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Determination of individual and average characteristics of native blood erythrocytes by the static spectral digital microscopy method

© V.A. Doubrovski[1], S.O. Torbin[1], I.V. Zabenkov[2]

[1] Saratov State Medical University (SSMU),
410000 Saratov, Russia
[2] Moscow Automobile and Road Construction State University (MADI),
125319 Moscow, Russia

e-mail: doubrovski43@yandex.ru

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The method of static (non-flow) spectral digital microscopy (SSDM) method to identify, to count and to determine the standard and individual characteristics of native blood erythrocytes is suggested. The object to study was the whole donor blood diluted by saline and placed into the counting Goryaev camber. Among the standard characteristics of erythrocytes, the following were determined: the concentration of erythrocytes in a blood sample RBC (Red Blood Cells concentration), the scatter of erythrocytes by volume RDW (Red cells Distribution Width), including RDW-SD and RDW-CV, hematocrit HCT, mean erythrocyte volume MCV (Mean Cell Volume). n addition, the possibility of measuring the average hemoglobin content in erythrocyte MCH (Mean Cell Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), as well as the total content of hemoglobin HGB in the blood sample (Hemoglobin) was investigated. The peculiarity of SSCM method proposed lies in the fact that it fundamentally allows to determine not only the general hematological characteristics of blood samples (RBC, RDW-SD and RDW-CV, HGB), but also the mean values of the characteristics of native blood erythrocytes (MCV, MCH, MCHC), and also, and most importantly, the individual characteristics of each erythrocyte. The latter permitted the authors to introduce a new type of erythrocyte characteristics ICV, ICH, ICHC (Individual Cell Volume, Individual Cell Hemoglobin, Individual Corpuscular Hemoglobin Concentration). In turn, this made it possible to obtain the histograms of hemoglobin distribution in erythrocytes of a blood sample along with the traditional distribution of their volumes, which can serve as an additional tool in the field of hemodiagnostics. Thus, the paper shows that SSCM method makes it possible to compile an individual metrological „passport“ for each erythrocyte of the blood sample under study — this is the main feature of this work.

Keywords: erythrocyte identification, erythrocyte counting, individual characteristics of erythrocytes, static spectral digital microscopy.

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Introduction

Clinical blood analysis is one of the most frequent laboratory medical tests. Its intrinsic part is a concentration determination of erythrocytes (RBC), hematocrit (HCT), as well as erythrocytes indices, that contain information on erythrocytes dimensions and hemoglobin content in them.

The first indices include: erythrocyte mean cell volume (MCV — measured in femtoliters, 10^{-15} l), the scatter of erythrocytes by their volume (RDW — red cells distribution width). There are two options of RDW calculation: 1) RDW-SD is measured in femtoliters, shows difference between the smallest erythrocyte of a sample and the biggest, 2) RDW-CV is measured in percents, shows how much the erythrocytes volume deviates from the mean value.

Indices, characterizing the hemoglobin content in erythrocytes, are: 1) mean corpuscular hemoglobin (MCH — measured in picograms, 10^{-12} g), 2) mean corpuscular hemoglobin concentration (MCHC) — measured in grams per liter.

Native blood analysis using static optical microscopy with the direct visual observation of a specimen through ocular or on a monitor screen allows to count erythrocytes, observe some morphological peculiarities of a specimen, but such approach is rather labour-intensive and time-consuming. Besides, it does not allow to fully perform the corresponding metrology with the examined blood sample.

Flow cytometry, beside the regular hematological parameters — concentration of erythrocytes, hematocrit and general hemoglobin content in blood, allowed to also define other important erythrocytes indices, such as above mentioned MCV, MCH, MCHC, RDW, etc. [1–4]. Disadvantages of such analyzers include complexity of their maintenance and high cost characteristics.

Development of computer technology stimulated an interest to static microscopic blood analysis methods and to creation of hematological analyzers of a new type, operating on a base of the digital optical microscopes [5–7]. However,

a smear, not blood solution, is usually studied in such analyzers, thus not allowing to determine the concentration of the formed elements and their other parameters. It should be noted, that at static digital microscopy method use, share of identified erythrocytes can reach 97% [8]. It is important to note, that there is not much articles, dedicated to erythrocytes count in native blood using static (not flow) method, beside [9] we managed to familiarize with [10,11] only.

Analysis shows, that most of the works, dedicated to automated identification, count and analysis of blood cells shape on microphotographs, use their morphological signs [6,7]. At the same time, as opposed to the flow hematological analyzers, these works do not use the spectral properties of the formed elements. Exactly this attempt of erythrocytes identification and their count was made in [12]. In this work we demonstrate, that the static spectral digital microscopy allows to identify red blood cells at the level of 97–98%, that is as good as the best results of the similar works of other authors. Moreover, such precision corresponds to the reliability degree of the modern commercially available flow hemoanalyzers.

This work can be considered as the development of [12]. Its purpose is to study a possibility of application of the static spectral digital microscopy (SSDM) method, proposed in [12], not just for identification and automated count of erythrocytes in native donor blood solution samples, but also for determination of such common metrological erythrocyte indices as MCV, MCH, MCHC, RDW, etc. Moreover, it seems that SSDM method will allow:

– to define the individual characteristics of each erythrocyte, such as ICV, ICH, ICHC (individual cell volume, individual cell hemoglobin, individual corpuscular hemoglobin concentration);

– to obtain the histograms of hemoglobin distribution in blood sample erythrocytes, that can be used as an additional tool in hemodiagnostics;

– to make an individual metrological „passport“ for each erythrocyte of the examined blood sample.

1. Experimental setup and samples preparation

1.1. Study objects

Four samples of the whole donor blood, diluted with physiological saline in a ratio of 1 : 100, were used as the study objects. The following parameters of erythrocytes and blood samples in general were preliminary determined using hemoanalyzer Sysmex XS 1000i (table 1).

1.2. Experimental setup

The examined blood samples were studied in transmitted light using digital optical microscope Lumam R-8 (fig. 1).

Light stream from the microscope lamp *5* transmitted through a light filters system *6, 7*, the solution of the examined blood sample, put into Goryaev chamber *8*, and entered the directly microscope optical system. Camera resolution was 1920 × 1200 pixels, while color depth — 12 bits. The important feature of this camera is a linear dependence of the registered signal (expressed in digital units) on intensity of the light, falling onto a photomatrix. Lens with magnification coefficient of 40× and numerical aperture of 0.65 was used for observing the microphotographs. The photographs resolution was 6900 pix/mm. During microscopic study of the blood samples per two photographs were made for each region of a microslide: one photo with application of an interference light filter on a wave length of 416 nm with band width of 10 nm (indicated as IF416). Another photo was made with the interference light filter IF620 on a wave length of 620 nm with the same band width. In both cases, beside the interference light filters, the broadband light filter of CZC23 type was used.

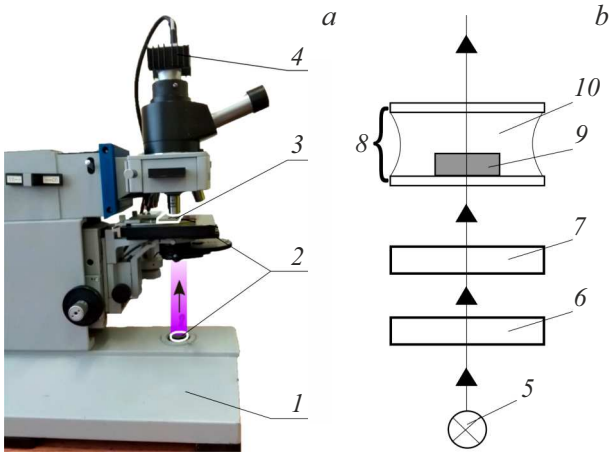


Figure 1. Experimental setup. General view (*a*): *1* — microscope Lumam R-8, *2* — optical filters system, *3* — Goryaev chamber, *4* — digital monochromatic camera Basler acA1920-40um. Fragment of the optical system (b): *5* — microscope halogen lamp KGM9-70KGM9-70, *6* — broadband cyan glass light filter, *7* — interference light filter IF416 or IF620, *8* — Goryaev chamber with native donor blood solution, *9* — erythrocyte model, *10* — plasma solution.

Table 1. Metrological characteristics of the study objects

№	Parameter	Size	Sample 1	Sample 2	Sample 3	Sample 4
A	RBC	$10^{12}/l$	4.56	4.47	5.25	4.85
	HGB	g/l	126	120	170	177
	HCT	%	36.9	34.9	45.8	45
B	MCV	fl	80.9	78.1	87.2	90
	RDW-CV	%	14.1	13.6	11.9	12.0
	RDW-SD	fl	41	37.5	37.9	39.5
C	MCH	10^{-12} g	27.6	26.8	32.4	35.8
	CHC	g/l	341	344	371	395

Filters spectra, as well as the hemoglobin spectrum, are presented in fig. 2, *a*. Also, the spectral characteristics of the microscope light source and digital camera sensitivity are presented in fig. 2, *b*.

Unlike other formed blood elements the erythrocytes have a natural sensibilizer — hemoglobin, that absorbs the light the most intensively in the region of 415 nm (Soret band — oxyhemoglobin). Therefore the interference light filter with close wave length of $\lambda = 416$ nm was used in the setup. Filter of CZC23 type is intended for prevention from the negative effect of IF416 light filter subsidiary passband and, as a result, improvement of selectivity of the optical signal, registered by the camera, in relation to hemoglobin content in erythrocyte. It should be noted, that the halogen lamp spectrum $I(\lambda)$, as well as the camera sensitivity $R(\lambda)$ in fig. 2, *b* are presented in relative units.

2. Erythrocytes identification principles

The first problem in the set of tasks on determination of erythrocytes characteristics is their identification and distinction among the formed elements of the analyzed blood sample. Erythrocytes identification principle, proposed in [12], is based on brightness comparison of pixels, belonged to erythrocytes, and pixels, corresponding to the photo image background. Such comparison is performed at two wave lengths: 416 and 620 nm. Application of the light filter IF416 is justified below in the sect. 4.3.1, application of the interference light filter IF620 is auxiliary — it is used for intensification of difference of the background brightness from erythrocyte photo image brightness, i.e. for improvement of erythrocyte identification certainty. Erythrocytes identification and count algorithm is described in detail in [12]. Certainty of erythrocytes recognition, achieved in [12], compared to flow cytometry method (hemoanalyzer Sysmex XS 1000i) is 97% minimum.

In this work for erythrocytes identification and count the same principles and software products are used, as in [12], but other blood samples are studied and, above all, the purpose of the work is broader than in [12] — determination of metrological characteristics of erythrocytes and blood samples in general. In this regard, some specific features of the experiments performing technique and methods of computer processing of the object photo images, related to the task, set in this work, will be described further.

3. Erythrocytes count and their geometric characteristics determination

3.1. Erythrocytes count

Spatial scanning of blood samples photo images allowed to differ by brightness the pixels, that belong to erythrocytes, from background pixels. Computer program [12] allowed:

Table 2. Numerical data required for quantitative evaluations performing

$I_0(620)$	0.57 arb. units
$I_0(416)$	0.094 arb. units
$I_0(620)/I_0(416)$	6.0
$T_{IF416}(416)$	31%
$T_{IF620}(620)$	38.4%
$T_{IF}(620)/T_{IF416}(416)$	1.24
$T_{ISZS23}(416)$	100%
$T_{CZC23}(620)$	43%
$T_{CZC23}(620)/T_{ISZS23}(416)$	0.43
$R(620)$	0.89
$R(416)$	0.68
$R(620)/R(416)$	1.3
α	5.36

— to differ the registered erythrocytes from other formed blood elements or artefacts and, as a result, to summarize the erythrocytes;

— in case of erythrocyte aggregates forming, to observe how many erythrocytes are included in a certain aggregate and to summarize them;

— to define the area, occupied with each registered erythrocyte;

— to define the registered erythrocyte size, but to prevent from error in its size defining, the cells, that are distanced from periphery for less than 5.5 μm, were excluded from the observation;

— to number and record the „address“ of each erythrocyte on a photo image (in pixels along coordinate axes) (fig. 3, *a*).

It should be noted, that in this work the erythrocytes „addresses“ recording was performed not just for elaboration of erythrocytes concentration in the examined sample, but also for arrangement of „passport“ for each erythrocyte with its metrological characteristics. After erythrocytes recognition on 10 micro photographs of blood solution their number was calculated on each photo image for each examined sample. Calculation of erythrocytes concentration in Goryaev chamber for each photo image was performed as per formula

$$\mathrm{RBC} = \frac{NR(\Gamma_{\mathrm{lens}} - \Gamma_{\mathrm{ad}})^2}{wzL}, \qquad (1)$$

where RBC — erythrocytes concentration, N — number of counted erythrocytes in a field of view for certain photo image, R — degree of blood dilution with physiological saline ($R = 0.01$), Γ_{lens} — lens magnification (40), Γ_{ad} — adapter magnification (*1*), w — camera sensor width (0.113 dm),

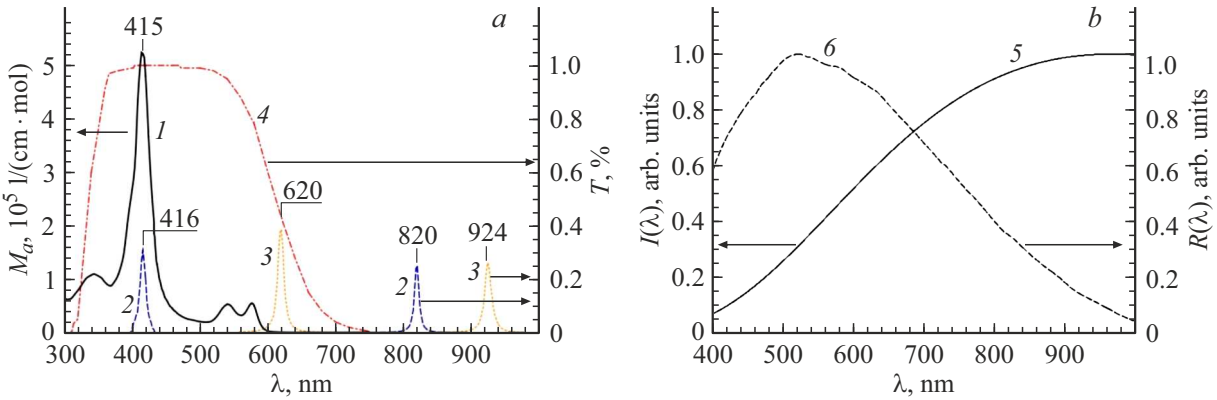


Figure 2. Spectral characteristics of hemoglobin and light filters (*a*): *1* — oxyhemoglobin molar absorption coefficient, *2, 3* — transmission coefficients of interference IF416 and IF620 and broadband CZC23 light filters respectively. Spectral characteristics (*b*): of halogen lamp KGM9-70 (calculated as per Planck formula at color temperature of 3000 K) (*5*) and spectral characteristic of Basler acA1920-40um (*6*) camera sensitivity (camera documentation).

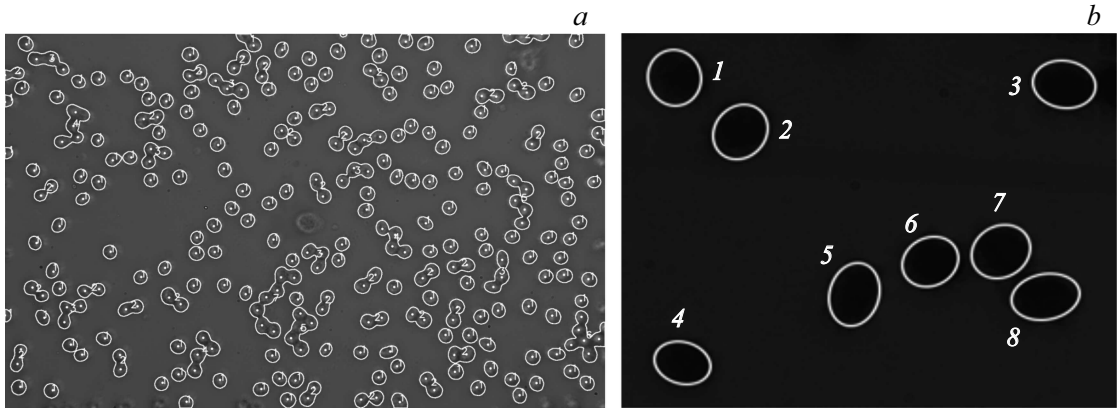


Figure 3. (*a*) The original photograph of the microslide of the native donor blood solution in the physiological saline (1 : 100) with the contours of the recognized erythrocytes drawn using software. The obtained erythrocyte centers are indicated with dots using software, while number of erythrocytes in each contour (aggregates) is indicated with numbers. (*b*) Fragment of photo image of erythrocytes of the blood sample № 2. For more contrast perception of erythrocytes on a general background: the selected erythrocytes are „contoured" artificially (the figure corresponds to a wave length of $\lambda = 416$ nm).

z — camera sensor height (0.071 dm), L — blood solution layer thickness in Goryaev chamber (0.001 dm).

Then RBC values count results as per formula (1) were averaged by 10 photo images of a certain blood sample. For example, the results of RBC erythrocytes concentration count for blood sample № 2 are presented below in sect. 5 and compared with the results of count for the same sample using flow technique.

Fig. 3, *b* is intended for discussion of a possibility of comparison of individual „passport" of each blood sample erythrocyte (sect. 5).

3.2. Erythrocytes geometric characteristics determination

Standard geometric characteristics of erythrocytes include erythrocyte mean cell volume (MCV), the scatter of erythrocytes by their volume (RDW), including RDW-CS and RDW-SD (sect. 1), hematocrit (HCT).

Recognition of pixels, related to erythrocytes (not background), allowed to determine the area (pix), occupied with each erythrocyte on this blood solution photo image surface. Obviously, with the known scale this area can be recalculated in units of μm^2. By approximating the observed erythrocyte area with a circle area, it is easy to observe the effective diameter of each erythrocyte d.

For calculation of the individual corpuscular volume (ICV) the formula [13] can be used:

$$\mathrm{ICV} = 0.16d^3. \qquad (2)$$

As noted above, each erythrocyte is numbered, its coordinates are known in pixels, its „passport" data can contain size and individual volume of a cell (ICV). As an example, the erythrocytes distribution by volumes for one of the blood sample photo images is presented in fig. 4.

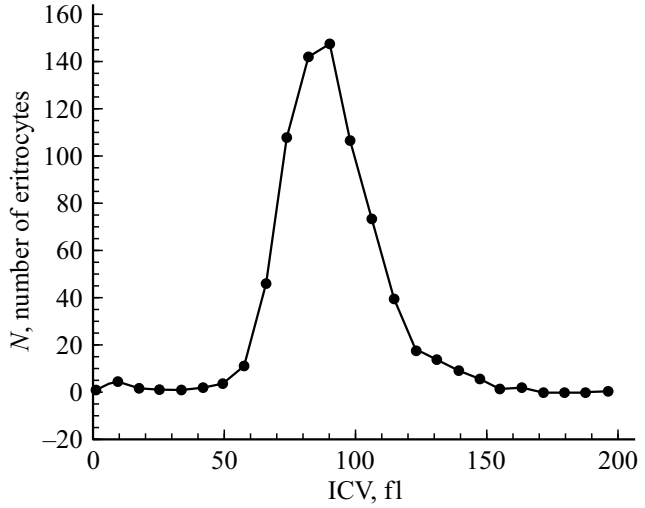


Figure 4. Erythrocytes distribution by volumes for blood sample № 1 (table 1). Total volume of erythrocytes picking for this blood sample on 10 photo images — 744.

By summarizing the ICV values of the erythrocytes of the analyzed photo image and by dividing the sum by erythrocytes number, the standard characteristic of blood sample (MCV) can be observed. However, it should be noted, that the proposed approach allows to define not only the mean blood parameter (MCV), but also the individual (ICV).

Based on results, similar to fig. 4, the parameters like RDW-SD and RDW-CV [13] can be defined for the examined blood samples (table 6 in sect. 5). Besides, by summarizing the observed volumes of erythrocytes (ICV) of this photo image with known corresponding volume of blood solution, as well as its dilution degree, the hematocrit (HCT) can be calculated (the results — below in table 6, sect. 5 compared to the results of flow cytometry from table 1).

Thus, it is shown, that the static spectral digital microscopy method allows not only to calculate the number of erythrocytes (RBC) in blood sample, but also to define the individual erythrocyte parameters (ICV), and, as a result, the common mean metrological values of red cells - MCV, RDW and HCT.

4. Principles of determination of erythrocytes characteristics, related to hemoglobin content

Standard erythrocyte characteristics, bearing the information on hemoglobin content, include HGB, MCH and MCHC. Historically, these parameters are defined in the following order: 1) determination of hemoglobin content in a blood sample volume unit (HGB) [g/l]; 2) with known erythrocytes concentration (RBC) $[l^{-1}]$, using HGB, it is possible to define the mean amount of hemoglobin in a cell (MCH) [g], 3) after determination of parameter MCH, with the known mean volume of erythrocytes (MCV) it is possible to get the mean hemoglobin concentration in erythrocyte MCHC = MCH/MCV [g/l]. It should be noted, that all erythrocytes in this case are assumed as equal, containing equal hemoglobin masses, that is highly doubtful.

One of the purposes of this work is to study a possibility of each erythrocyte individual characteristics determination (ICH — individual cell hemoglobin, ICHC — individual corpuscular hemoglobin concentration) based on static spectral digital microscopy. Obviously, when observing ICH and ICHC, the individual geometric characteristics of erythrocytes, described in sect. 3.2, will be used. Algorithm of our analysis will be opposite to traditional one: 1) determination of ICHC, 2) determination of ICH = ICHC×ICV and, finally, 3) HGB = ICH×RBC. But at the same time it should be noted, that all erythrocytes are considered individual, the opportunity arises to make „passport“ for each of them with a list of their own metrological characteristics. We emphasize, that this approach does not exclude determination of traditional mean parameters - HGB, MCH and MCHC.

4.1. Determination of relation of erythrocyte photo image brightness at two wave length

It was noted in section 2, that two photo types were made for microscopic study of blood samples: 1) using microscope lamp light stream, transmitted through sequence of two light filters — broadband CZC23 and interference IF416 with transmission maximum at a wave length of 416 nm (fig. 1, *b*); 2) using light filters CZC23 + IF620 ($\lambda = 620$ nm). These elements purpose and their characteristics are presented in sect. 1.2 and in fig. 2. Here we emphasize, that such characteristics, important for quantitative evaluations performing, of the experimental setup

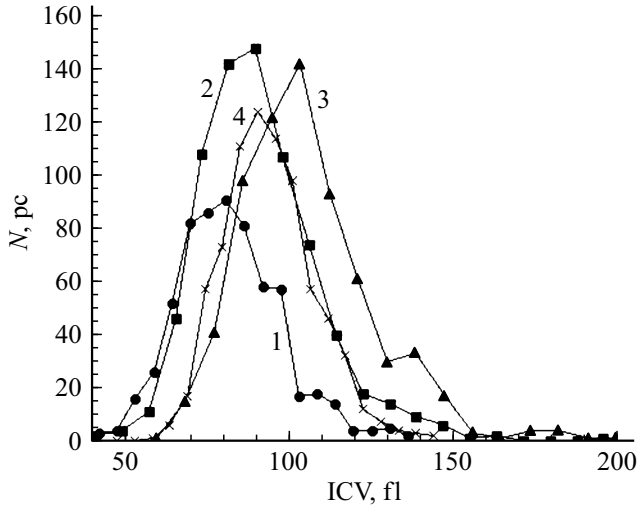


Figure 5. Distribution of erythrocytes of four blood samples by volume. Curve number corresponds with a blood sample number (table 1).

Table 3. Comparison of theoretical and experimental brightness relations $\frac{B_{rbc}(620)}{B_{rbc}(416)}$ for four examined native blood samples

№	From Table 1		D, μm	H, μm	$B_{rbc}(620)/B_{rbc}(416)$		K (12)
	MCHC, g/l	MCV, fl			Theory (11)	Experiment	
	1	2	3	4	5	6	7
1	341	80.9	7.97	1.63	15.15	1.39	0.092
2	344	78.1	7.87	1.60	14.93	1.41	0.094
3	371	87.2	8.17	1.67	16.98	1.47	0.087
4	393	90.9	8.28	1.69	18.95	1.49	0.079

elements (fig. 1), as spectral characteristics of sensitivity of camera Basler acA1920-40um, as well as spectrum of halogen lamp KGM9-70, are known to us and presented in fig. 2, *b* in relative units.

Therefore, considering linearity of light characteristics of photo camera Basler acA1920-40um for ICHC determination the relation of erythrocyte photo image brightness values at the above mentioned wave lengths $[B(620)/B(416)]$ was selected as the measurement parameter.

Generally the photo image pixel brightness is determined as

$$B = \int_{400}^{1000} I(\lambda)R(\lambda)\exp(-\mu_a(\lambda)h)d\lambda, \qquad (3)$$

where $I(\lambda)$ — spectral intensity of a light, transmitted through erythrocyte and falling onto photo camera matrix, $R(\lambda)$ — camera spectral sensitivity, μ_a — spectral coefficient of light absorption by hemoglobin, h — height of a layer, corresponding to analyzed erythrocyte thickness.

For the sake of simplicity we write the relation of brightness values $B_{rbc}(620)/B_{rbc}(416)$ as their peak values at the specified wave lengths at light transmission through erythrocyte (correction for integration by spectrum will be made further):

$$\left(\frac{B_{rbc}(620)}{B_{rbc}(416)} = \frac{B_0(620)}{B_0(416)}\exp(-(\mu_a(620) - \mu_a(416))h, \quad (4)\right.$$

where $\frac{B_0(620)}{B_0(416)}$ — relation of photo image brightness values without erythrocyte (background), „rbc" index in expression $\frac{B_{rbc}(620)}{B_{rbc}(416)}$ shows, that (4) is written for any single erythrocyte. Values of $\mu_a(620)$ and $\mu_a(416)$ — coefficients of light absorption by hemoglobin at wave lengths of 620 and 416 nm. Value of $\frac{B_0(620)}{B_0(416)}$ can be determined by analyzing the intererythrocyte space on photo image:

$$\alpha = \frac{B_0(620)}{B_0(416)} = \frac{I_0(620)}{I_0(416)}\frac{T_{IF620}(620)}{T_{IF416}(416)}\frac{T_{CZC23}(620)}{T_{ISZS23}(416)}\frac{R(620)}{R(416)}, \qquad (5)$$

where the relations of the following values at wave lengths of 416 and 620 nm are presented in (5): intensities of microscope lamp radiation $\frac{I_0(620)}{I_0(416)}$, coefficients of transmission of broadband cyan $\frac{T_{CZC23}(620)}{T_{ISZS23}(416)}$ and interference $\frac{T_{IF620}(620)}{T_{IF416}(416)}$ light filters, as well as spectral values of photo camera sensitivity $\frac{R(620)}{R(416)}$. It should be noted here, that value of $\frac{B_0(620)}{B_0(416)}$ is written in (5) without consideration of microscope lens achromatism, optical translucence and dispersion dependence of the coefficient of transmission of light by plasma solution (these factors will be examined in sect. 4.3.1). When calculating the value of α (5) we consider the parameters of the setup optical part, presented in table 2.

Numerical data of table 2: a) are based on diagrams of fig. 2, *b*) during calculation of the value of α the numerical integration (3) was performed by wave lengths, while integration limits corresponded to spectra of fig. 2.

Let's correlate $\mu_a(620)$ and $\mu_a(416)$ $[\text{cm}^{-1}]$ with molar coefficient of light absorption $M_a(\lambda)$, depending on light wave length:

$$\mu_a = 2.303\frac{M_a(\lambda)\text{ICHC}}{64500}, \qquad (6)$$

where number 64500 — molar weight of hemoglobin [g/mol]. Then (4) can be rewritten:

$$\frac{B_{rbc}(620)}{B_{rbc}(416)} = \frac{B_0(620)}{B_0(416)}\exp\left(\frac{-2.303(M_a(416) - M_a(620))\text{ICHC}}{64500}\right)h, \qquad (7)$$

where values of $M_a(416) = 521880$ and $M_a(620) = 942[(1/\text{cm})/(\text{l/mol})]$ [14]. Let?s define

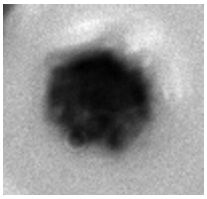
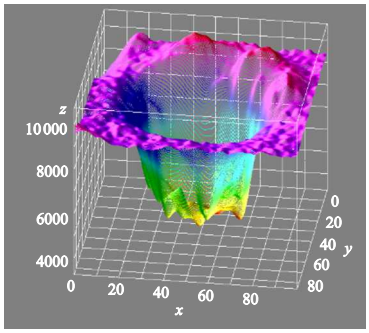
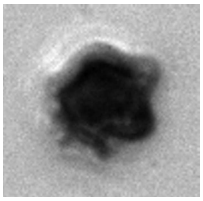
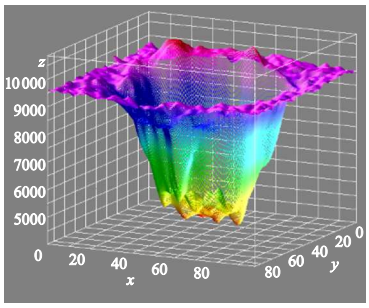
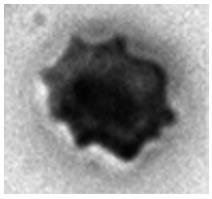
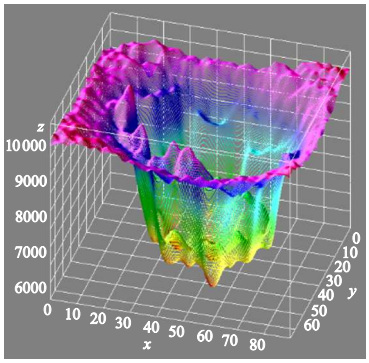
$$\beta(h) = \left(\frac{2.303(M_a(416) - M_a(620))}{64500}\right)h = \beta_0 h, \qquad (8)$$

where $\beta(h)$ — hemoglobin layer height function h. Substitution of the above mentioned values into (8) gives $\beta_0 = 18.6\,[(\text{cm}^{-1})(\text{l/g})]$. Considering (8) the expression (7) can be rewritten as

$$\left(\frac{B_{rbc}(620)}{B_{rbc}(416)}\right)_{theor} = \alpha\exp(\beta_0 h\text{ICHC}). \qquad (9)$$

It should be noted, that index „theor"should be added in the left part of (9). It shows, that as per (9) for certain values of α, β_0, h and ICHC the relation of brightness values $\frac{B_{rbc}(620)}{B_{rbc}(416)}$ should take a specific value.

Table 4. Characteristics of the selected erythrocytes, their photo and distribution of photo images brightness from coordinates

№	Parameters of erythrocyte and photo image	Erythrocyte photo	Pixels brightness dependence erythrocyte photo image on coordinates $B(x, y)$
	1	2	3
1	Diameter 8.2 μm ICH 11.11 pg 100 × 96 pixels; 16 bit		
2	Diameter 8.2 μm ICH 14.65 pg 98 × 96 pixels; 16 bit		
3	Diameter 6.1 μm ICH 5.45 pg 90 × 76 pixels; 16 bit		

However, in the experiment some non-considered factors (will be examined in sect. 4.3.1 and 4.3.2) can result in correction of (9). Therefore we proactively introduce some dimensionless constant coefficient K to (9) for theoretical results agreement with the experimental results. Then in the experiment we should get

$$\left(\frac{B_{\mathrm{rbc}}(620)}{B_{\mathrm{rbc}}(416)}\right)_{\mathrm{exp}} = K5.36\exp(18.6h\mathrm{ICHC}). \qquad (10)$$

Table 5. Example of individual „passport“ data of several erythrocytes of native blood sample № 2

Number of erythrocyte	ICV, fl	ICHC, gl	ICH, pg	H, μm
1	77	345	26.5	1.59
2	77	348	26.7	1.59
3	75	365	27.3	1.58
4	75	368	27.6	1.58
5	83	362	30	1.63
6	74	358	26.4	1.57
7	78	355	27.7	1.60
8	80	359	28.7	1.61

In relation (10) ICHC — the target value for each erythrocyte on the examined blood sample photo image, $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ the measurement value. Value of h — the height of effective layer, containing hemoglobin and proportional ti erythrocyte thickness — for different analyzed erythrocytes the values of h are different. Values of h were calculated as per formula $h = \frac{0.64d}{\pi}$, observed by equating the volume of the analyzed erythrocyte ICV (2) to the volume of its cylindrical model $V = \frac{\pi d^2 h}{4}$. At the same time diameter d is defined based on area of erythrocyte photo image at approximation as a circle. The results of h count for the selected erythrocyte (fig. 3, *b*) will be presented below in table 5.

It is obvious, that by measuring $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}$, it is possible to determine the hemoglobin concentration in erythrocyte ICHC (10), but the coefficient K remains unknown. To determine it, let?s turn to the experiment.

4.2. Calibration of hemoanalyzer model, based on spectral digital static microscopy method

As in commercially available flow hemoanalyzer, e.g. Sysmex XS 1000i, we use blood samples with pre-set mean erythrocyte parameters (table 1), particularly MCHC parameter. Similar to Sysmex XS 1000i we will call them controllers. Let?s write the relations (9) and (10) for them as

$$\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{theor}} = 5.36\exp(18.6H \times \text{MCHC}), \quad (11)$$

$$\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}} = K5.36\exp(18.6H \times MCHC). \quad (12)$$

Here, unlike (9), (10) H — the mean value of h for erythrocytes of this photo image, while „rbc“ index shows, that values of $B_{\text{rbc}}(620)$ and $B_{\text{rbc}}(416)$ are the mean brightness values of erythrocyte photo images over the variety of this photo image cells at the corresponding wave lengths.

It should be noted, that for application of (11) and (12) the value of K should be calculated regardless of these formulas, based on the mean characteristics of the controller erythrocytes. For that we modify the formula (2) to

$$\text{MCV} = 0.16D^3. \quad (13)$$

Substitution of MCV from table 1 to (13) allows to define the mean erythrocyte diameter D for any controller. Let?s set the mean erythrocyte volume (MCV) equal to the volume of the equivalent plane-parallel cylinder („tablet“) with diameter D and height H (fig. 1, *b*): $\text{MCV} = \left(\frac{\pi D^2}{4}\right)H$. The results of D and H values evaluation, as well as MCHC and MCV parameters, are presented in table 3.

As per table 3 the following is observed:

1) experimental values of $\frac{B_{\text{rbc}}620)}{B_{\text{rbc}}(416)}$ increase with MCHC parameter increase;

2) theoretical values of $\frac{B_{\text{rbc}}620)}{B_{\text{rbc}}(416)}$ (formula (11)) differ more than by an order from the experimental values (potential causes will be examined in sect. 4.3);

3) coefficient K is not constant, it depends on erythrocyte properties of blood sample; therefore further in calculations of individual parameters of the examined blood samples we will use the mean value of the observed values of $K_{\text{av}} = \frac{1}{n}\sum K_i = 0.088$, where $i = 1, 2, 3, 4$, and $n = 4$.

We will assume, that value of coefficient K_{av}, set based on analysis of controller erythrocytes photo image using blood sample parameters (tables 1 and 2), measured by flow cytometry, can also be used for the case, when ICHC characteristic is defined using SSDM method only. Them we modify the formula (10) to get this parameter for single, but any erythrocyte:

$$\text{ICHC} = \frac{\text{Ln}\left(\frac{\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}}{K_{\text{mean}}5.36}\right)}{18.6h}, \quad (14)$$

It is easily seen, that determination of ICHC (14) allows to observe two other parameters, related to hemoglobin content in blood sample — MCH and HGB.

4.3. Physical phenomena, conditioning the coefficient K, introduced for theoretical results agreement with the experimental results

Coefficients K (table 3) and, therefore, K_{av} were calculated as a result of comparison of the experimental values of $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}$ with the theoretically calculated for the blood samples with known parameters, e.g. for controller № 2 ($\text{MCHC} = 344$ g/l and $H = 1.60$). For analysis of the probing light beam intensity (brightness) transformation at transmission through the setup optical elements (fig. 1, *b*), as well as bio-object, let?s perform the analysis of numerical values of brightness relation $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}$ using this example. As per table 3, theoretically speaking the brightness relation $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{theor}}$ should be 14.93 (formula (11)), i.e. brightness $B_{\text{rbc}}(620)$ should exceed $B_{\text{rbc}}(416)$ more

than by order. This is related to three factors: 1) initially the microscope lamp intensity $I_0(620) > I_0(416)$ (fig. 2), 2) relations of light filters transmission coefficients, as well as camera sensitivity at the wave lengths in use (table 2); 3) significantly higher light absorption by hemoglobin at $\lambda = 416$ nm compared to $\lambda = 620$ nm (fig. 2).

However, in the experiment this relation was only $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}} = 1.41$. It means, that at light transmission through the setup optical system and bio-object some phenomena appear, resulting in attenuation of brightness relation at the selected wave lengths by a factor of $14.93/1.41 = 10.59$ and not considered by our model (it should be noted, that $K = 1/(10.59) = 0.094$, table 3). These factors, first of all, include the following: 1) disperse dependence of the coefficient of light transmission by plasma solution layer, located above settled erythrocyte in Goryaev chamber (fig. 1, *b*), 2) achromatism of microscope and translucence of its optics.

4.3.1. Influence of blood plasma solution dispersion and microscope optical elements on brightness relation $(B_{rbc}(620)B_{rbc}(416))_{exp}$

Intrinsic special photometric measurements showed, that for the whole blood plasma the relation $T(620)/T(416) = 1.97$, but with plasma dilution of 1 : 16 this relation is 1.05, while for blood (plasma) dilution, adopted in the article, of 1 : 100 the transmission coefficients are almost $T(620) \approx T(416)$ — no dispersion.

To determine the influence of achromatism, microscope optics translucence on brightness relation $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ the light stream transmission through erythrocyte should be excluded from examination. This means, that it is necessary to analyze the brightness values at wave lengths of 416 and 620 nm for „intererythrocyte“ spaces (background) for various blood samples. These sections correspond to plasma solution, for which at these blood dilutions, as shown above, there is almost no dispersion. Processing of background sections on photo images (fig. 3) showed, that within photo image the brightness values vary, therefore the mean value of background brightness on the image was determined by 10 selected „intererythrocyte“ zones. Besides, the mean background value was different for different blood samples. However, the result in general is the following: at transmission of light streams, formed by the microscope lamp and light filters system, through plasma solution and microscope optical system the brightness relation $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ in average by the four examined blood samples decreased by a factor of 6.12 ± 0.22 (deviation by samples from the average $\pm 3.6\%$). I.e. the microscope optical system dispersion changes the value of relation $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ to the opposite. To clarify: if brightness relation of the streams, probing the bio-object, was $\alpha = \frac{B_0(620)}{B_0(416)} = 5.36$ (formula (5) and table 2), i.e. $B_0(620) > B_0(416)$, then after attenuation of brightness relation $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ with the microscope by a factor of 6.12, the inverse inequality $B_0(620) < B_0(416)$ becomes true. These evaluations are true for the case of the light streams transmission through plasma solution and here, indeed, $B_0(620) < B_0(416)$.

Table 6. Comparison of erythrocyte characteristics of blood sample № 2, observed using methods of SSDM and flow hemocytometry

Parameters of erythrocytes	Method of static spectral digital microscopy	Method of flow hemocytometry
RBC	4.03E + 12	4.47E + 12
CV	78.5	78.1
CH	27.4	26.8
CHC	355	344
HGB	112	120
HCT	31.7	34.9
RDW SD	31	37.5
RDW CV	1.24	13.6

However, for light probing beams, transmitted through erythrocyte, the situation changes: due to high light absorption at a wave length of 416 nm the share of violet spectrum radiation decreases, while the relation $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ increases. This is evidenced by table 3: it shows, that for the blood sample № 2, examined as an example, without dispersion of the microscope optics the relation $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}$ should be 14.93 (formula (10)). But considering attenuation of this relation by microscope, its value will be $14.93/6.12 = 2.44$, i.e. $B_{\text{rbc}}(620)$ still exceeds $B_{\text{rbc}}(416)$, but just by a factor of 2.44. At the same time, as per table 3, actually in the experiment $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)} = 1.41$. It means, that beside the dispersion of the microscope optical system, there is another cause of actual reduction of brightness relation by a factor of $2.44/1.41 = 1.73$. What is it? Thus, at determination of ICHC of erythrocite (14) the attenuation coefficient $(1/K_{\text{av}})$ of the measurement value of relation $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}$ can be examined as a product of two attenuations:

$$\frac{1}{K_{\text{av}}} = \left(\frac{1}{K_1}\right)\left(\frac{1}{K_2}\right), \qquad (15)$$

where in this work $K_{\text{av}} = (1/10.59)$ (formula (11) — integral attenuation), $(1/K_1) = 6.12$ — attenuation by microscope due to dispersion properties of its optical elements, $(1/K_2) = 1.73$ — attenuation of the measurement value, which nature should be studied.

4.3.2. Optical phenomena at erythrocyte as a cause of reduction of brightness relation $\frac{B_{rbc}(620)}{B_{rbc}(416)}$

Let's examine, for instance, photo images of three erythrocytes (table 4) at higher magnification, than in fig. 3.

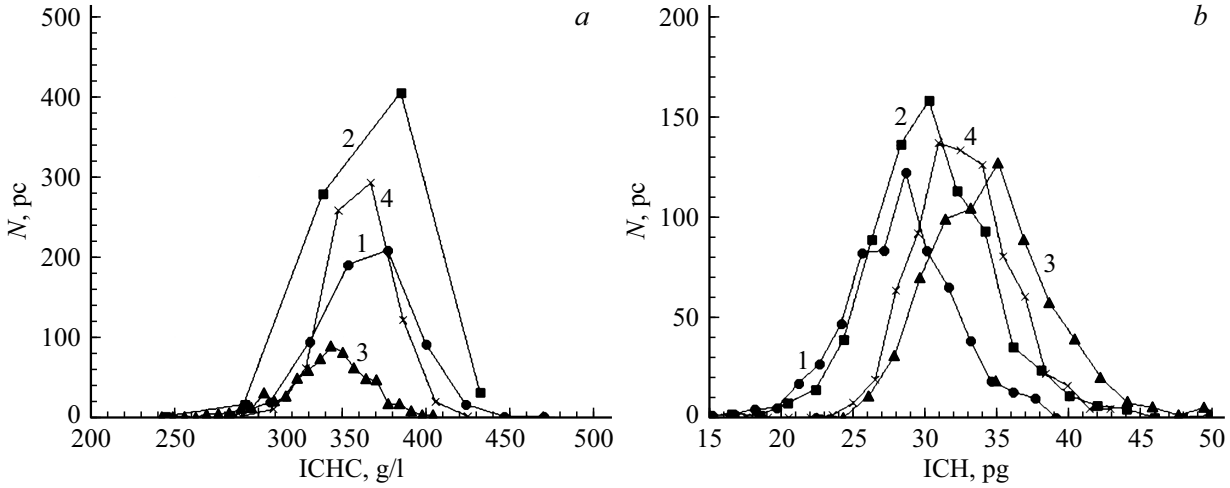


Figure 6. Hemoglobin distribution by erythrocytes of blood sample № 2: (a) and (b) — dependence of erythrocytes number N on value of ICHC and ICH respectively. Curve number corresponds with a blood sample number (table 1).

On all images in table 4 the erythrocytes had light glow along the edges (region with brightness above background brightness). It should be noted, that „piles" of glow at the edge of distributions $B(x, y)$ (column 3) correlate with the place of glows on erythrocytes photo (column 2). It seems, that the cause of the glow is mainly the light diffraction at erythrocytes edges. Then it is logical to assume that at reduction of erythrocytes sizes the relative contribution of the diffraction glow will be higher. I.e. the area of increased brightness relating to the whole erythrocyte area increases with erythrocytes sizes decrease. At the same time the calculated content of hemoglobin (MCH) should decrease. Indeed, the area of the third erythrocyte with minimum diameter is less than the areas of the previous two erythrocytes by a factor of 1.8 each, but hemoglobin content in it is less by a factor of 2−2.7. It should be noted, that for the latter, third erythrocyte, the dark section in its central part is minimum, that corresponds to MCH minimum.

It is possible, that beside the light diffraction on erythrocyte, the „lens effect"plays an important role: erythrocyte, due to its shape and difference of its refraction index from the same parameter for physiological saline, can be examined as a complex combination of the positive and negative lenses. We paid attention to this earlier during experimental study of the light beam transmission through erythrocyte structure [15], as well as during observance of leucocytes „luminescence" phenomenon at a task of the formed blood elements count using static microscopy method [9].

In general, such optical phenomena as erythrocyte diffraction and „lens effect" can act as the cause, resulting in the above mentioned attenuation of brightness relation $\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}$, technically equal to $(1/K_2) = 1.73$.

5. Experimental results

It is showed in section 4, that the static digital spectral microscopy method allows not only to define the individual geometric characteristics of erythrocytes (ICV), but also the individual characteristics of erythrocytes, related to hemoglobin content in them (ICHC, ICH and, as a result, MCHC, MCH, HGB). ICHC parameter was calculated as per formula (14) considering the experimental values of h and relations $\left(\frac{B_{\text{rbc}}(620)}{B_{\text{rbc}}(416)}\right)_{\text{exp}}$ for each examined erythrocyte (coefficient K_{av} is defined in sect 4.3.1). Also, it should be noted, that ICH = ICHC × ICV. As an example, the fragment of photo image of several random erythrocytes is presented in fig. 3, b, their „passport" data are presented in table 5.

As noted in sect. 3.2, summarizing of individual erythrocyte parameters allows to determine their mean values, defined using standard methods. The performed measurements and the corresponding calculations are presented in table 6; they are compared with the same parameters, but observed using flow cytometry method. For the purpose of the article volume reduction, the table 6 includes only results for blood sample № 2, the similar results are also observed for other three examined samples.

Table 6 shows, that static spectral digital microscopy (SSDM) method gives the results, similar to the flow gemocytometry results. At the same time it should be noted, that SSDM allows to obtain not only erythrocytes distribution by volume (fig. 5), but also the hemoglobin distribution by blood sample erythrocytes (fig. 6).

Conclusion

The method of static spectral digital microscopy (SSDM) is proposed for identification and automated count of ery-

throcytes in native blood solution samples. The main feature of SSDM method is the principal opportunity to determine the individual erythrocyte characteristics. Therefore the following new erythrocyte parameters are introduced: ICV (individual cell volume), ICH (individual cell hemoglobin), ICHC (individual corpuscular hemoglobin concentration). At the same time the erythrocytes are examined as different from each other, without any averaging of their parameters. Such approach allows to create the „passport" for each erythrocyte with the list of its metrological parameters.

It is experimentally demonstrated, that generalization of individual erythrocyte characteristics of this blood sample also allows to define the common metrological indices of this sample erythrocytes, such as MCV, MCH, MCHC and RDW. Comparison of these and other parameters of blood erythrocytes, observed using SSDM and flow cytometry methods, indicates their satisfactory agreement. At the same tine SSDM method has an advantage: it allows to get the histograms of ICH and ICHC distribution by erythrocytes. This property of the method can be used as an additional study tool both in hemodiagnostics and in the area of fundamental hematology.

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