Graduate School of Advanced Science and Engineering
Waseda University

博士論文概要

Doctoral Thesis Synopsis

論文題目

Thesis Theme

Optical detection of neuron connectivity by
random access two-photon microscopy

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Major in Department of Life Science and Medical Bioscience,
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December, 2015

A crucial step toward discovering brain function is observing the activity of local neural circuits and mechanisms which are ruling their communications. Although there exists a lot of knowledge about single neuron in both cellular and molecular views, it is not enough to disclose the mysteries of neuron circuits. Still there are many questions about neuron connectivity, direction of neuron connection, distribution and strength of connections, and hierarchy of information processing. To address these questions limitations should be overcome. Although electrical records from neurons are reliable in terms of temporal resolution, their poor spatial resolution has not been solved in addition to the lack of information about identity of recorded neurons. High spatial resolution measurement of intracellular Ca ion concentration ($[Ca^{2+}]_i$) has become popular for investigating activities of neuron population as a non-invasive optical approach. However, Ca monitoring is an indirect measure of action potentials, thus cannot accurately follow voltage transient of each action potential. And the slow kinetics of $[Ca^{2+}]_i$ hinders detection of direction of connections of each neuron pair. Membrane voltage tracer dyes fulfill the requirements in both spatial and temporal resolution, enabling to look into the complexity of neural circuits. A hybrid DiO/DPA voltage sensitive dye provides detection of membrane voltage change at sub millisecond resolutions with a high signal to noise ratio. In this study monitoring of fluorescence changes reflecting spontaneous firings in cultured hippocampal neurons was performed using this dye and an AOD driven random scanning two-photon microscope. The fast random scanning two-photon microscopy provided high spatiotemporal resolution with flexibility in point selection from multiple locations as well as advantages in reducing exposure light, scattering of laser light and unnecessary fluorescence emission. Reliability of the optical recordings was verified by parallel electrophysiological recordings and by evoking action potentials. Altogether, multi-point recordings were performed for longer than tens of minutes, which enabled determination of neuron connections and direction of connections. Time series of spikes of neural pairs were analyzed by means of a cross correlation method, revealing connectivity of pairs of neurons and direction of the connections of neurons.

This thesis is composed of five chapters with the following contents.

In the first chapter the background of this study and related studies are described in detail and the aim of this study is argued: In summary, many attempts have been made with electrophysiological approaches by means of multi-electrodes or microarrays for detection of neuron connectivity, which have enough temporal resolution and precision but are defective in spatial resolution. In addition extracellular recordings using multiple electrodes require spike sorting to identify neurons. Ca imaging overcomes these limitations in spatial information, but action potential detection by Ca imaging is an indirect measurement, which does not follow voltage transients accurately. Optical measurement of membrane potential with voltage sensitive dyes (VSDs), which is capable of sensing potential changes at sub millisecond order, is advantageous for direct recording of neuron population spiking and mapping neural connectivity patterns. Overview of VSDs are described in this chapter. A FRET-based VSD, DiO/DPA, is described in detail: DiO/DPA is comprised of DiO as a donor tracer and dipicrylamine (DPA) as a non-fluorescent mobile voltage-sensing acceptor which quenches the fluorescence emitted by the donor. This pair was selected in this study because of its high sensitivity, spatiotemporal resolution, and enough signal to noise ratio which is necessary to record high frequency action potentials in single trials.

Fast random scanning two-photon microscopy was used which provides high spatiotemporal resolution with flexibility in point selection and merit in reduced exposure light and unnecessary fluorescence emission. These features of

this microscopy were realized by adopting acousto-optic deflectors (AODs), which provide temporal resolution less than 100 μ s. The goal of this study was set to measure connectivity and direction of connections of neuron circuits.

In the second chapter materials and methods are described in detail, which are briefly summarized here: Methods of staining neuron networks with the DiO/DPA hybrid VSD was first established. The highly lipophilic nature of DiO made loading to neurons difficult. After testing several staining methods the gene gun labeling method was selected. DPA was introduced after staining with DiO with optimized concentration by bath application.

Another important aspect of this study was the adoption of an in-house made two-photon scanning microscope system. A femtosecond pulsed Ti:Sapphire laser was combined with two perpendicular AODs and an acousto-optic modulator (AOM) to conform random access scanning optics. Fluorescence was detected with two photomultiplier tubes operated in the photon-counting mode. This method enabled fluorescence detection from multiple locations in population of stained neurons at kilohertz order of frequency.

Primary culture of rat hippocampal neurons were used as a model neuron network. In order for neurons to keep firing spontaneously, 4-Aminopyridine (4AP) was added to the superfusate, which enabled stable recording of spike trains from multiple neurons for more than thirty minutes. For detection of action potential timing precisely a template matching method and Schmitt trigger method were tested. In the former method, all VSD recordings without noise filter were scanned with an action potential template which was obtained by averaging 46 action potentials recorded from a neuron with simultaneous electrophysiological recording. In the latter method optical traces were digitally filtered and peaks were detected with a pair of thresholds of fluorescence intensity with a threshold of duration.

In the third chapter results are described. In order to verify whether the system is capable of following action potentials, cultured neurons stained with DiO/DPA were patch-clamped and rectangular depolarization currents were applied to evoke action potentials. Simultaneous optical and electrophysiological recordings were performed. Reported timing of action potentials with the DiO/DPA DPA were in good accordance with results of electrophysiological recording.

DPA is known to distort/prevent action potential generation by increasing capacitive load of plasma membrane. Spiking responses of cultured neurons by exposure to stimulating solutions were inhibited by adding of DPA to the bath solution. The blocking effect of DPA was tested with various stimulating solutions (Picrotoxin, Mg^{2+} -free, high K^+ and 4AP), and time of blockade and recovery after wash of DPA were measured. 4AP (100 μ M) was chosen as a spike induction solution because of its longer effect under DPA and shorter latency for recovery.

Multi-point DiO/DPA optical recording was performed on cultured neurons stained with the DiO/DPA VSD. Action potential events were detected by the template matching method and the Schmitt trigger method. Time series of spike events were created for every neuron from the detected peak timing. Connectivity and direction of connections were investigated by calculating cross-correlation of the time series of action potentials between each neuron pair. The two peak detection methods resulted in similar sensitivity in the action potential detection and cross-correlation analysis. With the template matching method, 93 out of 151 analyzed neuron pairs from 27 experiments shared more than five spike

events each other. Out of the 93 neuron pairs 57 showed asymmetric cross-correlation, i.e., unidirectional connections. The rest of 36 neuron pairs may have their common presynaptic neurons.

Double staining of neurons with the DiO/DPA VSD and a red Ca indicator, Fura-red or Asante Calcium Red, was performed. These red Ca indicators did not interfere with the green fluorescence of the DiO/DPA VSD. Spike detection efficiencies were similar in many of the recordings, however in some recordings spikes detected with the VSD were

早稲田大学 博士（理学） 学位申請 研究業績書

(List of research achievements for application of doctorate (Dr. of Science), Waseda University)

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(As of February, 2016)

種 類 別 (By Type)	題名、 発表・発行掲載誌名、 発表・発行年月、 連名者(申請者含む) (theme, journal name, date & year of publication, name of authors inc. yourself)
Academic paper ▼	1. "Optical detection of neuron connectivity by random access two-photon microscopy", Nasrin Shafeghat, Morteza Heidarinejad, Noboru Murata, Hideki Nakamura, and Takafumi Inoue, Journal of Neuroscience Methods, in press
Academic paper	2. "Cyclin-dependent kinase 5 is required for normal cerebellar development", Ayumi Kumazawa, Naoki Mita, Motoyuki Hirasawa, Tomoko Adachi, Hiromi Suzuki, Nasrin Shafeghat, Ashok B. Kulkarni, Katsuhiko Mikoshiba, Takafumi Inoue, and Toshio Ohshima, Molecular and Cellular Neuroscience, 52:97-105, January 2013
Poster	3. "Habenula spike-triggered analysis of spike firing in hippocampal neurons", Nasrin Shafeghat, Hidenori Aizawa, Hitoshi Okamoto and Tomoki Fukai, Annual meeting of Society for Neuroscience, Chicago, 17-21 October 2015