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NIPKOW CONFOCAL IMAGING FROM DEEP BRAIN TISSUES

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One of the problems in imaging from brain tissues is light-scattering. Thus, multiphoton laser scanning microscopy is widely used to optically access fluorescent signals located deeply in tissues. Here we report that Nipkow-type spinning-disk one-photon confocal microscopy, which embodies high temporal resolution and slow photobleaching, is also capable of imaging tissues to a depth of up to 150 μm. Using a Nipkow-disk microscope, we conducted functional multi-cell calcium imaging (fMCI) of CA3 neurons from *in toto* intact hippocampal preparations and astrocytes from *in vivo* neocortical layer 1. This novel application of Nipkow-disk microscopy expands the potential usefulness of this type of microscopy and will contribute to our understanding of natural neuronal microcircuitry.

Keywords: Neocortex; neuron; optical imaging; single cell resolution; fluo-4.

1. Introduction

Experiments using intact brain tissues, such as *in vivo* and *en bloc* brain preparations, rather than sliced or cultured preparations, are important for understanding "more natural" brain functions. In widely used conventional confocal microscopy, however, the optically accessible depth is limited to only tens of micrometers below the preparation surface of tissues [16]. Thus, two-photon laser scanning microscopy is currently the first choice to access optical signals arising from deep tissues, because it can visualize neurons and glial cells even at depths of 500 μm [3, 13], and recently up to 900 μm [8]. Despite its high optical penetration, the usefulness is often dampened by its low time resolution, with frame rates typically ranging from 0.5 to 30 frames/second (fps). Therefore, if two-photon microscopy is used for functional multicell calcium imaging (fMCI), an optical technique that reconstructs action

potentials of multiple neurons through their somatic calcium dynamics, this slow image acquisition cannot resolve the temporal profiles of action potentials with millisecond precision.

Frame rates can be improved by Nipkow-type spinning-disk confocal microscopy, which has a time resolution of up to 2000 fps and can determine the timings of individual action potentials with high temporal precision [14]. Interestingly, we noticed, during our experiments using Nipkow-disk confocal microscopy, that this type of microscopy is able to obtain images at a depth of up to 150 μm. In this work, we compared the optical accessibility of Nipkow-disk microscopy with that of two-photon and conventional confocal microscopy. We then conducted high-speed fMCI in intact hippocampal preparations and detected action potential-dependent signals of CA3 neurons. Moreover, we applied this technique to *in vivo* imaging of neocortical layer I in anesthetized mice. The results showed that Nipkow-disk microscopy is applicable to wilder purposes than previously thought and could contribute greatly to functional neuroimaging.

2. Materials and Methods

Experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number, 19-41 and A21-6) and according to the University of Tokyo guidelines for the care and use of laboratory animals.

2.1. *Imaging apparatus*

Images were obtained with three methods; (i) Nipkow-disk confocal microscopy (CSU-X1, Yokogawa Electric, Tokyo, Japan) with a cooled CCD camera (iXonEM$^+$ DV897, Andor Technology, Belfast, UK), regulated by Solis software (Andor); (ii) conventional galvano-mirror-type confocal laser-scanning microscopy (FV1000, Olympus, Tokyo, Japan) with a photomultiplier tube detector (r7862, Hamamatsu Photonics, Shizuoka, Japan) regulated by FV10-ASW Version 1.7 (Olympus); (iii) two-photon laser-scanning microscopy (FV1000, Olympus) with a photomultiplier tube detector (r7862, Hamamatsu Photonics) regulated by FV10-ASW Version 1.7 (Olympus). All images were collected through a water-immersion objective lens (20×0.95 numerical aperture, Olympus) equipped with an upright microscope (BX61WI, Olympus). For one-photon imaging, fluorophores were excited at 488 nm with a laser diode (HPU50101PFS, FITEL, Tokyo, Japan) and visualized with a 507 nm long-pass emission filter. For two-photon imaging, fluorophores were excited at 840 nm with a mode-locked Ti:sapphire laser (a 100-fs pulse width, 80 MHz pulse frequency, Maitai, Spectra Physics, Mountain View, CA) and visualized with a 507 nm long-pass emission filter after blockade of the excitation light scattering with a 685 nm short-pass barrier filter.

2.2. *Assessment of optical accessibility in depth*

The morphology of neurons and neurites was visualized in brain slices prepared from mice expressing membrane-targeted green fluorescent protein (GFP) (line 21, thy1-GFP transgenic C57B6 mice), a gift from Dr. V. de Paola and Dr. P. Caroni [1]. Postnatal four-week-old mice (SLC, Shizuoka, Japan) were anesthetized with ether and decapitated. The brain was immersed in ice-cold sucrose-based artificial cerebrospinal fluid (aCSF) consisting of (in mM): 27 $NaHCO_3$, 1.4 NaH_2PO_4, 2.5 KCl, 7.0 $MgSO_4$, 1.0 $CaCl_2$, 222 sucrose, 0.5 ascorbic acid, bubbled with 95% O_2 and 5% CO_2. Horizontal entorhino-hippocampal slices of 400 μm thickness were cut using a vibratome (Vibratome 3000; Vibratome, St. Louis, MO) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution for 2 hours. The dentate gyrus was 3D-imaged at a Z-depth interval of 10 μm.

2.3. *Cerebrovascular imaging from the neocortex*

Male ICR mice (3 weeks old) were anesthetized with urethane (1.5 g/kg, i.p.) and fixed to a stereotaxic frame. To visualize blood vessels, 30–250 μl of 1% fluorescein isothiocyanate-labeled dextran (70 kDa) was intravenously injected into the tail vein to label the blood serum [6]. After decapitation, the brain was removed and immersed into physiological saline. The blood vessel was observed from the outside of the brain tissue using Nipkow-disk confocal.

2.4. *Calcium imaging from in toto hippocampus preparations*

fMCI from *in toto* hippocampus preparations was conducted using postnatal 9-to-13-day-old ICR mice. The mice were anesthetized with ether and decapitated. The brains were quickly removed and immersed into ice-cold sucrose-based aCSF consisting of (in mM): 27 $NaHCO_3$, 1.4 NaH_2PO_4, 2.5 KCl, 7.0 $MgSO_4$, 1.0 $CaCl_2$, 222 sucrose, and 0.5 ascorbic acid, bubbled with 95% O_2 and 5% CO_2. The whole hippocampal formation was surgically isolated from the surrounding structures, transferred in oxygenated aCSF consisting of (in mM) 126 NaCl, 26 $NaHCO_3$, 3.5 KCl, 1.24 NaH_2PO_4, 1.2 $CaCl_2$, and 10 glucose, and was maintained at room temperature for at least 30 mins. A hippocampal tissue was placed to a submerged imaging chamber (perfused at a rate of 10–15 ml/min with oxygenated aCSF at 30–32°C) and fixed to on a silicon bottom with 4–6 thin entomological needles so that the CA3 region surface became the upside of the preparation [5]. A micropipette (6–9 MΩ) filled with a dye solution was inserted into CA3 stratum pyramidale (50–100 μm below the surface) under the microscope control. The dye solution consisted of 1-mM Oregon Green 488 BAPTA-1AM (OGB1, Invitrogen, Eugene, OR), 100-μM sulforhodamine 101 (SR101, Invitrogen), 20% DMSO, and 20% Powerload^{TM} (Invitrogen), diluted in 150-mM NaCl, 2.5-mM KCl and 10-mM HEPES. A positive-pressure pulse (1–2 min, 0.03–0.3 bar) was applied through the pipette tip hole to eject the

dye solution. Neuronal activity was captured at 100–200 fps with Nipkow-disk confocal microscopy.

2.5. *In vivo calcium imaging of astrocytes*

For *in vivo* imaging, postnatal 9-to-21-day-old ICR mice were anesthetized with urethane (1.5 g/kg, i.p.), and the exposed skull was glued to a metal plate fixed to a stereotaxic frame. A craniotomy (1–2 mm diameter), centered at 0.5 mm posterior and 2.5 mm lateral to the lambda suture, was performed, and the dura was surgically removed. The surface of the brain was mounted with 2.0% agar. The exposed neocortical surface was treated with 2 μl of a dye solution containing 0.125% fluo-4AM (Invitrogen), 100-μM SR101, 20% DMSO, and 8% Pluronic F-127. During an incubation period of 45–60 min, the aspirated surface was covered with Gel Form (Pfizer Inc., Groton, CT). Using this method, the fluo-4-loaded cells were almost exclusively SR101-positive astrocytes [9]. After the unloaded dye was washed away with aCSF, the targeted area was gently mounted with 2% agar dissolved in aCSF and covered with a coverslip. Astrocytic calcium activity was captured at 1 fps using Nipkow-disk confocal microscopy, because the astrocytic activity kinetics is slow; the activity duration is typically 10–40 s [7, 10, 11].

3. Results

3.1. *Comparison of deep tissue imaging*

We assessed the optically accessible depth of Nipkow-disk confocal microscopy, two-photon microscopy, and conventional confocal microscopy, by comparing the fluorescence images of GFP-positive neurons in the dentate gyrus of acute hippocampal slices prepared from thy1-GFP mice (Fig. 1). The laser powers were fixed at 5 mW in all the microscope systems. The light permeation was the highest in two-photon microscopy, which was able to capture the GFP signal located at more than 200 μm below the preparation surface. On the other hand, confocal microscopy could image the GFP signal only within 80 μm in depth. Interestingly, Nipkow-disk confocal microscopy obtained the GFP fluorescence at more than 150 μm; although the contrast of the obtained images was not very sharp due to light scattering, they still maintained the single-cell resolution. The reason for this unexpectedly high optical accessibility is unclear. The speed of Nipkow-disk spinning did not significantly affect this optical permeability (Fig. 2). We took advantage of this competence and tried to utilize Nipkow-disk confocal microscopy for live calcium imaging from neurons and astrocytes located in relatively deep tissues of *in vitro* and *in vivo* preparations.

3.2. *In vitro Nipkow-disk confocal fMCI*

As to *in vitro* preparations, we chose the intact hippocampal formation [5]. The "*in toto*" preparations preserve intrinsic neuronal networks, providing unique experimental conditions that are appropriate to investigate more physiological patterns

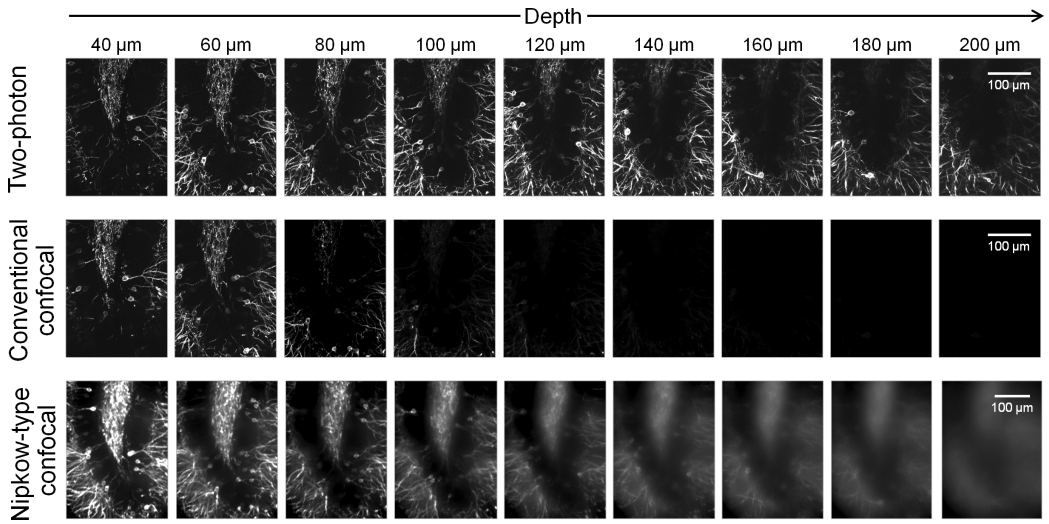
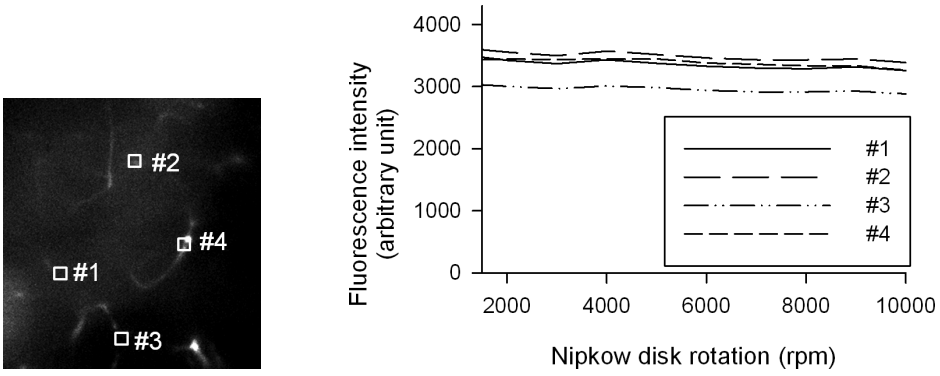


Fig. 1. Nipkow-disk confocal microscopy can image deeper tissues than commonly used conventional confocal microscopy. Images in different tissue depths were collected from of the dentate gyrus in the same hippocampal slice prepared from a thy1-GFP mouse and compared between Nipkow confocal microscopy (top), two-photon microscopy (middle), and conventional confocal microscopy (bottom). The numbers above images indicate the depths from the surface of the slice preparation.



(a) (b)

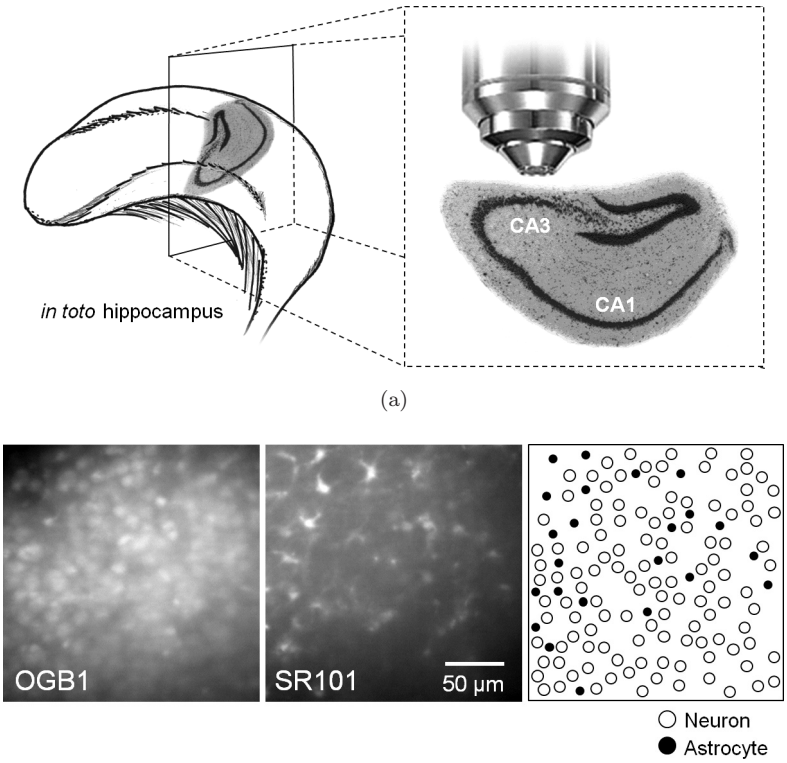
Fig. 2. No influence of the speed of Nipkow-disk spinning on deep imaging. (a) Blood vessels of the *en bloc* mouse cerebral cortex 100 μm below the brain surface. (b) Fluorescence intensity measured from four randomly selected regions of interest (ROI #1–4 in the panel a) was plotted against the disk rotation speed.

of the network activity, as compared to slice preparations in which axons and dendrites are severely cut during slicing. Unlike acute hippocampal slices, however, neurons in the *in toto* hippocampus are located deeply in the parenchyma and thus optically inaccessible from the outside of the preparations by conventional confocal microscopy. Fortunately, neurons in the CA3b region are located 80–200 μm below the ventral surface of the mouse hippocampus. Thus, we targeted these CA3 neurons

with Nipkow-disk confocal microscopy. The hippocampal formation was dissected from the mouse brain, and the fluorescent calcium indicator OGB1-AM was pressure-injected through a glass pipette into CA3 stratum pyramidale (Fig. 3(a)). SR101, a selective astrocyte marker [9], were simultaneously injected to discriminate neurons and astrocytes. Nipkow-disk confocal microscopy readily observed OGB1-loaded cells in CA3 stratum pyramidale (Fig. 3(b)). We thus conducted time-lapse calcium imaging from neuron populations at 100–200 fps and found that these neurons showed spontaneous spike activity (Figs. 3(c) and 3(d)).

3.3. *In vivo Nipkow-disk confocal fMCI*

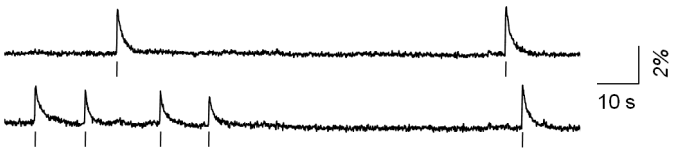
Next we sought to apply this technique to *in vivo* imaging (Fig. 4(a)). Mice were anesthetized with urethane and craniotomized above the primary visual cortex.



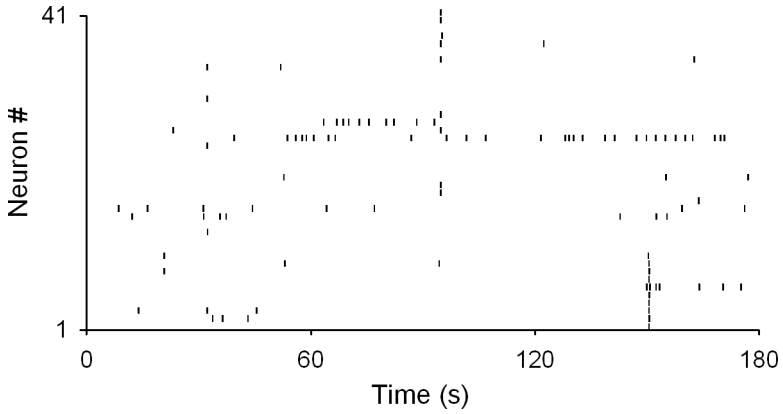
(a)

(b)

Fig. 3. *In vitro* Nipkow-disk confocal fMCI from CA3 neurons in *in toto* hippocampal preparations. (a) Schematic illustration of an *in toto* hippocampal preparation. Neurons located in CA3 stratum pyramidale was optically accessed through stratum oriens from the outside of the preparation. (b) Cell location was identified for neurons (white circles) and astrocytes (black circles), based on OGB-1 and SR101 double-stained images. (c) Two representative raw traces of the fluorescence intensity in neuronal somata. Vertical lines below each trace represent putative times of spikes emitted by these neurons. (d) Temporal patterns of the activities of 41 CA3 neurons simultaneously monitored from a hippocampal preparation. Each dot indicates the timing of a single activity of the corresponding neurons. Similar results were obtained for all 15 preparations tested.



(c)



(d)

Fig. 3. (*Continued*)

Fluo-4 was loaded into astrocytes in layer 1 by treating the cortical surface with fluo-4AM. We found that Nipkow-disk confocal microscopy could observe layer 1 astrocytes 80–100 μm below the pia (Fig. 4(b)). These astrocytes spontaneously exhibited calcium activities. The signal-to-noise ratio was sufficiently high (Fig. 4(c)) so that the time of individual activity could be determined (Fig. 4(d)).

4. Discussion

Biological tissues are highly light-scattering. In the brain, the light impermeability is mainly caused by myelin [2]. Therefore, infrared light is used to reach cells in deep tissues. The present study showed, however, that Nipkow-disk confocal microscopy is capable of accessing deeper tissues *in vitro* and *in vivo* than conventional confocal microscopy. Although images obtained by Nipkow were much more "blurred" than those by two-photon microscopy, the quality is still enough for fMCI. Thus, Nipkow has an advantage, in particular, for recording rapid biological events such as neuronal firing. In the scope of optics, this phenomenon is unexpected, even for company persons who designed or produced the Nipkow-disk confocal unit (personal communications in Yokogawa Electric). No reason or theoretical prediction has firmly explained this phenomenon so far.

Nipkow-disk confocal microscopy has several advantages over conventional confocal microscopy, i.e., higher speed imaging, slow photobleaching, and lower

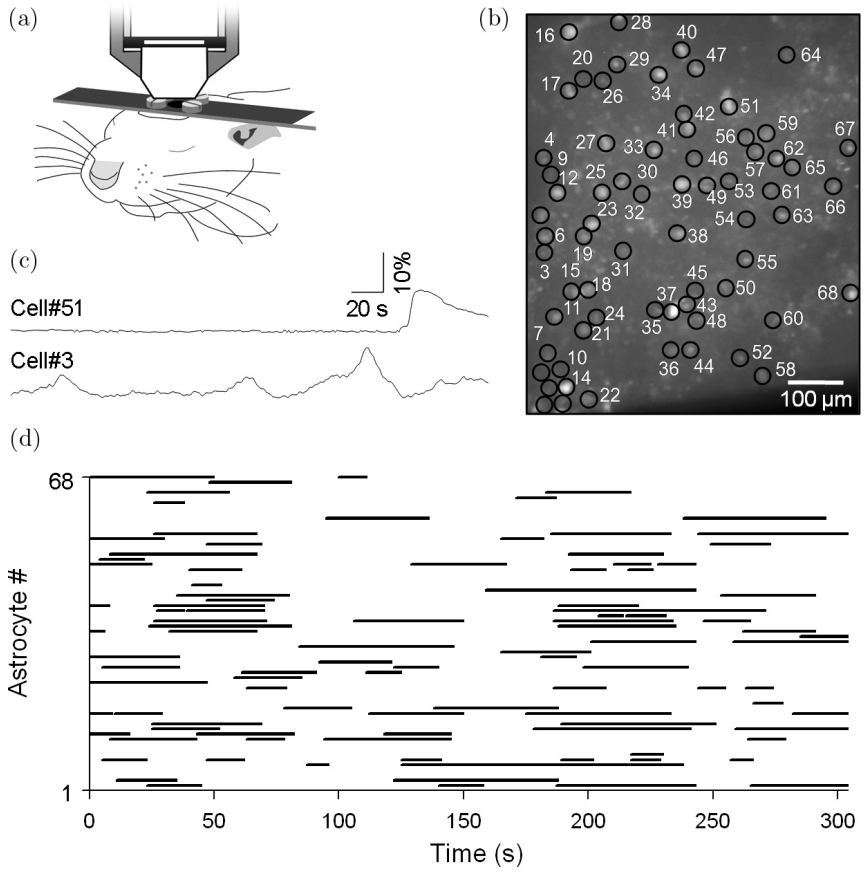


Fig. 4. *In vivo* Nipkow-disk confocal fMCI from astrocytes from primary visual cortex layer 1 of anesthetized mice. (a) The head of a urethane-anesthetized mouse were fixed to a stereotaxic frame, and fluo-4-loaded astrocytes were imaged *en masse* from neocortical layer 1. (b) Representative image of fluo-4-loaded tissues, including 68 astrocytes. (c) Two raw traces of the fluorescence intensity. These traces show slow calcium events typical of astrocytic activity dynamics. (d) Spatiotemporal patterns of calcium activities exhibited by 68 neocortical astrocytes. Each bar in the rastergram indicates the period during which the fluorescence increased above 3 × SDs of its baseline fluctuation. Similar results were obtained for all 7 mice tested.

phototoxicity [12]. These features are suitable, in particular, to fMCI, because the dynamics of action potentials are fast and also because neurons are highly vulnerable to damage. Nipkow-disk confocal fMCI is increasingly applied to monitor the population activity of neurons [4, 12, 14]. Although multiple unit recordings provide another opportunity to simultaneously record tens of neurons, the merits of fMCI include (i) no need for spike sorting algorithms (thus, no contamination of mathematical artifacts), (ii) simultaneous recordings from up to 10,000 neurons in a local circuit, (iii) identifiable locations and types of neurons (even neurons that are inactive during the recording period), and (iv) recording from electrically silent, but biologically active, cells, such as astrocytes [15]. With this respect, the present work

has advanced the potential usefulness of Nipkow-disk confocal fMCI in the field of neuroscience.

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