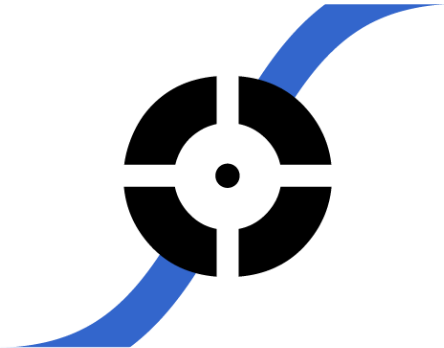
MANUAL
ROSETTA CALIBRATION V1.29

Rosetta Calibration 2
The problems 2
The solution 2
How does Rosetta Calibration work? 2
Applicability 2
Supported systems 3
Validation 4
Rosetta Calibration beads 4
Storage 4
Preparation 4
Measurement 4
Rosetta Calibration software 5
Requirements 6
Installation 6
Instructions 9
References 14
Contact 14
General 15
Support 15



Rosetta Calibration

Rosetta Calibration relates flow cytometry scatter signals to particle size.

The problems

Flow cytometry signals are expressed in arbitrary units, causing three major problems.

1. **Arbitrary units do not provide insight into the complex process of light scattering** [1]. For example, Figure 1A shows that for a particular flow cytometer 400-nm polystyrene beads scatter the same amount of light as 800 – 1,040 nm EVs. However, Figure 1B shows that for a different flow cytometer, 400-nm polystyrene beads correspond to EVs exceeding 2,600 nm in diameter. Thus, data representation in arbitrary units is misleading.
2. **Different flow cytometers provide different signals for the same sample** [2]. For example, Figures 1C and 1D show the forward versus side scatter intensity of extracellular vesicles (EVs) measured by different flow cytometers. Although both flow cytometers measured the same sample, the scatter intensity axes are different. Thus, data representation in arbitrary units precludes data comparison.
3. **Third, submicron particle measurements of the particle concentration in polydisperse samples depend on the detected size range** [3]. Figures 1E and 1F show that the measured particle concentration in polydisperse samples depends on the dynamic range of the flow cytometer. Therefore, any reported particle concentration should include the detected size range. However, data representation in arbitrary units preclude comparable particle size statements.

The solution

Rosetta Calibration converts the arbitrary units of light scattering signals measured by flow cytometry to the particle size in nanometers. With size we refer to diameter of the particle. Access to the particle size solves the three major problems with data representation in arbitrary units.

1. The particle size in nanometers gives insight into a key physical property of a particle, and in the capabilities of your flow cytometer.
2. The particle size can be compared between flow cytometers and laboratories.
3. The particle size can be used to report and compare the concentration of particles within a known size range.

How does Rosetta Calibration work?

Rosetta Calibration consists of beads and software. In three steps you can add the particle size to your flow cytometry data files:

1. Run the Rosetta Calibration beads.
2. Load the bead measurement in the Rosetta Calibration software.
3. Select the optical properties of your particles and click “Export”.

Applicability

Rosetta Calibration is particularly developed to determine the size of spherical particles <2,000 nm, such as extracellular vesicles and liposomes. One vial of Rosetta Calibration beads contains sufficient material to calibrate your flow cytometer 25 times. We recommend calibrating your flow cytometer weekly. If you change the settings on the scatter channels, you need to repeat the calibration.

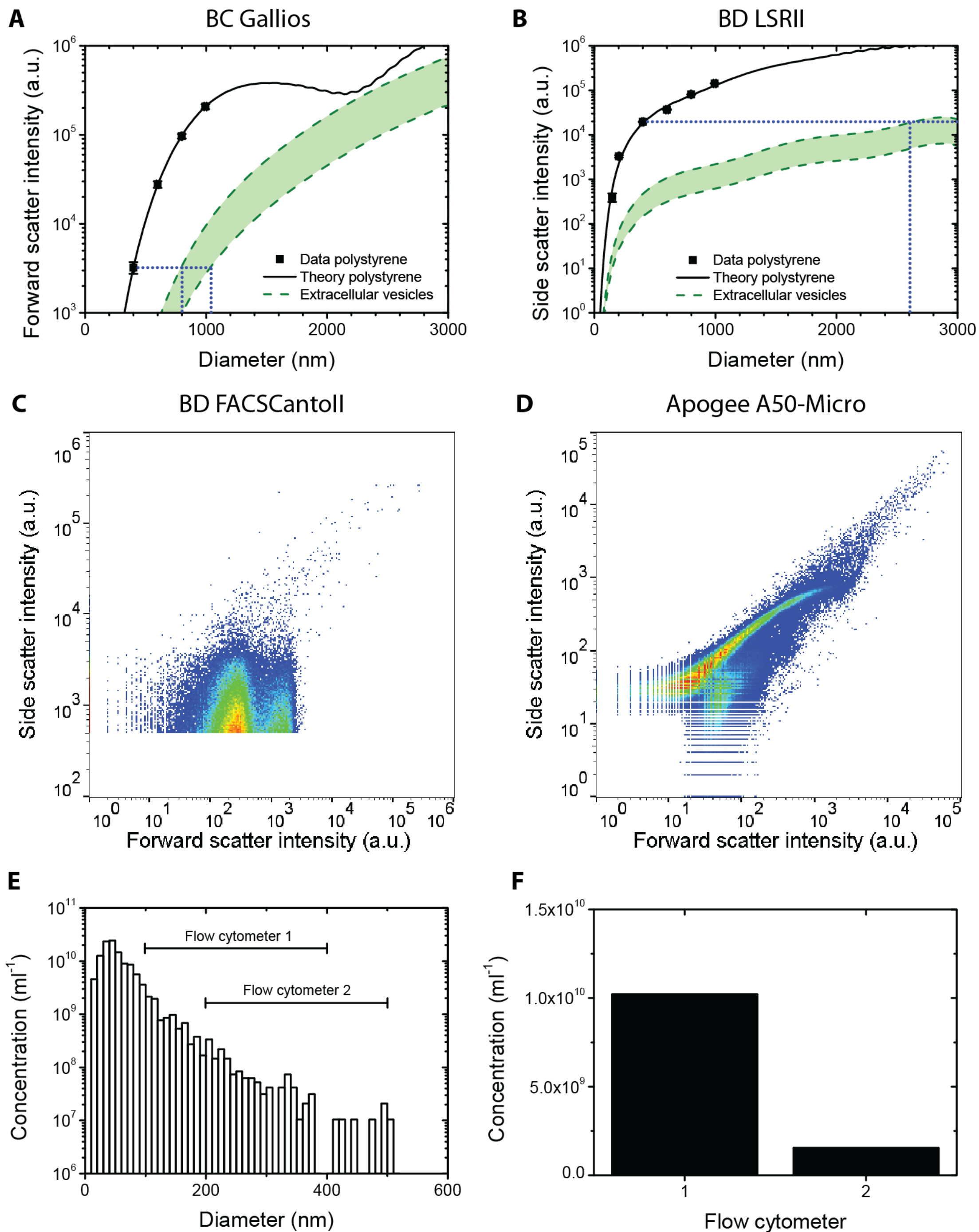


Figure 1. Problems of flow cytometry data representation in arbitrary units (a.u.). (A,B) Measured (symbols) and calculated (lines) scatter intensity versus diameter of polystyrene beads (black) and EVs (green) for a BC Gallios and BD LSRII. A 400-nm polystyrene bead corresponds to different EV sizes at different flow cytometers, showing that data representation in arbitrary units is misleading. **(C,D)** Forward versus side scatter intensity of extracellular vesicles (EVs) from a platelet concentrate measured by a BD FACSCantoII and an Apogee A50-Micro. Different scatter plots of the same samples show that data representation in arbitrary units precludes data comparison. **(E)** Concentration versus diameter for a typical EV sample and fictive dynamic ranges of two flow cytometers. **(F)** EV concentrations measured within the dynamic ranges of the flow cytometers in **(E)**. The dynamic range affects the measured concentration of EVs.

Supported systems

- Apogee A50-Micro
- Apogee A60-Micro
- Beckman Coulter Astrios
- Beckman Coulter CytoFlex
- Beckman Coulter Gallios
- Beckman Coulter Navios
- Becton Dickinson FACSAria
- Becton Dickinson FACSAria II
- Becton Dickinson FACSCalibur
- Becton Dickinson FACSCanto
- Becton Dickinson FACSCanto II
- Becton Dickinson FACSCelesta
- Becton Dickinson FACSVerse
- Becton Dickinson FACSymphony A1
- Becton Dickinson Influx
- Becton Dickinson LSR Fortessa
- Becton Dickinson LSR II
- Cytek Aurora
- Cytek Northern Lights
- Stratedigm S1000

Is your flow cytometer is not listed? Please contact us (support@exometry.com). Some flow cytometers require dedicated support or specific software updates.

Validation

The software is based on Mie theory, which is valid to describe light scattering from spherical and core-shell particles of all sizes. The Mie theory was published in 1908 and since then thoroughly validated. The calculus behind Rosetta Calibration is published [4]. The applicability of Rosetta Calibration to standardization was demonstrated in an international standardization study, wherein Rosetta Calibration was used to realize reproducible concentrations of extracellular vesicle measurements by 31 different flow cytometers [5].

Rosetta Calibration beads

Storage

Store the Rosetta Calibration beads in a refrigerator (2-8 °C).

Preparation

Please follow the steps below to prepare the Rosetta Calibration beads:

- Prepare the Rosetta Calibration beads within 30 minutes before use.
- Vortex the bottle for 10 seconds.
- Place one droplet from the dropper bottle into a flow tube.
- Add DI water to the flow tube till the volume is reached that you normally aspire when measuring a sample.
- As a reference: for flow cytometers with a fast flow rate (>10 μL min^{-1}), add one droplet to 560 μL of DI water. For flow cytometers with a slow flow rate (≤10 μL min^{-1}), add 1 droplet to 160 μL of DI water.
- Make sure that the total volume exceeds the minimal aspiration volume for your flow cytometer (air bubbles will disturb the calibration) and that at least >1,000 fluorescent marker beads are measured. For your information: the mixture contains ~10^7 fluorescent marker beads per mL and one droplet from the bottle is ~40 μL.
- Vortex the mixture for 10 seconds.

Measurement

Please follow the steps below to measure the Rosetta Calibration beads and your samples:

- Prepare Rosetta Calibration beads.
- Measure Rosetta Calibration beads and trigger on the most sensitive scatter

channel. For most flow cytometers, this is SSC/SC/LALS.

- Measure Rosetta calibration beads and your samples at the same scatter detector voltage and gain!
- For optimal calibration: configure the threshold and voltage of the scatter channel so that you detect the smallest beads in the mixture while retaining maximum dynamic range [6]. Rosetta Calibration software requires the detection of at least three but preferably more distinct peaks. It is no problem if one or more populations saturate your detectors.
- Set the voltage of the most sensitive green channel (e.g. FITC) so that you detect at least one population of green positive beads.
- Measure the beads and ensure that the (brightest) green positive bead population contains at least 1,000 counts.
- Measure the deionized water with the same settings to confirm that the background counts are low.
- If this is your first calibration, please send a copy of both .fcs files to support@exometry.com for a check.
- Measure your own samples. You may change the trigger channel to fluorescence. You may change the voltage/gain of all channels, except the calibrated scatter channel.
- If you do need to change the scatter channel settings, run the calibration again.
- The calibration is valid for a single voltage/gain setting for at least two months provided the system does not require realignment.

Error! Reference source not found. shows an example of the forward versus side scatter plot of the Rosetta Calibration beads.

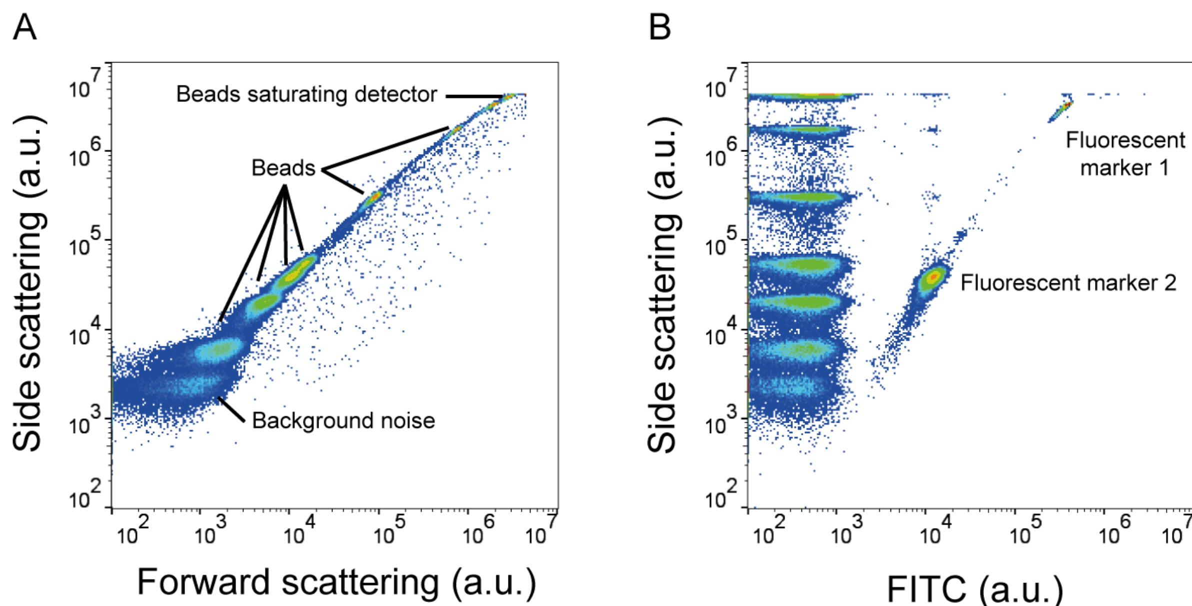


Figure 2 Rosetta Calibration beads measured by an Apogee A60-Micro. (A) Forward scatter (FSC) versus side scatter (SSC) intensity. (B) SSC intensity versus FITC fluorescence.

Rosetta Calibration software

Requirements

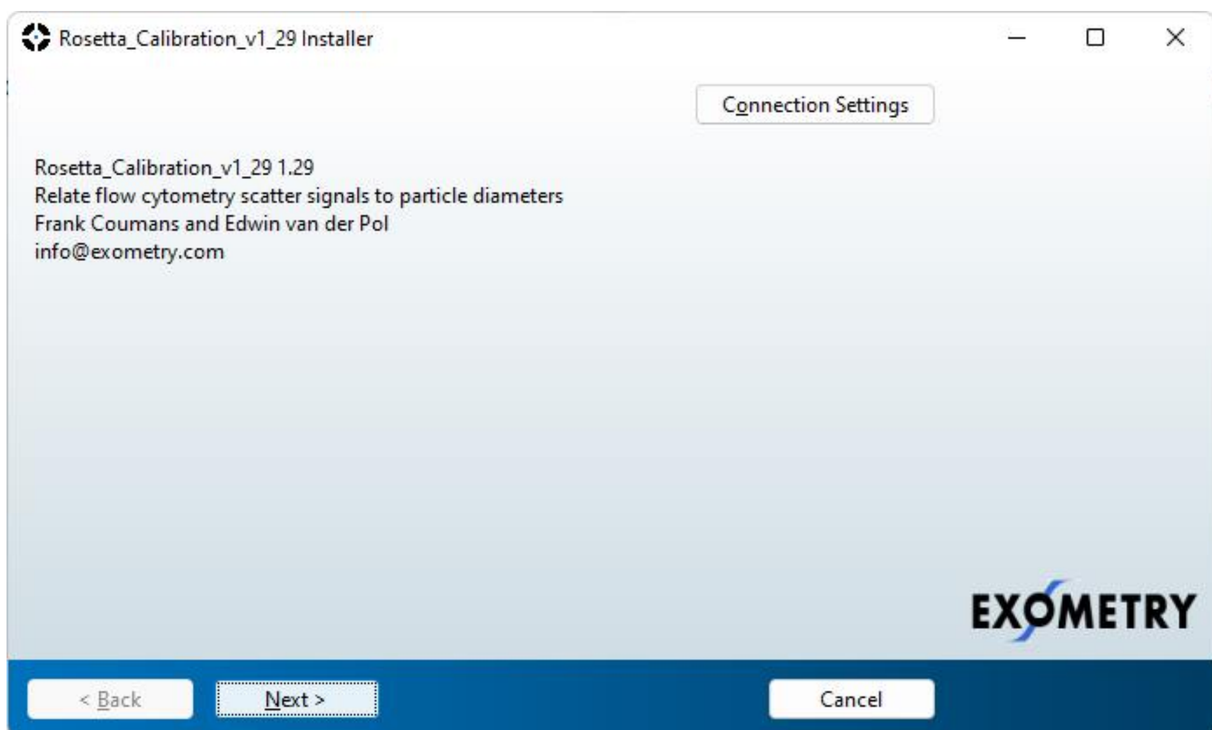
The system requirements of Rosetta Calibration software are:

- Windows 7 or higher
- 1.5 GB disk free space
- 2 GB RAM

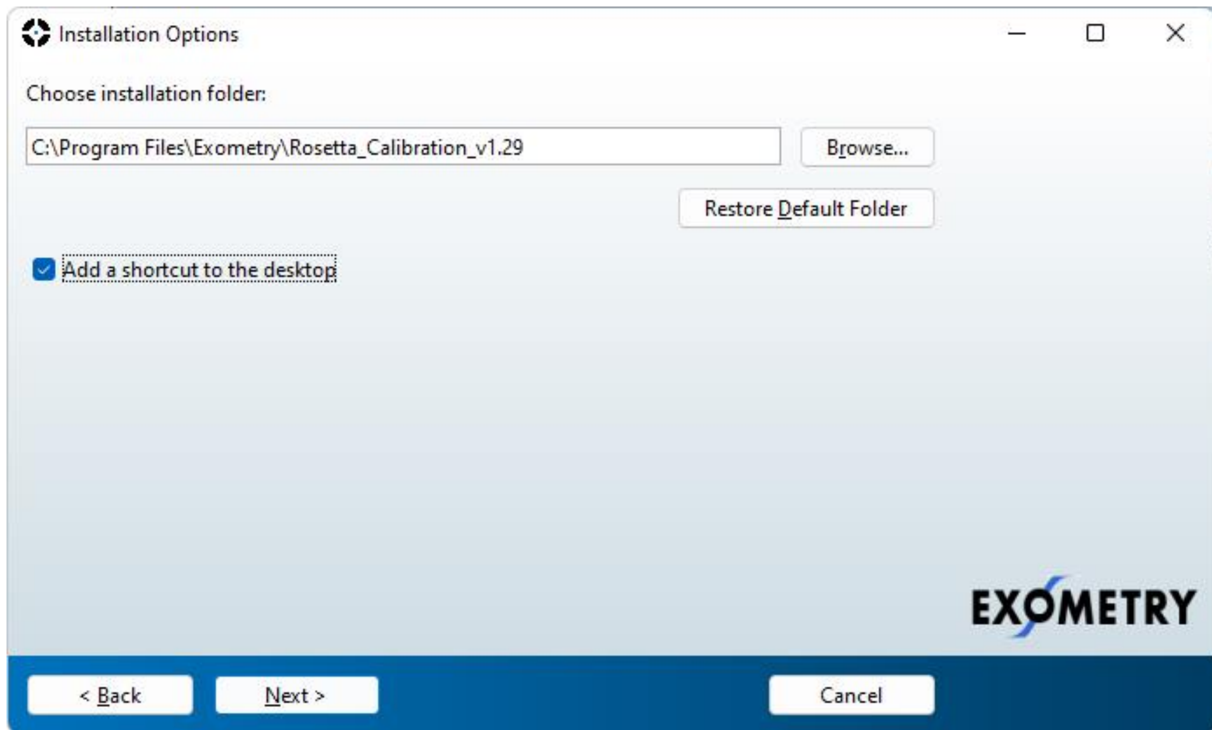
Installation

Please follow the steps below to install the Rosetta Calibration software.

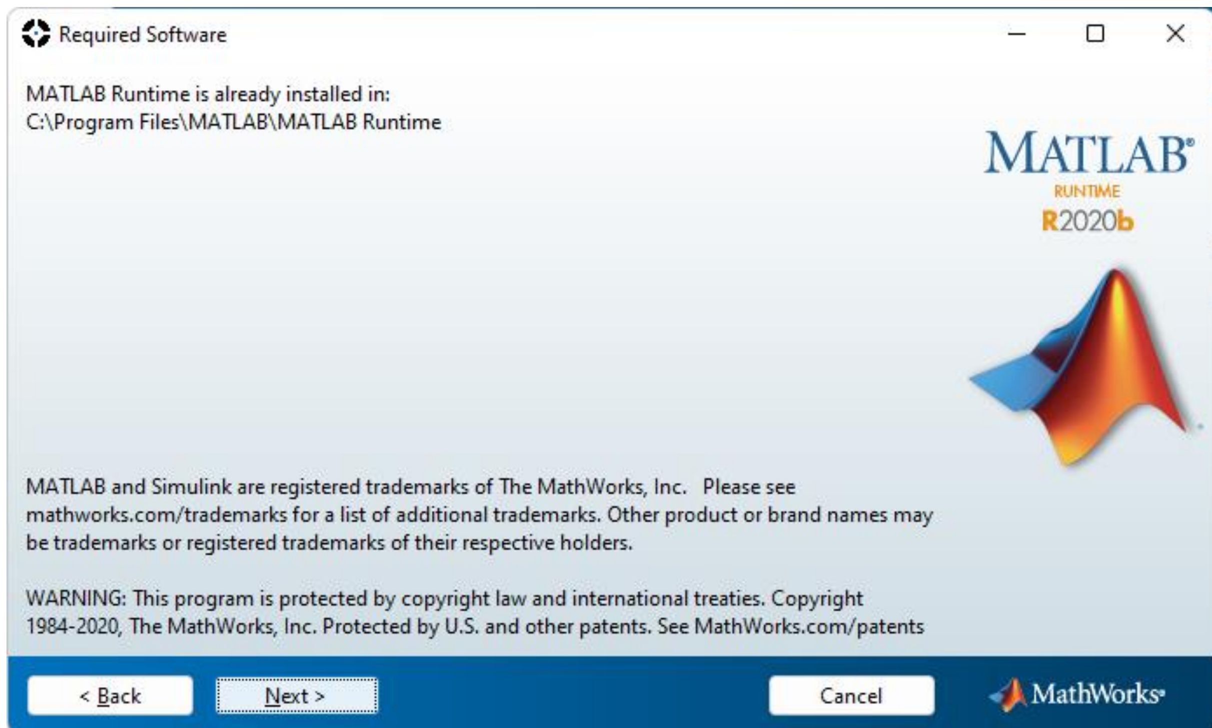
- Browse to exometry.com/downloads/
- Download Rosetta Calibration Setup.exe
- Give Windows permission to run the executable.
- Execute the downloaded .exe file as Administrator and follow the steps below.



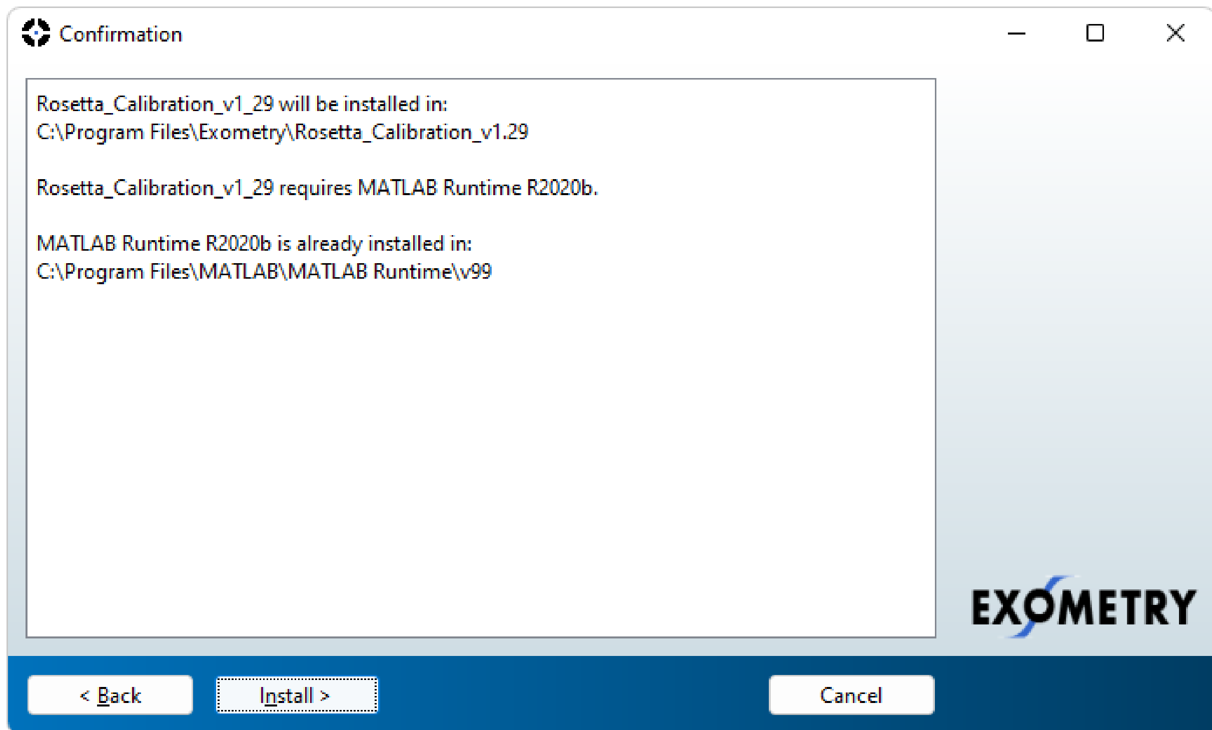
- Click “Next”.



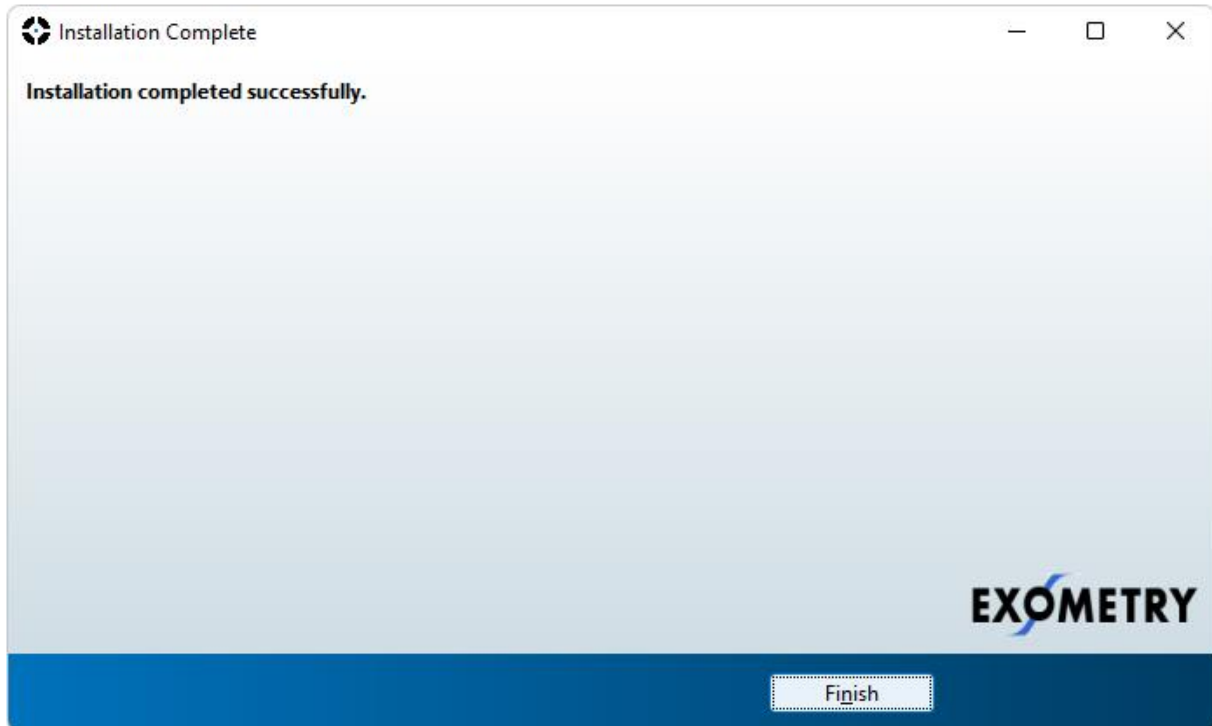
- Select “Add a shortcut to the desktop” and click “Next”.



- Click “Next”.



- Click “Install”.

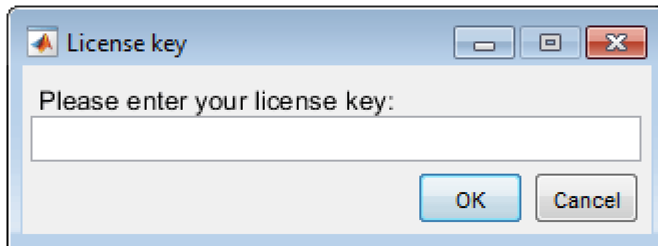


- Click “Finish”.
- The Rosetta Calibration software is installed.

Instructions

Please follow the instructions below to add particle sizes to your flow cytometry data files.

- Start the Rosetta Calibration software by clicking the icon on your desktop.



- The first time you start the Rosetta Calibration software, you will be asked for the license key. Enter your license key and click “Ok”.

Exometry - Rosetta Calibration v1.29
Status
Please open "Rosetta Calibration beads" file.
1. Calibrate
Open "Rosetta Calibration beads" file
Flow cytometer settings
Scatter detector
Rosetta Calibration kit: CAL003
Calibrate
2. Select sample calculation parameters
Mie model: solid sphere core/shell
Refractive index: 1.380
Refractive index shell:
Shell thickness:
Calculate
3. Export calibration and size distributions
Export
Rosetta Calibration settings
Rosetta Calibration Settings
Zoom horizontally
Scattering intensity (a.u.)
Counts
Scattering intensity (a.u.)
Diameter (nm)

- Click “Rosetta Calibration Settings” and verify whether the Rosetta Calibration kit version is correctly selected.

- Click “Open “Rosetta Calibration beads” file” to open the flow cytometry data file corresponding to the Rosetta Calibration beads measurement.

Settings

Flow cytometer settings

Flow cytometer name: Apogee A60-Micro

Flow cytometer type: Apogee A60-Micro

Forward scatter: 405SALS(Peak) - Small Ang... Wavelength: 405 nm

Side scatter: 405LALS(Peak) - Large Angl... Wavelength: 405 nm

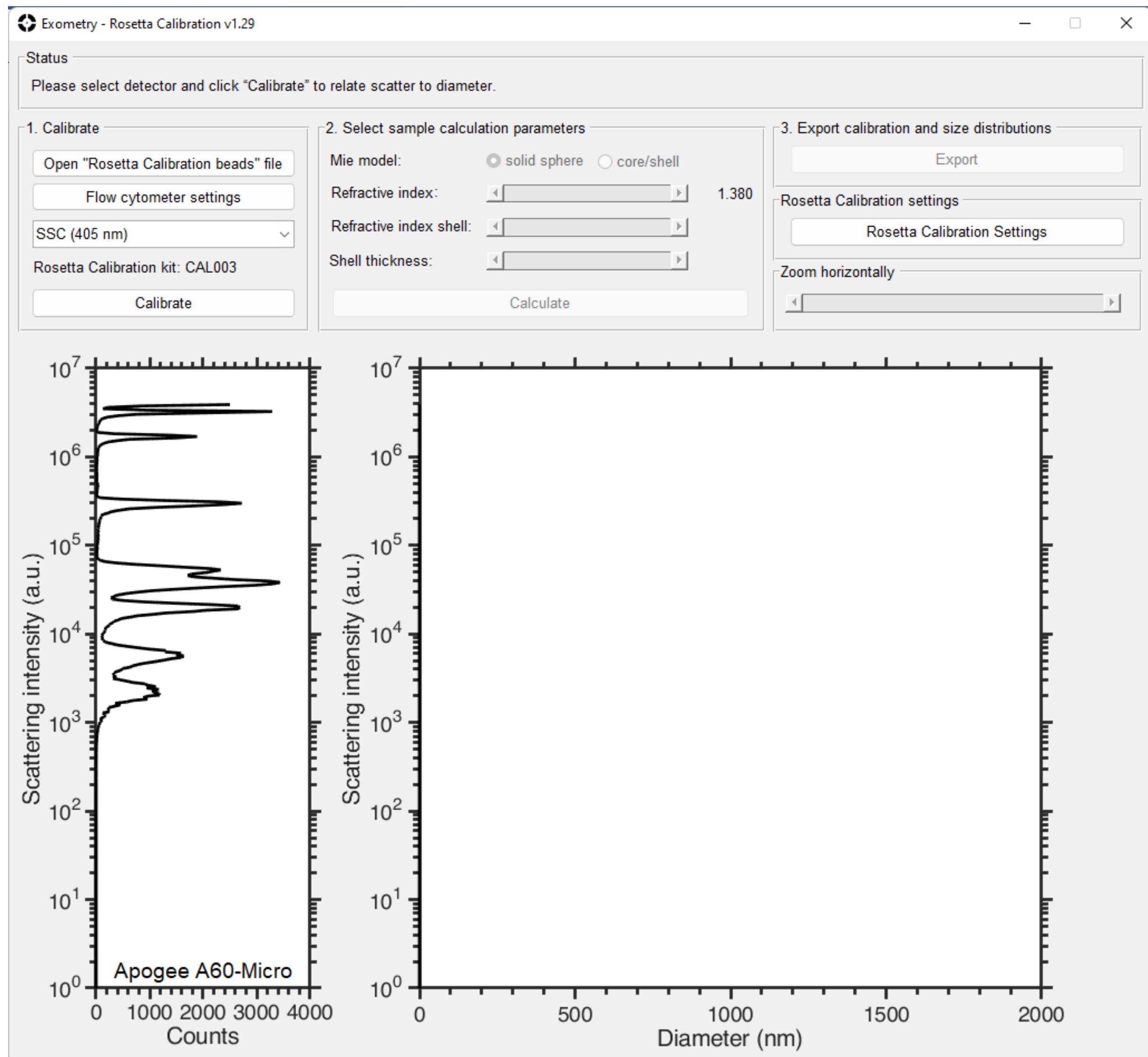
FITC: 405Grn(Peak) - Green Fluor...

Sheath fluid: Water PBS / saline

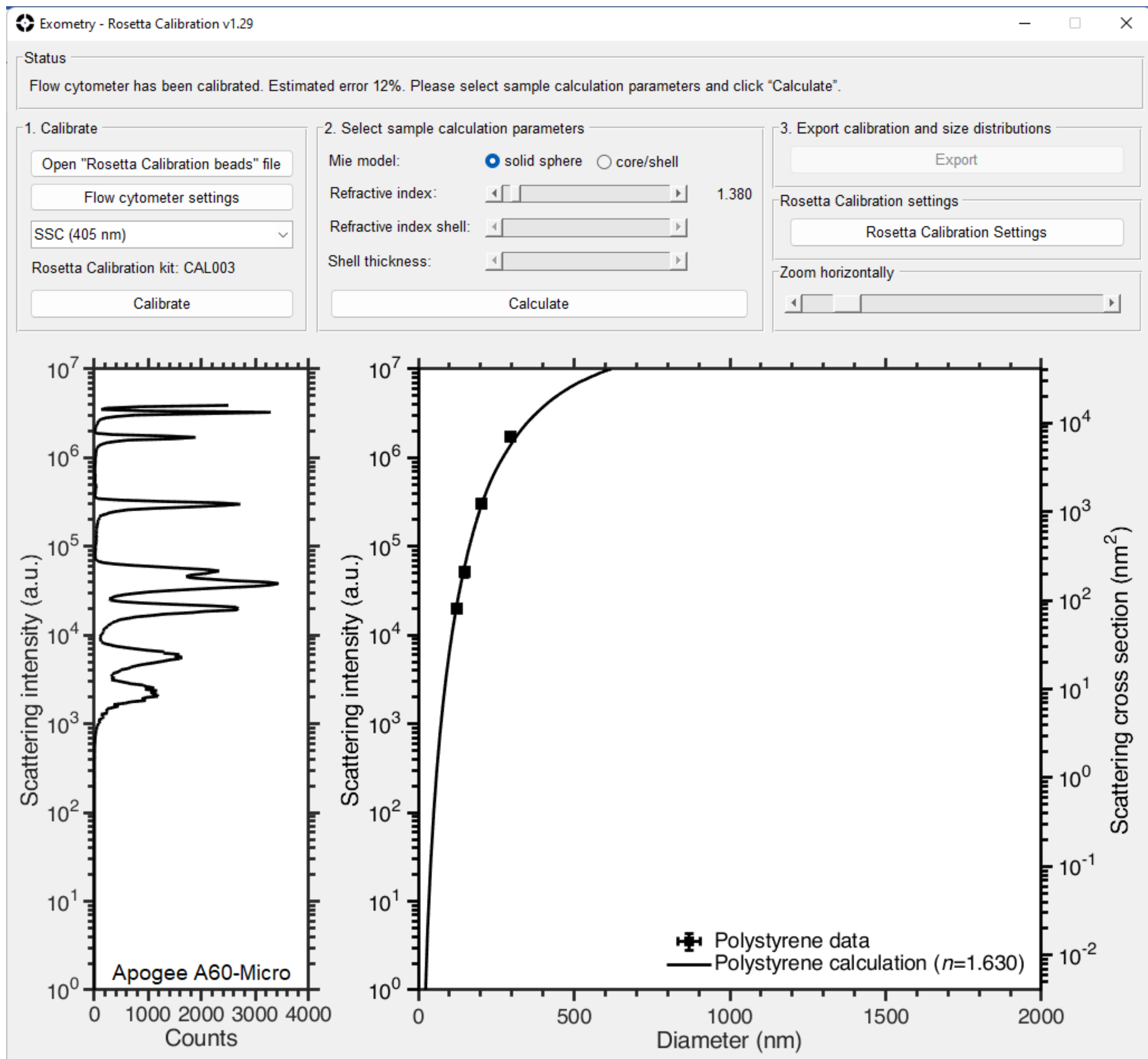
Rosetta Calibration assumes that particles are suspended in the same fluid as the sheath fluid.

Save

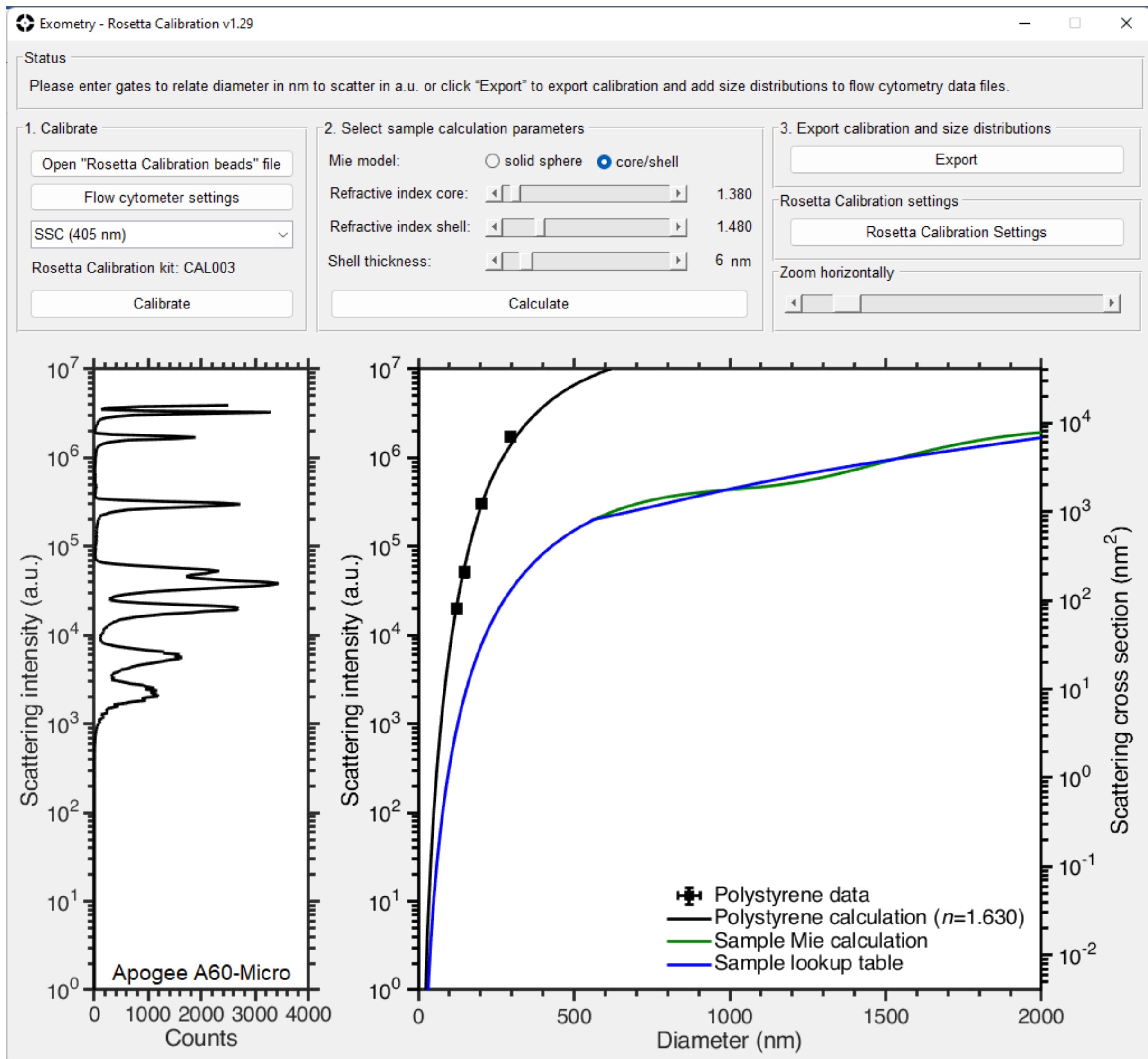
- The first time you calibrate your flow cytometer, a new window will be opened. The form contains specific settings for your flow cytometer.
- **Verify** that your flow cytometry acquisition software adds a unique name to the $CYT parameter in the saved .fcs files. The settings for your flow cytometer will be automatically stored based on the name provided by the $CYT parameter.
- **Verify** that the correct type of flow cytometer is selected.
- Select the forward scatter and side scatter channel that you want to calibrate. We recommend using the height parameters instead of the area or width parameters of your scatter channels.
- **Verify** the illumination wavelengths of the scatter detectors.
- Please select the most sensitive green channel (e.g. FITC).
- Click “Save”.
- Forward scatter channels may be disabled based on our knowledge of your type of flow cytometer.



- Select the scatter detector that you want to calibrate.
- The left graph shows a scatter histogram of the Rosetta Calibration beads.
- **Verify** that peak positions correspond to the peak positions of your own software.
- Click “Calibrate” to calibrate the selected scatter detector.



- After calibration, the right graph shows the scattering intensity in arbitrary units (a.u.) versus the diameter for polystyrene beads. Data points are represented by symbols. The line shows a fit based on Mie theory, taking into account the optical configuration of your instrument and the optical properties of the beads.
- To calculate the scattering versus diameter relationship for your own particles, adjust the calculation parameters to match the optical properties of your particles [1,2,5,7,8].
- For extracellular vesicles, we recommend [9]:
    - Core/shell model
    - Refractive index core: 1.38
    - Refractive index shell: 1.48
    - Shell thickness: 6 nm
- If you do not know the optical properties of your particles, please send an e-mail to support@exometry.com
- After adjusting the optical properties of your particles, click “Calculate”.



- The green line in the right graph shows the scattering intensity versus diameter relationship for the selected detector of your flow cytometer and your particles.
- If the green line contains oscillations, the blue dashed line will average out the oscillations to ensure that the relationship between scatter and diameter is unique.
- To add the size of your particles to your flow cytometry data files, click “Export” and select the folder where your files are stored. Rosetta Calibration software will use the blue dashed line to relate scatter to particle size. Rosetta Calibration software will never overwrite your original data files, but will add the particle sizes to a copy of your data files.
- Enjoy!

References

[1] van der Pol E, van Gemert MJC, Sturk A, Nieuwland R, van Leeuwen TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. *J Thromb Haemost* 2012; **10**: 919–30.

[2] van der Pol E, de Rond L, Coumans FAW, Gool EL, Böing AN, Sturk A, Nieuwland R, van Leeuwen TG. Absolute sizing and label-free identification of extracellular vesicles by flow cytometry. *Nanomed Nanotechnol Biol Med* 2018; **14**: 801–10.

[3] van der Pol E, Coumans FAW, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, Sturk A, van Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J Thromb Haemost* 2014; **12**: 1182–92.

[4] de Rond L, Coumans FAW, Nieuwland R, van Leeuwen TG, van der Pol E. Deriving extracellular vesicle size from scatter intensities measured by flow cytometry. *Curr Protoc Cytom* 2018; **e43**: 1–14.

[5] van der Pol E, Sturk A, van Leeuwen TG, Nieuwland R, Coumans FAW, Group I-S-VW. Standardization of extracellular vesicle measurements by flow cytometry through vesicle diameter approximation. *J Thromb Haemost* 2018; **16**: 1236–45.

[6] Shapiro HM, Leif RC. Practical flow cytometry. 4th ed. NJ: John Wiley & Sons, Inc.; 2003.

[7] Gardiner C, Shaw M, Hole P, Smith J, Tannetta D, Redman CW, Sargent IL. Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in extracellular vesicles. *J Extracell vesicles* 2014; **3**: 25361.

[8] van der Pol E, Coumans FAW, Sturk A, Nieuwland R, Van Leeuwen TG. Refractive index determination of nanoparticles in suspension using nanoparticle tracking analysis. *Nano Lett* 2014; **14**: 6195–201.

[9] van der Pol E, van Leeuwen TG, Yan X. Misinterpretation of solid sphere equivalent refractive index measurements and smallest detectable diameters of extracellular vesicles by flow cytometry. *Sci Rep* 2021; **11**: 24151.

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