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Three-Dimensional Digital Image Analysis of Immunostained Neurons in Thick Tissue Sections

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Abstract— **Detection and three dimensional reconstruction of cell structures from brightfield microscopy video clips using digital image processing algorithms is presented. While the confocal microscopy offers an efficient technique for three dimensional measurements, extensive and repeated measurements are still often better to be performed using permanent staining and brightfield microscopy. By processing of brightfield microscopy videos using automated and efficient digital image processing algorithms, the tedious task of manual analysis can be avoided. Our two-stage algorithm is applied for 1) cell soma detection and 2) identification of the 3D structure of entire neurons. To verify the results, we present 3D reconstructions of the detected cells.**

I. INTRODUCTION

Neurons typically have long processes (axons and dendrites) which run for a long distance out of the cell body. In order to have better three dimensional view about neurons of interest, the tissue is cut into much thicker sections (i.e. 50-100 μm) than what is used in routine histopathology (usually 5 μm). To visualize and to obtain analytic information of the structures inside these thick specimens at microscopic level, specific instrumentation is required. The development of confocal microscopy has been a great improvement in this field. However, in confocal microscopy, since the structures need to be stained using fluorescent dyes, extensive and repeated measurements of the specimens are often limited due to fading of the fluorescent molecules. Therefore, for long term inspection as well as for specimens aimed for correlated light and electron microscopy, the structures of interest are still better to be stained with permanent, i.e., light resistant dyes such as diaminobenzidine.

Although there has been a great development of different image processing tools to automatically study and analyze biological structures at two dimensional level, automatic detection and information processing of 3D data has still been limited [1] especially for the material studied using normal brightfield microscopy. For confocal images, automated image processing algorithms have been studied extensively (see, e.g., [2], [3]). Therefore, this paper studies an image processing system that utilizes sections stained for normal brightfield microscopic inspection. In this study we developed mainly two different image analyzing methods: 1) a method, which is utilized for automatic counting of the cell bodies, and 2) a method via which automatic detection of the whole structure of the neuron including dendrites and axon are performed.

For developing and testing the algorithm, we used video clips obtained by focusing through thick tissue sections using brightfield microscopy. Each frame in a video corresponds to an image captured with different focus settings, covering the entire depth of the sections. To solve the major problems in manual analysis of tissue sections, we implemented and tested digital image cytometry based analysis algorithms. The presented approach enables automatic detection of structures of interest. The collected data can be further used for neuron enumeration and for calculation of different numeric values characterizing the size, shape and dimensions of the neuronal cell bodies and their protrusions.

II. MATERIALS AND METHODS

A. Sample preparation and imaging

1) Tissue preparation: Wistar rats (n=6, 150-200 g), were deeply anesthetized with a mixture (0.4 ml/100 g, i.p.) of sodium barbiturate (Synopharm; concentration in the mixture 9.7 mg/ml), chloral hydrate (Merck, Dormstadt, Germany; 10 mg/ml), magnesium sulfate (Merck; 21.2 mg/ml), propylene glycol (Merck; 40%) and absolute ethanol (10%). Then, they were transcardially perfused first with physiological saline for 2-3 min and then with 300 ml of fixative containing 4% paraformaldehyde (P001; TAAB Laboratories Equipment, Aldermaston, Berks, UK), 0.05% glutaraldehyde (G002; TAAB Laboratories Equipment) and 0.26% picric acid (623; Merck) in 0.1 M phosphate buffer (PB), pH 7.4 for 30 min. The brains were removed from the skull and after extensive washing in PB, the brains were cut into 50 μm thick sections on a vibratome (Leica VT 1000S; Leica Instruments GmbH, Nußloch, Germany). Usage of the animal tissue was permitted by the National Laboratory Animal Center and were done according to the guidelines set by the Council of Europe and the State Provincial Office of Eastern Finland.

2) Immunohistochemical Staining: Immunostaining for calretinin and estrogen receptor alpha was performed on free-floating sections as described earlier [4], [5]. Briefly, sections were first incubated for 40 min in 0.05 M Tris-buffered saline (TBS), pH 7.4 that contained 10% normal goat serum (NGS) and 0.5% Triton X-100 solution. The sections were then

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washed in TBS containing 1% NGS and 0.5% Triton X-100 (TBST) for 10-15 min followed by incubation in rabbit anti-calretinin (CR, 1:8000; Swant 7969) or rabbit anti estrogen receptor alpha antibody (1:10000, Santa Cruz Biotechnology, CA, USA, catalog no. sc542; Pavao and Traish 2001) for 2 days at 4°C. This was followed by overnight incubation at 4°C in biotinylated goat anti-rabbit (BA1000; 1:300; Vector Laboratories, Burlingame, CA, USA). The sections were then incubated in avidin-biotinylated horseradish peroxidase complex (ABC; Vector Peroxidase Standard-ABC PK4000; 1:500; Vector Laboratories) for 3 h at room temperature. The sections were washed with TBST for 3 × 30 min after each antibody solution. The peroxidase reaction was developed by using 3'3-diaminobenzidine (DAB) as a chromogen (for calretinin), which yielded a brown reaction product or DAB intensified by ammonium nickel sulphate (for estrogen receptor alpha) giving dark-blue reaction product.

3) Section Embedding in Durcupan and Mounting on Objective Slides: After thorough washing in TBS, sections were embedded in Durcupan as described earlier [5]. Briefly, after rinsing with distilled water, sections were dehydrated in a series of ethanol (50%, 70%, 90%, 96% for 5 min in each and in absolute ethanol twice for 5 min) and propylene oxide twice for 5 min. The sections were then immersed in Durcupan (AMC; Fluka, Buchs, Switzerland). The sections were freely floating during the dehydration procedure and immersion in Durcupan. After 3 h at RT in Durcupan, the sections were transferred onto slides and covered with a coverslip. Durcupan was polymerized at 60°C for 24 h. As Durcupan embedding preserves well section thickness, better visualization and identification of individual cells while focusing through the section is enabled [5].

4) Microscopy and Digital Imaging: Sections were analyzed by using Optiphot-2 Nikon light microscope (Nikon Corp., Japan). Calretinin-immunoreactive cells in the entorhinal cortex and estrogen receptor alpha -immunoreactive cells in the amygdala were studied using 100x oil immersion objective (Plan NA 1.25). While focusing through the section, digital video clips were captured by using Nikon Coolpix 990 digital camera (Nikon Corp., Japan), which was attached to the microscope via MDC Lens 0.82-0.29x.

B. Automated Image Analysis

Manual quantification, being widely applied to analysis, is tedious of nature and prone to human errors [6]. Facilitation of the analysis phase forms the main motivation for using automated image analysis. In addition, automated image analysis offers several favorable features, such as enabling the quantification of several morphological features, the repeatability of analysis and the lack of human errors [7]. Our method for processing thick tissue video is here presented in two parts, namely, automated analysis of somata (cell bodies), and structure identification.

1) Counting of cell bodies: The analysis of the cell bodies in the video is performed using a two stage approach: first each frame is processed using thresholding [7] and mathematical morphology [8] to segregate and to detect soma of the cell bodies, and second the consecutive processed frames are compared to take into account the third dimension of the input data. As seen in Fig. 1 A, the somata are visible as darker spot-like structures. To locate pixels consisting of the somata, the original video frame is therefore binarized by thresholding with a user defined value. Due to the relatively high contrast of our test video material, thresholding with one global threshold value gives us an initial segmentation result: background is separated from the cell somata for the entire video. Since the intensity of the soma region is lower than the background, we invert the binarized image to obtain an image where 1's indicate soma pixels and 0's the background. Some isolated false pixels remain in the images, however. We remove these pixels by eroding the image with a 5×5 structuring element. After the binarization and erosion of each frame, we stack the frames to form a three dimensional matrix and apply dilation with a cubic $7 \times 7 \times 7$ structuring element. The dilation process minimizes the problems of global thresholding in the 2D processing phase, and makes the selection of a suitable thresholding value far less critical. The processing result is a binary three dimensional matrix of pixels, either somata or background.

2) Structure Identification: Identification of 3D structure for objects in thick tissue sections requires detection of areas that are in focus in each 2D video frame. Supposing the objects appearing in focus have sharp edges, we start the structure analysis by detecting the edges frame by frame. The edge detection, performed with Sobel edge detector [7], gives an initial segmentation for the regions of interest in each $m \times n$ frame. Next, the resulting binary objects are dilated with a 3×3 structuring element. From the detected in-focus objects, only those appearing in adjacent frames are included. This is done by performing set operations for three frames, which are the currently analyzed frame I_j, the previous frame I_{j-1}, and the following frame I_{j+1}. The objects that do not appear in all of these three frames, in other words, the objects for which the intersection of object area in I_{j-1}, I_j, and I_{j+1} is empty, will be ignored. Next, the resulting binary objects are thinned in order to make the slightly over-detected edges resemble more the size and shape of the actual biological object.

Following the initial segmentation and morphological operations in 2D, the $m \times n \times k$ binary matrix is further processed by using 3D operations. First, small objects are removed, since the edge detector is likely to find small objects that are not of interest. Finally, the resulting 3D matrix can be dilated with a $3 \times 3 \times 3$ structuring element in order to produce less scattered objects. However, in case the analysis result is preferred to have similar thin structure as the biological objects, this procedure may also be omitted.

III. ANALYSIS CASE STUDY

While estrogen receptor alpha-immunoreactivity is primarily present in the nucleus of the neurons, calretinin-immunoreactivity is filling up the whole neuron including dentritic and axonal profiles. Both of the studied antigens were heavily labeled at their actual locations. The automated

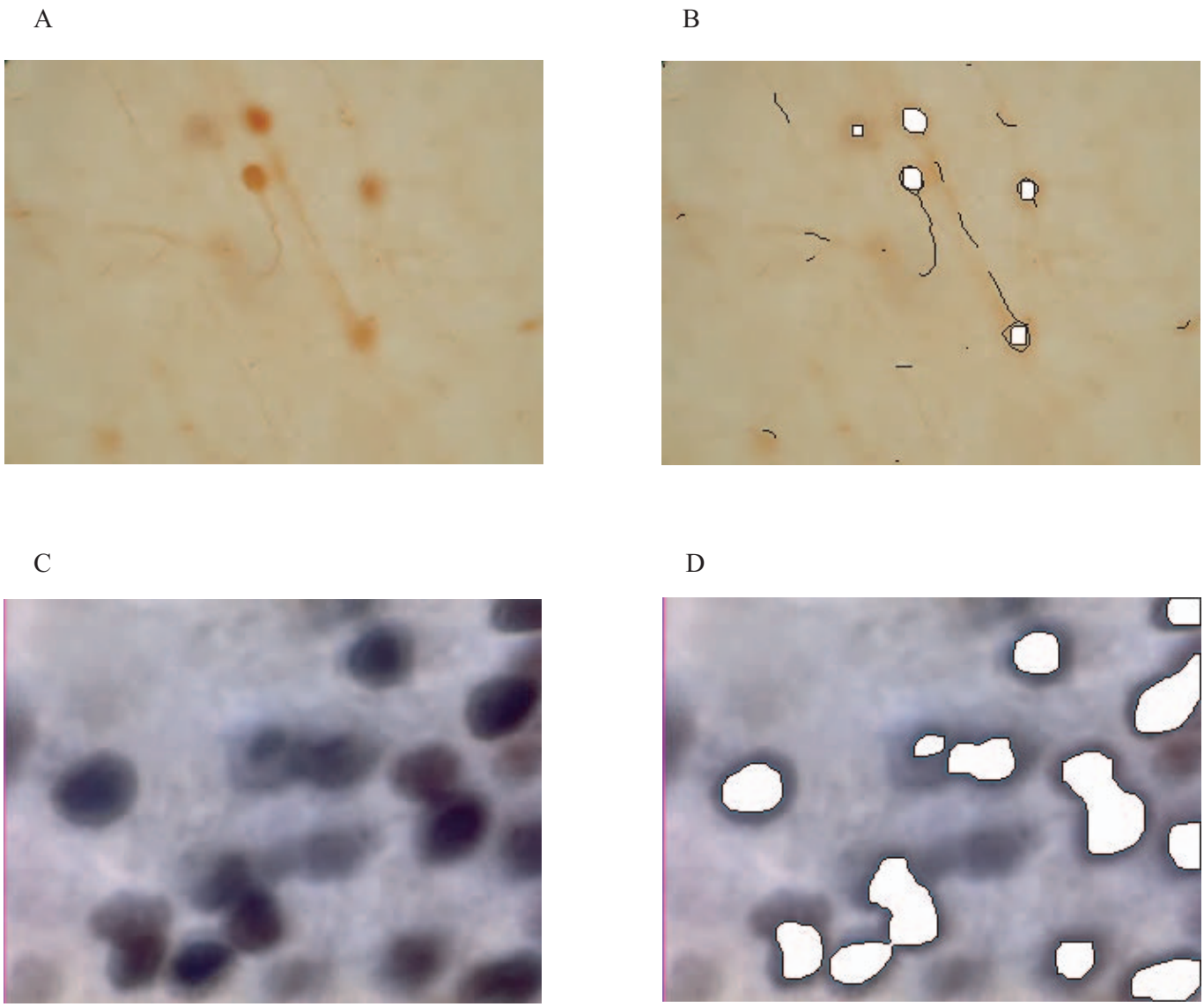

Fig. 1. Analysis results for single frames from the video clips. (A) The original frame of clip one. The objects of interest, calretinin-immunoreactive cell somata and dendritic profiles, are visible as objects darker than the background. Both in and out of focus objects can be seen. (B) The detected somata and dendrites that are in focus are shown with white and black superposed on the original frame. (C) One frame of clip two. (D) The detected somata.

cell body detection method was tested on video clips of thick tissue sections of both of these materials, one clip per material. The algorithm was run for both of the clips after which the 2D and 3D results were compared visually to the original videos. Although both of the videos were imaged using brightfield microscopy, properties of the clips differ significantly in order to test the versatility of the cell body detection algorithm. Fig. 1 presents an example frame from the first video clip (A) and the analysis result (B), where the cell somata and dendrites in focus are shown with white and black, respectively. For visualization purposes, the borders of the somata are also shown with black. The scattered appearance of the detected dendrites are due to the 3D nature of the object; long, thin threads are located throughout the whole tissue section, and our algorithm aims at detecting out of all visible objects only the ones appearing in focus.

An example frame of the second clip and the detection result can be seen in Fig. 1 parts C and D, respectively. Although the color and contrast of the video, and properties of the cells differ from the first video, the proposed algorithm yields satisfactory results after tuning of the thresholding value. Some of the somata are overlapping, but this problem can be overcome in the future by implementing different object separation algorithms in three dimensions. As estrogen receptor alpha was located only in the nucleus of the cell bodies and did not visualize the dendritic profiles of these neurons, therefore the cell structure identification was not applied to the video clip obtained from these cells.

An example of 3D structure reconstruction for the video clips are given in Fig. 2. The cell somata of the first video are illustrated in Fig. 2 A, and in Fig. 2 B both somata and dendrites are visible. The thin objects are the calretinin-

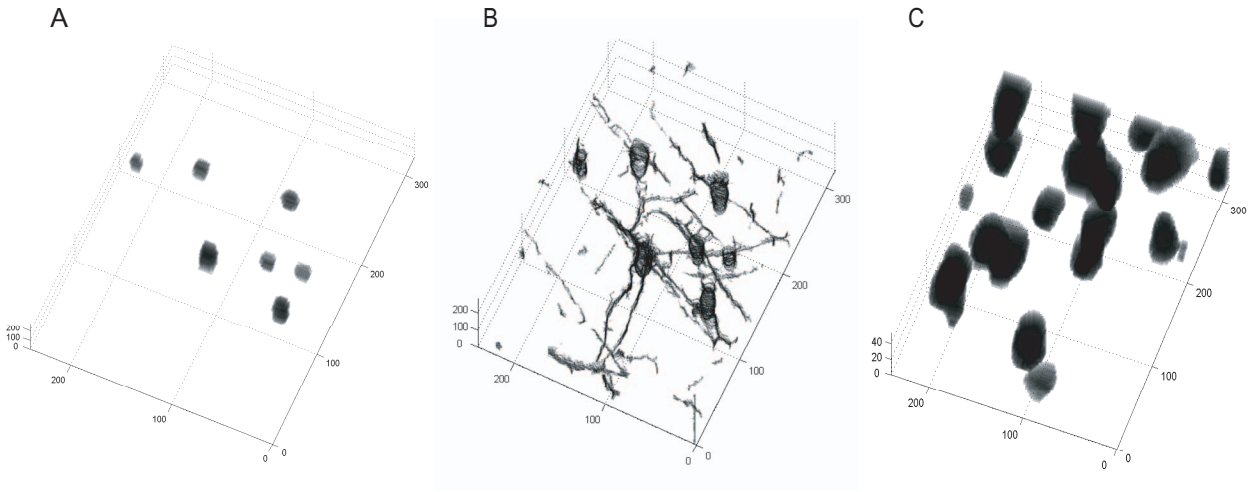

Fig. 2. Volumetric 3D reconstruction of the detection results. (A) An example of detected calretinin-immunoreactive somata. The locations of the somata are presented for each video frame. (B) An example of 3D structure identified for objects appearing in a tissue section video clip. The large nearly round, slightly elongated objects represent neuronal somata, and the thin threads are dendritic profiles of the calretinin-immunoreactive neurons. The clip consists of 280 frames, each having 320×240 pixels. (C) Detected somata of the second video clip. This clip consists of 54 frames.

immunopositive dendritic profiles extending from the cell body center i.e. soma (the larger nearly round profiles) of the neuron to different directions in 3D space. Fig. 2 C presents the detected somata of the second video clip. To validate the analysis results, an expert biologist (RM) has visually compared each 2D frame of the original videos to 2D slices taken from the 3D reconstruction result similarly as the results presented in Fig. 1.

IV. CONCLUSIONS

Digital image analysis based detection for thick tissue sections imaged with brightfield microscope was presented. The analysis method, strongly relying on morphological operations, has two objectives: 1) cell soma detection and 2) identification of the 3D structure for entire neuron in tissue. The method was applied on two separate thick tissue section video clips originating from two experiments, where sections of rat brains were imaged with normal brightfield microscope while focusing through the sections.

As the results show, our algorithm enables automated analysis of thick tissue sections. The proposed image analysis algorithms are capable of processing bright field microscopy video clips without any digital preprocessing of the video frames. While this is the case for the specific tissue clips presented here, some preprocessing steps such as background removal may be needed for low-contrast tissue clips.

In the future, the algorithms presented here will be modified in order to make them more robust and generic, and thus, more useful for practical laboratory analysis. For doing this, we will need to set up a high throughput test environment with a microscope equipped with remote controlled measurement table, to which our analysis platform would be connected. This will enable us to test the algorithms with a wide spectrum of different tissue samples and staining techniques. By enumerating the pixels of separate objects, we can automatically calculate several parameters required by different studies, e.g. volume, length, or width of the studied profile. To further improve the results, separation of overlapping objects can also be taken into account.

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