Beckman Coulter CytoFLEX Violet SSC: an Alternative to FSC PMT or Fluorescence in the Detection of Extracellular Vesicles

TECHNICAL INFORMATION BULLETIN

Principle of the Technique

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Introduction

Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). (1) The release of EVs has been reported in the pathologies of cancer (2-5), neurological, hematological (6), cardiovascular (7), autoimmune and rheumatologic diseases (8), and viral infections such as malaria (9). The study of EVs is gaining increasing interest within both the medical and scientific communities due to the diagnostic and therapeutic possibilities. However, the identification and classification of EVs has been problematic. Although advances in various fields, including microscopy, have addressed some of the preliminary hindrances, flow cytometry remains the dominant approach for the characterization of submicron cell- derived particles. The primary hurdle in analyzing particles at the submicron level has been to accurately represent their size distribution and light scatter profiles. Instrumentation thresholds were originally designed using whole blood as the standard, thereby excluding cellular measurement below 3um. Recently, flow cytometric technology has been developed to distinguish populations spanning the <400nm to 1um range. In this independent study, several of those technologies are evaluated and compared. As most of the hardware adjustments are accomplished by enhancements to the FSC parameter, the study will also evaluate the use of Violet SSC on Beckman Coulter's CytoFLEX as a novel approach to small particle detection. According to Mie theory, it is hypothesized that Violet SSC will give comparable results, as the lower wavelength will allow for detection of smaller particles.

Overview of Methods

Bangs Labs' Dragon Green Beads, Beckman Coulter's PCS controls and Spherotech's SPHERO Nano Fluorescent Particles will be acquired on several different instruments. Each of these instruments utilizes slightly differing hardware features to enhance the instruments ability for small particle detection. 192nm, 520nm and 780nm Dragon Green Beads were obtained from Bangs Laboratories, Inc. Dragon Green is an excellent spectral surrogate for fluorescein (488nm/530nm), and is suitable for use with fluorescein filter sets.

A photon correlation spectroscopy (PCS) latex, 5 x 15 mL, mixed kit was obtained from Beckman Coulter, Inc. 100nm, 200nm, 300nm and 500nm latex beads were chosen. PCS controls are non-labeled and are comparable in scatter profile to Dragon Green Beads.

SPHERO Nano Fluorescent Particles were obtained from Spherotech, Inc. 130nm fluorescent yellow particles are visualized in the fluorescein (488nm/530nm) channel and will be acquired for verification of <200nm detection.

For this flow cytometric assay, particles were chosen at the sizes listed above for later cell tracking. By using beads of differing sizes and fluorescent intensities, one can optimize the flow cytometer for cellular analysis. The bead sizes were chosen to be comparable to the size of the cells being analyzed. Therefore, all voltages, gains, and threshold settings were optimized for the both Dragon Green Beads and PCS controls to develop a relative size distribution matrix. The bead concentrations have been previously determined by serial dilution and subsequent measurement on Beckman Coulter's

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MoFlo Astrios EQ, MoFlo XDP with Propel Labs NanoView attachment, and Gallios flow cytometers in conjunction with manufacturer's specifications.

Instrumentation

MoFlo Astrios EQ

The Beckman Coulter MoFlo Astrios EQ is equipped with two FSC PMT pathways separated by a beam splitter (60/40 split). FSC1 is a direct laser beam pathway, with the FSC2 being directed at an angle from the beam splitter. Seven different and unique masks are provided to optimize particle identification and focus laser light to the photo multiplier tubes (PMTs). Due to this specific design, particles from 200nm to 30um can be identified (together or individually). In addition, The MoFlo Astrios EQ allows for triggering from any of the scatter parameters associated with the seven laser lines.

For this study, the beam splitter is removed from the FSC assembly. This allows the maximum amount of laser light to be detected through the FSC1 detector. In addition, a P1 mask was chosen after testing all the masks (data not shown). For small particle detection, the larger opening on the P1 mask allows for maximum resolution and dynamic range. Finally, 488 SSC was used for triggering. While not a manufacturer's recommended procedure, the Flow Core at Beth Israel Deaconess Medical Center has found this to be a viable and effective methodology.

MoFlo XDP with NanoView module

Propel Labs' NanoView forward scatter detector (FSC) was integrated onto a Beckman Coulter MoFlo XDP cell sorter. The NanoView design has improved the optical and electrical systems over the standard FSC for the purpose of extending the detection range down to < 200nm particles. The new optical system design utilizes a custom aspheric imaging lens that has been optimized to collect the scattered light from the core stream and image it onto a 200 micron pinhole. The collection angles in the forward scatter direction extend up to 18 degrees, which is double the maximum collection angle of a standard MoFlo FSC detector. The pinhole serves to align the system and remove the stray laser light that has not been generated by the particle of interest and greatly reduces the background light that is received at the detector. The NanoView design has further improved the detection system by replacing the photodiode with a much higher sensitivity PMT detector in the FSC path.

For this study, the largest available blocker bar was used (±6.3° - ±12.6° angles blocked as rotated from horizontal to vertical) and set to an approximate angle of 45°. 488 SSC was used as the trigger parameter.

Gallios with Kaluza G

Beckman Coulter Gallios is equipped with an enhanced wide angle scatter setting, listed as submicron on Kaluza G acquisition software. The FS photodiode sensor collects the laser light that is scattered at narrow angles to the axis of the laser beam. The forward angle light is filtered with a 488nm band pass before reaching the FS sensor to generate voltage pulse signals. The FS sensor set at submicron allows for the detection angle to be measured at 9° to 19°. The SS photodiode sensor collects the light that is emitted 90° from the laser excitation point. The emitted light is focused by gel coupling of the flow cell and the light is filtered by a 488nm band pass filter.

CytoFLEX

Beckman Coulter's CytoFLEX is equipped with custom fluidics and the ability to use Violet (405nm) Side Scatter (VSSC) as a trigger parameter. The CytoFLEX has the ability to both trigger off and analyze by VSSC. This is an important feature when considering Mie theory and its effects on small particle detection.

Mie theory predicts that the scattering cross section of a particle, and thus its scattering intensity is dependent on the wavelength of light, the angle of collection, and the size, shape, and refractive index of the particle.

Inside the WDM module, the fluorescence light is divided and tightly focused through a series of band pass filters and integrated optics, on to an array of ultra-low noise silicon photo detectors.

All other factors being equal, using a **shorter illumination wavelength** will result in an increase in scattering cross section, and thus more scattered light.

Therefore, using the VSSC parameter, Dragon Green Beads will be visible and distinct below 500nm as lower wavelengths of laser light allow for smaller particle size detection. Additionally, the CytoFLEX sheath delivery can be easily controlled through the software interface. The intuitive software control allows the user to manually control the sample speed to maximize the amount of laser interrogation at slower uL/min flow rates. Hydrodynamic focusing is also enhanced to limit the ability of particle clustering known as swarming (10).

CytoFLEX Background

The proprietary optical design includes an integrated optics flow cell and photo diode detection system. In addition, all lasers are integrated to present optimal excitation. Emission of light is directed into dedicated fiber optical arrays, minimizing light loss and maximizing sensitivity.

CytoFLEX does not use PMTs – rather, CytoFLEX is the first commercial flow cytometer to utilize photo diodes for fluorescence channel detection. Photo Diodes, are very robust, linear, and sensitive.

The Fiber Array Photo Diode (FAPD) provides low-noise detection with high quantum efficiency and minimum light loss ensuring high signal to noise ratio and optical resolution especially with small particle measurements and dim fluorescence detection. The technology has its origin from the fiber optical communication industry, where the term Wavelength Division Multiplexing or WDM, originated. The CytoFLEX detection module collects the emitted light from each of the laser paths through high-efficiency fiber optic coupling. Each optical fiber delivers emitted laser light by a given excitation laser source, to a wavelength specific WDM detection module. Enhanced detection capability is achieved by using reflective, band-pass only filters to collect light and provide modularity and consistent sensitivity for all channels.

Instrument Optimization

Gating and Analysis

The Dragon Green Bead size distribution protocol, previously established in Research Application Note: Setting up the Beckman Coulter CytoFLEX for detection of Extracellular Vesicles, was applied to assess and measure EVs. Scatter properties were analyzed to determine the most efficient parameters for EV analysis.

QC was performed according to manufacturer's recommendations. All Instrumentation and protocols were configured for small particle detection. While instrumentation differs, the protocol remained consistent throughout. However, due to the MoFlo XDP with NanoView and MoFlo Astrios EQ's alignment system, micrometers on the FSC attachments were adjusted to maximize FSC signaling. The CytoFLEX required a bandpass filter change; no alignment or manual adjustments were performed.

A stock solution of filtered PBS with 0.1% Tween-20 is prepared. 520nm, 780nm and 192nm beads are diluted with the PBS/0.1% Tween-20 solution, to a final concentration of $1.29*10^7$ beads/mL.

Prior to dilution, the stock solutions of the test particles were sonicated to eliminate clumps.

The following samples were run on the Beckman Coulter CytoFLEX for instrument optimization:

1. 780nm Dragon Green Beads
2. 520nm Dragon Green Beads
3. 192nm Dragon Green Beads
4. 520nm/780nm/192nm Dragon Green Beads Mixed

- 780nm Dragon Green Beads were acquired to set the Scatter properties for differentiation between beads and low-end noise. In addition, SSC properties were adjusted to maximize resolution and dynamic range. The largest size is chosen first for ease of particle identification and to prove instrument ability to analyze below 1um.
- 192nm Dragon Green Beads were acquired to test the ability of the instrument to differentiate between the particle and noise. As particle size decreases, instrument Noise populations will begin to overwhelm the Dragon Green Bead's signal. 192nm Dragon Green Beads were used as most instrument manufacturer's specifications quantify lowest detectable level of 300nm.
- 520nm Dragon Beads were acquired for accuracy of separation of 192nm and 780nm beads. This allows for visualization of dynamic range of the instrument.
- Gates were drawn to encompass the three distinct populations.
- Mixed Dragon Beads were acquired to ensure proper gating and maximum separation of bead populations for the determination of a relative sizing distribution matrix. Furthermore, previous analysis has determined the ability of larger particles to mask the existence of their smaller counterparts (data not shown). Therefore, mixed populations verify the ability to separate and distinguish multiple populations.
- Voltages, gains and threshold settings can be adjusted to maximize performance. However, it is strongly suggested that single bead populations be acquired again, if changes are made to settings. Instrument has been optimized; the template and settings are saved for future cellular experiments.
- In addition to the Dragon Green Beads, a sample of the stock PBS solution is acquired for quantification of the background contribution of the PBS (Figure 1).

Results

Both the MoFlo Astrios EQ and MoFlo XDP with NanoView cell sorters were peaked to maximize resolution and separation of populations. The CytoFLEX and Gallios did not require any adjustments to alignment (thresholds, voltages and/or gains were set accordingly).

NOTE: 780nm Dragon Beads were not acquired on the MoFlo Astrios EQ due to the 520nm distribution. For this particular instrument, the resolution and dynamic range were evident by acquiring only the 192nm and 520nm Dragon Green Beads. All three Dragon Green Bead populations were easily separated from background and one another.

Visual inspection of the resolution and dynamic range of the four instruments shows the CytoFLEX be to comparable to the NanoView and MoFlo Astrios EQ, and slightly better dynamic range compared to the Gallios (Figures 2,3).

- Initial results, using Dragon Green Beads, indicate that VSSC is a viable option for the detection of EVs when compared to FSC PMT enhancements.
- Dragon Green Beads fluorescence (488nm/530nm) was used for verification of populations (Figure 4).

To further investigate VSSC as an alternative to the conventional mechanisms of EV detection, PCS control beads of 100nm, 200nm, 300nm and 500nm were evaluated. Results are expected to be similar to Dragon Green Beads. However, PCS controls do not contain a fluorescent marker. Therefore, measurements will be based solely on Scatter detection. Furthermore, due to the minimal change in the dynamic range between 0.19um and 0.52um Dragon Green Beads on the Gallios, it will be excluded from further investigation.
Using the Dragon Green Beads saved protocols on each instrument, the PCS controls were acquired according to the Dragon Beads protocol listed above.

- Clear separation between the Noise and 200nm PCS control was evident in all instruments tested.
- Dynamic range was consistent in all instruments tested.
- Resolution of populations was consistent in all instruments tested (Figures 5,6).

Upon visual inspection and comparison of the Mean Fluorescent Intensities (MFI) of the PCS controls ranging in size from 200nm to 500nm, the CytoFLEX, NanoView, and MoFlo Astrios EQ performed equally well (Figure 7).

Therefore, further evaluation of the CytoFLEX VSSC method of EV detection was done.

As most flow cytometric instrumentation with FSC enhancements have specifications of 200nm as the lowest detectable size, the CytoFLEX VSSC detection option has shown to be a viable option in EV detection. However, past studies have shown the MoFlo Astrios EQ and NanoView to be capable of <200nm for EV detection. Therefore, the CytoFLEX will be tested using both 100nm PCS control and

130nm SPHERO Nano Fluorescent Particle. By using both a fluorescent and nonfluorescent particle, a determination of feasibility of VSSC for <200nm EV detection can be made. First, 100nm PCS control will be acquired to determine if the particles can easily be separated from noise without sacrificing dynamic range (Figure 8).

Using the VSSC detection option on the CytoFLEX allowed for the acquisition of 100nm PCS control particles. However, adjustments in the scaling and threshold were required. 130nm SPHERO Nano Fluorescent Particle and the 192nm Dragon Green Beads were acquired on the CytoFLEX after scaling and threshold were adjusted. The fluorescent characteristics of the two sizing particles were used to separate the two particle populations (Figure 9).

- The two related sizes are difficult to separate using the Scatter parameter alone.
- By using the differing fluorescent intensities, the 130nm and 192nm sizing particles can be differentiated (Figure 10).

PBS Alone

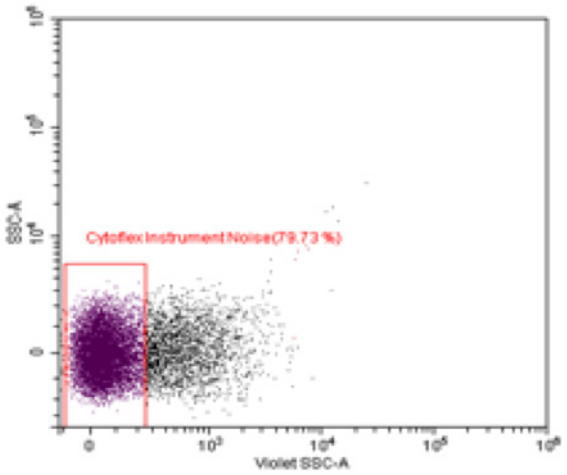


Figure 1. Stock PBS solution for quantification of the background contribution of PBS.

Dragon Green Beads CytoFLEX and Gallios

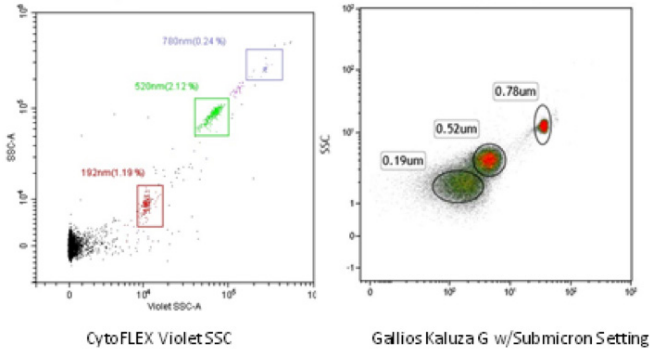
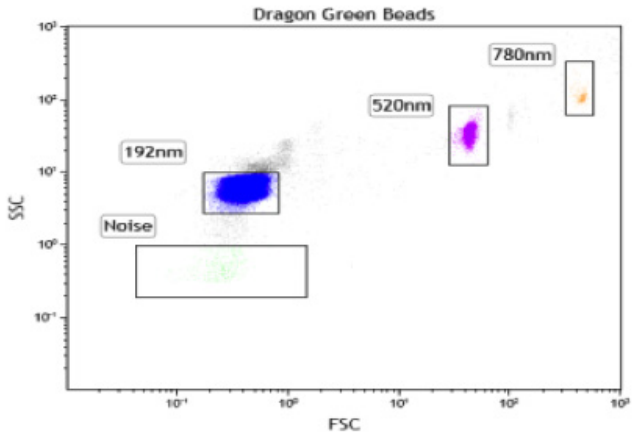
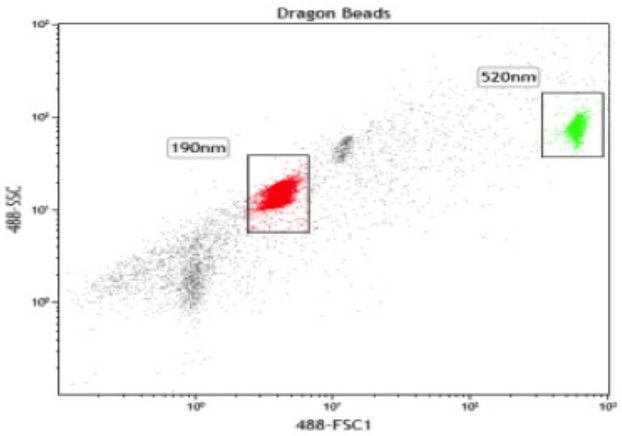


Figure 2 Verification of Dynamic Range by acquisition of 192nm, 520nm and 780nm Dragon Green Beads on CytoFLEX and Gallios.

Mixed Dragon Green Beads NanoView and Astrios EQ



NanoView 488 SSC trigger



Astrios EQ 488 SSC trigger/P1 Mask

Figure 3 Verification of Dynamic Range by acquisition of 192nm, 520nm and 780nm Dragon Green Beads on NanoView and 192nm and 520nm Dragon Green Beads AstriosEQ.

Dragon Green Beads Fluorescent Verification

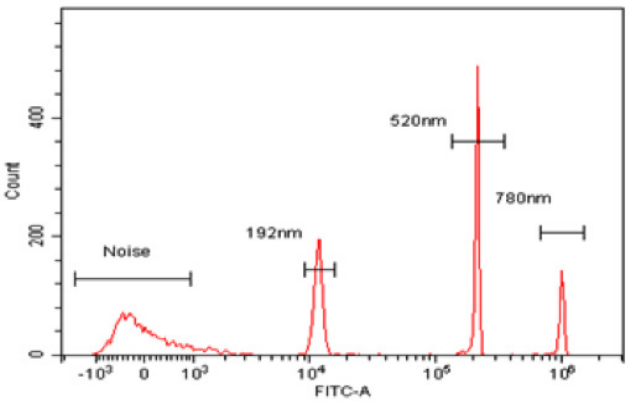


Figure 4 By using the fluorescent characteristics of the Dragon Green Beads, verification of size distribution based on fluorescent intensity can be used for data assurance.

PCS Controls CytoFLEX and AstriosEQ

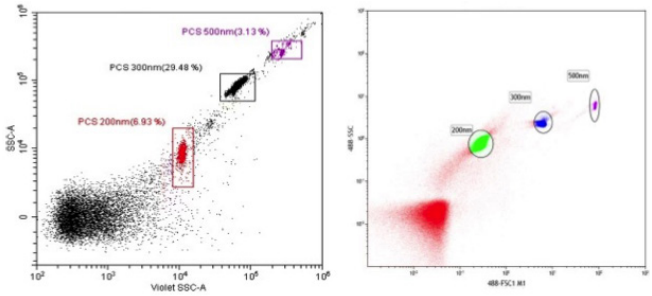


Figure 5 Photon Correlation Spectroscopy Control beads of 200nm, 300nm and 500nm were analyzed on the CytoFLEX and Astrios EQ. The data was used to compare size distribution across platforms using only scatter parameters.

PCS Controls MoFlo XDP w/NanoView

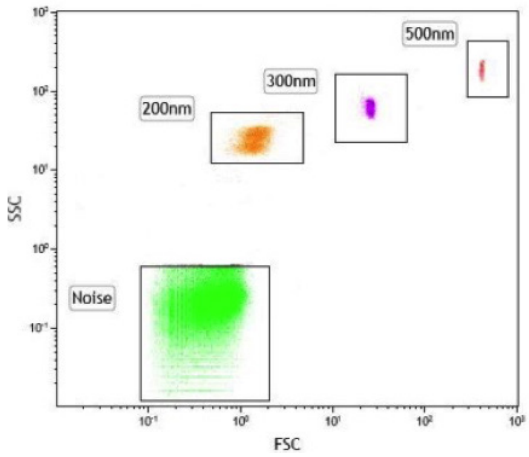


Figure 6 Photon Correlation Spectroscopy Control beads of 200nm, 300nm and 500nm were analyzed on the MoFlo XDP and compared to the Astrios EQ and CytoFLEX. The data was used to compare size distribution across platforms using only scatter parameters.

% Difference of MFI

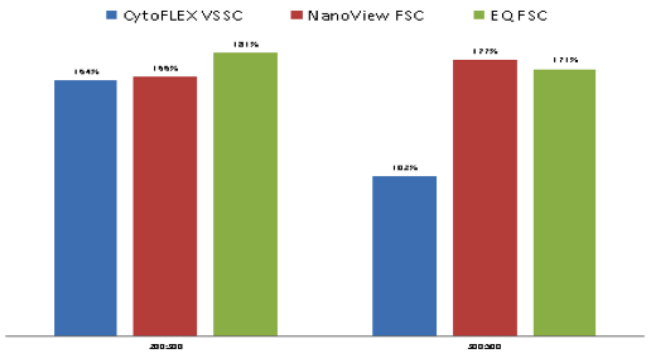


Figure 7 Measurement of Mean Fluorescent Intensity to calculate dynamic range and compare between the CytoFLEX and High End FSC modified Cell Sorters.

CytoFLEX 100nm PCS Control

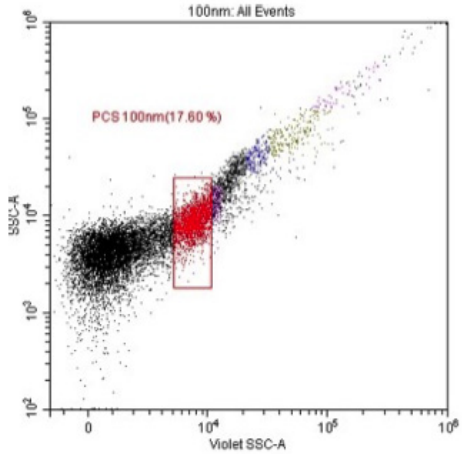


Figure 8 Photon Correlation Spectroscopy Control beads of 100nm in size were analyzed on CytoFLEX to establish a separation between instrument noise and particle.

130nm SPHERO Nano Fluorescent Particles and 192nm Dragon Green Beads

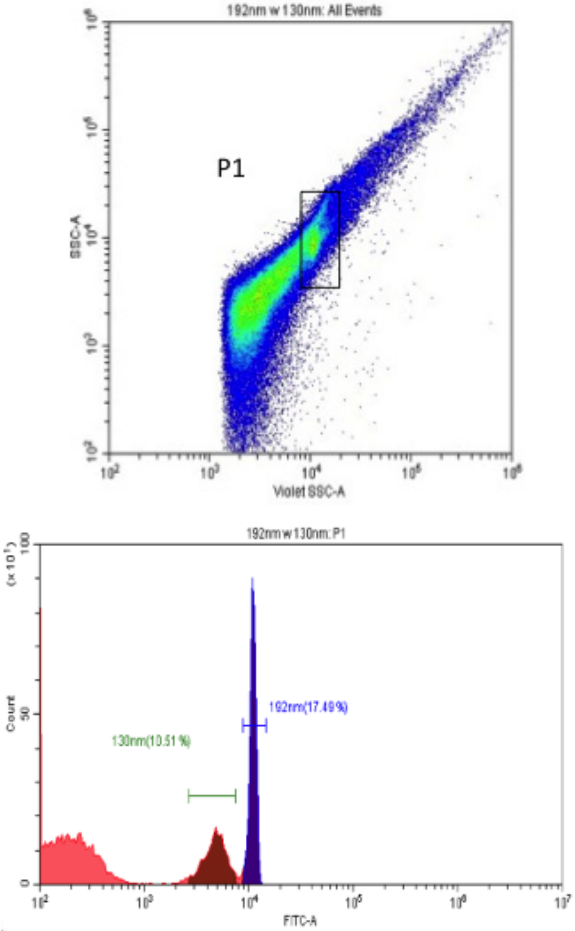


Figure 9 Nano Fluorescent particles and dragon beads 130nm and 192nm respectively were differentiated using a histogram of fluorescence intensities. The Scatter parameter alone was insufficient to separate the two bead samples.

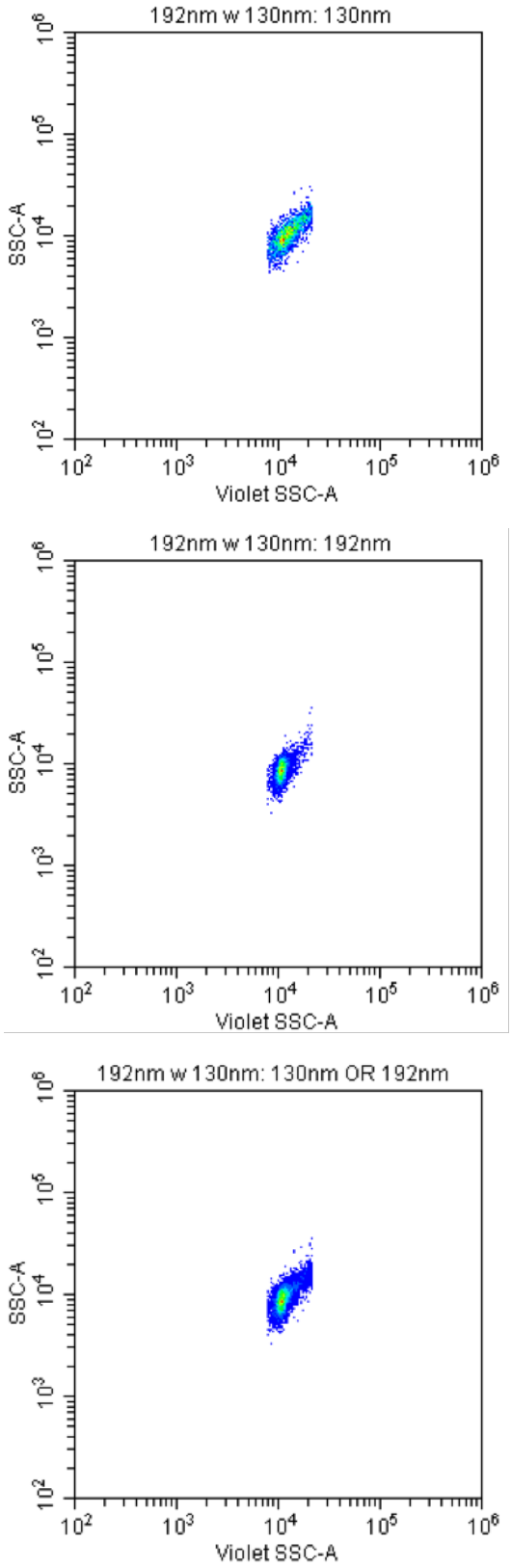


Figure 10 Back gating of the differing fluorescent intensities shows the inability to differentiate the populations via the scatter plots (Gated as listed in Figure 9). The 192nm DGBs mask the 132nm SPHERO Nano Fluorescent Particle.

Conclusion

The Beckman Coulter CytoFLEX's VSSC parameter has proven to be an effective tool for the detection of Extracellular Vesicles based on its ability to detect the different size beads as shown in the data. The ability to set the trigger and threshold using the VSSC parameter, allows the CytoFLEX to be comparable to its larger and more extensive counterparts.

By using methodologies and protocols previously established, the CytoFLEX was compared to three other flow cytometers where EV detection has been possible. Dragon Green Beads, PCS controls and Sphero Nano Fluorescent particles were acquired on a MoFlo Astrios EQ, MoFlo XDP with NanoView attachment, Gallios, and CytoFLEX. The CytoFLEX showed similarities in both resolution and dynamic range. However, there are limitations to the CytoFLEX VSSC detection system. Particles below 200nm can be resolved from the noise background to approximately the 100nm size range. Particles in the size range of 100nm to 200nm are not easily separated from one another on a Scatter Plot. Measuring differences in fluorescent intensity is the best means of separation.

The interest in the identification and detection of submicron particles has increased in recent years. The ability to study them has been hindered by available techniques to measure particles at sizes below 1um. Flow Cytometry has become an important tool in EV research with instrumentation being developed to identify particles at the submicron level. Instrumentation such as cytometers optimized to improve light scattering collection (11,12) and image cytometers (13). However, most equipment designed for the detection of EVs is expensive and complex. Hardware enhancements have focused around the development of the FSC PMT. While the FSC PMT enhancements have proven to enable the flow cytometer to detect particles <200nm in size, the instrumentation is not practical for all lab settings. In this comparison study, it has been shown that the CytoFLEX VSSC is compatible to 488nm SSC and to the results obtained from the AstriosEQ and NanoView enhanced FSC. The ability to resolve and distinguish the populations as effectively as its counterparts, has proven the CytoFLEX Violet SSC to be a viable alternative to the FSC PMT to detect EVs.

Methods based on the following Posters and Technical Papers

- Development of a Flow Cytometric technique for the study of Microparticles; Albert Mairuhu , R. Flaumenhaft, Vasilis Toxavidis, John Tigges
- NanoView: A Novel Approach To Microparticle Cell Sorting; J. Tigges, A, Vandergaw, V. Toxavidis
- Standardization of Flow Cytometry Instrumentation for the Analysis of Microparticles; J. Tigges, K. Groglio, E. Felton, M. Fahlberg, M. Schmelzle, R. Mairuhu, V. Toxavidis
- Microvesicle Detection and Cell Sorting; J. Tigges, V. Toxavidis
- MoFlo AstriosEQ – the New Standard in Forward Scatter and Fluorescence Dynamic Range Performance: a case study on microparticles ; V. Toxavidis, R. Sleiman, T. Reed, J. Tigges

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FLOW-990APP08.15-A