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To cite this version:

Arie Finkelstein, Hugues Talbot, Suat Topsu, Thérèse Cynober, Loïc Garçon, et al.. Comparison between a Camera and a Four Quadrant Detector, in the Measurement of Red Blood Cell Deformability as a Function of Osmolality. Journal of Medical and Bioengineering, 2013, 2 (1), pp.62-65. hal-00865916

HAL Id: hal-00865916

https://hal.science/hal-00865916v1

Submitted on 25 Sep 2013

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Comparison between a Camera and a Four Quadrant Detector, in the Measurement of Red Blood Cell Deformability as a Function of Osmolality

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***Abstract*— The ability of red blood cells (RBC) to deform under vascular conditions is essential for circulation. RBC deformability, measured at different shear rates and osmolalities, provides a useful way to evaluate RBC function and is used to diagnose several hereditary blood disorders.**

In clinical practice, ektacytometry has been used as a routine automated technique for measuring RBC deformability under shear stress at known osmolalities. RBC suspension is exposed to laser light and the resulting diffraction pattern is recorded. The mean deformability of the cells is characterized by the diffraction pattern. Our study is the first to compare the correspondence between two methods that measure diffraction simultaneously, on the same apparatus. Additionally, while others conducted studies under varying shear we used varying osmolalities.

A laser beam splitter produced two identical diffraction patterns, evaluated by synchronous data acquisition and analysis: One pattern was acquired by a digital camera and analyzed by image processing software. The other was analyzed using photodiode measurement at four fixed points and a microcontroller interface.

Data analysis resulted in two deformability vs. osmolality curves. Comparing these curves shows excellent overlap in shape with a clear difference in amplitude. Since routine patient curves are always compared to a normal control curve, the amplitue difference is not significant. Our results indicate that either method may be used for clinically-usable interpretation of RBC deformability, but also that additional studies are required in order to compare repeatability for both methods, and to demonstrate that the two curves overlap for a variety of pathologies.

***Index Terms* — Ektacytometer, ektacytometry, deformability, red blood cell, osmolality, diffraction, shear stress**

I. Introduction

The ability of red blood cells to deform is a crucial property allowing the cells to traverse capillaries narrower than their own diameter. Measurement of RBC deformability under shear stress with varying osmolality has permitted diagnosis of several hereditary disorders related to cell membrane or hemoglobin defects such as Spherocytosis, Eliptocytosis, or Stomatocytosis. RBC deformability depends on both cytosolic and membrane parameters and measurement of the osmotic

Manuscript received Jan 15, 2013; revised March 1, 2013.

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doi: 10.12720/jomb.2.1.62-65

deformability profile has been used to monitor patient treatment, aimed at normalization of RBC properties.

Current technology uses optical measurement of RBC population based on a technique called ektacytometry, originally developed in France by Bessis in the 1970s [1]. In this technique a red cell suspension is exposed to laser light and the diffraction pattern created by the laser beam going through the suspension is measured. When RBCs are exposed to a shear stress, they deform, which is in turn reflected by a change in the diffraction pattern of the laser beam. The measurement of the diffraction pattern therefore provides a quantitative measure for the average RBC deformability.

In this study we compared two measurement and calculation methods of average RBC deformability, both derived from the laser diffraction pattern. One method used a four quadrant silicon photodiode; the other used a CCD camera. Two distinct methods are used for the calculation of deformability from measured data.

II. Theoretical Background

Above a certain shear stress level, the flow of RBCs mixed in a viscous solution is laminar and they are oriented in a plane perpendicular to the shear gradient [2]. Their morphology changes with increasing shear from biconcave to tri-axial ellipsoid. Consequently, the diffraction pattern, created by the laser beam traversing the RBCs changes from a circle to an ellipse with its major axis oriented perpendicularly to the direction of flow [3],[4].

In ektacytometry, two main modes are used to characterize RBC deformability: In the first the cells are mixed with a high viscosity solution of normal physiological osmolality (for human plasma 290 mOsm/Kg) then increasing shear stress is applied to the mixture. The diffraction pattern therefore shows deformability as a function of shear stress at physiologic tonicity. In the second mode the shear stress is kept constant, but RBCs are mixed in a medium where osmolality is increased gradually. The diffraction pattern then shows deformability as a function of osmolality. This mode is called “osmotic gradient ektacytometry” or “Osmoscan”, and provides information on membrane stiffness, increased hemoglobin viscosity or reduced surface-area-to-volume ratio [5]. Both approaches have been used with different instrumentation and analysis methods of the diffraction pattern [6], [7], [8], and published reports have compared methods of the deformability measurements under changing shear stress [7], [8], [9], [10]. Both image analysis of the diffraction pattern and simple intensity measurement at discrete spots in the diffraction pattern have been used. Comparison of these different methods of analysis of the diffraction pattern, between different instrumentation, while changing osmolality is more complex as several factors affect the final result. Both the rate of osmolality change and the presence of hemolyzed cells in the population, as well as different shape changes as the result of water uptake or release may affect the diffraction pattern. In order to address this, and allow direct comparison of the two ways to measure the diffraction pattern, we designed a measuring device and method to measure the same cells simultaneously by the two methods. The approach uses the same laser source, same optics, same viscometer, same osmolality measurement sensor, same hydraulic system and gradient maker and the same solutions to measure the same cells at the same time. This eliminates virtually all potential errors that could result from differences between two similar but yet different samples measured on two different instruments.

III. Experimental Apparatus and Methods

Several studies use a camera and image analysis in ektacytometry [2], [11] and there exist two such commercial apparatus: Lorca [8] and RheoScan-D300 [7]. Our design employs a custom apparatus we built, based on a Technicon ektacytometer. Originally, RBC deformability was measured by projecting the diffracted laser beam on a mask with four equidistant holes behind which a four quadrant silicon detector was placed [12]. In order to compare image analysis with the deformation computed using the simpler four quadrant detector, we designed a setup that permitted simultaneous measurement of the same sample by the two methods consisting of a beam splitter (Thorlabs) placed in the path of the post-diffraction laser beam splitting the diffraction image into two identical intensity beams, 90 ° apart. The design used the optical bench and the transparent Couette viscometer of an ektacytometer with a 632.8 nm helium-neon laser source of 2 mW (Lasos, Germany). An ARM microcontroller board (Embedded Artists LPC2148) provided the interface to measure osmolality and temperature and to control the speed of the viscometer and the plumbing needed to create the osmotic gradient. A mixture of whole blood was introduced into the viscometer at a hematocrit of approximately 0.08% in phosphate buffered (pH 7.35) Polyvinylpyrrolidone (PVP, Sigma, St Louis) at 0.2 poise viscosity. The tonicity of the mixture was varied between 50 and 600 mOsmol/kg with NaCl gradient, and cells were exposed to a constant shear stress of 159.3 dyn/cm^2.

One image of the diffraction pattern was projected by the beam splitter on a mask with four holes, behind which was situated the four-quadrant detector. The other diffraction pattern was projected on a translucent screen, and behind it was placed a CCD camera. Data from both measurements were recorded by a computer equipped with an interface that allowed image analysis as well as signal detection of the quadrant detector.

The elongation index (EI), a measure of RBC mean deformability, was calculated from the signals of the four quadrant photodiodes measuring the projected diffraction pattern (Fig.1), using equation (1): where A and B are the signals on the long and short axis of the ellipsoid respectively. The use of two signals on each axis (A1, A2 and B1, B2) provides an average and compensates for slight difference in centering the beam on the mask.

$$EI = ((A1+A2)-(B1+B2)) / (A1+A2+B1+B2) \qquad (1)$$

In the case of the camera, we used an image analysis algorithm (modified PINK library functions [13]) to determine the elongation index by fitting the image, using isointensity curves, to an ellipsoid and determining the length of the short and long axis and EI= L/S as indicated in Fig. 1

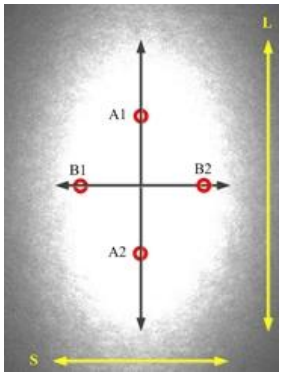

Figure 1. Screen shot of the diffraction image, indicating the position of the holes in the mask for signal detection by the quadrant detector (A1, A2, B1, B2) and the long (L) and short (S) axis of which the length is determined by the isointensity curves of the image analysis algorithm.

Both calculations of EI were performed simultaneously by a custom computer program as indicated in Fig. 2, and plotted as EI versus osmolality.

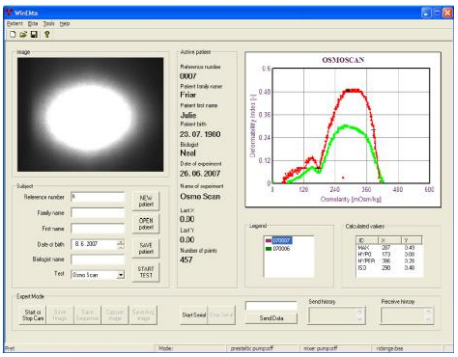

Figure 2. [14] Screen shot of the custom application to compare two different ways to measure EI from the diffraction pattern as described in figure one

The EI determined by the image processing (bottom curve) could be directly compared to the one determined by the quadrant detector (top curve).

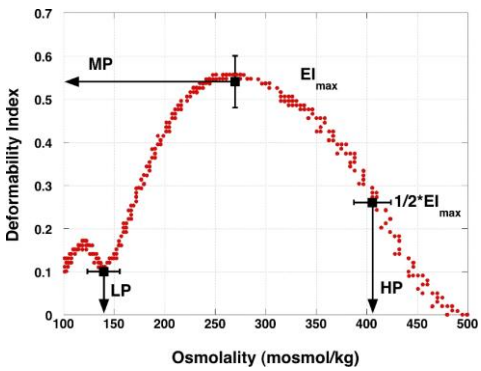

Figure 3. A typical osmotic deformability curve indicating the points used for comparison of the different measurement; the minimum at low osmolality (LP), the maximum deformability (MP), and the hypertonic osmolality (HP) at which EI= ½* EI max.. The use of these parameters simplifies data presentation and interpretation.

In order to facilitate the comparison of the results by these two methods, three indicator points are calculated and marked by the program on each curve, as shown in Fig. 3: Hypotonic point (LP), Maximum (MP) where elongation reaches its maximum (EImax), and Hypertonic point (HP). As indicated by the average and standard deviations, LP, MP and HP vary between different individuals.

IV. Results and Discussion

Comparison of the two curves clearly showed that they closely track each other in shape (Fig. 4). While the absolute value of the deformability index is different at isotonicity, the osmolality of the minimum (LP), the osmolality of the maximum (MP), and the decrease at higher osmolality are very similar using either method. The difference in amplitude can be explained by the fact that the diffraction pattern is not linear and light intensity ratio between the vertical and horizontal holes is higher than the diffracted ellipse's major to minor axes length ratio. Importantly, regardless of the method used, we find a minimum around 150 mOsmol, which has been shown to correlate with the osmolality at which approximately 50% of the RBC have hemolyzed [5], a maximum deformability around 290 mOsmol, and a sharp drop in deformation when the cell loses water under hyper osmolalities. Different samples from control individuals show slightly different results in LP, MP. IP and HP based on the individual characteristics of the donors. These shifts are very similar with either detection method. Similarly it can be expected that differences between control and patient samples show the same trends using either of the two methods. A direct comparison of patient samples on the same machine was not performed, but a study on a family with hereditary Elliptocytosis using either the camera based LORRCA Maxsis (Mechatronics, Hoorn) or the Quadrant diode based ektacytometer showed similar results [15]. Together these results indicate that either detection method identifies properly the change of RBC deformability over a large range of osmolalities and that neither method can be identified as being preferential to the other based on the final result.

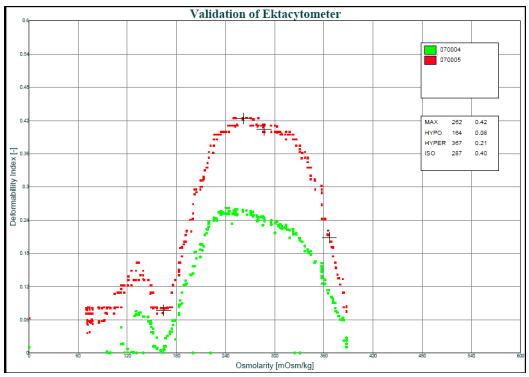

Figure 4. Experimental curve. The top curve was obtained by the four quadrant detector and the bottom one by the camera. The Y axis plots the elongation index against osmolality on the X axis. The dispersion of points at low osmolality is due to a low concentration of RBCs at the beginning of the experiment

V. Conclusions

Osmotic gradient ektacytometry is a very complex but valuable tool for the diagnosis of several (hereditary) RBC disorders. Our experiments lead us to conclude that either image analysis or the use of a quadrant detector result in clinically usable interpretation of RBC deformability.

Regardless of the analysis used, proper care of solution viscosity, temperature, pH, osmolality, and oxygenation is essential. However starting with the same samples our results show that the use of a very simple detection of intensity at discrete points of the diffraction pattern renders similar results as compared to a more complex and sophisticated analysis of the image.

We cannot exclude the possibility that changes in shape to the diffraction pattern under different conditions will add more information. However, in most cases in which laser diffraction is used to routinely measure RBC deformability, either for the diagnosis of hereditary Spherocytosis or Elliptcytosis, or to monitor changes in deformability as the result of treatment, a simple measurement of four points seems fully adequate. The added complexity of image collection in real time, comparison of thousands of images, use of image analysis algorithms, adds complexity and significant cost. The addition of variables such as the choice of various possible regions of the image for interpretation, gain, aperture size, exposure time, saturation, blooming effect and sensor sensitivity degradation seem unwarranted for routine measurements. The four quadrant detector is limited by the necessity for calibration and centering, but the simplicity, constant conditions of use and simple signal processing provide the design of a simple and highly cost effective instrument for routine measurement of RBC deformability. Additional studies are required to compare repeatability for both methods, and to demonstrate that the two curves overlap for a variety of pathologies.

Acknowledgments

This study was performed in the framework of a research contract between CHU de Bicêtre and ESIEE during 2006 and 2008 and partially funded by CHU de Bicêtre.

The authors wish to thank Michel Couprie for his valuable advice in image processing, Thibaut Barati and Olena Tankyevych for image processing software development, Ivan Swiac, Etienne Desjardins and Michel Pellegrin for GUI software development and Leon Heller for ARM microcontroller embedded software development.

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