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Multi-shape erythrocyte deformability analysis by imaging technique †

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Abstract

In their extensive circulation through the cardiovascular circuit, erythrocytes are forced to transform and assume different shapes depending on the varying flow conditions in different blood vessels. The present work aims to visualize various erythrocyte shape transformations by an in vitro microcirculatory model, and to visualize multi-shape erythrocyte deformability. The model used an in-house fabricated and inexpensive disposable micro flow channel to mimic certain in vivo conditions, and a fast frame rate video microscopic system to visualize the shape changes in erythrocytes. Results have shown the multi-shape transformation of erythrocytes as resting discoidal shape, asymmetrically deformed 'hat' and 'bullet-like' shapes, and axially deformed 'slipper- and spindle-like' shapes. Specific erythrocytes demonstrated shape transition and transformation while passing through the observed window. The obtained erythrocyte shaper were thoroughly analyzed based on the deformability index. Findings from the image processing techniques were significant ($P<0.001$) for the different shapes compared with the resting shape. The simple glass in-house fabricated microfludic channel allowed for multi-shape erythrocyte deformability characterization, many of which manifest in human circulation.

Keywords: Erythrocyte imaging; Microcirculatory model; Multi-shape deformability index

1. Introduction

Erythrocyte deformability in human beings is essential for the life-sustaining functions of blood circulation in the cardiovascular system. Red blood cell (RBC) survival within the circulation at 120 days is determined by its deformability. In fact, RBCs that do not meet the deformability requirements are retained in the spleen. Several studies have reported correlation of reduced erythrocyte deformability to a number of cardiovascular diseases and disorders, as well as according to their severity [1-10]. Reduced deformability is an important contributing factor in cardiovascular complications. Hence, erythrocyte shape and deformability measurement techniques assume significance in the prediction of cardiovascular dysfunctions.

Mammalian erythrocytes excessive surface areas as a means of holding internal contents or volume [1]. This allows the RBC to deform and assume various shapes in response to external flow forces. Apart from these geometrical features, other factors like the composition and viscosity of the internal fluid, the membrane bilayer and its skeletal proteins contribute to red cell deformability (RCD) [2]. As blood flows from arteries (large vessels) to the capillaries (micro-vessels), the flow changes from a homogeneous multi-profile to heterogeneous uniprofile condition, producing varying shear rates at different locations at the cardiovascular system [3]. This physiological flow causes rearrangement of the cellular components, which are predominantly composed of erythrocytes, to transform shape, deform, and align with the flow. RBCs may assume elongated or ellipsoidal shapes in larger vessels, while 'hats' and 'bullets' are more likely to appear in capillaries [5]. To visualize these deformed shapes under in vitro conditions, we developed the microfludic visualization system for deformability (μFVD), a system that not only allows imaging of deformed RBC shapes, but which also allows us to record their shape transformations could be recorded and measure their deformation index.

Blood rheology, both in in vivo and in vitro models, has been studied by several authors [5-10]. Hitherto, methods to measure erythrocyte deformability include viscosimetric, filtration technique, and micropipette aspiration. Despite their advantages, visualization of erythrocyte deformability was not possible. In the rheoscopic [8] method, deformation of cells at various shear rate can be directly visualized by a microscope, but even this could not visualize other shapes, except for elongated RBCs. In the recent years, the use of flow channel devices for deformability visualization has been reported. Microchannels were fabricated using different techniques to

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model capillary geometries, and then were used for in vitro blood flow and deformability visualization [5, 9]. Recently, a parachute-shape RBC was recorded in a transparent microchannel [5]. However, different deformed erythrocyte shapes, as well as the progression of their shape transformations, and the quantification of their dynamics, needs to be further elucidated in an in vitro flow. Therefore, this study focuses on the possibilities of recording various shapes of RBC deformation using an in-house disposable and fabricated microchannel.

2. Material and methods

2.1 Microfluidic visualization systems

Fig. 1 shows the schematic of (μFVD). The visualization system uses a basic clinical microscope (Olympus BX41, Japan) with 100x objective at high speed (max. 99 frames/sec), a CCD, a digital camera (Pixefly, PCO imaging, Germany), a PCI interface board, a computer, and a customized camera control software (CamWare V 2.5). The fluidic system employs an in-house fabricated microchannel, a programmable multi-syringe pump (NE 1600, NewEra Inc., USA), and tubing accessories. The flow of erythrocytes, which was controlled by a syringe pump, were viewed through a target window (100x100 micron c/s) using the microscope.

2.2 Sample preparation

Blood sample from healthy volunteers were obtained in a vacutainer (BD Vacutainer, USA) containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The samples were centrifuged (Felta 5, Hanil Industrial Co., Korea) at 3,000 RPM for 15 min. The supernatant plasma was separated, and the buffy coat on top of the cell was removed and discarded. An erythrocyte suspension of 1% hematocrit (hct) was prepared both in phosphate buffered saline (PH 7.4) and plasma. The experiments from these suspensions were carried out at a room temperature of 25°C.

2.3 Data acquisition

Erythrocyte suspensions were thoroughly mixed and loaded in the syringe and injected carefully through the tubing and onto the microchannel to ensure bubble priming. The syringe was then fixed onto a syringe pump. The microscope objective (100x) was adjusted to focus on the flowing cells. The customized camera software allowed recording of images at different exposures (1 μsec to 1 sec), and at a maximum of 99 frame/sec in the benign mode. Camera exposure was adjusted at a minimum value to obtain a clear image scene for a given microscopic illumination at prescribed aperture settings. With a fixed camera, microscope, and objective settings, the erythrocyte suspension flow, controlled by syringe pump program, was obtained. For a given flow, image sequences were acquired at an acquisition rate of 50 frames/sec, and such recordings were carried out for increasing flow rates. Meanwhile, resting, and non-deformed erythrocyte images were recorded in a similar manner, but with the flow nearly ceased, using the saline suspended erythrocyte.

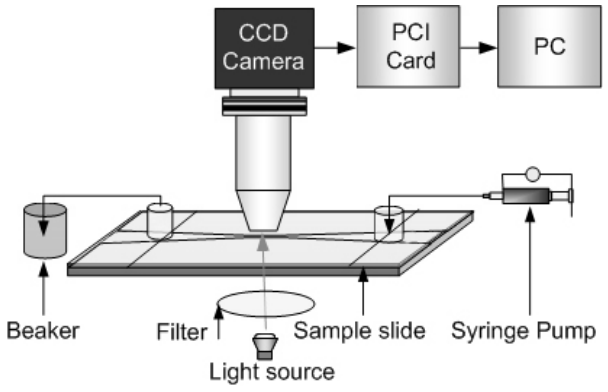

Fig. 1. Microfludic visualization system for erythrocyte deformability.

2.4 Image analysis

The digital images recorded were replayed at lower frame rates to enable better viewing. The reproduced image sequences of the deformed erythrocytes were saved for further analysis. Deformability index analysis by image processing was carried out using the Matlab software. Fig. 2 shows the flow chart of the image processing procedure while Fig. 3

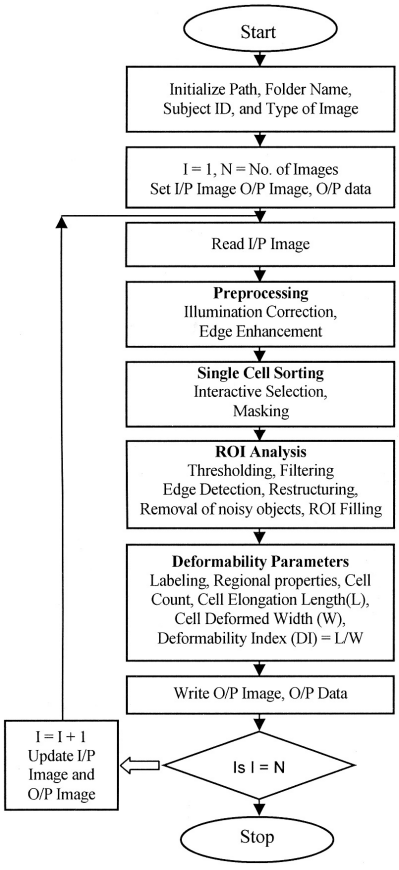

Fig. 2. Flow chart showing image processing for multi-shape deformability analysis.

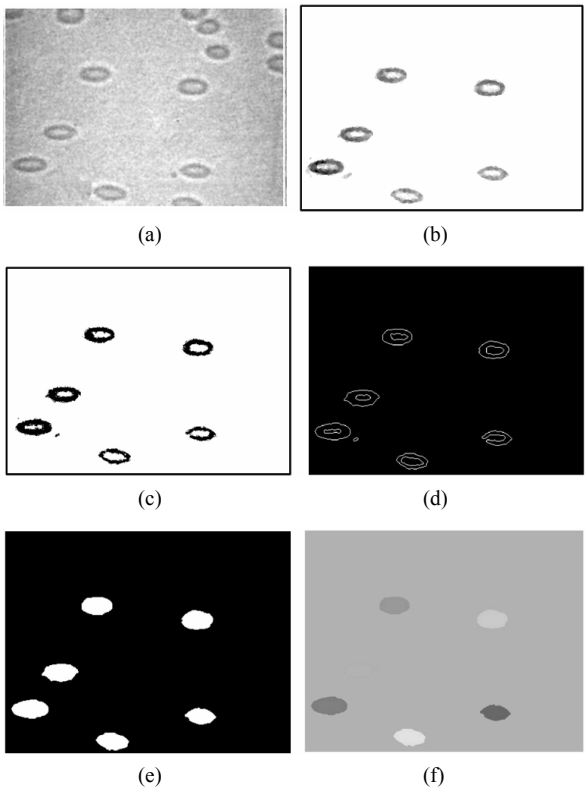

Fig. 3. Illustration of image processing steps for obtaining a labeled image to process deformability Index of erythrocytes. Original image after preprocessing (a), ROI Image developed by selecting erythrocytes and masking (b), Thresholded and filtered image (c), Edge detected image (d), and Restructured Image with line structuring element and noisy objects removed (e). Indexed image where each erythrocyte was uniquely labeled (f).

shows the sample images after performing the aforementioned different steps. The procedure involved pre-processing, single cell sorting, region of interest (ROI) analysis, and deformability parameter analysis. Pre-processing involved illumination correction by averaging the pixel intensity, as well as by edge sharpening using enhancement techniques. Fig. 3(a) shows the original image after performing this step. From these images, single cells were sorted out by avoiding the overlapping and interacting erythrocytes. Other regions of the image were masked off, and the ROI image was generated (Fig. 3(b)) in the cell sorting step.

In ROI analysis, the ROI image was further employed for threshold, median-filtered (Fig. 3(c)), and subjected to edge detection using Cranny's method. The edge-detected image (Fig. 3(d)) showed discontinuous edges. To overcome this, edge-detected images were reconstructed by dilation followed by erosion, applying the same line-structuring element of the morphological reconstruction techniques. This ensured restoration of continuous edges without increasing the size of the objects. Details of this method are similar to the discussion of a previous study [19]. Finally, for deformability parameter analysis, erythrocyte regions in the restructured image were filled with white pixels. Adopting the region properties function and image labelling technique, the cell count, as well as the elongation length (L), and deformed width (W), was determined. The erythrocyte deformability index was computed for each cell shown in the image.

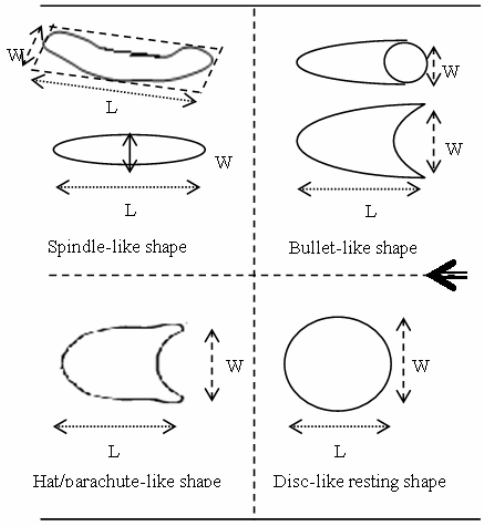

Fig. 4. Various shape schematics of RBC under flow and definition of their deformability index (DI =L/W). Binding box method was adopted for other elongated shapes in a different orientation.

2.5 Defining the deformability index

Under flowing conditions, erythrocytes align or deform in the direction of flow. Independent of their deformed shapes, they are essentially elongated in form resting in a biconcave-disc shape. Asymmetrically deformed cells (hat and bullet-shaped) orient their major axis perpendicular to the direction of flow. Meanwhile, the axially-deformed cells (slipper, spindle, or elliptical shapes), were oriented in parallel to the flow. For the different target shapes, deformability index is therefore defined as the ratio of cell-elongated length (L) to its deformed width (W) (Fig. 4).

3. Results

The erythrocytes shape transformation while deforming in a microchannel under flow conditions is shown in Fig. 5. Their corresponding deformability in terms of elongation length, deformed width, and deformability index are listed in Table 1. Apart from the resting/discoidal, four different shape transformations were observed; hat-, slipper-, spindle-, and bullet-like. The discoidal, non-deforming/resting erythrocytes (Fig. 5(a)) were obtained from the saline suspensions when the flow was near to stasis.

The hat-like transformati.on (also known as parachute shape) (Fig. 5(b)) manifest as asymmetrically-oriented cells depending on local shear variation. Each of the asymmetrically deformed and elongated erythrocytes assumed a slightly blunted conical or semicircular front with a cusp/dimple at the tail. Their shape resembles a hat (ex., as if they could be put

Table 1. Multi-shape deformability index of erythrocytes.

Shape of erythrocyte	Cell elongation length (L) (pixels)	Cell deformed width (W) (pixels)	Deformability index (DI)*
Non-deformed shape	35.3±1.16**	32.6±1.36	1.08 ± 0.06
Hat/Parachute shape	36.2±3.13	26.2±2.53	1.40 ± 0.21
Spindle/Elliptical /slipper shape	51.2±3.68	25.5±1.86	2.01 ± 0.15
Bullet shape	41.1±5.10	17.0±2.92	2.47 ± 0.50

** mean ± SD, No of samples=10, *$P<0.001$*

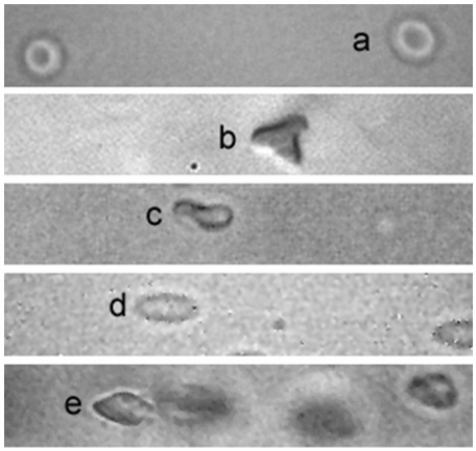

Fig. 5. Various shape transformations of erythrocyte observed during flow through in-house fabricated microchannel. Non-deformed disc-like shape (a), hat-like shape (b), slipper–like shape (c), spindle-like shape (d) and bullet like shape (e).

on) in the indentation of cusp.

Slipper-like shape (Fig. 5(c)) occurred in cells that would orient axially, and these were often found spinning during the flow. These cells showed up their cusp toward the top as if to allow foot insertion.

The spindle-like deformation (Fig. 5(d)) is also an elongation for axially-oriented cells with wider centre and tapering ends. Such transformed cells moved rapidly through the channel, showing no spinning or rotation.

Bullet-like transformation is another asymmetrical deformation similar to the hat type but with greater elongation. The front end narrowed to form a sharper tip (Fig. 5(e)) and the dimple indentation deepened at the rear with flaps around coming closer to it, which gave a nearly uniform width towards the rear end, appearing somewhat like a bullet. These cells move rapidly through the channel.

The multi-shape deformability index for erythrocyte transformations, as determined by the image processing, are shown in Table 1. The deformability index (DI) of deformed shapes showed significant variation ($p<0.001$) when compared to non-deformed/resting erythrocyte (control). Resting-shaped cells resembled a slightly elongated circle and hence had a deformability index close to unity. Elongation length progressively increased with the deformed shape, changing from hat- to bullet- to spindle. However, the increase in DI was observed when the shape changed from hat- to spindle- to bullet.

Although the spindle-shaped elongation lengths (L) was highest among all shapes, its DI was lesser compared with the bullet-shaped erythrocytes. This was because the spindle shapes had lower deformation along the width (W). Conversely, bullet-shaped erythrocyte had lesser elongation (L) compared with the spindle-shaped, and they showed greater deformability along the width.

4. Discussions and conclusions

Erythrocyte deformability is one of the critical factors in the successful delivery of essential oxygen replenishments for tissue metabolism and for the proper functioning of various organs in the human body. To survive this circulatory stress, erythrocytes may deform, align, adapt to flow and in the process, and transform into various shapes. The shapes that might occur in blood circulation are ellipsoidal shapes in arteries – hat/parachute-shaped in large capillaries (> 7 micron) and bullet-shaped small capillaries (< 5 micron) – due to the varying flow condition [4]. Some of these shapes were observed in microcirculation in the in vivo and in vitro experimental models [5-9]. Most in vitro erythrocyte visualization techniques involved single shape analysis, mostly, elliptical [8] and recently parachute-shaped erythrocytes [5]. The present study reports at least three erythrocyte shape transformations based on the analysis of their deformability using a microscopic imaging technique. Herein, shape transformations were demonstrated in a simple glass microchannel that was fabricated by using inexpensive materials like a glass slide, a transparency sheet, and a cover slip, which could be easily implemented.

Furthermore, the image processing procedure is capable of analyzing a batch of image sequences and hence allows analysis of every single-erythrocyte in a recorded sequence. Once the single-, and non-interacting erythrocytes are selected in an image, the program automatically carries out the rest of the analysis and directly provides the deformability data output. Aside from observing different erythrocyte shapes and analyzing their deformability, dynamic shape transformations can also be recorded using the presented μFVD technique. Specific image sequences have shown the changing shapes of the erythrocytes as they flipped, twisted, and turned during flows in accordance with local shear variation. Other dynamics that can be observed through target window are the changes in elongation length and deformation width, as well as the orientation during the traversal. Such twisting, turning, and flipping of cells may allow visualization of top-bottom and side surface profiles, which could be of value in examining morphological and surface modifications.

Measurement on erythrocytes deformability and its correlation to a disease is an emerging field in clinical hemorheology [3]. A direct and accurate, muti-shape erythrocyte deformability characterization, such as the one described above, thus assume significance for field application.

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