Available online at www.sciencedirect.com
ScienceDirect
Bioelectrochemistry 73 (2008) 92–96
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Application of digital holographic microscopy to investigate the sedimentation of intact red blood cells and their interaction with artificial surfaces

Ingolf Bernhardt [a,*], Lyubomira Ivanova [a], Patrik Langehanenberg [b], Bjoern Kemper [b], Gert von Bally [b]

[a] Laboratory of Biophysics, Faculty of Natural and Technical Sciences III, Saarland University, P.O.Box 151150, 66041 Saarbruecken, Germany
[b] Centre of Biomedical Optics and Photonics, University of Muenster, Robert-Koch-Strasse 45, 48129 Muenster, Germany

Received 10 July 2007; received in revised form 12 December 2007; accepted 13 December 2007
Available online 23 December 2007

Abstract

Red blood cells are able to undergo shape change from the "normal" discocyte to either echinocytes or stomatocytes depending on a large variety of membrane and cytoplasmic parameters. Such shape changes can be relatively fast (within seconds) during the sedimentation of the cells in suspension or after the cells are getting in contact with artificial surfaces. High resolution digital holographic microscopy has been applied to study these processes. This method represents a new set-up allowing a contact-less and marker-free quantitative phase-contrast imaging of living cells under conventional laboratory conditions. With the applied technique we were able to detect and analyse fast shape changes of red blood cells.

Keywords: Red blood cell; Erythrocyte; Shape change; Digital holographic microscopy; Cell surface interaction; Artificial surface

1. Introduction

Red blood cells (RBCs) from various mammalian species can have different resting shapes. The "normal" discocyte is able to transform to either echinocytes or stomatocytes depending on a large variety of membrane and cytoplasmic parameters. Our current understanding of the mechanism of the shape change is based on the "bilayer" couple mechanism developed by Sheets and Singer [1]. It assumes that an expansion of the inner or outer leaflet of the membrane leads to changes of the bilayer-based curvature since the two leaflets cannot separate from each other due to their coupling by hydrophobic interactions. It has been demonstrated that an expansion of one of the leaflets can occur after the transversal redistribution of the membrane phospholipids or after an insertion of amphiphilic compounds into the membrane. However, such a structural change of the membrane bilayer is a relatively slow process and occurs within minutes only.

On the other hand, a conformational change of integral membrane proteins could also lead to an expansion of one leaflet of the membrane bilayer relative to the other one and in turn results in a shape change. Such a possibility was speculated by Gimsa and Ried [2] interpreting the echinocytogenic effect of stilbene disulfonates (e.g. DIDS) and pyrimido-pyrimidines (e.g. dipyridamole), which are known inhibitors of the anion transport protein (band 3) in terms of a ligand-induced "recruitment" of band 3 protein to an outward-oriented conformation [3]. Recently we were able to demonstrate experimentally that the RBC shape depends on the conformation of the band 3 proteins, i.e. is affected by the change in the cross-sectional area of each band 3 monomer in the outer leaflet of the membrane. These investigations have been carried out with glutaraldehyde-fixed RBCs applying the AFM technique [4].

In comparison to the above mentioned process of lipid redistribution, the protein-based expansion of one of the membrane

* Corresponding author. Tel.: +49 681 3026689; fax: +49 681 3026690.
E-mail address: i.bernhardt@mx.uni-saarland.de (I. Bernhardt).

1567-5394/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2007.12.001

leaflets is relatively fast (within seconds). In addition, a very fast shape change can be observed when RBCs get in contact with a glass or artificial surface. To study the process of such rapid shape changes of living (non-fixed) RBCs we applied the high resolution digital holographic microscopy [5,6]. Using this method it is possible to perform time-resolved quantitative microscopic measurements of changes in the lateral and axial shape under different experimental conditions.

2. Materials and methods

2.1. Red blood cell preparation

Freshly drawn blood from healthy human donors was used for the experiments. The RBCs were isolated by centrifugation (2000 ×*g*, 5 min) at room temperature and the plasma and buffy coat removed by aspiration. The cells were washed three times with a physiological NaCl solution containing 145 mM NaCl, 10 mM glucose, 10 mM 4-(2-hydoxyethyl)-1-piperazineethane-sulfonic acid/NaOH (HEPES/NaOH), pH 7.4 at room temperature. The cells were re-suspended at a haematocrit of 0.01% on cover slips and immediately used for digital holographic microscopy measurements. Cover slips were coated with 0.01% poly-l-lysine (Sigma). Poly-l-lysine is widely used as an adhesive substance for cells in microscopy. In some experiments the cell shape was transferred to echinocytes by adding 10 μM 4,4″-diisothiocyanatostilbene-2,2″-disulfonic acid (DIDS) obtained from Sigma to the solution before transferring the cells on the cover slips.

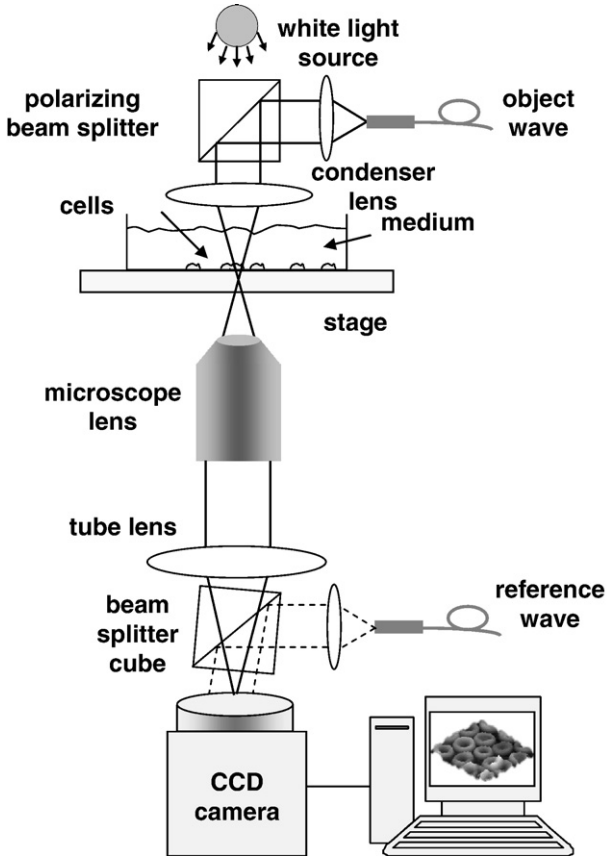

Fig. 1. Schematic setup of an inverse off-axis digital holographic microscopy system with transmitting light illumination of the sample.

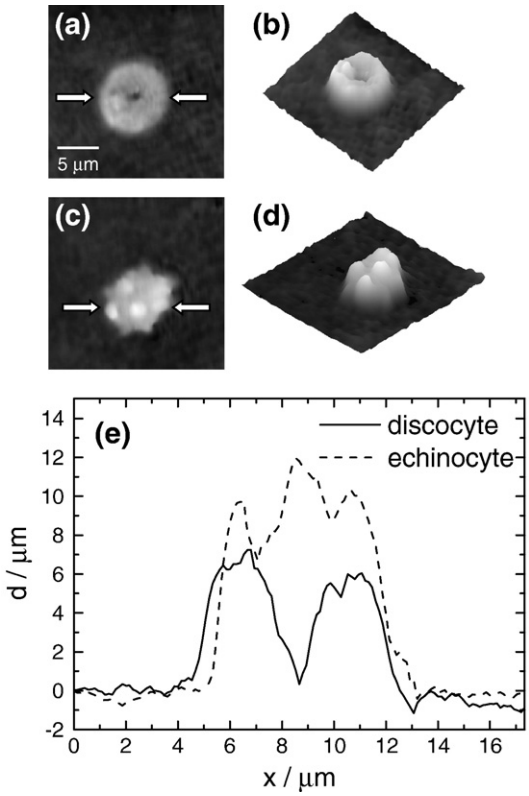

Fig. 2. Quantitative holographic phase contrast images of RBCs in physiological solution. The images (a), (b) indicate a "normal" discocyte, the images (c), (d) indicate an echinocyte; (shape change after the addition of 10 μM DIDS). (a), (c): grey level representations of the holographic phase contrast images; (b), (d): pseudo 3D plots of 2a and 2c; (e): cross-sections along the line marked with arrows in (a) and (c).

2.2. Setup for digital holographic microscopy

Holographic interferometric metrology is an established technique for technical non-destructive testing. For hologram recording the sample is illuminated by coherent laser light. The light that is reflected or transmitted by the sample is superimposed with a coherent reference wave. The resulting interferogram is recorded using a storage medium (e.g., photographic plate for classical holographic recording). In this way, holography permits the recording and reconstruction of wave fields in both, amplitude and phase. Digital holography is based on the classic holographic principle, with the difference that the hologram recording is performed by a digital image sensor, e.g. a CCD or CMOS camera. The subsequent reconstruction of the holographic images is carried out numerically with a computer. In connection with microscopy, digital holography provides contact-less, marker-free quantitative phase-contrast imaging that is suitable for modular integration into common microscopes [7,8]. The digital holographic feature of (subsequent) numerical focus adjustment opens up applications for multi-focus imaging. Thus, quantitative phase contrast metrology for high resolution investigation of reflective surfaces (sample dependent up to <5 nm in axial direction) and marker-free analysis of living cells [5,6,9] is enabled.

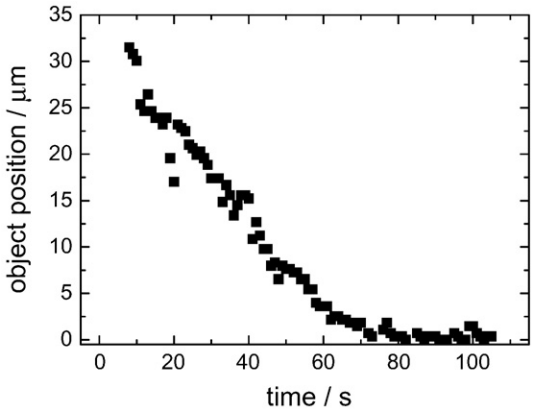

Fig. 3. Quantitative analysis of the sedimentation of a single human red blood cell in dependence on time. The z-displacement of a cell relatively to the z-position at the beginning of the experiment is obtained from subsequent digital holographic focus correction data.

Fig. 1 illustrates the applied setup of a digital holographic phase contrast microscopy system for the investigation of living cells [8]. The light of a frequency doubled Nd:YAG laser ($\lambda = 532$ nm) is divided into an object illumination wave (object wave) and a reference wave. Single mode optical fibres are applied for a variable light guidance. The illumination with laser light is performed by coupling the object wave into the microscope condenser. Thus, an optimized (Koehler-like) illumination of the sample is achieved. The reference wave is guided directly to an interferometric unit that is adapted to one of the microscope's camera ports. Holographic off-axis geometry is generated by a beam splitter that affects a slight tilt of the reference wave front against the wave front of the object wave. The interferogram that is formed by the superposition of object wave and reference wave is recorded by a CCD camera and transferred via an IEEE1394 ("FireWire") interface to an image processing system for the reconstruction and the evaluation of the digitised holograms. The magnification of the microscope lens (Zeiss Acroplan 63×, NA = 0.75) is chosen in such a way that the smallest imaged structures, given by the restriction of the Abbe criterion, are oversampled by the image recording device. In this way the maximum diffraction limited resolution of the optical imaging system is not decreased by the numerical reconstruction algorithm [5]. The hologram capturing time depends on the applied imaging device (here the CCD camera, typically: ms-range).

2.3. Reconstruction of digital holograms and evaluation of holographic phase contrast images

The reconstruction of the digitally recorded holograms is performed numerically. A spatial phase shifting based reconstruction method has been developed that is particularly suitable to digital holographic microscopy [5,6]. In connection with autofocus algorithms [10], automated multi-focus imaging during time-lapse measurements as well as automated quantitative focus tracking is enabled which is of main interest in this contribution.

With information about the integral refractive index of the specimen the digital holographically obtained quantitative phase

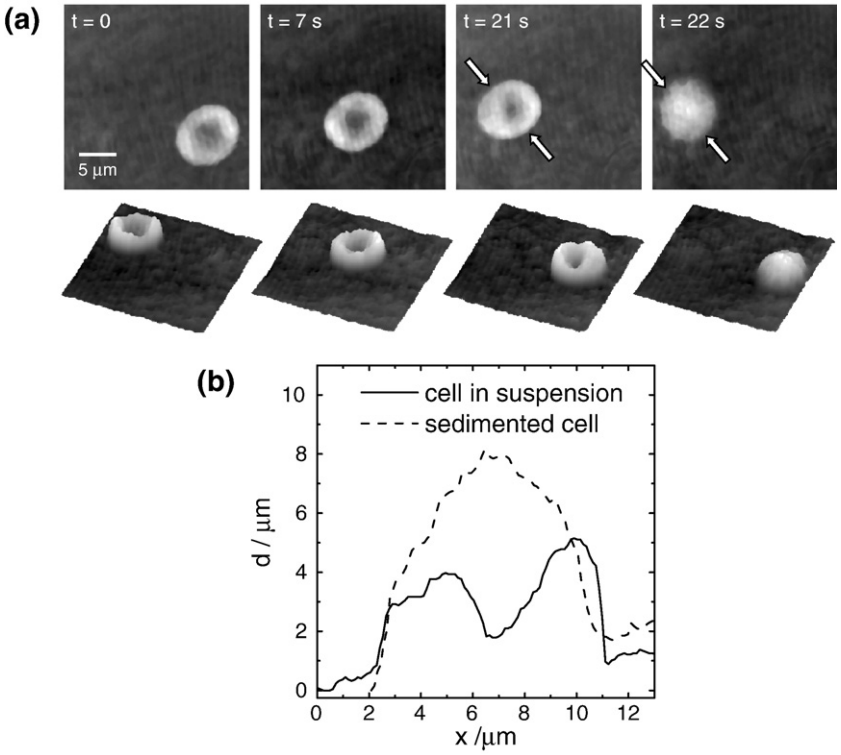

Fig. 4. Investigations on shape variations during sedimentation of a human red blood cell (in suspension) on a coated surface (63× microscope lens, NA = 0.75). (a): grey level coded phase contrast images (upper row) and pseudo 3D plots of the red blood cell before and after contact with the coated glass surface at $t = 22$ s, (b): cell thickness before and after the contact with the surface at the cross-sections that are marked in (a) by white arrows

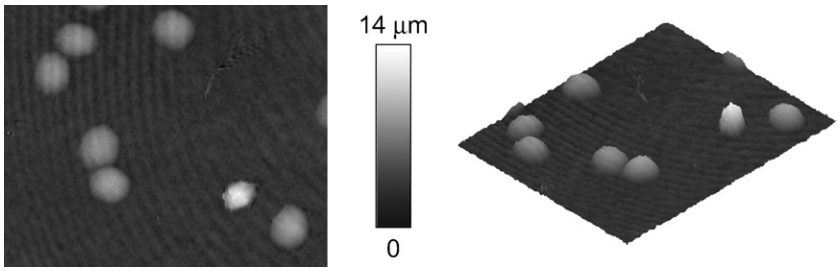

Fig. 5. Gray level representations of the holographic phase contrast images (left) and pseudo 3D plot (right) of sedimented red blood cells on the cover slip surface coated with poly-L-lysine.

contrast images can be applied for thickness and shape measurements of semi-transparent microscopic samples, such as living single cells [7,8,11]:

$$d = \frac{\lambda \Delta \varphi_{\text{cell}}}{2\pi} \cdot \frac{1}{n_{\text{cell}} - n_{\text{medium}}} \quad (1)$$

In Eq. (1) d represents the cell thickness and $\Delta\varphi_{\text{cell}}$ is the optical path length change of the cells with the integral refractive index n_{cell} to the surrounding medium with a refractive index n_{medium}.

3. Results

Fig. 2 illustrates the appearance of living RBCs in physiological solution in the quantitative digital holographic phase contrast images (Fig. 2a and c: gray level representations of the holographic phase contrast images; Fig. 2b and d: pseudo 3D plots of 2a and 2c). The images 2a and 2b represent a "normal" discocyte, whereas the images 2c and 2d indicate an echinocyte. The shape change has been obtained by adding 10 µM DIDS to the cell suspension in physiological solution before the cells were transferred to cover slips for imaging. Fig. 2e depicts the different cell shapes by cross-sections through cell thickness that have been calculated by application of Eq. (1) from the holographic phase contrast images which are marked in Fig. 2a and c by white arrows (n_{cell} estimated to be ≈1.4, [12]; n_{medium}=1.337, measured with an Abbe refractometer).

One particular advantage of using digital holographic microscopy is the possibility to track the sedimentation of a single cell by automated quantitative focus tracking. It also allows following the whole sedimentation process by observation of a single cell's z-position (object position) in relation to the position at the beginning of the experiment which is represented in Fig. 3. Fluctuations in the measured time dependence of the z-position values (e.g., at $t \approx 20$ s) are effected by convection within the cell suspension and mechanical instabilities of the experimental setup. Analysing the focus correction in dependence on time, a RBC sedimentation rate of about 3 mm/h has been estimated. Differences of the sedimentation rate have not been analysed for "normal" (discocytes) and DIDS-treated cells (echinocytes). In principal it should be also possible to present the sedimentation process as a 3D image. For this purpose, however, an enhanced algorithm has to be applied.

An important advantage of working with digital holographic microscopy is that it permits to investigate the process of interaction of single cells with the bottom surface of the sedimentation chamber (e.g., cover slip or petri dish). By application of suitable CCD cameras this process can be studied dynamically with a high frame acquisition rate. The shape transformation of a human RBC attaching the bottom surface of a cover slip coated with poly-L-lysine is shown in Fig. 4. The upper row of Fig. 4a shows the grey level coded phase contrast images of the RBC before surface contact at t=0, t=7 s, t=21 s and directly after the interaction with the surface at t=22 s. In the lower row, the corresponding pseudo 3D representations of the phase contrast images are depicted. Fig. 4b shows the change of the shape by the calculation of the cell thickness with Eq. (1) before (t=21 s) and after the contact with the glass surface (t=22 s) at the cross-sections through the holographic phase contrast image that are marked in Fig. 4a by white arrows.

It is of importance to notice that the application of this technique allows for the first time to follow such fast shape changes of cells after interaction with artificial surfaces with high temporal resolution. Until now analysis of shape changes have been performed after the cells where settled down on a surface. Only after some minutes the shapes have been categorized.

Fig. 5 shows on the left the quantitative holographic phase contrast image of several RBCs on a cover slip surface coated with poly-L-lysine after sedimentation and the corresponding grey level coded pseudo 3D representation. For all cells a mainly spherical shape is observed.

4. Discussion

Digital holographic microscopy is nowadays a versatile tool to investigate living cells (see e.g., [6,7]). In comparison to bright field or Nomarsky images this technique has the advantages of quantitative dynamic phase contrast imaging in combination with subsequent quantitative (autofocus) imaging of different object planes. In addition, the axial resolution is significantly higher than in classical optical microscopy [7]. Furthermore, in contrast to other imaging techniques, digital holographic microscopy is minimally invasive and enables temporal investigations of dynamic processes.

The presented results show that this technique can be applied to investigate dynamic processes of RBCs. For instance, the sedimentation process of the cells can be observed and the cells can be analysed and imaged during this process. In addition, fast shape changes of the cells during the sedimentation and/or after the cells are getting in contact with artificial surfaces can be investigated.

The shape change of the RBCs observed after the cells are attached to the poly-L-lysine coated glass surface is very fast (less than 1 s). Such a fast shape change can be also observed if the cells

are getting in contact with the uncoated glass surface of the cover slip. In this case echinocytes are formed (known as the "glass effect" in the literature [13,14], not shown). It seems evident that such fast shape changes are not based on lipid redistribution between the two membrane leaflets. It is more realistic to assume that the fast shape changes are due to changes of the conformation of major membrane proteins, as it was shown for conformational changes of the anion transport protein (band 3) [4].

In conclusion, digital holographic microscopy represents a new set-up allowing a contact-less and marker-free quantitative phase-contrast imaging of living cells under conventional laboratory conditions. Using this technique we were able to detect and analyse fast shape changes of red blood cells.

Acknowledgements

Financial support by the German Federal Ministry for Education and Research (BMBF) within the funding program "Biophotonics" and the European Network of Excellence "Nano2Life" is gratefully acknowledged.

References

[1] M.P. Sheets, S.J. Singer, Biological membranes as bilayer couples, A mechanism of drug erythrocyte interaction, Proc. Natl. Acad. Sci. U.S.A., 71, 1974, pp. 4457–4461.
[2] J. Gimsa, C. Ried, Do band 3 protein conformational changes mediate shape changes of human erythrocytes? Mol. Membr. Biol. 12 (1995) 247–254.
[3] J. Gimsa, Red cell echinocytogenesis is correlated to the recruitment of external band 3 conformations, Bioelectrochem. Bioenerg. 38 (1995) 99–103.
[4] T. Betz, U. Bakowsky, M.R. Müller, C.-M. Lehr, I. Bernhardt, Conformational change of membrane proteins leads to shape changes of red blood cells, Bioelectrochem. 70 (2006) 122–126.
[5] D. Carl, B. Kemper, G. Wernicke, G. von Bally, Parameter-optimized digital holographic microscope for high-resolution living-cell analysis, Appl. Opt. 43 (2004) 6536–6544.
[6] B. Kemper, D. Carl, J. Schneckenburger, I. Bredebusch, M. Schäfer, W. Domschke, G. von Bally, Investigation of living pancreas tumor cells by digital holographic microscopy, J. Biomed. Opt. 11 (2006) 034005.
[7] B. Kemper, D. Carl, A. Höink, G. von Bally, I. Bredebusch, J. Schnekenburger, Modular digital holographic microscopy system for marker-free quantitative phase contrast imaging of living cells, Proc. SPIE Int. Soc. Opt. Eng. 6191 (2006) 61910T.
[8] G. von Bally, B. Kemper, D. Carl, S. Knoche, M. Kempe, C. Dietrich, M. Stutz, R. Wolleschensky, K. Schütze, M. Stich, A. Buchstaller, K. Irion, J. Beuthan, I. Gersonde, J. Schnekenburger, New ways for marker-free life cell and tumor analysis, in: J. Popp, M. Strehle (Eds.), Biophotonics: visions for a better health care, Wiley, 2006, pp. 301–360.
[9] B. Rappaz, P. Marquet, E. Cuche, Y. Emery, C. Depeursinge, P.J. Magistretti, Measurement of the integral refractive index and dynamic cell morpholometry of living cells with digital holographic microscopy, Opt. Express 13 (2005) 9361–9373.
[10] P. Langehanenberg, B. Kemper, G. von Bally, Autofocus algorithms for digital-holographic microscopy, Proc. SPIE Int. Soc. Opt. Eng. 6633 (2007) 66330E.
[11] B. Kemper, S. Kosmeier, P. Langehanenberg, I. Bredebusch, W. Domschke, J. Schnekenburger, G. von Bally, Integral refractive index determination of living suspension cells by multi focus digital holographic phase contrast microscopy, J. Biomed. Opt. 15 (2007) 054009.
[12] B. Rappaz, A. Barbul, F. Charrière, J. Kühn, P. Marquet, R. Korenstein, C. Depeursinge, P. Magistretti, Erythrocytes volume and refractive index measurement with a digital holographic microscope, Proc. SPIE Int. Soc. Opt. Eng. 6445 (2007) 644509.
[13] R. Glaser, The shape of red blood cells as a function of membrane potential and temperature, J. Membr. Biol. 51 (1979) 217–228.
[14] L.E.G. Eriksson, On the shape of human red blood cells interacting with flat artificial surfaces – the "glass effect", Biochim. Biophys. Acta 1036 (1990) 193–201.