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Image-Based Red Cell Counting for Wild Animals Blood

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***Abstract*—An image-based red blood cell (RBC) automatic counting system is presented for wild animals blood analysis. Images with 2048x1536-pixel resolution acquired on an optical microscope using Neubauer chambers are used to evaluate RBC counting for three animal species (*Leopardus pardalis*, *Cebus apella* and *Nasua nasua*) and the error found using the proposed method is similar to that obtained for inter observer visual counting method, i.e., around 10%. Smaller errors (e.g., 3%) can be obtained in regions with less grid artifacts. These promising results allow the use of the proposed method either as a complete automatic counting tool in laboratories for wild animal's blood analysis or as a first counting stage in a semi-automatic counting tool.**

I. Introduction

BLOOD analysis is one of the most common test performed to support diagnosis. This well known analysis includes red blood cells (RBC), white blood cells (WBC) counting, evaluation of mean size and shape of cells. Various diseases and the general health status of a patient can be indicated from abnormally high or low counts in blood cells. The WBC count increase is used for detecting infectious diseases, inflammatory condition, and tissue damage. The WBC count decrease can indicate bone marrow failure, liver disease, and presence of toxic substances. For the RBC counting, a decrease would indicate anemia and chronic inflammation while an increase could point towards a renal tumor and organs overloaded with iron [1].

The microscopic-based evaluation was the introductory method for classifying and counting cells. In this method, an optical microscope is used to visualize a film, prepared using blood sample from a patient. Well trained laboratory personnel are then responsible for manually classifying and counting the cells of interest. While this method seems to be straightforward, it relies upon the expertise of the observer to classify the cells and might have results depending on the instantaneous capability of the technician to perform at his/her best performance potential. This reader-dependent performance varies along time mainly because visual quantification of cells through microscope is a repetitive and time-consuming task where the complexity and the unstructured nature of biological images present a unique set of challenges in data analysis and interpretation [2].

For providing faster and reader-independent counting, automatic counting equipments were developed. In 1949, Wallace Coulter disclosed a method for counting particles suspended in a fluid [3]. In this milestone method, a solution with suspended particles (e.g., blood cells) can be driven through a capillary while the electrical impedance is evaluated and used for determining the number and size of particles. This method soon became a reference for automatic complete blood cell (CBC) counting and is still largely used in blood analysis laboratories. In the 1970s, automated human blood equipments using microscopic image analysis with high throughput performance became commercially available [4], [5], however, there still exist aspects that need improvement [6].

While automatic counting equipment proliferated and the high demand for human blood analysis in most modern cities resulted in a cost effective analyses even when expensive equipments and consumables are used, this is not the case when wild animals are the "patients". For wild animals care centers and institutions such as Zoos, the variety of animal species abound and the number of blood analysis for each of the species, generally, does not economically justify the purchase of current equipments and associated consumables (i.e., hematology reagents) for blood analysis of that animal species. On the other hand, an even more important role might be played by the wild animal's blood analysis when compared to humans to allow the identification of diseases. While humans typically demonstrates symptoms and are able to communicate them to the clinician, wild animals do not usually present symptoms when in an ill state and diagnosis based only on the animal behavior is challenging. Wild animals might hide symptoms to avoid predators realizing they are in a disadvantageous self protection condition, emphasizing the need for analytical equipment targeting such animal species.

Setting the currently available equipments up for wild animal blood analysis is challenging due to the variety and the lack of information on the concentration, size and shape of the cells when considering many different species. Therefore, despite the technology available, it is still common to have those analyses made using the traditional method with visual inspection of microscopic images. In that method, a trained technician manually dilutes the blood

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sample and then, using a ruled chamber (e.g., Neubauer chamber) counts the cells of interest. The variations of size, shape and quantity among different species demands great knowledge from the technician and reliable results might be difficult to obtain.

II. Materials and Methods

A. Counting chamber preparation

Counting chambers with appropriate volumes and grids to facilitate counting through visual inspection are typically used. Neubauer, Burker and Fuchs-Rosenthal are well known chambers with accurate marked regions [7]. The Neubauer chamber, presented in Fig. 1, is used in this work because it includes regions adequate for counting both high or low concentration of elements of interest [8], [9], [10], [11].

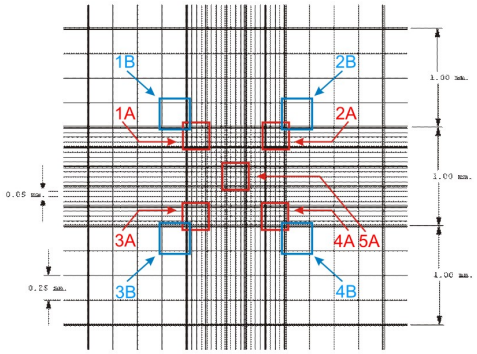


Fig. 1. Neubauer chamber with nine 1x1 mm^2 squares. The chamber depth is 0,1 mm resulting in a 0,9 mm^3 volume. The four squares in the corners are divided in 1/16 mm^2 regions (e.g., 1B square). The central square is composed by 1/25 mm^2 regions (e.g., 1A square) each on divided in 16 smaller regions with 1/400 mm^2.

Diluted blood is prepared at a 1:200 ratio using 10 µl of blood sample and 1990 µl of saline solution (NaCl at 0,9%). Diluted blood is placed in the chamber where volume (i.e., area and depth) is standardized. A counting value, RBC_{mm^2}, from the microscopic visual inspection of one of the 1 mm^2 regions in the chamber is obtained. The final RBC concentration (RBC_{mm^3}) is determined using Eq. 1 where d is the chamber depth, and b is the concentration of blood in the diluted blood introduced in the chamber.

$$RBC_{mm^3} = RBC_{mm^2} \cdot d \cdot b \qquad \text{Eq. (1)}$$

In a typical non automatic visual evaluation, the RBCs are counted in the 5 regions, i.e., 1A, 2A, 3A, 4A, and 5A, presented in Fig. 1 [12]. By multiplying the total count in these 5 squares by 5, the RBCs per mm2 (RBC_{mm^2}) is obtained.

B. Image acquisition

An Olympus BX41 optical microscope (Olympus Corporation, Tokyo, Japan) with a 20X planar objective was used. An Olympus DP12 digital camera (Olympus Corporation, Tokyo, Japan) with 2048x1536-pixel resolution is used to save images in lossless JPG (Joint Photographic Experts Group) compressed format.

Images for five internal regions (i.e., 1A, 2A, 3A, 4A, 5A) and four peripheral regions (i.e., 1B, 2B, 3B, and 4B) were registered using two focal setups. The first focal setup was adjusted to make the cells look dark and sharp (focus 1 - F1). The second focus (focus 2 - F2) position is set at 15 µm beyond the F1 position resulting in an image where cells present dark and blurry border with a bright interior.

C. Wild animals

Blood from three animal species where used in this work. Jaguatirica - *Leopardus pardalis*, Macaco Prego – *Cebus apella* and Quati - *Nasua nasua* with typical RBC volumetric concentration shown in Table I [12]. Three chambers for the *Leopardus pardalis* and *Nasua nasua* and two for *Cebus apella* were used during the RBC evaluation, totaling 8 wild animal blood samples.

TABLE I
Typical Values of RBC Counting

Animal species	RBC (millions cells/µl)	
	Male	Female
Leopardus pardalis	4.07-5.33	4.24-6.16
Cebus apella	4.34-5.48	3.47-5.33
Nasua nasua	4.21-5.35	3.88-4.26

D. Counting methods

Three counting methods were used for evaluating the proposed method.

1) Visual counting

Blood samples were visually counted using Neubauer chambers. The counting personnel from the Laboratório Ambiental Itaipu Binacional used the methodology described by Santos in [12].

2) Computer aided counting

A computer aided software was developed for enabling expert and non-expert personnel to evaluate the RBC counting. Images focused at F1 are presented and the user selects the region where he/she believes there is a RBC. Marked images including RBC counting value cells (x,y) coordinates are saved for later comparison. Counted cells are colored, as shown in Fig. 2, in real-time for diminishing the occurrences of non counted cells or cells counted twice. This on-the-fly counting feedback enables the user to promptly correct (1) a mark that was added in the absence of a cell or (2) a mark that was forgotten in the presence of a cell. By providing such error control, it is expected that this computer aided counting method generates more reliable results when compared to those from the typical visual assessment method made in laboratories where no image is registered. The counting was performed by an experienced

biochemist (E.B.) and by two lay collaborators (L.C.) following the visual count standard procedure recommended by the International Council for Standardization in Haematology (ICSH) [13]. The E.B.'s counting result is considered the ground truth in this paper.

3) Automatic Image-based counting

The proposed method uses images with different contrast captured at 2 focal settings, F1 and F2, as shown in Fig. 3. Gray scale image erosion is first applied to F1 image to evidence the cells overlapping lines in the chamber. An empirically defined threshold value is used to obtain segmented cells in binary images for F1 and F2. Blending

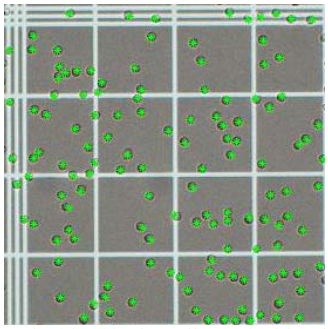
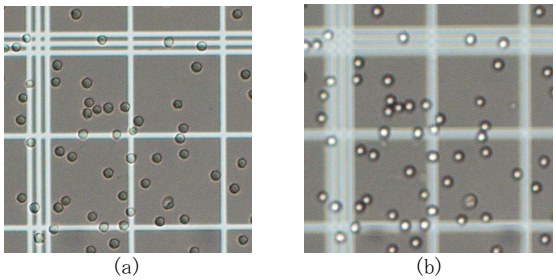


Fig. 2. Green marks identify the cells marked by the user with the computer aided counting software.

the two, occasionally different, segmented images results in smaller number of missed cells compared to using either one of the focal setups alone.

Central coordinates and area in pixels are determined for each one of the regions obtained. The reference value taken as a typical area value (A) is the median value of an ordered vector that includes the areas of all segmented regions. Every region within the area range of 0.5A to 1.5 A is considered cell to compensate for cell size variation and the grid lines Artifacts.



(a) (b)

Fig. 3. Image for Cebus apella's blood using focus (a) F1 and (b) F2.

III. Results and Discussion

Fig. 4 presents an image marked after using the automatic counting algorithm proposed. As shown in the Fig. 4, some RBCs are not counted and are missed by the automatic counting method due to either the threshold used or the

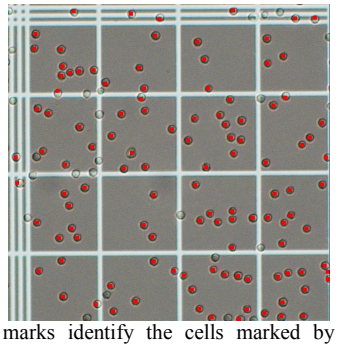


Fig. 4. Red marks identify the cells marked by the automatic counting method.

existence of a grid line in the chamber which reduces the contrast and decreases the capability of the method to count all cells.

Fig. 5 presents the results from all the blood samples for the 3 animals analyzed by the experienced biochemist (EB) considered as the reference and by two lay collaborators (LC1 and LC2) while using the computer aided counting method. The differences found between the EB and the two LC are mainly due to the region-of-interest (ROI) determination. Even though the ICSH protocol recommends to consider (1) any cell that touches a limiting ROI line and (2) only one of the horizontal and one of the vertical limiting lines as part of the ROI (e.g., top and left lines are part of ROI and bottom and right lines are considered outside of the ROI), it is challenging having every people considering the same cells as valid cells in a given ROI.

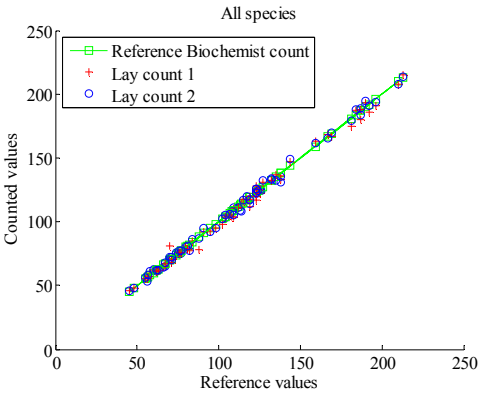


Fig. 5. Computer aided counting method results.

Fig. 6 presents the results from reference, visual and proposed automatic counting methods considering ROIs 1A, 2A, 3A, 4A, 5A for all 8 blood samples. The average percent error for the visual and automatic counting methods were 12,4% and 10,9%, respectively. Literature reports typical errors around 10% among laboratory personnel for the visual method [13]. The results presented for the automatic counting in the internal ROIs is equivalent to that error in the literature. Fig. 7 presents the results from reference and proposed automatic counting methods considering ROIs 1B, 2B, 3B, and 4B for all 8 blood samples. No visual counting results exist for these ROIs because only ROIs in the central part (i.e., A-labeled ROIs) of the Neubauer chamber are typically counted in the regular RBC analysis procedure.

The observed average error for external ROIs is smaller, i.e., 3%, than that seen in the internal ROIs for the proposed method. As discussed before, most of the missed cells in the

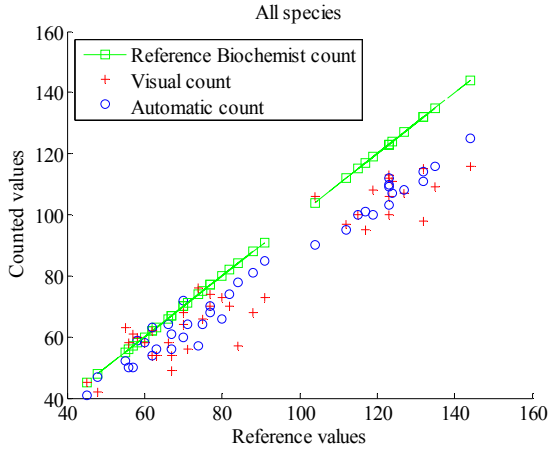


Fig. 6. Reference counting compared to the visual and proposed automatic counting results for the ROIs 1A, 2A, 3A, 4A, 5A.

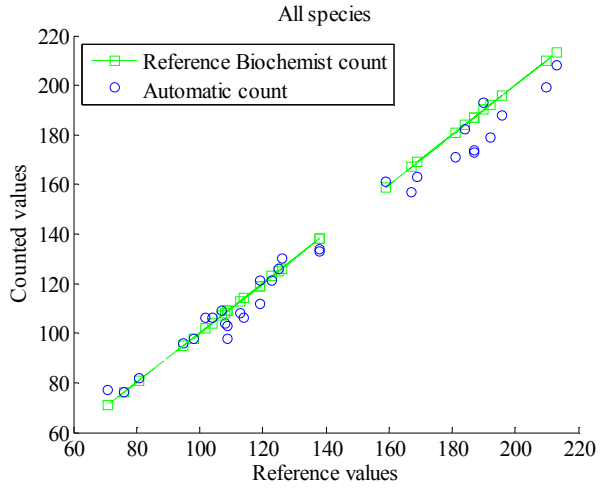


Fig. 7. Reference counting compared to proposed automatic counting results for the ROIs 1B, 2B, 3B, and 4B

proposed algorithm were those on the top of the grid lines. Therefore, smaller errors result from using the external ROIs, or eventually a chamber with less grid lines (e.g., Fuchs-Rosenthal).

IV. Conclusion

The proposed method, when using chambers with smaller number of grid lines, is able to provide results with errors smaller (i.e., 3%) than those errors reported for visual assessment (i.e., 10% [13]). Therefore, it has the potential for being used either as a complete automatic counting tool in laboratories for wild animals blood analysis or as a first counting stage in a semi-automatic counting tool. In this second option, the proposed method would be used to automatically find approximately 97% of cells and the user would be responsible for marking the missed cells, i.e., approximately 3%, in a second counting stage using the computer assisted tool presented in this paper. The use of this solution can improve the result accuracy, diminishes time for analysis and enable proper documentation by saving marked images.

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