Setting up the Beckman Coulter CytoFLEX for Detection of Extracellular Vesicles

TECHNICAL INFORMATION BULLETIN

Vasilis Toxavidis, Virginia Camacho, and John Tigges; Affiliation: BIDMC, CLS 932 – Flow Cytometry Core Facility, 3 Blackfan Circle, Boston, MA 02115

Introduction

There is great interest in both the medical and scientific communities in submicron cell-derived particles termed Extracellular Vesicles (EVs). A great difficulty in this field, however, has been the optimization and standardization of techniques to measure these small particles. Although competing techniques have been developed, flow cytometry is a convenient approach, for life science researchers. The hurdle in analysis has always been the ability to accurately measure the size characteristics of small particles; especially when only considering scatter properties. Flow Cytometry instrumentation was traditionally designed to perform whole blood analysis and, therefore, cellular measurements above 3µm. Particles detected below the 3µm "threshold" were considered to be debris. Due to advances in microscopy and the ability to identify and characterize than 1µm cellular particles, hardware upgrades to the scatter parameters of flow cytometry instrumentation have been developed to micro particle populations from <200nm to 1µm. However, the accuracy of these measurements and the validity of the results are frequently questioned due to insufficient reproducibility. In this study, previous methodologies to define a range for detection of EVs will be explored on Beckman Coulter's new CytoFLEX platform.

Technical Considerations of the CytoFLEX

The patent-pending 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 patent-pending Wavelength Division Multiplexing (WDM) detection module uses solid-state, high efficiency, low-noise Fiber Array Photodiode Detectors (FAPD), giving you exceptional resolution for more precise data and better detection of rare events.

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

BECKMAN COULTER

Life Sciences

coupling. Each optical fiber delivers emitted laser light by a given excitation laser source, to a wavelength specific WDM detection module. 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. Enhanced detection capability is achieved by using reflective, band-pass only filters to collect light and provide modularity and consistent sensitivity for all channels.

Materials and Methods

0.19μm, 0.52μm and 0.78μm 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. Many imaging applications rely on fluorescent microspheres for detection of binding events or signal enhancement. Addressable bead populations may be created with different intensities of fluorescence for the development of multiplexed flow cytometric assays, and small fluorescent spheres can function as reporters for ELISA-type assays. Fluorescent microspheres are also useful for fluid tracing, cell tracking, and phagocytosis studies.

For this project internally labeled Dragon Green fluorescent microspheres were used. Fluorescent microspheres are internally dyed using a solvent swelling/dye - entrapment technique.
Internal dyeing produces very bright and stable particles with typically narrow fluorescence CV's. With this strategy, surface groups remain available for conjugating ligands (proteins, antibodies, nucleic acids, etc.) to the surface of the bead, which is important for analyte-detection and immunoassay applications. Internally-dyed beads are also used extensively in imaging applications, as they offer a greater resistance to photobleaching.

For this flow cytometric assay, Dragon Beads were chosen at the sizes listed previously 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 Dragon Green Beads to develop a relative size distribution matrix. The bead concentrations have been previously determined by serial dilution of the beads and subsequent measurement on Beckman Coulter's MoFlo Astrios EQ, MoFlo XDP with Propel Labs NanoView attachment, and Gallios flow cytometers.

Instrument Optimization

Gating and analysis

The Dragon Bead size distribution protocol was applied to assess the CytoFLEX's ability to measure EVs. Scatter properties will be analyzed to determine the most efficient parameters for EV analysis. The CytoFLEX has the ability to both trigger off and analyze by Violet (405nm) Side Scatter (VSSC). For purposes of this study the VSSC will be used. Based on previous studies, a lack of hardware enhancements to the FSC parameter (a PMT or angle of light adjustment) does not allow for detection of particles below 0.5um (Figure 1).

Using the VSSC parameter, Dragon Green particles will be visible and distinct below 500nm as lower wavelengths of laser light are theorized to 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, of particles to maximize the amount of laser interrogation at slower μL/min flow rates. Hydrodynamic focusing is also enhanced to limit the ability of particle clustering. A stock solution of filtered PBS with 0.1% Tween-20 is prepared. 0.52, 0.78, and 0.19um 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 Dragon Green Beads were sonicated to eliminate clumps.

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

1. 0.78µm Dragon Green Beads

2. 0.52µm Dragon Green Beads

3. 0.19µm Dragon Green Beads

4. 0.52µm/0.78µm/1.01µm Dragon Green Beads Mixed

- 0.78µm Dragon Green Beads were acquired to set the Scatter properties for differentiation between beads and low-end noise. In addition, VSSC 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 1µm.

- 0.19µm 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. Also, 0.19µm Dragon Green Beads were used as most instrument manufacturer's specifications quantify lowest detectable level of 0.3µm.

- 0.52µm Dragon Beads were acquired for accuracy of separation of 0.19µm and 0.78µm 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.
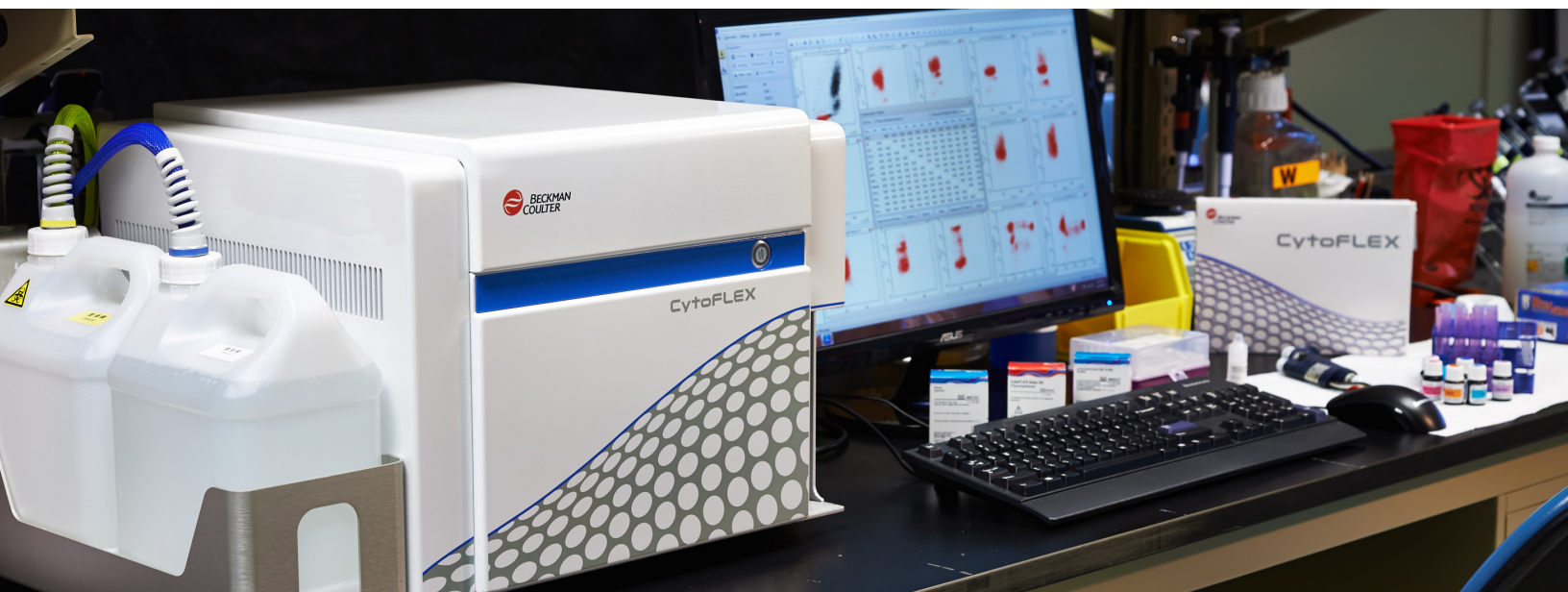
- Gain and threshold settings can be adjusted to maximize performance. However, it is strongly suggested that single bead populations be acquired again for quality assurance purposes.

- Instrument has been optimized and template and settings are saved for future cellular experiments.

Optimized Settings

- VSSC was set to a Log Area parameter and plotted against Blue (488nm) SSC set to a Log Area Parameter.

- Blue SSC setting was determined to be 350 units on the Gain

- FITC setting was determined to be 370 units on the Gain

- VSSC was placed at the following settings:
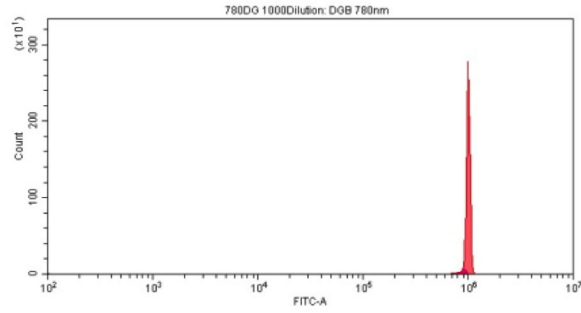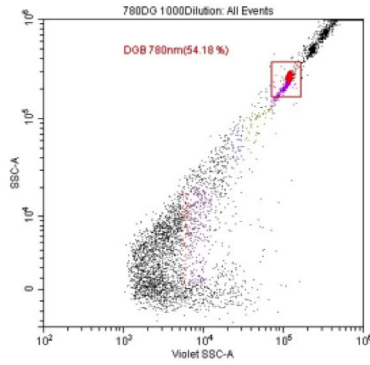 A. Gain units 22
  B. Threshold 2000

By using the fluorescent characteristics of the Dragon Green Beads, verification of size distribution based on fluorescent intensity can be used for data assurance.
In addition, a sample of the stock PBS solution is acquired for quantification of the background contribution of the PBS. All three populations were easily separated from background and from one another.
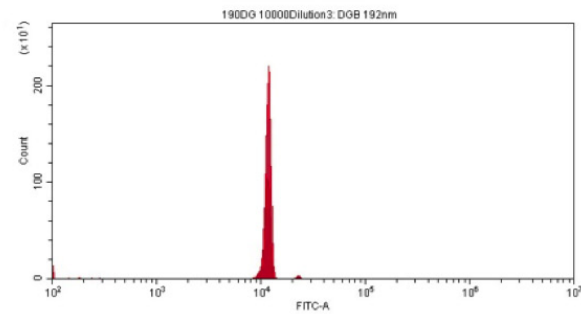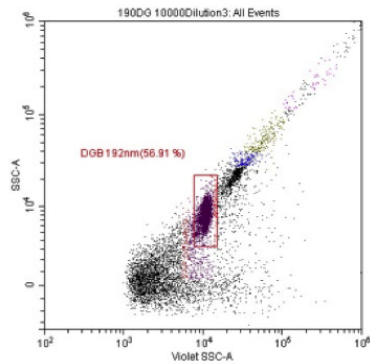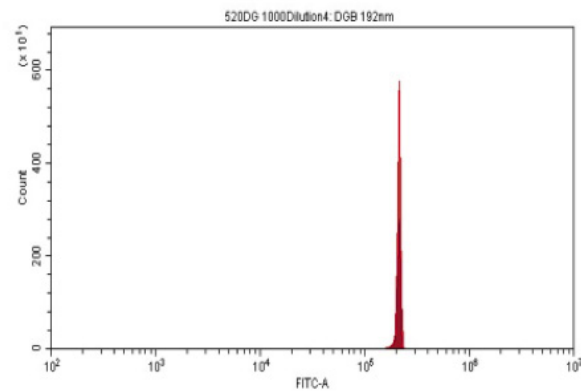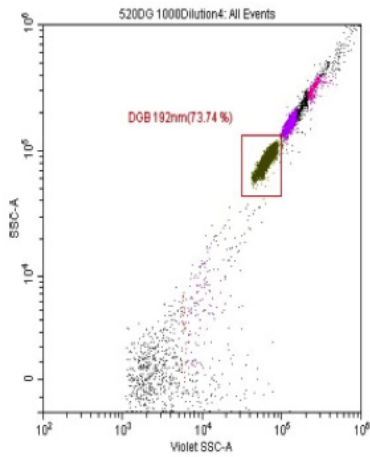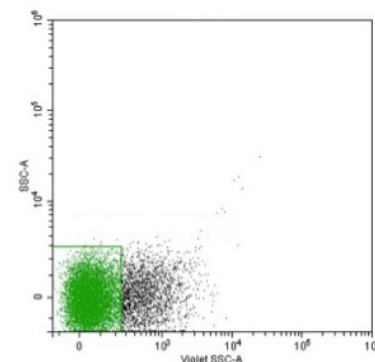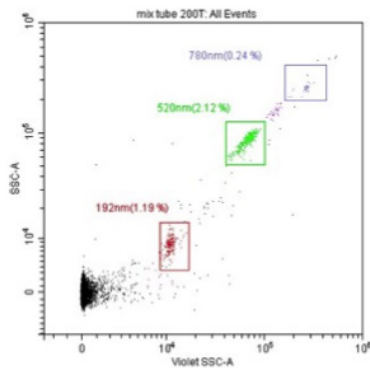
780nm Dragon Green Beads

192nm Dragon Green Beads

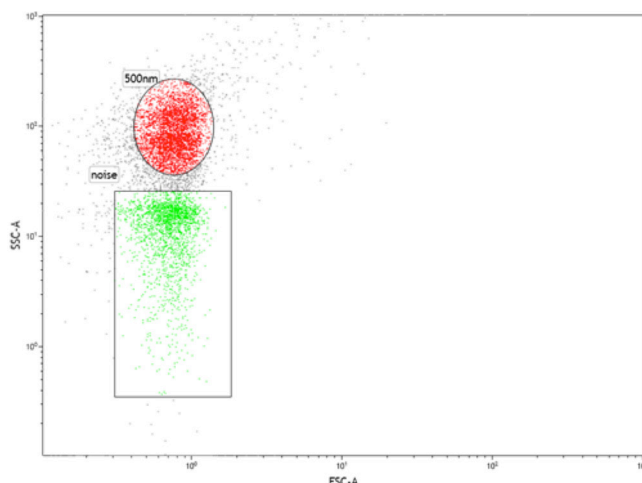# 520nm Dragon Green Beads

Mixed Dragon Green Beads and PBS Alone

Results

All samples were analyzed with either CytExpert* or Kaluza* software. As demonstrated in the previous graphs, the three different size Dragon Beads were identified, gated, and distinguishable from one another and from background. The settings were optimized and saved for later cellular applications. The ability to identify and easily differentiate the Dragon Bead populations based on size has lead to the optimization and standardization of settings for EVs.

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow cytometers.

Figure 1: Dynamic range of conventional Flow Cytometry instrumentation. Threshold set on 488nm SSC at the lowest possible setting.

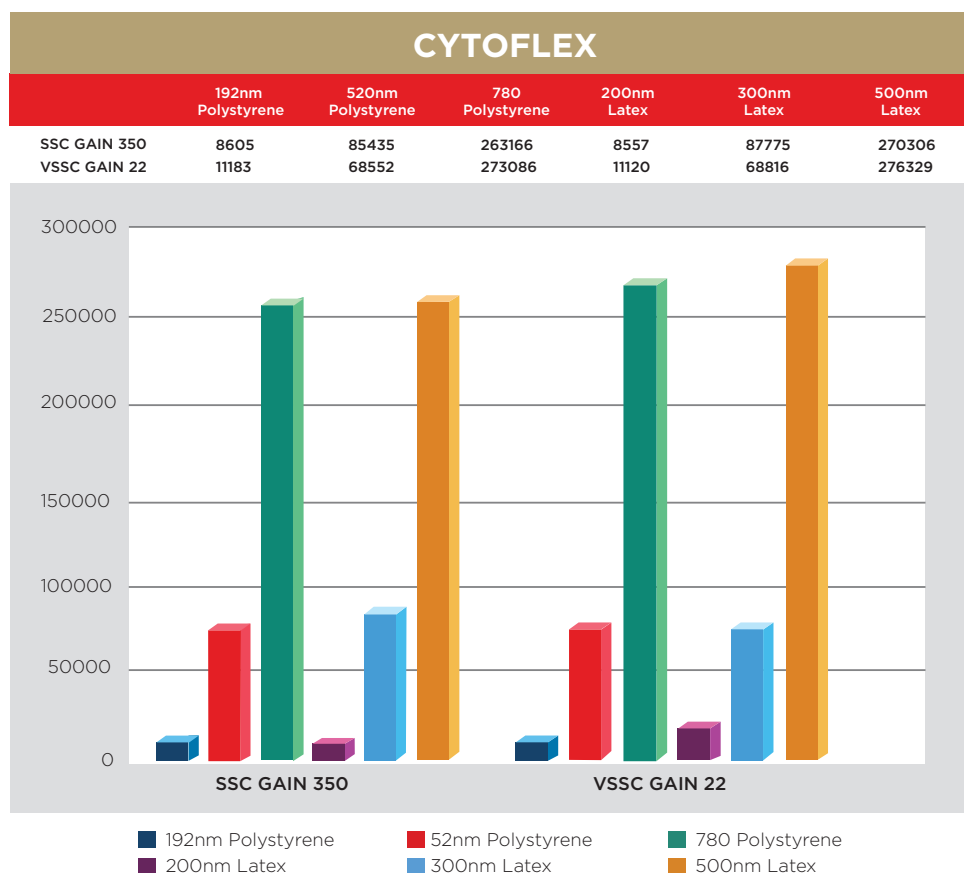| CYTOFLEX						
	192nm Polystyrene	520nm Polystyrene	780 Polystyrene	200nm Latex	300nm Latex	500nm Latex
SSC GAIN 350	8605	85435	263166	8557	87775	270306
VSSC GAIN 22	11183	68552	273086	11120	68816	276329

Table shows the differences between SSC and VSSC on the CytoFLEX. The actual MFI are consistent but the Gain values for VSSC are much lower compared to SSC Gain values.

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at **beckmancoulter.com**

FLOW-991APP08.15-A