

A methodology to study the deformability of red blood cells flowing in microcapillaries *in vitro*

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Summary. The deformability of red blood cells flowing in microvessels is essential to maintain optimal blood circulation and to allow gas transfer between blood and tissues. Here, we report on an experimental methodology to investigate the deformability of RBCs flowing in microcapillaries having diameter close to the average cell size. The microcapillaries are placed in a rectangular flow cell, where a suspension of RBCs, properly diluted in albumin-added ACD, is fed through a syringe under the action of a liquid head in the physiological range. Video microscopy images of the flowing RBCs are acquired at high magnification and later processed by an automated image analysis macro. It was found that RBCs from healthy donors exhibit the classical parachute shape observed *in vivo*. Furthermore, all the data of healthy RBC velocity vs liquid head are well represented by the same linear regression, independently on the donor. Preliminary results on β -thalassemia RBCs are also presented and show, on the average, a reduced velocity compared to healthy samples.

Keywords: erythrocytes, capillaries, erythrocyte deformability, microfluidics, thalassemia.

Riassunto (*Una metodologia per lo studio in vitro della deformabilità di globuli rossi in flusso attraverso microcapillari*). La deformabilità dei globuli rossi circolanti nei vasi di piccolo calibro è una proprietà essenziale per mantenere un flusso ottimale e per consentire gli scambi gassosi fra sangue e tessuti. In questo contributo viene descritta una metodica sperimentale per lo studio della deformabilità di globuli rossi in flusso attraverso microcapillari artificiali di diametro paragonabile alla dimensione cellulare media. I microcapillari sono collocati in una cella di flusso rettangolare, in cui una sospensione di globuli rossi, opportunamente diluiti in ACD addizionato di albumina, viene inviata mediante una siringa sotto l'azione di un battente liquido nel range fisiologico. Le immagini di globuli rossi in flusso sono acquisite ad elevato ingrandimento mediante video microscopia ottica ed elaborate successivamente attraverso routine di analisi dell'immagine. I globuli rossi da donatori sani mostrano la classica forma a paracadute osservata *in vivo*. Tutti i dati di velocità eritrocitaria in funzione del battente liquido per donatori sani sono inoltre ben rappresentati dalla stessa regressione lineare, indipendentemente dal donatore. Risultati preliminari su globuli rossi da pazienti affetti da β -talassemia mostrano in media una riduzione di velocità rispetto ai campioni da donatori sani, in accordo con la ridotta deformabilità di membrana associata a questa patologia.

Parole chiave: eritrociti, capillari, deformabilità eritrocitaria, microfluidica, talassemia.

INTRODUCTION

The deformability of red blood cells (RBCs) flowing in microvessels is essential to maintain optimal blood circulation and to allow gas transfer between blood and tissues, and is implicated in several physiopathological processes. RBC deformability has been the subject of a number of investigations in the literature. One of the main research directions in this area has been the design of flow cells somehow mimicking the fluidodynamic conditions experienced by RBCs in the microcirculation *in vivo*. The experimental methods so far reported in the literature include

sedimentation velocity of centrifuged blood [1], filtration through membranes with different porosity [2, 3], the time spent by RBCs in passing through a single membrane pore [4], blood viscosity as a function of the deformation velocity [5, 1], the aspiration of a single RBC in a micropipette [6, 7], the diffraction of RBC suspensions undergoing shear flow in a Couette rheometer (ektacytometer) [8], the separation between two microspheres adherent to RBC surface using optical tweezers [9-11]. A measurement associated, though indirectly, to RBC deformability is that of blood viscosity, which depends both

on plasma composition and on the properties and concentration of the suspended cells [12]. It was indeed found that blood viscosity is higher if RBCs are made less deformable (for example, by crosslinking with glutaraldehyde [13]). From the haemorheological point of view, one of the most popular techniques is the measurement of apparent viscosity of whole blood or RBC suspensions at different concentrations in artificial capillaries, usually made of glass or silica [14]. These studies include the pioneering work of Fåhræus [15] on the variation of apparent blood viscosity with capillary diameter. Even though this rheological approach is still followed, direct measurements of deformability of RBCs flowing in microcapillaries with size smaller than cell body are scarce in the literature. In fact, observations of flowing RBCs by optical microscopy have been mainly described for capillary diameters larger than cell size [14]. Data of RBC deformability in case of pathological cells are even more limited.

The progress of the experimental techniques has been paralleled by the development of theoretical analyses to model the ability of RBC to deform and flow through channels smaller than the size of the cells at rest. The deformability, defined as the extension of the cell body under the steady state application of a fluidodynamic stress, is mainly function of three variables: internal viscosity, surface/volume ratio, and viscoelasticity of the membrane [16, 17]; The structure of RBC membrane has been modelled on a microscale as a network of elastic elements [18, 19]. The relation between deformability and morphology has been also studied, focusing on the change from the usual biconcave shape to the one observed for echinocytes and stomatocytes [20]. Notwithstanding the recent applications of numerical simulation techniques [21] the modelling of RBC fluidodynamic behavior and the comparison with experimental data are still at a preliminary stage, especially for pathological situations, and further investigations are at order.

In fact, in spite of the progress in experimental techniques, from the clinical standpoint RBC deformability is still measured by quite approximate methods, for example by measuring the time of perfusion through filters with pores of a given size. The main limits of this technique are the difficult standardization of the methodology, the intrinsic variability of pore size, the lack of information concerning single cell deformability, and the limited quantitative results that can be so obtained. Therefore, the setup of novel experimental systems *in vitro* appears as a relevant research goal towards a deeper understanding of the biological significance of RBC deformability, especially in a physiopathological perspective.

In this work, we describe an experimental methodology to investigate RBC deformability in microcapillaries having internal diameter close to cell size. Our approach is based on direct visualization of flowing RBCs by video-enhanced microscopy and automated image analysis procedures to meas-

ure cell velocity and deformation. The experimental variables investigated in this work include flow rate, size and length of microcapillaries. RBCs from healthy donors and from patients suffering from β -thalassemia have been investigated; preliminary results are presented and discussed.

MATERIALS AND METHODS

Blood samples

Fresh venous blood was drawn from both healthy and heterozygous β -thalassemic consenting volunteers into Vacutainer tubes. All blood samples were used within 4h of collection. RBC viability was checked before each experiment by observing cell morphology under static conditions at high magnification (100x objective). Approximately 1 mL of whole blood was diluted with 100 mL of anticoagulant ACD (0.6% citric acid, 1.1% dextrose anhydrous, 2.3% sodium citrate, 96% water), 5 mL of human albumin and 5 mL of PBS (phosphate-buffered saline). Such level of dilution ensured optimal performance of the flow cell. The viscosity of the suspending fluid was measured by a Ubbelohde glass viscometer immersed in a water bath at 37 °C, and was equal to 0.8 mPa·s. The presence of RBCs at the dilution used in the experiments did not change significantly the value of fluid viscosity.

Experimental apparatus

The microcapillaries used in this work were either made of silica (with 5 and 6.6 μm ID, Polymicro Technologies) or embedded in a gel matrix. In the latter case, a 2% w/w agarose solution at ca. 90 °C was cast in a rectangular mold enclosing a 5 micron diameter gold-plated tungsten wire. After gelation, the wire was gently removed from the agarose slab, thus leaving a 5 μm microcapillary. The diameter and the length of all the capillaries used in this work were carefully measured by video microscopy. The measurements were carried out by filling the capillaries with an isorefractive fluid to avoid optical distortions.

The flow cell (a schematic is shown in *Figure 1*) was made of two Plexiglass plates separated by a rubber spacer. A window was cut in the bottom plate to allow insertion of a coverslip slide for observations at high magnifications with oil immersion objectives. The RBC suspension was fed to the flow cell through an input hole by a flexible tubing connected to a syringe. The suspension coming out from the flow cell through an output hole was collected by a plastic tubing in a glass reservoir placed on a vertical translating stage. The distance between the liquid menisci in the feeding syringe and the exit reservoir was adjusted by the translating stage. Such liquid head was measured during the experiment by imaging both the syringe and the exit reservoir with a CCD video camera against a graph paper background. This allowed to monitor the pressure differential in the course of the experiment.

The flow cell was placed on the motorized x-y stage of an inverted microscope (Zeiss Axiovert 100) equipped

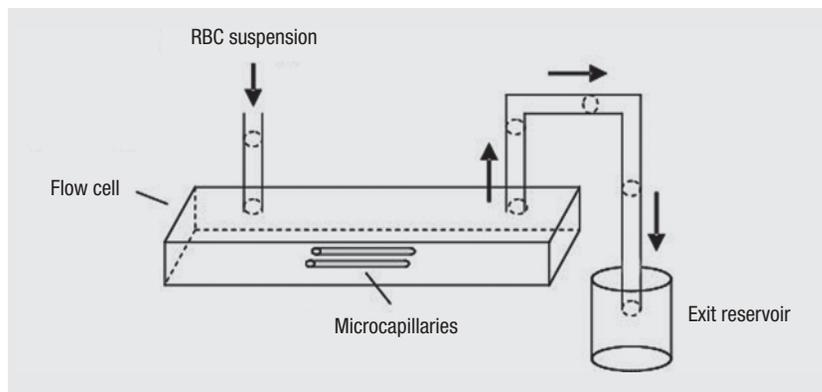


Fig. 1 | Schema of the flow cell.

with a motor assembly for focus control. Sample positioning was controlled by a custom LabView routine. In each experiment sample temperature was kept at 37 °C by enclosing the microscope and the flow cell assembly (including the feeding syringe and the exit reservoir) in a Plexiglass cage equipped with an air thermostating system based on a PID controller.

Images of the flowing RBCs were acquired by another CCD videocamera (Hitachi) and the whole experiment was recorded on video tape for reference (this recording was synchronized with that of the liquid head for later comparison). Real time image sequences (1000 images at a rate of 25 frames/s) were also digitized during the experiment by means of a frame grabber (National Instrument IMAQ PCI 1409) installed on a Pentium-based host PC and saved in computer memory for later analysis. The images were processed off-line by a macro calling standard image analysis routines from the library of a commercial package (Image Pro Plus 4.5). The fully automated macro operation allowed to isolate the subsets of images from each sequence where the passage of a cell could be identified and to determine the position of the flowing cell as a function of time. From these data RBC velocity was calculated as the slope of cell displacement vs time. Images of the flowing RBC were then saved in a database to evaluate the extent of cell deformation under the action of flow.

Images of RBCs at rest were also acquired to evaluate the cell size distribution. The measurements were carried out by pouring a drop of the dilute cell suspension between a microscope slide and a coverslip and acquiring images of several fields of view (the total number of counted cells was around 200). RBC size was measured by image analysis as the diameter of the cell body in the plane of observation (tilted cells were not considered in the measurements).

RESULTS

In a typical experiment, the pressure differential across the microcapillaries was regulated by adjusting the relative liquid levels in the syringe and the exit reservoir. The liquid head was initially set to 30 cm H₂O for about 5 minutes to fill the inlet and outlet tubings and the flow chamber. Then, the liquid head was brought

to 13 cm H₂O by lowering the position of the exit reservoir. The following decrease of the liquid head due to the emptying of the syringe and the filling of the exit reservoir was continuously recorded during the whole experiment by using the second CCD videocamera. From time to time, the syringe was refilled and the liquid head set back to 30 cm H₂O for about 10 minutes to prevent RBC sedimentation and the possible clogging of the outlet tubing around the exit hole.

As described in the previous section, real time image sequences were acquired throughout the experiment and processed off-line by the image analysis macro. The passage of an RBC through the microcapillary under observation was then associated to the current liquid head from the continuous recording of the distance between the two liquid levels in the syringe and the exit reservoir. A typical plot of cell displacement as a function of time in a silica microcapillary from a healthy donor sample is shown in *Figure 2a*, where the symbols refer to the data points and the solid line is the corresponding linear regression (the coefficients are also shown in the figure). Capillary length and diameter are 3.6 mm and 6.6 μm, respectively. It can be noticed that the data points follow quite closely a linear trend (the R² value of the regression is close to 1), thus showing that at the point of observation (which is located about halfway between the inlet and outlet microcapillary sections) RBC flow is under steady state conditions. The slope of the linear regression is the cell velocity, which is ca. 166 μm/s. An image of the flowing RBC is also shown in the inset of *Figure 2a*. The classical parachute shape, which is found *in vivo* [22], can be clearly observed in the image, and shows that the cells are indeed subjected to flow conditions similar to those experienced under physiological conditions. Similar shapes are also observed in the capillaries embedded in the agarose gels, as shown in *Figure 2b* (capillary ID is around 5 μm). It so appears that the parachute shape is due to the imposed fluidodynamics, and is essentially independent on the inner surface of the microcapillary.

The data analysis procedure illustrated in *Figure 2a* was systematically applied in a range of liquid heads from 1 to 10 mmHg. At each value of liquid head

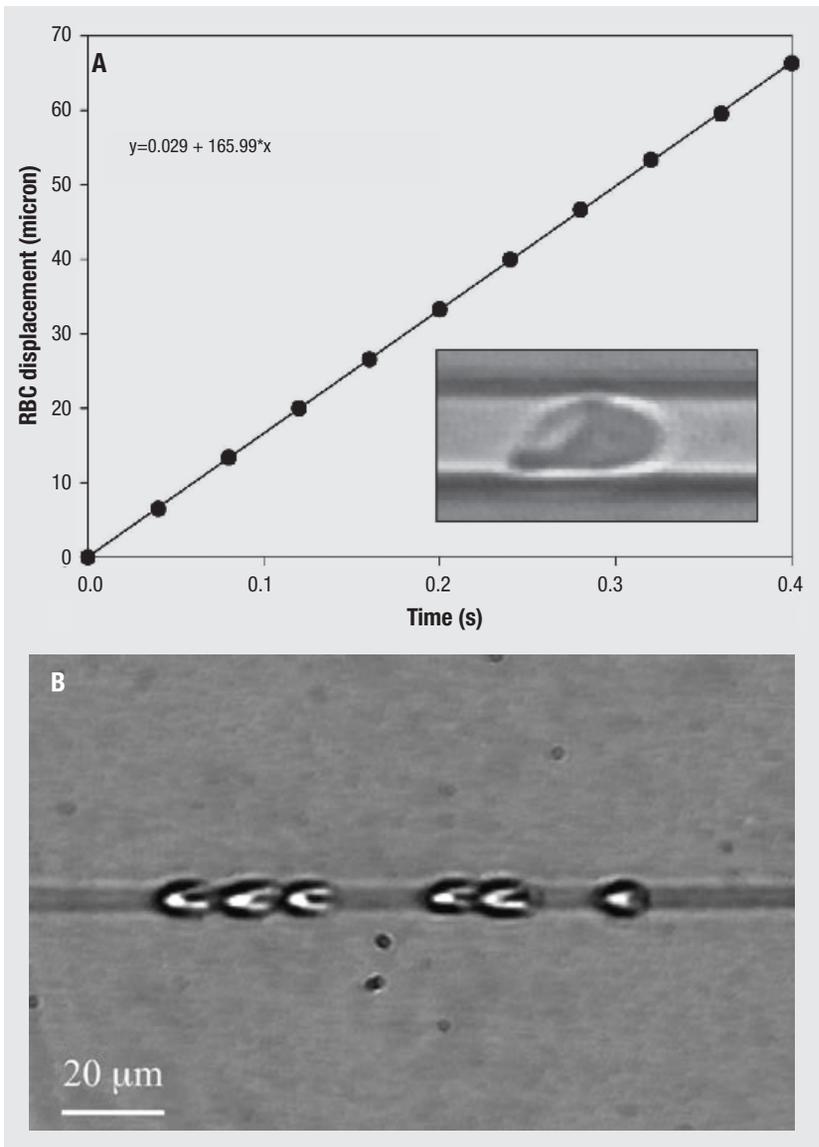


Fig. 2 | A) Cell displacement vs time in a microcapillary; B) images of RBCs flowing in a microcapillary embedded in agarose gel.

the velocity of 10-20 RBCs was measured. The results from three different healthy donors are plotted in *Figure 3a*. Each point in *Figure 3a* is the average value of RBC velocity from several measurements at the corresponding liquid head, and the error bars represent the standard deviation of the data. The three sets of points correspond to experimental runs from different donors with different capillary length from 3.6 to 4.9 mm, and the solid lines are linear fit to the data. All the data sets are well represented by the linear fit, and tend to zero at vanishing liquid head. This shows that the measured liquid head is dominated by the pressure drop between the ends of the microcapillaries, the other pressure losses (feeding and exit tubings, pressure drop due to converging flow to the capillary) being negligible.

In *Figure 3b* the same sets of data as in *Figure 3a* are scaled to the capillary length of 3.6 mm by assuming a direct proportionality between cell veloc-

ity and capillary length. It can be noticed that this scaling makes the three data sets superimpose each other, thus generating a “master curve” of healthy RBC velocity vs liquid head. In other words, the microcapillary flow behaviour of healthy RBCs does not depend on the donor, and it can be taken as a reference to be compared to pathological situations. The solid line shown in *Figure 3b* is the calculated value of the average fluid velocity $\langle v_z \rangle$ along the capillary axis z according to the classical equation

$$\langle v_z \rangle = \frac{\Delta P R^2}{8 \mu L} \quad (1)$$

where ΔP is the liquid head, R and L are capillary radius and length, respectively, and μ is the viscosity of the suspending liquid (which was measured by glass viscometry as described in the experimen-

tal section). The above equation is based on the assumption of well developed parabolic flow of a Newtonian fluid in a circular cross-section tube (Poiseuille flow), and does not contain any fitting parameters. The agreement between equation (1) and the experimental data of healthy RBC viscosity in *Figure 3b* is quite good. It shows that healthy RBCs move in a plug flow fashion inside microcapillaries of diameter comparable to cell size. The average RBC size at rest of the healthy samples was around $7 \mu\text{m}$ with a standard deviation of 0.5, as measured by optical microscopy and image analysis (see experimental section for details).

The measurements of RBC velocity vs liquid head from healthy donors were used as a control to evaluate the flow behavior of pathological blood samples. To exemplify the application of our methodology to pathological RBCs, we report preliminary data

from β -thalassemia blood samples. In *Figure 4*, the RBC velocity of four heterozygous β -thalassemia patients is plotted as a function of the liquid head. In the same figure, results from healthy RBCs are also shown for comparison. *Figure 4* shows that data from the β -thalassemia patients investigated in this work follow a linear trend passing through the origin. At variance with the healthy RBC results, however, β -thalassemia data do not superimpose on the same “master curve”, but rather exhibit different slopes, one being even coincident with the healthy case. The remaining three pathological data sets fall significantly below the velocity of healthy RBCs (the slopes are between 64% and 35% smaller), thus showing an increased hydrodynamic resistance in microcapillary flow. The lower panel in *Figure 4* shows representative images of flowing β -thalassemia RBCs from the four samples investi-

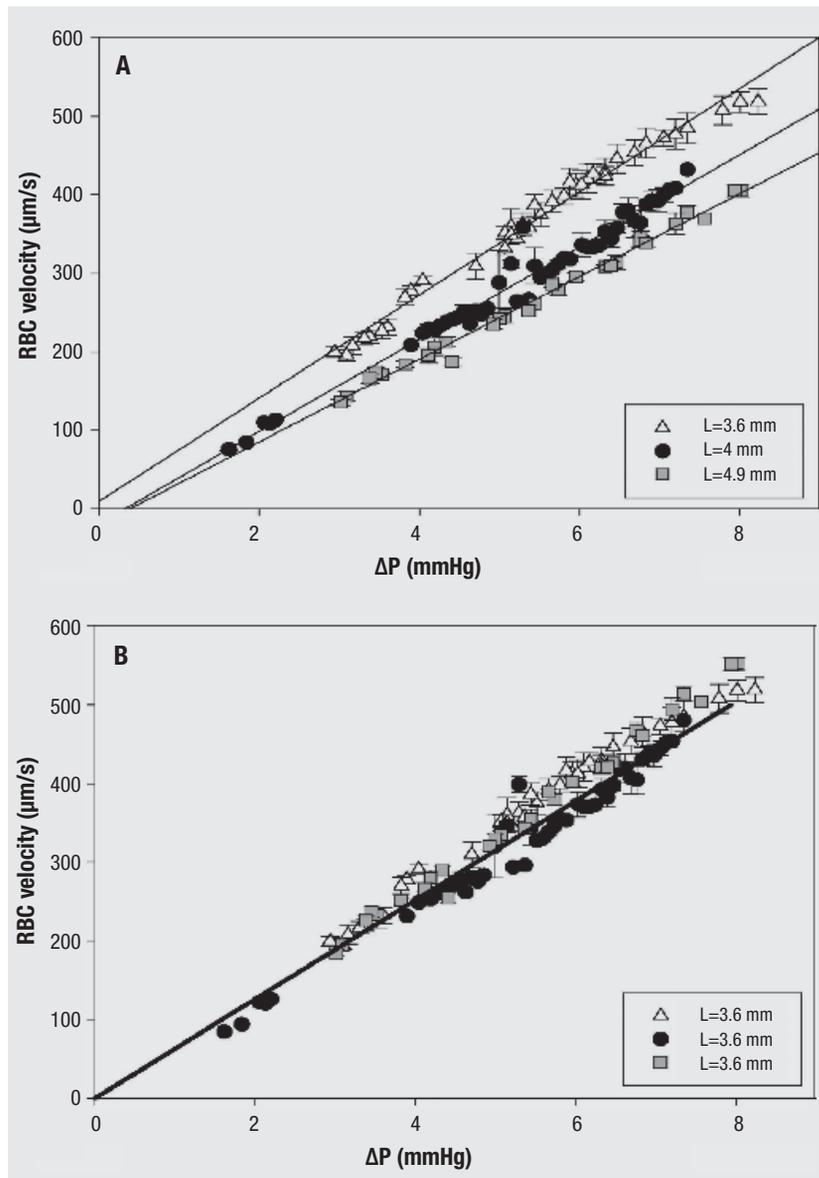


Fig. 3 | A) RBC velocity vs liquid head for three samples from healthy donors in microcapillaries of different lengths; B) same data as in A) rescaled to the same microcapillary length by assuming direct proportionality between RBC velocity and microcapillary length.

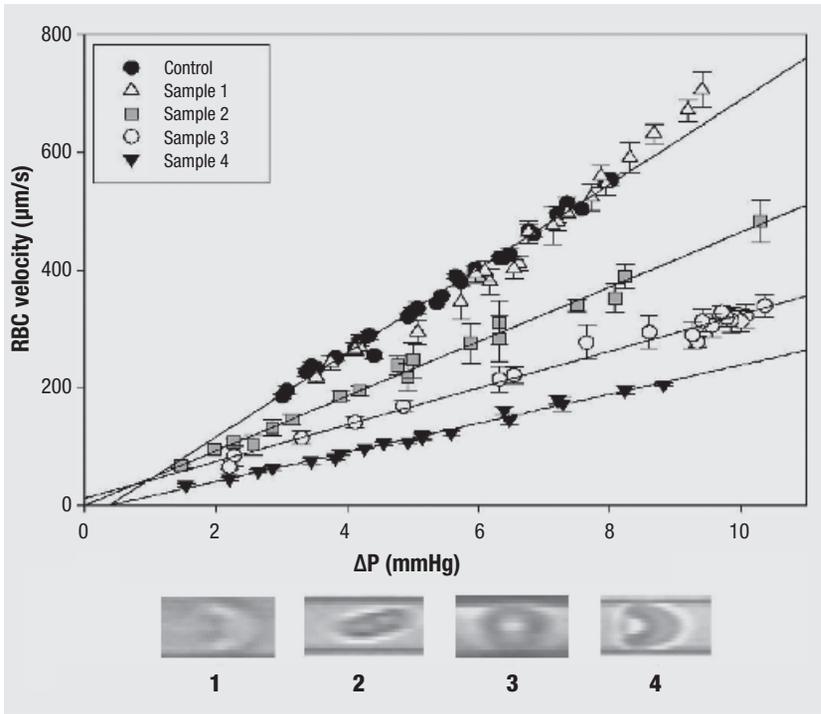


Fig. 4 | RBC velocity vs liquid head for four samples from β -thalassemia patients and one control from a healthy donor.

gated at a liquid head of ca. 6.5 mmHg. A parachute-like cell shape was observed in most images of the flowing RBCs, in analogy with the healthy samples. The RBC size distribution at rest was determined by video microscopy and image analysis for the β -thalassemia samples of Figure 4. The average size was slightly lower compared to the healthy case, going from 6.6 to 7.0. Some hematological data for the blood samples of Figure 4 are presented in Table 1. No simple correlation between RBC velocity and hematological parameters can be derived from Table 1. A systematic investigation to extend these preliminary results is currently in progress.

CONCLUSIONS

In this work, we describe an experimental methodology to investigate the flow behaviour of RBCs in microcapillaries of diameter comparable to cell size. Our approach is based on imaging deformed RBCs at high magnification by a video microscopy work-

station equipped with a motorized stage for precise positioning of the flow cell and a temperature control system. The acquired images are processed by image analysis techniques in an automated way to measure RBC velocity as a function of the imposed pressure differential. The performance of the apparatus was tested on blood samples from healthy donors, and it was found that RBC velocity is not dependent on the donor and is equal to the average fluid velocity in the microcapillary as calculated from the classical Poiseuille equation.

Preliminary results from heterozygous β -thalassemia patients are also reported, and show an average decrease of RBC velocity with respect to the healthy individuals. This is not unexpected, since it is well known that β -thalassemia is associated with an increased rigidity and a reduced mechanical stability of the cell membrane and cytoskeleton, leading to impaired RBC deformability [23]. Evidence of reduced deformability in β -thalassemia RBCs

Table 1 | Haematological data of the blood samples of Figure 4

Sample	Hb (g/dL)	HCT (%)	MCV (fL)	D* (\pm SD)
Control	13.2	42.1	85.6	6.9 (\pm 0.6)
1	15	47.7	75.5	6.3 (\pm 0.63)
2	8.8	30.1	65.6	6.5 (\pm 0.94)
3	13	40.8	60.1	7.0 (\pm 0.92)
4	13.9	43.8	72.2	6.4 (\pm 0.78)

D* is the RBC diameter in the plane of observation.
SD = standard deviation.

suspended in PBS viscosized with addition of dextran has been provided by laser diffractometry [24] in a flat glass cell. However, a direct visualization of RBC shape and velocity in a microcapillary of comparable size, such as in the present investigation, has not been reported so far to our knowledge. A further advantage of our approach is that it allows one to discriminate the flow deformability of RBCs from different patients and to give a quantitative evaluation of the difference with respect to the healthy case. A systematic work to elucidate the mi-

crocapillary flow behaviour of β -thalassemia RBCs and to correlate these results with clinically relevant parameters is currently in progress.

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