Supporting Information 1: "Calibration of flow cytometers enables reproducible measurements of extracellular vesicle concentrations and reference range establishment"

Interlaboratory comparison study protocol

Background

The concentration of extracellular vesicles (EVs) in liquid biopsies behold the promise of becoming new biomarkers for common diseases. Flow cytometry is a single-particle based detection technique capable of measuring the concentration of immuno-fluorescently stained particles in liquid biopsies. Stained EVs, however, have a broad size distribution, a broad light scattering distribution, and a broad fluorescence intensity distribution. Moreover, only a part of the EVs is sufficiently bright to exceed the lower detection limits of the detectors. As different flow cytometers have different lower detection limits, flow cytometers measure different EV concentrations. These EV concentrations can be compared only if the detection ranges can be expressed into comparable units by a calibration. The capability of measuring comparable EV concentrations within known detection ranges will improve the reproducibility of EV science and paves the road for clinical applications of EVs.

Study aim

To measure similar concentrations of EVs in a human plasma EV test sample (PEVTES) in different laboratories, within the same detection ranges. The detection ranges will be determined by relating the arbitrary units of light scattering to the diameter of EVs and by relating the arbitrary units of fluorescence to the number of molecules of equivalent soluble fluorophore (MESF). The flow rate will be calibrated with counting beads.

Protocol

This protocol contains Part A, Part B and Part C. Part A describes procedures that should be followed upon receipt of the packages and suggestions how to set up the flow cytometer. Part B contains a pre-run of two samples to confirm instrument performance. Part C contains the preparation and measurements of all samples. Part C is meant to be done once, but in case something may go wrong, there should be sufficient samples to run Part C a second time.

Protocol Part

A1. Requirements

-80^{o}C freezer for the test samples, 4^{o}C refrigerator for the beads

A2. Upon receipt of packages

The shipment contains a box with dry ice and a box without dry ice. Please check the content of the boxes upon arrival. If the PEVTESII samples have thawed, please report this back to us.

- Store calibration beads and quality control samples (Category A and B) in a 4 ^{o}C refrigerator
- Store the PEVTES samples in a -80 ^{o}C freezer

PEVTES samples were derived from blood of healthy donors but should be treated as if they are infectious. Safety cannot be guaranteed.

A3. Sample list including characteristics

Table 1. Sample list

Sample type	Sample ID	Description	Diameter (nm)	Dilution buffers	Volume (µL)	Aliquots	Fluorophore
Control	A1	MilliQ			10,000	1 tube	
Control	A2	dPBS			10,000	1 tube	
Control	A3	vCal™ Nanorainbow Beads	500	dPBS	400	1	Multifluor
Control	Buffer B3	BD staining buffer			4,000	4	
Calibration	B1	Rosetta Calibration beads CAL003	70-1000	MiliQ	400	1	FITC
Calibration	B2	BD APC MESF beads	2000	BD staining buffer	Tube	2 tubes	APC
Calibration	B3	BD PE MESF beads	2000	BD staining buffer	Tube	2 tubes	PE
Calibration	B4	Counting beads	217	MiliQ	100	1	
Calibration	B5	HOB1	216	MiliQ	100	1	
Calibration	B6	HOB2	280	MiliQ	100	1	
Calibration	B7	HOB3	351	MiliQ	100	1	
Calibration	B8	HOB4	396	MiliQ	100	1	
Test sample	C1	PEVTES	70-800	dPBS	100	2	APC, PE
Test sample	C2	Liposomes unstained	216	dPBS	100	1	
Test sample	C3	Liposomes APC	276	dPBS	100	1	APC

A4. Configuration of the flow cytometer

Flow cytometers differ and therefore there is no generic optimal configuration. As you are able to detect single 100-nm polystyrene beads by light scattering, we anticipate that you know how to configure your flow cytometer. The only criterium we have, is to trigger on light scattering, because many reference particles are not fluorescently stained. Regarding instrument sensitivity, use optimal sensitivity settings, but please make sure the background noise in the buffer control is acceptably low. As a rule of thumb, the background count rate of the buffer control should be <2.0% of the count rate of reference particles and EV samples. In this study, we aim for accuracy rather than sensitivity (e.g., by triggering excessively on background noise). Please find below some rules of thumb for configuring your flow cytometer:

- Sample flow rate
    - Please use the lowest flow rate possible, as long as the flow rate and flow cytometer operate in a stable way.
    - If your flow cytometer boosts the sample during sample loading, please ensure that the delay between boosting and data acquisition is sufficiently long to guarantee a stable flow rate.

- Measurement time

- Use a minimum measurement time of 120 seconds.
- Use the same measurement time for all samples.
- Make sure that the memory buffer is not limiting the total number of events that are measured during the set measurement time.
- Make sure that the minimum number of events of the reference particles in Table 6 (Measurement list) are measured.

- Detector voltages
  - This study involves the following detectors:
    - Most sensitive scatter detector
    - Allophycocyanin (APC), for test samples and reference particles
    - Phycoerythrin (PE), for test samples and reference particles
    - Green colour, like fluorescein (FITC), to recognize the marker beads in Rosetta Calibration
  - Strategies to determine the optimal voltage of APDs or PMTs can be readily found in literature, e.g., Maecker et al., Cytometry Part A. 2006.

- Trigger threshold detector
  - Please trigger on the most sensitive light scattering detector, which is typically (but not by definition) the SSC detector operating at the shortest wavelength.
  - Please do not use an OR triggering strategy.
  - In case, you are unsure about the most sensitive light scattering detector, please select the scatter detector where a dim bead, i.e., a bead that is close to the background noise, has the largest separation from the background noise. Alternatively, consult us at study@metves.eu.

- Trigger threshold value
  - Run a buffer only control, which should have an acceptable low count rate. As a rule of thumb, the background count rate of the buffer control should be <2.0% of the count rate of reference particles and EV samples.

Protocol Part B

Pre-run

To test the sensitivity of your flow cytometer, we would kindly ask you to perform this pre-run and send the fcs files to study@metves.eu for a quick check before continuing to running all samples (Section B2). You will get a response within one week.

Preparation

- Take MilliQ, dPBS, and Rosetta Calibration beads CAL003 out of the 4^{o}C refrigerator.
- Take the purified water and PBS that you normally use in your lab.
- Vortex the samples 10 seconds to mix them well.
- Dilute Rosetta Calibration beads CAL003 10-fold in MiliQ (Table 2).
- Perform the measurements as shown in Table 2. Please use the following syntax for the filenames: measurement number, followed by sample ID, followed by dilution +x, followed by sample name, all separated with an underscore. For example:
    - 01_A1_1x_MiliQ.fcs
    - 02_A2_1x_dPBS.fcs
    - 03_B1_10x_Rosetta_Calibration.fcs
- Send the fcs files to study@metves.eu and provide the following information:
    - Names of the most sensitive scatter detector, APC detector, and PE detector
    - Illumination wavelength of the most sensitive scatter detector
    - Sheath fluid
    - Flow rate. Please determine the flow rate with a procedure that is suitable for your flow cytometer.

Table 2. Pre-run

Measurement number	Sample ID	Description	Dilution factor x	Preparation
01	A1	MiliQ		300 µL MiliQ
02	A2	dPBS		300 µL dPBS
03	B1	Rosetta Calibration beads CAL003	10	30 µL Rb + 270 µL MiliQ
04		Own MiliQ		300 µL MiliQ
05		Own dPBS		300 µL dPBS

Protocol Part C

Preparation

- Take the samples (Tables 3, 4, 5) out of the 4^{o} C refrigerator.
- Vortex the samples 10 seconds to mix them well (applies to all samples).
- Prepare the dilutions (Tables 3, 4, 5). Watch out: dilution buffers differ per sample!
- If the dilution as suggested by us results in too little events, please decrease the dilution factor or increase the measurement time. Report the applied dilution in the filename.

Table 3. Sample Category A: Control samples.

Sample ID	Description	Dilution factor x	Preparation
A1	MiliQ		300 µL MiliQ
A2	dPBS		300 µL dPBS
A3	vCal™ Nanorainbow	10	30 µL vCal™ Nanorainbow + 270 µL dPBS

Table 4. Sample Category B: Calibration beads.

Sample ID	Description	Dilution factor x	Preparation
B1	Rosetta Calibration beads CAL003	10	30 µL Rosetta Calibration beads + 270 µL MilQ
B2	BD APC MESF beads		Add 500 µL BD staining buffer to 1 tube
B3	BD PE MESF beads		Add 500 µL BD staining buffer to 1 tube
B4	SNP Counting beads	16,000	2-step dilution 160x: 2 µL SNP + 318 µL MilQ 100x: 3 µL of 160x diluted SNP + 297 µL MilQ
B5	HOB1	3000	2-step dilution 100x: 3 µL HOB1 + 297 µL MilQ 30x: 10 µL of 100x diluted HOB1 + 290 µL MilQ
B6	HOB2	4000	2-step dilution 100x: 3 µL HOB2 + 297 µL MilQ 40x: 10 µL of 100x diluted HOB2 + 390 µL MilQ
B7	HOB3	3000	2-step dilution 100x: 3 µL HOB3 + 297 µL MilQ 30x: 10 µL of 100x diluted HOB3 + 290 µL MilQ
B8	HOB4	200	2 µL HOB4 + 398 µL MilQ

Table 5. Sample Category C: Test samples. For sample C1 (PEVTES), take one aliquot out of the -80 ºC freezer and thaw it for 1 minute in a 37 ºC warm water bath. Afterwards the samples can be stored at the bench at room temperature until the measurement. Although the bench stability of PEVTES is 6 hours, we highly recommend to minimize the time between the sample preparation and the measurement. **PEVTES samples were derived from blood of healthy donors but should be treated as if they are infectious. Safety cannot be guaranteed.**

Sample ID	Description	Dilution factor x	Preparation
C1	PEVTES	10	50 µL PEVTES + 450 µL dPBS
C1	PEVTES	30	20 µL PEVTES + 580 µL dPBS
C1	PEVTES	100	50 µL of 10x diluted PEVTES + 450 µL dPBS
C1	PEVTES	300	50 µL of 30x diluted PEVTES + 450 µL dPBS
C2	Liposomes unstained	2000	2-step dilution 100x: 3 µL Liposomes unstained + 297 µL dPBS 20x: 20 µL of 100x diluted Liposomes unstained + 380 µL dPBS
C3	Liposomes APC	300	2 µL Liposome APC + 598 µL dPBS

Run samples

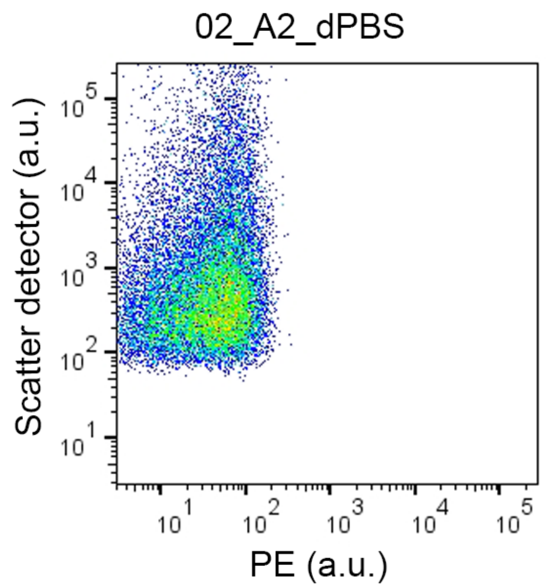
- Take into account our suggestions about configuring the flow cytometer (Section A4)
- Measure each sample according to the measurement list (Table 6) either manually or using an autosampler. Make sure samples are mixed well prior to the measurement, e.g., by vortexing in case of manual measurements or using suck and spit in case of automated measurements.
- If the dilution as suggested by us results in too little events, please decrease the dilution factor or increase the measurement time.
- Perform the measurements as shown in Table 6. Please use the following syntax for the filenames: measurement number, followed by sample ID, followed by dilution +x, followed by sample name, all separated with an underscore. For example:
    - 01_A1_1x_MiliQ.fcs
    - 02_A2_1x_dPBS.fcs
    - 03_A3_10x_Nanorainbow.fcs
- Send the fcs files to study@metves.eu and provide the following information:
    - Names of the most sensitive scatter detector, APC detector, and PE detector
    - Illumination wavelength of the most sensitive scatter detector
    - Sheath fluid
    - Flow rate. Please determine the flow rate with a procedure that is suitable for your flow cytometer.
- Notes:
    - dPBS is measured along the measurement series to check for carry-over and to check that background noise levels remain stable over time.
    - Please keep the detector settings the same throughout the entire experiment.
    - The aim of measurement 22 is to measure the noise of each detector
        - Background information: background noise affects the accuracy of calibrations. By triggering on the noise of the FITC detector, we can measure the background signal levels at all detectors used in the study (scatter, PE, APC), because the background signals are measured in the absence of a particle.
        - Measure MiliQ using the same settings as used for measuring all other samples, but while triggering at the noise of the FITC detector (or another detector that is not used within this study) instead of the most sensitive scatter detector. Set the trigger threshold value and/or voltage of the FITC detector value so that the count rate is within the same order of magnitude as the count rate for during sample measurements.
- In case BD APC MESF beads (B2) and BD PE MESF beads (B3) result in too little counts, this may be due to saturation of the scatter detector that is used as trigger detector. In this case, you may trigger at a less sensitive scatter detector or at the corresponding fluorescence detector for these 2 measurements only. Please keep the gain and voltage of the fluorescence detector the same as throughout the experiment.
- Depending on your flow cytometer, 10x and 30x diluted PEVTES (C1) may result in swarm detection and a high abort rate. This is no problem, because the aim of the PEVTES dilution series is to find the highest count rate without swarm detection.

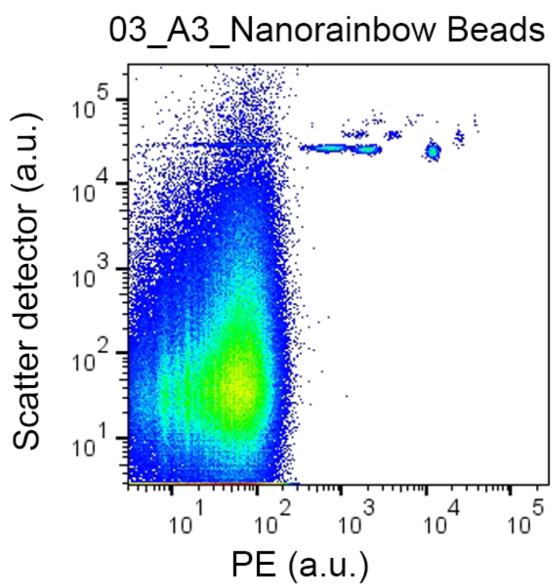
Table 6. Measurement list

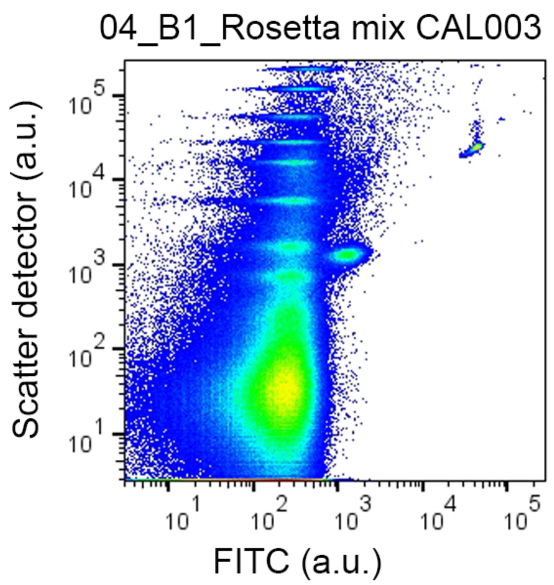
Measurement number	Sample ID	Description	Minimum number of events
01	A1	MilliQ	
02	A2	dPBS	
03	A3	vCal™ Nanorainbow Beads	>1,000 (per population)
04	B1	Rosetta mix CAL003	>1,000 (per population)
05	B2	BD APC MESF beads	>1,000 (per population)
06	B3	BD PE MESF beads	>1,000 (per population)
07	B4	Counting beads	>10,000
08	B5	HOB1	>1,000
09	B6	HOB2	>1,000
10	B7	HOB3	>1,000
11	B8	HOB4	>1,000
12	A2	dPBS	
13	C1	PEVTES 1:300	
14	C1	PEVTES 1:100	
15	C1	PEVTES 1:30	
16	C1	PEVTES 1:10	
17	A2	dPBS	
18	C2	Liposomes unstained	
19	C3	Liposomes APC	
20	A1	MilliQ	
21	A3	vCal™ Nanorainbow Beads	>1,000 (per population)
22	A1	MilliQ to trigger on noise (Section C2)	

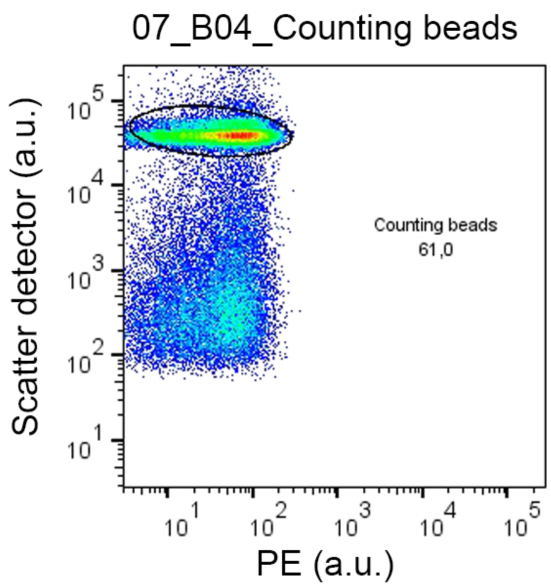
Supplement

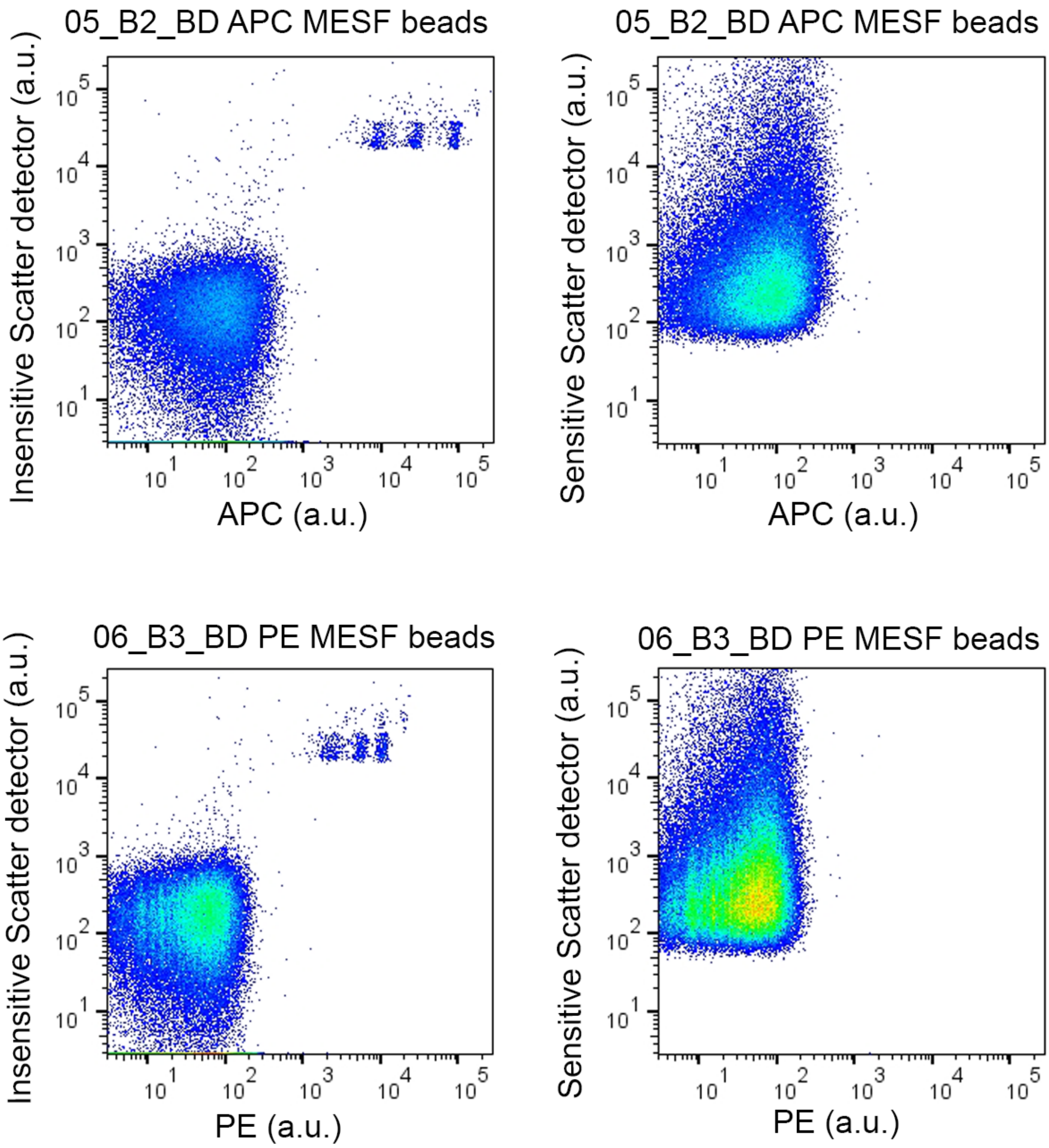
Example plots

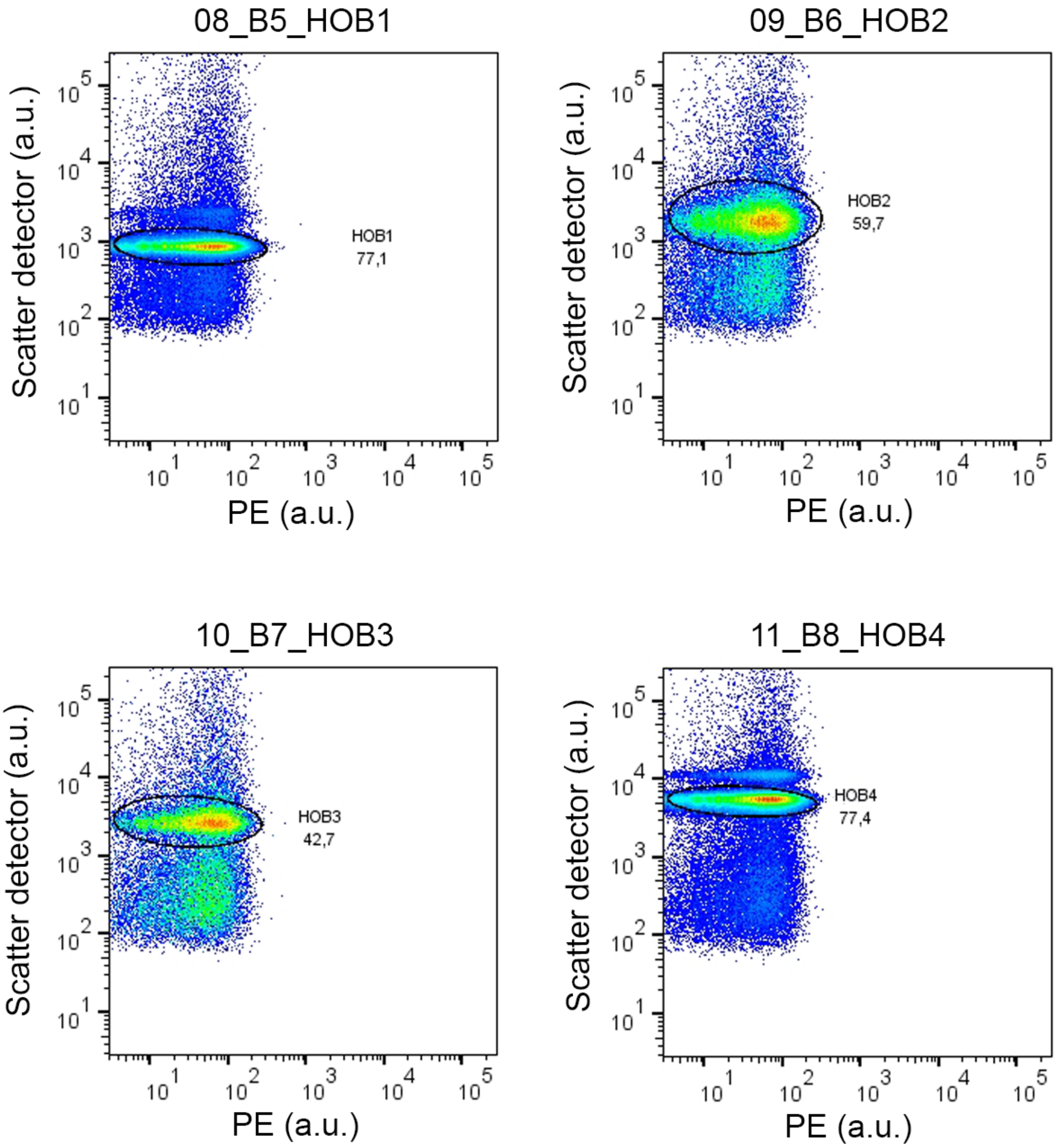


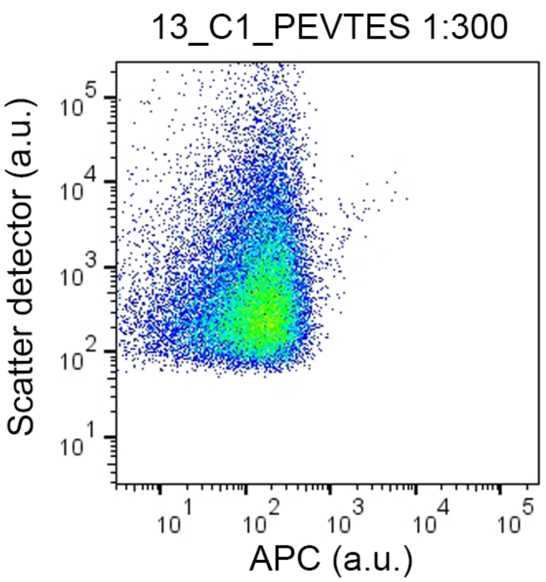







05_B2_BD APC MESF beads
Insensitive Scatter detector (a.u.)
APC (a.u.)
05_B2_BD APC MESF beads
Sensitive Scatter detector (a.u.)
APC (a.u.)
06_B3_BD PE MESF beads
Insensitive Scatter detector (a.u.)
PE (a.u.)
06_B3_BD PE MESF beads
Sensitive Scatter detector (a.u.)
PE (a.u.)


08_B5_HOB1
HOB1
77,1
09_B6_HOB2
HOB2
59,7
10_B7_HOB3
HOB3
42,7
11_B8_HOB4
HOB4
77,4
Scatter detector (a.u.)
PE (a.u.)
10^1
10^2
10^3
10^4
10^5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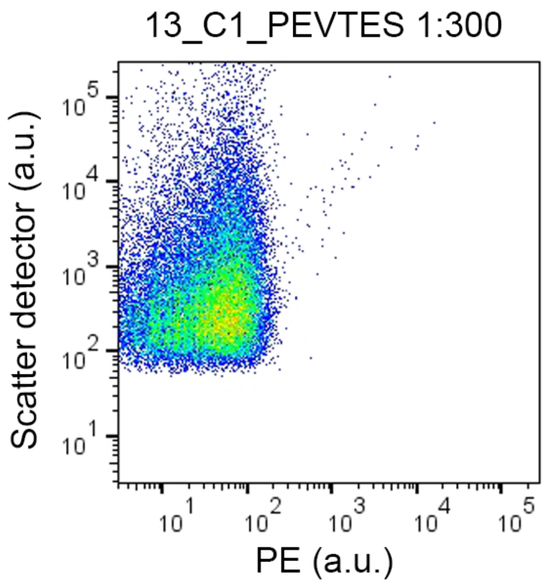
Scatter detector (a.u.)
APC (a.u.)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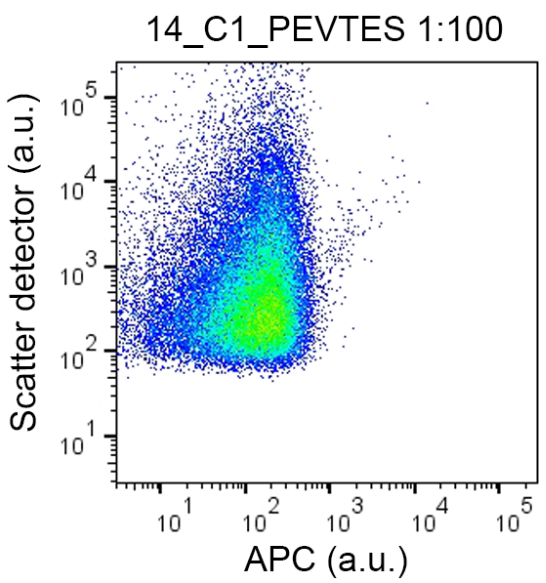
Scatter detector (a.u.)
PE (a.u.)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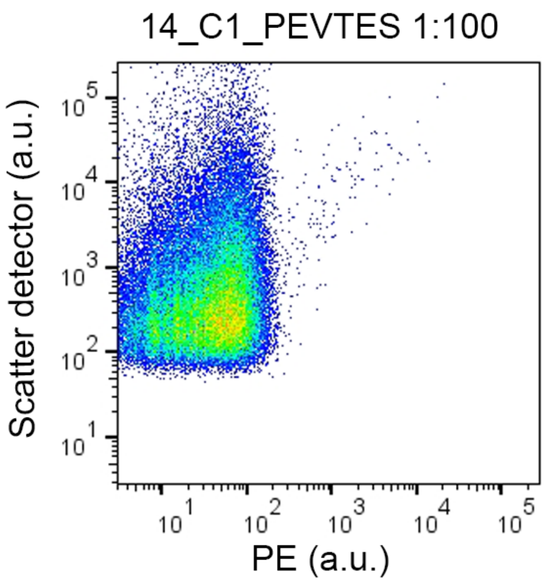
Scatter detector (a.u.)
APC (a.u.)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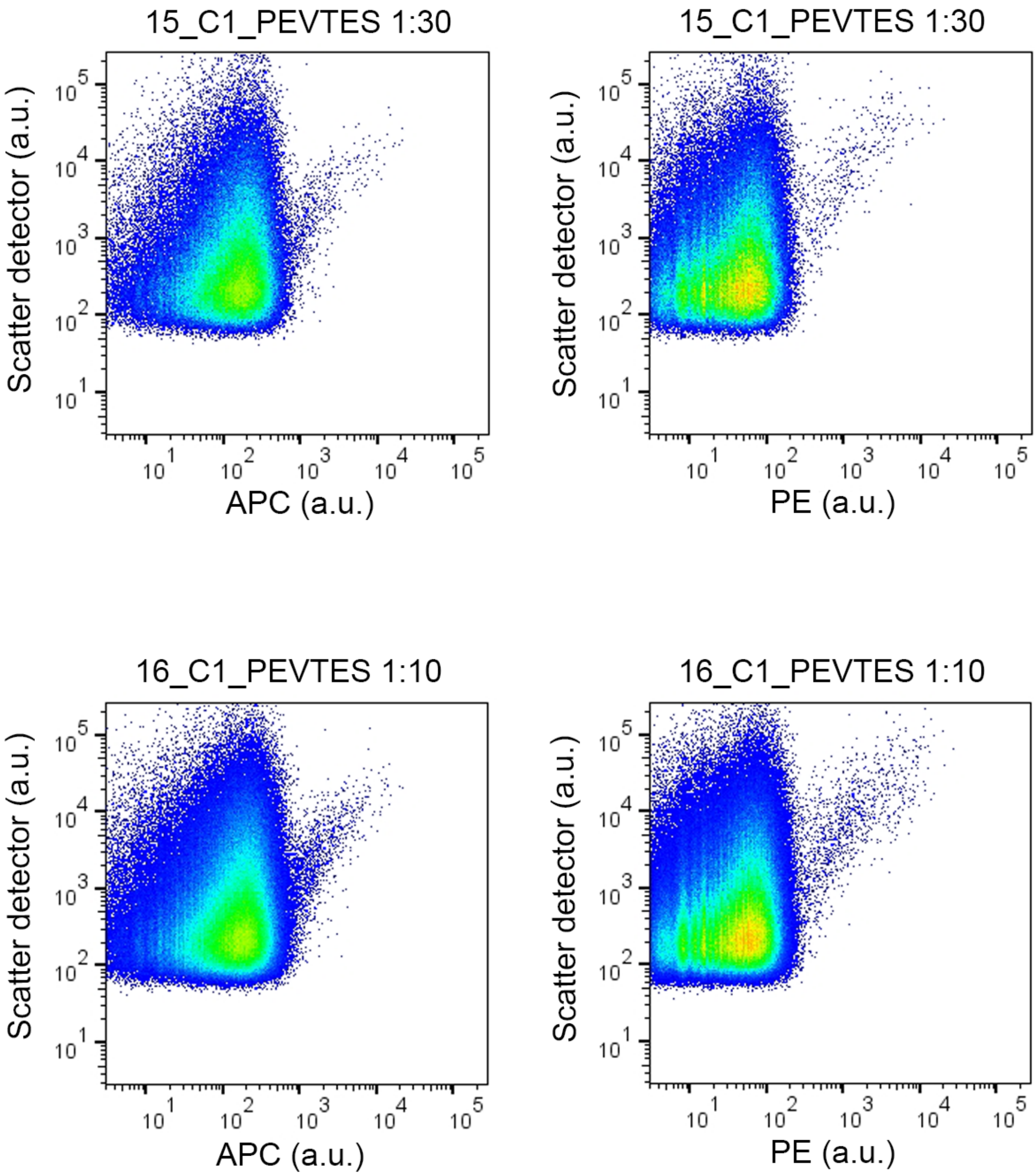
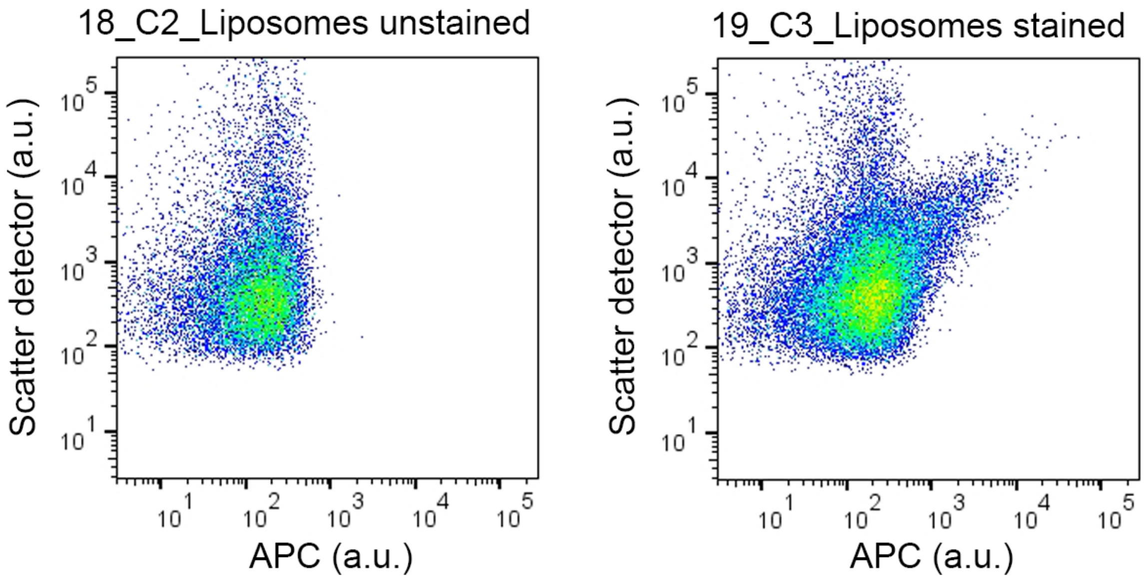
Scatter detector (a.u.)
PE (a.u.)


15_C1_PEVTES 1:30
Scatter detector (a.u.)
APC (a.u.)
15_C1_PEVTES 1:30
Scatter detector (a.u.)
PE (a.u.)
16_C1_PEVTES 1:10
Scatter detector (a.u.)
APC (a.u.)
16_C1_PEVTES 1:10
Scatter detector (a.u.)
PE (a.u.)


18_C2_Liposomes unstained
Scatter detector (a.u.)
APC (a.u.)
19_C3_Liposomes stained
Scatter detector (a.u.)
APC (a.u.)