Journal of Innovative Optical Health Sciences
Vol. 2, No. 1 (2009) 67–71

World Scientific
www.worldscientific.com

OBSERVING NEURONAL ACTIVITIES WITH RANDOM ACCESS TWO-PHOTON MICROSCOPE

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As a second messenger in signal transduction, calcium ion plays a very important role in neuronal information processing and integrating. Limited by the imaging technique, it is difficult to simultaneously perform deep tissue imaging and measure intracellular free calcium concentration ($[Ca^{2+}]_i$) in different compartments of neurons in brain slice noncollinearly. By means of random access two-photon microscopy, which provides high optical penetration into tissues and low photo damage, we successfully measured $[Ca^{2+}]_i$ of different parts of pyramidal neurons in neocortical layer V in rat brain slices with high spatial and temporal resolution. Combining the patch clamp technique, we stimulated the soma with depolarizing current and explored the dynamics of calcium in pyramidal neurons.

Keywords: Neuronal activity; two-photon microscopy; imaging.

1. Introduction

The nervous system uses ensembles of neurons to process and encode information. In the past years, extensive studies have been carried out on the soma and dendrite information integration, but how the information is processed and integrated is still undetermined.[1–4] While confocal and two-photon microscopes have been widely used in functional neuron imaging research, it has been found that the voltage-dependent characteristics in dendrite membranes may enhance the computational power of neurons.[5,6] Although confocal and two-photon microscopes provide good spatial resolution to identify the fine structure of neurons, such as dendrites and spines, its temporal resolution is not satisfactory in many functional imaging experiments. To better understand the mechanism of neuronal information integration, monitoring the activity of a single neuron by electrophysiology is essential, but not sufficient. As $[Ca^{2+}]_i$ is highly localized in neurons, it is comparably important to simultaneously monitor many parts of soma or dendrite interested to investigate some fast signaling. Besides, low signal-to-noise ratio (SNR) hampered us in deep tissue or *in vivo* imaging.[7–10] In order to improve imaging capabilities, a random access two-photon microscope was used to improve the spatial–temporal resolution and reduce the phototoxic and photo-bleaching; it will be much easier to repeat experiments and carry out long-time fluorescence recording.[11,12]

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2. Materials and Methods

2.1. *Slice preparation*

Acute neocortical slices were prepared as described.[13] In brief, Wistar rats at 10 to 15 days of post-natal age were anesthetized with sodium pentobarbital (50 mg/kg). After decapitation, the brain was dissected rapidly, fixed with glue to the stage of a vibratome (VTS1000, Leica), and submerged in ice-cold oxygenated (95% O_2 and 5% CO_2) ACSF (artificial cerebrospinal fluid) containing (in mmol/L): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose. Transverse slices (300–400 μm) were cut with a vibratome and stored in an incubation chamber for at least 1 hour at room temperature (22–25°C) before recording. During experiments, the individual slice was transferred to a submersion-recording chamber and perfused continuously with the above-oxygenated solution (1–3.0 mL/min) at room temperature. Slices were visualized with infrared optics using an Olympus microscope (BX61WI, Olympus) equipped with Normaski differential interference contrast optics and IR-CCD camera (IR1000, DTI).

2.2. *Electrophysiology*

Whole-cell patch recordings were made from neurons in cortex.[14,15] Recording pipettes were pulled from borosilicate capillaries (Sutter) using a Brown-Flaming micropipette puller (P-97, Sutter). The recording pipettes were routinely filled with a solution containing (in mmol/L): 125 K-gluconate, 15 KCl, 10 Hepes, 4 $MgCl_2$, 3 Mg-ATP, 0.3 Na-GTP, 10 Na_2-phosphocreatine, and 0.2 EGTA (pH 7.3 with KOH, 290–300 mOsm). The pipette resistance was 3–5 MΩ. The seal resistance formed with the soma membrane was $>$ 1 GV before the break-in into the whole-cell configuration. A Heka amplifier (EPC-9, HEKA) was used to record somatic membrane potential and inject current through the recording pipette. Stable recordings could last for 1–2 hours. Signals were sampled at 10 kHz. As usual, 100 μmol/L Fura-2 calcium dye was added to intracellular solution for simultaneous calcium imaging.

2.3. *Random access two-photon microscope*

The design of random access two-photon microscope was described previously.[16] A mode-locked laser is used (Mai Tai B B, Spectra-Physics). An electro-optic modulator is applied to adjust the laser power. When laser beam travels through acousto-optic defectors (AODs) (TS-XY, A-A), the acousto-optic effect takes place and forms an acoustic grating, then the laser is diffracted into a microscope (BX 61, Olympus). The fluorescence signal is recorded by a photomultiplier tube (PMT, 3896, Hamamatsu).

2.4. *Data analysis*

The random access two-photon images were analyzed by software written by MATLAB. The first 100 ms of the fluorescence before a stimulus were averaged to obtain the basal fluorescence, F_0. A region distant to any indicator-containing structure was chosen for subtraction of background fluorescence, F_B. The relative fluorescence changes were calculated as

$$\Delta F/F = (F - F_0)/(F_0 - F_B). \quad (1)$$

The decay time constant (τ) was obtained by fitting $\Delta F/F$ with a single exponential by using a least-squares-fit routine (IGOR, Wave Metrics).

3. Results

To demonstrate the structural and functional imaging properties of the random access two-photon microscope, we loaded pyramidal neurons in neocortical slices with calcium probe Fura-2, using a dye-filled micropipette. In whole-cell current recording, we injected 10 ms, 1 nA current at 1 Hz or 5 Hz to depolarize the neuron, and each injection elicited an action potential that propagated throughout the soma and dendrites. With the depolarization of the membrane, the voltage-dependent calcium channels opened, and the calcium ions entered the neuron, and bound to fluophores.

Simultaneously-recorded fluorescence signals from different compartments of neuron were shown in Fig. 1. Stable elicited fluorescence fluctuations and the corresponding action potentials can be easily identified at high SNR ($\Delta F/F_{\max} = 50\%$).

Previous experiments about modulation of calcium propagation between soma and dendrites were mainly done by the line-scan mode of microscope with low temporal resolution; besides, all measuring points must be coaxial; it limited the research on some fast signaling events. In comparison to line-scan mode, random access two-photon mode has

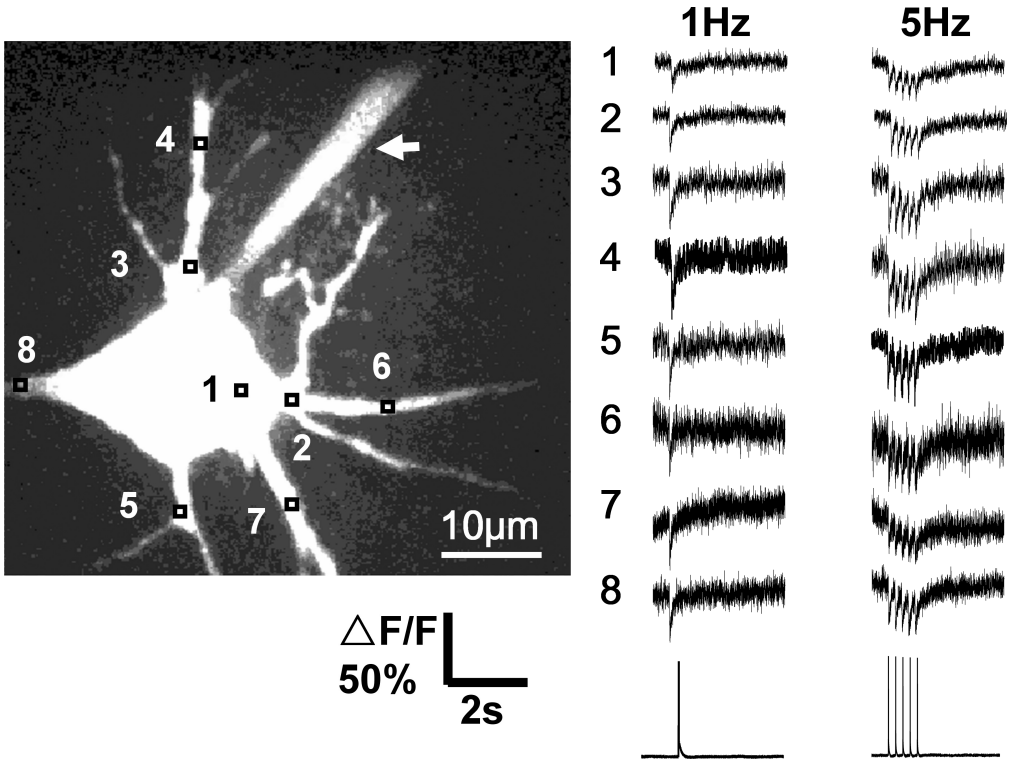
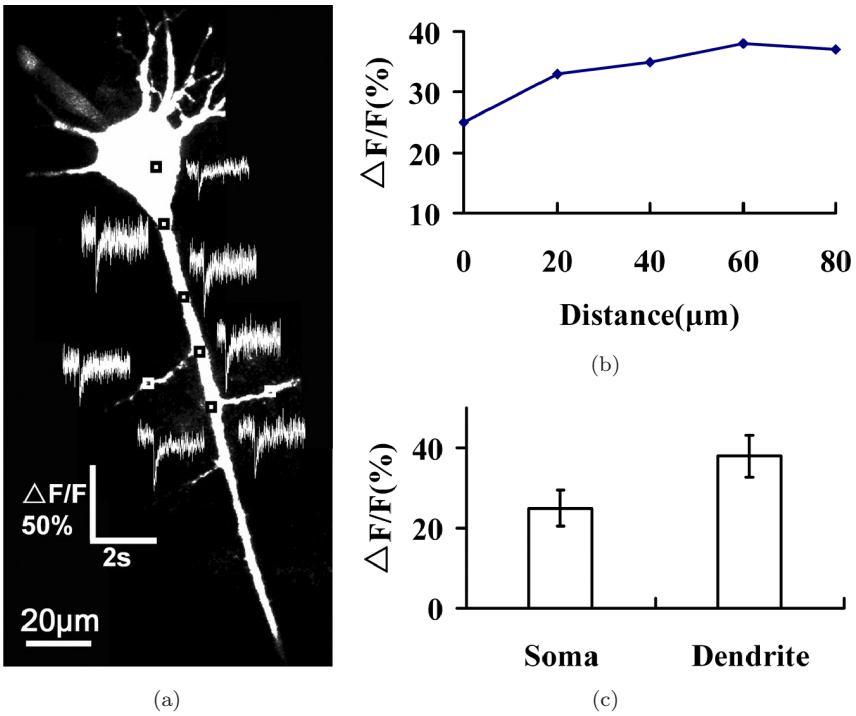


Fig. 1. Simultaneous monitoring of calcium changes in eight parts of a pyramidal neuron. (a) Whole-cell recording with a pyramidal neuron, the micropipette was filled with 100 μM Fura-2. The white arrow indicated the micropipette. (b) Fluorescence changes of different ROIs and APs (action potenial). Ten millisecond, 1 nA current was injected at 1 Hz and 5 Hz to depolarize the neuron and elicit action potentials.

the advantage of higher spatial–temporal resolution. Recent studies showed that most Ca^{2+} waves stopped near the soma–dendrite border.[17–22] There are two hypotheses of this phenomenon, mainly concluding about the IP_3 receptor and density, but the exact mechanism is still unclear.[23–25] In our study, we repeated the experiments and found significant attenuation near the soma–dendrite border. Furthermore, we discovered another interesting phenomenon: $[Ca^{2+}]_i$ increased significantly as calcium fluctuations propagate from soma to dendrite. When detecting a specific type of stimulation, the dendrites evoke $[Ca^{2+}]_i$ to increase and transmit the message to soma. In fact, a neuron has a wide range of dendrite branching; it regularly receives information from the dendrites. Thus it should have the abilities of processing and integrating the information; otherwise, it would be easily saturated. When calcium waves propagate from dendrite to soma, $[Ca^{2+}]_i$ attenuates notably, so a neuron is not easy to saturate and is able to integrate all the excitory and inhibitory information. In our study, when calcium waves propagate from soma to dendrite, we observed that the $[Ca^{2+}]_i$ increase in the dendrites (Fig. 2).

4. Discussion

In this study, we simultaneously recorded calcium signals from different parts of the pyramidal neurons in neocortical layer V in rat brain slices by the random access two-photon microscope. The results showed that the fluorescence of $[Ca^{2+}]_i$ increased significantly while calcium changes propagated from soma to dendrites. Previous work has proved that most Ca^{2+} waves stopped near the soma–dendrite border, so it is inferred that there was a kind of direction-dependent transmission mechanism at the initial part of the dendrite to perform the information processing and integrating course. When calcium fluctuations from large amount of branching dendrites gathered in soma, in case no protective information processing takes place, the soma is easy to saturate and make no response to other inputs.



(a) (b) (c)

Fig. 2. Simultaneous measuring $[Ca^{2+}]_i$ from soma and dendrite. (a) Neuron was loaded with Fura-2 using a micropipette. The $[Ca^{2+}]_i$ changes were recorded at five ROIs in a pyramidal neuron in response to 1 nA, 10 ms current injection. (b) $\triangle F/F$ (%) changes at different distances in (a). (c) Summary of results for 21 neurons of $\Delta F/F$ in soma and dendrite.

On the other hand, while calcium changes propagate from soma to dendrites, the signal is amplified to ensure the signal transmit to the distant termini in good condition. The recording of $[Ca^{2+}]_i$ in different compartments of neurons presented in this study may be helpful for the research of neuronal information integration and neuroscience computing. Surely, future research is necessary to explore its biochemical mechanism. The elaborate work of Sakmann also confirmed our experiments,[26] calcium fluctuations of soma and dendrite were carefully monitored by a slow-scan CCD camera in his experiment, however the mechanism of neuronal information integration is not considered.

We demonstrated a random access two-photon technique which is useful for simultaneous recording of different compartments of neurons in brain slices at high spatial and temporal resolution through functional neuronal imaging. For a one-photon confocal microscope, deep tissue imaging is impossible because of the opacity and scattering of the tissue, while these obstacles can be overcome by the random access two-photon microscope using a femtosecond pulse laser. Furthermore, the random scanning mode performs higher SNR than traditional line-scan mode, it has no coaxial requirement, but has a higher spatial resolution. In addition, due to its low phototoxic and photo-bleaching, it is especially convenient for long-time recording. Besides, it is convenient to combine this method with other techniques for more favorable usages. For example, combined with patch clamp technique, we can load specific structures and measure electrophysiological parameters, which is more persuasive and reliable[27−29]; combining the calcium probe of higher dissociation constant, it is probable to measure faster signaling. The work of random access two-photon microscope was also reported by Saggau;[30] nevertheless, the temporal dispersion is not compensated, as a result of lower two-photon fluorescence efficiency. In the future, we believe that random access two-photon technique presented in this paper may have a wide range of applications in life sciences.

References

1. Chance, F. S., Abbott, L. F. and Reyes, A. D., "Gain modulation from background synaptic input," *Neuron* **35**, 773–782 (2002).
2. Augustine, G. J., Santamaria, F. and Tanaka, K., "Local calcium signaling in neurons," *Neuron* **40**, 331–346 (2003).

3. Gabbiani, F., Krapp, H. G., Koch, C. and Laurent, G., "Multiplicative computation in a visual neuron sensitive to looming," *Nature* **420**, 320–324 (2002).
4. Yuste, R. and Tank, D. W., "Dendritic integration in mammalian neurons, a century after Cajal," *Neuron* **16**, 701–716 (1996).
5. Yuste, R. and Denk, W., "Dendritic spines as basic functional units of neuronal integration," *Nature* **375**, 682–684 (1995).
6. Seamans, J. K., Gorelova, N. A. and Yang, C. R., "Contributions of voltage-gated Ca^{2+} channels in the proximal versus distal dendrites to synaptic integration in prefrontal cortical neurons," *J. Neurosci.* **17**, 5936–5948 (1997).
7. Pawley, J. B., *Handbook of Biological Confocal Microscopy* (Plenum Press, New York, 1995).
8. Fan, G. Y., Fujisaki, H., Miyawaki, A., Tsay, R. K., Tsien, R. Y. and Ellisman, M. H., "Video-rate scanning two-photon excitation fluorescence microscopy and ratio imaging with cameleons," *Biophys. J.* **76**, 2412–2420 (1999).
9. Bewersdorf, J., Pick, R. and Hell, S. W., "Multifocal multiphoton microscopy," *Opt. Lett.* **23**, 655–657 (1998).
10. Kurtz, R., Fricke, M., Kalb, J., Tinnefeld, P. and Sauer, M., "Application of multiline two-photon microscopy to functional *in vivo* imaging," *J. Neurosci. Methods* **151**, 276–286 (2006).
11. Iyer, V., Losavio, B. E. and Saggau, P., "Compensation of spatial and temporal dispersion for acousto-optic multiphoton laserscanning microscopy," *J. Biomed. Opt.* **8**, 460–471 (2003).
12. Iyer, V., Hoogland, T. M. and Saggau, P., "Fast functional imaging of single neurons using random-access multiphoton (RAMP) microscopy," *J. Neurophysiol.* **95**, 535–545 (2006).
13. Gähwiler, B. H., Capogna, M. and Debanne, D., Organotypic slice cultures: a technique has come of age," *Trends Neurosci.* **20**(10), 471–477 (1997).
14. Larkum, M. E., Kaiser, K. M. M. and Sakmann, B., "Calcium electrogenesis in distal apical dendrites of layer 5 pyramidal cells at a critical frequency of back-propagating action potentials," *Proc. Natl. Acad. Sci.* **96**, 14600–14604 (1998).
15. Markram, H., Helm, P. J. and Sakmann, B., "Dendritic calcium transients evoked by single back-propagating action potentials in neocortical pyramidal neurons," *J. Physiol.* **485**, 1–20 (1995).
16. Lv, X. H., Zhan, C., Zeng, S. Q., Chen, W. R. and Luo, Q. M., "Construction of multiphoton laser scanning microscope based on dual-axis acousto-optic deflector," *Rev. Sci. Instrum.* **77**, 046101-3 (2006).
17. Watanabe, S., Hong, M., Ross, N. L. and Ross, W. N., "Modulation of calcium wave propagation in the dendrites and to the soma of rat hippocampal pyramidal neurons," *J. Physiol.* **575**, 455–468 (2006).
18. Koester, H. J. and Sakmann, B., "Calcium dynamics in single spines during coincident pre- and post-synaptic activity depend on relative timing of back-propagating action potentials and subthreshold excitatory postsynaptic potentials," *Proc. Natl. Acad. Sci.* **95**, 9596–9601 (1998).
19. Oakley, J. C., Schwindt, P. C. and Crill, W. E., "Dendritic calcium spikes in layer 5 pyramidal neurons amplify and limit transmission of ligand-gated dendritic current to soma," *J. Neurophysiol.* **86**, 514–527 (2001).
20. Larkum, M. E., Senn, W. and Lüscher, H. R., "Top-down dendritic input increases the gain of layer 5 pyramidal neurons," *Cerebral Cortex* **14**, 1059–1070 (2004).
21. Oviedo, H. and Reyes, A. D., "Variation of input–output properties along the somatodendritic axis of pyramidal neurons," *J. Neurosci.* **25**, 4985–4995 (2005).
22. Schiller, J., Schiller, Y., Stuart, G. J. and Sakmann, B., "Calcium action potentials restricted to distal apical dendrites of rat neocortical pyramidal neurons," *J. Physiol.* **505**, 605–616 (1997).
23. Bannister, N. J. and Larkman, A. U., "Dendritic morphology of CA1 pyramidal neurones from the rat hippocampus II Spine distributions," *J. Comp. Neurol.* **360**, 161–171 (1995).
24. Megias, M., Emri, Z., Freund, T. F. and Gulyas, A. I., "Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells," *Neuroscience* **102**, 527–540 (2001).
25. Jacob, S. N., Choe, C. U., Uhlen, P., DeGray, B., Yeckel, M. F. and Ehrlich, B. E., "Signaling microdomains regulate inositol 1,4,5-trisphosphate-mediated intracellular calcium transients in cultured neurons," *J. Neurosci.* **25**, 2853–2864 (2005).
26. Schiller, J., Helmchen, F. and Sakmann, B., "Spatial profile of dendritic calcium transients evoked by action potentials in rat neocortical pyramidal neurons," *J. Physiol.* **487**, 583–600 (1995).
27. Connors, B. W., Gutnick, M. J. and Prince, D. A., "Electrophysiological properties of neocortical neurons *in vitro*," *J. Neurophysiol.* **48**, 1302–1320 (1982).
28. Helmchen, F., Svoboda, K., Denk, W. and Tank, D. W., "*In vivo* dendritic calcium dynamics in deep-layer cortical pyramidal neurons," *Nat. Neurosci.* **2**, 989–996 (1999).
29. Haüsser, M., Major, G. and Stuart, G., "Differential shunting of EPSPs by action potentials," *Science* **291**, 138–141 (2001).
30. Saggau, P., "New methods and uses for fast optical scanning," *Current Opinion Neurobiol.* **16**, 543–550 (2006).