Analysis of Extracellular Vesicles by Flow Cytometry
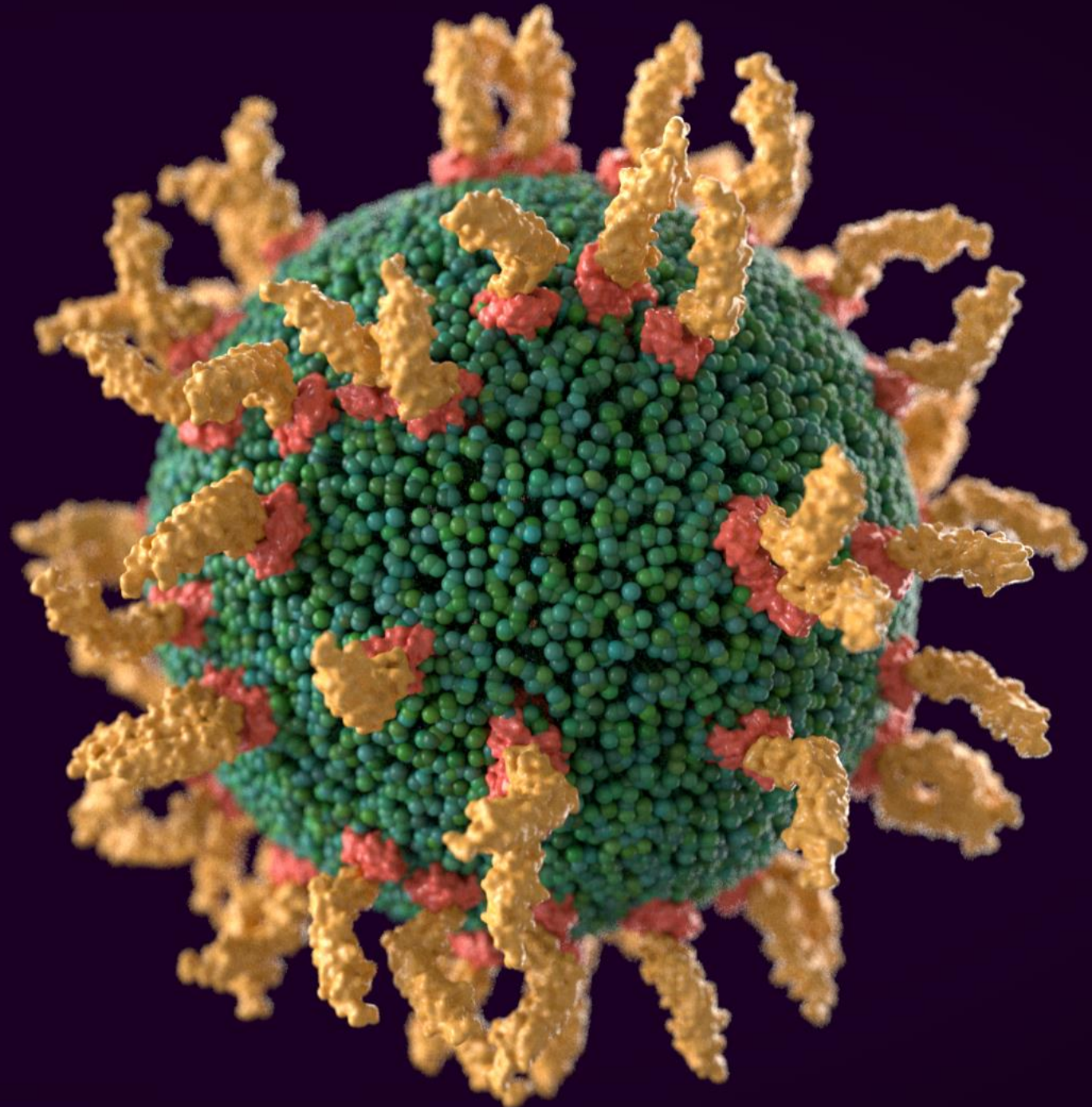
André Görgens, Ph.D
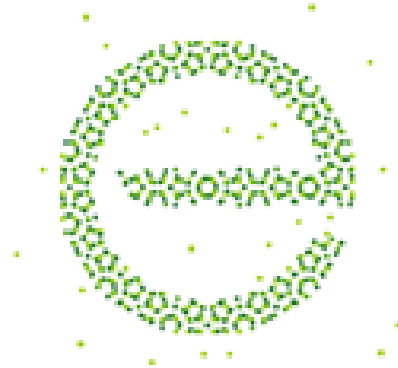
Karolinska Institutet
Stockholm, Sweden
Samir El Andaloussi Lab

@andregorgens
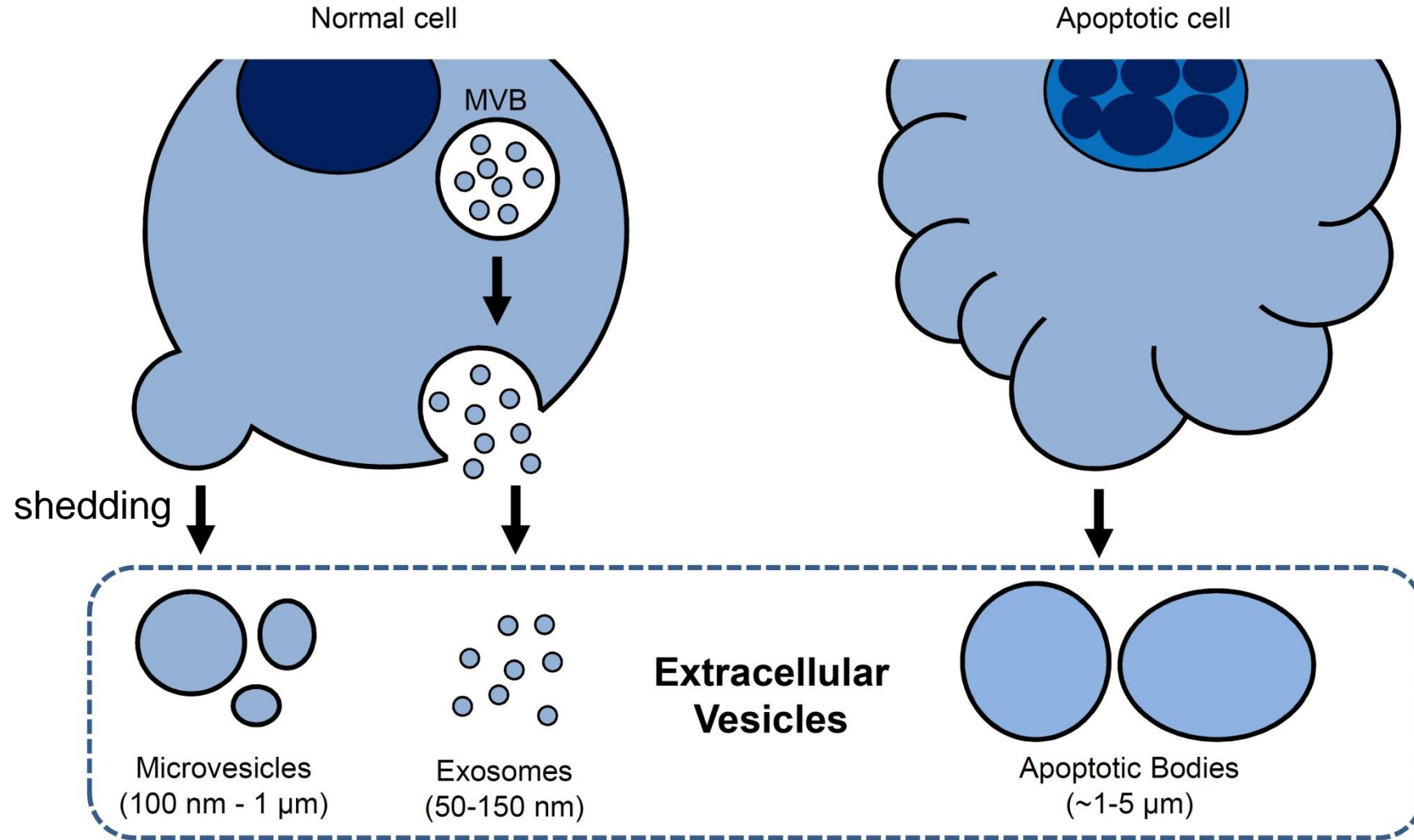
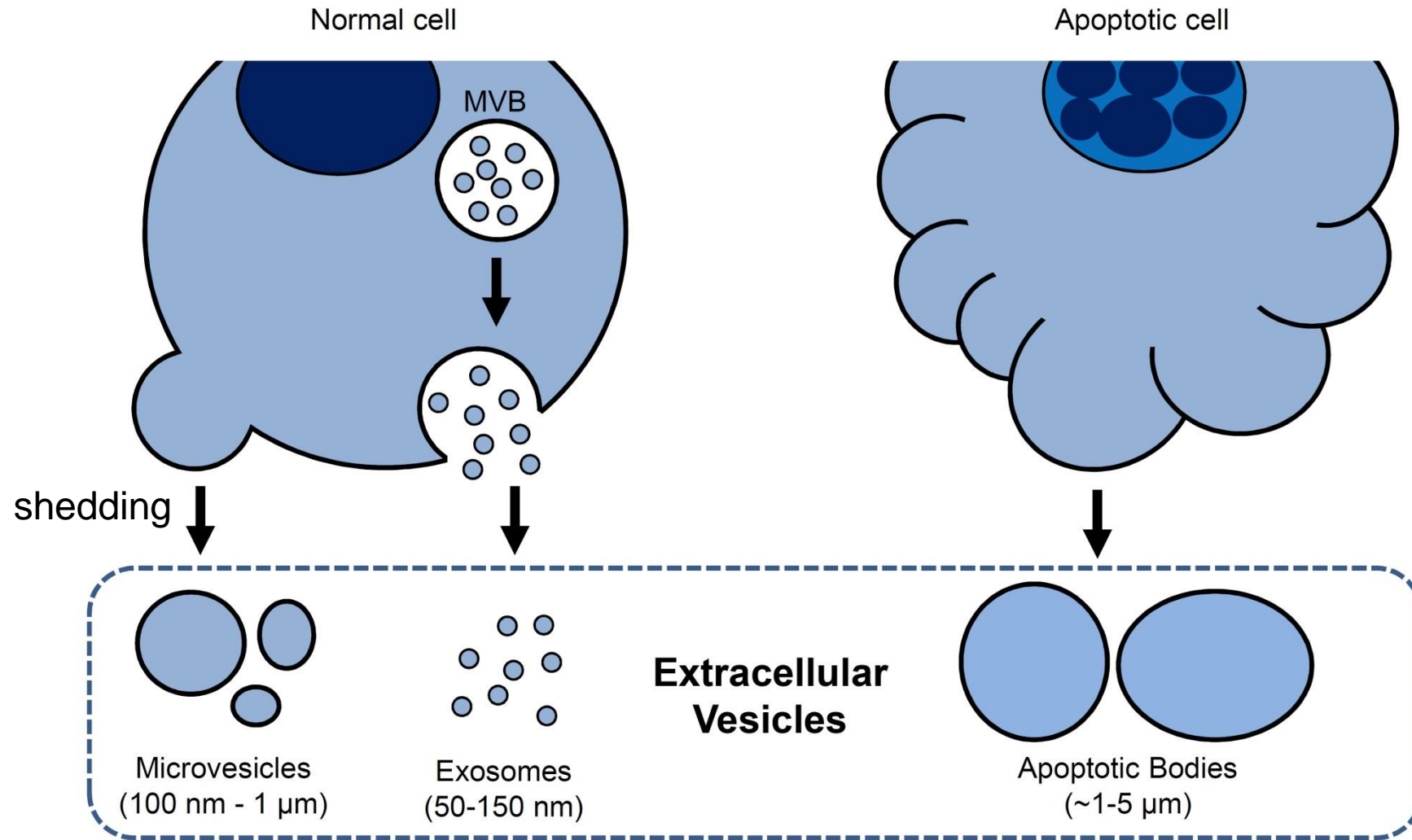# Conflict of Interest Disclosure

Consultant & Equity Interest:
Evox Therapeutics Limited, Oxford, UK,
a startup company developing engineered EVs
for therapeutic applications

Extracellular Vesicles (EVs)

Normal cell

MVB

Apoptotic cell

shedding

Extracellular Vesicles

Microvesicles
(100 nm - 1 μm)

Exosomes
(50-150 nm)

Apoptotic Bodies
(~1-5 μm)

EVs are secreted by a wide range of cells from different species.
EVs can be found in all body fluids.

Extracellular Vesicles (EVs)

Normal cell

Apoptotic cell

MVB

shedding

Extracellular Vesicles

Microvesicles
(100 nm - 1 µm)

Exosomes
(50-150 nm)

Apoptotic Bodies
(~1-5 µm)

Putative physiological functions: antigen presentation, intercellular communication, shuttling of nucleid acids between cells …

Extracellular Vesicles

- 50-150 nm vesicles secreted by all cells
- Found in all body fluids
- Central role in cell-cell communication
- Unique ability to convey various macromolecules

- **Therapeutic potential**
- **Diagnostic relevance**

- **Analysis of small EVs is challenging on multiple levels**

Source cell

Target cell

EL Andaloussi et al. Nat Rev Drug Discov 2013
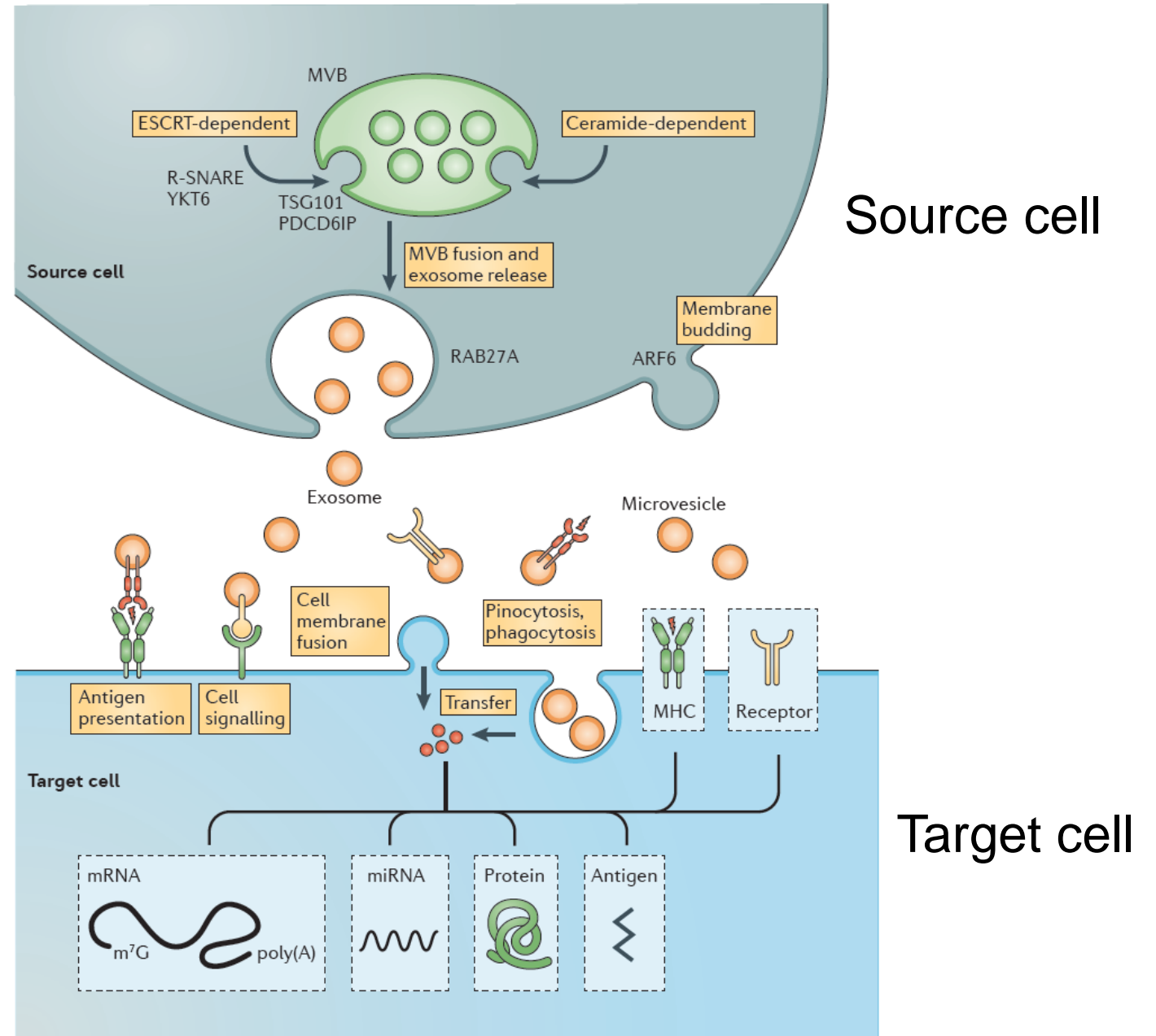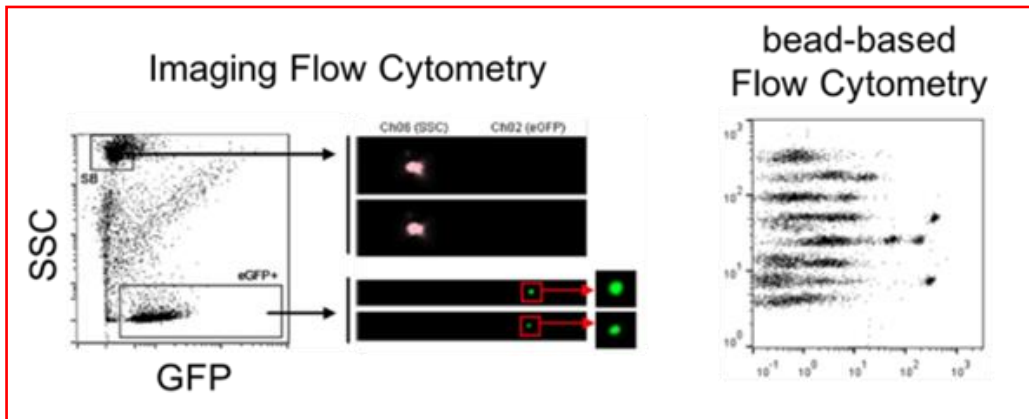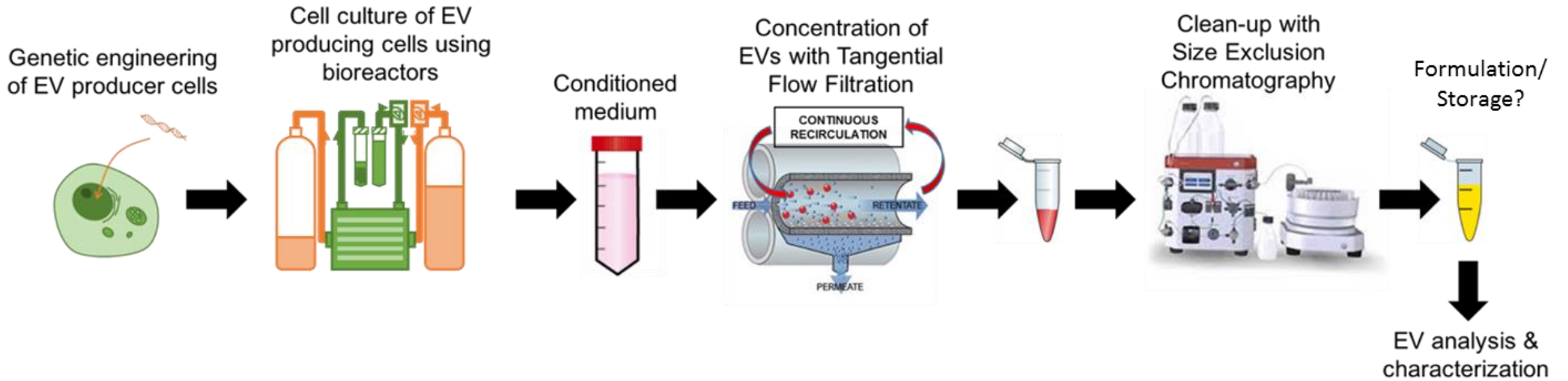
Extracellular Vesicles - Heterogeneity
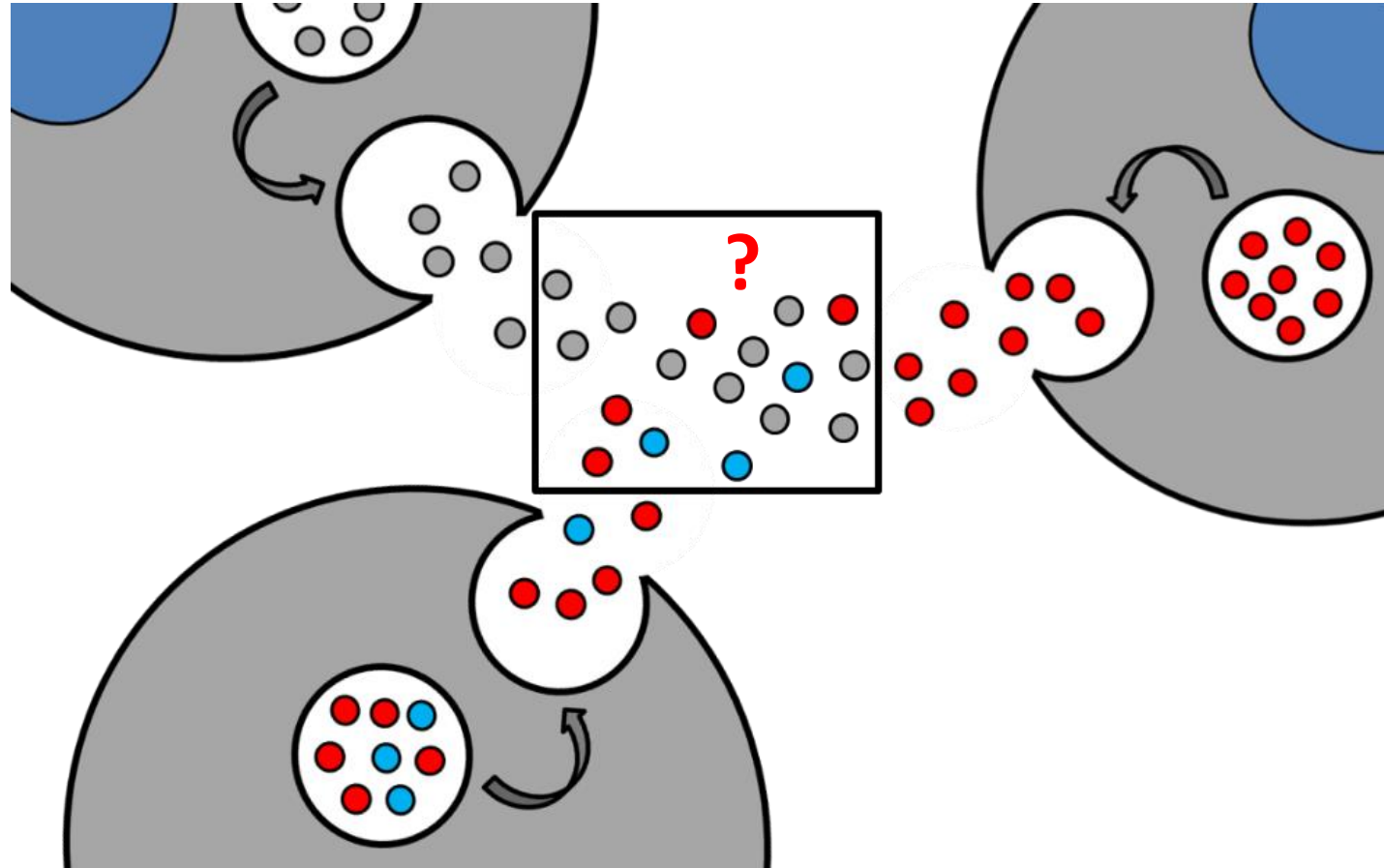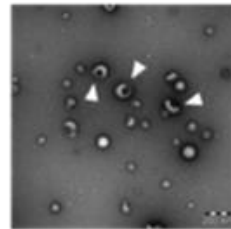

Objectives
- Understand EV heterogeneity
- Identify and define markers for EV subtypes
- Application: fractionation of subpopulations, functional testing
- Application: therapeutic and diagnostic use

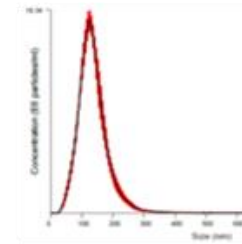→ **Reliable markers & optimized analytical methods required**

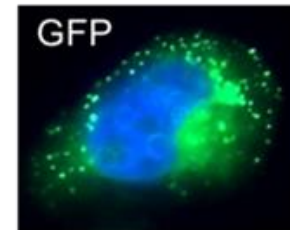# Exploratory EV engineering workflow (simplified)

Multiplex Bead-based Flow Cytometry

Single EV Imaging Flow Cytometry

Amnis Imagestream X MkII

Amnis Cellstream
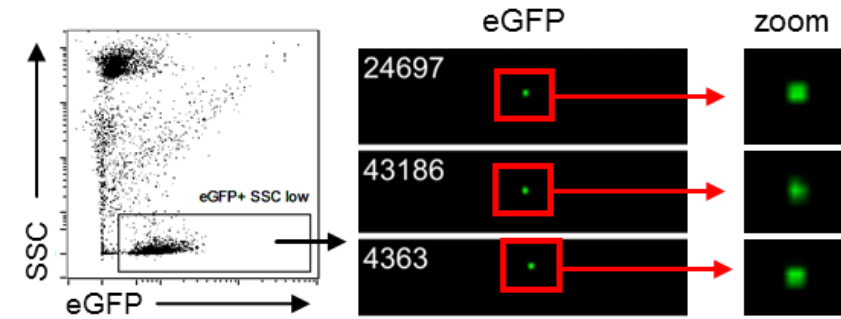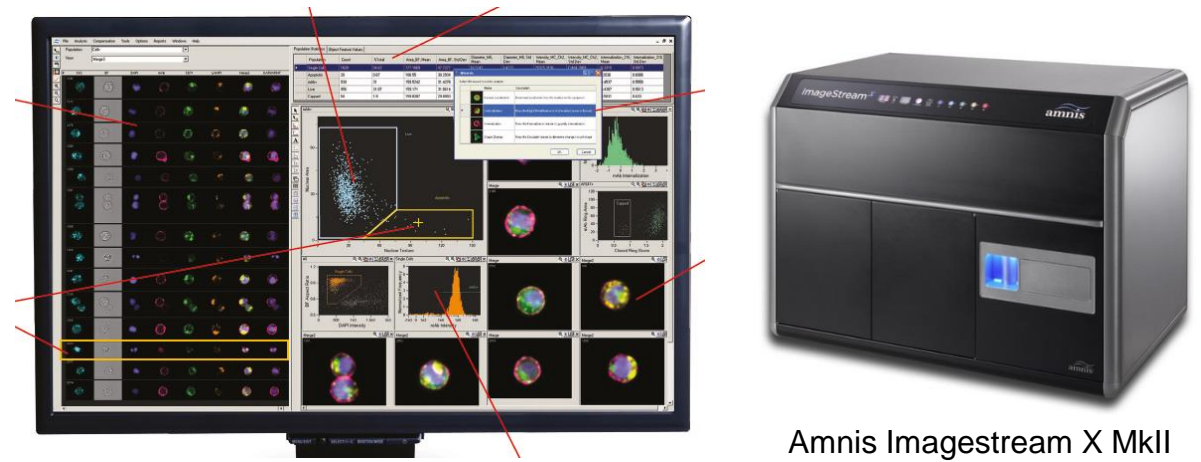
- Bead-based EV capture assay
- 39 multiplexed capture beads, antibody-based detection
- Bulk EV assay, **no** single EV analysis
- High throughput
- Can be used on most flow cytometers
- Semiquantitative

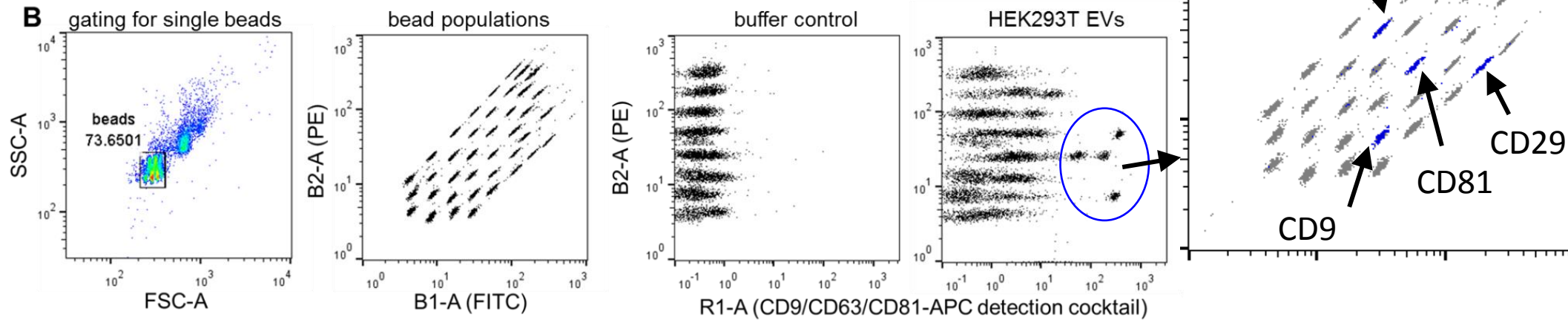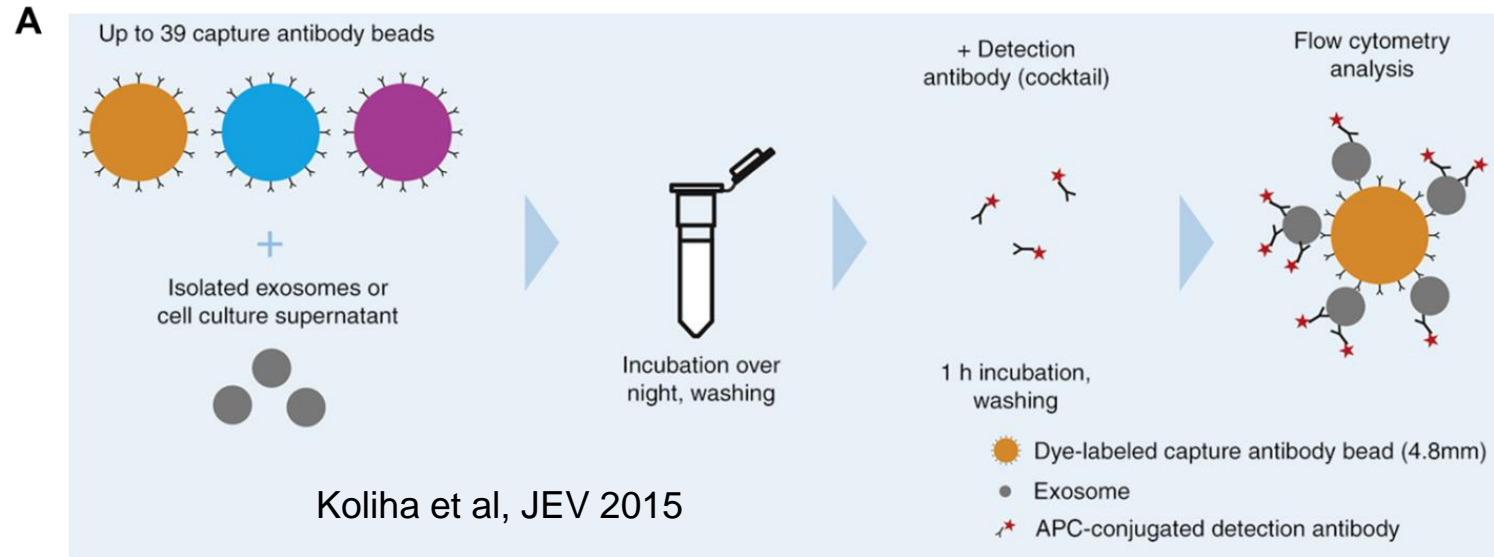Wiklander,…, Görgens, Frontiers in Immunology 2018

- CCD camera based signal detection
- Low sample rate, Time delay integration
- Multiple fluorescence parameters possible
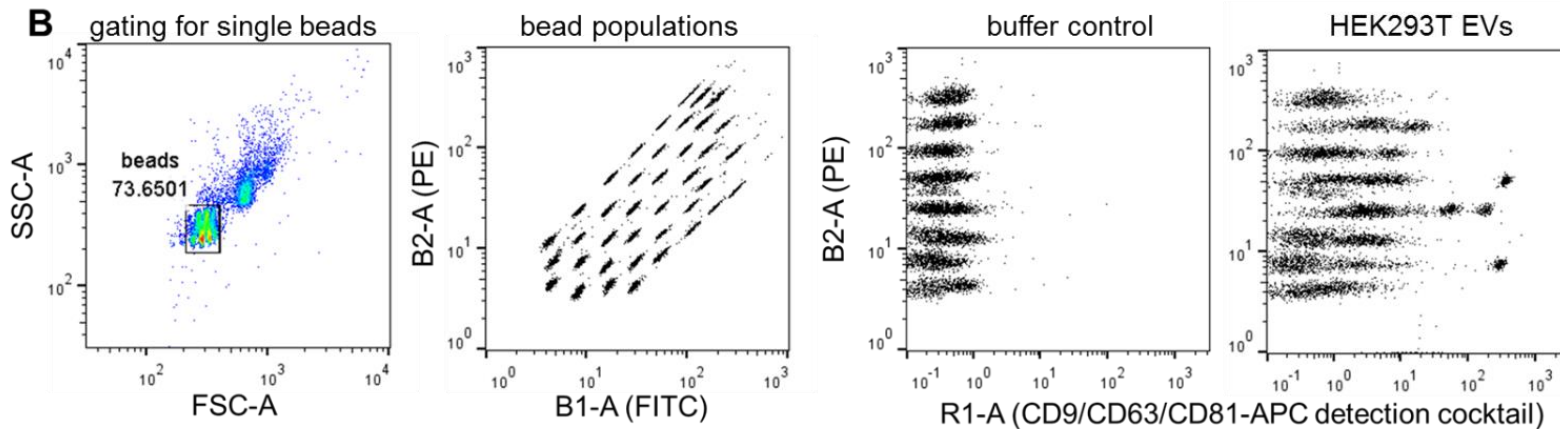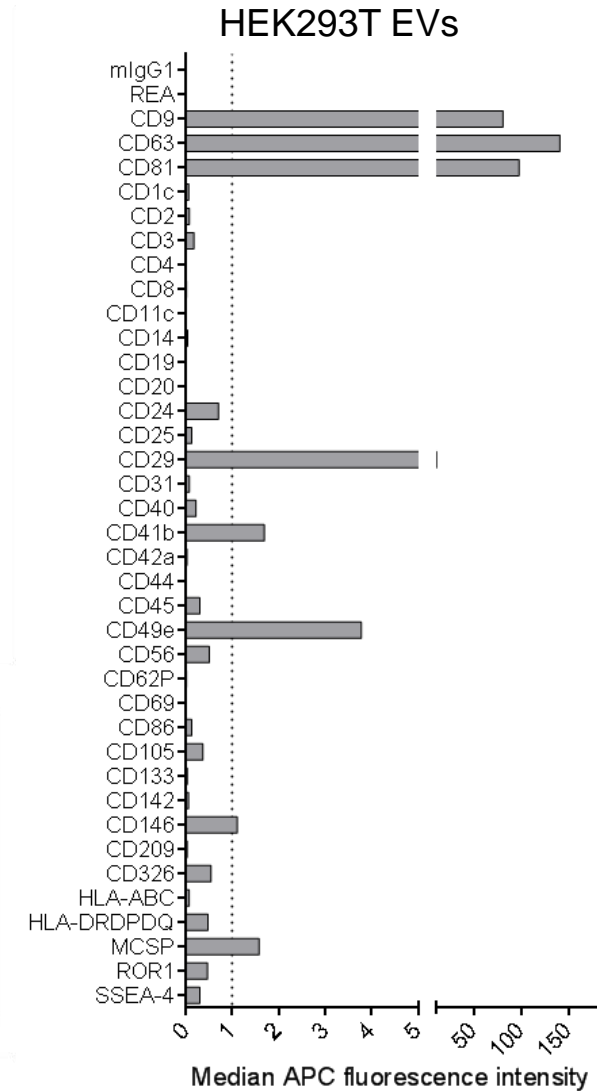- Facilitates single EV analysis
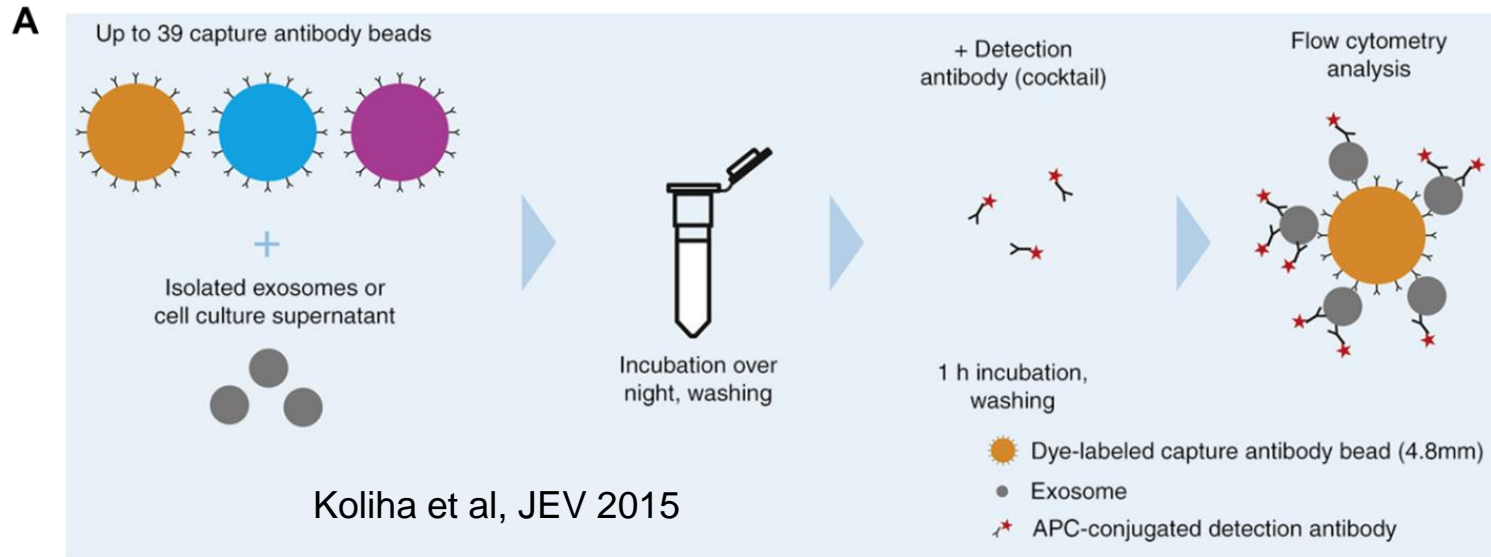- Highly quantitative

Görgens et al, JEV 2019

Multiplex Bead-based Assay for EV phenotyping

Koliha et al, JEV 2015

Wiklander,…, Görgens, Frontiers in Immunology 2018

Multiplex Bead-based Assay for EV phenotyping

Koliha et al, JEV 2015
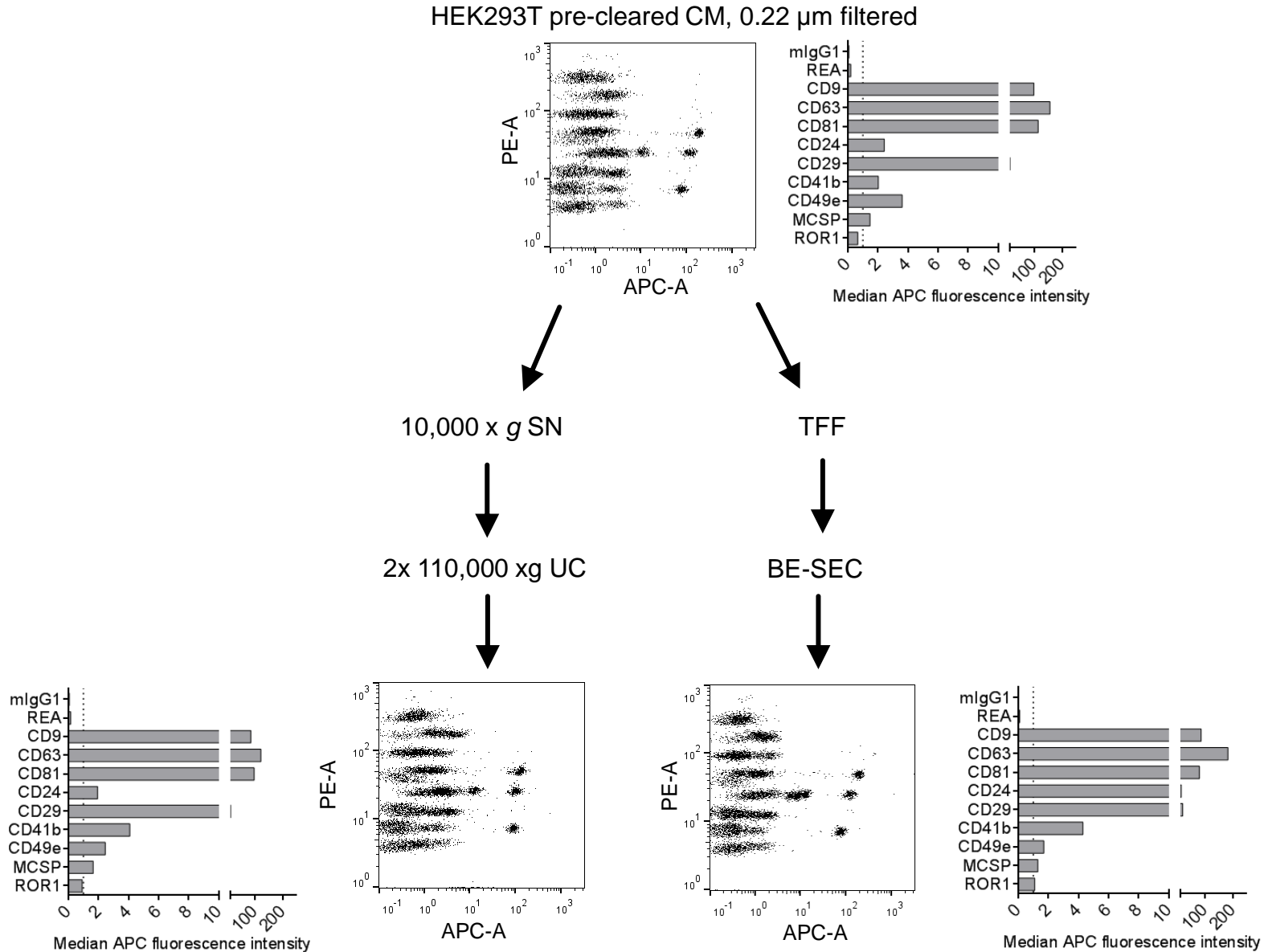
Wiklander,…, Görgens, Frontiers in Immunology 2018

Multiplex Bead-based Assay: How many EVs are required per assay?
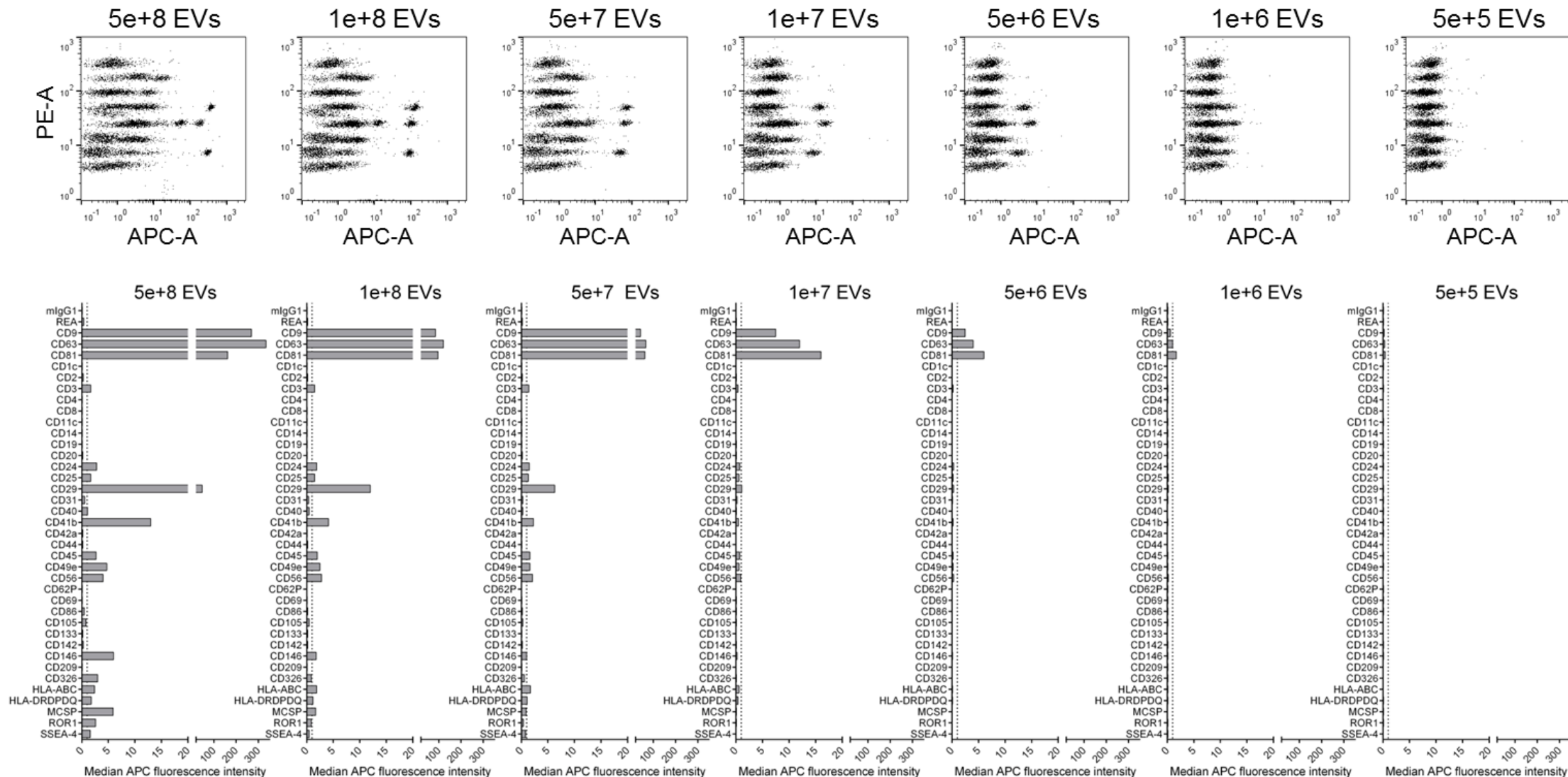
Detection for all samples: CD9+CD63+CD81. EVs derived from HEK293T cells

Wiklander,…, Görgens, Frontiers in Immunology 2018

Multiplex Bead-based Assay: Which sample types can be analyzed?

HEK293T pre-cleared CM, 0.22 µm filtered
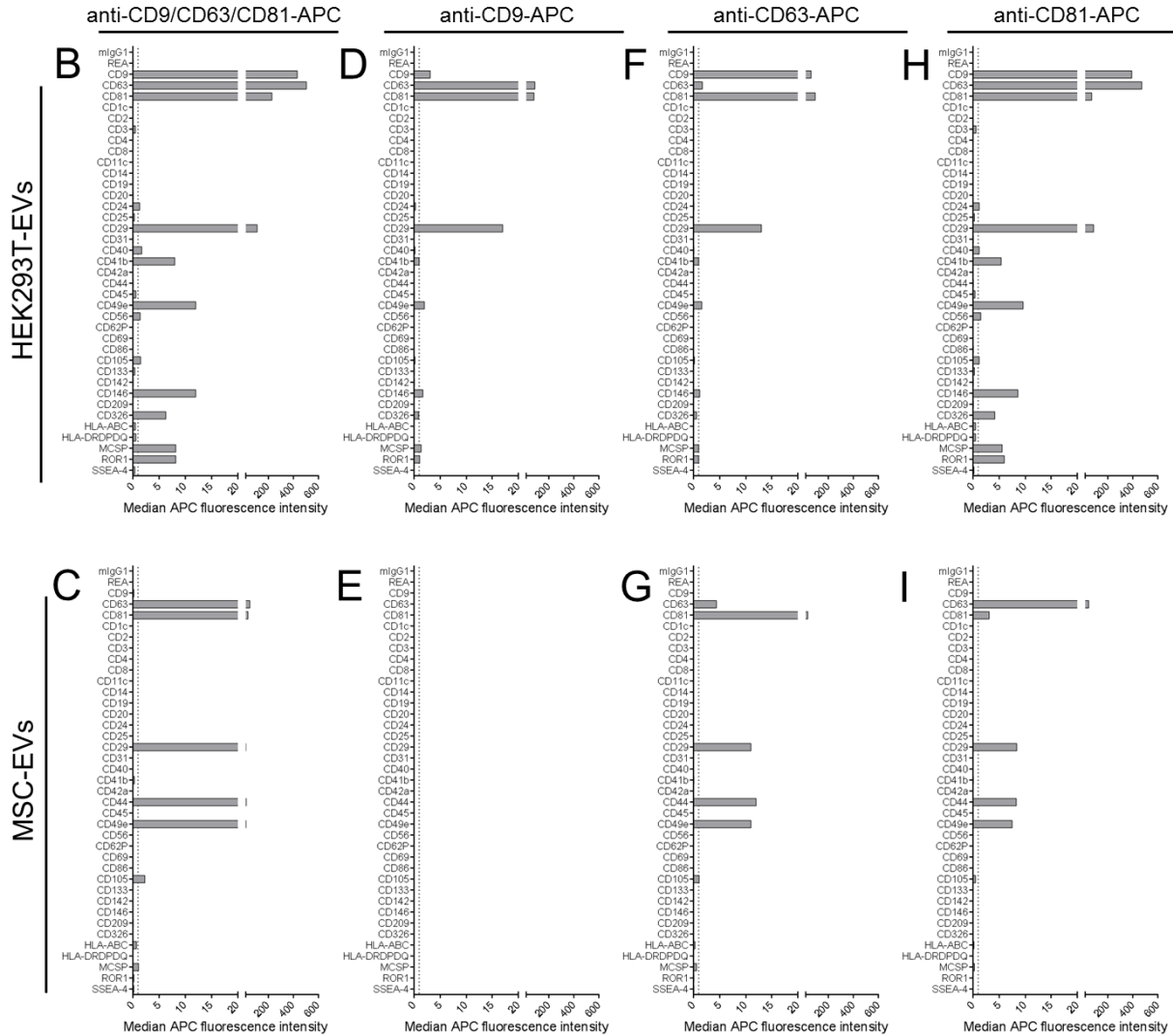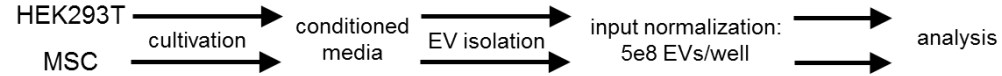

10,000 x *g* SN

TFF

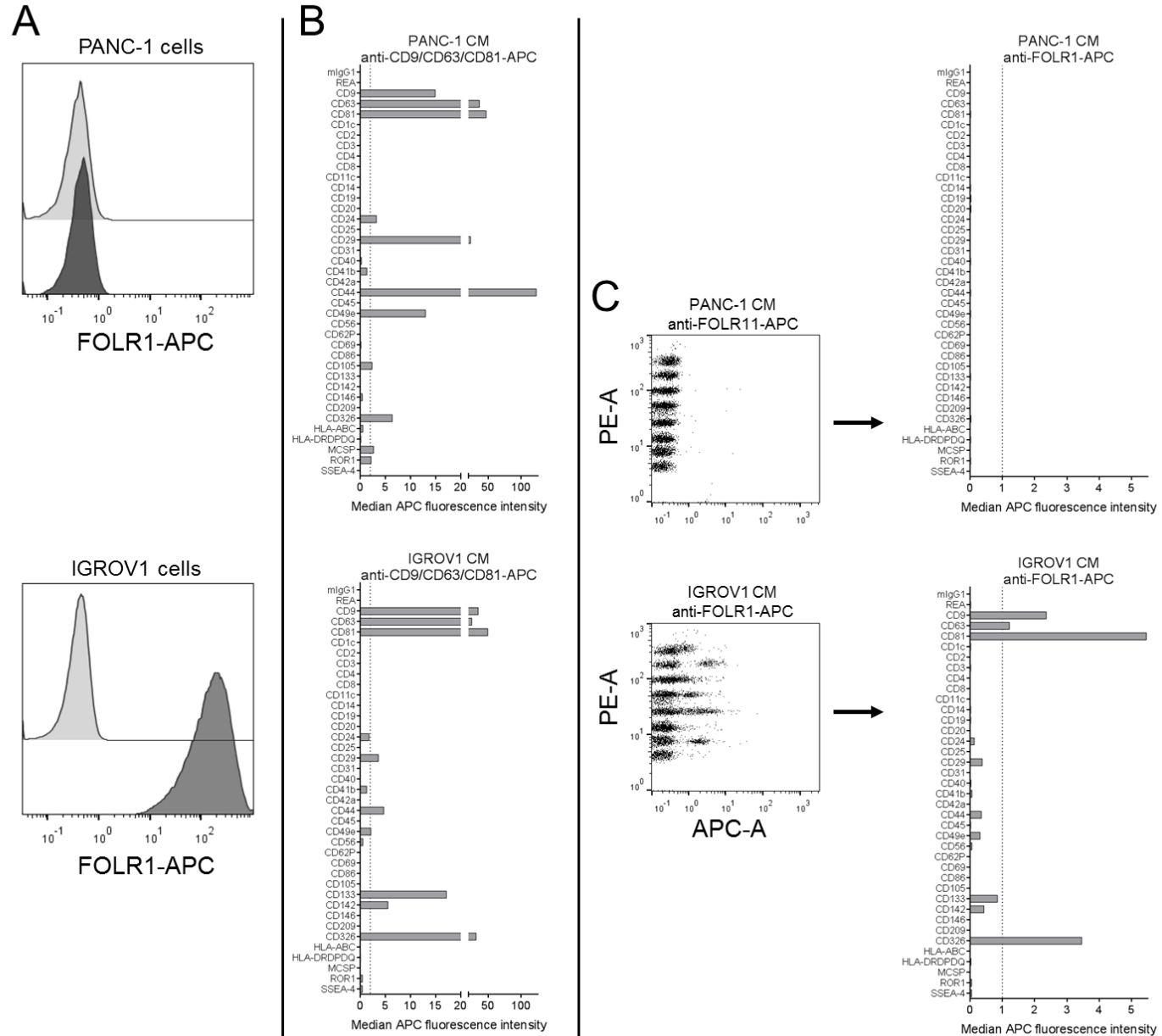2x 110,000 xg UC

BE-SEC

Detection for all samples: CD9+CD63+CD81

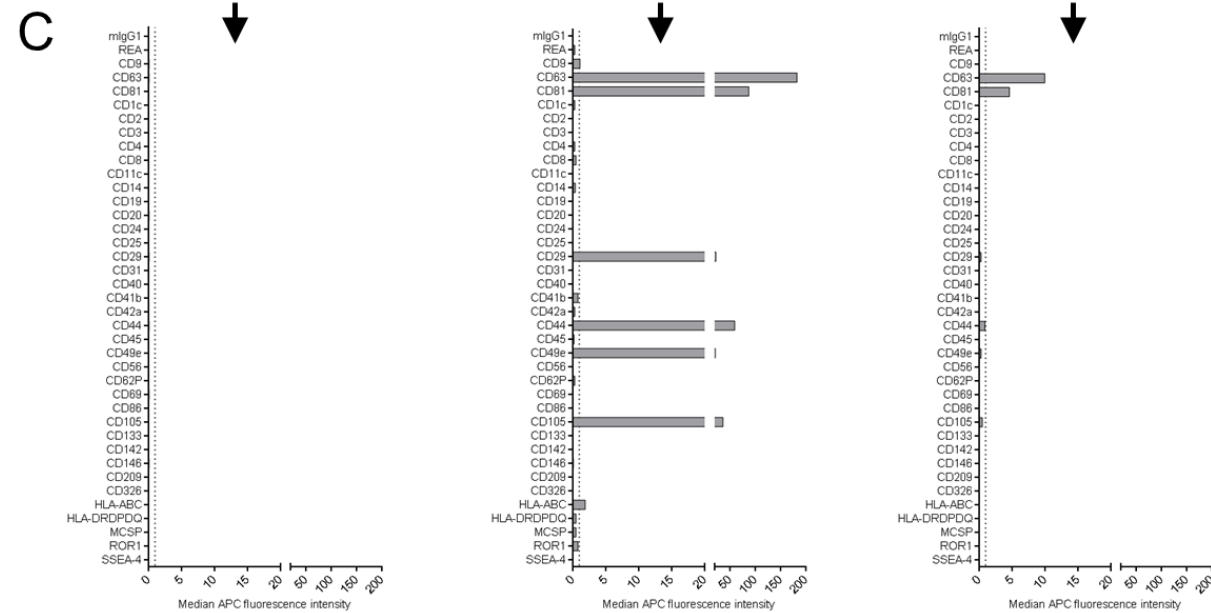Wiklander,…, Görgens, Frontiers in Immunology 2018

Comparison of EV surface signatures from different cell sources
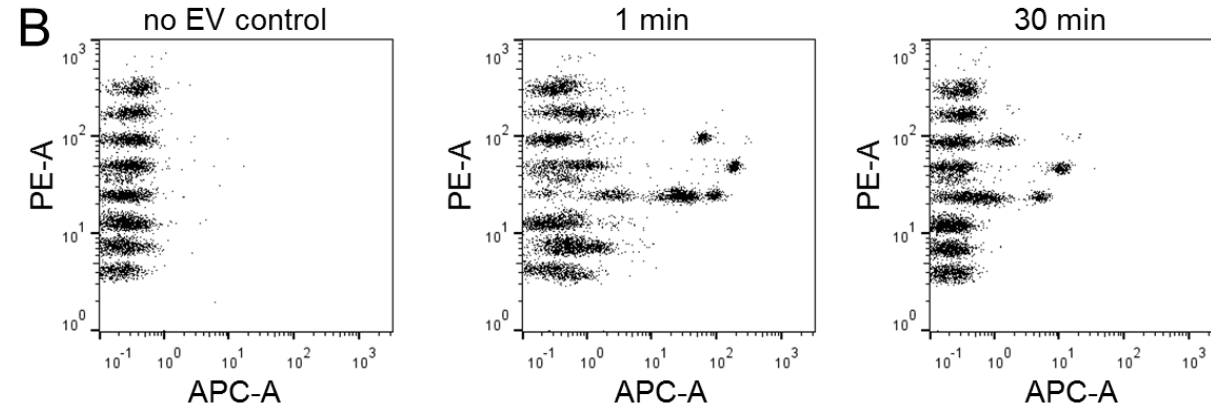
Usage of custom detection antibodies
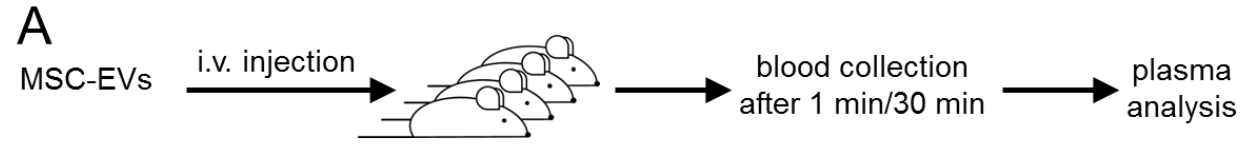

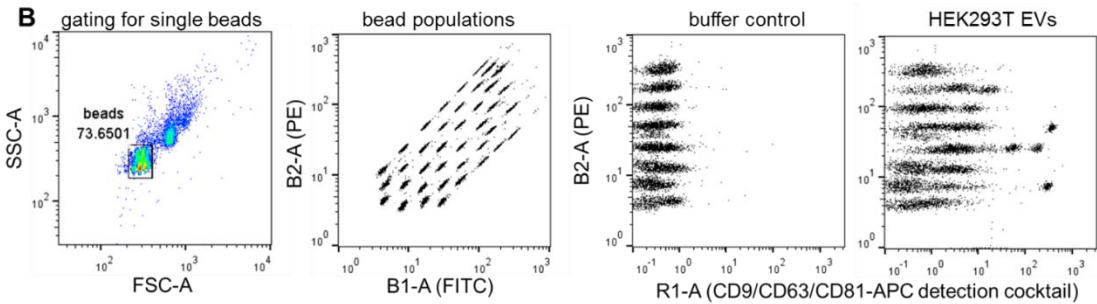Wiklander,…, Görgens, Frontiers in Immunology 2018

Detection of injected human EVs in mice

Detection for all samples: CD9+CD63+CD81

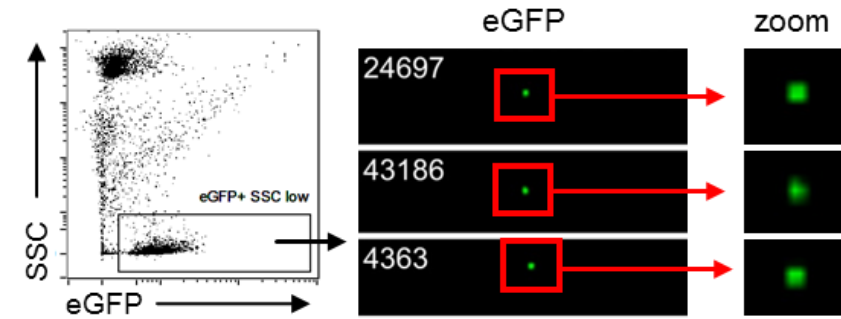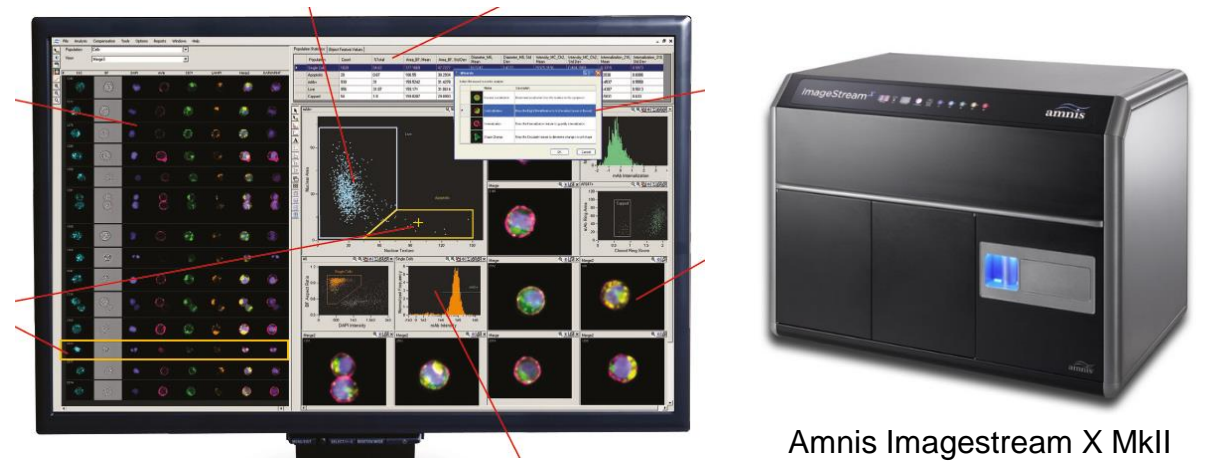Wiklander,…, Görgens, Frontiers in Immunology 2018

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Wiklander,…, Görgens, Frontiers in Immunology 2018

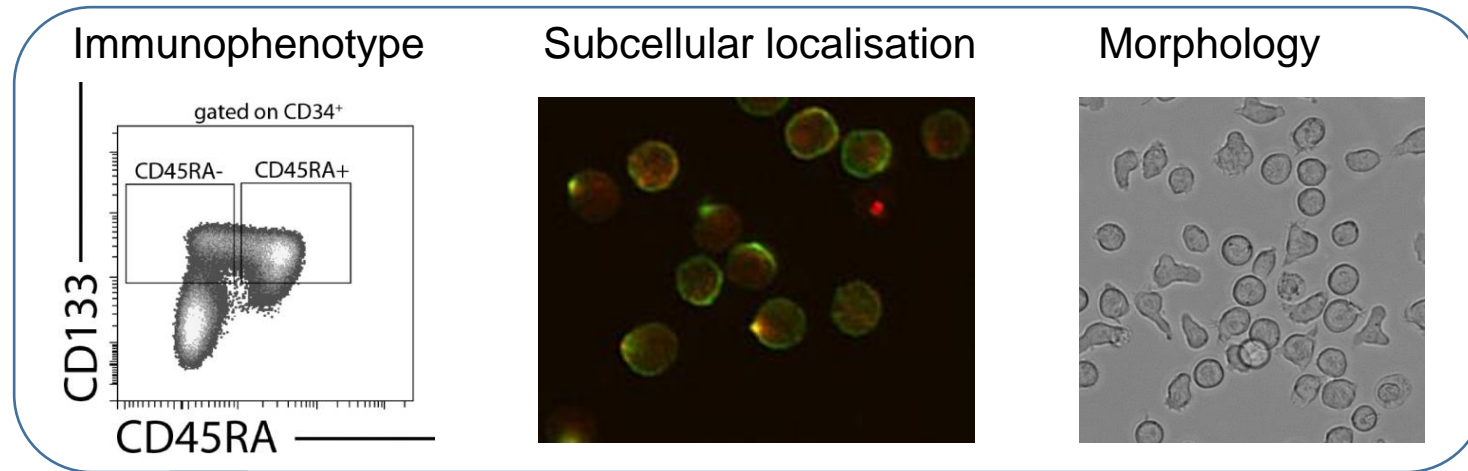# Single EV Imaging Flow Cytometry

Amnis Imagestream X MkII

- CCD camera based signal detection
- Low sample rate, Time delay integration
- Multiple fluorescence parameters possible
- Facilitates single EV analysis
- Highly quantitative

Görgens et al, JEV 2019

Amnis Cellstream

Imaging Flow Cytometry – Amnis ImageStream X MkII

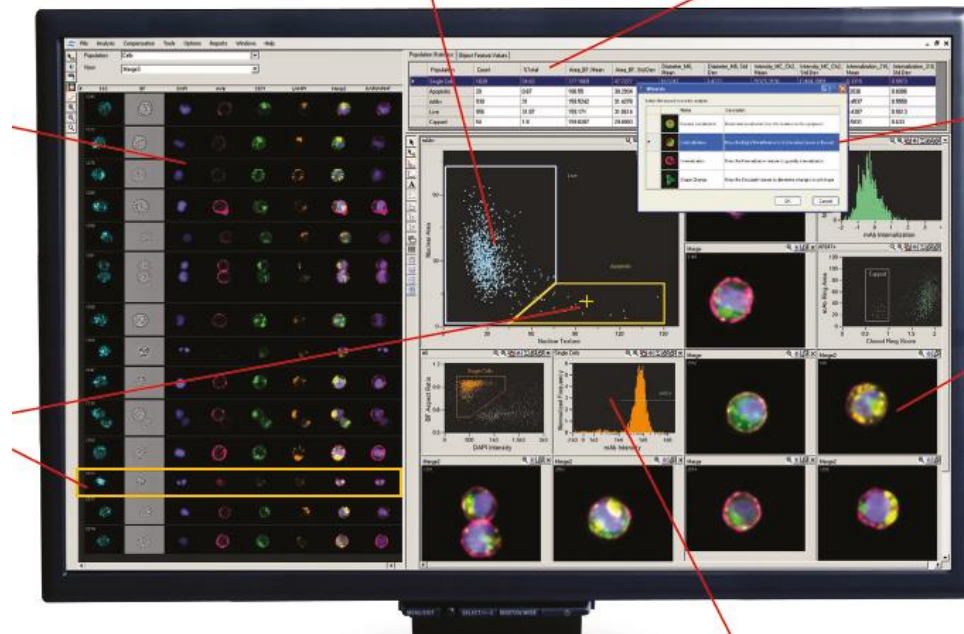Immunophenotype

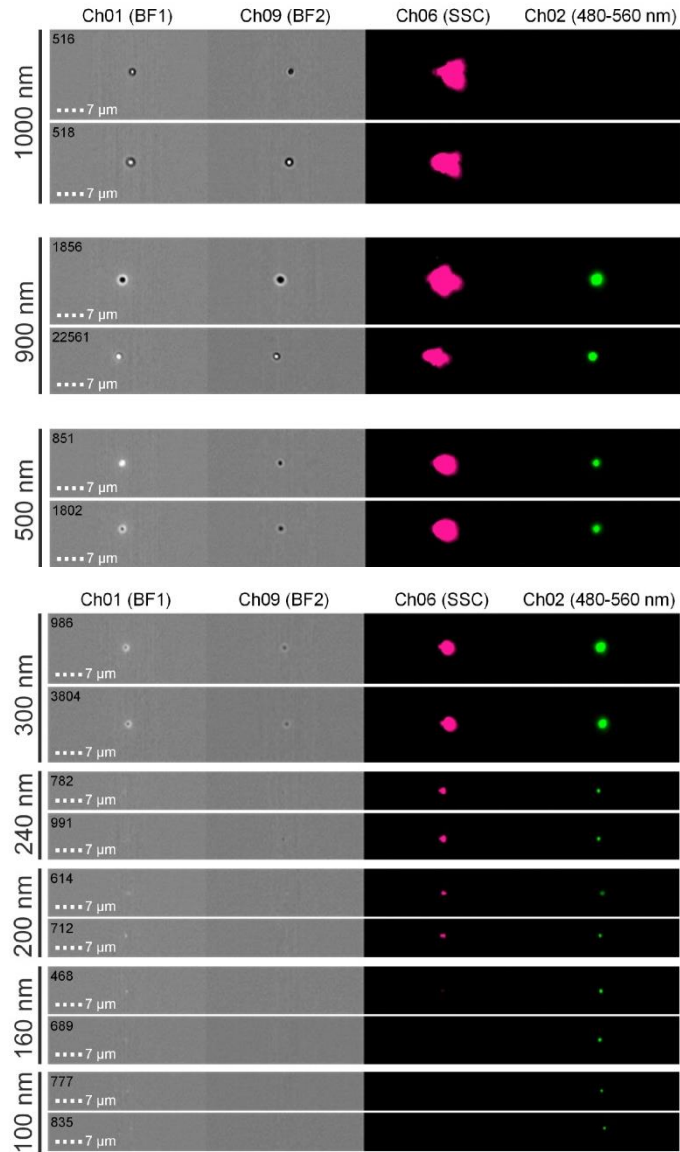Subcellular localisation

Morphology

Amnis Imagestream X MkII
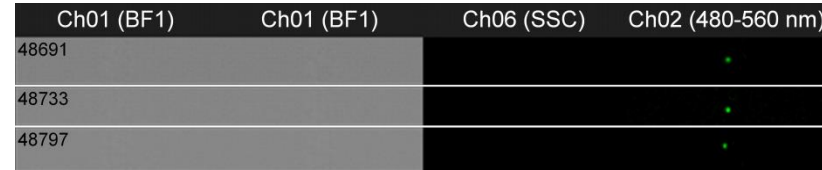
5 lasers
up to 12 channels

Triggering on all channels simultaneously (Size/SSC/FL)

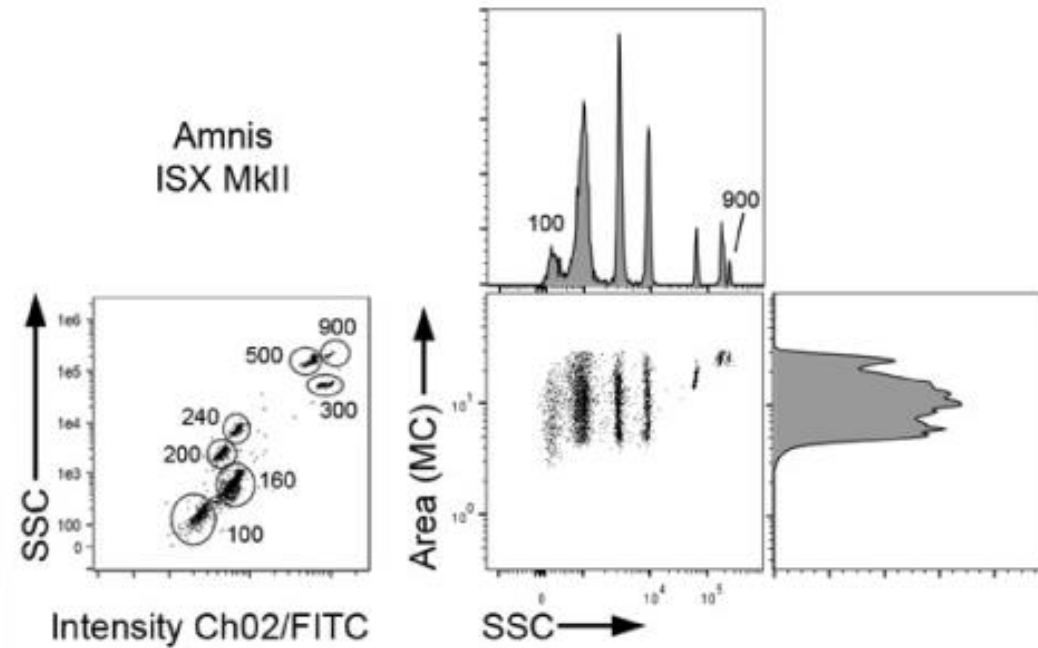# Submicron-Sized Beads as Reference Material

24 nm beads (Invitrogen)

✓ Beads

EVs?
Exosomes?
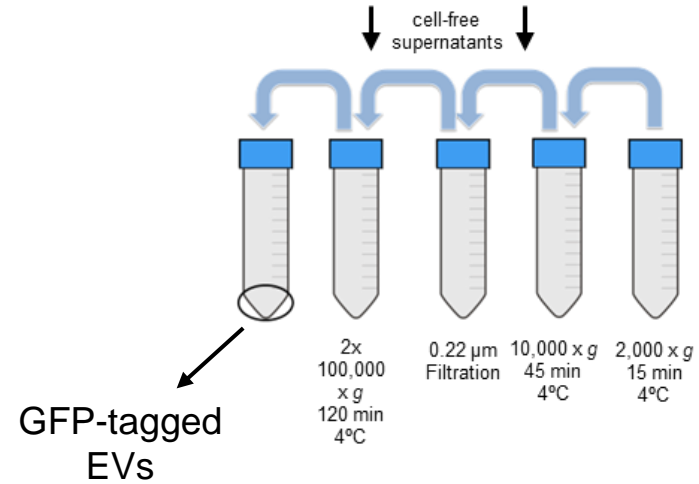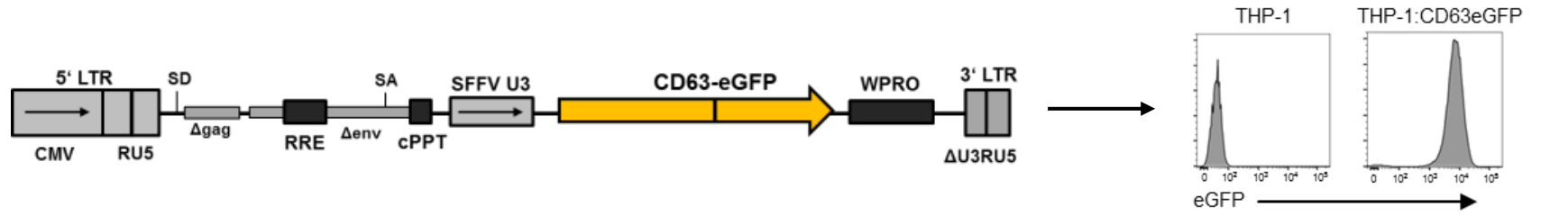
Amnis
ISX MkII

Görgens et al, JEV 2019

Imaging Flow Cytometry: CD63-eGFP EVs as Biological Reference Material

Görgens et al, JEV 2019
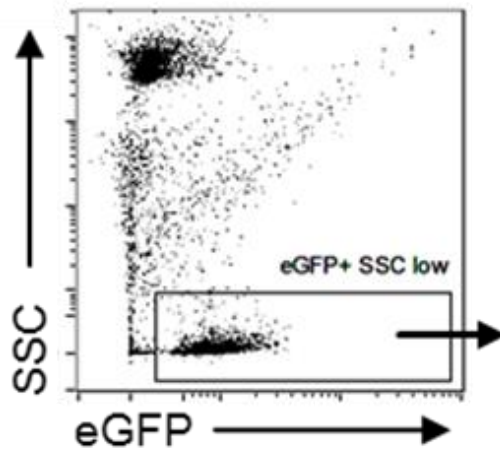
Imaging Flow Cytometry: CD63-eGFP EVs as Biological Reference Material

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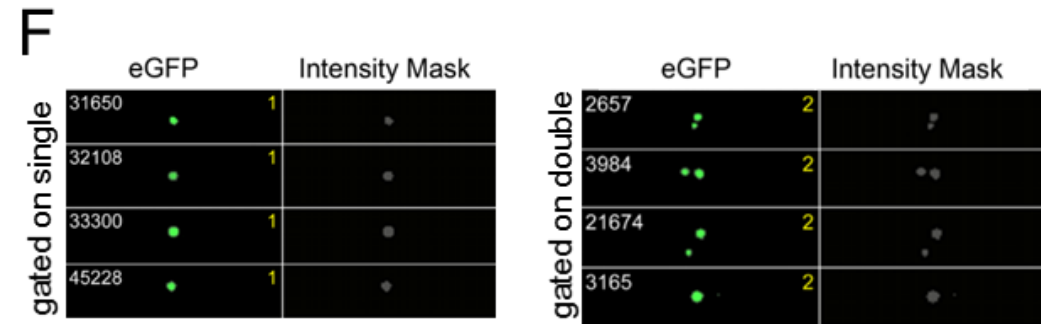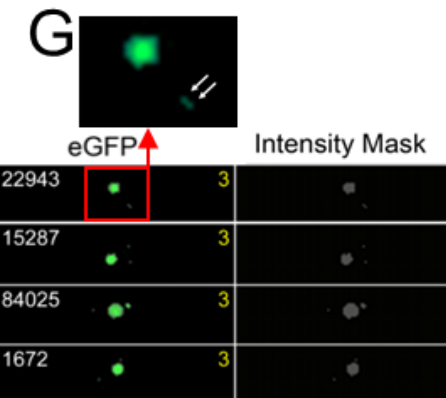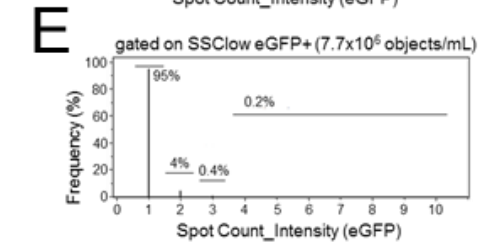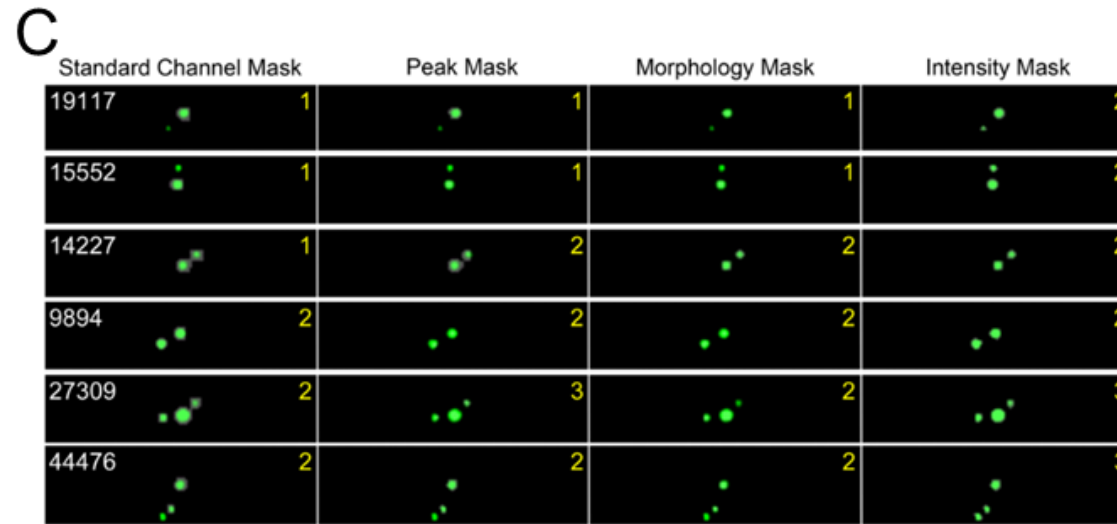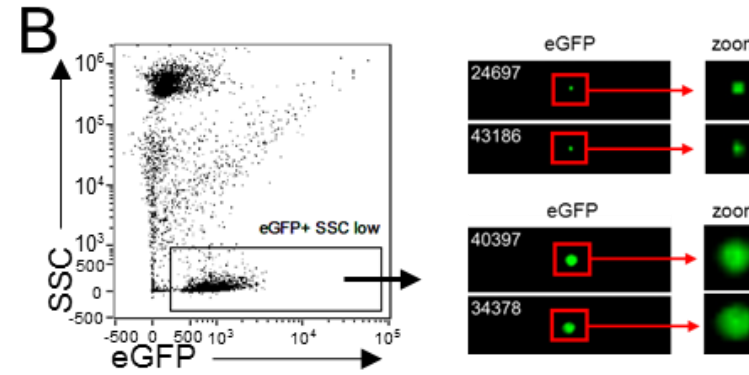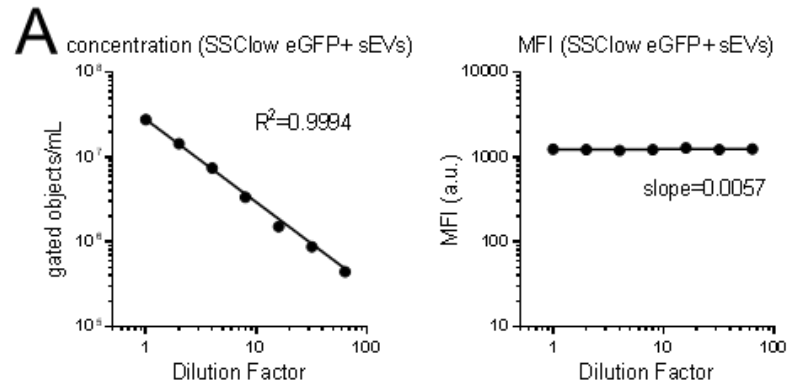
Evaluation of sEV isolation protocols/EV Subset Detection in Unprocessed Samples

Görgens et al, JEV 2019
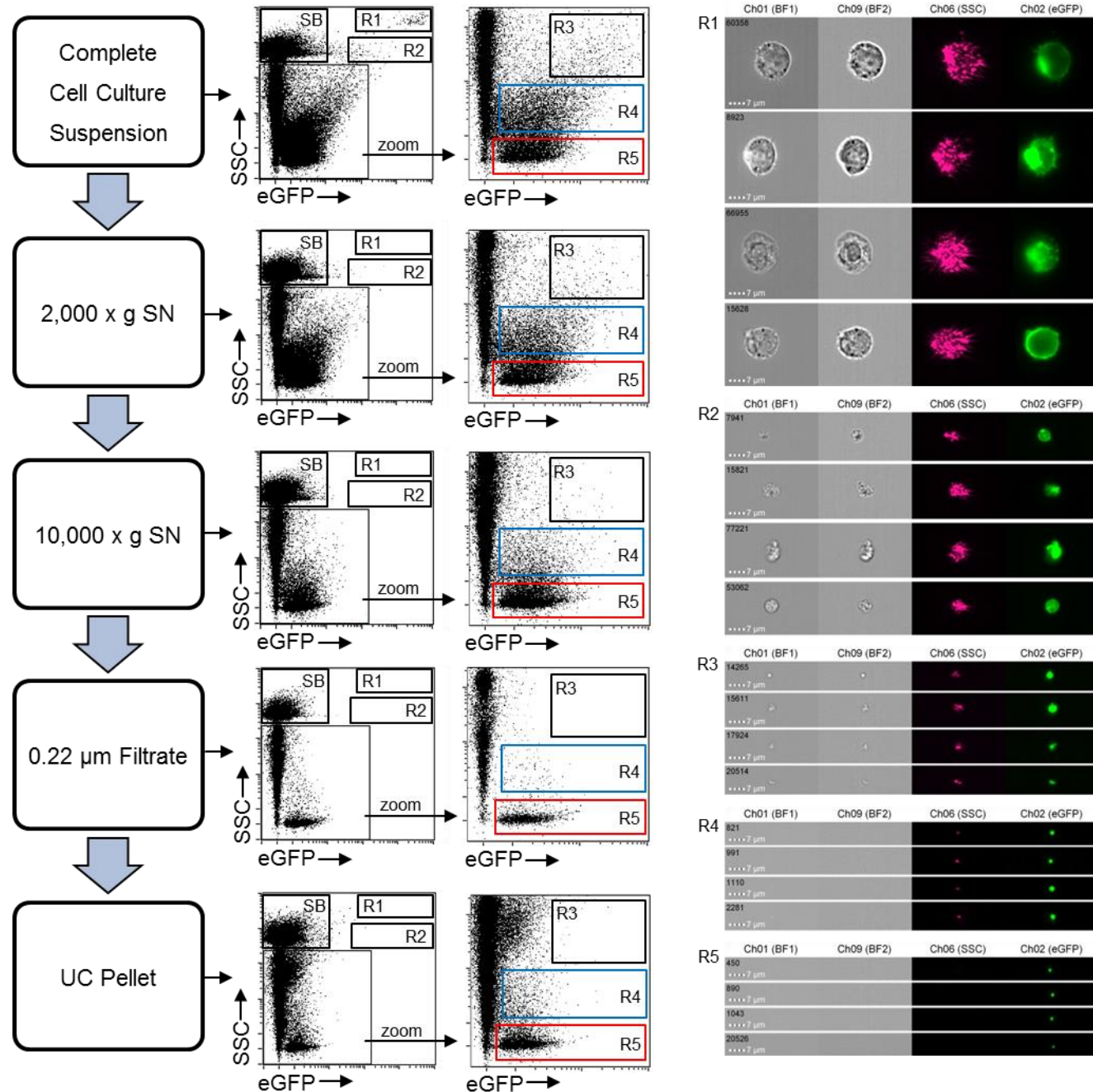
Imaging Flow Cytometry: Staining of surface markers with antibodies

Görgens et al, JEV 2019
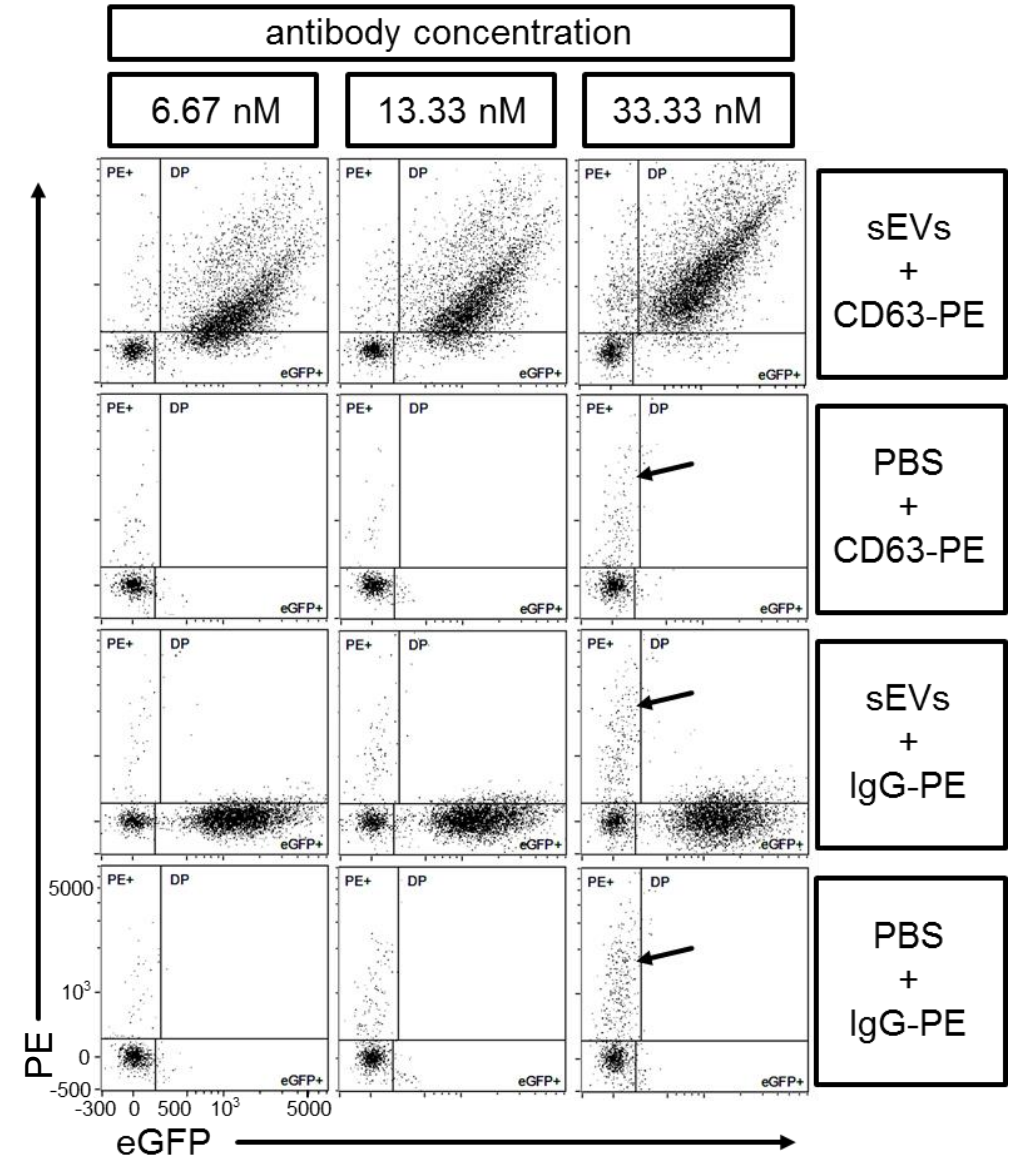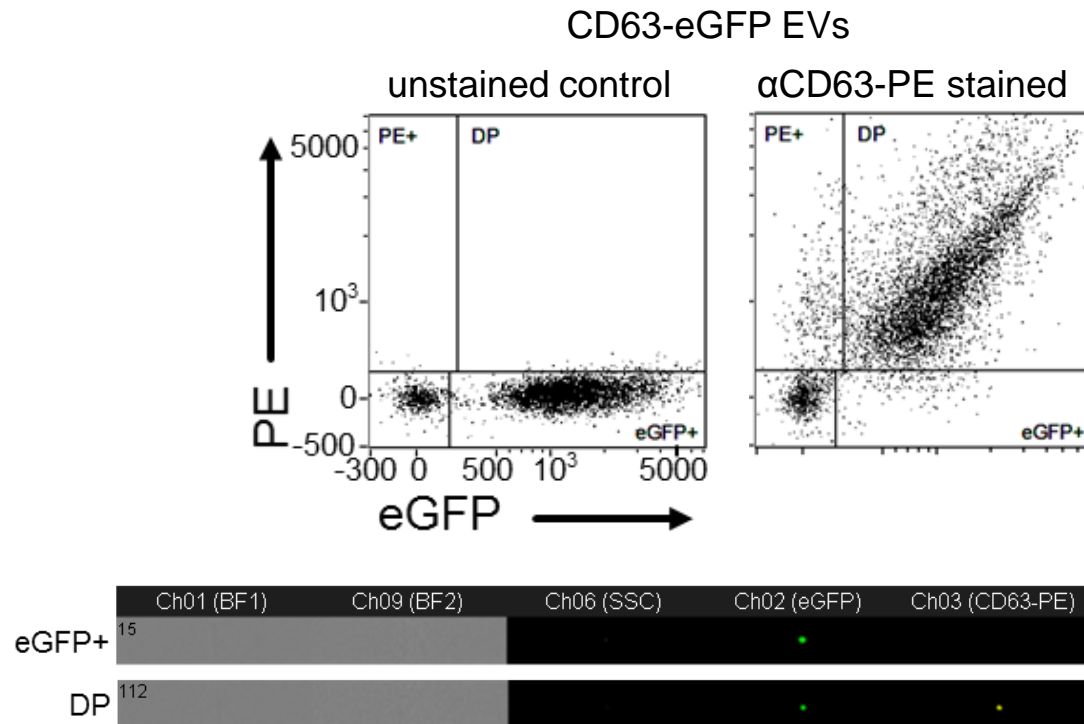
Imaging Flow Cytometry: EV Heterogeneity

Görgens et al, JEV 2019
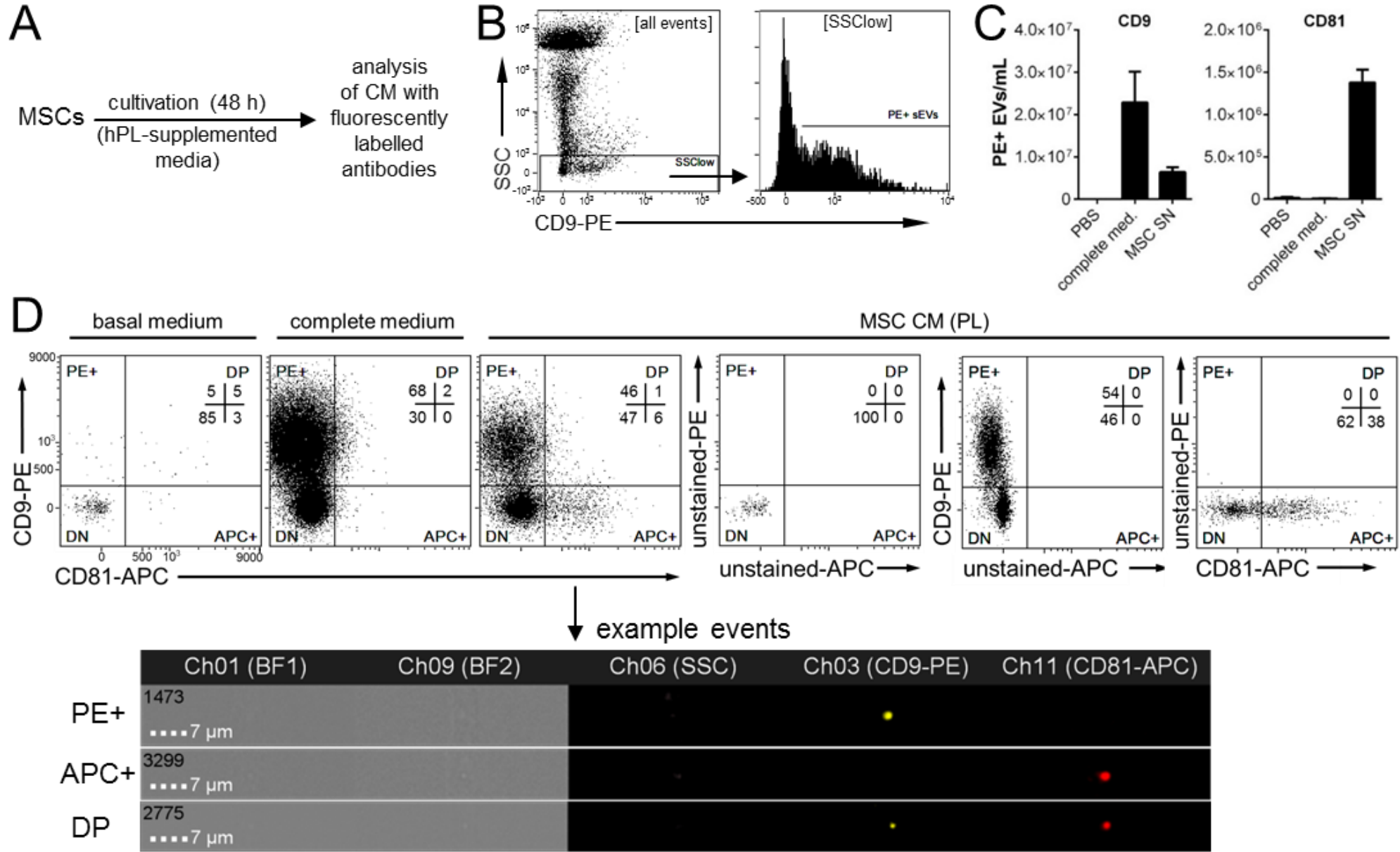
Imaging Flow Cytometry: Absolute Fluorescence Quantification

A

FITC MESF beads — Intensity_MC or NMC_Ch02 (Pk1, Pk2, Pk3, Pk4, Pk5)

PE MESF beads — Intensity_MC or NMC_Ch03 (Pk1, Pk2, Pk3, Pk4)
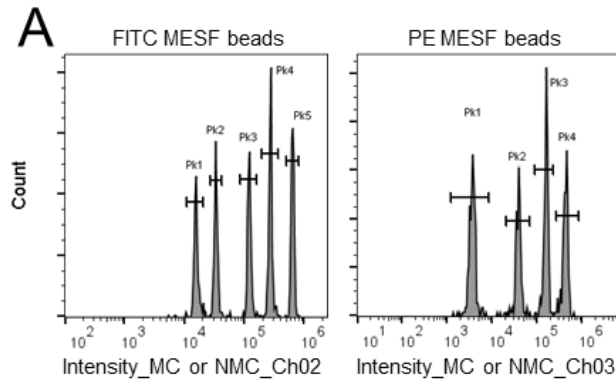
B

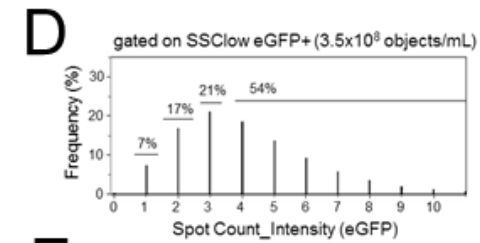| FITC MESF beads				
Peak	MFI	Log MFI	MESF	Log MESF
Pk1	15,364.03	4.1865	11,093	4.0450
Pk2	32,556.34	4.5126	38,718	4.5879
Pk3	116,688.06	5.0670	132,448	5.1220
Pk4	263,475.94	5.4207	361,327	5.5579
Pk5	615,222.88	5.7890	701,416	5.8460

PE MESF beads				
Peak	MFI	Log MFI	MESF	Log MESF
Pk1	3,567.94	3.5524	474	2.6758
Pk1	37,396.97	4.5728	5,359	3.7291
Pk3	153,446.53	5.1860	23,843	4.3774
Pk4	411,716.32	5.6146	62,336	4.7947

C

FITC MESF regression analysis
$y = 1.109x - 0.5077$
$R^2 = 0.9887$
(log MFI vs log MESF)

PE MESF regression analysis
$y = 1.0316x - 0.9866$
$R^2 = 0.9999$
(log MFI vs log MESF)

D uncalibrated data

MFI CD63-PE: 1037.45
MFI CD63eGFP: 902.89
(Intensity_MC or NMC_CD63-PE vs Intensity_MC or NMC_CD63eGFP)

E MESF calibrated data

Mean PE MESF: 174.41
Mean FITC MESF: 588.53
(PE MESF vs FITC MESF)
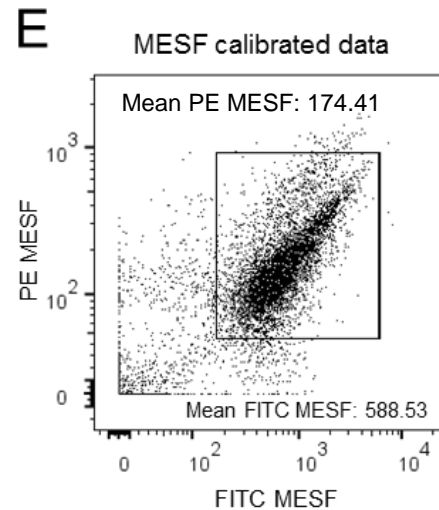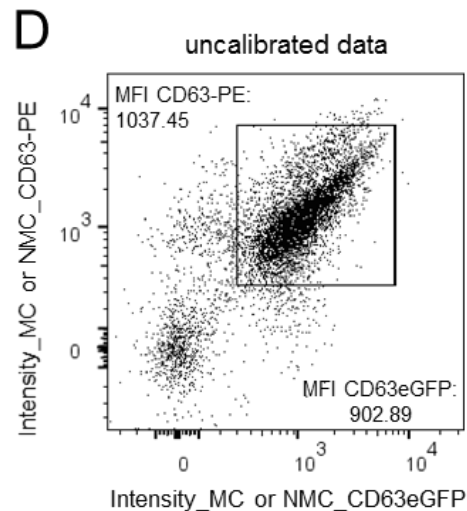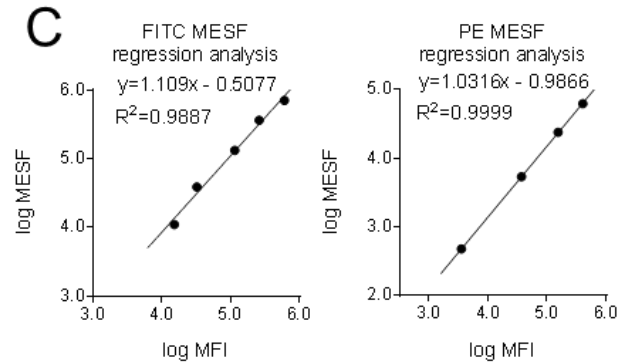
MESF = Molecules of Equivalent Soluble Fluorochrome
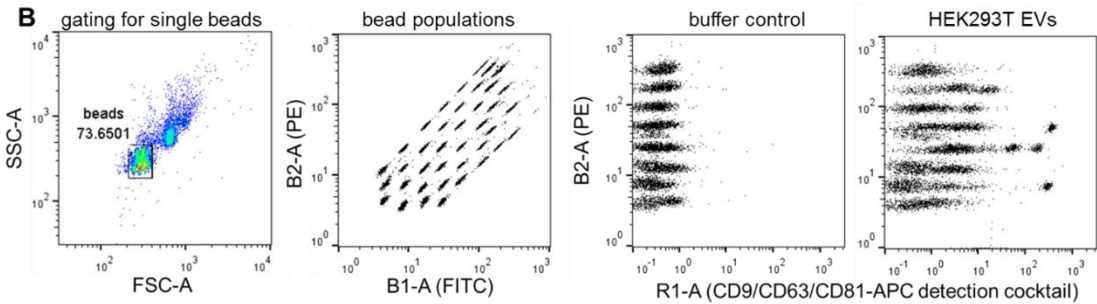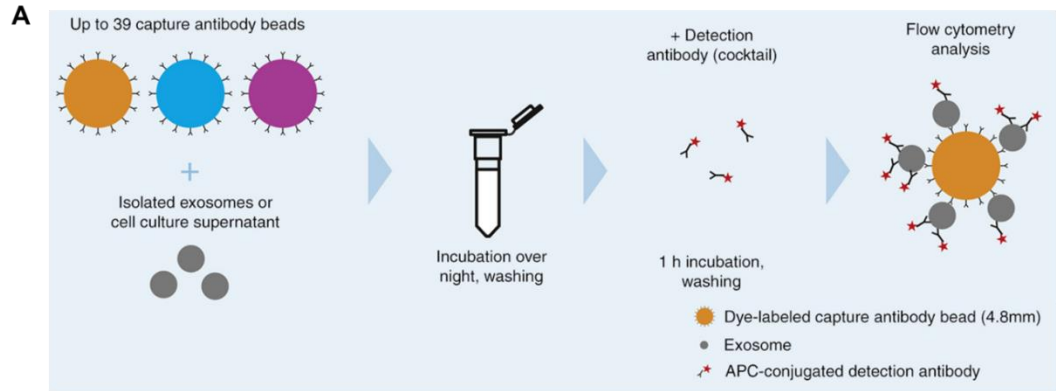
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Wiklander,…, Görgens, Frontiers in Immunology 2018
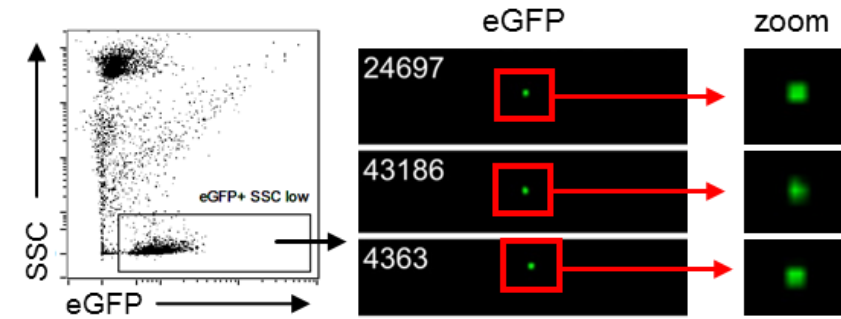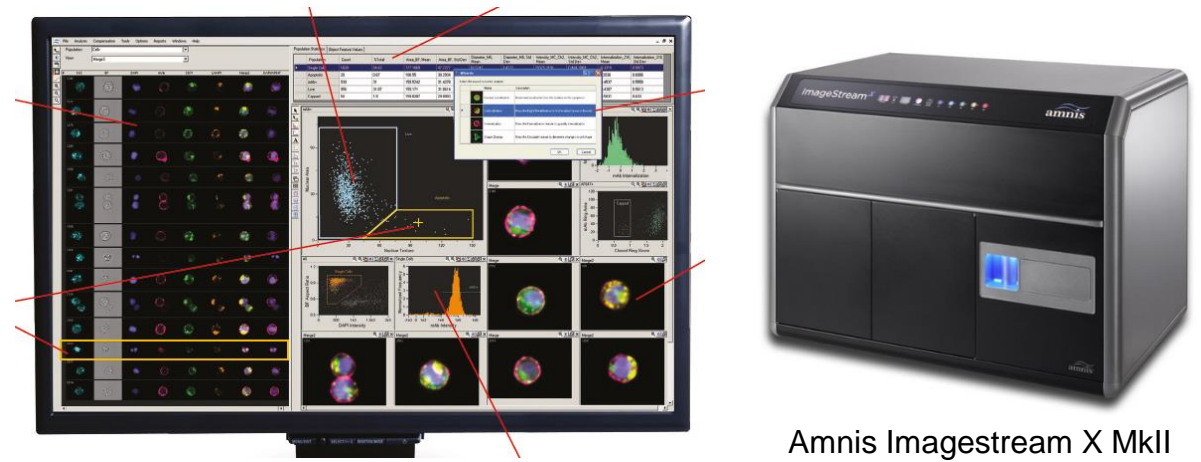
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Görgens et al, JEV 2019

Amnis Cellstream

Outlook: Assessment of EV surface signatures in biological fluids

Detection for all samples: CD9+CD63+CD81

Wiklander,…, Görgens, Frontiers in Immunology 2018

Acknowledgements
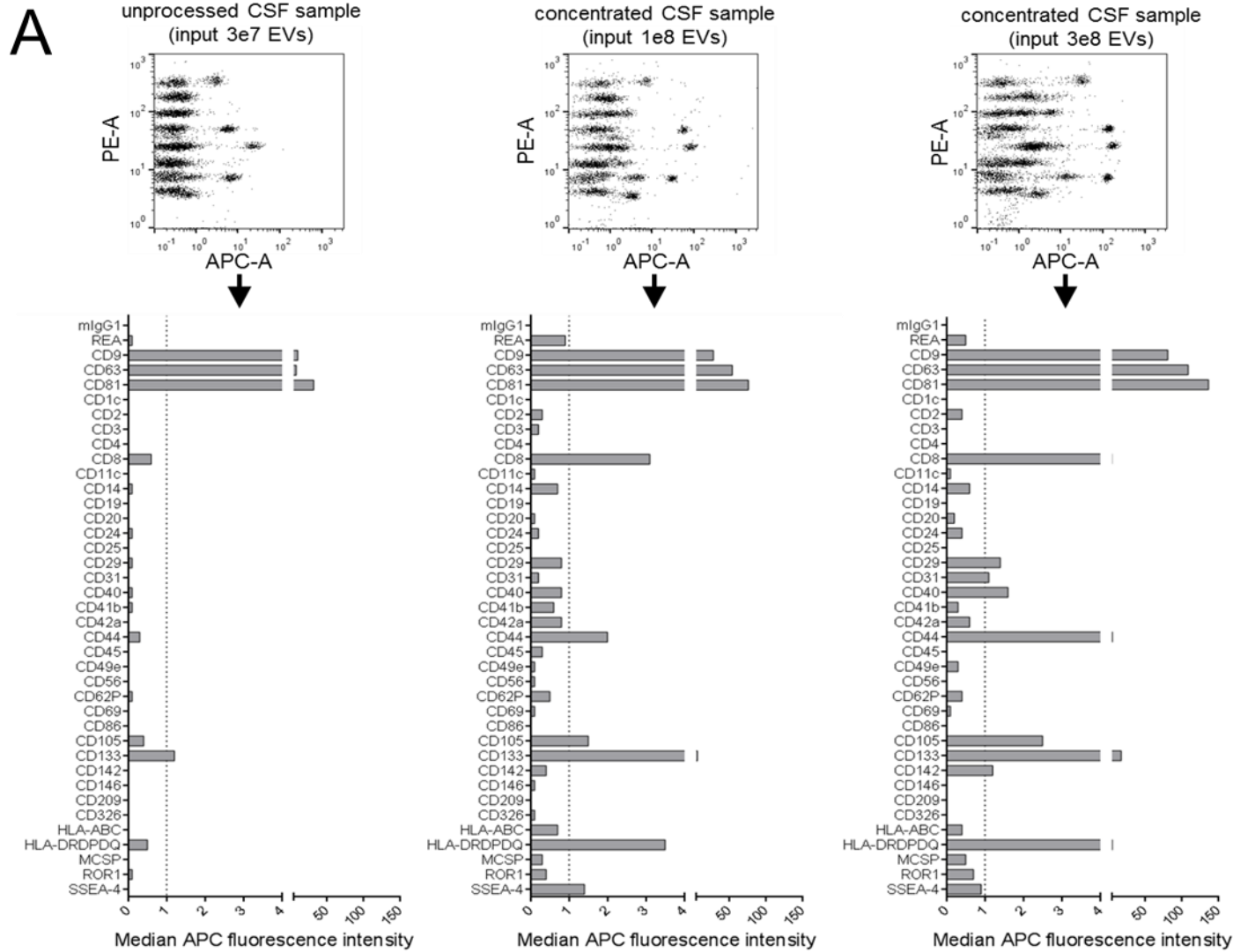
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Helmut Hanenberg

evox THERAPEUTICS

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www.evflowcytometry.org