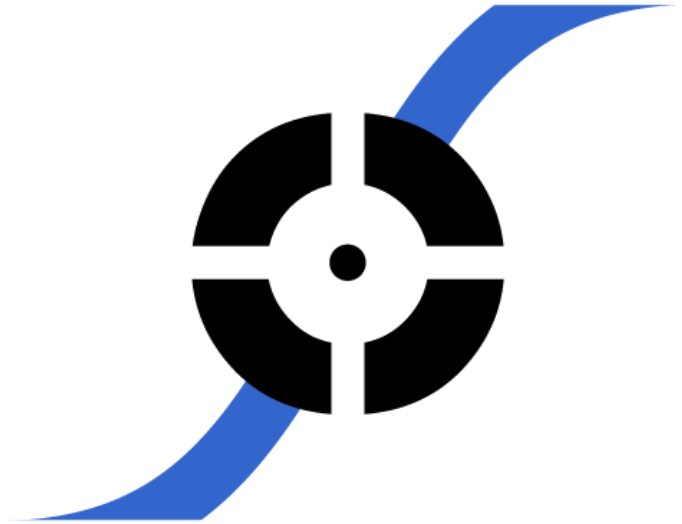
Manual Rosetta Calibration v1.04.00



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 given flow cytometer a 400 nm polystyrene bead scatters the same amount of light as 800-1,040 nm EVs. However, Figure 1B shows that at 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 plots are different. Thus, data representation in arbitrary units precludes data comparison.
3. Third, measurements of the particle concentration in polydisperse samples, such as EV samples and ocean water, are incomparable[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 precludes comparable particle concentration 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.
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. We currently support the following flow cytometers[4]:

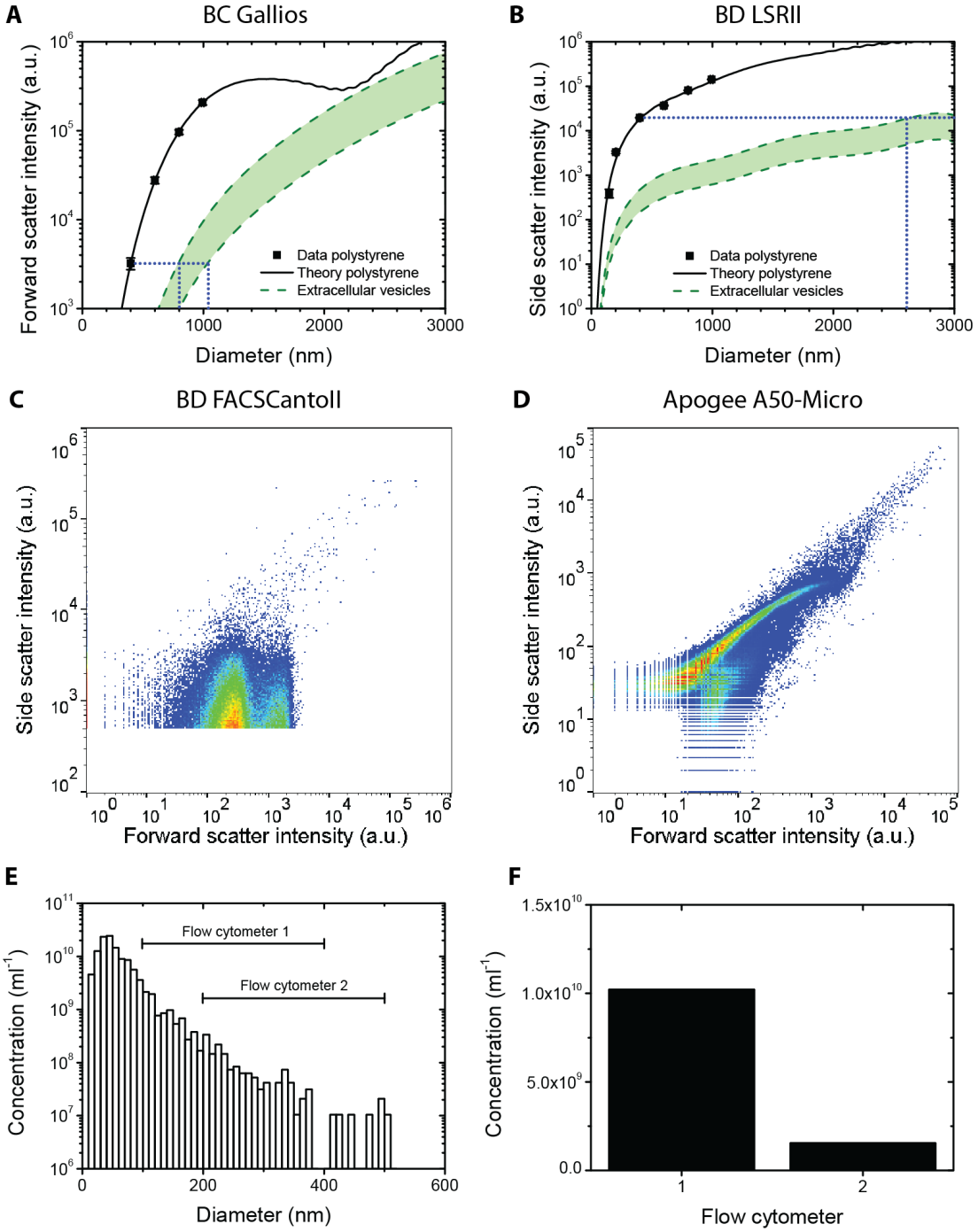


Figure 1. Problems of flow cytometry data representation in arbitrary units (a.u.). (A,B) Measured (symbols) and calculated (lines) scatter intensity versus diameter of polystyrene beads (black) and EVs (green) for a BC Gallios and BD LSRII. A 400 nm polystyrene bead corresponds to different EV sizes 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.

- 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 FACSVerse
- Becton Dickinson Influx
- Becton Dickinson LSR Fortessa
- Becton Dickinson LSR II
- Stratedigm S1000

If your flow cytometer is not listed above, please send an e-mail to support@exometry.com. We may support your flow cytometer, but dedicated support or specific software updates may be required.

Rosetta Calibration beads

Storage

Store the Rosetta Calibration beads in a refrigerator (2-6 °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 a volume of deionized water, so that:
  - The total counts of fluorescent marker beads is 1,000 during the measurement time. One droplet has a volume of ~40 µL and contains ~10^7 fluorescent marker beads per mL.
  - The total volume exceeds the minimal aspiration volume for your flow cytometer.
    - For most common flow cytometers and measurement settings, add 560 µL of deionized water to dilute the droplet 15-fold.
    - For nanoparticle flow cytometers, add 160 µL of deionized water to dilute the droplet 5-fold.
- Vortex the mixture for 10 seconds.

Measurement

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

- Run the prepared Rosetta Calibration beads.
- Trigger on the most sensitive scatter channel while measuring the Rosetta Calibration beads. For most flow cytometers, the side scatter channel has the highest sensitivity.
- Set the threshold and voltage of the most sensitive scatter channel so that you detect the smallest beads in the mixture while remaining as much as possible dynamic range[5]. Rosetta Calibration software requires the detection of at least three but preferably more distinct peaks.
- Set the voltage of the FITC channel so that you detect at least one population of FITC positive beads.
- Measure the prepared Rosetta Calibration beads and ensure that each 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 parameter to your favorite (fluorescence) channel as long as the settings of the scatter channels remain unchanged.

Figure 2 shows an example of the forward versus side scatter plot of the Rosetta Calibration beads.

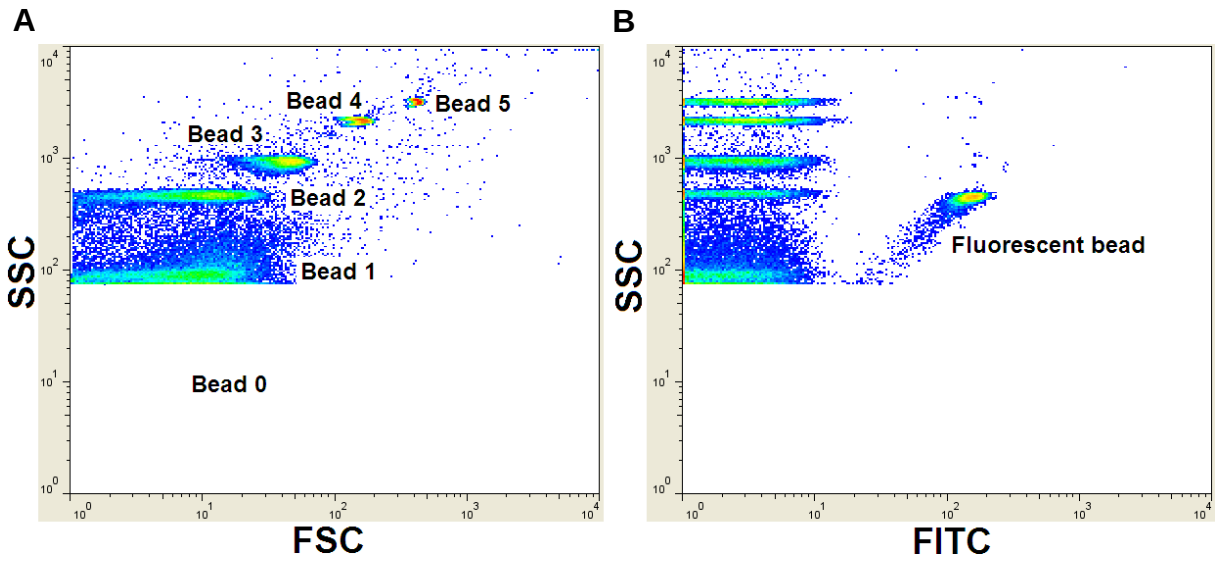


Figure 2 Rosetta Calibration beads measured by a FACSCalibur. (A) Forward scatter (FSC) versus side scatter (SSC) intensity. (B) SSC intensity versus FITC fluorescence. The signal of "Bead 1"is at the edge of the threshold, whereas the signal of "Bead 0" is below the threshold and therefore not visible. One fluorescent bead peak is separated from the non-fluorescent beads.

Rosetta Calibration software

Requirements

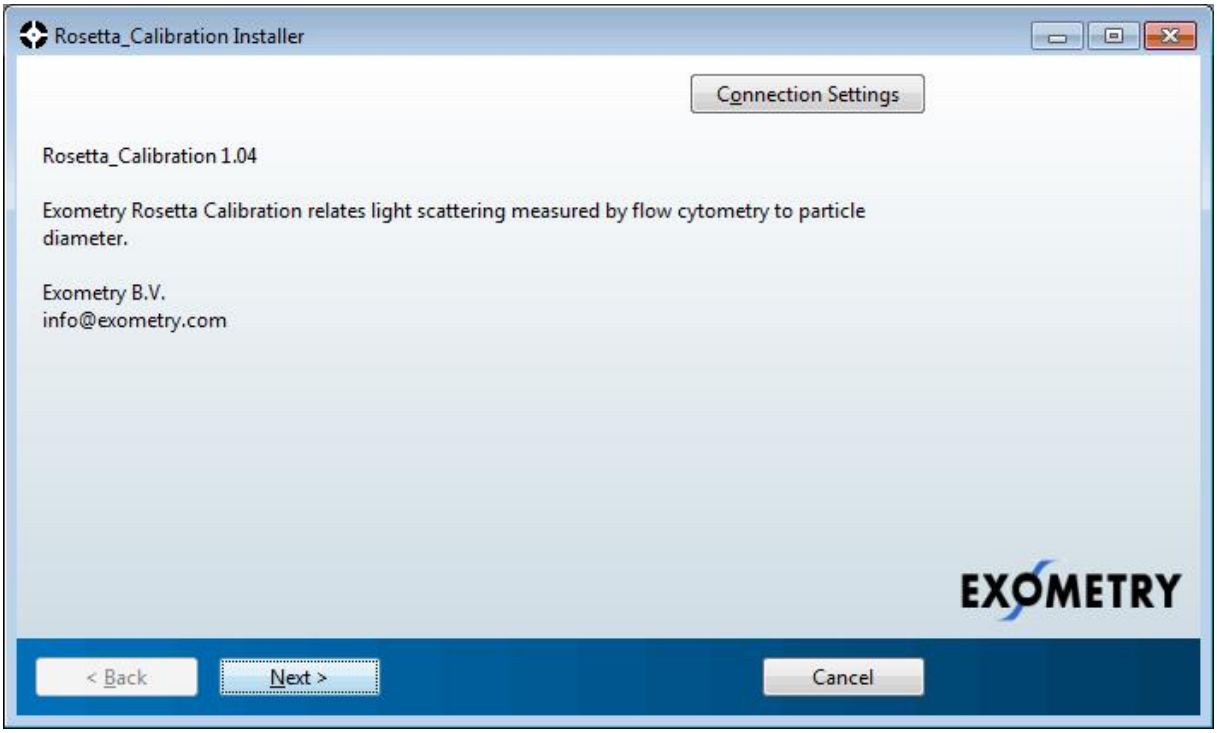
The system requirements of Rosetta Calibration software are:

- Windows 7 or higher
- 1 GB disk space
- 2 GB RAM

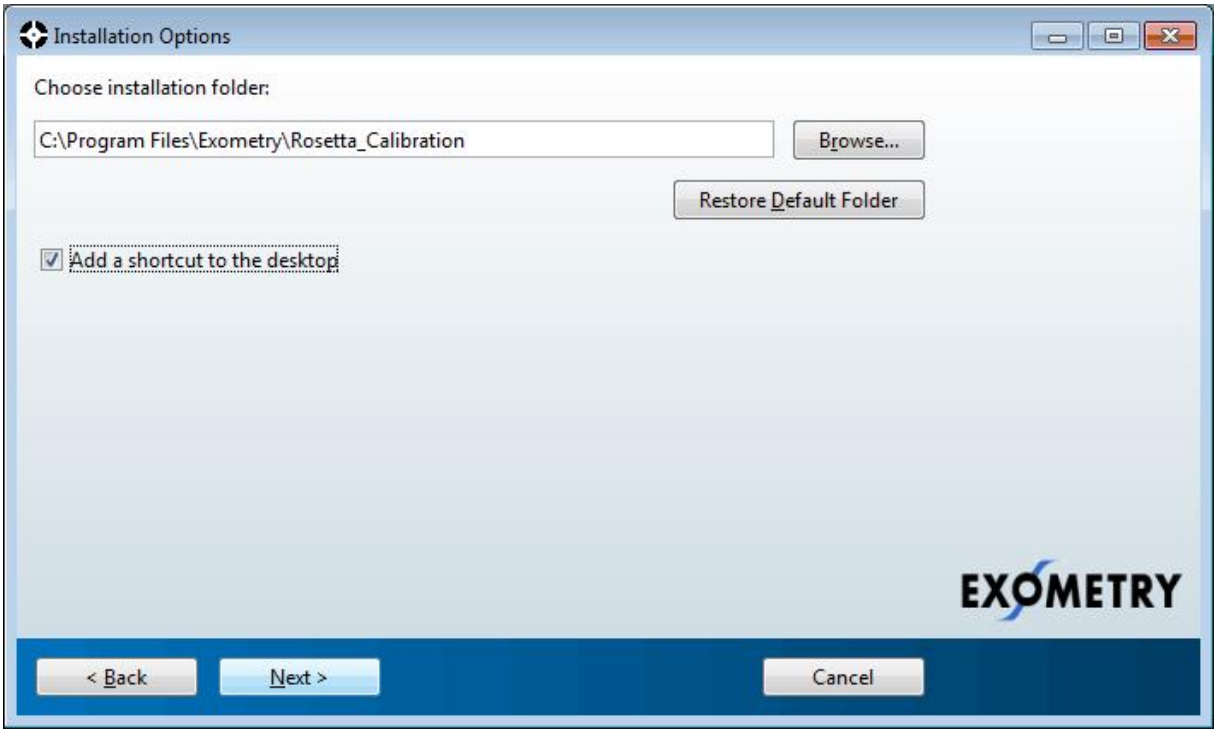
Installation

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

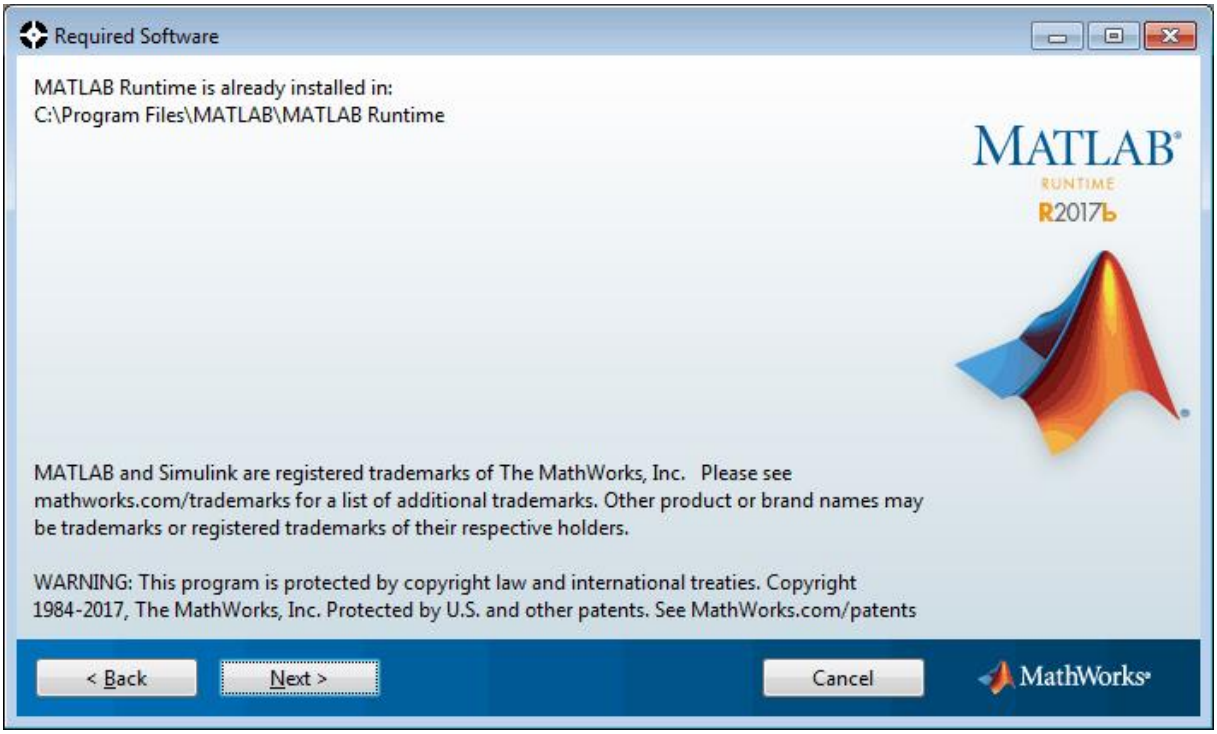
- Browse to exometry.com/downloads/
- Download the Rosetta Calibration software installer.
- Execute the downloaded .exe file as Administrator and follow the steps below.



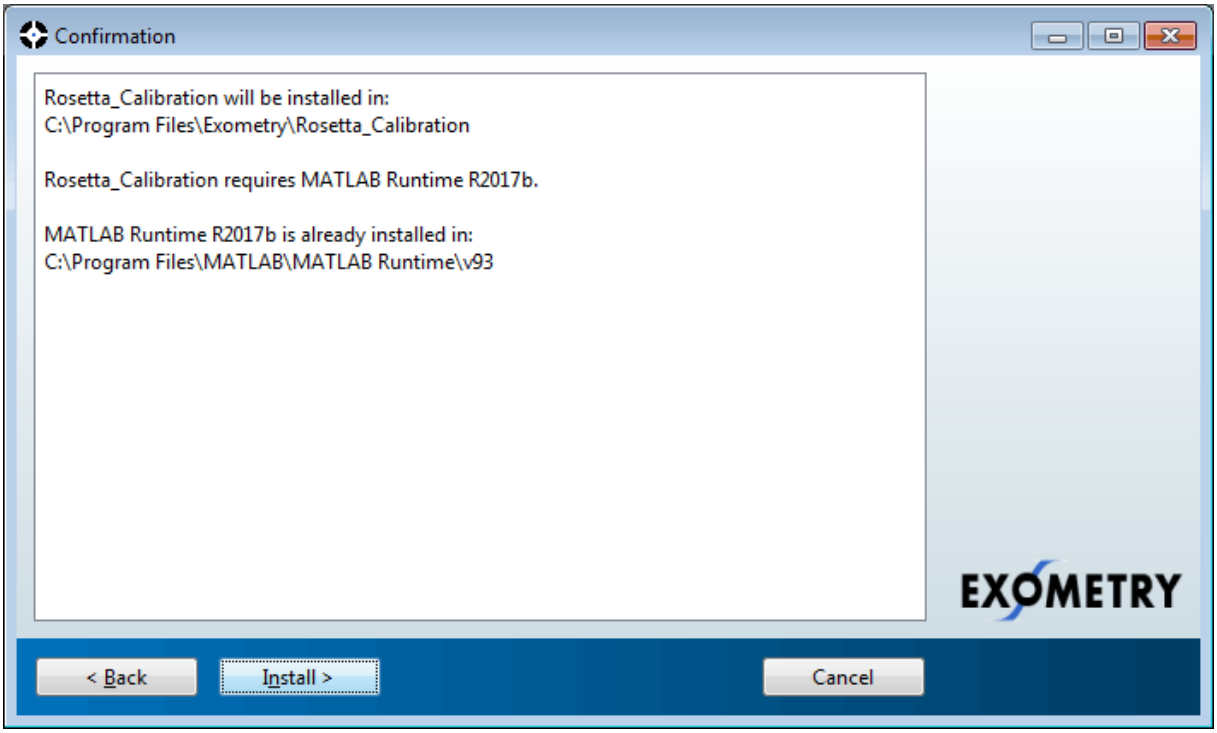
- Click “Next”.



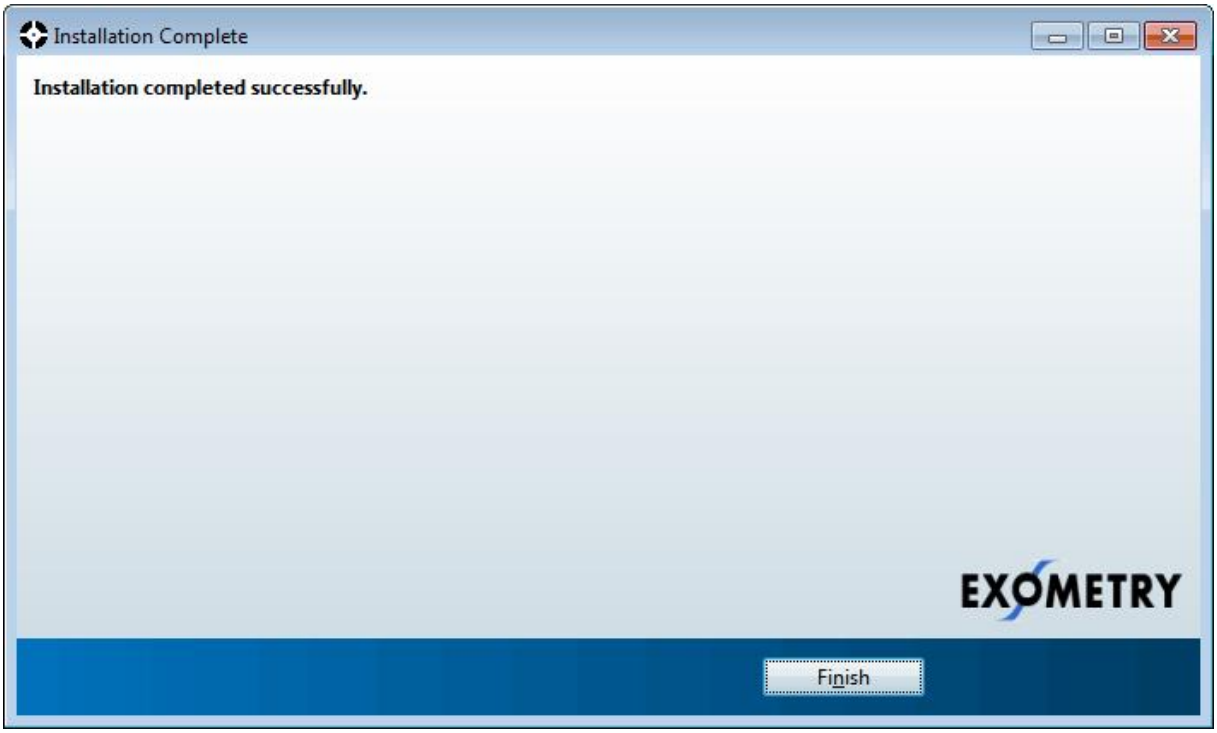
- Select “Add a shortcut to the desktop” and click “Next”.



- Click “Next”.



- Click “Install”.

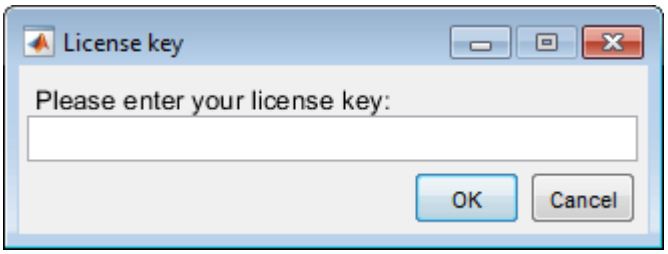


- Click “Finish”.
- The Rosetta Calibration software is installed.

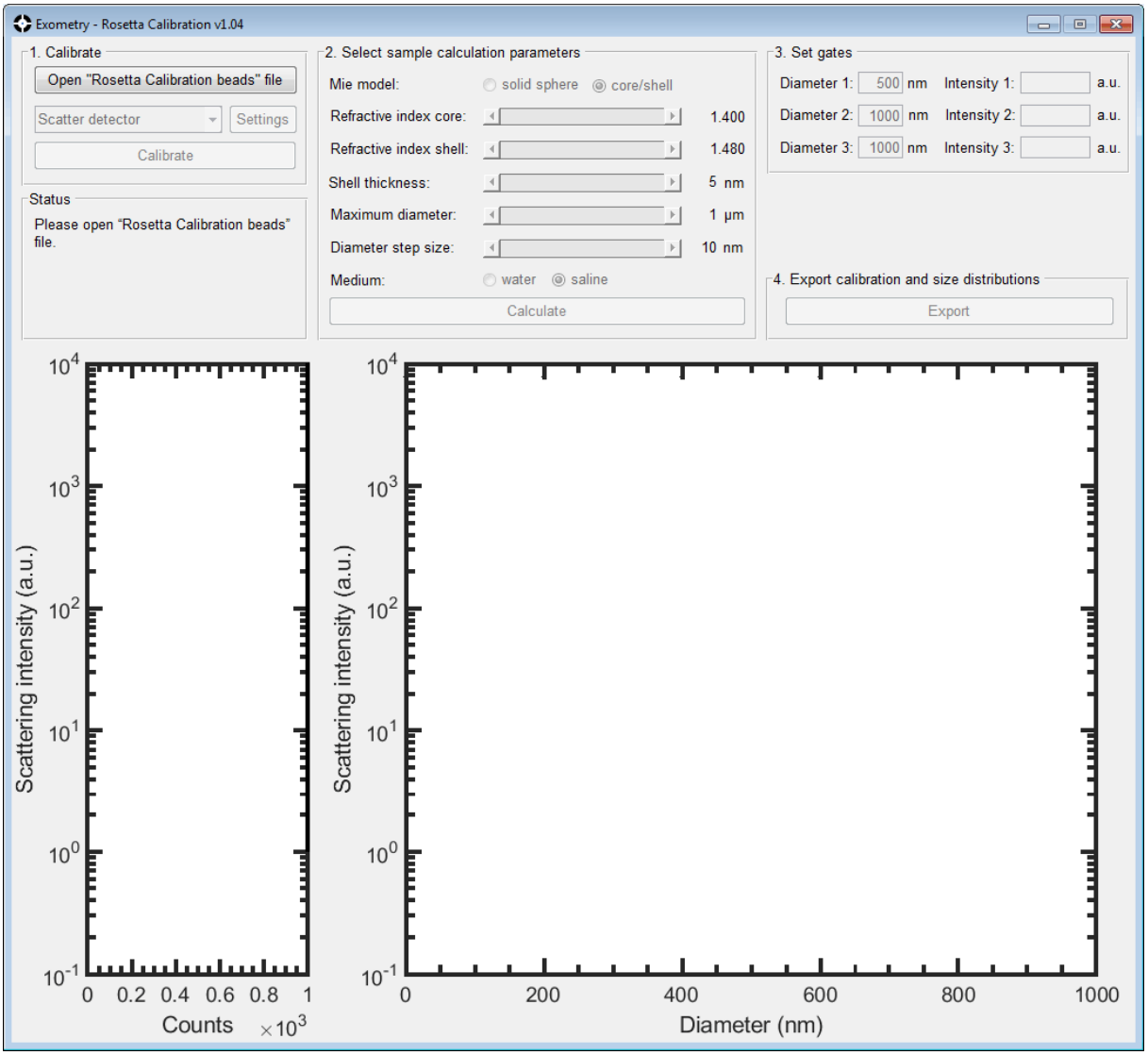
Instructions

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

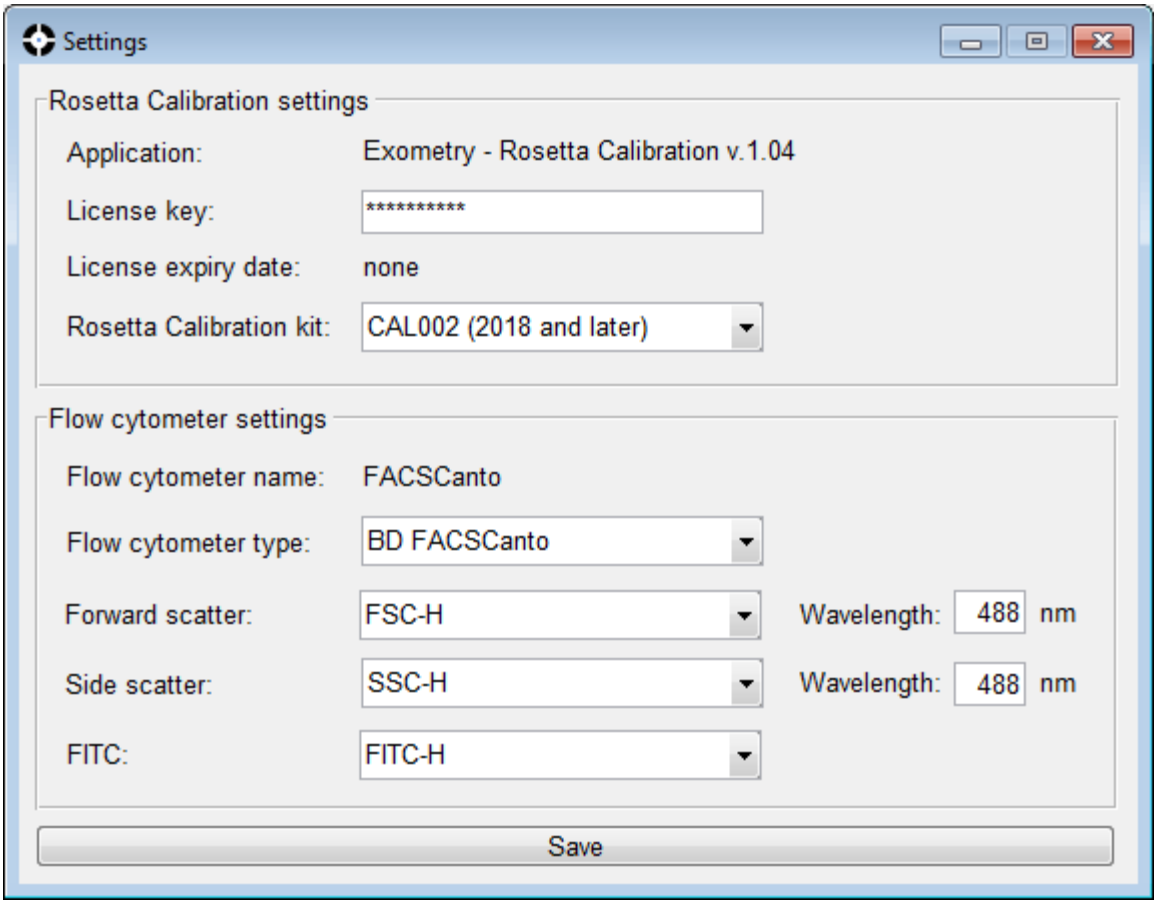
- Start the Rosetta Calibration software by clicking the icon on your desktop.



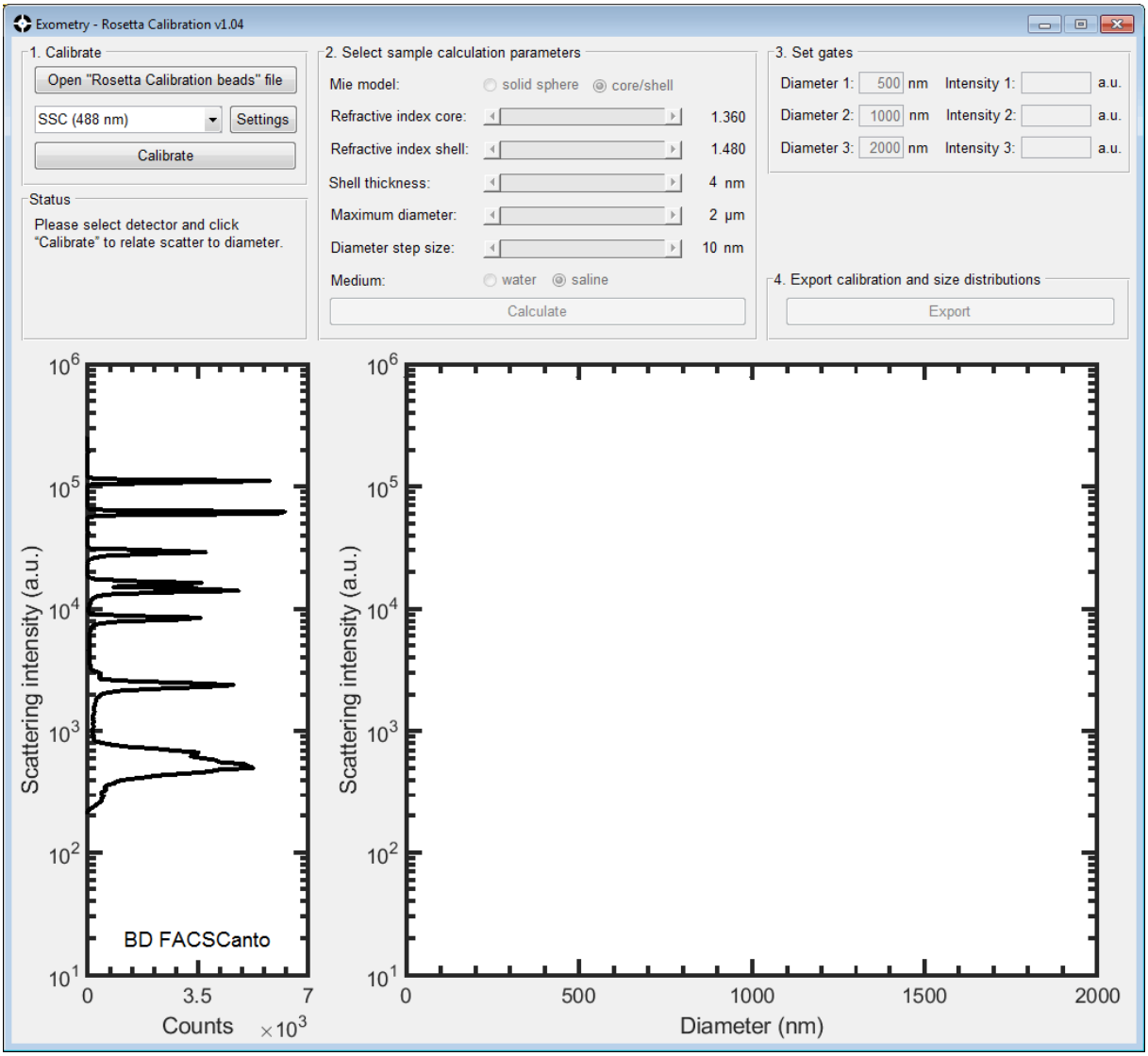
- The first time you start the Rosetta Calibration software, you will be asked for the license key. Enter your license key and click “Ok”.



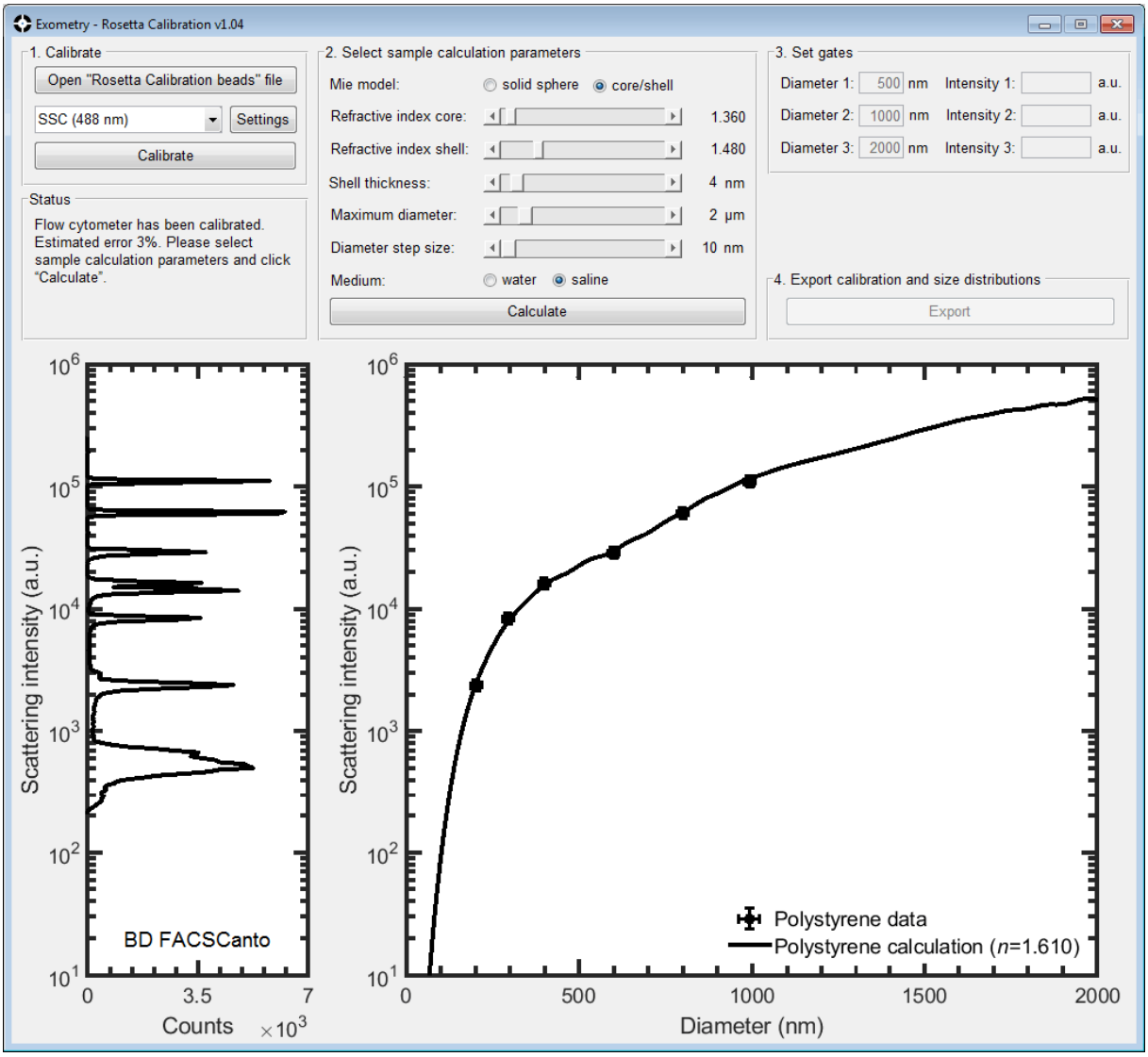
- Click “Open “Rosetta Calibration beads” file” to open the flow cytometry data file corresponding to the Rosetta Calibration beads measurement.



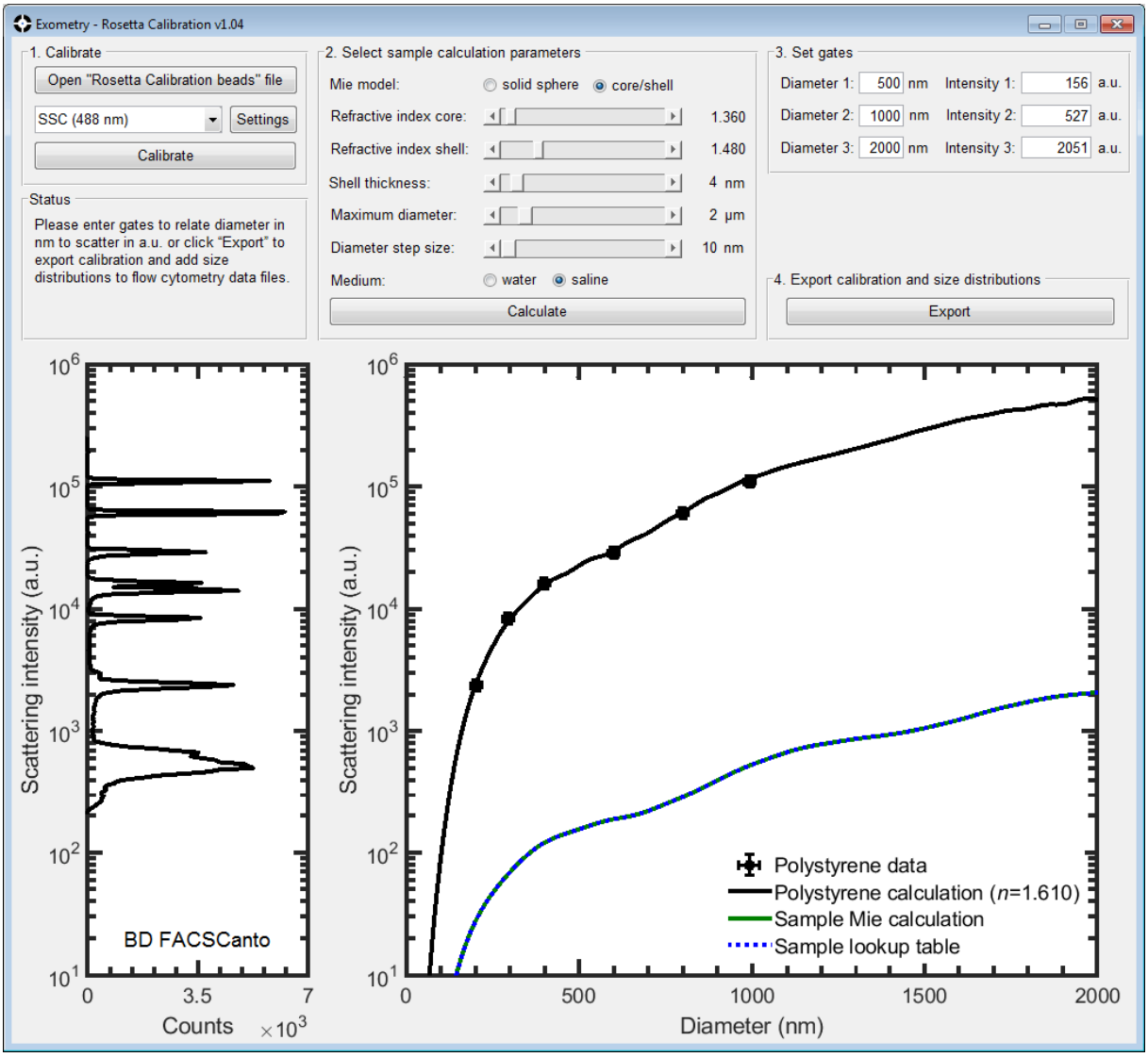
- The first time you calibrate your flow cytometer, a new window will be opened. The form contains specific settings for your flow cytometer.
- The settings for your flow cytometer will be automatically stored based on the name of the flow cytometer. Please make sure that your flow cytometry software defines a unique name for your flow cytometer(s).
- 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.
- Please verify the illumination wavelengths of the scatter detectors.
- Please select the FITC channel.
- 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[1,2,4,6,7].
- 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 set gates, you can provide three diameters in nanometers to lookup the corresponding scattering intensity in a.u. for the selected detector of your flow cytometer and your particles.
- 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 may extend the calculation range and 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, M. J. C.; Sturk, A.; Nieuwland, R.; Van Leeuwen, T. G.; Nieuwl, R.; Van Leeuwen, T. G. Single vs. Swarm Detection of Microparticles and Exosomes by Flow Cytometry. *J. Thromb. Haemost.* **2012**, *10* (5), 919–930.

(2) van der Pol, E.; de Rond, L.; Coumans, F. A. W.; Gool, E. L.; Böing, A. N.; Sturk, A.; Nieuwland, R.; van Leeuwen, T. G. Absolute Sizing and Label-Free Identification of Extracellular Vesicles by Flow Cytometry. *Nanomedicine Nanotechnology, Biol. Med.* **2018**, *14* (3).

(3) van der Pol, E.; Coumans, F. A. W.; Grootemaat, A. E.; Gardiner, C.; Sargent, I. L.; Harrison, P.; Sturk, A.; van Leeuwen, T. G.; 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* (7).

(4) van der Pol, E.; ISTH-SSC-VB Working group; Hau, C.; Sturk, A.; van Leeuwen, T. G.; Nieuwland, R.; Coumans, F. A. W. Standardization of Extracellular Vesicle Measurements by Flow Cytometry through Vesicle Diameter Approximation. *J. Thromb. Haemost.* (submitted).

(5) Shapiro, H. M.; Leif, R. C. *Practical Flow Cytometry*, 4th ed.; John Wiley & Sons, Inc.: NJ, 2003.

(6) Gardiner, C.; Shaw, M.; Hole, P.; Smith, J.; Tannetta, D.; Redman, C. W.; Sargent, I. L. Measurement of Refractive Index by Nanoparticle Tracking Analysis Reveals Heterogeneity in Extracellular Vesicles. *J. Extracell. vesicles* **2014**, *3* (August 2014), 25361.

(7) van der Pol, E.; Coumans, F. A. W.; Sturk, A.; Nieuwland, R.; Van Leeuwen, T. G. Refractive Index Determination of Nanoparticles in Suspension Using Nanoparticle Tracking Analysis. *Nano Lett.* **2014**, *14*, 6195–6201.

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