Micro flow cytometer with self-aligned 3D hydrodynamic focusing

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Abstract: A micro flow cytometer with a single step 3D hydrodynamic flow focusing has been developed. The proposed design is capable to create a single-file particle stream that is self-aligned with an integrated optical fiber-based detection system, regardless of the flow rate ratio between the focusing and core liquids. The design approach provides the ability to adjust the stream size while keeping the position of the focused stream centered with respect to the focusing channel. The device has been fabricated by direct micro milling of PMMA sheets. Experimental validation of the hydrodynamic sheath focusing effect has been presented and sample stream with tuneable size from about 18 to 50 μm was measured. Flow cytometry measurements have been performed by using 10–23 μm fluorescent particles. From the analysis of the signals collected at each transit event we can confirm that the device was capable to align and measure microparticles with a good coefficient of variance.

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Flow cytometers are very powerful tools in a number of fields, including cell biology, environmental monitoring and clinical analysis as they permit a fast and selective analysis of cells/particles suspended in liquids. Miniaturization of flow cytometers is becoming feasible thanks to the recent advances in microfluidics [1–3]. Microfluidic approaches have the potential advantages to provide cost-effective, disposable and portable chips with reduced sample and power consumption [4,5]. However, several requirements for a micro flow cytometer are needed to ensure functionalities which are comparable with conventional bench top flow cytometers. One of the major challenges in microfluidic approach is the integration of various fluidic manipulation components together with detection system on the same platform for complete on-chip analysis.

Hydrodynamic focusing system represents the core of the fluidic system of a flow cytometer and significant efforts are devoted to developing highly efficient particles focusing methods using planar microfluidic approaches [6]. Microfluidic strategies for focusing particles in a narrow stream include the use of sheathing fluids which can partially (two-dimensional (2D)) or totally (three-dimensional (3D)) wrap the inner stream [6,10–15]. In a 2D focusing scheme, the particles are focused in the center of the channel only in the horizontal direction. In this case the particles can spread in the vertical direction, even causing two or more particles to pass through the detection region at the same time. These events are undesirable since cause an increased variance of the detected signal and decrease the measuring accuracy. Moreover, cells clogging or damage often occurs when the biological sample moves closer to the channel walls. 3D hydrodynamic focusing of particles prevents these problems, since the particle stream is focused both in horizontal and in vertical direction. However the realization of 3D hydrodynamic focusing in a planar chip is not an easy task. Several designs capable of 3D hydrodynamic focusing have been developed in recent years. In order to realize a four-sided sheath flow focuser one possible approach relies...
on the use of four to six sheath inlets [16,17]. This focusing scheme has been successfully applied for flow cytometry measurements with reduced signal variance. Typical drawbacks of these configurations are the requirements of multiple sheath inputs to focus and control the particle stream with consequent complex device fabrication procedure and device operation. Other approaches have been proposed based on the manipulation of the focusing channel dimension, like grooves or array of contraction and expansion regions suitably manufactured along the channel walls [18,19]. These configurations require only few inlets, but do not offer the opportunity to fully control the shape and position of the core stream. Control over the focused flow strongly influences the reproducibility of each particle data and is crucial for a high throughput single particle analysis [20].

In this work we present the design and the experimental investigation of a microflow cytometer capable of focusing a particle stream by means of a simple and single step 3D hydrodynamic sheath focusing scheme. The present device presents the general design presented in [14] but provides the additional capability of a single particle analysis. In the proposed device, the particles were detected using two integrated optical fibers, self-aligned with the focused particles stream.

2. Microfluidic design

2.1 Design principle

The device comprised a hydrodynamic focusing manifold and a detection system constituted by two integrated optical fibers, as illustrated in Fig. 1. The 3D focusing is created in a single step by lateral flow coming from the two orthogonal microfluidic channels. The orthogonal channel is deeper than the sample channel; in this way some of the side fluids push the sample stream downward and upward away from the channel walls. The result is that the sheath fluid completely wraps around the sample stream. Moreover, due to the device symmetry, the sheath flows squeeze the stream symmetrically in both vertical and horizontal direction, forming a circular tight stream. The approach presented here is unique among other approaches in two respects. First, the sample flow size is tunable, making it possible to adjust the stream diameter according to the particle size to be analyzed. Second, the shape of the sample stream is always circular and placed at the center of the microfluidic channel regardless of the flow rate ratio between the focusing and core liquids. In this way the particles stream is self-aligned with the integrated detection system, ensuring an increased measurements accuracy.

![Fig. 1. Schematic of the microfluidic structure for 3D hydrodynamic flow focusing. In the illustration are also shown the optical fibers used for flow cytometry measurements.](image)

2.2 Fluid dynamics simulation

Fluid flow was simulated by solving the coupled Navier–Stokes and diffusion/convection fluid equations using a finite elements method. The width $w$ of the square central channel was fixed to $w = 127 \, \mu m$ in order to be compatible with standard optical fiber diameter. This
choice ensures the alignment of the fiber core with the vertical center of the channel, where the center of the sample stream is located. By simulations, the height of the side channels \( h = 4.4w \) was carefully selected in order to have the shape of the sample stream always circular and located in the center of the channel for any sample to sheath flow rate ratio (FRR). The sample flow rate was set to 0.5 ml/h and FRR was varied in a range between 1 and 16. The outlet was set to a fixed-pressure boundary condition. In the simulation the sample stream was water having a dye indicator (Rhodamine B) dissolved therein with a diffusion coefficient of \( 4 \times 10^{-10} \) m\(^2\)/s and the sheath as pure water. The concentration distribution was used in order to evaluate the sample stream size in the vertical and horizontal direction after the hydrofocusing region. The full width at half maximum (FWHM) of the width of the sample stream was evaluated as function of the FRRs. The position of the center and the shape of the focused stream was found to be insensitive to the FRRs.

3. Results and discussion

3.1 Device fabrication

A sketch of the fabricated device is shown in Fig. 2(a). The device was fabricated in poly(methyl methacrylate) (PMMA) using high precision computer numerical control micromilling machine. It was made in two halves of 3 mm-thick PMMA, bonded together with a solvent assisted thermal bonding at the end of the milling process. In order to improve integration and compactness, two grooves placed orthogonally to the focusing channel were fabricated to provide passive alignment of the collecting fibers. Channels were machined by means of 127 ± 12.7 \( \mu \)m diameter milling tool. The two halves are asymmetric in order to reduce alignment problem during bonding; the central channel and grooves for optical fibers were milled in one half and side channels were partially milled on both halves. The width and the height of the fabricated central channels were 134 \( \mu \)m and 120 \( \mu \)m, respectively. The fluid inlets were produced with a 500 \( \mu \)m diameter drill bit. The device requires two sheath inlets and one sample inlet. However, since the flow rates on two sides are equal, only two pumps were needed. This advance is highly desirable for simplicity of fabrication and operation and enables an easy integration with other microfluidic and optical components. It should be also highlighted that the two side channels can be connected to each other and to the sheath inlet. This is straightforward from a fabrication point of view and should further reduce the number of inlets to two.

3.2. Experimental investigation of three-dimensional sheath flow

The capability of the device of creating a three-dimensional sheath flow was experimentally demonstrated by imaging the fluid distribution at the output of the manifold. We used a fluid sample containing Rhodamine B dye dissolved in water at a concentration of 2mM and pure...
water as sheath fluids. Tubing system in polytetrafluoroethylene (PTFE) was connected to the inlets for pumping fluids in the channels. The fluids are injected in the channels by syringe pumps for precise control over flow rates. A collimated solid state laser emitting at 532 nm was directed perpendicular to the axis of the main channel near the channel end. To reject the excitation light from pump source, a band pass filter was used. In order to avoid lens effects due to the liquid surface tension, a thin glass slide (0.130 mm) was placed near to the output channel. However, the output channel was not sealed, in order to allow the fluid to flow out of the outlet. The fluorescence intensity distribution was imaged by using a microscope objective (20X) and a charge coupled device camera. The sheath flow rate was kept constant at 2 ml/hr and the sheath to sample flow rate ratio was changed in the range from 1 to 15. Images at various focusing ratios FRRs were measured [Fig. 3].

Fig. 3. Measured fluorescence intensity profile of focused stream under varying flow rate ratios. Orthogonally crossing dashed lines show the center of the focusing channel.

Sample flow was successfully focused into a circle placed in the center of the channel. A focused stream as small up to about 18 μm was measured. The fluorescence intensity profile of the captured images was compared with profile of fluid dynamically simulated concentration distribution. In Fig. 4(a) are shown the measured horizontal and vertical FWHMs versus FRR. Notably, the shapes of the distribution of fluids well agree with simulations. The measured horizontal versus the vertical FWHMs at different FRRs are shown in Fig. 4(b), where the line refers to the ideal case of a perfect circular shaped stream. The experimental points slightly spread around the line, indicating a good agreement with expected behaviour. As predicted, the sample size was controlled by varying FRRs, while its shape was nearly unaffected by changing FRRs. The influence of the FRR on the location of the center of the sample stream in the focusing channel was also experimentally evaluated. The center of mass of the focused stream at various FRRs was measured from the experimental images [20]. Images processing was performed with our image analysis program, written in MATLAB. Positions of center of mass in x and y direction are shown in Fig. 5 at various FRRs, where the axis origin coincides with the center of the focusing channel. As it can be noticed, the displacement of the center of the focused stream reach the maximum value of about 2 μm (in both x and y-direction) and it can be attributed to fabricated channel dimension that does not match perfectly with the simulated one.
4. Application: microflow cytometer

The proposed hydrodynamic focusing manifold forms the basis of a microflow cytometer with potential single particles analysis. Detection of particle transit is performed by means of two integrated optical fibers, self-aligned with the particle stream. Optical fibers (50 μm core diameter) with numerical aperture of 0.22 were cleaved and inserted into the grooves. This arrangement allows us to detect simultaneously two signal data from particles focused in the detection region: fluorescence and side scattering signal. Scattering signal is widely used for cell counting in flow cytometry, but it also improves device analytical performance by providing information on cell size and shape. Two set of measurements has been performed. with rhodamine B labeled fluorescent microparticles with different diameters. Micro particles used in the experiments had a diameter of 10 μm with std <0.3 μm and CV < 3% and a diameter of 23 μm with std <3 μm and CV< 13% with internally incorporated fluorescent labels (volume-stained). CV of fluorescent intensity was not furnished by the vendor. The capability of the proposed device to focus sample flow in a tight stream allows particles to pass the detection volume in single file. A focusing solution of water was used as sheath flow in both cases. Sample flow rate was set to 1.5 ml/hr during experiments, while focusing ratio was set to 14 and 4 for 10 μm and 23 μm-sized microparticles detection, respectively. The estimated stream size for FRR = 14, 4 was about FWHM = 18, 30 μm.
The experimental set-up for particles analysis is reported in Fig. 6. A collimated laser source at a wavelength of 532 nm was coupled into a single mode optical fiber with fiber axis perpendicular to the device plane. The exit ends of the collecting fibers were coupled to two avalanche photodiodes (APDs) connected to a digital oscilloscope. A band pass optical filter was placed in front of the fluorescence detector to reject pump contribution on fluorescent peaks detection. The particle’s transit through the detection volume gave rise to optical pulses in the signal collected by the fibers. The height and the width of each pulse are typically used for high throughput particles differentiation and both parameters were considered in this work for a statistical analysis. Figure 7 shows a typical diagram of fluorescent pulses detected by APDs modules. In Fig. 8 are shown histograms of the fluorescent and side scattered signals amplitude distributions against the number of events for 10 μm and 23 μm-sized microparticles. To assess the quality of the proposed device, the coefficients of variation (CVs) of both scatter and fluorescence amplitude were calculated. The CVs are calculated by a Gaussian fit of the histograms. Fluorescence amplitude CVs of 8.3% and 15.4% and scatter amplitude CVs of 12% and 18.6% were found for 10 and 23 μm particles, respectively. CV of a flow cytometer depends on several factors, including the quality of the optical excitation and of the detection system. High quality light source to uniformly interrogate the detection region and high numerical aperture detection optics have demonstrated to increase the CVs of a flow cytometer. For the purpose of this work, such a high quality illumination system and integrated focusing optic components are avoided as they increase costs and add complexity to the device. Furthermore, the use of optical fibers for excitation and collection permits a self-alignment of sample stream and the detection system. Typically CVs of the order 3% are obtained with bench top cytometer [21]. More recently, Frankowski et al [22] and Nawaz et al [23] used microfluidic chips with 3D hydrodynamic focusing with high numerical aperture free space optical scheme and a high-quality optical source to achieve CVs of about 3%. Even though the CVs of the proposed microflow cytometer are relatively high as compared to commercial benchtop cytometers, it is comparable and in some cases superior to other integrated versions [2], [24–28]. It is worth to be noted the work of Mao et al [29] that demonstrate superior CVs (6.3%) in a microflow cytometer with integrated single mode optical fibers to excite particles and detect the emitted fluorescence signals.

From results, higher CVs were observed for side scatter detection. Side scatter light is proportional to the overall size of the particle, but is also affected by the internal complexity (bulk scattering) and by the smoothness of the particle surface (surface scattering). Typically, even when relatively good CVs are obtained with fluorescence and forward scattering measurements, large CVs can arise in side scatter. This can be explained by Mie theory and is due to extreme sensitivity of the side scatter signal to small variations in the size of particles or internal granularity [21]. Such variations can be often found in beads due to imperfections in production process.

A further analysis has been performed on fluorescence pulse signals. The distributions of optical pulses width that represent the particle transit time and contain information about the size of particle have been evaluated. This parameter is often used to discriminate singlet, doublets or multiple particles in the detection region [30]. Hence, the analysis of transit time distribution represents another means to assess the 3D flow focusing quality. Low CV of the distribution of particles transit time indicates the ability of the 3D focusing to align particle in a single line and to prevent particles to spread in the vertical direction in the focusing channel. In Fig. 9 are shown histograms of the transit time against the number of events for 10 μm and 23 μm-sized microparticles. CVs of transit time distribution of 2% and 5.2% were found for 10 and 23 μm particles, respectively. These values are very good and in agreement with particle size CVs. The reported CVs are competitive among previously reported results and confirm the ability of the proposed device to focus particles successfully [15,30].
Fig. 6. Schematic illustration of the experimental set-up.

Fig. 7. Digital oscilloscope traces of fluorescent peak signals generated by APD module when detecting 10 \( \mu \text{m} \) particles.

Fig. 8. Frequency histogram of fluorescent and scattered light intensity of (a) 10 \( \mu \text{m} \) particles, (b) 23 \( \mu \text{m} \) particles.
5. Conclusion

This study has presented a microflow cytometer for particle scattering and fluorescence analysis. A narrow single-file stream of particles is achieved by hydrodynamic focusing effect based on a novel three-dimensional sheath focusing scheme. A focused particle stream with circular cross sectional shape and tuneable size is achieved by varying the sheath to sample flow rate ratio. Stream size with FWHMs varying from about 18 to 50 µm has experimentally demonstrated using fluorescence microscopy. The position of the particle stream in the focusing channel was found almost unaffected by changing FRR. Detection of fluorescence and scattering signal has been carried out by means of two integrated optical fibers, self-aligned with the focusing channel. The sample stream width can be adapted to the particle size by adjusting the hydrodynamic focusing ratio and two set of experiments have been carried out, each one using different particle size. The device provides low CVs, which demonstrate its ability to focus particles in a tight stream.