Techniques of image analysis for quantitative immunohistochemistry

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Abstract

The aim of this paper was to evaluate the usefulness of digital image analysis techniques to measure the amount and strength of immunohistochemical markers. The new method, based on the spatial visualization technique, was confronted with methods of colour sampling and grey scale thresholding. Examples of applications of the techniques for apoptosis and proliferation markers are also presented.

Key words: immunohistochemistry, image analysis, measuring expression of reaction.

Introduction

The use of immunohistochemistry in routine pathomorphological diagnosis brought a substantial methodological problem, related with an evaluation of the amount and strength of specific reaction. Often, specimens are evaluated qualitatively by assigning scores, based on appropriate criteria. The interpretation of such results is subjective and causes certain inconsistencies upon the evaluation process. In order to make immunohistochemical studies more objective, quantitative techniques, based on computer-assisted microscopy, have been developed. Our earlier attempts involved transforming colour images to grey scale. The area of reaction was then determined by thresholding segmentation as the number of pixels from the range of grey levels corresponding with the specific reaction [1]. Subsequent attempts employed colour thresholding by using commercial software, followed by either counting the total number of pixels of positive reaction [2] or calculating the cumulative signal strength of the evaluated image [3]. Typically, measurements in quantitative immunohistochemistry include the area fraction of colour pixels present in specific reaction in the evaluated material. However, processing and analysis of raw colour images is still difficult.

In this paper, we present the use of digital image analysis techniques to measure the amount and strength of immunohistochemical markers: bcl-2, caspase-3, Ki-67 and PCNA, in human thyroid and parathyroid glands.

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Material and methods

Our study was performed on specimens, obtained from goitres resected from patients with either focal chronic thyroiditis or hyperparathyroidism. Proliferating cells were detected by immunostaining for mouse monoclonal anti-Ki-67 antibodies (M7240, Dako) with dilution of 1/150 and mouse monoclonal anti-PCNA antibodies (M0879, Dako) with dilution of 1/400. Moreover, immunohistochemical stains were performed for primary mouse monoclonal antibodies against bcl-2 (M0887, Dako) with 1/40 dilution and polyclonal mouse anti-human caspase-3 (AF835; R&D Systems) with 1/1000 dilution. From each specimen, twenty colour images of 640x480 pixel resolution (at 400x magn.) were acquired with a light digital microscope (Motic Instruments) running under Motic Images v. 1.2 for Windows (Micro Optic Industrial Group Co).

Methods of segmentation of immunohistochemical reaction

Greyscale thresholding

Colour images were first converted to greyscale images and enhanced with the median filter. Then, the interval of grey shades, corresponding to the reaction, was defined and the area, occupied by the reaction, was extracted. The thresholding oper-

Table 1. Area fraction of the markers, calculated by using colour sampling and the spatial method.

Marker	Colour sampling method	Spatial visualization Technique	
	Area fraction	Area fraction	Colour intensity
caspase-3	0.04	0.05	134
bcl-2	0.27	0.26	83
Ki-67	0.02	0.02	134
PCNA	0.11	0.12	135

Figure 1. Micrographs of caspase-3 (left) and bcl-2 (right) in chronic thyroiditis.

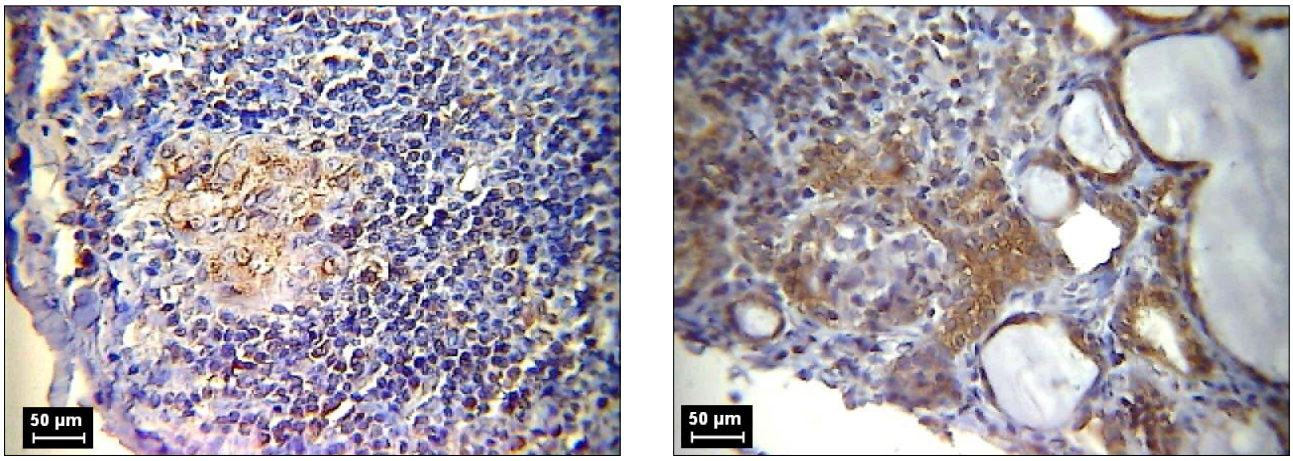
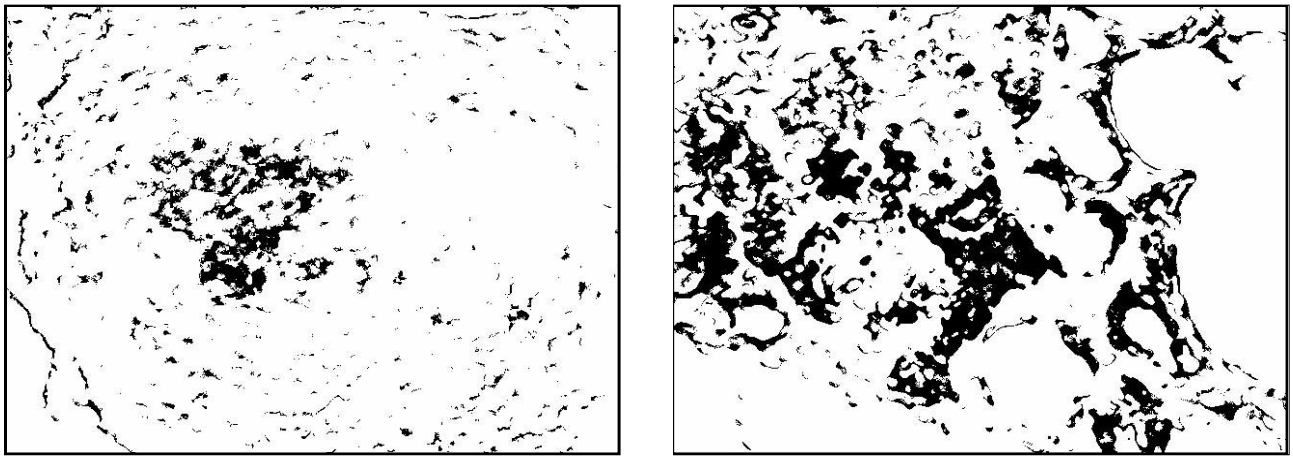


Figure 2. Pixels extracted from images in *Fig. 1* by using an "eye dropper" tool.



ation converted foreground pixels into black colour, while background pixels into white colour. Thus, the binary image represented the analyzed reaction. The area of positive reaction was estimated by the number of black pixels. The area fraction of positive reaction was determined as the percentage of black pixels in the binary image.

Method based on colour sampling

The area, occupied by the immunohistochemical reaction, was selected by colour sampling, using an "eye dropper tool" in commercial software (Figs 1-2). The number of selected pixels was read from the histogram of colours and their percentage per section was then determined.

Method based on spatial visualization of colour reaction

Colour images were processed in HSB (hue, saturation, brightness) colour space and extended to three-dimensional images by introducing the intensity of colour reaction as the third dimension (Fig. 3). For quantitative measurements of colour reaction, spatial images were linearly converted to 256 colours. Pixels in red and yellow colours corresponded to brown shades of the reactions analyzed in this study and

Figure 3. Spatial visualization of the images presented in *Fig. 1.*

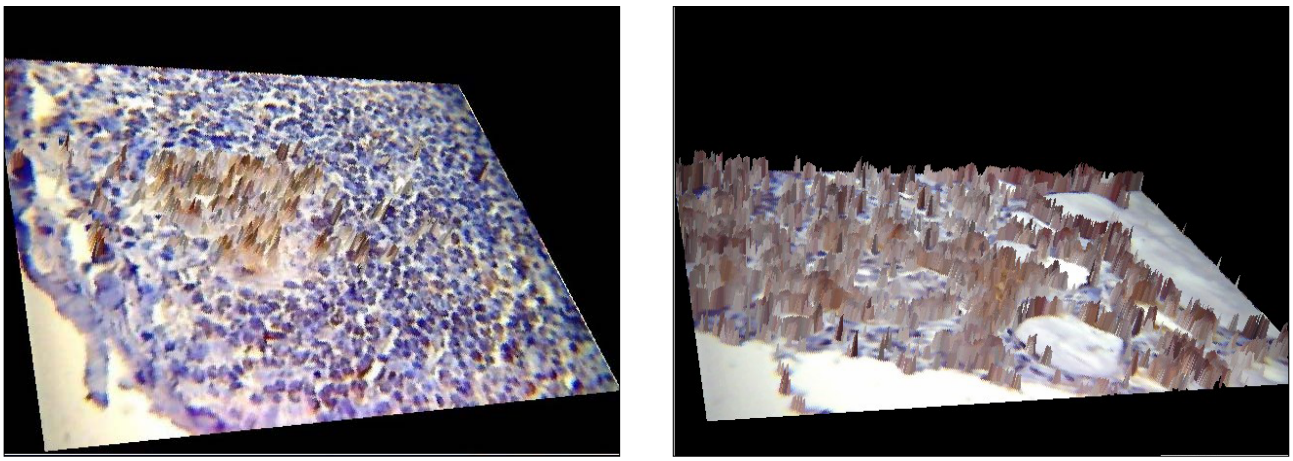


Figure 4. Segmented markers: caspase-3 (left) and bcl-2 (right).

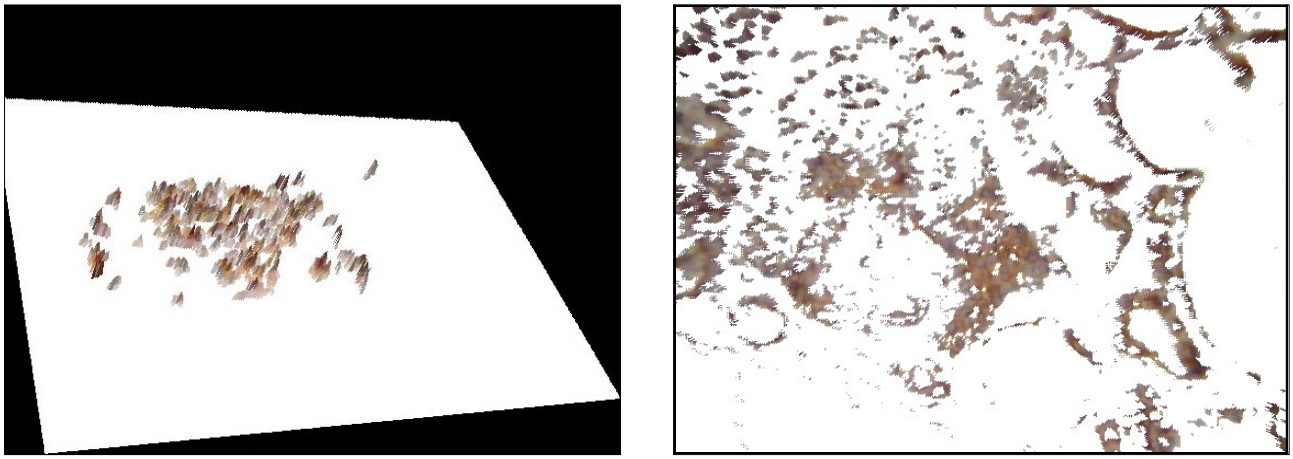
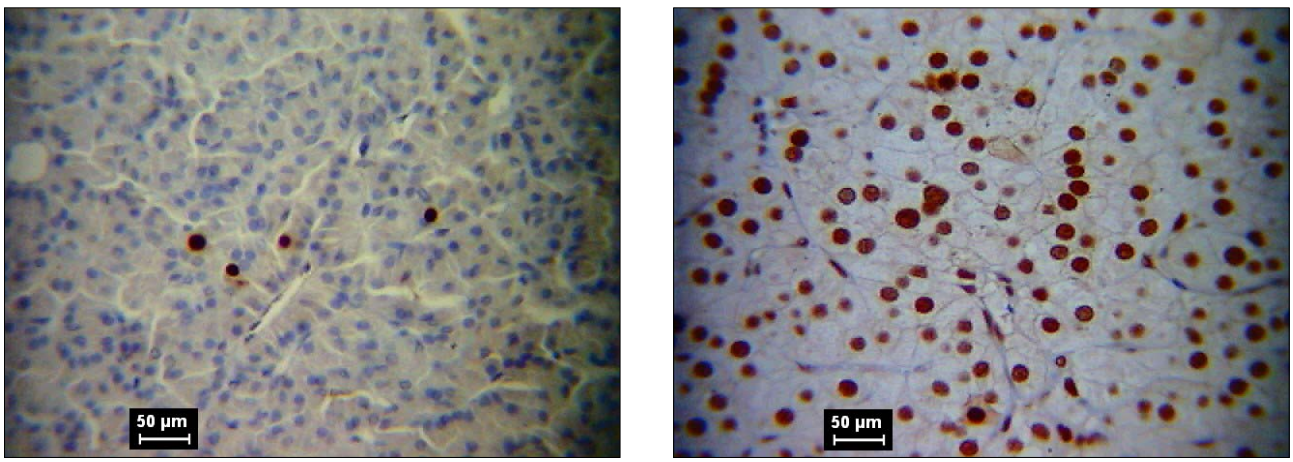


Figure 5. Micrographs of Ki-67 (left) and PCNA (right) in chronic parathyroiditis.



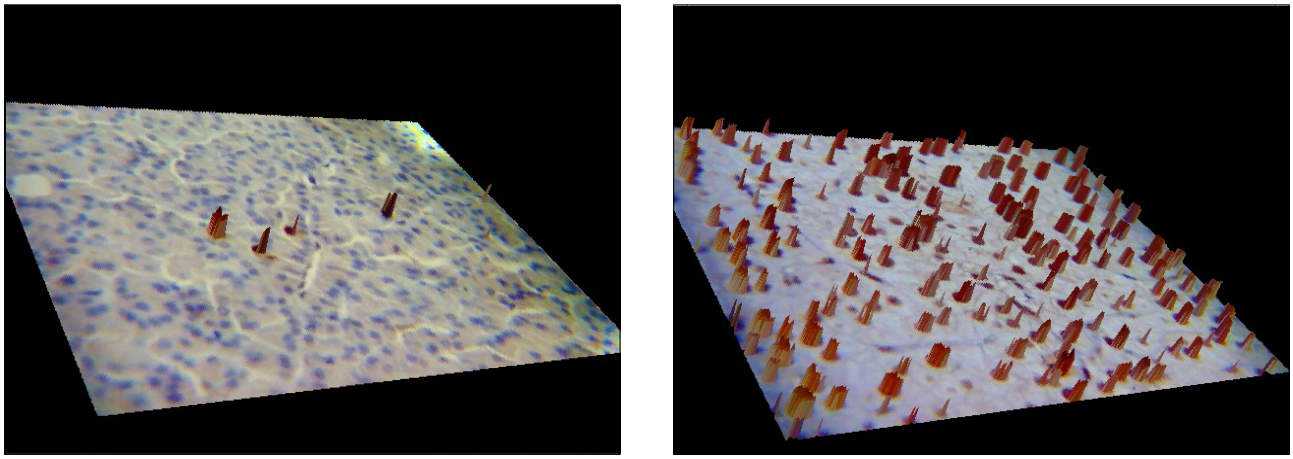
were used to assess the area, volume and the intensity of colour reaction. The spatial representation of the reaction was considered as a set of connected prisms and pyramids to determine the reaction total volume. The reaction total area was derived from the orthogonal projection of the prisms and pyramids onto the plane. The reaction intensity was derived from the volume/area ratio. Spatial image processing was performed by using a computer program, designed and programmed in C++ by Strzelczyk [4].

Results

Results of the segmentation, based on the spatial visualization technique, were consistent with the results obtained by colour sampling (Tab. 1).

However, the time required for the measurement with our new technique was repeatedly shorter. Greyscale thresholding was not performed for caspase-3 and bcl-2 stained specimens because discarding colour information caused weak-

Figure 5. Spatial representation of Ki-67 and PCNA images (see Fig. 5).



ness of the reaction recognition. Results of greyscale thresholding for Ki-67 and PCNA were also consisted with colour based methods, shown in Table 1.

Discussion

The advantages of our technique, based on spatial visualization, include a shorter time of processing and analysis, approx. 50 images per hour, including visual control of each image by an analyst [4]. Moreover, the use of the same filters of colours, brightness and saturation for the sequence of images, showing a similar brightness and saturation, makes the analysis more objective [3].

References

1. Kaczmarek E. Fuzzy logic for image processing and analysis of renal glomeruli observed in light, confocal and electron microscopes. In: Imaging Processing III: Mathematical methods, algorithms and applications. Chichester (England); 2001, p. 102-12.

2. Brelińska R, Kaczmarek E, Ostalska D. Kinetics of thymic stroma development in the foetal period. Folia Histochem. Cytobiol. 2001; 39: 195-6.

3. Matkowskyj K, Schonfeld D, Benya R. Quantitative immunohistochemistry by measuring cumulative signal strength using commercially available software Photoshop and Matlab. J Histochem Cytochem, 2000; 48: 163-74.

4. Nieruchalska E, Strzelczyk R, Woźniak A, Żurawski J, Kaczmarek E, Salwa-Żurawska W. A quantitative analysis of the expression of a-smooth muscle action in mesangioproliferative (GnMes) glomerulonephritis. Folia Morphol, 2003; 62: 451-3.