

A Microflow Cytometer on a Chip

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ABSTRACT

A rapid, automated, multi-analyte Microflow Cytometer is being developed as a portable, field-deployable sensor for on-site diagnosis of biothreat agent exposure and environmental monitoring. The technology relies on a unique method for ensheathing a sample stream in continuous flow past an interrogation region where optical fibers provide excitation and collect emission. This approach efficiently focuses particles in the interrogation region of the fluidic channel, avoids clogging and provides for subsequent separation of the core and sheath fluids in order to capture the target for confirmatory assays and recycling of the sheath fluid. Fluorescently coded microspheres provide the capability for highly multiplexed assays. Optical analysis at four different wavelengths identified six sets of the coded microspheres recognizing *Escherichia coli*, *Listeria*, and *Salmonella* as well as cholera toxin, staphylococcal enterotoxin B (SEB), and ricin, and assay results were compared with those of a commercial Luminex analysis system.

Keywords: flow cytometry, microfluidics, multi-analyte assay, coded microspheres, microflow cytometer

1. INTRODUCTION

Flow cytometers have established applications in many areas of analysis, ranging from environmental diagnostics to biomedical science. As the applications expand, the market for portable systems increase. Microfluidics offer the potential to reduce system size significantly, and a variety of microfluidic cytometers have been reported in the literature, as reviewed in Ateya et al.¹ In general, sheathing of the sample or “core” fluid is required to focus the particles uniformly in the laser beam. Because the power in the laser beam has a Gaussian distribution, particles distributed in different regions of the beam will be excited with varying efficiency; the more narrow the core stream, the more reproducible is the signal intensity. Since the nozzles typical of large flow cytometers are difficult to fabricate in microfluidic systems, we have developed an alternative method for sheathing the core stream that relies on grooves in the channel walls and only requires one or two sheath fluid inlets.² This sheathing system is easy to fabricate and can be fabricated in a variety of materials, including polymethylsiloxane (PDMS), polymethylmethacrylate, and glass, using soft lithography, CNC and laser milling, hot embossing, and standard lithography.

A feature of the microflow cytometer described here that sets it apart from most cytometer systems reported in the literature is the delivery of excitation light and collection of emissions using optical fibers embedded in the chip. Most investigators have used microscope objectives or other lenses, to deliver and collect light perpendicular to the substrate. We have simply placed the optical fibers in the substrate adjacent to the fluidic channel and rely on the fibers to focus the laser light on the core stream. Fluorescence and scattered light was collected by additional optical fibers placed at selected angles to the excitation fiber; the wavelengths measured were selected by using filters between the optical fibers and the photomultiplier tubes. While inserting the fibers into preformed alignment channels in the substrate does add an additional step to the fabrication, the resulting alignment is robust.

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2. METHODS

2.1 Materials

Streptavidin-conjugated phycoerythrin was supplied by Prozyme (San Leandro, CA, USA). Carboxy-functionalized microsphere sets 98, 81, 77, 56, 50, and 32 were purchased from Luminex Corporation (Austin, TX, USA). All microspheres were 5.6 μm diameter polystyrene microspheres coded with different amounts of two fluorescent indicator dyes. Llama IgG (anti-ricin) was purchased from Triple J Farms (Bellingham, WA, USA). Heat deactivated *Escherichia Coli* (*E. coli*), *Salmonella*, *Listeria*, goat anti-*E. coli*, goat anti-*Listeria*, and goat anti-*Salmonella* IgG were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Maryland). Rabbit anti-staphylococcal enterotoxin B (SEB) was kindly provided by the Naval Medical Research Center (Silver Spring, MD, USA). Cholera toxin was from Calbiochem (San Diego, CA) and rabbit anti-cholera toxin was from Biogenesis (Kinston, NH). Chlorotrimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO, USA). SU-8 photoresist was purchased from Microchem (Newton, MA, USA). PDMS (Sylgard 184) was purchased from Dow-Corning (Corning, NY, USA) and used according to manufacturer's instructions of 10:1 base to curing agent volume ratio. Polyetheretherketone (PEEK) tubing was purchased from Upchurch Scientific (Oak Harbor, WA, USA).

2.2 Device fabrication

The microflow cytometer channels were fabricated in polydimethylsiloxane (PDMS) using standard soft lithography procedures. Briefly, a master was created on a silicon wafer by sequentially spinning on layers of SU-8 photoresist and exposing with a photomask containing the desired channel geometries and features. After development, the master was treated with chlorotrimethylsilane vapor to prevent polymer adhesion. The master was placed into the bottom of a mold and PDMS was poured on top to make layers with a thickness of approximately 1 mm (bottom) or 10 mm (top). After curing, the PDMS was peeled from the master. Fluidic inlets were cored into the top layer of PDMS. Channels were located only in the top half of the device so that only the chevrons would need to be aligned before bonding. Channels were 130 μm high and 390 μm wide. The chevron-shaped grooves extended 65 μm into the top and bottom of the channels. The two PDMS pieces were then bonded together by oxygen plasma treatment. Optical fibers were cleaved and placed into their guide channels and carefully aligned with the channel walls. PEEK tubing sections (Upchurch Scientific, Oak Harbor, WA) with an internal diameter of 400 μm and a length of 13 mm were placed into the inlets. Finally, the optical fiber channels and PEEK inlets were sealed with PDMS cured at 50°C for 2 hours.

Diode lasers at 532 nm (GM32-10H, 10 mW, Intelite, Inc. Minden NV) and 635 nm (LAS-200-635-15, 15 mW, Lasermax Inc, Rochester, NY) provided the excitation light. A multimode "dump fiber" was positioned opposite the excitation fiber to minimize scatter from the excitation beam off the PDMS. Two fibers equipped with beam splitters collected the light signals from microspheres passing through the laser beams. Scatter from the microspheres was collected at 635 nm. Fluorescence was collected at 665 \pm 10 nm and 700 nm longpass filters for microsphere identification (665DF20, Omega Optical, Inc., Brattleboro VT, LL700, 700 nm longpass filter, Corion Corp., Franklin MA), and a 565 \pm 10 nm filter for detection of phycoerythrin fluorescence (565WB20, Omega Optical, Inc., Brattleboro, VT). The output of the PMTs was recorded using an A/D converter (NI USB-6251 M, National Instruments, Austin, TX) and data acquisition and data analysis software was written in LabWindows/CVI (National Instruments, Austin, TX).

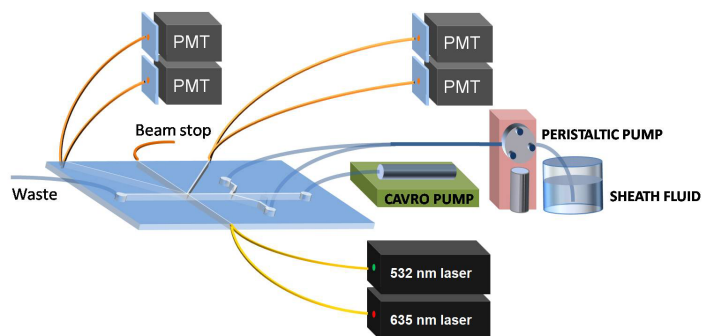


Figure 1. Diagram of optics and fluidics for the Microflow cytometer.

2.3 Assays

Multiplexed assays were performed using Luminex[®] microspheres and analyzed using both the microflow cytometer and the commercial Luminex[®] system. Earlier studies^{3,6} confirmed that six microsphere sets could be distinguished in the microflow cytometer based on the amounts of fluorescence of the microspheres at 665 nm and >700 nm. For this study, antibodies were attached to carboxy-functionalized microspheres (13 microsphere sets) using the standard two-step carbodiimide coupling chemistry protocol provided by the manufacturer. After removal of unbound antibody, the microspheres were resuspended in phosphate buffered saline (PBS) with 0.05% Tween-20 (PBST) and then stored in the dark at 4°C until used. For this experiment, the microsphere sets were coated with antibodies with the following specificities: cholera toxin, *Salmonella*, *Listeria*, ricin, *Escherichia coli*, and Staphylococcal enterotoxin B (SEB). The mixture of microsphere sets was incubated with concentrations of the analyte, washed, and then exposed to mixture of biotinylated tracer antibodies (5 µg/mL each). Excess tracer was removed by washing the microspheres over a filter plate. The microspheres were analyzed using the Luminex[®] flow cytometer or flowed through the microflow cytometer using a total sheath flow rate of 400 µL/min.

3. RESULTS AND DISCUSSION

Optical analysis at four different wavelengths identified the 13 sets of coded microspheres and could quantify target bound by the presence of phycoerythrin-bound tracer. The fluorescence values for the microsphere ID wavelengths were normalized by the light scatter at 635 nm in order to control for variation in illumination intensity. These measurements provided excellent discrimination of the microsphere sets used in this experiment (Figure 3).

The microcytometer detected targets such as *Escheria coli*, *Listeria*, *Salmonella*, cholera toxin, staphylococcal enterotoxin B (SEB), and ricin. The microsphere mixture was used to perform multiplexed sandwich immunoassays on samples spiked with bacteria or toxin, generating dose-response curves such as those in Figure 3. Assays with the microflow cytometer established limits of detection shown in Table 1.

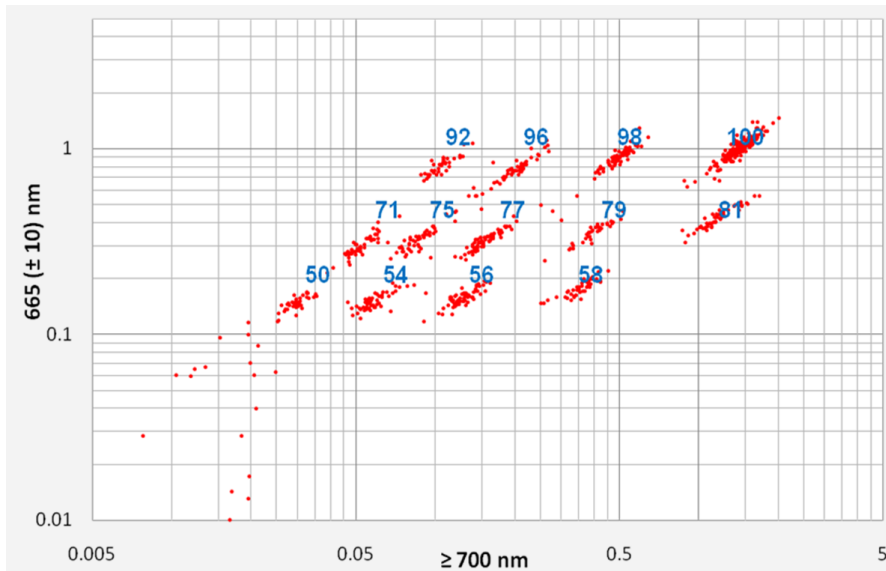


Figure 2. Two colors of fluorescence are plotted to identify each microsphere. The fluorescence units for the 705 and 665 nm signals divided by the 635 nm scatter signal are plotted on the x and y axes, respectively. Values segregate the microspheres into distinct populations.

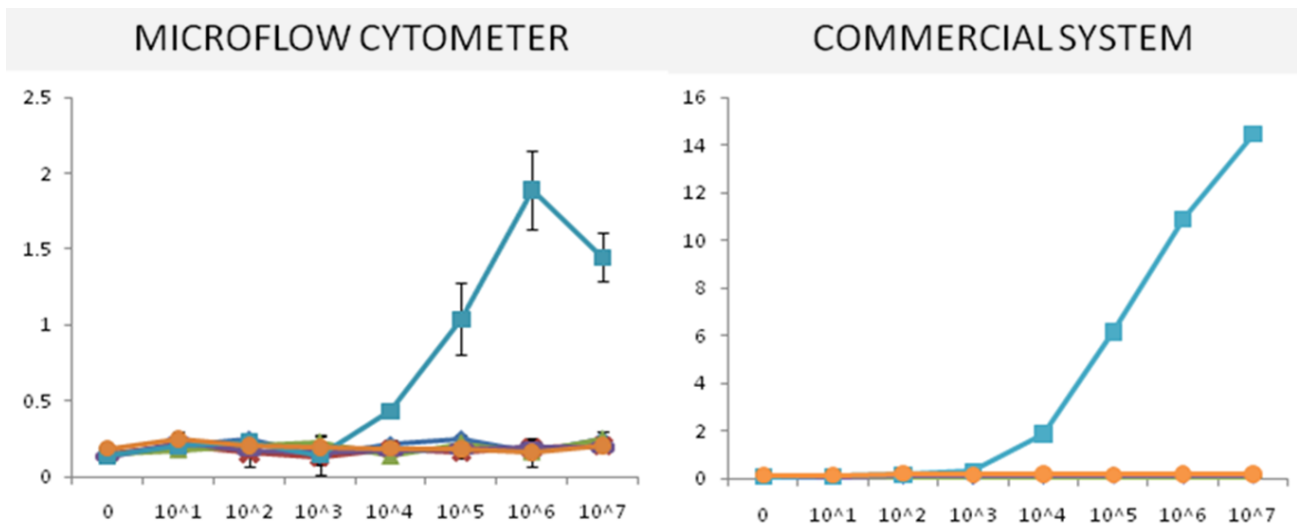


Figure 3. Dose response of *E. coli* comparing the microflow cytometer and the commercial system (Luminex); \blacklozenge = Salmonella, \times = Listeria, \blacktriangle = Cholera toxin, — = Ricin, \blacksquare = *E. coli*, \bullet = SEB).⁴

Table 1. Limits of detection for the three bacteria species and three toxins and their associated coefficients of variation were determined using the conventional benchtop cytometer (Luminex) and the Microflow Cytometer. Limits of detection were designated as the tested concentration of the antigen that produced a mean signal greater than the average of the mean signals from the five other microsphere sets that did not specifically bind the tested antigen (negative controls) plus 3 times the standard error. The coefficients of variation (CV) were determined for the analyses at the LODs.⁴

cytometer	bacteria (cfu/mL)			toxins (ng/mL)		
	<i>E. coli</i>	<i>Listeria</i>	<i>Salmonella</i>	cholera	SEB	ricin
Luminex (% CV)	10 ³ (1.0)	10 ⁶ (0.7)	10 ⁵ (2.7)	1.6 (1.0)	0.064 (2.5)	8.0 (5.6)
Microflow (% CV)	10 ⁴ (11)	10 ⁵ (14)	10 ⁵ (28)	0.32 (22)	1.6 (7.7)	1.6 (6.7)

3.1 Unsheathing and Recycling

Hydrodynamic focusing of one laminar stream by another has inspired new approaches for separations, optical components, biosensors, and cell analysis [3,5-8]. In nearly all of these publications, laminar flow is used to alter a flow stream irreversibly or redistribute components within parallel flow streams. The reversibility of these processes has received little attention. Here we demonstrate the capacity of hydrodynamic focusing to completely encircle a sample stream with a sheath fluid, reduce the diameter of the sample stream (as for cytometry), and then reverse the process both to recapture sample components with minimal dilution and to recycle the sheath fluid.

Using the reversibility in the laminar flow regime, we designed a microdevice that returns the sample stream to its original position with respect to the sheath streams. We quantified the reversibility by recapturing the beads as an indicator of particulates in a sample and a low molecular weight dye to visualize the exact position of the sample stream. We achieved 100% recapture of the sample stream and the recovered particles.

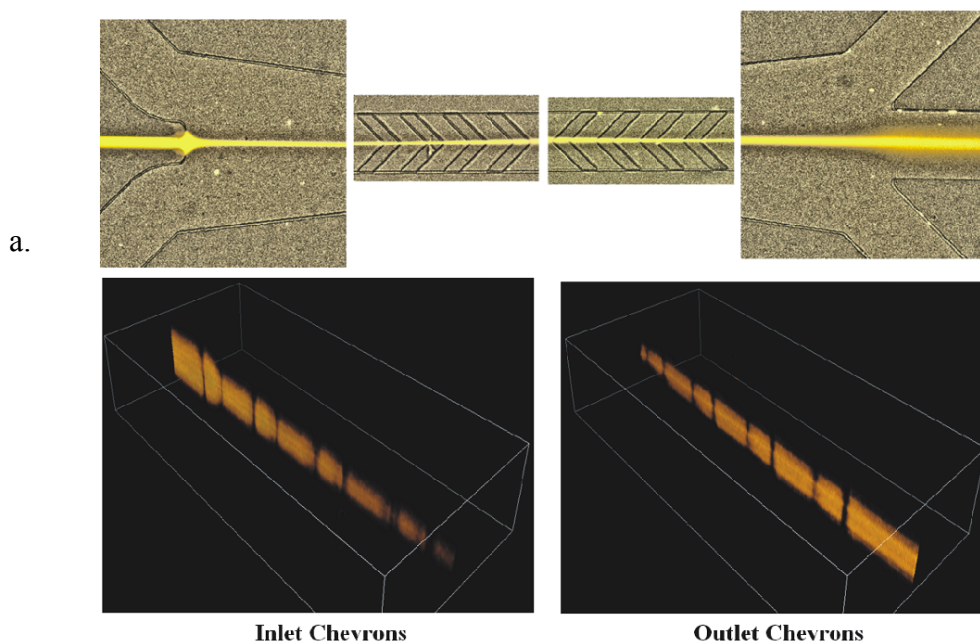


Figure 4. Images of the sheathing and unsheathing processes at 90% sheath fluid recycling. a) Top view of the inlet, inlet chevrons, reverse chevrons, and outlet sections of the microchannel. b) Confocal images of the sample fluid at the inlet and reverse chevrons. The sample height decreases upon passing through the inlet chevrons and increases by passing through each reverse chevron. The sample flow rate is 10 $\mu\text{L}/\text{min}$ and the sheath flow rate is 1100 $\mu\text{L}/\text{min}$.

4. CONCLUSIONS

We have constructed a microflow cytometer using chevron-shaped grooves to ensheath the sample stream with sheath fluid and optical fibers for delivering the excitation light and collecting signal at four wavelengths. Using the four signals, coded microspheres can be used to perform multi-analyte assays. The microflow cytometer is relatively simple to fabricate, robust and resistant to clogging.

The reversibility of the hydrodynamic focusing has application for automated processing of cells and particles, for long-term unattended use of flow cytometers, and for improved cell recovery after sorting of tumor and stem cells. For example, cells could be mixed with more rapidly diffusing dyes and then recaptured in their original volume. Cytometers with recycled sheath fluid could be used to monitor recycled drinking water for bacterial contamination on the space shuttle or to reduce the size and logistical burden of systems that monitor the air for release of biothreat agents.

5. ACKNOWLEDGEMENTS

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