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Algorithm for the use of ImageJ: Application in the detection of osmotic fragility human erythrocytes

Algoritmo para el uso de ImageJ: aplicación en la detección de fragilidad osmótica en eritrocitos humanos

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Abstract

The study of erythrocyte characteristics is carried out through different tests, including microscopy and image analysis. The purpose of this article is to develop a functional algorithm in the *ImageJ* software to identify the morphological characteristics of erythrocytes and to determine the variation in osmotic fragility when they are exposed to different conditions. The images were obtained using a Leica Aristoplan microscope and captured with a Leica DMC6200 camera connected to the equipment, and subsequently processed in *ImageJ*. The algorithm provides an orderly description of the process, from image calibration to the acquisition of quantitative results. This process includes calibration according to the microscope magnification, correction of possible image errors, and identification of cells to obtain parameters such as area, perimeter, roundness, and Feret's diameter. The application of the algorithm in *ImageJ* enables image processing that facilitates the morphological quantification of erythrocytes. The algorithm presented is efficient and reproducible, and can also be adapted according to user needs. Based on the analysis, it is concluded that the algorithm can be modified depending on specific requirements. Overall, the algorithm facilitates the use of *ImageJ* for the morphological quantification of erythrocytes.

Resumen

El estudio de las características de los eritrocitos se realiza mediante distintas pruebas, entre ellas la microscopía y el análisis de imágenes. El propósito de este artículo es desarrollar un algoritmo funcional en el software *ImageJ* para identificar las características morfológicas de los eritrocitos y determinar la variación de la fragilidad osmótica cuando estos son expuestos a distintas condiciones. Las imágenes utilizadas fueron observadas mediante un microscopio Leica Aristoplan y capturadas por una cámara Leica DMC6200 conectada al equipo, para posteriormente ser procesadas en el software *ImageJ*. El algoritmo describe de forma ordenada el proceso que se realiza desde la calibración de la imagen hasta la obtención de resultados cuantitativos. Este proceso incluye la calibración según el aumento del microscopio, la corrección de posibles errores en la imagen y la identificación de las células para la obtención de parámetros como el área, el perímetro, la redondez y el diámetro de Feret. La aplicación del algoritmo en *ImageJ* permite un procesamiento de imágenes que facilita la cuantificación morfológica de eritrocitos. El algoritmo presentado es eficiente y reproducible, además puede ser adaptado según las necesidades del usuario. Tras el análisis, se concluye que el algoritmo puede ser modificado según los requerimientos del usuario. En conjunto, el algoritmo facilita el uso de *ImageJ* para la cuantificación morfológica de eritrocitos.

Introduction

Microscopy was a determining and relevant factor for the development of research on living organisms throughout history. It began with Robert Hooke in 1665, when he invented apparatus with glass lenses to be able to observe, measure and record various phenomena, allowing him to be the first to describe the cell and observe the structure of tissues[1,2]. The microscope has become a universal tool used in different areas of research, for this reason, it gives infinite possibilities to study the morphology, composition, structure and other characteristics of various living organisms and inorganic matter.

The characteristics of the cell were studied for a long time; the first microscopic investigations of blood began with Marcello Malpighi, who described capillary structures and tissues, questioning the beliefs about blood composition of his time. Subsequently, Antonie van Leeuwenhoek and Jan Swammerdam looked under a microscope at the blood cells, which they called "globuli rubri" (red blood cells)[3].

With the development of new technologies, studies on red blood cells have been perfected, allowing their shape, size, density, diameter and color to be characterized. These properties are currently used for the diagnosis of various hematological diseases, such as anemia, including megaloblastic anemia[4].

Erythrocytes are blood cells present throughout the body, responsible for transporting oxygen to the tissues and eliminating carbon dioxide generated in cellular respiration[5].

A healthy erythrocyte is shaped like a biconcave disc with a depressed center, lacks a nucleus, and has a characteristic red color due to its high concentration of hemoglobin, an iron-rich protein. The average diameter of an erythrocyte in an adult person ranges from 7 to 8 µm[6].

The shape, dimensions and characteristics of erythrocytes observed under the microscope can be altered in the presence of diseases or changes in their physiological conditions. A clear example is sickle cell anemia, which causes the deformation of erythrocytes into a sickle-shaped, making it difficult for them to pass through small blood vessels and favoring hemolysis[7].

Among these diseases, many alterations can be detected by morphological analysis of erythrocytes, and different techniques have made it possible to identify different types of anemia according to cell shape. On the other hand, conditions such as polycythemia (erythrocytosis) can be recognized by elevated red blood cell counts. Likewise, the pathological classification of these cells can be carried out by microscopic observation[6,8,9].

To improve the visualization of blood cells under the microscope, various staining techniques have been developed to highlight their morphological characteristics. Among the most commonly used in hematology are Wright, Giemsa, and Leishman stains, each with specific applications. Wright staining, for example, is used in blood smears to differentiate cellular components, staining the nuclei blue and the cytoplasm pink[10,11].

Stains facilitate the visualization of cell structures under a microscope; However, to achieve a more precise quantification of these characteristics, images are captured using cameras attached to the microscope and their subsequent analysis with specialized software.

The analysis of the differences in the images obtained is essential to identify variations and changes in living systems or in structures that are difficult to perceive with the naked eye. Thanks to technological developments, it is now possible to perform three-dimensional reconstructions of structures using techniques such as high-resolution cryo-electron microscopy[12]. These advances have boosted science and allowed for a deeper understanding of the behaviors and characteristics of living systems.

Initially, computer programs were mainly used for image storage. Later, the first editing programs emerged, such as Adobe Photoshop[13], which was one of the first to be applied to microscopy for image editing. Later, more specialized programs were developed, such as *ImageJ* in 1997, derived from the NIH Image[14]. Currently, microscopy makes it possible to capture and store images or videos of the observed phenomena, which can be analyzed using specialized software. Among these, FIJI, *ImageJ*[15], Icy[16] and Orbit[17] stand out, which facilitate the identification and processing of structures, allowing them to be characterized more clearly[18].

ImageJ is one of the most prominent programs in image analysis and processing, widely used in the scientific field[19]. It allows a rigorous analysis of visual data and is especially useful in the study of biological systems thanks to its wide variety of tools[20]. Being open source and free, it can be used from any computer, which facilitates universal access. In addition, it has instructions and functions that make its use a simple and practical experience[21].

To make the most of free programs such as *ImageJ*, which facilitate information management and data analysis, it is necessary to standardize algorithms for the study of biological images, in order to simplify research and analysis of results. There are currently no standardized protocols that systematically describe the use of this program in the analysis of red blood cells, despite the fact that microscopic observation is a commonly used method for the detection of diseases. However, research has been carried out that performs cell counting on platforms such as *MATLAB*[22] and others that propose specific methods, such as the one proposed by Chadha et al.[23].

The objective of this article is to propose an algorithm for the analysis of erythrocyte images, which allows obtaining quantitative morphological parameters such as diameter, area, roundness and cell count through the use of *ImageJ software*.

Materials and methods

Patient preparation. Prior to taking samples, a complete blood count was performed in the laboratory of the General Hospital of La Paz to rule out hematological alterations that could affect the results. The individual had a hematocrit of 49 %, within the normal values for his age and gender[24]. Once the healthy status was confirmed, an informed consent was signed for their participation in the study, as established by the Bioethics Committee of the Bolivian Catholic University "San Pablo", La Paz campus. The research was carried out between November 2023 and March 2024.

Sample collection. Samples were obtained from the same individual to avoid data variability. A 3 mL sample of blood was taken daily for five consecutive days, at an ambient temperature of 25 °C, placed in tubes with EDTA as an anticoagulant (Vacuette, batch 454217).

Preparation of the sample prior to observation. The blood was processed according to the method of Alonso-Geli et al.[25]; It was centrifuged at 1000 g for 10 min to separate the plasma. Subsequently, 40 µL of the remaining globular package were taken and washed in 4 mL of sodium chloride (NaCl) solution (Scharlab S.L., Spain, batch 11285202). This procedure was repeated with 12 different concentrations of NaCl, 0.0, 0.1, 0.2, 0.25, 0.3, 0.32, 0.36, 0.4, 0.45, 0.55, 0.7 and 0.9 %. Finally, the samples were centrifuged again for 10 min in a centrifuge (Sigma, Germany).

With the erythrocyte package prepared, a drop was deposited on the slide, the sample was smeared and allowed to dry at room temperature (25° C) for 30 min[26].

To improve the visualization and distinction of the erythrocytes in each sample, Wright's Dip Stat staining kit (Chemical Corporation, United States, lot A187) was used, which contains four 250 mL solutions: fixative solution, eosinated stain, polychromatic stain, and rinse solution. The sample was stained according to the technique described by Li et al.[27]. Once the procedure was completed, it was left to dry for 1 h. Finally, a drop of dipex was added to preserve the preparation at room temperature and the coverslip was placed on top.

Observation of the sample under a microscope. The samples were observed under a Leica Aristoplan microscope (Germany) with 40x, 50x and 100x lenses. The images were captured using a Leica DMC6200 Pixel Shift camera (Germany), connected to the microscope. Images were recorded with each lens, selecting the field that would allow the cell characteristics to be differentiated more clearly. Once the observation was completed, the images were selected and coded for further analysis.

Sample analysis in ImageJ. The ImageJ program was used, downloaded from the network. Once installed, the application was entered on the computer. To explore its tools, the *File* tab → *Open Samples* was used, which displays a menu of images preselected by the program. To start the analysis of the samples, *File* → *Open* was selected and the corresponding image was loaded. The images analyzed were in TIFF format, which offers better quality for handling details; however, *ImageJ* accepts multiple formats such as PNG, GIF, and JPEG[28].

Once the image was selected, it was displayed on the screen. At the top of the window, below the toolbar, the file's name, format, resolution, color system, and size were displayed (Figure 1). Moving the cursor across the screen and selecting a point brought up the *x* and *y* coordinates of the selected area; by right or left click you could increase or decrease the size of the image window.

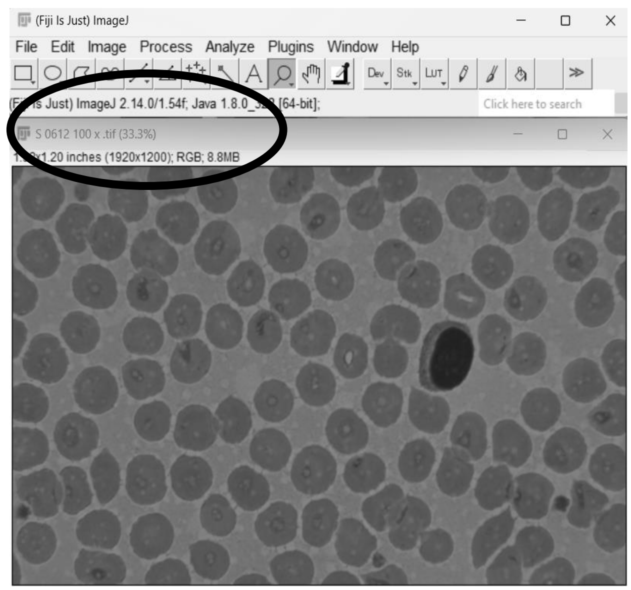

Figure 1 Screenshot of ImageJ software showing the available toolbar

Image calibration. To start working with the application, it is necessary to calibrate the image according to the measurements of the microscope. To do this, an image of an object with known dimensions must be captured during the observation, which will be used as a reference image. It is essential to have a reference image for each lens used.

The procedure consists of opening the reference image corresponding to the lens, selecting the line tool and drawing a straight line on the object whose measurement is known. Then, in the Analyze menu, choose the *Set Scale* option; in the *Known Distance* field, enter the known measurement, and in *Unit of Length*, select the corresponding unit, thus adjusting the scale to the actual size of the image to be analyzed. Once the calibration is done, the units will be displayed according to those set by the user. In images taken with the same lens, the initial calibration is maintained; however, when changing lenses, the calibration process must be repeated (Figure 2).

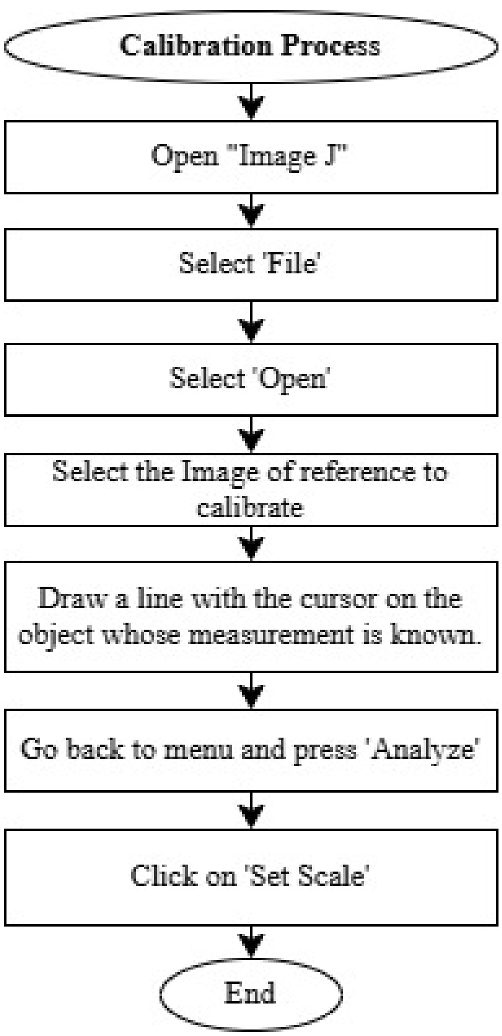

Figure 2 Image Calibration Process Algorithm in *ImageJ Software*

Image processing. Once the calibration is done, the image to be analyzed is opened. From the menu, select *Image* → *Adjust* → *Color Threshold*; if you don't want to make additional settings, the pop-up window closes, generating a red background in the image. Subsequently, from the menu, *Process* → *Binary* → *Make Binary* is selected (Figure 3), converting the image to black and white. The background should appear black and the cells white; if this does not happen, you should go to *Edit* → *Invert*, which inverts the colors and sets the background to black and the cells to white.

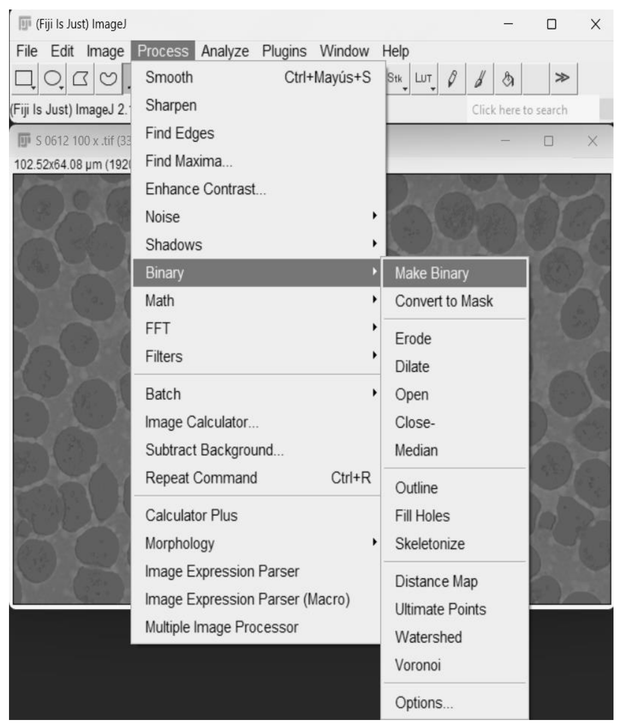

Figure 3 ImageJ Software Screenshot of a Step of the Image Processing Algorithm

If the cells cannot be distinguished from each other, because they are very close together, the Erode tool can be used, which reduces the contour of the white areas corresponding to the erythrocytes by one pixel. Finally, *Process* → *Binary* → *Fill Holes* is selected to ensure that the area calculation is complete (Figure 4).

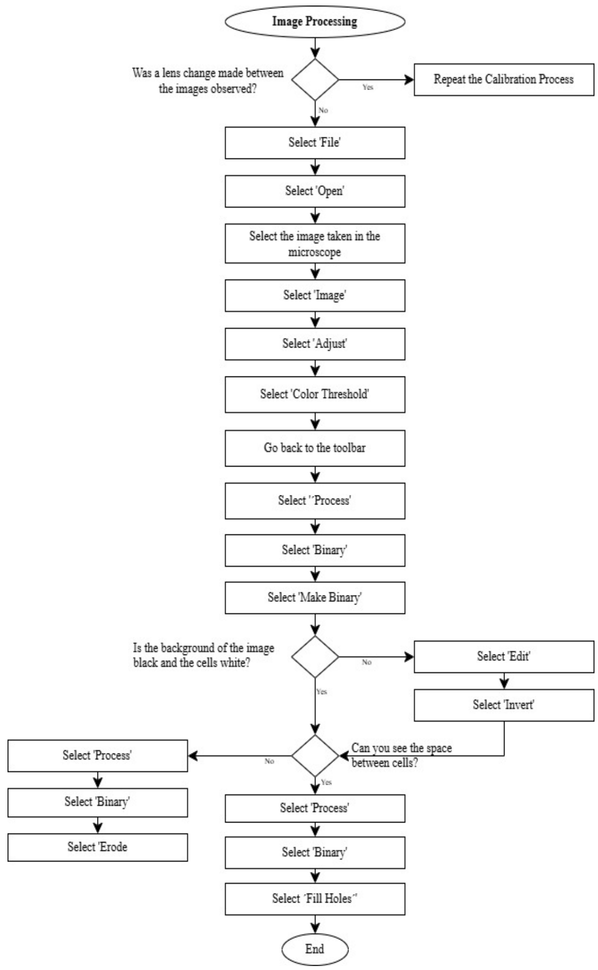

Figure 4 Image Processing Algorithm in *ImageJ Software*

Visualization of results. First, select the Analyze → Set Measurements option and ensure that the required options are enabled. In this case, the following were used: Area, which determines the area of the cell; *Perimeter*, which calculates the perimeter of each cell; *Shape Descriptors*, a parameter that indicates, in a range from 0 to 1, the similarity to a circular shape; *Feret's Diameter*, which measures the diameter in a specific direction; and *Add to Overlay*, which assigns a number to each cell in the image based on the count made. Finally, it is necessary to verify that in the *Redirect to* option so that the name of the image you are working on appears, to ensure that the processing is done correctly (Figure 5).

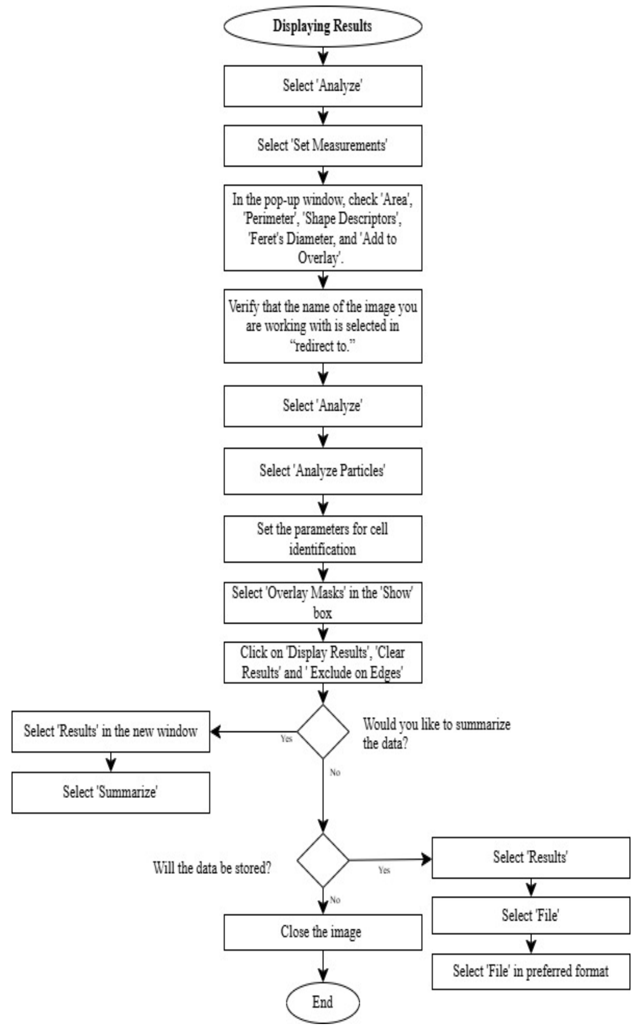

Figure 5 Algorithm for Performing Results Display in *ImageJ Software*

Particle analysis. The next step is to select *Analyze* → *Analyze Particles*. With this option it is possible to modify the parameters according to the needs of the analysis; in this case, the settings preset by the application were maintained: in *Size* from 0 to ∞, in *Circularity* from 0 to 1, and in *Show* Overlay Masks *was selected*. In addition, the *Display Results* (to display results), *Clear Results* (to avoid repetitions), and *Exclude on Edges* (to exclude incomplete cells at the edges of the image) options were enabled.

After completing this procedure, the image was shown with a black background and each cell was marked with a color and a number (Figure 6). Simultaneously, a table was generated with all the results. Inconsistent data can be adjusted by delimiting the parameters in *Set Measurements*.

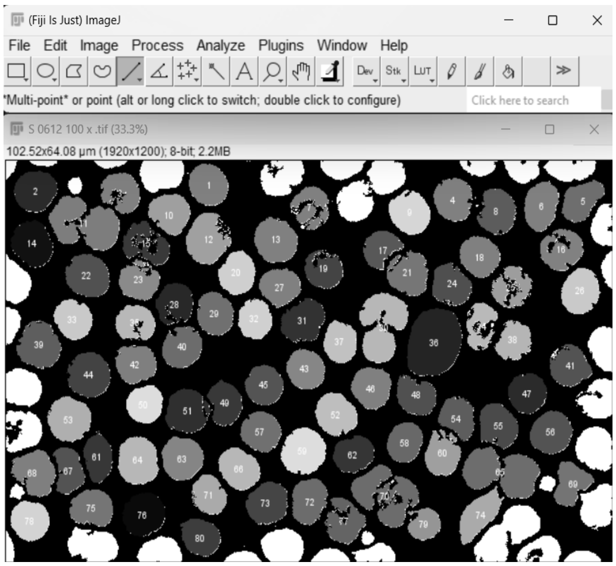

Figure 6 Results of the configurations used in the images to count the cells present

Summary and storage of results. Finally, you can get a summary of the data in the same table shown. Results → Summarize is selected, and the data will appear in summary form at the bottom. In Table 1 they are presented in a similar way to how they are shown in the software. Finally, if you want to save these results, you select *Results → File → Save,* which allows you to store them in Excel or TXT format.

Results

The final algorithm developed for implementation in the *ImageJ software* is the result of the process described in the previous stages (Figure 7). This algorithm facilitates the analysis of the images and allows obtaining quantitative morphological parameters of the erythrocytes observed, such as the area, perimeter, roundness and diameter of Feret. In addition, it seeks to optimize microscopy image processing, ensuring that the results are reproducible, accurate, and easy to interpret.

This algorithm seeks to facilitate the morphological analysis of erythrocytes and offer a tool adaptable to different types of biological samples with similar morphology. In this way, the use of *ImageJ* is simplified for researchers who require a standardized and functional procedure in cell analysis.

All values were automatically calculated by the program after the algorithm was applied. As can be seen, the standard deviations of the parameters of area, perimeter, circumference and Feret diameter are low, which shows an adequate consistency of the procedure and a correct delimitation of the image (Table 1). From this algorithm, a more specific analysis of the morphological variations of the samples under different osmotic conditions was carried out, applying the proposed method and following the methodology described in the document.

The analysis shows an upward trend in the average area of erythrocytes as the concentration of NaCl in the medium decreases (Table 2). At the same time, a decrease in the Feret diameter is observed. Standard deviation values are kept low relative to the averages, confirming the accuracy of the algorithm. These changes indicate that the method is capable of detecting morphological differences. Overall, the results show that the algorithm designed in *ImageJ* allows quantifying morphological parameters in a reproducible way, facilitating the obtaining of verifiable data under different observation conditions.

Tabla 1 Resultados de la muestra de sangre a 100x analizada en *ImageJ*

	Label	Area	Perim.	Circ.	Feret	FeretX	FeretY	FeretAngle	MinFeret	AR	Round	Solidity
77	Mean	0.013	0.509	0.674	0.147	861.882	581.474	100.048	0.121	1.212	0.843	0.942
78	SD	0.003	0.133	0.149	0.025	521.309	312.855	51.697	0.013	0.212	0.108	0.033
79	Min	0.01	0.396	0.168	0.122	20	19	8.315	0.088	1.006	0.443	0.834
80	Max	0.028	1.006	0.869	0.249	1792	1152	178.85	0.166	2.256	0.994	0.977

Tabla 2 Resultados de valores promedio de área y diámetro de eritrocitos a distintas concentraciones de NaCl (100x)

NaCl concentration (%)	100x magnification		
	Parameter	Area [μ^2]	Feret [μm]
0.9	mean	15.52315	5.05292
	SD	049921	0.09452
	Min	15.17016	4.98608
	Max	15.87615	5.11975
0.4	mean	25.96679	6.41365
	SD	6.15619	1.33875
	Min	18.07716	4.97799
	Max	40.60766	9.86242
0.32	mean	19.06149	6.06803
	SD	3.70165	0.5807
	Min	12.6247	5.14902
	Max	24.4628	7.09696

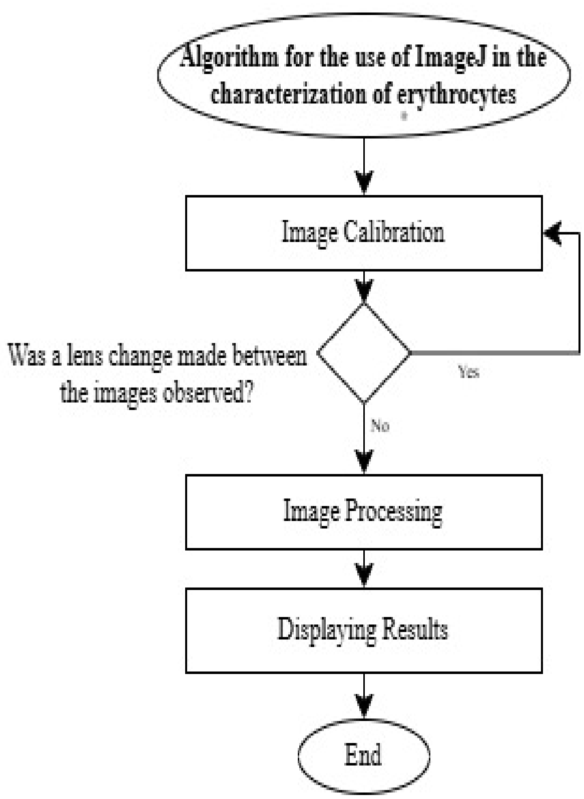

Figura 7 Algoritmo final para realizar la caracterización de los eritrocitos

Discussion

Many of the tools used for observation in *ImageJ* are also available in other programs. In the study by Mejía & Alzate[29], the analysis of erythrocytes according to their shape was performed on the *MATLAB* platform, where image processing and morphological characterization were carried out according to their shape and size. These authors point out that it is possible to use *MATLAB* to measure morphological parameters in microscopy. The preference for *ImageJ* over *MATLAB* lies in its accessibility and ease of use[30]. Unlike *MATLAB*, which requires a paid license and advanced programming skills as it is not specialized in image analysis, *ImageJ* is free, easy to install, and has a large community of users, as well as multiple tools aimed at analyzing biological images.

In accordance with these advantages, several authors have highlighted the relevance of open computational tools. Li et al.[31]. They conducted a review on the use of free platforms in the analysis of biomedical images, underlining their role in the standardization and accessibility of scientific methods. In addition, they pointed out that most studies lack parameters that allow replicating their procedures. These findings support the use of *ImageJ* as an effective and accessible platform, promoting the creation of reproducible algorithms for the morphological characterization of blood cells, especially in academic and research settings where reproducibility and low cost are essential.

The algorithm developed in *ImageJ* could be complemented in the future with *deep learning* strategies to create automated diagnostic systems that combine the precision of digital processing with the intelligent interpretation of biomedical images. Cakmak & Pacal[32] showed that artificial intelligence can be used in the diagnosis of anemia using *machine learning* techniques, taking advantage of the ability of these tools to detect morphological patterns and classify blood cells automatically, thus contributing to the early detection of hematological diseases.

In addition, recent advances in artificial intelligence have highlighted the potential of integrating image analysis algorithms with deep learning models. An example is the tool called *RedTell*, designed to interpret the morphology of erythrocytes[33]. Similarly, studies such as those by Foy et al.[34] and Ghosh et al.[35] have pointed out the usefulness of artificial intelligence to facilitate the analysis and collection of data on the morphological characteristics of erythrocytes. The algorithm used in this research is specific to measure morphological parameters of erythrocytes. However, its structure can be adapted to the analysis of cells with circular or quasi-spherical morphology. In future work it would be convenient to evaluate their performance in cells with different shapes or irregular morphology.

Finally, it is important to consider certain recommendations that prevent potential errors that can affect the performance of the algorithm. These include the correct preparation of the sample, such as the delicate performance of the smear[26]; if it does not allow you to observe areas where the cells are scattered, the image cannot be used, even with the *Erode* correction tool. The same happens when staining is not performed properly[27]. Another frequent error is related to the identification and delimitation of cell size: structures other than the object of study may appear in the image, such as leukocytes, which stand out after staining due to their size (Figure 1). This problem can be ruled out by selecting the Area parameter, limiting the component count. Also, the taking of the image for calibration must be precise, since it is decisive in the entire process[36].

In addition, correct calibration of the microscopy with the micrometer reticle is essential to ensure the reliability of the measurements. Each objective lens must be calibrated independently, as variations in magnification change the conversion scale from pixels to micrometers. Overall, the results and considerations presented reinforce the validity of the algorithm as a reliable tool for image analysis aimed at the morphological study of erythrocytes. The main contribution of this work lies in the standardization of a reproducible and accessible algorithm for any scientist who is starting out in image analysis using free software such as *ImageJ*, allowing accurate data to be obtained without resorting to high-cost programs. The implementation of this algorithm in *ImageJ* facilitates the study of hematological images and can be adapted to the analysis of cells with similar morphology, constituting a solid basis for future research. In this way, the proposed algorithm contributes to the standardization of the morphological analysis of erythrocytes in research.

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Conflicts of interest

This article was made with the samples belonging to the project work team so it does not present conflicts of interest.

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Ethical considerations

For the collection of blood samples, the approval of the bioethics committee of the Bolivian Catholic University "San Pablo" was obtained and the samples were taken with the signed consent of the donor.

Limitations in the research

In the research, the samples were not triplicated and samples were extracted from the same individual.

Permissions for publication

The research does not require any permission, the content of the research is original and has not been previously published.

Authors' contribution to the article

The writer of the article is *Natalia Agramont Morales*. The reviewer of the article and guiding professor is the *PhD. Georgina Chávez Lizárraga*. Those in charge of making the diagrams presented in the article were *Belén Araceli Flores Botello* and *Marcia Olga Carrasco Rosso*. *Daira Quenta* was in charge of the preparation of the samples. All authors performed data analysis for the laboratory.

Use of artificial intelligence

No artificial intelligence tools were used in the writing of this manuscript. Everything was written by the authors.

Imaging Disclosure

All figures, tables and diagrams were made by the authors, no artificial intelligence tool is used in their realization.

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