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Abstract. Blood smear is a crucial diagnostic aid. Quantification of both solitary and overlapping erythrocytes within these smears, directly from their whole slide images (WSIs), remains a challenge. Existing software designed to accomplish the computationally extensive task of hematological WSI analysis is too expensive and is widely unavailable. We have thereby developed a fully automated software targeted for erythrocyte detection and quantification from WSIs. We define an optimal region within the smear, which contains cells that are neither too scarce/damaged nor too crowded. We detect the optimal regions within the smear and subsequently extract all the cells from these regions, both solitary and overlapped, the latter of which undergoes a clump splitting before extraction. The performance was systematically tested on 28 WSIs of blood smears obtained from 13 different species from three classes of the subphylum vertebrata including birds, mammals, and reptiles. These data pose as an immensely variant erythrocyte database with diversity in size, shape, intensity, and textural features. Our method detected ~ 3.02 times more cells than that detected from the traditional monolayer and resulted in a testing accuracy of 99.14% for the classification into their respective class (bird, mammal, or reptile) and a testing accuracy of 84.73% for the classification into their respective species. The results suggest the potential employment of this software for the diagnosis of hematological disorders, such as sickle cell anemia.

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1 Introduction

Blood smear or peripheral blood film (PBF) is an integral hematological tool in the screening, diagnosis, and monitoring of disease progression and therapeutic response.^{1,2} Conventionally, PBFs are obtained by staining a drop of blood on a glass slide, whereupon an expert or a pathologist reviews it under a microscope³ and records any abnormalities in the blood cell morphology.⁴ This approach is time intensive,^{5,6} expensive,⁷ and subject to significant statistical variations,⁸ thereby resulting in insufficient throughput to meet the large daily patient influx for blood tests in medical laboratories. Thus, the development of automated image analysis methods for samples in the whole slide would be highly desirable.

A typical blood smear (Fig. 1) is traditionally divided into three basic regions (Fig. 2) such as (1) the feathered edge, located at the edge of the smear where cells are too scarce or mostly damaged, (2) the body or head of the smear, located at the region of blood drop deposition onto the slide, where most cells are overlapped, and (3) monolayer, a band of cells just interior of the feathered edge, where the cells are barely touching.² Ordinarily, for red blood cell (RBC) or erythrocyte examination, experts analyze just the monolayer,² which is deemed optimal for analysis due to the presence of cells that are neither too clumped nor damaged.⁹ However, this approach fails to extract the full spectrum of information present in the PBF, as it misses out on other regions within the smear that

may contain medically relevant information, such as erythrocyte aggregation.^{10,11}

Today, blood smear slides once prepared and stained, undergo high-resolution scanning and are archived as whole slide images (WSI).¹² Although numerous methods have been developed to quantify RBC morphology using randomly chosen patches¹³ from whole slides, the direct automated analysis of PBFs from entire WSIs remains relatively unexplored—presumably due to the large size of WSIs (~ 12 giga pixels each), the lack of a single fully automated software pipeline for processing them, and the extensive computational demands of the task.¹⁴

Another major factor that aggravates the inter- and intra-observer variabilities in all extant methods is cell overlap that produces a large variance in false negative results.¹⁵ Some methods attempt to handle the problem by excluding overlapping cells from the final analysis,^{16–19} but this reduces the sample size, thereby affecting the accuracy of the end result. Other prevalent methods for clump splitting, or the separation of touching or overlapping cells, include watershed-based,^{20,21} model-based,²² concavity-based,^{23,24} and morphology-based techniques.²⁵ These techniques, too, have many drawbacks. The watershed algorithm is known to over-segment the structures,²⁶ whereas concavity-based techniques are applicable to structures of specific shapes and sizes²⁷ and therefore cannot be used for the segmentation of morphologically irregular cells. Model-based techniques require template matching and

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Fig. 1 WSI of a peripheral blood film.

several tunable parameters, thereby deeming them computationally extensive.²⁸

Although several tools exist today, which perform automated analysis of RBCs,²⁹ the most popular being CellaVision's Advanced Red Blood Cell Software, such state-of-the-art software is expensive and is not widely available.³⁰ In sum, due to these limitations, progress in the field of computational hematology, and particularly the direct computational analysis of WSI, has been severely restricted.

To help address this situation, we have developed a novel automated method with minimal parameters that directly detect and quantify all erythrocytes (both solitary and overlapped) within the optimal area of a blood smear WSI, defined as all the regions within the WSI, and not just the monolayer, wherein the smear is well spread and the erythrocytes are barely touching. We employ a two-stage extraction system for the aforementioned optimal area detection: in the first stage, a quadratic discriminant analysis classifier³¹ extracts the optimal area in low resolution, and the second stage performs a high-resolution analysis to further eliminate unwanted regions. Finally, all erythrocytes (both solitary and overlapped) within this optimal area are quantified. Moreover, by employing entropy measurements and deep convolutional network models, we demonstrate that our method's inclusion of all erythrocytes within the optimal area leads to superior classification accuracy to that incorporating just the solitary cells or even all erythrocytes from the monolayer alone.

We validated our algorithm on blood smears from 13 different species within different classes of the subphylum vertebrate, including, birds, mammals, and reptiles. The results from three different models were compared. Model 1 comprised solitary cells from the monolayer, model 2 included all cells, both

Fig. 3 Algorithmic flowchart depicting our pipeline. All regions containing optimally spaced erythrocytes are extracted from the acquired WSI via the primary and secondary stages of optimal area extraction. This area is then examined in its highest magnification to separate overlapped erythrocytes into individual cells. These segmented cells along with the solitary ones are then fed as input in the form of individual images to the deep learner for classification into the respective species.

individual and overlapping, from the monolayer, and model 3 included all the cells from optimal area, as detected by our method. Model 3 showed a 93.4% and 46.6% increase in the number of cells when compared with models 1 and 2, respectively. As a result, the validation and testing accuracy of the classifier in classifying the species for model 3 was 92.8% and 84.7%, respectively, which was substantially higher than models 1 and 2, with testing accuracies of 76.5% and 82.0%, respectively, thereby proving our hypothesis that the confinement of analysis to the monolayer and the elimination of overlapping cells deteriorate the classification accuracy. Furthermore, for the classification task of the detected cells into their respective

Fig. 2 Depiction of high-resolution windows from different regions of the smear. The demarcation in red indicates the monolayer. The box in yellow depicts the clumped cells in the head region, blue depicts the barely touching cells in the monolayer, and green depicts the damaged cells in the feathered edge of the smear, respectively.

classes (bird, mammal, or reptile), which is a much easier task, resulted in a testing accuracy of 99.14% for model 3.

This dataset was specifically chosen as these species have diverged greatly over evolutionary time and their RBCs consequently vary tremendously in size and shape. In addition to the inherent interest in examining such diversity, we feel this variation provides an excellent opportunity to demonstrate the versatility of our approach.

2 Methodology

Our pipeline, as shown in Fig. 3, begins with the acquisition of the WSI of a peripheral blood smear. Next, this WSI is analyzed in low resolution to extract the aforementioned optimal area based on their difference in appearance in comparison with the rest of the slide. This stage will hitherto be referred as

the primary stage of optimal area extraction. The area detected by the primary stage is further refined by zooming into their highest magnification and discarding areas that contain too many overlapped or damaged cells. This stage, hitherto referred as the secondary stage of optimal area extraction, establishes the zone from which erythrocytes are to be detected and quantified. Once this optimal area is detected, they are analyzed in high resolution to separate overlapped erythrocytes into individual cells. The image containing these separated cells is then merged with that of the detected solitary cells to finally perform an ellipse fitting to obtain the cell boundaries while simultaneously performing a multiparametric feature extraction of each individual cell. Finally, each individual cell is extracted and fed to a deep convolutional neural network³² for classification into the respective species.

Fig. 4 Representative blood smear images. (a) Mammal, (b) Avian, and (c) Reptile.

Table 1 Erythrocyte sizes of species. Error indicates standard deviation.

Species	#WSI	Class or subphyllum	Area/cell (μm^2)	Major axis (μm)
<i>Testudo hermanni</i>	2	Reptile	179.90 ± 14.02	18.29 ± 1.14
<i>Shinisaurus crocodilurus</i>	2		191.95 ± 12.51	19.46 ± 0.87
<i>Ophisaurus apodus</i>	3		142.38 ± 13.10	18.05 ± 1.10
<i>Sistrurus catenatus</i>	2		184.65 ± 16.79	17.79 ± 0.68
<i>Mauremys annamensis</i>	2		223.78 ± 14.08	21.84 ± 1.31
<i>Iguana iguana</i>	2		126.37 ± 12.02	16.76 ± 0.93
<i>Crotalus cerastes</i>	3		178.30 ± 21.77	18.68 ± 1.29
		Average	175.33 ± 14.90	18.70 ± 1.05
<i>Dasyprocta leporina</i>	2	Mammal	44.63 ± 5.41	7.85 ± 0.57
<i>Addax nasomaculatus</i>	2		23.79 ± 2.91	5.70 ± 0.32
<i>Bison bison</i>	1		29.95 ± 4.41	6.48 ± 0.53
<i>Lontra canadensis</i>	1		34.42 ± 3.74	6.92 ± 0.44
<i>Saimiri sciureus</i>	2		37.07 ± 3.62	7.15 ± 0.58
		Average	33.97 ± 4.01	6.82 ± 0.48
<i>Rhamphastos swainsonii</i>	4	Bird	99.20 ± 7.11	13.74 ± 0.93

2.1 Image Acquisition and Dataset

The species chosen for this study has a wide variation in their RBC morphologies. Representative high-resolution blood smear images from a mammal, bird, and reptile are shown in Fig. 4. It is evident from these images that these erythrocytes vary in size and shape. In addition to this, a prominent nucleus is observed in some of them. The different species used along with their approximate erythrocyte area and major axis length are shown in Table 1. These results were obtained by extracting the area and major axis length of a few randomly chosen cells from each species.

Peripheral blood smears were obtained from the Buffalo zoo under the protocol approved by University at Buffalo and the Buffalo zoo. These slides were prepared as per the standard protocol, one for all mammals and another for all other vertebrates, using differential quick stain and fixed using methanol. These slides were then stained either red or purple. Imaging was conducted using a whole slide bright field microscope (Aperio, Leica, Buffalo Grove, Illinois) using a 40× objective with 0.75 NA. Pixel resolution is 0.25 μm . The digital images were analyzed in MATLAB as discussed below. The .svs images obtained from the scanner were analyzed in high-resolution using OpenSlide software.³³

2.2 Optimal Area Extraction

To extract the optimal area, the WSI is first scanned in low-resolution windows to select those containing optimally spaced RBCs based on the decision boundaries generated by a quadratic determinant analysis classifier.³⁴ These sections are then analyzed at high resolution to identify overlapping erythrocytes, which must be split, using each binary object size as a threshold criterion.

2.2.1 Optimal area extraction in low resolution

The WSI is first analyzed in low resolution to obtain the aforementioned optimal area. We define three distinct regions within the WSI (Fig. 2), such as clumped, scarce, and the optimal area. The clumped regions are defined as those where the cells are highly overlapped, unable to be separated, and are unsuitable for analysis. The scarce region is defined as the region where erythrocytes are spaced too far apart and show signs of morphological damage. The final region is where the spatial distribution of erythrocytes is optimal for morphological quantification, yielding the maximum amount of erythrocytes with the minimum amount of cell overlap.²

High-intensity regions in a blood smear could indicate the dense body of the smear, where cells are overcrowded, or an over-stained region or could simply be the result of an artifact (Fig. 2). As neither of these regions contribute to the RBC count, these high-intensity regions are avoided. Moreover, the region with optimally spaced erythrocytes is said to be uniform in texture, whereas the scarce region contains cells, which are often damaged,⁹ and also often contains accumulated platelet clumps,² thereby displaying a wide range of entropy locally. Hence, the local mean and entropy are the chosen features to identify the optimal area within the smear.

We divide the WSI into nonoverlapping image blocks (Fig. 5) and the local mean and entropy of the pixel intensities are calculated. Let $I(x, y)$ be the intensity at discrete coordinates (x, y) , where x and y indicate the exact pixel location, and

Fig. 5 A representative localized nonoverlapping window of size $W \times W$, where (x, y) gives the location of the center pixel of the window.

p_l^k ($\forall l \in \{1, 2, \dots, N-1\}$) is the probability associated with the gray level l for the k 'th block.³⁵ If the window of size $W \times W$ is centered at pixel (x, y) , the local mean for the k 'th block is given by

$$m_k = \frac{1}{W^2} \sum_{i=(x-\frac{W}{2})}^{(x+\frac{W}{2})} \sum_{j=(y-\frac{W}{2})}^{(y+\frac{W}{2})} I(i, j). \quad (1)$$

The local entropy of the k 'th block is given by

$$H_k = \sum_{l=0}^{N-1} p_l^k \log_2(p_l^k), \quad (2)$$

where N is the number of gray levels.

Using the mean and entropy calculated per window from the optimal, scarce, and the clumped area of the WSI in low resolution as feature vectors, the quadratic discriminant analysis classifier was trained on 13 WSIs from different species. It was

Fig. 6 Quadratic decision boundaries. The plot shows quadratic decision boundaries between the three regions of a representative WSI: clumped (black), scarce (blue), and optimal area (red).

Fig. 7 Visualization of the primary stage of optimal area extraction in low resolution. (a) WSI of a reptilian blood smear. (b) Optimal area detected by the classifier.

tested on the remaining 15 smears. A two-dimensional (2-D) scatterplot of these features and the corresponding decision boundary of the quadratic classifier of a sample test image (WSI of a reptile) is shown in Fig. 6. The windows identified as an optimal area by the classifier is shown in Fig. 7.

2.2.2 Optimal area refinement in high resolution

The optimal area obtained from the primary stage is then analyzed in high resolution to discard regions containing too many overlapping cells. To achieve this, the optimal area is analyzed in 256×256 blocks at $40\times$ magnification. First, the green channel of the 256×256 image is binarized using Otsu's thresholding.³⁶ Then, a metric describing the extent of overlap is computed from each resulting binary image by deducting first the binary image region from the corresponding convex hull image, and then computing a fraction between the resultant area and convex hull image area. A threshold on this extent

of overlap metric is used to either keep or eliminate the corresponding 256×256 image block. Image blocks below the threshold are considered to be containing damaged or extremely overlapping cells and are discarded.

Consider next the simulated images shown in Fig. 8 to discuss how the extent of overlap metric is computed. The simulated images shown in Figs. 8(a)–8(c) explain different extents of overlap between few cells. Assume that these simulated binary images of the cells are obtained after Otsu's thresholding. Images in the second row [Figs. 8(d)–8(f)] show the corresponding convex hull³⁷ images, and images in the third row [Figs. 8(g)–8(i)] show the images in the first row minus the respective images in the second row (i.e., the convex hull deficiency images). We see that the mismatch between the images in the second row and the first row increases with the extent of overlap between the cells. The fraction of this mismatch with respect to the respective convex hull region provides the metric describing the extent of overlap between the cells. We compute

Fig. 8 Metric indicating severity of overlap. The fraction of mismatch shown in the bottom row increases with an increase in the overlap. (a) Solitary cells, (b) slight overlap, (c) high overlap, (d) convex hull of (a), (e) convex hull of (b), (f) convex hull of (c), (g) convex hull deficiency image of (a), (h) convex hull deficiency image of (b), and (i) convex hull deficiency image of (c).

Fig. 9 Visualization of the secondary stage of optimal area extraction in low resolution.

this fraction for the binarized version of the primary stage image [Fig. 7(b)] and estimate the refined optimal region (Fig. 9) below a threshold fraction. This threshold was obtained based on 88.26% sensitivity and 89.09% specificity, where the sensitivity and specificity metrics were obtained via comparing the estimated refined optimal region with a hand-selected ground-truth. This ground-truth was obtained using WSIs with 10- μ m per pixel resolution, and conducted by the author Ms. Darshana Govind under the supervision of the coauthor Dr. John E. Tomaszewski.

The refined optimal area obtained is shown in Fig. 9. This area is next analyzed in high resolution to detect overlapping erythrocytes.

2.3 Separation of Solitary and Overlapped Cells

The estimated optimal area obtained from the previous stage is analyzed in high-resolution windows (Fig. 10) of size 256×256 to detect overlapping erythrocytes. First, the green channel is extracted from the image and subsequently, Otsu's threshold³⁶ is used to segment the image into foreground and background. The cells touching the image borders are removed as they provide only partial information.

Most species have a distinct set of morphological parameters defining their erythrocytes. The size of the cell is used as a threshold to determine if the detected structures are solitary cells or a cluster of cells. The latter needs to be separated prior to the quantification of their morphological parameters. Thus, using the area of a single cell as the threshold, the image is split into two, one containing just the solitary and the other containing the overlapped cells (Fig. 10).

Fig. 10 Separation of solitary and overlapped cells. (a) High-resolution window, (b) solitary cells, and (c) overlapped cells.

Fig. 11 Bottleneck detection.

2.4 Detection of Bottleneck Points in Overlapped Cells

The detected overlapped cells [Fig. 10(c)] need to be separated prior to their morphological quantifications. Therefore, for each clump, we identify the splitting points via bottleneck detection.²⁷ It identifies the most likely place to break and divide the clump into its constituent objects. For simplicity, we demonstrate the separation of clumps with just two cells.

Let $R(r_1, r_2)$ and $Q(q_1, q_2)$ represent two distinct points on the cluster boundary. The Euclidean distance, d , between R and Q is given by

$$d(R, Q) = \sqrt{(r_1 - q_1)^2 + (r_2 - q_2)^2}. \quad (3)$$

We define a cost function, C , as

$$C(R, Q) = \frac{d}{\min\{\text{length}(R, Q), \text{length}(Q, R)\}}, \quad (4)$$

where $\text{length}(R, Q)$ denotes the clockwise distance from R to Q along the cluster boundary. Let R^* and Q^* be the points along the cluster boundary that minimizes the cost C

$$(R^*, Q^*) = \arg \min_{R, Q} C(R, Q). \quad (5)$$

The bottleneck points are identified as R^* and Q^* (Fig. 11).

2.5 Segmentation of Clumped Cells

Our segmentation pipeline is shown in Fig. 12, demonstrating the determination of points that depict the bounds of the region

Fig. 12 Cell segmentation pipeline. (a) Clumped cells in grayscale with local image patch highlighted, (b) line drawn connecting the bottleneck points in the image patch to obtain the angle of tilt, (c) vertical alignment of the image patch, (d) horizontal line profile obtained along the midsection of the image patch is shown in red. Corresponding maximum-likelihood fit to determine the locations of local intensity peaks is shown in blue, (e) points depicting the boundaries of the overlapped region: bottleneck (R^* and Q^*) and side points (S and T), (f) binary image of the overlapped cells with convex hull highlighted, (g) subtraction of convex hull from original image, and (h) segmented cells.

of overlap. If the detected bottleneck points are assumed to indicate the vertical bounds of the overlapped region, the side points or the horizontal bounds of this region are to be estimated. Once the vertical and horizontal bounds of the overlapped region are determined, the convex hull of these points is obtained and subtracted from the original image, to separate the clumps into individual cells.

2.5.1 Cases of two cell overlap

Consider the case wherein two cells overlap with each other (Fig. 11). First, a small local image patch is extracted from around the two detected bottleneck points, R^* and Q^* [Fig. 12(a)]. The patch is then rotated for vertical alignment [Figs. 12(b) and 12(c)]. The horizontal intensity profile along the middle row of the resulting patch [Fig. 12(c)] are shown in red in Fig. 12(d). This profile resembles a mixture of two Gaussian curves, the means of which represent the extent of spread of the overlapped region [Fig. 12(e)]. To estimate this spread, we obtain the concentrated maximum-likelihood estimates³⁸ of the local spatial means of these Gaussian curves and use these estimates as the horizontal bounds or side points of the overlapped region. In detail, the local peaks here are modeled using mixture of two Gaussian curves, with unknown parameters as the intensity heights, means, and variances of the individual Gaussians. The measurement data [shown as “original” in Fig. 12(d)] are used to estimate the unknown parameters using maximum-likelihood estimation, assuming that Gaussian error between the measurement and the mixture of Gaussian model is distributed independently and identically in each pixel. For estimation, the likelihood is concentrated³⁸ on the nonlinear parameters (means and variances of the Gaussian curves) and estimated using the nonlinear parameter search

method by optimizing the concentrated likelihood. Once estimated, the resulting estimates are inserted in the original likelihood, and unknown intensity heights are estimated using the least squares estimation method. We denote the side points as S and T in Fig. 12(e). We then obtain the convex hull of the bottleneck points (R^* and Q^*) and the side points (S and T). The convex hull is highlighted in the binary image in Fig. 12(f). The convex hull is then subtracted from the original image [Fig. 12(g)] to yield the segmented cells [Fig. 12(h)].

2.5.2 Cases of multiple cell overlap

For multiple overlapping cells, the segmentation pipeline is repeated until all separated objects have an area less than that of the single cell area limit for the respective species, as shown in Table 1. The segmentation pipeline for multiple cell overlap is shown in Fig. 13.

2.6 Ellipse Fitting and Feature Extraction

The solitary and the segmented overlapped cells are combined, and an ellipse fitting is performed for each cell to detect the cell boundaries [Fig. 14(a)]. The coordinates, $[x_c, y_c]^T$, of the points on each ellipse are calculated as follows:

$$\begin{bmatrix} x_e \\ y_e \end{bmatrix} = \begin{bmatrix} x_c \\ y_c \end{bmatrix} + \begin{bmatrix} \cos(\theta) & -\sin(\theta) \\ \sin(\theta) & \cos(\theta) \end{bmatrix} \cdot \begin{bmatrix} a \cos(\phi) \\ b \sin(\phi) \end{bmatrix}, \quad (6)$$

where x_c and y_c are the centers of the ellipse, θ is the orientation, a and b are the half of the major and minor axes lengths, respectively, and ϕ is the parameter ranging from 0 to 2π radians. For the solitary cells, the parameters, $[x_c, y_c, \theta, a, b]^T$, are estimated

Fig. 13 Segmentation pipeline for multiple cell overlap. (a) Depicts a region of the smear containing a clump comprised of four cells, (b) Binary image of the cells, (c) Segmentation of clump after detection of bottleneck points and clump splitting as explained in Fig. 12, (d) Since the separated clumps are significantly larger than the average area of a single cell, the process is repeated until single cells remain, (e) Segmented cells in binary, and (f) Segmented cells in RGB.

Fig. 14 Performance of cell segmentation. (a) Our ellipse fitting-based individual cell segmentation after segmenting overlapping cells (b) segmentation based on optimizing an energy function based on overlapping cell edge strengths.²⁷

based on their binary mask, whereas for the segmented cells, the furthest side point [see Fig. 12(e)] from the centroid of a cell is connected to that cell via convex hull, and the parameters are subsequently estimated.

Tables 2 and 3 show some of the commonly used hand-selected features. Primarily, distinct morphological-, intensity-, and texture-based features are extracted from each cell.³⁵ We compute intensity-based features from each cell image, namely the average gray level and the average contrast, and the textural features, namely the measure of smoothness, third moment, and the entropy. Intensity- and texture-based features are commonly used as they help differentiate RBCs from lymphocytes or other cells or even artifacts present in the field of view as they are

significantly different in appearance. Apart from these features, we also compute the geometrical attributes of the cell, namely the area, perimeter, eccentricity, solidity, equivalent diameter, and major axis length and circularity of the cell. These features provide a geometrical signature for cells of different shapes and sizes. Although these hand-engineered features show a clear distinction between the different subphylum, they display a high degree of similarity among species. This intraspecies similarity, therefore, highlights the complexity of the task that necessitates the use of high-level features, such as those obtained with the help of a convolutional neural network. We, therefore, use the deep learner network to classify the cells into their respective species.

Table 2 Average of extracted intensity, and texture-based features per species. Error indicates standard deviation.

Species	Average gray level	Average contrast	Smoothness	Third moment	Entropy
<i>Ophisaurus apodus</i>	0.39 ± 0.03	0.106 ± 0	0.0112 ± 0	-26.76 ± 42.37	6.70 ± 0.10
<i>Mauremys annamensis</i>	0.44 ± 0.05	0.131 ± 0.01	0.0170 ± 0	-34.60 ± 102.97	6.93 ± 0.10
<i>Iguana iguana</i>	0.28 ± 0.05	0.108 ± 0.01	0.0119 ± 0	-76.31 ± 105.74	6.63 ± 0.16
<i>Crotalus cerastes</i>	0.42 ± 0.02	0.108 ± 0	0.0116 ± 0	-3.58 ± 56.47	6.74 ± 0.08
<i>Dasyprocta leporina</i>	0.32 ± 0.05	0.099 ± 0.01	0.0100 ± 0	-2.28 ± 2.97	6.51 ± 0.15
<i>Addax nasomaculatus</i>	0.27 ± 0.04	0.107 ± 0.01	0.0114 ± 0	-0.43 ± 1.33	6.52 ± 0.16
<i>Bison bison</i>	0.32 ± 0.03	0.097 ± 0	0.0095 ± 0	-0.61 ± 1.32	6.49 ± 0.11
<i>Saimiri sciureus</i>	0.36 ± 0.03	0.117 ± 0.01	0.0138 ± 0	1.34 ± 3.37	6.77 ± 0.16
<i>Rhamphastos swainsonii</i>	0.37 ± 0.12	0.094 ± 0.02	0.0094 ± 0	-14.14 ± 43.44	6.43 ± 0.49

Table 3 Average of extracted geometrical features per species. Error indicates standard deviation.

Species	Area (μm^2)	Eccentricity	Perimeter (μm)	Solidity	Circularity	Equivalent diameter (μm)	Major axis length (μm)
<i>Ophisaurus apodus</i>	132.82 ± 19.08	0.81 ± 0.06	43.57 ± 4.42	0.97 ± 0.04	1.16 ± 0.29	12.96 ± 0.97	17.32 ± 1.65
<i>Mauremys annamensis</i>	193.16 ± 45.07	0.77 ± 0.10	55.88 ± 10.16	0.93 ± 0.08	1.40 ± 0.67	15.54 ± 1.97	20.84 ± 3.43
<i>Iguana iguana</i>	121.93 ± 25.01	0.77 ± 0.11	44.42 ± 10.65	0.94 ± 0.06	1.38 ± 0.85	12.38 ± 1.34	16.41 ± 3.05
<i>Crotalus cerastes</i>	169.31 ± 22.48	0.76 ± 0.05	48.076 ± 4.81	0.97 ± 0.02	1.10 ± 0.22	14.63 ± 0.98	18.42 ± 1.48
<i>Dasyprocta leporina</i>	42.18 ± 6.29	0.44 ± 0.16	23.28 ± 2.27	0.95 ± 0.02	1.03 ± 0.14	7.30 ± 0.55	7.95 ± 0.83
<i>Addax nasomaculatus</i>	23.79 ± 3.10	0.35 ± 0.13	16.87 ± 1.36	0.97 ± 0.02	0.95 ± 0.09	5.49 ± 0.35	5.76 ± 0.48
<i>Bison bison</i>	28.78 ± 4.18	0.37 ± 0.14	18.51 ± 1.49	0.97 ± 0.01	0.95 ± 0.06	6.03 ± 0.43	6.36 ± 0.57
<i>Saimiri sciureus</i>	35.78 ± 4.98	0.42 ± 0.16	21.10 ± 1.78	0.96 ± 0.02	1.00 ± 0.13	6.73 ± 0.48	7.28 ± 0.73
<i>Rhamphastos swainsonii</i>	94.06 ± 21.01	0.71 ± 0.13	37.13 ± 5.95	0.95 ± 0.05	1.20 ± 0.36	10.87 ± 1.20	13.63 ± 2.04

2.7 Final Classification

The detected cells are individually cropped out and fed as an input to the GoogLeNet³⁹ deep learner network, implemented in Caffe,³² and accessible via DIGITs web wrapper (NVIDIA, Santa Clara, California).⁴⁰ For the analysis, $n = 7523/940/941$ (80%/10%/10%) images from the optimal area were used for training/validation/testing. We trained our network using two GPUs, an NVIDIA GeForce 1080 and Titan X Pascale. GoogLeNet is additionally regularized with dropout layer with 70% ratio of dropped outputs to guard against overfitting.³⁹ The network was trained for 30 epochs. A stochastic gradient descent solver was selected with base learning rate of 0.01, which was specified to decrease by a factor of 10 for every 10 epochs (33% of total training epochs). All other parameters used the default options specified by NVIDIA Caffe. As the GoogLeNet architecture accepts a fixed image size of 256×256 , each individual cell image was zero-padded and converted to an image of size 256×256 without causing any structural deformation of the RBC shape.

3 Results and Discussion

A total of 28 slides from 13 different species were used, as shown in Table 1. Among these, 13 slides were used for training the quadratic classifier for the detection of optimal region. For the remaining 15 slides, the optimal area was detected and the cells therein extracted and quantified.

3.1 Data Availability

All of the source code and images used to derive the results presented within this article are made freely available to the public. The data are held at goo.gl/sHZFhs.

3.2 Cells Detected

As mentioned previously, we compare the results from three different models. Model 1 includes the solitary cells alone from just the monolayer, model 2 includes all cells, both solitary and overlapping, from just the monolayer, and model 3 includes all the cells from the optimal area, as detected by model 3.

Table 4 Cell segmentation performance comparison.

Method	Sensitivity (%)	Specificity (%)
Our method	99.4	99.5
Energy optimization method ²⁷	95.6	99.9

As the optimal area covers far more area than the monolayer, our method detects higher number of cells than both models 1 and 2. Model 3 provides ~3.06 times more cells than model 1 and ~2.07 times more cells than model 1.

3.3 Feature Extraction of the Detected Cells

Distinct morphological- and intensity-based features were calculated from each of the detected cells (Tables 2 and 3).

3.4 Performance of Cell Segmentation

Our proposed computational pipeline has two new contributions than what is available in the literature. First, the definition and classification strategies of the optimal area in a blood smear. Second, segmentation of the overlapping cells. Regarding the former, we are the first one to propose the definition of optimal area, and thus, we refrain from conducting any performance comparison of the methodology. For the latter, we compare the performance of our segmentation with that established in the literature,²⁷ which is based on optimizing an energy function based on overlapping cell edge strengths. Clump splitting of cells is a known problem in any cell biology image analysis problem, and thus, our solution in this direction has a broad application range. Performance of the above-mentioned literature established method is shown in Fig. 14(b), describing visually, how the cell boundary is split, suggesting this literature established method is not very accurate in determining both cell boundaries simultaneously. Our methodology, which does bottleneck detection of clumped cells, splitting the cells based on the bottleneck points, and a subsequent ellipse fitting of the two cells, provides both cell boundaries. When we compare the performance of our method and the literature established method (Table 4), we find our method offers higher sensitivity than the literature established method while offering similar specificity in segmenting clumped cells, thus, capturing maximal cell detail. For this performance comparison, we obtained ground-truth by manually annotating the boundaries of all the cells.

3.5 Classification Results

As mentioned previously, the results from three different models were compared. For the classification of the detected cells into the respective species (see Table 2), the algorithms' results from three different models were compared. We used 4860 cells from model 1, 6411 cells from model 2, and 9404 cells from model 3, and splitted the cells similarly as discussed in Sec. 2.7. The validation accuracies for the models were 79.4%, 90.5%, and 92.8%, and the testing accuracies were 76.5%, 82.0% and 84.7%, respectively, thereby validating that our method extracts a significantly higher number of cell than the other models, thereby increasing the classification accuracy. Furthermore, for the classification of the detected cells into their respective

classes (birds, mammals, or reptiles), model 3 showed a testing accuracy of 99.14%.

4 Conclusion

The preceding results have demonstrated that the proposed pipeline can automatically detect the optimal area from the entire peripheral blood smear and segment the cells from clusters and classify them into their respective species categories. By means of such a pipeline, RBCs from peripheral blood smears can be detected and have their features extracted to diagnose diseases involving abnormal cells, such as sickle cell anemia. The proposed pipeline detects far more cells than that detected from just the monolayer, which is the current practice followed in clinics. Our method can, thereby, be potentially transferred to a clinic for efficient automated analysis for hematological disorders.

Disclosures

The authors have no financial interests or potential conflicts of interest to disclose.

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