Isolation and detection of extracellular vesicles as liquid biopsy biomarkers

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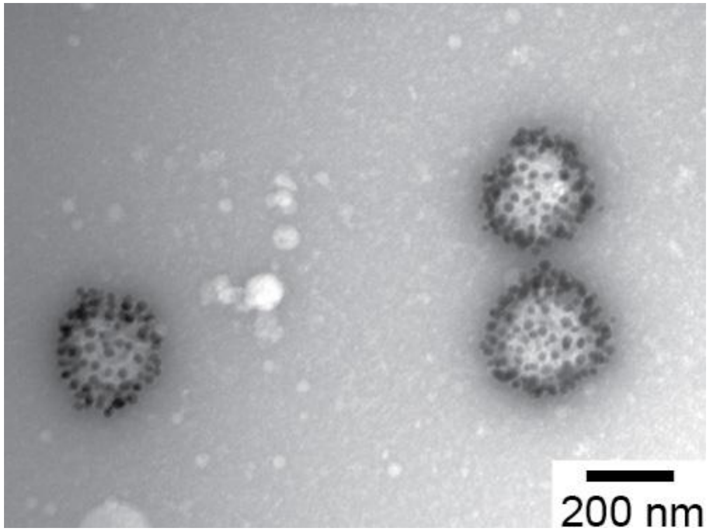
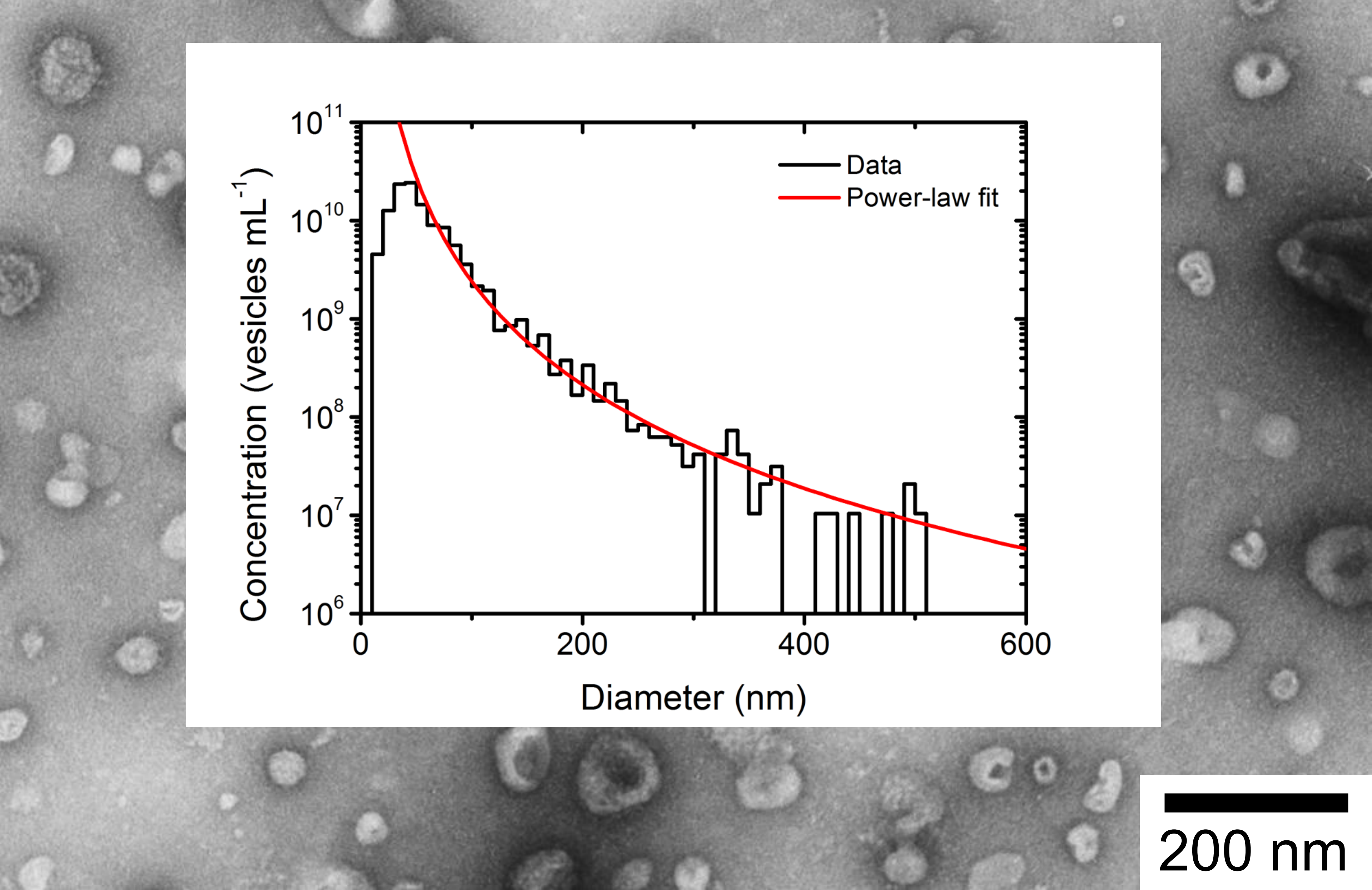
University Medical Centers

Outline

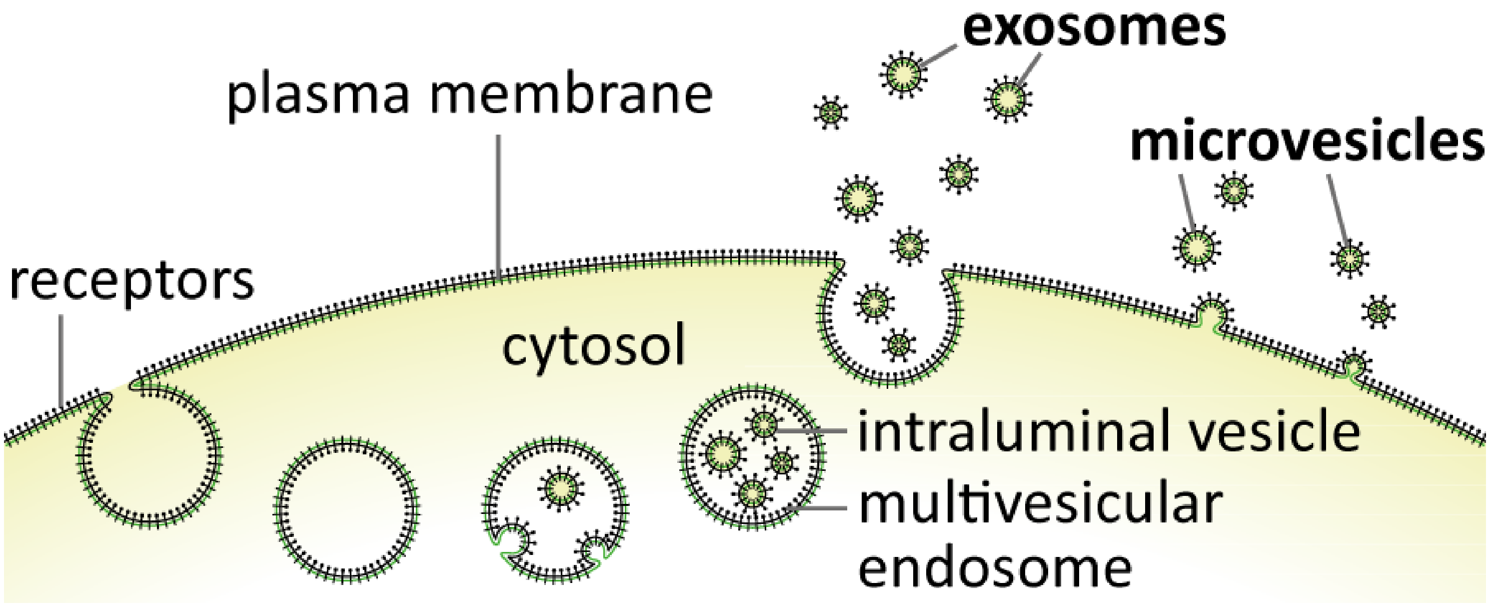
- Extracellular vesicles (EVs)
- EV collection and storage
- EV isolation
- EV detection
  - Ensemble techniques
  - Single particle techniques
    - Flow cytometry
    - Standardization



Extracellular vesicles



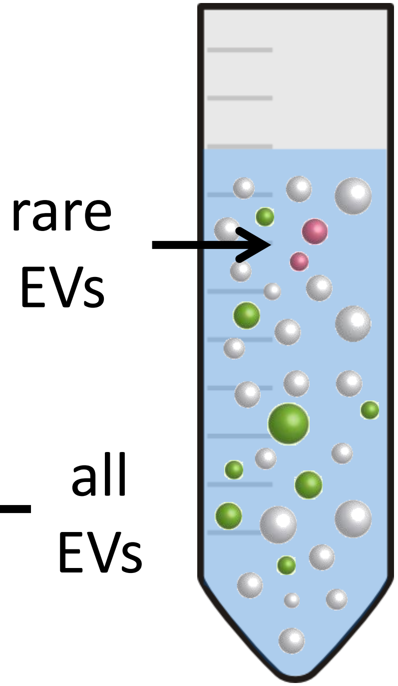
Extracellular vesicles



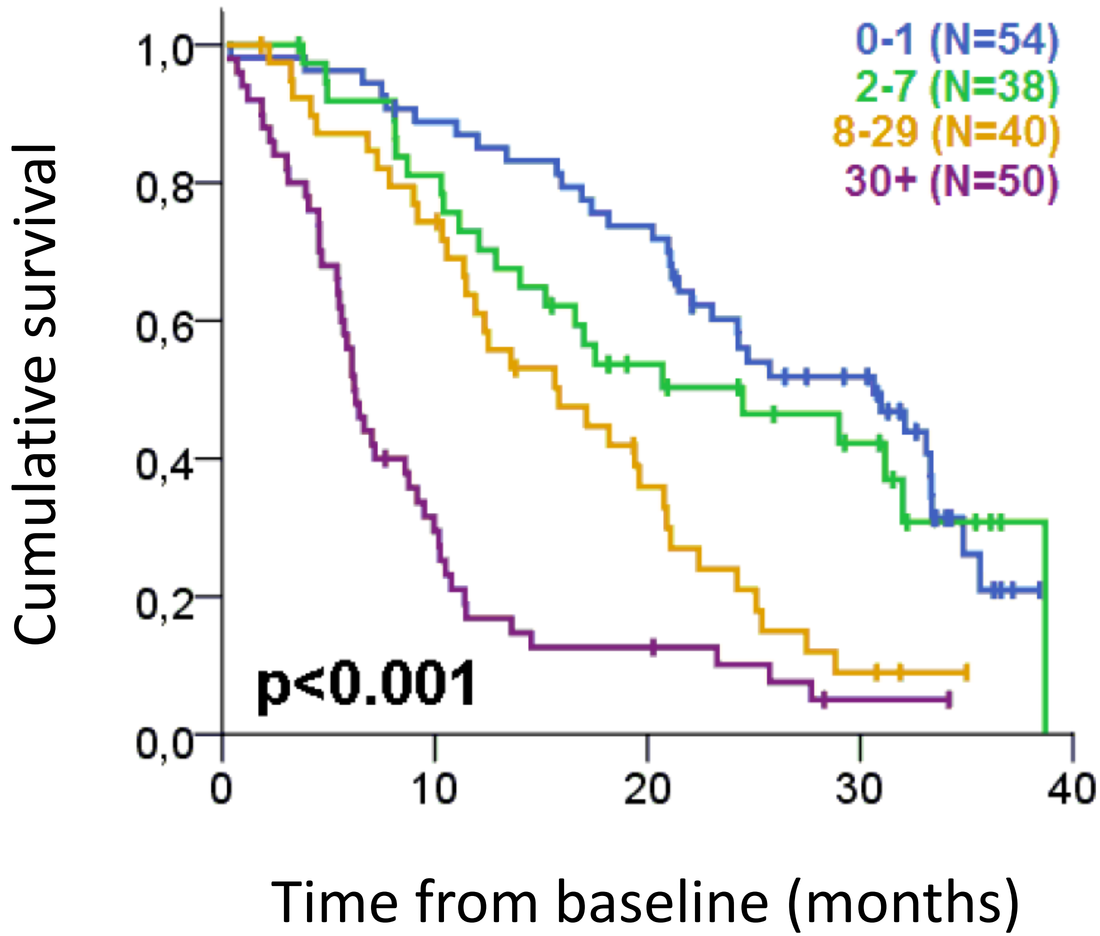
- Cells release EVs:
  biological nanoparticles with receptors, DNA, RNA
- Specialized functions
- Clinically relevant

EV-based "liquid biopsy"

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	$7.3 - 9.4 \cdot 10^{10}$

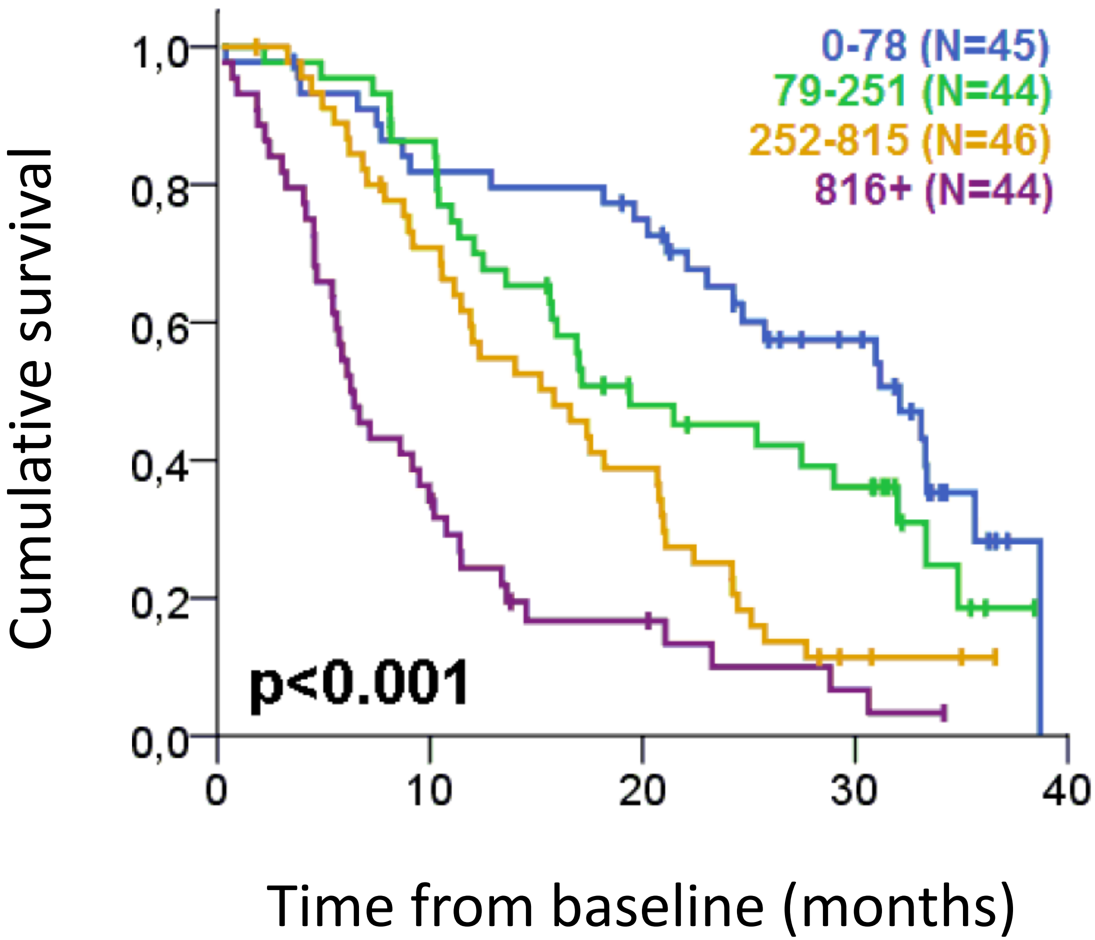


Circulating tumor cells predict overall survival



Coumans et al. *Ann Oncol* 2010

Circulating tumor microparticles predict overall survival



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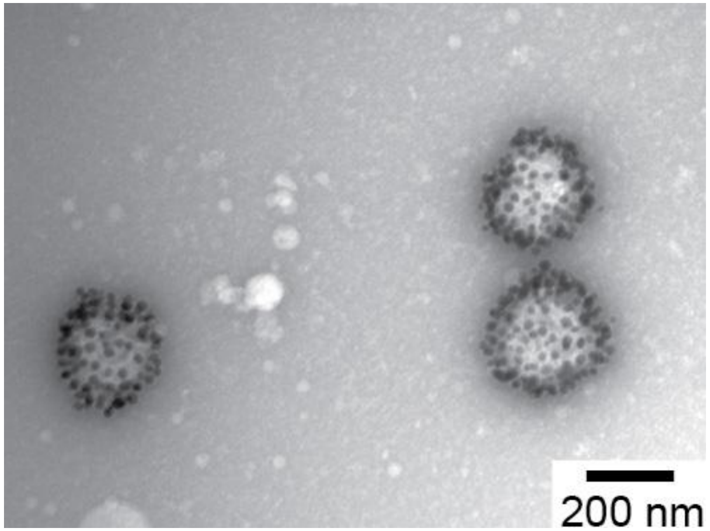
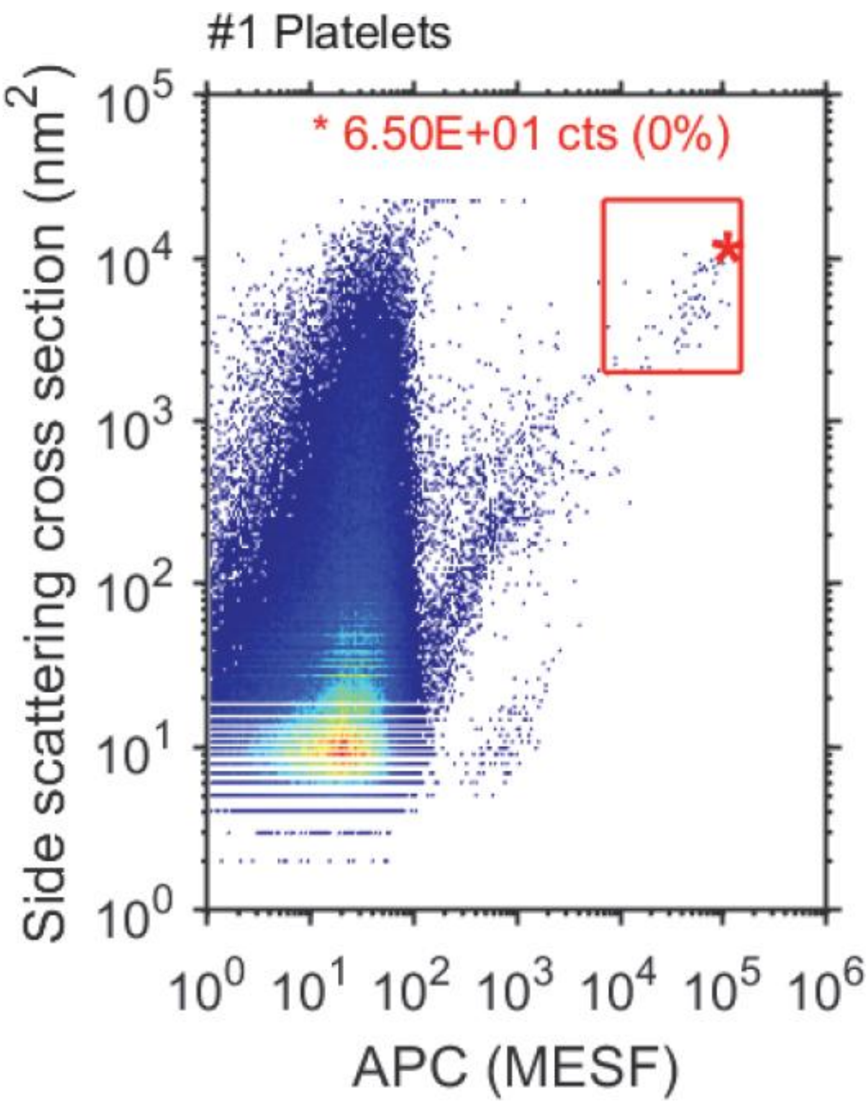


EV collection

- Body fluids contain EVs and cells
- Problem: cells release EVs after collection
- Possible solutions:
  - use large diameter (19 gauge) needle
  - use anticoagulant
  - keep tubes in vertical position
  - minimize time between collection and cell removal

EV storage

- EVs seem stable during freeze-thaw cycle
- Problem: freezing may break cells apart
- Common but inadequate solution:
  - remove all cells (including all platelets) by centrifugation



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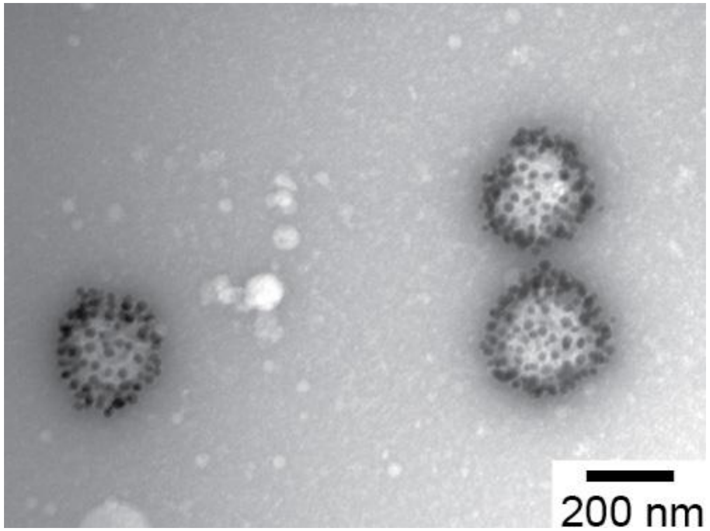
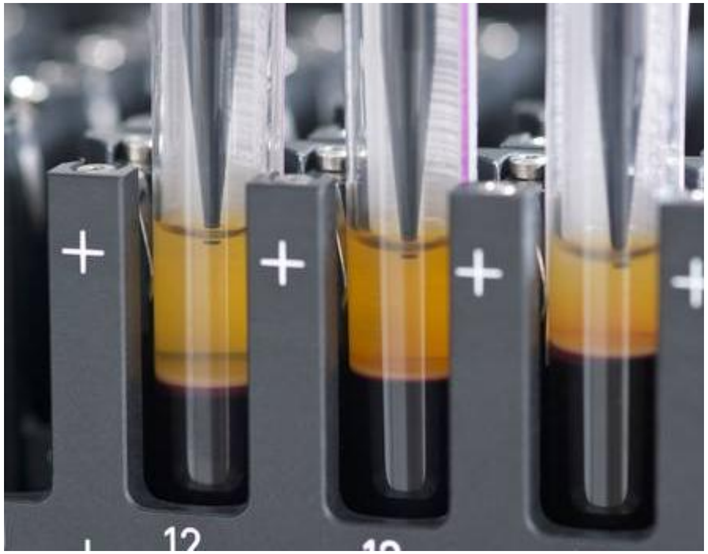
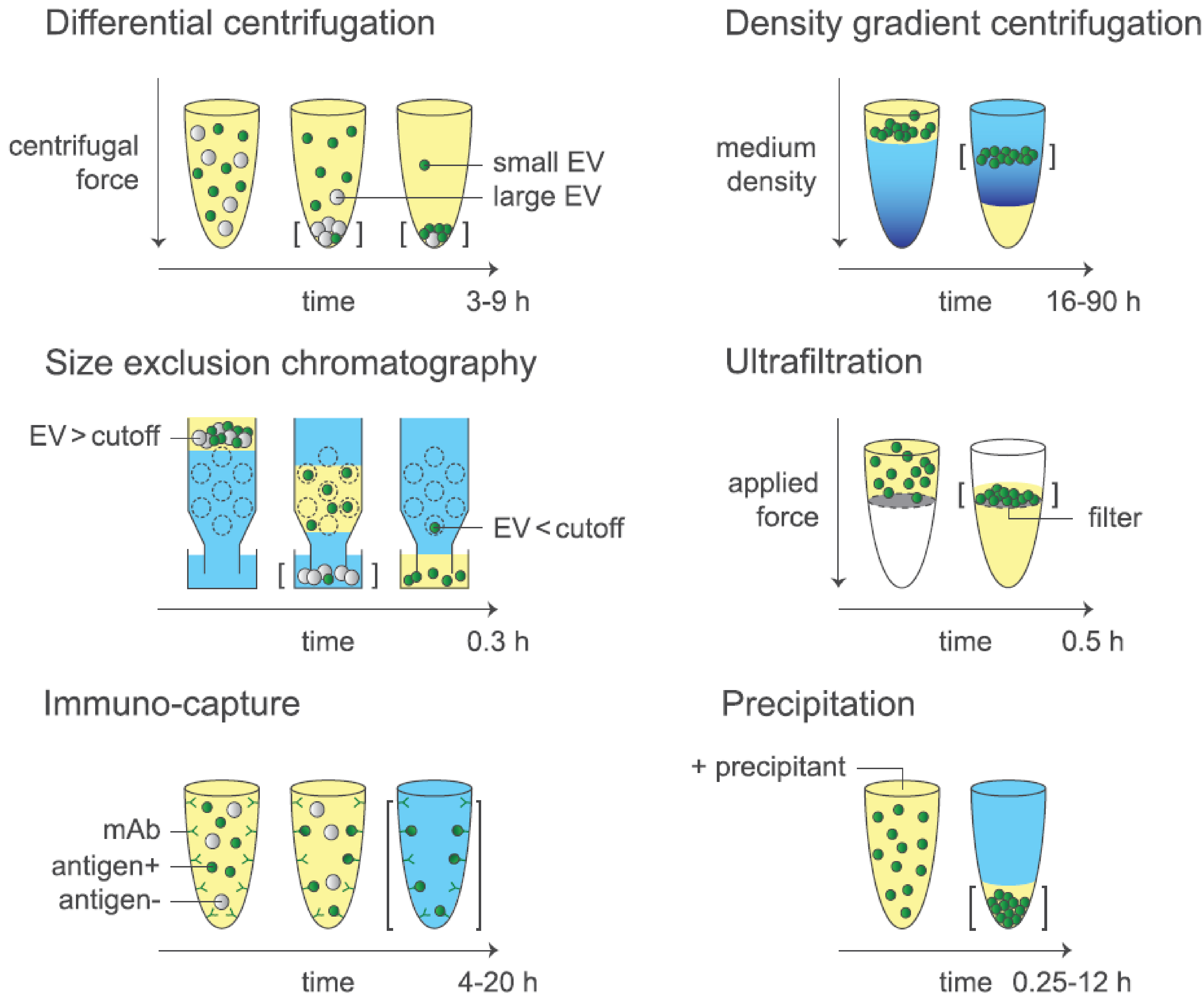


EV isolation

- Body fluids may contain
  - Proteins
  - Small RNAs
  - Lipoprotein particles
  - **EVs**
    - DNA
    - RNAs
    - Proteins
  - Cells (which release EVs)




Differential centrifugation
centrifugal force
small EV
large EV
time
3-9 h
Density gradient centrifugation
medium density
time
16-90 h
Size exclusion chromatography
EV > cutoff
EV < cutoff
time
0.3 h
Ultrafiltration
applied force
filter
time
0.5 h
Immuno-capture
mAb
antigen+
antigen-
time
4-20 h
Precipitation
+ precipitant
time
0.25-12 h

Current isolation techniques are inadequate

- Body fluids may contain
  - Proteins
  - Small RNAs
  - Lipoprotein particles
  - **EVs**
    - DNA
    - RNAs
    - Proteins
  - Cells (which release EVs)

- Overlapping physical properties
  - Size
  - Density
  - Refractive index
  - Biochemical composition

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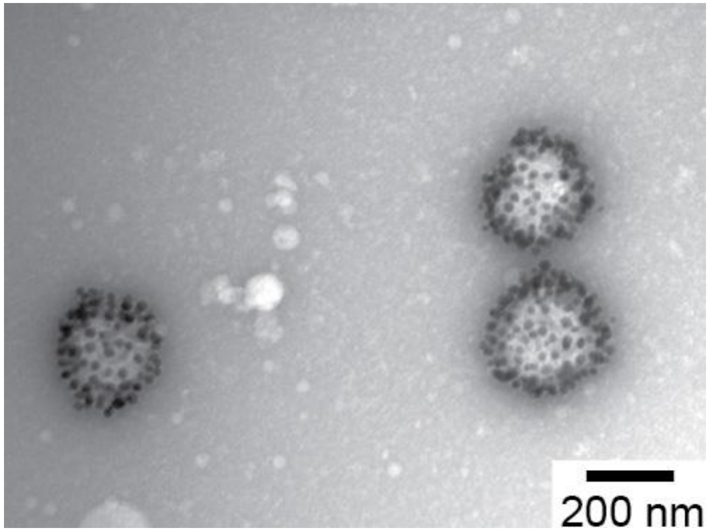
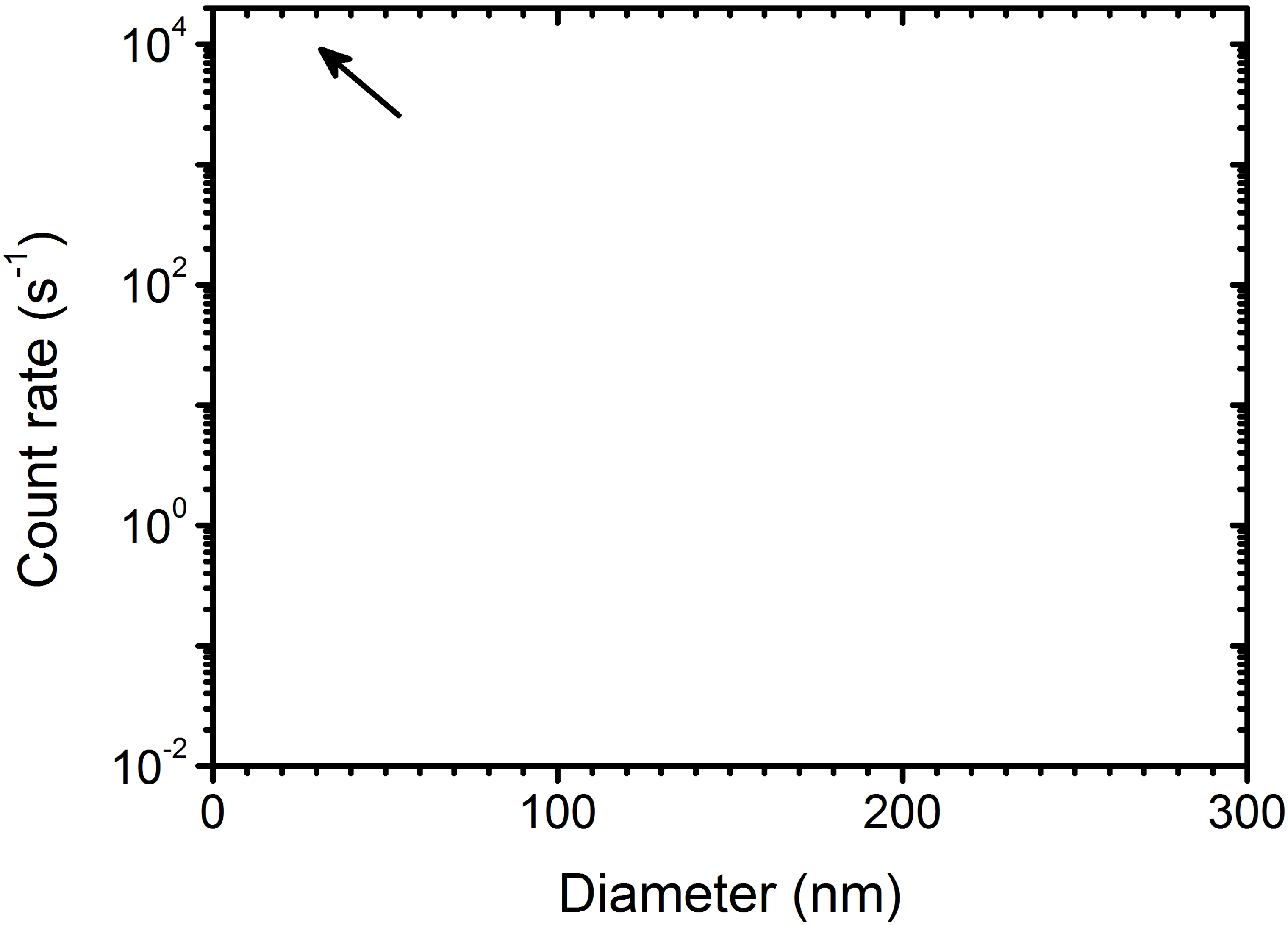
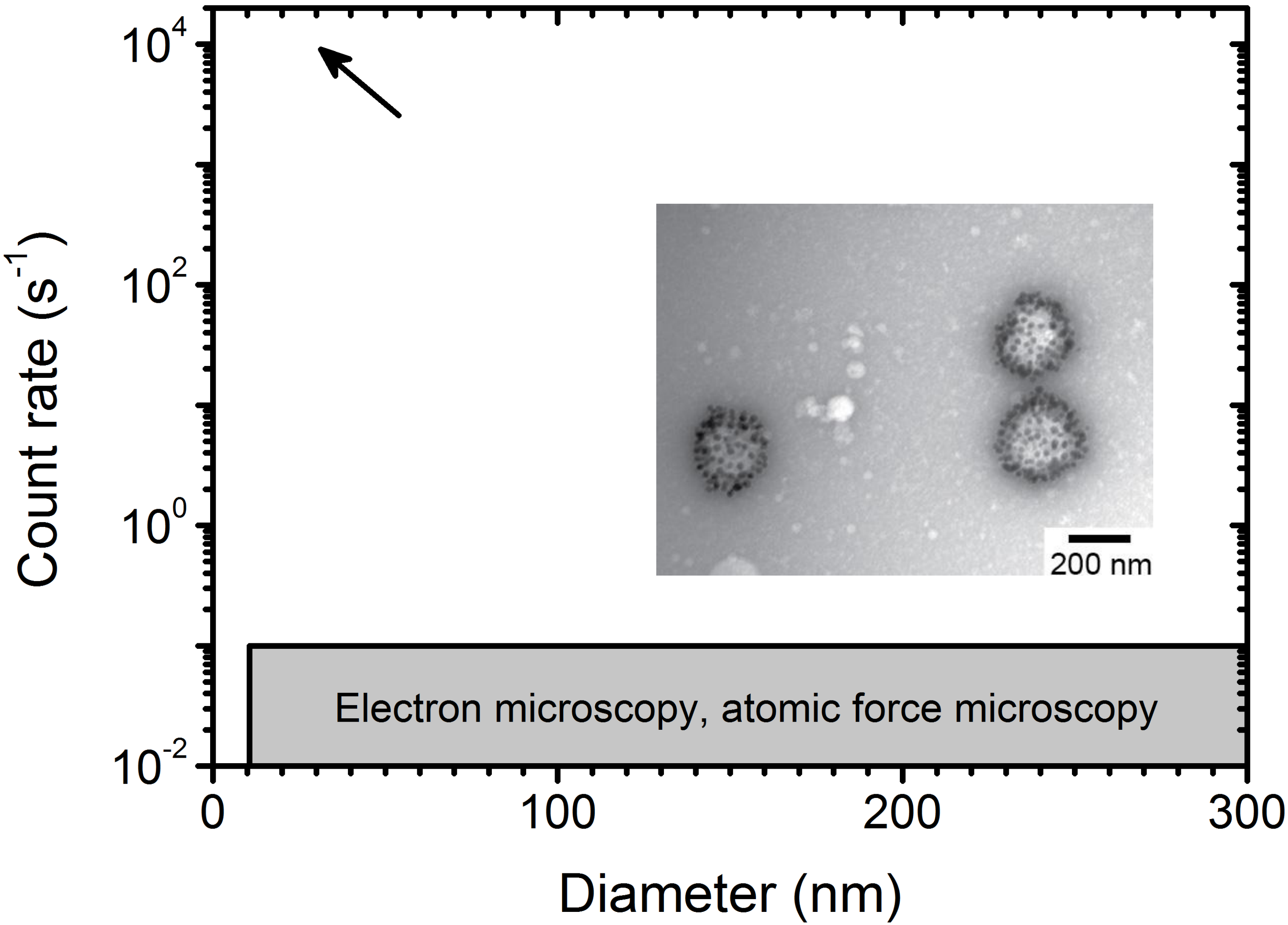


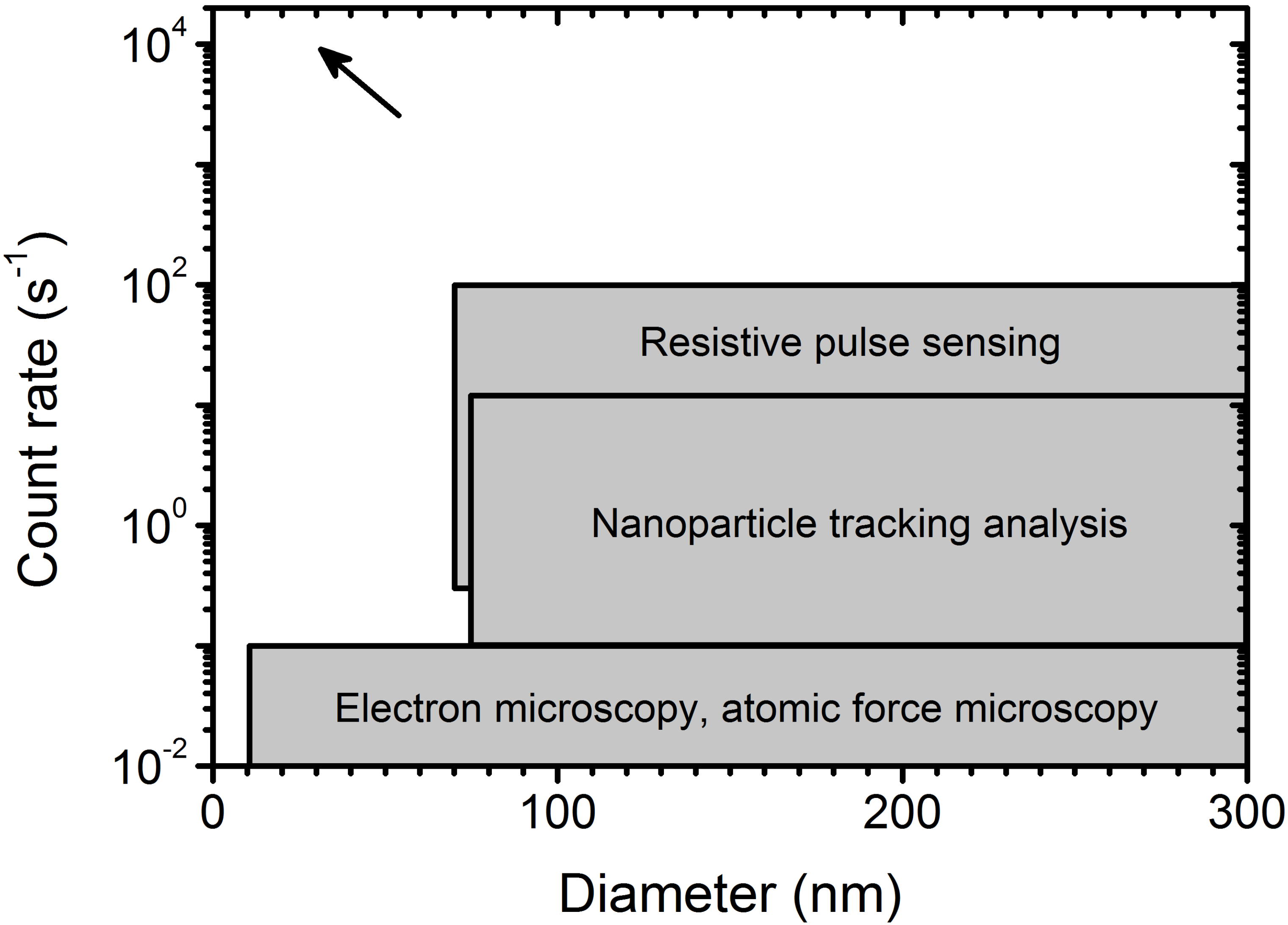
Types of detection techniques

- Ensemble techniques
  - Content
    - e.g. proteomics, genomics, western blot
  - Functional tests
    - e.g. fibrin generation test
- Single particle techniques
  - e.g. flow cytometry, nanoparticle tracking analysis, resistive pulse sensing, electron microscopy

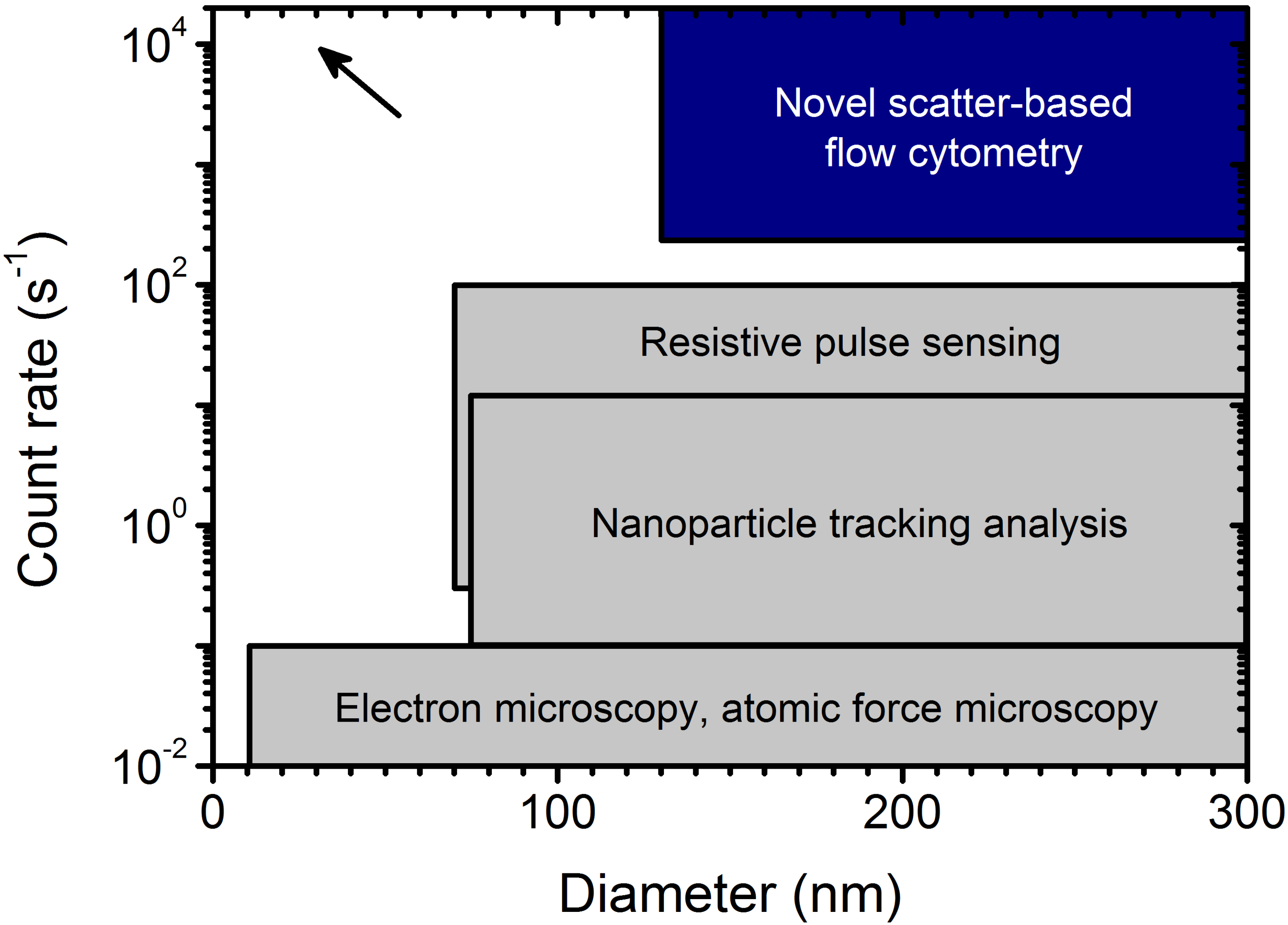


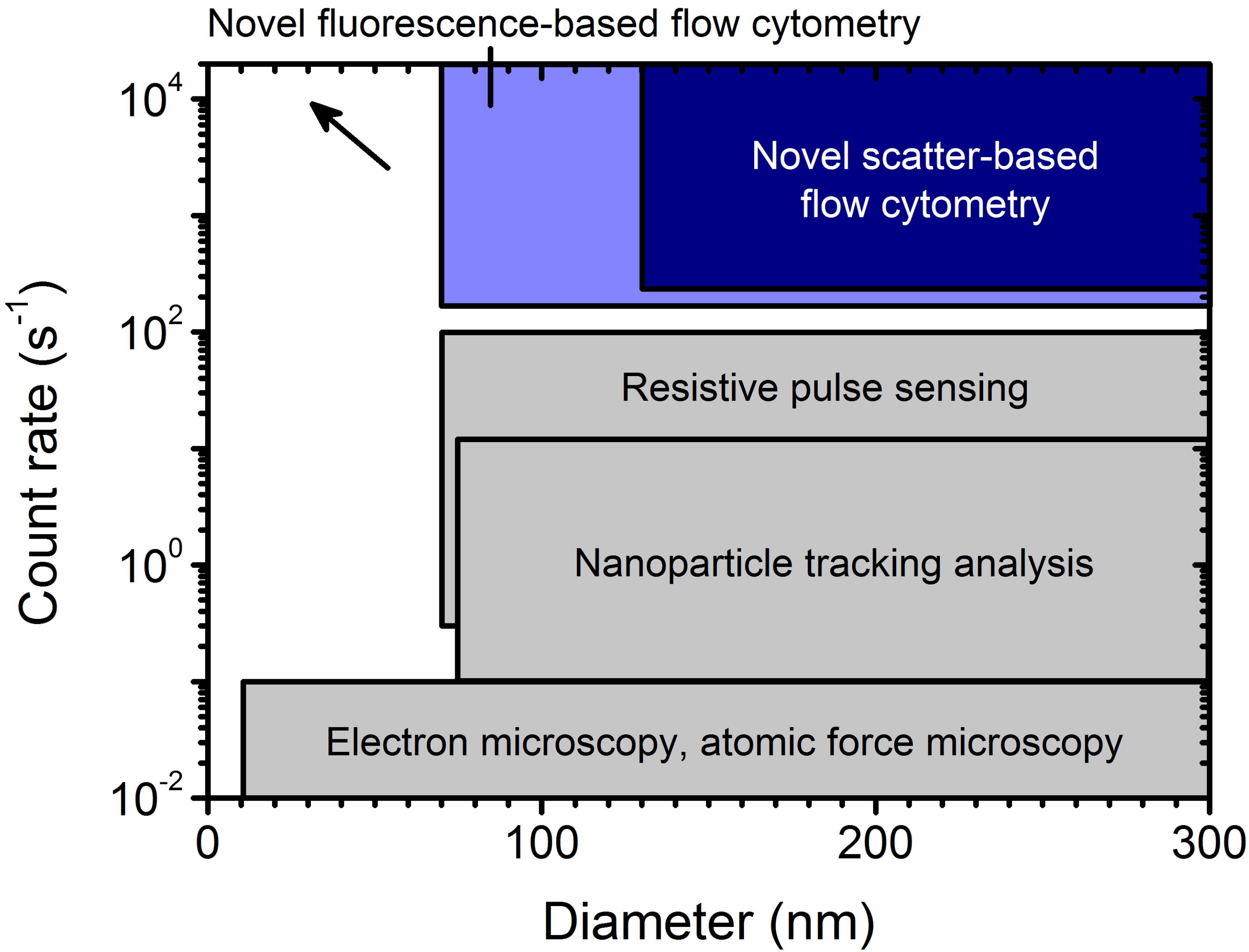
10^4
10^2
10^0
10^{-2}
Count rate (s^{-1})
0
100
200
300
Diameter (nm)

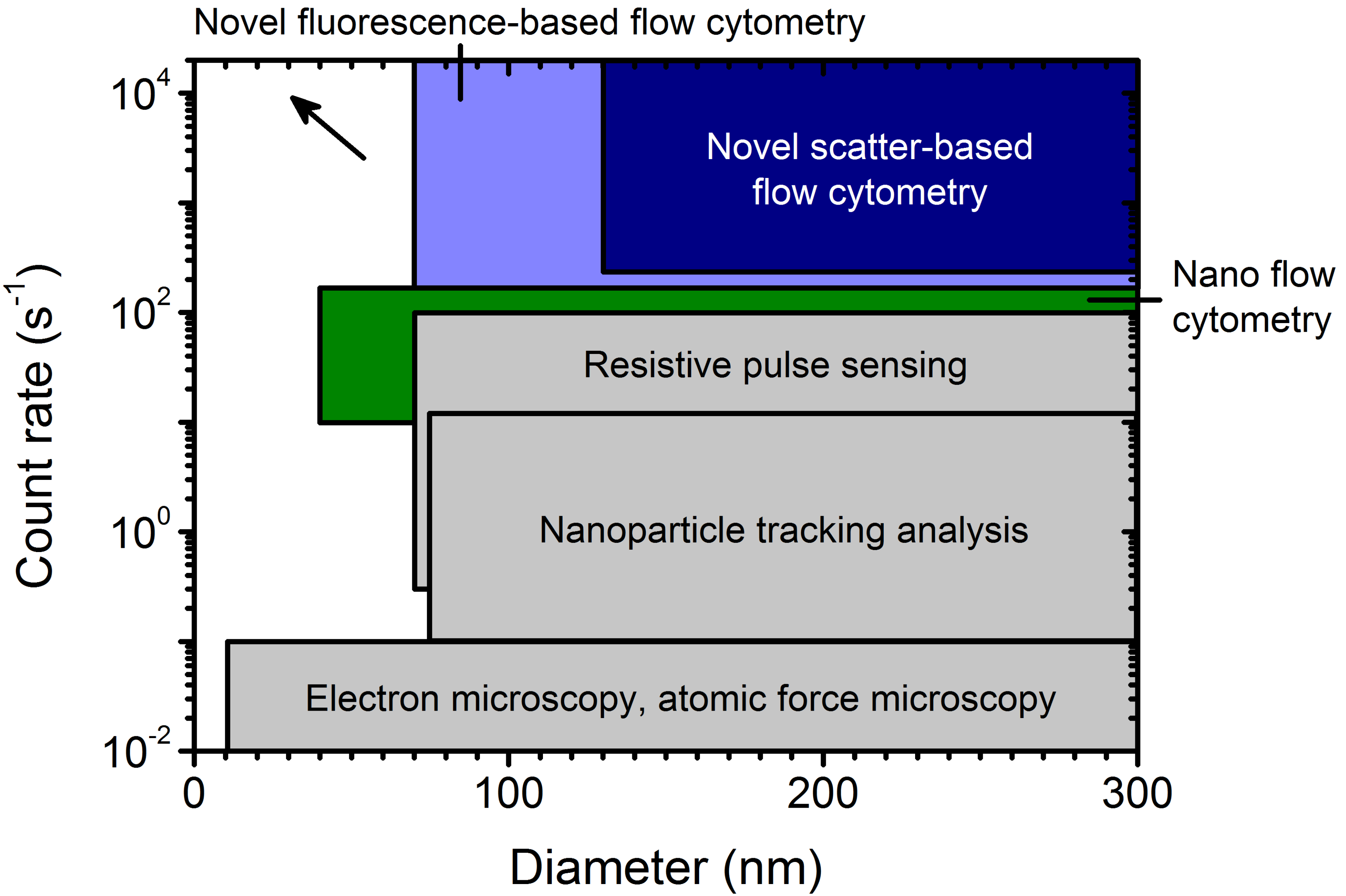

Count rate (s^{-1})
10^4
10^2
10^0
10^{-2}
0
100
200
300
Diameter (nm)
200 nm
Electron microscopy, atomic force microscopy



Count rate (s^{-1})
10^4
10^2
10^0
10^{-2}
Resistive pulse sensing
Nanoparticle tracking analysis
Electron microscopy, atomic force microscopy
0
100
200
300
Diameter (nm)

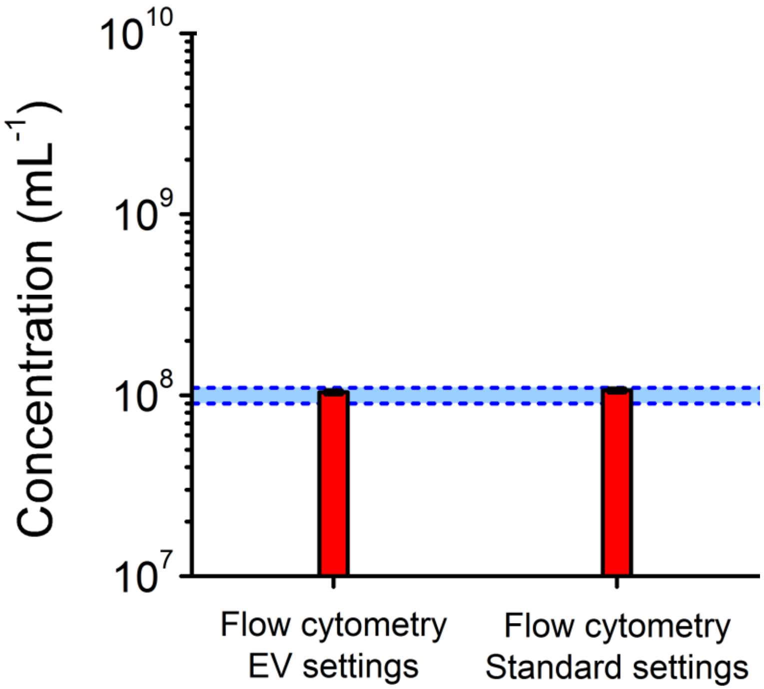

Novel scatter-based flow cytometry
Resistive pulse sensing
Nanoparticle tracking analysis
Electron microscopy, atomic force microscopy
Count rate (s^{-1})
10^4
10^2
10^0
10^{-2}
0
100
200
300
Diameter (nm)


Novel fluorescence-based flow cytometry
Novel scatter-based flow cytometry
Resistive pulse sensing
Nanoparticle tracking analysis
Electron microscopy, atomic force microscopy
Count rate (s-1)
10^4
10^2
10^0
10^-2
0
100
200
300
Diameter (nm)

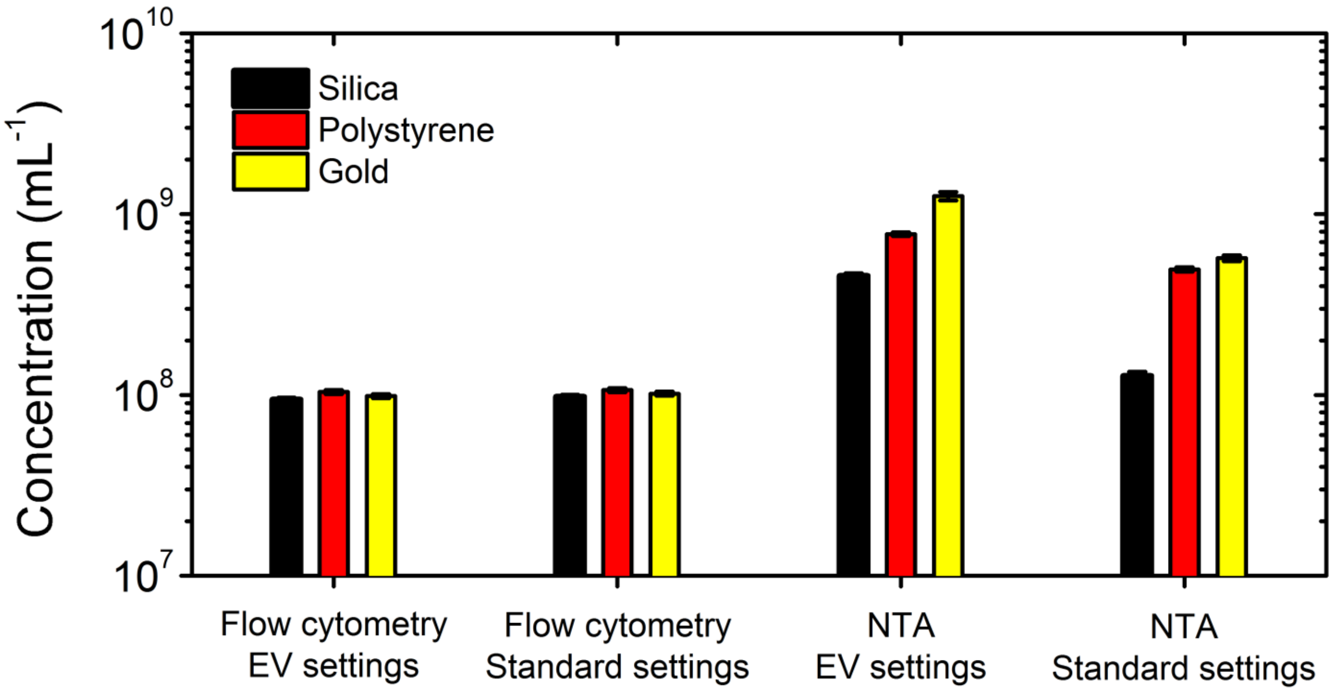


Coumans et al. *Circ Res* 2017

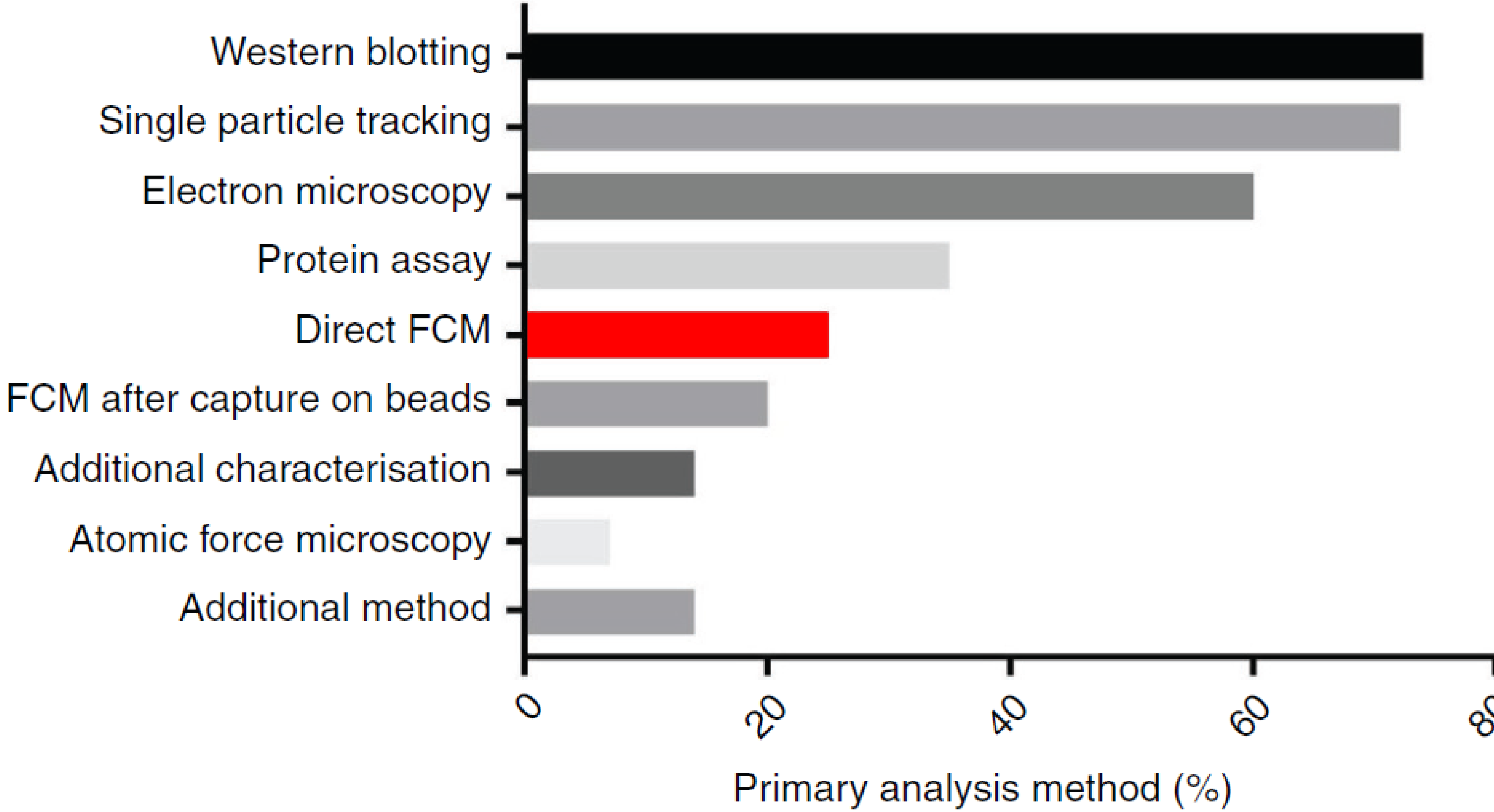
Flow cytometry versus nanoparticle tracking analysis



Flow cytometry versus nanoparticle tracking analysis



EV research using flow cytometry



Gardiner et al. *J Extracell Vesicles* 2016

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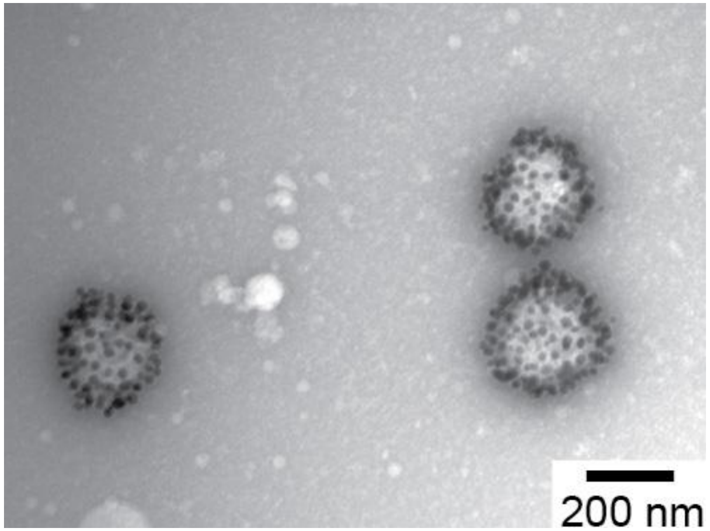
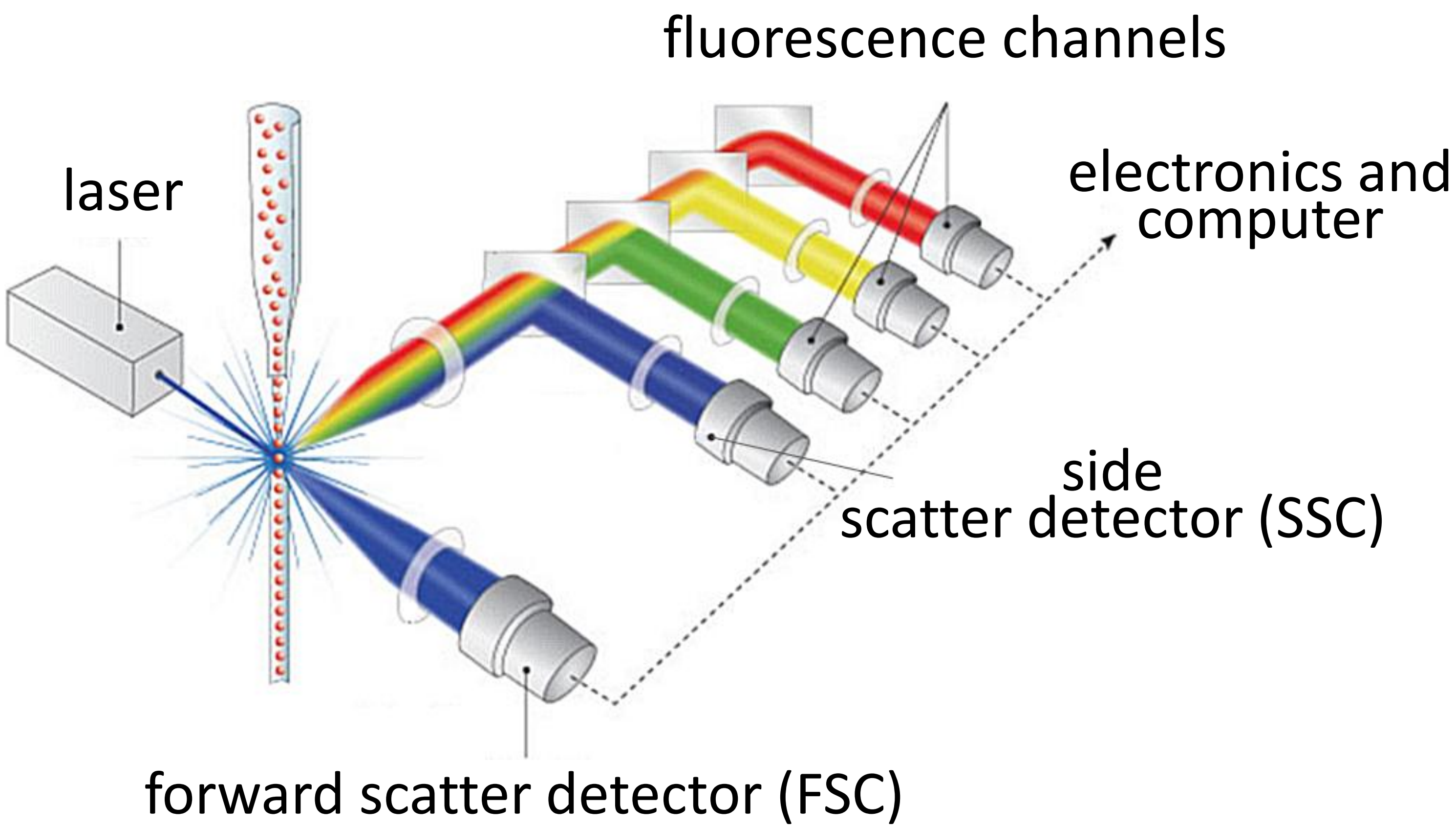
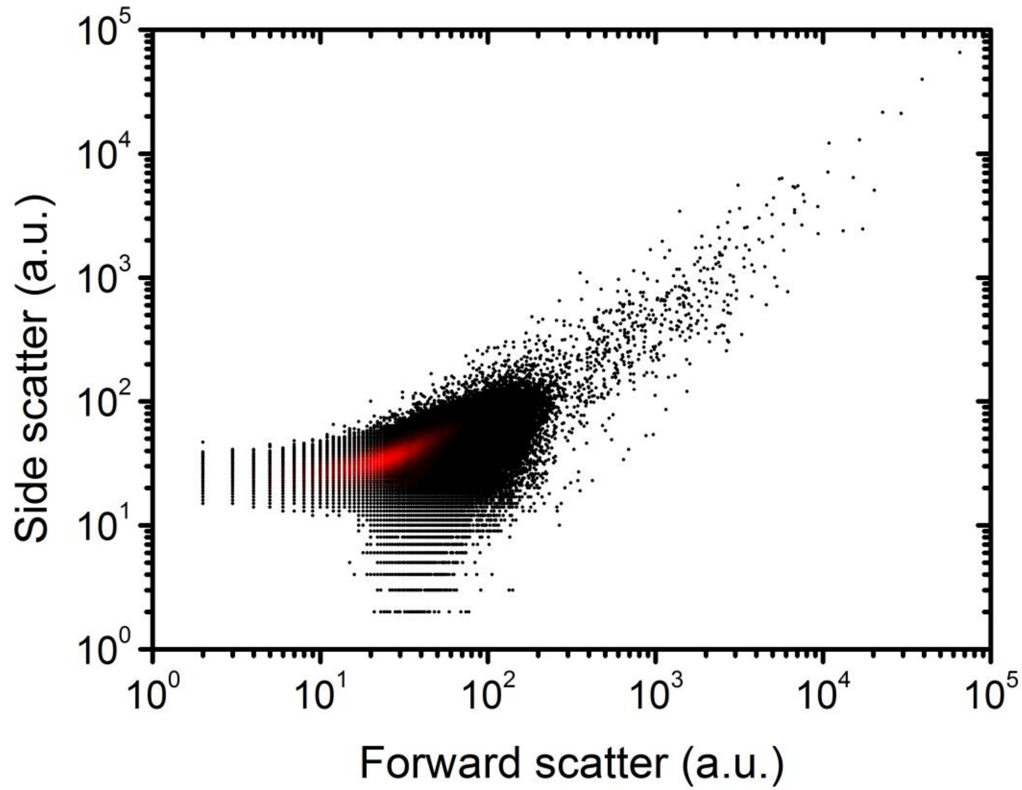


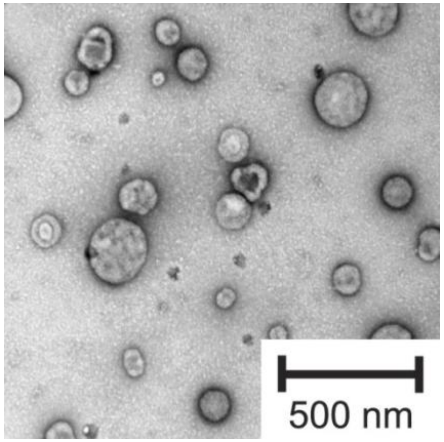
Flow cytometry



Problem: how to interpret and compare flow cytometry data?



?



Is a “bead size gate” a good idea?

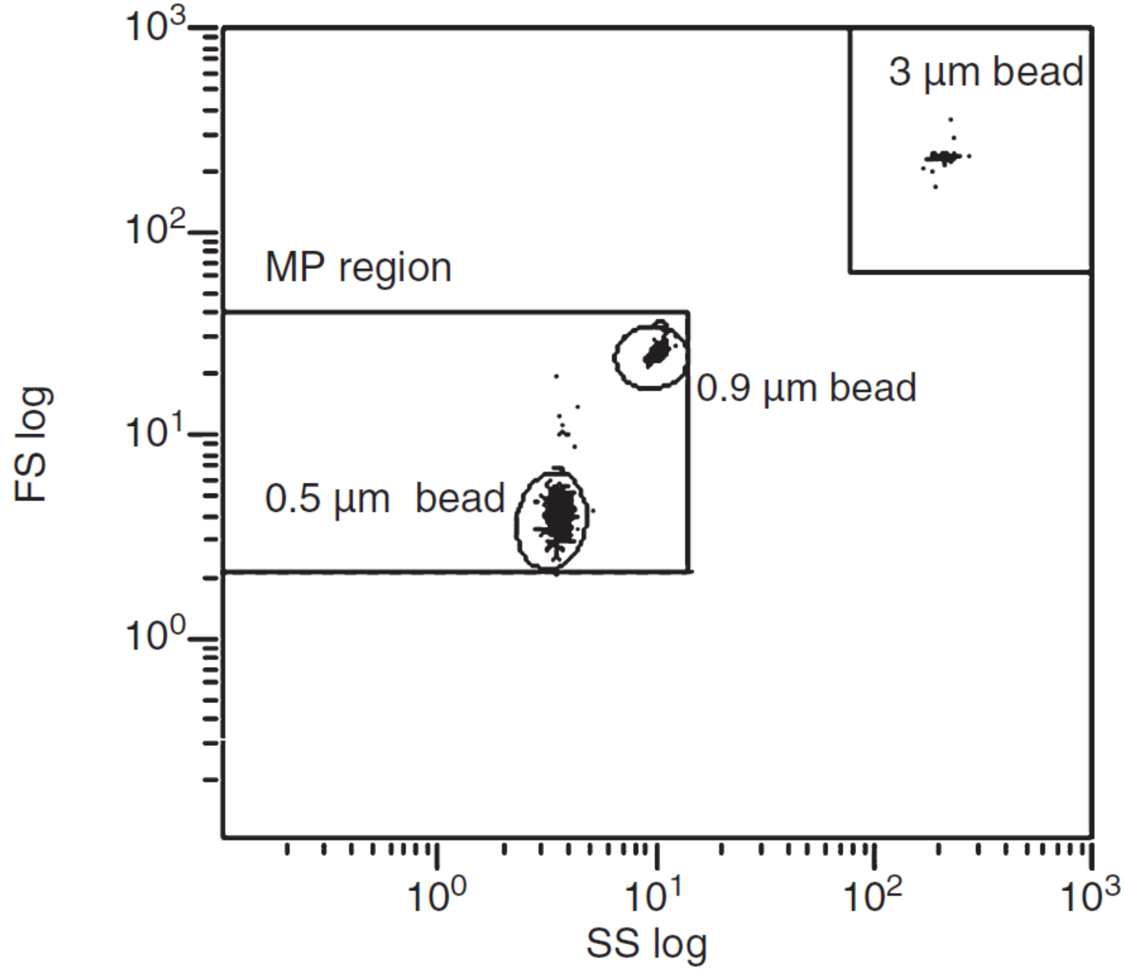
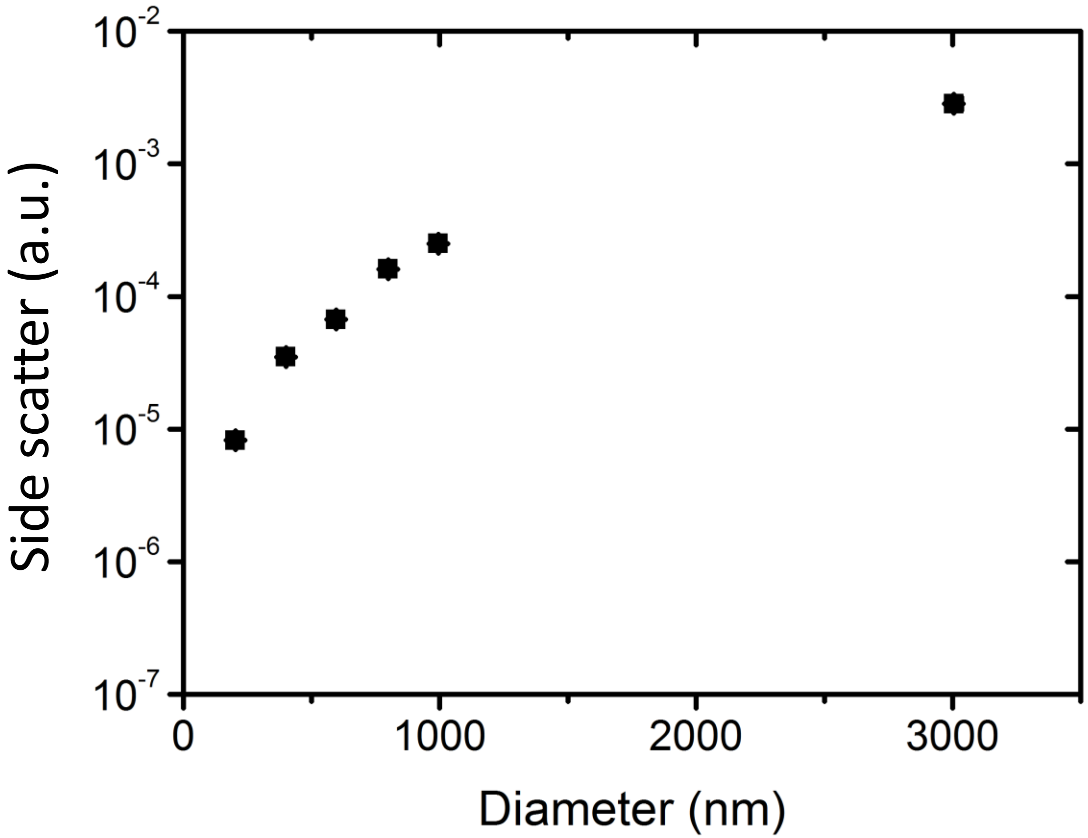
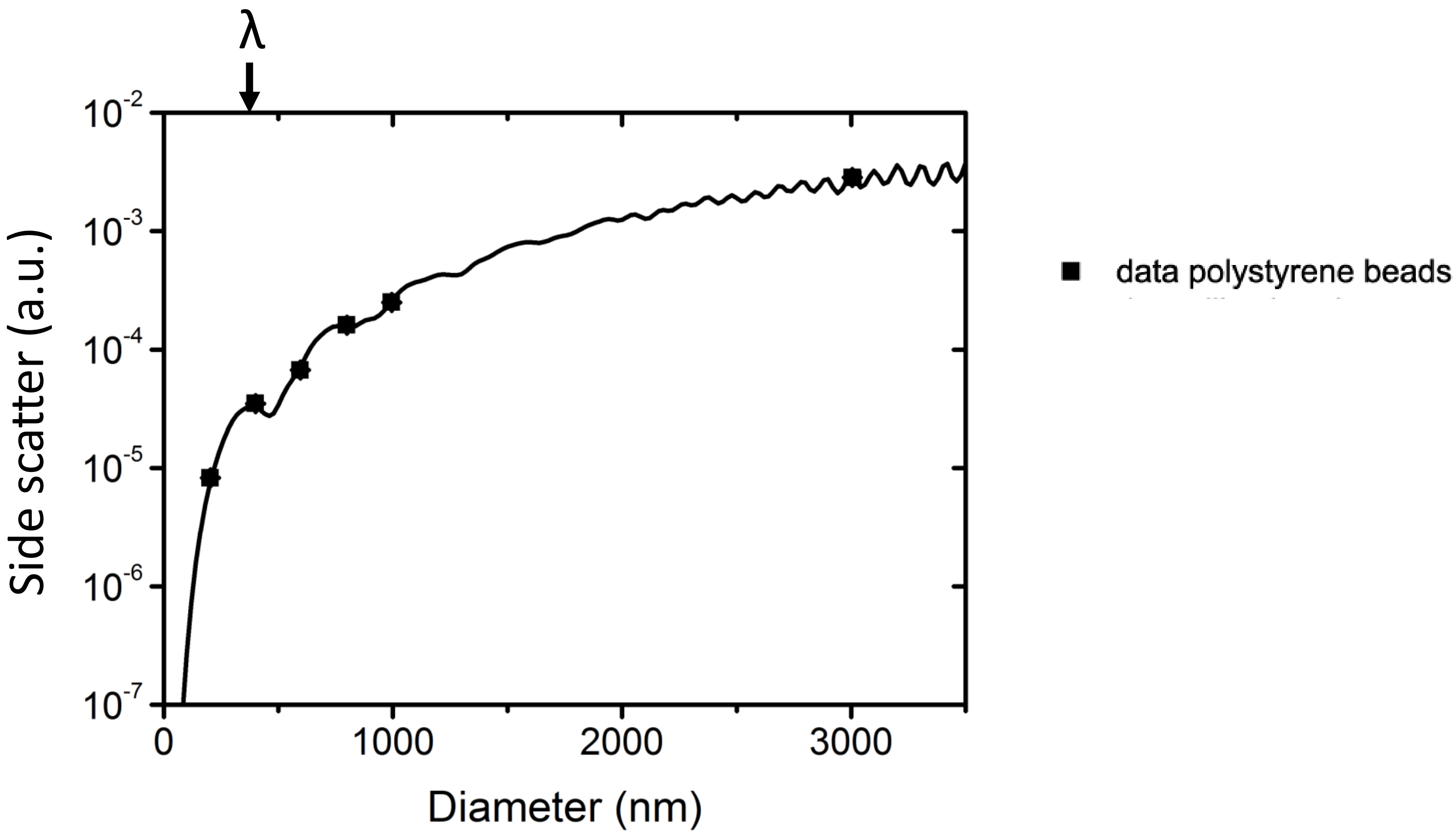


image adopted: Robert et al. *J Thromb Haemost* 2008

Relate scatter to diameter of beads

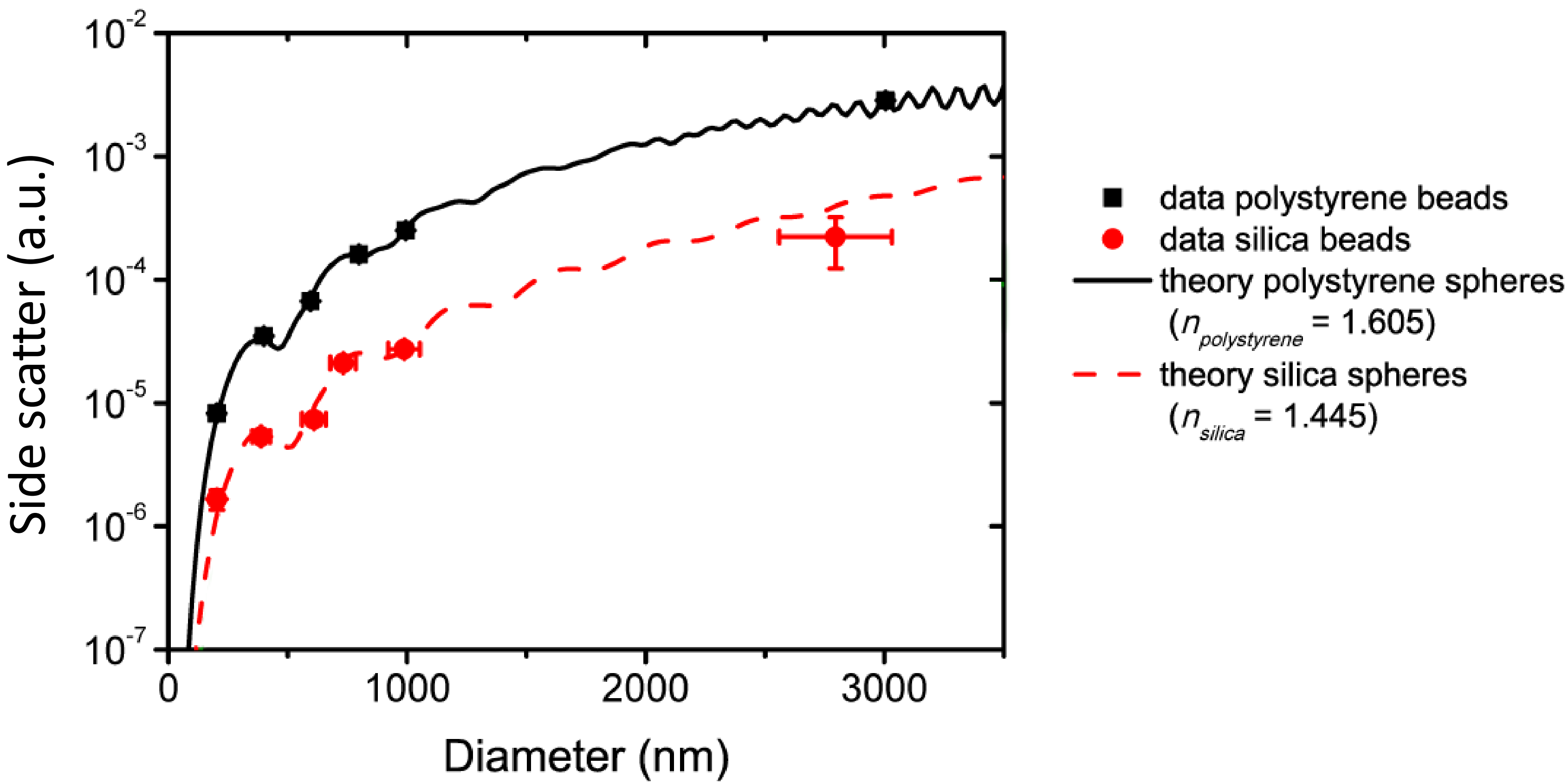


Relate scatter to diameter of beads

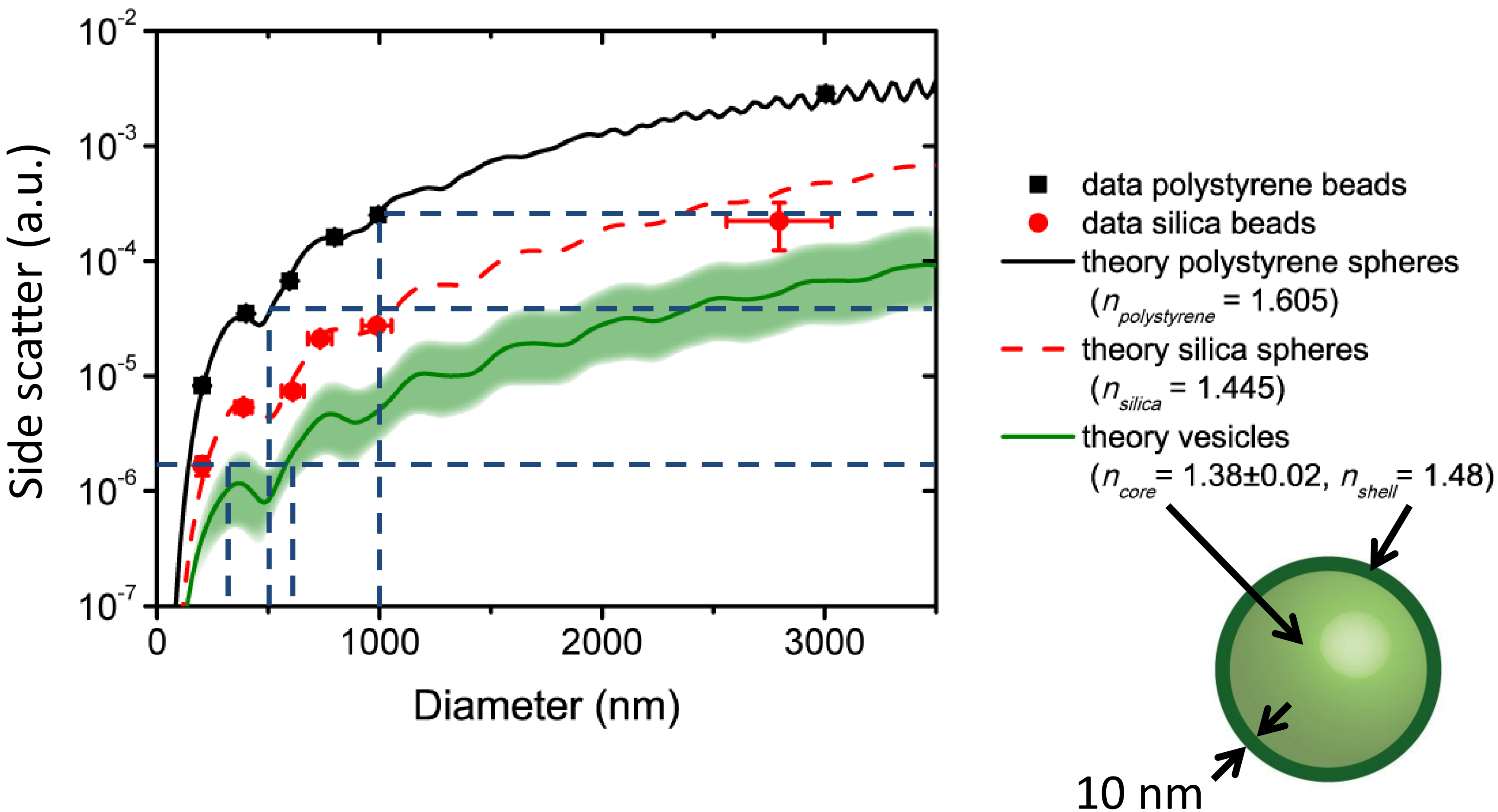


Mie based on scripts Mätzler (Bohren and Huffman)

Relate scatter to diameter of beads

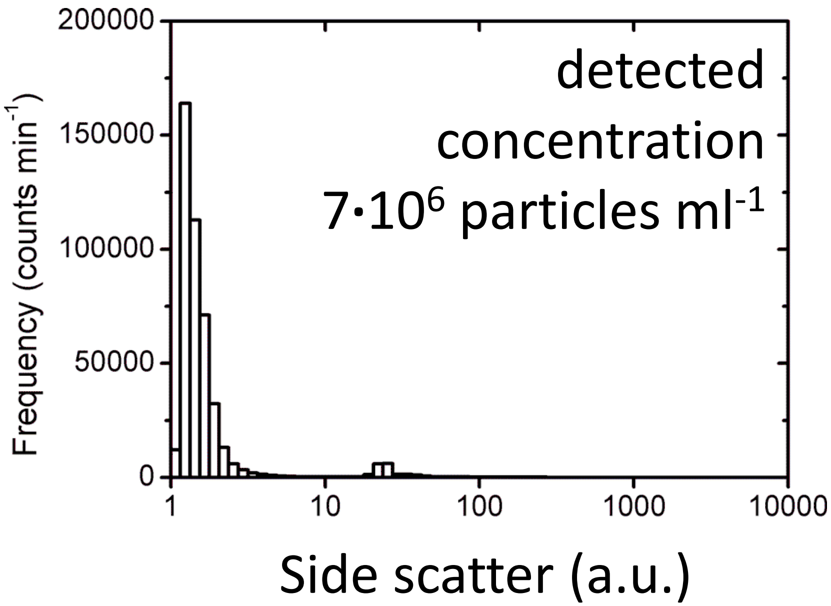


Relate scatter to diameter of vesicles

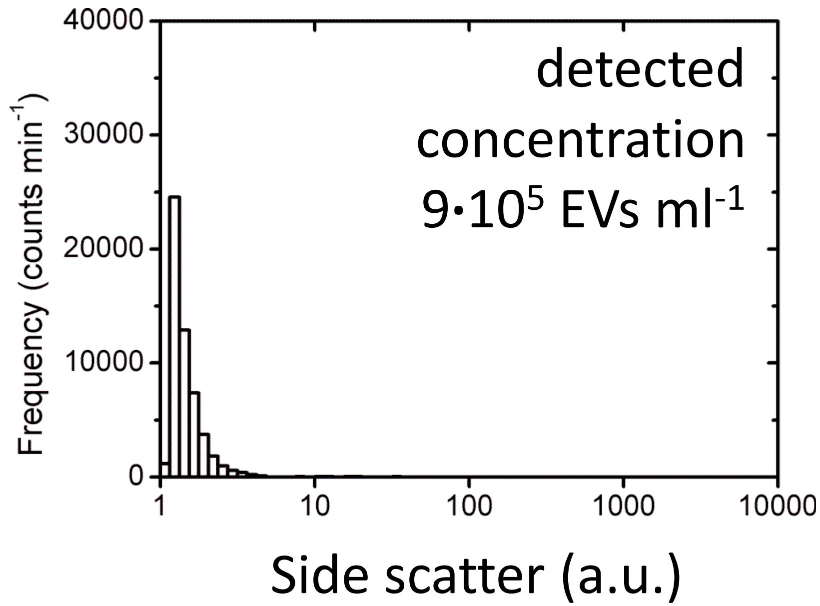


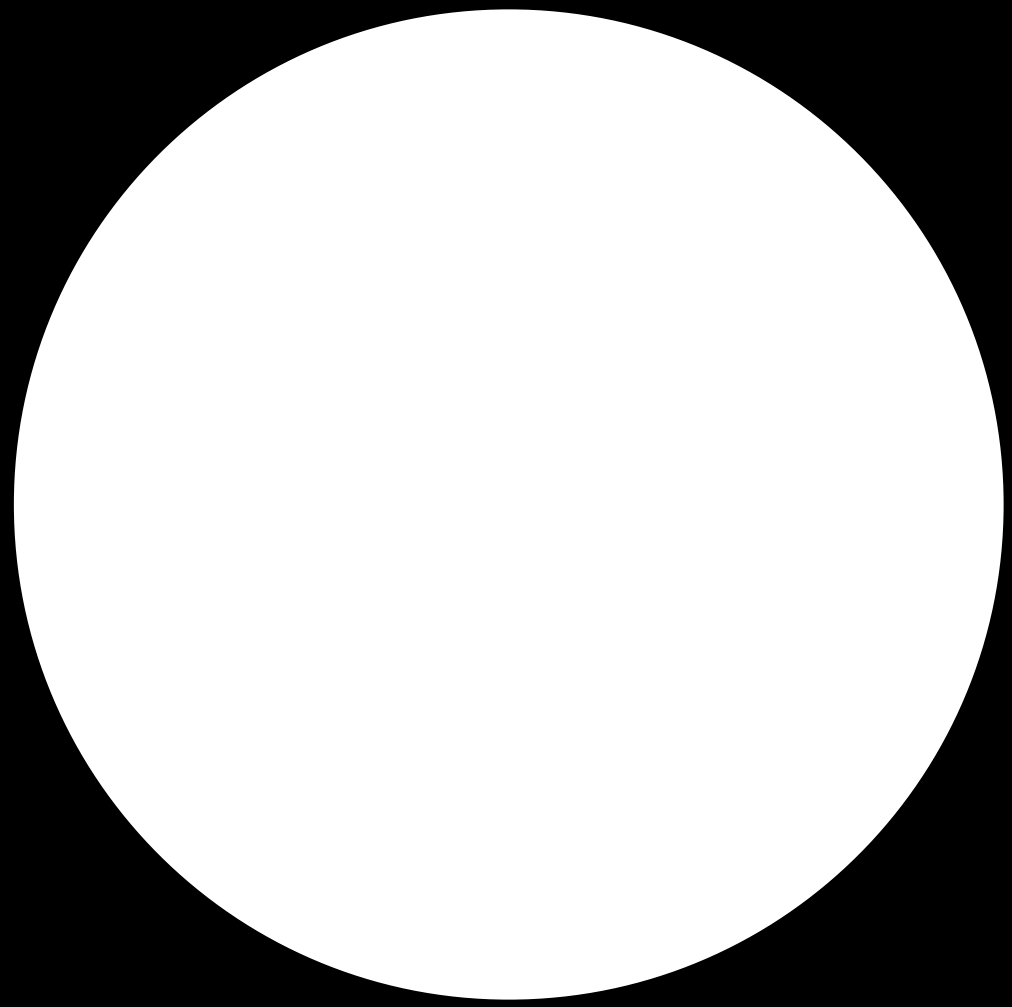
Particles that are too small to be detected generate a signal!

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

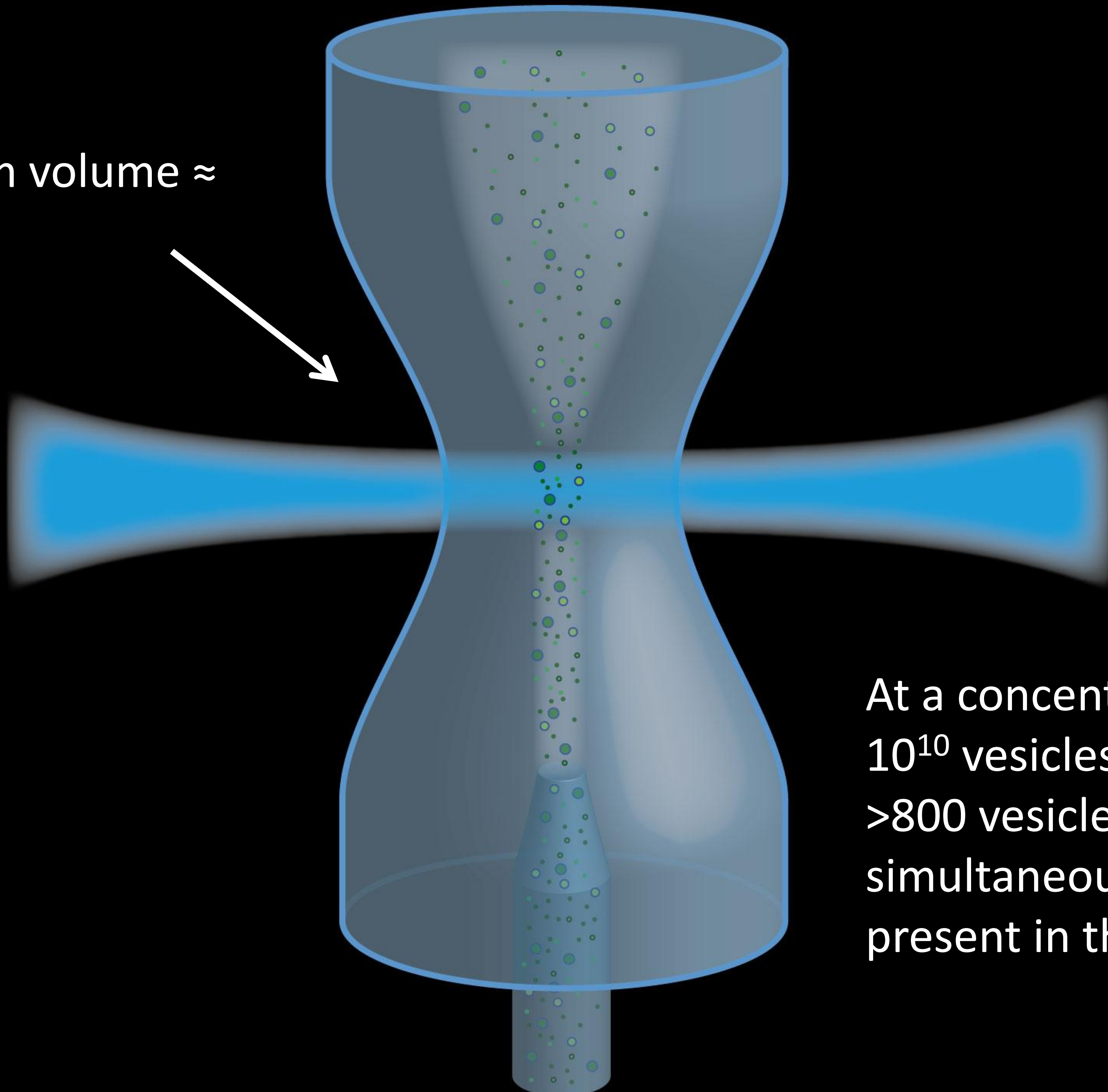


urine EVs <220 nm at concentration ≥ 10^{10} EVs ml^{-1}



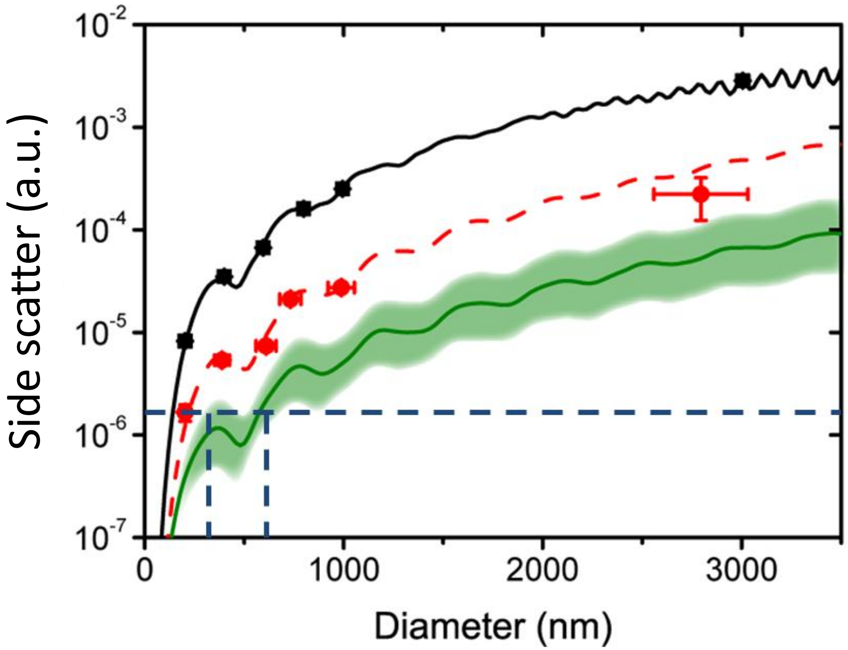


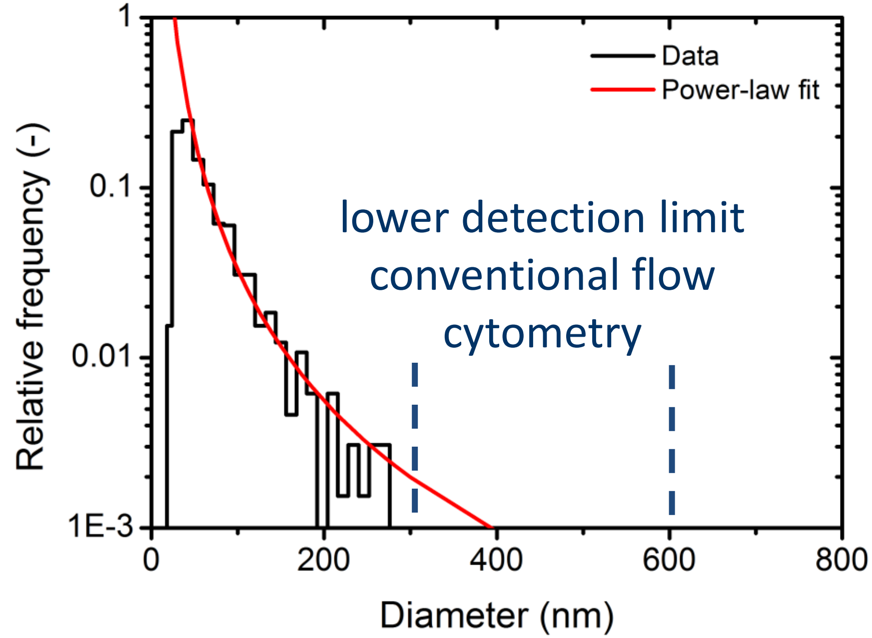
beam volume ≈
54 pl



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

Summary EV flow cytometry





- Single event signal attributed to scattering from *multiple* EVs ("Swarm detection")
- Conventional flow cytometry detects <1% of all EVs

van der Pol et al. *J Thromb Haemost* 2012

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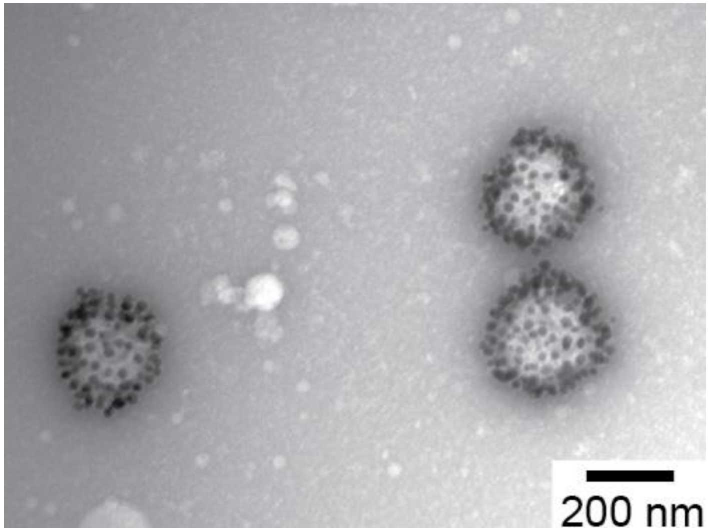


Standardization is boring (biologists, clinicians)

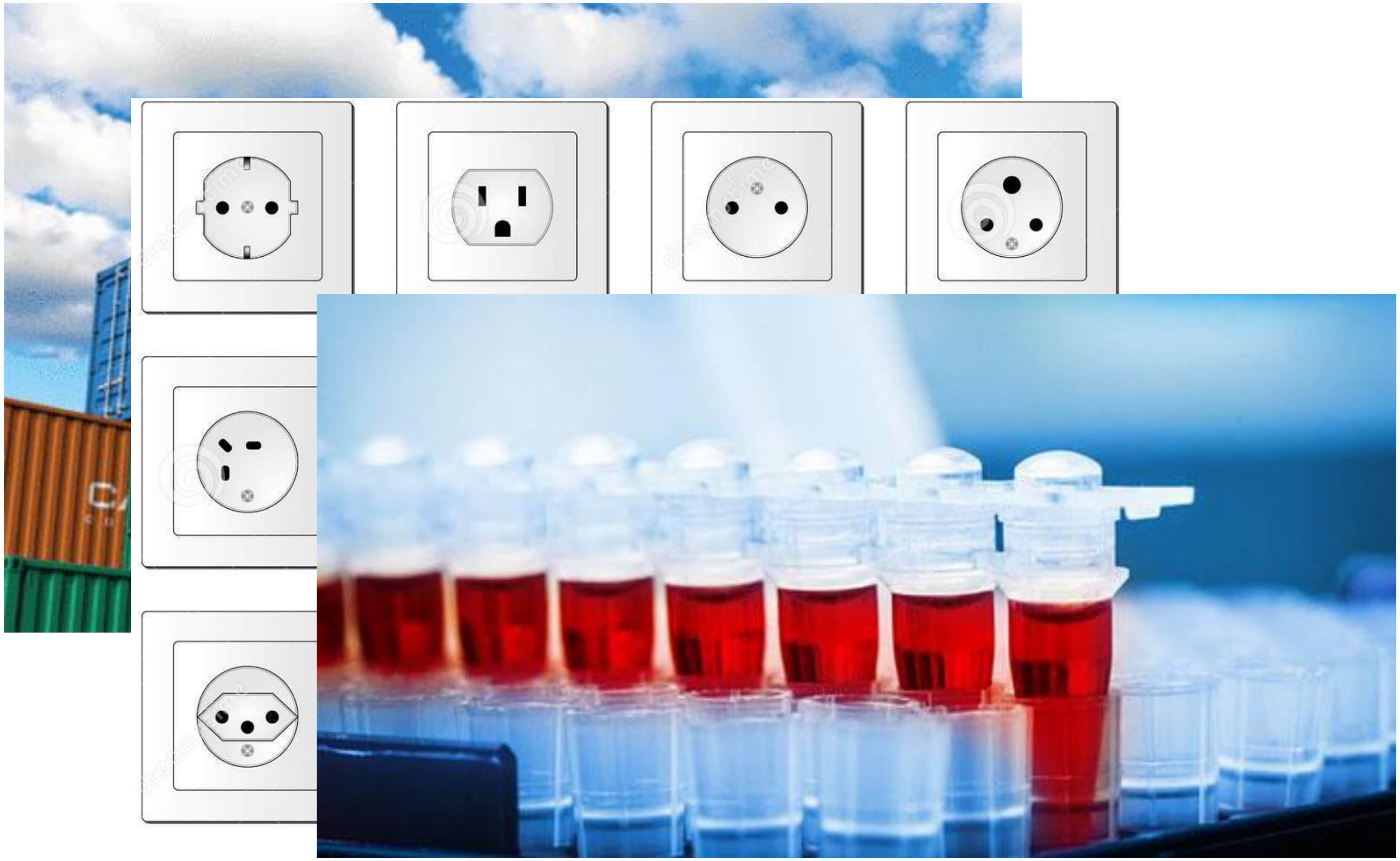
Standardisation is exciting (metrologists, physicists)

BESSYII

0.31 nm X-rays to size EV*
(flow cytometers typically use 488 nm light)

*Varga et al. *J Extracell Vesicles* 2014

Standardization is important (everybody)



Goal

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

Study comprises 33 sites (64 instruments) worldwide

Approach scatter-based standardization

- Measure EV reference sample and controls
- Scatter (a.u.) → diameter (nm)
  - Measure Rosetta calibration* beads
  - Rosetta calibration* software relates scatter to diameter and defines EV size gates
- Apply EV size gate to software (e.g. FlowJo) and report concentrations

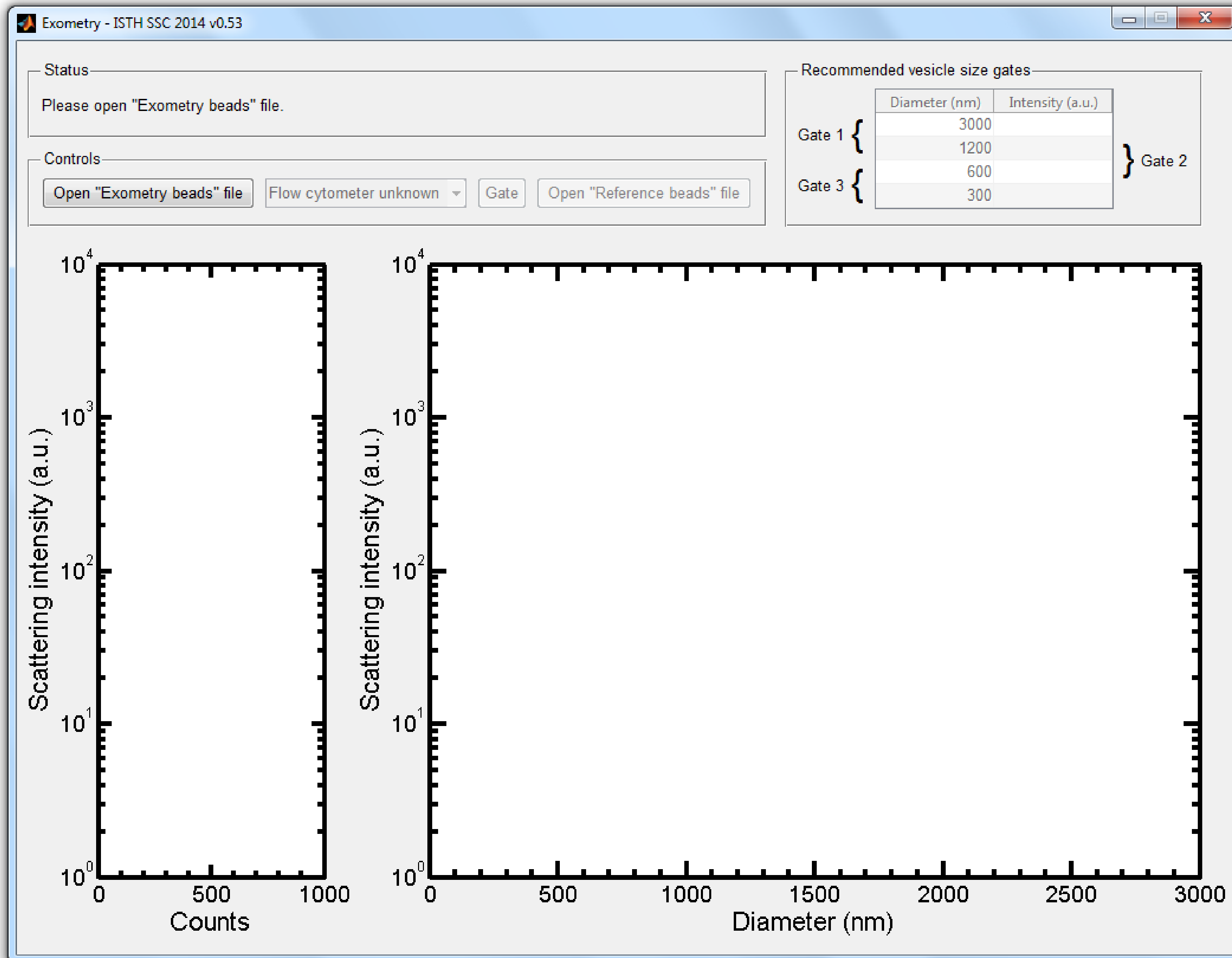
EXOMETRY

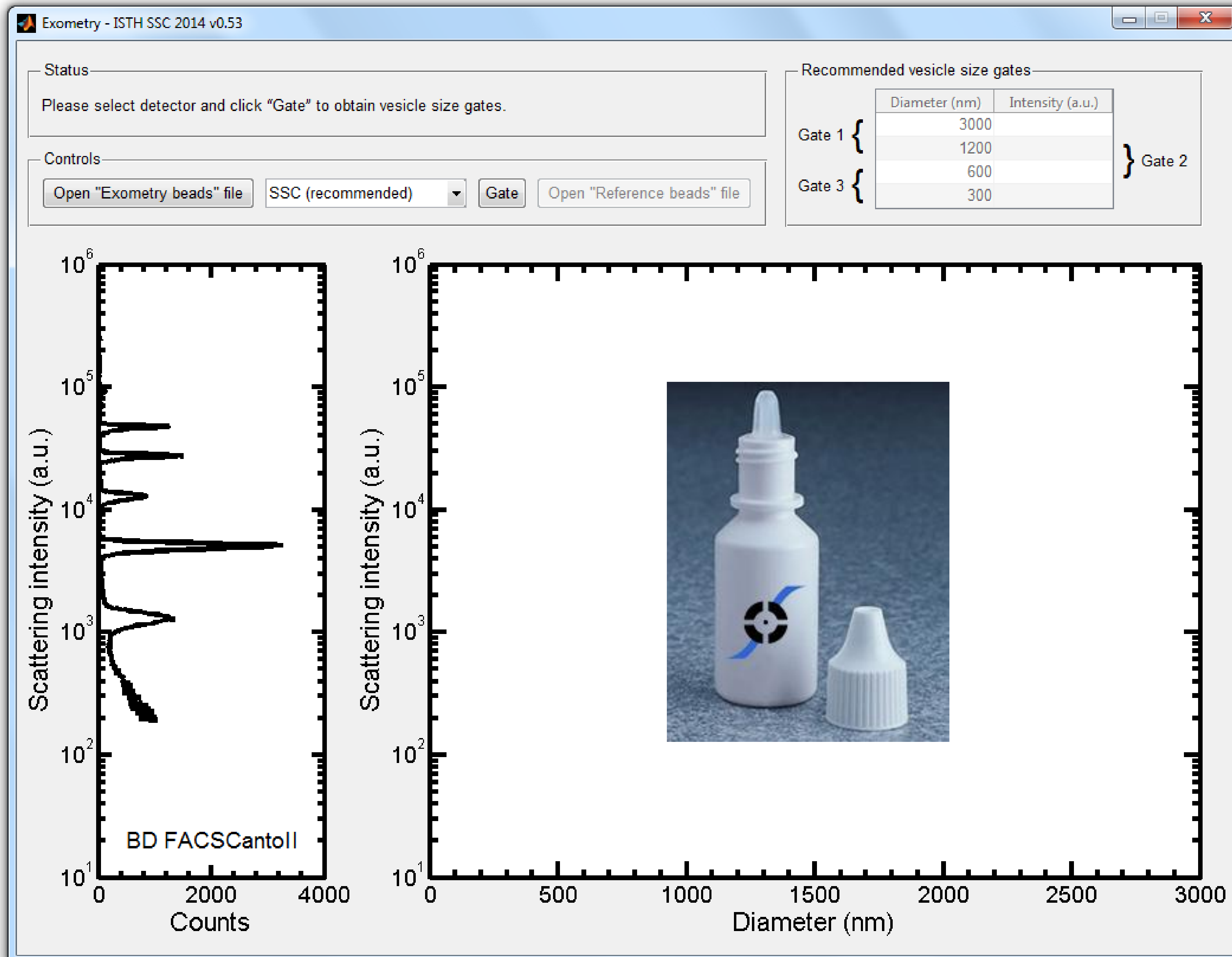
*Exometry.com

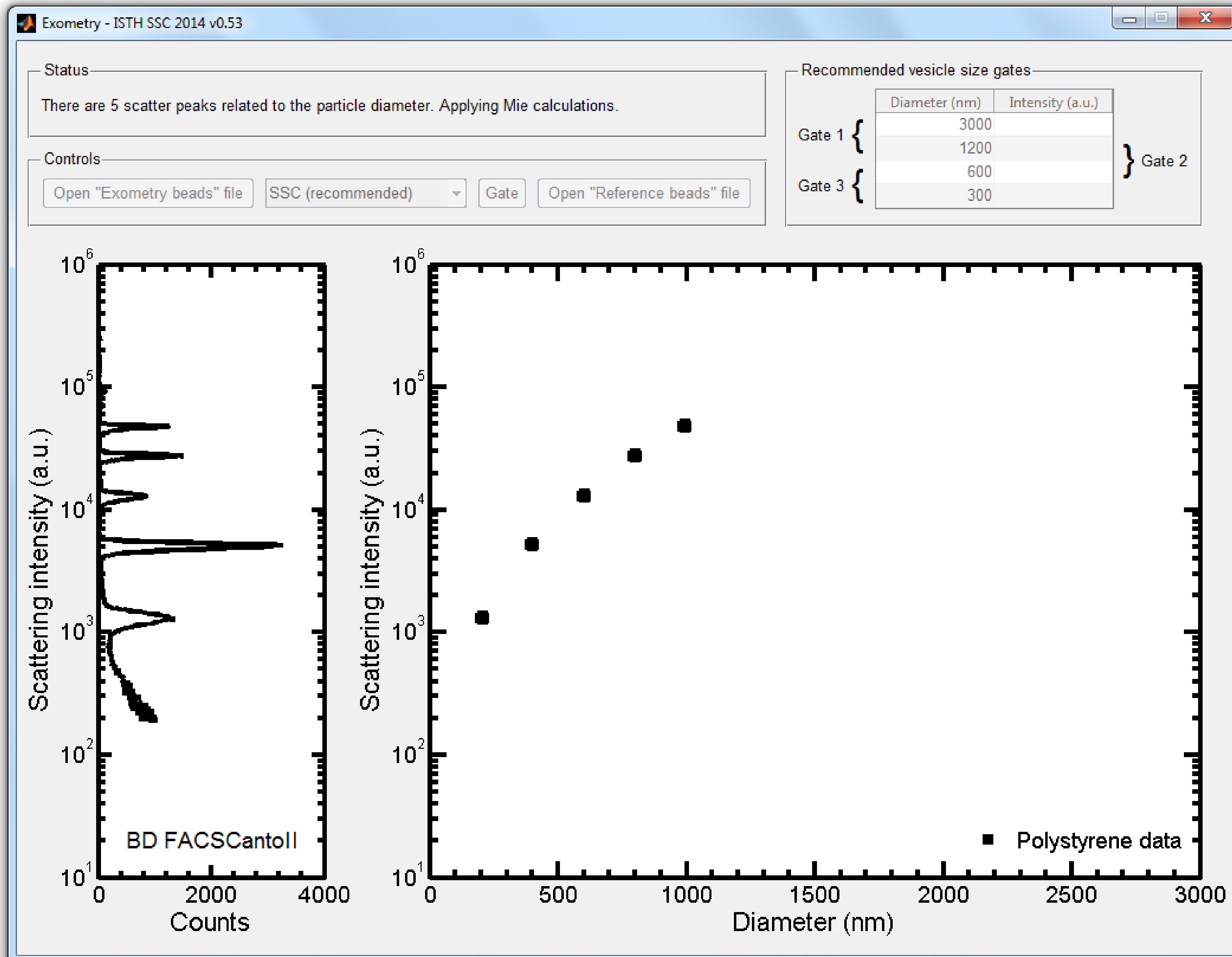
EV reference sample

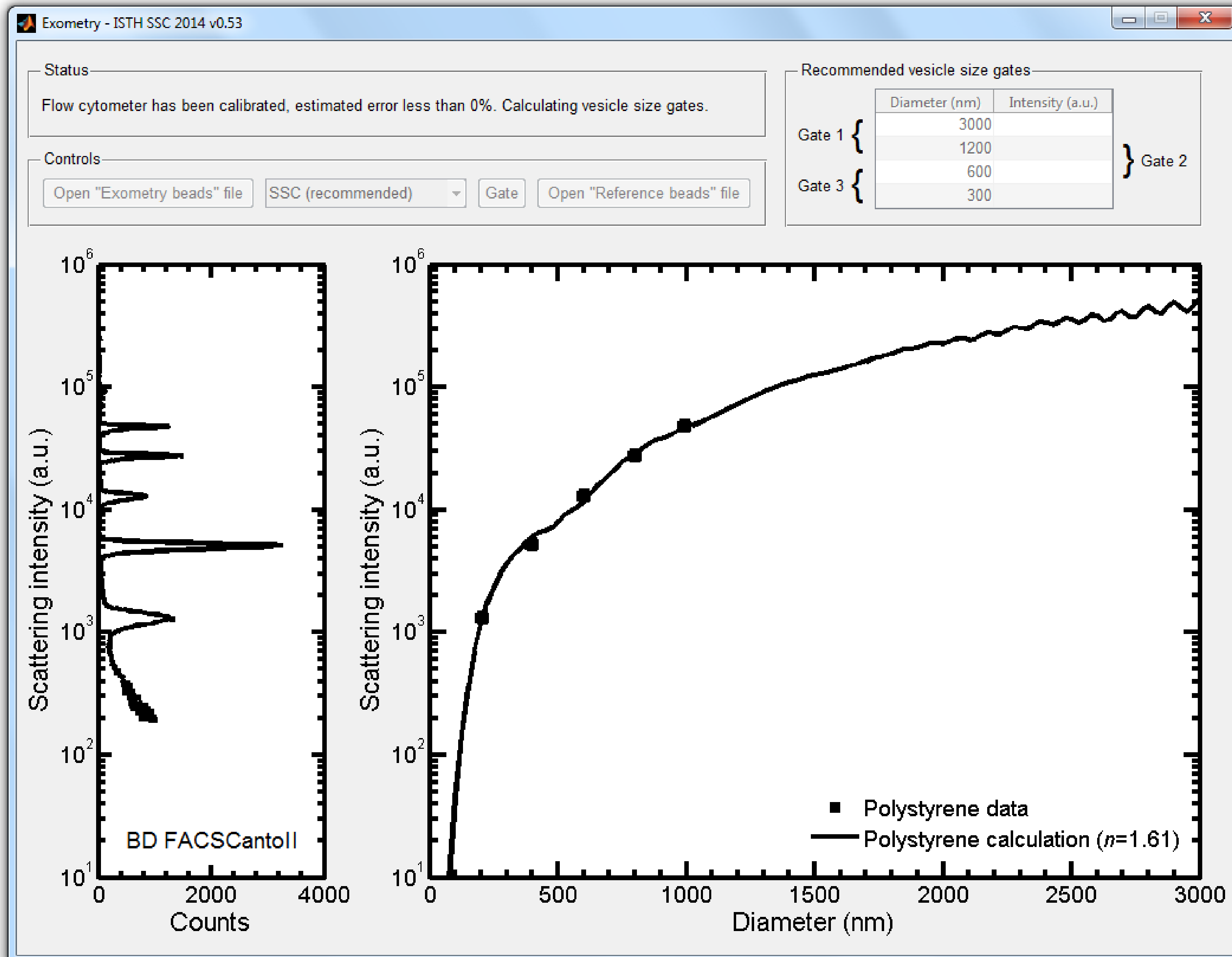
- Platelet (CD61-PE+) EVs from cell-free platelet concentrates

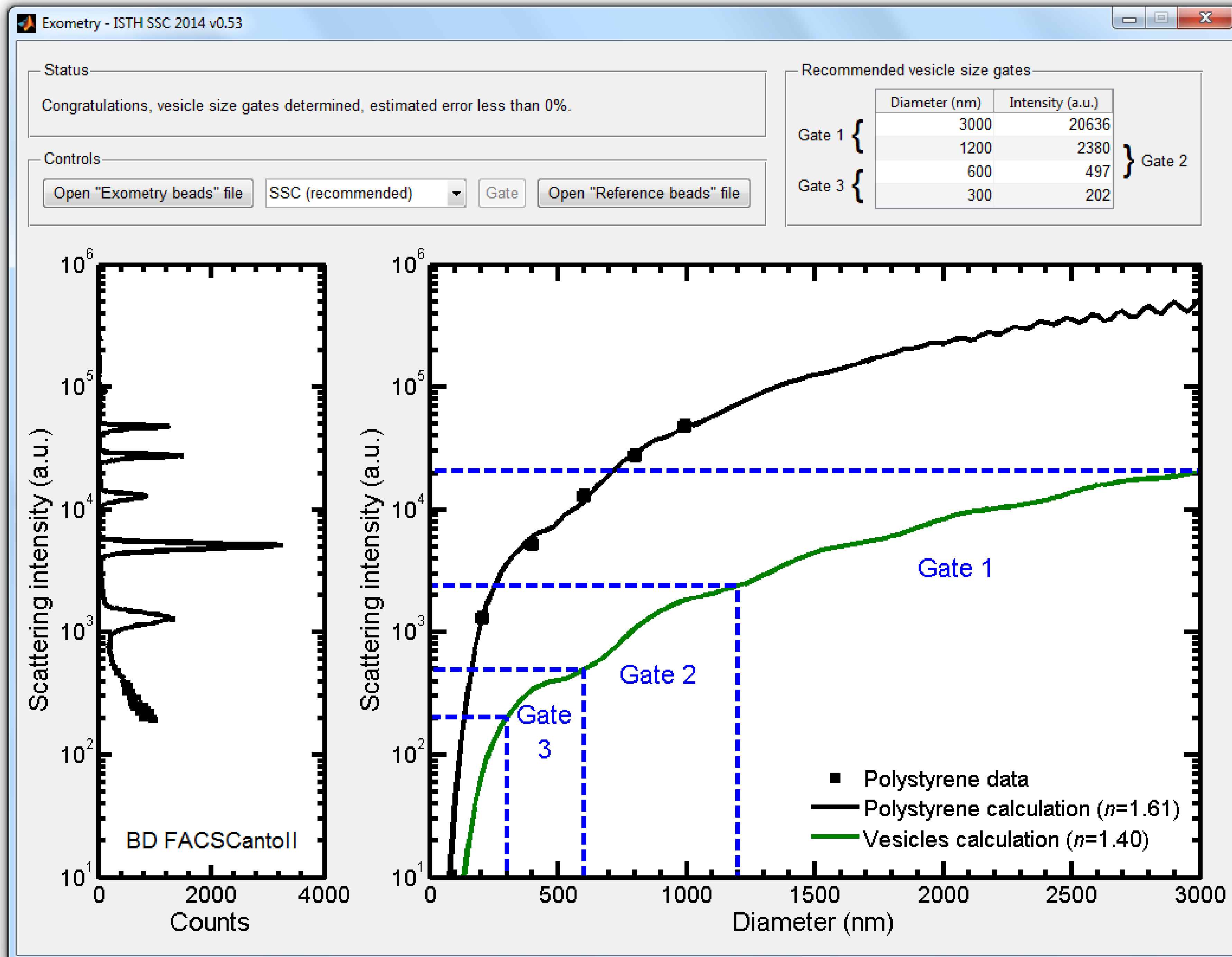
- Trigger on most sensitive scatter channel
- Exclude EVs similar to isotype

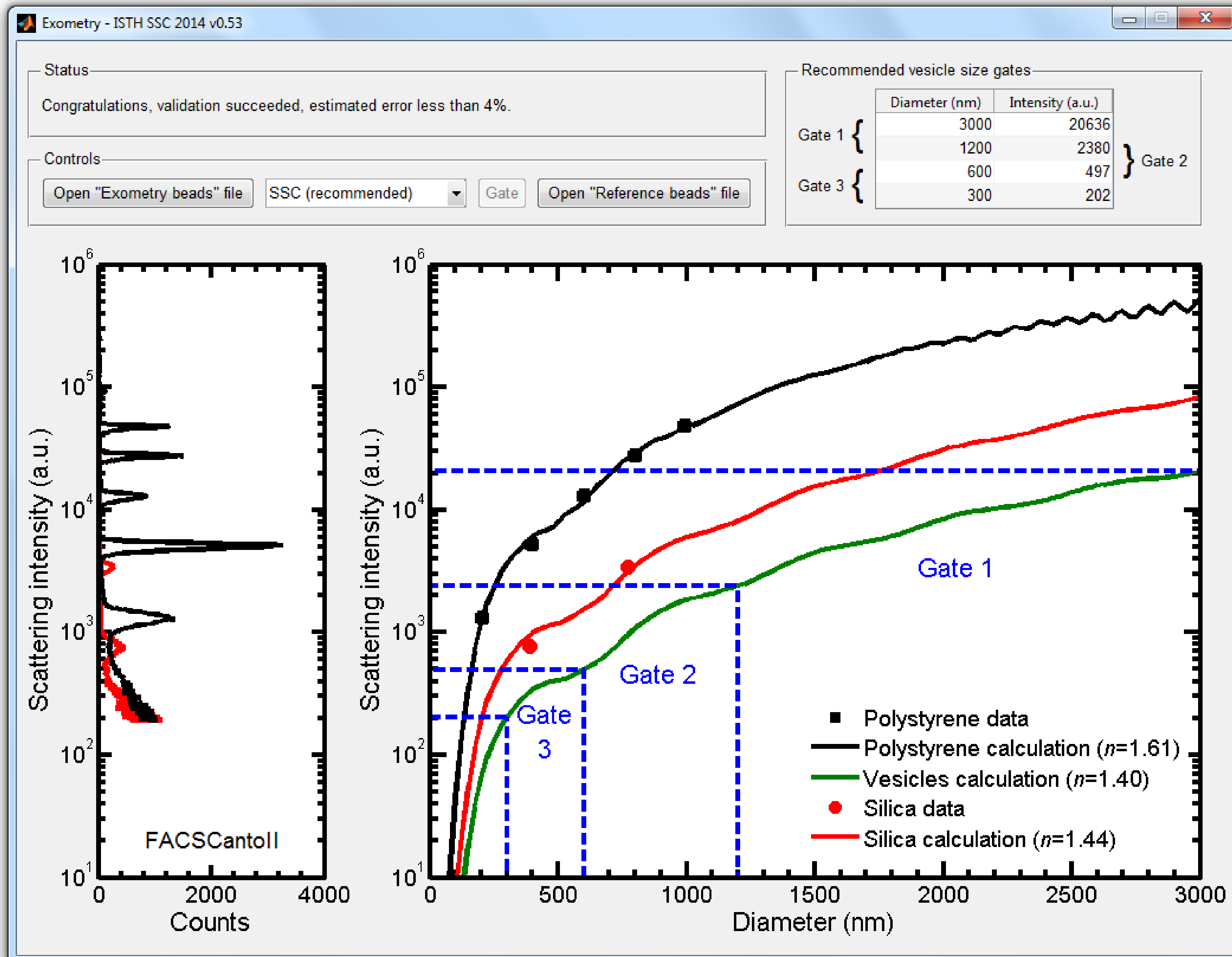

Exometry - ISTH SSC 2014 v0.53
Status
Please open "Exometry beads" file.
Controls
Open "Exometry beads" file
Flow cytometer unknown
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
Gate 2
Gate 3
3000
1200
600
300
Scattering intensity (a.u.)
Counts
0
500
1000
Scattering intensity (a.u.)
Diameter (nm)
0
500
1000
1500
2000
2500
3000


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
Gate 2
Gate 3
3000
1200
600
300
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)
0
2000
4000
500
1000
1500
2500
3000
10^1
10^2
10^3
10^4
10^5
10^6

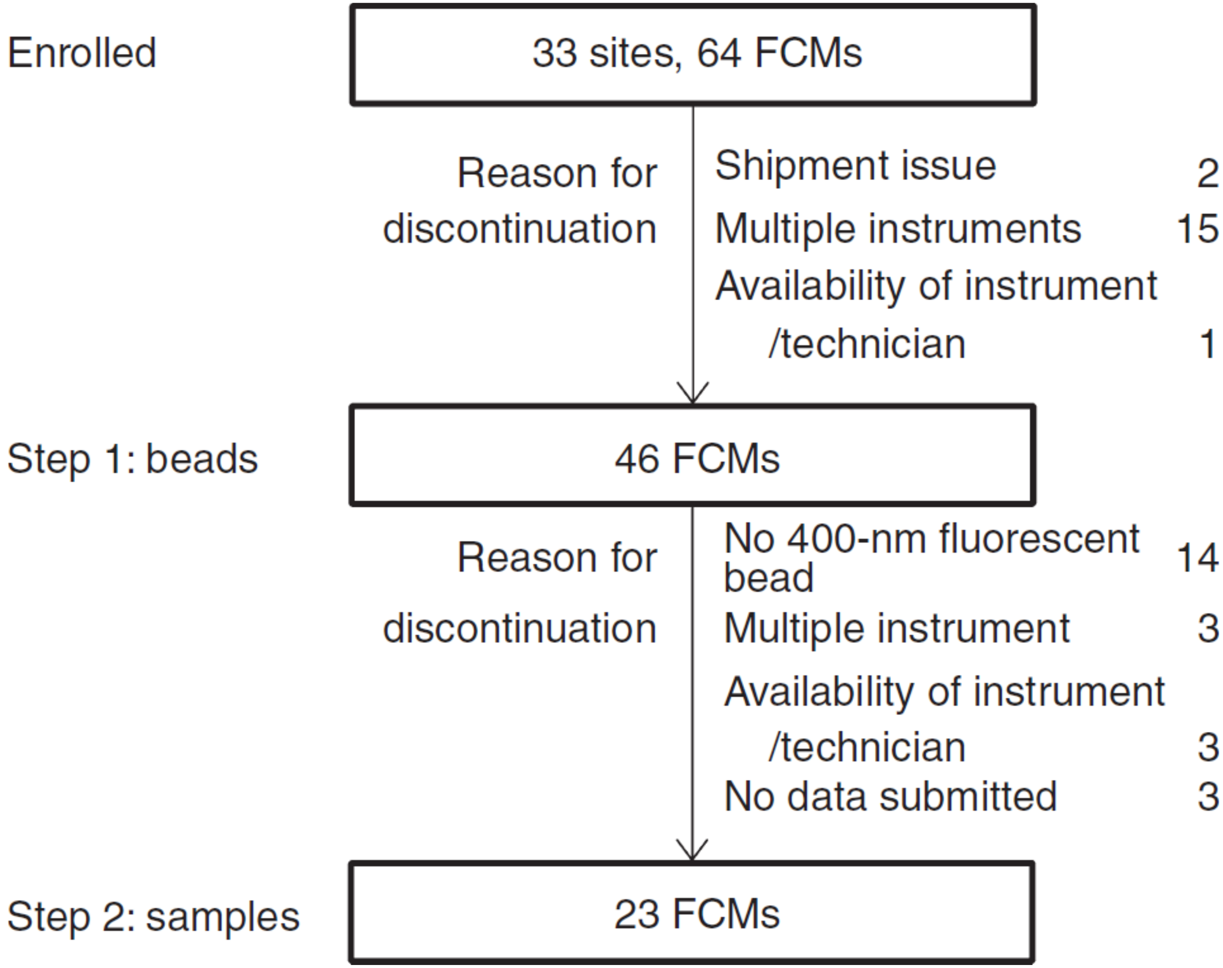

Exometry - ISTH SSC 2014 v0.53
Status
There are 5 scatter peaks related to the particle diameter. Applying Mie calculations.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
Gate 2
Gate 3
3000
1200
600
300
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)
Polystyrene data
0
2000
4000
500
1000
1500
2500
3000
10^1
10^2
10^3
10^4
10^5
10^6


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.)
Counts
BD FACSCantoII
Diameter (nm)
Polystyrene data
Polystyrene calculation (n=1.61)

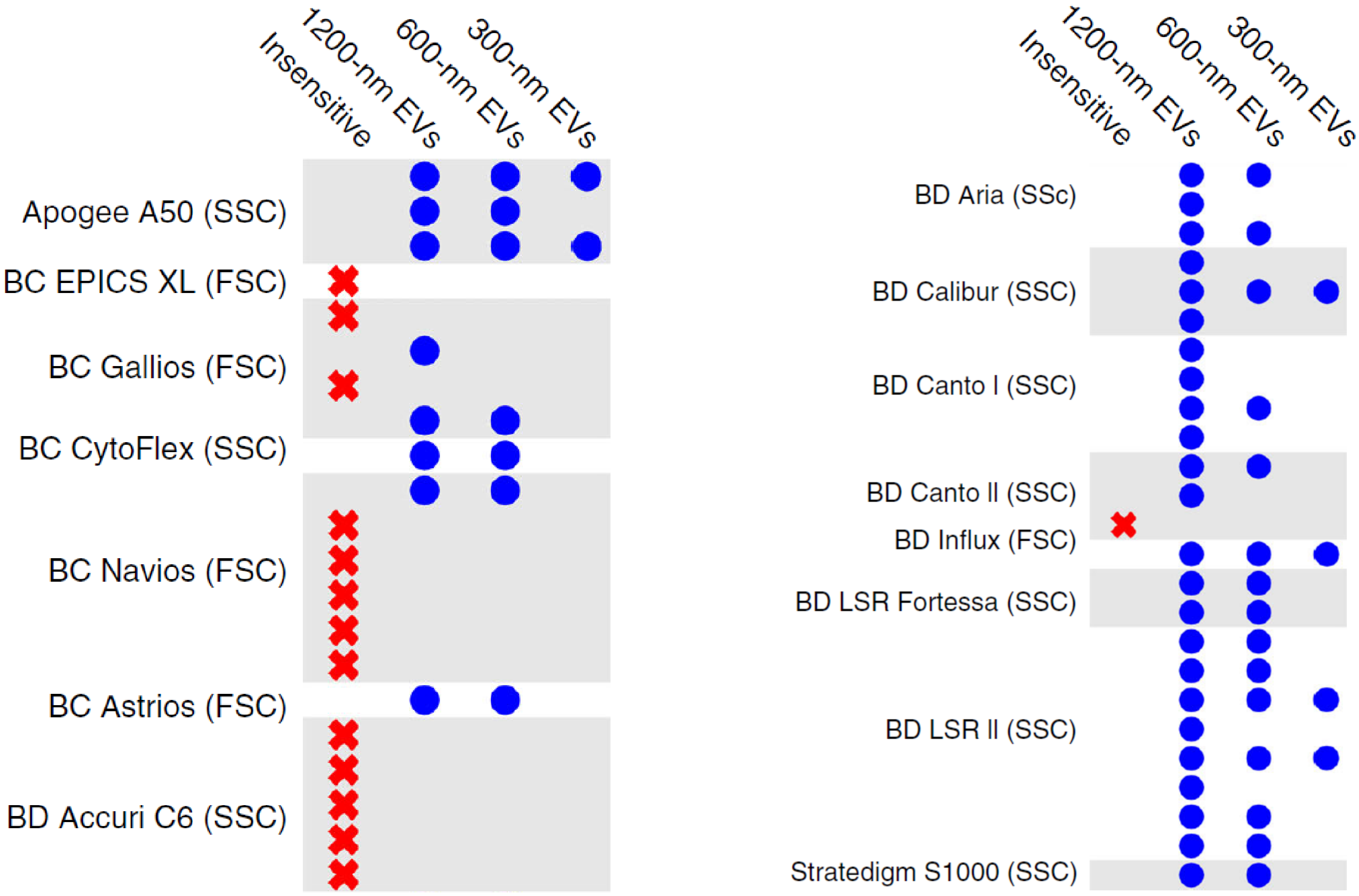

Exometry - ISTH SSC 2014 v0.53
Status
Congratulations, vesicle size gates determined, estimated error less than 0%.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
20636
1200
2380
Gate 2
Gate 3
600
497
300
202
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)
Gate 1
Gate 2
Gate 3
Polystyrene data
Polystyrene calculation (n=1.61)
Vesicles calculation (n=1.40)


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
Scattering intensity (a.u.)
Counts
FACSCantoII
Diameter (nm)
Gate 1
Gate 2
Gate 3
Polystyrene data
Polystyrene calculation (n=1.61)
Vesicles calculation (n=1.40)
Silica data
Silica calculation (n=1.44)

Exclusion of flow cytometers (FCM)

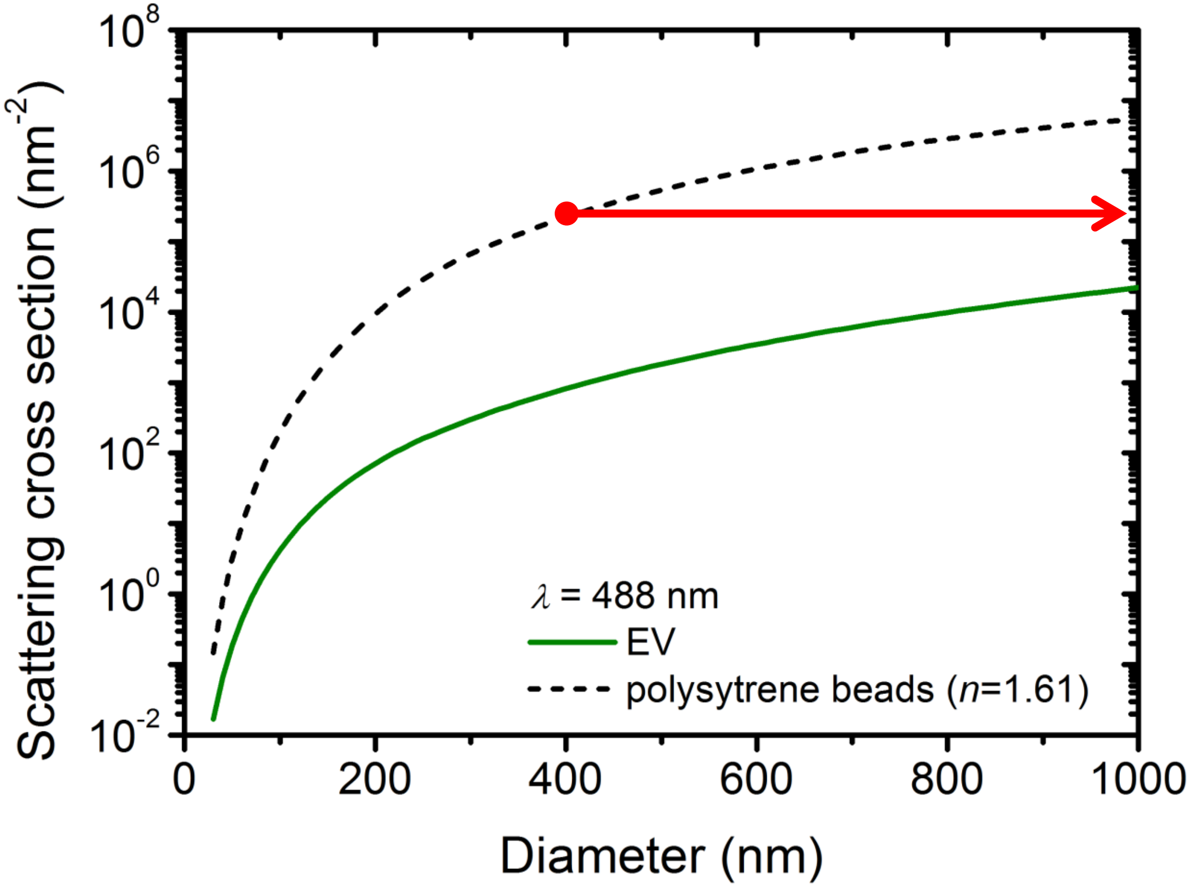


Sensitivity of 46 flow cytometers in the field

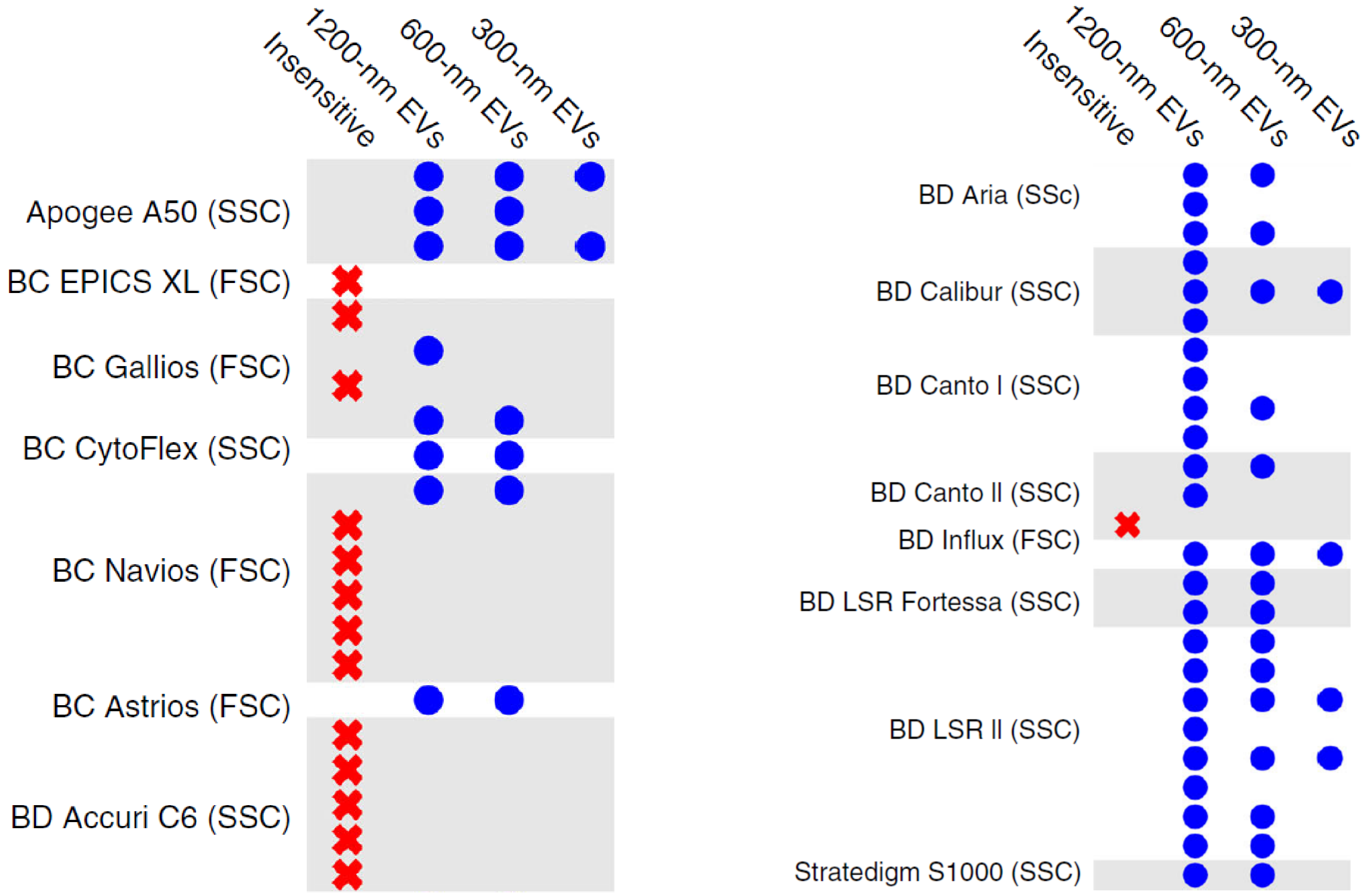


✖ = unable to detect 400 nm polystyrene beads

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



Sensitivity of 46 flow cytometers in the field



✖ = unable to detect EV < 1000 nm

Results

Method	CV* concentration (%)
No scatter gate	144
Traditional bead size gate	139
1,200-3,000 nm EV size gate	81
600-1,200 nm EV size gate	82
300-600 nm EV size gate	115

*CV: coefficient of variation (standard deviation / mean)

van der Pol et al. *J Thromb Haemost* 2018

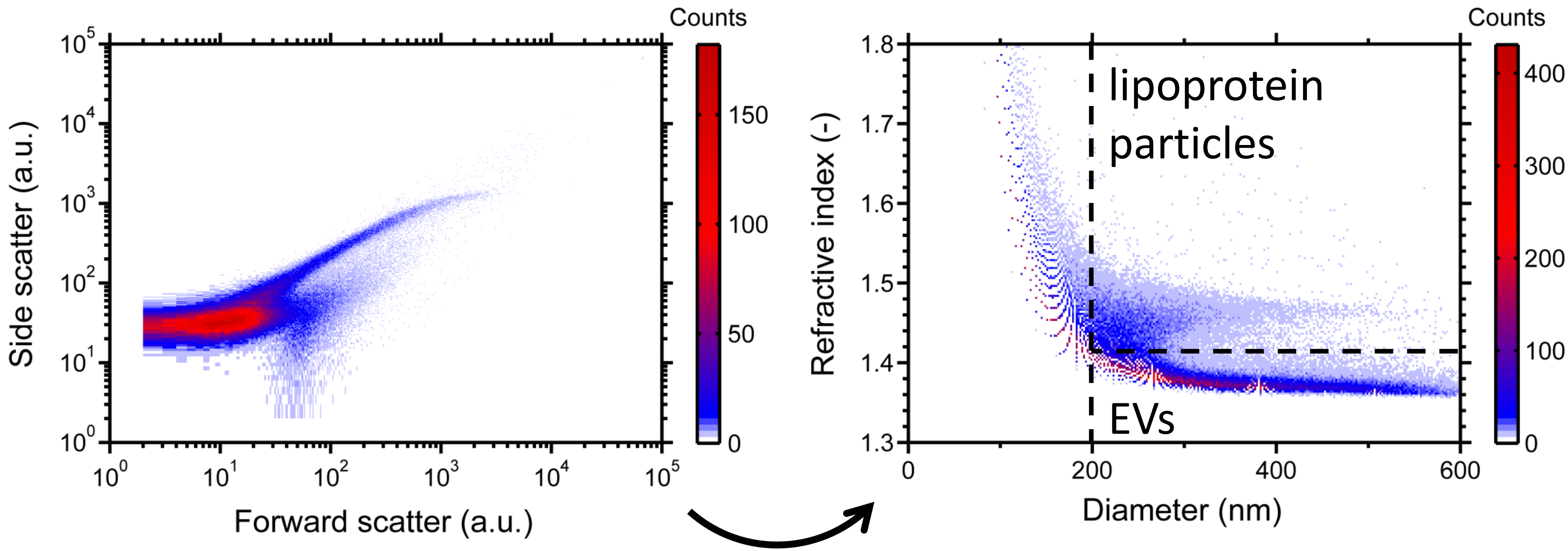
Conclusions standardization by sizing

- 24% of flow cytometers in study are unable to detect EVs by scatter-based triggering
- EV diameter gates by Mie theory improve reproducibility compared to no gate or bead diameter gate

Conclusions

- EVs are promising liquid biopsy biomarkers
- Collection and storing of EVs is tricky
- There is a need for better
  - EV isolation methods
  - EV detection techniques
    (faster, smaller, improved reproducibility)
- Standardize your EV measurements
  and report details

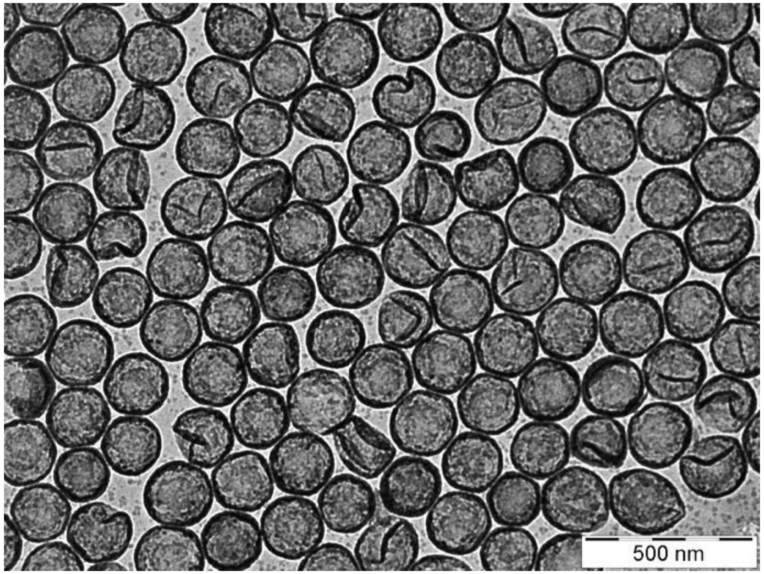
Outlook I

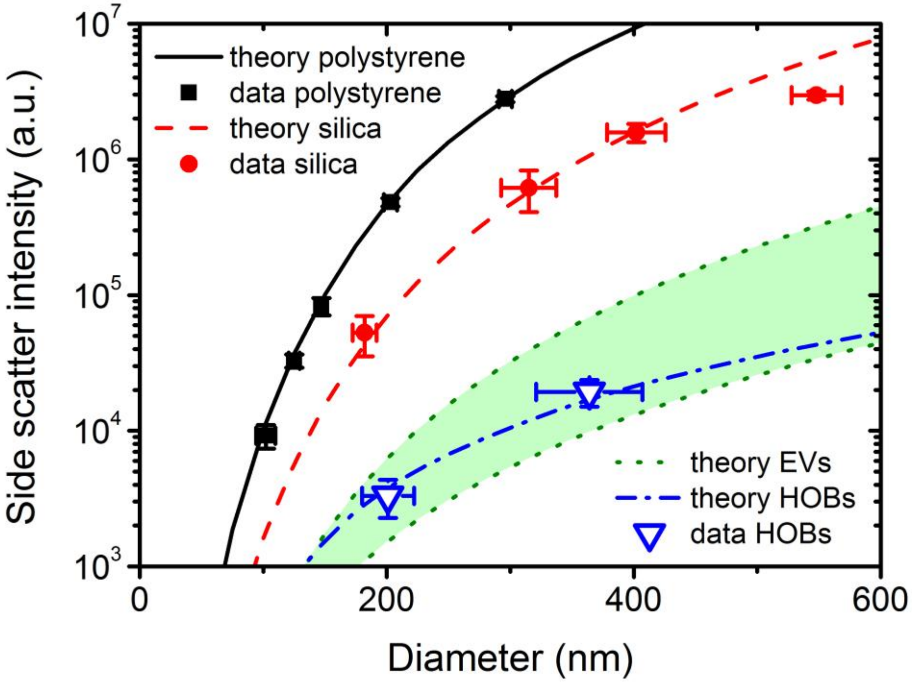


- With flow cytometry from arbitrary units to particle size *and* refractive index

Outlook *II*

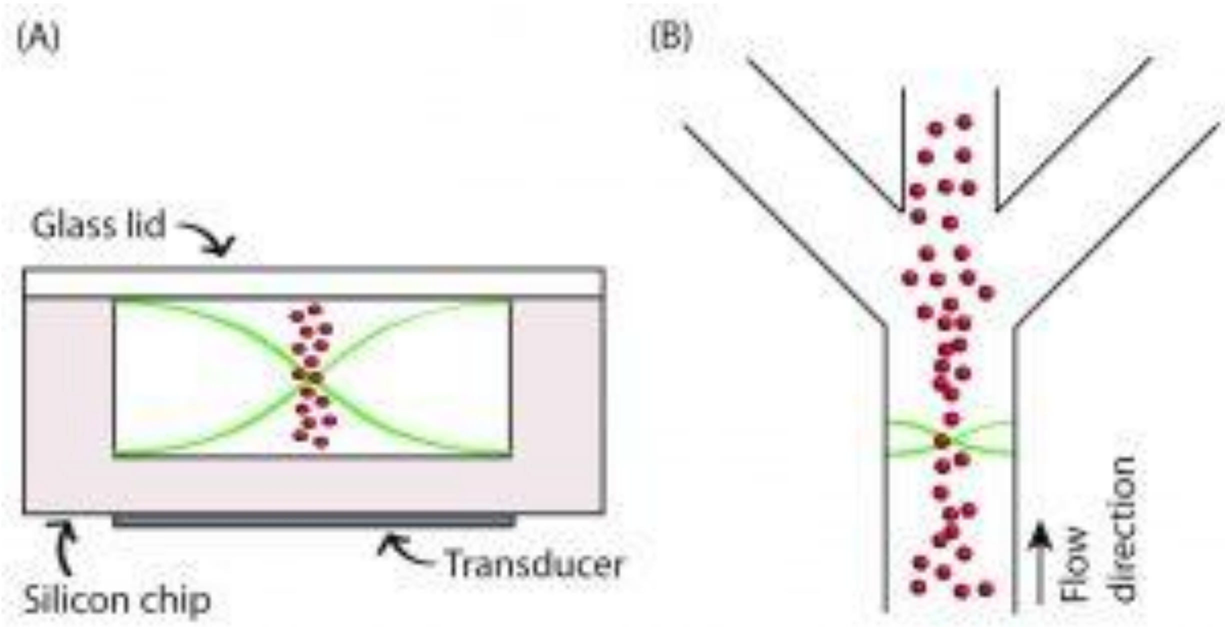
- One particle to calibrate all flow cytometry aspects





Outlook *III*

- How about acoustofluidic isolation of EVs?



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