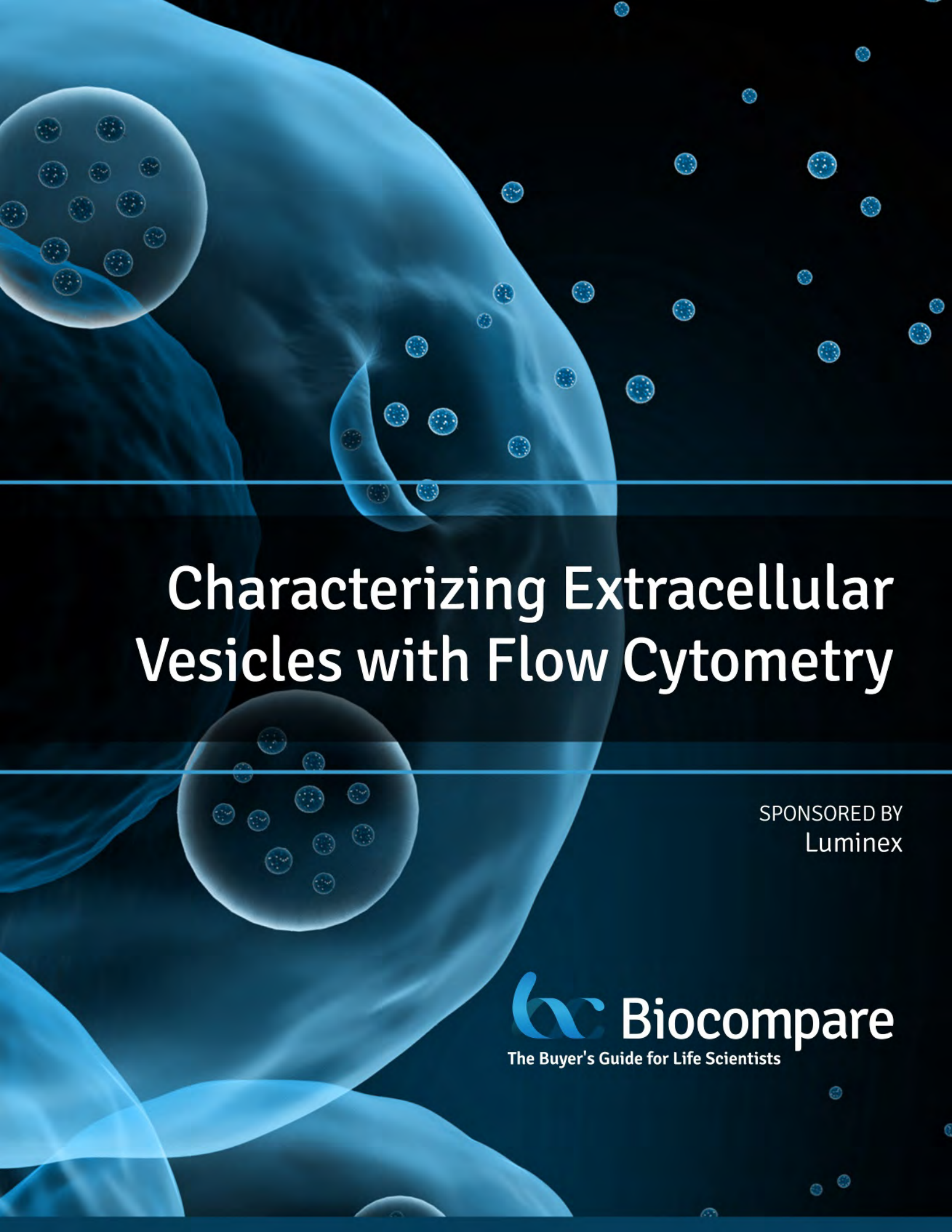

Characterizing Extracellular Vesicles with Flow Cytometry

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Extracting Maximum Data from Extracellular Vesicle Studies

Reliable methods for analyzing EVs are essential for the field to progress to the translational stage.

Angelo DePalma, Ph.D.

Extracellular vesicles (EVs) are membrane-derived structures that include exosomes, ectosomes, microvesicles, and apoptotic bodies.[1] Ranging in size from about 30 nm to 120 nm in diameter, exosomes are released through the exocytosis of multivesicular bodies, while ectosomes originate from the plasma membrane.[2]

Once released into the extracellular space, EVs enter body fluids. There, they interact with and transfer their molecular cargo to cells, thus influencing both physiological and pathological processes.[1] EVs are released by normal, healthy cells, but recent evidence suggests that EVs may serve as mediators in the pathogenesis of neurological, oncological,[3] vascular, hematological, and autoimmune diseases.[4]

Given the challenges in diagnosing, monitoring, and understanding diseases, and the potential participation of EVs in these conditions, protocols for isolating and analyzing EVs are essential for the field to progress to the translational stage.[5]

EV analysis

Reliably quantifying and characterizing EVs is challenging due to the particles' small size. High-magnification microscopy, such as electron microscopy, is often used for the in-depth study of specific EVs,[6] but microscopy is an inherently low-throughput technique. By contrast, flow cytometric EV analysis is a rapid, high-throughput technique suitable for characterizing discrete particles.

EV analysis using conventional PMT-based flow cytometers has been hampered by the dimensions of typical EVs compared with those of intact cells. A smaller size means a smaller refractive index, a property that factors into particle enumeration. Also, unlike cells that express thousands of copies of surface markers, EVs may express only tens of copies. Even when labeled with strongly fluorescent tags, label concentrations can be below the detection limits of conventional cytometers.[7]

Combined, these factors raise questions regarding the choice of detection method and the importance of using fluorescent markers. Another problem is validating that single vesicles are detected and not coincident events, which are known as swarms. Swarm detection is a common phenomenon that occurs when many small particles are reported as a single event, causing errors in concentration and intensity measurements.[8]

High sensitivity flow cytometry

To summarize, the issues with most traditional cytometry platforms for characterizing EVs are the small size of EVs and the low abundance of surface markers expressed on those EVs. To detect these small-sized particles and the low-signal markers, the Amnis® CellStream® Flow Cytometer and the ImageStream®X Mk II Imaging Flow Cytometer were used to analyze EVs.

Amnis® flow cytometers employ a charge-coupled device (CCD) using Time Delay Integration (TDI), which offers the advantages of high-throughput flow cytometry as well high-sensitivity detection of submicron particles.

The Amnis® TDI CCD camera technology preserves sensitivity and image quality, even with fast-moving particles, and captures multiple colors of fluorescence, as well as FSC and SSC with superior photonic

sensitivity. The effect is similar to physically panning a camera. TDI avoids image streaking despite signal integration times that are orders of magnitude longer than those of conventional flow cytometry.

Amnis® Flow Cytometers in Operation

Where conventional PMT-based cytometers have high amplification noise, TDI-CCD detection offers high sensitivity with low background, provided relevant study parameters are properly controlled. Controls that must be included for reliable results are buffer only, antibody/dye only, unlabeled EVs, and labeled EVs plus a detergent. These controls are in addition to the experimentally labeled EVs. Investigators should also run dilution series of their labeled EV samples to determine if swarming has occurred.

A multi-disciplinary research group, including scientists at Luminex and academic collaborators, examined the general suitability of the Amnis® CellStream® platform and the Amnis® ImageStream®X Mk II[9] to analyze small EVs, including exosomes, and the ability of the two systems to resolve populations of smaller EVs in particular.

Using antibody-labeling approaches, investigators showed that imaging flow cytometry was capable of detecting individual small EVs and could identify

distinct EV populations. They wrote that the technique "will help to significantly increase our ability to assess EV heterogeneity in a rigorous and reproducible manner, and facilitate the identification of specific subsets of small EVs as useful biomarkers in various diseases."[9]

Conclusion

EVs have been the subject of intense study in basic research and therapeutic and diagnostic medicine. Conventional approaches to understanding the role of EVs in disease and health, including microscopy and PMT-based flow cytometry, fall short on several fronts. Microscopy is slow, whereas flow methods, designed for particles larger than approximately 300 nm, miss too many details—particularly smaller EVs or EVs with rare surface markers. By utilizing a CCD-based image-acquisition detector similar to those employed in very high-end optical systems, the Amnis® platforms identify and characterize EVs with high sensitivity and specificity in a high-throughput manner.

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Immunophenotyping Extracellular Vesicles

Attempts to analyze EVs using traditional flow cytometers have been problematic.

Haley R. Pugsley, Ph.D., Bryan R. Davidson, and Phil Morrissey, Ph.D.

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 one 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 three 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 three 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.

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.

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.

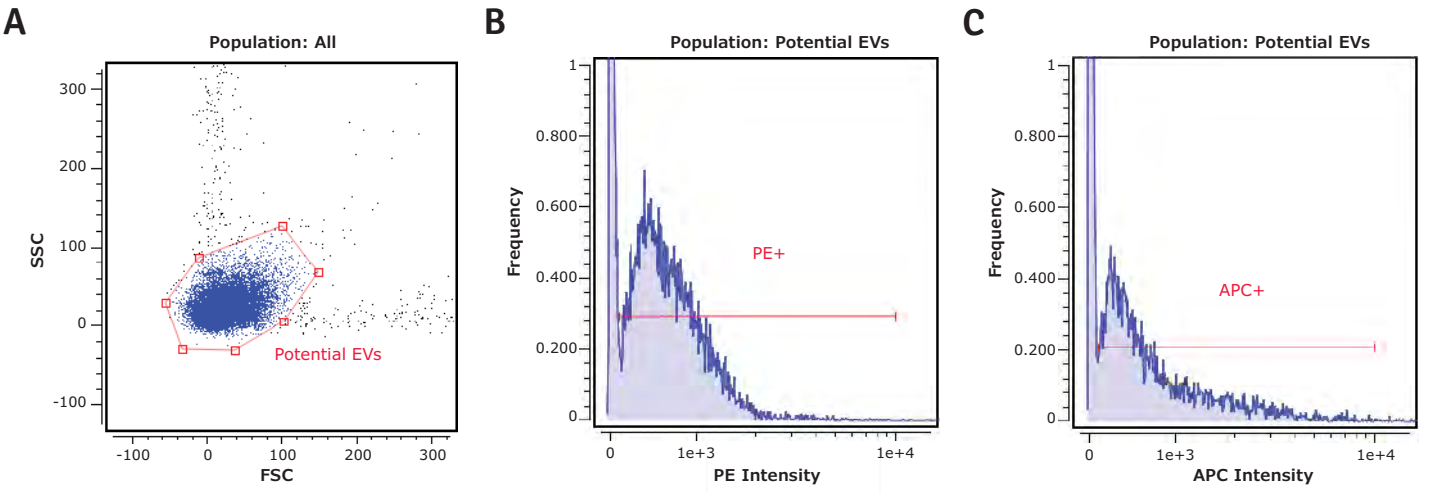

Figure 1. Gating strategy for identifying EVs. (A) A FSC vs. SSC plot was used to identify the potential EV population. The "potential EVs" gated population was used to identify PE+ events (B) and APC+ events (C).

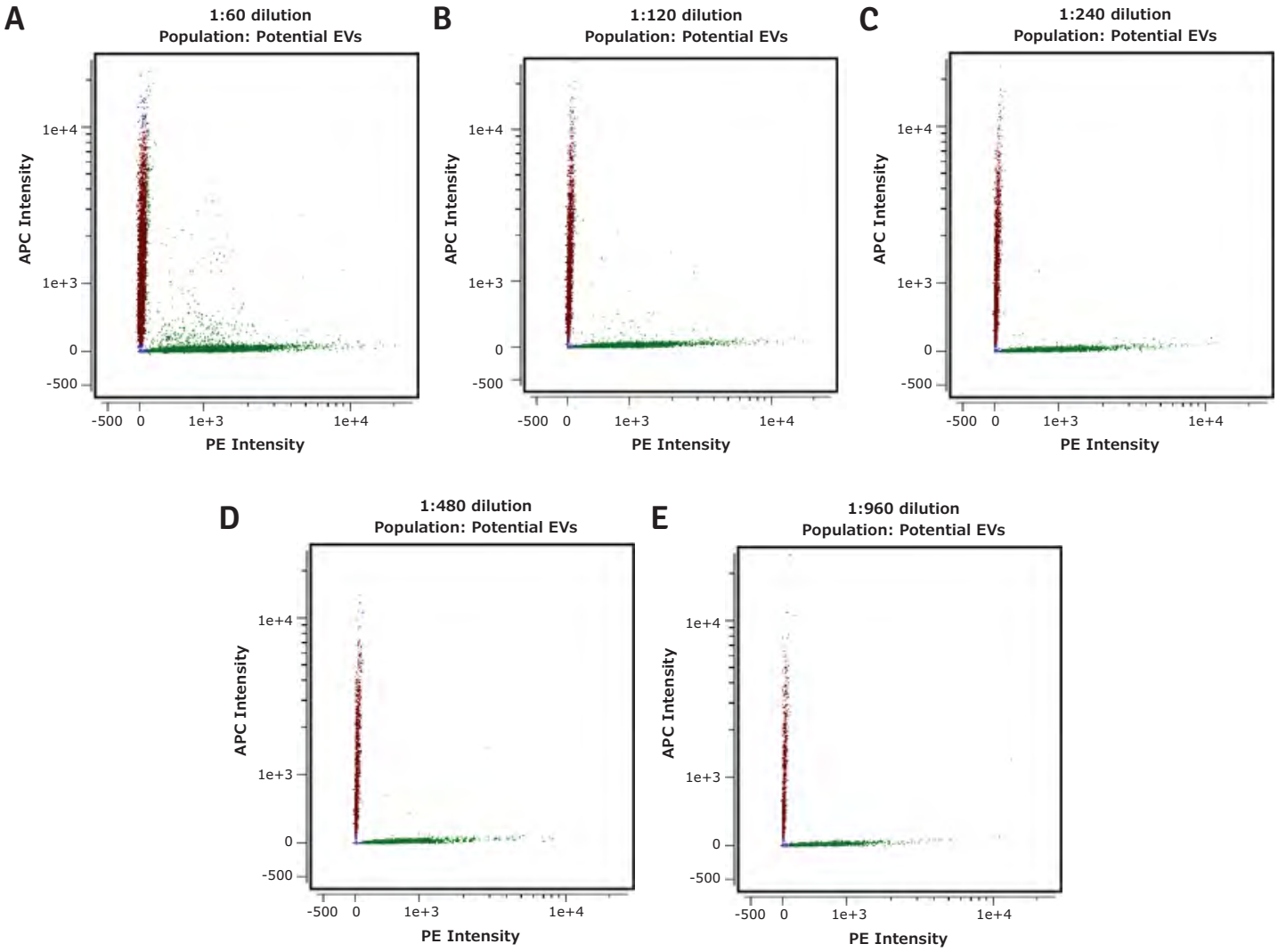

Figure 2. Bivariate dot plots for the dilution series of RBC-EVs and platelet-EVs labeled with CD235ab-PE and CD41-APC, respectively. PE+ events from Figure 1B are colored green, and APC+ events from Figure 1C are colored red. The dilutions are: (A) 1:60, (B) 1:120, (C) 1:240, (D) 1:480, and (E) 1:960.

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. Tables 1 and 2 show the average objects per µl and standard deviations for the PE+ and APC+ events in Figures 4A and 4B, respectively.

Summary

In this study, EVs derived from RBCs and platelets were immunophenotyped on the CellStream

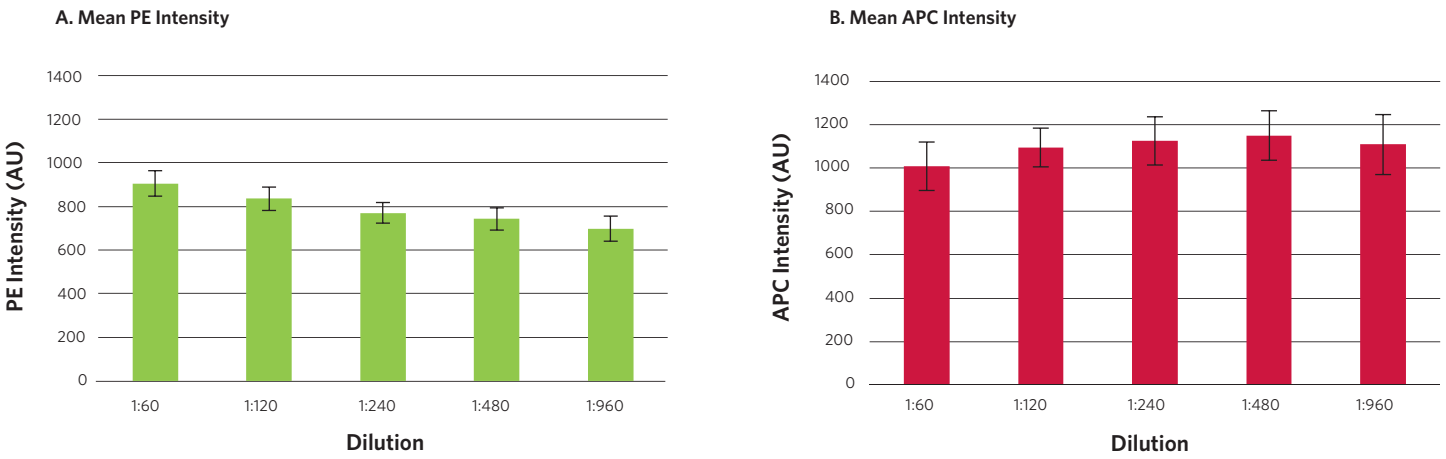

Figure 3. The mean fluorescence intensities for PE (A) and APC (B) across each dilution series. The mean fluorescent intensities are from the PE+ or APC+ gates in Figure 1.

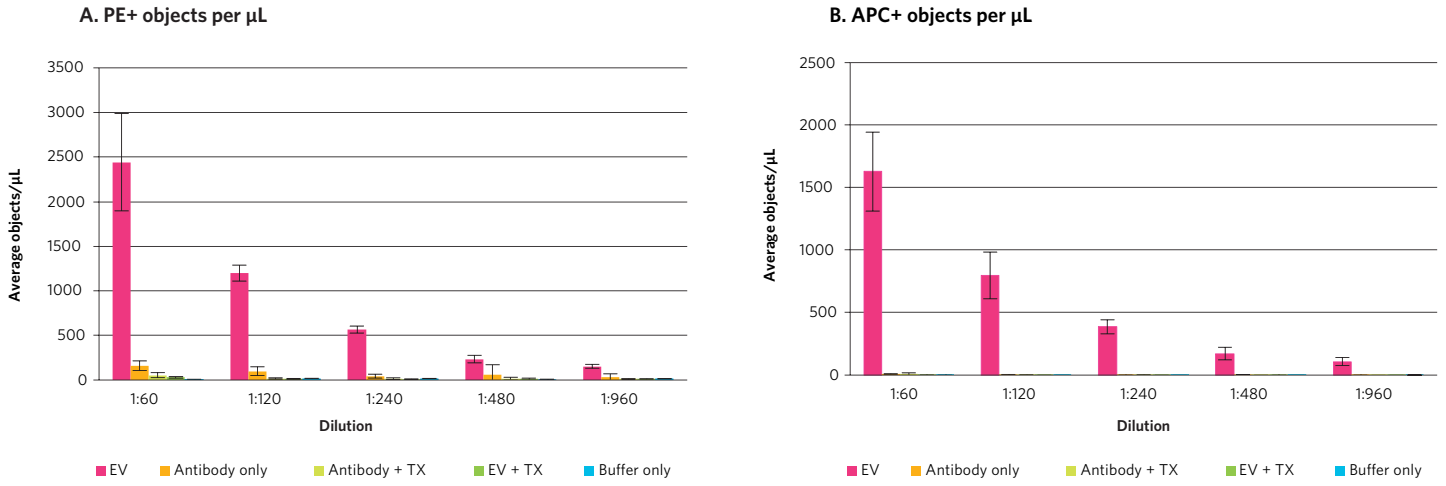

Figure 4. PE+ (A) and APC+ (B) objects per µl for the various experimental and control samples. Samples include labelled EVs, antibody only, antibody + Triton® X-100, labelled EVs + Triton X-100, and buffer only. The objects per µL are the events in the PE+ or APC+ gates shown in Figure 1.

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 three CellStream Flow Cytometers (Figure 3) and the objects per µL for all

Tables 1 and 2: Average objects per µl and standard deviations for the PE+ events (Table 1) and APC+ events (Table 2) in Figures 4A and 4B, respectively.

Table 1. 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

Table 2. 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

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

Thank you to John Nolan at Cellarcus Biosciences for providing the RBC and platelet EV samples and guidance.

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Single Vesicle Flow Cytometry to Count, Size, and Measure EV Cargo

A highly sensitive flow cytometer improves measurement of EV number, size, and surface cargo.

John P. Nolan, Ph.D.

Abstract

Extracellular vesicles (EVs) are gaining increased attention as intercellular signaling messengers, diagnostic biomarkers, and targeted therapeutics, but progress in the field is limited by the availability of quantitative EV analysis methods. Flow cytometry is an attractive platform for EV analysis, but conventional assays and instruments have poor specificity and sensitivity. To address this need, Cellarcus Biosciences has developed a quantitative and specific Vesicle Flow Cytometry (vFC™) assay that takes advantage of a new generation of sensitive flow cytometers. The vFC™ assay includes reagents, including stains, calibrators, and standards, as well as detailed sample preparation and analysis protocols that ensure quantitative and reproducible results. In this article, we describe the measurement of EV number, size, and surface cargo including tetraspanin and cell-specific markers using the Amnis® CellStream® (Luminex Corporation), a highly sensitive flow cytometer.

Introduction

Extracellular vesicles (EVs) are released by all cells, and they can carry molecular cargo to other cells, making them attractive targets as intercellular signal carriers,[1] disease biomarkers,[2] and therapeutic agents.[3] However, EVs are small, heterogeneous, and difficult to measure, and progress is limited by available EV analysis methods. Because EVs may originate from different compartments within a cell, and all cells can produce EVs, the EVs in a complex biofluid are extremely heterogeneous. Characterizing EV heterogeneity is a key step in understanding the function of EVs in biological or therapeutic contexts. It is also critical in identifying and quantifying EV subsets of diagnostic utility. EV heterogeneity confounds traditional biochemical (Western blot, ELISA, mass spectrometry) and molecular (PCR, sequencing) analysis, which report the total amount of target present but cannot resolve which EVs bear the target of interest.

Single nanoparticle analysis techniques such as resistive pulse spectroscopy (RPS)[4] and nanoparticle tracking analysis (NTA)[5] can estimate nanoparticle size distributions but are non-specific and cannot effectively measure cargo. The ideal single vesicle analysis method would not only count and size individual EVs but would also measure cargo abundance to be determined on a per vesicle basis, affording resolution of EV heterogeneity as well as revealing patterns of co-expression of different molecular cargos.

Flow cytometry is a powerful platform for single particle measurements, but conventional instruments and assays lack sensitivity and specificity.[6] Most conventional instruments were designed to measure lymphocytes, but EVs are orders of magnitude smaller and dimmer than cells. Moreover, the use of light scatter as a trigger makes it difficult to confidently resolve vesicles from other particles or background, and estimating vesicle size from light scatter intensity is fraught with complexity.[7,8] These limitations, plus a general lack of appropriate controls, calibration, and reporting, have produced a confusing literature on EV flow cytometry that is difficult to interpret. Recently developed guidelines for conducting and reporting EV flow cytometry measurements will improve this situation by alerting researchers to these potential artifacts and detailing procedures and controls to avoid them.[9]

Cellarcus Biosciences has addressed the need for quantitative and reproducible EV measurement via a Vesicle Flow Cytometry (vFC™) assay kit that can be used with a suitably sensitive flow cytometer. The Cellarcus vFC™ assay employs a fluorogenic lipid probe that provides membrane-selective detection of EVs using a fluorescence trigger and allows for estimation of vesicle size. The vFC™ assay kit also includes key calibrators and standards as well as protocols that include the necessary controls to ensure reproducible results. Here we demonstrate the vFC™ assay using the Amnis® CellStream®, an imaging-based flow cytometer that employs high efficacy photodetectors and time delay integration to achieve high sensitivity fluorescence detection.

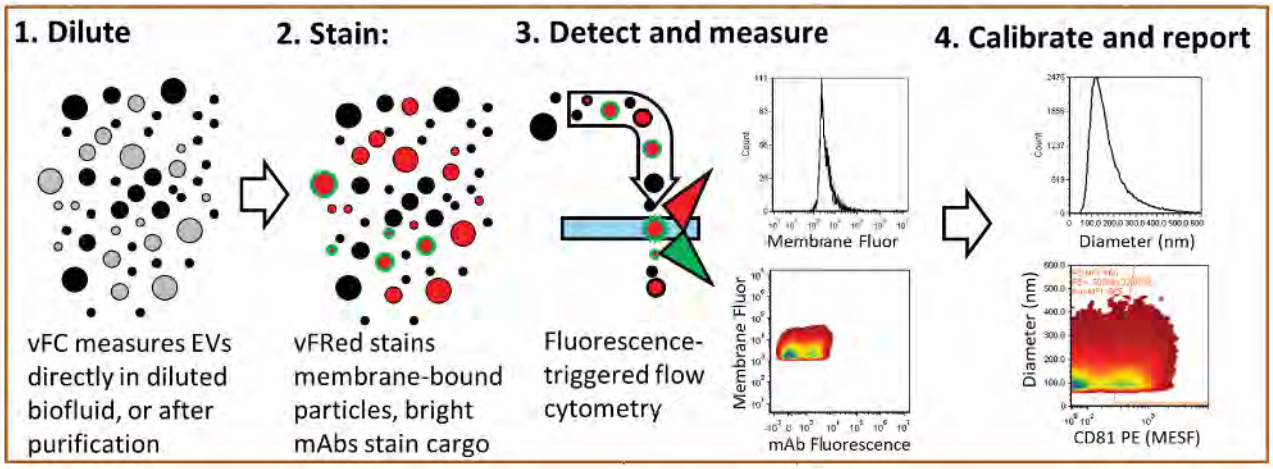

Figure 1. vFC™ assay workflow. The vFC™ assay is a simple, four-step assay that involves staining with the lipophilic probe vFRed™ and fluorescent antibodies, dilution, measurement on a sensitive flow cytometer, and data calibration and analysis. The vFC™ assay includes the necessary protocols, reagents, calibrators, standards, and analysis templates to ensure quantitative and reproducible data.

Materials and Methods

Cell culture and sample preparation

HEK 293T cells were cultured to 90% confluence in DMEM plus 10% FBS and 1% pen strep (37°C, 5% CO_2), at which point the cells were washed and cultured in serum-free DMEM. After 24 hours, media was collected, processed by centrifugation (2x 2,500 x *g*, 10 min) and further refined by centrifugal ultrafiltration (Amicon, 100KD MWCO). The retentate was aliquoted and stored at –80°C. Platelets (from expired concentrates) were washed extensively, treated with the calcium ionophore A23187 (10 µM) for one hour at 37°C, and pelleted by centrifugation (10,000k x *g*, 10 minutes), after which the supernatant was aliquoted and stored at –80°C.

Vesicle flow cytometry

EVs in these studies were characterized using a commercially available assay, vesicle flow cytometry (vFC™, Cellarcus Biosciences, Inc.), on the Amnis® CellStream® Flow Cytometer according to instructions provided with the kit. The vFC™ assay kit includes assay workflows, reagents, standards, protocols and analysis templates to facilitate quantitative and reproducible results. Briefly, samples were diluted in Vesicle Staining Buffer (Cellarcus Biosciences) and stained with vFRed™ along with one or more vTag™ antibodies in a total volume of 50 µL in a 96-well plate. Samples incubated for one hour at ambient temperature. After staining, samples were diluted 30-fold in Vesicle Staining Buffer to reduce background and analyzed on the Amnis® CellStream® Flow Cytometer.

The Amnis® CellStream® Flow Cytometer was configured in small particle mode with forward and side scatter turned off. Each sample was run for 120 seconds at sample volumetric flow rate of 3.5 µL/minutes (7 µL total). The fluorescence axes were calibrated using FITC Quantum MESF beads (Bangs Labs) and PE Quantibrite Beads (BD Biosciences). All other channels were calibrated using vCal™ nanoRainbow beads (Cellarcus Biosciences) or vCal™ antibody capture beads (Cellarcus). Data were analyzed using the included vFC™ analysis template which runs on the free reader version of FCSExpress (De Novo Software).

Results

Vesicle detection and sizing

vFC™ uses a fluorogenic membrane probe, vFRed™, to detect and size vesicles. This probe is non-fluorescent in aqueous buffer, but in the presence of membranous vesicles it intercalates into the lipid-bilayer and becomes fluorescent. A synthetic lipid vesicle that is included in the vFC™ kit, Lipo100™, serves as a positive control and size standard (Figure 2A). Lipo100™ was prepared by extrusion through a nanopore filter to produce a uniform vesicle population and was characterized by orthogonal methods including RPS and NTA. When stained in accordance with the protocol and measured on the Amnis® CellStream® Flow Cytometer, the fluorescence of the Lipo100™ (Figure 2B) is proportional to its surface area. This relationship (Figure 2C) can be used to estimate surface area and diameter of EVs in unknown samples, assuming EVs are spherical in shape (Figure 2D).

Assay Controls

vFC™ includes several controls to assess the specificity of the assay and improve the reproducibility of the reported data. One issue common to many assays used by EV researchers is the presence of non-EV contaminants in datasets. To confirm measurement of individual vesicles, it is recommended to

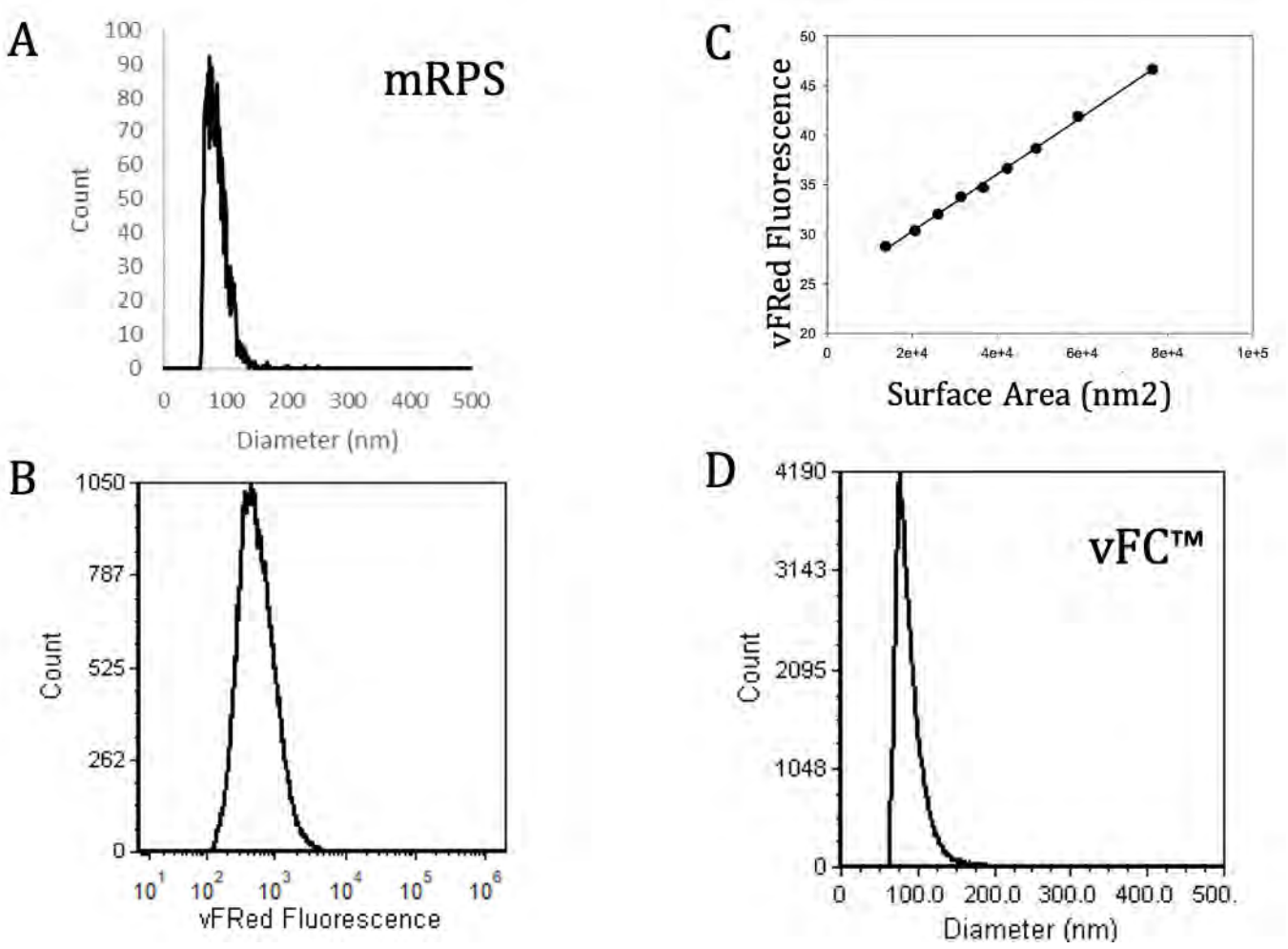

Figure 2. vFC™ size calibration. (A) A vesicle standard (Lipo100™), whose population size distribution been extensively characterized by orthogonal methods including RPS and NTA, is stained with vFRed, and the corresponding fluorescence distribution is measured (B). Linear regression (C) of the vesicle standard surface area distribution versus its fluorescence distribution calculates the surface area (nm2) per fluorescence unit, allowing the size distribution (D) of the vesicle population to be estimated from its fluorescence.

perform a detergent treatment, which will solubilize vesicles and should be used accordingly to qualitatively assess the specificity of an EV characterization assay. vFC™ includes a detergent treatment control. In Figure 3A, the reduction of events following detergent treatment confirms the vesicular nature of events measured in the assay.

A second problem attributable to the small size of EVs and limited sensitivity of some approaches is reporting of data derived from many EVs as a single EV analysis. In flow cytometry, this is referred to as coincidence (and sometimes "swarm" in the EV literature) and can be evaluated by serial dilution. In the absence of coincidence, the number of particles

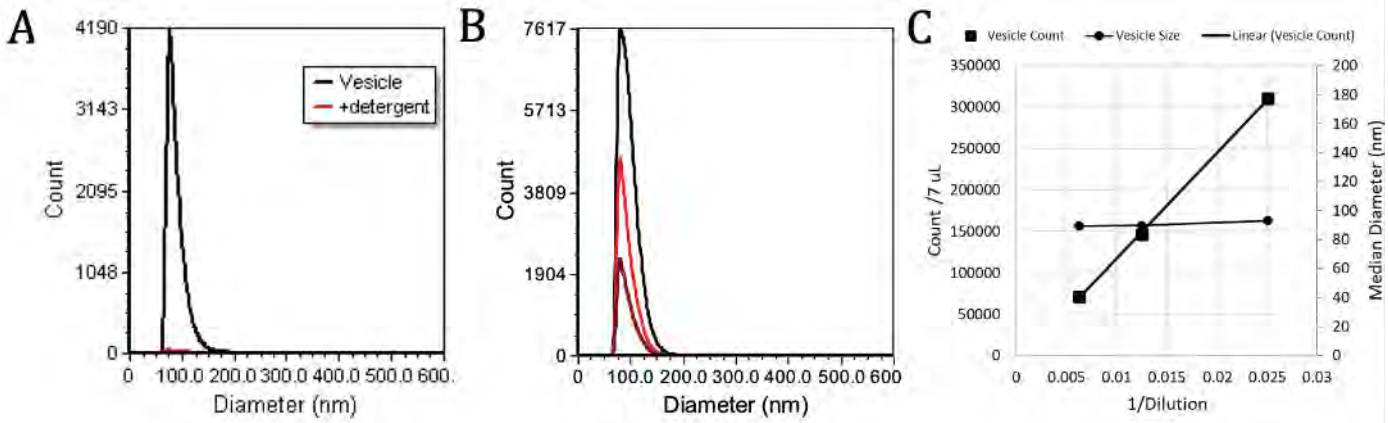

Figure 3. Essential controls to ensure EV specificity. (A) Detergent treatment shows that the detected events are detergent-labile, confirming their vesicular nature. (B) Serial dilution of sample demonstrates assay dynamic range and lack of coincidence. (C) Vesicle counts decrease in proportion to dilution, but the vesicle size estimate is independent of dilution, consistent with single particle analysis.

detected in a measured volume should decrease in proportion to the dilution, but the measured brightness of those events should not change. The vFC assay protocols ensure that the median EV diameter, which is derived from vFRed™ fluorescence, is consistent over a range of sample dilutions (Figure 3C), indicating the measurement of single vesicles without observable evidence of coincidence.

Measurement of EV surface markers

Measuring EV-associated cargo is a major objective of EV researchers, and vFC™ immunofluorescence is an effective way to measure EV surface cargo. The considerations for immunofluorescence of EVs are similar for measuring cells, including the careful titration of antibodies to determine optimal stain concentrations and the use of appropriate controls and calibration. vFC™ immunofluorescence protocols, paired with antibodies validated for EV analysis such as vTag™ antibodies, provide a no-wash, standardized, and quantitative approach to ensuring reproducible, interpretable data. vFC™ and supporting protocols address considerations for immunofluorescence of EVs. Many of these are similar to those made when measuring cells, including the careful selection and titration of antibodies to determine optimal staining conditions and the use of appropriate controls and calibration steps.

Tetraspanins are a class of proteins frequently associated with EVs. While there are more than 20 known tetraspanin molecules, CD9, CD63, and CD81 are the most commonly measured. Although they are expressed heterogeneously and not on every EV, they are useful and are often included as a marker to evaluate the vesicular nature of EV characterization data. vFC™ is available with a cocktail of vTag™ tetraspanin antibodies in a variety of fluorescent conjugates to measure tetraspanin expression on EVs. Some example data is provided in Figure 4 demonstrating heterogeneity commonly observed in measuring tetraspanins on EVs from different cell types. PLT EVs stain brightly and uniformly for tetraspanins, HEK 293T EVs are an example of EVs with lower tetraspanin expression, and Lipo100™ are an antigen-negative control and show no tetraspanin signal. In surveys of more than a dozen cell types, including primary and

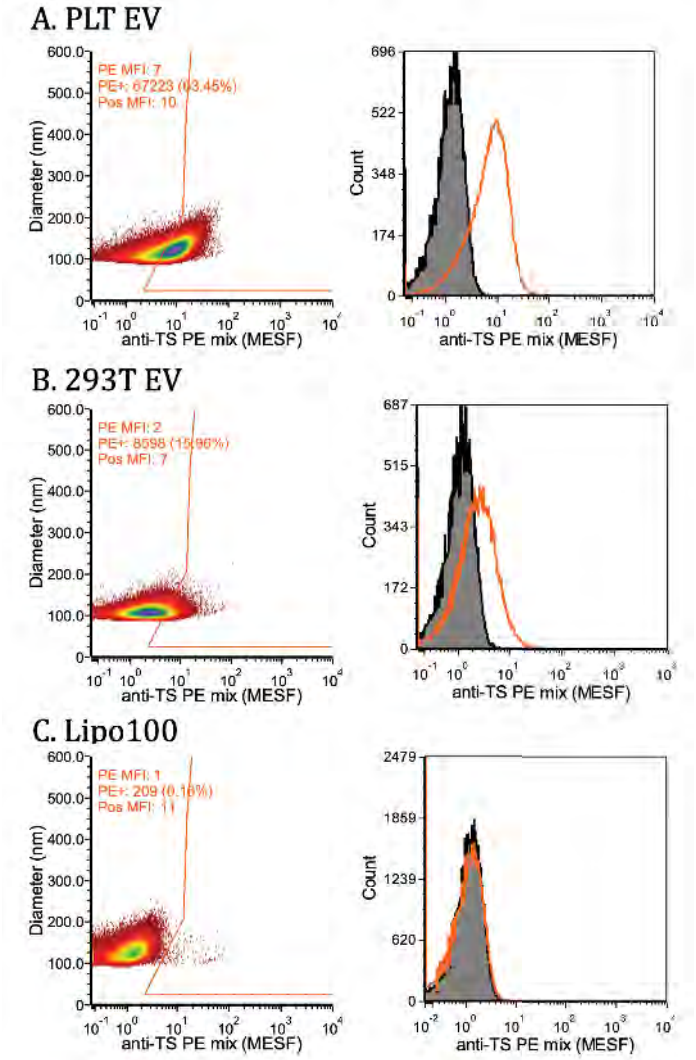

Figure 4. Immunofluorescence of common EV markers. (A) PLT EVs, (B) HEK 293T EVs, and (C) Lipo100™ were stained with the tetraspanin cocktail containing a mixture of vTag™ CD9 PE, CD63 PE, and CD81 PE antibodies and analyzed using the Amnis® CellStream® Flow Cytometer. The gray histograms show the negative controls.

cultured cells, we have observed striking differences in tetraspanin expression between cell lines and among EVs from a single cell type.

Profiling individual tetraspanins on cells is an area of active interest as it can vary greatly due to cell type, sample preparation, or other pre-analytical variables. Tetraspanin profiling can be achieved via multicolor antibody staining. Proper panel design is critical to achieving optimized, interpretable results in these assays due to the small size of EVs and the dim signals being measured. Quantification is also important to generate reproducible, comparable results across instruments or time. To demonstrate multicolor staining of EVs to profile tetraspanin expression via vFC™, a panel of CD9 APC, CD63 PE, and CD81 PE-Cy7 vTag™ antibodies was developed and optimized to stain PLT EVs, HEK 293T EVs, and Cellarcus's vCal™ antibody capture beads with calibrated binding capacities (Figure 5). The use of antibody capture beads, together with PE MESF calibration, allows the fluorescence intensity of each fluorochrome to be calibrated in units of antibodies bound per vesicle (ABV), enabling quantitative assessment of expression of all three tetraspanins. These data further illustrate the heterogeneity of EVs from different cell types. The predominant tetraspanin on PLT EVs is CD9, while HEK 293T EVs mainly express CD81. Surveys of EVs from different cell types reveal considerable heterogeneity in tetraspanin expression, and the "tetraspanin profile" may provide clues to an EV's origins or functional significance.

Beyond tetraspanins, cell type–specific markers are useful when, for example, attempting to identify specific subsets of EVs, especially in complex biofluids like plasma. The most common cell types in blood are red blood cells (RBCs) and platelets (PLTs), and highly abundant EVs from these sources could be expected in plasma. RBCs express abundant CD235ab (glycophorin) while PLTs express high levels of CD41, and these cell surface markers are tightly associated with their respective cell lineages. They are thus useful cell-specific markers. EVs prepared from RBCs and PLTs also express these markers. A multicolor panel that includes CD235ab

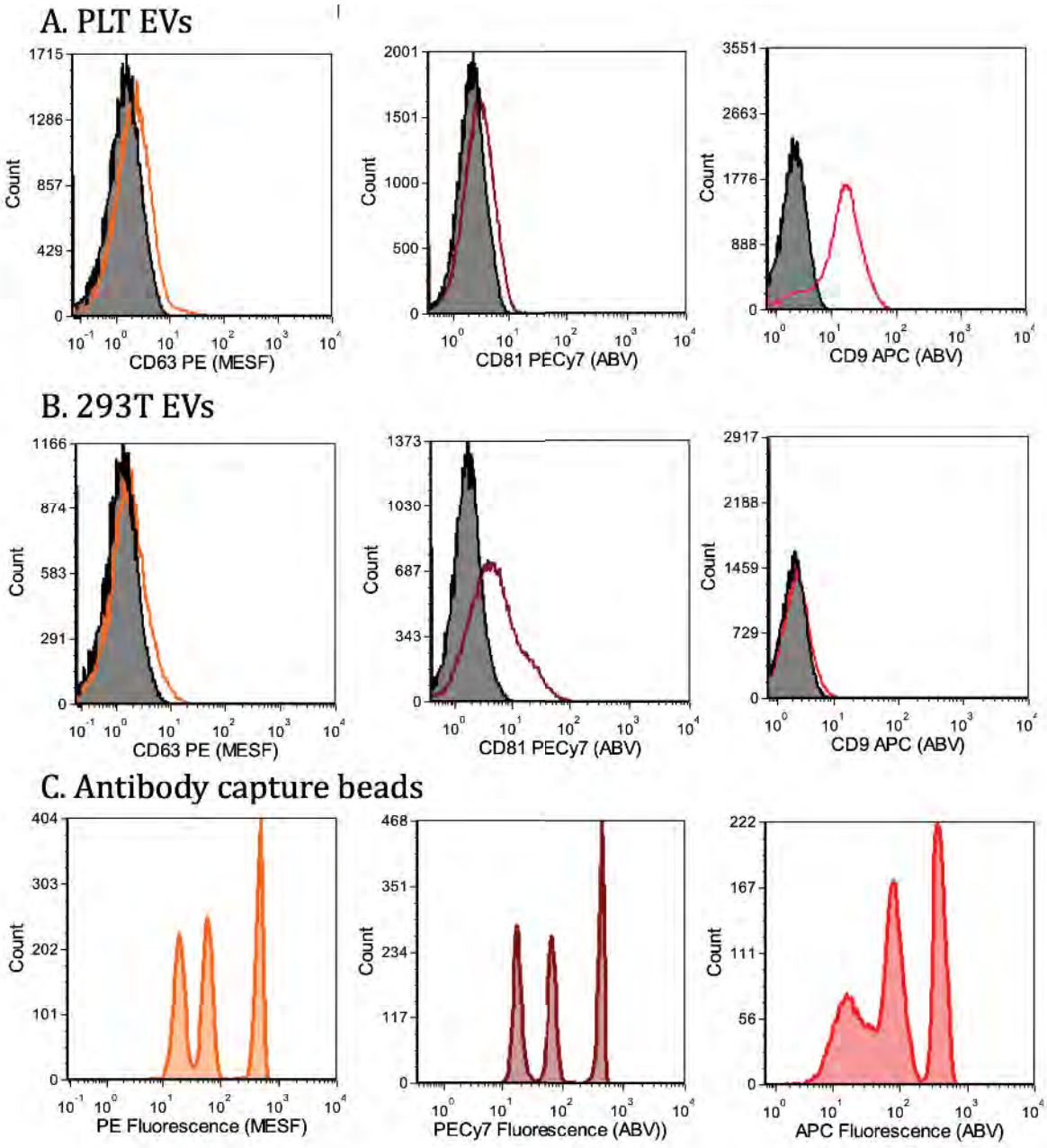

Figure 5. Multicolor tetraspanin panel. A multicolor panel of CD9 APC, CD63 PE, and CD81 PE-Cy7 vTag™ antibodies was used to stain (A) PLT EVs, (B) HEK 293T EVs, and (C) calibrated antibody capture nanobeads. Antibody capture beads enable EV fluorescence to be expressed in units of antibodies per vesicle (ABV). The gray histograms in A and B show the negative controls.

PE and CD41 BV421 vTag™ antibodies illustrates the utility and specificity of these markers in distinguishing EV subsets that might be found in a biofluid (Figure 6). Combinations of cell-specific EV markers and unique tetraspanin profiles may compose the most useful biomarker panels.

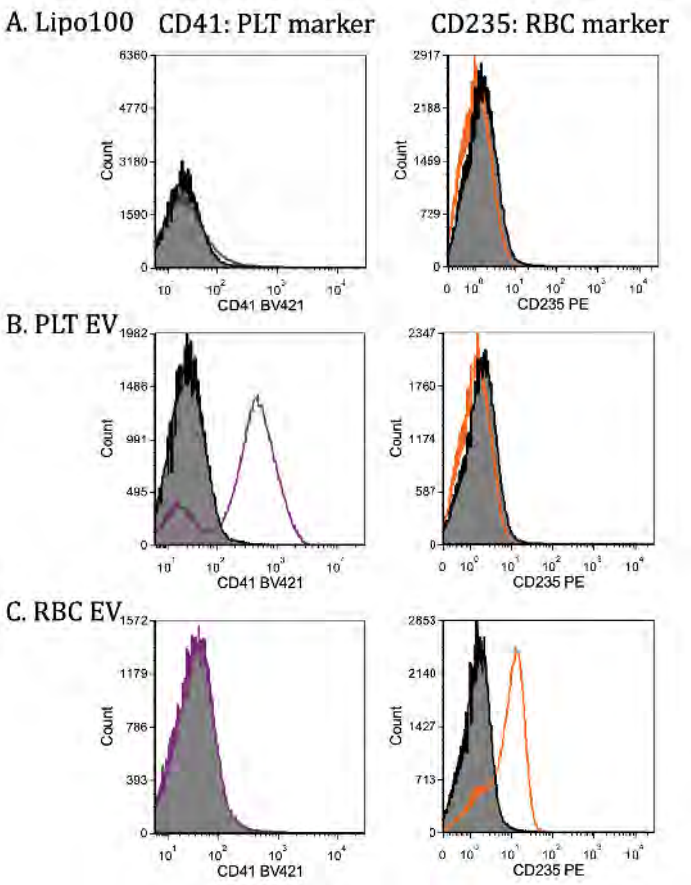

Figure 6. Immunofluorescence of cell-specific EV markers. Lipo100™ (A), PLT EVs (B), and RBC EVs (C) were stained with a cocktail of anti-CD41 BV421 and CD235 PE and analyzed using the Amnis® CellStream® Flow Cytometer. The specificity of the markers to each cell type is conserved in EVs derived from those cells. The gray histograms show the negative controls.

Conclusions

Progress in understanding the origins, functional roles, and translational significance of EVs is limited by our ability to quantitatively and specifically measure individual EVs and their cargo. Vesicle flow cytometry (vFC™), performed using a sensitive flow cytometer such as the Amnis® CellStream®, provides the ability to count, size, and measure molecular cargo quantitatively, sensitively, and reproducibly.

The vFC™ assay kit includes the necessary reagents, including stains, calibrators and standards, as well as assay and analysis protocols that include the necessary controls and calibration to ensure specific and quantitative measurements. These include controls to assess the specificity of EV detection (including buffer- and reagent-only controls to evaluate background), detergent treatment to demonstrate vesicle lability, and serial dilution to demonstrate absence of coincidence and assay dynamic range. The vFC™ kit also includes controls and calibrators for EV immunofluorescence including antigen-negative vesicles, antigen-positive EVs, and fluorescence intensity and antibody binding standards to enable quantitative reporting of results.

By employing optimized, standardized, and validated sample preparation and data analysis protocols, together with the appropriate use of calibrators and standards, the sensitivity of a new generation of high-sensitivity flow cytometers can be harnessed to develop a predictive understanding of the roles of EVs in health and disease.

About the author

John P. Nolan, Ph.D. is founder and CEO of Cellarcus Biosciences, Inc., San Diego, CA.

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Detection of Extracellular Vesicles

IFC protocol combines high fluorescence sensitivity, low background, image confirmation ability, and data analysis tools.

Uta Erdbrügger, MD, Sabrina La Salvia, Ph.D, and Joanne Lannigan

The following study was conducted by the authors noted above and has not been independently validated by Luminex.

Introduction

Extracellular vesicles (EVs) have gained significant interest as potential biomarkers and bio-activators in health and disease. These submicron vesicles are believed to transfer proteins, lipids, and nucleic acids, thus facilitating communication between cells. The detection of this wide range of EVs is challenged by their small and heterogeneous size range, high concentration, low refractive index, and heterogeneity in composition and morphology. This protocol uses imaging flow cytometry (IFC) to detect EVs, as it combines high fluorescence sensitivity, low background, image confirmation ability, and powerful data analysis tools. As EVs carry markers of their parent cells, IFC can also be used to identify EV origin via targeted and high-throughput phenotyping.[1]

Methods

The current study characterizes and enumerates circulating EVs in human blood. EVs were isolated by differential centrifugation from fresh citrated blood. A low centrifugation speed of 2,000g for 15 minutes was used to remove cells, debris, and larger particles, followed by higher speed of 21,000g for 30 minutes to generate an EV pellet. Note that the specific EV isolation protocol depends on the scientific question and type of bodily fluid being investigated and is not discussed in this application note. In this study, EVs of platelet vs. endothelial origin and composition were detected using CD31 (a platelet adhesion molecule), and annexin V (AnV; a membrane marker found on circulating EVs), respectively.

Basic IFC protocol

1. **Settings.** All lasers of the ImageStream®X Mk II System are set to full power, including the scatter laser (Figure 1). Magnification is set to 60X, and the core size is reduced to 7 µm. Channels not used are deselected and 'remove beads' is unchecked. Samples are loaded and acquired for two minutes (or specific fixed time for all samples). The lowest speed is used. The image gallery presents events that will be collected when the appropriate population is chosen for display.

2. **Acquisition gates.** Set on low scatter signals that are 2-3 decades lower than speed beads (Figure 2). However, gates can be set on different scatter populations. The low scatter population is mostly used for analysis and to exclude the speed beads. Using a higher scatter cannot rule out the inclusion of some debris, small cells, or speed beads. If only large EVs are of interest, lowering the scatter laser power may be advised; however, this runs the risk of missing the smaller EVs. IFC has the ability to confirm each EV morphology, which helps to set the proper scatter range.

3. **Compensation controls.** Single-stained EV samples are measured per standard protocol.

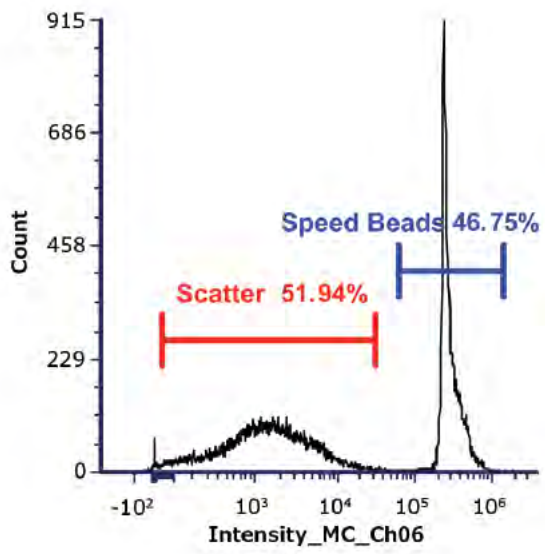


Figure 2. Example of scatter range.

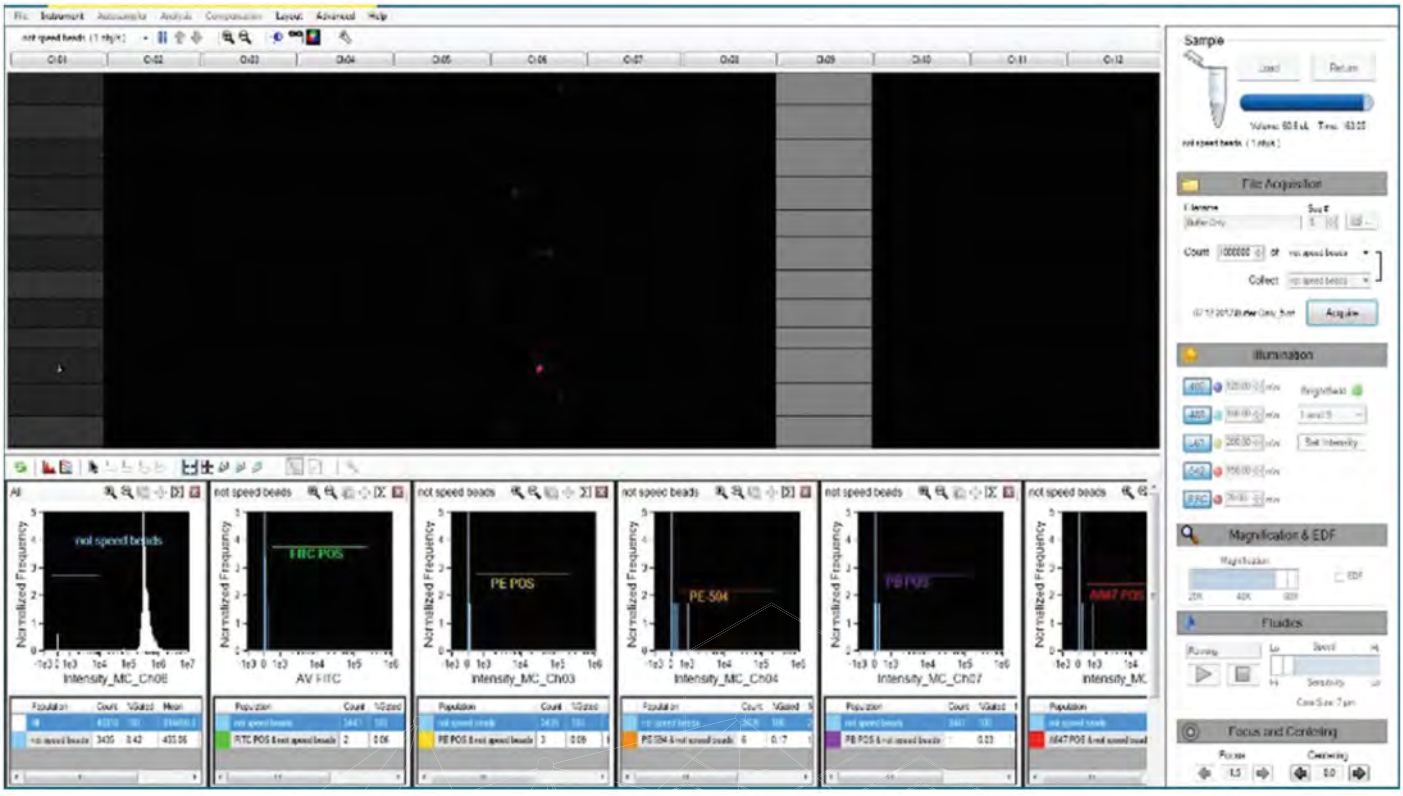


Figure 1. Screenshot example of INSPIRE® acquisition software measuring buffer only.

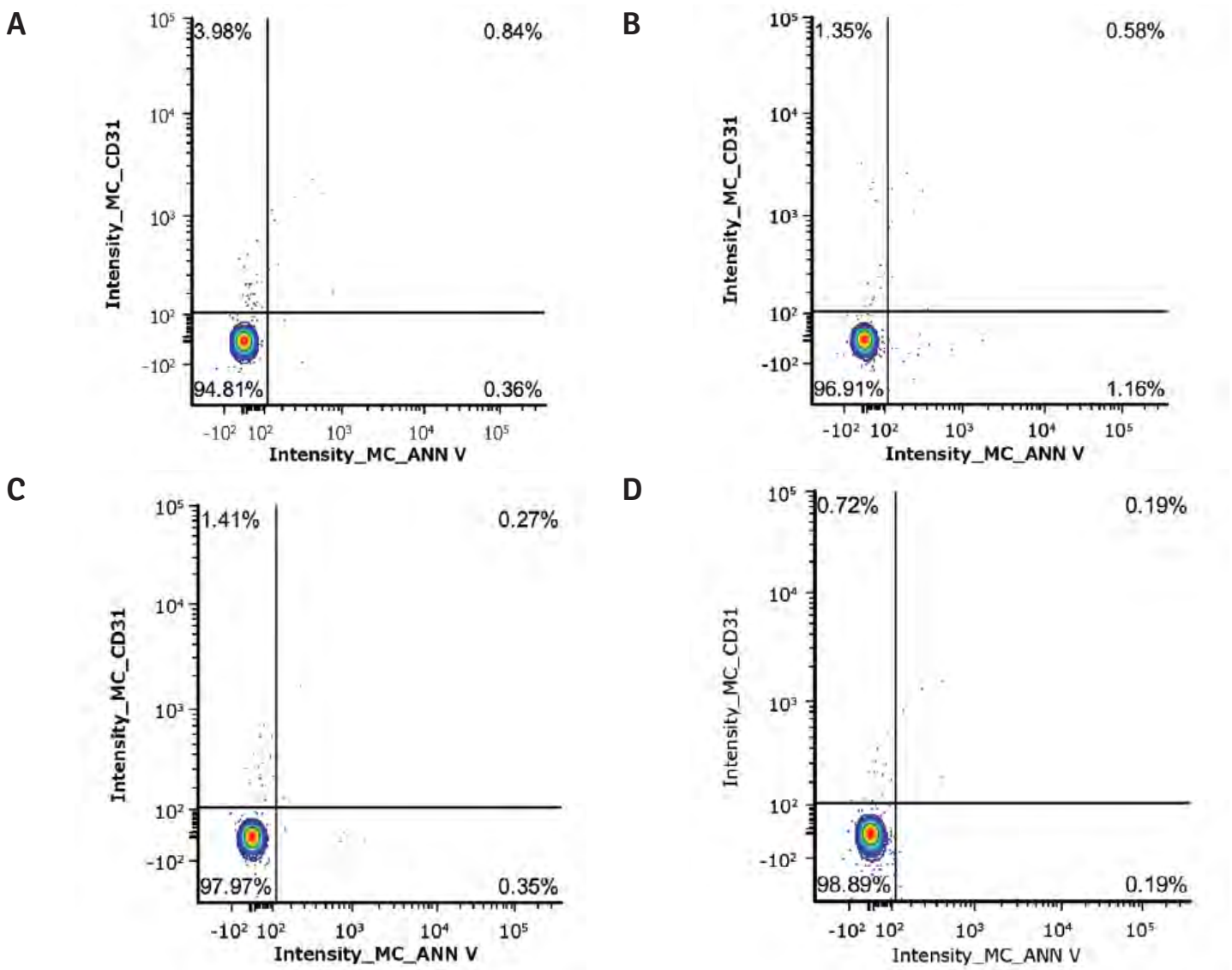


Figure 3A. Buffer only (collected for 2 minutes or a fixed time for all samples). Buffers should be filtered with a 0.1 µm filter to remove any particulates. Ideally, the sheath fluid should also be filtered similarly.

Figure 3B. Buffer plus reagents (e.g., antibody or dye). Emerging evidence shows that antibodies—in particular, larger fluorophores— and some lipophilic dyes can mimic the appearance of EVs by aggregation or micelle formation. This control is needed to rule out such artifacts. All reagents should be added to the buffer at the same concentration used experimentally and collected for 2 minutes, or a fixed time, for all samples.

Figure 3C. EV sample plus detergent (0.01% SDS or other detergents; lipid-based samples only). NOTE: Type and concentration of detergent required might be different for different EV populations of different compositions and origins.2

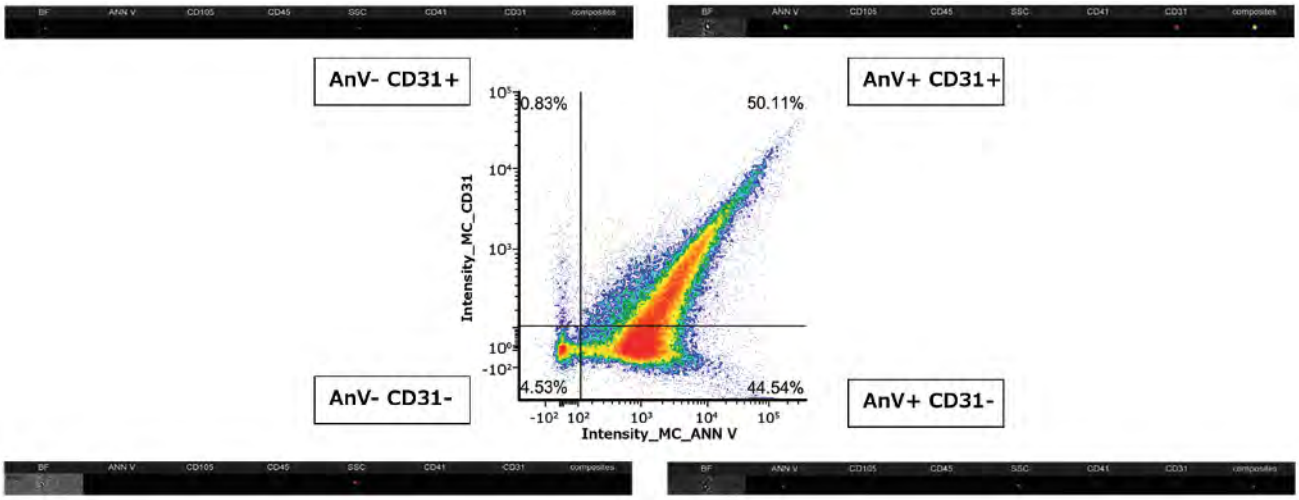
Figure 3D. Determine positive EVs by testing unlabeled EVs. An unlabeled control sample can be used to establish the gating of subpopulations, as in this example with CD31 and AnV markers.

4. **Other important controls.** Controls should be reported for each protocol and in each publication. Some examples are shown in Figure 3.

5. **Analyzing EV samples with antibodies for endothelial and membrane markers.** Compensation matrix is used per standard protocol. EV count can be determined by the volumetric method as summarized in this example (Figure 4) using CD31 APC (platelet endothelial cell adhesion molecule, PECAM-1) and AnV FITC staining as markers.

6. **Fluorescent calibration.** Specific fluorophore beads (e.g., from Bangs Laboratory), whose intensities have been calibrated in units of mean equivalent soluble fluorochromes (MESF) are used to calibrate the fluorescence scale in units of MESF. This allows fluorescence expression across different platforms in terms of a standard unit of measure. In addition, these beads can be used to calculate the resolution limit (i.e., the number of fluorescent molecules needed to detect a signal above background).

A. IFC images analysis.



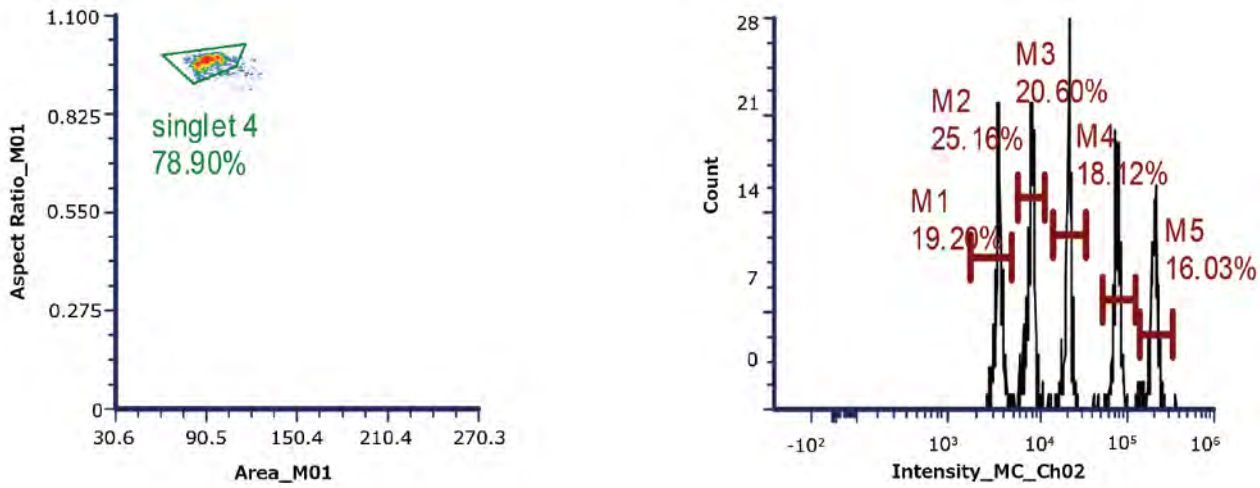
B. IFC statistical analysis.

Intensity_MC_ANN V, Intensity_MC_CD31

Population	Count	Objects/mL
Low Scatter	10,998	5,943,163
AnV-CD31- & Low Scatter	2,915	1,575,225
AnV-CD31+ & Low Scatter	1,492	806,256
AnV+CD31+ & Low Scatter	5,297	2,862,424
AnV+CD31- & Low Scatter	1,232	665,755

Figure 4. Example EV sample.

A. Singlet gate (left) and a histogram (right) of different beads tested.



B. Information about the channel calibration provided by FCS Express 6.

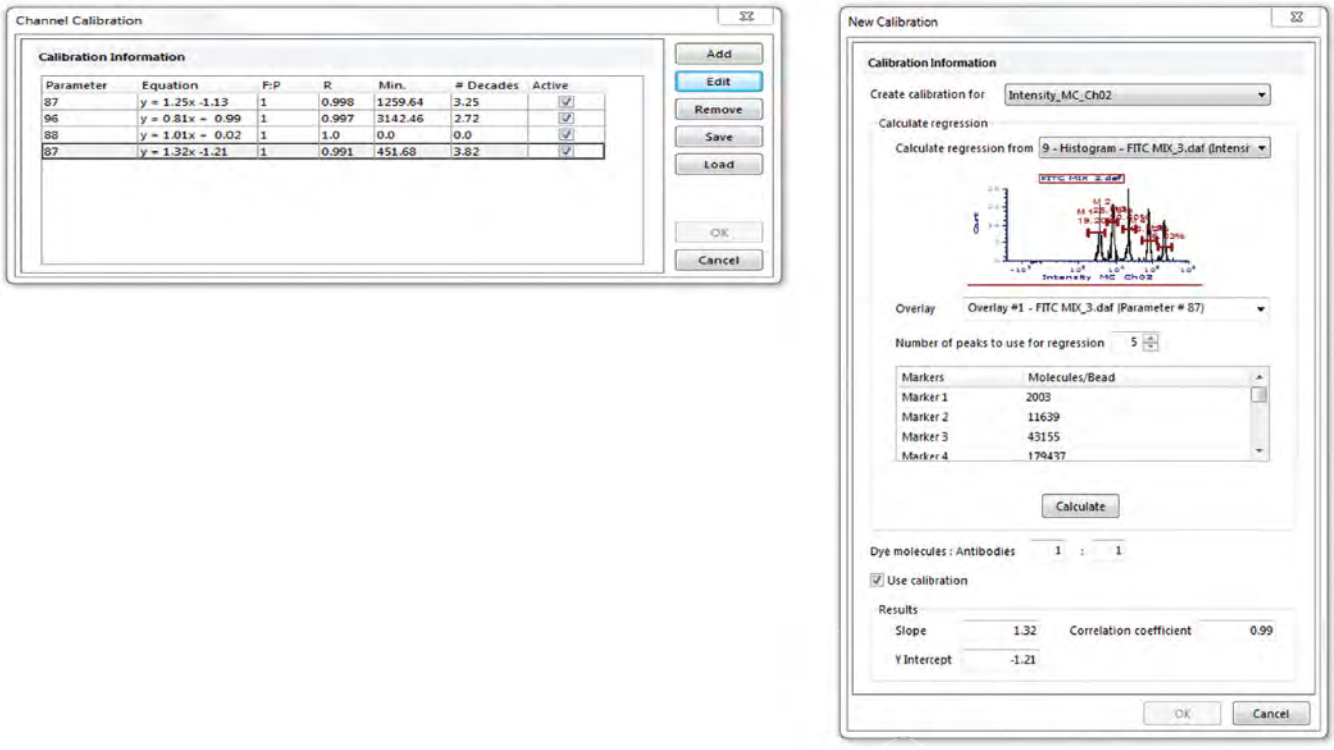


Figure 5. Example of calibration bead gating.

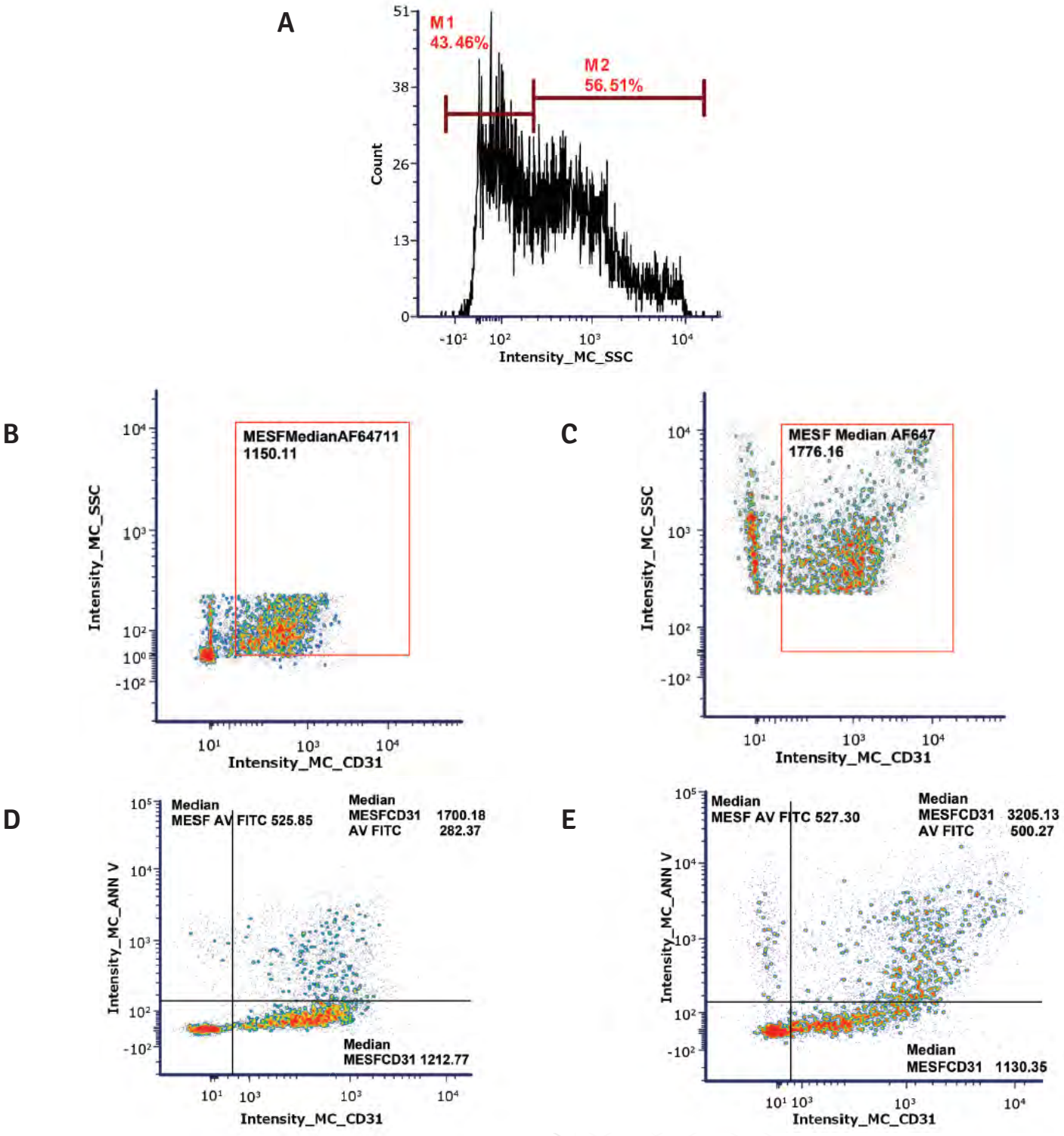


Figure 6. Example EV sample.

MESF beads should be vortexed and used undiluted as their concentration is generally low. All lasers used in EV studies are set to maximum power except the scatter laser, which is set to 5mW due to the increased scatter of polystyrene beads. At least 2,000 bead events are acquired for each bead. The .rif files are exported as FCS files from the ImageStream^X Mk II System using IDEAS® Software. Using FCS Express 6 (De Novo™ Software), bead intensities from the singlet gate (Area Ch01 vs. Aspect ratio Ch01) are displayed in a histogram (channel calibration option is turned off; Format/Specific Option) (Figure 5). Markers are drawn around each intensity population. Using FCS Express Channel Calibration under Tools, MESF values (provided by the bead manufacturer) are entered for each peak and then, using the calculate button, linear regression is calculated. This calculation can be saved and loaded into subsequent experiment layouts to be used in converting median fluorescent intensity statistics to MESF values. Alternatively, one can calculate linear regression manually by plotting the log of median intensity vs. the log of the MESF value.

Figure 6A gives an example of two gates for low and high scatter. Figures 6B and 6C show that the high scatter gate for CD31 has a slightly higher median MESF value vs. the low scatter gate (1,776 vs. 1,160, respectively). Figures 6D and 6E show the different median MESF values for each quadrant of a dot plot when double staining with CD31 (PECAM-1) APC and AnV FITC. The values are slightly different but in the same range, from 200-1,300. In comparison, whole cells have MESF values, measured using the same technique, of approximately 100,000 to 200,000.[2]

Considerations

A. Imaging flow cytometry offers targeted phenotyping. Still, other tools for characterizing EV size (e.g., nanosight technology or tunable resistive pulse sensing), morphology (e.g., cryo-electron microscopy), density (densitometry), and/or protein content (e.g., Western blotting) must be used to achieve a comprehensive analysis of EVs.[1,3]

B. Incorrect particle phenotyping caused by coincident events has the potential to lead to false conclusions about the biology of EVs. Quantitative features in IDEAS Software can help to identify these false positive events.[1] In general, swarming (high levels of coincidence) is less common using the ImageStreamX Mk II platform; however, concentrations greater than 108 particles per uL should be diluted. Multiple EV images per frame indicate the concentration is too high.

C. The following resulting information should be reported:

- All details of sample source and isolation
- Size range of EVs
- Total EV concentration per mL of biological fluid
- Concentration of phenotypic EV subsets per mL
- MESF values of fluorescent positive populations
- Representative images

Conclusions

Imaging flow cytometry for EV detection combines increased fluorescence sensitivity, low background, image confirmation ability, and powerful data analysis tools. Measurement of EVs as small as 100 nm is possible in a high-throughput manner. MESF experiments should be performed as they provide additional information about the fluorophore/antigen density and allow comparison between other studies and instruments used. Best practices for evaluating EVs are still in development and are forthcoming.

About the authors

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Amnis® ImageStream®X Mk II Flow Cytometer High Gain Mode for Increased Sensitivity in the Detection of Small Particles

With this setting, even more EVs and virus particles can be detected.

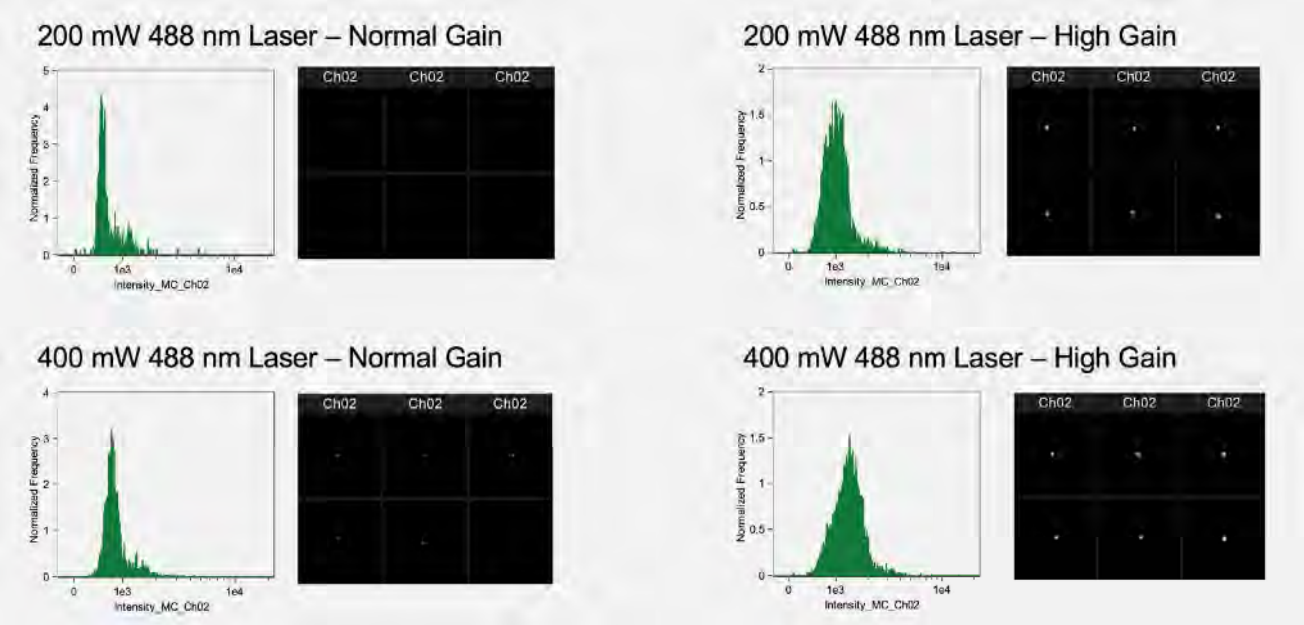
Haley R. Pugsley, Ph.D, María Gracia García-Mendoza, Ph.D, and Bryan R. Davidson

High Gain mode for the Amnis® ImageStream®X Mk II Flow Cytometer is designed to detect small, dim particles such as extracellular vesicles (EVs) and viruses. In High Gain mode, the time-delay integration (TDI) CCD camera at the heart of the Amnis® Technology is adjusted to a higher gain setting to maximize signal while minimally increasing the noise, allowing for increased sensitivity and increased signal from small particles. In addition to increasing the gain, the object detection thresholds and masking have been adjusted to better identify small objects like EVs and viruses. High Gain mode is designed to work at 60X and at slow speed. With the addition of a 400 mW 488 nm laser and an increase in photonic sensitivity, even more EVs and virus particles can be detected.

Example 1: Murine leukemia virus-sfGFP reference particles show an increase in object detection.

MV-M-sfGFP reference particles from ViroFlow Technologies, Inc., are inactivated murine retroviruses produced in mouse cells that express superfolder green fluorescent protein (sfGFP) on the outer surface of the viral envelope. They are small fluorescent reference particles for flow cytometry with a size of ~120 nm. In this experiment, the MV-M-sfGFP particles were reconstituted in 0.1 µm filtered water and diluted 1:400 in PBS. The samples were acquired for three minutes at four collection settings: Normal Gain 200 mW 488 nm laser power; Normal Gain 400 mW 488 nm laser power; High Gain 200 mW 488 nm laser power; and High Gain 400 mW 488 nm laser power. Figure 1A shows the intensity histograms for

A



B

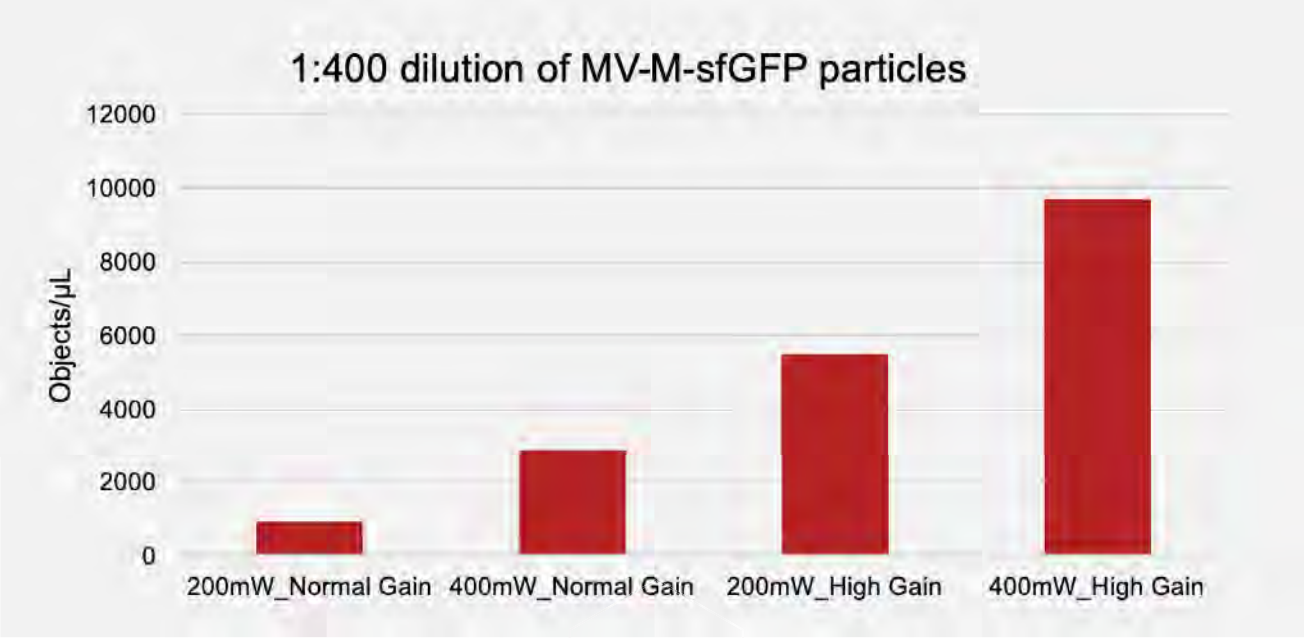


Figure 1. A) Histograms and representative images from the mean intensity for each of the collection settings. All images used the same display settings. B) MV-M-sfGFP diluted at 1:400 in PBS show an increase in objects per µL detected with increased laser power and increased gain settings.

channel 2 (GFP channel) and the associated images from the mean intensity for the different gain and power settings. Note, as more dim objects are detected by the system when using increased laser power and/or High Gain, the mean intensity for the GFP+ population will not necessarily double because the dim particles that were not previously detected will lower the overall average intensity. Figure 1B shows an increase in GFP+ objects detected with increased laser power as well as increased camera gain.

Example 2: 0.22µm fluorescent beads demonstrate an increase in photonic sensitivity.

To illustrate the increase in fluorescent signal using High Gain with the 400 mW 488 laser, 0.22µm yellow SPHERO™ Nano Fluorescent particles were acquired on the ImageStreamX Mk II in Normal Gain and High Gain modes using the 488 nm laser at 200 mW and 400 mW. Figure 2 shows a clear increase in the mean intensity with both High Gain and the 400 mW 488 nm laser. In this example, 2,000 bead images were collected, and the mean fluorescent intensity for channel 2 was reported.

Example 3: HEK293-derived EVs pre-labeled with CFSE show no swarm detection.

HEK293-derived EVs from Exosomics S.p.A pre-labeled with carboxyfluorescein succinimidyl ester (CFSE) were serially diluted (1:10, 1:50, and 1:100) in PBS and data was acquired for three minutes at four collection settings: Normal Gain 200 mW 488 nm laser power; Normal Gain 400 mW 488 nm laser power; High Gain 200 mW 488 nm laser power; and High Gain 400 mW 488 nm laser power. The dilution series was performed to verify the absence of swarming (swarm detection or coincident events), which happens when multiple objects such as EVs are captured as a single event.

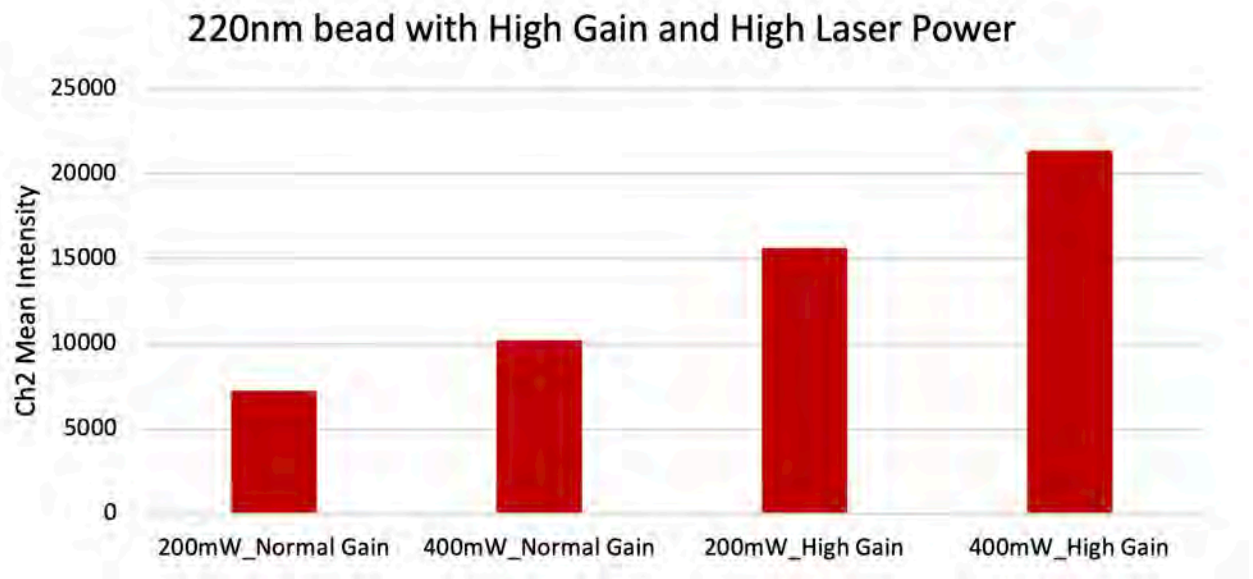


Figure 2. Increased fluorescent signal using High Gain mode and a 400mW 488nm laser on 220nm yellow SPHERO™ Nano Fluorescent particles.

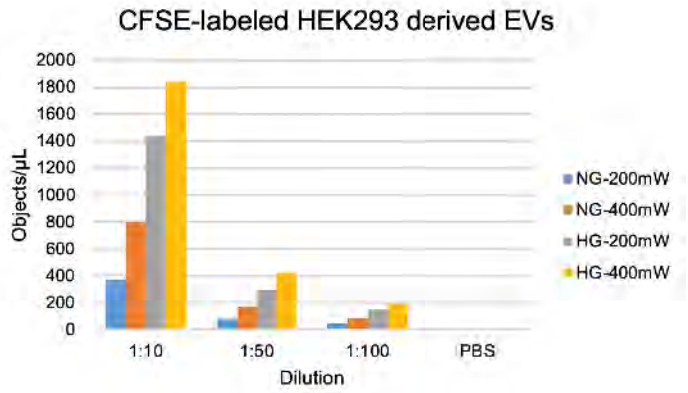


Figure 3. CFSE-labeled, HEK293-derived EVs show increased objects per µL when detected at High Gain with 400 mW 488 nm laser power. CFSE-labeled, HEK293-derived EVs dilution series and collection settings: Normal Gain (NG) with 200 mW 488 nm laser power; Normal Gain (NG) with 400 mW 488 nm laser power; High Gain (HG) with 200 mW 488 nm laser power; and High Gain (HG) with 400 mW 488 nm laser power.

Figure 3 summarizes the detected objects per µL for the CFSE-labeled, HEK293-derived EVs and the PBS control. These results show the dilution series concentration decreases as expected, indicating that swarm detection was not occurring. This trend held true for the different collection settings. Analysis of the various collection settings shows an increase in CFSE+ events, with both an increase in laser power and an increase in camera gain.

Example 4: RBC-derived EVs labeled with anti-CD235ab-PE show how antibodies can be used to immunophenotype EVs.

EVs derived from red blood cells (RBCs) purchased from Cellarcus Biosciences were labeled for one hour at room temperature with anti-CD235ab-PE (BioLegend). After labeling, the samples were serially diluted (1:15, 1:30, and 1:60) in the Cellarcus Biosciences vFC™ Staining Buffer. The PE-labeled EV samples were acquired for three minutes at four collection settings: Normal Gain 200 mW 488 nm laser power; Normal Gain 400 mW 488 nm laser power; High Gain 200 mW 488 nm laser power; and High Gain 400 mW 488 nm laser power. Control samples for antibody-only, buffer-only, and detergent controls were similarly diluted in vFC™ Staining Buffer and acquired in the same manner as the EV samples. Labeled EVs were incubated in 0.1% Triton™ X-100 for 10 minutes to break down the EVs. Figure 4A summarizes the objects per µL for the PE-labeled, RBC-derived EVs. The detected objects per µL linearly decreased as expected for the dilutions, indicating there was no swarm detection occurring, and there is a clear increase of PE+ objects detected with both increased laser power and increased gain settings. The control samples for the antibody-only and detergent controls are shown in Figures 4B and 4C, respectively. The vFC™ Staining Buffer control had zero PE+ objects per µL detected (data not shown). While the antibody-only controls and detergent controls show the same trend as the labeled EVs, there is a clear difference in the number of objects per µL compared to the PE-labeled EV samples, validating the EV population.

Summary

These four examples demonstrate the use of High Gain mode and the high-powered 488 nm laser on the ImageStreamX Mk II. The 0.22µm yellow fluorescent beads showed a consistent increase in signal intensity with increased laser power and increased gain. The EV and virus examples all showed an increase in positive objects detected for both increased 488 nm laser power and increased gain settings. Using serial dilutions, we were able to verify the absence of swarming. The RBC-EV data demon-

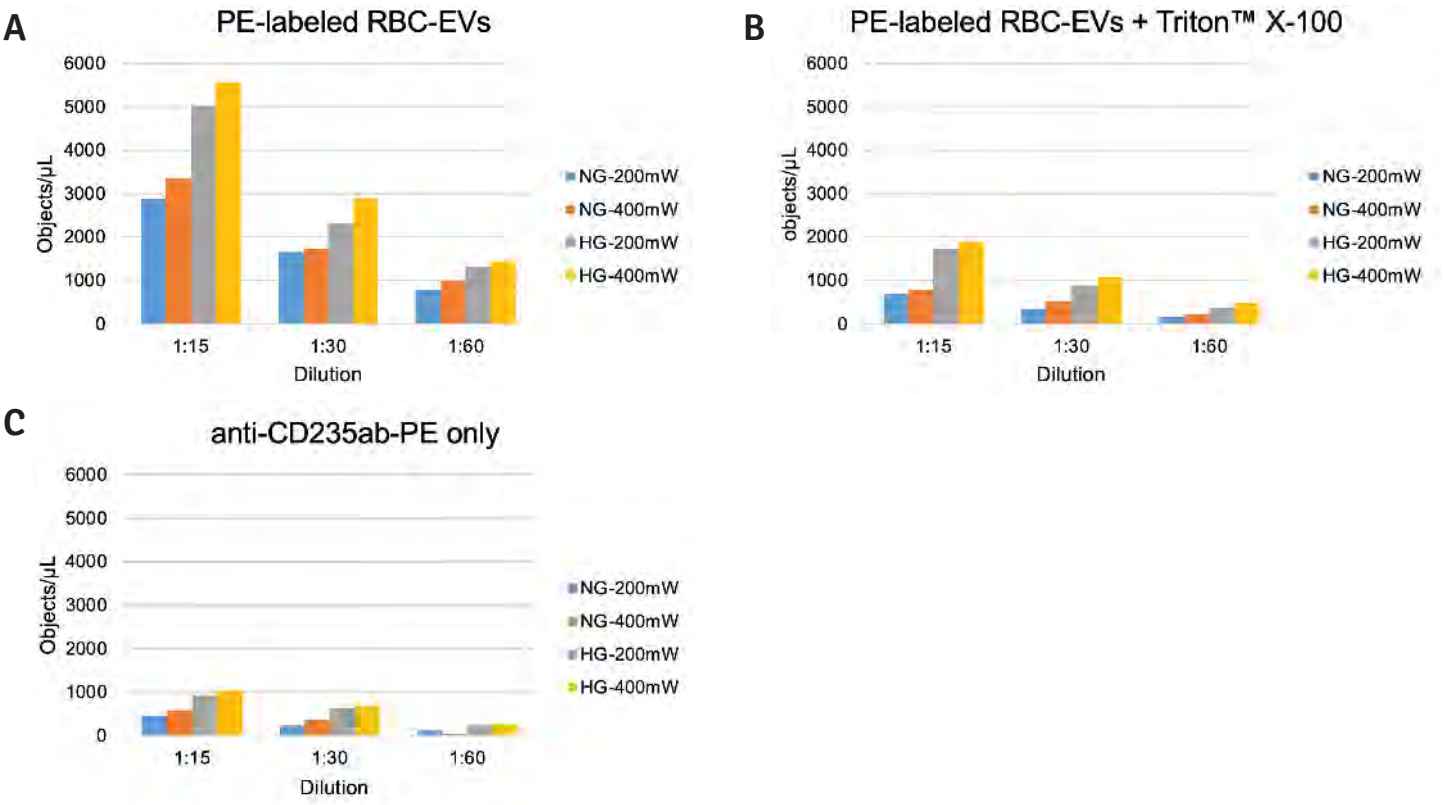


Figure 4. PE-labeled, RBC-derived EVs show an increase in objects per µL when detected at High Gain with 400mW 488 nm laser power. The bar graphs show the dilution series for A) PE-labeled, RBC-derived EVs, B) Triton™ X-100 detergent controls breaking down the labeled EVs, and C) Antibody-only controls for the four collection settings: Normal Gain (NG) with 200 mW 488 nm laser power; Normal Gain (NG) with 400 mW 488 nm laser power; High Gain (HG) with 200 mW 488 nm laser power; and High Gain (HG) with 400 mW 488 nm laser power.

strates the ability to use antibodies that specifically identify the type of EV, with the potential to use multiple fluorochromes to characterize the EVs further, or measure multiple EV types in a single sample.

Acknowledgments

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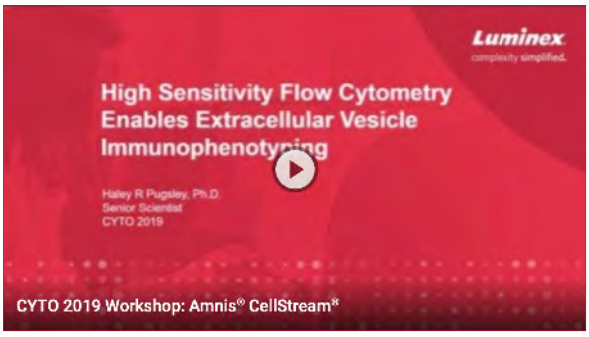

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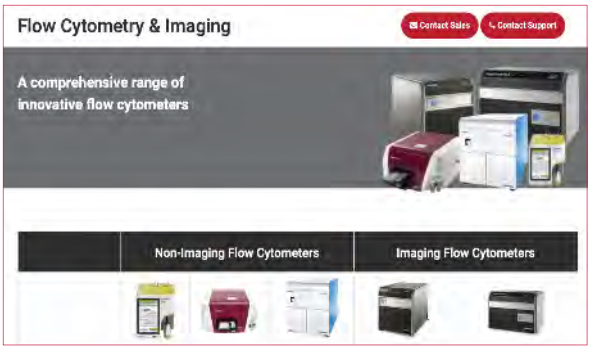

Imaging FC