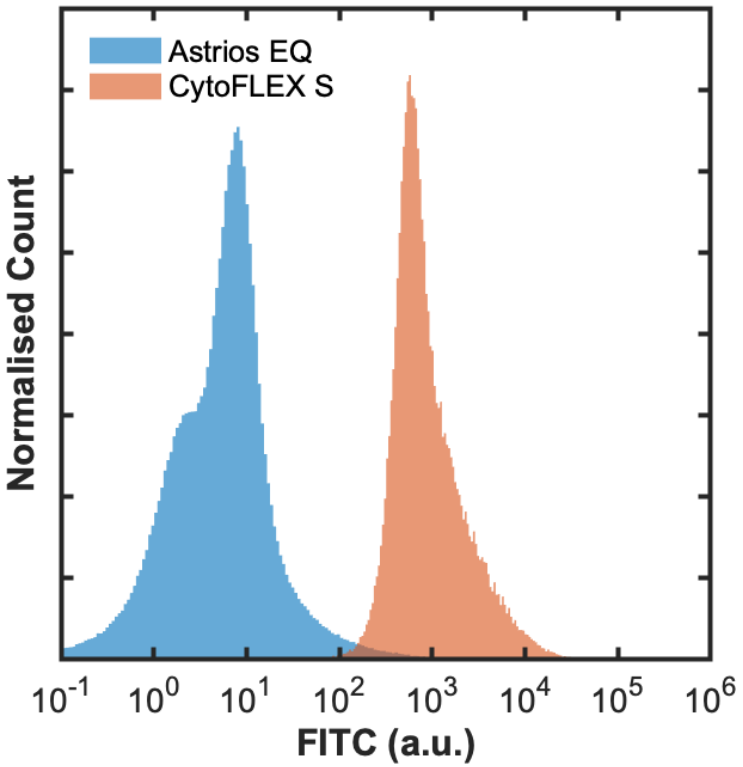
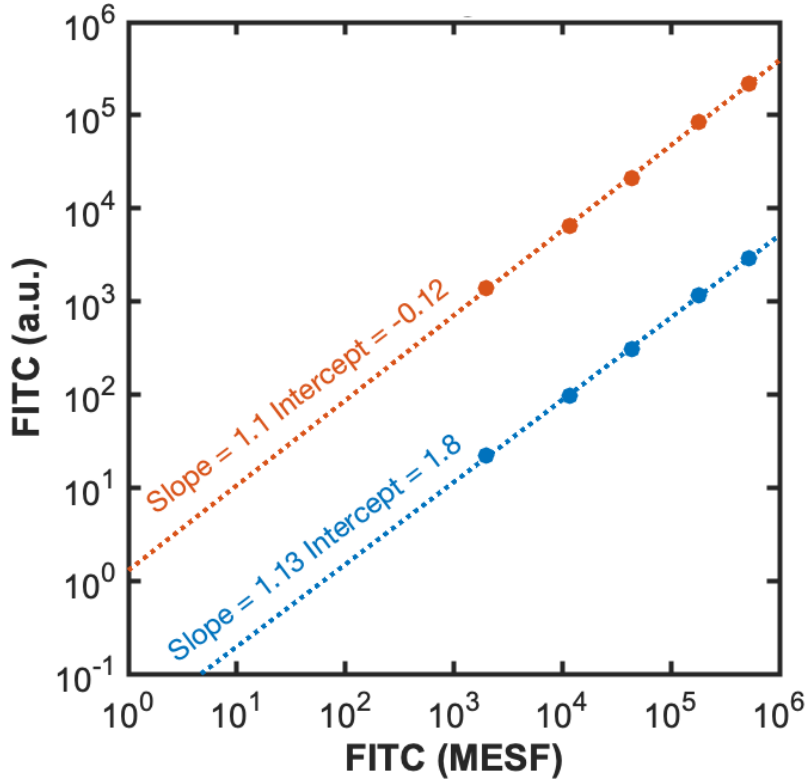
Calibrate fluorescence

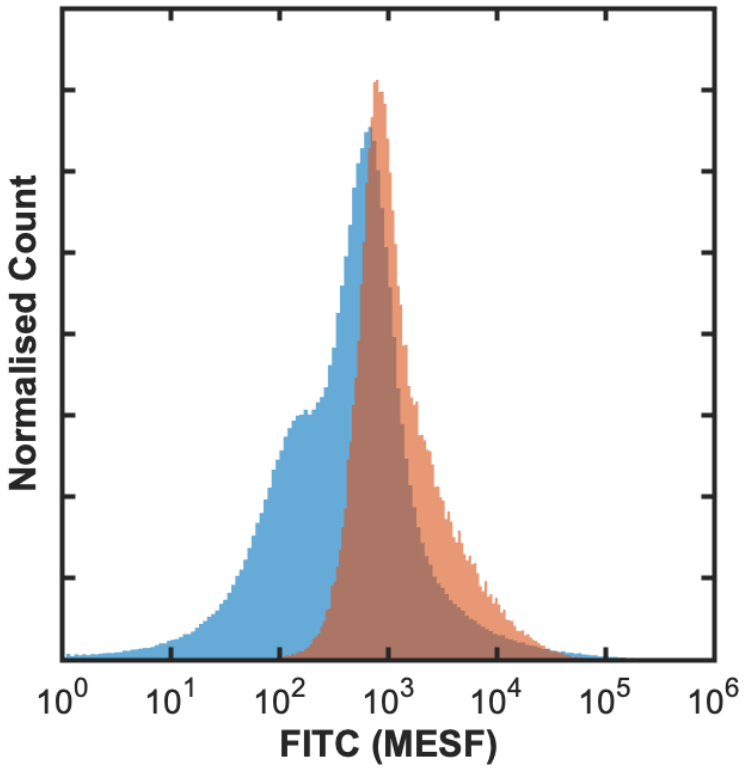
Before calibration



Calibration

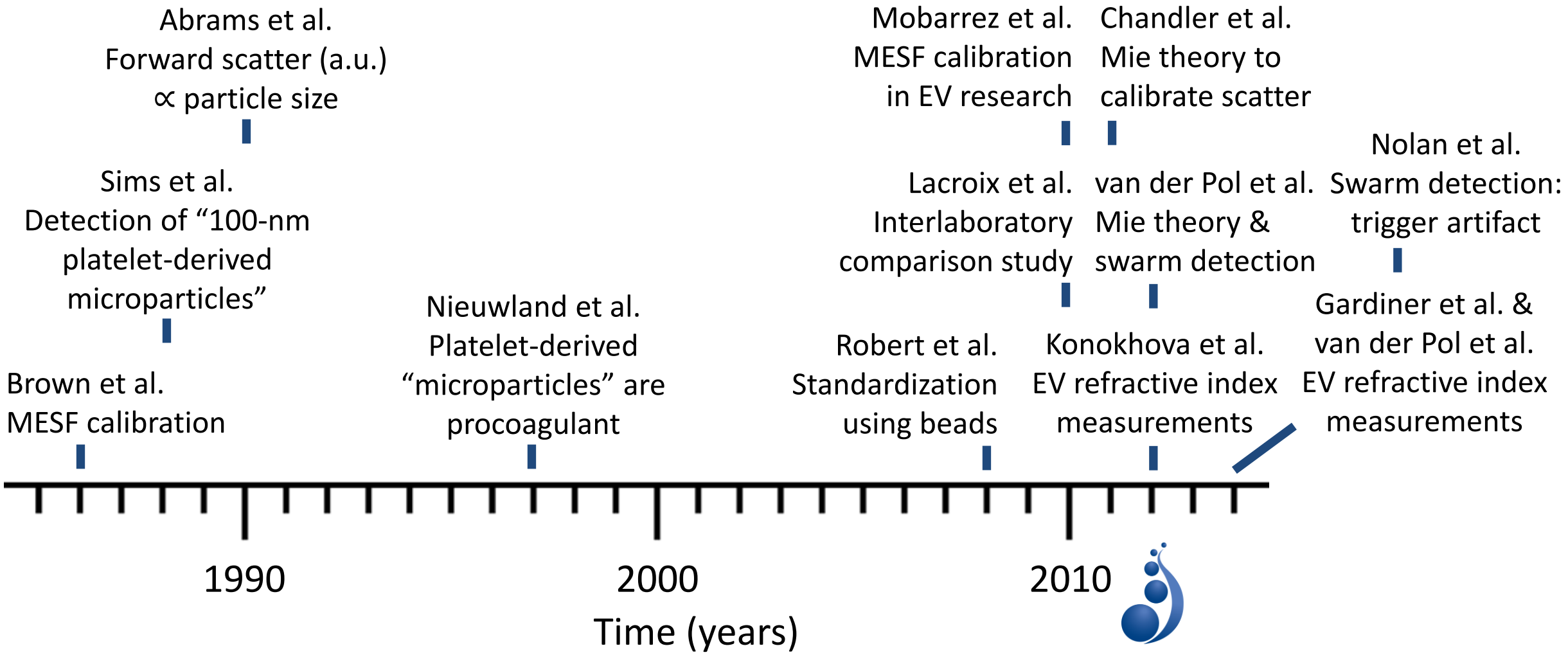


After calibration



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

A history of extracellular vesicle detection by flow cytometry



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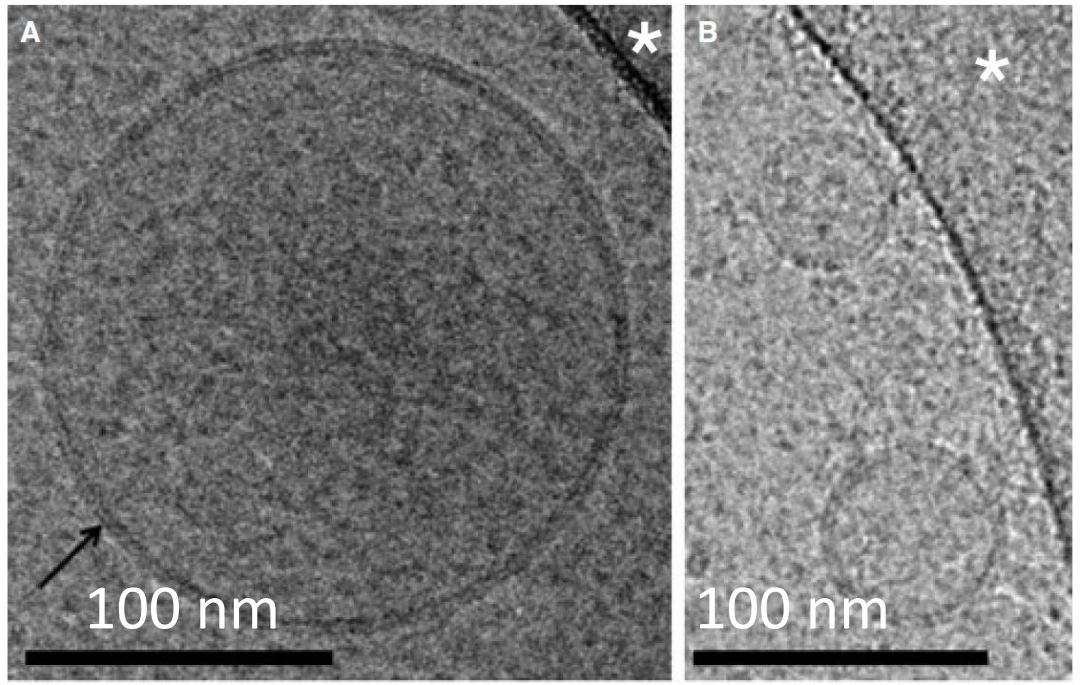


Zhu et al.
24-nm silica
beads detection

van der Pol et al.
Size distribution
of EVs in urine

Arraud et al.
Size distribution
of EVs in plasma

2015

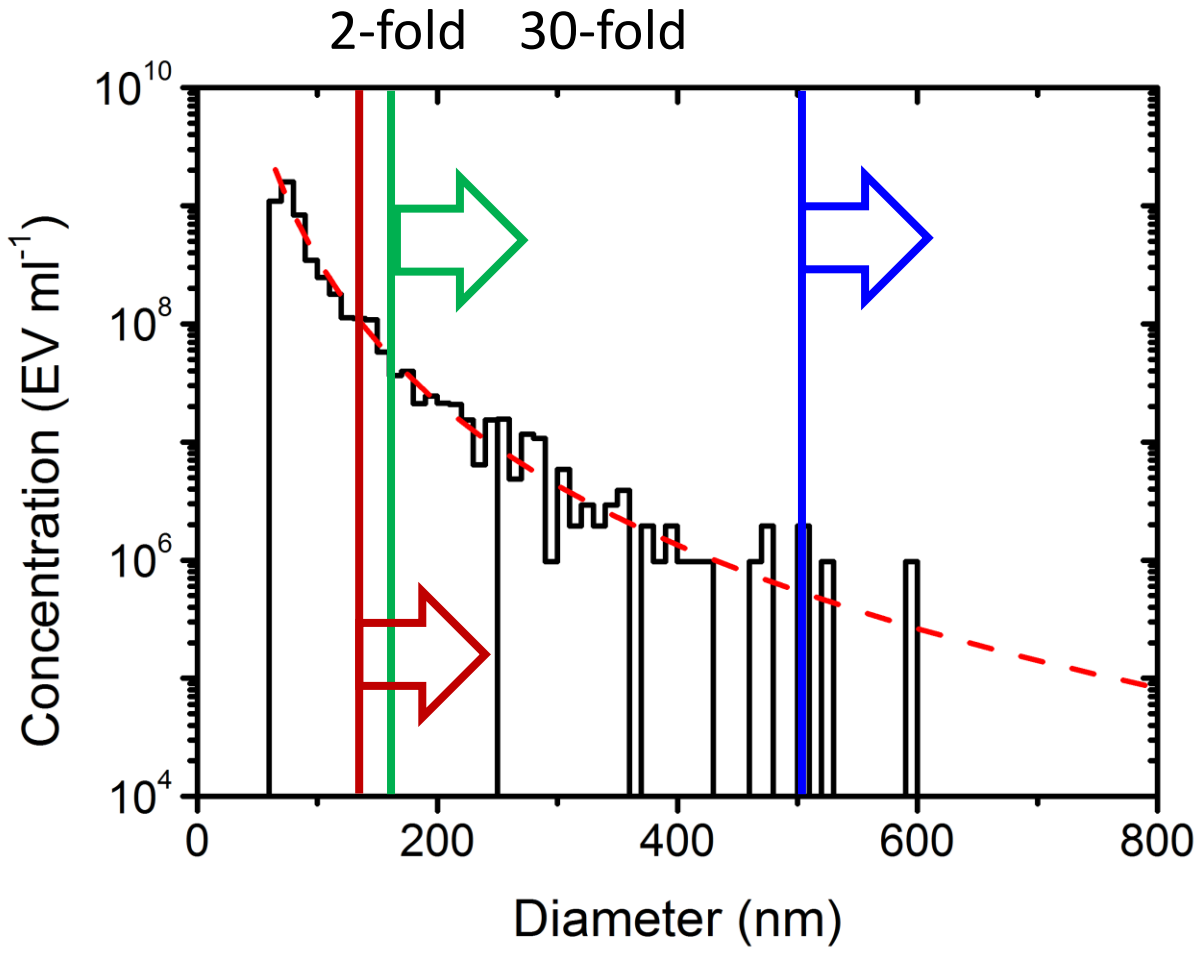
2020

Time (years)

Disclosure: this story is incomplete

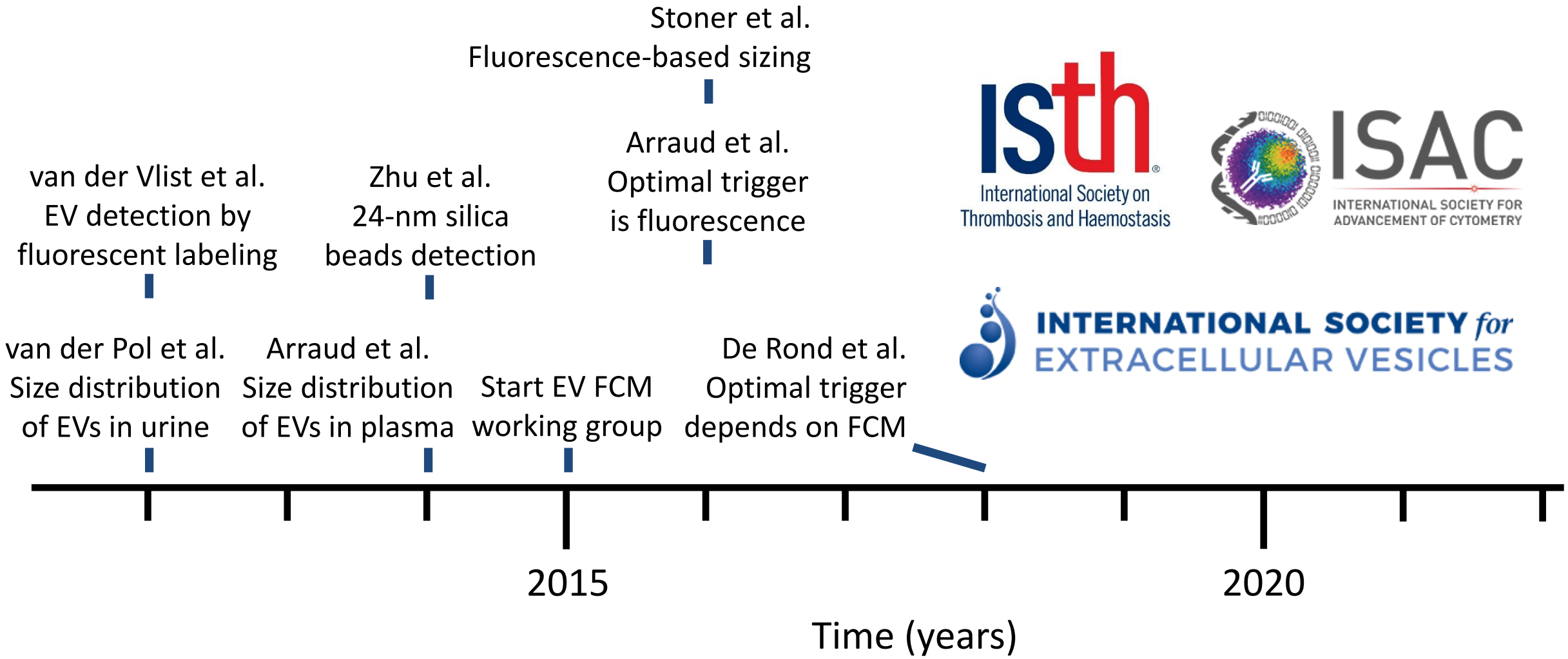
EVs: extracellular vesicles; Figure: Arraud et al. *J Thromb Haemost* 2012

Measured concentrations of extracellular vesicles depend on lower limit of detection



power-law relation*

A history of extracellular vesicle detection by flow cytometry



ISTH International Society on Thrombosis and Haemostasis

ISAC INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

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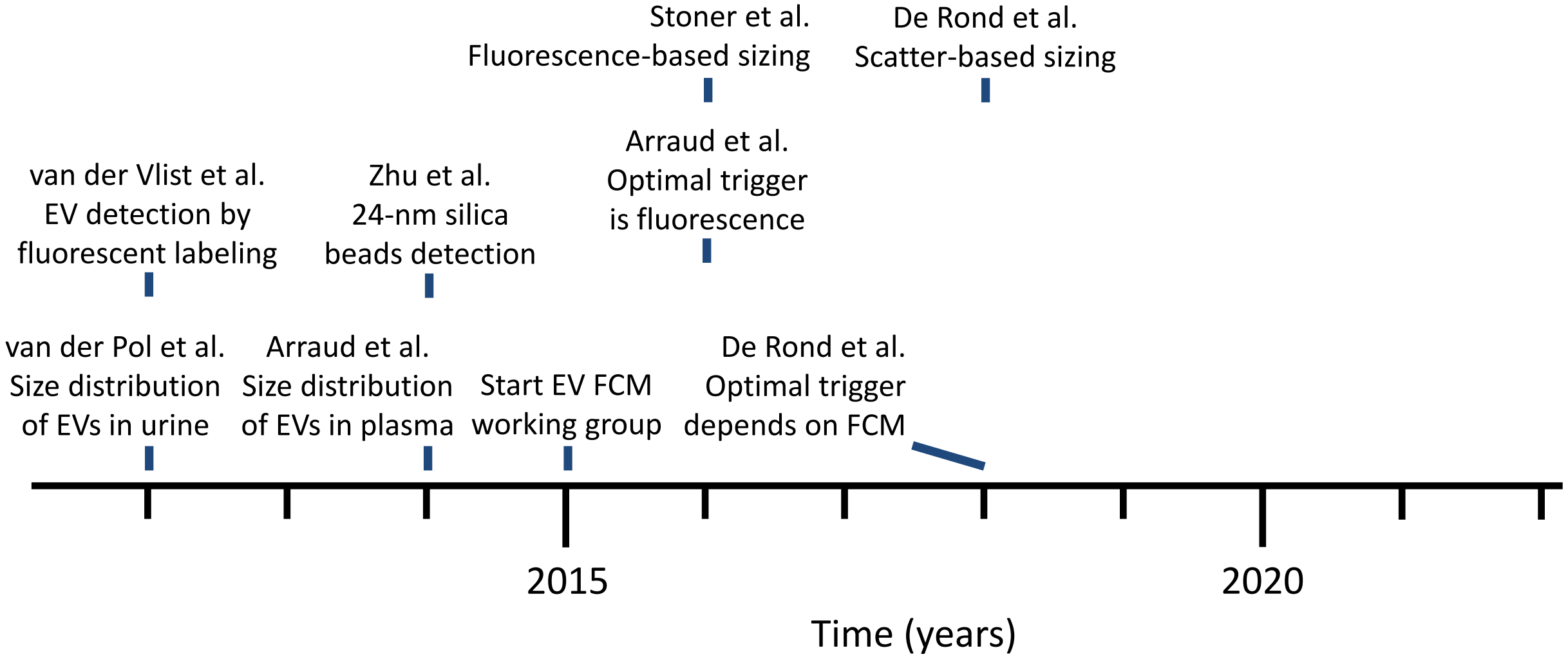
EVs: extracellular vesicles; FCM: flow cytometry

A history of extracellular vesicle detection by flow cytometry



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Turn your flow cytometer into a particle size analyzer!

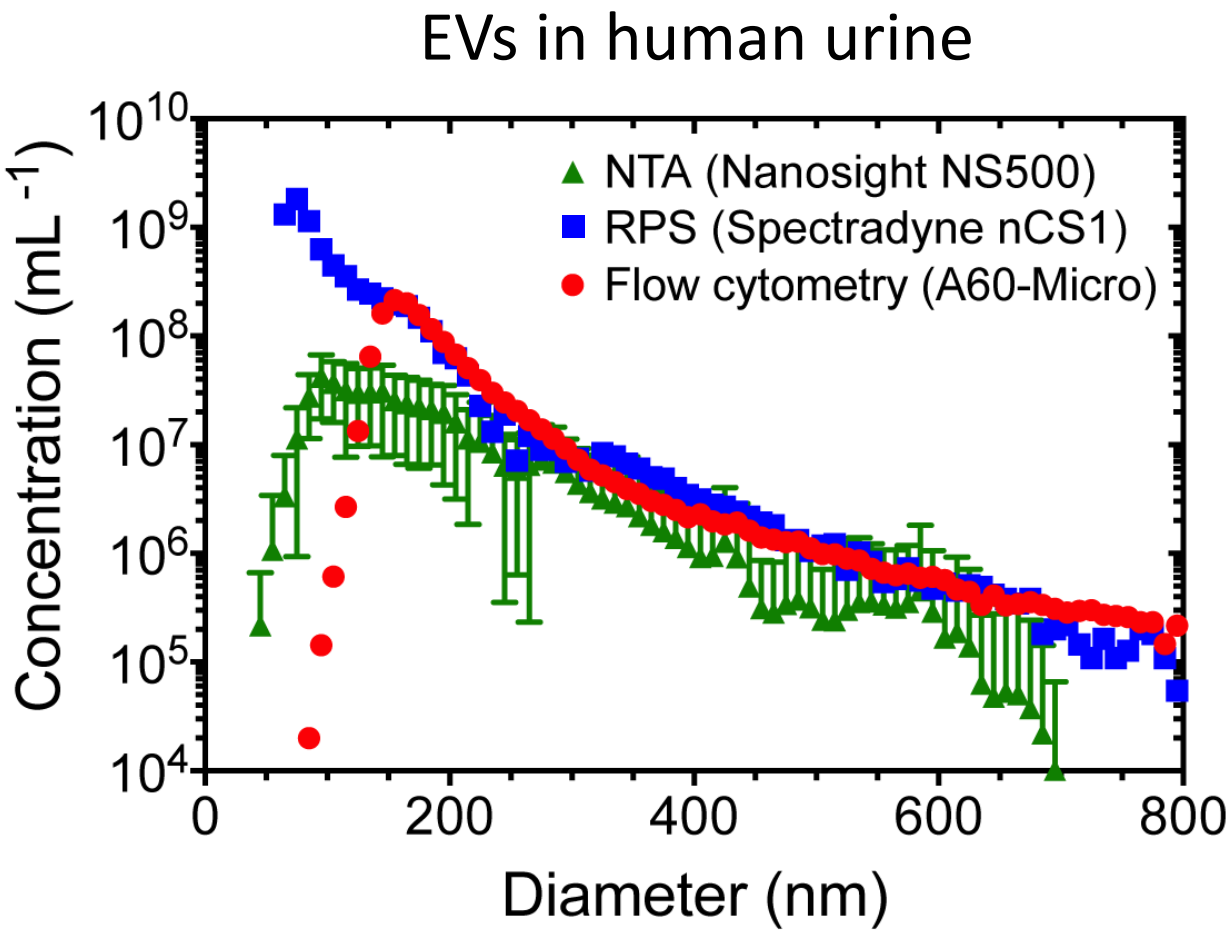
Deriving Extracellular Vesicle Size From Scatter Intensities Measured by Flow Cytometry

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)

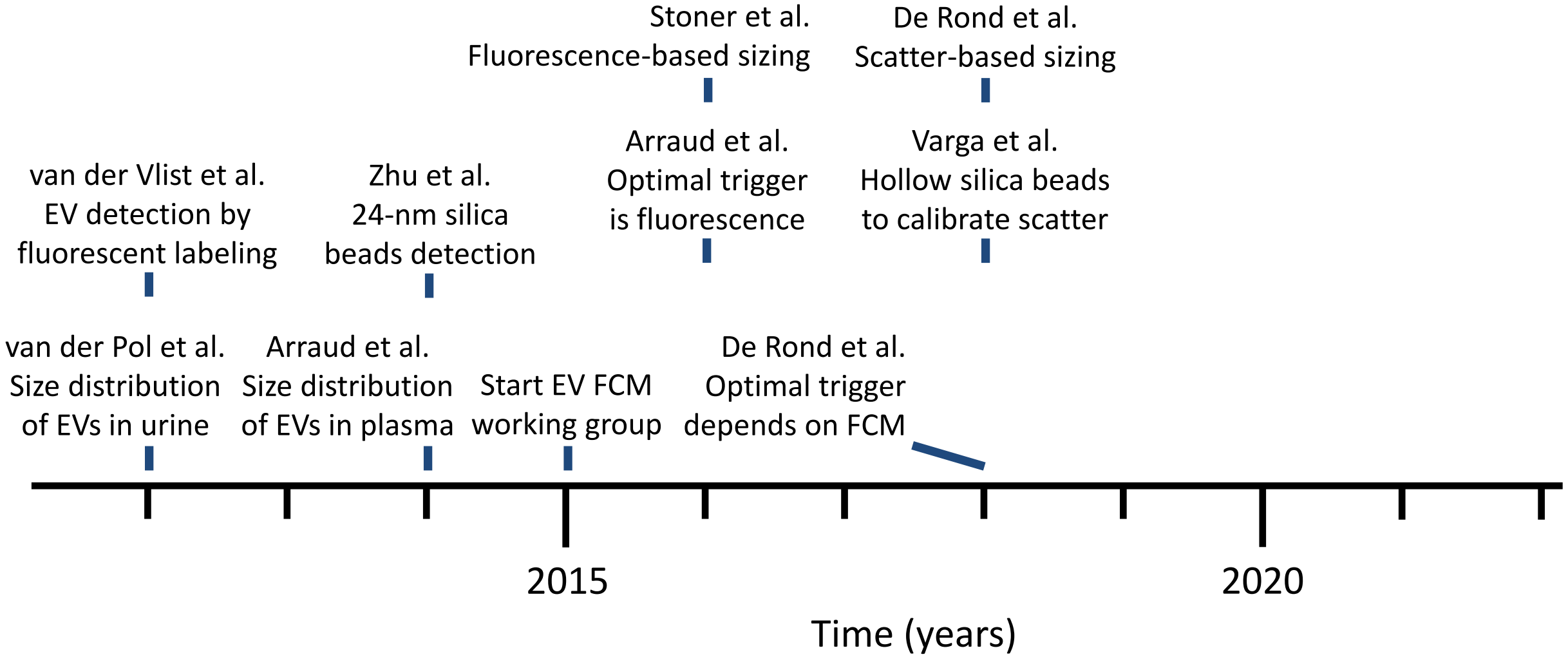
- Easy
- Cheaper than daily quality control with polystyrene beads
- More accurate than NTA (flow cytometer dependent)

Example of scatter-based sizing of EVs



Data and figure by Michael Cimorelli, manuscript in prep.

A history of extracellular vesicle detection by flow cytometry



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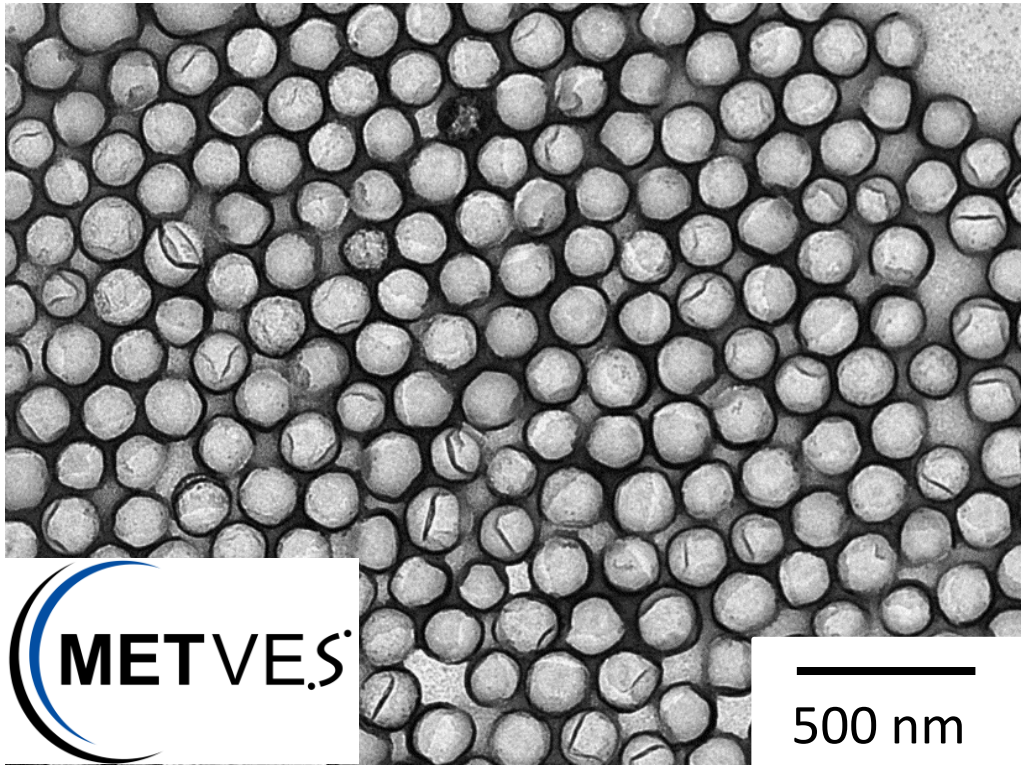
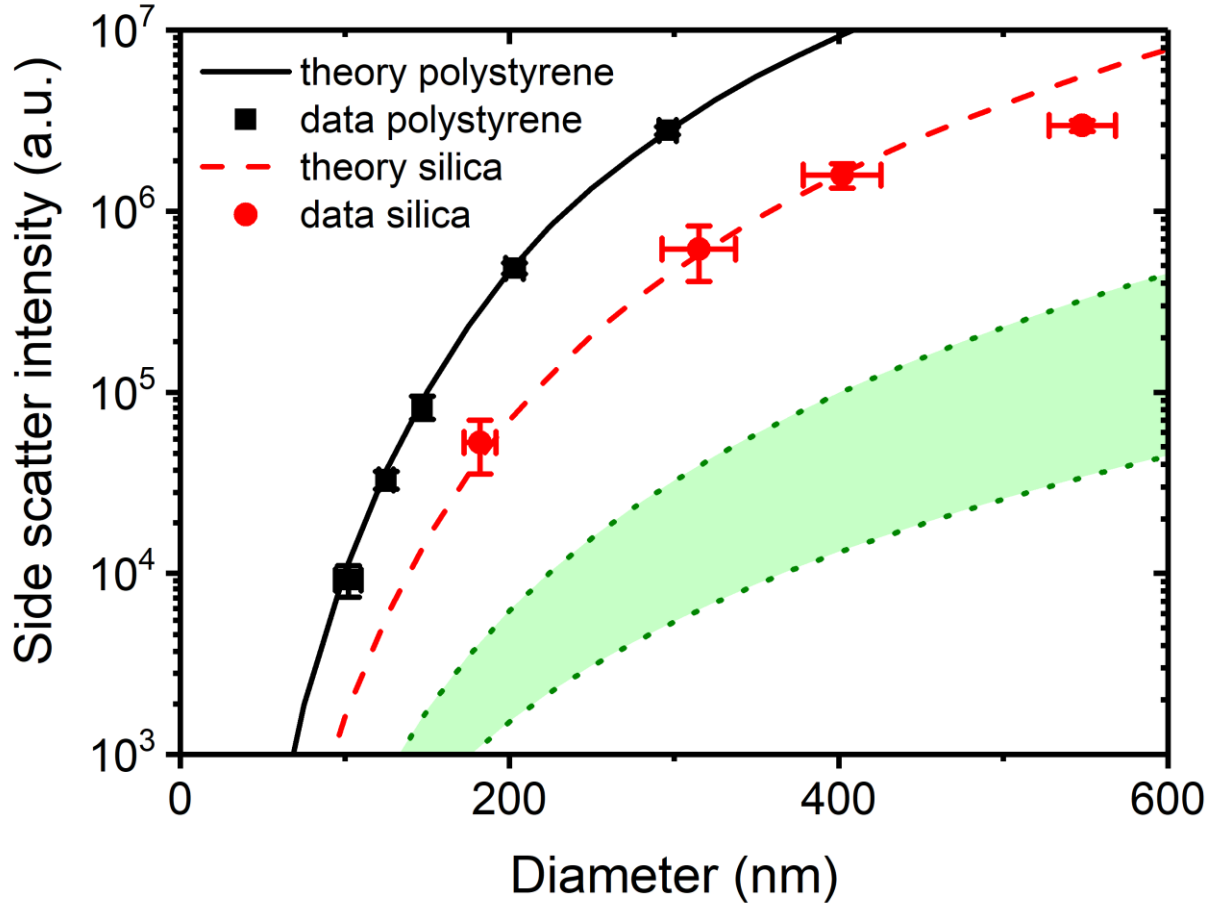
EVs: extracellular vesicles; FCM: flow cytometry

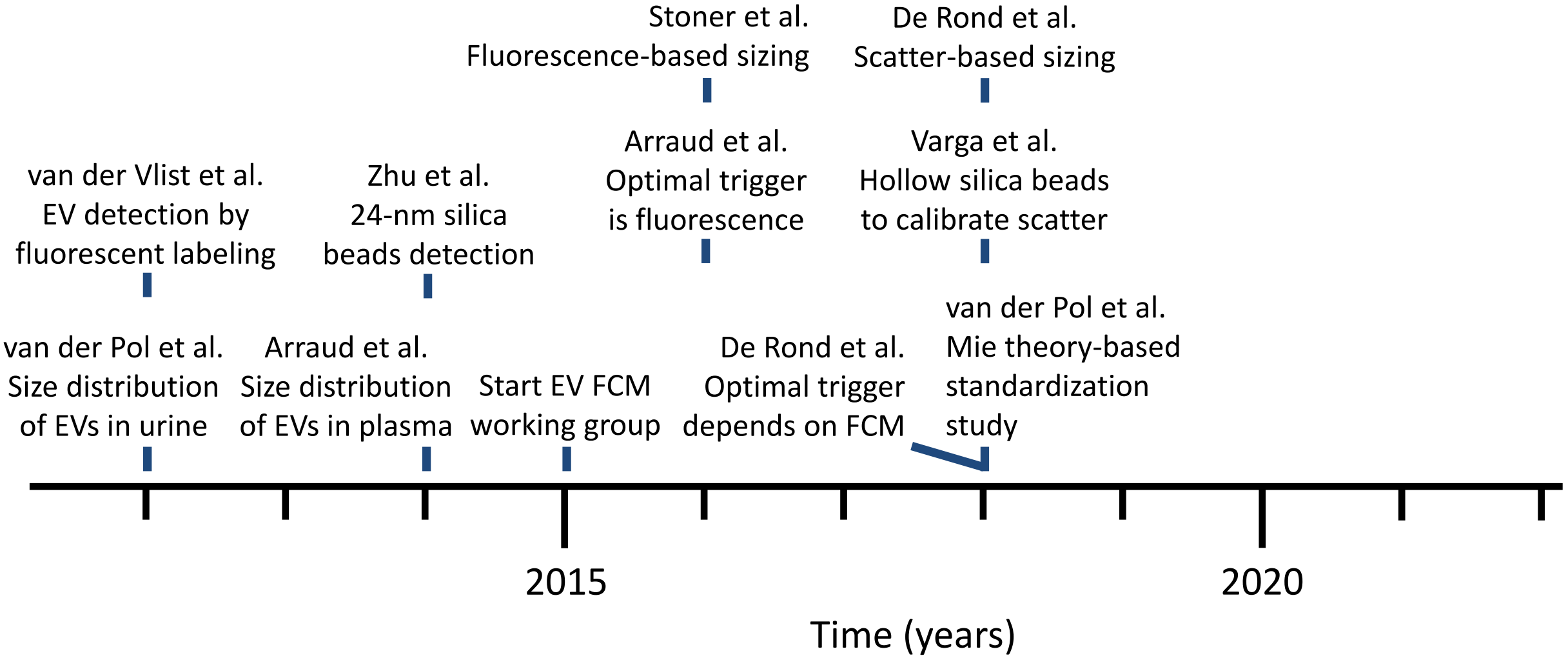
Hollow organosilica beads confirm scatter-diameter relationship for EVs





Varga et al. *J Thromb Haemost* 2018

A history of extracellular vesicle detection by flow cytometry



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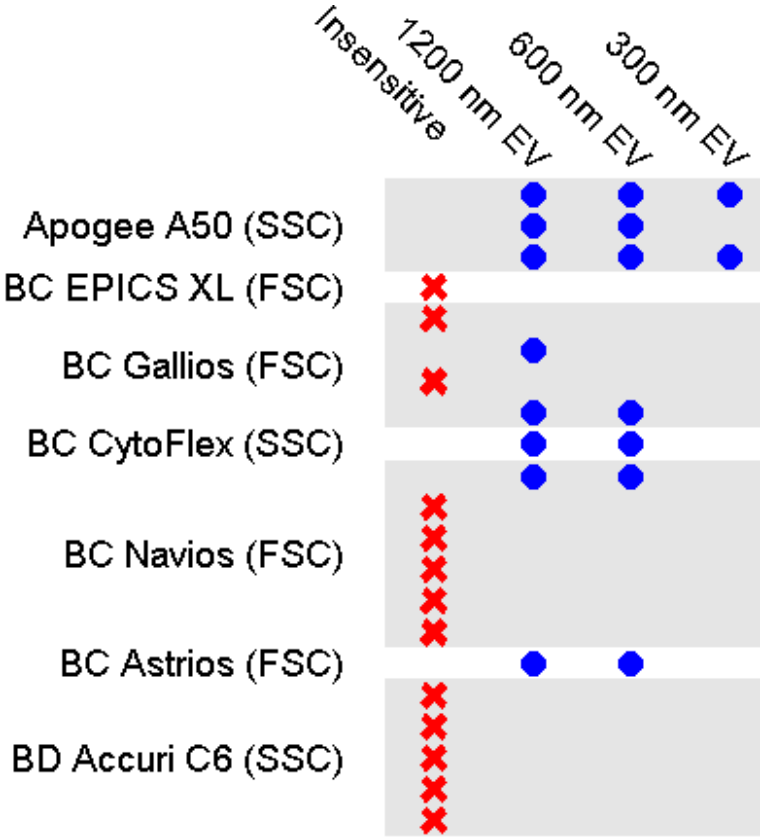
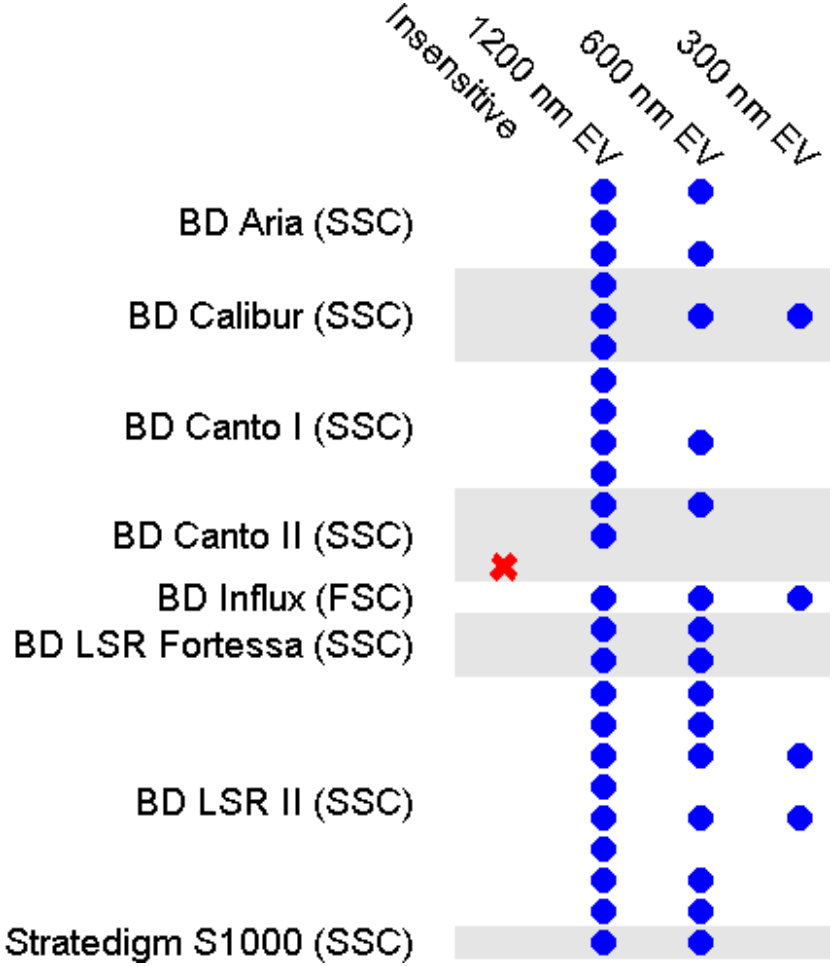
EVs: extracellular vesicles; FCM: flow cytometry

Study comprises 33 sites (64 instruments) worldwide

2014-2018

Scatter sensitivity of 46 flow cytometers in the field





× = unable to detect 400 nm fluorescent polystyrene beads

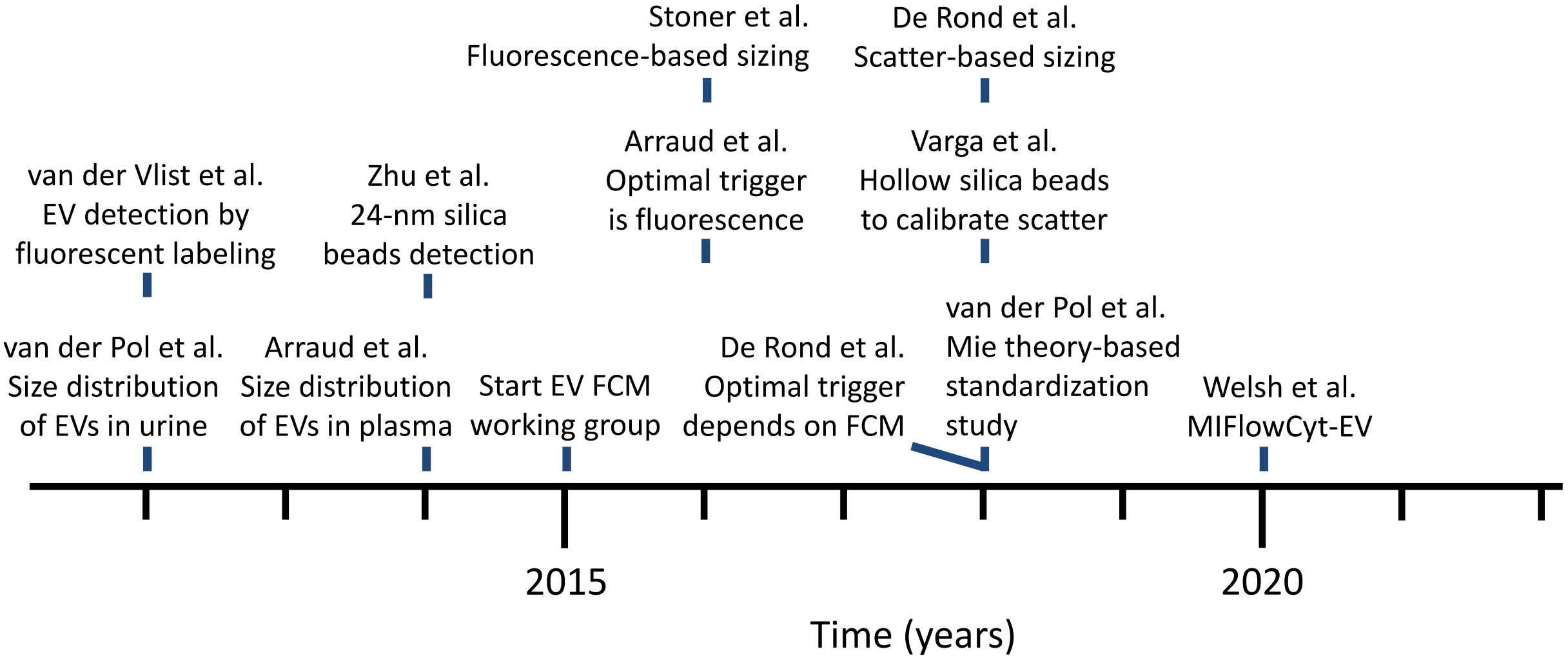
Reproducibility of 1200-3000 nm EVs, 31 FCMs

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

Requires improvement!

%CV = standard deviation / mean * 100%

A history of extracellular vesicle detection by flow cytometry



Disclosure: this story is incomplete

EVs: extracellular vesicles; FCM: flow cytometry

Reporting

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

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

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Solution: MIFlowCyt-EV

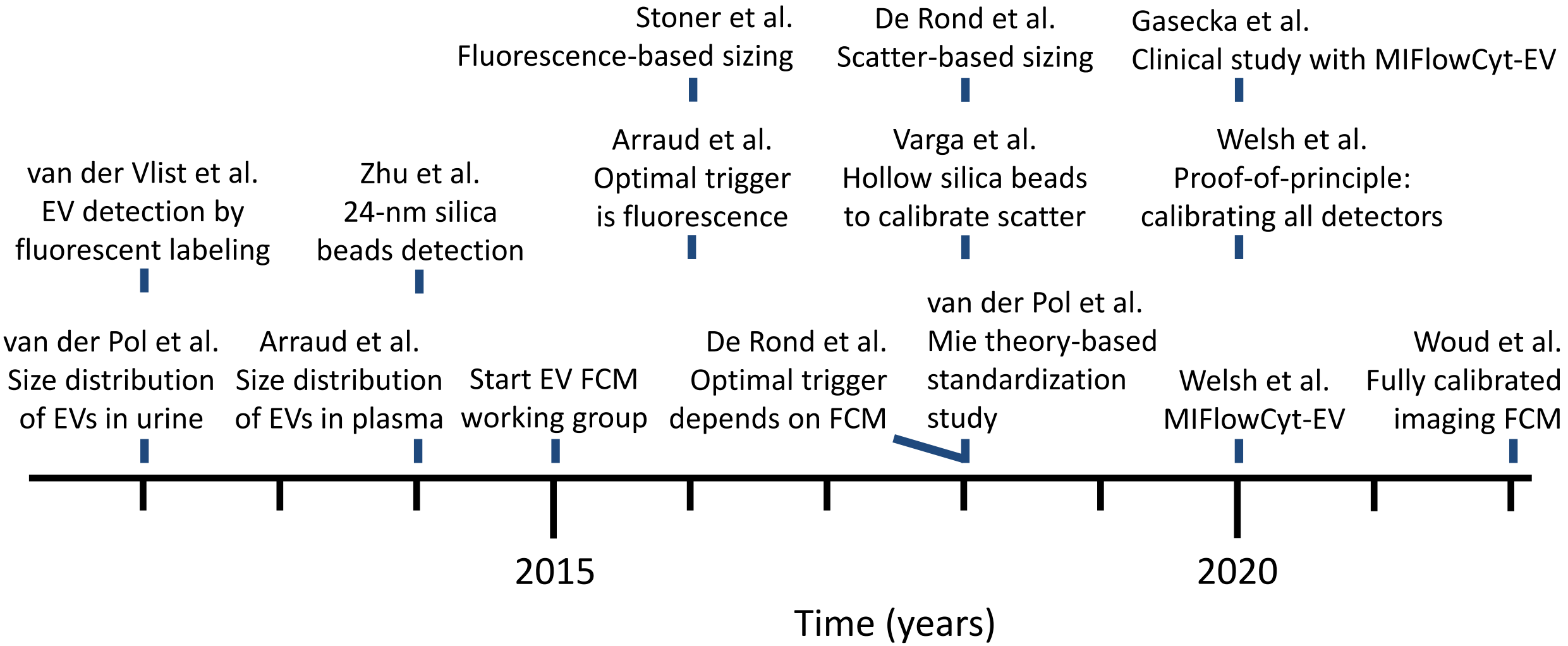
	Category	Components	Objective
1	Preanalytical variables & experimental design	1.1. Report preanalytical variables conforming to MISEV guidelines* 1.2. Report experimental design according to MIFlowCyt guidelines*	Reproducibility
2	Sample preparation	2.1. Sample staining* 2.2. Sample washing steps* 2.3. Sample dilution*	Reproducibility
3	Assay co	3.5. Single-stained controls* 3.6. Procedural controls** 3.7. Serial dilution* 3.8. Detergent-treated EV samples	Proof of single vesicle detection
4	Instrume & data	reshold(s)* ntification (µL min^{-1} / µL)* MESF/ERF units)* m^2)	Standardization
5	EV chara	lume approximation ation ximation	Advanced standardization
6	FC data reporting	6.1. Complete MIFlowCyt checklist* 6.2. Calibrated channel detection range 6.3. EV number concentration 6.4. EV brightness	Reproducibility
7	FC data sharing	7.1. Share data to public repository	Reproducibility

This is not a guideline!
Do not just cite this!

Instead, fill-out the MIFlowCyt-EV template

Welsh et al. *J. Extracell. Vesicles* 2020

A history of extracellular vesicle detection by flow cytometry



Disclosure: this story is incomplete

EVs: extracellular vesicles; FCM: flow cytometry

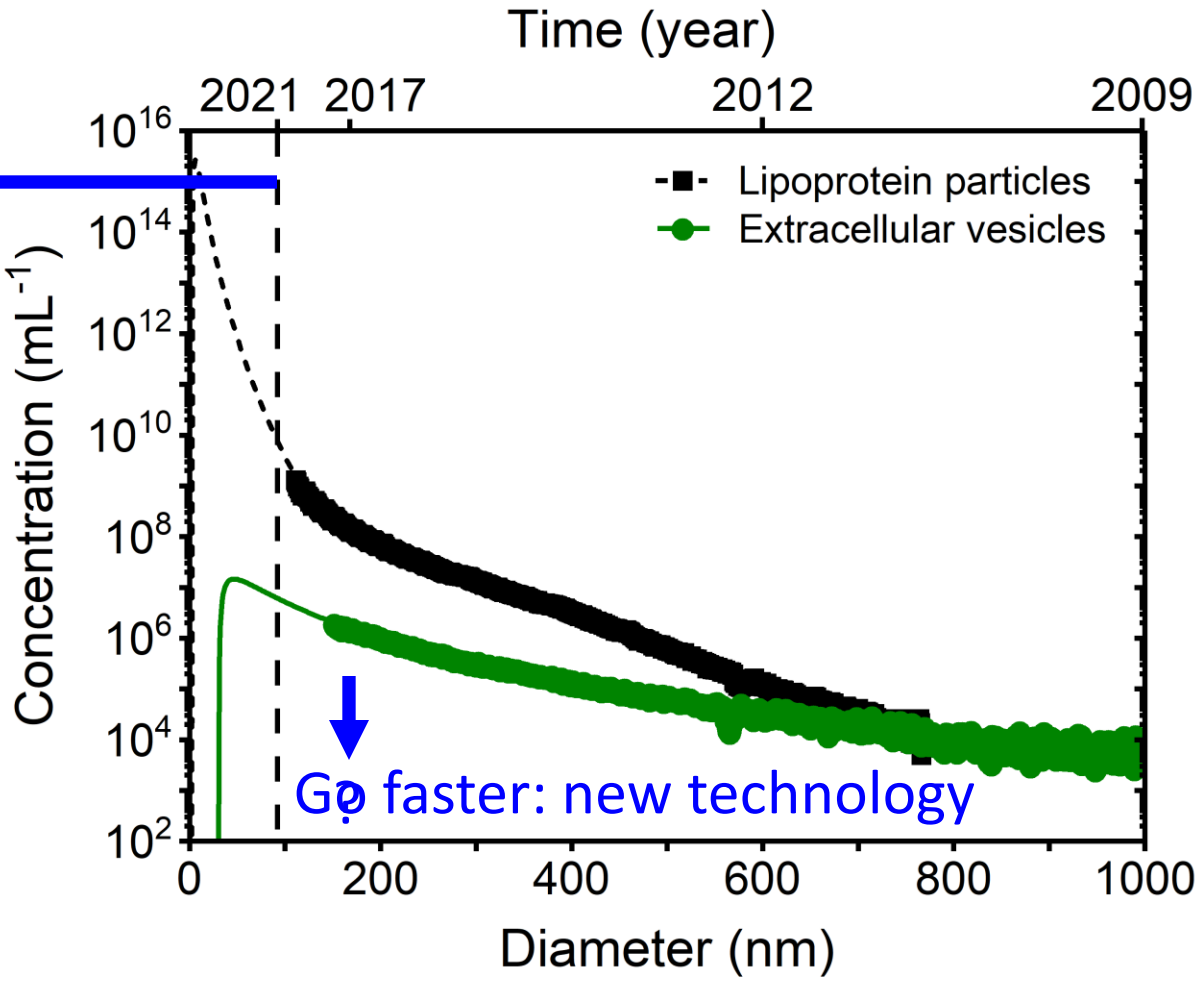
Outline

- ✔ Flow cytometry basics: parler la même langue

- ✔ A history of… *extracellular vesicle detection*
- The future of… *by flow cytometry*

The future of EV detection by flow cytometry

Go smaller:
Nano FCM
Imaging FCM



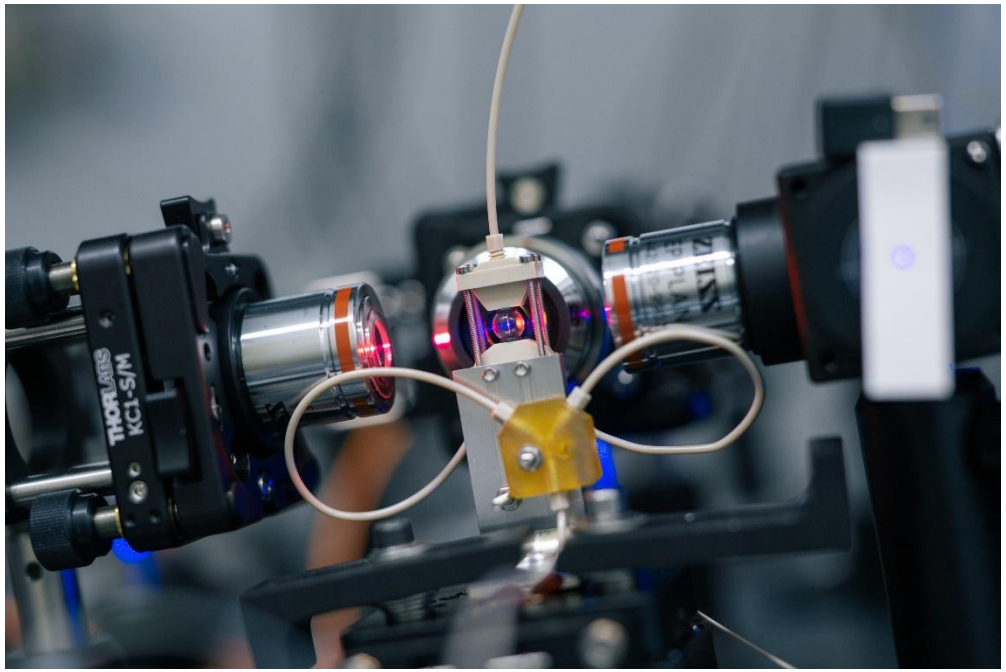
The future of EV detection by flow cytometry

- New instruments with similarities to flow cytometry
  - Resistive pulse sensing + fluorescence detection (ARC, Spectradyne)
  - Metrological flow cytometer (METVES II)

Metrological flow cytometer

- Will measure
  - Diameter in nm
  - Refractive index
  - Shape
  - Fluorescence intensity in standard units
  - Sample volume

traceably, including an uncertainty



Kuiper et al. *Mikronek* 2022
Image courtesy of Martine Kuiper (metves.eu)

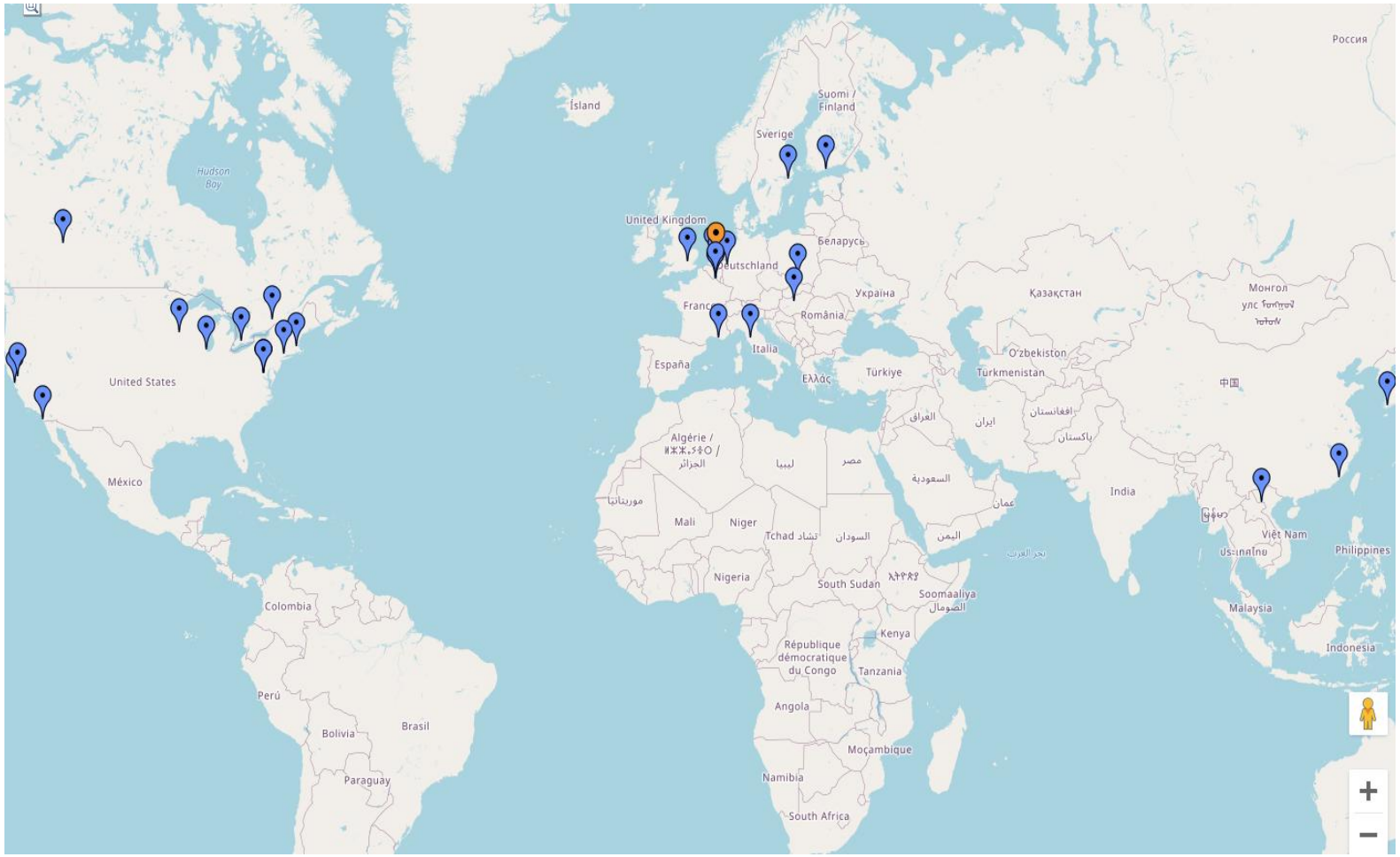
Expected soon

- Welsh, van der Pol & the EV FCM working group: *EV flow cytometry compendium* (under revision)

- METVES II interlaboratory comparison study
  - calibrate all flow cytometry aspects

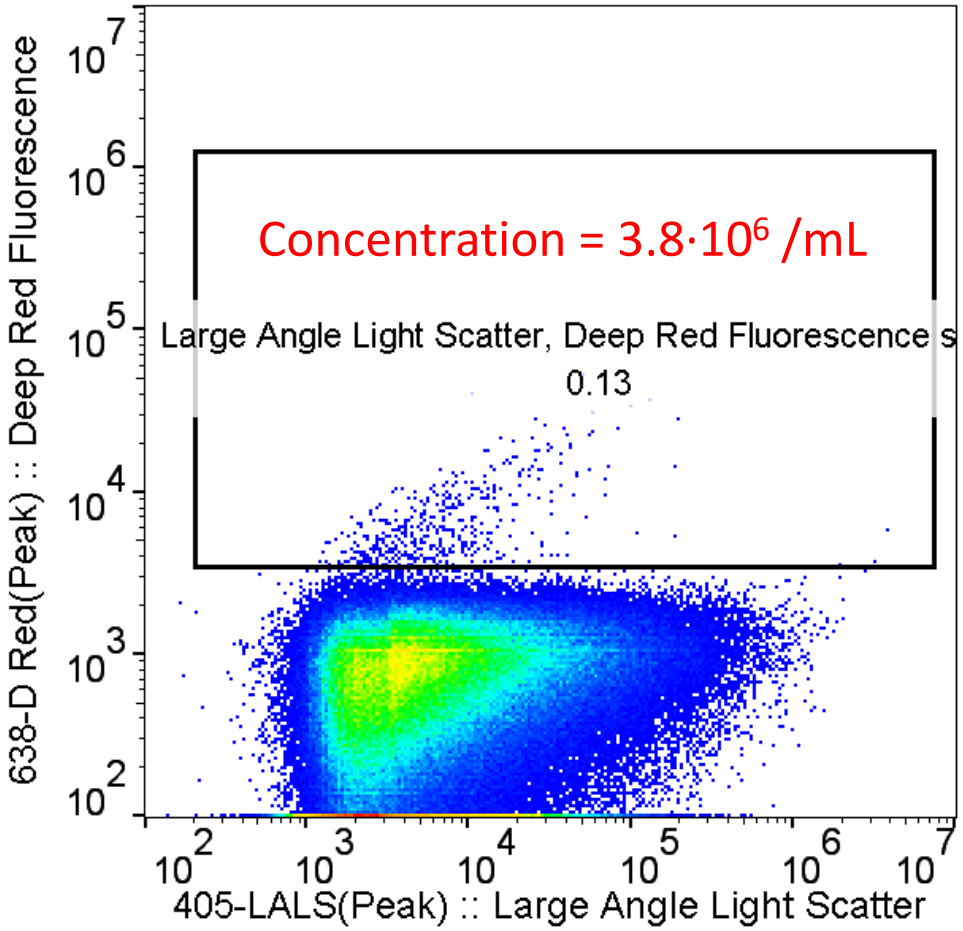
- Reference values of EVs in human blood plasma

METVES II: 23 participants and 35 flow cytometers

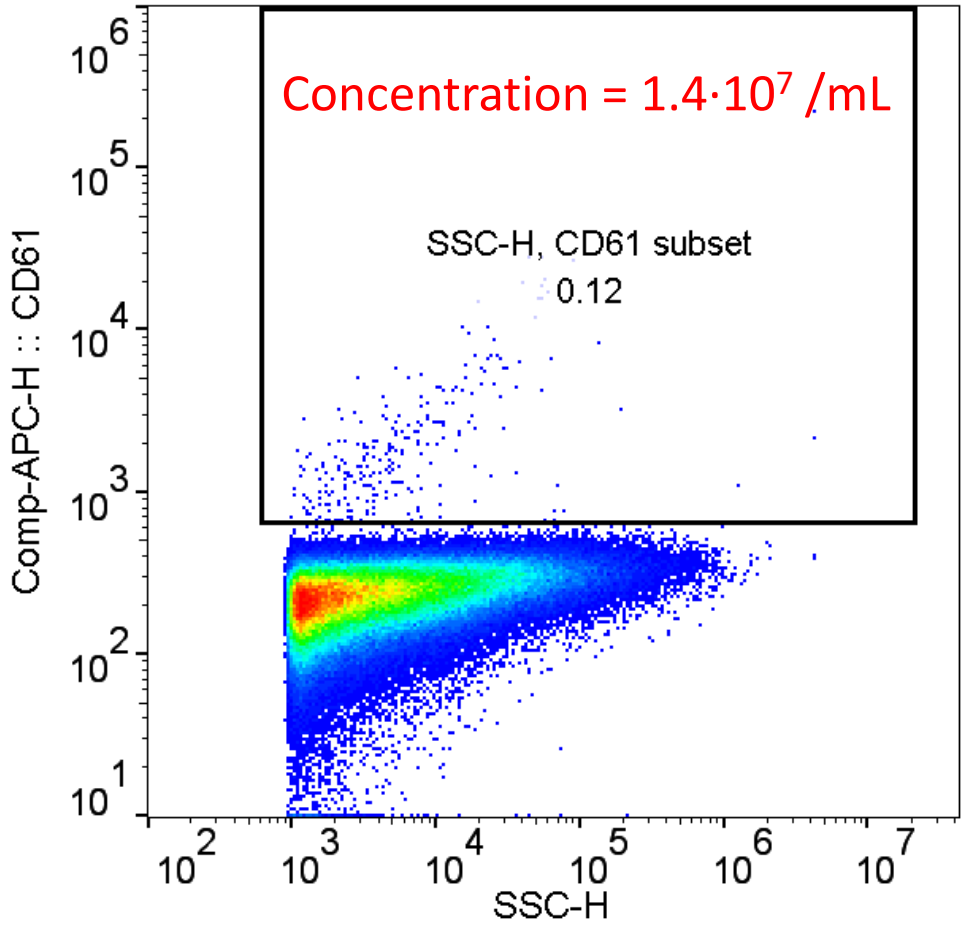


Without calibration, data do not reproduce

Apogee A60-Micro



Cytek Aurora

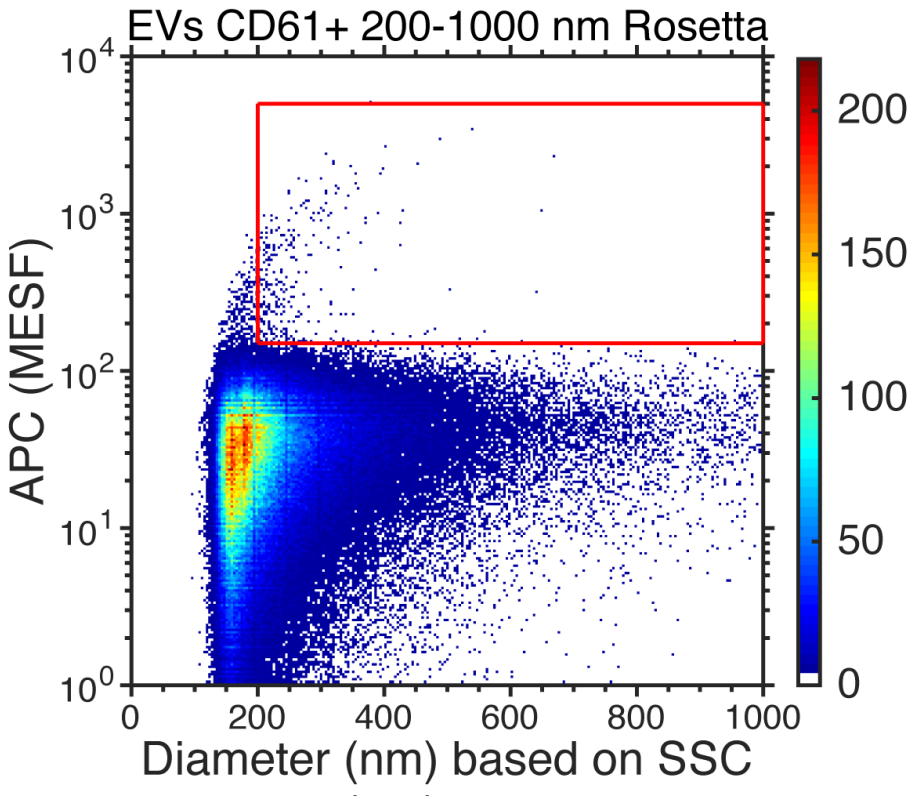


EV test sample prepared by Britta Bettin, Amsterdam UMC

Calibration improves data reproducibility

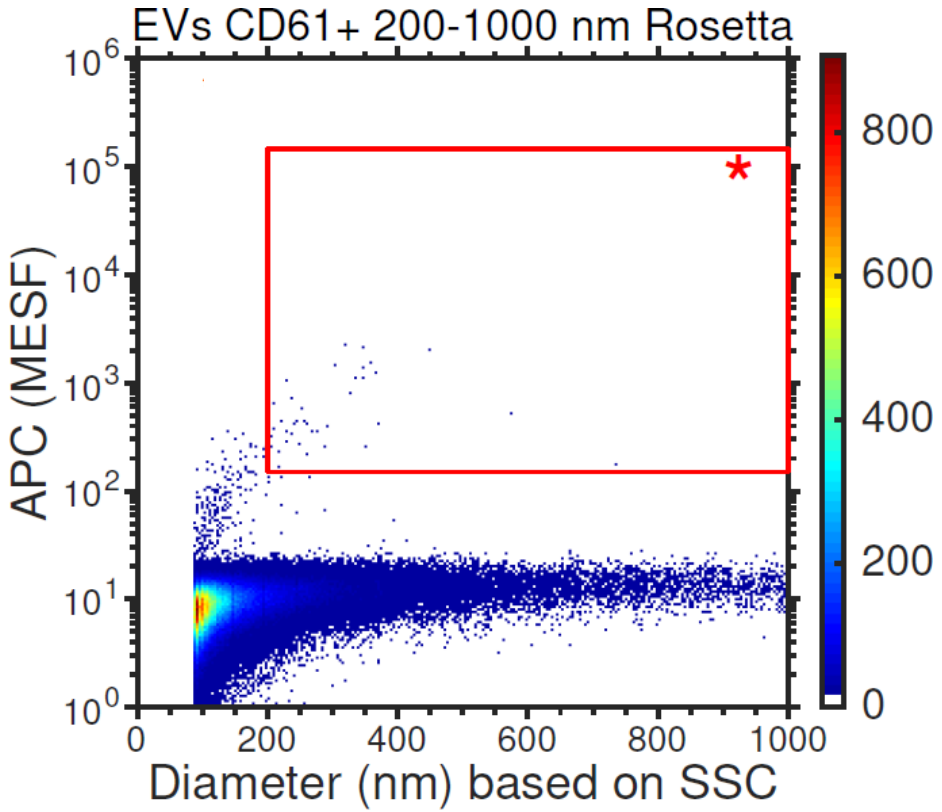
Apogee A60-Micro

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



Cytek Aurora

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



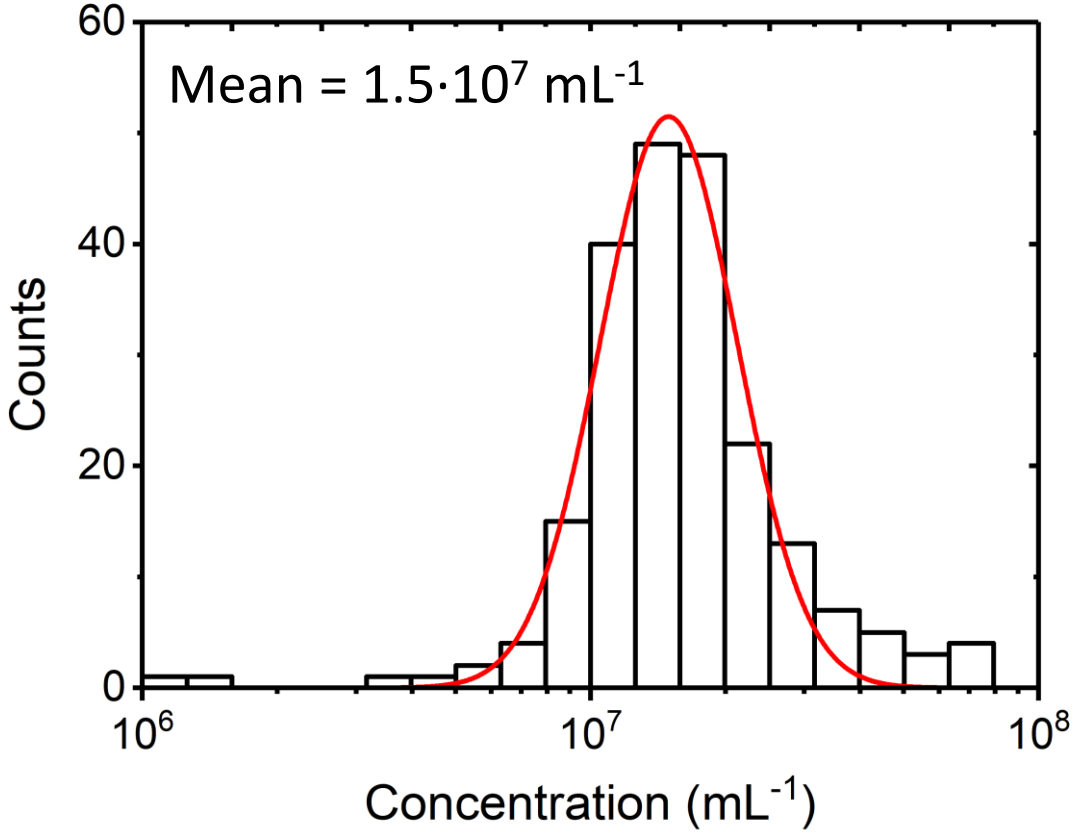
EV test sample prepared by Britta Bettin, Amsterdam UMC

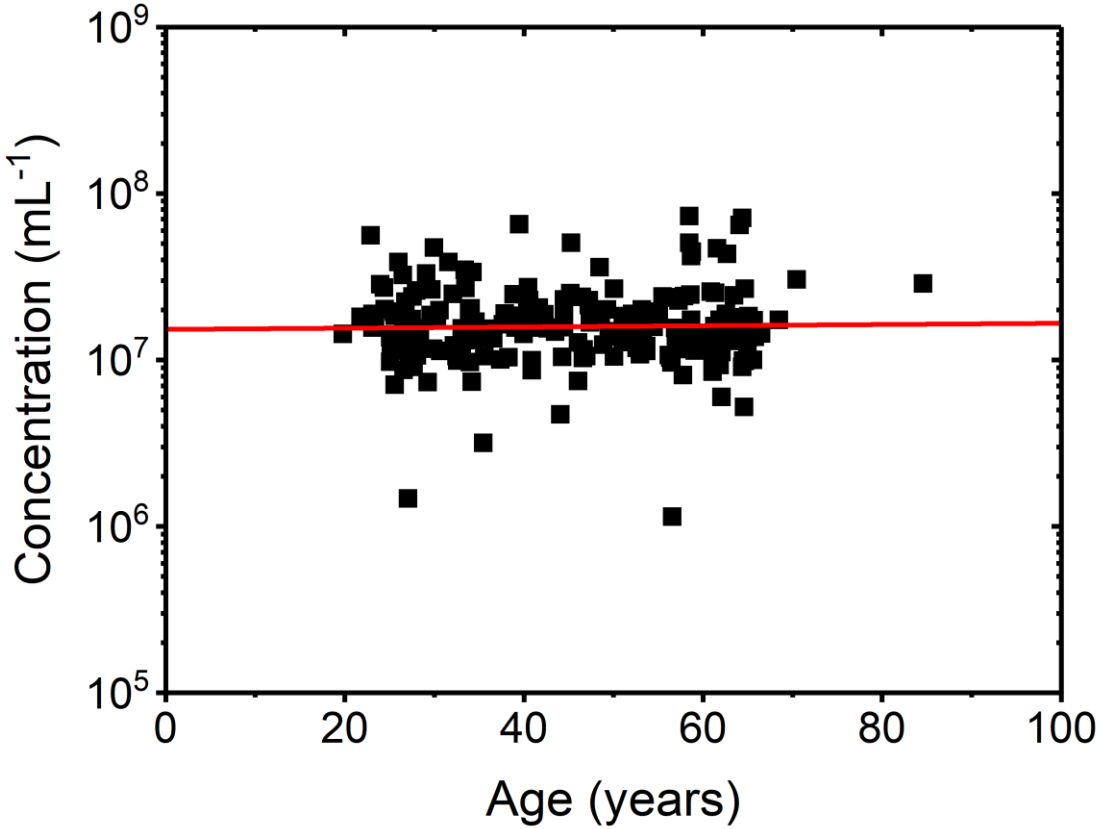
Reference values of EVs in human plasma

- 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





Summary

- Calibrated, reproducible and well-controlled flow cytometry measurements of EVs are in place
- Read the *EV flow cytometry compendium*, calibrate, report and…

…start exploring!

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- Amsterdam University Medical Centers
  - Vesicle Observation Center
- EV flow cytometry working group
  - Joshua Welsh
- Hungarian Academy of Sciences
  - Zoltan Varga

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

- Funding
  - EURAMET project 18HLT01 (METVES II)