Standardisation of extracellular vesicle measurements

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International Society on Thrombosis and Haemostasis

EXOMETRY

METVES

July 12th, 2016

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

- standardisation
  - relevance
  - extracellular vesicle (EV) measurements
  - recent developments
- quantitative light scattering to standardise EV measurements by flow cytometry
- outlook: size and refractive index determination by flow cytometry

Standardisation is boring (most biologists)

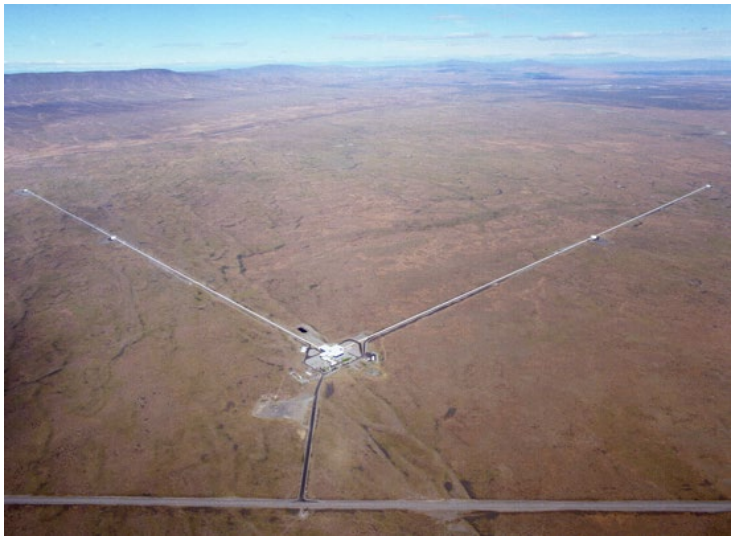
Standardisation is exciting (metrologists, physicists)

BESSYII

0.31 nm X-rays to size EV

(flow cytometers typically use 488 nm light)

LIGO



measure length variations of 10^{-9} nm

Standardisation

- allows comparison of results between instruments and laboratories
- allows investigation and validation of biomarkers
- allows development of reliable biorepositories

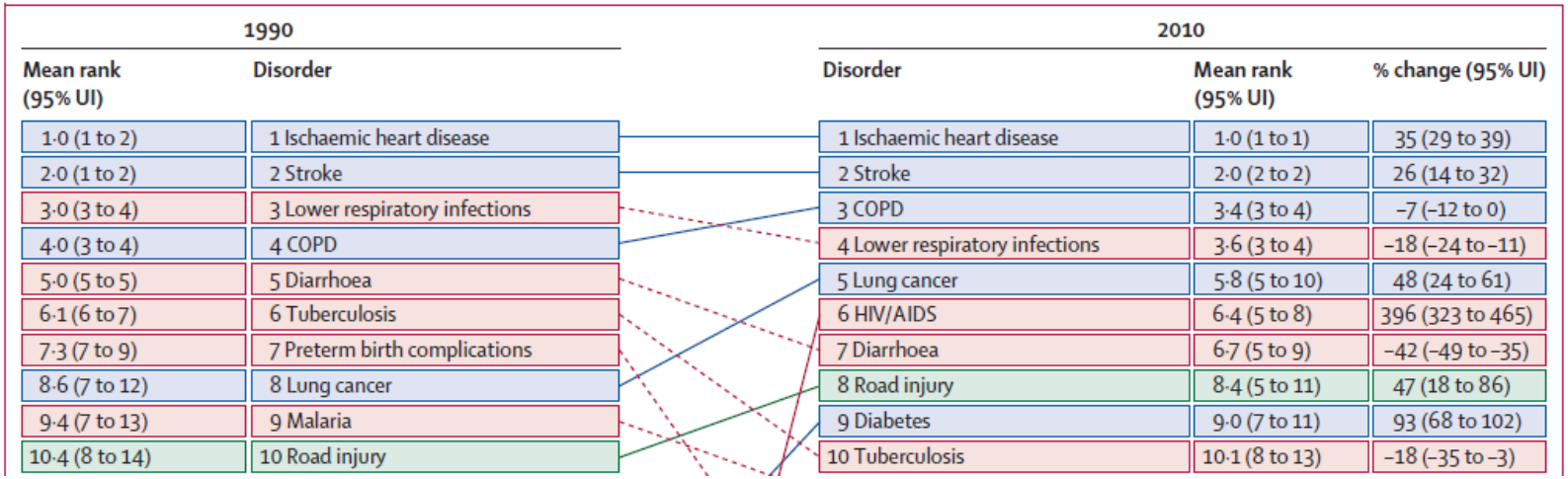
image: biorepositoryresources.com

What happens without standardisation?

an ISTH example

Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010

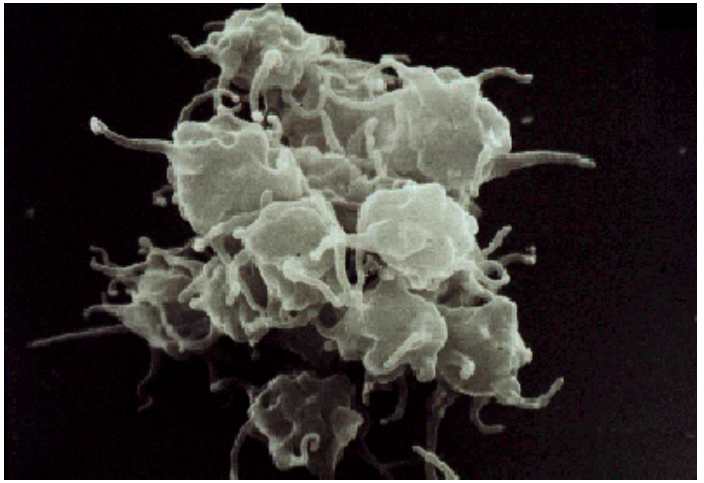
Rafael Lozano, Mohsen Naghavi, Kyle Foreman, Stephen Lim, Kenji Shibuya, Victor Aboyans, Jerry Abraham*, Timothy Adair*, Rakesh Aggarwal*, Stephanie Y Ahn*, Mohammad A AlMazroa*, Miriam Alvarado*, H Ross Anderson*, Laurie M Anderson*, Kathryn G Andrews*, Charles Atkinson*, Larry M Baddour*, Suzanne Barker-Collo*, David H Bartels*, Michelle L Bell*, Emelia J Benjamin*, Derrick Bennett*, Kavi Bhalla*, Boris Bikbov*, Aref Bin Abdulhak*, Gretchen Birbeck*, Fiona Blyth*, Ian Bolliger*, Soufiane Boufous*, Chiara Bucello*, Michael Burch*, Peter Burney*, Jonathan Carapetis*, Honglei Chen*, David Chou*, Sumeet S Chugh*, Luc E Coffeng*, Steven D Colan*, Samantha Colquhoun*, K Ellicott Colson*, John Condon*, Myles D Connor*, Leslie T Cooper*, Matthew Corriere*, Monica Cortinovis*, Karen Courville de Vaccaro*, William Couser*, Benjamin C Cowie*, Michael H Criqui*, Marita Cross*, Kaustubh C Dabhadkar*, Nabila Dahodwala*, Diego De Leo*, Louisa Degenhardt*, Allyne Delossantos*, Julie Denenberg*, Don C Des Jarlais*, Samath D Dharmaratne*, E Ray Dorsey*, Tim Driscoll*, Herbert Duber*, Beth Ebel*, Patricia J Erwin*, Patricia Espindola*, Majid Ezzati*, Valery Feigin*, Abraham D Flaxman*, Mohammad H Forouzanfar*, Francis Gerry R Fowkes*, Richard Franklin*, Marlene Fransen*, Michael K Freeman*, Sherine E Gabriel*, Emmanuela Gakidou*, Flavio Gaspari*, Richard F Gillum*, Diego Gonzalez-Medina*, Yara A Halasa*, Diana Haring*, James E Harrison*, Rasmus Havmoeller*, Roderick J Hay*, Bruno Hoen*, Peter J Hotez*, Damian Hoy*, Kathryn H Jacobsen*, Spencer L James*, Rashmi Jasrasaria*, Sudha Jayaraman*, Nicole Johns*, Ganesan Karthikeyan*, Nicholas Kassebaum*, Andre Keren*, Jon-Paul Khoo*, Lisa Marie Knowlton*, Olive Kobusingye*, Adofo Koranteng*, Rita Krishnamurthi*, Michael Lipnick*, Steven E Lipshultz*, Summer Lockett Ohno*, Jacqueline Mabweijano*, Michael F MacIntyre*, Leslie Mallinger*, Lyn March*, Guy B Marks*, Robin Marks*, Akira Matsumori*, Richard Matzopoulos*, Bongani M Mayosi*, John H McAnulty*, Mary M McDermott*, John McGrath*, Ziad A Memish*, George A Mensah*, Tony R Merriman*, Catherine Michaud*, Matthew Miller*, Ted R Miller*, Charles Mock*, Ana Olga Mocumbi*, Ali A Mokdad*, Andrew Moran*, Kim Mulholland*, M Nathan Nair*, Luigi Naldi*, K M Venkat Narayan*, Kiumarss Nasseri*, Paul Norman*, Martin O'Donnell*, Saad B Omer*, Katrina Ortblad*, Richard Osborne*, Doruk Ozgediz*, Bishnu Pahari*, Jeyaraj Durai Pandian*, Andrea Panozo Rivero*, Rogelio Perez Padilla*, Fernando Perez-Ruiz*, Norberto Perico*, David Phillips*, Kelsey Pierce*, C Arden Pope III*, Esteban Porrini*, Farshad Pourmalek*, Murugesan Raju*, Dharani Ranganathan*, Jürgen T Rehm*, David B Rein*, Guiseppe Remuzzi*, Frederick P Rivara*, Thomas Roberts*, Felipe Rodriguez De León*, Lisa C Rosenfeld*, Lesley Rushton*, Ralph L Sacco*, Joshua A Salomon*, Uchechukwu Sampson*, Ella Sanman*, David C Schwebel*, Maria Segui-Gomez*, Donald S Shepard*, David Singh*, Jessica Singleton*, Karen Sliwa*, Emma Smith*, Andrew Steer*, Jennifer A Taylor*, Bernadette Thomas*, Imad M Tleyjeh*, Jeffrey A Towbin*, Thomas Truelsen*, Eduardo A Undurraga*, N Venketasubramanian*, Lakshmi Vijayakumar*, Theo Vos*, Gregory R Wagner*, Mengru Wang*, Wenzhi Wang*, Kerrianne Watt*, Martin A Weinstock*, Robert Weintraub*, James D Wilkinson*, Anthony D Woolf*, Sarah Wulf*, Pon-Hsiu Yeh*, Paul Yip*, Azadeh Zabetian*, Zhi-Jie Zheng*, Alan D Lopez†, Christopher J L Murray†‡*



1990		2010		
Mean rank (95% UI)	Disorder	Disorder	Mean rank (95% UI)	% change (95% UI)
1·0 (1 to 2)	1 Ischaemic heart disease	1 Ischaemic heart disease	1·0 (1 to 1)	35 (29 to 39)
2·0 (1 to 2)	2 Stroke	2 Stroke	2·0 (2 to 2)	26 (14 to 32)
3·0 (3 to 4)	3 Lower respiratory infections	3 COPD	3·4 (3 to 4)	−7 (−12 to 0)
4·0 (3 to 4)	4 COPD	4 Lower respiratory infections	3·6 (3 to 4)	−18 (−24 to −11)
5·0 (5 to 5)	5 Diarrhoea	5 Lung cancer	5·8 (5 to 10)	48 (24 to 61)
6·1 (6 to 7)	6 Tuberculosis	6 HIV/AIDS	6·4 (5 to 8)	396 (323 to 465)
7·3 (7 to 9)	7 Preterm birth complications	7 Diarrhoea	6·7 (5 to 9)	−42 (−49 to −35)
8·6 (7 to 12)	8 Lung cancer	8 Road injury	8·4 (5 to 11)	47 (18 to 86)
9·4 (7 to 13)	9 Malaria	9 Diabetes	9·0 (7 to 11)	93 (68 to 102)
10·4 (8 to 14)	10 Road injury	10 Tuberculosis	10·1 (8 to 13)	−18 (−35 to −3)

1. Ischaemic heart disease
2. Stroke

Platelet aggregation



J Physiol. (1963), **168**, *pp.* 178–195
With 14 *text-figures*
Printed in Great Britain

THE AGGREGATION OF BLOOD PLATELETS

BY G. V. R. BORN AND M. J. CROSS
From the Department of Pharmacology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C. 2

(*Received* 3 *December* 1962)

Light transmission aggregometry (LTA) is not rocket science

- blood collection
- centrifugation
- stirring at 37 ˚C
- adding an agonist
- measure change in light transmission

Journal of Thrombosis and Haemostasis, **7**: 1029 DOI: 10.1111/j.1538-7836.2009.03458.x

OFFICIAL COMMUNICATION OF THE SSC

Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH

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Conclusions: This is the largest and most detailed survey of LTA practices ever undertaken. It shows a very high variability in LTA practices worldwide, and, as a consequence, methodological standardization is necessary. The information gathered in this survey will be helpful in the development of ISTH methodological guidelines for LTA.

Journal of Thrombosis and Haemostasis, **11**: 1183–1189 DOI: 10.1111/jth.12231

OFFICIAL COMMUNICATION OF THE SSC

Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH

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Recommendations of the working party on standardization of light transmission aggregometry, of the SSC subcommittee of platelet physiology of ISTH

The recommendations of the Working Party on standardization of LTA include 70 statements, which have been grouped into eight sections: (i) clinical usefulness of LTA; (ii) pre-analytical variables; (iii) blood collection; (iv) preparation of PRP and platelet-poor plasma (PPP); (v) assessment of PRP quality; (vi) methodology; (vii) choice of agonists; and (viii) evaluation and reporting of results.

Lessons learned?

- major clinical problem
- easy assay (LTA)
- >50 year later: no standardisation
- no comparison of results possible between instruments and laboratories
- no reference materials (“standards”)
- no validated assays
- no multi-centre trials

***EV are not platelets*. Do we need any standardisation of EV measurements?**

EV diameter in human plasma

Diameter (nm)	Method	Reference
20-50	EM	Wolf, 1967
200-800	EM	Turiák, 2011
180	EM	György, 2011
80	AFM	Dragovic, 2011
30-1,000	Cryo-EM	Brisson, 2014

EV concentration in human plasma

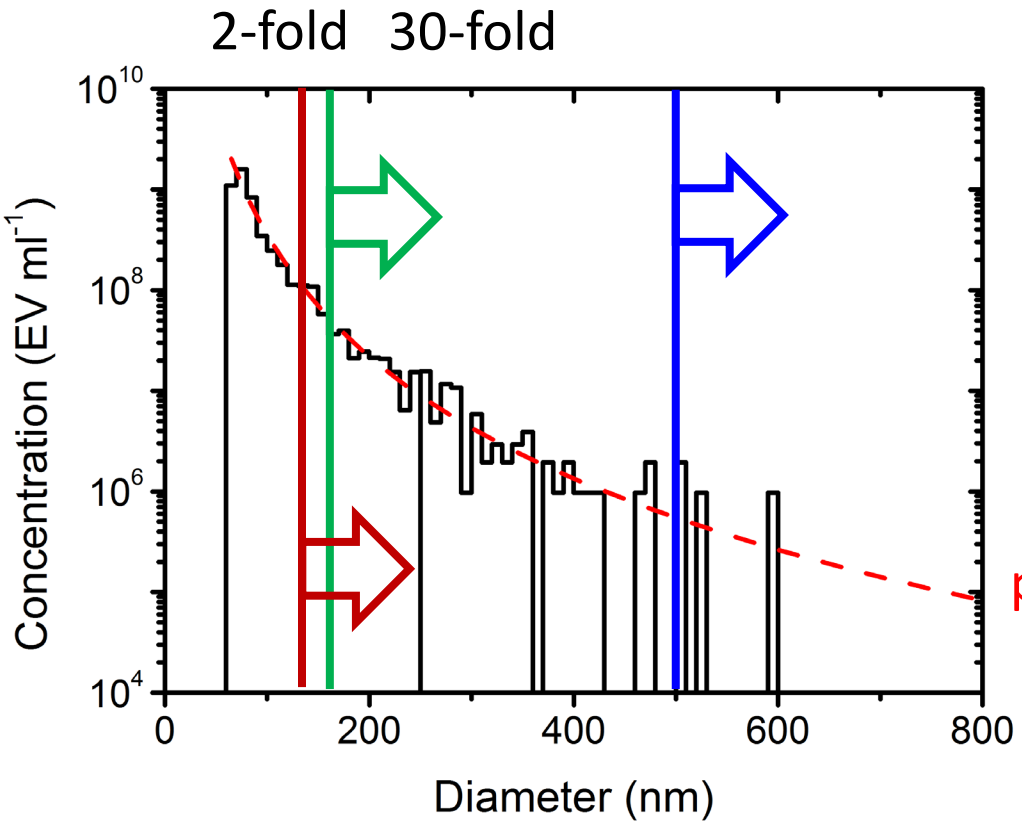
Concentration (mL^{-1})	Method	Reference
1.1×10^4 - 7.0×10^8	AFM	Yuana, 2010
5.8×10^6 - 9.3×10^6	FCM	Biro, 2003
1.5×10^8 - 2.5×10^8	TRPS	De Vrij, 2013
1.2×10^{10} - 1.5×10^{12}	NTA	Dragovic, 2011

Yes: we do need standardisation of EV measurements

Why is standardisation of EV measurements difficult?

- EV originate from complex fluids
- EV are small (diameter <100 nm for >80% of EV)
- EV differ
  - 25-fold in diameter
  - 20,000-fold in volume
  - 300,000-fold in concentration
  - 10,000,000-fold in scattered light
- instruments differ >100-fold in sensitivity
- signals are in arbitrary units

Problem: instruments differ in sensitivity

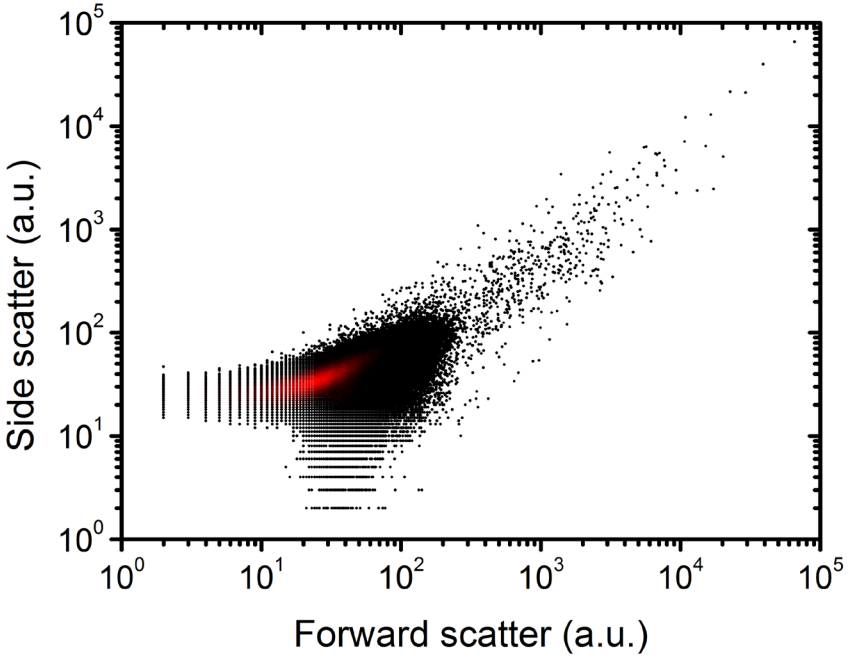


power-law relation*

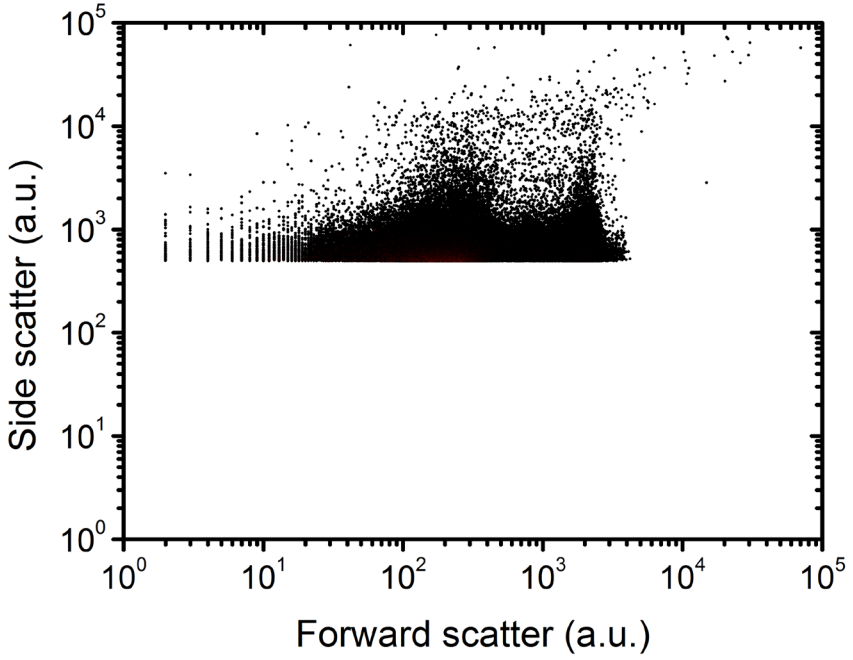
*van der Pol et al. *JTH* (2014)

Problem: signals are in arbitrary units

same population of erythrocyte EV



Apogee A50-micro



Becton Dickinson FACSCanto II

EV standardisation in 2016

A standardized method of determining the concentration of extracellular vesicles using tunable resistive pulse sensing. Vogel R et al, 2016 submitted

Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. Hisada Y et al. *Thromb Res* 2016;**139**:90-7

Standardisation of flow cytometry measurements

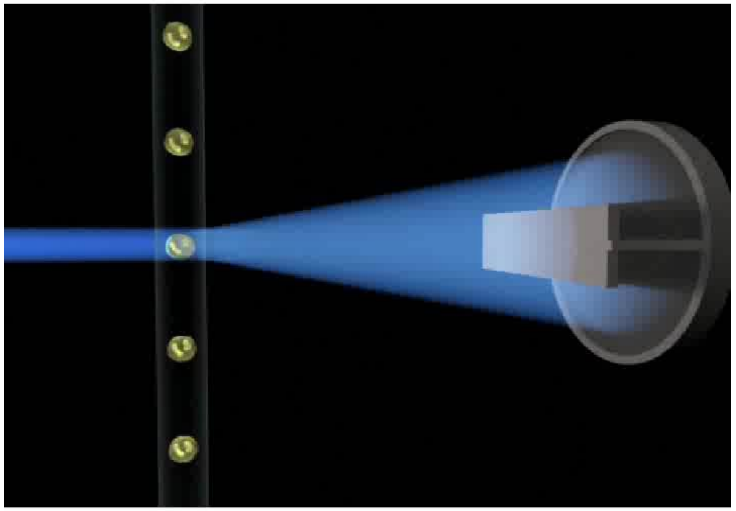
- fluorescence-based standardization (ISEV-ISAC-ISTH)
  - Nolan et al. 2016
- scatter-based standardization (ISTH)
  - *Robert S et al. JTH 2008*
  - *Cointe S et al. 2016, submitted*
  - *Coumans FAW, et al. 2016, in prep*

- common goal, no competition
- combine advantages of approaches

Outline

- standardisation
  - relevance
  - extracellular vesicle (EV) measurements
  - recent developments
- quantitative light scattering to standardise EV measurements by flow cytometry
- outlook: size and refractive index determination by flow cytometry

Flow cytometry



- flow cytometry is the most commonly used method to count EV

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

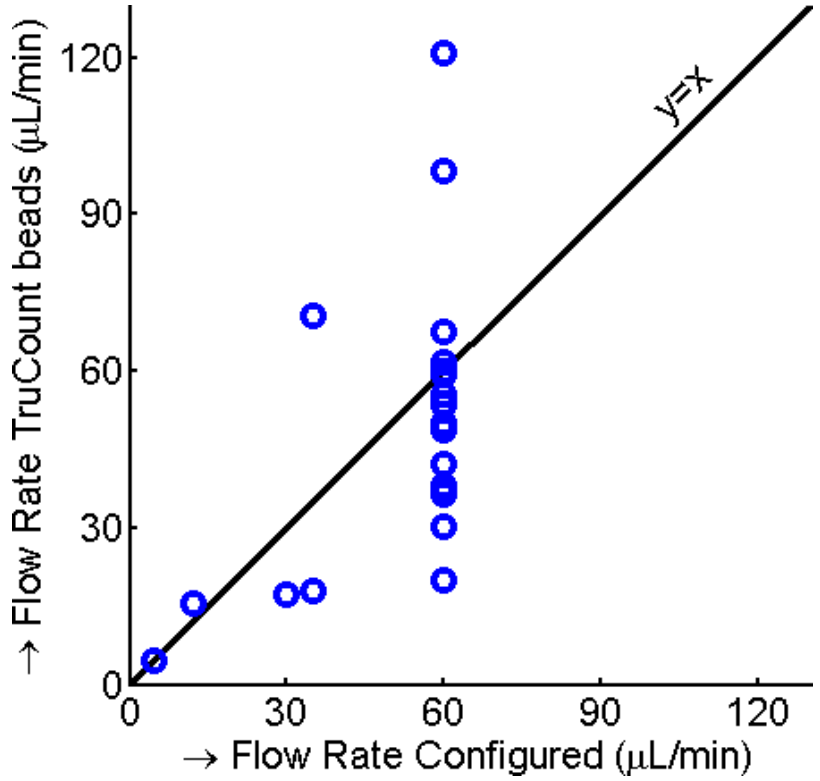
- erythrocyte EV from blood bank concentrate
  - CD235a-FITC labeled
  - trigger on most sensitive scatter channel
  - exclude EV similar to isotype

Flow cytometry standardisation 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

Determine flow rate

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

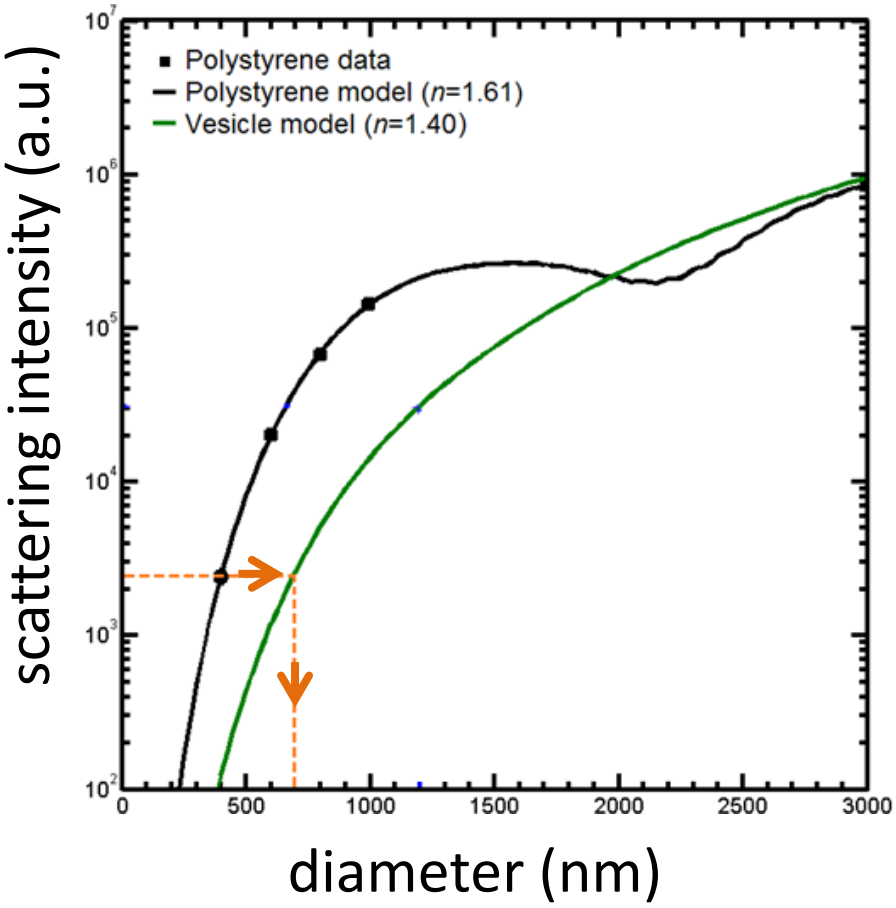


Flow cytometry standardisation approach

- 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

Earlier ISTH studies: gate on polystyrene beads

BC Gallios (forward scatter)

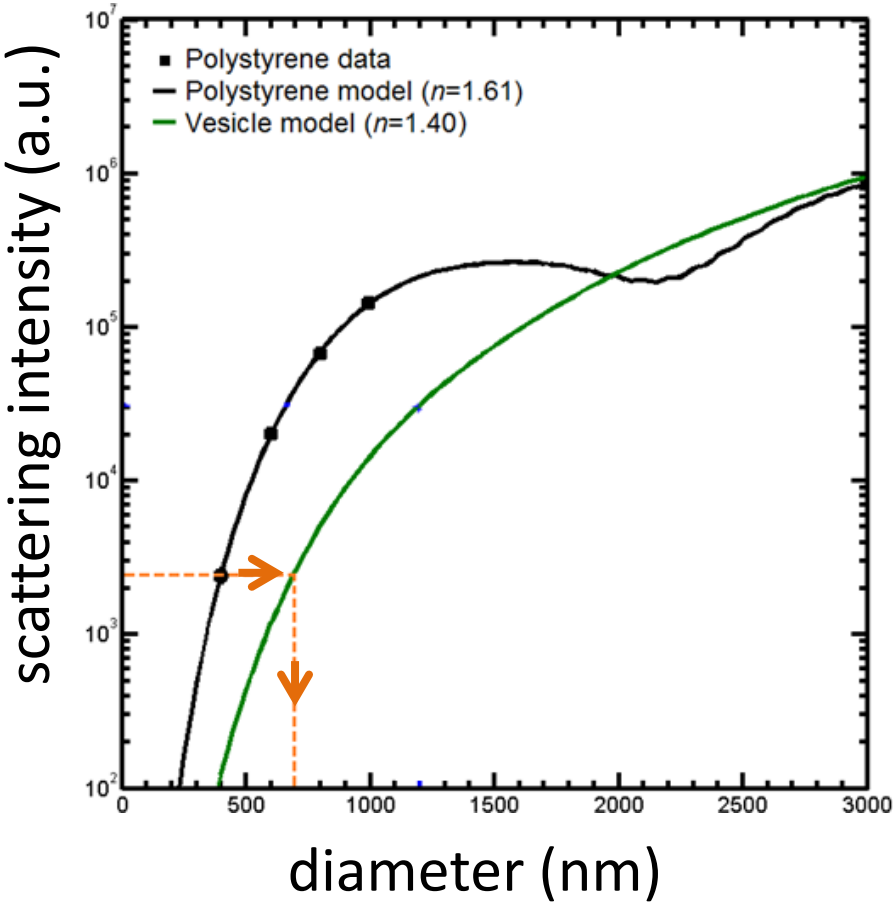


Robert et al. *JTH* (2008)

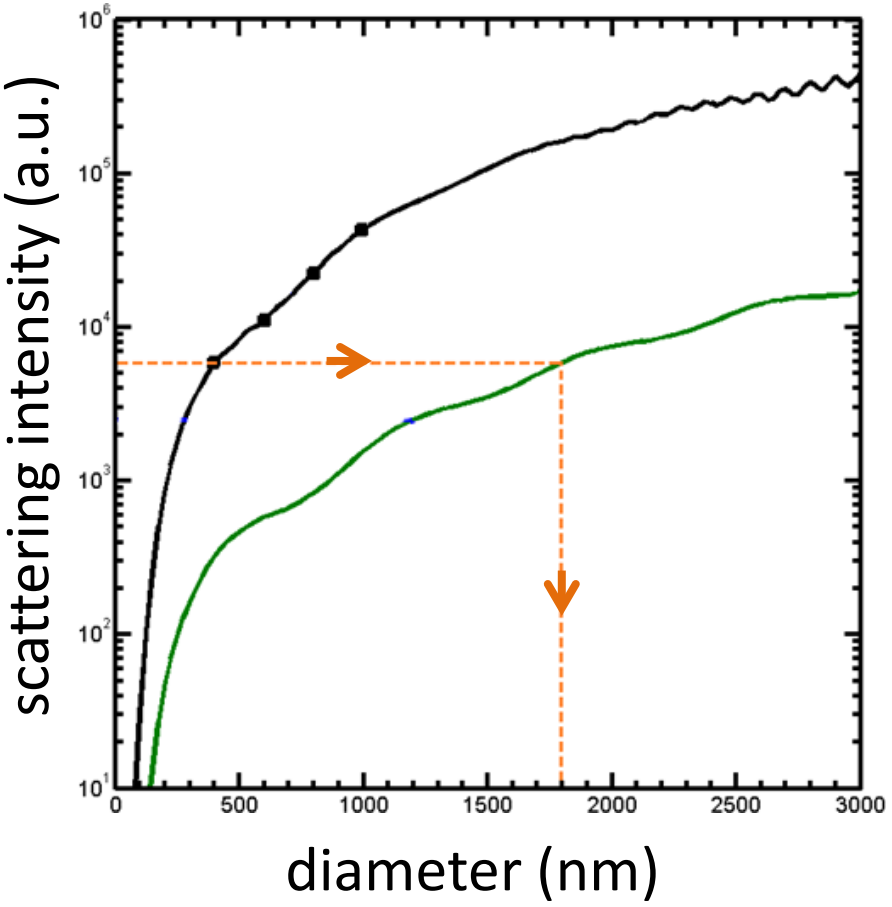
Lacroix et al. *JTH* (2010)

Earlier ISTH studies: gate on polystyrene beads

BC Gallios (forward scatter)

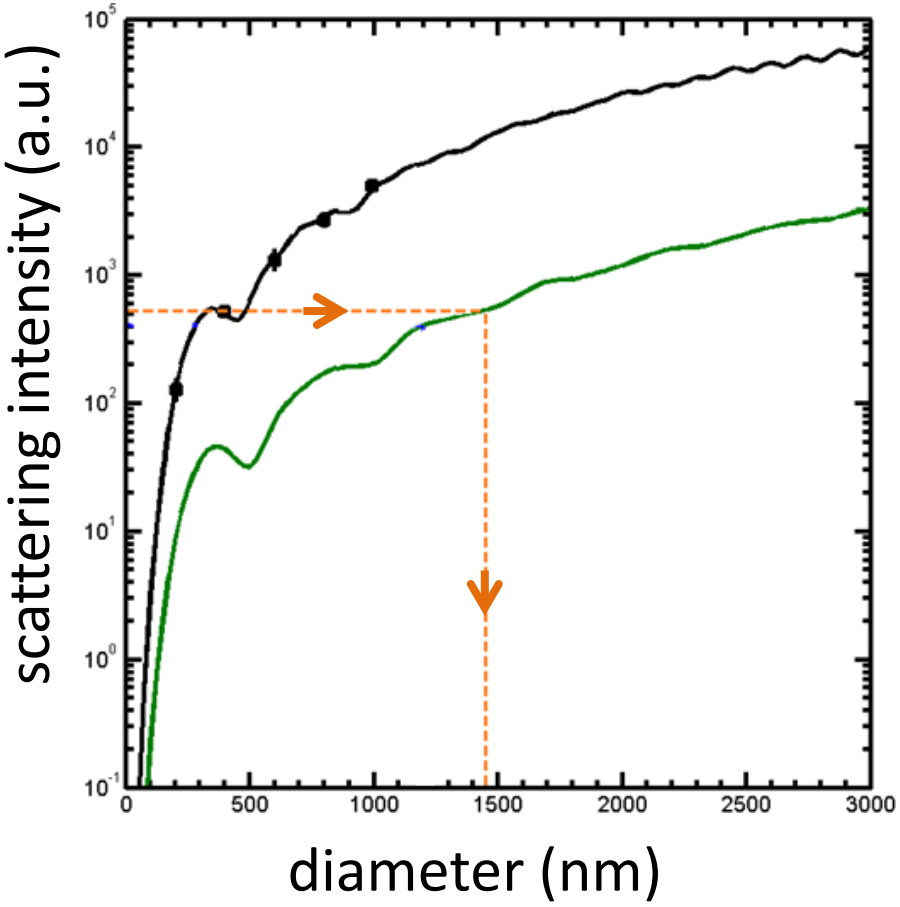


BD LSR II (side scatter)

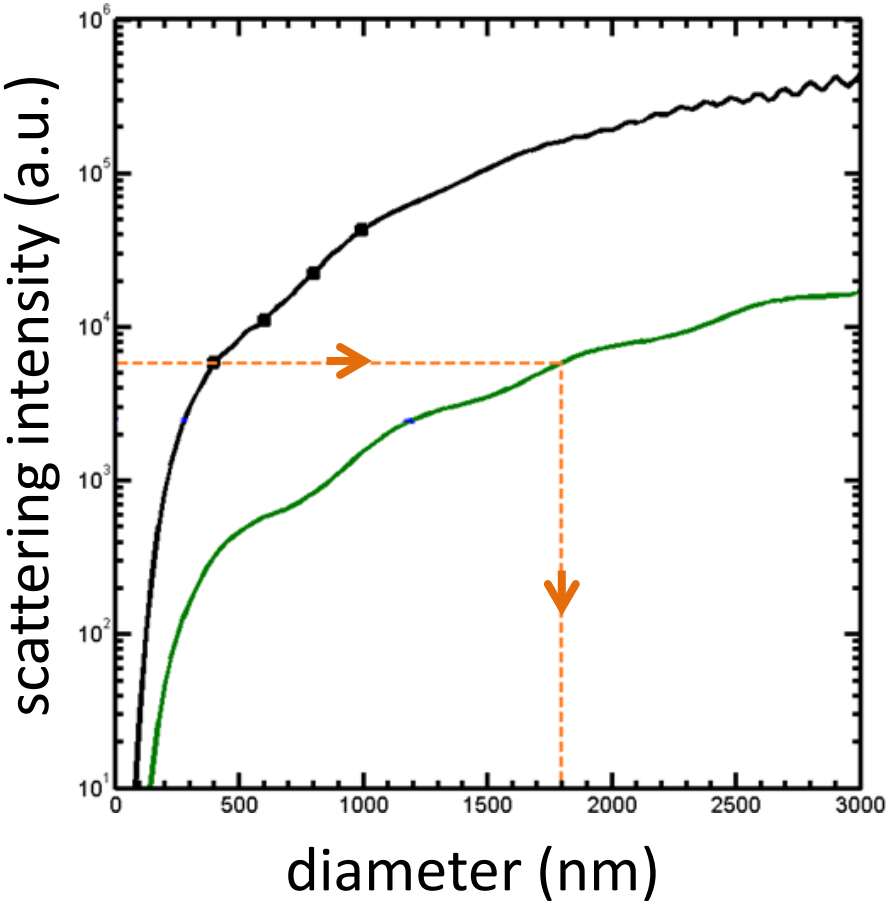


Earlier ISTH studies: gate on polystyrene beads

BC Astrios MoFlo (side scatter)



BD LSR II (side scatter)

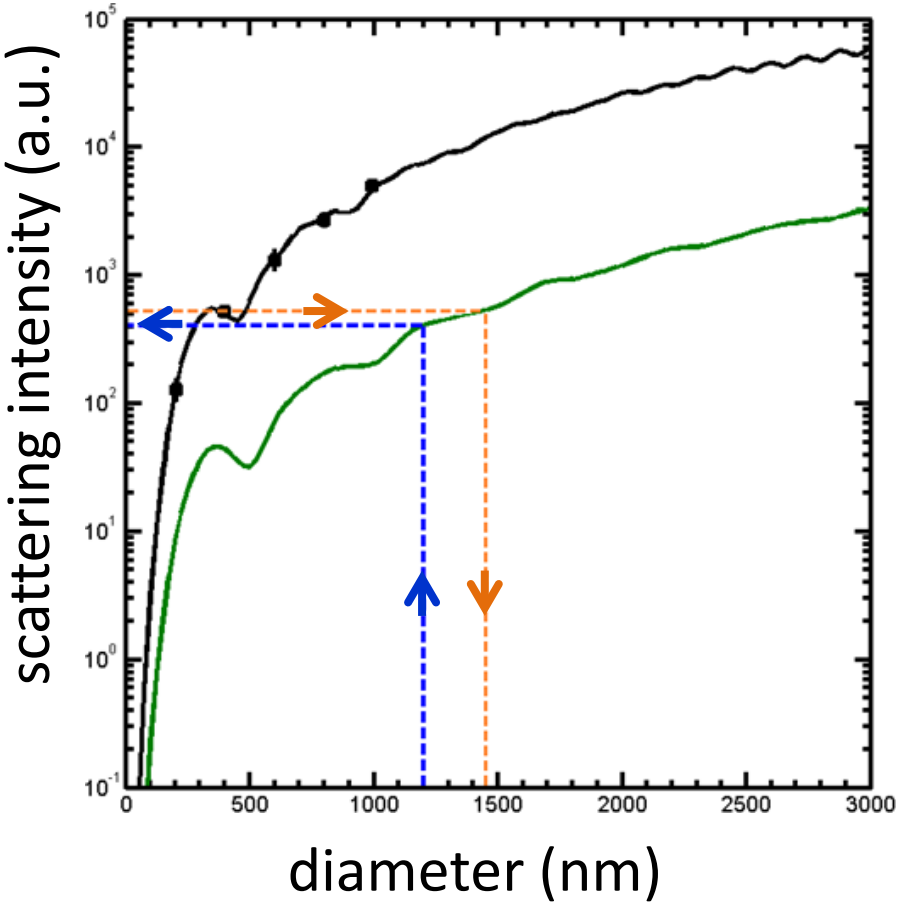


Robert et al. *JTH* (2008)

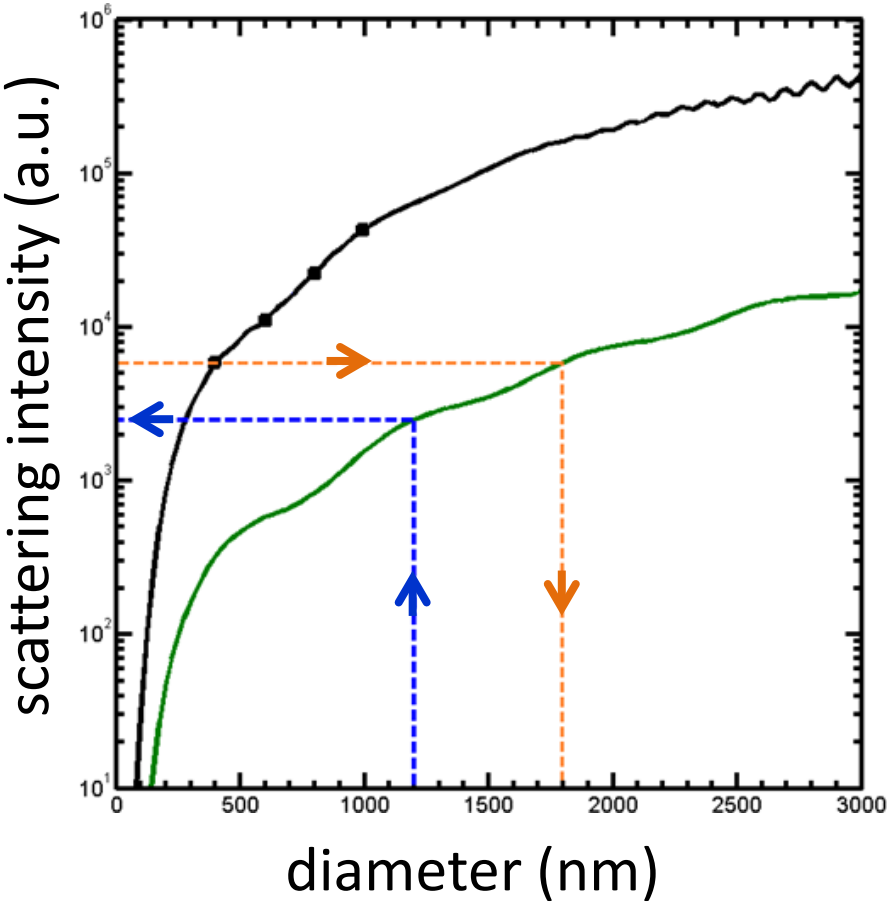
Lacroix et al. *JTH* (2010)

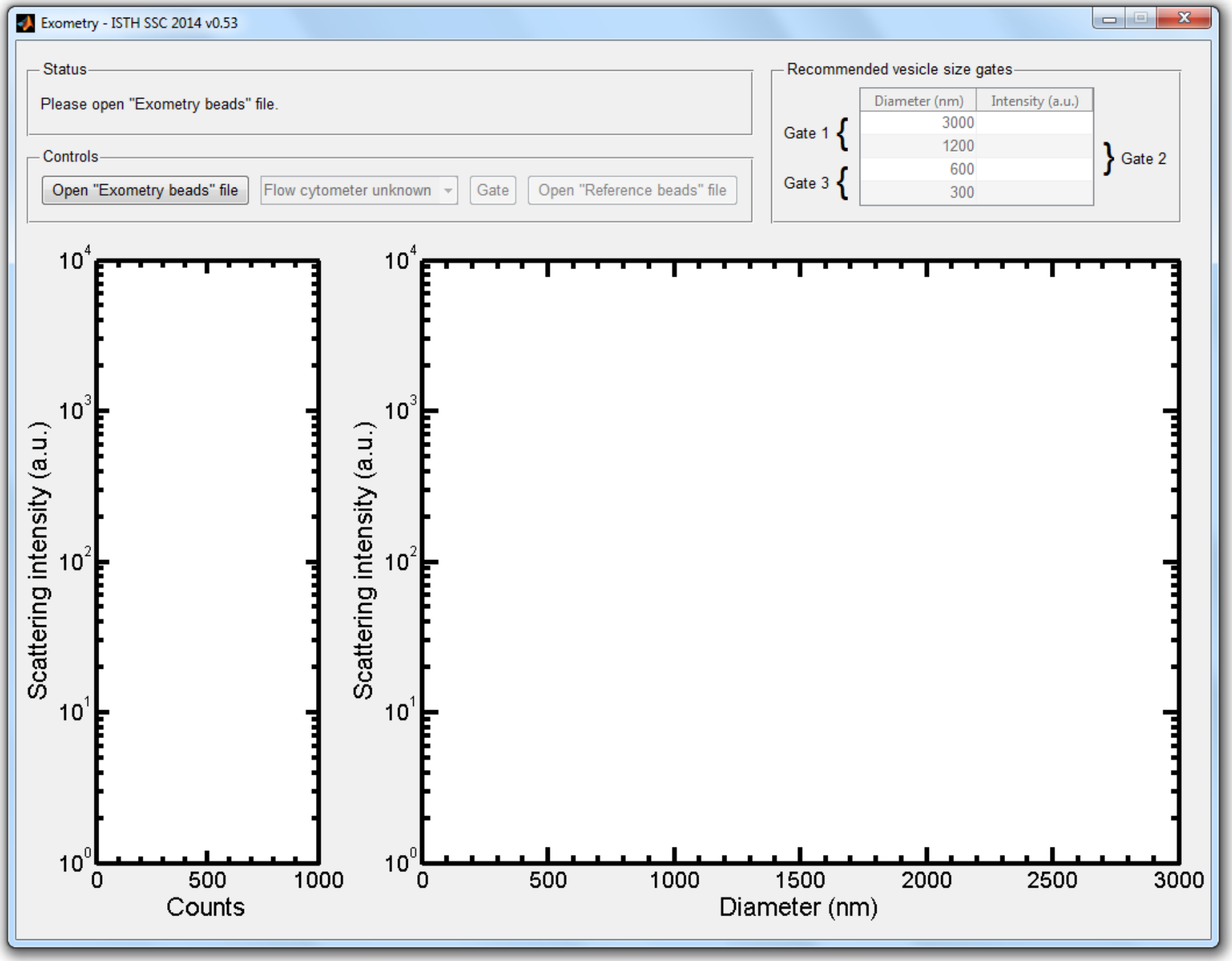
2016: relate scatter (a.u.) to diameter (nm)

BC Astrios MoFlo (side scatter)



BD LSR II (side scatter)




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

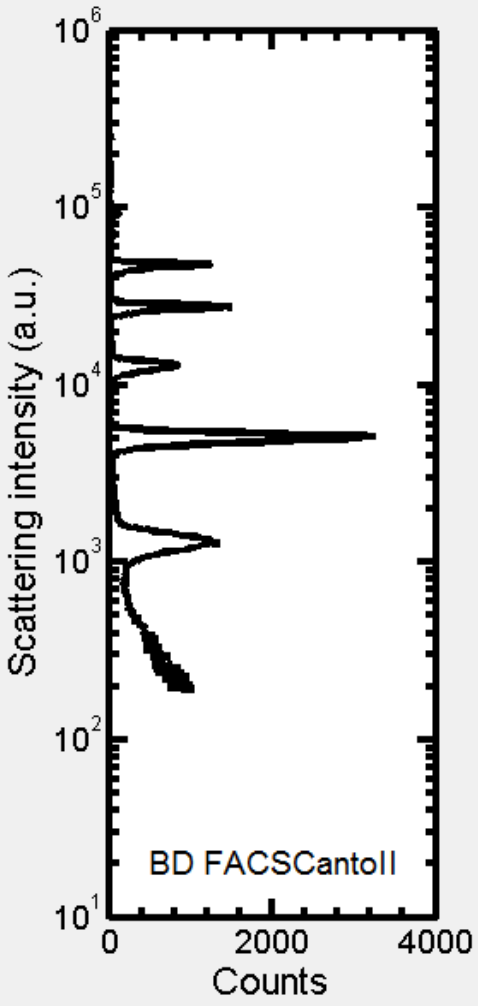
Please select detector and click "Gate" to obtain vesicle size gates.

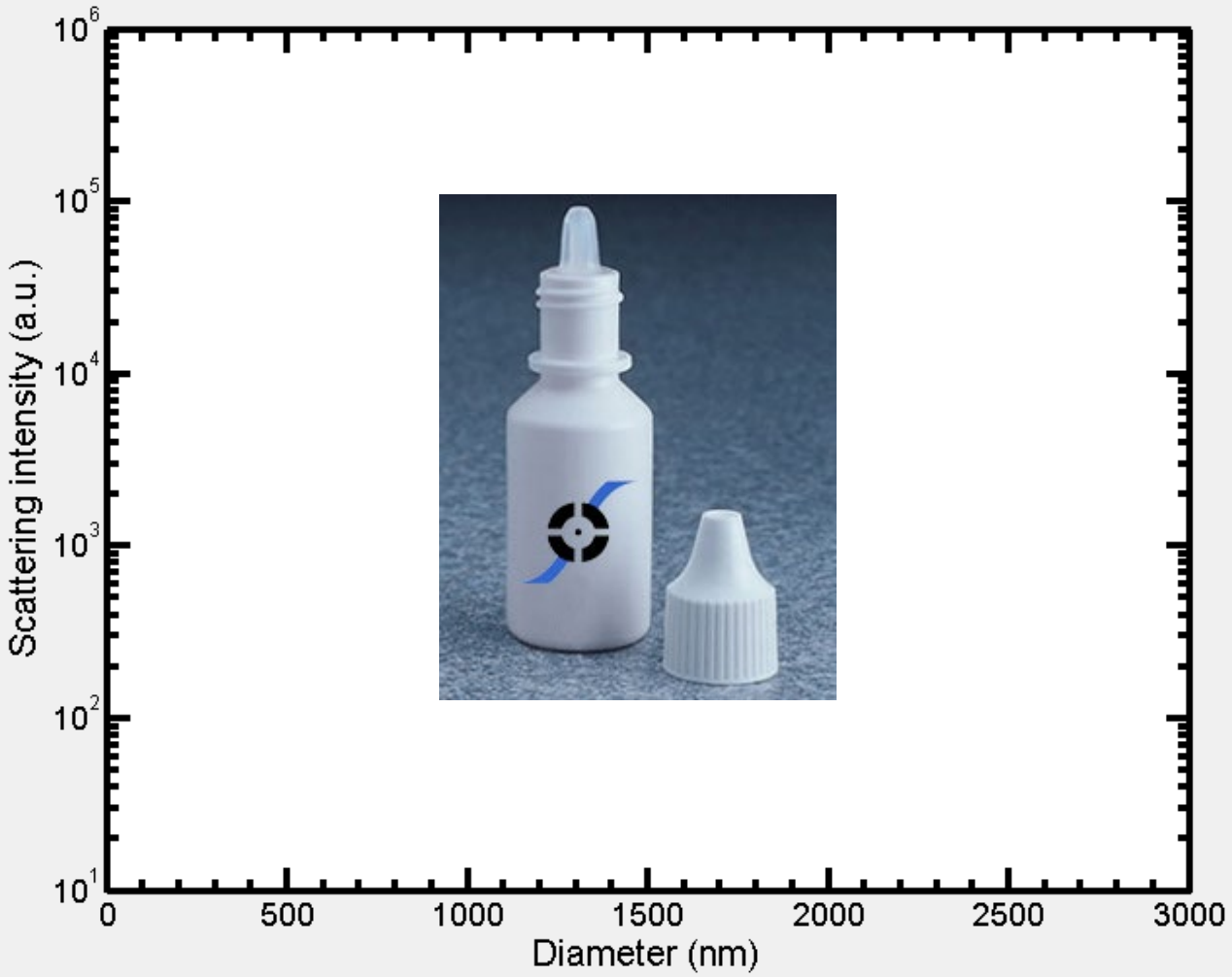
Controls

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.

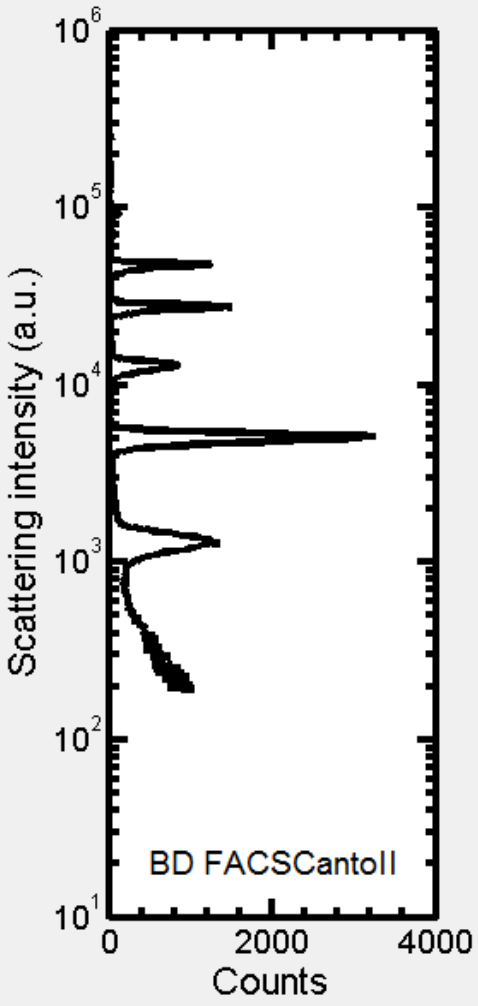
Controls

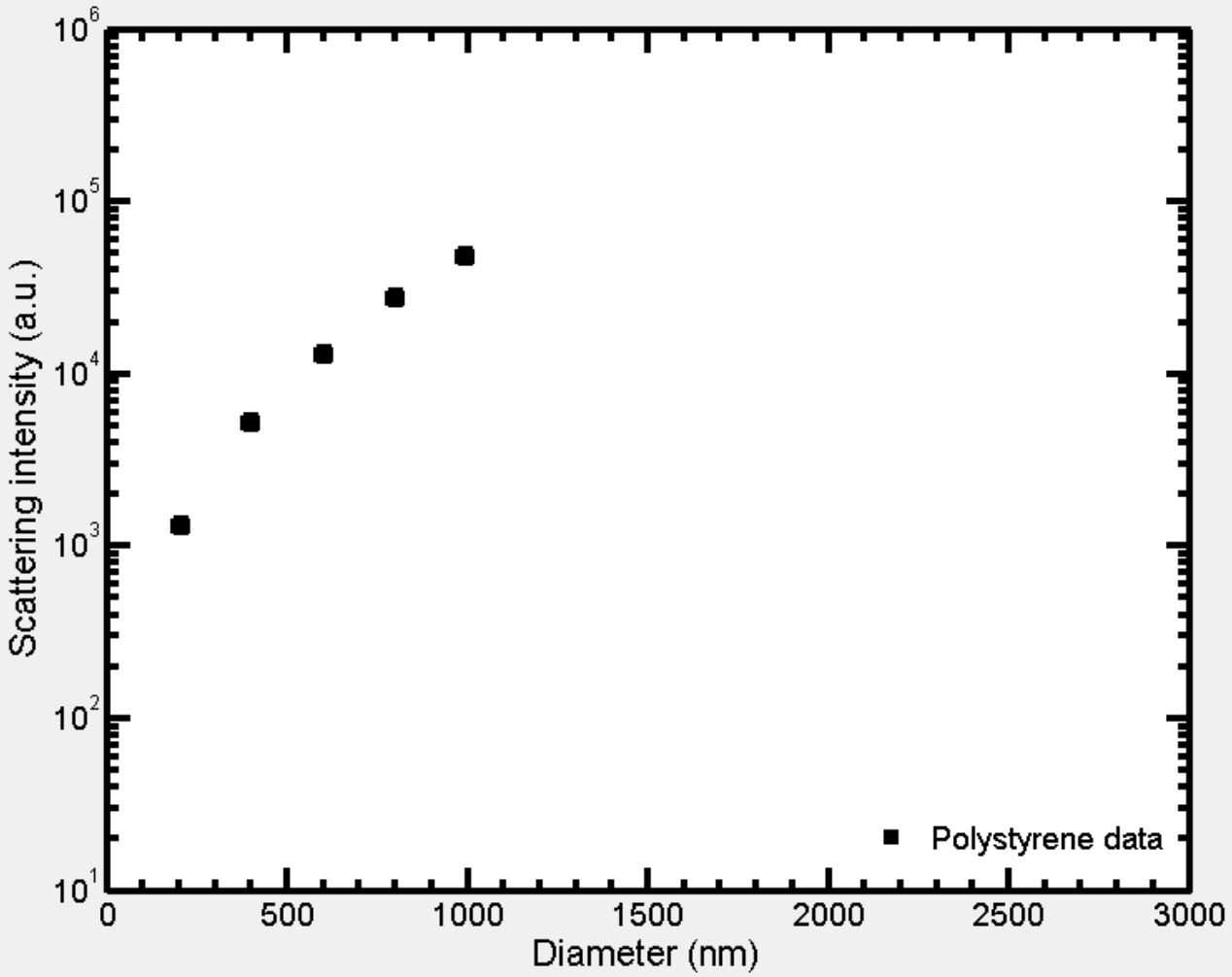
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

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

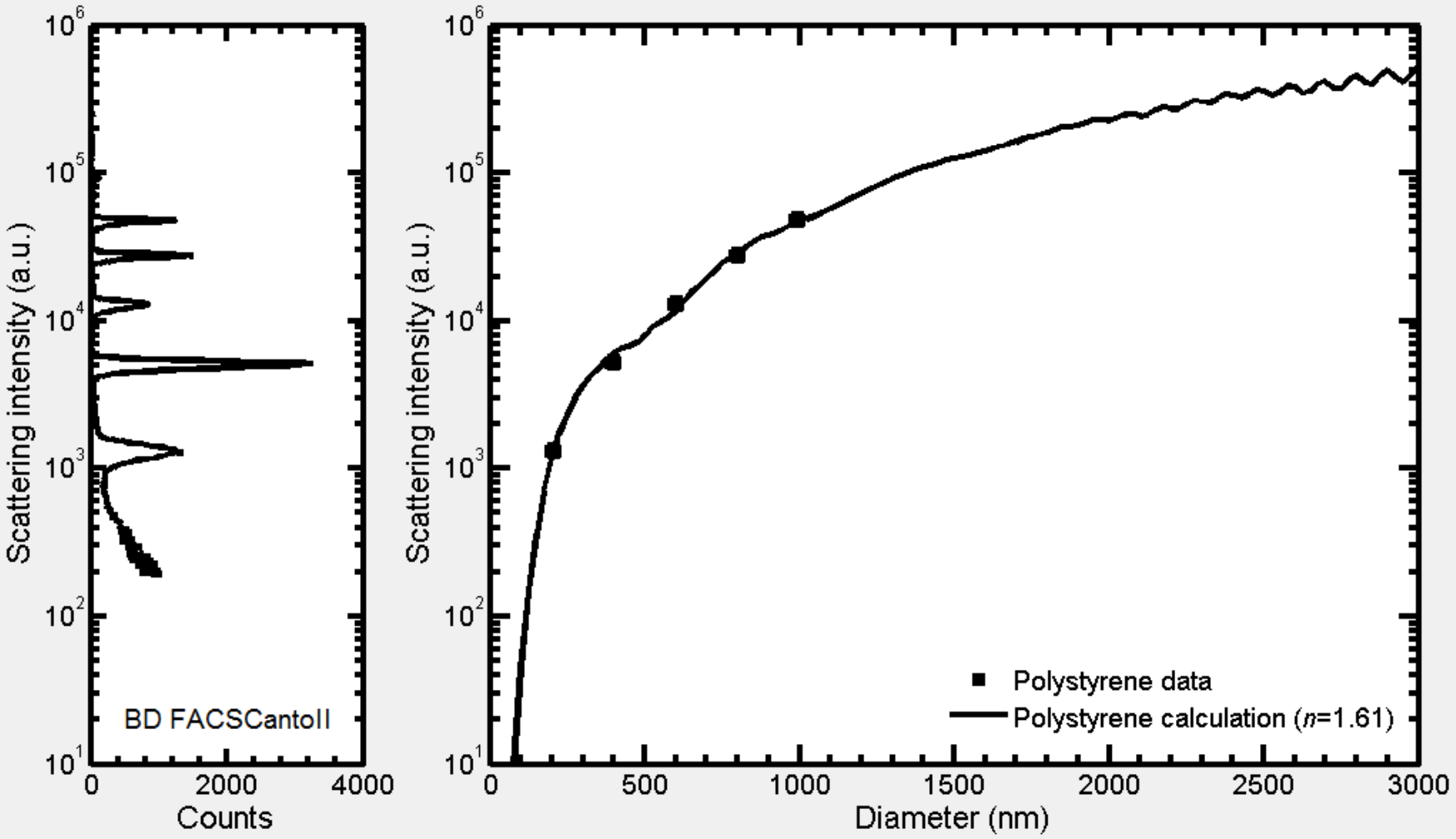
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.

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

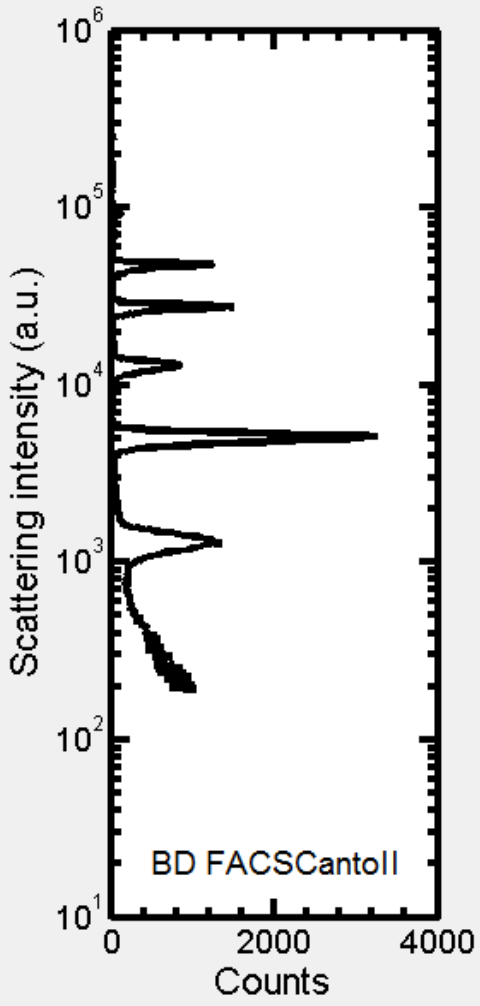
Congratulations, vesicle size gates determined, estimated error less than 0%.

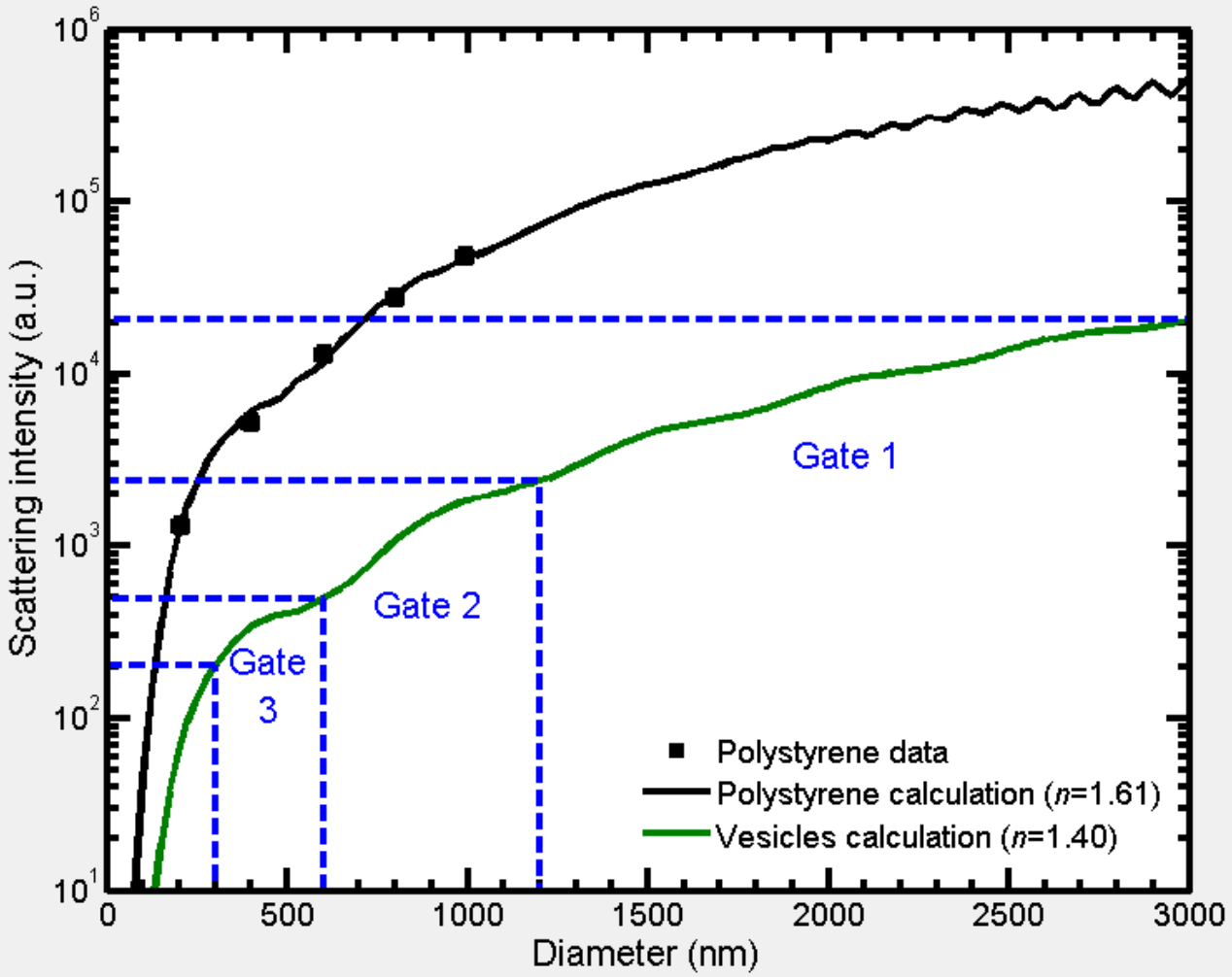
Controls

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

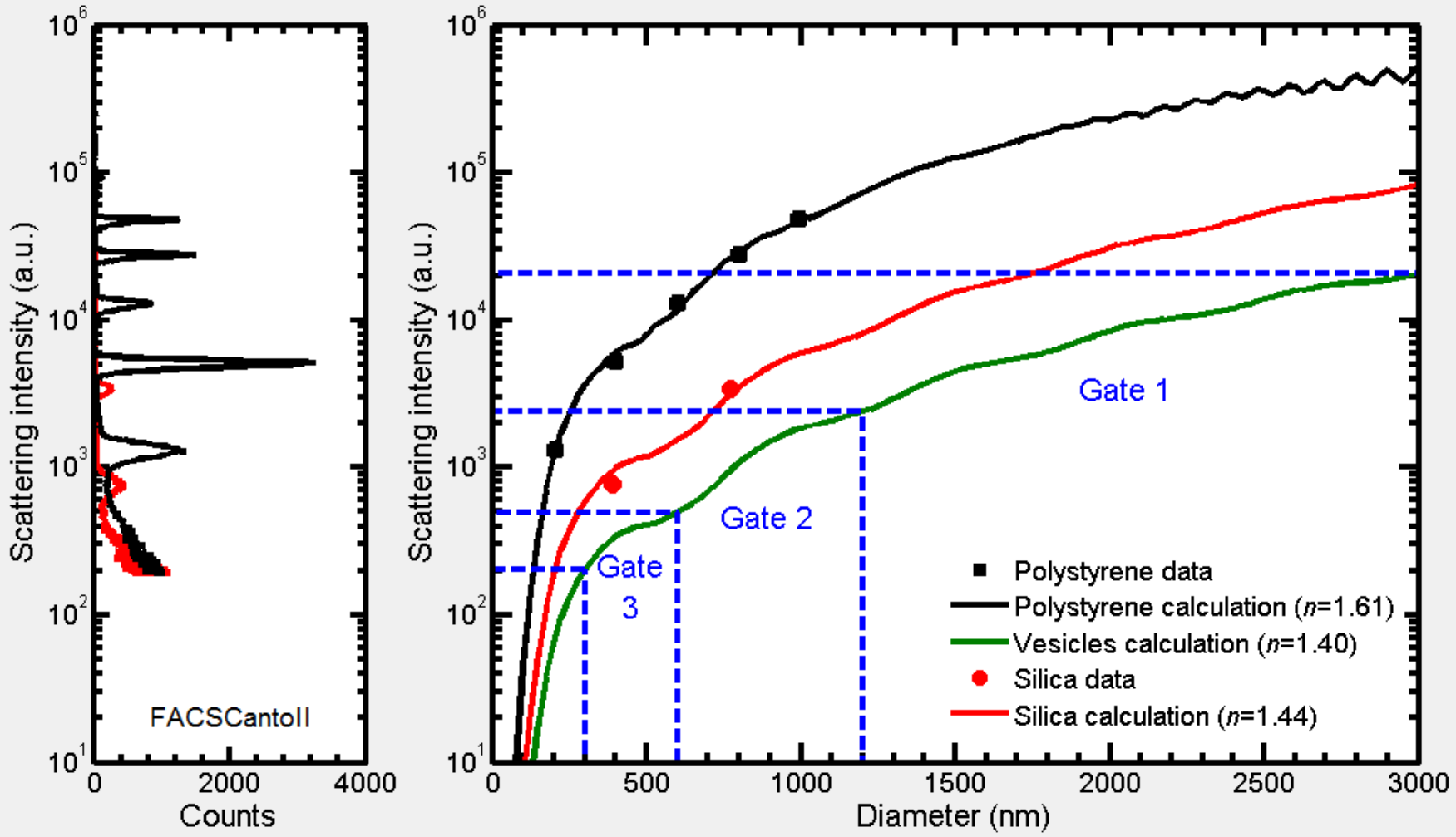
Congratulations, validation succeeded, estimated error less than 4%.

Controls

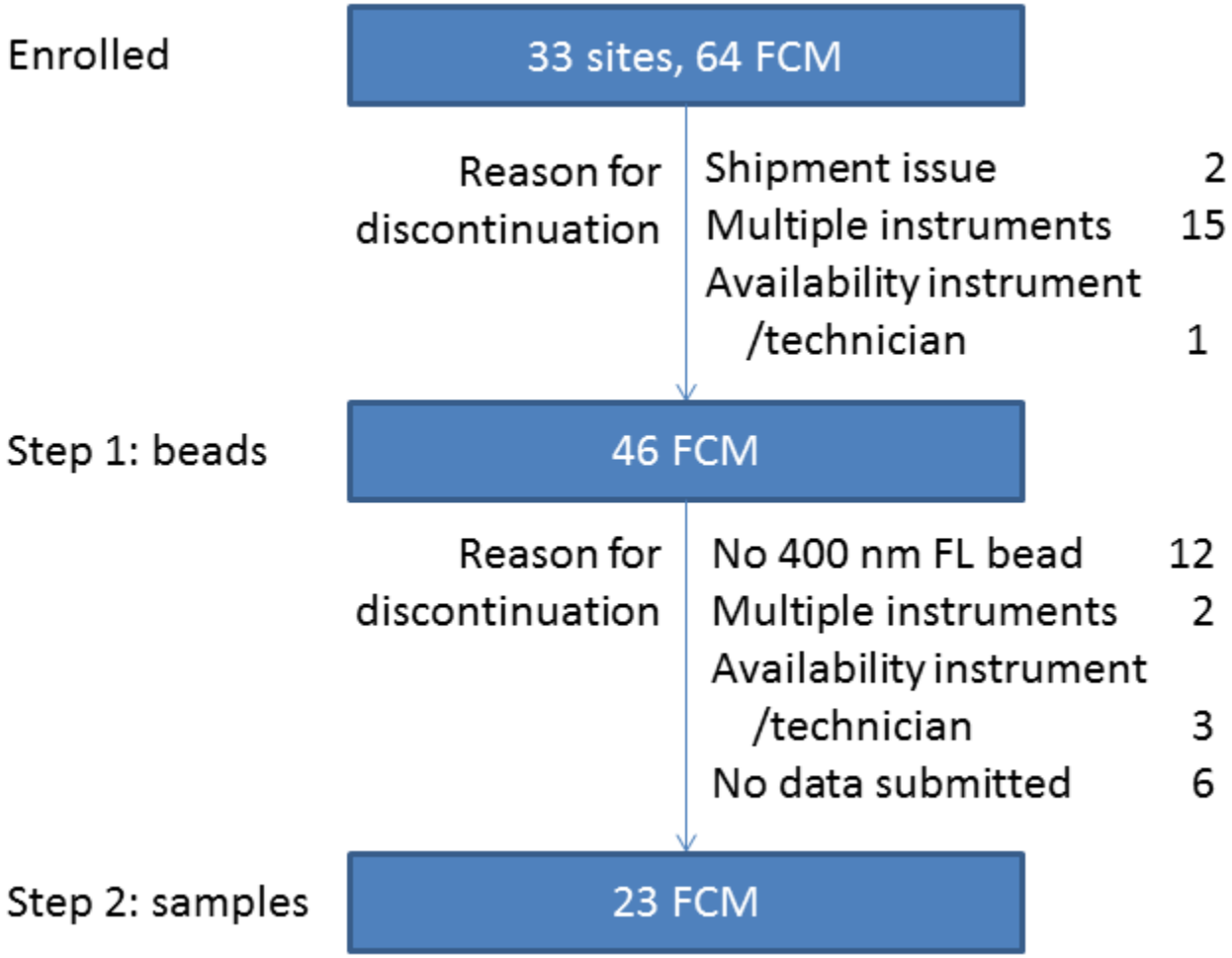
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

Recommended vesicle size gates

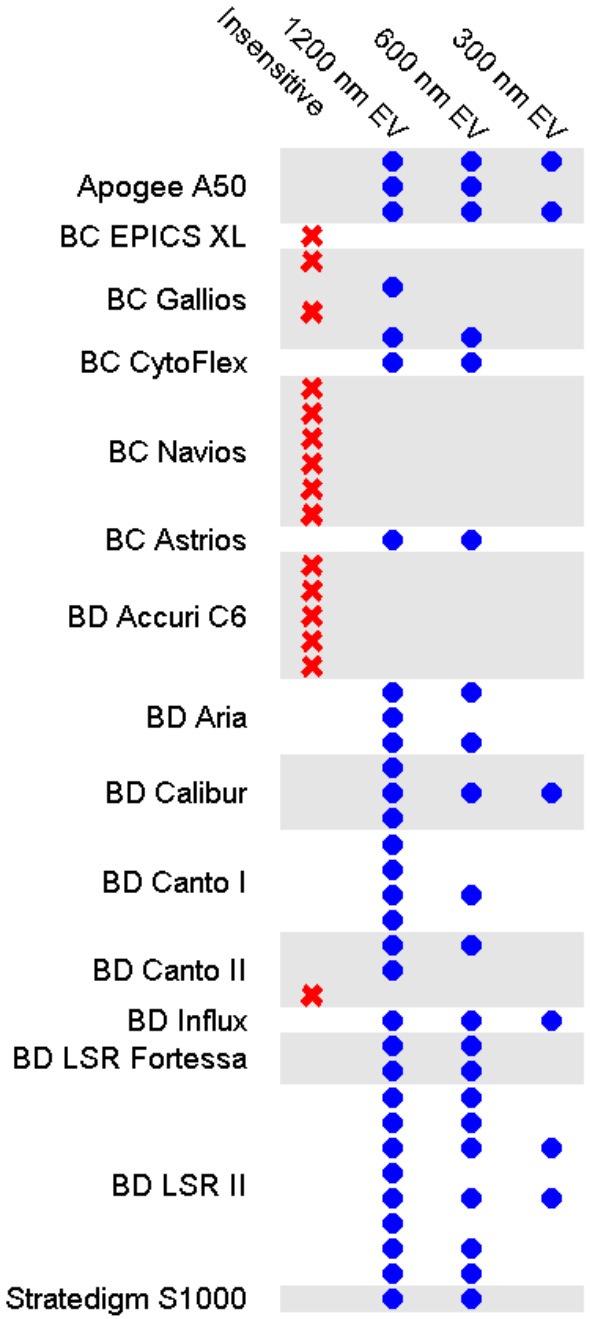
	Diameter (nm)	Intensity (a.u.)	
Gate 1	3000	20636	
	1200	2380	Gate 2
Gate 3	600	497	
	300	202	



Exclusion of flow cytometers (FCM)



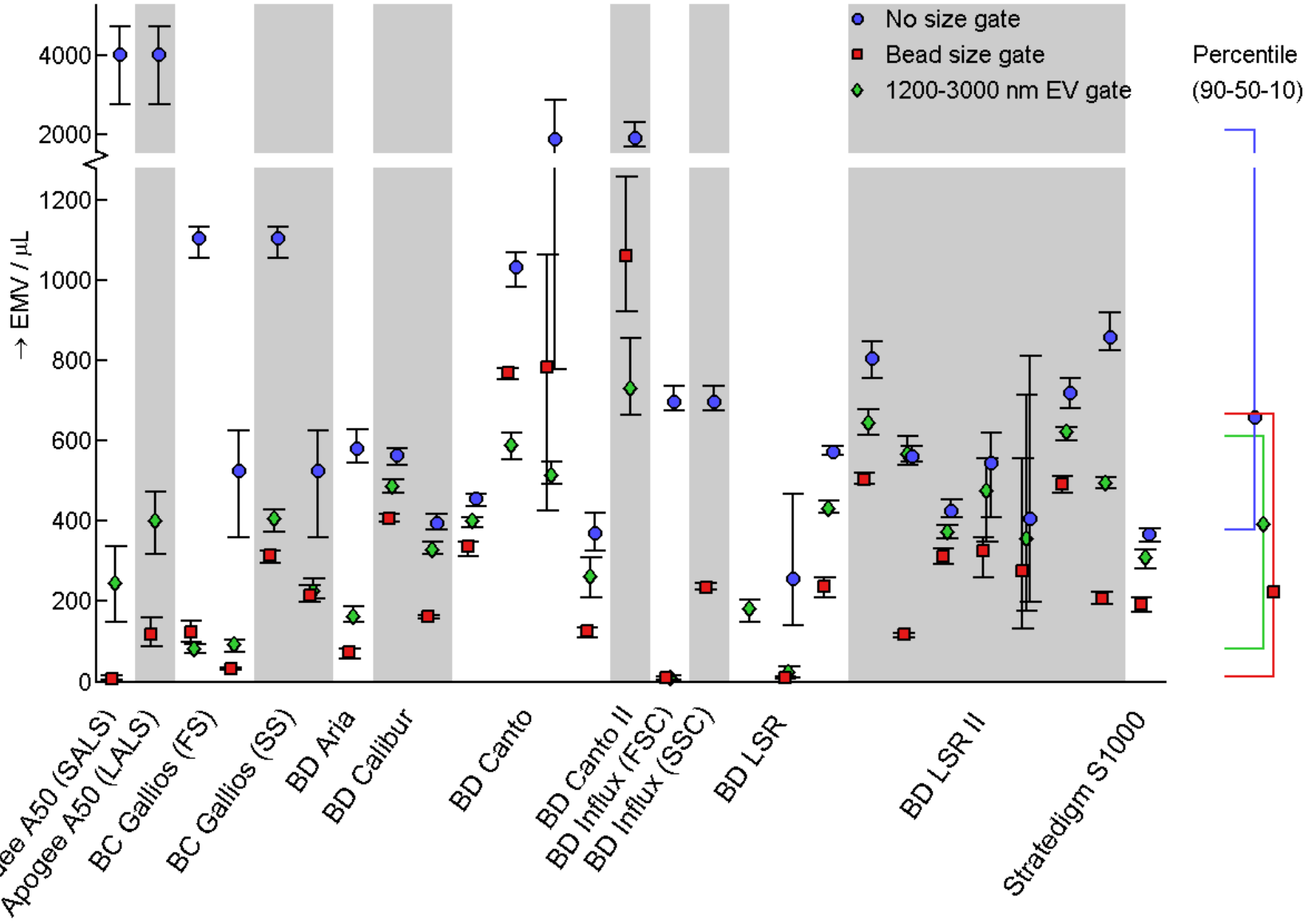
Instrument sensitivity



Flow cytometry standardisation 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

Reproducibility of 1200-3000 nm EV



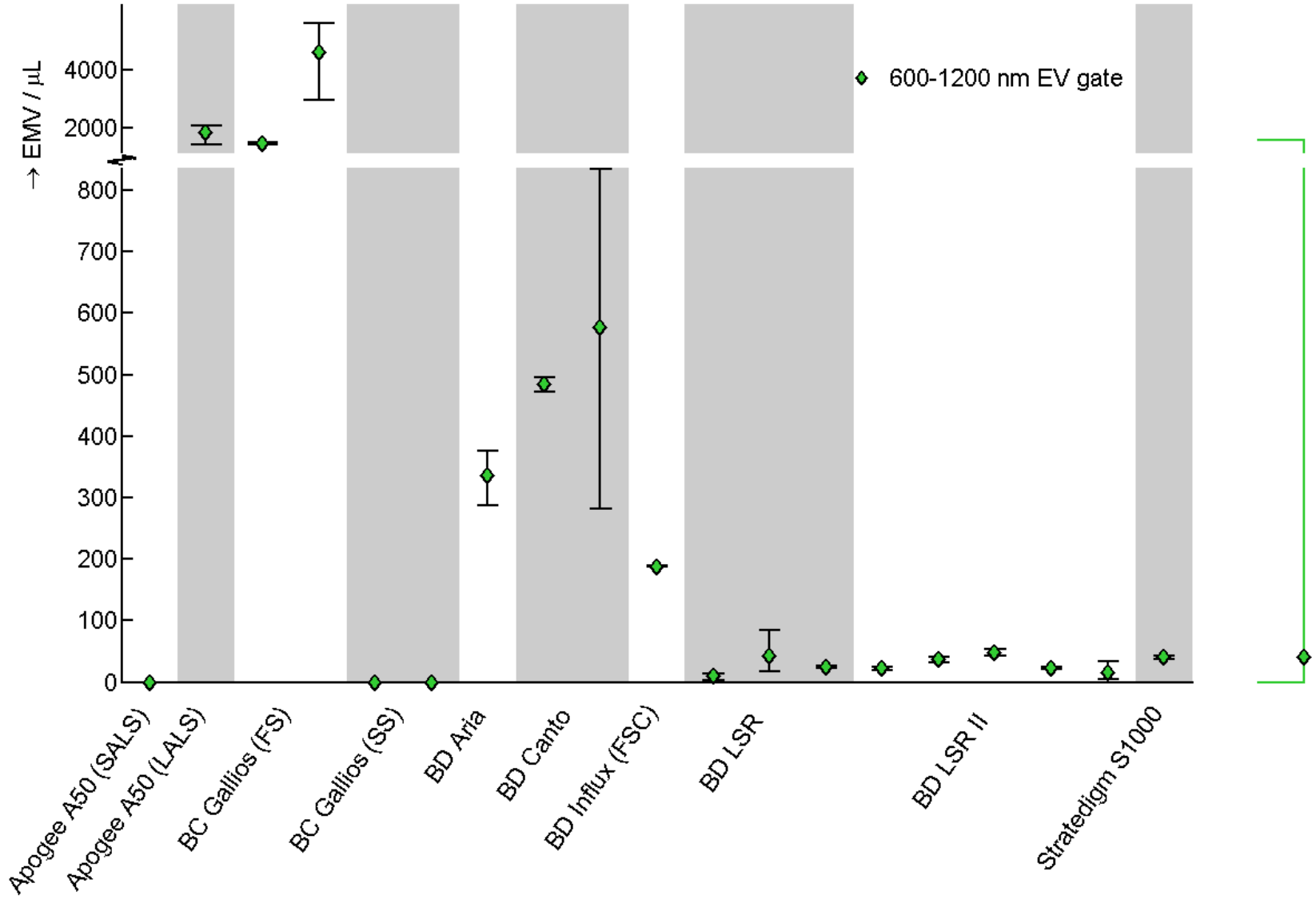
Reproducibility of 1200-3000 nm EV

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

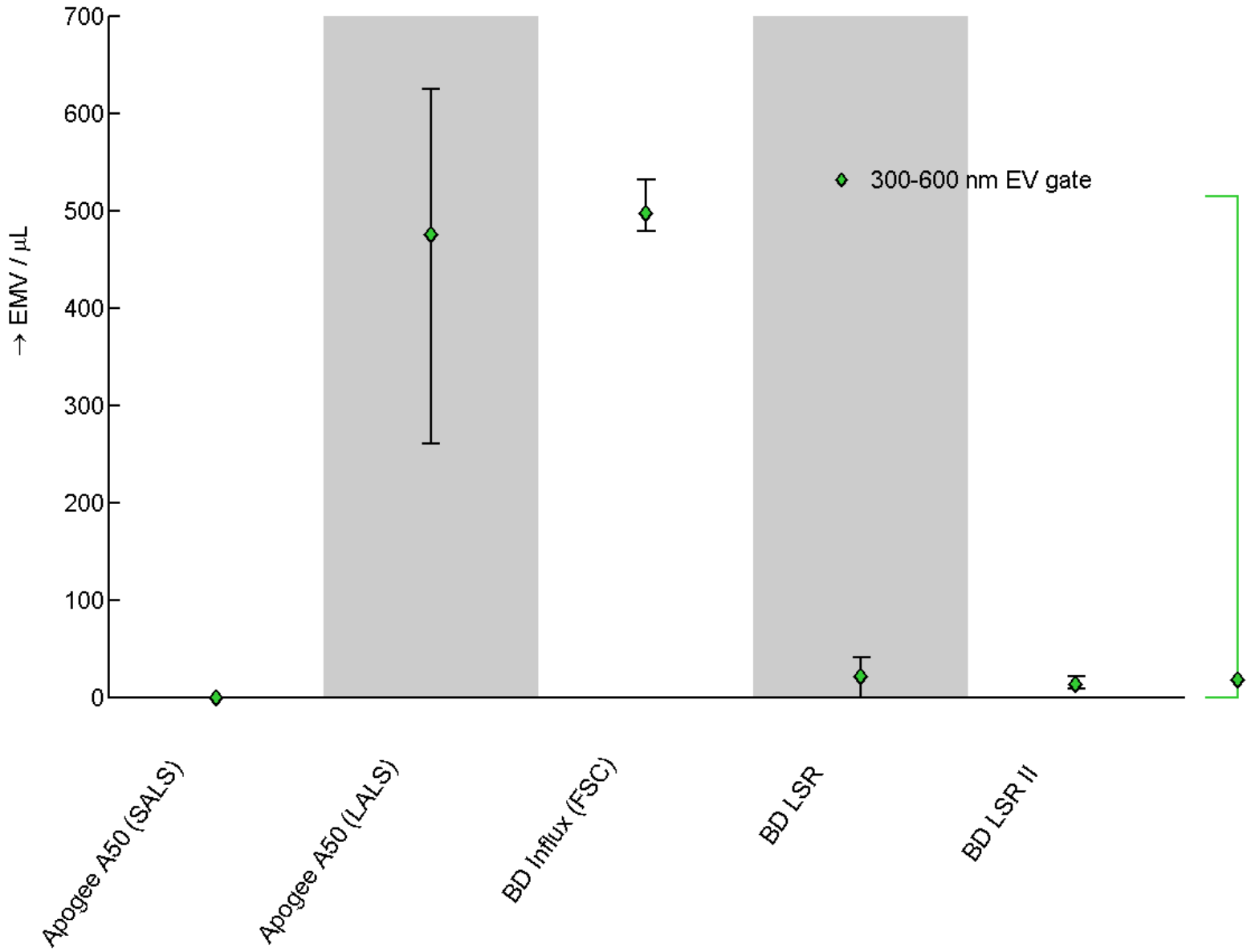
%CV = standard deviation / mean * 100%

Preliminary results

Reproducibility of 600-1200 nm EV



Reproducibility of 300-600 nm EV



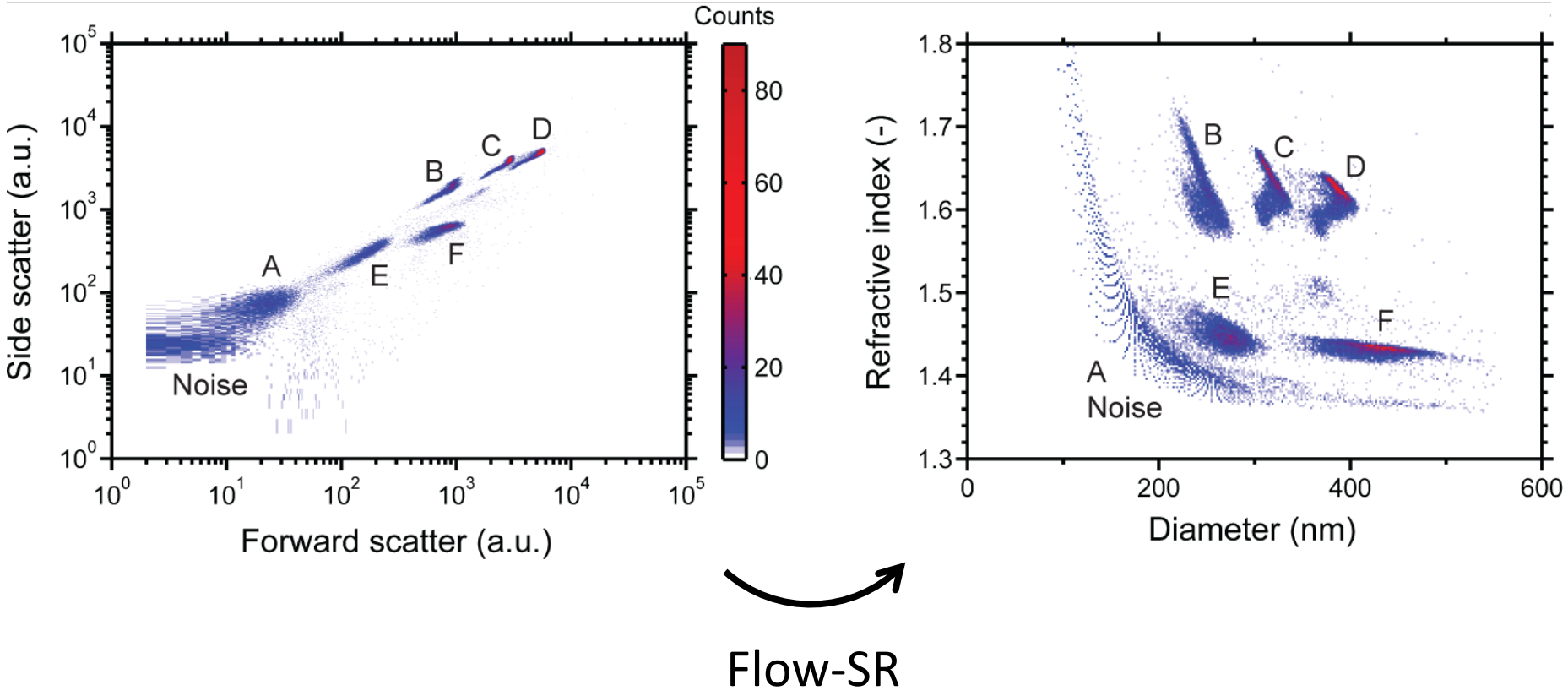
Conclusions

- standardisation of EV measurements is essential
- flow cytometry
  - 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%)

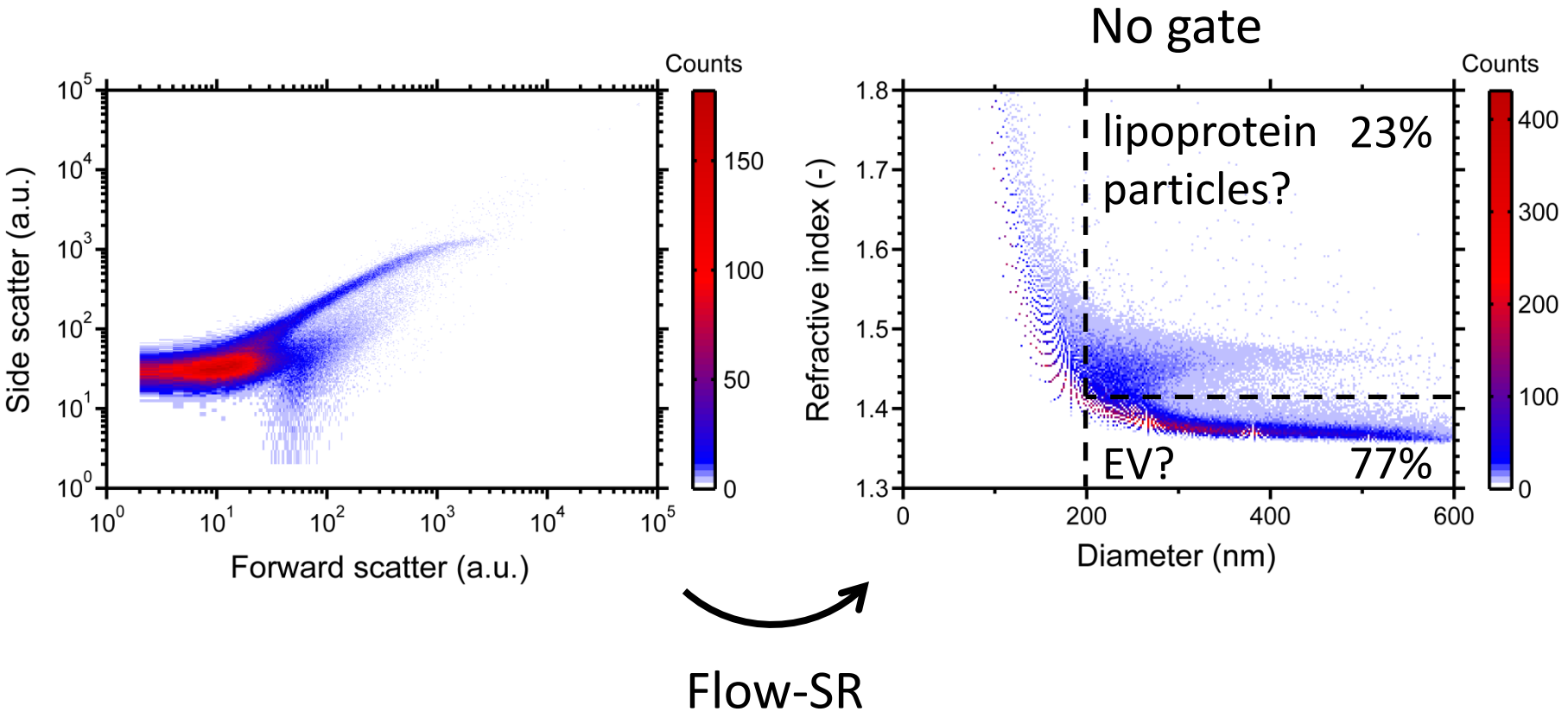
Discussion

- 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

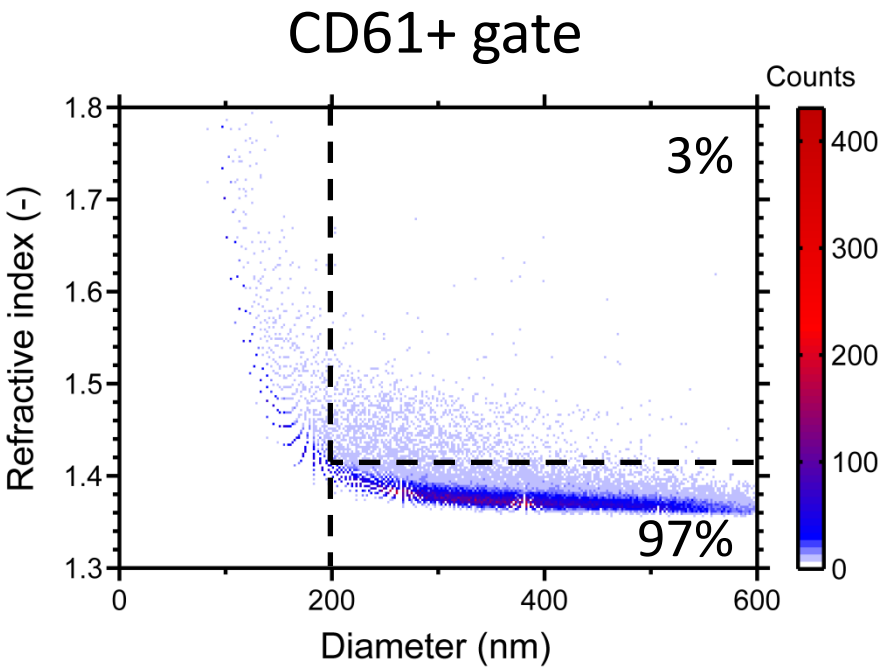
Outlook: size and refractive index determination by flow cytometry

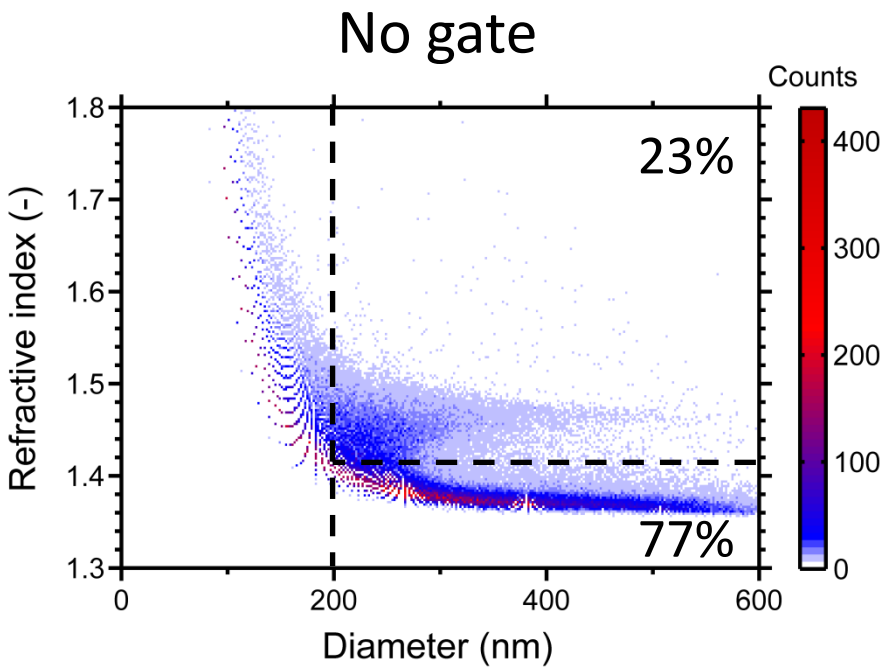


Supernatant of outdated platelet concentrate



Supernatant of outdated platelet concentrate





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

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- Info: edwinvanderpol.com

Vesicle Observation Center

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EXOMETRY

METVES

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