An Imaging Flow Cytometry-based Methodology for the Analysis of Single Extracellular Vesicles in Unprocessed Human Plasma

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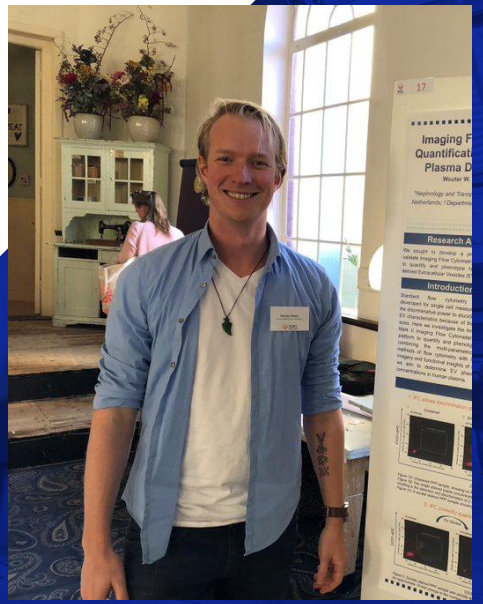

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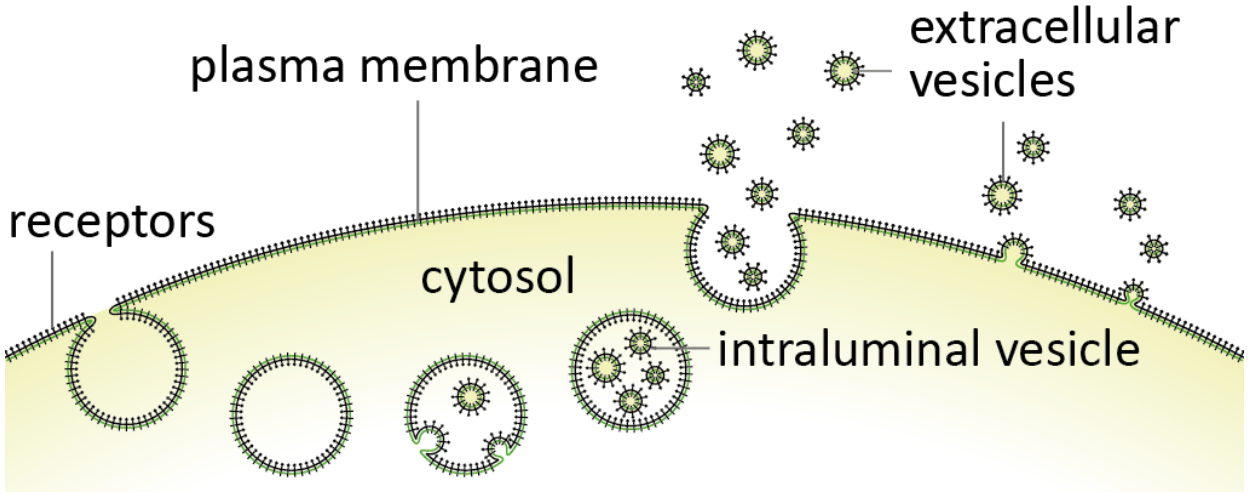
Conflict of Interest

Edwin van der Pol is co-founder and stakeholder of Exometry B.V.

Other authors declare no funding-related or other conflicts of interest.

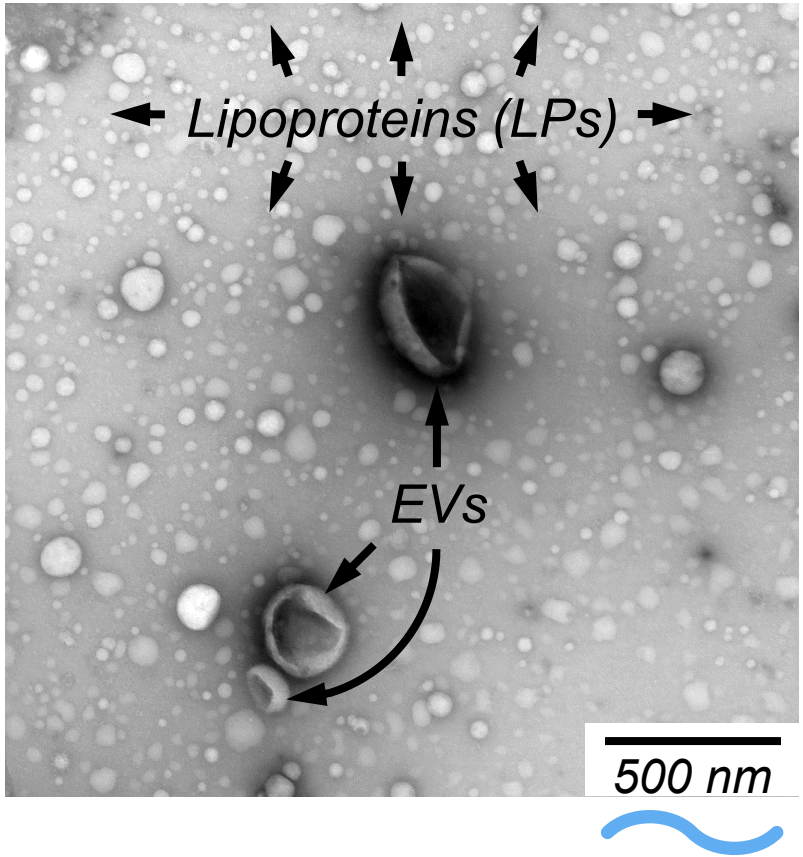
Extracellular vesicles (EVs)

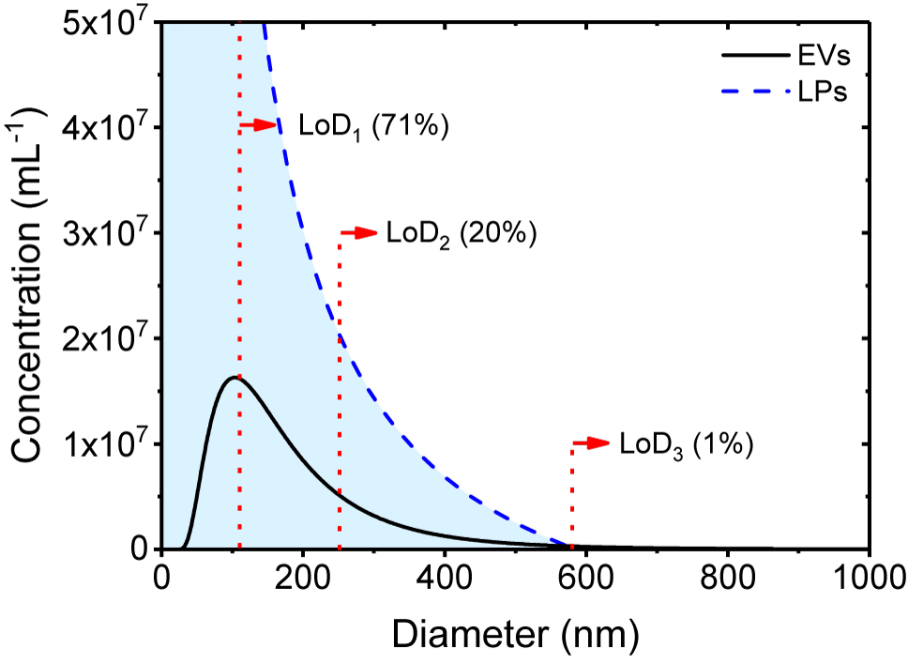


van der Pol, E., et al., Pharmacol Rev 2012

- Biological nanoparticles with receptors, DNA, RNA
- Reflect the status of their cell of origin
- Clinically relevant

Challenges of characterizing EVs from human plasma





Aim

To measure single EVs **directly** in diluted, labelled human plasma using Imaging Flow Cytometry (IFCM)...

Source: Unsplash.com

...reflecting the status of the individual ‘as is’

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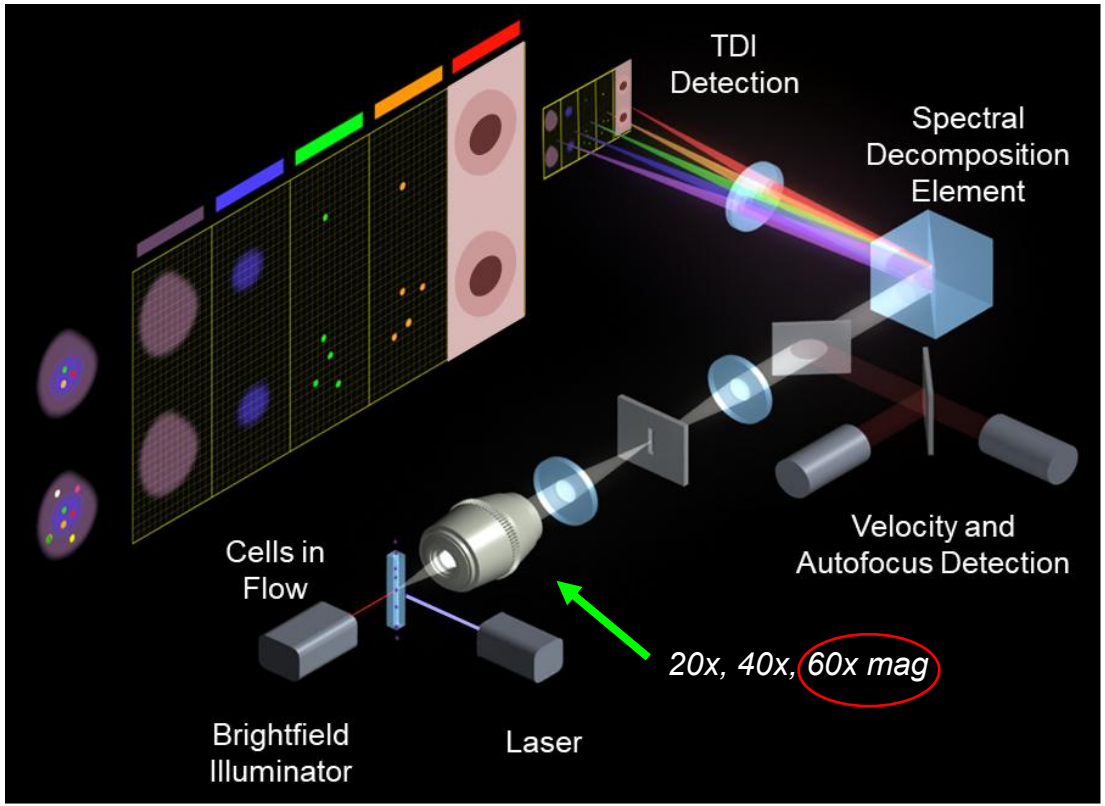
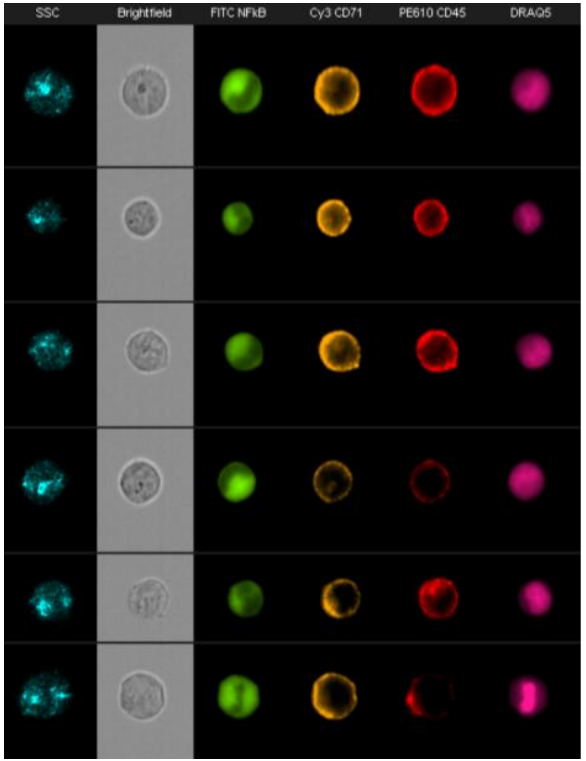
Imaging Flow Cytometry *(ImageStreamX MKII)*



Multi-Spectral / Multi-Mode Imagery



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	Category	Components	Objective
1	Preanalytical variables & experimental design	1.1. Report preanalytical variables conforming to MISEV guidelines* 1.2. Report experimental design according to MIFlowCyt guidelines*	Reproducibility
2	Sample preparation	2.1. Sample staining* 2.2. Sample washing steps* 2.3. Sample dilution*	Reproducibility
3	Assay controls	3.1. Buffer-only* 3.2. Buffer with reagents* 3.3. Unstained controls* 3.4. Isotype controls** 3.5. Single-stained controls* 3.6. Procedural controls** 3.7. Serial dilution* 3.8. Detergent-treated EV samples	Proof of single vesicle detection
4	Instrument calibration & data acquisition	4.1. Trigger channel(s) and threshold(s)* 4.2. Flow rate & volumetric quantification (μL min^{-1} / μL)* 4.3. Fluorescence Calibration (MESF/ERF units)* 4.4. Light Scatter Calibration (nm^2)	Standardization
5	EV characterization	5.1. EV diameter/surface area/volume approximation 5.2. EV refractive index approximation 5.3. Epitope number approximation	Advanced standardization
6	FC data reporting	6.1. Complete MIFlowCyt checklist* 6.2. Calibrated channel detection range 6.3. EV number concentration 6.4. EV brightness	Reproducibility
7	FC data sharing	7.1. Share data to public repository	Reproducibility

Welsh, J.A. et al. MIFlowCyt-EV J. Extracell. Vesicles 2019

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Outline: MIFlowCyt-EV

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J. Extracell. Vesicles 2019

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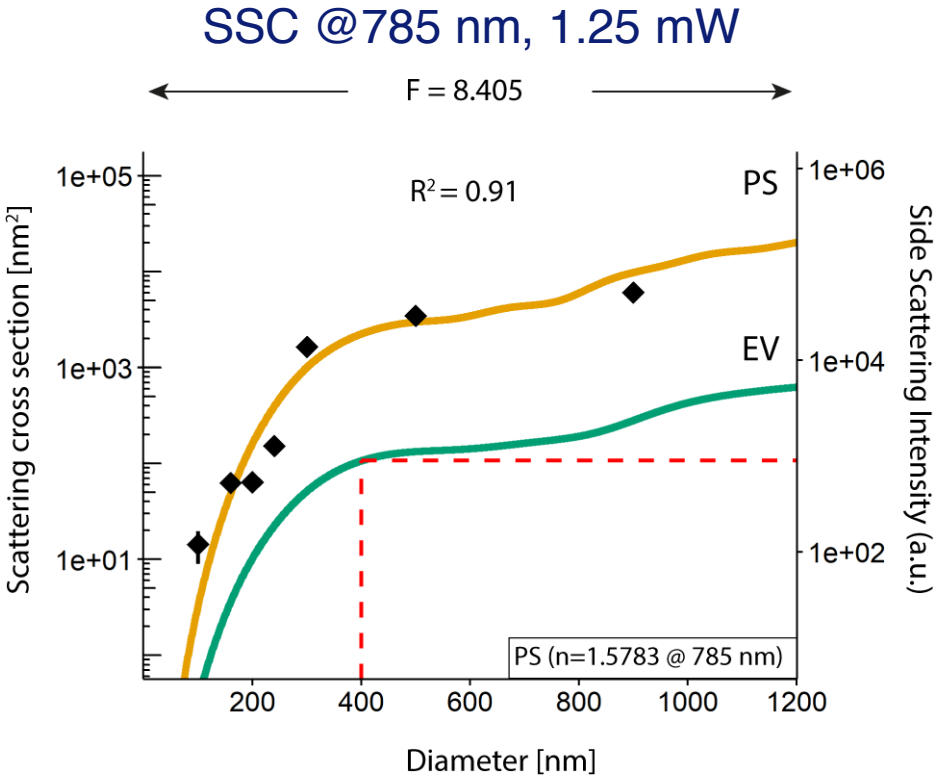
IFCM SSC Size Calibration

Acquired 7 sizes of PS Beads:

- 100 nm
- 160 nm
- 200 nm
- 240 nm
- 300 nm
- 500 nm
- 900 nm

Use Mie Theory to calibrate for size:



Woud W.W. et al., Communications Biology 2022

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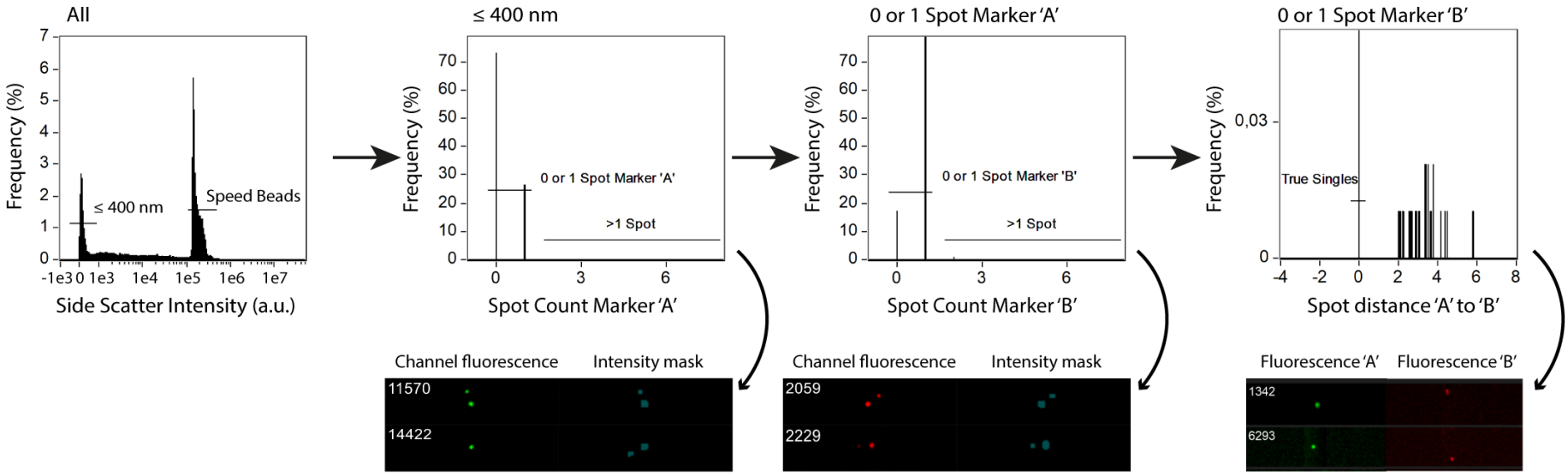
Gating Strategy

Selection of single fluorescent EVs ≤ 400 nm in plasma:



Tip from Haley Pugsley: not intensity mask but system mask

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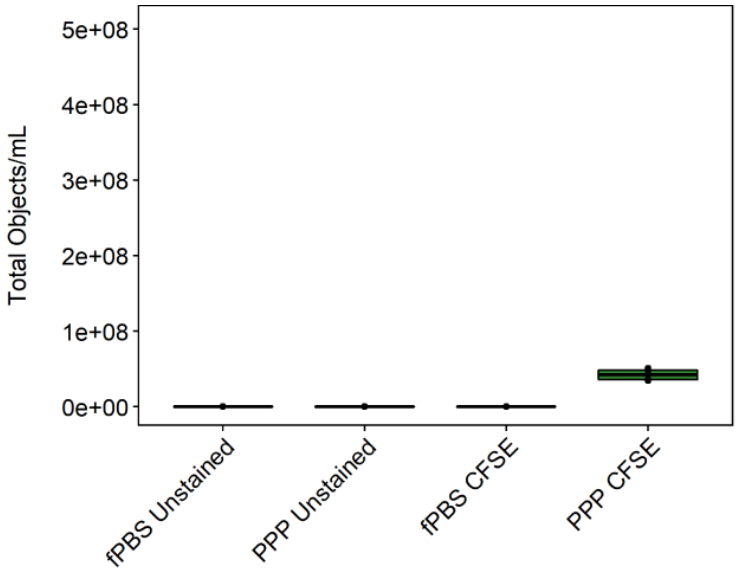
Background Analysis

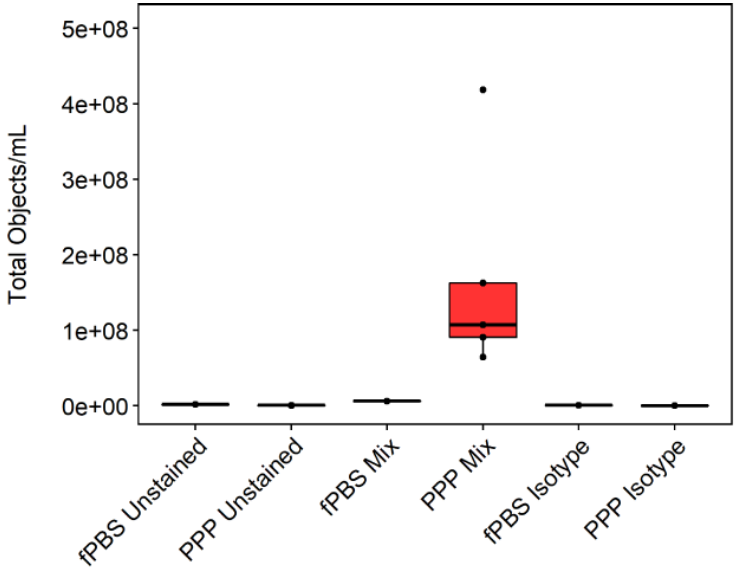
No washing steps are performed →
Discrimination of EVs from fluorescent background signals

CFSE+ events



~100-fold

Tetraspanin+ (CD9, CD63, CD81) events



~28- to 350-fold

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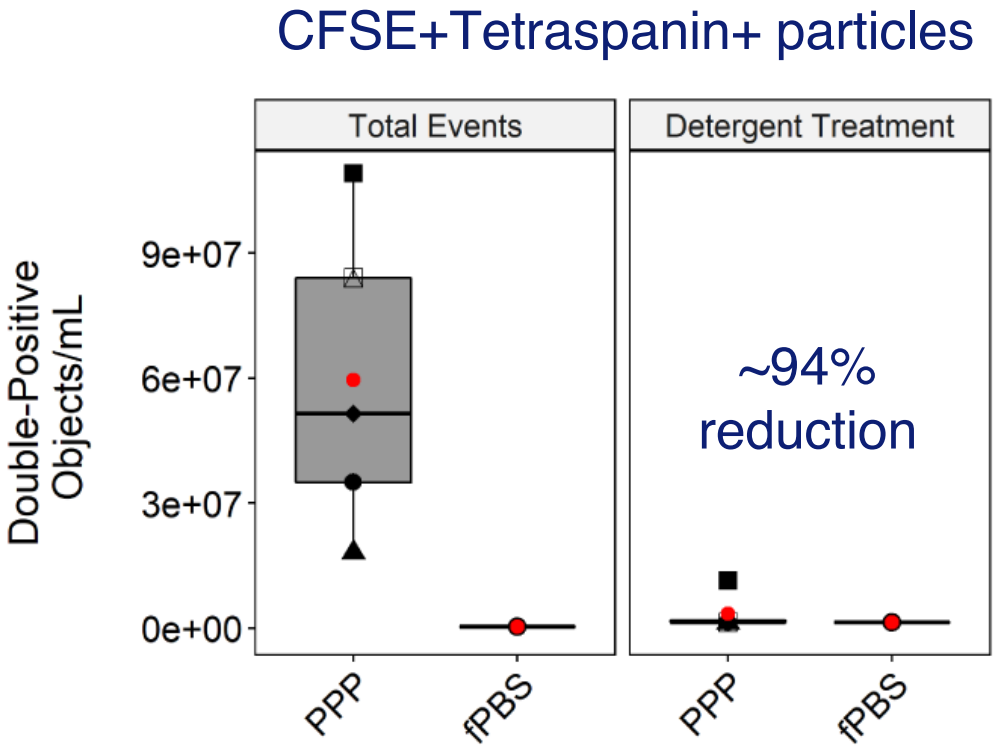
Detergent Treatment *(10% (v/v) TritonX-100)*



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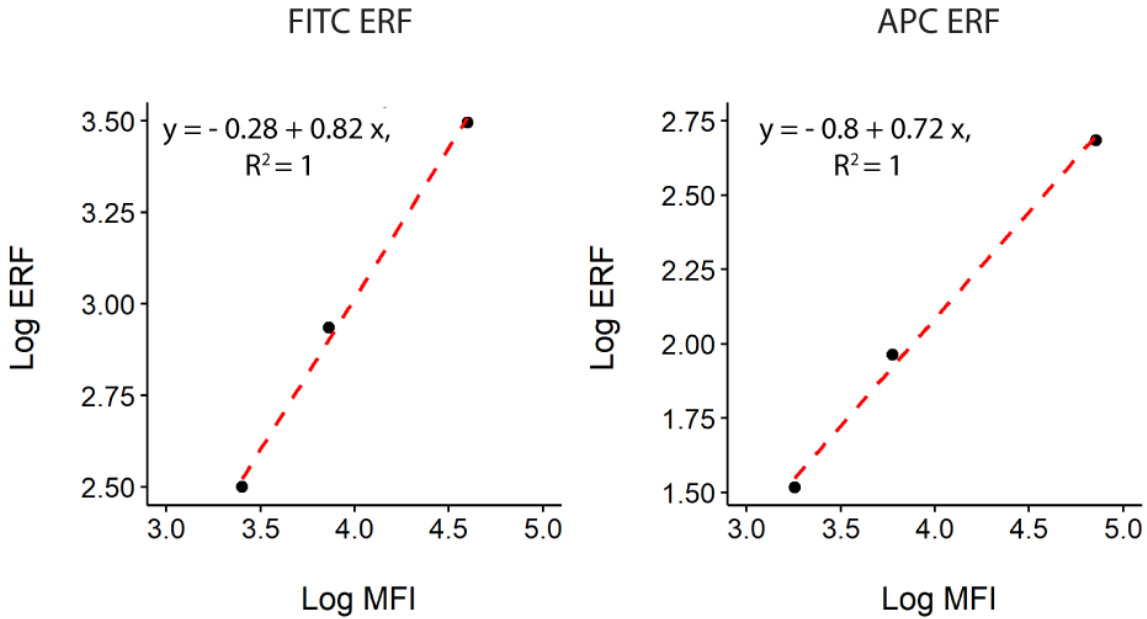
Fluorescent Calibration

500 nm Rainbow Calibration Particles with known reference values in terms of **Equivalent Reference Fluorophores (ERF)**



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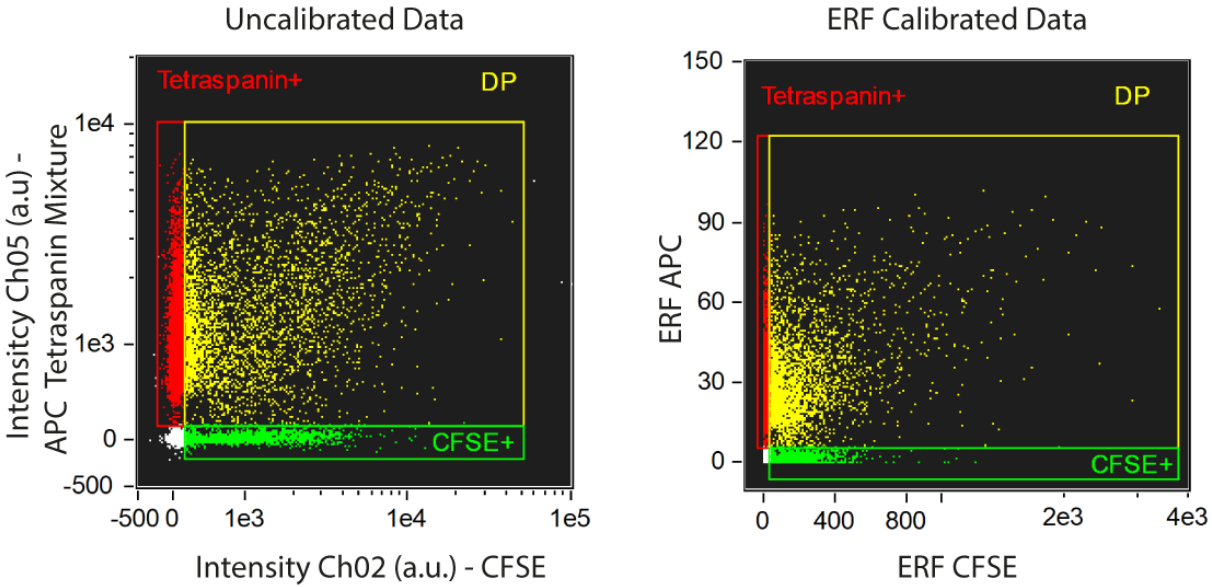
Fluorescent Calibration

Conversion of fluorescent intensities (a.u.) into ERF values



Lower - Upper fluorescent thresholds:

CFSE: 35.40 – 3776 ERF

APC: 6.40 – 123 ERF

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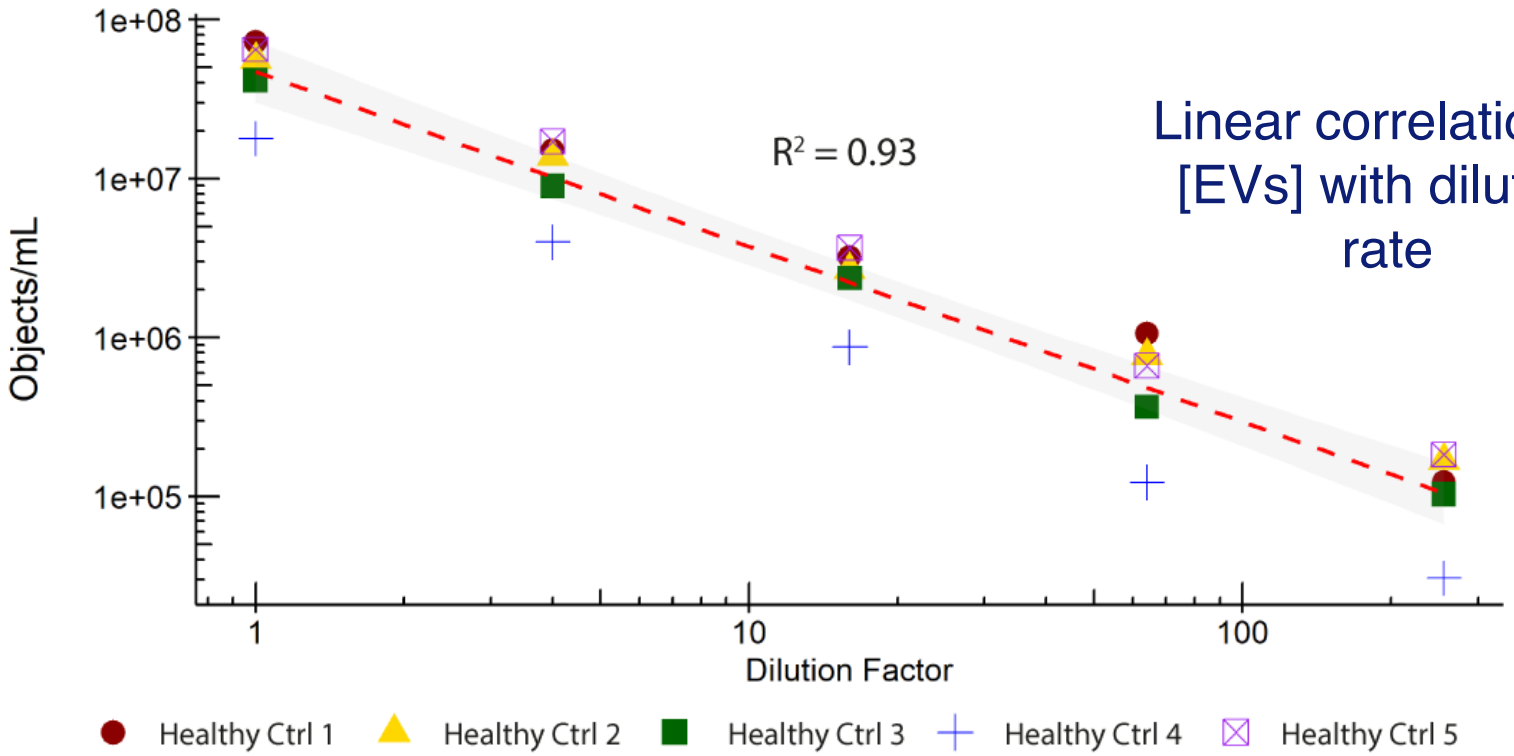
Testing EV coincidence occurrence – Serial Dilution



Linear correlation of [EVs] with dilution rate

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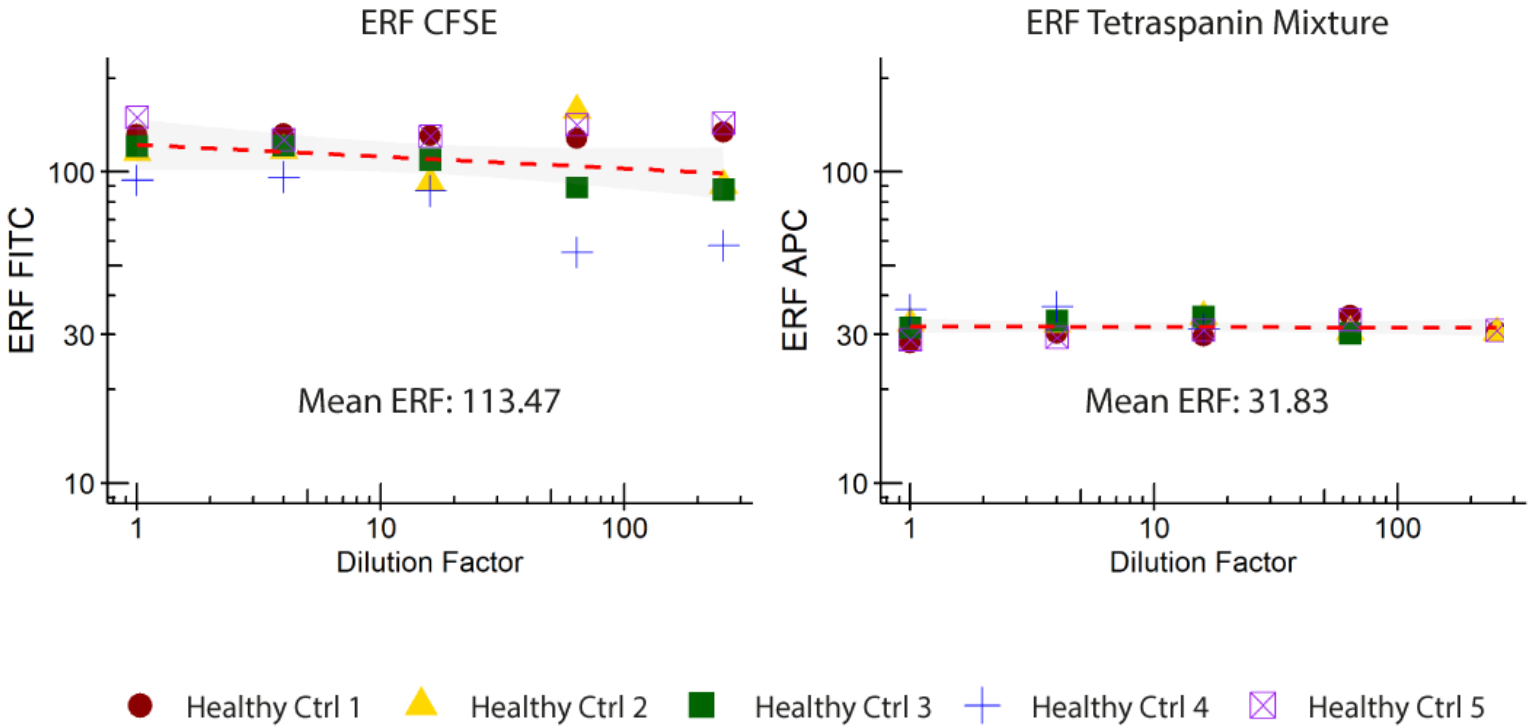
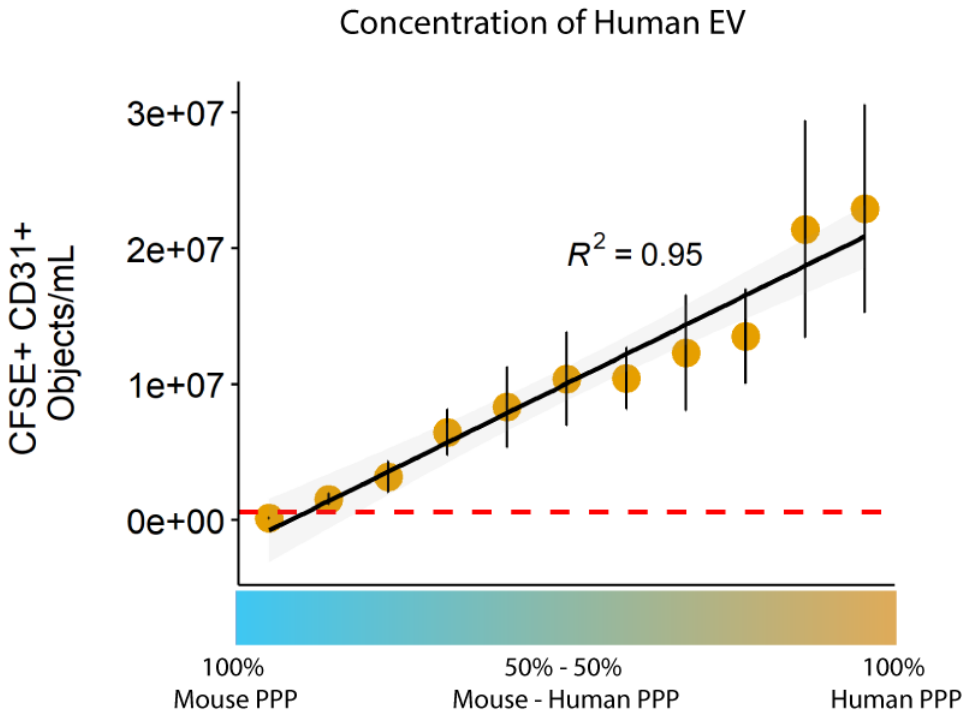
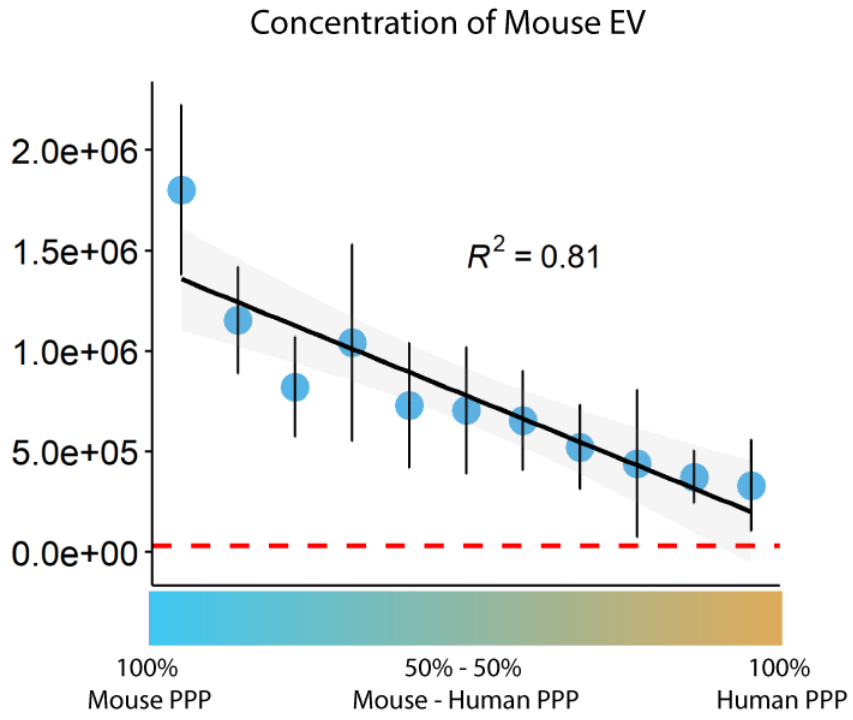
Testing EV coincidence occurrence – Serial Dilution



Stable Fluorescent Signals

IFCM facilitates specific EV subset analysis in contaminated/diluted PPP samples





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Conclusions

- Developed a **standardized** IFCM-based methodology
- Measure EVs **directly** in diluted platelet poor plasma
- High specificity (human / mouse)
- Showing the status of an individual “as is”

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Acknowledgement

Erasmus MC Transplant Institute
Wouter W. Woud
Ana Merino
Karin Boer
Martin Hoogduijn
Carla C. Baan
Wenda Verschoor

LUMINEX
Peter Rhein
Haley Pugsley

Amsterdam University Medical Center
Erik Mul

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