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Ultramicroscopy 86 (2001) 107–112

ultramicroscopy

www.elsevier.nl/locate/ultramic

Quantification of red blood cells using atomic force microscopy

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Received 12 July 2000

Abstract

For humans the sizes and shapes of their red blood cells are important indicators of well being. In this study, the feasibility of using the atomic force microscope (AFM) to provide the sizes and shapes of red blood cells has been investigated. An immobilisation procedure has been developed that enabled red blood cells to be reliably imaged by contact AFM in air. The shapes of the red blood cells were readily apparent in the AFM images. Various cell quantification parameters were investigated, including thickness, width, surface area and volume. Excellent correlation was found between the AFM-derived immobilised mean cell volume (IMCV) parameter and the mean cell volume (MCV) parameter used in current haematological practice. The correlation between MCV and IMCV values has validated the immobilisation procedure by demonstrating that the significant cell shrinkage that occurs during immobilisation and drying does not introduce quantification artifacts. Reliable IMCV values were obtained by quantifying 100 red blood cells and this typically required 3–5 AFM images of 100 μm × 100 μm area. This work has demonstrated that the AFM can provide in a single test the red blood cell size and shape data needed in the assessment of human health.

Keywords: Atomic force microscopy; Red blood cells; Quantification

1. Introduction

For humans the sizes and shapes of their red blood cells are important indicators of well being. Red blood cells from a healthy person appear circular and biconcave with only a very small proportion having an irregular outline. They are essentially uniform in size with a mean cell volume (MCV) in the range 82–95 fl [1]. The MCV for a healthy individual remains quite constant and can be used for reference purposes. In contrast, biconcave red blood cells with MCV values above 95 fl (i.e. macrocytic) are a common finding for persons with megaloblastic anaemia [2]. Furthermore, changes in red blood cell shape, which may consist of changes in outline or cross-section, result from a wide range of conditions, e.g. sickle cell anaemia, thalassaemia and obstructive liver disease, and have been well classified [3].

Within current clinical haematology practice, automatic analysers are used to determine inter alia the MCV of red blood cells within an in vitro sample. The automatic analysers also determine the size distribution of the red blood cells and flag abnormalities such as double populations. The shapes of red blood cells are determined manually

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0304-3991/01/$ - see front matter
PII: S0304-3991(00)00081-4

by optical microscopy of a stained, dried blood film. Notwithstanding the widespread use of this methodology, there are clinical conditions where the results of the two tests are either inconclusive or in conflict. In essence, the latter difficulties arise from the lack of a single test that can provide unambiguous size and shape data and also from the inability of either of the above tests to provide reliable data on the thickness of the red blood cells. The aim of this work was to determine the feasibility of using atomic force microscopy in a single test to provide both size and shape data of red blood cells.

Since its invention in 1986 [4], the atomic force microscope (AFM) has found widespread application within biological science (e.g. [5–7]) and has been used to a limited extent, when compared to nucleic acids and lipids for example, to image red blood cells. Such work has generally fallen into two categories: studies of whole cells (e.g. [8–11]); and studies of the cytoskeleton of the inner surface of the membrane of lysed cells (e.g. [12–14]). In the context of this paper, the work of Zachée et al. [15] is of particular interest as they used the AFM to confirm the adverse effect of the splenic environment on hereditary spherocytosis. This appears to be the first application of the AFM to medical diagnosis and was based on observing with the AFM changes in the shape of a patient's red blood cells before and after splenectomy. In a further study, Zachée et al. [16] used the AFM to confirm the existence of uremic echinocytes, i.e. red blood cells with characteristic features, in the blood film of a patient with anaemia that arose from chronic renal failure.

2. Experimental

2.1. Immobilisation of red blood cells

A number of methods have been developed to immobilise red blood cells for subsequent optical microscopy or scanning electron microscopy. Some of these methods have in turn been either adopted or adapted for atomic force microscopy of red blood cells. If the red blood cells are to be imaged by AFM in vitro then the cells simply have to be attached to a suitable substrate. However, if they are to be imaged in air then the cells must also be fixed so that they remain stable. For the work reported here a reliable method of immobilising red blood cells in vitro was not available. Instead, we concentrated on identifying methods of immobilising and fixing red blood cells in air that did not introduce artifacts in the AFM images and thereby in the subsequent quantification. Details of that study [17] will be reported elsewhere.

The following method was found to give AFM images that were free of artifacts. A fresh blood sample was taken and mixed with an anticoagulant (EDTA) and a full blood count was performed with an automated analyser to obtain the MCV value for the sample. A 4 μl aliquot of the blood sample was manually spread onto a polished microscope glass slide to create the blood film. The film was then air-fixed rapidly by waving the slide vigorously. The film was examined under the optical microscope and regions with a single layer of red blood cells but without undue overlap of neighbouring cells were marked using a lens-mounted inking device.

2.2. Atomic force microscopy

Atomic force microscopy was performed in air on the blood films using a commercial AFM[1] in contact mode. The glass slide carrying the blood film was mounted onto the XY stage of the AFM and the integral video camera was used to locate the regions of interest that had been marked as described above. Microfabricated contact mode silicon cantilevers[2] with a spring constant of approximately 0.3 N/m were used. The force applied to the sample during imaging was typically 20 nN. Repeated scanning of the same red blood cells confirmed that no physical damage occurred during atomic force microscopy. For quantification purposes 100 μm × 100 μm area AFM images were obtained at a line scan rate of 2 Hz.

Non-overlapped, red blood cells within the AFM images were quantified using the software

[1] Explorer model, ThermoMicroscopes, Sunnyvale, CA.
[2] Point Probe type, NanoSensors GmbH, Wetzlar, Germany.

provided with the instrument.[3] Before quantification was carried out, the AFM images were planar levelled using the commercial software. Using the line analysis function of the software, the average width and the average height (i.e. thickness) of the red blood cells were determined. Using the particle analysis function of the software, the surface area and the volume of each red blood cell were determined. In order to avoid confusion with the MCV parameter the volume of each RBC and the mean volume measured from our AFM images are termed the immobilised cell volume (ICV) and the immobilised mean cell volume (IMCV), respectively. The ICV is enlarged in the AFM image by tip imaging and by the fact that the tip cannot reach the underside of the red blood cell. To overcome such artifacts, we considered measuring the volume of the top half of the cell and then scaling up appropriately. However, it was evident from line sections through immobilised red blood cells that the central depression of some immobilised red blood cells was lower than the half-height of that cell and that this variable 'collapse' of cells would undermine such an approach to quantification. Thus, the immobilised cell volumes reported here are enlarged by the tip imaging artifacts described above. An analysis [17] of 280 red blood cells from a series of AFM images obtained for the same blood sample showed that the IMCV and its standard deviation stabilised to within $\pm 3\%$ when the number of red blood cells quantified exceeded about 40. In this work, 100 red blood cells were quantified per sample and this typically required 3–5 $100\,\mu m \times 100\,\mu m$ area AFM images.

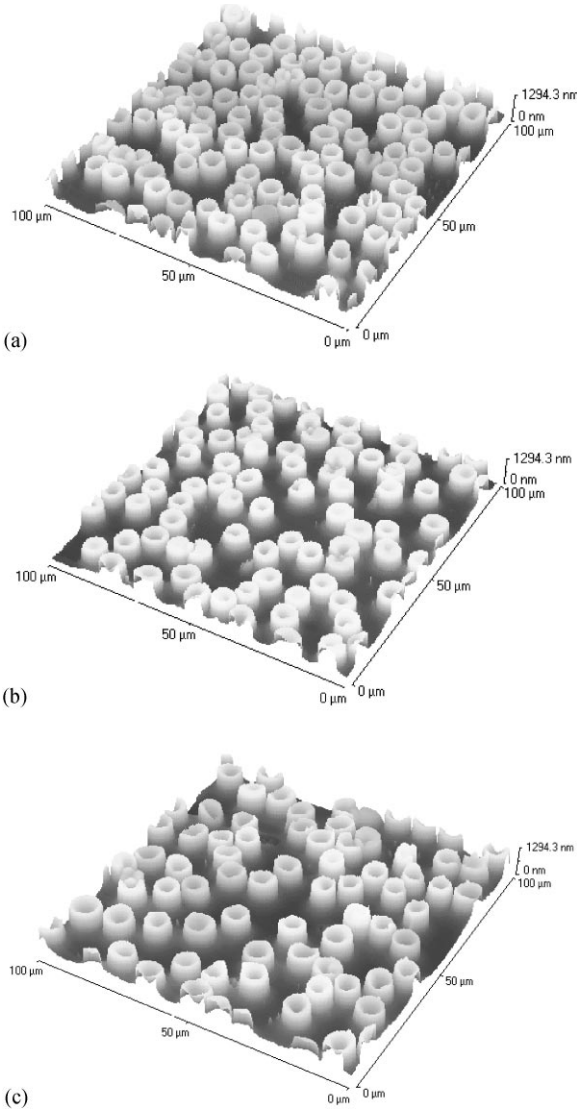

Fig. 1. Three-dimensional view AFM images ($100\,\mu m \times 100\,\mu m$) of (a) a microcytic blood film (MCV = 73.7 fl), (b) a normal blood film (MCV = 85.5 fl) and (c) a macrocytic blood film (MCV = 109.7 fl).

3. Results and discussion

Blood films from three individuals whose red blood cell populations were microcytic (MCV = 73.7 fl), normal (MCV = 85.5 fl) and macrocytic (MCV = 109.7 fl) were imaged by AFM and quantified as described above. Fig. 1 shows, by way of example, three-dimensional view AFM images ($100\,\mu m \times 100\,\mu m$) for the three different samples. It is clear that the biconcave shape of the red blood cells can be readily discerned from these images. However, due to the natural variations in size within any population of red blood cells it is not so easy to discriminate visually between these samples.

Fig. 2 shows histograms of frequency versus ICV for 100 red blood cells from each of the

[3] SPMLab Version 3.06.06.

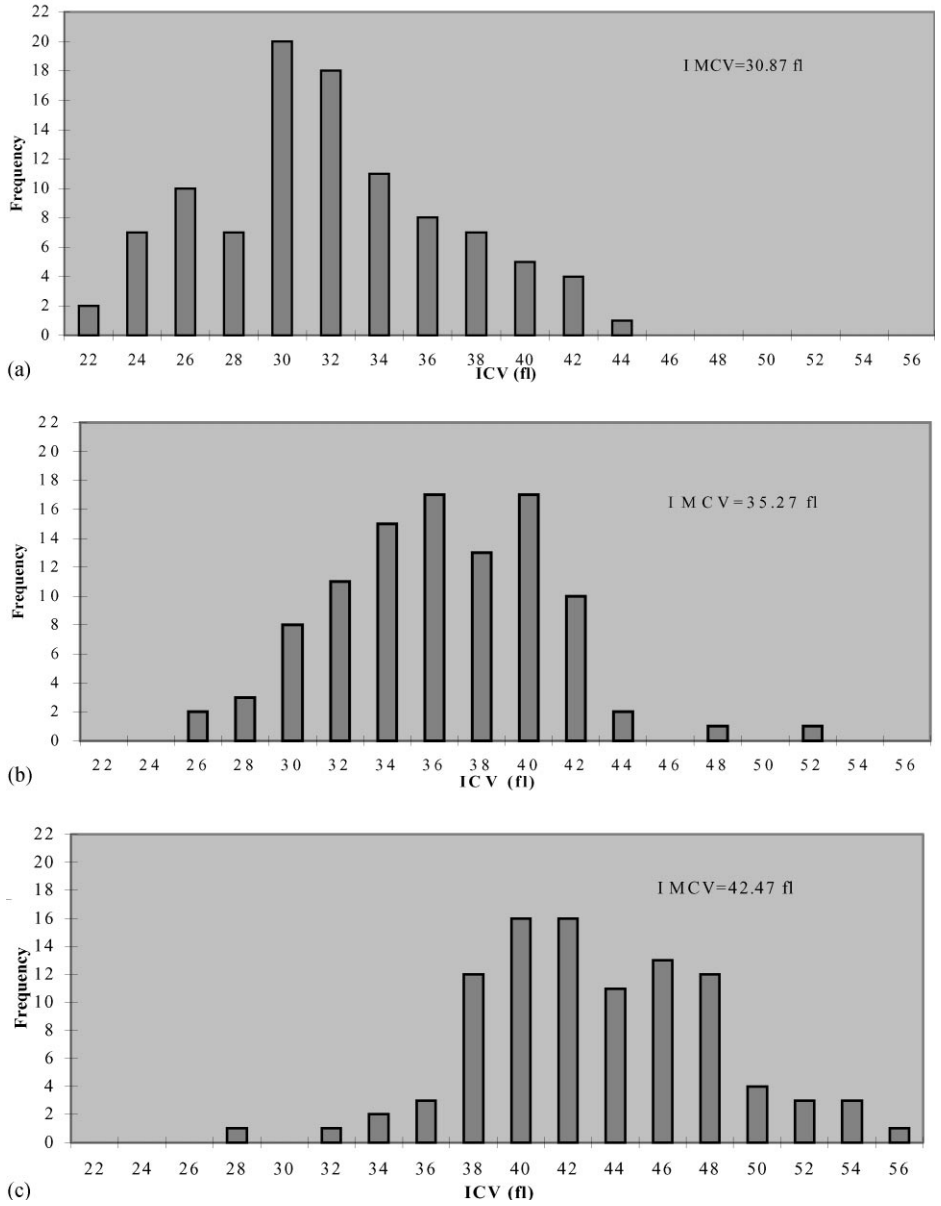

Fig. 2. Histograms of frequency versus ICV ($n = 100$ red blood cells) for (a) a microcytic blood film (MCV = 73.7 fl), (b) a normal blood film (MCV = 85.5 fl), (c) a macrocytic blood film (MCV = 109.7 fl).

microcytic, normal and macrocytic blood films. It is clear from these data that the distributions of the ICV values and the mean ICV values for each type of blood film are well separated and that the microcytic, normal and macrocytic conditions can be clearly distinguished.

The average height, average width, average surface area and IMCV for each of the three blood films are plotted against MCV in Fig. 3. The correlation of IMCV with MCV is excellent. While the correlation of average width is very good it should be noted that this parameter does not

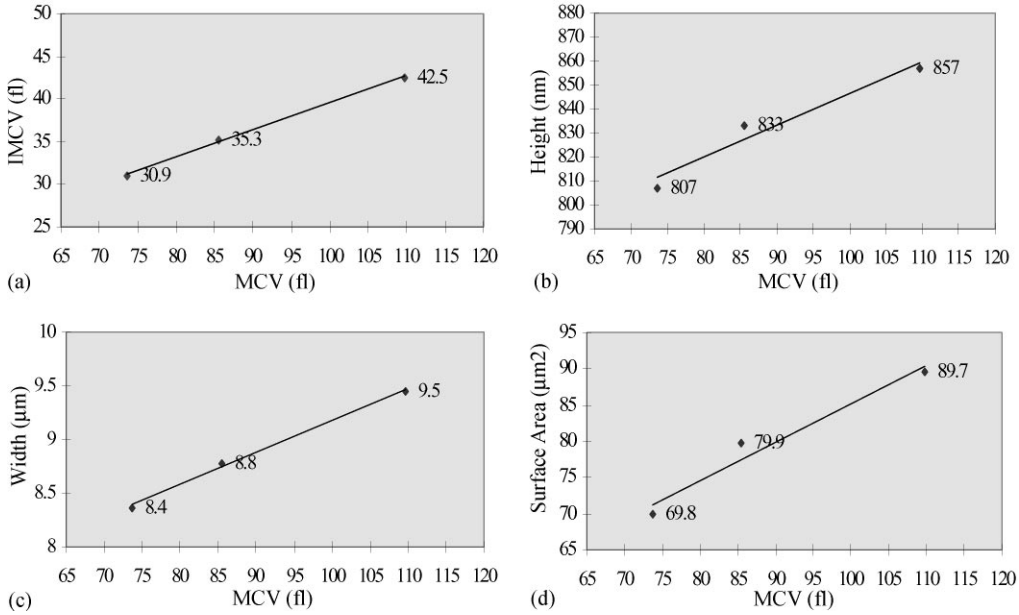

Fig. 3. Graphs of (a) IMCV, (b) average height, (c) average width and (d) average surface area versus MCV for 100 red blood cells from microcytic (MCV = 73.7 fl), normal (MCV = 85.5 fl) and macrocytic (MCV = 109.7 fl) blood films.

respond to changes in the cross-section of the red blood cell whereas IMCV does. For the three blood films quantified in this work the red blood cells had biconcave shapes.

4. Conclusion

An immobilisation procedure has been developed that enabled red blood cells to be reliably imaged by contact AFM in air. The shapes of the red blood cells were readily apparent in the AFM images. Excellent correlation was found between the AFM-derived IMCV parameter and the MCV parameter used in current haematological practice. The correlation between MCV and IMCV values has validated the immobilisation procedure by demonstrating that the significant cell shrinkage that occurs during immobilisation and drying does not introduce quantification artifacts. Reliable IMCV values were obtained by quantifying 100 red blood cells for each sample and this typically required three to five AFM images of $100\,\mu m \times 100\,\mu m$ areas. This work has demonstrated that the AFM can provide in a single test the red blood cell size and shape data needed in the assessment of human health. Further work is underway to extend the number of patient samples and to examine the correlation of IMCV with MCV for red blood cells with shapes other than biconcave.

Acknowledgements

The authors wish to thank Dr. P. Cotter and Mr. B. Joyce, Cork University Hospital, for providing the blood samples used in the work reported here. Financial support for part of this work was provided by Enterprise Ireland under the Applied Research Programme. The authors also thank the reviewers for their suggestions on improving the manuscript.

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