

Detection of extracellular vesicles: size does matter

Edwin van der Pol^{1,2}

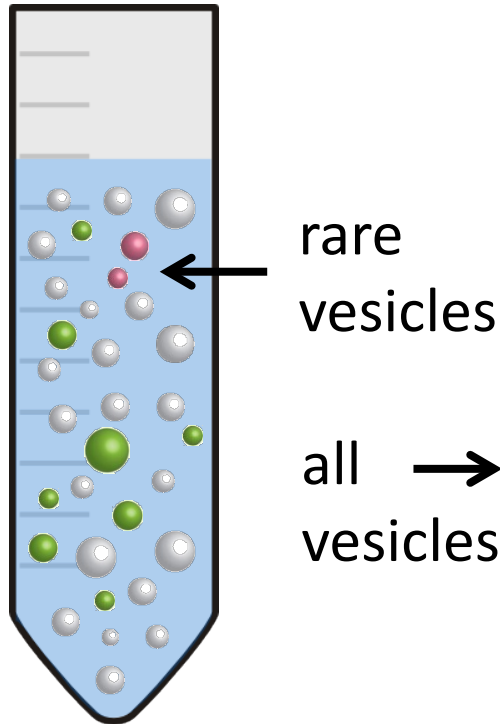


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Introduction



Hematology parameter	Concentration (vesicles mL ⁻¹)
Platelet vesicle count	2.3 – 6.2 · 10 ⁹
Erythrocyte vesicle count	7.0 – 8.6 · 10 ¹⁰
Reticulocyte vesicle count	3.9 – 15.6 · 10 ⁸
Leukocyte vesicle count	6.2 – 16.4 · 10 ⁷
Total vesicle count	7.3 – 9.4 · 10 ¹⁰

- goal: determine the concentration of a vesicle subpopulation

Outline

- ✔ goal
 - ✔ determine concentration of a vesicle subpopulation
- challenges
 - determine vesicle concentration and size
 - detect vesicles by flow cytometry
- solutions
 - vesicle refractive index
 - standardization of flow cytometry measurements
- outlook
 - future role of metrology

Vesicle concentration determination factsheet

- reported concentration in plasma:
 $10^4 - 10^{12}$ vesicles mL^{-1}
-

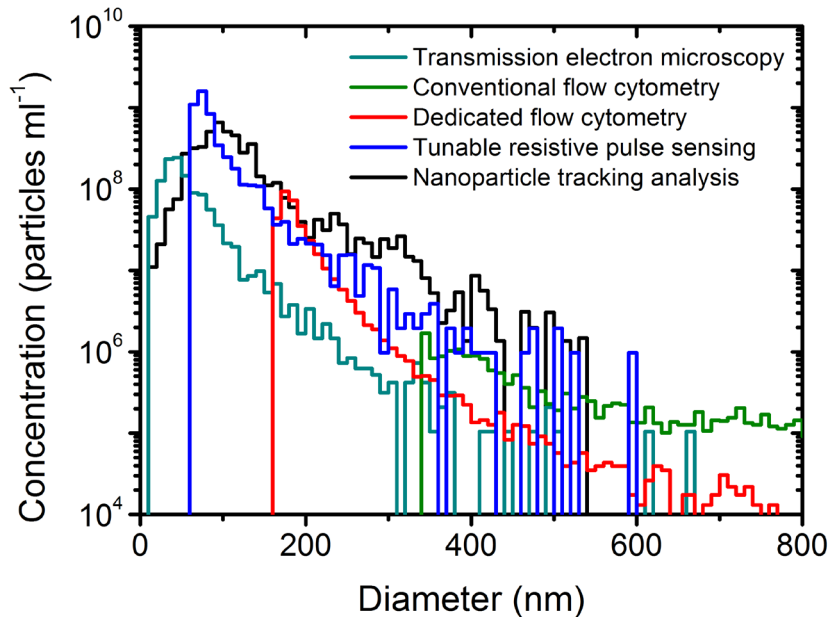
- diameter <100 nm for $>80\%$ of vesicles
 - vesicles differ
 - 25-fold in diameter
 - 20,000-fold in volume
 - 300,000-fold in concentration
 - 10,000,000-fold in scattered light
 - vesicles originate from complex fluids
-

- instruments differ >1 order of magnitude in sensitivity

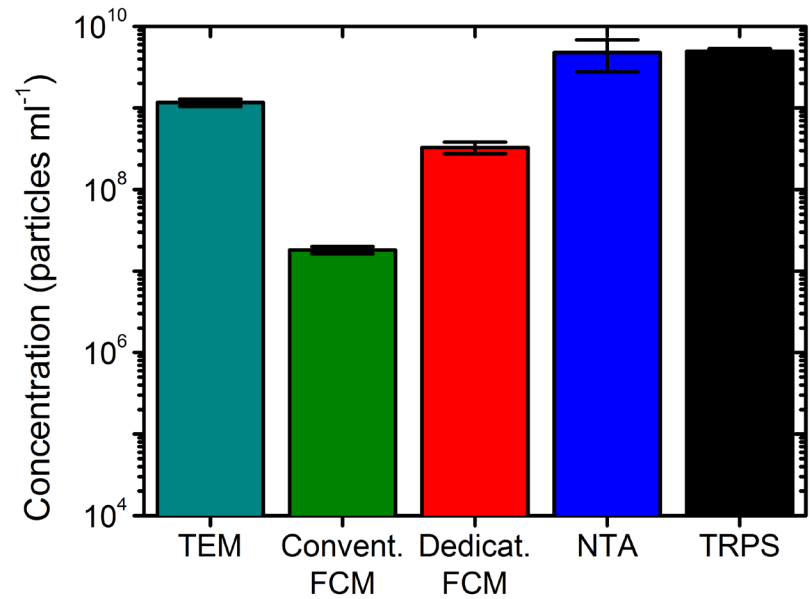


Concentration and size of urinary vesicles

size distribution

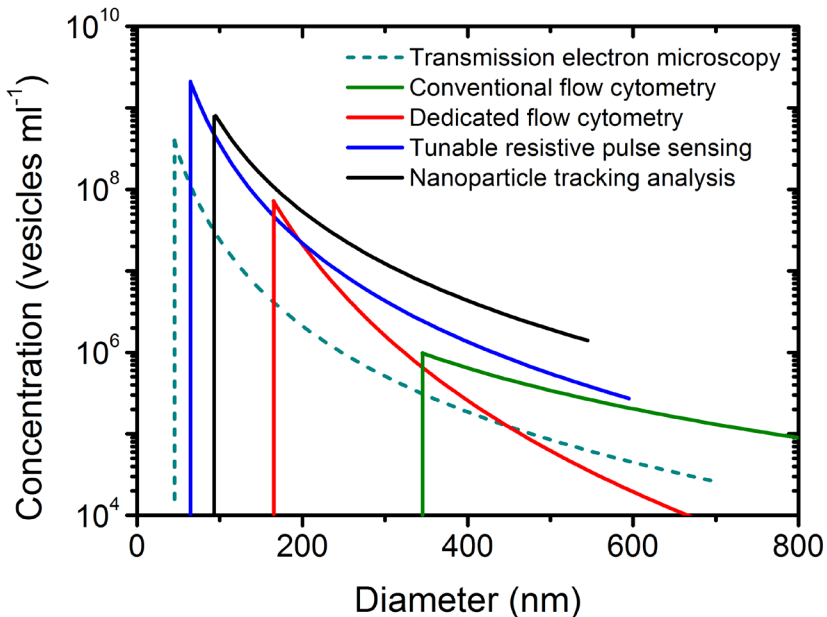


total concentration

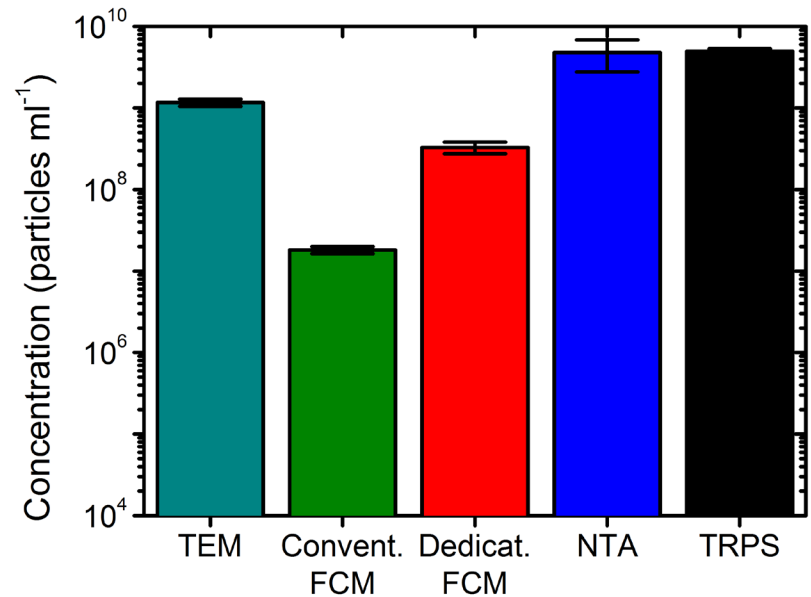


Concentration and size of urinary vesicles

size distribution fitted



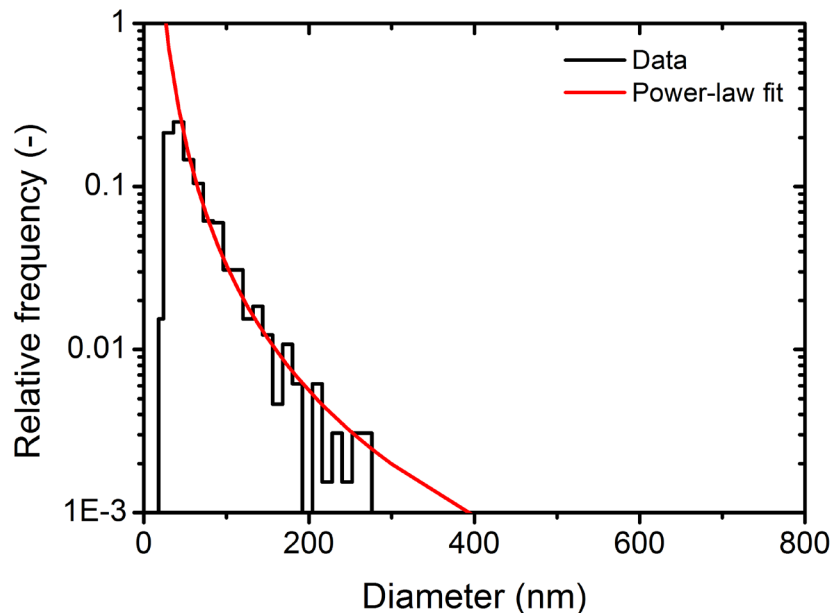
total concentration



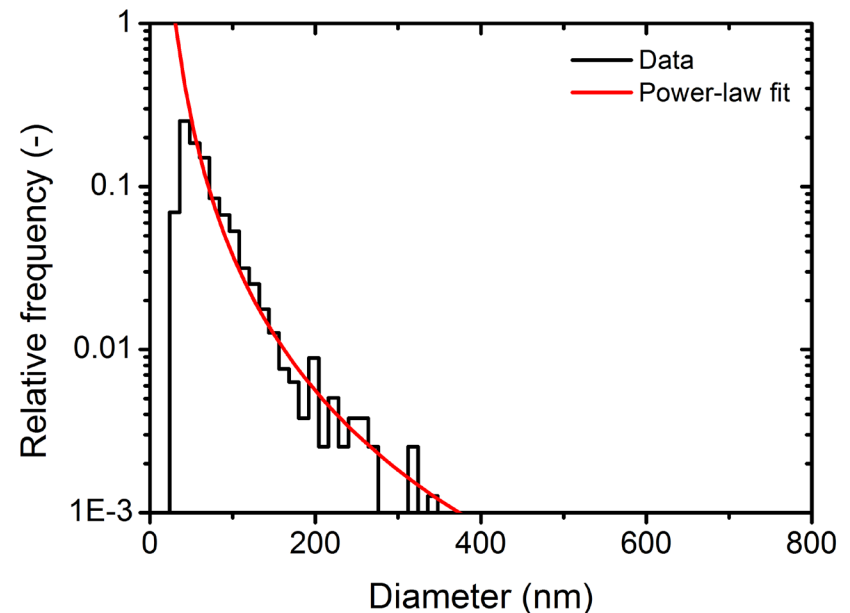
- size distribution: power-law function
- smallest detectable size affects concentration

Platelet and erythrocyte vesicle size and concentration by atomic force microscopy*

Erythrocyte vesicles in air



Platelet vesicles in air



- size distribution: power-law function!

Outline

✔ goal

- ✔ determine concentration of a vesicle subpopulation

● challenges

- ✔ determine vesicle concentration and size
 - detect vesicles by flow cytometry

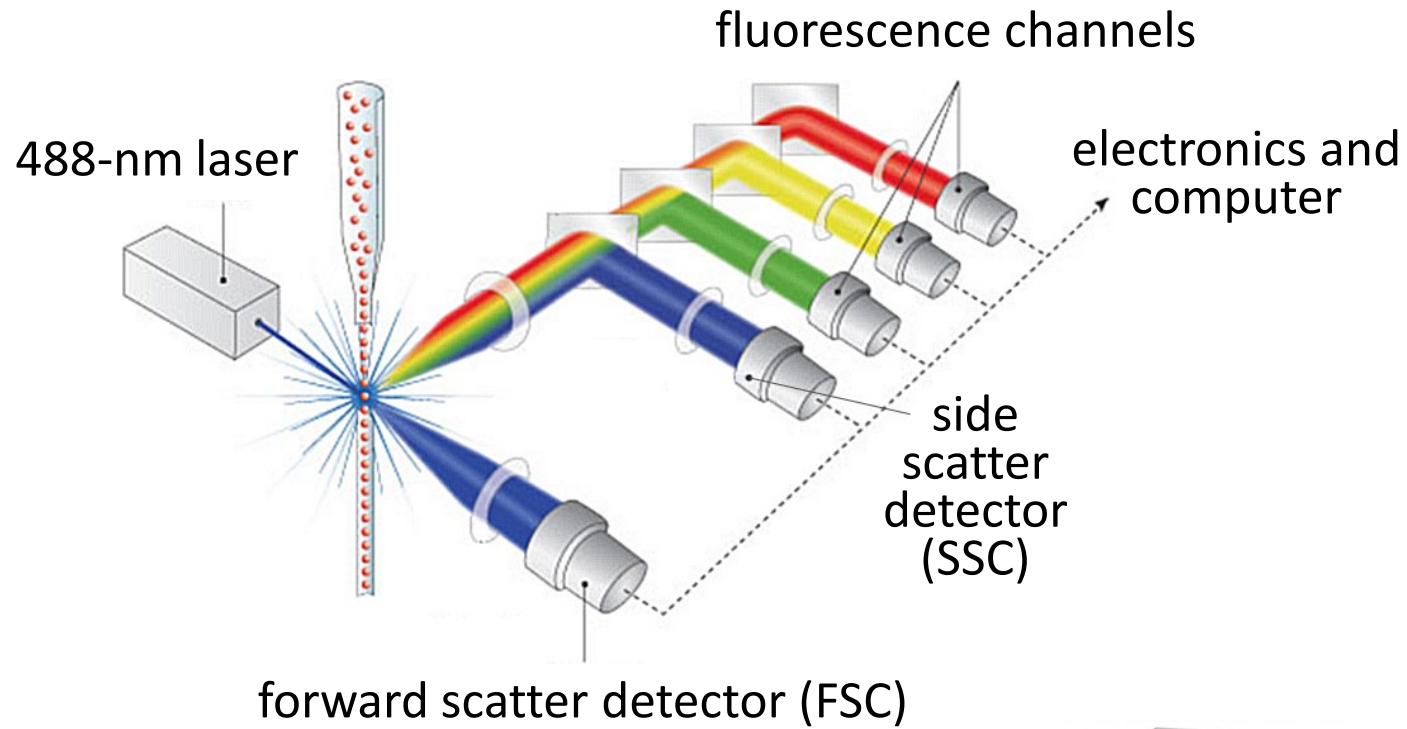
● solutions

- vesicle refractive index
- standardization of flow cytometry measurements

● outlook

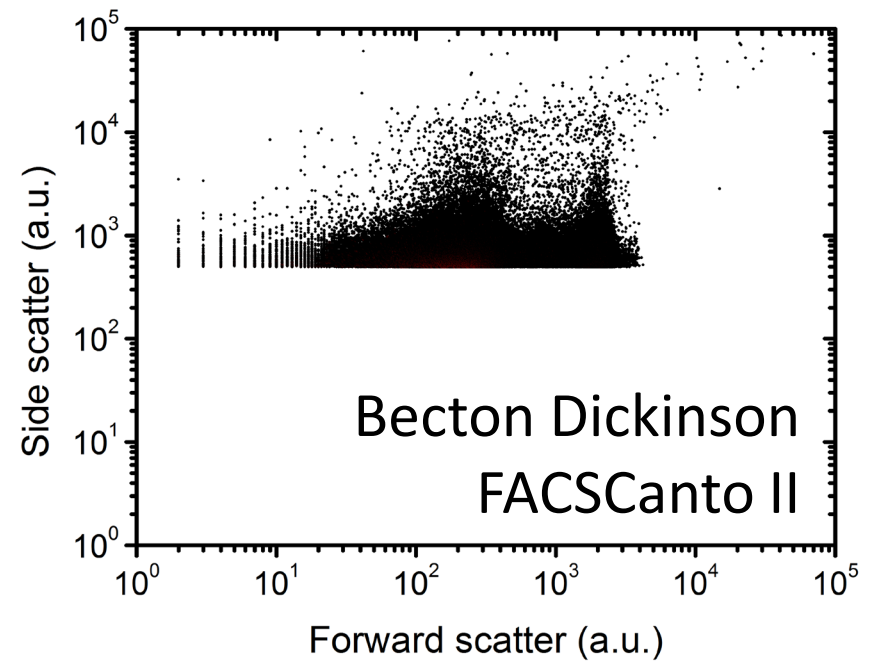
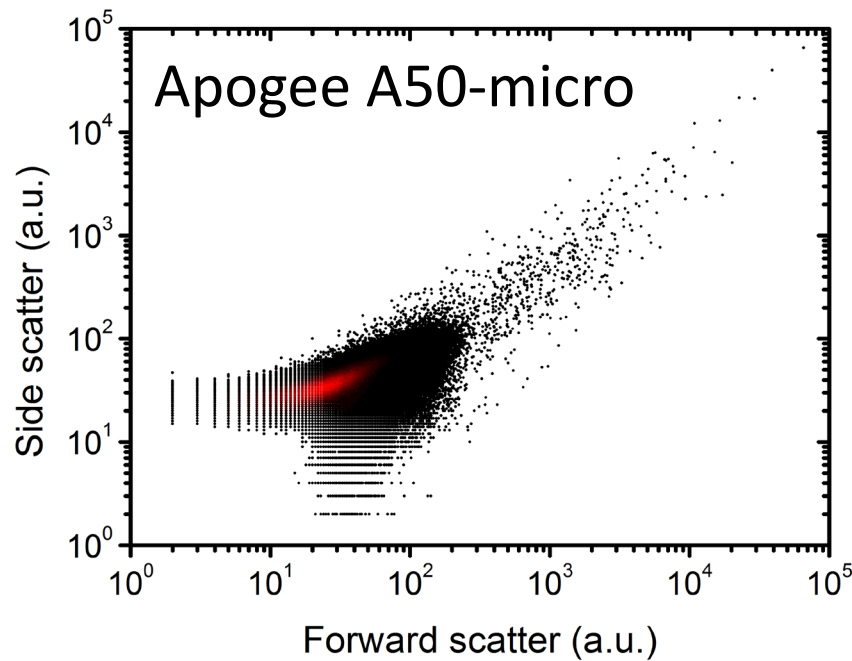
- future role of metrology

Vesicle detection by flow cytometry



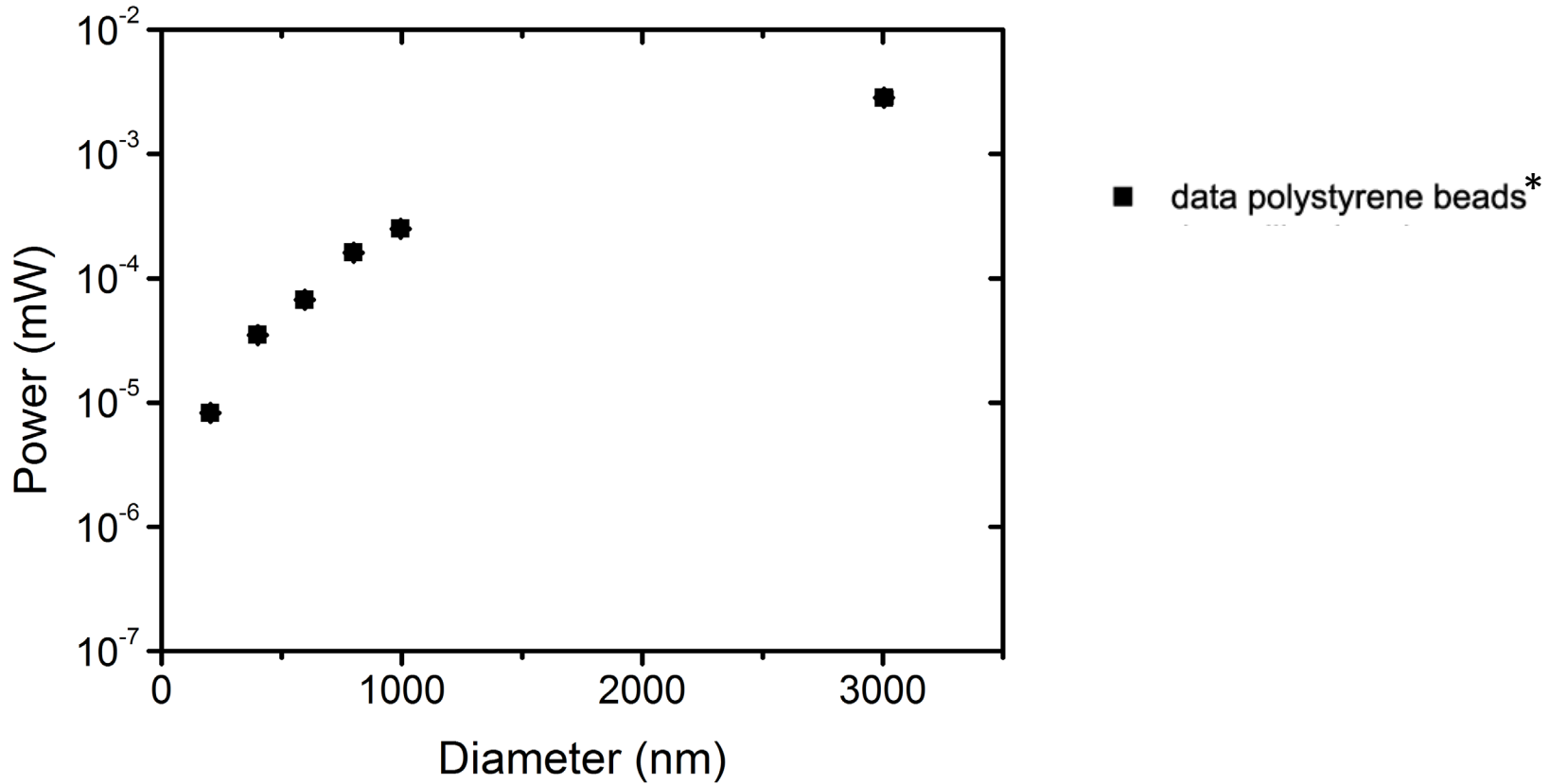
Problem: arbitrary units hamper data interpretation and comparison

same population of erythrocyte vesicles

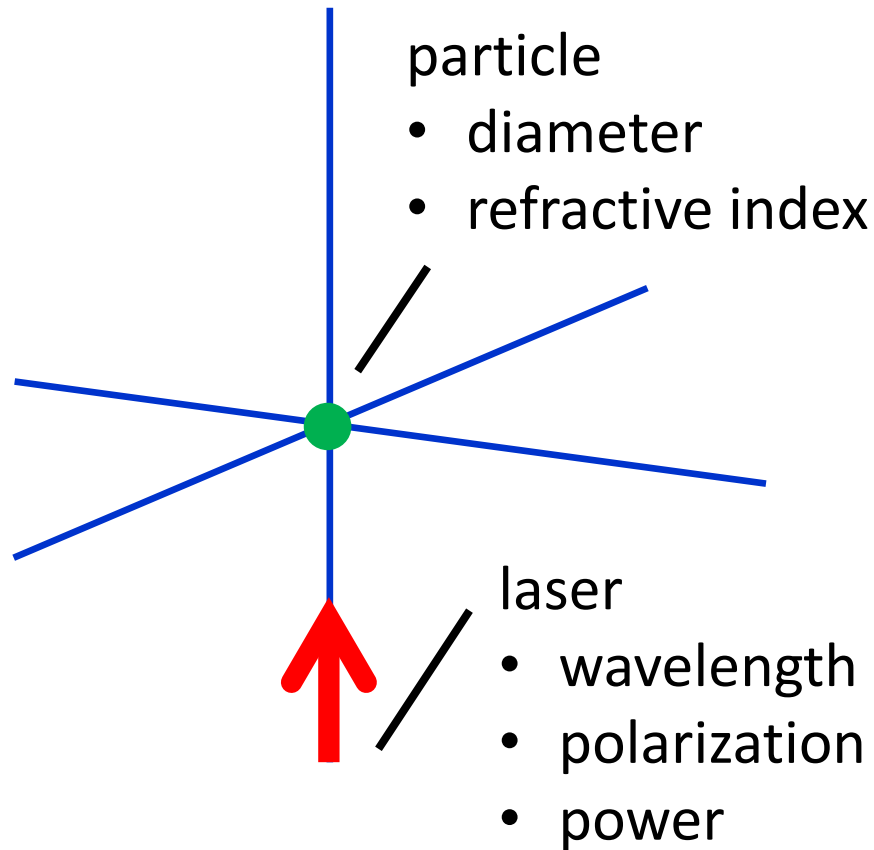


- goal: relate scatter to particle diameter

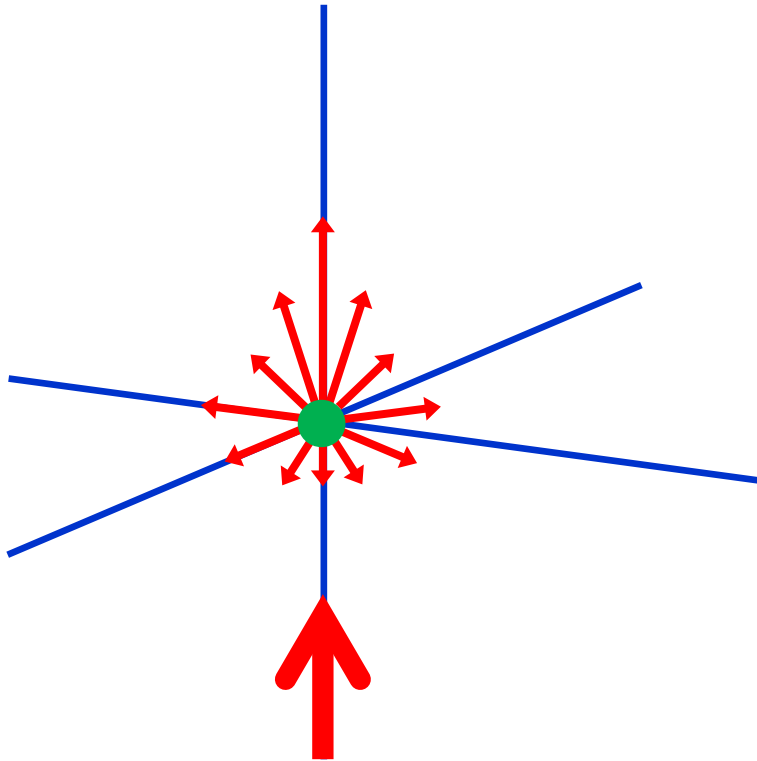
Relate scatter to diameter of beads



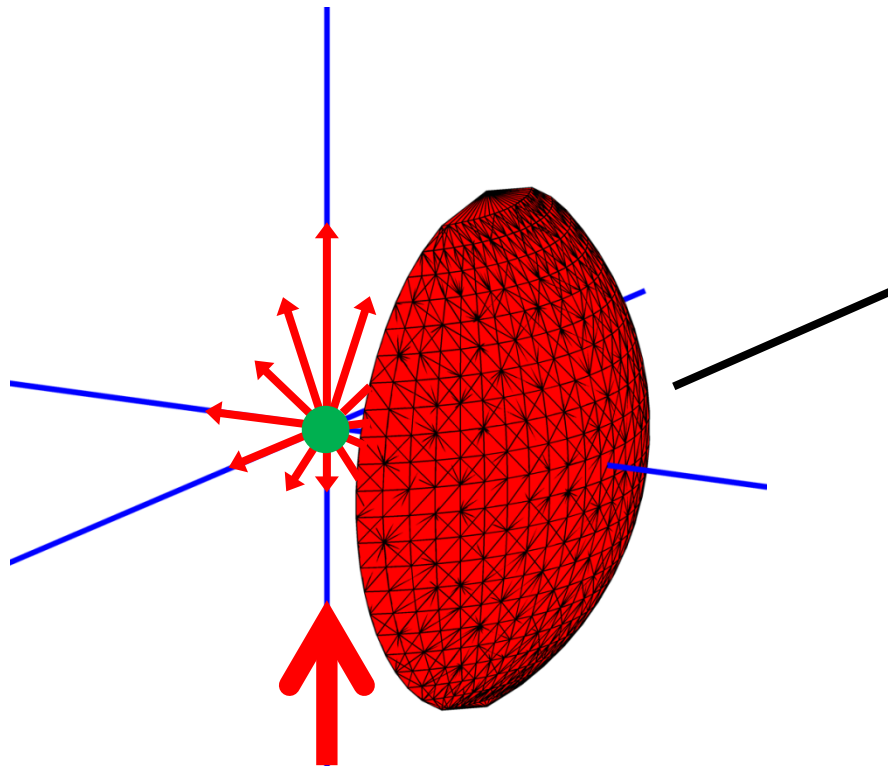
Relate scatter to diameter of particles with Mie theory



Relate scatter to diameter of particles with Mie theory



Relate scatter to diameter of particles with Mie theory

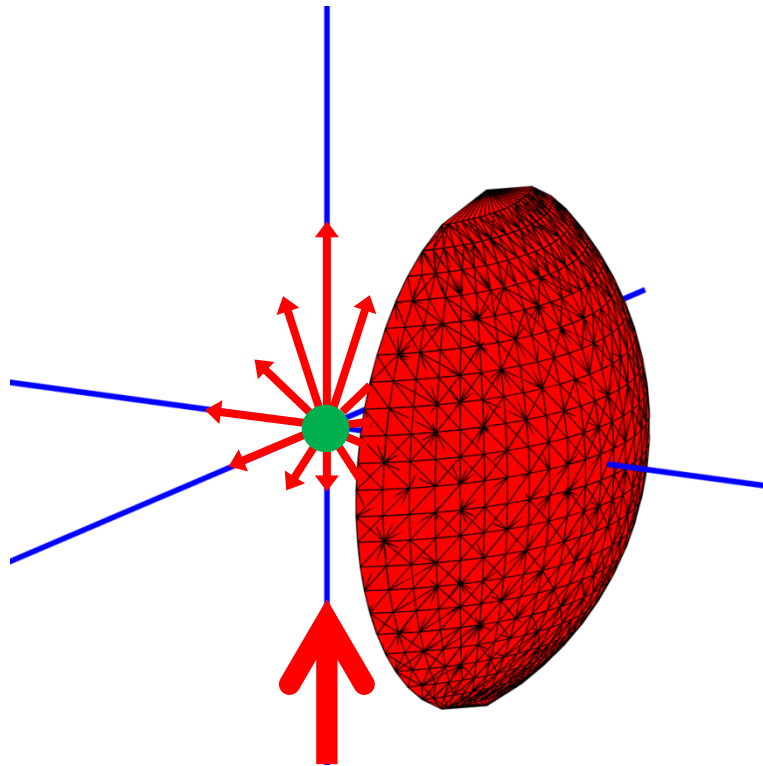


optical configuration

- collection angles
- collection efficiency
- obscuration bar
- diaphragm
- mirror

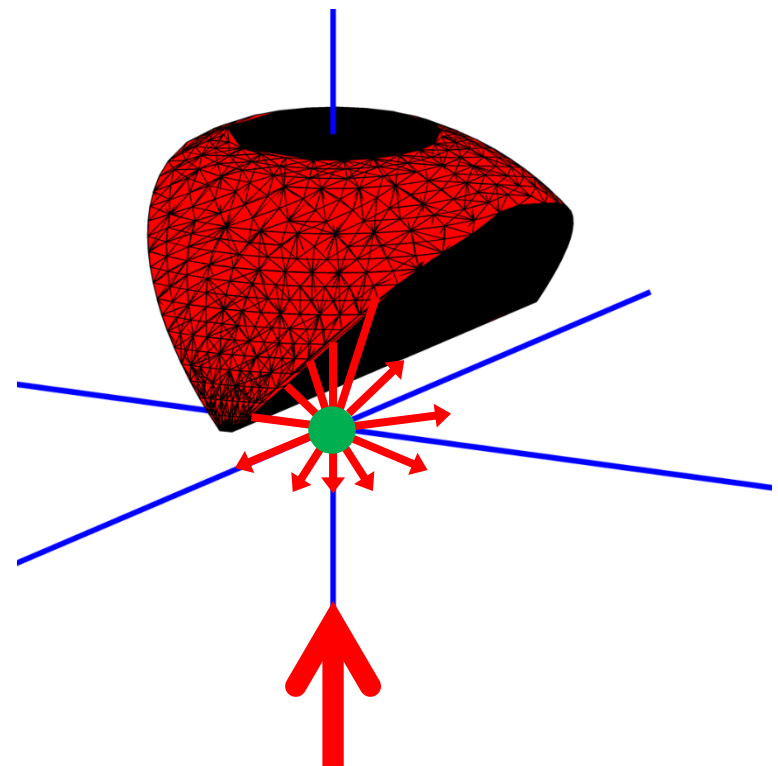
Relate scatter to diameter of particles with Mie theory

Becton Dickinson FACSCalibur



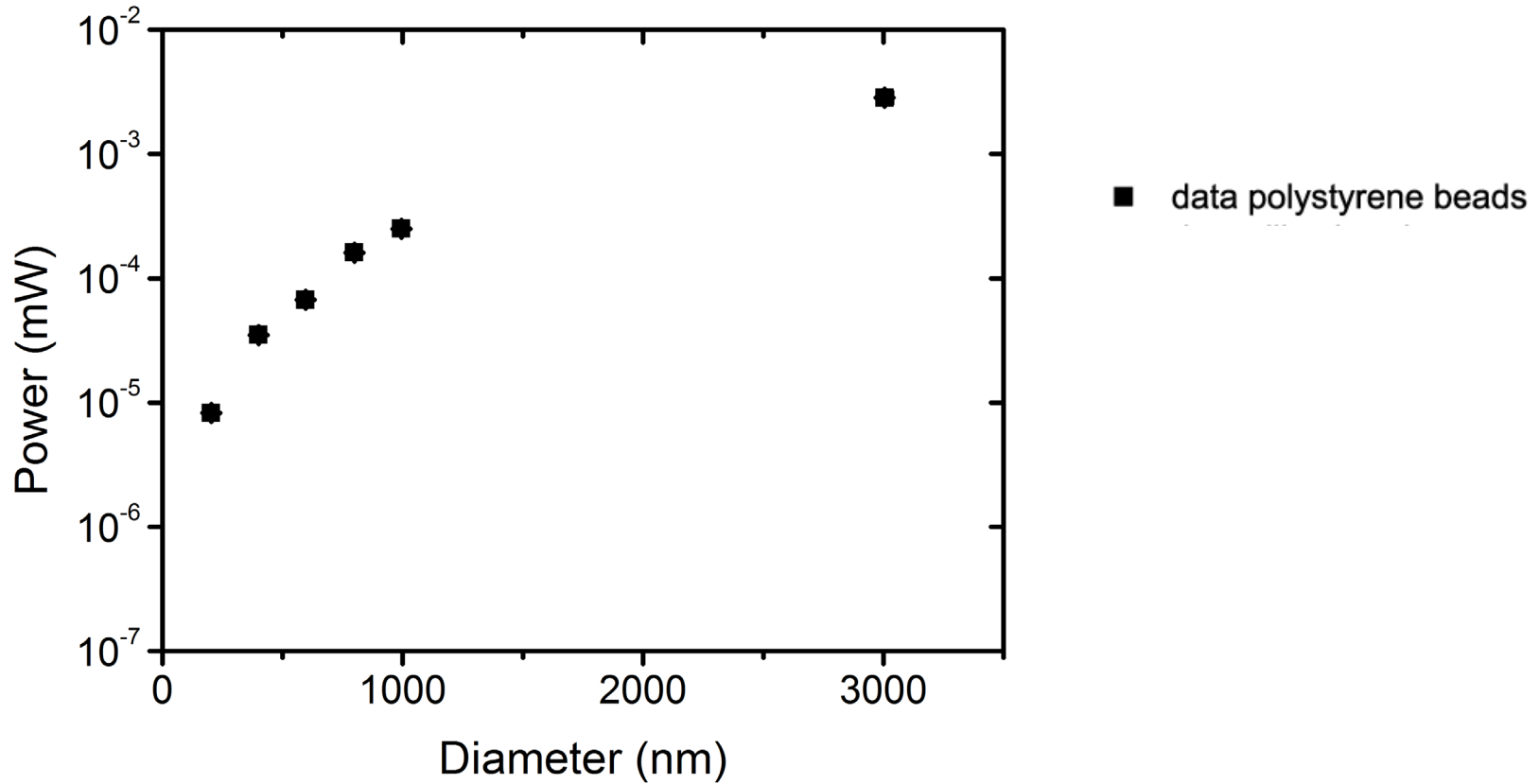
Side scatter

Apogee A50-micro

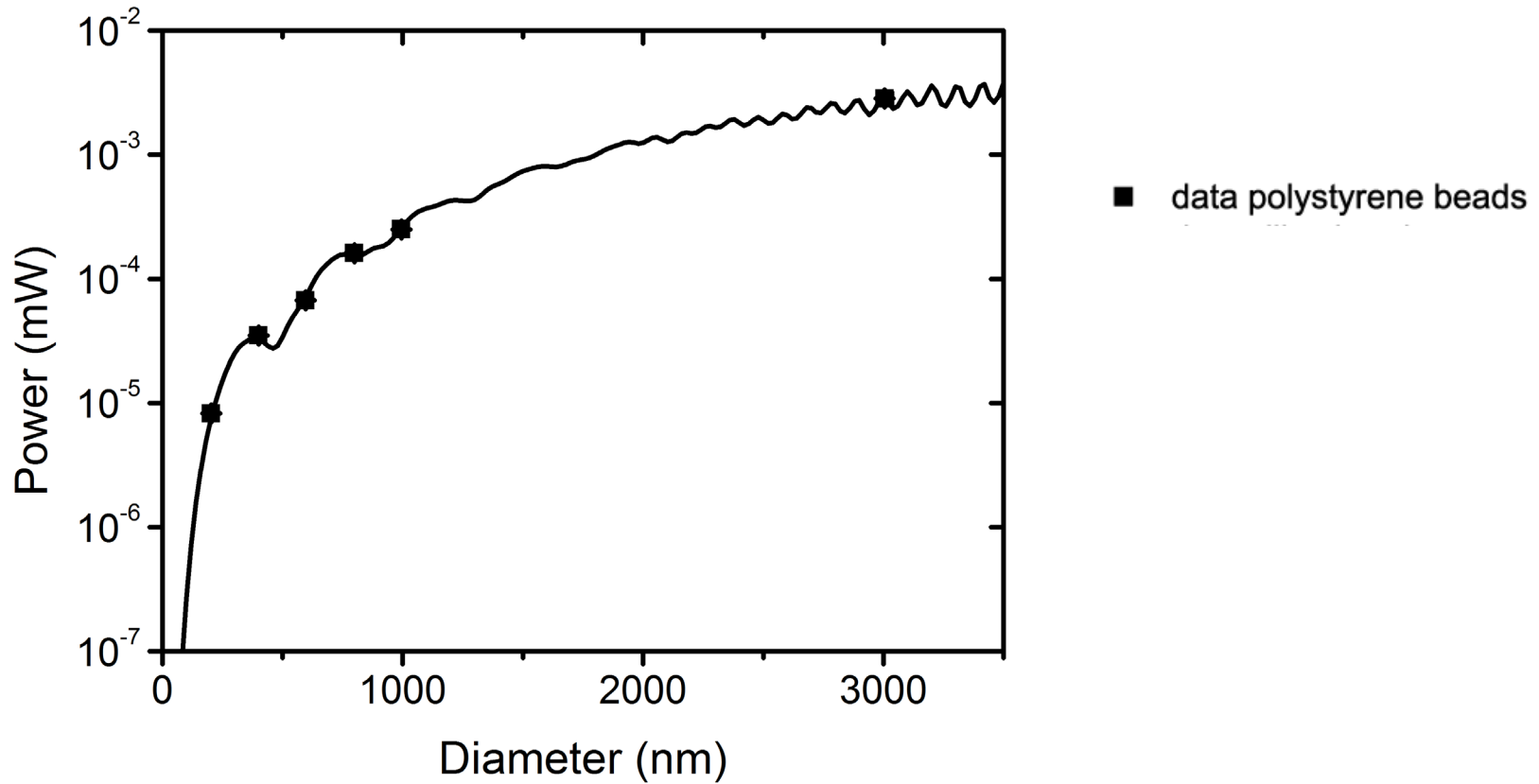


Forward scatter

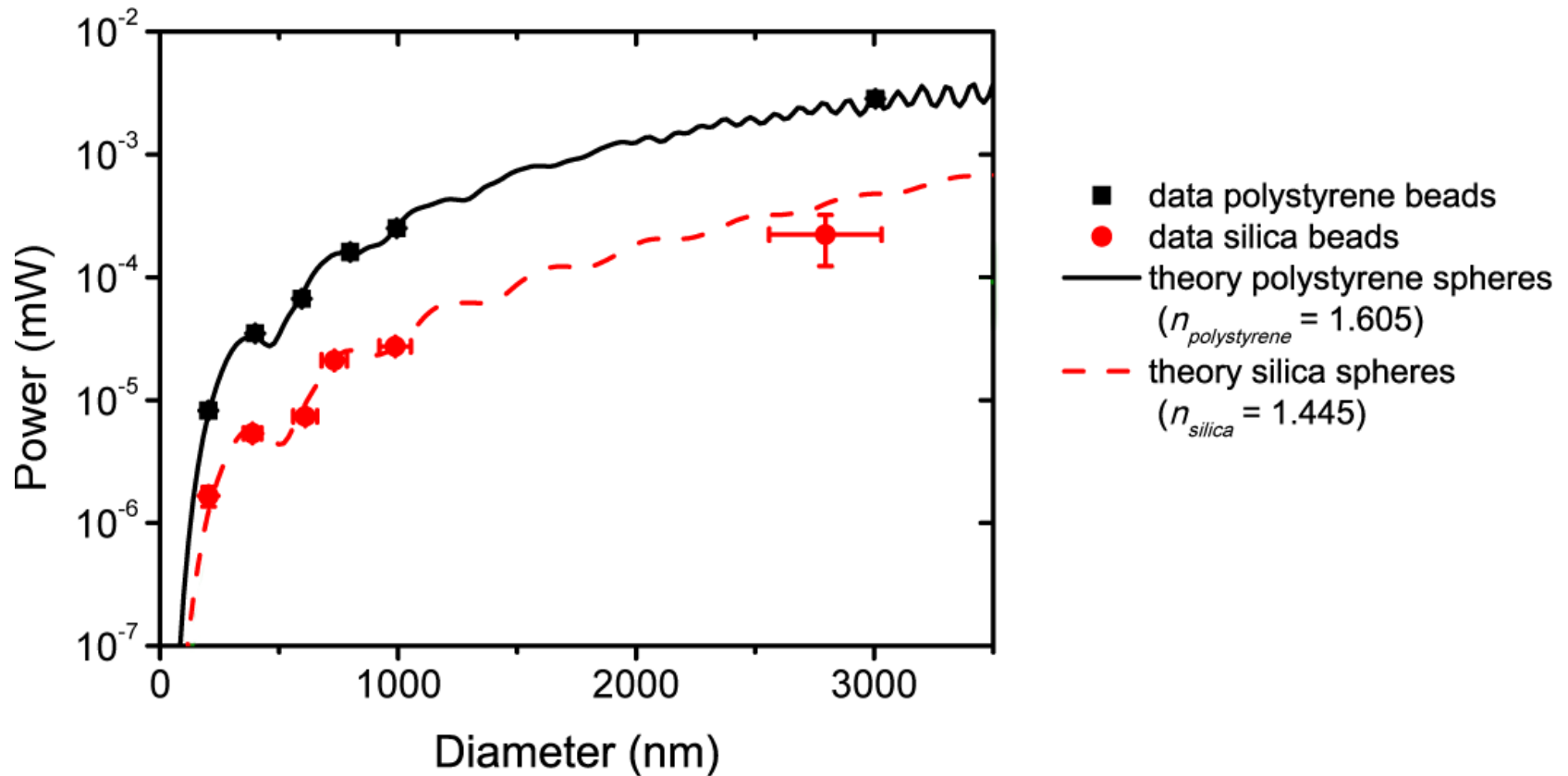
Relate scatter to diameter of beads



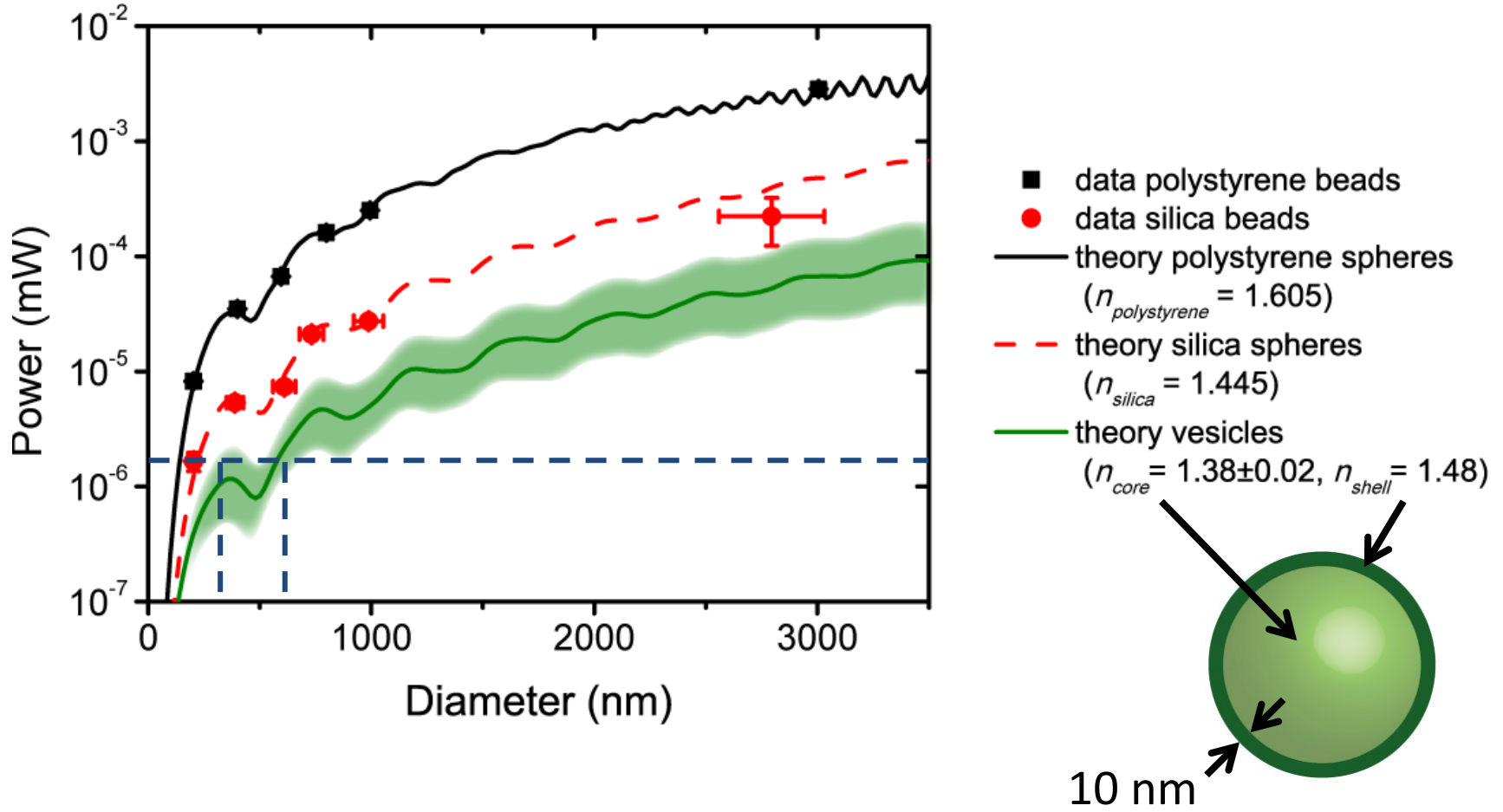
Relate scatter to diameter of beads



Relate scatter to diameter of beads

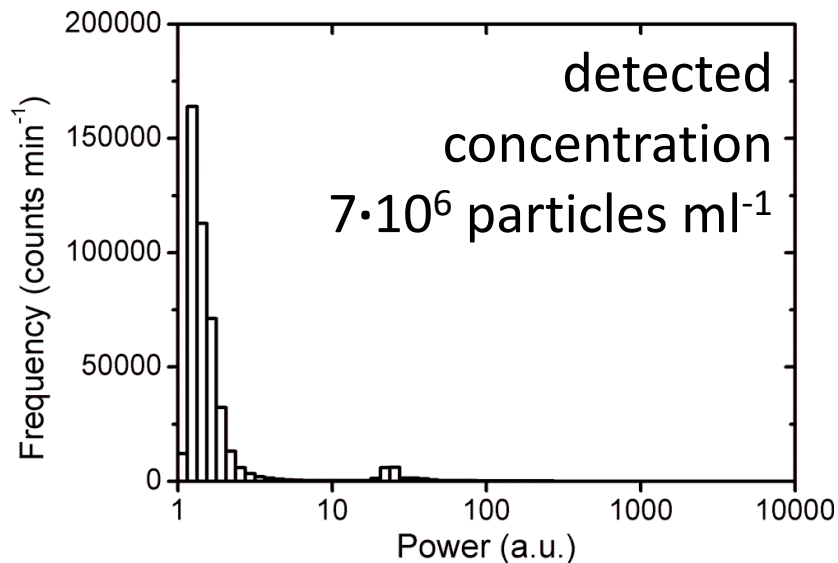


Relate scatter to diameter of vesicles

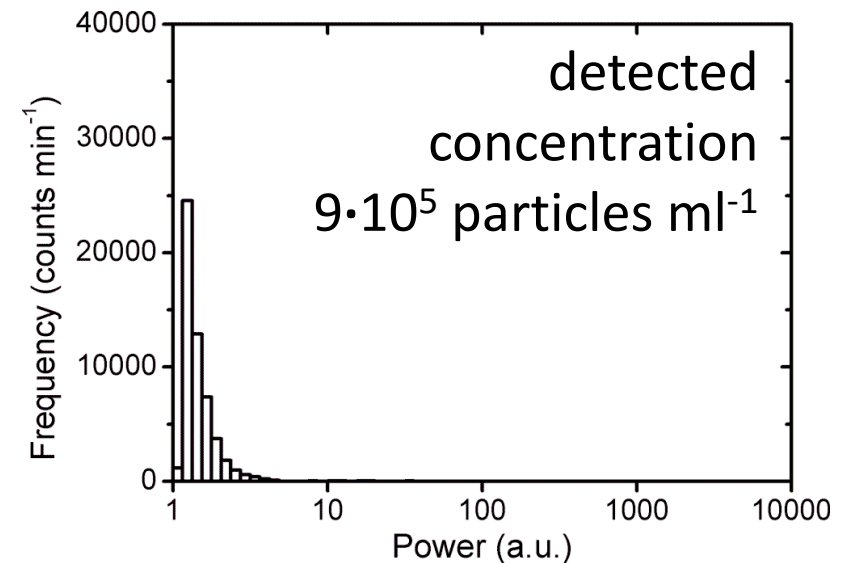


Particles that are too small to be detected generate a signal!

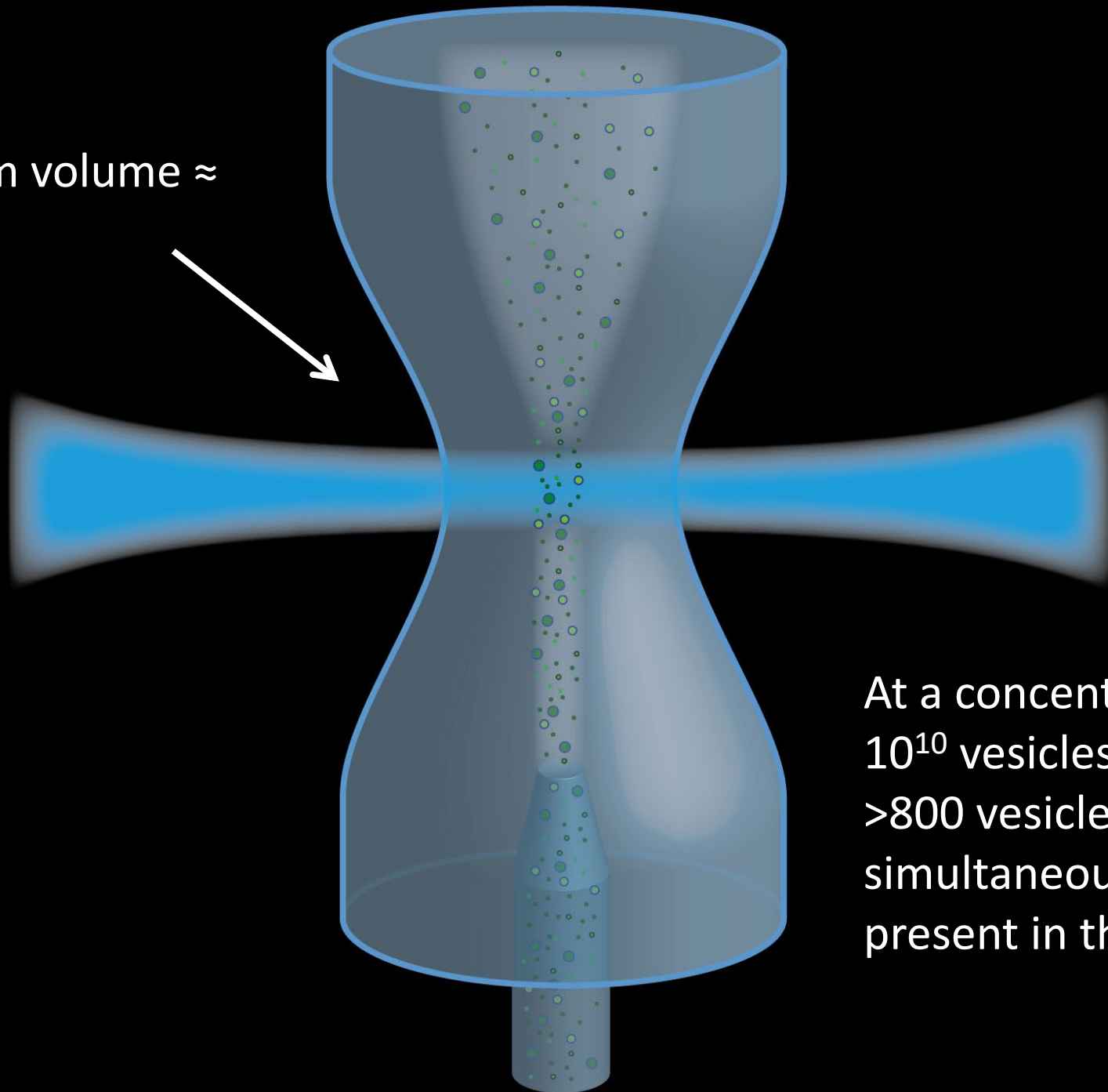
89 nm silica beads at
concentration 10^{10} beads ml^{-1}



urine filtered with 220 nm filter
concentration $\geq 10^{10}$ vesicles ml^{-1}



beam volume \approx
54 μ l



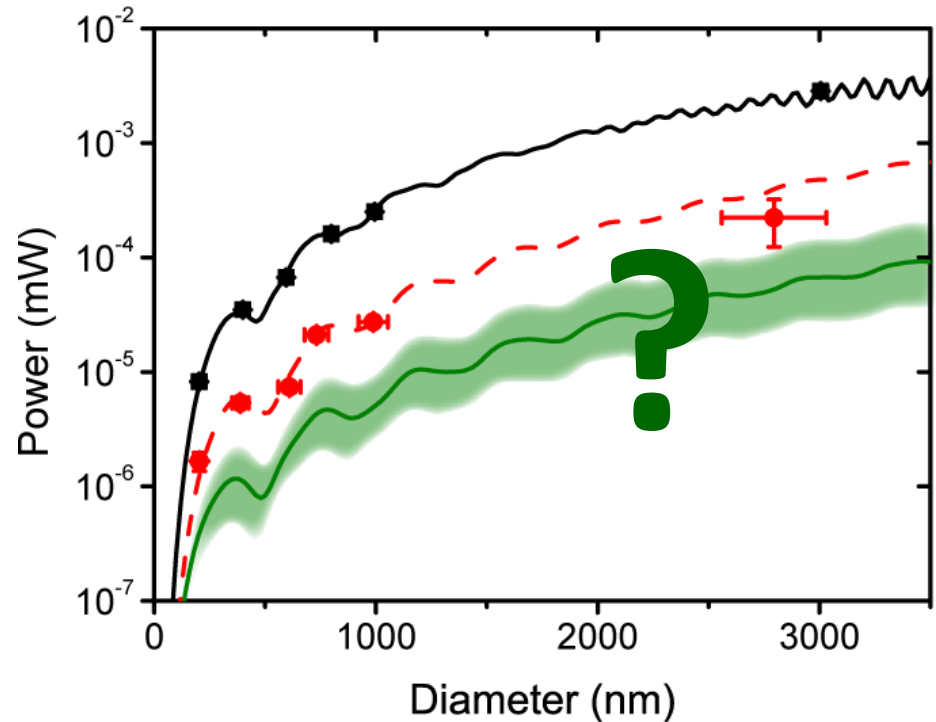
At a concentration of 10^{10} vesicles ml^{-1} ,
>800 vesicles are
simultaneously
present in the beam.





Conclusion vesicle detection by flow cytometry

- single event signal attributed to scattering from *multiple* vesicles
- scattering power related to diameter and refractive index for *single* beads and vesicles



Outline

✔ goal

- ✔ determine concentration of a vesicle subpopulation

✔ challenges

- ✔ determine vesicle concentration and size
- ✔ detect vesicle by flow cytometry

● solutions

- vesicle refractive index
- standardization of flow cytometry measurements

● outlook

- future role of metrology

Nanoparticle tracking analysis for refractive index determination

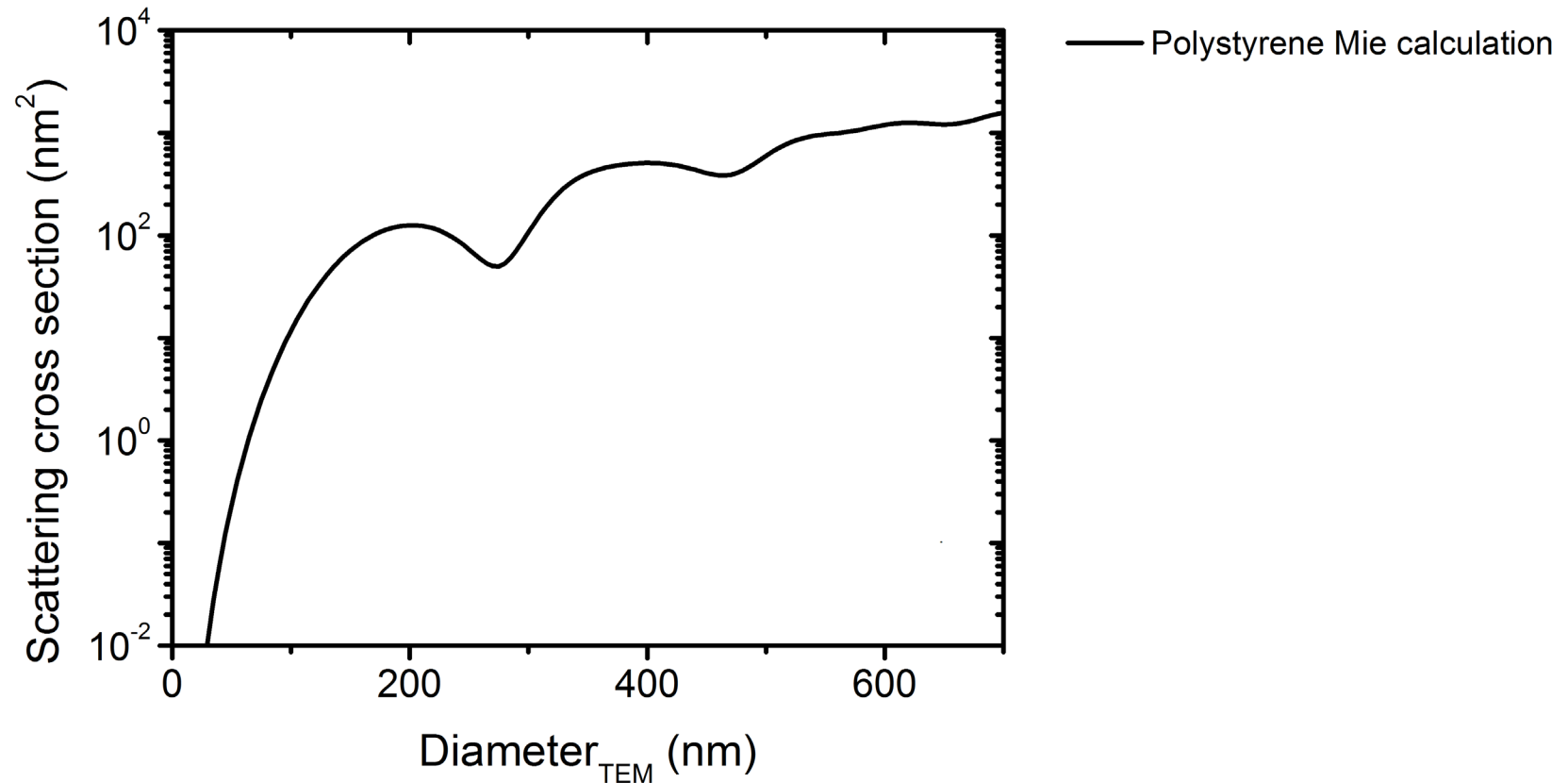


- obtain **particle diameter** d by tracking the Brownian motion of single particles (Stokes-Einstein equation)
- measure **scattering power** P
- derive particle **refractive index** $n(P, d)$ from Mie theory

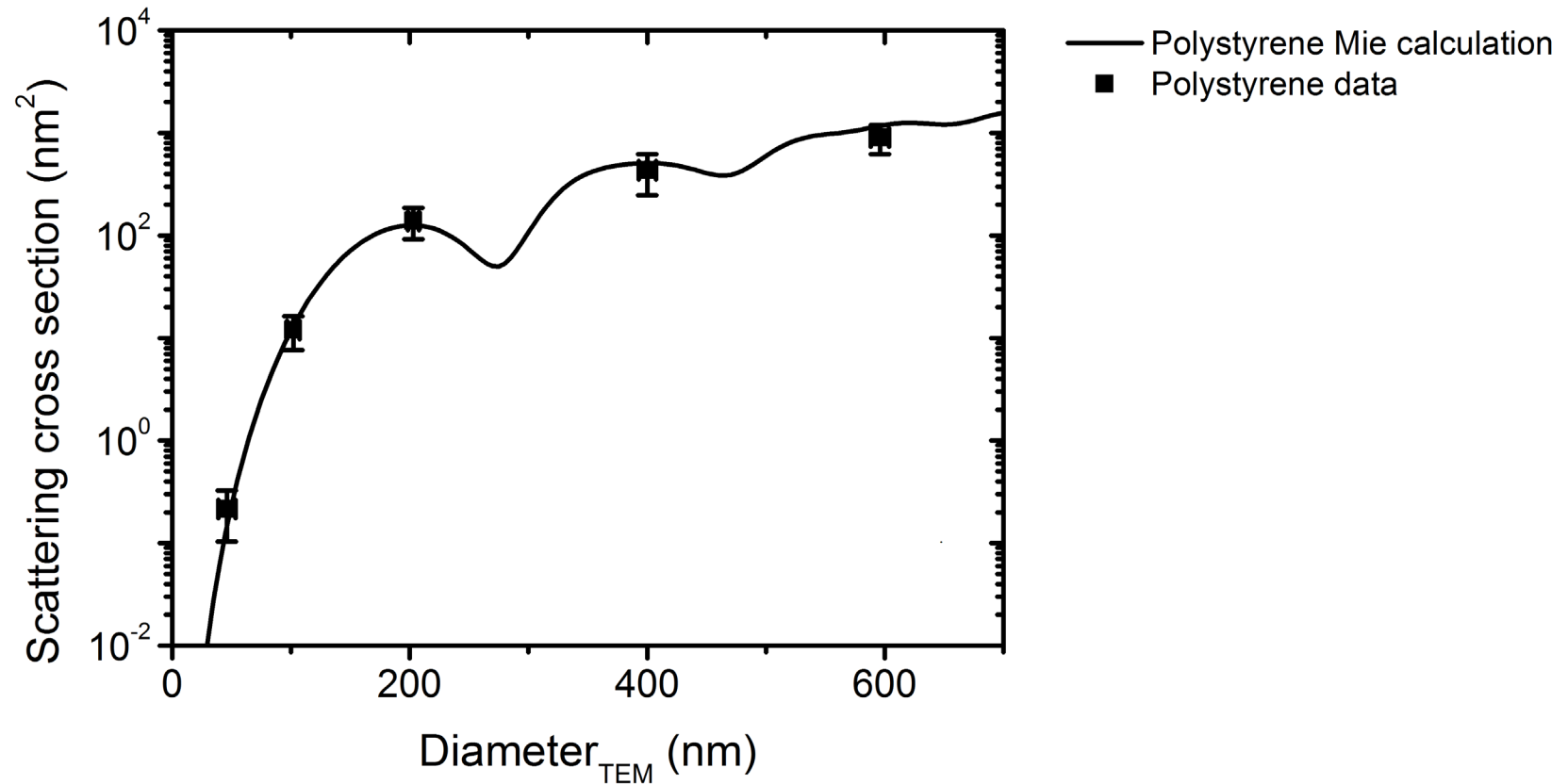
Refractive index determination - approach

- calibration
 - calculate light scattering by Mie theory
 - measure light scattering of beads
- validation
 - determine refractive index of beads mixture
- application
 - determine refractive index of vesicles

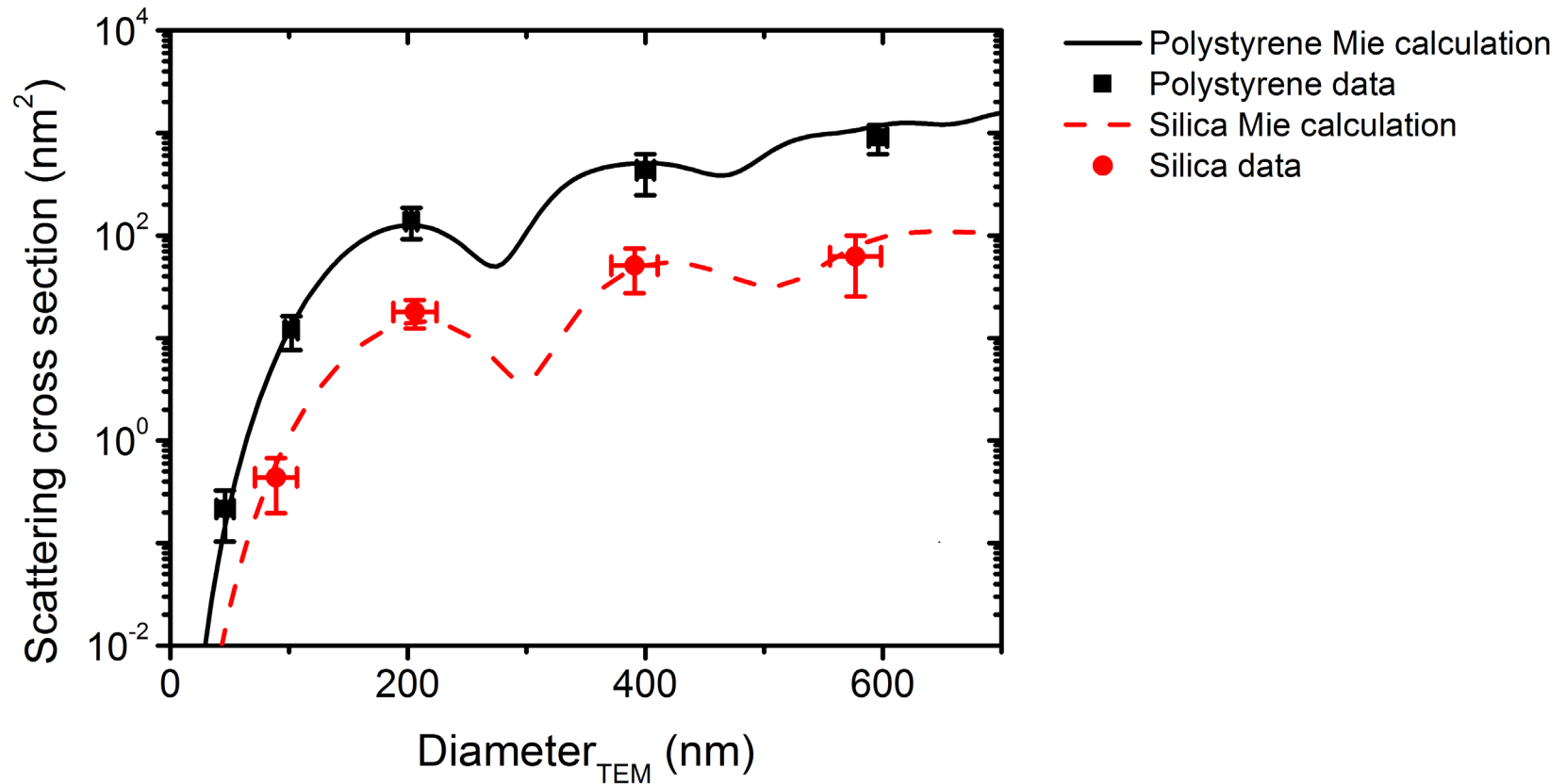
Results - scattering cross section vs. diameter of polystyrene beads by Mie theory



Results - scattering cross section vs. diameter of polystyrene beads



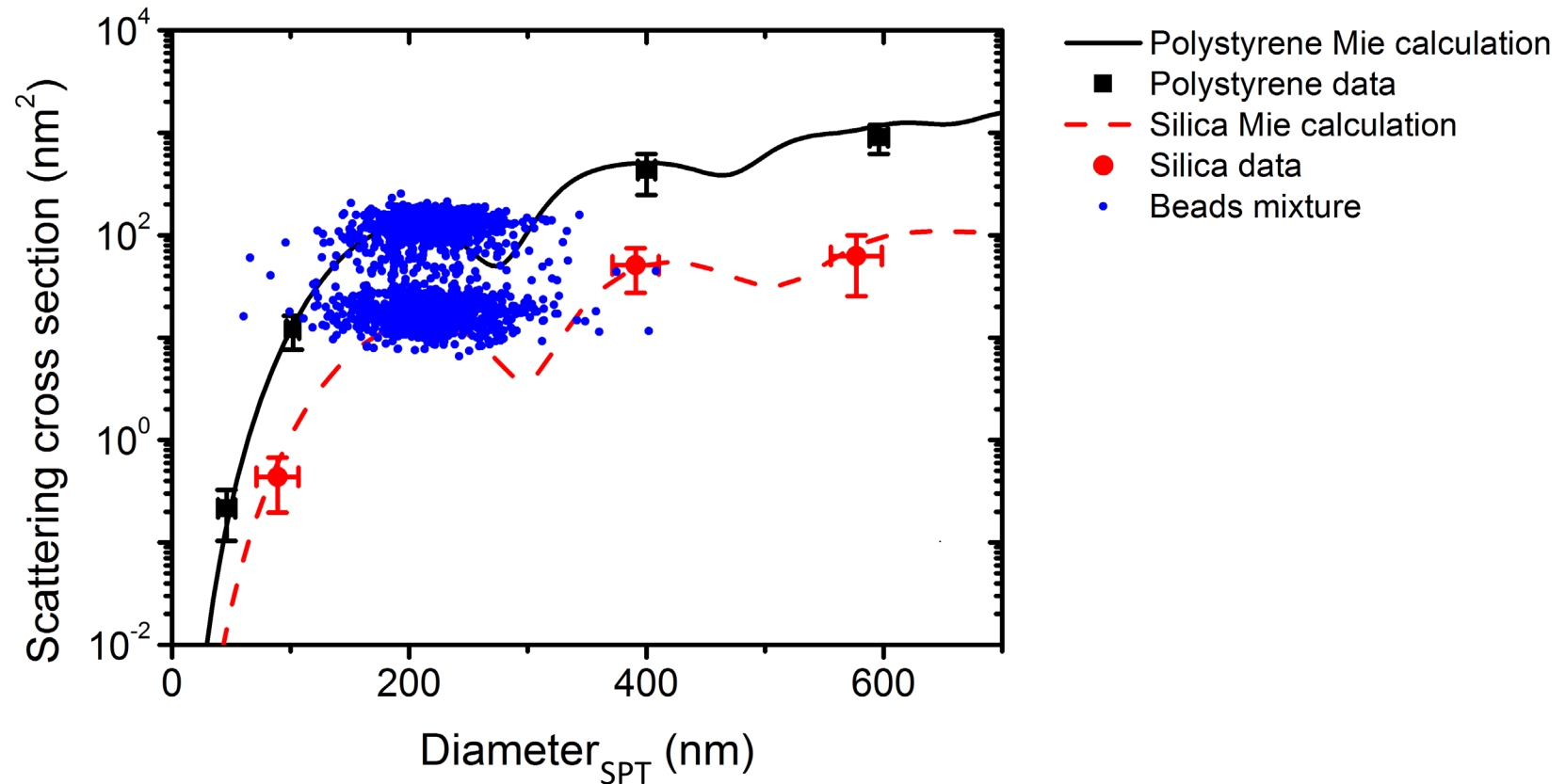
Results - scattering cross section vs. diameter of polystyrene and silica beads



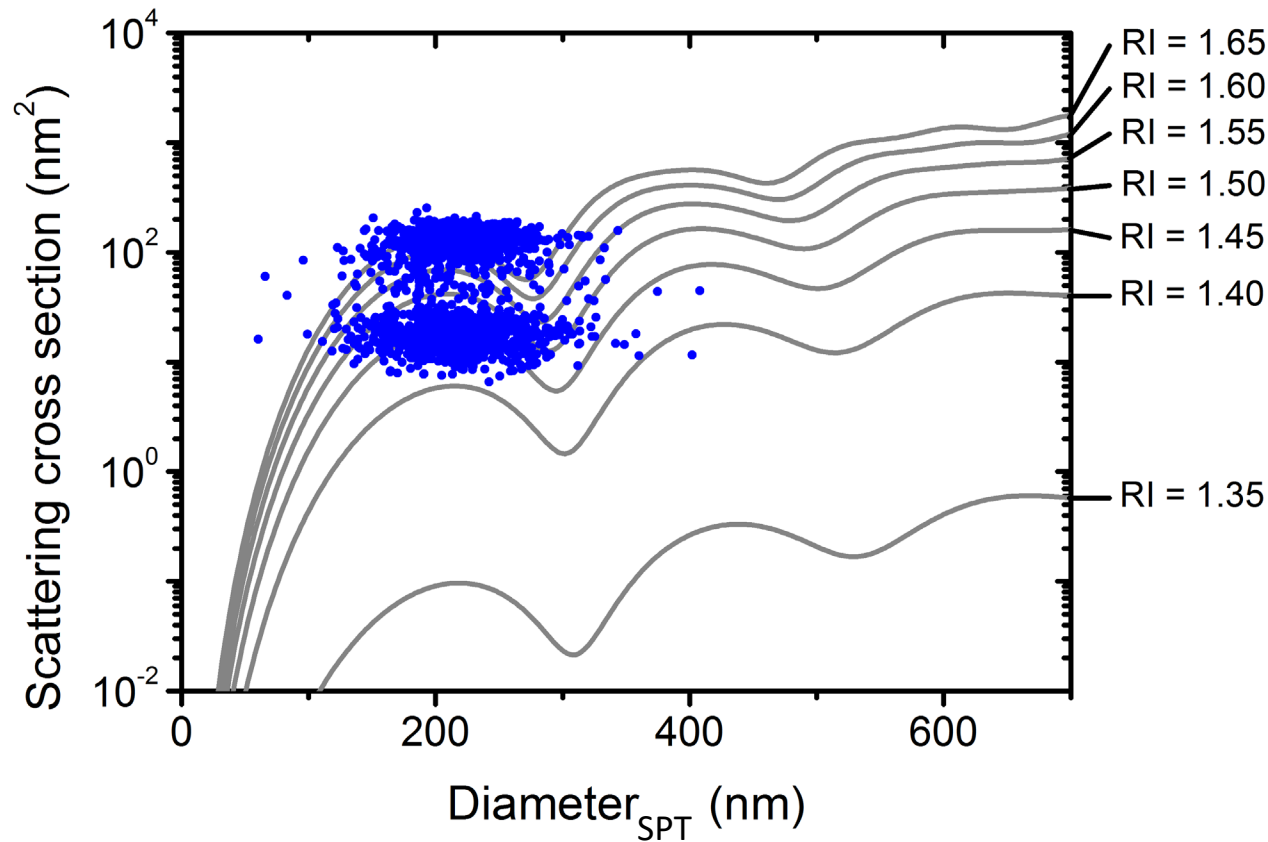
Refractive index determination - approach

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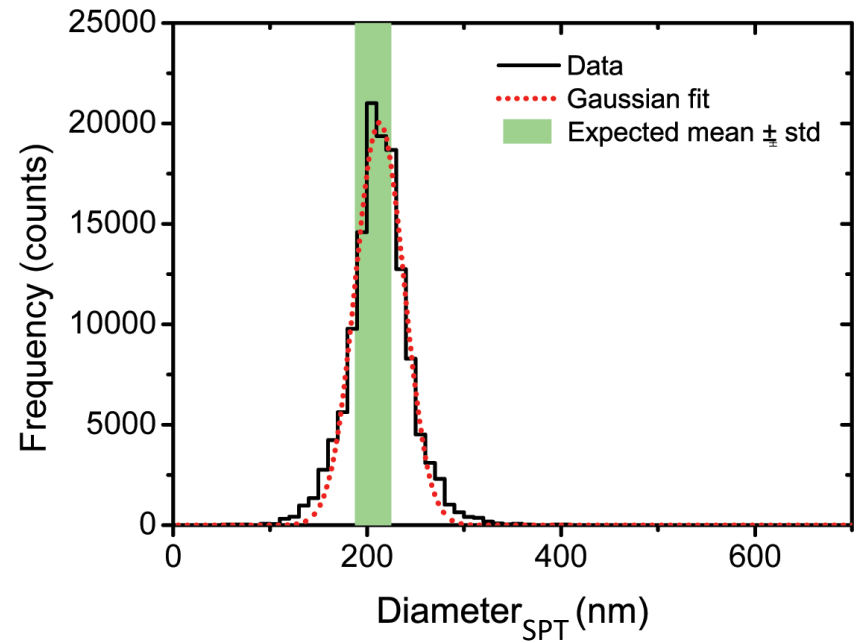
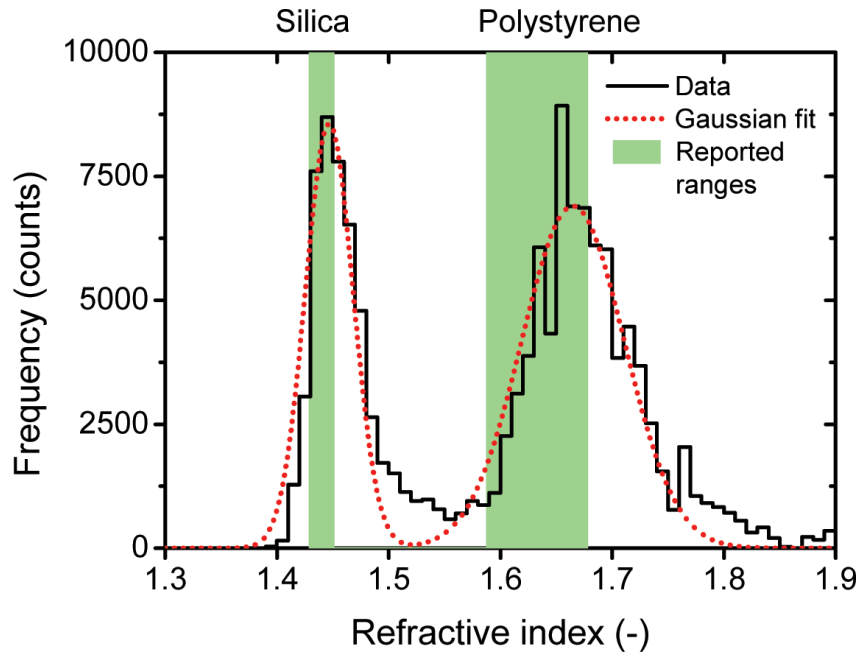
Results - scattering cross section vs. diameter of a mixture of polystyrene and silica beads



Results - scattering cross section vs. diameter of a mixture of polystyrene and silica beads



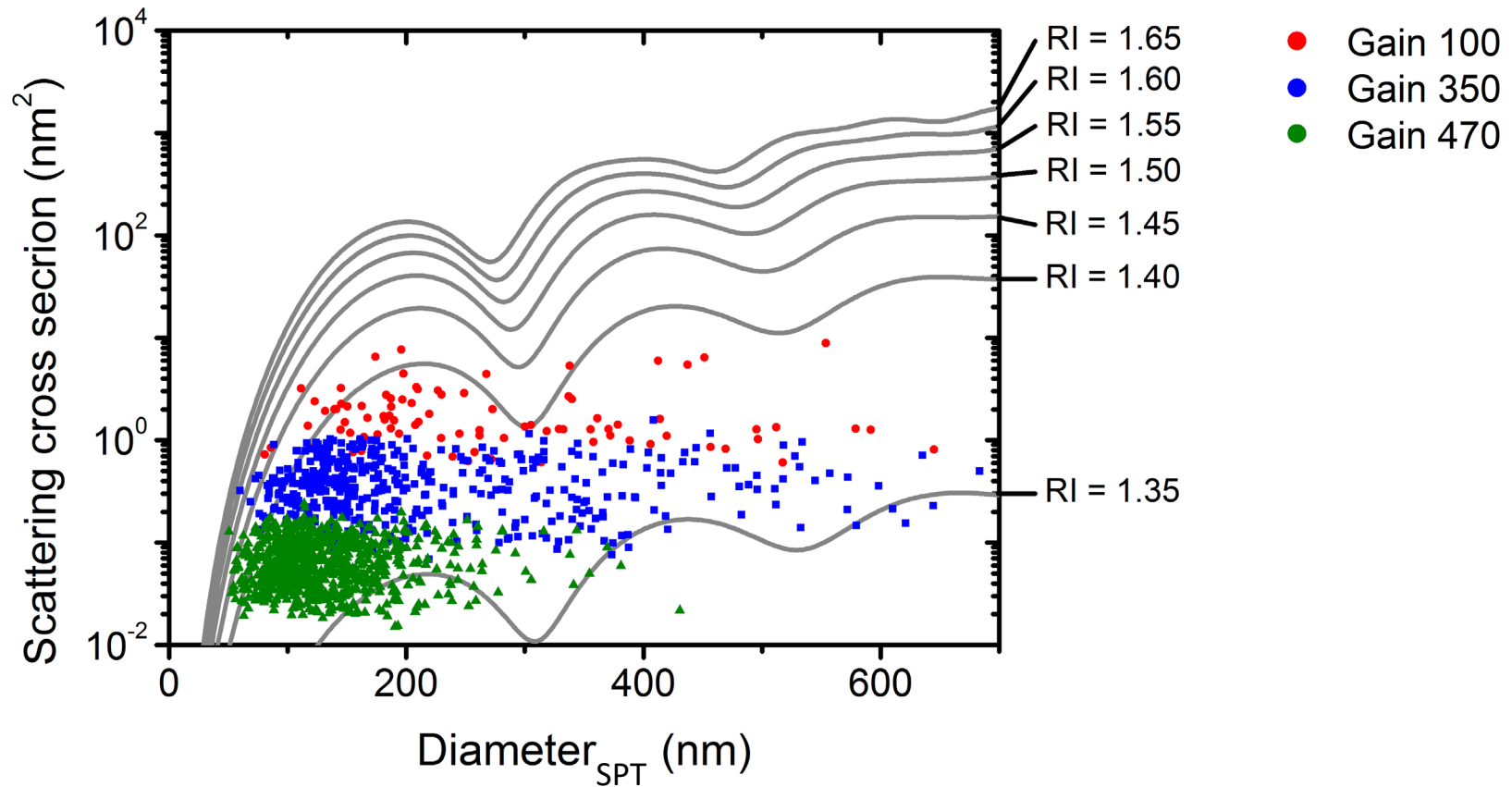
Results - refractive index and size distribution of a mixture of polystyrene and silica beads



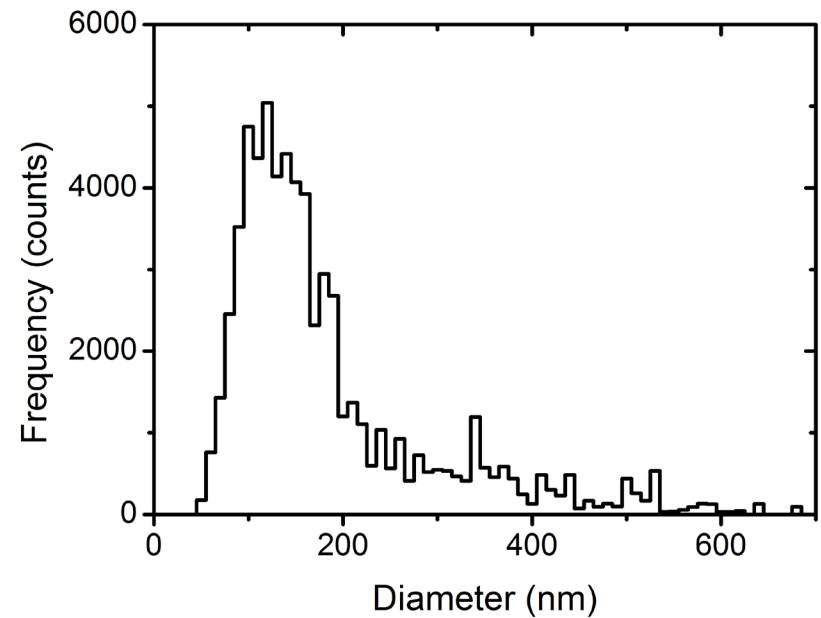
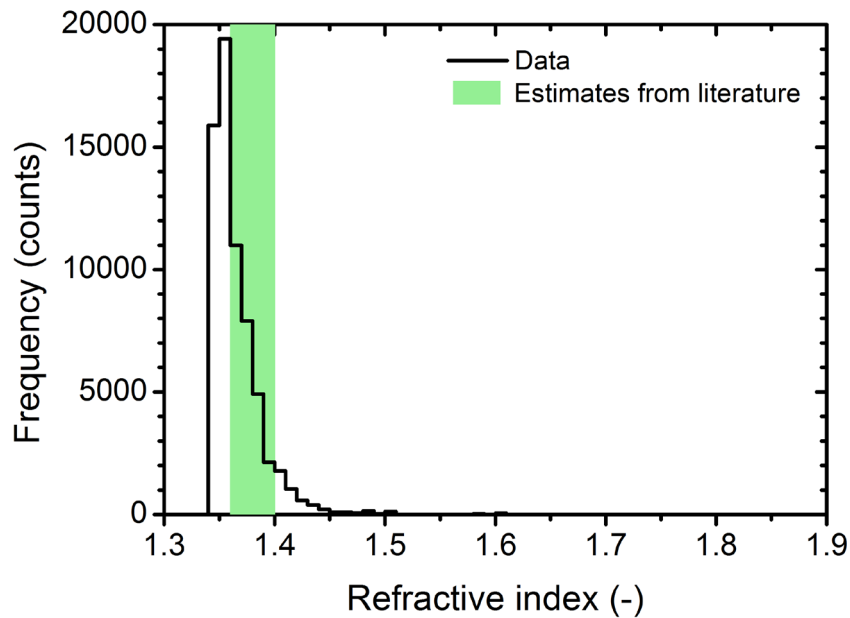
Refractive index determination - approach

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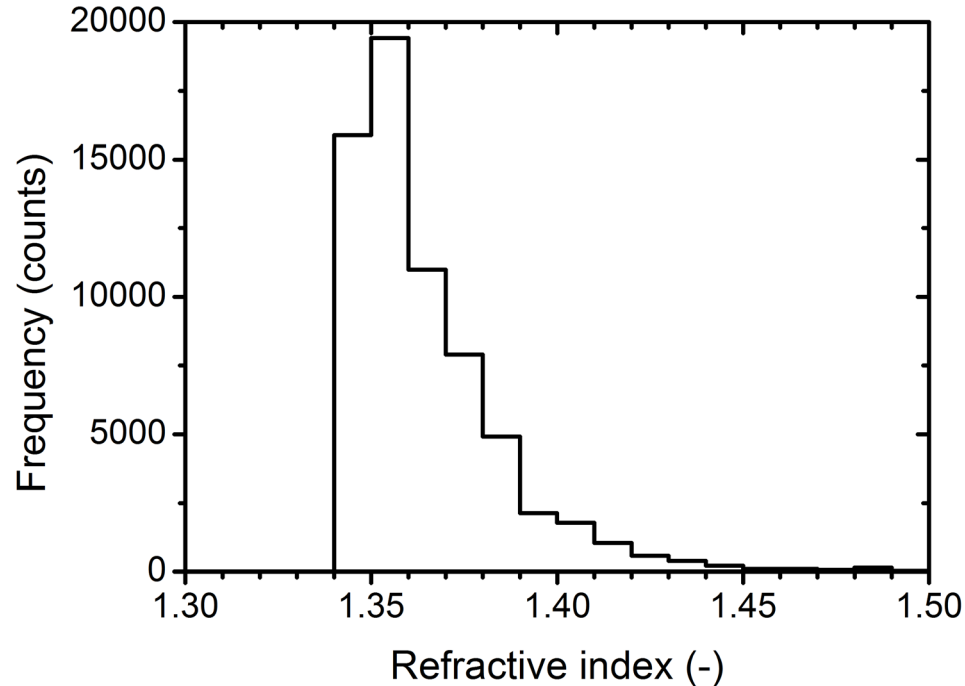
Results - scattering power versus diameter of urinary vesicles



Results - size and refractive index distribution of urinary vesicles



Conclusions refractive index of vesicles



- nanoparticle tracking analysis can be used to determine the refractive index of nanoparticles
- mean refractive index of urinary vesicles is 1.37

Outline

✔ goal

- ✔ determine concentration of a vesicle subpopulation

✔ challenges

- ✔ determine vesicle concentration and size

- ✔ detect vesicles by flow cytometry

● solutions

- ✔ vesicle refractive index

- standardization of flow cytometry measurements

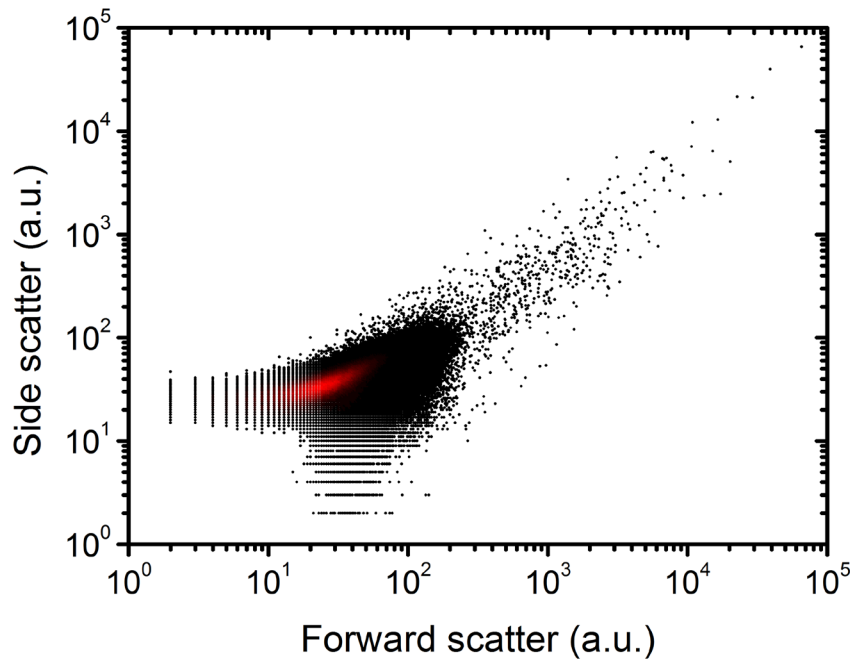
● outlook

- future role of metrology

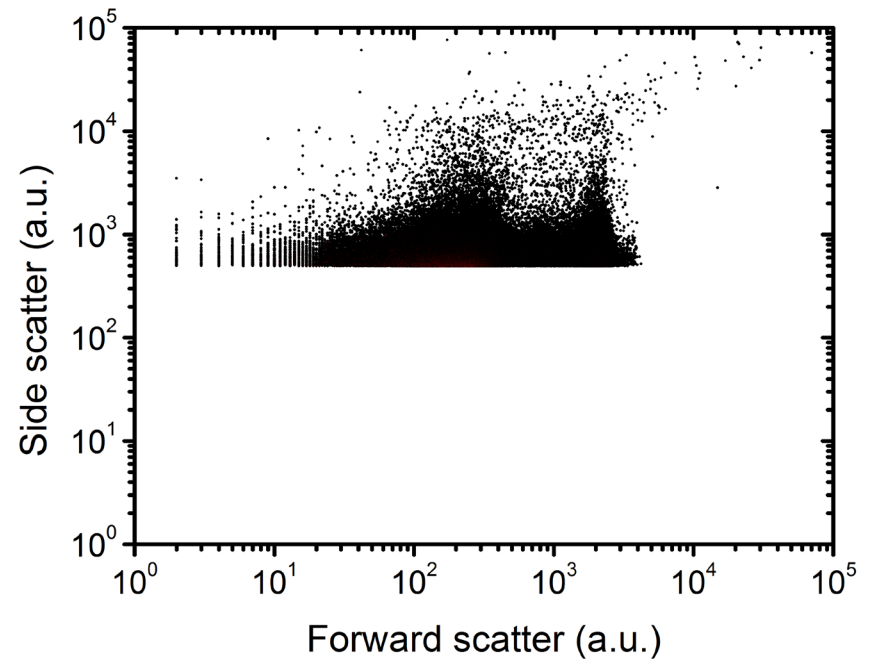


Problem 1: arbitrary units (solved!)

same population of erythrocyte vesicles



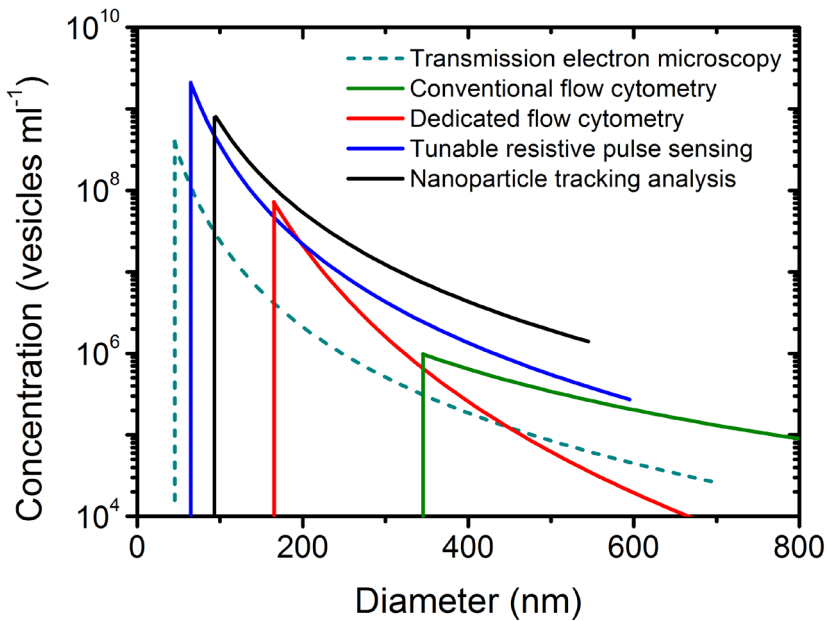
Apogee A50-micro



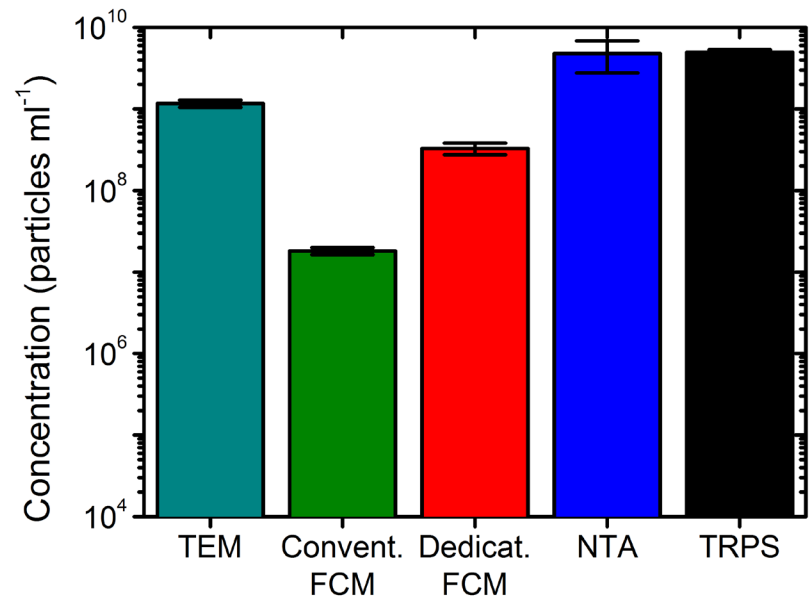
Becton Dickinson FACSCanto II

Problem 2: instruments differ in sensitivity

size distribution fitted



total concentration



Goal

- reproducible concentration of
 - erythrocyte vesicles
 - lactadherin-FITC, CD235a-PE labeled
 - platelet vesicles
 - lactadherin-FITC, CD61-PE labeled
- using different flow cytometers

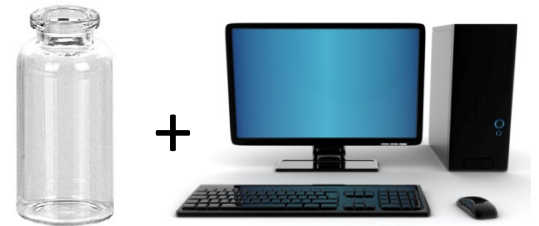


Study comprises 30 sites (50 instruments) worldwide



Approach

- determine flow rate
- determine size gates
 - measure well-characterized beads
 - obtain scatter to diameter relation



- measure vesicle samples and controls
- derive vesicle concentrations

Status

Please open "Exometry beads" file.

Controls

Open "Exometry beads" file

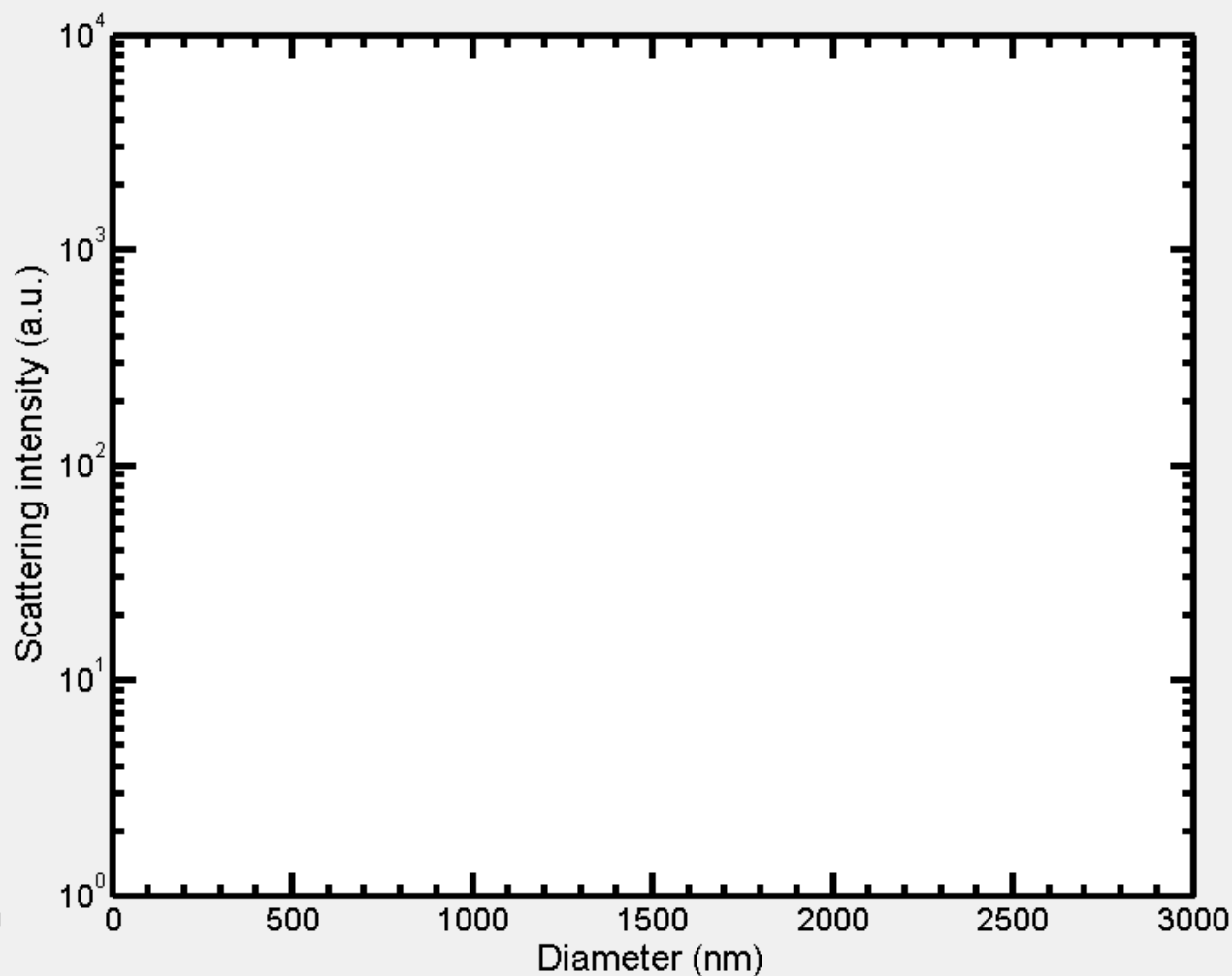
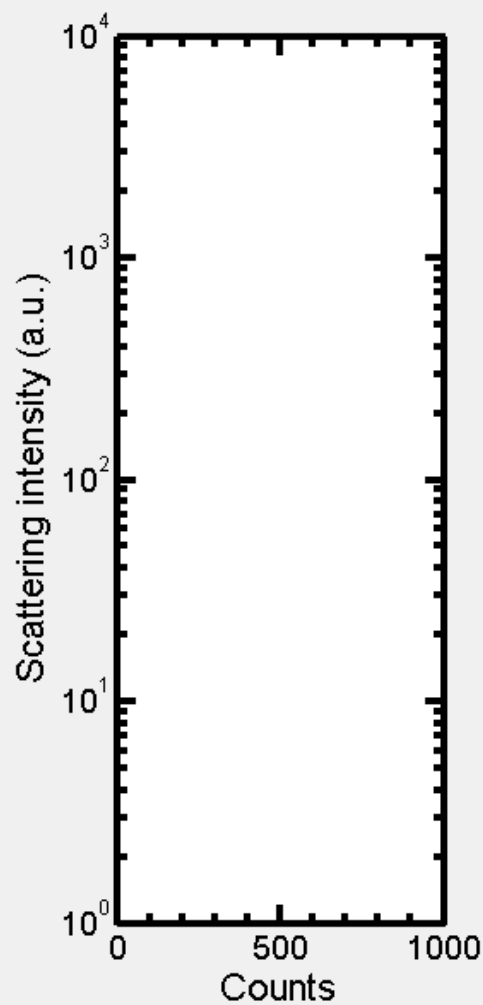
Flow cytometer unknown

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

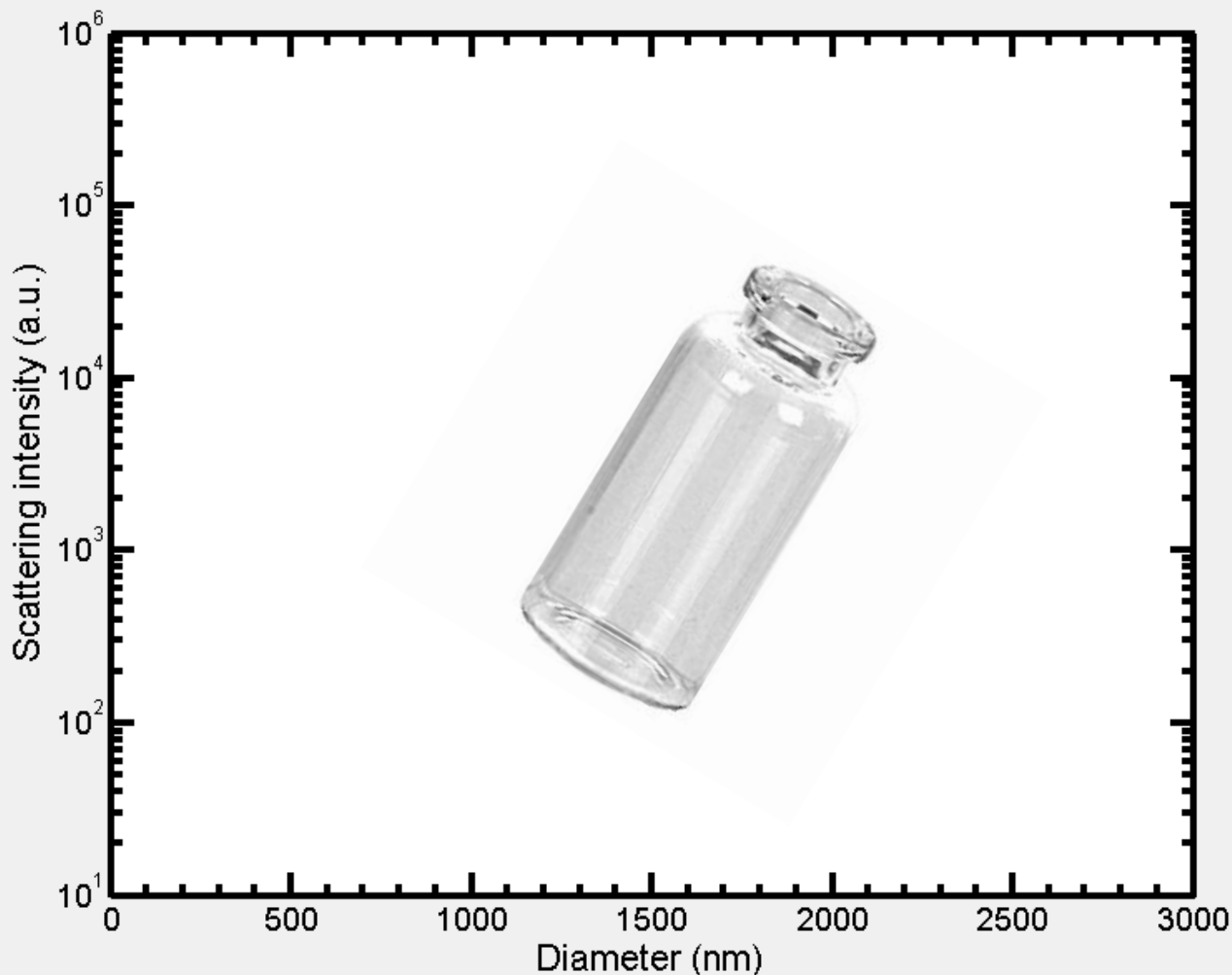
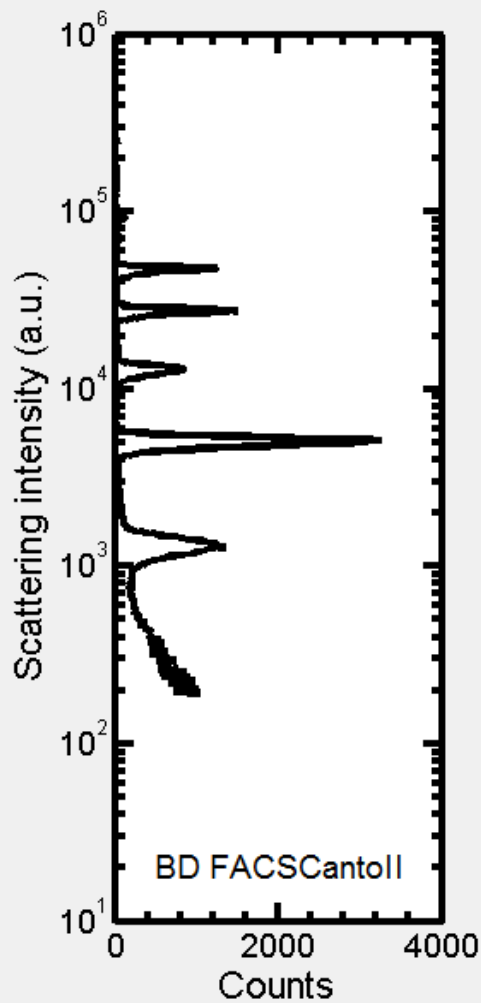
SSC (recommended)

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

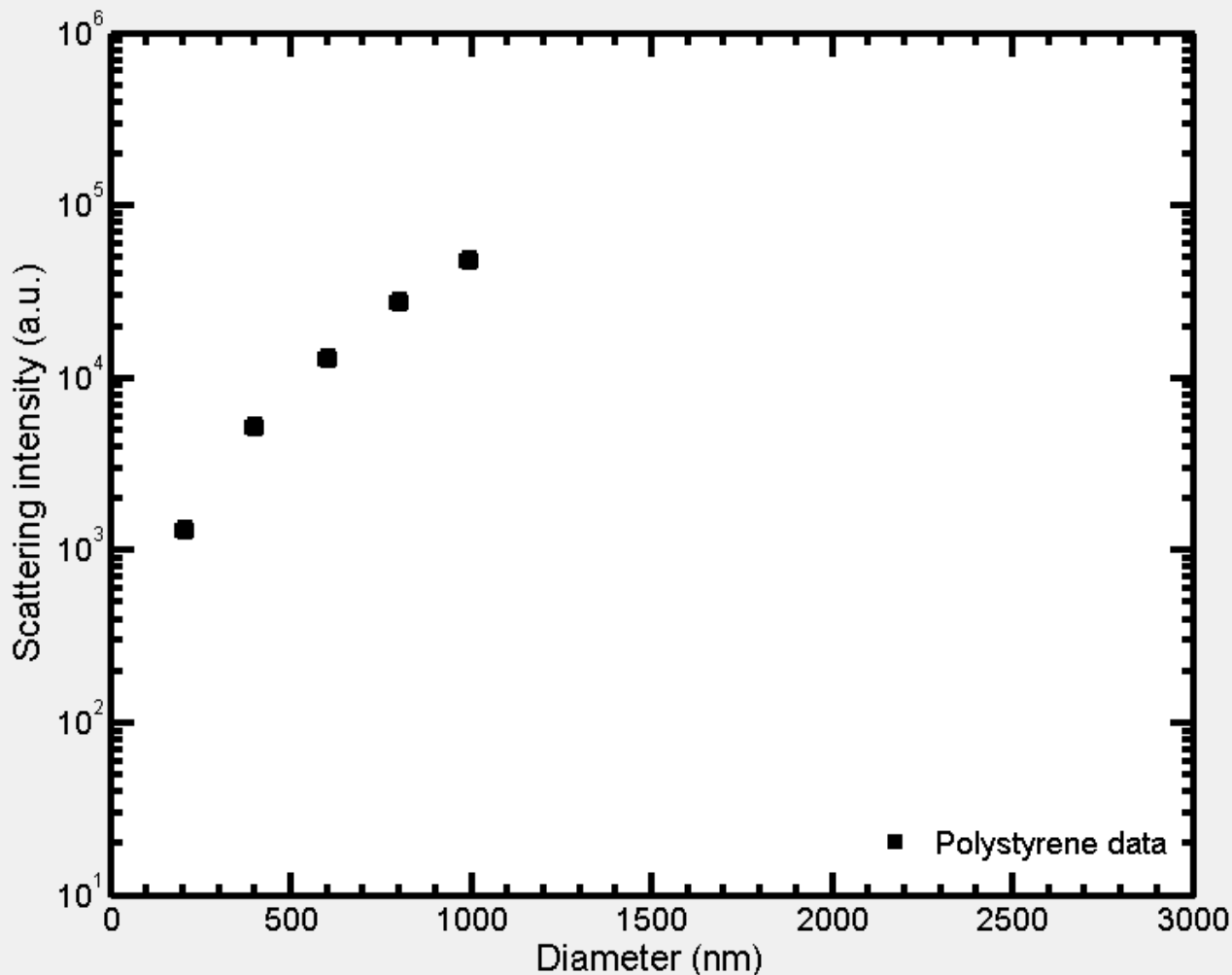
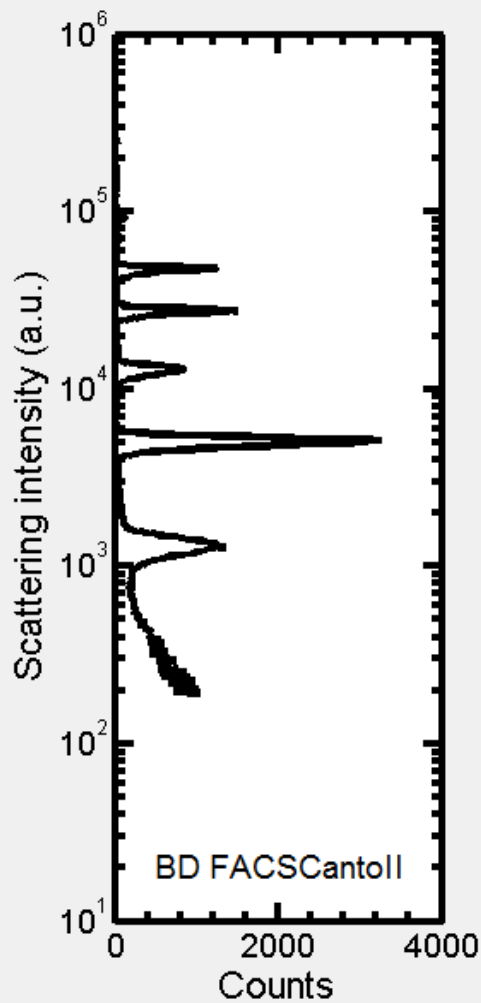
SSC (recommended)

Gate

Open "Reference beads" file

Recommended vesicle size gates

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Gate 1 {	3000		} Gate 2
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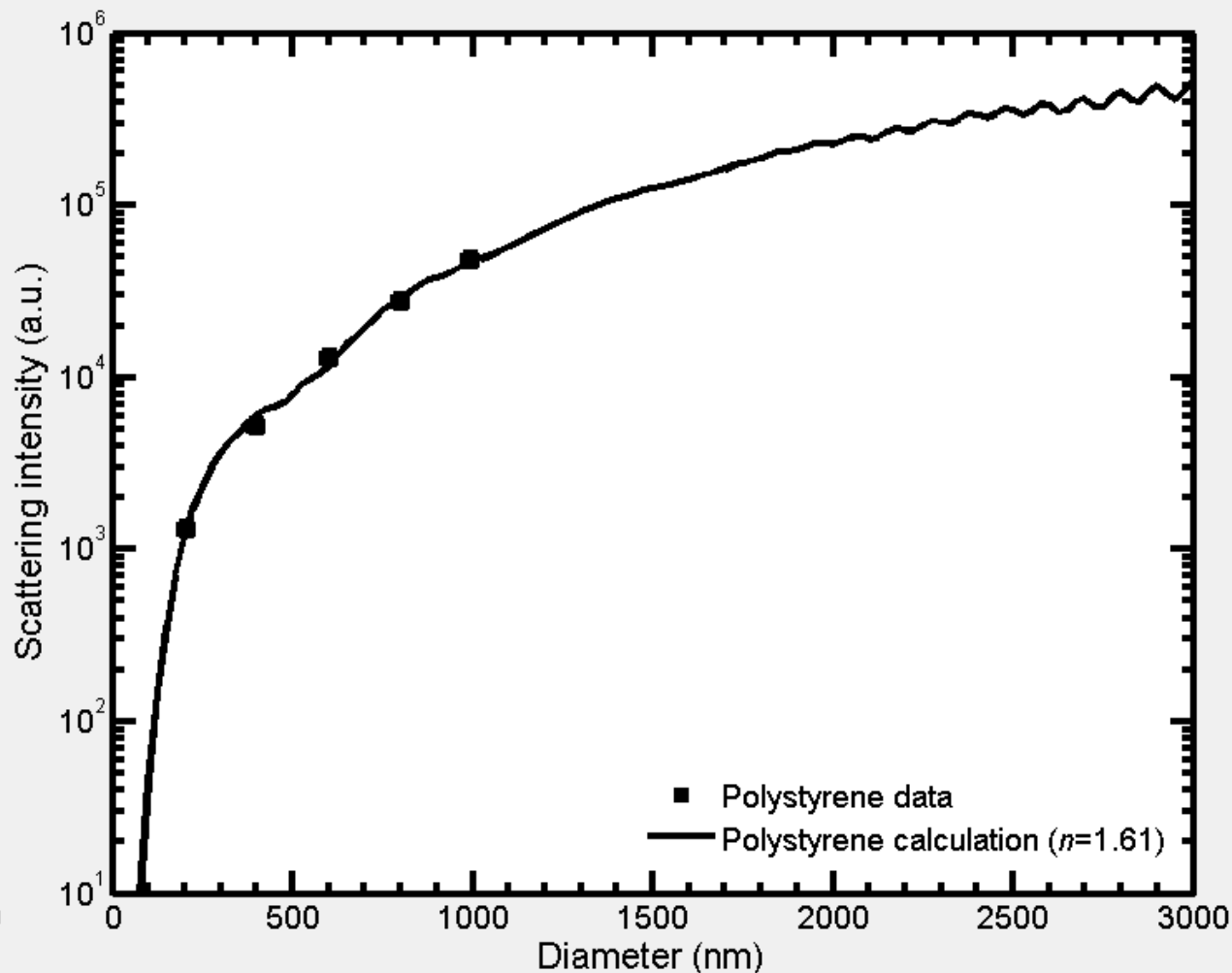
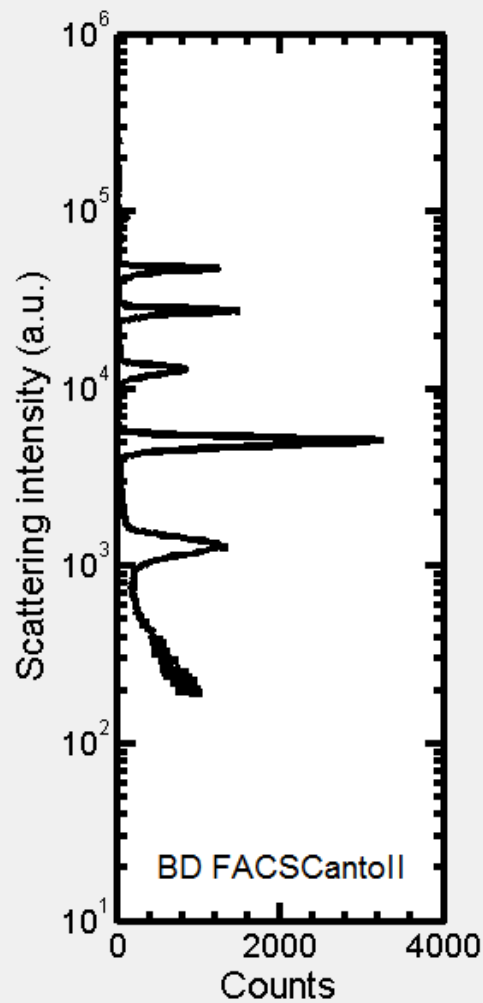
Status

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

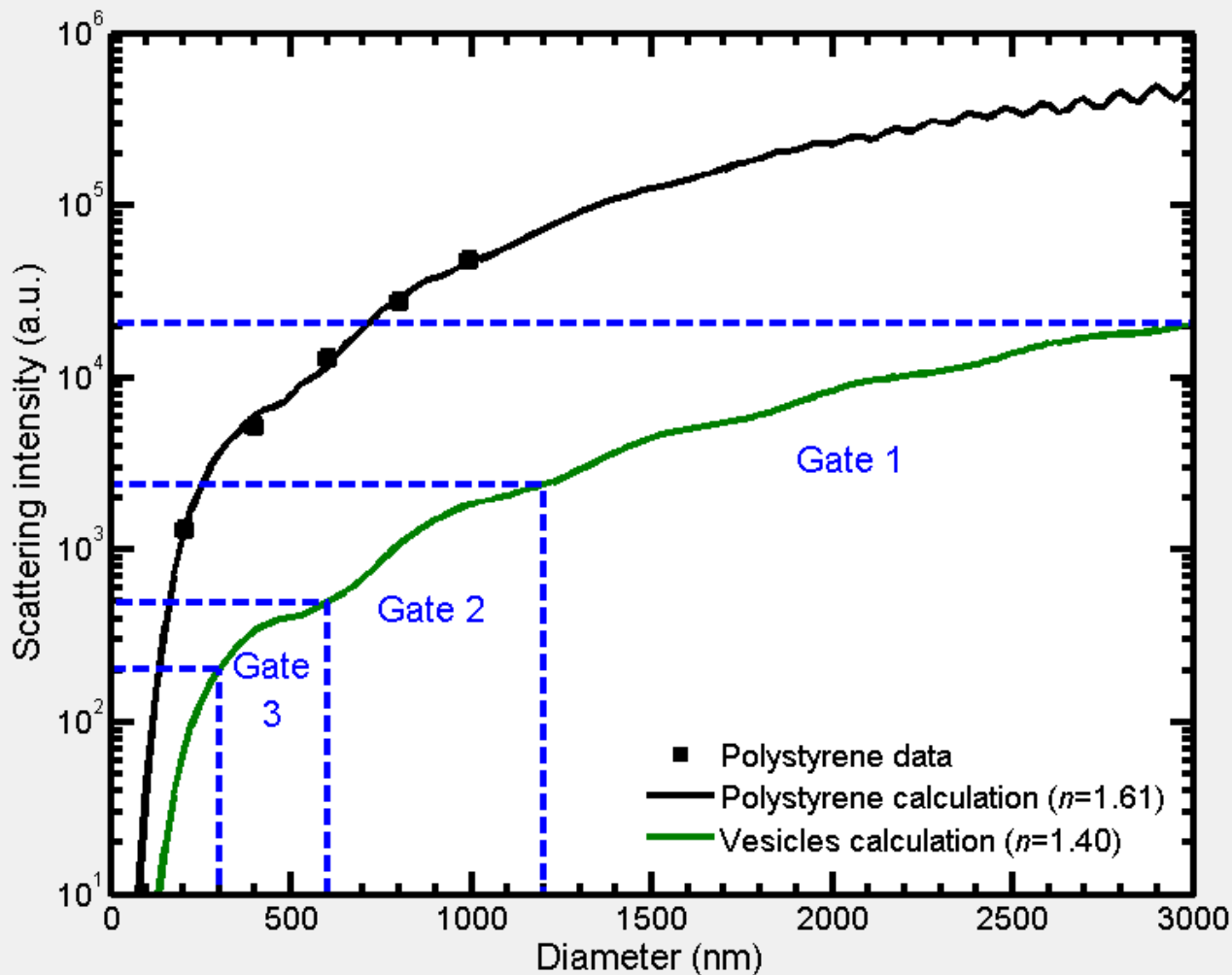
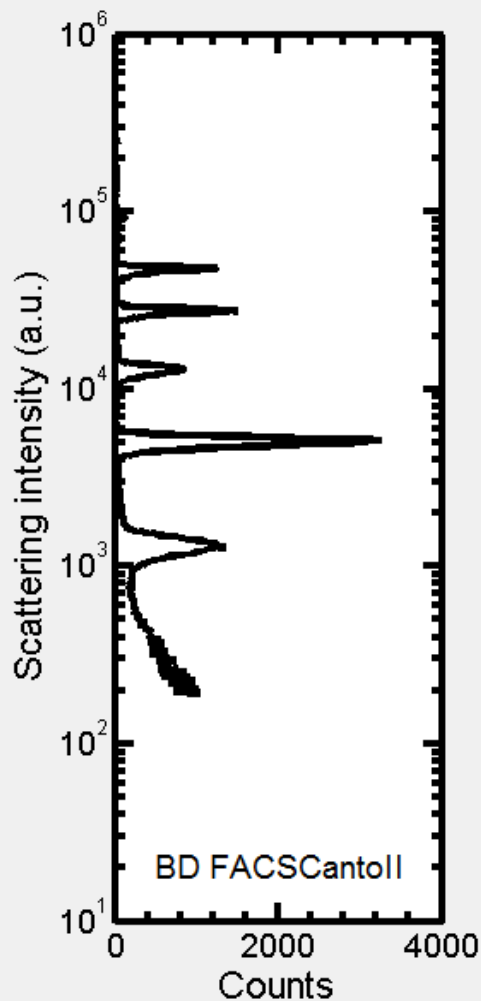
SSC (recommended)

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

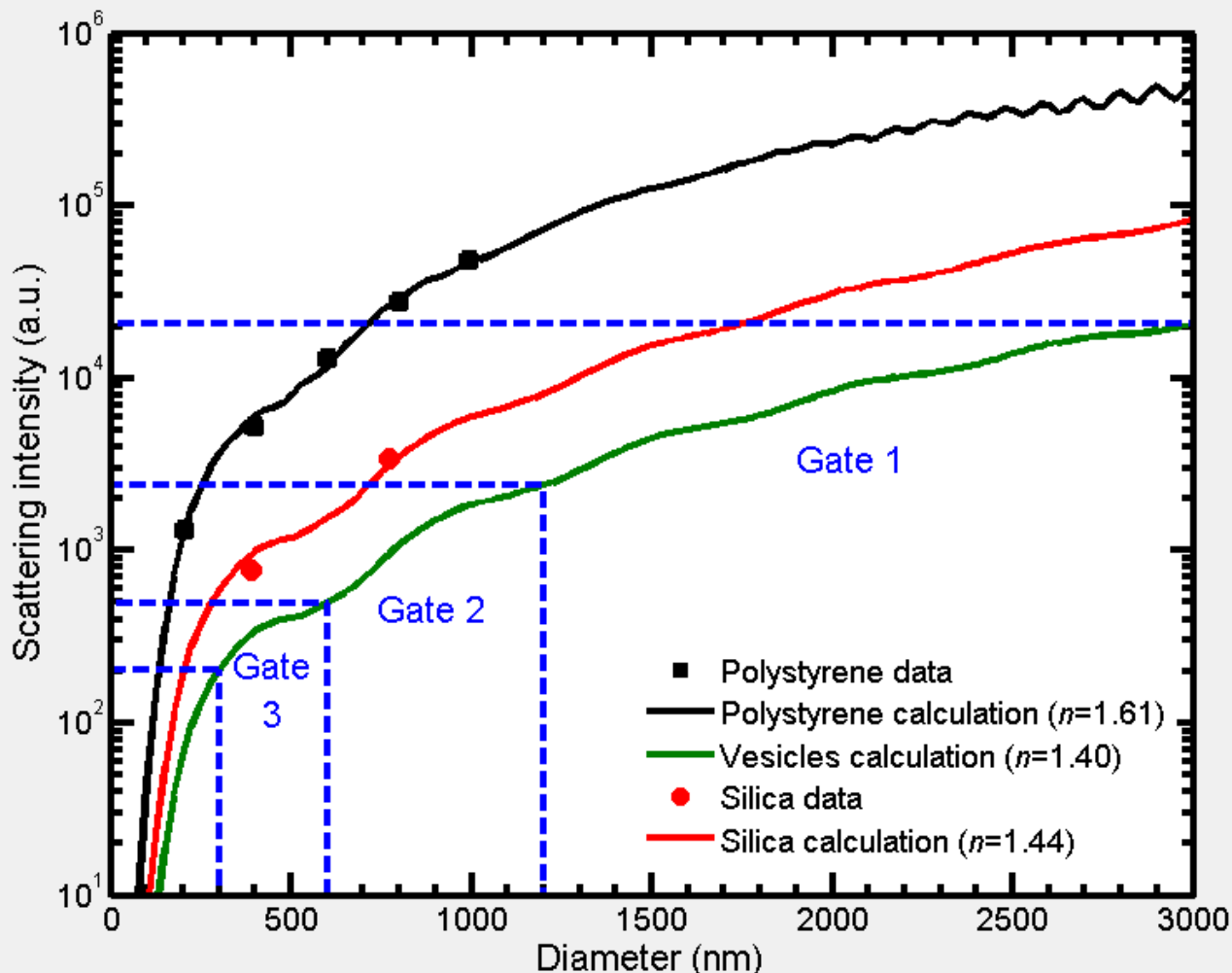
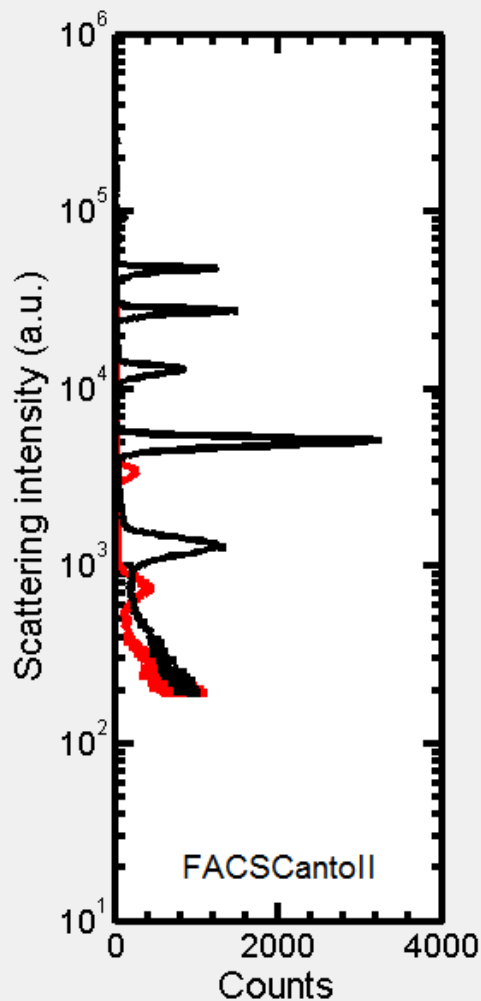
SSC (recommended)

Gate

Open "Reference beads" file

Recommended vesicle size gates

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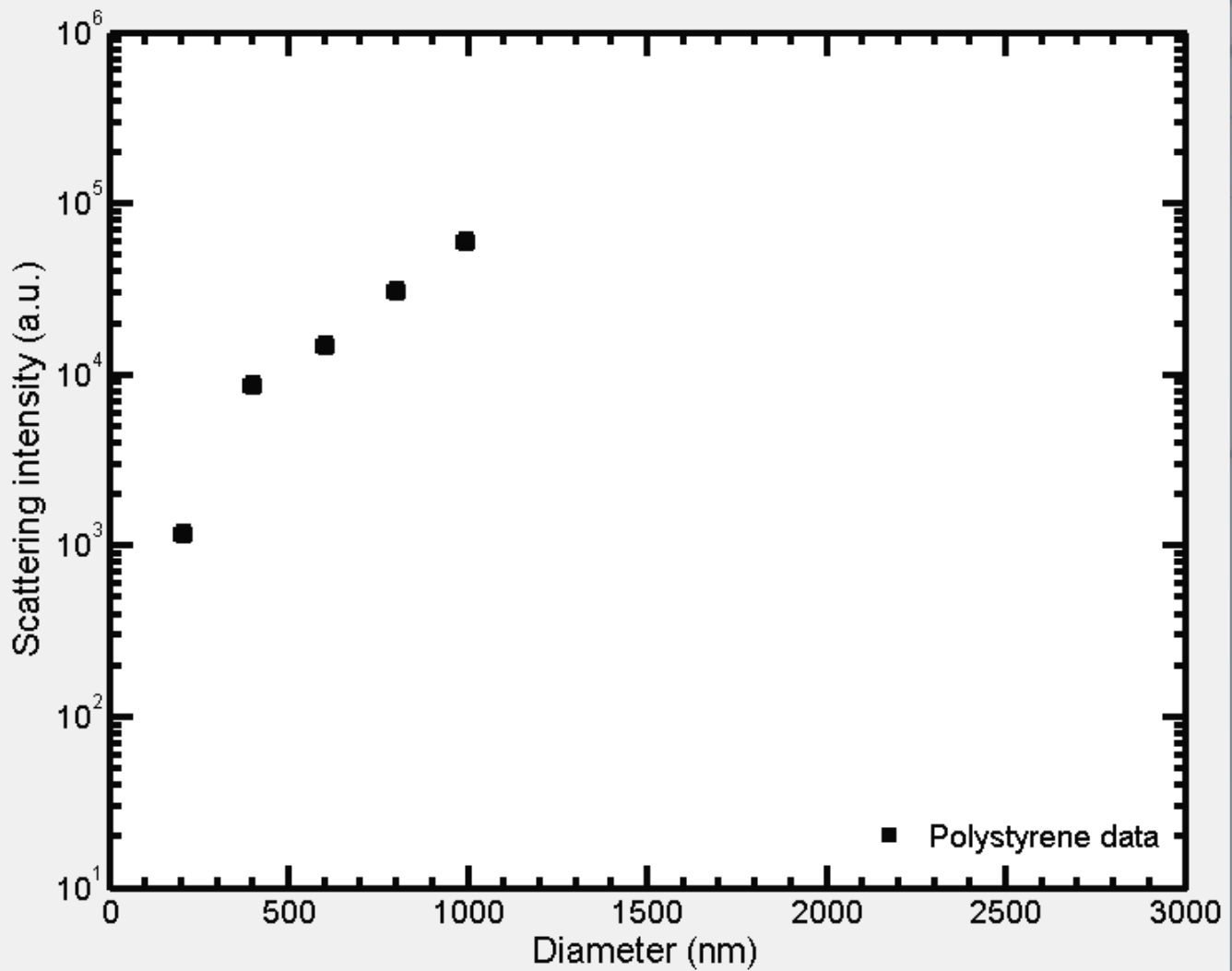
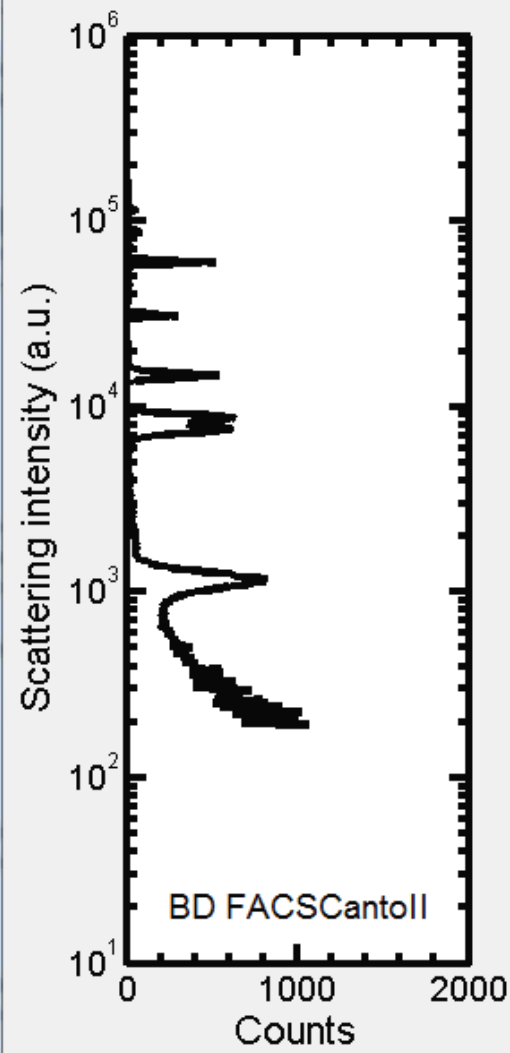


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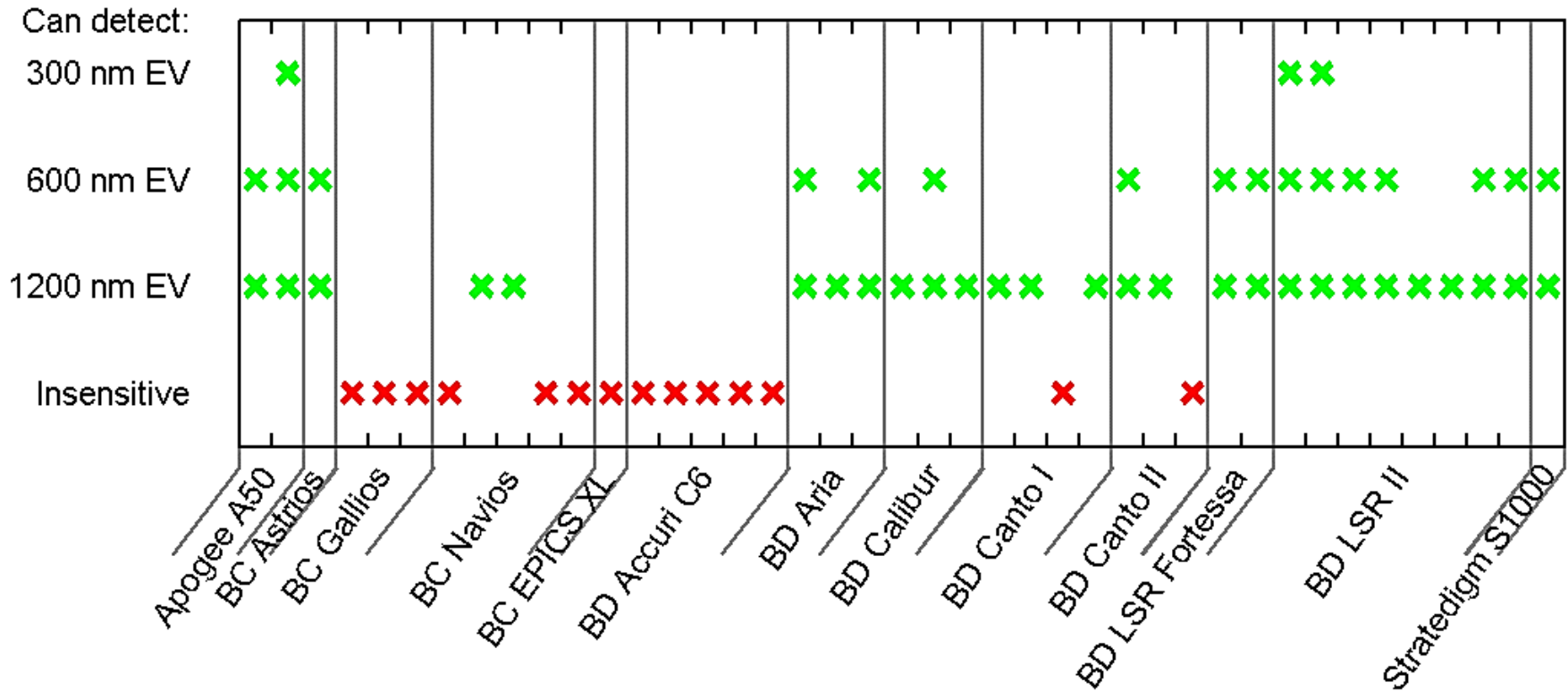
Controls

Recommended vesicle size gates

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

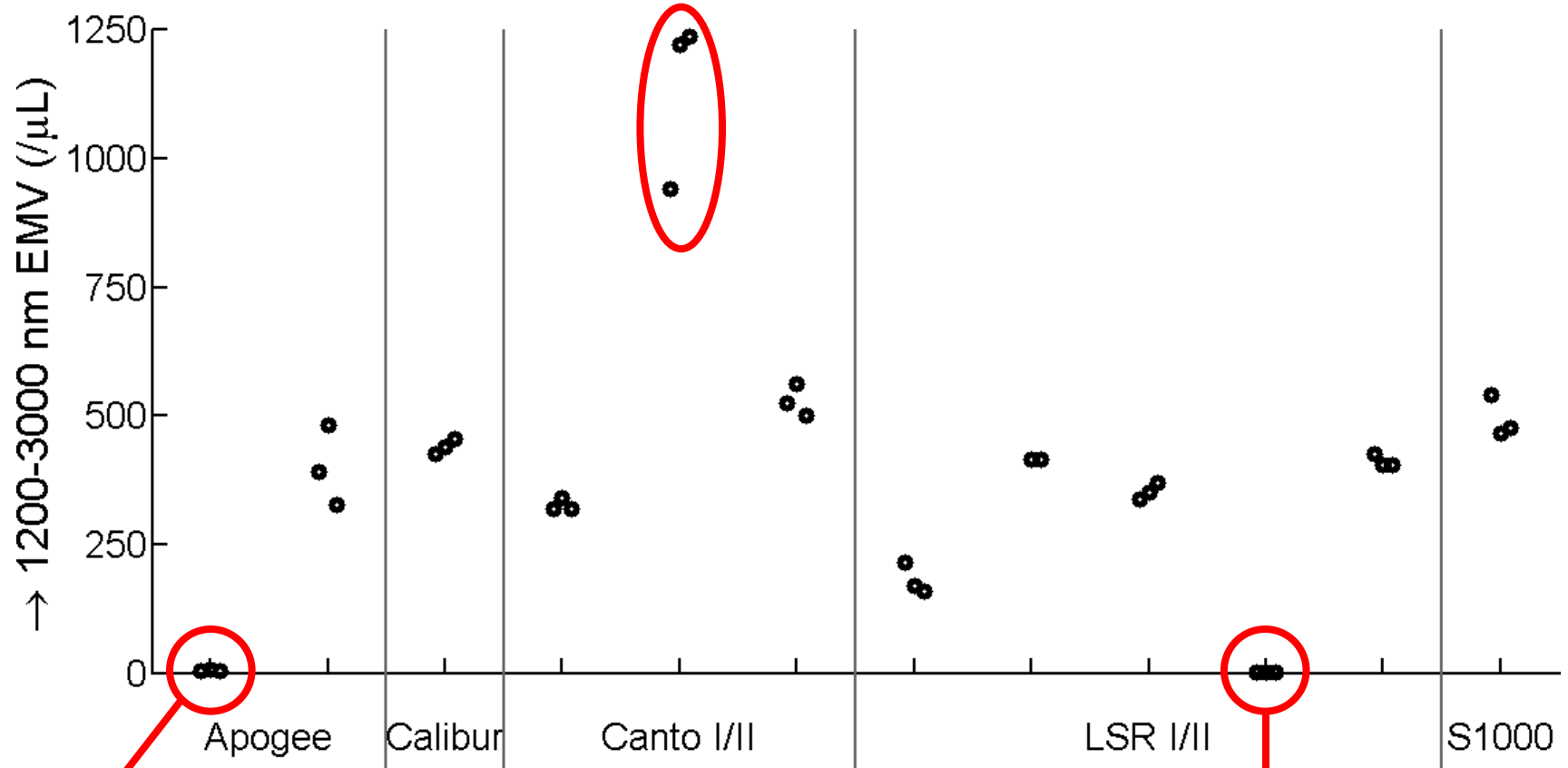


Results – sensitivity of 41 systems



Results – erythrocyte EV concentration in gate 1

error in flow rate

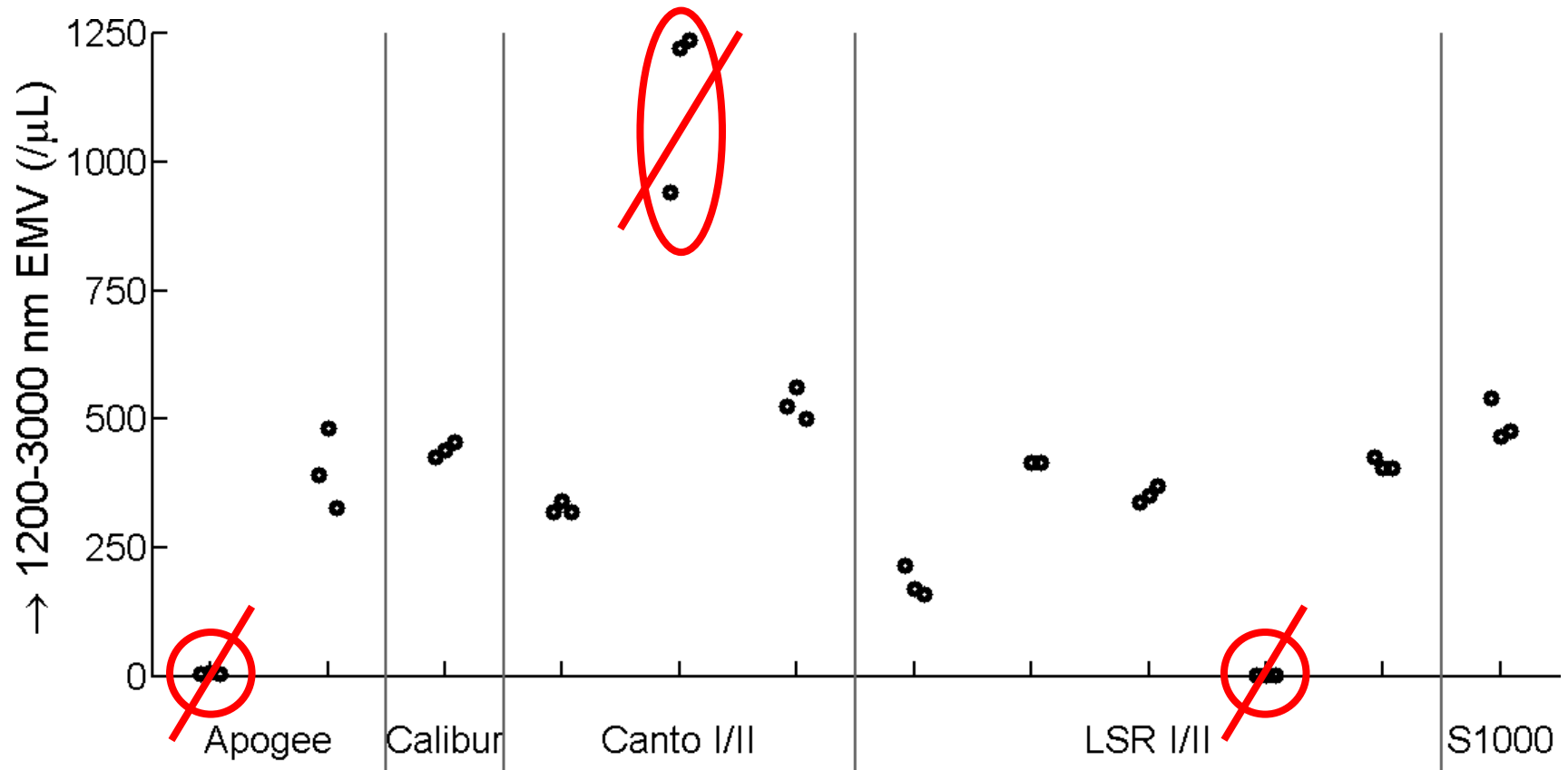


error in Mie fit

?

Results – erythrocyte EV concentration in gate 1

coefficient of variation (CV, without 3 outliers) = 26%

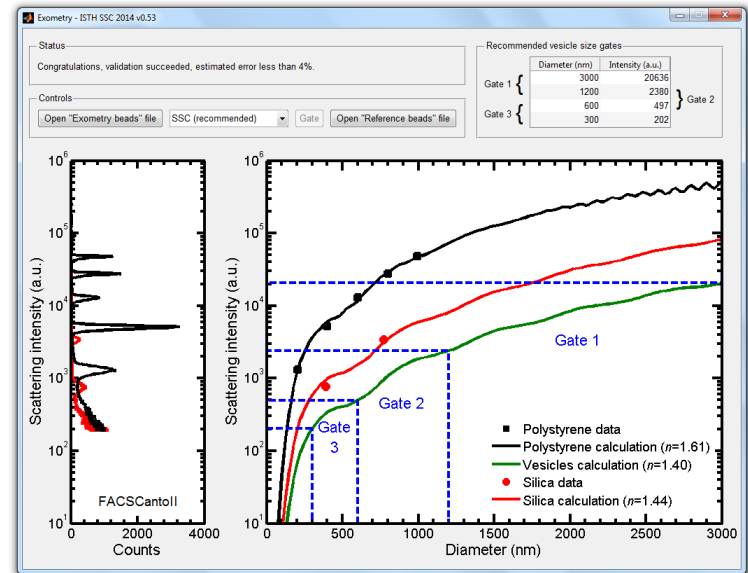


Conclusions flow cytometry standardization

- beads + software developed to set a vesicle size gate for all flow cytometers
- *preliminary* CV of **26%** on the concentration of erythrocyte vesicles of 1200 – 3000 nm
- **34%** of the systems neither detect 400 nm fluorescently labeled polystyrene beads nor vesicles

Summary

- ✔ goal
 - ✔ determine concentration of a vesicle subpopulation
- ✔ challenges
 - ✔ vesicle concentration requires minimum size
 - ✔ detect vesicles by flow cytometry
- ✔ solutions
 - ✔ vesicle refractive index < 1.40
 - ✔ standardization of flow cytometry measurements
- outlook
 - future role of metrology



Outlook

- ✓ goal *fluorescence?*
 - ✓ determine **concentration** of a vesicle subpopulation
- ✓ challenges *concentration reference materials?*
 - ✓ vesicle **concentration** requires minimum size
 - ✓ detect vesicles by flow cytometry
- ✓ solutions *(low) refractive index reference materials?*
 - ✓ vesicle **refractive index** < 1.40
 - ✓ standardization of **flow cytometry** measurements
- outlook
 - *future role of metrology*

Acknowledgements

- Academic Medical Center
 - Biomedical Engineering & Physics
 - Ton van Leeuwen
 - Frank Coumans
 - Laboratory Experimental Clinical Chemistry
 - Rienk Nieuwland
 - Guus Sturk
- Exometry
- European Association of National Metrology Institutes (EURAMET)
 - The European Metrology Research Programme (EMRP) is jointly funded by the EMRP participating countries within EURAMET and the European Union
- International Society on Thrombosis and Haemostasis

Software: exometry.com
Info: edwinvanderpol.com

