Development of an Efficient Method for Classifying Large Numbers of Dendritic Spines Using Confocal Microscopy

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Key words: dendrite, dendritic spine, confocal microscope

Spines are protrusions from the dendrites of a neuron and receive synaptic inputs from other neurons. They are classified by their length, head diameter, and neck diameter into four types as seen in figure 1: Filopodium, thin, stubby, and mushroom spines\textsuperscript{1)}. Mushroom and stubby spines are considered to have stronger inputs whereas thin spines and filopodia are assumed to have weak inputs or be in the process of synapse formation\textsuperscript{2)}.

Recent findings in neuroscience have pointed to the importance of spines on the overall behavior of the human brain\textsuperscript{3)}.

Figure 1  The Classification of Dendritic Spines
Dendritic spines are classified according to its spine length, head diameter, and neck diameter\textsuperscript{1)}. Filopodium are spines that are over 3 µm in length without a wide head. Thin are spines that are not long as the filopodium cutoff length but have a larger spine length than its diameter. Stubby spines have a longer diameter than its spine length. Mushroom spines have a bigger head diameter than its neck diameter.
ders such as Down Syndrome and Tuberous Sclerosis are reported to show distinct spine patterns that deviate from normal trends\(^9\). Patients with Down syndrome are known to have spines with large heads\(^9\) and longer necks while having poorer synaptogenesis in childhood and stronger synapse atrophy in adulthood compared to normal subjects\(^9\). Tuberous Sclerosis is known to cause an increase in spine density because spine pruning is impaired\(^9\). Further analysis with animal experiments is necessary to clarify the link between spine morphology and pathology. There have been attempts to reveal the spine morphology of model mice with neurological disorders using electron microscopes. Although remarkable advances in electron microscopy have allowed for analysis of multiple synapses in three dimensions\(^7\), large-scale analysis of neuronal spines remain impractical. Development of new observational technologies such as structured illumination microscopy combined with machine learning has been proposed to observe spines with electron microscopy grade resolution in high volume\(^8\), but these require new observational equipment which limit institutions that can do such research.

To overcome these limitations, we investigated an efficient approach using confocal microscopy and computer software to make simultaneous analysis of spines throughout the dendrite possible. Although images acquired from confocal microscopy have a significantly lower resolution than images acquired from electron microscopy, immunofluorescent markers allow spines to be clearly visible for three-dimensional analysis under confocal microscopy as seen in figure 2a. We used 300 µm slices of B6 mouse including the somatosensory cortex and injected biocytin into a few pyramidal neurons on the slice. After fixation of the slices with 4% paraformaldehyde, the slices were incubated with streptavidin conjugated with a fluorescent label (Alexa Flour\(^\text{®}\) dye). Then, we observed the structures of the labeled neurons under a confocal laser scanning microscope. The full protocol is available on figure 3.

Further, we employed the following clearing reagents to increase the quality of the confocal images for 3D reconstruction. We used CUBIC–L solution\(^9\) to clear the tissue before applying streptavidin. We applied SCALE S4\(^10\) solution as the medium to observe the tissues under the microscope. Both solutions preserve tissue structure and prevent light scattering within the tissues which are necessary to create high-quality three-dimensional reconstruction of spines.

The compiled confocal images such as one shown

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**Figure 2** Images and Models of the Dendrites
(a) Image of the fully labeled neuron using biocytin and streptavidin on the 300-µm-thick coronal slice taken with a confocal microscope. (b) In high-magnification image, dendritic spines in the layer of the neocortex are clearly observed. (c) A three-dimensional model was reconstructed from the data acquired by the IMARIS software using three-dimensional modeling software Blender. (d) A portion of the reconstructed three-dimensional model of a dendrite with four different spines is shown.
Figure 3  Protocol used to prepare brain slices for confocal microscopy.

The figure provides the exact protocol to visualize dendritic spines under a confocal microscope. Biocytin was injected inside the neuron using a micropipette, and the slice containing the injected neuron was incubated in 4% paraformaldehyde for 24 hours at 4°C. The well solution was changed to 50% dilution of the CUBIC-L solution and was incubated for 20 minutes at room temperature. Next, the well solution was changed to the full CUBIC-L solution and was incubated for 40 minutes at room temperature. After incubation, the slice was washed 3 times inside the well using PBS at room temperature. Fluorescent streptavidin with a 1/200 dilution in PBS was incubated after wash for 2 days at 4°C. The slice was, again, washed with PBS 3 times inside the well at room temperature. After the wash, the slice was placed on a slide enclosed with SCALE S4 before observation under the confocal laser scanning microscope.

Figure 4  Algorithm for Spine Classification

The figure shows the workflow for processing each set of filament data. We defined a spine as any filament over 0.4 µm. A neck was defined as the ratio of the head diameter against the neck diameter exceeding 1.1. A tall aspect ratio was defined as the ratio of the spine length against the head diameter exceeding 2.5. A wide head was defined as the head diameter exceeding 0.5 µm. Lastly, the cutoff between filopodium and thin spines were set at 3 µm.
in figure 2b were processed with IMARIS. Measurement data of each spine from the software were exported into a spreadsheet file. Using the spine length, head diameter, and neck diameter data on the spreadsheet file, each neuron was redrawn into three dimensional models on the graphic software Blender as seen in figure 2c and more closely in figure 2d. The measurement data were also used to classify each spine. Spines were classified using its spine length, head radius, and neck radius according to the algorithm seen in figure 4).

We are currently working on this novel method to overcome the shortcomings of previous methods to analyze spine morphology. Compared with the reported techniques using electron microscopy, this method allows for quick analysis of thousands of spines at once. This method opens the door for large scale analysis of spine morphology on multiple neurons. The approach developed in the present study allows rapid extraction of abnormalities in spine morphology in neurological diseases and will contribute to the further understanding of the pathogenesis.

Acknowledgements

This work was performed at the Institute for Diseases of Old Age under Juntendo University Graduate School of Medicine. I wish to thank Eri Arikawa-Hirasawa and Aurelien Kerever for their advice on the experimental design. I also would like to thank Fumihito Saitow and Hidenori Suzuki from Nippon Medical College for injecting the Biocytin in the pyramidal neurons on the sample slices.

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