Supplemental information

Data S1: Further details samples and distribution

Outdated platelet concentrates were diluted 2-fold with phosphate buffered saline (PBS; 0.15 M NaCl, 1.23 mM $Na_2HPO_4.2H_2O$ and 0.21 mM $NaH_2PO_4.2H_2O$; pH 7.4), and then further diluted in acid citrate dextrose (0.085 M trisodium citrate, 0.11 M glucose, 0.071 M citric acid; pH 4.4) in a ratio of 5:1 (v/v). Cells were removed by double centrifugation at 1,550 g for 20 minutes at 20 °C [1, 2]. The platelet-depleted supernatants were collected, 1 mL aliquots were pipetted in Eppendorf vials with a screw lid, aliquots were snap-frozen in liquid nitrogen (-196 °C) and stored at -80 °C. Each participant received reagents and frozen EV samples. Participants stored the aliquots at -80 °C and the reagents at 4 °C.

All participants were given the same protocol, see Data S4 at the end of the supplemental information for details. On the day of measurement, antibodies and lactadherin were diluted in PBS and then centrifuged at 19,000 *g* for 5 minutes at room temperature to obtain protein aggregate depleted supernatants [3]. The concentration of reagents was 5.2 µg/mL for Lactadherin-FITC (Haematologic Technologies, Essex Junction, VT), 0.65 µg/mLfor CD61-PE (VIPL2, BD Biosciences, San Jose, CA), and 1.8 µg/mL for IgG1-PE (Mouse BALB/c IgG_1, κ, BD Biosciences). The mismatch in isotype concentration is because BD initially did not provide concentration data, the IgG1 concentration was set to match the fluorescence background of CD61-PE. Lactadherin targets phosphatedylserine as well as integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$. Fluorescence minus one controls showed no detectable interference between CD61-PE (integrin β_3) and lactadherin-FITC. After centrifugation, the conjugate supernatant was transferred to a new Eppendorf vial.

Data S2: Erythrocyte EV samples

Together with the platelet extracellular vesicles (EVs) samples, we also distributed erythrocyte EVs, also referred to as erythrocyte microvesicles (EMV). To isolate EMVs, outdated erythrocyte concentrates were diluted 2-fold with phosphate buffered saline (PBS). Cells were removed by double centrifugation at 1,550 g for 20 minutes at 20 °C before snap freezing and further processing analogous to platelet EV samples. Instead of staining with CD61-PE, EMVs were stained with 1.7 µg/mL CD235a-PE (JC159, Dako, Glostrup, Denmark).

Reproducibility of erythrocyte EV measurements

Five gates were applied to analyze the EMV samples. The range of CD235a+ EMVs was 48 to 5,327 (CV 105%) for no gate, 0 to 1,197 (CV 93%) for the bead diameter gate and 3 to 582 (CV 60%, 1,200-3,000 nm EV), 0 to 984 (CV 205%, 600-1,200 nm EV), and 2 to 690 (CV 86% 300-600 nm EV), see Supplemental Figs. S1 and S2. It should be noted that in the EMV samples there was a group of CD235a-PE positive particles that was clearly separated from the noise for scatter and fluorescence. Selecting only the population clearly separated from the noise in fluorescence would presumably outperform any strategy with an additional gate on scatter.

Data S3: Mie model fitting and extrapolation

Mie theory describes light scattering of spheres of all size parameters by solving Maxwell's equations [4]. We use software (Rosetta Calibration, Exomety, The Netherlands) based on the MATLAB (v7.13.0.564) scripts of Mätzler [5] to obtain the amplitude scattering matrix

elements, which describe the relation between the incident and scattered field amplitudes of a sphere. Our model is extensively described in earlier publications [6, 7] and incorporates particle diameter and refractive index, refractive index of the medium, and illumination wavelength, polarization, and collection angles of flow cytometer detectors. For the refractive index of PBS, we added 0.002 to the dispersion relation of water measured at 20.0 °C [8, 9]. For polystyrene, we use the dispersion relation measured at 20.0 °C [10]. Because the exact material composition of silica beads is unknown, we measured the refractive index of the silica beads using two flow cytometers by solving the inverse light scattering problem with Mie theory. We obtained the refractive index of the reference silica beads to be 1.444 at 488 nm and 1.445 at 405 nm. Because the dispersion relation for EVs is unknown, we assumed an effective refractive index of 1.40 for all wavelengths for EVs.

Because the optical alignment of a flow cytometers deviates from the specified alignment, the collection angles are described by fixed and fit parameters. The fixed parameters are derived from manufacturer specifications and include a weighting function to account for the transmission efficiency of light propagating through a lens with high numerical aperture (NA) [4] and the angle between the optical axis of the lens and the propagation direction of the laser, which is 0 degree for most forward scatter detectors (FSC) and 90 degrees for most side scatter detectors (SSC).

Fit parameters are the NA of the lens, the dimension of the blocker bar, and a linear scaling factor to scale the power of scattered light to the arbitrary units of the detector. At least N+1 bead peaks were used to fit N variables. Fit parameters are allowed to vary within boundaries relative to the manufacturer specified values. The fit boundaries were determined empirically, and are ± 8% from the specified NA for SSC, ± 16% from the specified collection angle for FSC, and ± 80% from the specified blocker bar dimensions. Because the model always converges by varying the blocker bar dimensions, the boundaries of the blocker bar dimensions are not strictly needed. The linear scaling factor has no boundaries because signals are provided in arbitrary units. Intellectual property does not allow disclosure of more details about the BC Astrios and Apogee A50-Micro. To ensure that a misidentification of a bead peak does not lead to a false theoretical description, we required the coefficient of determination, which is a statistical measure of how well theory describes data, to exceed 0.93. Figure S5 shows the Mie models for all FCM that measured samples.

The illumination wavelength is selectable for all instruments that have multiple known scatter illumination wavelengths.

Table S1: Diameter of silica beads determined by flow cytometers (FCMs) that measured extracellular vesicles from platelet and erythrocyte samples

Bead diameter	***772 nm***	***391 nm***
FCM	***Estimated***	***Estimated***
Apogee A50 micro	*747*	*323*
BC Gallios	*758*	
	748	
BD Aria	*770*	
BD Calibur	*792*	
	790	*363*
BD Canto	*777*	*371*
	774	*361*
	766	*390*
	763	
BD Canto II	*762*	*331*
BD Influx	*791*	*394*
LSR	*697*	*381*
	693	*393*
LSR II	*775*	*381*
	726	*380*
	756	*368*
	735	*370*
	742	*387*
	734	
S1000	*785*	*396*
Mean	*756*	*373*
Standard deviation	*28.0*	*21.7*
Coefficient of variation (%)	*3.7*	*5.8*
Maximum	*792*	*396*
Minimum	*693*	*323*

Supplemental figure S1

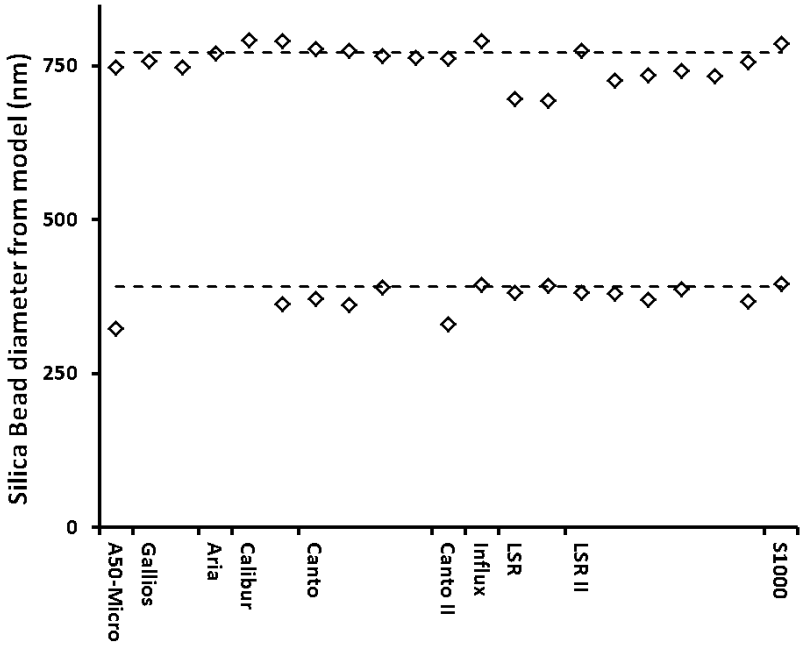


Figure S1: Estimated size of silica beads compared to bead diameters determined by transmission electron microscopy (TEM).

Supplemental Figure S2

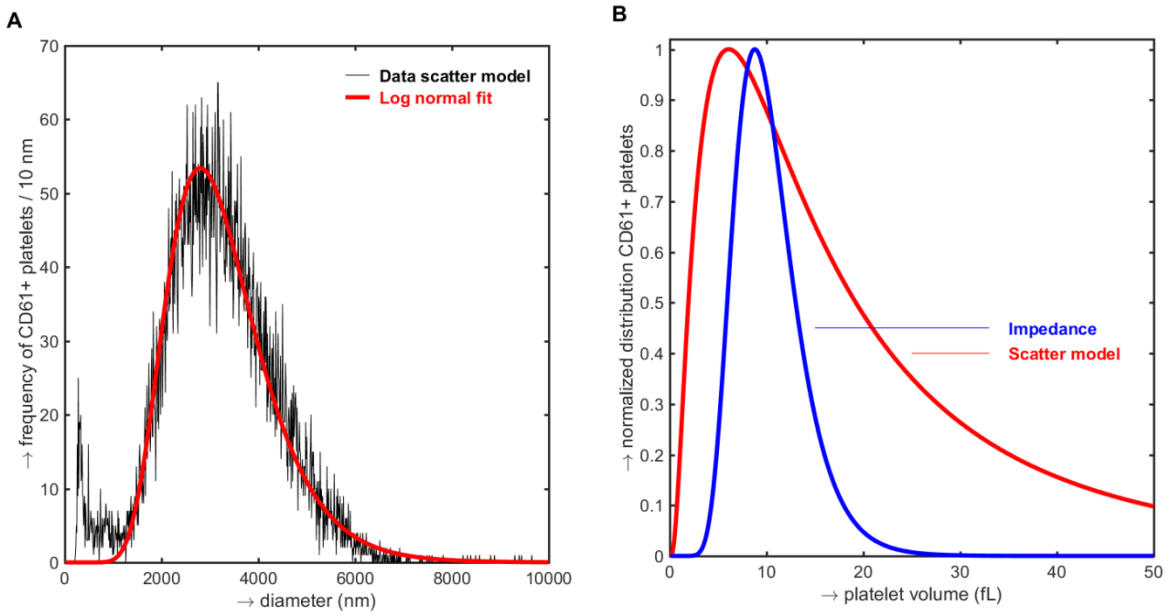


Figure S2: The volume distribution of platelets by the scatter model compared to a hematology impedance analyzer. Panel A shows the diameter distribution of CD61+ platelets in plasma prepared 45 minutes after (citrate-anticoagulaed) blood collection, and measured on SSC for a FACSCanto II (BD, San Jose, CA). The red line shows a log normal fit, from which the platelet volume is derived in **panel B** (red line), assuming a spherical platelet. This platelet volume distribution is compared to the platelet volume distribution determined with an impedance based Sysmex XN-9000 (blue line; Kobe, Japan). The mode volume is 30% underestimated by the model (6.2 vs 8.9 fL on impedance), the mean platelet volume is 2-fold overestimated (21.4 vs 10.4 fL on impedance) and the volume distribution is almost 3-fold overestimated (sigma 0.91 vs 0.33 on impedance). These differences may be attributed to the discoid shape of platelets, and/or the detection on SSC of resting platelets containing dense granules that are sufficiently large to contribute to the side scatter signal.

Supplemental figure S3

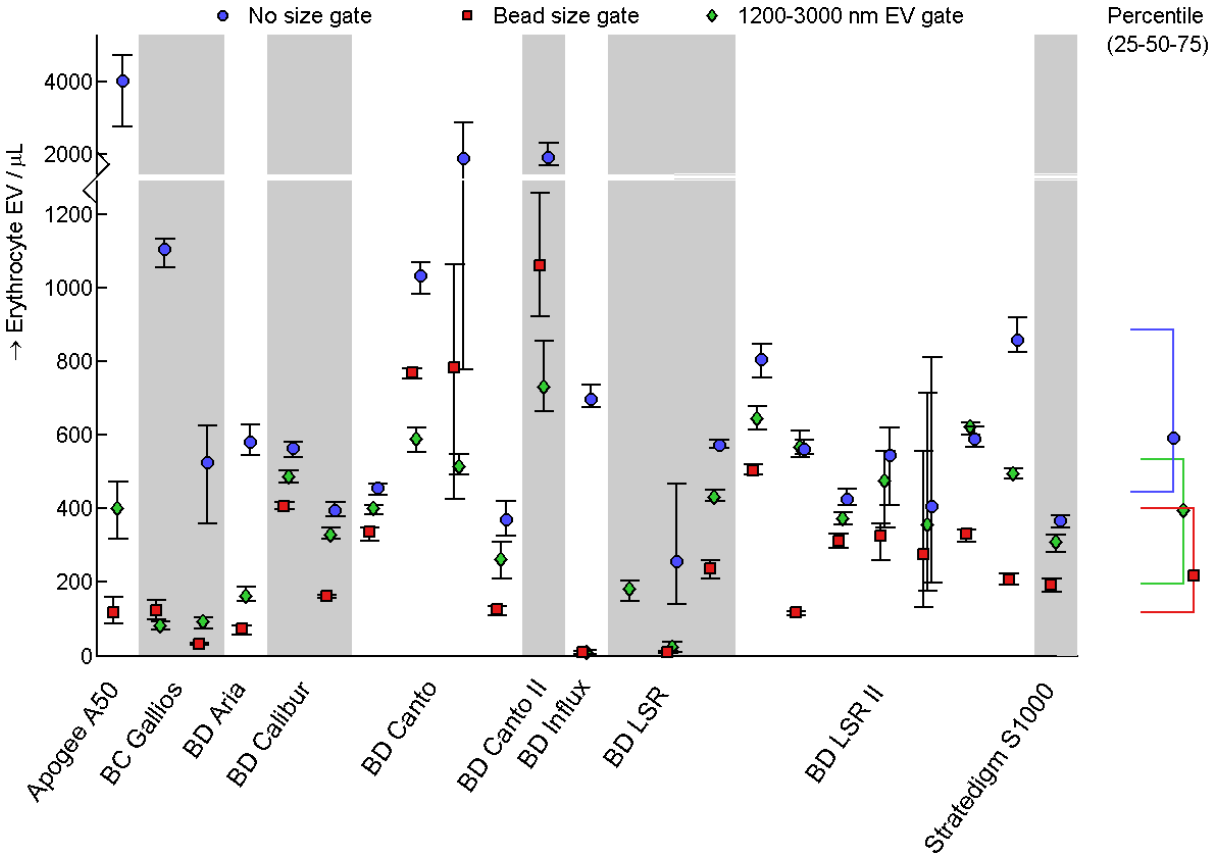


Figure S3: Determined concentration of extracellular vesicles (EVs) from erythrocytes on different flow cytometers for three different gating strategies. In "fluorescence only gate" method, events are included that have a CD235A-PE signal above the isotype control (CD235a-PE+). In the "bead size gate" method, CD235a-PE+events are included if their scatter signal is in between that of 400-800 nm polystyrene beads for side scatter FCMs and in between that of 600-1,000 nm polystyrene beads for forward scatter FCMs. In the 1,200-3,000 nm EV diameter gate method, CD235a-PE+ events are included if their scatter signal is in between the signal of 1,200-3,000 nm EV. The markers show the minimum, maximum and median of three measurements. On the right, the 25, 50 and 75 percentiles for all methods are shown.

Supplemental figure S4

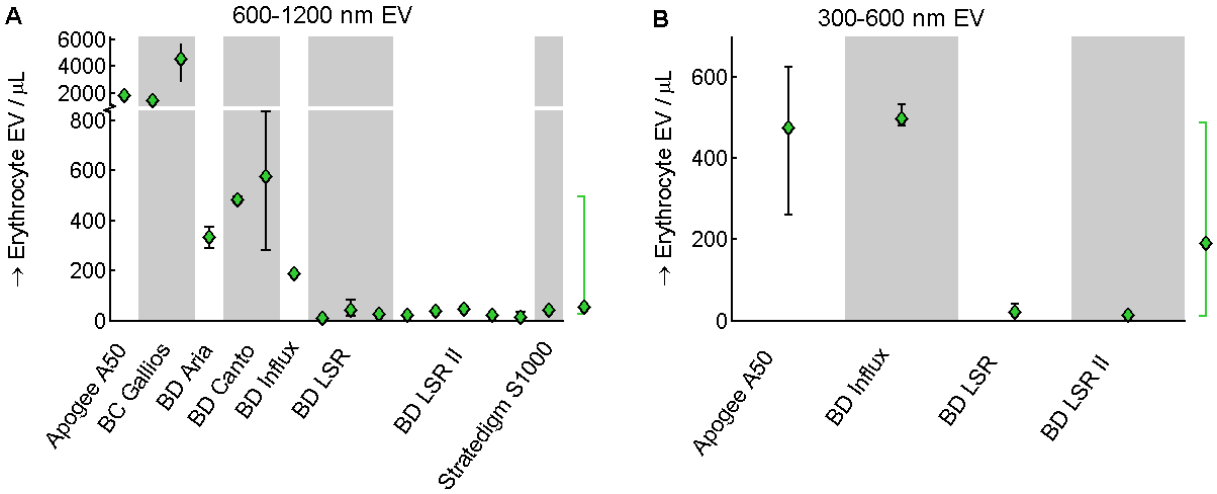
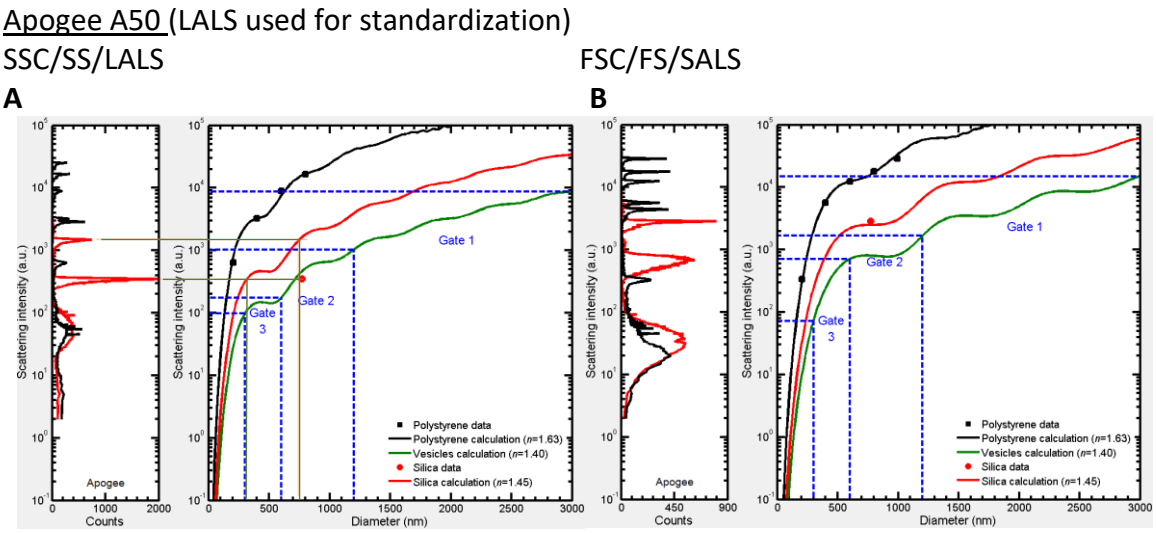


Figure S4: Determined concentration of erythrocyte EV on different flow cytometers for 600-1,200 nm EV and 300-600 nm EV. Brackets to the right indicate 25, 50 and 75 percentiles of all data, marker and whiskers indicate median and max/min concentration of three repeats.

Figure S5: Size to scatter relationships for FCM that measured platelet EV and erythrocyte EV samples. Side by side panels show the diameter-scatter model for side scatter (lefthand side) and forward scatter (righthand side). Each panel contains two figures, the left hand figure a histogram of the measured calibration and validation bead mixes. The Rosetta calibration bead mix is shown with a black line, the validation bead mix is shown in the red line. From these histograms, the software identifies bead peaks with predefined bead diameters, shown as black and red dots in the right-hand panel. The diameter to scatter model is fit to the Rosetta calibration data (black line), and by changing the particle refractive index (shown as 'n = …' in the legend) the resulting model is extrapolated for silica validation beads (red line), and extracellular vesicles (green line). The silica bead peaks were mis-identified in panel A and F, here orange lines show the manual correction that was applied for the respective data in Fig. S3, and Tab. S1.

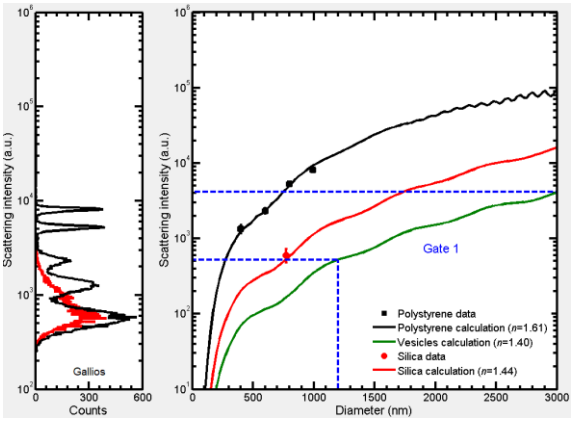


BC Gallios (FS used for standardization)

SSC/SS/LALS

FSC/FS/SALS

C



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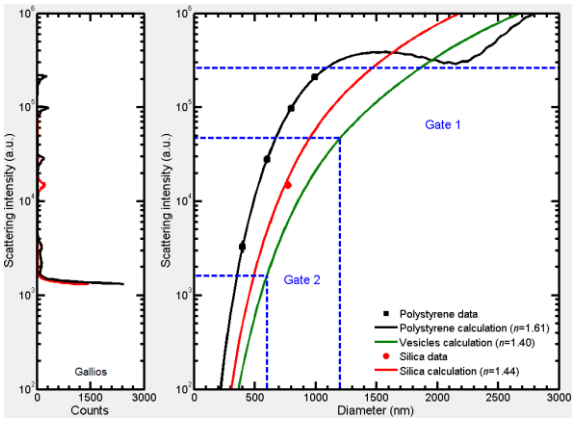

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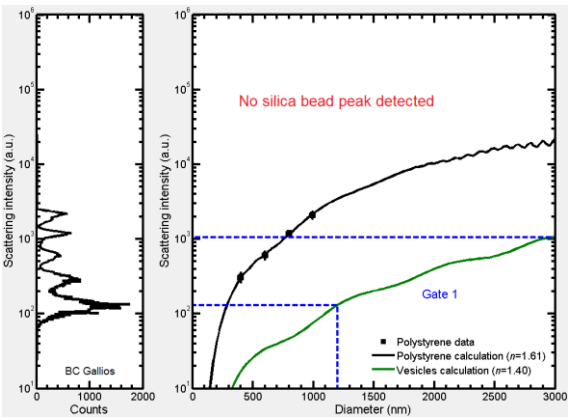

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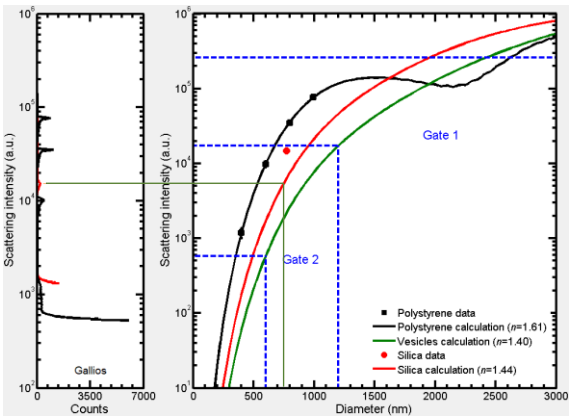
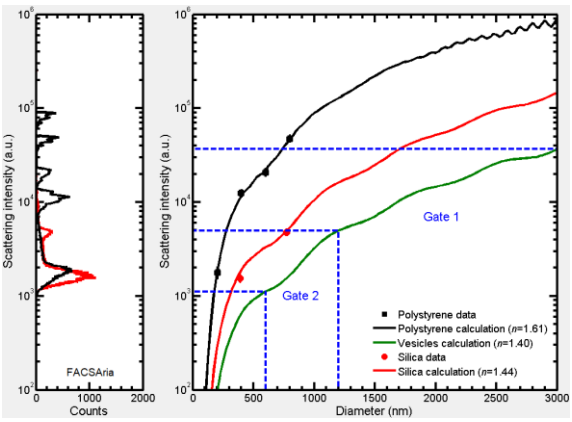


BD Aria

SSC/SS/LALS (used for standardization)

FSC/FS/SALS

G



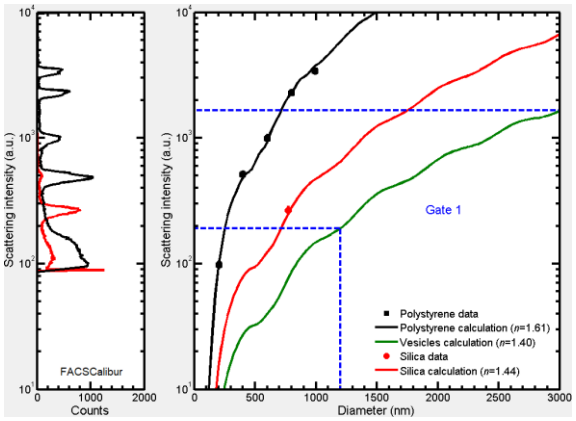
H

<3 bead diameters distinguished for FSC

BD Calibur

SSC/SS/LALS (used for standardization) FSC/FS/SALS

I **J**



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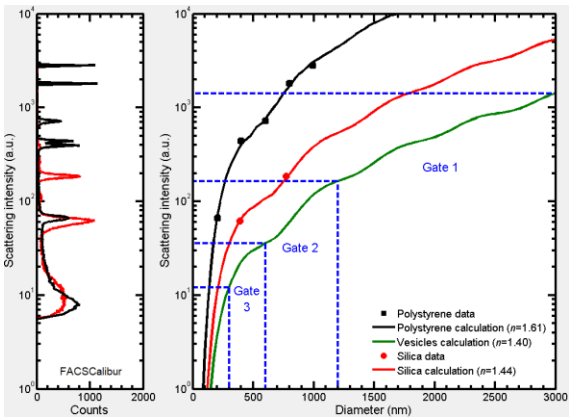
K **L**



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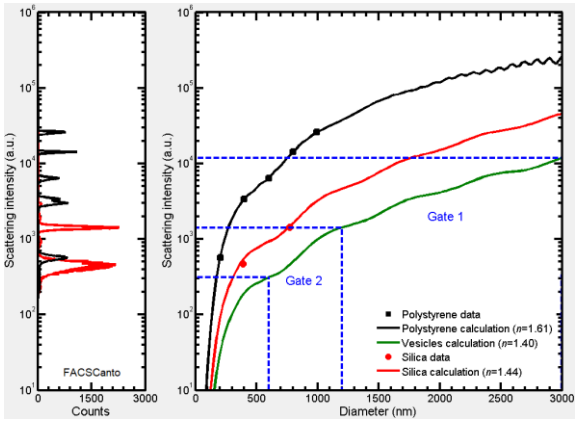
BD Canto

SSC/SS/LALS (used for standardization) FSC/FS/SALS

M **N**



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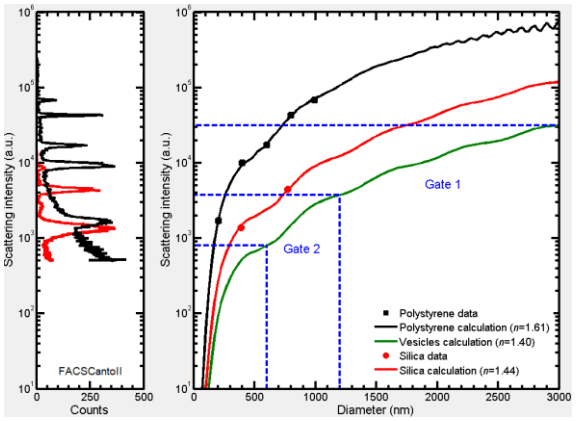
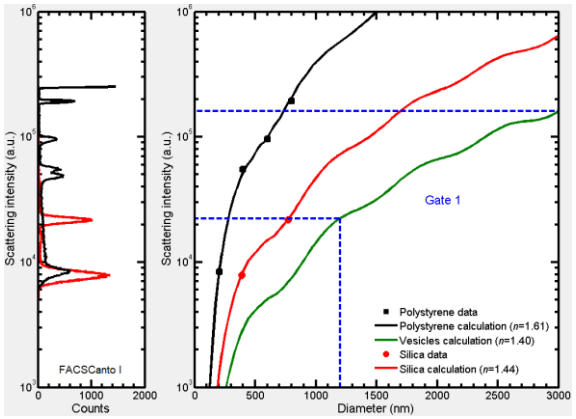
O **P**



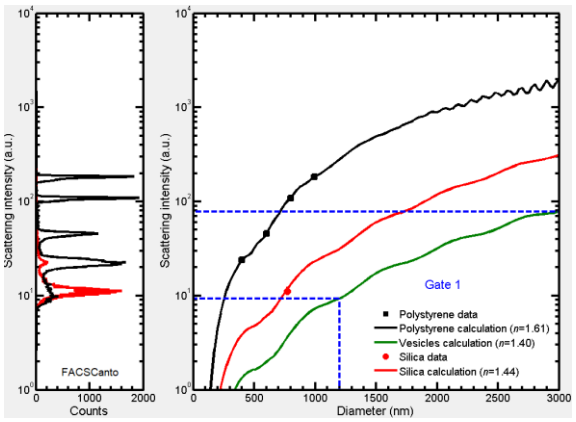
<3 bead diameters distinguished for FSC

Q **R**



<3 bead diameters distinguished for FSC

S **T**

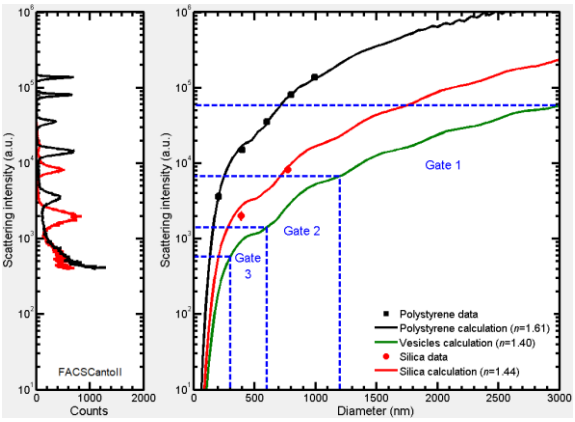


<3 bead diameters distinguished for FSC

BD Canto II

SSC/SS/LALS (used for standardization)

U



FSC/FS/SALS

V

<3 bead diameters distinguished for FSC

BD Influx

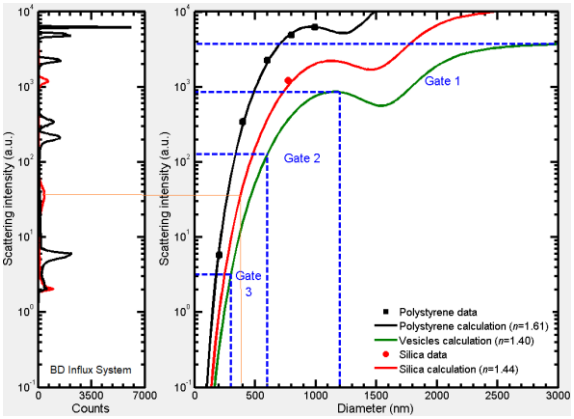
SSC/SS/LALS

W

SSC model not available at time of study

FSC/FS/SALS (used for standardization)

X



BD LSR

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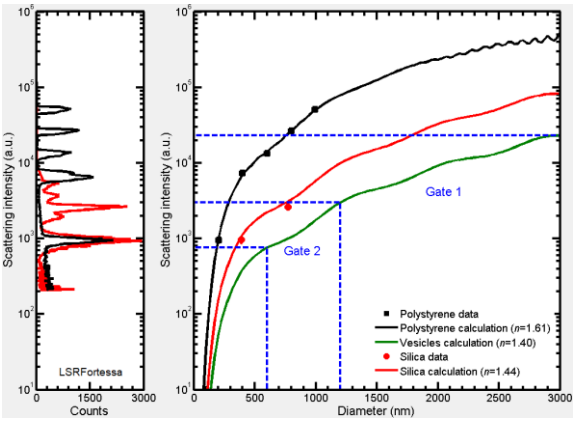
SSC/SS/LALS (used for standardization) FSC/FS/SALS

Y **Z**



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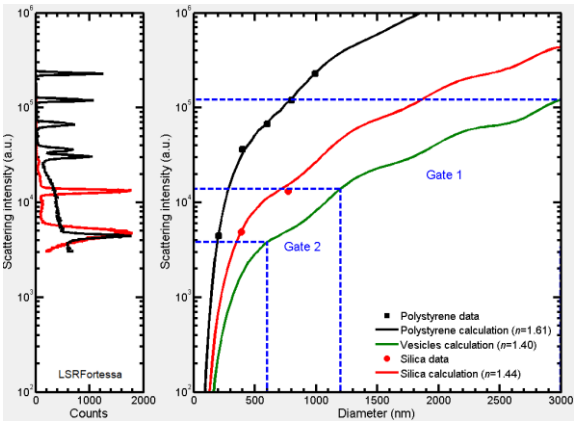
AA **AB**



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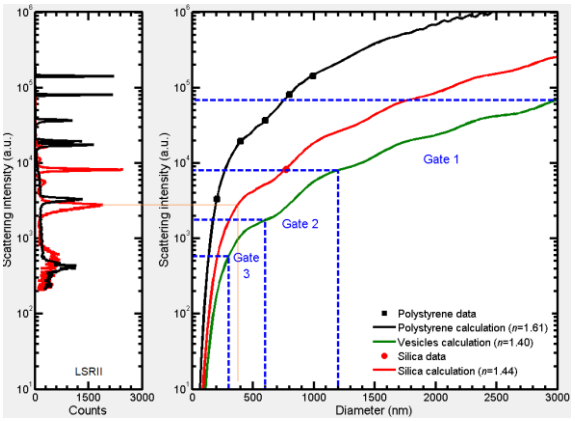
BD LSR II

SSC/SS/LALS (used for standardization) FSC/FS/SALS

AC **AD**



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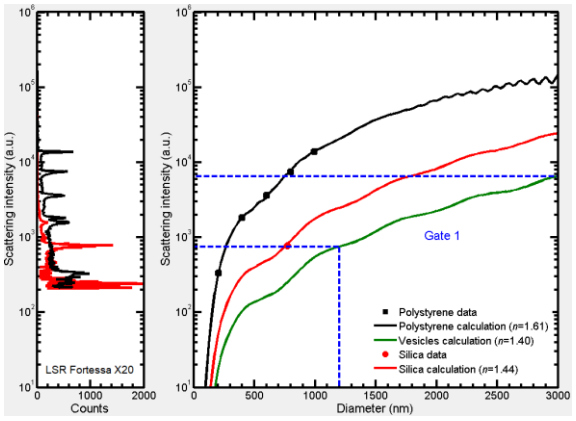

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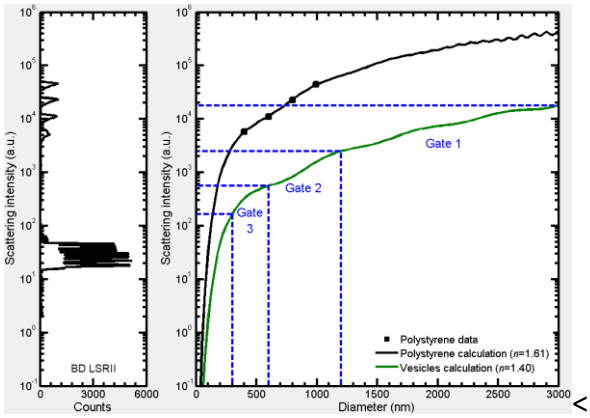
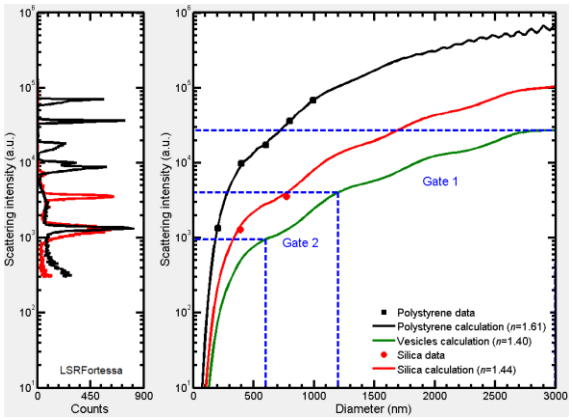
AG **AH**



<3 bead diameters distinguished for FSC
Reference bead mix file unreadable

AI **AJ**



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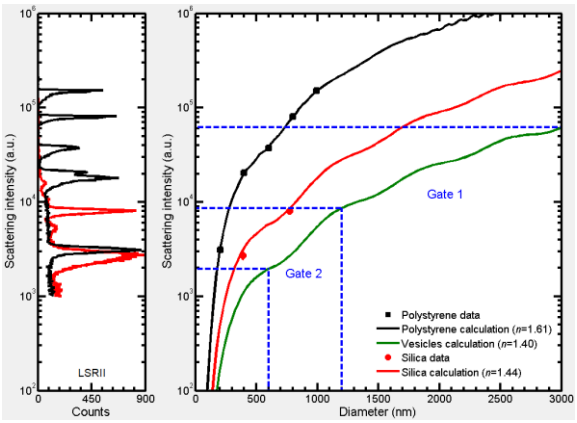
AK **AL**



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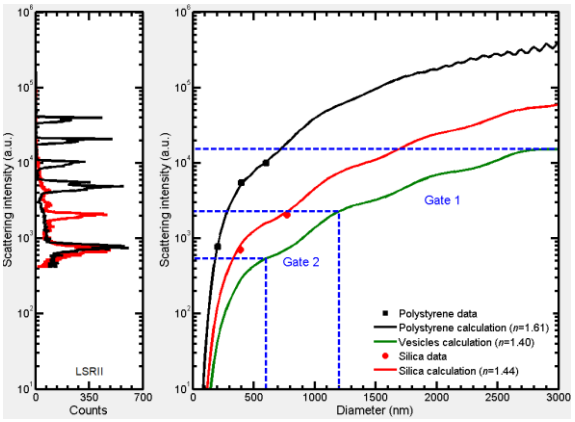
AM **AN**



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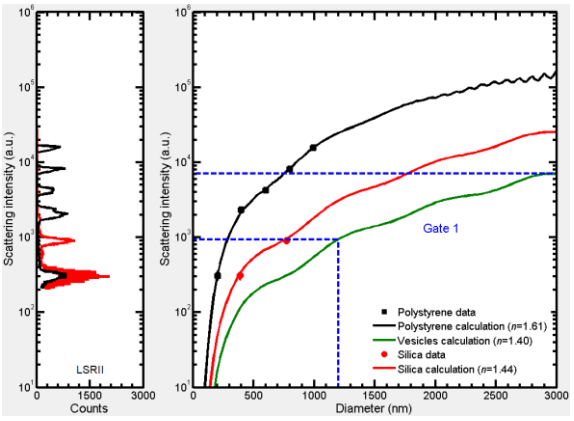
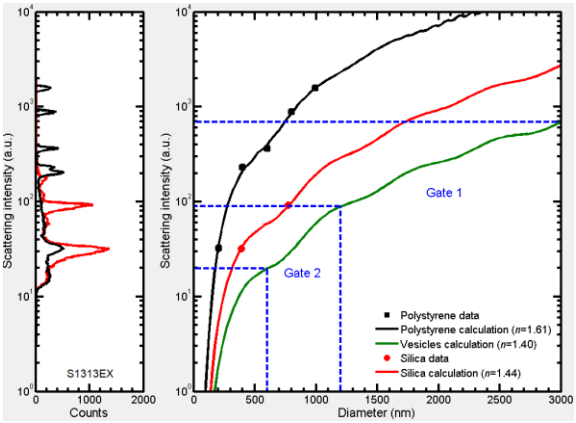


<3 bead diameters distinguished for FSC

Stratedigm S1000

AQ **AR**



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Data S4: Measurement protocol

Protocol overview

***Background*.** Reproducibility of vesicle concentration will be determined by laboratories that employ flow cytometry to detect extracellular vesicles. To obtain maximum reproducibility, each system will be calibrated by beads and Mie theory prior to use. The samples measured will include beads as well as platelet microparticles (PMP) and erythrocyte microvesicles (EMV) from thrombocyte concentrates or red blood cell concentrates, respectively.

Flow cytometry enables simultaneous detection of scatter and fluorescence signals of individual particles. The scatter signal depends on the sample (size, refractive index and shape) and the instrument (illumination and collection geometry). Calibration with two sizes of polystyrene beads leads to a relatively small improvement in reproducibility. Theoretical simulations show that this is mainly caused by the difference in refractive index between polystyrene beads and vesicles (1.62 vs. ~1.40). The influence of this difference in refractive index can be eliminated by application of Mie theory to signals from polystyrene beads, and thus determine the gate required for a desired size of vesicles.

Aim. To determine the reproducibility of vesicle concentration after flow cytometry calibration.

Methods. We will measure (i) 100-1000 nm calibration beads supplied by Exometry, (ii) the number of Lactadherin-FITC+CD61-PE+ vesicles in PMP, (iii) the total number of particles in PMP, (iv) the number of Lactadherin-FITC+CD235a-PE+ vesicles in EMV (concentrate),and (v) the total number of particles in EMV. Controls will be isotype controls, dilution buffer, polystyrene and silica beads. Concentration will be obtained from the flow rate and determined for vesicles with size of 300-600 nm, 600-1200 nm, and 1200-3000 nm. Users will configure their flow cytometers such that the forward, and/or side scatter channels are not saturated, the isotype control is in the first decade for the FITC and PE fluorescent channel. Once configured, all samples will be measured with the same settings. Some of the bead signals should saturate in the scatter channel, fluorescence from the calibration vial may saturate the detector.

Detailed protocol

Site requirements

A -80°C freezer is needed!

Flow cytometer, 2-8 °C refrigerator, vortex, pipettes, centrifuge

Upon receipt of package

The package contains a box with dry ice and one without dry ice. Please check the contents upon receipt (quantity on labels on zip-lock bags):

Dry ice:	EMV samples (vials with red caps)	
	PMP samples (vials with blue caps)	
No dry ice:	Lactadherin-FITC	(dilute 1:10)
	IgG1-PE	(dilute 1:30)
	CD61-PE	(dilute 1:10)
	CD235a-PE	(dilute 1:60)
	Exometry beads (10x concentrated)	
	Silica beads (10x concentrated), called reference beads in protocol.	
	Dilution buffer (PBS-citrate 0.32%)	

The samples in the box with dry ice should be stored in -80°C upon receipt. The other box contains beads and staining reagents. Spin the reagents briefly to remove any reagent from the caps, and then store all beads and reagents in the refrigerator, 2-8°C.

EMV and PMP samples were derived from healthy donors, however, their safety cannot be guaranteed, and these samples should be treated as if they are infectious.

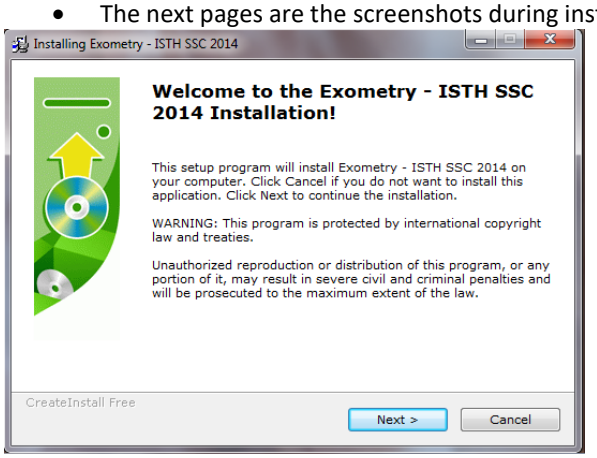
Preparation

1. Exometry and reference beads

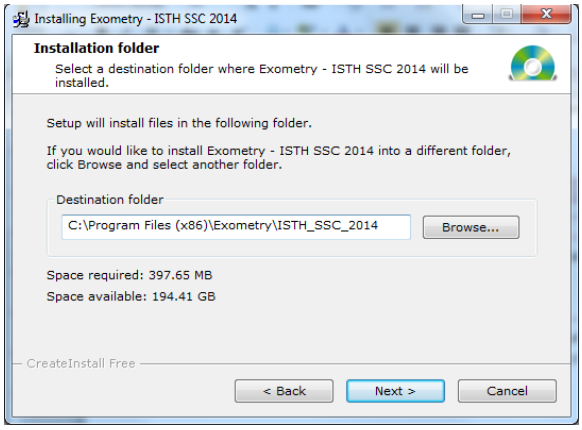
Before you prepare samples, please install the software.

1.1 Preparing the software

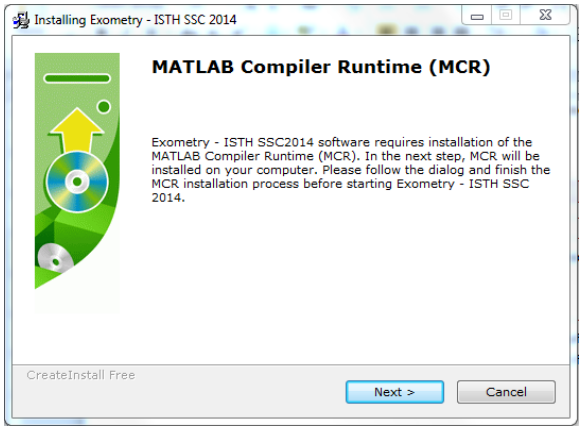
- Go to www.exometry.com and download the calibration software
- Install the program **as administrator** on a windows PC. It's most convenient if installed on the flow cytometer computer, but not necessary.
- The next pages are the screenshots during installation:



Press next



Press next



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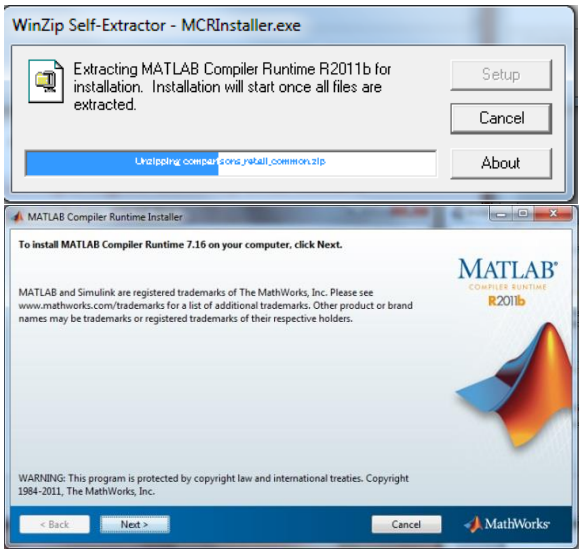
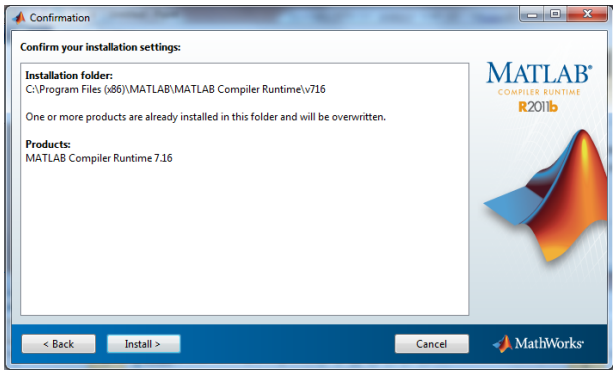
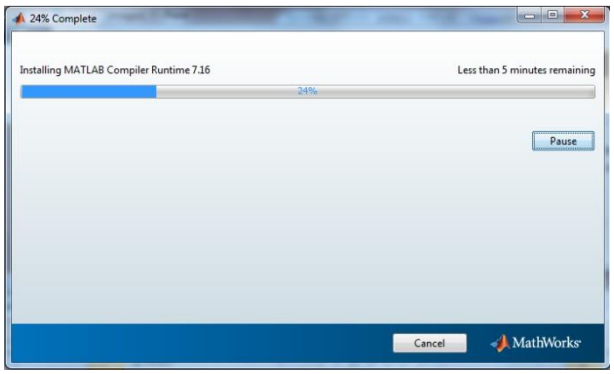
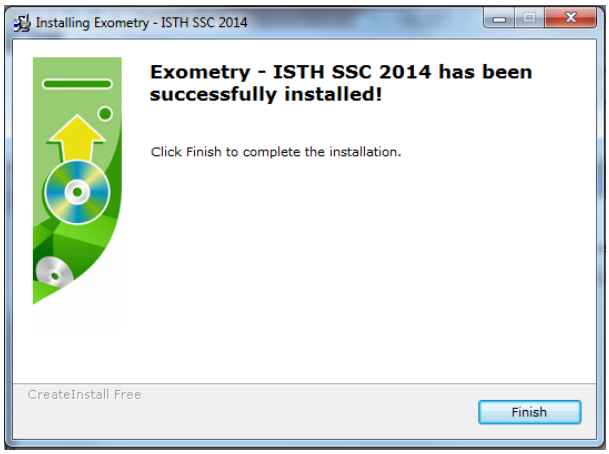


Press next



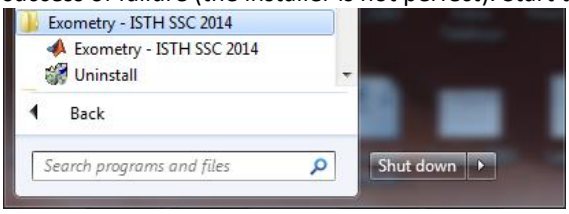
Press Install



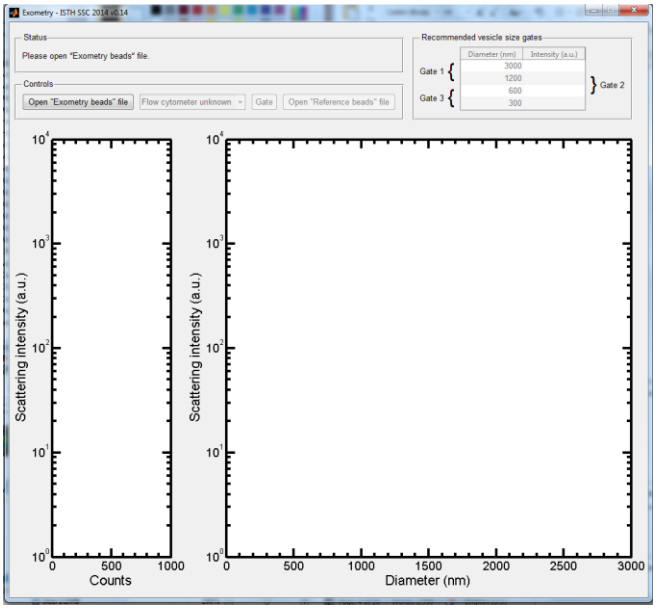


Press Finish

- The "Exometry – ISTH SSC 2014 has been successfully installed" screen will be shown regardless of success of failure (the installer is not perfect). Start the program to verify installation.



The software should start and look like this:



1.2 Preparing the Exometry bead mixture

- **Prepare the calibration beads within 30 minutes before use**
- Vortex the bottle labeled "Exometry Beads" for 10 seconds
- Place one drop from the dropper bottle into a flow tube
- Add 550 µL of deionized water and vortex to mix

1.3 Preparing the reference reference bead mixture

- **Prepare the reference beads within 30 minutes before use**
- Vortex the bead vial for 10 seconds
- Pipette 40 µL from the bead vial into a flow tube
- Add 550 µL of deionized water and vortex to mix

1.4 Checking the software

- **At this time (October 12th 2014), the software does not support the following systems:**
  - **Some of the BC systems (data storage on in FCS 2.0 and 3.0 format)**
  - **Milipore Guava**
- **Software version v0.50 or higher should be used for the study**
- Take the Exometry bead mix. Configure the flow cytometer with the flow rate in Table 2, and with the SSC/FSC gains such that the largest bead in the mix does not saturate the detector. Trigger on forward or side scatter. Set the FITC gain so that you see a separate group of FITC positive beads. **If you don't see the FITC positive beads the software will not be able to fit the size vs scatter relationship. This has been a problem with many of the Beckman Coulter systems tested.** Try to improve the SNR by adjusting the gains, cleaning the system, etc. If you cannot improve the system to the point where you can see the FITC positive beads, please contact us (f.a.coumans@amc.uva.nl, or +31205668977).
- Acquire data for 120 seconds

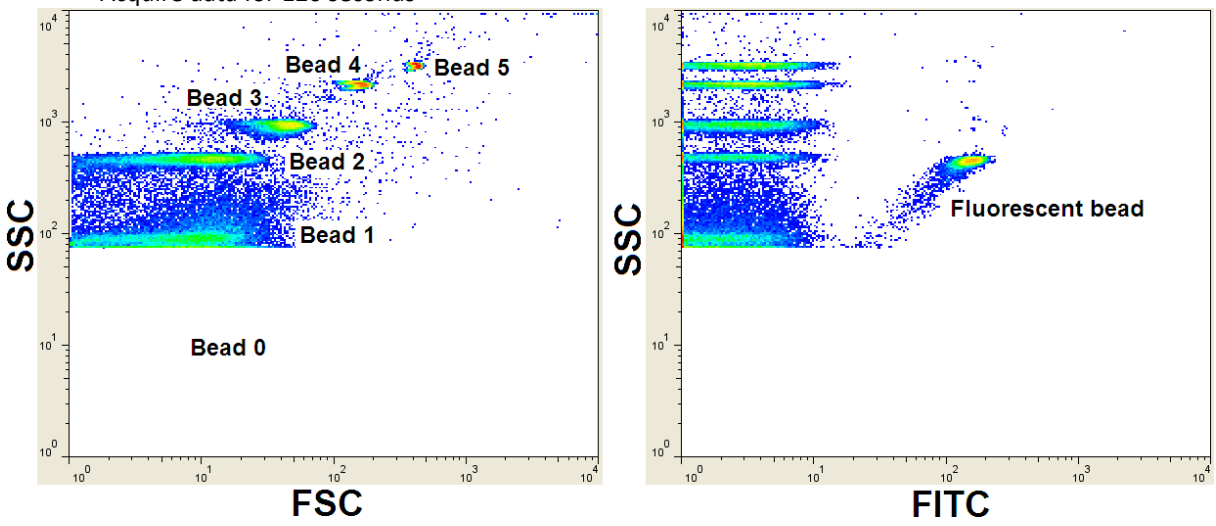


Figure 1: Exometry bead mix measured on FACSCalibur. The panel on the left shows the forward vs side scatter plot, the panel on the right shows the fluorescence vs side scatter plot. "Bead 1"is at the edge of the threshold, the signal from "Bead 0" is well below the threshold. The fluorescent bead peak is well separated from the non-fluorescent beads.

- Load the reference bead mix and acquire data for 120 seconds

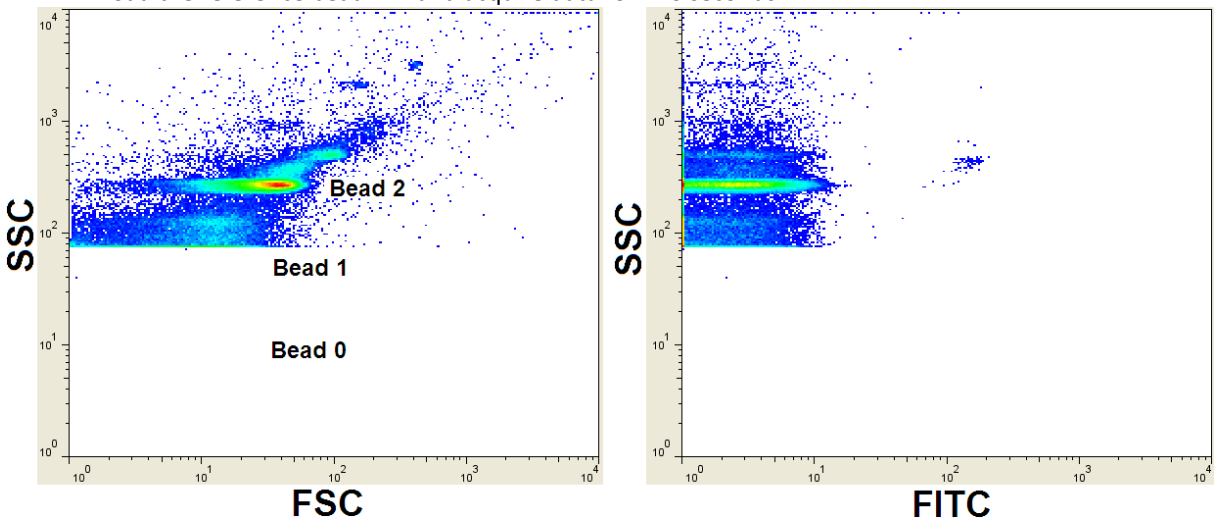


Figure 2: Reference bead mix measured on FACSCalibur. Only the largest bead is detectable on this system, both bead 0 and 1 are below the threshold.

- If needed, export both FCS files to the computer with the Exometry software

- Load the Exometry fcs file (1a, numbers refer to the screenshot in figure 3). The software will default to the best scatter channel for an instrument in the default configuration, if you have a modified instrument, select the optimal scatter channel (1b). Press gate (2), load the Reference beads file) (3). The software should present you with a view as shown in figure 3. The estimated fit error is shown in the status window (10). The black line (4) should pass through the found Exometry bead peaks (5), the red line (6) should pass through the found reference bead peaks (7). The green line (8) is the scatter to size relationship for vesicles with a refractive index of 1.40. The gates that will be set for this instrument are shown in the table in the upper right corner (9). The FACSCalibur instrument in Figure 3 lacks the scatter sensitivity to detect vesicle sizes from 600 nm, therefore, no gate is determined for this size.

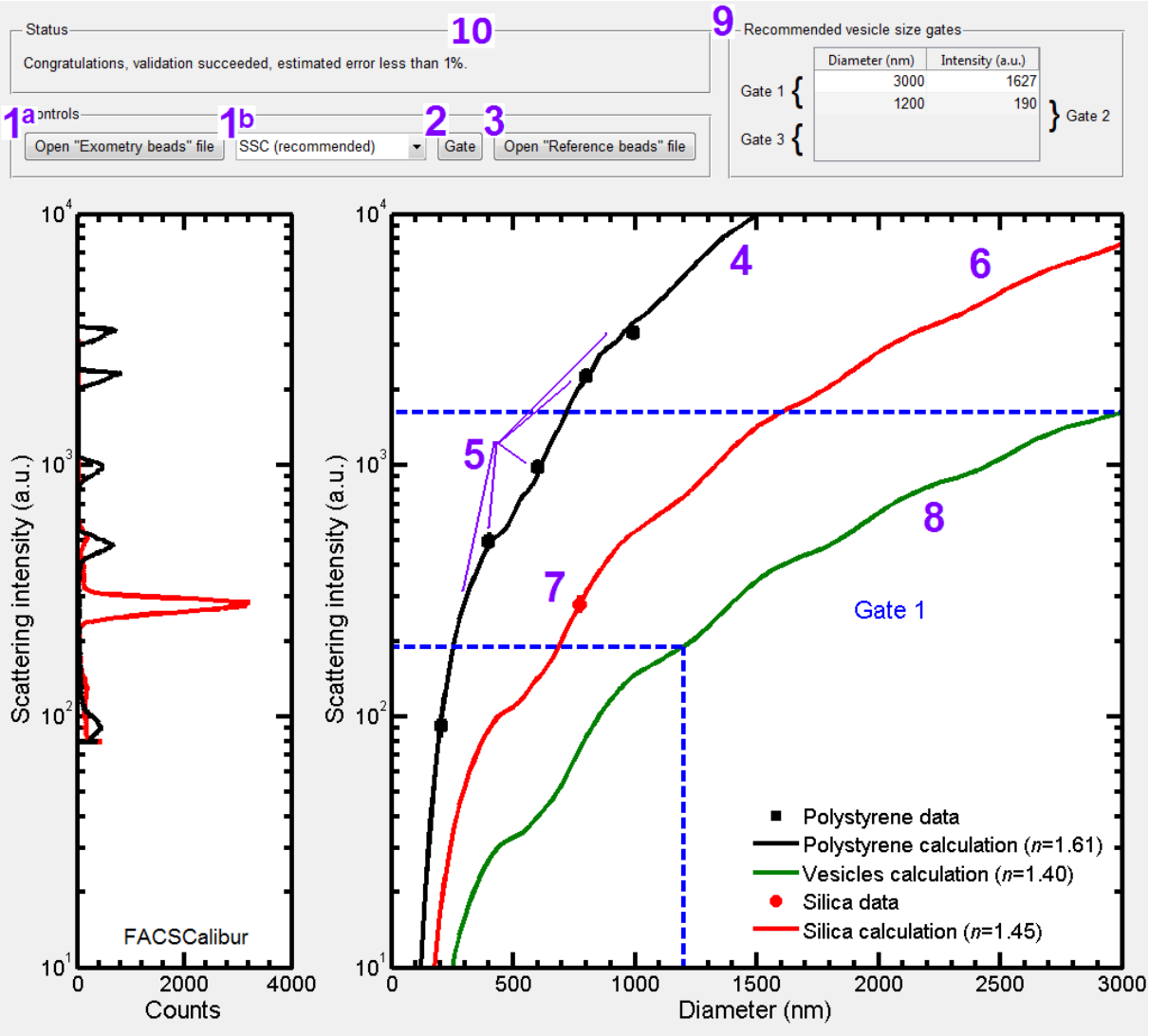


Figure 3: Exometry calibration software, see text for description

Possible failures:

- **You need to be able to configure the instrument in such a way that the signal from the fluorescent beads exceeds the noise in the scatter signal.** If you have any issues with this, please perform a flow cell clean, change gain settings, etc. If you do not succeed, it will not be possible to calibrate your instrument. Further measurements will not result in any data. Do not measure samples on this instrument.
- The validation on the reference beads may fail due to issues with the peak finding algorithm.
- If you encounter one of the two failures above, or you have any questions, contact us and do not measure samples on this instrument. Please send us the fcs files for both bead mixes and a screenshot of the software. Use Wetransfer or Dropbox of the files are too large for email.

2. *Sample staining*

Please use pipettes with valid calibration, and use pipettes that are appropriate for the volume that needs to be pipetted.

2.1 Preparing reagents

- You need 7 eppendorf tubes for this part
  - To 36 uL diluent, add 4 µL CD61-PE
  - To 58 uL diluent, add 1 µL CD235a-PE
  - To 42.5 uL diluent, add 1.5 µL IgG-PE
  - To 56 uL diluent, add 4 µL Lactadherin-FITC
- Centrifuge the **diluted** CD61-PE, CD235a-PE, IgG-PE, and Lactadherin-FITC for 5 minutes @ 19.000 x g at 20 °C
- Pipette 25 µL from each vial into a new Eppendorf (50 uL for Lactadherin-FITC), discard the remainder

2.2Preparing samples

- Take one tube with PMP sample and one tube with EMV sample from the freezer
- Thaw samples on melting ice for 1 hour
- Mix thawed sample and check for clumps

Table 1: Sample preparation volumes (buffer = supplied dilution buffer)

		Reagent 1	Reagent 2			
Label	Sample	Lact-FITC	CD61-PE	CD235a-PE	IgG-PE	Add at end
1 Exometry beads	Exometry beads					See 1.2
2 Reference beads	Reference Beads					See 1.3
3 Diluent						600 µL buffer
4 PMP-isotype	PMP				X	550 µL buffer
5 PMP-diluent	Buffer	X	X			550 µL buffer
6 PMP-sample 1	PMP	X	X			550 µL buffer
7 PMP-sample 2	PMP	X	X			550 µL buffer
8 PMP-sample 3	PMP	X	X			550 µL buffer
9 EMV-isotype	EMV				X	550 µL buffer
10 EMV-diluent	Buffer	X		X		550 µL buffer
11 EMV-sample 1	EMV	X		X		550 µL buffer
12 EMV-sample 2	EMV	X		X		550 µL buffer
13 EMV-sample 3	EMV	X		X		550 µL buffer
14 TruCount	TruCount tube					550 µL buffer
15 DI Water						1000 µL DI water

2.3 Staining procedure

A total of 15 flow tubes will be prepared for measurement on the flow cytometer. Table 1 provides an overview of the steps and volumes required.

Tube 1, 2 prepare as described in 1.2/1.3

Tube 3 add 600 uL dilution buffer to flow tube

Tube 4-13:

Preparation:

- Place 100 uL of PMP (Blue caps) and 700 uL of buffer in an Eppendorf – mark diluted PMP
- Place 100 uL of EMV (Red caps) and 700 uL of buffer in another Eppendorf - mark diluted EMV

For each tube

- Pipette 5 µL of reagent 1 in the flow tube (see table 1)
- Pipette 5 µL of reagent 2 in the flow tube (see table 1), vortex briefly
- Place 40 uL of the diluted sample in each tube (see Table 1)
- Incubate for 15 minutes in dark, room temperature
- Add 550 µL of dilution buffer to all tubes, vortex briefly

Tube 14 add 550 uL dilution buffer to TruCount tube

Tube 15: add 1000 uL DI water to flow tube, weigh tube and record total mass. Cover the tube to prevent evaporation. (You will weigh this tube again at the end of measurements)

Configuring the flow cytometer

Note: after measuring the Exometry beads, the settings of the FSC/SSC should not be changed. If you do change settings, re-measure all samples!

These instructions are written for advanced flow operators, as the differences between the operation of different flow cytometers preclude an exact description. If you have any questions about the protocol, please contact me at f.a.coumans@amc.uva.nl.

- Please configure your flow cytometer with
    - 60 uL/min flow speed (check table 2). Slower speeds are permitted if this improves the performance of your instrument. If you do select a slower speed, please increase the measurement time accordingly.
    - gains at 1
    - trigger on SSC/FSC, threshold high enough to keep event rate around or below a quarter of the maximum event rate (see table 2).
    - scales on logarithmic, if possible collect the max height (collect area if height not available) for all scatter and fluorescence detectors
    - If you can choose a scatter wavelength, set it at 488 nm
    - To collect for 120 seconds (make sure the maximum total collected events is set high enough to allow completion of a 120 second measurement). If you chose a slower speed than 60 uL/min, please increase the time per measurement to compensate for this increase (e.g. if you chose 30 uL/min, measure for 240 seconds). Do not exceed 360 seconds per measurement, even if the speed you selected was smaller than 20 uL/min.

Table 2: Suggested instrument configurations

Brand	Name	SSC/FSC *	Flow rate *	Max event rate
Amnis	Imagestream Mark II	SSC	~60 uL/min	5.000/s
Apogee**	A50	LALS	10 uL/min	100.000/s
	A50-micro	LALS	10 uL/min	100.000/s
BD	FACSCalibur	SSC	Hi (~60 uL/min)	4.000/s
	FACSAria	SSC	8 (~60 uL/min)	70.000/s
	FACSAria II	SSC	8 (~60 uL/min)	70.000/s
	FACSCanto	SSC	Med (~60 uL/min)	10.000/s
	FACSCanto II	SSC	Med (~60 uL/min)	10.000/s
	LSR II (Fortessa)	SSC	Hi (~60uL/min)	20.000/s

	LSR w/ enhanc FSC	FSC	Hi (~60uL/min)	20.000/s
	LSR (Fortessa)	SSC	Hi (~60uL/min)	20.000/s
	Accuri C6	FSC	Med (~35uL/min)	10.000/s
	FACSVerse	SSC	High sensitivity	35.000/s
	FACSJazz	SSC?	~60 uL/min	20.000/s
	Influx w/ enhanc. FSC	FSC	~60 uL/min	100.000/s
BC	FC-500	FSC?	~60 uL/min	3.300/s
	Epics XL	FSC?	~60 uL/min	3.300/s
	Gallios	FSC	~60 uL/min	25.000/s
	Navios	FSC	~60 uL/min	25.000/s
	Astrios	FSC	~60 uL/min	100.000/s
Stratedigm	S1000	SSC	~60 uL/min	10.000/s

* Note 1: this table is a guideline based on technical specifications as far as available. Whether you trigger and calibrate on SSC or FSC is ultimately up to you. The flow rate of various systems is poorly specified, our aim is to measure 120 µL of sample at a flow rate in the order of 60 µL/s. However, for the Apogee systems this rate leads to an unacceptable degradation of performance. This may be true for other systems as well, use your own judgment. **If you decide to choose another flow rate than recommended, let us know that you did!**

** Note2: The apogee systems are not reliable at flow rates above 10 uL/min due to fluidics design. For each measurement on an Apogee system, please increase the measurement time three-fold. The mass discharge measurement (tube 15) is not possible with Apogee.

- Configure the detector voltages for optimal measurement according to your lab procedure. For reference our approach is:
    - Load the Exometry bead sample
    - Optimize the SSC/FSC voltages to achieve maximum sensitivity for the smallest detectable bead. (see figure 1). **Note:** if your system can detect the smallest bead in the mix, you may saturate the largest beads, which is no problem.
    - Load the PMP isotype control
    - Set the voltage on FITC and PE to have the auto-fluorescent/background signal from the isotype in the first decade.
    - Change the detection threshold (SSC/FSC) if the events/s exceeds 25% of the maximum events per second for your flow cytometer (see table 2).
    - Check the settings with the EMV isotype control

Run Samples

- Vortex briefly prior to each measurement.
- Load the Exometry beads and collect the sample for 120 seconds. **Note:** there are many bead sizes in this vial, some could saturate the detector. This is no problem, do not change the flow cytometer settings to avoid saturation of the bead signals. The fluorescent bead (see figure 1) needs to be detected! If needed, export the data file for this measurement in FCS format, and complete the instructions on page 4 to verify that your instrument can be calibrated. Do not measure samples if you cannot calibrate your system.
- Run tubes 2-13 with the same settings**.** Label the samples in software with the corresponding name in table 1.
- Change the trigger channel to the FITC channel and set the appropriate trigger level.

- Measure tubes 3-13 again. Prefix the software label with F, for example "3 Diluent" becomes "F3 Diluent".
- For tube 14 (TruCount), change the trigger back to scatter. You may need to change the voltages on the forward/side scatter to prevent saturation of these 3 µm beads. Remember to vortex and acquire for 120 seconds. A sample flow rate of 60 µL/min corresponds to 14540 beads. See figure 4 for an example of a TruCount bead measurement.
- For tube 15 (Weighted tube) load the tube, press start immediately, acquire for 10 minutes, and remove tube immediately after the measurement. Weigh it again after the measurement to determine the sample flow rate.

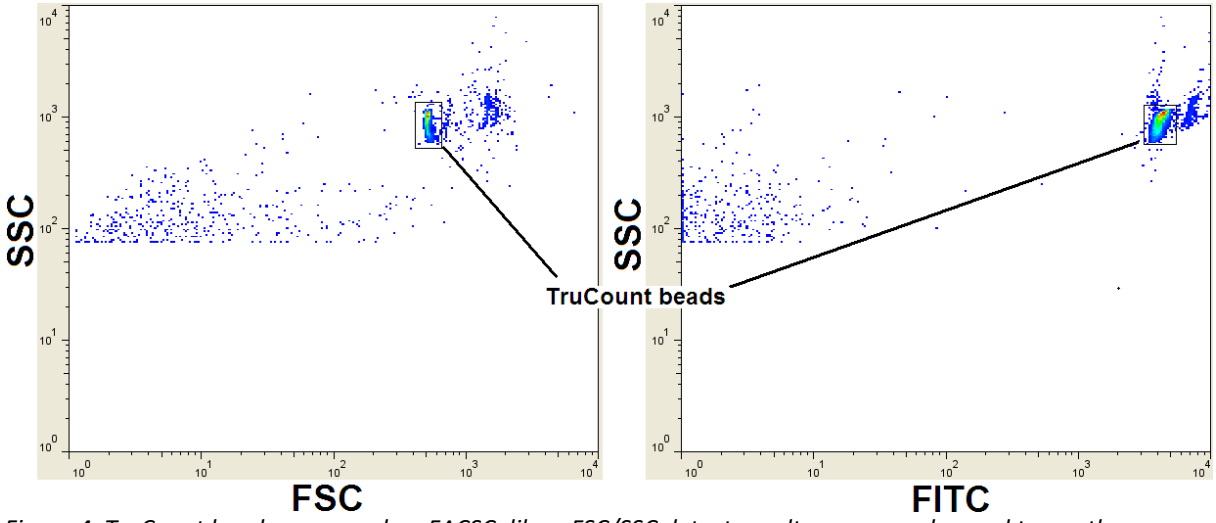


Figure 4: TruCount beads measured on FACSCalibur. FSC/SSC detector voltages were changed to see these beads. This is the only sample that may be measured with different FSC/SSC detector voltages, all other samples should be measured with the same voltages!

Data analysis

- Start the program Exometry.exe, load the file with the calibration measurement, and follow the instructions on the screen.
- The program returns the signals corresponding to a vesicle size of 300, 600, 1200, and 3000 nm.
- Set gates to collect vesicles with a size of 300-600 (gate 3), 600-1200 (gate 2), and 1200-3000 (gate 1) nm. **Note:** if your flow cytometer is not suitable for the size determination of 300-600 nm or 600-1200 nm vesicles, do not set a gate for this size.
- Set the gate on the isotype control at the edge of the autofluorescent/isotype signal level.
- Set up your measurement worksheet and a table with the following outcomes for tubes 4-13 (see figures 5-14 for an example):
    - Gate 1: 1200-3000 nm scatter
    - Gate 2: 600-1200 nm scatter
    - Gate 3: 300-600 nm scatter
    - Gate 4: CD61/CD235a+ & Gate 1
    - Gate 5: CD61/CD235a+ & Gate 2
    - Gate 6: CD61/CD235a+ & Gate 3
    - Gate 7: Lactadherin & Gate 4
    - Gate 8: Lactadherin & Gate 5
    - Gate 9: Lactadherin & Gate 6
- Analyze all samples with the same gates, and fill in the provided spreadsheet

Return data to us

- Send the spreadsheet and all FCS files via dropbox/wetransfer to f.a.coumans@amc.nl.

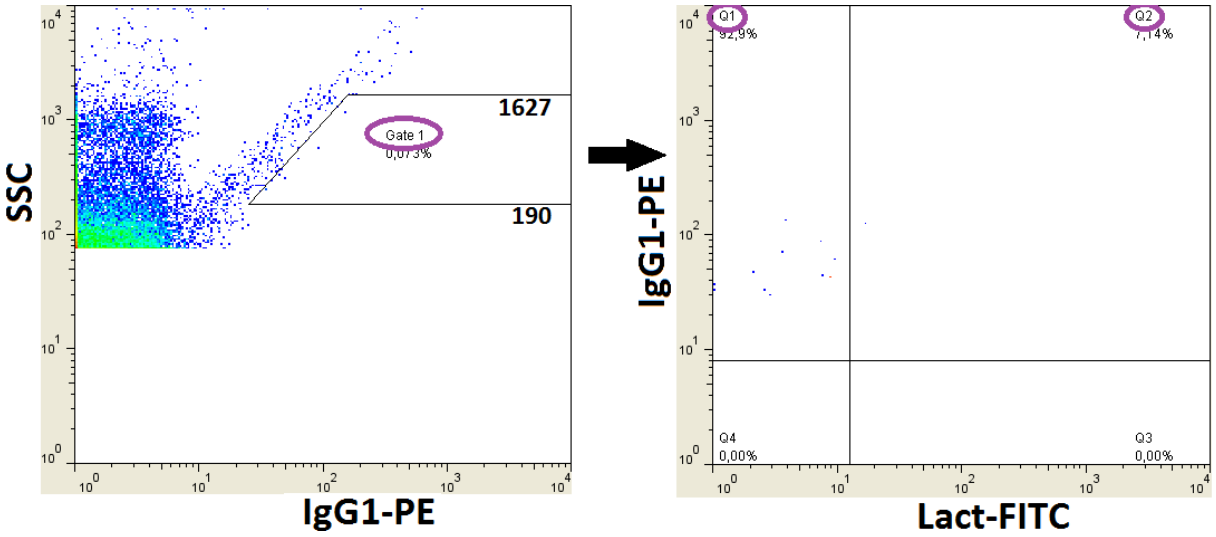


Figure 5: Analysis example on PMP isotype control measured on FACSCalibur. Gate 1 is set on side scatter from 190-1627, the values from figure 3 (the table indicated with 9). The events within gate 1 are then shown in a scatter plot. The number of CD61+ events with size from 1200-3000 nm is the total in Q1&Q2, the number of Lactadherin+CD61+ events with size from 1200-3000 nm is the total in Q2.The left edge of the gate is set to exclude the autofluorescent

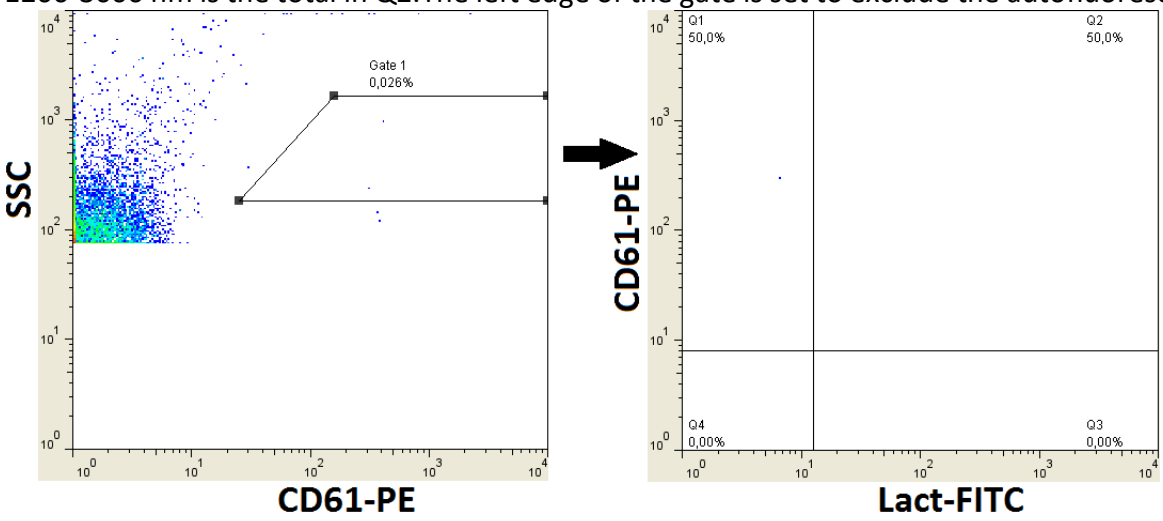


Figure 6: Analysis example on PMP diluent control measured on FACSCalibur. Gates were copied from figure 5.

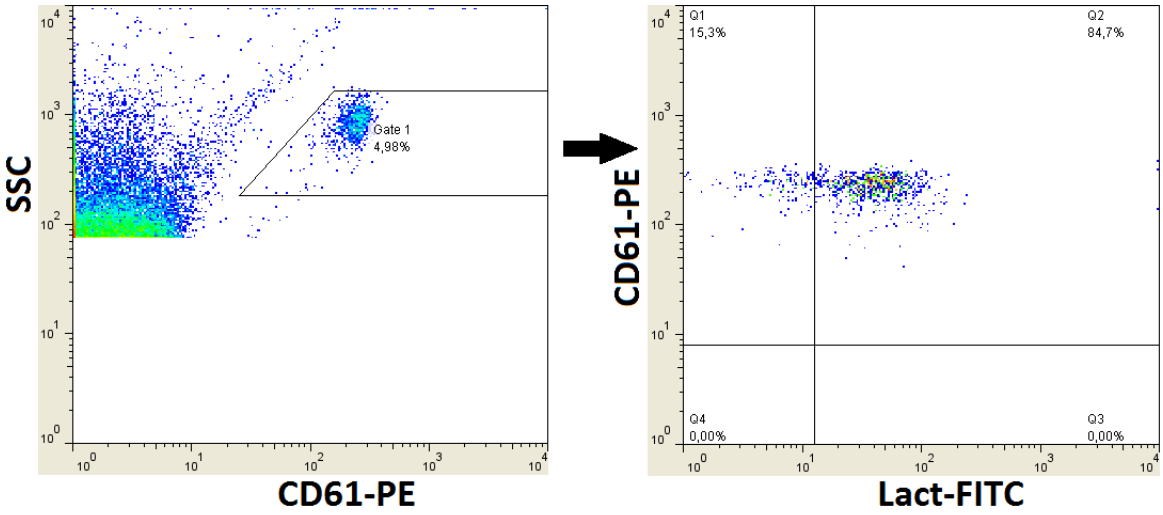


Figure 7: Analysis example on PMP sample measured on FACSCalibur. Gates were copied from figure 5.

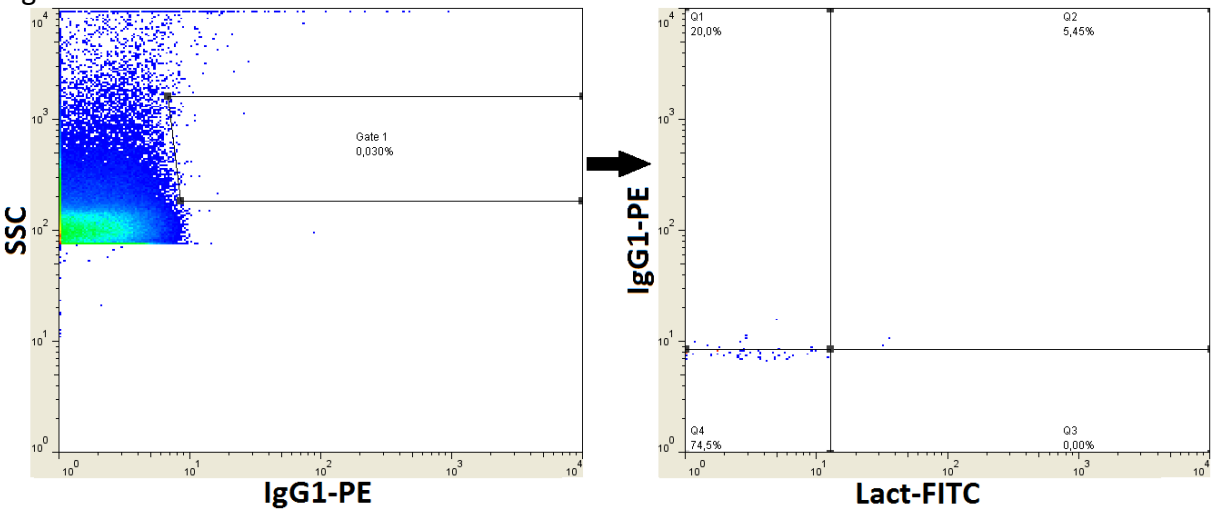


Figure 8: Analysis example on EMV isotype control measured on FACSCalibur. Gate 1 was set as in figure 5, quadrant was set based on isotype control.

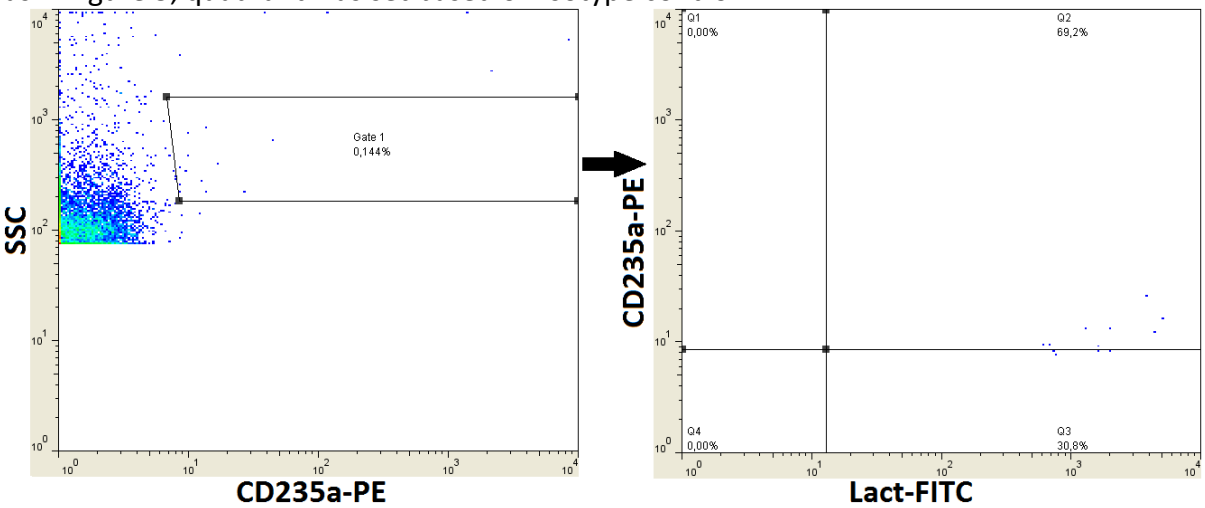


Figure 9: Analysis example on EMV diluent control measured on FACSCalibur. Gates were copied from figure 8.

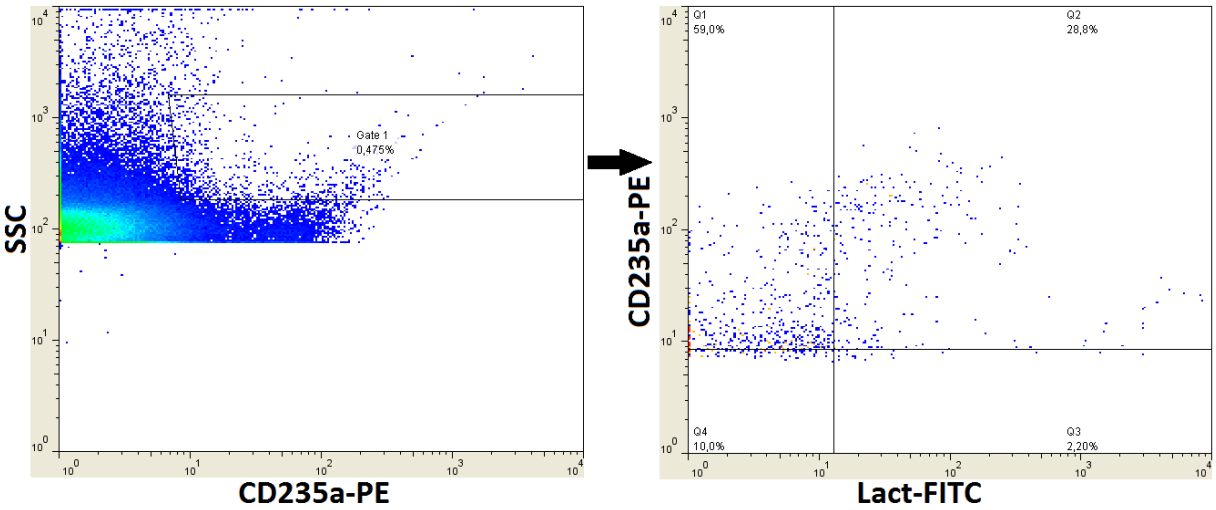


Figure 10: Analysis example on EMV sample measured on FACSCalibur. Gates were copied from figure 8.

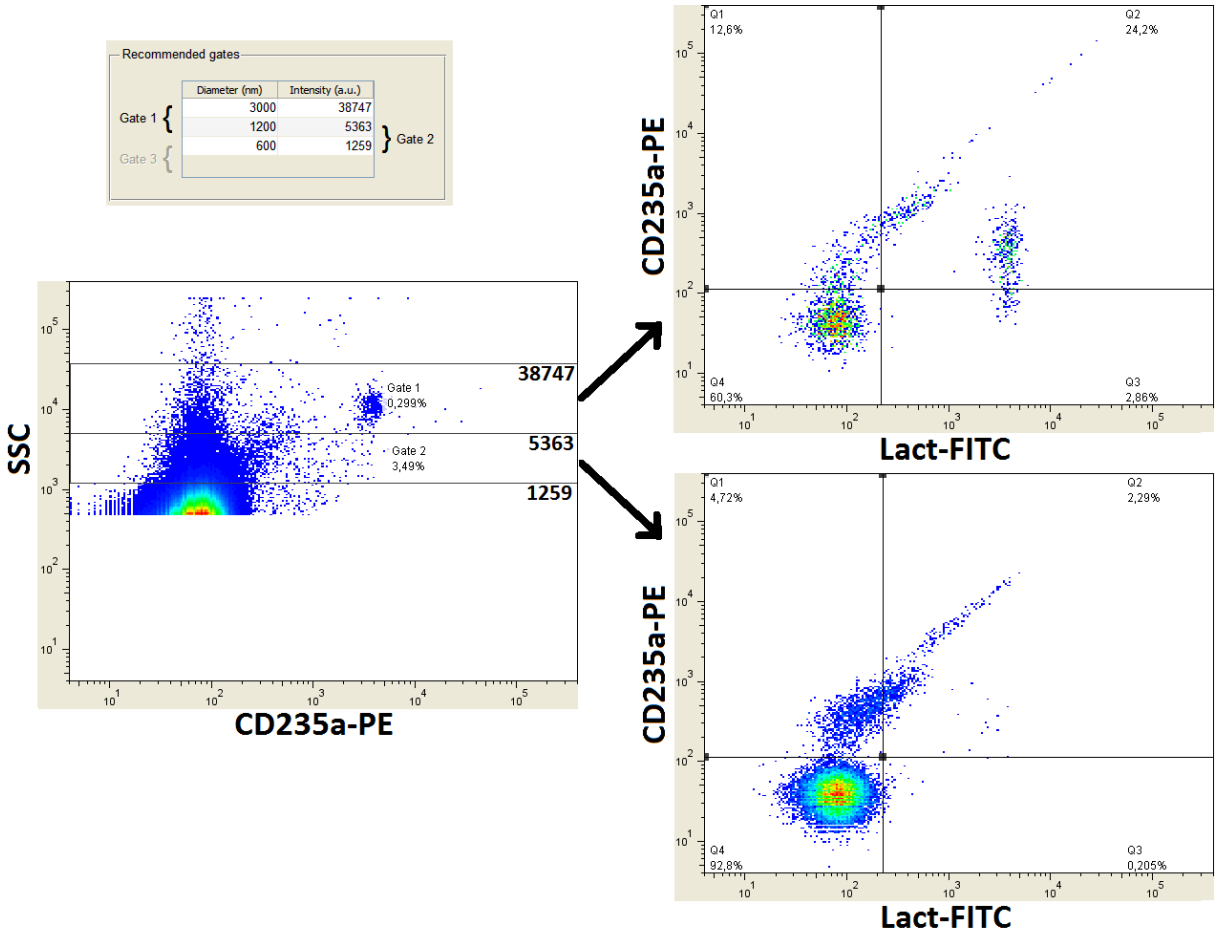


Figure 11: Gating with multiple gates. For our FACSCanto II, the Exometry software finds two gates. To perform this analysis, we apply the two gates on scatter within the thresholds indicated in the software, and analyze the fluorescence signals separately.

Site info

Name of lab	Academic Medical Center	**Please enter the appropriate information in the shaded cells**
City, Country	Amsterdam, Netherlands	
Analyst	Chi Hau	
Flow cytometer	FACSCalibur	
Date of analysis	28/4/2014	

Calibration

Estimated error exometry bea	1	%
Estimated error silica beads	2	%
Number of gates determined	1	1/2/3

Instrument configuration

Scatter channel	SSC	FSC/SSC	
Scatter laser wavelength	488	nm	
Configured flow rate	~60	uL/min or set value	med
Scatter trigger threshold valu	200		
Fluorescence trigger threshol	25		

Measurement results

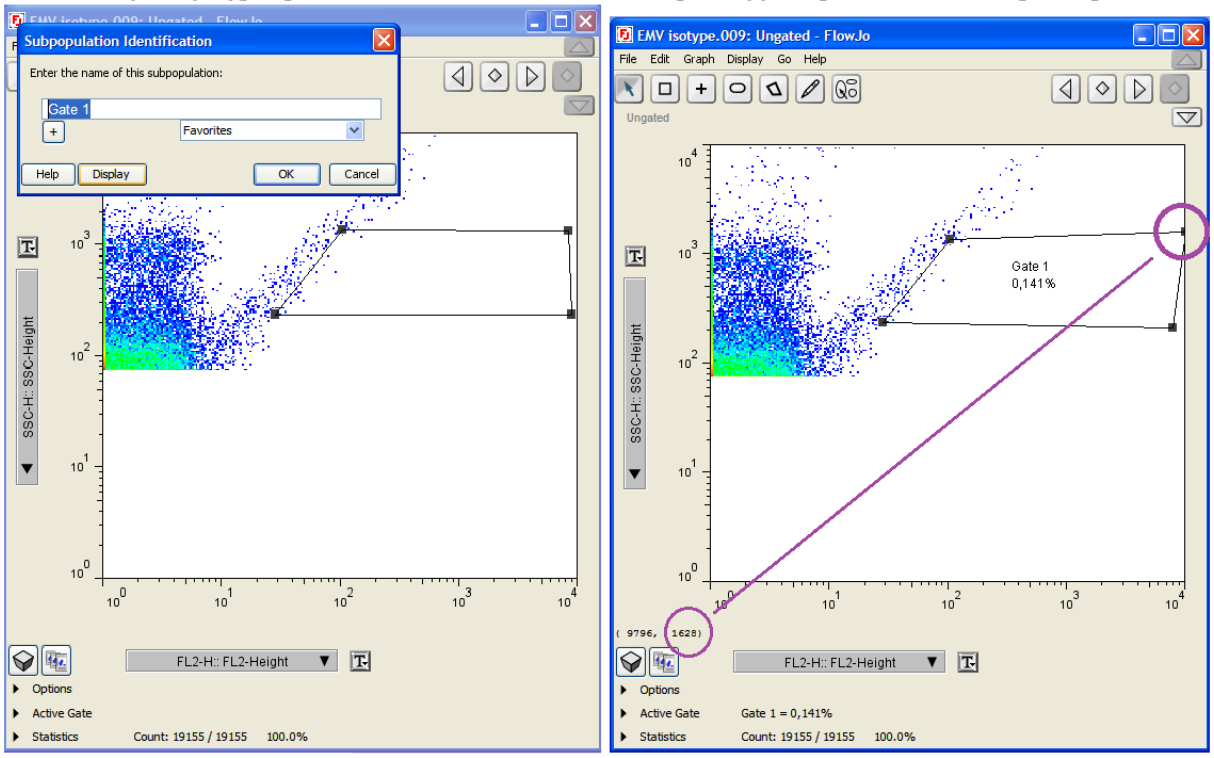
		Lactadherin+ & (CD61 or CD235a)+			(CD61 or CD235a)+			Total	Acquisition time (s)
Tube	Name	gate 1	gate 2	gate 3	gate 1	gate 2	gate 3	events	
1	1 Exometry beads								120
2	2 Silica beads								120
3	3 Diluent								120
4	4 PMP-isotype	fig 5 Q2	leave	leave	fig 5 Q1&2	leave	leave	All events	120
5	5 PMP-diluent	fig 6 Q2	blank if	blank if	fig 6 Q1&2	blank if	blank if	before	120
6	6 PMP-sample 1	fig7 Q2	no gate 2	no gate 3	fig7 Q1&2	no gate 2	no gate 3	gating	120
7	7 PMP-sample 2	"			"				120
8	8 PMP-sample 3	"			"				120
9	9 EMV-isotype	fig 8 Q2			fig 8 Q1&2				120
10	10 EMV-diluent	fig 9 Q2			fig 9 Q1&2				120
11	11 EMV-sample 1	fig 10 Q2			fig 10 Q1&2				120
12	12 EMV-sample 2	"			"				120
13	13 EMV-sample 3	"			"				120
F-3	F-3 Diluent								120
F-4	F-4 PMP-isotype								120
F-5	F-5 PMP-diluent								120
F-6	F-6 PMP-sample 1								120
F-7	F-7 PMP-sample 2								120
F-8	F-8 PMP-sample 3								120
F-9	F-9 EMV-isotype								120
F-10	F-10 EMV-diluent								120
F-11	F-11 EMV-sample 1								120
F-12	F-12 EMV-sample 2								120
F-13	F-13 EMV-sample 3								120
14	14 TruCount	Number of beads in bead gate			fig 4, all beads in gate			ul/min	120
15	15 DI Water	Mass start		mg	Mass end		Flow rate	0	600

Comments

Figure 12: Data entry template. Please fill in the shaded cells. If no gate 2 or 3 is indicated in the Exometry software, leave these fields blank.

1. Draw a 4 point polygon gate

2. Drag the upper right corner to the right height



3. Drag the bottom right corner to the right height

4. Adjust the left corners to the edge of the isotype

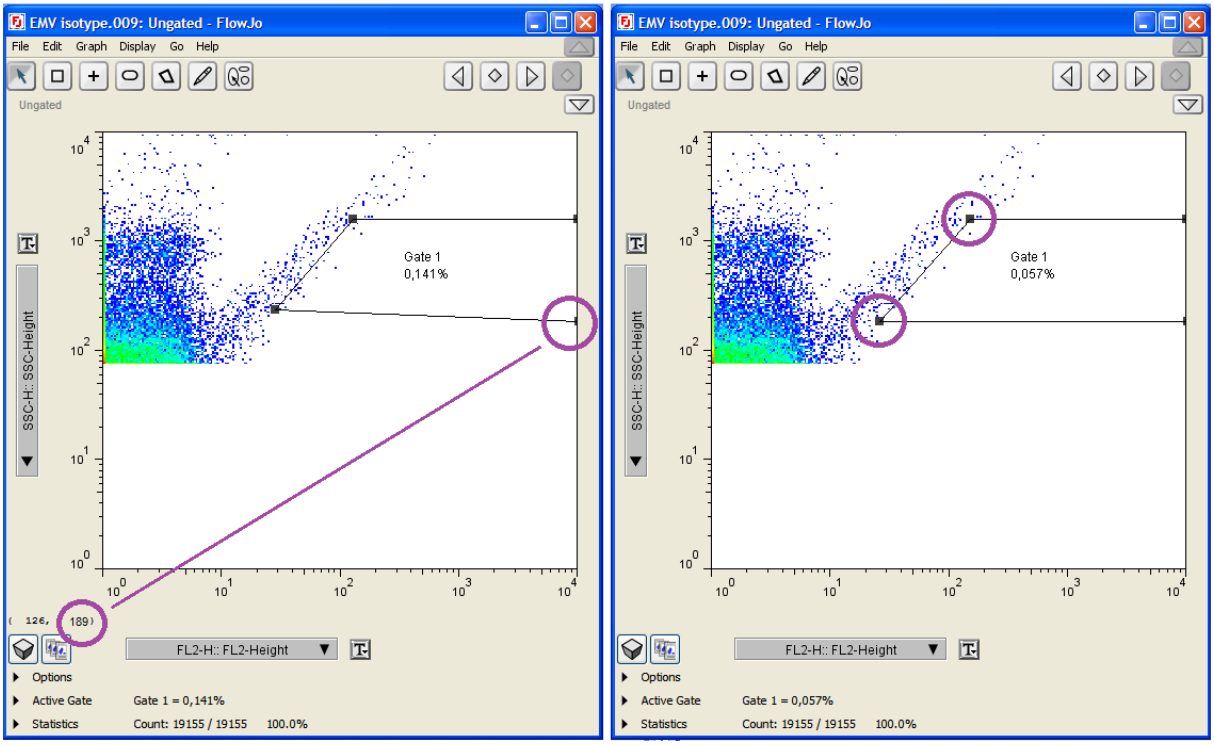
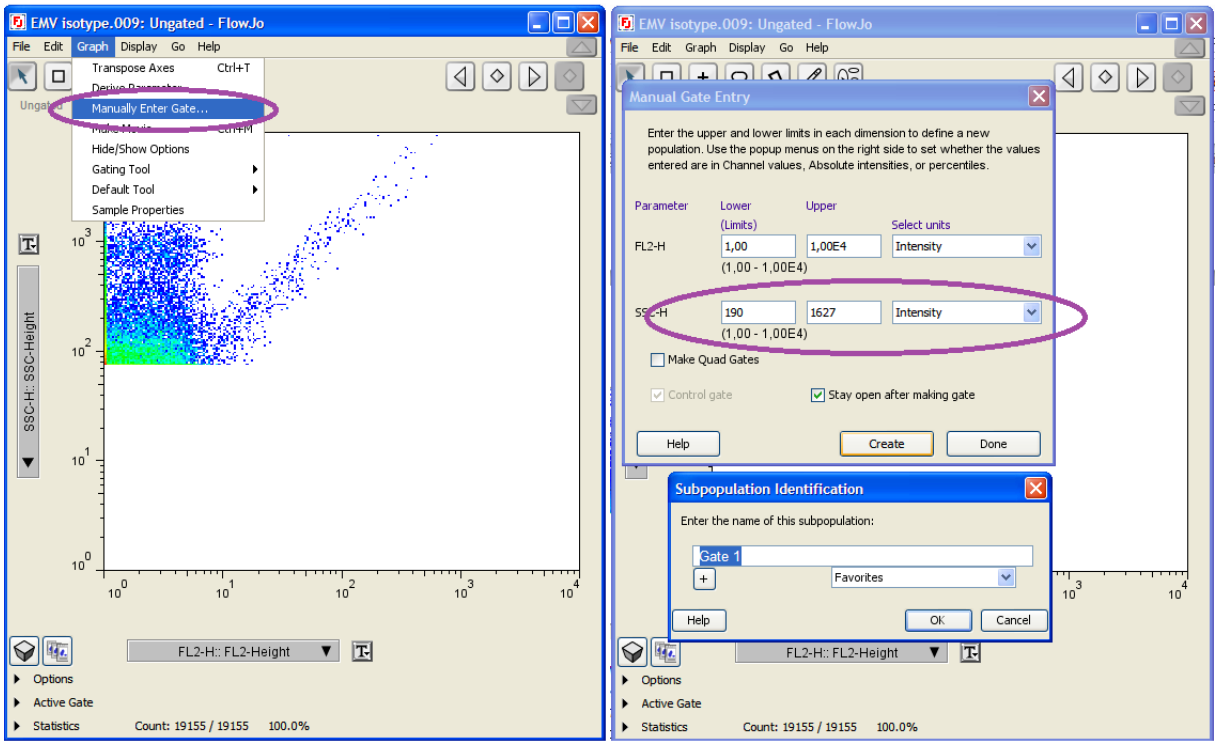


Figure 13: Method 1 for setting an SSC gate in FlowJo.

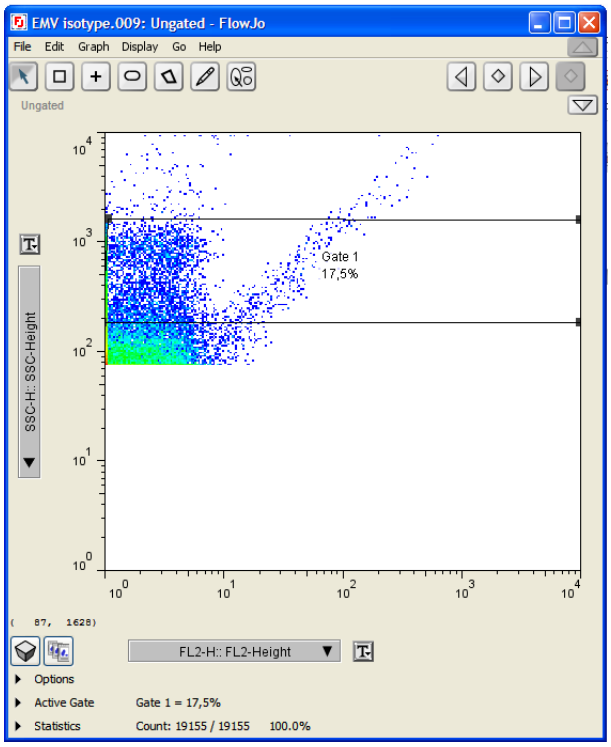
1. Select Manually enter gate

2. Enter gate values ht corner to the right height



3. Create gate ttom right corner to the right height

4. Draw polygon gate using the gate boundaries



Update image

Figure 14: Method 2 for setting an SSC gate in FlowJo

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