FLUORESCENT LABELING, SENSING AND DIFFERENTIATION OF LEUKOCYTES FROM UNDILUTED WHOLE BLOOD SAMPLES

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Abstract: In this paper, we demonstrated leukocyte labeling, sensing and differentiation from undiluted human whole blood samples with microfabricated devices. A challenging issue in leukocyte sensing from blood samples is the required high dilution level, which is used mainly to prevent interference from the overwhelmingly outnumbered erythrocytes. Dilution is undesirable for micro hemacytometers. It not only increases sample volume and processing time, but also requires mixing and buffer storage for on-chip implementation. Unlike commercial bulk instruments and previous efforts by other groups, we completely eliminated the requirement for dilution by staining leukocytes specifically with fluorescent dye Acridine Orange (AO) in undiluted human whole blood and then sensing them in microfluidic devices. Green fluorescent signal centered at 525nm was used for leukocyte count and red fluorescent signal centered at 650nm was used for leukocyte differentiation. Throughput of one hundred leukocytes per second was achieved, which means operation time for one sample only requires several seconds.

Keywords: Leukocyte, Fluorescent, Sensing, Differentiation, Acridine Orange.

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1. INTRODUCTION

Leukocytes respond to toxic, infectious and inflammatory process to defend tissues and eliminate disease process or toxic challenge. Accurate and prompt counting and differentiation of leukocytes is critical for diagnostics of infection, leukemia or allergy, monitor bone marrow function or the body's response to various treatments [1]. Currently it is performed either manually or by macroscopic automatic machines. Manual blood analysis requires making blood smear or use hemacytometer and then counting by a trained professional. Automatic blood counters are usually based on flow cytometry and employ a combination of electrical impedance sensing, light scattering measurement and chemical or immunostaining. Micro blood counters promise to provide a point-of-care solution, which costs less and be more accurate than manual count.

Particle (bead, erythrocyte and cultured cell) sensing has been demonstrated, for example, by electrical impedance sensing [2], light scattering detection [3] and fluorescent sensing [4-6] in micro systems. All of these previous studies use diluted samples. Here we report human leukocyte counting and differentiation with microfabricated devices from undiluted human blood. Dilution is normally required as one of the sample preparation steps in blood cell counting and differentiation. Dilution is used to prevent coincidence effect, reduce the risk of sample clogging and sometimes as a natural result of either chemical hemolysis process to remove erythrocytes or fixation to preserve leukocyte properties. To avoid hemolysis, various fluorescent dyes that stain specifically for leukocytes can be used. Acridine orange (3,6-dimethylaminoacridine) binds strongly to double stranded DNA with shifted excitation maximum wavelength of 502nm and emission maximum wavelength of 525nm (green). It also binds strongly to RNA and single stranded DNA, with shifted excitation maximum to 460nm and emission maximum to 650nm (red). Because mature erythrocytes lack DNA and RNA, after AO staining, only leukocytes generate signals and normal erythrocytes do not interfere. Leukocyte sensing can be achieved by using the strong signal from the green fluorescent channel. Differentiation can be achieved by analyzing the signal from the red fluorescence channel since different types of leukocytes have different cytoplasm compositions [7-9]. Red fluorescence

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intensity emission of lymphocytes is lower than that of non-lymphocytes.

2. FABRICATION

The device was fabricated using soft lithography. PDMS and glass were chosen as the materials to make the device because of ease of fabrication and the excellent optical properties in the wavelengths used in this study. The channel structure was molded on a 1cm by 1cm PDMS block. To accommodate the working distance of the optical lens, the thickness of the PDMS block was controlled to be less than 3mm. The channel depth was 16μm.

As in Fig. 1, a filter array close to inlet filters out particle contaminates, erythrocyte rosettes and other large particle aggregates to prevent clogging in detection zone. Sheath flow channel hydrodynamically focuses sample flow into the detection region. In our design, the ratio of cross sectional area of sheath flow to core sample flow was ten to one. The channel width of the detection zone was 50μm. Considering the parallel flow profile, the width of the focused sample flow should be less than 5μm.

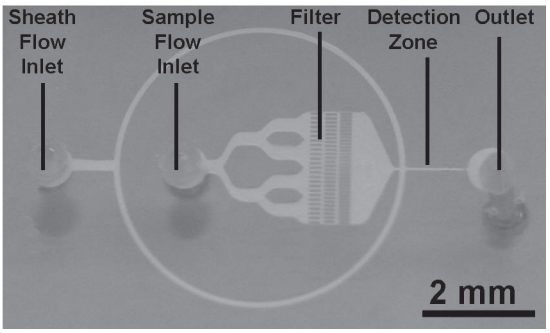

Fig. 1 Top view of fabricated device.

3. RESULTS AND DISCUSSION

The fluorescent sensing was demonstrated with a laser induced fluorescent (LIF) system (Fig. 2). An Ar laser with central wavelength of 488nm was used as the excitation source. CCD camera, photodiode and/or photon multiplication tube (PMT) were used as detectors.

The system was first calibrated with green fluorescent beads. Under the testing flow rates, the center position of the beads was limited within 0.7μm at the detection region, which confirms the effectiveness of the hydrodynamic focusing. Each bead generated a flash on videos taken by the CCD camera. With a focused laser beam as in Fig. 3(a), the beads created a enlarged light circle as in Fig.3(b). Only a single bead normally appeared in each image. With diffused laser illumination as in Fig.3(c), the trace of the bead could be identified Fig.3(d). Distinctive peaks with high SNR was recorded by the photodiode (Fig.4) at 525nm.

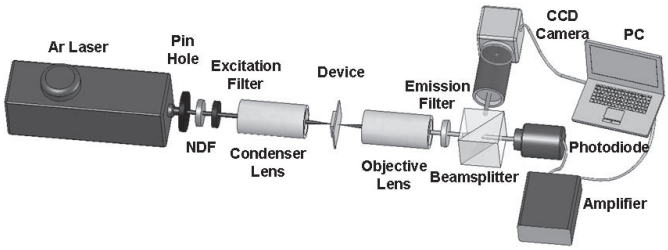

Fig. 2 Optical system setup. For sensing at two wavelengths simultaneously, the CCD camera was replaced by a PMT with two emission filters in front of two detectors.

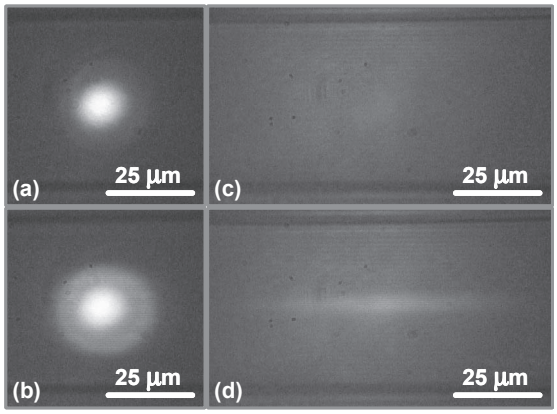

Fig. 3 Images of 5μm beads flow taken by CCD camera. (a) and (c) were images of background. (b) and (d) were images of beads passing by. The laser spot in (a) showed the position of the focused laser spot. (c) and (d) were images of with diffused laser illumination. (d) showed the trace of the bead.

For blood samples, fluorescent dye AO and anticoagulant EDTA was added into blood collection tubes. To achieve high signal to noise ratio, it is important to measure the optimal acridine orange concentration for leukocyte staining. Because of the autofluoresence of acridine orange and its absorption by erythrocytes

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and platelet, higher concentration of acridine orange increased the background noise. Lower concentration did not provide enough contrast to distinguish leukocytes from background. 10μg/mL was found experimentally to give the highest signal to noise ratio when viewed using a fluorescent microscope.

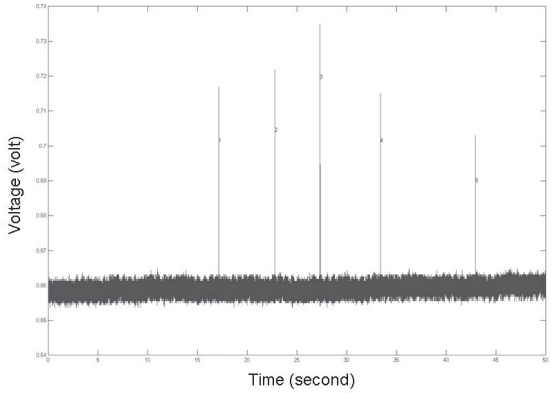

Fig. 4 5μm fluorescent beads detection with photodiode detector.

AO stained whole blood was introduced with th e central flow and 10x PBS diluted to 1x with Fico ll Phage Plus was used as sheath flow solution. Fi g.5 shows the images taken by the CCD camera. Although the signals were weaker compared with those of the 5μm beads, they could clearly be dete cted by the photodiode at both green and red wave lengths (Fig.6). The histogram of the signal intens ity detected at 525nm (Fig.7) shows a signal peak which tails off in high signal intensity range. Red fluorescent signal at 650nm had two peaks in the h istogram (Fig.8). The peak of the lower signal int ensity is dominated by lymphocytes and the peak of higher signal intensity is contributed by monoc ytes and granulocytes.

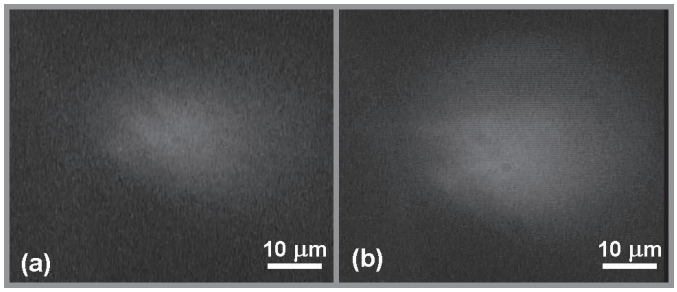

Fig. 5 Images of whole blood cell sample taken by CCD camera. (a): background image showing focused laser beam. (b): signal from a leukocyte.

In our testing, the maximal throughput was about a hundred leukocytes per second with PMT detector. Under the maximal throughput, the total time for counting a minimum of 200 leukocytes was a couple of seconds, which is adequate for leukocyte counting in complete blood count test. The overall system throughput of the current system is limited by the bandwidth of the built-in preamplifier of the PMT.

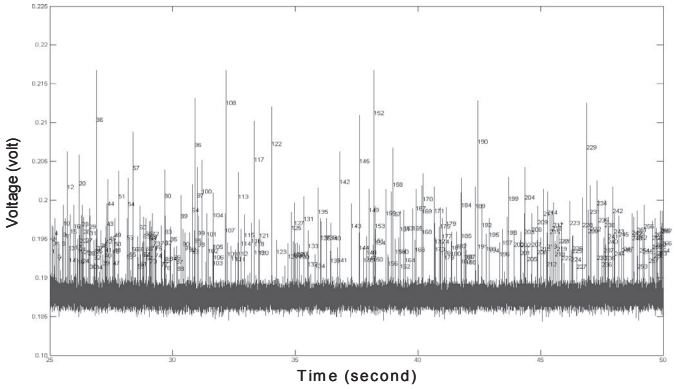

Fig. 6 Time traces of amplified photodiode signal of AO stained undiluted whole blood with green emission filter centered at 525nm. Peaks are labeled.

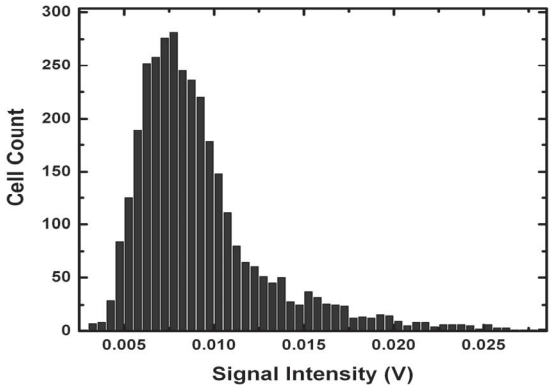

Fig. 7 Histogram of signal intensity from photodiode detector with green emission filter centered at 525nm.

The time constant of photobleaching was determined by measuring the fluorescence change from single acridine orange stained leukocyte inside the channel under laser illumination. The time constant fell in seconds range. The time for one cell to pass the detection zone in our testing was roughly 30ms for low flow rate and 1ms for high flow rate. So photobleaching was not a

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problem in our experiments.

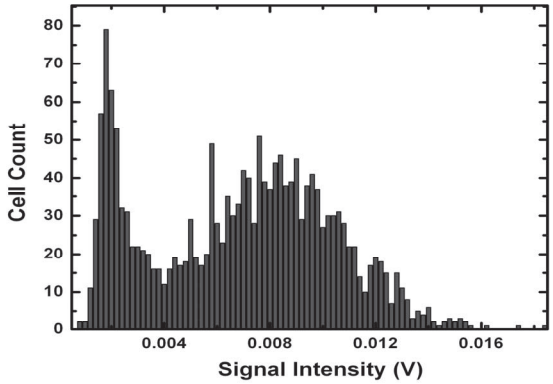

Fig. 8 Histogram of signal intensity from photodiode detector with red emission filter centered at 650nm.

Counting and differentiation leukocytes from undiluted blood were enabled by labeling leukocytes specifically and the microfluidic implementation. Handling blood samples without dilution not only greatly reduced sample volume and processing time, but also eliminate on chip mixing and buffer storage completely. Fluorescent dye and anticoagulant can be coated on the device wall so no mixing and storage of dilution buffer are required. Blood cell sedimentation is no longer a concern thanks to the high throughput. Enough counts can be collected for one sample in seconds. We also tested this technique successfully in straight microfluidic channels without hydrodynamic focusing (results not shown here). In that case, flow control can be minimal since the detection does not require stable flow rate. These advantages are especially attractive for point of care devices.

4. CONCLUSION

We fabricated a microfluidic device and used the LIF system to demonstrate leukocyte counting and differentiation from undiluted whole human blood. Leukocytes were specifically labeled by with fluorescent dye acridine orange with optimal final concentration of 10mg/mL. Green fluorescent channel centered at 525nm was used for leukocyte counting and red fluorescent channel centered at 650nm was used for leukocyte differentiation. Maximal throughput of 100 leukocytes per second was achieved. The photobleaching time constant of acridine orange in the current detection system was measured to be in seconds range.

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