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Techniques using ImageJ for histomorphometric studies

Técnicas de uso do ImageJ para estudos histomorfométricos

Uso de técnicas en ImageJ para estudios histomorfometricos

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Abstract

Computational histomorphometry is an available and easy tool that has been used in the assessment of morphophysiological tissue changes, offering greater scientific reliability to the data, as well as facilitating the automation process. The present work aimed to describe the application of the methodology of the free software ImageJ for morphological evaluation of fish tissues. For this, micrographs of histological sections of the intestinal tract of fish stained with Periodic Acid-Schiff (PAS) were used as a model. The images were analyzed for variables of length, width, and tissue area and, number of cells or molecules. The application of computational histomorphometry demonstrated efficiency in the evaluation of histological structures of the intestine of fish supplemented with probiotics, contributing to the improvement of image analysis techniques in animal tissue models.

Keywords: Histology; Morphology; Tissues; Software imageJ.

Resumo

A histomorfometria computacional é uma ferramenta disponível e de fácil utilização, que tem sido utilizada na avaliação de alterações morfofisiológicas dos tecidos, oferecendo maior confiabilidade científica aos dados, além de facilitar o processo de automação. O presente trabalho teve como objetivo descrever a aplicação da metodologia do software livre ImageJ para avaliação morfológica de tecidos de peixes. Para isso, utilizou-se fotomicrografias de cortes histológicos do trato intestinal de peixes corados com Periodic Acid-Schiff (PAS) foram utilizadas como modelo. As imagens foram analisadas quanto às variáveis de comprimento, largura e área do tecido e número de células ou moléculas. A aplicação da histomorfometria computacional demonstrou eficiência na avaliação das estruturas histológicas do intestino de peixes, contribuindo para o aprimoramento das técnicas de análise de imagens em modelos de tecidos animais.

Palavras-chave: Histologia; Morfologia; Tecidos; Software imageJ.

Resumen

La histomorfometría computacional es una herramienta disponible y fácil que se ha utilizado en la evaluación de cambios morfofisiológicos tisulares, ofreciendo mayor confiabilidad científica a los datos, además de facilitar el proceso de automatización. El presente trabajo tenido como objetivo describir la aplicación de la metodología de uso del software libre ImageJ en la evaluación morfológicos en tejidos de peces. Para ello, se utilizaron como modelo micrografías de cortes histológicos del tracto intestinal de peces teñidos con Ácido Periódico-Schiff (PAS). Las imágenes se analizaron en busca de variables de longitud, ancho y área de tejido y número de células o moléculas. La aplicación de histomorfometría computacional demostró eficiencia en la evaluación de estructuras histológicas del intestino de peces, contribuyendo a la mejora de las técnicas de análisis de imagen en modelos de tejidos animales.

Palabras clave: Histología; Morfología; Tejidos; Software imageJ.

1. Introduction

The approach of the histological structures of the gastrointestinal tract of fish allows application of this knowledge in the understanding of animal biology, morphophisiology, ethology and pathology, helping the development of food production chains, contributing to the rational use and with greater sustainability of environmental services (Tawfiek et al, 2017). Likewise, knowledge of the morphology of the digestive system in various species of fish is useful for research on food management and preparation of appropriate diets for adaptation and well-being in captivity (Rotta 2003; Moawad, Tawfiek & Awaad 2017; Vidal et al, 2019). The technology associated with microscopy has advanced on the world stage, enabling biological studies of images which provide the analysis of quali-quantitative data increasingly accessible, faster and more accurate (Schindelin, Rueden, Hiner & Eliceiri 2015).

The use of digital images as a scientific tool has collaborated with life scientists and through the application of image processing techniques to analyze data (Schindelin et al, 2015). The Digital Image Processing (PDI) of histomicrographs can be defined as computational technique or method that has as a premise the performance of several operations on the captured image, to improve it and obtain useful data (Carson & Hladik 2009; Gonzalez & Woods 2010; Guo & Zhang 2019).

According to Sengar, Dutta & Sarkar (2017) there are several steps for making and manipulating images, including acquisition, pre-processing, processing, analysis, and data extraction. The use of the PDI for a histomorphometric analysis consists, initially, in the capture of the image to be evaluated by a device (sensor), which can be a high-resolution digital camera, attached to the composite optical microscope (Schnell et al, 2020; Silva et al, 2016), that permits the picture to enlarge more than 2000 X (Novaes et al, 2007; Dias 2008; Novaes et al, 2010; Wang, Ka & Chen 2014).

The histomicrographs obtained by the sensors, in general, must go through pre-processing which allows the correction of geometric distortions and promotes the filtering of noise coming from the camera, performing transformations and improvements (Burger & Burge 2008; Hannickel et al, 2012; Casero et al, 2017; Passos et al, 2020).

In this work, we sought to focus on the ImageJ software as a study of how open source software promotes a tool interface, demonstrating an abundance of methods and approaches for analyzing digital images that can be easily accessible to scientific community (Schindelin et al, 2015; Caicedo et al, 2017). Therefore, the objective of this work was to demonstrate the use of the ImageJ Software for histomorphometric studies promoting accurate and automated measurements.

2. Computational Tool for Histomorphometric Evaluation

There are many technical and technological advances in the computational area that help all areas of knowledge (Passoni et al, 2014), emphasizing that the use and applicability of software and technologies can bring benefits to science, economics and society (Santos & Kalid, 2020). Through advances in hardware, software, and applications, several possibilities for using digital image processing have been created (Russ 2015). A tool that has been used in image analysis is morphometry, which is a word originated from the Greek for ‘measures a shape’, employed to methodically measure structures based on morphology and patterns of morphometry (Søreide et al, 2009) is an interactive tool that allows obtaining variables that can be confirmed by statistics (Di Leva, Bruner & Widhalm 2012). In this same understanding, Mojekwu & Anumudu (2015) complement that “the morphometry can be defined as a set of procedures to analyze a variability in the size and shape of organs and organizations.”

The application of these technological innovations has been reported in several areas of science, but mainly human medicine (Oliveira, Oliveira & Pinheiro 2010; Sousa, Vasconcelos

& Soares 2012; Rodrigues et al, 2013; Eberhardt et al, 2016; Jeffcoate, Musgrove & Lincoln, 2017; De Laffitte Alves et al, 2018; Weber & Santos 2019) and veterinary (Ginel et al, 2002; Egan, Brennan & Pignolo 2012), agronomy, zootechnics and biological sciences (Passos et al, 2020; Karachle & Stergiou 2010; Zur & Klement 2015; Meurer et al, 2007; Valente et al, 2017; Grune et al, 2018) and hematology (Jatobá et al, 2018). In addition to these, there are reports in the literature of material engineering (Hannickel et al, 2012), soil sciences (Passoni et al, 2014) and immunology.

In the medical and biological sciences, there are many possibilities for using ImageJ: (i) in the evaluation of normal or altered anatomical structures of organisms, among them: Atotal = total area of intestinal villi, A = height (distance: apex/muscular layer), ATotal = Total height (Apex to terminal portion of tunic serosa), LA, M, B = width (Apical, Average and Basal), EE = thickness of the epithelium, ETM = thickness of the muscular tunica, MCC = average of goblet cells per villus intestinal, area and changes in the liver and gonads (Yilmaz, Genc & Genc 2007; Mello et al, 2012; Mello et al, 2013; Salwa et al, 2013; Lima, 2014; Santos et al, 2015; Maftuch et al, 2018; Abdel-Azizet al, 2020; Dawood et al, 2020), thickness of the gastro-intestinal mucosa layer (Rodrigues, Saturnino & Fernades 2017; Nimet, Amorim & Delariva 2018) thickness of the muscle layers (Kihara, Ohba & Sakata 1995; Molina, Pelissari & Feirhmann 2009; Shiraishii et al, 2009), and, (ii) in the analysis of the amount of glycogen in the liver parenchyma (Gawish Saa-Eda, Omar & Sarhan 2013). The data should always be compared with control groups (standard), in order to establish improvements in the morphophysiological and nutritional conditions that allow the highest quality and quantity in zootechnical performance in animal husbandry (Fornel & Cordeiro-Estrela 2012; Salman & Giachetto 2014).

3. Use Of Imagej and Histomorphometric Analysis

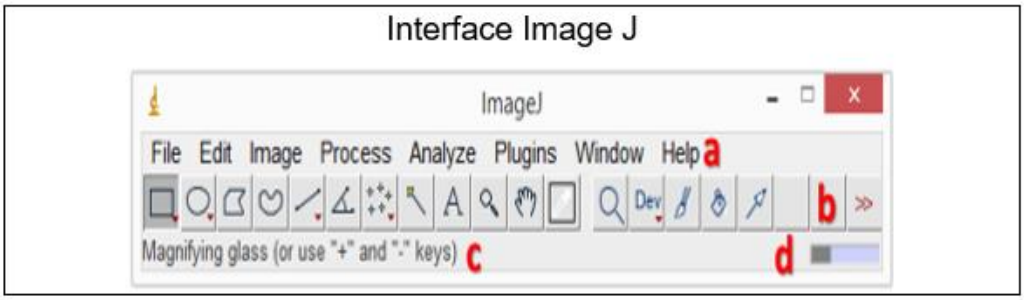
According Barbosa, Silva & Mendes (2014), ImageJ is a free Java Software developed by Wayne Rasband at the National Institute of Health (Bethesda, MD, USA), that has been applied to histological analysis (Egan et al, 2012). It has a simple and practical interface on its toolbar (Figure 1) and through plug-ins it allows users to employ various types of metric analysis, such as frequency distribution, distances, areas, perimeters, circumferences, counts, and angles (Zur & Klement 2015).

Note in Figure 1 that the commands are highlighted with red letters, and each one has a role during the PDI and the measurements that will be performed by the software. For each

click, there is a step to be taken for the inclusion of the image, its duplication, calibration, exposure of the image size bar, equalization, segmentation and specific measurements.

commands for the PDI are shown: ImageJ graphical interface (letters in red - a - command bar, b - toolbar, c - status bar, d - progress bar.

Figure 1. After the first step opening ImageJ, the window below will open, and the initial.



Source: Authors.

Morphological analysis and tissue changes in animals have been carried out through histomicrographs, which are acquired by capturing images of histological slides via cameras coupled to optical microscopes (Novaes et al, 2007; Silva et al, 2016; Moawad et al, 2017).

The digital processing of histomicrograph technique needs a software, such as the ImageJ, whose images are captured from an optical microscope with attached cameras (Novaes et al, 2007; Ferreira & Rasband 2012; Venter & Niesler 2019). There is a wide range of features and applicability of ImageJ and its Plugins, for PDI and morphometric studies. ImageJ is in the public domain, free of charge, for consolidated use by the scientific community, mainly for histological structures (Papadopulos et al, 2007; Venter & Niesler 2019).

ImageJ enables descriptive and quantitative measurements with confidence and greater accuracy, as the researchers' observations concerning measurements can vary due to personal factors (Oliveira et al, 2010). The use of ImageJ software in histomorphometry goes beyond the simple fact of measuring, ensuring that data and measurements are correct and constant among observers (Oliveira et al, 2010, Zur & Klement 2015). In qualitative and quantitative research approaches, it is important to consider the possibility of measurements subjectivity, which would be understood as an analytical-explanatory systemic concern of the real (Majeed et al, 2019).

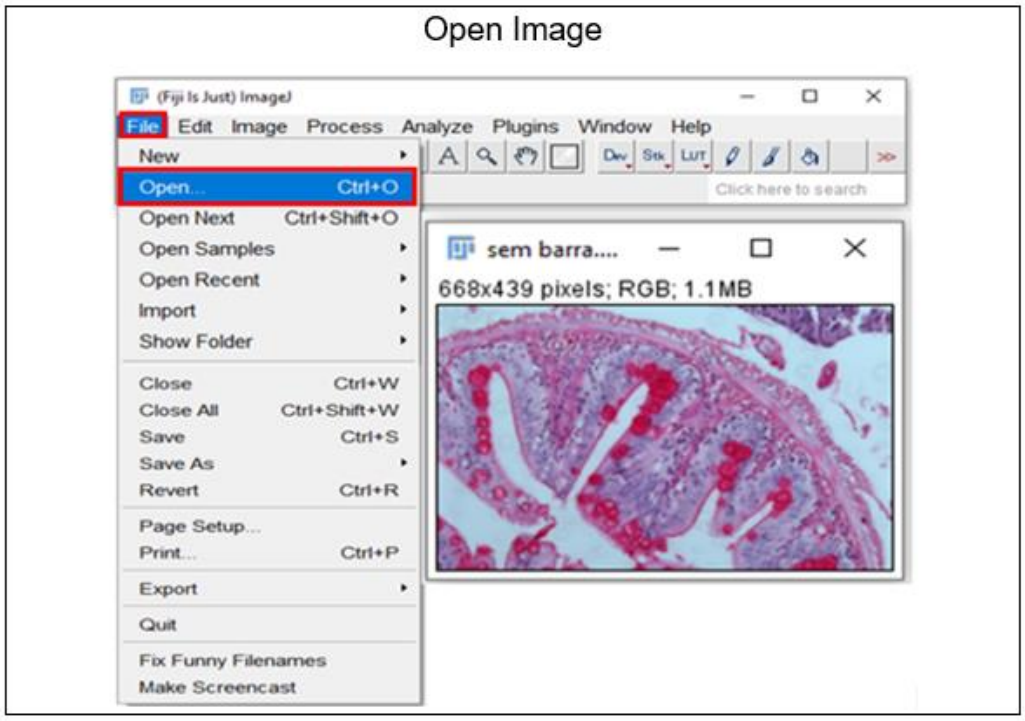
The ImageJ Software provides several resources and complementary tools that support the analysis of histomicrography. It is possible to use the selection tools and colors adjustment

according to histochemical staining, manual and automated measurements, image scales, segmentation, and filters (Burger & Burge 2008). There are add-ons of native resources in ImageJ, such as macros and specific auxiliary plug-ins for morphometric studies (Ferreira & Rasband, 2012).

4. Methodology for Computational Histomorphometry Using ImageJ

The application of histomorphometry using ImageJ was performed with a histological slide fragment of fish intestine (Teleostei: Characidae – *Astyanax altiparanae* Garutti & Britski, 2000), placed in the animal pathology laboratory collection of Catholic University Dom Bosco (UCDB). The slide was stained with PAS (Periodic Acid-Schiff) and observed by a light microscope under the 10x lens. The microphotography PDI was initiated by opening (Open) the file command on the ImageJ toolbar (File) (Figure 2).

Figure 2. In this step, the image was opened from the stored archive on the device and the indicated command was used: Open file command of the histomicrography of the lambari (*A. altiparanae*) intestine.

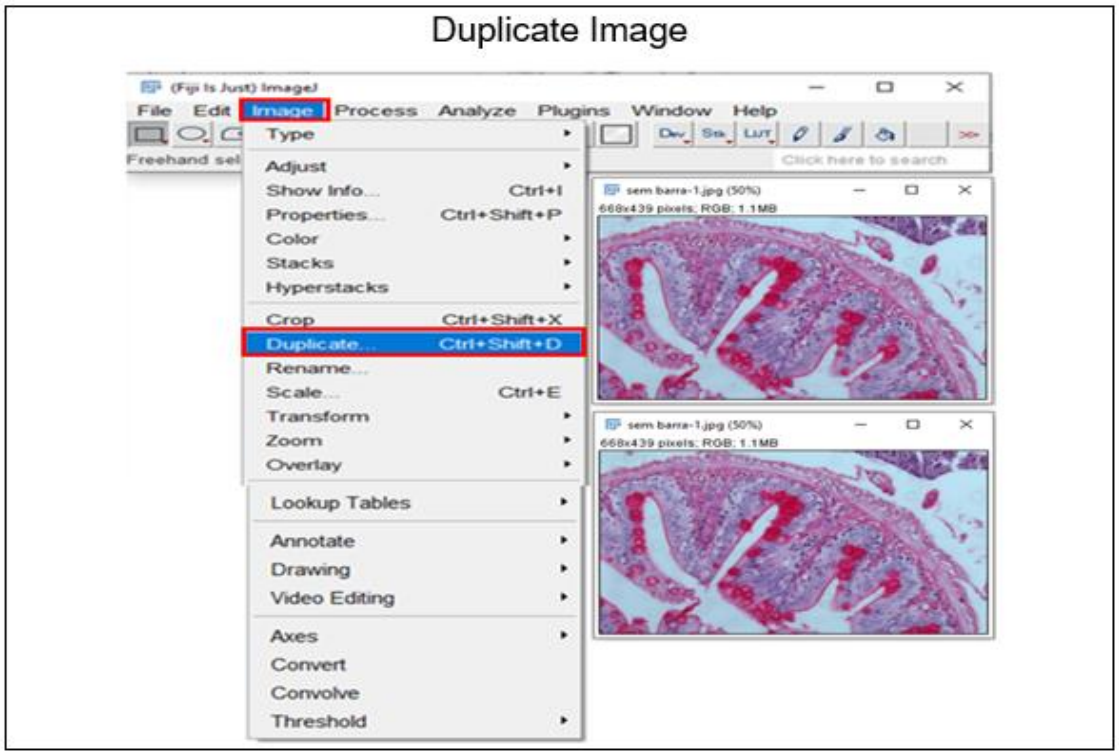


Source: Authors.

In the previous step, in the beginning of the works, the image to be studied was inserted. From the file on the device, this command indicating (open) in the figure allow opening in the format that the illustration is saved.

After opening, the image was duplicated using the command image and duplicate, in order to avoid changes in the original image that goes through the PDI (Figure 3).

Figure 3. Duplication of the image of lambari (*A. altiparanae*) intestine slide, using the command Image and Duplicate.



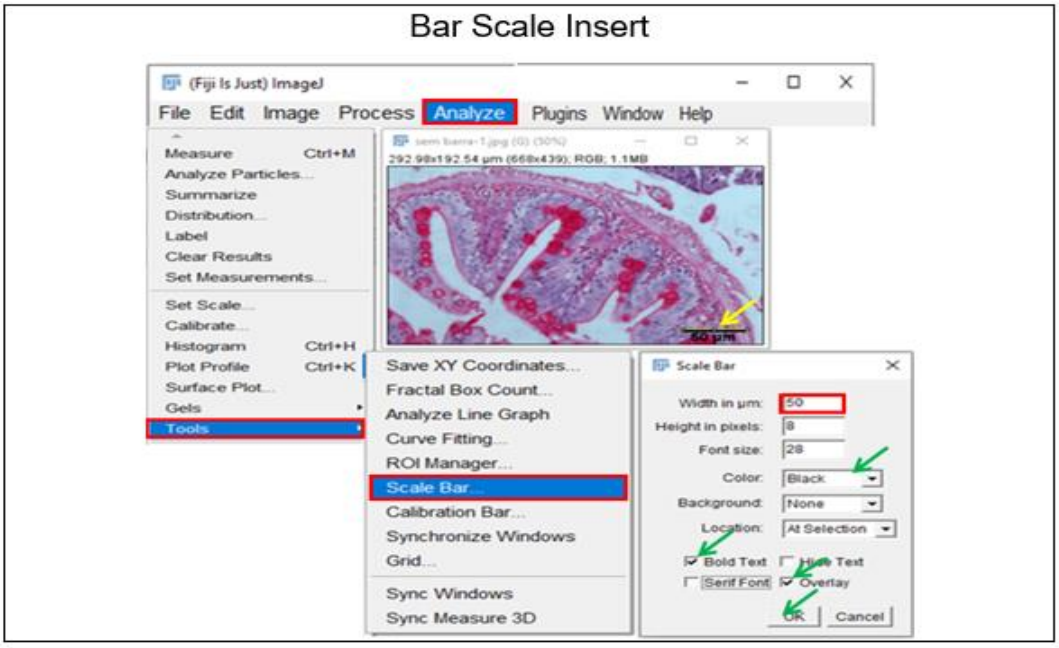
Source: Authors.

The original opened image is digitally processed and can be modified and saved in another format (from JPGE to TIFF). In the previous step, it is suggested to duplicate the image, so it retains the original characteristics of the image.

Subsequently, the images must be calibrated, that is, establish a known metric reference in order to position a metric object or instrument providing a comparison with other areas of tissues, cells, and substances, regardless of material (Russ 2015; Noronha, de Medeiros & Pereira 2018). The Straight command is used to draw a line at the bottom of the image and define the metric units to be worked on, according to the needs of the investigation (Analyze -> Set Scale). In the opened window, insert the magnification value of the image in the known distance option (Known Distance). Next to the Unit of length, type the unit of measurement

(um = µm), select Global, and then, Ok. (Figure 4).

Figure 4. Calibration of image the lambari (*A. altiparanae*) histomicrograph, using the following commands: Analyze and Set Scale. Demote the image according to its original size during capture under the microscope, ensuring the accuracy of the measurements.



Source: Authors.

In the previous step, the calibration is relevant. From there, all measurements take as reference the size of the image informed by the user, and the results appear with the units in line. The size inserted during this calibration comes from the magnification of the image in the microscope in which it was photographed and depends on the amplitude of the lenses and objectives. Once calibrated, the scale (Scale Bar) is added to the image obtained during the magnification and visualization under the microscope (Figure 5).

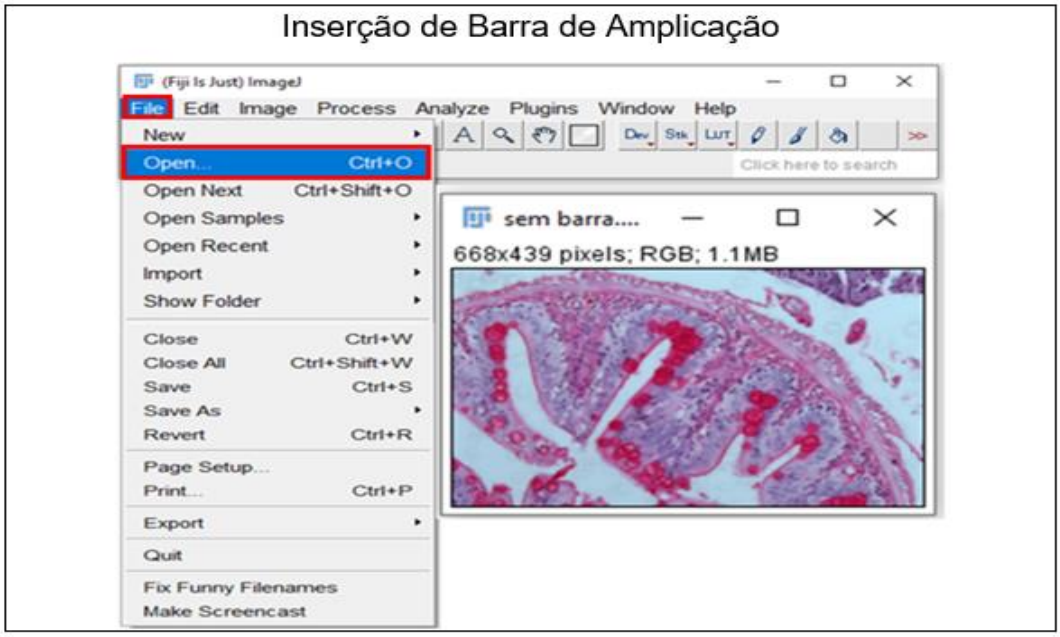
At this moment, the scales must be inserted in the original figure, so that the viewer can have notion of dimension. Observe the bar and the unit number within the figure, which takes part of the image to be measured.

The height and thickness measurements (Length Measurements) of the image tissues are performed using the Straight subcommand, click on the image with the left mouse and cross the two ends of the extension to be measured (Figure 6).

In the previous figure the yellow lines represent the measurements made. Note that each of them receives a calibration number and the corresponding values in the table. During the measurements of histological structures, the next measurement should be started, maintaining

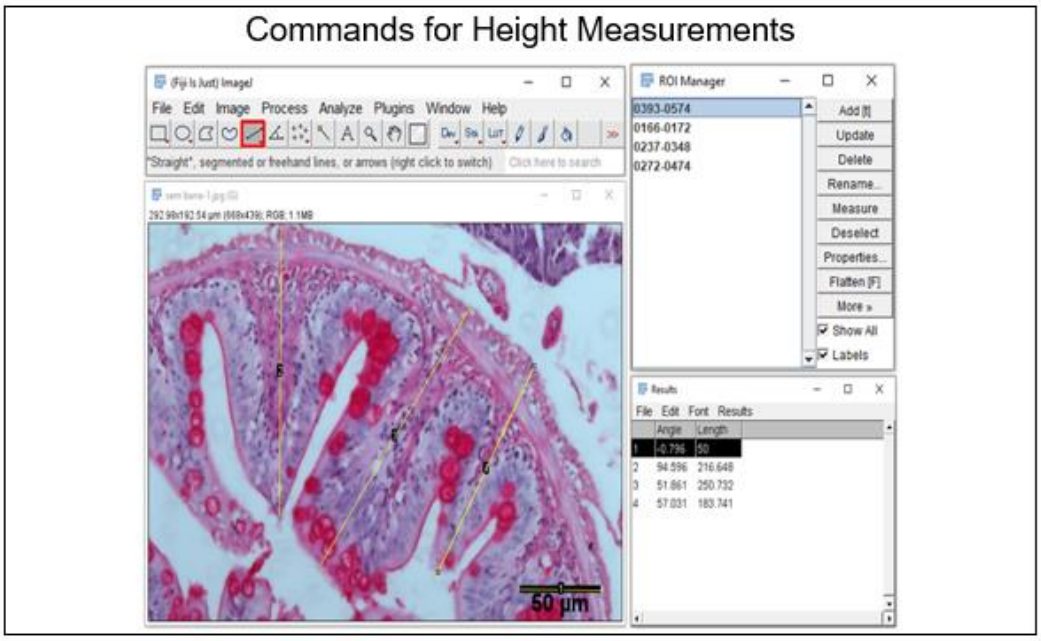
a pattern of proximity to the point chosen for the beginning and end, taking as reference the previous point.

Figure 5. Scale bar insertion in the image of the lambari (*A. altiparanae*) intestine slide. The image size scale must be introduced using the indicated commands: Analyze.



Source: Authors.

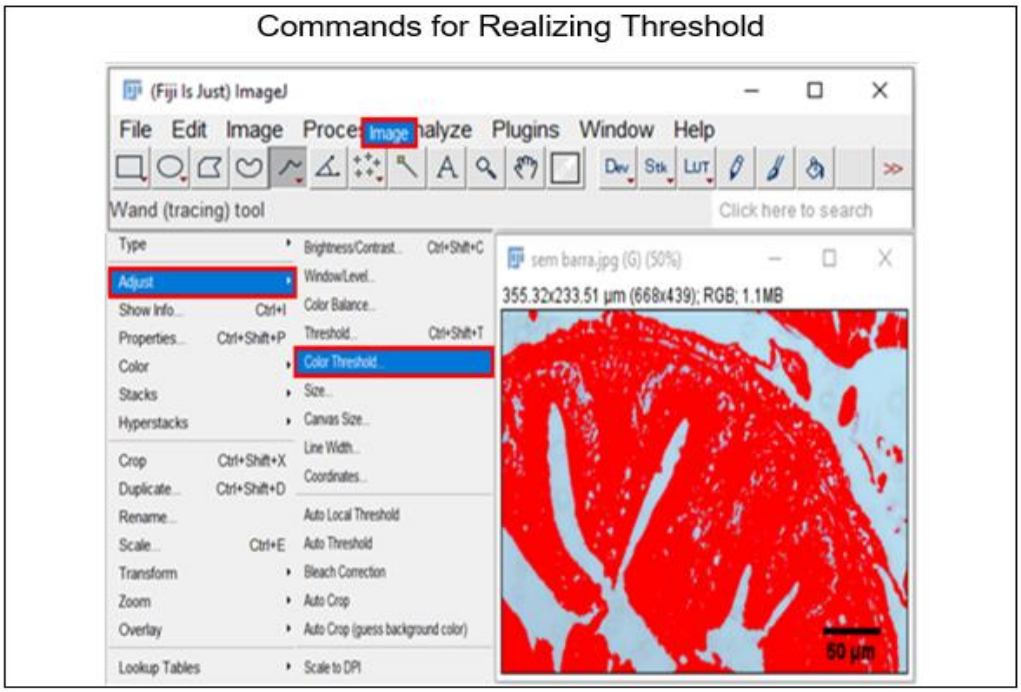
Figure 6. Steps to measure the height of intestinal villi of lambari (*A. altiparanae*). Start clicking in the Length measurements command, then Straight subcommand and create the measurement lines (in yellow) by crossing the two ends of the extension of the structure to be measured.



Source: Authors.

Each measurement can be automatically numbered and shown in a dialog box (ROI Manager) (Figure 6). In that same window click on Measure to measure the selected regions, which will be shown in a new result window in Length, being able to save each step. Note that the first marked measurement corresponds to the "magnification bar" and should be discarded as data for analysis. To measure the total area of histomicrography, the simple method using ImageJ is used. Initially, the duplicated image (Image -> Duplicate) (Figure 3) and the image area to be measured is selected, proceed with its binarization/manual segmentation by the commands/subcommand in the ImageJ toolbar (Image -> Adjust-> color Threshold) for image color equalization (Figure 7). Binarization is used to segment the images with different colors, aiming to separate different areas.

Figure 7. Commands for processing the equalization (binarization/segmentation) of the lambari (*A. altiparanae*) intestine histomicrograph: Image -> Adjust-> color Threshold.

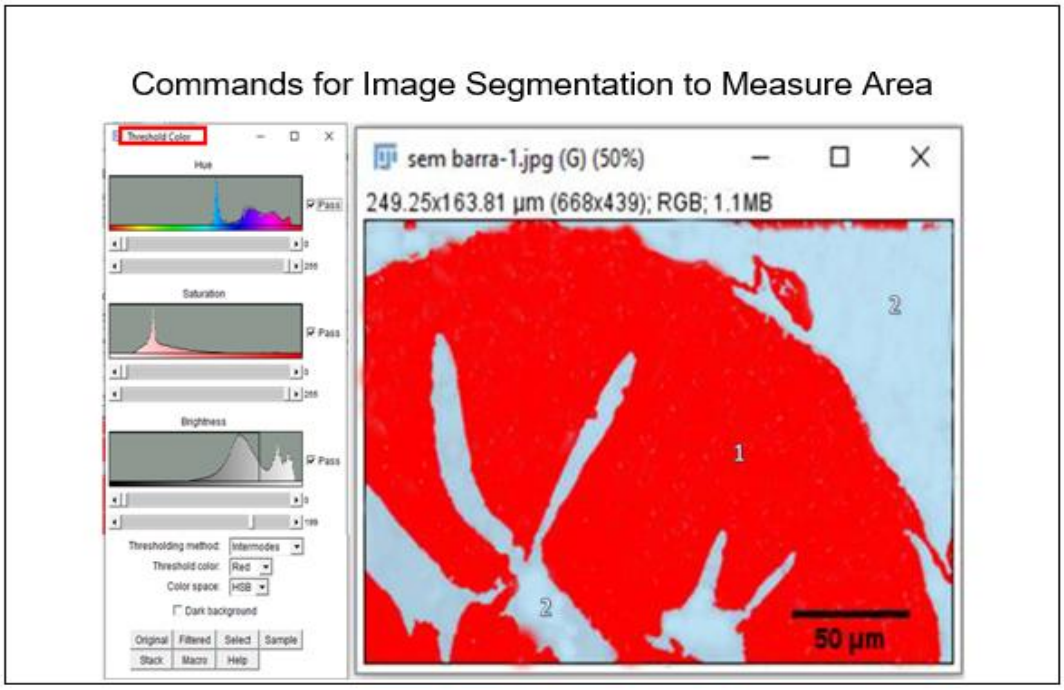


Source: Authors.

Area measurements are always preceded by preparation, so that different regions can be distinguished. In the previous figure, note the Threshold command, which allows image equalization or binarization.

After opening the color threshold window, the graphics can be adjusted to the necessary tones to form two distinct areas 1 and 2, one of this is prepared to be measured (Figure 8).

Figure 8. Image segmentation using Threshold command for - 1: measured portion – villous area; 2: light area of the lambari (*A. altiparanae*) intestine histomicrograph. The red layer is the area to be measured.



Source: Authors.

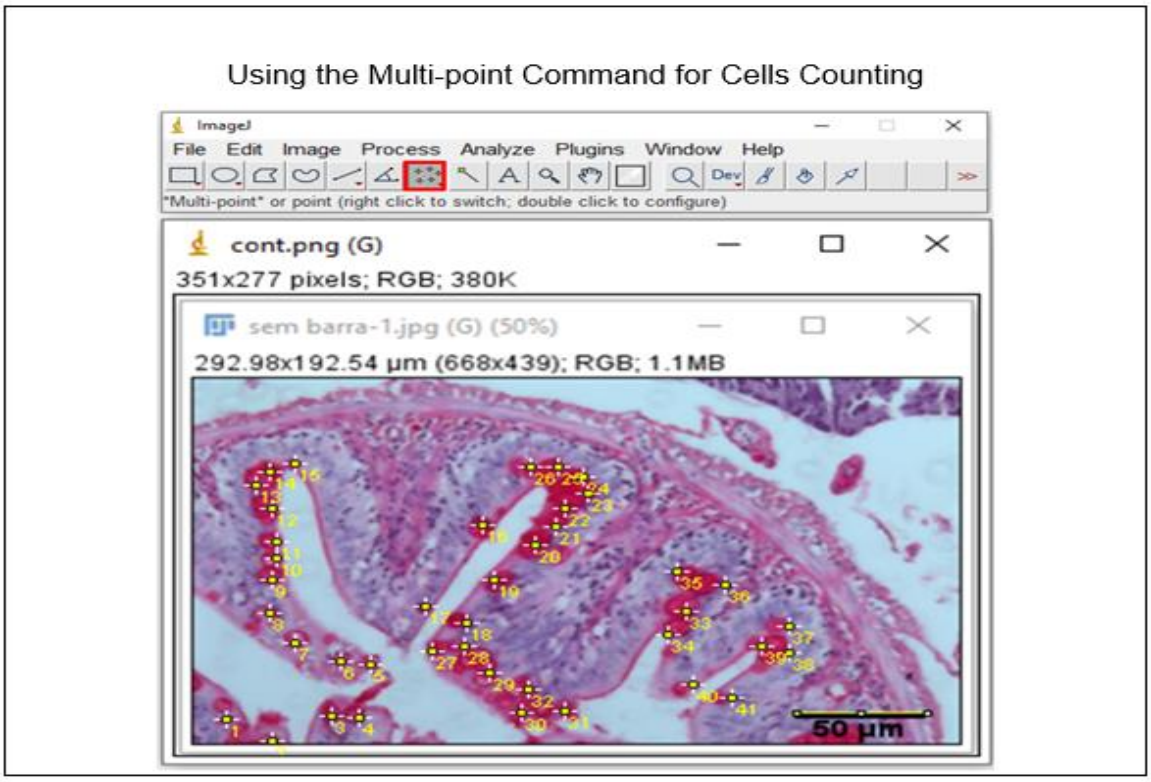
The binarization or equalization allowed to separate (fragment) the previous image into two distinct regions, as indicated (1 and 2), that is, a light and a dark (white and red). See in the figure that the red portion corresponds to the chosen area to be measured.

The equalization procedure permits to select the region of the object (Image) of interest for measurement (Figure 8) (Li & Plataniotis, 2014). The measurements can be calculated using the Analyze -> Measure function (Figure 8), which in this example was applied to the height of the intestinal villus of the lambari (*A. altiparanae*).

The stains used in slides submitted to histochemical treatments are read according to their colors (pixels), and it is possible to segment them according to the types of tissues and or cells, clusters of biomolecules in the respective layers. The characteristics of histomicrography are stained by histochemical processes that enable to easily measure each tissue area separately. For this, through the Thresholding Tool, it is possible to segment the image and separate each tissue area to measure the desired measures. This function categorizes in pixels the different tissue intensities, continuous or discontinuous cells or molecules, by calorimetric selection and amount of gray, and through Brightness, the amount of different tones of the image and the Smooth Tool overlap the pixels.

There are histochemical methods for staining cell structures (organelles and cells) and biomolecules that serve as a parameter for assessing normal or altered tissues, functioning as a marker of interest (Laplante, 2018). The most practical way to use and apply ImageJ and the counting functions can be found using the Multi-Point Toolbar command with the click of a mouse, mark what you want to count. Goblet cells were individually marked and counted, displayed by yellow points with numbers that correspond to the quantity of cells (Figure 9).

Figure 9. Counting function of goblet cells in histomicrography of the lambari (*A. altiparanae*) intestine. Note the yellow points that represent each goblet cell individually marked using mult-point bar. Each mark has a number and the last one corresponds to the total quantity of cells.



Source: Authors.

The marked areas in the figure correspond to the counted units. In this way, the amount of any particle in the tissues can be measured. Using the same histomicrograph as an example, other measurements can be made using ImageJ, such as the thickness of villi, epithelia, brush edges and muscle tunics, among others.

5. Final Considerations

The use of Image J software has applications in the most diverse areas, biological, agronomic and medicine (human and veterinary). Many studies can be performed with a focus on morphometry, allowing qualitative and quantitative measurements to be made from living tissues and microorganisms. For future work, molecular, cellular, tissue and organ measurements of the most varied species are suggested. Interpretations of images with Image J, can assist in the search for nutritional efficacy, medications and also in tissue injuries (pathologies).

The use of ImageJ has proved to be a complementary resource to assess tissue morphology and can be applied in several models, providing the use of safe tools for image analysis and monitoring, especially the performance of appropriate management in animal husbandry, contributing to environmental health and efficiency in animal production.

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