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Fluorescent Imaging on a Microfluidics Chip for Quantification of Leukocyte Count

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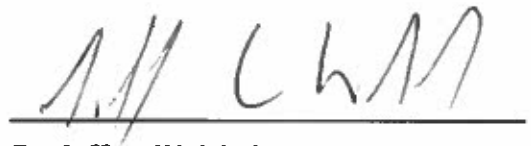

Dr. Jeffrey Wolchok

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Summary

We have demonstrated a method for conducting a leukocyte count in whole blood using a microfluidics chip and epi-fluorescence setup. Leukocyte counts provide physicians with a wealth of information about a patient's medical condition and as such are routinely completed for many hospital visits. Miniaturization of this diagnostic tool may enable physicians to provide healthcare in resource-limited settings, where patients would otherwise not receive this test. The microfluidics chip was fabricated in polydimethylsiloxane (PDMS) using soft-film lithography. Following further processing and cleaning, the PDMS mold is exposed to UV-ozone for surface activation, and then sealed with a glass coverslip to create an enclosed chip. The epi-fluorescence microscope was constructed using a blue LED light source, excitation and emission filters, dichroic mirror, and objective lens. Prior to imaging leukocytes in whole blood, the optimal linear flow velocity in the microfluidics channel had to be determined to achieve minimal motion blur and sufficient signal-to-noise ratio. This was done by conducting a series of flow rate experiments in which fluorescent microspheres were seeded in phosphate-buffered saline (PBS) and pumped at various volumetric pump rates while simultaneously imaged with the epi-illuminating fluorescence microscop. These images were analyzed using ImageJ to determine average linear velocity of bead flow as it passed the image sensor. Values from this experiment were used to pump leukocytes at an optimal rate for image acquisition. Minimal pre-processing of the sample was completed with an anticoagulation agent, which prevents clogging within the channel, and proflavine, a fluorophore used to stain the nuclei of the leukocytes for imaging. The processed blood sample was then pumped into the chip and imaged simultaneously. Image data was gathered and processed to separate between populations of leukocytes based on nuclear morphology. In this particular

system, a 3-part differential can be completed to distinguish between monocytes, lymphocytes, and granulocytes.

1. Introduction

Blood assessments are an essential diagnostic tool for physicians because they contain a significant amount of information regarding a patient's health. In particular, the white blood cell (WBC) count, which quantifies leukocyte subpopulations, is able to detect a variety of conditions that are associated with quantitative abnormalities in leukocyte count (1, 2). Leukocytes are nucleated white blood cells involved in the immune system functions of the body and they are subdivided into granulocytes and agranulocytes. Granulocytes, which contain granules within the cytoplasm, are further subdivided into neutrophils, basophils, and eosinophils. Agranulocytes, which are mono-nucleated cells with a clear cytoplasm, are comprised of lymphocytes and monocytes (3). Deviation from normal leukocyte levels can be indicative of a range of medical conditions from allergies and bacterial infections to anemia and autoimmune disease (1, 3, 4, 5, 6). The multitude of conditions that a blood count can help diagnose has made it a tool that many physicians depend upon, and they recommend that patients get regular blood assessments (4, 7).

Current methods used for blood count testing employ automated hematology analyzers. These devices are primarily based on principles of flow cytometry, although some systems, such as the HemoCue WBC, yield results based on leukocyte concentrations within a specified range. One main disadvantage of this system is its inability to provide information about the different subpopulations of leukocytes (i.e. differential count). Furthermore, HemoCue WBC cannot provide reliable data beyond the functioning concentration range (8). Other hematology analyzers, which do utilize flow cytometry, include the Chempaq XBC, the Backman Coulter LH 750, and the Sysmex XE-2100 (8, 9). These three devices employ the method of electrical impedance, which

is based on the Coulter Principle, and they function by detecting changes in electrical signals. The processed sample, which is an electrolyte solution containing blood cells, is passed through an aperture (or detection zone) and the hematology device senses changes in impedance as cells flow by (2, 10, 11). This impedance shift is proportional to the volume of the cell passing through. Although this method enables volume-based differentiation, one major disadvantage is the effect of coincidence. This is an error which occurs when multiple suspended cells appear in the sensing zone simultaneously, preventing accurate separation between cell types (10). To address this issue, many clinicians conduct hemolysis as an additional pre-processing step (6). Another option is to couple this impedance technique with the optical method of measuring light scattering, which is applied in the design of the latter two devices mentioned above (Backman Coulter LH 750 and Sysmex XE-2100) (9). Light scattering is also a volume-dependent differentiation method in which the cell volume is a function of light intensity; however, other parameters such as the cell's surface conditions and overall shape complicate this relationship, rendering light scatter measurements an imprecise determinant of cell volume (10). Another main disadvantage of both light scattering measurements and impedance-based analysis is that neither method can detect differences in cytological morphology (7), which is a reliable visual indicator for differences in leukocyte types. These limitations in the current metrics for determining leukocyte count supplicate further development in the design of hematology analyzers.

The frequent use of leukocyte counts as a diagnostic tool in regular hospital visits (1,12) as well as in emergency care settings (4) not only requires reliability of yielded results but also better accessibility to the tool, rapid output of the diagnostic information, and reduced complexity of the device interface. Presently employed hematology analyzers are large, bulky, and expensive instruments, which confines their use to settings such as clinical laboratories and research facilities

(2, 4, 12, 13). Additionally, handling and maintenance of these benchtop hematology devices is often complex and requires trained personnel (4, 14). Other procedure related disadvantages include the use of large reagent and sample volumes (4, 6, 12) and the lengthy turnaround time for test results (2, 4, 8). To address these disadvantages, miniaturization of hematology analyzers for point-of-care testing is currently being investigated by many research laboratories. One common route many researchers have explored is the use of microfluidics devices to perform diagnostic assays for blood count (4). Microfluidics refers to systems that interact with fluids at the micrometer level (10-100 um) and it entails the manipulation of these fluids to perform analytical tasks in the areas of chemistry, biology, and biomedical sciences (15, 16, 17). Current prototypes include the lab-on-a-chip Coulter counter (11), the microflow cytometer for fluorescent dye assay (12), the laser-induced fluorescent (LIF) detection system (6), the microfluidics impedance cytometer (2, 18), and other microfluidic devices using label-free methods (7, 14).

We developed a custom system comprised of a microfluidics chip and epi-illuminating fluorescence microscope which can be used to analyze blood and perform a three-part differential leukocyte count. This system (shown in Figure 1) is designed to use minimal preprocessing with fluorescent staining and provide information on leukocyte nuclear morphology for accurate classification. The microfluidics chip is fabricated from polydimethylsiloxane (PDMS), a flexible elastomeric material with tunable surface properties and ideal for replica molding (16, 17). To create the microfluidics chip, we employed the technique of

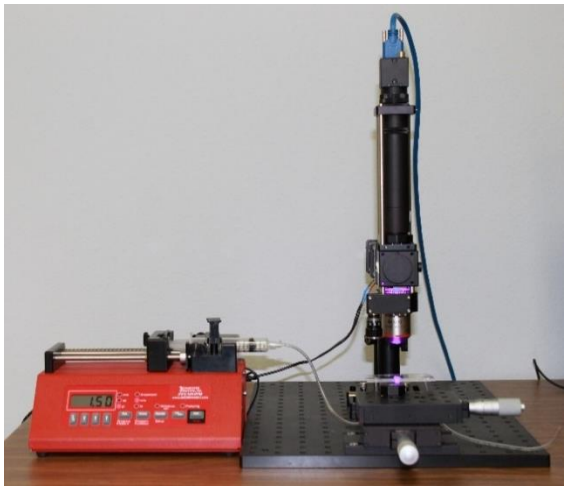

Figure 1. Full setup of microfluidic chip and epi-illuminating fluorescence system with syringe pump.

soft-film lithography to transfer the microfluidics channel pattern from a silicon wafer onto PDMS. The pattern for the microchannel is created using a computer-aided design (CAD) program and then printed onto a transparency which acts as the photo mask for the contact lithography process (17). During the fabrication process, the surface properties of PDMS are exploited to induce an irreversible seal between the PDMS and a glass coverslip. This irreversible seal is achieved using UV/Ozone surface activation by which polar groups are introduced to the surface of the PDMS, making it very reactive. Once the PDMS is in contact with the glass coverslip (which is also exposed to UV/Ozone surface activation), a tight covalent bond is formed between the two surfaces (17).

Manipulation of fluids within the microfluidics device can be complex since fluid flow in microsystems is influenced strongly by surface properties of the device. Control over fluid flow can be achieved via capillary effects, an electric field, a magnetic field, or a pressure gradient (15). In our system, an automated syringe pump (BS-8000 120V, Braintree Scientific, USA) with tunable volumetric flow rates is used to mechanically pump samples through the microfluidics device. Samples are inserted into the tubing and pumped at specific volumetric flow rates. However, due to limitations imposed by the imaging system, fluid flow must be set at a low rate in order to capture useful image data with reduced motion blur. In order to determine this required volumetric rate, we employed the technique of particle imaging velocimetry (PIV) to determine the approximate linear flow rate of fluid flow as it passes by the CCD sensor (15). Fluorescent microspheres were seeded into a solution with PBS and flown through the microchannel at various volumetric rates. The microspheres were simultaneously illuminated and imaged at specified parameters for exposure time and gain. The series of images captured were processed using ImageJ to track the movement of the microspheres in the fluid over time. This data was converted into a

linear flow rate of the mobile particles, which could be used as an estimate of the linear flow rate of the fluid. This conversion was done using pixel calibration with a resolution target by which the dimensions of the image's pixels were converted into physical dimensions of the object. Linear flow rate values were found at various volumetric pump rates and this data was used as a reference for determining what volumetric flow rate to use for blood samples. However, flow rate manipulation of biological fluids can be rather complex due to presence of protein interactions. The effects of these interactions becomes amplified in a microsystem due to increased surface contact (15). To mitigate these effects, PBS solution was pumped through the microfluidics chip prior to injecting the blood sample. This procedure creates a hydrophilic coating over the microchannel of the PDMS device, making the channel more traversable with reduced flow-inhibiting effects from the hydrophobic PDMS surface. Additionally, another set of protein interactions specific to blood complicates its flow properties; this set of interactions is referred to as coagulation. During coagulation, platelets aggregate, making blood more viscous and resistant to flow. To minimize the effects of this process, blood was heparinized before introducing it into the microchannel.

Additional pre-processing was completed in order visualize the nucleus of the leukocytes in whole blood. Leukocytes were stained with proflavine dye which intercalates with DNA (19) and highlights the structure of the nucleus. Using proflavine eliminates the need for red blood cell lysing because proflavine specifically stains leukocytes and no other artifacts. As the processed blood sample is pumped through the microchannel, it is continuously illuminated and imaged. The illumination from the light source excites the proflavine, inducing low-energy light emissions (i.e. fluorescence). The sensor captures the fluorescence emissions and creates an image to display on-screen. However, due to the low intensity of the emission light, the proflavine-stained leukocytes

can appear faint. Moreover, since the blood sample is mobile, the image can appear blurred. To correct these issues, parameters on the camera are adjusted through the associated computer program, FlyCap. Specifically, gain and exposure time are controlled to display an image with an optimal signal-to-noise ratio and minimal motion blur.

Using these proflavine-stained leukocyte images, a 3-part differential blood count can be conducted using an automated program to classify the leukocytes based off of thresholding and size discrimination.

2. Material and Methods

2.1. Epi-Illuminating Fluorescent Microscope Construction

The epi-illuminating fluorescent microscope enables visualization of the nuclear morphology and this information can be used in performing an accurate differential count. As shown in Figure 2, a 455 nm blue LED (LEDsupply, USA) delivers light through an excitation filter (Chroma, USA), which transmits light of wavelengths less than 450 nm. This filtered light then impinges on a 475 nm dichroic mirror (Chroma, USA), which reflects light of wavelengths below its cutoff value. The filtered blue light then passes through a 20X, 0.5 NA objective lens (Nikon, Japan), illuminating the leukocytes stained with proflavine. Proflavine has a peak fluorescence excitation and emission of 444 nm and 508 nm, respectively. When the light hits the specimen, the proflavine molecules are excited and emit green light (i.e. fluorescence), which has a wavelength of approximately 508 nm. The fluorescence light is then collected by the same 20X objective lens and is transmitted through an

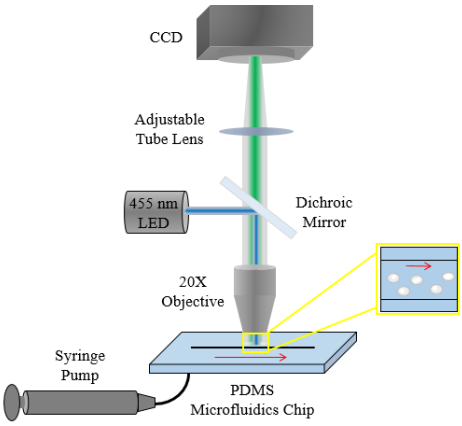

Figure 2. Schematic image of epi-illuminating fluorescence microscope.

emission filter. The use of a single objective lens for light transmission and collection invokes principles of epi-illumination. The filtered light is then transmitted through the dichroic mirror and projected on to the sensor of the CCD. In our studies we have used the Flea3 Fl3-U3-32S2M-CS (Point Grey, Candad), a monochrome camera capable of video acquisition at a frame rate of 60fps. The CCD capture images and displays them through the FlyCap program (Point Grey, Canada).

2.2. Polydimethylsiloxane (PDMS) Microfluidics Chip Construction

In order to create the PDMS microchannel, a master mold must first be fabricated. The process of photolithography is implemented to design a silicon wafer (University Wafers, USA) with the microchannel pattern deposited on it. Then pre-polymer is poured over the master to replicate the microchannel pattern on the PDMS. This pre-polymer mix consists of a precise 10:1 ratio of elastomer and curing agent (Dow Corning, USA). Approximately 65 g of the elastomer base is thoroughly mixed with 6.5 g of the curing agent. Then the mixture is degased in a vacuum chamber to eliminate bubbles that could interfere with the microchannel pattern. Once the pre-polymer is degased, it is poured over the master, degassed once more, and then

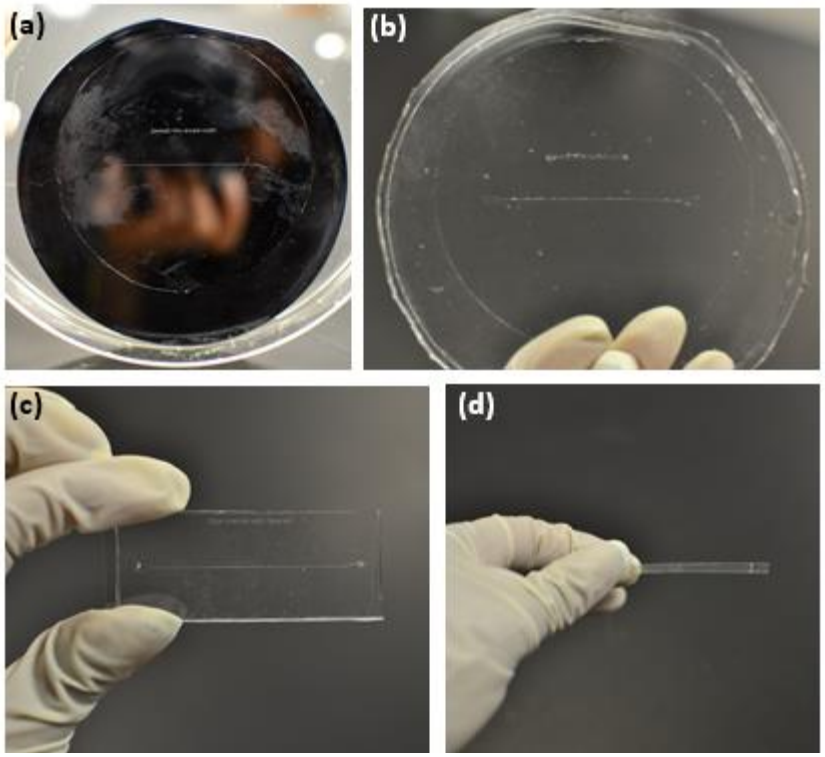

Figure 3. (a) Silicon master wafer. (b) Cured PDMS with microchannel imprint. (c) PDMS chip cut to the dimensions of the glass coverslip. (d) Side view of PDMS chip which has a thickness between 3-4 mm.

placed in the oven at 60°C to cure for 3 hours. After the PDMS is fully cured, it is separated from the master wafer, cut to the dimensions of the glass coverslip, and cleaned in a 50/50 mixture of ethanol (Sigma-Aldrich, USA) and water using sonication (Figure 3). Holes are punched into the inlet and outlet ports of the channel so that tubing can later be inserted to facilitate entry and exit of the fluid. Next, the glass coverslip used for sealing the microchannel is cleaned in three different solutions of acetone, methanol, and isopropyl (Sigma-Aldrich, USA). Then both the PDMS chip and glass coverslip are completely air dried and placed in a UV/Ozone chamber for approximately 15 minutes. After surface activation, the PDMS and glass coverslip are bonded together to enclose the microchannel.

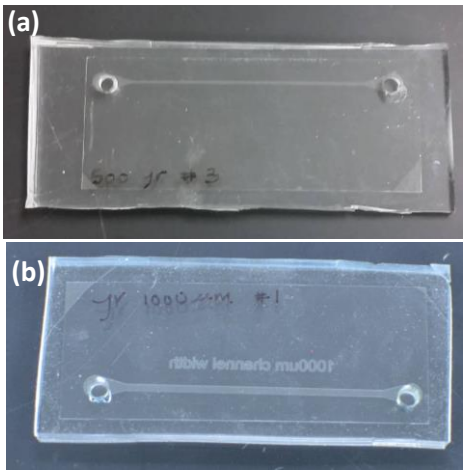

Figure 4. (a) Fully fabricated 500 µm-width and (b) 1000 µm-width microchannels.

Two different sizes of microfluidics chips were created to assess changes in fluid flow properties. The two microchannels (displayed in Figure 4) vary in dimensions, with one channel having a width of 500 um and the other 1000 um (Figure). The length and depth of both channels are approximately 4 cm and 50 um, respectively.

2.3. Quantification of Linear Flow Rate

In order to acquire valuable image data of mobile leukocytes flowing through the microfluidics channel, we needed to quantify linear velocity of fluid flow through the channel. Having an appropriate flow rate will yield images with an optimal signal-to-noise ratio and minimal motion blur. To acquire flow rate measurements, 200 µL of fluorescent microspheres (FluoSpheres, Life Technologies) were spun down and resuspended in a solution of 15% glycerol and PBS. The bead solution was pumped through the microchannel and imaged using the PointGrey camera. Glycerol

was used to prevent beads from separating out of the solution and to maintain bead suspension during flow. Using FlyCap, the imaging interval was set to capture a series of TIFF images every 100 ms for 2 s. The bead solution was pumped at various volumetric flow rates and each set of data acquired was processed using ImageJ. Figure 5 displays an example of the data that is analyzed to track microsphere flow over a series of consecutive images.

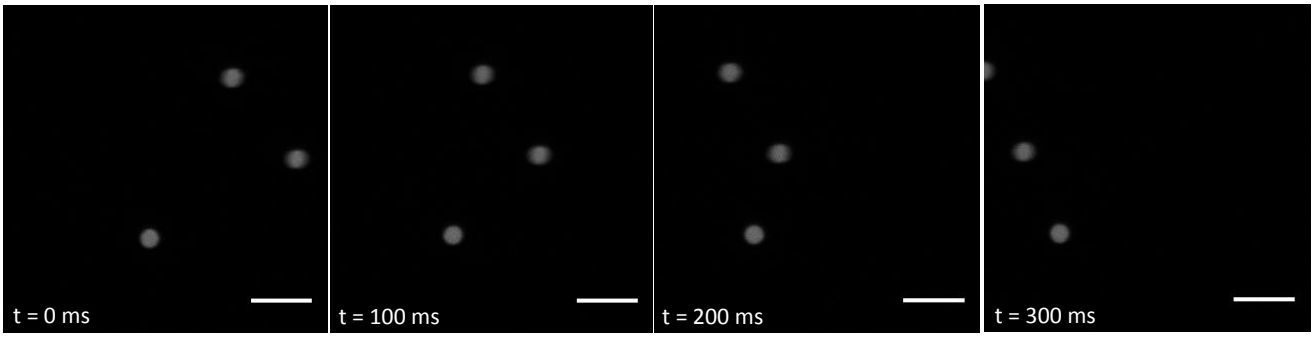

Figure 5. Consecutive images of bead flow across the focal plane of a 4X objective in a 500 µm channel. Images were captured at 5 ms exposure time and 0 dB gain. Scale bar is 50 µm.

2.4. Leukocyte Imaging in Whole Blood

Blood sample collection was approved by the Institutional Review Board at the University of Arkansas, and samples from healthy participants were obtained using fingerprick procedure. Using a pipette, 20 µL of blood was extracted and mixed with 15 µL of 2 IU/ mL heparin and 20 µL 0.02% (w/v) proflavine in a micro centrifuge tube. Prior to pumping the blood sample, 20 µL of PBS was rinsed through the microfluidics channel to ensure clear passage of sample. 20 µL of the prepared blood sample was drawn from the micro centrifuge tube and inserted into the tubing connected to the microfluidics chip. The tubing was then secured to the syringe pump, and the desired flow parameters were set on the pump. The blood sample was transported through microchannel while simultaneously imaged with the PointGrey camera.

3. Results

3.1. Quantification of Linear Flow Rate

Linear flow rate of the microspheres was calculated by converting from pixels per millisecond to millimeters per second using pixel calibration. The pixel to micron ratio is used to convert values of pixels to values of physical distance travelled by the microspheres. The physical rate values were plotted against their corresponding volumetric flow rate, as shown in Table 1. Results from the experiment indicate that an increase volumetric pump rate will yield an increase linear flow rate of the fluid in both channel types; however, the degree to which the linear flow rate increases varies. In the 500 µm channel, linear flow rate increased more rapidly than linear flow rate within the 1000 µm channel.

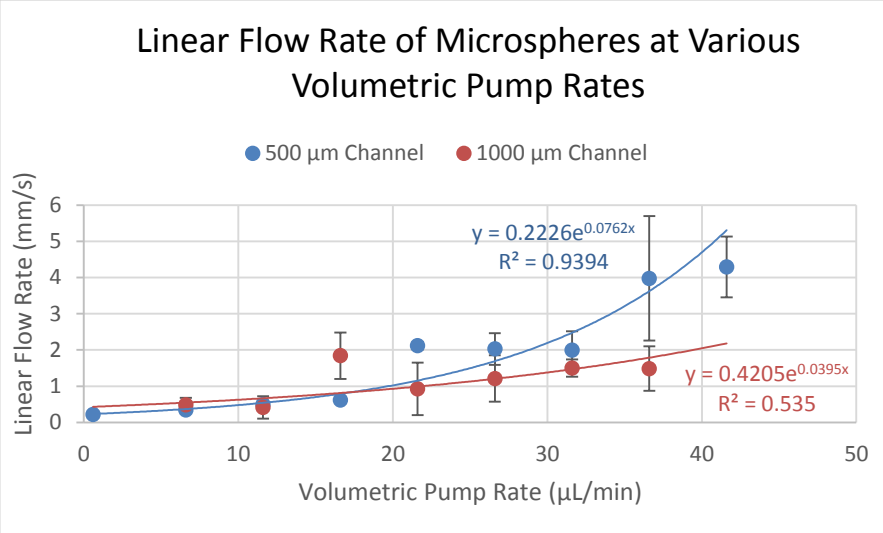

Table 1. Flow rate data from the 500 µm and 1000 µm channels. Comparison shows that the 500 µm channel experiences greater increase in linear flow rate than the 1000 µm channel.

3.2. Leukocyte Imaging in Whole Blood

Prior to imaging whole blood in our microfluidics device, static images of proflavine-stained blood were taken to assess the ability of our imaging system to visualize nuclear morphology. Figure 6 displays a comparison of proflavine-stained and Giemsa-stained leukocytes.

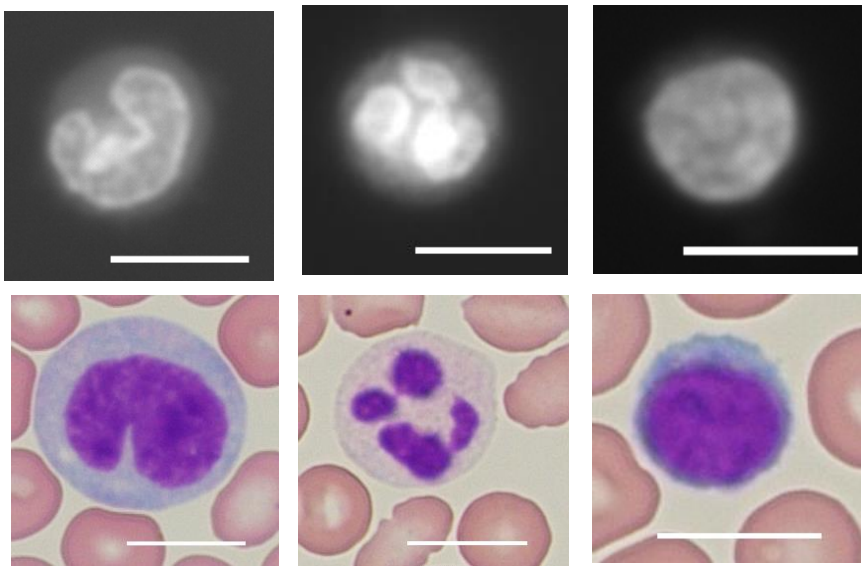

Figure 6. (a) Monocyte, (b) neutrophil, and (c) lymphocyte stained with proflavine and imaged at 100 ms exposure and 0 dB gain with a 40X objective. (d) Monocyte, (e) neutrophil, and (f) lymphocyte stained with Giemsa and imaged at 10 ms exposure with a 60X oil objective. Scale bars are 10 µm.

Using flow rate data from the microsphere experiment, proflavine-stained leukocytes in whole blood were flown in a PDMS microfluidics chip. The whole blood was injected after initially rinsing the microchannel with PBS and flown at designated volumetric rates to achieve a linear flow rate that would produce clear images. Figure 7 displays an array of images of a mobile neutrophil flowing through a microchannel. In order to enhance classification, a custom detection algorithm is currently under development. This algorithm can identify leukocytes using

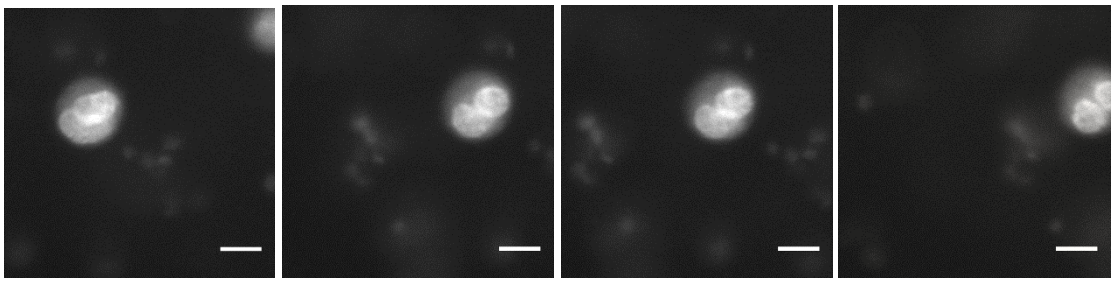

Figure 7. Images of a rolling neutrophil exposing its lobulated nucleus. Image taken at 10 ms exposure and 20 dB gain with a 40X objective. Scale bars are 15 um.

thresholding and size discrimination. Figure 8 displays flowing leukocytes that are boxed by the algorithm.

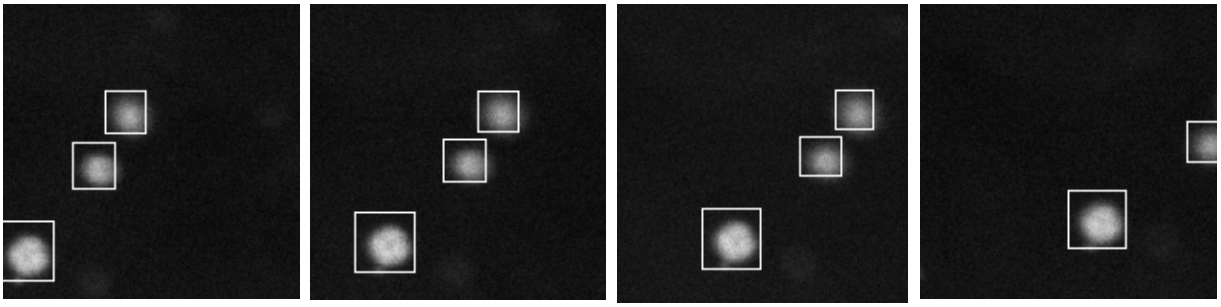

Figure 8. Whole blood flown at 1.5 µL/min in 1000 µm channel was imaged using a 20X objective at 50 ms intervals with 5 ms exposure and 22 dB of gain.

4. Discussion

Our lab developed and validated the use of a microfluidics chip and custom epi-illuminating imaging system for blood screening. The microfluidics channel was constructed using PDMS, an inexpensive and accessible elastomer. Despite these advantages, the PDMS channel posed some challenges due its incompatibility with biological analytes. These challenges were further amplified due to using a microfluidics system. Microflow is complex due to the increased importance of surface forces (15). The surface chemistry of PDMS is hydrophobic which can inhibit flow of hydrophobic components, such as proteins and cells. To remedy this, surface modification must be made to render the surface irreversibly hydrophilic because PDMS exposed to air is unstable and can revert to being hydrophobic in approximately 30 minutes (17). Additional modifications can be made in manipulation of the flow. When conducting experiments at low volumetric rates, the mechanical pump infrequently had issues maintain continuous flow of fluid. This often led to large deviations in flow rate values for specific volumetric pump rates. To alleviate this, alternative methods for flow manipulation can be used, such as electro-osmotic flow, which can yield a more uniform distribution of flow (15).

Results from leukocyte imaging in whole blood yielded clear nuclear morphology data. Figure 6 displays a mobile neutrophil flowing across the plane of a 40X objective. One unique aspect of imaging mobile leukocytes is that we are able to visualize various angles of the nucleus due to rolling of the leukocyte. This will enable a more accurate differentiation between leukocytes since it permits further review of the morphology. Using these images, classification of leukocyte subpopulations can be conducted to perform a 3-part differential count of granulocytes, monocytes, and lymphocytes. More importantly, with a program to track mobile leukocytes, as depicted in Figure 7, an automated leukocyte count can be performed to enable rapid and accurate blood screening.

With further development, the applications of this device are far-reaching with the potential to improve point-of-care diagnostics in homecare settings or in areas with low resources and/or trained personnel. Our particular technique of utilizing a single staining reagent coupled with reduced pre-processing can substantially reduce the associated costs, time, and complexity of performing a blood assessment.

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