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www.KineticRiver.com
info@KineticRiver.com
+1 (650) 439-7413

The *Delaware*: A Flow NanoCytometer™ for Nanoparticle Analysis

Giacomo Vacca[1], Richard Hanson[1], Giovanni Contini[2], Paolo Cappella[3], Anna Sannino[4], Romeo Bernini[4]

[1]R&D, Kinetic River Corp., Mountain View, CA, United States; [2]Cytoflowservice Division, E.M.C2 Srl, Rome, Italy; [3]E.M.C2 Srl, Saronno, Italy (currently at Champions Oncology srl, Bresso, Italy); [4]IREA, National Research Council, Naples, Italy

BACKGROUND

Detection and characterization of sub-micron biological entities, such as exosomes, extracellular vesicles (ECVs), liposomes, and micelles, represents an important next frontier in both research and clinical applications. The main obstacles to nanoparticle analysis in flow cytometry are (i) the small particle sizes and (ii) the short time available for interrogation, which, combined, result in exceedingly small scattering and fluorescent signals. Commercial hardware either modified or tailored for this problem has so far been underwhelming in terms of both speed of analysis and ease of use. There is unmet demand for flow cytometry systems that deliver nanoparticle analysis without compromising usability and throughput.

Fig. 1. *The* Delaware *Flow NanoCytometer™.*

MATERIALS & METHODS

We have developed a new cell analyzer, the *Delaware* Flow NanoCytometer™ (**Fig. 1**), designed for sensitive detection and characterization of sub-micron particles (biological or otherwise). The *Delaware* is based on the architecture of our *Potomac* modular flow cytometry platform (CYTO 2017; CYTO 2021), with design modifications specifically intended to enhance nanoparticle sensitivity. The custom *Potomac* used for this work, installed at IREA-CNR in Naples, Italy, used collinear 50-mW 375- and 405-nm excitation (Pavilion Integration), and can additionally accommodate an external 266-nm laser, while the *Delaware* has up to five lasers (in this work, Toptica 120-mW and DTR 800mW 405-nm, and Coherent 300-mW 488-nm, 50-mW 532-nm, and 25-mW 640-nm). The detection modules have user-changeable Semrock beamsplitters and filters and use high-NA collection lenses. The platform offers two scattering channels and from two to eight fluorescence channels, all equipped with photomultiplier tubes (Hamamatsu PMTs). Ultrastable sheath flow for core stream control is established with our previously introduced *Shasta* fluidic control system (CYTO 2021) and monitored using our *Cavour* flowcell monitoring module (**Fig. 2**). The analyzer is operated using our *Panama* flow cytometry software for instrument control and data visualization.

For this work, filtered distilled water (Tito Menichelli), double filtered through a 0.10 um PVDF syringe filter (AG Advangene), was used as a sheath fluid and as a sample diluent. Four populations of polystyrene beads 0.1-0.3 (0.22) µm, 0.4-0.6 (0.45) µm, 0.7-0.9 (0.88) µm, and 1.0-1.9 (1.33) µm (Spherotech, NFPA-52-4K) were diluted in filtered water. Silica nanoparticles (Non-functionalized Colloidal Silica Nanospheres, Alpha Nanotech) with diameters of 20, 50, and 100 nm were used undiluted. Samples were recorded using 375 nm, 405 nm, and combined 375 + 405 nm mode. PMT voltages were optimized using Red Nile beads (Spherotech, FP-3056-2). Sheath fluid pressure was set to 5.3 psi and sample pressure to 3.8 psi. Area (A), height (H), and width (W) linear PMT signals were acquired on FSC, SSC, FL2 (466 ± 20 nm), FL3 (535 ± 25 nm) and FL4 (698 ± 35 nm). Data were analyzed using FlowJo software v.10.8 by applying a gating strategy using time gate and singlet gate.

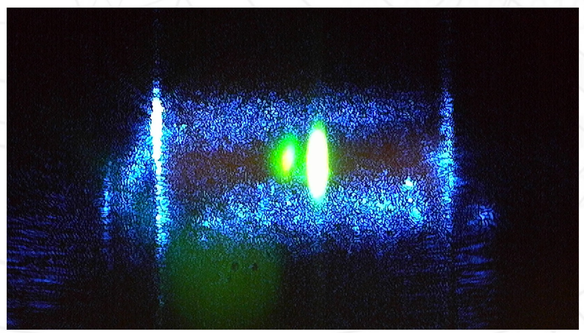

Fig. 2. *The interrogation region as captured by the* Cavour*, a built-in, always-on flowcell monitoring module used to verify laser alignment, establish core stream size, and troubleshoot fluidic issues.*

RESULTS

Results from detection sensitivity assessment using polystyrene microspheres are shown in **Fig. 3**. With either individual 375-nm [panel (**a**)] or 405-nm excitation [panel (**b**)], the 880-nm and 1,330-nm populations are easily resolved; however, the 450-nm and 220-nm populations overlap significantly or completely. On the other hand, using both 375- and 405-nm excitation in combination [panel (**c**)] produces clear resolution of all four populations. This is not likely due to the double amount of optical power delivered as excitation, since elsewhere (see below) a single laser was able to resolve smaller particles. The likely explanation is that the deep Mie scattering minima from 375- and 405-nm excitation partially cancel out in the combination experiments. Further work, including modeling of the collection geometry, would help elucidate this.

The refractive index of silica microspheres (n = 1.47 at 375 nm) is closer than that of polystyrene (n = 1.63 at 375 nm) to that of relevant biological entities (n = 1.37–1.39) and therefore provides a better estimate of performance with exosomes and EVs. Our system was able to distinguish, using 375-nm excitation, 100- and 50-nm silica particles from 20-nm ones (**Fig. 4**). The limit of detection for silica in water was found to be between better than 50 nm.

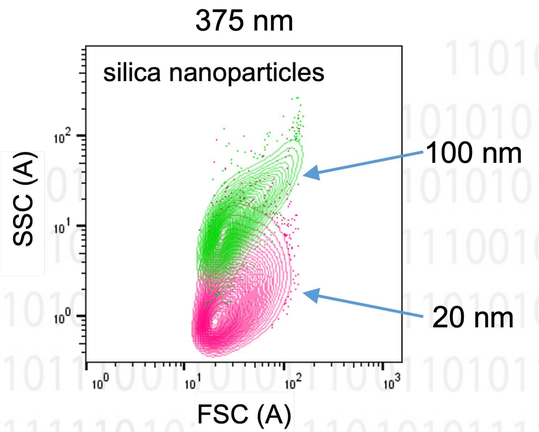

Fig. 4. *Colloidal silica nanoparticles were measured with 375-nm excitation. The platform was able to resolve nanospheres using forward and side scatter, with a Limit of Detection (LOD) below 50 nm.*

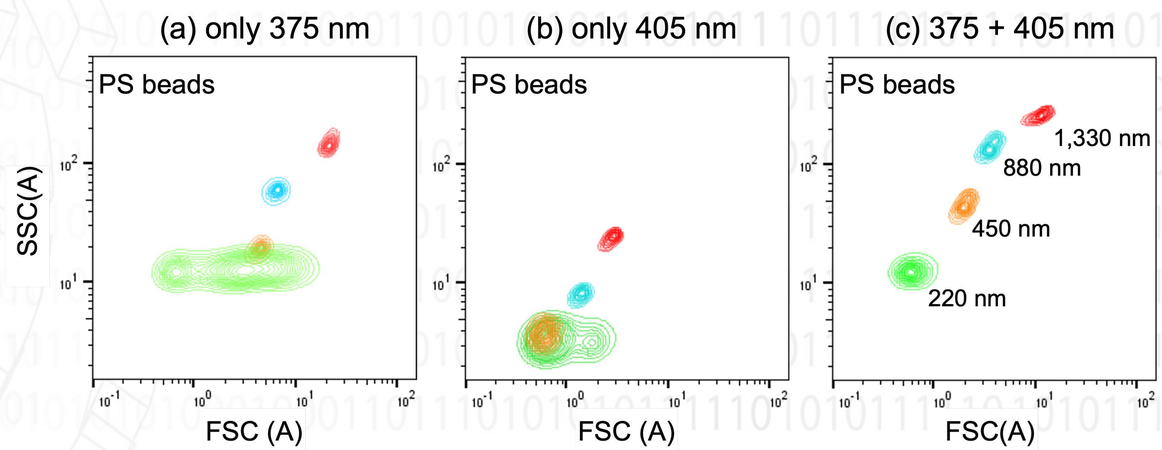

Fig. 3. *Resolution of sub-micron particles. Polystyrene microparticle were analyzed using (a) only 375-nm, (b) only 405-nm, and (c) both 375 and 405 nm excitation. Combined, collinear excitation produced the best resolution across the size range measured.*

CONCLUSION

We have demonstrated the integration of powerful multicolor excitation sources, an optical system designed to maximize light collection, an ultrastable fluidic control module, and a highly flexible yet intuitive graphical user interface. The *Delaware* Flow NanoCytometer combines ease of use with advanced nanoparticle sensitivity to offer users a powerful new tool for exosome and EV research.

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