

Quantitative light scattering to standardize flow cytometry measurements of extracellular vesicles

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Frank Coumans

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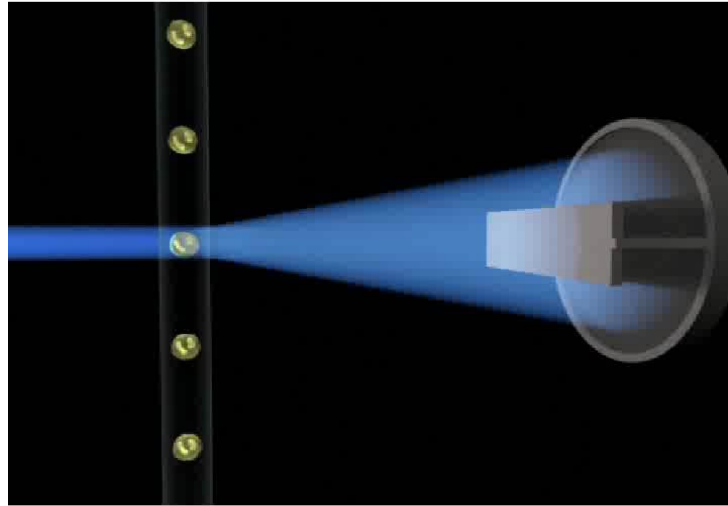
*Vesicle Observation Center, Academic Medical Center,
University of Amsterdam, The Netherlands*



Conflicts of interest

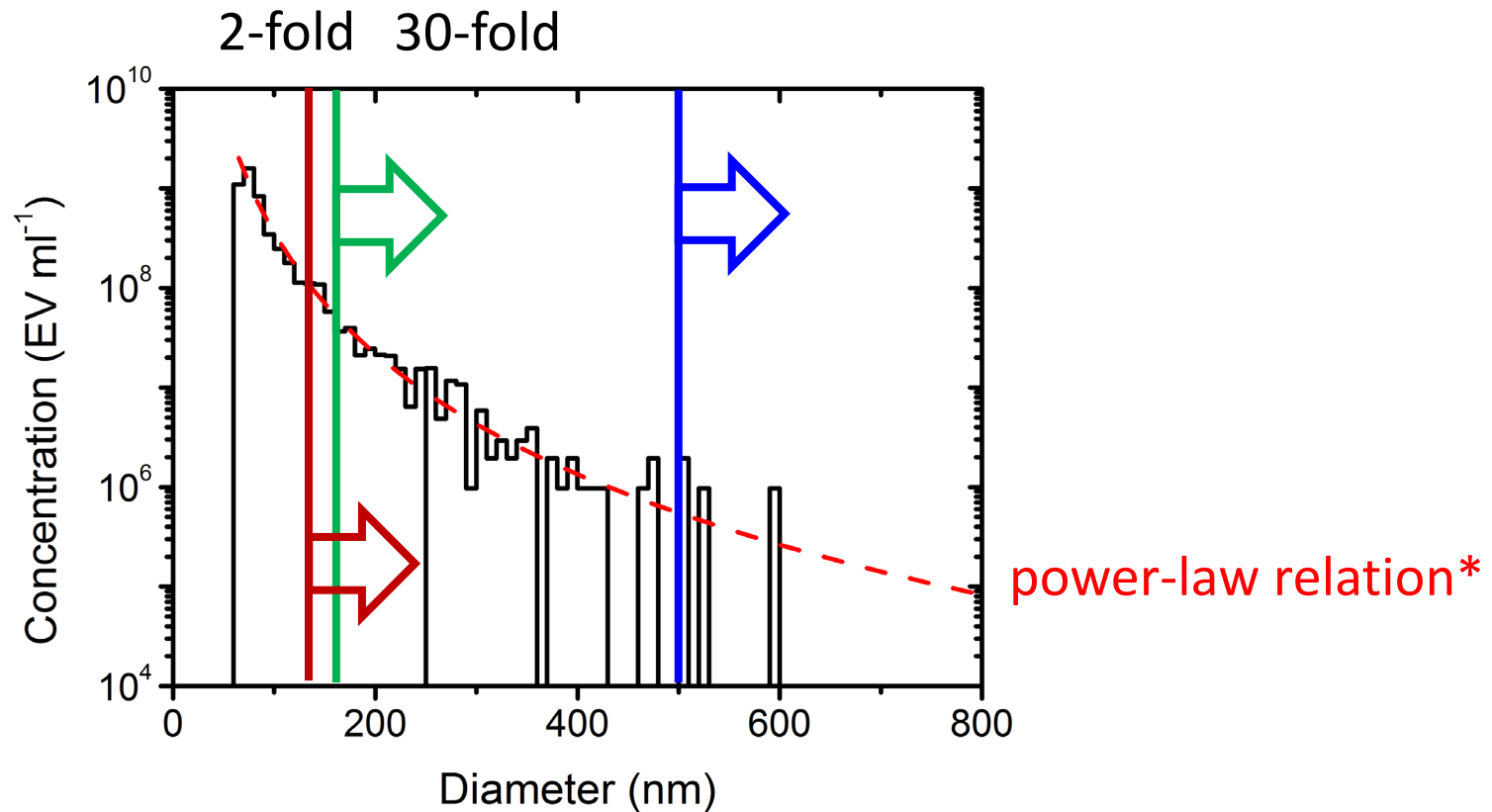
- Edwin van der Pol and Frank Coumans are cofounder and stakeholder of **EXOMETRY**

Introduction



- extracellular vesicles (EV) are potential biomarkers
- flow cytometry is commonly used to count EV
- reported concentrations of EV in plasma differ $>10^6$ -fold*
- standardization required

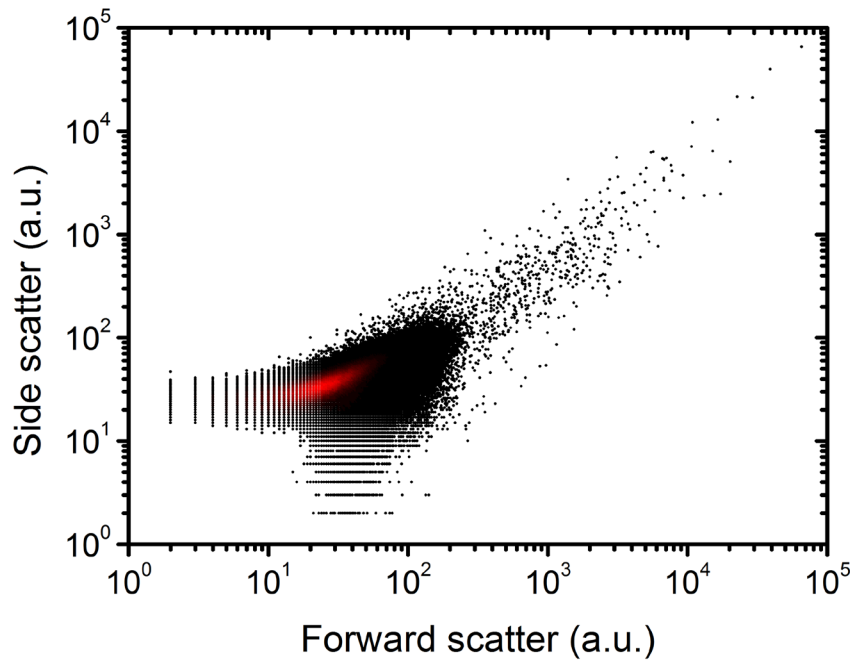
Problem 1: instruments differ in sensitivity



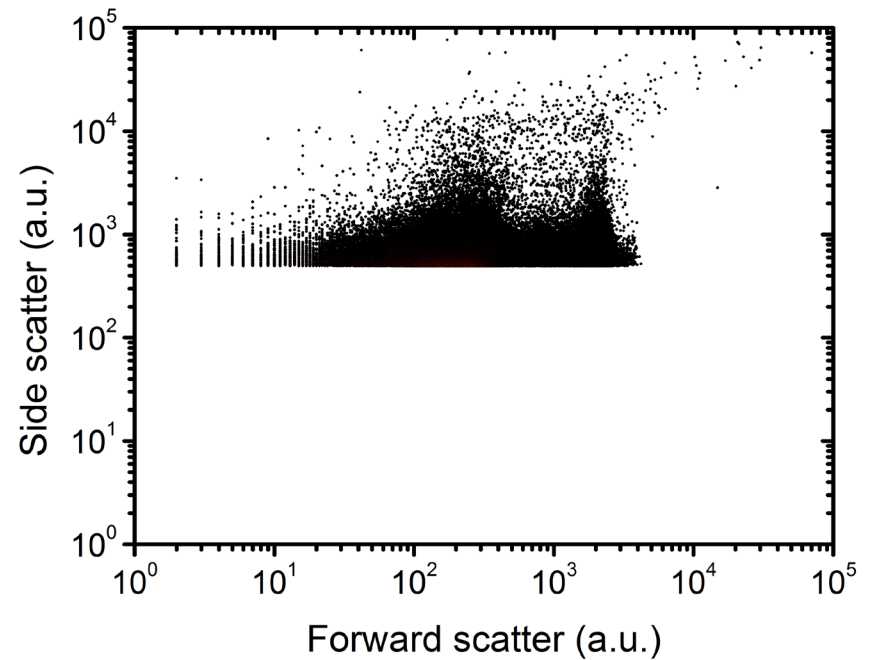
*van der Pol et al. *JTH* (2014)

Problem 2: arbitrary units

same population of erythrocyte EV



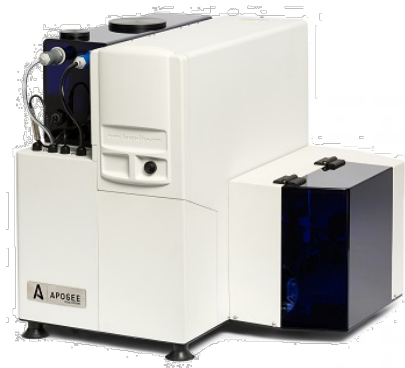
Apogee A50-micro



Becton Dickinson FACSCanto II

Goal

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



Study comprises 33 sites (64 instruments) worldwide



Approach

- 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

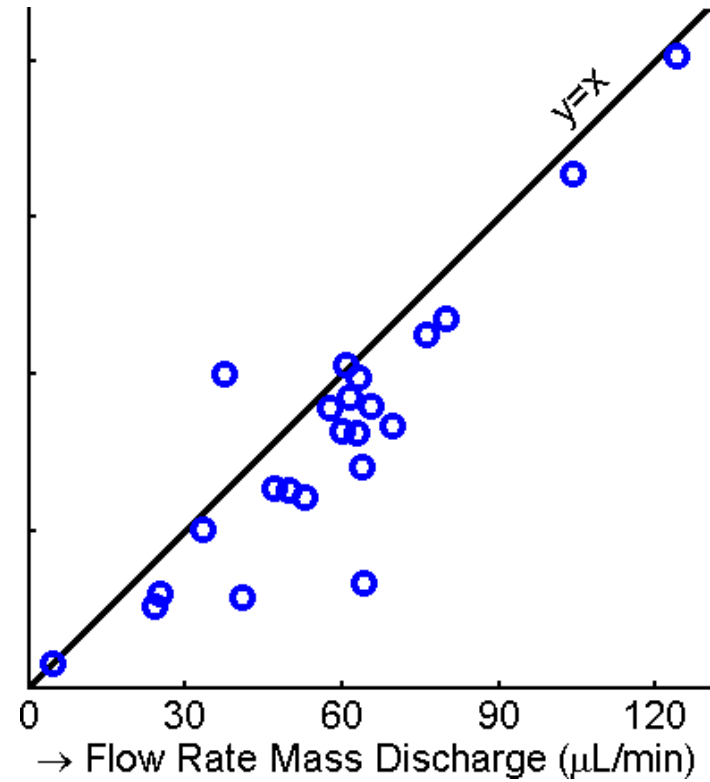
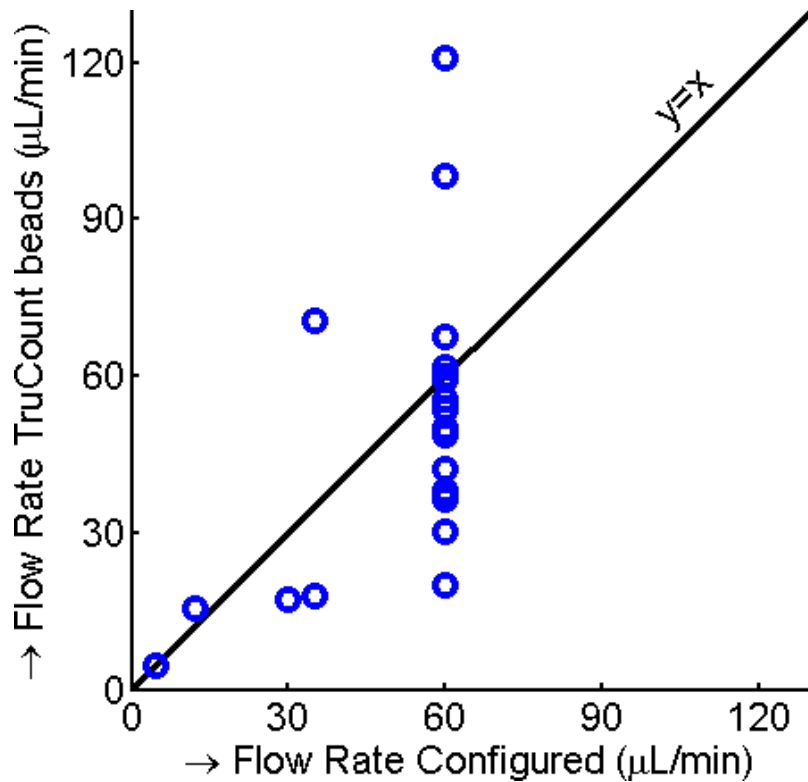


Approach

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Determine flow rate

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

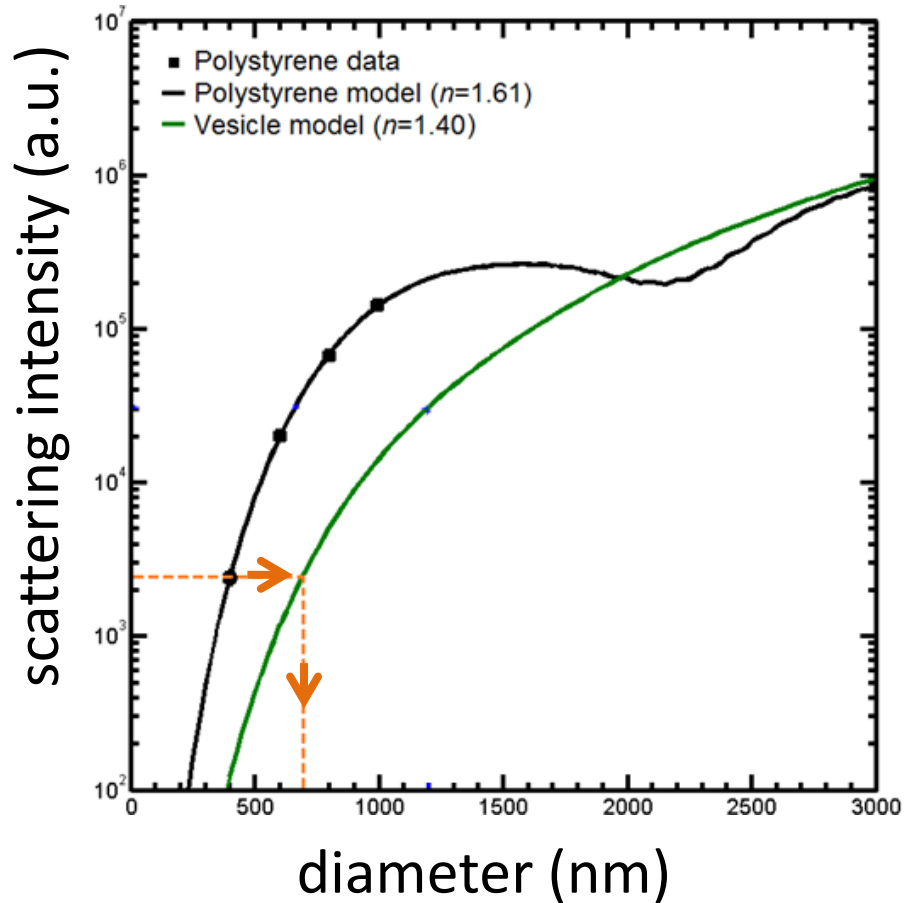


Approach

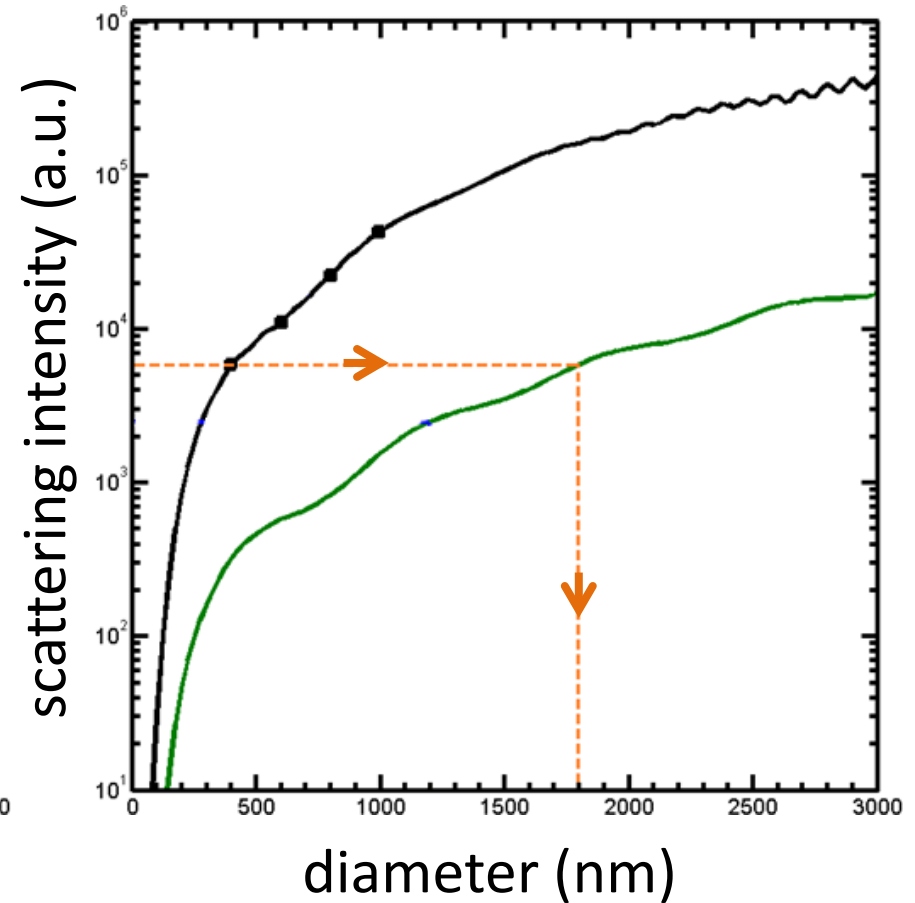
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Earlier ISTH studies: gate on polystyrene beads

BC Gallios (forward scatter)

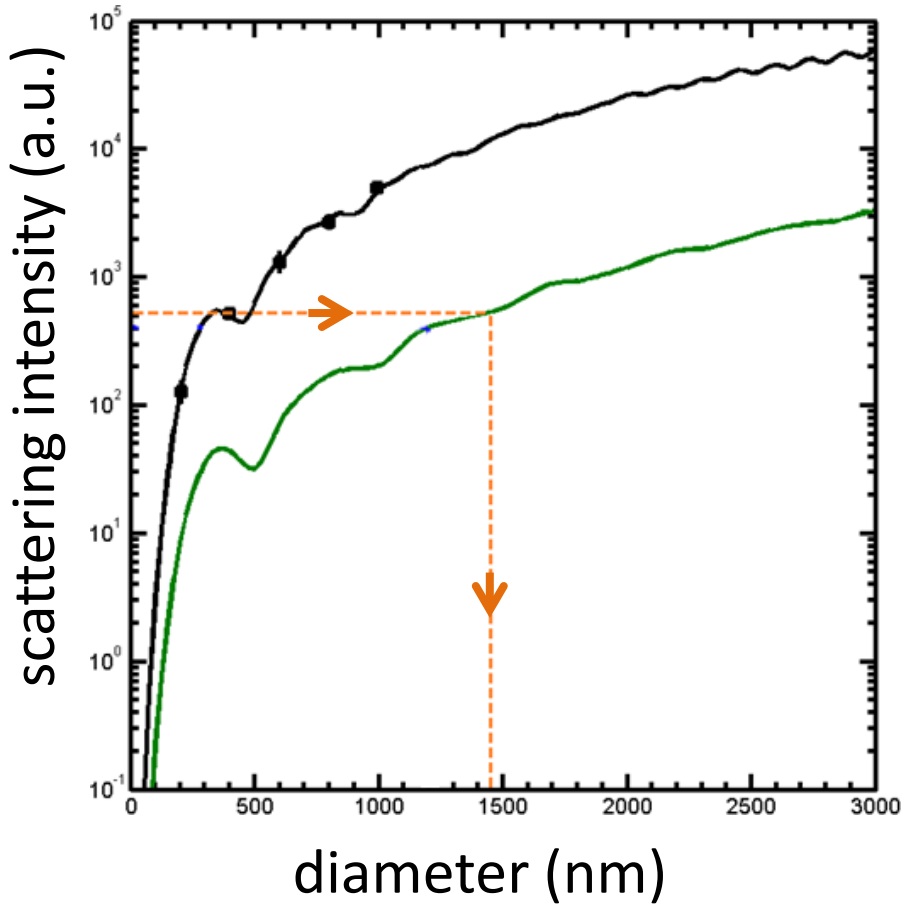


BD LSR II (side scatter)

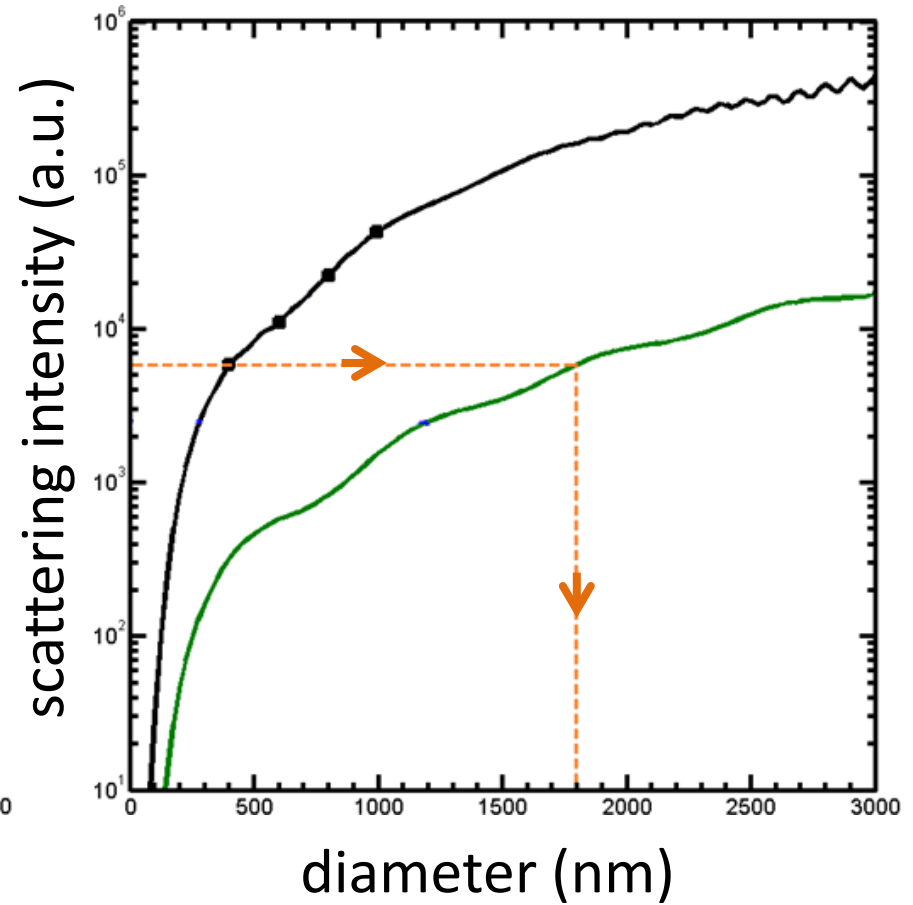


Earlier ISTH studies: gate on polystyrene beads

BC Astrios MoFlo (side scatter)

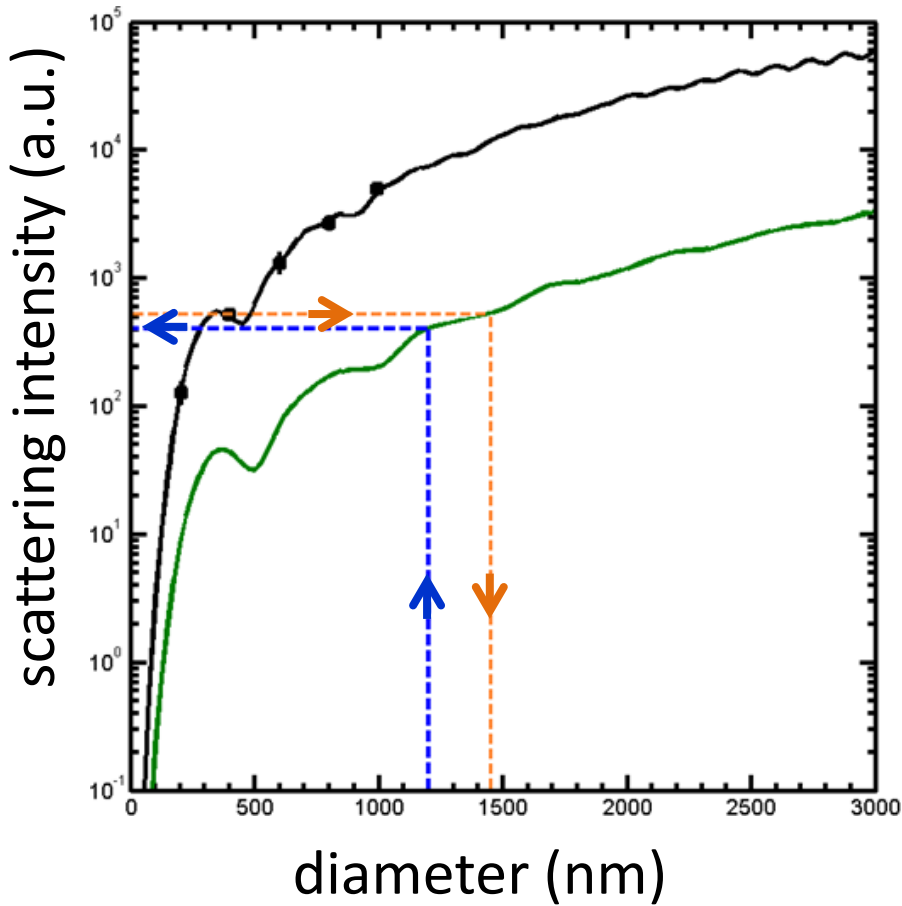


BD LSR II (side scatter)

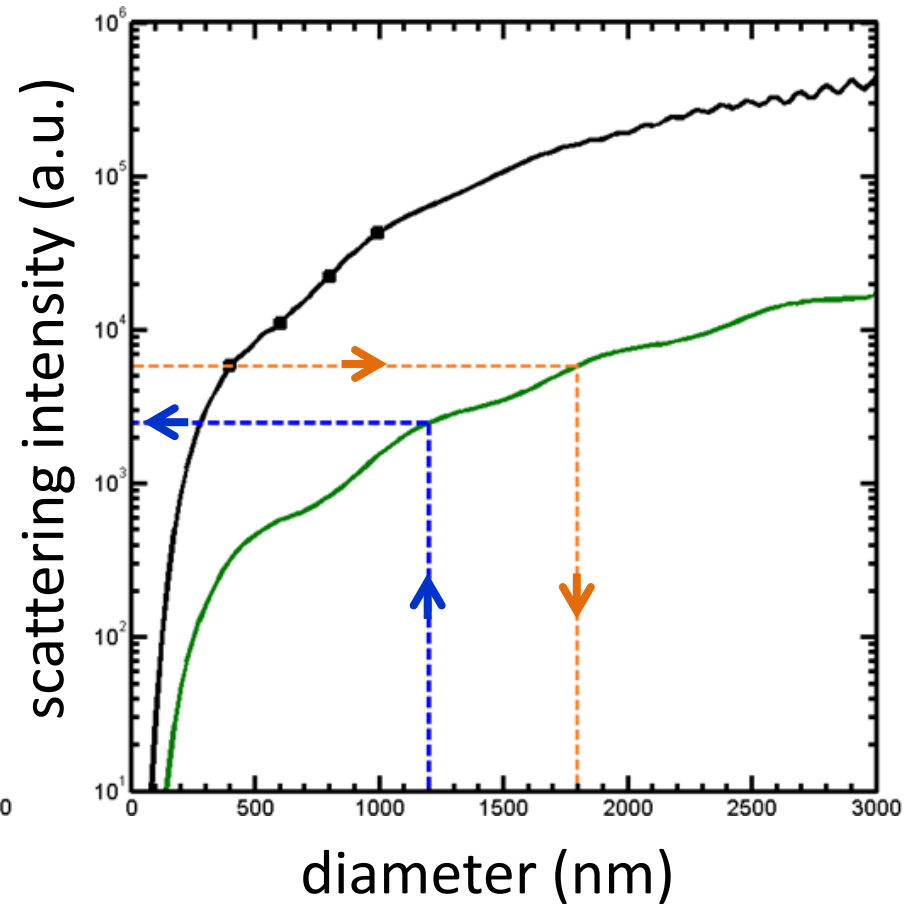


2016: relate scatter (a.u.) to diameter (nm)

BC Astrios MoFlo (side scatter)



BD LSR II (side scatter)



Status

Please open "Exometry beads" file.

Controls

Open "Exometry beads" file

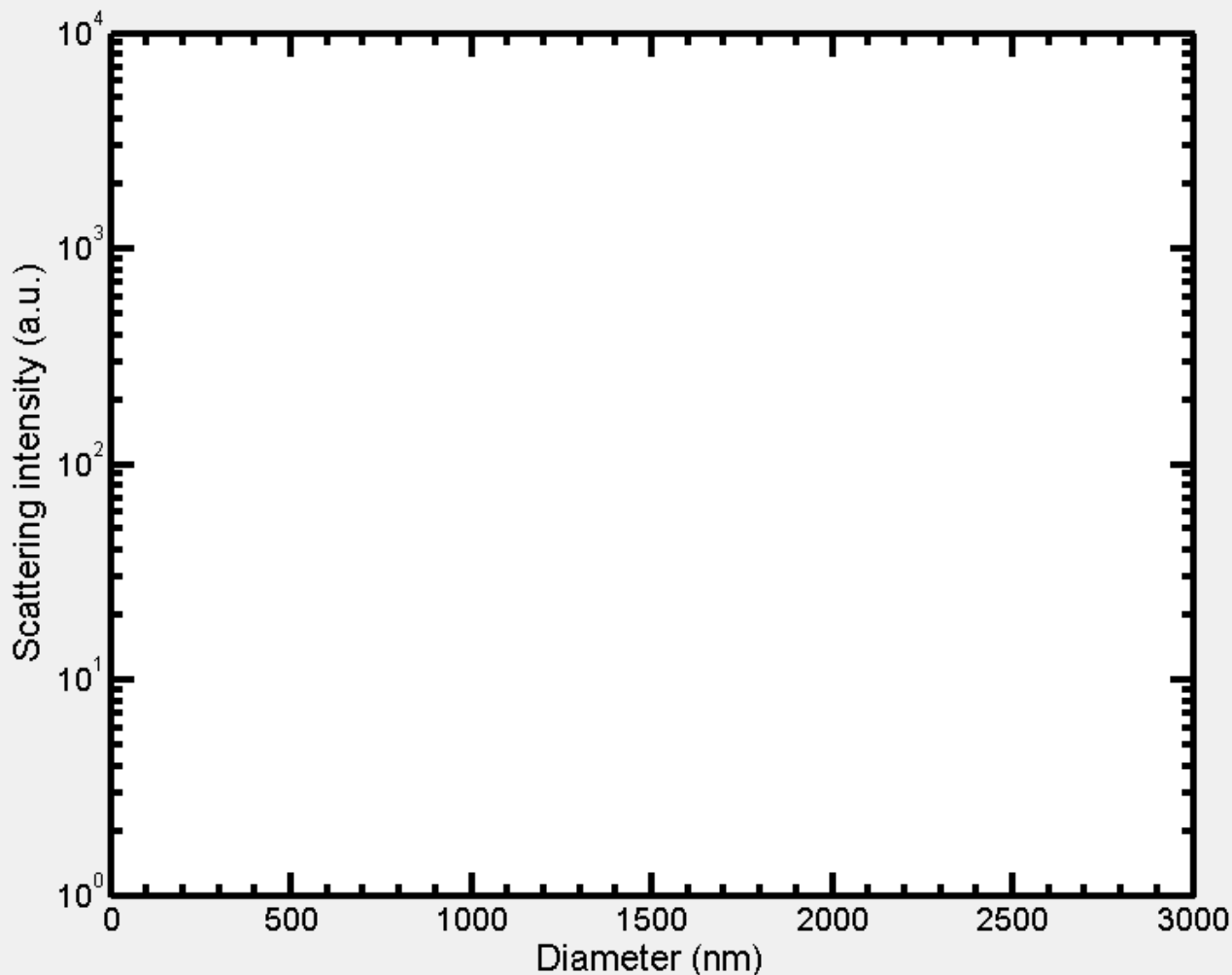
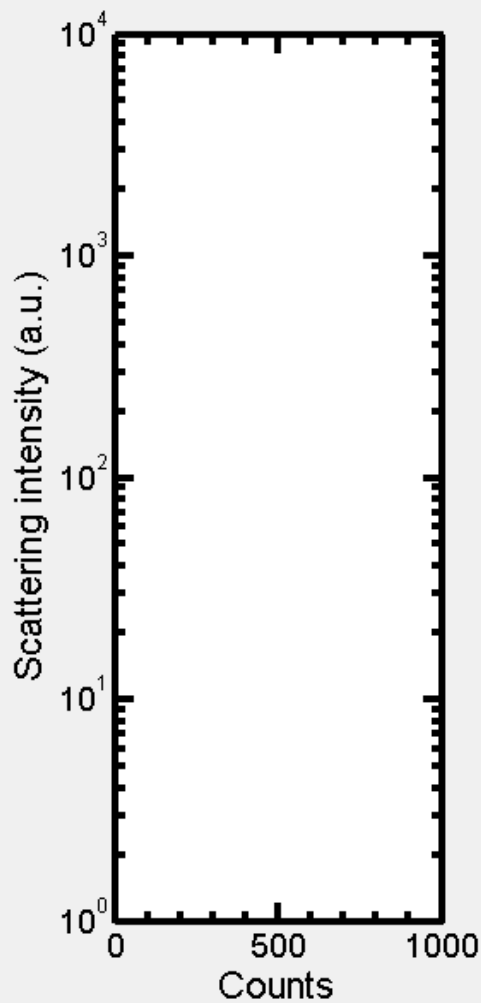
Flow cytometer unknown

Gate

Open "Reference beads" file

Recommended vesicle size gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000		} Gate 2
	1200		
Gate 3 {	600		
	300		



Status

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

Controls

Open "Exometry beads" file

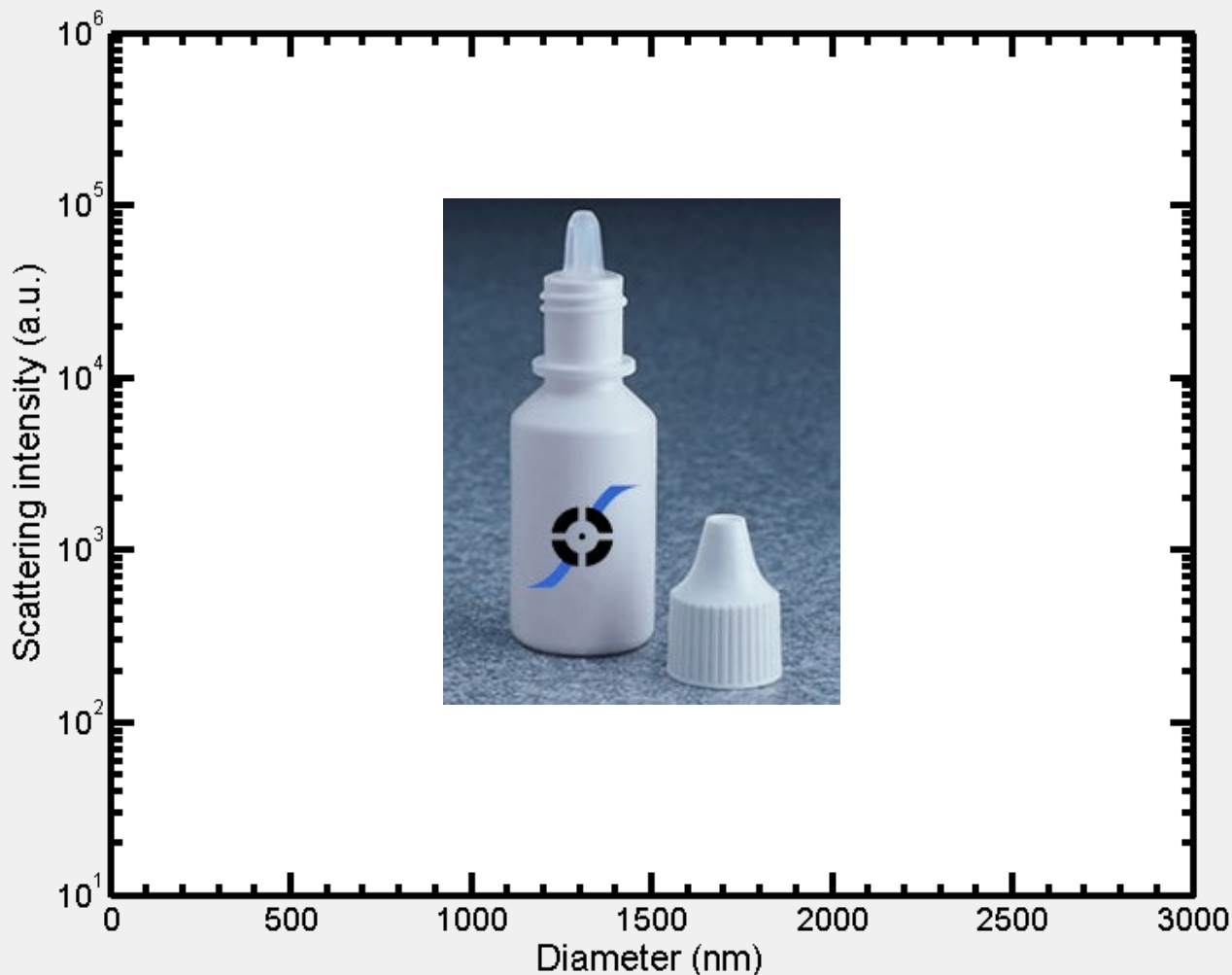
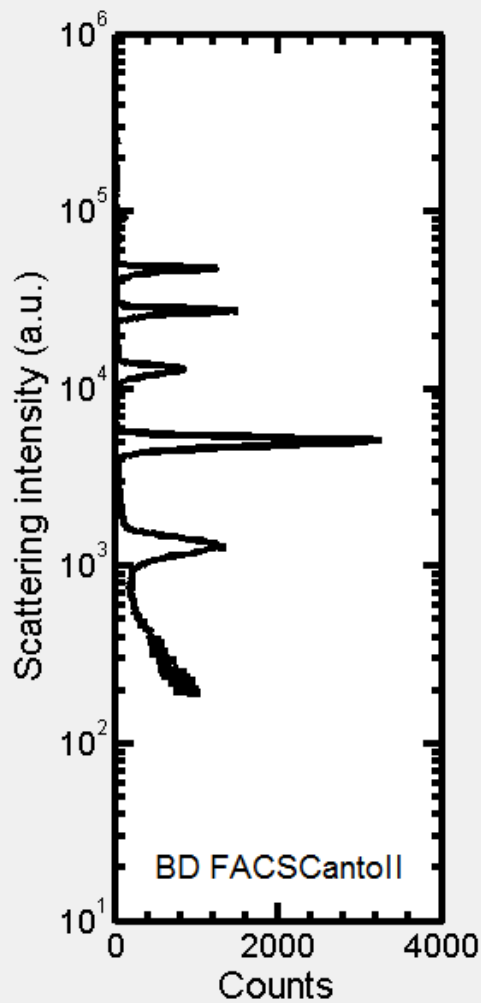
SSC (recommended)

Gate

Open "Reference beads" file

Recommended vesicle size gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000		} Gate 2
	1200		
Gate 3 {	600		
	300		



Status

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

Controls

Open "Exometry beads" file

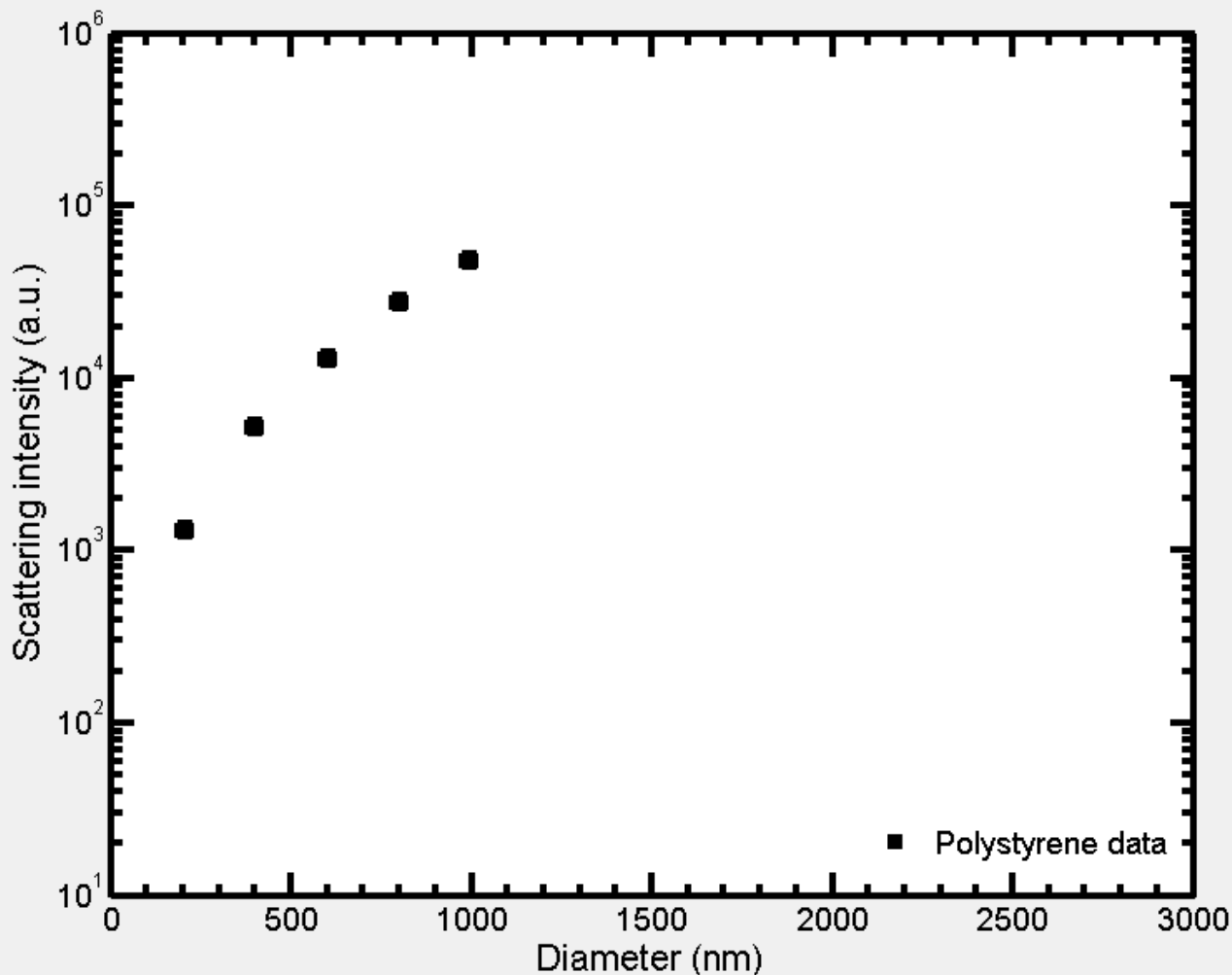
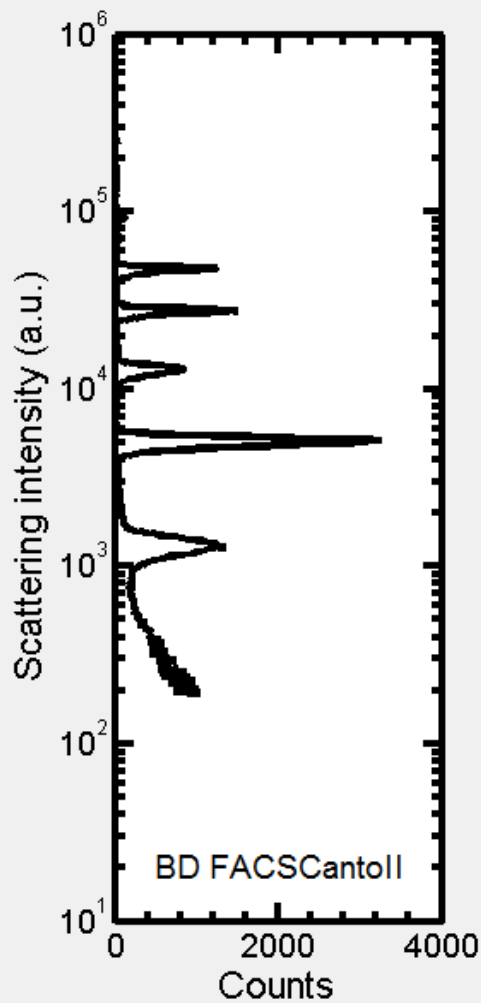
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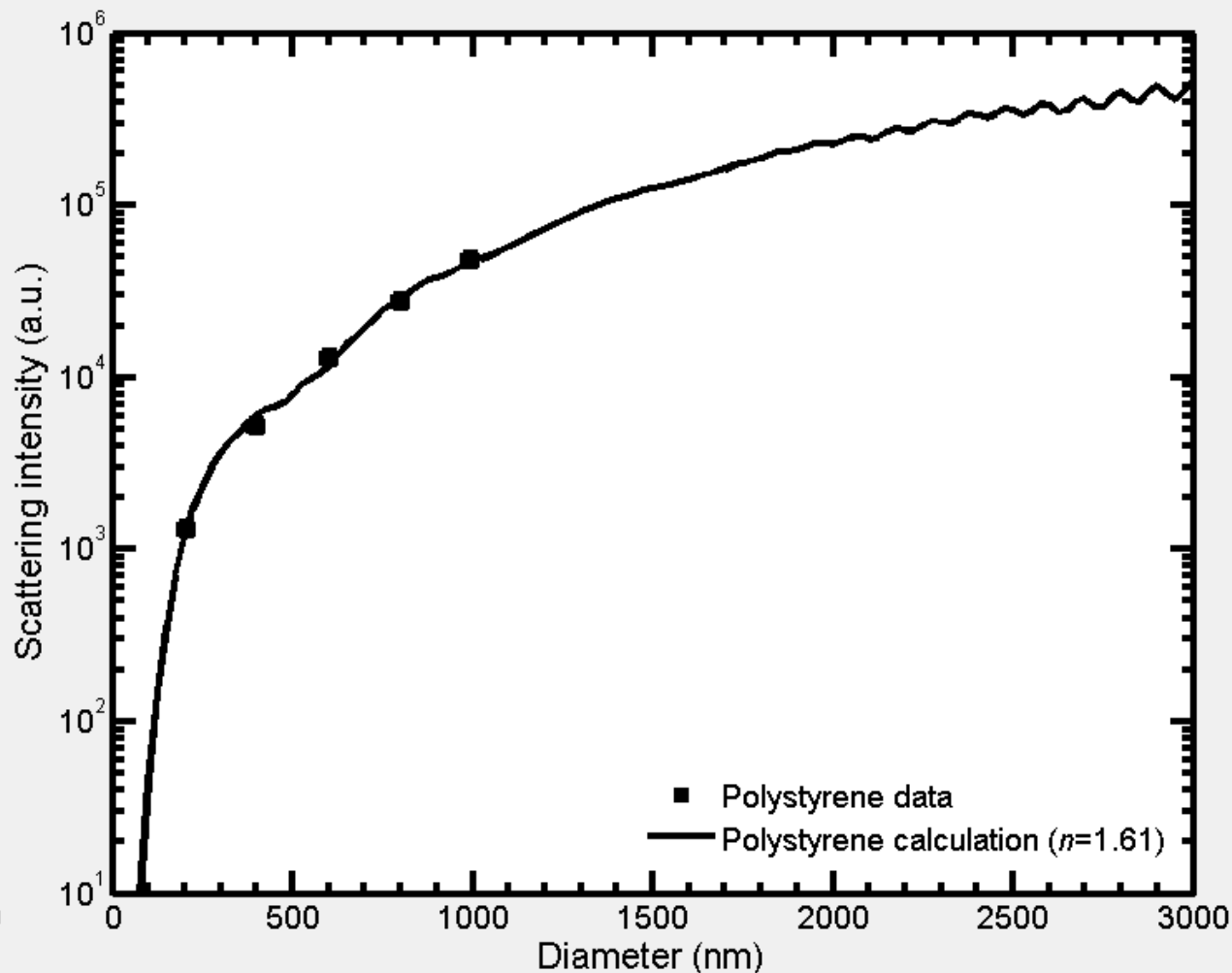
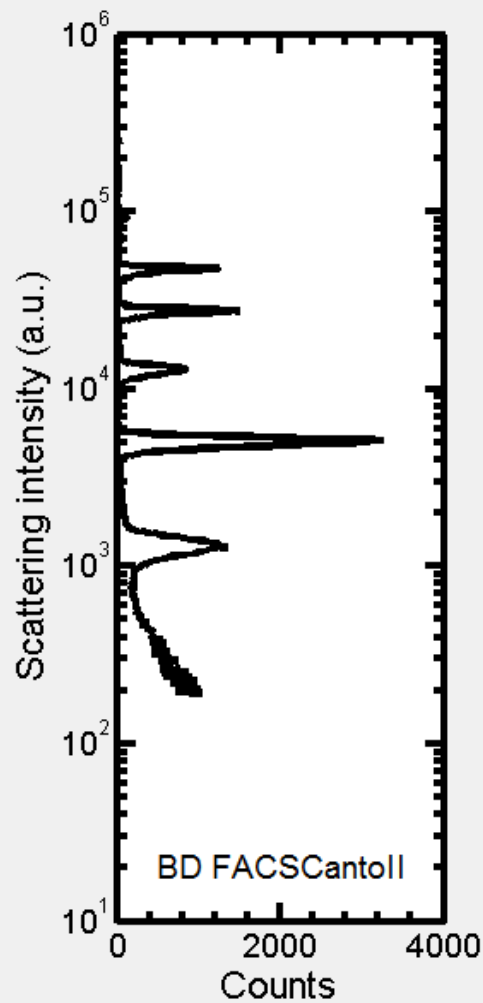
Status

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

Controls

Recommended vesicle size gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000		} Gate 2
	1200		
Gate 3 {	600		}
	300		



Status

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

Controls

Open "Exometry beads" file

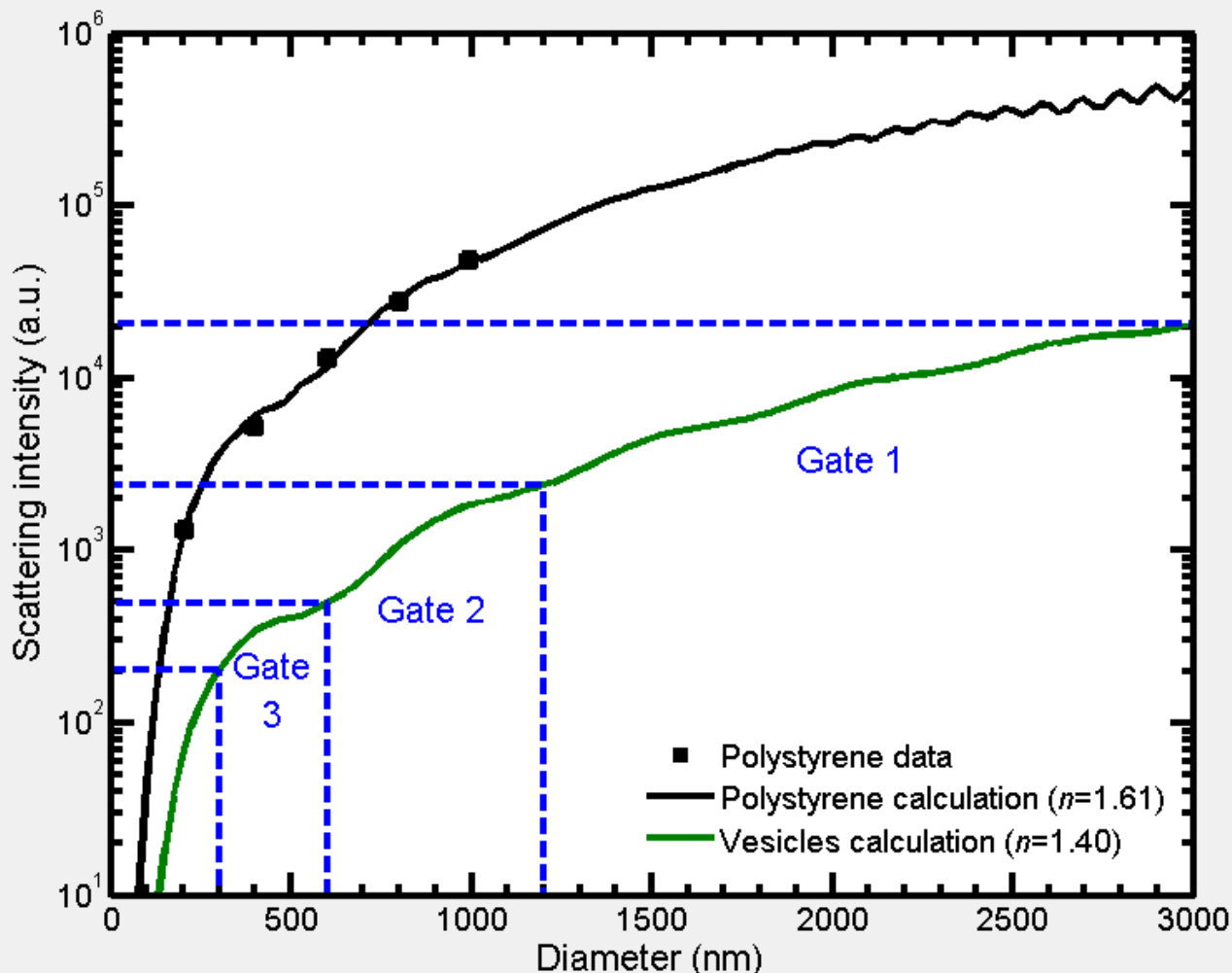
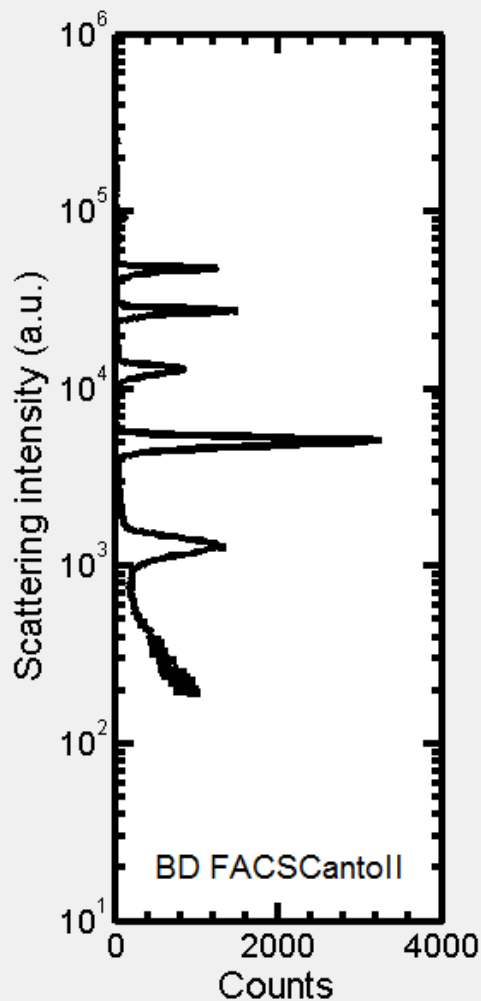
SSC (recommended)

Gate

Open "Reference beads" file

Recommended vesicle size gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000	20636	} Gate 2
	1200	2380	
Gate 3 {	600	497	}
	300	202	



Status

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

Controls

Open "Exometry beads" file

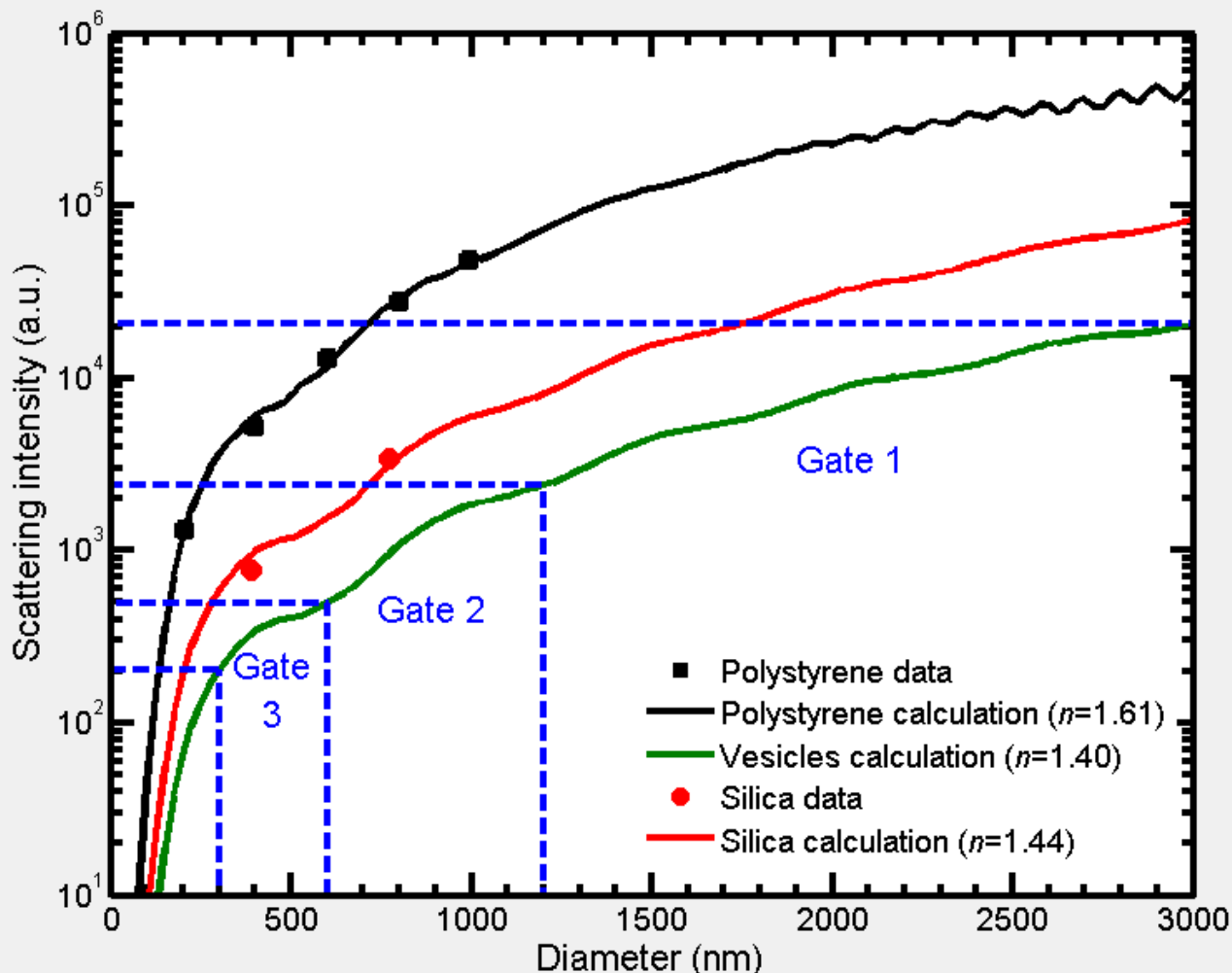
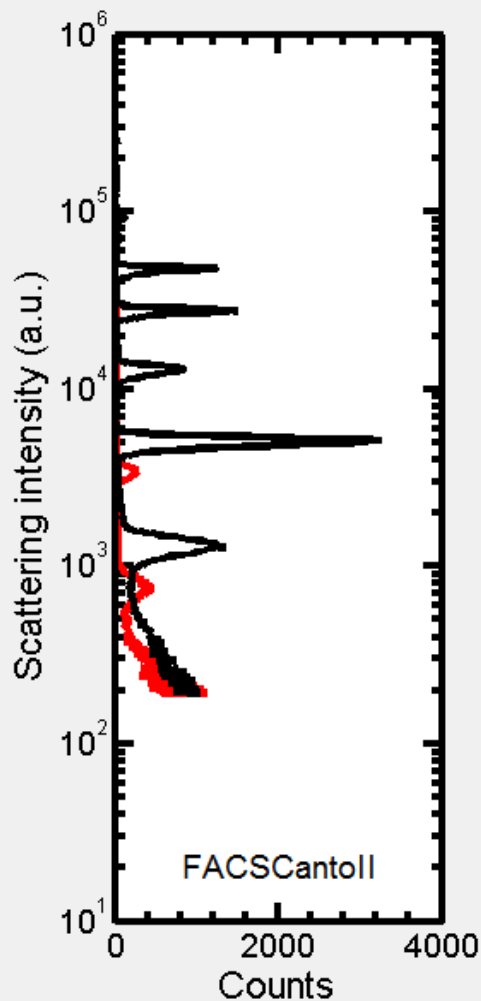
SSC (recommended)

Gate

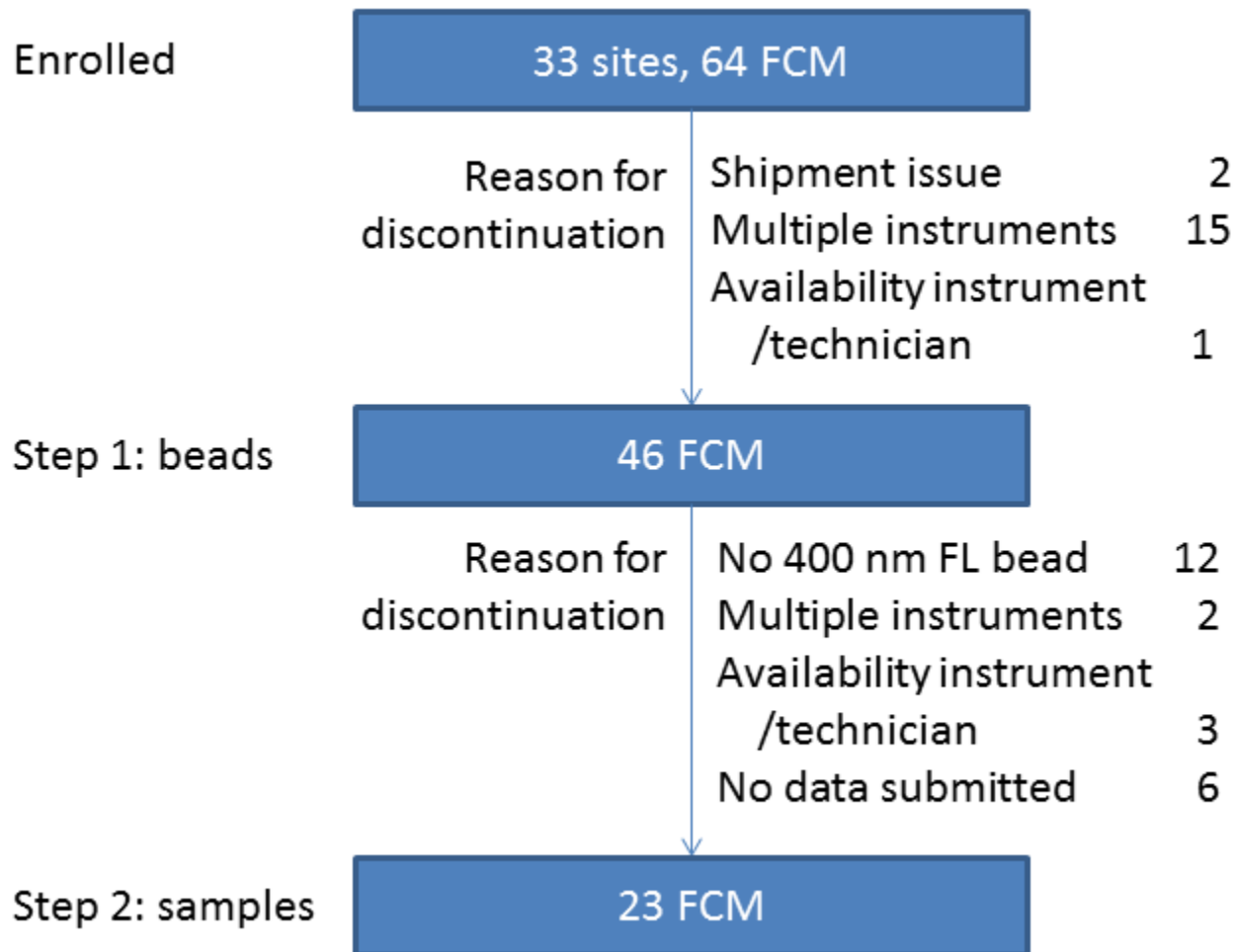
Open "Reference beads" file

Recommended vesicle size gates

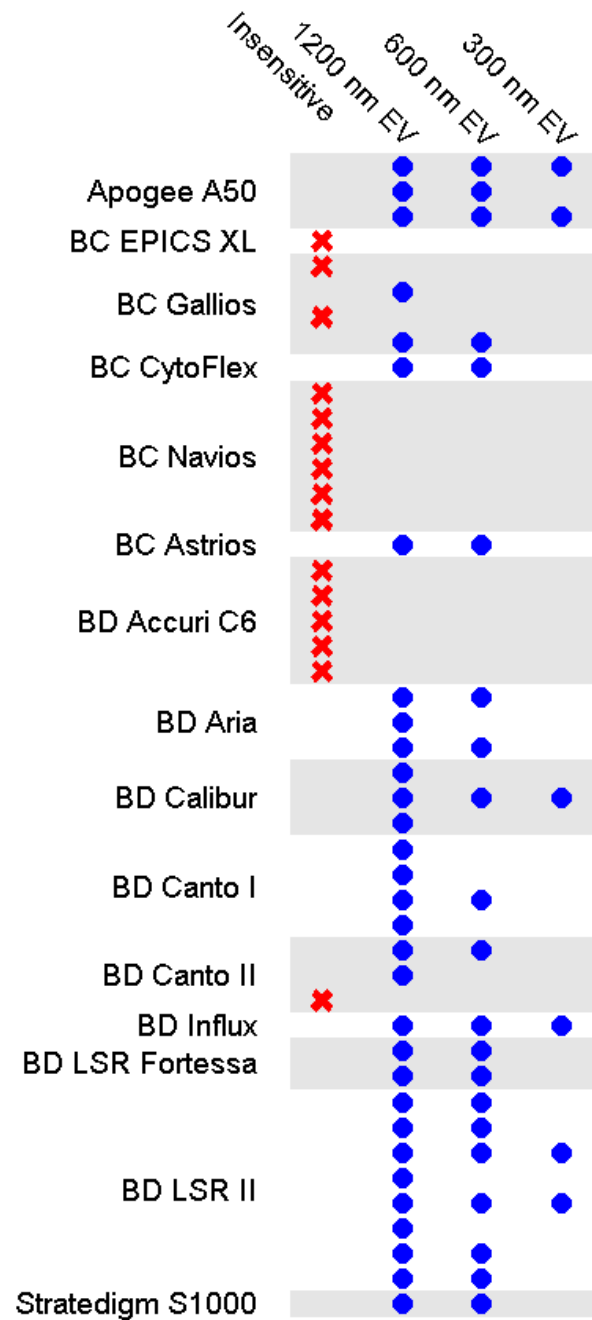
	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000	20636	} Gate 2
	1200	2380	
Gate 3 {	600	497	}
	300	202	



Exclusion of flow cytometers (FCM)



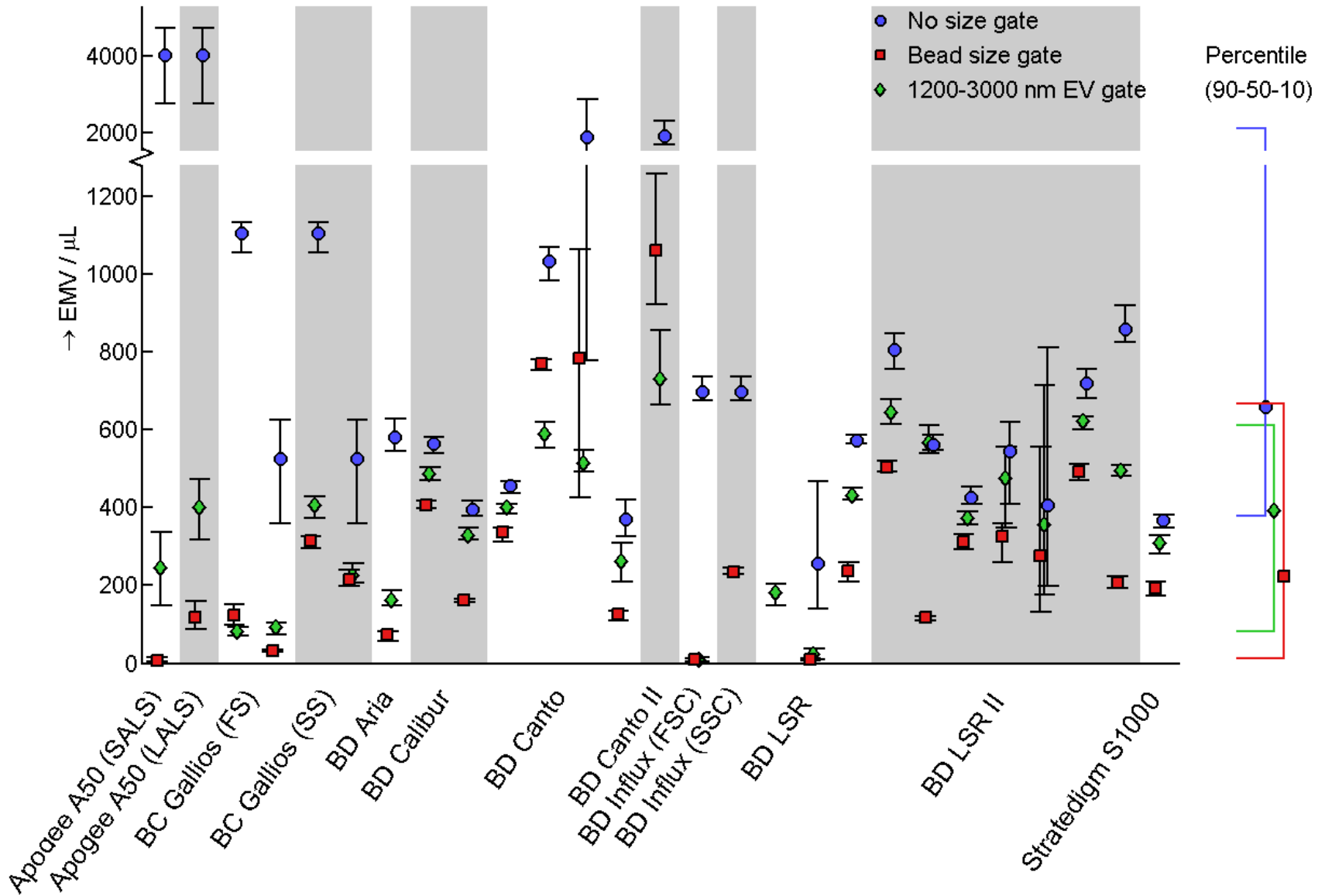
Instrument sensitivity



Approach

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Reproducibility of 1200-3000 nm EV



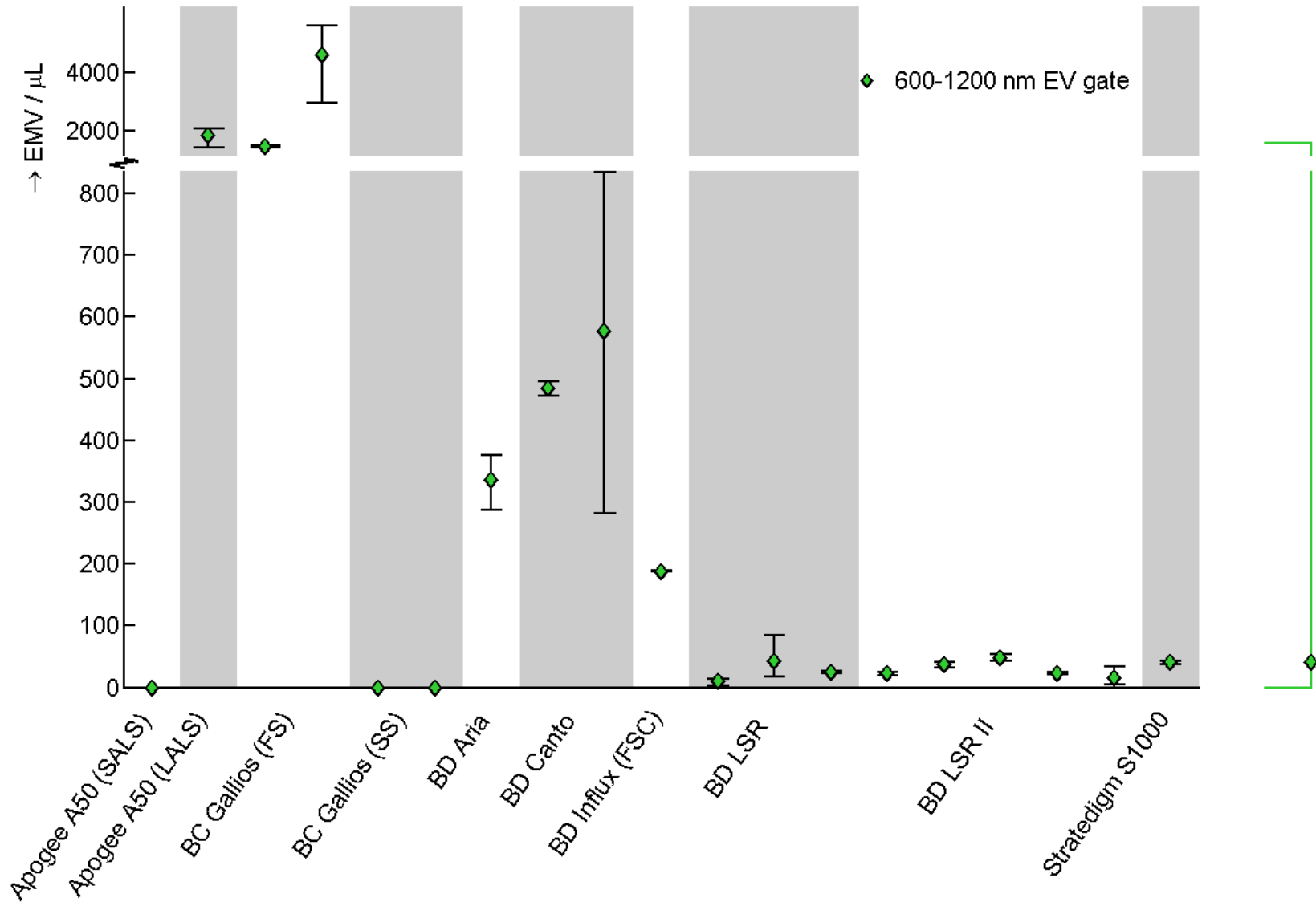
Reproducibility of 1200-3000 nm EV

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

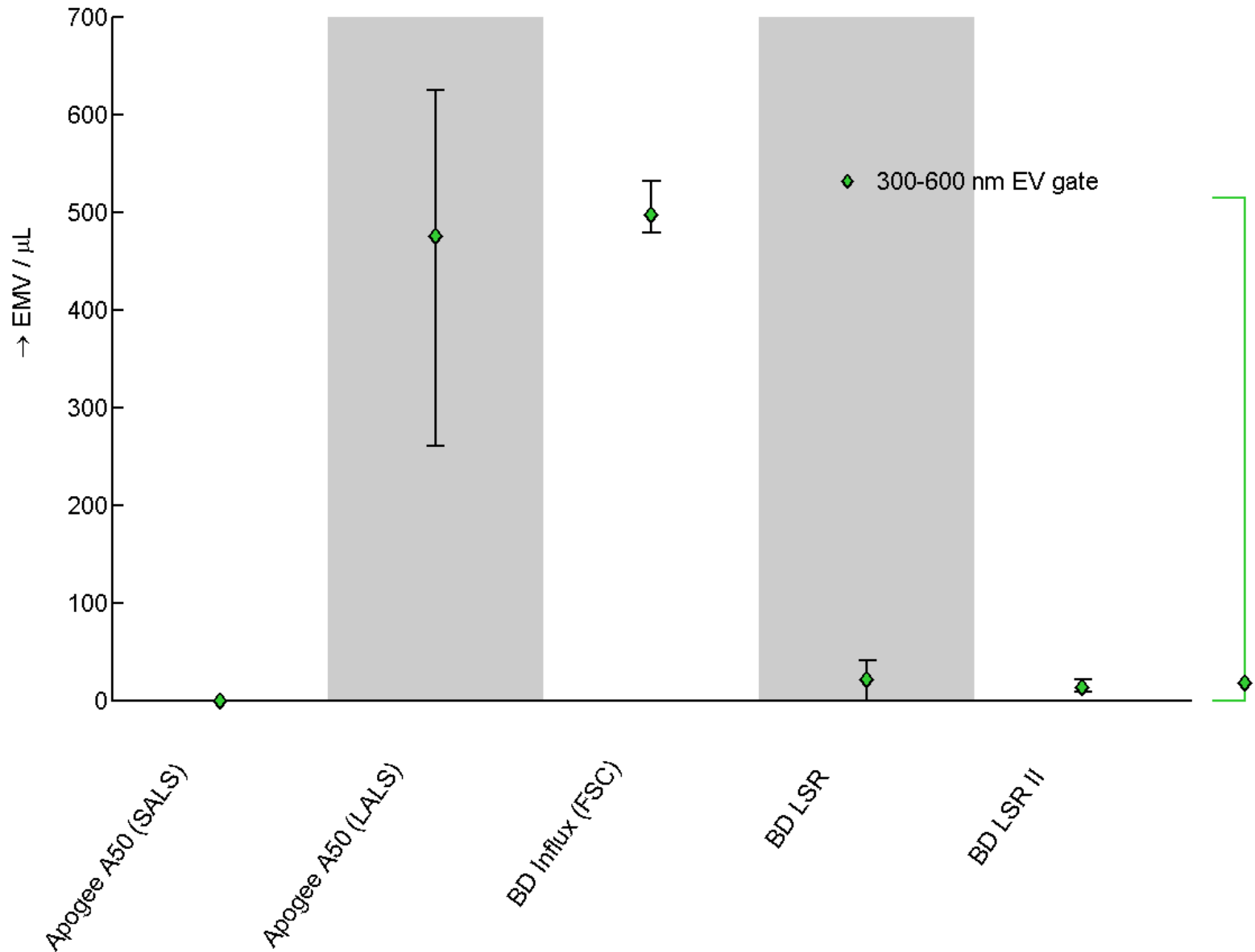
%CV = standard deviation / mean * 100%

Preliminary results

Reproducibility of 600-1200 nm EV



Reproducibility of 300-600 nm EV



Conclusions

- 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

- 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

Acknowledgements

- Academic Medical Center
 - Frank Coumans
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 - European Association of National Metrology Institutes (EURAMET)
- Software and beads by exometry.com
- Info: edwinvanderpol.com

