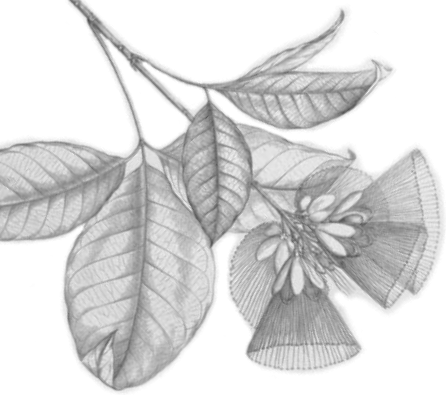

Microscopic Image Segmentation to Quantification of Leishmania Infection in Macrophages

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ABSTRACT

The determination of infection rate parameter from *in vitro* macrophages infected by *Leishmania* amastigotes is fundamental in the study of vaccine candidates and new drugs for the treatment of leishmaniasis. The conventional method that consists in the amastigotes count inside macrophages, normally is done by a trained microscope technician, which is liable to misinterpretation and sampling. The objective of this work is to develop a method for the segmentation of images to enable the automatic calculation of the infection rate by amastigotes. Segmentation is based on mathematical

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morphology in the context of a computer vision system. The results obtained by computer vision system presents a 95% accuracy in comparison to the conventional method. Therefore, the proposed method can contribute to the speed and accuracy of analysis of infection rate, minimizing errors from the traditional methods, especially in situations where exhaustive repetitions of the procedure are required from the technician.

Keywords: Amastigote Count; Image Segmentation; Environmental Degradation.

Leishmaniasis in the tegumentary and visceral clinical forms are anthropozoonosis caused by protozoa of the genus *Leishmania* and transmitted by females of some phebotomine insects species (Conceição-Silva and Alves 2014). They are worldwide prevalent neglected diseases with one million cases registered in the last five years and 20,000 deaths per year (WHO 2010).

Currently, environmental degradation such as changes in water temperature, irrigation, deforestation, climate change and drug resistance are important factors contributing to the spread of leishmaniasis (Akhoundi et al. 2016; Abrantes et al. 2018; Oryan and Akbari 2016). It is difficult to control the leishmaniasis because the fact there is no vaccine for the protection of humans and domestic animals (Da-Cruz and Pirmez 2005). The treatment of infected individuals is the main control parameter, since preventive measures are not efficient (Anversa et al. 2018).

Currently, the use of leishmanicidal drugs, such as antimonials, initially proposed by Brazilian physician Gaspar Vianna (Vianna 1912; Almeida et al. 2003), remains the first choice for treatment. New drug tests for the treatment of leishmaniasis require several stages of evaluation. Initially, the selective power of the drug[13] and the efficiency in controlling the parasite load[14] are evaluated in comparison to the conventional drugs (Sifontes-Rodríguez et al. 2015).

The development of mathematical methods is important for improving the quality of treatment using the therapeutic dose control and the optimization of laboratory protocols from the reduction of time costs (Catharina et al. 2017; Leal et al. 2012).

[13] Ability to be toxic to the parasite without damaging the host cell.
[14] Measured by the infection rate (the percentage of infected macrophages) and infection index (mean value of intracellular amastigotes among infected macrophages).

Biomedical research and diagnosis of diseases based on image analysis is an important strategy to minimize uncertainties in the dosage of drugs to combat disease and aid in the testing of new drugs (Catharina et al. 2017). Manual procedures of laboratory routine such as macrophage count can be replaced by automated procedures with gain of patient waiting time to receive the appropriate medication and cost reduction (Leal et al. 2012).

Automated procedures for counting macrophages involve different steps in the analysis of a digital image (Yazdanparast et al. 2014). Image segmentation is a stage of constantly evolving image analysis and consists of dividing the digital image into sets of pixels or regions in order to simplify the image for the analysis. In general, the success or failure of the analysis depends on the segmentation step (Petrou and Petrou 2010). Result of segmentation is a set of contours extracted from the image where each of the pixels of the same region is similar to some computational property such as color, intensity, texture or continuity (Snyder and Qi 2017).

Neves and collaborators propose an algorithm to locate the positions of macrophages and parasites in fluorescence images of *Leishmania*-infected macrophages. Their strategy is primarily based on blob detection, clustering, and separation using concave regions of the cells' contours. They claimed have achieved better performance in the automatic annotation for *Leishmania* infections in comparison with other approaches that analyse fluorescence images (Neves et al. 2014).

Ouertani and collaborators developed an automatic segmentation method to locate fluorescent Promastigote parasite. They developed segmentation algorithms for the removal of the background of the microscopic image and grouping in regions to remove noise and locate parasites in the fluorescent image (Ouertani et al. 2014).

Farahi and collaborators provide an automatic segmentation method of *Leishmania* parasite based on digital color microscopic images, captured from bone marrow samples (Farahi et al. 2015). The algorithm is based on the Chan and Vese method that ignores the edges (Chan and Vese 1999).

Taheri and collaborators developed a photography-based technique and an algorithm for extracting a three dimensional map of leishmaniasis lesion using a two dimensional image. The method finds the depth in the estimate of the blur of the image caused by a lens (Taheri et al. 2017).

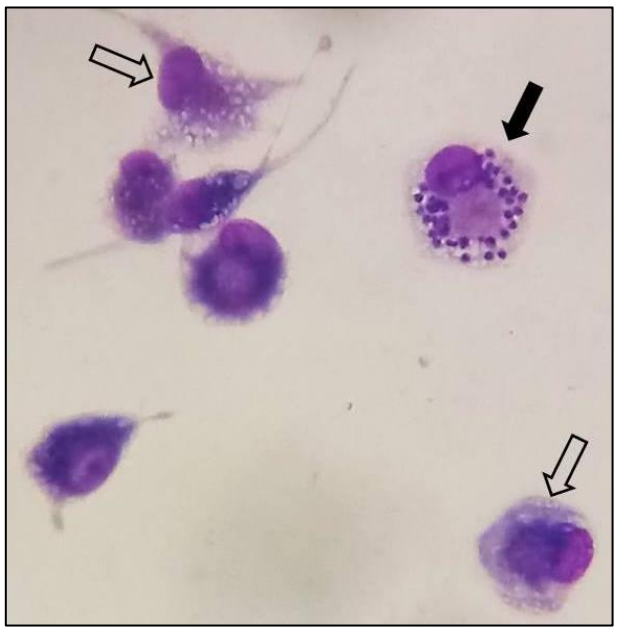
This work presents an algorithm for robust segmentation of images based on mathematical morphology to enable counting of macrophages and intracellular amastigotes. Algorithm is able to handle with high variability of shapes and textures typical of optical light microscope images.

MATERIAL AND METHODS

This work is related to the development of an algorithm for automatic annotation of *Leishmania* parasite in macrophages. The dataset are microscope images from macrophage monolayer culture. The samples are produced from rapid and focused panoptic method with $100x$ objective in immersion oil. The culture is prepared at the Interdisciplinary Laboratory of Medical Research (LIPMED) of the Oswaldo Cruz Foundation (FIOCRUZ). A Nikon eclipse 80i optical light microscope is used for imaging the samples.

Image segmentation process begins with the generation of color images in Red, Green, and Blue (RGB) space of $1024x1280$ pixels and with 16 bit precision in each color channel. Morphological mathematical operations are applied only on channel *B* (blue) due to the coloration used in the preparation of the samples for the images acquisition.

Figure 01. Microscopy blade with Leishmania-infected macrophages. Full arrow indicates infected macrophage with intracellular amastigotes and empty arrows indicate the non-infected macrophages.



Source: FIOCRUZ (2018)

Figure 01 shows an example of the image set used for segmentation of macrophages. The background of the image or parts of the image that does not contain macrophages or *Leishmania* is characterized by regions of homogeneous colors with light tones. Macrophages define complex, larger regions, resulting from the presence of nucleus, depicted by dark regions and cytoplasm that has

characteristic texture. The *Leishmania* of interest are arranged in the cytoplasmic region of the macrophages as dark regions, however, of smaller area when compared to the macrophages nuclei.

The methodology proposed in this work starts by detecting the structures that make up the image based on its connectivity. To that end, the image is first submitted to a smoothing operator with Gaussian filter in order to remove edges from noisy structures that make up the image background. Since the original image $I(x, y)$ and $H(u, v)$ is the Gaussian filter, the smoothed image is obtained by the convolution operator

$$G(x, y) = I(x, y) * H(u, v) = \sum_{u,v \in H} [\, I(x - u, y - v) \cdot H(u, v) \,] \tag{1}$$

where $H(u, v) = (2\pi\sigma^2)^{-1/2}\, exp\{-\frac{(u+v)^2}{2\sigma^2}\}$ and σ^2 is the variance of the Gaussian filter. The smoothing process results in an image with less influence of noise, prevailing macroscopic structures that make up the image. Then the background segmentation is obtained by binarizing the image so that $B(x, y) = 1$ if $G(x, y) \leq T$, and $B(x, y) = 0$ if$G(x, y) > T$, where $B$is the binary image and $T$is the binarization threshold that separates light regions (object of interest) from background.

Assuming that the objects of interest are connected regions, the binary image is subjected to the process of filling holes (holes, depression) to remove background pixels inside the light regions. The fill algorithm is the complement of the morphological reconstruction by dilation, resulting from the recursive morphological operation of geodesic dilations of the Bimage with the structuring element$S$using the marker F as the starting point (Gonzalez et al. 2008). We define F as a binary image of the same size as B, called the marker image constructed as $F(x, y) = I - B(x, y)$. If (x, y) is a pixel on the edge of image B, and $F(x, y) = 0$,otherwise. The geodetic dilation of size 01 of image B is denoted by $D_B^{(1)}(F) = (F \oplus S) \cap B$, with $\oplus$ as dilation operator, and $D_B^{(n)}(F) = D_B^{(1)}\left(D_{B^c}^{(n-1)}(F) \right)$ is recursive definition of dilation operation, considering $n > 1$.Binary image is denoted by Hequal to B, free of holes

$$H = \left[R_{B^c}^{D}(F)\right]^c \tag{2}$$

where B^c is the complement of image B and $R_{B^c}^{D}(F) = D_{B^c}^{(k)}(F)$ is the morphological reconstruction operation by dilation with k such that $D_{B^c}^{(k)}(F) = D_{B^c}^{(k+1)}(F)$.

Identification of regions is achieved by applying the pixel labeling method according to their connectivity. Let X_n^0 a binary matrix, of the same size as H,formed by zeros except for a position corresponding to a point of the *n*th connected region of H,the iterative procedure

$$X_n^k = (X_n^{k-1} \oplus S) \cap H. \quad (3)$$

results in the *n*th image. The procedure ends when $X_n^k = X_n^{k-1}, k = 1, 2, 3, \ldots$

Regions X_n, whose area is larger than A_M are considered macrophages. For detection of amastigotes the same method is applied considering a minimum area of A_L, but only in the regions of the image that are occupied by the identified macrophages. For the results presented in this work, the values are: $T_M = 45000$ and $A_M = 10000$; the binarization threshold and minimum area for the segmented regions in the macrophages detection stage $T_L = 30$ and $A_L = 300$; the binarization threshold and minimum area for the segmented regions in the detection stage of *Leishmania*.

The parameters used are defined empirically during the image analysis process. The segmentation process is executed with different parameters values and the results analyzed until the best outcome. In the result and discussion section is presented the parameters used to obtain the results and the implication to use the same values in other images to obtain similar results. Definition of values to these parameters can be considered as a contribution of this work.

The algorithm was developed at the Scientific Computing Laboratory (*Laboratório de Computação Científica*, LCC) of the Pontifical Catholic University of Goiás using Matrix Laboratory (MATLAB).

RESULTS AND DISCUSSION

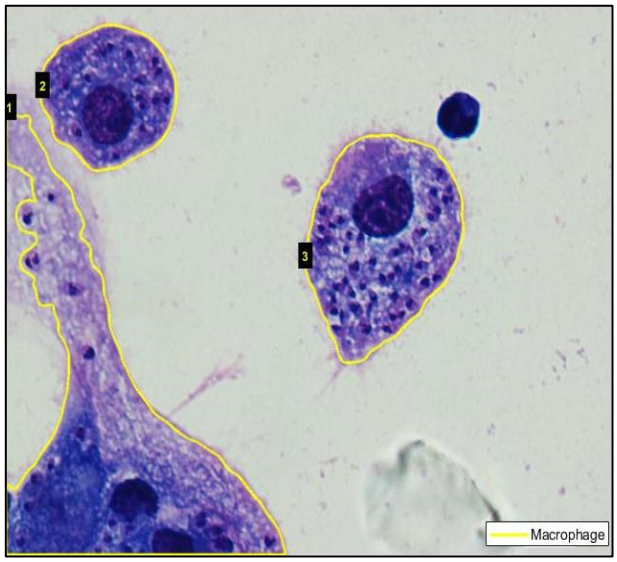
The results presented are related to the detection and counting of macrophages and amastigotes in images obtained from different visual fields containing a certain confluence. To illustrate the results, Figure 02 depicts the detection of macrophages. It shows a yellow outline delimiting the edges of each macrophages. Each macrophage was identified from one to three, starting from left to right. It is also possible to visualize two other forms above and below the macrophage three, referring to artifacts of technique of blade attachment. This figure is used as an example of quantitative analysis to all results obtained from the method.

Macrophage 1 is detected as a single form. However, it is not possible to determine whether there are different macrophages or a single macrophage activated macrophage. Thus, in agreement with what occurs in conventional counting, macrophage 1 is not included in the analysis because it is not possible to determine whether there are different macrophages or a single activated macrophage. Thus, according to conventional counting, macrophage 1 is not included in the automatic analysis.

Figure 03 shows the results of the detection and counting of *Leishmaniasis* into macrophages 2 and 3 of Figure 02. In Figure 3(a), it is possible to verify that the algorithm detected all 15 *Leishmania* contained in macrophage 02. Figure 3(b) shows that the algorithm detected 45 *Leishmania*, while in manual counting it was detected 42 for the same image. In this case the algorithm obtains three false positives because some regions of the image have similar characteristics of coloration and area.

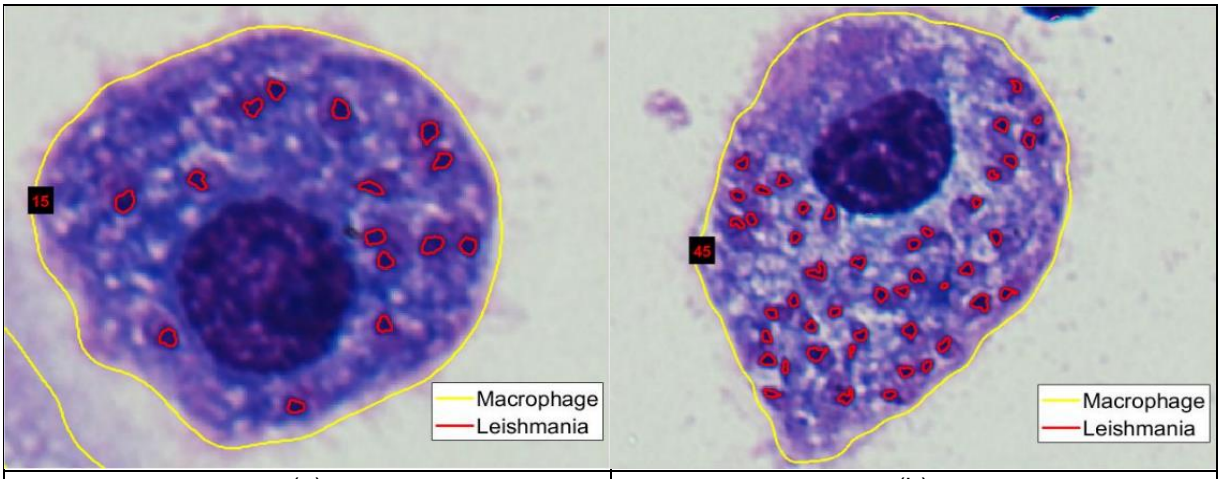
Proposed algorithm is executed for three more images resulting from different visual fields. Considering all the macrophages reliable for analysis, the algorithm obtained 95% accuracy compared to conventional counting done by a specialist.

Figure 02. Microscopy blade with Leishmania-infected macrophages for automatic segmentation of macrophages.



Source: FIOCRUZ (2018)

Figure 03. Result of detection and counting of Leishmania amastigotes within macrophages. (a) macrophage within 15 Leishmania parasite detected by the algorithm; (b) macrophage within 45 Leishmania detected by the algorithm.



(a) (b)

Source: FIOCRUZ (2018)

CONCLUSION

The automated microscope image analysis is an important tool to improve the speed and accuracy in the detection of disease. It is possible to analyze tens of thousands of images per day, avoiding subjective analysis that can occur in the traditional visual examination and minimize false positive derived from external interferences.

In this work, an algorithm was proposed for automatic counting of macrophages and intracellular *Leishmania* in microscopy images based on mathematical morphology in the context of computer vision. Macrophages and *Leishmania*, are connected structures formed by shades of similar pixels that differ in relation to their area and location on the sample, it was found that it is possible to differentiate and count the objects of interest to further determine the rate of infection by *Leishmaniasis*.

At the current stage of the algorithm, results obtained approximate the results produced by specialists in manual counting with the advantage of being automated and performed in a shorter time when compared to the manual counting process. In future works, it is intended to obtain more images and maintain a direct relationship with the experts for the fine tuning of the algorithm. Next, a method will be developed to calculate the *Leishmania* infection index or infection rate for the development of a mobile application to be used in different locations to aid in many applications like search for new anti-*Leishmania* drugs or anti-*Leishmania* vaccines.

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Segmentação de Imagem Microscópica para a Quantificação de Infecção por Leishmania em Macrófagos

RESUMO

A determinação de parâmetros como taxa de infecção em monocultura de macrófagos cultivados *in vitro* com *Leishmania* é fundamental no estudo de candidatos vacinais e novos fármacos para o tratamento de leishmanioses. O método convencional que consiste na contagem de amastigotas no interior de macrófagos, normalmente é realizada por um especialista treinado em microscopia óptica, o que está sujeito a erros de interpretação e amostragem. O objetivo do trabalho é desenvolver um método para a segmentação de imagens como etapa preliminar para o cálculo automático da taxa de infecção por amastigotas. A segmentação é baseada em morfologia matemática no contexto de um sistema de visão computacional. Os resultados obtidos pelo método computacional demonstraram acerto de 95% quando comparados ao método convencional. Conclui-se que a metodologia computacional baseada na segmentação de imagem como pré-requisito para o cálculo de taxa de infecção, pode contribuir para a rapidez e a precisão na obtenção dos resultados e na minimização de erros cometidos no método tradicional, especialmente em situações em que exaustivas repetições do procedimento são exigidas ao observador.

Palavras-Chave: Contagem de Amastigotas; Segmentação de Imagem; Degradação Ambiental.

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