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Improved Flow Cytometric Light Scatter Detection of Submicron-Sized Particles by Reduction of Optical Background Signals Ger Arkesteijn[1,2], Sten Libregts[2], Esther Nolte-t Hoen[2], Marca Wauben[2]

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Introduction: Boosted by the increased interest in extracellular vesicles (EV), flow cytometric measurement of sub-micron particles has gained particular attention during the last 5 years. However, due to the physical properties of sub-micron particles, light scatter signals are overlapping with background signals. This hampers light scatter-based EV measurements. Our goal in this study is to properly measure scatter from sub-micron particles by maximizing signal to background ratio (s/b). We focused our research on the specific reduction of optical background, while keeping the reduction of specific signal to a minimum. To pursue this goal we inserted pinholes of various sizes in the optical path of the FSC detector and tested them in conjunction with a series of differently sized blocker bars.

Methods: For all measurements we used an BD Influx jet in air flow cytometer that was optimized for the detection of submicron-sized particles. Based on fluorescence triggering, we measured 100 nm and 200 nm fluorescent polystyrene (PS) beads and defined and gated the position of the bead-clusters in the FSC-SSC dot plots. Next, we measured the same beads while sequentially placing pinholes with sizes of 30 μm, 50 μm, 100 μm, 200 μm, 400 μm, and 700 μm in the FSC light path. The relationship between pinhole size and blocker bar size was subsequently tested by placing blocker bars with sizes of 2.5 mm, 3.5 mm, 5 mm, 8 mm, and 10 mm. Under all conditions, we adjusted the PMT gain to match the exact position of the beads with the predefined gates of the standard configuration. This provided us with a visual tool to observe the increase or decrease of the s/b in the resulting dot plots.

Results: We observed a clear relation between pinhole size and s/b with an optimum at 200 μm and 100 μm. In a range of large to small pinholes, we experienced an increasing degree of difficulty in aligning the machine and keeping the measuring spot in focus of the pinhole, especially with pinholes of 100 μm and smaller. When we next tested the different blocker bar sizes in combination with the different pinholes we found that the use of a 200 μm sized pinhole in combination with a 8 mm blocker bar resulted in the most optimal separation between scatter signal and background. Using this configuration, background signals in the FSC channel were strongly reduced, allowing us to use FSC triggering instead of fluorescence triggering to resolve 100 nm PS beads above background signals.

Conclusion: To enhance light scatter-based detection of submicron-sized particles, reduction of optical background signals can be accomplished by adjusting the optical path of the FSC detector with a smaller pinhole and larger blocker bar. This allows FSC triggered measurements of sub-micron particles with minimal amount of interfering optical background. Our findings apply to the BD Influx and we anticipate that they most likely apply to other types of cytometers as well, although optimal sizes of the blocker\bar and pinhole will probably differ per instrument type.

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New Strategies for the Detection of Leukocyte-Derived Microvesicles by Fluo-Sensitive Flow Cytometry

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Background: Among extracellular vesicles, leukocyte-derived microvesicles (LMVs) have emerged as a potential biomarker in patients with atherosclerosis, diabetes, or sepsis. However, their measurement by current flow cytometry remains a challenge because of their scarcity, small size, and low antigen density. Only few specific markers have been identified so far to detect LMVs in human plasma samples. The goal of our study was therefore to evaluate new strategies based on a broad panel of antigen specificities and size exclusion chromatography (SEC) to reduce the FCM background noise for the detection of LMVs using a fluo-sensitive flow cytometer (CytoFLEX, Beckman Coulter).

Methods: To that aim 64 myeloïd specificities were tested on purified MVs generated from human circulating monocytes and neutrophils on resting or stimulated conditions. Titration curves were determined on MVs for all antibodies. Selected antibodies for LMV detection were evaluated both in saline buffer and plasmatic matrix with serial dilutions in spiking experiments. SEC was evaluated as a new strategy to remove the excess of unlabeled antibodies. Performance of CytoFLEX and Gallios (Beckman Coulter) to measure LMVs were compared using the Megamix standardization approach (BioCytex).

Results: As a result, 11 and 7 antibodies detected at least 20% of neutrophil- or monocyte-derived MVs, respectively. Some specificities such as CD15, CD11b and CD45 were previously reported to measure LMVs by flow cytometry but most of them were unknown specificities. Interestingly, performances can be improved combining several antibodies, allowing to detect up to 90% of neutrophil-MVs and 70% of monocyte-MVs. Detection of LMVs was not impeded by plasmatic environment as shown by spiking experiments in MV-free plasma. LMV measurement was linear down to 20 MVs/μl. Importantly, washing strategy by SEC dramatically decreases the fluorescent background noise and significantly increases LMVs detection by 90% +/- 120% and 170% +/- 160% for neutrophil- and monocyte-MVs respectively compared to classical no wash condition. Impact of SEC depends on the concentration of the tested antibody and its primary performance. Finally, a significant increase in LMVs counts was found when LMV detection was compared between current instrument (Gallios) and the new fluo-sensitive flow cytometer.

Conclusion: Altogether, combining new specificities to detect LMVs, use of SEC to reduce the background noise and new fluo-sensitive instrument significantly improves the measurement of LMVs by flow cytometry. These methodological progresses represent key steps toward the use of extracellular vesicles as relevant biomarker in clinical practice.

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Detection of Lipoprotein Particles among Blood Microvesicles by Scanning Flow Cytometry

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Introduction: Lipoproteins constitute a significant part of submicron particles in human blood. The largest lipoproteins are

chylomicrons (CMs) with size ranging from 100 nm to 1 micron. Blood microvesicles (MVs) have approximately the same size range, but can be distinguished from chylomicrons structurally. In contrast to MVs, CMs have at least two distinct features, which can be observed by electron microscopy: They are denser and have no lipid bilayer. Unfortunately, flow cytometry is not able to directly detect these differences. However, estimates of size and especially refractive index (RI) of submicron particles in plasma, which can be derived from measured angle-resolved light-scattering profiles through the solution of the inverse light-scattering problem, may serve to distinguish these two classes of particles, CMs and MVs. We demonstrate the performance of scanning flow cytometry in such measurements and its ability to identify and characterize lipoproteins in blood plasma.

Method: We studied particles in fresh platelet-rich plasma (PRP) obtained by precipitation, thus minimizing any artifacts of sample manipulations. PRP samples were analyzed with a scanning flow cytometer (SFC), which measures angle-resolved light-scattering profiles (LSPs) of individual particles in a flow. We processed the measured LSPs using the previously developed method to identify spherical submicron particles in PRP, and to characterize them both by size and RI. For direct observation of changes in CM fraction we analyzed several samples, including ones obtained after more than 12 h fasting and at different time periods after a meal. Production of MVs was stimulated by platelet activation with ADP and collagen.

Result: Compared to the fasting sample postprandial PRP demonstrates a much larger amount of particles with RI values close to that of lipids, confirming that the majority of detected spherical submicron particles are CMs. By contrast, the activation of platelets does not lead to a substantial increase of the detected events probably due to the fact than majority of MVs remain below the SFC detection limit.

Conclusion: The majority of submicron particles detected by the SFC in platelet-rich plasma represent lipoproteins, whereas MVs are still hard to detect and characterize. The latter is due to the low RI of MVs resulting in low signal-to-noise ratio of angle-resolved light-scattering signals.

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Resolving the Heterogeneity of Extracellular Vesicles by Flow Cytometry and Imaging Flow Cytometry

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Extracellular vesicles (EVs) can be harvested from cell culture supernatants and from all body fluids. They can be roughly classified based on their size and origin as exosomes (70–150 nm) which are released when multivesicular bodies fuse with the plasma membrane and microvesicles (100 nm to 1 μm), which are formed by the outward budding of the plasma membrane. In addition to these different EV subtypes, it is nowadays commonly accepted in the field that there is a much higher degree of EV heterogeneity within these two subgroups. The content, the protein composition and the surface signature of EVs vary and are likely to be dependent on the cell type source, the cell's activation status and multiple other parameters. Until today, no specific markers to discriminate

even exosomes from microvesicles have been identified, and only few EV surface markers have been related to specific cell sources. In general, the question of heterogeneity in EV samples is rarely addressed at the experimental level, mainly due to the lack of qualified methods to analyze multiple parameters of single EVs. However, the identification of specific vesicular surface markers will be of high relevance to further understand the molecular content and related functions of subsets of EVs.

In the last few years, we and others developed different multi-parameter methods for flow-cytometric analysis of EVs, including bead-capturing methods. Of note, we recently optimized an imaging flow cytometry-based method and demonstrated its use to analyze multiple parameters on single exosomes in heterogeneous samples. Now, we have started to apply those flow cytometric approaches to analyze EVs derived from various sources, including cell lines of mesenchymal, epithelial, endothelial, and hematopoietic origin. First, we are applying a multiplex bead-based method to screen for new EV surface markers. In a second step, we will validate newly identified markers at the single vesicle level by using imaging flow cytometry. Here, we present preliminary results obtained, and with this study we expect to further unravel heterogeneity of EVs and identify new and cell source specific EV surface signatures.

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The Cell Atlas

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Compartmentalization of biological processes is a fundamental principle of eukaryotic cells that enables multiple processes to occur in parallel. Resolving the spatial distribution of the human proteome at a subcellular level greatly increases our understanding of human biology and disease. A high-resolution map of the human cell has been generated, the Cell Atlas, part of the Human Protein Atlas database (www.proteinatlas.org). Using an antibody-based approach, the in situ localization of more than 12,000 human proteins at a single-cell level covering 32 subcellular structures has been determined, enabling 13 major organelle proteomes to be defined. The high spatial resolution allows identification of novel protein components of fine cellular structures such as the cytokinetic bridge, nuclear bodies, as well as rods and rings. An integrative approach to data generation includes strict validation criteria using gene silencing, paired antibodies, and fluorescently tagged proteins. The Cell Atlas reveals that approximately half of all proteins localize to multiple compartments and many proteins show variation at the single-cell level in terms of protein abundance or spatial distribution. Here we discuss the importance of spatial proteomics for cell biology, including the citizen science effort "Project Discovery," and present the content of the Cell Atlas, as well as the path ahead to define the spatiotemporal organization of the human proteome at a subcellular level.

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Super-Resolution Microscopy as a Tool to Study Dynamic Biological Events in the Endoplasmic Reticulum

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The endoplasmic reticulum (ER) is an expansive, membrane-enclosed organelle that plays crucial roles in numerous cellular