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Abstract. We present a real-time method to measure the amplitude of thermal fluctuations in biological membranes by means of a new treatment of the defocusing microscopy (DM) optical technique. This approach was also applied to study the deformation of human erythrocytes to its echinocyte structure. This was carried out by making three-dimensional shape reconstructions of the cell and measuring the thermal fluctuations of its membrane, as the cell is exposed to the anti-inflammatory drug naproxen and as it recovers its original shape, when it is subsequently cleansed of the drug. The results showed biomechanical changes in the membrane even at low naproxen concentration (0.2 mM). Also, we found that when the cell recovered its original shape, the membrane properties were different compared to the nondrugged initial erythrocyte, indicating that the drug administration-recovery process is not completely reversible. [DOI: 10.1117/1.JBO.17.10.106013]

Keywords: red blood cells; erythrocyte; membrane dynamics; bright field microscopy.

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1 Introduction

Human erythrocytes red blood cells (RBCs) are cells with a flattened disc shape (discocytes) formed by a lipid bilayer attached to a two-dimensional cytoskeleton. The lipid bilayer is mainly composed of proteins and phospholipids, which are asymmetrically distributed.[1] It is well known that various external agents, i.e., drugs, or changes to the pH of the medium and temperature, can alter the structure of erythrocytes, leading to a large number of anomalous forms.[2–4] Among these, the best known are echinocytes, characterized by the presence of protuberances in the membrane,[3] and stomatocytes, typified by a cup-like shape.[4]

RBCs deformation induced by different drugs is particularly important in the study of several medical diseases.[5] According to Sheetz and Singer's bilayer-couple hypothesis,[6] the asymmetric distribution of phospholipids allows the membrane to respond differently to different drugs. This is explained by the fact that phosphatidylserine,[7] located in the inner leaflet of the bilayer, is negatively charged. Therefore, cationic drugs will be attracted and will bind primarily to this layer of the membrane, which will expand, generating a stomatocyte-type deformation. On the other hand, anionic drugs will be repelled by phosphatidylserine molecules and then interleave in the outer layer of the membrane, expanding it and inducing a discocyte to an echinocyte transformation. These deformations are produced at a constant volume and the erythrocyte can recover its original form through the removal of the drug from the medium.[3,4,8,9]

Drugs effects in RBCs have been studied principally through Electron Microscopy. These observations have led to a significant understanding of the morphologies with a very high concordance with theoretical models.[10,11] However, these experiments have studied shape changes in fixed and static conditions only. In order to achieve a more thorough understanding of the action of an external agent on the erythrocyte membrane, it is important to know the alterations of membrane mechanical properties and their dynamics. Therefore, *in vivo*, noninvasive and dynamic RBC studies have been the subject of significant attention.

A well known method for performing these studies is based on the analysis of membrane thermal fluctuations (flickering)[12,13] with optical techniques. According to theoretical models,[12–16] thermal fluctuations depend on parameters of membrane mechanics, such as the elastic bending modulus and the shear modulus. Examples of techniques based on measuring thermal fluctuations are reflection laser probing,[17] point dark-field microscopy,[18] Hilbert phase microscopy,[19] digital holographic microscopy,[20] and defocusing microscopy (DM)[21] (for a detailed description of these and other techniques see Ref. 22). In the case of discocytes, much of the research has been about the relationship between biomechanical properties and thermal fluctuations, but only recently have these been studied related to the anomalous forms caused by external agents.[23,24]

In this work, we analyzed the amplitude of thermal fluctuations of erythrocyte membranes during the transformation from discocyte to echinocyte. In particular, we induced this process using the antiinflammatory drug naproxen. This drug has been widely investigated in terms of its pharmacological action, but less is known about its effects on cell membranes and particularly in human erythrocytes.[25] These fluctuations are measured during the deformation process, as the drug concentration rises,

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and during the recovery process, where the echinocyte is reverted to its original morphology due to a decrease in the concentration of the drug in the medium.[3] The analysis of amplitude of fluctuations after RBCs recover their original shape provides new information about the reversibility of the process. For a complete description of the deformation-recovery cycle, we additionally analyze the erythrocyte shape and volume through three dimensional reconstructions.

For this purpose, DM was used.[21] DM is an optical technique based on the visualization and analysis of contrast images of transparent objects (e.g., biological organisms) seen out of focus, using a bright field microscope. This technique has proven to be very powerful with a large number of applications, such as the characterization of fluctuations in living cells, mainly macrophages, allowing to measure their optical and mechanical properties,[26,27] as well as time periods involved with phagocytosis events.[28] In the study of human erythrocytes, DM has also been used to make three-dimensional reconstructions,[29] and to measure mechanical parameters of membranes,[30] through image analysis in a wide range of focal positions.

We have improved the DM technique by means of a large defocus approximation on the theoretical model. This approach made it possible to obtain average values for the amplitude of thermal fluctuation by measuring contrast images fluctuations in a single defocus position. This leads us to obtain a simple, accurate and real-time method to detect alterations in a single human erythrocyte.

2 Method Description

2.1 *Three-Dimensional Shape Reconstruction*

DM is a technique based on the fact that transparent objects (phase object) become visible in a bright field microscope when defocused. The image contrast is defined as $C(\vec{\rho}) = [I(\vec{\rho}) - I_0]/I_0$, where $I(\vec{\rho})$ is the intensity of the light going through the object and I_0 is the intensity of the background. The two-dimensional vector $\vec{\rho}$ denotes the transverse position in the object mid-plane. We defined the defocus Δf as the focal position in relation to the mid-plane of the RBC (where there is a minimum contrast). The contrast of a defocused image is given by Ref. 27:

$$C(\vec{\rho}) = \frac{\Delta n \Delta f}{n} \nabla^2 h(\vec{\rho}), \tag{1}$$

where Δn is the difference between the refractive index of the object n and that of the medium n_0, $h(\vec{\rho})$ is the total thickness function of the object and $\nabla^2 h(\vec{\rho})$ is its curvature.

This equation is valid only for small defocus and small wave vectors $\vec{q}$, more precisely for $q^2 \Delta f / 2k \ll 1$. If we consider the most curved part of a typical RBC, $q \sim 2$ rad/μm and $k \asymp 12.9\ \mu m^{-1}$ for red light ($\lambda = 0.65\ \mu m^{-1}$), therefore $\Delta f \ll 2k/q^2 \asymp 6.5\ \mu m$.

Solving Eq. (1) for $h(\vec{\rho})$ it is possible to obtain three-dimensional reconstructions from which its volume can be directly calculated.

$$h(\vec{\rho}) = \frac{n}{\Delta n \Delta f} \mathcal{F}^{-1} \left[\frac{\mathcal{F}[C(\vec{\rho})]}{q^2} \right], \tag{2}$$

where $\mathcal{F}$ is the transverse Fourier transform. As the nonuniformity of the background acquires importance when carrying out the Fourier transform, it is convenient to subtract two images in two different defocus positions.[29] In this case Eq. (2) takes the form

$$h(\vec{\rho}) = \frac{n}{\Delta n (\Delta f_1 - \Delta f_2)} \mathcal{F}^{-1} \left[\frac{\mathcal{F}[C_1(\vec{\rho}) - C_2(\vec{\rho})]}{q^2} \right], \tag{3}$$

where C_1 and C_2 are the image constrast on the defocus Δf_1 and Δf_2, respectively.

2.2 *Measurements of Amplitude of Thermal Fluctuations*

In accordance with the mathematical model for the DM procedure[21] a propagation of the angular spectrum is carried out in the paraxial approximation of the light that crosses a phase object and the optical system of a microscope. The phase object is represented as two flat interfaces, 1 and 2, situated in the positions P_1 and P_2 in relation to an arbitrary plane. The interfaces 1 and 2 are characterized by the thickness functions $h_1(\vec{\rho})$ and $h_2(\vec{\rho})$, respectively. If it is considered that the interfaces of the object fluctuate in time causing contrast fluctuations of the defocused image, i.e., the thickness function is time-dependent $h_{1,2}(\vec{\rho}) \to h_{1,2}(\vec{\rho}, t)$, then an expression for the mean square fluctuation for any defocus position can be obtained[30]

$$\langle \Delta C^2(z_f) \rangle = \frac{(\Delta n k_0)^2}{\pi} \int_0^{q_m} q \mathrm{d}q \left[|u_1(q)|^2 \sin^2 \left(\frac{z_f - p_1}{2k} q^2 \right) + |u_2(q)|^2 \sin^2 \left(\frac{z_f - p_2}{2k} q^2 \right) \right], \tag{4}$$

where z_f is the focus position relative to an arbitrary plane and k_0 and k are the wave numbers in a vacuum and in the medium, respectively. $u_1(q)$ and $u_2(q)$ are the fluctuation spectrums of each interface, with regard to its equilibrium state as $\tilde{u}_{1,2}(\vec{\rho}, t) = h_{1,2}(\vec{\rho}, t) - \langle h_{1,2}(\vec{\rho}, t) \rangle$, where $\langle \cdot \rangle$ denotes temporal average.

To obtain Eq. (4), the diffracted electric field was expanded to first order (valid for $\Delta n k_0 u_{1,2} \ll 1$). The maximum wave number collected q_m is given by the experimental conditions, where $q_m = k\mathrm{NA}$, with NA being the numerical aperture of the objective lens.

By varying the focus position and adjusting Eq. (4), it is possible to quantify the amplitude of fluctuations of each interface separately.[30] As the aim of this study is to measure amplitude of thermal fluctuations quickly, it is convenient to carry out measurements from one defocus position only. In order to achieve this, the asymptotic limit $z_{f \to \infty}$ is considered in Eq. (4),

$$\langle \Delta C^2_{z_{f \to \infty}} \rangle = \frac{(\Delta n k_0)^2}{2\pi} \int_0^{q_m} \mathrm{d}q q [|u_1(\vec{q})|^2 + |u_2(\vec{q})|^2] = 2(\Delta n k_0)^2 \langle u^2 \rangle, \tag{5}$$

where it was assumed that the average amplitude of fluctuations is equal in both interfaces $\langle u^2 \rangle = \langle u_1^2 \rangle \approx \langle u_2^2 \rangle$. Equation (5) enables us to obtain average values for amplitude of thermal fluctuations of biological membranes, measuring the mean square fluctuation of contrast. This can be carried out quickly, through measurements from only one highly defocused position. In general, for the RBC membrane, the asymptotic

condition of Eq. (5) is completely satisfied for $z_f - (p_1 + p_2)/2 > 3\ \mu m$, as shown in Fig. 1. In the asymptotic limit the measurements are independent of the defocus positions. This is an advantage of this method, because small focus drifts, due to thermal or mechanical factors, do not affect the result of the measured fluctuation. Additionally, it does not require high accuracy in the setting of the defocused position, in contrast with the method presented in Ref. 30.

3 Experimental Details

3.1 *Experimental Setup*

The experimental setup consists of a Nikon inverted optical microscope, Eclipse Ti-U. The light source is a halogen lamp with a transmission filter centered on 650 ± 5 nm (Andover Corporation) to avoid damaging the erythrocytes. The microscope uses the Köhler illumination system and a Nikon Plan Apochromatic oil immersion objective (100X) with numerical aperture NA = 1.4, which is mounted on a Mad City Labs C-focus system for automatic focus drift correction and nanometric focusing with the Nano-F100 lens nanopositioner (precision of 5 nm), see Fig. 2(a). Visualization is carried out by means of a Retiga EXI Fast 12-Bit CCD camera.

Computer software was developed to integrate the nanopositioner control and the CCD camera. This software enables data acquisition and processing to make three-dimensional reconstructions and measurements of amplitude of fluctuations in real time.

3.2 *Sample Preparation*

RBCs were obtained from a healthy donor under no pharmacological treatment. The cells were centrifuged (1000 rpm for 10 m) and the supernatant was discarded and replaced by the same volume of a solution of Phosphate Buffer Saline (PBS) 1X pH 7.4 with 1 mg/mL of Bovine Serum Albumine (BSA). The whole process was repeated three times. RBC solution was prepared diluting the washed blood 20 times in a solution of PBS and BSA. Sodium naproxen solution was prepared in the same preparation of PBS and BSA, at different concentrations.

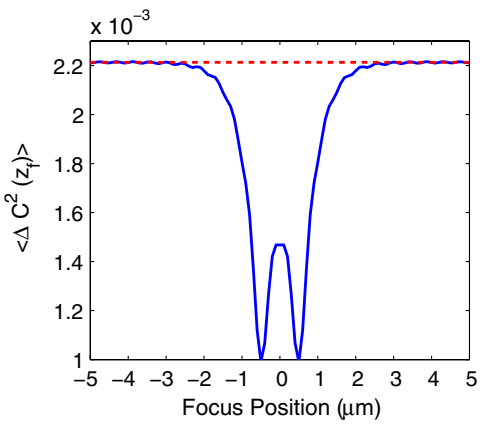

Fig. 1 Solid (blue) line simulate Eq. (4) with $(\Delta n k_0)^2/\pi = 1$, $p_1 = 0.5\ \mu m$ and $p_2 = -0.5\ \mu m$. The fluctuation spectrum was considered as $|u_{1,2}(q)|^2 = 1/(aq^4 + c)$ (typical expression in membrane fluctuation models),[12] with $a = 10$ and $c = 10000$ (typical values for RBC membranes[30]). The dashed (red) line represents the asymptotic limit ($z_f \to \infty$) calculated in Eq. (5). In this figure, the defocus position is considered in relation to the mid-plane of the object $\Delta f \equiv z_f - (p_1 + p_2)/2$. For this consideration the asymptotic condition is fulfilled for $\Delta f > \pm 3\ \mu m$.

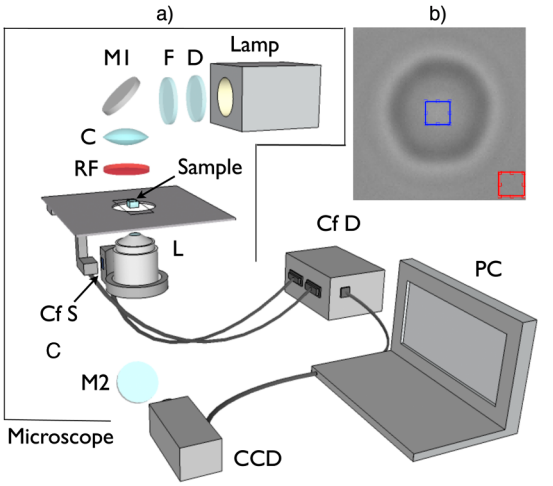

Fig. 2 (a) Experimental Setup. *D* is a diffusor, *F* is a neutral color balance filter, M1 is a mirror. RF is a red transmission filter, *C* is a condenser lens. Cf S and Cf D are the nanopositioner system and its control driver. *L* is a microscope objective lens. (b) RBC 4 μm defocused image. The central (blue) square is the fluctuation measuring region and the right bottom (red) square is the background reference region.

3.3 *Experimental Procedure*

In order to carry out the analysis, 1.7 mL of the RBCs solution was placed in an acrylic cuvette, and visualized at the microscope. After that, a morphologically normal erythrocyte was selected with the developed software. The concentration of naproxen in the solution was gradually varied between 0 to 1.4 mM in the reconstruction experiments and between $0 - 1$ mM for the fluctuation experiments, in steps of 0.2 mM. Each incremental step of the drug was done by extracting 200 μL of the RBCs solution and adding 200 μL of naproxen solution, taking care to not disturb the selected RBC. Once the erythrocyte reached a maximum drugged stage, the concentration of naproxen of the sample was diluted. This was done by removing 200 μL of the RBC-naproxen solution and adding 200 μL of PBS plus BSA repetitively until a drug concentration of approximately zero mM was reached. We performed fluctuation measurement and three-dimensional reconstructions after each drug concentration change. Before we performed these measurements, we waited 1 min in order to stabilize the RBC shape.

3.4 *Data Acquisition and Analysis*

The image contrast in Eqs. (1) and (4) is defined with regard to intensity. Therefore the CCD camera must be calibrated to recognize the equivalence between the level of gray in the image observed and the intensity on the image plane (for further details see Ref. 21). Considering this correction, the contrast of an image can be defined as

$$C = \frac{N - N_0}{N_0}, \tag{6}$$

where N and N_0 are the gray levels of the image and the background, respectively. The contrast fluctuation is given for

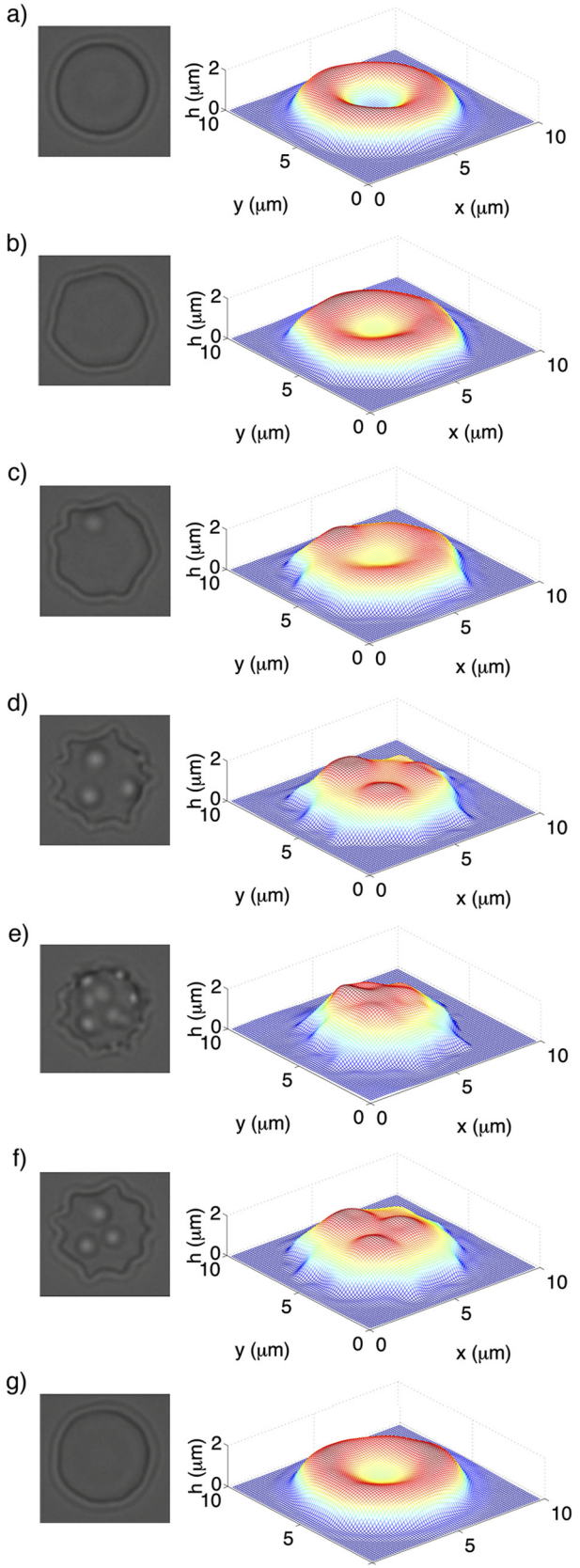

Fig. 3 Defocused images $\Delta f = 1\ \mu m$ and its three dimensional reconstruction. (a) RBC under normal conditions. (b)–(e) Administration of naproxen. (f)–(g) Recovery process. Naproxen concentrations (a) 0 mM, (b) 0.4 mM, (c) 0.8 mM, (d) 1.0 mM, (e) 1.4 mM, (f) 0.7 mM, (g) 0.1 mM. The RBC mean volume considering the reconstruction from a) to g) was $105 \pm 5\ \mu m^3$.

$\Delta C = C - \langle C \rangle$. Then, the mean square contrast fluctuation is $\langle \Delta C^2 \rangle = (\langle N^2 \rangle - \langle N \rangle^2)/N_0^2$.

To make three-dimensional reconstructions two images were captured in the defocus positions 1 and $-1\ \mu m$, for each drug concentration. These focus values fulfill the small defocusing approximation of Eq. (1). Using Eq. (6) the contrast of the images was obtained and three-dimensional shape reconstruction was directly calculated from Eq. (3).

To obtain the square amplitude of fluctuations $\langle u^2 \rangle$, we placed the RBC at $\Delta f = 4\ \mu m$, value that fulfills the approximation of $z_f \to \infty$ of Eq. (5) and does not generate diffraction rings in the central zone of the erythrocyte. By measuring $\langle \Delta C^2 \rangle$, the square amplitude of fluctuations were directly obtained from Eq. (5). The measurements are averaged temporally and spatially by taking 10-second videos, recorded at 55 fps, in a central area of the selected erythrocyte of: $1.2 \times 1.2\ \mu m$ (approximately flat membrane), see Fig. 2(b).

4 Results and Discussion

Gradual deformations of the erythrocytes were observed in relation to the naproxen concentration in the solution. The stage in which the drug enters the membrane is shown in Fig. 3(a) to 3(e), and the recovery stage, where the drug leaves the membrane, is shown in Fig. 3(f) to 3(g). Although shape deformation seems to be instantaneously, we waited 1 min after measurement in order to be sure of the stability of the shape. Three-dimensional reconstructions made it possible to clearly observe the changes in shape and estimate the volume in each stage, showing that the latter remains approximately constant at $105 \pm 5\ \mu m^3$, in accordance with the hypothesis of Singer and Shetlz.[6]

In addition, we have determined the average amplitude of fluctuations $u_{\mathrm{rms}} = \sqrt{\langle u^2 \rangle}$ of the erythrocyte membrane as a function of drug concentration (see Fig. 4). Specifically, Fig. 4(a) shows the drug administration process and Fig. 4(b) shows the recovery process; both are for the same single RBC, although this behavior was observed in every analyzed cell. The value of u_{rms} obtained for initially nondrugged erythrocytes was consistent with those measured by other techniques, in the range between 10 to 30 nm.[13,20,30] The errors bar of the plotted data is an estimation of the method precision and represent statistical errors due to the fact of working with 10-second videos. If we take longer videos, the errors bars will decrease but the technique will be slower. These errors were calculated by the u_{rms} standard deviation obtained from 10 consecutively recorded videos for each drug concentrations.

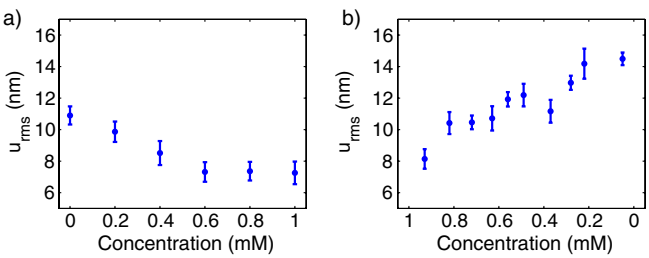

Fig. 4 Average amplitude of thermal fluctuations u_{rms} for the processes of drug administration, (a), and recovery (b). The points correspond to the fluctuations obtained using Eq. (5). The error bars correspond to the standard deviation of 10 measurements carried out consecutively for each concentration.

The relaxation time of thermal fluctuations, for purely bending modes, varies[12] with the inverse of the cube of the wave number q^3, while $\langle u^2 \rangle$ varies with q^4. Therefore, the fastest modes have the smallest amplitudes. Consequently, the measured amplitude of thermal fluctuations depends on the camera frame rate. For typical RBCs and camera frame rates above 300 fps, the amplitude of fluctuations remain constant. For a camera frame rate of 55 fps, the u_{rms} values of Fig. 4(a). and 4(b). are under-estimated[31] by about 12%.

The process in which the drug enters the membrane produces a decrease in the amplitude of thermal fluctuations, Fig. 4(a). In the recovery process, when the drug leaves the membrane, the amplitude of fluctuations unexpectedly increases to a value higher than the initial value, Fig. 4(b). Amplitude of thermal fluctuations decreases in the echinocyte state is a result observed in Ref. 24. However, we observed this decrease even for low concentrations of the drug: 0.2 mM [lower concentration than showed in Fig. 3(b)], when changes in morphology are barely visible. As the amplitude of thermal fluctuations of RBC membrane are inversely proportional to the bending elastic modulus of the membrane,[12,15] the behavior observed Fig. 4 can be explained by the fact that the naproxen molecules increase the rigidity of the membrane as they intercalate between the phospholipids. For the reverse process, the rigidity decreases until it reaches a value lower than the initial value. This can be due to the outgoing drug producing a higher degree of disorder in the distribution of the phospholipids on the membrane.

5 Conclusions

This study presents a new approach for the DM technique that enables real time measurements for amplitude of thermal fluctuations of erythrocyte membranes by image contrast analysis in a highly defocused position. The technique was applied to study the RBCs morphological changes, specifically from discocyte to echinocyte condition. During this process we observed changes in the morphology but not in the volume of the cell. These results are consistent with those observed in Ref. 25 when the concentration of drug increases the RBCs change their shape and the echinocytes transformation was observed.

The negative naproxen charge allows it to intercalate in the outer layer of the RBC membrane, modifyng its organization and therefore its mechanical properties. For this reason, when the erythrocyte acquires an echinocyte shape, it was possible to observe a decrease in amplitude of thermal fluctuations starting at low concentrations of naproxen (0.2 mM).

The interesting thing about these results is that in the recovery stage, even when the original morphology was ostensibly recovered, the biomechanical parameters, characterized by the thermal fluctuations, did not return to their original state. This leads us to conclude that the membrane does not recover its original properties, presumably because the phospholipids organization becomes more disrupted. This last conclusion corroborates the work done in Ref. 9, where an increase of choline-cointaining phospholipid concentration of a shape-recovered RBC after a Mg^{2+} depletion was observed, compared with the control, suggesting a change in the membrane lipidic distribution.

DM, as it was applied, provides a high degree of utility in the study of the action of drugs and changes in external factors on cells, in addition to being a useful tool for identifying pathologies that affect biological membranes. This technique has the following advantages: it is easy to apply in the context of an experiment; it allows results to be obtained rapidly; and, finally, it is a noninvasive technique for a single cell study. As a continuation of this work, we will investigate the modifications in the biomechanics of erythrocytes caused by various drugs, applied in low concentrations of <0.1 mM so as to be within therapeutic limits, as well as the effects due to the temperature control at which these processes occur.[32]

Acknowledgments

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References

1. S. J. Singer and G. L. Nicolson, "The fluid mosaic model of the structure of cell membranes," *Science* **175**(4023), 720–731 (1972).
2. W. D. Stein and F. Bonner, *Cell Shape : Determinants, Regulation, and Regulatory Role*, Academic Press, San Diego (1989).
3. G. Brecher and M. Bessis, "Present status of spiculed red cells and their relationship to the discocyte-echinocyte transformation: a critical review," *Blood* **40**(3), 333–344 (1972).
4. B. Deuticke, "Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment," *Biochim. Biophys. Acta.* **163**(4), 494–500 (1968).
5. H. Dumez et al., "Human red blood cells: rheological aspects, uptake, and release of cytotoxic drugs," *Crit. Rev. Clin. Lab. Sci.* **41**(2), 159–188 (2004).
6. M. P. Sheetz and S. J. Singer, "Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocytes interactions," *Proc. Natl. Acad. Sci. U. S. A.* **71**(11), 4457–4461 (1974).
7. J. M. Boon and B. D. Smith, "Chemical control of phospholipid distribution across bilayer membranes," *Med. Res. Rev.* **22**(3), 251–281 (2002).
8. B. Isomaa, H. Hagerstrand, and G. Paatero, "Shape transformations induced by amphiphiles in erythrocytes," *Biochim. Biophys. Acta.* **899**(1), 93–103 (1987).
9. G. Pages, T. W. Yau, and P. W. Kuchel, "Erythrocytes-shape reversion from echinocytes to discocytes: kinetic via fast-measurement NMR diffusion-diffraction," *Magn. Reson. Med.* **64**(3), 645–652 (2010) .
10. G. Lim, M. Wortis, and R. Mukhopadhyay, "Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer-couple hypothesis from membrane mechanics," *Proc. Natl. Acad. Sci.* **99**(26), 16766–16769 (2002).
11. A. Iglic, "A possible mechanism determining the stability of spiculated red blood cells," *J. Biomech.* **30**(1), 35–40 (1997).
12. F. Brochard and J. F. Lennon, "Frequency spectrum of the flicker phenomenon in erythrocytes," *J. Phys.-Paris* **36**(11), 1035–1047 (1975).
13. H. Strey, M. Peterson, and E. Sackmann, "Measurement of erythrocyte membrane elasticity by flicker eigenmode decomposition," *Biophys. J.* **69**(2), 478–488 (1995).
14. N. S. Gov and S. A. Safran, "Red blood cell membrane fluctuations and shape controlled by atp-induced cytoskeletal defects," *Biophys. J.* **88**(3), 1859–1874 (2005).
15. N. S. Gov, "Active elastic network: cytoskeleton of the red blood cell," *Phys. Rev. E. Stat. Nonlin. Soft Matter Phys.* **75**(1), 011921 (2007).
16. T. Auth, S. A. Safran, and N. S. Gov, "Fluctuations of coupled fluid and solid membranes with application to red blood cells," *Phys. Rev. E. Stat. Nonlin. Soft. Matter. Phys.* **76**(5), 051910 (2007).
17. V. L. Kononenko and J. K. Shimkus, "Coherent versus noncoherent optical probing of dynamic shape fluctuations in red blood cells," *Proc. SPIE* **3732**, 326–335 (1999).
18. S. Tuvia et al., "Mechanical fluctuations of the membrane skeleton are dependent on F-actin ATPase in human erythrocytes," *J. Cell Biol.* **141**(7), 15511561 (1998).
19. G. Popescu et al., "Erythrocyte structure and dynamics quantified by Hilbert phase microscopy," *J. Biomed. Opt.* **10**(6), 060503 (2005).

20. B. Rappaz et al., "Spatial analysis of erythrocyte membrane fluctuations by digital holographic microscopy," *Blood Cells Mol. Dis.* **42**(3), 228–232 (2009).
21. U. Agero et al., "Defocusing microscopy," *Microsc. Res. Tech.* **65**(3), 159–165 (2004).
22. V. L. Kononenko, "Characterization of red blood cells' rheological and physiological state using optical flicker spectroscopy," in *Advanced Optical Flow Cytometry: Methods and Disease Diagnoses*, V. V. Tuchin, Ed., pp. 155–210, Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim, Germany (2011).
23. D. Szekely, T. W. Yau, and P. W. Kuchel, "Human erythrocyte flickering: temperature, ATP concentration, water transport, and cell aging, plus a computer simulation," *Eur. Biophys. J.* **38** (7), 923–939 (2009).
24. Y. Park et al., "Measurement of red blood cell mechanics during morphological changes," *Proc. Natl. Acad. Sci. U. S. A.* **107**(15), 6731–6736 (2010).
25. M. Manrique-Moreno et al., "Effects of the nonsteroidal anti-inflammatory drug naproxen on human erythrocytes and on cell membrane molecular models," *Biophys. Chem.* **147**(1–2), 53–58 (2010).
26. U. Agero et al., "Cell surface fluctuations studied with defocusing microscopy," *Phys. Rev. E. Stat. Nonlin. Soft. Matter. Phys.* **67**(5), 051904 (2003).
27. J. Coelho Neto et al., "Measuring optical and mechanical properties of a living cell with defocusing microscopy," *Biophys. J.* **91**(3), 1108–1115 (2006).
28. J. Coelho Neto et al., "Real-time measurements of membrane surface dynamics on macrophages and the phagocytosis of leishmania parasites," *Exp. Cell Res.* **303**(2), 207–217 (2005).
29. L. G. Mesquita, U. Agero, and O. N. Mesquita, "Defocusing microscopy: an approach for red blood cell optics," *Appl. Phys. Lett.* **88**(13), 133901 (2006).
30. G. Glionna et al., "Tomography of fluctuating biological interfaces using defocusing microscopy," *Appl. Phys. Lett.* **94**(19), 193701 (2009).
31. Private communication with Paula M. Roma, (July 2012).
32. M. J. Gallardo, S. Etcheverry, and C. Saavedra, "Propranolol increase thermal fluctuations in red blood cells membranes at therapeutic concentrations," (in preparation).