Microfluidic device for label-free measurement of platelet activation

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In this work we demonstrate a new microfluidic method for the rapid assessment of platelet size and morphology in whole blood. The device continuously fractionates particles according to size by displacing them perpendicularly to the fluid flow direction in a micro-fabricated post array. Whole blood, labeled with the fluorescent, platelet specific, antibody PE-anti-CD41, was run through the device and the positions of fluorescent objects noted as they exited the array. From this, histograms of platelet size were created which show marked increases in size after exposure to thrombin or a temperature of 4 °C. We infer that the well known morphological changes that occur during activation are causing the observed increase in size.

1 Introduction

Hemostasis in humans is a vital process that has three functions: to maintain blood as a fluid while circulating, to stop bleeding and blood loss at the site of injury by forming a clot, and to ensure the eventual removal of the clot once healing is complete. This requires holding a balance between conflicting tendencies where any exaggeration or deficiency in one can lead to excessive and life threatening clotting or bleeding.[1]

Platelet function is impaired in a number of hereditary diseases and by some common cardiology drugs. Also, disseminated intravascular coagulation is a dangerous condition which has been linked with a variety of underlying causes. We believe that an inexpensive and simple to use device for platelet analysis would find numerous applications and while commercial products exist for platelet function analysis, no product meets all clinical needs and no method profiles platelet morphology in a timely manner.

Platelets are one component of the hemostatic system. In blood they are small enucleated (no nucleus) discs. Frojmovic *et al.*[2,3] made optical measurements of fixed, freely rotating platelets in citrate-anticoagulated blood. In two separate studies, they measured the large diameters to be 3.6 ± 0.7 and 3.2 ± 0.5 microns, and the thickness to be 0.92 ± 0.34 and 1.1 ±0.2 microns, respectively. Some 2 to 10% of the platelets were echinocytes, spherical objects with irregular surfaces and spiny or tenticle-like objects extending from the cell (pseudopods), with the remainder being "irregular forms." On dried blood smears, platelets appear smaller, 2 to 3 microns in diameter.[4] Without careful handling platelets change shape, making quantitative study of their morphology challenging.

Platelets function by aggregating and adhering to tissue. During aggregation, platelets change shape from discocytes to echinocytes and experience a host of physiochemical changes; this process is collectively called activation.

Rapid platelet and clotting analysis for point of care diagnostics is a growing area of interest. Diagnosing platelet function and condition can help assess a person's risk of excessive bleeding prior to surgery, help monitor a patient's response to certain blood disorder and cardiology drugs, and determine the viability of platelets for transfusions. There are numerous tests for platelet function. Traditional tests include chemical detection methods, electron microscopy, measurements of bleeding time, aggregation in response to agonists, high-shear platelet function, and retraction forces during clotting.[5] Various new platelet function tests are available to automate some of the traditional tests and measure new aspects of platelet function such as protein and mRNA content.[5] Flow cytometry is also being used to analyze platelets. While many of the new platelet function tests could be considered microfluidic, there are few examples of platelet work in the typical microfluidics literature.[6–8,25]

Despite the importance of platelet morphology, the authors are not aware of any automated systems to assess morphological changes. Electron microscopy gives the best picture of morphology, but sample sizes are low, the preparation required for imaging is extensive, and electron microscopes are very expensive. Here we present a microfluidic device that measures platelet size. We demonstrate an increase in the apparent size of platelets after exposure to thrombin or refrigeration at 4 °C. Given the well known morphological change that occurs during refrigeration[9] (a conversion from discocyte to echinocyte), we hypothesize that the increase in apparent size in our device is a result of this morphological change. In contrast to much of the blood and cell literature, here size refers to a linear dimension, rather than volume.

This work follows previous work using microfluidic devices to continuously fractionate particles by size, including blood cells.

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Table 1 Device specifics in microns. The separation column in Array 1 is 1160 μm wide. The separation column in the platelet fractionation array, Array 2, is 885 μm wide. Array 2 is composed of eleven sections

ε	Post diameter	Gap	Length of section	Lateral displacement	Observed critical size
1/42	20	17	7308	−222	5.0
1/100	20	6	7800	78	2.3
1/50	20	6	2600	52	2.3
1/29	20	6	2263	78	2.6
1/22	20	6	1145	52	3.0
1/18	20	6	937	52	3.4
1/15	20	6	780	52	3.6
1/12	20	6	624	52	4.0
1/10	20	6	520	52	4.3
1/8	20	6	415	52	4.6
1/12	20	8	672	84	4.8
1/10	20	8	559	84	5.3

Huang *et al.*[10] introduced the deterministic lateral displacement (DLD) method and showed astounding size resolution with polystyrene beads. Other work[11,12] used the method to separate red blood cells and white blood cells in whole blood. In this work we focus on platelets and show clear differences in platelet size as a result of exposure to agonists. We will briefly describe the physics of the deterministic lateral displacement principle, then describe the device, followed by the results for platelets.

2 Methods

We determine cell size by deterministic lateral displacement (DLD), a uniquely microfluidic method where fluid flows through an array of posts under low Reynolds number conditions. This method has been described numerous times in the literature[10,11,13–15] and will not be discussed in depth here. In the low Reynolds number regime, viscous forces dominate over inertial forces and fluid flow is laminar, *i.e.* not turbulent. A DLD array consists of nothing more than an array of pillars or posts, having rows and columns. Each subsequent row is shifted horizontally by a fraction of the post-post spacing relative to the previous or upstream row. This row shift divided by the period of the array is called the row shift fraction (epsilon or ε). Epsilon is the slope that the columns in the post array form relative to the fluid flow direction. This establishes two modes for particles to travel through the array: (1) particles smaller than a critical size follow the fluid, and (2) particles larger than the critical size follow the slope ε. This creates a continuous mode of separation for large and small particles separated by an angle $\theta = \text{atan}(\varepsilon)$. Typical angles are 1 to 6°.

The critical particle size is affected by the size of the gap between the posts and the row shift fraction, ε. By injecting a narrow stream of particles, using a hydrodynamic jet, into a series of arrays with increasing critical particle size, it is possible to separate over a range of sizes. This creates a correlation between size and position where the largest particles are displaced the most, and the smallest particles are not displaced at all.

As described previously[15] there is a limit to the range of particle sizes that can be separated in a single array. The smallest particle that can be separated is about 4 times smaller than the gap. This makes separation of platelet-sized objects impossible in an array with gaps large enough to pass most white blood cells.[11,15]

To separate red blood cells and platelets, the larger white blood cells must be prevented from clogging the separation array. Upstream of the platelet fractionation arra (Array 2) we use another array (Array 1) to divert particles larger than 5 microns into a non-clogging path that runs parallel to the platelet fractionation array (Fig. 1). Array 1 and the alternate path have gaps of 17 and 20 microns respectively. Array 2 has a minimum gap of 6 microns and is composed of a series of eleven arrays, each with increasing critical particle size so that increasing lateral displacement correlates with increasing size. Table 1 gives details of the array.

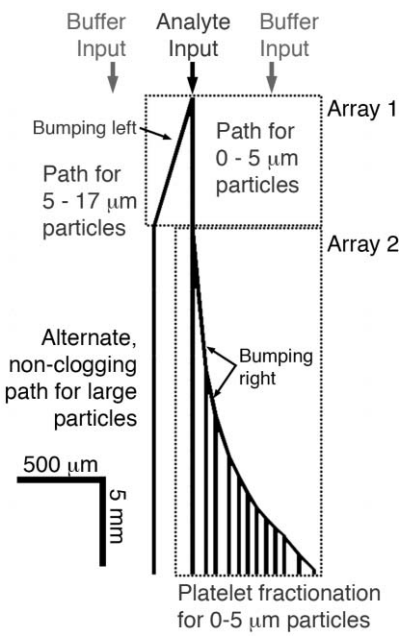

Fig. 1 Diagram of ideal particle paths in our device. Particles that would clog the lower portion of the device are moved to the left by Array 1 into a 20 micron gap channel that runs parallel to but is not in connection with the platelet fractionation array, Array 2. In the platelet fractionation array, increasing lateral displacement correlates with increasing size. Note that the vertical dimension is compressed ten times to the horizontal dimension for the purposes of this figure.

The entire device fits on a standard glass microscope slide (25 by 75 mm). The distance between the buffer input and the outputs is 6.3 cm. Fig. 1 shows the ideal paths for all particles that enter the device as they branch off the narrow input stream. Particles larger than 17 microns are sufficiently rare in blood not to cause any apparent jamming or build up in any part of the device.

The device consists of a PDMS (polydimethylsiloxane) mold sealed to a fluorosilane-coated silicon wafer backplane that had been cleaved to the size of a standard glass slide. The mold was created by standard photolithography on a silicon substrate, followed by an 18 micron deep silicon etch. This was cleaned, baked at 1000 °C for 3 h, and coated with a fluorosilane. PDMS was poured over the master mold and squished under a 25 by 75 mm glass slide, then allowed to cure for 2 h at 70 °C. The PDMS-coated glass slide was separated from the master mold using a razor blade and transfered to the backplane. Holes for fluid connections had been sandblasted into the silicon-wafer backplane. After assembly, the chip was soaked in a solution of deionized water containing 2 g L^{-1} pluronic F108 (BASF, Florham Park New Jersey) and placed under vacuum for at least 2 h to remove trapped air bubbles from within the device.

Blood was collected from volunteers by venipuncture in 5 mL ACD (anticoagulant citrate dextrose) Vacutainer collection tubes (BD Franklin Lakes, NJ). Volunteers provided written consent and the project had IRB approval. Samples were measured as soon as possible, which was not more than 4 h after blood collection.

3 Experiments

Fluid is driven through the device by positive air pressure applied to both the buffer and blood input ports by an electronically controlled regulator. The running buffer was AutoMACS buffer (Miltenyi Biotech, Auburn California). When blood enters the device (typically at about 0.8 nL min^{-1}), all the white blood cells are diverted into the alternate non-clogging path (Fig. 2 left image). White blood cells have previously been observed to behave as particles larger than 5 microns in diameter in DLD arrays.[11,12] A fraction of platelets enter the alternate non-clogging pathway, and the remainder enter the platelet fractionation array.

To determine the platelet size distribution, fluid is driven through the device at 2 psi (14 kPa). This provides a suitable rate of cells for counting and a suitable residence time in front of the camera to be imaged. Five parts ACD whole blood are incubated with one part PE-conjugated antihuman CD41 (ebioscience.com), for 20 min at room temperature prior to running in the microdevice. No wash step is required at this concentration of label. The device is mounted on an inverted fluorescence microscope with high pressure mercury lamp illumination. Video is captured by a Hamamatsu silicon intensified-target video camera and recorded onto a Sony miniDV cassette recorder.

We record the lateral positions of bright streaks at the end of the platelet fractionation array as the platelets travel through a set of 22, 40 micron period, parallel channels. These channels facilitate counting and binning of the cells for the histograms.

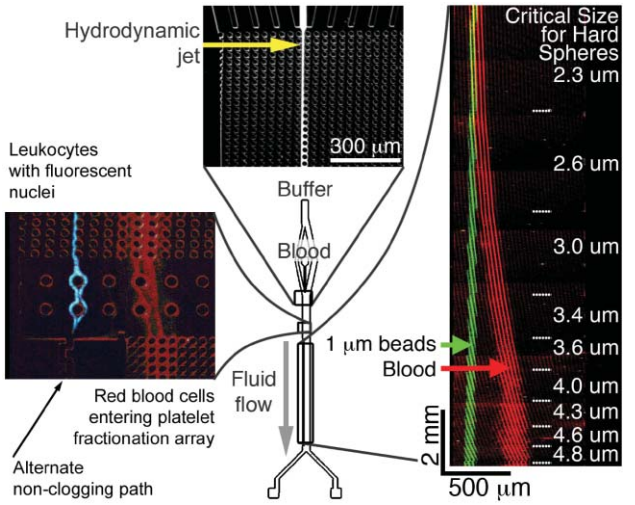

Fig. 2 Micrographs of various particles in the device at three locations. In the device the fluid flows from top to bottom. Top: A hydrodynamic jet of beads entering the first separation array. Left: White blood cells (blue) separated from other cells after the first separation array and immediately before entering the alternate non-clogging path. Right: Overlayed images of a stream of 1 micron fluorescent beads (green), and whole blood (red). (Compare to Fig. 1 to assist in interpretation.) The 1 micron beads are not displaced by the separation array at any point. The image is compressed vertically 4 times with respect to the horizontal dimension. The numbers 2.3 to 4.8 are the approximate measured critical particle sizes in microns for hard spheres. Notice that the whole blood, composed mostly of red blood cells, travels at an angle to the flow (following the direction of the column of the posts) until the section with a 4 micron critical particle size. After that, the blood cells travel vertically in the average fluid flow direction.

In the same image we record the large cells that have travelled through the alternate non-clogging path.

The behavior of deformable, non-spherical particles in a DLD array is significantly more complex than that of hard spheres. The shear forces, which result from gradients in the fluid velocity around a particle, may result in complex motions including tumbling and shape change.[16] This suggests that such particles may appear, in the microfluidic device, to be different sizes depending on their orientation as they pass through the gap. Because of these effects the term hydrodynamic size is used to refer to particle size as measured by a DLD array.

Fig. 3 shows a calibration measurement of particle size *vs.* channel number. This was done by flowing rigid polystyrene beads of known sizes (2.3, 2.7, 3.1 and 4.35 microns) through the device and noting their exit positions.

3.1 Thrombin-induced platelet activation

In this section we show that thrombin-induced activation causes an increase in the apparent size of platelets. *In vivo*, thrombin is released at the site of tissue damage and causes coagulation. We prepared control and thrombin-activated blood samples using the procedure given by Leytin *et al.* in 2000[17]† The activated sample is exposed to 1 NIH unit mL^{-1} human

† Leytin *et al.* gives a detailed description of the protocol which uses ACD blood mixed with the fibrin polymerization inhibitor GPRP (SigmaAldrich.com) to prevent aggregation.

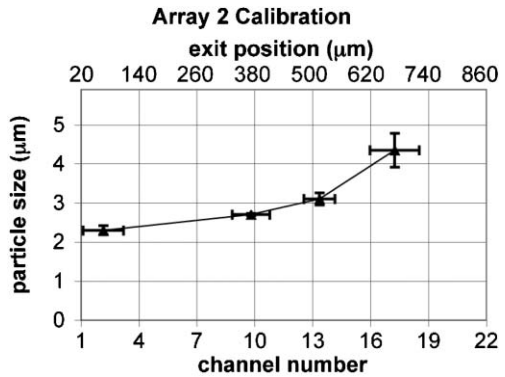

Fig. 3 Plot of the particle size *vs.* the exit position (measured from the left column edge) and channel number. Horizontal error bars are one standard deviation in each direction, as determined by fitting a Gaussian to the fluorescence bead intensity profiles. Vertical error bars are determined from the coefficient of variation specified by the manufacturer.

thrombin (SigmaAldrich.com) while the control sample is not exposed to thrombin. Both blood samples are then fixed in paraformaldehyde.

We verify activation in the same way as Leytin *et al.*, by measuring the expression of CD62p. They observed that with increasing doses of thrombin, on average platelets bind more CD62p. Fig. 4 shows the level of CD62p expression for the control and thrombin-activated populations as measured by flow cytometry. For flow cytometry, FITC-conjugated CD41 was used simultaneously to identify platelets. Activation is defined as events which are both CD41+ and CD62p+ (CD62p expression >294, as shown in Fig. 4).

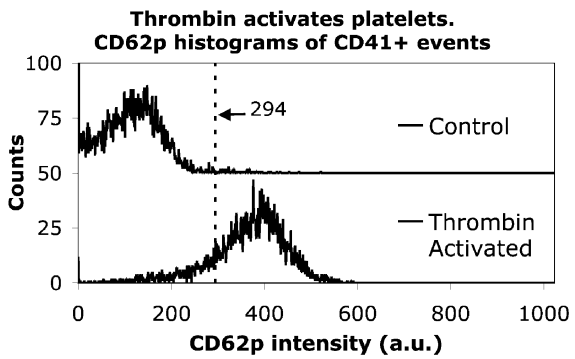

Fig. 4 Thrombin treatment of whole blood causes activation of platelets, evaluated by CD62p expression of $CD41^+$ events in a FACScan flow cytometer. In the control sample, 1.1% of platelets are activated. In the thrombin-activated sample, 87% of platelets are activated.

The sizes of platelets in these two samples were measured in the microfluidic device. CD41 is used to identify platelets in the microdevice, but no activation specific marker, such as CD62p is used. The point of this research is to demonstrate a purely mechanical method of measuring platelet activation.

We created an exit position histogram for platelets (identified by CD41 fluorescence), and observed an increase in hydrodynamic size in the sample treated with thrombin. The value plotted in the exit position histograms of Fig. 5A and C represents the fraction of cells that travelled through each channel. The large cells that have travelled through the alternate non-clogging path are plotted at negative 50 microns on the horizontal axis. All charts are normalized.

Fig. 5A shows the exit position histograms for 4 experiments conducted at room temperature, where the mean and standard deviation (as error bars) for each bin are plotted. The platelets at negative 50 microns were separated by Array 1 and travelled down the alternate non-clogging path. They are behaving as particles larger than 5 microns. Fig. 5B shows the same data, re-binned to show the size histogram. All data has been normalized, and more than 400 platelets were counted in each experiment. Each experiment takes between 1 and 2 min to collect the positions of the 400 platelets.

In the thrombin-activated sample there is a 10-fold reduction in the smallest platelets (under 2.4 microns in diameter), and a 2.5-fold increase in the number of platelets larger than 4.8 microns compared to the control samples. We can calculate a mean using the centers of the hydrodynamic size histogram bins (somewhat arbitrarily assuming the upper limit of the last bin in Fig. 5B and D is 7 microns). The average hydrodynamic size of the control population platelets is 3.1 microns. This compares to the long dimensions of the platelets measured by Frojmovic *et al.* in 1976 and 1978[2,3] of 3.4 microns, see the Introduction. The activated sample measurement (mean size of 4.4 microns) gives sizes that are larger than that reported by Frojmovic *et al.* It is possible that some of the largest objects are platelet–platelet and platelet–cell aggregates.

Numerous studies have been devoted to the analysis of platelet morphology changes upon activation. Activation involves a change from discoid state to spherical cells with pseudopods (spiny or tenticle-like projections from the cell wall) called echinocytes. Cold storage also causes activation and morphological changes, though these morphological changes are reversible upon re-warming.[9] The morphological changes are also experienced by nearly all platelets.[18] Upon re-warming, about 70% returned to a normal disc shape. In contrast to what we observe here, by light microscopy the transition from disc to sphere appears as a reduction in size.

This leads to the question: Is there an increase in size upon activation? Using the Coulter principle, Bull and Zucker[19] observed an increase in platelet volume of 24% upon cold storage. Converting these volumes to spheres, the reported difference corresponds to a 180 nm increase in diameter on a 2.3 micron sphere, about 8% in diameter. So there is an increase in volume, but only a very small increase in diameter. A slightly smaller increase in volume was observed for platelets exposed to 10 units mL^{-1} thrombin. This increase in volume is not large enough to be the sole source of the approximately 1.3 micron, or roughly 40% increase in hydrodynamic size (diameter) that we measure. Given the drastic morphological changes that occur upon exposure to either thrombin or cold temperatures it is likely that this morphological change is somehow responsible for most of the increase in hydrodynamic size that we observe here and in the following section.

3.2 Temperature-induced changes

Because temperature is also known to induce morphological changes in platelets, we also examined the platelet size

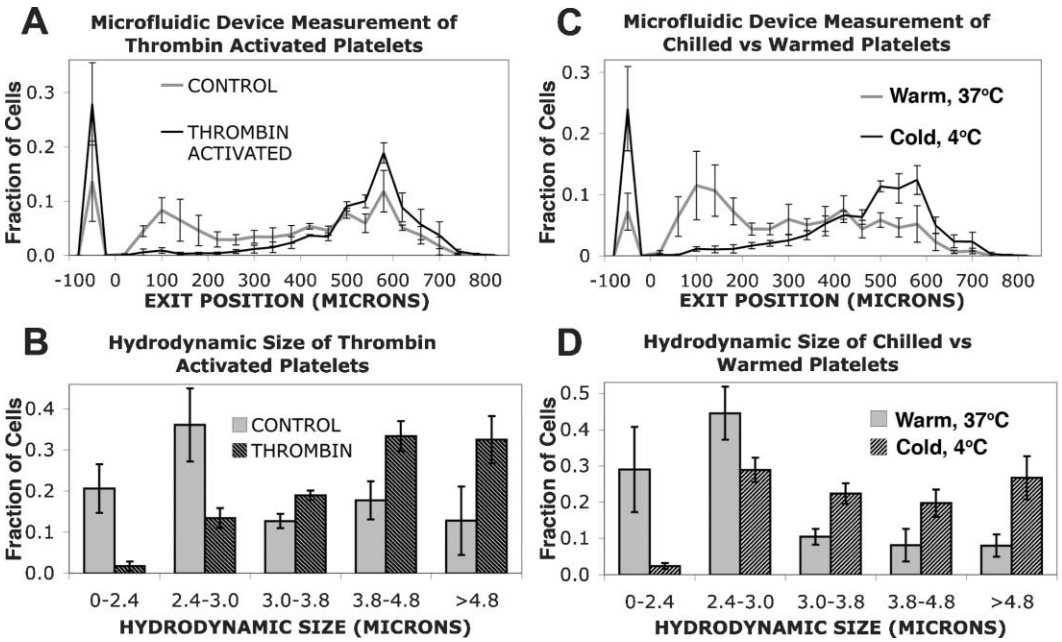

Fig. 5 Microdevice exit position and hydrodynamic size histograms for thrombin induced and temperature induced platelet activation. In both cases a significant increase in platelet size is observed after activation.

distribution as measured by our chips on blood which was heated or cooled prior to measurement. Again we show that temperature induced activation causes a marked increase in the hydrodynamic size of platelets.

Here we conduct two separate experiments, one where the blood is warm and one where the blood is cold during the measurement. The labeled blood and the microfluidic device were either refrigerated at 4 °C or placed in a 37 °C incubator for at least 30 min prior to running the experiment. Reports[18] indicate that the activation-induced shape change is mostly complete after 10 min and is complete after 30 min. After 30 min in either 4 or 37 °C, the microdevice was placed on the microscope (which is at room temperature), where the sample is loaded, and the external plumbing connected. The experiment was then immediately run, and completed within 3 min. Because the 2 mm thick device is mounted onto a 1 cm thick acrylic adaptor, which was heated or cooled with the chip, we think the thermal mass of the adaptor prevents the device and blood from changing temperature significantly during the three minutes of the experiment.

Fig. 5C and D shows the exit positions histograms and hydrodynamic size histograms for the warm and cold platelets. The experiment was repeated 5 times on 5 different devices. The error bars in (C) represent the standard deviation observed for each bin across all five experiments, in (D) they are the standard deviation of the re-grouped data.‡ As with thrombin exposure, the cooling-induced an increase in the hydrodynamic size of the platelets. There is a sharp reduction in the fraction of the smallest platelets and at least a doubling of the number of the platelets in the largest group. The mean sizes are 2.7 microns in the warmed sample and 4.0 microns in the chilled sample. This result is very similar to that observed when thrombin is used to activate the cells. This suggests that the morphologic change from discoid to spheroid, that is known to occur in both cases, is causing the change in hydrodynamic size. The increase in the fraction of large (>4.8 microns) platelets is not expected to be caused by aggregation as no evidence of aggregation is seen in the cited studies of temperature-induced morphological changes.

3.3 Discussion

Comparing Fig. 5A, B to 5C, D, the effect of activation is nearly identical. Fig. 6 shows how the mean hydrodynamic size of the platelets increases with decreasing temperature and when exposed to thrombin. The common thread in both experiments is a morphological change, and we believe that this is manifest as an increase in hydrodynamic size.

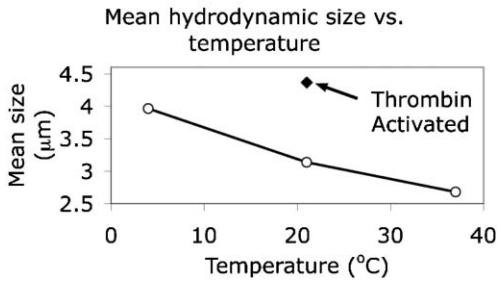

Fig. 6 Plot of mean platelet hydrodynamic size *vs.* temperature, including the thrombin activated sample.

‡ Exit positions 20–80 microns correspond with 0–2.4 microns in hydrodynamic size; positions 100–480 with 2.4 to 3.0; positions 520–560 with 3.0–3.8; positions 600–680 with 3.8–4.8; positions 720–880 plus the negative 50 position with >4.8.

It is possible that the discocyte cell behaves as a particle with size proportional to its narrow dimension, and when it converts to a spherocyte with pseudopods the size increases. However this is hard to reconcile with the observation that most discocytes behave as particles greater than 2.4 microns thick, when 1 micron is their typical thickness. The pseudopods themselves may be responsible for the large size measured here and/or some other interaction between the complex fluid flow field and the platelet's shapes may be causing this effect. The authors are not completely satisfied with this explanation and neither should you be. Nevertheless, the method appears capable of distinguishing and separating platelets based on morphology.

Are the results representative of most individuals? Information on platelet size variation among healthy individuals indicates that discocytes have a coefficient of variation in diameter of around 20%.[2,3] Here we see a 40% increase in mean size with thrombin induced activation and a 48% increase in size with temperature induced activation. The measured effect is larger than the expected variation across healthy individuals, so it is possible that diagnostic information could be obtained immediately and without a control sample. The volume throughput is quite small, less than a nanolitre per minute or about 200 platelets per minute, but at this rate is still fast enough to give statistically relevant information in a few minutes. Further increases in speed could be achieved by using a device with a deeper etch, and by improving the optical sensitivity to the passing fluorescent cells.

The morphological analysis of platelets could further be used to evaluate conditions in which platelets are known to participate. For example, platelets play a major role for the development of microvascular hyperpermeability associated with sepsis.[20] Evaluation of early changes in platelets may help to predict disseminated intravascular coagulation (DIG) which can lead to severe health problems.[21] A diagnosis of DIG should always prompt a search for an underlying medical disorder, including sepsis, severe trauma, solid and hematological malignancies, obstetrical complications, and vascular disorders.[22,23]

4 Summary

In this paper we have shown evidence that the microfluidic device measures hydrodynamic size, a parameter associated with morphological change and activation of platelets. This suggests that the device may be able to provide rapid diagnostic information about the haemostatic condition of a blood sample without the use of an activation specific label or marker. This is especially significant because chemicals that report activation status do not respond equally to all routes to activation,[24] and characterizing the fluorescence from a marker requires additional complexity in the device. These diagnostic measures may be useful in a wide range of medical procedures and diagnostics which involve platelets.

Acknowledgements

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