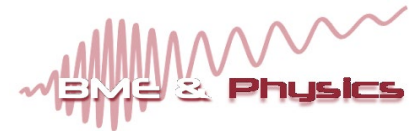


Standardization of vesicle detection by flow cytometry using traceable beads and optical scattering theory

Edwin van der Pol^{1,2}, Frank Coumans^{1,2}



Yuana Yuana¹, Auguste Sturk¹,
Ton van Leeuwen², Rienk Nieuwland¹



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



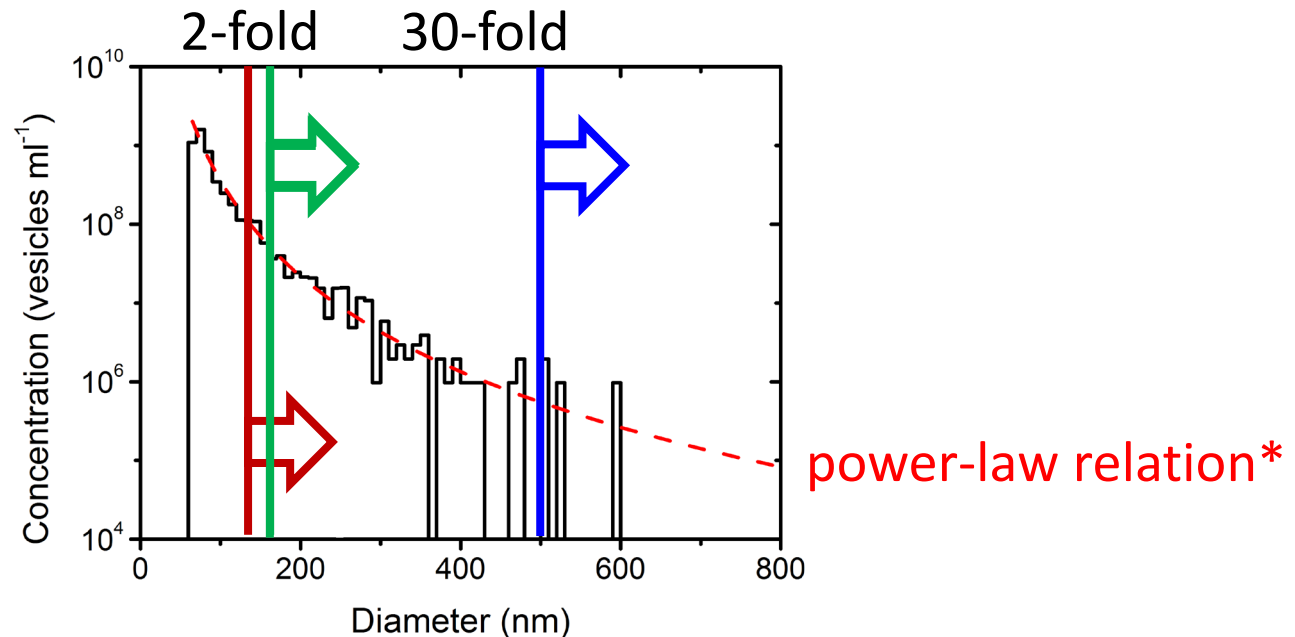
Introduction

- reliable vesicle concentration is essential
 - pathological conditions
 - count rare vesicles
- flow cytometry is widely used to count single vesicles

Extracellular vesicles (EV)	
Platelet vesicles	? X 10 ¹² /L
Erythrocyte vesicles	? X 10 ¹¹ /L
Monocyte vesicles	? X 10 ¹⁰ /L

Problem

- vesicle concentrations in plasma between 10^5 to 10^{10} vesicles/mL with flow cytometry



- primary cause: instruments differ in sensitivity

Goal

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



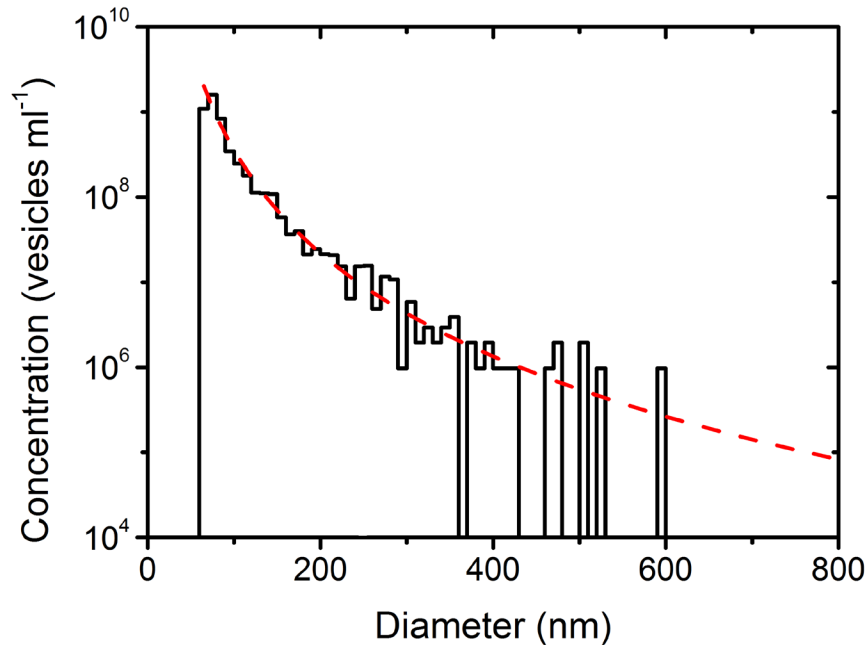
Study comprises 30 sites (60 instruments) worldwide



- funded by the Scientific Standardization Committee of the International Society on Thrombosis and Haemostasis

Requirements to determine the vesicle concentration

- knowledge of the flow rate
- well-defined size range
- all vesicles within the size range are detected



Approach

- determine flow rate
- relate scatter to diameter* (stand alone)
 - measure METVES-characterized beads mixture
 - *Exometry* software obtains scatter to diameter relation
 - *Exometry* software provides vesicle size gates
- apply vesicle size gate to software (e.g. FlowJo)
- measure vesicle standard

- preliminary results

* van der Pol and Coumans et al., JTH (2013)

Determine flow rate

- mass discharge
 - weighting the sample volume aspirated during 10 minutes
- TruCount beads
 - 3 measurements with a sample with a known concentration of beads

Approach

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- relate scatter to diameter (stand alone)
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- apply vesicle size gate to software (e.g. FlowJo)
- measure vesicle standard

- preliminary results

Status

Waiting for .fcs file to read

Controls

Open calibration file

Detector:

Flow cytometer unknown

Calibrate

Open validation file (optional)

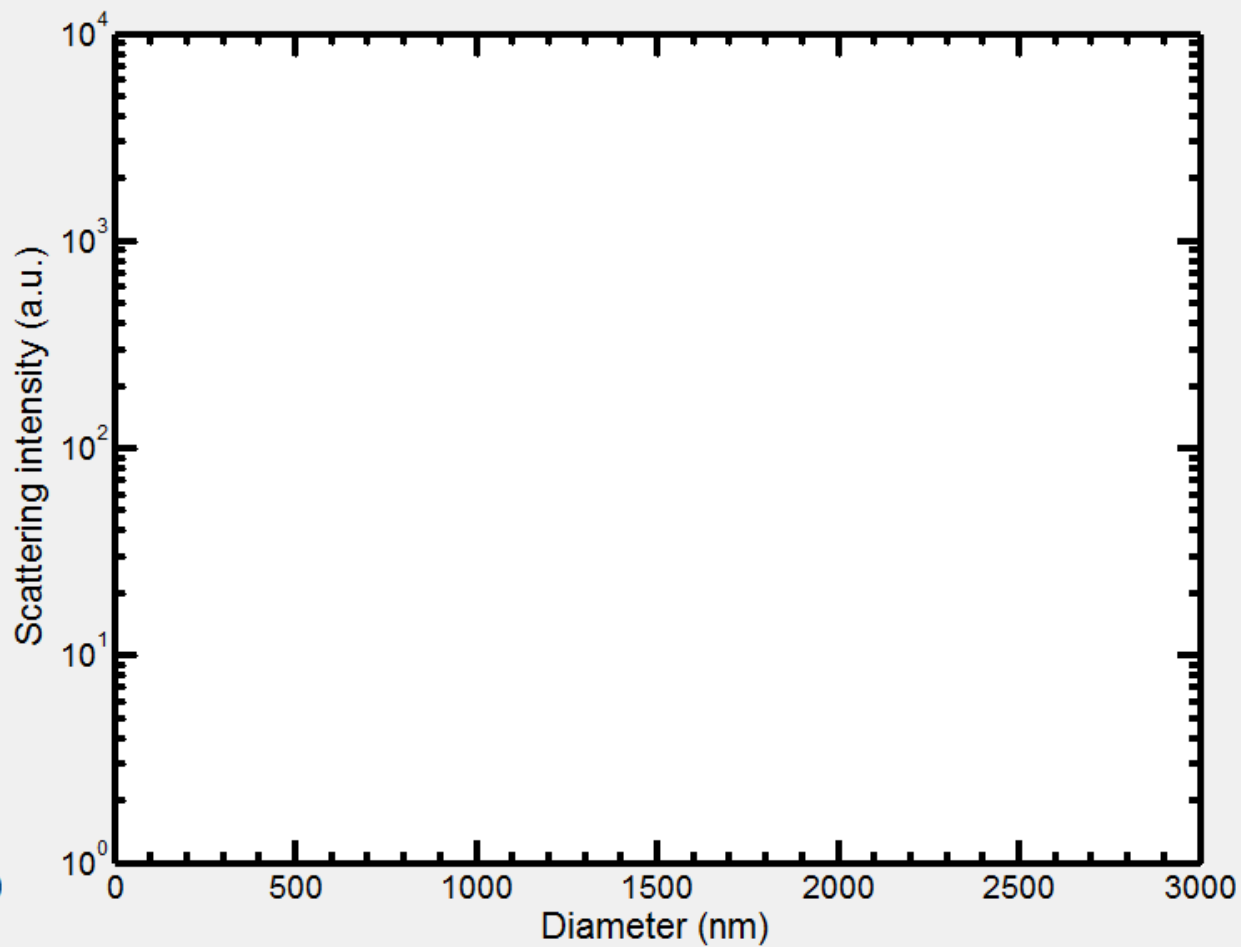
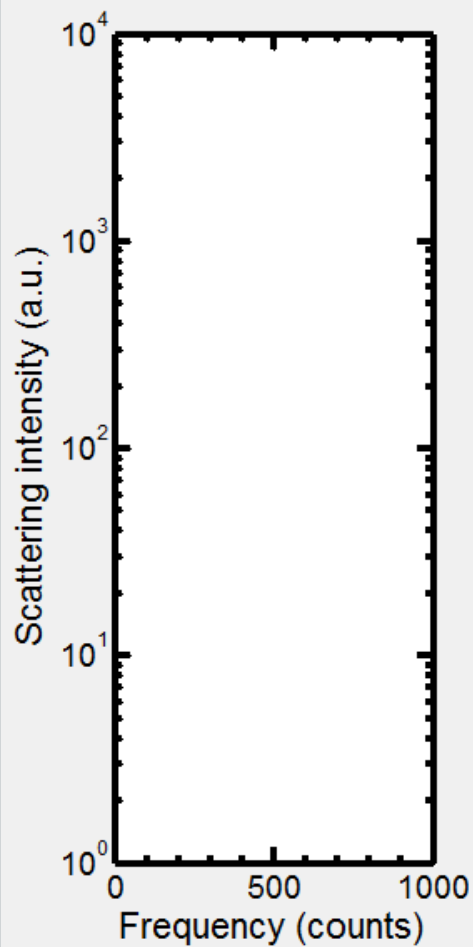
Refractive index:

1.40

Gate

Recommended gates

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



Status

Please select a flow cytometry calibration file.

Recommended gates

Wavelength (nm)	Intensity (a.u.)
3000	
1200	
600	
300	

} Gate 2

Please select the flow cytometry calibration file.

Look in: Temp

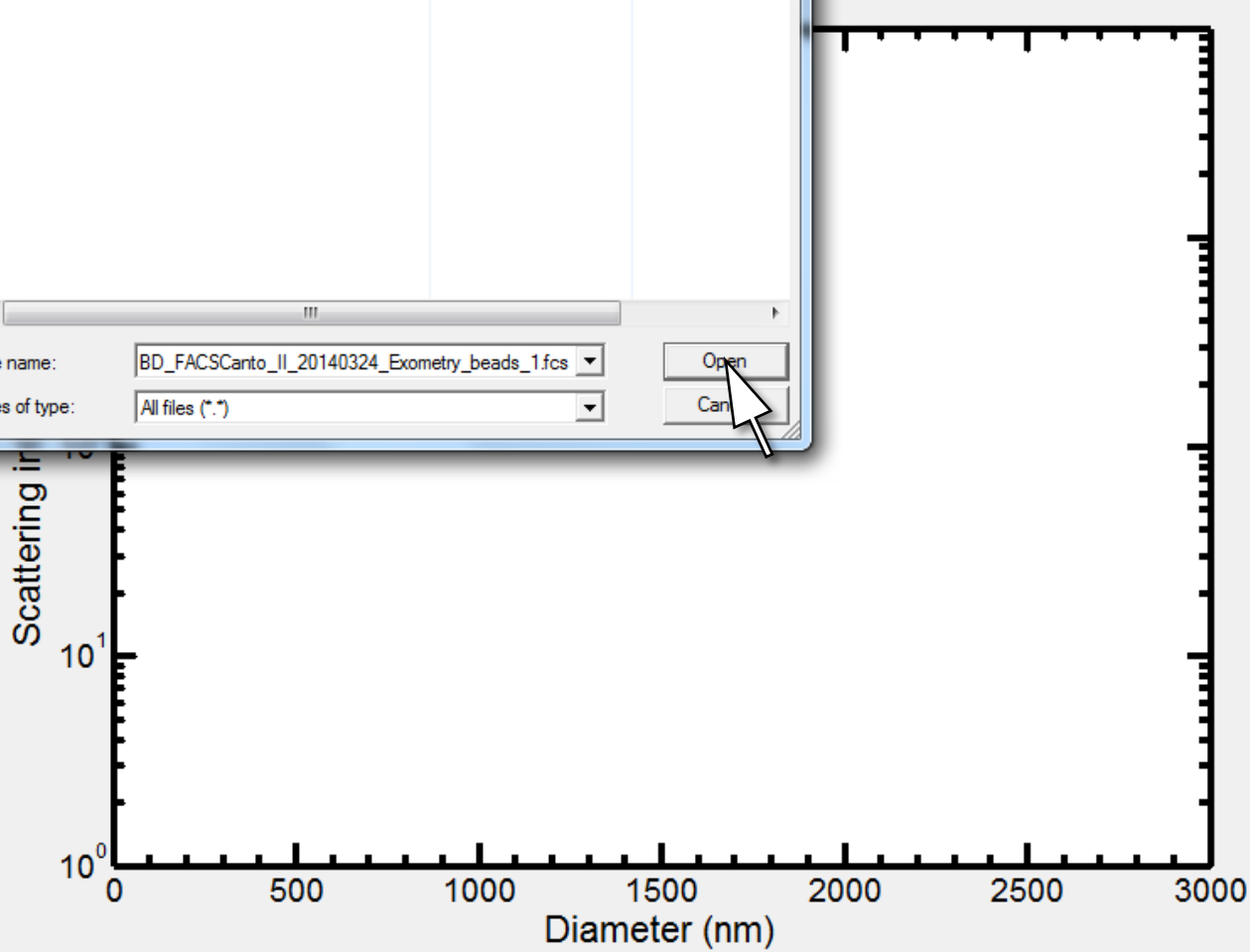
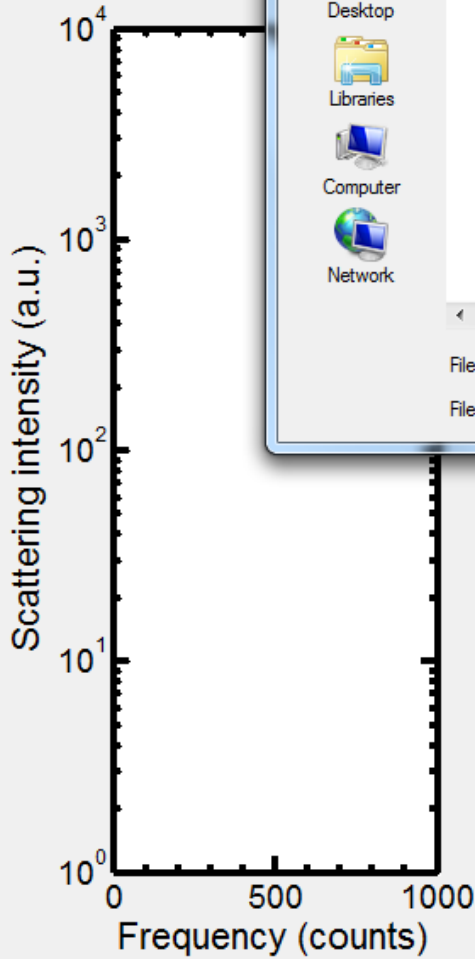
Name	Date modified	Type
BD_FACSCanto_II_20140324_Exometry_bead...	24-3-2014 13:39	FCS File

File name: BD_FACSCanto_II_20140324_Exometry_beads_1.fcs

Files of type: All files (*.*)

Open

Cancel



Status

Waiting for .fcs file to read.

Controls

Open calibration file

Detector:

SSC (recommended)

Calibrate

Open validation file (optional)

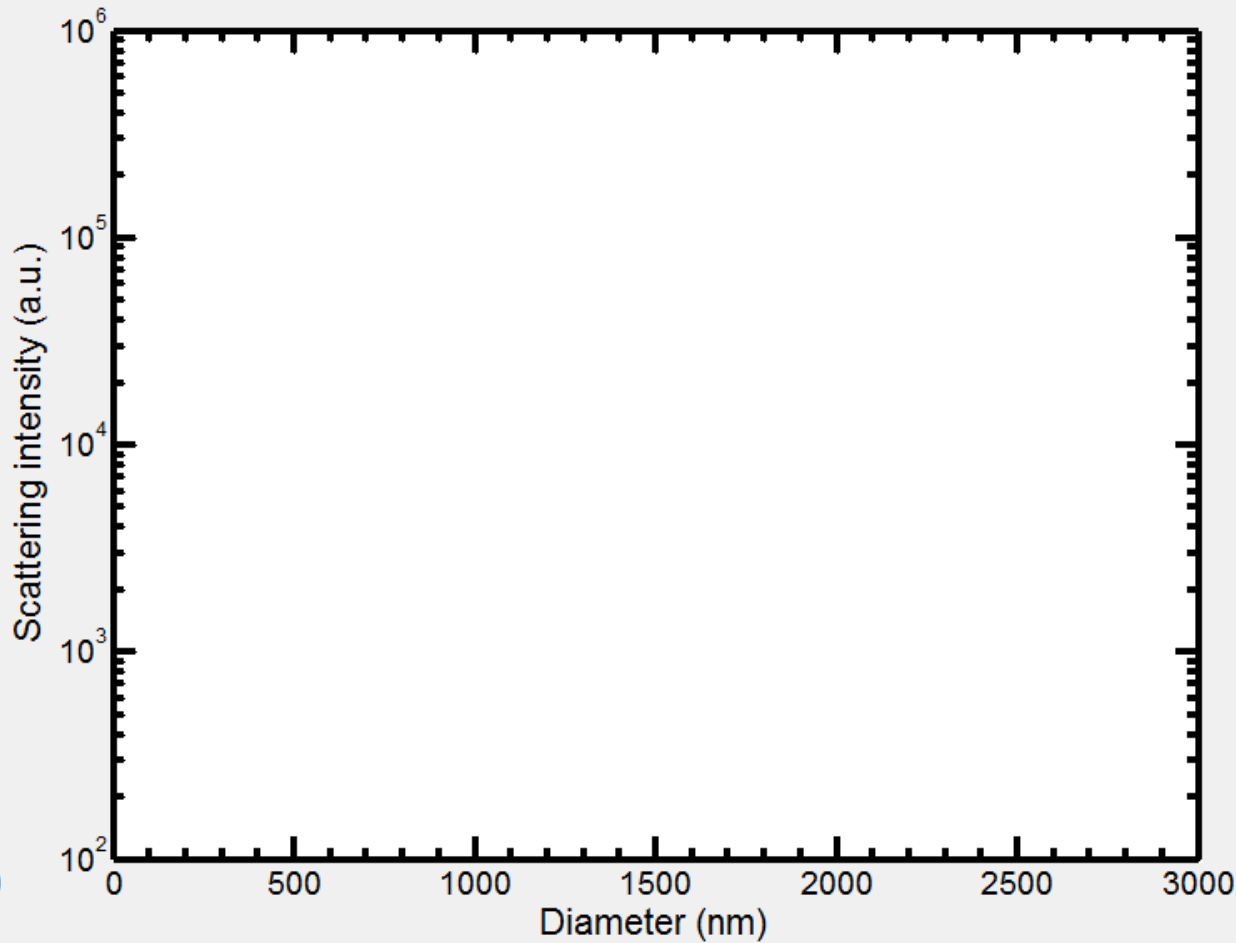
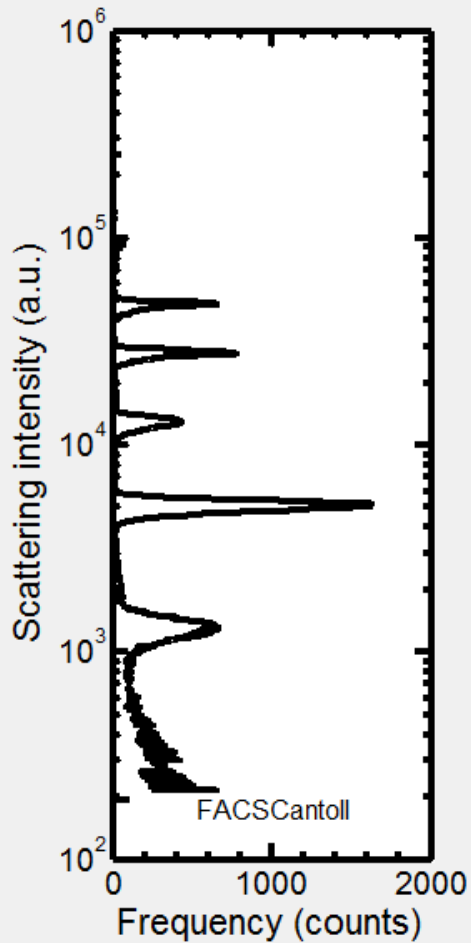
Refractive index:

1.40

Gate

Recommended gates

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



Status

Waiting for .fcs file to read.

Controls

Open calibration file

Detector:

SSC (recommended)
 SSC (recommended)
 FSC

Calibrate

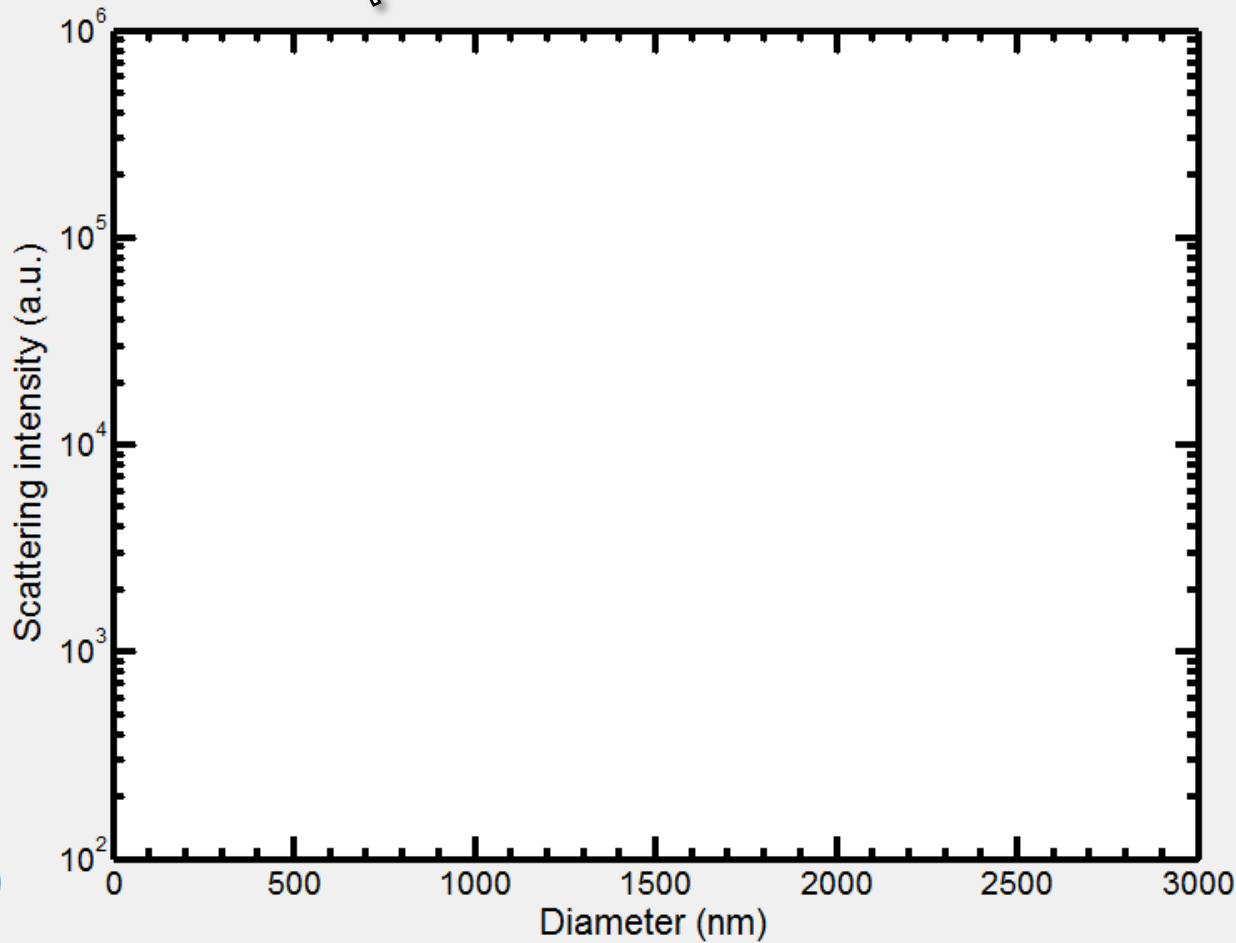
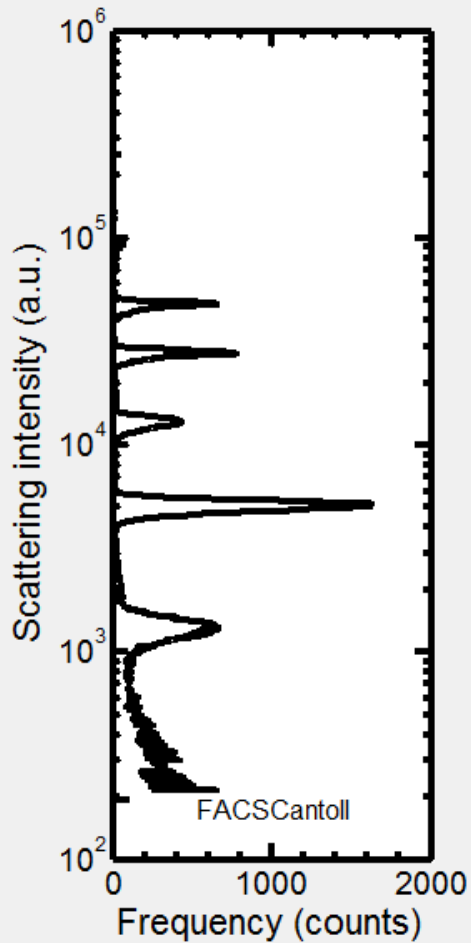
Open validation file (optional)

Refractive index:

Gate

Recommended gates

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



Status

Waiting for .fcs file to read.

Controls

Open calibration file

Detector:

SSC (recommended)

Calibrate

Open validation file (optional)

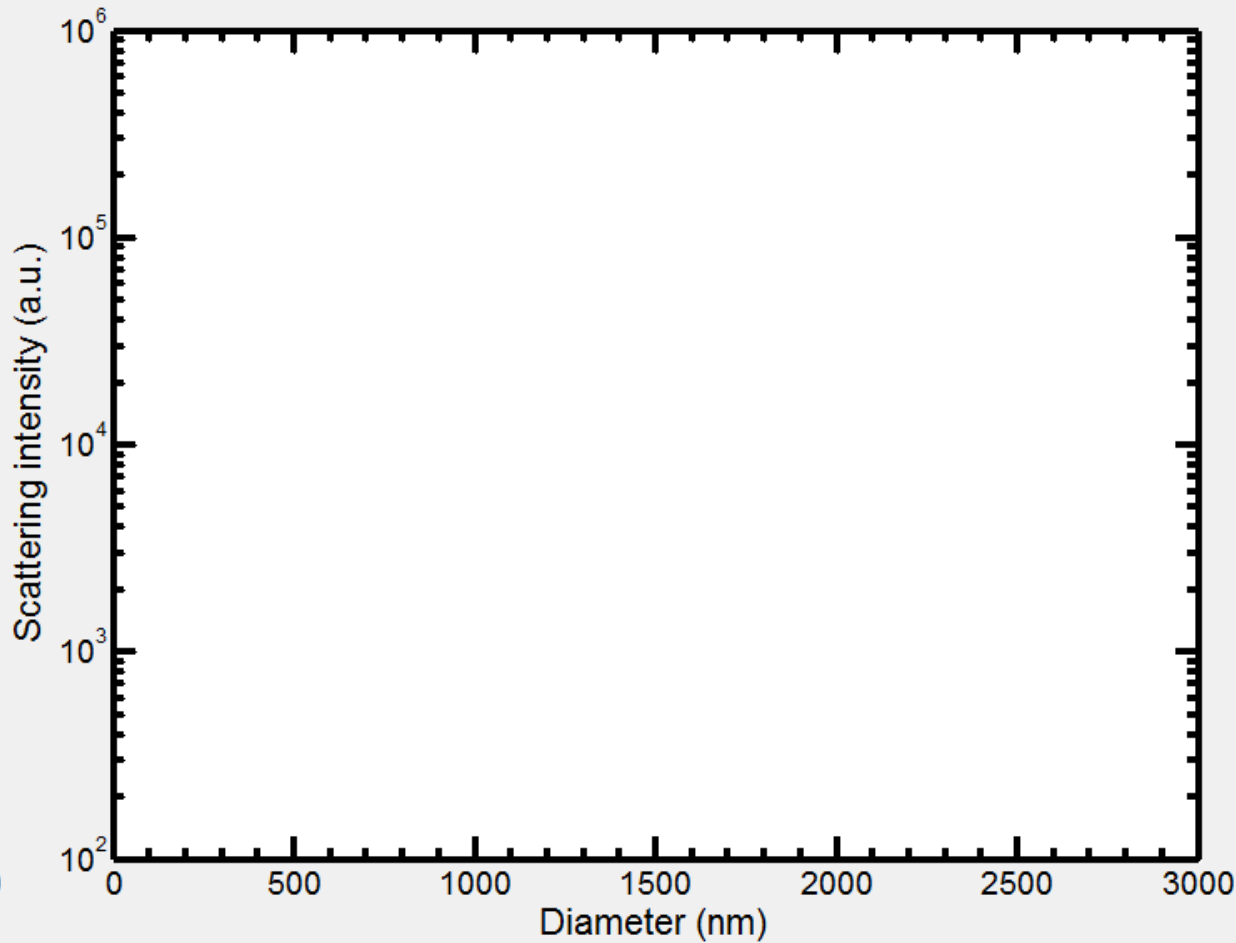
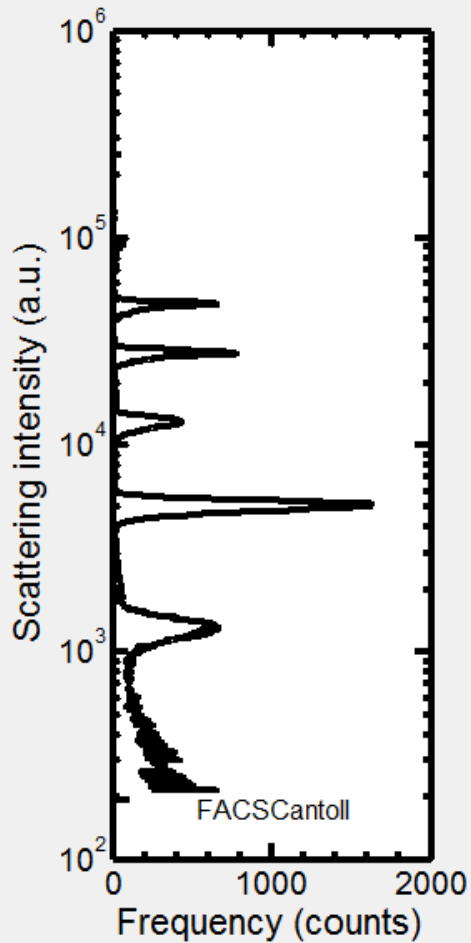
Refractive index:

1.40

Gate

Recommended gates

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



Status

There are 5 scattering peaks related to their diameter. Applying Mie calculations.

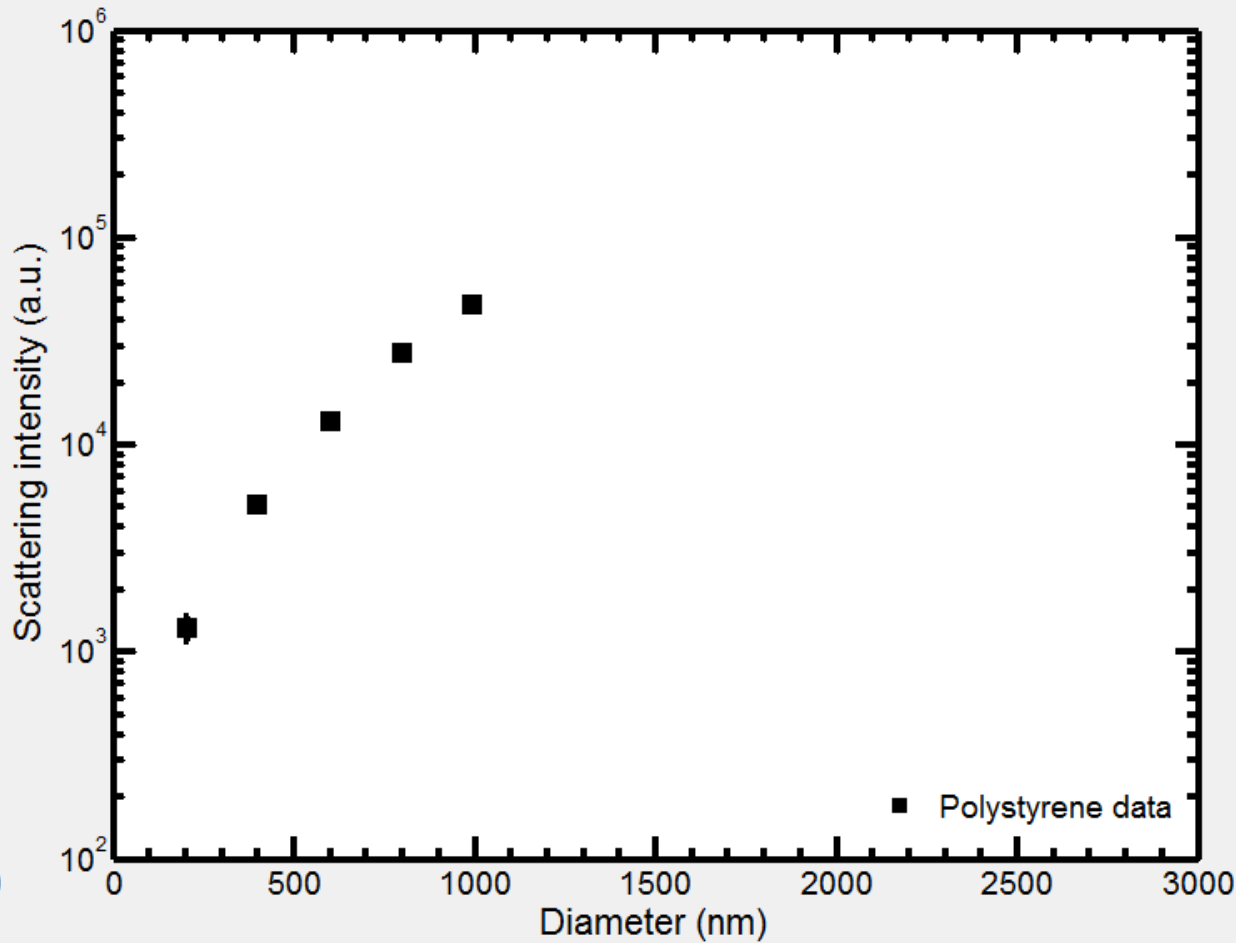
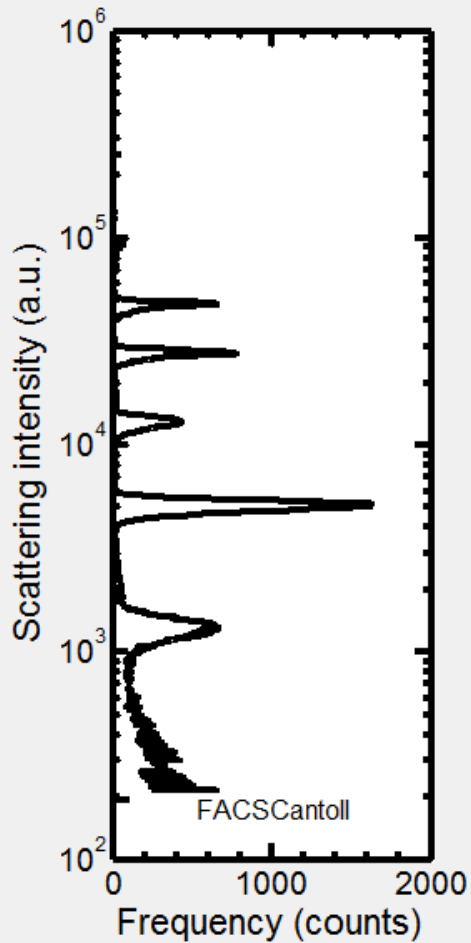
Controls

Detector:

Refractive index:

Recommended gates

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



Status

Congratulations, your flow cytometer has been calibrated with an accuracy of 99%.

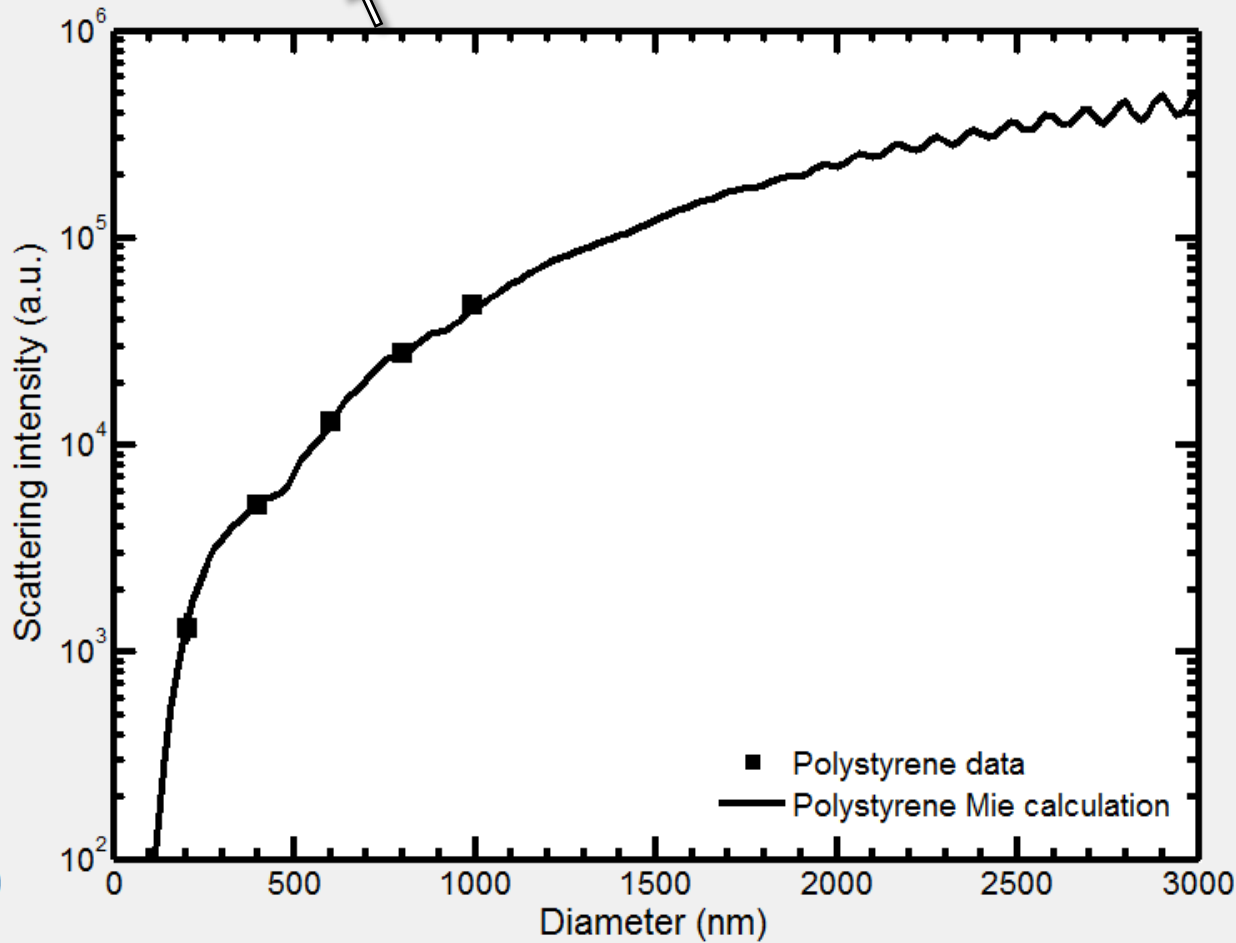
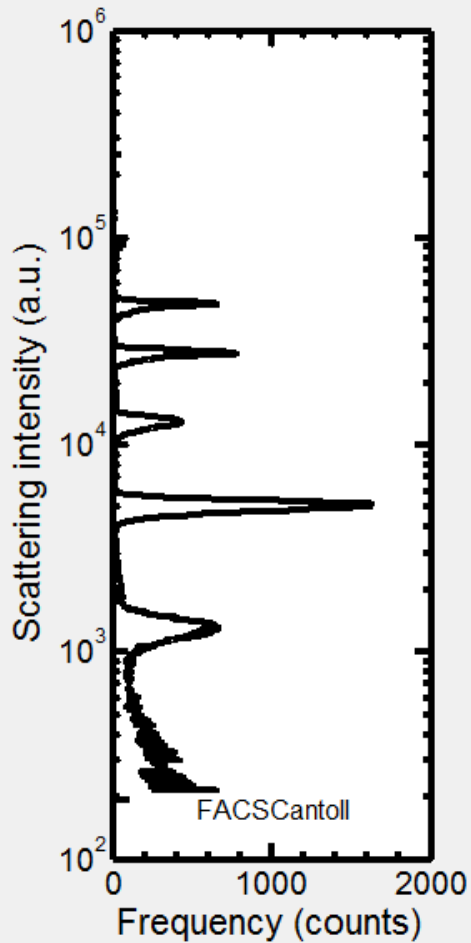
Controls

Detector:

Refractive index:

Recommended gates

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



Status

Congratulations, your flow cytometer has been calibrated with an accuracy of 99%.

Controls

Detector:

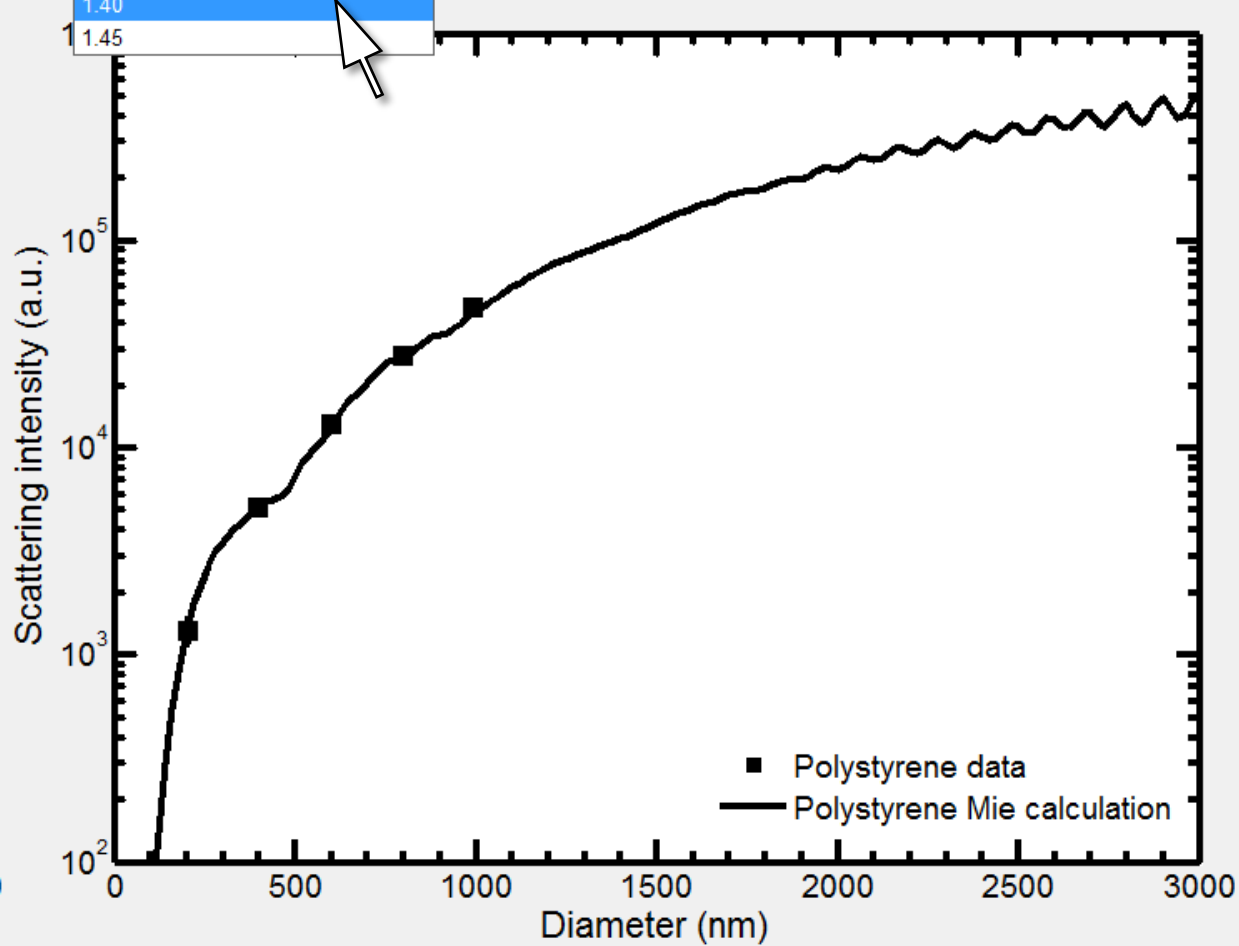
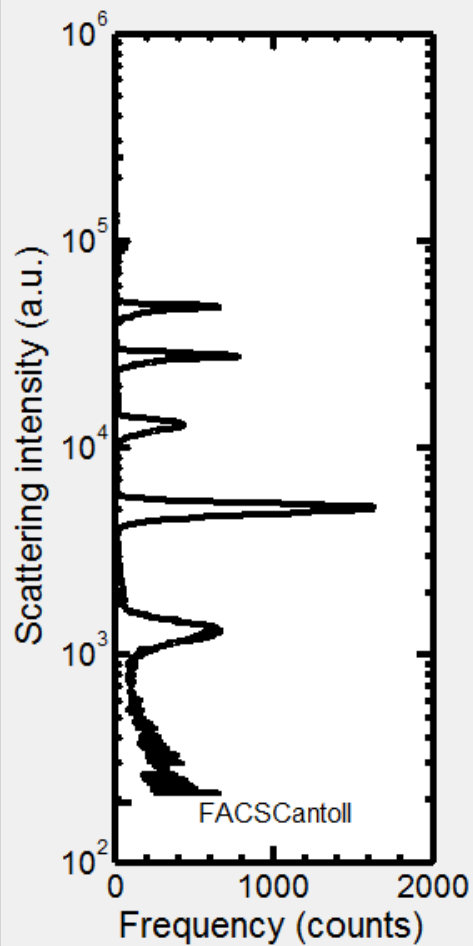
SSC (recommended)

Refractive index:

1.40

Recommended gates

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



Status

Congratulations, your flow cytometer has been calibrated with an accuracy of 99%.

Controls

Open calibration file

Detector:

Calibrate

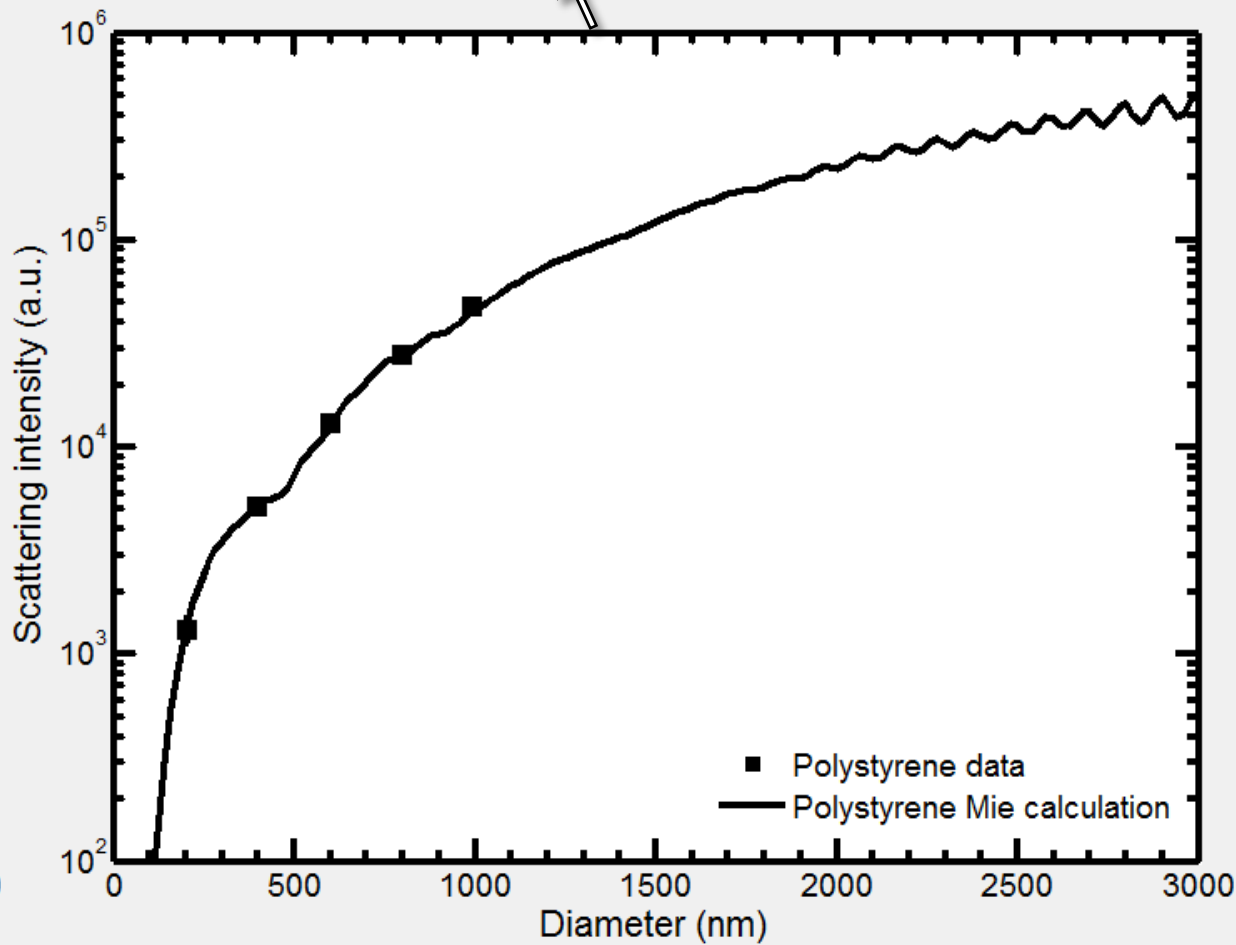
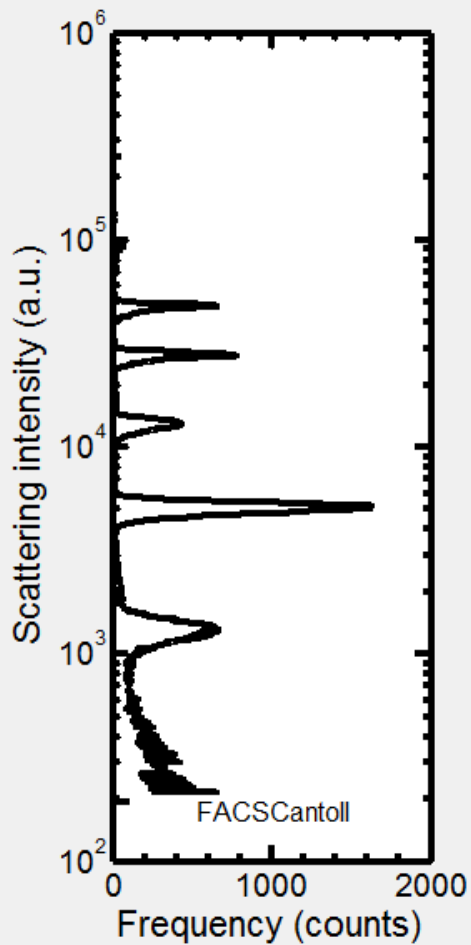
Open validation file (optional)

Refractive index:

Gate

Recommended gates

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



Status

Gates are applied for particles with a refractive index of 1.4.

Controls

Detector:

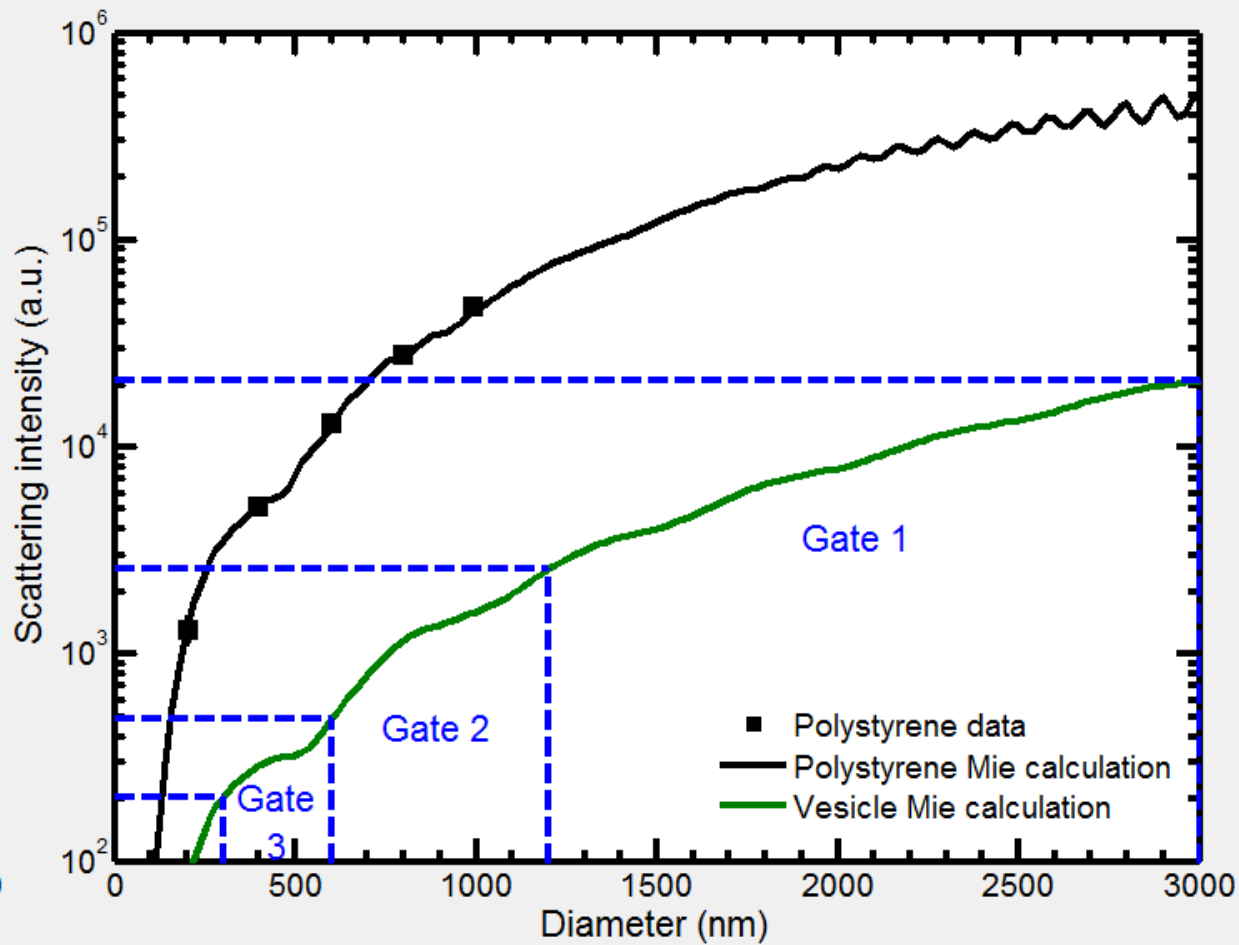
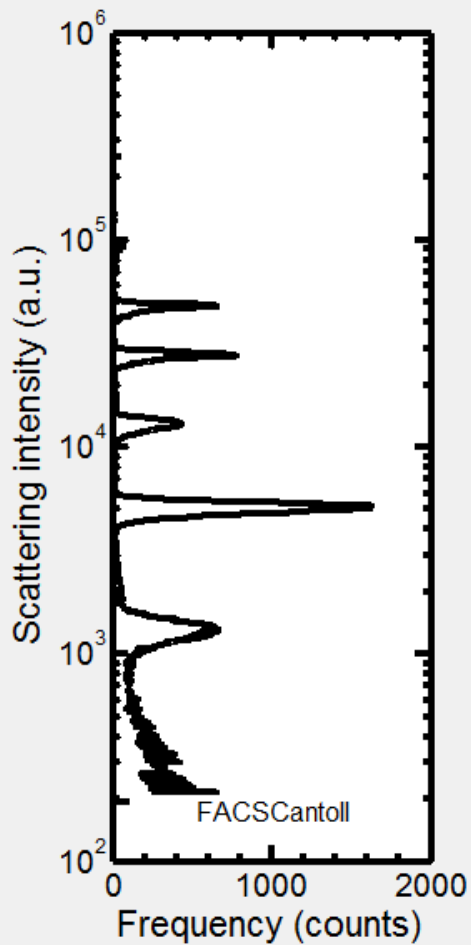
SSC (recommended)

Refractive index:

1.40

Recommended gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000	21040	} Gate 2
	1200	2577	
Gate 3 {	600	484	}
	300	204	



Approach

- ✓ determine flow rate
- ✓ relate scatter to diameter (stand alone)
 - ✓ measure METVES-characterized beads mixture
 - ✓ *Exometry* software obtains scatter to diameter relation
 - ✓ *Exometry* software provides vesicle size gates
- apply vesicle size gate to software (e.g. FlowJo)
- measure vesicle standard

- preliminary results

Status

Gates are applied for particles with a refractive index of 1.4.

Controls

Recommended gates

Diameter (nm)	Intensity (a.u.)
3000	21040
1200	2577
600	484
300	204

Gate 1 {

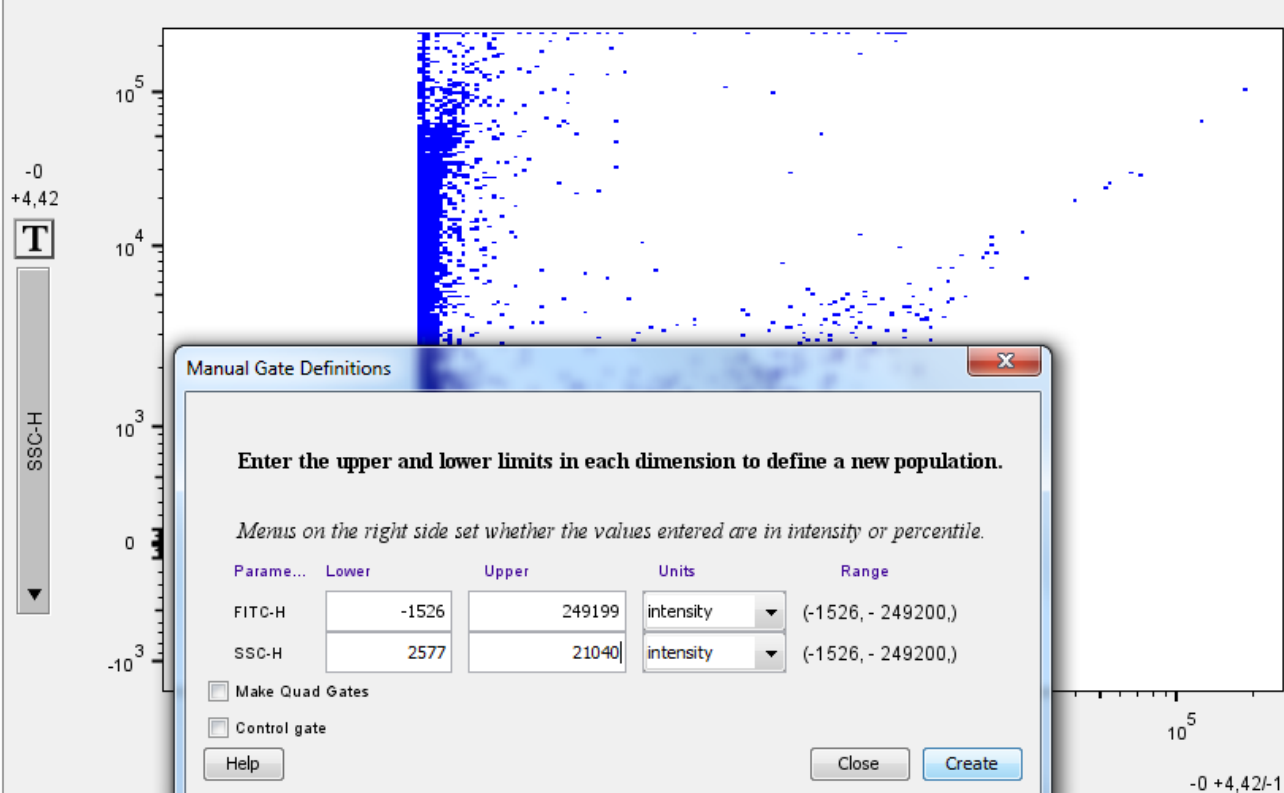
} Gate 2

Specimen_001_PMP sample 1.fcs: Ungated - FlowJo

File Edit Graph Display Go Help



>



Manual Gate Definitions

Enter the upper and lower limits in each dimension to define a new population.

Menus on the right side set whether the values entered are in intensity or percentile.

Parame...	Lower	Upper	Units	Range
FITC-H	-1526	249199	intensity	(-1526, - 249200,)
SSC-H	2577	21040	intensity	(-1526, - 249200,)

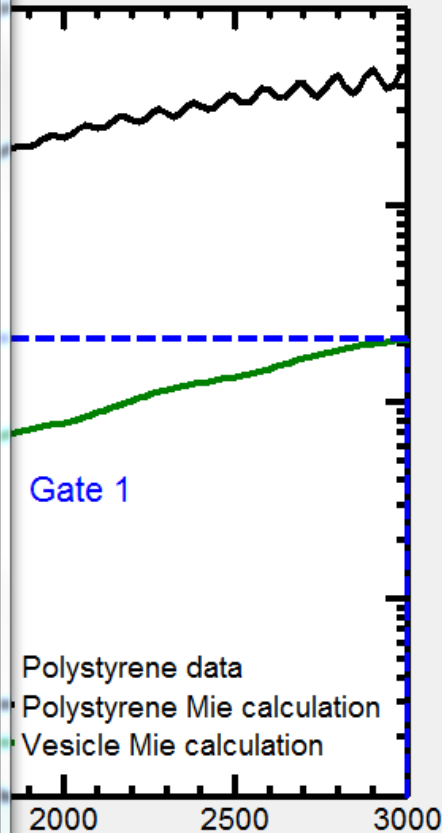
Make Quad Gates

Control gate

Help

Close

Create



Gate 1

Polystyrene data

Polystyrene Mie calculation

Vesicle Mie calculation

FITC-H



Options

Active Gate

Σ Statistics - Count: 1303986 / 1303986 100%

Exometry v0.12

Status
Gates are applied for particles with a refractive index of 1.4.

Recommended gates

Diameter (nm)	Intensity (a.u.)
3000	21040
1200	2577
600	484
300	204

Gate 1 { } Gate 2

Specimen_001_PMP sample 1.fcs: Ungated - FlowJo

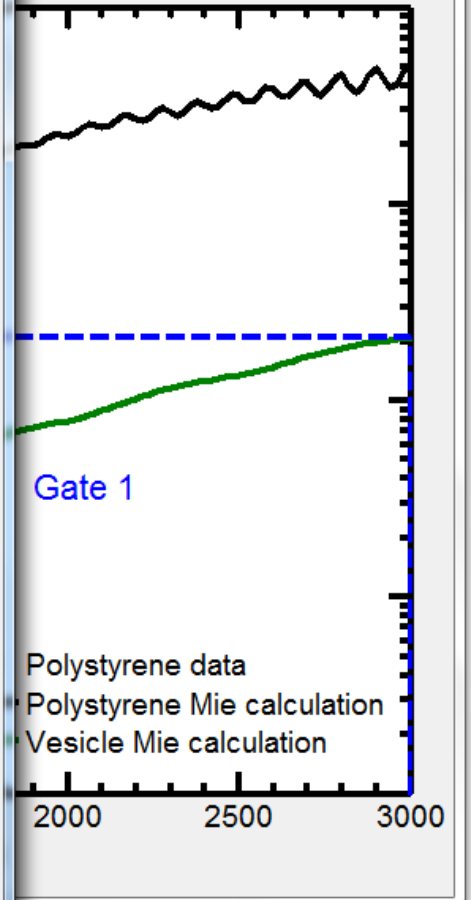
File Edit Graph Display Go Help

SSC-H

Gate 1, EV, 1200-3000 nm
0.92

FITC-H

Statistics - Count: 1303986 / 1303986 100%



Status

Gates are applied for particles with a refractive index of 1.4.

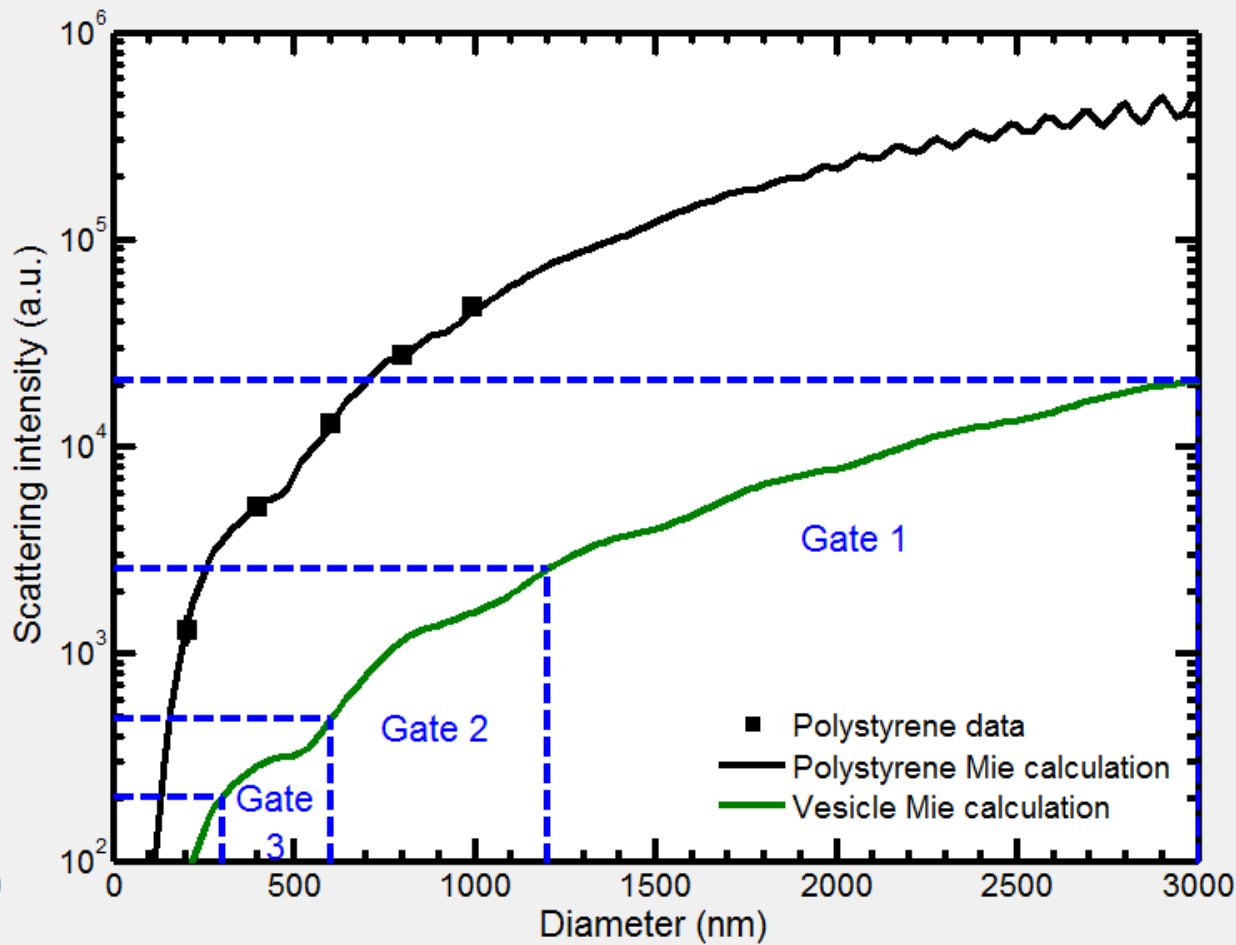
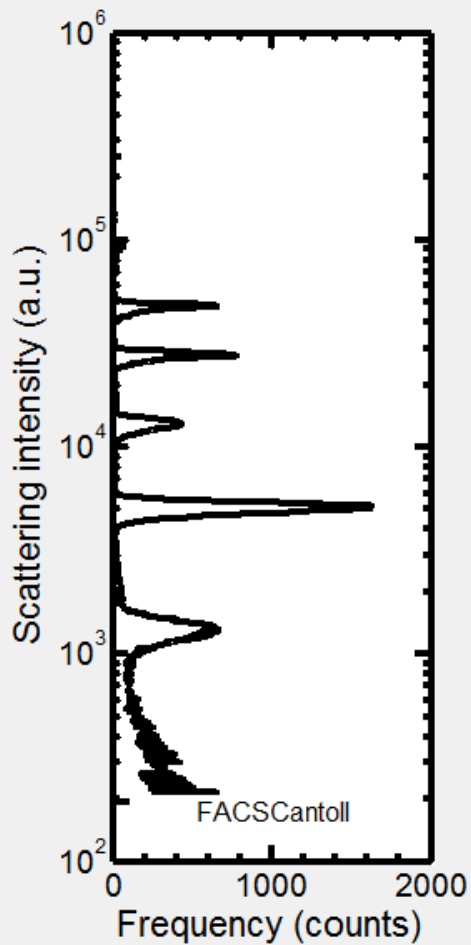
Controls

Detector:

Refractive index:

Recommended gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000	21040	} Gate 2
	1200	2577	
Gate 3 {	600	484	}
	300	204	



Status

Gates are applied for particles with a refractive index of 1.4.

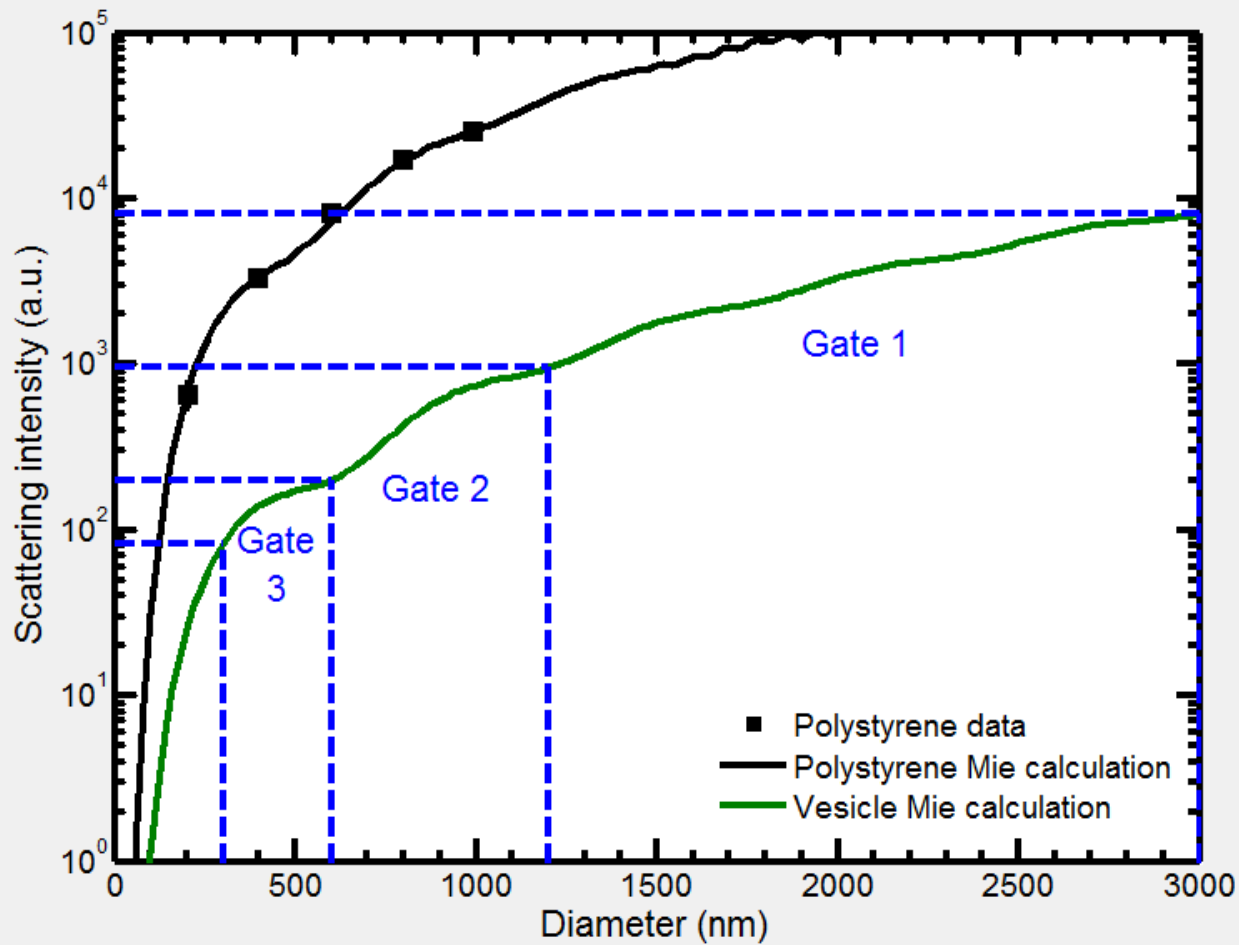
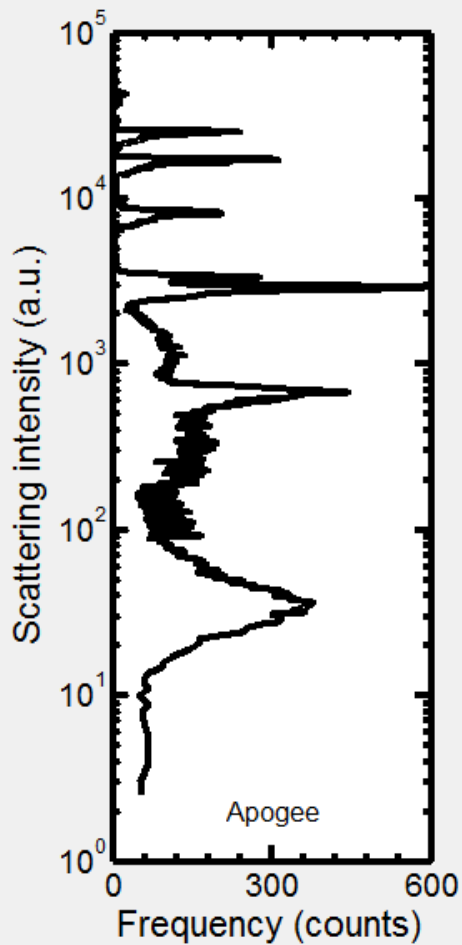
Controls

Detector:

Refractive index:

Recommended gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000	8125	} Gate 2
	1200	952	
Gate 3 {	600	198	}
	300	82	



Status

Gates are applied for particles with a refractive index of 1.4.

Controls

Detector:

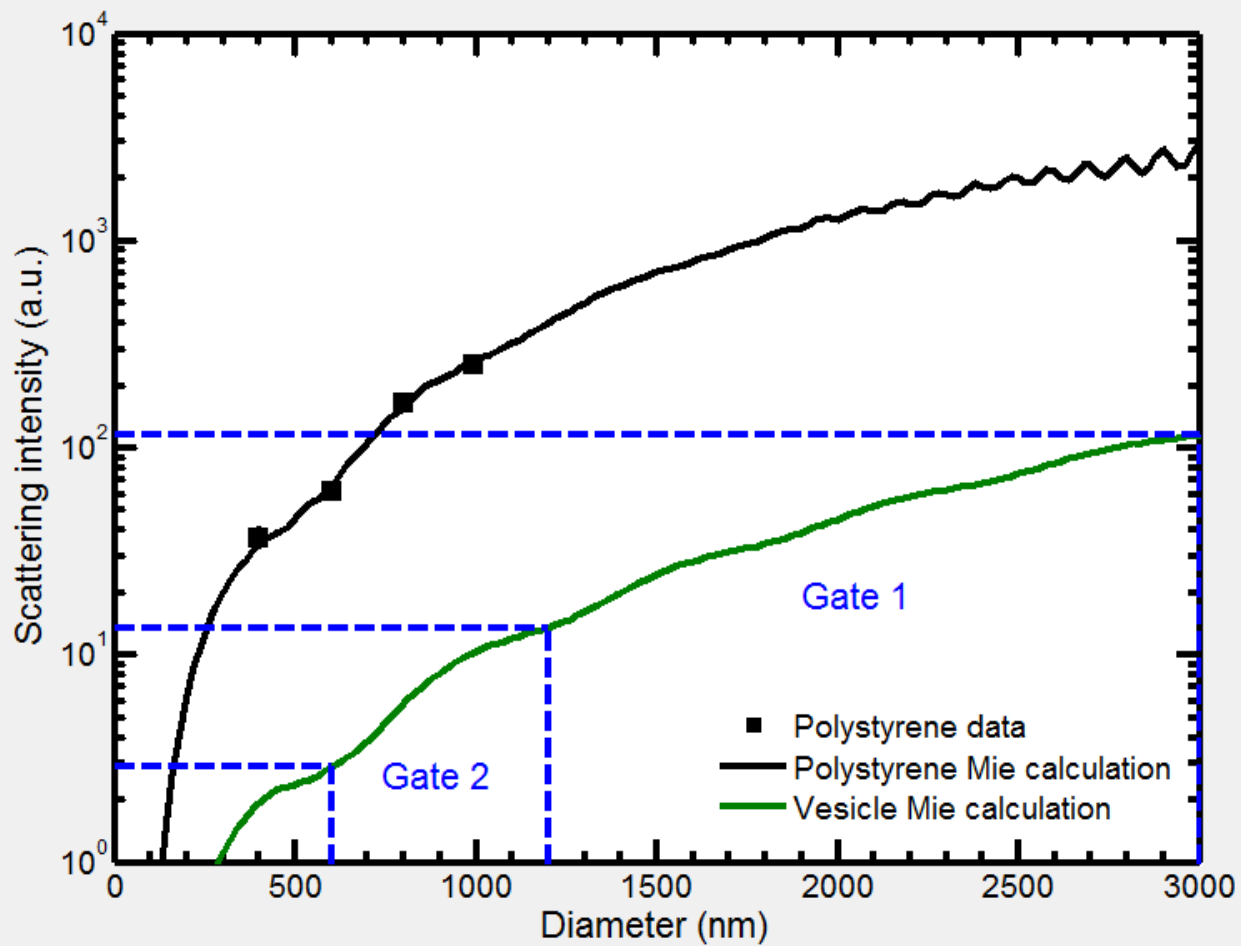
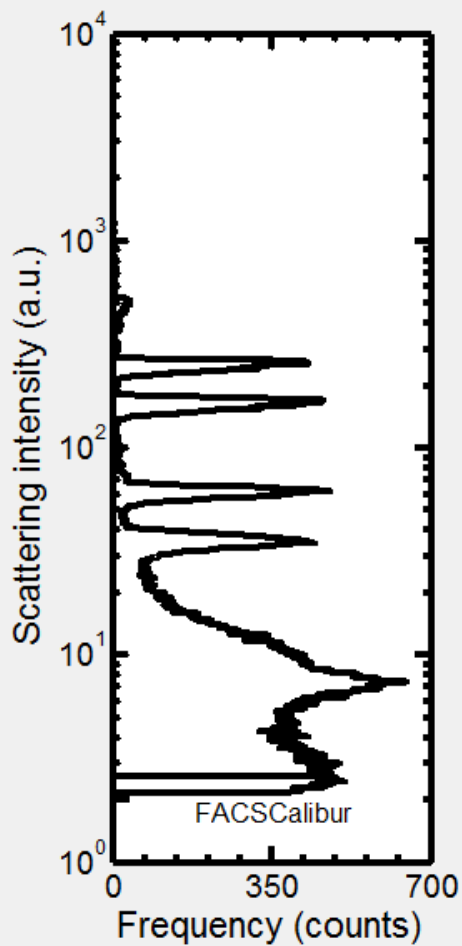
SSC (recommended)

Refractive index:

1.40

Recommended gates

	Diameter (nm)	Intensity (a.u.)	
Gate 1 {	3000	116	} Gate 2
	1200	14	
Gate 3 {	600	3	}



Approach

- ✔ determine flow rate
- ✔ relate scatter to diameter (stand alone)
 - ✔ measure METVES-characterized beads mixture
 - ✔ *Exometry* software obtains scatter to diameter relation
 - ✔ *Exometry* software provides vesicle size gates
- ✔ apply vesicle size gate to software (e.g. FlowJo)
- measure vesicle standard

- preliminary results

Measure vesicle standards

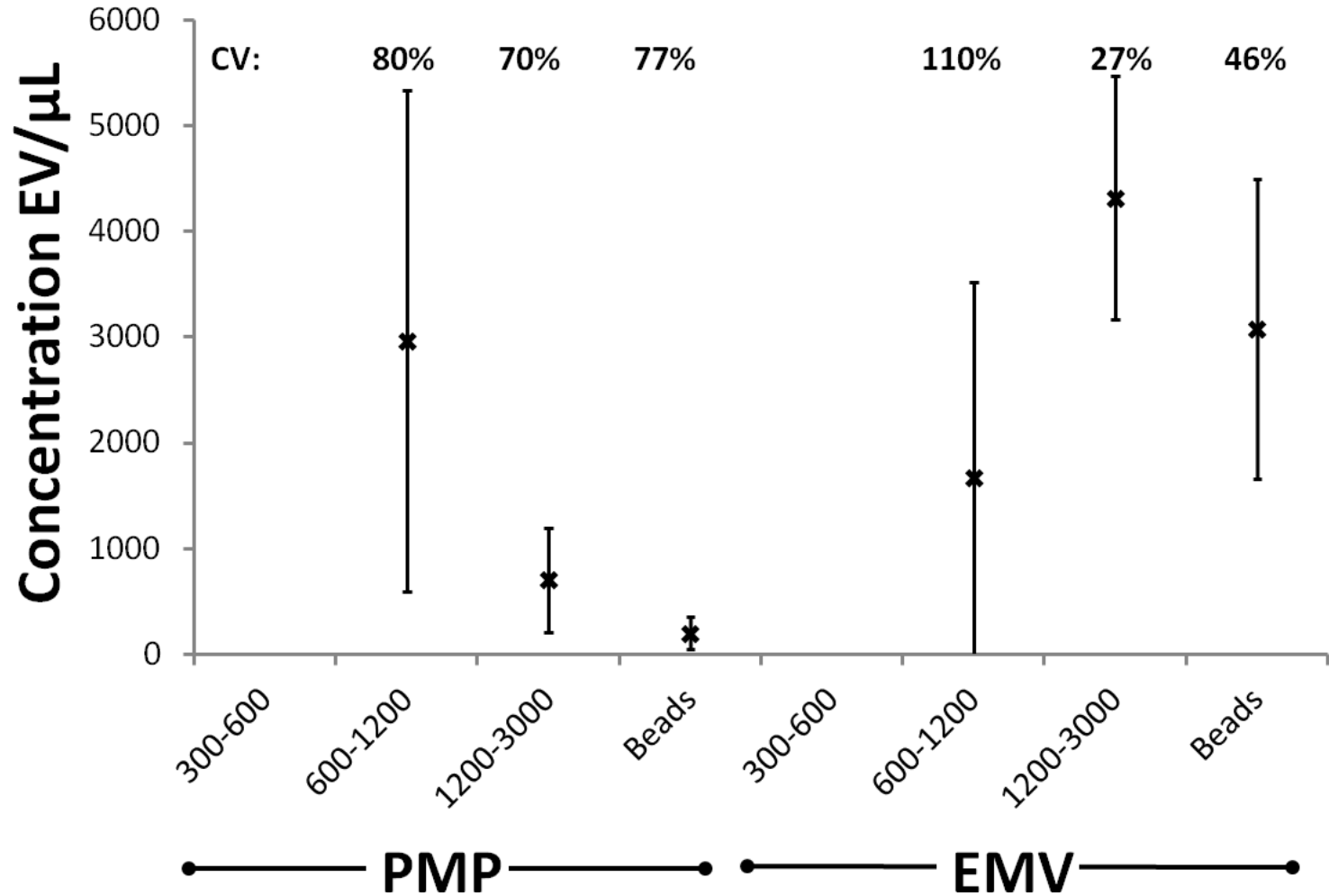
- Platelet vesicles ($n=3$)
 - lactadherin-FITC, CD61-PE labeled
 - isotype control
 - diluent
- Erythrocyte vesicles ($n=3$)
 - lactadherin-FITC, CD235a-PE labeled
 - isotype control
 - diluent

Measure vesicle standards

Canto II (flowrate 53,9 uL/min)

Phenotype	Lactadherin+ & (CD61 or CD235a)+			
	300-600	600-1200	1200-3000	400-800
PMP sample 1		759	102	27
PMP sample 2		1344	101	30
PMP sample 3		458	74	28
PMP isotype		0	0	0
PMP diluent		144	28	13
EMV sample 1		560	507	194
EMV sample 2		525	582	229
EMV sample 3		933	673	276
EMV isotype		0	3	5
EMV diluent		162	46	15
TruCount	11683			

Preliminary results



Conclusions

- reliable vesicle concentration by flow cytometry is essential and requires
 - knowledge of the flow rate
 - well-defined size range
- international standardization study is ongoing
- preliminary results show a CV improvement of 19% for erythrocyte vesicles compared to previous standards

Progress indicator



Acknowledgements

- Academic Medical Center
 - Laboratory Experimental Clinical Chemistry
 - Anita Böing
 - Chi Hau
 - Frank Coumans
 - Guus Sturk
 - Rienk Nieuwland
 - René Berckmans
 - Ton van Leeuwen
 - Yuana Yuana
 - Biomedical Engineering and Physics
- 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

More on vesicle detection:
edwinvanderpol.com

