# Characterization of Extracellular Vesicles by Flow Cytometry: A Methodological Approach

Metro Flow

October 2016

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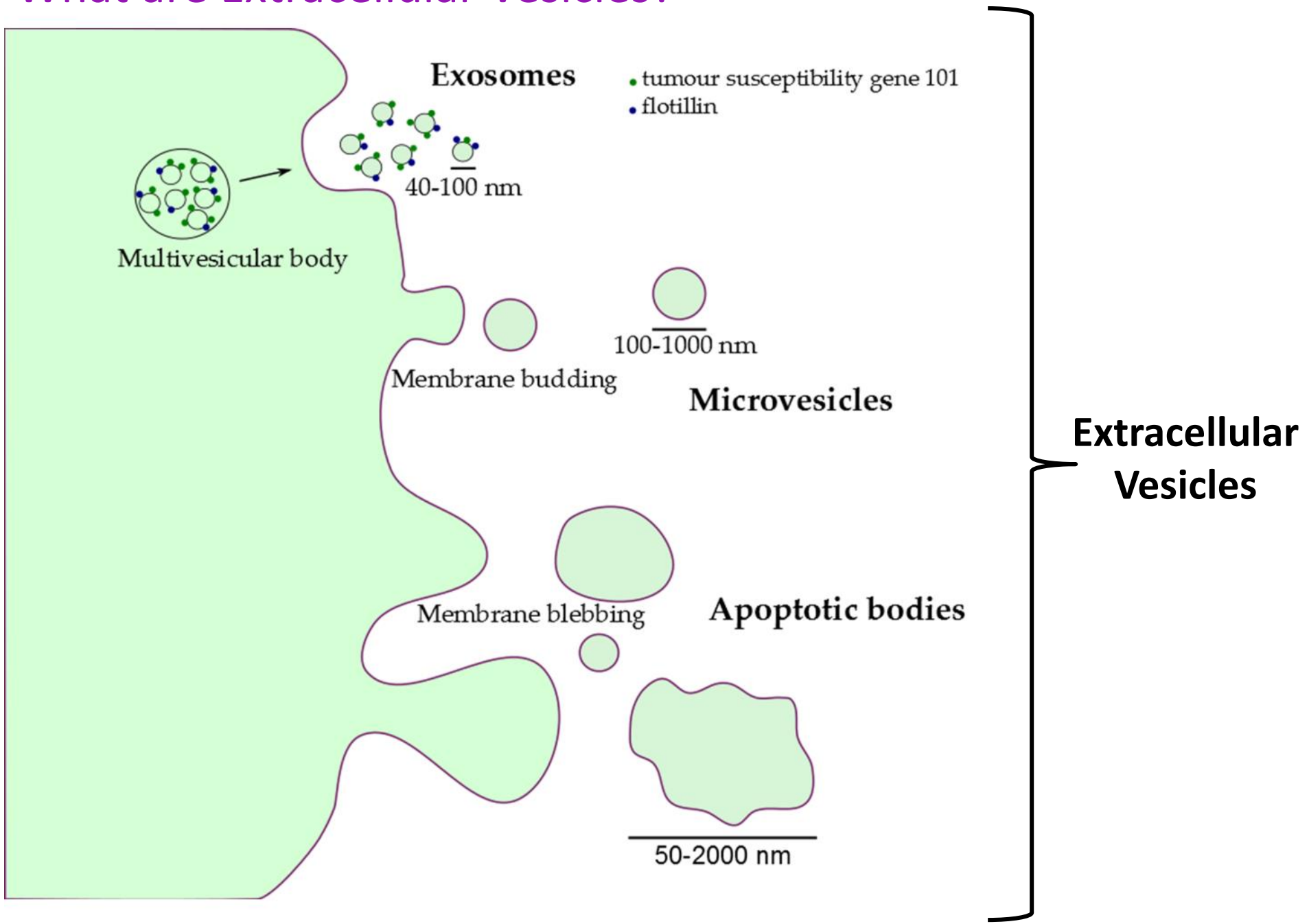
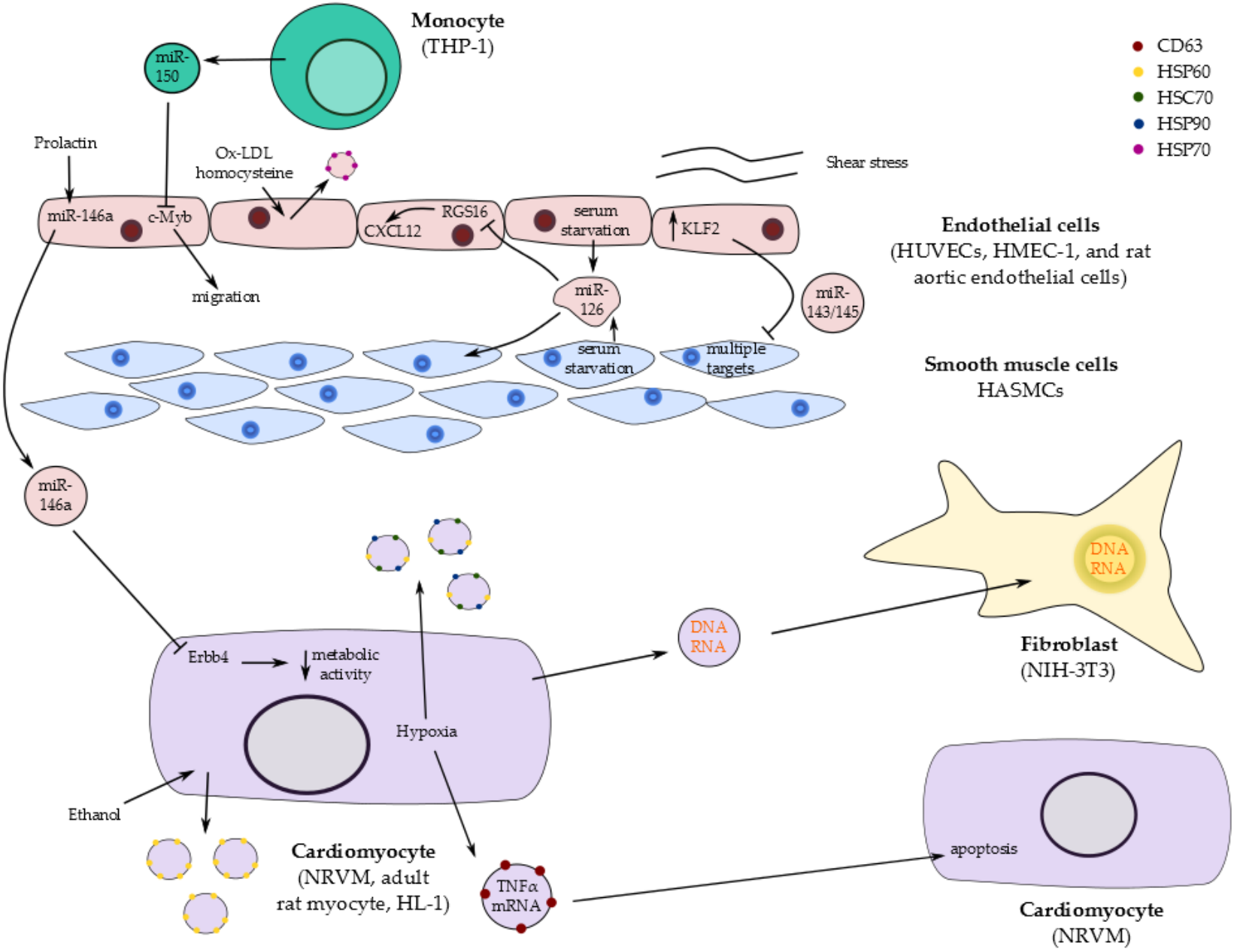
# What are Extracellular Vesicles?




Monocyte
(THP-1)
miR-150
CD63
HSP60
HSC70
HSP90
HSP70
Prolactin
Ox-LDL
homocysteine
Shear stress
miR-146a
c-Myb
migration
CXCL12
RGS16
serum starvation
KLF2
Endothelial cells
(HUVECs, HMEC-1, and rat aortic endothelial cells)
miR-126
miR-143/145
serum starvation
multiple targets
Smooth muscle cells
HASMCs
miR-146a
DNA
RNA
Fibroblast
(NIH-3T3)
Erbb4
metabolic activity
Hypoxia
DNA
RNA
Ethanol
Cardiomyocyte
(NRVM, adult rat myocyte, HL-1)
TNFα mRNA
apoptosis
Cardiomyocyte
(NRVM)

# Why Study Extracellular Vesicles (EVs)?

- EVs and their cargo as functional biomarkers
  - Extant in many biofluids: blood, csf, saliva, etc.
- Can contain a wide array of protein, RNA, and lipids that is capable of transferring between cell types
  - microRNAs, piRNAs, tRNA fragments, etc.
- Work between our labs has identified several EV-bound miRNAs associated with heart failure

# Current technologies for analyzing EVs

- Dynamic Light Scatter
- Nanoparticle Tracking Analysis
- Tunable Resistive Pulse Sensing
- Microscopy
- Flow Cytometry
  - Non-fluorescent, non-antibody approach
  - Analysis of whole plasma
  - Sorting of populations for downstream analysis

# Why (nano)Flow? : Biological Validity

- Extant methods have benefits but alter EV character
  - YFP-CD41 transgenic mice
  - Centrifugation gradients
  - Labeling methods can get 100-200nm resolution
    - Lack of universal EV marker
- Minimal manipulation of samples
  - Less bias
  - Point of care diagnostic potentials

# Why Flow? (nanoFlow)

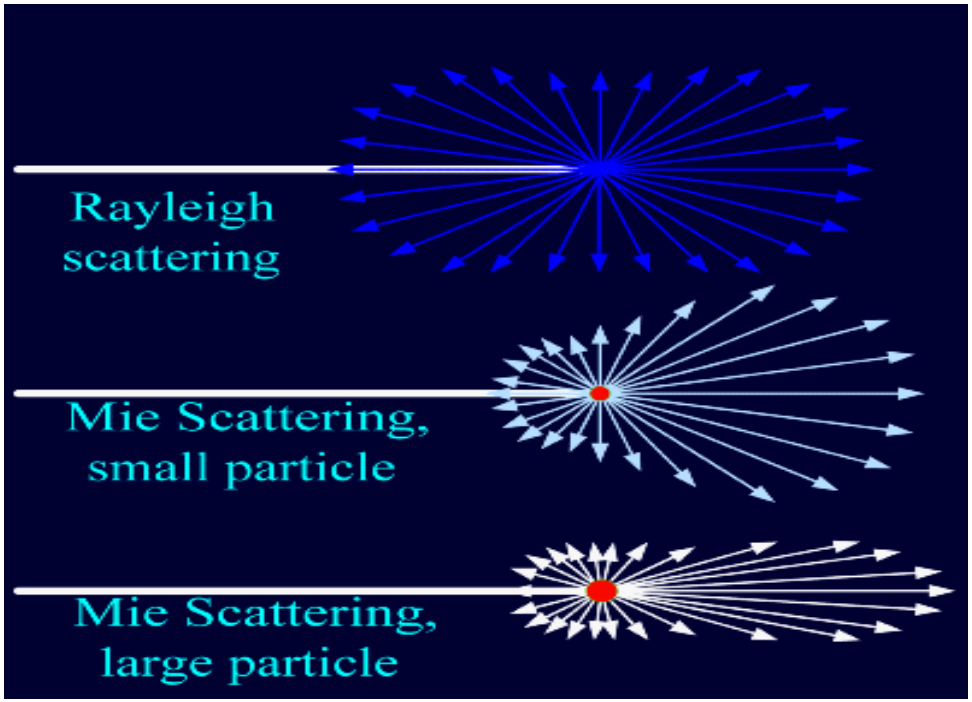
- Because we love flow!

- High content and high throughput way to examine these populations

# Limitations:

- Size and overlapping size distribution
- Particle polydispersity
- Overall low refractive index

# It's all about Mie!



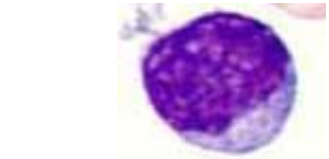

- Mie theory predicts the scattering cross section of a particle, and thus its scattering intensity
- It is dependent on the wavelength of light, the angle of collection, and the size, shape, and RI of the particle

# Eliminating Noise

- Higher laser powers
- More precise optics
  - Forward scatter masks that change
- Filtration of sheath fluid

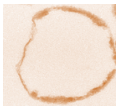
# Traditional FSC vs SSC

- Threshold is set to eliminate events measuring <1um, hence all Extracellular Vesicles are considered cellular debris. Small Particle Detection requires lower threshold capacities.

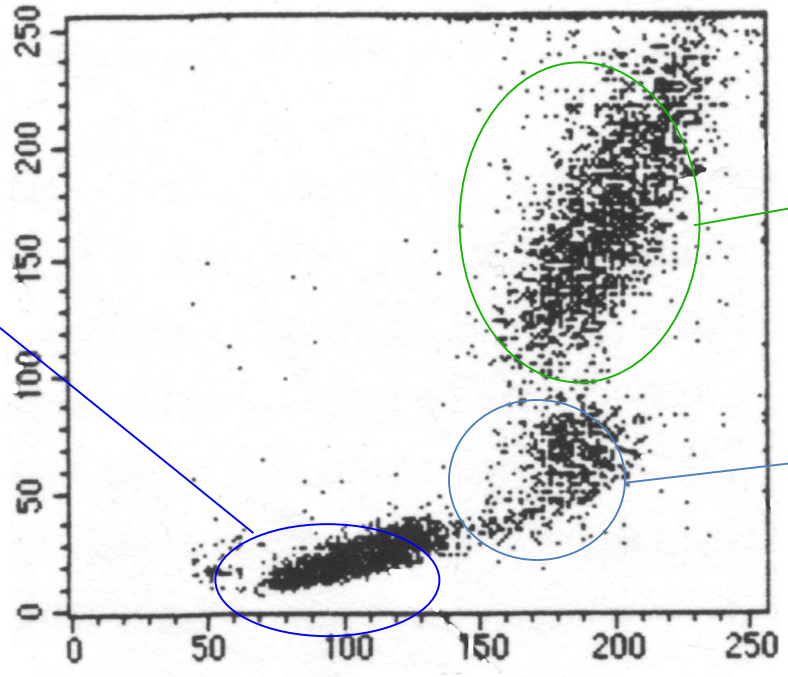


Lymphocytes

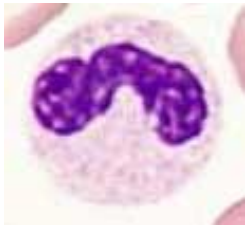
**EVs/SPD**



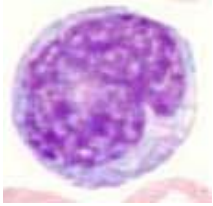
**RBCs, Debris, Dead Cells**




Granulocytes



Monocytes



# Flow Cytometric Analysis of EVs

- Instrumentation designed with enhanced low-end resolution and data processing
  - FSC resolution <1um
  - SSC resolution <500nm
  - Different FSC masks enhance resolution and dynamic range
  - Enhanced scatter angle, greater laser power at flow cell, high end dichroic mirrors and band pass filters for better signal to noise resolution

# Flow Cytometric Analysis of EVs

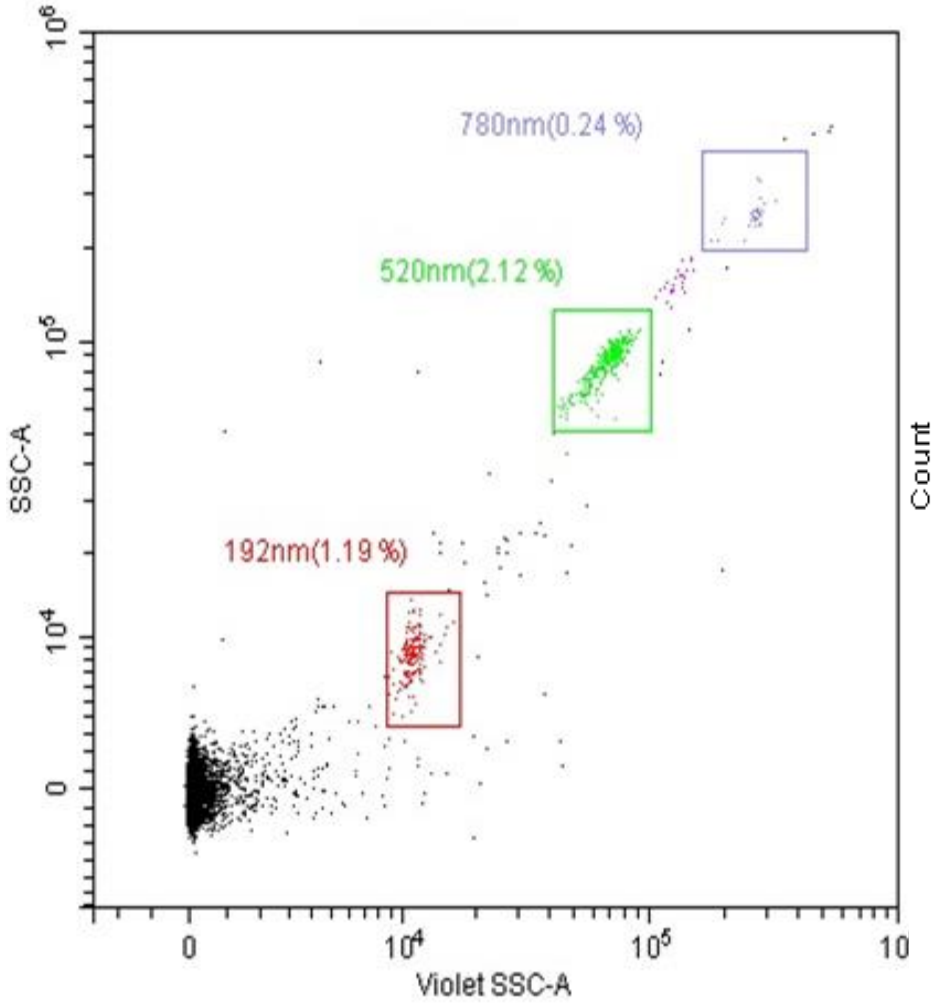
- Ability to threshold off of any parameter
  - Threshold can be set minimally (0.003)
  - Ability to exclude noise without losing small particles
  - Visualize particles <1um

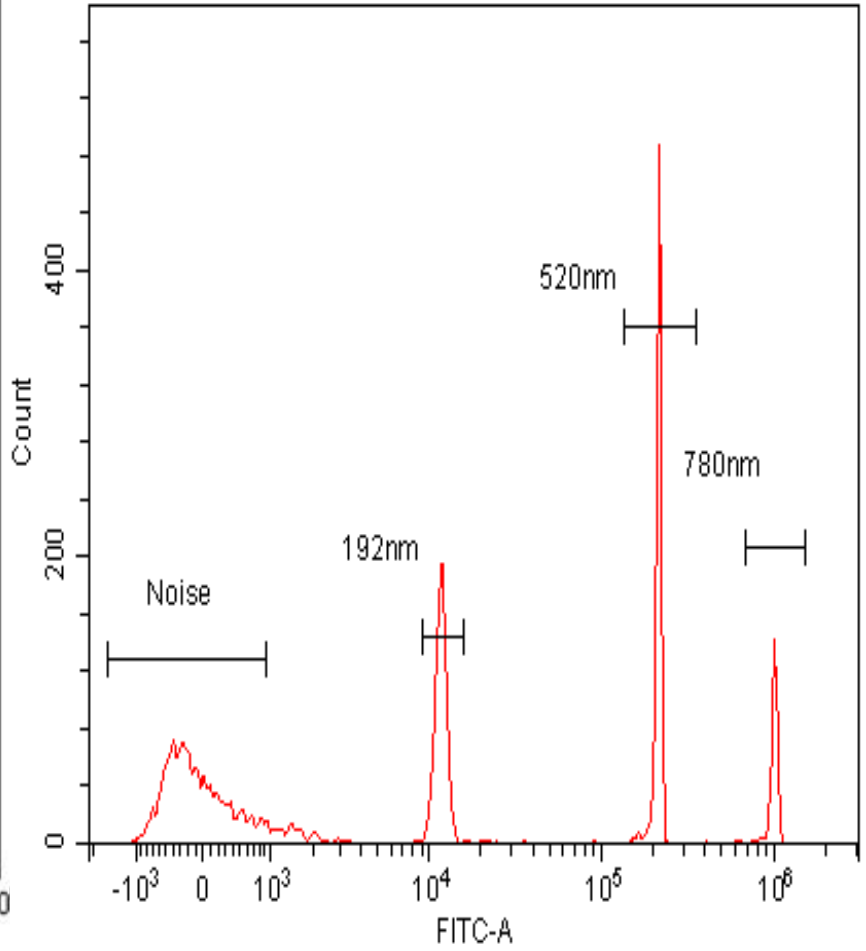
- Specific range of controls are available for instrument optimization and standardization. However, this is by no means PERFECT!!!

# Validation / Troubleshooting

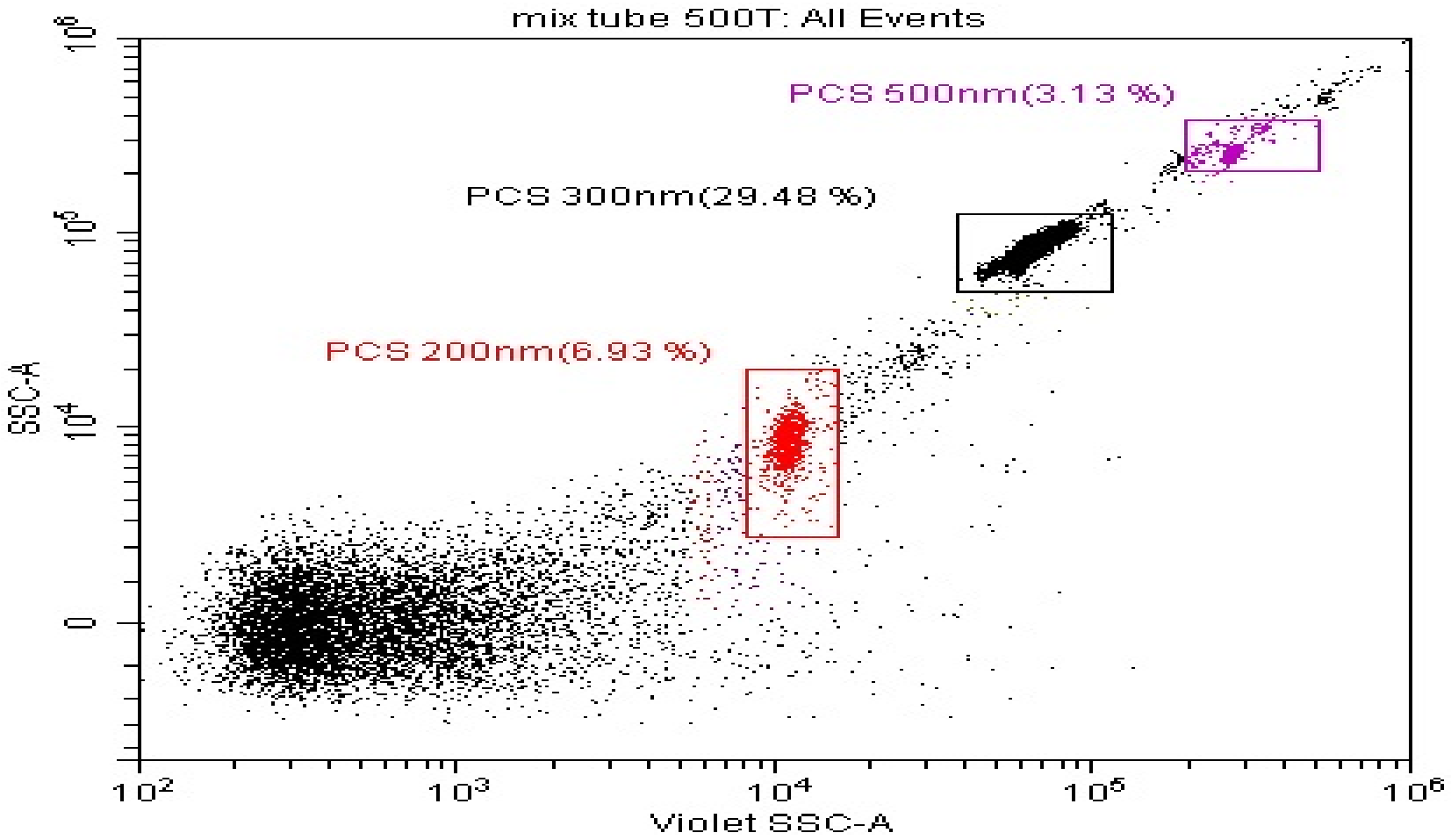
- One tool for assessing the size distribution of EVs is the bead sizing curve. This curve is generated prior to EV analysis
  - 100nm, 200nm, 300nm, 500nm PCS
  - 192nm, 520nm, 780nm Dragon Green
- PCS controls are comparable in scatter profile to Dragon Green Beads

# Dragon Green Beads

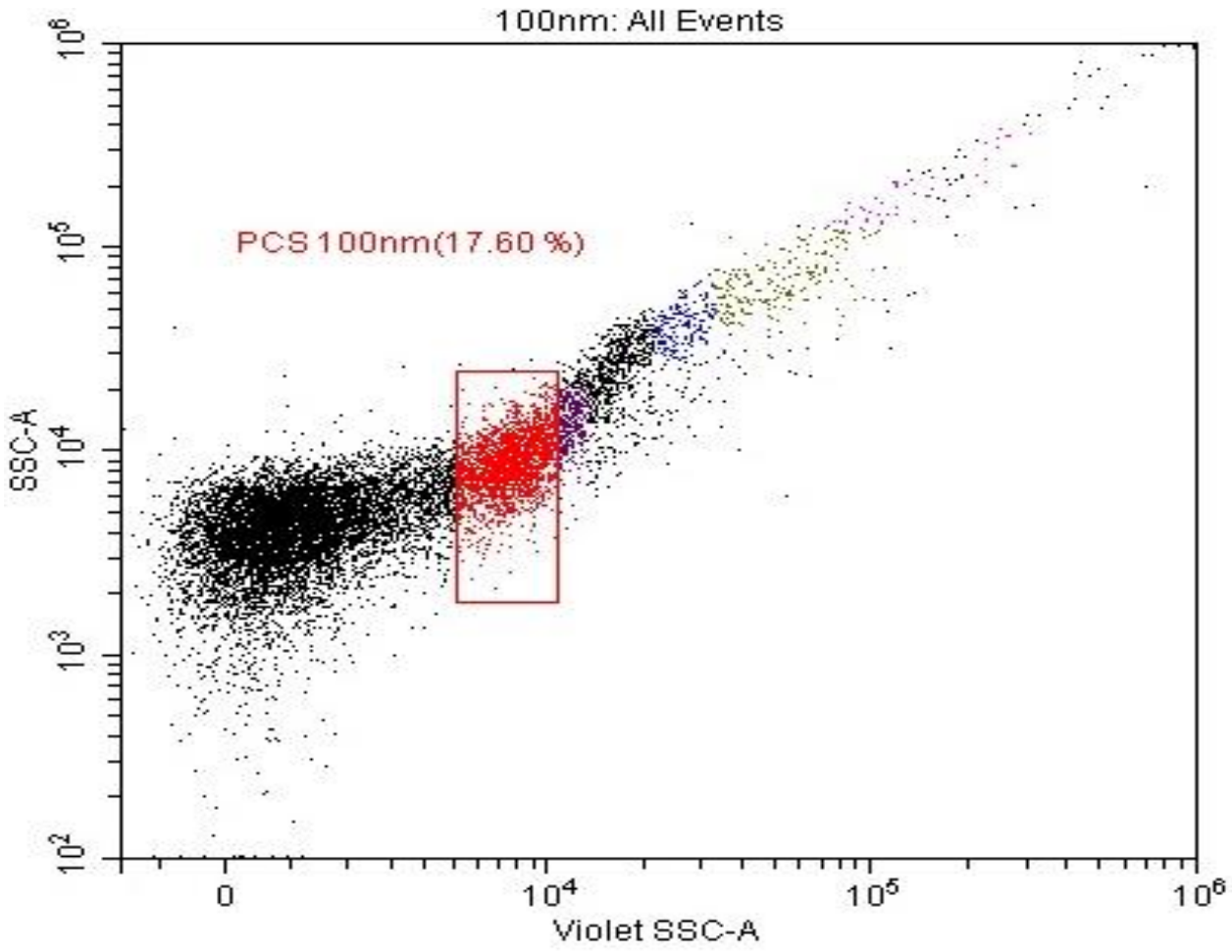


# PCS Controls Instrument Comparison

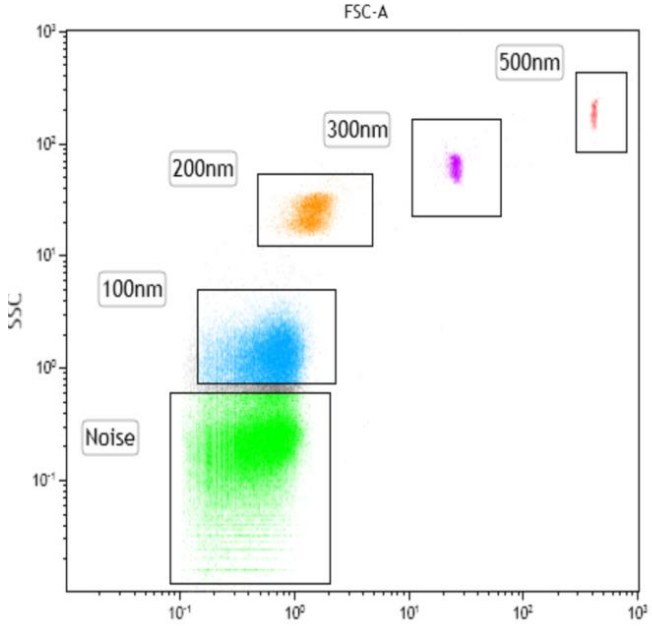


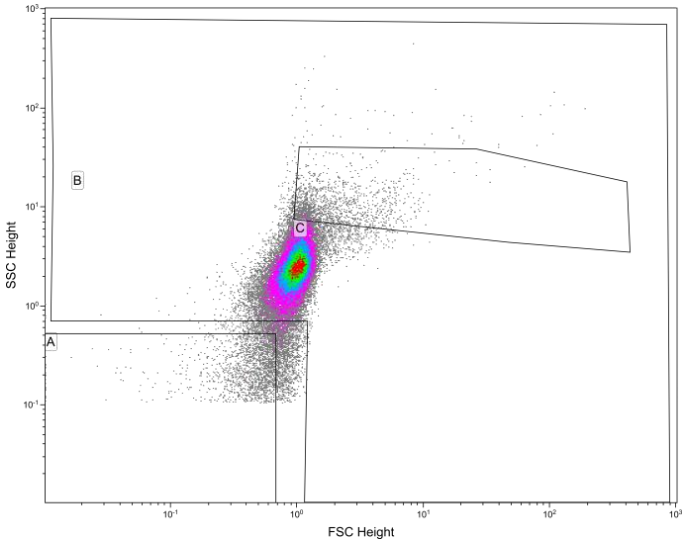
# 100nm PCS control

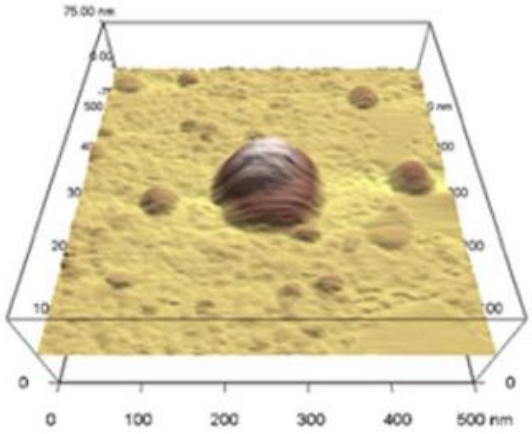


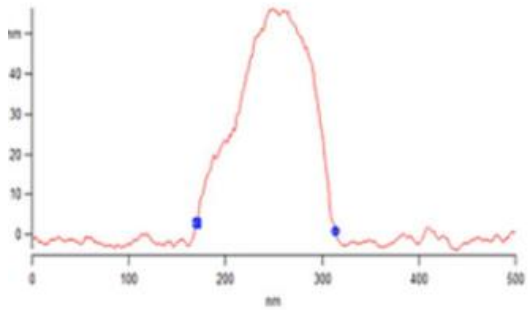
Questions?

# Flow Cytometers are capable of seeing particles down to 100 nm in size: Beads



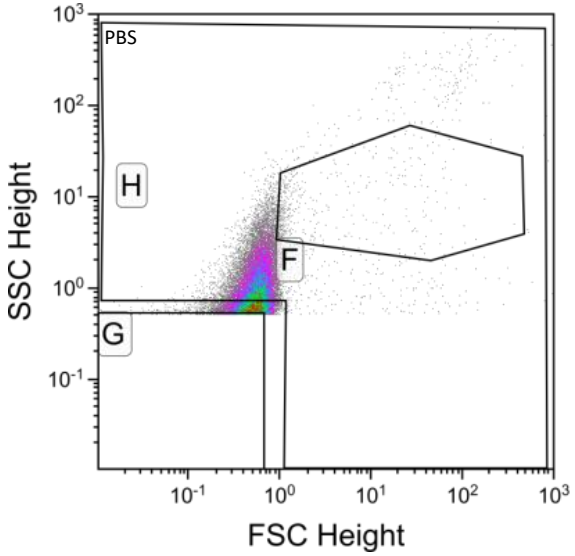



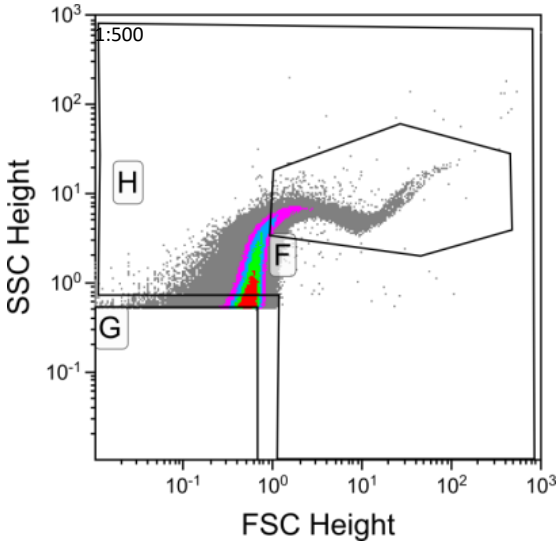



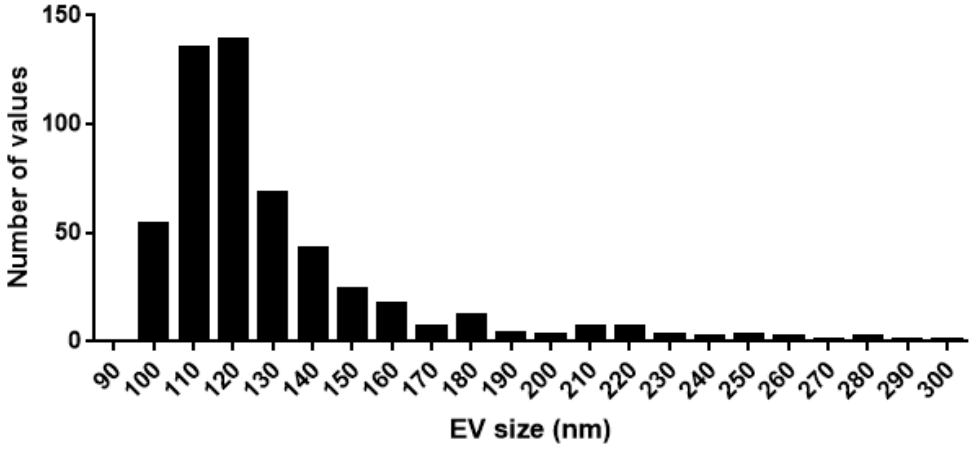



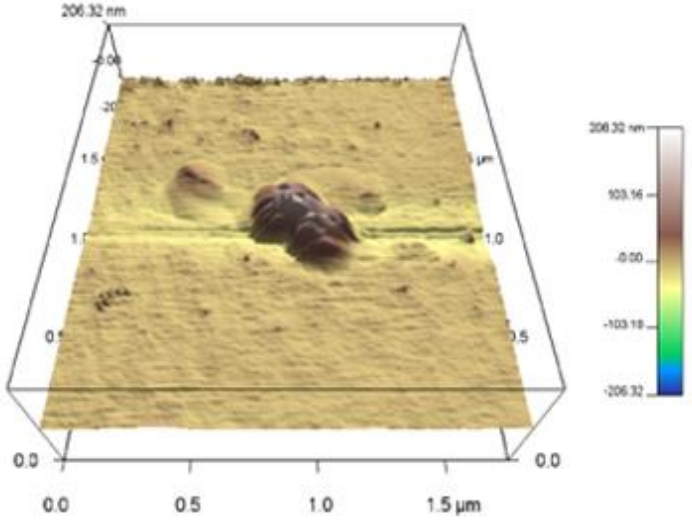

Work conducted on a Beckman Coulter MoFlo XDP with Nanoview module

# Analysis of plasma EVs by flow cytometry

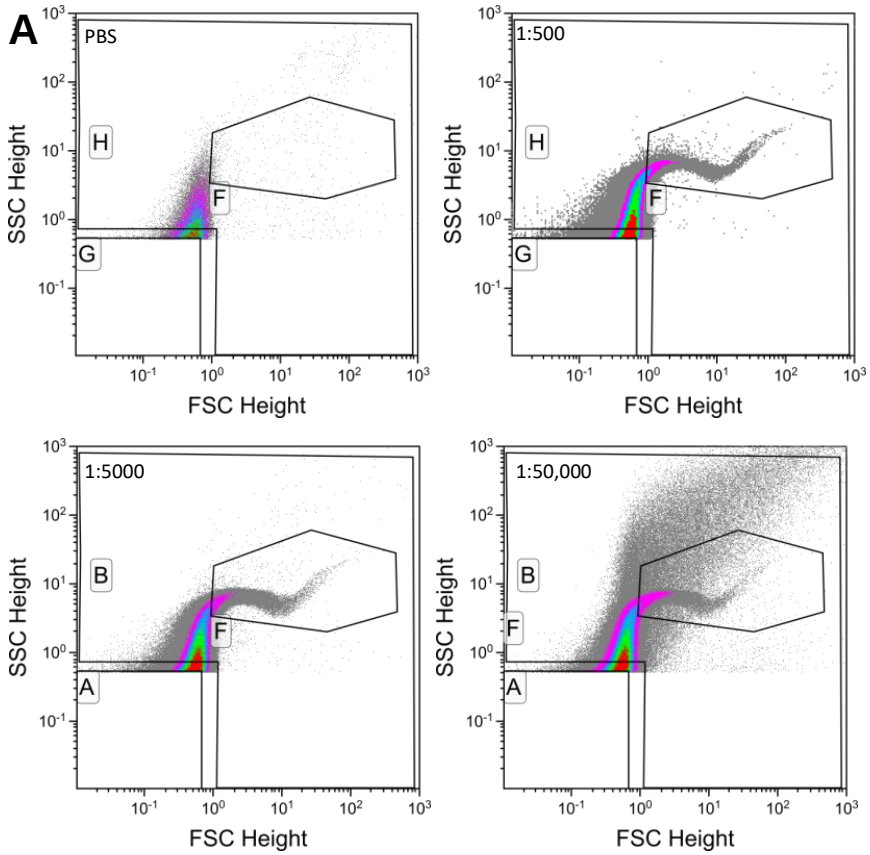


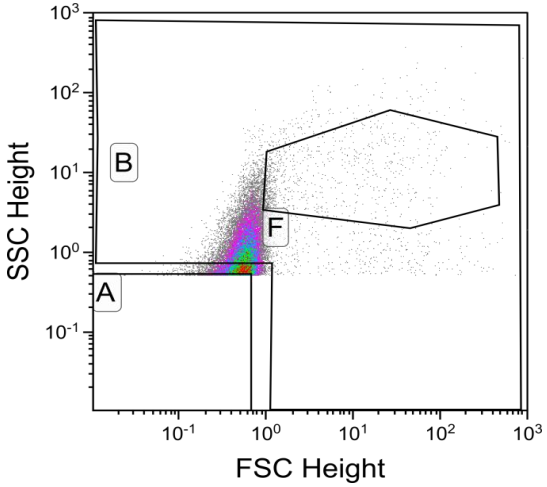



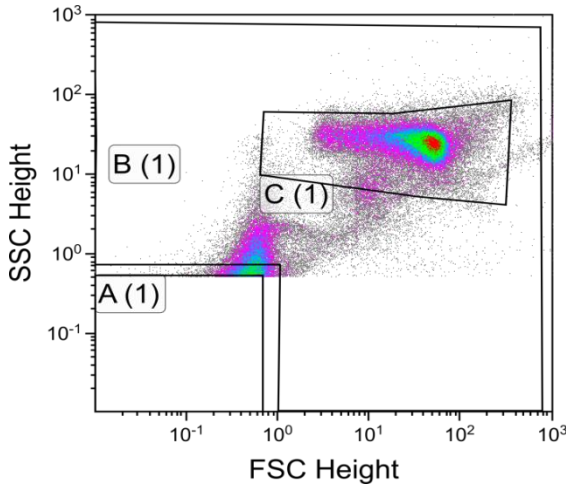
# The Dreaded Swarming



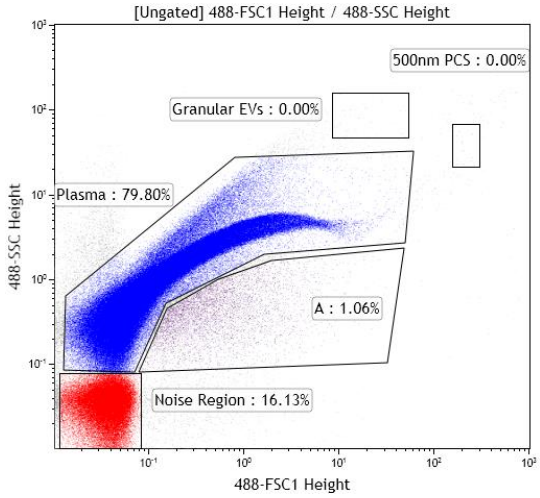

| Sample | % Gated | Number of events | Mean Acquisition Time (s) |
|---|---|---|---|
| PBS | 99.93 | 30,079 | |
| Plasma 1:100 | 99.84 | 499,214 | 8.18 |
| Plasma 1:500 | 99.91 | 499,558 | 12 |
| Plasma 1:5000 | 99.91 | 499,538 | 213.83 |
| Plasma 1:50,000 | 98.94 | 494,718 | 367.44 |

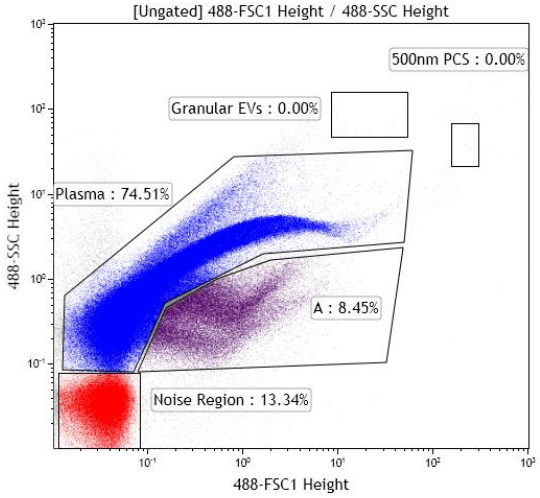
# A couple of other things we learnt…..




Some tubes shed particles the same size as EVs!




Hemolysis makes things look funny

# Validation/Troubleshooting: Take Homes

- Container material contamination
- Sample handling
  - Hemolysis
  - Unprocessed whole blood kept on ice
  - Container material contamination
- Shortcomings
  - Only able to measure numerousness + size

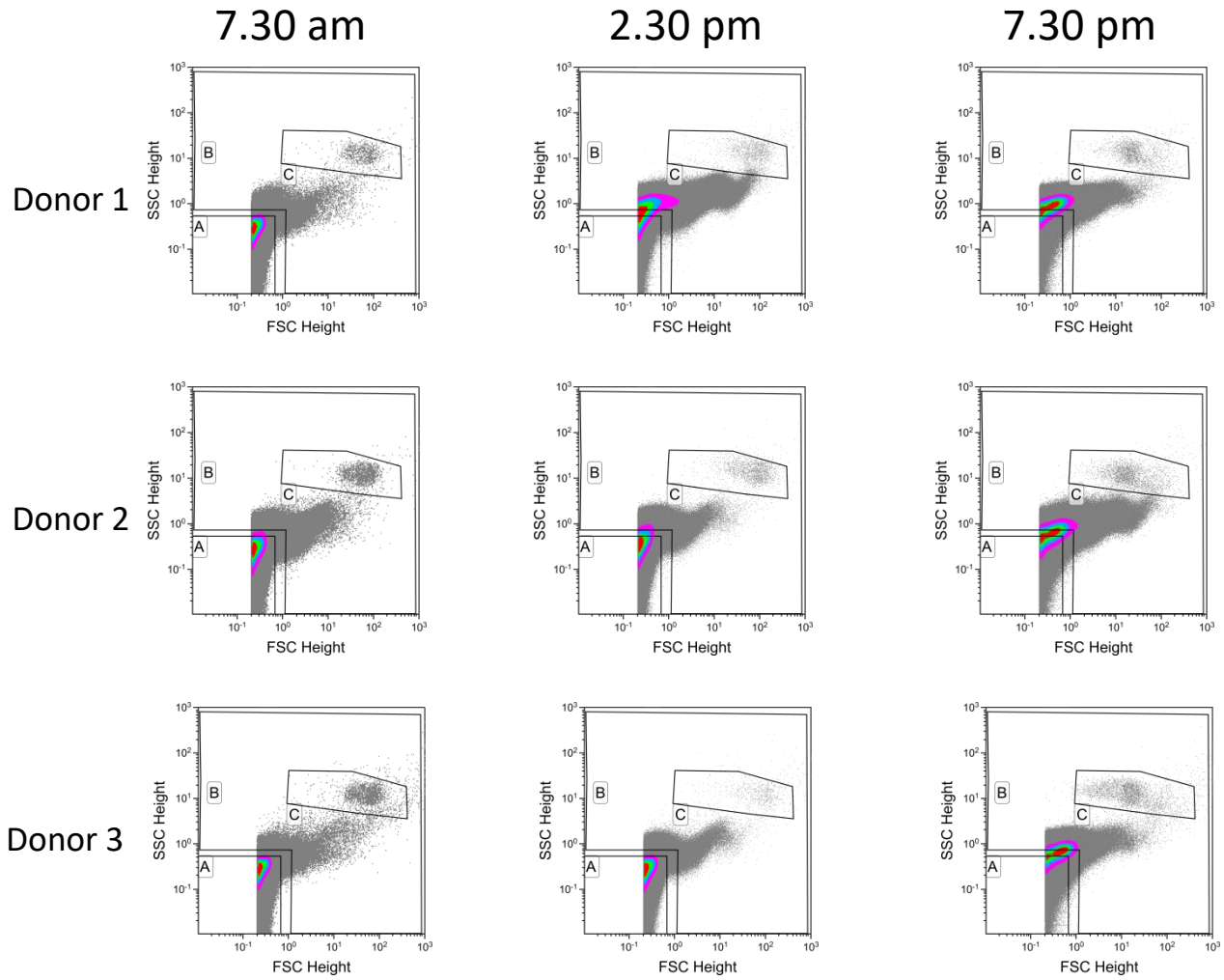
# CVD Applications and Considerations

- **Normal physiology: circadian variation in EVs and exercise-induced EV release**
- Disease: Plasma EVs from heart failure patients
- Murine models for tracking EV origins

# Normal Physiology of Circulating EVs

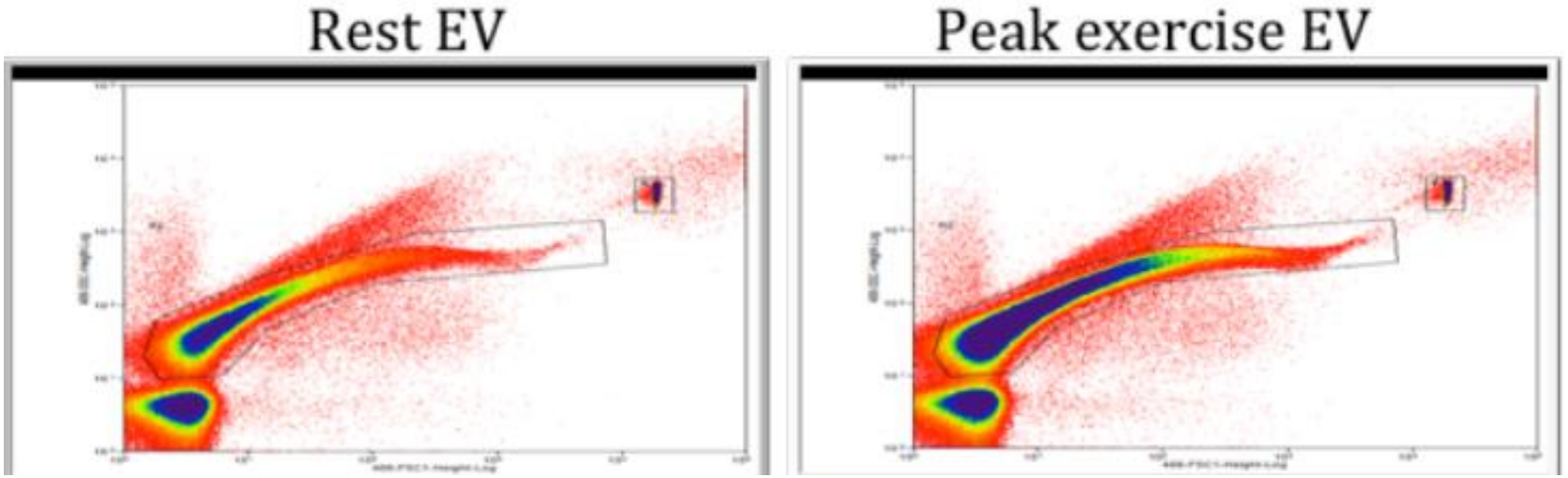
- Very little currently known on ‘normal’ levels and regulation of EVs
- Both circadian rhythms and exercise affect expression of circulating factors in the blood
- Important to consider these fluctuations when developing EVs as biomarkers
  - As a part of an NIH common fund to examine EV circadian biology (Das and Ghiran)

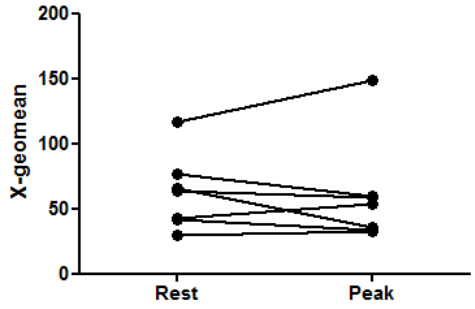
# Circulating plasma EVs vary in relative size and number during the day

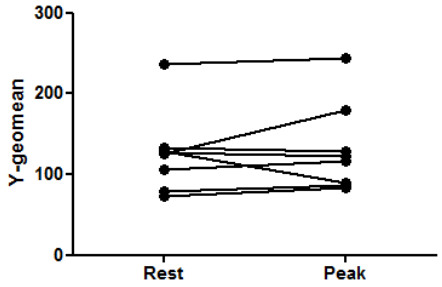


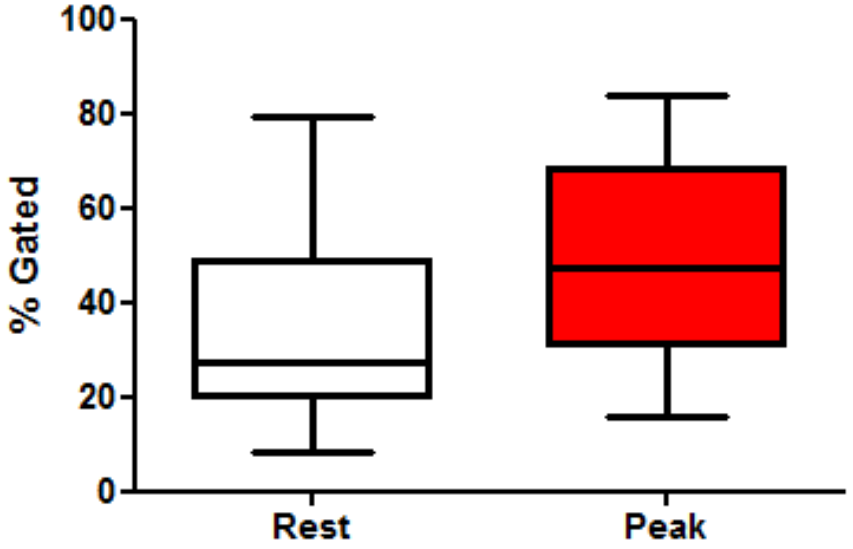

| | Forward Scatter Geo Mean | | | Side Scatter Geo Mean | | |
|---|---|---|---|---|---|---|
| **Donor #** | 7.30 am | 2.30 pm | 7.30 pm | 7.30 am | 2.30 pm | 7.30 pm |
| **1** | 0.77 | 0.43 | 0.90 | 0.76 | 0.86 | 1.30 |
| **2** | 0.86 | 0.65 | 0.51 | 0.74 | 0.84 | 1.00 |
| **3** | 0.56 | 0.41 | 0.74 | 0.81 | 0.90 | 0.94 |

# Exercise induces EV release into the circulation




Work conducted on a Beckman Coulter MoFlo Astrios EQ

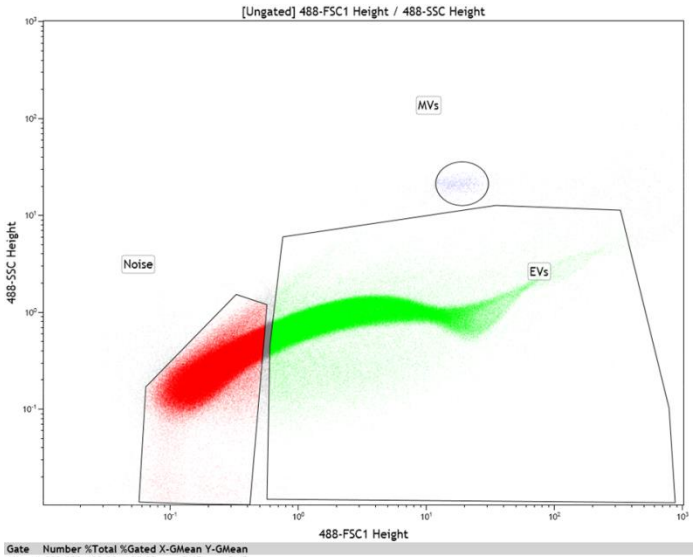
# CVD Applications: Flow + Other

- Normal physiology: circadian variation in EVs and exercise-induced EV release
- **Disease: Plasma EVs from heart failure patients**
- Murine models for tracking EV origins

# EVs from Patients with Heart Failure

- Blood collected from inside the heart (coronary sinus) and periphery during procedure
- Plasma EVs analyzed by flow cytometry and compared to control (SVT) patients
- EVs isolated by iodixanol-sucrose gradient

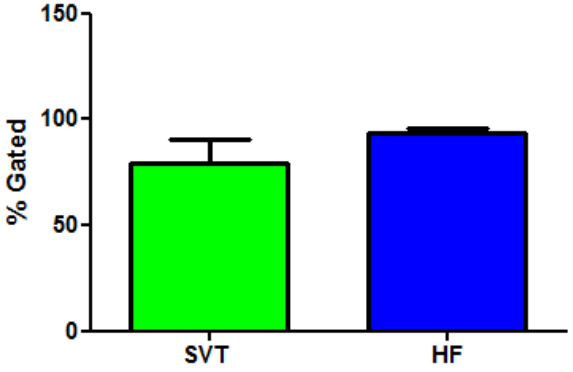
## Control patient

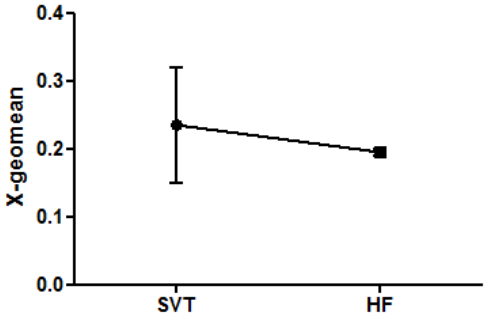


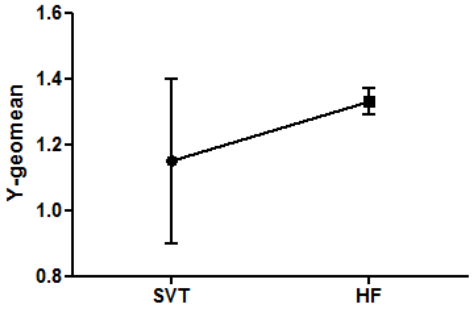

| Gate | Number | %Total | %Gated | X-GMean | Y-GMean |
|---|---|---|---|---|---|
| All | 1,000,000 | 100.00 | 100.00 | 0.65 | 0.45 |
| EVs | 416,709 | 41.67 | 41.67 | 2.31 | 0.83 |
| MVs | 575 | 0.06 | 0.06 | 17.58 | 20.73 |
| Noise | 546,649 | 54.66 | 54.66 | 0.25 | 0.28 |

## Heart Failure patient


| Gate | Number | %Total | %Gated | X-GMean | Y-GMean |
|---|---|---|---|---|---|
| All | 1,000,000 | 100.00 | 100.00 | 0.48 | 0.42 |
| EVs | 324,510 | 32.45 | 32.45 | 1.51 | 0.80 |
| MVs | 19 | 0.00 | 0.00 | 28.78 | 19.88 |
| Noise | 632,165 | 63.22 | 63.22 | 0.26 | 0.29 |



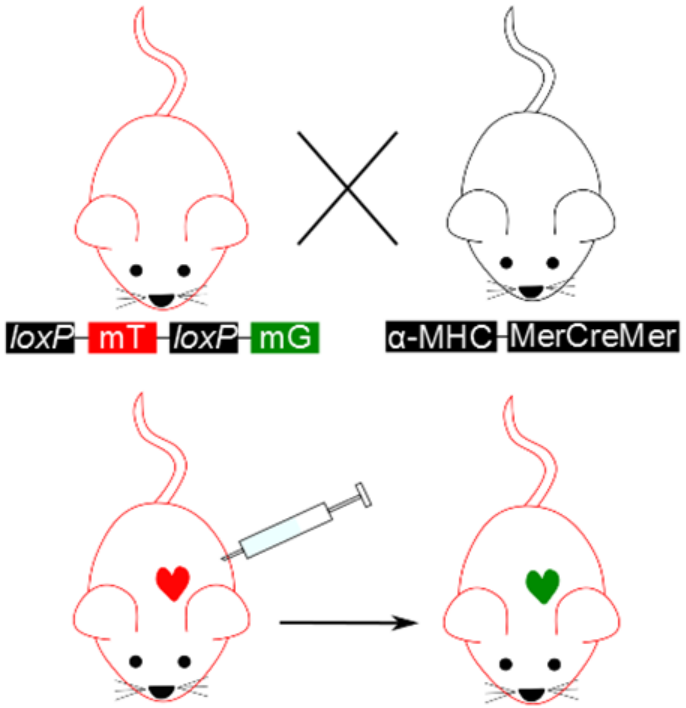



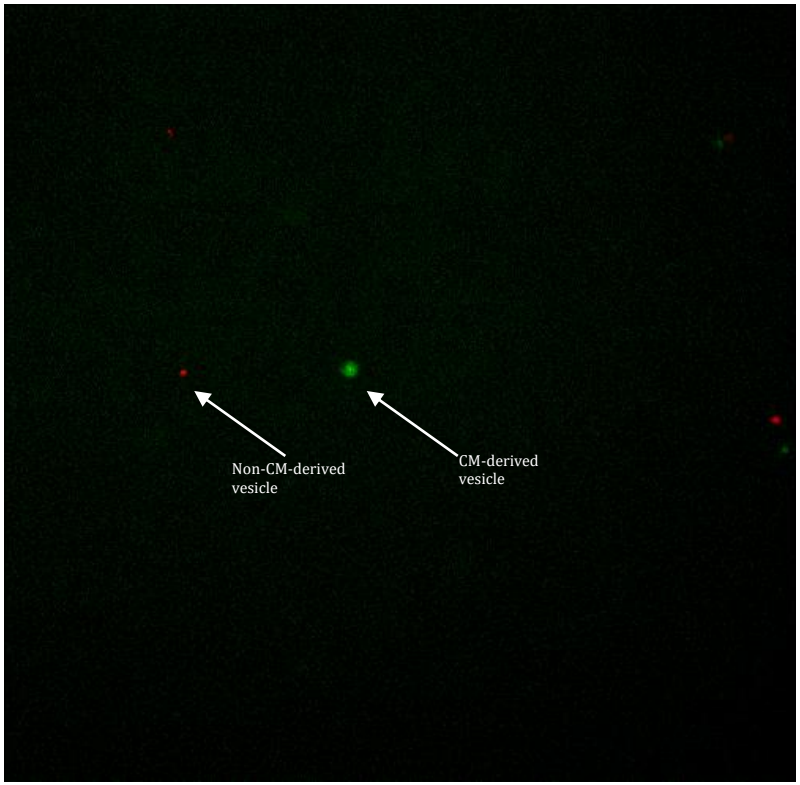
# CVD Applications

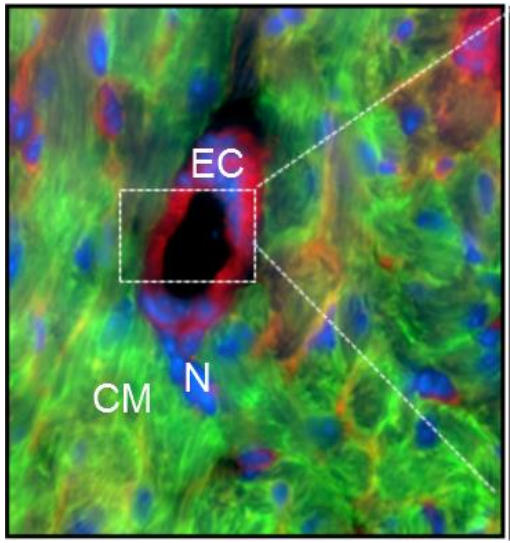
- Normal physiology: circadian variation in EVs and exercise-induced EV release
- Disease: Plasma EVs from heart failure patients
- **Murine models for tracking EVs**

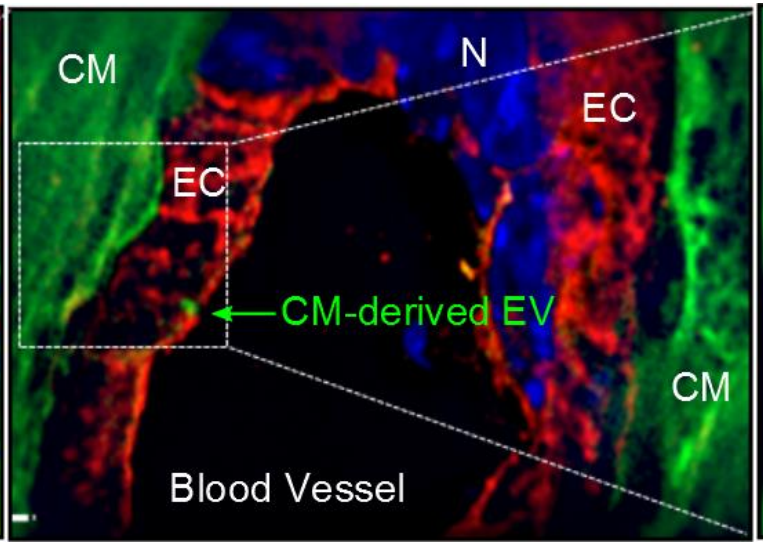
# Fluorescently labelled EVs in vivo

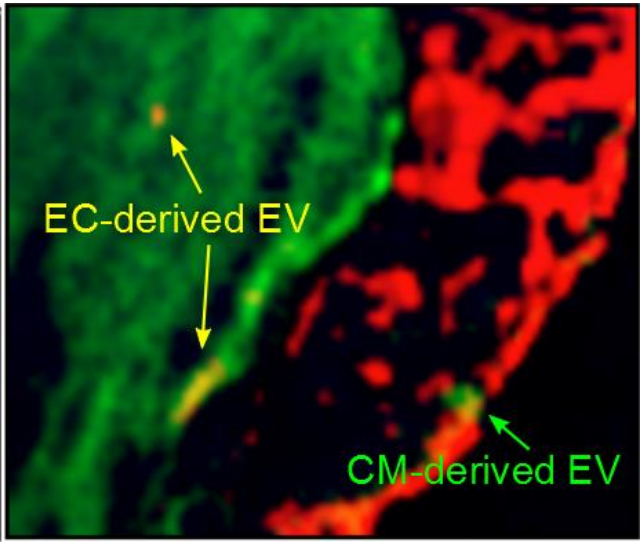
- EVs in circulation originate from numerous cell types
- Currently there are no concrete markers for EVs from different cell types
- We are utilizing the Rosa mT/mG mouse line to track EVs in vivo

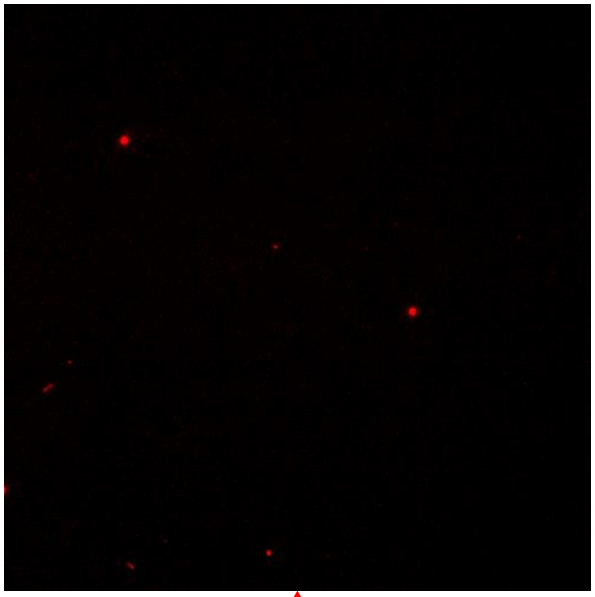


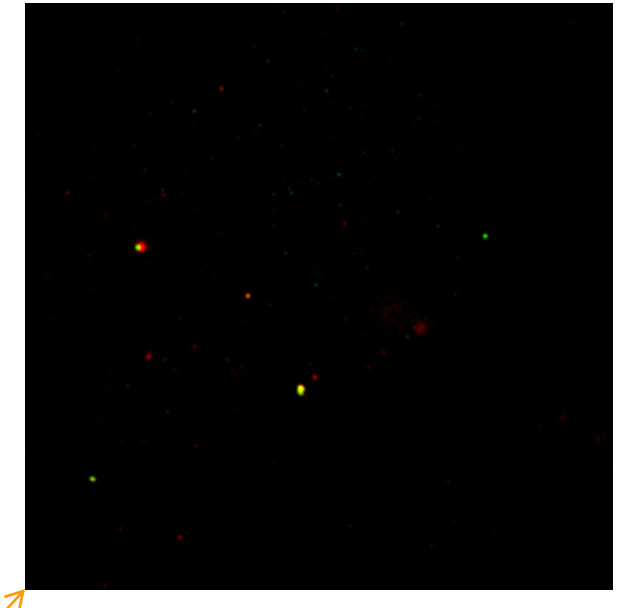

Non-CM-derived vesicle
CM-derived vesicle

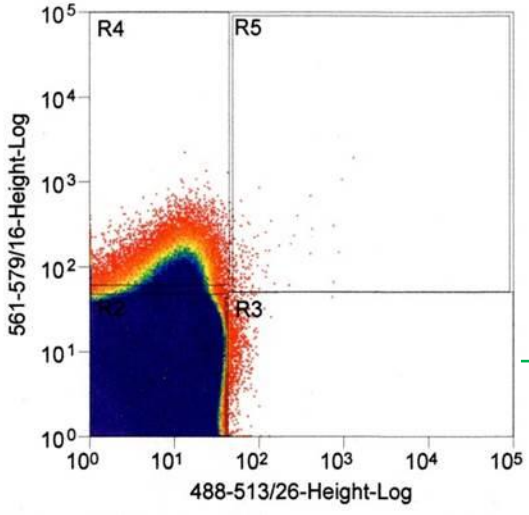

EC
CM
N

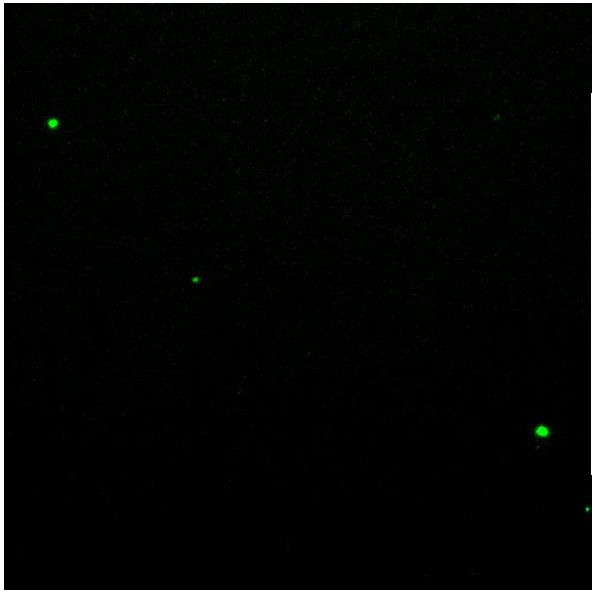

CM
N
EC
EC
CM-derived EV
CM
Blood Vessel

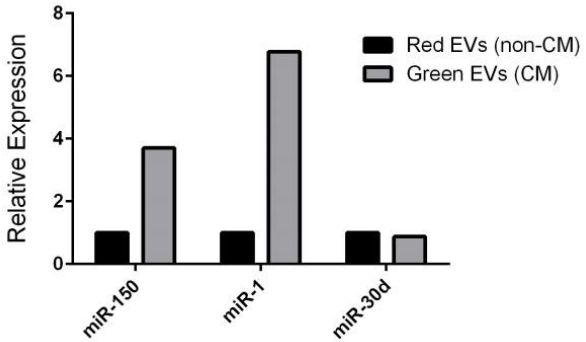

EC-derived EV
CM-derived EV






R4
R5
R3
561-579/16-Height-Log
488-513/26-Height-Log
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
$10^5$




Relative Expression
0
2
4
6
8
Red EVs (non-CM)
Green EVs (CM)
miR-150
miR-1
miR-30d

# In Summary

- Effective detection, characterization and sorting of EVs down to 100 nm in size using flow cytometry
  - Advantageous over extant methods but has limitations
- Profiles of these EVs (size, character, number) are biologically pertinent
  - Pathologically and physiological applications: circadian rhythm and exercise
- Utility for study of modified EVs
  - tissue-specific EVs from transgenic murine models

# Acknowledgements

**MGH**
David Milan
Ling Xiao

**Das Lab:**
Saumya Das
Ravi Shah
Bridget Simonson
Vinita Subramanya
MC Chan
Fernando Contreras
Ismail Haisam
Alefiyah Rajabali
Olivia Ziegler

**Beth Israel Deaconess Medical Center**

**BIDMC Flow Cytometry Core**
Vasilis Toxavidis
John Tigges
Virginia Camachio
Eric Zigon

**Ghiran Lab:**
Ionita Ghiran
Joe Khoory
Jessica Estanislau
Shulin Lu
Tony Velasquez