



Original article

An image processing software applied to oral pathology

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ABSTRACT

This study presents the development and evaluation of an image processing software for computer-assisted cellular structure counting. The proposed software consists of a set of processing and analytical tools which allows its use in several applications of cell and cellular structure counting. A particular application in AgNOR quantitative analysis is presented. The knowledge obtained from experienced pathologists has been codified in a sequence of processing steps in order to allow automatic estimation of the mean number of AgNORs per cell in ameloblastomas. The performance of the presented software in such application was verified by comparing the data provided by visual analysis, by two observers previously calibrated and under supervision of two experienced specialists (Group 1) and by the computer program (Group 2). No statistical difference was observed ($p < 0.05$) between the two groups. The use of the proposed method in AgNOR applications permitted attainment of accurate and precise data without the difficulties frequently found in the traditional visual analysis method (time, training and subjectivity). The developed software is an interesting tool as an aid in the study (estimation of the number of cells and cellular structures) of malignant and benign neoplasms.

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Introduction

Currently, computerized image systems have been increasingly applied as useful tools in oral health, aiding in clinical diagnosis and research, as they are easy-to-use and not time consuming. These systems use computational methods for shape analysis and evaluation of the number of cells and their components, guaranteeing standardization of the processes and minimizing dependence on the experience and knowledge of the specialists [1,2,5,6,12,13].

Studies on neoplasms, one important area of research in oral pathology, benefit from such technology, as they rely on cellular components counting, an important procedure to identify different types of lesions. Depending on the number of certain cellular components, it is possible to estimate the cell potential for proliferation.

Many methods can be used to estimate tumor proliferation capability; one of them is the evaluation of the mean number of (silver-staining) nucleolar organizer regions (NOR–AgNOR) per nucleus in a given histological section. The NORs are DNA segments responsible for the transcription of ribosomal RNA [4]. They

are closely related to protein synthesis and, as a consequence, with the increase of cellular metabolism [10]. Evaluation of AgNOR mean number is laborious and tedious; hence, automated systems for such application are in great demand.

This work presents an image analysis software for cellular components counting, showing its characteristics and potentialities and describing its performance in an AgNOR counting problem.

Materials and methods

The software developed in this study is a general image analysis system and processing tool for oral pathology applications. Its latest version presents a friendly user interface, allowing the user to obtain different types of information from a selected image. It is a flexible platform that incorporates several techniques for image analysis and processing [7], such as: histogram equalization, thresholding, segmentation, border enhancement, morphological operators, clustering, two-dimensional filters, and gray-level transformation. Its different techniques can be joined to integrate specific-application routines. Some facilities for image manipulation are: bitmap and JPEG formats, pixel (picture element) information (RGB values), erasure, zoom, undo, and area selection.

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AgNOR image analysis and application

The presented general image analysis system was used to develop a specific automated routine to evaluate the mean number of AgNORs per cell in histological samples of ameloblastomas (odontogenic tumors). The process of developing such a routine consisted of three main parts: (a) acquisition of knowledge (definition of a set of rules for counting AgNORs) [3]; (b) testing image processing techniques in AgNOR sections; (c) assembling the selected procedures in a specific routine.

The analysis of AgNOR sections showed the following characteristics: (a) cellular nuclei are observed as yellow stained regions with non-homogeneous pattern; (b) AgNORs are brownish dots inside nuclei; (c) sometimes cellular nuclei can be very close or even joined to one another, due to the thickness of the obtained slice; (d) extra-cellular matrix and cytoplasm are yellow-blue colored non-homogeneous regions; (e) there are dark granulated elements associated to the image background.

The proposed method for AgNOR counting comprises two main tasks: (a) nuclei identification and counting, and (b) AgNOR identification and counting. The first task consists of the following steps:

- *Segmentation by color thresholding*: information about the color of each pixel determines if it will be maintained or rejected. In this first stage, only pixels belonging to the cellular nuclei must be kept. When this process is finished, image color information is discarded.
- *Artifact rejection*: after image segmentation, several remaining pixels do not belong to nuclei regions. A recursive algorithm for counting pixels is used to reject large scale clusters of pixels and small scale ones, as the cellular nuclei of a given kind of cell have maximum and minimum limits for its area.
- *Nuclei counting*: the same recursive routine for counting pixels is used for counting the remaining clusters (nuclei). Each set of joined pixels (cluster) receives a numeric label, determining each individual nucleus and the total number of clusters.

Evaluation of the AgNOR number followed the same steps previously described but with different parameters. The only difference between both stages was that in AgNOR analysis, an extra rejection stage characterized by a Boolean AND operation between the processed AgNOR and nuclei images was performed. Such procedure facilitates the identification of false-positive AgNORs that do not belong to any nuclei. At the end of this procedure, only nuclei with at least one AgNOR were registered.

Case selection and staining procedure

To verify the performance of the developed system, we selected 10 cases of ameloblastomas from the archives of the Diagnosis Center for Oral Diseases of the Federal University of Pelotas, Brazil (Fig. 1). Formalin-fixed, paraffin-embedded tissues were cut at 3 μm of thickness and placed on glass slides with 3-aminopropyltriethoxy-silano. The silver staining was applied according to the method of Ploton et al. [8] modified by Rivero et al. [9]. Slides were mounted in Permount[®]. Digital images were captured by a JVC TK-1270/RGB camera, mounted on a microscope with 1000 \times magnification. The optical resolution is 8533.37 μm^2 for a [24-bit 640 \times 480 image].

Quantitative and statistical analysis

Quantitative analysis of the AgNORs was performed in two ways: (1) visual analysis; (2) automated analysis.

Ten different cases of ameloblastomas were selected, and 10 fields with about 100 cells were available in each case. Visual anal-

Table 1

Comparison between the mean value and standard deviation of the number of AgNORs per cell obtained by two observers previously calibrated and under supervision of experienced specialists (Group 1) and the computer program (Group 2).

Counting method	Mean value	Standard deviation
Visual analysis	2.04	0.18
Automated analysis by the software	2.02	0.25

ysis was performed at the microscope (with the help of a graticule mounted on the ocular) by two graduate students previously calibrated and under supervision of two experienced specialists.

In the automated analysis procedure, two fields were randomly chosen for each case and were used for determining the optimum settings of the proposed software (testing group) and eight fields for evaluation (validation group). This task was performed by a user having general information about the description of the AgNOR staining aspects and cellular pattern, provided by the specialists. Once the optimum parameters had been obtained, the automated counting procedure was applied to the remaining eight fields (validation group). To determine statistical difference between visual and automated procedures, Student's *t*-test with 95% confidence interval ($p < 0.05$) was used.

Results

The present version of the proposed software was implemented in C++ for Microsoft Windows. Special attention was given to the user's interface and picture manipulating facilities. Auxiliary tools are available for semi-automated analysis in order to refine the obtained results. However, they were not used in the presented application in order to demonstrate the results of a fully automated analysis.

Slide preparation of the ameloblastomas cases revealed AgNORs as black dots within cell nucleus (yellow stained regions), while extracellular matrix and cytoplasm can be found as yellow and blue regions in the image background, respectively (Fig. 2A).

In Fig. 2, the main steps of the nuclei automated segmentation in a section of ameloblastoma are shown. Firstly, a typical histological sample stained by AgNOR is presented. Secondly, preliminary segmentation, based on a color map, was done. After this process, color information was discarded. Some regions were artificially presented in intermediary gray-level in order to indicate false-positive nuclei that will be disregarded in the next processing step by an artifact rejection tool. The aim of this step was to reject regions that had a much larger area than expected for a nucleus. In Fig. 2C, we can see the result after the rejection routine was applied. In the same figure, very small areas that will be rejected by a second artifact rejection routine are shown (artificially presented in intermediary gray-level), because nuclei present a minimum expected area. Information about minimum and maximum area was obtained during the acquisition of knowledge stage with the help of experienced pathologists. Finally, nuclei identification was performed. AgNOR identification followed the same steps, but it was submitted to different parameters of color information and maximum and minimum sizes. Fig. 3 shows the original AgNOR image and the result of the automated nuclei and AgNOR identification process.

Results obtained from the visual analysis at the microscope were compared with those obtained by the automated method in the validation group (ten cases, eight fields, and approximately 800 cells) (Table 1). No significant statistical difference was found between both methods (Student's *t*-test with $p < 0.05$).

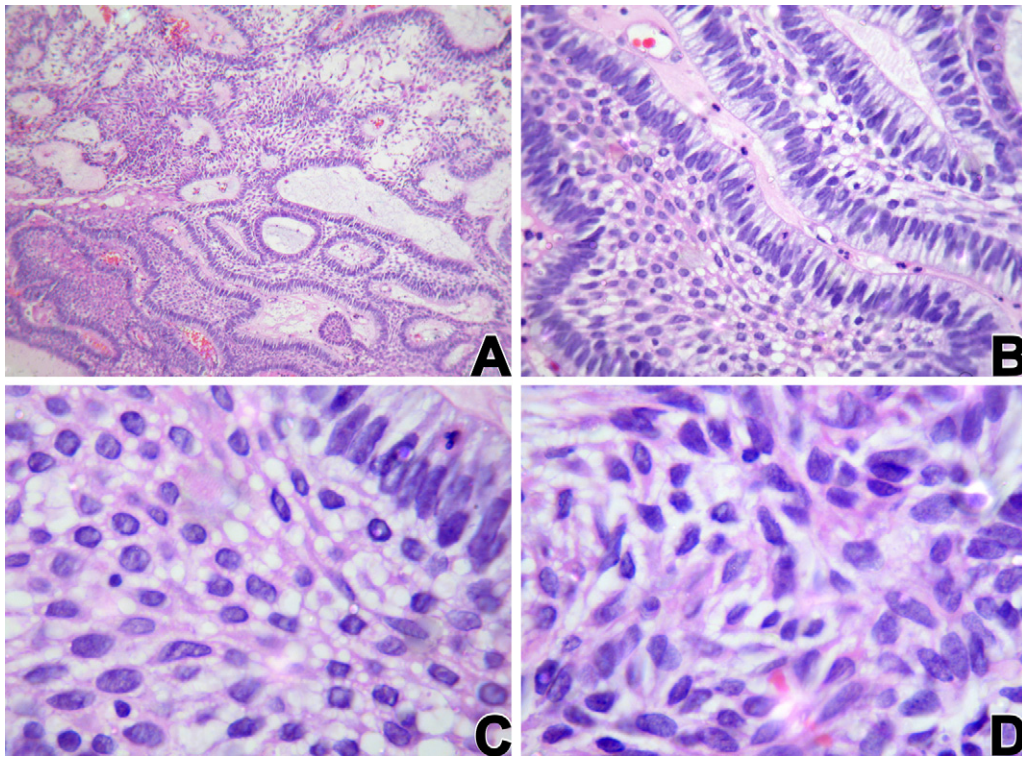


Fig. 1. Hematoxylin and eosin (HE) staining of ameloblastoma: (A) islands and cords of odontogenic epithelium within a fibrous stroma (100 \times). (B) Tumor island showing peripheral palisade cells – with typically nuclei displaced away from the basement membrane – surrounding a central area of stellate-shaped cells (400 \times). (C) Details of the central region of the islands (1000 \times). (D) Another area of the same tumor, showing the diversity of cellular shapes (1000 \times).

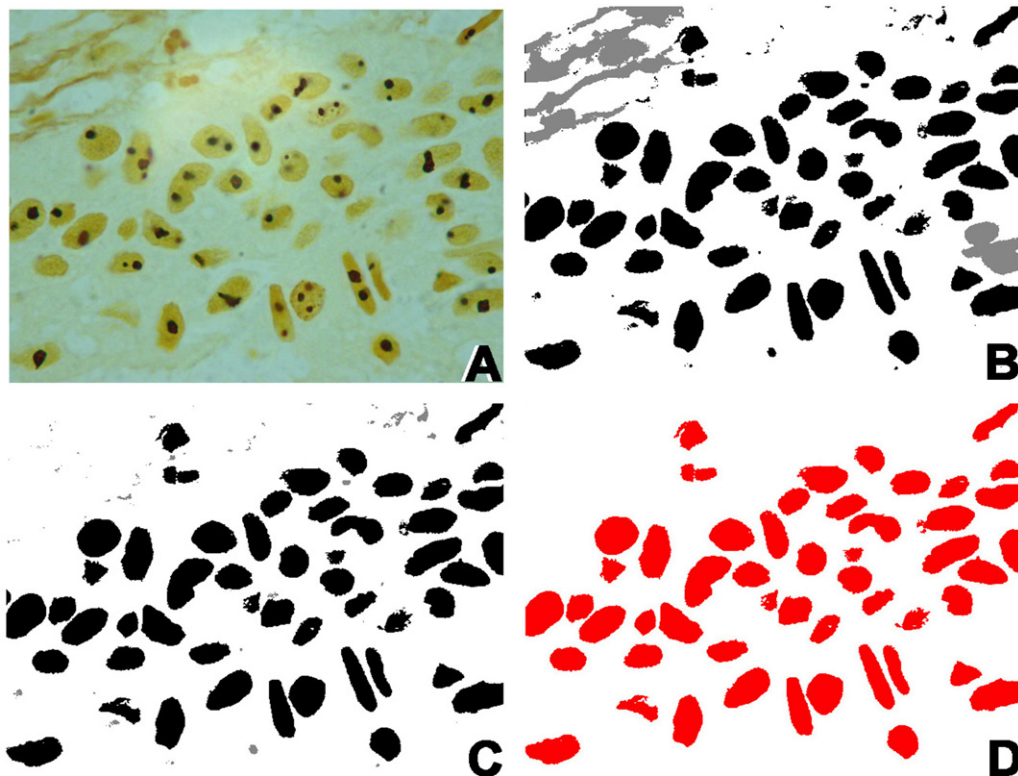


Fig. 2. Processing steps related to nuclei identification: (A) AgNOR coloration of an ameloblastoma section. (B) Preliminary segmentation and identification of large artifacts (gray areas will be rejected). (C) Large artifact rejection performed and small artifacts identification (gray areas). (D) Segmented nuclei.

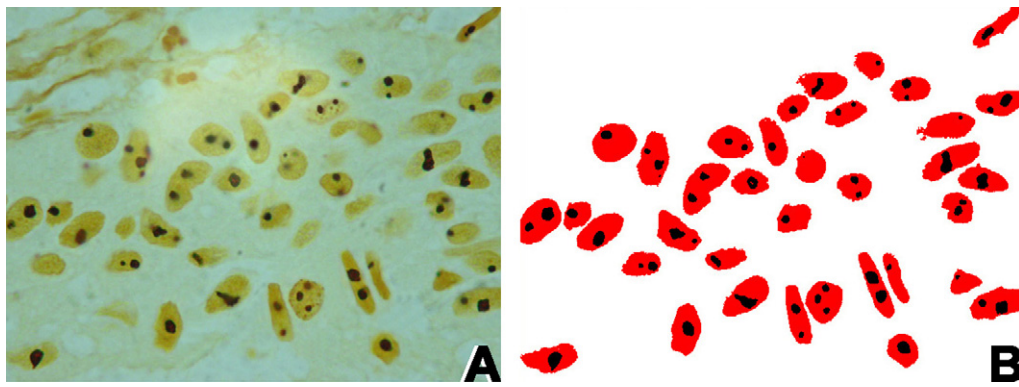


Fig. 3. Developed software: (A) original AgNOR image; (B) automated nuclei and AgNOR identification.

Discussion

The AgNOR staining is a low-cost and easy-to-use technique that can be used in the study of cellular metabolism and investigation of tumors [8,9]. However, counting a large number of stained structures using the visual (traditional) method can be very time-consuming. Slowinska-Klenka et al. [11] pointed out many advantages in using computerized systems for counting AgNORs, such as: standardization of the analysis procedure, reduction of individual bias, and reproducibility of the results. However, such systems must be used only as auxiliary tools in the diagnosis provided by the pathologists.

The performance of the proposed method resulted in a standard deviation of about 1%, compared with the visual analysis method in which it was only 1.3% greater. This preliminary result indicates the potential of the proposed method. For a complete validation a large number of tests should be performed.

The implemented artifact rejection routines were demonstrated to be of great importance in the segmentation process, discarding joined-nuclei and extraneous pigmentations that could interfere and lead to counting errors.

According to the obtained data, the proposed methodology presented a similar counting efficacy compared to the visual analysis performed by two observers previously calibrated and under supervision of experienced specialists. Semi-automated procedures (with user's help) can improve the obtained performance but with an increase in the processing time.

The complete process of automatic analysis of a standard image used in our study took less than 80 s in an Intel 2.2 GHz processor. However, in this study, it was not our aim to provide the fastest possible implementation of the algorithm, but instead, to demonstrate the possibilities and advantages of using mathematical algorithms to aid pathologists in the study of tumors through an easy-to-use and low-cost system.

A future version of this software will incorporate morphological tools in order to provide shape analysis (area, perimeter) and classification of cellular structures.

Conclusion

This study presented an image processing software for oral pathology applications. A specific application in AgNOR quantitative analysis was presented in order to demonstrate its potentialities. The obtained data showed no significant statistical difference between the visual analysis performed by two observers

previously calibrated and under supervision of experienced specialists and a user assisted by the computer program. The use of this software allowed the analysis to be done in a shorter time, as well as providing accurate and precise results. Therefore, we concluded that this software may be an interesting tool for helping oral pathologists in the estimation of the number of cells and cell structures, which can be used for different purposes, such as the study of neoplasms, as presented here.

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