



# The effects of incubation media on the assessment of the shape of human erythrocytes by flow cytometry: a contribution to mathematical data interpretation to enable wider application of the method

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## Abstract

Flow cytometry (FC) analysis of erythrocyte shape and related biomechanical properties, such as osmotic fragility, have not moved from a research tool to regular clinical testing. The main reason is existing evidence that various pre-analytical factors influence the mathematical interpretation of the data obtained. With an aim to contribute to the standardization and broaden the use of FC for human erythrocyte shape assessment, freshly prepared peripheral blood erythrocytes isolated from healthy donors were incubated in iso and hypo-osmotic solutions (pure saline, saline with potassium and calcium, and phosphate buffered saline) and examined by FC using values of forward scatter (FSC) and side scatter (SSC). Kurtosis, skewness, Pearson's second skewness coefficient of dissymmetry (PCD), and spherical index, calculated from FSC distributions, were used for the erythrocyte shape evaluation. In all isotonic media FSC distribution and FSC-based morphology parameters showed huge inter-individual and inter-medium variation. With decreasing osmolality, in all media and samples, the size of the erythrocytes increased, and swelling index and kurtosis decreased. However, changes in skewness and PCD were influenced by the medium used and the sample tested. Compared to FSC, SSC signal in isotonic and its change in hypotonic media showed lower inter-individual variation and was not influenced by the type of medium. We propose a spherical index and kurtosis as FSC-based indicators of erythrocyte shape. As more resistant to the influence of the preanalytical treatment, SSC data appeared to be unfairly neglected for the assessment of erythrocyte shape, in comparison to the usually employed FSC data.

**Keywords** Human erythrocytes · Flow cytometry · Osmotic swelling · Morphology · Sphericity · Multimodal distribution

## Introduction

Erythrocyte shape and corresponding deformability are well-known critical parameters for understanding the functionality of these cells for oxygen transport (Diez-Silva et al. 2010). Furthermore, since red blood cells possess life spans of 100–120 days in humans, alteration of their morphology and biomechanical characteristics, also represents a good indicator of their bidirectional interaction with various endogenous and exogenous factors involved in the progression of not just hematological diseases (Greer et al. 2009), but many other acute and chronic pathological states/diseases, such as sepsis, alcoholism, postoperative states, terminal renal failure, thermal injuries, hypophosphatemia, malaria, diabetes mellitus, and intracerebral hemorrhage

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(Piagnerelli et al. 2007; Greer et al. 2009; Diez-Silva et al. 2010).

Among methods for the analysis of erythrocyte morphology at the single-cell level, microscopy is most often used in both research and clinical laboratories. The microscopy related assays are considered as a gold standard but require manual characterization of more than 100 cells per sample by specially trained personnel, which is labor- and time-consuming (Pinto et al. 2019). On the other hand, flow cytometry can investigate, on a single cell level, a large number of cells in a very short period of time (up to 40,000 cells per second) (Mach et al. 2010). Flow cytometry methods have been used in clinical diagnostics of leukocyte disorders for decades (Greer et al. 2009). On the other hand, it is still not routinely used in clinical laboratories for screening of erythrocyte morphology, osmotic behavior, and surface marker expression. The main reason for this is the specific biconcave shape of erythrocytes, which is observed in the bimodal histogram for the forward scattering cross-section signal (FSC), primarily due to the possibility of different cell spatial orientation during the analysis (Pinto et al. 2019), as well as a stretching by hydrodynamic forces (Gienger et al. 2019). However, even in the case of cell types which are conventionally analyzed by flow cytometry for clinical application (e.g. malignant hematopoietic and lymphoid cells) and with technological advancement in terms of the greater multiparameter capabilities of commercial flow cytometers in recent years, strategies to extract maximum information from an  $n$ -dimensional space and to provide clear, simple representation of data, have become a major challenge (Robinson et al. 2012). The real challenge nowadays is to build flow cytometry data “mining tools that extract relevant information in an objective, precise, reproducible, and comprehensive way” (Pedreira et al. 2013). In other words, especially in the case of erythrocytes that possess specific morphological complexity, new procedures for flow cytometer data manipulation, statistical analysis, modelling and interpretation are prerequisites for easier, more objective, and robust interpretation of flow cytometry data both in biomedical research and clinical diagnostic laboratories. Hopefully, these needs are beginning to be recognized as demonstrated by the recent very comprehensive study of Gienger et al. (2019) on the assessment of erythrocytes deformability in their own developed flow cytometer. Such a custom-made flow cytometer allowed detailed estimation of erythrocyte morphology by a mathematical approach that is based on accurate optical modeling with modeling of rheological and mechanical properties of cells in a macroscopic flow geometry (Gienger et al. 2019).

Quantitative data extracted from histograms obtained even in commercially available flow cytometers (i.e. interpeak distance and height) are shown to strongly depend on erythrocyte shape and distributions of cell size (Pinto et al.

2019). These data are already proposed as a clinical marker for altered morphology and deformability of erythrocytes in conditions such as terminal renal failure, diabetes mellitus, sepsis or acute inflammatory state (Piagnerelli et al. 2003, 2007; Won and Suh 2009; Ahlgrim et al. 2013; Yamamoto et al. 2014). The studies mentioned represent the first attempts to standardize flow cytometry for the analysis of erythrocyte morphology, through the introduction of different measures for erythrocyte shape evaluation, such as Pearson’s second skewness coefficient of dissymmetry, kurtosis, and spherical index (Piagnerelli et al. 2007; Ahlgrim et al. 2013). However, these studies were performed using different flow cytometry devices, different measurement protocols (flow rate, number of analyzed cells...), and pre-analytical treatment of erythrocytes samples, so the results could not be directly compared or the analysis could not be widened to other applications. Such data stress the fact that each research or diagnostic laboratory establishes its own “in-house” standardized protocols for the analysis of erythrocytes using flow cytometry, which eventually leads to a huge waste in research activity. Without standardized guidelines for flow cytometry analysis of erythrocytes, one can still need to adjust the experimental steps prior and during the flow cytometry analysis, as proposed by Daskalakis et al. (2014) for the flow cytometric analysis of platelets.

Besides the chosen flow velocity (Ahlgrim et al. 2013), the step regarding erythrocyte sampling and preparation has been also stressed as important for the flow cytometry analysis of erythrocyte osmotic fragility (Nobre et al. 2018). In this study, we employed a constant flow rate that is already proved to induce as low as possible shear stress on erythrocyte deformation in flow cytometric studies for the investigation into the erythrocyte shape with clinical relevance (Piagnerelli et al. 2007). However, we varied the pre-analytical handling with isolated erythrocytes by means of three different incubation media and osmolalities, to widen the potential application of flow cytometry for evaluation of erythrocyte functionality. Specifically, we investigated the effects of widely used, iso- and hypo-osmotic solutions (pure saline, saline with potassium and calcium, and phosphate buffered saline) on healthy erythrocytes, through monitoring the values of forward scatter (FSC) and side scatter (SSC) parameters. In any standard cytometric analysis, it is generally accepted that FSC data (light scattered near the forward direction) are principally related to size, while SSC signals (scattering at a right angle) represent the surface granularity, shape i.e. morphological complexity of a cell (de Grooth et al. 1987; Ghosh et al. 2016). As generally accepted in the literature regarding light scattering by erythrocytes (Piagnerelli et al. 2003, 2007; Won and Suh 2009; Ahlgrim et al. 2013; Yamamoto et al. 2014), from the obtained FSC data different mathematical measures for erythrocyte shape evaluation were assessed. Although unfairly neglected in FC

analysis of erythrocytes, SSC data originate from cell shape/morphology, i.e. inevitably contribute to the determination of complete scattered signal and were one of the research objectives in this study.

Thus, the obtained results might be an important contribution to the mathematical interpretation and standardization of flow cytometry analysis for human erythrocyte quality assessment during blood storage or immediately after blood collection or erythrocyte filtration before transfusion. Also, this study might help in defining adequate hypoosmotic media in which erythrocytes become spherical and suitable for the further testing of erythrocyte antigen expression by flow cytometry, providing the full potential for application of the method.

## Materials and methods

### Erythrocytes

This study was performed at the Institute for Medical Research at the University of Belgrade, Serbia using freshly peripheral blood erythrocytes isolated from buffy coats of five healthy men aged 40–50 years as blood donors, obtained at the Institute of Blood Transfusion and Hemobiology at the Military Medical Academy in Belgrade, Serbia. The buffy coats as blood fraction enriched in leucocytes represent wasted material obtained after fractional centrifugation of whole blood (as described in Vučetić et al. 2018). The peripheral blood erythrocyte parameters (hematocrit, hemoglobin concentration, erythrocytes concentration and volume distribution width), were in the reference range for healthy men (Table 1). Since the study was designed in such a way that it does not include a new method or instrument, and it uses buffy coats which are wasted blood material, the ethics committee of the Institute for Medical Research, University of Belgrade, Serbia granted the exemption for the particular ethical approval.

Centrifugation of the buffy coats containing erythrocytes, at 2500 rpm for 20 min at 4 °C, was performed in Megafuge 1.0R, Heraeus centrifuge (Langensfeld, Germany). After the centrifugation, plasma and leucocytes were carefully discarded by vacuum aspiration. The pelleted erythrocytes were resuspended in isotonic (0.9%, w/v) sodium chloride solution (Hemofarm, Vršac, Serbia), washed twice via centrifugation, and finally resuspended in isotonic NaCl solution (*Natrii chloridi infundibile*; Hemofarm, Vršac, Serbia), phosphate buffered saline pH 7.2–7.4 (PBS; 0.8% NaCl, 0.02% KCl, 0.02% KH<sub>2</sub>PO<sub>4</sub>, 0.115% Na<sub>2</sub>HPO<sub>4</sub>) (Sigma-Aldrich Chemie, Steinheim, Germany), or isotonic Ringer's solution (0.86% NaCl, 0.03% KCl, 0.33% CaCl<sub>2</sub>) (*Natrii chloridi infundibile compositum*; Hemofarm, Vršac, Serbia) to the hematocrit values of 0.6–0.7 L/L. All further analyses were done within three hours of the blood collection.

### Osmotic fragility test

The osmotic fragility of human erythrocytes was determined by the method of Beutler (1990). Briefly, packed erythrocytes (20 µL) were added in tubes containing decreasing concentration of NaCl solution (0.9%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%; 5 mL per tube) and in one tube with distilled water. The tubes were gently mixed and incubated at room temperature for 20 min. Then, the samples were centrifuged at 2000 rpm for 10 min and the supernatants were collected. Optical density at 540 nm (OD<sub>540</sub>) of the supernatant was measured by Ultrospec 3300pro spectrophotometer (AmershamBioscience, Uppsala, Sweden). The extent (%) of hemolysis (% HE) was calculated using the following equation:

$$\%HE = \frac{OD_{540} \text{ in hypotonic NaCl solution}}{OD_{540} \text{ hemolysis in water}} \times 100$$

The osmotic fragility curve was plotted from the %HE values by Boltzmann sigmoidal function using Origin Pro

**Table 1** Basic indices of healthy men peripheral blood erythrocytes

	Erythrocytes (× 10 <sup>12</sup> /L)	Hematocrit (L/L)	Hemoglobin (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	RDW (%)
Reference range <sup>a</sup>	4.52–5.90	0.41–0.53	135–175	80–100	25.4–34.6	310–360	11.0–15.5
Sample 1	4.92	0.45	148	91	30.1	330	11.8
Sample 2	5.10	0.45	158	88	31.0	350	11.3
Sample 3	4.87	0.43	146	88	30.0	340	12.5
Sample 4	4.95	0.46	163	93	32.9	350	12.5
Sample 5	5.01	0.46	154	92	30.7	330	12.3
mean ± SD	4.97 ± 0.09	0.45 ± 1	154 ± 7	91 ± 2	30.9 ± 1.2	340 ± 1	12.1 ± 0.5

Peripheral blood erythrocyte indices were analyzed as a part of the blood donors' regular medical check-ups

<sup>a</sup>According to Greer et al. (2009)

2018 software (Origin Lab Corporation, Northampton, MA, USA). From the plotted curve the concentration of NaCl that induced 50% of hemolysis ( $H_{50}$  values) and slope factor were calculated. The derivative osmotic fragility curves obtained using the principle of hemolytic increment were plotted as well.

### Mechanic fragility test

The mechanic resistance of erythrocytes was determined by a method described in Kostić et al. (2015) adjusted to U bottom 96 well microtiter plate. Erythrocytes suspended in isotonic (0.9%, w/v) sodium chloride solution were additionally diluted 10 times in isotonic (0.9%, w/v) sodium chloride or distilled water to the hematocrit 0.06–0.07 L/L. The diluted erythrocytes suspension was added in six wells of the plate, in a volume of 200  $\mu$ L per well. In three of six wells, two glass beads with diameter of 3 mm were added. Hemolysis of erythrocytes in the wells without beads reflected the level of spontaneous hemolysis. As a measure of total hemolysis, 200  $\mu$ L of hemolysate (erythrocyte suspension with 0.06–0.07% hematocrit, diluted ten times in distilled water) was added in three well. The plates were incubated for 90 min, at room temperature under permanent shaking on Biosan M12-Rocker Shaker (Riga, Latvia). After the incubation, the plate was centrifuged for 10 min, at 2500 rpm, at room temperature. The supernatants in a volume of 100  $\mu$ L were transferred to a new plate and  $OD_{540}$  was read using the microplate reader. Mechanic fragility index (MFI) was calculated using the following formula:

$$MFI(\%) = \frac{OD_{540} \text{ well with mechanical stress} - OD_{540} \text{ well without mechanical stress}}{OD_{540} \text{ well hemolysis in water} - OD_{540} \text{ well without mechanical stress}}$$

### Phase contrast microscopy

The erythrocytes morphology was analyzed by an Olympus CKX 41 inverted phase-contrast microscope (Olympus Europa Holding GmbH, Hamburg, Germany). Photomicrographs were made using Quick PHOTO Camera 2.3 software (PROMICRA, Prague, Czech Republic).

### Flow cytometry

To analyze the morphology and osmotic swelling of human erythrocytes by flow cytometry, a new protocol was introduced. This protocol was based on determining forward scatter (FSC) and side scatter (SSC) parameters of the erythrocytes, after the short time incubation in a series of solutions with decreasing molarity (Table 2). Although the detection system of flow cytometer in forward direction is primarily designed for fluorescent signal and the majority of the

**Table 2** Osmolality of isotonic and hypotonic solutions used for the flow cytometry analysis of erythrocyte osmotic swelling

mM	%	Osmolality (Osmol/kg)		
		0.9% NaCl	PBS	RR
155	100	0.281	0.279	0.284
139	90	0.256	0.251	0.258
124	80	0.227	0.226	0.229
108	70	0.201	0.198	0.202
93	60	0.168	0.171	0.166

Osmolality determined with Osmomat 030 cryoscopic osmometer (Gonotec GmbH, Germany)

PBS phosphate buffered saline pH 7.2–7.4, RR Ringer's solution, (%) percentage of 155 mM isotonic solution

forward scattered laser beam is blocked by the beam stop, some of the forward scattered laser light is still detectable and can be used for data analysis providing certain information on the erythrocytes sample which is already proven in studies of Piagnerelli et al. 2003, 2007; Won and Suh 2009; Alhgrim et al. 2013; Yamamoto et al. 2014; Pinto et al. 2019.

In brief: 5  $\mu$ L of packed erythrocytes was added to 2 mL of isotonic or hypotonic solution. The suspension was incubated at room temperature for 20 min. The erythrocytes forward scatter (FSC) and side scatter (SSC) values were determined by BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lake NJ, USA), using Cell Quest Pro (Becton Dickinson) and NovoExpress (ACEA, San Diego, CA, USA) software. FSC and SSC channels correspond to forward and side scattering at 488 nm. A number of 100,000 events per sample were analyzed using 0.2  $\mu$ L/s (Piagnerelli et al. 2007) flow rate. Under these conditions acquisition speed was approximately 5000 events/s. FSC and SSC were acquired via logarithmic amplification, as Nombre et al. (2018) reported for the flow cytometric analysis of erythrocytes osmotic fragility. The intensities of FSC and SSC from each individual event were detected and assigned to one of 256, 512 or 1024 quantity classes, and presented in FSC/SSC dot plots or in FSC/Events counts and in SSC/Events counts histograms, as relative values in 4 decades logarithmic scale.

### Statistics

Statistical analysis of the results was carried out using IBM SPSS Statistic Version 25 software. Kurtosis and skewness of FSC distribution were determined for the 400–1000 quantity range with an aim to exclude (rarely formed) aggregates and cell debris. Pearson's second skewness coefficient of dissymmetry (PCD) was calculated by the formula:

$$PCD = \frac{3(\text{mean} - \text{median})}{\text{standard deviation}}$$

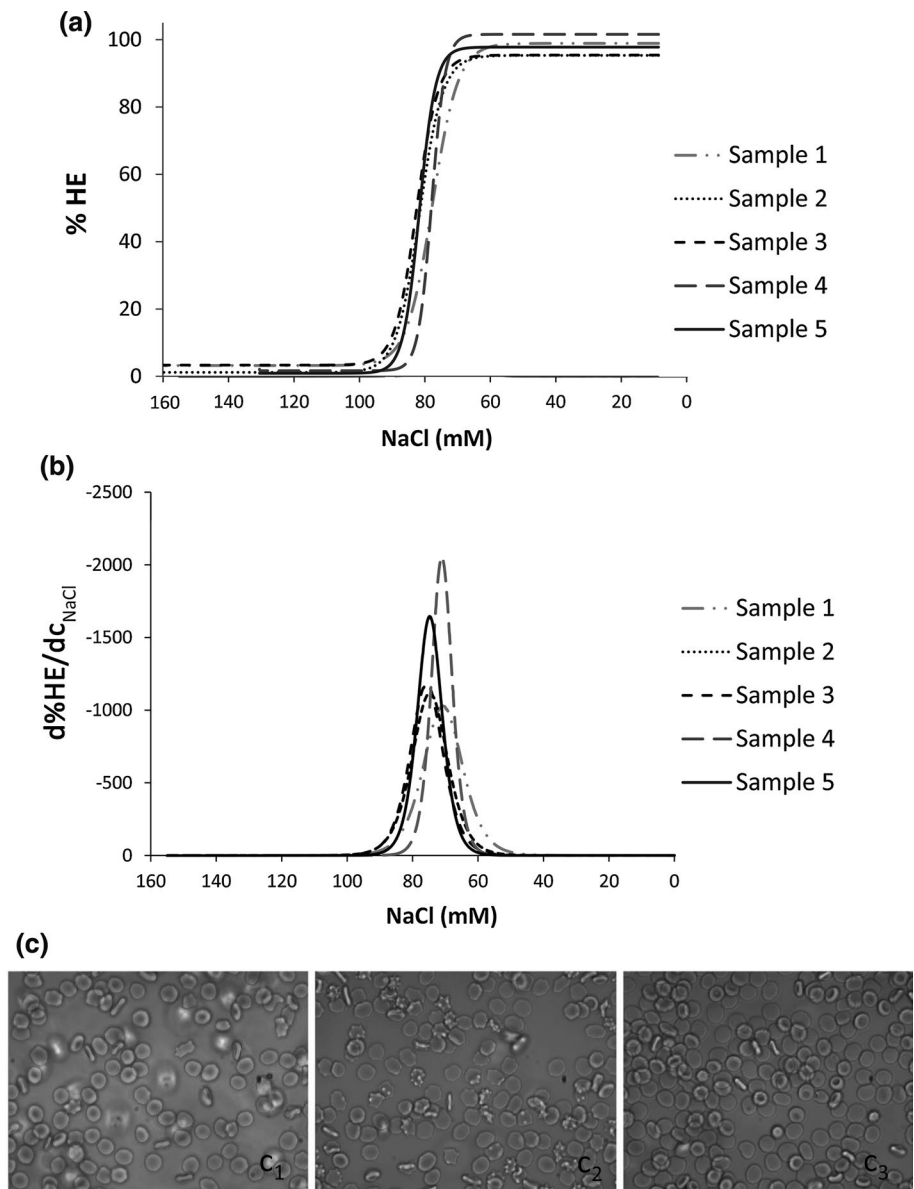
The results are expressed as mean ± standard deviation. The statistical significance of differences between the groups was determined with ANOVA with repeated measures (within-subjects ANOVA). Differences with *p* values of < 0.05 were considered significant. Correlations between analyzed parameters were tested by linear regression analysis.

### Results

Prior to the flow cytometric analysis, osmotic and mechanic resistances of human erythrocytes were estimated by conventional spectrophotometric assays. Cumulative osmotic fragility curves of all erythrocyte samples were of sigmoidal shape (Fig. 1a). Calculated  $H_{50}$  values for erythrocytes from five healthy volunteers were  $73.40 \pm 2.08$  mM (range 71.07–75.71 mM) and were within the reference range for human erythrocytes (Greer et al. 2009). The slope factor for these hemolysis curves was  $3.13 \pm 0.71$  mM<sup>-1</sup> (range 2.10–4.01 mM<sup>-1</sup>).

Calculation of the first derivative of the function (Fig. 1b) showed that the erythrocytes osmotic fragility distribution

**Fig. 1** **a** Cumulative osmotic fragility curves of human erythrocytes. The curves were obtained by fitting the experimental data using a Boltzmann sigmoidal function. **b** Derivative osmotic fragility curves. **c** Representative phase contrast micrographs (magnification ×400) of human erythrocytes in isotonic saline (*c*<sub>1</sub>), isotonic phosphate buffered saline pH 7.2–7.4 (PBS) (*c*<sub>2</sub>), and isotonic saline with addition of potassium and calcium (Ringer’s solution) (*c*<sub>3</sub>)



was close to Gaussian distribution and corresponded to the osmotic distribution of healthy adult erythrocytes (Gordiyenko et al. 2004).

MFI of analyzed erythrocytes was very low (0.1%) indicating that the erythrocytes were not predisposed to lysis when exposed to mechanical stress. Obtained  $H_{50}$  values within the physiological range, along with demonstrated low MFI indicated that the erythrocytes were suitable to be used for the flow cytometry analysis. Additionally, the erythrocytes morphology analyzed using phase-contrast microscopy (Fig. 1c) revealed that in isotonic saline and Ringer's solution erythrocytes were of normocytic morphology (Fig. 1c<sub>1</sub>, c<sub>3</sub>), but in PBS four of five erythrocyte samples demonstrated also the presence of crenated cells, i.e. cells of echinocytes/acanthocytes morphology, besides cells of normal morphology.

Flow cytometry analysis was performed on erythrocytes incubated in isotonic, and a series of corresponding hypotonic saline solutions (Table 2). For all examined erythrocyte samples and incubation solutions, FSC/SSC dot plots, FSC/cell counts and SSC/cell counts were obtained. Representative FSC/SSC dot plots, FSC/cell counts, and SSC/cell counts reflecting extreme inter-individual variations of examined samples of erythrocytes of healthy donors after incubation in PBS are presented in Fig. 2.

The morphological differences in size distribution of erythrocytes resuspended in three different isotonic solutions (pure saline, PBS, and Ringer's solution), determined by phase-contrast microscopy were also observed by flow cytometry. The differences occurring due to normal, physiological inter-individual variations of healthy human erythrocytes were also reflected in FSC and SSC signal distribution (Fig. 3) and the FSC median value for total erythrocytes as an estimated measure of erythrocyte size (Table 3; Supplementary material).

In all isotonic saline solutions, the erythrocyte FSC signal showed a multimodal distribution, with two or more partially overlapped peaks (Fig. 3). The multimodal distribution of the erythrocyte FSC signal occurred due to ellipsoid, biconcave shape of erythrocytes (Piagnerelli et al. 2003, 2007; Ahlgrim et al. 2013), as well as random orientation of the red blood cells around the flow direction and a stretching by hydrodynamic forces (Gibaud 2015; Gienger et al. 2019). A ratio of median values of two maxima with the highest FSC values (cells within the gate in Fig. 2a<sub>1</sub>; Supplementary material) was used as a measure of the erythrocyte sphericity i.e. spherical index. Besides inter-individual variations in the spherical index, the influence of the chemical composition of solvent was also observed, and the erythrocytes resuspended in PBS had the highest spherical index (Table 3).

Beside spherical index, we analyzed three measures of erythrocyte sphericity based on the distribution of FSC signal: kurtosis, skewness and PCD (Piagnerelli et al. 2003, 2007;

Ahlgrim et al. 2013). Results in Table 3 showed that kurtosis and PCD values had enormous inter-individual variability and that the FSC distribution was left-skewed for all erythrocyte samples in PBS, and for four of five samples in isotonic NaCl and Ringer's solution.

The distribution of SSC values of erythrocytes resuspended in any isotonic solution was also multimodal (Fig. 3). More than 70% of erythrocytes had low SSC values, and their SSC distribution was relatively tight and close to the Gaussian distribution in shape. For the erythrocytes with higher SSC values, the SSC distribution was broader. Compared to the FSC values, SSC signal distribution was less influenced by the chemical composition of isotonic solutions (Fig. 3, Table 3).

In the next step of the investigation, erythrocytes were exposed to a series of hypotonic solutions and their swelling was analyzed using flow cytometry. Data obtained showed that 139, 124, 108, and 93 mM solutions did not provoke hemolysis of red blood cells (Fig. 4), but induced their osmotic swelling.

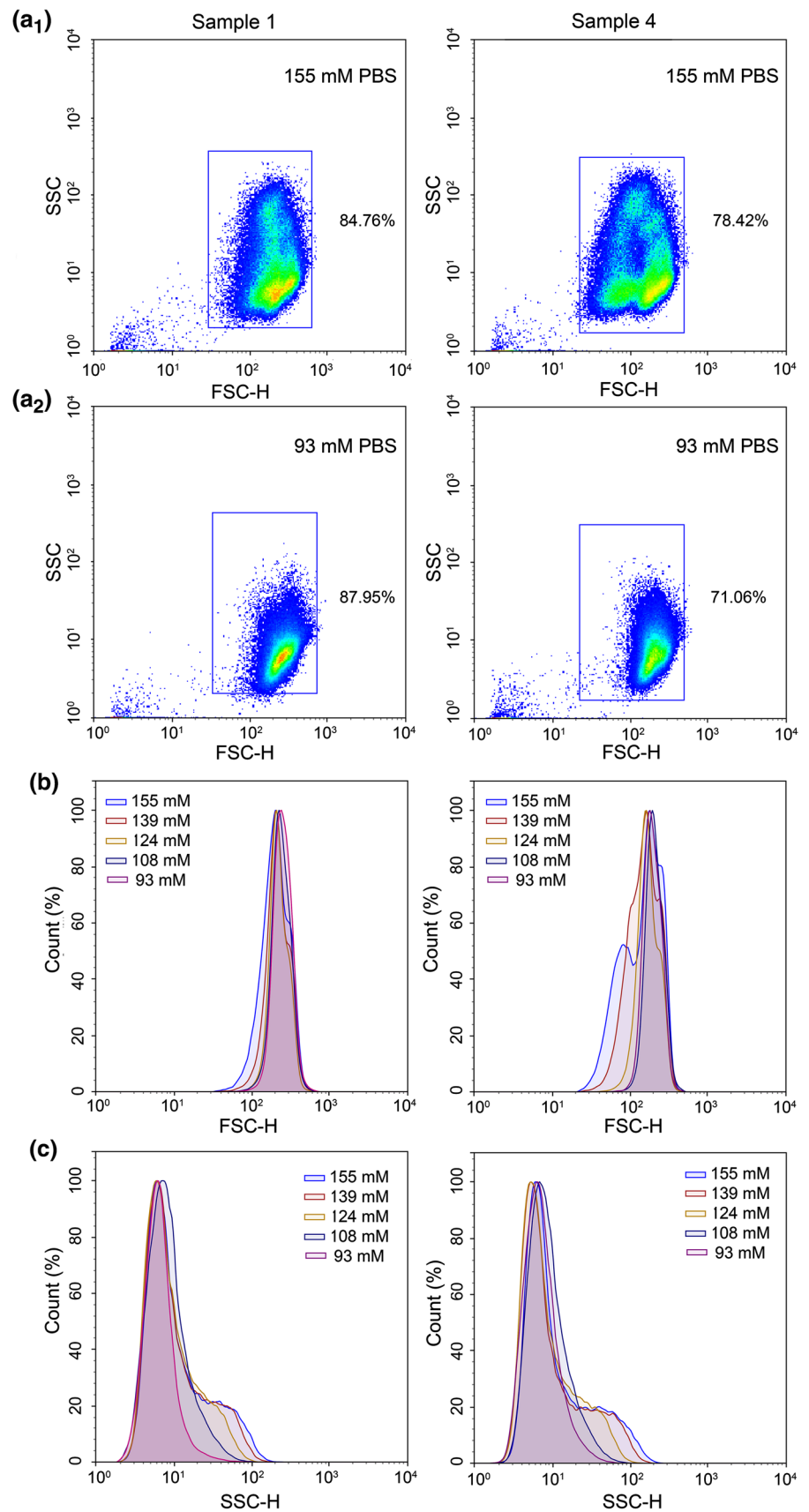
Erythrocyte swelling in a series of solutions with decreasing molarity was accompanied by changes in both FSC and SSC distribution, as shown in representative histograms for erythrocytes incubated in PBS (Fig. 2). The intensity of the observed alterations was influenced by both, the chemical composition of the solvent (saline) and erythrocyte donor. The osmotic swelling impact on FSC values in all examined solvents was reflected as a significant decrease in the spherical index (Fig. 5), a significant increase in the cell size (Fig. 6) and a significant decrease in kurtosis (Table 4). The sphericity index in all examined 124 mM hypotonic solutions was approximately 20% lower than in corresponding isotonic solutions (Fig. 5). In 108 mM solutions FSC distribution was unimodal (Fig. 2), thus the swelling index could not be calculated. Swelling of erythrocytes in the hypotonic solutions was also identified by a gradual increase in their size, where for instance in all 93 mM incubation media the erythrocyte size increased by 30% on average (Fig. 6). Hypoosmotic swelling of erythrocytes in 93 mM solutions was accompanied by a decrease of kurtosis in all incubation media used (with the highest increase in 93 mM NaCl), and a decrease in the PCD values only in 93 mM PBS. A decrease in the negative skew was detected only in PBS (Table 4).

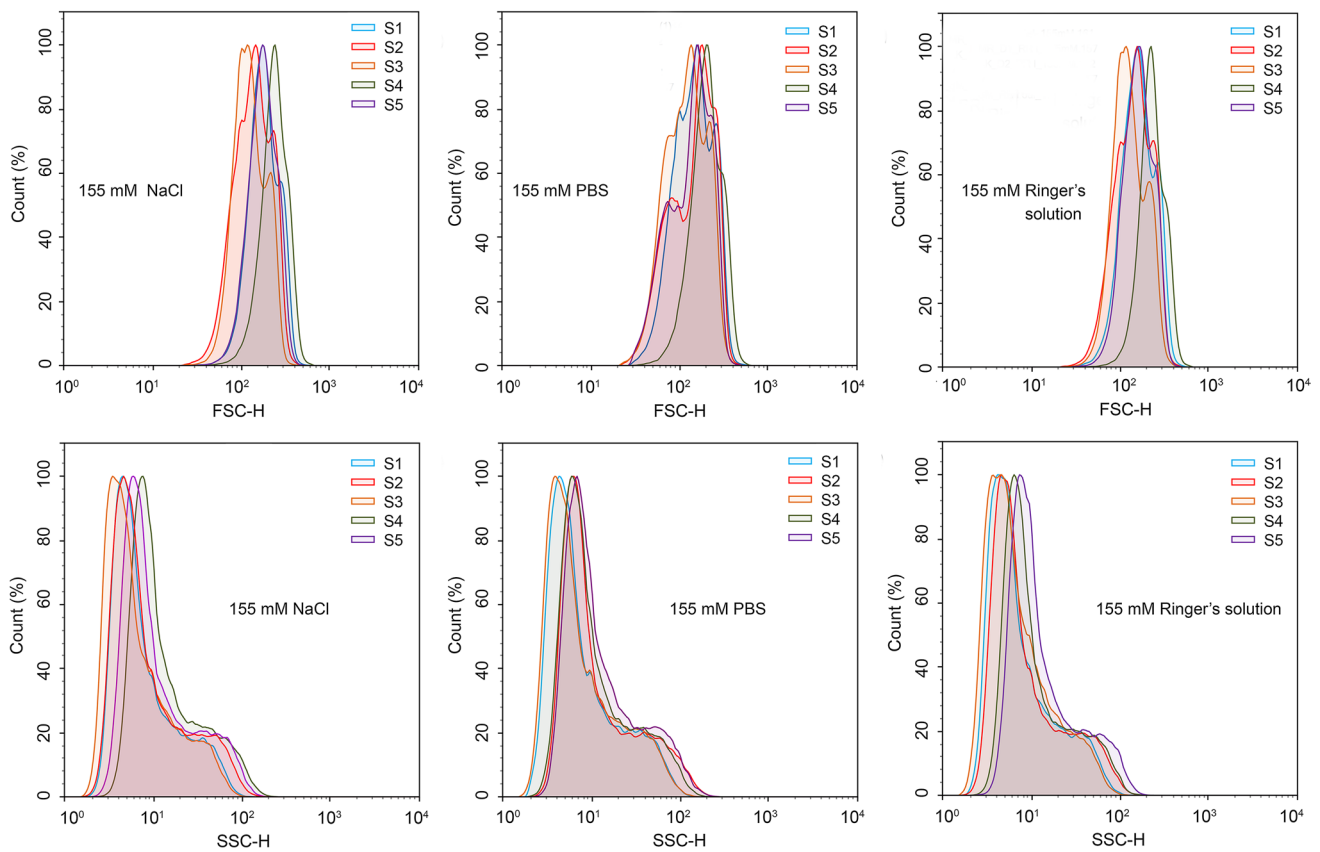
The swelling of erythrocytes was also identified by a gradual increase in the percentage of the cells having lower SSC values and a gradual decrease in the percentage of those with higher SSC values (Figs. 7 and 8). In 93 mM solution, more than 95% was within the cells with low SSC values.

## Discussion

Nowadays almost all specialized clinics and research institutes are equipped with flow cytometers allowing rapid analysis of multiple chemical and physical characteristics

**Fig. 2** Flow cytometry analysis of human erythrocytes: **a** representative flow cytometry FSC/SSC dot plots of human erythrocytes in isotonic (155 mM) (**a<sub>1</sub>**) and hypotonic (93 mM) phosphate buffered saline (PBS) (**a<sub>2</sub>**); intact cells are within rectangular gates. **b**, **c** Representative FSC/cell count and SSC/cell count histograms of intact human erythrocytes in isotonic and hypotonic (139, 124, 108 and 93 mM) PBS pH 7.2–7.4





**Fig. 3** Flow cytometric FSC and SSC distribution of five healthy human erythrocytes samples in three different isotonic solutions: pure NaCl (155 mM NaCl), phosphate buffered saline (155 mM PBS) and NaCl with potassium and calcium (155 mM Ringer's solution)

of single cells (Brown and Wittwer 2000). With regard to the analysis of erythrocytes, the use of flow cytometry has been relatively limited in comparison with the wide variety of flow cytometric techniques for leucocyte characterization (Yamamoto et al. 2014). A few studies have proposed the use of flow cytometry for the examination of expression of selected surface and intracellular antigens of erythrocytes (Rolfes-Curl et al. 1991; Gutowski et al. 1991; Fauré et al. 2019), detection of intracellular parasites in erythrocytes (Vimonpatranon et al. 2019), and investigation of erythrocyte morphology and osmotic properties (Piagnerelli et al. 2003, 2007; Won and Sun 2009; Ahlgrim et al. 2013; Yamamoto et al. 2014). The newest studies even claim the advantage of flow cytometry over conventional methods for the diagnosis of hereditary spherocytosis (Ciepiela et al. 2018; Arora et al. 2018). Such finite application of flow cytometry in erythrocyte examination is primarily due to the specific ellipsoid, biconcave morphology of these cells in mammals, as well as the possibility of their random orientation around the flow direction and a stretching by hydrodynamic forces, consequently resulting in bimodal, multimodal or “shifted” distributions of FSC signal (Rolfes-Curl et al. 1991; van de Bos et al.

1992; Piagnerelli et al. 2003, 2007; Won and Sun 2009; Ahlgrim et al. 2013; Yamamoto et al. 2014, Gibaud 2015, Gienger et al. 2019). An additional issue in flow cytometry analysis is that erythrocytes in peripheral blood are not of the same age and size, and even in a healthy person, an overlapping of FSC signal between the smallest red cells (microcytes) and platelets may occur (Kim and Ornstein 1983). Such distribution of FSC signal might influence fluorescence signal during the analysis of erythrocyte markers expression (Wagner and Fleigel 1998). In this study we have used packed erythrocytes isolated from buffy coats of blood donors, which means a sample of erythrocytes without plasma and leucocytes and platelets. Nevertheless, the basic hematological parameters are determined from the whole blood. As comprehensively reported in Gibaud (2015), there are numerous factors in terms of erythrocyte sample properties that could affect the obtained results in blood analyzers. This observation in the case of blood analyzers could be “translated” to sample behavior in the flow cytometer, i.e. FSC/SSC distribution. Among the “restrictions”, besides the small sample size, cell–cell interaction and viscosity of the tested sample can be the explanation for the nonexistent effect of MCV on the FSC (or SSC)



**Table 3** Flow cytometry analysis of morphological characteristics of erythrocytes resuspended in three isotonic solutions: 0.9% NaCl, phosphate buffered saline pH 7.2–7.4 and saline with addition of potassium and calcium (Ringer's solution)

	FSC					
	Size <sup>a</sup>					
	NaCl	PBS		RR		
Sample 1	611	593		606		
Sample 2	579	558		567		
Sample 3	577	550		537		
Sample 4	556	565		538		
Sample 5	568	557		538		
Mean ± SD	578 ± 18	565 ± 15		557 ± 15		
	Spherical index <sup>b</sup>			Kurtosis <sup>c</sup>		
	NaCl	PBS	RR	NaCl	PBS	RR
Sample 1	1.59	1.80	1.63	0.95	0.31	0.79
Sample 2	1.83	2.15	1.95	0.01	−0.61	−0.25
Sample 3	1.68	1.99	1.78	0.10	−0.77	−0.18
Sample 4	2.03	2.13	1.99	−0.58	−0.83	−0.52
Sample 5	2.01	2.22	2.05	−0.55	−0.81	−0.51
Mean ± SD	1.83 ± 0.20	2.06 ± 0.17 <sup>b</sup>	1.88 ± 0.17	−0.01 ± 0.62	−0.54 ± 0.48 <sup>c</sup>	−0.13 ± 0.54
	Skewness <sup>d</sup>			PCD <sup>e</sup>		
	NaCl	PBS	RR	NaCl	PBS	RR
Sample 1	−0.58	−0.43	−0.37	−0.20	−0.15	0.04
Sample 2	−0.24	−0.22	−0.18	0.02	−0.18	0.05
Sample 3	−0.36	−0.41	−0.24	−0.12	−0.57	−0.08
Sample 4	−0.23	−0.43	−0.28	−0.14	−0.69	−0.25
Sample 5	0.02	−0.13	0.06	0.15	−0.19	0.23
mean ± SD	−0.28 ± 0.22	−0.32 ± 0.14	−0.20 ± 0.16	−0.06 ± 0.14	−0.36 ± 0.26 <sup>e</sup>	−0.00 ± 0.18
	SSC					
	% low SSC <sup>f</sup>			% High SSC <sup>g</sup>		
	NaCl	PBS	RR	NaCl	PBS	RR
Sample 1	73.0	76.8	77.0	26.6	23.0	23.0
Sample 2	83.8	81.1	83.8	15.9	17.9	15.6
Sample 3	76.5	73.6	74.0	23.4	25.9	25.8
Sample 4	72.8	74.4	75.5	27.1	25.5	24.0
Sample 5	78.1	75.29	78.8	21.8	24.6	21.0
Mean ± SD	76.8 ± 4.0	77.1 ± 3.1	77.8 ± 3.4	23.0 ± 4.1	23.4 ± 2.9	21.9 ± 3.5

FSC parameters defined based on the FSC distribution, SSC parameters defined based on the SSC distribution, NaCl 0.9% NaCl, PBS phosphate buffered saline, RR Ringer's solution, PCD Pearson's coefficient of dissymmetry

ANOVA results:

<sup>a</sup>Size: within group  $p=0.041$ ; pairwise comparison: there were no significant differences between used isotonic solutions

<sup>b</sup>Spherical index: within group  $p=0.004$ ; pairwise comparison: Spherical index in PBS was significantly higher than spherical index in and 0.9% NaCl ( $p=0.014$ ) and Ringers' solution ( $p<0.001$ )

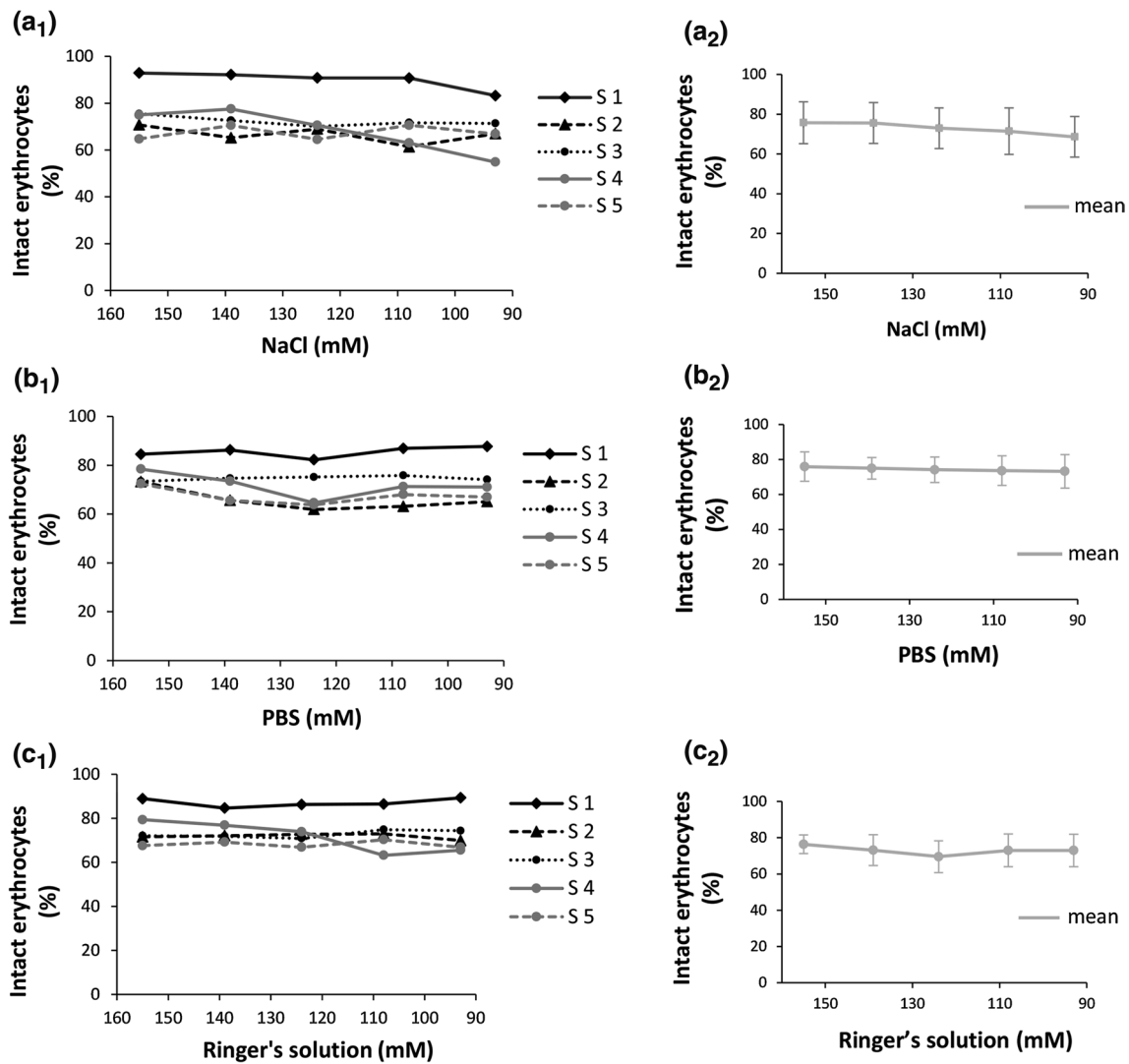
<sup>c</sup>Kurtosis: within group  $p=0.001$ ; pairwise comparison: Kurtosis in PBS was significantly different than kurtosis in and 0.9% NaCl ( $p=0.035$ ) and Ringers' solution ( $p=0.006$ )

<sup>d</sup>Skewness: within group  $p>0.05$

<sup>e</sup>PCD: within subject  $p=0.04$ ; pairwise comparison: PCD in PBS was significantly lower than PCD in Ringer's solution ( $p=0.013$ )

<sup>f</sup>Low SSC: within group  $p>0.05$

<sup>g</sup>High SSC: within group  $p>0.05$



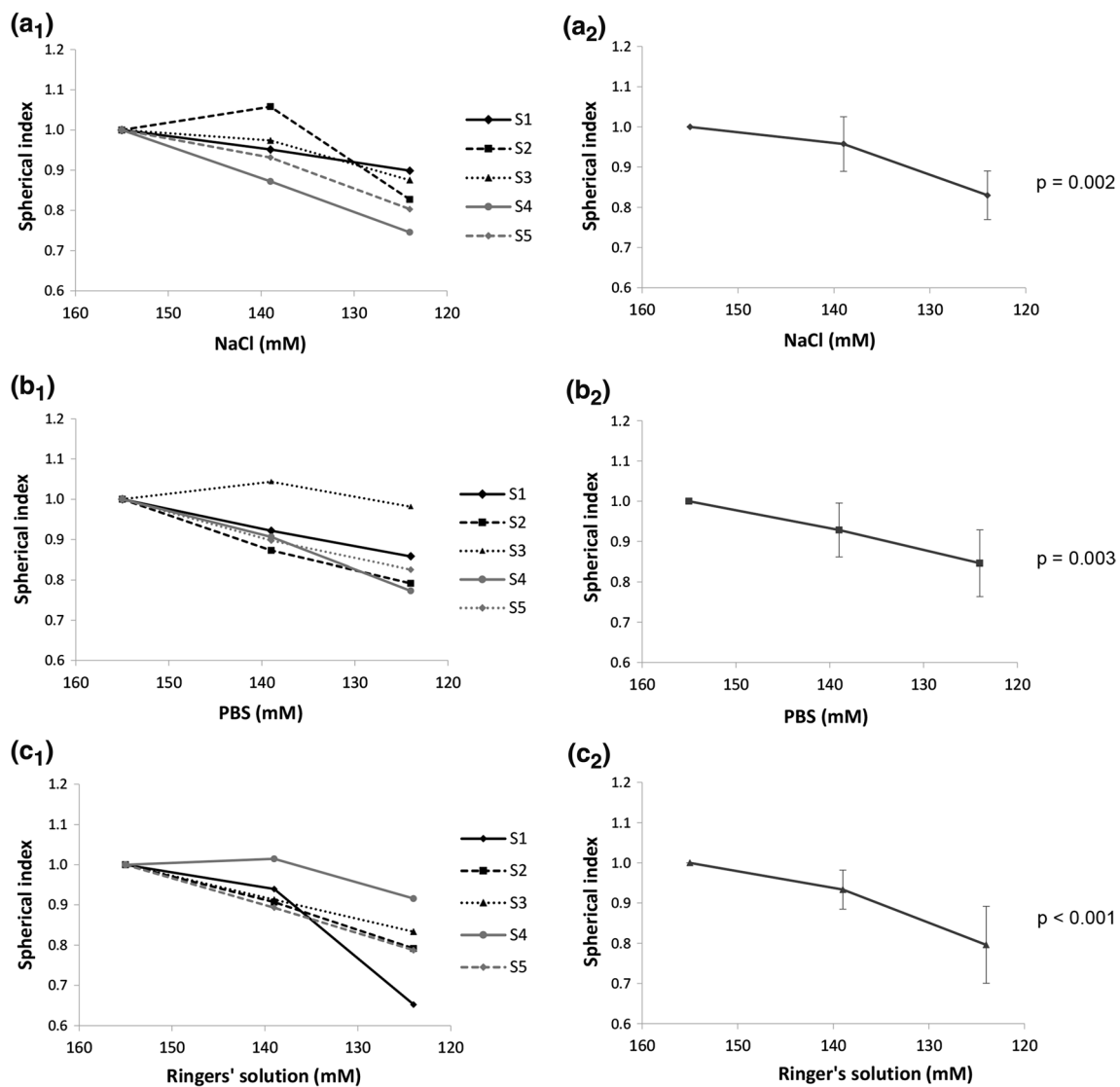
**Fig. 4** Flow cytometry analysis of human erythrocytes: percentages of intact erythrocytes (cells within gates in Fig. 2) in isotonic and hypotonic saline (NaCl) (a), phosphate buffered saline pH 7.2–7.4

(PBS) (b), and saline with addition of potassium and calcium (Ringer's solution) (c). Individual results (a<sub>1</sub>, b<sub>1</sub>, c<sub>1</sub>) and mean  $\pm$  SD (a<sub>2</sub>, b<sub>2</sub>, c<sub>2</sub>) values are given

data. Namely, as demonstrated in Gibaud (2015), the interaction between cells might have an important effect on the orientation phenomenon and in the measurement of the cell volume in the blood analyzer. The interaction of red blood cells happening in the injection tube notably could have a significant effect on the resulting orientation in the micro-orifice. Aside from this indirect effect, some of the recent experimental measurements show that two red blood cells can stay very close during their travel in the blood analyzer (Gibaud 2015). Furthermore, the viscosity of the sample strongly affects cell deformation and reorientation in blood analyzers (Gibaud 2015), which is a parameter known to be of great importance for FSC/SSC distribution in flow cytometers. Since it is well-known that removal of plasma proteins directly affects viscosity and

cell–cell interactions (Kwaan 2010), this could be one of the reasons for the unnoticed, but expected effect i.e. correlation of FSC/SSC distribution with basic hematological parameters in our study.

Piagnerelli et al. (2003,2007) were among the first to provide a clinically relevant application of flow cytometric FSC signal of erythrocytes. In the studies mentioned, the authors used skewness, expressed as Pearson's coefficient of dissymmetry, as a statistic measure of erythrocyte sphericity and introduced a new indicator of erythrocyte morphology determined as a spherical index. Piagnerelli et al. (2003) demonstrated that the erythrocytes of adult patients with diabetes mellitus, sepsis, or terminal renal failure, possess a more spherical shape than the erythrocytes of healthy volunteers. Ahlgrim et al. (2013) revived the knowledge about



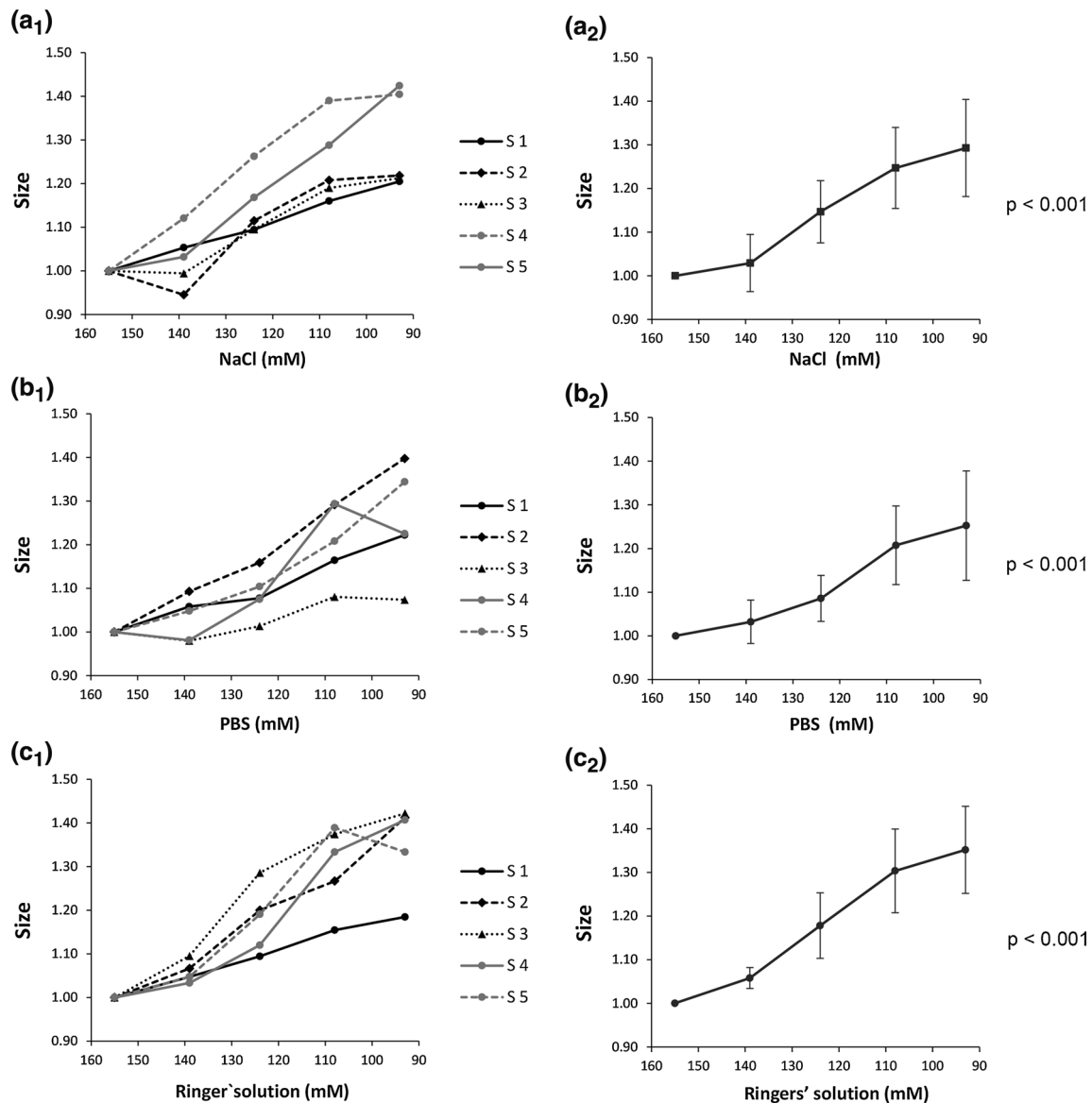
**Fig. 5** Osmotic swelling of human erythrocytes analyzed by flow cytometry: effect of decreasing molarity of pure saline (a), phosphate buffered saline pH 7.2–7.4 (b), and saline with addition of potassium and calcium (Ringer's solution) (c) on erythrocytes spherical index.

The analyzed cells are within gate presented in Fig. 2. The value of spherical index in 155 mM solution is set as 1. Individual results (a<sub>1</sub>, b<sub>1</sub>, c<sub>1</sub>) and mean ± SD value (a<sub>2</sub>, b<sub>2</sub>, c<sub>2</sub>) are given

erythrocyte FSC histograms analysis and proposed kurtosis as a better statistical parameter for the assessment of shape of erythrocytes compared to spherical index.

In line with the existing knowledge on FSC distribution variation induced by different processing of erythrocytes prior to, or during the analysis, such as the use of buffers of various osmolalities, labeling by antibodies or variation of applied velocities (Rolfes-Curl et al. 1991; Ahlgrim et al. 2013), we have examined the influence of commonly used media for erythrocyte pre-treatment (isotonic NaCl, PBS and Ringer's solution) on FSC/SSC distribution data and their implication for the assessment of erythrocyte shape via determination of spherical index, PCD and kurtosis.

Besides inter-donor variability, we showed that the FSC distribution of healthy human erythrocytes varied by analyzed incubation media (isotonic NaCl, PBS and Ringer's solution) from the “true” bimodal distribution [as described in the studies of Piagnerelli and co-workers for native erythrocytes (Piagnerelli et al. 2003, 2007)] to so-called unimodal distribution, but with two or more partially overlapped FSC signals (Ahlgrim et al. 2013). The calculated spherical index was dependent on the chemical composition of isotonic media, and it was highest in isotonic PBS. The spherical index in isotonic PBS obtained in our study ( $1.98 \pm 0.15$ ) was lower than values recorded for erythrocytes in isotonic PBS recorded by Piagnerelli et al. (2007) ( $2.72 \pm 0.47$ ) and



**Fig. 6** Osmotic swelling of healthy human erythrocytes analyzed with flow cytometry: effect of decreasing molarity of saline (a), phosphate buffered saline pH 7.2–7.4 (b), and saline with addition of potassium and calcium (Ringer's solution) (c) on erythrocytes

size. The analyzed cells are within presented gates in Fig. 2. The cell size in 155 mM solution is set as 1. Individual results (a<sub>1</sub>, b<sub>1</sub>, c<sub>1</sub>) and mean ± SD value (a<sub>2</sub>, b<sub>2</sub>, c<sub>2</sub>) are given

more similar to the values given in Ahlgrim et al. (2013) ( $2.17 \pm 0.22$ ) and Piagnerelli et al. (2003) ( $1.95 \pm 0.32$ ). We believe that the lower value sphericity indices demonstrated in our study were not a consequence of shear stress-induced fragmentation of erythrocytes (Cokelet and Goldsmith 1991), since the applied flow rate of 12  $\mu\text{L}/\text{min}$  allows the erythrocytes to rotate freely without deformation (Piagnerelli et al. 2003).

In the present study, we showed enormous inter-individual variations in PCD and kurtosis values. This was different from the study of Ahlgrim et al. (2013) where the PCD and kurtosis were more homogenous. Such a difference could be

explained by different buffer compositions used in the studies. Ahlgrim et al. (2013) worked with PBS/2 mM EDTA with higher osmolality (0.335 Osm/kg), and with cells labeled with “isotype control” mouse IgG-FITC. Obtained values for skewness and PCD different from zero in this study were in accordance with previous reports (Rolfes-Curl et al. 1991; van de Bos et al. 1992; Wagner and Flegel 1998; Piagnerelli et al. 2003, 2007; Ahlgrim et al. 2013; Yamamoto et al. 2014), showing the asymmetric distribution of human erythrocyte FSC signal distribution.

According to Ahlgrim et al. (2013), the sphericity is a distinct individual characteristic of erythrocytes and can be

**Table 4** Hypoosmotic swelling induced changes in human erythrocyte sphericity

	$\Delta$ Kurtosis <sup>a</sup>			$\Delta$ Skewness <sup>b</sup>			$\Delta$ PCD <sup>c</sup>		
	NaCl	PBS	RR	NaCl	PBS	RR	NaCl	PBS	RR
Sample 1	−6.82	−0.92	−1.50	0.38	−0.18	−0.17	−0.25	−0.18	0.01
Sample 2	−3.47	−1.60	−2.67	0.48	0.19	0.24	0.10	−0.17	0.04
Sample 3	−1.35	−1.25	−1.61	0.02	−0.05	0.38	−0.04	−0.61	0.08
Sample 4	−7.08	−1.35	−1.25	1.48	−0.33	−0.10	0.08	−0.75	−0.14
Sample 5	−2.96	−0.82	−0.44	0.67	−0.15	−0.13	0.17	−0.37	−0.16
Mean $\pm$ SD	−4.33 $\pm$ 2.51	−1.19 $\pm$ 0.32	−1.49 $\pm$ 0.80	0.61 $\pm$ 0.54	−0.10 $\pm$ 0.19	0.05 $\pm$ 0.25	0.01 $\pm$ 0.17	−0.42 $\pm$ 0.26	−0.04 $\pm$ 0.11

$\Delta$  Difference between the values of erythrocyte sphericity parameters in isotonic (155 mM) and hypotonic (93 mM) solutions, *PBS* phosphate buffered saline, *RR* Ringer's solution, *PCD* Pearson's coefficient of dissymmetry

ANOVA results:

<sup>a</sup> $\Delta$  Kurtosis: within group  $p=0.049$ ; pairwise comparison: there were no significant differences between used isotonic solutions

<sup>b</sup> $\Delta$  Skewness: within group  $p>0.05$

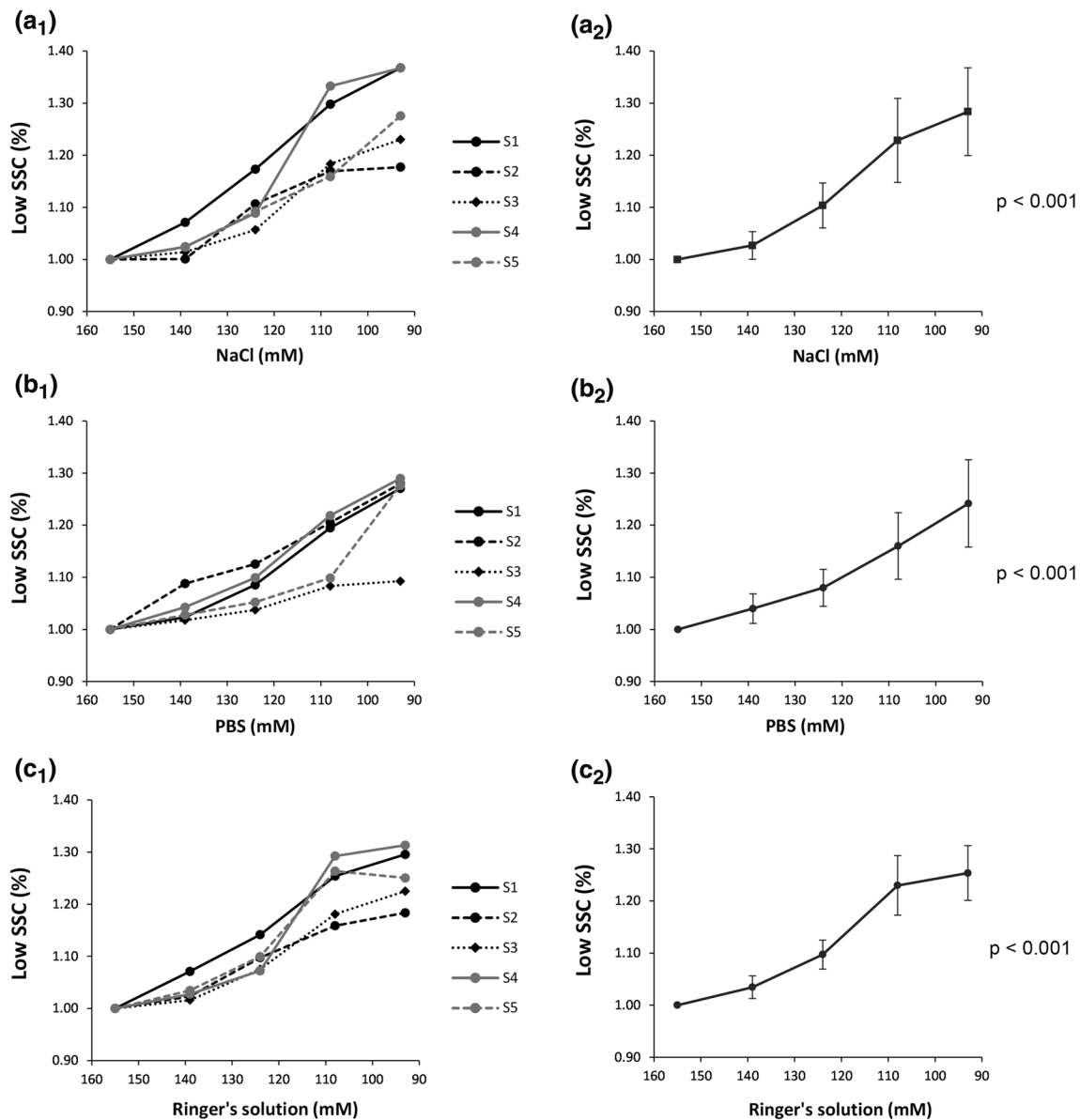
<sup>c</sup> $\Delta$  PCD: within group  $p=0.017$ ; pairwise comparison: pairwise comparison: there were no significant differences between used isotonic solutions

used for longitudinal monitoring of diseases accompanied by changes in sphericity (sepsis, other acute inflammatory states, diabetes, terminal renal failure; Piagnerelli et al. (2007)). Also, Ahlgrim et al. (2003) proposed the monitoring of erythrocyte sphericity as an additional method for the detection of autologous blood transfusion in anti-doping. We believe that the longitudinal monitoring of erythrocyte sphericity might be of importance in the analysis of erythrocyte storage induced lesions and can be used as an additional method in routine quality control of erythrocyte concentrate. Inter-individual differences in erythrocyte indices/parameters calculated from the distribution of FSC signal might not be an exclusive consequence of their intrinsic characteristic. Namely, it is known that time-dependent changes in erythrocytes shape exist (Piagnerelli et al. 2003), as well as the variations in quality assurance of the flow cytometer used (Wagner and Flegel 1998). Although we finished our sample testing within the same period of time, the time-dependent effect, as well as an effect of small distinction in ambient temperature on the FSC based parameters cannot be excluded.

The complementary phase contrast microscopy in our study revealed that PBS induced pronounced changes in the analyzed erythrocyte morphology. The mechanism of PBS induced changes in the organization of cell membrane and cytoskeleton components is not elucidated yet. It is possible that phosphate ions, acting as chelators of divalent cations, influence calcium-dependent regulation of the spectrin/actin interaction (Tanaka et al. 2006). The echinocyte morphology can be induced by calcium depletion with EDTA (Burr Cells (Echinocytes)—LabCE.com 2020), but also by loading the erythrocyte with calcium ionophore A23187 (Reinhart and Chien 1987). The echinocyte transformation might also be induced as a result of the anionic amphiphilic nature of

phosphate ions in PBS (Stasiuk et al. 2009), or by disturbing phosphate transmembrane transport (Reinhart et al. 1986). Regardless of the molecular mechanisms, the observed effect of PBS can be of great importance knowing that it represents the most frequently used buffer for the preparation of cells for flow cytometry analysis. Whether the echinocyte inducing effect of PBS might be eliminated by adding 0.2% albumin, as described by Reinhart et al. (2015) remains to be verified in a future study. Using the advanced configuration of flow cytometry equipped with an imaging system able to capture single-cell at flow, it was recently demonstrated that quantification of the prevalence of spherocytosis could provide an important morphological measure of the quality of stored blood products as well (Pinto et al. 2019).

Back in 1983, Kim and Ornstein (1983) proposed an isovolumetric spherizing of human erythrocyte performed with sodium dodecyl sulfate detergent in very low concentration, as a method to eliminate the flow cytometry signal “errors” caused by the biconcave shape of erythrocytes. The isovolumetric spherizing resulted in a tighter, unimodal, FSC distribution and allowed identification of the simultaneous presence of microcytes and normocytes without overlapping of their FSC peaks. Other research groups used the osmotic swelling of erythrocytes as a method to eliminate the flow cytometry signal “errors”. Thus, osmotic swelling of erythrocytes in 103 mM saline was applied in morphological analysis of erythrocyte chimerism in  $\alpha$ -thalassemic mice (van de Bos et al. 1992). Osmotic swelling of human erythrocytes in hypotonic 0.65% NaCl, 3 mM Tris buffer, pH 7.4 (205–210 mOsm) was necessary to obtain accurate flow cytometric data on the level of surface expression of sialic acid, the level of membrane-bound autologous IgG and IgM antibodies, and identification of senescent cells (Rolfes-Curl et al. 1991). The osmotic swelling precedes hemolysis, and

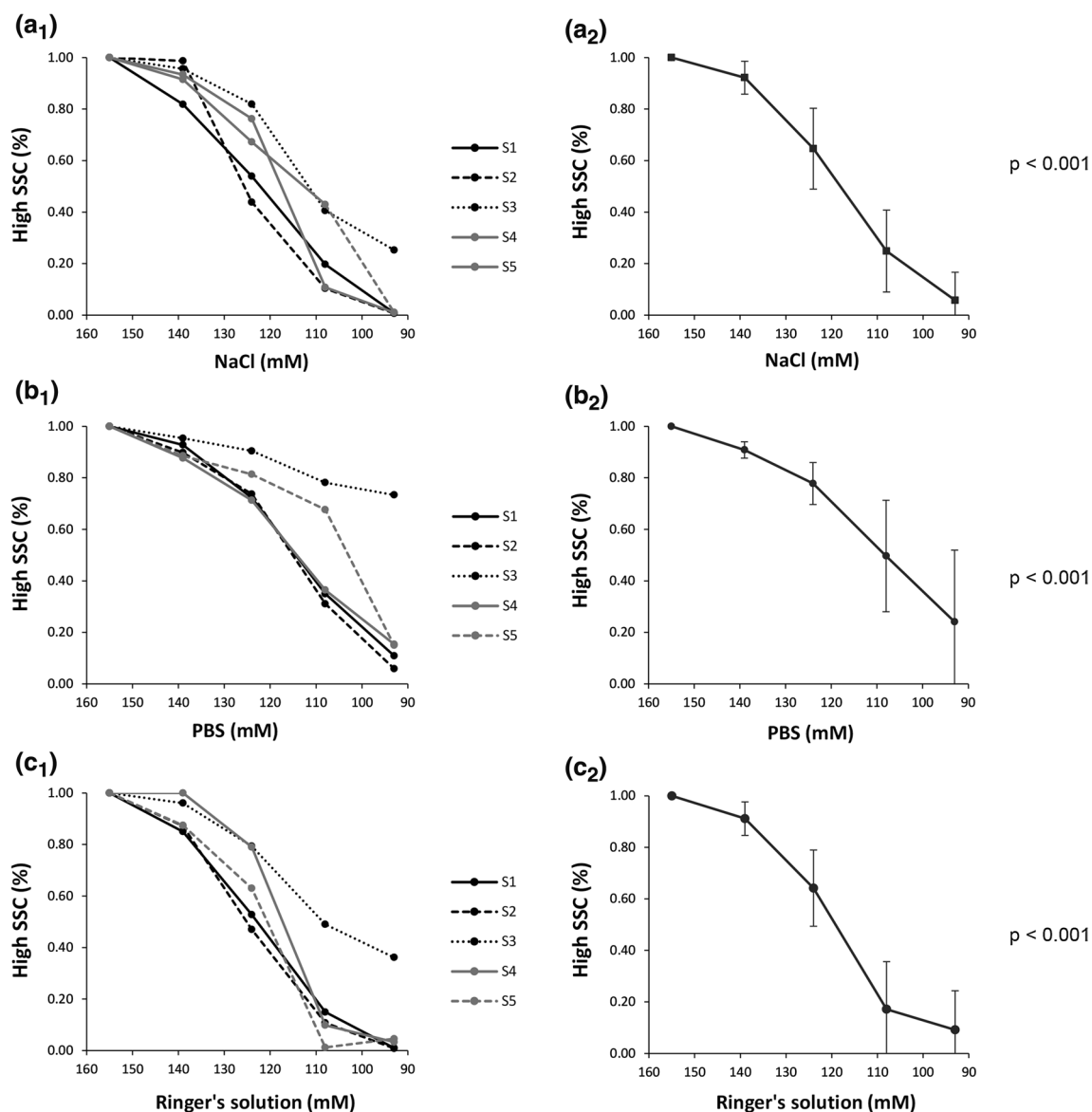


**Fig. 7** Osmotic swelling of healthy human erythrocytes analyzed by flow cytometry: effect of decreasing molarity of saline (a), phosphate buffered saline pH 7.2–7.4 (b), and saline with addition of potassium and calcium (Ringer's solution) (c) on erythrocytes having low SSC

value. The analyzed cells are within gates in Fig. 2. The percentage of erythrocytes with low SSC in 155 mM solution is set as 1. Individual results ( $a_1$ ,  $b_1$ ,  $c_1$ ) and mean  $\pm$  SD value ( $a_2$ ,  $b_2$ ,  $c_2$ ) are given

to prevent a loss of osmotically sensitive cells, the swelling should be performed in non-hemolysis-inducing hypotonic saline. Yamamoto and co-workers (2014) detected a gradual decrease in the number of intact human erythrocytes in serial dilutions of PBS from 100 to 50% (77.5 mM); afterwards the number of intact erythrocytes decreased slowly, then sharply in 40% PBS (62 mM), whereas in 30% (46.5 mM) almost all erythrocytes were lysed. In our study, 100% hemolysis was not detected by flow cytometry even in 40 mM PBS (our unpublished data). Opposite to the results of Yamamoto et al. (2014) we did not detect a decrease in

the number of intact erythrocytes in any of the used media, in concentrations ranging from 155 to 93 mM. The different storage conditions of erythrocytes (e.g. several hours at 25 or 37 °C) or incubation period (e.g. 24 h at 37 °C) and type of anticoagulants used are already well-established pre-analytical variables that are reported to have an impact on the diagnostic capability of flow cytometry for osmotic fragility analysis (Won and Sun 2009; Nobre et al. 2018). Nobre et al. (2018) showed that the type of anticoagulants and storage time might influence the result of flow cytometry analysis of osmotic fragility. However, we think that the discrepancy



**Fig. 8** Osmotic swelling of healthy human erythrocytes analyzed with flow cytometry: effect of decreasing molarity of saline (a), phosphate buffered saline pH 7.2–7.4 (b), and saline with addition of potassium and calcium (Ringer’s solution) (c) of the popula-

tion of erythrocytes having low SSC value. The analyzed cells are within gates in Fig. 2. The percentage of erythrocytes with high SSC in 155 mM solution is set as 1. Individual results (a<sub>1</sub>, b<sub>1</sub>, c<sub>1</sub>) and mean ± SD value (a<sub>2</sub>, b<sub>2</sub>, c<sub>2</sub>) are given

between our results and results of Yamamoto et al. (2014) is not related to the erythrocyte storage time because in both studies the experiments were performed with fresh erythrocytes (stored at 4 °C) and finished within a maximum of 3 h. This discrepancy might be rather a result of working with different flow cytometers and/or a difference in the flow rate. Yamamoto et al. (2014) did not state the flow rate used and did not discuss the well-known effect of shear stress on erythrocyte fragmentation (Cokelet and Goldsmith 1991).

Yamamoto and co-workers (2014) showed that healthy human erythrocytes swelled in hypoosmotic PBS, and the linear increase in their FSC values amounted to 4% in

80% PBS, 10% in 60% PBS and 14% in 50% PBS. This FSC increase was less than that detected in our study (29 ± 10% in 60% PBS; 25 ± 11% in 60% NaCl, and 35 ± 9% in 60% Ringer’s solution). As we mentioned above, we cannot compare the results of these two studies since different experimental set-ups were applied. Piagnerelli et al. (2003) showed that the osmotic swelling of healthy erythrocytes in 60% NaCl buffered with 3 mM Tris, pH 7.4 (202 mOsm) was detected by (1) changing in shape of FSC/cell count histograms from typical bimodal with two distinct peaks to histograms with overlapping peaks, and (2) decreasing the values of sphericity index

by approximately 20%. These authors also showed that erythrocytes of septic patients reach maximal sphericity in isotonic solution (PBS, 286 mOsm) and have a lower capacity to swell in the hypotonic, Tris-buffered, saline (sphericity index decreased by approximately 7% only) (Piagnerelli et al. 2003). In our experimental set-up, in 124 mM the sphericity index decreased by  $17 \pm 5\%$  in pure saline, by  $15 \pm 7\%$  and by  $20 \pm 9\%$  in saline with calcium and potassium, respectively. In our study, in 108 mM used media (198–202 mOsm), FSC signal distribution was unimodal and swelling was detected based on the increase in FSC values for the entire erythrocyte population. The unimodal and more tight distribution of FSC signal resulted in an increase in kurtosis, as was described in Ahlgrim et al. (2013). Although we detected this increase in all incubation media, it is not clear why it was the most prominent in pure saline.

The reports of analysis of erythrocyte flow-cytometric SSC characteristics are very scarce. Trpković et al. (2010) showed that hemolysis induced by fullerene (C60) nanoparticles can be monitored by changes in both, FSC and SSC values, reflecting erythrocyte size and surface topology, respectively. To the best of our knowledge, the effect of osmotic swelling of erythrocytes on SSC data was not previously studied. We showed that, regardless of the used isotonic solution, just over 20% of erythrocytes had high SSC values. The percentage of these erythrocytes decreased with the decrease of the solvent molarity, up to 93 mM solution in which the erythrocytes were almost absent. We believe that the SSC parameter, in line with additional optimization of the molarity and chemical composition of hypotonic solutions, should also be considered as a novel parameter for the analysis of erythrocyte osmotic swelling.

The results of our study and the pieces of evidence available from the literature, prove that there is a need to harmonize the laboratory procedures for the flow cytometry analysis of morphology and osmotic characteristics of healthy human erythrocytes. Besides already known affecting parameters, including the protocol for isolation and preparation of erythrocytes (anticoagulants, washing steps...), the protocol for acquiring flow cytometry data, and the maximum duration of measurements that would not impair erythrocyte physicochemical characteristics, this study revealed that the osmolality and chemical composition of cell buffers, which allows maximum swelling without hemolysis, are variables of great importance as well. This study is a small step contribution toward inter-laboratory standardization and mathematical interpretation of flow cytometry analysis data on osmotic properties of erythrocytes and might help in defining a hypoosmotic solvent in which erythrocytes become spherical and suitable for the analysis of surface antigen expression. However, a larger population of the sample should be foreseen in the future studies.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00249-021-01527-3>.

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**Author contribution** The study conception and design was performed by ID, SM and VI. Material preparation and data collection was performed by ID, AS, SM, IM and IR. Data were analyzed by ID, SM and VI. Contribution to reagents/materials/analysis tool was performed by DV, MK, and DM. The first draft of the manuscript was written by ID and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethics approval** Since the study was designed in such a way that it does not include a new method or instrument, and it uses buffy coats which are wasted blood material, the ethics committee of the Institute for Medical Research, University of Belgrade, Serbia granted the exemption for the particular ethical approval.

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