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Life Sciences

Nanoscale Sorting with the CytoFLEX SRT Cell Sorter

Nanoscale flow cytometry is one of several techniques employed in the characterization of submicron particles. Biological particles often explored in this size range include bacteria, viruses, extracellular vesicles, and exosomes. This analysis tool has become more prevalent due to its high-throughput and multiparametric capabilities. It has been demonstrated previously that the Beckman Coulter CytoFLEX Platform Analyzers are capable of detecting 80 nm polystyrene nanoparticles using the Violet Side Scatter parameter. Due to the optical design components, wavelength division multiplexing, avalanche photodiodes, and diode lasers, the CytoFLEX flow cytometer has been shown to have an enhanced level of light scatter and fluorescence sensitivity [1]. With optical engineering advancements and the increase in the use of flow cytometry to analyze EVs, there have been efforts to standardize nanoscale flow cytometry methods. This rigor and ease of applying the power of high-throughput, single-particle characterization, which significantly contributed to the advances in cellular immunology, could then by applied to EV research.

While these efforts have made progress for nanoscale flow cytometry analysis, nanoscale sorting is still a complex technique that is not commonly used. It has been previously demonstrated on a MoFlo Astrios that nanoscale vesicles could be sorted with efficiencies ranging from 25 to 45% [2]. However, the MoFlo Astrios design is a large format with complex workload requirements for setting up and running a nanoscale sort.

Due to the complementary design of the CytoFLEX SRT cell sorter and the CytoFLEX S cell analyzer (both use APD detectors, WDM, and VSSC detection [1]), performance characteristics are expected to be similar. Thus, it is expected that nanoscale particle sorting is feasible. In addition, the automated sort setup and minimal instrument setup necessary to perform nanoscale flow cytometry may enable the CytoFLEX SRT cell sorter to be used as a tool for standardized and reproducible (sorting) experiments.

In the following experiments, we describe the ability of the CytoFLEX SRT cell sorter to sort nanoscale samples. Our results show an increased efficiency in the sorting of nanoparticles compared to existing reports and that the CytoFLEX SRT cell sorter can sort several types of nanoparticles.

Materials & Methods

Instrument Setup

Daily startup and QC were performed as directed in the CytoFLEX SRT cell sorter Instructions for Use. In brief, after turning on the instrument the system start-up program is run, and the nozzle is inserted. After start-up is completed the CytoFLEX Daily QC fluorospheres are placed onto the sample station and the instrument is instructed to run QC. After passing QC the instrument is configured for Side Scatter (SSC) detection using the Violet laser, as described in Brittain et al. Since the optical decks of the CytoFLEX S flow cytometer and the CytoFLEX SRT cell sorter are complimentary in design this procedure is valid on both instruments. This is performed as easily as swapping the 405/10 filter and 450 filter WDM positions, thus eliminating the Pacific Blue channel and creating the Violet Side Scatter (VSSC) channel. Re-swapping the filters will enable reversal to the original configuration.

Preparation of NIST beads, fluorescent beads, VLP, bacteria

All NIST beads (Polysciences, Inc., Warrington, PA) were prepared as described in FCMPASS - Acquisition and gating of light scatter reference materials V.2 [3]. Briefly, each stock bottle was mixed and then diluted to a concentration of $1x10^7$ in 500 µL of DPBS. Beads were then acquired to a total of 10,000 events in the designated gate using the VSSC configuration on the CytoFLEX SRT cell sorter.

eGFP and tdTomato tagged VLP were prepared and provided by Dr. Rahm Gummuluru. In brief, the expression plasmid HIV-1 pGag-eGFP and/or pGag-tdTomato which expresses a Gag-enhanced green fluorescent protein (eGFP) and/or tdTomato fluorescent protein fusion protein, has been described previously (Cat # ARP-11468 NIH HIV Reagent Program, Division of AIDS, NIAID, NIH; contributed by Marilyn D. Resh and George Pavlakis). HIV Gag-eGFP/tdTomato, virus-like particles (VLPs) were generated via calcium phosphate mediated transfections of HEK293T cells. Upon harvest, VLP-containing supernatant was clarified of cell debris, passed through a 0.45-µM filter, and pelleted through a 20% sucrose cushion. The p24 Gag content of VLPs was determined by an ELISA.

Bacillus subtilis strains were constructed by transformation into *B. subtilis* 168 *trpC2* (PB2) and its derivatives unless otherwise noted. *B. subtilis* strains were transformed with 5–10 µL of plasmid DNA or 1–2 µL of genomic DNA using the two-step method. Genomic DNA was prepared using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Cultures were grown in LB (Lennox).

Post-sort analysis

All microscopy images were taken with an Olympus BX62 microscope (Olympus Corporation, Tokyo, Japan). Images were captured using an Andor Sona camera all at 40.0x objective with immersion oil. Both the FITC single-channel and Texas Red single channel were used with the exposure set to 100 ms.

Results

In this section we are describing the sorting of various entities of different size, with decreasing size and increasing complexity. The detailed methods are described in the previous section. The diversity of sorting experiments described here aim at demonstrating the versatility of the CytoFLEX SRT cell sorter.

Single Bacterium Sorting (>250 µm)

A sample of YFP labeled activated *Bacillus subtilis* was analyzed using the FITC (525/40) channel. Positive bacteria were gated and then a single bacterium was sorted onto a slide and verified by fluorescence microscopy. The test was performed to verify the ability of the machine to sort a relatively small biological (2-6 µm in length and 0.25–1.0 µm in diameter) with a refractive index similar to that of an EV at 1.38 [4].

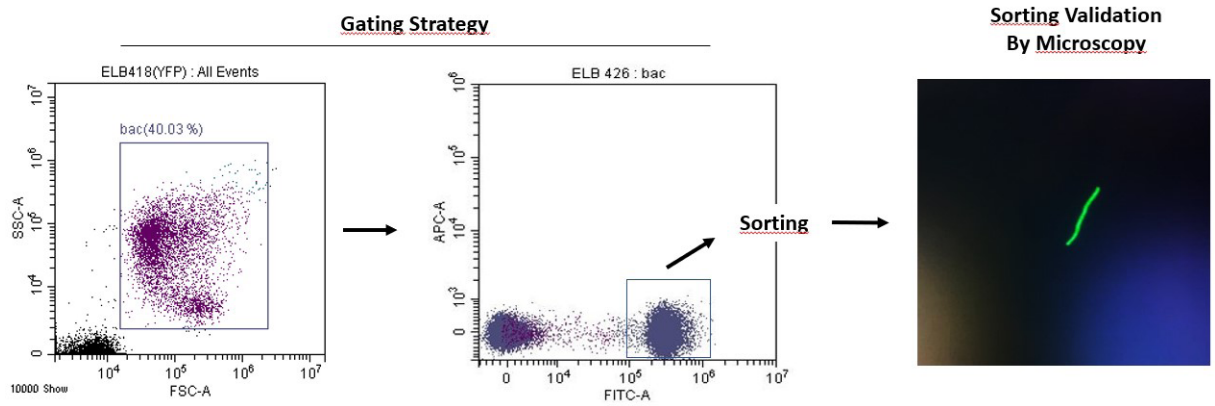

Figure 1. *B. subtilis* test sort. Cultured bacteria were sorted with sort regions to remove debris and doublets, and finally for YFP positive bacteria (VSSC Threshold 1650/Gain 300, FITC-750, SSC-100). Following deposition on a glass slide and visualized using an Olympus BX62 microscope. Images were captured using an Andor Sona camera all at 40x objective with immersion oil.

Sorting of 100nm YG Beads

Next, 100 nm YG Polystyrene beads were mixed with 150 nm non-fluorescent beads. The 100 nm beads were sorted five onto a slide and a single bead onto a slide. The threshold was set low enough (VSSC Threshold 3000/Gain 300, FITC-400, SSC-500) to visualize the two bead populations and cut out electronic and fluidic noise events. This test sort was performed to determine if the instrument would be capable of sorting nanoparticles using a non-biological standard.

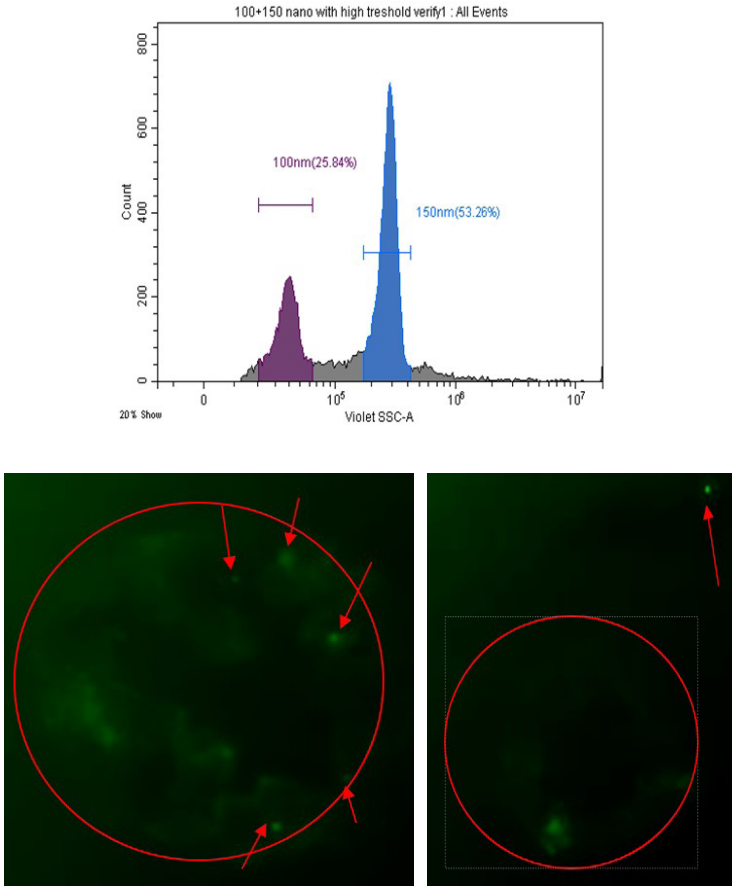

Figure 2. Sorting of 100 nm YG beads. 100 nm beads were separated using the 100 nm region on the VSSC parameter **(top)**. Following deposition on a glass slide, the beads were imaged at 40X magnification **(bottom)**. Sorts for both 5 beads per spot **(left)** and 1 bead per spot **(right)** were performed. Arrows on each image indicate the location of 100 nm beads. Note the high levels of background due to the salt crystals formed by drying sheath.

Sorting of eGFP and tdTomato labeled VLP

The eGFP and tdTomato tagged VLP particles (100-120 nm, RI 1.369 [5]) serve as further biological controls. These are well-characterized nanoparticles with a bright signal. Samples were analyzed on VSSC trigger before switching to their respective fluorescence trigger that was optimal for each fluorescent protein. VSSC threshold was initially set using NIST beads as described in the FCM_{PASS} protocol [6]. A minimum of 100,000 events were collected. The collection tubes were then concentrated using an Amicon filter and re-analyzed on the instrument. Results are shown in figures 3 and 4.

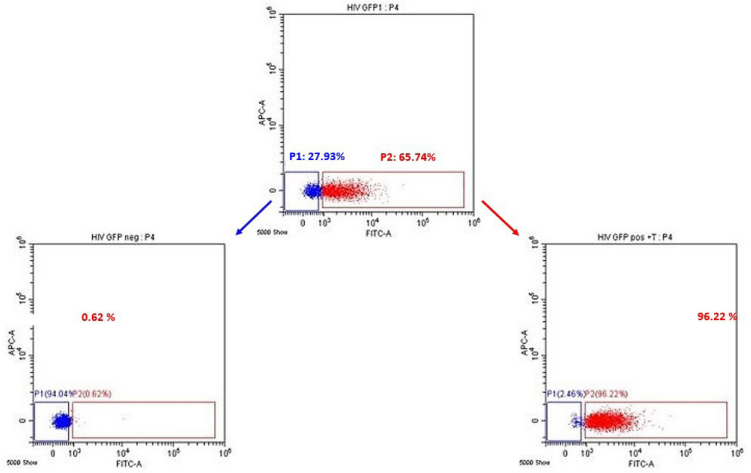

Figure 3. Sorting of GFP+ and GFP- virus like particles. The positive collection tube **(bottom right panel)** showed >96% of events in the previously determined positive gate **(top middle panel)**. The negative collection tube **(bottom left panel)** showed l <1% in the positive gate.

Since the goal of these experiments is to multiplex molecular beacons for different miRNAs, the eGFP and tdTomato VLP samples were combined to test if the different fluorescent populations could be separated on the SRT with high purity. After sorting into FITC positive and PE positive gates collection tubes were analyzed to check for purity. The tdTomato positive population showed 97% purity and the GFP positive population showed 98% purity. Results are shown in figure 4.

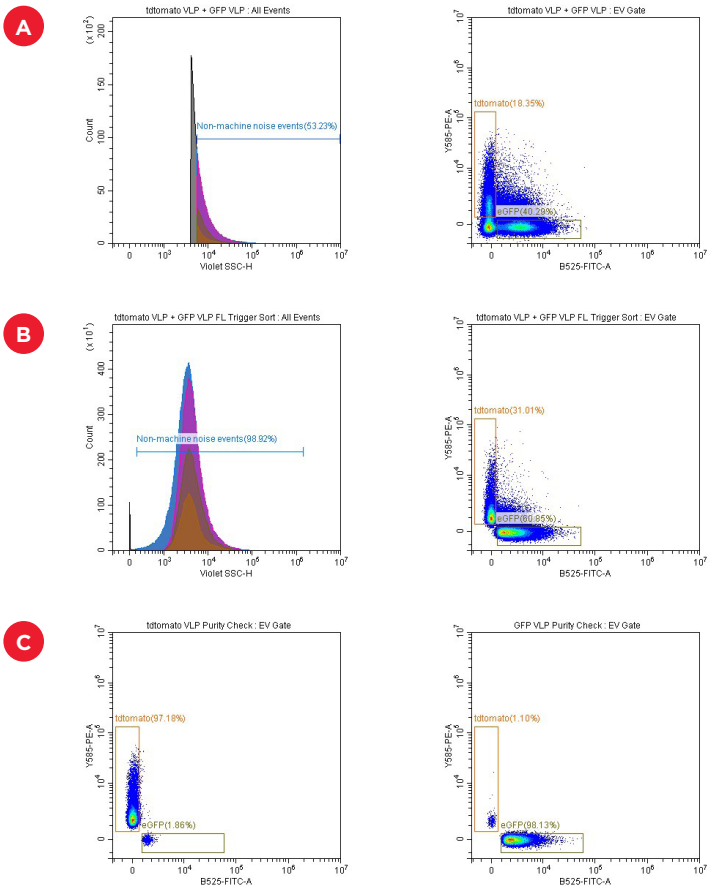

Figure 4. Sorting of tdTomato and GFP VLPs. (A) Gain and threshold: VSSC 200, SSC 400, GFP 700, tdTomato 500. Threshold VSSC 4000. **(B)** Gain and threshold: VSSC 200, SSC 400, GFP 700, tdTomato 500. Threshold GFPH 1000 and tdTomato 1000. **(C)** Recovery of sorted populations based on B. Machine noise was determined by running sample buffer only to allow for threshold adjustment to remove the majority of noise.

Conclusion

Several techniques are used for the analysis and characterization of nanoscale biologicals. As the use of flow cytometry in the field of nanoscale inquiry is increasing and the need for isolating specific particles is pressing, we have tested the possibility to perform sorting of EVs. In this application note, we are reporting analysis and sorting of different nanoscale particles using the CytoFLEX SRT cell sorter. Taken together the data presented here clearly shows that the CytoFLEX SRT cell sorter is capable of sorting particles well under 1 micron in diameter. Experiments aiming at sorting bacteria, virus-like particles, and beads generated results of high quality. We are able to demonstrate high purity sorting of nanoscale particles in an instrument that has considerably less complexity than other published flow cytometry-based solutions.

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