

IMAGES IN BIOMEDICINE

## A quantitative method for analysing neuron networks marked by acetylcholinesterase histochemistry

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Enzyme histochemistry is frequently used in classical morphological studies for the qualitative analysis of neuronal networks. However, this procedure does not readily provide quantitative results. Two new alternative approaches based on digital image processing techniques were explored and the data quality compared. The preliminary results explored the feasibility of these approaches in the applied setting.

**Key words:** image analysis, morphometry, composition, histochemistry, neuronal networks.

### Un método cuantitativo para analizar redes neuronales marcadas por histoquímica para acetilcolinesterasa

El análisis cualitativo de las redes neuronales por histoquímica enzimática se usa comúnmente en los estudios morfológicos tradicionales. Una limitante de este tipo de estudios consiste en la dificultad de obtener resultados cuantitativos. Este artículo presenta dos técnicas originales de procesamiento de imágenes para realizar estudios cuantitativos y un análisis comparativo entre ellas. Los resultados preliminares presentados permiten verificar la utilidad de la metodología aplicada.

**Palabras clave:** análisis de imágenes, morfometría, composición, histoquímica, redes neuronales.

Quantitative morphological studies are currently being carried out in biological sciences. However, they are time-consuming and need a fair level of expertise in handling them. Digital image processing techniques have been employed when analysing biomedical samples in an attempt to solve this kind of problem; they also enable improving characteristics of images of interest, more information thus being obtained from them.

The difficulty of obtaining morphometric data from tissue samples from different focal planes appears when analysing enzymatic histochemistry multi-focal images. Immunohistochemical and cytochemical techniques are particularly employed in neuron network studies. Samples are

analysed by observing a sample's first plane and verifying whether it is positive for the marker used. In the case of enzyme histochemistry, a sample's neurons of interest are stained for identifying their ramifications (1-3), thus obtaining morphological information about stained neurons and the distribution of their ramifications.

One of the greatest difficulties regarding this procedure is tracing the ramifications' pathway through the different planes. This is because a ramification's position may change considerably between each plane and reference points cannot be established for following them. It thus becomes difficult to obtain morphometric data from the regions of interest, such as their area and number of ramifications. Image processing can be used for solving this kind of problem; this paper presents a new procedure aimed at doing so, which employs image superimposition and colour equalisation techniques. The method is applied on images captured from different focal planes for obtaining

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Recibido: 08/08/03; aceptado: 11/08/04

a complete image of the microscopic field where the object of interest is present.

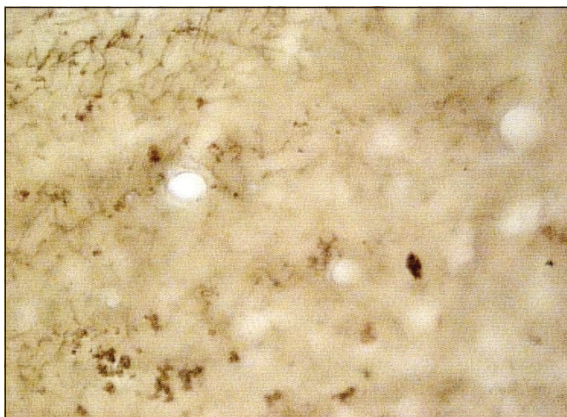
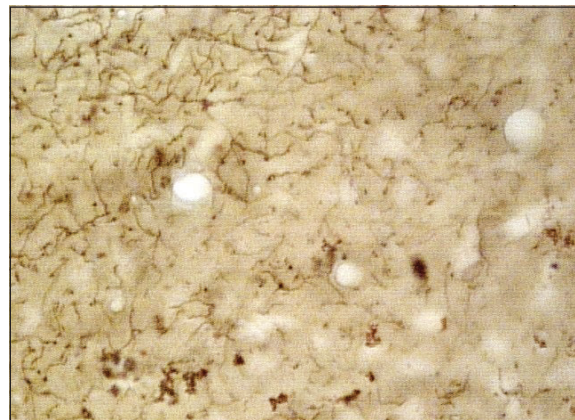
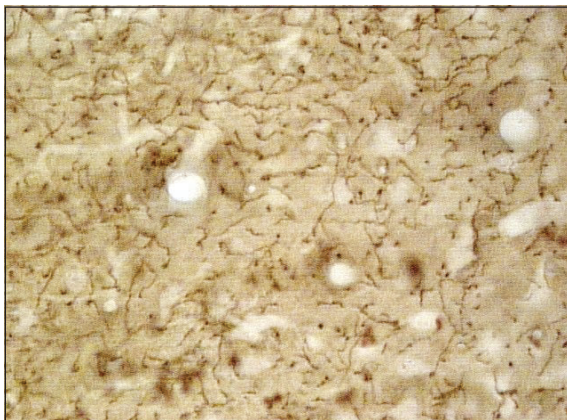
A 50 to 100  $\mu\text{m}$  thick rat brain vibratome slice was used for developing the proposed method. The section was positive for an acetylcholinesterase (Ach) reaction. This reaction was demonstrated by the presence of dark-brown stained neuron ramifications as can be seen in figure 1. Four of the slide's fields were observed by light-microscope (Zeiss Axiophot) and three images from each field were captured, corresponding to three different focal planes. A CCO Iris/RGB video camera, coupled to the microscope and connected to a personal computer, was used for image acquisition. Ramifications of their pathways were drawn with Microsoft Paint software. KS300 (Kontron Elektronik System) (4) and Ohwahalmagen (Universidad Nacional de Colombia's Ohwaha Research Group) (5) software were used for developing the methodology. Figure 1

presents the original images employed in developing the technique.

Two techniques were developed for tracing neuron ramifications through different planes. Their main difference consisted of the original image remaining unmodified in the first whilst colour information was modified for emphasising the structures in the images in the second.

#### First technique

This technique was developed using KS300 software. A composite operation was initially carried out for following the path through different images (6). Composition techniques allowed images to be superimposed for integrating them into just one where transparencies and different dispositions from each image could be observed (7). The composite image technique allows a composite image of several images to be formed by giving a new value to each pixel (i.e., piece of



**Figure 1.** Three different focal planes from the same rat brain field were stained using acetylcholinesterase histochemistry; bar length = 50  $\mu\text{m}$ . Dark brown neuron ramifications demonstrate that tissue was positive for an acetylcholinesterase reaction.

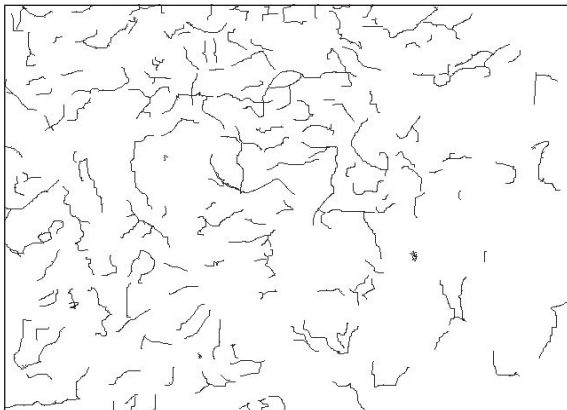
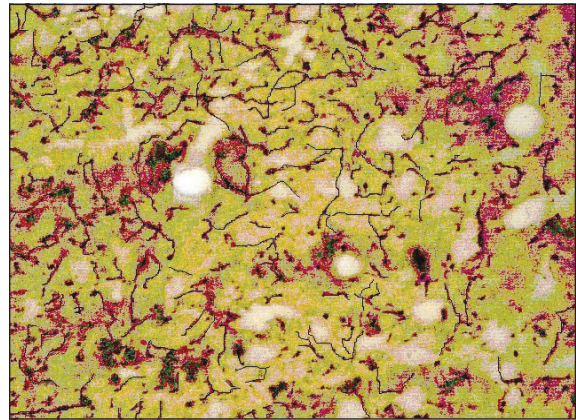
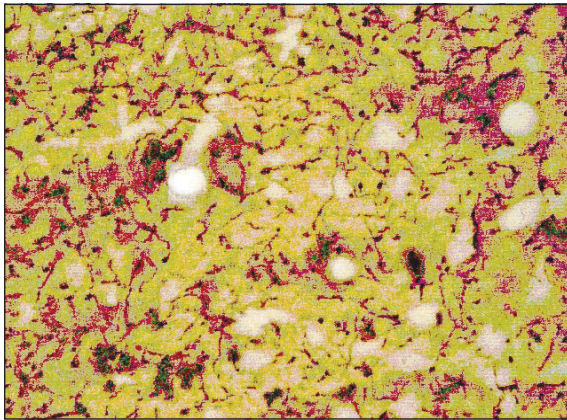
information). This value (denominated alpha or transparency channel) changes from zero (0) to one (1), where zero indicates a totally transparent image and one an image which is totally opaque. The opacity of each image thus becomes modified by giving its transparency channel a value of  $\alpha=0.5$ ; an OVER composite image operation is then carried out. The result is shown in figure 2A. The stained neuron ramifications were then manually delineated in black, using the mouse and a light pen as shown in figure 2B. The colour image thus became monochromatic once it had been binarised. Thresholding methods binarise an image within regions according to a grey level used as threshold (7); in this case, the low and high threshold values were 0.0 (red), 2.255 (green) and 0.255 (blue) according to the channels of the RGB camera used. Pixels below the threshold (belonging to the background) thus became white and pixels above the threshold (belonging to the

region of interest) became black. The resulting neuron ramifications are presented in figure 2C.

**Second technique**

Figure 2A shows that the resulting composite image has a lower definition respecting the original ones. Some ramifications have therefore become lost and others are difficult to define. This is because the composite operation includes an implicit contrast reduction producing a loss of information.

Automatic colour equalisation is applied to the original images for reducing loss of contrast. As the human eye is more sensitive to contrast than to an image's complete intensity, it perceives less information when the image has poor intensity distribution. Histogram equalisation is an image transformation technique that redistributes its intensities in trying to equalise a histogram (7).



**Figure 2.** Sequence of images resulting from applying the first image processing technique. A. Composition of three images on figure 1 by using KS300 software's AND function. B. Manual delineation. C. Final binarised image. Bar length = 50  $\mu$ m



Equalisation allows original image contrast to be improved before carrying out the composite image operation. Once the composite image has been constructed, network paths are easier to follow. Six colour equalisation techniques were tested (RGB, CMY, CMYK, VIO, HSV and CIELAB) to obtain the best contrast. Each equalisation technique corresponded to a different pixel colour range. The best results were obtained with the YIQ colour model, agreeing with previous work done on digital image processing (8).

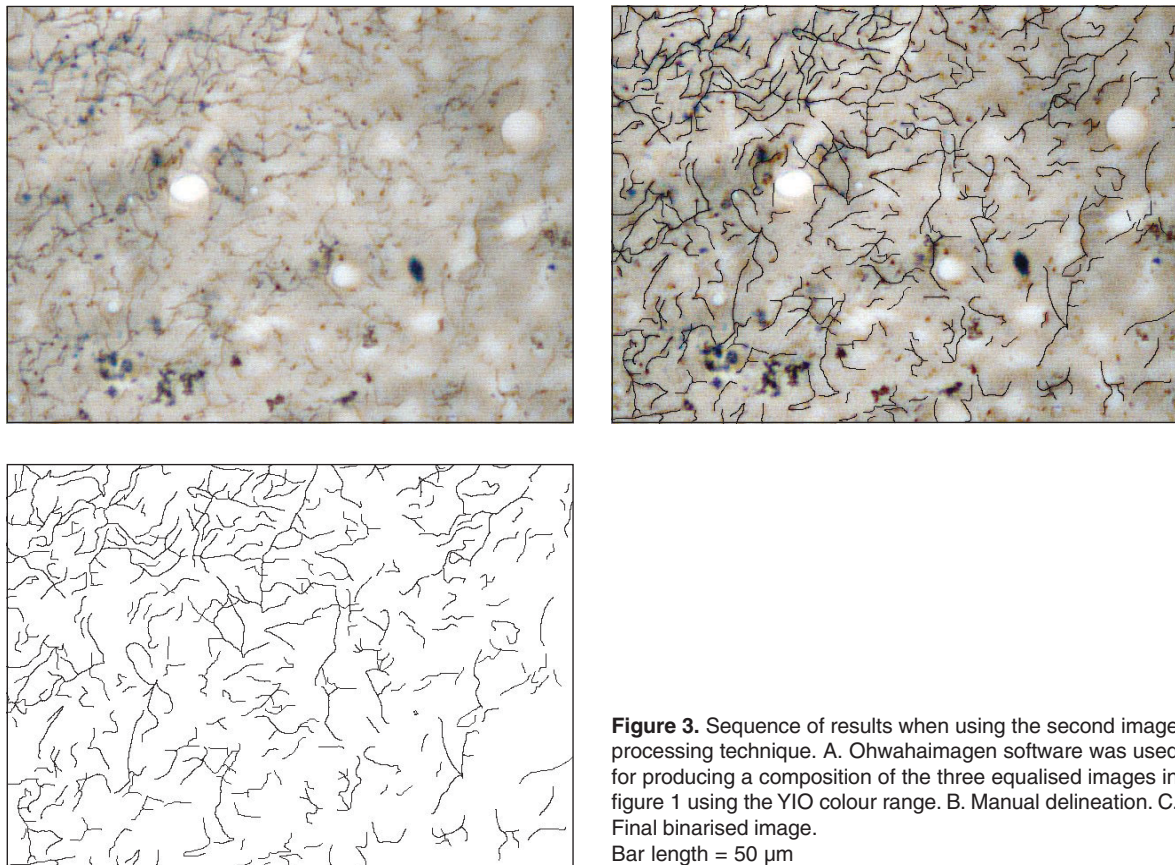
This technique's first step therefore consisted of equalising each original image by using the YIQ range; the result is presented in figure 3A. The first technique was then applied; figures 3B and 3C show the resulting images.

Positive neuron ramification areas were obtained in each binarised image for comparing the results obtained with both techniques. The composition

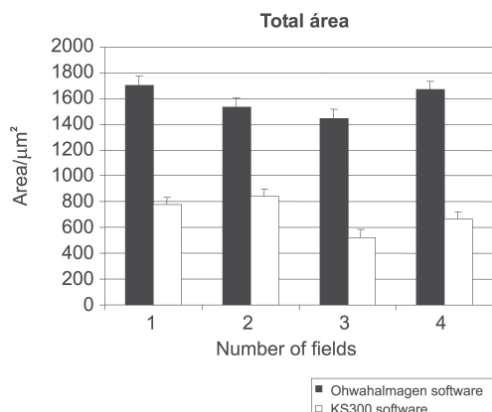
image technique allowed regions of interest to be defined; the second technique made this easier. Figure 4 shows that the second technique allowed a larger area of stained neuron networks to be recognised; best sensitivity and precision was thus shown with respect to the former.

### Conclusions

Two new techniques for identifying neuron network pathways using thick rat brain slices have been presented in this paper. The second technique allowed better definition of neuron ramifications to be identified. This technique enables high accuracy to be used when identifying neuron pathways and areas to be measured reproducibly. It can be used in quantitative enzyme histochemistry tissue studies, but it could be further extended to other fields. Quantitative studies can thus complement classical qualitative morphological techniques.



**Figure 3.** Sequence of results when using the second image processing technique. A. Ohwahaimagen software was used for producing a composition of the three equalised images in figure 1 using the YIO colour range. B. Manual delineation. C. Final binarised image. Bar length = 50  $\mu$ m



**Figure 4.** Histogram showing positive neuron ramification area within the evaluated microscopic fields.

### Acknowledgements

We would like to thank the Instituto Nacional de Salud's Neuroscience Laboratory and Hernán Hurtado for supplying us with the histological slides employed in the research. We are also grateful to the Universidad Nacional de Colombia's Ohwaha Image Processing and Computer Graphics Research Group for helping us use the Ohwahalmagen software.

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