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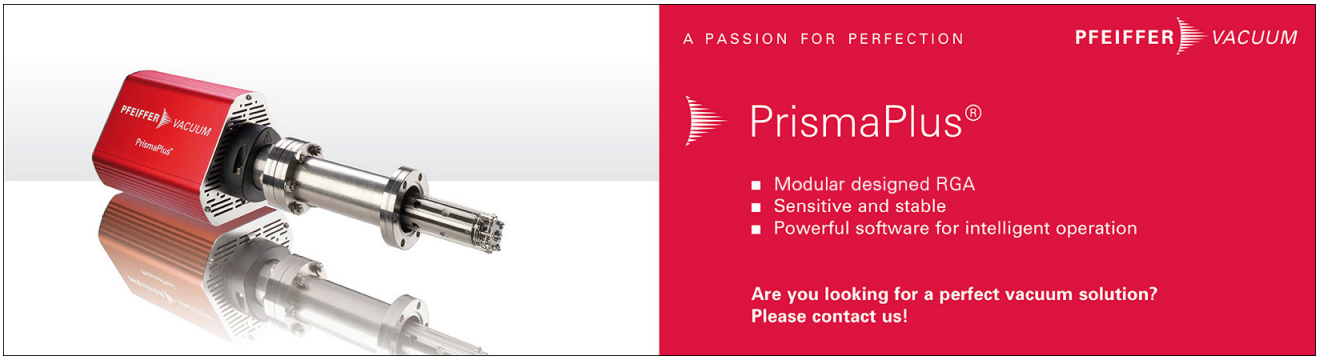

Development of a regeneration-type neural interface: A microtube guide for axon growth of neuronal cells fabricated using focused-ion-beam chemical vapor deposition

Takayuki Hoshino[a)]
Graduate School of Information Science and Technology, The University of Tokyo, Engineering Building 6, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan; Research Fellowships, Japan Society for the Promotion of Science, 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan; and CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi-shi, Saitama 332-0012, Japan

Akinori Ozasa
CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi-shi, Saitama 332-0012, Japan and LASTI, University of Hyogo, 3-1-2, Koto, Kamigori-cho, Ako-gun, Hyogo 678-1201, Japan

Reo Kometani
Research Fellowships, Japan Society for the Promotion of Science, 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan; CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi-shi, Saitama 332-0012, Japan; and LASTI, University of Hyogo, 3-1-2, Koto, Kamigori-cho, Ako-gun, Hyogo 678-1201, Japan

Takafumi Suzuki
Graduate School of Information Science and Technology, The University of Tokyo, Engineering Building 6, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Shinji Matsui
CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi-shi, Saitama 332-0012, Japan and LASTI, University of Hyogo, 3-1-2, Koto, Kamigori-cho, Ako-gun, Hyogo 678-1201, Japan

Kunihiko Mabuchi
Graduate School of Information Science and Technology, The University of Tokyo, Engineering Building 6, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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The authors' goal is to develop a neural interface system that enables connection of the human nervous system with external devices and allows transmission of information in both directions. One way to connect interface to neurons is a regenerative electrode, where the electrode is placed between the two cut ends of a nerve. The cut nerve fiber can regenerate through channels in a metallic electrode on a two-dimensional (2D) plane of the regenerative electrode. As this type of electrode enables both the recording of signals from a single nerve fiber and the stimulation of a single nerve fiber, attempts have been made to develop it using traditional 2D microfabrication techniques. However, it is difficult to process such electrodes with these 2D microfabrication techniques, particularly the high-density integrated structure of the electrical wiring. The authors previously designed a neural interface system—a type of regenerative electrode consisting of an electrode segment and many carbon microtubes for guiding the regenerating axons of the neuronal cells—that can be made through three-dimensional processing. In the current study, the authors have fabricated a prototype of the guiding segment of the electrode (which was previously reported) and evaluated the biocompatibility of diamondlike carbon (DLC) made by focused-ion-beam chemical vapor deposition (FIB-CVD) and carbon microtubes *in vitro*. The microtubes were fabricated using FIB-CVD. DLC was deposited with a scanning 30 keV Ga+ ion beam in an atmosphere containing phenanthrene ($C_{14}H_{10}$). The scanning patterns were determined by a computer-aided-design system before the fabrication. The beam was scanned over a Au-coated glass capillary or polycarbonate membrane to deposit DLC and form the carbon microtubes. For observation of the axon growth through the microtubes, the authors fabricated bifurcated carbon microtubes with an inner diameter of 3–6 μm on the glass capillary and straight microtubes on the polycarbonate membrane. The fabricated microtubes were immersed in a culture medium containing nerve growth factor and PC12 cells were cultured inside the capillary and on the membrane to evaluate whether they could extend axons through the microtubes. Here, the authors show that cultured rat phaeochromocytomas adhered to and survived for days on a DLC surface while preserving their morphology provided that the adhesion molecule poly-D-lysine was used as an organic substance to anchor the cells to the DLC surface. As a result, cells spread and neurites projected on the DLC area, suggesting that the DLC had little cytotoxic effect. © *2006 American Vacuum Society.* [DOI: 10.1116/1.2359730]

I. INTRODUCTION

The regenerative neural interface is one type of neural interface for the peripheral nerve system.[1,2] This type of interface has been studied for single unit recording of neural activity from chronic moving animals since the 1960s.[3,4] Such an interface enables us to interconnect between the peripheral nerve system and external devices. The principle underlying this sort of interface device is that the axons of severed peripheral nerves can be regenerated from the proximal nerve stump to the distal nerve stump. The regenerative electrode uses this characteristic of peripheral nerves to connect itself to a single nerve fiber. The connection between a nerve fiber and a channel of the regenerative electrode can be created through the regeneration of nerve fibers through microholes acting as channels of the regenerative electrode. In previous studies, this type of electrode has been used to record signals from sensory or motor nerves and to stimulate nerves.[2,5] These electrodes, called sieve electrodes, were fabricated on a two-dimensional surface of a silicon or plastic substrate through photoprocessing. Bradley *et al.* fabricated a sieve electrode with 214 holes and five 5 μm diameter recording sites on a Si wafer using a micromachining process and recorded peripheral nerve activity.[6] Mensinger *et al.* recorded chronic activity using a similar interface with nine recording sites which were 5–20 μm in diameter.[5] Ideal interface devices of this sort will need to have a large number of channels in a microscopic substrate, though, and such devices have yet to be reported. The electrode should be designed to enable a high microhole fill factor that enables the axon of the nerve cell to regenerate well, so the area available for electrical wiring is limited to the extremely small amount of space between the microholes. One solution to this problem was proposed in our previous study.[7] Our proposed design uses three-dimensional electrical wiring and microtubes to guide the regenerating nerves. Regenerating nerves can spread through spaces positioned to facilitate the connection of the wiring to the nerve fiber. The development of such devices, though, has been impeded by lack of the three-dimensional processing techniques needed to fabricate an electrode structure in three-dimensional space which will let us avoid the electrical wiring problem. In our previous studies, we investigated multimaterial micro-/nanoscale three-dimensional processing using focused-ion-beam chemical vapor deposition (FIB-CVD).[7–9] This technique enabled fabrication of structures with an overhang or inverse-tapered shape in micro-/nanospace, and these structures could be made of several kinds of materials such as diamondlike carbon (DLC), tungsten, Pt, or SiO_2 in a single process. A focused Ga+ ion beam accelerated at 30 keV was used to irradiate a substrate within a precursor gas atmosphere, for example, phenanthrene ($C_{14}H_{10}$) or tungsten hexacarbonyl [$W(CO)_6$], to deposit the materials. A beam scanning controller was developed and optimized for fabrication of a three-dimensional structure using a computer-aided-design system. The minimum diameter of the deposition resolution was 80 nm. We could produce complicated nanostructures in microscopic space using this technique. We demonstrated that fabricated microtubes made of DLC or tungsten could be used as guidance tubes of the regenerative axons of severed peripheral nerves. In this article we report on our evaluation of the biocompatibility of the microtubes and axon extension toward them using cultured neuronal cells. Here, we show that cultured rat phaeochromocytomas adhered to and survived for days on a DLC surface while preserving their morphology provided that the adhesion molecule poly-D-lysine (PDL) is used as organic substance to anchor the cells to the DLC surface. PDL improved adhesiveness between the cell membrane and the solid surface through its electrical charge.

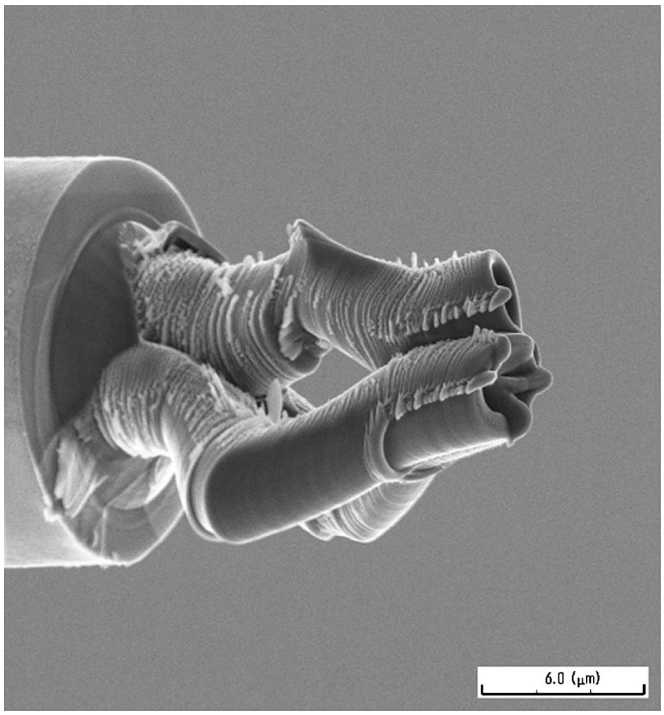

FIG. 1. Bundle of DLC microtubes attached to the edge of a glass capillary. These had an inner diameter of about 2 μm.

II. THIN-FILM DLC

100×100 μm^2 thin-film DLC of 0.1 μm in thickness was deposited on a glass slide using a FIB system (SMI-9200, SII Nano Technology Inc.) with 13 nA/μm^2 of beam current density and 30 kV Ga ion beam in 1×10^{-4} Pa phenanthrene gas. A glass slide without DLC was prepared as a control. The slides were coated with poly-D-lysine (P6407, Sigma) to add a cellular adhesiveness. PC12 cells were grown on these glass slides in Neurobasal medium (GIBCO) containing 10% fetal bovine serum, 5% horse serum, 7.35 mg/l L-glutamic acid, and 338 mg/l L-glutamine. The PC12 cells, from a rat phaeochromocytoma cell line, were triggered to differentiate into neuronlike cells and to generate neurite outgrowth within several hours through the addition of nerve growth factor (NGF) to the culture medium. This setup was incu-

a)Electronic mail: takayuki_hoshino@ipc.i.u-tokyo.ac.jp

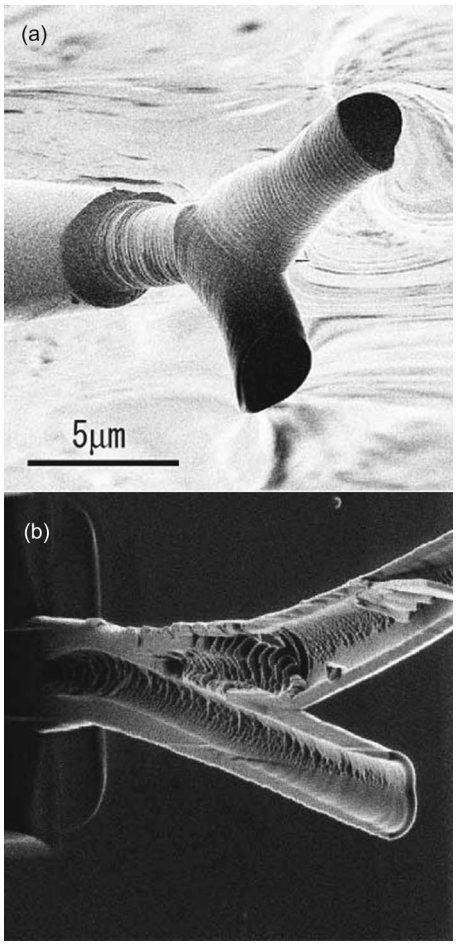

FIG. 2. Bifurcated microtube attached to a glass capillary. The inner diameter of the end of the microtube was about 3 μm.

bated for 1 day at 37 °C in an atmosphere of 5% CO_2 and then 40 ng/ml of NGF was added to the medium.

III. CARBON MICROTUBES

The microtubes were fabricated using the FIB-CVD system described above and a three-dimensional beam scanning system that we developed. This system could use two types of precursor gas: phenanthrene or tungsten hexacarbonyl. Three gas nozzles led into the specimen chamber for the precursor gas. The two nozzles for phenanthrene faced each other 150 μm above the substrate surface and 300 μm apart. The one nozzle for tungsten hexacarbonyl was placed 150 μm above the substrate surface. In this study, we used phenanthrene gas at 1×10^{-4} Pa in the sample chamber to form the DLC structure. DLC could be used for three-dimensional deposition on the substrate at room temperature. The diameter of a pillar irradiated with a spot beam at 1–20 pA was about 100–150 nm.

In this study, *in vitro* testing was done on the PC12 cells as neuronal cells. The PC12 cells extended axons that were 1–3 μm in diameter which were observed in scanning electron microscopy (SEM) images of cells contained in the NGF. Therefore, microtubes designed to be 2–8 μm in diameter should be suitable for the axons of PC12 cells (though many medullated nerves larger than PC12's have been observed *in vivo*).

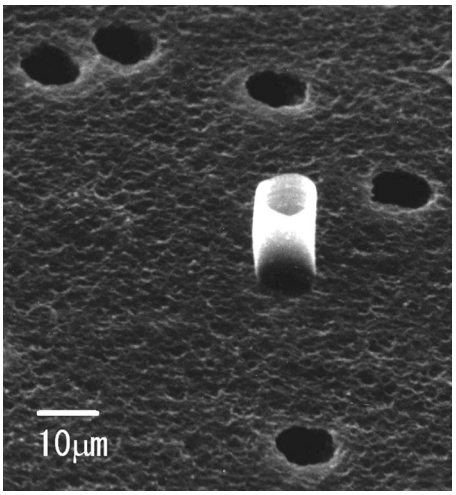

FIG. 3. Carbon microtube on a porous polycarbonate membrane with pore diameter of 8 μm. The microtube was about 14 μm in height and 8 μm in inner diameter as observed by SEM.

Microtubes were attached at the tips of glass capillaries and on polycarbonate membranes to evaluate the biocompatibility using cultured cells. Before microtube fabrication, 15 nm thick Au layer was sputtered onto the membranes. The process time needed to produce each microtube was about 2 h. Figure 1 shows a bundle of three DLC microtubes attached to the end of a glass capillary. The inner diameters of the microtubes were about 2 μm. Figure 2 shows a bifurcated microtube with a 3 μm inner diameter attached on the end of a glass capillary. A cross section of the bifurcated microtube is shown in Fig. 2(b). The fluidic channel of the bifurcated microtube was open throughout the tube length. Straight microtubes that were about 14 μm high and had an 8 μm inner diameter were produced on a 10 μm thick porous polycarbonate membrane with 8 μm diameter pores (Fig. 3).

IV. *IN VITRO* TEST OF MICROTUBES

We performed two types of experiments using cultured cells to evaluate the biocompatibility of the microtubes and axon extension through the microtube. Glass capillaries with bifurcated microtubes and polycarbonate membranes with straight microtubes were used for the culturing.

Each capillary was 10 mm long and gradually becomes finer with the inner diameter decreasing from 1 mm to about 3 μm at the end of the fine tips. A schematic of the experiment is shown in Fig. 4. The capillary with the microtube and its acrylic holder were sterilized by ultraviolet light on a clean bench before the *in vitro* test. PC12 cells were cultured in a glass capillary coated with PDL to improve cell adhesion. The PC12 cells inside the glass capillary with the microtube were immersed in a medium (previously described) in a culture dish, and they were incubated with 50 ng/ml of NGF at 37 °C, 5% CO_2 for 7 days.

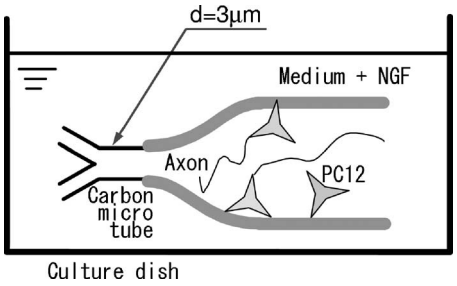

FIG. 4. Schematic of the *in vitro* test using a glass capillary with a bifurcated microtube. The glass capillary and the microtubes were immersed in a culture medium with 50 ng/ml of NGF added. PC12 cells were cultured in the capillary.

The polycarbonate membranes, each with four straight microtubes, were incubated with 200 ng/ml of NGF. The straight microtubes attached to a membrane were placed at the bottom of inserter wells (Corning: Transwell) as shown in Fig. 5. These wells were inserted into a 24-well plate, leaving space between the microtube and the bottom of the plate. PC12 cells were cultured inside the inserter wells with the microtubes. The cells were immersed in the medium with 200 ng/ml of NGF. There was a NGF concentration gradient from below the membrane and above it. The NGF medium under the membrane with NGF was changed every 1–2 days. The cells were incubated at 37 °C, 5% CO_2 for 10 days. The cells on the membranes were fixed in 4% *para*-formaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature and then rinsed gently in PBS. The fixed cells were dehydrated by a graded series of ethanol dilutions, and then immersed in 100% *t*-butanol. The *t*-butanol was then freeze-dried at 4 °C. After sputter coating with Pt–Pd, the cells were examined by SEM.

V. RESULTS

The axons of cells on the DLC thin film were observed by an optical microscope one day after the NGF was added (Fig. 6). The cells extended the axons on DLC and around it. Its activity was as high as for the control.

Figure 7 shows an example of the results from a glass capillary observed with an inverted optical microscope. Rounded PC12 cell bodies, whose diameters were about

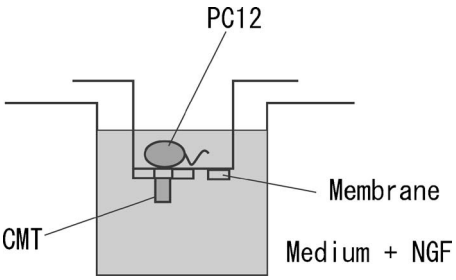

FIG. 5. Schematic of the *in vitro* test using polycarbonate membranes with straight microtubes. The membranes and the microtubes were immersed in a culture medium with 200 ng/ml of NGF added. PC12 cells were cultured on the membranes.

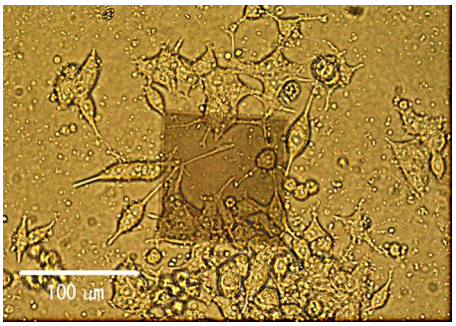

FIG. 6. Axons of PC12 extend on DLC thin film ($0.1\times100\times100$ μm^3 fabricated by FIB-CVD) at day 2 (day 0: beginning; day 1: addition of 200 ng/ml NGF; day 2: optical observation). The sharpness of this image was increased by digital image processing.

10 μm, adhered to the inner surface of the glass capillary. These cells were differentiated into neuronal cells through the addition of NGF. The shapes of the cells changed into projecting shapes, as shown in Fig. 7(b). Neurite outgrowth of PC12 cells at the tip of the glass capillary was observed, but the viability of the PC12 cells was not good and almost no axon outgrowth was observed. On the other hand, the axons at the thick end of the capillary grew extremely well.

Figure 8 shows the underside of a membrane with an axon extension toward the carbon microtube, observed by SEM. The PC12 cells were differentiated into neuronal cells through the addition of NGF, and sufficient extension of the axons was observed. The cell on the underside of the mem-

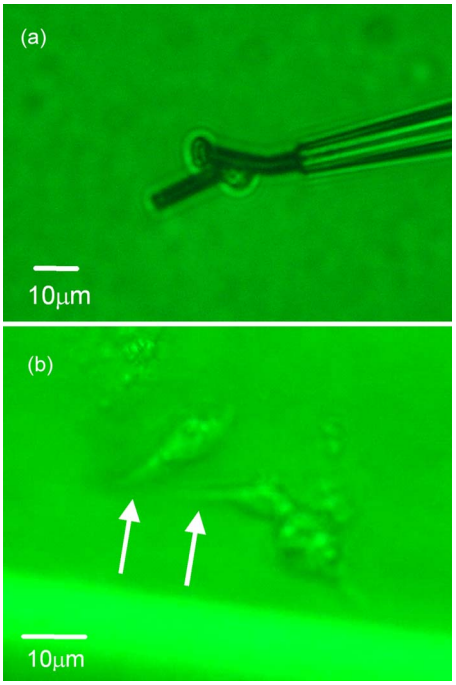

FIG. 7. Bifurcated microtube in the medium and PC12 cells in a glass capillary observed by optical microscope: (a) bifurcated microtube; (b) differentiated PC12 cells at the tip of the glass capillary. The neurite was observed, but axon extension was not observed.

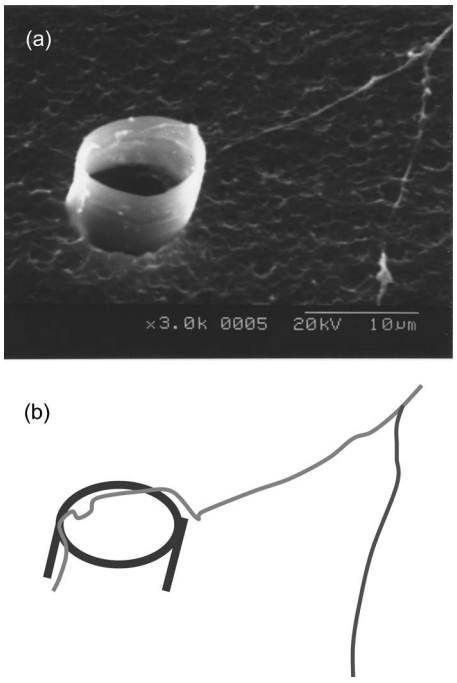

FIG. 8. Axon extension on a microtube attached to a polycarbonate membrane. (a) SEM image of the microtube and the axon. (b) Schematic of SEM image in (a). The axon grew from the upper right of (a) to the microtube, extended along the inner surface of the microtube, and then exited from it.

brane extended its axon into one of four microtubes (from the upper right of this figure), and then the axon grew outside of the microtube.

VI. DISCUSSION

The characteristics of materials used to make instruments for implantation into a living body are an important consideration when developing an implant system such as a neural interface. Since our goal is to develop a three-dimensional neural interface, we previously investigated the fundamental characteristics of DLC fabricated by FIB-CVD techniques.[10,11] DLC fabricated by FIB-CVD has a high contact angle and is a hydrophilic material.[12] We investigated the cell adhesion of cultured cells on DLC surface, and found that it was low.[9] In the current study, to ensure cell adhesion so that we could observe axon extension, we coated PDL onto the DLC, polycarbonate, and glass as a cell adhesive. PDL did not increase the hydrophilia, but it increased cell adhesion by creating an electrical charge on the material surface. The surviving cells quickly aggregated and formed clusters on the nontreated DLC surface on a glass slide (no data shown). The DLC deposited on a glass slide with PDL allowed cells to attach themselves and commence neuritic processes. No significant difference in morphology could be observed between the cells grown on DLC and those on the glass area. The PDL was deposited as a thin layer and increased cell adhesiveness only through its electric charge. The PDL did not diminish the toxicity of the solid surface. Therefore, the spreading of cells and neurite outgrowth on the DLC area suggested that the DLC had little cytotoxic effect. Therefore any small amount of gallium from the ion beam that remained tens of nanometers below the surface of DLC fabricated by FIB-CVD apparently affected the axon growth a little or not at all. However, this single morphological result is not enough to prove in a statistically significant way that this type of DLC has low cytotoxicity. To assess a greater number of samples and larger sample area, we must increase the deposition throughput of FIB-CVD by optimizing fabrication.

Insufficient extension of the PC12 cell axons in the capillary suggests insufficient medium diffusion in such a narrow space, and we believe the diameter and length of the capillary and microtube will be important factors if the microfluid channel is used as an axon guiding structure in an implantable human interface. One solution to this problem is for the microtubes to have holes in their lateral surface. Our FIB-CVD technique can be used to fabricate microtubes with such a complicated porous structure. Porous structures or wire-frame structures will enhance the diffusion of O_2 and the nutrients of a medium in such a narrow space.

Our finding that PC12 cell extended its axon onto a microtube located on a polycarbonate membrane suggests that DLC microtubes made by the FIB-CVD technique offer good biocompatibility. In the experiment using polycarbonate membranes, the axon grew on the microtube, although any cellular adhesive material was not coated to this setup. This showed that the bare DLC microtube was biocompatible and had some cellular adhesiveness. The reason axon extension through the microtubes did not occur was probably insufficient guidance for the axons through the microtubes. There are some mechanisms that can be used for axon guidance, such as neurotropic factors (NGF, etc.), surface patterns (grooves, pillars, etc.), or guidance cues. Thus, although the NGF concentration in the early phase had a gradual gradient between above the membrane and below it, the NGF diffused through the medium with time. A scaffold to enhance the axon extension was not especially designed for the microtubes, so the axons would have only a small likelihood of navigating through the microtubes. The application of guidance cue and a scaffold inside the microtubes should be considered in future evaluation of axon growth into microtubes. To investigate whether these conditions improve neurite outgrowth and to quantify the biocompatibility of the microtubes, samples of greater number and larger size structures should be fabricated. Therefore deposition throughput of FIB-CVD will have to be increased by optimizing fabrication.

In this study, we investigated the biological characteristics of a single microtube using cultured cells *in vitro* and were able to evaluate the fundamental characteristics of microtubes using this setup. However, we did not investigate the characteristics of an integrated system where a large number of multichannel microtubes with electrodes work as a neural interface. The long processing time needed to produce the tubes would make it difficult to fabricate a large structure within a practical time. An improved beam scanning strategy and increasing the precursor gas pressure around the deposi-

tion point will significantly raise the deposition rate, but this will not be enough to enable the fabrication of such large, complex structures. It may be necessary to combine three-dimensional FIB-CVD fabrication with a micromachining technology such as stereolithography or a microelectromechanical system technique.

VII. CONCLUSION

We used focused-ion-beam-induced deposition of DLC to form microtubes on a culturing device and evaluated the biocompatibility of the microtubes using living cells. DLC fabricated by FIB-CVD has sufficiently low cytotoxicity for growing axon *in vitro*, and PDL coating improved the weak cellular adhesiveness so that the axon could extend onto the DLC. This low cytotoxicity indicates that the microtubes can be used as guides for regenerative electrode. Our findings suggest that our FIB-CVD technique of three-dimensional DLC deposition will be suitable for forming microtubes for axon guidance, though the problems of ensuring axon guidance and a sufficient supply of nutrients and oxygen remain to be solved.

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