Development of a Lab on a Chip Flow Cytometer
Portable and Affordable Flowcytometer for Point of Care Diagnostics in Rural Areas

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Abstract: We have developed a lab prototype of a microfluidic flow analyzer, which is capable of quick and efficient analysis of biological samples. Low cost and portability makes it suitable for point of care diagnostics in rural area of developing countries. A significant size reduction has been achieved by choosing a microfluidic flow and pumping system, micro-electronic components, integrated circuits boards, and fiber optics. A two dimensional microfluidic chip fabricated with nanolithography technique integrates the fluidics and optics into a single platform. Forward scatters (FSC), side scatter (SSC) and fluorescence (FL) are measured from polystyrene beads as well as from different live cells. Overall dimension achieved for the final prototype is 39 cm x 22 cm x 10 cm.

1 INTRODUCTION

Among the existing methods for biological fluid analysis, flow cytometry is always preferred for its functionality and capability of detailed analysis (Shapiro 1995). It is a non destructive way of knowing the physical and biochemical properties of biological samples based on optical detection. In addition to applications in biomedical research field for immunology (Chattopadhyay et al. 2006; Mohan et al. 2015), single cell analysis (Chattopadhyay and Roederer 2012; De Rosa 2012; Telford et al. 2012) and molecular biology (Nunez 2001; Chattopadhyay et al. 2006), it is also being availed in a clinical environment (Glencross et al. 2002). As far as haematological diseases are concerned, it is very important to check the status and progression of disease in a regular basis. Taking the case of rural areas in developing counties, many of them are struggling with communicable and non communicable diseases of severe nature like AIDS, leukaemia etc. Lack of proper diagnostics and treatment facilities is one of the major reasons for unsolved health issues. This has been a motivation to researchers in the field of biomedical devices for the past few decades (Martinez et al. 2008; Martinez et al. 2010). There have been many efforts to develop a portable and cost effective flow cytometer, which can be used in primary health care centres in rural area (Tung et al. 2004; Wang et al. 2004; Chattopadhyay et al. 2006; Mao et al. 2009; Mao et al. 2012). Numerous studies have proved that the transportation and handling of blood sample can significantly affect test results while on spot tests can improve reliability. In addition to rural communities in need of testing for HIV health monitoring, many urban localities throughout India, will benefit from access to a low-cost and more instrument.

Conventional models of a flow cytometer are as expensive as $10,000 and work only in centralised facilities of major health care centres (Shapiro 1995). Bulkiness and complexity of the instrument demands proper maintenance and well trained expertise to operate the machine. Qualified people have to go through special training for using the machine, analyzing the data and make reports. In addition to that, fixing any functional failure and troubleshooting requires access to technicians. All these add a considerable cost to the maintenance.

Our approach for designing a portable and low cost flow cytometer involved understanding the major factors that cause the bulkiness and complexity of the conventional system, and methods

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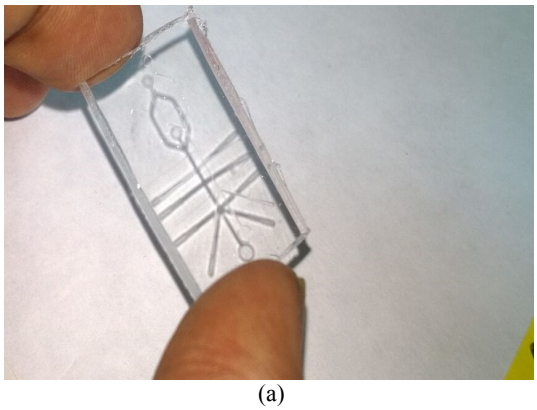
to reduce them. A conventional flow cytometer design encompasses three major disciplines of technology, namely are fluidics, optics and electronics. Fluidics deals with guiding the sample inside the machine once it is loaded. Around 2-5 mL of the sample fed into the inlet of the machine is sucked in by fluidic pumps through the respective tubing. This sample is then subject to a 3 dimensional fluid focusing technique, called hydrodynamic focusing (HDF). A guiding fluid will flow around the sample keep the flow focused to a certain diameter. Pumping of the sheath fluid is controlled such that the sample stream makes a single file flow of cells or particles down the flow. This flow then undergoes optical interrogation with one or more lasers. Collection of various signals from the sample is achieved with the detectors kept at different angles with respect to the incident laser. Most of the incident light gets scattered in the forward direction, without much interaction with the internal structures of the cell. This signal collected between 0° and 20° gives information regarding the cell size and is called forward scatter (FSC). Internal complexity information of the cell is given by the scatter at larger angles 45° -90° , called side scatter (SSC) (De Rosa et al. 2001). While the FSC and SSC data together described the physical and structural properties of cells, we can label the cells with specific fluorescent tags and identify them by detecting the emitted fluorescence (FL). Many of them have multicolour detection for which a bunch of additional lasers and detectors are used. Although the functionality is high, bulkiness, complexity and cost is also high because of the design. The optical unit working in free space domain consumes considerable amount of space and it also requires several components like lenses, prisms etc. to guide the light in a desired way. Such a system would also be highly sensitive to dust and other disturbances in a primary health care (PHC). Use of several detector units such as photomultiplier tubes (PMT), and Avalanche photo diodes (APD) with high gain, again increases both the complexity as well as power consumption.

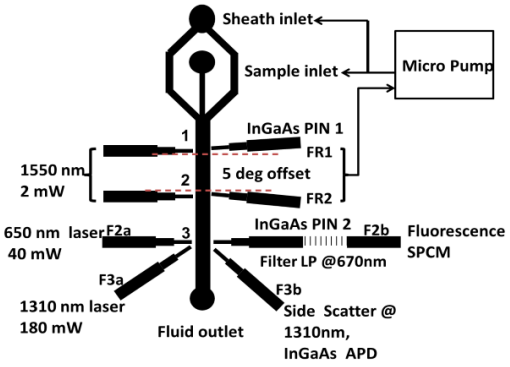
In this work, what we describe is a miniature flow analyser as a lab-on-chip; combining principles of optics, flow cytometry, microfluidics device fabrication, and nano electronics to allow rapid cell analysis and quantification. After minimal preparation, the sample flows through a microfluidic device in which a chosen laser detects the presence of specific biomarkers e.g. CD4 in case of HIV immune health monitoring. Data is collected and processed on a small electronics board. The device gives the user a clear, concrete measure of the level of these biomarkers in patient samples. This technology can be adapted to cell culture assays, detection of water contamination, platelet and other blood cell counts and oncology tests. The proposed device will need an investment of only USD 5000, uses a smaller sample volume, is highly portable and is cheaper and easier to maintain and upgrade.

2 DESIGN AND FABRICATION

Reduction of size and cost being the major concerns of the design, we have carefully chosen each component for fluidics, optics and electronics. Microfluidic pumps and chip have played a role in reducing the physical volume considerably. Microfluidics is generally chosen for the fact that its channel dimensions are well suited to the dimensions of cells being analyzed (El-Ali et al. 2006; Young and Beebe 2010; Thompson et al. 2014). The heart of the design is a microfluidic chip with rectangular flow channels and grooves for optical fiber insertion. Devices were fabricated with the commonly used PDMS (Poly dimethyl Siloxane) (Tung et al. 2004) as well as PMMA (Polymethyl methacrylate). PDMS based devices were fabricated using the photolithographic technique on silicon wafer. Mixture of PDMS and cross linking agent in 10:1 ratio was poured onto the Silicon master and baked in a hot oven at $70^{\circ C}$ for 6 hours. The mould (Figure 1a) was peeled off from the silicon and bonded with onto a glass slide after plasma treatment. Inlets and outlets for tubing were punched at the respective positions before the bonding. Sample fluid and the sheath fluid are fed into their respective inlets through silicon tubing. Once the flow is started from the respective micro pump, (Dolomite) the fluids will get into the main fluidic channel of width 130 μm. Flow rates are controlled such that the sample stream attains a desired width depending on the size of the particle being investigated. The sheath fluid guides the sample sideways by developing a laminar flow. Required volume of samples and reagents are tested to be 100 to 500 μl in such a device. The microfluidic chip also has grooves meant for inserting optical fibers for light illumination and detection. These groves are located at a distance of 2 mm from the fluidic junction (x) in a direction normal to the main flow channel. Particles in the flow will undergo the optical interrogation at specific locations in this manner. Customised optical fibers with tapered/ len sed tips (Lase Optics Inc.) serve to illuminate and



(a)



(b)

Figure 1: (a) Microfluidic device fabricated with PDMS (b) Micro flow analyser setup design describing the functions of each fiber channels. The fluidic channel and fiber grooves are sown as thick lines.

collect light. The lensed single mode fiber helps to focus the light to 15-20 µm dia. at a distance of 150 µm, which is the distance from the fiber groove inner end to the centre of the fluidic channel as shown in Figure 2. Fiber at the collection end has a hemispherical lens with a higher numerical aperture, to increase the collection efficiency. Detection fibers are chosen to be multimode, since it is required to maximize the amount of light being collected. Losses due to presence of an air gap were eliminated by filling the fiber groove with an index matching fluid (IMF) of refractive index 1.4. The complexity with free space optics and components was thus avoided by availing of lensed fibers and self-guided fiber grooves.

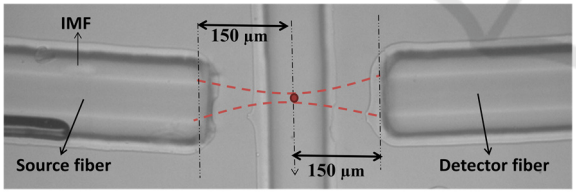


Figure 2: Top view of the microfluidic chip depicting optical interrogation of particles in flow channel.

Figure 1b, shows the complete design of the instrument including three lasers and four detectors. First two pairs of fiber grooves FR1 and FR2 are meant for collecting the FSC signal at +50 and -50 respectively. A diode laser at 1550 nm with fiber coupled output was used as a common source for FR1 and FR2. Output power of 2 mW is split into nearly equal powers by using a 50:50 fiber splitter and fed to the tapered fibers that were fit into the grooves. A cell passing through the fluidic channel gets interrogated at point 1 and 2, and the FSC is collected by separate fiber coupled InGaAs p-i-n by correlating PIN1 and PIN2 outputs during the flow. A feedback system connected to the micro pump is designed to monitor and control the flow rates. Point 3 in figure 1b is the junction where FL and SSC are being collected. Our choice laser and detector for fluorescence excitation were according to the biomarker being used. In this work we have used a 650 nm source at 2 mW, and a single photon counting module (SPCM - Perkin Elmer) for detection of fluorescence signal at 5°. SSC is also detected at the same point in the device using a 1310 nm, 180 mW laser source. Fiber grooves F3a and F3b are used to make 90° between the source and detector. We have used an avalanche photodiode (APD), as the scattered signal is too weak to be detected with a PIN diode. The design is such that one can customize the combination of lasers and detectors to be used for a given experiment. Desired combinations of F2a, F2b, F3a and F3b can be used for measuring different parameters of interest.

3 EXPERIMENT AND RESULTS

3.1 Flow Control and Manipulation

Having established hydrodynamic focusing in the microfluidic chip, and interrogated cells at different locations down the channel, it was important for us to know the extent of correlation between the events different sizes (Sigma Aldrich). Original samples of 1 million/mL concentration were fed to the sample inlet and DI water was used as the sheath fluid. Sample and sheath were flowed at the rate of 10 µL/min and 30 µL/min respectively. The lag time of the particle, as it travels from FR1 to FR2 was observed for different flow rates. A reduction of lag time was found as the flow rate as increased as

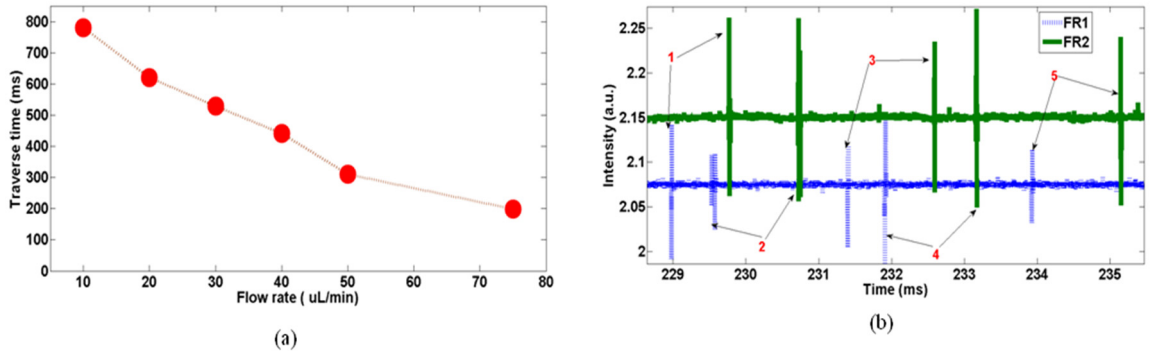


Figure 3: (a) Experimentally observed traverse time (time delay) between FR1 and FR2, as a function of flow rate. (b) Intensity versus time plot showing the correlation of events occurring in FR1and FR2 channel. 5 pair of events are identified and marked.

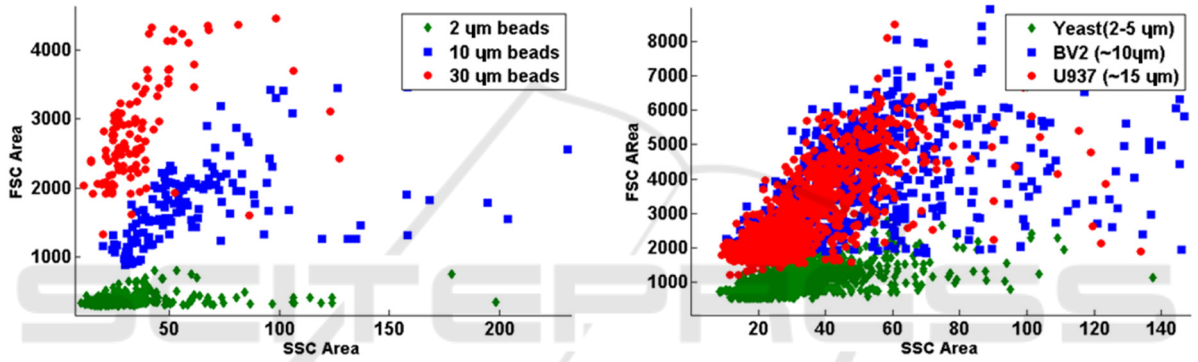


Figure 4: (a) SSC versus FSC scatter plot of different size beads. (b) Scatter plot for cells of different sizes. Area of FSC signal is plotted against the area of SSC signal in both cases.

shown in Figure 3a. Observing the trend between the two parameters also helped in validating the concept of flow control using the velocity measurement and feedback system. FSC events detected at both the channels are found to be reasonably correlated. Figure 3b shows an instance of flow with polystyrene beads. We have FR1 and FR2 locations separated by a distance of 2 mm, and the corresponding time delay observed in the data is around 1ms. Time delay measured from this data allows one to estimate the flow rate of the sample. Correlation measure between FR1 and FR2 yield a reliable metric for use as flow control.

3.2 Size based Isolation of Beads and Cells

The primary testing of the FSC and SSC detection was carried out with commercially available polystyrene beads of 2 µm, 10 µm and 30 µm standard sizes. Therefore, we validated the detection of scattering from different sized live cells such as yeast (3-5 µm), U937 (~10 µm) and BV2 (~15 µm). Figure 4a shows the isolation of different sized beads plotting the area of FSC signal with that of SSC signal. We can see that the beads are reasonably separated in terms of FSC amplitude. A similar plot of different types of cells is shown in figure 4(b). We could see a considerable difference in the FSC area of the yeast cells from the other two cell groups which are not properly resolved. The sample stream is flowing under a two dimensional (2D) flow focusing condition in which the sample fluid is getting focused only in the lateral directions. This lateral focusing also allows the particles to distribute along the height, 150 µm of the flow channel. Hence it never achieves a complete single file flow in a 2D device. Studies have shown to have improved results when we use 3D flow focusing methods (Rosenauer et al. 2011; Shivhare et al. 2016) (Mao et al. 2009).

3.3 Cell Labelling and Detection of Fluorescence

Our setup for fluorescence detection system consists of a 650 nm laser source which can yield 30 mW of power. Collection of the signal was made at 135^0 with respect to the source direction. The choice of laser wavelength and filter (670 nm long pass) was optimized for APC - Cy5.5 dye, which has a maximum excitation at 650 nm and emission peak at

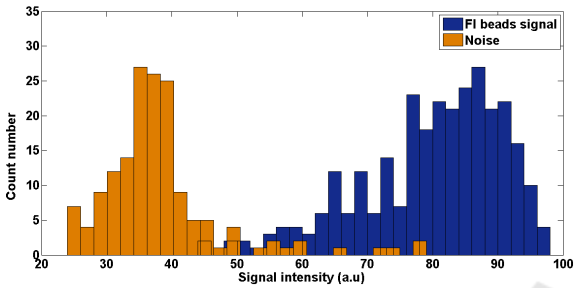


Figure 5: Spectrums of Cy 5 fluorophore and APC - Cy 5.5 used for beads and cells respectively.

680 nm. Figure 5 shows the spectra of Cy5 and APC – Cy 5.5. Fluorescent beads (Bangs Laboratory) were customized to have Cy5 dye which has excitation and emission at 630 nm 670 nm respectively. Figure 6 is showing the capability of

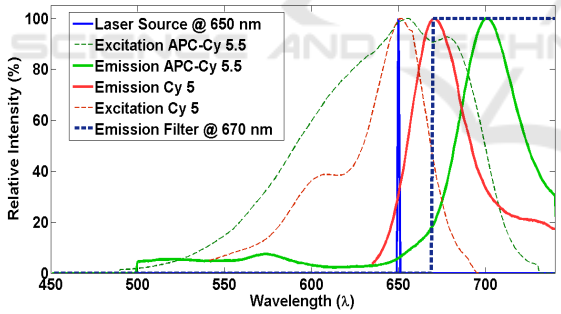


Figure 6: Histogram of fluorescent beads detected over the noise. The fluorescent signal intensity is well separated from the noise histogram.

the system to separates out the intensity of fluorescent beads from the noise. Having detected the fluorescence signal from beads, we moved on to measure the FSC and FL signals simultaneously from live cells.

3.3.1 Sample Preparation – Staining of Peripheral Blood Mononuclear Cells with CD4- AP - Cy 5.5

We prepared a both stained and unstained samples of PBMC (Himedia laboratory) with a concentration of 1 million /100 µl. The sample was spun down at 1500 rpm and $4^{\circ C}$ for 3 minutes. Blocking buffer (1% BSA, 0.01 % Azide and 1X PBS) was added to it after removal of media. One million cells were counted, washed and kept for 10 minutes of room temperature incubation on adding 2.5 µg of true stain. It was taken and washed again in blocking buffer. 15 µl of CD4 APC Cy 5.5 were then added into 100 µl of reaction volume. After doing one more incubation for 20 minutes in ice, the cells were washed in buffer (1X PBS, 0.01 % Azide). Same count of cells was taken for unstained sample after doing the washing. Both the samples were tested in Guava analyzer and the micro flow analyser simultaneously. Figure 7 shows the comparison of counts obtained in Guava and our micro flow analyser. Out of 10000 events in the Guava, 7500 were giving positive staining results, making the

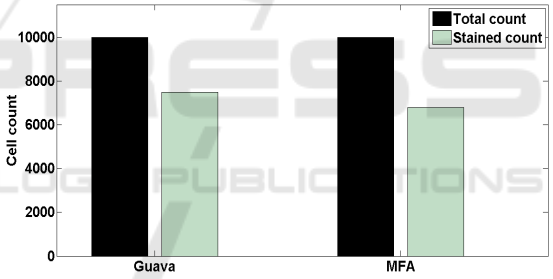


Figure 7: Comparison of detection efficiency of MFA and Guava with CY5 stained PBMC cells.

ratio 1.33. We have compared this number to the ratio of FSC events to FL events detected in micro flow analyzer. And it came to be as close as 1.4. Results with PBMC were found to be encouraging to continue the experiments with human blood, since the system has proved it's the ability to pick up the staining levels of PBMCs. In the following section we showcase the hardware of lab prototype which includes the optics, fluidics and electronics parts of the system.

3.4 Product Packaging

We have been able to encase the entire setup of our micro flow analyzer in the form of a portable instrument (Figure 6(d)) along with user friendly software. Lasers with infrared wavelengths being

available at a lower cost, we decided to use them for FSC and SSC detection. The fluorescence wavelengths being dependent on the sample (fluorophore) being used, the current version was designed for APC Cy 5.5, and we used a 650 nm laser for excitation. To test any other sample, the excitation wavelength and filters can be changed. A wavelength tunable laser will also serve this purpose for testing variety of samples. All the lasers and detectors were integrated onto a single board as shown in figure 8(b). Use of optical fibers to guide light has helped in saving physical space. All the sources and detectors were spliced with lensed fibers and were guided right till the point of interrogation. The specially designed grooves for fibers in the microfluidic chip kept the fiber in place well aligned to the microfluidic channel. Microfluidic piezo pumps (Dolomite) greatly contributed to the reduction of sample volume as well as the instrument size. Electrical signals from the detectors were converted by a 10 bit analog to digital converter (ADC) and digital data was transferred to the 16 bit micro-controller. Data communication with the computer was achieved over a UART interface. We have demonstrated a compact packaging method for the instrument which is enclosed with a black acrylic box as shown in Figure 8(d). The components were arranged in two stacks supported by acrylic plates and metallic posts. The bottom plate consists of the electronic board with lasers and detectors, microcontroller board and power supply. Top plate has the microfluidic chip, micro pump and controllers etc. (Figure 8(c)). Optical fibers spliced to the detectors of bottom plate are brought to the top plate through given slots. The whole package has been finally covered with a compatible lid of same material. Containers for sample, sheath and wash fluids were kept in the small outer box attached to the package. One can load the sample at this point so that it will be taken in through the tubing connected with the micro pump. Figure 8 shows different parts of the final package. Connecting ports for power supply and USB connectivity are also given at one side of the box. The software for real time data collection and analysis has been designed on Python in which user is allowed to control the lasers and detectors from a graphical user interface.

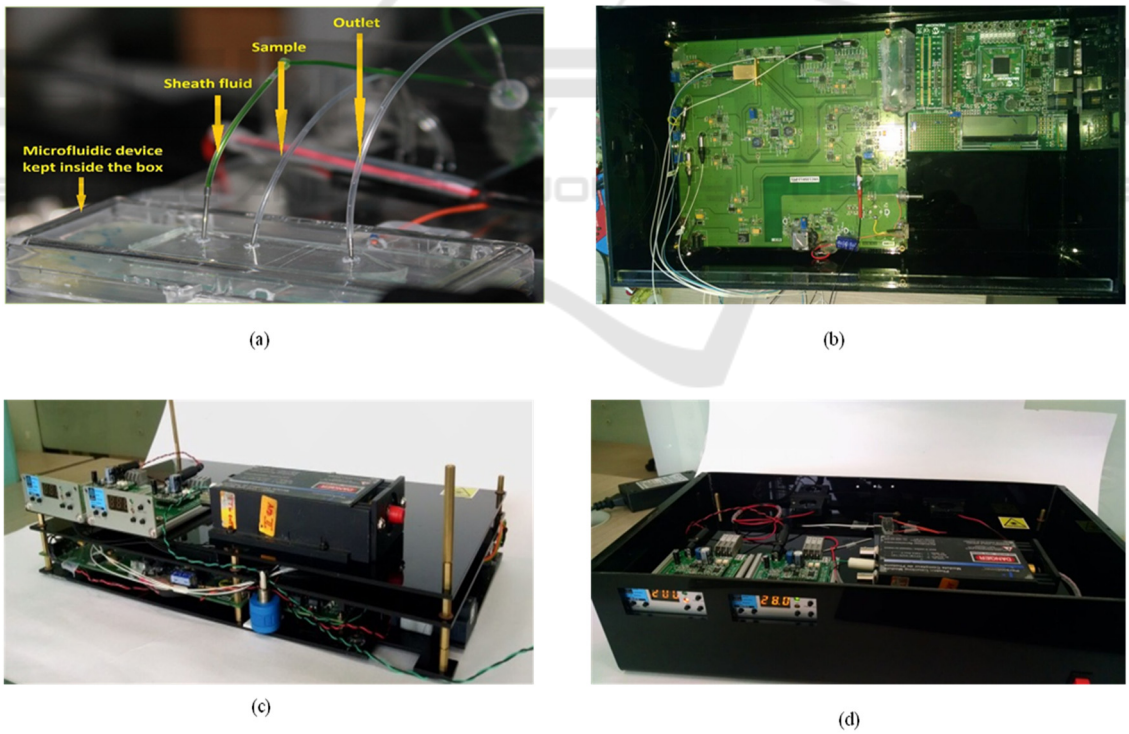


Figure 8: (a) Microfluidic system of the Micro flow analyser, comprising the microfluidic chip, piezo pump and tubing. (b) Bottom plate of the instrument consisting of integrated electronic board of lasers and detectors, microcontroller board, and power supply unit. (c) Top plate with pump controllers, SPCM and microfluidic system. (d) Complete final look of the instrument packaged in a black acrylic enclosure of size 39 cm x 22 cm x 10 cm.

4 CONCLUSIONS

We have developed a laboratory scale prototype, which is in the final stages of optimization. We have successfully tested this prototype for microfluidic, optics and electronics integration along with flow rates for beads and cells testing. We have also optimized the fluorescence testing with beads and PBMC cells with the final integration. This novel design and integration has been patented in US and South Africa (Saiyed et al. 2016). Currently we are working on alternative methods for 3D flow focusing since presently employed methods requires complex fabrication techniques.

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