***In-situ* guidance of individual neuronal processes by wet femtosecond-laser processing of self-assembled monolayers**

Hideaki Yamamoto,^{1,2,a)} Kazunori Okano,^{3,4} Takanori Demura,⁵ Yoichiro Hosokawa,⁴ Hiroshi Masuhara,⁴ Takashi Tani,^{2,5} and Shun Nakamura¹

¹*Faculty of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei-shi, Tokyo 184-8588, Japan*

²*Nanotechnology Research Center, Waseda University, 513 Waseda Tsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan*

³*Kansei Fukushi Research Center, Tohoku Fukushi University, 6-149-1 Kunimigaoka, Aoba-ku, Sendai-shi, Miyagi 989-3201, Japan*

⁴*Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma-shi, Nara 630-0192, Japan*

⁵*School of Fundamental Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan*

(Received 6 August 2011; accepted 21 September 2011; published online 17 October 2011)

In-situ guidance of neuronal processes (neurites) is demonstrated by applying wet femtosecond-laser processing to an organosilane self-assembled monolayer (SAM) template. By scanning focused laser beam between cell adhesion sites, on which primary neurons adhered and extended their neurites, we succeeded in guiding the neurites along the laser-scanning line. This guidance was accomplished by multiphoton laser ablation of cytophobic SAM layer and subsequent adsorption of cell adhesion molecule, laminin, onto the ablated region. This technique allows us to arbitrarily design neuronal networks *in vitro*. © 2011 American Institute of Physics. [doi:10.1063/1.3651291]

Establishment of an “artificial neuronal circuit,” a simple *in vitro* model that mimics the *in vivo* architecture and function of neural circuits, are expected to facilitate the study of network properties of neurons in a brain and their computational role. Various attempts have been made to pattern neurons and their processes using pre-designed templates of polypeptides, proteins, or self-assembled monolayers (SAMs).^{1–4} Yet arbitral fabrication of a neuronal circuit with defined connectivity has not been demonstrated with the conventional approaches, primarily due to the complexity of neurons, having two types of neurites, i.e., axons and dendrites. Controlling inter-neuronal connectivity requires distinctive guidance of the neurites.

Previously, it has been shown that an axon and dendrites of single neurons can be distinctively elongated by patterning multiple adhesion molecules on a template surface,^{5–7} but fabrication of a designed network of neurons has never been demonstrated with this approach. In this context, a novel approach, where individual neurites are guided and connected to other cells *in-situ*, i.e., while the cells are being cultured, is promising. This approach is akin to building an electronic circuit on a stripboard and enables us to design a living neuronal circuit by guiding a selected neurite to a preferred site of any target neuron with an arbitrary pathway.

Two lines of methods are available to realize the *in-situ* guidance of neurites. One way is attract tip of neurites with, for example, optical forces,^{8–10} and the other is to locally modify culture surfaces *in-situ*^{11–17} so that novel adhesive region is presented for neurites to extend. Considering the ability to concurrently process multiple targets, the latter method is more favourable for our purpose. Several methods, including agarose-gel patterning with infrared laser,^{12,13}

electrochemical decomposition of surface bound proteins,¹⁴ UV-activation of photoreactive SAMs,¹⁵ oxidative-activation of electroactive SAMs,¹⁶ and femtosecond (fs)-laser processing of SAMs,¹⁷ have been proposed for the latter, and among these methods, fs-laser processing is a promising method for realizing our goal.

Under microscope, an effective multiphoton absorption of a focused fs-laser induces three-dimensionally selective laser ablation at the laser focal point in transparent multilayered materials such as a cell culture system. Therefore, local modification of a SAM layer can be accomplished with sub-cellular, μm -scale accuracy, even when cell culture medium and transparent substrate exist over and beneath the SAM layer.¹⁷ Here, we show that neurites of cultured neurons can be guided individually and arbitrarily using the fs-laser processing. We first arrayed primary neurons on a predesigned template of organosilane SAMs, and then applied the fs-laser processing to guide their neurites to a desired site with an arbitrary pathway.

The template for arraying primary neurons were fabricated by patterning aminosilane ((3-trimethoxysilylpropyl) diethylenetriamine; DETA) and octadecylsilane (*n*-octadecyltrimethoxysilane; ODS) SAMs on a Pyrex glass slide.¹⁸ DETA and ODS SAMs served as cytophilic and cytophobic regions, respectively. Primary neurons were obtained from chick forebrain at embryonic day 8. The dissociated neurons were seeded at a density of 3×10^4 cells cm^{-2} and cultured in M199 medium supplemented with 10% fetal bovine serum, 10% N9, 100 ng ml^{-1} 7S-nerve growth factor, and antibiotics.¹⁹ Fig. 1 shows a phase-contrast image of primary neurons arrayed on the DETA/ODS SAM template. The neurons adhered selectively on the 15 μm -diameter circles of DETA SAM and grew neurites along the DETA lines (0.2

^{a)}Electronic mail: h-yama@cc.tuat.ac.jp.

FIG. 1. A phase-contrast image of primary neurons cultured on a DETA/ODS SAM template for two days. DETA SAM pattern is depicted in the inset (black region). The neurons adhered selectively onto the DETA SAM region and grew neurites along the line. Scale bar: 50 μm .

and 1 μm -wide lines were used interchangeably). The neurites elongated until they reached the end of the line and stopped growing without extending further.

Next, the neurites of arrayed neurons were guided by scanning a focused fs-laser (wavelength: 800 nm, pulse duration: 120 fs, repetition rate: 1 kHz, power: 0.5 mW) to modify the cytophobic ODS region. Neurons that adhered to the DETA region and elongated at least one neurite after two days of culture were subjected to the subsequent laser experiment.¹⁸ Prior to laser irradiation, laminin-1, which is an extracellular matrix protein and promotes neuronal adhesion to substrates, was added to the culture medium at a concentration of 10 $\mu\text{g ml}^{-1}$. Fig. 2(a) shows primary neurons cultured on the DETA/ODS SAM template for two days and the same region just after the laser was scanned between DETA regions, respectively. A local increase of refractive index in the glass slide was observed at the laser-scanning line,²⁰ and the laser trace could be confirmed with phase-contrast microscopy. After a 24 h incubation, neurites of two neurons, which originally adhered on DETA sites, elongated along the scanning line.

High affinity of neurites to the laser-irradiated region was confirmed by observing neurite extension on an irregular, zigzag scanning line. As shown in Fig. 2(b), a neuron extended its neurite precisely along the zigzag line pattern. This result clearly presents that the neurite guidance by the laser processing was not an artefact but was accomplished by the surface modification caused by the fs-laser irradiation. In addition, we confirmed that the inherent property of a neurite to branch was conserved on the scanning line fabricated by the laser processing. As shown in Fig. 2(c), a neuron on a cell adhesion site extended and branched its neurite along two scanning lines whose edges cross perpendicularly at an edge on a DETA line. The longest neurite extension observed so far is 34 μm . Once neurites elongated on the laser-scanning line, they remained stably on the line for at least four additional days. These results imply that our method can be employed to interconnect cultured neurons at an arbitrary distance by designing the pathway for neurite growth, which should greatly aid studies on signal propagation within a neuronal network.

FIG. 2. *In-situ* guidance of neuronal processes. (a) Consecutive images of a neurite growing on a laser-scanning line. From left to right: Before laser scanning, and at 0 h (focused on the laser-scanning line) and 24 h after scanning laser between the top and bottom cell adhesion sites. Black arrows indicate the site of laser scanning. Illustration of the DETA/ODS patterns and the laser-scanning line is shown in the right-most panel. The dotted line represents the laser-scanning line. (b and c) Other representative images of neurite guidance. Left column: Phase-contrast images focused on neurons, observed 24 h after the laser scan. Center column: The same field focused on the laser traces. Right column: Illustration of the DETA/ODS patterns and the laser-scanning line. Scale bar: 50 μm .

The neurite guidance with fs-laser lithography required laminin-1 to be present in the medium. Fig. S1 show neurons cultured for 24 h after laser lithography in the culture medium without addition of 10 $\mu\text{g ml}^{-1}$ laminin-1.¹⁸ Even though the laser was irradiated to interconnect the separated DETA regions, none of the tested neurites grew along the scanning line ($n = 0$ of 68 scan lines). Cell culture for longer time periods (48 or 72 h) did not change the result. This indicates that addition of cell adhesion molecule, laminin-1, to the medium is necessary to successfully guide neurites with the laser processing.

Furthermore, selective adsorption of laminin-1 to the laser-scanning line was confirmed by imaging dye-labeled laminin-1.¹⁸ (Fig. 3(a)) Laminin-1 adsorbed selectively on the laser irradiated region, strongly implying that proteins in cell culture medium adsorb extensively onto the laser-scanning line. On the basis of these results, a possible mechanism of the laser-induced neurite guidance is speculated as follows (Fig. 3(b)): (i) the SAM and surface-bound proteins are modified and/or decomposed by the multiphoton laser ablation, (ii) proteins in medium, including the adhesion molecule laminin-1, subsequently adsorb onto the irradiated region, and finally, (iii) neurites elongate on the region.

Further work is in progress to improve the success rate of the *in-situ* guidance, which is currently estimated to be approximately 14% ($n = 16$ of 115 scan lines).¹⁸ We are also currently searching for optimal cell adhesion molecule to be added in the culture media in place of laminin-1. Finally, we must assign whether a neurite is an axon or a dendrite in the

FIG. 3. (Color online) (a) Selective adsorption of laminin onto the laser-irradiated region. Top row: Confocal (left) and light scattering (right) images of fluorescein-labelled laminin on a laser-irradiated region ($30 \times 30 \mu\text{m}^2$). Bottom row: No autofluorescence was detected before the application of laminin. Scale bar: $30 \mu\text{m}$. (b) Schematic illustration of a possible mechanism of the neurite guidance. Roman numerals correspond to steps (i)-(iii) described in the text.

process of the guidance. This can be accomplished, for example, by fluorescently labelling axons with FM-dyes.²¹ These results will be reported soon.

In conclusion, we demonstrated an *in-situ* guidance of individual neurites from neurons arrayed on a template surface. By locally modifying the cytophobic surface with focused fs-laser, the cytophobic region was converted into a cytophilic region. This modification required cell adhesion molecule laminin-1 to be present in the culture medium during fs-laser irradiation, implying that re-adsorption of

proteins in the medium takes place after laser irradiation. The method presented here opens up a new possibility towards artificially designing a neuronal network *in vitro*.

The authors acknowledge Ms. Mie Matsubara and Ms. Sayaka Sakaguchi of the Nara Institute of Science and Technology for setting up equipment. This work was supported by the Research Fellowships for Young Scientists (HY) and by the Grant-in-Aid for Scientific Research (B) (No. 20310075) (KO) from the Japan Society for the Promotion of Science.

¹B. C. Wheeler and G. J. Brewer, *Proc. IEEE* **98**, 398 (2010).

²A. K. Vogt, G. Wrobel, W. Meyer, W. Knoll, and A. Offenhäusser, *Biomaterials* **26**, 2549 (2005).

³A. K. Vogt, F. D. Stefani, A. Best, G. Nelles, A. Yasuda, W. Knoll, and A. Offenhäusser, *J. Neurosci. Methods* **134**, 191 (2004).

⁴C. Wyart, C. Ybert, L. Bourdieu, C. Herr, C. Prinz, and D. Chatenay, *J. Neurosci. Methods* **117**, 123 (2002).

⁵A. A. Oliva, Jr., C. D. James, C. E. Kingman, H. G. Craighead, and G. A. Banker, *Neurochem. Res.* **28**, 1639 (2003).

⁶P. Shi, K. Shen, and L. C. Kam, *Dev. Neurobiol.* **67**, 1765 (2007).

⁷J. T. Nevill, A. Mo, B. J. Cord, T. D. Palmer, M.-m. Poo, L. P. Lee, and S. C. Heilshorn, *Soft Matter* **7**, 343 (2011).

⁸A. Ehrlicher, T. Betz, B. Stuhmann, D. Koch, V. Milner, M. G. Raizen, and J. Käs, *Proc. Natl. Acad. Sci. USA* **99**, 16024 (2002).

⁹S. K. Mohanty, M. Sharma, M. P. Panicker, and P. K. Gupta, *Opt. Lett.* **30**, 2596 (2005).

¹⁰D. J. Carnegie, T. Čížmár, J. Baumgartl, F. J. Gunn-Moore, and K. Dholaria, *J. Biophotonics* **2**, 682 (2009).

¹¹J. Robertus, W. R. Browne, and B. L. Feringa, *Chem. Soc. Rev.* **39**, 354 (2010).

¹²Y. Sugio, K. Kojima, H. Moriguchi, K. Takahashi, T. Kaneko, and K. Yasuda, *Sens. Actuators B* **99**, 156 (2004).

¹³I. Suzuki, Y. Sugio, Y. Jimbo, and K. Yasuda, *Lab Chip* **5**, 241 (2005).

¹⁴H. Kaji, K. Tsukidate, T. Matsue, and M. Nishizawa, *J. Am. Chem. Soc.* **126**, 15026 (2004).

¹⁵J. Nakanishi, Y. Kikuchi, T. Takarada, H. Nakayama, K. Yamaguchi, and M. Maeda, *J. Am. Chem. Soc.* **126**, 16314 (2004).

¹⁶W. Luo and M. N. Yousaf, *J. Am. Chem. Soc.* **133**, 10780 (2011).

¹⁷K. Okano, D. Yu, A. Matsui, Y. Maezawa, Y. Hosokawa, A. Kira, M. Matsubara, I. Liao, H. Tsubokawa, and H. Masuhara, *ChemBioChem* **12**, 795 (2011).

¹⁸See supplemental material at <http://dx.doi.org/10.1063/1.3651291> for Fig. S1, details on experimental methods, and a discussion on the success rate.

¹⁹S. R. Heidemann, M. Reynolds, K. Ngo: P. Lamoureux, in *Neurons: Methods and Applications for the Cell Biologist*, edited by P. J. Hollenbeck and J. R. Bamberg (Academic, California, 2003), p. 51.

²⁰K. M. Davis, K. Miura, N. Sugimoto, and K. Hirao, *Opt. Lett.* **21**, 1729 (1996).

²¹H. Jiang, W. Guo, X. Liang, and Y. Rao, *Cell* **120**, 123 (2005).