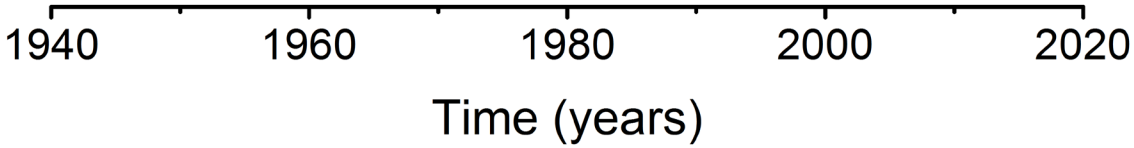
Detecting cell-derived vesicles with cell counters: from artefact to clinical act

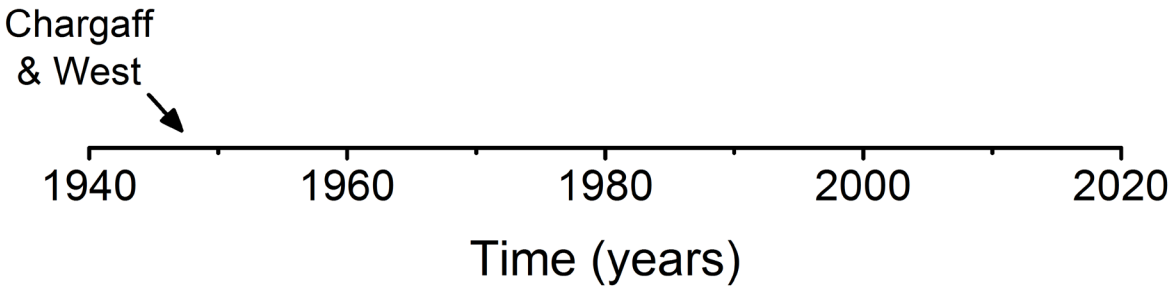
Edwin van der Pol

September 25th, 2017

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


1940
1960
1980
2000
2020
Time (years)

“cell-free plasma contains a subcellular factor that promotes clotting of blood”



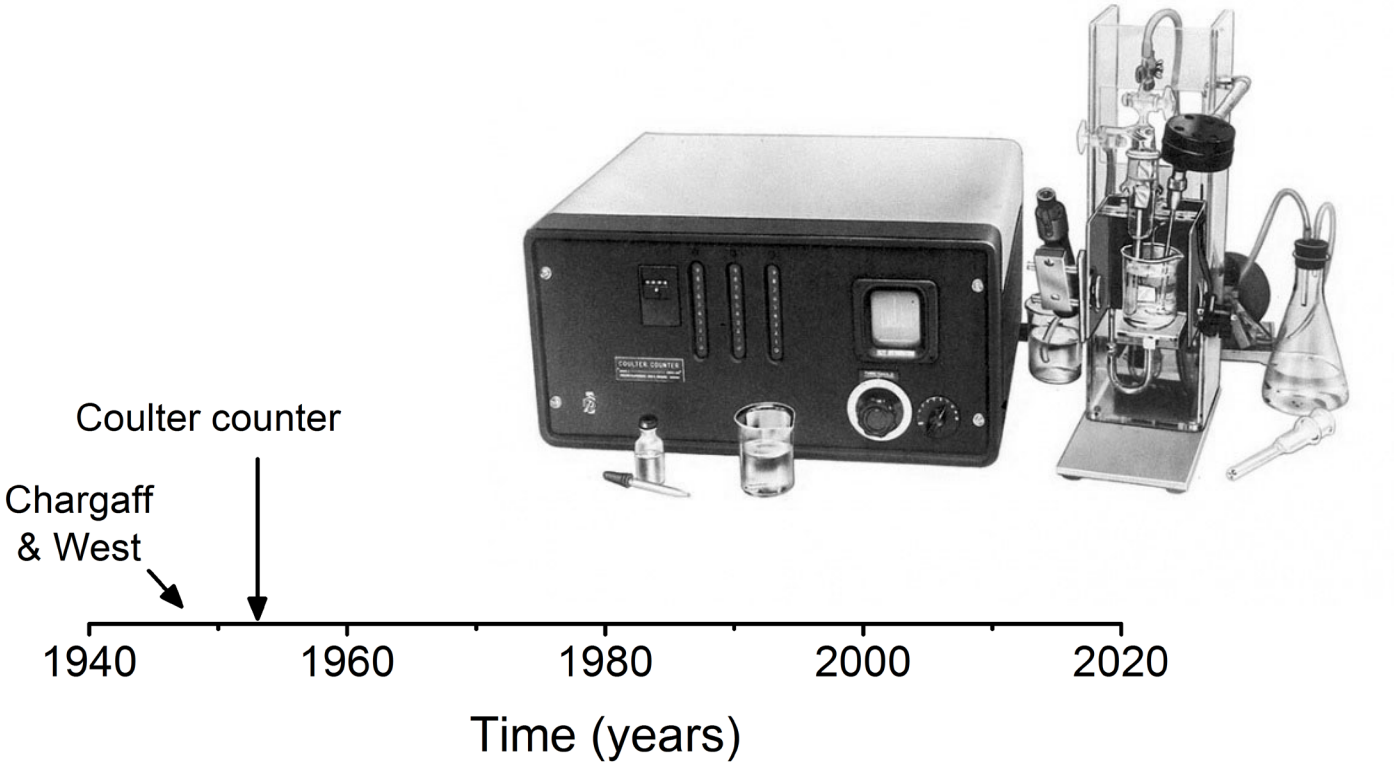
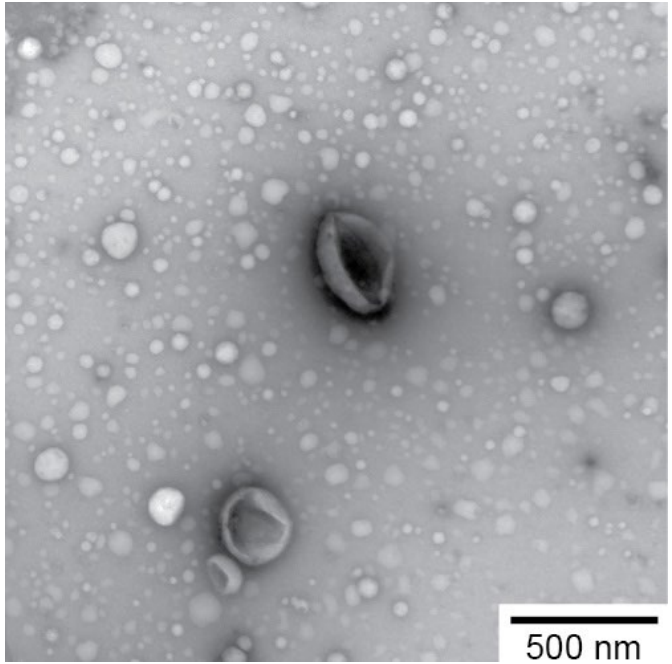
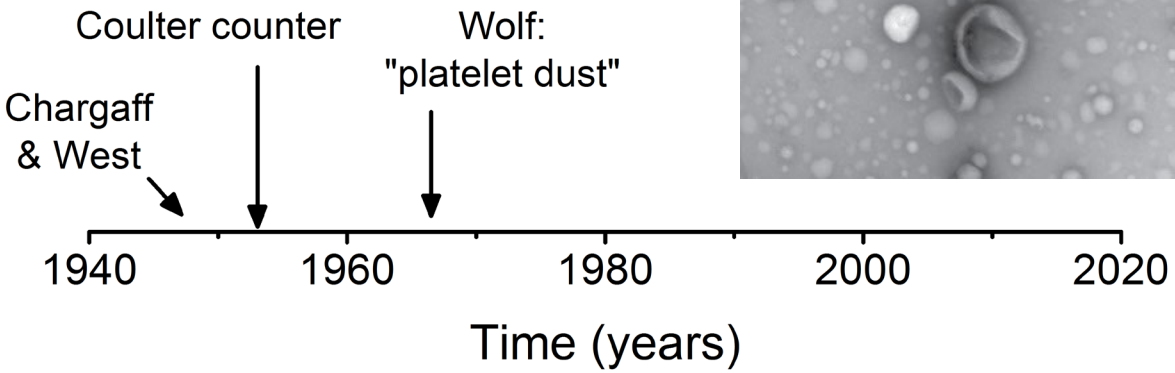
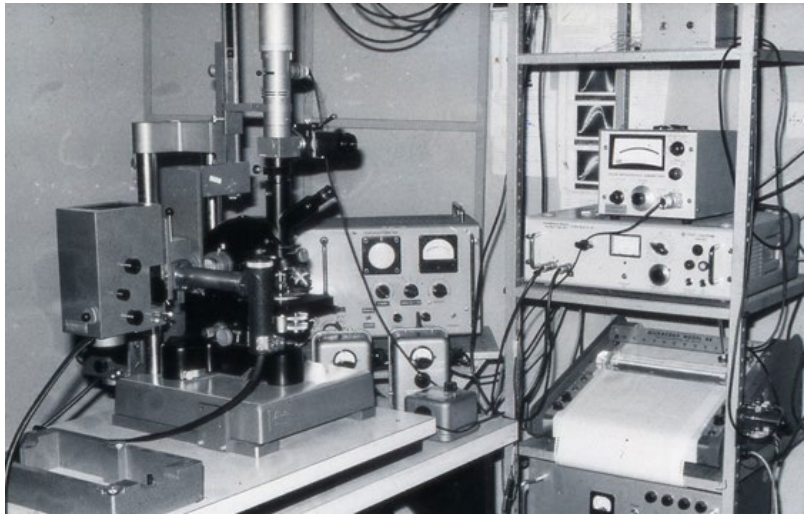


Image: gatech.edu

“small vesicles promote clotting of blood”







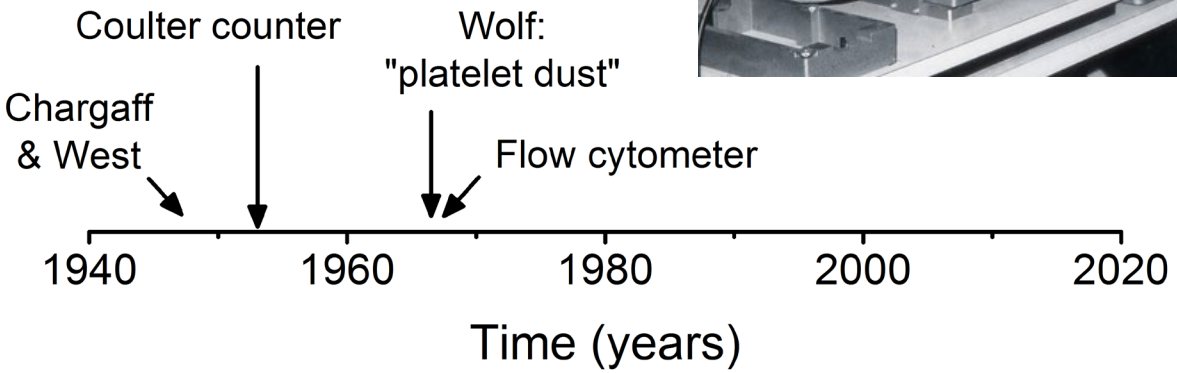


Image: purdue.edu

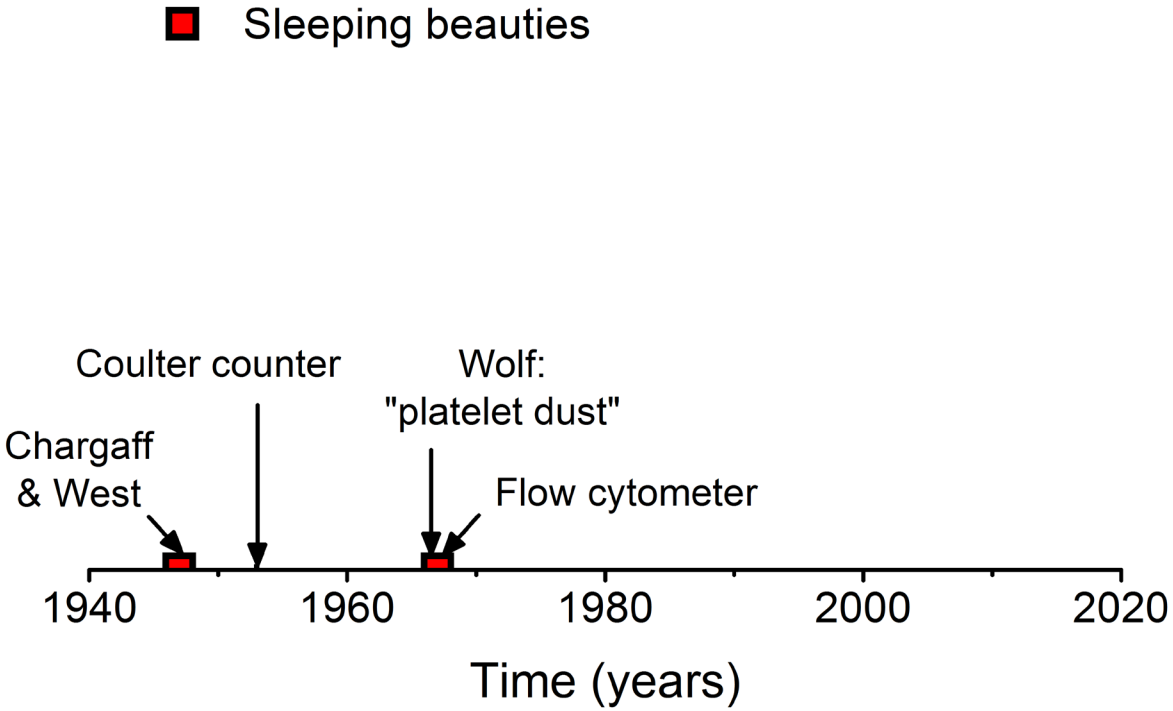
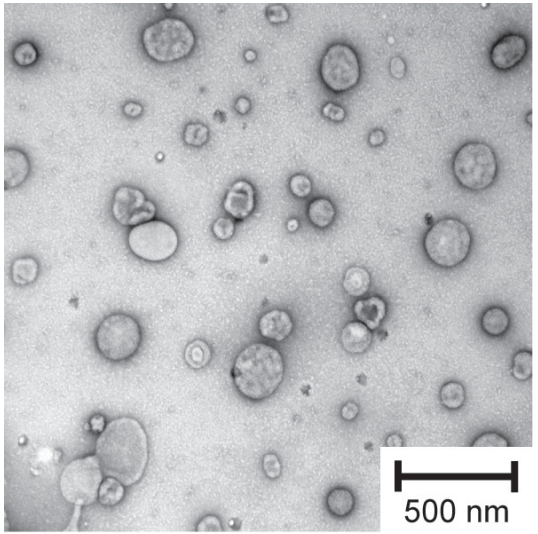
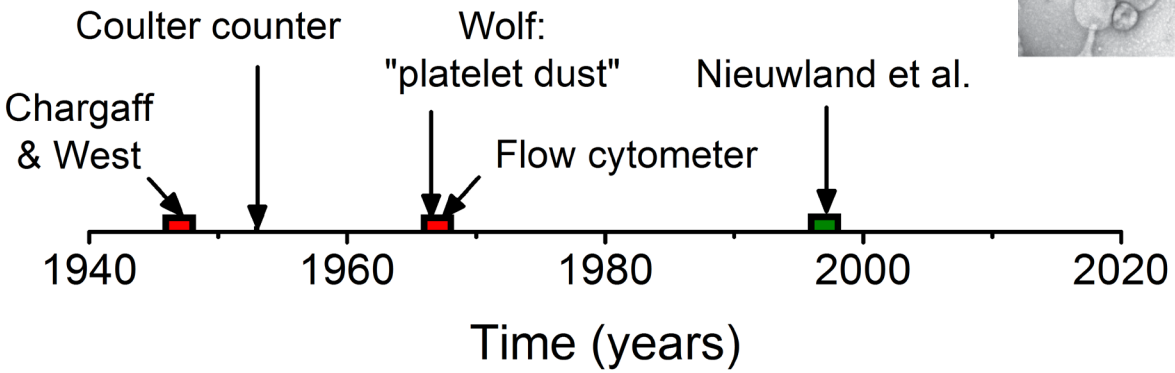


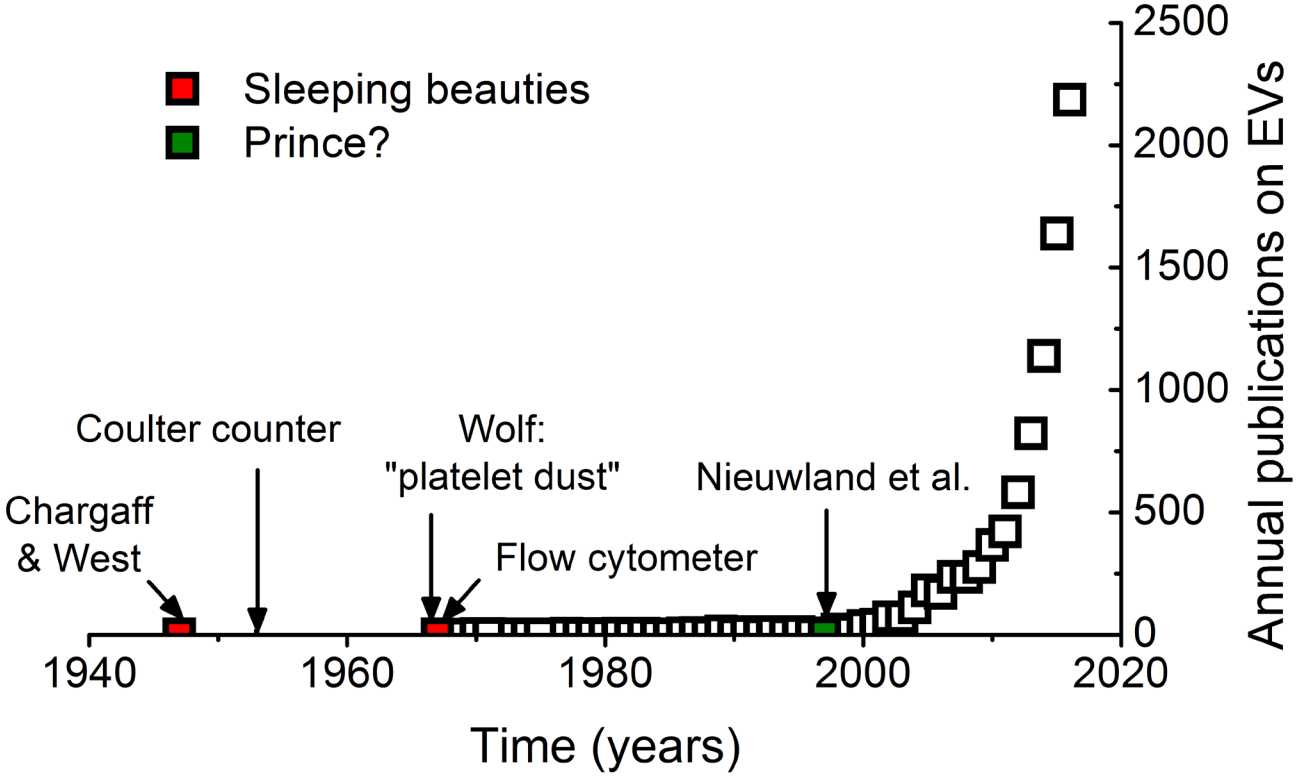
Image: purdue.edu

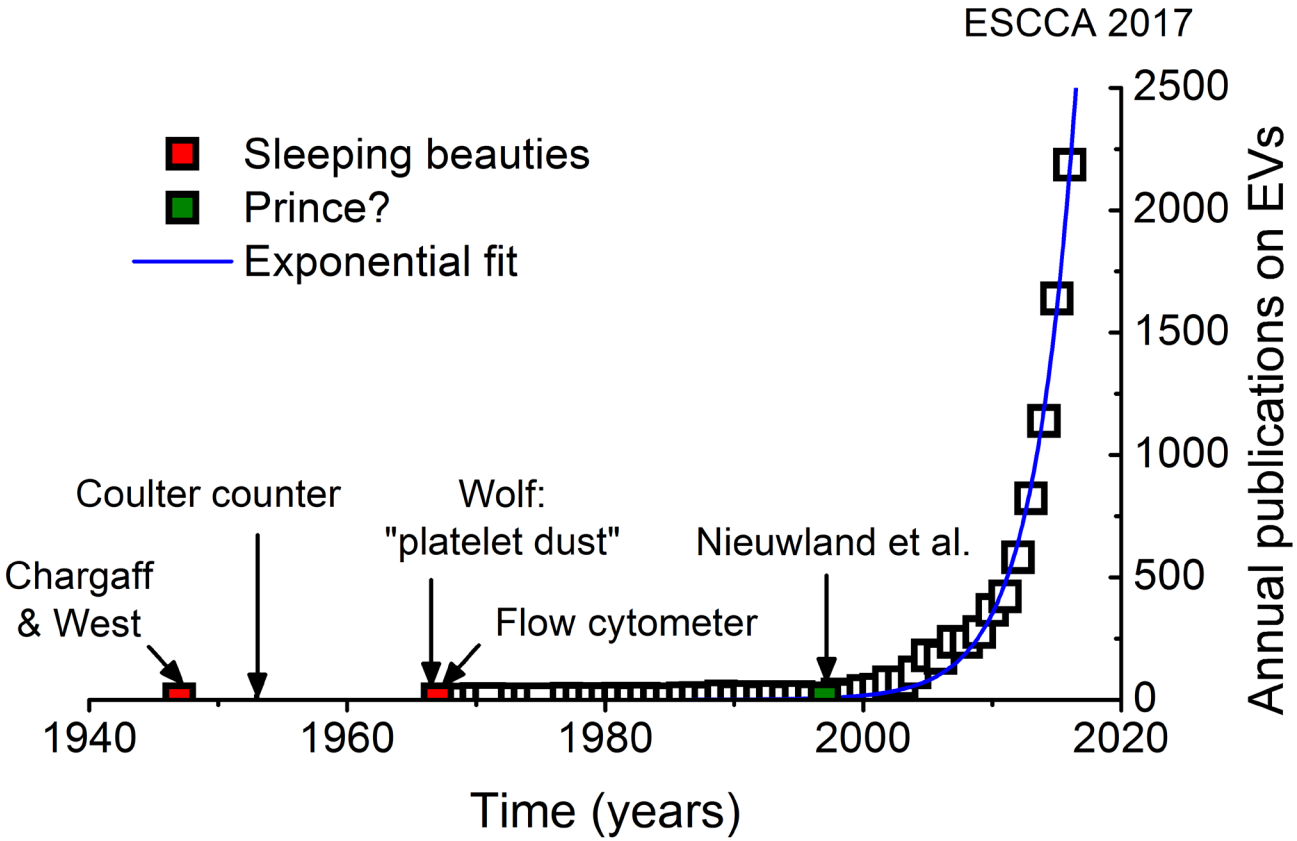
Sleeping beauties

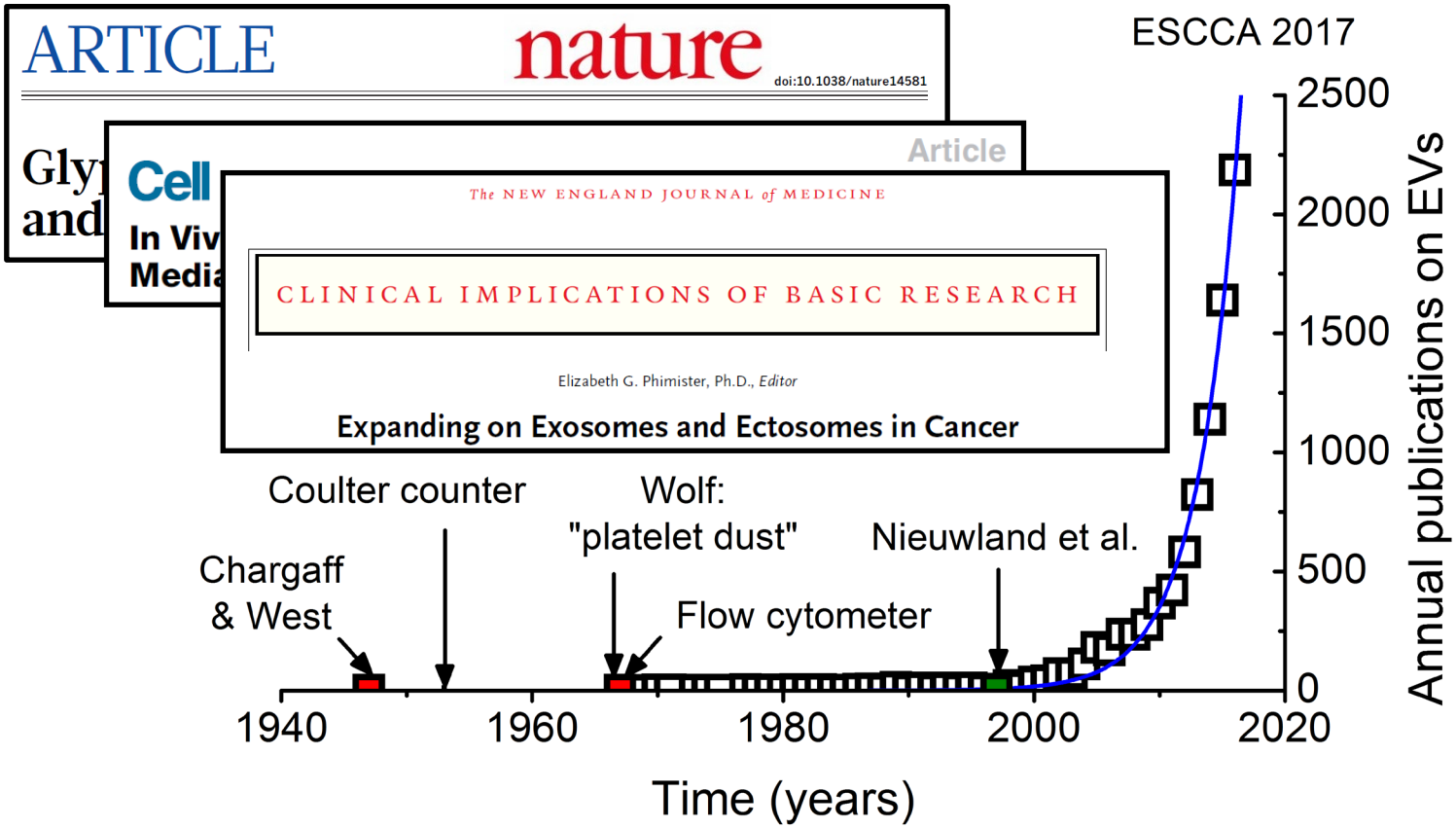
Cells release extracellular vesicles (EVs) in body fluids





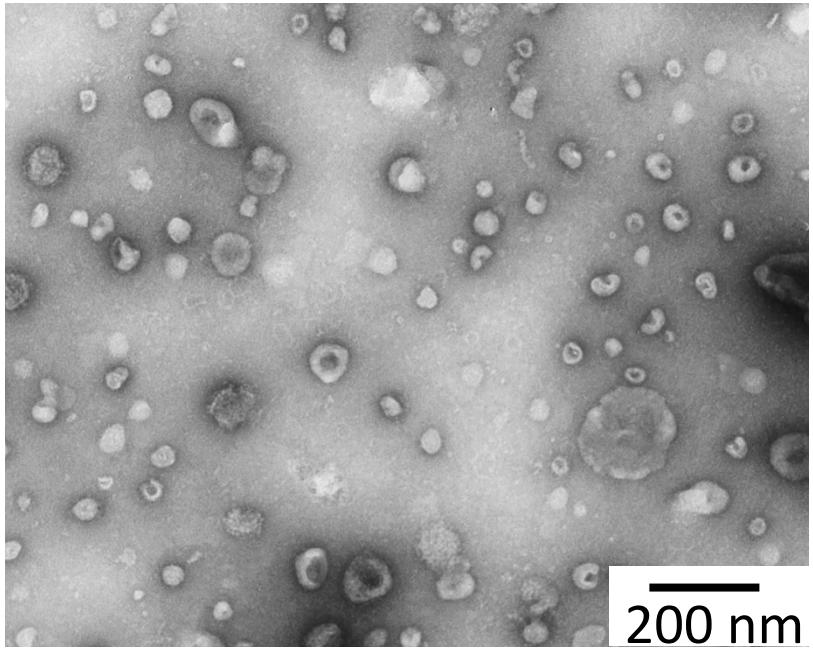

Sleeping beauties
Prince?
Coulter counter
Wolf:
"platelet dust"
Nieuwland et al.
Chargaff
& West
Flow cytometer
1940
1960
1980
2000
2020
Time (years)
2500
2000
1500
1000
500
0
Annual publications on EVs


ESCCA 2017
Sleeping beauties
Prince?
Exponential fit
Coulter counter
Wolf:
"platelet dust"
Nieuwland et al.
Chargaff
& West
Flow cytometer
2500
2000
1500
1000
500
0
Annual publications on EVs
1940
1960
1980
2000
2020
Time (years)


ARTICLE
nature
doi:10.1038/nature14581
Article
The NEW ENGLAND JOURNAL of MEDICINE
CLINICAL IMPLICATIONS OF BASIC RESEARCH
Elizabeth G. Phimister, Ph.D., Editor
Expanding on Exosomes and Ectosomes in Cancer
ESCCA 2017
Coulter counter
Wolf:
"platelet dust"
Nieuwland et al.
Chargaff
& West
Flow cytometer
1940
1960
1980
2000
2020
Time (years)
0
500
1000
1500
2000
2500
Annual publications on EVs

Why extracellular vesicles (EVs) are interesting

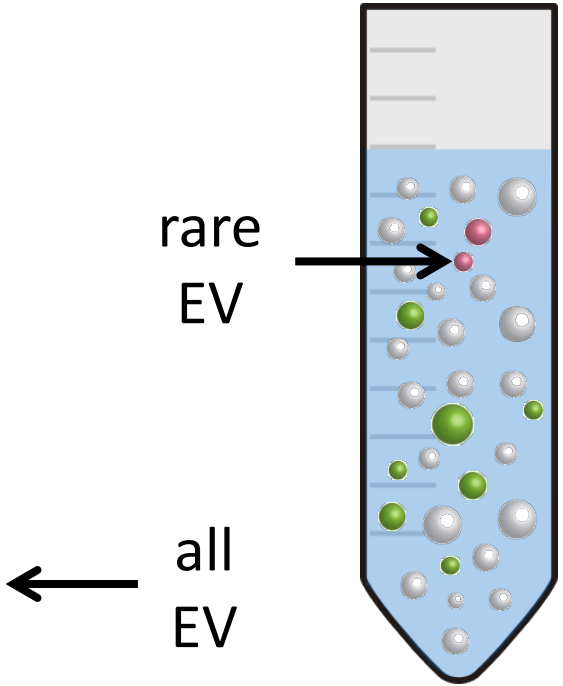
- cells release EVs in body fluids to
  - transport waste
  - communicate
- EVs are biomarkers for diseases
  - cancer
  - thrombosis
  - preeclampsia



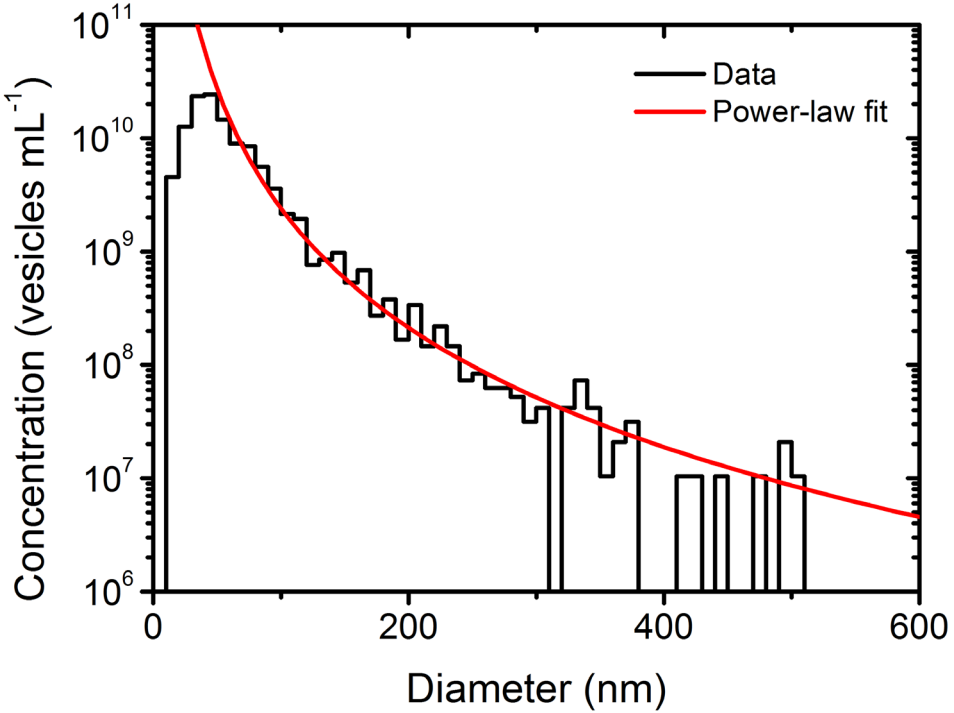
van der Pol et al. *Pharmacol.Rev.* 2012

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

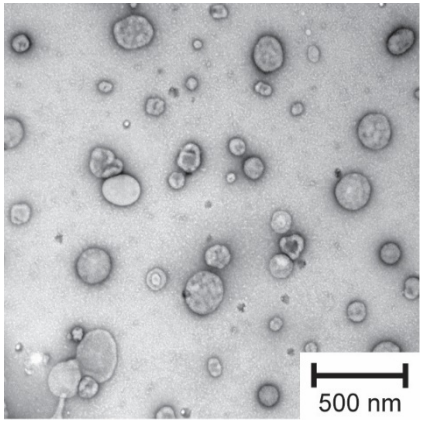


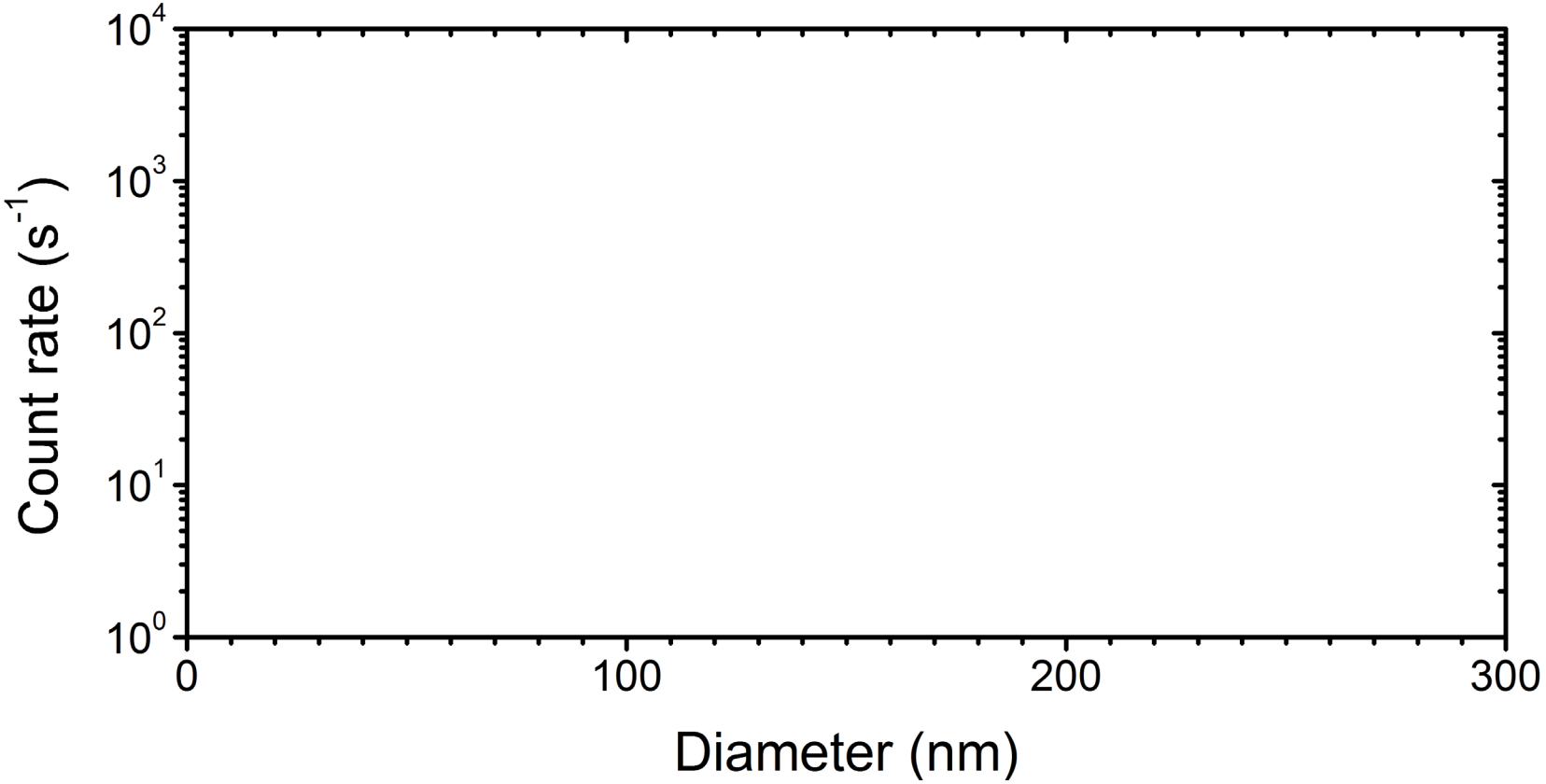
Problem: EVs are small and heterogeneous

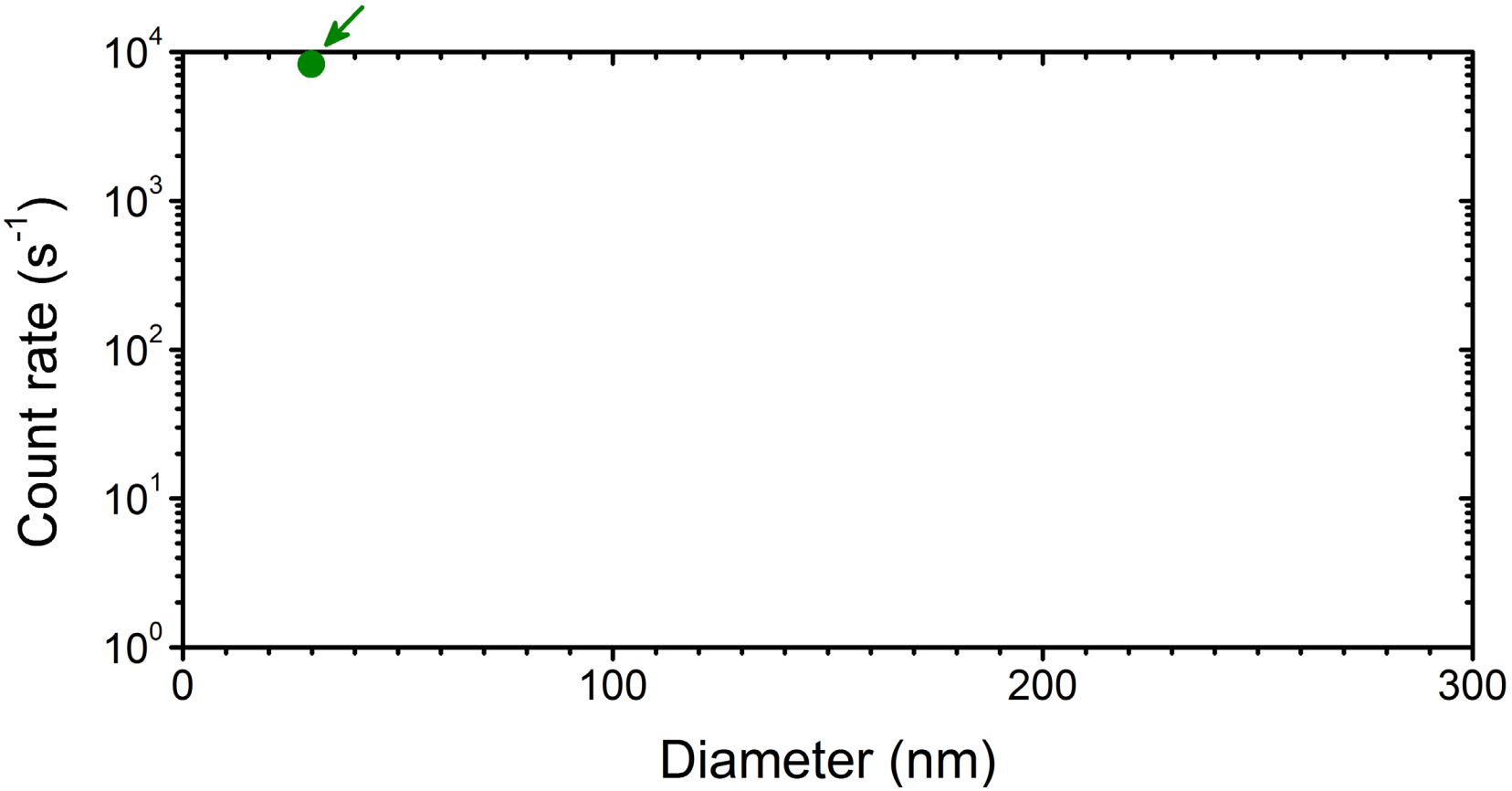


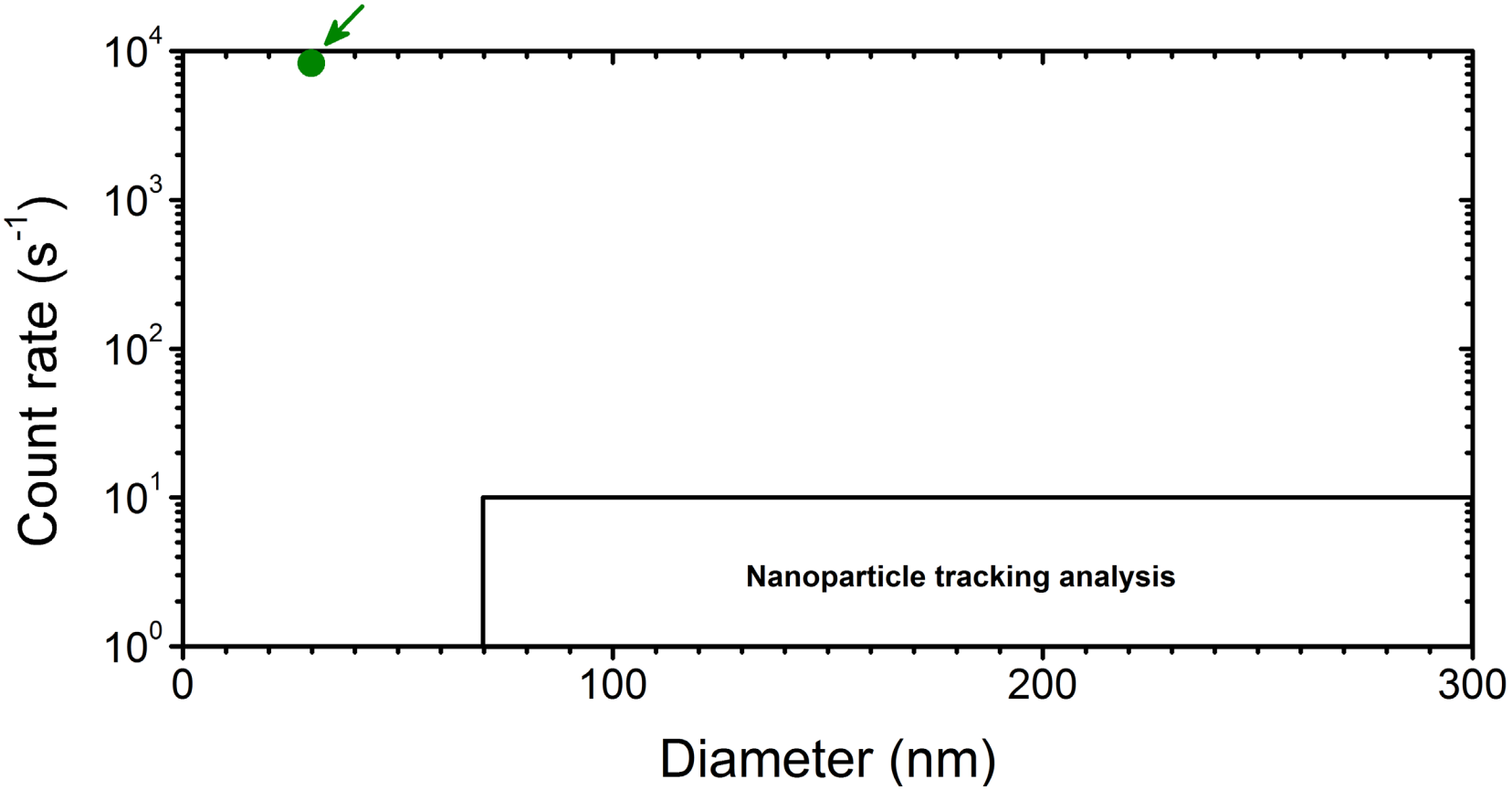
Outline

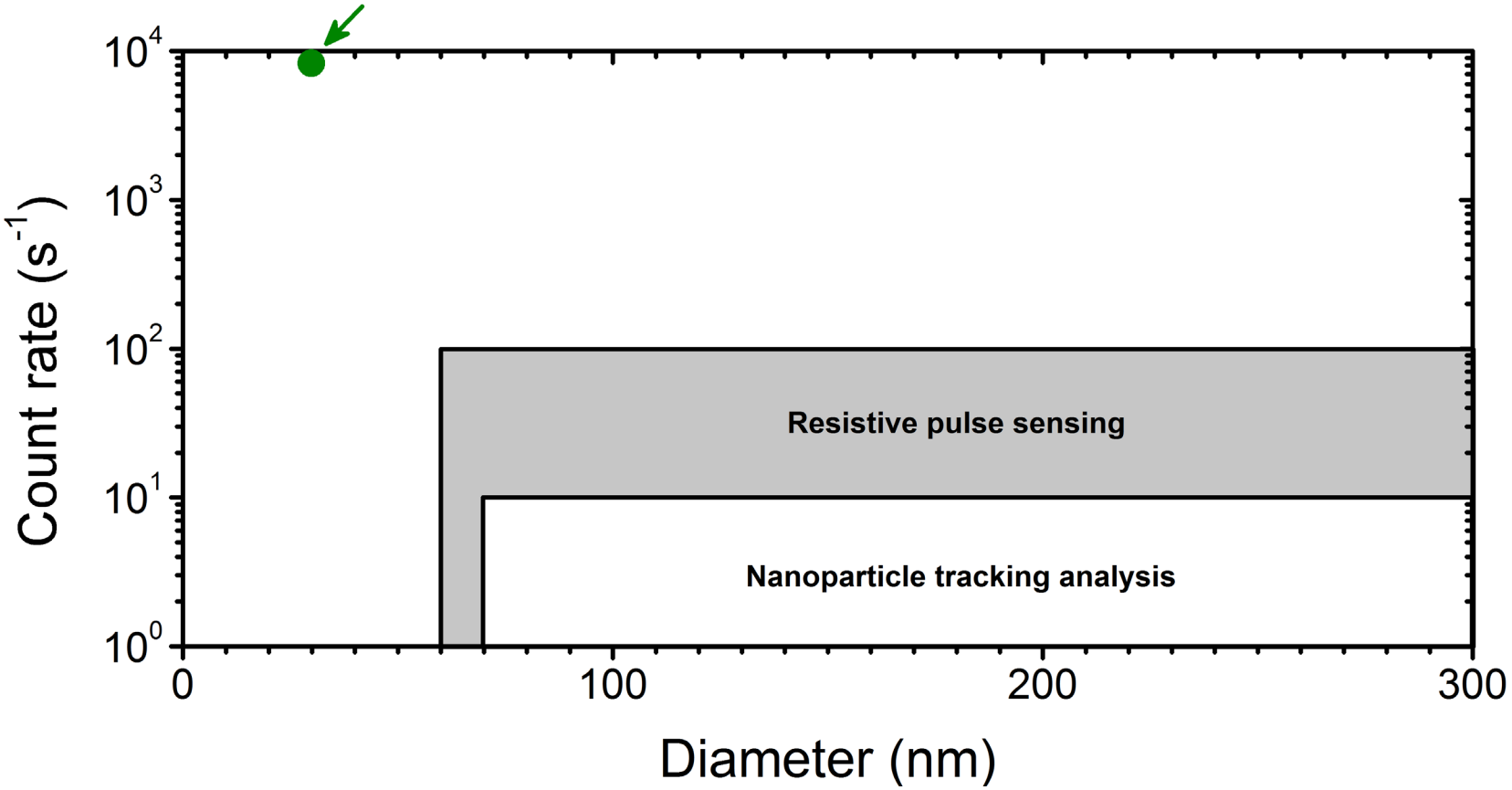
- ✔ Extracellular vesicles (EVs)
- Single EV detection methods
- Single EV flow cytometry
  - *From artefact…*
  - *to clinical act*
    - Standardization by size determination
    - Standardization by size and refractive index determination



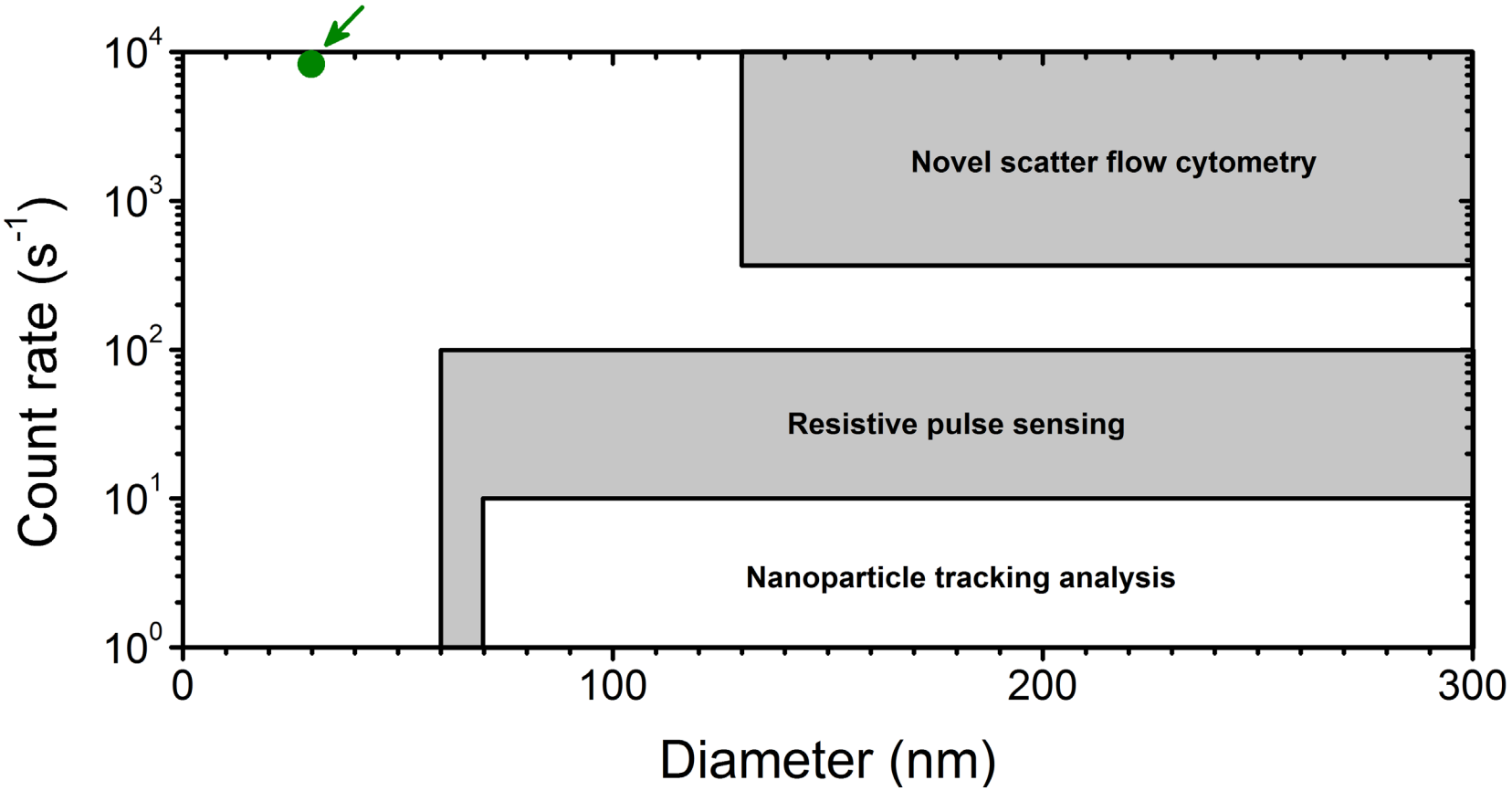

Count rate (s^{-1})
10^4
10^3
10^2
10^1
10^0
0
100
200
300
Diameter (nm)


Count rate (s^{-1})
10^4
10^3
10^2
10^1
10^0
0
100
200
300
Diameter (nm)

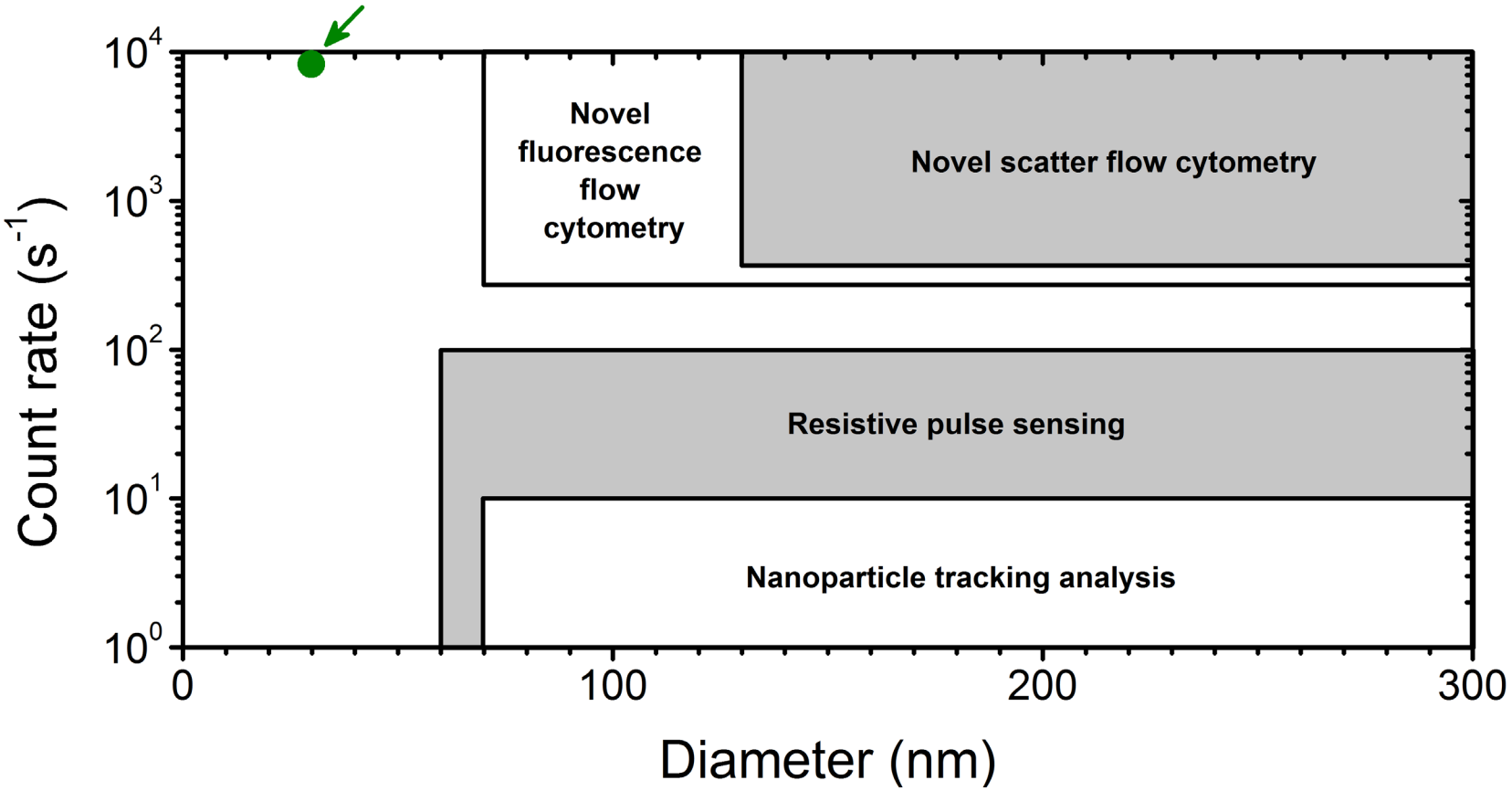

Count rate (s^{-1})
10^4
10^3
10^2
10^1
10^0
0
100
200
300
Diameter (nm)
Nanoparticle tracking analysis

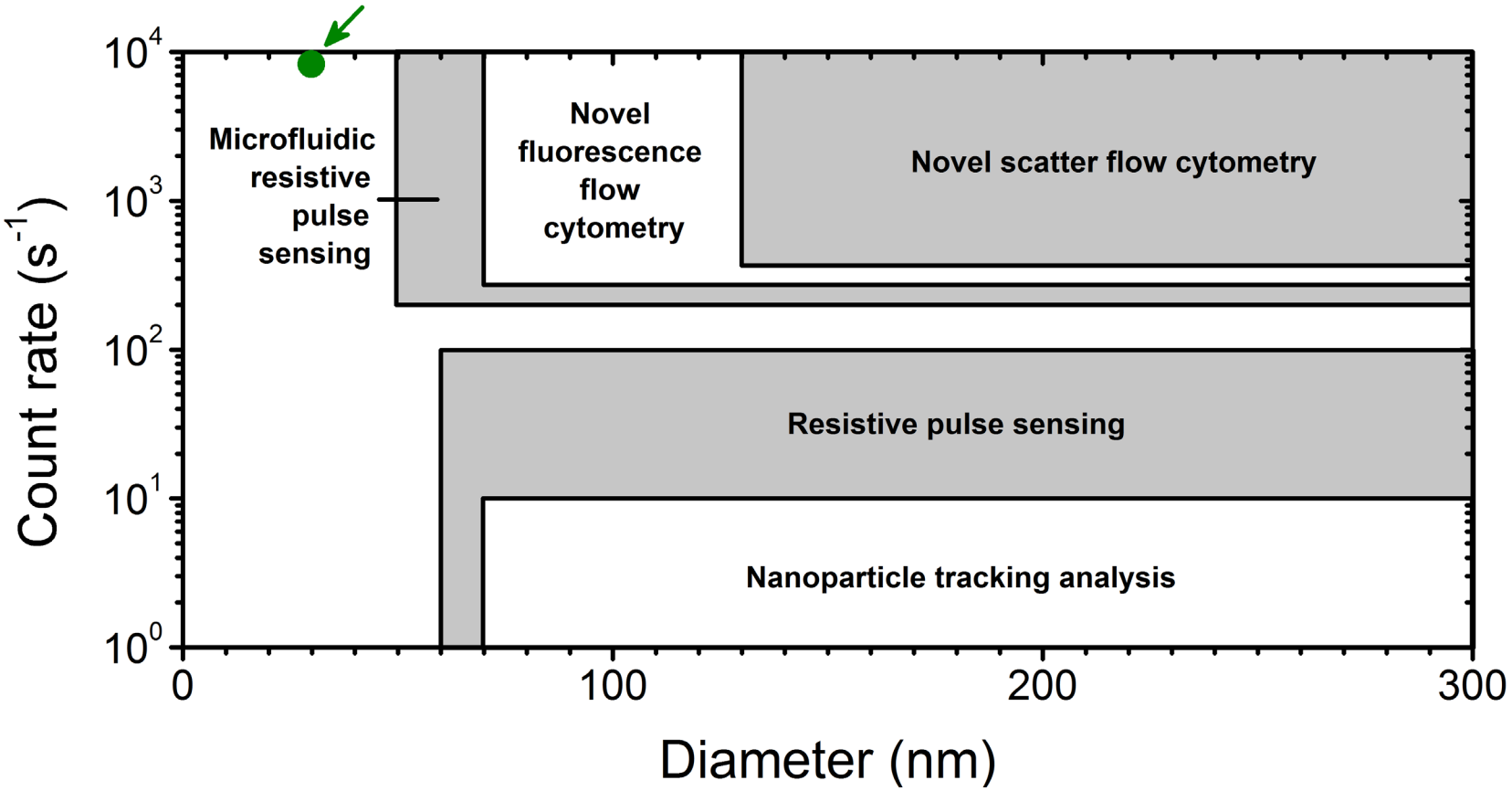


Coumans et al. *Circ.Res.* 2017

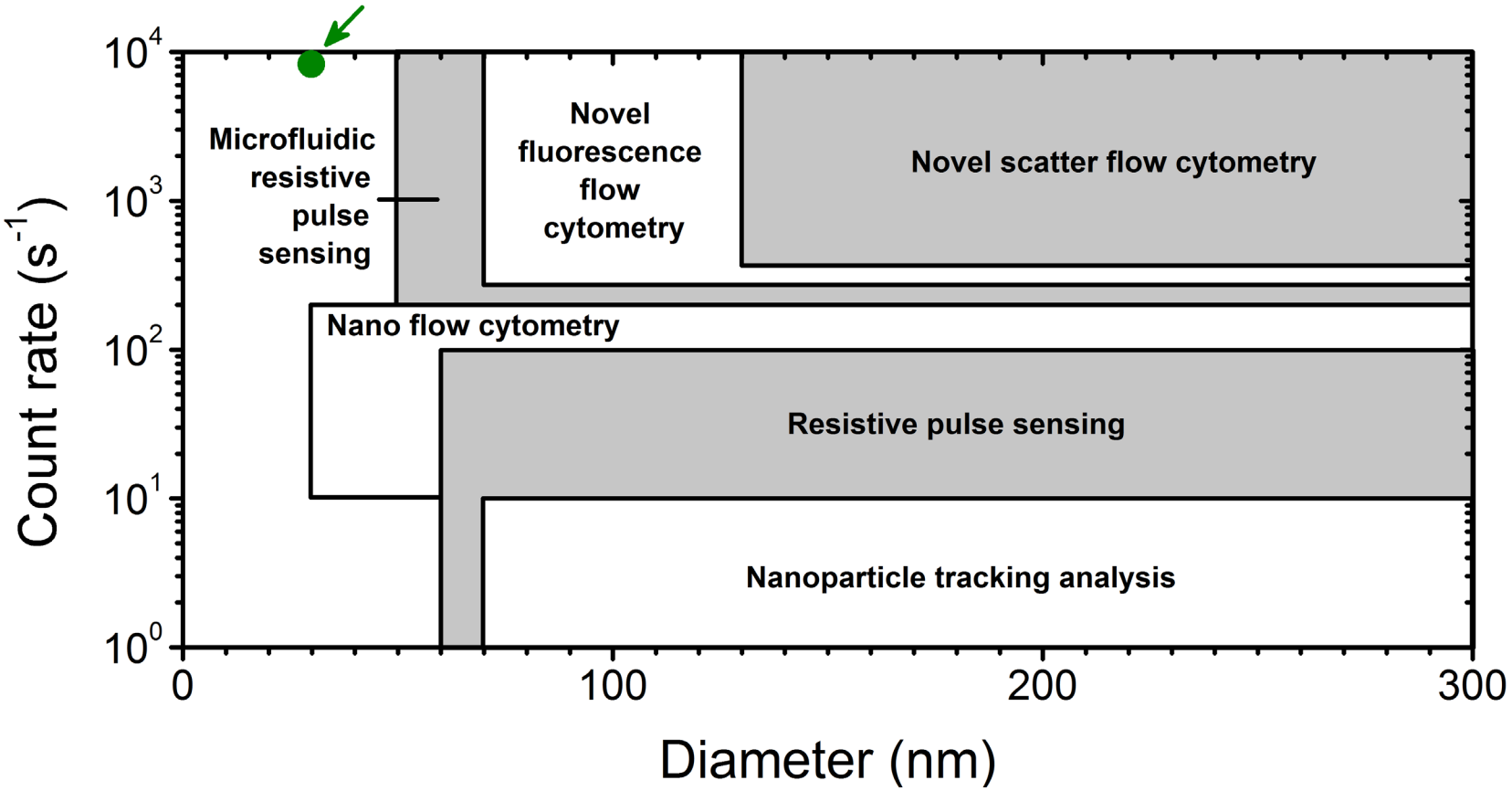


Novel scatter flow cytometry: van der Pol et al. *JTH* 2014


Count rate (s^{-1})
10^4
10^3
10^2
10^1
10^0
0
100
200
300
Diameter (nm)
Novel fluorescence flow cytometry
Novel scatter flow cytometry
Resistive pulse sensing
Nanoparticle tracking analysis

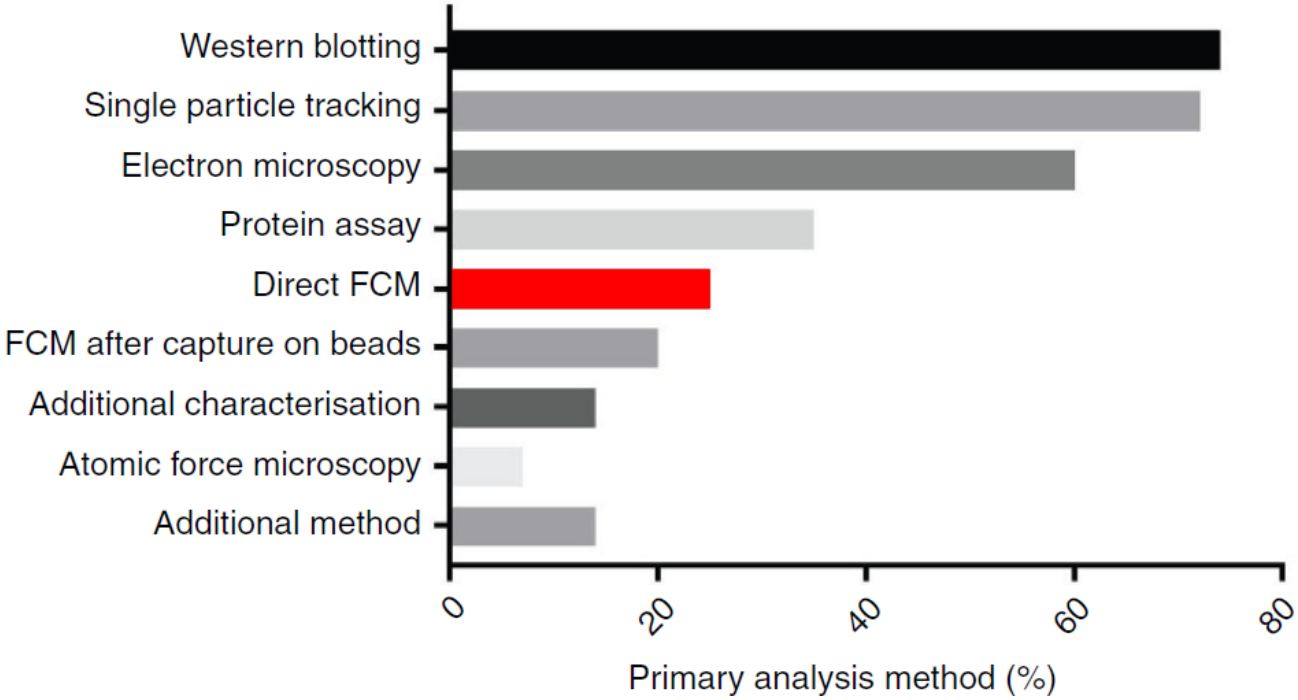


Microfluidic resistive pulse sensing: Fraikin *Nature Nanotech.* 2011



Nano flow cytometry: Zhu et al. *ACS Nano* 2014

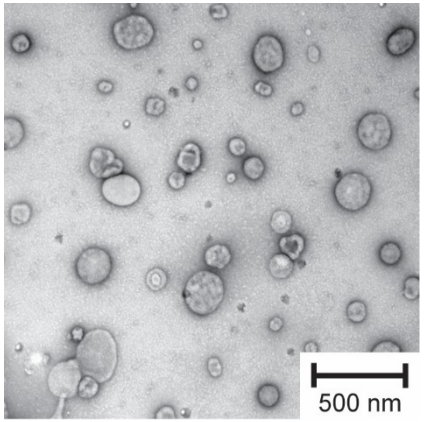
EV research using flow cytometry



Gardiner et al. *JEV* 2016

Outline

- ✔ Extracellular vesicles (EVs)
- ✔ Single EV detection methods
- ● Single EV flow cytometry
  - ➢ *From artefact…*
  - ➢ *to clinical act*
    - ▪ Standardization by size determination
    - ▪ Standardization by size and refractive index determination



Flow cytometry

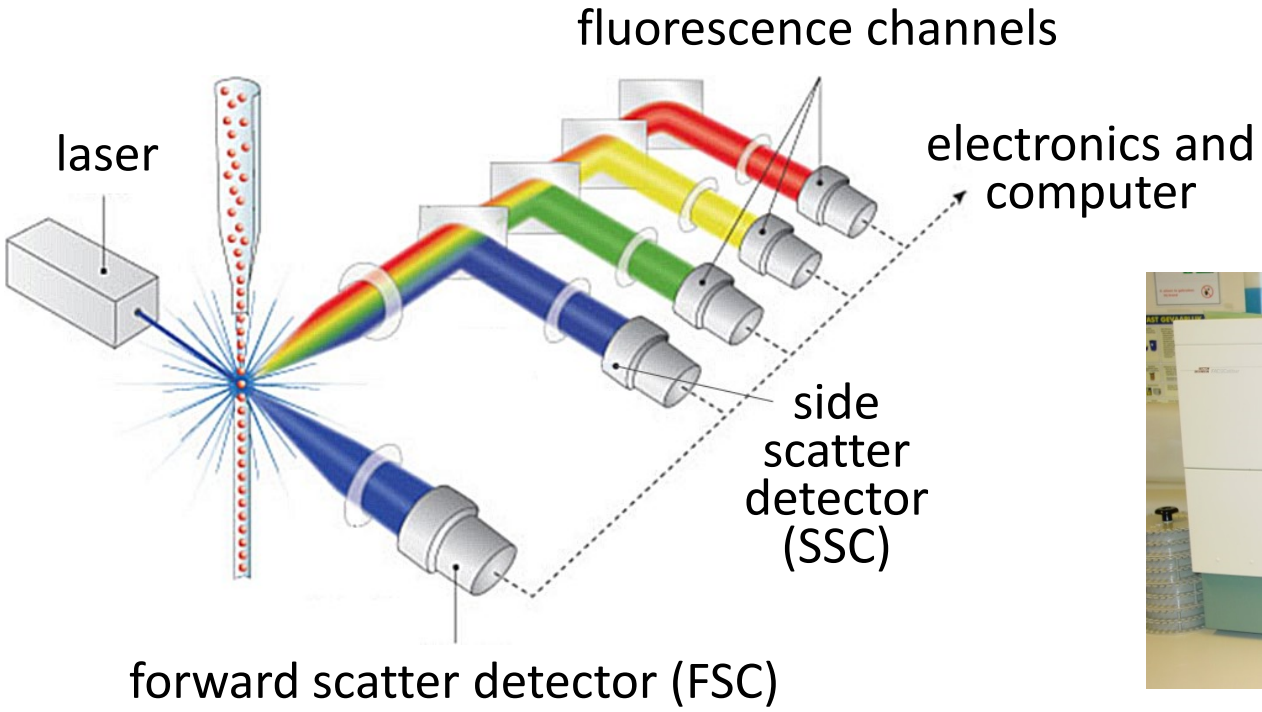
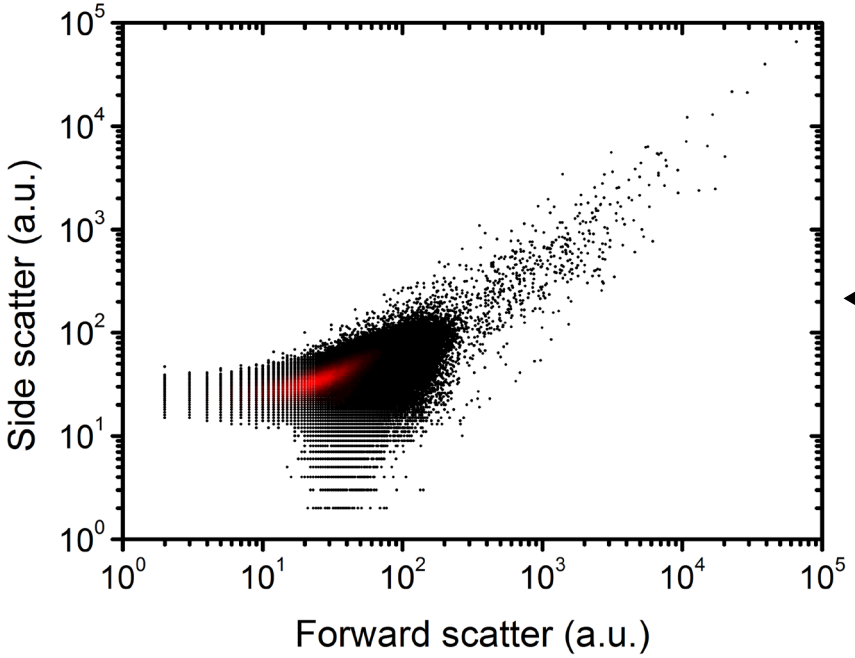
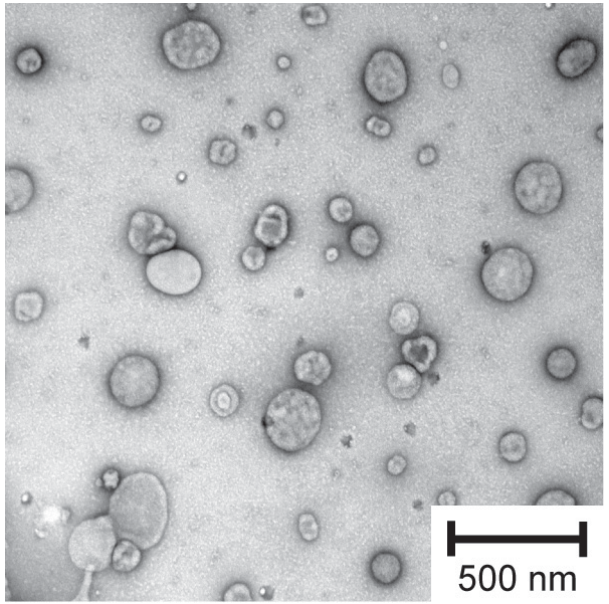


image: semrock.com

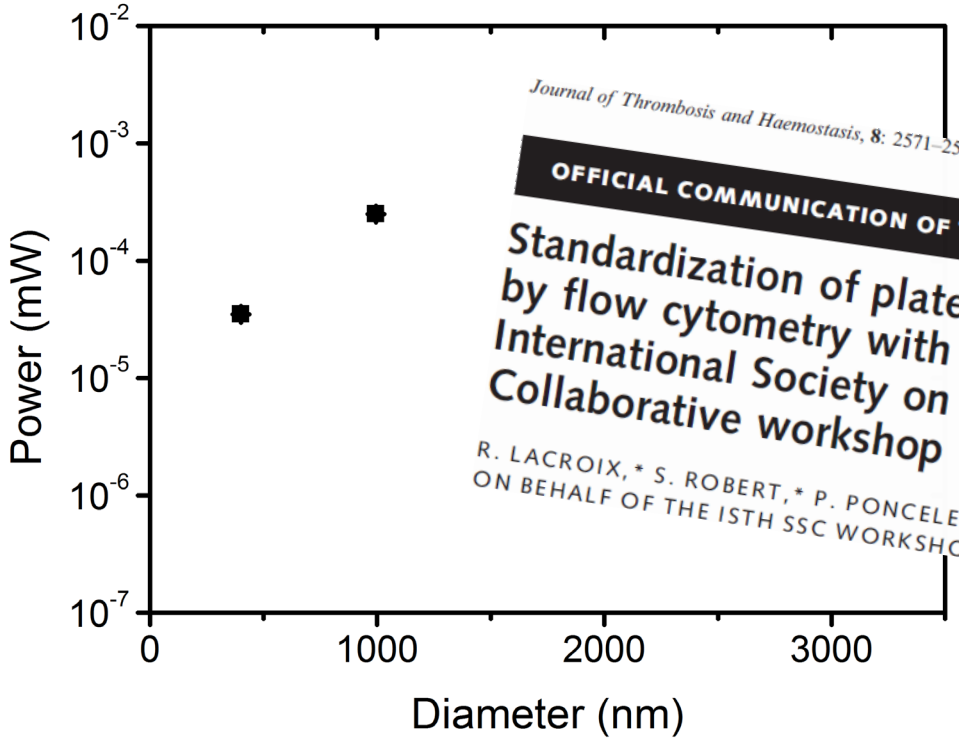
How do we know that we are counting EVs?



?



Set gate with polystyrene beads



Journal of Thrombosis and Haemostasis, 8: 2571–2574

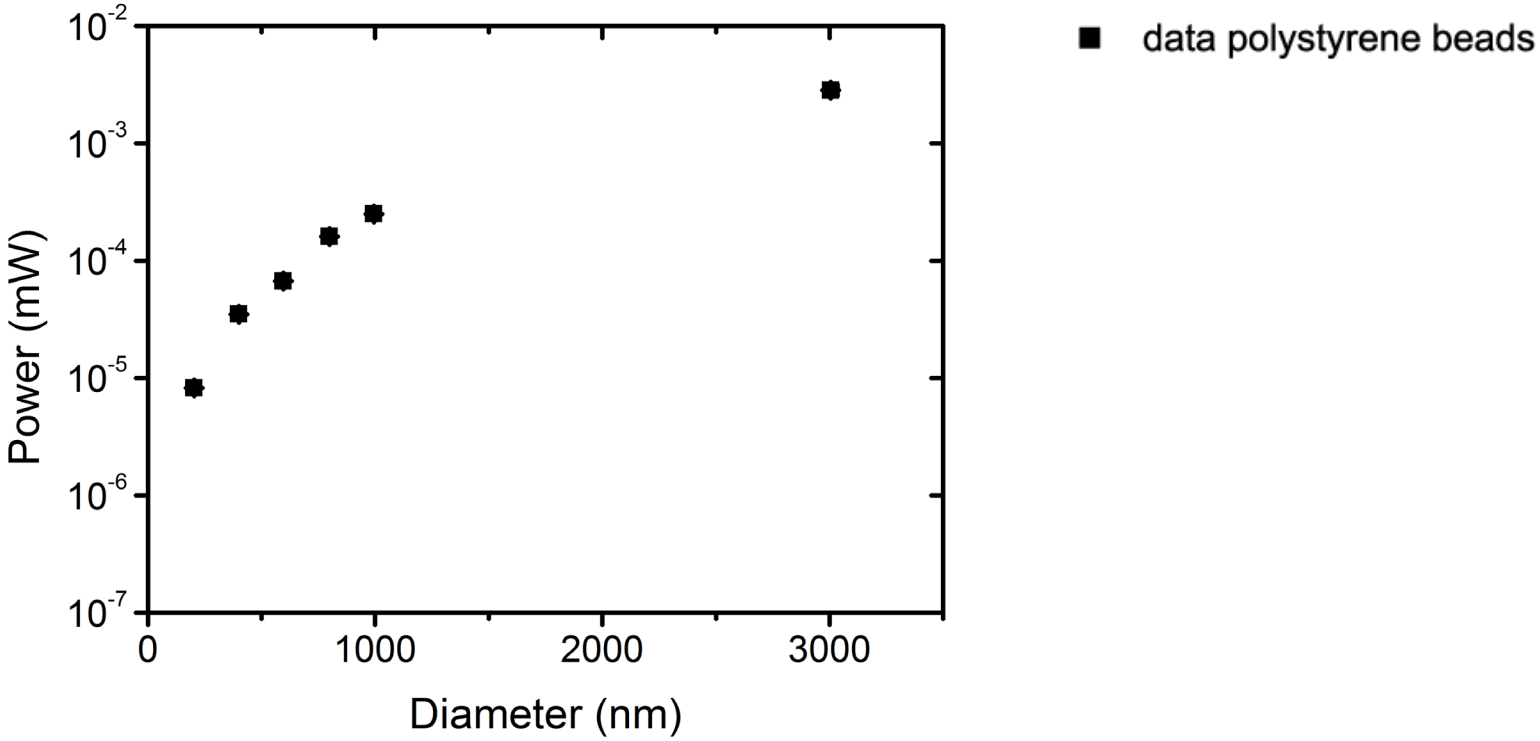
OFFICIAL COMMUNICATION OF THE SSC

DOI: 10.1111/j.1538-7836.2010.04047.x

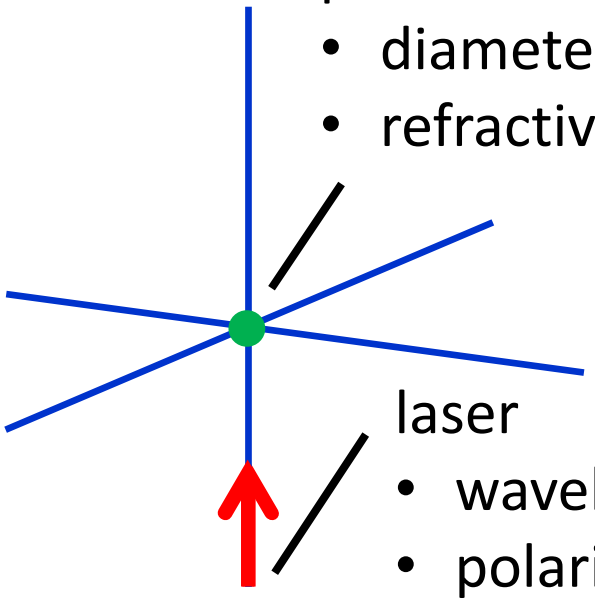
Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop

R. LACROIX,* S. ROBERT,* P. PONCELET,† R. S. KASTHURI,‡ N. S. KEY‡ and F. DIGNAT-GEORGE*
ON BEHALF OF THE ISTH SSC WORKSHOP

Relate scatter to diameter of beads



Relate scatter to diameter of beads with Mie



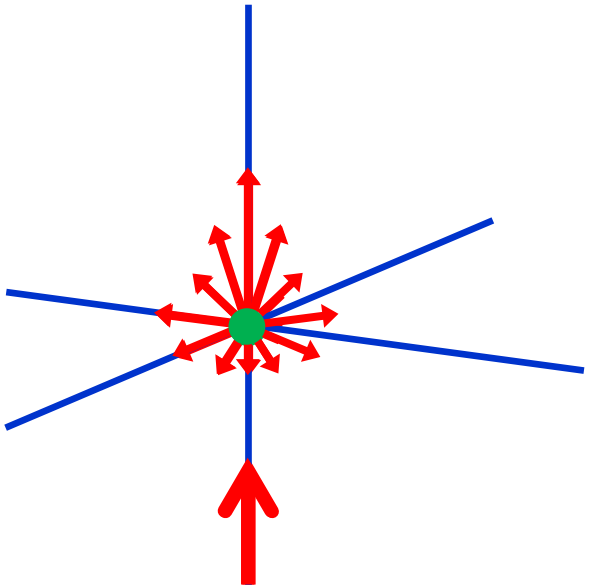
particle

- diameter
- refractive index

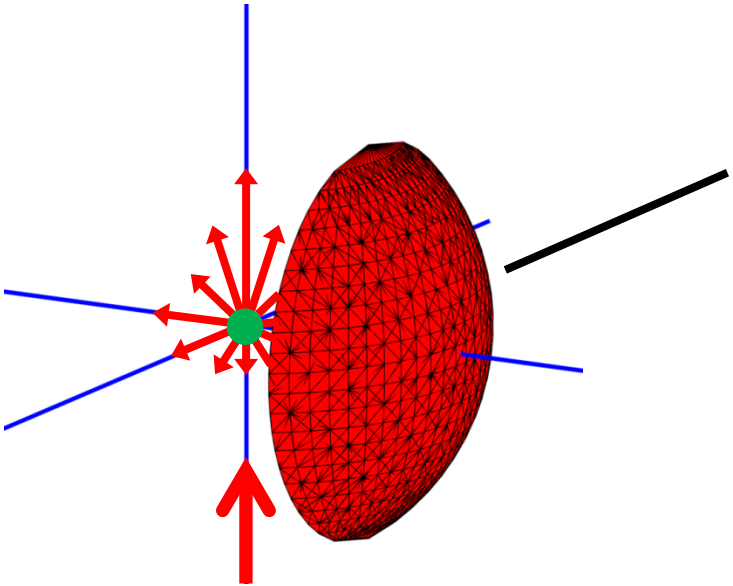
laser

- wavelength
- polarization
- power

Relate scatter to diameter of beads with Mie



Relate scatter to diameter of beads with Mie

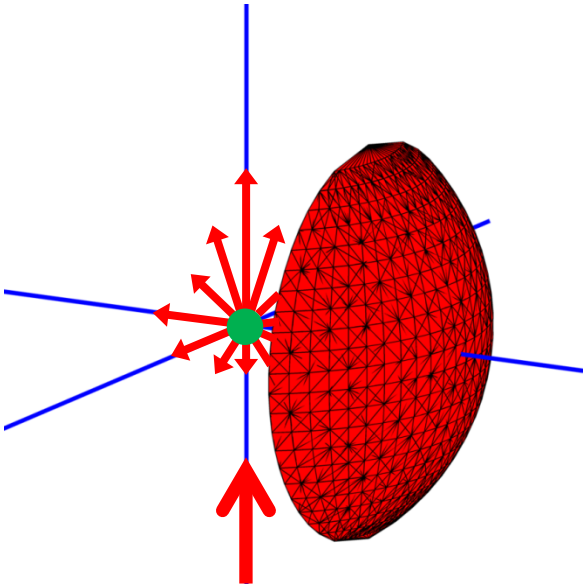


optical configuration

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

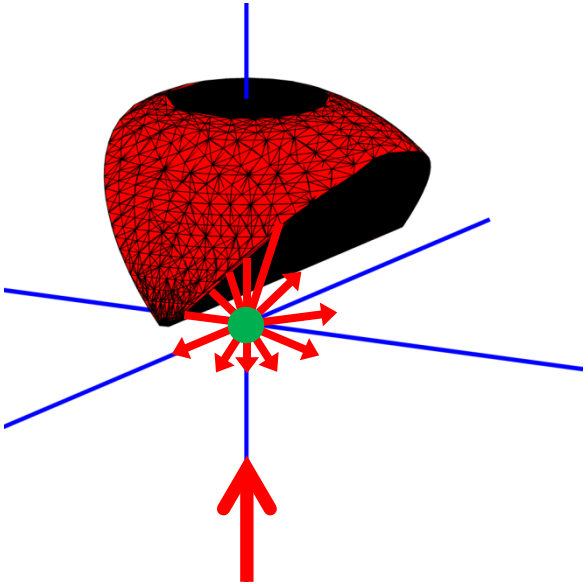
Relate scatter to diameter of beads with Mie

Becton Dickinson FACSCalibur



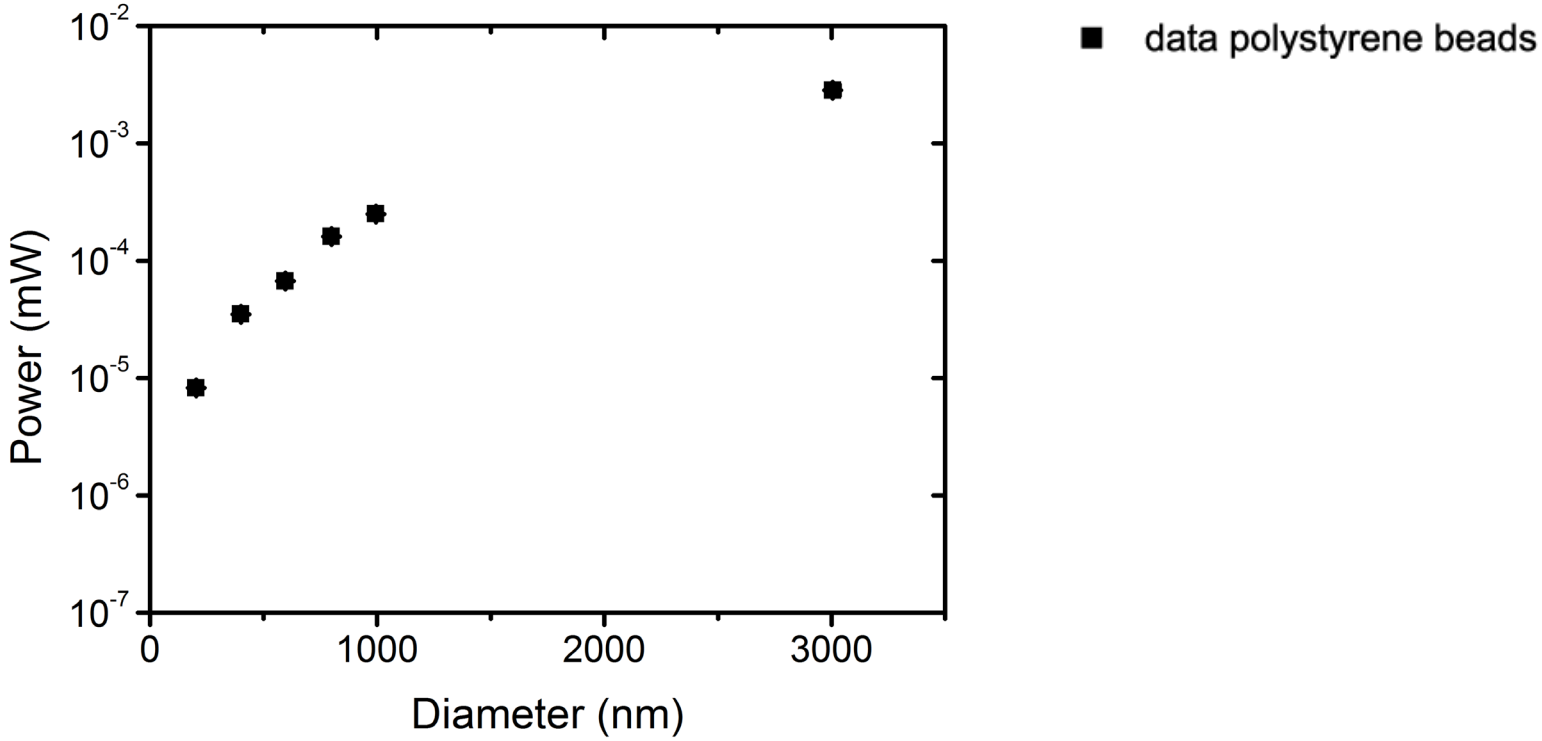
Side scatter

Apogee A50-micro

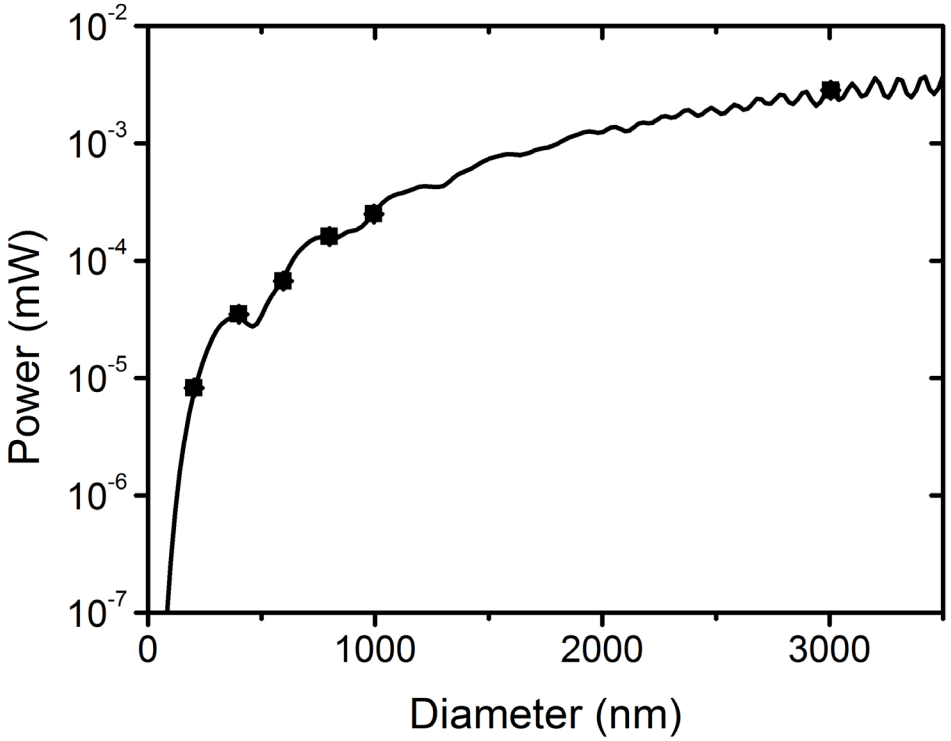


Forward scatter

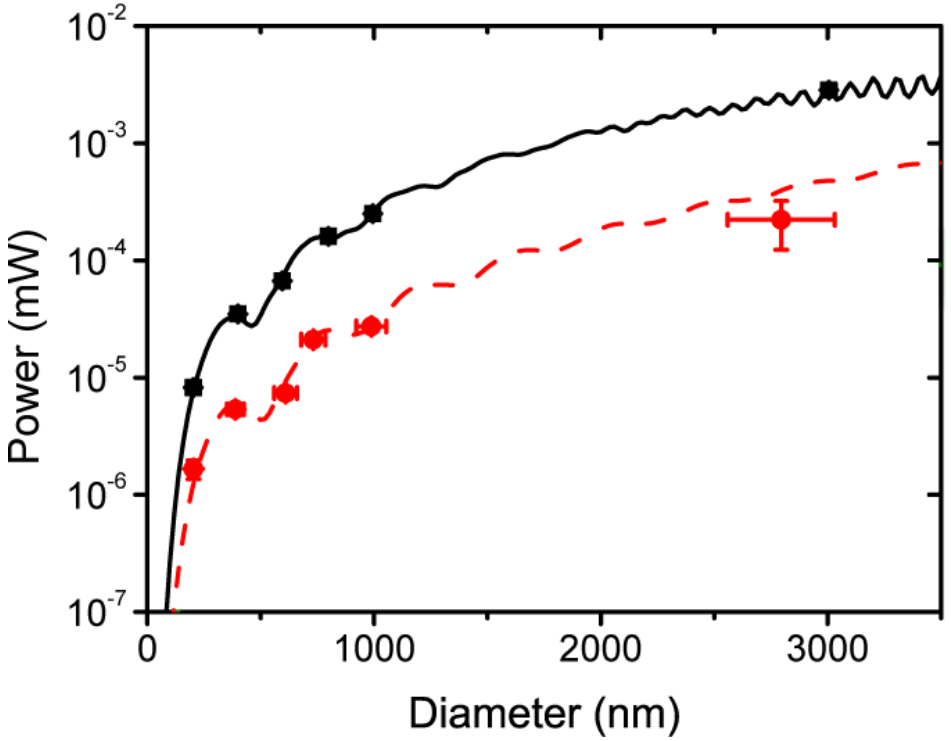
Relate scatter to diameter of beads

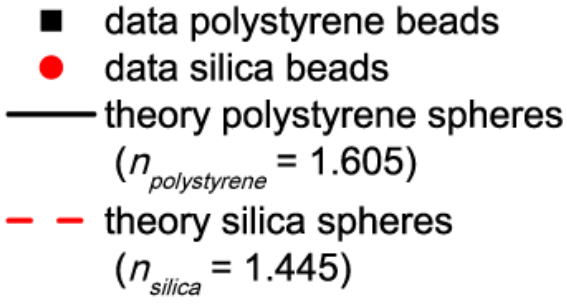


Relate scatter to diameter of beads

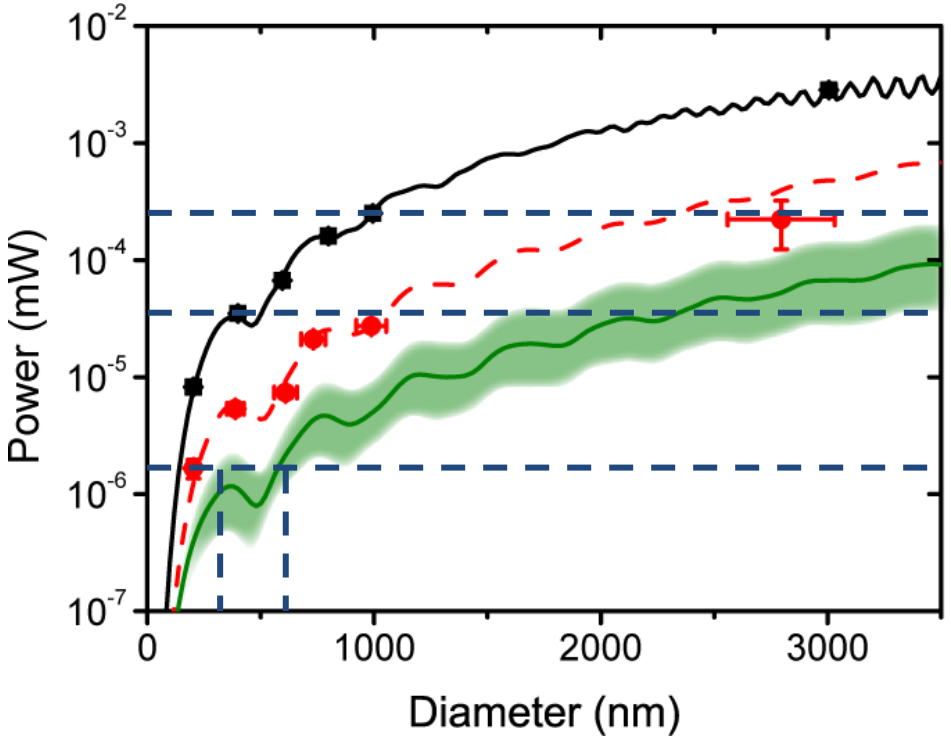


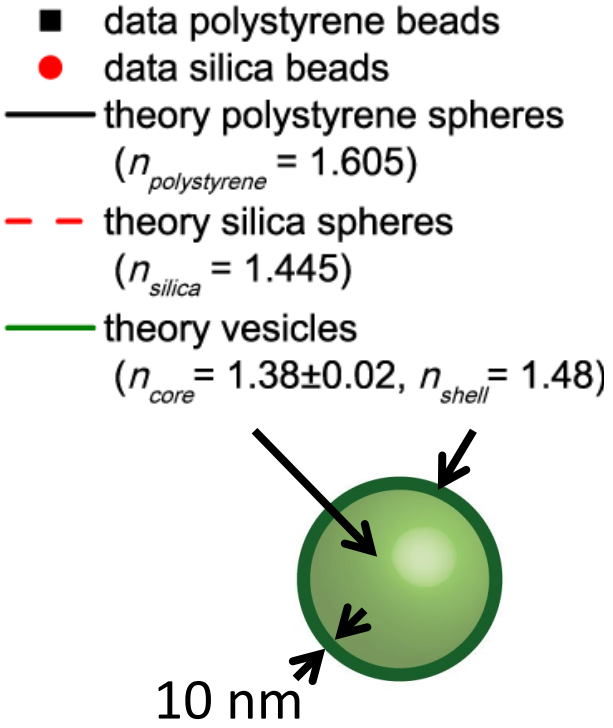
Relate scatter to diameter of beads





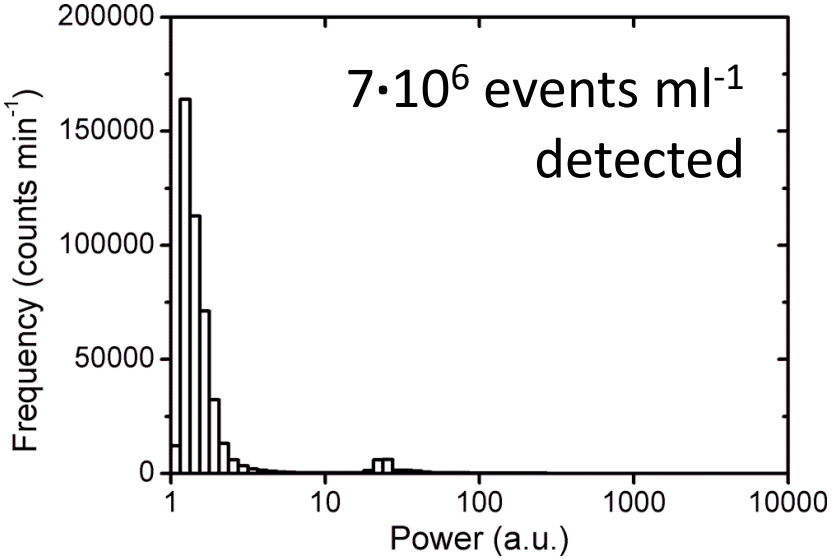
Relate scatter to diameter of EVs



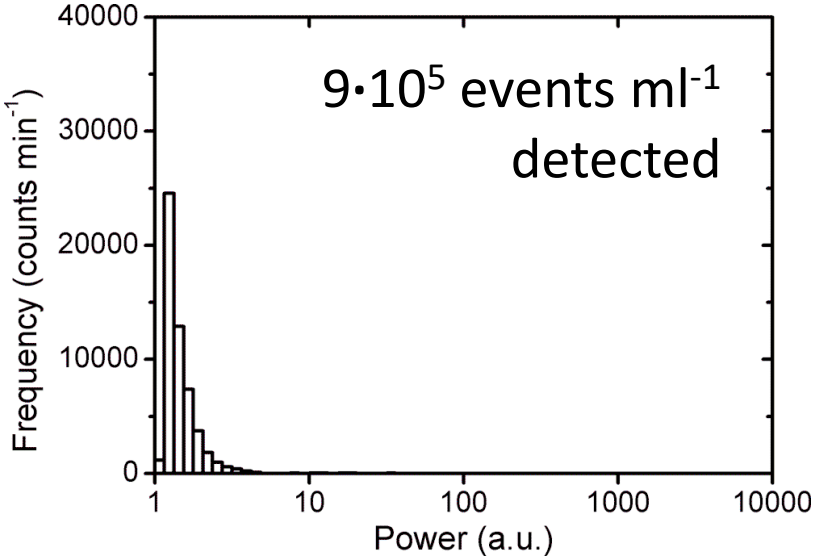


Particles below detection limit are detected

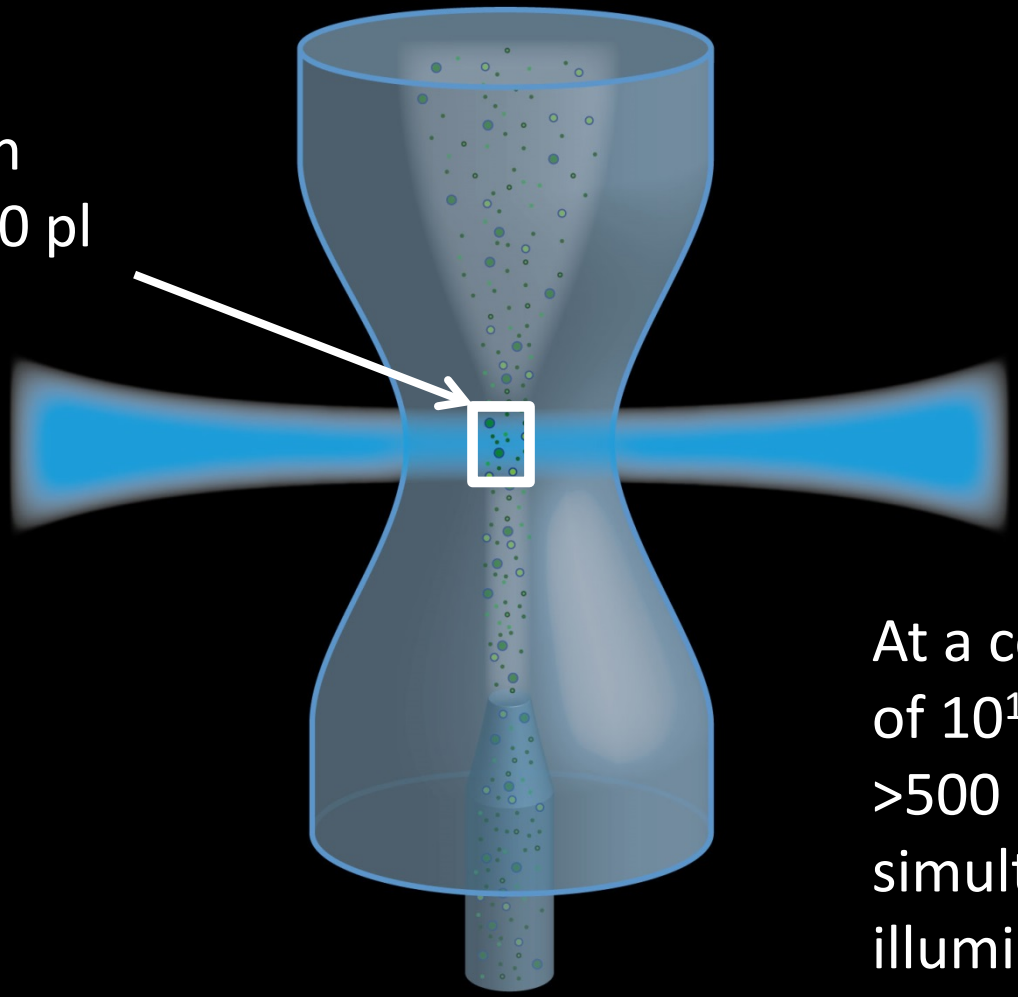
89 nm silica beads
(10^{10} ml^{-1})



220 nm filtered urine
(10^{10} EVs ml^{-1})



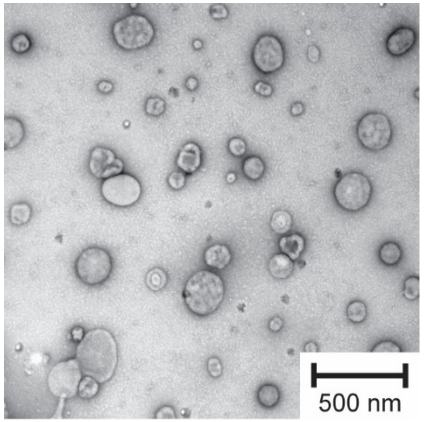
illumination
volume ≈ 50 pl



At a concentration of 10^{10} EVs ml^{-1}, >500 EVs are simultaneously illuminated

Swarm detection

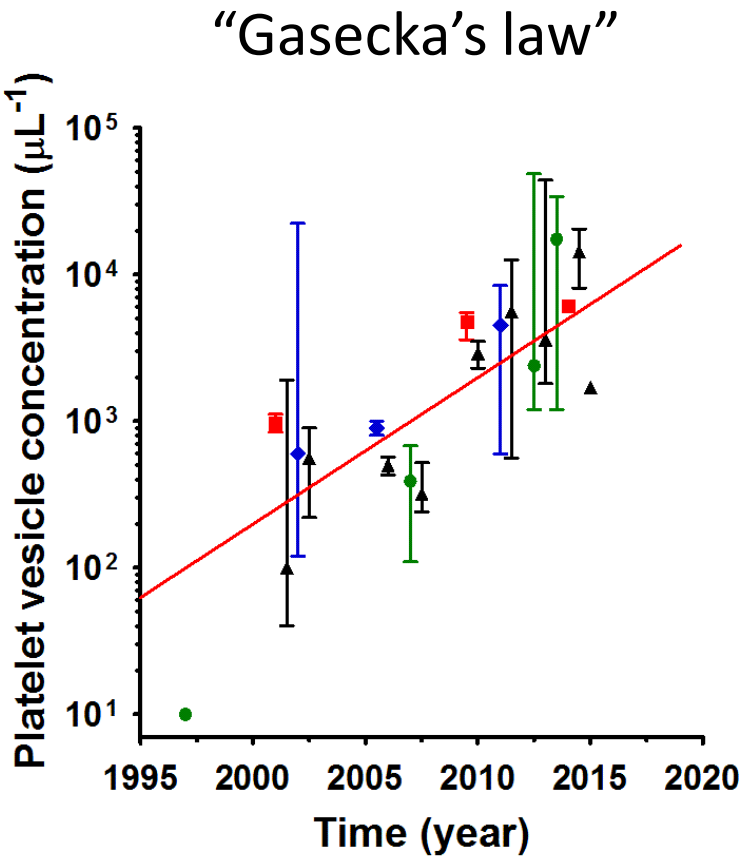
Outline



- Extracellular vesicles (EVs)
- Single EV detection methods
- Single EV flow cytometry
  - *From artefact…*
  - *to clinical act*
    - Standardization by size determination
    - Standardization by size and refractive index determination

Clinical act

- reported concentrations of plasma EV differ >10^6-fold
- clinical data cannot be compared
- standardization required



Standardization is boring (biologists, clinicians)

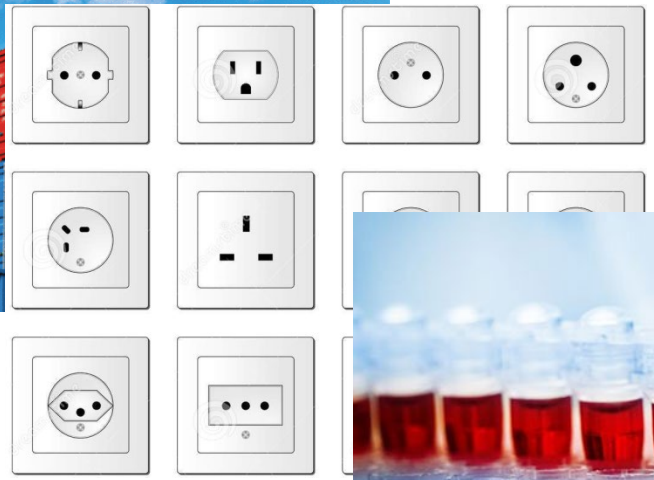
Standardization is exciting (metrologists, physicists)

BESSYII synchrotron

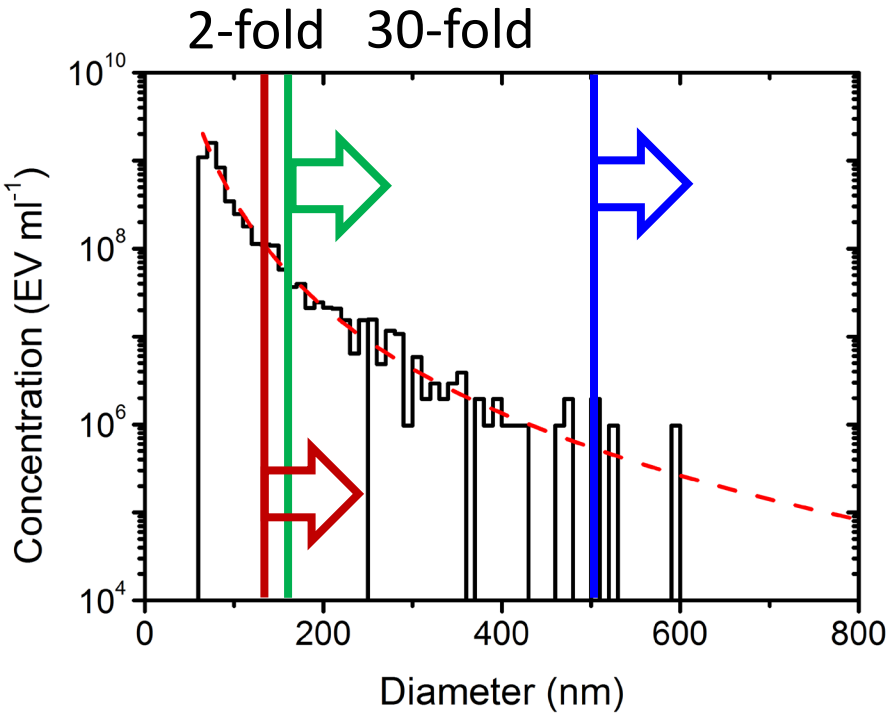
0.31 nm X-rays to size EVs (flow cytometers typically use 488 nm light)

Varga et al. *J.Extracell.Vesicles* 2014

Standardization is important (everybody)



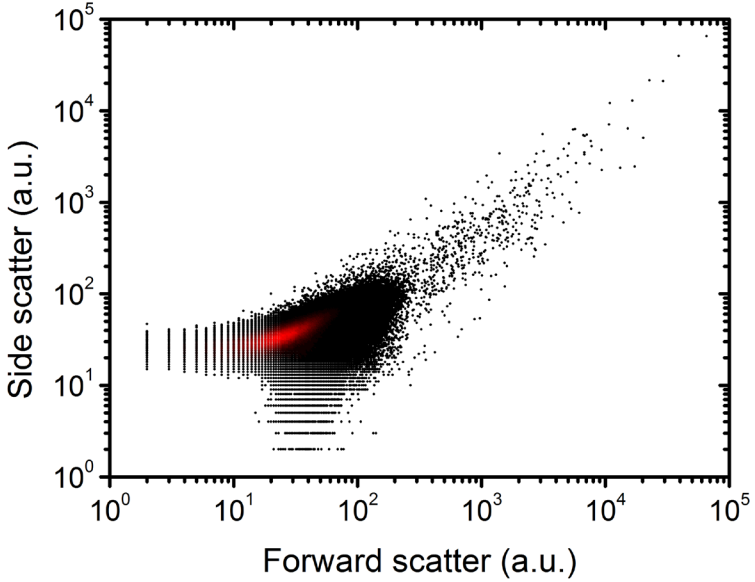
Problem 1: instruments differ in sensitivity



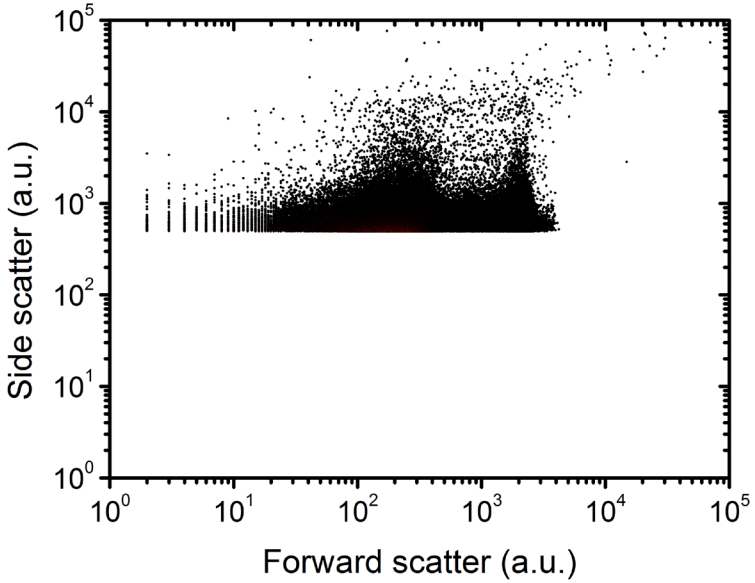
power-law relation*

Problem 2: arbitrary units

same population of erythrocyte EVs



Apogee A50-micro



Becton Dickinson FACSCanto II

Goal

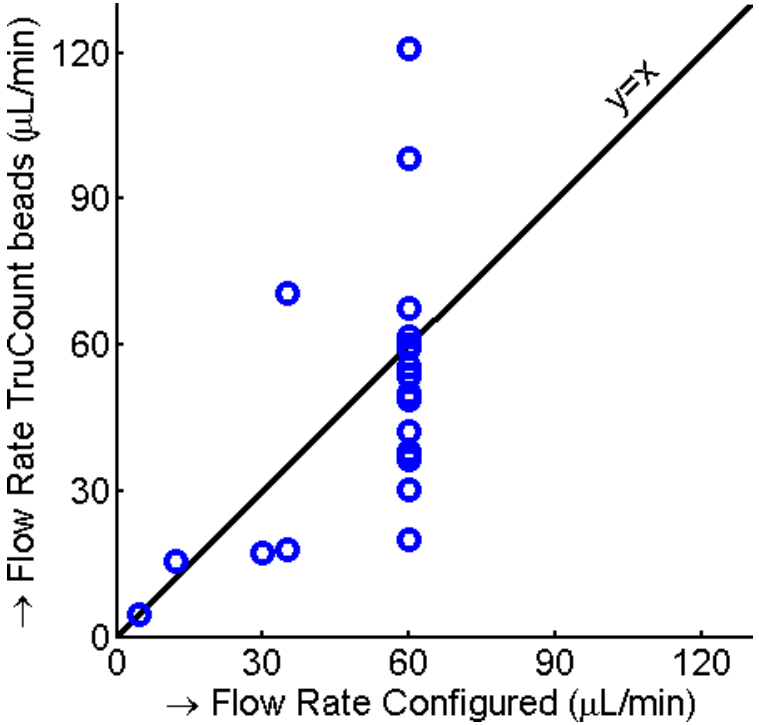
- obtain reproducible measurements of the EV concentration using different flow cytometers

Study comprises 33 sites (64 instruments) worldwide

Approach scatter-based standardization

- determine flow rate
- scatter (a.u.) → diameter (nm)
- measure EV reference sample
- apply EV size gate to software (e.g. FlowJo)
- report concentrations

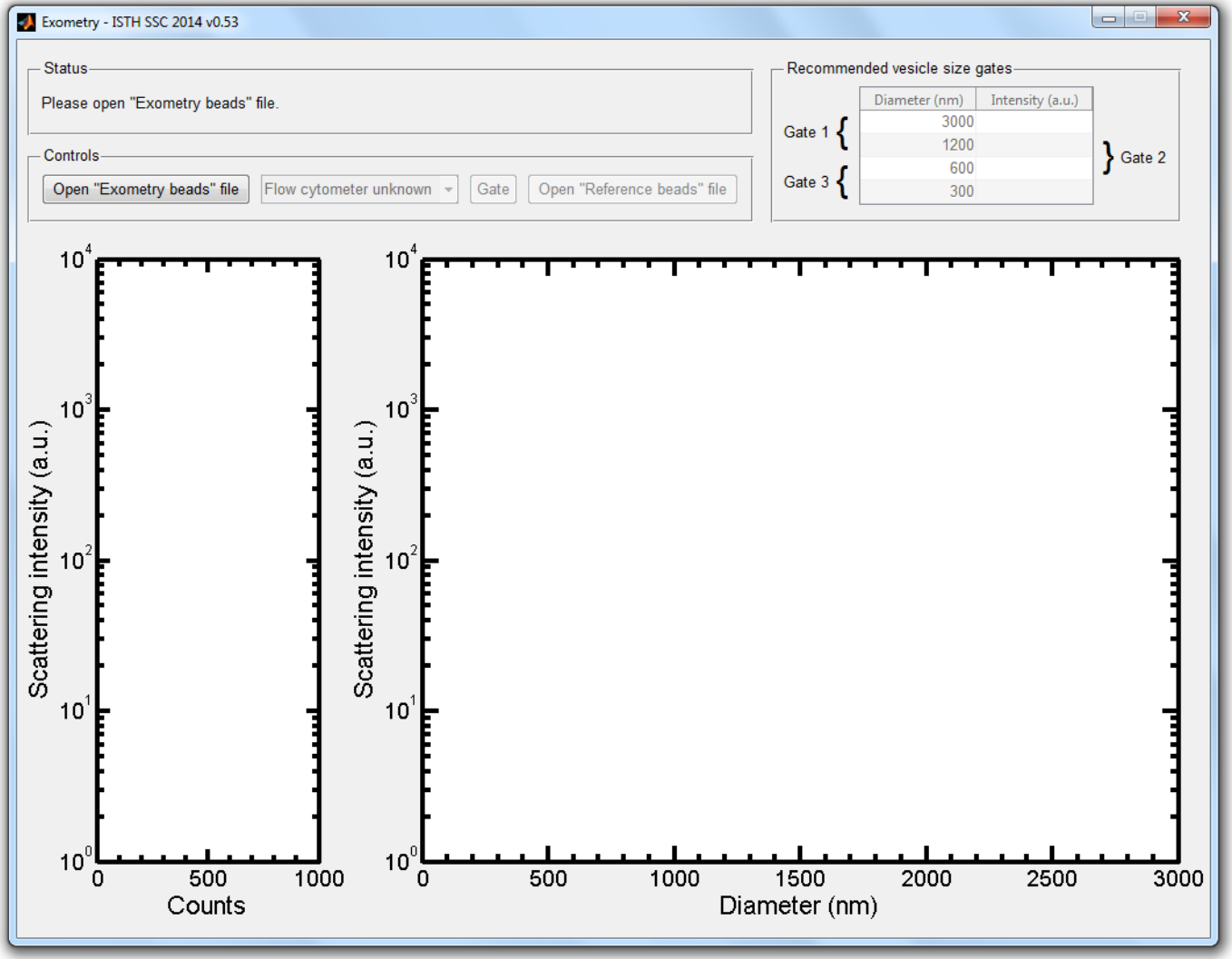
Determine flow rate

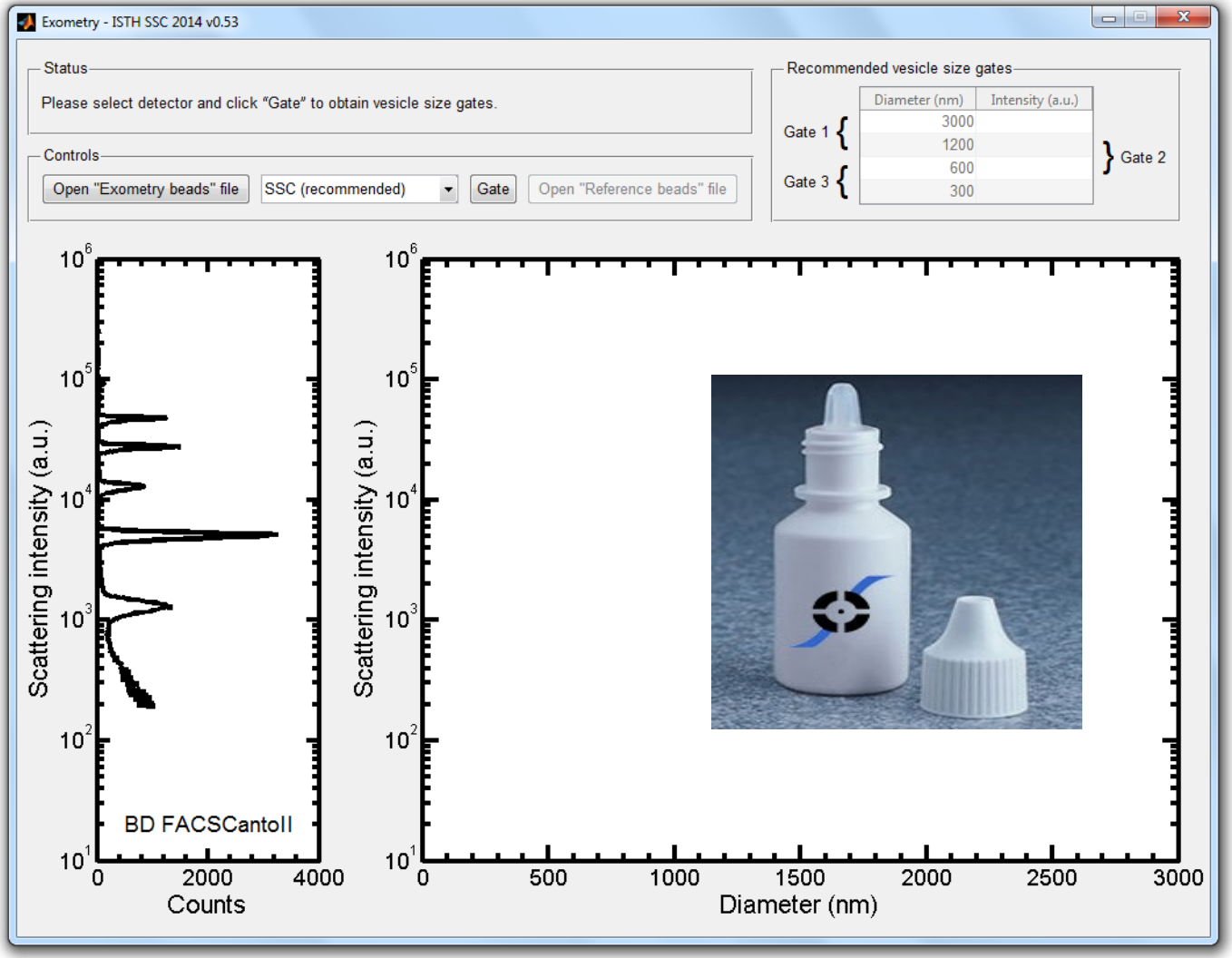


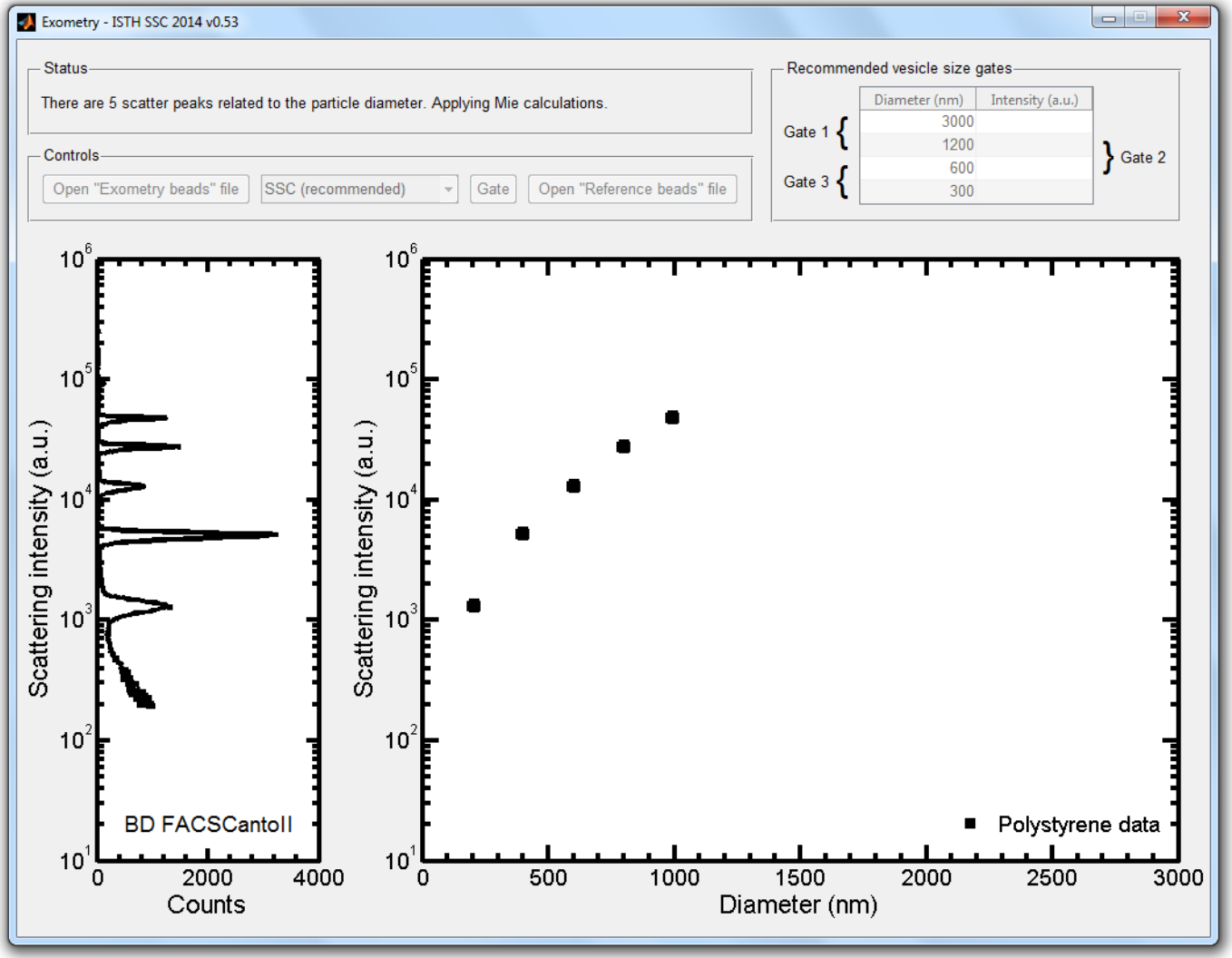
$$\text{concentration} = \frac{\#\ \text{of EV}}{\text{flow rate} \times \text{measurement time}}$$

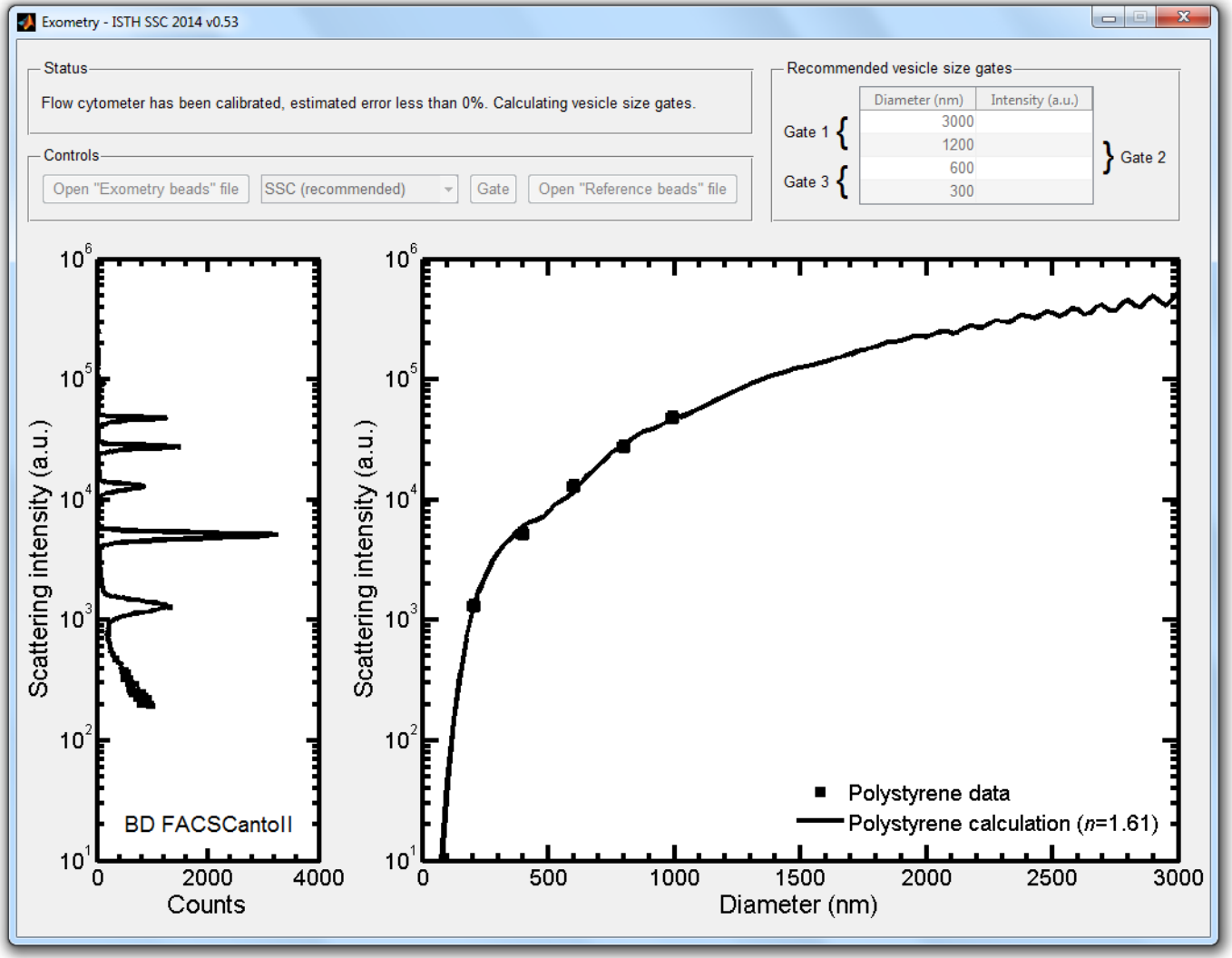
Approach scatter-based standardization

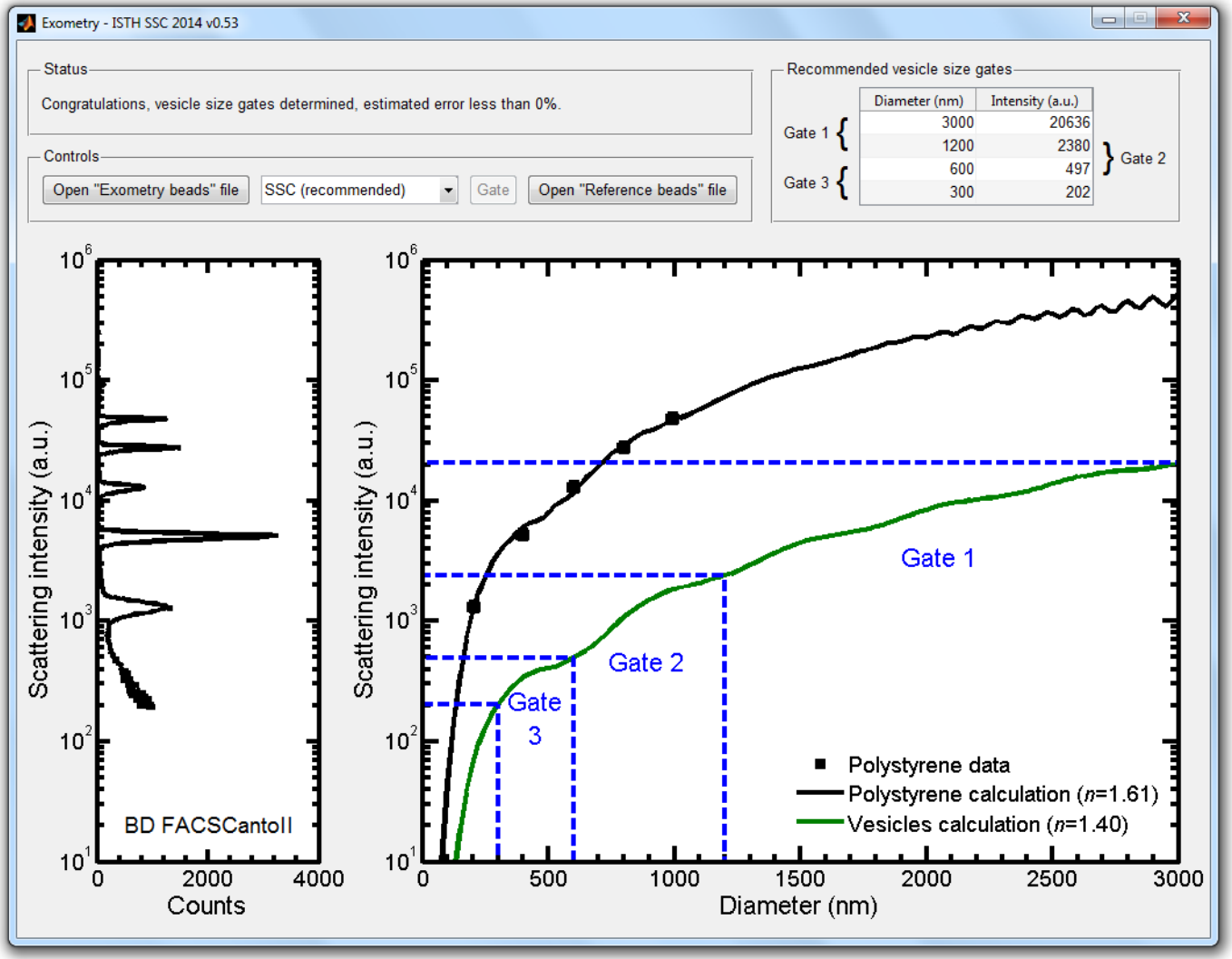
- ✔ determine flow rate
- scatter (a.u.) → diameter (nm)
- measure EV reference sample
- apply EV size gate to software (e.g. FlowJo)
- report concentrations

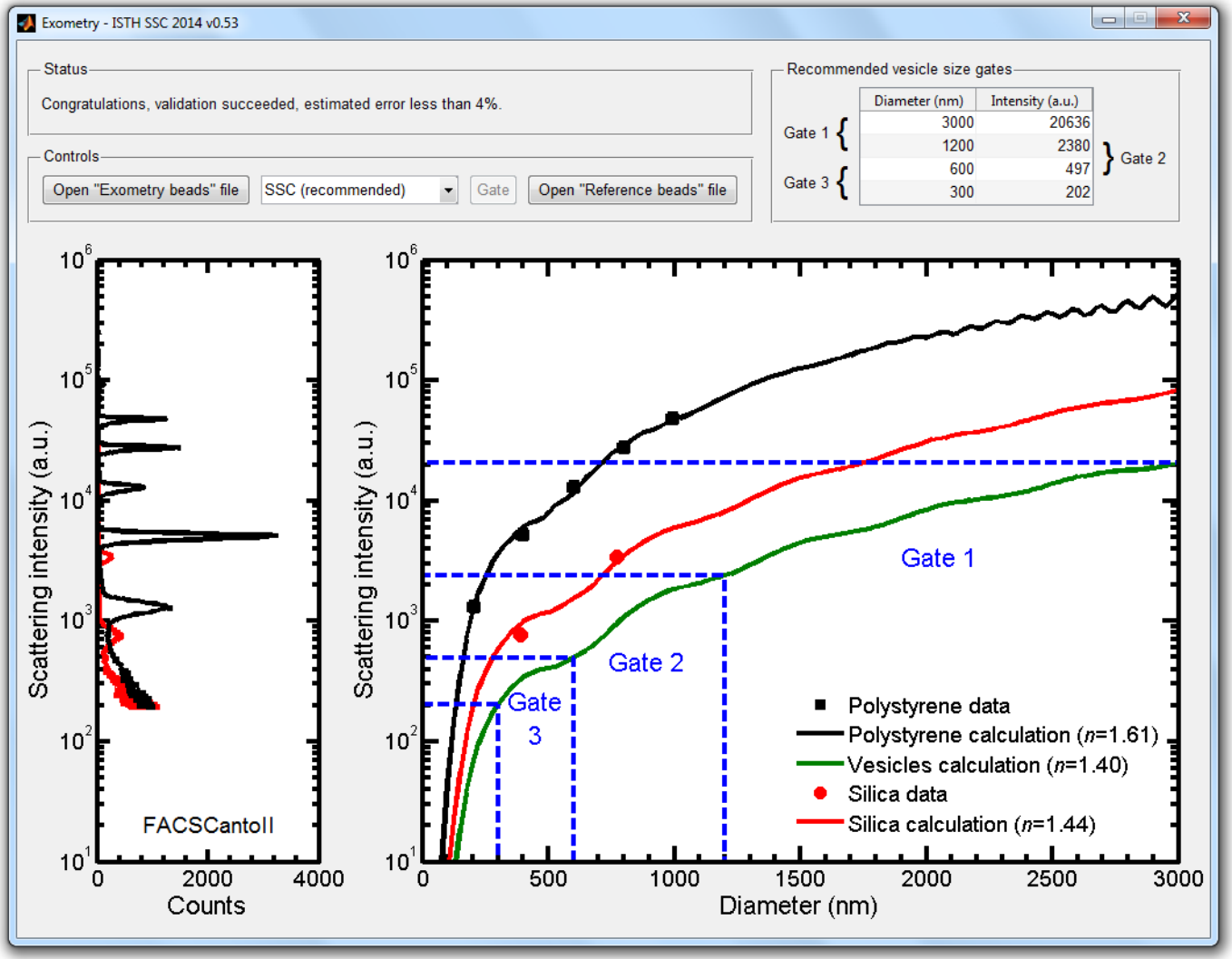

Exometry - ISTH SSC 2014 v0.53
Status
Please open "Exometry beads" file.
Controls
Open "Exometry beads" file
Flow cytometer unknown
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
Counts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

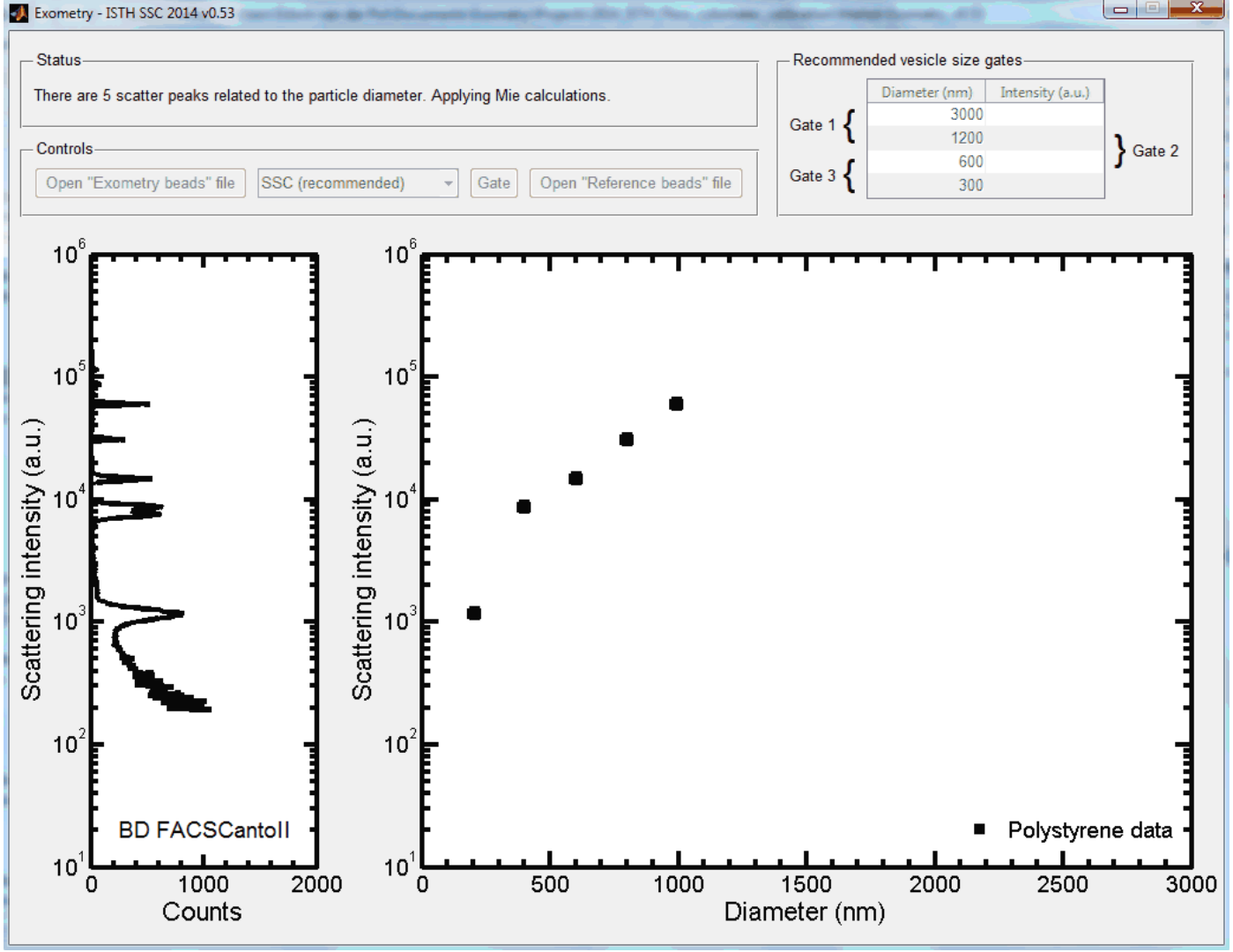

Exometry - ISTH SSC 2014 v0.53
Status
Please select detector and click "Gate" to obtain vesicle size gates.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)


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 2
Gate 3
600
300
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)
Polystyrene data

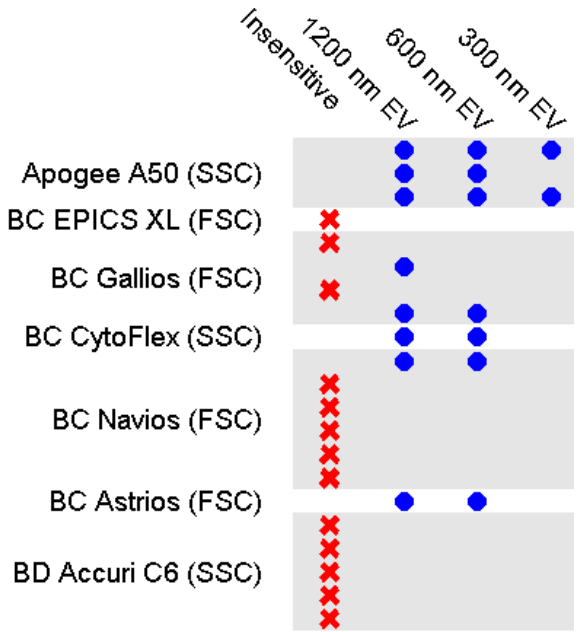

Exometry - ISTH SSC 2014 v0.53
Status
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
1200
Gate 2
Gate 3
600
300
Scattering intensity (a.u.)
Counts
BD FACSCantoII
Diameter (nm)
Polystyrene data
Polystyrene calculation (n=1.61)

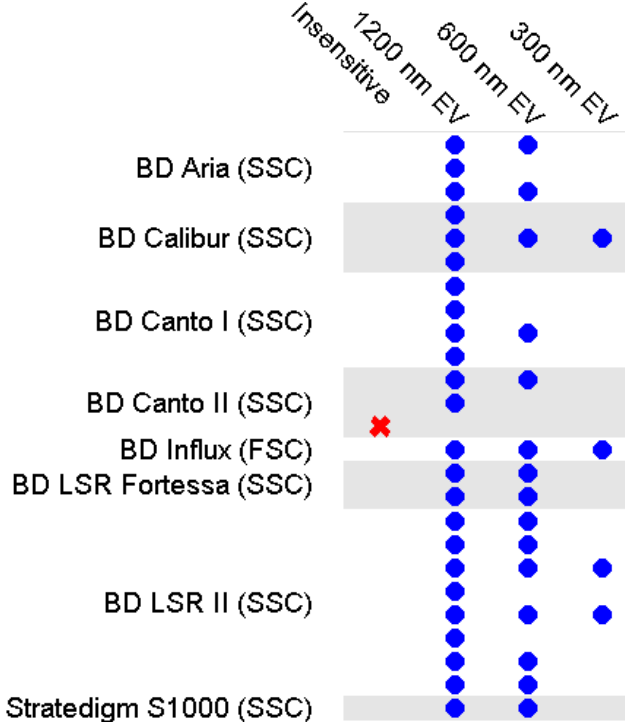

Exometry - ISTH SSC 2014 v0.53
Status
Congratulations, vesicle size gates determined, estimated error less than 0%.
Controls
Open "Exometry beads" file
SSC (recommended)
Gate
Open "Reference beads" file
Recommended vesicle size gates
Diameter (nm)
Intensity (a.u.)
Gate 1
3000
20636
1200
2380
Gate 2
Gate 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.)
FACSCantoII
Counts
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)
Diameter (nm)


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 2
Gate 3
600
300
Scattering intensity (a.u.)
BD FACSCantoII
Counts
Diameter (nm)
Polystyrene data

Sensitivity of 46 flow cytometers in the field





✖ = unable to detect 400 nm fluorescent polystyrene beads

van der Pol et al. submitted to *JTH*

Approach scatter-based standardization

- ✔ determine flow rate
- ✔ scatter (a.u.) → diameter (nm)
- measure EV reference sample
- apply EV size gate to software (e.g. FlowJo)
- report concentrations

Reproducibility of 1200-3000 nm EV

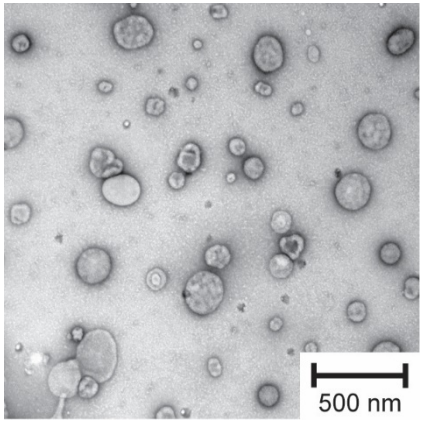
%CV	All
Gate on beads	74%
Gate on EV size with light scatter theory	59%

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

Conclusions standardization by sizing

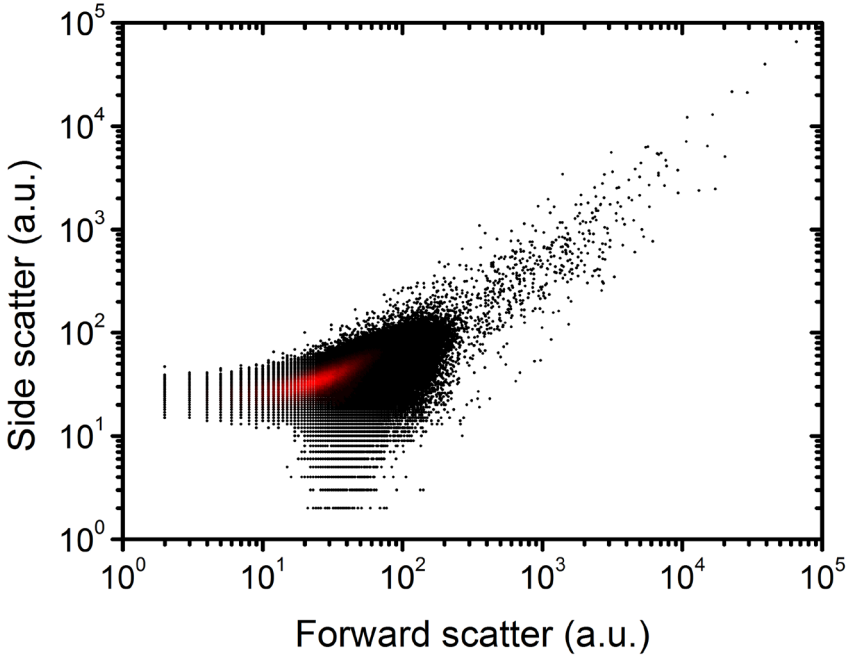
- flow rate calibration is essential
- many flow cytometers used in EV research do not detect EV by scatter-based triggering
- EV size gate by Mie theory (CV=59%) leads to better reproducibility than classical bead gate (CV=74%)

Outline



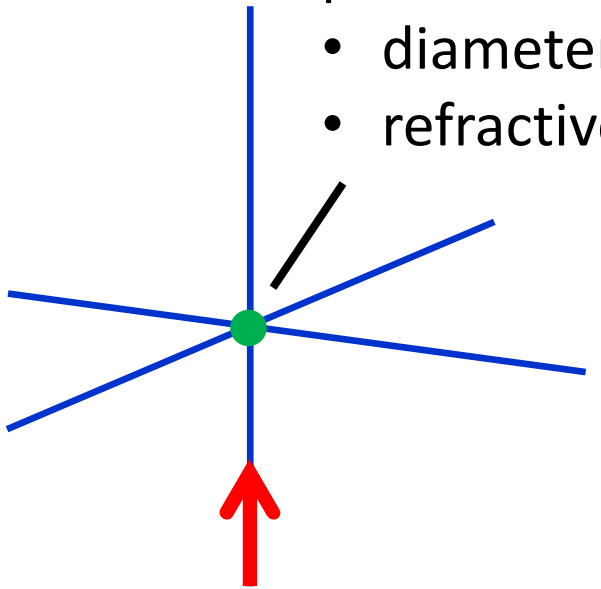
- Extracellular vesicles (EVs)
- Single EV detection methods
- Single EV flow cytometry
  - *From artefact…*
  - *to clinical act*
    - Standardization by size determination
    - Standardization by size and refractive index determination

Goal: from a.u. to size and refractive index

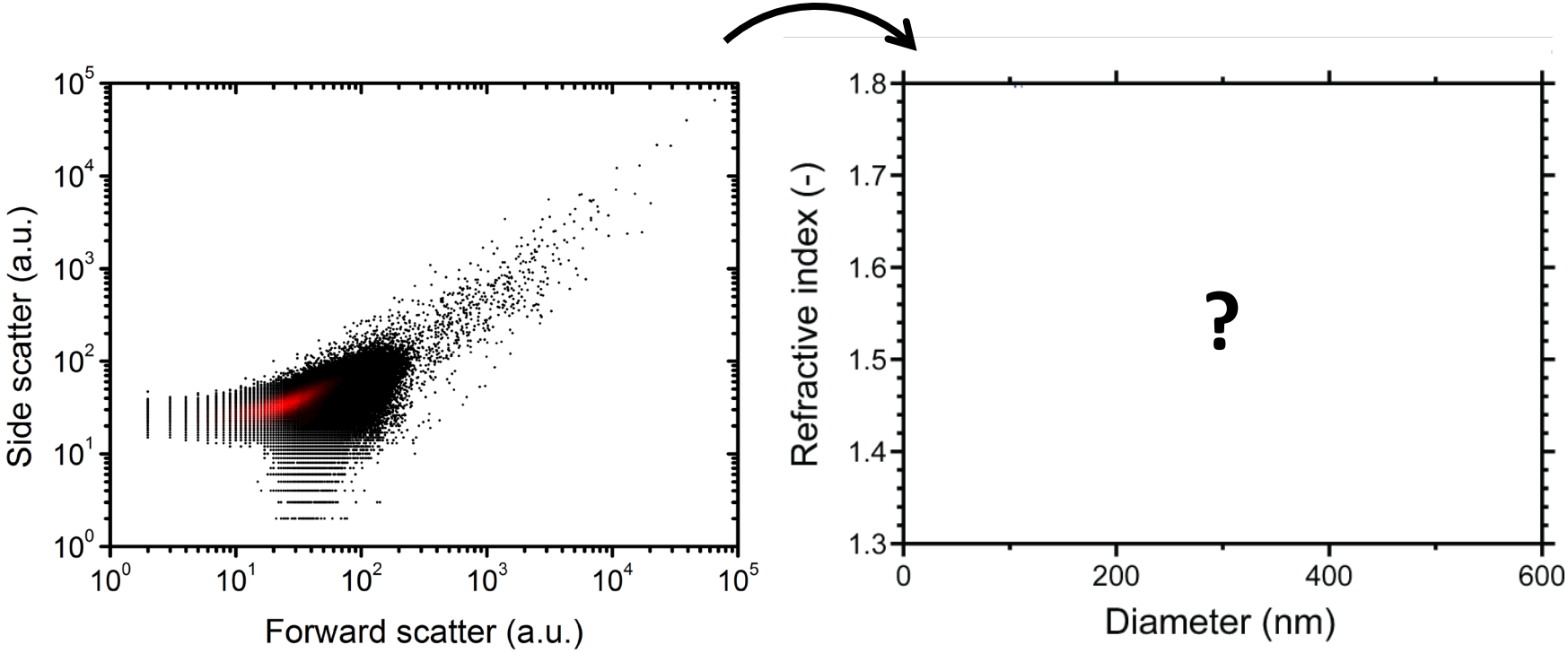


particle

- diameter
- refractive index



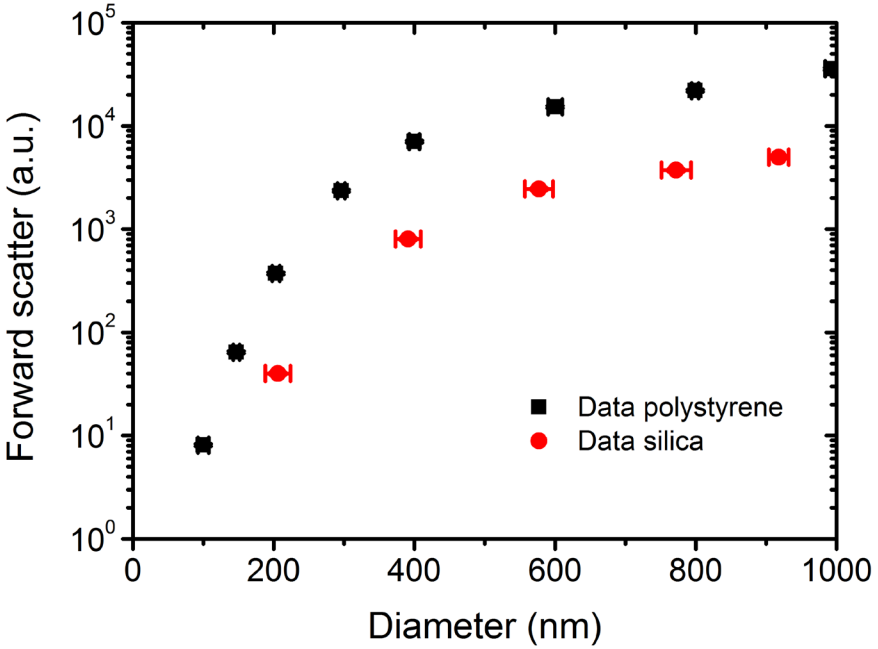
Goal: from a.u. to size and refractive index

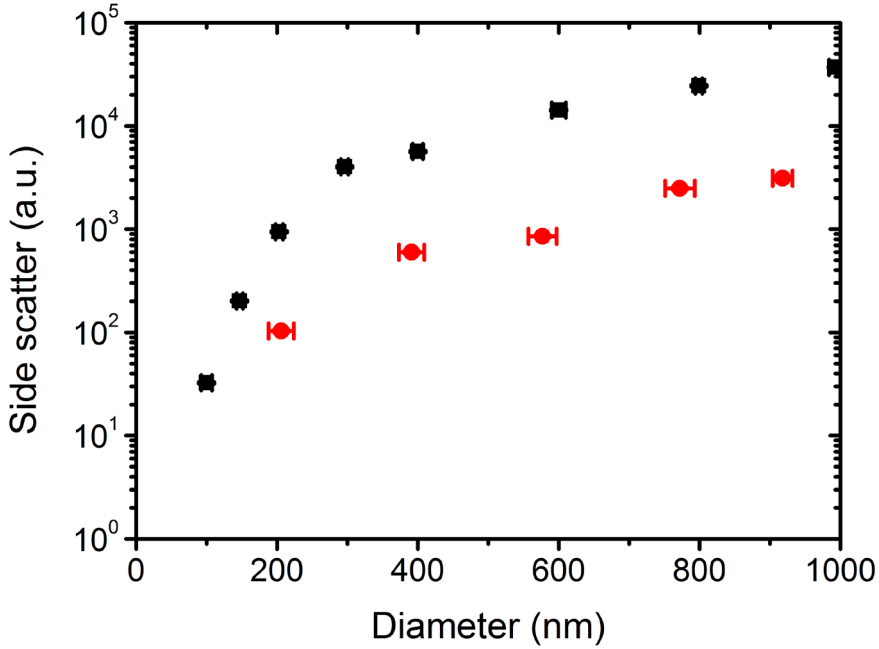


Approach size and refractive index determination

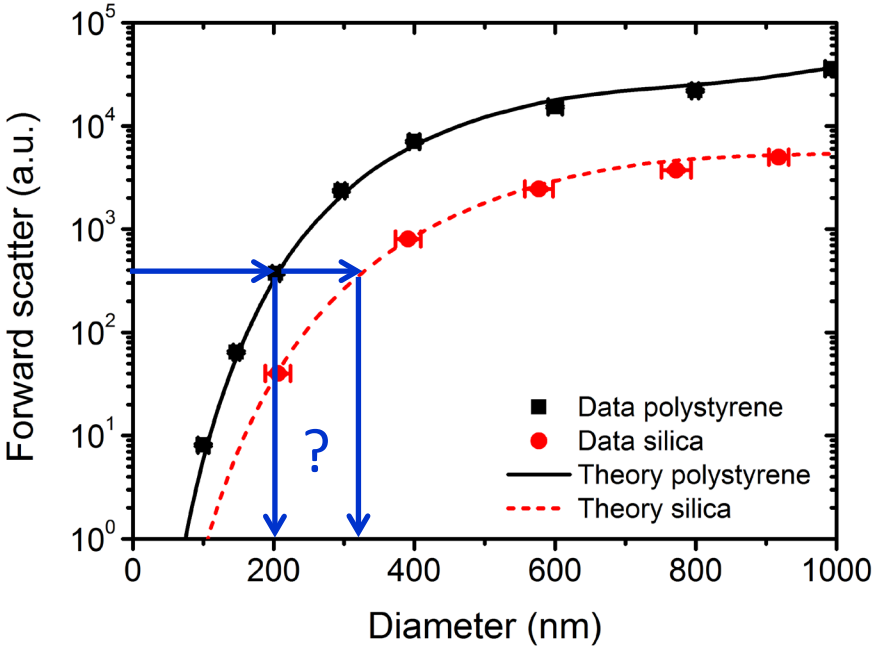
- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

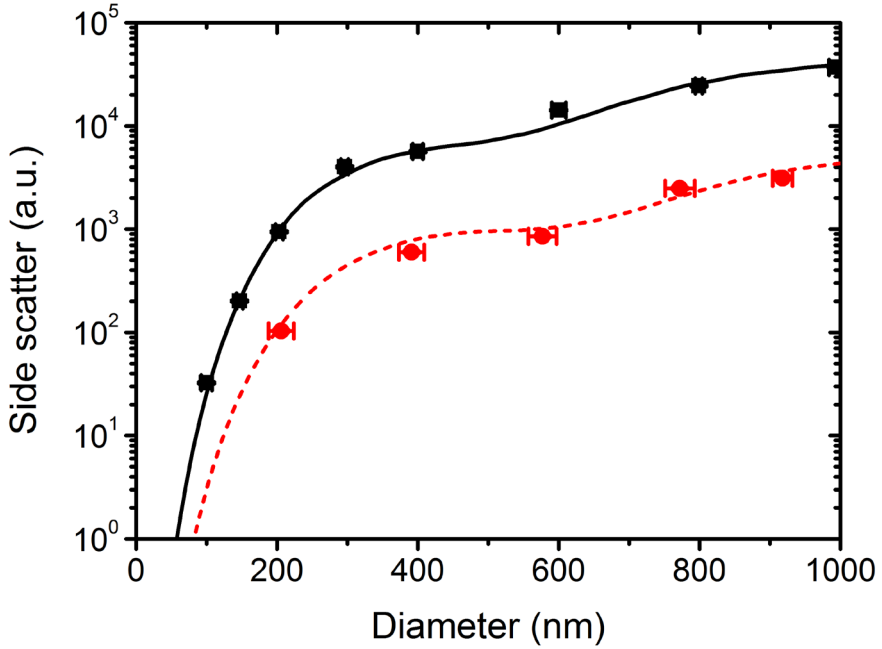
Calibrate forward scatter and side scatter



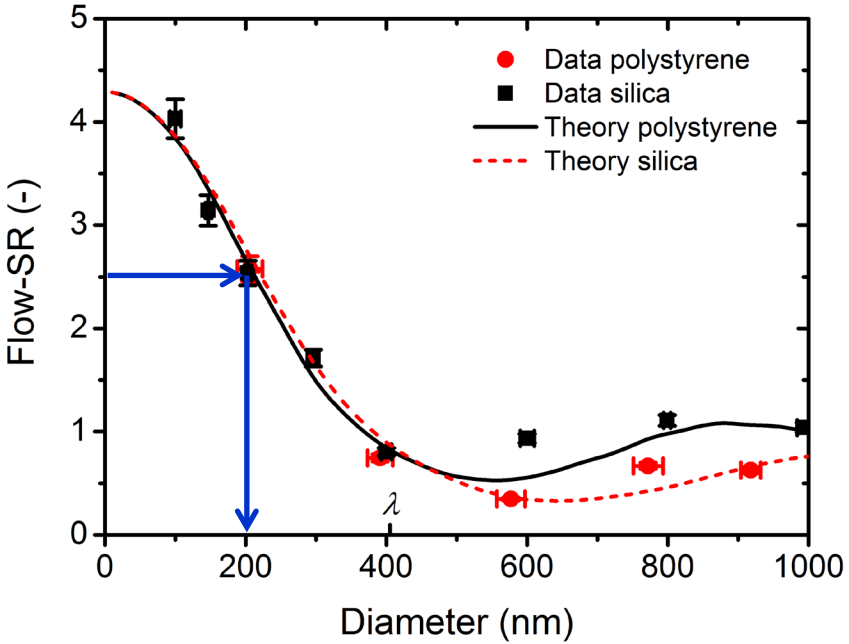


Calibrate forward scatter and side scatter



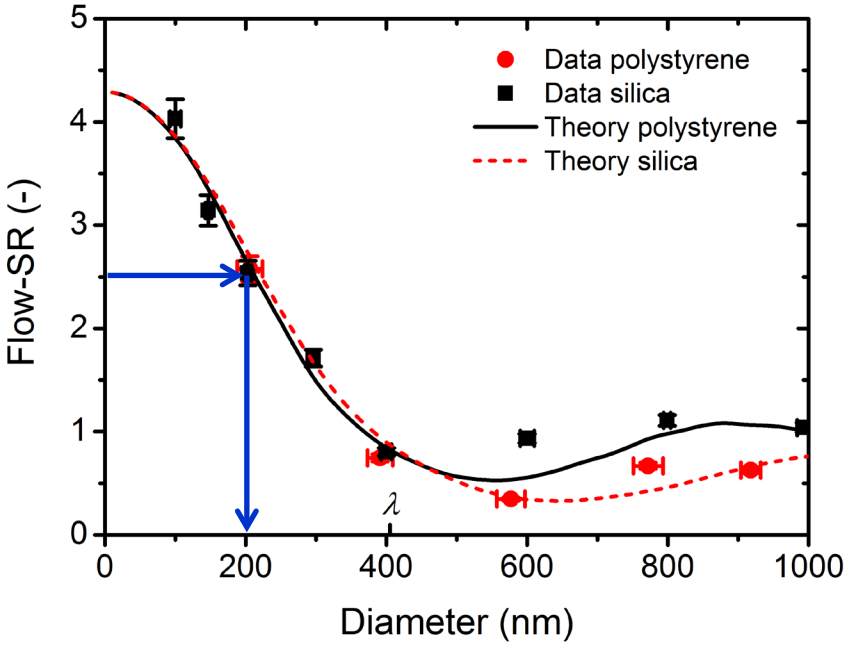


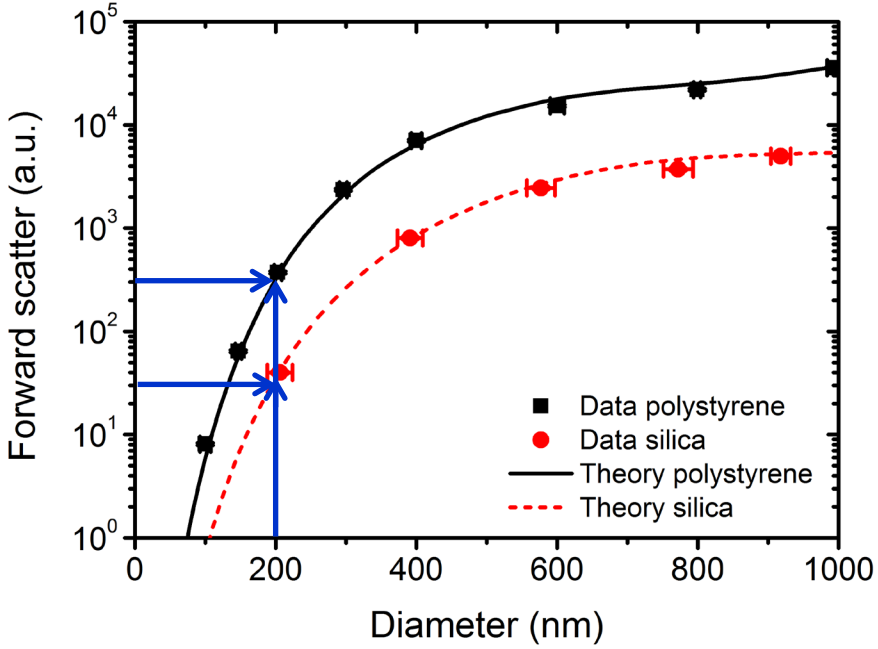
Derive size from Flow-SR



$$\text{Flow-SR} = \frac{\text{side scatter}}{\text{forward scatter}}$$

Derive size from Flow-SR

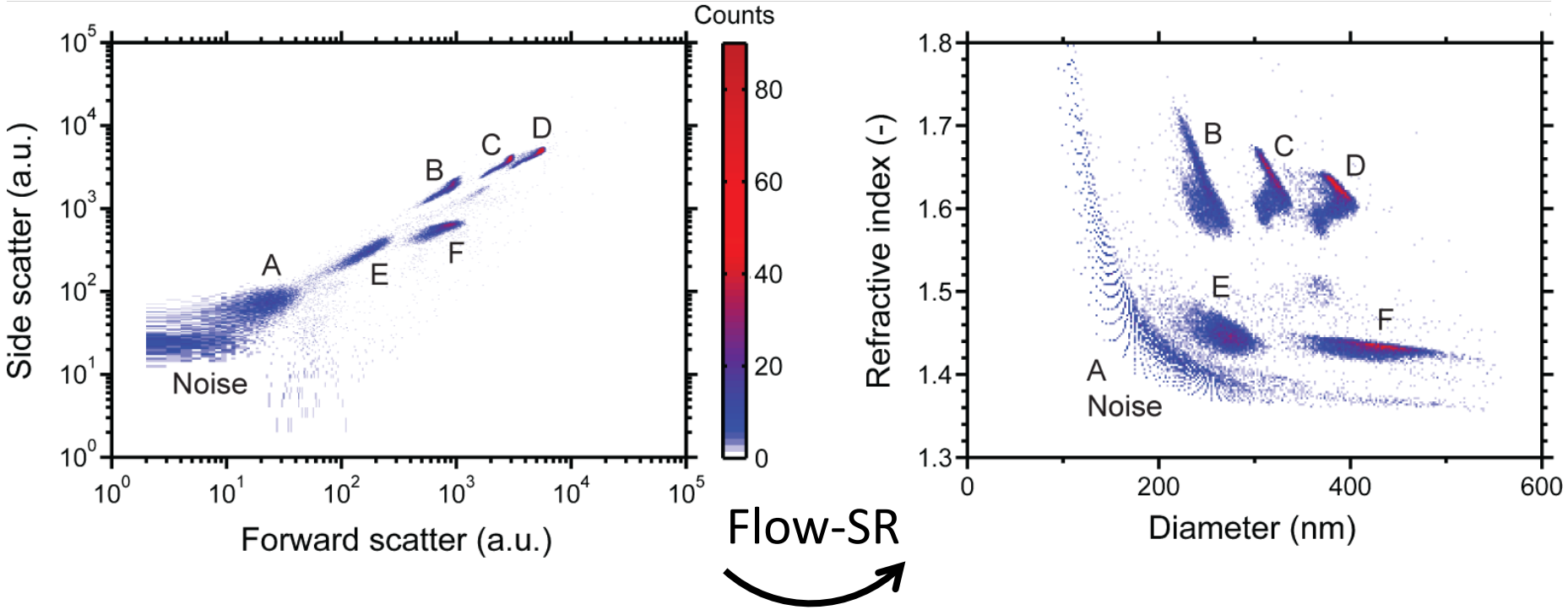




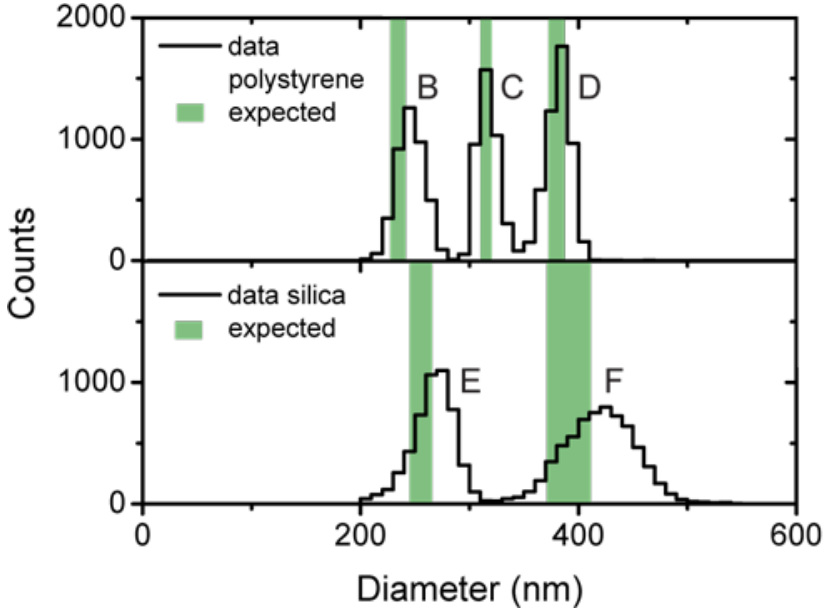
Approach size and refractive index determination

- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

Validate Flow-SR with a beads mixture

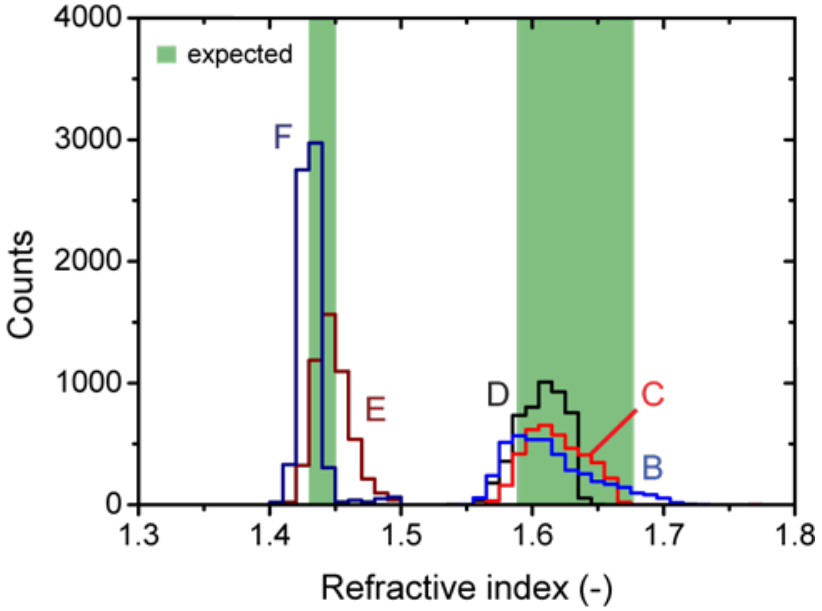


Validate Flow-SR with a beads mixture



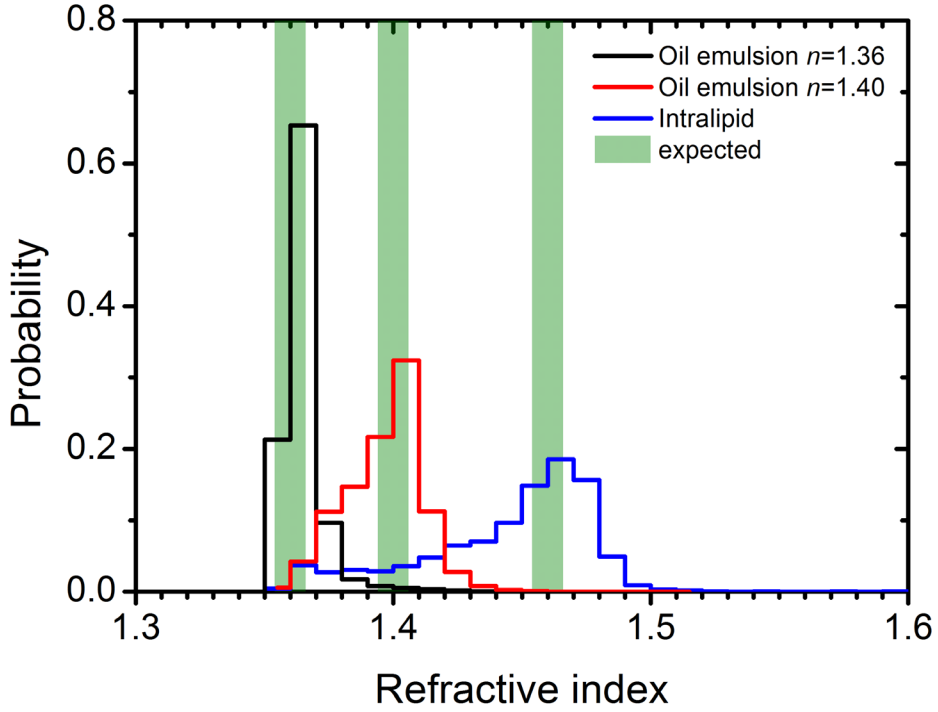
measurement error < 8%

CV < 8%



CV < 2%

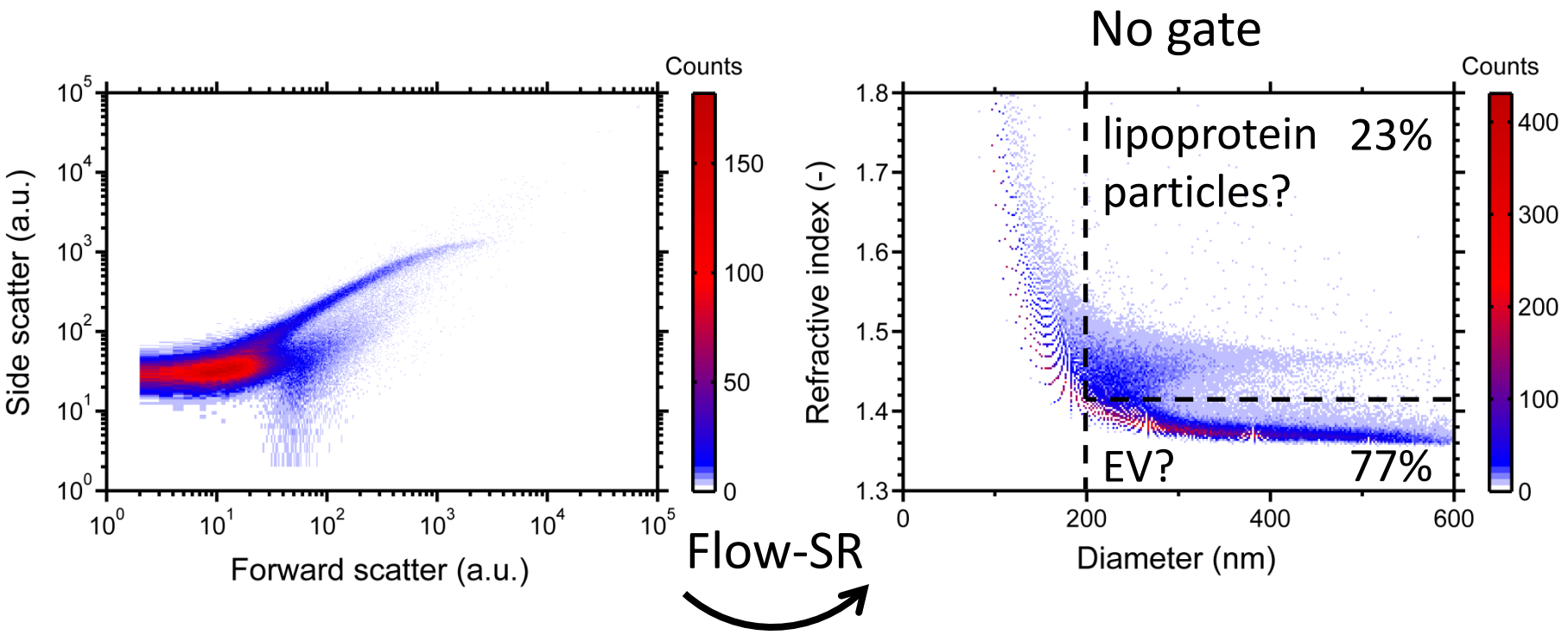
Validate Flow-SR with oil emulsions



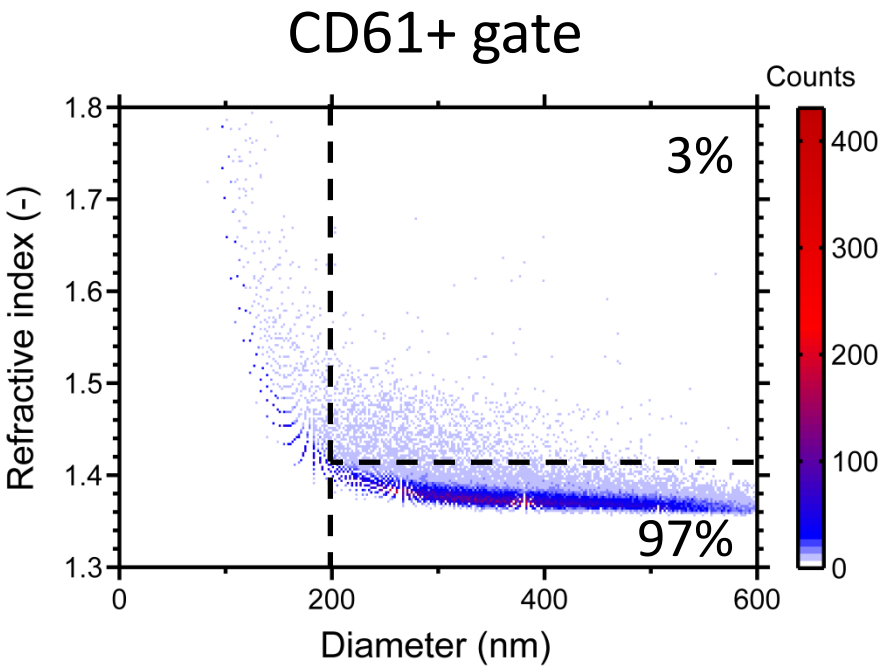
Approach size and refractive index determination

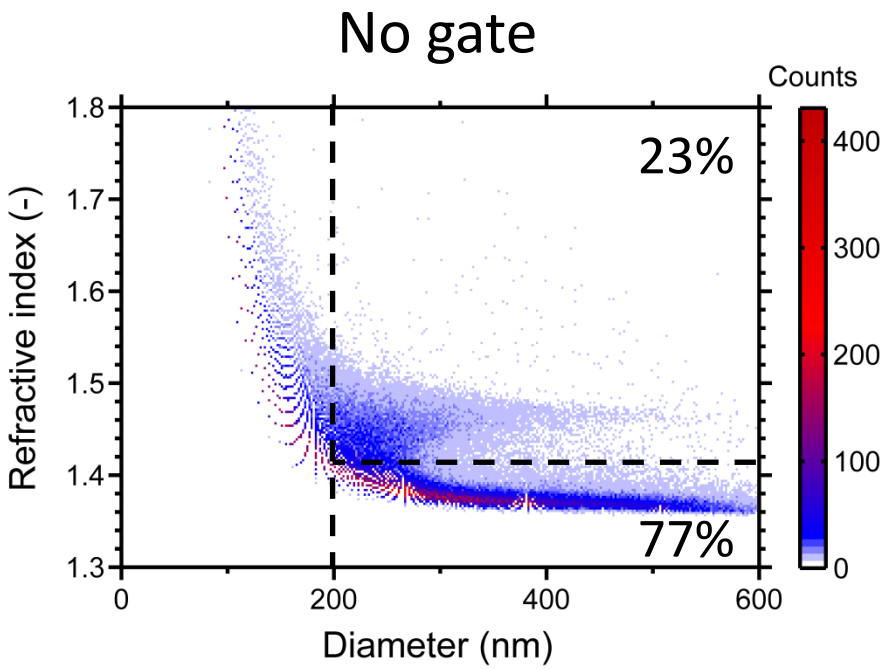
- calibrate instrument (Apogee A50-micro)
  - calibrate FSC and SSC
  - derive size from Flow Scatter Ratio (Flow-SR = SSC/FSC)
  - derive refractive index from size and FSC
- validate Flow-SR
  - beads mixture
  - oil emulsion
- apply Flow-SR
  - EV and lipoprotein particles from blood

Supernatant of outdated platelet concentrate

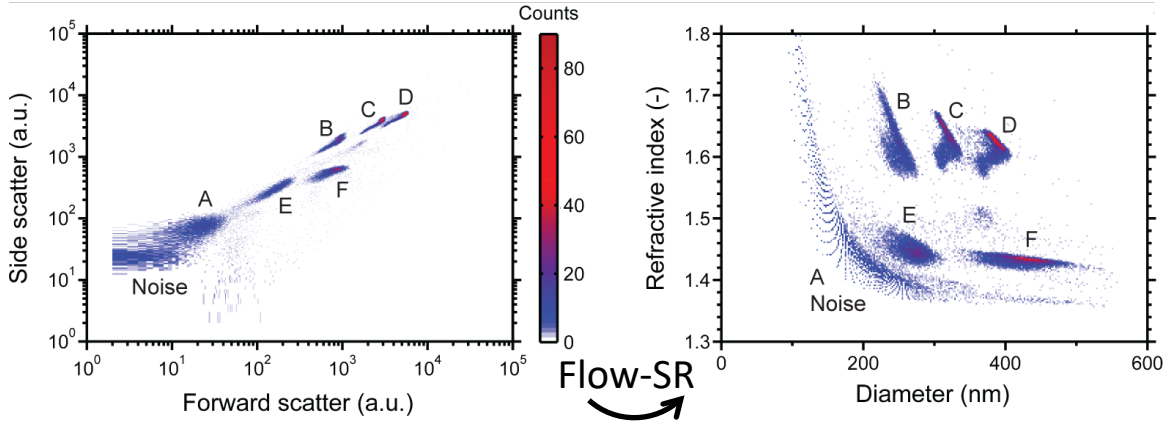


Supernatant of outdated platelet concentrate





Conclusions Flow-SR



- Flow-SR enables size and refractive index determination of nanoparticles by flow cytometry
  - data interpretation and comparison
  - label-free identification

Summary

- body fluids contain extracellular vesicles (<1 µm)
  - biomarkers for disease
  - difficult to detect
- flow cytometry is promising for clinical applications of vesicles, but requires improvements:
  - sensitivity at high throughput (30 nm vesicles at 10^4 s^{-1})
  - standardization
  - vesicle identification

Acknowledgements

- Academic Medical Center
  - Biomedical Engineering & Physics
    - Ton van Leeuwen
    - Frank Coumans
  - Laboratory Experimental Clinical Chemistry
    - Rienk Nieuwland

- More information
  - edwinvanderpol.com
  - exometry.com
  - metves.eu
  - isev.org