Flow cytometry standardization by size and refractive index determination

Edwin van der Pol

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Vesicle Observation Center, Academic Medical Center, University of Amsterdam, The Netherlands

Conflicts of interest

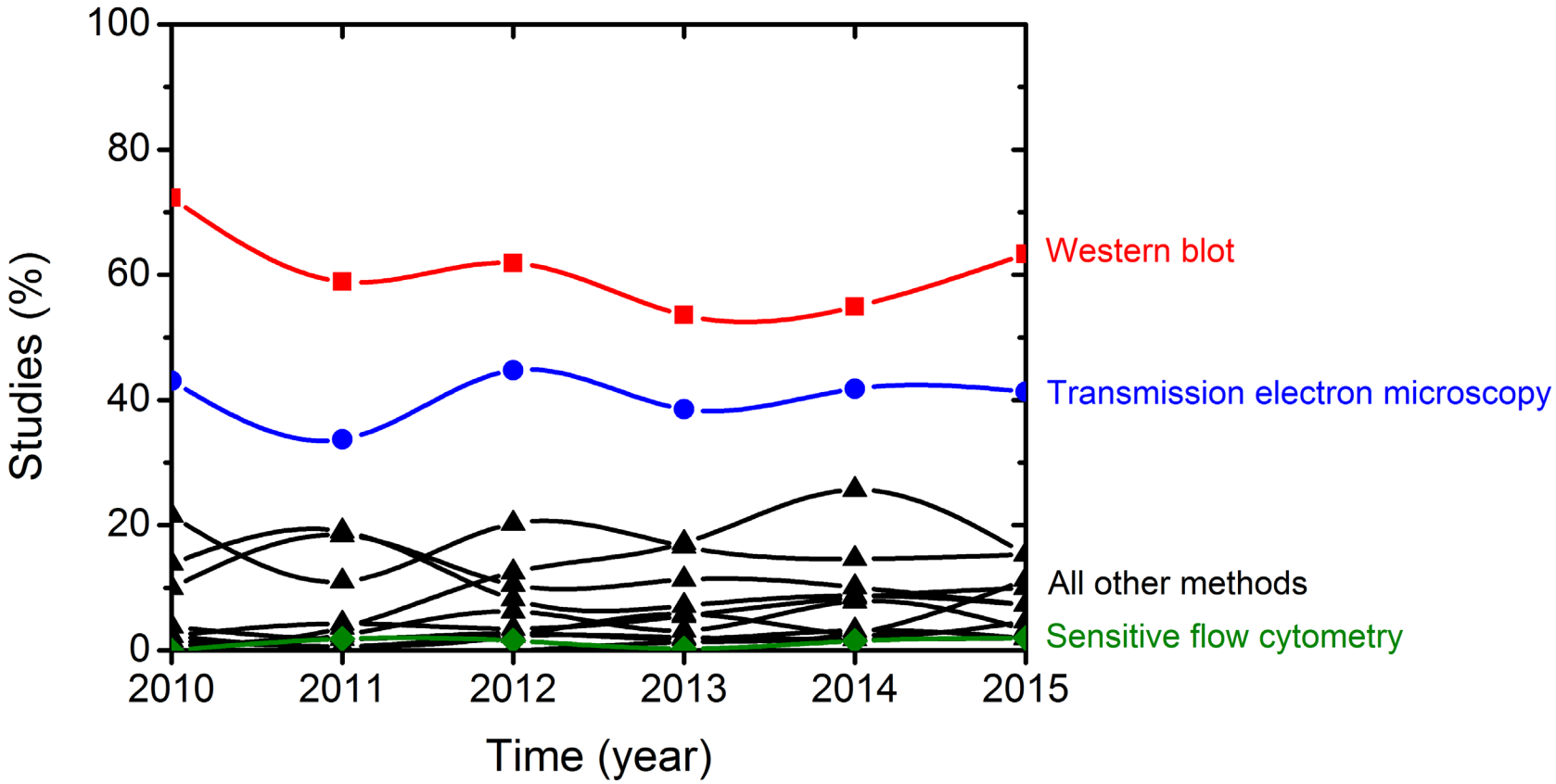
- Edwin van der Pol and Frank Coumans are cofounder and stakeholder of EXOMETRY

Outline

- standardization of flow cytometry measurements on extracellular vesicles
  - motive
  - by size determination
  - by size and refractive index determination
- Summary


Extracellular vesicles (EV)
200 nm

How do we study EV?



Motivation to detect single EV

- EV are heterogeneous
- study the contribution of all EV, including rare EV

Flow cytometry

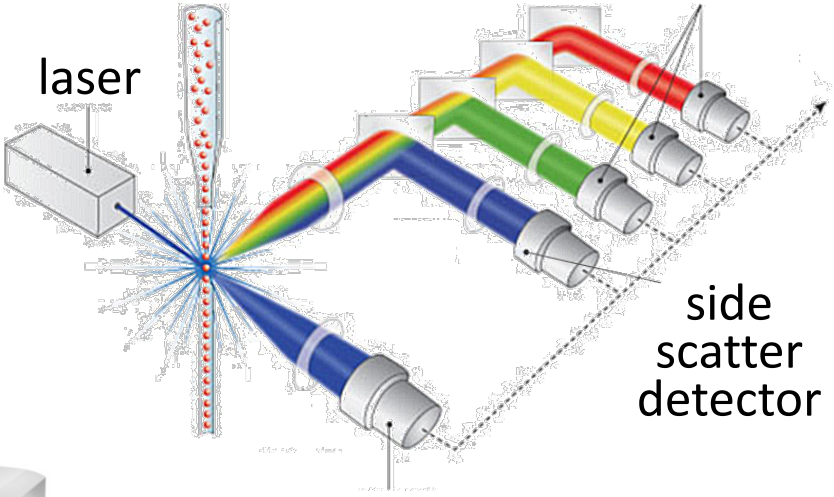


image: semrock.com

Standardization is boring (biologists, clinicians)

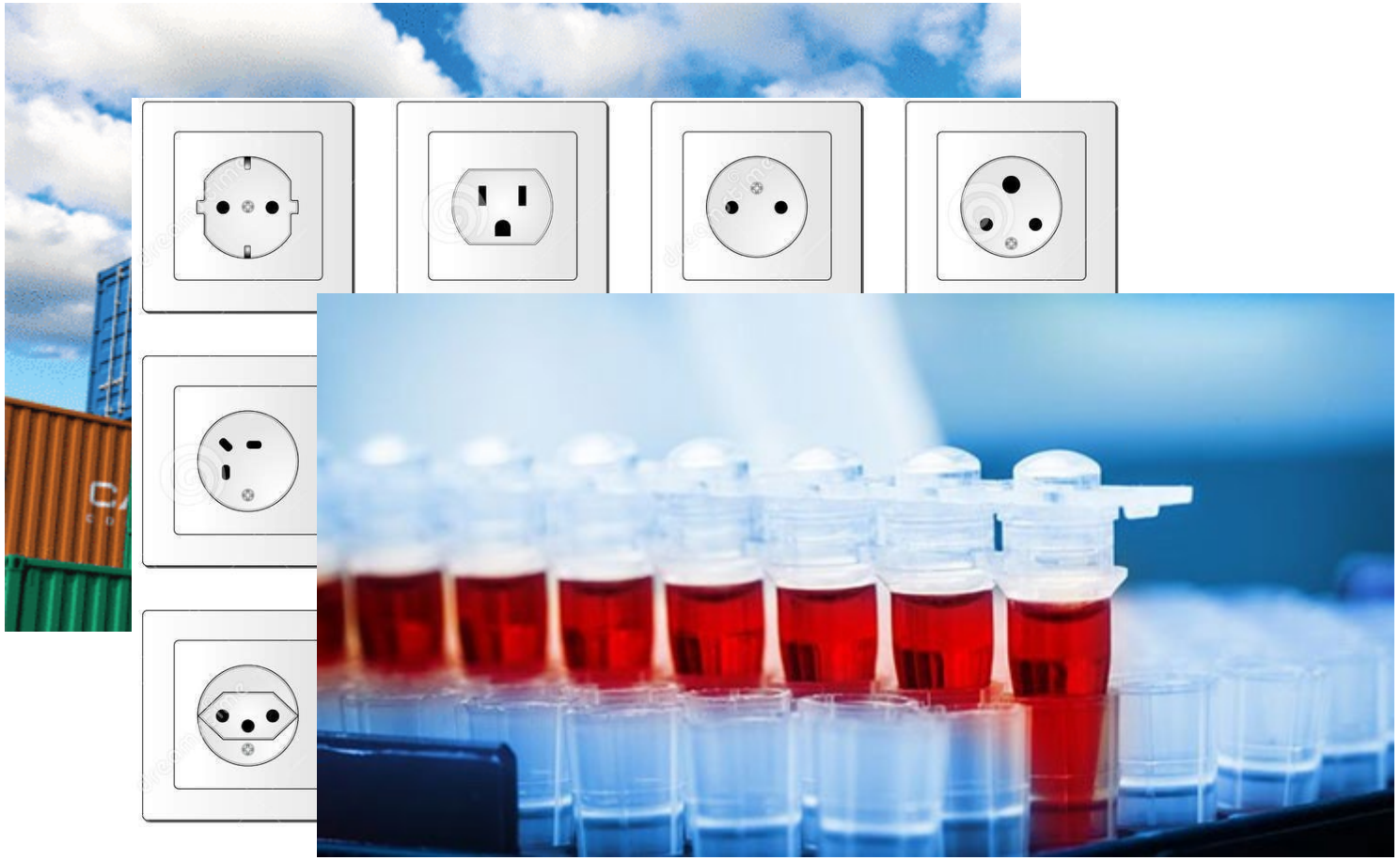
Standardisation is exciting (metrologists, physicists)

BESSYII

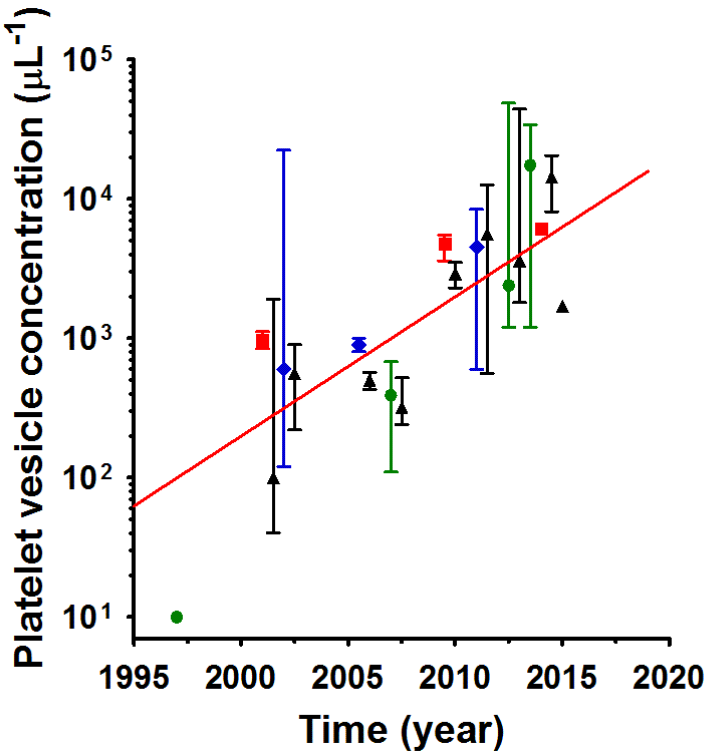
0.31 nm X-rays to size EV*
(flow cytometers typically use 488 nm light)

*Varga et al. *J.Extracell.Vesicles* 2014

Standardization is important (everybody)



Standardization is difficult (EV-field)



"Gąsecka's law"

- reported concentrations of plasma EV differ >10^6-fold
- clinical data cannot be compared

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  - ✔ motive
  - ➢ by size determination
  - ➢ by size and refractive index determination
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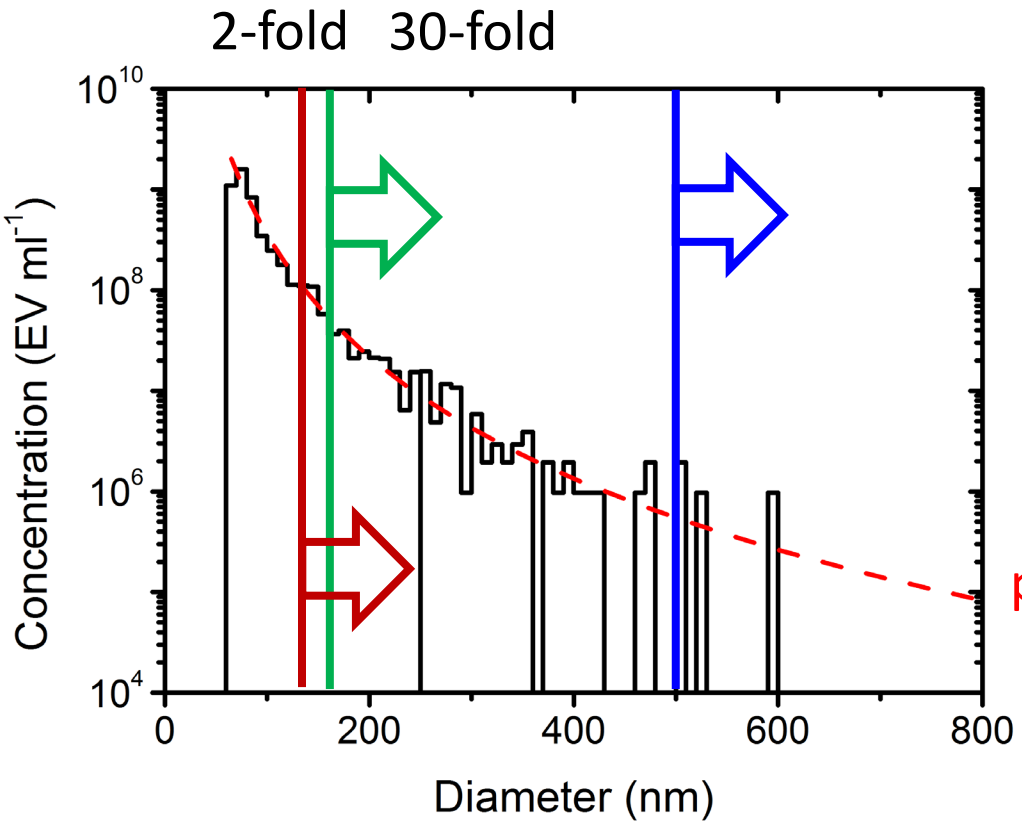
Problem 1: instruments differ in sensitivity

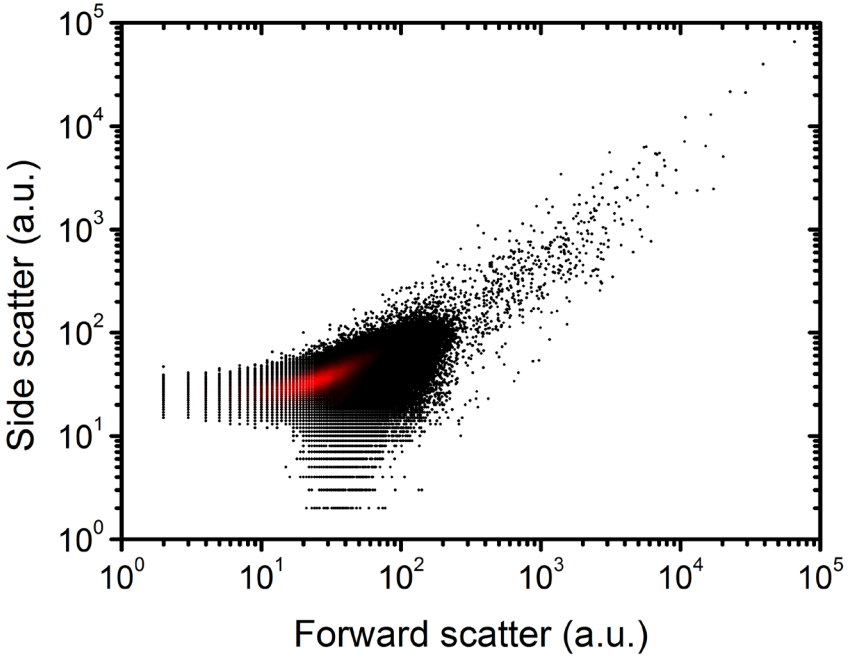


power-law relation*

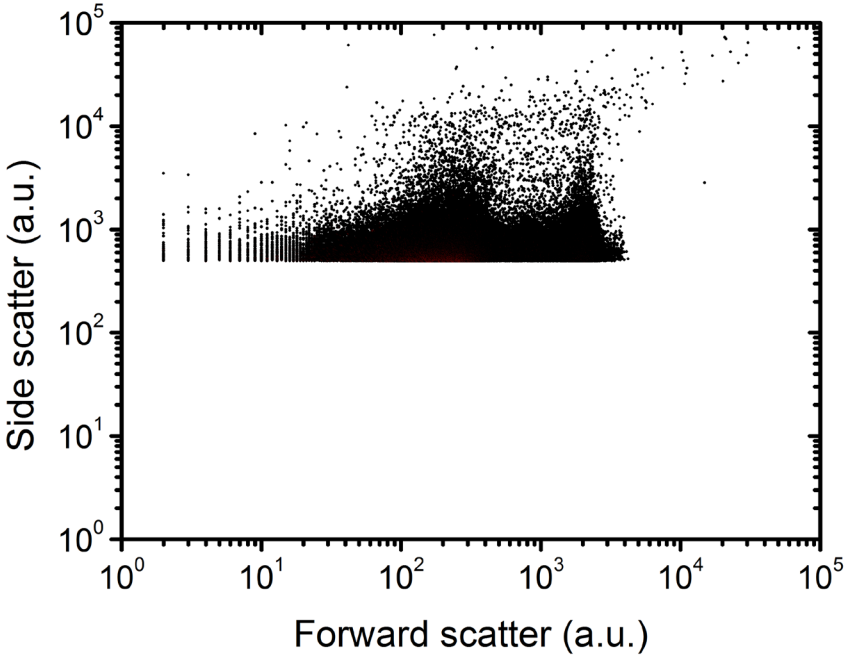
*van der Pol et al. *JTH* (2014)

Problem 2: arbitrary units

same population of erythrocyte EV



Apogee A50-micro



Becton Dickinson FACSCanto II

Goal

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

Study comprises 33 sites (64 instruments) worldwide

METVES

International Society on Thrombosis and Haemostasis

Vesicle Observation Center

EXOMETRY

Approach scatter-based standardization

- measure EV reference sample and controls
- determine flow rate
- scatter (a.u.) → diameter (nm)
  - measure METVES-beads
  - Exometry software obtains scatter to diameter relation
  - Exometry software provides EV size gates
- apply EV size gate to software (e.g. FlowJo) and report concentrations

EV reference sample

- erythrocyte EV from blood bank concentrate
  - CD235a-FITC labeled
  - trigger on most sensitive scatter channel
  - exclude EV similar to isotype

Approach scatter-based standardization

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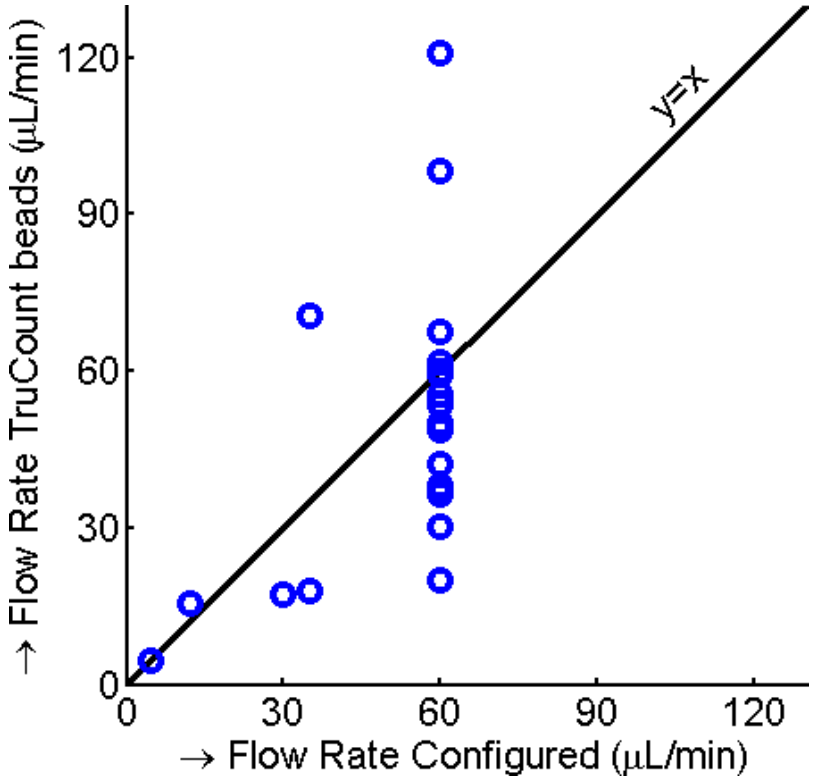
Determine flow rate

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



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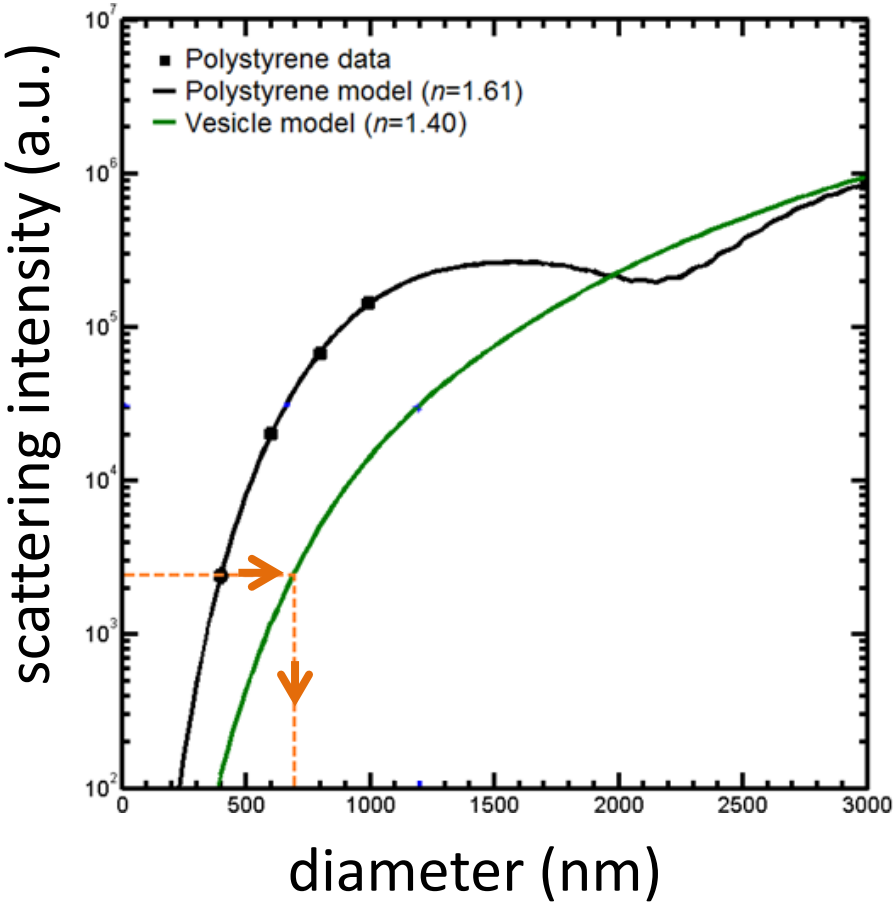
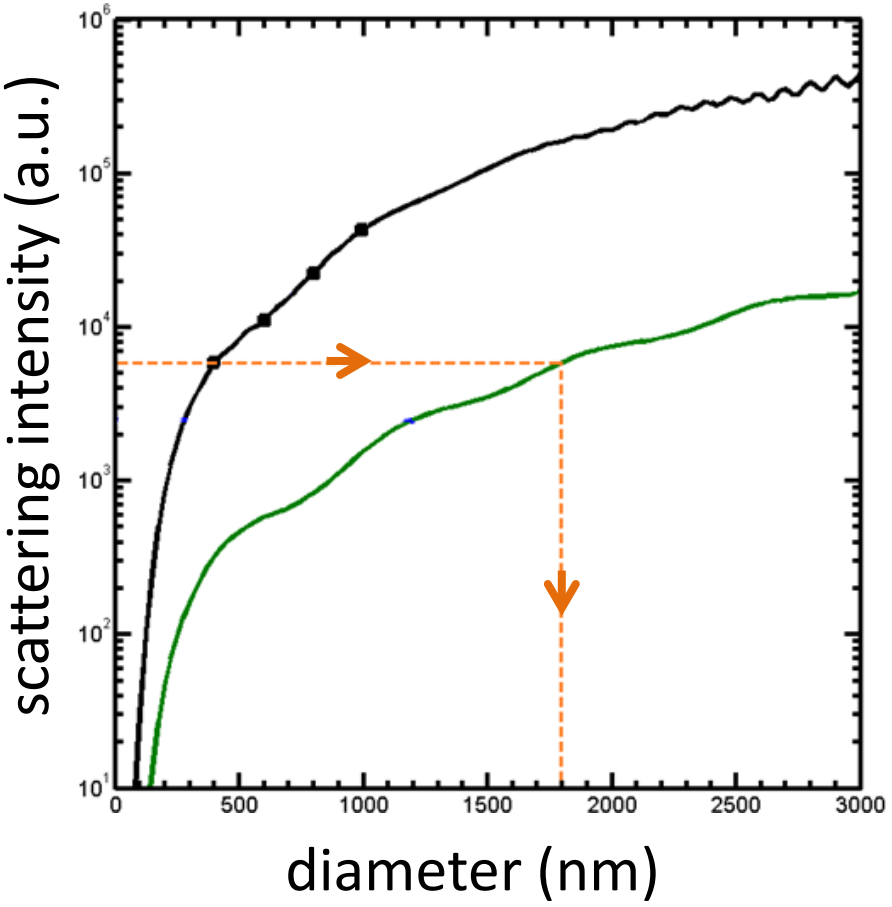
Earlier approach: gate on polystyrene beads

BC Gallios (forward scatter)



BD LSR II (side scatter)



Robert et al. *JTH* (2008)
Lacroix et al. *JTH* (2010)

Earlier approach: gate on polystyrene beads

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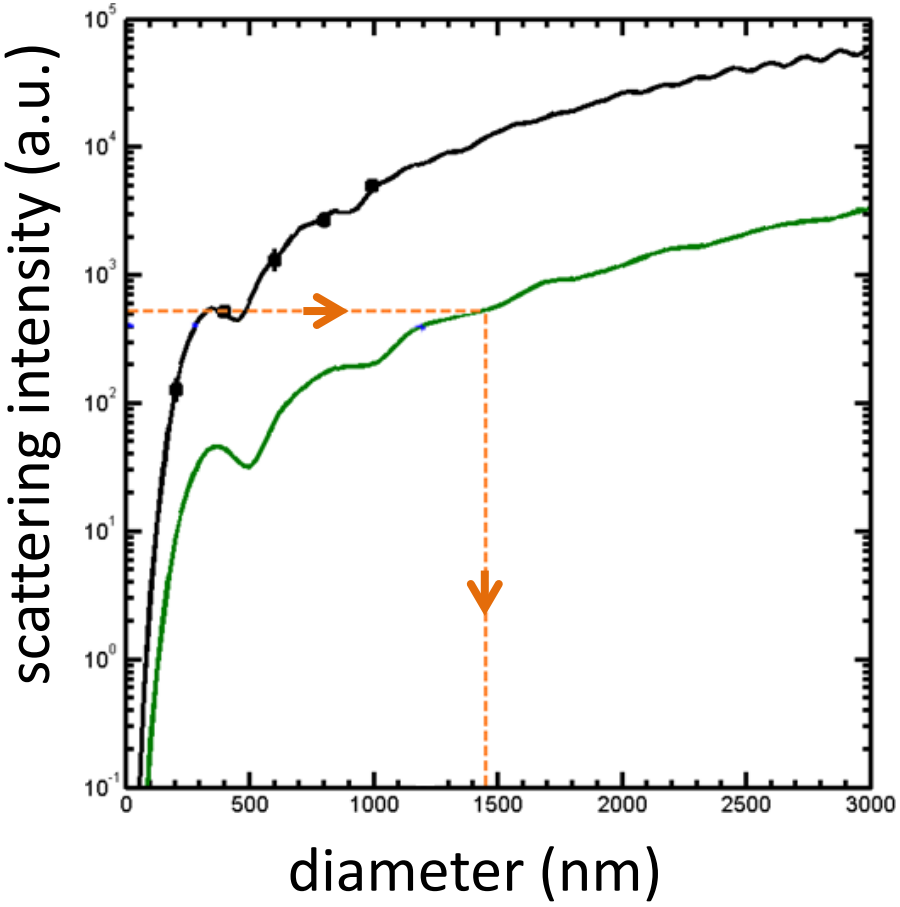

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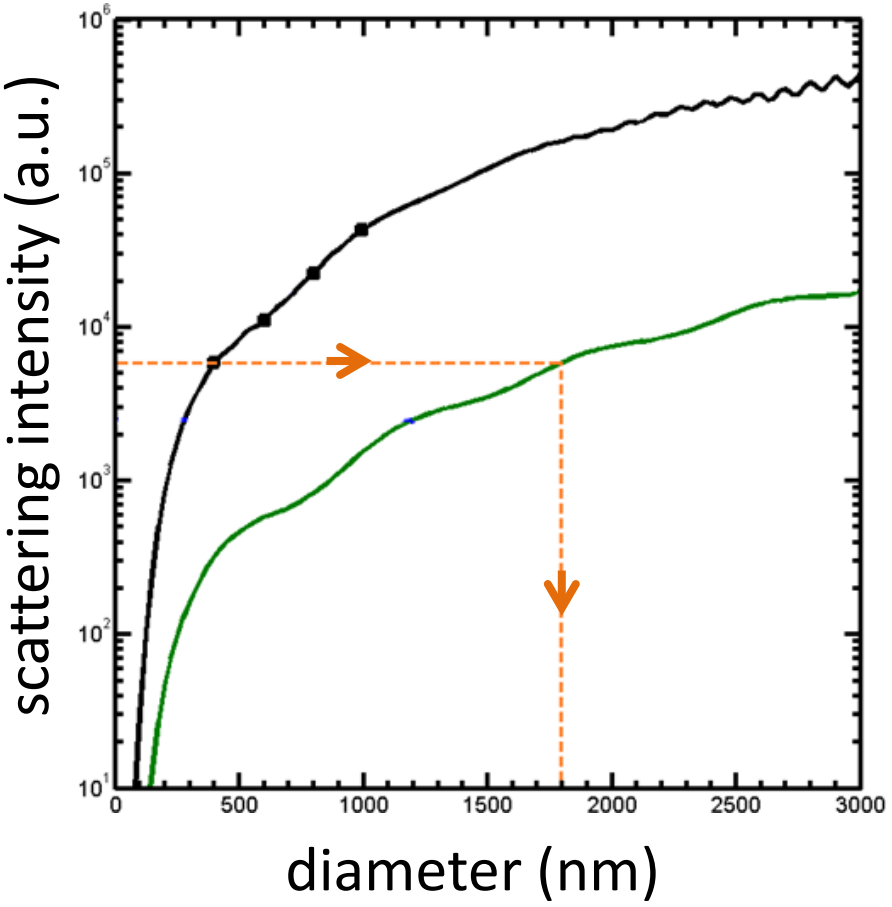
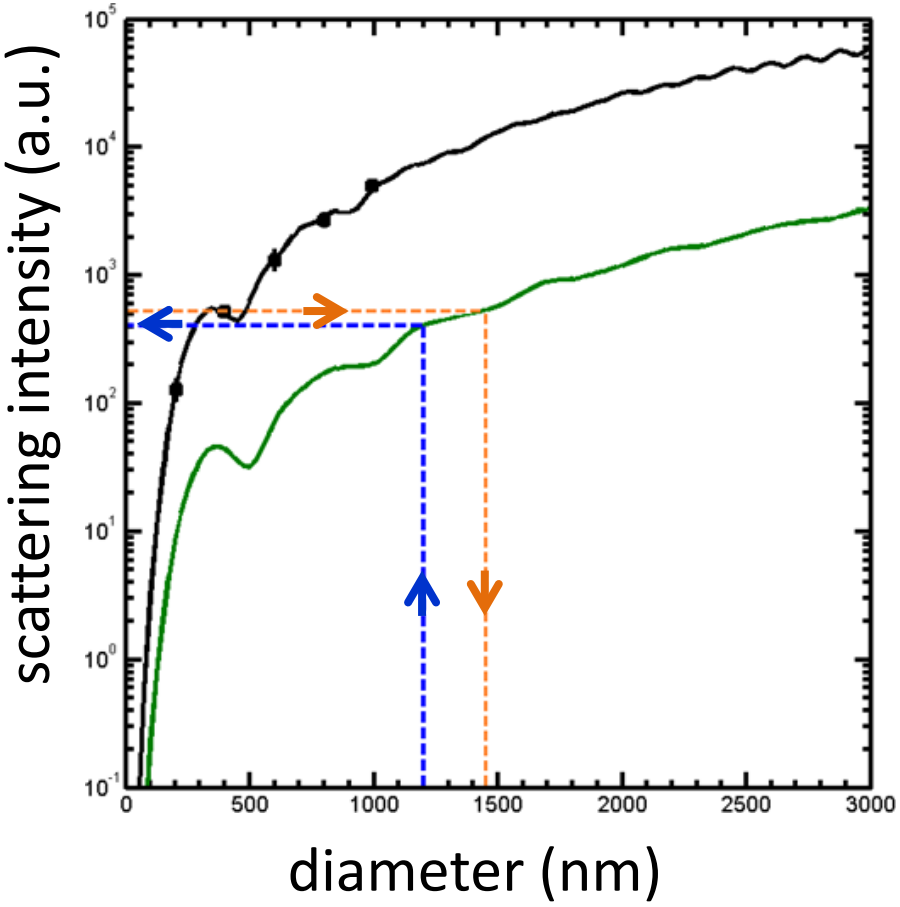


Robert et al. *JTH* (2008)
Lacroix et al. *JTH* (2010)

2016: relate scatter (a.u.) to diameter (nm)

BC Astrios MoFlo (side scatter)



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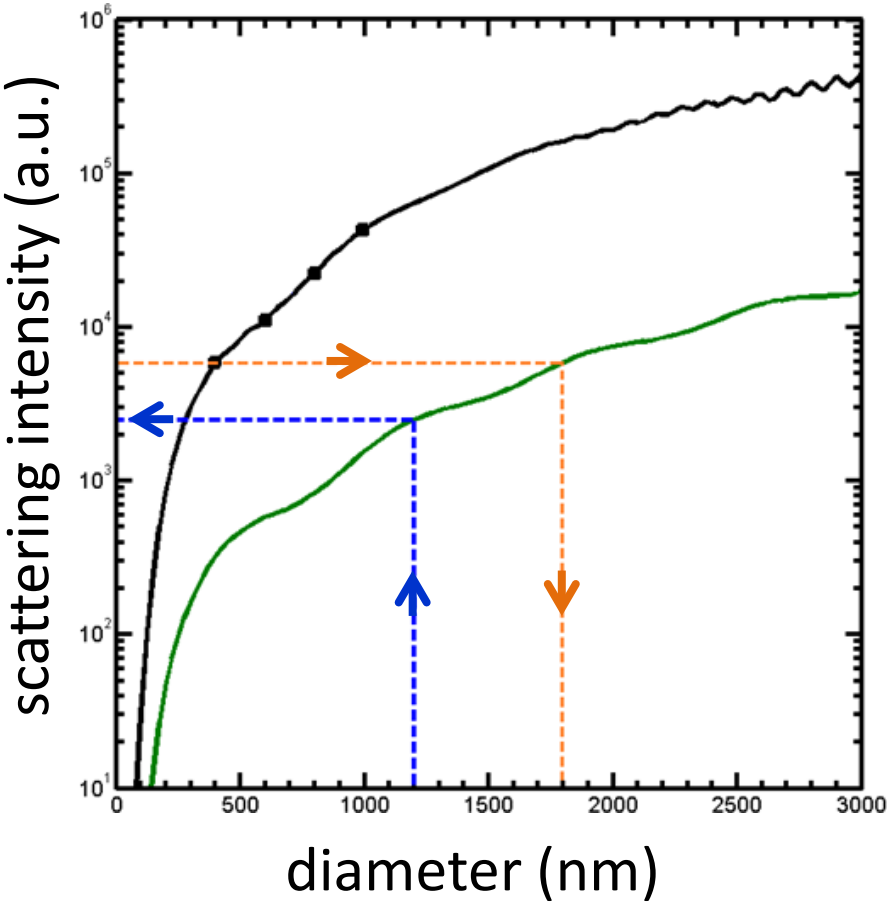

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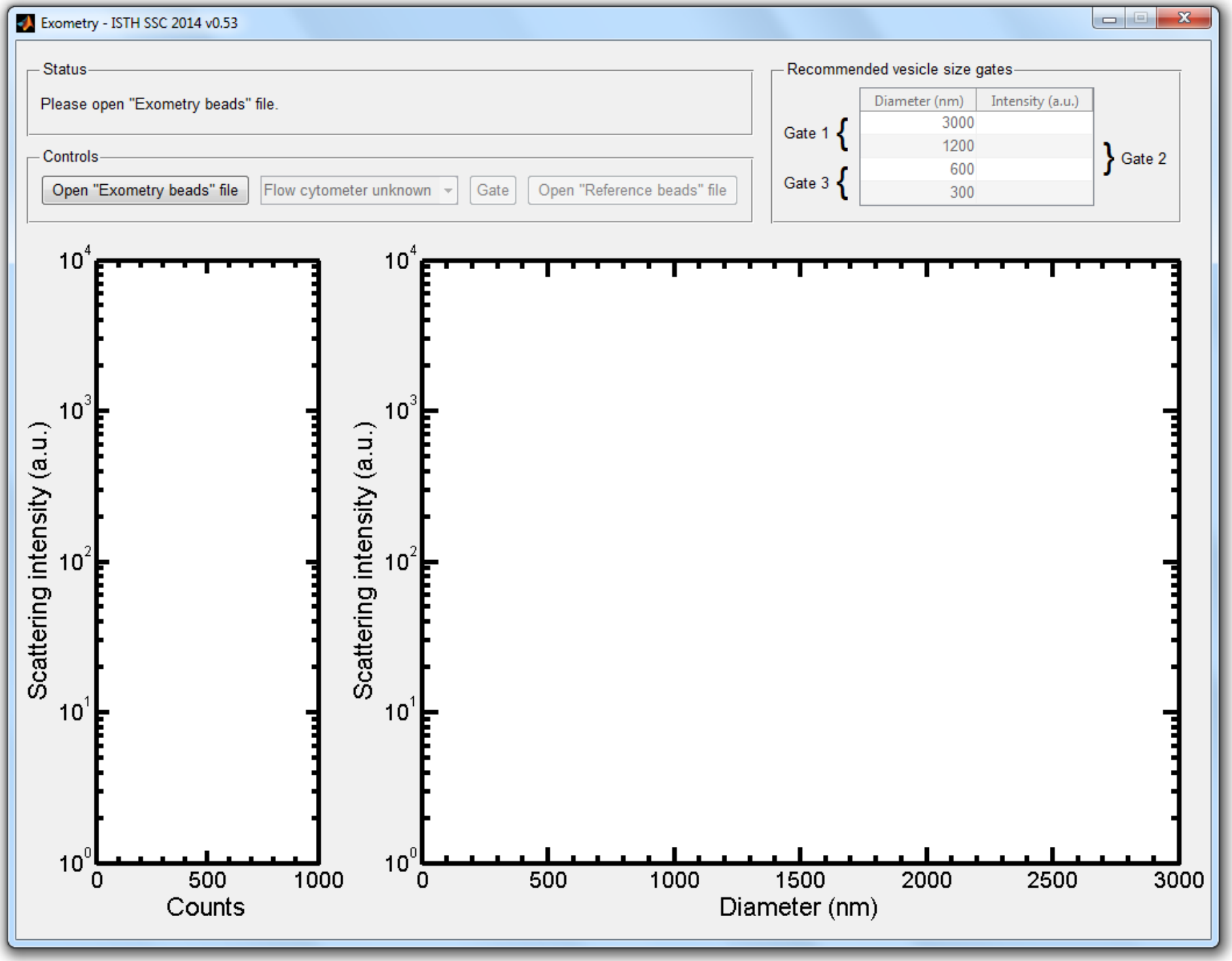

Exometry - ISTH SSC 2014 v0.53
Status
Please open "Exometry beads" file.
Controls
Open "Exometry beads" file
Flow cytometer unknown
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
10^0
10^1
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

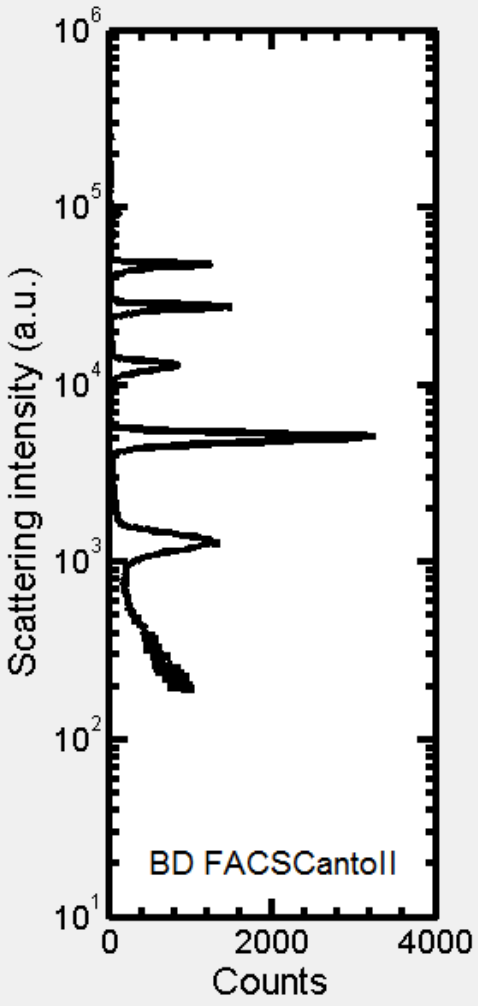
Please select detector and click "Gate" to obtain vesicle size gates.

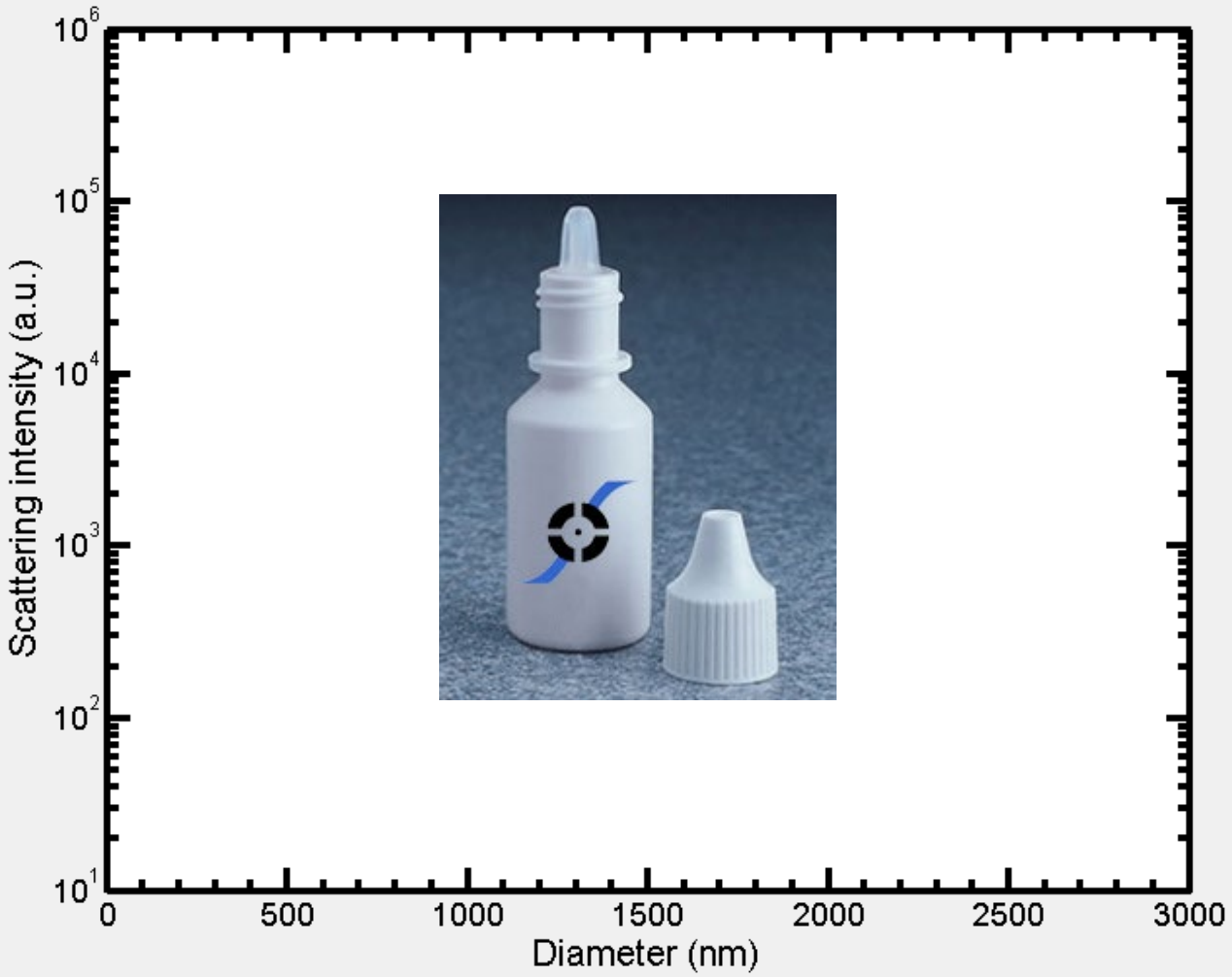
Controls

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.

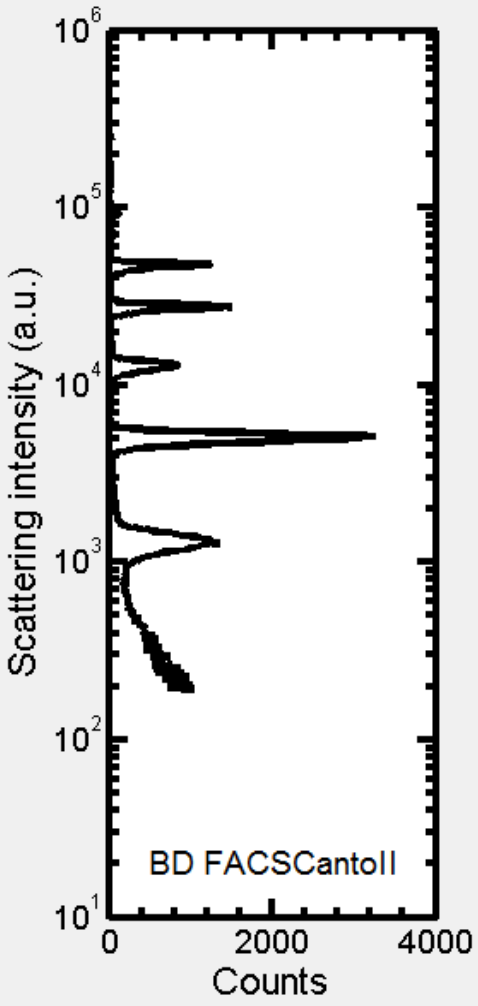
Controls

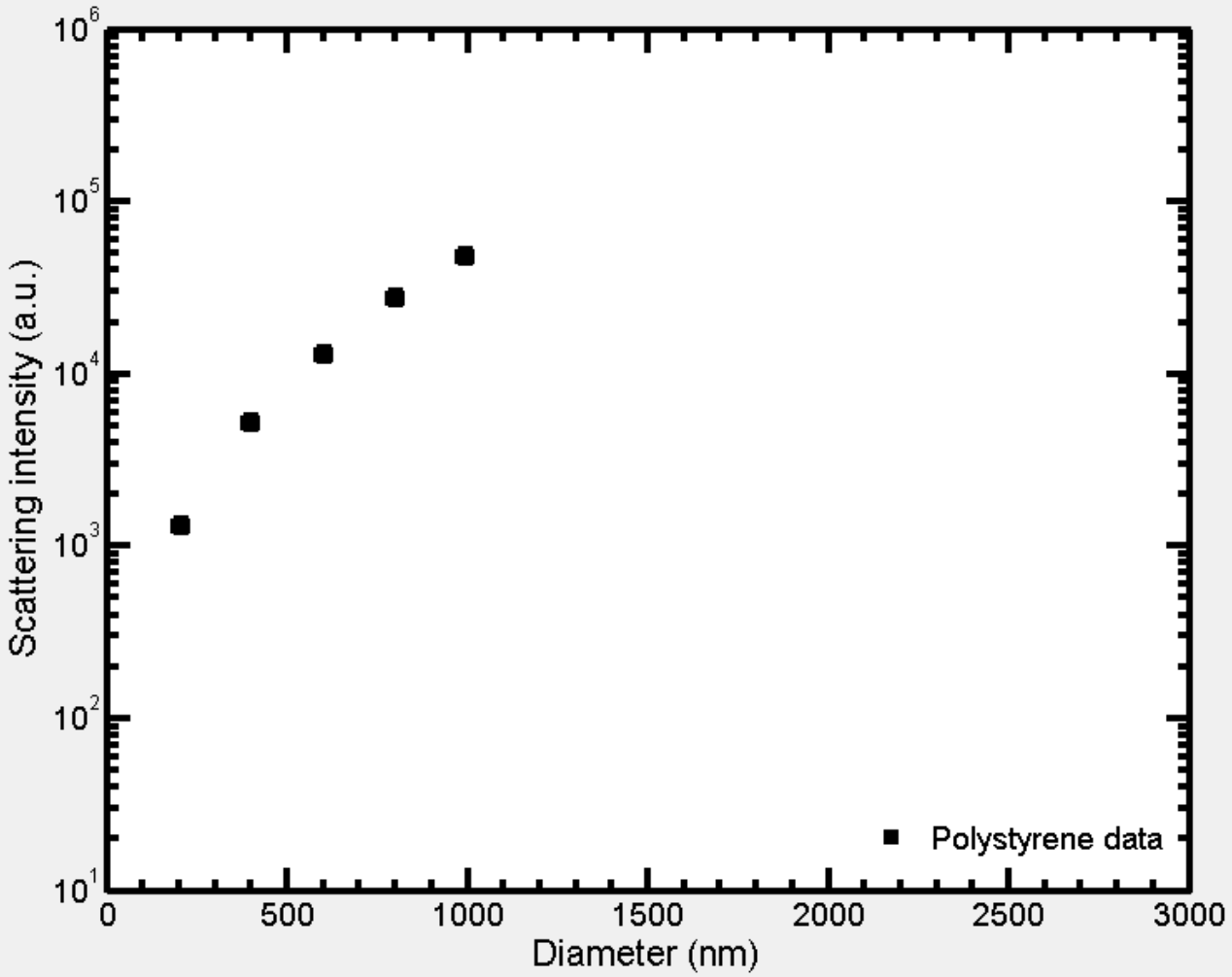
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

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

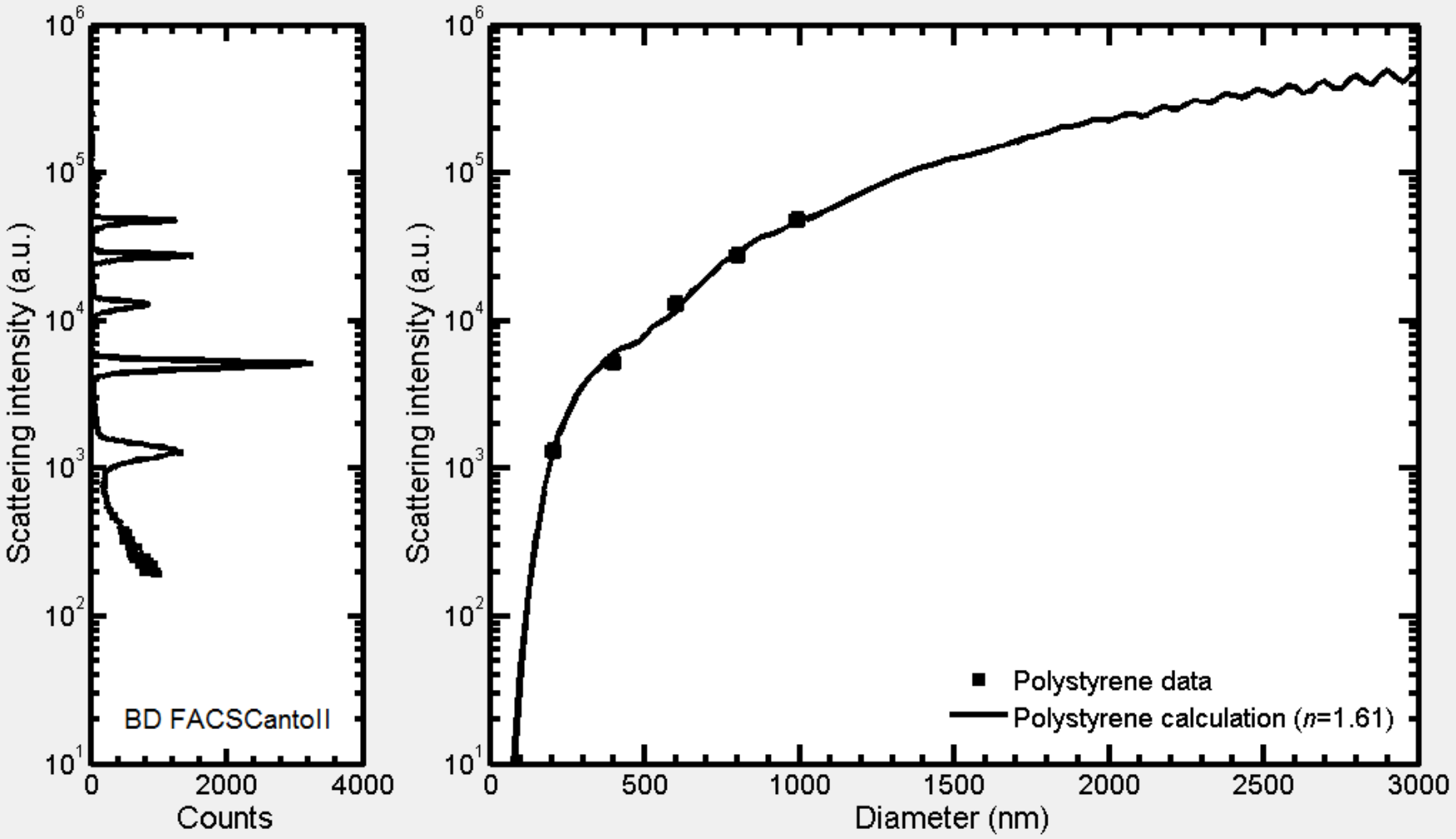
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.

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

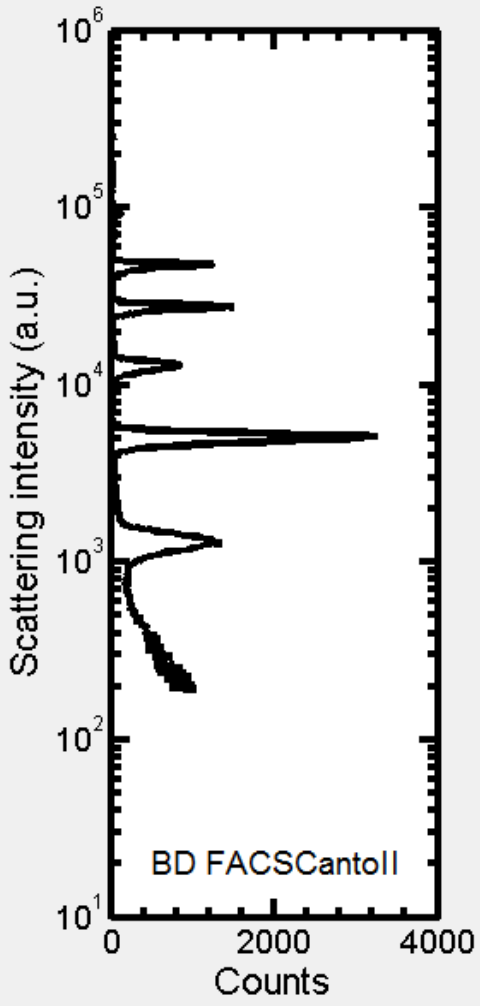
Congratulations, vesicle size gates determined, estimated error less than 0%.

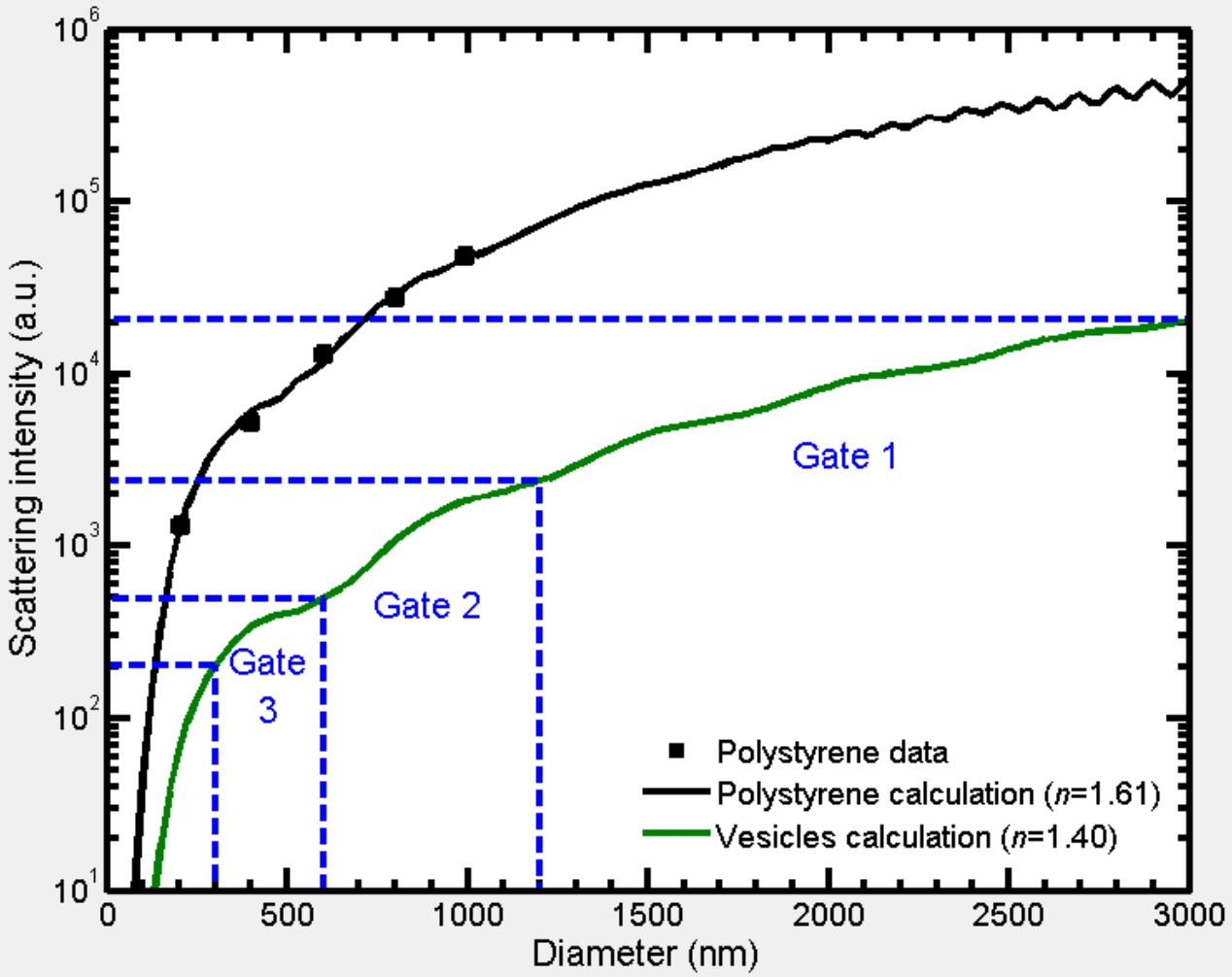
Controls

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	
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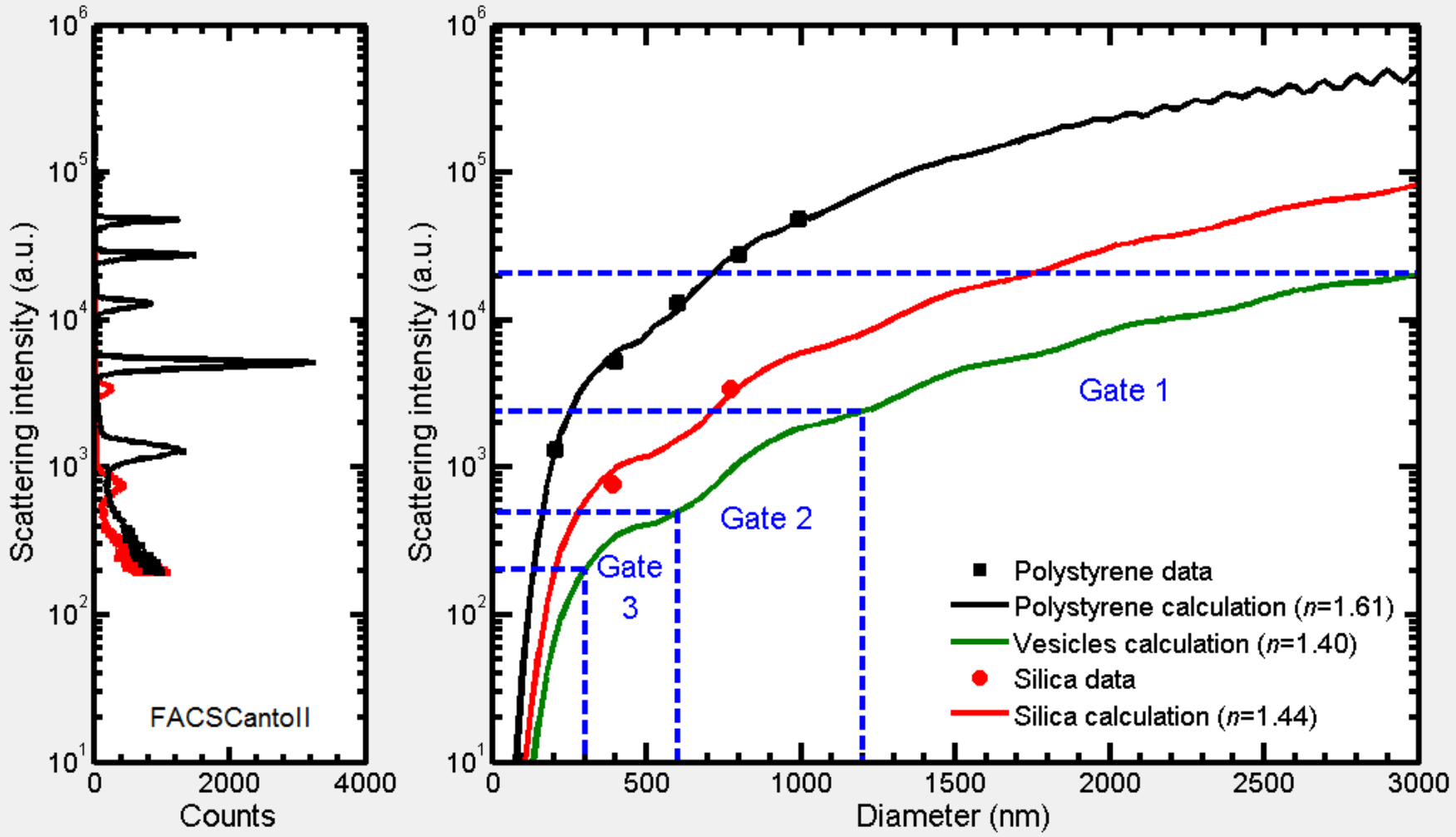

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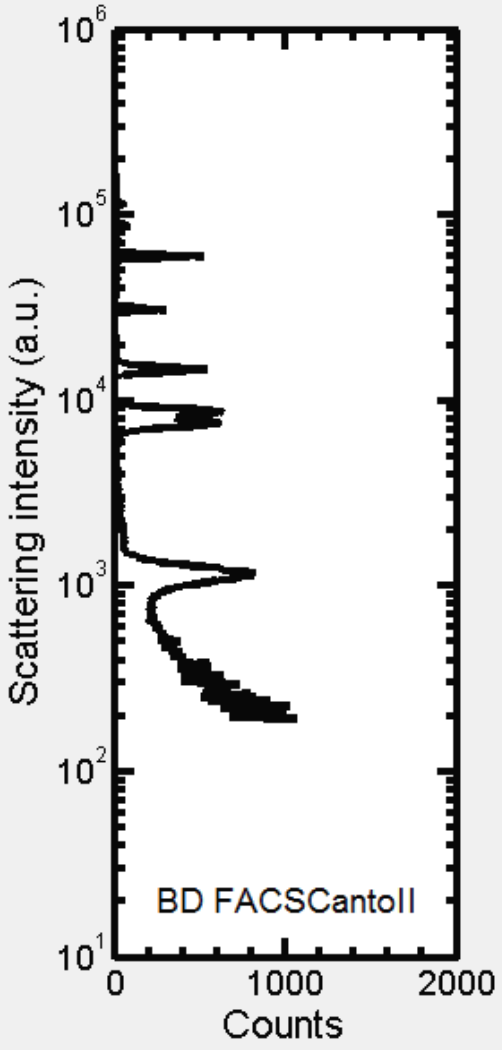
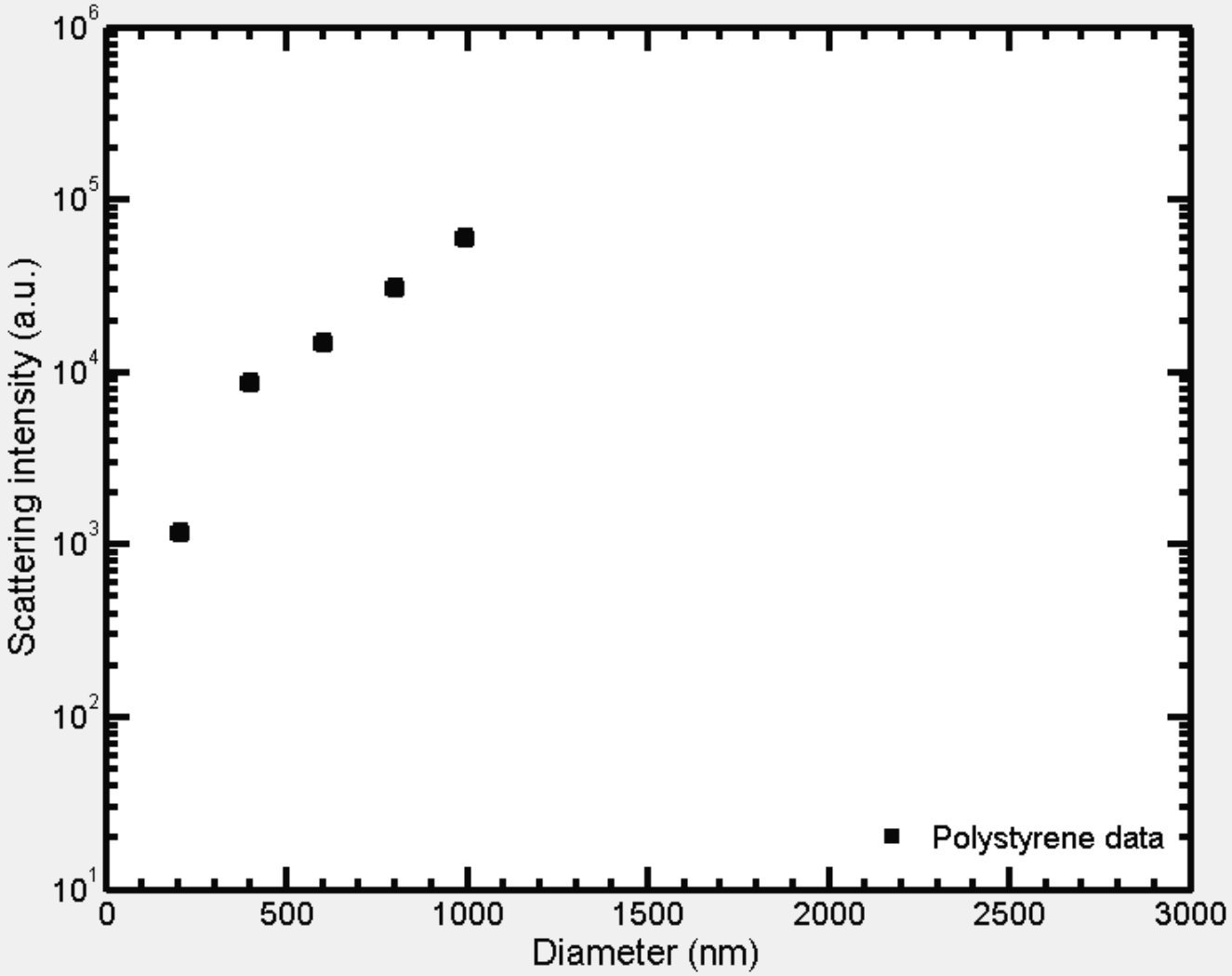
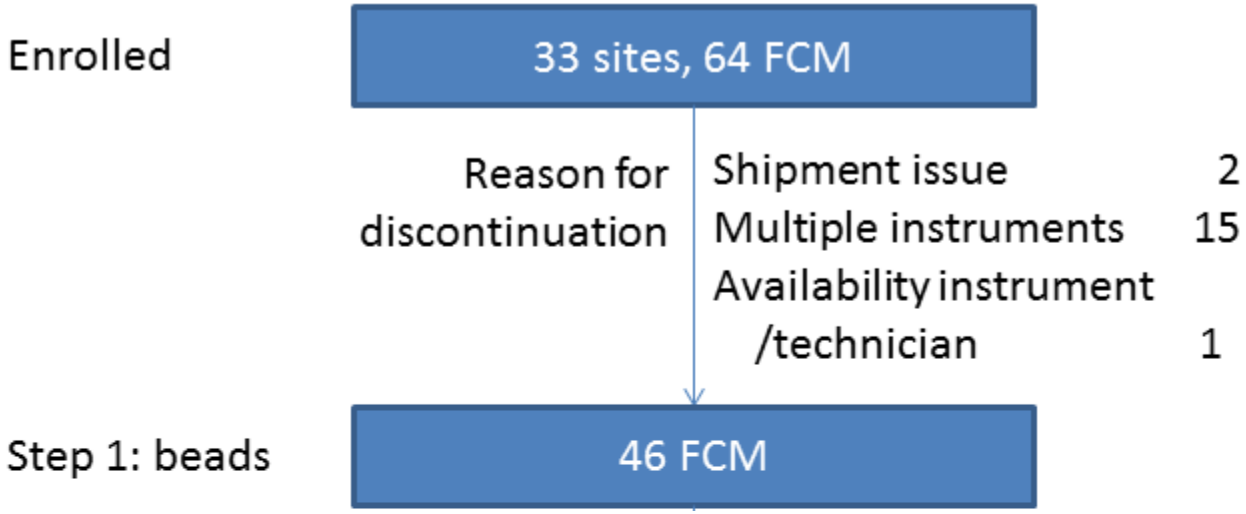
	300	

Gate 2



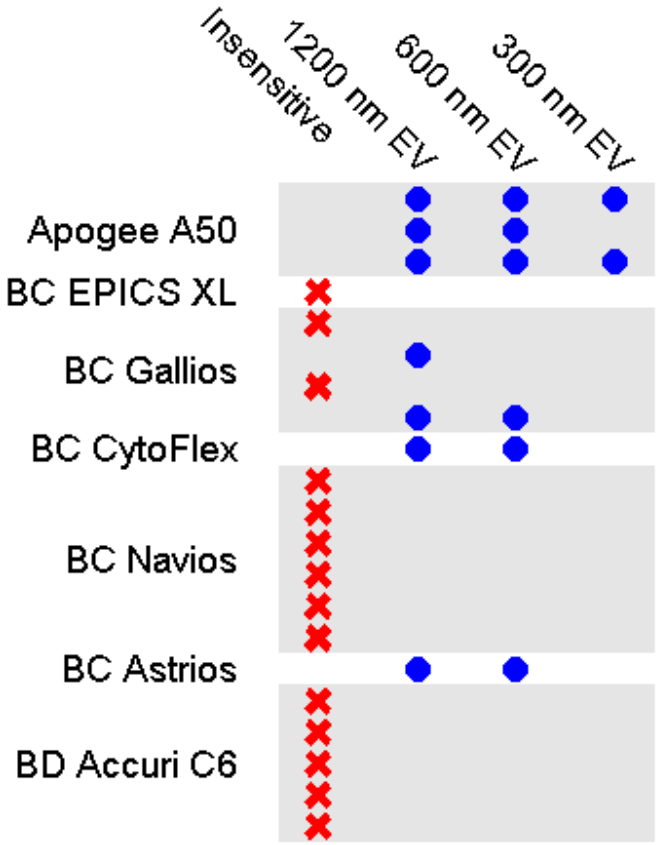


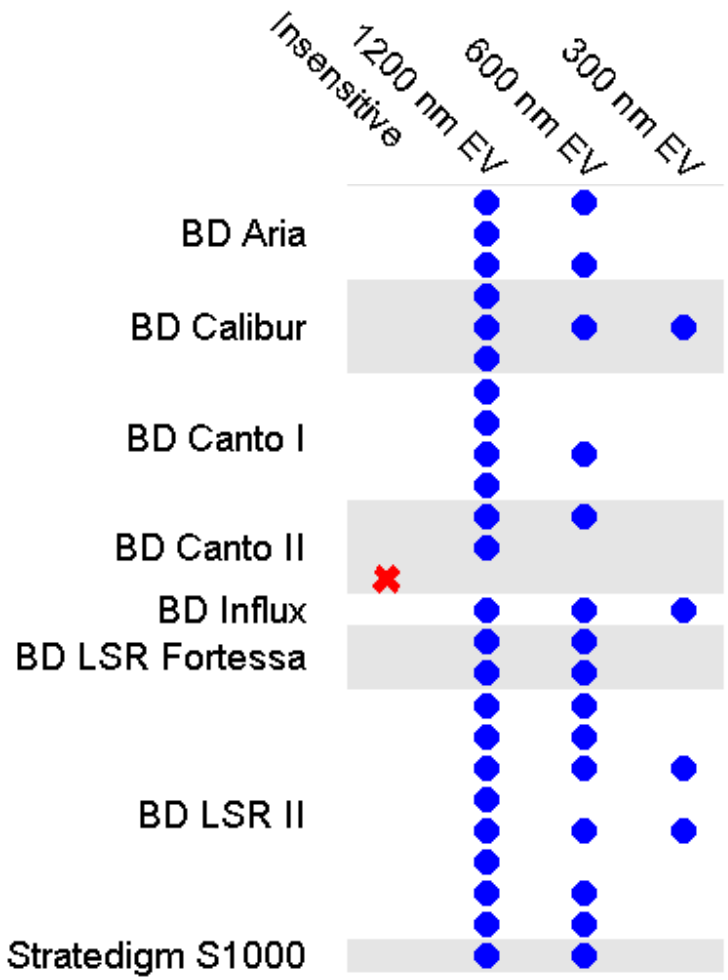
Exclusion of flow cytometers (FCM)



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

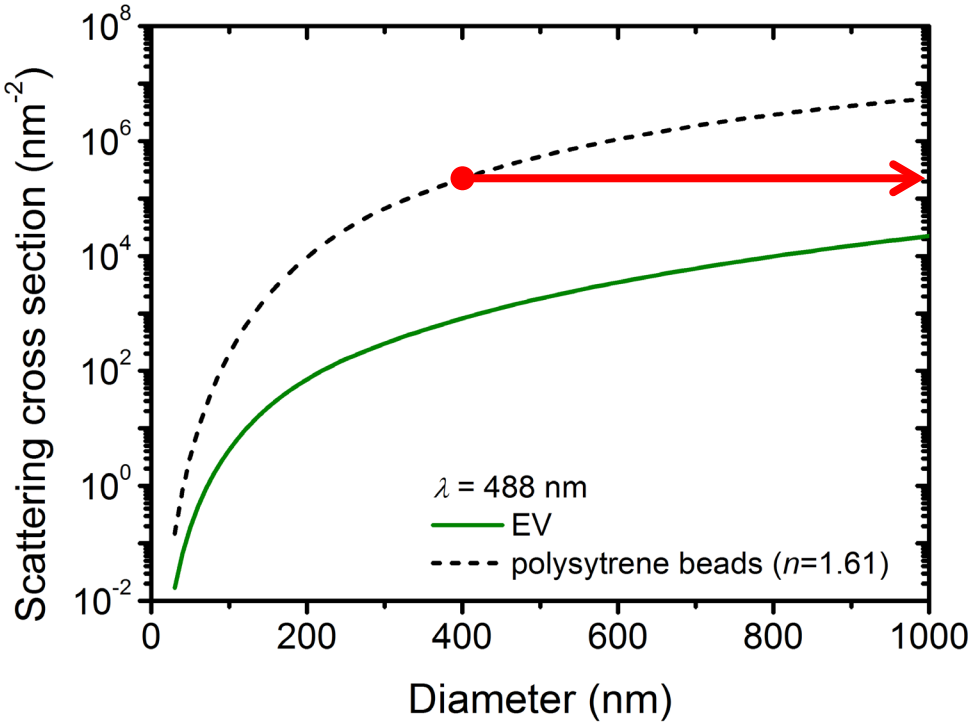
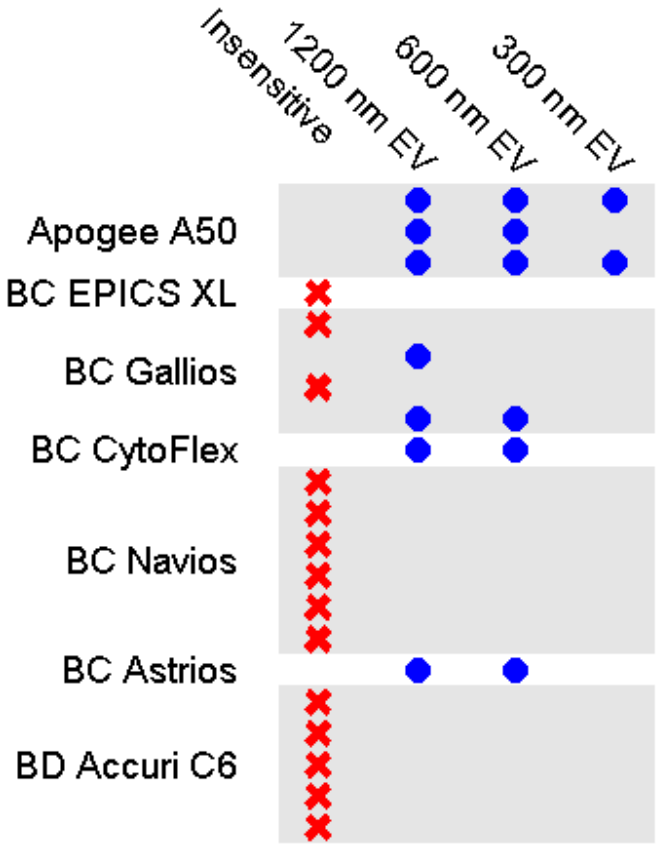
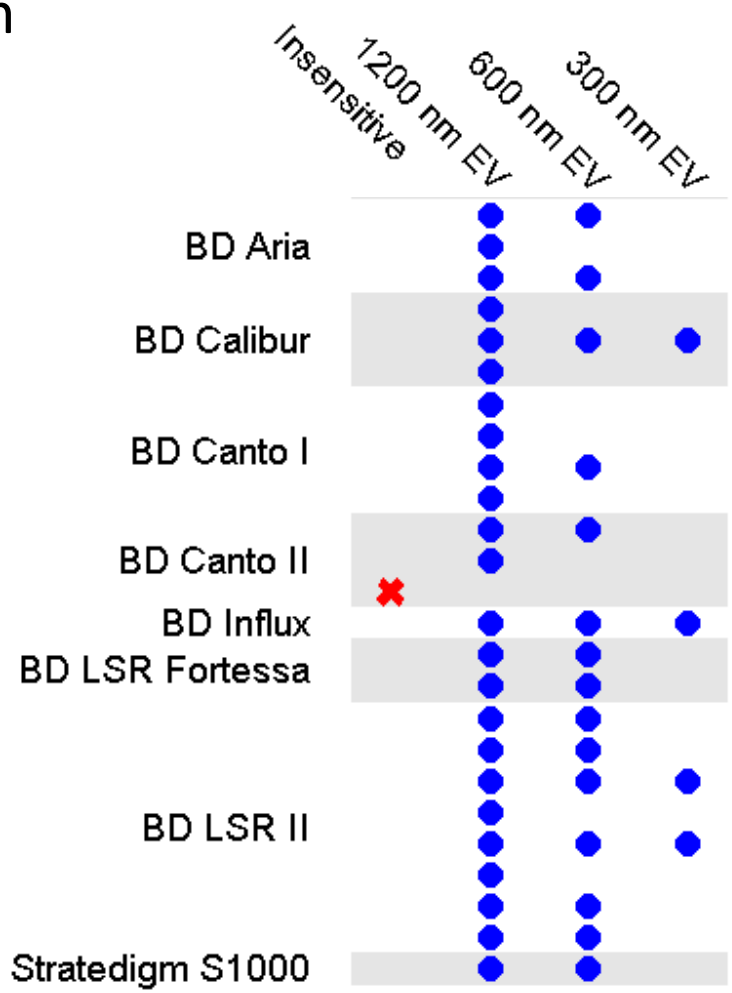


image: Issman et al. *PLoS ONE* 2013
van der Pol et al. *Nano Lett.* 2014

Sensitivity of 46 flow cytometers in the field

✖ = unable to detect EV < 1000 nm





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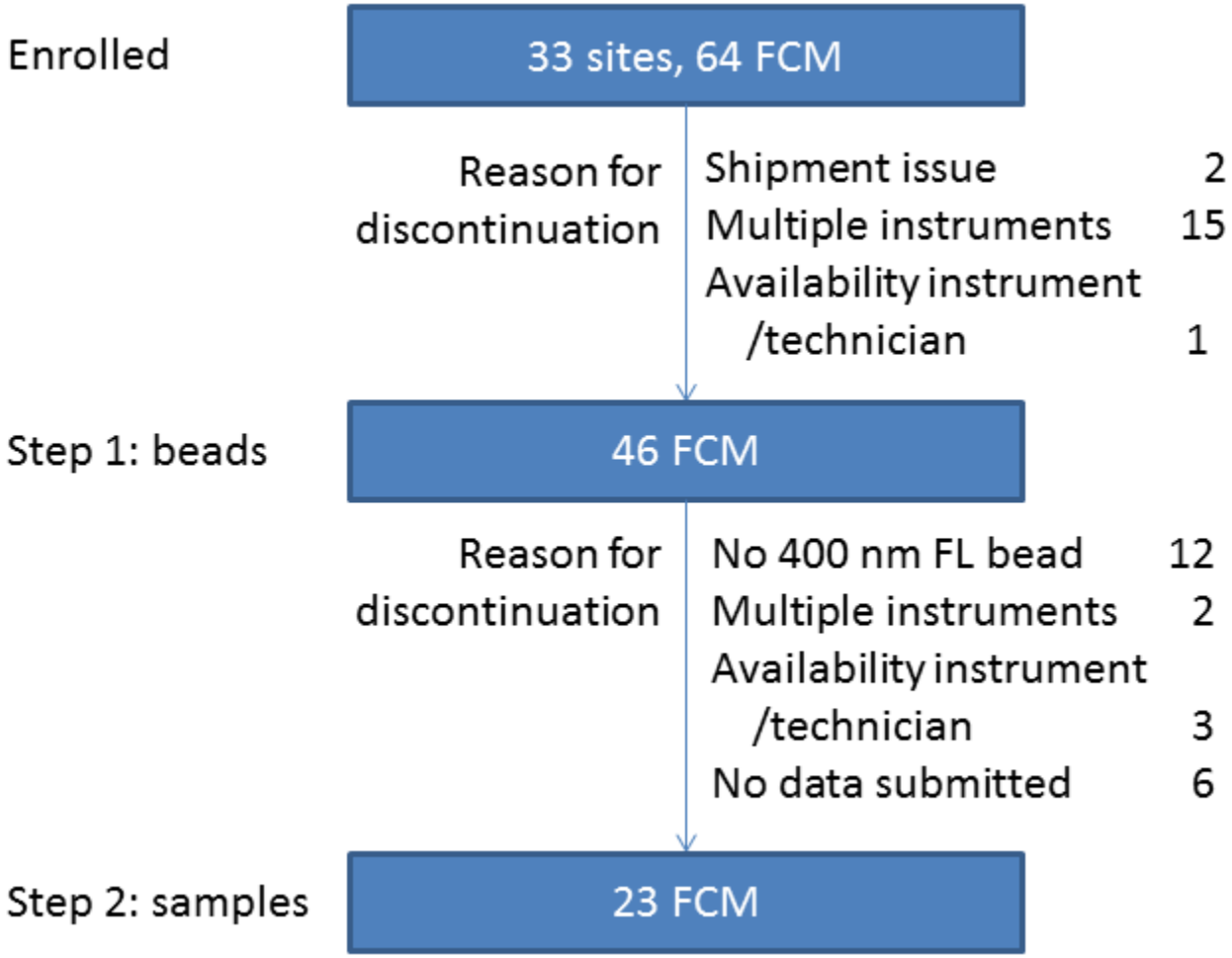

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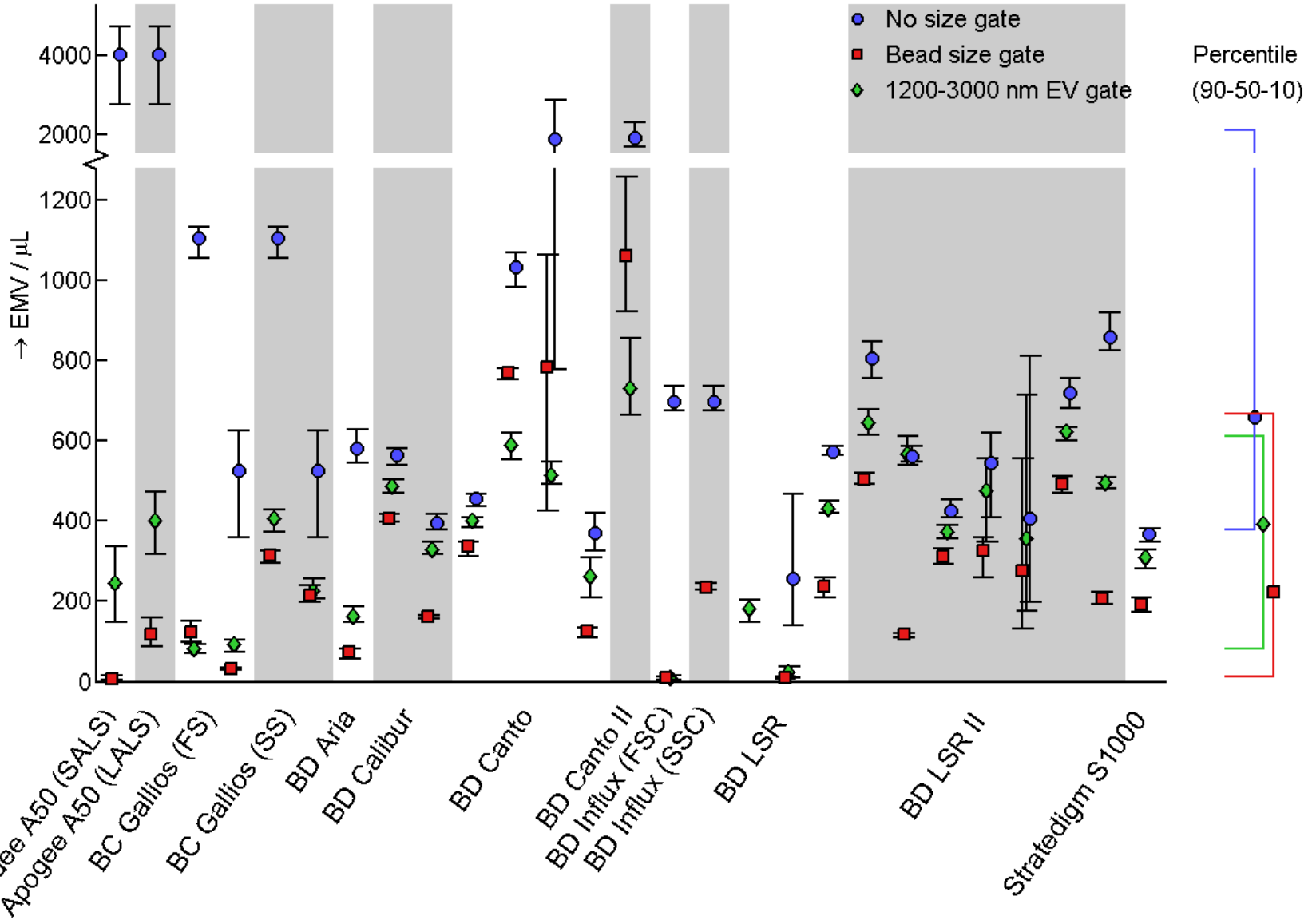
Reproducibility of 1200-3000 nm EV



Reproducibility of 1200-3000 nm EV

%CV	All	Side scatter only	Forward scatter only
Gate on beads	74%	60%	80%
Gate on EV size with light scatter theory	59%	42%	92%

%CV = standard deviation / mean * 100%
Preliminary results

Conclusions standardization by sizing

- flow rate calibration is essential
- many flow cytometers used in EV research do not detect EV by scatter-based triggering
- EV size gate by Mie theory (CV=59%) leads to better reproducibility than gate on beads (CV=74%)

Discussion standardization by sizing

- assumption of EV size gate by Mie theory
  - EV have similar refractive index of 1.4
- discrepancy between FSC and SSC
  - due to incorrectly selected refractive index?
- standardization of EV sizes <1200 nm ineffective

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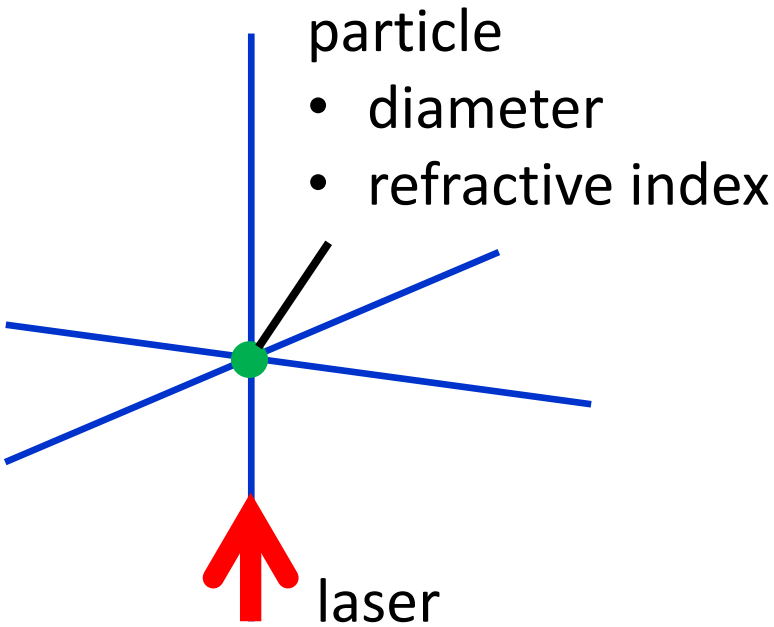
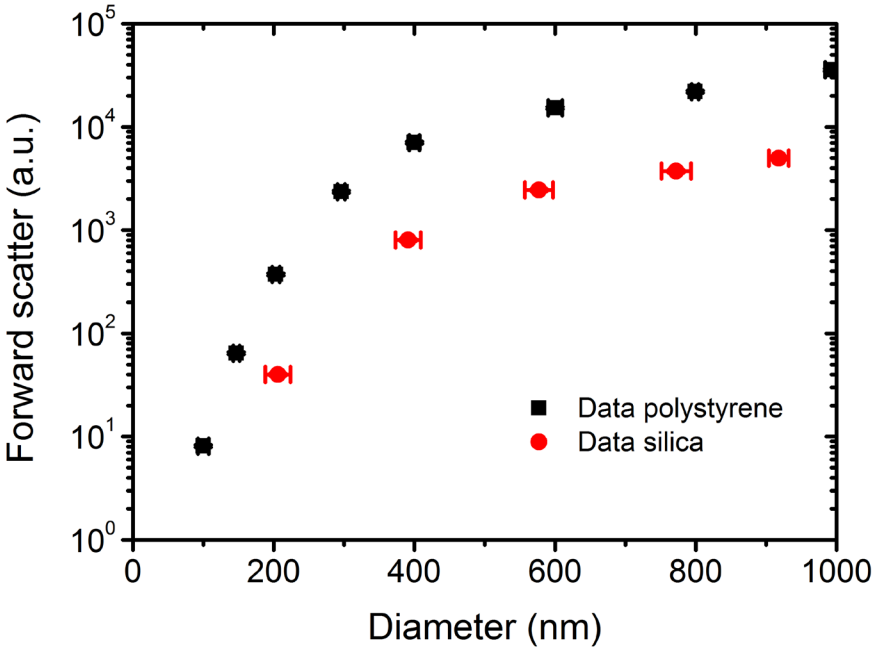
Size *and* refractive index determination of EV

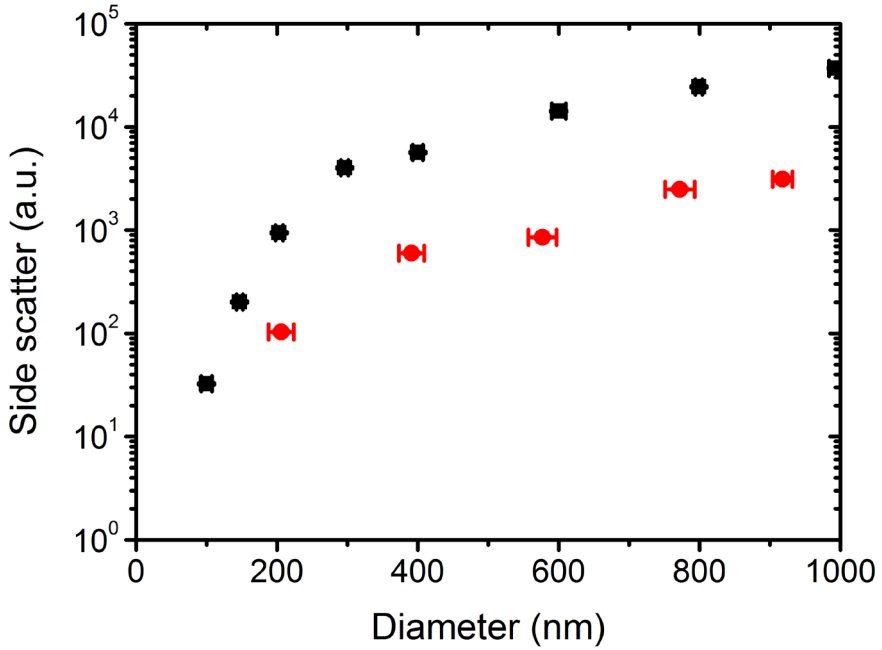


Approach

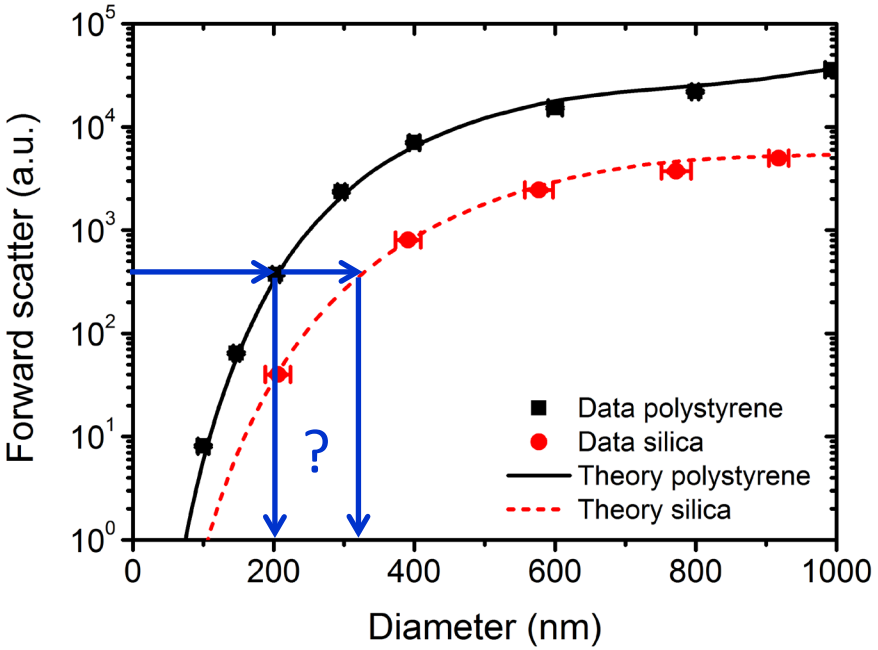
- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

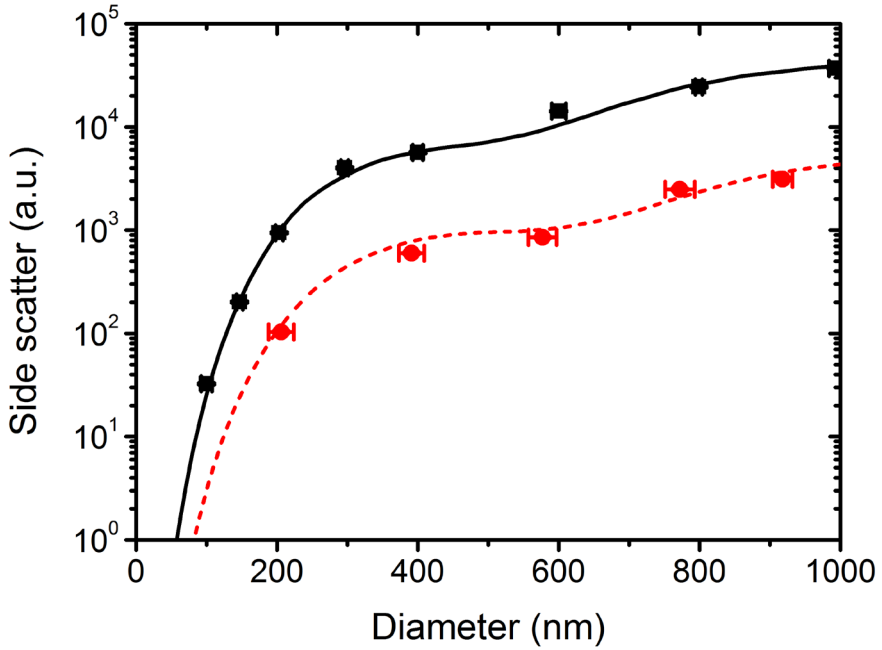
Calibrate forward scatter and side scatter





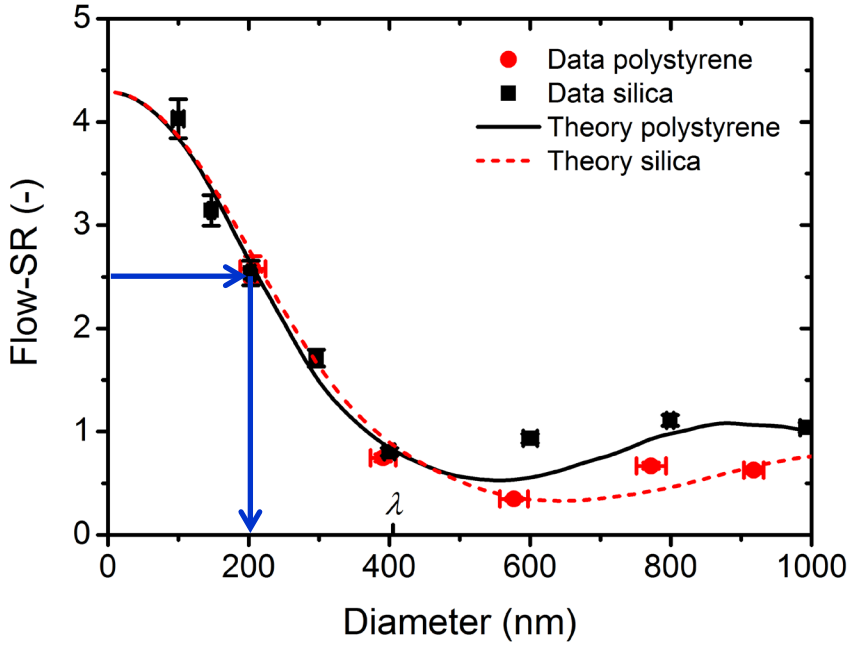
Calibrate forward scatter and side scatter





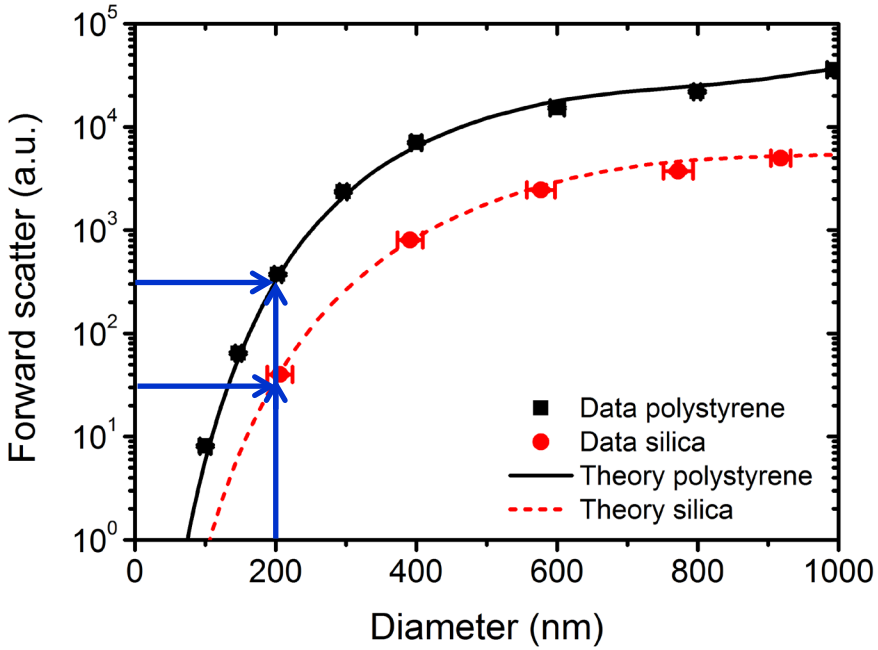
$$\text{Flow-SR} = \frac{\text{side scatter}}{\text{forward scatter}}$$

Derive size from Flow-SR



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

Derive refractive index from size and FSC



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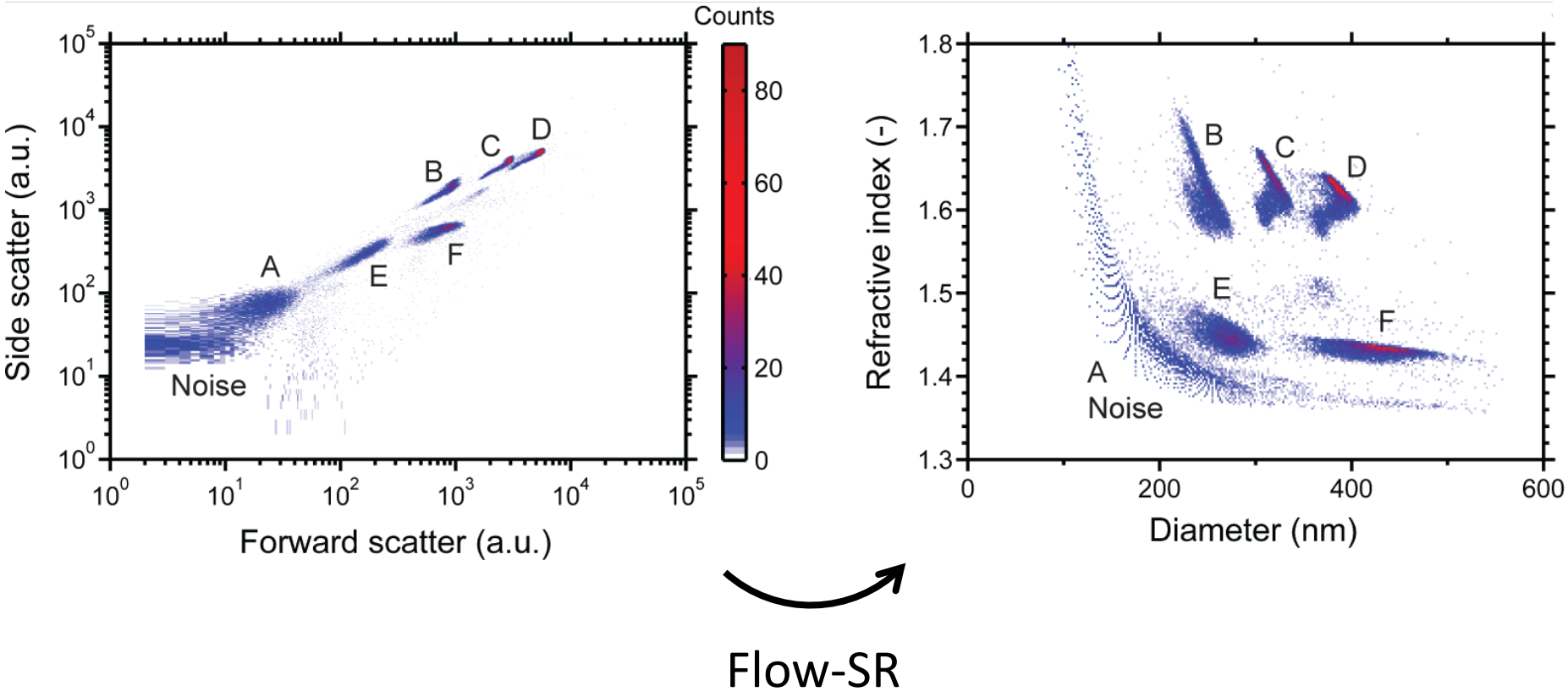
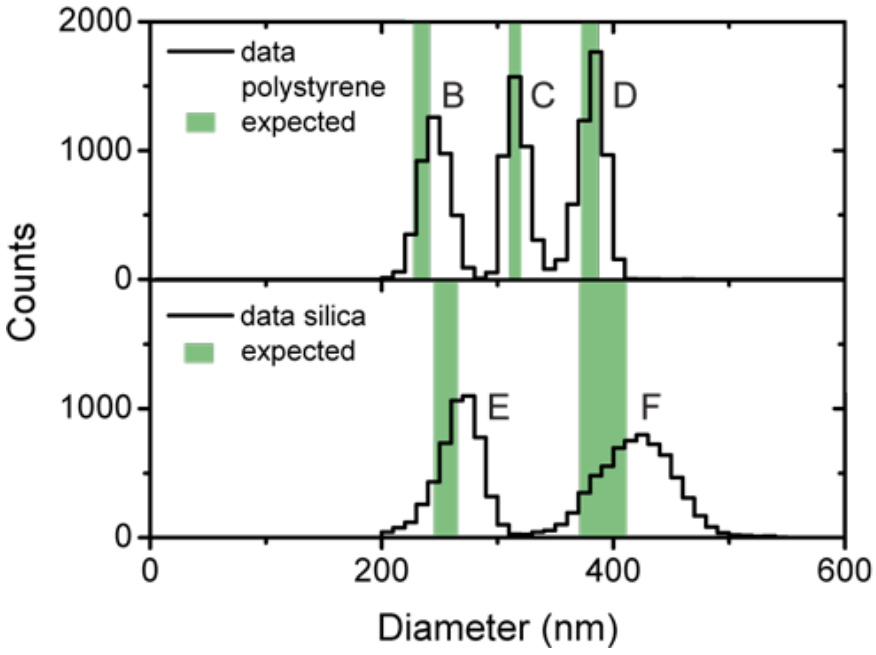
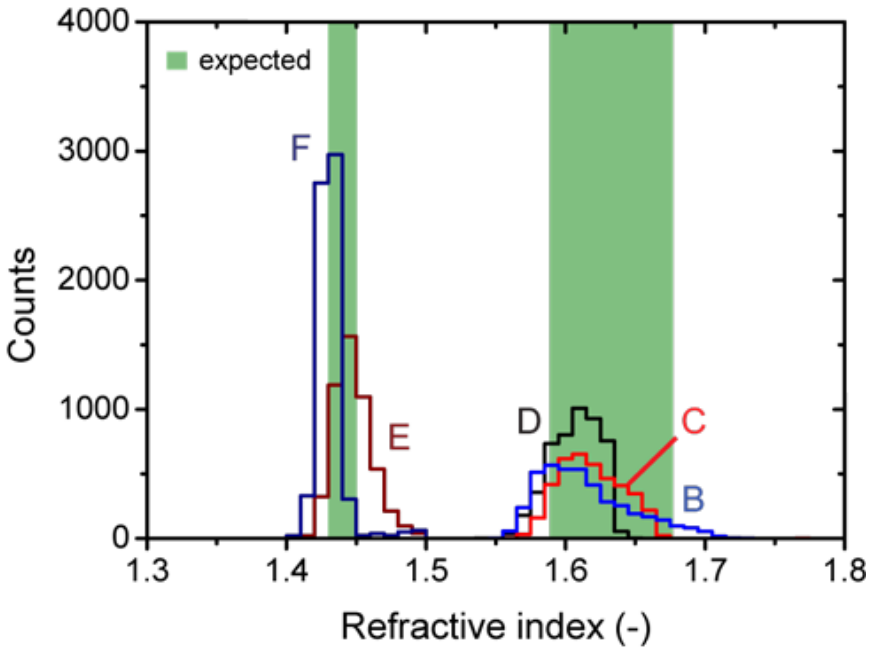
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

Validate Flow-SR with a beads mixture



Validate Flow-SR with a beads mixture



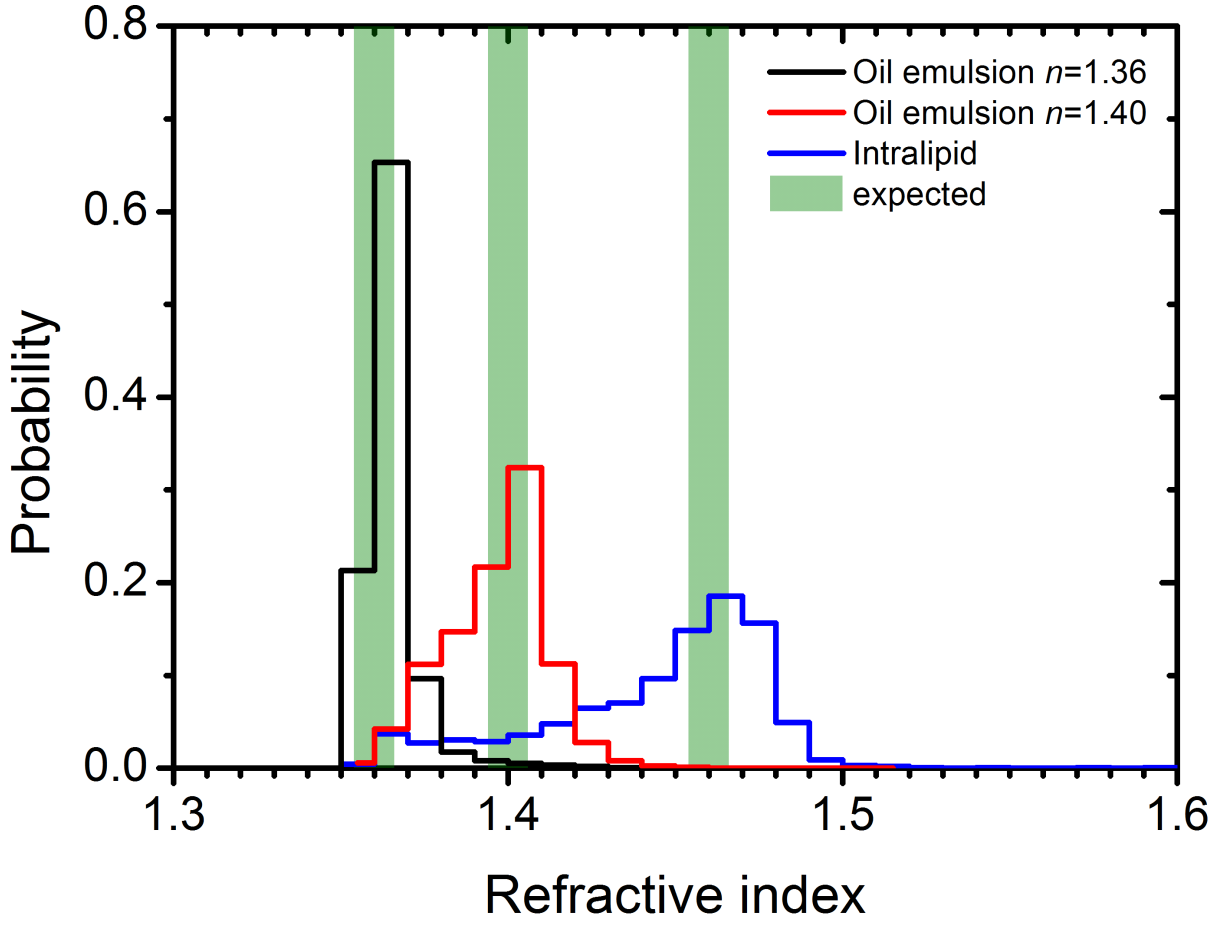


measurement error < 8%

CV < 8%

CV < 2%

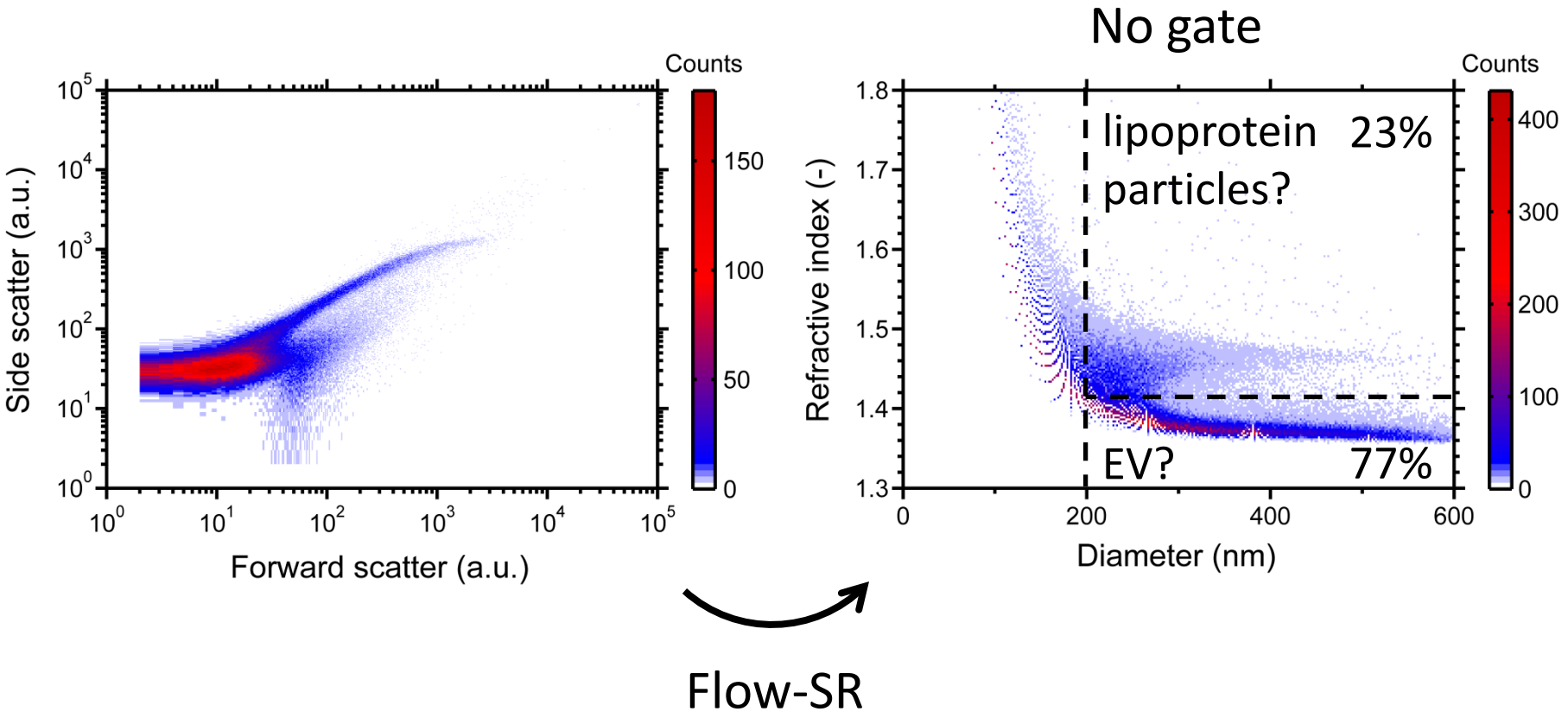
Validate Flow-SR with oil emulsions



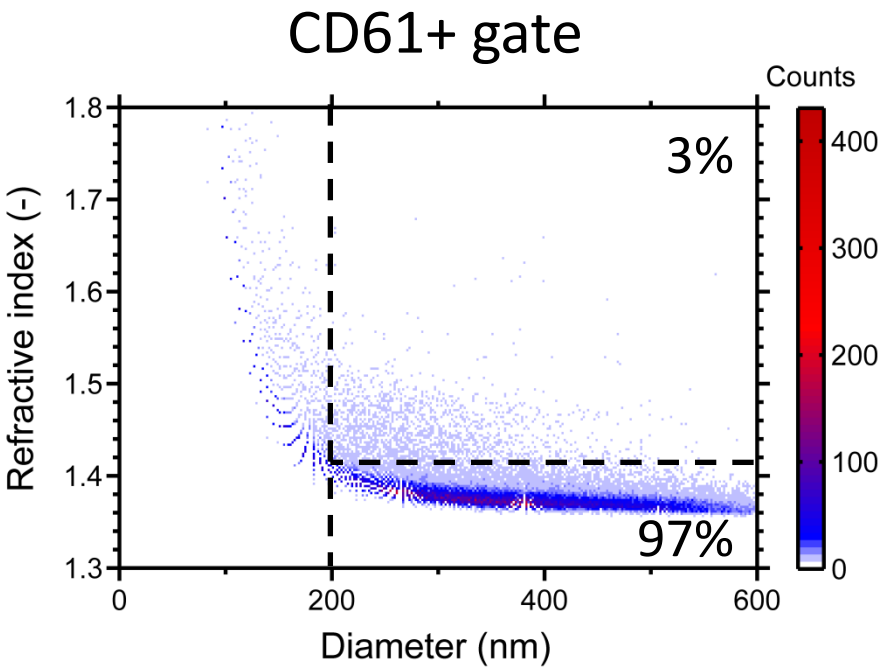
Approach

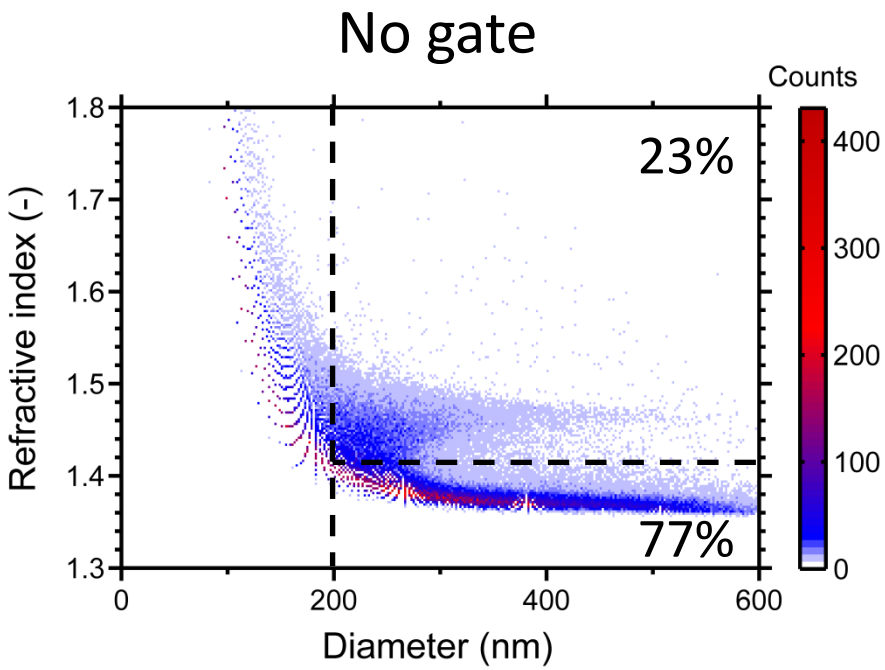
- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

Supernatant of outdated platelet concentrate



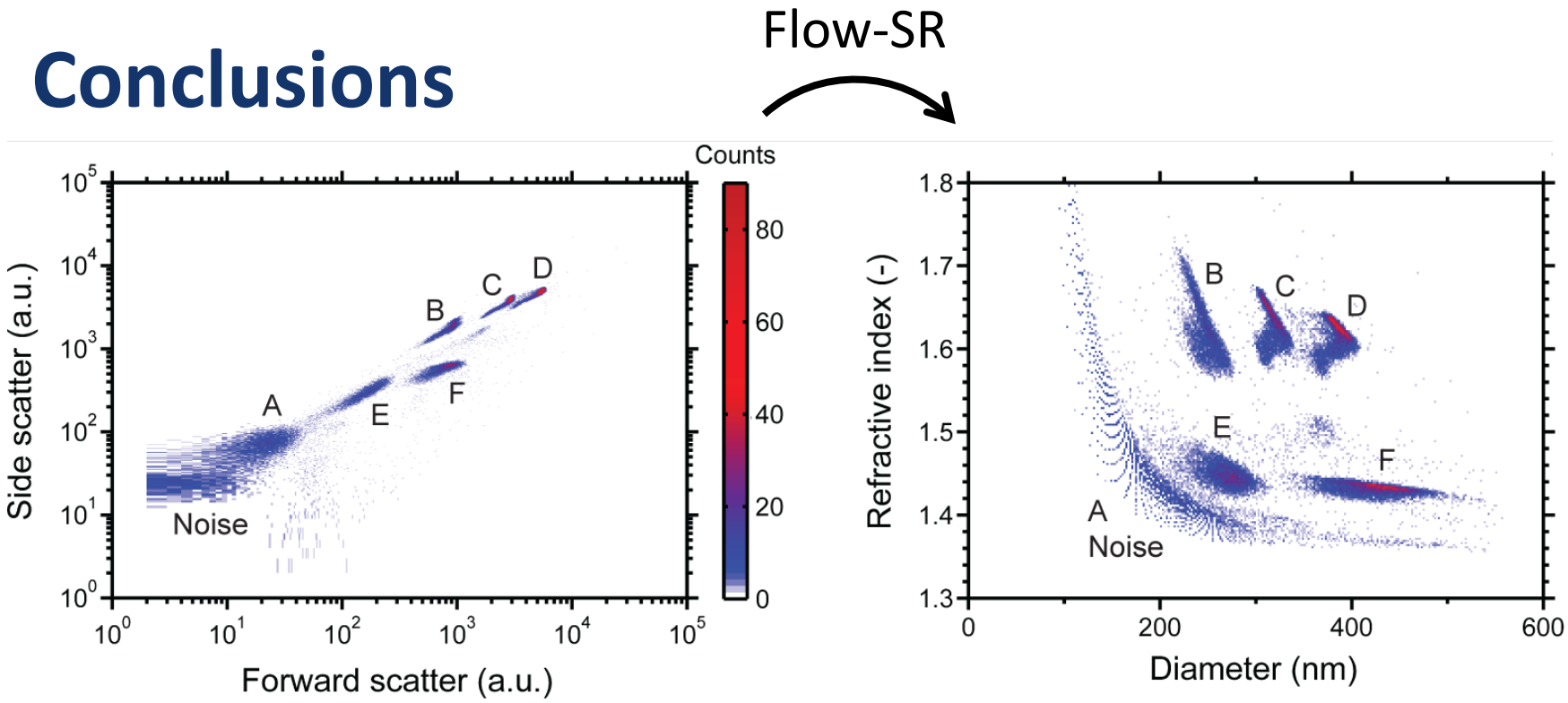
Supernatant of outdated platelet concentrate





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

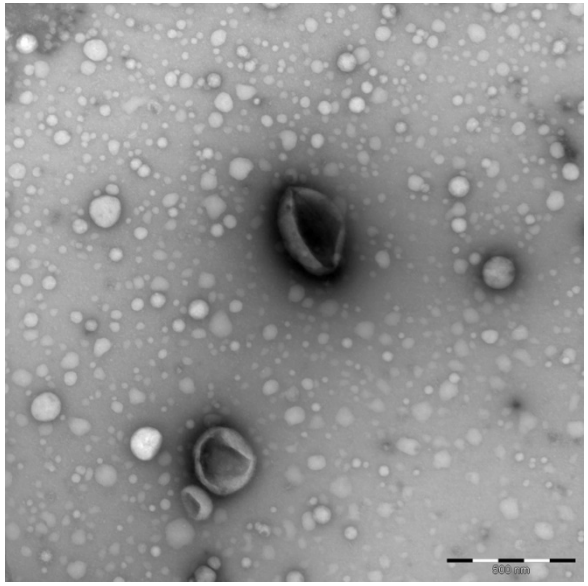
Conclusions



- Flow-SR enables size and refractive index determination of nanoparticles by flow cytometry
  - data interpretation and comparison
  - label-free identification

Summary

- extracellular vesicles contain clinical information
- standardization is essential
- scatter provides valuable information
  - size
  - refractive index



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- More info: edwinvanderpol.com