

UNIVERSIDADE VILA VELHA-ES
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

**DETECÇÃO DE APOPTOSE EM TECIDO RENAL DE RATOS
EMPREGANDO MICROSCOPIA LUZ E SOFTWARE DE ANÁLISE DE
IMAGEM LIVRE COM SUBSEQUENTE APRENDIZAGEM DE
MÁQUINA**

NAYANA DAMIANI MACEDO

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Dissertação apresentada à Universidade
Vila Velha, como pré-requisito do
Programa de Pós-Graduação em Ciências
Farmacêuticas, para a obtenção do grau
de Mestra em Ciências Farmacêuticas.

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Orientador

" Na vida, não vale tanto o que temos, nem tanto importa o que somos. Vale o que realizamos com aquilo que possuímos e, acima de tudo, importa o que fazemos de nós. "

Chico Xavier

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RESUMO

MACEDO, Nayana Damiani, M.Sc., Universidade Vila Velha - ES, janeiro de 2017.
Detecção de apoptose em tecido renal de ratos empregando microscopia luz e software de análise de imagem livre com subsequente aprendizagem de máquina. Orientador: Dominik Lenz.

O presente estudo propõe uma abordagem automatizada empregando aprendizado da máquina na quantificação de células em vias de morte celular de acordo com a fragmentação do DNA. Utilizou-se um total de 17 imagens de amostras de lâminas histológicas de rim de ratos Wistar machos. As lâminas foram fotografadas na objetiva de 40x utilizando microscópio Axio Zeiss Vert.A1 com câmera acoplada Anisio Cam MRC Zeiss e software Zen 2012. As imagens foram analisadas utilizando CellProfiler (versão 2.1.1) e CellProfiler Analyst, ambos softwares de código aberto. Dos 10.378 objetos, 4.970 (47,9%) foram identificados como TUNEL positivos, e 5.408 (52,1%) foram identificados como TUNEL negativo. Em média, os valores de sensibilidade e especificidade da abordagem de aprendizagem da máquina foram 0,80 e 0,77, respectivamente. Citometria de imagem forneceu uma alternativa analítica quantitativa para os métodos qualitativos tradicionais mais comumente empregados nos estudos.

PALAVRAS-CHAVE: Citometria de tecido; Detecção automatizada; Análise de imagem; Aprendizado da máquina; Análise de tecido.

ABSTRACT

MACEDO, Nayana Damiani, M.Sc., University of Vila Velha – ES, janeiro de 2017.

Objective detection of apoptosis in rat renal tissue sections using light microscopy and free image analysis software with subsequent machine learning. Advisor: Dominik Lenz.

The current study proposes an automated machine learning approach for the quantification of cells in cell death pathways according to DNA fragmentation. A total of 17 images of kidney histological slide samples from male Wistar rats were used. The slides were photographed using an Axio Zeiss Vert.A1 microscope with a 40x objective lens coupled with an AxioCam MRC Zeiss camera and Zen 2012 software. The images were analyzed using CellProfiler (version 2.1.1) and CellProfiler Analyst open-source software. Out of the 10,378 objects, 4,970 (47,9%) were identified as TUNEL positive, and 5,408 (52,1%) were identified as TUNEL negative. On average, the sensitivity and specificity values of the machine learning approach were 0.80 and 0.77, respectively. Image cytometry provides a quantitative analytical alternative to the more traditional qualitative methods more commonly used in studies.

KEYWORDS: Tissue cytometry; Automated detection; Image analysis; Machine learning; Tissue analysis.

1 INTRODUCTION

Histopathology involves the study of changes and anomalies in cells and biological tissues that can potentially lead to illness. In clinical medicine, histopathology is primarily used for biopsies⁽¹⁾, where all of the biological material collected is permanently fixed onto a glass slide, stained and analyzed⁽²⁾. Histopathological studies aim to identify and classify different illnesses⁽³⁾.

Since the discovery of histological staining techniques in the 19th century⁽⁴⁾, the majority of histopathological diagnostics are performed by manually analyzing hematoxylin and eosin stained tissue with light microscopy⁽⁵⁾. Between 1970 and 1980, the use of morphometric analysis in pathologically altered tissues became common⁽⁶⁾. Currently, the manual analysis of tissues is the most commonly used method for pathological diagnosis. Accurate diagnosis using this method requires a highly experienced analyst^(3,7). Although the traditional methods have been very valuable, they have their limitations: the human eye has different brightness and contrast sensitivities, which can result in subjective analyses; the interpretations and/or conclusions using this approach can vary across analysts^(8,9); and this method is often time-consuming and tedious^(5,9,10,11,12).

In the past two decades, exceptional advances have prompted the search for new biological analysis tools⁽¹³⁾. Significant progress has been made in establishing automated histopathological images for analysis⁽¹⁴⁾.

Image cytometry is a computerized imaging technique that can be used to analyze and evaluate involved in pathologies in human biopsy samples⁽¹⁵⁾. This tool has been used to classify cells based on the recognition of standards⁽¹⁶⁾. Due to the increased availability of software that allows the manipulation and analysis of histological images, image cytometry has been gaining increasing amounts of attention in the medical and scientific fields⁽¹⁷⁾. Among the available programs, CellProfiler is free open-source software that detects objects in images and evaluates several parameters of cell recognition⁽¹⁸⁾. This software allows the user to simultaneously identify and measure a variety of biological objects^(19,20) in images in a reproducible and automatic manner^(18,20,21).

In developed countries, image cytometry has been successfully used as a quality control method for cytological diagnostics and to complement routine laboratory diagnoses⁽²²⁾. Some researchers have found that this technique requires smaller samples, consumes smaller amounts of reagent^(23,24,25,26) and is reproducible^(8,27,28,29,30). Because there is no sample loss during image cytometry, the analysis can be repeated many times⁽²⁵⁾.

The current study proposes an automated machine learning approach for the quantification of cells in cell death pathways selected using DNA fragmentation.

2 MATERIAL AND METHODS

2.1 Ethical declaration

The histological slides were provided by the Federal University of Espírito Santo (UFES) and were approved by the Ethics Committee CEUA/UFES (Protocol No. 050/2013) (ATTACHMENT).

2.2 Slide preparation

Internucleosomal DNA fragmentation was detected in situ using the terminal deoxynucleotidyl transferase (TdT)-mediated uridine triphosphate (dUTP) nick end-labeling (TUNEL) assay (TUNEL POD kit, Roche Diagnostics, Germany). Paraffin sections were dehydrated and xylene-based mounted under glass cover slips. Human placenta was used as the positive control. The same procedures were applied to both the negative and positive controls, with the exception that the negative control was incubated in the absence of the TdT enzyme. To visualize the staining, the sections were treated with 3,3-diaminobenzidine tetra hydrochloride (DAB; Sigma Chemical Co., St. Louis, MO, USA). The specimens were then lightly counterstained with Mayer's hematoxylin, dehydrated, and mounted with xylene-based mounting media under glass cover slips. The content of the positive staining area was averaged for ten randomly chosen fields across the cortex and the medulla.

2.3 Materials

A total of 17 images of kidney histological slide samples from male Wistar rats were used.

2.4 Image acquisition

The slides were photographed using an Axio Vert.A1 Zeiss microscope with a 40x objective lens coupled with an AxioCam MRC Zeiss camera and Zen 2012 software. The images of each slide were saved in the *.tif format and stored individually in separate folders and properly identified by the user.

2.5 Image analysis

The images were analyzed using CellProfiler (version 2.1.1) and CellProfiler Analyst (version 2.0) open-source software(<http://www.cellprofiler.org/>). CellProfiler has a set of configurable modules for image analysis, which are executed consecutively in a pipeline. The pipeline of each analysis can be stored and used for future projects. The pipeline used in this study, employed 6 key modules, described in Table 1.

Table 1.CellProfiler pipeline.

Module	Operation
1- Load Images	Identify and load the *.tif images Name the loaded objects: i1
2- Color To Gray	Select the input image: i1 Conversion method: split Convert green to gray Name the output image: i2
3- Image match	Operation: Invert. Select the first image: i2
4- Identify Primary Objects	a) Identify an object of interest: nuclei b) Maximum and minimum area: 10-40 c) Threshold strategy: adaptive d) Method: manual
5- Measure Objects size/shape	Measurement object: nuclei
6- Filter Objects	a) Name the output objects: nuclei2

b) Select the objects to filter: nuclei

7- Measure ObjectIntensity

a) Select the objectsto measure: nuclei2

1) Identify which images will be loaded for processing. 2) Convert the RGB channel (red, green and blue) to shades of grey because the software does not recognize colored images. 3) This module performs operations to reverse the intensity of the objects: dark under the light background for bright objects on a dark background. 4) Identify components of interest in the images. "Object", is used as a generic term in CellProfiler to refer to a specific characteristic identified in the image, in this case, the nuclei. 5) Exclude all objects that do not fulfill the user-defined limits in step 4 (form factor smaller than 0.55). 6) Identify the objects of interest. This tool extracts the intensity characteristics of each object. The intensity is measured for all image and object combinations. 7) This module calculates several parameters of the recognized objects, such as size, shape, perimeter, extension and others.

After identifying and analyzing the object of interest, the data were exported to CellProfiler Analyst, using a database in the SQLite format (Figure 1). This format allows only the data of interest to be exported.

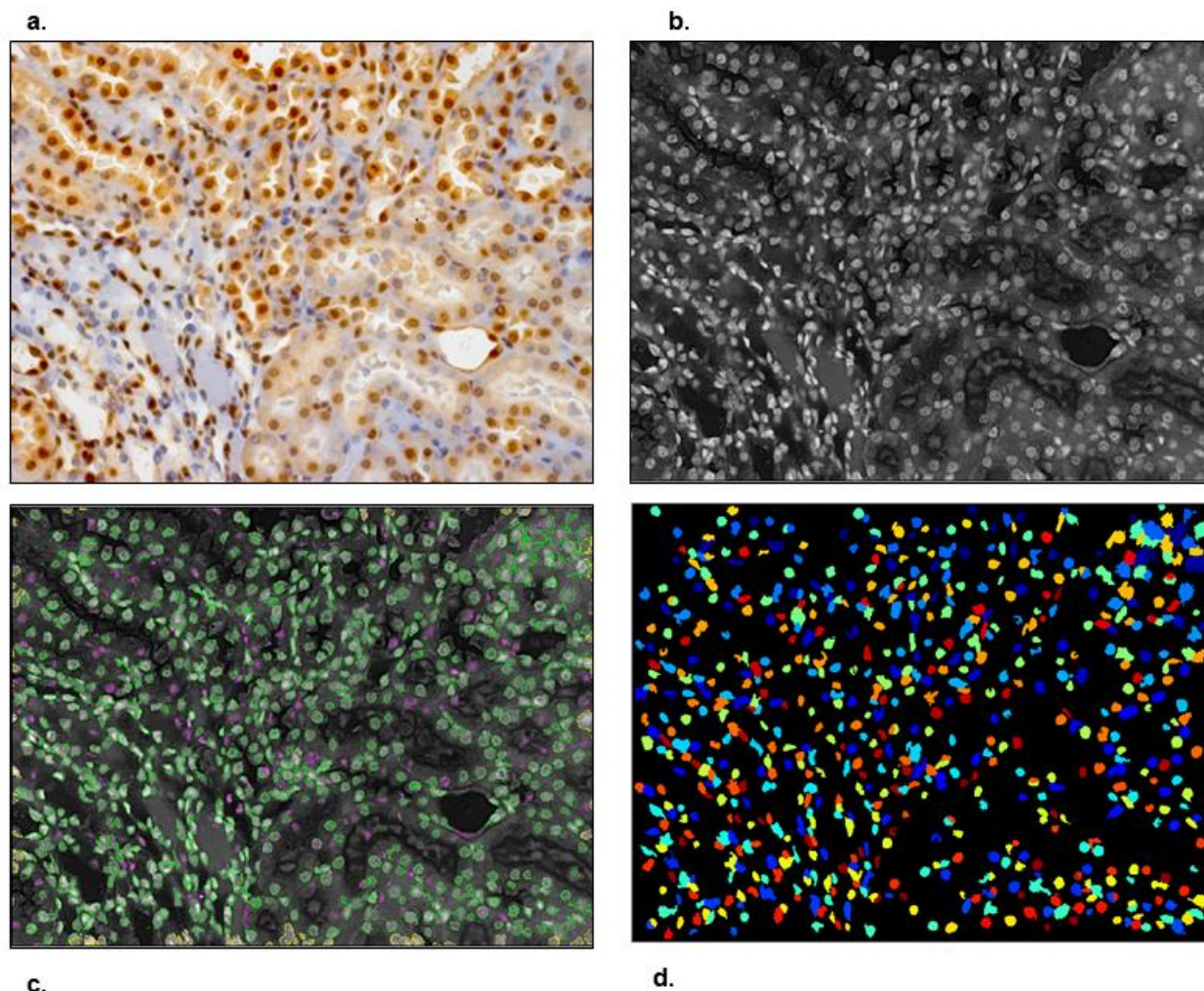


Figure 1: Object identification using the pipeline

a) The original image loaded into the program. **b)** Image with inverted intensity, showing the bright objects on a dark background. **c)** Objects identified according to the maximum and minimum size and shape are shown in green; objects that were not within the established parameters are shown in pink. **d)** The identified objects from picture c, each colored point represents one identified nucleus. Note that the identification area was assigned to only the green objects; the objects that do not have compatible information were excluded (pink).

2.6 Machine learning

After the data were converted into the SQ-lite format, they were loaded in to the Cellprofiler Analyst software for further classification. TUNEL labeling (region labeling via dUTP-mediated deoxynucleotidyl transferase terminal)

detected objects that contained fragmented DNA. The objects were classified as either “TUNEL positive” or “TUNEL negative” based on the presence or absence of fragmented DNA.

The classification grouped the sample objects that possessed similar characteristics. The information obtained using machine learning involved a software training procedure to recognize the patterns. Subsequently, the sensitivity and specificity of the images were calculated.

The CellProfiler Analyst possess tools such as “Score All” and “Score Image” that allow the quantitative and qualitative analyses, respectively, of the image objects. Using “Score All”, we were able to quantify the total number of objects in the sample set as well as the TUNEL labeled and unlabeled objects. The “Score Image” tool was used to visualize the classification. The data presented by each application were directly connected to the information provided to the software by the analyst.

2.7 Statistical analysis

The Bland-Altman test was applied using Sigma Plot (version 12.5, Systat Software Inc.) to evaluate the similarity between the manual (gold standard) and automated counts. A receiver operator characteristic(ROC) curve was plotted to show the progress of the machine learning process.

3RESULTS

CellProfiler was used to create the pipeline and process the images. Subsequent classification was conducted using CellProfiler Analyst. The “Score All” tool (CellProfiler Analyst) was used to obtain the number of objects in each class present in the samples, i.e., the quantitative result. Out of the 10.378 objects, 4.970 (47.89%) were identified as TUNEL positive and 5.408 (52.11%) as TUNEL negative.

On average, the machine learning procedure had a sensitivity of 0.80 and a specificity of 0.77. The sensitivity and specificity were calculated as follows: 100 TUNEL positive objects were selected and analyzed using Cellprofiler Analyst to calculate the sensitivity, and 100 TUNEL negative objects were selected to calculate the specificity.

Subsequently, the “Score Image” tool was used to identify and correct cellular classification errors. This process involved a qualitative comparison between the original image and the classified image based on the information provided to the program.

The results obtained using “Score Image” – CellProfiler Analyst were very similar to the original image (Figure2). This tool allows the analyst to qualitatively verify the analysis. After the sample objects were counted and classified accurately, the software generated reliable results.

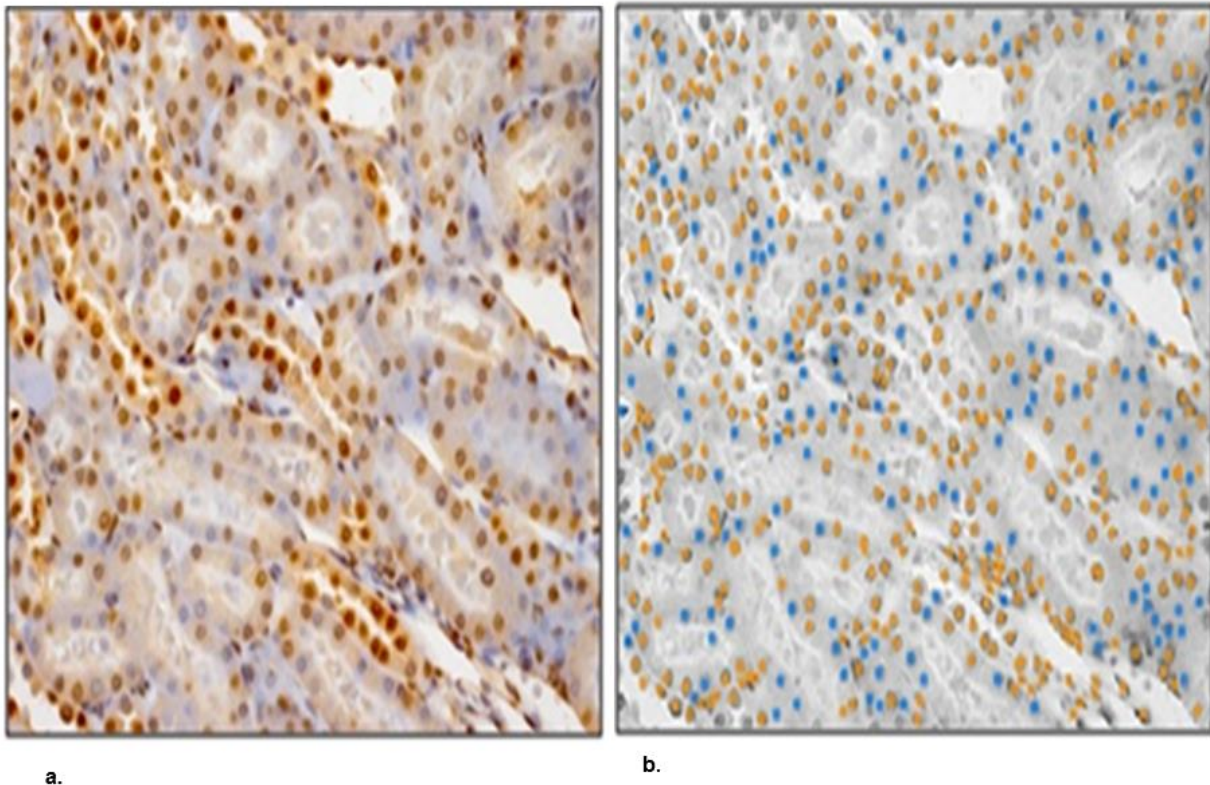


Figure 2: Classification based on the “Score Image” tool in CellProfiler Analyst.
a) The original image loaded into the program; the brown colored objects are stained with TUNEL (TUNEL positive), while the blue objects are stained with Harris Hematoxylin (TUNEL negative). **b)** Classification according to “Score Image”: The TUNEL positive nuclei appear orange, and the TUNEL negative nuclei appear blue.

The ROC curve (Figure 3) represents the progress of the machine learning process. Initially, 10 objects from each group were added to the training set. Ten more objects were added to each class of the training set, and the sensitivity was recalculated. This process was repeated until the training set contained 100 objects in each group. The area under curve (AUC) is 59%.

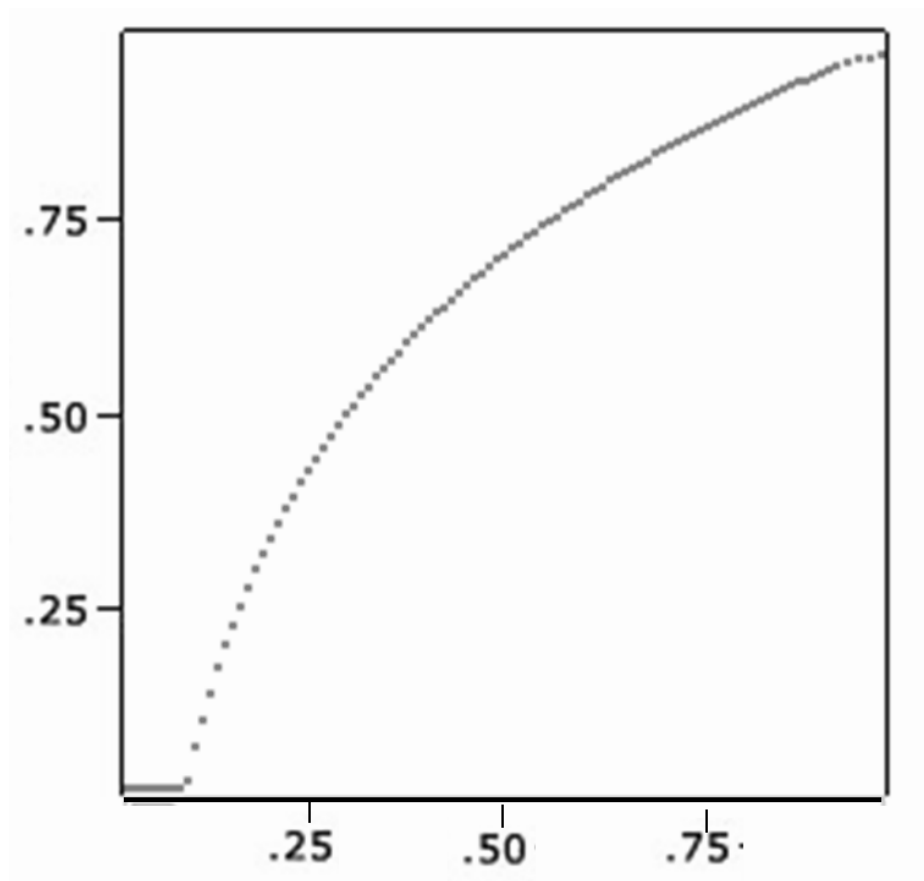


Figure 3: ROC curve

X-axis: 1 - specificity; Y-axis: sensitivity

The Bland-Altman test (Figure 4) was used to assess the similarity between the manual and automated counts. The mean difference between the two counts was 0.11 (middle line). All of the events were within ± 1.96 standard deviations of the average (lower and upper lines). There were no systematic biases in the comparisons as there were both positive and negative results, i.e., the automated count was either the same, higher or lower than the manual count.

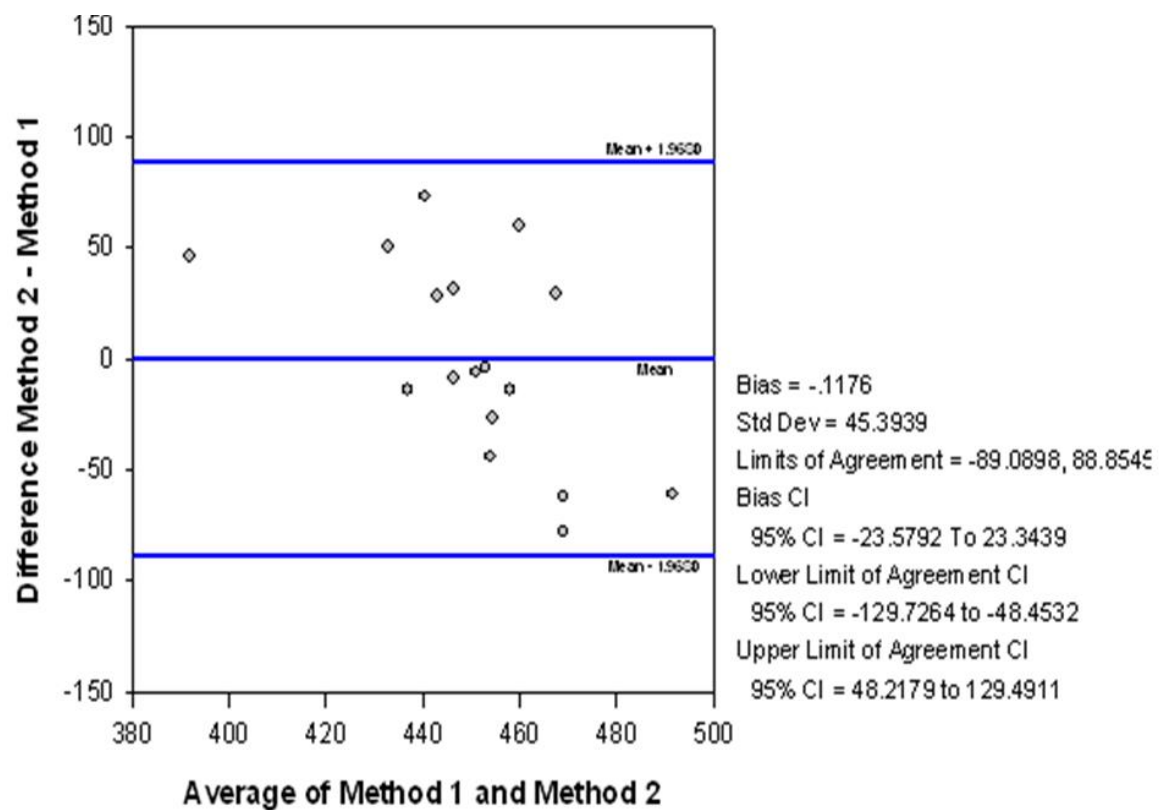


Figure 4: Bland-Altman graph

The mean of the two methods is plotted on the X-axis; the difference between the two counting methods is plotted on the Y-axis. The entire sample was between +1.96 SD and -1.96 SD.

4DISCUSSION

According to Hedvat (2010)⁽²⁸⁾, the most significant advantage of image cytometry is its ability to perform a variety of analyses using large populations of cells⁽²⁹⁾. Whether assessing the number of cells or the chromogen intensity, repeated evaluations of a region of interest using the automated quantification system produce results that are both reliable and reproducible^(11,18,20,21,27).

Furthermore, it has been shown that this tool can be used to supplement existing methods or as an alternative method, as the traditional methods are often costly due to the requirement for expensive equipment that requires regular maintenance and the need for specialized trained professionals^(7,31). Image analysis provides a quantitative tool that can be used for histological examination. Implementing image analysis routinely can reduce the variability of opinions⁽³²⁾.

Krajewska and collaborators⁽⁸⁾ used algorithms to characterize cellular processes associated with cell death. Similar to the present study, they also used TUNEL as a marker and applied the algorithm in tissue sections with fibrosis. They found that the quantitative results indicated a significant increase in apoptosis markers in the treated tumors when compared to the untreated tumors. In 2014, Yeh and collaborators⁽³³⁾ obtained quantitative measures and demonstrated that the calculation of the maximum size of the nuclei can be used to distinguish renal cell carcinomas. Consistent with these results, our study successfully differentiated the stained from the unstained nuclei implementing a similar step ("Identify Primary Objects").

Combining their analysis with CellProfiler, Buzin and collaborators⁽²¹⁾ claimed that it is possible to replace specific markers of apoptosis and necrosis without losing the reproducibility of the image analysis, which resulted in cost reduction. According to Carpenter and colleagues (2006)⁽³⁰⁾ the CellProfiler was able to simultaneously measure characteristics, such as size, shape, intensity and texture in a variety of cells. Horvath and collaborators (2011)⁽³⁴⁾ used this software to identify cells and extract their characteristics.

The efficiency of automated algorithms depends on several factors: the nature of the histological structures of interest, the classification to be performed and the preparation and staining of the samples⁽¹¹⁾. Once recognized, the cell

differentiation and/or segmentation become more noticeable when the material is dispersed⁽¹⁶⁾ than when the nuclei are very close together in the tissues⁽³⁾.

Studies by González et al., 2012⁽³⁵⁾, Chan et al., 2011⁽³⁶⁾, and Yang et al., 2004⁽³⁷⁾, among others, have contributed to cytopathology by adopting automated image systems. This application extends beyond cytopathology. For example, Milord and colleagues (2001)⁽³⁸⁾ applied nuclear morphometry as a parameter in examining carcinoma in situ slides to differentiate bladder carcinoma from other urothelial lesions. Rexhepaj and collaborators⁽²⁷⁾ proposed the recognition of melanoma from non-melanoma cells by combining image cytometry and pattern recognition. Image cytometry has also been used to detect and evaluate pre-malignant alterations in the head and neck region⁽³⁹⁾. Recent studies and the growing interest in this field indicate the relevance and potential of computer-assisted quantification. In 2015, Rogojanu and colleagues⁽⁴⁰⁾ described new software that allows a detailed morphometric analysis of cancer tissues that reduced the analysis time, enabled the quantitative analysis of data and enhanced the reproducibility.

Combining morphometry and automated computer or statistics-based tissue analysis demonstrates that quantitative histology is a promising and challenging field⁽⁴¹⁾.

5CONCLUSION

The combination of image analysis, light microscopy and free access software has significant clinical potential. Light microscopy and software are accessible in most laboratories. Moreover, the implementation of image cytometry provides a quantitative alternative to the qualitative approach traditionally used by analysts. The improvement of applied technologies and their advantages enhance the potential of image cytometry applications in the medical and scientific fields.

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ATTACHMENT



UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
CENTRO DE CIÊNCIAS DA SAÚDE
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

CERTIFICATE

We certify that the Protocol **050/2013** related to research project entitled **"Effects of G-CSF in an experimental model of renal ischemia/reperfusion.***, under responsibility of **Breno Valentim Vassalo**, is in accordance with the ethical principles of animal experimentation adopted by this Ethics Committee on Animal Use (CEUA-UFES), being approved at the regular assembly in december 20, 2013.

*Title as supplied by author.

Vitória (ES), december 20, 2013.

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