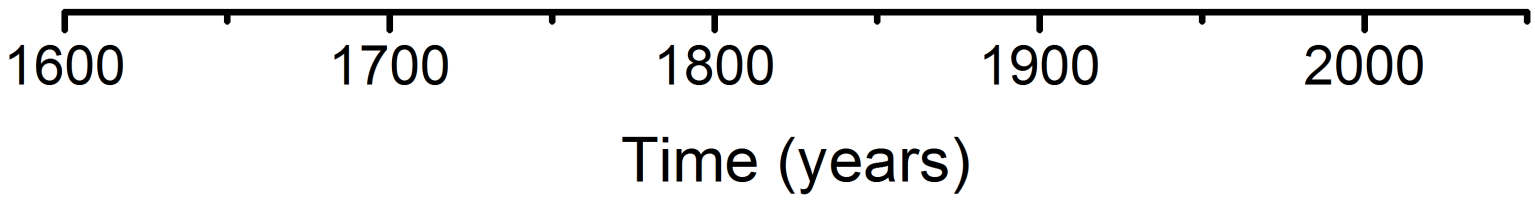
Detection of extracellular vesicles by flow cytometry: size does matter

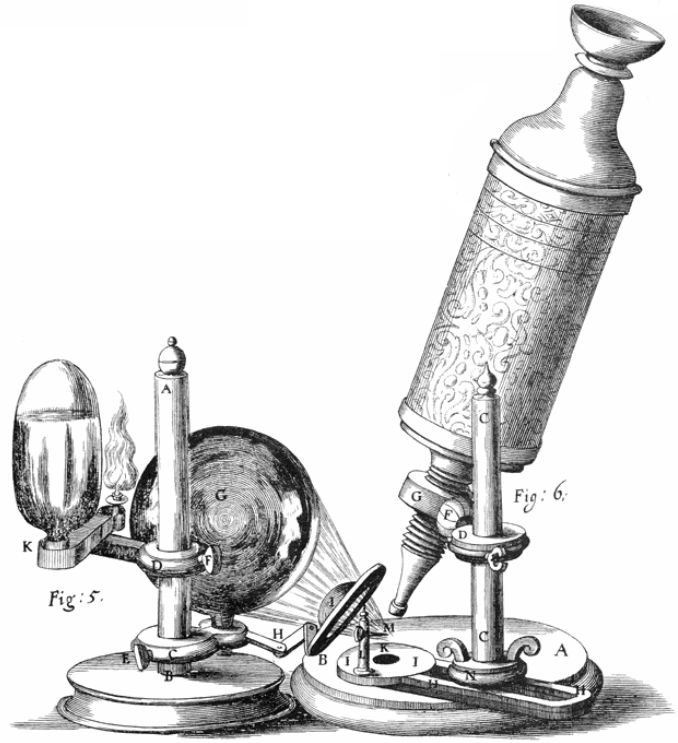
Edwin van der Pol

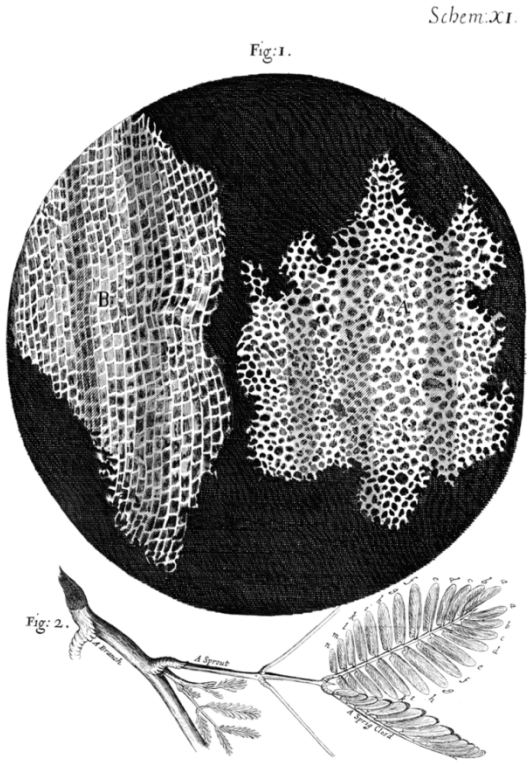
Vesicle
Observation Center

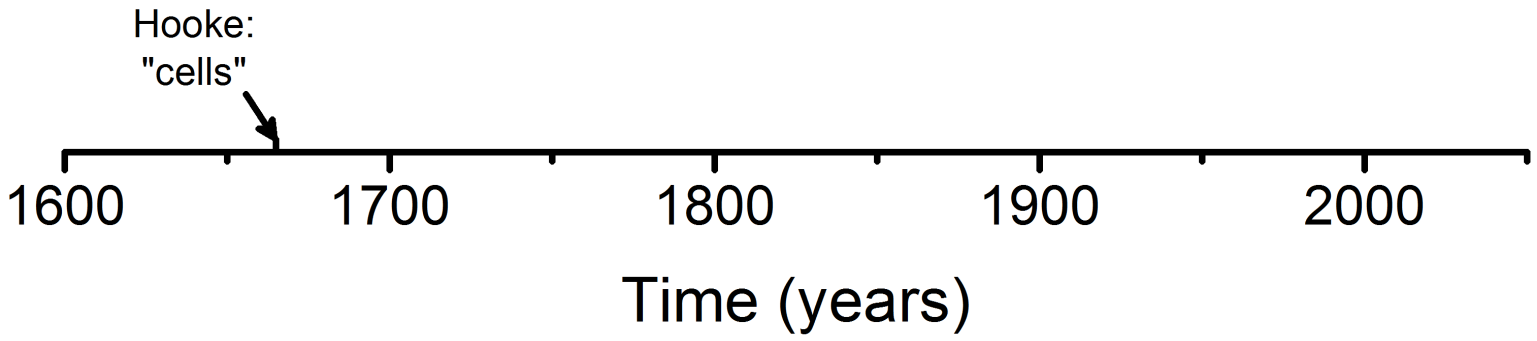
November 6th, 2018

Amsterdam UMC
University Medical Centers

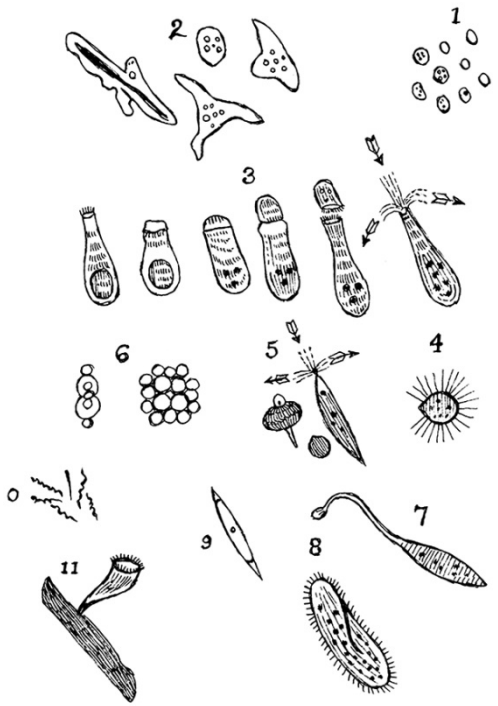

1600
1700
1800
1900
2000
Time (years)







images: R. Hooke *Micrographia* 1665



van Leeuwenhoek:
"animalcules"

Hooke:
"cells"

1600 1700 1800 1900 2000

Time (years)

image: A. van Leeuwenhoek *Royal society* 1675

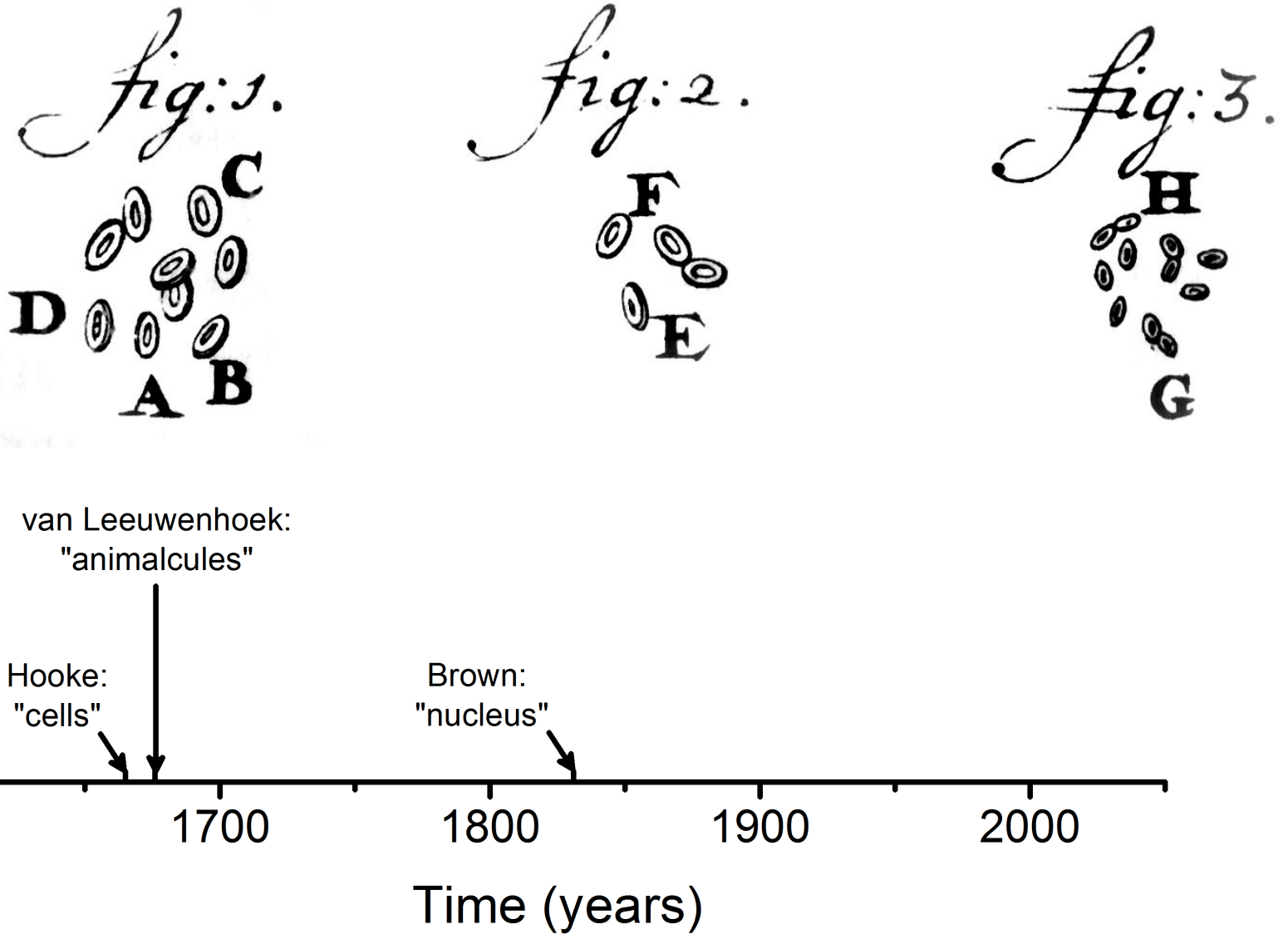


image: A. van Leeuwenhoek *Opera Omnia* 1719

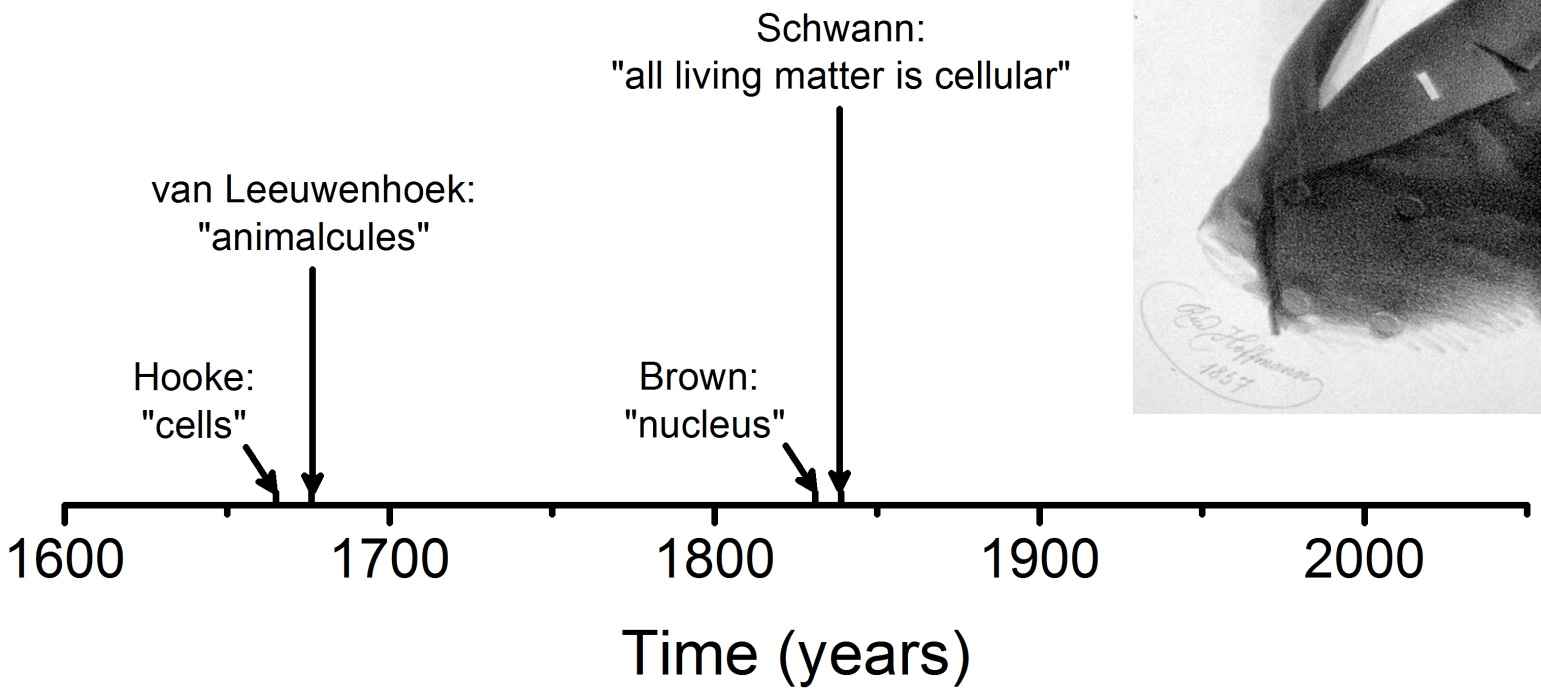


image: Österreichische Nationalbibliothek (Vienna)

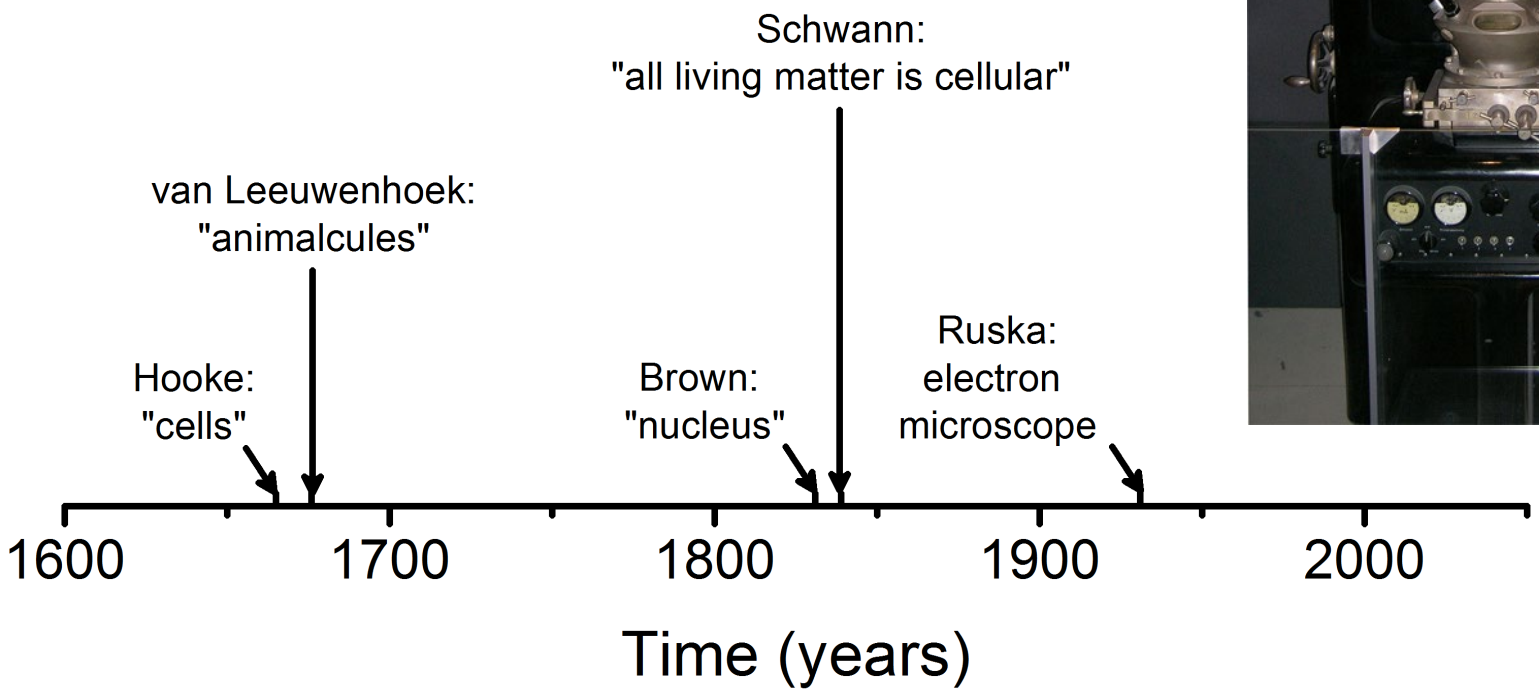
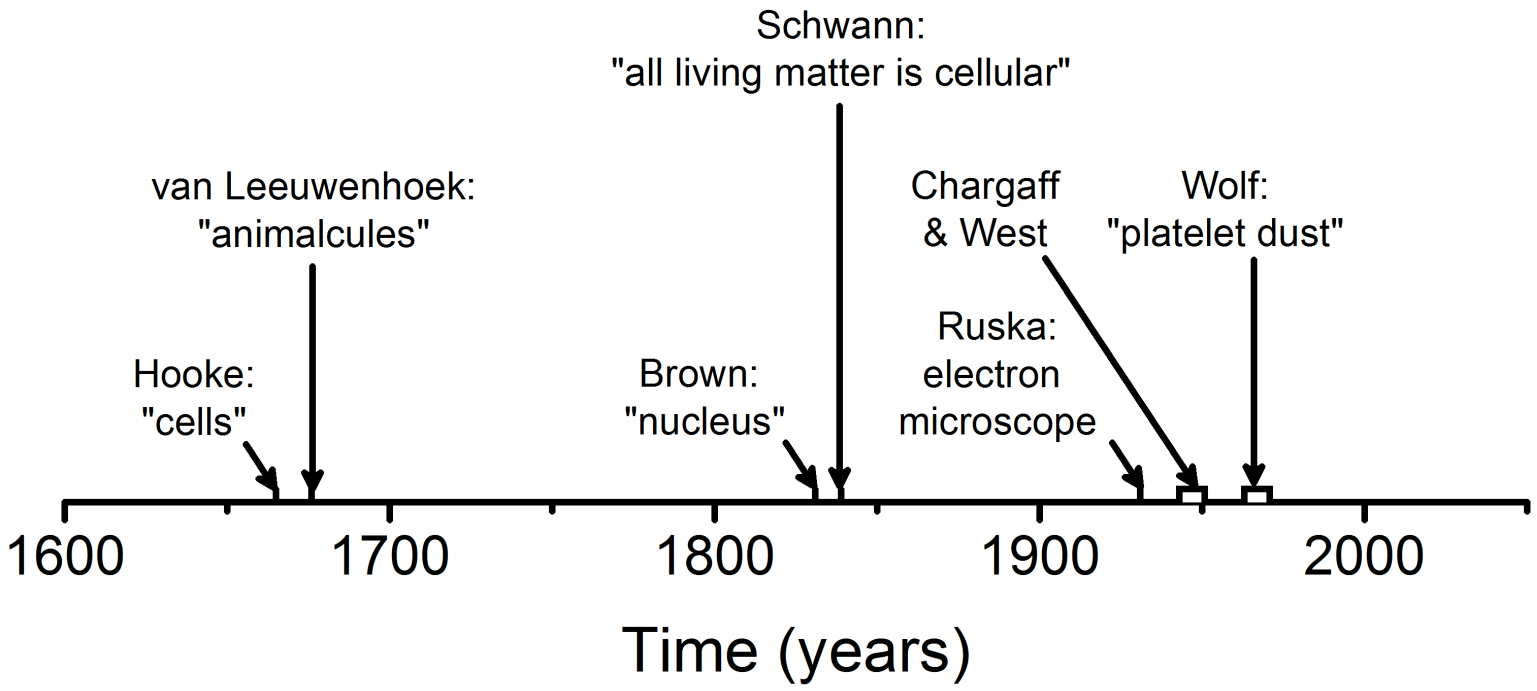


image: Deutsches Museum

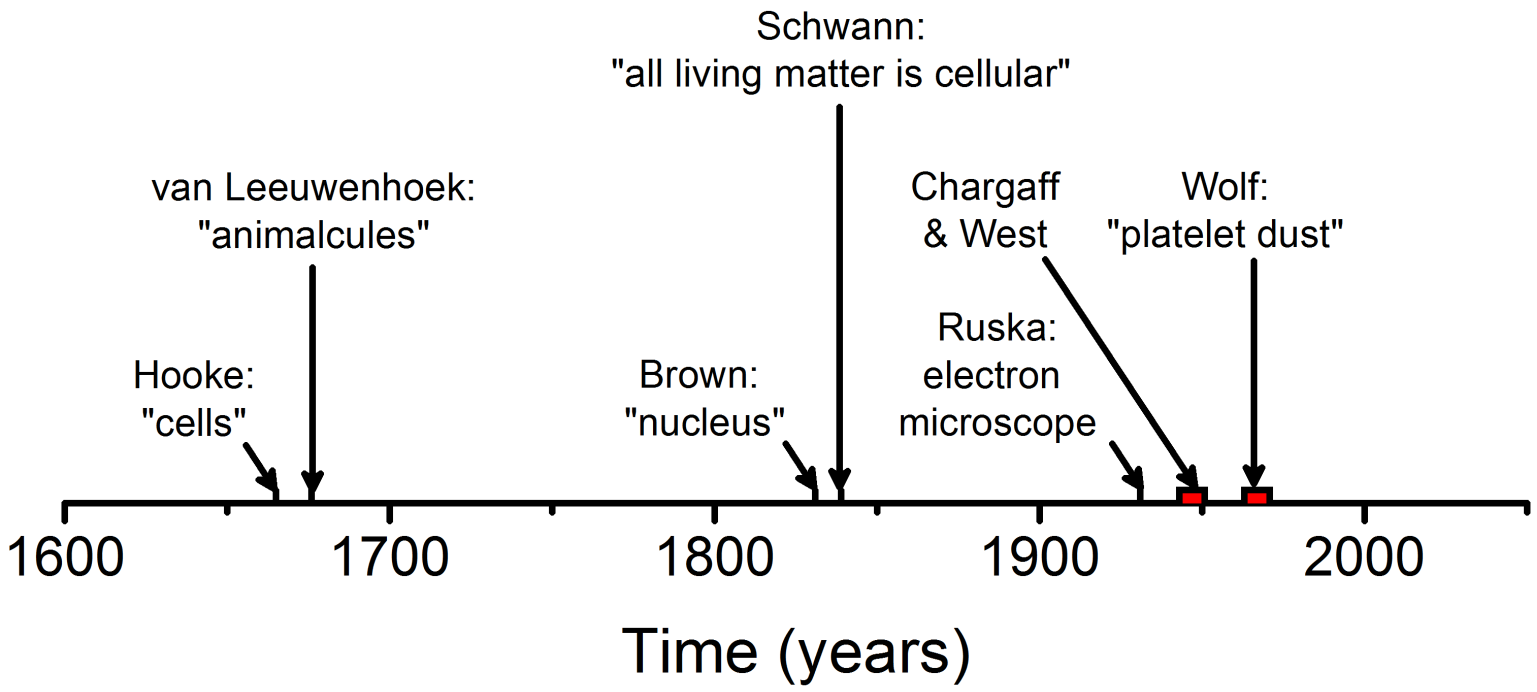
SUMMARY

Fresh plasma freed of intact platelets can be shown to contain minute particulate material (platelet-dust) which can be separated by ultracentrifugation.

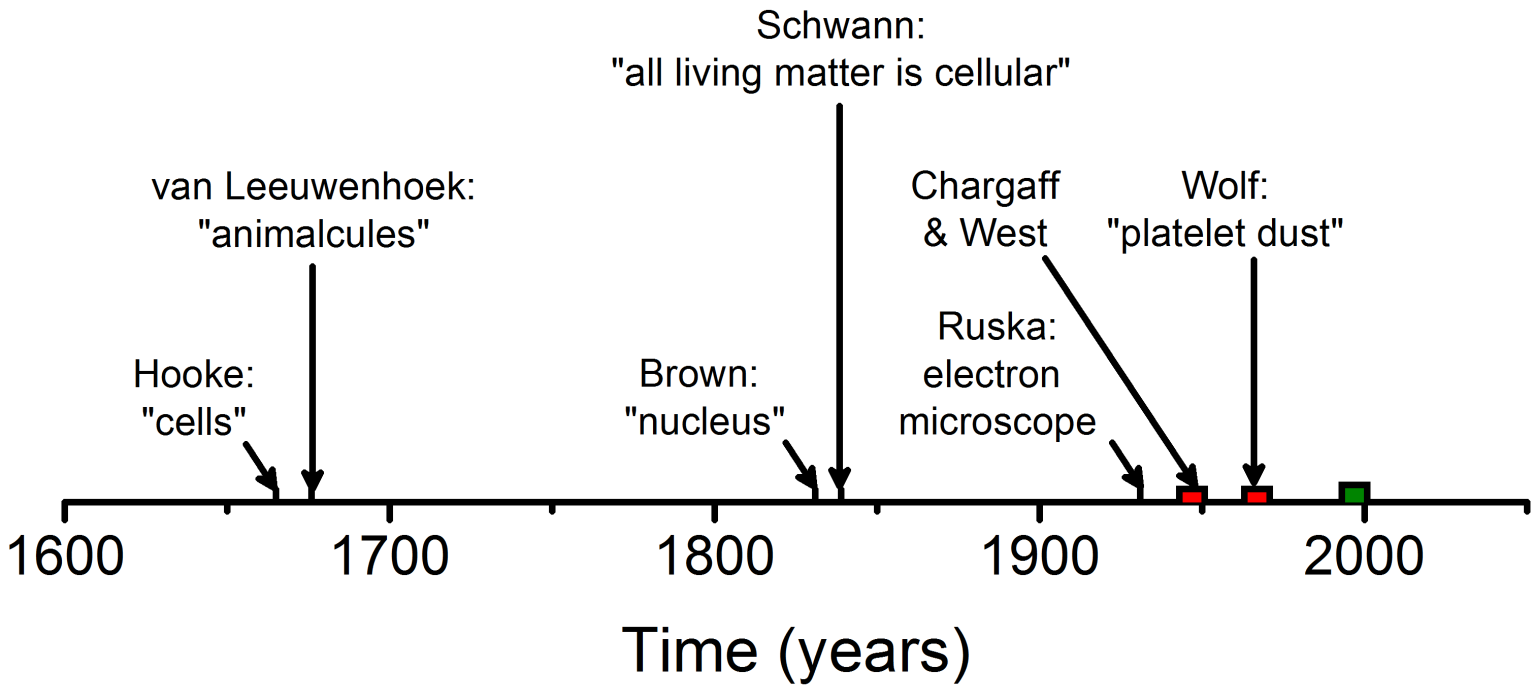


summary: Wolf *Brit.J.Haemat.* 1967

- Sleeping beauties



- Sleeping beauties
- Prince?



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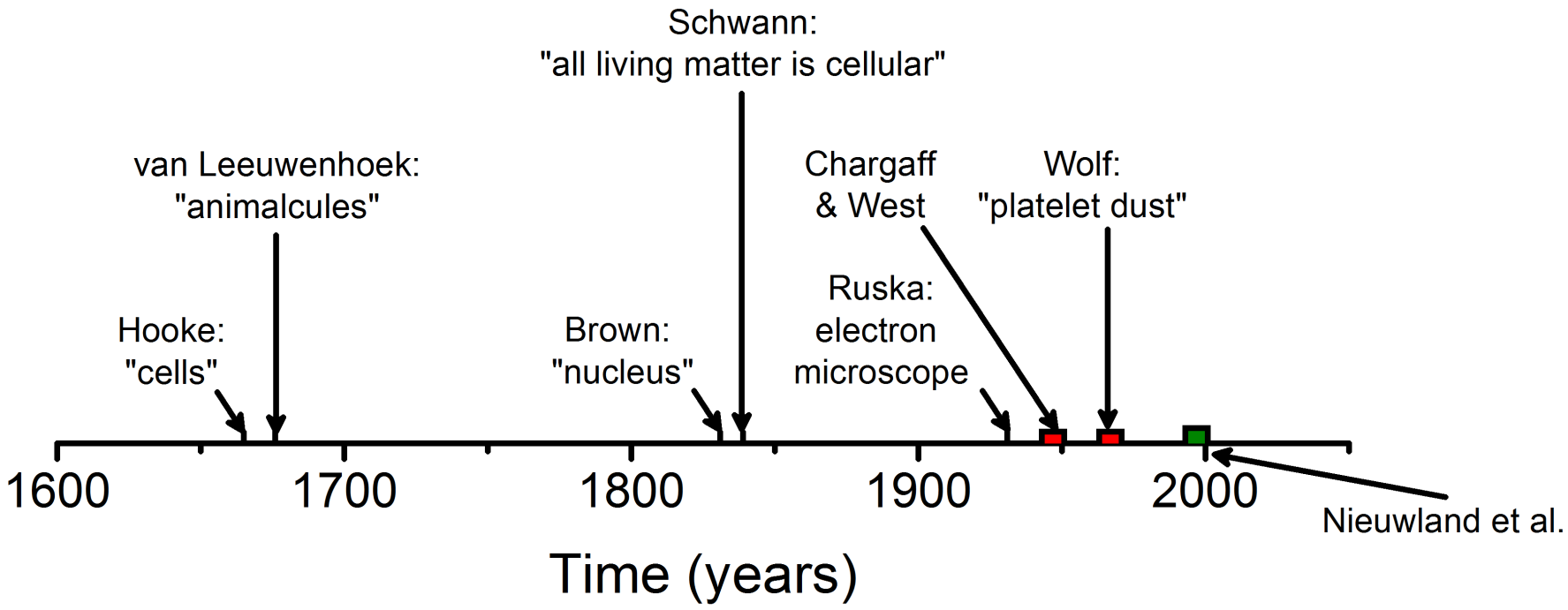

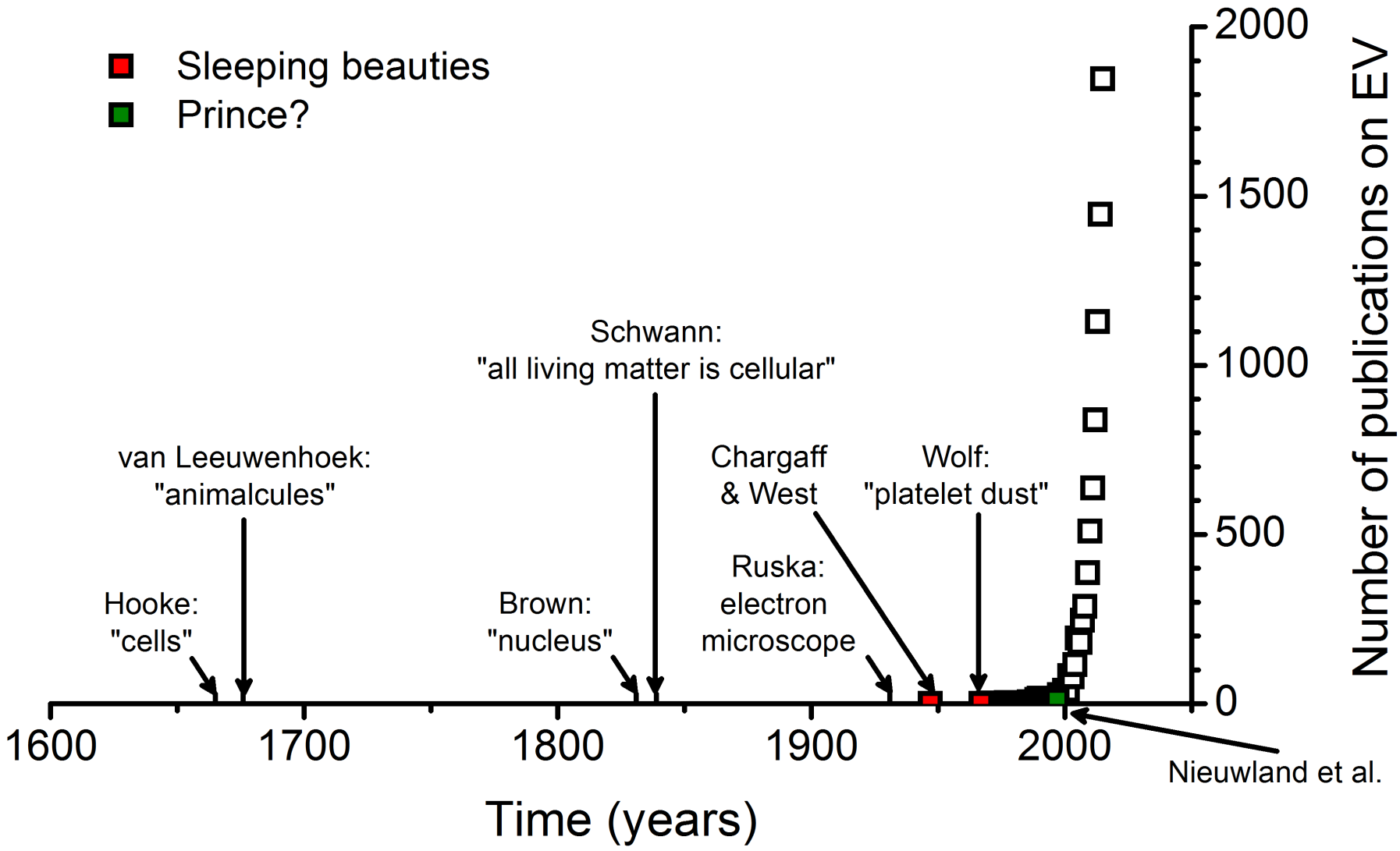
Sleeping beauties
Prince?
Schwann:
"all living matter is cellular"
van Leeuwenhoek:
"animalcules"
Chargaff
& West
Wolf:
"platelet dust"
Ruska:
electron
microscope
Hooke:
"cells"
Brown:
"nucleus"
Nieuwland et al.
2000
1500
1000
500
0
Number of publications on EV
1600
1700
1800
1900
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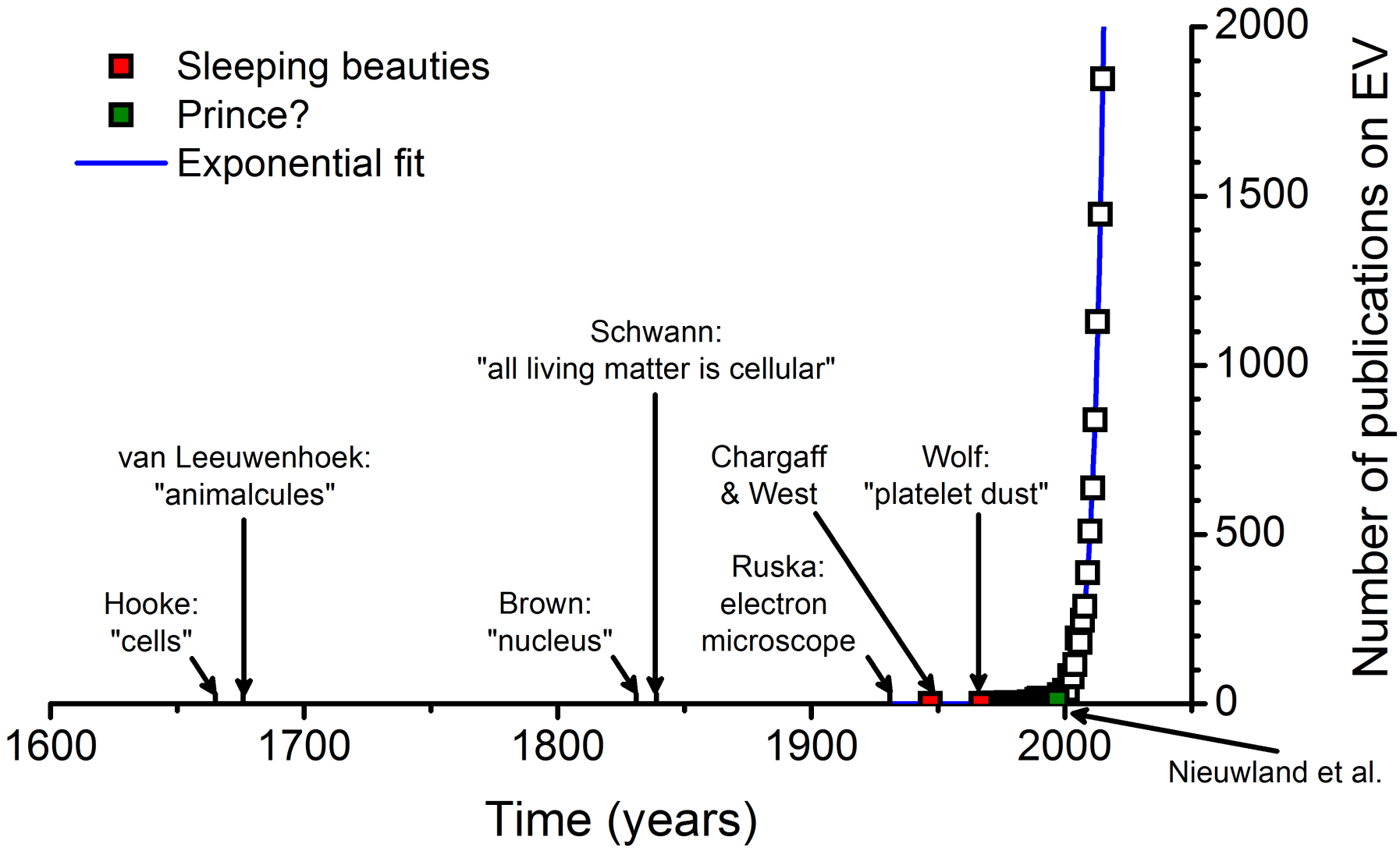
Time (years)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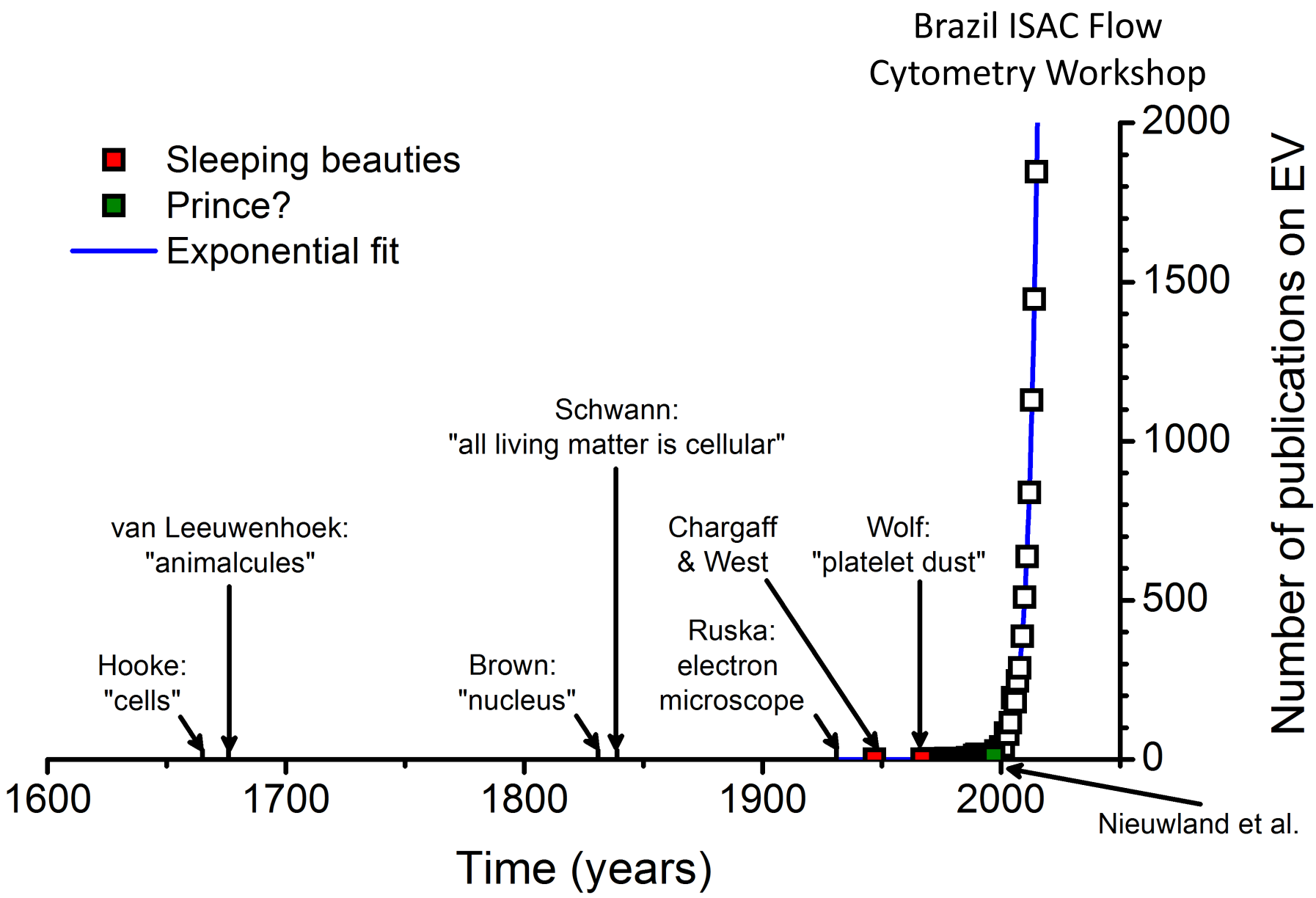
Brazil ISAC Flow
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2000
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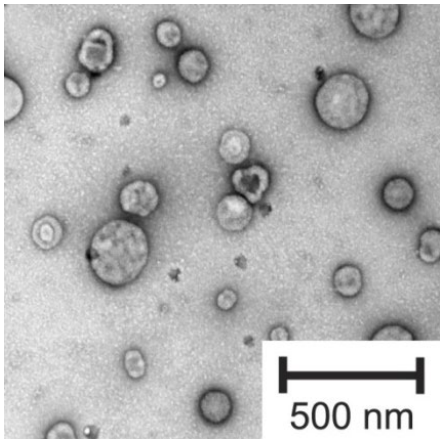
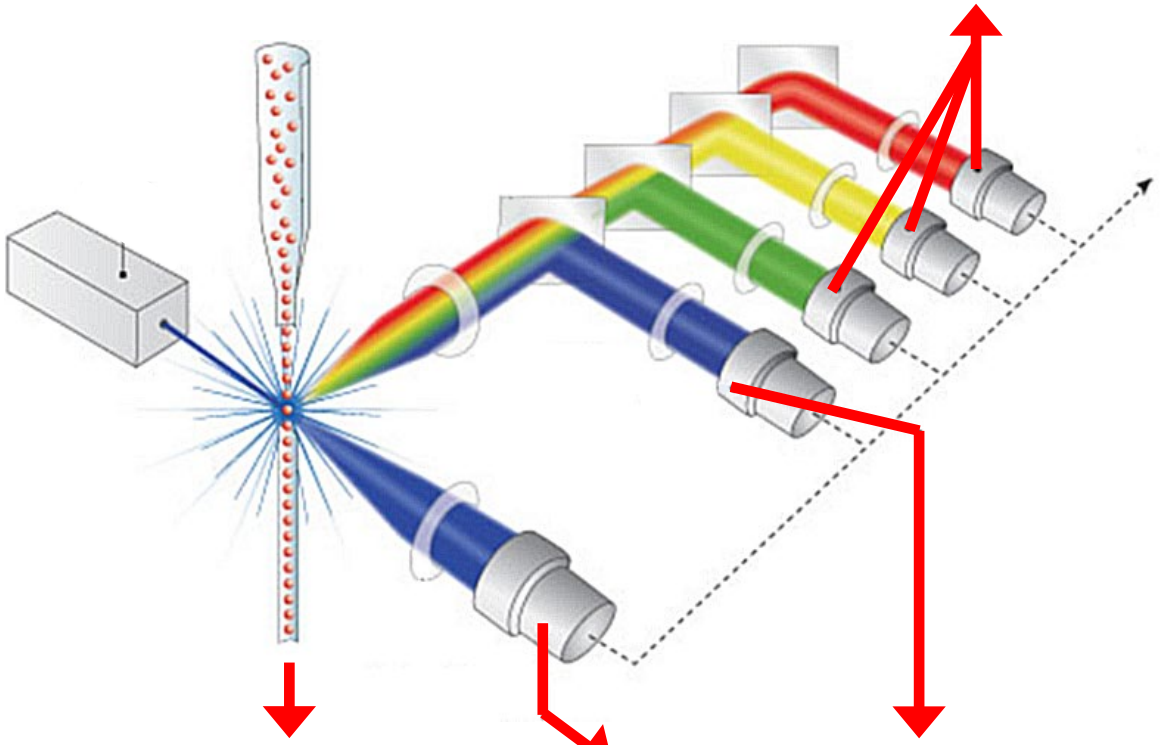
Outline

1. Extracellular vesicles (EVs)



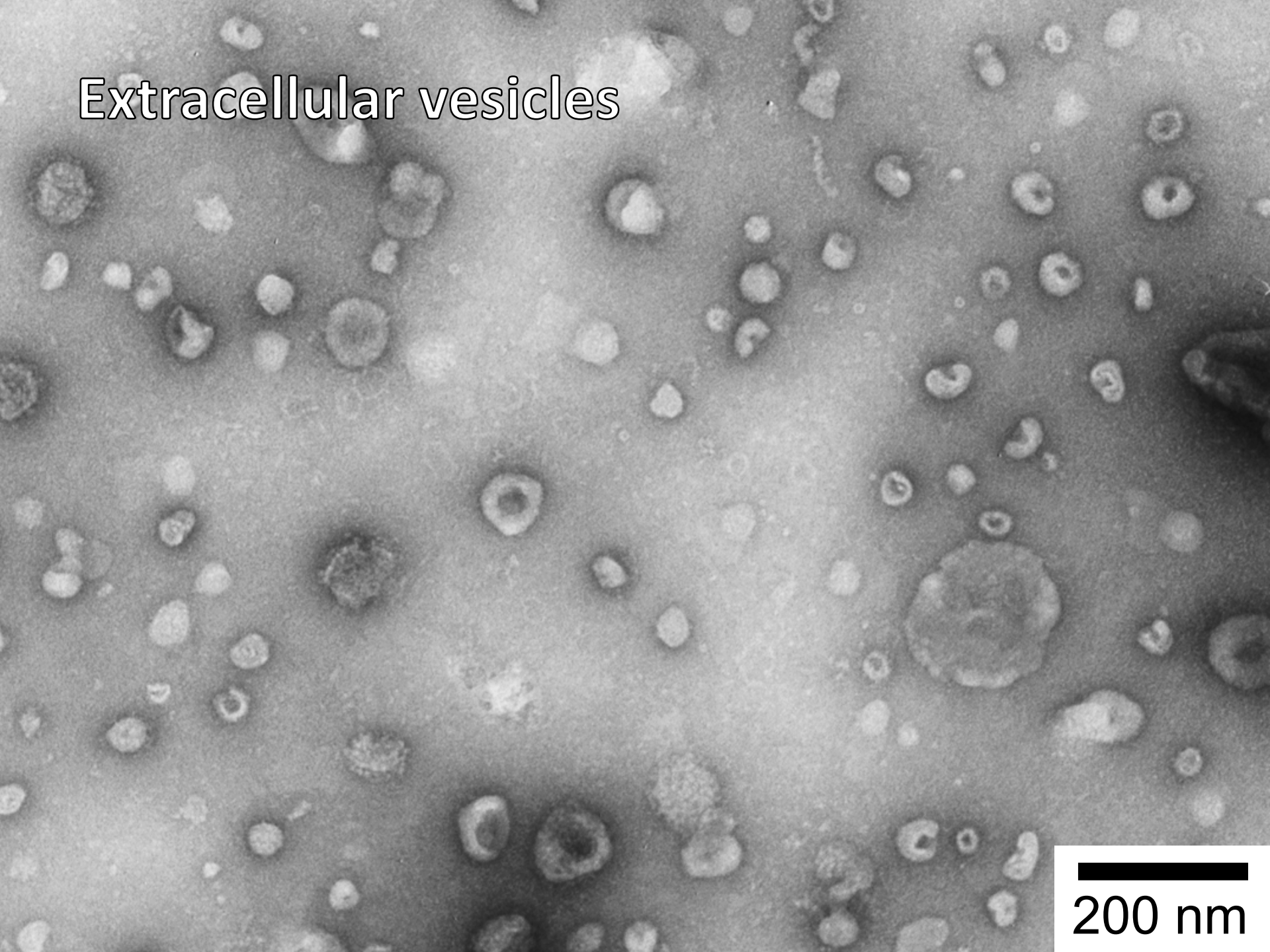
3. Fluorescence



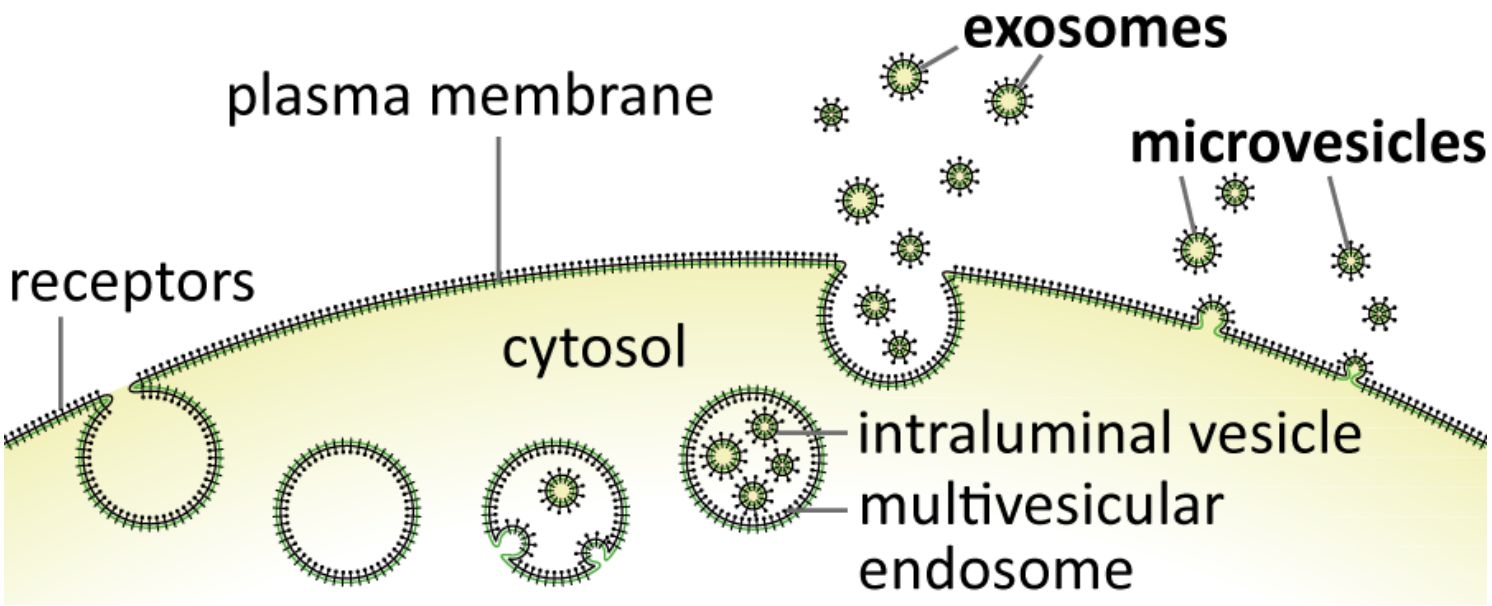
4. Flow rate

2. Light scatter

image: semrock.com


Extracellular vesicles
200 nm

Extracellular vesicles

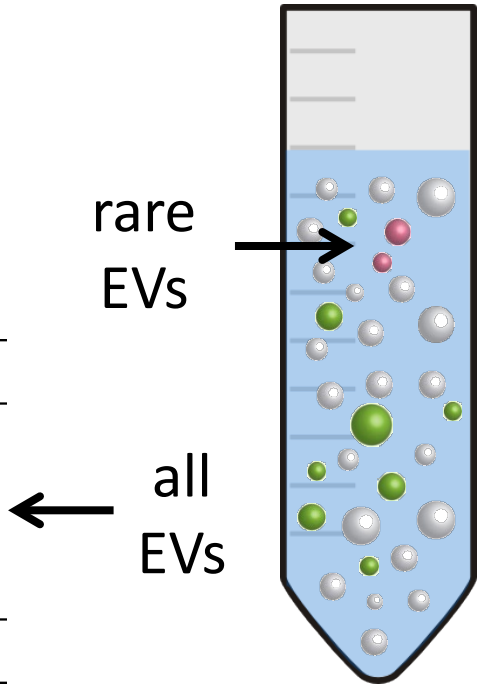


- Cells release EVs:
  biological nanoparticles with receptors, DNA, RNA
- Specialized functions
- Clinically relevant

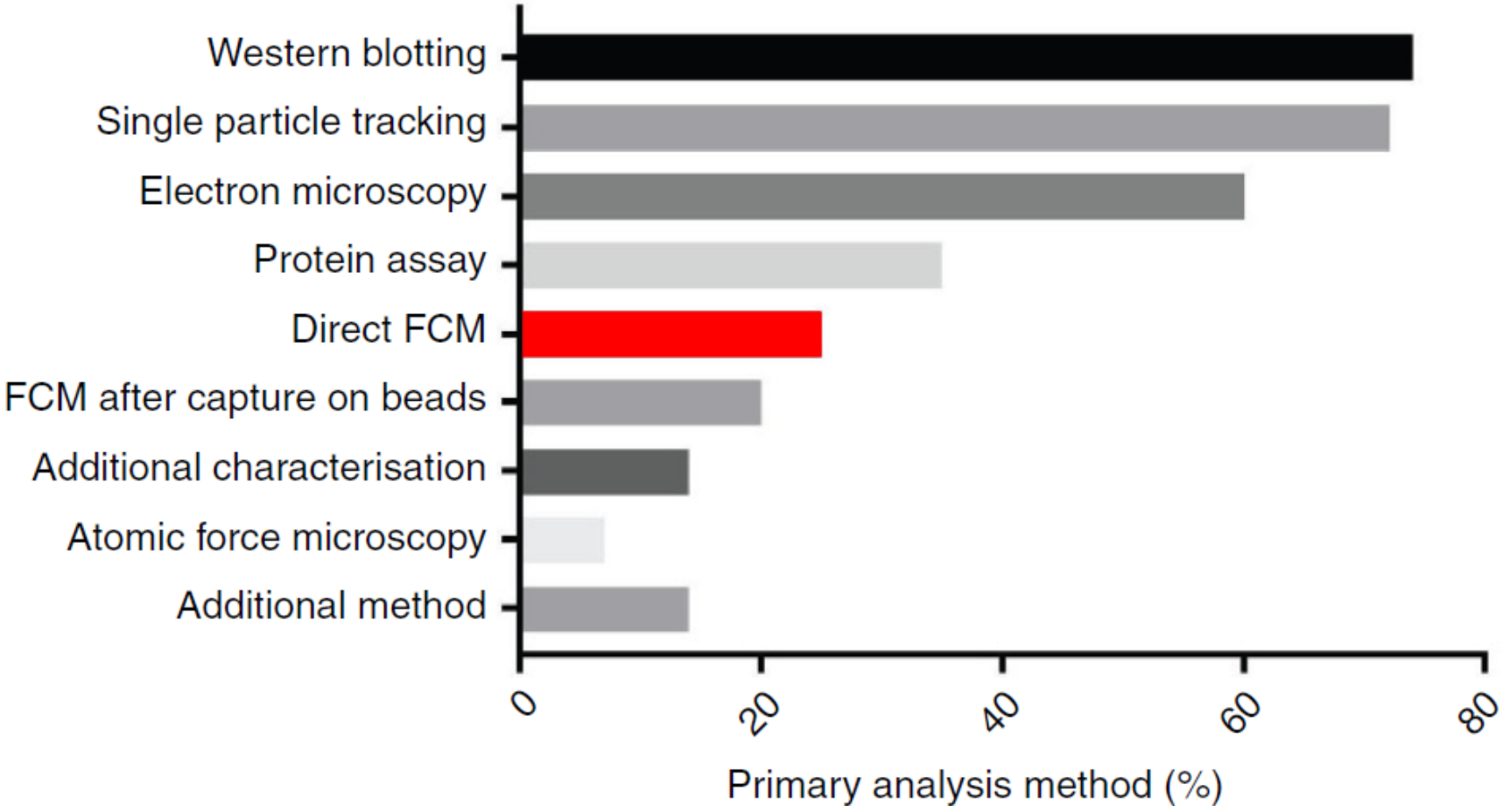
van der Pol et al. *Pharmacol Rev* 2012

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	$7.3 - 9.4 \cdot 10^{10}$



EV research using flow cytometry

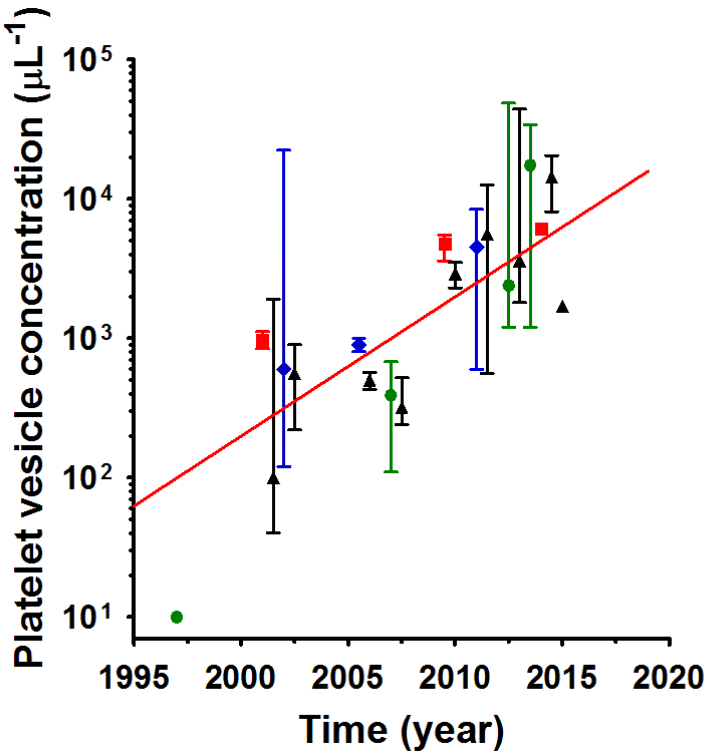


Gardiner et al. *J Extracell Vesicles* 2016

Motivation to detect EVs by flow cytometry

- EVs are heterogeneous
  - Flow cytometry can differentiate EV types
- Study all (also rare) EVs
  - Flow cytometry is fast (>10,000 events s^{-1})

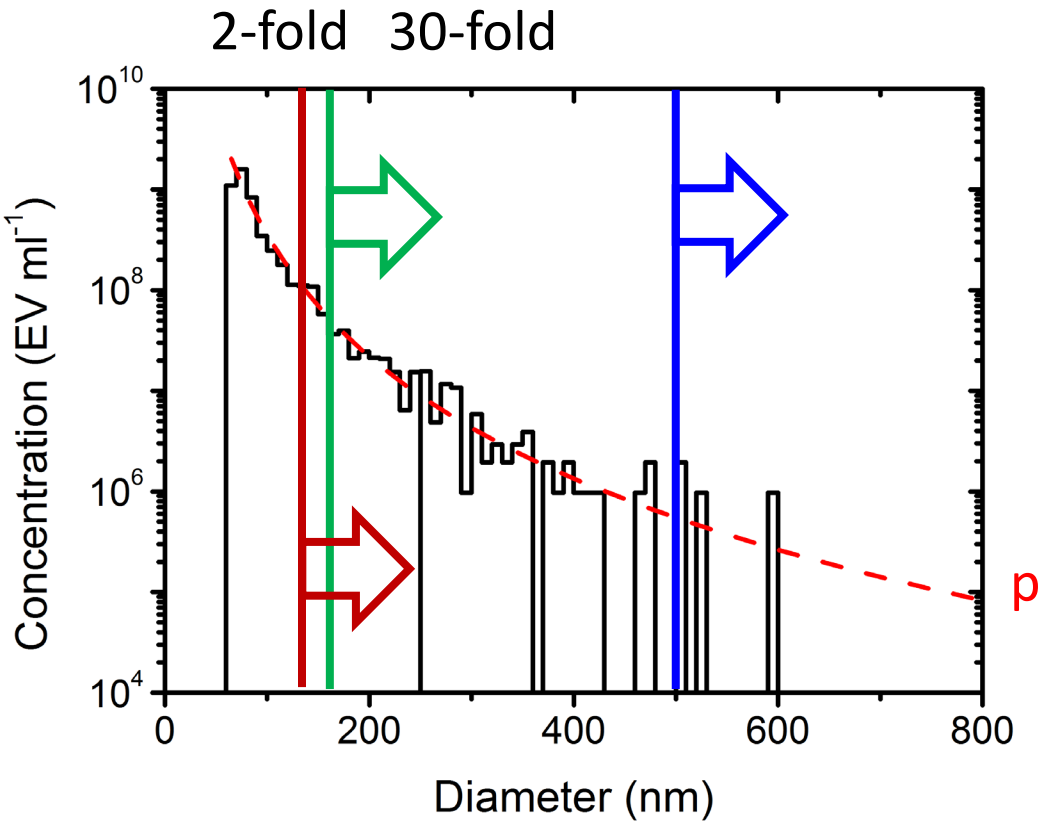
Problem: EV flow cytometry is difficult



"Gąsecka's law"

- Reported concentrations of plasma EVs differ >10^6-fold
- Clinical data cannot be compared

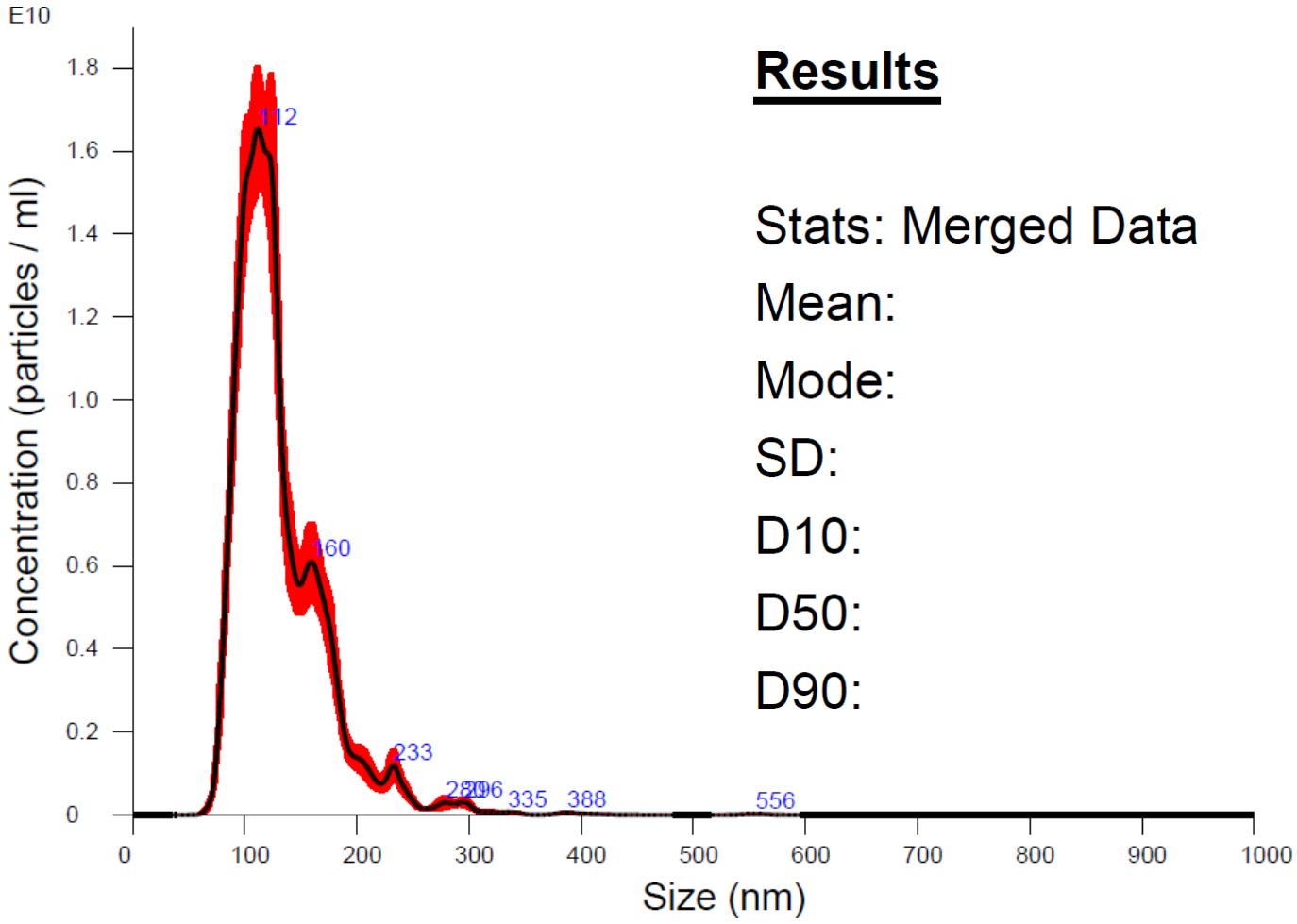
Detection of EVs: size does matter



power-law relation*

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

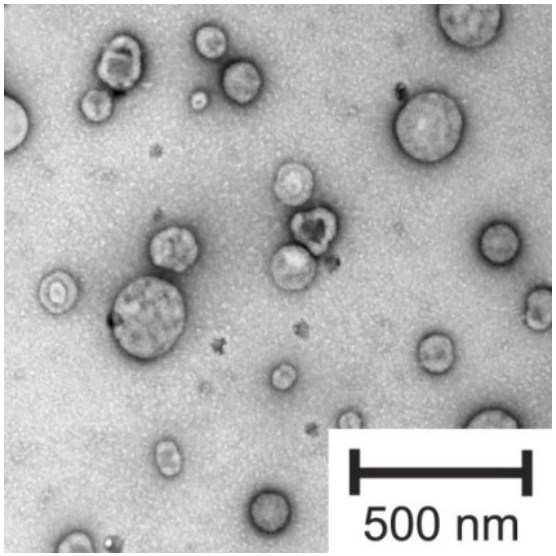
What is this and what is wrong?

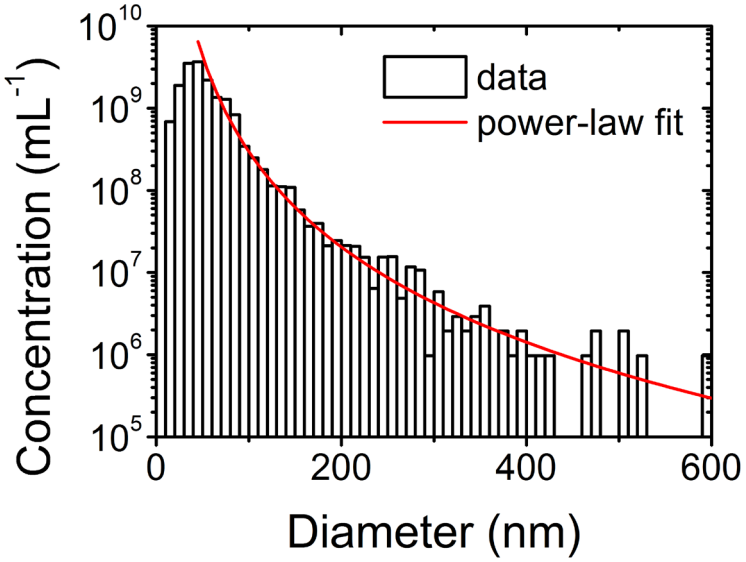


Results

Stats: Merged Data	
Mean:	131.2 nm
Mode:	111.9 nm
SD:	41.7 nm
D10:	91.2 nm
D50:	119.8 nm
D90:	178.5 nm

Summary extracellular vesicles (EVs)





- Body fluids contain EVs with clinical information
- Flow cytometers can identify EV populations
- Size distribution and detection limit determine measured concentration: apply statistics carefully!

Outline

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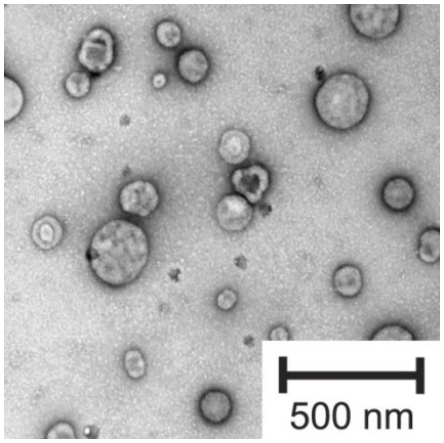

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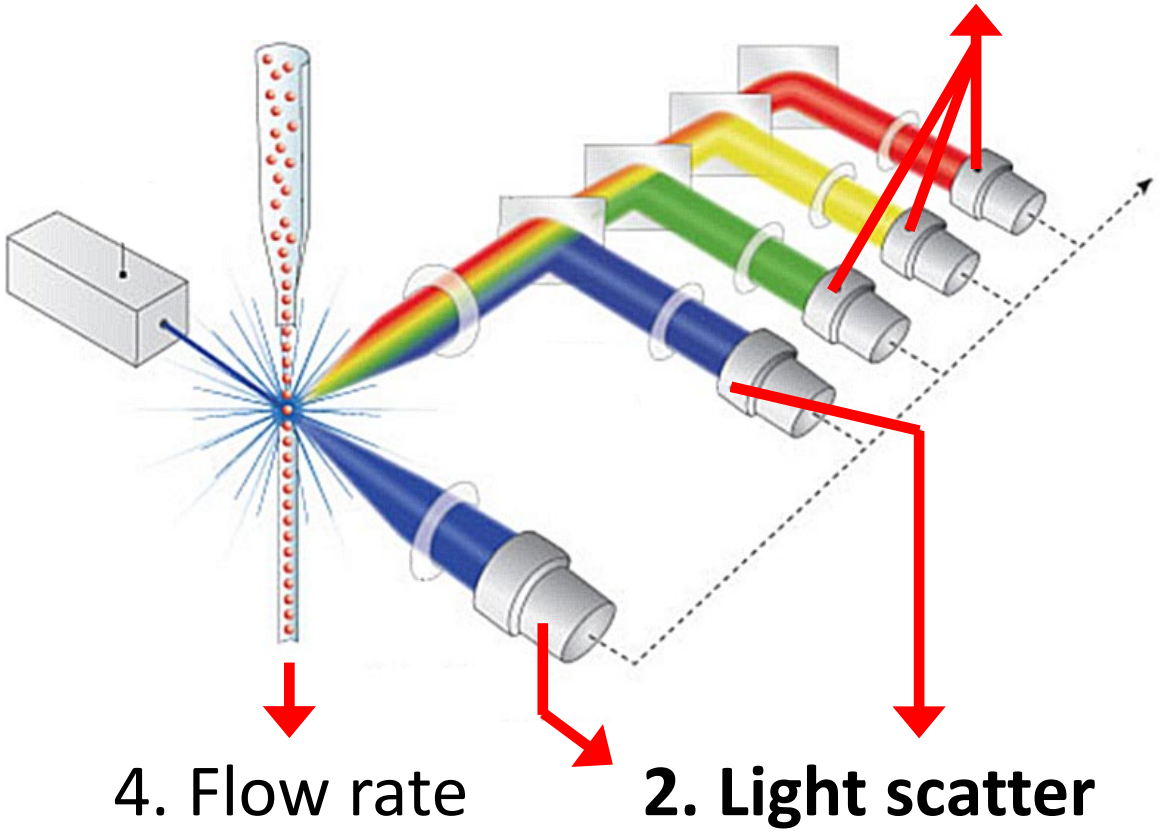
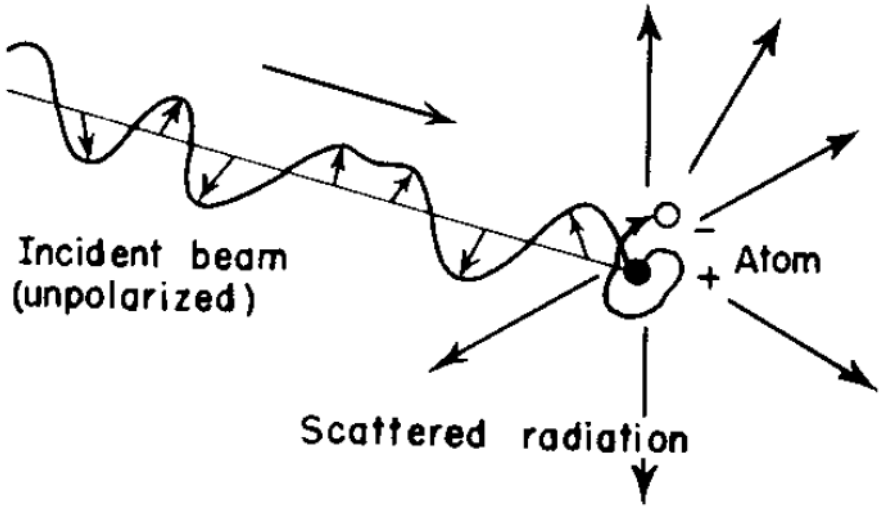


4. Flow rate

2. Light scatter

image: semrock.com

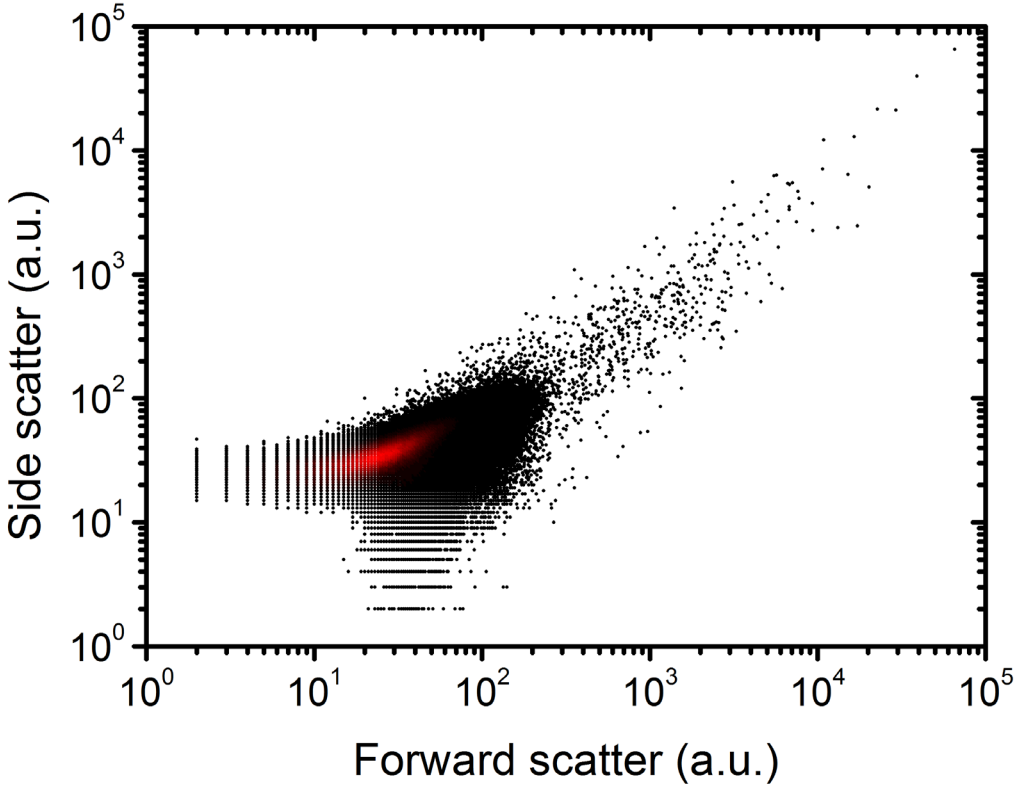
Outline light scatter



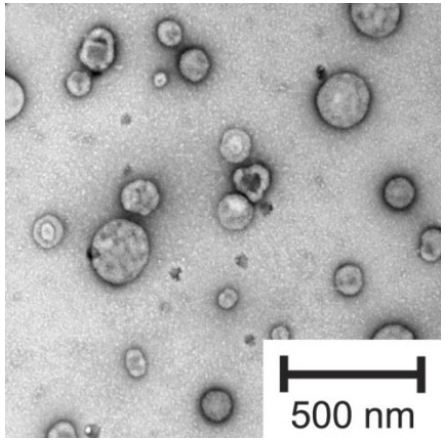
- Flow cytometry detection of EVs with
  - **one scatter detector**
  - two scatter detectors
- Standardization

image: Feynman lectures on physics

Goal: use scatter to interpret EV flow cytometry data



?



Is a “bead size gate” a good idea?

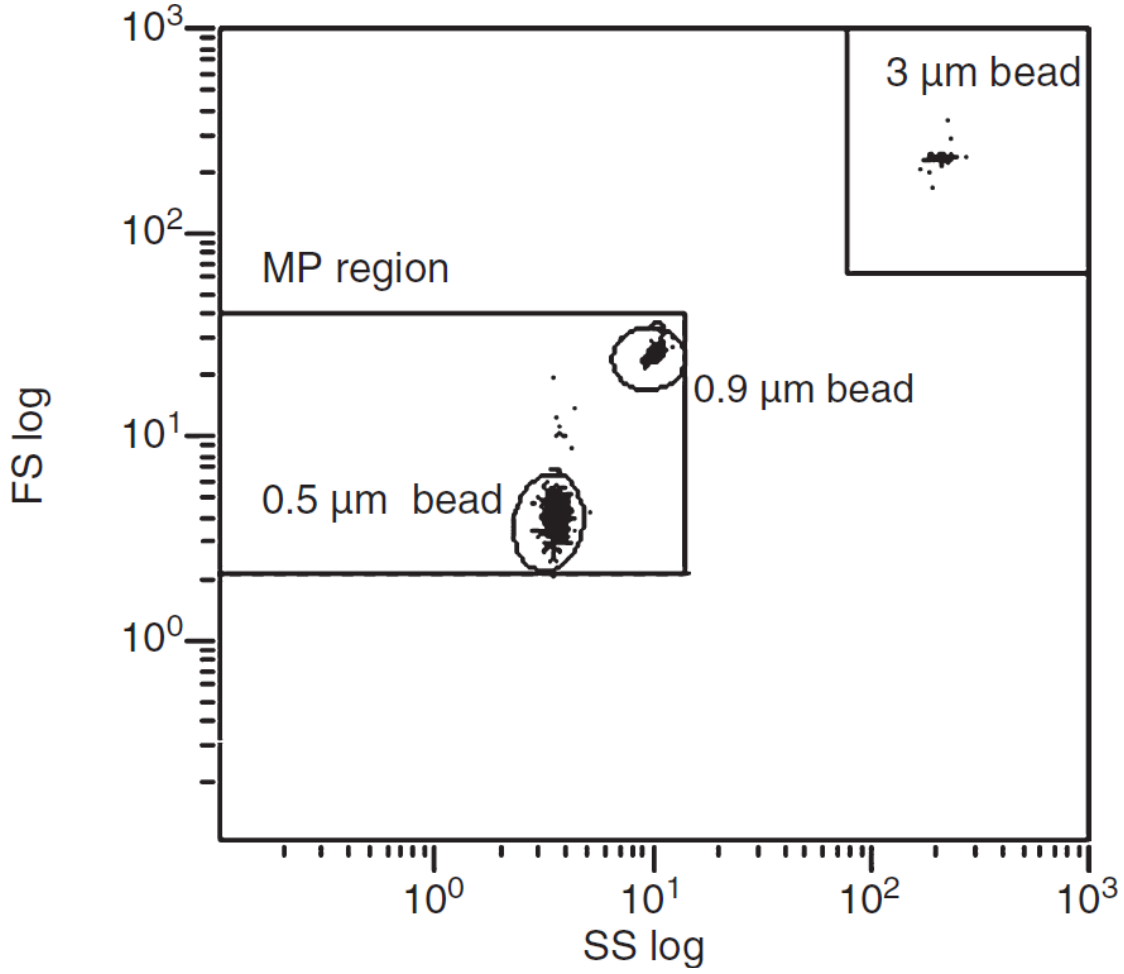
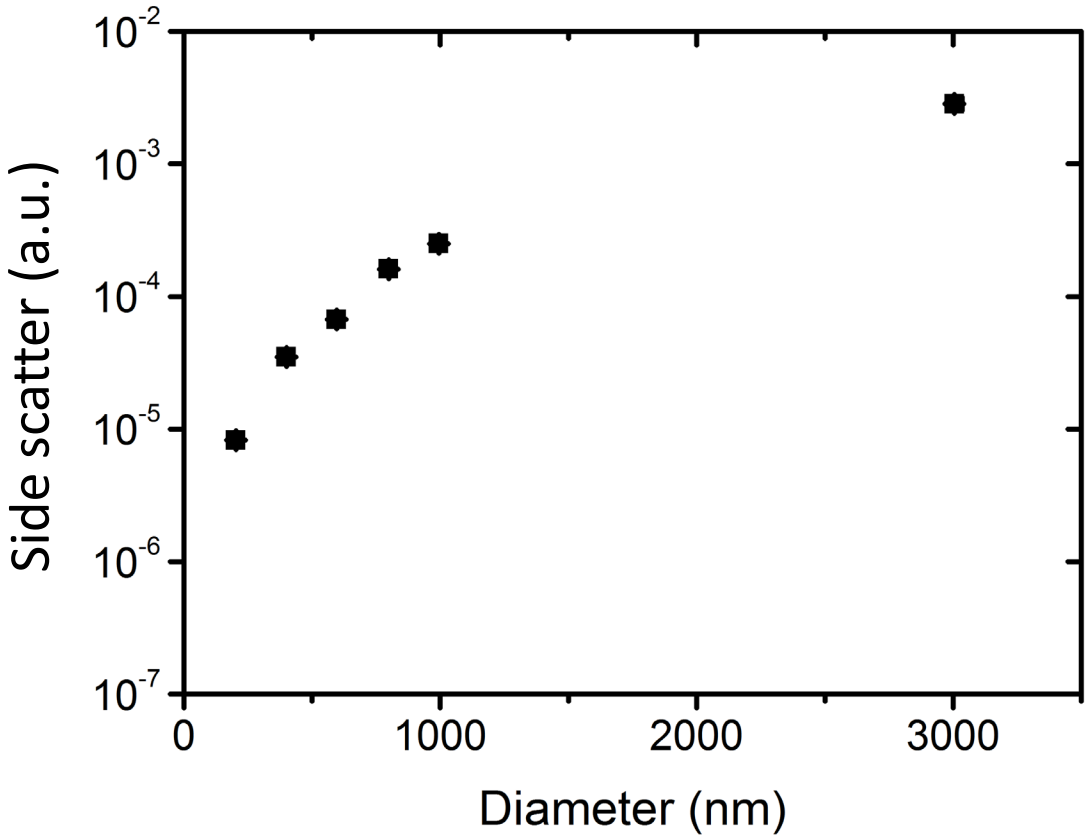


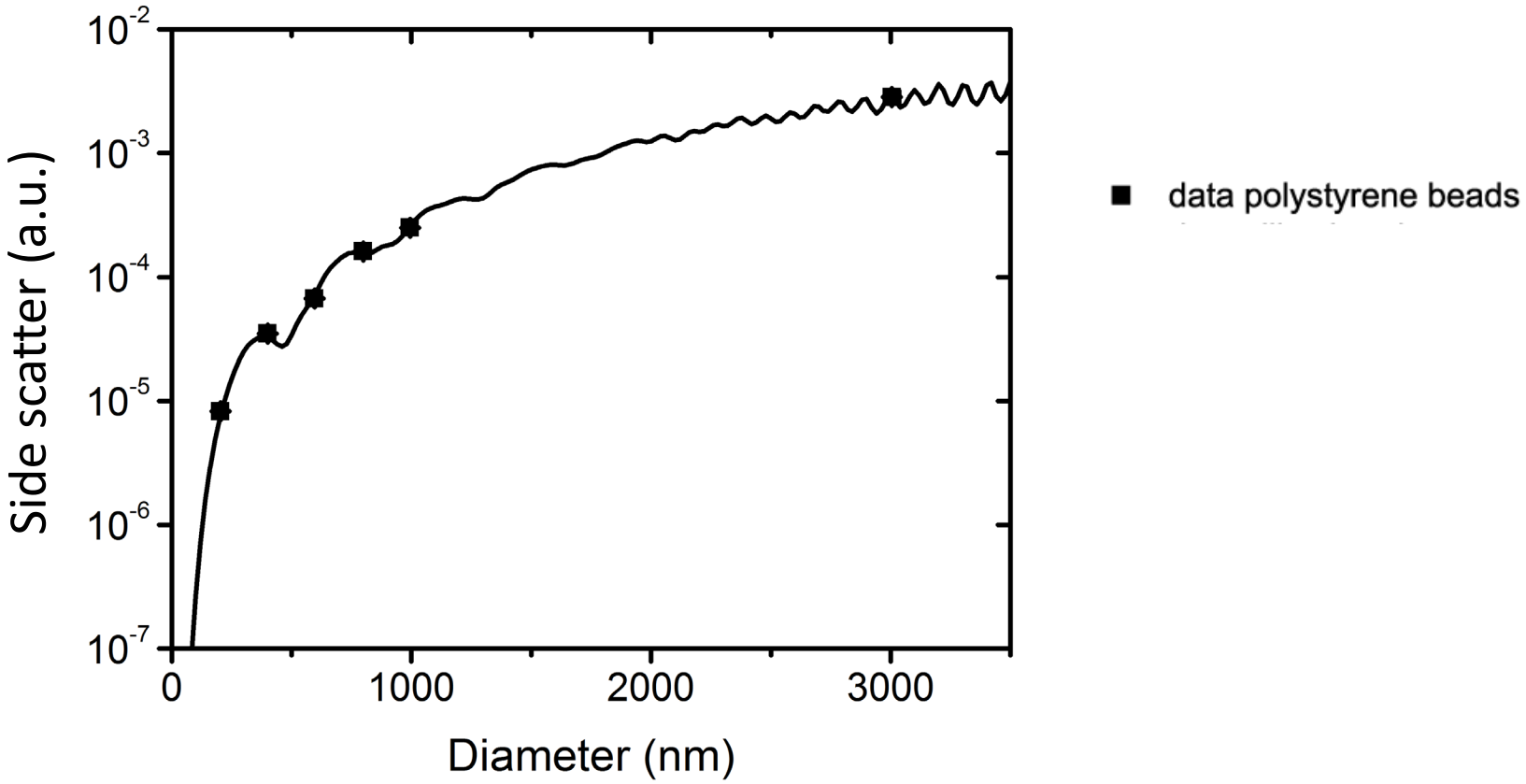
image adopted: Robert et al. *J Thromb Haemost* 2008

Relate scatter to diameter of beads



■ data polystyrene beads

Relate scatter to diameter of beads



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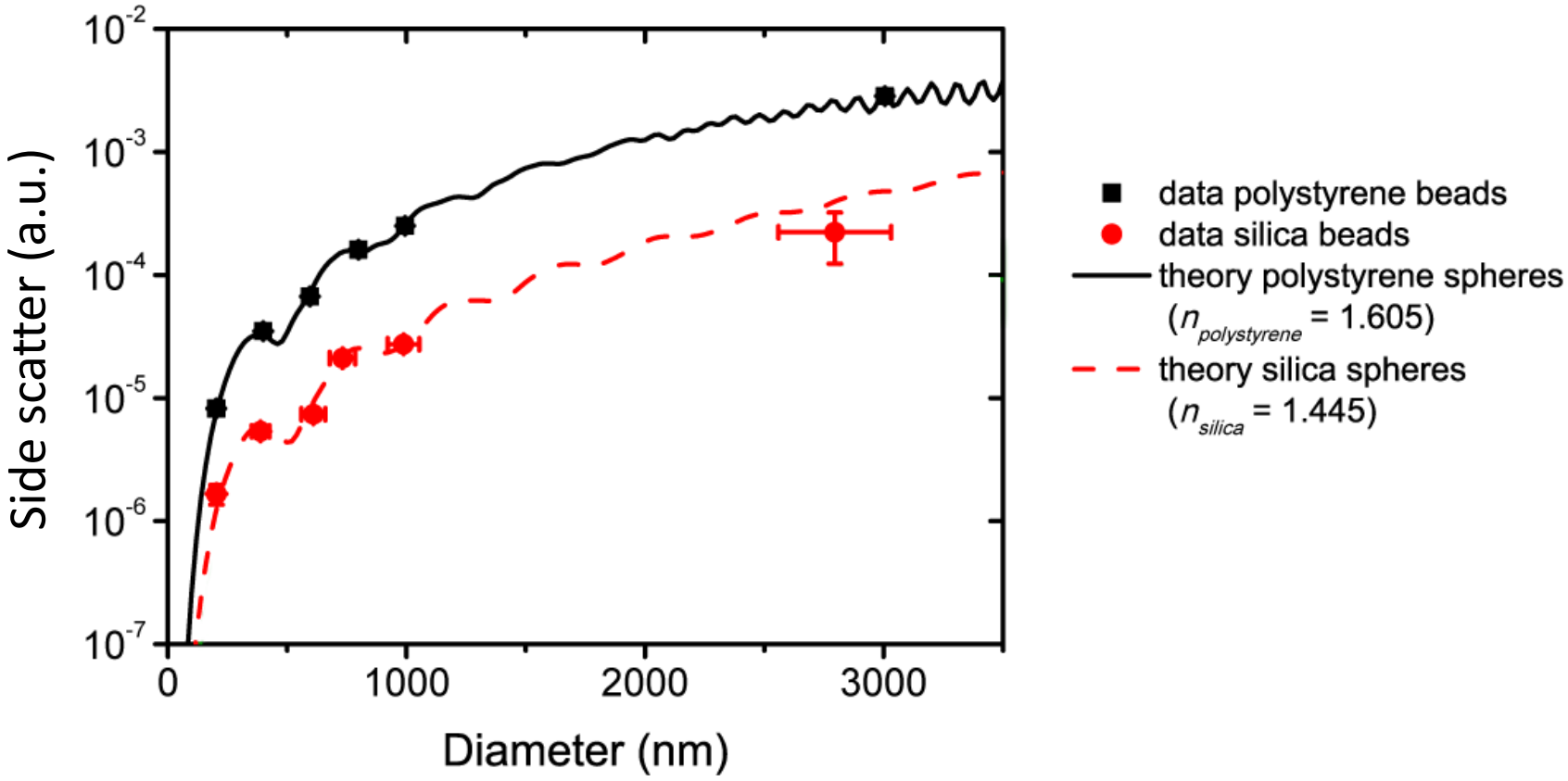
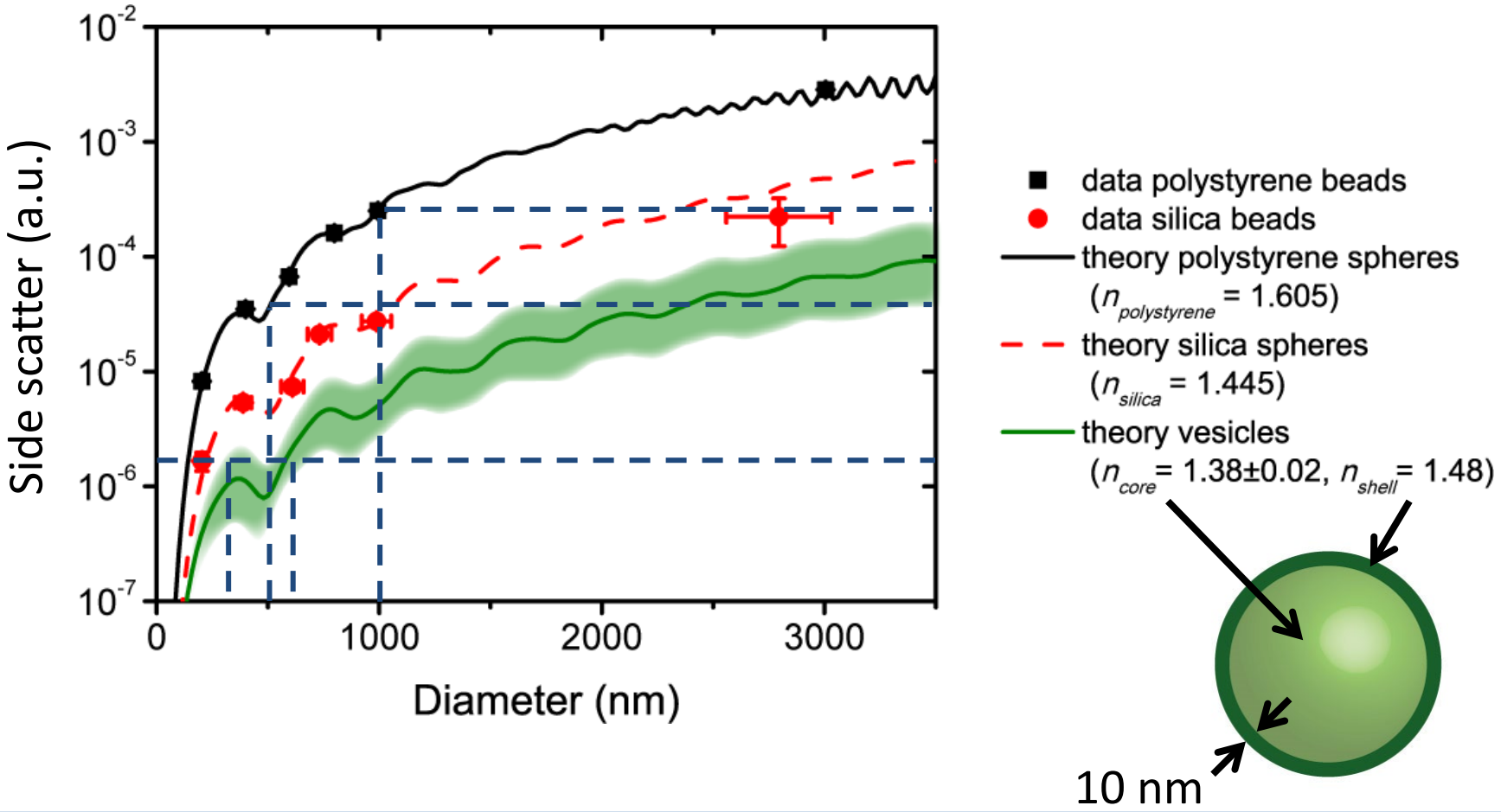
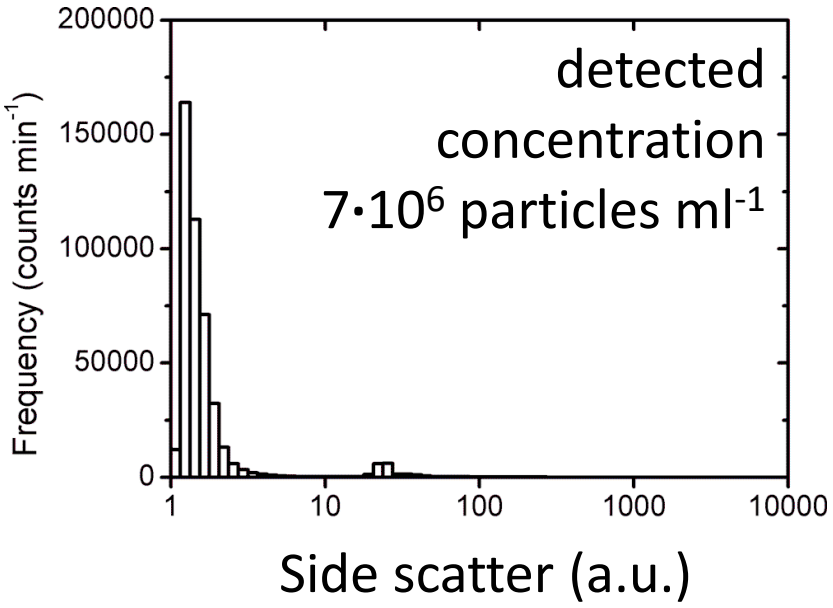


Relate scatter to diameter of vesicles

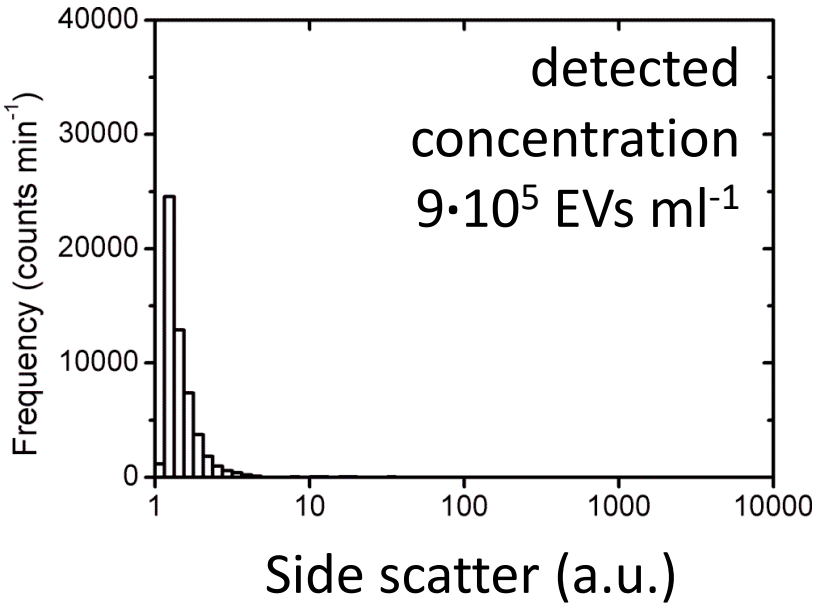


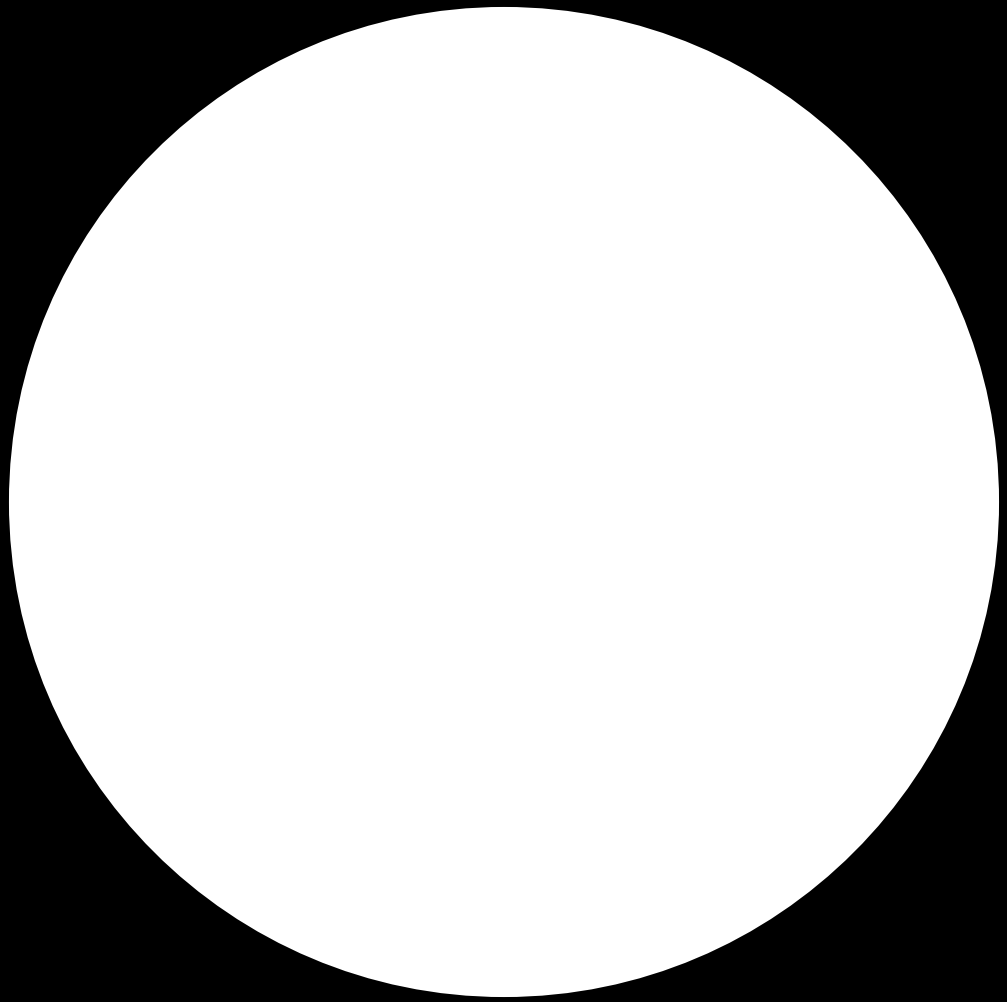
Particles that are too small to be detected generate a signal!

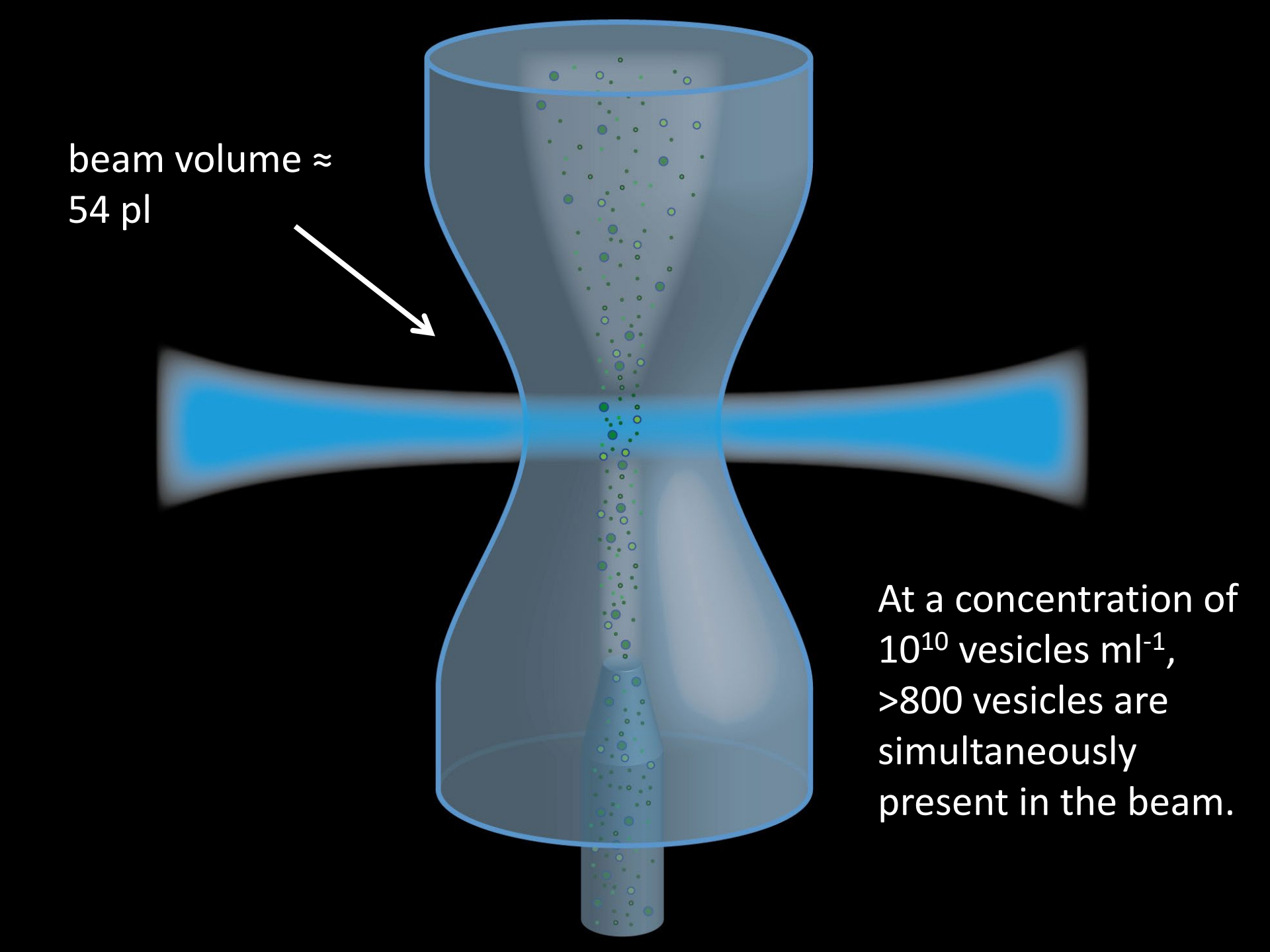
89 nm silica beads at concentration 10^{10} particles ml^{-1}

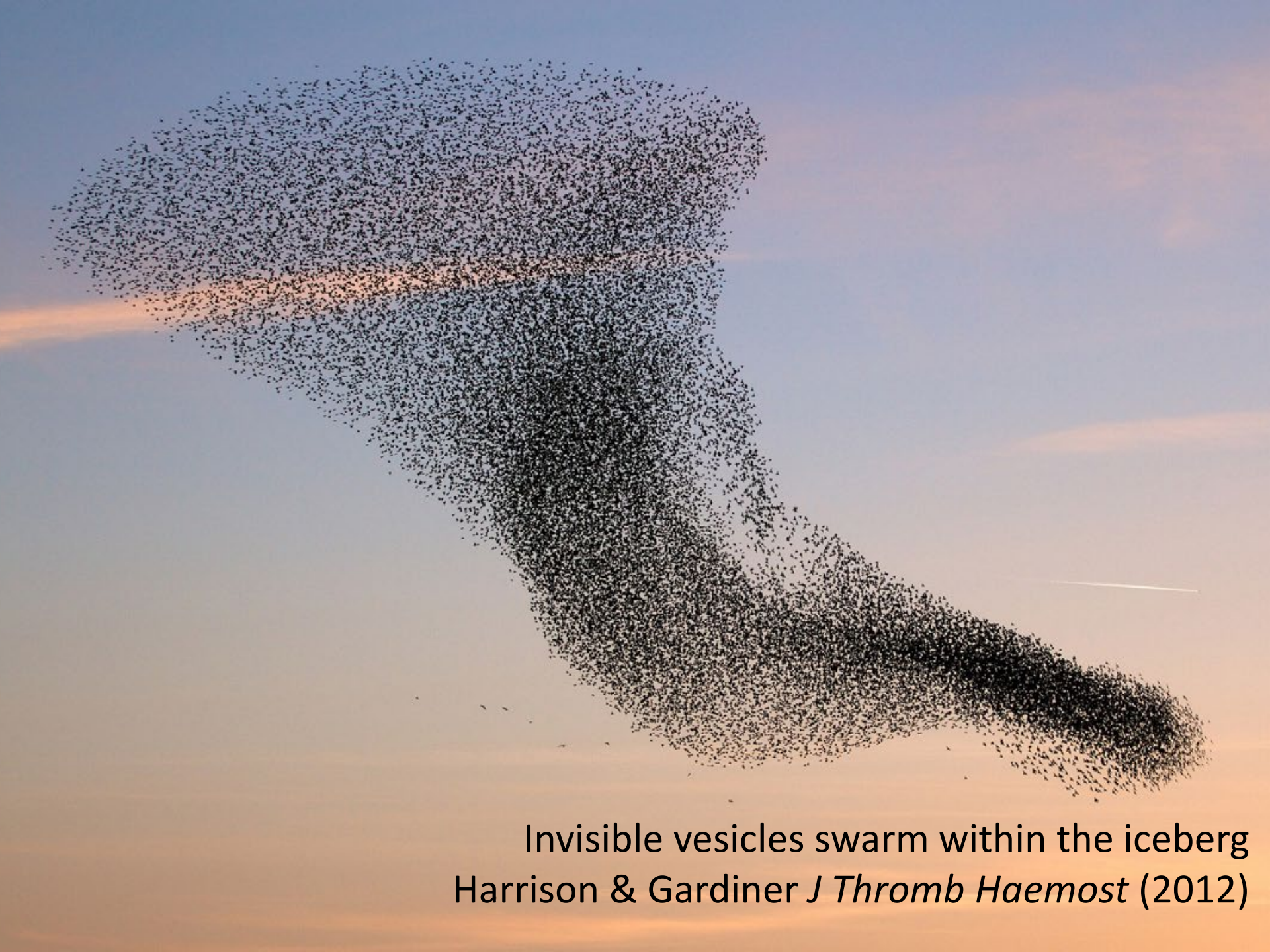


urine EVs <220 nm at concentration ≥ 10^{10} EVs ml^{-1}



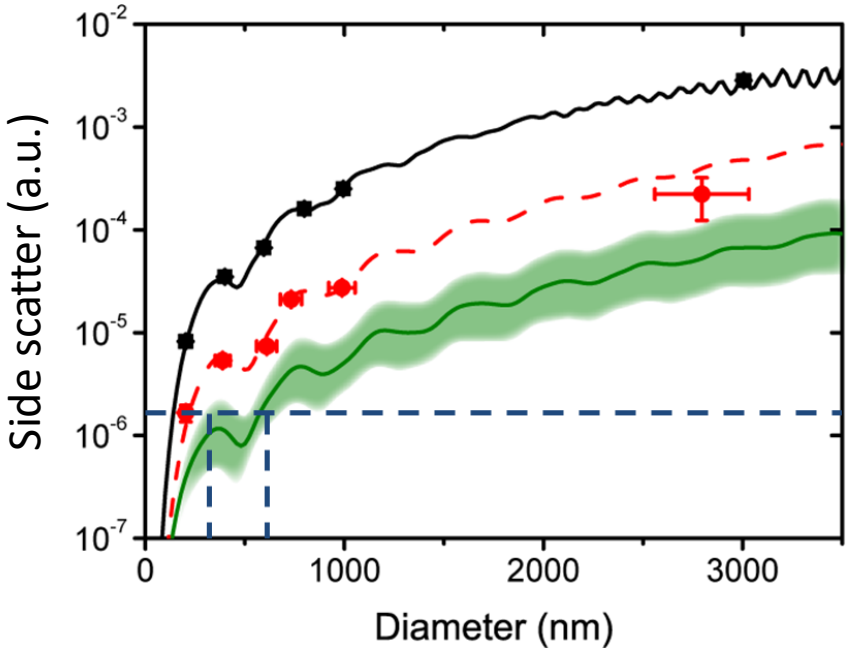


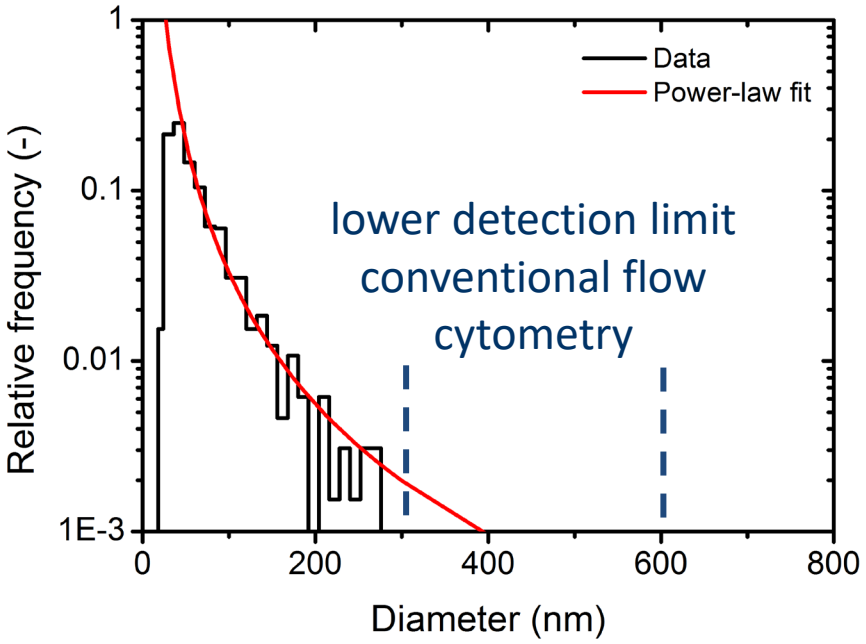

beam volume ≈
54 pl
At a concentration of
10^{10} vesicles ml^{-1},
>800 vesicles are
simultaneously
present in the beam.



Invisible vesicles swarm within the iceberg
Harrison & Gardiner *J Thromb Haemost* (2012)

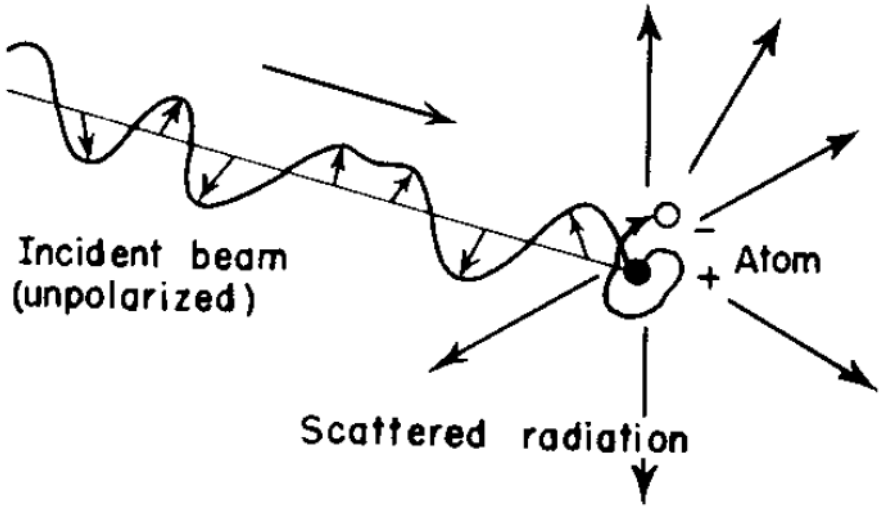
Summary EV detection with 1 scatter detector





- Single event signal attributed to scattering from *multiple* EVs ("Swarm detection")
- Conventional flow cytometry detects <1% of all EVs

Outline light scatter

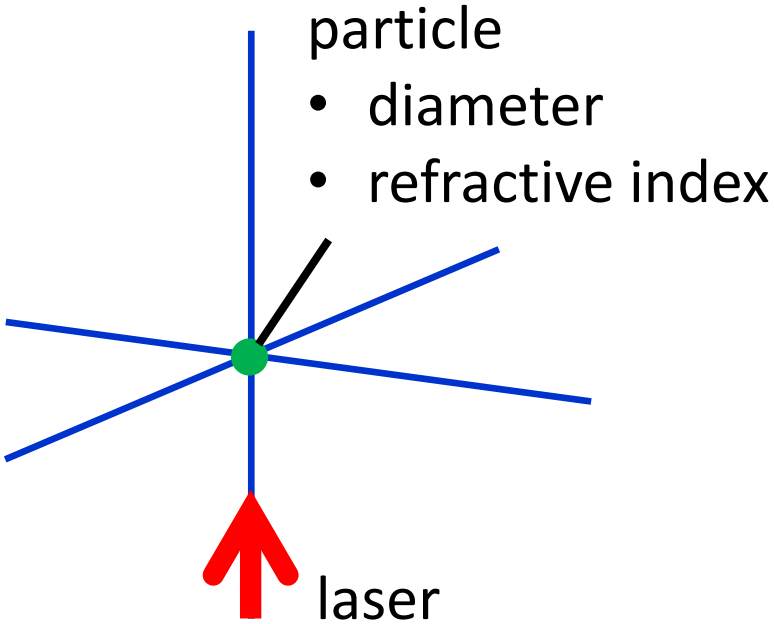


- Flow cytometry detection of EVs with
  - one scatter detector
  - **two scatter detectors**
- Standardization

image: Feynman lectures on physics

Goal

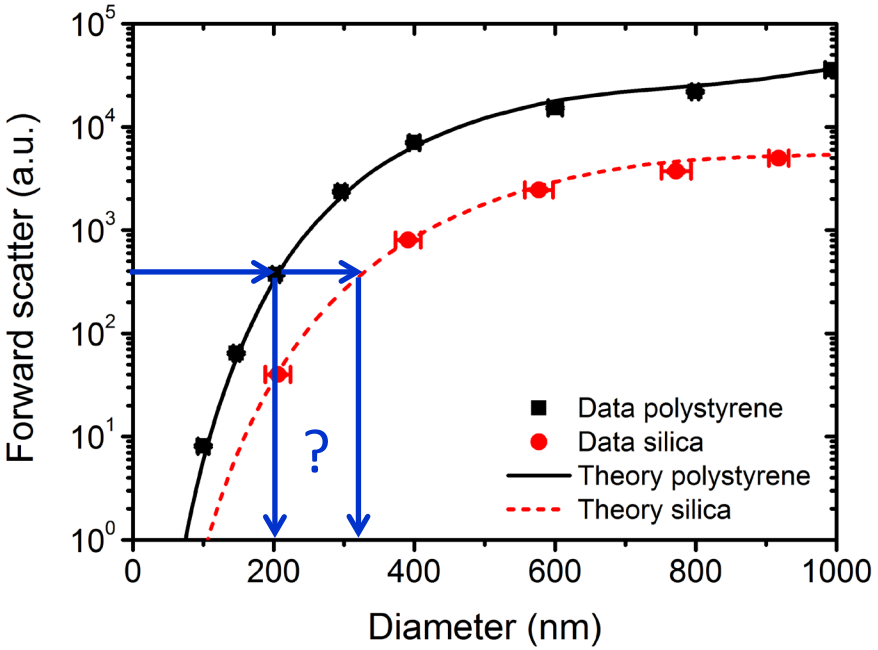
- Obtain physical properties of particles from flow cytometry scatter signals

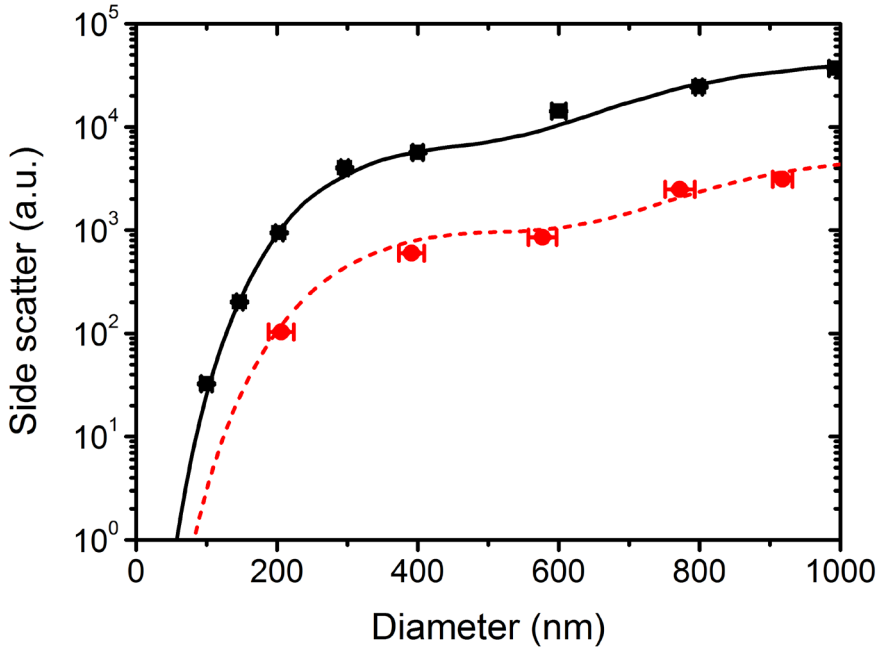


Approach

- Calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- Validate Flow-SR
  - beads mixture
  - oil emulsion
- Apply Flow-SR
  - EV and lipoprotein particles from blood

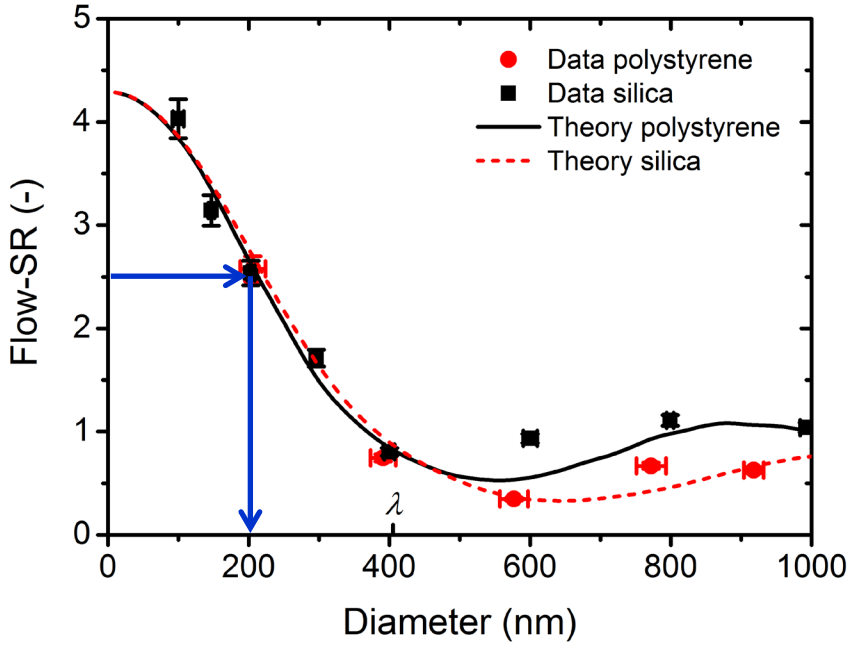
Calibrate forward scatter and side scatter





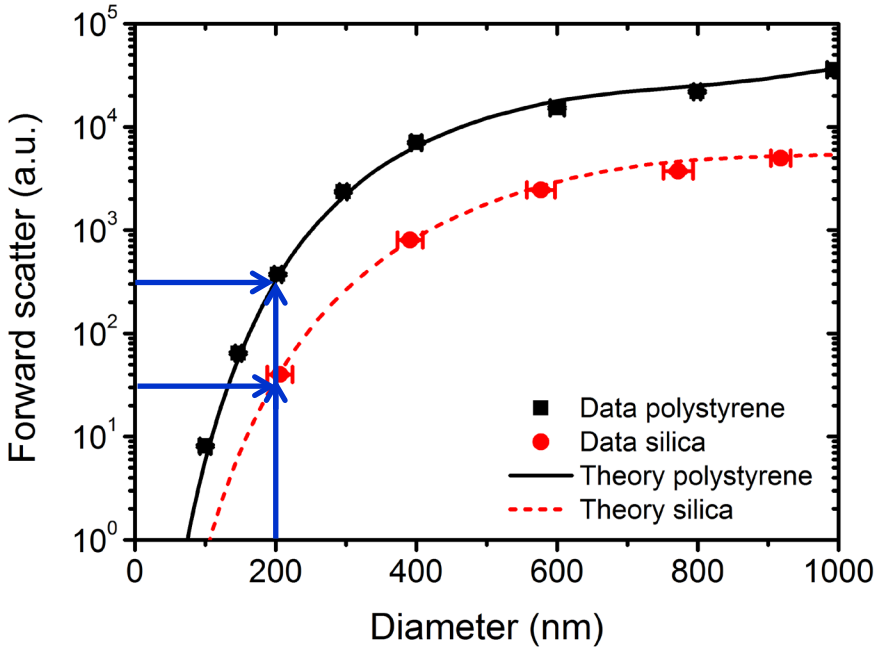
$$\text{Flow-SR} = \frac{\text{side scatter}}{\text{forward scatter}}$$

Derive size from Flow-SR



$$\text{Flow-SR} = \frac{\text{side scatter}}{\text{forward scatter}}$$

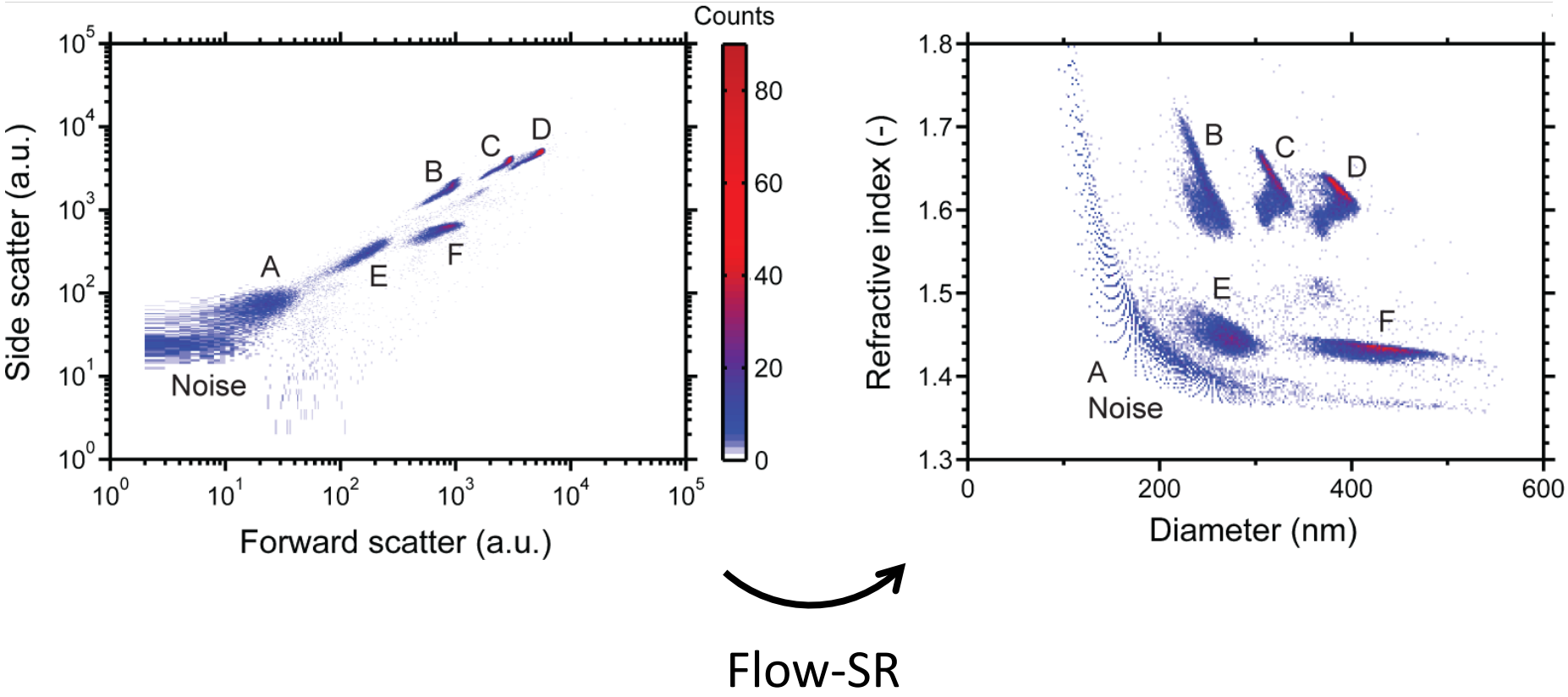
Derive refractive index from size and FSC



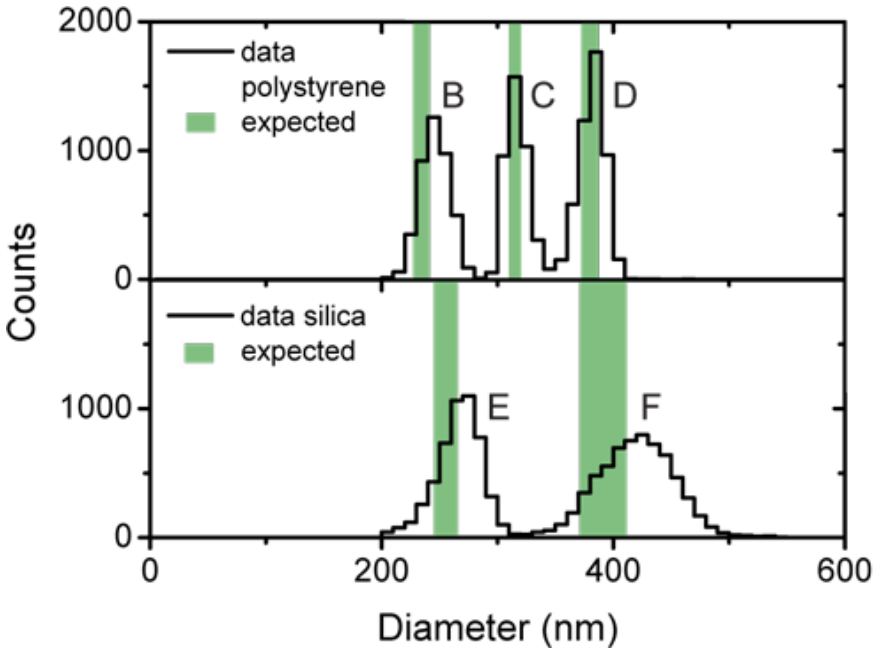
Approach

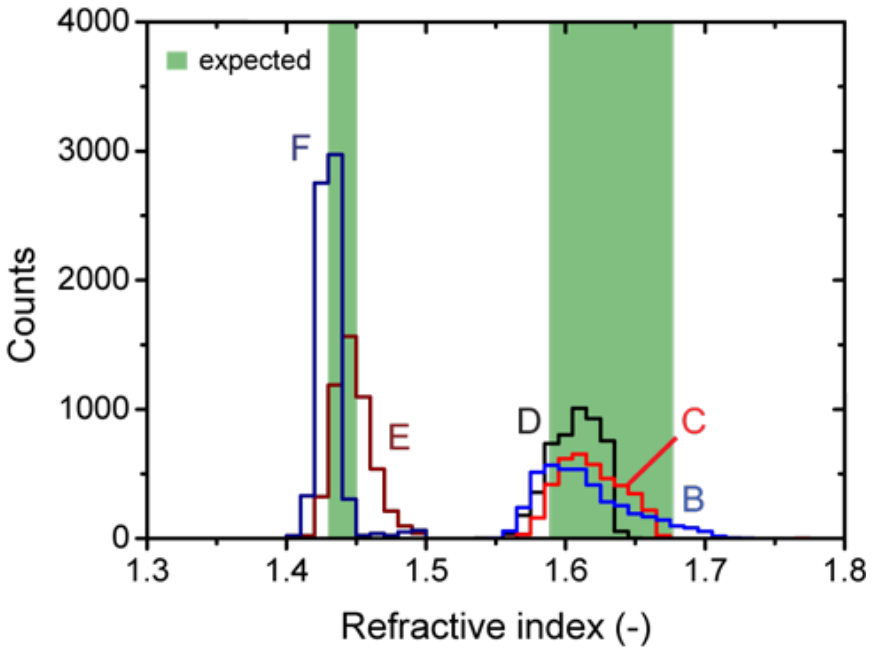
- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

Validate Flow-SR with a beads mixture



Validate Flow-SR with a beads mixture



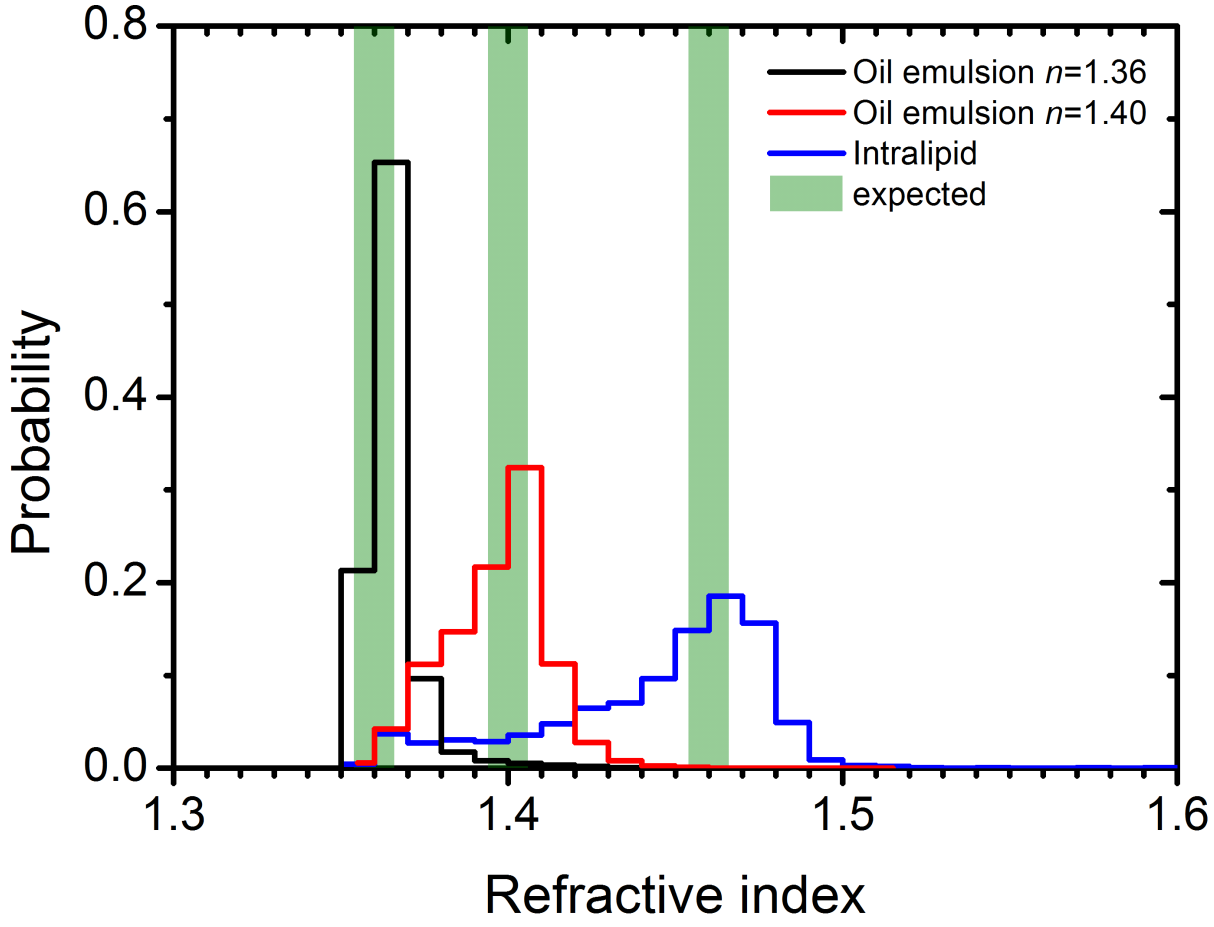


measurement error < 8%

CV < 8%

CV < 2%

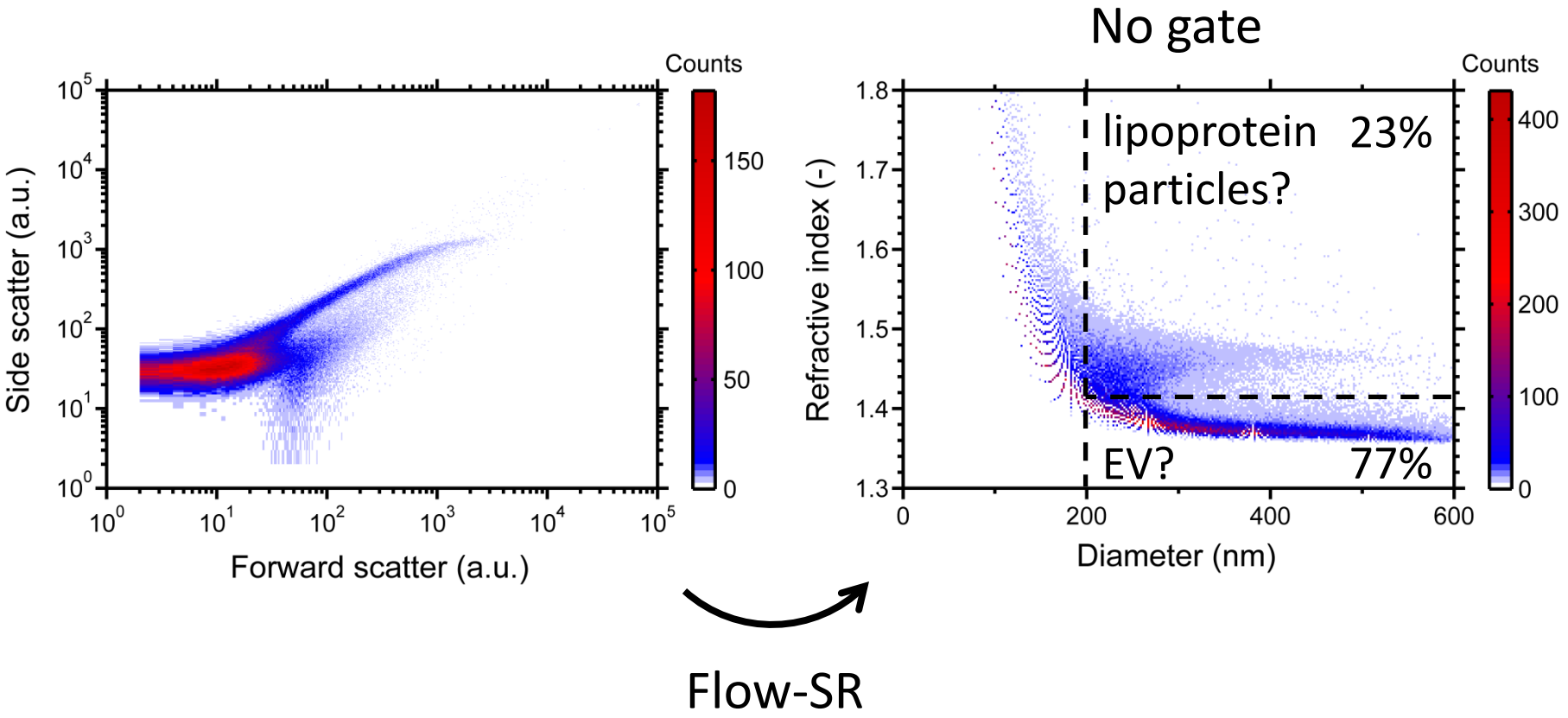
Validate Flow-SR with oil emulsions



Approach

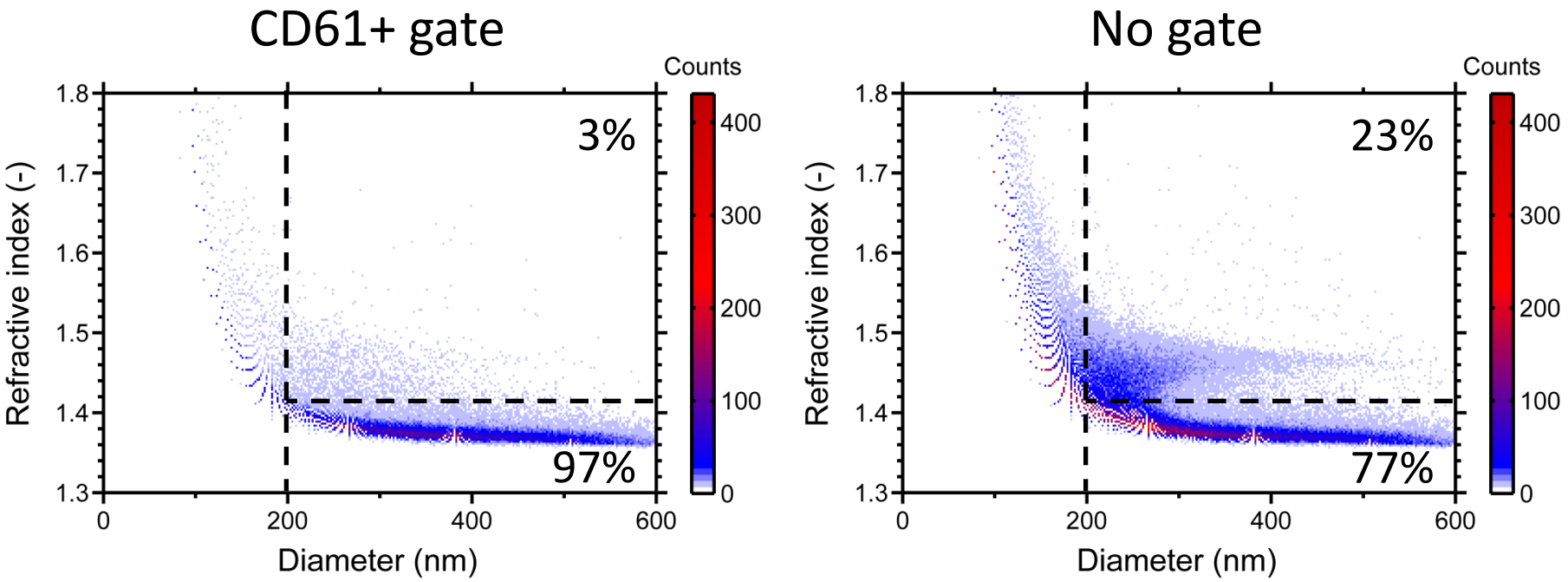
- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

Supernatant of outdated platelet concentrate



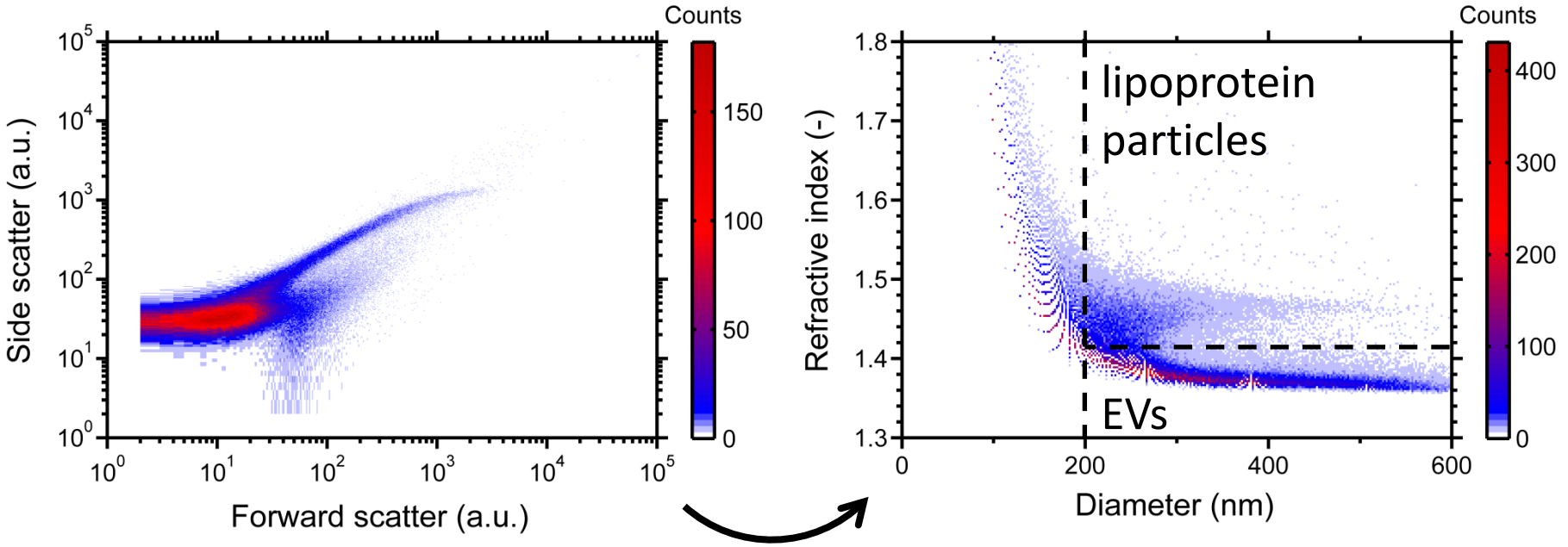
centrifuged 3-fold, 1550 × *g*, 20 min

Supernatant of outdated platelet concentrate



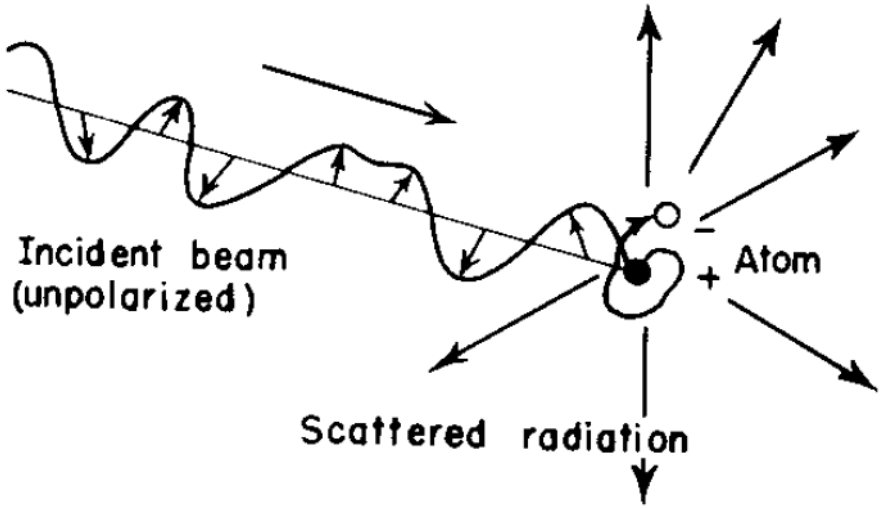
- Median refractive index platelet EVs >200 nm = 1.37

Summary EV detection with 2 scatter detectors



- Flow-SR enables size and refractive index determination of nanoparticles by flow cytometry
  - data interpretation and comparison
  - differentiate EVs and lipoprotein particles

Outline light scatter



- Flow cytometry detection of EVs with
  - one scatter detector
  - two scatter detectors
- **Standardization**

image: Feynman lectures on physics

Standardization is boring (biologists, clinicians)

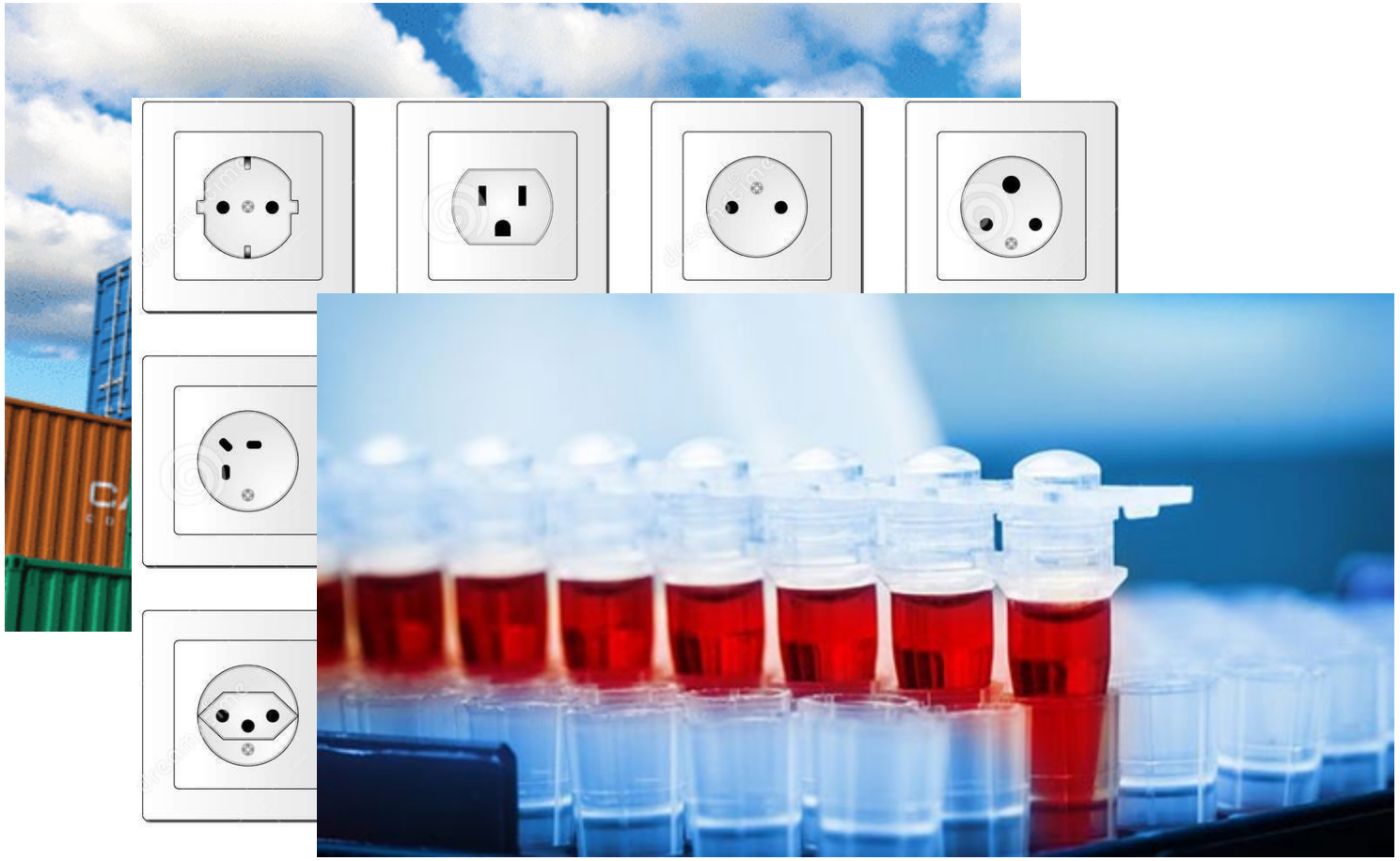
Standardisation is exciting (metrologists, physicists)

BESSYII

0.31 nm X-rays to size EV*
(flow cytometers typically use 488 nm light)

*Varga et al. *J Extracell Vesicles* 2014

Standardization is important (everybody)



Goal

- obtain reproducible measurements of the EV concentration using different flow cytometers

Study comprises 33 sites (64 instruments) worldwide

Approach scatter-based standardization

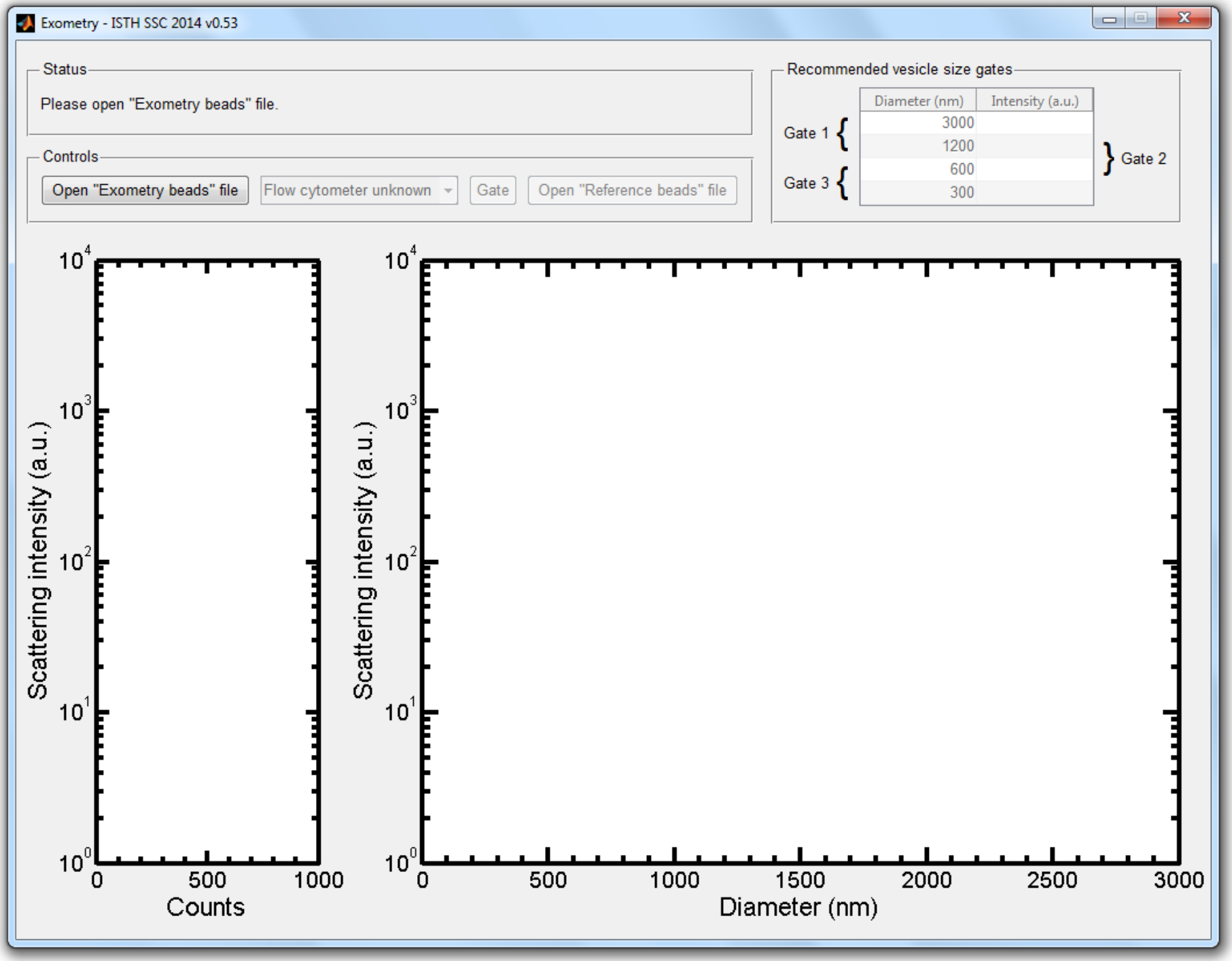
- Measure EV reference sample and controls
- Scatter (a.u.) → diameter (nm)
  - Measure Rosetta calibration* beads
  - Rosetta calibration* software relates scatter to diameter and defines EV size gates
- Apply EV size gate to software (e.g. FlowJo) and report concentrations

*Exometry.com

EV reference sample

- Platelet (CD61-PE+) EVs from cell-free platelet concentrates

- Trigger on most sensitive scatter channel
- Exclude EVs similar to isotype


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
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
10^0
10^1
10^2
10^3
10^4
0
500
1000
Counts
Scattering intensity (a.u.)
10^0
10^1
10^2
10^3
10^4
0
500
1000
1500
2000
2500
3000
Diameter (nm)

Exometry - ISTH SSC 2014 v0.53

Status

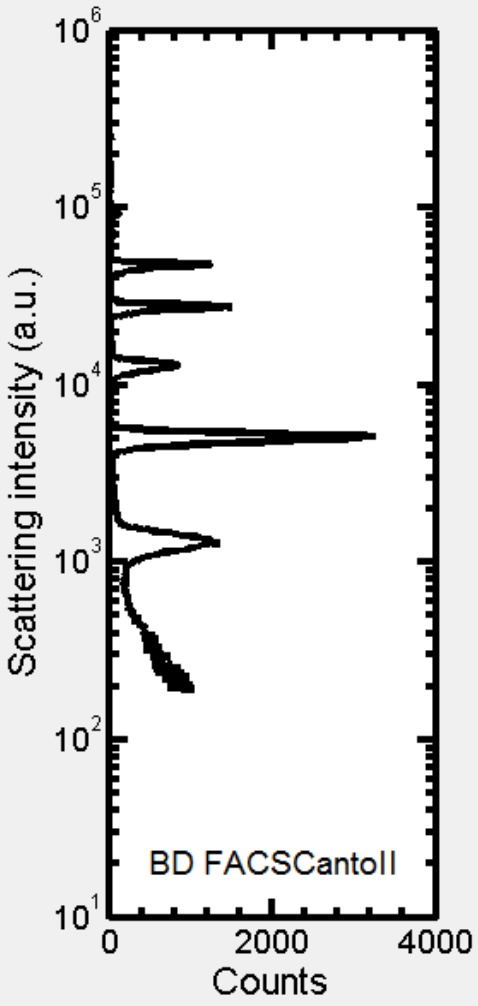
Please select detector and click "Gate" to obtain vesicle size gates.

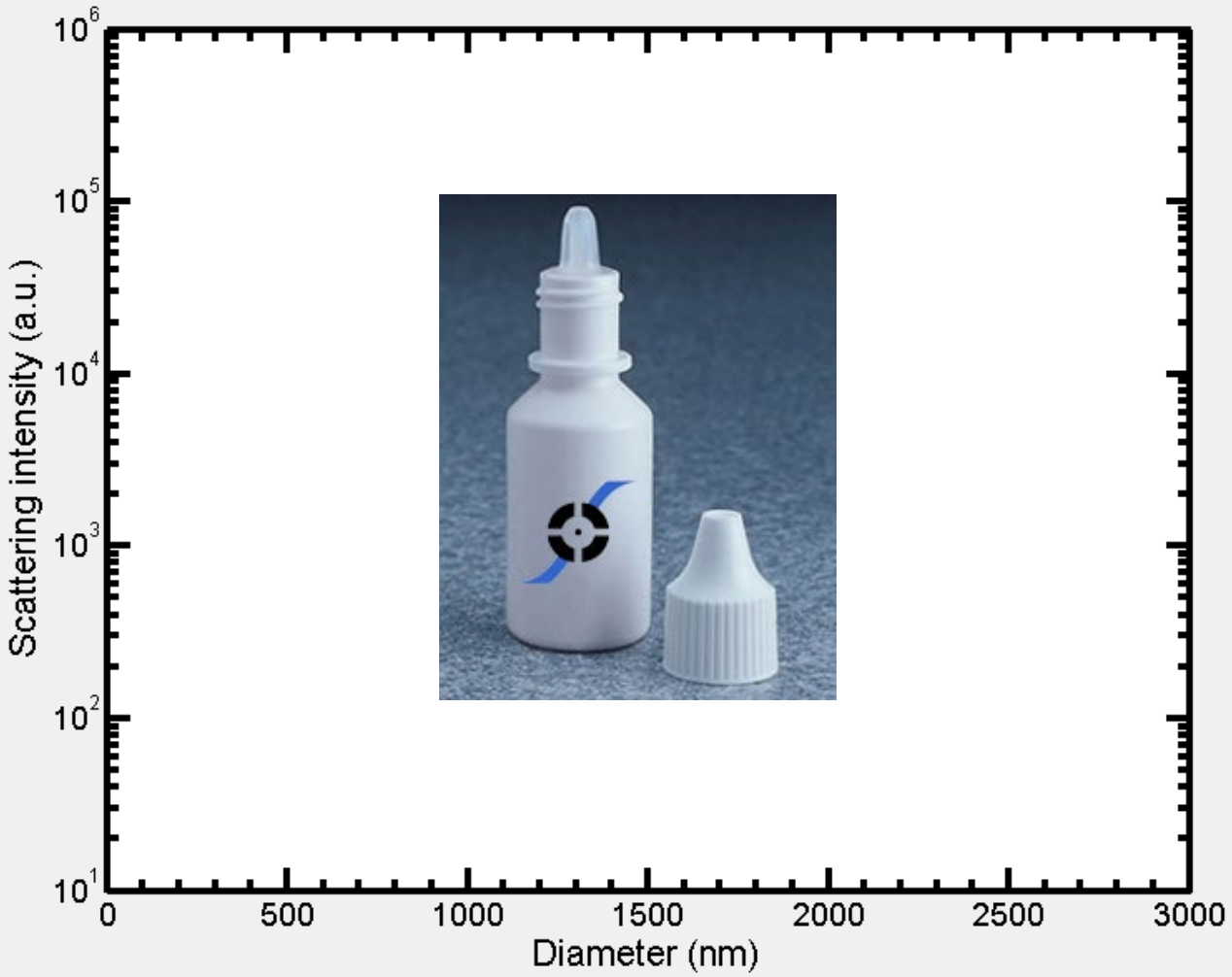
Controls

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	





Exometry - ISTH SSC 2014 v0.53

Status

There are 5 scatter peaks related to the particle diameter. Applying Mie calculations.

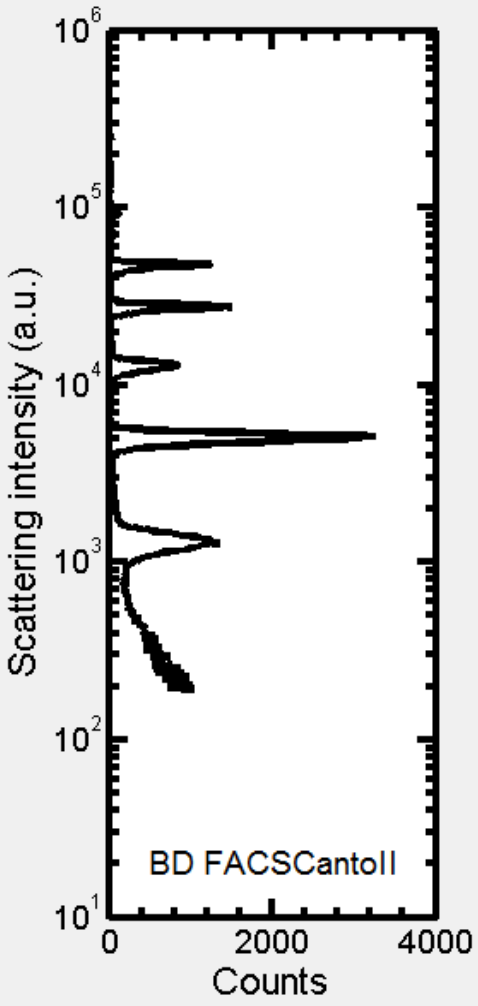
Controls

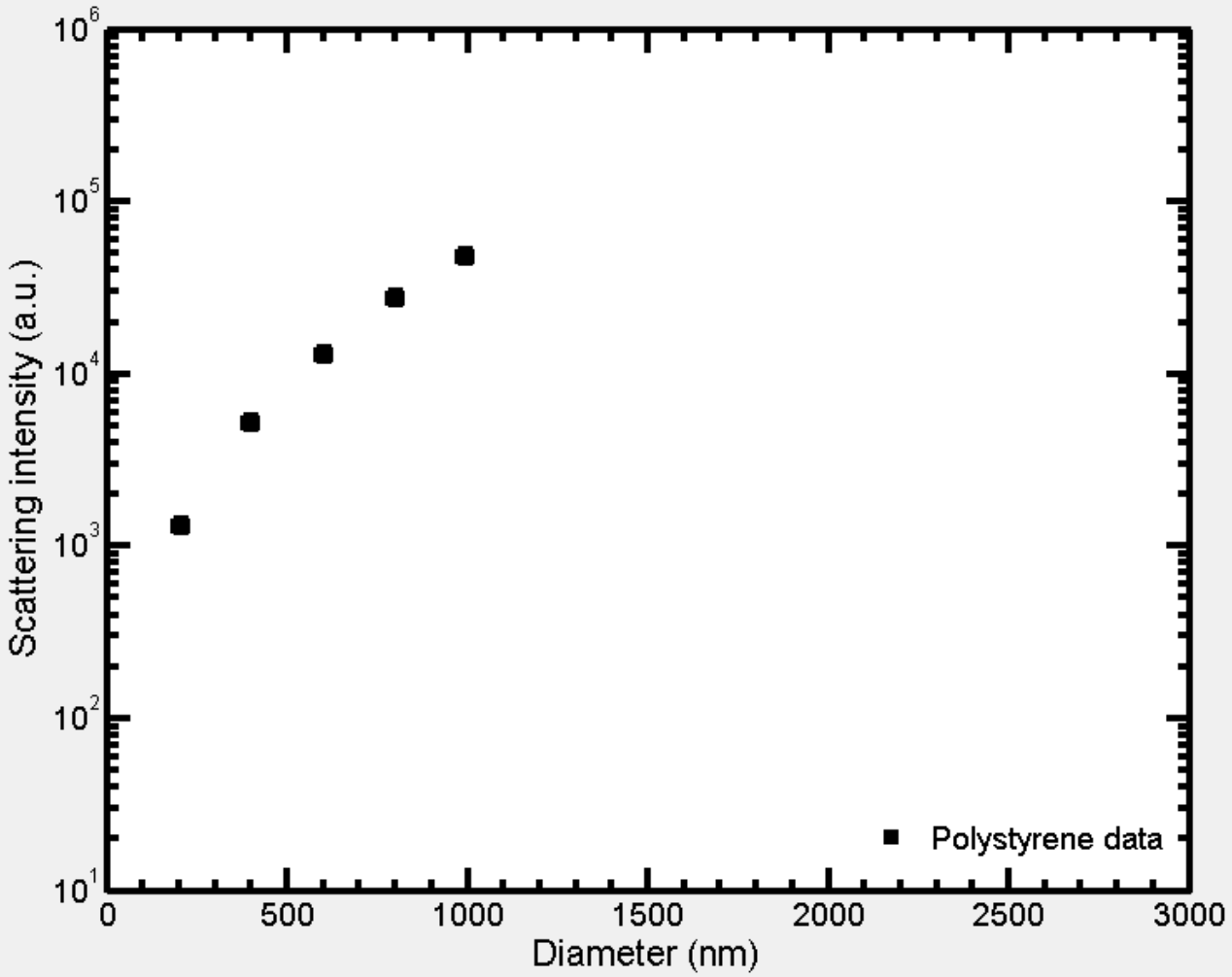
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

Recommended vesicle size gates

	Diameter (nm)	Intensity (a.u.)
Gate 1	3000	
	1200	
Gate 3	600	
	300	

Gate 2





Exometry - ISTH SSC 2014 v0.53

Status

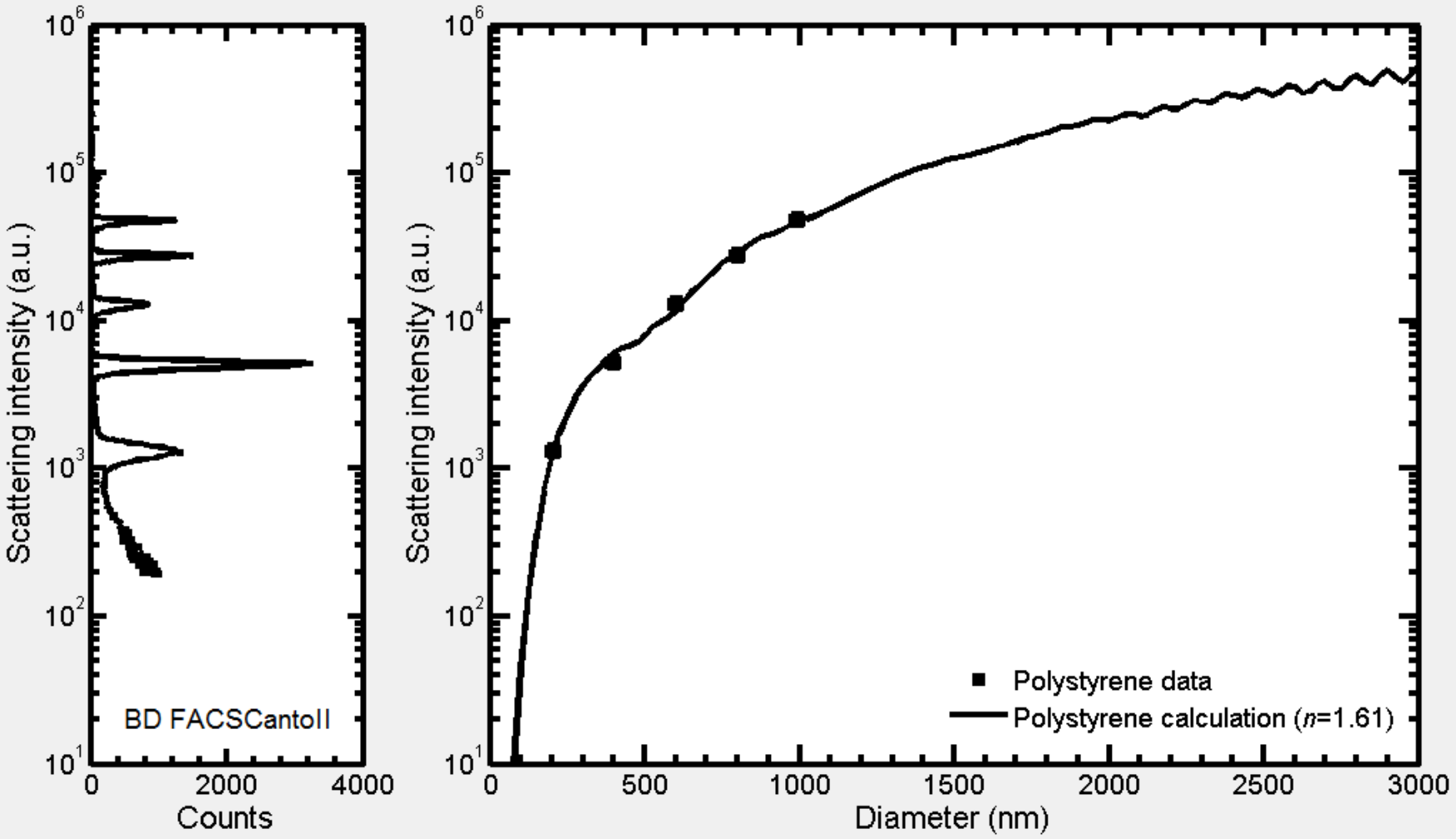
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.

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	



Exometry - ISTH SSC 2014 v0.53

Status

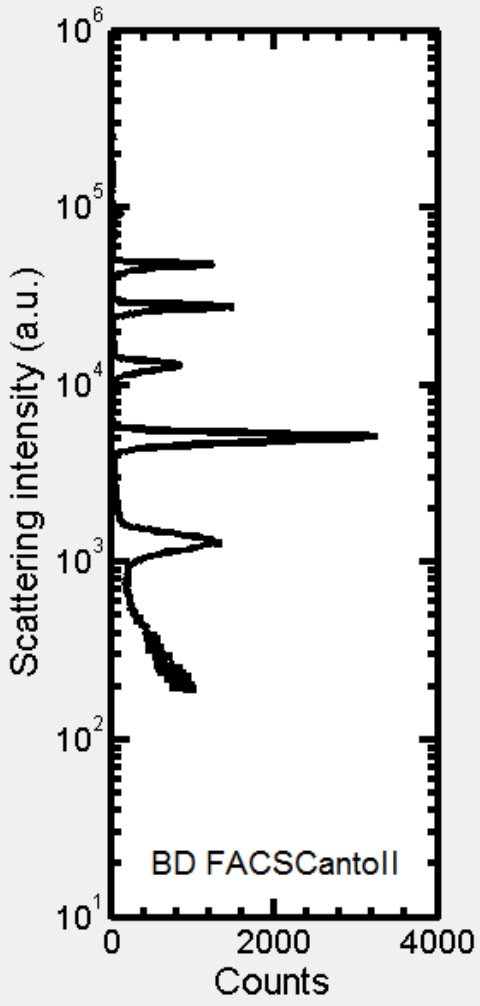
Congratulations, vesicle size gates determined, estimated error less than 0%.

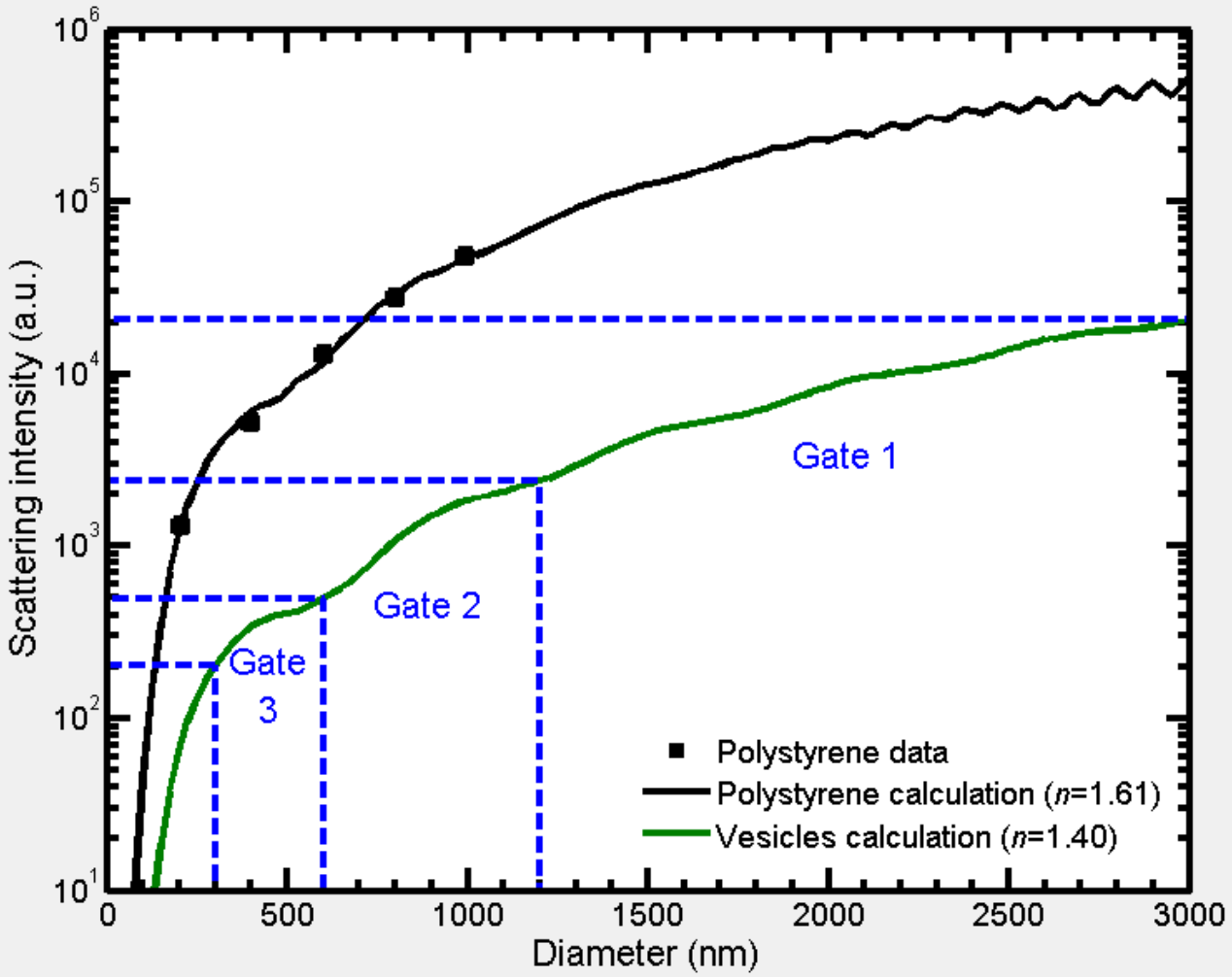
Controls

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	





Exometry - ISTH SSC 2014 v0.53

Status

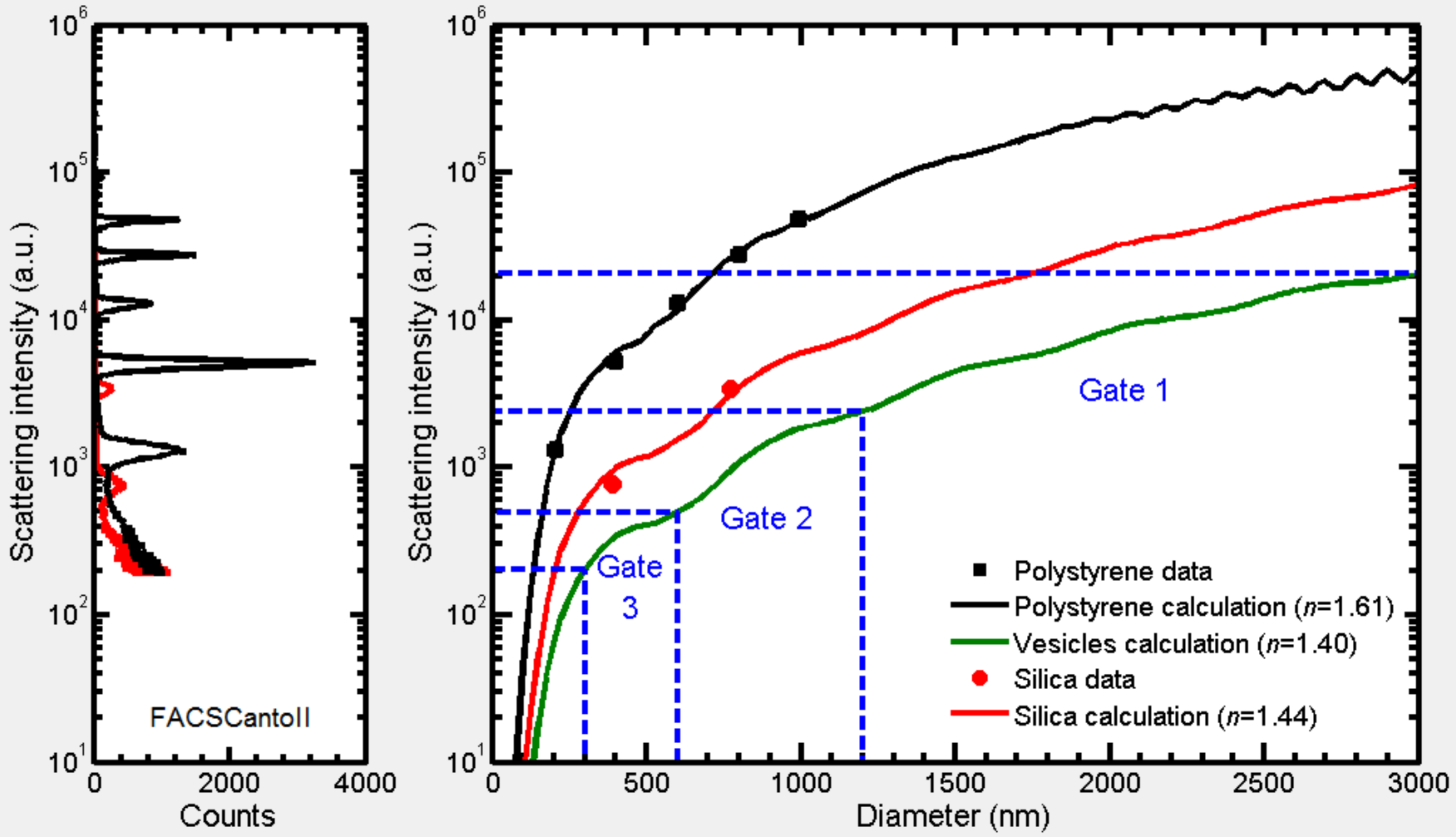
Congratulations, validation succeeded, estimated error less than 4%.

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	



Exometry - ISTH SSC 2014 v0.53

Status

There are 5 scatter peaks related to the particle diameter. Applying Mie calculations.

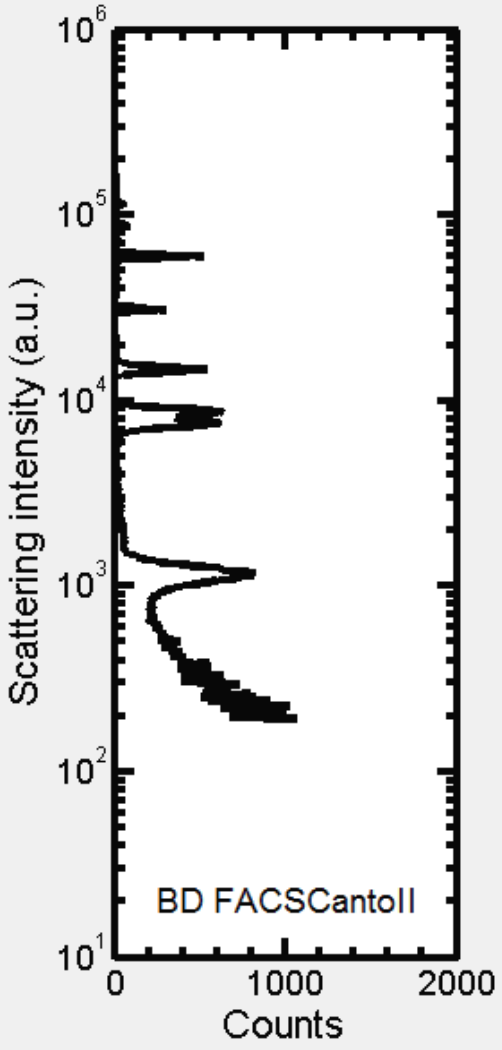
Controls

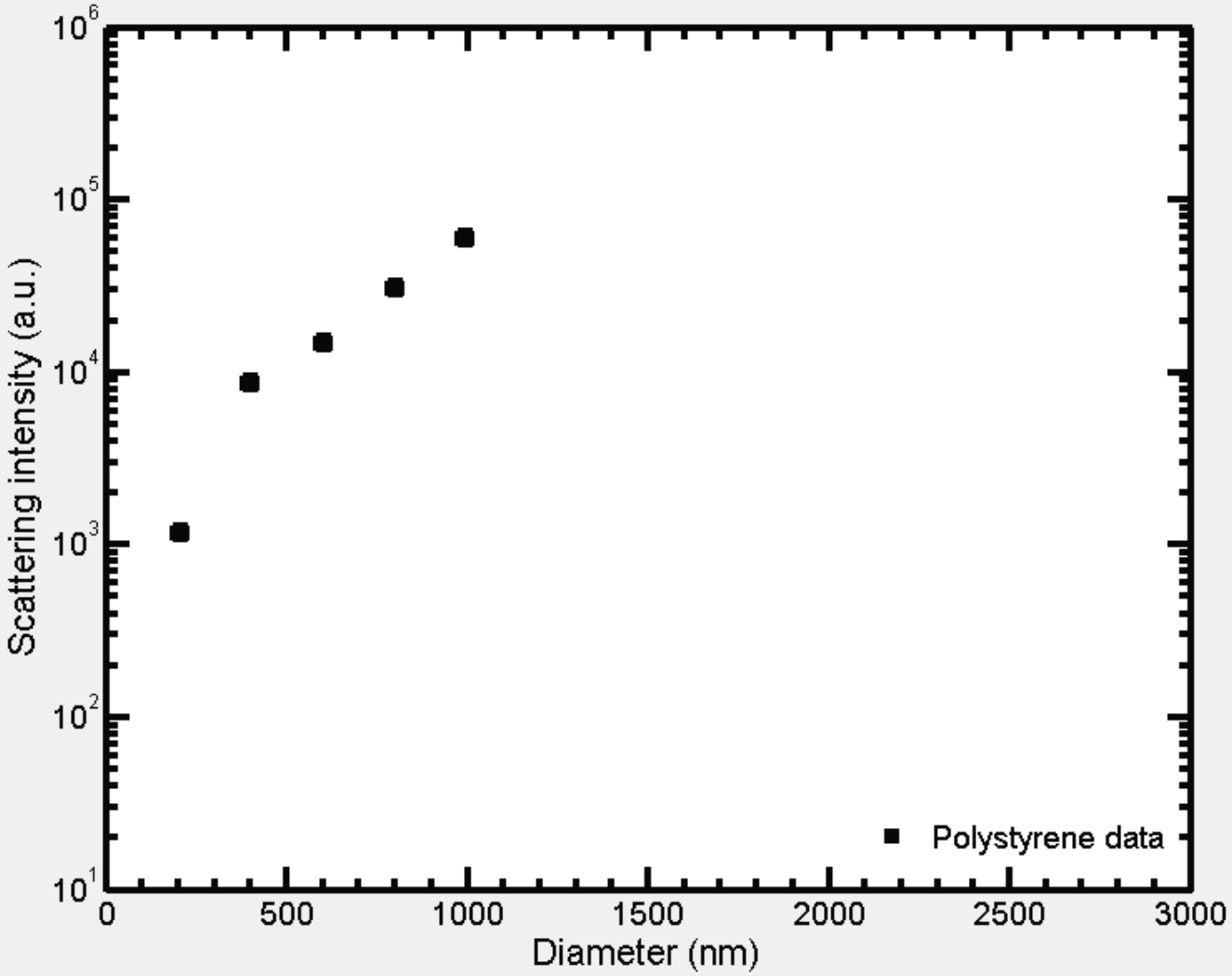
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

Recommended vesicle size gates

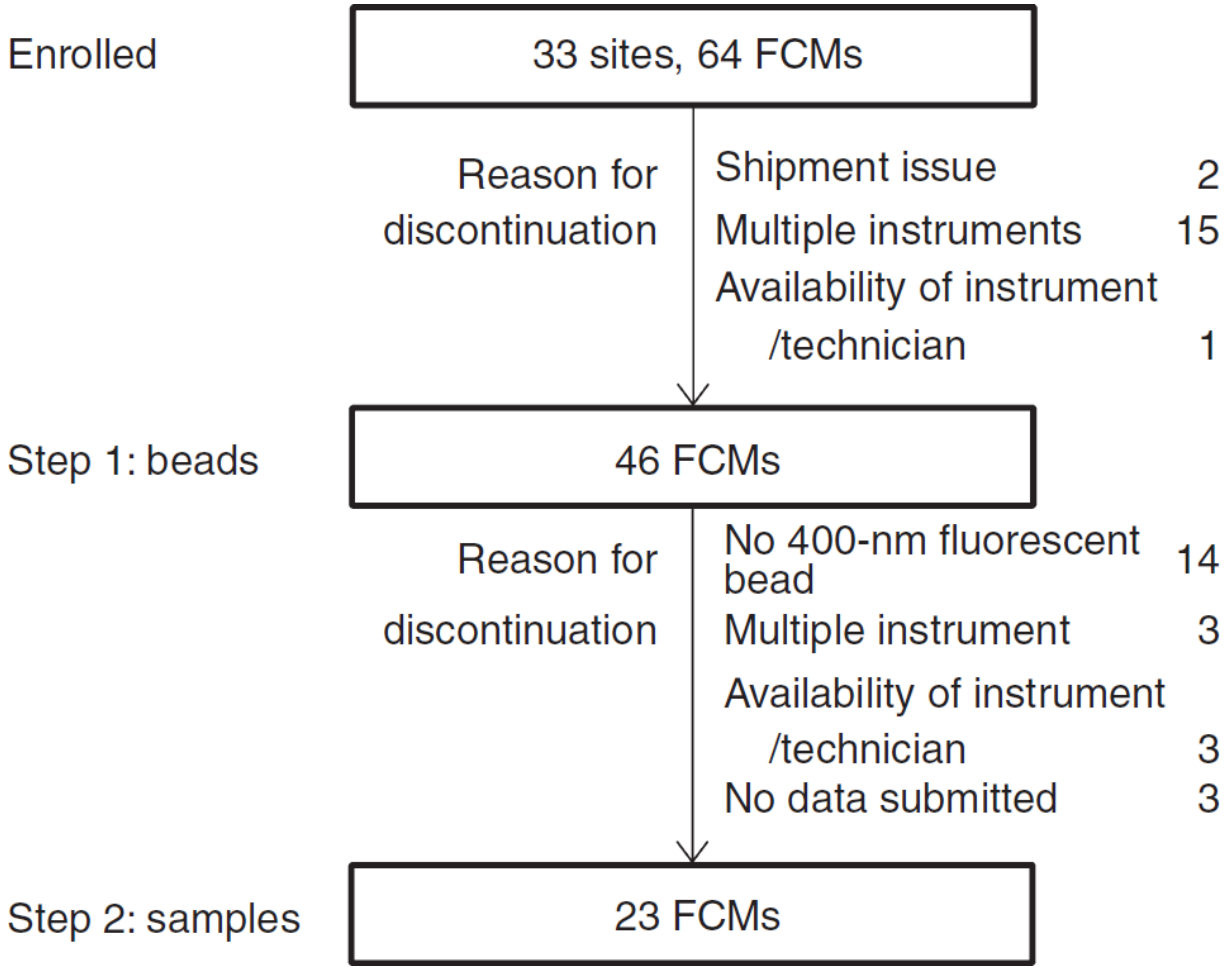
	Diameter (nm)	Intensity (a.u.)
Gate 1	3000	
	1200	
Gate 3	600	
	300	

Gate 2

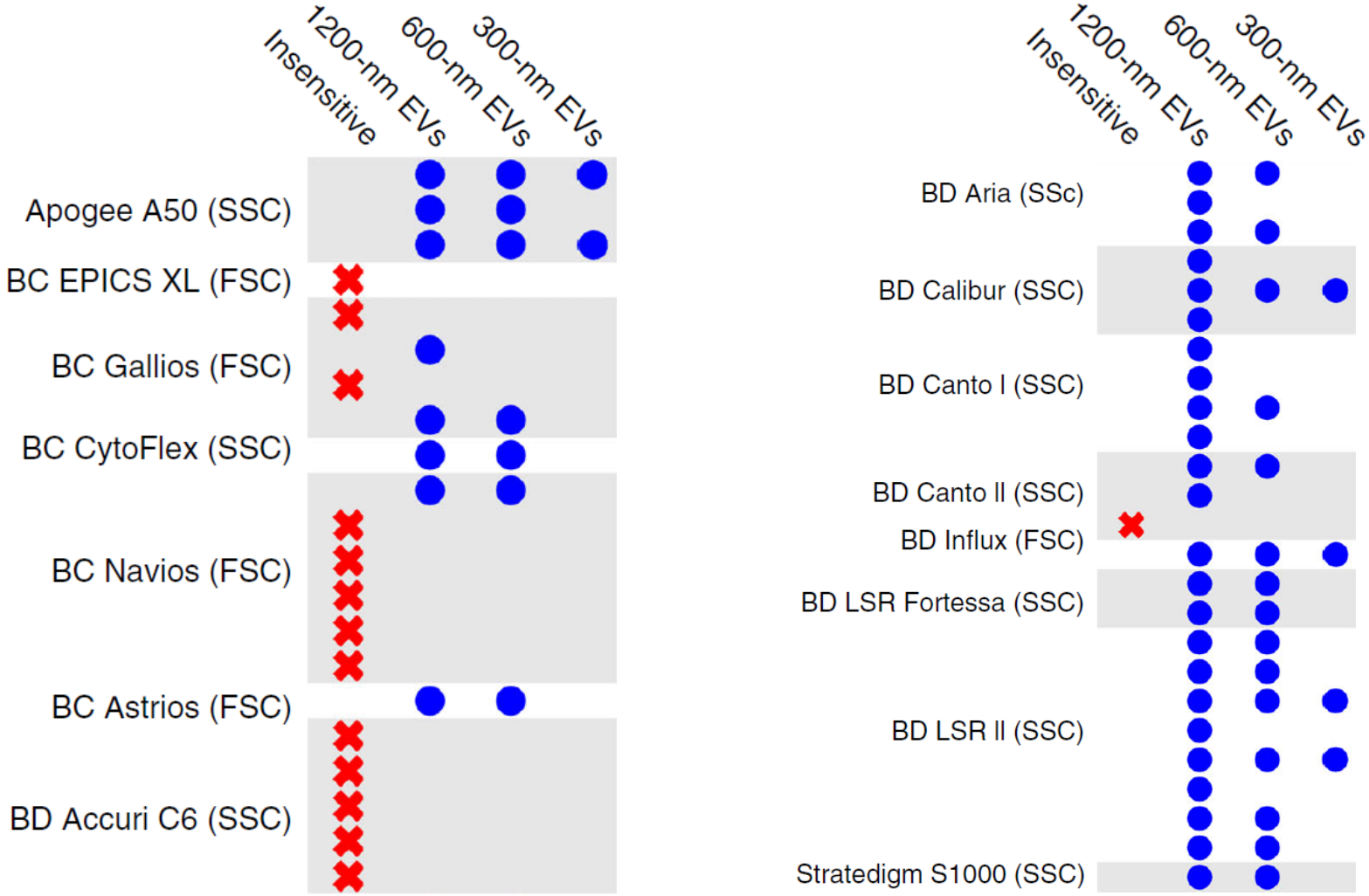




Exclusion of flow cytometers (FCM)

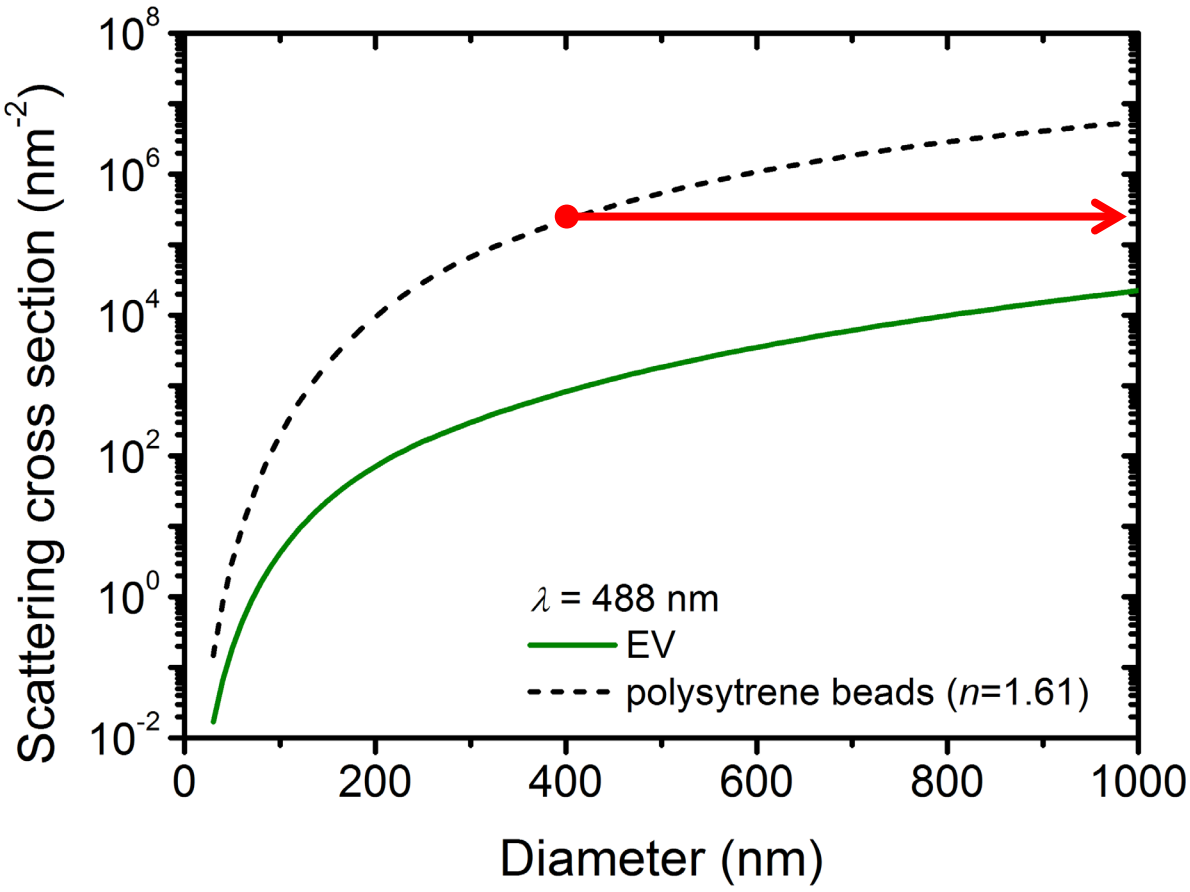


Sensitivity of 46 flow cytometers in the field

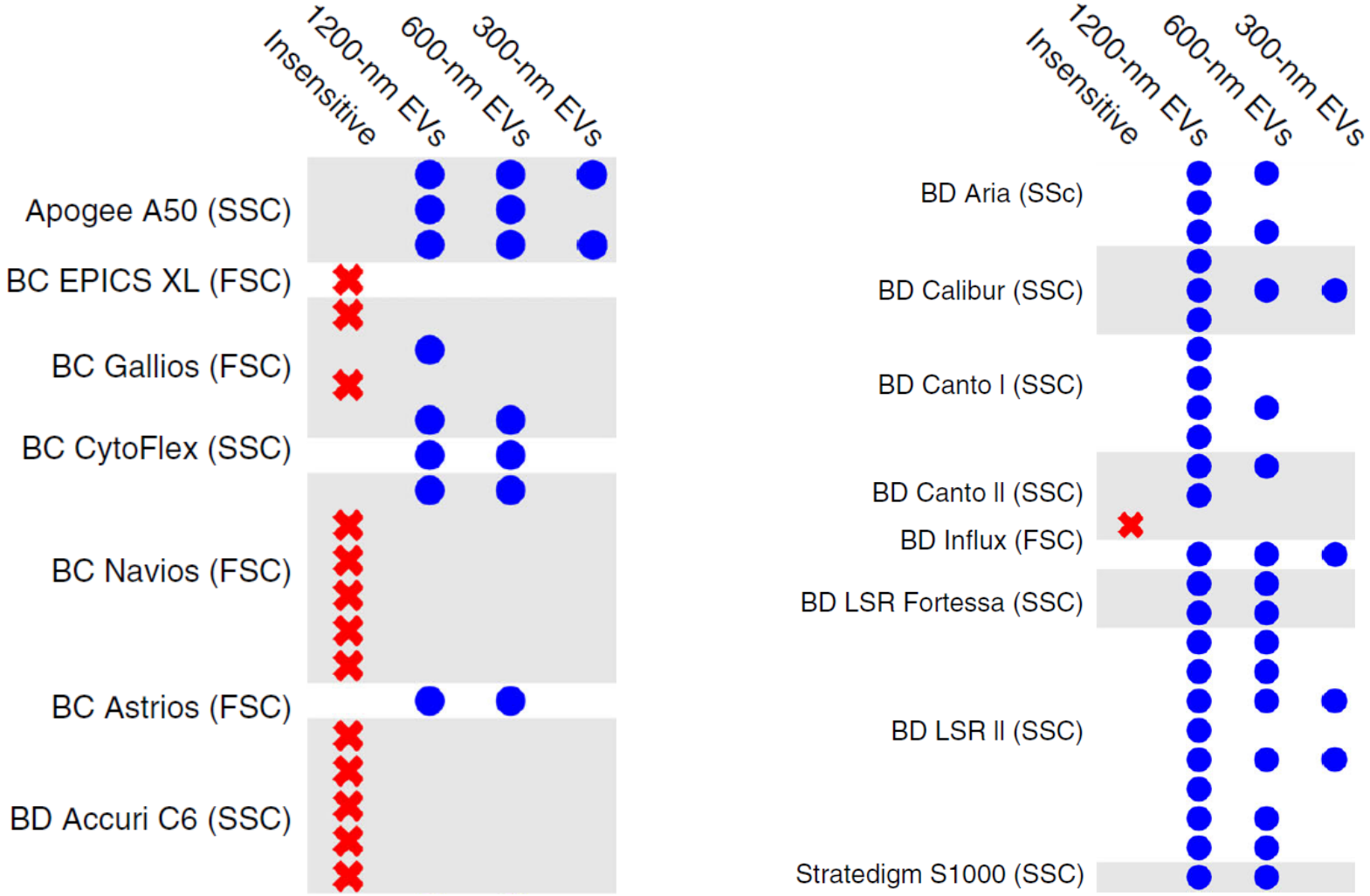


✖ = unable to detect 400 nm polystyrene beads

400 nm polystyrene beads scatter more than 1,000 nm EV



Sensitivity of 46 flow cytometers in the field



✖ = unable to detect EV < 1000 nm

Results

Method	CV* concentration (%)
No scatter gate	144
Traditional bead size gate	139
1,200-3,000 nm EV size gate	81
600-1,200 nm EV size gate	82
300-600 nm EV size gate	115

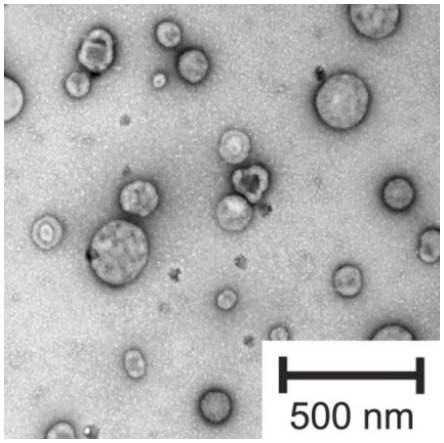
*CV: coefficient of variation (standard deviation / mean)

Conclusions standardization by sizing

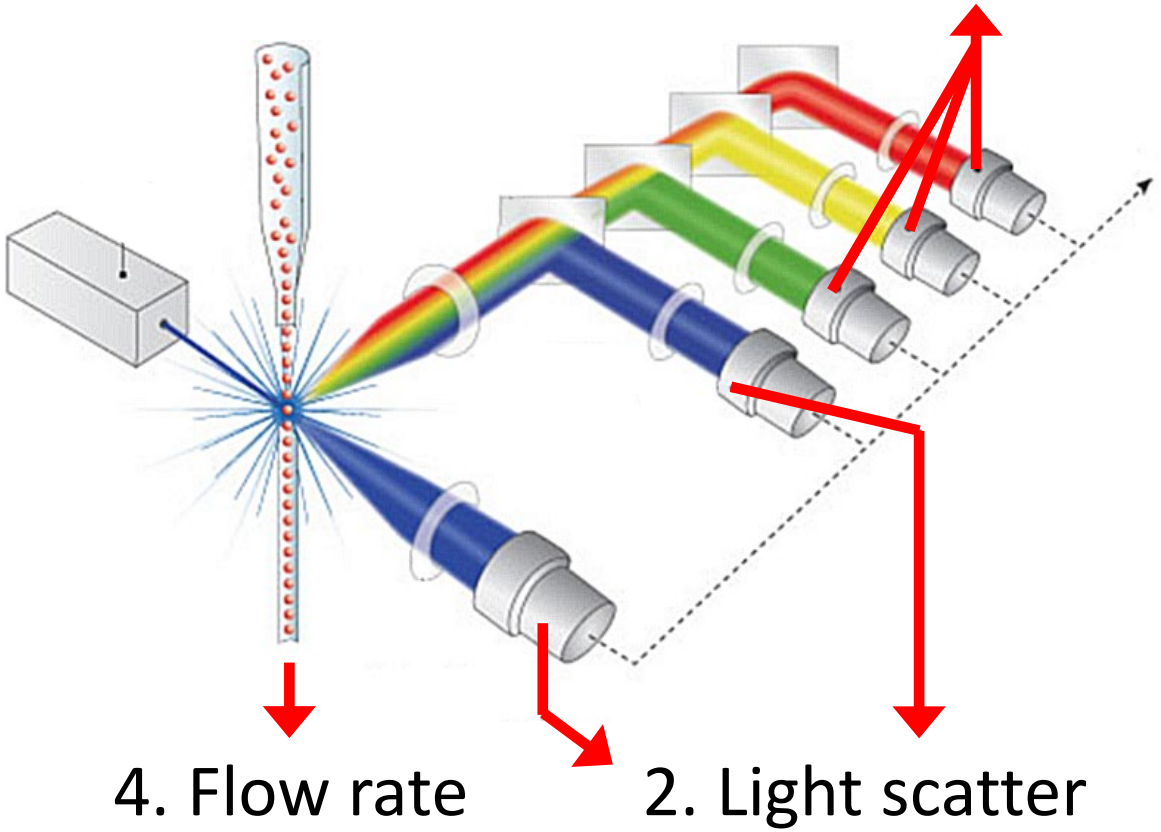
- 24% of flow cytometers in study are unable to detect EVs by scatter-based triggering
- EV diameter gates by Mie theory improve reproducibility compared to no gate or bead diameter gate

Outline

1. Extracellular vesicles (EVs)



3. Fluorescence



4. Flow rate

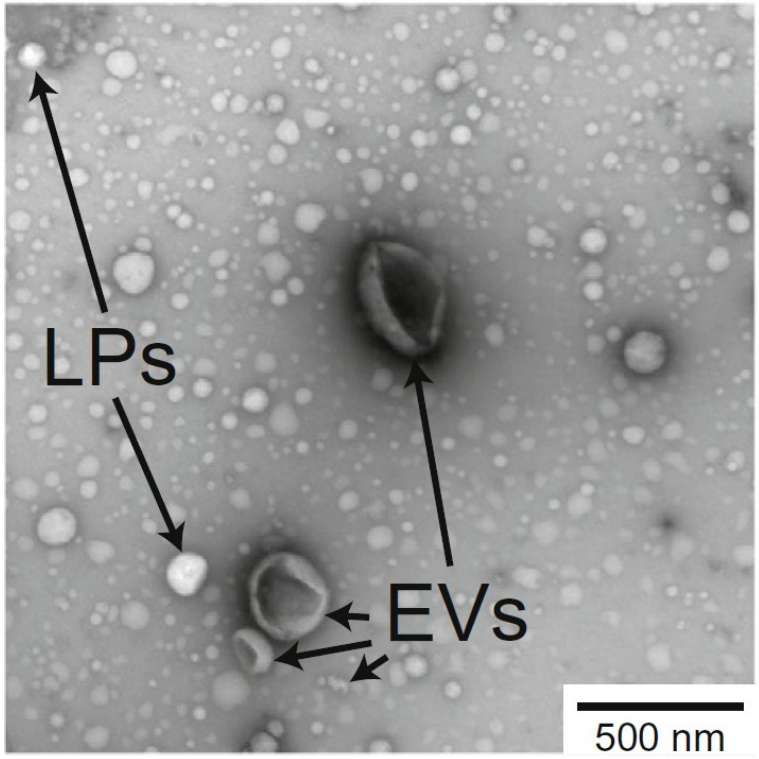
2. Light scatter

image: semrock.com

Fluorescence

- Please ask Dr. Zosia Maciorowski
- Label EVs
  - Antibodies
  - **Membrane dyes?**

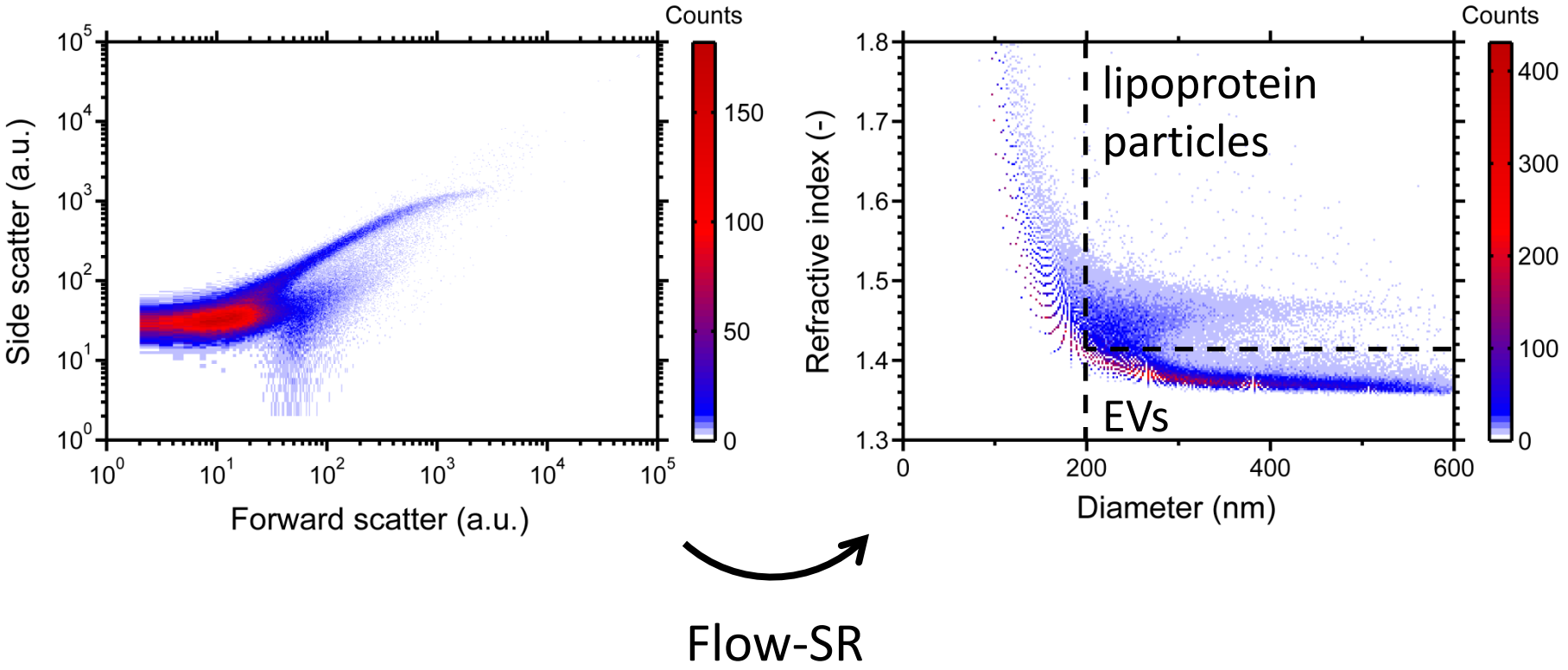
How specific do generic dyes label EVs?



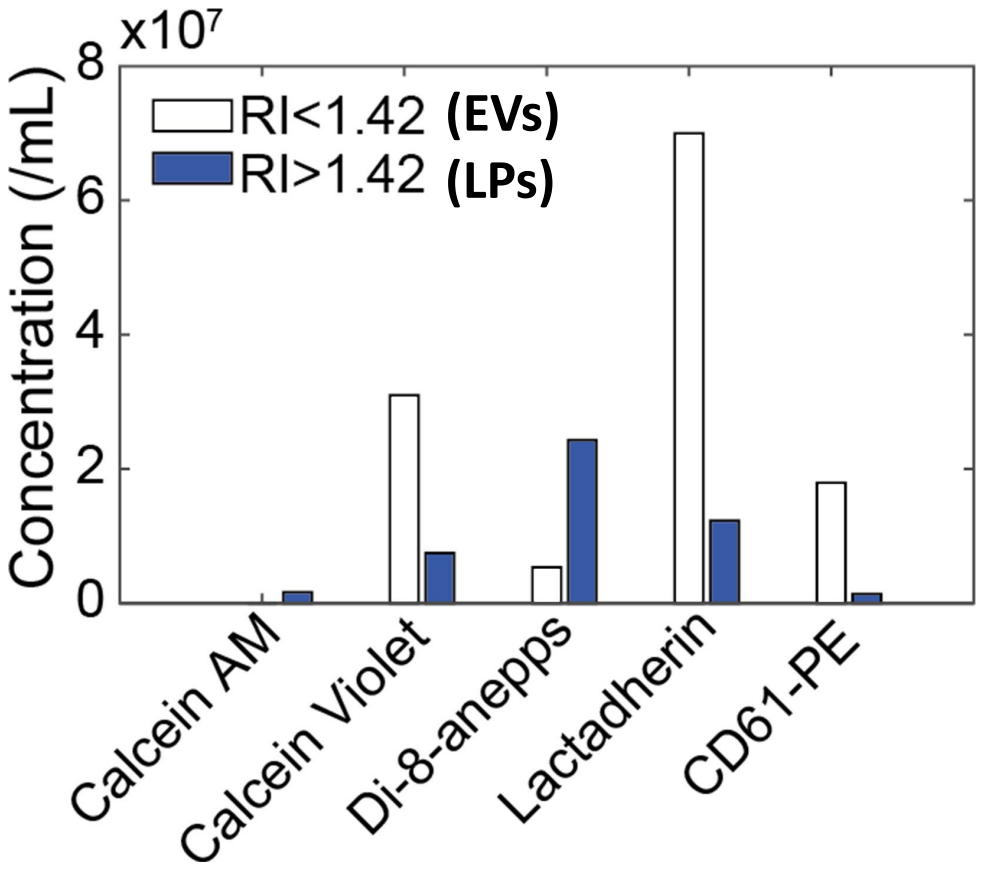
- blood contains ~1,000 lipoprotein particles (LPs) for each EV*

*Dragovic et al. *Nanomedicine* 2011

Method: Flow-SR



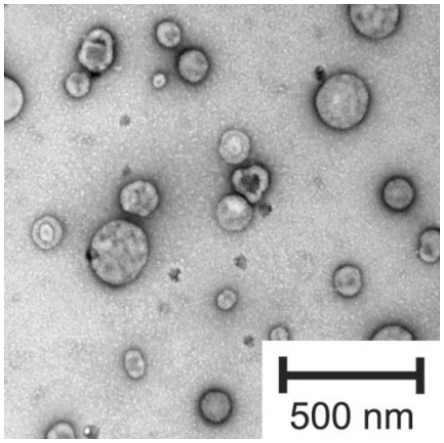
Specificity of generic dye



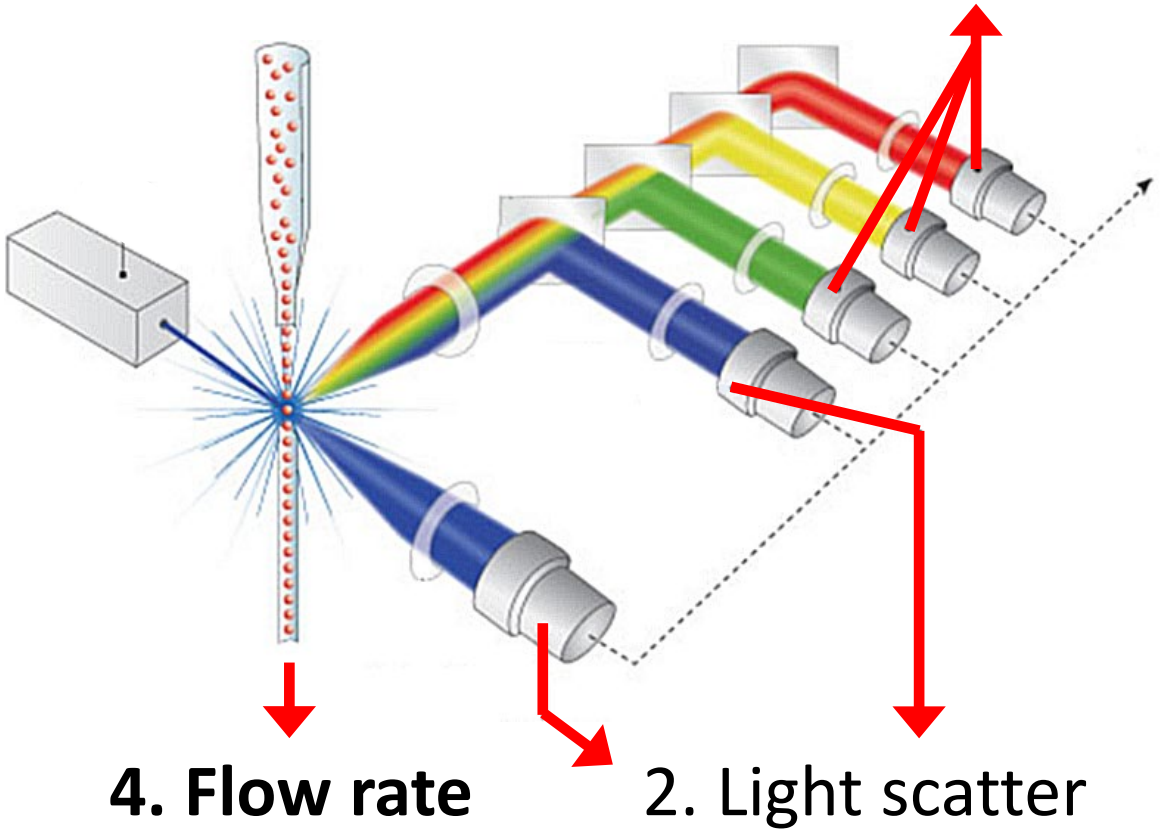
Label	LP fraction (%)
Calcein AM	
Calcein violet	19
Di-8-anepps	82
Lactadherin	15
CD61-PE	7

Outline

1. Extracellular vesicles (EVs)



3. Fluorescence



4. Flow rate

2. Light scatter

image: semrock.com

Study comprises 33 sites (64 instruments) worldwide

METVES

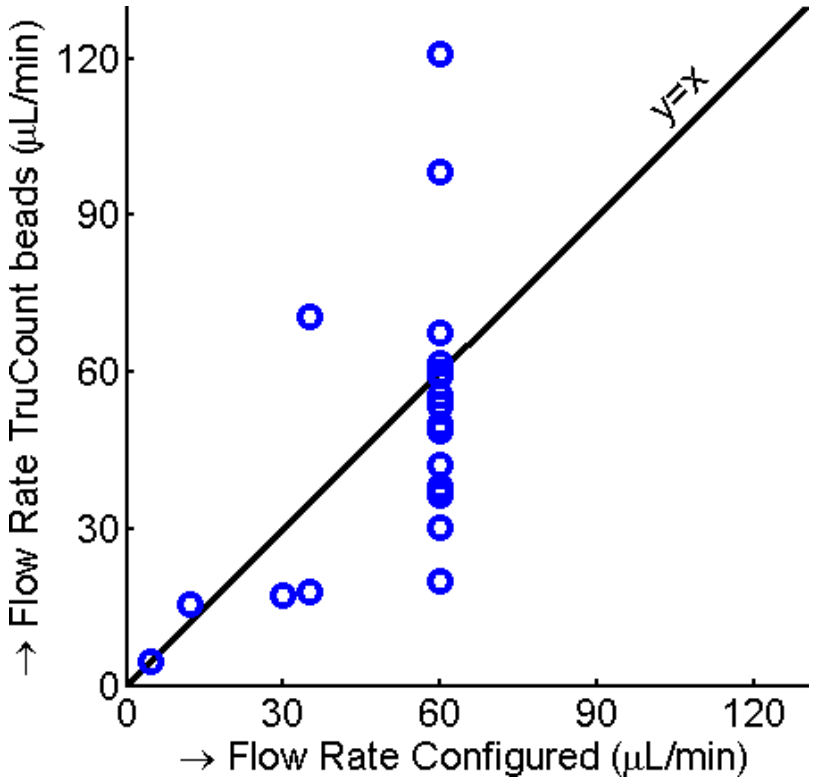
International Society on Thrombosis and Haemostasis

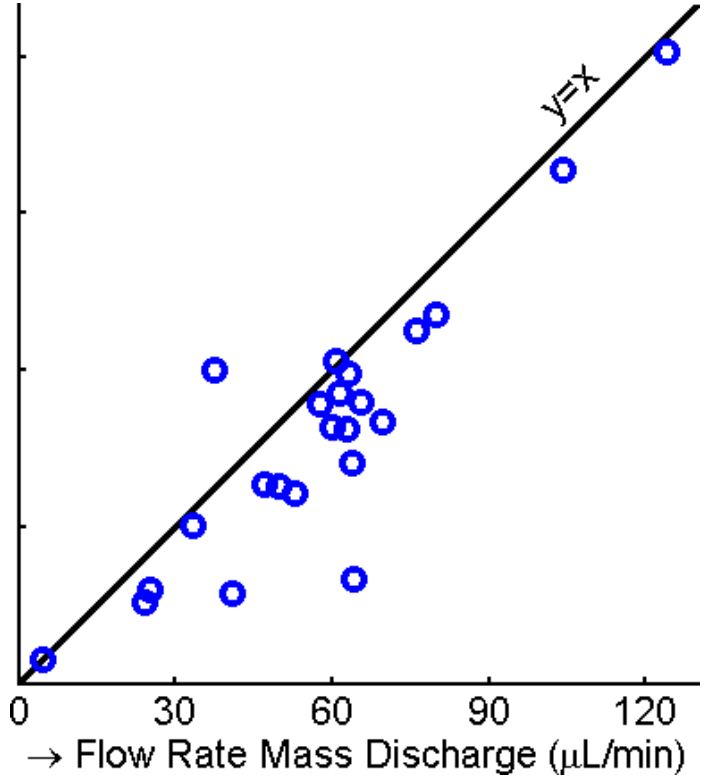
Vesicle Observation Center

EXOMETRY

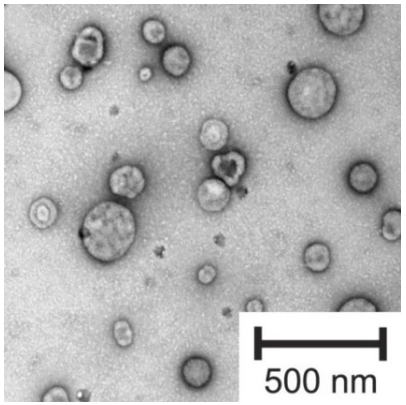
Determine flow rate

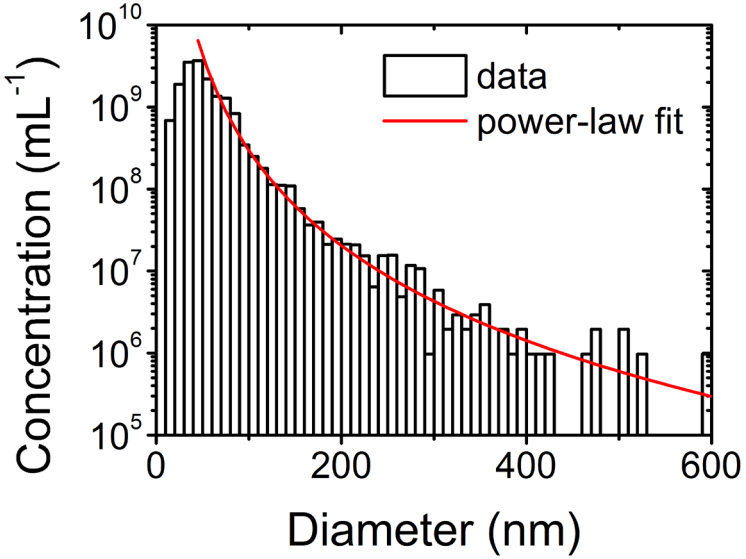
$$\text{concentration} = \frac{\#\ \text{of EV}}{\text{flow rate} \times \text{measurement time}}$$





Conclusions





- Detection of extracellular vesicles by flow cytometry: size does matter!
- Consider each flow cytometry aspect
  - Scatter
  - Fluorescence
  - Flow rate

Acknowledgements

- Vesicle Observation Center Amsterdam University Medical Centers
  - Ton van Leeuwen
  - Rienk Nieuwland
  - Frank Coumans
  - Leonie de Rond
- Software and beads by exometry.com
- More info: edwinvanderpol.com