Single Vesicle Flow Cytometry (vFC™)

JOHN NOLAN, CEO CELLARCUS BIOSCIENCES

SELECTBIO EV-BASED DIAGNOSTICS, DELIVERY & THERAPEUTICS

FEBRUARY 18, 2020

Workshop Overview

- MIFlowCyt EV – Minimum Information Guidelines for Reporting EV FC Methods and Results
- Vesicle Flow Cytometry (vFC™) overview
- Instrument considerations and setup
- Assay protocol
- Data analysis protocol
- Live demo

Beckman Coulter CytoFlex

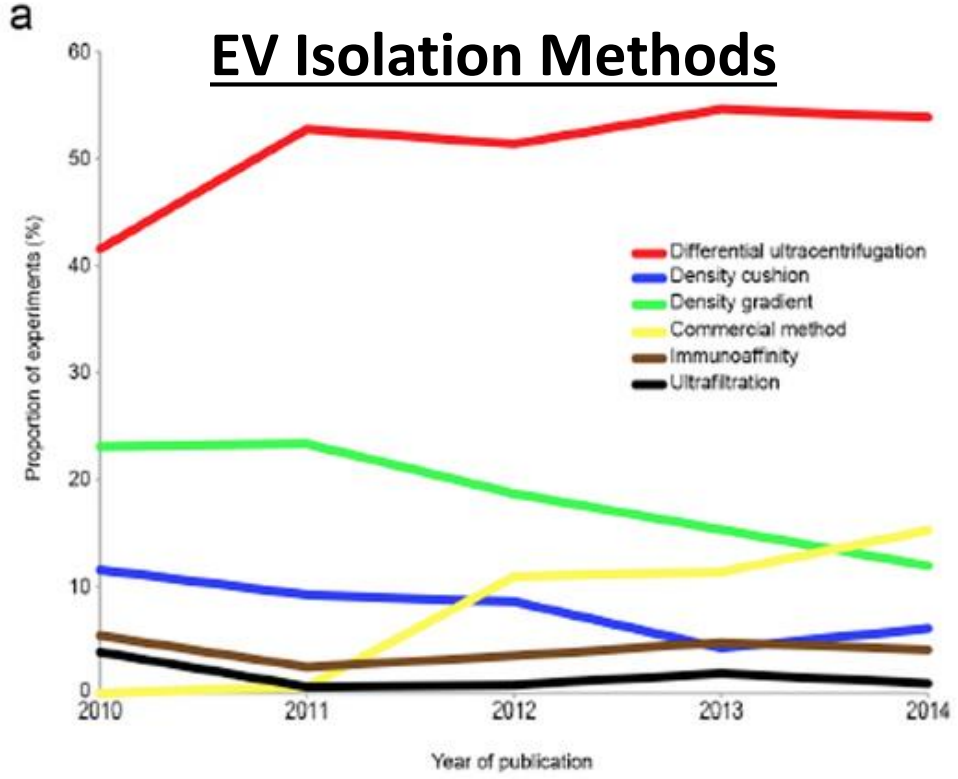
228 | VOL.14 NO.3 | MARCH 2017 | NATURE METHODS

EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research

EV-TRACK Consortium*

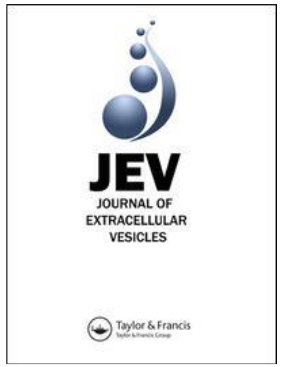
We argue that the field of extracellular vesicle (EV) biology needs more transparent reporting to facilitate interpretation and replication of experiments. To achieve this, we describe EV-TRACK, a crowdsourcing knowledgebase (http://evtrack.org) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice.

EV Isolation Methods



The 1,742 experiments that are recorded in EV-TRACK report 190 unique isolation methods and 1,038 unique protocols to retrieve EVs from biofluids (**Supplementary Tables 2** and **3**). Differential ultracentrifugation (dUC) is the most popular method (45% of all experiments), but with variable parameters selected by researchers, even for experiments handling a similar sample type. For cell culture supernatant ($n = 813$ experiments using dUC), 218 unique combinations of centrifugation steps and final pelleting times are recorded, along with a

MISEV 2018: EV Characterization



Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines

EV Quantification
- Particle number
- Total protein (lipid, RNA)

Protein composition
- EV markers
- Non-EV markers
- “Small EV” markers

Non-protein components

Single vesicle analysis
- Imaging
- Impedance
- Flow cytometry

Protein topology
- Exofacial
- Cytoplasmic

Conventional FC of Individual EVs: Pitfalls

Lack of sensitivity, specificity

Lack of appropriate calibration

Difficulty of standardization

Irreproducibility

Artifacts!!!

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

Cytometry Part A • 79A: 990–999, 2011

Fluorescent Particles in the Antibody Solution Result in False TF- and CD14-Positive Microparticles in Flow Cytometric Analysis

Hans Christian D. Aass,* Reidun Øvstebø, Anne-Marie S. Trøseid, Peter Kierulf, Jens Petter Berg, Carola Elisabeth Henriksson

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

Cytometry Part A • 83A: 242–250, 2013

Calcium-Phosphate Microprecipitates Mimic Microparticles When Examined with Flow Cytometry

Michael C. Larson,[1,2] * Maia R. Luthi,[3] Neil Hogg,[1] Cheryl A. Hillery[2,4]

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

MIFlowCyt

A Trigger Channel Threshold Artifact in Nanoparticle Analysis

John P. Nolan,* Samuel A. Stoner

Journal of Extracellular Vesicles

SHORT COMMUNICATION

High-speed centrifugation induces aggregation of extracellular vesicles

Romain Linares[1], Sisareuth Tan[1], Céline Gounou[1], Nicolas Arraud[1] and Alain R. Brisson[1,2]*

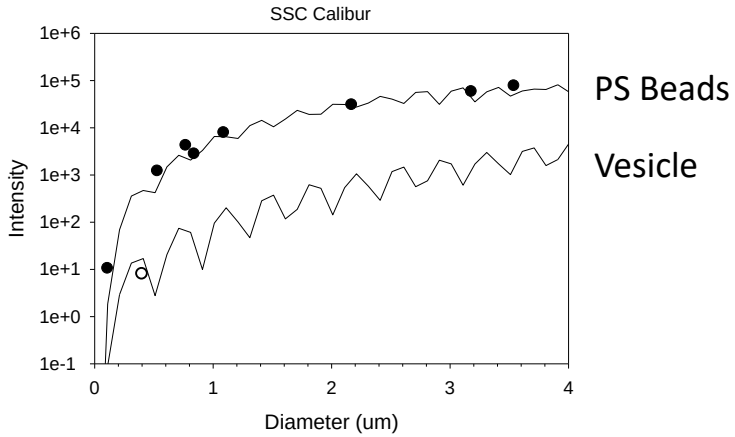
[1]Molecular Imaging and NanoBioTechnology, University of Bordeaux, Pessac, France; [2]Institut Universitaire de France, Paris, France

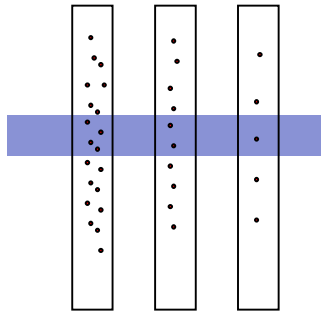
Limitations of Conventional FC for EV Analysis

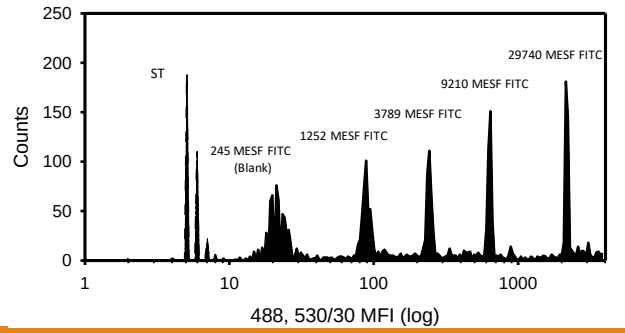
1. Light scatter as a trigger channel
   - Depends on size, shape, λ, collection angle, refractive index
   - Well described by Mie theory
   - Vesicles scatter 10-100x <beads

2. Coincidence (aka “swarm”)
   - Depends on [EV], probe volume
   - Frequency is readily calculated
   - Can be identified/eliminated by dilution

3. Fluorescence sensitivity and calibration
   - Required for data/methods sharing
   - Well-established protocols, reagents
   - Not widely practiced







ISEV-ISAC-ISTH EV FC Working Group

- Need for high throughput EV analysis
- Knowledge on biological samples and EV isolation
- Development of EV analysis technology
- Many end-users

- Technology development
- Education
- Strong connections with Industry
- Interface with users

SSC on Vascular Biology

- Development of guidelines for plasma EV isolation
- Standardization of plasma EV analysis and functional assays (e.g. coagulation)
- Many end-users

Marca Wauben, Ger Arkesteijn, Sten Libregts, EstefaniaLozano Andres (Utrecht)

Rienk Nieuwland, Edwin van der Pol, Frank Coumans, Leonie de Rond (Amsterdam)

John Nolan, Erika Duggan (San Diego)

Jennifer Jones, Aizea Morales-Kastresana, Joshua Welsh (Bethesda)

Joanne Lannigan, Uta Erdbrugger (Charlottesville)

Alain Brisson (Bordeaux)

Romaric Lacroix, Stpahne Robert, Fracoise Dignat-George (Marseilles)

John Tigges, Ionita Ghiron, Vasilis Toxivaidis (Boston)

Bernd Giebel, Andre Goergens, Tobias Tertel (Essen)

James Higgenbotham, Bob Coffey (Vanderbilt)

An Hendrix, Oliver de Wever (Ghent)

Xiaomei Yan (Xiamen)

ISEV-ISAC-ISTH EV FC Working Group

- Need for high throughput EV analysis
- Knowledge on biological samples and EV isolation
- Development of EV analysis technology
- Many end-users

- Technology development
- Education
- Strong connections with Industry
- Interface with users

SSC on Vascular Biology

- Development of guidelines for plasma EV isolation
- Standardization of plasma EV analysis and functional assays (e.g. coagulation)
- Many end-users

JOURNAL OF EXTRACELLULAR VESICLES
2020, VOL. 9, 1713526
https://doi.org/10.1080/20013078.2020.1713526

Taylor & Francis
Taylor & Francis Group

OPEN ACCESS Check for updates

MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments

Standardized Methods and Results Reporting

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	**Confirm single EV 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**

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

1.	Buffer only	background
2.	Buffer with reagents	background
3.	Unstained controls	autofluorescence
4.	Isotype controls	Fc receptor binding
5.	Single stained controls	positivity, compensation
6.	Procedural controls	unexpected artifacts
7.	Serial dilution	coincidence
8.	Detergent-treatment	vesicle lability

Workshop Overview

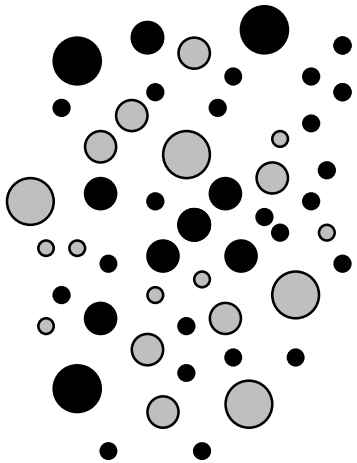
- MIFlowCyt EV – Minimum Information Guidelines for Reporting EV FC Methods and Results
- Vesicle Flow Cytometry (vFC™) overview
- Instrument considerations and setup
- Assay protocol
- Data analysis protocol
- Live demo

Beckman Coulter CytoFlex

Vesicle Flow Cytometry (vFC™)

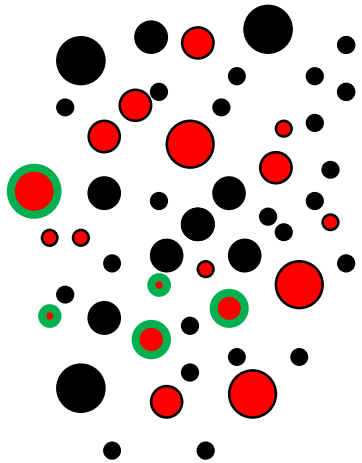
CELLARCUS BIOSCIENCES

1. Dilute



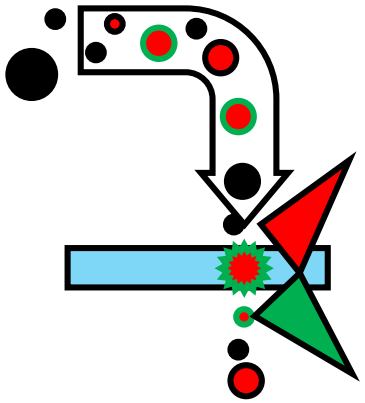
vFC measures EVs directly in diluted biofluid, or after purification

2. Stain:

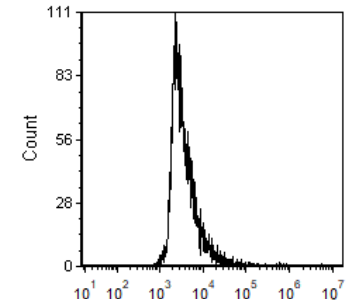


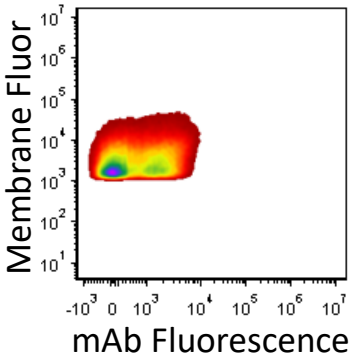
vFluorRed selectively stains membrane-bound particles

3. Dilute and measure

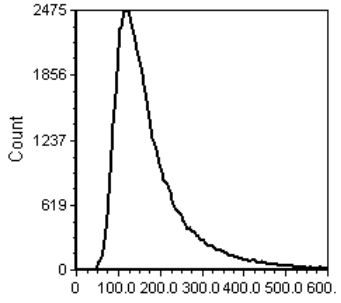


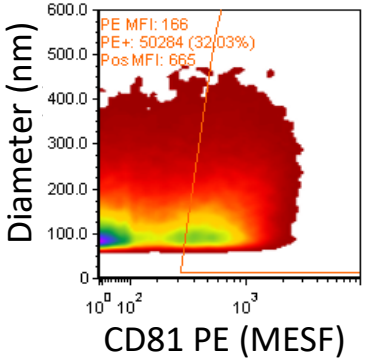
Fluorescence-triggered flow cytometry





4. Calibrate and report





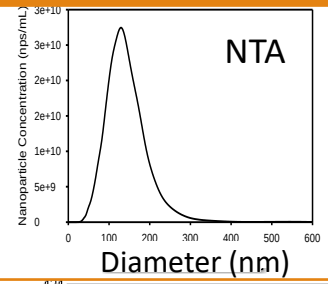
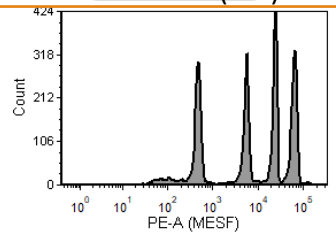
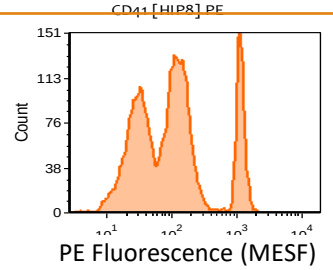
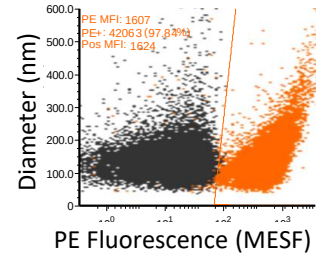
- Membrane probe provides specificity
- Homogeneous assay: no wash steps
- Measures EVs directly in biofluid: no isolation/purification required

- Uses commercially-available flow cytometers
- Lab automation-compatible

- Sensitive and specific detection: vesicle size to ~70 nm, cargo to >25 molecules
- Calibrated measurements for inter-lab, longitudinal, cross-platform comparisons

vFC™: Standards for EV Analysis

ELLARCUS BIOSCIENCES

Measurement	Standard	Uses	Data	
Vesicle size	Lipo100™ : synthetic vesicle, extruded through nanopore filters, extensively characterized	Calibrate VFC measurements, Immunofluorescence negative control		Lipo100™ Vesicle Size Standard
Fluorescence intensity	vCal™ MESF beads: Polymer beads (800 nm) with calibrated levels of fluorescence	Calibrate fluorescence (MESF units) Enable cross-platform fluorescence measurements		vCal™ MESF calibration beads
Antibody binding	vCal™ mAb binding beads: Polymer beads (800 nm) with calibrated mAb capture capacity	Qualify antibody conjugates, Calibrate antibody binding, Enable cross-platform measurements		vCal™ mAb capture beads stained with PE-anti-CD41
Cell-derived EVs	EVs prepared from specific cells types expressing characteristic cargo	Cargo expression positive control, size and concentration standard, enable cross-platform measurements		vCal™ RBC EVs staining with PE-anti-CD235ab

vFC™: Getting Started

CELLARCUS BIOSCIENCES

Instrument evaluation, configuration and set up

- Protocol 0 – Essential standards to qualify and calibrate an instrument

Sample preparation and staining

- Protocol 1 – Sample serial dilutions to establish assay dynamic range, EV concentration, lack of coincidence/swarm
- Protocol 2 – EV counting, sizing and cargo analysis

Data processing and analysis

- Gating
- Diameter estimation
- Immunofluorescence calibration
- Batch export of data to spreadsheet, summary and plots to PPT/PDF

Instrument performance

vCal™ nanoRainbow beads
- 0.5 nm multifluorophore, multipeak beads
- Cross calibrated vs MESF, vesicle standards
- Fixed concentration: counting standard

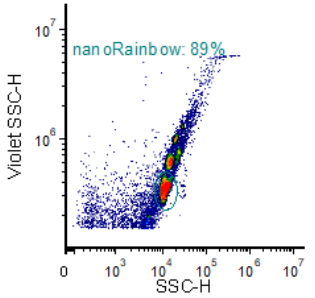
Protocol and template evaluates:
- Laser alignment
- Vesicle detection performance
- Immunofluorescence calibration
- Volumetric measurement

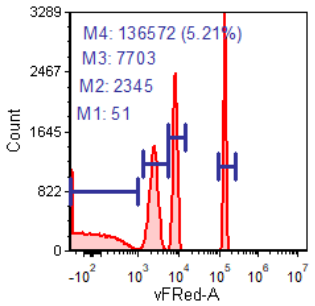
Beckman Coulter CytoFlex

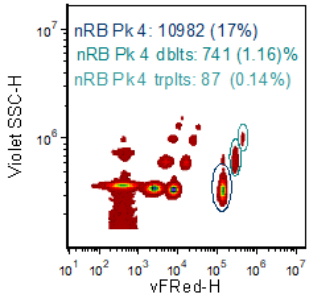
vCal nanoRainbow Beads -
Instrument Characterization and Calibration Report

CELLARCUS BIOSCIENCES

File: 01-Tube-A12.fcs
Sample: nanorainbow
Instrument: CytoFLEX LX AS38003
Volume:5275.157 Gated:56612 Abort: 1930
Sample Concentration:

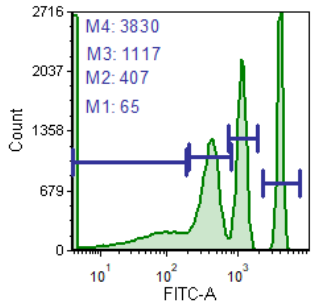


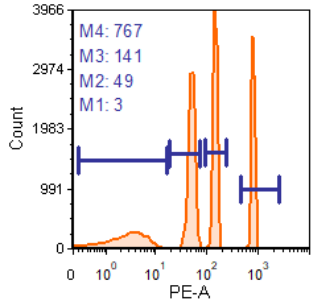




Parameter	Marker	# of Events	% of gated cells	Median	Arithmetic Mean	CV	95%-ile	Standard Deviation
vFRed-A	M1	12917	23	51	85	285	547	242
vFRed-A	M2	15752	28	2345	2385	22	3274	516
vFRed-A	M3	14225	25	7703	7730	11	9167	852
vFRed-A	M4	10934	19	136572	136203	5	146067	7096

	A	B	C
1	Pos med	2345	
2	Neg med	51	
3	Neg sd	242	
4	**Sep Index**	**18.99**	
5			
6			
7			
8			



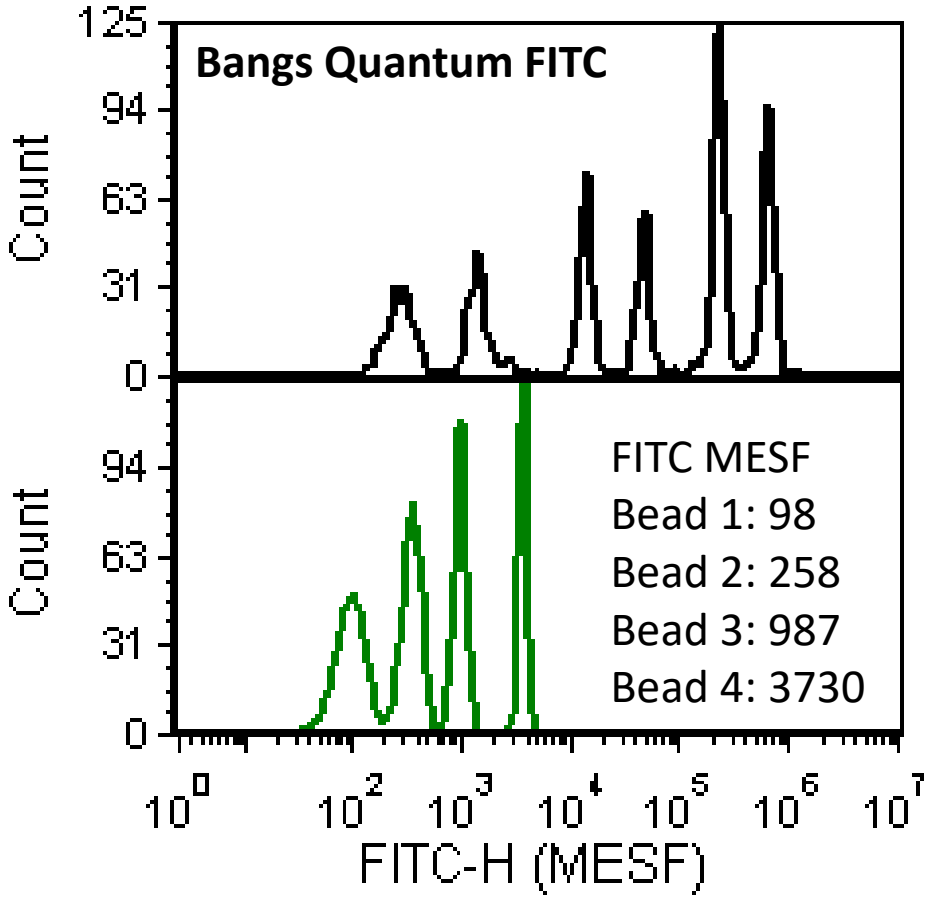


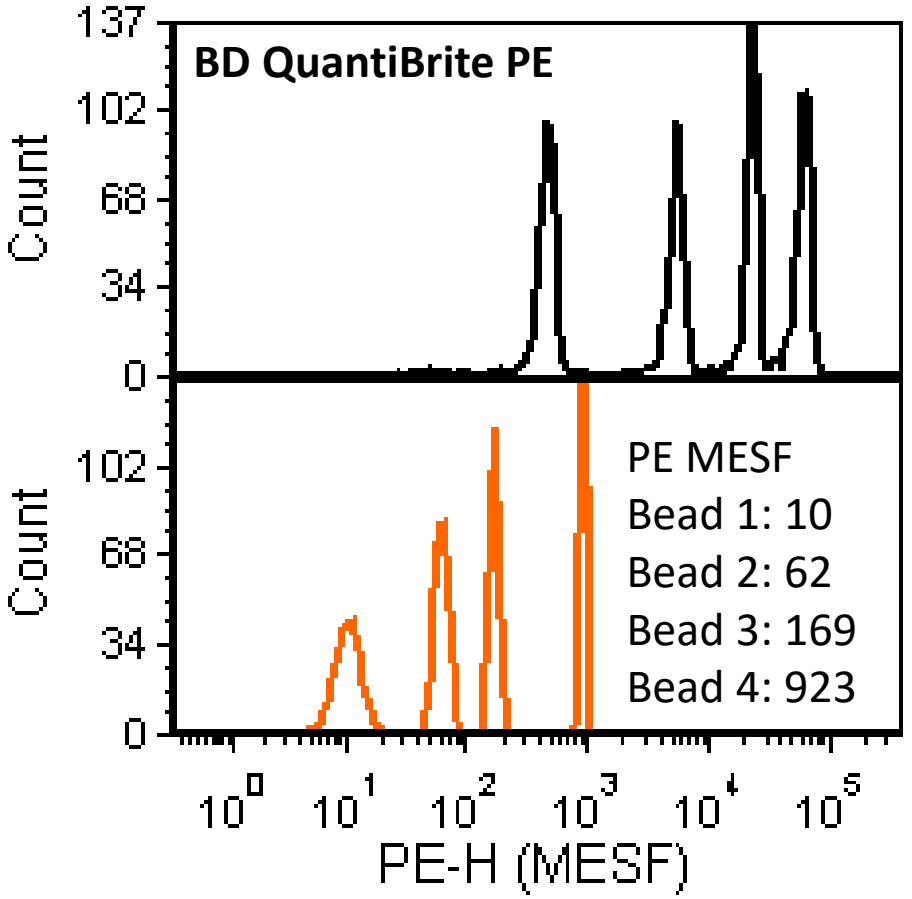
Marker	# of Events	Median	Arithmetic Mean	95%-ile
M1	7799	65	74	172
M2	15736	407	416	627
M3	14192	1117	1119	1372
M4	10941	3830	3826	4258

Marker	# of Events	Median	Arithmetic Mean	95%-ile
M1	7386	3	3	8
M2	15873	49	49	61
M3	14251	141	141	158
M4	10949	767	767	823

vCal™ nanoRainbow beads

MESF Cross Calibration





vFC™: Getting Started

CELLARCUS BIOSCIENCES

Instrument evaluation, configuration and set up

- Protocol 0 – Essential standards to qualify and calibrate an instrument

Sample preparation and staining

- Protocol 1 – Sample serial dilutions to establish assay dynamic range, EV concentration, lack of coincidence/swarm
- Protocol 2 – EV counting, sizing and cargo analysis

Data processing and analysis

- Gating
- Diameter estimation
- Immunofluorescence calibration
- Batch export of data to spreadsheet, summary and plots to PPT/PDF

CELLARCUS BIOSCIENCES

Vesicle Flow Cytometry Analysis Kit

FOR VESICLE COUNTING AND SIZING

Protocol 2-1: Measuring EV Surface Cargo Demo

Purpose

This Protocol demonstrates vFC by counting, sizing, and measuring surface marker (tetraspanin) expression of an EV standard.

Kit Component	Size	Store	Notes
vFRed™, membrane stain (100x)	1 x 50 uL	2-8°C	
Lipo100™ Standard (10x)	2 x 50 uL	2-8°C	
EV Lysing Solution (1000x)	1 x 25 uL	2-8°C	
vFC™ Staining Buffer (1x)	1 x 100 mL	2-8°C	
Anti-Tetraspanin (TS) Mix [PE] (10x)	1 x 20 tests	2-8°C	
PLT EV standard (10x)	1 x 50 uL	2-8°C	

Materials to be provided by User

Gloves
Microwell plate (Sartstedt 82.1583.001)
Pipettes (5 uL – 300 uL)
Pipette tips

Protocol 2-1: Measuring EV Surface Cargo

Materials

a. Gloves
b. Microwell plate
c. vFRed™ Membrane Stain (100x)
d. VFC Staining Buffer, 2 mL
e. Lipo100™ Standard (10x)
f. EV standard (10x)
g. Fluorescent antibody (FL mAb, 10x)
h. EV lysing solution

Procedure

Prepare Working Solutions

1. Prepare 200 uL 10x vFRed™ working solution (5 uL per well) by adding 20 uL vFRed™ (100x) to 180 uL VFC Staining Buffer (for 4 samples plus controls)
2. Prepare 10x Vesicle Lysing Solution by adding 5 uL to 495 uL Staining Buffer (500 uL)

Prepare Samples

3. Dilute sample to between ~$1x10^6$ and $1x10^8$/uL in VFC Staining Buffer in a microfuge tube and mix well.

Note: For new samples with unknown concentrations, see Protocol 1.

4. Place 35 uL of VFC Staining Buffer into individual wells (see Protocol 2 Plate Map).
5. Add 5 uL of FL mAb (or buffer for no mAb samples)
6. Add 5 uL of diluted samples and standards to designated wells.
7. Add 5 uL of 10x vFRed™ to each well (expect row H), mix by pipetting up and down.
8. Incubate for 60 minutes in the dark at RT.

Dilute and Read

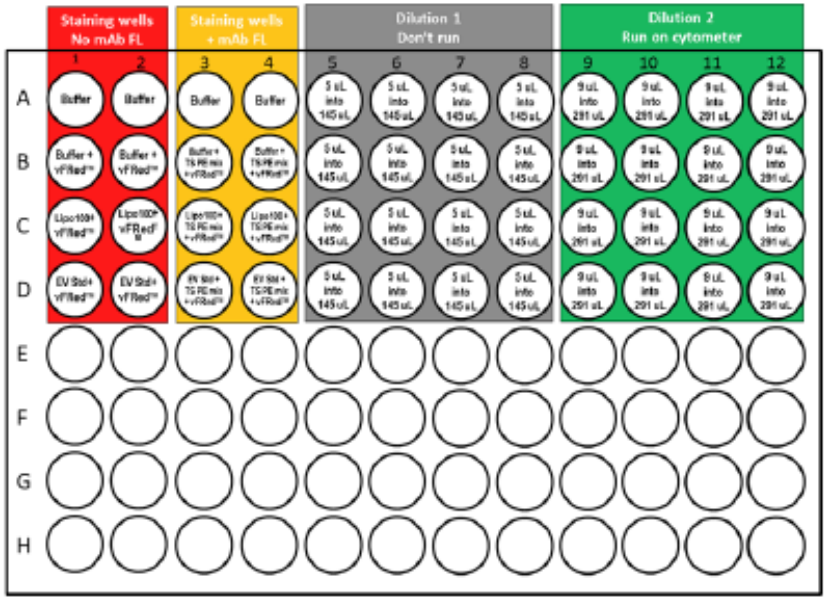
9. Place 145 uL Staining Buffer into wells in Columns 5-8, and 291 ul into wells in Columns 9-12.

10. Transfer 5 uL of stained sample from wells in Columns 1-4 into wells in Columns 5-8 and mix by pipetting up and down (Dilution 1).
11. Transfer 9 uL of Dilution 1 into the well in Columns 9-12 and mix (Dilution 2).
12. Run on Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 uL/min).

Detergent Sensitivity

1. Following the first post-stain dilution (Step 10), add 5 uL 10x Vesicle Lysing Solution to desired Staining Wells (eg wells A1-D4) and incubate 10 minutes.
2. Dilute and read as above.

Protocol 2-1 Plate Map



vFC™: Getting Started

Instrument evaluation, configuration and set up

- Protocol 0 – Essential standards to qualify and calibrate an instrument

Sample preparation and staining

- Protocol 1 – Sample serial dilutions to establish assay dynamic range, EV concentration, lack of coincidence/swarm
- Protocol 2 – EV counting, sizing and cargo analysis

Data processing and analysis

- Gating
- Diameter estimation
- Immunofluorescence calibration
- Batch export of data to spreadsheet, summary and plots to PPT/PDF

N:\Nolan_Lab\Raman_FC_Data\Cytoflex S\VFC Report v0.756 BCFlex 111219 CDW demo.fey - FCS Express 6 Flow

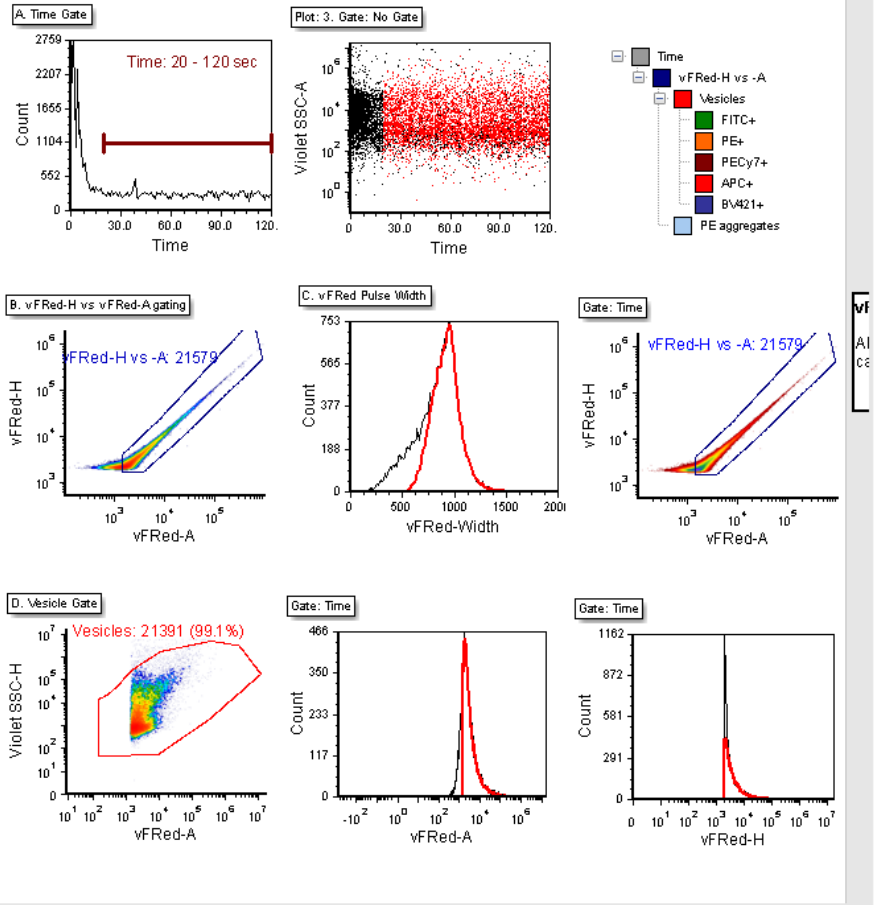
File Home Insert Design Gating Batch Format Text Data Tools Quality View

General Size Fill Border/Line Authorizations — Object Options
Gates To Display Parameters To Display Data Image Options — Data Options
Overlays Axes Title Legend 3D Specific Options — Plot Options
Markers — 1D Options
Quadrants Color Levels — 2D Options
Statistics — Statistic Options
Change Plot Type — Change
Override Authorizations — Override

vFC™ Gating

ELLARCUS BIOSCIENCES

Experiment:
Sample: PLT +hu TS mix PE+Detergent
Well name: 02-20191113-H9.fcs
Trigger threshold:vFRed 1900 ABT: 386 ()
Instrument: CytoFLEX LX AS38003



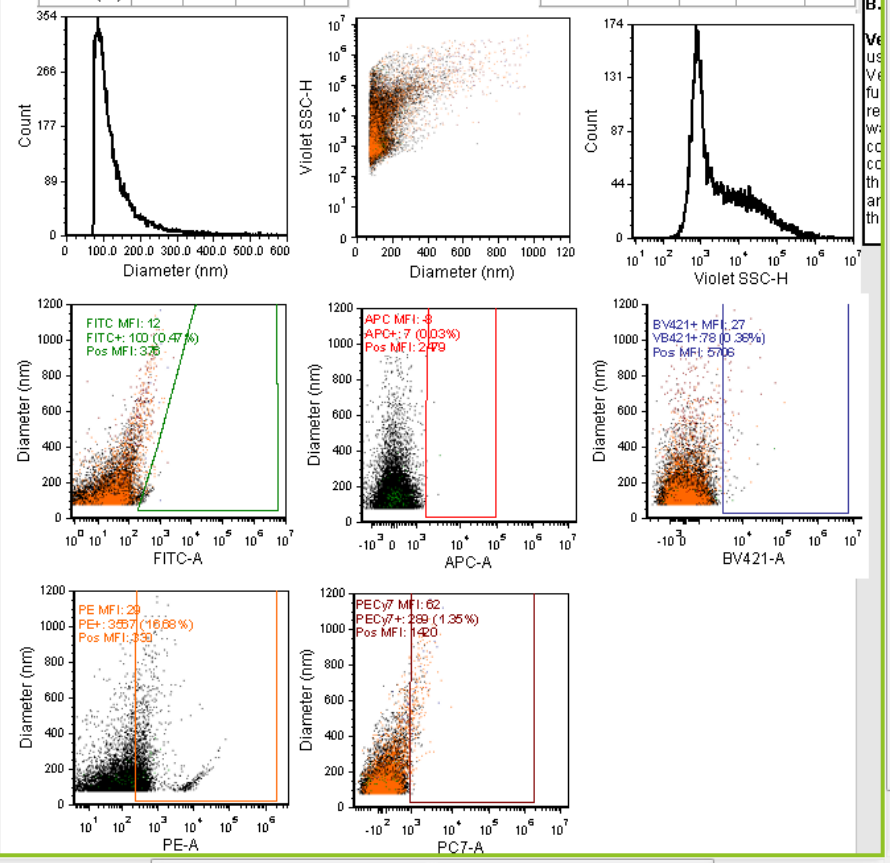
vFC™ Report

ELLARCUS BIOSCIENCES

Experiment:
Well name: 02-20191113-H9.fcs
Sample: PLT +hu TS mix PE+Detergent
Volume:
Instrument: CytoFLEX LX AS38003

Parameter	# of Events	Median	Arithmetic Mean	CV
Diameter (nm)	21391	109.37	139.89	71.46

Parameter	# of Events	Median	Arithmetic Mean	CV
Violet SSC-H	21391	1696.20	31351.03	471.80



Data List

Iteration	File Name	SMID	$ETIM	$DATE
1	02-20191113-A9.fcs	buffer no dye	11:04:01	13-Nov...
2	02-20191113-B9.fcs	buffer +VFRed	11:06:15	13-Nov...
3	02-20191113-C9.fcs	Lipo100 +VFRed	11:08:29	13-Nov...
4	02-20191113-D9.fcs	PLT +VFRed	11:10:43	13-Nov...
5	02-20191113-E9.fcs	buffer no dye	11:12:57	13-Nov...
6	02-20191113-F9.fcs	buffer no dye	11:15:11	13-Nov...
7	02-20191113-G9.fcs	buffer +VFRed ...	11:17:25	13-Nov...
8	02-20191113-H9.fcs	PLT +hu TS mix ...	11:19:39	13-Nov...
9	02-20191113-A10.fcs	buffer no dye	11:21:54	13-Nov...
10	02-20191113-B10.fcs	buffer +VFRed	11:24:08	13-Nov...
11	02-20191113-C10.fcs	Lipo100 +VFRed	11:26:22	13-Nov...
12	02-20191113-D10.fcs	PLT +VFRed	11:28:36	13-Nov...
13	02-20191113-E10.fcs	buffer no dye	11:30:50	13-Nov...
14	02-20191113-F10.fcs	buffer no dye	11:33:04	13-Nov...
15	02-20191113-G10.fcs	buffer +VFRed ...	11:35:18	13-Nov...
16	02-20191113-H10.fcs	PLT +hu TS mix ...	11:37:32	13-Nov...
17	02-20191113 hu TS mix PE-A11.fcs	buffer no dye	11:39:47	13-Nov...
18	02-20191113 hu TS mix PE-B11.fcs	buffer +VFRed	11:42:01	13-Nov...
19	02-20191113 hu TS mix PE-C11.fcs	Lipo100 +VFRed	11:44:15	13-Nov...
20	02-20191113 hu TS mix PE-D11.fcs	PLT +VFRed	11:46:29	13-Nov...
21	02-20191113-E11.fcs	buffer no dye	11:48:43	13-Nov...
22	02-20191113-F11.fcs	buffer no dye	11:50:57	13-Nov...
23	02-20191113-G11.fcs	buffer no dye	11:53:11	13-Nov...
24	02-20191113-H11.fcs	buffer no dye	11:55:25	13-Nov...
25	02-20191113 hu TS mix PE-A12.fcs	buffer no dye	11:57:40	13-Nov...
26	02-20191113 hu TS mix PE-B12.fcs	buffer +VFRed	11:59:55	13-Nov...
27	02-20191113 hu TS mix PE-C12.fcs	Lipo100 +VFRed	12:02:09	13-Nov...
28	02-20191113 hu TS mix PE-D12.fcs	PLT +VFRed	12:04:23	13-Nov...
29	02-20191113-E12.fcs	buffer no dye	12:06:37	13-Nov...
30	02-20191113-F12.fcs	buffer no dye	12:08:51	13-Nov...
31	02-20191113-G12.fcs	buffer no dye	12:11:05	13-Nov...
32	02-20191113-H12.fcs	buffer no dye	12:13:19	13-Nov...
33	02-20191113 +VFRed-A1.fcs	buffer	13:43:38	13-Nov...
34	02-20191113 +VFRed-A2.fcs	MV-M-Zero dilu...	13:45:52	13-Nov...
35	02-20191113 +VFRed-A3.fcs	MV-M-DsfGFP ...	13:48:06	13-Nov...
36	02-20191113 +VFRed-A4.fcs	MV-M-sfGFP dil...	13:50:20	13-Nov...
37	02-20191113 +VFRed-A5.fcs	buffer no dye	13:54:04	13-Nov...
38	02-20191113 +VFRed +GFP_PE-B1.fcs	buffer	13:56:19	13-Nov...
39	02-20191113 +VFRed +GFP_PE-B2.fcs	MV-M-Zero dilu...	13:58:33	13-Nov...
40	02-20191113 +VFRed +GFP_PE-B3.fcs	MV-M-DsfGFP ...	14:00:47	13-Nov...
41	02-20191113 +VFRed +GFP_PE-B4.fcs	MV-M-sfGFP dil...	14:03:01	13-Nov...
42	02-20191113 +VFRed +GFP_PE-B5.fcs	buffer no dye	14:05:16	13-Nov...

3/16 Log Instructions Gating Report Report II All_dens All_cdot VSSC v FL

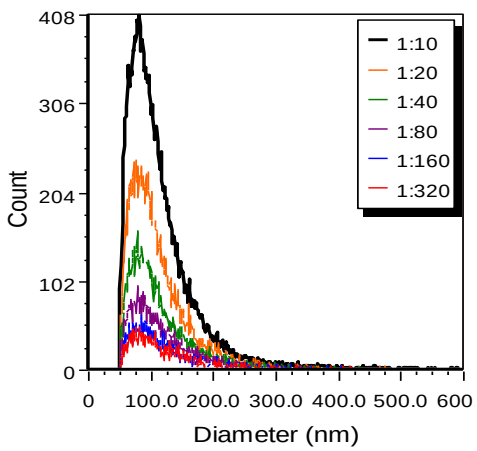
4/16 Log Instructions Gating Report Report II All_dens All_cdot VSSC v FL

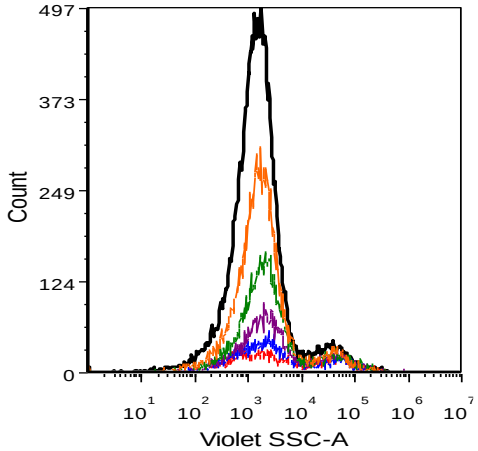
90% Zoom No panel is active Background tasks have completed Logged in as: Default Warnings Modified

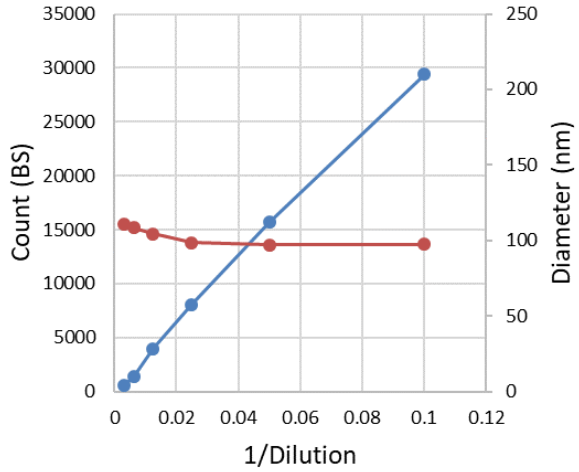
vFC™: Controls

Dilution Series

Establishes dynamic range and allows assessment of coincidence (“swarm”) artifact (multiple EVs).

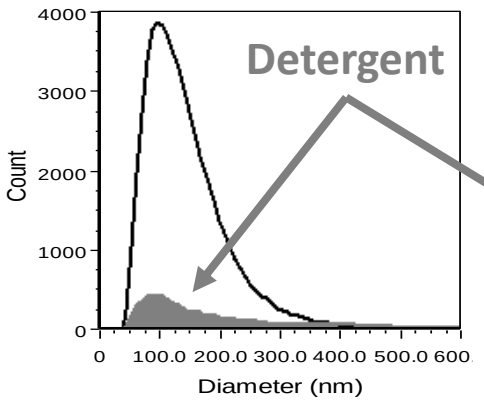


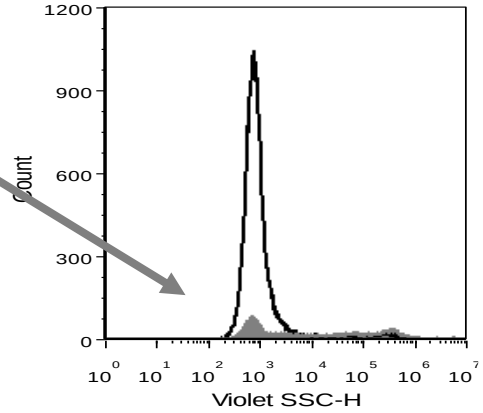


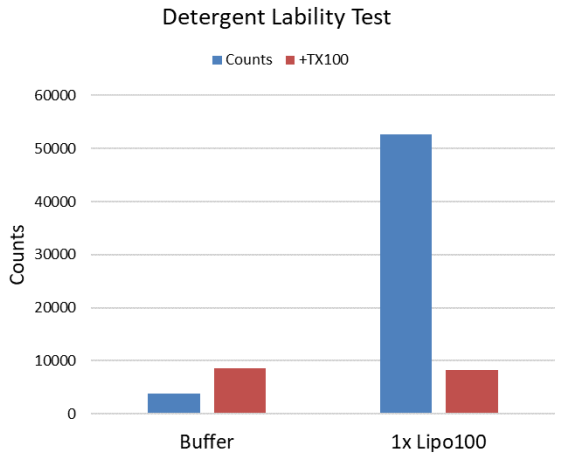


Detergent treatment

Detergent solubilizes EV and other vesicles, confirming their vesicular nature

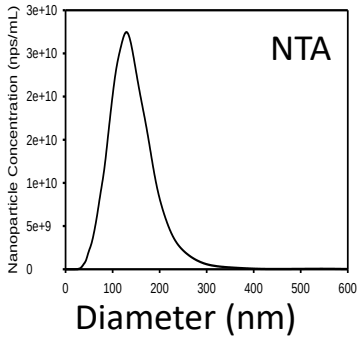


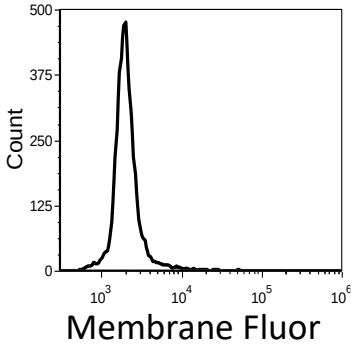


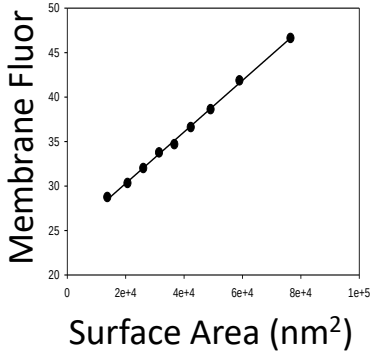


vFC™: EV Size and Light Scatter

EV Size Calibration





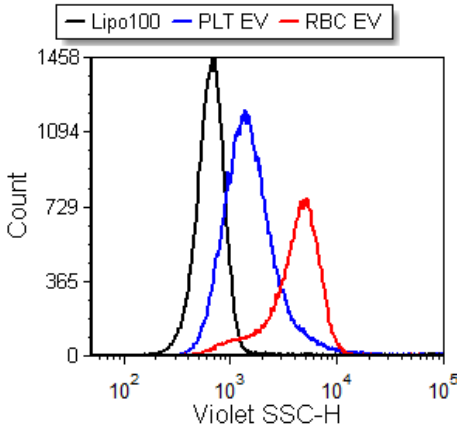


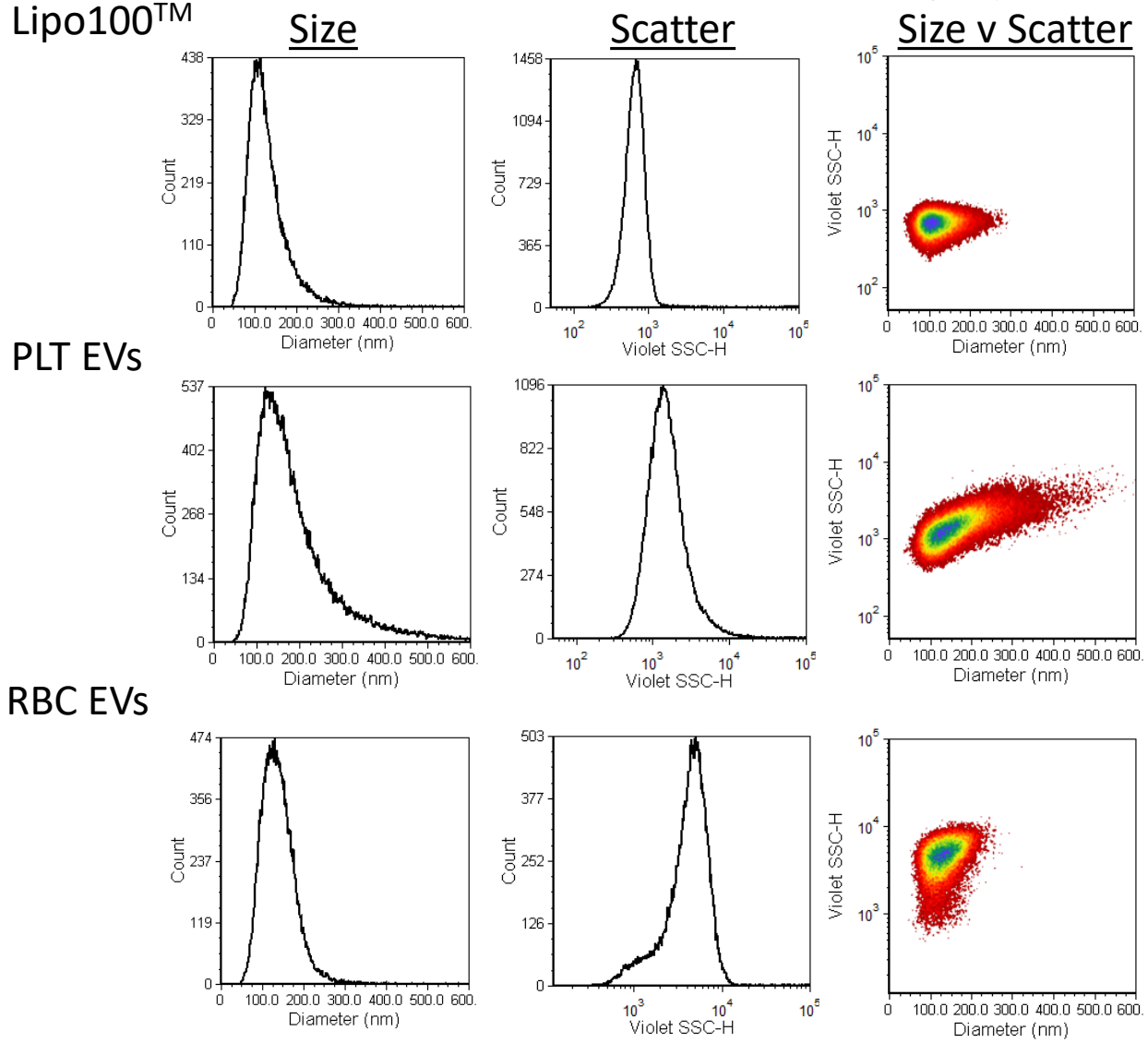
Lipo100™ is characterized by several orthogonal methods (NTA, RPS EM) and serves as a vesicle size standard

EV Light Scatter

EV light scatter is a complex function of particle size and refractive index.

Light scatter is different for EVs from different sources, likely indicating differences in refractive index due to differences in EV cargo





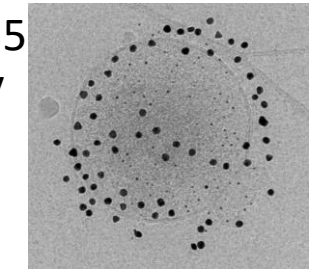
EV Standards: Blood cell-derived EVs

Liposome

No antigens

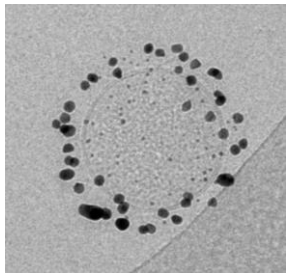
RBC EVs

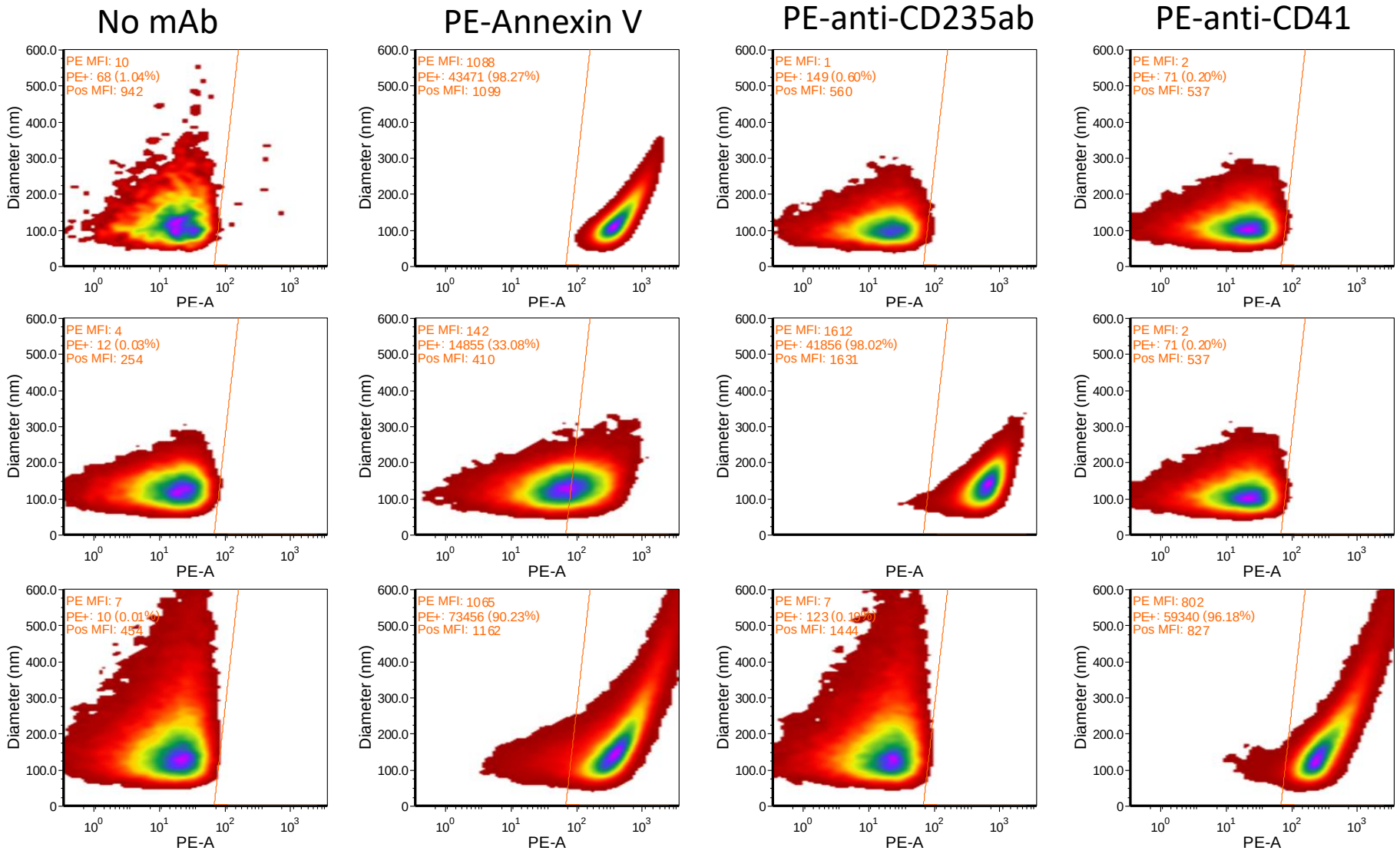
- CD235
- AnnV



PLT EVs

- CD41
- AnnV

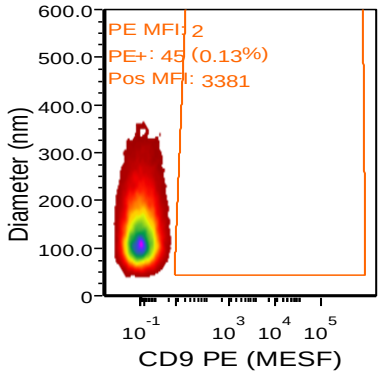




vFC™: EV Immunofluorescence

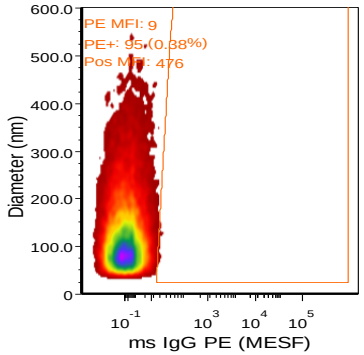
Negative controls

Lipo100™



Lipo100 bears no antigen, and provides a reference for background and threshold for positivity

ms IgG PE



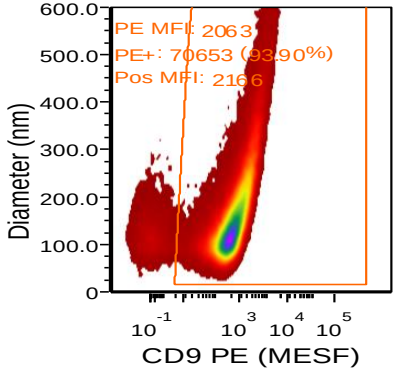
Isotype control tests for presence of Fc-mediated IgG binding

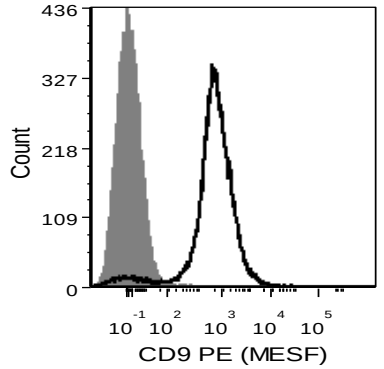
Positive Controls

Cell-derived EVs with characterized expression of specific markers

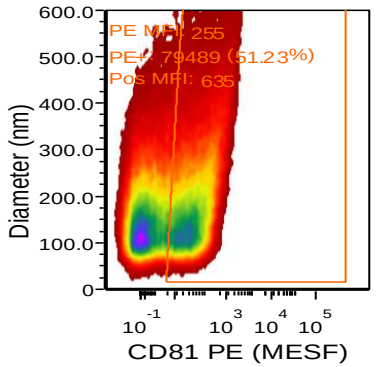
Examples:

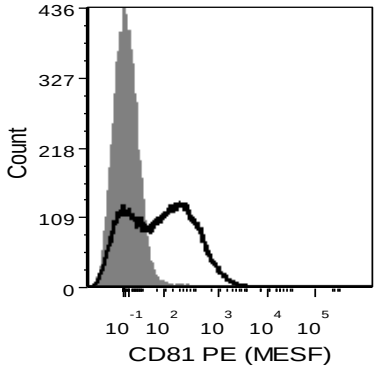
CD9 on PLT EVs





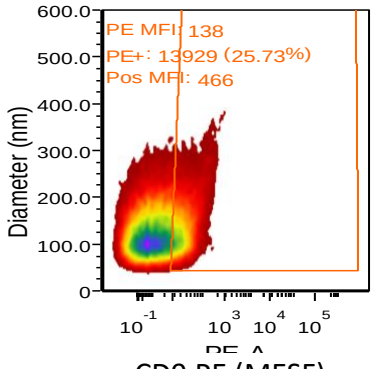
CD81 on 293T EVs





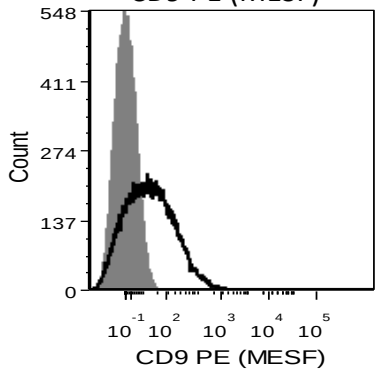
Analysis

Depending on the goals of the measurement, immunofluorescence can be expressed as:



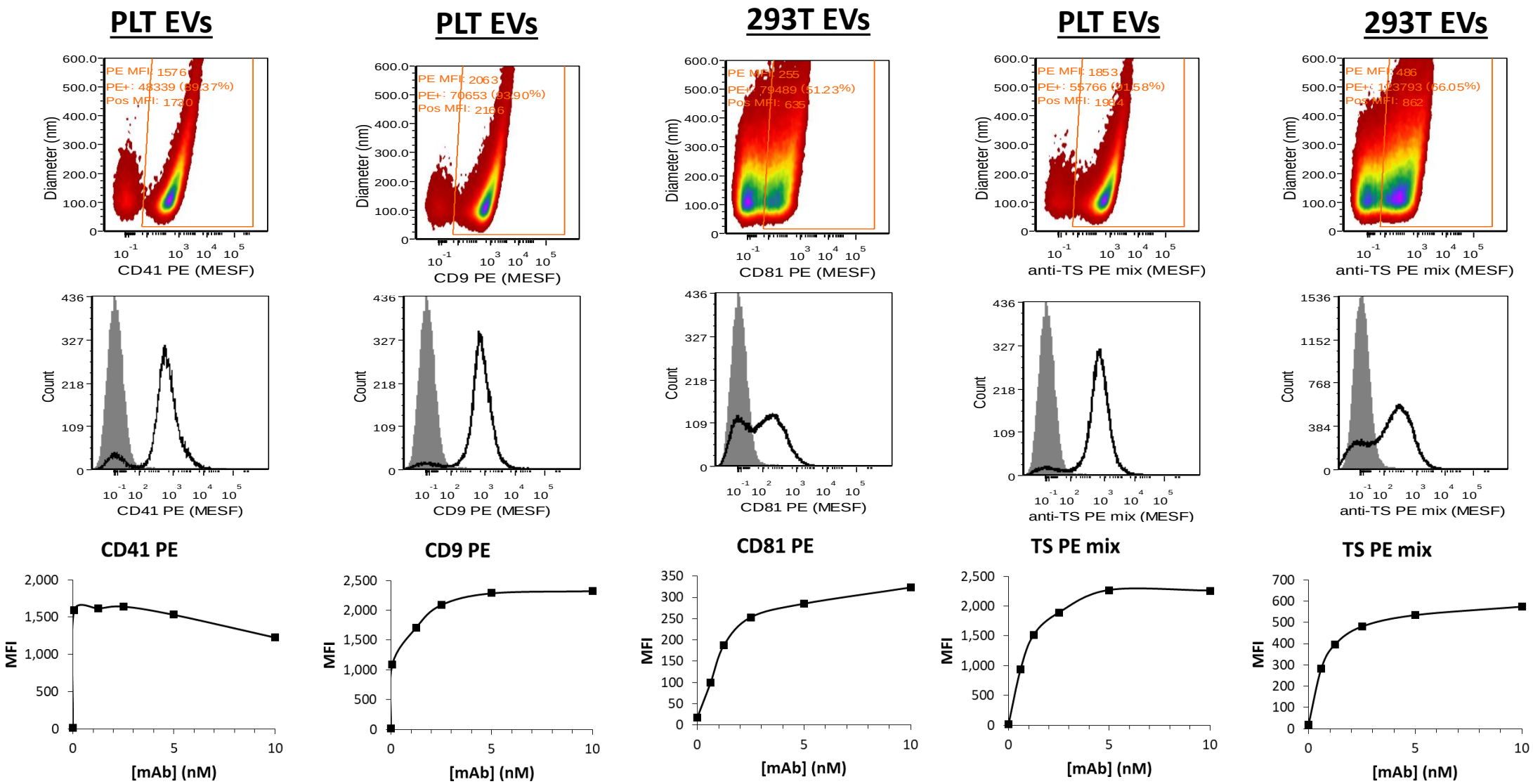
the number “positive” above a defined threshold

or



the population median fluorescence intensity (MFI)

vFC™: Antibody Titration



vFC™: EV Analysis Assay kit

ELLARCUS BIOSCIENCES

Instrument evaluation, configuration and set up

- Protocol 0 – Essential standards to qualify and calibrate an instrument

Sample preparation and staining

- Protocol 1 – Sample serial dilutions to establish assay dynamic range, EV concentration, lack of coincidence/swarm
- Protocol 2 – EV counting, sizing and cargo analysis

Data processing and analysis

- Gating
- Diameter estimation
- Immunofluorescence calibration
- Batch export of data to spreadsheet, summary and plots to PPT/PDF

CELLARCUS BIOSCIENCES

Vesicle Flow Cytometry Analysis Kit

FOR VESICLE COUNTING AND SIZING

Protocol 2-1: Measuring EV Surface Cargo Demo

Purpose

This Protocol demonstrates vFC by counting, sizing, and measuring surface marker (tetraspanin) expression of an EV standard.

Kit Component	Size	Store	Notes
vFRed™, membrane stain (100x)	1 x 50 uL	2-8°C	
Lipo100™ Standard (10x)	2 x 50 uL	2-8°C	
EV Lysing Solution (1000x)	1 x 25 uL	2-8°C	
vFC™ Staining Buffer (1x)	1 x 100 mL	2-8°C	
Anti-Tetraspanin (TS) Mix [PE] (10x)	1 x 20 tests	2-8°C	
PLT EV standard (10x)	1 x 50 uL	2-8°C	

Materials to be provided by User

Gloves
Microwell plate (Sartstedt 82.1583.001)
Pipettes (5 uL – 300 uL)
Pipette tips

Protocol 2-1: Measuring EV Surface Cargo

Materials

a. Gloves
b. Microwell plate
c. vFRed™ Membrane Stain (100x)
d. VFC Staining Buffer, 2 mL
e. Lipo100™ Standard (10x)
f. EV standard (10x)
g. Fluorescent antibody (FL mAb, 10x)
h. EV lysing solution

Procedure

Prepare Working Solutions

1. Prepare 200 uL 10x vFRed™ working solution (5 uL per well) by adding 20 uL vFRed™ (100x) to 180 uL VFC Staining Buffer (for 4 samples plus controls)
2. Prepare 10x Vesicle Lysing Solution by adding 5 uL to 495 uL Staining Buffer (500 uL)

Prepare Samples

3. Dilute sample to between ~$1x10^6$ and $1x10^8$/uL in VFC Staining Buffer in a microfuge tube and mix well.

Note: For new samples with unknown concentrations, see Protocol 1.

4. Place 35 uL of VFC Staining Buffer into individual wells (see Protocol 2 Plate Map).
5. Add 5 uL of FL mAb (or buffer for no mAb samples)
6. Add 5 uL of diluted samples and standards to designated wells.
7. Add 5 uL of 10x vFRed™ to each well (expect row H), mix by pipetting up and down.
8. Incubate for 60 minutes in the dark at RT.

Dilute and Read

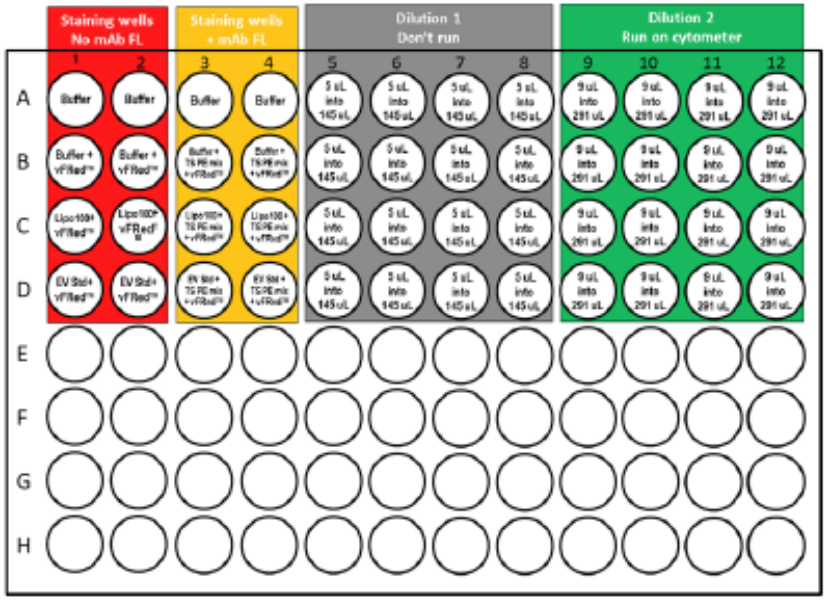
9. Place 145 uL Staining Buffer into wells in Columns 5-8, and 291 ul into wells in Columns 9-12.

10. Transfer 5 uL of stained sample from wells in Columns 1-4 into wells in Columns 5-8 and mix by pipetting up and down (Dilution 1).
11. Transfer 9 uL of Dilution 1 into the well in Columns 9-12 and mix (Dilution 2).
12. Run on Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 uL/min).

Detergent Sensitivity

1. Following the first post-stain dilution (Step 10), add 5 uL 10x Vesicle Lysing Solution to desired Staining Wells (eg wells A1-D4) and incubate 10 minutes.
2. Dilute and read as above.

Protocol 2-1 Plate Map



N:\Nolan_Lab\Raman_FC_Data\Cytoflex S\VFC Report v0.756 BCFlex 111219 CDW demo.fey - FCS Express 6 Flow

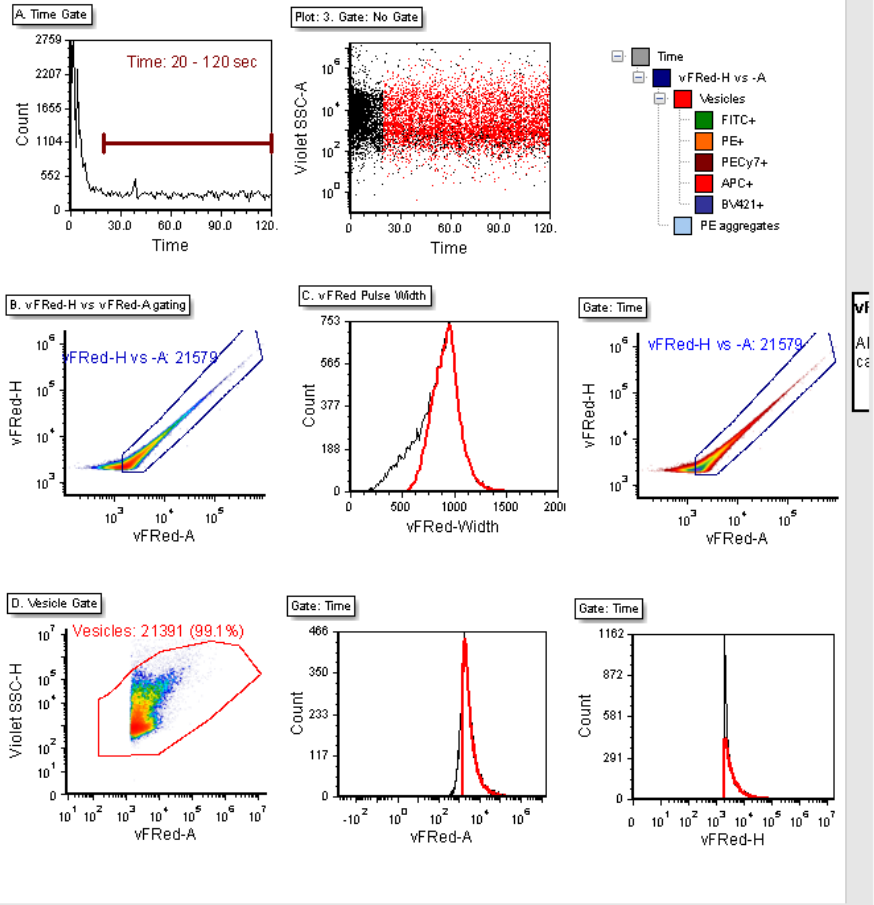
File Home Insert Design Gating Batch Format Text Data Tools Quality View

General Size Fill Border/Line Authorizations — Object Options
Gates To Display Parameters To Display Data Image Options — Data Options
Overlays Axes Title Legend 3D Specific Options — Plot Options
Markers — 1D Options
Quadrants Color Levels — 2D Options
Statistics — Statistic Options
Change Plot Type — Change
Override Authorizations — Override

vFC™ Gating

ELLARCUS BIOSCIENCES

Experiment:
Sample: PLT +hu TS mix PE+Detergent
Well name: 02-20191113-H9.fcs
Trigger threshold:vFRed 1900 ABT: 386 ()
Instrument: CytoFLEX LX AS38003



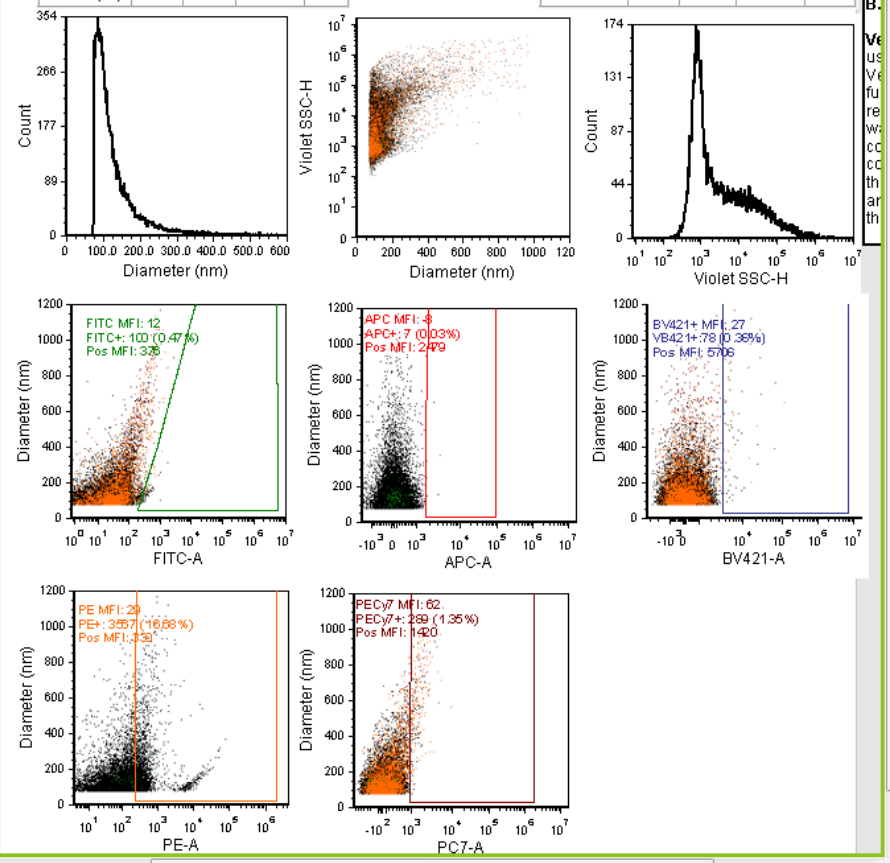
vFC™ Report

ELLARCUS BIOSCIENCES

Experiment:
Well name: 02-20191113-H9.fcs
Sample: PLT +hu TS mix PE+Detergent
Volume:
Instrument: CytoFLEX LX AS38003

Parameter	# of Events	Median	Arithmetic Mean	CV
Diameter (nm)	21391	109.37	139.89	71.46

Parameter	# of Events	Median	Arithmetic Mean	CV
Violet SSC-H	21391	1696.20	31351.03	471.80



Data List

Iteration	File Name	SMID	$ETIM	$DATE
1	02-20191113-A9.fcs	buffer no dye	11:04:01	13-Nov...
2	02-20191113-B9.fcs	buffer +VFRed	11:06:15	13-Nov...
3	02-20191113-C9.fcs	Lipo100 +VFRed	11:08:29	13-Nov...
4	02-20191113-D9.fcs	PLT +VFRed	11:10:43	13-Nov...
5	02-20191113-E9.fcs	buffer no dye	11:12:57	13-Nov...
6	02-20191113-F9.fcs	buffer no dye	11:15:11	13-Nov...
7	02-20191113-G9.fcs	buffer +VFRed ...	11:17:25	13-Nov...
8	02-20191113-H9.fcs	PLT +hu TS mix ...	11:19:39	13-Nov...
9	02-20191113-A10.fcs	buffer no dye	11:21:54	13-Nov...
10	02-20191113-B10.fcs	buffer +VFRed	11:24:08	13-Nov...
11	02-20191113-C10.fcs	Lipo100 +VFRed	11:26:22	13-Nov...
12	02-20191113-D10.fcs	PLT +VFRed	11:28:36	13-Nov...
13	02-20191113-E10.fcs	buffer no dye	11:30:50	13-Nov...
14	02-20191113-F10.fcs	buffer no dye	11:33:04	13-Nov...
15	02-20191113-G10.fcs	buffer +VFRed ...	11:35:18	13-Nov...
16	02-20191113-H10.fcs	PLT +hu TS mix ...	11:37:32	13-Nov...
17	02-20191113 hu TS mix PE-A11.fcs	buffer no dye	11:39:47	13-Nov...
18	02-20191113 hu TS mix PE-B11.fcs	buffer +VFRed	11:42:01	13-Nov...
19	02-20191113 hu TS mix PE-C11.fcs	Lipo100 +VFRed	11:44:15	13-Nov...
20	02-20191113 hu TS mix PE-D11.fcs	PLT +VFRed	11:46:29	13-Nov...
21	02-20191113-E11.fcs	buffer no dye	11:48:43	13-Nov...
22	02-20191113-F11.fcs	buffer no dye	11:50:57	13-Nov...
23	02-20191113-G11.fcs	buffer no dye	11:53:11	13-Nov...
24	02-20191113-H11.fcs	buffer no dye	11:55:25	13-Nov...
25	02-20191113 hu TS mix PE-A12.fcs	buffer no dye	11:57:40	13-Nov...
26	02-20191113 hu TS mix PE-B12.fcs	buffer +VFRed	11:59:55	13-Nov...
27	02-20191113 hu TS mix PE-C12.fcs	Lipo100 +VFRed	12:02:09	13-Nov...
28	02-20191113 hu TS mix PE-D12.fcs	PLT +VFRed	12:04:23	13-Nov...
29	02-20191113-E12.fcs	buffer no dye	12:06:37	13-Nov...
30	02-20191113-F12.fcs	buffer no dye	12:08:51	13-Nov...
31	02-20191113-G12.fcs	buffer no dye	12:11:05	13-Nov...
32	02-20191113-H12.fcs	buffer no dye	12:13:19	13-Nov...
33	02-20191113 +VFRed-A1.fcs	buffer	13:43:38	13-Nov...
34	02-20191113 +VFRed-A2.fcs	MV-M-Zero dilu...	13:45:52	13-Nov...
35	02-20191113 +VFRed-A3.fcs	MV-M-DsfGFP ...	13:48:06	13-Nov...
36	02-20191113 +VFRed-A4.fcs	MV-M-sfGFP dil...	13:50:20	13-Nov...
37	02-20191113 +VFRed-A5.fcs	buffer no dye	13:54:04	13-Nov...
38	02-20191113 +VFRed +GFP_PE-B1.fcs	buffer	13:56:19	13-Nov...
39	02-20191113 +VFRed +GFP_PE-B2.fcs	MV-M-Zero dilu...	13:58:33	13-Nov...
40	02-20191113 +VFRed +GFP_PE-B3.fcs	MV-M-DsfGFP ...	14:00:47	13-Nov...
41	02-20191113 +VFRed +GFP_PE-B4.fcs	MV-M-sfGFP dil...	14:03:01	13-Nov...
42	02-20191113 +VFRed +GFP_PE-B5.fcs	buffer no dye	14:05:16	13-Nov...

3/16 Log Instructions Gating Report Report II All_dens All_cdot VSSC v FL

4/16 Log Instructions Gating Report Report II All_dens All_cdot VSSC v FL

90% Zoom No panel is active Background tasks have completed Logged in as: Default Warnings Modified

Summary

Vesicle Flow Cytometry (vFC™) provides:

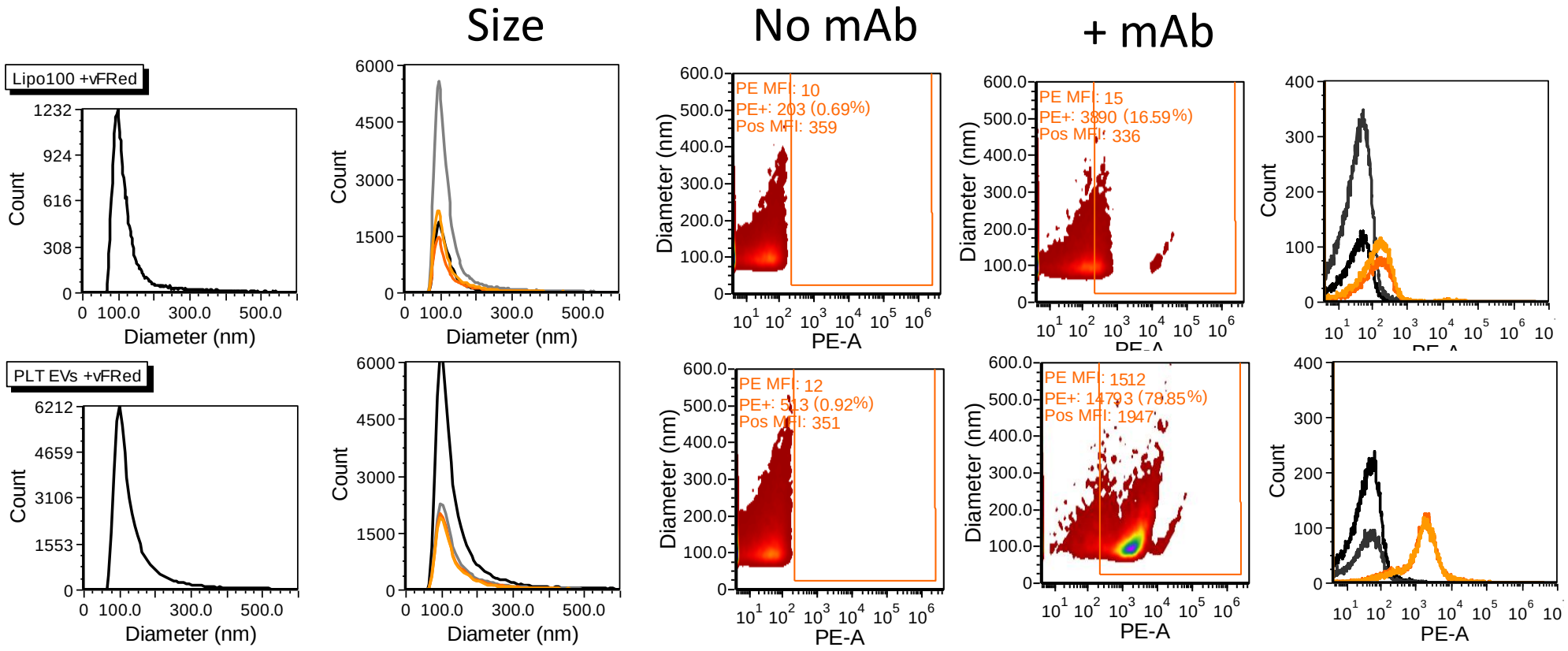
- Selective fluorescence detection of membrane particles
- Surface area-based size estimates
- Quantitative cargo immunofluorescence
- Calibrators and standards, controls
- Standardized sample preparation and data analysis protocols
- Unambiguous data interpretation and cross lab comparisons

Demo and discussion

vFC™: Immunofluorescence

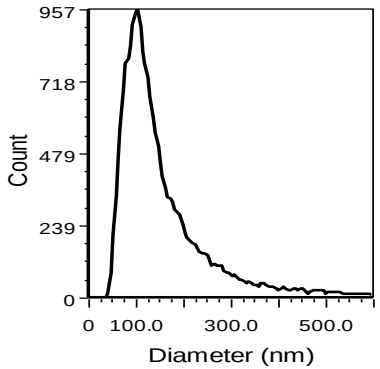
Lipo100

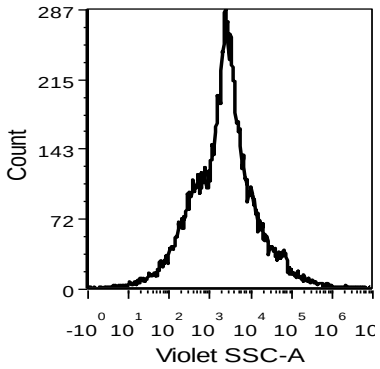
PLT EVs

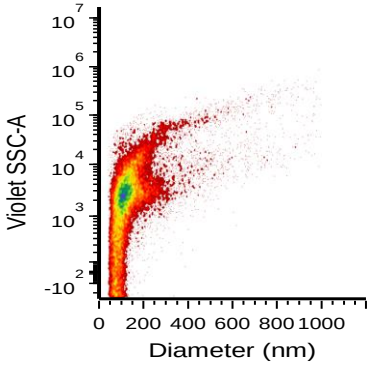


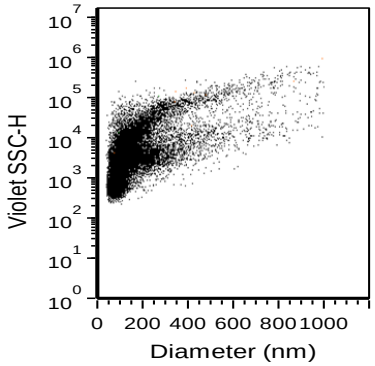
vFC of Plasma EVs

Human Plasma (ISTH)
Citrated, spun 2x @2500xg, 15 min
Freeze aliquots







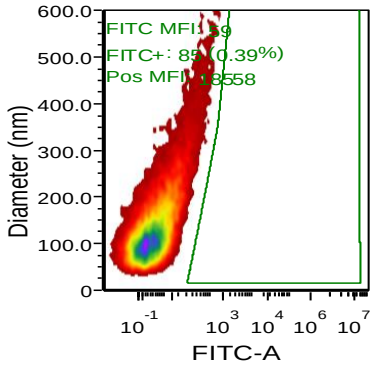


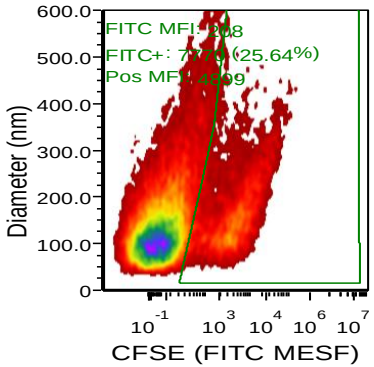
Lipoproteins have higher refractive indices

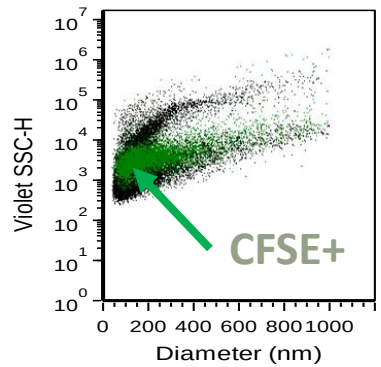
Plasma vFC protocol:

1. Dilute
2. Stain: membrane probe, volume marker, surface marker

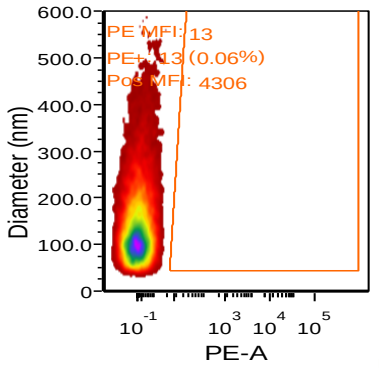
CFDA-SE:
Esterase-based staining of EV volume

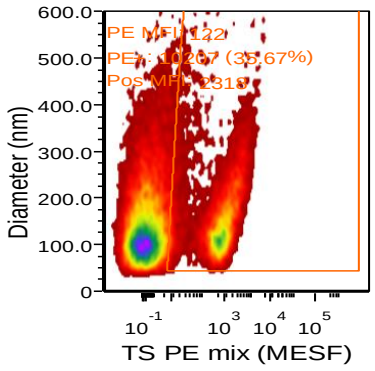


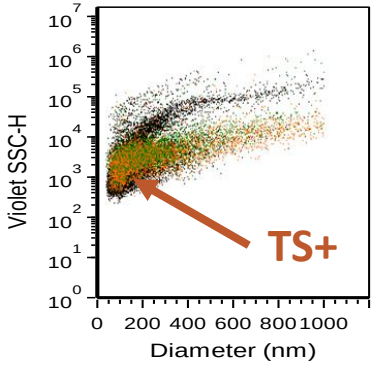


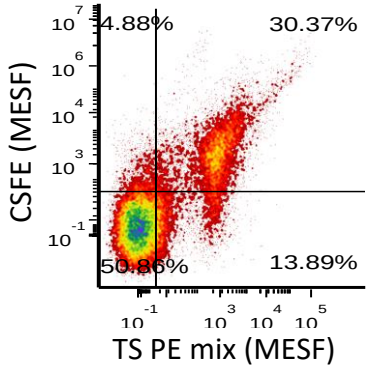


Anti-TS PE mix:
CD9 PE
CD63 PE
CD81 PE









vFC of Plasma EV Subtypes

Plasma vFC protocol:

1. Dilute
2. Stain: membrane probe, volume marker, surface marker cocktail

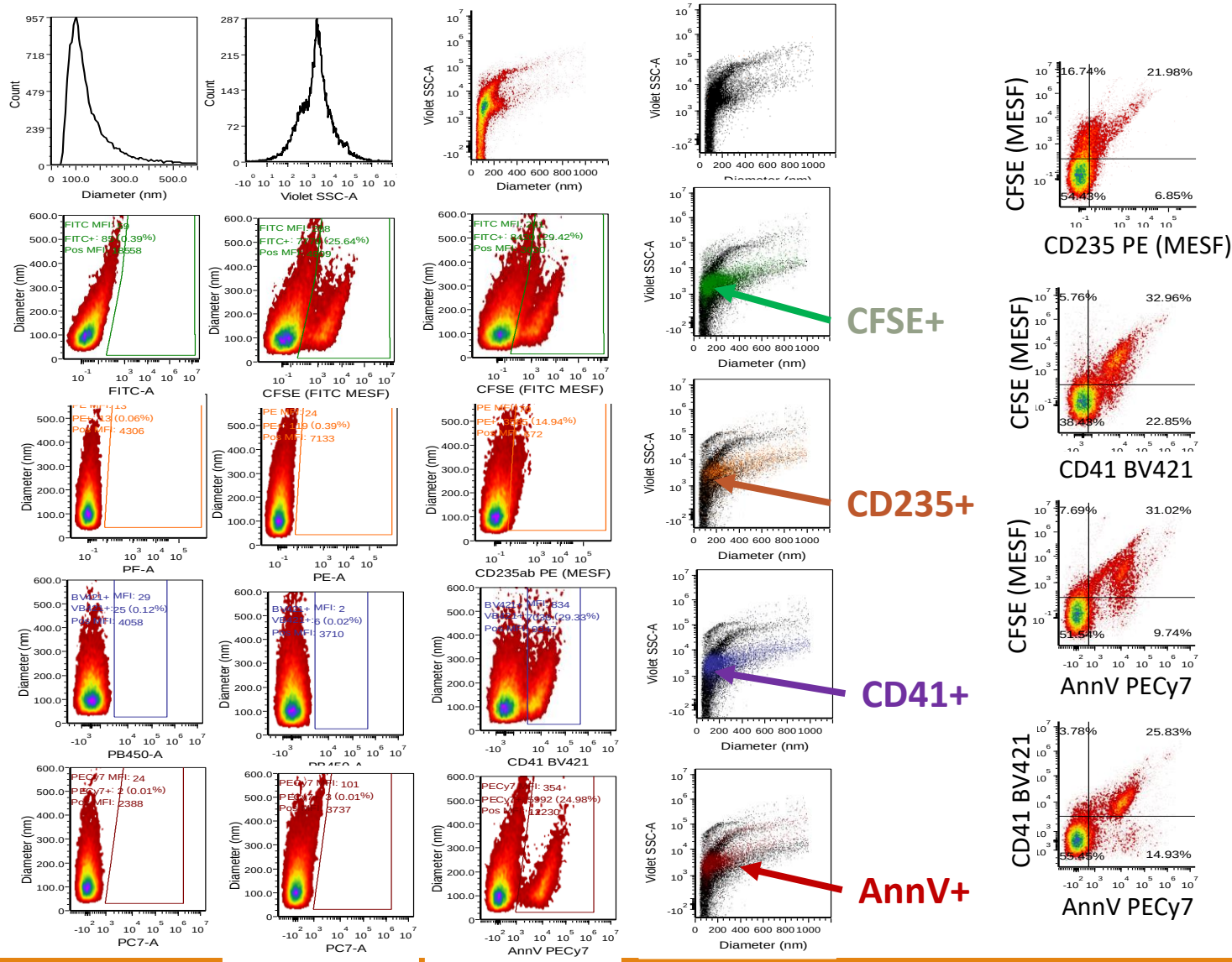
vFRed: Membrane marker

CFSE: Esterase/ volume marker

CD235 PE: Glycophorin (RBCs)

CD41 BV421: α2 integrin (PLTs)

AnnV PECy7: Exposed phosphatidyl-serine



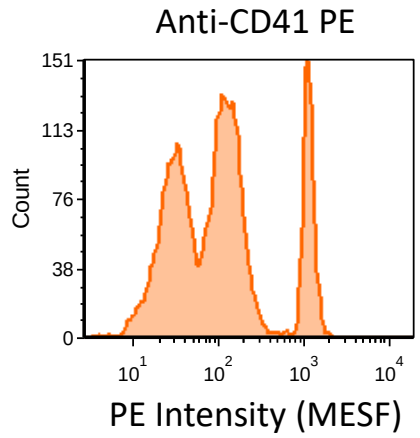
Questions?

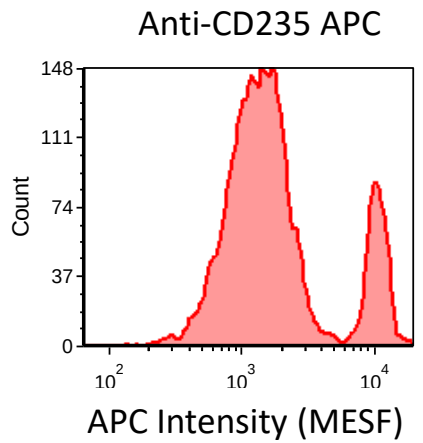
Multicolor EV panel development

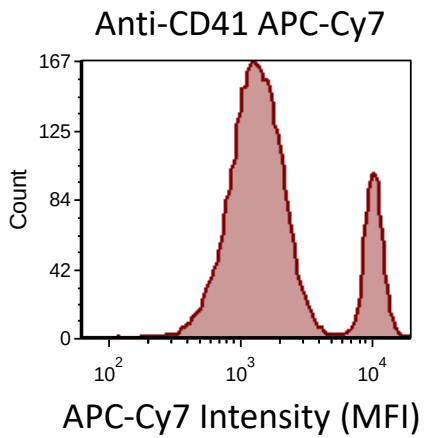
A. Antibody capture beads
- Blank
- 200 IgG
- 1200 IgG

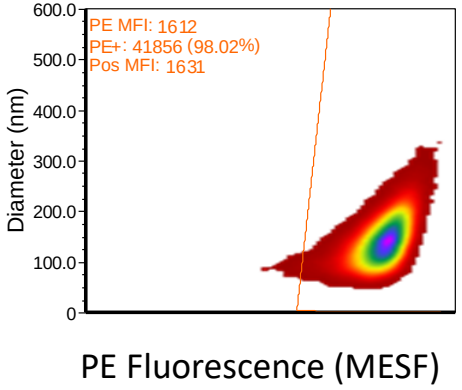
B. RBC EVs
- CD235 PE
- CD235 APC

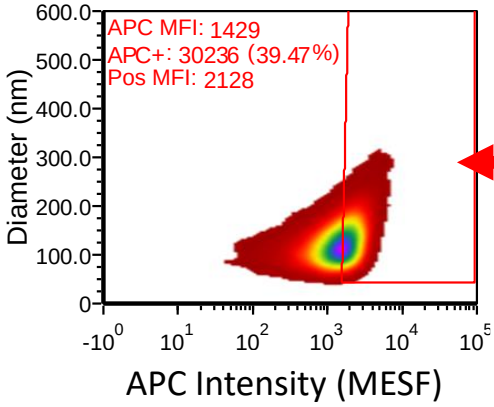
C. PLT EVs
- CD41 PE
- CD41 APCCy7



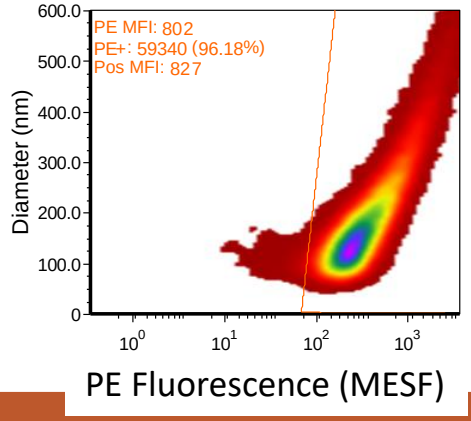








APC not as bright as PE, but still usable for CD235 on RBC EVs



APCCy7 is too dim to detect CD41 on PLT EVs