Engineering

Industrial & Management Engineering fields

Okayama University *Year* 2004

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IEEE IST 2004 – International Workshop
on Imaging Systems and Techniques
Stresa, Italy, 14 May 2004

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Abstract *– The neuronal organization of the hippocampus is extensively studied. However, the synaptic connections between neurons of the hilus and neurons of the granule cell layer are still debate. In the present study, we utilized automated micromanipulation technique combined with visual image processing to analyze the synaptic connections between hilar mossy cells or mossy fibers, and granule cells or basket cells in the granule cell layer. The stereoscopic microscope image of the dentate gyrus of coronal slice in adult rat brain was converted into a binary image. The area of the dentate gyrus is detected by genetic algorithms. This was matched with the template image made from a microscopic photo of cresyl violet-stained coronal section of the rat brain by parallel and rotation matching. A glass microelectrode inserted into the apex of the dentate gyrus by the micromanipulation system. Lipophilic fluorescent tracer DiI or DiD was injected into the hilus by a nanoinjector.*

Keywords *– Image Processing , Micromanipulation, Neuronal Organization, Microinjection.*

I. INTRODUCTION

The hippocampus is a prominent brain structure which plays an important role in memory and learning. Its cytoarchitecture is relatively simple compared to that of cerebral cortex, and its intrinsic and extrinsic neuronal connections are well known. However, its intrinsic synaptic connections, especially that between dentate granule cells and hilar interneurons, are still debate.

To examine neuronal reorganization induced by brain insults, a precise anatomical study combining immunofluorescent tracing and visual image processing are required. Visual image processing is widely utilized in experimental neuroanatomy, and computer-based morphological analysis is an indispensable technique in neuroscience.

Although the injection of fluorescent tracers are generally done manually under microscopic observations, an automated micromanipulation technique combined with computer-based visual image processing may be helpful to reduce labor. In addition, an analysis of fluorescently traced neurites by visual image processing may be useful to elucidate the structure of normal and reorganized neuronal circuits because the hippocampus has highly branched, three-dimensional neuronal organization. In the present study, lipophilic fluorescent dyes DiI and DiD were injected into small regions of the hilus of the rat brain slice by an automated micromanipulation to analyze synaptic connections of the hilar neurons with granule cells and interneurons in the dentate granule cell layer. We selected the hilar region near to the apex of the granule cell layer as the target because it was easy to determine distances from the apex to the target from its characteristic triangular morphology.

II. METHODS

A Detection method of injection area

Figure1 shows the microinjection system. The system consists of a stereoscopic microscope, two CCD cameras, a micromanipulator and a personal computer. A target is set on the stage of the microscope. The cameras are mounted to the eye pieces of the microscope. The micromanipulator has three degrees of freedom. The microelectode is set to the micromanipulator. The (X, Y, Z) coordinate is set as shown in Fig. 1. The Y axis is perpendicular to the stage of the microscope. The XZ plane is parallel to the stage. The left image and the right image inputted to the personal computer that performs image processings to direct the microelectrode and the target in the images. The distance between the microelectrode and the target is measured three dimensionally by the stereovision method. The microinjection system guides the microelectrode to reach the injected point within the target. The measurement of the distance between the tip position of the microelectrode and the injected point, and the movement of the microelectrode are carried out alternately to reduce the distance to 0.

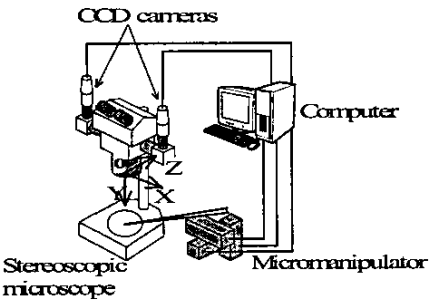

Fig.1 Microinjection system.

Fig. 2 Dentate gyrus of rat brain.

Fig. 3 Dentate gyrus after region segmentation.

Fig. 4 Extracted area of the dentate gyrus.

A coronal slice of adult Sprague-Dawley rat brain of (5mm in thickness) was placed in a recording chamber filled with phosphate-buffered saline. The stereoscopic microscope color image of the hippocampus was converted into a black and white image and the noises were eliminated by a median filter. Noises of the microscopic image were largely eliminated by this procedure (Fig.2). The threshold for binary image was determined by the mean concentration and the standard deviation calculated from the dentate gyrus. By the region segmentation, the area of the dentate gyrus is extracted. In the brain slice shown in Fig. 2, the mean concentration was 92.93 and the standard deviation was 5.39 respectively. Figure 3 shows the dentate gyrus after region segmentation. The small rigions in Fig. 3 is eliminated and the area of the dentate gyrus is detected as shown Fig.4. In this image, noises were eliminated. The triangular-shaped apex of granule cell layer was clearly visualized because the dentate granule cell layer was large enough compared to small noises dispersed in hilus and molecular layer of the dentate gyrus, and CA3.

B Detection of the apex of the dentate granule cell layer by genetic algorithms.

For successful automated micromanipulation, the apex region of the dentate gyrus shown in Fig. 4 should be matched with the template image. A binary image of the template is shown in Fig. 5. At first, the template image was made from microscopic photos of cresyl violet-stained coronal section of the rat brain (Fig.6). The area of the dentate gyrus was selected from microscopic photo image. A clearly defined image of the dentate gyrus was obtained by this processing because the granule cells were strongly stained with cresyl violet compared to adjacent molecular layer and hilar region. It is suitable for the template because the images which reflected morphological characteristics of the dentate gyrus.

The reference pattern is positioned in the binary image as shown in Fig.7. A correlation value between the reference pattern and the binary image that is shown in Fig.4 is estimated. When the reference pattern and the pattern of the black pixels in the binary image is equals to each other, the correlation value is near to 1. If the reference pattern and the binary image is not match up each other the correlation value becomes small. Therefore, if the correlation value is small, the reference pattern is moved and the correlation value is estimated again. Finally, the position of the reference pattern where the correlation value becomes near to 1 can be detected. After the detection of the position, an angle of the reference pattern is detected in the same way.

Additionally, it is needed to reduce the detection process time to perform the microinjection at a realistic rate.

However, these detection process requires a long time because the enormous amount of calculation is necessary. Therefore, we developed the detection algorithm using the genetic algorithm.

A search area is 64× 64 pixels in the center of the image. Angle is selected from 0°, 45°, 90°, 135°, 180°, 225°, 270°and 315°.

Fig. 8 shows the structure of a gene. The size of the gene is set at 15 bits, and each bit is the value of 0 or 1.The position in row, the position in column is indicated by 6 bits And the angle of the reference pattern is indicated by 3 bits of gene.

The position and the angle of the template was detected as shown in Fig.9.

The maximum correlation coefficient was 0.330375 when the center of the template image was located at i=311 j=174.

Fig. 5 Template for the apex of the dentate gyrus.

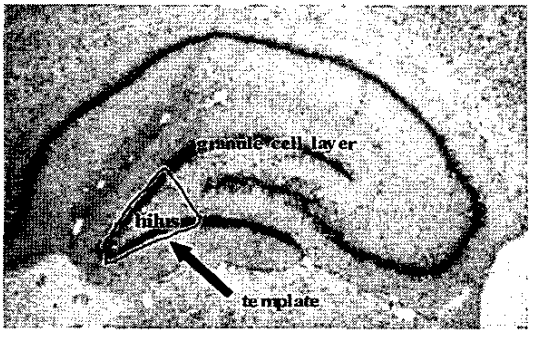

Fig. 6 Cresyl violet-stained coronal section of the rat brain.

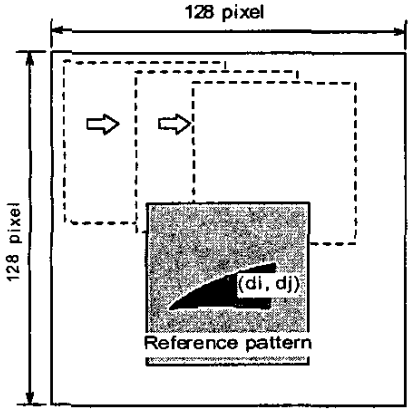

Fig.7 Detection method of the dentate gyrus.

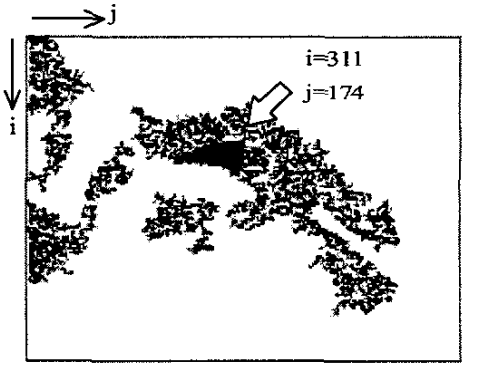

Fig. 9 Result of template matching.

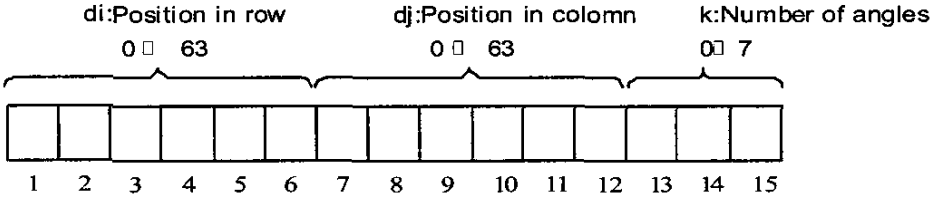

Figure 8 Structure of a gene.

Fig. 10 Detected triangular-shaped apex of the dentate gyrus where lipophilic tracers are to be injected.

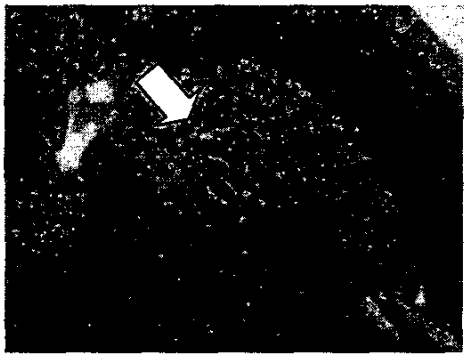

Fig. 11 Coronal slice injected lypophilic tracer into the apex of the dentate gyrus. The injected site is indicated by arrow.

III. RESULTS

The tip of microelectrode was successfully inserted into the target region and DiI or DiD was slowly injected by a nanotransjector (Fig. 10, Fig. 11).

In the present study, we injected lipophilic tracer into 4% paraformaldehyde-fixed brain slices because lipophilic tracers were incorporated into plasma membrane are slowly dispersed along the outer layer of plasma membrane and provide sharp morphological image. Moreover, lipophilic tracers can be injected into 5-mm brain slice was processed as described in the methods. By using an automated micromanipulation device, the tip of microelectrode was successfully inserted into the apex of dentate gyrus by 6 to 8 steps of movements within 2 to 3 minutes, and 0.5 to 1 μl of lipophilic tracer was injected into granule cell layer (Fig. 10, Fig. 11). The injected slices were returned into fixative and

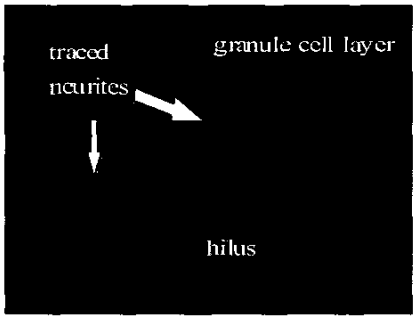

Fig. 12 Confocal microscopic image of axons of the hilar neurons traced with DiD. The traced neurites are indicated by arrow.

stocked at room temperature or 4□ under light-protected condition. Morphological properties of neurons in granular cell layer, molecular layer, and hilus was analyzed by confocal laser scan microscope (Zeiss) more than two months after the injection. The hilar neurons were beautifully traced and their processes extending into the granule cell layer was clearly identified (Fig. 12)

IV. CONCLUSIONS

We have shown that image processing and micromanipulation could be utilized for lipophilic tracer injection into the hilus of the dentate gyrus of rat brain. Local injection of tracers has been done by microscopic visual inspection and manual control of microelectrode. However its general use is limited because sophisticated techniques and much burdens are required to operate micromanipulator. Especially when tracers, chemoatractants or neurotrophic factors must be injected into the areas with exact distance from the implanted neurons or regions to where growth cones of axons or dendrites are introduced, it is difficult to inject them in unfixed, unstained brain slice. Our system may be useful in these experimental studies.

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