Morphological analysis of red blood cells by polychromatic interference microscopy of thin films

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Abstract. Red blood cells (RBC) distribution width (RDW) is a promising hematological parameter with broadapplications in clinical practice; in various studies RDWhas been shown to be associated with increased risk of heart failure (HF) in general population. It predicts mortality and other major adverse events in HF patients. In this report new method of RDWmeasurement is presented. It's based on interference color analysis of red blood cells in blood smear and further measurement of its optical thickness. Descriptive statistics of the of the RBC optical thickness distribution in a blood smear were used for RDW estimation in every studied sample. Proposed method is considered to be avoiding type II errors and minimizing the variability of measured RDW.

1. Introduction

Red blood cell (RBC) distributional width (RDW) is a numerical measure of the variability in size of circulating erythrocytes. It is a hematological parameter of an increasing interest due to its prognostic value in various cardiovascular disorders [1,2].

Routine clinical methods of RDW measurement are performed in different hematology analyzers. RDW is a parameter, which lacksinternational standardization: analyzers from different manufacturers may produce different RDW values in the same blood samples. On one hand, different measuring technologies and algorithmic approaches used by hematology analyzers can lead to differences in RDW. On the other hand upper mentioned differences may result from unpredictable flow induced blood cell deformations [2].

This paper represents the tentative results of an alternative RDW measurement method development. We consider it to be avoiding type II errors and minimizing the variability of measured parameter.

Proposed method is based on coherent effects in conventional optical microscopy of microobjects and allows to measure object'soptical thickness.The method is based on white light interferometry, as a non-contact measurement technique and on interference color analysis of microscopic images of studied objects. The quantitative relationship between interference colors of image and object optical thickness is used in various fields of optical measurements [3]. Presented method of RDW measurement uses optical microscope and color digital camera to capture interference microscopic

images of the studied object, which thickness can be determined by the color analysis of captured image of the object in every pixel of captured image.

2. Red cell Distribution Width (RDW)

The clinicians have recently witnessed massively growing interest in the RDW, especially after progressive increase of data demonstrated prognostic significance of RDW in heart failure patients. Usually RDW is defined as the standard deviation of the RBC volume distribution curve, but practically is reported as a percentage relative to mean RBC volume.

In our study RDW was calculated as following:

$$RDW = \frac{\sigma}{M} \cdot 100\%, \tag{1}$$

where σ is standard deviation of measured thicknesses of RBC and M is mean value of RBC thickness in studied blood samples.

3. Method of measurement of RBC optical thickness

The object of the study was RBC monolayer prepared on highly reflective surface. This RBC monolayer can be considered as a thin transparent film. The incident on the film light is reflected by both upper and lower boundaries of the film. If optical thickness of film is less than half of length of longitudinal coherence of incident light interference of the light is observed. If light source of polychromatic radiation is used interference colors and hues are appeared in image of the film [4]. These colors and hues mainly depend on optical thickness of illuminated film. Therefore interference color analysis can be used for measurement of optical thickness of thin film.

We use interference color analysis for estimation of RBC monolayer optical thickness with further going RDW calculation.To be more specific the main principle of developed method is comparison of image interference color of research object with color of numeric simulated interference pattern. Method of optical thickness measurement of thin film consists of three main steps:

- simulation of interference pattern;
- registration and processing interference image of research objects;
- computer analysis of registered images and estimation of object's optical thickness.

Physical process of formation and recording of interference pattern in case of polychromatic illumination of thin film can be considered with taking in to account spectral properties of light source and spectral sensitivity of recording photo elements of digital camera. We used the spectral representation for oscillations of interfering light waves to describe influence of spectral properties of light and matrix photodetector on the forming of interference patternof thin film. This representation allows using light spectrum of the source in an explicit form. Using this representation the interfering fields can be defined as

$$E_1(t) = \int_0^\infty r_1(\omega) \cdot g(\omega) \cdot \exp(i\phi_1) \cdot \exp(i\omega t) d\omega, \tag{2}$$

$$E_2(t) = \int_0^\infty r_2(\omega) \cdot g(\omega) \cdot \exp(i\phi_2) \cdot \exp\left(i\omega \left(t - \frac{\Delta}{c} \right) \right) d\omega, \tag{3}$$

where $g(\omega)$ is amplitude frequency spectrum of incident on the thin film light, $r_1(\omega)$ and $r_2(\omega)$ are amplitude reflection coefficients of the thin film's boundaries, ϕ_1 and ϕ_2 are phase shifts of the reflected light waves, Δ is optical path difference of the interfering light waves reflected by boundaries of the film.

The interference equation in spectral representation can be obtained using equation (2) and (3) in the following form

$$I(\Delta) \sim \left\langle \left| E_1 + E_2 \right|^2 \right\rangle = \int_0^\infty r_1^2(\omega) \cdot g^2(\omega) \cdot d\omega + \int_0^\infty r_2^2(\omega) \cdot g^2(\omega) \cdot d\omega + \\ + 2 \cdot \mathrm{Re}\left(\exp(i(\phi_1 - \phi_2)) \int_0^\infty r_1(\omega) \cdot r_2(\omega) \cdot g^2(\omega) \cdot \exp\left(i\omega \frac{\Delta}{c} \right) \right) \cdot d\omega \quad , \tag{4}$$

where angular brackets $\langle \cdots \rangle$ define time averaging operation due to temporary inertia of the photodetector. Equation (4) allows computing and simulating color interference pattern in polychromatic light.

To consider process of color calculating of interference images we used the RGB (Red, Green, Blue) color model. This choice is explained by using this color model in system of image recording by digital matrix photodetectors. In this model color image consists of three monochromatic patterns that are called chrominance channels [5]. Each of the pattern is registered in monochromic light - red, green and blue. The color in any pixel of resultant image is formed by combination of intensity level in each chrominance channel.

To create RGB image we should calculate three matrix arrays. Each of them describes interference pattern that is formed after transmission of interfering fields through on of the color light filters in matrix photodetector. So the spectral transmission of color light filter should be introduced into equation (4) and it will look as following

$$I(\Delta) = \int_0^\infty r_1^2(\omega) \cdot f_{ch}^2(\omega) \cdot g^2(\omega) \cdot d\omega + \int_0^\infty r_2^2(\omega) \cdot f_{ch}^2(\omega) \cdot g^2(\omega) \cdot d\omega + \\ + 2 \cdot \mathrm{Re}\left(\exp(i(\phi_1 - \phi_2)) \int_0^\infty r_1(\omega) \cdot r_2(\omega) \cdot f_{ch}^2(\omega) \cdot g^2(\omega) \cdot \exp\left(i\omega \frac{\Delta}{c} \right) \right) \cdot d\omega \quad , \tag{5}$$

where $f_{ch}(\omega)$ is amplitude transmission spectrum of corresponding chrominance channel ch.

Assuming that $r_1(\omega) = r_2(\omega) = Const = 1/2$ and ignoring multiple light reflections in the film, equation (5) can be reduced to the form that is used in computer simulation of interference pattern. This equation also can be changed to the form in terms of wavelengths λ:

$$I_{ch}(\Delta) \sim \int_0^\infty f_{ch}^2(\lambda) \cdot g^2(\lambda) \cdot \left(1 + \cos\left(\frac{2\pi}{\lambda} \Delta + \pi \right) \right) \frac{d\lambda}{\lambda^2} . \tag{6}$$

In our work simulation was performed in range of optical thickness of 0 – 2000 nm. Light spectrum of the light source can be calculated using Planck radiation law [6] for temperature of the radiation 2500 K. The values of wavelengths are over the range of 0 – 1000 nm. For spectral amplitude transmission of light filters $f_{ch}(\omega)$ we use Gaussian function

$$f_{ch}(\lambda) = \exp\left(\frac{(\lambda_{0ch} - \lambda)^2}{\Delta\lambda_{ch}^{\ 2}} \right), \tag{7}$$

where λ_{0ch} is central wavelength of transmission spectrum of chrominance channel; $\Delta\lambda_{ch}$ is width of spectrum contour of the channel.

Parameters of transmission spectrum of chrominance channel are presented in table 1.

Table 1. Parameters of transmission spectrum of chrominance channel

Channel	λ_{0ch}, nm	$\Delta\lambda_{ch}$, nm
Red	610	39
Green	540	56
Blue	460	58

The resultant interference signal and color interference pattern for thin linear optical wedge are shown on figure 1. This interference image demonstrates color dependence vs optical thickness in range of 0 – 2000 nm.

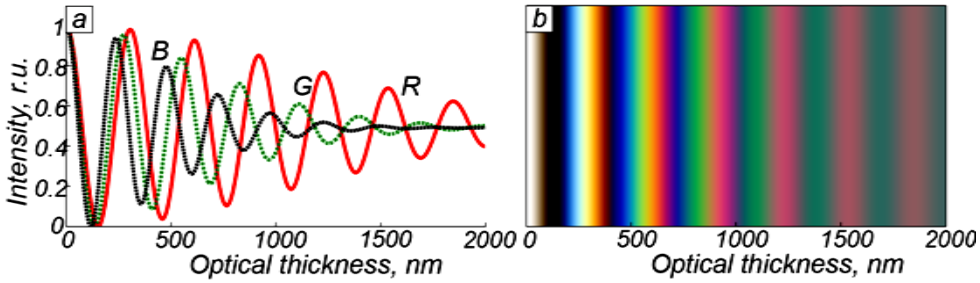

Figure 1. Results of numerical simulation: simulated interference signal for each chrominance channel (a), color interference image of thin linear optical wedge (b).

Speaking about studied object (RBC monolayer prepared on reflective substrate), it is necessary to take into account properties of this reflective substrate. In case of preparing blood smear on glass substrate light reflection from lower boundary object-glass is negligibly small. So interference colors of object images are practically undetectable. The usage of substrate with highly reflective surface, for instance polished silicon plate as in our work, instead of a glass platecould significantly increase light reflection from lower object boundaryand enable to obtain interference images of the RBC with high color contrast.

Interference RBC images were obtained by means of the optical microscope Axio Imager 2 (Carl Ceiss, Germany) with microobjectiveEpiplan-Neofluar 100x/0.75 HD DIC and captured by digital camera Nikon D80.Examples of these imagespresent in figure 2.Captured interference image were stored as a digital image andoptical thickness of RBC was measured.

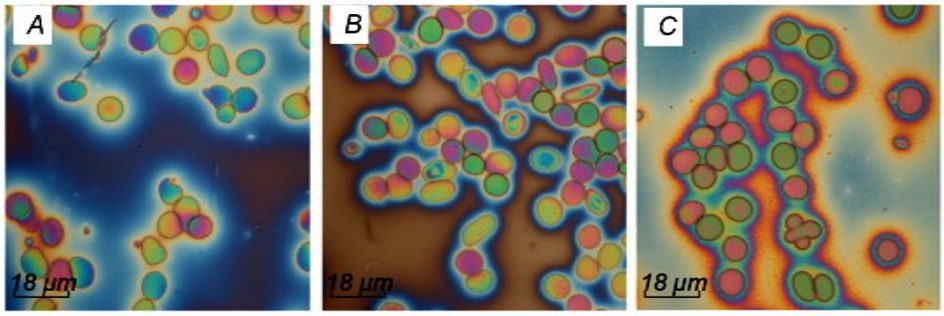

Figure 2. Interference images of formed blood elements (primary red blood cells) obtained by optical microscopy (Samples A, B and C respectively).

Main idea of the procedure for determining of the optical thickness is comparison of color in chosen pixel of experimental image with computer simulated interference scale. The pixel of image is characterized by three values of light intensity (in RGB chrominance channel).To measure optical thickness in this pixel it is necessary to compare color in the pixel with simulated color scale. Determination on this scale the point with similar color values and using known relationship between color and optical thickness one can be measured RBC optical thickness of in each pixel of experimental image.

4. Results

All blood samples in our work are obtained from patients with chronic heart failure (Stage IIa, 3NYHA functional class) during acute hyperglycemia test at base point (0, 10 and 60 min points).

Interference images of studied samples obtained by optical microscope are shown on figure 2. Image *A* captures from a blood smear prepared at base point (before 40% glucose solution administration), images *B* and *C* – from blood smear prepared at 10 and 60 minutes after 40% glucose solution administration respectively. Measurements of RBC optical thickness were performed in the

central zone of the blood smear. Histograms of optical thickness distribution in each samples present in figure 3. Red cell Distribution Width was calculated, results are presented in figure 3.

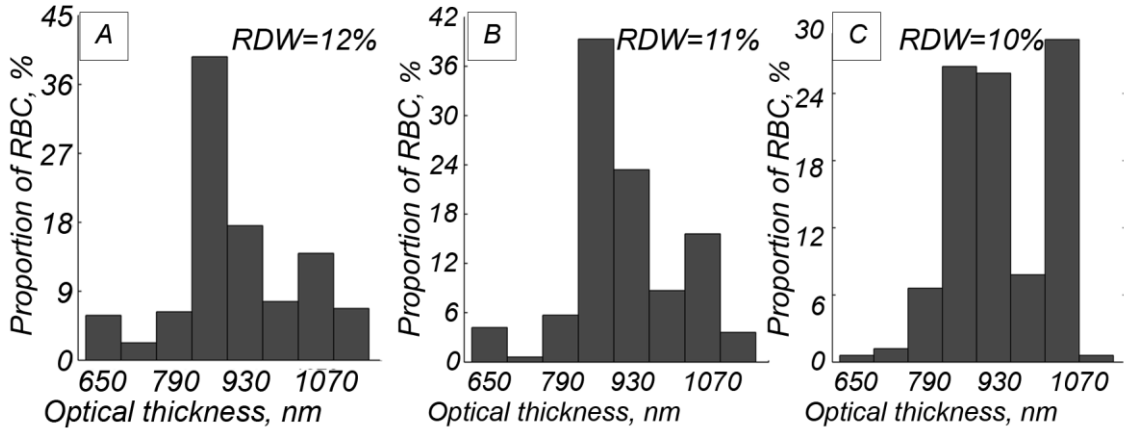

Figure 3. Optical thickness distribution in researched blood samples.

To validate obtained results we compare received data with those obtained by reference method – blood test using automatic hematology analyzers ABS Micros and Datacell-16. Optical thickness of RBC reveal strong positive correlation with MCV (mean cell volume), that is a measure of the average volume of a red blood cell and RDW ($R = 0.84$, 0.79 $p < 0.05$). Variability of studied method was 0.2 compare to 0.5 and 0.4 (automatic hematology analyzers ABS Micros and Datacell-16).

5. Conclusion

Received data elucidate interference microscopy as a promising method for RDW assessment in routine clinical practice. Among obvious merits of proposed method the simplicity of experimental set-up and procedure of computer image processing should be mentioned. Thickness profile measurement of thin blood smears by white light interferometry allows receiving more stable results. This method is potentially high sensitive promising tool to assess morphological and functional RBC properties as a prognostic biomarkers in HF patients.

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