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Immunophenotyping Extracellular Vesicles Using Amnis® Technology

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Introduction

Only recently has the importance of extracellular vesicles (EVs) as key mediators of intercellular communication been appreciated. EVs are membrane derived structures that include exosomes, microvesicles, and apoptotic bodies. In particular, 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 EVs in a reproducible and reliable manner is difficult due to their small size (exosomes range from 30 nm to 100 nm in diameter). Although EV analysis can be performed using high magnification microscopy, this technique has a very low throughput. Attempts to analyze EVs using traditional PMT-based flow cytometers has been hampered by the limit of detection of such small particles and their low refractive index.

To overcome these limitations, we employed the Amnis® CellStream® Flow Cytometer, which contains the Amnis® Time Delay Integration (TDI) image capturing system. This detection technology allows the CellStream® Instrument to combine the advantages of high throughput flow cytometry with high sensitivity to submicron particles.

In this study, the CellStream Flow Cytometer was used to immunophenotype EVs derived from red blood cells (RBCs) and platelets.

Methods

EVs were isolated from blood as follows: Erythrocytes (RBCs) and platelets were washed and treated with calcium ionophore (A23187) to induce vesiculation. The resulting vesicles were isolated via centrifugation.

Prepared EV samples were simultaneously incubated for 1 hour at room temperature with anti-CD235ab-PE (BioLegend) and anti-CD41-APC (BioLegend) to label RBC-derived EVs and platelet-derived EVs, respectively. Samples were then serially diluted (1:60, 1:120, 1:240, 1:480, and 1:960) in PBS buffer.

Data were acquired using the CellStream Flow Cytometer for 3 minutes per sample. The 488 nm and 642 nm lasers were run at 100% laser power and no thresholding was used. Samples were run in duplicate on 3 separate CellStream instruments. Data were analyzed with the CellStream system's integrated analysis software.

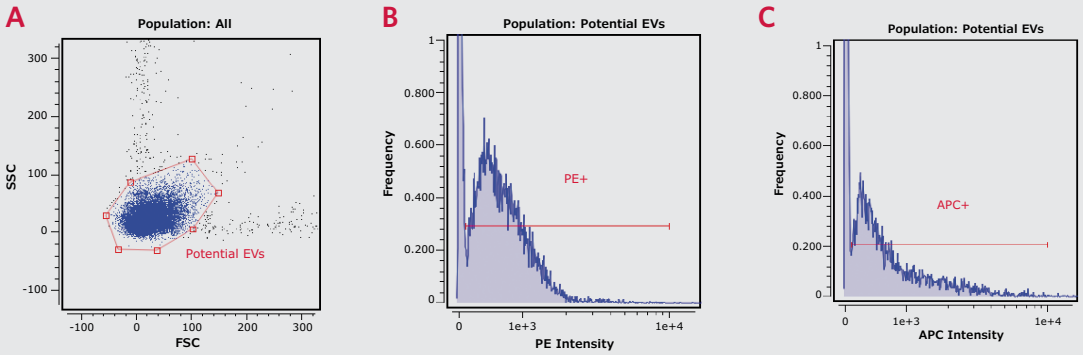
Control samples were collected for antibody only and buffer only; detergent controls were collected for the antibody-labeled EV samples and for antibody-only samples, which were incubated in 0.1% Triton® X-100 (TX) for 10 minutes. All controls were similarly diluted in PBS and run on the CellStream System in the same manner as the EV samples.

Results

Identification of Potential EVs

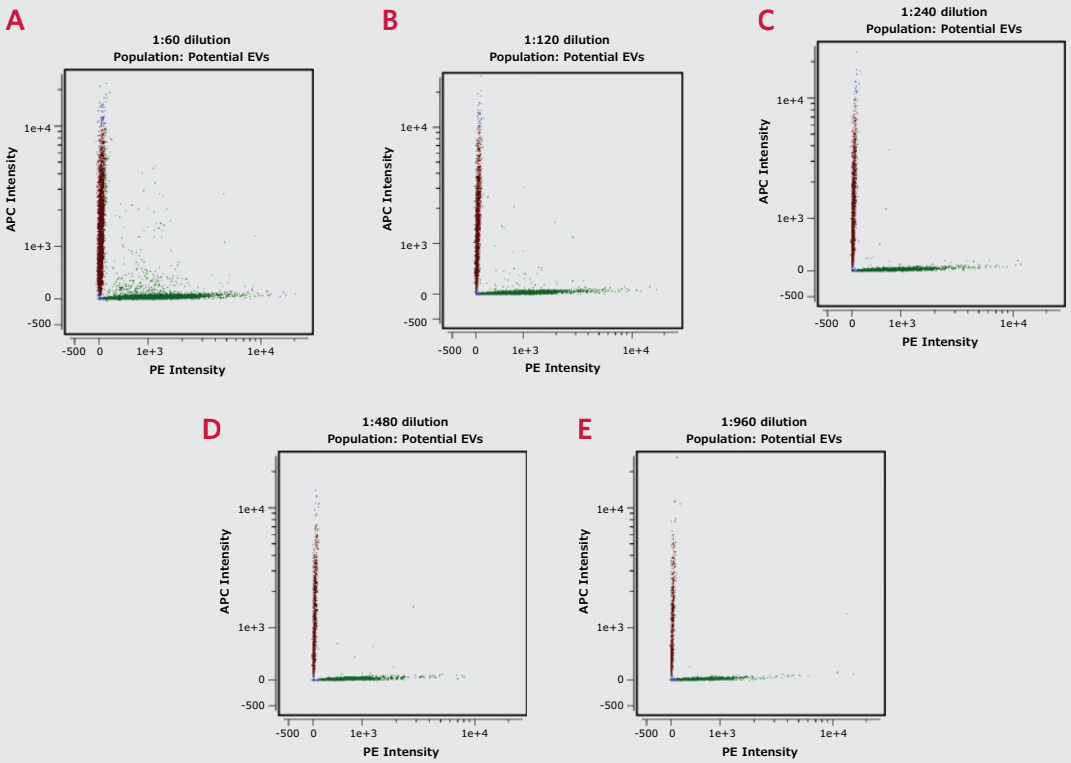
To identify potential EVs, a gate was set using an SSC vs. FSC plot (Figure 1A). Using this "Potential EVs" gated population, (B) PE-positive (PE+), and (C) APC-positive (APC+) events were gated. Objects in the PE+ gate were the EVs labeled with CD235ab-PE, and objects in the APC+ gate were EVs labeled with CD41-APC.

Figure 1.



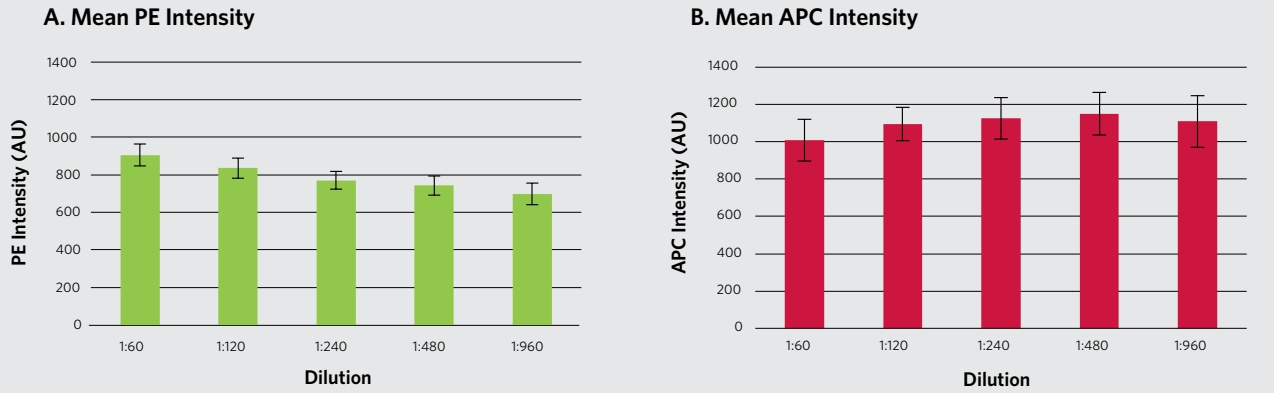
Bivariate dot plots for the dilution series of RBC-EVs and platelet-EVs labeled with CD235ab-PE and CD41-APC, respectively, are shown in Figure 2. PE+ events from Figure 1B are colored green, and APC+ events from Figure 1C are colored red.

Figure 2.



To verify detection of single EV particles and confirm swarm detection was not occurring, serial dilutions were performed. If single EV particles are being detected, the positive EV events will linearly decline while the fluorescence intensity of the positive events remains constant. Figure 3 illustrates the mean fluorescence intensities for PE (A) and APC (B) across each dilution series. The mean fluorescent intensities are from the PE+ or APC+ gates in Figure 1. There was no compensation of the data.

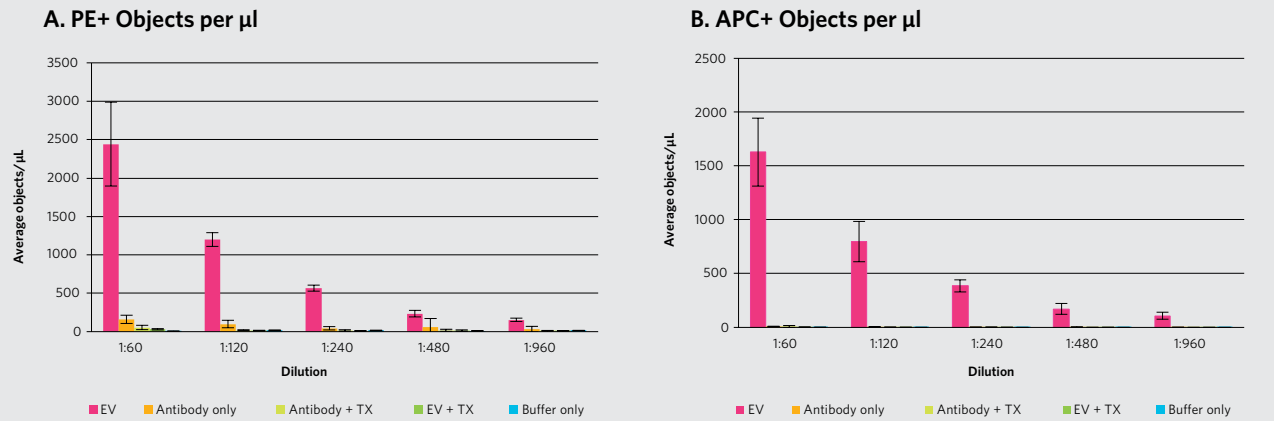
Figure 3.



Detection of EVs on the CellStream® Flow Cytometer

PE+ and APC+ objects per µl for the various experimental and control samples are shown in Figure 4 (A, B): labelled EVs, antibody only, antibody + Triton® X-100, labelled EVs + Triton X-100, and buffer only. The objects per µL are the events in the PE+ or APC+ gates shown in Figure 1. The tables below show the average objects per µl and standard deviations for the PE+ (C) and APC+ (D) events in Figures 4A and 4B, respectively.

Figure 4.



C. Average PE+ Objects/µl

Dilution	EVs		Antibody Only		Antibody + TX		EV + TX		Buffer Only	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
1:60	2442	546	160	54	55	28	30	8	5	4
1:120	1199	89	99	49	17	7	13	5	19	1
1:240	566	39	44	22	15	9	9	3	17	1
1:480	235	43	64	108	18	14	13	9	9	1
1:960	154	21	32	37	10	5	10	5	15	2

D. Average APC+ Objects/µl

Dilution	EVs		Antibody Only		Antibody + TX		EV + TX		Buffer Only	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
1:60	1629	316	9	2	9	9	3	0	1	1
1:120	798	187	4	1	2	1	1	1	0	0
1:240	386	56	2	1	2	1	1	0	0	0
1:480	172	50	2	3	1	1	1	1	0	0
1:960	109	33	1	1	0	0	1	0	0	0

Summary

In this study, EVs derived from RBCs and platelets were immunophenotyped on the CellStream Flow Cytometer. RBC- and platelet-derived EVs were labeled simultaneously with CD235ab-PE and CD41-APC antibodies. CD235ab is specific to RBC-derived EVs, and CD41 is specific to platelet-derived EVs. By performing simple gating on PE+ and APC+ events, we were able to separate out the RBC-derived EVs from the platelet-derived EVs (Figures 1 and 2). The average mean fluorescence intensity from 3 CellStream Flow Cytometers (Figure 3) and the objects per µL for all the EV and control samples (Figure 4) are shown. The high sensitivity of the CellStream Flow Cytometer makes it an excellent platform for measuring and immunophenotyping EVs.

Acknowledgements

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