Application Note

Cell Analysis

Agilent
Trusted Answers

Detection of Small Particles by Flow Cytometry

Analysis of small particle beads and extracellular vesicles

Authors

Lauren Jachimowicz,
Yan Lu, Peifang Ye, and
Garret Guenther
Agilent Technologies, Inc.

Abstract

This application note describes the ability of the Agilent NovoCyte Quanteon flow cytometer to resolve small particles, including cell-derived extracellular vesicles (EVs) and beads. The NovoCyte Quanteon, equipped with advanced detectors, provides high-sensitivity side scatter (SSC) and fluorescence detection to give superior results to those of traditional flow cytometers.

Introduction

The importance of cell-derived EVs has been recognized in multiple biological processes, and is a topic of increasing relevance in biomedical research. EVs are cell-derived membranous vesicles released from cells under normal and pathological conditions. This includes exosomes, microvesicles, and apoptotic bodies according to their cellular origin and size. Exosomes are 50 to 150 nm-sized particles of endocytic origin. Microvesicles bud from the cell surface and range in size from 50 nm to 1 µm, and apoptotic bodies are 0.8 to 5 µm vesicles released from cells undergoing programmed cell death. EVs are important for intracellular communication and have been found in blood, other biological fluids, and cultured cell supernatant.

EVs have been associated with a wide range of conditions including cancer metastasis, blood disorders, cardiovascular disease, infectious disease, and others. Their therapeutic potential as a cell free treatment for cancer and other pathologies has also been extensively investigated. Current EV detection methods use techniques such as Western blotting and ELISA to determine bulk protein concentration in purified particles, which prevents readout of individual samples. Inclusion of flow cytometric assays for small particle research would enhance current detection methods, with the capability to analyze indvidual particles in significant numbers, as well as measure surface protein levels. However, traditional flow cytometers have difficulty resolving EVs due to their small diameter and variable composition. Technological advancements in flow cytometry, such as the NovoCyte Quanteon, has increased the capacity to detect small particles, such as EVs. This investigation determines the ability of the NovoCyte Quanteon flow cytometer to resolve small particles, using beads and cell-derived EVs.

NovoCyte Quanteon can detect small particle beads with sensitive SSC detection

To determine the capability of the NovoCyte Quanteon to resolve small particles, we first performed analysis of three commercially available small particle bead mixes:

- Bang's laboratories NIST traceable size standards
- ApogeeMix beads
- BioCytex-Plus FSC and SSC hybrid microspheres (Figure 1)

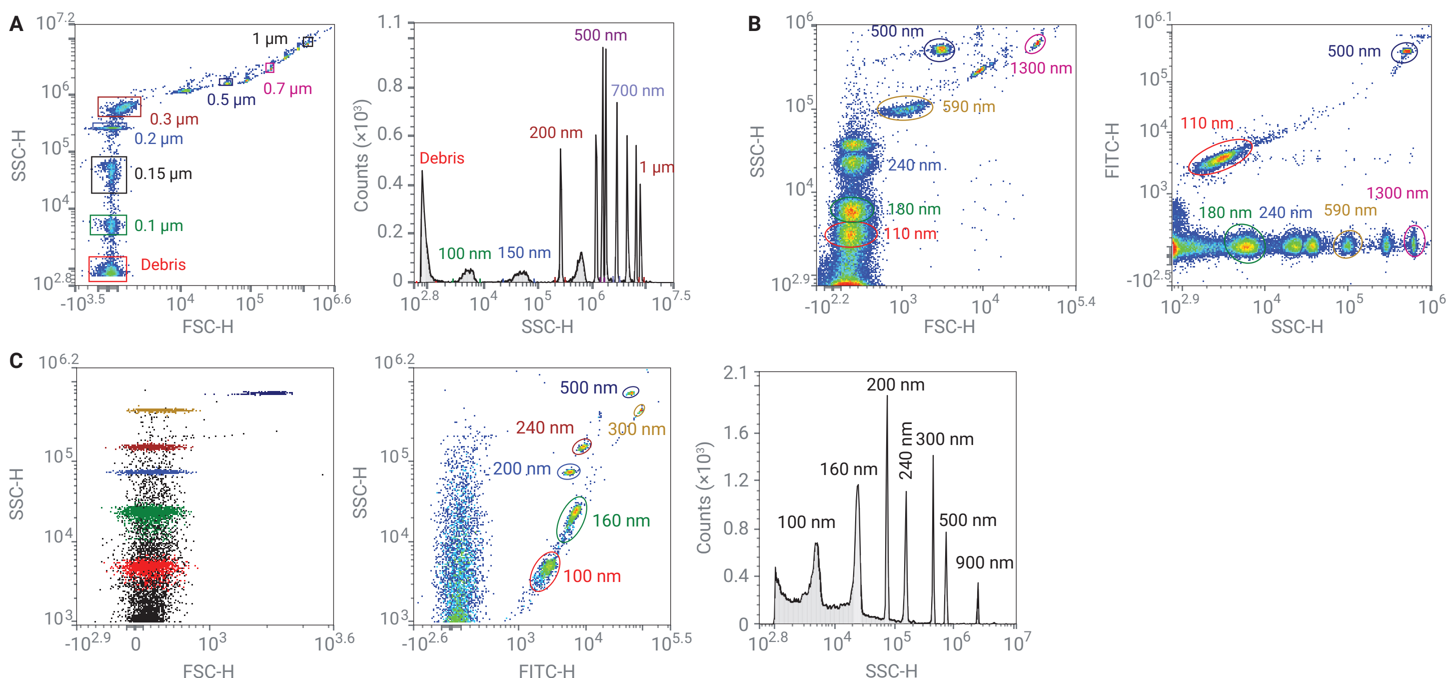
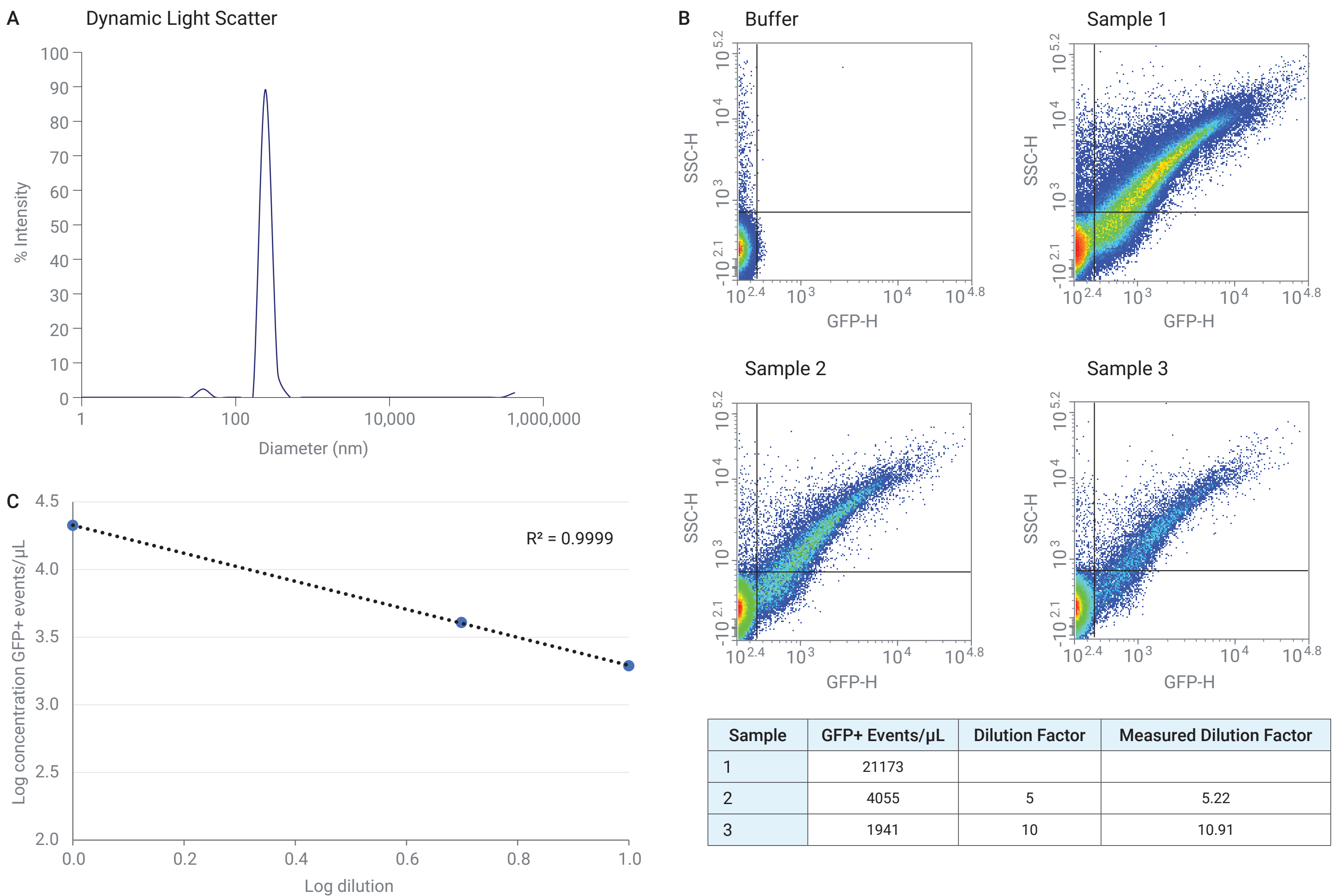


Figure 1. Small particle bead detection on the Agilent NovoCyte Quanteon. Three commercially available small particle beads were used to demonstrate the high sensitivity of the NovoCyte Quanteon side scatter detection (SSC): Bang's Laboratories NIST traceable size standards (A), ApogeeMix beads (B), and BioCytex Megamix-Plus (C). Particles as small as 100 nm were easily resolved from the background. Instrument settings: SSC-H gain 1,000, FSC-H gain 1,000, threshold SSC-H greater than 1,000.

These each consist of beads ranging in size from 100 to 1,300 nm. Equipped with an avalanche photodiode for side scatter detection (SSC) instead of the traditional photodiode detector, the NovoCyte Quanteon was able to resolve beads as small as 100 nm using SSC alone.

Sensitive resolution of small biological particles by flow cytometry

The refractive index (RI) of membrane vesicles is different than particle beads, therefore testing the capability of the NovoCyte Quanteon to detect more biologically relevant particles was important. We analyzed purified lipoparticles derived from HEK293T GFP-expressing cells, the release of which is induced by Murine leukemia virus (MLV). These lipoparticles are approximately 240 nm determined by dynamic light scattering (Figure 2A). Lipoparticles were separated from background (Figure 2B) by SSC as well as GFP fluorescence. The NovoCyte Quanteon features a syringe pump enabling aspiration and dispension of precise sample volumes for direct absolute counting of lipoparticles. Figure 2C shows the results of a serial dilution of MLV induced lipoparticles. It is especially important with small particle detection to ensure that single particles are being analyzed. If particles are run at too high of a concentration, the particles can be too close together and multiple particles may be detected as a single event. This phenomenon is known as the coincidence or swarm effect. Individual particles that would normally register below the detection threshold are detected as a cumulative event, enabling them to reach the trigger threshold. If not controlled for, this effect distorts the data, altering the detected sample concentration and phenotype. Therefore, diluting the sample enough to ensure



Sample	GFP+ Events/µL	Dilution Factor	Measured Dilution Factor
1	21173		
2	4055	5	5.22
3	1941	10	10.91

Figure 2. MLV-induced lipoparticle detection using the Agilent NovoCyte Quanteon. (A) Dynamic light scattering (DLS) of lipoparticles to determine size. (B) SSC-H versus GFP-H plots of serially diluted lipoparticle fraction. Sample 1: 1:1000, Sample 2: 1:5000, Sample 3: 1:10,000. (C) Absolute count of GFP+ events obtained by volumetric counting of the sample dilution series, which is described in the text. The table shows the absolute event count and the actual versus the measured dilution factor. Instrument settings: SSC-H gain 900, threshold FITC-H greater than 220, 14 µL/min.

single particle detection is essential. One way to control for the coincidence is to perform serial dilutions of the sample. If there is no coincidence occurring, a linear decrease in sample concentration should be observed with dilutions. Based on the measured absolute event count the actual dilution factor correlated well with the calculated dilution factor (Figure 2C), confirming that the events detected were not due to coincidence. These data demonstrate the capacity of the NovoCyte Quanteon to detect small membranous particles.

Platelet activation increases released EV in plasma

Upon activation, platelets release procoagulant EVs, for example during thrombosis or inflammation. These particles range from 50 nm to 1 µm. However, most plasma-derived EVs are 100 to 250 nm.

To induce EV release, platelets were activated with ADP. After activation, total EVs were isolated through ultracentrifugation, and stained with anti-CD9, anti-CD61 antibodies, and Annexin V to label EV (Figure 3).

CD61 is a highly prevalent surface receptor on platelets, and thus identifies platelet-originated EV, while Annexin V and CD9 are commonly used as universal markers for EV. EV concentration increased by more than five-fold after platelet activation with ADP (Figure 3C). The highest concentration of EV was identified by expression of CD9 (Figures 3A and 3B). Most CD9+ EV also expressed Annexin V and CD61, which confirms that they are derived from platelets. A lower concentration of EVs were identified by surface expression of CD61 or Annexin V, which demonstrates the heterogeneity of EVs present in the plasma. To confirm that the particles detected are membrane vesicles and not protein aggregates or other nonmembranous structures, samples were incubated with a detergent, Triton-X-100, for one hour to disrupt the plasma membrane and ultimately eliminate any vesicles present in the sample. Only background events were present after Triton-X-100 addition, confirming that the events detected were EVs. In summary, platelet-derived EVs are easily detected using the NovoCyte Quanteon by either platelet or EV-specific markers.

Colorectal cancer cell line releases EV under normal growth conditions

EV secreted from tumor cells are important cell mediators in the tumor microenvironment, and play an important role in tumor growth and metastases. Detecting cancer-derived EV is important to learn more about their composition and function.

Cancer-derived EVs were purified from the supernatant of a colorectal cancer cell line, SW480, after 48 hours of culture by ultracentrifugation (Figure 4). The EVs were then identified by detecting the surface expression of known EV markers CD9, CD81, and Annexin V with specific antibodies (Figure 4A). EVs positive for CD9, CD81, and Annexin V were all detected, while CD9+ EVs were present at higher concentrations than the others. Next, co-expression of surface markers was examined in each population (Figure 4B). This demonstrates the heterogeneity of EV populations and the importance of single particle analysis, as the variation of surface protein expression in EVs would not be realized through bulk analysis of protein content. As a control, EVs were also incubated with the detergent Triton-X-100 to induce lysis of all membranous vesicles (Figure 4C). In summary, the detection of EVs derived from cancer cell lines is facilitated by the NovoCyte Quanteon and does not require far-reaching adjustment of cytometer settings or instrument modifications.

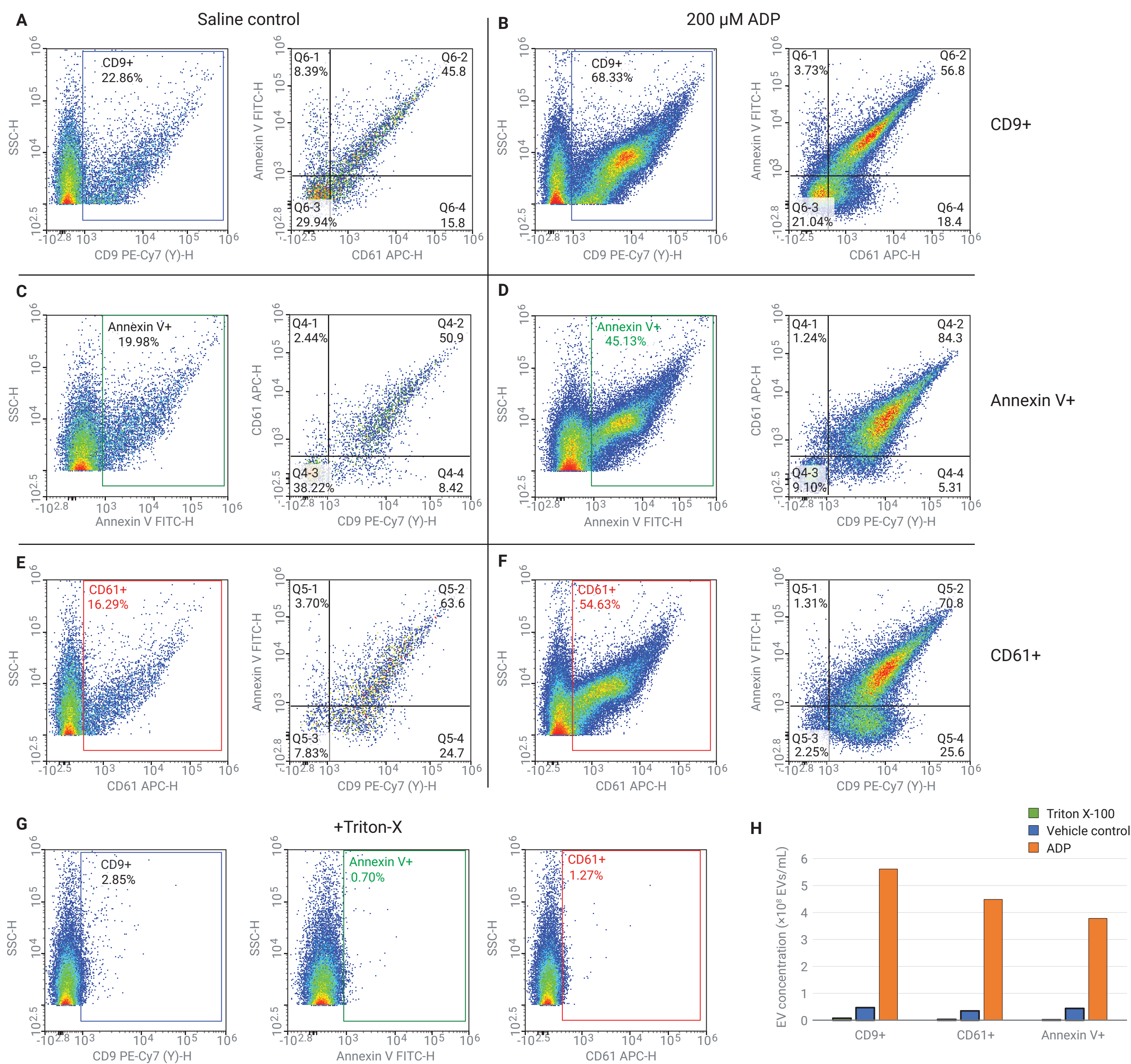


Figure 3. Increased EV release by platelet activation can be resolved by a flow cytometer. Subject's blood was collected using sodium citrate blood collection tubes. Whole blood was treated either with 200 µm ADP or saline alone for 30 minutes. EVs were then isolated by centrifugation: 2,500 × g 15 minutes twice, then the supernatant was centrifuged at 110,000 × g for 90 minutes twice. This was followed by staining with αCD9 PE-Cy7, αCD61 APC antibodies, and Annexin V FITC. EVs were identified by CD9 (A, B), Annexin V (C, D), and CD61 (E, F) in the saline-treated control (A, C, E) or ADP treated samples (B, D, F). (G) Identified EVs were analyzed for co-expression of other EV markers (A–F, right plots). A portion of the vesicles was incubated with 1% Triton-X-100 on ice for one hour. (H) Graph with EV concentration under the conditions described in the text. Instrument settings: SSC-H gain 1,000, threshold SSC-H greater than 1,000.

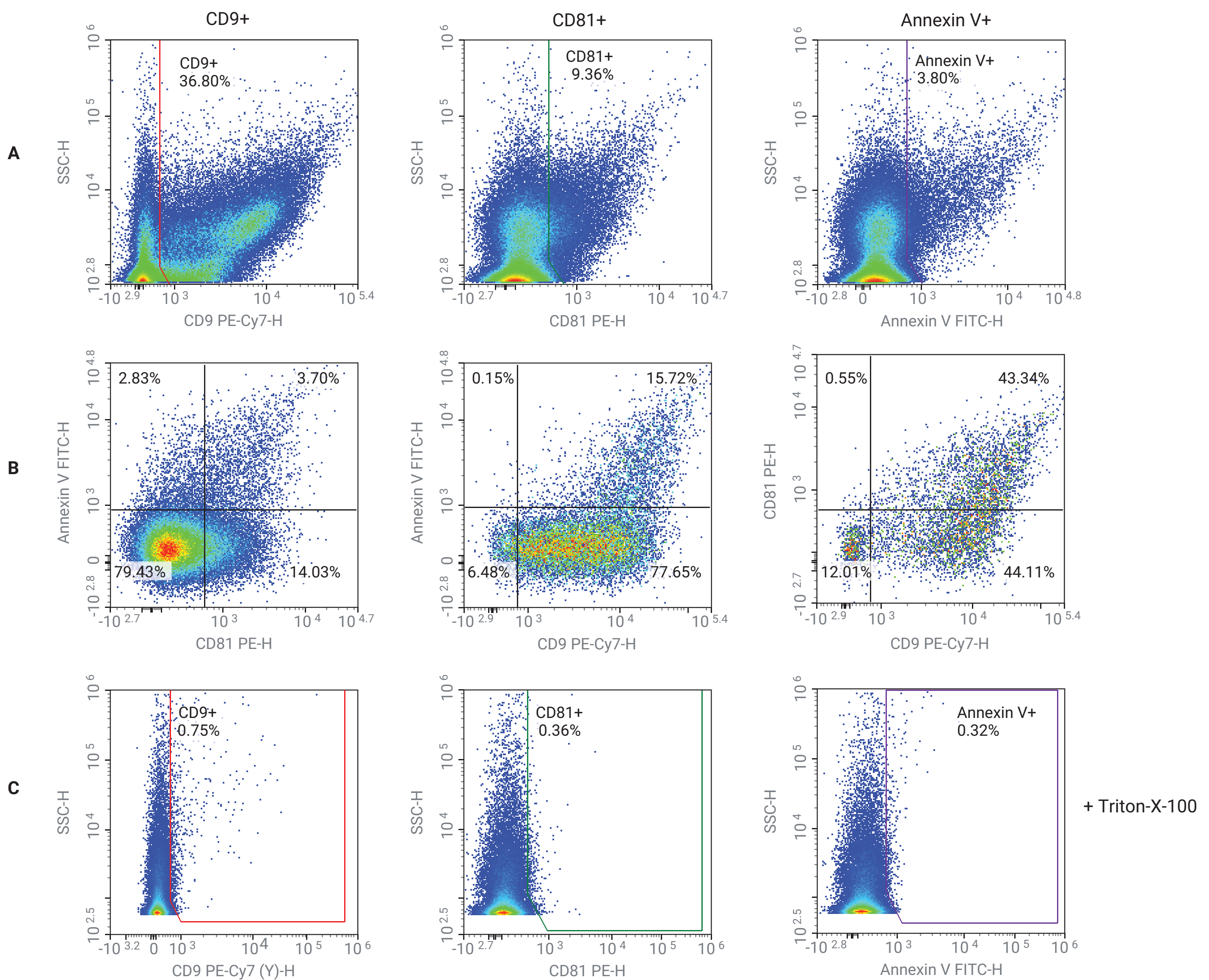


Figure 4. SW480 colorectal cancer cell-derived extracellular vesicles. SW480 cells were cultured to 70 to 80% confluency, media was then removed, and cells were washed twice with PBS. Fresh media were added that had been ultracentrifuged at 110,000 × g overnight. Cells were cultured for an additional 48 hours before the supernatant was collected. Cell culture supernatant was centrifuged at 300 × g for 10 minutes, then left at 4 °C overnight. The supernatant was then centrifuged at 2,000 × g for 20 minutes, followed by centrifugation at 110,000 × g for 90 minutes. The pellet was washed with PBS then centrifuged at 110,000 × g for 90 minutes. The pellet was stained with αCD9 PE-Cy7, αCD81 PE, and Annexin V FITC antibodies. (A) EVs were identified by CD9 (red), CD81 (green), or Annexin V (purple). (B) EVs were analyzed for co-expression of various EV markers. (C) A portion of the vesicles were incubated with 1% Triton-X-100 on ice for one hour. Instrument settings: SSC-H gain 1,000, threshold SSC-H greater than 600.

Conclusion

EVs are released from various cells and found in biological fluids and cell culture supernatant. They are easy to obtain from subjects, and carry important biological information. They are potential biomarkers for cancer, infectious disease, and other pathological conditions. Recent studies have indicated their value for disease research. The Agilent NovoCyte Quanteon flow cytometer, equipped with advanced detectors, provides high-sensitivity SSC and fluorescence detection, overcoming many of the obstacles of small particle detection in traditional flow cytometers.

References

1. Shao, H. *et al.* New Technologies for Analysis of Extracellular Vesicles. *Chem. Rev*. **2018**, *118(4)*, 1917–1950.
2. Aatonen, M. T. *et al.* Isolation and Characterization of Platelet-Derived Extracellular Vesicles. *J. Extracell. Vesicles* **2014**, *3*.
3. Osteikoetxea, X. *et al.* Differential Detergent Sensitivity of Extracellular Vesicle Subpopulations. *Org. Biomol. Chem*. **2015**, *13(38)*, 9775–9782.
4. https://www.ncbi.nlm.nih.gov/pubmed/29754178 Tickner, J. A. *et al*. EV, Microvesicles/MicroRNAs and Stem Cells in Cancer. *Adv. Exp. Med. Biol*. **2018**, **1056**, 123–135.
5. Xu, R. *et al.* Extracellular Vesicle Isolation and Characterization: Toward Clinical Application. *J. Clin. Invest*. **2016**, *126(4)*, 1152–1162.
6. Aatonen, M. T. *et al.* Isolation and Characterization of Platelet-Derived Extracellular Vesicles. *J. Extracell. Vesicles* **2014**, *3*.

www.agilent.com/chem

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019
Printed in the USA, October 29, 2019
5994-1203EN