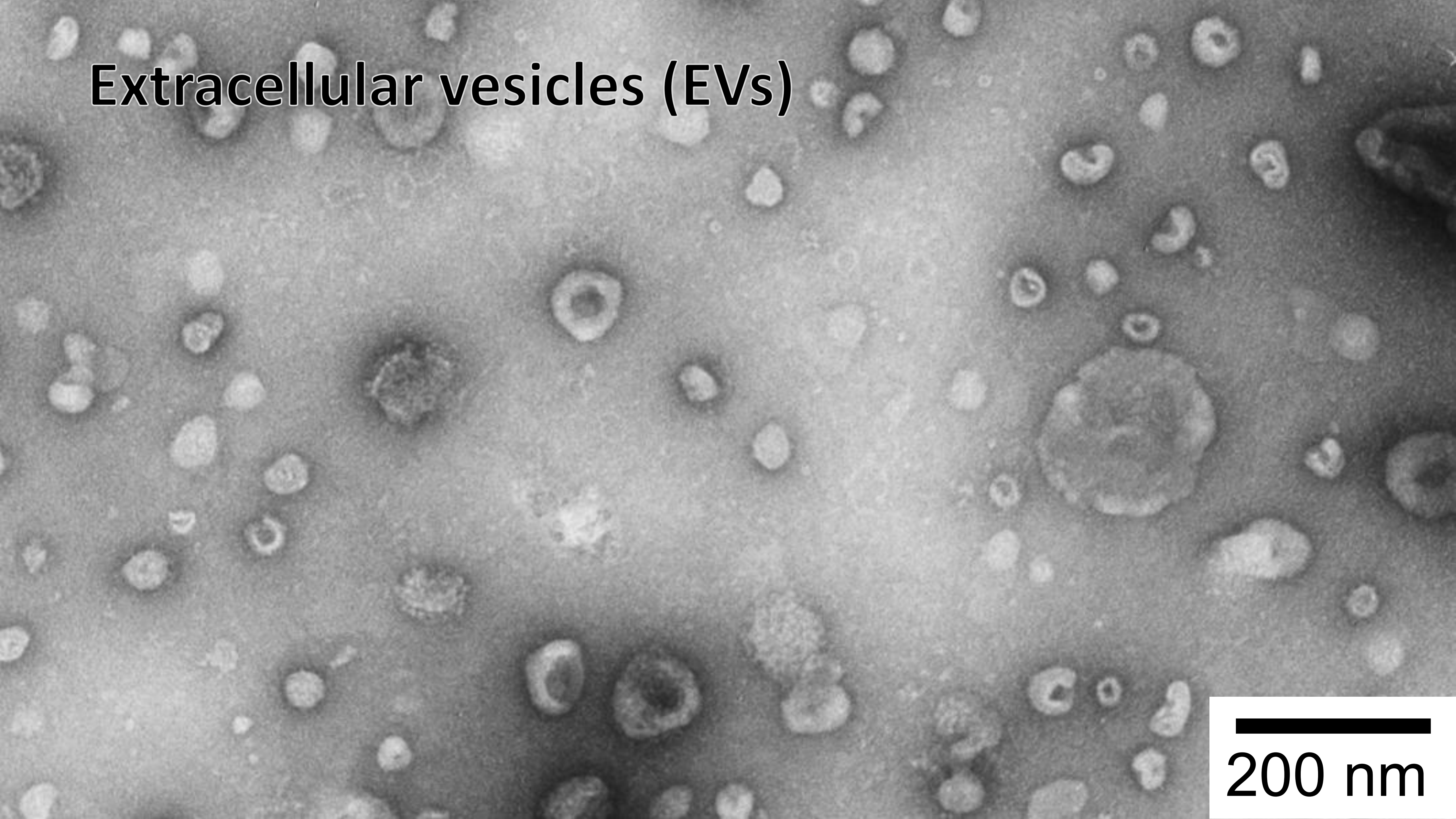
Are extracellular vesicles in liquid biopsies the source of my signals?

Edwin van der Pol

March 1st, 2022

Amsterdam UMC
University Medical Centers

Vesicle
Observation Center



Extracellular vesicles (EVs)
200 nm

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

Fictive values, PhD thesis van der Pol *UvA* 2015

Problem: to isolate envisioned biomarker

- Biomarkers in human blood plasma include
  - circulating cell-free DNA
  - circulating tumor cells
  - extracellular vesicles (EVs)
  - miRNAs within EVs
  - platelets

Outline

- Part 1: Elucidate how centrifugation affects the purity of liquid biopsy-based biomarkers
- Part 2: Why bother about remaining platelets
- Summary and outlook

Part 1: to elucidate how…

Centrifugation Affects the Purity of Liquid Biopsy-Based Tumor Biomarkers

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Cytometry PART A

ISAC International Society for Advancement of Cytometry

Journal of Quantitative Cell Science

Biomarkers / contaminants may affect signal

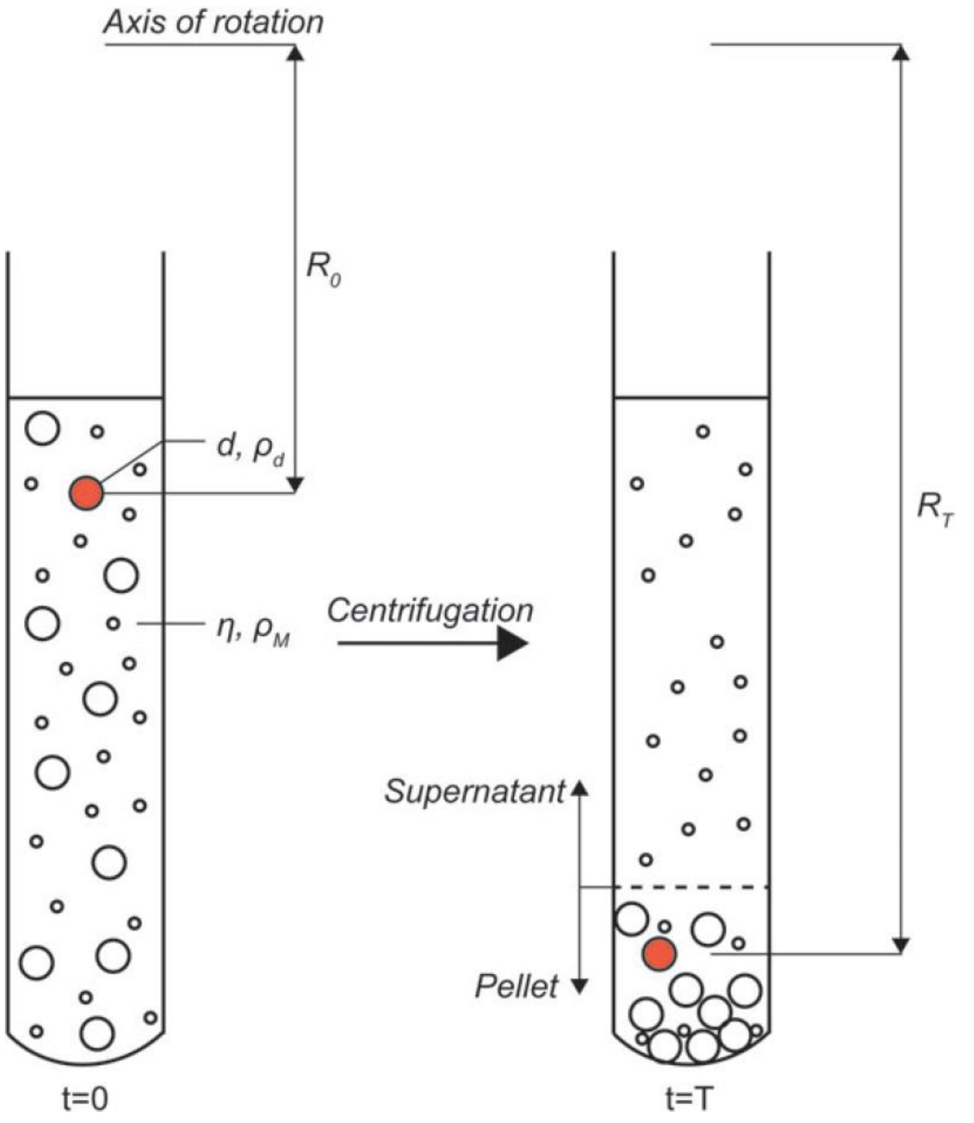
	Antigen exposure	RNA composition	DNA composition
Circulating cell-free DNA			Biomarker
Circulating tumor cells	Biomarker	Contaminant	Contaminant
Extracellular vesicles (EVs)	Biomarker		Contaminant
miRNAs within EVs		Biomarker	
Platelets	Biomarker	Biomarker	

- After centrifugation, biomarkers / contaminants may affect signal, leading to misinterpretation of results

Stokes model in MATLAB

$$R_T = R_0 e^{\frac{d^2(\rho_P - \rho_M)}{18\, S\, \eta}\omega^2 T}$$

R_T	position after time T
R_0	starting position
d	particle diameter
ρ_P	particle density
ρ_M	particle mass
S	shape factor
η	viscosity
ω	angular velocity
T	centrifugation time

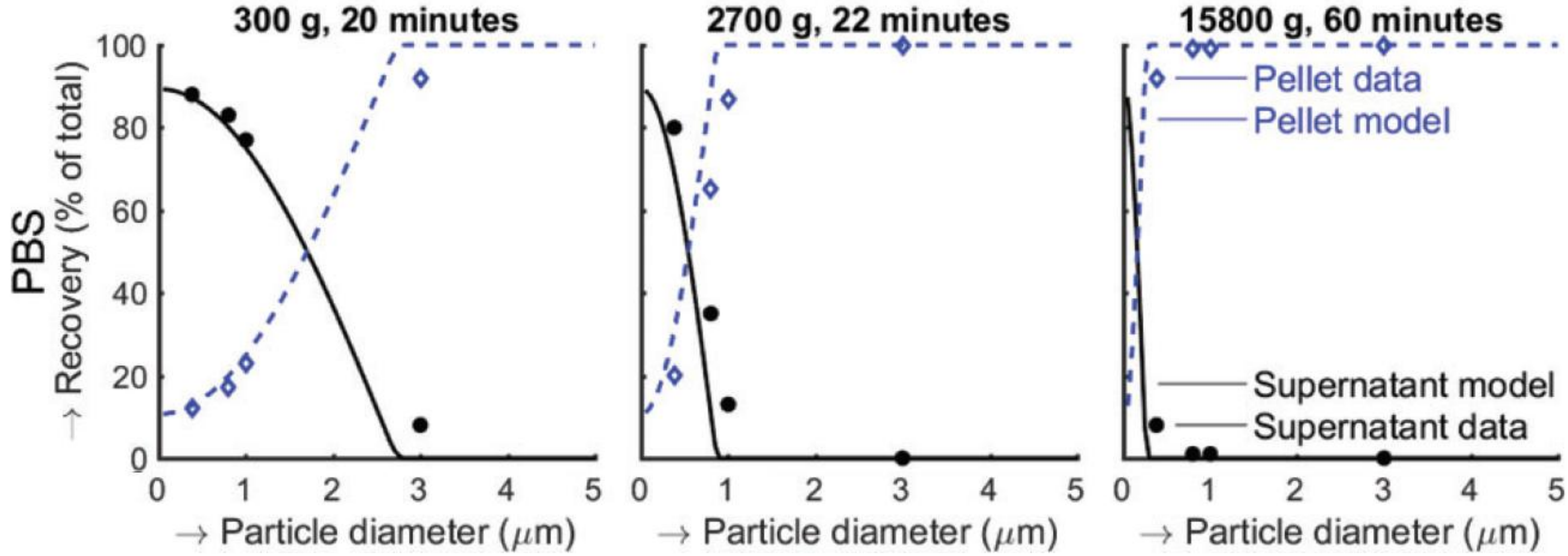


Centrifuges and tubes

Rotina 380R
57.462 Sarstedt tube

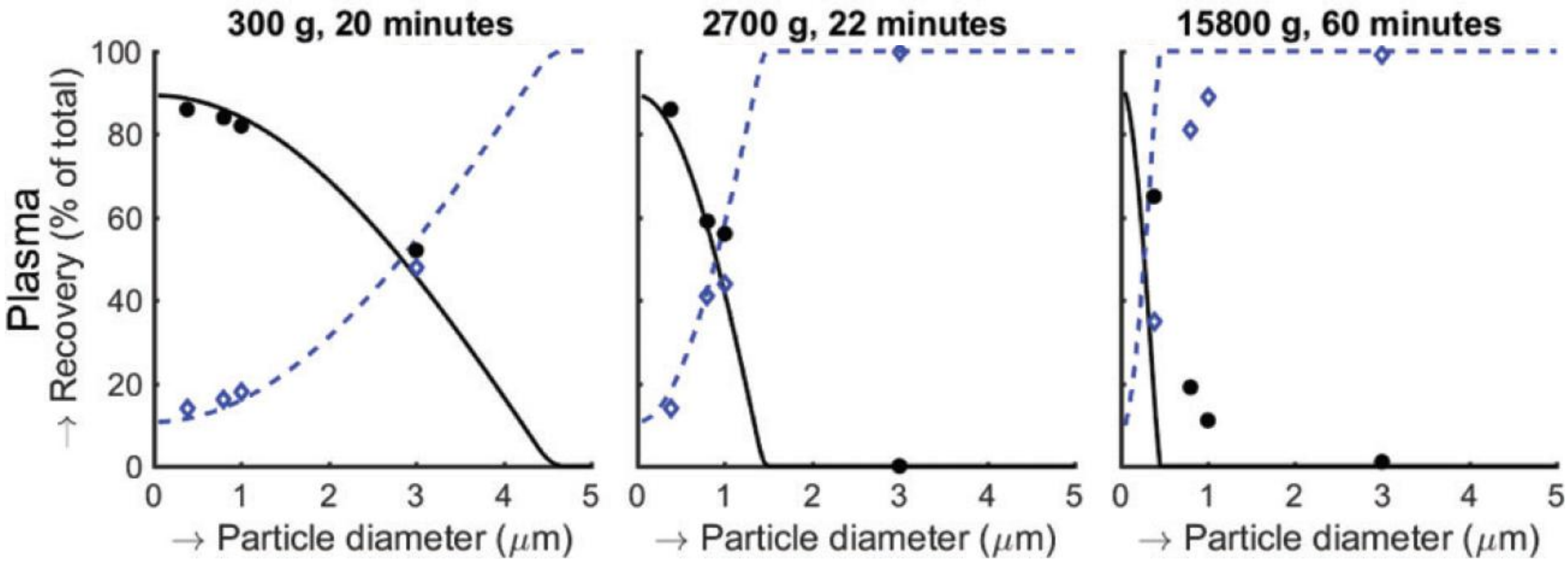
Optima L-80 XP, SW41 Ti rotor
344059 BC tube

Stokes model validation: PBS



PBS: phosphate buffered saline
Rikkert et al. *Cytom Part A* 2018

Stokes model validation: human blood plasma



Mean errors < 8%

Maximum error = 20%

Predict purity in biomarker studies

Biomarker	Protocol summary	Reference
Circulating cell-free DNA (Speicher)	WB 200·*g*, 10 min, 1,600·*g*, 10 min → (3 mm) Sup. 1,600g, 10 min → (3 mm) Sup	Heidary et al. *Breast Cancer Res* 2014
Circulating cell-free DNA (Dawson)	WB 820·*g*, 10 min → (5 mm) sup. → 20,000·*g*, 10 min → (10 mm) sup.	Dawson et al. *N Engl J Med* 2013
Circulating tumor cells	WB 7.5 mL + 6.5 mL PBS → 800·*g*, 10 min → (10 mm) pellet	Cristofanilli et al. *N Engl J Med* 2004
Extracellular vesicles		
miRNAs within extracellular vesicles	WB 900·*g*, 7 min → (5 mm) sup. 2,500·*g*, 10 min → (5 mm) sup. 500·*g*, 10 min → (5 mm) sup.	van Eijndhoven et al. *JCI Insight* 2017
Platelets	WB 120·*g*, 20 min → (3 mm) sup. 360·*g*, 20 min → (10 mm) pellet, wash 2×	Best et al. *Cancer Cell* 2015

sup.: supernatant; WB: whole blood
PBS: phosphate buffered saline

Stokes model sample properties

TYPE AND PROPERTY	VALUE USED IN MODEL
CTCs	
Density (g/ml)	1.053
Diameter (μm)	Uniform. 8–20
Platelets	
Density (g/ml)	Norm., mean 1.069, SD 0.0053
Diameter (μm)	Lognorm., median 2.4, SD 0.6
Shape factor	2.0
EVs	
Density (g/ml)	Core 1.060, 8 nm shell 1.155
Diameter (μm)	Uniform. 0.05–1
ccfDNA	
Density (g/ml)	1.7
Diameter (μm)	≤0.05
PBS	
Density (g/ml)	1.004
Viscosity (mPa s)	1.193
Plasma	
Density (g/ml)	1.0253
Viscosity (mPa s)	1.75

Rikkert et al. *Cytom Part A* 2018

Predicted recovery (%) of centrifugation protocols

Biomarker	ccfDNA	CTCs	EVs > 1.0 µm	EVs 0.2-1.0 µm	EVs < 0.2 µm	Platelets
ccfDNA (Speicher)	82.0	0.0	2.4	63.4	82.0	0.05
ccfDNA (Dawson)	48.4	0.0	0.0	6.0	49.0	0.00
CTCs	13.3	100.0	81.1	16.7	13.3	39.4
EVs						
miRNAs within EVs	56.9	0.0	0.7	39.7	57.0	0.01
Platelets	2.2	0.0	21.7	3.2	2.2	71.1

ccfDNA: circulating cell-free DNA; CTC: circulating tumor cells
EVs: extracellular vesicles; Rikkert et al. *Cytom Part A* 2018

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Plasma preparation protocol in EV research

ORIGINAL ARTICLE

Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol

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and F. DIGNAT-C
*UMR1076 INSERM-A
and ‡Biocytex, 140, C

Methodological Guidelines to Study Extracellular Vesicles

Frank A.W. Coumans, Alain R. Brisson, Edit I. Buzas, Françoise Dignat-George,
Esther E.E. Drees, Samir El-Andaloussi, Costanza Emanueli, Aleksandra Gasecka,
An Hendrix, Andrew F. Hill, Romaric Lacroix, Yi Lee, Ton G. van Leeuwen, Nigel Mackman,
Imre Mäger, John P. Nolan, Edwin van der Pol, D. Michiel Pegtel, Susmita Sahoo,
Pia R.M. Siljander, Guus Sturk, Olivier de Wever, Rienk Nieuwland

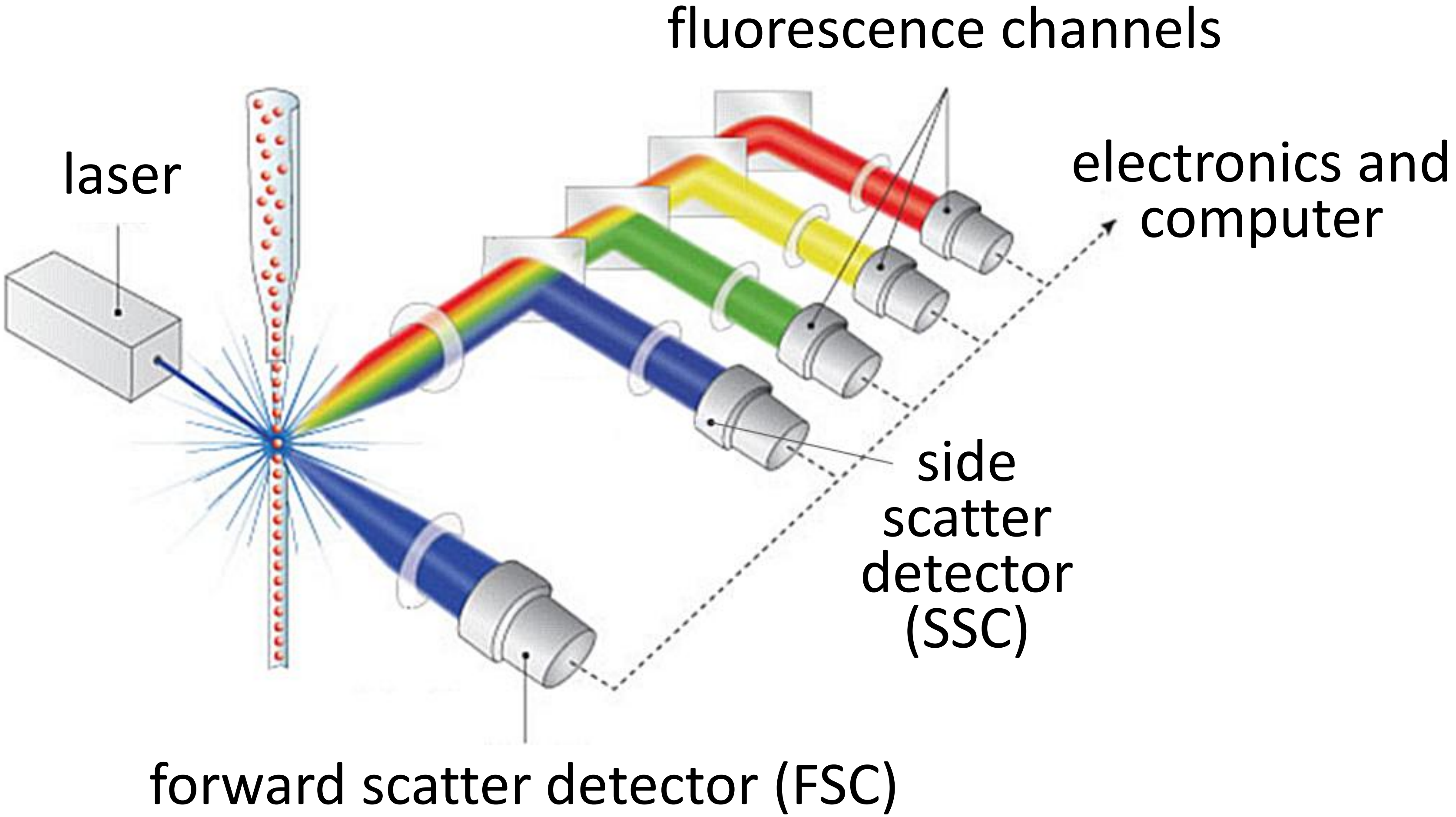
Lacroix et al. *J Thromb Haemost* 2012
Coumans et al. *Circ Res* 2017

Plasma preparation protocol in EV research

- Remove platelets by 2 subsequent centrifugations steps of 2500·*g* for 15 minutes
- …
- Quantify residual platelets in platelet-free plasma

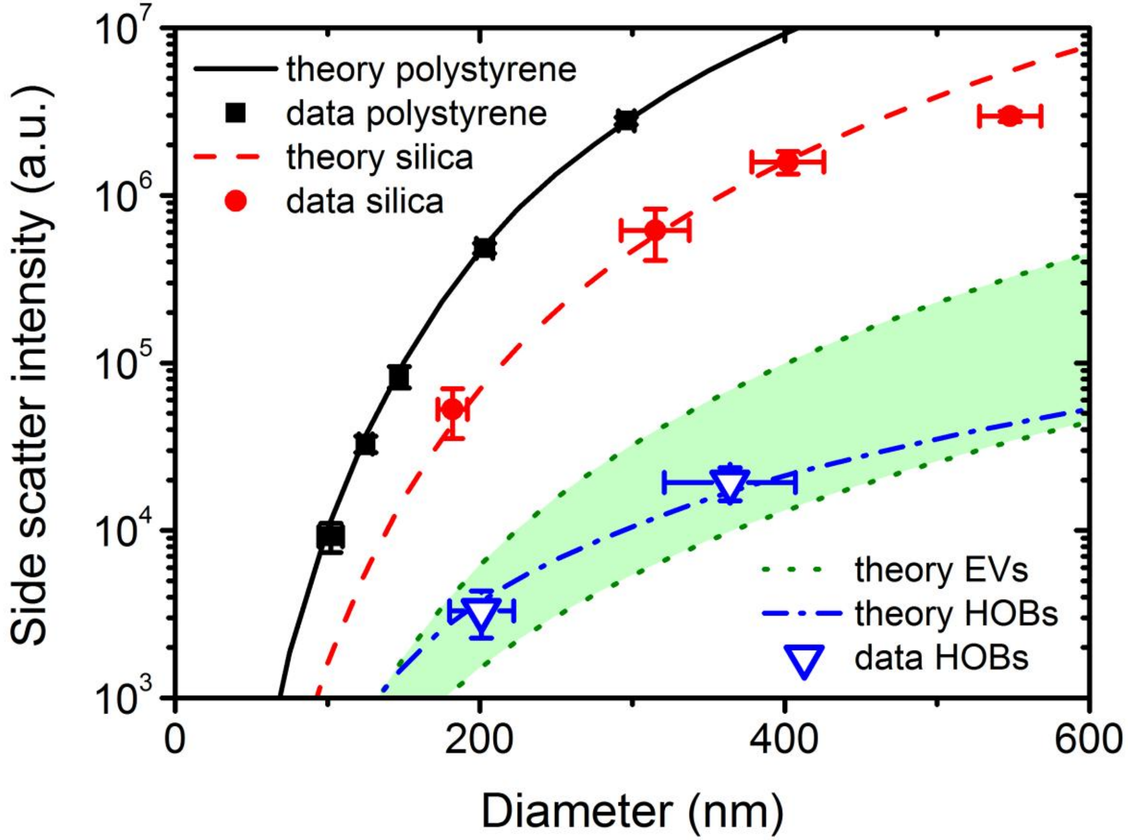
Coumans et al. *Circ Res* 2017

Flow cytometry

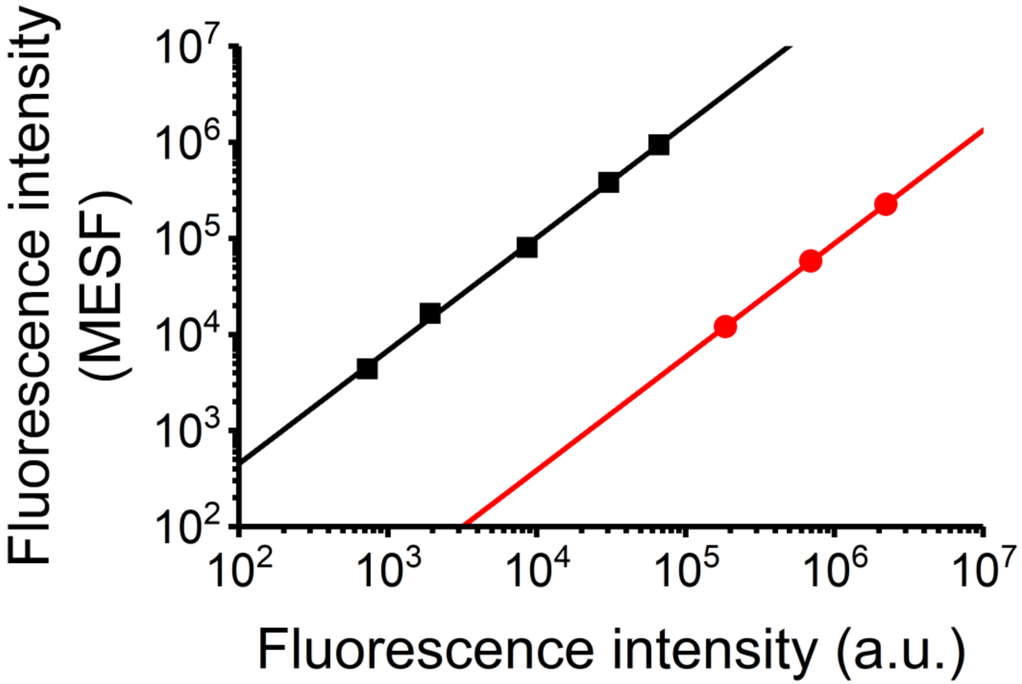


Calibrate flow cytometer

Scatter (a.u.) → diameter (nm)

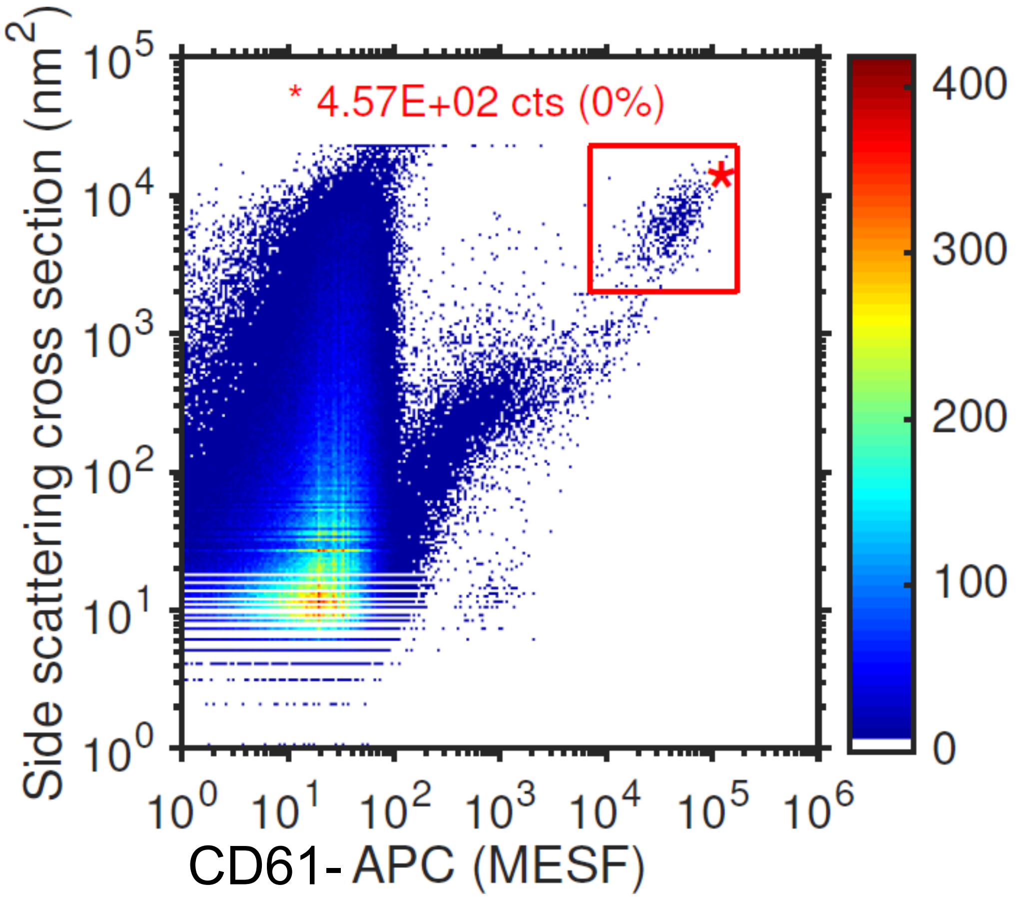


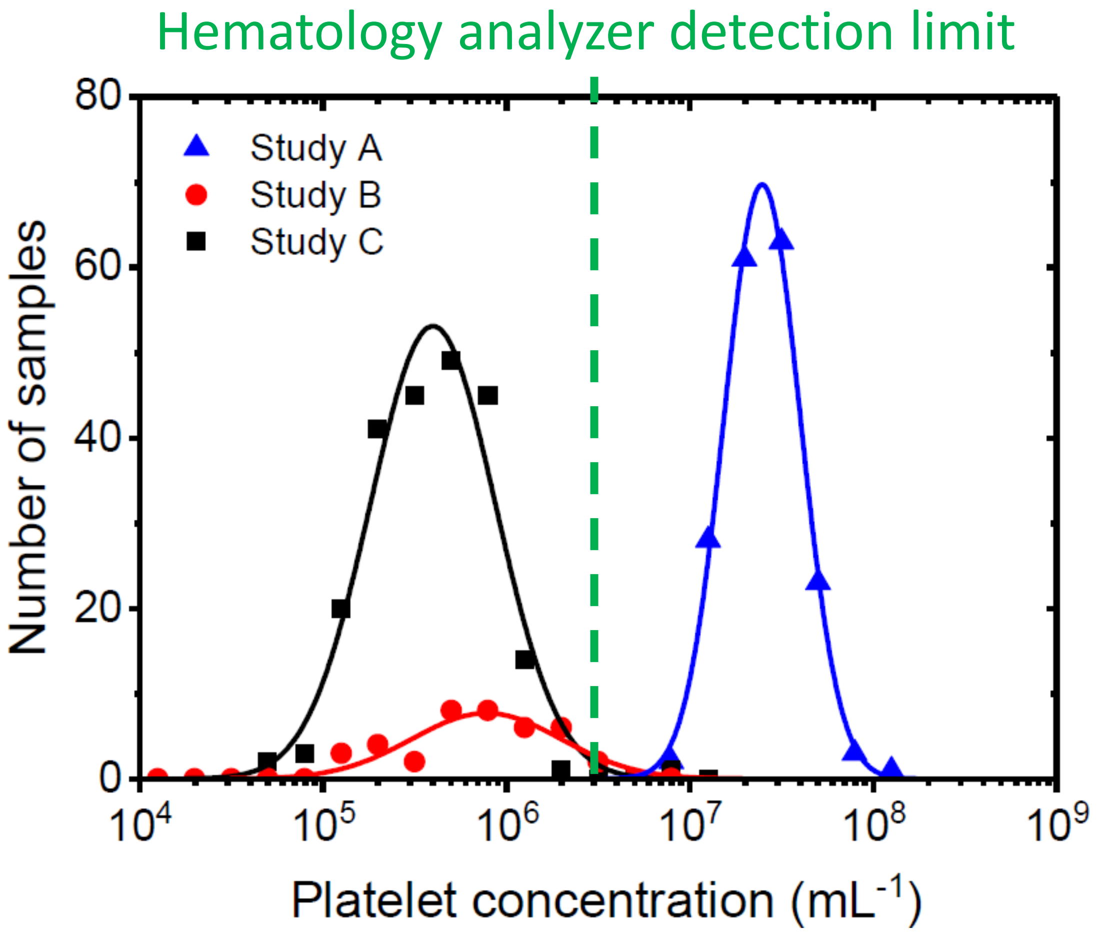
Fluorescence (a.u. → MESF)



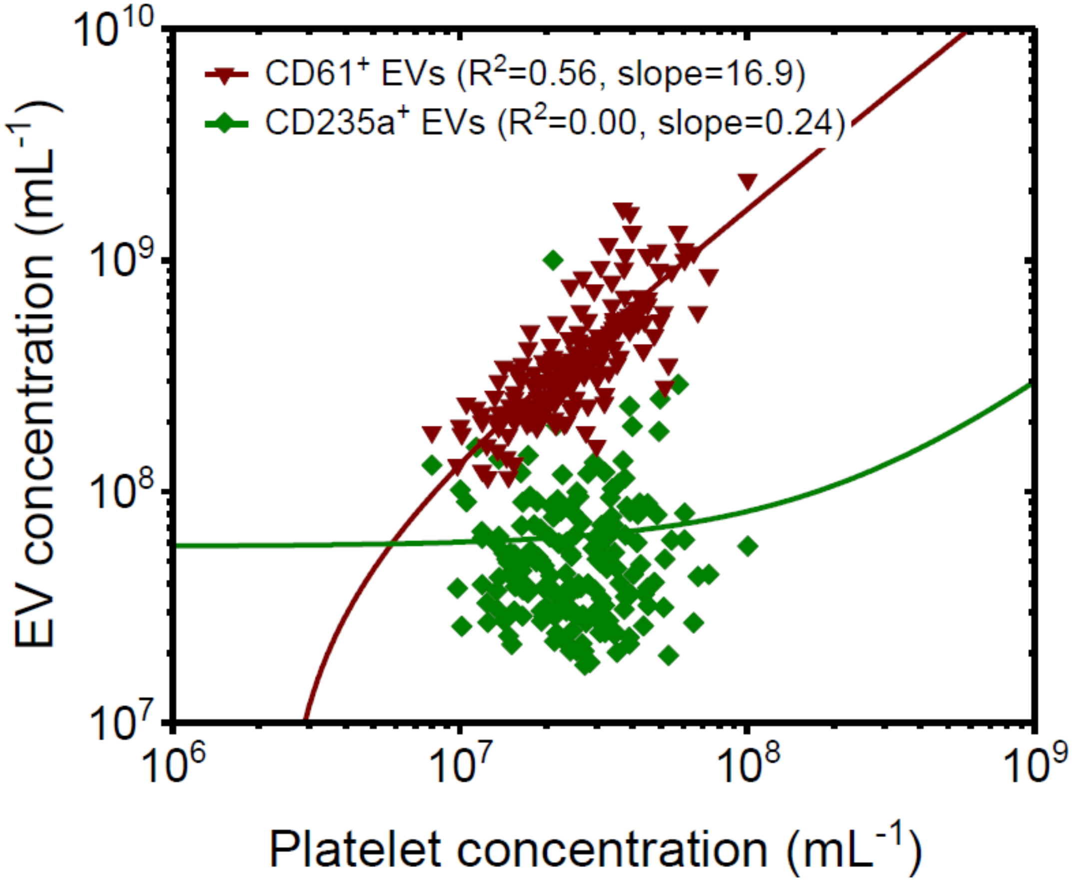
de Rond et al. *Curr Protoc Cytom* 2018
Welsh et al. *J Extracell Vesicles* 2020

“Platelet-free plasma” is not platelet-free





Why bother about residual platelets? #1

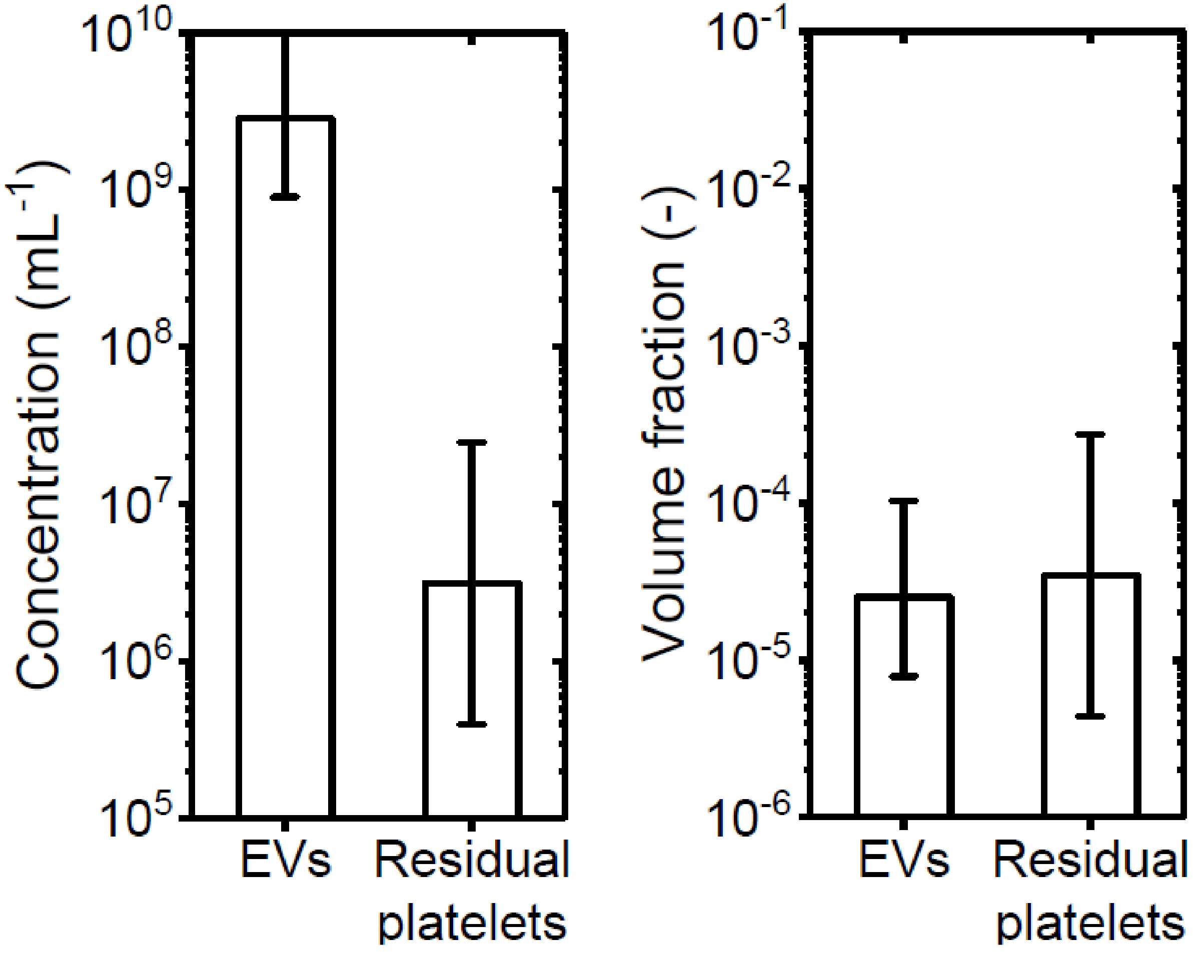


Lower detection limits:

EV diameter	> 160 nm
CD61-APC	> 150 MESF
CD235a-PE	> 123 MESF

- After freeze-thawing, we measure 17 CD61+ EVs for each remaining platelet

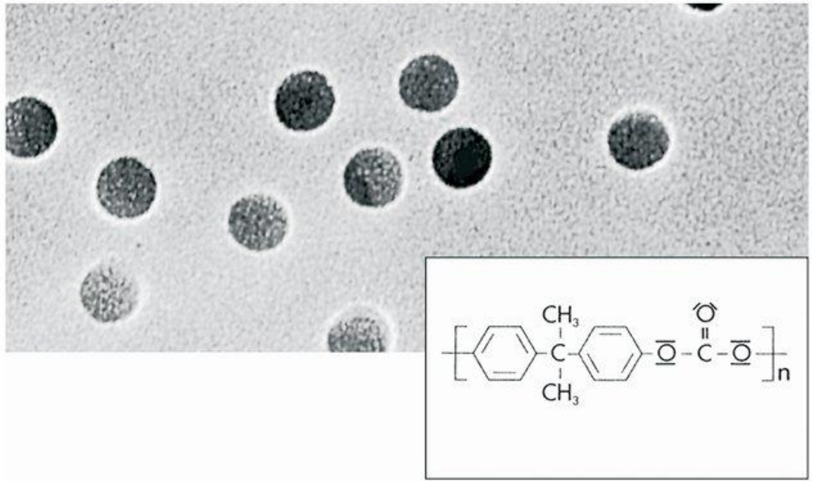
Why bother about residual platelets? #2

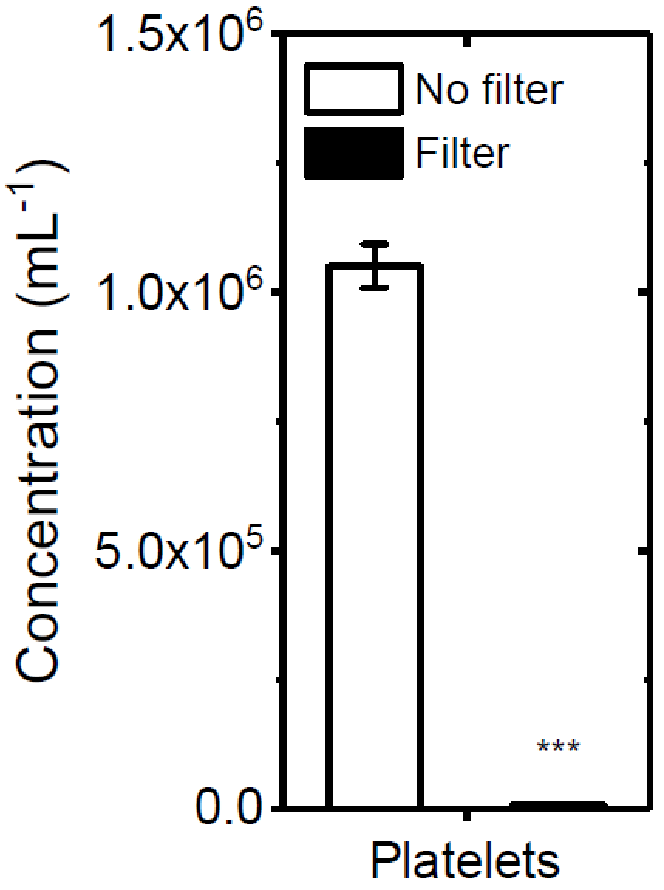


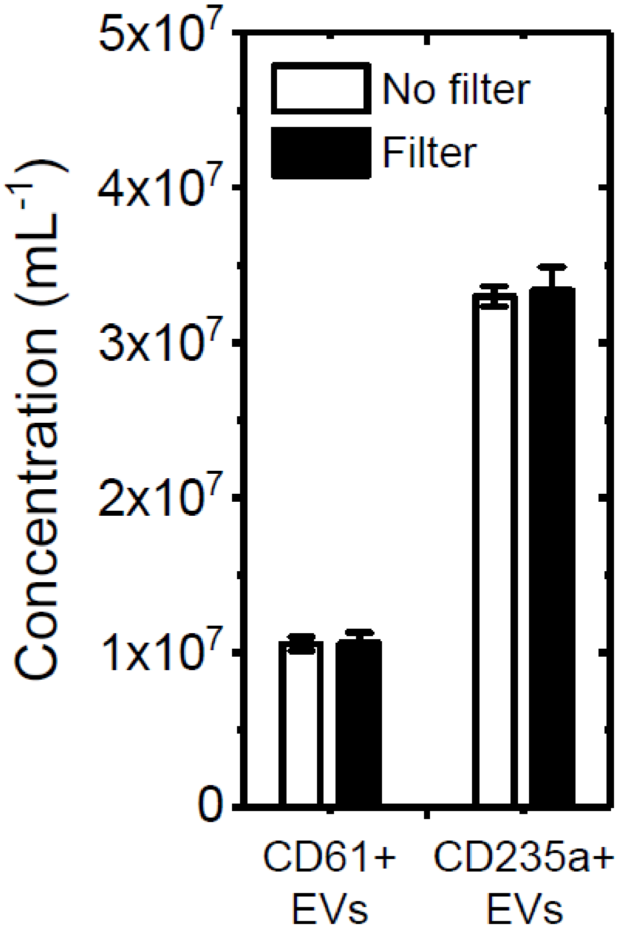
- Volume fraction of EVs and remaining platelets in plasma is similar

Possible procedure to remove remaining platelets

Track etch polycarbonate filters







Left image: fishersci.se
Right data: Britta Bettin (Amsterdam UMC)

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Summary and outlook

- Centrifugation affects the purity of biomarkers
- Models + experiments help understanding biomarker isolation methods
- Plasma prepared via the “ISTH protocol” contains platelets, which likely affect downstream analyses
- Calibration and quality controls are required to confirm purity of biomarkers

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- Amsterdam University Medical Centers
  - Biomedical Engineering & Physics
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  - edwinvanderpol.com
  - evflowcytometry.org
  - metves.eu

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