Image-Based Flow Cytometry and Angle-Resolved Light Scattering to Define the Sickling Process

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Abstract
Red blood cells (RBCs) from sickle cell patients exposed to a low oxygen tension reveal highly heterogeneous cell morphologies due to the polymerization of sickle hemoglobin (HbS). We show that angle-resolved light scattering approach with the use of image-based flow cytometry provides reliable quantitative data to define the change in morphology of large populations of RBCs from sickle cell patients when the cells are exposed for different times to low oxygen. We characterize the RBC morphological profile by means of a set of morphological and physical parameters, which includes cell shape, size, and orientation. These parameters define the cell as discocyte, sickle, elongated, as well as irregularly or abnormal RBC shaped cells, including echinocytes, holly-leaf, and granular structures. In contrast to microscopy, quick assessment of large numbers of cells provides statistically relevant information of the dynamic process of RBC sickling in time. The use of this approach facilitates the understanding of the processes that define the propensity of sickle blood samples to change their shape, and the ensuing vaso-occlusive events in the circulation of the patients. Moreover, it assists in the evaluation of treatments that include the use of anti-sickling agents, gene therapy-based hemoglobin modifications, as well as other approaches to improve the quality of life of sickle cell patients. © 2019 International Society for Advancement of Cytometry

Key terms
computational flow cytometry; red blood cells; sickle cell disease; HbS polymerization; light scattering; precision and personalized patient-oriented medicine

SICKLE Cell Anemia, an inherited red blood cell (RBC) disorder, was defined as the first molecular disease (1). The simple point mutation in beta globin generates a hemoglobin molecule that polymerizes under low oxygen tension changing the discoid morphology of the RBC to the shapes that gave the disorder its name, an altered the cell rigidity, altered cell surface, adhesion, a short life span, and intravascular hemolysis and inflammation (2–8). Hence, the process of morphology change (sickling) of RBC from sickle cell patients (SS-RBCs) is the fundamental pathophysiological hallmark of the vasculopathy, and organ damage that defines sickle cell disease (SCD). Perhaps more importantly, the rate by which sickling takes place is significant. A decrease in the rate of polymerization-induced sickling is thought to have a profound effect on the clinical outcome of patients. Rapid deoxygenation combined with morphology assessment of large numbers of cells to provide high-throughput data assessments of the heterogeneous cell population is therefore important. Microscopy does not provide the information to describe large numbers of highly heterogeneously shaped RBC observed in a typical blood film (9). The blood smearing technique might also lead to alter morphological RBC structures especially for irregularly or abnormally shaped cells, and induces error-prone procedure for the classification of the “true” morphology profile of RBCs. Hence, fixing of
the morphology of the cell population is meaningful. In contrast to light microscopy, conventional flow cytometers (FCs) can detect cells of interest that are fluorescently labeled, and provide automatic cell sorting by capturing the scattered light and fluorescence intensity values (10). However, the optical signature of conventional FCs is based on the light scattered from individual particles at fixed observation angles only (e.g., forward scattering [FSC-A] and side scattering [SSC-A]), and yields limited information about morphology and biophysical properties of cells. Cell segmentation methods such as CellProfiler (11), Fiji (12), CellSegm (13), and deep convolutional neural networks (dCNNs) (14), as well as clustering algorithms based on k-means, t-SNE, and SPADE techniques (15) have been used to analyze cellular structures in the data obtained by light microscopy or flow cytometry. However, the performance of these algorithms to define morphology depends on proper initiation of labeled parameters by hand, and extensive algorithm training, of a large number of bright- and dark-field images. This has not been shown to be practical for the assessment of large populations of RBC with a highly heterogeneous morphology that is different from sample to sample. Imaging flow cytometry (IFC) combines the single cell image-based screening of microscopy with the high-throughput speed of conventional FCs (16–20). IFC can provide information regarding cellular structures, such as cell size, shape, orientation, signal intensity, location, and texture for each cell with high statistical validity through bright- and dark-field channels, which require no staining procedures, and fluorescence channels. In a recent publication, van Beers et al. (21) revealed the first use of IFC to measure normal and abnormal RBCs obtained from bright-field image data under hypoxic condition (2% oxygen) at one time sickling point after the onset of deoxygenation.

In this study, we investigated the changes in time as cells undergo deoxygenation. We aimed to show that a quick and well-defined deoxygenation, morphological fixation, together with a computational Nyström-type meshless discretization morphology framework, can be used to follow the rate of sickling with an imaging FC. This approach improves the automated, high-throughput classification performance of the heterogeneous morphological RBC shapes as they develop in time in SCD blood. The combination of Thickness Minimum, Length, and Area features as a Sickle Index shape feature obtained from a user-defined custom mask in the IFC data enhances the potential of the optimal discrimination between normal, intermediate, and sickle cell region boundaries in the deoxygenated SS-RBCs. Ten thousand events of SS-RBCs deoxygenated for 0, 2, 5, 10, and 20 min. were analyzed with the use of a cell classifier set at a minimum area of 30 μm² to exclude 1 μm nonfluorescent polystyrene beads (speed beads). First, focused cells from all events were selected from the BF channel by the Gradient RMS feature as a criterion of BF Gradient RMS feature above 30 arbitrary units. Focused cells were further masked using a user-defined custom mask, which is a combination of the Range, System, and Object function masks, to reduce noise and artifacts arising from objects that change feature properties resulting from more than one cell in a frame. This user-defined custom mask was visually determined to better fit the actual contour of the BF region as compared to the default M01 mask (Fig. 2), and this approach was used for all subsequent feature calculations. For the determination of a parent population, a bivariate plot of BF Area versus BF Aspect Ratio was used and a region was created to select a single cell population in order to exclude small particles, doublets, and aggregates. This parent population was then used for subsequent assessment of morphological SS-RBC subpopulations and determination of distinguishing feature sets. Details about masks and criterion of features for the classification of deoxygenated SS-RBCs used throughout this article are given in “Results” section.

**Materials and Methods**

**Sample Preparation and Image Data Collection**

Whole blood (50 μl) was added at time zero to 1 ml of buffer (HEPES-buffered saline [HBS] with 2% bovine serum albumin [BSA], pH 7.4) equilibrated in a Tonometer (model 1237, Instrumentation laboratory, Bedford, MA) at a set oxygen tension of 1% oxygen (PO₂ = 8 mmHg). A regular spin (10 s on, 10 s off) cycle was applied to rapidly equilibrate the RBC with the oxygen tension applied. At different time points, 10 μl fractions were automatically collected and fixed by addition to HBS with 2% paraformaldehyde and 0.5% glutaraldehyde. Samples were stored and subsequently measured using the ImageStream® Mark II flow cytometer (Amnis Corporation, Seattle, WA, part of Millipore Sigma). This study was approved by the UCSF Benioff Children’s Hospital Oakland’s IRB. Data representing at least 10,000 intact single RBC were collected based on bright-field (BF) cell images in focus (gated on 30–70 gradient root mean square [RMS]) for events 50–200 Area and 0.3–1.0 Aspect Ratio in Amnis INSPIRE software.

**IFC Data Analysis in IDEAS**

IFC data exploration and analysis software (IDEAS® version 6.2.188.0, Amnis Corporation, part of Millipore Sigma) was used for the determination of parent populations, such as normal, intermediate, and sickle cell region boundaries in the deoxygenated SS-RBCs. Ten thousand events of SS-RBCs deoxygenated for 0, 2, 5, 10, and 20 min. were analyzed with the use of a cell classifier set at a minimum area of 30 μm² to exclude 1 μm nonfluorescent polystyrene beads (speed beads). First, focused cells from all events were selected from the BF channel by the Gradient RMS feature as a criterion of BF Gradient RMS feature above 30 arbitrary units. Focused cells were further masked using a user-defined custom mask, which is a combination of the Range, System, and Object function masks, to reduce noise and artifacts arising from objects that change feature properties resulting from more than one cell in a frame. This user-defined custom mask was visually determined to better fit the actual contour of the BF region as compared to the default M01 mask (Fig. 2), and this approach was used for all subsequent feature calculations. For the determination of a parent population, a bivariate plot of BF Area versus BF Aspect Ratio was used and a region was created to select a single cell population in order to exclude small particles, doublets, and aggregates. This parent population was then used for subsequent assessment of morphological SS-RBC subpopulations and determination of distinguishing feature sets. Details about masks and criterion of features for the classification of deoxygenated SS-RBCs used throughout this article are given in “Results” section.
Angle-Resolved Light Scattering Technique, Muller Boundary Integral Equation

The analysis of a light scattering pattern by an individual RBC has been reported by using various electromagnetic numerical methods (10, 22–29) and integral equation methods. In this article, a boundary integral equation method (Muller Boundary Integral Equation [MBIE]), which enables fast and efficient procedures for spherical and nonspherical biological particles with a Nyström-type meshless discretization, was used. When the boundary is smooth and has a regular 2π-periodic parametrization, the Nyström-type discretization (30–33) using trigonometric approximation of integrand functions is convenient, as it leads to exponential convergence of numerical solution. The time factor $e^{-jwt}$ is assumed and suppressed throughout the article. The numerical code developed in the work is available upon request from the corresponding author.

The boundary integral equation method (Muller Boundary Integral Equation [MBIE]), which enables fast and efficient procedures for spherical and nonspherical biological particles with a Nyström-type meshless discretization, was used. When the boundary is smooth and has a regular 2π-periodic parametrization, the Nyström-type discretization (30–33) using trigonometric approximation of integrand functions is convenient, as it leads to exponential convergence of numerical solution. The time factor $e^{-jwt}$ is assumed and suppressed throughout the article. The numerical code developed in the work is available upon request from the corresponding author.

The shapes of SS-RBCs are modeled as a homogeneous dielectric particle with boundaries by means of the Gielis formula (34):

$$r(\theta) = \left( \frac{1}{a} \cos \left( \frac{m\theta}{4} \right) \right)^{\alpha} + \left( \frac{1}{b} \sin \left( \frac{m\theta}{4} \right) \right)^{\beta} ; \quad a, b > 0.$$  

where $r$ is the radial coordinate; $\theta$ is the polar angle; $a > 0$ and $b > 0$ are the size coefficients of the shape structure; $m$ is the radial symmetry number; and $\alpha, \beta,$ and $\gamma$ are shape coefficients. Although parameters in the formula have a substantial meaning on the modeling of the shapes, they are not simple numbers to provide more insight into our understanding of each individual coefficient (34) combined to create the shape. However, broadly, the radial symmetry (or the number of vertices) is adjusted by the parameter $m$, inside the sine and cosine functions. By changing the value of $m$, the number of sides of a shape can be adjusted. The parameter $\alpha$ determines sharpness or flatness of corners and convexity of sides in the cell shape. The parameters $\beta$ and $\gamma$ determine whether the structure is inscribed or circumscribed in the shape (e.g., $\beta, \gamma < 2.0$ for sub-polygon or $\beta, \gamma > 2.0$ for super-polygon structures) (35). Thus, the modeling of the cell structure is depending on the combination of the $a, b, m, \alpha, \beta,$ and $\gamma$ coefficients in the formula. The MBIEs for unknown equivalent currents at the boundary of scatterer (36) are formulated as follows:

$$ \int_{L} K_{11}(\bar{r}, \bar{r}') U(\bar{r}') \, ds' = \int_{L} K_{12}(\bar{r}, \bar{r}') V(\bar{r}') \, ds' = U^{\text{inc}}(\bar{r}).$$

$$ \frac{p_{1} + p_{2}}{2p_{e}} V(\bar{r}) + \int_{L} K_{21}(\bar{r}, \bar{r}') U(\bar{r}') \, ds'$$
$$- \int_{L} K_{22}(\bar{r}, \bar{r}') V(\bar{r}') \, ds' = V^{\text{inc}}(\bar{r}), \quad \bar{r} \in L.$$  

where $\bar{r} = (x, y)$ and $\bar{r}' = (x', y')$ are the integration and observation points, respectively. $U$ corresponds to the field component $E_{z}$ in the inner domain $D_{i}$. Moreover, $V(\bar{r}')$ is the limit value of the normal derivative of the total field on the closed cross-section contour $L$ of the scattered object from the inner side of it, $d'$ is the elementary arc length, and the constants are $p_{1e} = 1/\mu_{0e}$ in the E-polarization case. The primary field and its normal derivative are defined as $U^{\text{inc}}$ and $V^{\text{inc}}$, respectively.

The kernel functions of the MBIE are defined as follows:

$$K_{11}(\bar{r}, \bar{r}') = \partial G_{1}(\bar{r}, \bar{r}')/\partial \bar{n}' - \partial G_{2}(\bar{r}, \bar{r}')/\partial \bar{n}'.$$  

$$K_{12}(\bar{r}, \bar{r}') = G_{1}(\bar{r}, \bar{r}') - (p_{1} / p_{e}) G_{2}(\bar{r}, \bar{r}').$$

$$K_{21}(\bar{r}, \bar{r}') = \partial^{2} G_{1}(\bar{r}, \bar{r}')/\partial \bar{n}' \partial \bar{n}' - \partial^{2} G_{2}(\bar{r}, \bar{r}')/\partial \bar{n} \partial \bar{n}'.$$

$$K_{22}(\bar{r}, \bar{r}') = \partial G_{1}(\bar{r}, \bar{r}')/\partial n - (p_{1} / p_{e}) \partial G_{2}(\bar{r}, \bar{r}')/\partial n.$$  

where $G_{1}(\bar{r}, \bar{r}') = (i/4) H_{0}^{(1)}(k_{o} r_{e} \bar{r} / r_{e} \bar{r}')$ are the Green functions of the corresponding media; $k_{e} = k_{o} / \sqrt{\varepsilon_{0} \mu_{0}}$ and $k_{i} = k_{o} \sqrt{\varepsilon_{0} \mu_{0}}$, where $k_{o}$ is the free-space wavenumber, and $\rho = |r - r'|$ is the distance between the points $r$ and $r'$, and $H_{0}^{(1)}(.)$ is the Hankel function of the first kind and zero-order.

For the case of piece-smooth contour (37,38), we approximated unknown equivalent currents in Eqs. (4)–(7) with a step-constant function. Following Smotrova et al. (32), we apply the following quadrature formula:

$$\int_{0}^{2\pi} \ln \left( \frac{4 \sin \frac{\theta}{2}}{\theta} \right) f(t, \theta) \, d\theta \approx \sum_{l=0}^{2N-1} P_{l}^{N}(t) f(t, \theta).$$  

where $f$ has a logarithmic singularity, and

$$P_{l}^{N}(t) = \frac{2\pi}{N} \sum_{m=1}^{N-1} \cos \left( \frac{m(t-t_{l})}{m} \right) - \frac{\pi}{N^{2}} \cos \{N(t-t_{l})\};$$

$$t_{l} = \pi l / N, \quad l = 0,1, \ldots , 2N-1.$$  

$$\int_{0}^{2\pi} f(t, \theta) \, d\theta \approx \left( \frac{\pi}{N} \right) \sum_{l=0}^{2N-1} f(t, \theta).$$

We obtain the following matrix equation block after such a discretization:

$$\left( I + K \right) \left( \begin{array}{c} U \vspace{0.1cm} \\
V \vspace{0.1cm} \end{array} \right) = \left( \begin{array}{c} U^{\text{inc}} \vspace{0.1cm} \\
V^{\text{inc}} \vspace{0.1cm} \end{array} \right).$$

where the vectors of unknowns are $U = \{ U(t) \}_{l=0, \ldots, 2N-1}$ and $V = \{ V(t) \}_{l=0, \ldots, 2N-1}$, and $I$ is a unit $4N \times 4N$ matrix, and the matrix $K$ has a block structure:

$$K = \left( \begin{array}{cc} K_{11}(t_{l}, t_{l}) & K_{12}(t_{l}, t_{l}) \\
K_{21}(t_{l}, t_{l}) & K_{22}(t_{l}, t_{l}) \end{array} \right).$$

where $k, l = 0, 1, \ldots, (2N-1)$. In Eqs. (4)–(11), the quantities $(\bar{r}, \bar{r}'), (\bar{r}', \bar{r})$, and $(n, n')$ are the scalar products of the corresponding vectors. Assuming that the variables $t$ and $\theta$ in
parametric form correspond to $\vec{r}$ and $\vec{r}'$, the distance between two points on the boundary contour is $\rho = \left( (x(t) - x(t'))^2 + (y(t) - y(t'))^2 \right)^{1/2}$, and the outer normal vector in the boundary is $\vec{n} = \left( \frac{\alpha}{\rho}, \frac{\beta}{\rho} \right)$, where $R(t)$ is the Jacobian matrix as $I(t) = \sqrt{(dx/dt)^2 + (dy/dt)^2}$.

Each $K_{ij}$ is a $2N \times 2N$ matrix:

$$K_{ij} = \left[ P_{ki}(t)K_{ji}(t) + \frac{\pi}{N}K_{ji}(t)\right] I(t). \quad (13)$$

Functions $K_{ij}^{1,2}$ take the following values:

$$K_{ij}^1 = \begin{cases} K_{ij}^1 = 0, & \text{if } \rho = 0, \\ \frac{1}{4\pi} \left[ J_0(k_i\rho) - \frac{P_i}{P_e}J_0(k_e\rho) \right], & \text{otherwise} \end{cases} \quad (15)$$

$$K_{ij}^2 = \begin{cases} k_i^2J_2(k_i\rho) - k_e^2J_2(k_e\rho) \frac{d\rho}{\rho^2}(\vec{\rho} \cdot \vec{n}) \frac{d\rho}{\rho^2}, & \text{otherwise} \end{cases} \quad (16)$$

$$K_{ij}^2 = \begin{cases} i \frac{1}{4\pi} \left[ \frac{1}{4\pi} \left[ \frac{P_i}{P_e} \right] H_0^{(1)}(k_i\rho) - H_0^{(1)}(k_e\rho) \right], & \text{otherwise} \end{cases} \quad (17)$$

$$K_{ij}^2 = \begin{cases} K_{ij}^2 = 0, & \text{otherwise} \end{cases} \quad (18)$$

For the far-field computation, we use the asymptotic expansion of the Hankel function for large arguments (39), leading to the following expression:

$$U_{\text{far}}(\theta, \varphi) = \int_{1}^{\infty} e^{-ik_e(\cos \theta + \sin \phi)} \left\{ ik_e(\vec{n} \cdot \vec{r}) U(r) + \frac{P_e}{\partial \rho} U(r) \right\} dl. \quad (21)$$

where $\theta$ is a scattering angle and $\phi$ is an angle of incidence.

An angle-resolved light scattering pattern is calculated as follows:

$$I_{\text{far}}(\theta, \varphi) = \frac{|U_{\text{far}}(\theta, \varphi)|^2}{4k_e}. \quad (22)$$

Since the area of a side scattering pulse (SSC-A) in FC is detected at approximately $90^\circ$ to the laser beam at large angles (13–26$^\circ$), a side scattering pattern is calculated as:

$$\text{SSC-A}(\varphi) = 10\log \left\{ \sum_{\theta = 90^\circ + \varphi - 13^\circ}^{90^\circ + \varphi + 13^\circ} I_{\text{norm}}(\theta, \varphi) \right\}, \quad (23)$$

where

$$I_{\text{norm}}(\theta, \varphi) = \frac{I_{\text{far}}(\theta, \varphi)}{\min I_{\text{far}}(\theta, \varphi)}, \quad \varphi \in [0^\circ, 360^\circ]. \quad (24)$$

**RESULTS**

**Optical Modeling of SS-RBCs**

From the image data collected, six representative cell morphological states were chosen from a SS-RBC population obtained from IFC bright- and dark-field data, reproducing them with Eq. 1 (see Fig. 1A). RBC shape models ranging from biconcave to elongated, and to typical sickle cell shapes are modeled as a homogenous dielectric particle with losses, whose shape is determined by choosing the corresponding $a$, $b$, $m$, $\alpha$, $\beta$, and $\gamma$ coefficients as follows: Discocyte ($a = 0.99$, $b = 0.99$, $m = 2$, $\alpha = 1$, $\beta = 8$, and $\gamma = 8$), Granular ($a = 1.2$, $b = 2.4$, $m = 5$, $\alpha = 3$, $\beta = 24$, and $\gamma = 4$), Holly-leaf ($a = 1.2$, $b = 2.4$, $m = 7$, $\alpha = 1.3$, $\beta = 1.3$, and $\gamma = 2.1$), Echinocyte ($a = 2.1$, $b = 2.1$, $m = 7$, $\alpha = 3$, $\beta = 6$, and $\gamma = 6$), Elongated ($a = 1.07$, $b = 1.07$, $m = 2$, $\alpha = 24$, $\beta = 128$, and $\gamma = 128$), and Sickle ($a = 1$, $b = 1$, $m = 2$, $\alpha = 36$, $\beta = 128$, and $\gamma = 512$) (see Fig. 1B). We performed numerical electromagnetic simulations based on the light scattering technique in order to investigate the relationship between cell morphology (e.g., cell shape, size, and orientation) and refractive index on the scattering pattern. Numerical simulations were carried out in the wavelength of 632.8 nm. The refractive index spectra of SS-RBCs were used in the range of $n_r = 1.6 \times 10^{-3}$ and
1.36 \leq n_r \leq 1.41 \text{ whereas the refractive index of the host medium, } n_e \text{ was considered to be 1.335. We assume that dielectric function, } \varepsilon_i(\lambda) \text{ has a nonzero positive imaginary part (} \sqrt{\varepsilon_i} = n_r + i\eta). \text{ Cell rotations were taken in the range of 0° and 360° with 4° interval range for each SS-RBC structure. In Figure 1C, we calculated the angular far-field distributions of the scattered E-polarized light for three sample cell orientations: } \varphi = 0° \text{ (face-on incidence), } \varphi = 45° \text{ (oblique incidence), and } \varphi = 90° \text{ (rim-on incidence). The simulation results indicate that the scattering cross-section values have a remarkable change depending on cell types and orientation in the range of side scattering (SSC-A) pattern.}

**Figure 1.** Representative IFC images and simulated optical signature of SS-RBCs upon deoxygenation. (A) Representative images of deoxygenated SS-RBCs acquired by bright-field (BF/Ch-01) and dark-field (SSC/Ch-06) channels in IFC, obtained using the IDEAS® software. BF objects were masked using an investigator-defined custom mask (System 80, Object Tight, Range-Area 350–1,200, and Range-Aspect Ratio 0.3–1). Scale bar = 7 μm. (B) Two-dimensional cross-sections of SS-RBC shape models obtained from Eq. (1). (C) The simulated light scattering profile of individual SS-RBCs with the use of MBIE method at } n_r = 1.38 \text{ and E-polarization conditions. [Color figure can be viewed at wileyonlinelibrary.com]}

Sickling Defined by Image-Based and Light Scattering Flow Cytometry
Finding an Optimal Shape Quantification Factor for Deoxygenated SS-RBCs in IFC

In order to determine the optimal settings for normal, intermediate, and sickle cell region boundaries in deoxygenated SS-RBCs, a user-defined custom mask including the Range, System, and Object function masks was used to increase the possibility of analyzing one cell per frame in BF channel whereas a default M01 mask contains a small particle in the masked area, resulting in distorted feature values (Fig. 2A). The Range mask was created using the System 80, Range Area 350–1,200 pixels and Range Aspect Ratio 0.3–1 masks to reach a tighter fit to the actual contour of the BF region, and thus providing an accurate measurement of cellular information for the individual cell. This range is slightly different as compared to the RangeSystem mask at van Beers and coworkers (18) which indicates that Area varies between 350 and 5,000 pixels, and Aspect Ratio is in the range of 0–1. The combination of Shape Ratio (that is, minimum thickness of the masked object divided by the length) and Area features as a Sickle Index shape feature (= Shape Ratio×1,000/Area) enhances the selection of the “true” sickle cell region boundary from other delineated SS-RBC phenotypes (Fig. 2B). The well-known highly heterogeneous morphologies of SS-RBC under low oxygen can be distinguished as sickle, elongated, intermediate, and normal cells in the range of 0–20 Sickle Index whereas the intermediate cells would be located in the sickle cell region boundary at Shape Ratio versus Circularity (that is, the degree of the deviation of mask from a circle) feature (Fig. 2C). The quantitation of percent morphological RBC subpopulations were analyzed with the use of “Shape Ratio versus Circularity” feature obtained from the default M01 mask and the user-defined custom mask as Shape RatioDefault (M01) Mask, and Shape RatioCustom Mask legends, and Shape Ratio feature with a cut-off range (21) as a Shape RatioCut-off level, Custom Mask legend and “Sickle Index versus Circularity” feature obtained from the user-defined custom mask as a Sickle IndexCustom Mask legend (Fig. 3A). Candidate sickle cell region boundaries were selected at Shape Ratio < 0.6 and Circularity < 10 on the default M01 and user-defined Range mask, and at Shape Ratio feature with a cut-off of below 0.5 on the combination of default and custom BF mask (21) and at Sickle Index < 7 and Circularity < 7 on the custom BF mask as our proposed shape feature criteria for deoxygenated SS-RBCs over a period of 0, 2, 5, 10, and 20 min. The “Ratio Sickled to Normal” is calculated as the percentage of sickled cells divided by the percentage of normal cells on the defined mask. We observed that the Sickle Index feature enables better identification of sickle cells especially for SS-RBCs after 5 min. of deoxygenation. Moreover, we performed the “Ratio Abnormal to Normal” analysis, which is calculated as the percentage of abnormal cells (thus, sickle + intermediate

Figure 2. Masking and gating strategy to identify normal, intermediate, and sickle cell region boundaries in deoxygenated SS-RBCs. (A) Representative BF images of SS-RBCs overlaid with the respective boundary masks (cyan color). The left column shows the BF image with no mask. The second column shows the default M01 mask, which contains a small particle in the masked area. The third column reveals the user-defined custom mask, Range(System(Object(M01, Ch01, Tight), Ch01, 81), 350–1,200, 0.3–1). This custom BF mask reaches a tighter fit to the actual contour of the BF region, resulting in an accurate measurement of cellular information for the individual cell. (B) Normal, intermediate, and sickle cell region boundaries are identified with the use of “Shape Ratio versus Circularity” and “Sickle Index versus Circularity” features obtained from the user-defined custom BF mask. (C) The representative sample of Shape Ratio and Sickle Index feature values are shown in the upper left side of each BF image. [Color figure can be viewed at wileyonlinelibrary.com]
cells) divided by the percentage of normal cells obtained from the defined mask, in Figure S1. It is noted that at time zero, when the cells are not exposed to low oxygen, around 10% and 25% of the cells have sickle and abnormal (sickle + intermediate) cell structures obtained from the criterion of our proposed feature, respectively. However, the difference between sickle and abnormal cell region boundaries obtained from Shape Ratio feature with a cut-off range (21) is smaller than of our proposed feature since sickle cells would be seen in the undefined region boundaries, resulting in lower estimates of the percentage of “true” sickle cells in SCD. When cells were exposed to air after 20 min., this number is regained (not shown). These abnormal cells have the typical morphology of irreversible sickled cells (ISC), generated in the circulation of the patient. For completeness, the quantitation of “number of sickled and abnormal cells” and “percentage of sickled and abnormal cells” that are measured for each reaction time of deoxygenation is provided in Table S1.

Figure 3. Morphological/physical response of normal and sickle RBCs of SCD as a function of exposure time to low oxygen acquired by IFC. (A) The quantitation of “percent sickled cells” and “ratio sickled to normal cells” using Shape Ratio Default (M01) Mask, Shape Ratio Custom Mask, Shape Ratio Cut-off level, Custom Mask, and our proposed shape feature (Sickle Index Custom Mask) criteria. (B) Morphological/physical parameters obtained with the criterion of Sickle Index feature on the user-defined Range mask as a function of reaction time of deoxygenation. The dots in each case represent the mean value, whereas the vertical error bars represent the standard deviation of the corresponding cellular information. [Color figure can be viewed at wileyonlinelibrary.com]
We examined additional features used in the BF analysis such as Cell Diameter, Area, Orientation, and SSC-A (that is, 10log(Side Scattering Intensity)) in IFC to assess the effect of exposure time during the deoxygenation procedure on the morphological/physical properties obtained with the criterion of our proposed shape feature (Fig. 3B). The cell diameter, area, and side scattering intensity of sickle cells are significantly higher than that of normal cells after 5 min. of deoxygenation. However, among the first 5 min. of deoxygenation, there is no statistically significant difference in the cellular features. We observed that sickle cells are characterized by limited angular width, and are growing in a certain direction much longer than the diameter of the normal cell while normal cells appear to have originated from multiple domains with homogenous growth directions. Moreover, the comparison plots of the mean morphological/physical features obtained with the criterion of our proposed shape feature and Shape Ratio feature with a cut-off range (21) as a function of reaction time of deoxygenation, and also as a function of normal and sickle RBCs are provided in Figure S2 and S3, respectively. Specifically, mean values of cellular information for normal and sickle RBCs obtained from our proposed method are higher than of van Beers et al.’s (21) approach. For completeness, we also report the quantitation of the

**Figure 4.** Angle-resolved light scattering approach in IFC allows for label-free, noninvasive classification of morphological RBC subpopulation region boundaries in the deoxygenated SS-RBCs. (A) The scatter histogram plots of measured SSC-A patterns as a function of cell diameter and orientation for SS-RBCs at t = 0 and 20 min. of deoxygenation obtained from bright- and dark-field images in IFC. (B) The difference plots of the simulated SSC-A patterns between (Left) Discocyte and Elongated, and (Right) Discocyte and Sickle shaped RBC models. (C) The comparison between simulated and measured SSC-A patterns for the case of 1.36 ≤ n_r ≤ 1.41 and average radius, d = 8 μm in normal and sickle cell region boundaries of SS-RBCs. [Color figure can be viewed at wileyonlinelibrary.com]
measured cellular information by means of mean ± standard deviations (SD) range obtained with the criterion of features used in this study in Table S2.

Optimization of Normal and Sickle Cell Region Boundaries in High-Throughput IFC with Angle-Resolved Light Scattering Approach

We further considered the angular information on the light scattering pattern to improve the accuracy of classifying “true” normal, intermediate, and sickle RBCs since it is uncertain to find a specific range of a shape quantification feature extracting the exact population of sickle cell and other delineated RBC phenotypes. Additional features used in the BF analysis were coupled with SSC intensity measurements using the default M06 mask. Figure 4A shows measured SSC-A intensity values as a function of cell diameter and orientation for SS-RBCs at 0 and 20 min. of deoxygenation. There is an increase in the cell diameter as the pattern of the SSC-A increased at 20 min. of deoxygenation. In order to provide more insight into our understanding of normal and sickle cell region boundaries from measured SSC-A pattern, we calculated the angular far-field distribution obtained from MBIE, and modified the predicted light scattering dataset to consider minimal and maximal changes on the scattering pattern as a normalization procedure, and then converted the normalized pattern into SSC-A pattern to make the comparison with the measured IFC data. First, we performed the light scattering analysis to investigate the effect of cell type and refractive index on the SSC-A pattern for the case of 1.36 ≤ nr ≤ 1.41 and average radius, d = 8 μm in the Discocyte, Elongated, and Sickle shape RBC models (Fig. 4B). The minimum difference of SSC-A patterns between Discocyte and Elongated shaped RBC models was observed at around nr = 1.39 whereas that of between Discocyte and Sickle shaped RBC models was found at around nr = 1.36 or 1.39 depending on the cell orientation. We compared the predicted and measured SSC-A patterns for the case of average radius, d = 8 μm in normal and sickle cell region boundaries. Then, we applied the statistical analysis performed using GraphPad Prism 7.0a in order to find optimal refractive index values for normal or sickle cell region boundaries from SSC-A pattern. For illustrative purposes, the data are shown in the mean ± SD range in Figure 4C. For consistency, all data are treated as nonparametric. Between the data differences are tested with the Kruskal-Wallis or Dunn’s multiple comparisons test. One-sample tests were performed with the Wilcoxon signed-rank test. From the multiple comparison tests, we selected the refractive index value with respect to the criteria of “nonsignificant” and “minimum value of mean rank difference.” The refractive index values were found as nr = 1.39 and nr = 1.37 for normal and sickle cell region boundaries of SS-RBCs, respectively (Fig. 4C).

DISCUSSION

The polymerized state of hemoglobin S (HbS) has been characterized by a double nucleation mechanism at the molecular scale (40). The process by which the polymer distorts the normal RBCs of SCD patients is complex and leads to diverse morphological shapes depending on the configuration of the intracellular HbS polymer affected by physiological conditions, such as temperature, pH, rate of the deoxygenation, and mean corpuscular hemoglobin concentration (41–43). The presence of molecules such as HbF or HbA as the result of hydroxyurea treatment or transfusion will further affect the morphology diversity of the RBC population when exposed to low oxygen. In addition, bloods collected from patients and not exposed to low oxygen contain a fraction of abnormally shaped RBC classified as ISC. Previous studies have classified the morphology of SS-RBCs as “discocyte” (cells with a nearly normal appearance), “intermediate cells” (irregularly or abnormal shaped cells, such as echinocyte, holly-leaf, and granular structures), “sickle”, and “elongated” types under deoxygenation states (41,42).

The analysis of a light scattering pattern by an individual RBC has been reported by using various electromagnetic numerical methods such as Mie-series (22), physical-geometric-optics (23), discrete-dipole (10) approximations, anomalous diffraction (24), Lorenz-Mie (25), and discrete sources method (26) theories, finite-difference time-domain (FDTD) (27), T-matrix (28), multilevel fast multipole algorithm (29) and integral equation methods. In this article, a boundary integral equation method (MBIE), which enables fast and efficient procedures for spherical and nonspherical biological particles with a Nystrom-type meshless discretization, was used to solve biological cells in health and disease conditions. In our case, when the boundary is smooth and has a regular 2π-periodic parametrization, the Nystrom-type discretization (30–33) using trigonometric approximation of integrand functions is more convenient, as it leads to exponential convergence of numerical solution.

Although it has been previously reported that the mean values of Hb content for sickle RBCs are measured higher than that for normal RBCs (44), Byun et al. (45) reported that the refractive index value of HbS is not different from the refractive index of Hb. However, Finch et al. (46) mentioned that the refractive index and absorption coefficient exhibit low value when the incident light is parallel to the deoxygenated HbS molecules in the fiber compared with normal condition. Moreover, the refractive index spectra of the malaria parasite Plasmodium falciparum RBCs are provided in the range of 1.36–1.41 (47), but the refractive index spectra for individual SS-RBCs from sickle cell patients exposed to a low oxygen tension are unknown. Hence, we used the refractive index spectra of SCD in the range of 1.36–1.41 in this study in order to determine biophysical properties of individual SS-RBCs by means of cell diameter, size, and orientation on SSC-A pattern. Our results showed that the morphological/physical features of the cell are correlated with the cell orientation, shape, and diameter parameters on the predicted and measured scattering patterns.

Conventional FCs is limited to scatter measurements at fixed observation angles in the forward and side scattering detectors which in turn limits the cellular information that could be gained. Additional optical detectors in FCs could be utilized to enhance cellular specificity, and to achieve a better
understanding of the scattering cross-section values to provide more quantitative results for the biological questions.

High-throughput IFC allows for simultaneous phenotypic and function studies in the customized creation features and masks, which provides a cell-by-cell prediction of cellular information and highly sensitive selection of parent populations from all events. A curved object such as a sickle shaped cell is more accurately obtained using Thickness, Length, and Area features instead of Height and Width features, which can be used to separate rectangular shaped objects. It is often not known which morphological shape quantification feature is invaluable to distinguish heterogeneous shapes of SS-RBCs in IFC. Since small particles in the same frame cannot be removed from the default M01 mask, normal and intermediate cells can be classified in the range of sickle cell region boundary, resulting in distorted sickle cell percentage of SS-RBCs. Hence, a default M01 mask cannot be optimal for determining accurate cellular features in the deoxygenated SS-RBCs. Moreover, intermediate cells would be located in the criterion of Shape Ratio < 0.6 and Circularly < 0.10 as a sickle cell region boundary on the user-defined custom mask, thus leading to overestimating the percentage of sickle cells. In a recent publication, van Beers et al. (21) reported that Shape Ratio feature with a cut-off range improves the accuracy of the classified cell structures as abnormal, normal, and undefined region boundaries in SS-RBCs. However, sickle cells can be seen in the undefined region boundaries, resulting in lower estimates of the percentage of sickle cells. Thus, the combination of Thickness Minimum, Length, and Area features as a Sickle Index shape feature created with a user-defined custom mask enables better automated quantification of SS-RBCs partially following deoxygenation and hemoglobin polymerization, resulting in improvement of image analysis in mask generation and feature selection in IFC data.

In this study, we show that a novel label-free, computational sickle cell classification framework based on the boundary integral equation method with the use of image-based flow cytometry data improves the accuracy of classification performance of “true” normal, intermediate, and sickle RBCs in deoxygenated SS-RBCs. By using a rapid deoxygenation allows the measure of sickling in time. Our results show the rate of sickled cell structures as abnormal, normal, and undefined region boundaries in SS-RBCs. Thus, the combination of Thickness Minimum, Length, and Area features as a Sickle Index shape feature created with a user-defined custom mask enables better automated quantification of SS-RBCs partially following deoxygenation and hemoglobin polymerization, resulting in improvement of image analysis in mask generation and feature selection in IFC data.

CONFLICT OF INTEREST
The authors declare no competing financial interests.

LITERATURE CITED

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