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Life Sciences

Optimizing EV Analysis with a CytoFLEX nano flow cytometer and FCM$_{PASS}$™

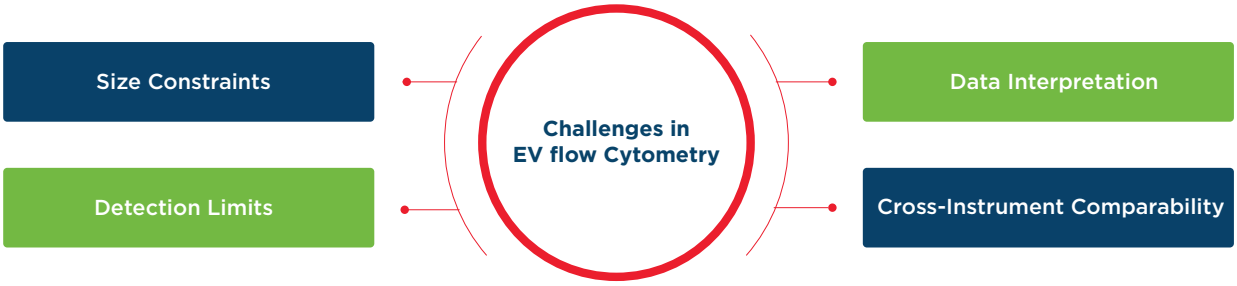
Flow cytometry is a key method for analyzing small particles like extracellular vesicles (EVs). With more than 5,000 EV-related articles published annually, EVs show significant promise in clinical and biochemical research. However, EV studies are challenging and often inconsistent due to detection limits, lack of robust methods, and unclear data interpretation. Converting scatter parameters from arbitrary to standardized units can help compare results across cytometers. To address this, we present a solution: CytoFLEX nano flow cytometer paired with FCM$_{PASS}$™ software, designed for accurately detecting and analyzing small particles.

The CytoFLEX nano flow cytometer features a Violet Side Scatter (VSSC1) detector, detecting particles as small as 40 nm polystyrene NIST (National Institute of Standards and Technology) reference particles, and up to 1000 nm using the VSSC2 channel. For cross-platform comparisons, we calibrate scatter data to nanometers using Mie-modeling software, following Minimum Information about a Flow Cytometry Experiment - Extracellular Vesicles (MIFlowCyt-EV) and Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines. FCM$_{PASS}$ software facilitates fluorescence and light scatter calibration, optimization, and reporting of nanoparticle flow cytometry data to the highest standards.

Challenges in EV Analysis Using Flow Cytometry

EVs and other sub-micron particles are small and heterogeneous, making them difficult to measure accurately. Understanding the biogenesis, fate and diversity of EVs is crucial for human health. Therefore, we need sensitive and reliable methods to analyze individual EVs. Flow cytometry, which can count single particles and measure their components precisely, is promising for analyzing individual EVs. However, conventional flow cytometers and their assays, which are designed for larger cells, have several limitations for counting EVs, such as:

- Due to the small size (30 to 1,000 nm, mostly 100-150 nm), EVs are hard to measure. These particles often hit the detection limits, making it difficult to distinguish them from background noise and other particles.
- The data is specific to that instrument's design and reported in arbitrary values, making it impossible to accurately compare it across different flow cytometers.

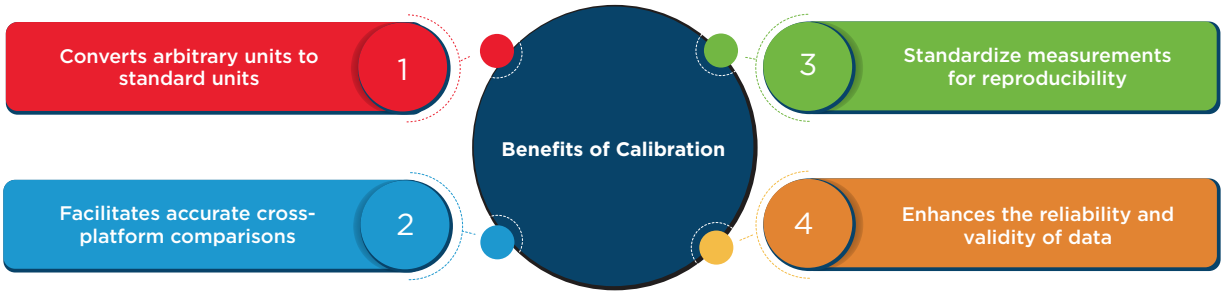


Therefore, EV measurement by flow cytometry requires careful tuning of the equipment and detailed measurements. This ensures consistent results over time and allows for comparison between different instruments. To address these challenges and support data comparison across experiments, the MIFlowCyt-EV guidelines were authored using a consensus process from authors representing three difference scientific societies. Following the MISEV and MIFlowCyt-EV guidelines, researchers report crucial details about sample staining, EV detection, measurement, and experimental design in EV flow cytometry studies. While the guidelines offer a framework for reporting flow cytometer settings and readouts in standard units, they do not provide detailed steps for achieving this standardization, especially for data calibration.

Note: For more detailed information MIFlowCyt-EV guidelines, please refer to the references at the end of this application note.

Calibration in EV Flow Cytometry: Ensuring Consistent and Comparable Data

Calibration converts arbitrary units into standard units to ensure consistent and comparable data across different flow cytometers. Due to varying sensitivities and dynamic ranges, instruments might detect small particles like EVs differently. Fluorescence calibration involves plotting known bead fluorescence against their arbitrary unit intensities and drawing a regression line. The slope and intercept of this line convert arbitrary values to a calibrated scale, like molecules of equivalent soluble fluorophore (MESF), antibody bound per cell (ABC), or equivalent reference fluorophore (ERF).



Calibration for light scatter signals is complex due to factors like particle size, composition, refractive index, and the instrument's scatter detection angle and optimization. Direct comparison to measurements from polystyrene beads alone is insufficient for calibration. Instead, Mie-modeling software is needed to accurately standardize scattering channels. This ensures reproducibility and accurate cross-platform comparisons of measurements, regardless of the instrument's optical setup.

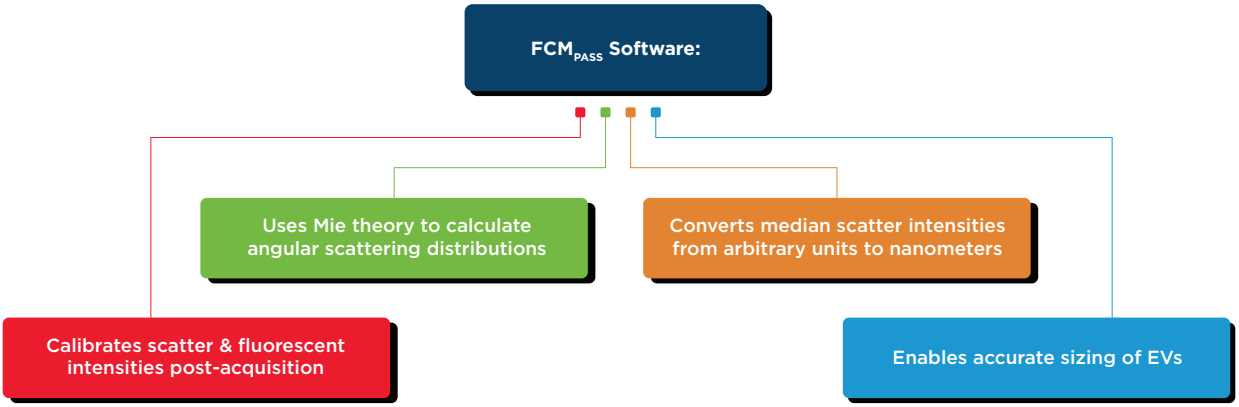
CytoFLEX nano Flow Cytometer: Advanced Detection and Calibration for Small Particles

The CytoFLEX nano flow cytometer, the latest in the CytoFLEX family, was specifically designed for high-sensitivity detection of small particles like EVs and nanoparticles. It features advanced light scatter detection capabilities, using two VSSC channels that preserve a wide dynamic range of detection. Additionally, it includes three additional side scatter channels for blue, yellow, and red lasers, enabling detailed analysis of particles as small as 40 nm based on polystyrene refractive index (RI). With up to six fluorescent channels available, the CytoFLEX nano flow cytometer enables comprehensive characterization and significantly expands research possibilities for small particles.

We also facilitate the use of Mie-modeling software for users to calibrate scatter and fluorescent intensities using FCM_{PASS}. Based on Mie theory, FCM_{PASS} calculates the angular scattering of particles, solid spheres (e.g., polystyrene beads) and core-shell particles like EVs. This helps determine the effective scattering cross-section for flow cytometers. By understanding the relationship between particle size, refractive index, and light scattering, users can accurately report the size of sub-micron particles, standardize measurements, and identify particles without labels using the CytoFLEX nano flow cytometer. The software converts median scatter intensities in arbitrary units to size in nanometers.

This method is explained in the application note CytoFLEX nano: Calibrating Light Scatter Data to Diameter using FCM_{PASS} and is one among the several methods researchers use to estimate size from scatter intensities (MFI).

Note: Beckman Coulter does not endorse any specific method for converting flow cytometry data to size; this method is shown only as an example.



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Here is a summary of the steps from the white paper for calibrating CytoFLEX nano flow cytometer light scatter data to standard units using FCM_{PASS}:

1. Objective

The primary goal is to calibrate arbitrary units of VSSC data into standard units (nanometers) for particle analysis using the CytoFLEX nano flow cytometer and FCM_{PASS} software.

2. Key Assumptions for Light Scatter Calibration

- Light scatter calibration beads must have specified diameter, variance and refractive index described using a method other than light scatter.
- Accurate bead sizing and reasonable refractive indices are crucial; NIST-traceable size standards are recommended.
- Fluorescently hard-dyed beads are not recommended due to their approximate size and complex refractive indices.
- Accuracy depends on the number and diameter of bead populations used. At least five beads ranging from 80-600 nm should be used.

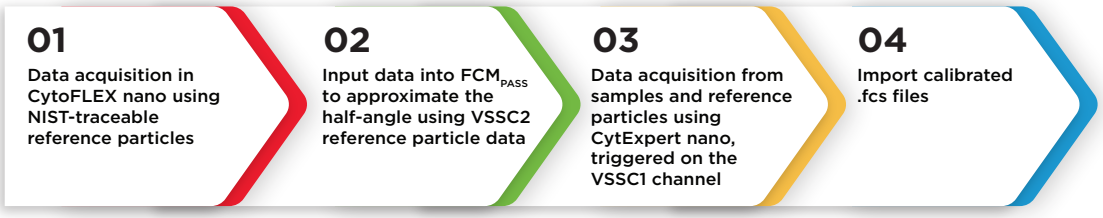
3. Considerations for VSSC1 and VSSC2 Channels in CytoFLEX nano flow cytometer

- VSSC1: The increased sensitivity in the 40 nm to 150 nm range (polystyrene RI) and limited dynamic range of the VSSC1 channel make its half-angle approximation imprecise, as particles with higher RI, such as polystyrene, saturate earlier than biological or silica particles with lower RI.
- VSSC2: The CytoFLEX nano flow cytometer light scattering detectors share the same half-angle. The VSSC2 channel, with a broader dynamic range of 80 nm to 1000 nm (polystyrene RI), can estimate the half-angle, which can then be used to calibrate the VSSC1 channel data effectively.

4. Materials Needed:

- CytoFLEX nano flow cytometer (Beckman Coulter PN: C80351)
- CytExpert nano software (Version 1.2.0.34 and later)

- FCM_{PASS}™ software (Version 5.0.7 and later)
- CytoFLEX nano QC Scatterspheres (Beckman Coulter PN: C85323)
- CytoFLEX nano QC Fluorospheres (Beckman Coulter PN: C85324)
- CytoFLEX nano Multi-intensity Fluorospheres (Beckman Coulter PN: C92889)
- Demo Kit with NIST-traceable Reference Particles (40 nm, 80 nm, 125 nm, 200 nm, 300 nm, and 450 nm)
- Ultrapure or HPLC-grade water (Thermo Fisher PN: W8-1)
- PBS (Cellarcus PN: CBS4B-100ml)
- 20 nm filter (Cytiva PN: 6809-2002)
- 12x75mm Borosilicate Glass Tubes (VWR PN: 47729-570)



5. Steps for Calibration:

- Data Acquisition: Use CytExpert nano software to acquire data from EV samples and at least four sizes of NIST-traceable PS reference particles of known size and RI ranging from 80 nm – 1000 nm, triggering on the VSSC1 channel.
- Half-angle Approximation: Approximate the side scatter detector half-angle using Mie-modeling with at least 4 sizes of NIST-traceable polystyrene reference particles. This step is crucial for accurate calibration.
- Calibration with FCM_{PASS} Software: Use FCM_{PASS} to calibrate VSSC1 data using the half-angle approximated from the VSSC2 channel.
- Import Calibrated Data: Import the calibrated CytoFLEX nano .fcs files back into CytExpert nano software for further analysis.

6. Important Considerations:

- User must know the RI of the particle being interrogated.
- FCM_{PASS} software allows for manual input of core-shell and solid sphere RIs.
- Ensure the fit confidence of the half-angle is above 90%.
- Validate the calibration by checking bead sizes against expected values.

Conclusion:

Standardizing CytoFLEX nano flow cytometer data into standard units enhances the value and comparability of small particle research data. This process aligns with guidelines provided by MISEV and MIFlowCyt-EV to ensure repeatability, verifiability and traceability in scientific research involving EVs and other submicron particles.

References:

1. Edwin van der Pol, Joshua A. Welsh, Rienk Nieuwland. Minimum information to report about a flow cytometry experiment on extracellular vesicles: Communication from the ISTH SSC subcommittee on vascular biology. J Thromb Haemost. 2022 Jan;20(1):245-251.

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3. Joshua A Welsh, Ger J A Arkesteijn, Michel Bremer, et al. A compendium of single extracellular vesicle flow cytometry. J Extracell Vesicles. 2023 Feb;12(2):e12299.

4. Joshua A Welsh, Edwin Van Der Pol, Ger J A Arkesteijn et al. MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments. J Extracell Vesicles. 2020 Feb 3;9(1):1713526.