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

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HIGHLIGHTS

- Voltage sensitive dye and a random-access two-photon microscope were combined to detect action potentials in multiple cultured neurons.
- Long-duration recording up to 100 min yielded enough number of spike events for analysis of synaptic connections.
- Cross-correlation analysis of neuron pairs clearly distinguished synaptically connected neuron pairs and the direction of connection.

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ABSTRACT

Background: Knowledge about the distribution, strength, and direction of synaptic connections within neuronal networks are crucial for understanding brain function. Electrophysiology using multiple electrodes provides a very high temporal resolution, but does not yield sufficient spatial information for resolving neuronal connection topology. Optical recording techniques using single-cell resolution have provided promise for providing spatial information. Although calcium imaging from hundreds of neurons has provided a novel view of the neural connections within the network, the kinetics of calcium responses are not fast enough to resolve each action potential event with high fidelity. Therefore, it is not possible to detect the direction of neuronal connections.
New method: We took advantage of the fast kinetics and large dynamic range of the DiO/DPA combination of voltage sensitive dye and the fast scan speed of a custom-made random-access two-photon microscope to resolve each action potential event from multiple neurons in culture.
Results: Long-duration recording up to 100 min from cultured hippocampal neurons yielded sufficient numbers of spike events for analyzing synaptic connections. Cross-correlation analysis of neuron pairs clearly distinguished synaptically connected neuron pairs with the connection direction.
Comparison with existing method: The long duration recording of action potentials with voltage-sensitive dye utilized in the present study is much longer than in previous studies. Simultaneous optical voltage and calcium measurements revealed that voltage-sensitive dye is able to detect firing events more reliably than calcium indicators.
Conclusions: This novel method reveals a new view of the functional structure of neuronal networks.

Abbreviations: 4AP, 4-aminopyridine; ACSF, artificial cerebrospinal fluid; AM, acetoxymethyl; AOD, acoust-optic deflector; Ca, calcium; $[Ca^{2+}]_i$, intracellular calcium concentration; DIV, days *in vitro*; DiO, dioctadecyl-3,3,3,3-tetramethylindocarbocyanine percholorate; DPA, dipicrylamine; DMSO, dimethyl sulfoxide; GEVI, genetically encoded voltage indicator; VSD, voltage sensitive dye.

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1. Introduction

Although many attempts have been made to determine the topology and direction of neuronal connections in neuronal networks by monitoring neuronal activities, satisfactory techniques have yet to be developed. There is a long history of electrophysiological techniques, which provide high temporal resolution with high fidelity. The multi-electrode array method allows for detection of activities of hundreds of neurons. However, it lacks the single-cell level resolution and capability for morphological identification of recorded neurons, which is why spike sorting is required for neuronal identification (Takahashi et al., 2003; Buzsáki, 2004; Vigneron and Chen, 2014). Conversely, optical methods provide high spatial resolution and identification of each neuron. Recording intracellular calcium ion (Ca^{2+}) concentration ($[Ca^{2+}]_i$) has become popular for investigating activities of neuron populations (Grienberger and Konnerth, 2012) and has revealed neuronal connection maps (Stetter et al., 2012). However, $[Ca^{2+}]_i$ monitoring is an indirect measure of action potentials and, thus, cannot accurately follow voltage changes in each action potential. Additionally, the slow kinetics of $[Ca^{2+}]_i$ hinders the detection of each neuron connection direction.

Optical measurements of membrane potential with voltage-sensitive dyes (VSDs) are capable of sensing potential changes at a sub-millisecond order, and are promising in terms of spatial and temporal resolutions. VSDs have successfully enabled the recording of action potential events from a population of neurons in a single trial, without averaging, in invertebrate (Grinvald et al., 1977; Wu et al., 1994) and vertebrate peripheral nervous systems (Neunlist et al., 1999; Obaid et al., 1999). However, single trial spontaneous action potential recordings in the central nervous system have only recently been reported in single neurons (Pagès et al., 2011) and multiple neurons (Yan et al., 2012) *via* newly developed VSDs and advanced optical devices. This can be attributed to the relatively poor signal-to-noise ratio of the majority of VSDs compared to Ca indicators, and also to the slow frame rate of laser scanning microscopes required for fluorescence imaging.

Random-scanning two-photon microscopy provides high temporal and spatial resolution with flexibility in point selection, reduced total exposure light, reduced scattering of laser light and the elimination of unnecessary fluorescence emission (Bullen and Saggau, 1999; Salome et al., 2006; Katona et al., 2012). The acousto-optic deflector (AOD) adopted in microscopy helps attain a temporal resolution >10 kHz. The AOD also allows access to any point of the field of view within microseconds. By adding a third AOD for *z*-axis adjustment, three dimensional random access is capable without reducing time resolution (Reddy and Saggau, 2005; Katona et al., 2012). Recording spontaneous action potentials from multiple neurons was first made possible using random-access two-photon microscopy with a new generation of VSDs (Yan et al., 2012). However, a key feature necessary for neuronal network determination is still missing, *i.e.*, long-duration recording. There has been no study showing stable action potential recording at single cell resolution for extended periods longer than 10 min using two-photon microscopy. No less than 10 min of neuron activity recording is necessary to determine the neuron connections and connectivity direction or for monitoring mode transitions in connections of neuron networks using pharmacological interventions. Because excitatory interactions between neurons in the central nervous system are generally weak (Mason et al., 1991; Deuchars et al., 1994; Markram et al., 1997; Thomson and Deuchars, 1997; Thomson et al., 2002), the accumulation of many spike events is needed (Schwindel et al., 2014).

In this study, the action potentials of multiple-cultured hippocampal neurons, a simple neuron network model system (Ivenshitz and Segal, 2010; Sporns, 2010), were recorded using the DiO/DPA VSD and an AOD-driven random scanning two-photon microscope. DiO/DPA is a VSD comprised of dioctadecyl-3,3,3,3-tetramethylindocarbocyanine percholorate (DiO) and dipicrylamine (DPA) with fast sub-millisecond kinetics and a large dynamic range (Chanda et al., 2005; Bradley et al., 2009; Fink et al., 2012). With this experimental setup, we succeeded in stably recording spike trains of multiple neurons for more than 30 min. Accumulated spike events enabled us to analyze neuron connections and the direction of connections between pairs of neurons in a robust manner.

2. Materials and methods

2.1. Experimental animal and neuronal cultures

Animal care was in accordance with guidelines outlined by the Institutional Animal Care and Use Committee of Waseda University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Waseda University (2011-A068). Throughout all experimental procedures, efforts were made to minimize the number of animals used and their suffering. The primary culture from Wistar rat hippocampi was prepared according to a standard method (Bannai et al., 2009). Primary cultured neurons at 9–24 days *in vitro* (DIV) were used.

2.2. Electrophysiology

Artificial cerebrospinal fluid (ACSF) consisting of (in mM) 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose bubbled with a mixture of 95% O_2 and 5% CO_2 was superfused throughout experiments. The temperature was maintained at 29–35°C. For patch-clamp recording glass pipettes of 5 MΩ tip resistance was pulled from borosilicate glass capillaries (Sutter Instrument, Navato, CA, USA), which were filled with an internal solution consisting of (in mM): 130 K gluconate, 5 KCl, 5 NaCl, 10 HEPES, 0.4 EGTA, 1 $MgCl_2$, 4 Na_2ATP, and 4 NaGTP (pH 7.25, 300 mOSm). Cells were identified using differential interference contrast (DIC) optics and a charge-coupled device camera (CFW-1612M, Scion Corp, Frederick, MD, USA). Patch-clamp recordings were performed using a Multiclamp 700B amplifier (Molecular Device, Sunnyvale, CA, USA). Analogue signals were filtered at 10 kHz using a built-in low-pass Bessel filter in the amplifier and fed into an interface device (ITC-18, HEKA Elektronik, Lambrecht, Germany). The membrane potential was maintained at −60 mV in either the voltage- or current-clamp mode.

2.3. Optical configuration

A custom-made two-photon scanning microscope was used to monitor fluorescence of VSD and calcium indicators equipped with a femtosecond pulsed Ti:Sapphire laser (λ = 930 nm, average output = 500–700 mW, Tsunami, Spectra Physics Japan, Tokyo, Japan) pumped by a green laser (λ = 532 nm, 7.5 mW, Millenia, Spectra Physics Japan). An upright microscope (BX51WI, Olympus, Tokyo, Japan) was used in combination with laser optics. A 20× water immersion lens (numerical aperture = 1.0, XLUMPlan FL, Olympus) was used. Laser spot radius was 0.35 μm on the target using this objective lens. In-house TI Workbench software, written by T.I. and running on a Mac computer (Mac Pro, Apple, Cupertino, CA, USA), controlled all devices and recorded electrophysiological and optical data.

We adopted the optical design of the AOD-driven scanning system developed by the L. Bourdieu group (Salome et al., 2006). For the overall settings, refer to Fig. 1 of Salome et al. (2006) with the following details. The laser beam was deflected by two orthogonal AODs (DTSXY-400-850.950, AA Opto-Electronic, Orsay,