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A Methodological Approach for Characterization of Extracellular Vesicles: Small-Particle Flow Cytometry

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Flow cytometry is an advantageous tool for the analysis and characterization of extracellular vesicles (EVs) because of its robust statistical power and its multiparametric capabilities. The goal of nanoscale flow cytometry analysis and nanoscale sorting, termed Small Particle Flow Cytometry (SPFC), is to accurately represent the size distribution and scatter profiles of these vesicles. As with conventional flow cytometry, the suspended vesicles pass through a chamber and are hydrodynamically focused. As vesicles pass through, the laser light is refracted and scattered in all directions. Their scatter properties are measured by detectors; concurrently fluorescent dyes can be used to tag various properties of interest.

Characterization of extracellular vesicles (EVs) is greatly impeded by several factors: their size (below 100nm), their overlapping size distribution, particle polydispersity, and an overall low refractive index (Orzoco, Van Der Pol). Because conventional flow cytometers are not equipped with necessary optical capacities to reach this lower threshold, instruments that are used for SPFC typically have specific adaptations. In light of these enhancements, there are a number of modifications to the conventional form of analysis that should be considered.

Here we describe a comprehensive methodology for the set up and standardization of EV analysis using SPFC. Controls of different size ranges, fluorescent intensities, and materials can be used to set up distribution curves that are then used for instrument optimization and as a reference guide. Using these controls, FACS instruments can be primed for the detection, analysis and sorting of specific EV populations. This allows for cross platform comparison and the ability to monitor both Quality Control (QC) and Quality Assurance (QA). The method here will describe the use of nanoparticles to optimize a flow cytometer for small particle detection. It will also outline the procedures necessary to recover EVs for downstream applications.

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Limitations of Microvesicle Analysis with Light-Scattering Flow Cytometry

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Background: Light-scattering is the most widely used method for microvesicle's (MVs) analysis, in particular, in flow cytometry (FC). Still, complete understanding and interpretation of these measurements is lacking. The key open questions are: (1) how sensitive are standard measurements of scattering in forward (FS) and side directions (SS) for MV detection? (2) Which MV's characteristics can potentially be estimated from these signals? (3) What is the reliability of these estimates (especially, of FS-based sizing) given uncertainties in MV's refractive index (RI) and shape (possibility of their aggregation) and commonly incomplete knowledge of the instrument optical configuration: (4) What are the options, when FS and SS fail, i.e. do not provide sufficient information?

Methods: Light-scattering measurements from individual particles (polystyrene microspheres and MVs in platelet-rich plasma) were performed using a Scanning Flow Cytometer (SFC) fabricated by CytoNova Ltd (Novosibirsk, Russia, http://cyto.kinetics.nsc.ru), which provides simultaneous measurement of angle-resolved light-scattering profiles (LSPs) and standard FS and SS signals. Light-scattering simulations for particles with spherical and non-spherical (aggregates of spheres) shapes were performed using the Mie theory and the discrete-dipole approximation, respectively. Transmission electron microscopy was used for control sizing of polystyrene microspheres.

Results: We developed a general method to estimate collection angular ranges for FS and SS signals, based on measurement of preliminary characterized microspheres and consequent non-linear fitting. Applied to SFC, this method allowed us to determine detection limits in terms of size and RI. In particular, FS is capable to detect single MVs in a more broad range than SS. Additionally, when both FS and SS signals are above noise level, we demonstrated that size and RI can be deducted. However, the the solution is unique only for some combinations of particle characteristics, even if particle sphericity is assumed, and this region complexly depends on a specific instrument. By contrast, LSP-based characterization is always unique and accounts for particle shape. Moreover, when combined with FS and SS measurements it results in excellent precision – typically tens nanometers for size and several thousandths for RI.

Conclusions: Accurate estimation of the angular range of collected light-scatter data is a necessary practice for MV analysis with any flow cytometer. However, even at its best, a combination of FS and SS signals leads to reliable determination of size and RI of single spheres only in a limited range of those characteristics. Enhanced MV analysis, including their accurate identification and characterization by shape, size and RI, is only possible with additional light-scattering signals, such as LSPs measured by a SFC.

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Identifying Exosome Binding and Internalization in Blood Cell Subsets by Imaging Flow Cytometry

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Only recently has the importance of extracellular vesicles as key mediators of intercellular communication been appreciated. Extracellular vesicles are membrane derived structures that include exosomes, microvesicles and apoptotic bodies. Exosomes have been shown to transfer molecules between cells and have the potential to transfer signals between cells. Exosomes are released under normal physiological conditions; however, they are also believed to serve as mediators in the pathogenesis of neurological, vascular, hematological and autoimmune diseases as well as cancer. Quantifying and characterizing exosomes in a reproducible and reliable manner has been difficult due to their small size (50 – 100 nm in diameter). Exosomes analysis can be done using high-magnification microscopy however this technique has a very low throughput. Attempts to analyze exosomes using traditional flow cytometers has been hampered by the limit of detection of such small particles and low refractive index. To overcome these limitations we have employed multispectral imaging flow cytometry that has the advantage of combining high throughput flow cytometry with higher sensitivity to small particles and the added benefit of imaging that can provide visual confirmation of particle integrity and characterization. In this study we use multispectral imaging flow cytometry to investigate the interaction of exosomes with white blood cells. Exosomes derived from different cell types will be investigated for their preferential interactions with blood cell subsets by combining immunophenotyping with morphological parameters to measure their binding and internalization.