Single extracellular vesicle analysis: nanoparticle tracking analysis, tunable resistive pulse sensing, and scatter-based flow cytometry

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Frank Coumans

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Vesicle Observation Center

International Society on Thrombosis and Haemostasis

EXOMETRY

METVES

October 26th, 2016

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

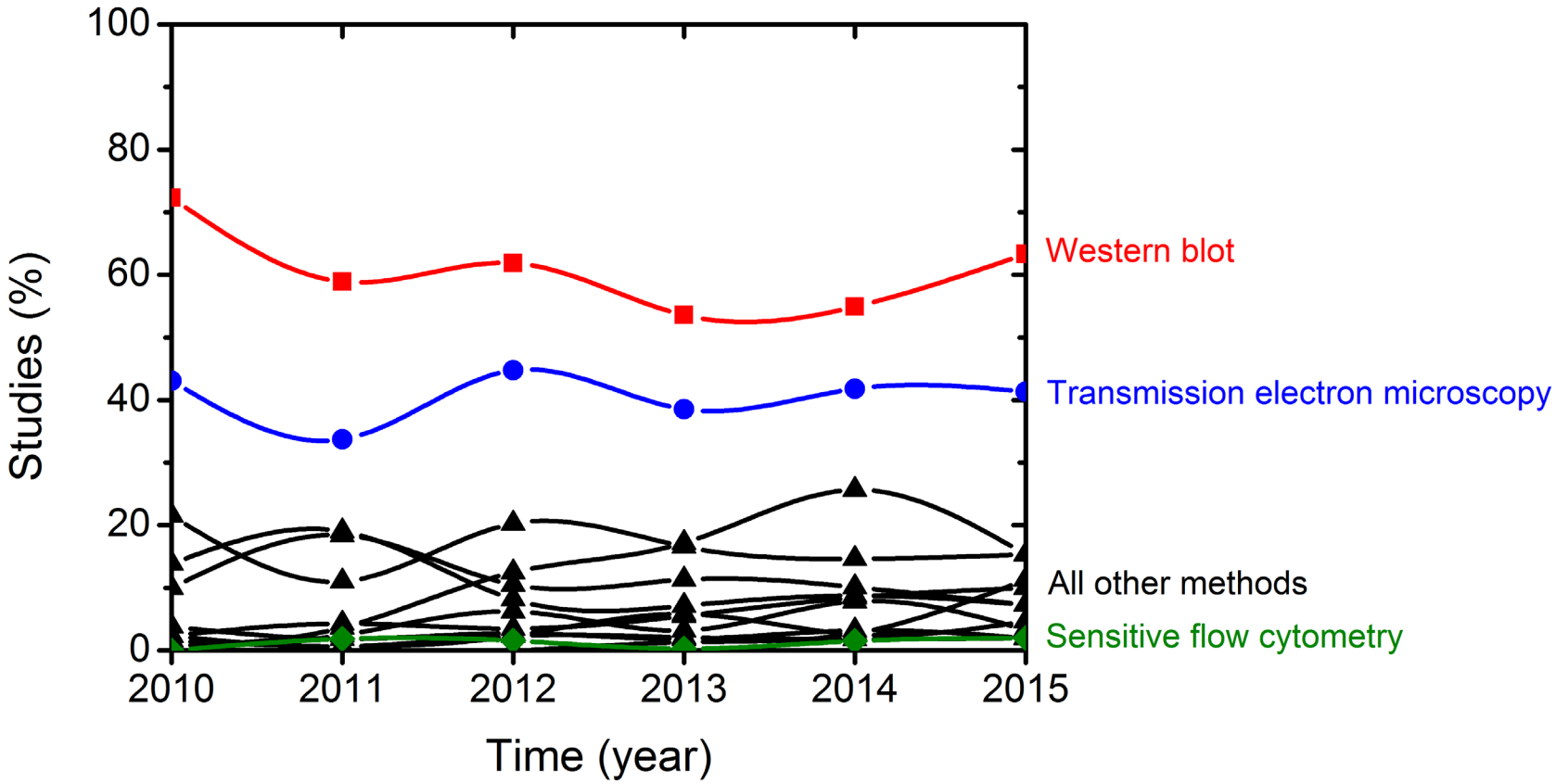
Conflicts of interest

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

Single extracellular vesicle (EV) analysis

- motivation
- requirements
- detection methods
  - nanoparticle tracking analysis (NTA)
  - tunable resistive pulse sensing (TRPS)
  - flow cytometry
- scatter-based standardization of flow cytometry measurements
- summary

How do we study EV?



Motivation to detect single EV

- EV are heterogeneous
- study the contribution of all EV, including rare EV

Summit of EV characterization

- know the origin and function of *each* EV

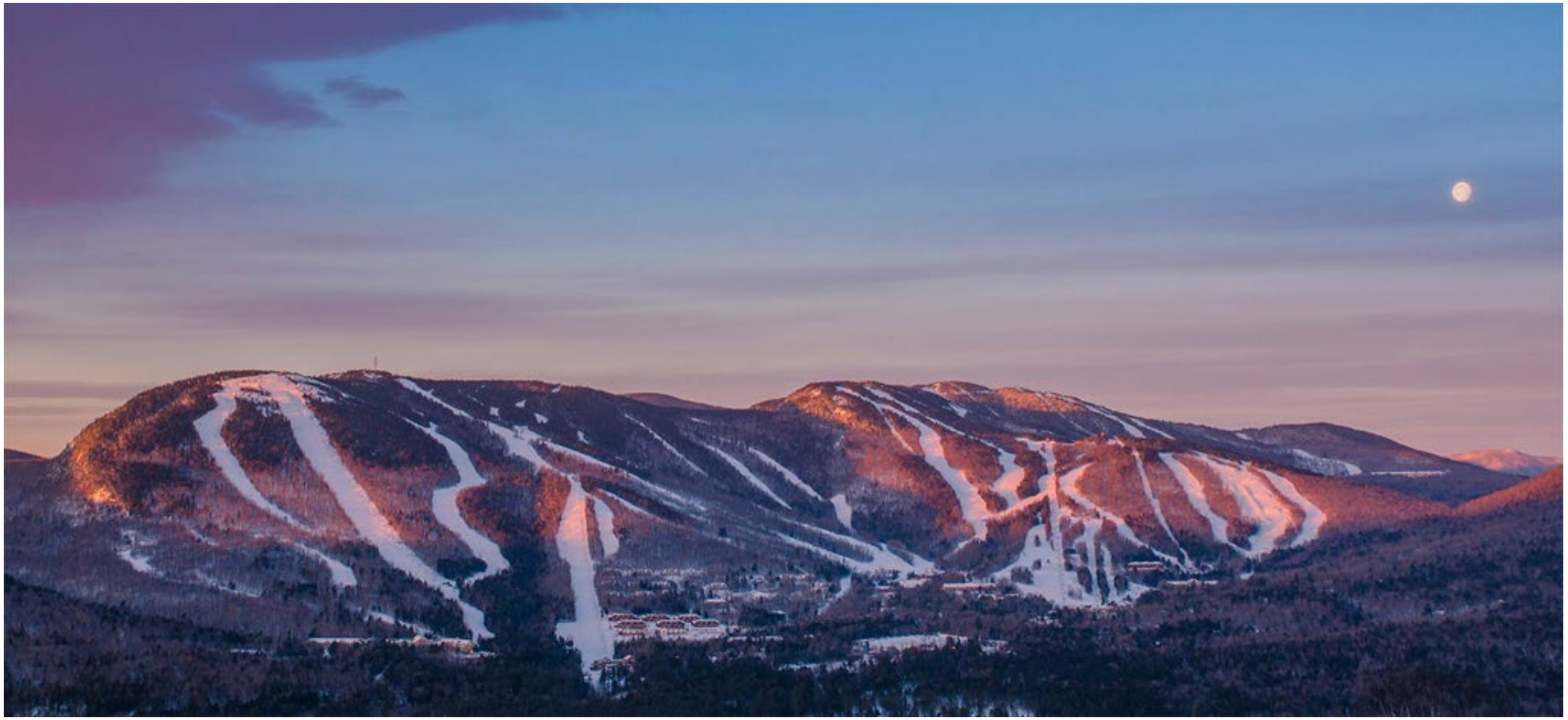


image: sundayriver.com

Requirements for single EV detection

"know the origin and function of *each* EV"

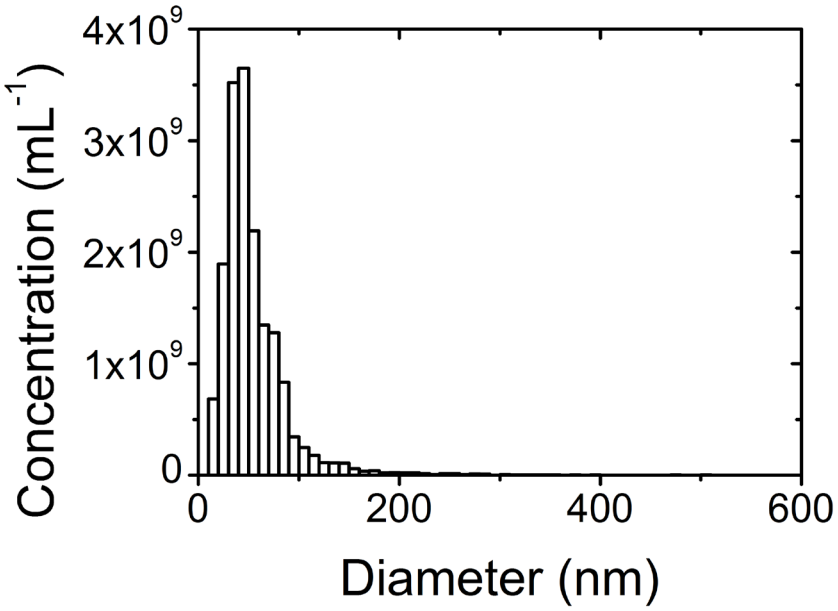
- count rate
  - blood tube volume = 3 mL
  - EV concentration* = $4 \cdot 10^7$ mL^{-1}
  - number of EV = 10^8
  - measurement time = minutes
  - count rate = 10^5 s^{-1}

*Arraud et al. *JTH* 2014

Requirements for single EV detection

"know the origin and function of *each* EV"

- count rate = 10^5 s^{-1}
- sensitivity = 30 nm EV



Requirements for single EV detection

"know the origin and function of *each* EV"

- count rate = 10^5 s^{-1}
- sensitivity = 30 nm EV
- identify EV

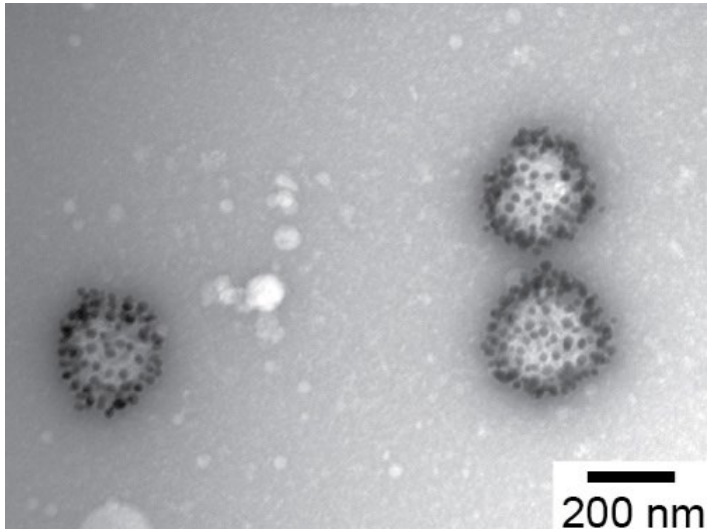
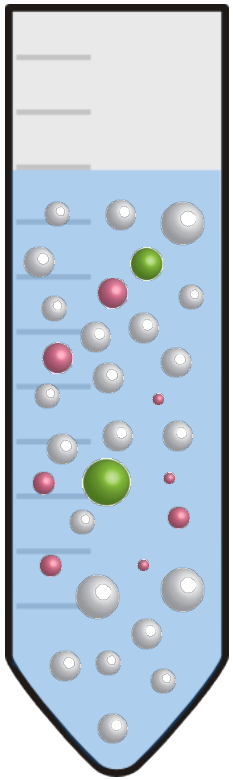


image: L. Rikkert
Vesicle Observation Center, Academic Medical Center, Amsterdam

Requirements for single EV detection

"know the origin and function of *each* EV"

- count rate = 10^5 s^{-1}
- sensitivity = 30 nm EV
- identify EV
- known detection volume



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Nanoparticle tracking analysis

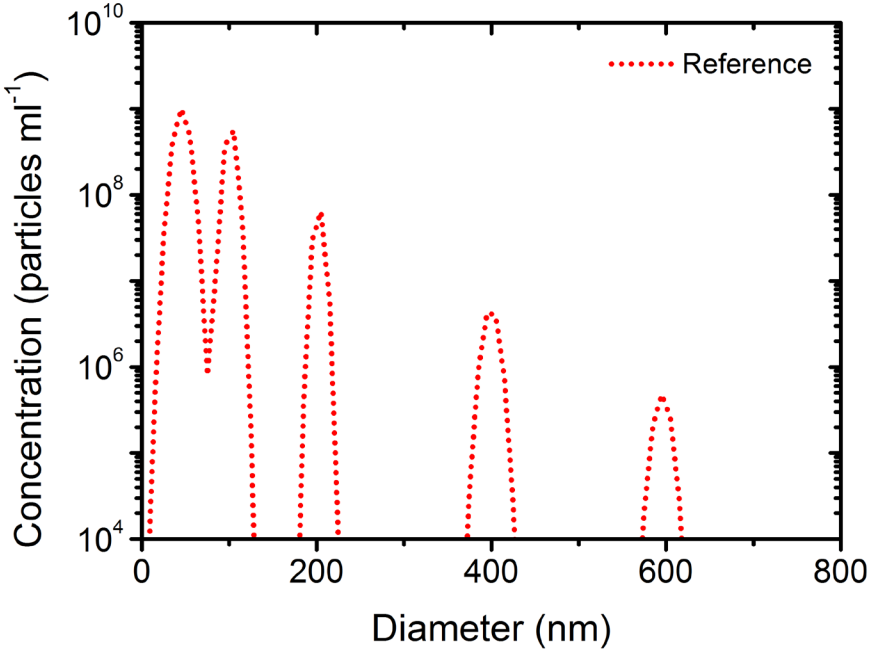
diameter from
Brownian motion

- maximum count rate: ~10 EV/s
- sensitivity: 70-90 nm EV
- identification: 1 fluorescence channel
- detection volume “known”: 1 order of magnitude error

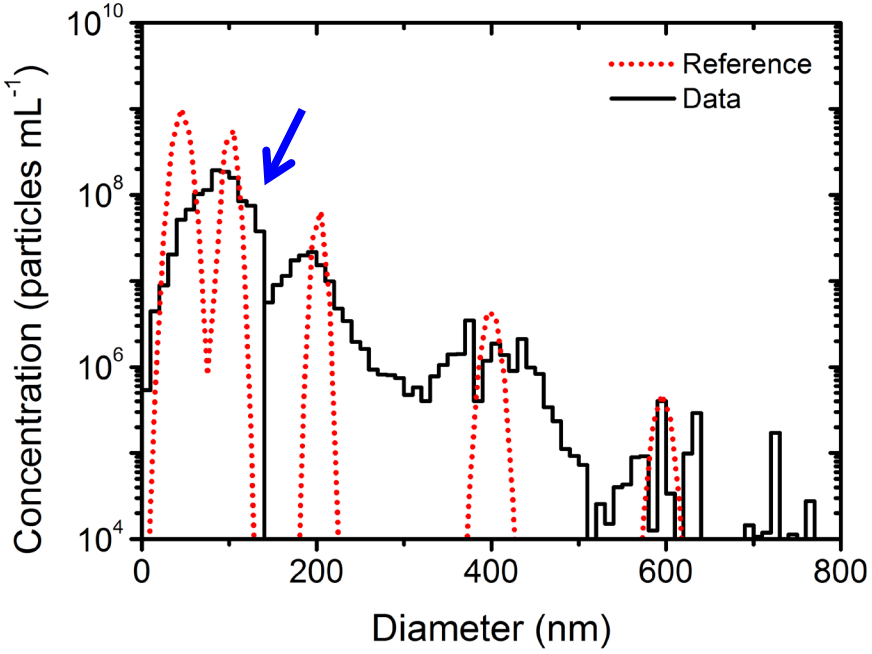
Gardiner et al. *JEV* 2013, van der Pol et al. *JTH* 2014

Based on Nanosight NS500. Other manufacturer: ParticleMetrix (ZetaView)

Reference sample of polystyrene beads

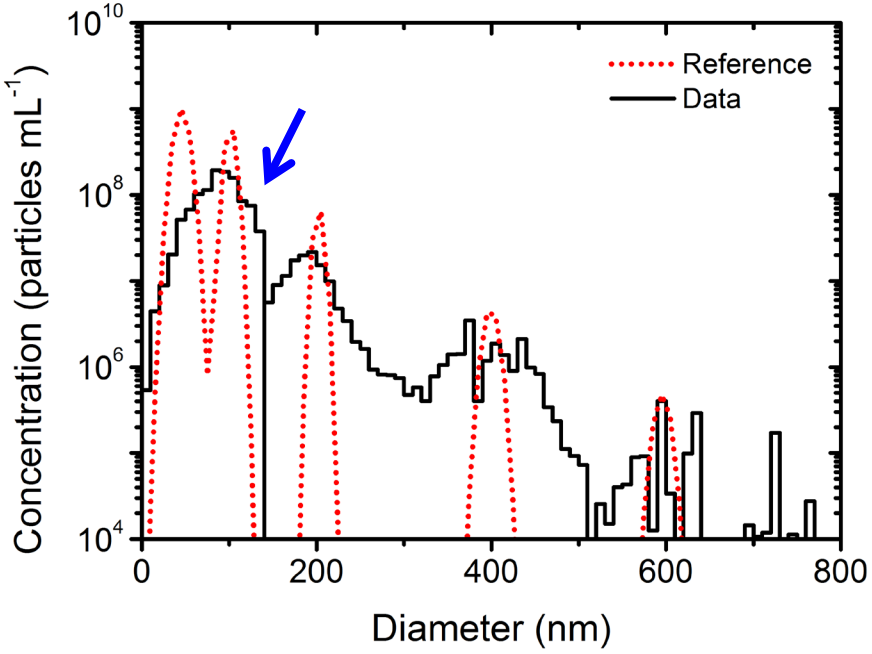


Accuracy and precision of NTA

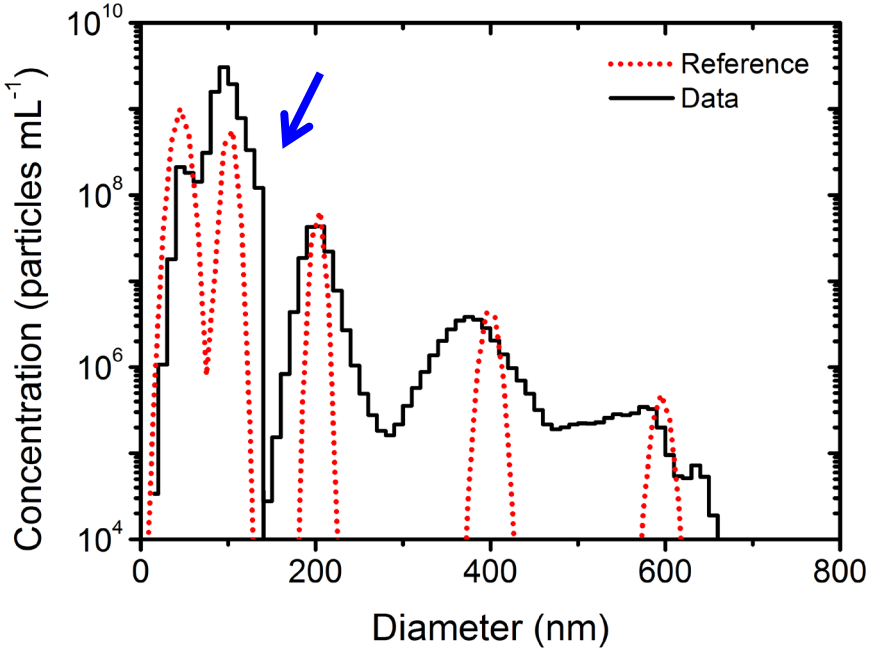


Nanosight software version 2

Accuracy and precision of NTA



Nanosight software version 2



Nanosight software version 3

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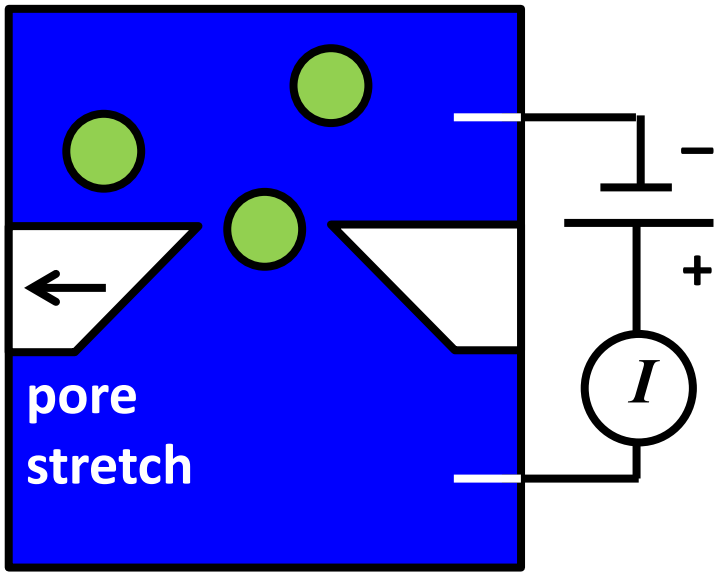
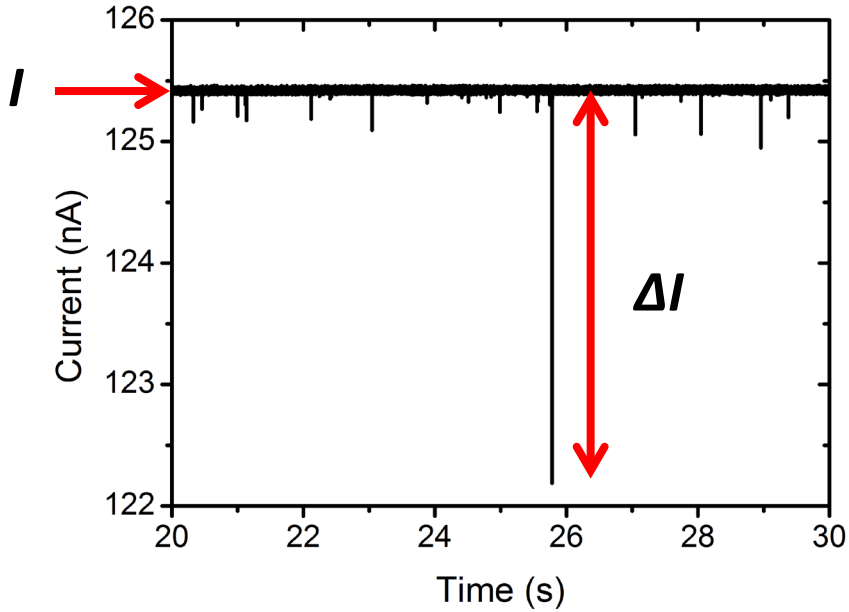
(Tunable) resistive pulse sensing

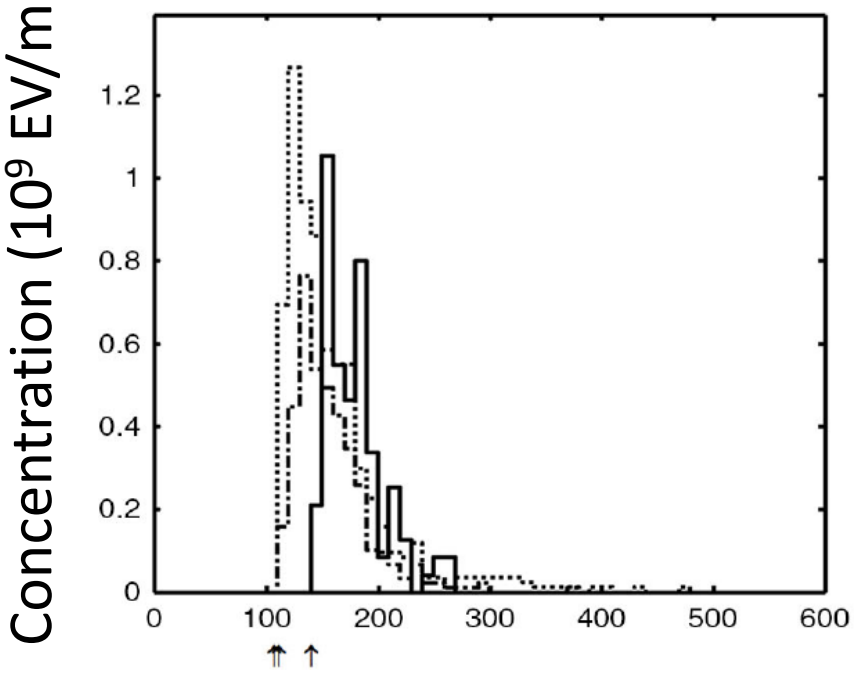




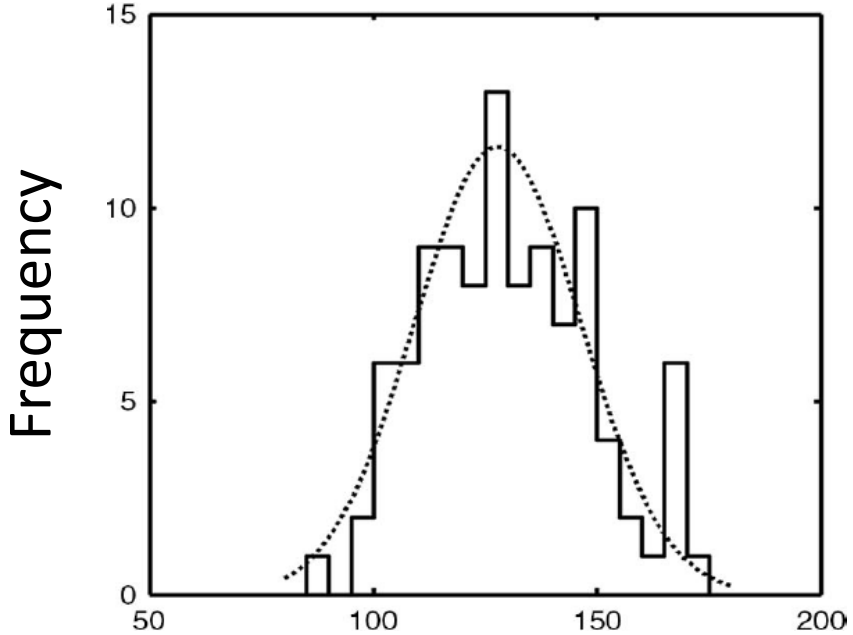
- maximum count rate: ~100 EV/s
- sensitivity: 70-100 nm EV
- identification: electrophoretic mobility
- detection volume calibrated with beads

Reproducibility of TRPS: perhaps too tunable?

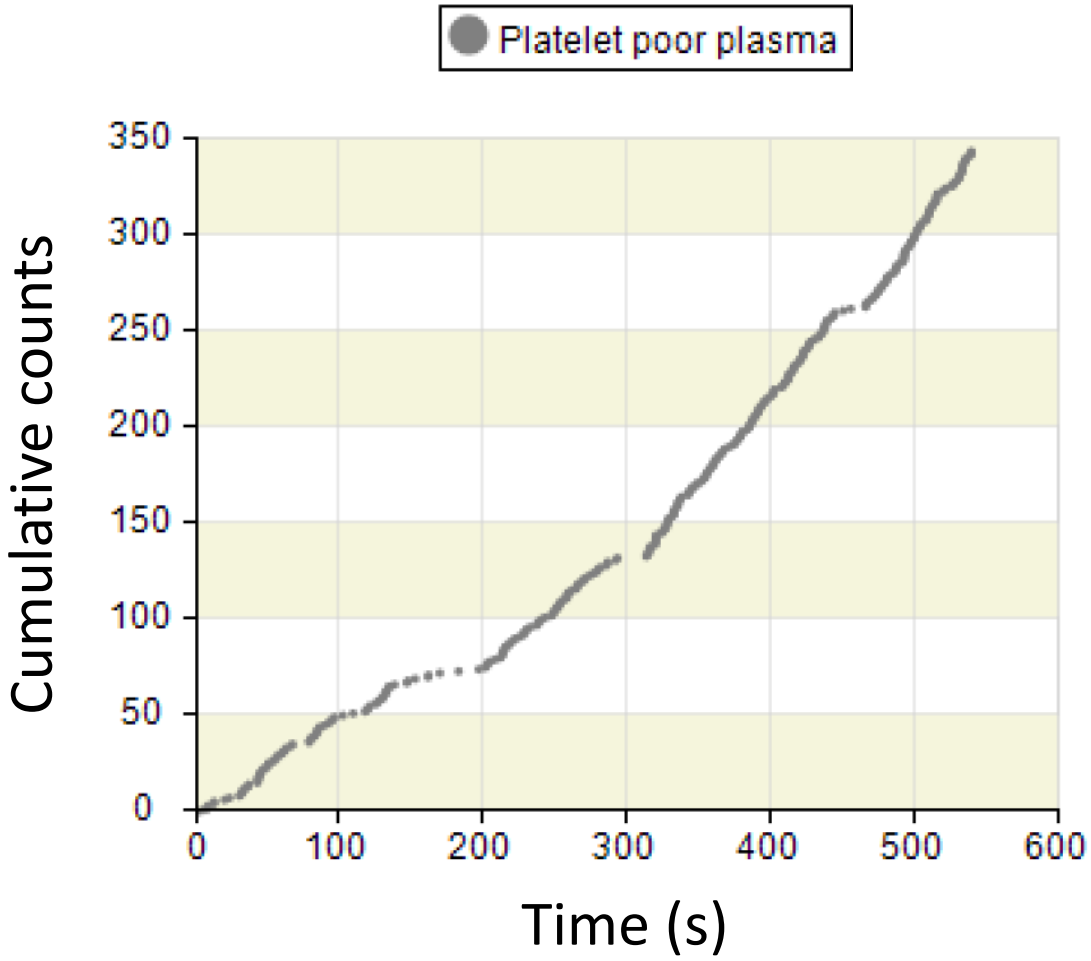
3 measurements of urinary reference sample



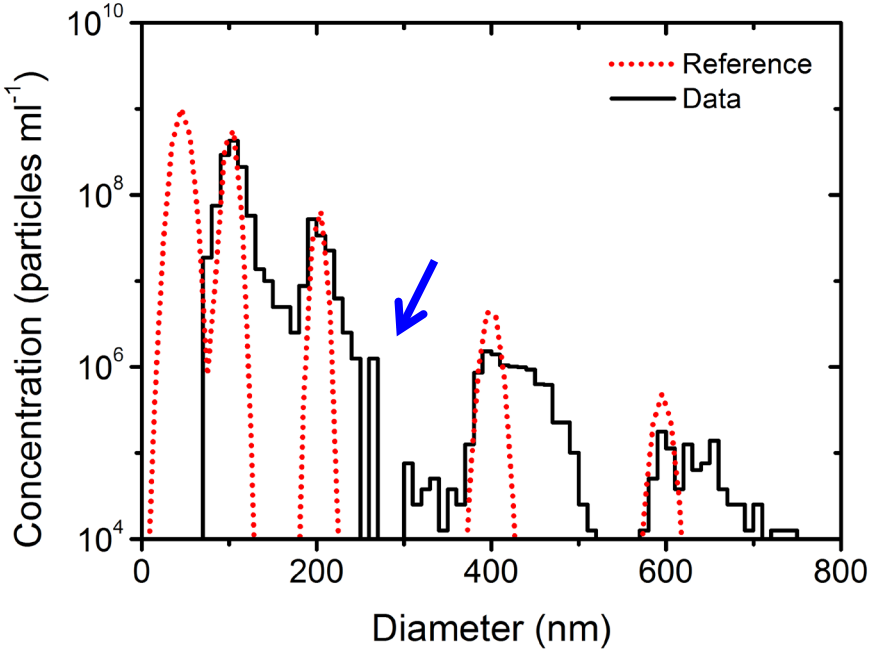
102 measurements of urinary reference sample



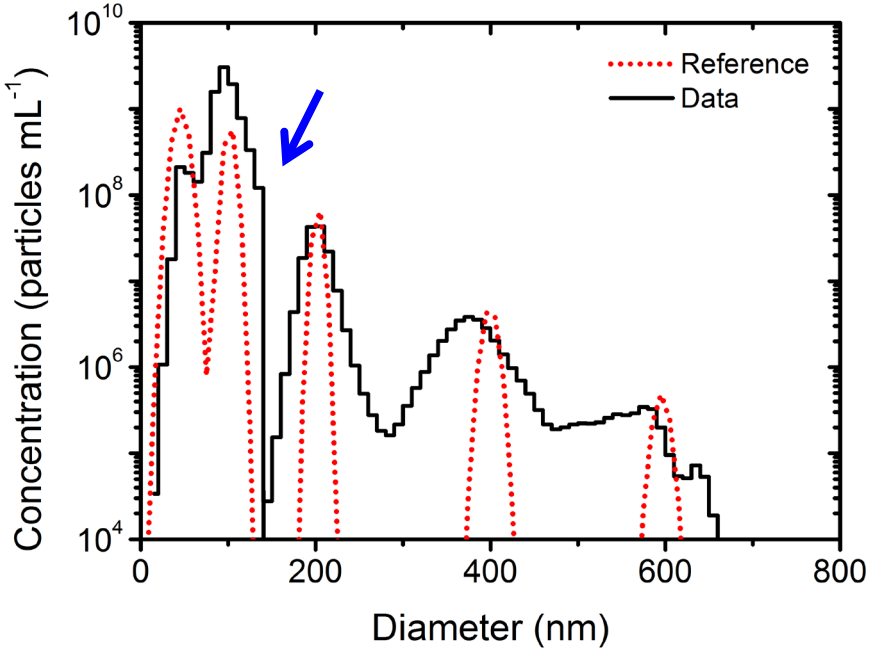
Practical limitation of TRPS: pore clogging



Accuracy and precision of TRPS vs. NTA



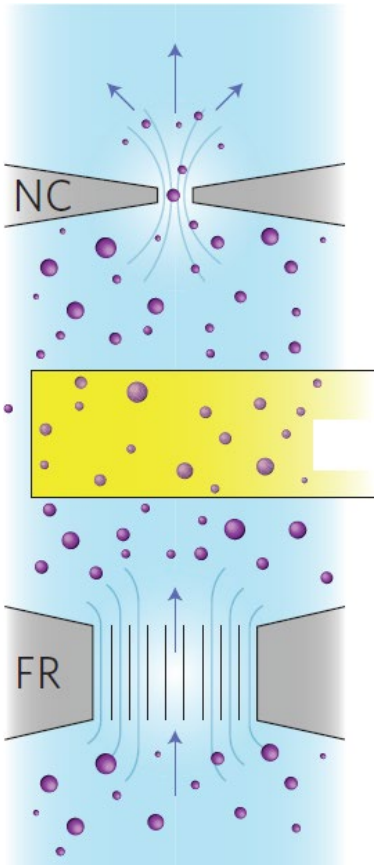
TRPS
(qNano)



NTA
(Nanosight software v.3)

New RPS development: Spectradyne

fixed pore in PDMS
pre-calibrated chip
pre-filtration



- maximum count rate: ~10,000 EV/s
- sensitivity: ~50 nm EV
- identification: none
- detection volume calibrated with beads

image adopted from Fraikin et al. *Nat. Nanotechnol.* 2011
spectradynellc.com

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Flow cytometry

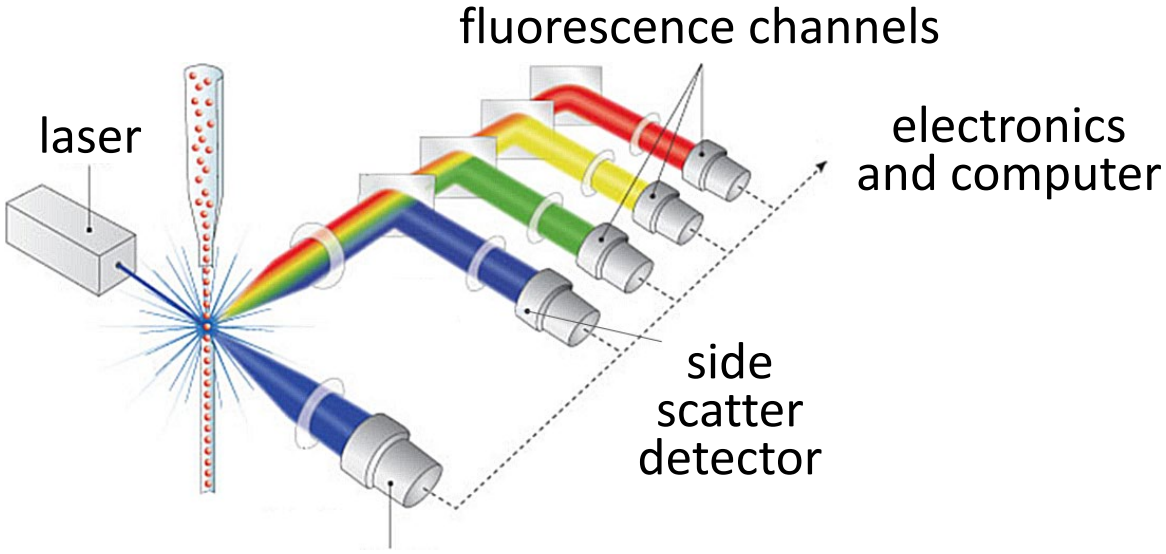
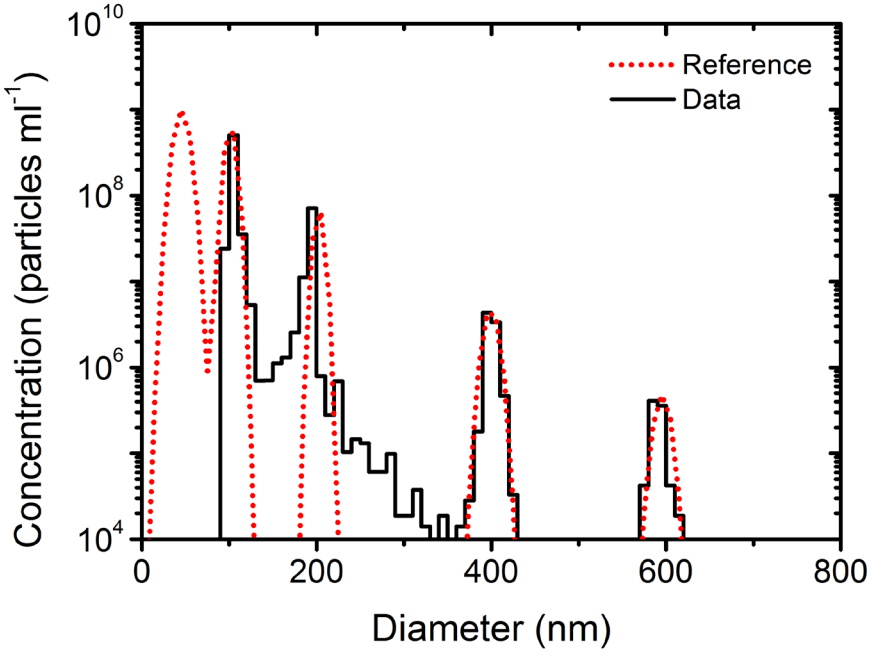


image:
semrock.com

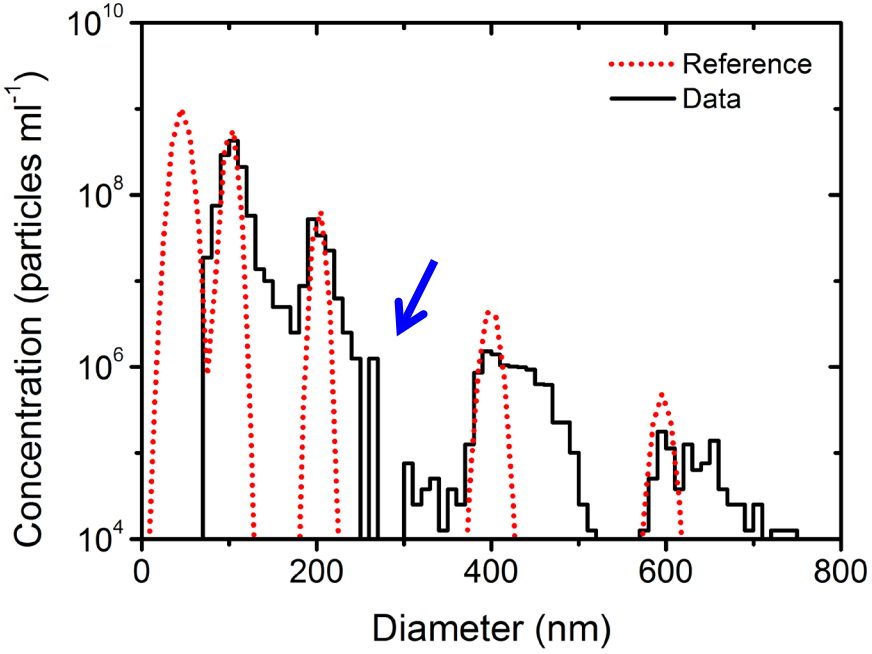
- maximum count rate: 1,000-100,000 EV/s
- sensitivity: ~50-200 nm EV*
- identification: multiplex fluorescence
- detection volume known with ~10% error

* Zhu et al. *ACS Nano* 2014, van der Pol et al. *JTH* 2014
van der Vlist et al. Nature Protocols 2012, Stoner et al. *Cytometry A* 2016

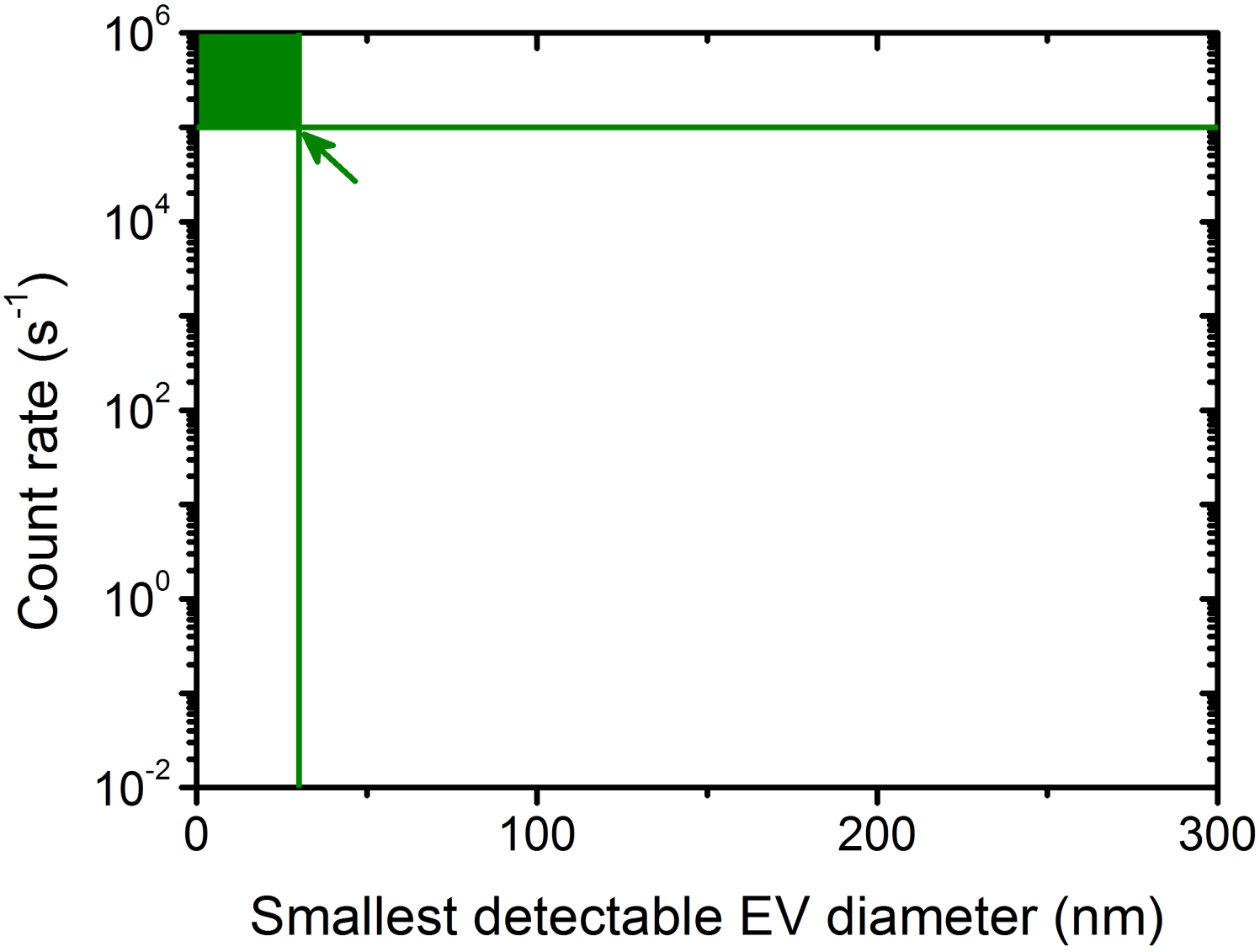
Accuracy and precision of flow cytometry

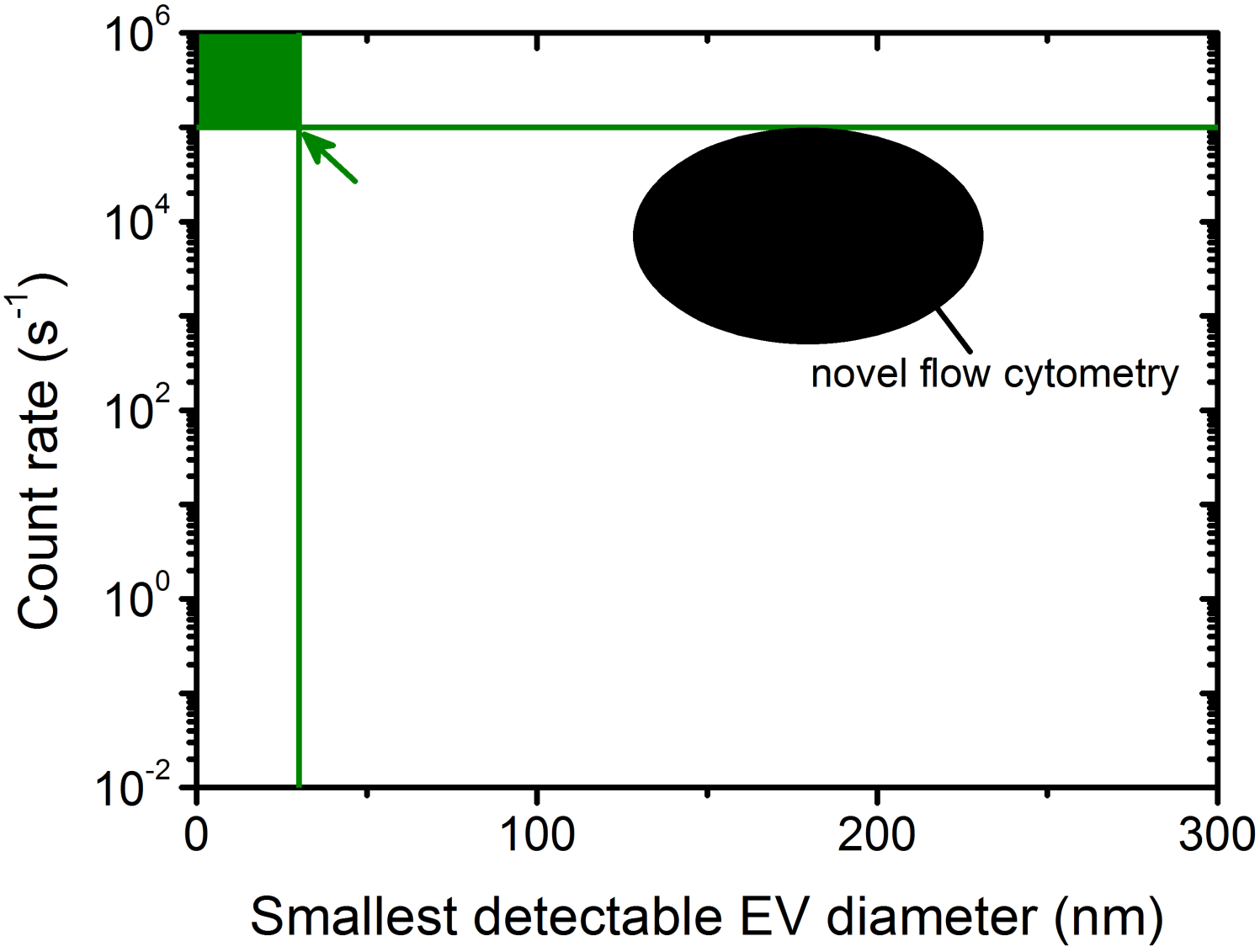


Flow cytometry
(Apogee A50)



TRPS
(qNano)


10^6
10^4
10^2
10^0
10^{-2}
Count rate (s^{-1})
0
100
200
300
Smallest detectable EV diameter (nm)


10^6
10^4
10^2
10^0
10^{-2}
Count rate (s^{-1})
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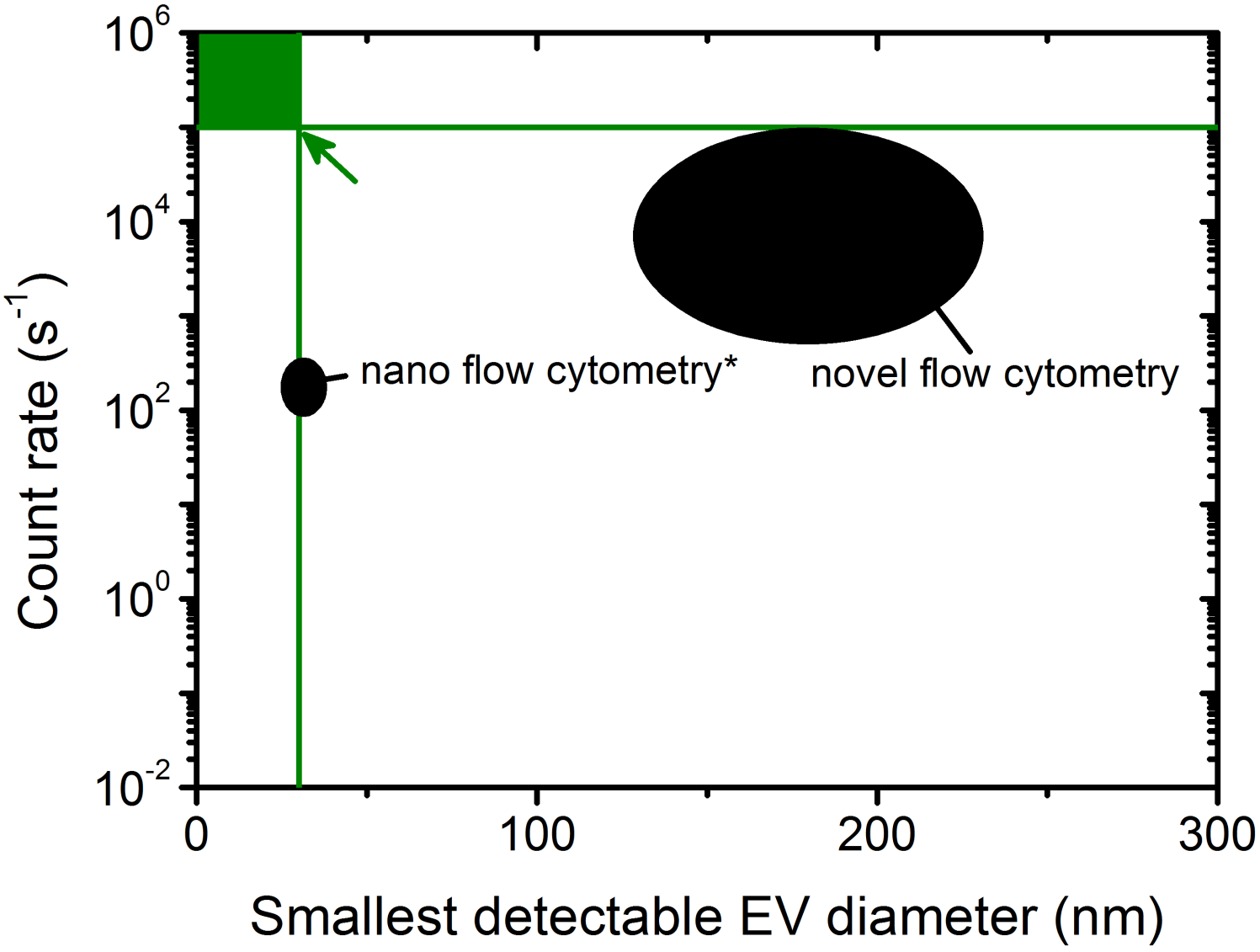
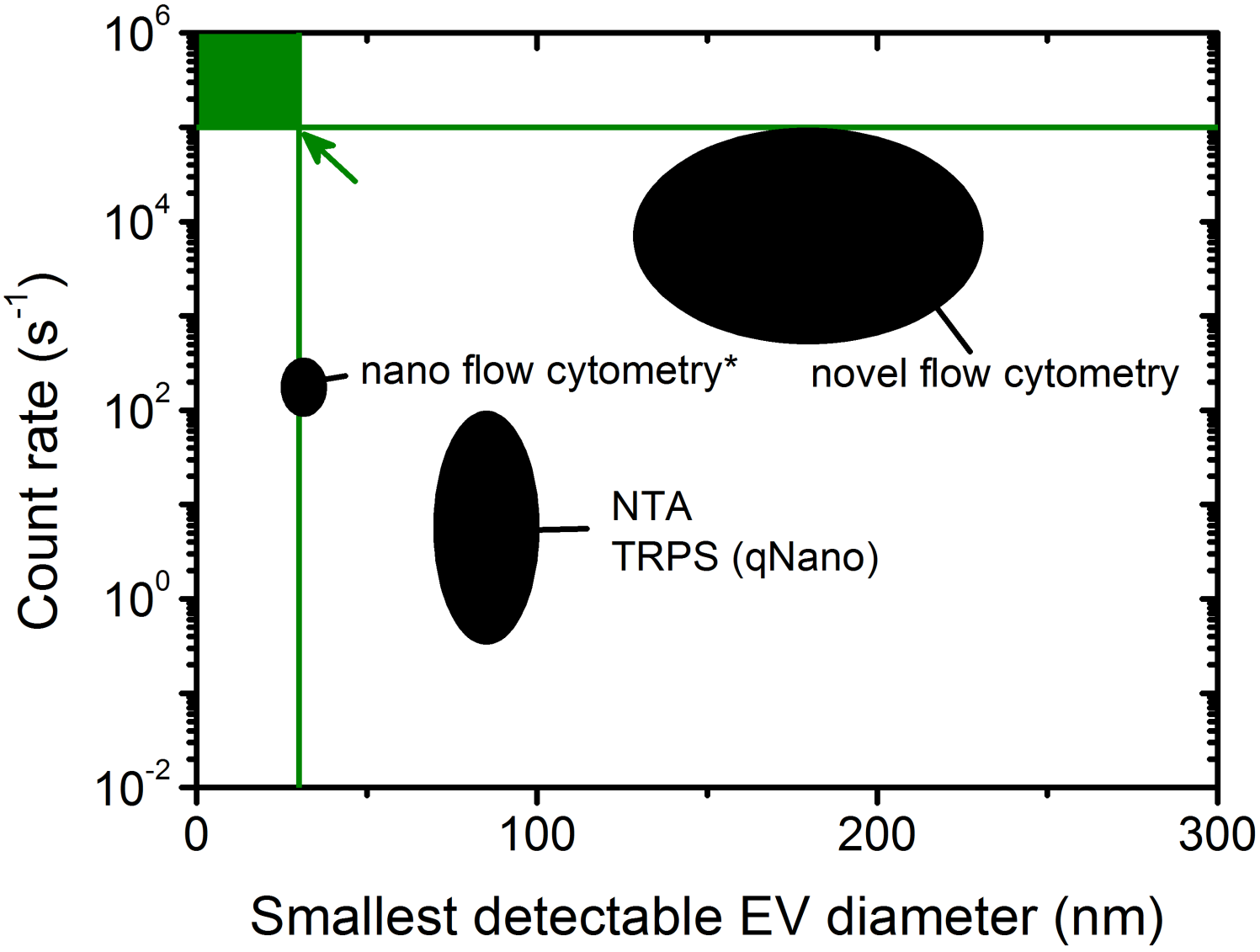
Smallest detectable EV diameter (nm)
novel flow cytometry



* Zhu et al. *ACS Nano* 2014



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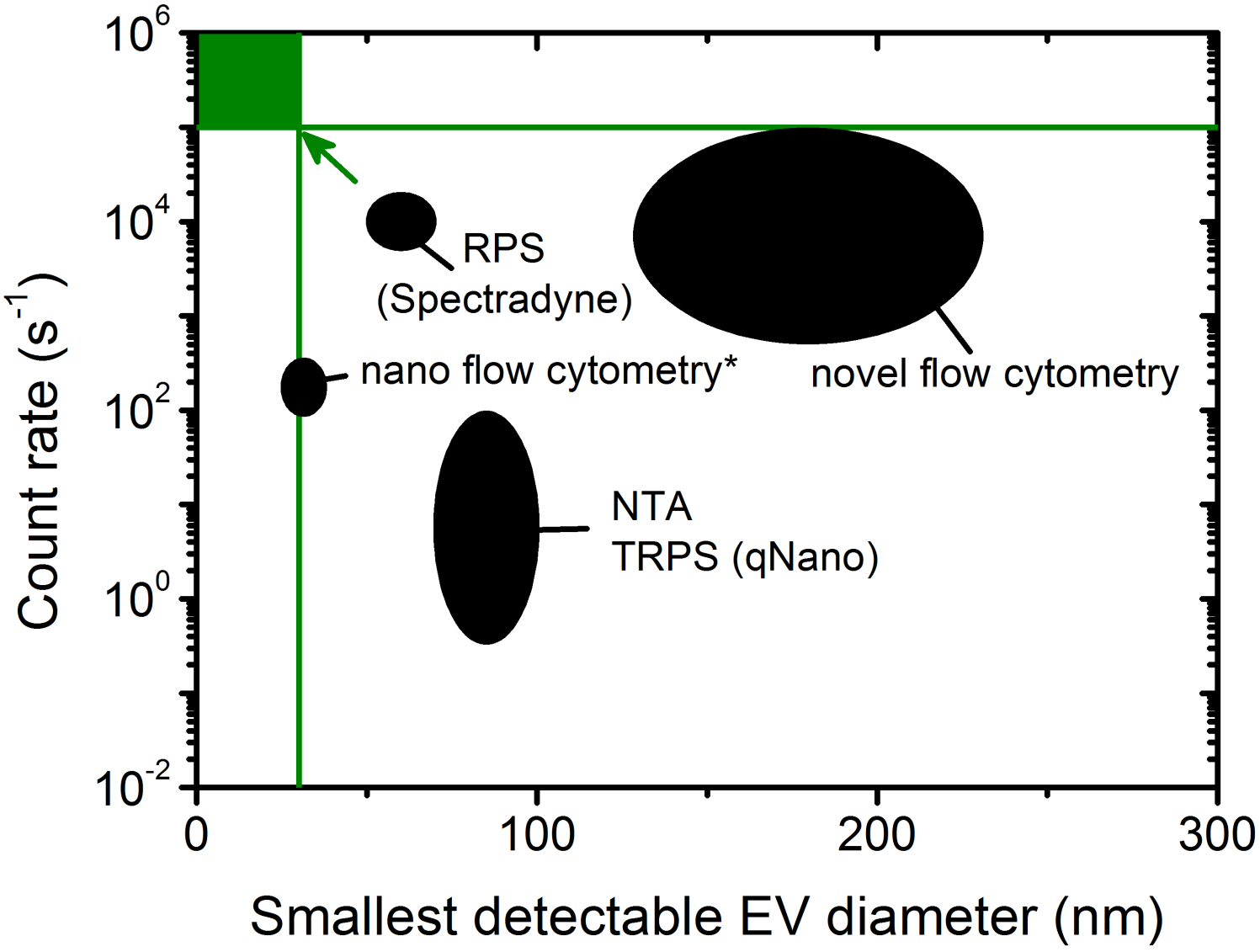

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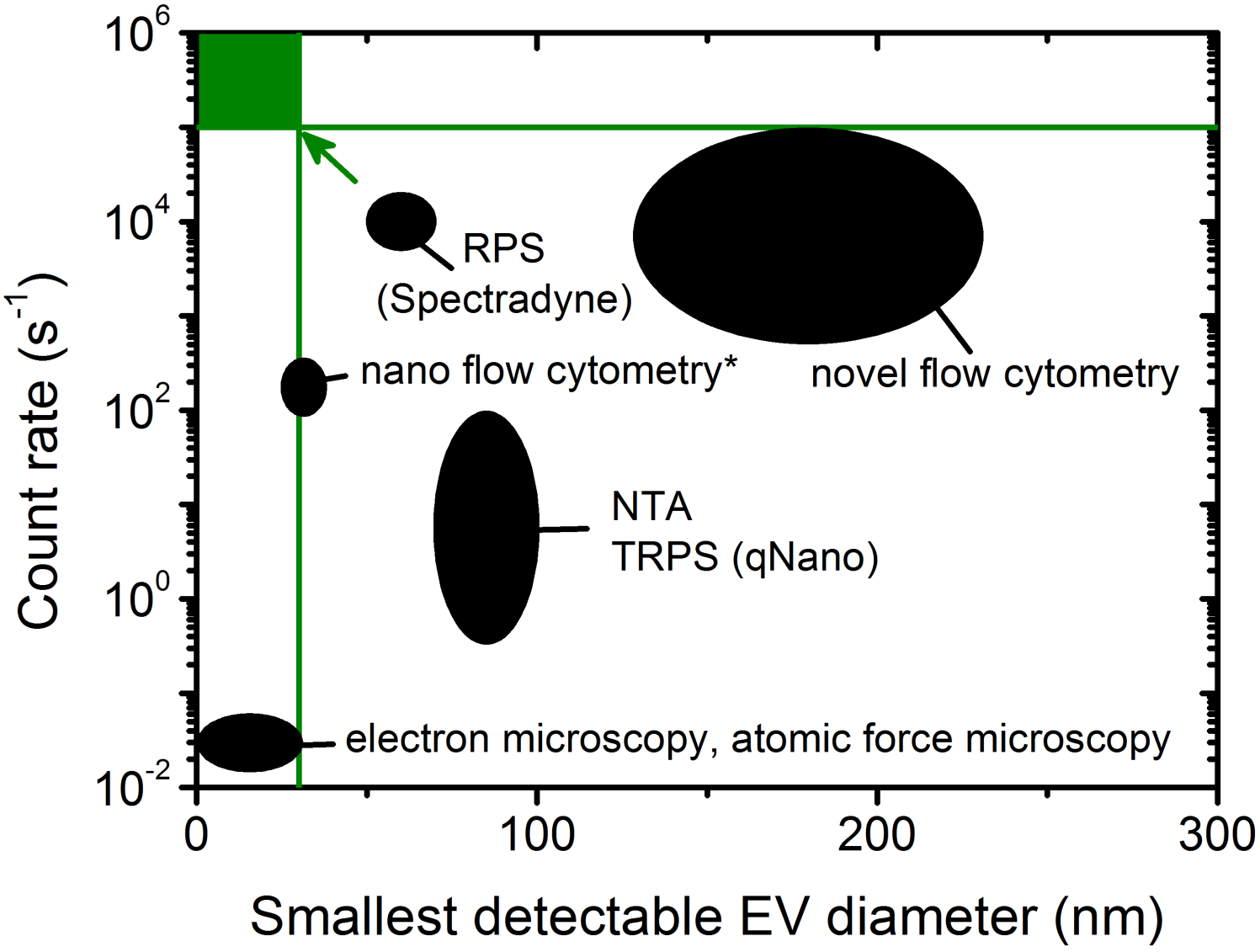

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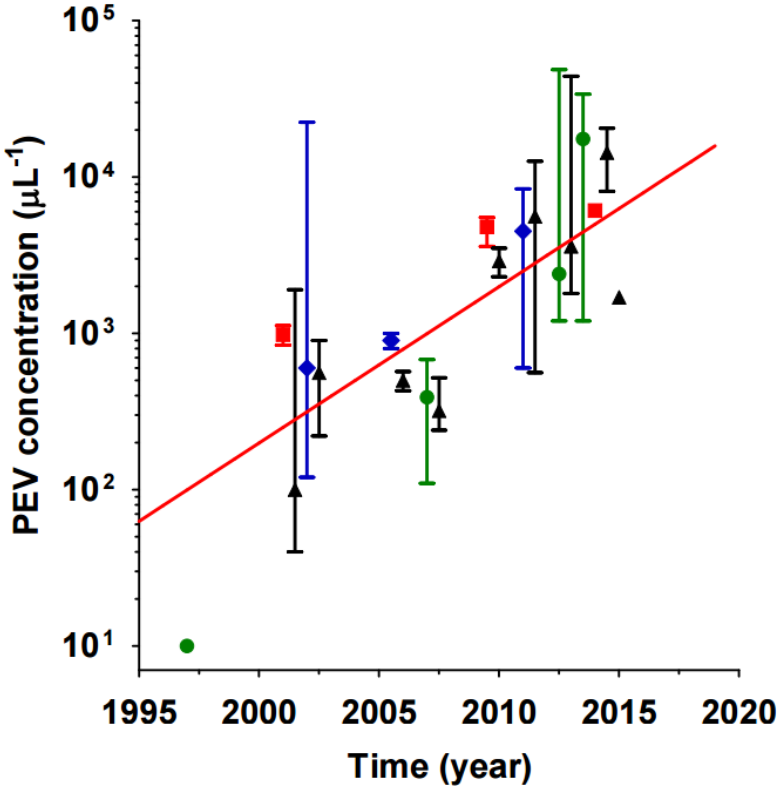
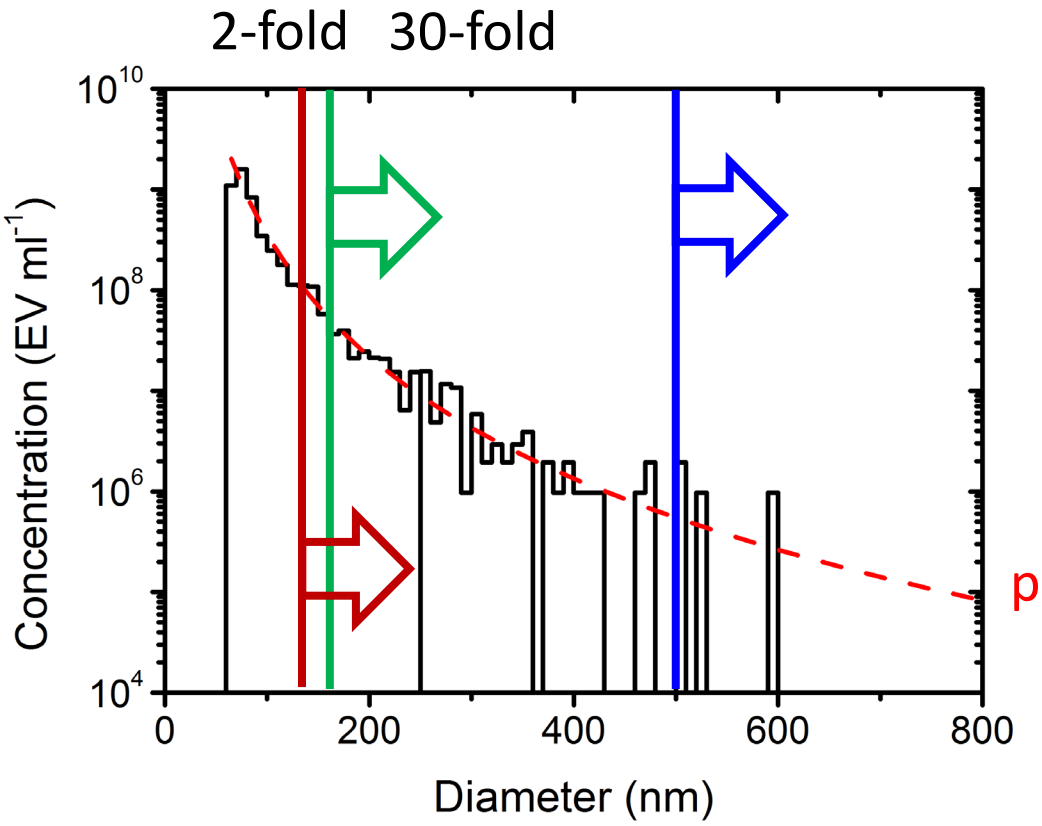
Motivation of flow cytometry standardization



"Gąsecka's law"

- compare clinical EV data
- reported concentrations of plasma EV differ >10^6-fold

Problem 1: instruments differ in sensitivity

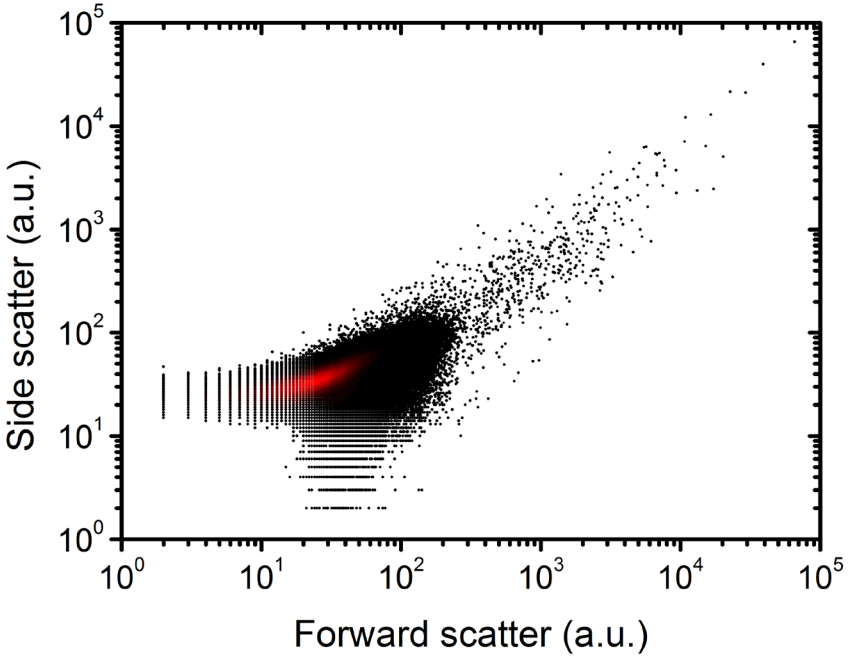


power-law relation*

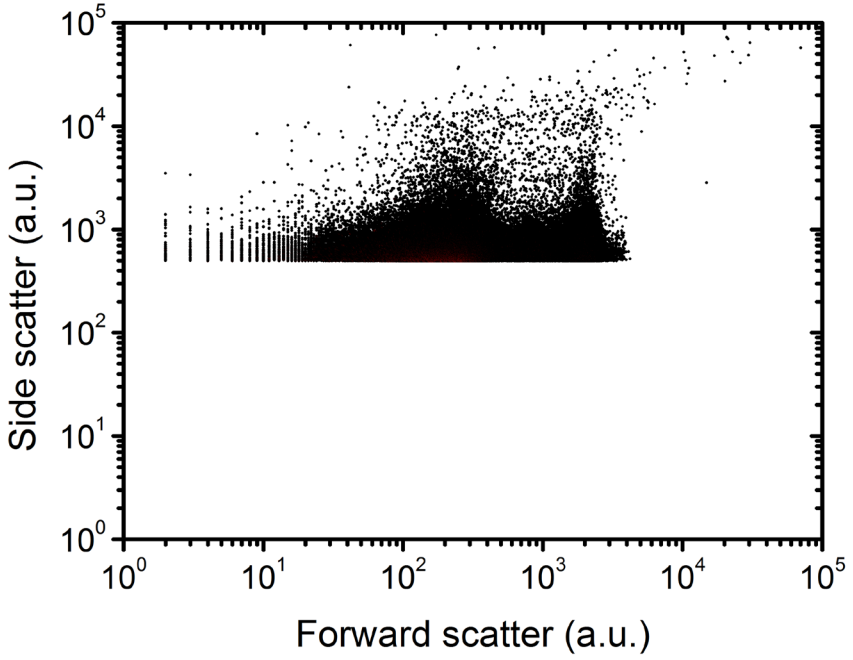
*van der Pol et al. *JTH* (2014)

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

METVES

International Society on Thrombosis and Haemostasis

Vesicle Observation Center

EXOMETRY

Approach scatter-based standardization

- 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

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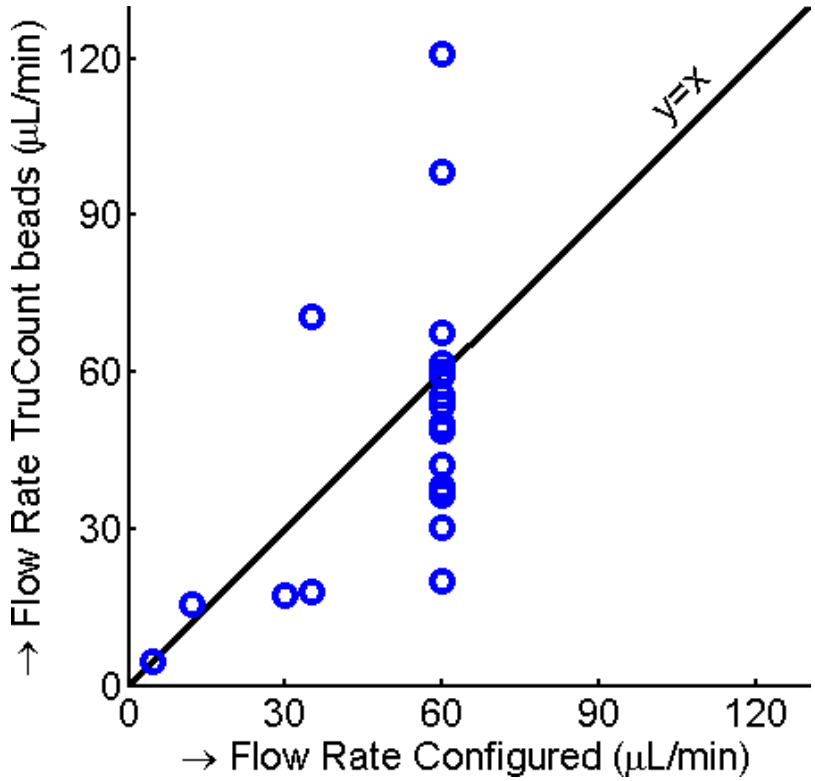
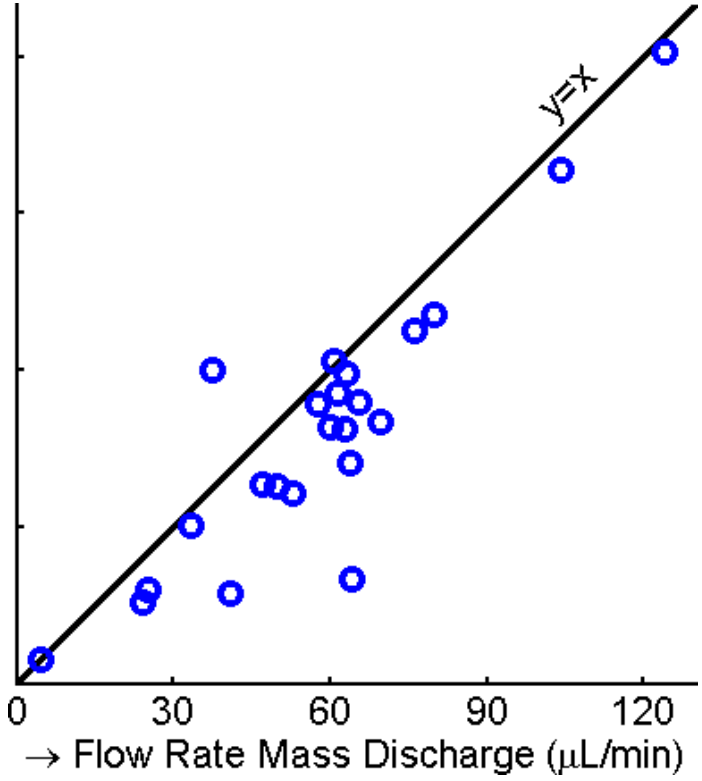
Determine flow rate

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





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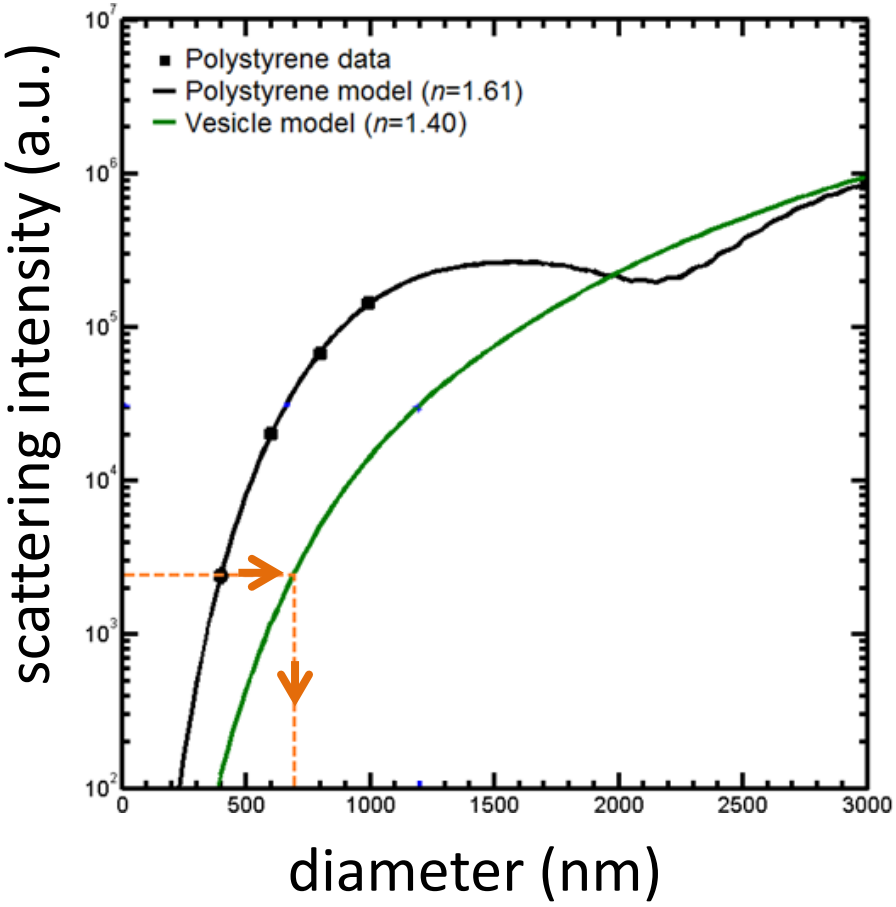
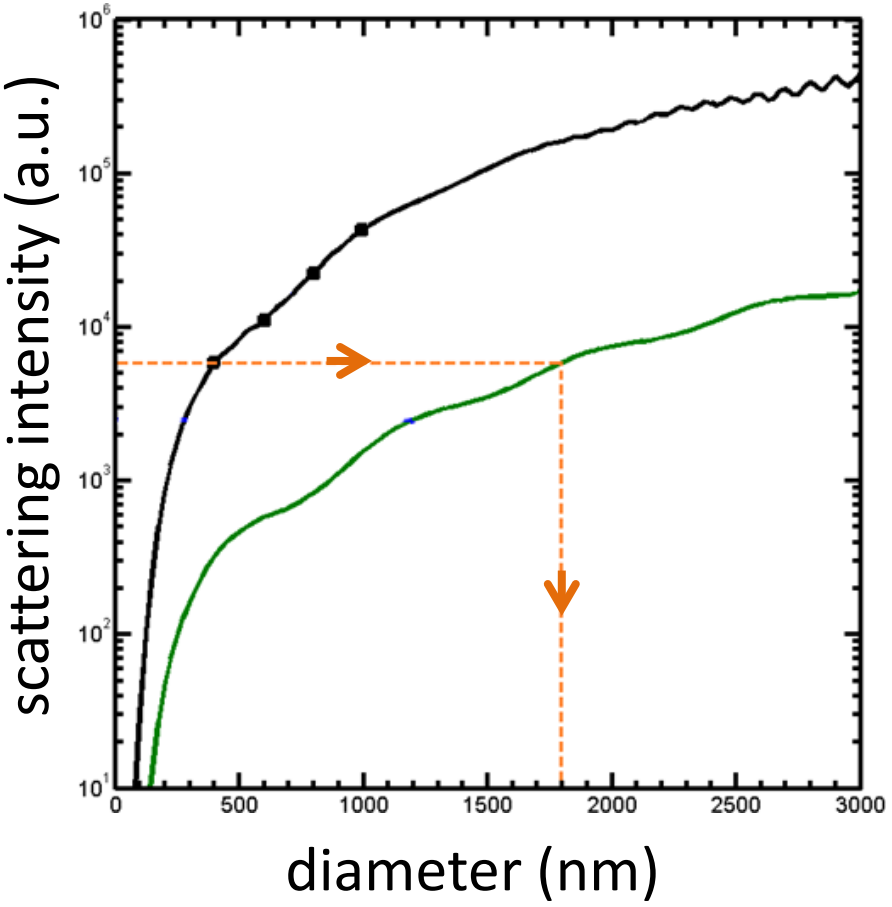
Earlier approach: gate on polystyrene beads

BC Gallios (forward scatter)



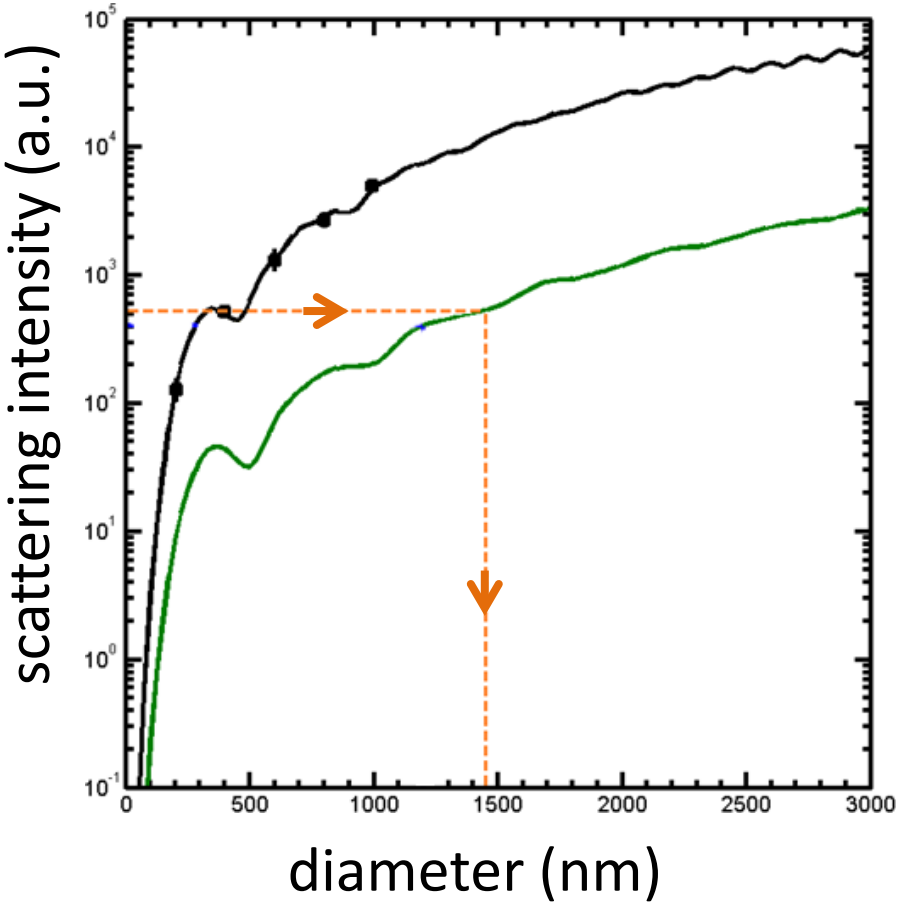
BD LSR II (side scatter)



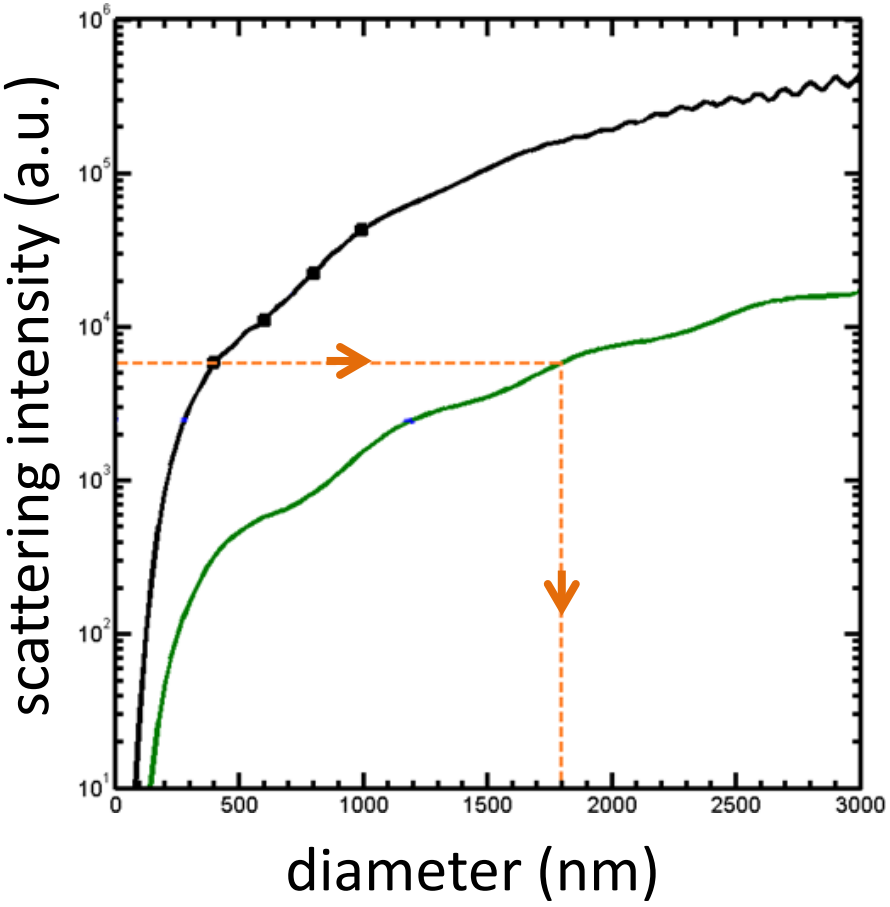
Robert et al. *JTH* (2008)
Lacroix et al. *JTH* (2010)

Earlier approach: gate on polystyrene beads

BC Astrios MoFlo (side scatter)



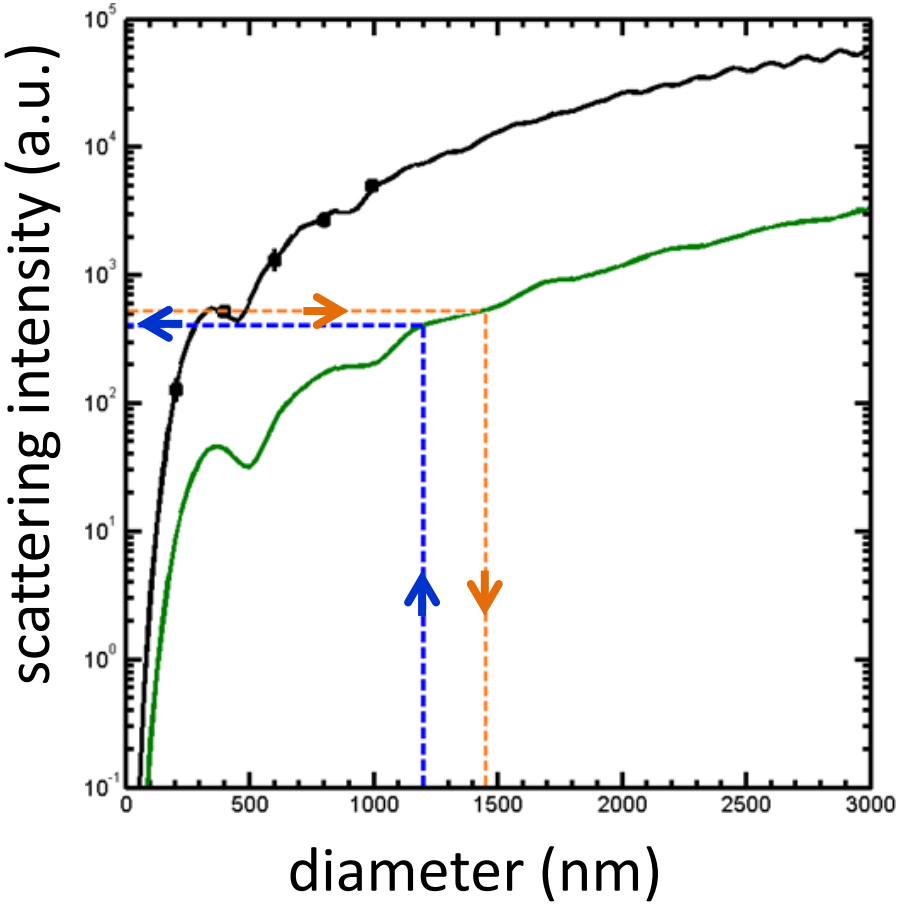
BD LSR II (side scatter)



Robert et al. *JTH* (2008)
Lacroix et al. *JTH* (2010)

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

BC Astrios MoFlo (side scatter)



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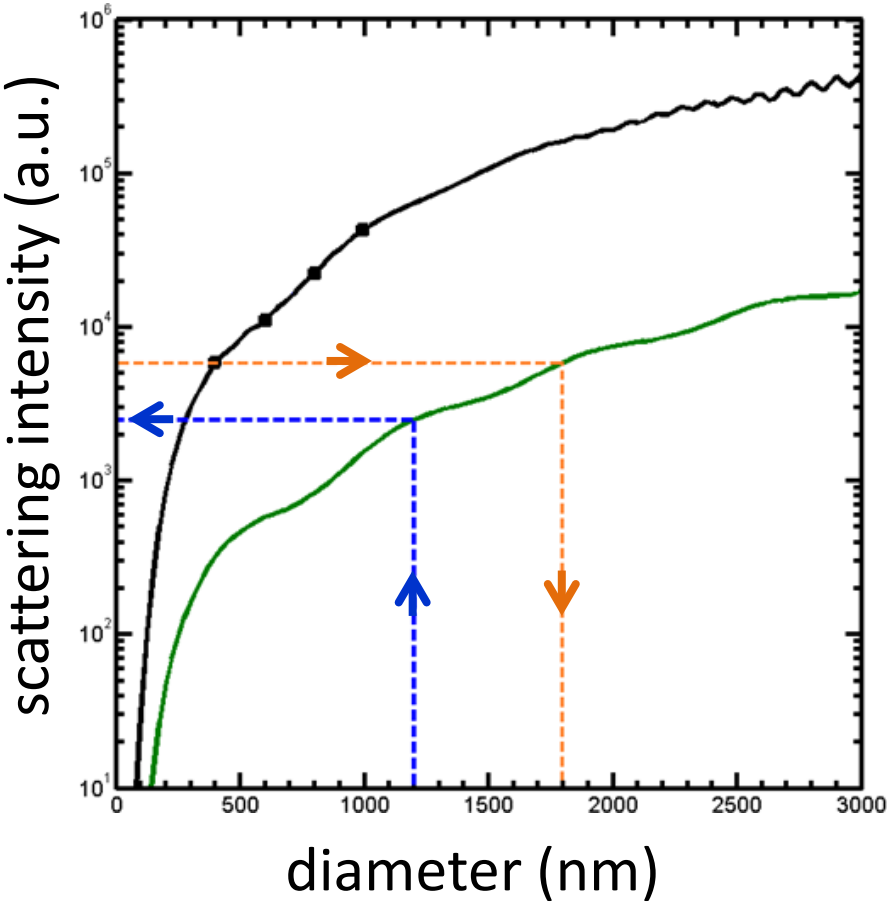

Robert et al. *JTH* (2008)
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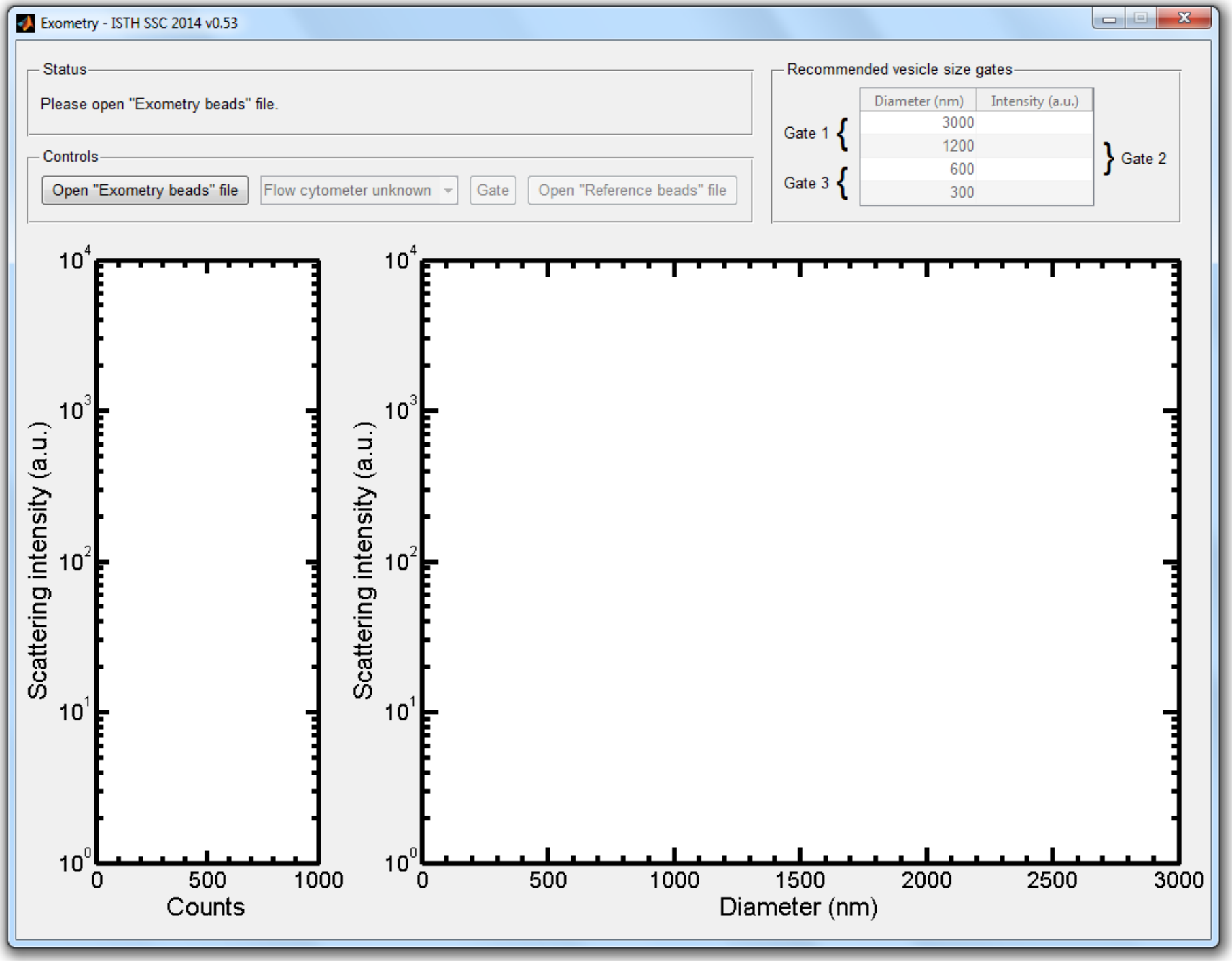

Exometry - ISTH SSC 2014 v0.53
Status
Please open "Exometry beads" file.
Controls
Open "Exometry beads" file
Flow cytometer unknown
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
Counts
Diameter (nm)

Exometry - ISTH SSC 2014 v0.53

Status

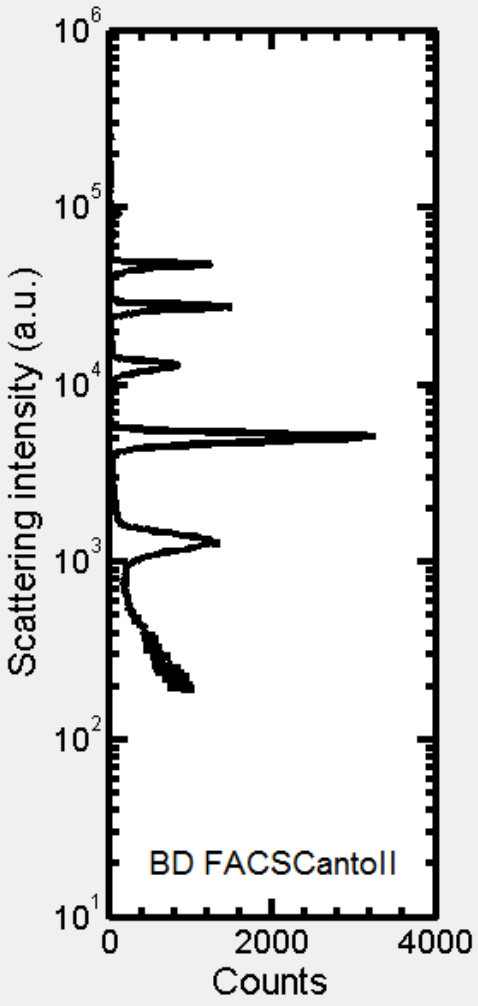
Please select detector and click "Gate" to obtain vesicle size gates.

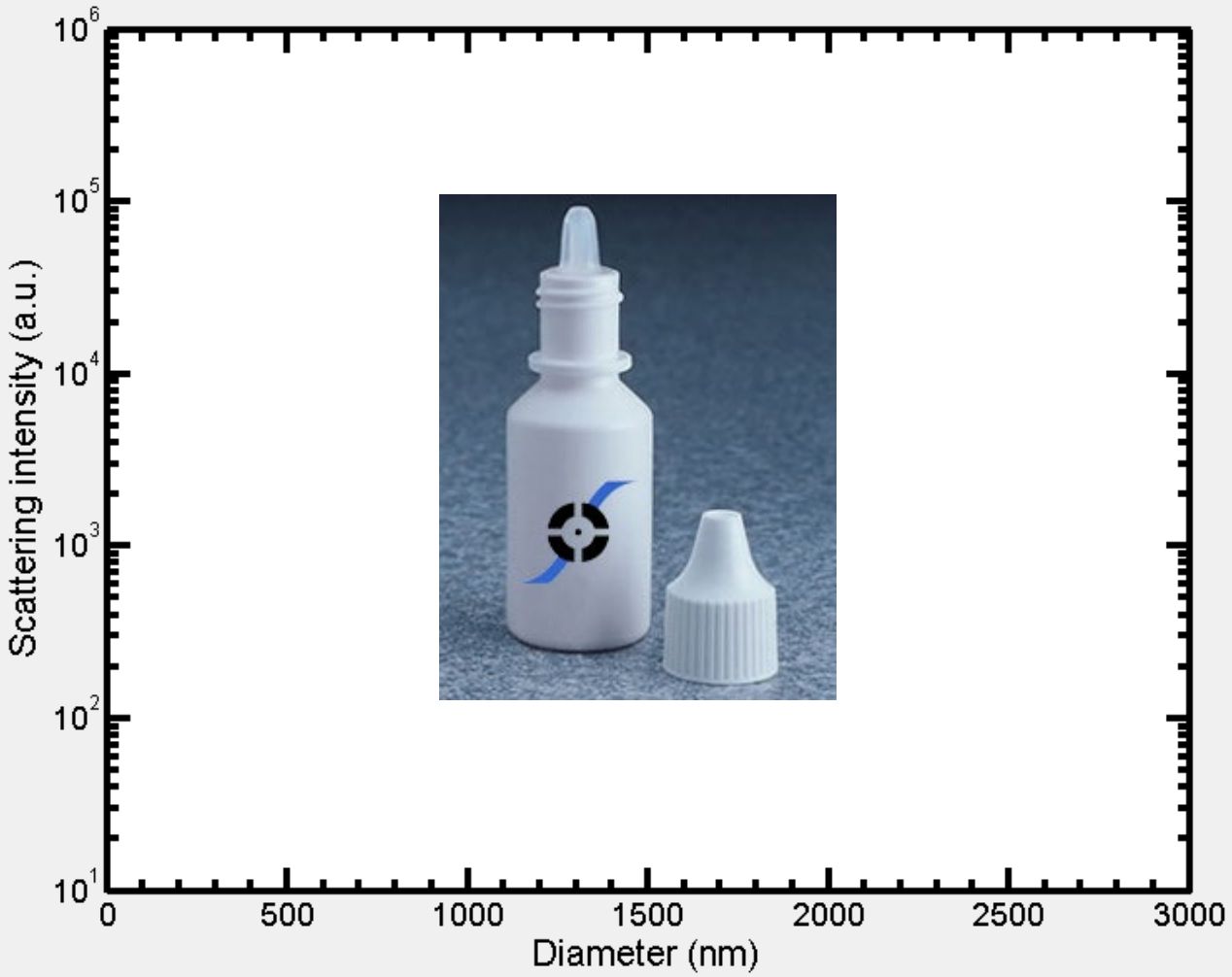
Controls

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.

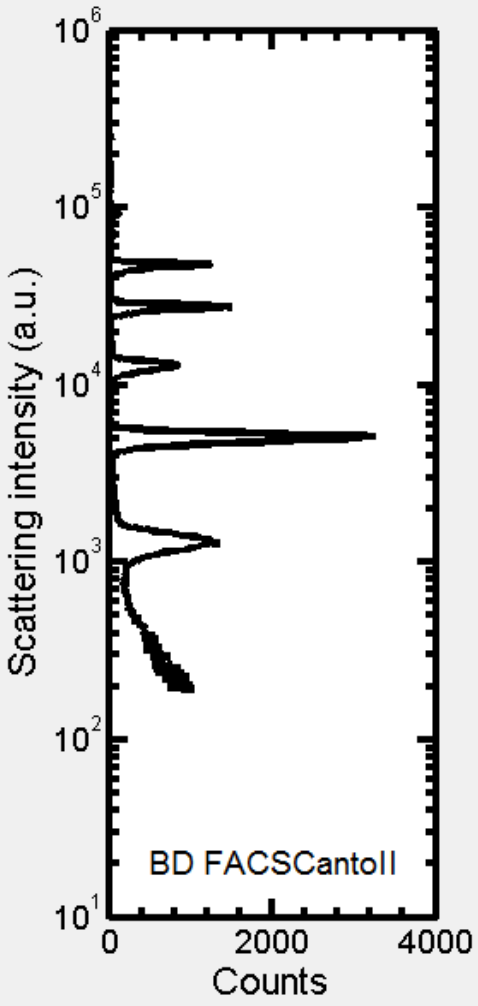
Controls

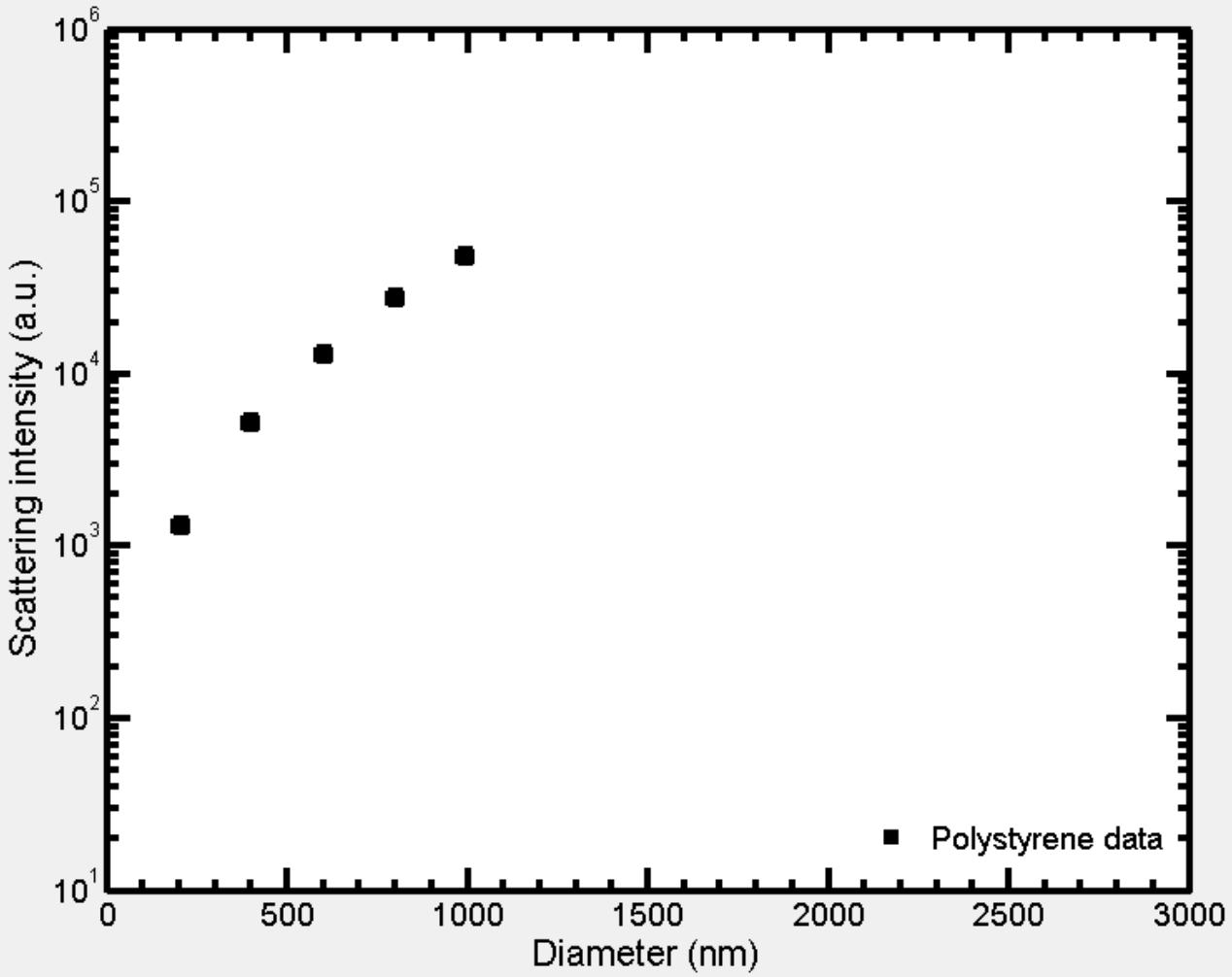
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

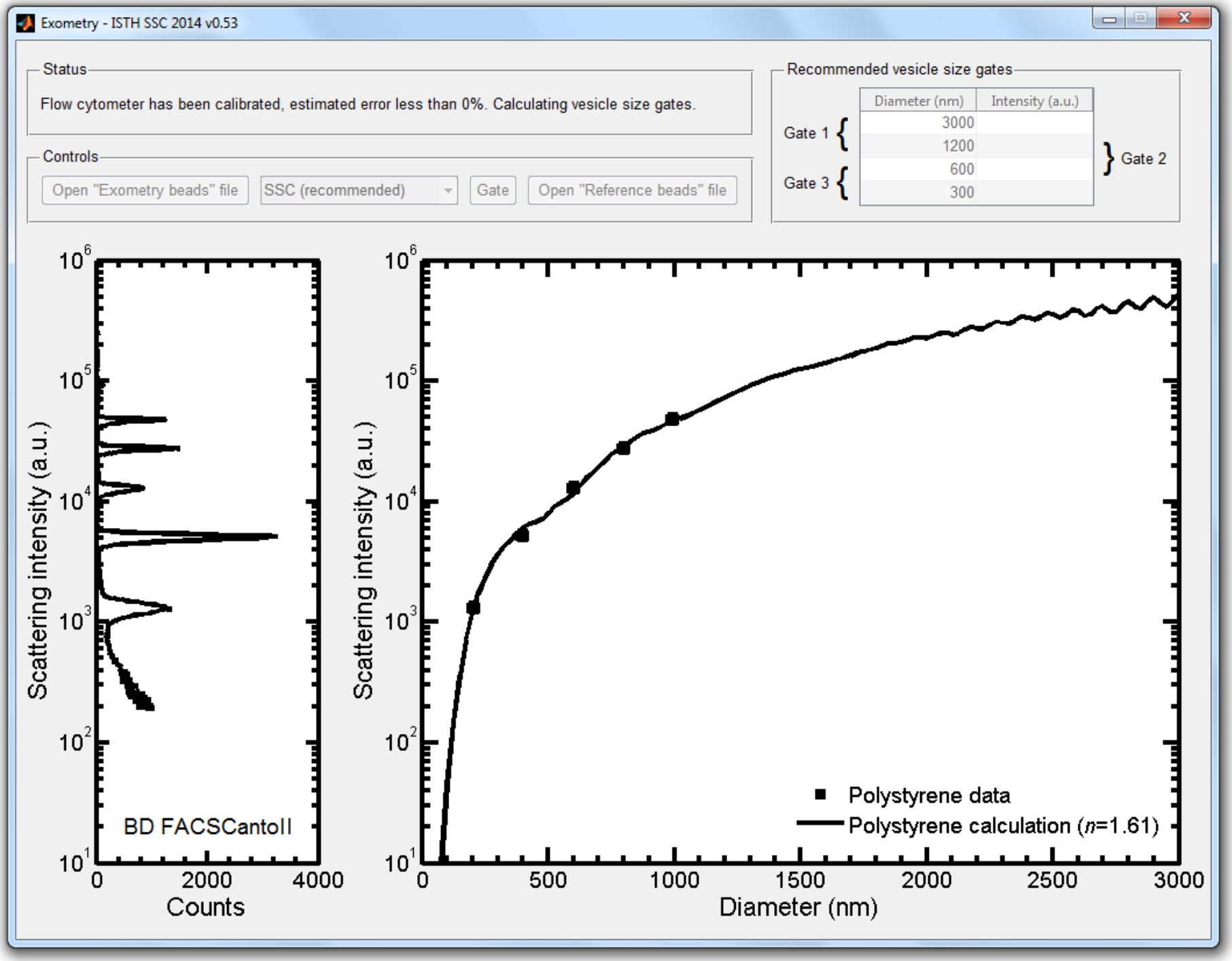
Recommended vesicle size gates

	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
Scattering intensity (a.u.)
BD FACSCantoII
Counts
Diameter (nm)
Polystyrene data
Polystyrene calculation (n=1.61)

Exometry - ISTH SSC 2014 v0.53

Status

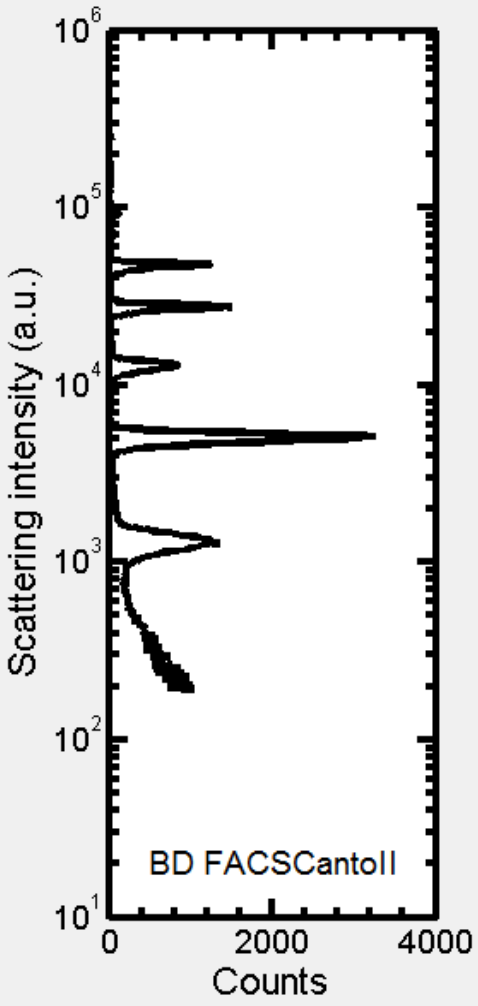
Congratulations, vesicle size gates determined, estimated error less than 0%.

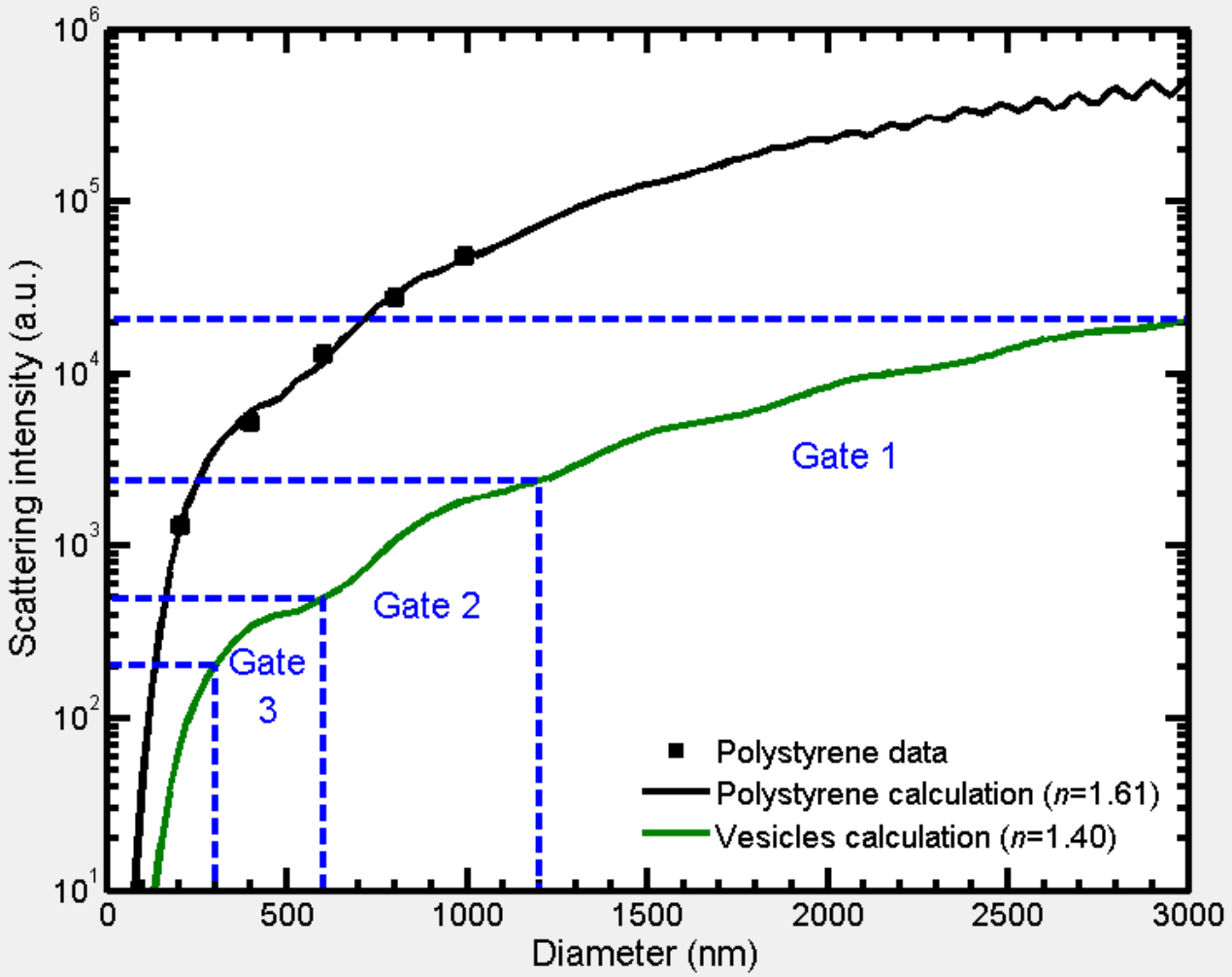
Controls

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

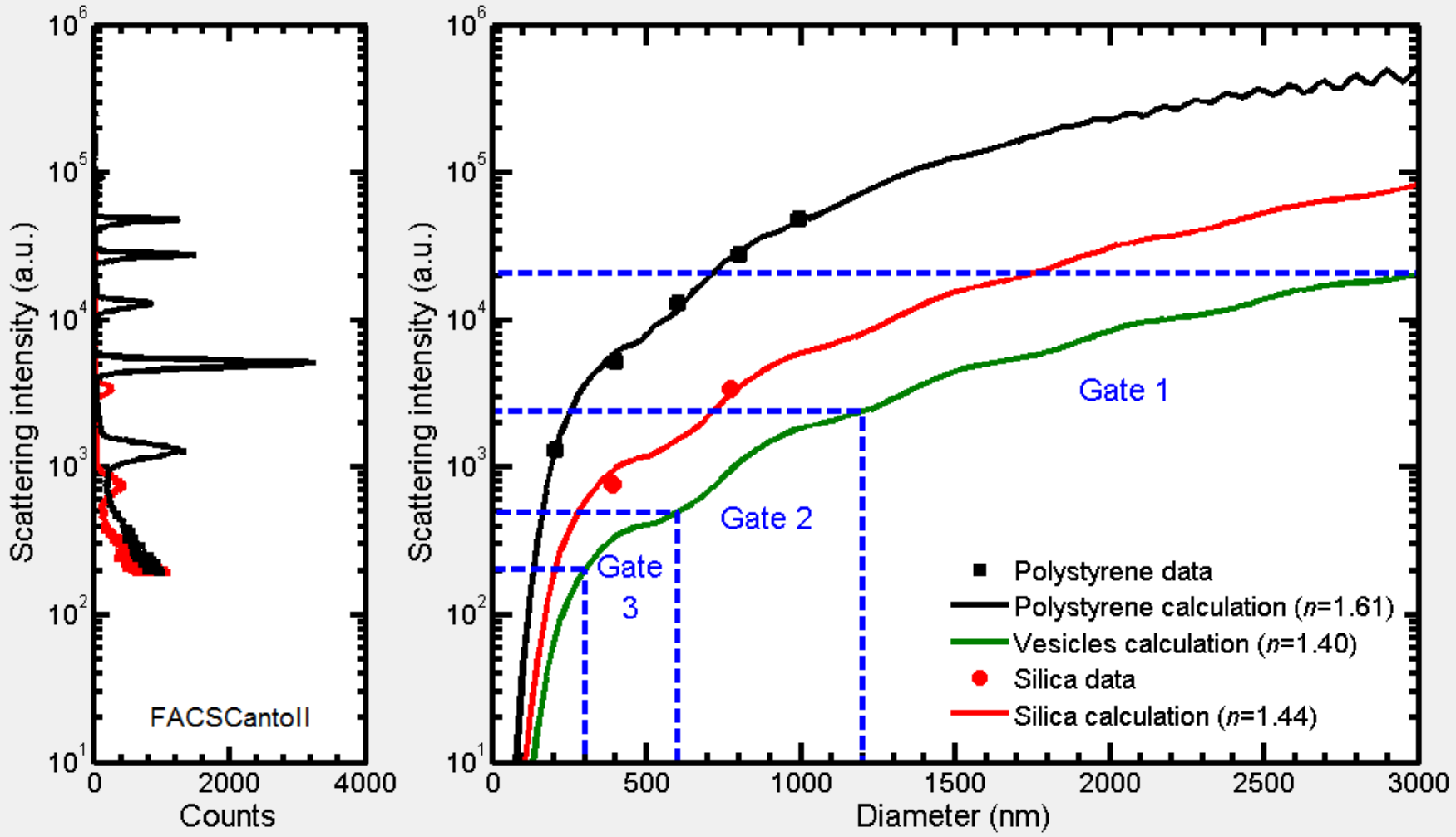
Congratulations, validation succeeded, estimated error less than 4%.

Controls

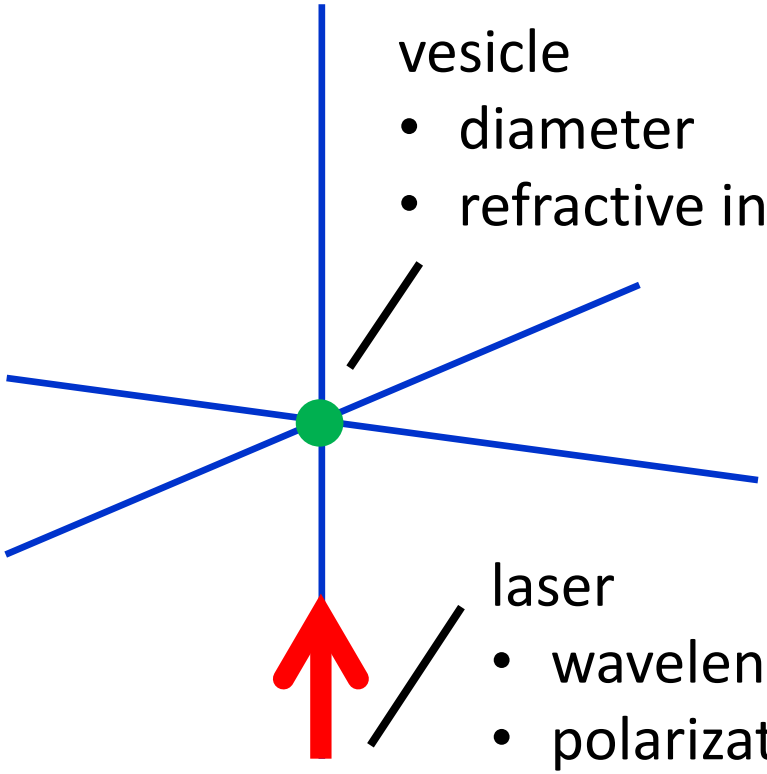
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

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?

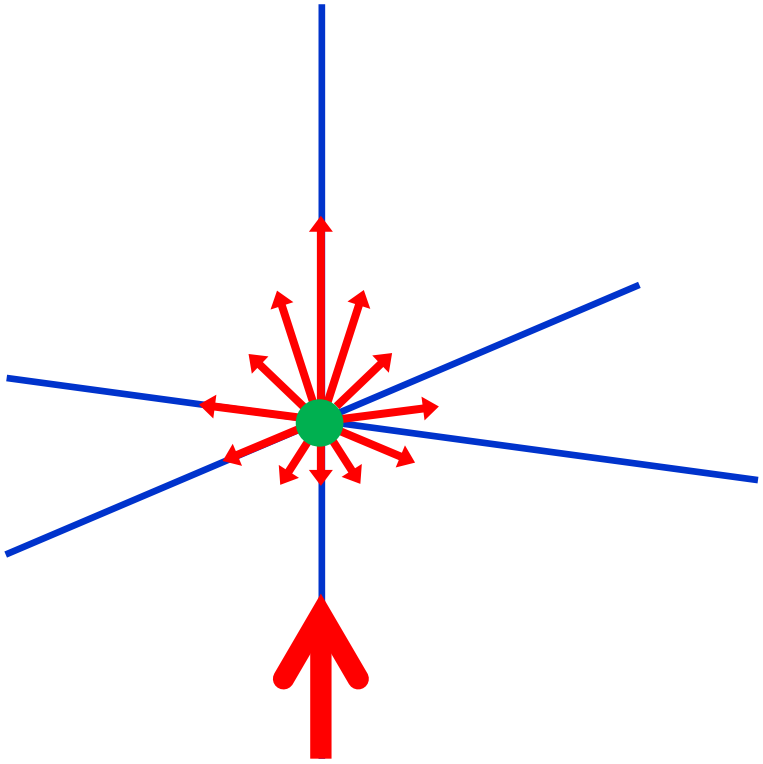


vesicle
- diameter
- refractive index

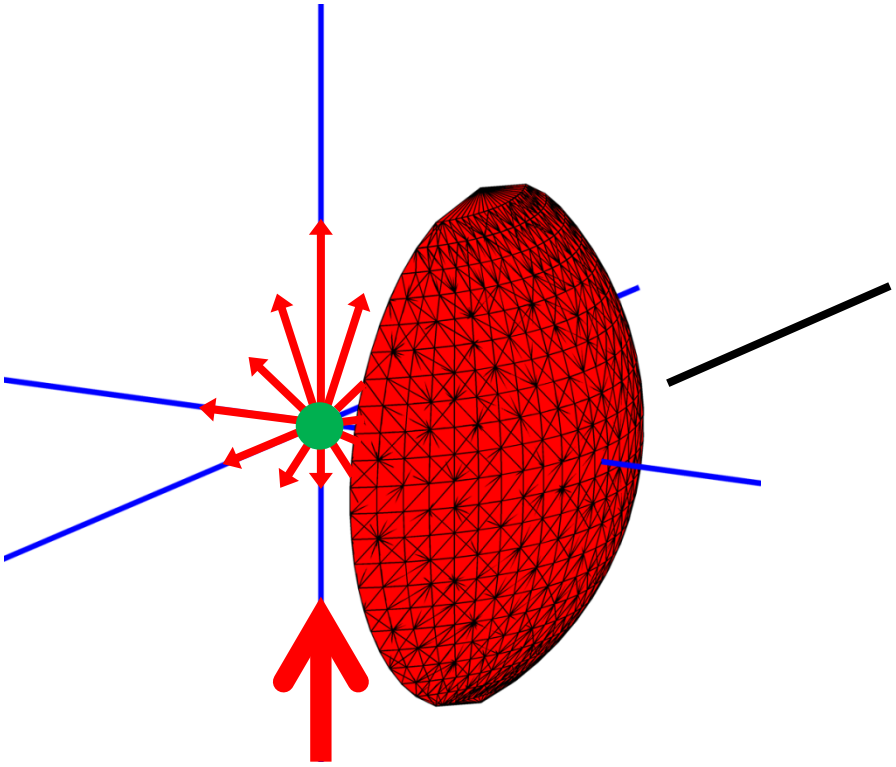
laser
- wavelength
- polarization
- power

Facts
- Mie theory in MATLAB
- ~1,000 lines of code

What does the software do?



What does the software do?

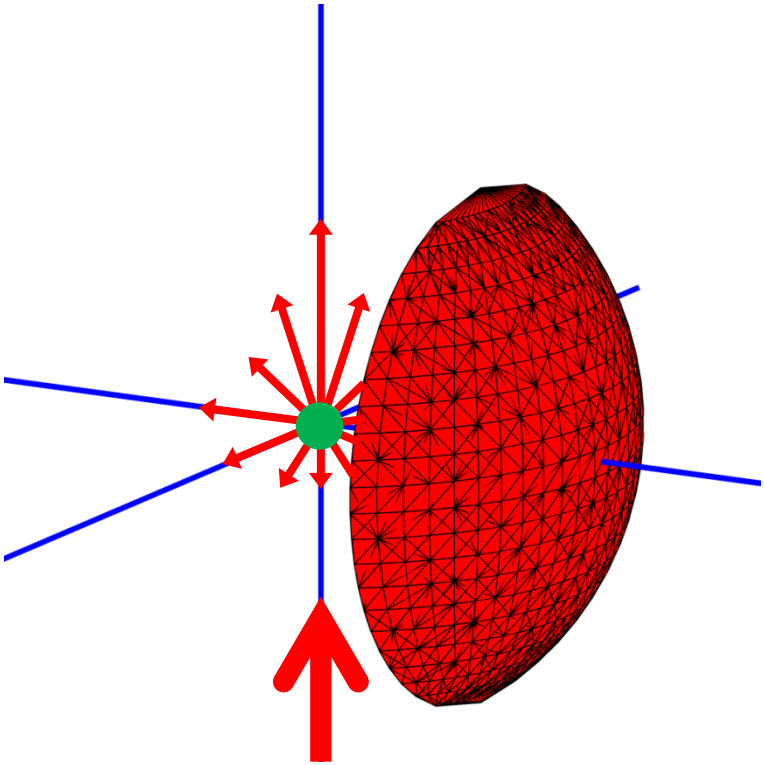


optical configuration

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

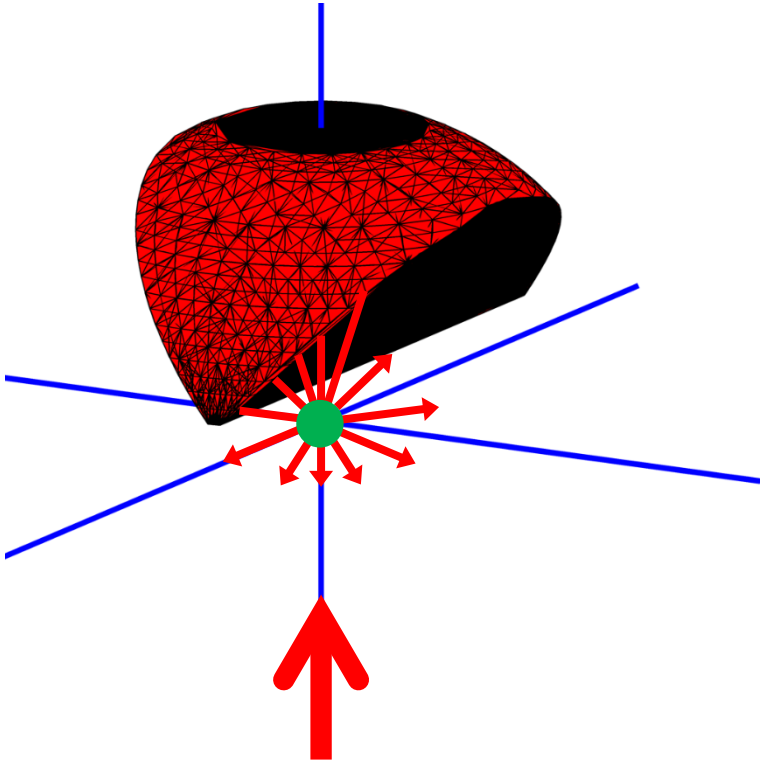
What does the software do?

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.

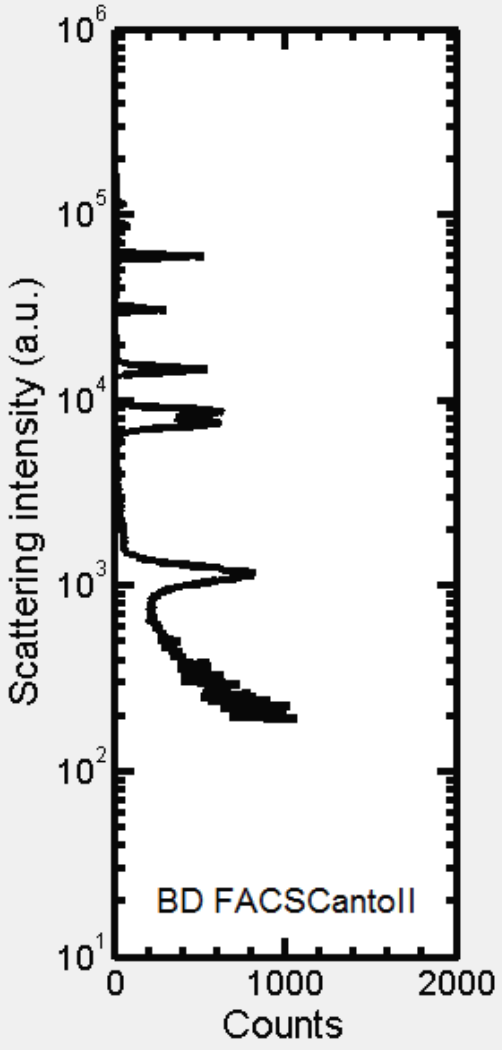
Controls

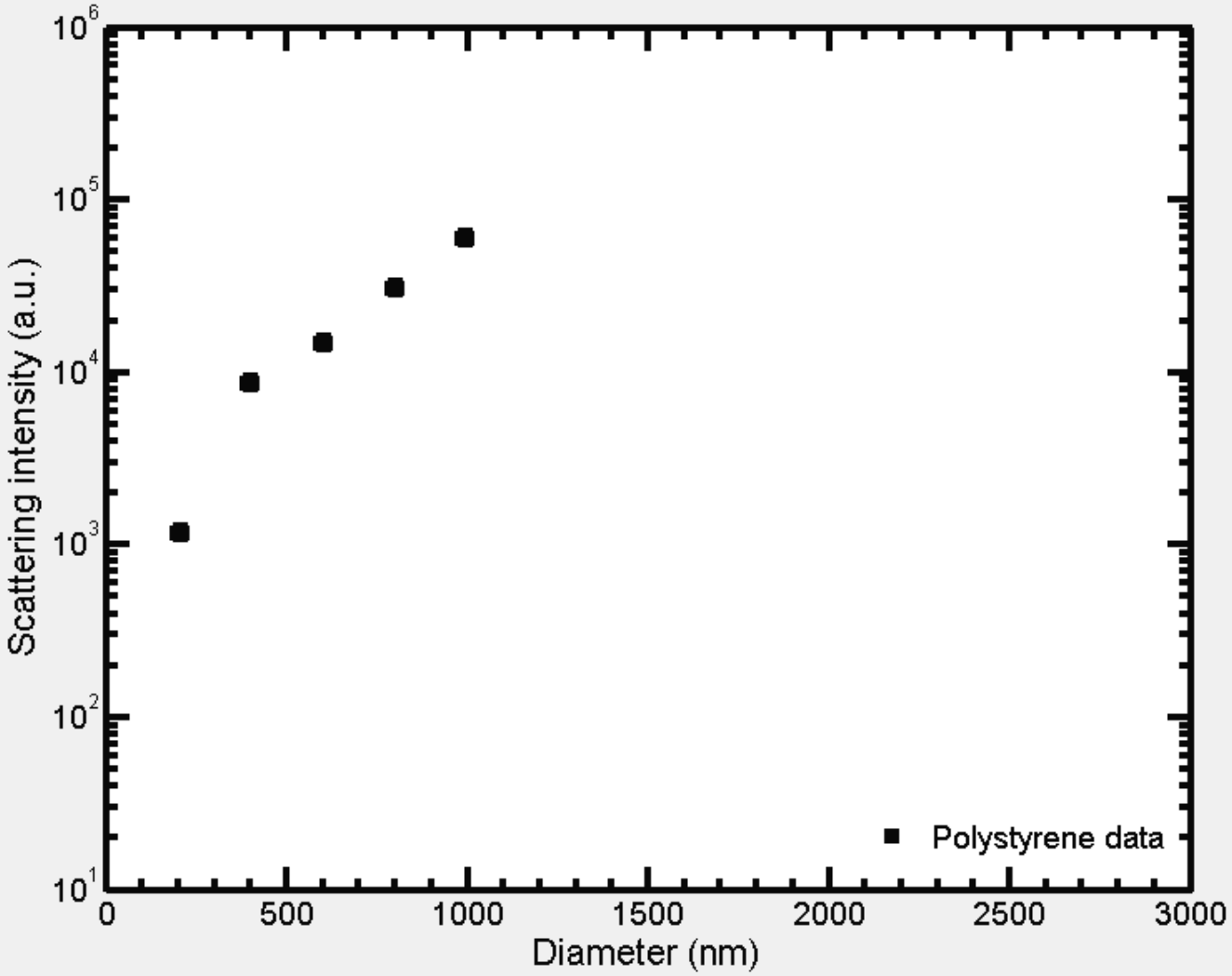
Open "Exometry beads" file | SSC (recommended) | Gate | Open "Reference beads" file

Recommended vesicle size gates

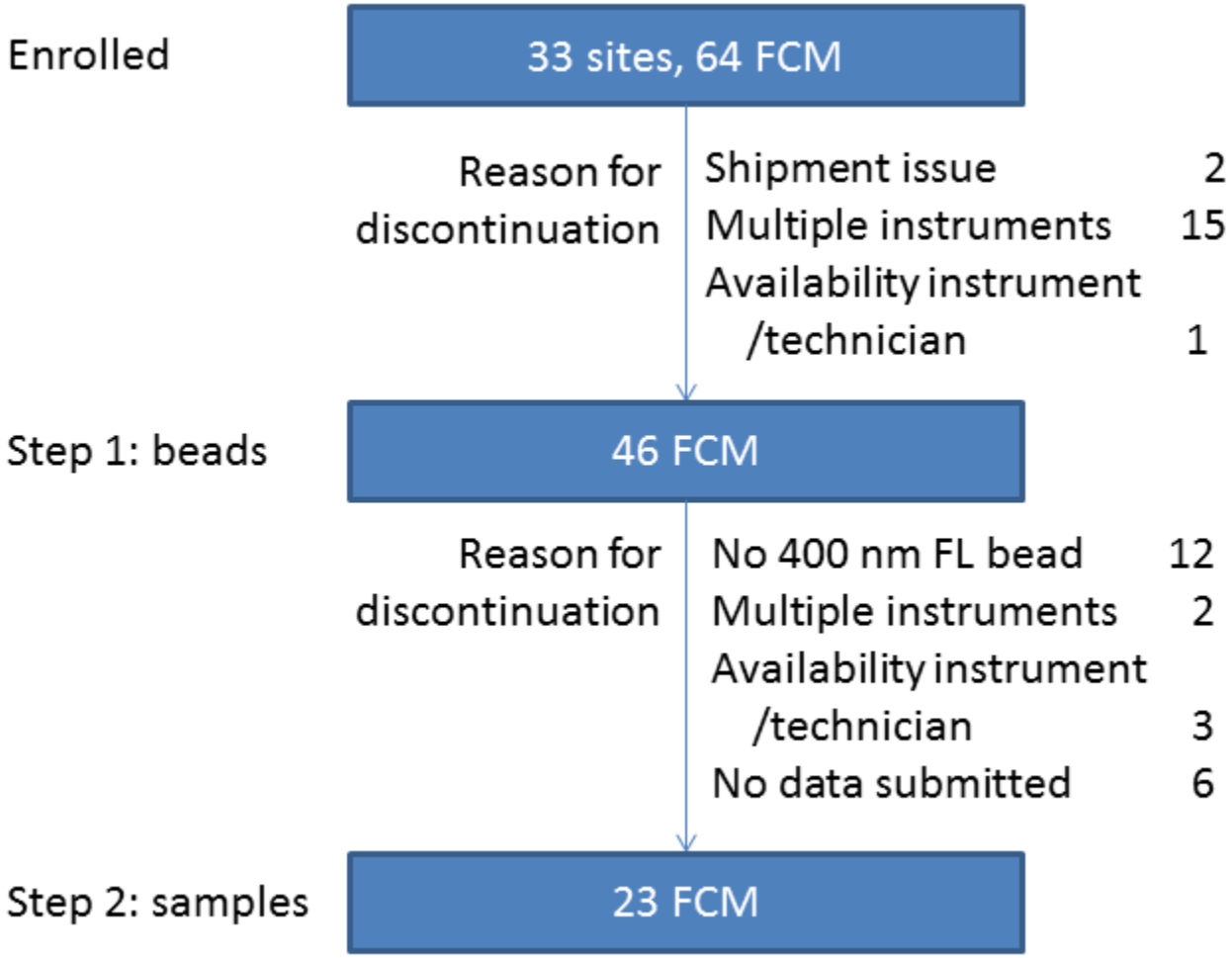
	Diameter (nm)	Intensity (a.u.)
Gate 1	3000	
	1200	
Gate 3	600	
	300	

Gate 2



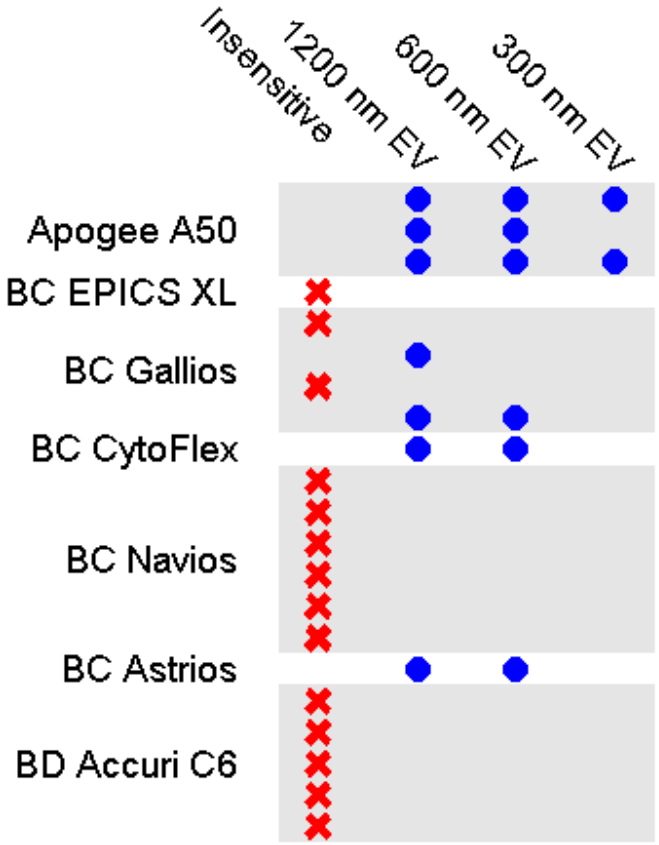


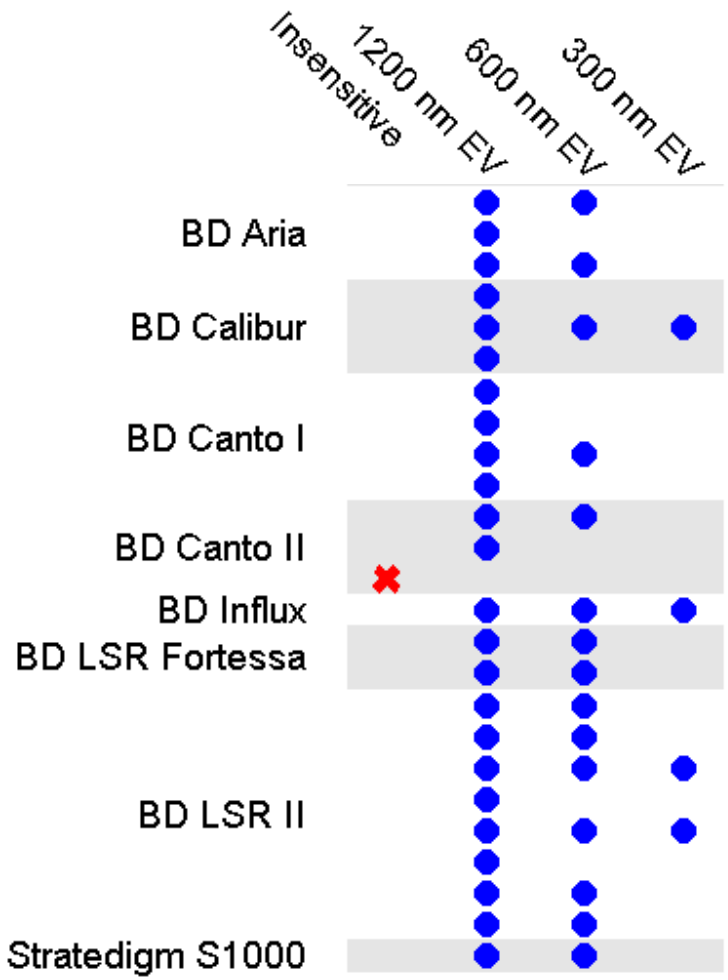
Exclusion of flow cytometers (FCM)



Sensitivity of 46 flow cytometers in the field

✖ = unable to detect 400 nm polystyrene beads





400 nm polystyrene beads scatter more than 1,000 nm EV

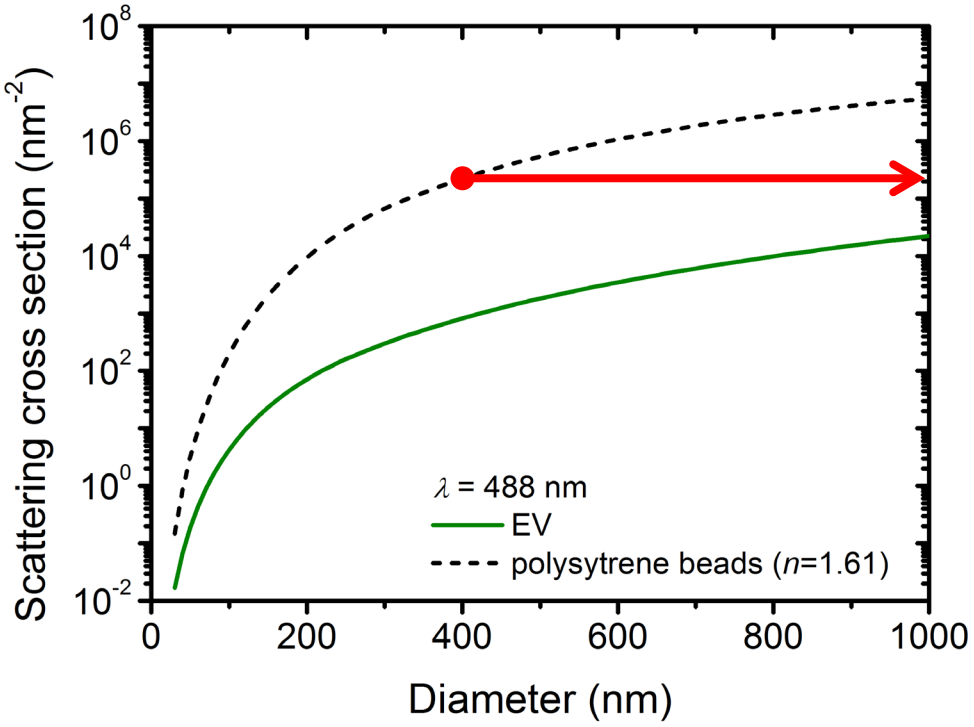
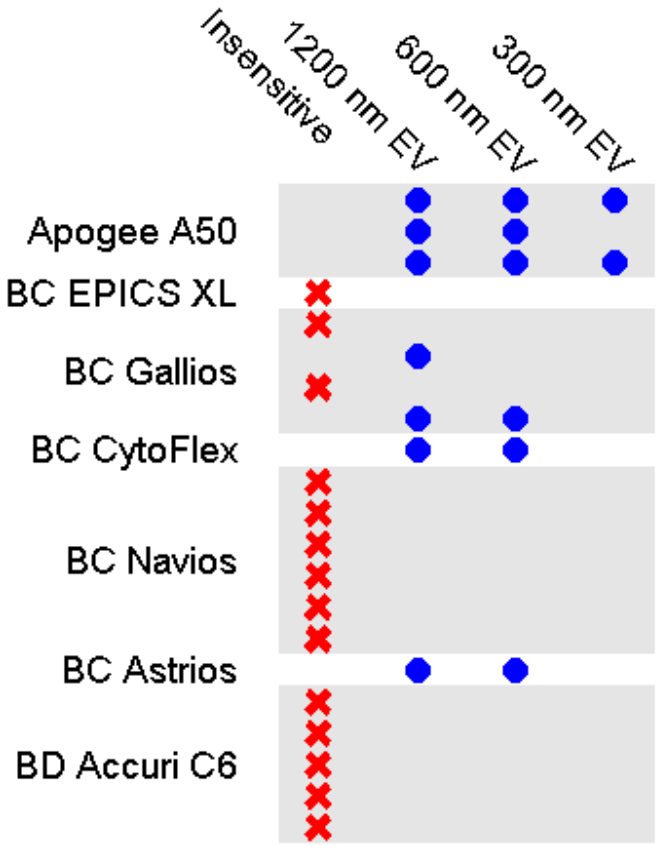
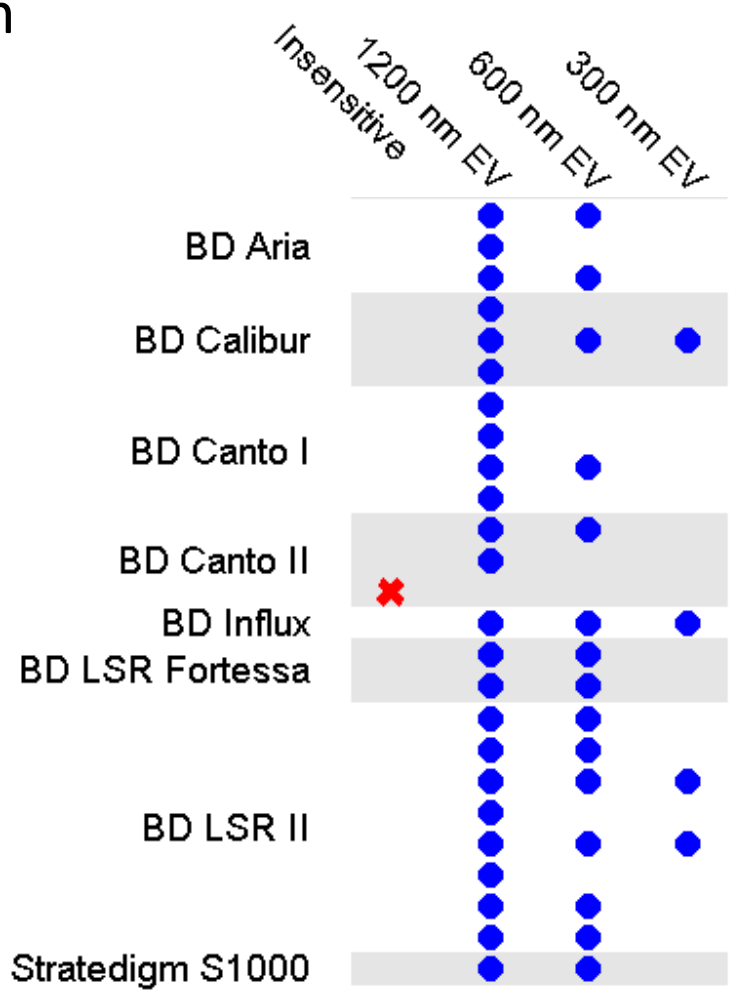


image: Issman et al. *PLoS ONE* 2013
van der Pol et al. *Nano Lett.* 2014

Sensitivity of 46 flow cytometers in the field

✖ = unable to detect EV < 1000 nm

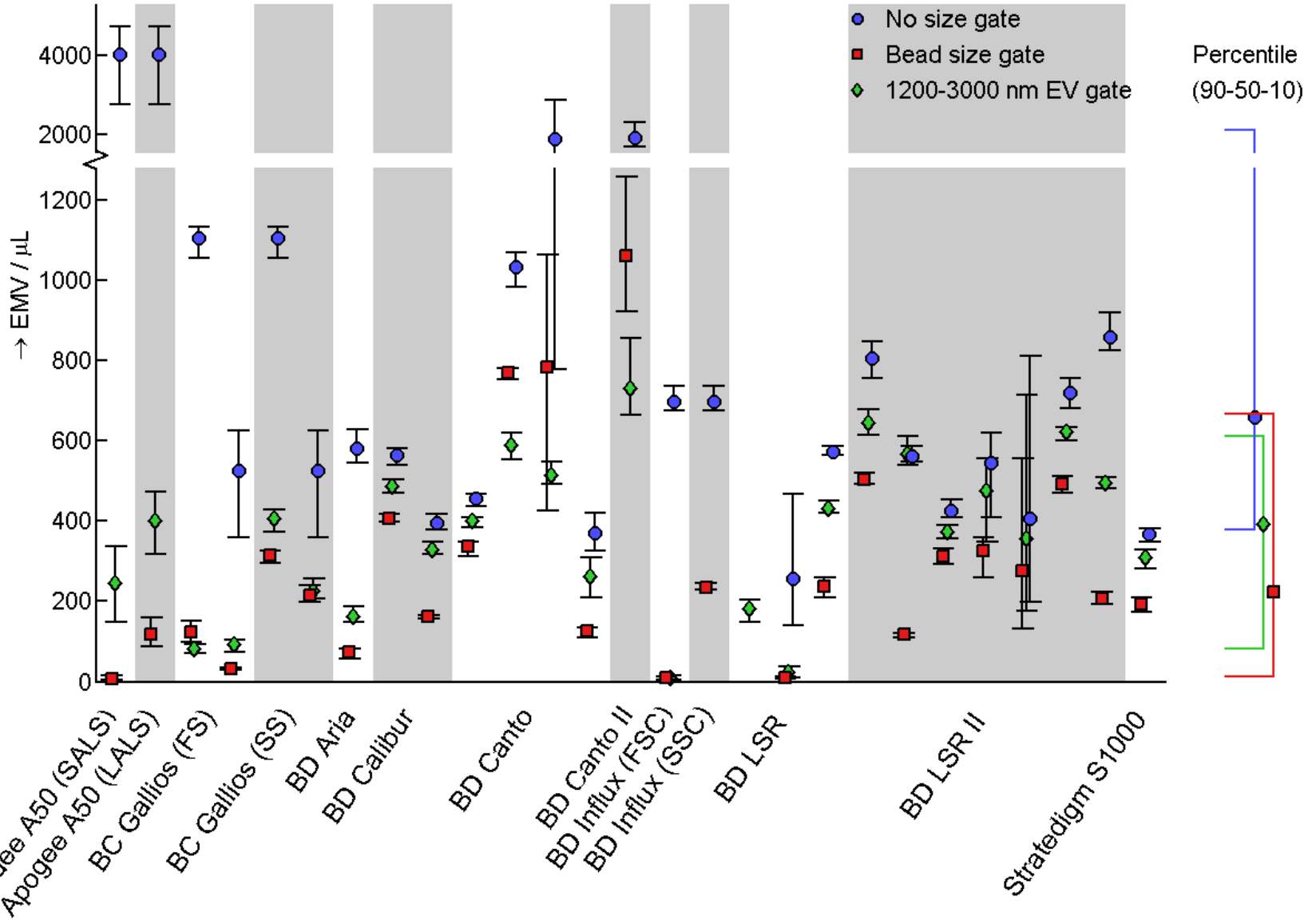




Approach scatter-based standardization

- 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

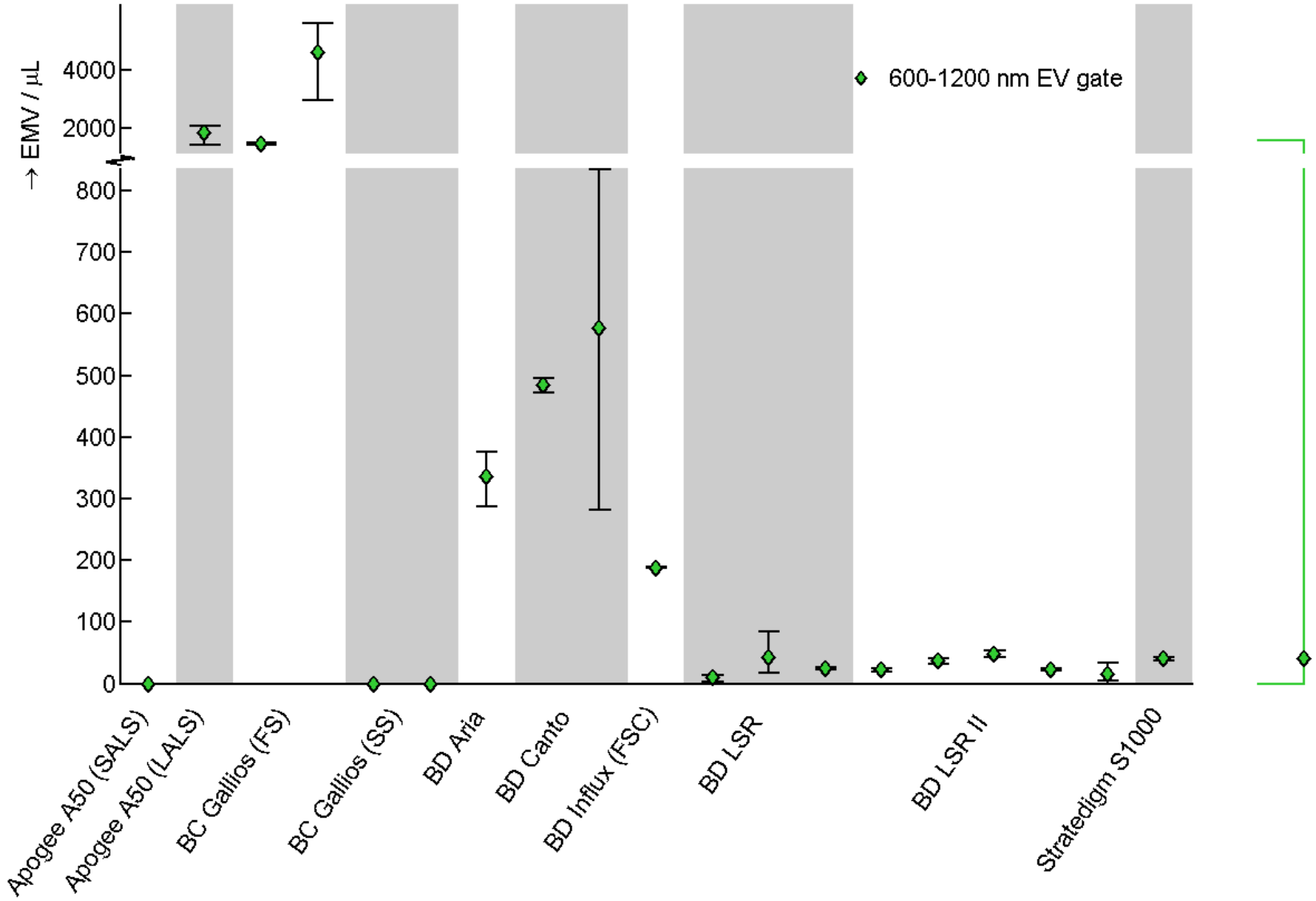


Reproducibility of 1200-3000 nm EV

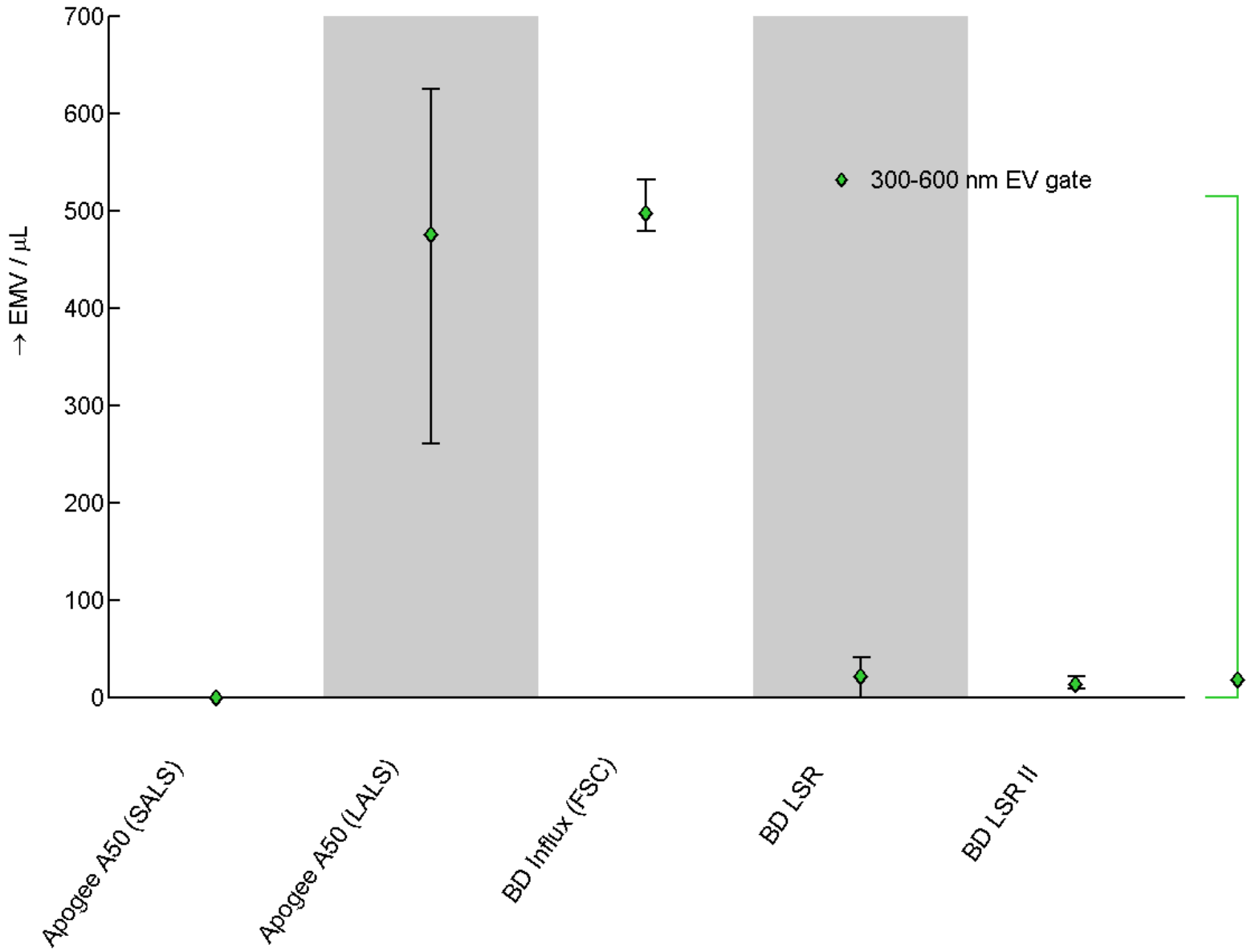
%CV	All	SSC only	FSC only
Gate on beads	74%	60%	80%
Gate on EV size with light scatter theory	59%	42%	92%

%CV = standard deviation / mean * 100%
Preliminary results

Reproducibility of 600-1200 nm EV



Reproducibility of 300-600 nm EV



Conclusions scatter-based standardization

- 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 scatter-based standardization

- 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

Single extracellular vesicle (EV) analysis

- motivation
- requirements
- detection methods
  - nanoparticle tracking analysis (NTA)
  - tunable resistive pulse sensing (TRPS)
  - flow cytometry
- scatter-based standardization of flow cytometry measurements
- summary

Summary

- heterogeneity of EV requires single EV analysis
- sensitive flow-based techniques are superior to NTA and TRPS
- scatter-based standardization of flow cytometry measurements
  - provides insight (EV diameter)
  - improves reproducibility (CV=59%)
  - is compatible with fluorescence approaches for next standardization initiatives

Outlook

Hematology parameter	Concentration (vesicles mL^{-1})
Platelet vesicle count	$2.3 - 6.2 \cdot 10^9$
Erythrocyte vesicle count	$7.0 - 8.6 \cdot 10^{10}$
Reticulocyte vesicle count	$3.9 - 15.6 \cdot 10^8$
Leukocyte vesicle count	$6.2 - 16.4 \cdot 10^7$
Total vesicle count	$7.3 - 9.4 \cdot 10^{10}$

Acknowledgements

- Vesicle Observation Center
  Academic Medical Center
  University of Amsterdam

- Laboratory Experimental Cancer Research, Ghent University
  - Olivier de Wever
  - An Hendrix

- Software and beads by exometry.com

- More info: edwinvanderpol.com

Vesicle
Observation Center