128

Label-Free Identification and Characterization of Cell-Derived Microparticles with Scanning Flow Cytometry

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Background: Cell-derived microparticles (MPs) released from cells during stress conditions, including activation and apoptosis, are present in peripheral blood and body fluids and constitute a heterogeneous population of particles highly variable in size, composition, concentration, cellular origin, and functional properties. MPs are involved in a variety of physiological processes and may serve as potentially useful diagnostic or prognostic biomarkers. Increasing scientific and clinical interest to MPS makes the reliable detection and analysis of single MPs, which is extremely complicated by their small sizes (0.1-1 μm) and low refractive indices, of great importance and motivates development of new techniques and methods. However, flow cytometry is still the most widely applied optical method to detect MPs in clinical samples, even though it is greatly hampered by lack of appropriate standards for MPs isolation, triggering and gating, and size estimation.

Methods: We developed a flow-cytometry based method for identification and characterization of cell-derived MPs from light scattering. Using scanning flow cytometer (SFC) we measure angle-resolved light-scattering profiles (LSPs) and side scattering (SSC), also used as triggering signal, of individual MPs in plasma. Since a homogeneous sphere is an adequate optical model of MPs, we separate MPs from other non-spherical plasma constituents, including platelets, by measured LSPs on the basis of their agreement with theoretical fits, using the Mie theory. Thus, solving the inverse scattering problem by fitting measured LSPs of individual MPs, we not only identify them from other particles but also characterize MPs by their size and refractive index, using additional light-scattering data from SSC for better accuracy.

Results: We prepared three different samples containing cell-derived MPs from a human blood using different methods for MP isolation and applied the developed characterization method. The major part of detected MPs fell in the range of 400-800 nm for size and 1.46-1.50 for the refractive index. The median precisions in determination of size and refractive index of single MPs were 7 nm and 0.0018, respectively.

Conclusions: We present a method to identify MPs in platelet-rich plasma among other plasma particles, unambiguously separate them from optical and electronic noise and multiple detection events, without MP staining and polystyrene-bead gating procedure, and to determine size and refractive index of individual MPs solely from light-scattering signals. The only limitation of this approach is size detection limit, which is currently about 400 nm, but could be potentially decreased down to 150 nm.

129

Nanoparticle Flow Cytometry of Individual Extracellular Vesicles?

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Background: Extracellular vesicles (EVs), membranous particles shed and/or secreted from cells, are thought to play a role in normal physiology and disease. EVs are generally 100-200 nm in diameter, and can be shed from the cell surface (ectosomes) and secreted from internal compartments (exosomes) of many different cell types. The combination of small size and heterogeneous origins make EVs extremely difficult to measure. We have developed a Nanoparticle Flow Cytometer and associated reagents and protocols to better detect these small, dim particles.

Methods: EVs were obtained from supernatants of rat aortic endothelial cells (RAEC) cultures or rat plasma. EVs were separated from cell debris and soluble protein by low (two cycles of 2.5K xg, 5 minutes) and high (20k x g, 4 hours) speed centrifugation, respectively, and resuspended in 0.1 um filtered dPBS. Purified EVs were stained with an optimized concentration (determined by titration) of the fluorogenic lipid probe, di-8ANEPPS (red fluorescence), and DyLight 488-labeled cell surface markers and analyzed using the NFC with 488 nm excitation (150 mW) triggered on red fluorescence. Intensity-calibrated reference beads were used for calibration of fluorescence measurements. Liposomes and EVs were also analyzed using nanoparticle tracking analysis, providing an independent estimate of nanoparticle size.

Results: Fluorescence triggered detection of the membrane-bound lipid probe enabled measurement of individual liposomes and EVs less than 80 nm in diameter. Dilution experiments show an expected decrease in detected events with no change in intensity, indicating these measurements are not compromised by coincident occurrence of multiple EVs in the measurement volume. We labeled annexin V and antibodies against cell surface markers with an F/P of 2-4 to maximize the brightness of these reagents, and were able to detect their binding to EVs with a limit of resolution of ~800 FITC MESF. We find that only a fraction of EVs from RAEC cultures are annexin V positive, and that neither the number of EVs nor the AnnV+ fraction changes significantly with 24 hour treatment with TNF α or IL-1β. By contrast, LPS treatment of rats did result in a significant increase in plasma EVs.

Conclusions: Fluorescence triggered detection of EVs stained with a fluorogenic lipid probe enable robust and quantitative enumeration and sizing of individual EVs. Serial dilution of the sample can be used to ensure that the pervasive artifact of coincidence is not affecting the data, and the surface area staining of the lipid probe gives results that are comparable to the diameter estimates provided by NTA. Brightness-optimized reagents can greatly improve the detection of low abundance surface markers. We are now working to implement additional fluorescence channels to allow more parameters to be measured on individual EVs.

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130

Small Particle Analysis by Flow Cytometry

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Single particle analysis by flow cytometry of cells and other particles larger than 1μm has become quite routine over the past forty years or so and is an essential tool in many fields of medicine and research. However, for the past few years there has been an increased interest in analyzing submicron particles by flow cytometry. For these applications, flow cytometers are being pushed to the limits of their sensitivity in order to detect not just small bacteria, but also microparticles, exosomes, liposomes and even viruses. Unfortunately, the instruments, techniques and procedures commonly used to analyze cells of several microns in size, do not always work as well for submicron particles and therefore the data obtained from the small particles may be incorrect or misleading.