Quantitative light scattering of extracellular vesicles for flow cytometry standardization

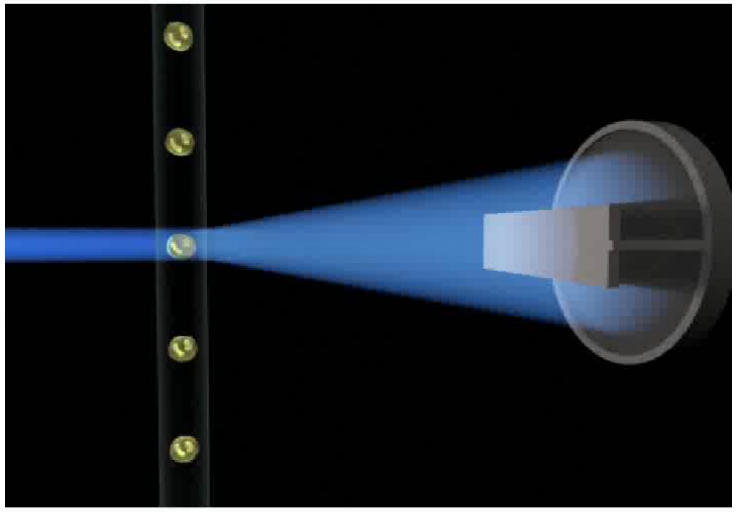
Edwin van der Pol[1,2]

Rienk Nieuwland[2], Auguste Sturk[2],
Ton van Leeuwen[1], and Frank Coumans[1,2]

April 23rd, 2015

[1]Biomedical Engineering and Physics; [2]Laboratory Experimental Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands;

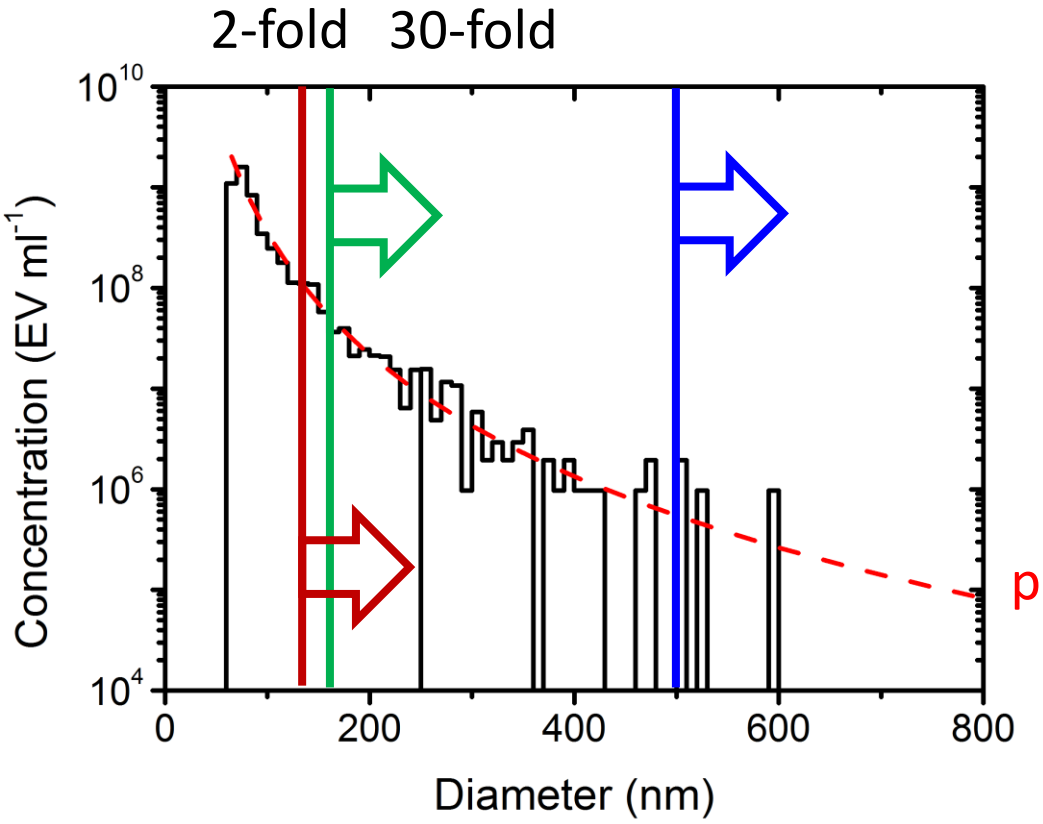
Introduction



- reported concentrations of EV in plasma differ >10^6-fold*
- standardization required
- most widely used method is flow cytometry

*van der Pol et al. JTH (2014)

Problem 1: instruments differ in sensitivity



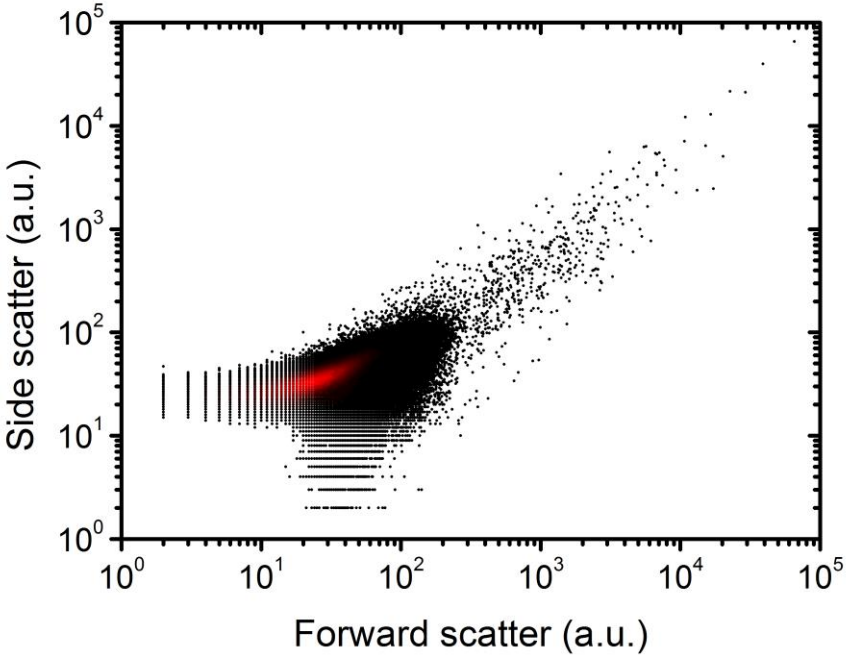
power-law relation*

- primary cause: instruments differ in sensitivity

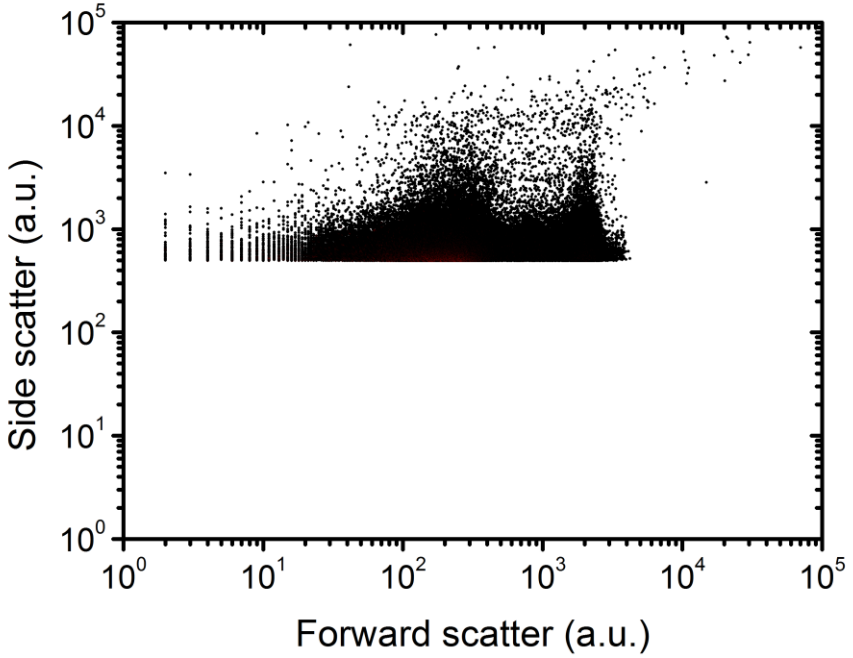
*van der Pol et al. JTH (2014)

Problem 2: arbitrary units

same population of erythrocyte EV



Apogee A50-micro



Becton Dickinson FACSCanto II

Goal

- reproducible concentration of
  - erythrocyte EV
    - ~~lactadherin-FITC~~, CD235a-PE labeled
  - platelet EV
    - lactadherin-FITC, CD61-PE labeled

  using different flow cytometers

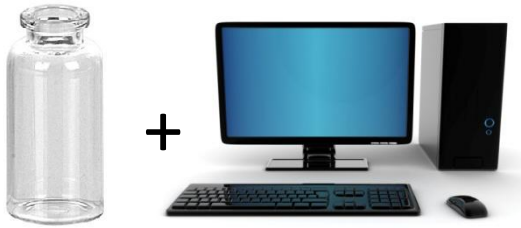





Study comprises 30 sites (50 instruments) worldwide

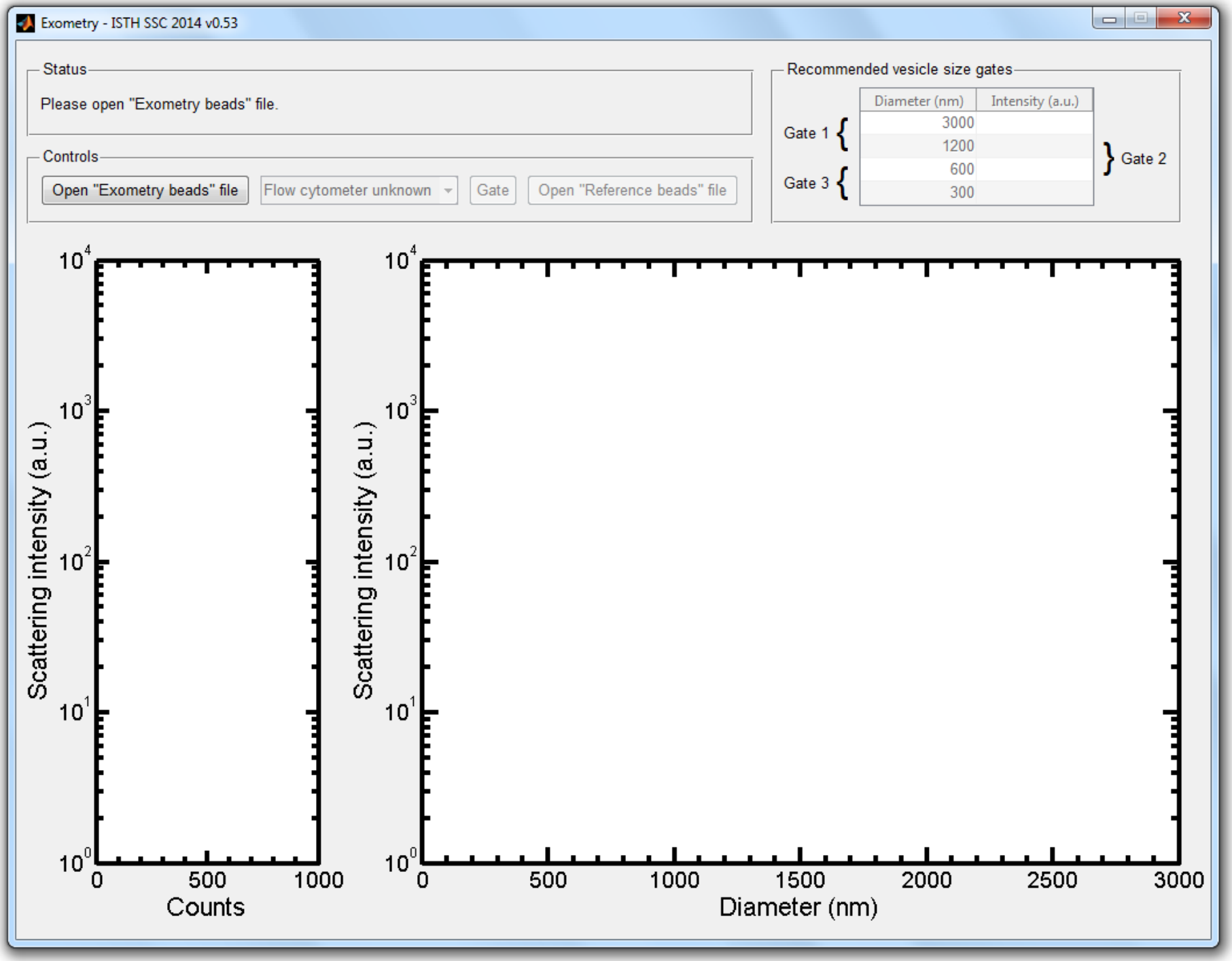
Approach

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



- measure EV samples and controls

- derive EV concentrations


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

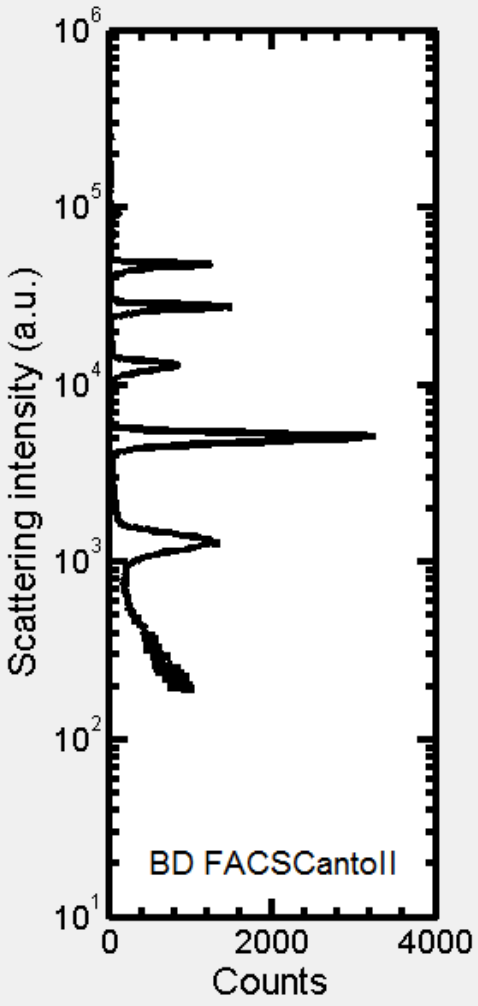
Please select detector and click "Gate" to obtain vesicle size gates.

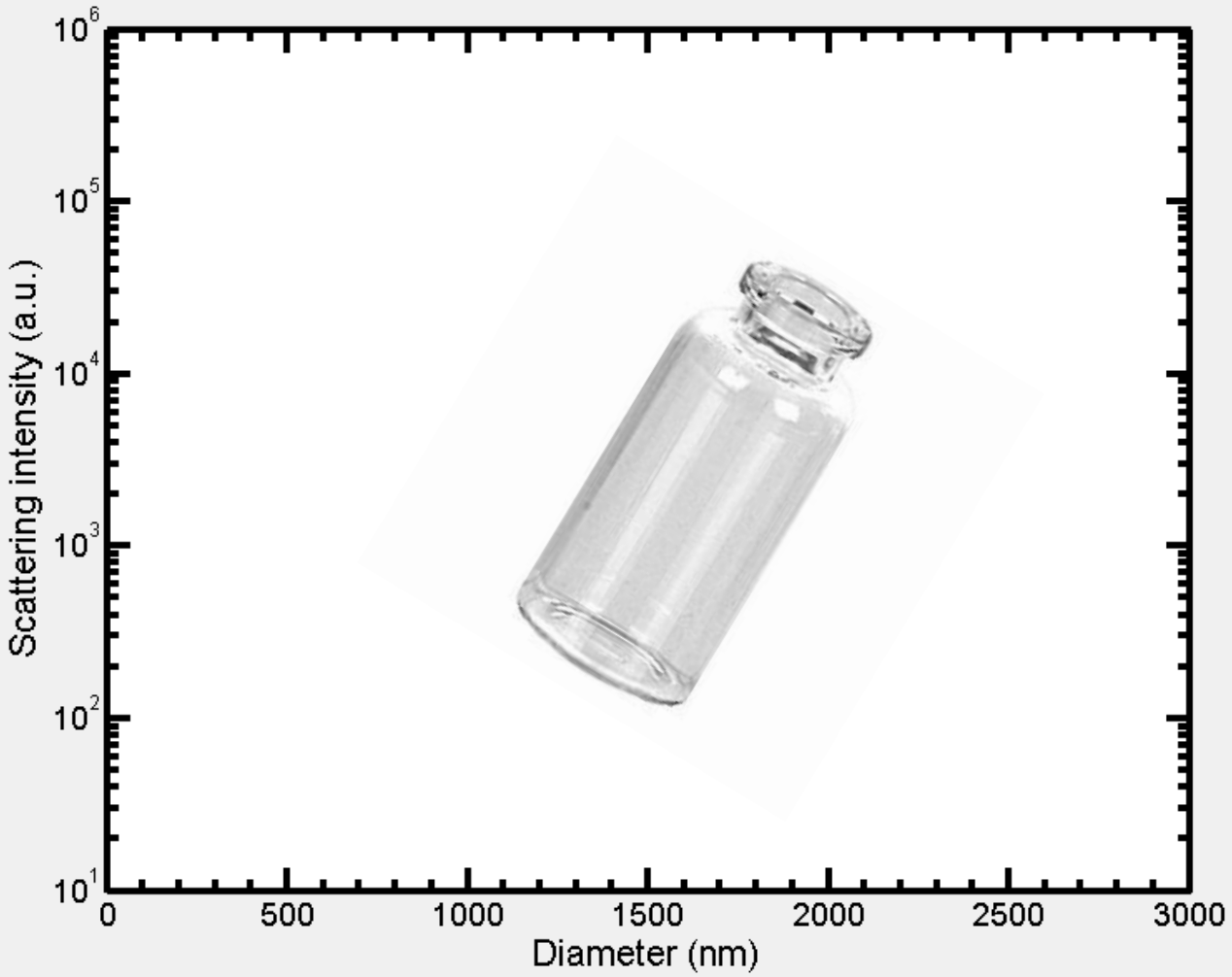
Controls

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.

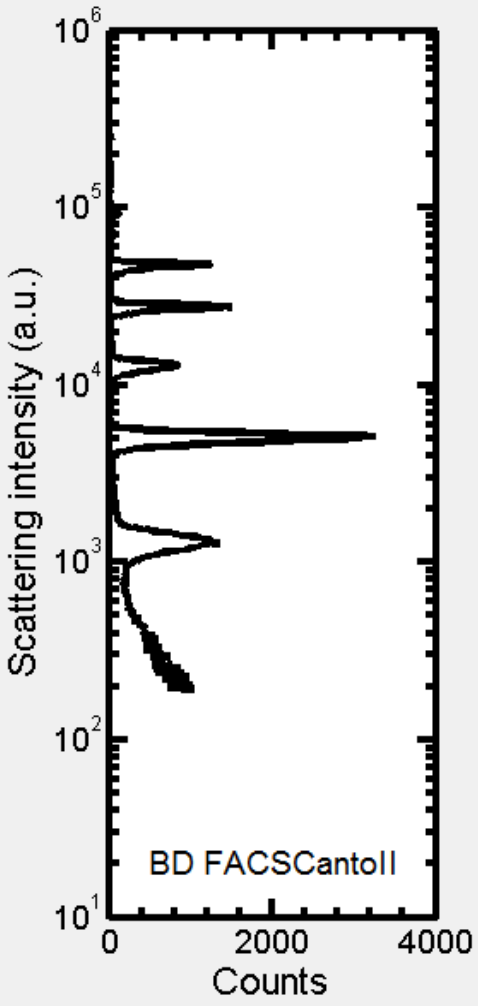
Controls

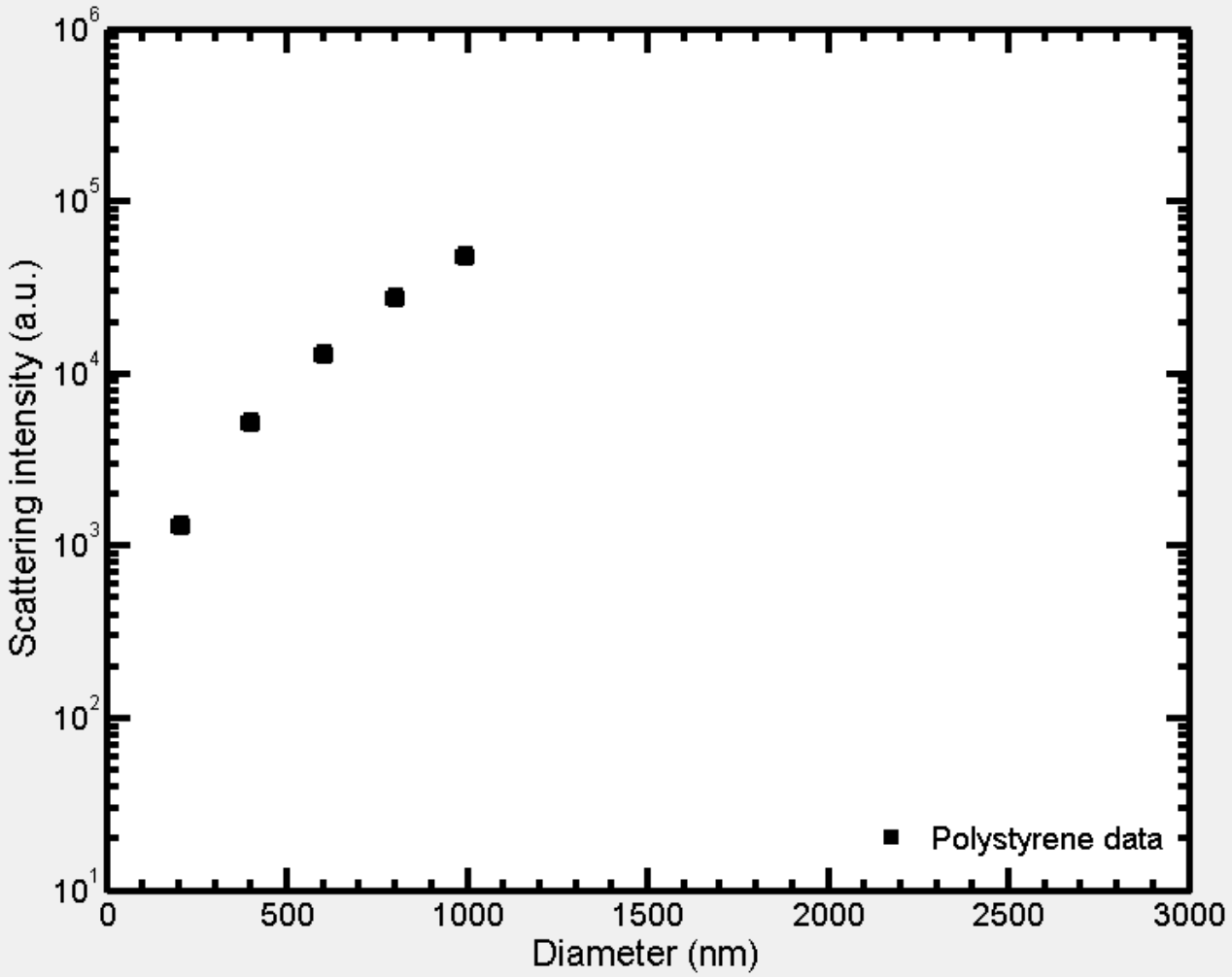
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

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

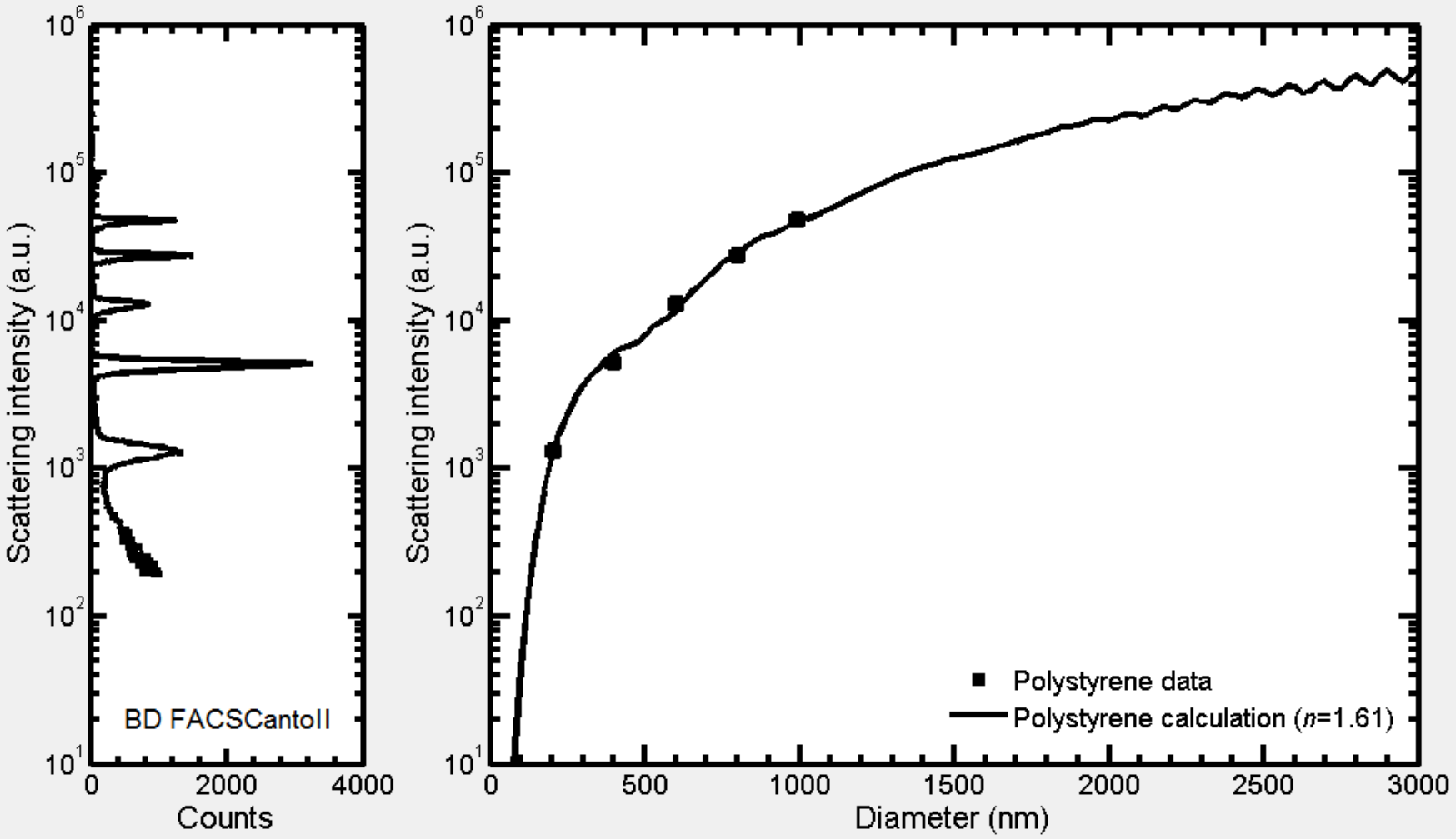
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.

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

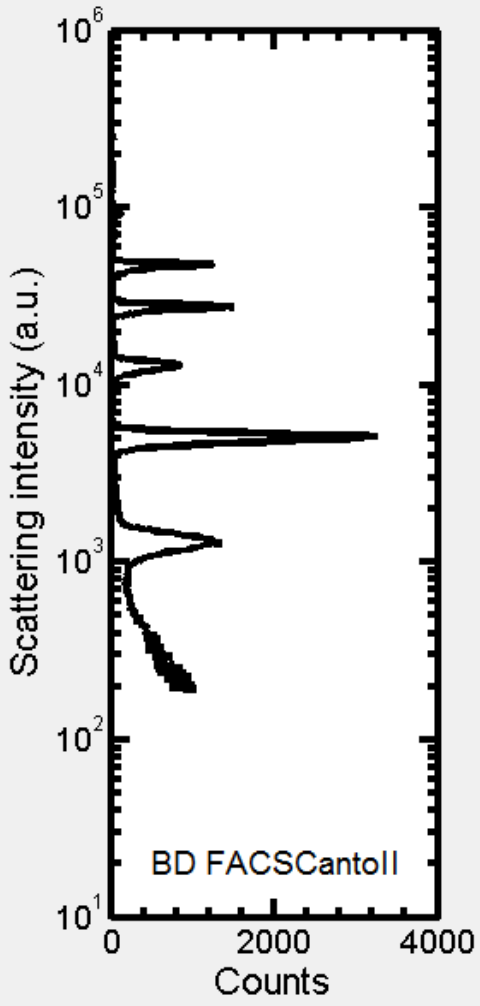
Congratulations, vesicle size gates determined, estimated error less than 0%.

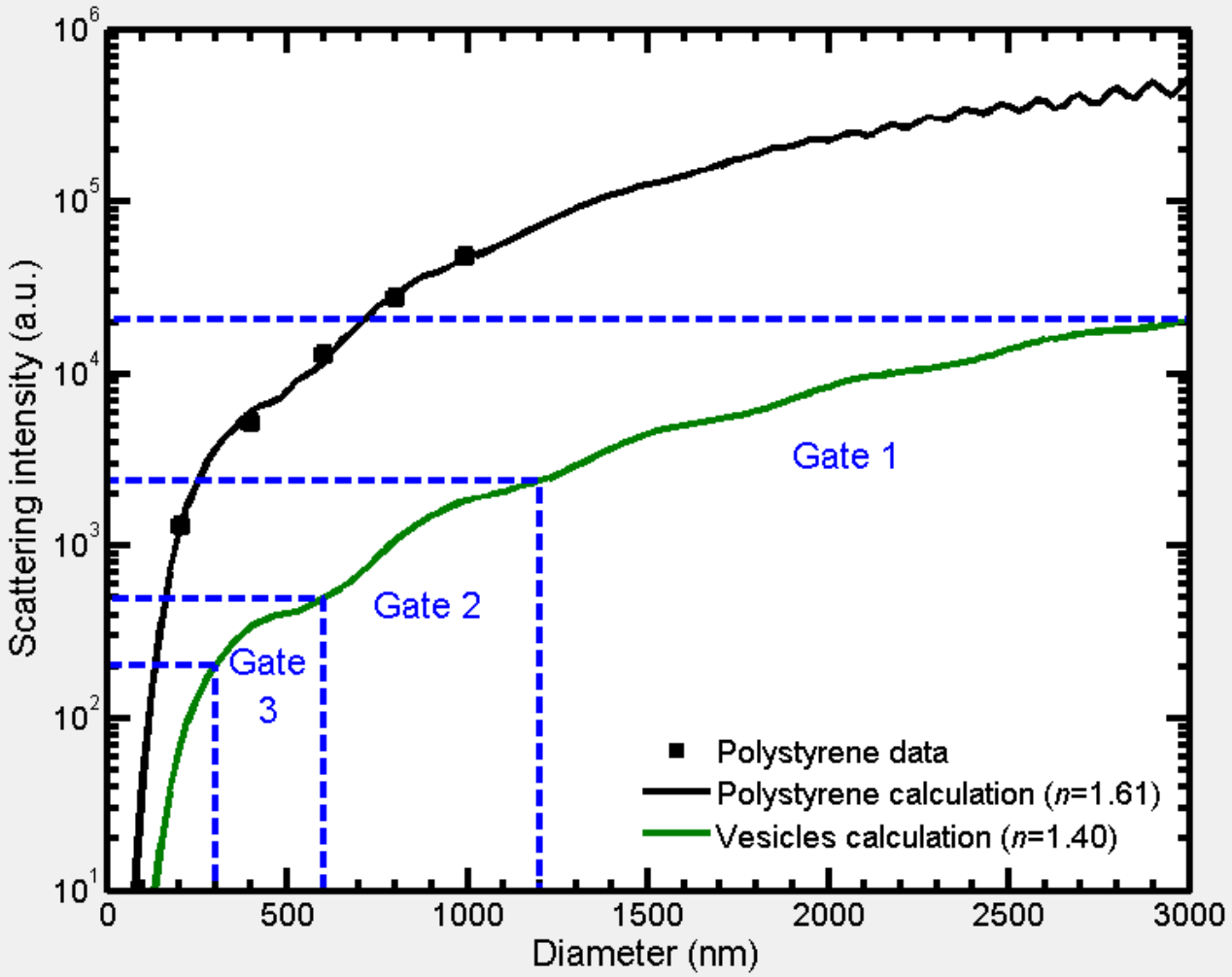
Controls

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

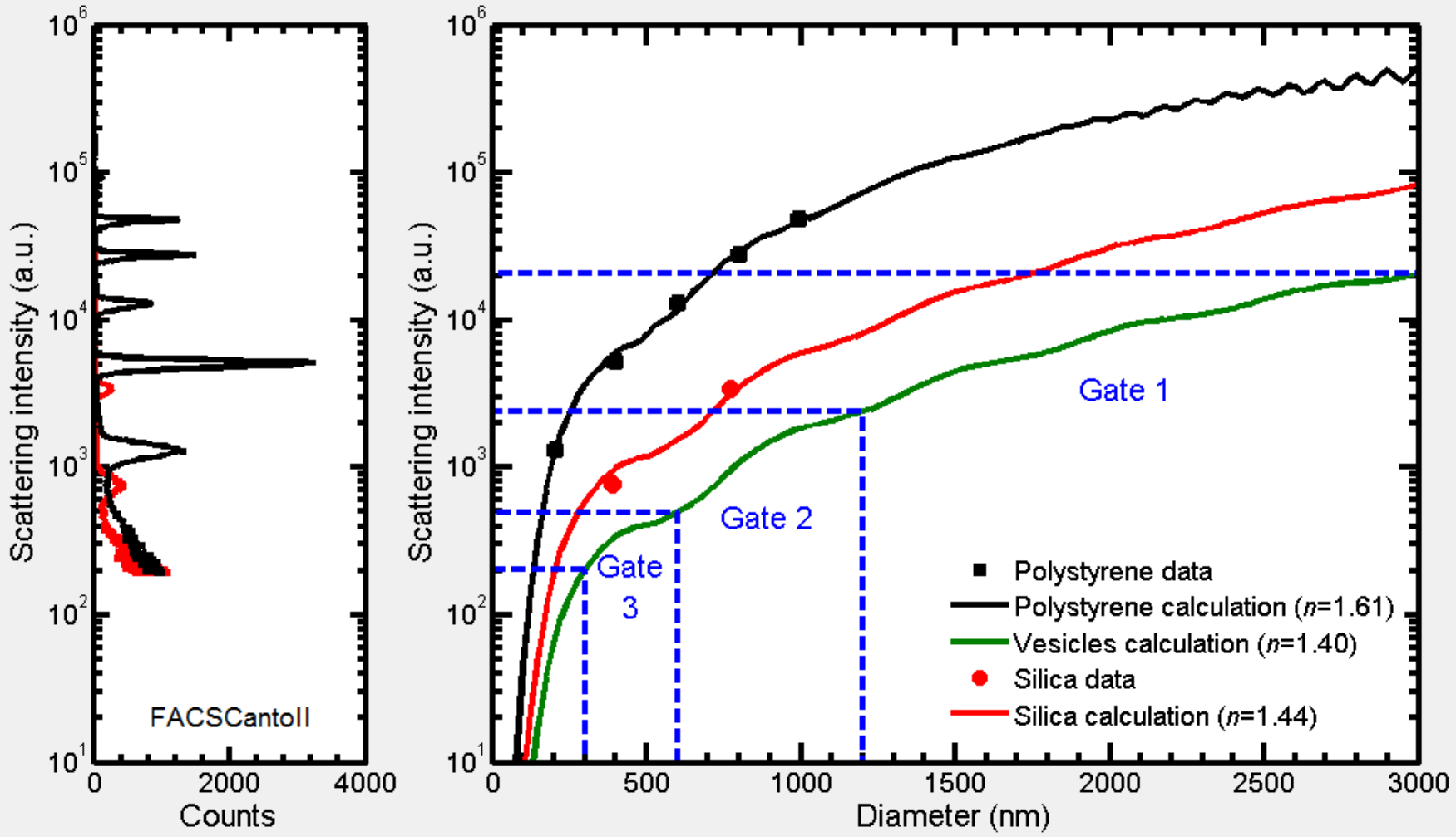
Congratulations, validation succeeded, estimated error less than 4%.

Controls

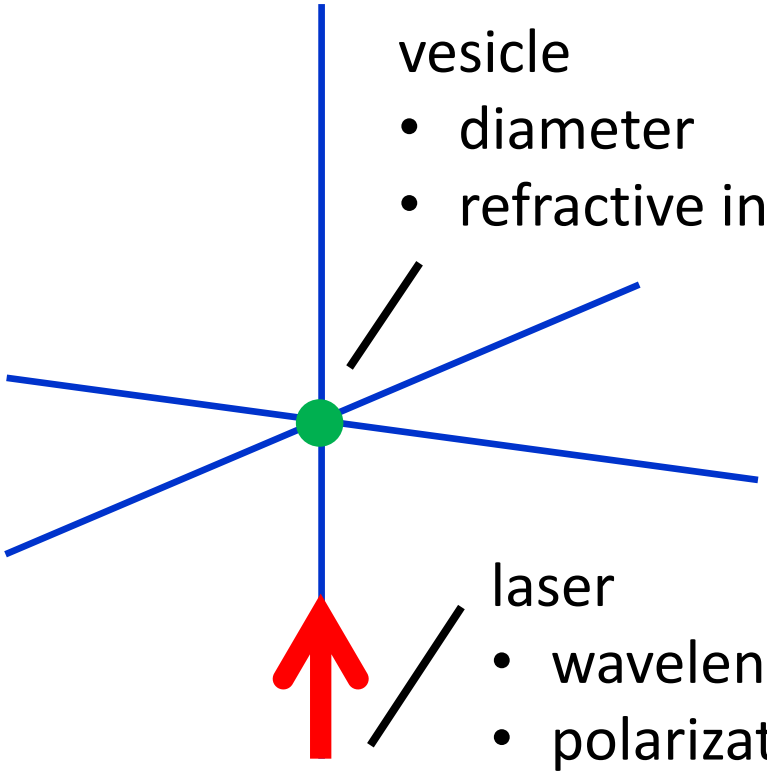
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

Recommended vesicle size gates

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



What does the software do?

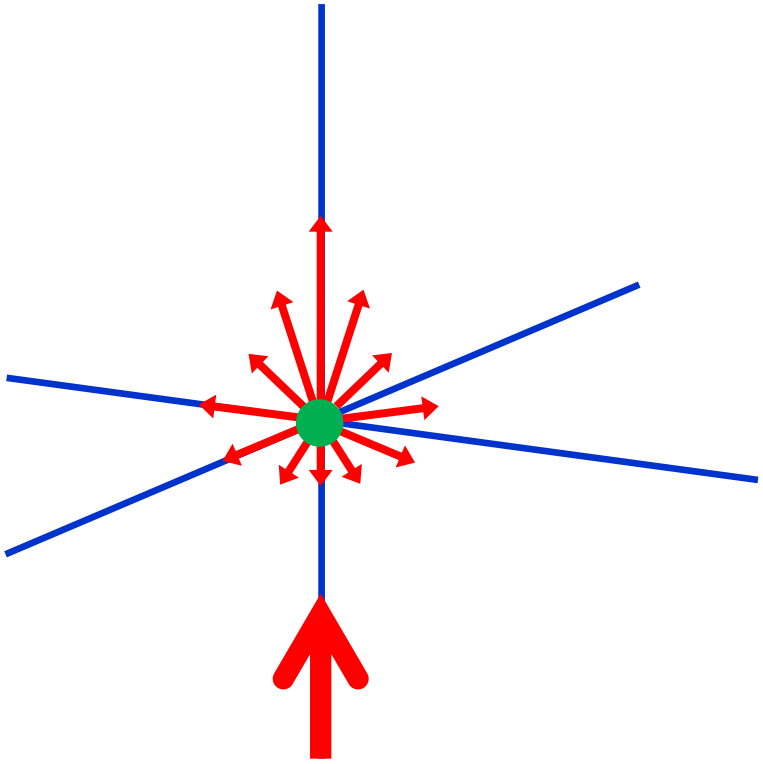


vesicle
- diameter
- refractive index

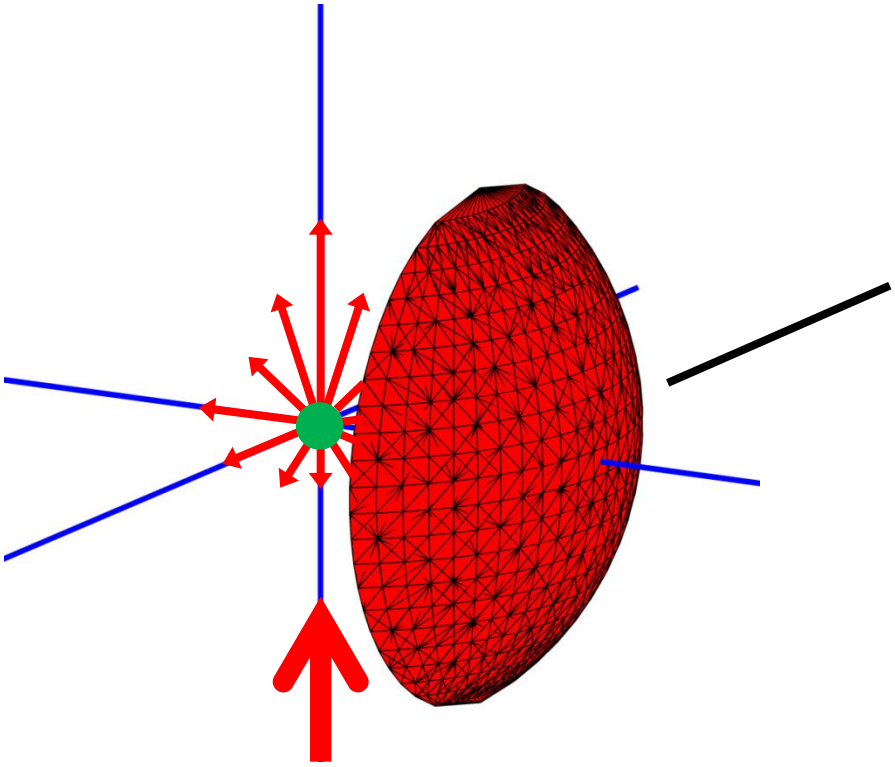
laser
- wavelength
- polarization
- power

Facts
- Mie theory in MATLAB
- 866 lines of code
- 1,410 comment lines
- 342 times used

What does the software do?



What does the software do?

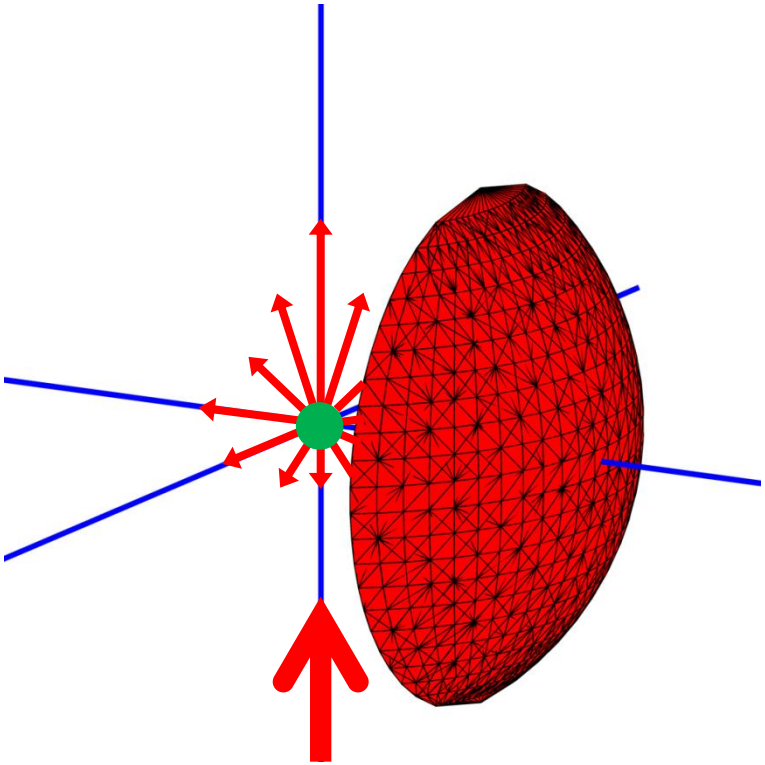


optical configuration

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

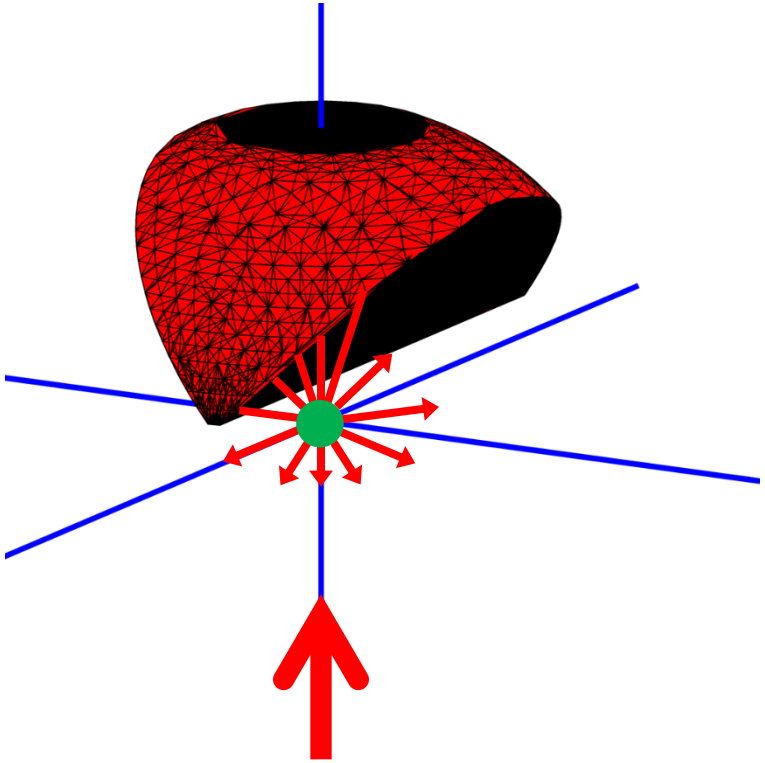
What does the software do?

Becton Dickinson FACSCalibur



Side scatter

Apogee A50-micro



Forward scatter

Exometry - ISTH SSC 2014 v0.53

Status

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

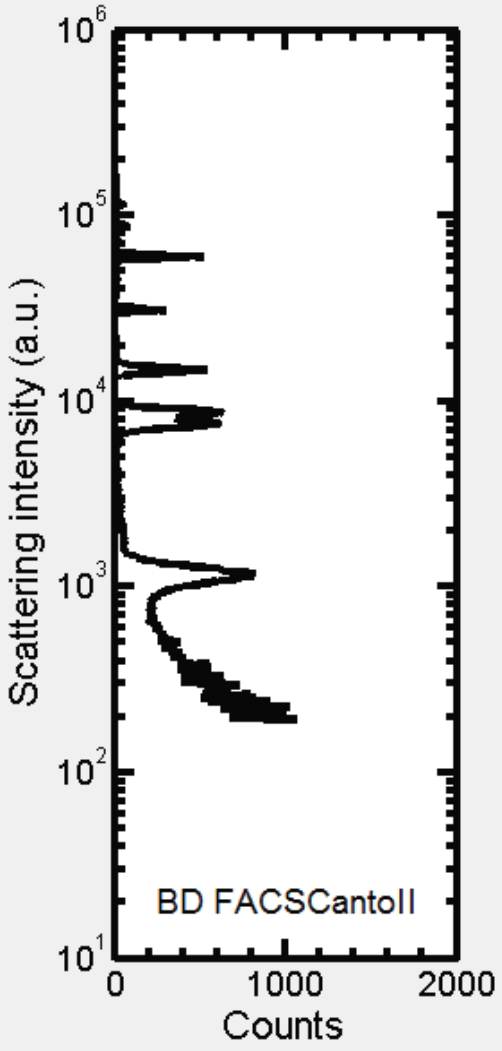
Controls

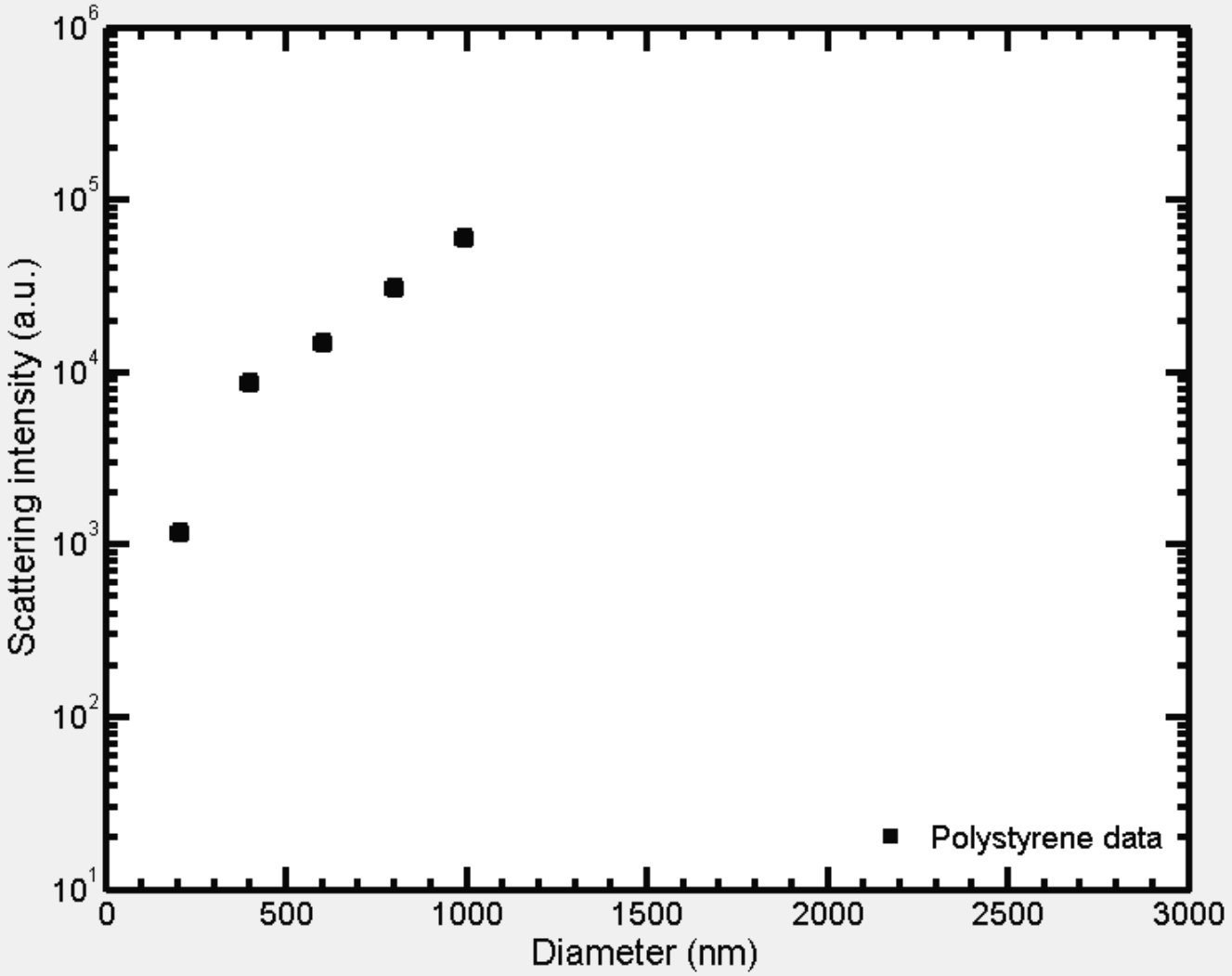
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

Recommended vesicle size gates

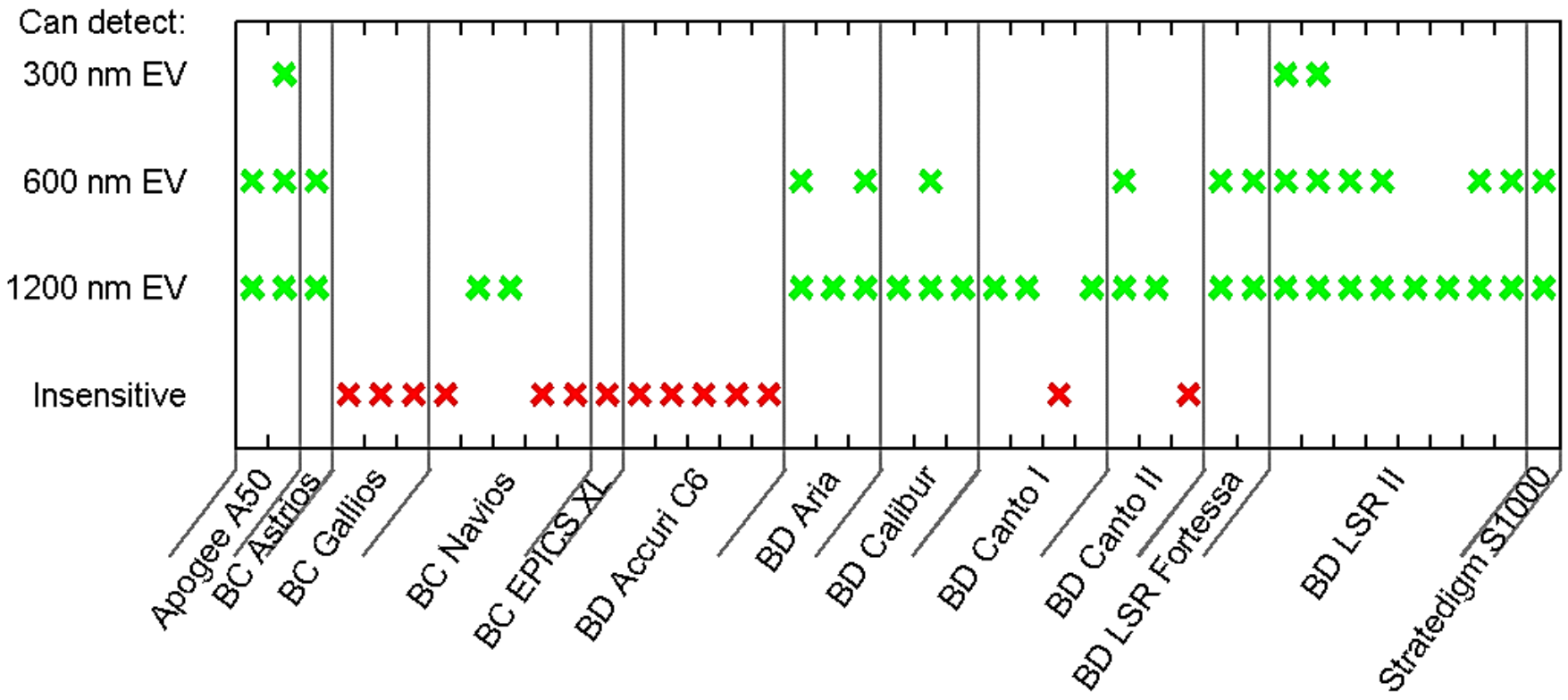
	Diameter (nm)	Intensity (a.u.)
Gate 1	3000	
	1200	
Gate 3	600	
	300	

Gate 2

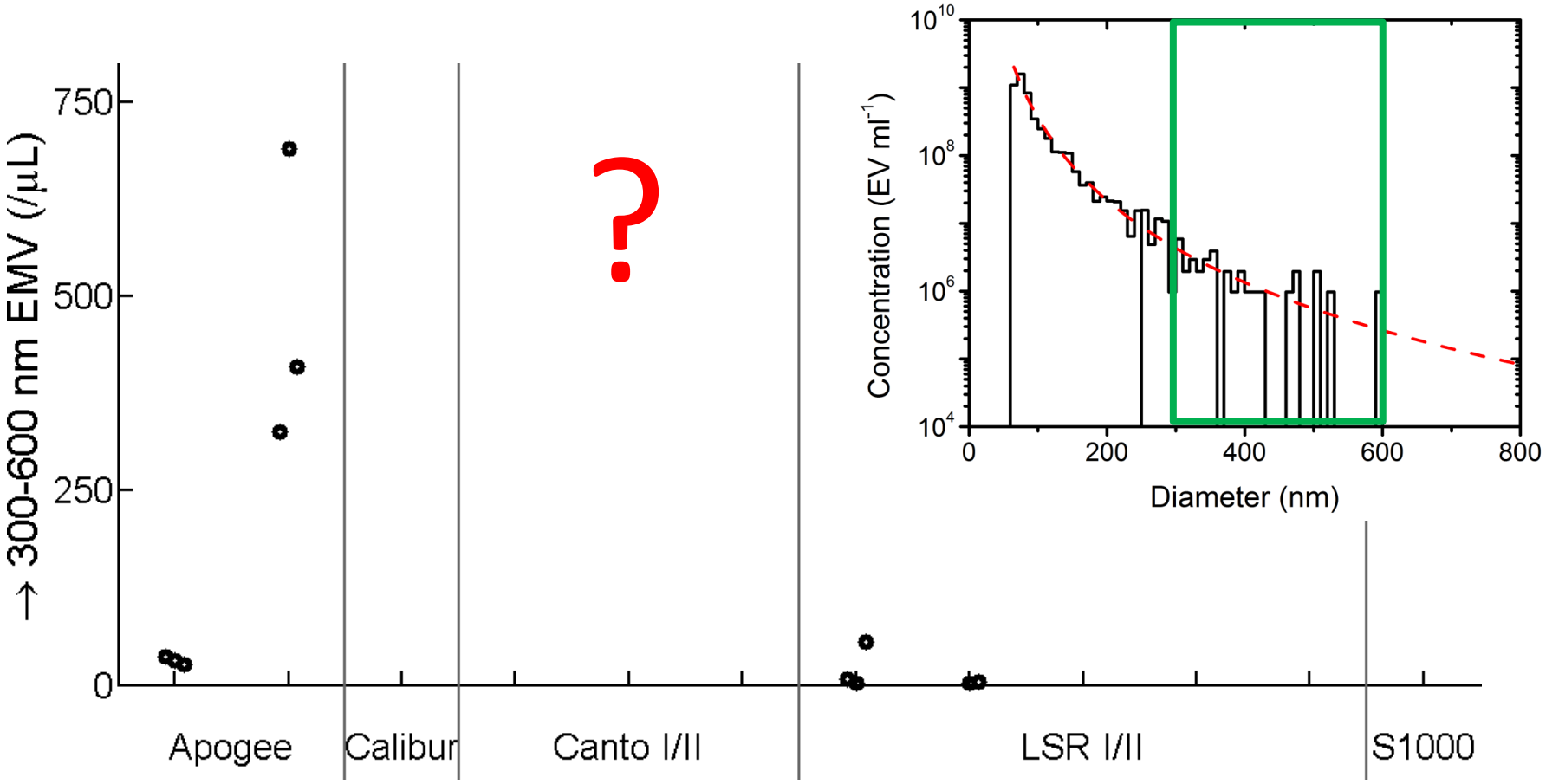




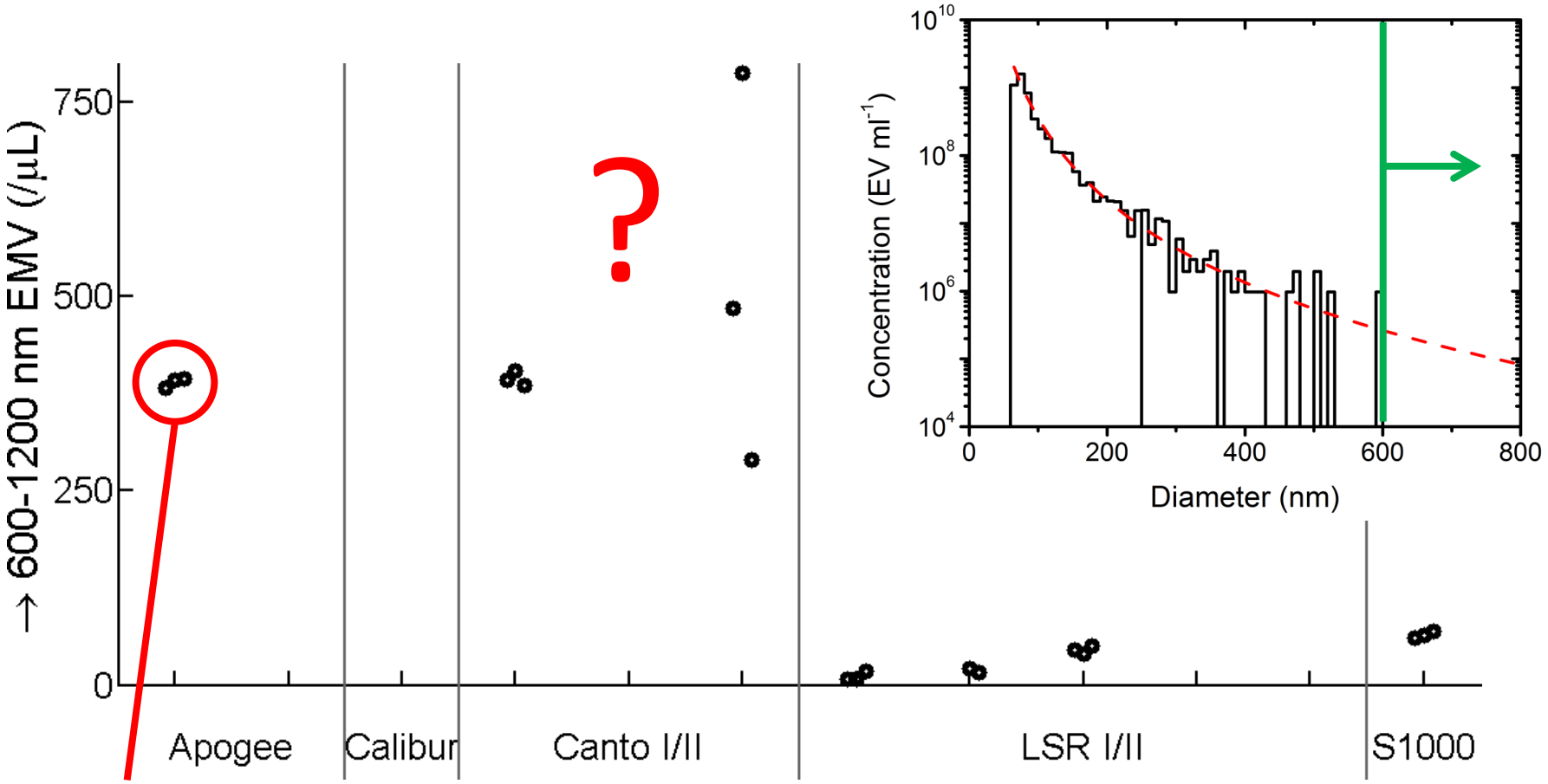
Results – sensitivity of 41 systems



Results – erythrocyte EV concentration in gate 3

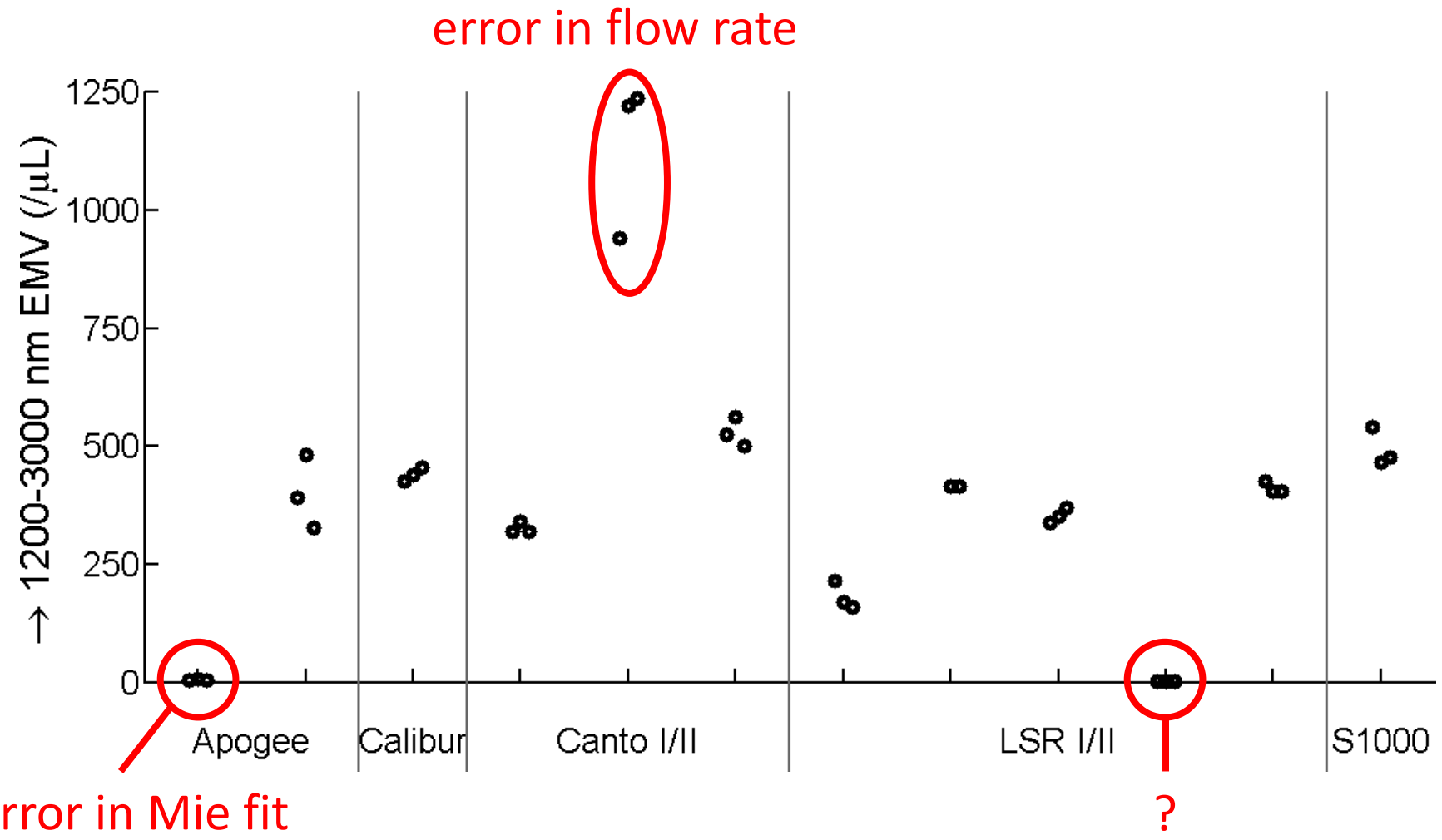


Results – erythrocyte EV concentration in gate 2



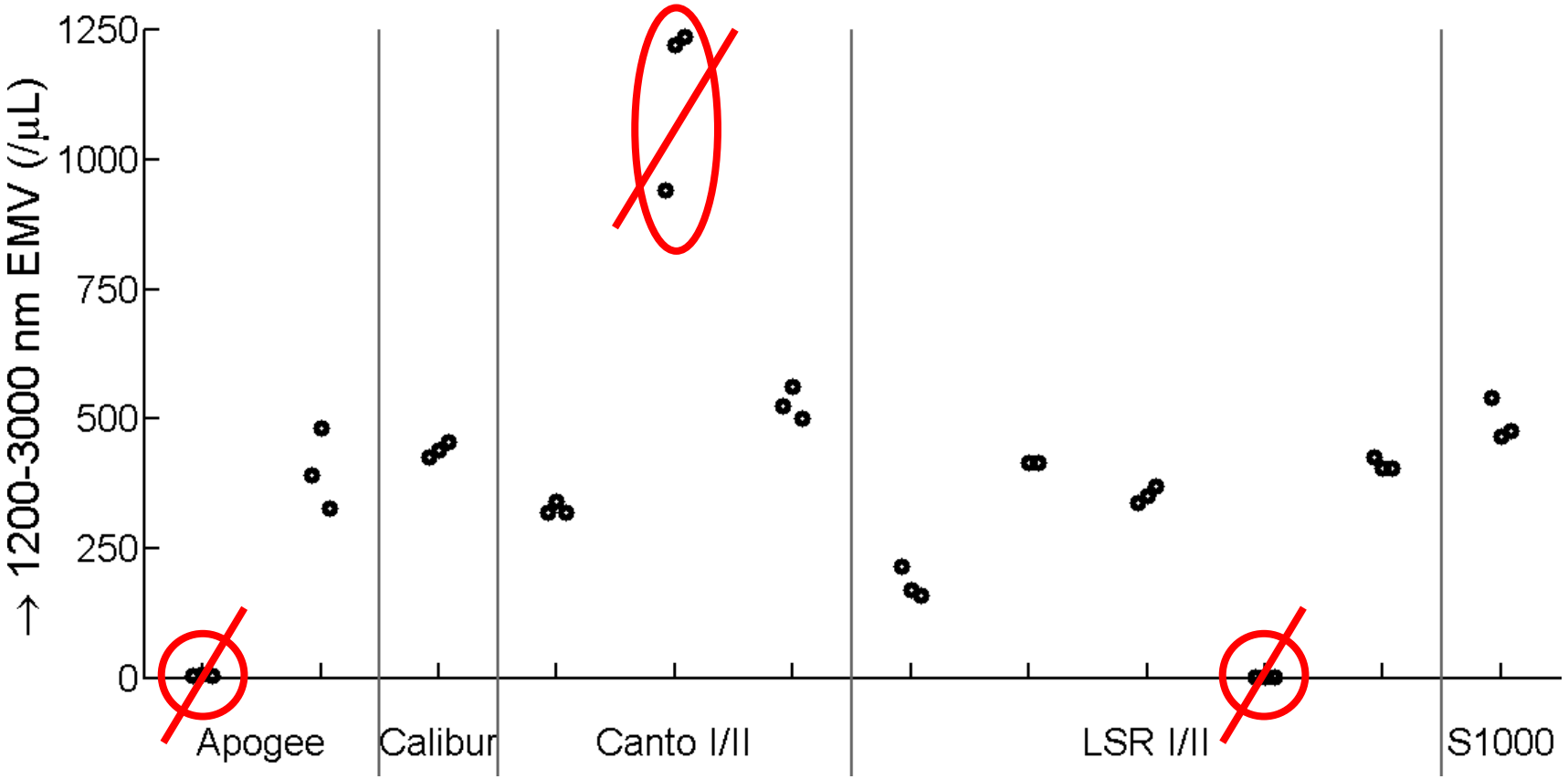
error in Mie fit

Results – erythrocyte EV concentration in gate 1



Results – erythrocyte EV concentration in gate 1

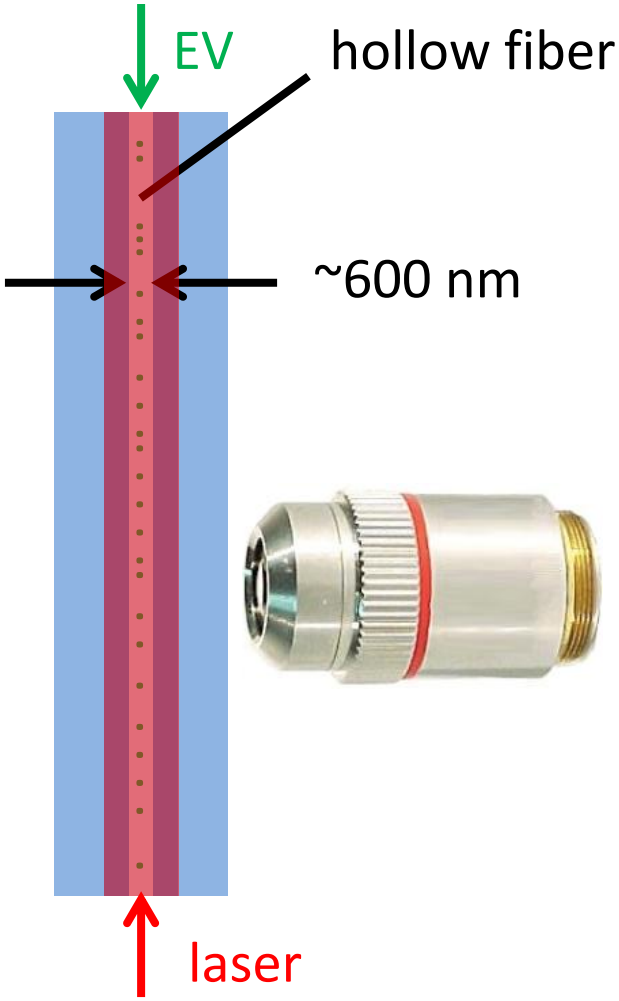
coefficient of variation (CV, without 3 outliers) = 26%



Conclusions

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

Outlook: Nano-EPics



- contact: Sanli Faez
  faez@physics.leidenuniv.nl
- fiber: Heraeus Quartz Glass

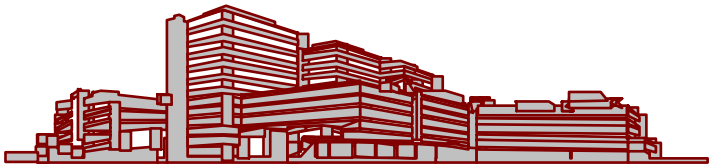
- scattering of single…
  - 35-70 nm urinary EV (this video)
  - 19 nm polystyrene beads (submitted paper Faez et al.)
  - contact: Sanli Faez (faez@physics.leidenuniv.nl)

Acknowledgements

- Academic Medical Center
  - Frank Coumans
- University of Leiden
  - Sanli Faez
- Heraeus Quartz Glass
  - Stefan Weidlich
  - Markus Schmidt

Software: exometry.com
Info: edwinvanderpol.com

- 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



Improvements

- flow rate
  - TruCount beads versus mass discharge
- Mie model
  - refractive index of EV
  - particles with shell
  - no unique solution for scatter to diameter relationship
- gating
  - criteria for setting fluorescence gate
  - software limitations for entering gates