Detection of extracellular vesicles by flow cytometry: size does matter

Edwin van der Pol

November 6th, 2018
Hooke: "cells"

Time (years)

images: R. Hooke *Micrographia* 1665
van Leeuwenhoek: "animalcules"

Hooke: "cells"

Brown: "nucleus"
van Leeuwenhoek: "animalcules"

Hooke: "cells"

Brown: "nucleus"

Schwann: "all living matter is cellular"
- van Leeuwenhoek: "animalcules"
- Hooke: "cells"
- Schwann: "all living matter is cellular"
- Brown: "nucleus"
- Ruska: electron microscope

Time (years):
- 1600
- 1700
- 1800
- 1900
- 2000

image: Deutsches Museum
SUMMARY

Fresh plasma freed of intact platelets can be shown to contain minute particulate material (platelet-dust) which can be separated by ultracentrifugation.

Sleeping beauties

van Leeuwenhoek: "animalcules"

Hooke: "cells"

Brown: "nucleus"

Schwann: "all living matter is cellular"

Chargaff & West

Ruska: electron microscope

Wolf: "platelet dust"

1600 1700 1800 1900 2000
Time (years)
Sleeping beauties
Prince?

van Leeuwenhoek: "animalcules"
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Ruska: electron microscope
Wolf: "platelet dust"

1600 1700 1800 1900 2000

Time (years)
Brazil ISAC Flow Cytometry Workshop

- Sleeping beauties
- Prince?
- Exponential fit

van Leeuwenhoek: "animalcules"
Hooke: "cells"
Brown: "nucleus"
Schwann: "all living matter is cellular"
Chargaff & West
Ruska: electron microscope
Wolf: "platelet dust"
Nieuwland et al.

Time (years)

Number of publications on EV
0 500 1000 1500 2000
Outline

1. Extracellular vesicles (EVs)

2. Light scatter

3. Fluorescence

4. Flow rate

image: semrock.com
Extracellular vesicles
Extracellular vesicles

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

van der Pol et al. *Pharmacol Rev* 2012
**EV-based “liquid biopsy”**

<table>
<thead>
<tr>
<th>Hematology parameter</th>
<th>Concentration (vesicles mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet vesicle count</td>
<td>2.3 – 6.2 $\cdot 10^9$</td>
</tr>
<tr>
<td>Erythrocyte vesicle count</td>
<td>7.0 – 8.6 $\cdot 10^{10}$</td>
</tr>
<tr>
<td>Reticulocyte vesicle count</td>
<td>3.9 – 15.6 $\cdot 10^8$</td>
</tr>
<tr>
<td>Leukocyte vesicle count</td>
<td>6.2 – 16.4 $\cdot 10^7$</td>
</tr>
<tr>
<td>Total vesicle count</td>
<td>7.3 – 9.4 $\cdot 10^{10}$</td>
</tr>
</tbody>
</table>

![Image showing rare and all EVs in a liquid biopsy sample](image-url)
EV research using flow cytometry

- Western blotting
- Single particle tracking
- Electron microscopy
- Protein assay
- Direct FCM
- FCM after capture on beads
- Additional characterisation
- Atomic force microscopy
- Additional method

Primary analysis method (%)
Motivation to detect EVs by flow cytometry

- EVs are heterogeneous
  - Flow cytometry can differentiate EV types
- Study all (also rare) EVs
  - Flow cytometry is fast (\(>10,000\) events s\(^{-1}\))
Problem: EV flow cytometry is difficult

- Reported concentrations of plasma EVs differ $>10^6$-fold
- Clinical data cannot be compared

“Gąsecka’s law”

Gasecka et al. *Platelets* 2016
Detection of EVs: size does matter

*van der Pol et al. *J Thromb Haemost* 2014
What is this and what is wrong?

Results

Stats: Merged Data
Mean: 131.2 nm
Mode: 111.9 nm
SD: 41.7 nm
D10: 91.2 nm
D50: 119.8 nm
D90: 178.5 nm
Body fluids contain EVs with clinical information
Flow cytometers can identify EV populations
Size distribution and detection limit determine measured concentration: apply statistics carefully!
Outline

1. Extracellular vesicles (EVs)
2. Light scatter
3. Fluorescence
4. Flow rate
Outline light scatter

- Flow cytometry detection of EVs with
  - one scatter detector
  - two scatter detectors
- Standardization
Goal: use scatter to interpret EV flow cytometry data
Is a “bead size gate” a good idea?
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

![Graph showing scatter versus diameter of beads]

- **Data**
  - Polystyrene beads
  - Silica beads

- **Theory**
  - Polystyrene spheres ($n_{polystyrene} = 1.605$)
  - Silica spheres ($n_{silica} = 1.445$)
Relate scatter to diameter of vesicles

- Side scatter (a.u.)
- Diameter (nm)

- Data polystyrene beads
- Data silica beads
- Theory polystyrene spheres ($n_{polystyrene} = 1.605$)
- Theory silica spheres ($n_{silica} = 1.445$)
- Theory vesicles ($n_{core} = 1.38 \pm 0.02$, $n_{shell} = 1.48$)

10 nm
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 $\geq 10^{10}$ EVs ml$^{-1}$

![Graphs showing detected concentration](image)
beam volume ≈ 54 pl

At a concentration of $10^{10}$ vesicles ml$^{-1}$, >800 vesicles are simultaneously present in the beam.
Invisible vesicles swarm within the iceberg
Summary EV detection with 1 scatter detector

- 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
Outline light scatter

- Flow cytometry detection of EVs with
  - one scatter detector
  - **two scatter detectors**
- Standardization
Goal

- Obtain physical properties of particles from flow cytometry scatter signals

- particle
  - diameter
  - refractive index

laser
Approach

- 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

Flow-SR = \frac{\text{side scatter}}{\text{forward scatter}}
Derive size from Flow-SR

Flow-SR = \frac{\text{side scatter}}{\text{forward scatter}}
Derive refractive index from size and FSC
Approach

- 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

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

Flow-SR

No gate

lipoprotein particles? 23%

EV? 77%
Supernatant of outdated platelet concentrate

CD61+ gate

No gate

- Median refractive index platelet EVs >200 nm = 1.37
Summary EV detection with 2 scatter detectors

- Flow-SR enables size and refractive index determination of nanoparticles by flow cytometry
  - data interpretation and comparison
  - differentiate EVs and lipoprotein particles

van der Pol Nanomedicine 2018
Outline light scatter

- Flow cytometry detection of EVs with
  - one scatter detector
  - two scatter detectors
- Standardization
Standardization is boring (biologists, clinicians)
Standardisation is exciting (metrologists, physicists)

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

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.) $\rightarrow$ 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.com
**EV reference sample**

- Platelet (CD61-PE+) EVs from cell-free platelet concentrates
- Trigger on most sensitive scatter channel
- Exclude EVs similar to isotype
Please select detector and click "Gate" to obtain vesicle size gates.

Recommended vesicle size gates:

<table>
<thead>
<tr>
<th>Gate</th>
<th>Diameter (nm)</th>
<th>Intensity (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gate 1</td>
<td>3000</td>
<td>1200</td>
</tr>
<tr>
<td>Gate 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gate 3</td>
<td>600</td>
<td>300</td>
</tr>
</tbody>
</table>

Scattering intensity (a.u.) vs. Counts

Scattering intensity (a.u.) vs. Diameter (nm)

BD FACSCantoll
There are 5 scatter peaks related to the particle diameter. Applying Mie calculations.

**Recommended vesicle size gates**

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</tr>
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<td>300</td>
</tr>
</tbody>
</table>
Flow cytometer has been calibrated, estimated error less than 0%. Calculating vesicle size gates.

Recommended vesicle size gates:

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<td>Gate 3</td>
<td>600</td>
<td>300</td>
</tr>
</tbody>
</table>
Congratulations, vesicle size gates determined, estimated error less than 0%.

<table>
<thead>
<tr>
<th>Gate 1</th>
<th>Gate 2</th>
<th>Gate 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (nm)</td>
<td>Intensity (a.u.)</td>
<td>Diameter (nm)</td>
</tr>
<tr>
<td>3000</td>
<td>20636</td>
<td>1200</td>
</tr>
<tr>
<td>600</td>
<td>497</td>
<td>300</td>
</tr>
</tbody>
</table>

BD FACSCantoll

- Polystyrene data
- Polystyrene calculation ($n=1.61$)
- Vesicles calculation ($n=1.40$)
Congratulations, validation succeeded, estimated error less than 4%.

Recommended vesicle size gates:

<table>
<thead>
<tr>
<th>Gate 1</th>
<th>Diameter (nm)</th>
<th>Intensity (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3000</td>
<td>20536</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>2380</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>497</td>
</tr>
<tr>
<td>Gate 2</td>
<td>300</td>
<td>202</td>
</tr>
</tbody>
</table>

Graphs showing scattering intensity against diameter with gates for Polystyrene, Vesicles, and Silica data.
Exclusion of flow cytometers (FCM)

- Enrolled: 33 sites, 64 FCMs
  - Reason for discontinuation:
    - Shipment issue: 2
    - Multiple instruments: 15
    - Availability of instrument/technician: 1

- Step 1: beads: 46 FCMs
  - Reason for discontinuation:
    - No 400-nm fluorescent bead: 14
    - Multiple instruments: 3
    - Availability of instrument/technician: 3
    - No data submitted: 3

- Step 2: samples: 23 FCMs
Sensitivity of 46 flow cytometers in the field

- □ = unable to detect 400 nm polystyrene beads

<table>
<thead>
<tr>
<th>Flow Cytometer</th>
<th>1,200-nm EVs</th>
<th>600-nm EVs</th>
<th>300-nm EVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apogee A50 (SSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>BC EPICS XL (FSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>BC Gallios (FSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>BC Cytoflex (SSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>BC Navios (FSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>BC Astrios (FSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>BD Accuri C6 (SSC)</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

- □ = able to detect 400 nm polystyrene beads

- □□ = able to detect 400 nm polystyrene beads with high efficiency

- □□□ = able to detect 400 nm polystyrene beads with very high efficiency
400 nm polystyrene beads scatter more than 1,000 nm EV

![Graph showing scattering cross section vs. diameter for EV and polystyrene beads at 488 nm wavelength.](image)
Sensitivity of 46 flow cytometers in the field

- Apogee A50 (SSC)
- BC EPICS XL (FSC)
- BC Gallios (FSC)
- BC CytoFlex (SSC)
- BC Navios (FSC)
- BC Astrios (FSC)
- BD Accuri C6 (SSC)
- BD Aria (SSC)
- BD Calibur (SSC)
- BD Canto I (SSC)
- BD Canto II (SSC)
- BD Influx (FSC)
- BD LSR Fortessa (SSC)
- BD LSR II (SSC)
- Stratedigm S1000 (SSC)

※ = unable to detect EV < 1000 nm
Results

<table>
<thead>
<tr>
<th>Method</th>
<th>CV* concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No scatter gate</td>
<td>144</td>
</tr>
<tr>
<td>Traditional bead size gate</td>
<td>139</td>
</tr>
<tr>
<td>1,200-3,000 nm EV size gate</td>
<td>81</td>
</tr>
<tr>
<td>600-1,200 nm EV size gate</td>
<td>82</td>
</tr>
<tr>
<td>300-600 nm EV size gate</td>
<td>115</td>
</tr>
</tbody>
</table>

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

1. Extracellular vesicles (EVs)
2. Light scatter
3. Fluorescence
4. Flow rate
Fluorescence

- Please ask Dr. Zosia Maciorowski
- Label EVs
  - Antibodies
  - Membrane dyes?
How specific do generic dyes label EVs?

- blood contains ~1,000 lipoprotein particles (LPs) for each EV*

*Dragovic et al. Nanomedicine 2011
Outine

1. Extracellular vesicles (EVs)
2. Light scatter
3. Fluorescence
4. Flow rate
Determine flow rate

\[
\text{concentration} = \frac{\# \text{ of EV}}{\text{flow rate} \times \text{measurement time}}
\]
Conclusions

- Detection of extracellular vesicles by flow cytometry: size does matter!
- Consider each flow cytometry aspect
  - Scatter
  - Fluorescence
  - Flow rate
Acknowledgements

- Vesicle Observation Center
  Amsterdam University Medical Centers
  - Ton van Leeuwen
  - Rienk Nieuwland
  - Frank Coumans
  - Leonie de Rond

- Software and beads by exometry.com

- More info: edwinvanderpol.com